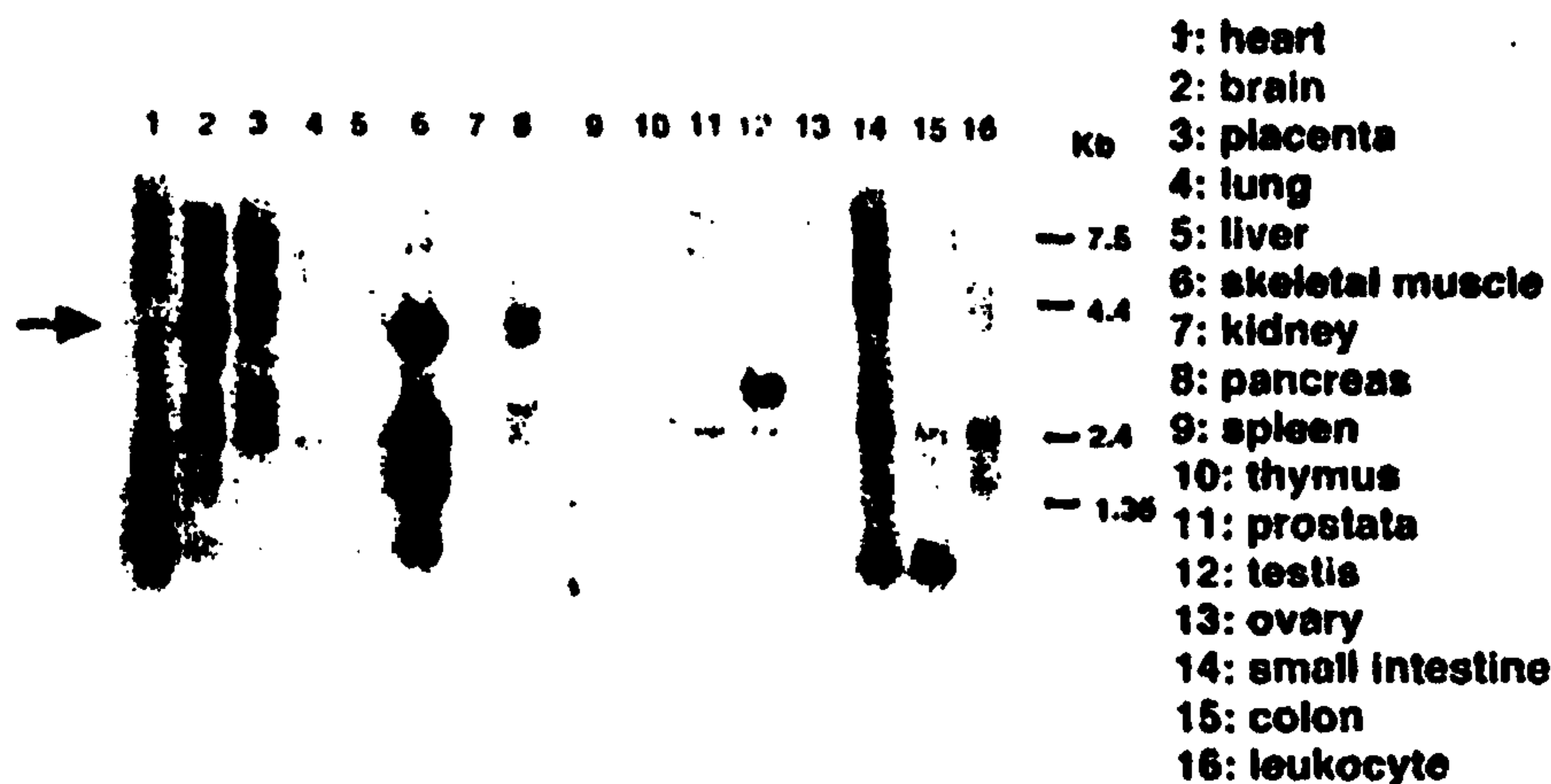




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(51) Int.Cl.⁶ C12N 15/12, C12Q 1/68, C07K 14/47, C07K 16/18, C12P 21/08
(30) 1997/11/14 (9/313211) JP
(54) **PROTEINE NEURALISEE, POLYNUCLEOTIDE CODANT
CETTE DERNIERE, ET ANTICORPS RECONNAISSANT
CETTE PROTEINE**
(54) **NEURALIZED PROTEIN, POLYNUCLEOTIDE ENCODING
THE SAME, AND ANTIBODY RECOGNIZING THE SAME**



(57) Selon l'invention, un gène responsable d'une mutation nerveuse au niveau d'un site dans le dixième chromosome humain présentant fréquemment une délétion a été identifié. L'invention concerne également une protéine (un facteur de mutation nerveuse) codée par ce gène. Elle concerne en outre un anticorps contre cette protéine. Parce qu'il présente une grande homologie avec un gène neuralisé de mouche drosophile, ce gène humain responsable de cette mutation nerveuse est un homologue humain du gène neuralisé. L'invention concerne également un gène neuralisé de souris, une protéine codée par ce gène et un anticorps contre cette protéine.

(57) A gene causative of nerve mutation located in a site in the human tenth chromosome frequently suffering from deletion and having been identified; a protein (a nerve mutation factor) encoded by this gene; and an antibody against this protein. Because of showing a high homology with a Drosophila neuralized gene, this human gene causative of nerve mutation is a human homolog of the neuralized gene. Also, provision is made of a mouse neuralized gene; a protein encoded by this gene; and an antibody against the same.

ABSTRACT

This invention identify a site which is missing with high frequency in Chromosome 10, as well as a causative gene for nervous mutation which is located at the site, and provides said gene. The invention also provides a protein (nerve-mutating factor) that the gene encodes. Furthermore, the invention provides an antibody against the protein. Specifically, this invention provides a human nerve mutating causative gene which is present at the site which is missing with high frequency in human Chromosome 10. The human nerve mutating causative gene is highly homologous to the neuralized gene of *Drosophila* and is a human homologue of the neuralized gene. The invention also provides a neuralized gene in mouse. In addition, the invention provides a protein that the gene encodes, as well as an antibody against said protein.

DESCRIPTION

NEURALIZED PROTEINS, POLYNUCLEOTIDES CODING THE
PROTEINS, AND ANTIBODIES RECOGNIZING THE PROTEINS

5 TECHNICAL FIELD

This invention relates to homologous proteins of
the neuralized proteins of *Drosophila* as well as to
polynucleotides encoding the homologous proteins. It
also relates to antisense polynucleotides of said
10 polynucleotides. Additionally, it relates to
antibodies recognizing the homologous proteins.

BACKGROUND ART

Among vertebrate animals, human beings are living
15 things that have the most highly differentiated nervous
system. Its analysis, however, lags behind due to the
complicated mechanism. Especially, concerning tumors
that develop in the cerebral nervous system, their
analysis has been behind those of other tumors. In the
20 cerebral nervous system, even benign tumors, not to
mention malignant ones, may sometimes be life
threatening to patients depending on the locations at
which the tumors develop. Therefore, progress in
elucidation of the development mechanism of cerebral
25 nerve tumor has been awaited.

Up to the present time, it has been known that

tumors in the cerebral nervous system, especially, the abnormality in malignant glioma arise from aberration of Chromosome 10. However, the relationship between the actual aberration such as the deficiency of Chromosome 10 and clinical symptoms has not been clarified.

On the other hand, in *Drosophila*, many mutants have been obtained during the analysis of their neural development, and the genetic analysis thereof has advanced. As a result, the following have, thus far, been suspected to be causative genes for the mutations: "Notch," "Delta," "Big Brain," "Neuralized," "Master Mind," "Almondex," and "Enhancer of Split," etc.

Among the aforementioned genes, human homologues have been acquired with respect to "Notch," "Delta," and "Enhancer of Split."

DISCLOSURE OF INVENTION

With a view to elucidating tumors in the cerebral nervous system and the aberration of Chromosome 10, an object of this invention is to identify sites in Chromosome 10 at which the frequency of deficiency is high, to identify causative genes for nerve mutation, and to provide said genes. It is also an object of the invention to provide proteins (nerve-mutating factor) that the genes encode and further to provide antibodies

against the proteins.

