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(54) **HYBRID PROTEINS THAT MIGRATE RETROGRADELY AND TRANSYNAPTICALLY INTO THE CNS**

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(57) **ABSTRACT**

The non-toxic proteolytic C fragment of tetanus toxin (TTC peptide) has the same ability to bind nerve cells and be retrogradely transported through a synapse as the native toxin. A hybrid protein encoded by the IacZ-TTC gene fusion retains the biological functions of both proteins in vivo, i.e. retrograde transynaptic transport of the TTC fragment and β-gal enzymatic activity. After intramuscular injection, enzymatic activity could be detected in motoneurons and connected neurons of the brainstem areas. This strategy is useful for the delivery of a biological activity to neurons from the periphery to the central nervous system. Such a hybrid protein can also be used to map synaptic connections between neural cells.
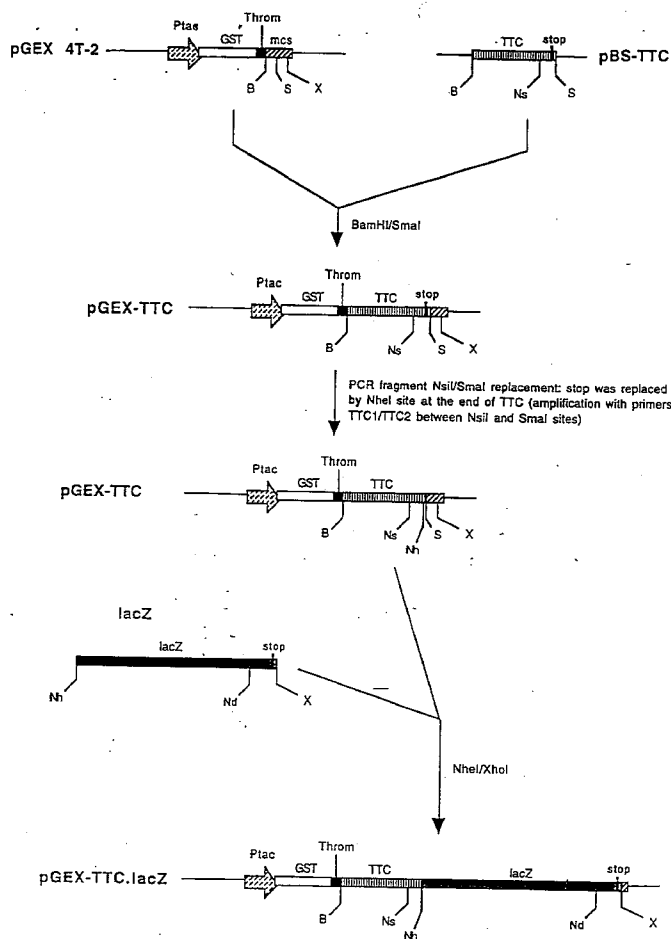
SK-TTC -> Genes

DNA sequence   1600 b.p.   ggaaacagctat ... gtcgttttacaa   linear

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   1 ggaaacagctatgaccatgattacgccaagctcgaaattaaccctcactaaagggaacaaaagctggagctcggtacccg 80

  81 ggccacc ATG GTT TTT TCA ACA CCA ATT CCA TTT TCT TAT TCT AAA AAT CTG GAT TGT TGG 141
   1         M   V   F   S   T   P   I   P   F   S   Y   S   K   N   L   D   C   W   18

 142 GTT GAT AAT GAA GAA GAT ATA GAT GTT ATA TTA AAA AAG AGT ACA ATT TTA AAT TTA GAT 201
  19  V   D   N   E   E   D   I   D   V   I   L   K   K   S   T   I   L   N   L   D   38

 202 ATT AAT AAT GAT ATT ATA TCA GAT ATA TCT GGG TTT AAT TCA TCT GTA ATA ACA TAT CCA 261
  39  I   N   N   D   I   I   S   D   I   S   G   F   N   S   S   V   I   T   Y   P   58

 262 GAT GCT CAA TTG GTG CCC GGA ATA AAT GGC AAA GCA ATA CAT TTA GTA AAC AAT GAA TCT 321
  59  D   A   Q   L   V   P   G   I   N   G   K   A   I   H   L   V   N   N   E   S   78

 322 TCT GAA GTT ATA GTG CAT AAA GCT ATG GAT ATT GAA TAT AAT GAT ATG TTT AAT AAT TTT 381
  79  S   E   V   I   V   H   K   A   M   D   I   E   Y   N   D   M   F   N   N   F   98

 382 ACC GTT AGC TTT TGG TTG AGG GTT CCT AAA GTA TCT GCT AGT CAT TTA GAA CAA TAT GGC 441
  99  T   V   S   F   W   L   R   V   P   K   V   S   A   S   H   L   E   Q   Y   G   118

 442 ACA AAT GAG TAT TCA ATA ATT AGC TCT ATG AAA AAA CAT AGT CTA TCA ATA GGA TCT GGT 501
 119  T   N   E   Y   S   I   I   S   S   M   K   K   H   S   L   S   I   G   S   G   138

 502 TGG AGT GTA TCA CTT AAA GGT AAT AAC TTA ATA TGG ACT TTA AAA GAT TCC GCG GGA GAA 561
 139  W   S   V   S   L   K   G   N   N   L   I   W   T   L   K   D   S   A   G   E   158

 562 GTT AGA CAA ATA ACT TTT AGG GAT TTA CCT GAT AAA TTT AAT GCT TAT TTA GCA AAT AAA 621
 159  V   R   Q   I   T   F   R   D   L   P   D   K   F   N   A   Y   L   A   N   K   178

 622 TGG GTT TTT ATA ACT ATT ACT AAT GAT AGA TTA TCT TCT GCT AAT TTG TAT ATA AAT GGA 681
 179  W   V   F   I   T   I   T   N   D   R   L   S   S   A   N   L   Y   I   N   G   198

 682 GTA CTT ATG GGA AGT GCA GAA ATT ACT GGT TTA GGA GCT ATT AGA GAG GAT AAT AAT ATA 741
 199  V   L   M   G   S   A   E   I   T   G   L   G   A   I   R   E   D   N   N   I   218

 742 ACA TTA AAA CTA GAT AGA TGT AAT AAT AAT AAT CAA TAC GTT TCT ATT GAT AAA TTT AGG 801
 219  T   L   K   L   D   R   C   N   N   N   N   Q   Y   V   S   I   D   K   F   R   238

 802 ATA TTT TGC AAA GCA TTA AAT CCA AAA GAG ATT GAA AAA TTA TAC ACA AGT TAT TTA TCT 861
 239  I   F   C   K   A   L   N   P   K   E   I   E   K   L   Y   T   S   Y   L   S   258

 862 ATA ACC TTT TTA AGA GAC TTC TGG GGA AAC CCT TTA CGA TAT GAT ACA GAA TAT TAT TTA 921
 259  I   T   F   L   R   D   F   W   G   N   P   L   R   Y   D   T   E   Y   Y   L   278

 922 ATA CCA GTA GCT TCT AGT TCT AAA GAT GTT CAA TTG AAA AAT ATA ACA GAT TAT ATG TAT 981
 279  I   P   V   A   S   S   S   K   D   V   Q   L   K   N   I   T   D   Y   M   Y   298

 982 TTG ACA AAT GCG CCA TCG TAT ACT AAC GGA AAA TTG AAT ATA TAT TAT AGA AGG TTA TAT 1041
 299  L   T   N   A   P   S   Y   T   N   G   K   L   N   I   Y   Y   R   R   L   Y   318

1042 AAT GGA CTA AAA TTT ATT ATA AAA AGA TAT ACA CCT AAT AAT GAA ATA GAT TCT TTT GTT 1101
 319  N   G   L   K   F   I   I   K   R   Y   T   P   N   N   E   I   D   S   F   V   338

1102 AAA TCA GGT GAT TTT ATT AAA TTA TAT GTA TCA TAT AAC AAT AAT GAG CAC ATT GTA GGT 1161
 339  K   S   G   D   F   I   K   L   Y   V   S   Y   N   N   N   E   H   I   V   G   358

1162 TAT CCG AAA GAT GGA AAT GCC TTT AAT AAT CTT GAT AGA ATT CTA AGA GTA GGT TAT AAT 1221
 359  Y   P   K   D   G   N   A   F   N   N   L   D   R   I   L   R   V   G   Y   N   378

1222 GCC CCA GGT ATC CCT CTT TAT AAA AAA ATG GAA GCA GTA AAA TTG CGT GAT TTA AAA ACC 1281
 379  A   P   G   I   P   L   Y   K   K   M   E   A   V   K   L   R   D   L   K   T   398

1282 TAT TCT GTA CAA CTT AAA TTA TAT GAT GAT AAA AAT GCA TCT TTA GGA CTA GTA GGT ACC 1341
 399  Y   S   V   Q   L   K   L   Y   D   D   K   N   A   S   L   G   L   V   G   T   418

1342 CAT AAT GGT CAA ATA GGC AAC GAT CCA AAT AGG GAT ATA TTA ATT GCA AGC AAC TGG TAC 1401
 419  H   N   G   Q   I   G   N   D   P   N   R   D   I   L   I   A   S   N   W   Y   438

1402 TTT AAT CAT TTA AAA GAT AAA ATT TTA GGA TGT GAT TGG TAC TTT GTA CCT ACA GAT GAG 1461
 439  F   N   H   L   K   D   K   I   L   G   C   D   W   Y   F   V   P   T   D   E   458

1462 GGA TGG ACA AAT GAT TAA acagattgatatgttcatgacatatgcccgggatcctctagagtcgacctcgaggg 1535
 459  G   W   T   N   D   *                                                           464

