

Promoter for the Expression of Foreign Genes in Neuronal Cells

The invention relates to a promoter for the expression of foreign genes in neuronal cells.

A promoter represents a regulatory starting sequence, important for the gene expression, within the genome of any organism; it determines whether, how and to what extent the transcription of a gene takes place in messenger RNA (mRNA). There are “strong” and “weak” promoters (i.e., those that bring about the formation of numerous or less numerous mRNA transcripts of the gene), as well as constitutive (constantly active), inducible (i.e., those that control the transcription based on certain conditions) and tissue-specific promoters.

The neuron-specific Thy1 promoter of the mouse was already used in multiple ways to produce transgenic mice that express foreign genes in their central nervous system. Such mouse strains represent important instruments, in particular for studying molecular mechanisms of neurodegenerative diseases as well as for the development of these potential therapies.

The mThy1 promoter is an atypical promoter without TATA-box. It is composed of two identical promoter nonamers, in which in each case, a short, non-translated Exon follows (Exons 1a and 1b). The Intron A, which is followed by the first translated Exon (Exon 2), is connected to Exon 1b. After that, the Exons 3 and 4 follow, in each case separated from one another by an additional Intron (Introns B and C). This sequence (first promoter-nonamer up to and including Exon 4) is flanked by non-translated regions (5'- and 3'-UTR), whereby the 3'-UTR contains the poly-A-signal. It is assumed that the

5'-UTR, the Exons 1a and 1b, as well as the Intron A have regulatory properties that can also be responsible for the neuron-specific expression of the promoter (E. Spanopoulou et al., *Mol. Cell. Biol.* 1988; 8: 3847-3856 and 1991; 11 (4): 2216-2228).

In addition to the neuronal expression, the original mThy1 promoter also showed an expression in the thymus. The thymus expression could be excluded by the deletion of the range from Exon 2 to Exon 4 (H. A. Ingraham and G. A. Evans, *Mol. Cell. Biol.* 1986; 6 (8): 2923-2931; M. Vidal et al., *EMBO* 1990; 9 (3): 833-840).

The mThy1 promoter with deletion of Exons 2-4 was and is used for control of the transgenic expression in the generation of transgenic animals to express the corresponding transgene specifically in the neurons. Thus, for example, transgenic mice that express a mutated alpha-synuclein protein under mThy1-promoter control found in a rare congenital form of Parkinson's disease have similar symptoms to the corresponding patients (H. van der Putten et al., *J Neurosci.* 2000; 20 (16): 6021-9; B. Sommer et al., *Exp Gerontol.* 2000; 35 (9-10): 1389-403). Similar murine animal models exist for certain human tauopathies, in which mutated forms of the protein Tau that is associated with the microtubule are found (J. Gotz et al., *J Biol Chem.* 2001; 276 (1): 529-34). Animal models of Alzheimer's disease, in which amyloid precursor proteins (APP, the precursor protein of the beta-amyloid, which forms the so-called plaque in the brains of Alzheimer patients) are expressed or over-expressed under Thy1 control, are very commonly used. In this case, both normal amyloid (E. Masliah and E. Rockenstein, *J Neural Transm Suppl.* 2000; 59: 175-83) and mutated amyloid can be formed from known congenital forms of Alzheimer's dementia (J. Davis et al., *J Biol Chem.* 2004; 279 (19): 20296-306).

The Thy1 promoter has decisive drawbacks, however. The considerable size of the Thy1 promoter makes it difficult to handle for genetic engineering works, in particular for the incorporation of major gene constructs.

In view of the fact that the viral vectors that can also be used for the generation of transgenic animals can take up only limited amounts of foreign DNA, the promoter size considerably limits the size of the gene to be transferred. Also, the Thy1 promoter is often not strong enough to produce in the transgenic animals an expression of the foreign gene to a desirable extent.

Therefore, the object according to the invention was to modify the known mThy1 promoter structure, such that

- a) The promoter size is reduced such that its use for genetic engineering works is facilitated and the transfer of larger foreign genes or gene fragments with stable vectors is made possible,
- b) The expression of this foreign DNA in the thus produced transgenic mice is increased significantly, and
- c) The expression of the transgene to be controlled by the promoter is carried out in a neuron-specific manner.