Specifically, this invention provides a human nerve mutating causative gene which is present at the sites which are missing with high frequency in human
5 Chromosome 10. It also provides a murine nerve mutating causative gene having homology with the human nerve mutating causative gene. These genes are highly homologues to the neuralized gene of *Drosophila* and are, respectively, human and murine homologues of the
10 neuralized gene. This invention has revealed that the neuralized gene previously known in *Drosophila* also exists in vertebrate animals, more specifically mammals such as human and mouse.

The invention also provides antisense
15 polynucleotides comprising base sequences that are complementary to the aforementioned base sequences.

In addition, this application discloses a method for producing a neuralized protein. Specifically, a method is disclosed whereby a transformant to which
20 neuralized cDNA has been introduced is allowed to produce a neuralized protein. The neuralized proteins produced by the method are also disclosed. Additionally, there are disclosed a method for producing a variant of the neuralized protein where one
25 or more amino acids within the amino acid sequence of the neuralized protein have been substituted, deleted

or subject to addition, as well as the variant of the neuralized protein produced by the production method.

Further, this invention discloses antisense polynucleotides comprising base sequences that are complementary to polynucleotides encoding the neuralized proteins or to polynucleotides encoding variants of the neuralized proteins. Although antisense polynucleotides are embraced by polynucleotides, they are referred to as "antisense polynucleotide(s)" in this specification particularly where the polynucleotides comprising base sequences for an antisense strand are clearly meant. Representatives of the antisense polynucleotide are antisense DNAs and antisense RNAs.

Still further, the invention discloses a polynucleotide that is a part, which comprises not less than 12 bases, or the whole of a polynucleotide encoding the neuralized protein or a variant thereof.

This polynucleotide, if it is a part of the coding region, can be used in producing a partial length protein of the neuralized protein or of a variant of the neuralized protein. It can also be used as a probe.

Further, the invention discloses an antisense polynucleotide that is a part, which comprises not less than 12 bases, or the whole of an antisense polynucleotide of the neuralized protein or a variant

thereof.

This antisense polynucleotides is able to respectively inhibit biosynthesis of the neuralized protein or that of the variant of the neuralized protein. It can also be used as a probe.

Furthermore, the invention discloses polynucleotides that are obtained by chemically modifying the aforementioned polynucleotides, including antisense polynucleotides.

Moreover, the invention discloses that through an analysis by the northern blot hybridization using DNA probes, mRNA of the neuralized can be detected in various tissues or cell strains of human or mouse and that mRNA of the neuralized is naturally expressed in these animals.

In addition, the invention discloses a human homologous protein of the neuralized protein encoded by a human homologous gene of the neuralized gene: it is also a homologous protein of the *Drosophila* neuralized protein. Here, in this specification the neuralized proteins in various vertebrate animals are indicated collectively where no names of the animals, from which the proteins are derived, are mentioned. The aforementioned neuralized proteins can be produced by transformants to which the neuralized genes have been introduced.

The invention encompasses a method whereby a polynucleotide encoding the human neuralized, a polynucleotide encoding the murine neuralized, or a part of the foregoing comprising not less than 12 bases is used as a probe to obtain a neuralized cDNA from a cDNA library of any other vertebrate animal, preferably a mammal, as well as encompasses the mammalian neuralized cDNA obtained by the method.

Here, the probes that can be used are a cDNA fragment comprising a base sequence of high homology portion between the base sequence of the human neuralized cDNA and the base sequence of the murine neuralized cDNA, a polynucleotide obtainable from the base sequence of said cDNA fragment, including single-stranded DNA (which contains antisense strands) and RNA against the cDNA or chemically modified ones thereof. If the probes are allowed to hybridize with the cDNA libraries of other animals, neuralized cDNAs of said animals could be acquired.

Further, the invention provides neuralized proteins that are homologous proteins of the human neuralized protein and the murine neuralized protein which the aforementioned cDNAs (the human neuralized cDNA and the murine neuralized cDNA) encode.

Still further, the invention provides antibodies that recognize the aforementioned neuralized proteins

and variants thereof. In this application, there is disclosed that the antigenicity of the neuralized proteins, namely antibodies, can be prepared from the neuralized proteins.

5 Additionally, the invention discloses the locations of the human neuralized gene on its chromosomes.

BRIEF DESCRIPTION OF DRAWINGS

10 Fig. 1 is a photograph of electrophoresis showing the results of analysis of expression of the neuralized gene with respect to individual human tissues according to northern blot hybridization.

15 Fig. 2 is a photograph of electrophoresis showing the results of analysis of expression of the neuralized gene with respect to a normal human cerebral tissue (three samples), an astrocytoma tissue (anaplastic astrocytoma: one sample, and glioblastoma: three samples), and glioma cell strains according to northern
20 blot hybridization.

 Fig. 3 is a photograph of electrophoresis showing the results of analysis of expression of the neuralized gene with respect to a normal human cerebral tissue, medulloblastoma, and glioma cell strains according to
25 RT-PCR.

BEST MODE FOR CARRYING OUT THE INVENTION

The homologous protein of the *Drosophila* neuralized protein according to this invention is not limited to a protein comprising the amino acid sequence set forth in SEQ ID NO: 1 in the Sequence Listing or in
5 SEQ ID NO: 3 in the Sequence Listing. The invention encompasses proteins encoded by cDNAs obtainable from the cDNA libraries of various animals: the probes used to obtain the cDNAs are polynucleotides encoding those
10 proteins, polynucleotides comprising parts of the coding regions of the foregoing, especially the base sequences set forth in SEQ ID NO: 2 or in SQ ID NO: 4 in the Sequence Listing or partial base sequences of the coding regions of the base sequences.

15 A highly conserved base sequence between the human neuralized gene and the murine neuralized gene is, for example, a sequence from G at position 576 to C at position 938 of the base sequence of the human neuralized cDNA set forth in SEQ ID NO: 2 in the
20 Sequence Listing: it corresponds to from the 82nd base to the 446th base of the base sequence of the murine neuralized cDNA set forth in SEQ ID NO: 4 in the Sequence Listing.

25 DNA comprising this base sequence can be used as a probe to obtain the full-length neuralized gene from the cDNA library of an objective animal, following the

manipulations described below.

(1) DNA which serves as the probe is labeled with a Takara MEGA LABEL kit (Registered Trademark: available from Takara Shuzo Co. Ltd.) as exactly
5 described in its manual.

(2) Next, a DNA reaction solution having the composition described below is prepared; and after incubating this solution at 37 ° C for 30 min, it was subjected to heat treatment at 70 ° C for 10 min,
10 causing the enzyme to be deactivated.

DNA probe (2pmol/ μ l)	2 μ l
10-Fold phosphorylation buffer	2 μ l
[γ - ³² P]ATP (370 MBq/ml)	5 μ l
(available from Amersham Pharmacia Biotech Inc.)	
T4 polynucleotidekinase	1 μ l
Total	10 μ l

15

(3) A SEPHADEX G-25 (Registered Trademark: available from Amersham Pharmacia Biotech Inc.)
20 equilibrated with TE50 (50 mM Tris-HCl, pH 8.0, 1 mM, EDTA) is packed into a 1.5-ml Polyprep column (available from Bio-Rad Laboratories Inc.) to give a bed volume of 1 ml. The DNA reaction solution that has
25 been heat-treated in step (2) is loaded onto the column.

(4) Subsequently, the column is eluted with 200 μ

1 of T50E four times, and a labeled DNA probe is
obtained from the fraction eluted by the second 200 μ l.

(5) The above-obtained labeled DNA probe fraction
of 150 μ l is hybridized to the cDNA probe of a plaque
5 fixed on a nitrocellulose membrane under the conditions
described below: Fixation of a cDNA library onto a
nitrocellulose membrane can be carried out according to
the method as described in *Molecular Cloning*, 2nd ed.;
Cold Spring Harbor Laboratory Press: 1989; 9.38-9.40.