1536 ggggcccggtacccaattcgccctatagtgagtcgtattacaattcactggccgtcgttttacaa                1600
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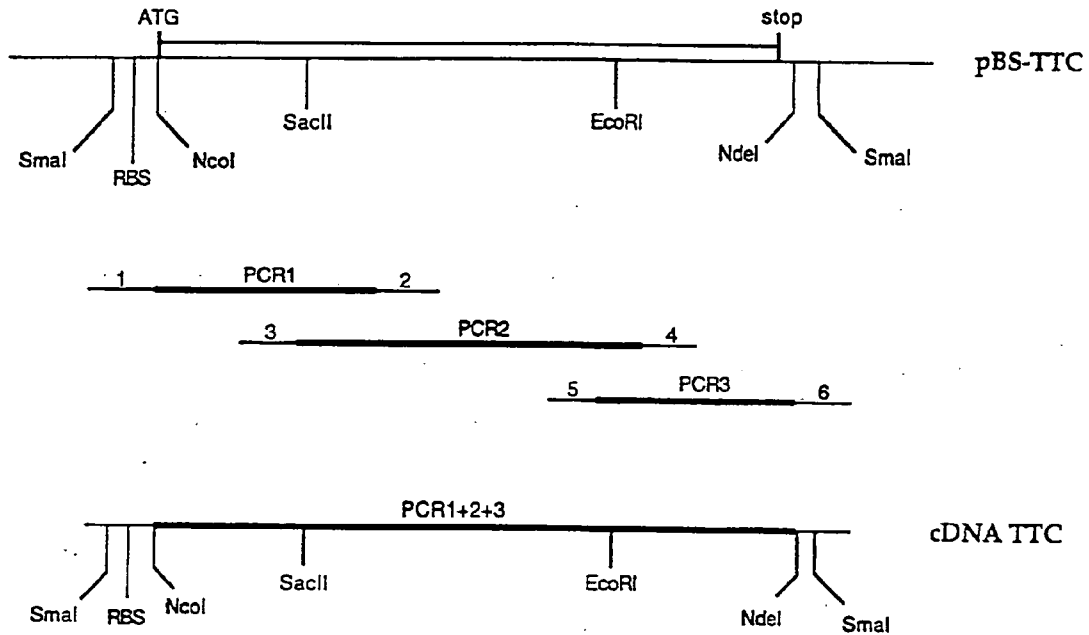
FIGURE 1

TTC cDNA isolation:

The TTC cDNA was isolated from a Clostridium Tetani strain using Polymerase Chain Reaction. We used a three times PCR to generate three overlapping fragments respectively of 465 bp (PCR1; primer 1: 5'-CCC CCC GGG CCA CCA TGG TTT TTT CAA CAC CAA TTC CAT TTT CTT ATT C-3' & primer 2: 5'-CTA AAC CAG TAA TTT CTG-3'), of 648 bp (PCR2; primer 3: 5'-AAT TAT GGA CTT TAA AAG ATT CCG C-3' & primer 4: 5'-GGC ATT ATA ACC TAC TCT TAG AAT-3') and of 338 bp (PCR3; primer 5: 5'-AAT GCC TTT AAT AAT CTT GAT AGA AAT-3' & primer 6: 5'-CCC CCC GGG CAT ATG TCA TGA ACA TAT CAA TCT GTT AAA TC-3'), and each fragment was sequentially cloned into pBluescript KS+ to produce plasmid pBS-TTC. The upstream primer 1 contained the Ribosome Binding Site (RBS) and translation initiation signals.
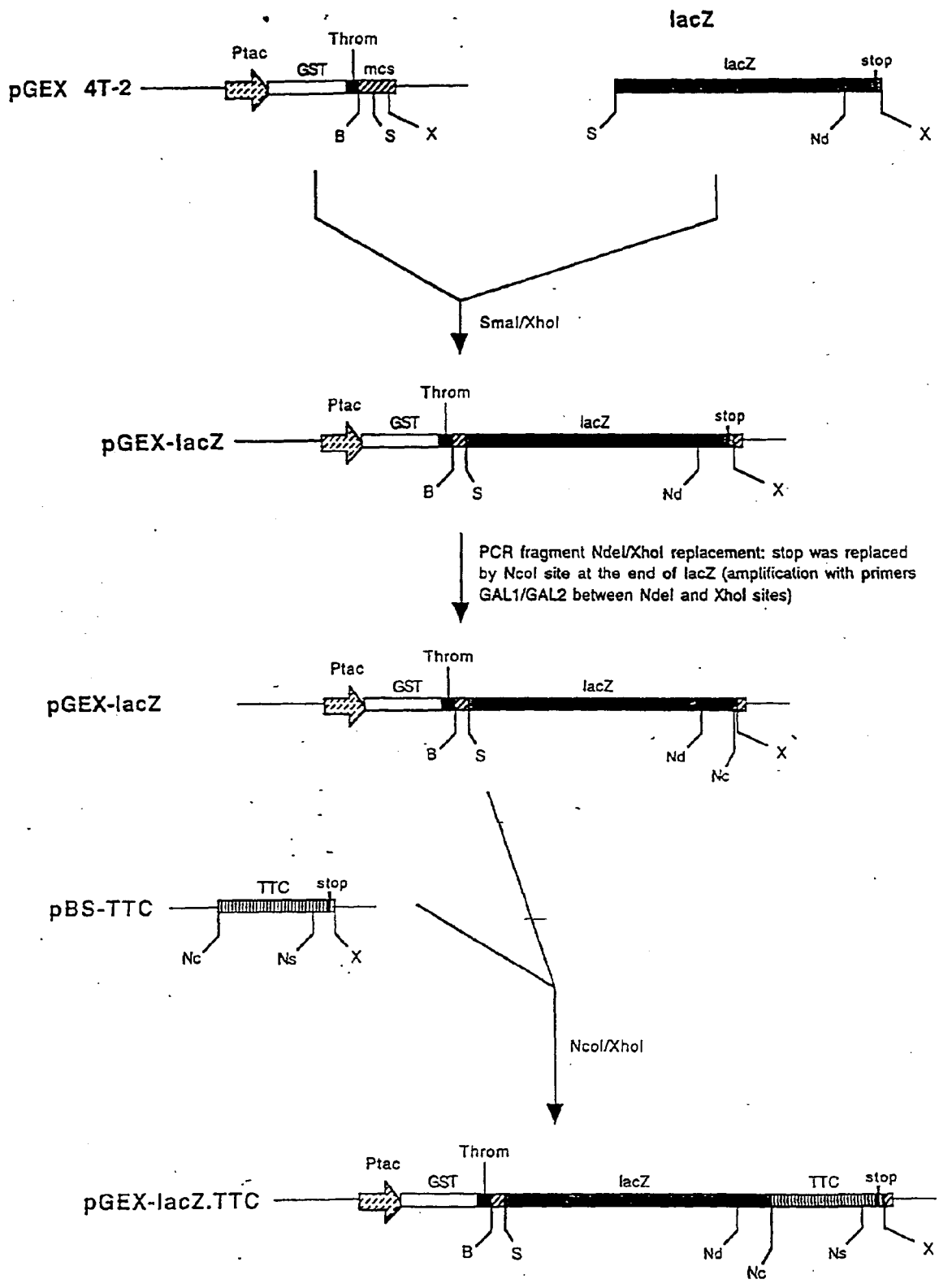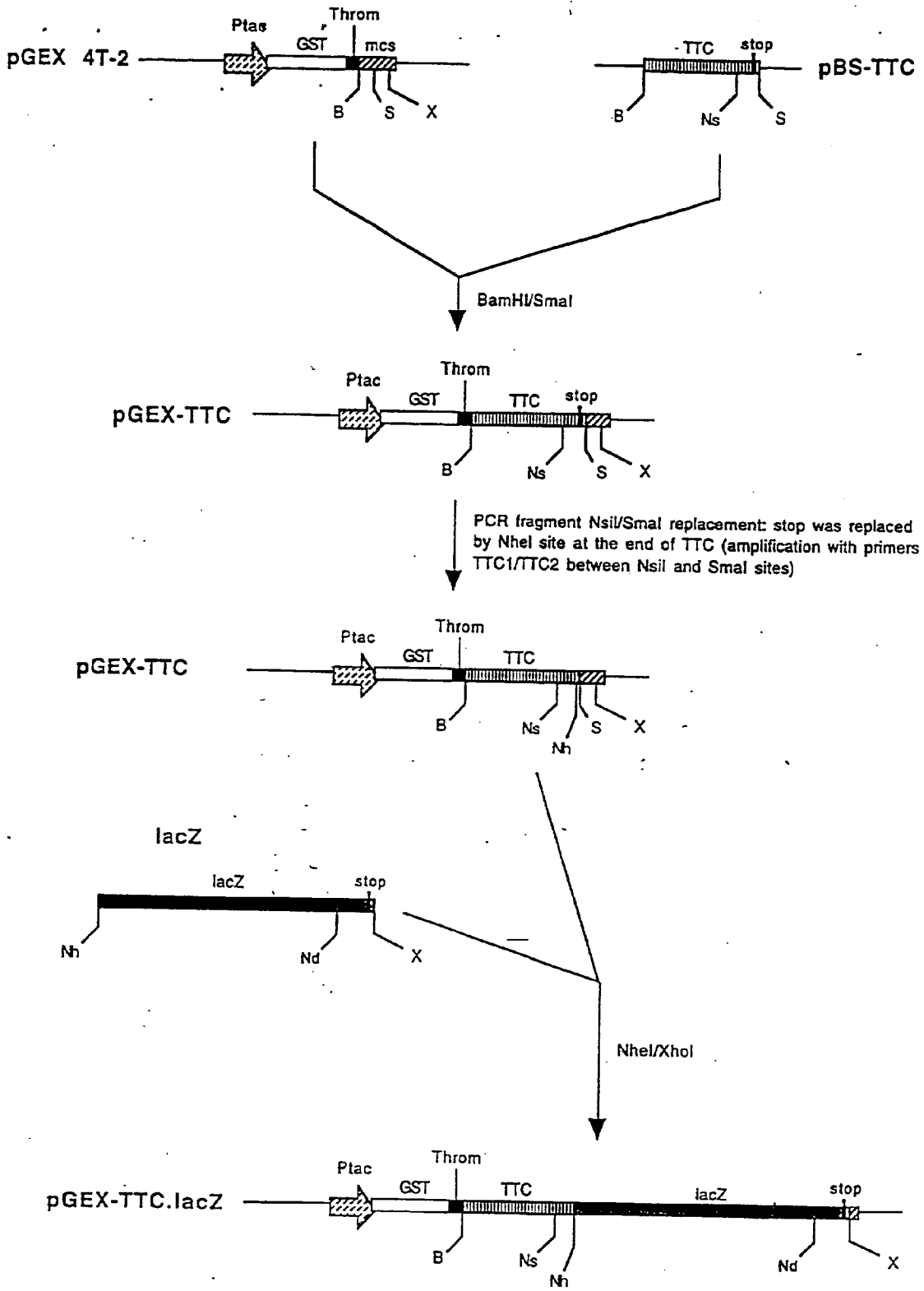
FIGURE 2