It has now turned out that the above-described object can be achieved by the design of a promoter that is altered by genetic engineering, which contains only certain (original or slightly altered) partial sequences of the mThy1 promoter in combination with other regulatory elements.

The invention relates to a promoter that comprises a nucleic acid sequence that is derived from Thy-1 mammals and that brings about the neuron-specific transcription of a

heterologous nucleic acid sequence that is located 3'-downward from the promoter both in mammal and in non-mammal cells.

The invention furthermore relates to a nucleic acid sequence that consists of the sections

- (a) The mThy1 5'-"untranslated region" (UTR), the 20bp sequence that is located in front of 5'-UTR, and the sequence of the first nonameric promoter that consists of 9 nucleotides and flanking regions of the mouse-Thy-1- gene
- (b) The sequence of the Exon 1a of the mouse-Thy-1 gene
- (c) The sequence of the second nonameric promoter that consists of 9 nucleotides of the mouse-Thy-1 gene
- (d) The sequence of the Exon 1b of the mouse-Thy-1 gene
- (e) The complete or partial sequence of the Intron A of the mouse-Thy-1 gene
- (f) The sequence of a poly A signal
- (g) The mammal-Thy-1-gene-promoter sequence, which hybridizes with the sequence that is described under (a), (b), (c), (d), (e) and (f), whereby the promoter sequence, if it is combined with a heterologous gene sequence, transcribes the latter in a neuron-specific manner both in mammal cells and in non-mammal cells.

The invention furthermore relates to a nucleic acid construct, comprising an expression cassette, which contains the sections (a), (b), (c), (d), (e), (f) and (g) characterized according to claim 2 as well as a heterologous nucleic acid sequence, which is positioned between the sections (e) and (f) and is associated operatively with the latter.

The invention furthermore relates to advantageous configurations of this nucleic acid construct as they are disclosed according to claims 4 to 8.

The invention relates to an isolated cell or cell line that comprises the promoter according to claim 1.

The invention also relates to an isolated cell or cell line that comprises the nucleic acid sequence according to claim 2. In an advantageous way, this isolated cell or cell line comprises a nucleic acid construct with the features according to claims 3 to 8.

The invention relates to a transgenic non-human animal, comprising the promoter according to claim 1.

The invention also relates to a transgenic non-human animal, comprising the nucleic acid sequence according to claim 2. In this case, in an advantageous way, the transgenic non-human animal comprises a nucleic acid construct according to one of claims 3 to claim 8.

The invention relates to a process for gene expression in neuronal and non-neuronal cells, which contains the transformation/transfection of the cells with a vector that comprises the nucleic acid sequence according to claim 2 or a nucleic acid construct according to one of claims 3 to 8.

The invention relates to advantageous configurations of this process with the features according to claims 16 to 18.

The invention relates to the use of a vector that comprises the nucleic acid sequence according to claim 2 or a nucleic acid construct according to one of claims 3 to 8 for the production of injection solutions, which are suitable for injection into the central nervous system or into the cerebrospinal fluid.

The invention relates to the use of a vector that comprises the nucleic acid sequence according to claim 2 or a nucleic acid construct according to one of claims 3 to 8 for the production of injection solutions for treating neuronal disorders, such as stroke, ischemia, epilepsy, Parkinson's disease, Alzheimer's disease, Huntington's disease, brain and spinal cord trauma, and amyotrophic lateral sclerosis.

The invention relates to the use of a vector that comprises the nucleic acid sequence according to claim 2 or a nucleic acid construct according to one of claims 3 to 8 for the production of injection solutions for treating neurogenetic disorders.

The invention relates to a kit for the expression of recombinant gene products that comprise isolated cells or cell lines according to one of claims 9 to 11.

The invention relates to a pharmaceutical agent that comprises a promoter according to claim 1.

The invention also relates to a pharmaceutical agent that comprises a protein, which is coded by the nucleic acid sequence according to claim 2.

