Title: ALTERED ION CHANNEL PROTEINS

Abstract: Altered ion channel proteins having acquired sensitivity to gating agents are disclosed. The invention is directed towards those ion channels that are refractory to gating agents. The altered ion channel proteins are of use in identifying modulators of the unaltered ion channel. Host cells, vectors and screening assays are also disclosed.
Altered ion channel proteins

The present invention concerns altered ion channel proteins. In particular, the present invention concerns altered ion channel proteins having acquired sensitivity to a gating agent, that is an agent that activates the ion channel protein (or slows or otherwise inhibits the rate of inactivation), thereby permitting the flow of ions via the channel into and/or out of the host cell expressing the altered ion channel protein. Other objects and advantages of the present invention will become apparent from the description below.

Voltage-gated sodium ion channels are responsible for the rising phase of the action potential and as such, play a key role in mediating electrical activity in excitable tissues. The sodium ion channel is activated in response to depolarisation of the membrane. This causes a voltage-dependent conformational change in the ion channel, from a resting, closed conformation to an open, active conformation, the result of which increases the membrane permeability to sodium ions (1, 2). In the case of voltage-gated sodium ion channels the activation of the ion channels is usually transient (10-50ms) since the ion channels rapidly inactivate (i.e. switch to a confirmation which is unable to conduct sodium ions) during a sustained membrane depolarisation. This rapid activation and inactivation of sodium conductance causes the rapid rising phase of the action potential in excitable cells.

Voltage-gated sodium ion channels comprise a multisubunit complex consisting of a large (230-270kDa) highly glycosylated alpha (α) subunit which is usually associated with one or two of the smaller beta (β)-subunits (β1, β2 and β3) (3, 4). The alpha subunits of voltage-gated sodium ion channels form a large multigene family that has expanded over recent years and at least ten different genes have now been identified in mammals (5-13). This alpha subunit consists of four homologous domains (DI-IV) each containing six potential α-helical transmembrane segments (SI-S6) which make-up the pore forming region. Domains critical for the function of the channel are highly conserved throughout the family of voltage-gated sodium ion channels. These include the S4 voltage sensors, the loop between domains III and IV which is involved in the
inactivation of the ion channel and the SSI and SS2 segments of the extracellular loop between transmembrane regions S5 and S6, which are responsible for the channels vestibule and ion selectivity (14-16). β subunits appear to have a role in altering the kinetics of the sodium ion channel during activation and inactivation gating. Expression of the β subunits has been associated with an increase in peak current and a role in trafficking of the α subunit to the membrane (17-20).

The most potent blocker of voltage gated sodium ion channels is the puffer fish toxin, tetrodotoxin, (TTX). While most voltage-gated sodium ion channels are inhibited by low nanomolar concentrations of TTX, there are three ion channels which are only inhibited by micromolar concentrations of TTX. These are the major cardiac ion channel (H1 or SKM2) and the sensory neurone specific ion channels, SNS/PN3 (here designated SNS1) and SNS2/NaN (3, 7, 8, 12, 13).

Sensory neurones of mammalian dorsal root ganglion (DRG) transmit sensory information from the periphery to the central nervous system and are known to express at least three distinct kinetic types of voltage-gated sodium currents (21, 22). The small diameter neurones co-express a rapidly inactivating, fast TTX sensitive current and a slowly activating and inactivating TTX resistant sodium current. The larger diameter cells only express TTX sensitive sodium currents that have intermediate activation and inactivation kinetics (23, 24). This electrophysiological analysis has now been supported by molecular distribution studies, which suggest that there is a dynamic expression of voltage-gated sodium ion channels in DRG neurones that can change during development, response to injury and upon exposure to inflammatory mediators (25-28). The small diameter neurones are unmyelinated and are involved in the transmission of pain impulses, these neurons are the c-fibres or nociceptive neurones (29).

Recent experimental evidence has associated and implicated sodium currents with the chronic pain and hypersensitivity pathologies of both inflammatory and neuropathic origin. For example in the small diameter nociceptive neurones, hyperalgesic agents such as prostaglandin E2 (PGE2) and serotonin enhance TTX resistant sodium currents and decrease the threshold for inactivation (30, 31). Neuronal injury produces dramatic changes in sodium ion channel expression and distribution, for example accumulation of TTX sensitive sodium ion channels at
the neuroma of lesioned axons is thought to be responsible for formation of ectopic discharges (32, 33). Following human peripheral nerve injury there is accumulation of sodium ion channels at the site of injury (34). Following brachial plexus injury in human patients the sensory neuron specific ion channel α-subunits, SNS/PN3 and SNS2/NaN, redistribute in the DRG neurons (35). There is an acute decrease of SNS/PN3 and SNS2/NaN protein in the sensory cell bodies of DRG whose central axons have been avulsed from the spinal cord and an increase in the channel proteins in nerve fibres. Recent studies in the rat have also demonstrated that there is a change in the localisation of SNS/PN3 after nerve injury (36). In each case the neuronal hyperexcitability that results is highly likely to contribute to the induction and maintenance of this sensitised state. It follows that voltage-gated sodium ion channels in sensory neurones may provide a highly tractable and attractive target for the development of novel analgesic and anti-hypersensitivity agents, and thus indicated in diseases such as neuropathic pain, inflammatory pain, migraine and headache, multiple sclerosis and other demyelinating conditions (see ref. 37).

This supposition is supported by the observation that anaesthetic, anticonvulsant and antiarrythmic drugs, each with sodium ion channel blocking activity, can produce analgesia. For example, it has been recognised that sub-anaesthetic doses of lignocaine and bupivocaine elevate pain thresholds in man (38, 39). In addition the anticonvulsant agents, phenytoin, carbamazepine and the class Ia antiarrythmic agent mexiletene are used clinically for neuropathic pain (40-42). The anticonvulsant lamotrigine is also weakly analgesic (43). In order to discover novel voltage-gated Na⁺ ion channel modulating agents it would be desirable to carry out a high-throughput plate-based screen. In order to determine the ability of an agent to inhibit a sodium ion channel it is necessary to determine the ability of the ion channel to open and to conduct Na⁺ ions in the presence of the agent. However, the physiological method of gating these ion channels by membrane depolarisation is difficult to reproduce in a high throughput plate-based assay format.

It is established that α-scorpion toxins (α-ScTx) bind to a common extracellular site on brain Na⁺ ion channels thus inhibiting rapid inactivation, and that domains I and IV of the brain Na⁺ ion channel α-subunits are implicated in
α-ScTx binding (44, 45). The ability of α-ScTx to prevent inactivation of the brain sodium channels provides a convenient method to gate these Na⁺ ion channels in plate-based assays appropriate for high-throughput drug screening (46). Mammalian cells expressing the brain Na⁺ ion channel to be assayed are plated in multi-well plates and pre-incubated in a fluorescent membrane potential-sensitive dye (DiBAC(4)(3)). At resting membrane potentials, the ion channels are believed to be slowly cycling between the closed, open and inactivated configurations (upon the membrane depolarisation that causes physiological gating the rate of transition from closed to open to inactivated is believed to increase). Upon addition of α-ScTx to the multi-well plates the transition from the open to inactivated conformation is inhibited resulting in a slight membrane depolarisation. This initial depolarisation in turn causes more channels to open and not inactivate. Thus a cascade occurs that leads to a pronounced membrane depolarisation which causes a change in the fluorescence of the membrane potential-sensitive dye. This fluorescence change can be detected on a fluorometric imaging plate reader (FLIPR, Molecular Devices, Sunnyvale, CA, USA; 47). If the cells are pre-incubated with agents that inhibit the Na⁺ channel the fluorescence change upon addition of α-ScTx is reduced or completely ablated in a concentration-dependent manner. This method has allowed the activity of the brain Na⁺ ion channels to be assayed in a plate-based system.

However, the present inventors have found that α-ScTx does not inhibit inactivation of the SNS Na⁺ ion channel suggesting that the α-ScTx binding site is not conserved between the brain and SNS Na⁺ ion channels. Thus a similar high throughput plate-based assay using α-ScTx cannot be easily developed to assay the activity of the wild-type SNS Na⁺ ion channel. This presents a barrier to the rapid HTS identification of SNS Na⁺ ion channel inhibitory compounds that would potentially be effective analgesic and anti-hypersensitivity drugs.

In U.S.5,858,713 there is disclosed a voltage-sensitive sodium ion channel having a mutation that renders it permeable to calcium. The purpose of the mutation is to allow detection of calcium flow with readily available calcium
sensitive dyes. There is no teaching to mutate an ion channel to make it sensitive to a gating agent. Furthermore, it is believed that the mutation of '713 is introduced into the pore itself which brings with it the risk that the ion flow detected is not an accurate representation of the in vivo situation with the unmodified ion channel.

All references referred to throughout this specification are expressly and entirely incorporated herein by reference.

It is an object of the present invention to provide altered ion channel proteins that may be used in identifying modulators, particularly antagonists, of the unaltered ion channel protein. Such modulators may be used in the preparation of pharmaceutical compositions for the treatment of a range of diseases or disorders, such as those mentioned supra, e.g. pain and hypersensitivity.

In accordance with the present invention there is provided an altered ion channel protein (e.g. altered voltage sensitive sodium ion channel) having acquired sensitivity to a gating agent. The terms "ion channel protein" and "ion channel" are used interchangeably, unless the context suggests otherwise.

The term "altered ion channel protein" or "altered ion channel" is intended to be understood in a structural sense as opposed to implying that for any given example, a particular process has necessarily been followed in its production. For example, an ion channel protein produced by expression of a mutagenic oligonucleotide of the invention which in turn has been produced by e.g. PCR driven amplification of an ancestry mutagenic oligonucleotide, is an altered ion channel protein according to the present invention, even though the oligonucleotide it is directly produced from has not in itself undergone alteration in a process sense.

By the term "acquired sensitivity" we mean that the altered ion channel protein has substantially increased sensitivity to a gating agent compared to the unaltered ion channel protein. The increased sensitivity is at least two fold, more typically, at least 10 fold, preferably at least 100 fold.
The present invention provides means for conferring on an altered ion channel protein, sensitivity to gating agents in circumstances where the unaltered ion channel protein has no or little sensitivity to such agents or a particular gating agent of interest. This enables investigation of the altered ion channel protein, particularly when expressed in a host cell, and identifying modulators of the unaltered ion channel protein for use in the preparation of pharmaceutical compositions for the treatment of diseases or disorders.

This may be achieved by identifying within the primary amino acid sequence (or the polynucleotide e.g. DNA encoding it) of the unaltered, gating agent insensitive ion channel protein that region equivalent to a gating agent sensitive region in a family member. This may be achieved, at least in part, by e.g. sequence alignment studies.

Once identified, the gating agent insensitive region of the unaltered ion channel protein may be changed to have a primary amino acid sequence corresponding (e.g. identical or similar/conservative change) to the gating agent sensitive region of the family member. Polynucleotides encoding the altered ion channel protein may be generated by site-directed mutagenesis with mutagenic oligonucleotides being employed using a single-stranded (e.g. M13 bacteriophage based techniques) or double stranded template (e.g. Chameleon™ or Quikchange™ – Stratagene™). After verifying each mutant by sequencing, the mutated polynucleotide is excised and inserted into a suitable vector so that the altered ion channel protein can be expressed by a host cell (although mechanically based carriers are not excluded). Where a plurality of contiguous polynucleotide bases of the unaltered ion channel protein are changed (i.e. a cassette mutation), splice overlapping extension PCR may be employed.

Thus in a general scheme, an altered ion channel protein according to the invention may be produced as follows:
(a) identifying the binding site of a gating agent in the primary amino acid
sequence of a gating agent sensitive family member;
(b) aligning the sequence of step (a) with the sequence of the gating agent
insensitive ion channel protein;
(c) comparing the sequence of the gating agent binding region of the sensitive
family region with its corresponding region in the gating agent insensitive ion
channel protein;
(d) producing an oligonucleotide encoding an altered gating agent insensitive ion
channel protein having a gating agent binding region identical or similar to the
gating agent binding region of the gating sensitive member;
(e) incorporating the oligonucleotide of step (d) into a vector;
(f) introducing the vector of step (e) into a host cell;
(g) permitting the cell of step (f) to express said altered ion channel.