10

(i) Prehybridization

6xSSC

5xDenhardt's

0.05% sodium pyrophosphate

15 100 μ g/ml denatured herring sperm DNA

0.5% SDS

Total amount of solution: 50 ml

Reaction temperature: 37 ° C

Reaction time: 1 hr

20

(ii) Hybridization

6xSSC

1xDenhardt's

0.05% sodium pyrophosphate

100 μ g/ml denatured herring sperm DNA

25 1×10^6 cpm/ml cDNA probe

Total amount of solution: 50 ml

Reaction temperature: 42 ° C

Reaction time: 18 hr

(6) The nitrocellulose membrane having undergone hybridization is once washed under each of the following conditions:

(i) 6xSSC, 0.1% SDS 500 ml

Temperature: 40 ° C

Time: 20 min

(ii) 3xSSC, 0.1% SDS 500 ml

Temperature: 42 ° C

Time: 20 min

(7) The washed nitrocellulose membrane is exposed to an X-ray film such as Kodak XR-5 film at -80 ° C overnight and an autoradiograph is taken.

(8) The positions of positive plaques are determined from the resultant autoradiograph and the corresponding plaques on agar are recovered into SM solution.

(9) The recovered plaques are allowed to undergo plaque formation again on a NZY agar medium according to a standard method and they are fixed on a nitrocellulose membrane.

(10) Steps (5)-(9) are repeated three times to make the positive plaques into a single one. The plaque is then recovered and suspended in 100 µl of SM solution to stabilize the phage. cDNA that will be

isolated from the plaque is the neuralized cDNA.

3. Large Scale Preparation of the Neuralized cDNA

(1) The phage of plaque suspended in SM solution, 50 μ l, is mixed with 20 μ l of Y1090r *E. coli* and allowed to stand at 37 ° C for 15 min.

(2) Subsequently, the solution mixed in step (1) is transferred to a 10-ml NZY medium containing 100 μ g/ml ampicillin and culturing is carried out at 37 ° C for 6 h.

(3) Centrifugation is carried out at 8,000 rpm for 5 min, and the supernatant is recovered.

(4) To the supernatant is added 1 ml of 5 M NaCl and 1.1 g of polyethylene glycol 6000, achieving their dissolution.

(5) The solution is placed on ice for 1 h, and then, centrifugation is carried out at 10,000 rpm at 4 ° C for 20 min.

(6) The precipitate is recovered and is suspended in 700 μ l of SM solution.

(7) To this is added 500 μ l of chloroform and stirring is done to dissolve the remaining *E. coli*.

(8) Centrifugation is carried out at 5,000 rpm for 10 min and the aqueous layer is recovered.

(9) To this is added each 1 μ l of RNaseA (1 mg/ml) and DnaseI (5 mg/ml) (both available from Sigma). After allowing to stand at 37 ° C for 1 h, 600 μ l of

20% polyethylene glycol 6000 (0.8 M NaCl) is added and it is allowed to stand on ice for 30 min.

(10) Centrifugation is carried out at 15,000 rpm at 4 ° C for 20 min and the precipitate is recovered.

5 (11) To this precipitate are added 500 μ l of SM solution, 50 μ l of 5M NaCl, and 50 μ l of 0.5 M EDTA. Further, 400 μ l of phenol is added and stirring is done to dissolve the phage and to liberate the cDNA.

10 (12) The solution is centrifuged at 15,000 rpm for 5 min, and then, the aqueous layer is recovered. To this is added 1 ml of ethanol, centrifugation is carried out at 15,000 rpm for 20 min, and the liquid layer is discarded.

15 (13) The precipitate is washed with 1 ml of 70% ethanol and is dissolved in 100 μ l of TE solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to obtain a DNA solution.

4. Determination of the Base Sequence of Neuralized cDNA

20 The full base sequence of the neuralized cDNA is determined using an autosequencer, according to the dyeterminator method.

25 When the result of sequencing reveals that a DNA containing the full length of the coding region (which hereinafter may be referred to as "the full-length DNA") has not been obtained, a combination of DNAs is

selected so as to cover the coding region, and the DNAs are cut at the suitable restriction enzyme sites, which are contained in their overlapping parts, and are linked together, whereby the full-length DNA can be
5 obtained. Alternatively, primers are designed in such a manner as to contain the full length of the coding region. The primers are then used in performing PCR with the template of a cDNA library from an objective vertebrate animal, preferably a mammal: this can result
10 in obtaining the full-length DNA.

5. Determination of the Amino Acid Sequence

From the base sequence determined in step 4, the amino acid sequence of the neuralized cDNA is to be determined.

15 6. Preparation of Transformants

Neuralized cDNAs obtained above in a similar manner to step 9 in Example 1 are inserted into suitable vectors such as a TA cloning vector, and then, the vector can be introduced into a host such as *E.*
20 *coli* to prepare a transformant.

Natural or artificial mutations make it possible to alter a polynucleotide without changing the principal function of a polypeptide that the polynucleotide encodes. (For example, the method as
25 described in *Molecular Cloning*, 2nd ed.: 15.1-15.113.) Concerning the neuralized of this invention, this

method also allows the preparation of a protein comprising an amino acid sequence obtained by making substitution, deletion or addition of one or more amino acids in the amino acid sequence set forth in SEQ ID NO: 1 or in SEQ ID NO: 3 in the Sequence Listing: namely, a variant of the neuralized protein.

When the variants are prepared by the site-directed mutagenesis technique, a single operation can normally substitute, delete, or add several amino acids. Repetition of this operation plural times can further substitute, delete, or add more amino acids, and thus, desirable variants can be prepared. It is preferred that homology between the neuralized of this invention and its variant be not less than 75% at an amino acid level; more preferably not less than 90% and most preferably not less than 95%.

The amino acid sequences for variants of the neuralized proteins according to this invention can be determined by the base sequences of genes encoding said variants. For example, it is feasible through commercially available programs such as GENETIX-MAC (Registered Trademark: available from Software Development Inc.).

Because of degeneracy in genetic codes, it is possible to substitute at least a part of bases in the base sequence of a polynucleotide with other kinds of

bases without altering the amino acid sequence of a polypeptide produced by the polynucleotide. Therefore, by a polynucleotide of this invention encoding the neuralized protein is meant that which contains all
5 patterns of degeneracy.

DNA comprising the base sequence set forth in SEQ ID NO: 2 in the Sequence Listing is the human neuralized cDNA existing in nature; and DNA comprising the base sequence set forth in SEQ ID NO: 4 in the
10 Sequence Listing is the murine neuralized gene existing in nature.

This invention encompasses polynucleotides and derivatives thereof comprising the base sequences of antisense strands for the coding regions of
15 polynucleotides that encode the neuralized proteins. The antisense polynucleotide is able to hybridize to a polynucleotide encoding the neuralized, and if the polynucleotide to which it is hybridized is one of the coding region, it is able to inhibit the biosynthesis
20 of a polypeptide that said polynucleotide encodes.

It is preferred that the antisense polynucleotide for the inhibition of biosynthesis of the polypeptide comprise not less than 15 bases. On the other hand, when a full-length polynucleotide is to be incorporated
25 into cells, it is unsuitable if its length is too long. In cases where an antisense polynucleotide is to be

incorporated into cells and is allowed to inhibit biosynthesis of the neuralized protein, it is advisable that an antisense polynucleotide be used which comprises not less than 12 bases but not more than 30
5 bases, preferably not less than 15 bases but not more than 25 bases, and more preferably not less than 18 bases but not more than 22 bases.

The antisense polynucleotides of this invention or parts thereof encompass all of those in which a
10 plurality of nucleotides comprising bases, phosphoric acid, and sugars are bonded, including those not present in nature; their representatives are antisense DNAs and antisense mRNAs.

With respect to the antisense polynucleotides of
15 this invention, a variety of derivatives can be obtained by using the antisense technology known in the art, which have enhancement in binding strength with objective DNAs or mRNAs, tissue selectivity, cell permeability, nuclease resistance, and intracellular
20 stability.

In view of the ease of hybridization, it is thought to be advantageous that antisense polynucleotides or derivatives thereof having the base sequences complementary to those of the regions which
25 form stem loops be designed. The antisense polynucleotides of this invention or derivatives

thereof are capable of forming stem loops if necessary.