FIGURE 3

FIGURE 4

pCMV.LACZ.TTC -> Graphic Map

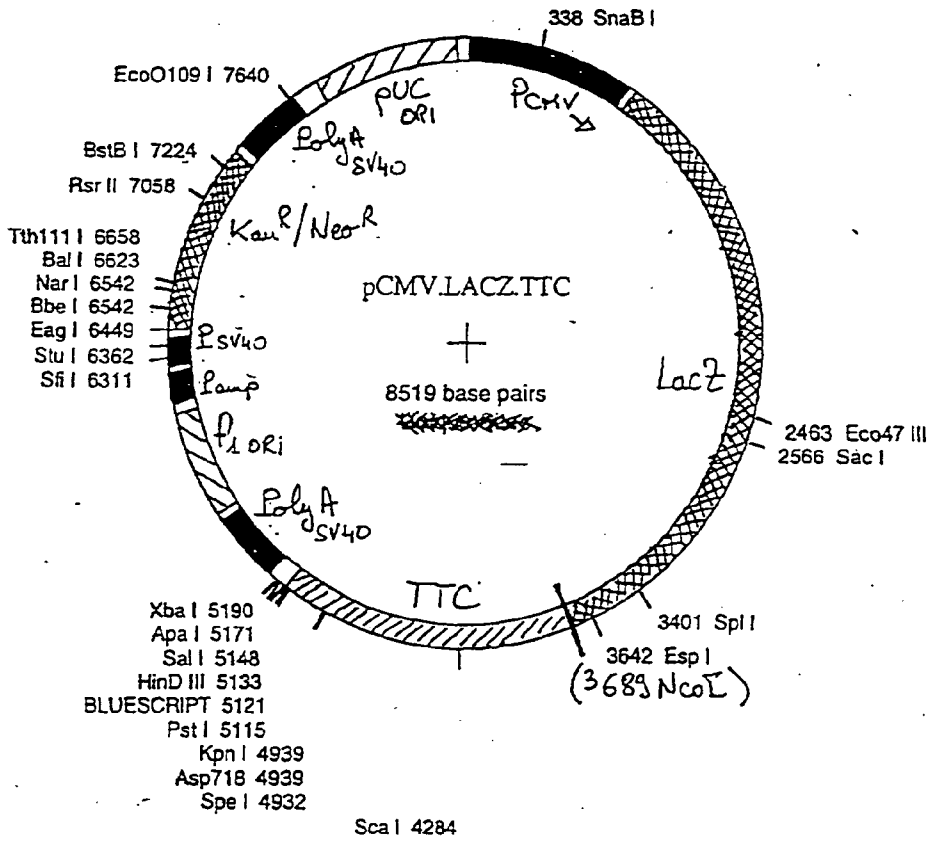DNA sequence   8519 b.p.   tagttattaata ... accgccatgcat   circular

338 SnaB I

EcoO109 I 7640

pUC ORI

PCMV

BstB I 7224

PolyA SV40

Rsr II 7058

Tth111 I 6658
Bal I 6623
Nar I 6542
Bbe I 6542
Eag I 6449
Stu I 6362
Sfi I 6311

Kan R / Neo R

pCMV.LACZ.TTC

8519 base pairs

P SV40

P amp

f1 ORI

PolyA SV40

Xba I 5190
Apa I 5171
Sal I 5148
HinD III 5133
BLUESCRIPT 5121
Pst I 5115
Kpn I 4939
Asp718 4939
Spe I 4932

Sca I 4284

Lac Z

2463 Eco47 III
2566 Sac I

TTC

3401 Spl I

3642 Esp I
(3689 NcoI)

FIGURE 5

HYBRID PROTEINS THAT MIGRATE RETROGRADELY AND TRANSYNAPTICALLY INTO THE CNS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of Provisional Application No. 60/055,615, filed Aug. 14, 1997 and Provisional Application No. 60/065,236, filed Nov. 13, 1997. The entire disclosure of each of these provisional applications is relied upon and incorporated by reference herein.

BACKGROUND OF THE INVENTION

[0002] This invention relates to the use of part of tetanus toxin for delivering a composition to the central nervous system of a human or animal. This invention also relates to a hybrid fragment of tetanus toxin, a polynucleotide that hybridizes with natural tetanus toxin, and a composition containing the tetanus toxin fragment as an active molecule. Further, this invention relates to a vector comprising a promoter and a nucleic acid sequence encoding the tetanus toxin fragment.

[0003] Tetanus toxin is produced by *Clostridium tetani* as an inactive, single, polypeptide chain of 150 kD composed of three 50 kD domains connected by protease-sensitive loops. The toxin is activated upon selective proteolytic cleavage, which generates two disulfide-linked chains: L (light, 50 kD) and H (heavy, 100 kD) [Montecucco C. and Schiavo G. Q. Rev. Biophys., (1995), 28: 423-472].

[0004] Evidence for the retrograde axonal transport of tetanus toxin to central nervous-system (CNS) has been described by Erdmann et al. [Naunyn Schmiedebergs Arch Phamacol., (1975), 290:357-373], Price et al. [Science, (1975), 188:945-94], and Stoeckel et al. [Brain Res., (1975), 99:1-16]. In each of these studies, radiolabeled toxin was found inside membrane bound vesicles. Another property was the transynaptic movement of tetanus toxin that was demonstrated first by autoradiographic localization of $_{125}$I-labeled tetanus toxin in spinal cord interneurons after injection into a muscle [Schwab and Thoenen, Brain res., (1976), 105:218-227].

[0005] The structure of this tetanus toxin has been elucidated by Belting et al. [J. Biol. Chem., (1977), 252:187-193]. Papain cleaves the tetanus toxin in two fragments:

[0006] the C terminal part of the heavy chain, 451 amino acids, also called fragment C; and

[0007] the other part contained the complementary portion called fragment B linked to the light chain (fragment A) via a disulfide bond.

[0008] European Patent No. EP 0 030 496 B1 showed the retrograde transport of a fragment to the CNS and was detected after injection in the median muscle of the eye in primary and second order neurons. This fragment may consist of "isofragments" obtained by clostridial proteolysis. Later, this fragment B-II$_b$ was demonstrated to be identical to fragment C obtained by papain digestion by Eisel et al. [EMBO J., 1986, 5:2495-2502].

[0009] This EP patent also demonstrated the retrograde transport of a conjugate consisting of a I$_{bc}$ tetanus toxin fragment coupled by a disulfide bond to B-II$_b$ from axonal endings within the muscle to the motoneuronal perikarya and pericellular spaces. (The I$_{bc}$ fragment corresponds to the other part obtained by papain digestion as described above by

Helting et al.). There is no evidence that this conjugate was found in second order neurons. The authors indicated that a conjugate consisting of the fragment B-II$_b$ coupled by a disulfide bond to a therapeutic agent was capable of specific fixation to gangliosides and synaptic membranes. No result showed any retrograde axonal transport or a transynaptic transport for such conjugate.

[0010] Another European Patent, No. EP 0 057 140 B1, showed equally the retrograde transport of a fragment II$_c$ to the CNS. As in the European Patent No. EP 0 030 496 31, the authors indicated that a conjugate consisting of the fragment II$_c$ and a therapeutic agent was capable of specific fixation, but no result illustrated such allegation. This fragment II$_c$ corresponds to the now called fragment C obtained by papain digestion.

[0011] Francis et al. [J. Biol. Chem., (1995), 270(25): 15434-15442] just led an in vitro study showing the internalization by neurons of hybrid between SOD-1 (Cu Zn superoxide dismutase) and a recombinant C tetanus toxin fragment by genetic recombination. This recombinant C tetanus toxin fragment was obtained from Halpern group. (See ref. 11).

[0012] Moreover, Kuypers H. G. J. M and Ugolini G. [TINS, (1990), 13(2):71-75] indicated in their publication concerning viruses as transneuronal tracers that, despite the fact that tetanus toxin fragment binds to specific receptors on neuronal membranes, transneuronal labeling is relatively weak and can be detected only in some of the synaptically connected neurons.

[0013] Notwithstanding these advances in the art, there still exists a need for methods for delivering compositions into the human or animal central nervous system. There also exists a need in the art for biological agents that can achieve this result.

SUMMARY OF THE INVENTION

[0014] This invention aids in fulfilling these needs in the art. More particularly, this invention-provides a method for in vivo delivery of desired composition into the central nervous system (CNS) of the mammal, wherein the composition comprises a non-toxic proteolytic fragment of tetanus toxin (TT) in association with at least a molecule having a biological function. The composition is capable of in vivo retrograde transport and transynaptic transport into the CNS and of being delivered to different areas of the CNS.

[0015] This invention also provides a hybrid fragment of tetanus toxin comprising fragment C and fragment B or a fraction thereof of at least 11 amino acid residues or a hybrid fragment of tetanus toxin comprising fragment C and fragment B or a fraction thereof of at least 11 amino acid residues and a fraction of fragment A devoid of its toxic activity corresponding to the proteolytic domain having a Zinc-binding motif located in the central part of the chain between the amino acids 225 and 245, capable of transferring in vivo a protein, a peptide, or a polynucleotide through a neuromuscular junction and at least one synapse.

[0016] Further, this invention provides a composition comprising an active molecule in association with the hybrid fragment of tetanus toxin (TT) or a variant thereof. The composition is useful for the treatment of a patient or an animal affected with CNS disease, which comprises delivering a composition of the invention to the patient or animal. In addition, the composition of this invention may be useful to

elicit a immune response in the patient or animal affected with CNS, which comprises delivering a composition of the invention to the patient or animal.

[0017] Moreover, this invention provides polynucleotide variant fragments capable of hybridizing under stringent conditions with the natural tetanus toxin sequence. The stringent conditions are for example as follows: at 42° C. for 4 to 6 hours in the presence of 6×SSC buffer, 1×Denhardt's Solution, 1% SDS, and 250 μg/ml of tRNA. (1×SSC corresponds to 0.15 M NaCl and 0.05 M sodium citrate; 1×Denhardt's solution corresponds to 0.02% Ficoll, 0.02% polyvinyl pyrrolidone and 0.02% bovine serum albumin). The two wash steps are performed at room temperature in the presence of 0.1×SCC and 0.1% SDS.

[0018] A polynucleotide variant fragment means a polynucleotide encoding for a tetanus toxin sequence derived from the native tetanus toxin sequence and having the same properties of transport.