It will be apparent that many variations particularly with respect to the strict
order presented here are possible.

Determination of the gating agent binding region can be achieved by a number
of routes known or apparent to those skilled in the art. For example, the binding
region may be well characterised in the literature. In the event that the binding
region is not known or poorly characterised then the skilled person has a number
of options available. As one example, synthesise of a radiolabelled gating agent
and incubation of this with cells expressing said ion channel protein or
alternatively with purified ion channel protein will result in a radiolabelled gating
agent-ion channel protein complex which can be proteolytically digested and the
fragments probed with e.g. antibodies to isolate the ion channel protein fragment -
radiolabelled gating agent complex. Once isolated, the complex can be sequenced
to obtain a general picture of the binding region. Further information is then
available by site-directed mutagenesis (for example alanine scanning site-directed
mutagenesis) of the fragment to identify those bases critical in the binding of the
gating agent. From this a comprehensive picture of the binding region can be
built up.
The term "family" is used herein to indicate a group of proteins which share substantial sequence similarities, either at the level of the primary sequence of the proteins themselves or at the level of the DNA encoding them. The sequence similarities may extend over the entire protein/gene or may be limited to particular regions or domains. Similarities may be based on nucleotide/ amino acid sequence identity as well as similarity (for example, those skilled in the art recognise certain amino acids as similar and identify differences based on switches of similar amino acids as conservative changes). Some members of a protein family may be related in the sense that they share a common evolutionary ancestry. The members of a protein family do not necessarily share the same biochemical properties or functions though their similarities are usually reflected in common functional features. The criteria by which protein families are recognised are well known in the art and include computer analysis of large collections of sequences at the level of DNA and protein as well as biochemical techniques such as hybridisation analysis and enzymatic assays (see for example Pearson and Lipman (1988) PNAS USA, 85:2444). The term "family" embraces "homologue".

The term "homologue" is used herein in two distinct senses. It is used sensu stricto to define the corresponding protein from a different organism (i.e. a species variant or orthologue) in which case there is a direct evolutionary relationship between the protein and its homologue. The term is also used sensu lacto to define a protein which is structurally similar to a given ion channel protein. In this sense, homology is recognised on the basis of purely structural criteria by the presence of amino acid identities and/or conservative amino acid changes (as set forth by Dayhoff et al, Atlas of protein structure, vol. 5, National Biomed Dd’n, Washington D.C., 1979).

Preferably, the unaltered ion channel protein has at least 35% identity with the gating agent sensitive family member or homologue. However preferably the ion channel protein has at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, more preferably, at least 90%, 95%, 96%, 97%, 98% or even 99% identity.

The present invention is of particular use in conferring gating agent sensitivity on fast voltage gated ion channel proteins which are insensitive or otherwise
refractory to gating agents. Preferred examples are sodium ion channel proteins such as the sensory neurone specific (SNS) family as disclosed in WO 99/47670 (SNS2) WO 97/01577 (here termed SNS1) and purportedly so in Jeong et al (2000; ref. 48). The reader is specifically referred to each reference. Particularly preferred is SNS1 as disclosed in WO 97/01577 which is insensitive to α-scorpion toxin. Thus in accordance with the present invention there is provided an altered sodium ion channel protein, particularly SNS1 ion channel protein having acquired sensitivity to a gating agent, particularly α-scorpion toxin. The altered SNS ion channel protein comprises a region corresponding to (e.g. identical or similar) to a sodium ion channel protein family member that has sensitivity to α-scorpion toxin such as the brain I, II or III sodium ion channel protein. The alteration takes place in the SNS α subunit.

α-scorpion toxin sensitive and insensitive family members are shown in table 1 below.

Table 1.

<table>
<thead>
<tr>
<th>α-ScTx sensitive voltage-gated sodium channels</th>
<th>α-ScTx insensitive voltage-gated sodium channels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain type I (Na\textsubscript{1.1})</td>
<td>SNS/PN3 (Na\textsubscript{1.8})</td>
</tr>
<tr>
<td>Brain type II (Na\textsubscript{1.2})</td>
<td>SNS2/NaN (Na\textsubscript{1.9})*</td>
</tr>
<tr>
<td>Brain type III (Na\textsubscript{1.3})</td>
<td></td>
</tr>
<tr>
<td>Brain type VI (Na\textsubscript{1.6})</td>
<td></td>
</tr>
<tr>
<td>hNE-Na/PN1 (Na\textsubscript{1.7})*</td>
<td></td>
</tr>
<tr>
<td>SkM1 (Na\textsubscript{1.4})*</td>
<td></td>
</tr>
<tr>
<td>H1/SkM2 (Na\textsubscript{1.5})*</td>
<td></td>
</tr>
</tbody>
</table>

In accordance with the present invention there is provided an altered rat SNS1 ion channel protein wherein positions 1554 to 1569 inclusively are identical to or similar to positions 1607 to 1620 inclusively in rat brain II sodium ion channel protein so as to confer gating agent sensitivity on said altered rat SNS1 ion
channel protein to α-scorpion toxin. Host cells expressing said altered rat SNS1 ion channel protein as hereinbefore described are also provided.

In accordance with the present invention there is provided an altered human SNS1 sodium ion channel protein having gating agent sensitivity particularly to scorpion toxin. Positions 1552 to 1566 inclusively of said altered human SNS1 sodium ion channel protein comprises a plurality of contiguous bases derived from a gating agent sensitive region of a gating agent sensitive family member such as a brain sodium ion channel (e.g. I, II or III). The term “gating agent sensitive region” meaning that region responsible for conferring gating agent sensitivity on the gating agent sensitive family member.

In accordance with the present invention there is provided an altered mouse SNS1 sodium ion channel protein having gating agent sensitivity particularly to α-scorpion toxin. Positions 1553 to 1567 inclusively of said altered mouse SNS1 sodium ion channel protein comprises a plurality of contiguous bases derived from a gating agent sensitive region of a gating agent sensitive family member such as a brain sodium ion channel.

In accordance with the present invention there is provided an altered rat SNS1 sodium ion channel protein having gating agent sensitivity particularly to scorpion toxin. Positions 1554 to 1569 inclusively of said altered rat SNS1 sodium ion channel protein comprises a plurality of contiguous bases derived from a gating agent sensitive region of a gating agent sensitive family member such as a brain sodium ion channel or similar thereto.

The term “derived from” is intended to define not only a source in the sense of it being the physical origin for the material but also to define material which has structural and/or functional characteristics which correspond to those of material which does originate from the source. Thus a “plurality of contiguous bases derived from a gating agent sensitive region” need not necessarily have been purified from the gating agent sensitive family member.
It is preferred that the unaltered, gating agent insensitive ion channel protein is mammalian, more preferably rat (protein is SEQ.I.D.NO:1, DNA is SEQ.I.D.NO:2), mouse (protein is SEQ.I.D.NO:3, DNA is SEQ.I.D.NO:4) or human (protein is SEQ.I.D.NO:5, DNA is SEQ.I.D.NO:6) of which human is most preferred. Particularly preferred is a human sodium ion channel protein such as SNS1 or SNS2. The alteration to the gating agent insensitive amino acid region of the unaltered ion channel protein may take the form of a single base change or a plurality of base changes (which plurality of bases maybe contiguous). Indeed, a single amino acid change may in some circumstances be insufficient for conferring gating agent sensitivity on the altered ion channel protein. For example when the primary amino acid sequences of the D-IVS3 to D-IVS4 region of voltage-gated Na\(^+\) channel family members are aligned (Fig. 1), the acidic glutamic acid residue at 1613 (brain IIa) is conserved across the rat brain channels whereas in the SNS channels it is neutral (rat SNS) or basic (rat SNS2). The glutamic acid residue at 1616 (brain IIa) is also highly conserved across the brain channels, whereas in rat SNS it is basic. The present inventors hypothesised that increased affinity \(\alpha\)-ScTx binding to a recombinantly expressed altered SNS1 channel with a \(\alpha\)-ScTx binding site similar to that of the brain Na\(^+\) channels would allow the channel to be gated in a manner similar to the brain Na\(^+\) channels in a \(\alpha\)-ScTx-FLIPR assay. This would afford a previously unavailable opportunity to develop a high throughput screen for SNS channel blockers.

Three altered rat SNS ion channel proteins were constructed in an attempt to confer an \(\alpha\)-ScTx binding site with affinity equivalent to that seen in the brain IIa Na\(^+\) channel protein. The present inventors reasoned that if the high binding affinity of the brain IIa Na\(^+\) channel for ScTx was attributable to non-identical amino acids of the IVS3-S4 loop together with neighbouring amino acids in IVS3 segment (49), then the most likely candidates for changes in the rat SNS gene product were the amino acids at position 1560 and/or 1563. However, if the tertiary amino acid context of these residues was also important, then the entire non-identical region from amino acid 1554 to 1569 in rSNS would need to be changed so as to correspond to (e.g. identical or similar) with amino acids 1607 to
that form the conserved α-ScTx binding site in the brain Na\(^+\) channels. The three altered SNS ion channel proteins were tested for α-ScTx sensitivity by whole cell patch-clamp electrophysiology. Only the altered ion channel protein with the entire putative α-ScTx binding site substituted from amino acid 1553 to 1569 inhibited inactivation in the presence of α-ScTx. This altered ion channel enables the development of a plated-based assay for the SNS Na\(^+\) channel protein and potentially other α-ScTx insensitive Na\(^+\) channel proteins. Thus those skilled in the art are taught that a single base change in the unaltered gating agent insensitive ion channel protein in a position equivalent to a single base in a family member that is gating agent sensitive may not be sufficient to confer acquired sensitivity on the altered ion channel protein, even if the change corresponds to the single base of the family member. In such circumstances, one should consider a plurality of changes such as a cassette mutation in a polynucleotide sequence encoding the unaltered ion channel protein.

The alteration is achieved through the provision of polynucleotide(s) encoding the altered ion channel protein which is then inserted into a suitable vector as outlined above. It will be apparent to those skilled in the art that due to the degeneracy of the genetic code, many variations of the polynucleotide are possible. Such changes may be by way of addition, substitution, deletion, insertion of one or more polynucleotide bases.

The term “region” includes a single base or a plurality of bases, which plurality may or may not be contiguous. It is preferred that the number of alterations introduced into the primary amino acid sequence to confer acquired gating agent sensitivity is kept to a minimum, i.e. as few changes as are necessary. This ensures that the resulting functional character of the altered ion channel protein reflects as far as possible the unaltered ion channel protein.

Suitable vectors may be a plasmid, phage, minichromosome or transposon of which plasmid is particularly preferred. The vector may include other regulatory elements such as a promoter, operator, activator, repressor and/or enhancer, transcription, translation, initiation and termination sequences. They may also contain sequence encoding any of various tags (e.g. to facilitate subsequent
purification of the expressed protein such as affinity (e.g. His) tags. Where the altered ion channel protein is a sodium ion channel protein, particularly SNS1, vectors of the present invention may also comprise polynucleotide sequences encoding the unaltered β subunit of the sodium channel protein. Thus the present invention specifically contemplates expression of an altered α subunit of a sodium ion channel particularly SNS1, either alone or together with one or more β sodium ion channel subunit(s) to form an altered sodium ion channel protein.

Particularly preferred are vectors which comprise a regulatory element or elements operably linked to the mutagenic polynucleotide (e.g. DNA) of the present invention to provide expression thereof at suitable levels (e.g. at a level which facilitates its use in drug screening assays).