It is also expected that such polynucleotides as having sequences complementary to those in the vicinity of the translation start codon or at a ribosomal binding site, the capping site or a splicing site are generally provided with a great expression inhibitory effect. Thus, the great expression inhibitory effect is expected for the antisense polynucleotides or derivatives thereof according to this invention that contain the genes encoding the neuralized or that contain sequences complementary to those of mRNAs in the vicinity of the translation start codon, or at a ribosomal binding site, the capping site or a splicing site.

At present, generally known derivatives are preferably those which have enhancement in at least one of nuclease resistance, tissue selectivity, cell permeability, and binding strength. Most preferably, the polynucleotide derivatives are those which have phosphorothioate bonds as their skeleton structures. The polynucleotides or derivatives thereof according to this invention also encompass derivatives having these functions and structures.

If the antisense polynucleotides of this invention are of the natural type, they can be prepared either by synthesis using a chemical synthesizer or by the PCR

method which employs the genes encoding the neuralized
as templates. Also, among the derivatives, there are
ones that can be chemically synthesized, such as those
of the methylphosphonate type and the phosphorothioate
5 type. In these cases, operations are conducted
following the manual attached to the chemical
synthesizer and the resultant synthetic products are
purified by a HPLC method using reverse phase
chromatography or the like; thus, the objective
10 antisense polynucleotides or derivatives thereof can be
obtained.

The polynucleotides encoding the neuralized,
antisense polynucleotides thereof, or polynucleotides
being parts of the foregoing (polynucleotides
15 comprising base sequences of not less than 12
consecutive bases) according to this invention can be
used as probes for screening neuralized genes from cDNA
libraries and the like. Where the species of an
objective animal is the same as the species of an
20 animal from which the polynucleotide that serves as the
probe has been derived, portions in the non-coding
region can even be used. Here, those having GC
contents of from 30 to 70% can preferably be used.
Especially preferred are polynucleotides comprising
25 base sequences of not less than 15 consecutive bases.
Said polynucleotides to be used as probes may be

derivatives thereof. It is normally recognized that sequences having base numbers greater than those described above are those having specificities. With respect to cDNA libraries that are used in screening
5 with the aid of said probes, those prepared from mRNAs can preferably be used. A group of cDNAs selected from these cDNA libraries by random sampling may be employed as samples for screening. Commercially available cDNAs are also usable.

10 For example, DNA having a base sequence comprising 12 or more consecutive bases within the base sequence set forth in SEQ ID NO: 2 or in SEQ ID NO: 4 in the Sequence Listing, or a polynucleotide (antisense polynucleotide) capable of hybridizing to said DNA can
15 be used as the probe to screen the neuralized gene from a cDNA library or the like.

The polynucleotides encoding the neuralized, antisense polynucleotides thereof, or polynucleotides comprising parts of the foregoing according to this
20 invention are used as the probes to carry out northern blot hybridization with respect to mRNAs derived from various tissues: this enables the detection of any tissue where mRNA derived from the neuralized gene has expressed.

25 In the chemical synthesis of DNAs or RNAs, chemical modification such as methylation or

biotinylation of their side chains and substitution of
O of their phosphoric acid group portions with S is
well known. For example, when the DNA set forth in SEQ
ID NO: 2 in the Sequence Listing is chemically
5 synthesized, it is possible to carry out the chemical
modification and to synthesize a DNA different from the
DNA shown in the Sequence Listing per se.

cDNAs can also be labeled with radioisotopes even
if they are obtained from cDNA libraries.

10 Accordingly, DNAs and RNAs of this invention
encompass the chemically modified DNAs, RNAs, and
antisense polynucleotides within their scope. The
chemically modified DNAs or RNAs are able to exert both
the function of encoding proteins and the function as
15 probes; the chemically modified antisense
polynucleotides are able to exert both the function of
inhibiting the biosynthesis of probes or proteins and
the function as probes.

According to methods known in the art, it is
20 possible to introduce plasmids into suitable hosts such
as *E. coli* and then to obtain transformants.
Transformants to which the neuralized genes of this
invention have been introduced are grown and the genes
are amplified, or proteins are allowed to express to
25 prepare the neuralized proteins or variants thereof,
which steps are all possible. Subsequently, the

prepared products are recovered, and if necessary, manipulations such as concentration, solubilization, dialysis, and various chromatographic techniques are conducted; thus, it is possible to purify the
5 neuralized proteins or variants thereof according to this invention.

A variety of textbooks are available for the cultivation of transformants. According to methods known in the art, it is also possible to prepare the
10 neuralized proteins or variants thereof on the basis of the base sequences as described in this invention. At this juncture, any of bacteria such as *E. coli*, yeast, and animal cells can be used as a host. Particularly, animal cells are preferable. For the introduction of
15 genes into cells, the liposome method, electroporation, and the like can be used. Particularly, it is preferable to use nuclear microinjection.

The methods for purifying the neuralized proteins or variants thereof from the resultant cultured
20 products include immunoprecipitation, salting out, ultrafiltration, isoelectric precipitation, gel filtration, electrophoresis, a variety of affinity chromatography (e.g., ion-exchange chromatography, hydrophobic chromatography and antibody chromatography),
25 chromatofocusing, adsorption chromatography, and reverse phase chromatography; and purification may be

carried out by selecting them as appropriate.

Also, in the production stage the neuralized proteins or variants thereof to be produced may be prepared by transformants as fusion peptides with other polypeptides. In this case, the purification step requires a manipulation that slices out the neuralized proteins or variants thereof through treatment with a chemical substance such as bromocyanogen or with an enzyme such as protease.

As Example 3 illustrates the antigenicity of the neuralized protein, this invention reveals the following: an antibody can be readily obtained by immunizing an animal, other than human and the animal from which the neuralized protein is derived, with the neuralized protein produced by the aforementioned method or with an oligonucleotide comprising an amino acid sequence that is specific for the neuralized protein. Consequently, the antibodies of the invention that recognize the neuralized proteins (which hereinafter may be referred to as "the neuralized antibodies") encompass, within their scope, those which can be obtained by immunosensitization of animals, other than human and the animals from which the neuralized proteins are derived, with the neuralized proteins and which can be confirmed to recognize the neuralized proteins by western blotting, ELISA,

immunostaining (e.g., staining tissues of lyophilized specimens or paraffin specimens), or the like.

For an immunogen, part of a protein is used although it is conjugated to other carrier protein such as bovine serum albumin, which is a well-known method. The part of a protein may be synthesized using a peptide synthesizer. Here, the part of a protein is preferably eight amino acid residues or more.

It is well known that if polyclonal antibodies against substances which have manifested their antigenicity can be obtained by immunosensitization, monoclonal antibodies are produced by the use of hybridomas employing lymphocytes of the immunized animals. (*Antibodies; A Laboratory Manual; Cold Spring Harbor Laboratory Press: 1988; Chapter 6.*) Accordingly, the neutralized antibodies of this invention encompass monoclonal antibodies within their scope.

In this invention, the antibodies encompass active fragments thereof. The term "active fragments" means the fragments of antibodies that possess antigen-antibody reactivity; and specifically named are $F(ab')_2$, Fab' , Fab , Fv , etc.

For example, $F(ab')_2$ results if an antibody of this invention is digested with pepsin, and Fab results if digested with papain. $F(ab')_2$ is reduced with a reagent such as 2-mercaptoethanol and alkylated with

monoiodoacetic acid to give Fab'. Fv is an active fragment of monovalent antibody obtained by binding the heavy chain variable region and the light chain variable region through a linker.

5 Chemeric antibodies are obtained by retaining these active fragments and substituting other parts with fragments from other animals.

The antibodies of this invention can be used in the elucidation of functions such as expression of the
10 neuralized as well as in the elucidation of formation mechanism of malignant tumors.

For the detection of the neuralized, mentioned are methods using antibodies and those utilizing enzymatic reactions.

15 For the method using antibodies, specifically mentioned are as follows: (1) a technique to detect the neuralized by the use of a labeled neuralized antibody; and (2) a technique to detect the neuralized by the use of a neuralized antibody or a secondary labeled
20 antibody thereof. As the label, radioisotopes (RI), enzymes, avidin, biotin, and fluorescent substances such as FITC and Rhodamine are to be utilized, for example.