[0019] In addition, the invention provides a vector comprising a promoter capable of expression in muscle cells and optionally an enhancer, a nucleic acid sequence coding for the fragment of tetanus toxin of the invention or an amino acid variant fragment of the invention associated with a polynucleotide coding for a protein or a polypeptide of interest. In a preferred embodiment of the invention the promoter can be the CMV promoter and preferably the CMV promoter contained in pcDNA 3.1 (In Nitrogen, ref. V790-20), or the promoter β actin as described in Bronson et al. (PNAS, 1996), 93:9067-9072).

[0020] In addition, the invention provides a vector comprising a promoter capable of expression in neuronal cells or in precursors (such NT2(hNT) precursor cells from Stratagen reference #204101) and optionally an enhancer, a nucleic acid sequence coding for the fragment of tetanus toxin of the invention or an amino acid variant fragment of the invention associated with a polynucleotide coding for a protein or a polypeptide of interest. In a preferred embodiment of the invention the promoter can be β actin (see the above reference). These vectors are useful for the treatment of a patient or an animal infected with CNS disease comprising delivering the vector of the invention to the patient or animal. In addition, these vectors are useful for eliciting immune responses in the patient or animal.

[0021] One advantage of the present invention comprising the fragment of tetanus toxin. (fragment A, B, and C) is to obtain a better transport of the fragment inside the organism compared with fragment C. Another advantage of the composition of the invention is to obtain a well defined amino acid sequence and not a multimeric composition. Thus, one can easily manipulate this composition in gene therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] This invention will be more fully described with reference to the drawings in which:

[0023] FIG. 1 shows the DNA sequence and amino acid sequence of the TTC fragment cloned in pBS:TTC.

[0024] FIG. 2 shows the details of construct pBS:TTC.

[0025] FIG. 3 depicts pGEX:lacZ-TTC construct.

[0026] FIG. 4 shows construct pGEX:TTC-lacZ.

[0027] FIG. 5 depicts the details of the construct pCMV:lacZ-TTC.

DETAILED DESCRIPTION

[0028] Tetanus toxin is a potent neurotoxin of 1315 amino acids that is produced by *Clostridium tetani* (1, 2). It prevents the inhibitory neurotransmitter release from spinal cord interneurons by a specific mechanism of cell intoxication (for review see ref 3). This pathological mechanism has been demonstrated to involve retrograde axonal and transynaptic transport of the tetanus toxin. The toxin is taken up by nerve endings at the neuromuscular junction, but does not act at this site; rather, the toxin is transported into a vesicular compartment and travels along motor axons for a considerable distance until it reaches its targets. The transynaptic movement of tetanus toxin was first demonstrated by autoradiographic localization in spinal cord interneurons after injection into a muscle (4). However, previous studies of transynaptic passage of tetanus toxin from motoneurons were limited by the rapid development of clinical tetanus and death of the experimental animal (4, 5, 6).

[0029] A fragment of tetanus toxin obtained by protease digestion, the C fragment, has been shown to be transported by neurons in a similar manner to that of the native toxin without causing clinical symptoms (7, 8, 9, 10). A recombinant C fragment was reported to possess the same properties as the fragment obtained by protease digestion (11). The fact that an a toxic fragment of the toxin molecule was able to migrate retrogradely within the axons and to accumulate into the CNS led to speculation that such a fragment could be used as a neurotrophic carrier (12). A C fragment chemically conjugated to various large proteins was taken up by neurons in tissue culture (13) and by motor neurons in animal models (ref. 12, 14, and 15). According to the invention the fragment of tetanus toxin consists of a non-toxic proteolytic fragment of tetanus toxin (TT) comprising a fragment C and a fragment B or a fraction thereof of at least 11 amino acid residues or a non-toxic proteolytic fragment of tetanus toxin (TT) comprising a fragment C and a fragment B or a fraction (thereof of at least 11 amino acids residues and a fraction of a fragment A devoid of its toxic activity corresponding to the proteolytic domain having a zinc-binding motif located in the central part of the chain between the amino acids 225 and 245 (cf. Montecucco C. and Schiavo G. Q. Rev. Biophys., (1995), 28:423-472). Thus the fraction of the fragment A comprises, for example, the amino acid sequence 1 to 225 or the amino acid sequence 245 to 457, or the amino acid sequence 1 to 225 associated with amino acid sequence 245 to 457.

[0030] The molecule having a biological function is selected from the group consisting of protein of interest, for example, for the compensation or the modulation of the functions under the control of the CNS or the spinal cord or the modulation of the functions in the CNS or the spinal cord, or protein of interest to be delivered by gene therapy expression system to the CNS or the spinal cord. The proteins of interest are, for example, the protein SMN implicated in spinal muscular atrophy (Lefebvre et al., Cell; (1995), 80:155-165' and Roy et al., Cell, (1955), 80:167-178); neurotrophic factors, such as BDNF (Brain-derived neurotrophic factor); NT-3 (Neurotrophin-3); NT-4/5; GDNF (Glial cell-line-derived neurotrophic factor); IGF (Insulin-like growth factor) (Oppenheim, Neuron, (1996), 17:195-197; Thoenen et al., Exp. Neurol., (1933), 124:47-55 and Henderson et al., Adv. Neurol., (1995), 68:235-240); or PNI (protease nexin I) promot-

3

ing neurite outgrowth (this factor can be used for the treatment of Alzheimer disease (Houenou et al., PNAS, (1995), 92:895-899)); or SPI3 a serine protease inhibitor protein (Safaei, Dev. Brain Res., (1997), 100: 5-12); or ICE (Interleukin-1β converting Enzyme) a factor implicated in apoptosis, to avoid apoptosis (Nagata, Cell, (1997), 88:355-365); or Bcl-2, a key intracellular regulator of programmed cell death (Jacobson, M. D. (1997), Current Biology, 7:R277-R281); or green fluorescent protein (Lang et al., Neuron, (1997), 18:857-863) as a marker; enzyme (ex: β-Gal); endonuclease like I-SceI (Choulika A., et al. (1995), Molecular and Cellular biology, 15 (4):1968-1973 or CRE (Gu H., et al. (1994), Science, 265:103-106); specific antibodies; drugs specifically directed against neurodegenerative diseases such as latero spinal amyotrophy. Several molecules can be associated with a TT fragment.

[0031] In association means an association obtained by genetic recombination. This association can be realized upstream as well as downstream to the TT fragment. The preferred mode of realization of the invention is upstream and is described in detail; a downstream realization is also contemplated. (Despite Halpern et al., J. Biol. Chem., (1993), 268(15):11188-11192, who indicated that the carboxyl-terminal amino acids contain the domain required for binding to purified gangliosides and neuronal cells.)

[0032] The desired CNS area means, for example, the tongue which is chosen to direct the transport to hypoglossal motoneuron; the arm muscle which is chosen to direct the transport to the spinal cord motoneurons.

[0033] For this realization of transplantation of a neuron to the CNS or the spinal cord see Gage, F. H. et al. (1987), Neuroscience, 23:725-807, "Grafting genetically modified cells to the brain: possibilities for the future."

[0034] The techniques for introducing the polynucleotides to cells fare described in U.S. Pat. Nos. 5,580,859 and 5,589,466, which is relied upon and incorporated by reference herein. For example, the nucleotides may be introduced by transfection in vitro before reimplantation in area of the CNS or the spinal cord.

[0035] A chemical linkage is considered for a particular embodiment of the invention and comprises the association between the TT fragment and a polynucleotide encoding the molecule of interest with its regulatory elements, such as promoter and enhancer capable of expressing said polynucleotide. Then the TT fragment allows the retrograde axonal transport and/or the transynaptic transport, and the product of the polynucleotide is expressed directly in the neurons. This chemical linkage can be covalent or not, but preferably covalent performed by thiolation reaction or by any other binding reaction as described in "Bioconjugate Techniques" from Gret T. Hermanson (Academic press, 1996).

[0036] The axonal retrograde transport begins at the muscle level, where the composition of interest is taken up at the neuromuscular junction, and migrates to the neuronal body of the motoneurons (which are also called the first order neurons) in the CNS or spinal cord. First order neurons mean neurons that have internalized the composition of interest, and thus in this case, correspond to motoneurons.

[0037] The transynaptic retrograde transport corresponds to interneuron communications via the synapses from the motoneurons, and comprises second order neurons and higher order neurons (fourth order corresponding to neurons in the cerebral cortex).

[0038] The different stages of the neuronal transport are through the neuromuscular junction, the motoneuron, also called first order neuron, the synapse at any stage between the neurons of different order, neuron of order second to fourth order, which corresponds to the cerebral cortex.

[0039] In one embodiment of this invention, it is shown that a β-gal-TTC (TT-fragment C) hybrid protein retains the biological activities of both proteins in vivo. Therefore, the hybrid protein can undergo retrograde and transneuronal transport through a chain of interconnected neurons, as traced by its enzymatic activity. These results are consistent with those of others who used chemically conjugated TTC, or TTC fused to other proteins (12, 13, 14, 15). In these in vitro analyses, the activity of the conjugated or hybrid proteins was likewise retained or only weakly diminished. Depending on the nature of the TTC fusion partner, different types of potential applications can be envisioned. For example, this application can be used to deliver a biologically active protein into the CNS for therapeutic purposes. Such hybrid genes can also be used to analyze and map synaptically connected neurons if reporters, such as lacZ or the green fluorescent protein (GFP; 29) gene, were fused to TTC.

[0040] The retrograde transport of the hybrid protein may be demonstrated as follows. When injected into a muscle, β-gal activity rapidly localized to the somata of motoneurons that innervate the muscle. The arborization of the whole nerve, axon, somata and dendrites can easily be visualized. However, in comparison to the neurotropic viruses, the extent of retrograde transneuronal transport of the hybrid protein from the hypoglossal neurons indicates that only a subset of interconnected neurons is detected, although most areas containing second-order interneurons have been identified by the β-gal-TTC marker. Transneuronal uptake is mostly restricted to second order neurons. In such experiments, when a limited amount of a neuronal tracer is injected into a muscle or cell, only a fraction will be transported through a synapse, thereby imposing an experimental constraint on its detection. Presently, the most efficient method, in terms of the extent of transport, relies on neurotropic viruses. Examples include: alpha-herpes viruses, such as herpes simplex type 1 (HSV-1), pseudorabies virus (PrV), and rhabdoviruses (24, 25). Viral methods are very sensitive because each time a virus infects a new cell, it replicates, thereby amplifying the signal and permitting visualization of higher order neurons in a chain. Ultimately, however, one wants to map a neuronal network in an in vivo situation such as a transgenic animal. Here, the disadvantage of viral labeling is its potential toxicity. Most viruses are not innocuous for the neural cell, and their replication induces a cellular response and sometimes cell degeneration (24). Furthermore, depending on experimental conditions, budding of the virus can occur leading to its spread into adjoining cells and tissues.