The invention furthermore relates to a pharmaceutical agent that comprises a nucleic acid construct according to one of claims 3 to 8.

Possible methods of implementing the invention can be represented as follows:

Starting from the mThy1-expression cassette (Moechars et al. 1996, EMBO J. 15(6), 126574), which has an original size of about 8.2 kb and which contains an Xho I linker instead of the mThy1 Exons 2 to 4, major deletions were put in place with the aid of restriction endonucleases, and then minor insertions were performed, so that at the end, a promoter cassette that was only 1.6 kb was produced.

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In the first step, the mThy1-expression cassette was cleaved with Sma I, and thus the area adjoining the mThy1 5'-UTR on the 5'-end was removed. The construct that was produced, in which the eGFP reporter gene (coded for a Green Fluorescent Protein, which is harmless to mice and serves as a fluorescence-microscopic marker) was integrated 3'-terminally from mThy1 Intron A, was studied by means of transfection in two neuronal cell lines (SH-SY5Y and Neuro-2A) in promoter activity (eGFP expression).

In a second step, the sequence was digested with the restriction enzymes Nco I and Nde I, by which parts of the mThy1 Intron A, the control gene eGFP, the mThy1 3'-UTR together with the mThy1 Poly A signal and the 3' flanking mThy1-sequence ranges were removed. Instead of this, a sequence that consists of the eGFP sequence and the "late SV40 Poly A Signal" was ligated with the reduced mThy1-promoter sequence. The above-mentioned steps are depicted in Fig. 1, the correlation diagram mThy1-gene sequence (A) / mThy1 promoter cassette (B) / mTUB promoter cassette (C). The mThy1 part is labeled above the respective figure, and the non-mThy1 part is labeled below it.

The thus produced sequence, excluding the eGFP-gene sequence, was referred to as an mTUB-promoter (mouse Thy1 Usable for Brain expression; for sequence, see Fig. 3). Also here, the functionality was confirmed by means of transfection in SH-SY5Y and

neuro-2A cells and control of the eGFP expression in comparison to eGFP design under the control of the unaltered mThy1 promoter or the promoters for human Platelet-Derived Growth Factor (human PDGF) and cytomegalovirus (CMV), as shown below.

Promoter properties can be different in vitro and in vivo. Thus, e.g., the inherent neuron-specific mThy1 promoter produces a strong expression of the eGFP reporter gene after transfection of the hamster ovarian cell line CHO-K1 with an mThy1/eGFP construct. On the one hand, to be able to characterize broadly the promoter mTUB according to the invention, and, on the other hand, to achieve the second part of the object according to the invention (neuron specificity), a transgenic mouse strain (mTUB-eGFP-tg) was generated, which expresses eGFP under the control of the mTUB promoter. Figure 4 shows fluorescence micrographies of brain sections of this mouse strain. In the neurons of the transgenic mouse strain, a clear eGFP expression could be detected. Sections through the most varied organs of the transgenic mouse strain showed no eGFP-positive body cells except for eGFP-positive nerve cells. It can clearly be shown that the mTUB promoter expresses the gene, which is under its control, in a neuron-specific manner.

The mTUB-eGFP-transgenic mouse strain was generated as follows, whereby for microinjection, the promoter-gene construct was incorporated in an insulator cassette (egg- β -globin insulator sequences), which is to facilitate the expression and protect from foreign regulation (plasmid pC2xINS, JSW Research).

1. Production and Preparation of the Vector for Microinjection:

- Starting plasmid = pmTUB-eGFP (JSW Research)

- Digestion with Not I, Pvu I and Nde I for obtaining the promoter-insert construct
- mTUB-eGFP construct has Not I- or Nde I-ends; Nde I-end is made up with polymerase I Klenow fragments
- Incorporation of the mTUB-eGFP construct in the insulator vector pC2xINS that is opened with Not I and Pml I
- Sequencing of the cloning transitions
- Removal of the vector sequences (pKO Backbone) and linearization of the insulator-mTUB-eGFP-insulator construct with Mlu I,
- Gel elution of the linear construct “insulator-mTUB-eGFP insulator” (7280 bp)