For the method utilizing enzymatic reaction,
25 mentioned are, for example, the identification technique of immune reaction molecules which employs

ELISA, immunoagglutination, and western blotting, and those analogous to the foregoing.

EXAMPLES

5 Hereinbelow, this invention will be illustrated in more detail, but it should not be limited to the following examples.

EXAMPLE 1

10 I. Identification of the Position of Deficiency of Chromosome 10 in Malignant Glioma

(1) Preparation of Cells

15 Forty-six cases of human malignant glioma tissue and peripheral blood of malignant glioma patients were collected. The peripheral blood was collected in 5 cc into blood-collecting tubes with the addition of EDTA, and leukocyte nuclear cells were recovered by specific gravity centrifugation using FIRECALL-BAKE (available from Amersham Pharmacia Biotech Inc.). With respect to
20 the tissue, tissue sections (5 mm square) removed in operations or samples from biopsy were treated with collagenase and trypsin to prepare cell suspensions.

(2) Preparation of DNAs

25 DNAs were extracted from the resultant cell suspensions using a Xeagen Blood and Cell Culture DNA

kit (available from Xeagen Inc.) according to its protocol as attached.

(3) Microsatellite Analysis

5 The resultant DNAs were analyzed using primers which were dye-labeled microsatellite markers of 16 sites along the long arm of Chromosome 10.

Consequently, in malignant gliomas of grade III and grade IV frequently noted was the loss of heterogeneity (LHO) of genes in the regions containing 10 D10S540 and D10S566 among the microsatellite markers along the long arm of Chromosome 10.

(4) STS Mapping

Then STS mapping around D10S566 was carried out using the panel of whole genome radiation hybrid and 15 YAC clones. The result revealed the presence of D10S566 in 10q25, as well as the positional relationship of STS markers in a vicinity thereof.

II. Identification of Neuralized Gene

1. Searching of EST Data Base

20 From the sequences registered (EST) in EST Data Base on Internet (a date base in which the sequences of mRNA fragments have been registered:

<http://www.ncbi.nlm.nih.gov/i.html>), EST existing in the vicinity of from 10q24 to 10q25 was searched, and 25 H82634 was hit. When the localization of this EST was determined by PCR with the radiation hybrid panel and

YAC clones, it was found out that H82634 was positioned between D10S540 and D10S566 on human Chromosome 10. A possibility thus arose that a gene containing H82634 is the causative gene for malignant glioma.

5 2. Searching of TIGR Data Base

From the sequences registered in TIGR Data Base on Internet (a date base in which the sequence of mRNA fragments have been registered:

10 <http://www.ncbi.nlm.nih.gov/i.html>), sequences having
homology with H82634 were searched for. The result
revealed that H82635 was a sequence complementary to
H82634. Based on these two sequences, base sequence A
described below (the base sequence set forth in SEQ ID
NO: 23 in the Sequence Listing) was determined.

15 Base Sequence A:

CAGCTGGACG CCCCGNTGNA GGCCGTAGAC GTCCACCAGG GCCCAGAGCG
GGTCGGCCGT GCGGACCCGC TGAAGAACAG CATAACAGCC GAGTCGTTGA
TGCGGTGGAA GACACGGCCC TTCTTGTTCA CCCAGAATGC GATGATGTTG
CCCTCATTTG CAAACTCCTC AGGCAGCGCT NTGGCCCAGA AGCCACTCTG
20 GGACACCAGG TTCGGGGCAG GCGTACTTGG GCAGCGAGTC AGGGTGGATG
CGGGACGGGT CCTTGCTGGT GAAGCCAGCC GCAGGCCCCG CTCCAGCACT
GCTTCTTGGT GATCTTCAGC CTGACTTGCT CGTAGATGAG GACCGGGCGG
TTGCTGAAGG TGATGNCGTT GCAGAAGCTG GCCTGCCTCT TGNACAGCCT
TGTGGCTGAG GTCCATGAGG ATCTGGGAGC CCTTGGTGTG CGGGTGGAAAG
25 AGCAGCGGCG TGGCTGGGAG CCCCCGCTGG GCAGCACTGC CGGACAGTGC
TTCTGCTTGT GGTGGCATCG GTGAGAAGTG ACGGGGAAGG GGCCCCCGAT

AGAGTCGTGG AGAGTGCTCC GGGTGAT

3. Synthesis of Primers

To obtain a DNA fragment of base sequence A
5 described above, the following primers were synthesized.
Primer P1, 5'-TAGACGTCCA CCAGGGCCCA GAC-3' (the base
sequence set forth in SEQ ID NO: 5 in the Sequence
Listing), as a 5'-primer and Primer P2, 5'-CGGAGCACTC
TCCACGACTC TAT-3' (the base sequence set forth in SEQ
10 ID NO: 6 in the Sequence Listing), as a 3'-primer were
designed and synthesized with a DNA synthesizer (Model
392 available from ABI). The synthesized primers were
adjusted to 20 pmol/ μ l with distilled water. The
synthesis of primers as will be described below was
15 carried out in the same manner.

4. PCR

Human fetus brain cDNA library (available from
CLONTECH Laboratories Inc.) was used as a cDNA library
and PCR was performed under the following conditions:

20

CDNA	0.8 μ l
dNTPmix(each 2.5 mM)	0.6 μ l
Primer P1	0.6 μ l
Primer P2	0.6 μ l
25 3.3xPCR Buffer	4.5 μ l
25 mM MgCl ₂	0.72 μ l

Distilled water	6.68 μ l
Total	14.5 μ l

Mineral oil (15 μ l) was overlaid on the
5 aforementioned composition and it was allowed to stand
at 94 °C for 5 min. Then, Taq polymeraseXL, 0.5 μ l,
(Registered Trademark: available from Takara Shuzo Co.
Ltd.) was added. The following cycle was repeated 35
times to allow reaction: at 94 °C for 30 sec, at 60 °C
10 for 30 sec, and then at 72 °C for 1 min. Finally, the
extension reaction of fragment was carried out at 72 °C
for 10 min, thus completing PCR.

After the reaction, the PCR product was subjected
to electrophoresis on a minigel of 1.5% agarose gel.
15 An about 0.6 kb band that was believed to be the gene
encoding the neuralized protein was sliced off, and the
PCR product was recovered. Furthermore, a part of the
recovered product was again subjected to the minigel
electrophoresis described above, which ascertained that
20 a band of about 0.6 kb appeared. This DNA fragment
will be referred to as "Fragment A" hereinafter.

4. Incorporation into a Vector

Fragment A was subjected to subcloning under the
following conditions, using a pCRIITA cloning vector
25 kit (available from Invitrogen Corp.).

	Sterilized distilled water	5 μ l
	10x Ligation buffer	1 μ l
	pCRII vector	2 μ l
	DNA fragment	1 μ l
5	T4DNA ligase	1 μ l
	Total	10 μ l

Reaction was carried out at 14 ° C overnight to yield a ligation mixture.

10 5. Transformation

Transformation was performed using a TA cloning kit.

(i) To *E. coli* cells (50 μ l) on ice were added 2 μ l of 0.5 M 2-mercaptoethanol and the ligation mixture prepared in step 4 described above, and it was allowed to stand for 30 min. Subsequently, it was incubated at 42 ° C in a hot bath while being shaken at 225 rpm.

(ii) Next, the *E. coli* cells were spread over a LB agar plate to which ampicillin, X-Gal, and IPTG had been added. Incubation was done at 37 ° C for 18 h and colonies in white and in blue appeared.

6. Miniscale Culturing

Four white colonies were picked up from the aforementioned plate and each colony was put in a tube in which 3 ml of LB medium (ampicillin added) was present. This was shake-cultured at 37 ° C overnight.

7. Miniscale Preparation

A Plasmid was prepared according to the alkali method (for example, a method as described in *Molecular Cloning; A Laboratory Manual*, 2nd ed; 1.25-1.28, *vide supra*)

8. DNA Sequencing

Plasmid (1 μ l) prepared in step 7 described above was taken up and diluted with 99 μ l of TE. Absorbance at 260 nm (A260) was measured and DNA values were computed: the computation was based on that the A260 value being 1.0 was taken as 50 μ g/ml. The plasmid was diluted with TE buffer so that DNA reached 1 μ g/ml based on its A260 values. The base sequence of Fragment A was determined by the dye-terminator method, using an autosequencer (Model 373S available from ABI).