[0041] Differences in mechanisms of transneuronal migration could also account for the restricted number of neurons labeled by β-gal-TTC. Matteoli et al have provided strong evidence that the intact tetanus toxin crosses the synapses by parasitizing the physiological-process of synaptic vesicle recycling at the nerve terminal (22). The toxin probably binds to the inner surface of a synaptic vesicle during the time the lumen is exposed to the external medium. Vesicle endocytosis would then presumably provide the mechanism for internalization of the toxin. Because the TTC fragment is known to mimic the migration of the toxin in vivo, it could therefore direct the fusion protein along a similar transynaptic pathway.

4

If this hypothesis is confirmed, it would strongly suggest that synaptic activity is required for the transneuronal transport of β-gal-TTC. Therefore, only active neuronal circuits would be detected by the hybrid protein. The possible dependence of β-gal-TTC on synaptic vesicle exocytosis and endocytosis could be further investigated, since techniques are now available to record synaptic activity in neural networks in vitro (30). In contrast, the transneuronal pathway of neurotropic viruses has not yet been elucidated and could be fundamentally different, involving virus budding in the vicinity of a synapse. Finally, the transneuronal transport of the hybrid protein might depend on a synaptic specificity, although the tetanus toxin is not known to display any (7, 23). It is therefore likely that a virus would cross different or inactive synapses. In summary, the restricted spectrum of interneuronal transport, in addition to its non-toxicity, make the β-gal-TTC hybrid protein a novel and powerful tool for analysis of neural pathways.

[0042] One advantage of the fusion gene of the invention, for neuronal mapping is that it derives from a single genetic entity that is amenable to genetic manipulation and engineering. Several years ago, a technique based on homologous recombination in embryonic stem cells was developed to specifically replace genes in the mouse (31, 32). This method generates a null mutation in the substituted gene, although in a slightly modified strategy, a dicistronic messenger RNA can also be produced (33, 34). When a reporter gene, such as *E. coli* lacZ, is used as the substituting gene, this technique provides a means of marking the mutated cells so that they can be followed during embryogenesis. Thus, this technique greatly simplifies the analysis of both the heterozygote expression of the targeted gene as well as the phenotype of null (homozygous) mutant animals.

[0043] Another advantage of this invention is that the composition comprising the fusion gene may encode an antigen or antigens. Thus, the composition may be used to elicit an immune response in its host and subsequently confer protection of the host against the antigen or antigens expressed. These immunization methods are described in Robinson et al., U.S. Pat. No. 5,43,578, which is herein incorporated by reference. In particular, the method of immunizing a patient or animal host comprises introducing a DNA transcription unit encoding comprising the fusion gene of this invention, which encodes a desired antigen or antigens. The uptake of the DNA transcription unit by the host results in the expression of the desired antigen or antigens and the subsequent elicitation of humoral and/or cell-mediated immune responses.

[0044] Neural cells establish specific and complex networks of interconnected cells. If a gene were mutated in a given neural cell, we would expect this mutation to have an impact on the functions of other, interconnected neural cells. With these considerations in mind, a genetic marker that can diffuse through active synapses would be very useful in analyzing the effect of the mutation. In heterozygous mutant animals, the cells in which the targeted gene is normally transcribed could be identified, as could the synaptically connected cells of a neural network. In a homozygous animal, the impact of the mutation on the establishment or activity of the neural network could be determined. The feasibility of such an in vivo approach depends critically on the efficiency of synaptic transfer of the fusion protein, as well as its stability and cellular localization.

[0045] Another extension of the invention is to gene therapy applied to the CNS. This invention provides for uptake of a non-toxic, enzyme-vector conjugate by axon terminals and conveyance retrogradely to brainstem motoneurons. A selective retrograde transynaptic mechanism subsequently transports the hybrid protein into second-order connected neurons. Such a pathway, which by-passes the blood-brain barrier, can deliver macromolecules to the CNS. In fact, pathogenic agents, such as tetanus toxin and neurotropic viruses, are similarly taken up by nerve endings, internalized, and retrogradely transported to the nerve tell somata. In such a scenario, the lacZ reporter would be replaced by a gene encoding a protein that provides a necessary or interesting activity and/or function. For example, the human CuZn superoxide dismutase, SOD-1, and the human enzyme β-N-acetylhexosaminidase A, HexA, have been fused or chemically coupled to the TTC fragment (13, 16), and their uptake by neurons in vitro was considerably increased and their enzymatic functions partially conserved. Combined with the in vivo experiments described here using β-gal-TTC, a gene therapy approach based on TTC hybrid proteins appears to be a feasible method of delivering a biological function to the CNS. However, ways have to be found to target the TTC hybrid proteins, which are likely to be sequestrated into vesicles, to the appropriate subcellular compartment. Such a therapeutic strategy could be particularly useful for treating neurodegenerative and motoneuron diseases, such as amyotrophy lateral sclerosis (ALS, 35), spinal muscular atrophies (SMA, 36, 37), or neurodegenerative lysosomal storage diseases (38, 39). Injection into selected muscles, even in utero, could help to specifically target the appropriate neurons. In addition, such an approach would avoid the secondary and potentially toxic effects associated with the use of defective viruses to deliver a gene (40, 41).

EXAMPLES

Example 1

Plasmid Constructions

[0046] (A) TTC Cloning:

[0047] Full length TTC DNA was generated from the genomic DNA from the *Clostridium Tetani* strain (a gift from Dr. M. Popoff, Institut Pasteur) using PCR. Three overlapping fragments were synthesized: PCR1 of 465 by (primer 1: 5'-CCC CCC GGG CCA CCA TGG TTT TTT CAA CAC CAA TTC CAT TTT CTT ATT C-3' and primer 2: 5'-CTA AAC CAG TAA TTT CTG-3'), PCR2 of 648 by (primer 3: 5'-AAT TAT GGA CTT TAA AAG ATT CCG C-3' and primer 4: 5'-GGC ATT ATA ACC TAC TCT TAG AAT-3') and PCR3 of 338 by (primer 5: 5'-AAT GCC TTT AAT AAT CTT GAT AGA AAT-3' and primer 6: 5'-CCC CCC GGG CAT ATG TCA TGA ACA TAT CAA TCT GTT TAA TC-3'). The three fragments were sequentially introduced into pBluescript KS+ (Stratagene) to give pBS:TTC plasmid. The upstream primer 1 also contains an optimized eukaryotic Ribosome Binding Site (RBS) and translational initiation signals. Our TTC fragment (462 amino acids) represents the amino acids 854-1315 of tetanus holotoxin, i.e. the carboxy-terminal 451 amino acids of the heavy chain, which constitute the fragment C plus 11 amino acids of the heavy chain that immediately precede the amino terminus of the fragment C. The DNA sequence and amino acid sequence of the TTC fragment cloned in pBS:TTC is shown in FIG. **1**. The construct pBS:TTC is shown in FIG. **2**.

[0048] (B) pGEX:lacZ-TTC:

[0049] pGEX:lacZ was obtained by cloning a SmaI/XhoI lacZ fragment from the pGNA vector (a gift from Dr. H. Le Mouellic) into pGEX 4T-2 (Pharmacia). PCR was used to convert the lacZ stop codon into an NcoI restriction site. Two primers (upstream: 5'-CTG AAT ATC GAC GGT TTC CAT ATG-3' and downstream: 5'-GGC AGT CTC GAG TCT AGA CCA TGG CTT TTT GAC ACC AGA C-3') were used to amplify the sequence between NdeI and XhoI, generating pGEX:lacZ(NcoI) from pGEX:lacZ. pGEX:lacZ-TTC was obtained by insertion of the TTC NcoI/XhoI fragment into pGEX:lacZ(NcoI), fusing TTC immediately downstream of the lacZ coding region and in the same reading frame. FIG. 3 shows the details of the pGEX:lacZ-TTC construct.

[0050] (C) pGEX:TTC-lacZ:

[0051] pBS:TTC was modified to change NcoI into a BamHI restriction site (linker 5'-CAT GAC TGG GGA TCC CCA GT-3') at the start of the TTC DNA, to give pBS:TTC (BamHI) plasmid. pGEX:TTC was obtained by cloning. The TTC BamHI/SmaI fragment from pBS:TTC(BamHI) into pGEX 4T-2 (Pharmacia). PCR was used to convert the TTC stop codon into an NheI restriction site. Two primers (upstream: 5'-TAT GAT AAA AAT GCA TCT TTA GGA-3' and downstream: 5'-TGG AGT CGA CGC TAG CAG GAT CAT TTG TCC ATC CTT C-3') were used to amplify the sequence between NsiI and SmaI, generating pGEX:TTC(NheI) from pGEX:TTC. The lacZ cDNA from plasmid pGNA was modified in its 5' extremity to change SacII into an NheI restriction site (linker 5'-GCT AGC GC-3'). pGEX:TTC-lacZ was obtained by insertion of the lacZ NheI/XhoI fragment into pGEX:TTC(NheI), fusing lacZ immediately downstream of the TTC coding region and in the same reading frame. The details of the construct of pGEX:TTC-lacZ are shown in FIG. 4.

[0052] (D) pCMV:lacZ-TTC:

[0053] pCMV vector was obtained from pGFP-C1 (Clontech laboratories) after some modifications: GFP sequence was deleted by a BglII/NheI digestion and relegation, and SacII in the polylinker was converted into an AscI restriction site (linkers 5'-GAT ATC GGC GCG CCA GC-3' and 5'-TGG CGC GCC GAT ATC GC-3').

[0054] pBluescript KS+ (Stratagene) was modified to change XhoI into an AscI restriction site (linker 5'-TCG ATG GCG CGC CA-3'), giving pBS(AscI) plasmid. pBS:lacZ-TTC was obtained by cloning a XmaI lacZ-TTC fragment from pGEX:lacZ-TTC into pBS(AscI). pCMV:lacZ-TTC was obtained by insertion of the lacZ-TTC XmnI/AscI fragment into pCMV vector at the XhoI and AscI sites (XhoI and XmnI was eliminated with the clonage), putting the fusion downstream of the CMV promotor. FIG. 8 shows the details of the construct pCMV:lacZ-TTC. Plasmid pCMV:lacZ-TTC was deposited on Aug. 12, 1997, at the Collection Nationale de Cultures de Microorganisms (CNCM), Institut Pasteur, 25, Rue de Docteur Roux, F-75724, Paris Cedex 15, France, under Accession No. 1-1912.