Any of a wide variety of regulatory elements may be used and may be regulatable for example being inducible. The term “operably linked” refers to a condition in which portions of a linear polynucleotide sequence are capable of influencing the activity of other portions of the same linear sequence, for example, a ribosome binding site is operably linked to a coding sequence if it positioned so as to permit translation. The vector may be inserted into the host cell according to any conventional method. It is preferred that the altered sodium ion channel has acquired sensitivity to α-scorpion toxin although the acquired sensitivity may be towards one or more of the following gating agents; Veratridine, batrachotoxinin, aconitine, grayanotoxin, sea anemone, β scorpion toxin, brevetoxin, ciguatoxin, deltamethrin or cypermethrin.

It is preferred that the altered ion channel protein is amenable to ion flow by its natural ion to better reflect the in vivo situation as opposed to the approach taken in US 5,858,713.

Host cells harbouring the vectors of the present invention (and hence expressing the altered ion channel proteins) are preferably eukaryotic, more preferably mammalian, most preferably human, e.g. a human cell such as a HEK 293 cell. Also preferred are mammalian cell lines such as neuroblastoma. For screening purposes it is preferred to use a human cell line so as to best represent the in vivo
situation. Vectors of the present invention maybe inserted into a host cell by way of transfection according to techniques well known or apparent to those skilled in the art. Liposomes and other mechanical carriers are not excluded.

Host cells expressing the altered ion channel protein may then be used in assays for identifying modulators of the unaltered ion channel protein. The modulator may be an agonist, antagonist or mimetic. Preferably the modulator is an antagonist.

By altering the ion channel protein so as to render it sensitive to a gating agent, those skilled in the art will recognise that in drug screening assays, particularly for an antagonist, the ability of a candidate agent to modify activation of the altered ion channel protein when in the presence of the gating agent is indicative of a potential modulator of the unaltered ion channel protein in vivo. Such candidate agents may be identified and further selected and/or modified (i.e. developed) to produce a modulator of the unaltered ion channel protein in vivo (particularly for use in treating a mammalian patient such as a human).

Further selection and/or modification criteria may include one or more of the following considerations; Potency as a ion channel protein modulator, selectivity (towards the target unaltered ion channel protein), pharmacokinetics (e.g. ADME in animal models), in vitro electrophysiology characteristics (e.g. on dorsal sensory neurones or spinal nerve preparations), in vivo activity in appropriate animal models of disease (e.g. chronic constriction injury, model of neuropathic pain, carageenan model of inflammatory pain), chemical synthesis, stability and formulation characteristics of the candidate agent, safety and toxicology (acute and chronic) studies in animals. Development may then proceed to proof of concept studies in man before clinical trials begin. It is particularly preferred that where the goal is to develop a modulator of SNS1 or SNS2 ion channel proteins, a candidate modulator which is capable of modulating the altered SNS ion channel protein in vitro does not also modulate activation of the unaltered cardiac sodium ion channel protein in vitro is selected or vice-versa. The aim of which is to minimise potential side effects in vivo as a result of unwanted or inappropriate
interaction of a SNS ion channel protein modulator with the cardiac sodium ion channel and vice-versa.

Thus in accordance with the present invention there is provided a method of identifying an ion channel protein modulator, particularly an antagonist of ion channel protein activation, which ion channel protein (which is preferably a sodium ion channel, such as SNS1 or SNS2) is insensitive or otherwise refractory to known gating agents in vitro which method comprises the steps of;

(a) providing a mutagenic oligonucleotide that encodes an altered ion channel protein having a region identical or similar to a region responsible for conferring gating agent sensitivity on a gating agent sensitive family member of said unaltered ion channel protein such that said altered ion channel protein has acquired sensitivity to said gating agent;

(b) contacting said altered ion channel protein with said candidate modulator in the presence of a gating agent (for example by expressing said altered ion channel protein in a host cell then incubating said host cell with said candidate modulator in the presence of said gating agent);

(c) identifying a candidate ion channel modulator which modifies activation of said altered ion channel protein by said gating agent (for example by measuring activation of said altered ion channel protein using membrane potential imaging techniques by said gating agent in both the presence and absence of said candidate ion channel modulator);

(d) and optionally synthesising and/or purifying said ion channel modulator of step (c).

Illustrative of commercially available assays that may be employed in identifying a candidate ion channel modulators include Voltage Imaging Plate Reading (VIPR, Aurora Biosciences Corporation), Fluorometric Imaging Plate Reading (FLIPR, Molecular Devices) or the method involving bioluminescence resonance energy transfer (BRET) as disclosed in WO 99/66324 and to which the reader is specifically referred.

Ion channel protein modulators identified by (or identifiably by) said method are also provided. Medicaments, typically pharmaceutical compositions, comprising
said modulator are also provided. Such pharmaceutical compositions generally
comprise a therapeutically effective amount of the modulator, usually in unit
dosage form together with a pharmaceutically acceptable carrier, excipient and the
like as known and called for by accepted pharmaceutical practice. Use of a
modulator in the manufacture of a medicament for the treatment of pain and/or a
hypersensitivity disease or disorder and methods of treating the same are
provided.

In accordance with the present invention there is also provided an altered
mammalian cardiac sodium ion channel protein having acquired sensitivity to a
gating agent.

In accordance with a further aspect of the present invention there is provided a
polynucleotide capable of encoding an altered ion channel protein as hereinbefore
described. Also provided are polynucleotides capable of hybridising to said
encoding polynucleotides. Preferably such hybridisable polynucleotides hybridise
under stringent conditions according to methods well known to those skilled in the
art. The exact conditions employed are a matter of choice for the skilled person
but stringency may be manipulated by varying temperature and salt conditions.
Further guidance may be sought from standard textbooks in the art such as
Sambrook J et al, Molecular Cloning: A laboratory manual, 2nd Edtn, Cold Spring
Harbour Labs.

Exemplification

In the figures:

**Figure 1:** Alignment of SNS, brain, cardiac and neuroendocrine sodium channels
across the conserved α-ScTx binding site of the brain channels between domains
IVS3 and IVS4. The two conserved amino acids are underlined while the
extracellular end of the IVS3 domain is indicated (/).
Figure 2: Alignment showing the amino acid sequence substituted into rSNS1 from brain II (in bold) to generate the rSNS-bII mutant (SNS mutant 1). The two conserved amino acids are underlined while the extracellular end of the IVS3 domain is indicated (/).

Figure 3: DNA alignments showing the single base pair changes made to generate the single and the double amino acid substitutions (SNS mutants 2 and 3 respectively).

Figure 6: Analysis of α-ScTx sensitivity of wild type rSNS1, human brain IIA and the three novel rSNS1 mutants by patch-clamp electrophysiology.

Figure 7: Analysis of α-ScTx sensitive SNS mutant 1 in a FLIPR DiBAC assay.

These Examples are intended to illustrate the invention and not to limit the scope of the appended claims.

Cloning of rat SNS into mammalian expression vector pCIN5:

The rat SNS cDNA (rSNS1) was cloned by screening a rat DRG lambda ZAP express cDNA library (constructed by GW; Clontech vector) using a colony hybridisation protocol, and by RT-PCR performed on rat DRG total RNA. This resulted in 7 fragments spanning the coding region which were then assembled in the vector, pBluescript SK⁺ (Stratagene). This plasmid was then modified by site directed mutagenesis to correct a number of PCR derived mutations and to insert a deleted nucleotide. An upstream ATG was removed before the rSNS1 coding region was engineered into the mammalian expression vector pBK-CMV (Stratagene) using NotI and KpnI restriction sites, to give pBK-CMV-rSNS1 (Fig. 4a).

The rat SNS1 coding region was then subcloned into a pBR322-based shuttle vector pBRLT7 using the NotI and KpnI enzyme restriction sites to give pBRLT7-rSNS1 (Fig. 4b). The NotI and MluI sites were then used to transfer the coding region into an IRES mammalian expression vector, pCIN5 (GW) which was
digested with NotI and Ascl (MluI compatible) restriction enzymes, to give pCIN5-rSNS1 (Fig. 4c).

**Constructing the rSNS1-rBII chimeric α-ScTx binding domain in the rSNS cDNA by splice overlap extension PCR:**

98mer reverse-complementing oligonucleotide primers NC1
(5’CGACTTCATAGTGATCCATTGTGGAAATGTGTTTCTCGCG
AGCTGATAGAGAAGTAGTTCTCGTCCCGACACTTCTTCCGGGTCATCC
GTC3’) (SEQ.ID.NO:7) and NC2
(5’GACGGATGACCGGAGAGTGCTCGGGGACACGAAATACTTCTTCTAT
CAGCTCCCGGAGAAACATTTCTACTAAATGGACAGGATCACCACATGAA
GTGC3’) (SEQ.ID.NO:8)

were designed as a fusion of the rat SNS1 and rat brain II sodium channel (underlined) sequences. PCR reactions were carried out on the rat SNS template using primer pairs NC1-SNS2 (TCACTGAGGTCCAGGGCTTTCCCTTC) (SEQ.ID.NO:9) and NC2-SNS17 (CCTCATCCTTCTTGTGACTGT) (SEQ.ID.NO:10), and the eLongase amplification system (Life Technologies) according to manufacturer’s instructions. The two PCR fragments were mixed and joined by further PCR using the primer pair SNS2-SNS17. This mutant PCR fragment, designated mutant 1 (Fig.2 shows relevant amino acid changes), was then digested with Xhol and Kpni restriction enzymes, and replaced the Xhol-Kpni wild type SNS1 fragment of pBK-CMV-rSNS1 (Fig. 4a). The entire mutant coding region (rSNS1-BII) was excised by HindIII and Kpml restriction enzymes, and inserted into the BstXI-NsiI restriction sites of the IRES mammalian expression vector, pCIN5-PL (GW) by blunt end ligation. The resulting construct was designated pCIN5-rSNS1-rBII and the encoded mutant protein designated rSNS1 mutant 1.
Constructing the point mutations in the rat SNS cDNA by site directed mutagenesis:

The rat SNS1 coding region was sub-cloned into the plasmid pBK-CMV (Stratagene) as described previously, to give pBK-CMV-rSNS1 (Fig.4a). Complementary oligonucleotide primer pairs were designed which incorporated the required single and double nucleotide mutations respectively.

The sequence of the primers for mutant 2 were:
SCTX1 5' GAGTCTGCTGTTTTCTGAAATCCTAAGTCACTGG 3'
(SEQ.I.D.NO:11)
SCTX2 5' CCAGTGACTTAAGGATTTCAGAAACAGCAGACTC 3';
(SEQ.I.D.NO:12)

and for mutant 3 were:
SCTX3 5'
GAGTCTGCTGTTTTCTGAAATCCTGAGTCACTGGAAAACCTAC 3'
(SEQ.I.D.NO:13)
SCTX4 5'
GTAGTCTTCCAGTGACTCAAGGATTTCAGAAACAGCAGACTC 3'
(SEQ.I.D.NO:14)

The mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). For each mutagenesis reaction 50ng of pBK-CMV-rSNS1 was combined with 125ng of each complementary primer, 1µl of dNTP (25mM of each NTP), 5µl of reaction buffer, ddH₂O to a final volume of 50µl and 1µl of Pfu DNA polymerase (2.5U/µl).

Temperature cycling was performed on a PTC-200 DNA engine thermal cycler (GRI) using the following protocol:

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55°C</td>
<td>1 minute</td>
</tr>
</tbody>
</table>
Following temperature cycling, 1µl of DpnI restriction enzyme (10U/µl) was added to each amplification reaction and incubated in a water bath at 37°C for 1 hour, then 1µl of each reaction was transformed into 50µl E. coli XL2-Blue supercompetent cells.

The transformed bacterial cell suspension plus 450µl of NZY+ broth was pre-incubated for 1 hour at 28°C with shaking at 225rpm, before the entire volume was plated out onto LB agar plates supplemented with 50µg/ml of ampicillin. The plates were then incubated for 18 hours at 28°C. Plasmid DNA was purified from overnight cultures of single colonies picked from these plates using the miniprep spin column protocol (Qiagen). The mutations, designated mutant 2 and 3 for the single and double mutations respectively (Fig.3), were then verified by sequencing the plasmid DNA.