2. Microinjection of the Linear Construct

For the microinjection, the linear insulator-mTUB-eGFP-insulator construct was isolated from a 0.8% TAE-agarose gel without ethidium bromide, purified and diluted with microinjection buffer to the extent that 1000 DNA molecules were present per injection volume. The injection was carried out according to standard protocol (Brem et al., 1985). To this end, 3-week-old hormone-treated female CB6F1 mice were paired with C57BL/6 males in the preliminary area. The resulting fertilized CB6F1 (B6) oocytes were removed and cultivated until two clear pronuclei were visible. The purified DNA construct was then injected into the pronucleus, followed by an overnight cultivation until the two-cell stage was achieved. The two-cell embryos were

then implanted in pseudo-pregnant foster mice. After 18-19 days, the babies were born.

The control gene eGFP was used to be able to characterize promoter properties and transgene expression as quickly and simply as possible. The eGFP gene can be replaced by any other gene that is to be expressed in a neuron-specific manner (see Fig. 3, sequence of the mTUB promoter cassette).

In this case, the functional areas are correlated to the following nucleotide ranges in the sequence:

1 – 222:	3'-Part of the mThy1 5'-UTR
223 – 291:	1. Nonameric mThy1 promoter and flanking regions
292 – 345:	mThy1 Exon 1a
537 – 545:	2. Nonameric mThy1 promoter
592 – 733:	mThy1 Exon 1b
346 – 1378:	5'-Part of mThy1-Intron A
1379 – 1392:	Polylinker I
1393 – 2112:	Control gene eGFP (not part of the disclosure)

The coding sequence, which extends from nucleotide 1393 to 2112, stands for the reporter gene eGFP, which is not part of the disclosure. The late SV40 poly A sequence, which can be replaced by any other poly A sequence, follows the reporter gene sequence.

2113 – 2130:	Polylinker II
2131 – 2350:	Late SV40 Poly A signal

The table below shows a comparison of the mTUB promoter according to the invention with the unaltered murine Thy1 promoter, as well as the promoters of the human Platelet-Derived Growth Factor (hPDGF) and the cytomegalovirus (CMV). Both SH-SY5Y and neuro-2A cells were transfected with the eGFP-reporter gene under control of the respective promoter, and the transfection efficiency (TE in %) as well as the mean fluorescence intensity (MFI in random units), both measured by means of FacScan, were compared (transfection efficiency in power = (fluorescent cells/non-fluorescent cells) x 100).

	mTUB-eGFP (TE-MFI)	mThy1-eGFP (TE / MFI)	hPDGF-eGFP (TE / MFI)	CMV-eGFP (TE / MFI)
SH-SY5Y	5% / 13	Not Measurable/Not Measurable	0.2% / 16	10% / 110
Neuro-2A	79% / 293	9% / 25	52% / 46	86% / 201

Thus, the mTUB promoter in both neuronal cell lines is far superior to the mThy1 promoter both with respect to the transfection efficiency and the expression intensity. In SH-SY5Y cells, mTUB achieves 25 x-higher transfection efficiency than the hPDGF promoter with approximately the same average signal intensity, and in neuro-2A cells, mTUB achieves 1.5 x-higher transfection efficiency than the hPDGF promoter in the case of approximately 6 x average signal intensity. The transfection efficiency is comparable to that of the CMV promoter in the case of clearly higher average signal intensity (in Neuro-2A).

In Fig. 4, fluorescence-microscopic images of sections of the brain of the transgenic mouse strain mTUB-eGFP-tg are shown in comparison to the corresponding non-transgenic strain C57Bl6 ntg. In the brain sections, the eGFP-expressing neurons of

the transgenic mouse are discerned quite clearly in comparison to the eGFP-negative brain section of the non-transgenic mouse.