Example 2

Purification of the Hybrid Protein

[0055] The *E. coli* strain SR3315 (a gift from Dr. A. Pugsley, Institut Pasteur) transfected with pGEX:lacz-TTC was used for protein production. An overnight bacterial culture was diluted 1:100 in LB medium containing 100 μg/ml ampicillin, and grown for several hours at 32° C. until an OD of 0.5

was reached. Induction from the Ptac promoter was achieved by the addition of 1 mM IPTG and 1 mM MgCl$_2$ and a further 2 hrs incubation. The induced bacteria were pelleted by centrifugation for 20 min at 3000 rpm, washed with PBS and resuspended in lysis buffer containing 0.1M Tris pH 7.8, 0.1M NaCl, 20% glycerol, 10 mM EDTA, 0.1% Triton-X100, 4 mM DTT, 1 mg/ml lysozyme, and a mixture of anti-proteases (100 μg/ml Pefablok, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM benzamidine). After cell disruption in a French Press, total bacterial lysate was centrifuged for 10 min at 30000 rpm. The resulting supernatant was incubated overnight at 4° C. with the affinity matrix Glutathione Sepharose 4B (Stratagene) with slow agitation. After centrifugation for 5 min at 3000 rpm, the matrix was washed three times with the same lysis buffer but without lysozyme and glycerol, and then three times with PBS. The resin was incubated overnight at 4° C. with Thrombin (10 U/ml; Sigma) in PBS in order to cleave the β-gal-TTC fusion protein from the Glutatione-S-transferase (GST) sequence and thereby elute it from the affinity column. Concentration of the eluted fusion protein was achieved by centrifugation in centricon X-100 tubes (Amicon; 100,000 MW cutoff membrane).

[0056] Purified hybrid protein was analyzed by Western blotting after electrophoretic separation in 8% acrylamide SDS/PAGE under reducing conditions followed by electrophoretic transfer onto nitrocellulose membranes (0.2 mm porosity, BioRad). Immunodetection of blotted proteins was performed with a Vectastaln ABC-alkaline phosphatase kit (Vector Laboratories) and DAB color development. Antibodies were used as follows: rabbit anti-β-gal antisera (Capel), dilution 1:1000; rabbit anti-TTC antisera (Calbiochem), dilution 1:20000. A major band with a relative molecular mass of 180 kDa corresponding to the β-Gal-TTC hybrid protein was detected with both anti-β-Gal anti-TTC antibodies.

Example 3

Binding and Internalization of Recombinant Protein in Differentiated 1009 Cells

[0057] The 1009 cell line was derived from a spontaneous testicular teratocarcinoma arising in a recombinant inbred mouse strain (129×B6) (17). The 1009 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and passaged at subconfluence. In vitro differentiation with retinoic acid and cAMP was perforted as described (18). Eight days after retinoic acid treatment, cells were used for the internalization experiments with either the hybrid protein or β-gal.

[0058] Binding and internalization of the β-Gal-TTC fusion were assessed using a modified protocol (16). Differentiated 1009 cells were incubated for 2 hrs at 37° C. with 5 μg/ml of β-Gal-TTC or β-Gal protein diluted in binding buffer (0.25% sucrose, 20 mM Tris acetate 1 mM CaCl$_2$, 1 mM MgCl$_2$, 0.25% bovine serum albumin, in PBS). The cells were then incubated with 1 μg/ml Pronase E (Sigma) in PBS for 10 min at 37° C., followed by washing with proteases inhibitors diluted in PBS (100 μg/ml Pefablok, 1 mM benzamidine).

[0059] The cells were fixed with 4% formalin in PBS for 10 min at room temperature (RT) and then washed extensively with PBS. β-gal activity was detected on fixed cells by an overnight staining at 37° C. in X-Gal solution (0.8 mg/ml X-Gal, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 4 mM MgCl$_2$ in PBS). For electron microscopy,

the cells were further fixed in 2.5% glutaraldehyde for 18 hrs, and then processed as described (19).

[0060] For immunohistochemical labeling, cells were fixed with 4% paraformaldehyde in PBS for 10 min at RT then washed extensively with PBS, followed by a 1 hr incubation at RT with 2% BSA/0.02% Triton X-100 in PBS. Cells were co-incubated in primary antibodies diluted in 2% BSA/0.02% Triton X-100 in PBS for 2 hrs at RT. Antibodies used were a mouse anti-neurofilament antibody (NF 200 Kd, dilution 1:50; Sigma) or the rabbit anti-TTC antibody (dilution 1:1000). The labeling was visualized using fluorescent secondary antibodies: Cy3, goat anti-rabbit IgG (dilution 1:500; Amersham) or anti-mouse IgG with extravidin-FITC (dilution 1:200; Sigma). Cells were mounted in moviol and visualized with epifluorescence.

Example 4

In Vivo Recombinant Protein Injection

[0061] 14-week old B6D2F1 mice were obtained from IFFA-CREDO. The animal's tongue muscle was injected using an Hamilton syringe (20 μl per animal) while under general anesthesia with 3% Avertin (15 μl/g of animal). The protein concentration was 0.5 to 5 μg/μl in PBS; therefore, mice received approximately 10 to 100 μg per injection. Animals were kept alive for 12 hrs to 48 hrs post-injection to permit migration of the injected protein, and in no case were any tetanus symptoms detected. The mice were sacrificed by intracardiac perfusion with 4% paraformaldehyde in PBS while under deep anesthesia. Brains were harvested; rinsed in PBS and incubated in 15% sucrose overnight at 4° C., then mounted in tissue-tek before sectioning, 15 μm thick slices using a cryostat.

Example 5

Histology, Immunohistology, and X-Gal Staining

[0062] For in toto X-Gal staining of the dissected brain and tongue, mice (10 animals) were sacrificed and fixed as described above. The brain was further cut with a scalpel along a median plane and directly incubated for 12 hrs in X-Gal solution.

[0063] For immunohistology, sections were incubated In a 1:5000 dilution of anti-TTC antibody in 2% BSA/0.02% Triton X-100 in PBS overnight at 4° C. after nonspecific antibody binding sites were blocked by a 1 hr incubation in the same buffer. Antibody detection was carried out using the Vectastain ABC-alkaline phosphatase kit with DAB color development. For X-Gal staining, sections were incubated in X-Gal solution and counterstained for 30 sec with hematoxylin 115 (v/v) in PBS. Histology on adjacent sections was done after X-Gal staining, using a 30 sec incubation in hematoxylin/thionin solution. All sections were mounted in moviol before eight microscopy analysis.

Example 6A

Internalization of the β-Gal-TTC Fusion Protein by Neurons In Vitro

[0064] Differentiation of 1009 cells with retinoic acid and cAMP in vitro yields neuronal and glial cells (18, 20). X-Gal staining or immunolabeling were performed after incubation with the β-gal-TTC fusion protein or with either the β-gal or TTC proteins alone. Only when the hybrid protein was incubated with differentiated 1009 cells was a strong X-Gal staining detected in cells having a neuronal phenotype. No signal was detected when β-gal alone was incubated under the same conditions. A similar X-Gal staining pattern was obtained after pronase treatment of the cells to remove surface bound proteins, indicating that the hybrid protein had been internalized. The intracellular localization of the hybrid protein was further confirmed by electron microscopic analysis of X-Gal-stained cells. Furthermore, the enzymatic activity observed in axons seemed to be localized in vesicles associated with filaments, which is in agreement with previous work on TTC fragment or native tetanus toxin (14, 21, 22). Co-labeling with anti-TTC and anti-neurofilament antibodies revealed that β-gal activity co-localized with TTC fragment in neuronal cells. No glial cells were labeled with either antibody.

Example 6B

Internalization of the TTC-β-Gal Fusion Protein by Neurons In Vitro

[0065] The method used for the internalization was identical to that described in Example 6 above. The results show efficiently internalization of the hybrid as in Example 6 above.

Example 7

Retrograde Transport of the Hybrid Protein In Vivo

[0066] To study the behavior of the β-gal-TTC protein in vivo, the hybrid protein was tested in a well characterized neuronal network, the hypoglossal system. After intramuscular injection of β-gal-TTC protein into the mouse tongue, the distribution of the hybrid protein in the CNS was analyzed by X-Gal staining. Various dilutions of the protein were injected and sequential time points were analyzed to permit protein transport into hypoglossal motoneurons (Xll), and its further transneuronal migration into connected second order neurons.

[0067] A well-defined profile of large, apparently retrogradely labeled neurons was clearly evident in the hypoglossal structure, analyzed in toto at 12 hrs post-injection. A strong labeling was also apparent in the hypoglossal nerve (Xlln) of the tongue of the injected mice. At the level of muscle fibers, button structures were observed that might reflect labeling of neuromuscular junctions where the hybrid protein was internalized into nerve axons. These data demonstrate that the β-gal-TTC hybrid protein can migrate rapidly by retrograde axonal transport as far as motoneuron cell bodies, after prior uptake by nerve terminals in the tongue. This specific uptake and the intraaxonal transport are similar to the properties that have been described for the native toxin (6, 21, 23).

[0068] Transport of the hybrid protein was examined in greater detail by analyzing X-Gal-stained brain sections. Motoneurons of the hypoglossal nucleus became labeled rapidly, with 12 hrs being the earliest time point examined. Most of the label was confined to neuronal somata, the cell nuclei being unlabeled. The intensity of the labeling depends upon the concentration of the β-gal-TTC protein injected: when 10 μg of protein was injected, only the hypoglossal somata were detected, whereas with 25 to 50 μg a fuzzy network of dendrites was visualized; transynaptic transfer was detected with 100 μg of hybrid protein. An identical distribution of label was observed then brain sections were immunostained with

7

an anti-TTC antibody, demonstrating that β-gal and TTC fragment co-localize within cells. Finally, injection of β-gal alone did not result in labeling of the hypoglossal nuclei and therefore confirms that transport of the hybrid protein is TTC-dependent. Labeling with an anti-TTC antibody was less informative than detection of β-gal activity; for instance, the nerve pathway to the brain could not be visualized by anti-TTC immunostaining. At 18 hrs post-injection, labeling was observed in the hypoglossal nuclei: all motoneuron cell bodies and the most proximal part of their dendrites were very densely stained. In contrast, no labeling was ever detected in glial cells adjoining Xll motoneurons or their axons. Our results are in accordance with others who reported an identical pattern of immunolabeling after injection of the TTC fragment alone (9). Transneuronal transfer is detectable after 24 hrs. An additional 24 hrs and beyond did not yield a different staining.