**Analysis of the α-ScTx sensitivity of the novel rSNS1 mutants.**

Mammalian cells (either HEK293, A, C, D & E or CHO, B) were stably (A, B & C) or transiently (D & E) transfected with expression constructs (Figures 4 & 5) for the wild-type or mutant sodium ion channel α-subunits indicated. Whole-cell patch clamp recordings were made from these cells using standard methods (50). Briefly, cells were grown on a glass coverslip, placed into a recording chamber (0.5 ml volume) and superfused with an extracellular recording solution at 2 ml min⁻¹. The extracellular recording solution contained (in mM): 140 NaCl, 4.7 KCl, 1.2 MgCl₂, 1 CaCl₂, 11 glucose, 5 HEPES (pH 7.4). Patch electrodes had resistances of 2 to 6 MΩ when filled and the pipette-filling solution contained (in mM): 120 CsF, 15 NaCl, 10 Cs-EGTA (ethylene glycol-bis(β-aminoethyl ester) N,N',N'-tetra acetic acid Cs salt), 10 HEPES. Currents were recorded at room temperature using an Axopatch 200A amplifier (Axon Instruments Inc.). Currents were elicited by a step depolarisation to +20 mV (A), -10 mV (B) or 0 mV (C, D & E) from a holding potential of -90 mV, which in each case yielded the peak Na⁺ current.
in the representative cells. Current traces were initially recorded prior to the addition of α-ScTx (control) after which 10μg/ml α-ScTx (from Leiurus quinquestriatus from North Africa, Sigma-Aldrich Co. Ltd.) was washed on and the subsequent current traces recorded using the same command protocol as for the control trace (figure 6).

The effect of α-ScTx on the inactivation kinetics of each α-subunit is visible in the representative current traces shown in Figure 6. The inactivation kinetics of wild-type SNS (fig. 6A), mutant 2 (fig. 6D) and mutant 3 (fig. 6E) were unaffected by the addition of 10μg/ml α-ScTx, thus indicating that the substitution of conserved amino acids in the α-ScTx binding site alone is insufficient to confer α-ScTx-sensitivity on the rSNS1 ion channel. However, addition of α-ScTx to both the brain II (fig. 6B) and SNS mutant 1 (fig. 6C) gave pronounced inhibition of fast inactivation. These results indicate that substitution of the entire region of rSNS from amino acids 1554 to 1569 with the amino acids that form the conserved α-ScTx binding site in the brain Na⁺ ion channels is necessary to confer α-ScTx-sensitivity on the SNS ion channel.

Assay of rSNS1 activity in a plate-based assay using rSNS1 mutant 1.

The generation of an SNS mutant that is sensitive to α-ScTx (rSNS1 mutant 1) has allowed the development of a plate-based assay similar to those that have been developed for the Na⁺ channels (46). Addition of α-ScTx to cells expressing the SNS mutant 1 ion channel, that have been pre-loaded with the fluorescent dye, DiBAC(4)3, will cause a depolarisation that can be detected as a change in the fluorescence on FLIPR (46, 47). Representative data from plate based assays carried out on a Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices Ltd.) is shown in Figure 7.

The expression plasmids pCIN5-rSNS1wt and pCIN5-rSNS1-mutant1 were transfected into HEK293 cells by electroporation and stable HEK293 clonal cell lines were isolated following selection in 800μg/ml Geneticin G418 (Life Technologies). Two cell lines that functionally expressed the SNS proteins were identified by electrophysiology (one expressing wild type rSNS1 and one expressing the α-ScTx-sensitive rSNS1 mutant 1) and these
cell lines were used for the FLIPR assays described here. The two cell lines were plated in black, clear bottomed 96 well assay plates (Costar®, Corning Inc.) at a seeding density of $5 \times 10^4$ cells per well and incubated for 48 hours prior to assay (37°C). Native HEK293 cells were also plated as a negative control.

The cells were loaded with the membrane potential sensitive dye, DiBAC(4(3)) 5µM, Molecular Probes) either in the presence or absence of 30µg/ml α-ScTx in low sodium buffer (5mM NaCl, 140mM tetramethyl ammonium-Cl, 5mM KCl, 2mM CaCl$_2$, 0.8mM HEPES, 10mM D-glucose, pH7.4, plus 0.5mM Brilliant Black dye; 100µl per well) at 37°C for 30 minutes. The plates were transferred to the FLIPR and the cell-loaded DiBAC fluorescence (excited at 488nm) assayed during and following addition of 100µl of sodium add-back buffer (145mM NaCl, 5mM KCl, 2mM CaCl$_2$, 0.8mM HEPES, 10mM D-glucose, pH7.4, plus 0.5mM Brilliant Black dye) to each well. An increase in the fluorescence of the membrane potential sensitive dye corresponds to the membrane depolarisation caused by the sodium current passing through the channel (if it has been gated by the α-ScTx) (46, 47).

Figures 7A and 7B show the representative traces of changes in fluorescence in control HEK293, HEK293 rSNS1 wild type (wt) and HEK293 rSNS1 mutant 1 ion channel cells following 30 minutes incubation in the absence (A) or presence (B) of 30µg/ml α-ScTx. Similar responses as those in the representative traces were obtained in duplicate wells. In the absence of α-ScTx preincubation, only a small depolarisation occurs in each of the 3 cell types upon addition of sodium. This depolarisation does not correspond to SNS ion channel expression since it occurs in the native HEK293. However, on addition of sodium following preincubation with 30µg/ml α-ScTx the HEK293 rSNS1 mutant 1 (the α-ScTx-sensitive rSNS1 mutant) gives a significant increase in fluorescence that corresponds to membrane depolarisation indicating that the α-ScTx has gated the mutant ion channel to allowing sodium to flow through. The wild type rSNS1 and native HEK cells fail to give this increase in fluorescence indicating that the α-ScTx only gates the ScTx-sensitive mutant ion channel. Thus mutating of the rSNS1 ion
channel in order to change its sensitivity to a gating agent (in this case \(\alpha\)-ScTx) has successfully enabled analysis of the channel's activity in a plate-based assay.

In order to determine whether this plate-based assay could be used to identify inhibitors of the ion channel's activity, we preincubated the rSNS1 mutant 1 cells with 30\(\mu\)g/ml \(\alpha\)-ScTx plus or minus a sodium ion channel inhibitor, tetracaine (100\(\mu\)M). Figure 7C, shows that the ion channel inhibitor prevents the change in DiBAC(4)3 fluorescence that is observed in the absence of inhibitor (control). This demonstrates that rSNS1 mutant 1 can be used to screen for compounds/agents that inhibit the ion channel which could be potentially therapeutic. This plate-based assay has the advantage that it could be adapted to high throughput, thus enabling the rapid analysis of many compounds/agents for SNS inhibitor activity.
References:


Claims

1. An altered ion channel protein having acquired sensitivity to a gating agent.
2. A protein according to claim 1 which is a voltage sensitive sodium ion channel.
3. A protein according to claim 2 wherein the sodium ion channel is a sensory neurone specific (SNS) ion channel.
4. A protein according to claim 3 wherein the SNS is SNS1 or SNS2.
5. A protein according to claim 4 wherein the SNS is SNS1.
6. A protein according to claim 3, 4 or 5 wherein the gating agent is α-scorpion toxin.
7. A protein according to claim 2 wherein the gating agent is selected from the group consisting of;
   Veratridine, batrachotoxin, aconitine, grayanotoxin, sea anemone, β-scorpion toxin, brevetoxin, ciguatoxin, deltamethrin, cypermethrin.
8. A protein according to any preceding claim wherein the protein is mammalian.
9. A protein according to claim 8 wherein the protein is rat, mouse or human.
10. A protein according to claim 9 wherein the protein is human.
11. A host cell expressing the protein according to any preceding claim.
12. A cell according to claim 11 which is eukaryotic.
13. A cell according to claim 12 which is mammalian.
14. A cell according to claim 13 which is human.
15. A cell according to claim 14 which is HEK293 or a neuroblastoma.
16. A polynucleotide capable of encoding a protein according to any one of claims 1 to 10.
17. A vector comprising a polynucleotide of claim 16.
18. Use of a protein according to any one of claims 1 to 10 in a screening assay for the identification of modulators capable of modulating an unaltered gating agent insensitive ion channel protein.
19. Use of a modulator identified by the assay of claim 18 in the manufacture of a medicament for the treatment of pain, inflammation or hyper-sensitivity.
20. A method of treating a mammalian patient, particularly human comprising the step of administrating a therapeutically effective amount of a modulator identified by the assay of claim 18.

21. A method for the production of an altered ion channel protein having acquired sensitivity to a gating agent which method comprises the steps of:
(a) identifying the binding site of a gating agent in the primary amino acid sequence of a gating agent sensitive family member;
(b) aligning the sequence of step (a) with the sequence of the gating agent insensitive ion channel;
(c) comparing the sequence of the gating agent binding region of the sensitive family region with its corresponding region in the gating agent insensitive ion channel;
(d) producing an oligonucleotide encoding an altered gating agent insensitive ion channel having a gating agent binding region identical or similar to the gating agent binding region of the gating sensitive member;
(e) incorporating the oligonucleotide of step (d) into a vector;
(f) introducing the vector of step (e) into a host cell;
(g) causing the cell of step (f) to express said altered ion channel.

22. A method of identifying a modulator of a ion channel protein which has no or little sensitivity to a gating agent which method comprises the steps of:
(a) producing a host cell expressing an altered ion channel protein having acquired sensitivity to said gating agent;
(b) contacting said ion protein of step (a) with a candidate agent;
(c) measuring ion channel protein activation in the presence of said agent;
(d) selecting said agents which modify the activation of step (c);
(e) optionally synthesising and/or purifying said agent of step (d);
(f) optionally chemically modifying said agent of step (e) to improve one or more of the following features: selectivity, pharmacokinetics, potency, toxicological profile, stability; formulation characteristics.

23. A method of claim 22 wherein the modulator is an antagonist.

24. An altered rat SNS1 ion channel protein wherein positions 1554 to 1569 inclusively are identical to or similar to positions 1607 to 1620 inclusively in
rat brain II sodium ion channel so as to confer gating sensitivity on said altered rat SNS1 ion channel protein to α-scorpion toxin.

25. An altered mouse SNS1 sodium ion channel protein having gating agent sensitivity particularly to scorpion toxin wherein positions 1553 to 1567 inclusively of said altered mouse SNS1 sodium ion channel protein comprises a plurality of contiguous bases derived from a gating agent sensitive region of a gating agent sensitive family member such as a brain sodium ion channel or similar thereto.