9. 5'-Extension

On the basis of the base sequence of Fragment A, Primer P3: 5'-AGCACTGCCG GACAGTGCTT CTG-3' (the base sequence set forth in SEQ ID NO: 7 in the Sequence Listing) was synthesized. Employing this primer and a human fetus brain cDNA library as a template, single PCR was performed under the following conditions:

25	cDNA	1 μ l
	dNTPmix	0.6 μ l
	Primer P3	1 μ l

3.3x PCR buffer	4.5 μ l
25 mM MgCl ₂	0.72 μ l
Distilled water	6.68 μ l
Total	14.5 μ l

5

Mineral oil (15 μ l) was overlaid on the
aforementioned composition and it was allowed to stand
at 94 °C for 5 min. Then, Taq polymeraseXL, 0.5 μ l,
(Registered Trademark: available from Takara Shuzo Co.
Ltd.) was added. The following cycle was repeated 50
times to allow reaction: at 94 °C for 30 sec, at 60 °C
for 30 sec, and then at 72 °C for 1.5 min.

Subsequently, the extension reaction of fragment was
carried out at 55 °C for 2 min and finally at 72 °C for
10 min, thus completing PCR.

Next, this PCR product was used as a template and
second PCR was performed. Primer P4, 5'-GTGGTGGCAT
CGGTGAGAAG TGA-3' (the base sequence set forth in SEQ
ID NO: 8 in the Sequence Listing) was synthesized.
Employing this primer and Primer T3, 5'-ATTAACCCTC
ACTAAAG-3' (the base sequence set forth in SEQ ID NO: 9
in the Sequence Listing), as a 5'-primer, PCR was
performed under the following conditions:

25	PCR product	1 μ l
	dNTPmix	1 μ l

	Primer P4	1 μ l
	Primer T3	1 μ l
	3.3x PCR buffer	7.5 μ l
	25 mM MgCl ₂	1.2 μ l
5	Distilled water	11.8 μ l
	Total	24.5 μ l

Mineral oil (20 μ l) was overlaid on the
aforementioned composition and it was allowed to stand
10 at 94 °C for 5 min. Then, Taq polymeraseXL, 0.5 μ l,
(Registered Trademark: available from Takara Shuzo Co.
Ltd.) was added. The following cycle was repeated 40
times to allow reaction: at 94 °C for 30 sec, at 60 °C
for 30 sec, and then at 72 °C for 1 min. Subsequently,
15 the extension reaction of fragment was carried out at
55 °C for 2 min and finally at 72 °C for 10 min, thus
completing PCR. This DNA fragment will be referred to
as "Fragment B" hereinafter.

10. 3'-Extension

20 On the basis of the base sequence of Fragment A,
Primer P5: 5'-GGCCGTGTCT TCCACCGCAT CAA-3' (the base
sequence set forth in SEQ ID No: 10 in the Sequence
Listing) was synthesized. Employing this primer and
human fetus brain cDNA library as a template, single
25 PCR was performed under the following conditions:

	CDNA	1 μ l
	dNTPmix	0.6 μ l
	Primer P5	1 μ l
	3.3x PCR buffer	4.5 μ l
5	25 mM MgCl ₂	0.72 μ l
	Distilled water	6.68 μ l
	Total	14.5 μ l

Mineral oil (15 μ l) was overlaid on the
10 aforementioned composition and it was allowed to stand
at 94 °C for 5 min. Then, Taq polymeraseXL, 0.5 μ l,
(Registered Trademark: available from Takara Shuzo Co.
Ltd.) was added. The following cycle was repeated 50
times to allow reaction: at 94 °C for 30 sec, at 60 °C
15 for 30 sec, and then at 72 °C for 1.5 min. Finally,
the extension reaction of fragment was carried out at
72 °C for 10 min, thus completing PCR.

This PCR product was used as a template and second
PCR was performed. Primer P6, 5'-CTCGGCTGTT ATGCTGTTCT
20 TCA-3' (the base sequence set forth in SEQ ID NO: 11 in
the Sequence Listing) was synthesized. Employing this
primer and Primer T7, 5'-AATACGACTC ACTATAG-3' (the
base sequence set forth in SEQ ID NO: 12 in the
Sequence Listing), as a 5'-primer, PCR was performed
25 under the following conditions:

	PCR Product	1 μ l
	dNTPmix	1 μ l
	Primer P6	1 μ l
	Primer T7	1 μ l
5	3.3x PCR buffer	7.5 μ l
	25 mM MgCl ₂	1.2 μ l
	Distilled water	11.8 μ l
	Total	24.5 μ l

10 Mineral oil (20 μ l) was overlaid on the
aforementioned composition and it was allowed to stand
at 94 °C for 5 min. Then, Taq polymeraseXL, 0.5 μ l,
(Registered Trademark, available from Takara Shuzo Co.
Ltd.) was added. The following cycle was repeated 40
15 times to allow reaction: at 94 °C for 30 sec, at 60 °C
for 30 sec, and then at 72 °C for 1 min. Finally, the
extension reaction of fragment was carried out at 72 °C
for 10 min, thus completing PCR. This DNA fragment
will be referred to as "Fragement C" hereinafter.

20 11. DNA Sequencing

Direct sequencing was performed on Fragments B and
C by the dye-terminator method, relaying on Primer P4
and Primer P6.

12. 3'-Extension

25 On the basis of the base sequence of Fragment C,
Primer P7: 5'-GTGGACGCCT CGCAGCCGCT TTG-3' (the base

sequence set forth in SEQ ID NO: 13 in the Sequence Listing) and Primer P8: 5'-CGATGAGTGC ACCATTGCT ATG-3' (the base sequence set forth in SEQ ID NO: 14 in the Sequence Listing) were synthesized. Employing first
 5 Primer P7 and then a human fetus brain cDNA library as a template, single PCR was performed under the following conditions:

	CDNA	1 μ l
10	dNTPmix	0.6 μ l
	Primer P7	1 μ l
	3.3x PCR buffer	4.5 μ l
	25 mM MgCl ₂	0.72 μ l
	Distilled water	6.68 μ l
15	Total	14.5 μ l

Mineral oil (15 μ l) was overlaid on the aforementioned composition and it was allowed to stand at 94 °C for 5 min. Then, Taq polymeraseXL, 0.5 μ l, (Registered Trademark: available from Takara Shuzo Co. Ltd.) was added. The following cycle was repeated 50
 20 times to allow reaction: at 94 °C for 30 sec, at 60 °C for 30 sec, and then at 72 °C for 1.5 min. Finally, the extension reaction of fragment was carried out at
 25 72 °C for 10 min, thus completing PCR.

This PCR product was used as a template and second

PCR was performed. With the use of Primers P8 and T3

PCR was performed under the following conditions:

	PCR product	1 μ l
5	dNTPmix	1 μ l
	Primer P8	1 μ l
	Primer T3	1 μ l
	3.3x PCR buffer	7.5 μ l
	25 mM MgCl ₂	1.2 μ l
10	Distilled water	11.8 μ l
	Total	24.5 μ l

Mineral oil (20 μ l) was overlaid on the
aforementioned composition and it was allowed to stand
15 at 94 °C for 5 min. Then, Taq polymeraseXL, 0.5 μ l,
(Registered Trademark: available from Takara Shuzo Co.
Ltd.) was added. The following cycle was repeated 40
times to allow reaction: at 94 °C for 30 sec, at 60 °C
for 30 sec, and then at 72 °C for 1 min. Finally, the
20 extension reaction of fragment was carried out at 72 °C
for 10 min, thus completing PCR. The DNA fragment thus
obtained will be referred to as "Fragment D"
hereinafter.