Example 8

Transneuronal Transport of the Hybrid Protein

[0069] Second order interneurons, as well as higher order neurons that synapse with the hypoglossal motoneurons, have been extensively analyzed using conventional markers, such as the wheat germ agglutinin-horseradish peroxidase complex (WGA-HRP) or neurotropic viruses such as alpha-herpes (24) and rhabdoviruses (25). An exhaustive compilation of regions in the brain that synaptically connect to the hypoglossal nucleus has also been described recently (25). In this invention, the distribution of the β-gal-TTC fusion depended on the initial concentration of protein injected into the muscle and the time allowed for transport after injection. Up to 24 hrs post-injection, labeling was restricted to the hypoglossal nuclei. After 24 hrs, the distribution of second order transneuronally labeled cells in various regions of the brain was consistent and reproducible. Even at longer time points (e.g. 48 hrs), labeling of the hypoglossal nucleus remained constant. At higher magnification, a discrete and localized staining of second-order neurons was observed, suggesting that the hybrid protein had been targeted to vesicles within cell somata, synapses and axons. A similar patchy distribution was previously described for tetanus toxin and TTC fragment alone (14, 21, 22).

[0070] Intense transneuronal labeling was detected in the lateral reticular formation (LRF), where medullary reticular neurons have been reported to form numerous projections onto the hypoglossal nucleus (26, 27). β-gal activity was detected bilaterally in these sections. Label led LRF projections formed a continuous column along the rostrocaudal axis, beginning lateral to the hypoglossal nucleus, with a few neurons being preferentially stained in the medullary reticular dorsal (MdD) and the medullary reticular ventral (MdV) nuclei. This column extends rostrally through the medulla, with neurons more intensely labeled in the parvicellular reticular nucleus (PCRt, caudal and rostral). After 48 hrs, cells in MdD and PCRt were more intensely stained. A second bilateral distribution of medullary neurons projecting to the hypoglossal nucleus was detected in the solitary nucleus (Sol) but the labeling was less intense than in the reticular formation, presumably because relatively few cells of the solitary nucleus project onto the hypoglossal nucleus (26). However, no labeling was found in the spinal trigeminal nucleus (Sp5), which has also been shown to project onto the hypoglossal nucleus (26). Transynaptic transport of the β-gal-TTC protein was also detected in the pontine reticular nucleus caudal (PnC), the locus coeruleus (LC), the medial vestibular

nucleus (MVe) and in a few cells of the inferior vestibular nucleus (IV). These cell groups are known to project onto the hypoglossal nucleus (25), but their labeling was weak, probably because of the greater length of their axons. A few labeled cells were observed in the dorsal paragigantocellular nucleus (DPGi), the magnocellular nucleus caudal (RMc), and the caudal raphe nucleus (R); their connections to the hypoglossal nucleus have also been reported (25). Finally, labeled neurons were detected bilaterally in midbrain projections, such as those of the mesencephalic trigeminal nucleus (Me5), and a few neurons were stained in the mesencephalic central gray region (CG). These latter nuclei have been typed as putative third order cell groups related to the hypoglossal nucleus (25).

[0071] Neurons in the motor trigeminal nucleus (Mo5) and the accessory trigeminal tract (Acs5) were also labeled, along with a population of neurons in the facial nucleus (N7). However, interpretation of this labeling is more ambiguous, since it is known that motoneurons in these nuclei also innervate other parts of the muscular tissue, and diffusion of the hybrid protein might have occurred at the point of injection. Conversely, these nuclei may have also projected to the tongue musculature via nerve XII, since neurons in N7 have been reported to receive direct hypoglossal nerve input (28). This latter explanation is consistent with the fact that labeling in these nuclei was detected only after 24 hrs; however, this point was not further investigated.

[0072] Altogether, the data summarized in Table 1 clearly establish transneuronal transport of the β-gal-TTC fusion protein from the hypoglossal neurons into several connected regions of the brainstem.

TABLE 1

Transneuronal transport of the lacZ-TTC fusion from the XII nerve: labeling of different cells types in the central nervous system.		
Cell groups	12-18 hrs	24-48 hrs
First order neurons		
First category:		
XII, hypoglossal motoneurons	++	+++
Second category:		
N7, facial nu	–	++
Mo5, motor trigeminal nu	–	++
Acs5, accessory trigeminal nu	–	+
Second order cell groups		
MdD, medullary reticular nu, dorsal	–	++
MdV, medullary reticular nu, ventral	–	+/–
PCRt, parvicellular reticular nu, caudal	–	++
PCRt, parvicellular reticular nu, rostral	–	++
Sol, solitary tract nu	–	+
DPGi, dorsal paragigantocellular nu	–	+/–
PnC, pontine reticular nu, caudal	–	+
RMc, magnocellular reticular nu	–	+/–
R, caudal raphe nu	–	+/–
MVe, medial vestibular nu	–	+
IV, inferior vestibular nu	–	+/–
LC, locus coeruleus	–	+
Me5, mesencephalic trigeminal nu (*)	–	+
CG, mesenphalic central gray (*)	–	+/–

(*) Represents second order cell groups that also contain putative third order neurons (see text).
–, no labeling;
+ to +++, increased density of label;
+/– weak labeling.
16 animals were analysed for the 12-18 hrs p.i. data; 6 animals were analysed for the 24-48 hrs p.i. data.

8

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SEQUENCE LISTING

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aagctggagc tcggtacccg gccacc atg gtt ttt tca aca cca att cca ttt          114
                              Met Val Phe Ser Thr Pro Ile Pro Phe
                               1               5

tct tat tct aaa aat ctg gat tgt tgg gtt gat aat gaa gaa gat ata          162
Ser Tyr Ser Lys Asn Leu Asp Cys Trp Val Asp Asn Glu Glu Asp Ile
 10                  15                  20                  25

gat gtt ata tta aaa aag agt aca att tta aat tta gat att aat aat          210
Asp Val Ile Leu Lys Lys Ser Thr Ile Leu Asn Leu Asp Ile Asn Asn
                 30                  35                  40

gat att ata tca gat ata tct ggg ttt aat tca tct gta ata aca tat          258
Asp Ile Ile Ser Asp Ile Ser Gly Phe Asn Ser Ser Val Ile Thr Tyr
             45                  50                  55

cca gat gct caa ttg gtg ccc gga ata aat ggc aaa gca ata cat tta          306
Pro Asp Ala Gln Leu Val Pro Gly Ile Asn Gly Lys Ala Ile His Leu
         60                  65                  70

gta aac aat gaa tct tct gaa gtt ata gtg cat aaa gct atg gat att          354
Val Asn Asn Glu Ser Ser Glu Val Ile Val His Lys Ala Met Asp Ile
     75                  80                  85

gaa tat aat gat atg ttt aat aat ttt acc gtt agc ttt tgg ttg agg          402
Glu Tyr Asn Asp Met Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg
 90                  95                 100                 105

gtt cct aaa gta tct gct agt cat tta gaa caa tat ggc aca aat gag          450
Val Pro Lys Val Ser Ala Ser His Leu Glu Gln Tyr Gly Thr Asn Glu
                110                 115                 120

tat tca ata att agc tct atg aaa aaa cat agt cta tca ata gga tct          498
Tyr Ser Ile Ile Ser Ser Met Lys Lys His Ser Leu Ser Ile Gly Ser
             125                 130                 135

ggt tgg agt gta tca ctt aaa ggt aat aac tta ata tgg act tta aaa          546
Gly Trp Ser Val Ser Leu Lys Gly Asn Asn Leu Ile Trp Thr Leu Lys
         140                 145                 150

gat tcc gcg gga gaa gtt aga caa ata act ttt agg gat tta cct gat          594
Asp Ser Ala Gly Glu Val Arg Gln Ile Thr Phe Arg Asp Leu Pro Asp
     155                 160                 165

aaa ttt aat gct tat tta gca aat aaa tgg gtt ttt ata act att act          642
Lys Phe Asn Ala Tyr Leu Ala Asn Lys Trp Val Phe Ile Thr Ile Thr
170                 175                 180                 185

aat gat aga tta tct tct gct aat ttg tat ata aat gga gta ctt atg          690
Asn Asp Arg Leu Ser Ser Ala Asn Leu Tyr Ile Asn Gly Val Leu Met
                190                 195                 200

gga agt gca gaa att act ggt tta gga gct att aga gag gat aat aat          738
Gly Ser Ala Glu Ile Thr Gly Leu Gly Ala Ile Arg Glu Asp Asn Asn
             205                 210                 215

ata aca tta aaa cta gat aga tgt aat aat aat aat caa tac gtt tct          786
Ile Thr Leu Lys Leu Asp Arg Cys Asn Asn Asn Asn Gln Tyr Val Ser
         220                 225                 230

att gat aaa ttt agg ata ttt tgc aaa gca tta aat cca aaa gag att          834
Ile Asp Lys Phe Arg Ile Phe Cys Lys Ala Leu Asn Pro Lys Glu Ile
     235                 240                 245

gaa aaa tta tac aca agt tat tta tct ata acc ttt tta aga gac ttc          882
Glu Lys Leu Tyr Thr Ser Tyr Leu Ser Ile Thr Phe Leu Arg Asp Phe
250                 255                 260                 265

tgg gga aac cct tta cga tat gat aca gaa tat tat tta ata cca gta          930
Trp Gly Asn Pro Leu Arg Tyr Asp Thr Glu Tyr Tyr Leu Ile Pro Val
                270                 275                 280

gct tct agt tct aaa gat gtt caa ttg aaa aat ata aca gat tat atg          978
Ala Ser Ser Ser Lys Asp Val Gln Leu Lys Asn Ile Thr Asp Tyr Met
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tat ttg aca aat gcg cca tcg tat act aac gga aaa ttg aat ata tat    1026
Tyr Leu Thr Asn Ala Pro Ser Tyr Thr Asn Gly Lys Leu Asn Ile Tyr
        300              305              310

tat aga agg tta tat aat gga cta aaa ttt att ata aaa aga tat aca    1074
Tyr Arg Arg Leu Tyr Asn Gly Leu Lys Phe Ile Ile Lys Arg Tyr Thr
        315              320              325