26. An altered human SNS1 sodium ion channel protein having gating agent sensitivity particularly to scorpion toxin wherein positions 1552 to 1566 inclusively of said altered human SNS1 sodium ion channel protein comprises a plurality of contiguous bases derived from a gating agent sensitive region of a gating agent sensitive family member such as a brain sodium ion channel (e.g. I, II or III) or similar thereto.
Figure 1.

rSNS1  L S I G S L L F S A I L /K S L E N Y F S P T L F /R V I  
(bold 1554-1569, unde rlined 1560, 1563)

(bold 1607-1620; underlined 1613, 1616)


hNeNa  L S I V G M F L A D L I /E . . T Y F V S P T L F /R V I  

hH1  L S I V G T V L S D I I /Q . . K Y F F S P T L F /R V I  

D-IVS3/  

/D-IVS4

A = alanine/neutral  E = glutamic acid/acidic  
R = arginine/basic  D = aspartic acid/acidic
Figure 2

Mutant 1: (rSNS/rBII amino acids 1554-1569/1607-1620 substitution).

rSNS1  ...L S I G S L L F S A I L /K S L E N Y F S P T L F /R V I ...
rbraII ...L S I V G M F L A E L I /E ... K Y F V S P T L F /R V I ...
Mutant 2: (A1560E)

5' ... GAGTCTGCTGTTTTCTGAAATCCTTAAGTCACTGG... 
     CTCAGACGACAAGAGCTTTAGGAATTTCAGTGACC... 5' 
     S L L F S E I L K S L

Mutant 3: (A1560E/K1563E)

5' ... GAGTCTGCTGTTTTCTGAAATCCTTGAGTCACTGGAAGACTAC... 
     CTCAGACGACAAGAGCTTTAGGAACTCAGTGACCTTTTGATG... 5' 
     S L L F S E I L E S L E N Y
Figure 4a

pBK-CMV-rSNS1
10339 bps

rSNS1 coding
Figure 4b

pBRLT7-rSNS1

9737 bps

'rSNS1 coding
Figure 4c

pCIN5-rSNS1

11105 bps

rSNS1 coding
Figure 5

<Diagram of pCIN5-rSNSBII plasmid showing restriction enzyme sites and other genetic elements.>
Figure 6

A. rSNS1 wt

B. Brain IIA wt

C. rSNS1 mutant 1

D. rSNS1 mutant 2

E. rSNS1 mutant 3
A. No ScTx preincubation

B. 30µg/ml α-ScTx preincubation

C. HEK293 rSNS1 mutant 1 with 30µg/ml α-ScTx preincubation.
VGIIFFTYYIIISFLIVVNMYIAVILENFNVATEESTEPLSEDDFDMFYETWEKFDPEA
TQFIAFSALSDFASTLSPRLRIPKNPMQQNILQMDLPLVPGKIHCLUDIFLFAFTKNVLGE
SGELDSLKTNMEEKFMATNLSKASYEPIATTLTRWKQEDLSATVIQKAYRSYMLHRSLTL
SNTLHVPRAEEDGVSLPGEGVTFMFANSGLPDKSETASATSFPPSYDSVTRGLSDRANINPSSSMQNEDEVAAKEGNSPGPQ
1  CTTCCCCAAG AAGAATGAGA AGATGGAGCT CCCCTTTTGC TCCGTGGGAA
51  CTACCAATTT CAGAGCGGTC ACTCCAGAGT CACTGGCAGA GATCGAGAA
101  CAGATTGCTG CTCACCGCGC AGCCAAAGAG GCCAAAGACC AGCACAGGAG
151  ACAGGAGGAC AAGGGCGAGA AGCCATGGGC TCAGCTGGAC TGAAGAGCCT
201  GTAACCAGCT GCCCAAGTTC TATGTTGAAC TCCAGCGAGA ACTGGTCGGG
251  GACCCCCTTG AAGAAGCAGGA CCATTTTGAC AGCACACACG GCACATTCAT
301  GGTGTTGAAAT AAGGACAGGA CACATTCCAG ATTCAGTGCC ACTGGGGCCC
351  TGAGGCTCTT CAGTCCCTTC AACCCTGATA GAAGAACAGC CATCAAAGTG
401  TCTGTCCTTT CACTTCTCC ATATTCGATCC ACCATCACTA TTTTGGTCAA
451  CTGCGTGTGC ATGACCCGGA AGTATCTGCC AGAGAAAGTGC GAGTAGCCTT
501  TCACCTGTCA TTACACCTTC GAGGCTTGAG TTAAGATAGT GGAAGAGGGG
551  TTTTGTACTA ATGAGTTCCAC TTATTCCTGCA GATCCGGTTGAA ACTGCGTGGG
601  CCTTCACTGTC ATACCTTGCC GTATGTAGG TGCAAGCGATA GACCTCCGAG
651  GAATCTCAGG CCTGCCGGACA TTCCAGATTC TGAGACGGCT GAAAACCTTT
701  TCCTGTATCC CAGGACTGAA GTCACTCGTG GAGGCCCTGA TCCACTCAGT
751  GAGAGAGCTG GCCGACGTGA CTATCCTCAC AGTCTTCTGC TGAGAGCTCT
801  TCAGGCTTGGT GGGCTGACAG CTCTTTAAGG GGAACCTTAA GAACAAATGC
851  ATCAGGAACG GAAACAGATCC CCACAAGGCT GACAAACCTCT CATCTGAAAT
901  GGCAGAAATAC ATCTTTTATCC AGCTTGTGAC TACGGATCCC TTACTTGTCG
951  GCAATGGGTC TGAATGCTGGT CACTGCCCTG GAGGTATGTCT TGCCCTGAAA
1001  ACTCTGACA ACCCCGATTT TAACCTACCC AGCTTGGATT CCTTTCGTTG
1051  GGCAATTCTC TCACACCTTC GCCTACGTAC GCAGGACTGCC TGGGAGCCGC
1101  TGTACCGACA GACACTCGCG GCTTCTGGGA AAATGTACAT GGTCTTTTTC
1151  GTGCTGTTTA TTTTCTTTGGA ATCGTCTTAC GTGGTAATTT TGATCTTGGC
1201  CGTGTCACCC ATGGCGTATG AAGAGCAGAG CCAGGCAACA ATGGCAGAAA
1251  TCGAAGCCCA GAAAAAAGAG TTCCAGGAAG CCGTGAGGT GCTGCAGAG
1301  GAAACAGGAG TGCTGCGGACG CCGTGGGATT GACAGCACCCT CGCTCCAGTC
1351  CCACAGTGGG TCACCTTATAG CCTCCAAAAGC GCAATGAG AGAAGACCCA
1401  GGCTGAAAATC AAGGGTGCTC AGGGCGTCGA GGATGACAA CAGGTCACCC
1451 CAATCTGACC CTGAAACCCA GGGCCGGATG TCTTTCCTAG GCCTGTCCTTC
1501 AGGAAAGACGC AGGGCTAGCC AAGGCGCATGT GTTCCAATCTC CGAGACGCCCA
1551 GCCAAAGCAT CTCATTTTCT GACGGGATCA CGGATGATGG GGTCTTTCAC
1601 GGAAGACCAGG AAAAGGCTAGC AGGTTCATA TTGCTGGGCA GGGTGTGCTTG
1651 GCAGACAGGT CCACTCCCCA GGAGCCTCAG TCTCAGTCCA CCCAACCCTTG
1701 GCCGTAGACA TGGAGAGAACG GGAAGCTCGC GAAGTCCCAC ATGGTACCTT
1751 ACCGCTTGGAG CCGCTGAAAG CCCGCGACTT GACACTACAG GCGAGAAGAG
1801 CTTTCTGCTT GCGGGGTACT TGAAGCAACC TGTCCAGAAG CAAGGAGGCCA
1851 TGAGGCGTTGT CAGTATACGT ACTCTCTGCA TTGGAGGAGCT TGAAGAGTCT
1901 AAGCTGAAGT GCCACCCCTG CTTGACAGAC TTTGCTTGA AGATATCTGT
1951 CTGGGAGTGC TGCCCCAAGT GAGAGAAGTT CAAGATGCGC CTGGTCTGAGC
2001 TGTTGACTGA CCCCTTCGCA GAGCTTACCA TCACCCCTCG CATCCTGTGGG
2051 AACACCCTCT CTATGCGCAT GGAGACTACG CCCATGACCG ATGCGTCTCGA
2101 TGCCATGCTT CAAGGCGGCA ACATGTCTTT CACGTGTTTT TTCACAATGG
2151 AGATGGCCCTT CAAGATCATT GCCTGTGCAC CCTACTATTA CTTCAGAAG
2201 AAGGATGATA TCTTCTGACTG TGTCATCGTC ACCGTTAGCC TCTTGGAGCT
2251 GAGCGCATCC AAGAAGGCGCA GCCGTGTCTGT GCTCGTACCC TCCGCTTTCG
2301 TGCCGGGTCTT CAAGCTGCGCC AAGTCCTCGGC CCACCCCTGA CACCCCTCATC
2351 AAGATCATCG GGAACCTCGT GGGGCGCTCG GGCAACCTGA CTTTATCTCT
2401 GGCATCTACG GTCTTGATCT TGCCCTCTGT GGGAAGACAG CTTCCTCAG
2451 AGGACTACGG GTTCCGCGAAC CAGGGCGTCT CCGTGGGAAA CGGCGGAGAG
2501 CTCCGCTGCG ACATGTGACT CTCTTCCCAT TCTTCCCTGG TCGTCTTTCG
2551 AATCCTCTGC GGGAGTGGA TCGAGAAGAT GTGGGTCTGC ATGGAGGTCA
2601 GCCAAAGAAC CATCTGCCCT ATCCCTCTCT TGACCTGTAG GGTGCTGGGC
2651 AACCTAGTGG TGCTCAACAC CTCTCATGCT TTACTGCTGA ACTCCTTCAG
2701 CCGGGACAAC CTACCGGCTC CAGAGGATGA GGGGAGGTTG AAACACTTGCC
2751 AGTTAAGACT GGCCAGGATC CAGGTACTTG GCCATCGGGCC CAGCAGGGGC
2801 ATGCGCCAGTT ACATCAGCAG CCACTGGCAG TCCGCTGCG CCAAGGTGGA
2851 GACCCAGCTG GCCATGAGAAC CCCACTCAC CAGCTCAGAG GCAAGAACCC
2901 ACATTGGCACC TGATGCTGTC AGTGTCTGCA TGGGGAACTT GCAAAAGCCA
2951 GCTCTCAGTA GCCCCAAGGA GAACCACGGG GACTTCATCA CTGATCCCAA
3001 CGTGTGGGTC TCTGTGCCCA TTGTCGAGGG GGAATCTGAC CTCGACGAGC
3051 TCGAGGAAGA TATGGAGCGG GCTTCGCAGA GCTCCTGGCA GGAAGAGGAC
3101 CCAAGGGGAC AGCAGGAGCA GTTGCCACAA GTCCAAAAGT GTGAAAACCA
3151 CCAGGCAGCC AGAAGCCACG CCTCCATGAT GTCTCCTCGAG GACCTGGCTC
3201 CATACCTGGA TGAGAGCTGG AAGAGGAAGG ATAGCCCTCA GGTCCCTGCC
3251 GAGGGAGTTGG ATGACACAGAG CTCCCTGAG GGCAGACAGG TGGACTGCCC
3301 GGAACCAAGAG GAAATCTCGA GGAAGATCCC CGAGCTGGCA GATGACCTGG
3351 ACGAGGCCGA TGACTGTGTC ACAGAAGGCT GCACCTGCGC CTGTCCTGTC
3401 TGCAACGTGA ATACTAGCAAA GCTGCTCTGG GCCACAGGGCT GGCAGGCTGC
3451 CAAGACCTGC TACCAGCATC TGAGAGACAG CTGGTGTGAG AGTTTCATCA
3501 TCTTCATGAT CCTGCTCACG AGTGAGGAGC TGGCCTTTGA GGTATACTAC
3551 CTGGAAGAGA AAGACCAGGT GAAGCTCGGT CTGGAGTACA CTGACCGAGT
3601 GTTCACCTTC ATCTTCGCTT TTGAGATGCT GCTCAAGTGG GTAGCCCTATG
3651 GCTTCAAAAA GTATTCCACC AATGCGCTGT GCTGGCTGGA CTTTCCTATT
3701 GTGAAACATCT CCTGCAACAG CCTCATAGCG AAGATCCTTG AGTATTTCCA
3751 GGGGCGCTCC ATCAAAGCCC TCTGAAGCTC CCGTGCCTCC CGACCGCTGC
3801 GGGCCTTGTC TGAGATGCGA GGGATAGGAG TAGTGGTGGA TGCCCCTCTG
3851 GGCGGCCATCC CCTCCATCAT GAACGTCCTC CTGGCTCTGCC TCACTCTCTG
3901 GCTCATCTTC AGCATCATGG GCGTAAACCT CTTCGCGCGG AAATTTTCCA
3951 AGTGCCTGCA CACCAGAAT AACCCATTAT TCCACGTAAT CCCAAGCTGA TCGACAGATG
4001 GTGAAATAACA AGTGCTAGY TCACAATCAA AACGCAACCG GCCACTCTTT
4051 CTGGGTCAAC GTGAGATCG AACTCGACCA CGTGCCTATG GGCTACCTCG
4101 CACTTCTTCA GGTGGAACCC TTCAAGGGCT GGAAGACAT AATGTATGCA
4151 GCTGTGTGAT TCCGGAGAGAT CAACAGTCAG CCTAACTGGG AGAACAACCTT
4201 GTACATGCTC CGTACTCTTG TCATTCTCAT CATTTCCGGT GGCTTCTTCA
4251 CGCTGAATCT CTTGGTTTGGG GTGATAACTG ACAACTTCAA CCAACAGAAA
4301 AAAAGCTAG GAGGCCAGGA CATCTCATG ACAGAAGAGC AGAAGAAGTA
4351 CTACATTGCC ATGGAAGAGC TGGGCTCCAA GAAACCCCA GAGCCCATCC
4401 CACGGCCCCT GAATAAGTAC CAAGGCTTCG TGTTTGACAT CGTGACCGAG
4451 CAAGCCTTTG ACATCATCAT CATGGTTTCG ATCTGCCTCA ACATGATC
4501 CAGTATGGTG GAGACCGACG AGCGAGCCGA GGAGGAAGACG AAGGTTCTGG
4551 GCAGAATCAA CCAGTCTTTT GTGGCCGCTT TCACGAGCGA GTGTGTGATG
4601 AAGATGTTGC CCCTGCGACA GTACTAATTC ACCAAGCGGT GGAAACGTGGT
4651 CGACCTCATA GTGGTGATCC TGTCACATTG GAGTTGCTGT TTTTCTGCAG
4701 TCCTTAAGTC ACTGGAANAC TACTTCTCCC GCAGGCCTTT CCAGCTCATC
4751 CGTCTGGCCA GGATCGCAGC CCACCTCAGG CTGATCCGAG CAGCCAAAGG
4801 GATTGCGACG CTGTCCTTTC CCCTCATGAT GCCTCGCCG GCCTCTTCTC
4851 ACATCGGCCG CCTCCCTCTTC CTCGTCATGT TCATCTACTC CATCTCTCCC
4901 ATGGCCAGCT TCGTAAAGG GTGGAGCGAG GGCCGCATCG ACAGCATGTT
4951 CAACCTCAAG ACCTTTGGCA ACAGCATGCT GTGCCTGTTC CAGATCACCA
5001 CTCGCGCGGT CGTGGACGCC CTCTCAGCAG CCTCTCAGCA CCAGGGCCCT
5051 CCCTACTACG GCCCACAACCT GCCAAACAGC AAGGCGTCCC GGGGAACTG
5101 CGGAGACCGC GCCGTTGGCC ATCTCTTTTC CACCACTACC ATCATCATCT
5151 CCTTCTCTCAT GTGGTGCAAC ATGTACATCG CAGTGATTCT GGAGAACCTC
5201 AACGTGGCAG CGGAGGAGGAC CGGAGGGCC CTGAGCGAGG ACAGCTTCGA
5251 CATGTTCTAT GAGACCTTGG AGAAGTTCGA CGCGAGGCC ACCCAGTCTCA
5301 TTGCCTTTTC TGCCCTCTCTCA GACTTGCGGG ACAGCTCTCC CGGCCCTTTT
5351 AGAATCCCA AAACCAACAA GAATATATTA ATCCAGATGG ACCTGCGGT
5401 GGTCCCCGGG GATAAGATCC ACTGCTGGGA CATCCTTTTT GCCCTCACAA
5451 AGAACGTCTT GGGAGAATCC GGGAGGTTTG ACTCCCTGAA GACCAAATAG
5501 GAAAGAGAAGT TTATGGCAGC CAATCTCTCC AAGACATCCT ATGAACCAAT
5551 AGGCCACACC CTCCGGTGGA AGCAGAGAAGA CCTCTGAGGCC ACAGTCATTC
5601 AAAAAAGGCTA CGGAGAGCTA ATGCTGCAAC GCTCCTTTGAC AACTTCAAAC
5651 ACCCTGCAAT TGCCCCAGGCG TGAGGAGGAT GGGGTGTCAC TTCCCCGGGA
5701 AGGCTACGTT ACAAATCCAG CAAAACAGTG AGCTCGCGAC AAATCAAGAAA
5751 CTGCTCTCTGC TACGCTTTTC CGCCCATCCT ATGAACAGTG CACCAGGGGC
5801 CTGAGTGACC CCGCAAACT ATACCCATCT AGCTCAATGC AAAATGAAAG
5851 TGAGGTGCCT GCTAAGGAAG GAACAGCCCC TGGACCTCAG TGAAGGCCACT
5901 CAGGCATGCA CAGGGCAAGGT TCCATATGTCT TTCTCTGCTG TGCTAACTCC
TTCCCTCTGG AGGTGGCACC AACCTCCAGC CTCCACCAAT GCATGTCACT
GGTCATGGTG TCAGAACTGA ATGGGACAT CTTTGAGAAA GCCCCCACCC
CAATAGGAAT CAAAAGCCAA GGATACTCCT CCATTCTGAC GCCCTTTCGG
AGTCCCAAGA AGATGTCATT GCTCCCTTCT GTTTGTGACC AGAGACGTGA
TTCACCAACT TCTCGGAGCC AGAGACACAT ACCAAGACT TTTCTGCTGG
TGTCGGGCAG TCTTAGAGAA GTCACTAGGG GGTGAGCAGT GAGAATTAGG
GTGTCATGC CTGCAATGTC ACAGCTGCCG GACAAATACCT GTGAGTCGTC
CATTTAAATT AATATTTTTA AAGTTAAAAA AAAAAAAAAA AAAAA
MEFPFSGVGTNFRRTPTESLAEIKQIAAHRAAKGRTKQGRQK