Claims:

1. Promoter that comprises a nucleic acid sequence that is derived from Thy-1 mammals and that brings about the neuron-specific transcription of a heterologous nucleic acid sequence that is located 3'-downward from the promoter both in mammal and in non-mammal cells.
2. Nucleic acid sequence that consists of the sections
 - (a) The mThy1 5'-"untranslated region" (UTR), the 20bp sequence that is located in front of 5'-UTR, and the sequence of the first nonameric promoter that consists of 9 nucleotides and flanking regions of the mouse-Thy-1- gene
 - (b) The sequence of the Exon 1a of the mouse-Thy-1 gene
 - (c) The sequence of the second nonameric promoter that consists of 9 nucleotides of the mouse-Thy-1 gene
 - (d) The sequence of the Exon 1b of the mouse-Thy-1 gene
 - (e) The complete or partial sequence of the Intron A of the mouse-Thy-1 gene
 - (f) The sequence of a poly A signal
 - (g) The mammal-Thy-1-gene-promoter sequence, which hybridizes with the sequence that is described under (a), (b), (c), (d), (e) and (f), whereby the promoter sequence, if it is combined with a heterologous gene sequence, transcribes the latter in a neuron-specific manner both in mammal cells and in non-mammal cells.
3. Nucleic acid construct, comprising an expression cassette, which contains the sections (a), (b), (c), (d), (e), (f) and (g) that are characterized according to

claim 2 as well as a heterologous nucleic acid sequence that is positioned between the sections (e) and (f) and is associated operatively with the latter.

4. Nucleic acid construct according to claim 3, wherein the section (g) is a therapeutic gene sequence.
5. Nucleic acid construct that comprises the nucleic acid sequence according to claim 2 as a component of a plasmid.
6. Nucleic acid construct according to claim 3, wherein the heterologous nucleic acid sequence between the sections (e) and (f) codes for a protein.
7. Nucleic acid construct that comprises a vector and a nucleic acid molecule with the nucleic acid sequence according to claim 2.
8. Nucleic acid construct according to claim 7, wherein the vector is a virus or is derived from a virus.
9. Isolated cell or cell line that comprises the promoter according to claim 1.
10. Isolated cell or cell line that comprises the nucleic acid sequence according to claim 2.
11. Isolated cell or cell line that comprises a nucleic acid construct according to one of claims 3 to 8.
12. Transgenic non-human animal that comprises the promoter according to claim 1.
13. Transgenic non-human animal that comprises the nucleic acid sequence according to claim 2.
14. Transgenic non-human animal that comprises a nucleic acid construct according to one of claims 3 to claim 8.

15. Process for gene expression in neuronal and non-neuronal cells, which contains the transformation/transfection of the cells with a vector that comprises the nucleic acid sequence according to claim 2 or a nucleic acid construct according to one of claims 3 to 8.
16. Process according to claim 15, wherein the vector for the gene transport is mixed with a polymeric carrier substance.
17. Process according to claim 16, wherein a cationic polymer and/or a lipid is (are) used as a polymeric carrier substance.
18. Process according to claim 16, wherein polyethylenimine is used as a polymeric carrier substance.
19. Use of a vector that comprises the nucleic acid sequence according to claim 2 or a nucleic acid construct according to one of claims 3 to 8 for the production of injection solutions, which are suitable for injection into the central nervous system or into the cerebrospinal liquid.
20. Use of a vector that comprises the nucleic acid sequence according to claim 2 or a nucleic acid construct according to one of claims 3 to 8 for the production of injection solutions for treating neuronal disorders, such as stroke, ischemia, epilepsy, Parkinson's disease, Alzheimer's disease, Huntington's disease, brain and spinal cord trauma, and amyotrophic lateral sclerosis.
21. Use of a vector that comprises the nucleic acid sequence according to claim 2 or a nucleic acid construct according to one of claims 3 to 8 for the production of injection solutions for treating neurogenetic disorders.

22. Kit for the expression of recombinant gene products that comprise isolated cells or cell lines according to one of claims 9 to 11.
23. Pharmaceutical agent that comprises a promoter according to claim 1.
24. Pharmaceutical agent that comprises a protein, which is coded by the nucleic acid sequence according to claim 2.
25. Pharmaceutical agent that comprises a nucleic acid construct according to one of claims 3 to 8.