cct aat aat gaa ata gat tct ttt gtt aaa tca ggt gat ttt att aaa    1122
Pro Asn Asn Glu Ile Asp Ser Phe Val Lys Ser Gly Asp Phe Ile Lys
330              335              340              345

tta tat gta tca tat aac aat aat gag cac att gta ggt tat ccg aaa    1170
Leu Tyr Val Ser Tyr Asn Asn Asn Glu His Ile Val Gly Tyr Pro Lys
                350              355              360

gat gga aat gcc ttt aat aat ctt gat aga att cta aga gta ggt tat    1218
Asp Gly Asn Ala Phe Asn Asn Leu Asp Arg Ile Leu Arg Val Gly Tyr
                365              370              375

aat gcc cca ggt atc cct ctt tat aaa aaa atg gaa gca gta aaa ttg    1266
Asn Ala Pro Gly Ile Pro Leu Tyr Lys Lys Met Glu Ala Val Lys Leu
            380              385              390

cgt gat tta aaa acc tat tct gta caa ctt aaa tta tat gat gat aaa    1314
Arg Asp Leu Lys Thr Tyr Ser Val Gln Leu Lys Leu Tyr Asp Asp Lys
        395              400              405

aat gca tct tta gga cta gta ggt acc cat aat ggt caa ata ggc aac    1362
Asn Ala Ser Leu Gly Leu Val Gly Thr His Asn Gly Gln Ile Gly Asn
410              415              420              425

gat cca aat agg gat ata tta att gca agc aac tgg tac ttt aat cat    1410
Asp Pro Asn Arg Asp Ile Leu Ile Ala Ser Asn Trp Tyr Phe Asn His
                430              435              440

tta aaa gat aaa att tta gga tgt gat tgg tac ttt gta cct aca gat    1458
Leu Lys Asp Lys Ile Leu Gly Cys Asp Trp Tyr Phe Val Pro Thr Asp
            445              450              455

gag gga tgg aca aat gat taaacagatt gatatgttca tgacatatgc    1506
Glu Gly Trp Thr Asn Asp
        460

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                20                  25                  30

Thr Ile Leu Asn Leu Asp Ile Asn Asn Asp Ile Ile Ser Asp Ile Ser
        35                  40                  45

Gly Phe Asn Ser Ser Val Ile Thr Tyr Pro Asp Ala Gln Leu Val Pro
    50                  55                  60

Gly Ile Asn Gly Lys Ala Ile His Leu Val Asn Asn Glu Ser Ser Glu
65                  70                  75                  80

Val Ile Val His Lys Ala Met Asp Ile Glu Tyr Asn Asp Met Phe Asn
                85                  90                  95
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Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser
            100                 105                 110

His Leu Glu Gln Tyr Gly Thr Asn Glu Tyr Ser Ile Ile Ser Ser Met
        115                 120                 125

Lys Lys His Ser Leu Ser Ile Gly Ser Gly Trp Ser Val Ser Leu Lys
    130                 135                 140

Gly Asn Asn Leu Ile Trp Thr Leu Lys Asp Ser Ala Gly Glu Val Arg
145                 150                 155                 160

Gln Ile Thr Phe Arg Asp Leu Pro Asp Lys Phe Asn Ala Tyr Leu Ala
                165                 170                 175

Asn Lys Trp Val Phe Ile Thr Ile Thr Asn Asp Arg Leu Ser Ser Ala
            180                 185                 190

Asn Leu Tyr Ile Asn Gly Val Leu Met Gly Ser Ala Glu Ile Thr Gly
        195                 200                 205

Leu Gly Ala Ile Arg Glu Asp Asn Asn Ile Thr Leu Lys Leu Asp Arg
    210                 215                 220

Cys Asn Asn Asn Asn Gln Tyr Val Ser Ile Asp Lys Phe Arg Ile Phe
225                 230                 235                 240

Cys Lys Ala Leu Asn Pro Lys Glu Ile Glu Lys Leu Tyr Thr Ser Tyr
                245                 250                 255

Leu Ser Ile Thr Phe Leu Arg Asp Phe Trp Gly Asn Pro Leu Arg Tyr
            260                 265                 270

Asp Thr Glu Tyr Tyr Leu Ile Pro Val Ala Ser Ser Ser Lys Asp Val
        275                 280                 285

Gln Leu Lys Asn Ile Thr Asp Tyr Met Tyr Leu Thr Asn Ala Pro Ser
    290                 295                 300

Tyr Thr Asn Gly Lys Leu Asn Ile Tyr Tyr Arg Arg Leu Tyr Asn Gly
305                 310                 315                 320

Leu Lys Phe Ile Ile Lys Arg Tyr Thr Pro Asn Asn Glu Ile Asp Ser
                325                 330                 335

Phe Val Lys Ser Gly Asp Phe Ile Lys Leu Tyr Val Ser Tyr Asn Asn
            340                 345                 350

Asn Glu His Ile Val Gly Tyr Pro Lys Asp Gly Asn Ala Phe Asn Asn
        355                 360                 365

Leu Asp Arg Ile Leu Arg Val Gly Tyr Asn Ala Pro Gly Ile Pro Leu
    370                 375                 380

Tyr Lys Lys Met Glu Ala Val Lys Leu Arg Asp Leu Lys Thr Tyr Ser
385                 390                 395                 400

Val Gln Leu Lys Leu Tyr Asp Asp Lys Asn Ala Ser Leu Gly Leu Val
                405                 410                 415

Gly Thr His Asn Gly Gln Ile Gly Asn Asp Pro Asn Arg Asp Ile Leu
            420                 425                 430

Ile Ala Ser Asn Trp Tyr Phe Asn His Leu Lys Asp Lys Ile Leu Gly
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Cys Asp Trp Tyr Phe Val Pro Thr Asp Glu Gly Trp Thr Asn Asp
    450                 455                 460
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aatgatatta tatcagatat atctgggttt aattcatctg taataacata tccagatgct     180

caattggtgc ccggaataaa tggcaaagca atacatttag taaacaatga atcttctgaa     240

gttatagtgc ataaagctat ggatattgaa tataatgata tgtttaataa ttttaccgtt     300

agcttttggt tgagggttcc taaagtatct gctagtcatt tagaacaata tggcacaaat     360

gagtattcaa taattagctc tatgaaaaaa catagtctat caataggatc tggttggagt     420

gtatcactta aaggtaataa cttaatatgg actttaaaag attccgcggg agaagttaga     480

caaataactt ttagggattt acctgataaa tttaatgctt atttagcaaa taaatgggtt     540

tttataacta ttactaatga tagattatct tctgctaatt tgtatataaa tggagtactt     600

atgggaagtg cagaaattac tggtttagga gctattagag aggataataa tataacatta     660

aaactagata gatgtaataa taataatcaa tacgtttcta ttgataaatt taggatattt     720

tgcaaagcat aaatccaaa agagattgaa aaattataca caagttattt atctataacc     780

ttttaagag acttctgggg aaacccttta cgatatgata cagaatatta tttaatacca     840

gtagcttcta gttctaaaga tgttcaattg aaaaatataa cagattatat gtatttgaca     900

aatgcgccat cgtatactaa cggaaaattg aatatatatt atagaaggtt atataatgga     960

ctaaaattta ttataaaaag atatacacct aataatgaaa tagattcttt tgttaaatca    1020

ggtgatttta ttaaattata tgtatcatat aacaataatg agcacattgt aggttatccg    1080

aaagatggaa atgcctttaa taatcttgat agaattctaa gagtaggtta taatgcccca    1140

ggtatccctc tttataaaaa aatggaagca gtaaaattgc gtgatttaaa aacctattct    1200

gtacaactta aattatatga tgataaaaat gcatctttag gactagtagg tacccataat    1260

ggtcaaatag gcaacgatcc aaatagggat atattaattg caagcaactg gtactttaat    1320

catttaaaag ataaaatttt aggatgtgat tggtactttg tacctacaga tgagggatgg    1380

acaaatgatt aa                                                       1392
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     pCMV.LACZ.TTC

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     pCMV.LACZ.TTC

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1-11. (canceled)

12. A method for in vivo delivery of a desired composition into human or animal central nervous system (CNS) or spinal cord, wherein the method comprises administering to the human or animal a composition comprising a vector containing nucleotides encoding, a non-toxic, proteolytic fragment of tetanus toxin (TT) in association with at least a molecule having a biological function and said composition is capable of in vivo retrograde axonal transport and transynaptic transport into the CNS or the spinal cord of the human or animal and of being delivered at different areas of the CNS or the spinal cord, wherein the vector is capable of in vivo expression in a muscle.

13. The method according to claim 12, wherein said vector comprises a promoter and an enhancer capable of expressing the nucleotides contained in said vector in the muscle.

14. The method according to claim 13, wherein said vector is the plasmid pCMV-LacZ-TTC which has been deposited at the C.N.C.M. on Aug. 12, 1997, under the registration number I-11912.

15. The method according to claim 12, wherein said vector is administered into the muscle.

16. The method according to claim 12, wherein the molecule is a nucleotide encoding for a protein or a polypeptide linked chemically to the fragment of tetanus toxin and being transported and expressed directly in neurons.

17-26. (canceled)

27. A method for in vivo delivery of a desired composition into human or animal central nervous system (CNS) or spinal cord, wherein the method comprises administering to the human or animal a composition comprising a cell or a vector containing nucleotides encoding a non-toxic, proteolytic fragment of tetanus toxin (TT) in association with at least a molecule having a biological function and said composition is capable of in vivo retrograde axonal transport and transynaptic transport into the CNS or the spinal cord of the human or animal and of being delivered at different areas of the CNS or the spinal cord, wherein the cell or vector is capable of in vivo expression in neuronal cells or precursor of neuronal cells and wherein said cell is reimplanted into the CNS or spinal cord.

28. The method according to claim 27 wherein said cell or vector comprises a promoter and an enhancer capable of expressing the nucleotides contained in said cell in neuronal cells or precursors of neuronal cells.

29. The method according to claim 27, wherein the molecule is a nucleotide encoding for a protein or a polypeptide linked chemically to the fragment of tetanus toxin and being expressed directly in neurons.

30. The method according to claim 27, wherein the molecule is a nucleotide encoding for a protein or a polypeptide linked chemically to the fragment of tetanus toxin and being expressed directly in neurons.

31. (canceled)

* * * * *