eck

DKSEKPRPQLDLKACNQLPFRFYGELPAELVGEPELEDLDYPFSTHRTFILLNKSRTISRFT

SATWALWFSPNFLNLRTAIKTVSVHWSFISITVTILVNCVMTRTDLPKLEYVFVT

YTFREALIKILARGFCLNEFTYLRDPWNLDFSITLAYVGAAVLDLRGISLRTFVRPLRA

LKTVsVIPKLKIVGALVHSVRKLAVTITLTVFCLSFVLFALVGQLFKGNLKNKCIKNGT

DPHKADNLSEMAEDEFIPIGPTTDPLCNGSADAGHCNPYVCQPTPDPNPDFYNTFSDFS

FAWAFSLFLRMLMTDWSWELYQQLRLASGMKMYMFFVLVLIFLGSFYLVLNLILAVVMTAY

EEQSQATAIAEAEKKEKFQEEAEVQLQKEQEVEAALGIDTTSFYSHSGSPLASKNANERR

PRVKSRYSEGSTDNRSPQDSPNYQRMSFLGLSSGRARRASHGVSFHFRAQDQVDVSPD

GILDDGVFHHGDQESPSSSILRLGRAGAQAGPLPRSPLPQSPNPGRKHGKQGQLGMPG

AAGTPEGPALDAAGQKFNLSAGLYNEPFRAQRAMSYSISMTSVEELEESKLCPPLC

SFAQKYLWECCPKKRKFKMVLLELVTDPFALLLTTLTICVNVTFMAMEHYMPMDAFA

MLQAGNIVFTVFMTMEMAKIIAFDYYFYQKWNIFDCVIVTSVLLSASKGSGLSV

LRSLRLRVRKFLAKSWPTLNLKIKIIGNSVAGLNLTFLAIIVFFALVGKQLLENY

GCRRDGVSVWNEGKLRWHMCDFFHFSFLVVFRLCWEIENMWMCMEVSNQYICLTLFLT

VMVLGNNLVLNLFIALLNSFADNLTAPEDEDDGEVNNLQLARIQVGLHRASRAFTSY

IRSHCRRFWPKVETQLGMKPPLTSCKVHIAITADVNAAYGGNTKPALSGPKENHGDIF

TDPNVWVSVPJEAEGISDLDELEDEDEQASQSSWQEEKQPQELLIPQPCQCENHQAARS

PTSGMSSDALPYGERWKRKDPQVPSEAVIDTSSSEGSTVCDPDEEILRKIPALE

DLDEPDCCFTGCECTRCCPKCVNKSPTSDTRQVKRTCYRIEHSVFSFIIFMILLS

SGTLAFEDNYLEEKPVRKVSTLEYTDRVFTFIVFEMLLKKWYAYGFKKYFTNAWCWDLFL

IVNISLTSILAIKILEYSDVASIKALRTLRALRPLRALSERFEGMRVVVDALVGAIPSIMN

VLLVCLIFLWLSFIMGVNLFAFGRSROCVDTRSNPSVSTFVNNKSDCHNQNNTGHFF

WVVNKNVFDNVAMGYYLLQTVATKGWMDIMAAYVDSRDINSQPNWESLAMYLFVF

IIIFGFFTLNLVFVGIIDNPNQKTKKLGQDIFMETEEQKYYNAMKKLGKSKQPQPIS

PLNKYQGFVFIDIVTRQAFDIIMLVDLCNITMMVTDNQSEEKTVKLRGNNQFFVAF

TGECVMKFALRQYYFTNGWNFDIVFIVVILSIALKSLSEYFSPFFVFVRILRA

RIGRLRLRAAKGIRLFFALMMALPFLNIALLFLVMFIYSIFGMSFANVIDAG

IDDMFNFKTFGNSMLCLFQITTASGWGDLSSPINLGPYCPDNRRNSNGSKGNCGSPA
VGILFFTTYIIISFLIVNVNMYIAVILENFNVATEEESTEPLSEDDFDMFYETWEKFDPEA
TQFIAFSALSDFDAFTLSGPLRIPKPNQNILIQMDLPLVPGDHKIHCIDILFAFTKENVLGE
SGELDSLKTNMEEKFMATNLSKASYEPLATTLRCKQEDISATIQKAYRNMLQRLML
SNTLHPRAEEDGVSLPKGGYVTMMANDNGGLPDKSETASATSFPPSYESVTRGLSDRA
NINTSSSMQNEDEVTAKEGNSPGPQ
SEQ.I.D.NO:4