13. DNA Sequencing

25 Direct sequencing was performed on Fragment D by
the dye-terminator method, relaying on Primer P8.

14. Formation of a Full-Length DNA

On the basis of the base sequences for Fragments A, B, C, and D, a sense primer, 5'-AGAGCAGCAG AGGTGGCTGC ACT-3' (the base sequence set forth in SEQ ID NO: 15 in the Sequence Listing) and an antisense primer, 5'-GGCTTGTTCC TCAGCTGGGA CTG-3' (the base sequence set forth in SEQ ID NO: 16 in the Sequence Listing) were designed. Employing this and a human fetus brain cDNA library as a template, PCR was performed under the following conditions:

	CDNA	1 μ l
	dNTPmix	1 μ l
	Sense primer	1 μ l
15	Antisense primer	1 μ l
	3.3x PCR buffer	7.5 μ l
	25 mM MgCl ₂	1.2 μ l
	Distilled water	11.8 μ l
	Total	24.5 μ l

20

Mineral oil (15 μ l) was overlaid on the aforementioned composition and it was allowed to stand at 96 °C for 5 min. Then, Taq polymeraseXL, 0.5 μ l, (Registered Trademark: available from Takara Shuzo Co. Ltd.) was added. The following cycle was repeated 40 times to allow reaction: at 94 °C for 30 sec, at 60 °C

25

for 30 sec, and then at 72 °C for 1.5 min. Finally,
the extension reaction of fragment was carried out at
72 °C for 10 min, thus completing PCR. This full-
length cDNA was sequenced by the dye-terminator method.

5 It was then ascertained that the objective cDNA
containing the full-length coding region had been
obtained. This gene was determined to be a human
homologue gene of the neuralized gene of *Drosophila*.
This base sequence is shown in SEQ ID No. 2 in the
10 Sequence Listing. The amino acid sequence of human
neuralized protein that this gene encodes is shown in
SEQ ID NO: 1 in the Sequence Listing.

15. Preparation of a Transformant

The full-length human neuralized cDNA obtained
15 above was inserted into the BamHI-KpnI site of
pBTM116HA: an 83mer linker containing a HA tag had been
inserted into the EcoRI-Pst site of a plasmid vector
pBTM116HA. This plasmid vector was introduced to *E.*
coli DH5 α to prepare the transformant.

20

EXAMPLE 2 Expression of the Neuralized mRNA in Various Tissues (1)

Each 2 μ g of poly(A)+RNA (mRNA) from a variety of
human tissues and poly(A)+RNA (mRNA) from a variety of
25 human cells was blotted to a membrane: Human Multiple
Tissue Northern Blot I, II and Human Cancer Cell Line

Multiple Tissue Northern Blot (available from CLONTECH Laboratories, Inc.). Fragment A labeled with ^{32}P -CTP using a Random Primed Labeling kit (Registered Trademark: available from Takara Shuzo Co. Ltd.) was
5 hybridized to the membrane under high stringency conditions by following the description in *Molecular Cloning: A Laboratory Manual* 2nd ed; p 7.39-7.52. An analysis by northern blot hybridization was conducted.

Results on the respective tissues are shown in Fig.

10 1. It was understood that the neuralized gene is expressed in brain and muscle tissues.

The results showed no clear expression with respect to any of the cells.

15 From the above results, it is thought that the neuralized gene and the neuralized protein are nerve-specific molecules.

EXAMPLE 3 Expression of the Neuralized mRNA in Various Tissues (2)

20 Total RNAs were extracted from a normal human cerebral tissue (three samples), astrocytoma tissues (anaplastic astrocytoma: one sample, and glioblastoma: three samples), and a glioma cell strain by acid-guanidinium thocyanate-phenol-chloroform method. These
25 RNAs were subjected to electrophoresis on an agarose gel, and then they were stained with ethidium-bromide.

The total RNAs were denatured with 6% formaldehyde-1% agarose gel and transcribed onto a nylon membrane (Hybond ®: available from Amersham Pharmacia Biotech Inc.).

5 Hybridization was performed by allowing reaction at 42 ° C overnight in the following buffer:

α -³²P-dCTP labeled probe (the full-length human neuralized cDNA)

10 50 mM HEPES (pH 7)

 0.75 M NaCl

 50% Formamide

 3.5% SDS

 5xDenhardt's

15 2mM EDTA

 0.1% SDS

 200 µg/ml Salmon sperm DNA

 After the membrane was washed in the same buffer
20 with 2xSSC and 0.1% SDS at 55 ° C for 20 min, it was further washed with 1xSSC and 0.1% SDS at 55 ° C for 20 min.

 Subsequently, the membrane was exposed by being irradiated with X-ray at -80 ° C for three nights.

25 The result is shown in Fig. 2. As is apparent from Fig. 2, expression of the human neuralized mRNA

was noted in the normal human cerebral tissue and the anaplastic astrocytoma, whereas it was not noted in the glioblastoma and the glioma.

5 EXAMPLE 4 Analysis of Variation of the Human Neuralized
in Malignant Astrocytoma

1. Synthesis of cDNA

Total RNAs were extracted from a normal brain tissue, medulloblastoma Med-3, and glioma cell strains
10 (U87MG, U251MG, U138MG, Hs638, TG98, A172, SF126,
U373MG, U105MG, NP1, NP2, and RBR17T).

Each 5 μ g of the total RNAs was incubated in 20 μ l of the following composition at 37 ° C for 1 h and a cDNA was synthesized.

15 Reaction Solution:

50 mM Tris-HCl (pH 8.3)

10 mM DTT

10 mM KCl

0.5 mM dNTP

20 30 μ g RNAsin (available from GIBCO BRL Inc.)

150 μ g SuperscriptII reverse transcriptase
(available from GIBCO BRL Inc.)

2. RT-PCR

PCR was performed on 2 μ g of the reverse
25 transcriptase product at 30 cycles with a Geneamp PCR
System 9600 (available from Perkin-Elmer Ceutus Inc.),

using 25 μ l of rTth DNA polymerase (available from Applied Biosystems Inc.).

A set of the following primers was used as primers for PCR.

5 neuS-13: 5'-AGCTGGTGCTCCCGGACTGTCTG-3' (the base sequence set forth in SEQ ID NO: 19 in the Sequence Listing)

10 neuAS-stop: 5'-AGCTGGTGCTCCCGGACTGTCTG-3' (the base sequence set forth in SEQ ID NO: 20 in the Sequence Listing)

In order to ascertain that the cDNA synthesis had been successfully carried out, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified using the following primers.

15 GAPDH sense primer: 5'-AAGGCTGGGGCTCATTTGCAG-3' (the base sequence set forth in SEQ ID NO: 21 in the Sequence Listing)

20 GAPDH antisense primer: 5'-CCAAATTCGTTGTCATACCAGG-3' (the base sequence set forth in SEQ ID NO: 22 in the Sequence Listing)

The amplified products were subjected to electrophoresis on a 2% agarose gel.

25 The result is shown in Fig. 3. As is apparent from the figure, the human neuralized was amplified in the normal human cerebral tissue, the medulloblastoma, and U87MG, whereas it was not amplified in the glioma

cells other than U87MG.

EXAMPLE 5 Preparation of Antibodies Recognizing the Neuralized Protein

5 1. Preparation of Antigens

Peptide A and Peptide B described below were synthesized.

10 Peptide A: a peptide comprising 21 amino acids of from the 123rd Leu to the 143rd Cys of the amino acid sequence of the neuralized protein set forth in SEQ ID NO: 1 in the Sequence Listing.

15 Peptide B: a peptide comprising an amino acid obtained by adding cysteine (Cys) to the N-terminus of 20 amino acids of from the 272nd Pro to the 291st Asp of the amino acid sequence of the neuralized protein set forth in SEQ ID NO: 1 in the Sequence Listing.

20 Addition of cysteine was done to effect conjugation with hemocyanin from *Macroschisma sinensis* (KHL). Iwaki Glass Co. Ltd. was commissioned for their synthesis.

25 The synthesized peptides (2 mg) were conjugated to 2 mg of maleimido-KLH (available from Pierce Chemical Co.). The reaction was carried out according to the method as described in the manual for a kit from Pierce Chemical Co.

2. Immunization

For each antigen (Peptide A and Peptide B), 100 μ l of an antigen solution (1 μ g/ml), 0.5 ml of PBS and 0.5 ml of a Freund's complete adjuvant (available from Difco Inc.) were taken in a syringe and mixed to
5 prepare an emulsion, which was subcutaneously inoculated to a rabbit at four places on its back.