1 AAGAGTGTTAA ATCCCTCCCCCC AGAAGGAATG AGAAGATGGA GTTCCCTTTT
51 GGTTCCGTGG GAATACACCA A TTCAGACGG T TCACTCCAC AGTGCTCGGC
101 AGAGATCAGG AAGCAAGATCG CTGCCCAACG GCGCCGCAAG AAGGCGAGAA
151 CTAAGCAAAAG AGACAAAGAG AGAACAGGTG AGAAGGCCAG GCCTCGAATTG
201 GACTTGAAGG C CCTGTAACCA GCTGCCCGAG TTCTATGGCC AGCTCCCGAC
251 AGAGCTGAC GGCGAGCCCC TGGAGGACCT GTGATCTTTT TACAGCACAC
301 ACAGGACATT CATACTGTTG AATAAAGACCA GACCATTCTC CAGATTCAGT
351 GCCACTTGGG CTCTGTTGCT CTTCAGCTCC TTCAACCCTGA TCAGAAGAAC
401 AGCCACTCAA GTGTCGCTTC ACTCTCTTGT CTCCCATATT ATACCTGTCA
451 CATAATCTTG CAAAAGGTTG TGCGATGACC GAACTGTCTT CCCAGAGAAA
501 CTGCGATATG TCTTCACCTGT TGTTTACACC TTCGAGGGCTC TGATAAAGAT
551 ACTGCGAAGA AGGTTTATCT TTAATATTCC ACTATCTTCT CGAGATCCCT
601 GGAACCTGGCT GAACCTCAGT GTCTATACCT TGGCATACGT GGGTGCGAGC
651 GTAGACCTCC GAGGAATCTC AGGCCTGCGG ACATCCGACG TTCTCAGGGC
701 CCTGAAGACT GTTCTCTGTA TCCAGAGGACT GAAAGGGTCG GTGGGAGCCC
751 TGATCCCCAC AGTGAGGAAG CTGGGCGACG TGACCATCCT CACAGTCTTC
801 TGCGCTGAGT GCTTTCGCCT GTGGGGGCTC CAGCTCTCTCA AGGGGAACTT
851 CAAGAATAAA TGCAATTAAGA AGCGCAAGA TCCGCACAAG GCTGACAATC
901 TTCGTACCTGA AATGGCAGAA GACATCTTCA TCAAGGCCGG TACTACGGAT
951 CTCTGTTTGT GTGGCAATAG GTCTGTATGCT GGGCACTGCC CTATATGTTA
1001 TGTCGCTCAG AAAAACTCCTG ACAAACCAGGA TTITAAACTAC ACCAGCTTTG
1051 ATTCCTTTGC GTGGCGGTTC CTCTCAGCTT GTGGGTCTCAT GAGCAGAGAC
1101 TTCTGGGAAAC GGCTGTACCA GCAGACACCT CGGGTCCCG GAAATAATGA
1151 CATGGTCTTT TTTGTCTGGT CTATTTTCT TGGATCATTTC TACCTGGTCA
1201 ATTTGATCTT GGCTGTGGTG ACCATGGCAT ATGAGGAACA GAGCCAGGCA
1251 ACAATTGCAG AAAGAAGAGC AGAAGAAAAA AAGTTCAGGC AGGCCCTCGA
1301 GGTGCCTGCAA AAAAGAAGAG AGGGTGTGGC AGCGCTGGGA ATGGACACAA
1351 CTTCTTATTAA TTTTCCACAGC GGATCACCCT TAGGCTCCCA AAAAGCCAAT
1401 GAGAGAAGAC CCAGGGTGAAT GCAGAAGGTG TCGAAGGGCT CCACAGATGA
CAACAGATCA CCACAATCCG ACCCTTACAA CCAGCGCAGG ATGTCCCTCC
TAGGCCTTTCC TCTGGAAGAG CCGAGGGGCTA GCCACGGCAGG TGTGGTCCAC
TTCCAGACAC CCAGCCAAGA TGTCCTATTT CTTGATGGGA TCTGGGACGA
TGGGGTCTTT CATGGGATAC AGGAAAGCCG TCGAAAATTCC ATATTTGCTGG
GCAGGGGGTGC CGGCCAGGCA GTGCTCTTCC CCAGAACTGC ACTGCCCTCAG
TCCCCCAAACC CTGGCGGTAA ACATGAAAAA GAGGGACAGC TGGGAATGCC
CACTGGTGAA CTTGCCGCTG GAACGCCTGA AGGCCCGGCA CTGAGATGCTG
CAGGACAGAA GAACCTCTCTG TCTGCAAGGCT ACTGGAATGA ACCTTTCCGA
GCACAGAGGG CAATGAGTGT GTGCACTATAC ATGACTCTTG TCATTGAGGA
ACTGGAAGAA TCTAAACGTGA AGTGGCCACC CTGCTTATGC AGCTTTCGCC
AAAAATATCT GATATGGGAA TGCTGCCCA ACTGGAGAA AATCCAAAAATG
GTGCCTCTCG AACTGGTGAC TGACCCCTTC GCAGAGCTTA CCATCACCT
TGCACTTTGTG GTGAATACCG TCTTATCGCC CATGGAACAC TACCCCATGA
CTGATGCTTT CGATGCAGCT CTCAAGCAGG GCAACATTTG CTCACTGTG
TTTTTACAGA TGGAGATGGC CTCAAGATCATTGCTTTG ACCCGTACTA
CTACTTCCAG AAGAAATGGGA ACACTCTTCGA CTGTGTCTATT GTCACCGTA
GCCTGCTTGA GCTGAGTGCAC TCCAAAAGGG GCAGCCCTATC TGCTGGCTCGT
TCCTCAGCCT GTTCTTCTGGT CTTCAGACTG GCCAAGTCTG GCCCCACCCCT
GAACATGCTC ATCAAAGATCA TCGGGAAACTC TGTTGGGGGC CTGGGCAACC
TGACCTTCTC CTTGGCCATC ATGTCTTTCAT CTTTTGCCCT GTGGGGAAAG
CAGCTCTCTC CAGAAGACTA TGGGTGCCGC AAGGATGGCG TCTCGTGTGTG
GAATGGGTAG AAGCTGCGCT GGCACATGTG TGACTTCTTC CATTTCTTCC
TCGGTGGTCT TGCGATTTCC TGCGGGAGGT GGATCGAGAA CATGTTGGGTC
TGCAATGGAGG TCAGCCAGAA ATCATCTGC CCTACCCCTT TCTTGACAGT
GATGGTGCTA GCGAACATGGG TGGTGCTCAA CTTTTTATG GCTTTTACTGC
TGAGTCCGCAA GACCTCAGG CCCCAGAGGA TGACCGGGAG
TGAGAACACT TGCAAGTTAGC ACTGCGCCAGG ATTCAGGTAC TGGGCCATCG
GGCCAGTCGG GCCATTACAA GTTACATCAG AAGCCATTGC CGGTTCCTTT
GGCCCAAGGTT GGAGACCAAG CTGGGGATGA AACCCCATC CACCAGCTGC
AAAGTTGAGA ACCACATTGC TACTGATGCT GTAATGCTG CAGTGGGGAA
2951 CCTGACAAAG CCAGCTCTTA GTGGCCCCAA GAGAATCAC GGGGACTTCA
3001 TCACTGATCC TAACGTTGGA GTCTCTTGCC CATTGCTGGA GGGGAGTCCC
3051 GACCTTGATG AGTCGAGAGA AGTAATGGAAG GAGCCTCTCTG AGAGCTCTTG
3101 GCAGAAGAAG AGCCCCAAAAG GGCAGCAGGA GCTGCTGCCA CAAGTCTAAA
3151 AGTGTTGAAAA TCACCCAGCG CAGCAAGGCC CAACCTCAGGG GATGGTCTCT
3201 GAAGACCTGG CTCCTATACCT GGGGAGAGA TGAAAAAGGA AGGATAACCC
3251 TCAGGGCTCCT GCGAGGGAGA TGATGACACG AGGCCTCTCC GAGGGAAGCA
3301 CGTTGGACGT CCCGGACCACG AGGGAGATCC TGAGAGACGT TGCTGAGCTG
3351 GCAGAGGATGC TGAGAGGACG CGATGACTGT TCACAGAAGA GCTGCACTCG
3401 CCGCTGTCCG TCTGCAAAAG TGAACCAACG TAAATGCTCT TGAGGCCACAG
3451 GCTGCGCAGGT GCGACAAAA CGTTCACGCTC TTGAGGACGA CAGCTGGTCT
3501 GAGAGTGTTTA TCATCTCTTC CATGGTCTGCT AGCAGTGGAA CGCTGCGCCTT
3551 TGAGAGAATAAC TACCTGGAGA AGAAACCCGG AGTGAAGTCT GTGCTGAGT
3601 ACACTGACCG AGTGGTCACC TCTATCTTTG TATTCAGAGT GTGCTCAAG
3651 TGGTGGCTT ATGGCTTCAA AAAATATTTTC ACAATGCTCT GGTGCTGGCT
3701 GGAACCTCCTC ATCGTGAAACA TCTCCCTCAC AAGGCTCTAC AACAAGATCC
3751 TCAGATGATTC AGACGGTGCC TCCATCAAAG CCGTCCGAC TCTCCGTCGC
3801 CTCCGGCGCG TCGGCGCCTC GCCTGATGTC GAAGGCTAGA GGTAGTGGT
3851 GGATGCCTTG GTGGGCGGCA TCCCCTCATC CATGAAGGTC TCTCTCGTCT
3901 GCCTCATCTT CGCTGCTCATC TCCAGCATCA TGGGTGGGAA CCTCTCCGCC
3951 GGAAATTTT CGAGATGTGT CGACACCAGA AGCAACCAT TTTCCGTCGT
4001 GAATTCGACA TCTGTGAAATA ACAATGCTGA GTGTCAAAAT CAAAACAAATA
4051 CGGCCACATT CCTCTGGGTT AAGCCTCAAAG TCAAATCTGC CAACGTGCGCT
4101 ATGGGCTTCC TCGGTCTTCT CGAGGGTGGC ACCTCAAAG GCTGGATGGA
4151 CATTATGTAT GCAGCTGTCG ATTCCTGAGA TATCAACAGT CAGCXXAATT
4201 GGGAGGAGAG CCTGTACATG TACCTATACT TGTCGTCCTT CATCAATTTT
4251 GGTGGCTTCT TCACGCCTGA TCTCTTGCTG GGAGTTCACTA TCAGAACACTT
4301 CAATCAACAG AAAAAAAAAAT TGGGGGCGCA GAGCATCTTC ATGACGGGAAG
4351 AAAAAAAATA ATATTAATTT GCCATGAAAA AACTGGGGCTA CAAAAACCCC
4401 CAAAAGCCCC TCCACCAGGC TCTGGAATAAG TACCAGGCTG TGTGTTTGA
CATTTGTACC AGGCAAGGCT TTAGACATCAT CATCATGGT TCTCATCTGCC
TCAACATGAT CACCATGATG GTGGAGACCG ACAATCAGAG CGAGGAGAG
ACGAAAGTTC TGGCCAGAAT CAACACGTATTT TTGATGCGGG TCTTCAAGGG
CGAGTGTGTTG ATGAAAGTTG TCGCCGTCGG ACAGTATTAC TCTCAACAAAG
GCTGAAGATGT GTTCCACCTT ATGGTGTTGA TTCTGTCCAT TGCGAGTCTG
TTGTCTCTGA CAATCTCTAA GTCACTAGAA AGTTACTCTT CCCCACGGTT
CTTCCCGCAGTC ATCCGCTTGG CGAGATCGG CGCCATCCTC AGGCTGATTAC
GAGCAGCCAA GGGGATTCGC ACAGCTGCTT TCGCCCTCAT GATGTCCCTG
CCCGCCTCTT TCAACATCGC TCTCCCTCCT TCTCCGTCGTA GTGTCATCTA
CTCCTACCTTC GGCATGGCCA GCTTCGCTAA TGTCATAGAT GAGGCTGGCA
TCGACGACAT GTTCAACTTC AAGACCTTTG GCAACAGCAT GCTGTGCCCCT
TTCAGATCA CCAACGTGCC GGGCTGGGAT GGCTCTTCTA GCCCCTACCT
CAACACAGGA CCCCCACTG GGCACCCCAA CCGGCCCAAAC AGCAATGGCT
CCAAGGGGAA TTGTGGAAGC CGACGGTGAG GCACTCCTCT TCTCACACC
AACATCATCA TCCTCTCTTG CATCGTGGTC AACATGTACA TTGCAGTGAT
CTGGGAGAAC TCTCAATGTGG CCACAGAAGA GAGCAGGAGC CCCCTAGAGG
AGGACGACTT TGACATGTTC TATGAGACCT GGGAGAAGTT TGACCCGGGAG
GCCACCCAGT TCATTGGCTT TCCTGCCCCC TCAGACTTGG CAGACACACT
CTCCGGCCCT CTAGAAATCC CAAAACCTAA TCAGAATATA TTAATCCAGA
TGACCTGCC TCCTGTGGCC CGGAGATAAGA TCCACTGGTT GGACACTTCC
TTTGCCTTCA AAAAAGATGT CTTGGGAGAGA TCTGGGAGAT TGAGATTCTCT
GAAGACTAAT ATGGGAGAGA AGTTTATGGC AACTAATCTT TCPAAACGAT
CCTATGAACC AAATGCAAAC ACCCTCCGGT GCAAGCAGGA AGACATCTCA
GCCACCATTTC TTTAAAGGCG CTATCGGAAAC TACATGTGGC AACGCTTCTT
GATGCTCTCC AACACCCTGC ATGTTGCCAG GGCTGAGGAA GATGGCGGTG
CAGGGGGCTAT GTTACATTTA TGCCAAATGA CAACGGGTGG
CTCCCAGACA AATCAGAAAC TGCTCTGTCT ACGTCTTCCC CACCATCTCA
TCAGGGCCGCC ACCAGGGGCC TGAGTGCAGG GCCCAACATT AACACATCTA
GCTCAATGCA AAATGAAGAT GAAGTCCTG CTAGGAAAGG GAATAAGCCCT
GGACCTCAGT GAAGACACTC CAGCATGCAC GGCGCGGGTT CTAATGTCTT
5951 TTCTGGGTG GCTGACCTTT TCCTTCTAGA GATGGCACCA ACCTACAGCC
6001 TCCACCGATG CATGTCACG GTCATGTTGT CAGAGCTGAA TAGGGACATC
6051 TTGGAGAAAG CCCCCACCCC AATAGGAATC AAAAGCCAAG AATACTCCCC
6101 CACCTTTGACA TCCTCTCTGA ACTTTCAAGA GATGACATCG CTTTGTGTTG
6151 TTGTGACCA GAGATGTGAT TTACCAAATT CTCAGAAACCA GGACTTCCAG
6201 AGACACATGG CAAGGACATT TCTGGTGGTC TCAGGCAATC TTTGAGAAGT
6251 TGCATAGGGG TGGACTGTGC GCATAGGGGT GGCACGTGTA ATGAGGGTTT
6301 GCATGACTGC ATGCTCGCAG TTGCGGACA ATACCTGAGG GTCGGCCATT
6351 AAAATTGATA TTTTAAAGG TTA
SEQ ID NO: 6

1 ATGGAAATTCC CCATTTGATC CCTCGAAACT AACAACCTCC GTGCTTTTAC
51 TCCCGAGTCA CTGGTGAGAGA TAGAGAGAGA ATTTGCTGCA AAGCGGAAAG
101 CAAAGAAAGAC CAGAGAGAGA CTTAGGAGAC GAGAAGCA ACAAGAGAAG
151 CCTCGGGCCCG AGCTGGACCTTT GAAAGCTGCTG AACCAGCTGC CCAAGTTCTTA
201 TGGTGAGCTC CCAGGAGAAC TGATTGGGGAG GGGCTCTGGAG GATCTAGATC
251 CGTTTCTACAG CACACACCGG ACATTTTATGG TGCTGAACAA AGGGAGAGAG
301 ATTTCCCAGT TTAGTGACCAT CCGGGCCCCGT GGGCTTATTA GTTCTCTTCAA
351 CCTGATCAAG AGAAGGCGCA TCAAGTGTCC TGTCACACTG TGGTTCAGTT
401 TATTATTAC GGTACTATT TTGTGTTATG TGTTGTCAT GACCCGAACT
451 GACCTCCAG AGAAAATTGA ATATGTCTTC ACTGTCATTT ACACCTTTGA
501 AGGCTTGTAGA AAGATACGGA CAAAGGGGATT TTGCTTAAAT GAGTTGACGT
551 ACCTGAGAGA TCCCTGGAGT GTGCTGATT TTACGCTCAT TACCCCTGCA
601 TAGTTGGCA CAGCAATAGA TCTCGGTGGG ATCTCAGGCC TGGGGAGCATT
651 CAGAGTTTCTG AGAGCTTAA AAAAAAGTTTC TGATGCTCAAA GGGCTGAAAGG
701 TCATTGTGGG GGCCCTGATT CACTGATGA AGAAACTGGG TGATGTCACCC
751 ATCCTCACCA TCTCTCCTCT AAAGTTTTTT TGCTTTGGTTGG GGCTGCAACT
801 CTTCAAGGGC AACCTCAAAG ATAAATGTGT CAAGATGAC ATGGGCTGTC
851 ATGAGACAAC CAACACTCTA TCTCAGAGAA AACCAGATAT CTACATAAAAT
901 AAGCGGGCCA CTTGTTAGCC CTTACTGTGT GGCAATGGAT CTGACTCAGG
951 CCACTGCCCCT GATGGTTATA TCTGCTTAA AACTTCTGAC AACCAGTTT
1001 TTAACATACAC CAGCTTTTGT TCTTTCGCTT GGGCTTTTCT CTCATCTGTC
1051 CCGCTCTAGA CACAGGATTC CTGGGAACGC CTCTACAGAC AGACCCCTAG
1101 GACTTTCTGGG AAAATCTATA TGATCTTTTT TTGCTACTGA ACTCTCTTG
1151 GATCTTTCTA CCGGGTCACAG TCTGCTCTGG GCTGATGTAC CATGGGCCGTAT
1201 GAGGAGCGCA ACCAGGCAAC CACTGATGAA ATGGAAAGCAA AGGAGAAAG
1251 GTTCCAGGAG GCCCTCGAGA TGCTCCGGAAG GGAGCAGGAG GTGCTAGCGAC
1301 CACTAGGGAT TGACAAACAC TCTCTCCTACT CCCACAAATTG ATCACCCTTATA
1351 ACCTCCTAAAA ATGCGCTTGA GAGAAGGCAT AGAATAAAGC CAAGAGTGTC
AGAGGGGTCC AAGAGAAGACA CAAGATCACC CCGCTCTGAT CCTTACAAC
AGGCGAGGAT GTCTTTTCTA GGGCTCGCT CTGGAAAAACG CCGGGCTAGT
CATGCGAATG TGGTACCCTT CCGTCCCTT GGCCGAGATA TCTACCTCCC
TGAGGGAGTC ACAGATGATG GAGTCTTTCC TGGAGACCAC GAAAGCCATC
GGGGCTCTCT CGGCTGGGTT GGGGTGTGCTG GCCAGCAAGG CCCCCTCCCT
AGAAAGCCTCT TCTCCTCAACG CAGCAACCTT GACTCCAGGC ATGGGAAGA
TGAACACCAA CCAGGGCGCA CTAAGTGAAT GTTGCTCAGT TGCCCCCGA
TCTCGGCATT CGATGCAAGA CAAAAGAGGA CTTCTTGTGC ACGAAATATC
TAGATGAAC TTTCCGGGCG CCAAGGGGGA ATGAGTTTG TGAGTATC
AACCTTCGTC CTTGAGGAAC TCGAGAAGTC TGAACAAAG TGCCCCACCT
GCTGGACCA CAGTTCGAGA CAGTCTCGAA TCTGGGATG GTGCTCCCAT
TGGTGTAAGGC TCAAAGACAT TTTCTTTGGG CTTGTGACGG ATCCCTTTGC
AGAGTCACCC ATACCTTTGT GCCATCGTGTT GAAACACTAT TCTATGGCCA
TGGAGCAGCA TGGCATGAGC CCTACCTTCG AAGGCAATGC CCAGATAGGC
AACATCGTCT TTACCATTATT TTCTACTGCT GAAATGCTT GCATCCATAT
TGCTTTCGAC CCATACTATT ATTTCCAGAA GAAGTGGAAT ATCTTTGACT
GCATCATGCT CAGTCGAGAT CTTCTAGAC TGGGCCTGGG CAAAGAGGGA
AGCCTGTCTG TGCTGCGGAAG CTCCGGTCTG CTGGCCGTAT TCAAGCTTGCC
CAAATCTCTG CGACACCTTAA ACACACTCAT CAAGATCATC GGAACACTAG
TGGGGGCACCT GGGGAACCTCC ACCATCATCC TGGCCATCAT TGTCTTTGTC
TTGTCTTCTG TGGGAAAGGA GCCTCTAGGG GAAAACCTAC GTAACAAACGG
AAAAATATC TCCGGCAGCC ATGAAGACTG GCCCCTGGTG CACATGCAACG
ACTCTTCGCA CTCTTCCTTC ATTTGCTCTTCGTATCC TGGAGAGTGG
ATTGAGAACA GTGGGCCTGT CATGGAAGTT GGCCAAATAA CCATATGCCT
CAGCTTCTTC TGACGGTGTA TGGTGCTTGG GAACCTGGTG GTGTTAACC
TGTCATCGCG CCTGCTATTG AAACCTTTCA GTGCTGACAA CCTCAGAGCC
CGGAGGAGCG ATGGGGAGGGT GAAACACTCTG CAGTTGGCCC GGCCACCGGAT
CCAGGCTTTT GGCCACATCA CCAACAGGCC TTTGCGACGC TTCTTCAGCA
GTCCTGGCC ATTCAGCGAA CAAAAGGGCA AGCCCTAGCT GGTGTGAAA
CTCCACTCTC CCAGCTCCAA GGCTGAGAAC CACATGCTCG CCAACACTGC
TTTGTCTTTT GACATCGTGA CCAGACAAGC TTTTGACATC ACCATCATGG
TCCTCATCTG CTCACACATG ATCACCATGA TGTTGGAGAC TGATGACCAA
AGTGAAGAAA AGACGAAAAT TCTGGGCAAA ATCAACCAGT TCTTTGTGGC
CGTCTTCACA GCCGAATGTG TCAATGAGAT GTTGCCTTTG AGGCCAGACT
ACTTCAAAAA TGGTGGAAAT GTTGTGTAGT CATATTGATG GGTTCCTCCTC
ATTGCGAGCC TGATTTTTTC TGCAATTCCT AAATCAGCTC AAAGTTACTT
CTCCCCAACGC CTCTTCAGAG TCAATCGGCTT GGCCCCAATT GGCCGACATCC
TCAGACTGAT CCGAGCGGCC AAGGGGATCC GCACACTGCT CTGTGGCCTC
ATGATGTTCC TGCGTGGGCT TTTAACACTC GGGTGTTGCA TATCTCTTTGT
CATGTTCATC TACTCCATCT TCGTAGTTGTC CATATTTCCT CATGTGAGGT
GGAGGGCTTG CATCGACGAC ATGGTTCACT TCAGAAGCTT CGCAAGAACGC
ATGCTGTGCA TCTTCCAGAT TACCAAGTGC GGCGGGTGGA ATGGCCTCCT
CAGCCCCATC CTCAACAGAG GGCCCCCTGT CTGTGACCCC AATCTGGCCA
ACAGCAATGG CACCAAGGG ACTGTTGGGA GCCAGCGCGT AGGCATCATC
TTCTCACCAC CCTACATCAT CATCTCTCTC TCTGTCCTGG TCAACATGTA
CATTGCAGTG ATTTCTTGAG ACTTCAATGT GGCACCGGAG GAGAGACCTG
AGCCTCTGAG TGAGGACGAC TTTGACATGT TCTATGAGAC CTGGGAGAAG
TTTGGACCAG AGGCCACTCA GTTATTACC TTTTCTGTC TCTGGACTT
GTCAGACACT CTCTCTGTCG CCCTGAGAAAT CCCAAAACC AATCGAAATA
TACTGATCCA GATGGACCTG CTTTGGTTCC CTGGAGATAA GATCCACTGC
TTGGACATCC TTTTTGCTTT CACCAAGAAT GTCTTAGGAG AATCCGGGGA
GTGGATACCT CTGAAGCGCA ATATGGAGGA GAAGTTATG GCAACTAATC
TTTAAAAATC ATCTTAGAA CCAATAGCAA CACTCTCCG ATGGAAGCAA
GAAGACATTT CAGCCACTGT CATTAAAAAG GCCATCGGA GCTATGTGCT
TTGGACATCC TTTTTGCTTT CACCAAGAAT GTCTTAGGAG AATCCGGGGA
GTGGATACCT CTGAAGCGCA ATATGGAGGA GAAGTTATG GCAACTAATC
TTTAAAAATC ATCTTAGAA CCAATAGCAA CACTCTCCG ATGGAAGCAA
GAAGACATTT CAGCCACTGT CATTAAAAAG GCCATCGGA GCTATGTGCT
GCACCGCTCC ATGGCACTCT CTAACACCC ATGTGTGCAC AGAGCTGAGG
AGAGGGCTGC ATCACTCCCA GATGAAAGTT TTGGTCATT CACAGCAAAT
5701 GAAAAATTGTG TACTCCAGA CAAATCTGAA ACTGCTTCTG CCACATCAATT
5751 CCCACCGTCC TATGAGAGTG TCACTAGAGG CCTTAGTGAT AGAGTCAACA
5801 TGAGGACATC TAGCTCAATA CAAAATGAAG ATGAAGCCAC CAGTATGGAG
5851 CTGATTGCC CTCGGCCCTA GTGA