One week later, a second immunization was carried out. The adjuvant was changed to a Freund's incomplete adjuvant (available from Difco Inc.) from the second
10 time, after which the immunization was done. The other manipulations were the same as those in the first time. After the second time, immunization was done six times in total at intervals of one week.

3. Purification of Antibodies

15 One week after the final immunization, blood was collected. After this blood was allowed to stand at room temperature for 3 h and sufficient coagulation was effected, centrifugation was carried out at 3,000 rpm for 5 min to recover the supernatant (serum).

20 This serum was salted out by the addition of ammonium sulfate to bring the final concentration to 50%. This sample was centrifuged and fractions containing an antibody were subjected to precipitation. Subsequently, the precipitate was dissolved in PBS and
25 was further dialyzed against PBS.

Then, the antibody was purified by affinity

chromatography, using a Protein G Sepharose column (Registered Trademark: available from Pharmacia Inc.). As a result, a total amount of 370 mg of IgG fractions was obtained.

5 The IgG fractions were purified by affinity chromatography, using a prepared column: the column was prepared by binding the peptide used in immunization to NHS-activated Sepharose (available from Pharmacia Inc.) according to its attached manual. As a result, a total
10 amount of 5 mg of a purified antibody was obtained.

4. Determination of Antibody Titers

The titers of the resultant antibodies were measured by ELISA.

(1) Each of the antigen preparations (Peptide A
15 and Peptide B) was diluted with PBS to give a concentration of 25 μ g/ml, and it was fractionally poured to each well of a 96-well ELISA plate (XENOBIND[®]: available from XENOPORE Corp.) at 50 μ l, which was allowed to stand at 4 ° C overnight.

20 (2) The antigen preparation was discarded and BLOCKACE (Registered Trademark: available from Dainippon Pharmaceuticals Co. Ltd.) diluted fourfold with distilled water was fractionally poured to each well at 200 μ l. Blocking was carried out by allowing
25 it to stand at room temperature for 2 h.

(3) The blocking solution was discarded and the

purified antibody was added as a primary antibody. A serially (in two-fold) PBS-diluted solution of the purified antibody was fractionally poured to each well at 50 μ l so that its concentration could successively reach 10 μ g/ml, 5 μ g/ml, 2.5 μ g/ml, etc. from the first row to the 12th row of the 96 wells. The concentration of the antibody in the 12th row proved to be about 0.5 μ g/ml. In addition, IgG purified from the blood of a rabbit that was not immunized was used as control. After each antibody was fractionally poured, reaction was allowed to proceed at room temperature for 1 h.

(4) The antibody solution was discarded and the plate was washed with 0.05% Tween20/PBS four times. Next, a biotinylated rabbit IgG antibody (available from Vector Laboratories, Inc.) that had been 1000-fold diluted with PBS, which served as a secondary antibody solution, was fractionally poured to each well at 50 μ l. Subsequently, reaction was allowed to proceed at room temperature for 30 min.

(5) After the secondary antibody was discarded, the plate was washed with 0.05% Tween20/PBS four times. An ABC solution diluted 1000-fold (available from Vector Laboratories, Inc.) was fractionally poured to each well at 50 μ l, and then it was allowed to stand at room temperature for 30 min.

(6) After the ABC solution was discarded, the plate was washed with 0.05% Tween20/PBS four times. OPD (orthophenylenediamine)-H₂O₂/PBS was fractionally poured to each well at 100 μ l. After the reaction was
5 quenched with 2N sulfuric acid upon sufficient coloring, absorbance at 490 nm was measured with a microplate reader (available from Bio-Rad Inc.).

From this result, the antibody obtained by immunization of Peptide A (which hereinafter may be
10 referred to as "Antibody A") and the antibody obtained by immunization of Peptide B (which hereinafter may be referred to as "Antibody B") were both higher in their titer compared to the control.

15 EXAMPLE 6 Western Blotting

(1) The coding region of human neuralized cDNA was inserted into the BamHI-KpnI site of pBTM116HA: an 83mer linker containing a HA tag had been inserted into the EcoRI-Pst site of the plasmid vector pBTM116HA.
20 This plasmid vector was introduced to COS cells.

(2) The COS cells to which the neuralized cDNA had been introduced, the cells of its parent strain (cells to which no neuralized cDNA had been introduced), a human brain tissue, and a human muscle tissue were
25 subjected to dissolution after recovery of the cells and tissues. 1×10^6 cells were used and a Human Brain

Protein Medley (available from CLONTECH Laboratories Inc.) was used for the brain tissue. As a cell lysate, 68 mM Tris-HCl, 14% glycerol, 3% SDS, 0.1 M DTT, 10 μ g/ml soybean trypsin inhibitor, 1 μ g/ml aprotinin, 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 1 μ g/ml leupeptin were used. The cell lysate (100 μ l) was added to a cell pellet, and it was dissolved with thorough stirring. After boiling for 10 min, centrifugation was carried out at 15,000 rpm for 10 min and the supernatant was recovered.

(3) Ten microliters of the supernatant was subjected to electrophoresis on a 10-20% gradient gel.

(4) After electrophoresis, proteins on the gel were transferred onto nitrocellulose membrane using a Trans Blot System (available from Marisol Inc.).

(5) The nitrocellulose membrane was immersed in 10% skim milk/PBS and 0.1% Tween20 and allowed to stand for 1 h to effect blocking. Subsequently, it was washed with 0.3% Tween20/PBS for 5 min twice.

(6) After each of the anti-neuralized antibodies prepared in Example 3 was diluted with PBS to give a concentration of 1 μ g/ml, it was added to the nitrocellulose membrane and allowed to react at room temperature for 40 min. Subsequently, washing was done with 0.3% Tween20/PBS for 5 min three times.

(7) Next, a peroxidase-labeled anti-rabbit IgG

(available from Amersham Pharmacia Biotech Inc.) was diluted with PBS 20000-fold and it was added to the nitrocellulose membrane and further allowed to react at room temperature for 40 min. Subsequently, washing was
5 done with 0.3% Tween20/PBS for 5 min three times.

(8) Next, only 5 ml of the coloring solution from an ECL kit (available from Amersham Pharmacia Biotech Inc.) was added to the nitrocellulose membrane and it was allowed to react for 1 min. Then, this membrane
10 was exposed to an X-ray film for 20 sec and the film was developed, after which a photograph was taken.

From this result, when either of the antibodies was used, a band for the neuralized protein was detected in any of the cell lysates of the COS cells,
15 the brain tissue, and the muscle tissue. This ascertained that Antibody A and Antibody B both recognize the neuralized protein.

EXAMPLE 7 Tissue Staining

20 Employing Antibody A and Antibody B, immunostaining was conducted on a tissue of human nervous system and a human brain tissue, according to the following manipulations.

(1) The human brain tissue was put in a compound
25 and a frozen block was prepared. The block was made into a frozen section, using a cryostat, and the

section was placed on a slide glass.

(2) Next, the frozen tissue section was subjected to 10% buffered formalin treatment for 10 min to effect fixation. Then, washing was done with PBS (pH 7.5) for 5 min three times. Further, 0.3% H₂O₂/methanol treatment was done for 30 min and washing was done with PBS twice.

(3) Over the tissue on the slide glass was placed 0.15% donkey normal serum/PBS and blocking was conducted by allowing it to stand at room temperature for 1 h.

(4) Antibodies A and B diluted with PBS (to give a concentration of 1 µg/ml) were added to the tissues on the slide glasses, respectively. After allowing them to react at room temperature for 1 h, the slide glasses were washed with PBS (pH 7.5) for 5 min three times.

(5) A biotinylated rabbit IgG antibody (available from Amersham Pharmacia Biotech Inc.) diluted with PBS 1000-fold was added to the tissues. They were allowed to react at room temperature for 1 h. Then, washing was done with PBS (pH 7.5) for 5 min three times. After addition of a peroxidase-labeled avidin-biotin complex solution and reaction for 40 min, washing was done with PBS for 5 min three times.

(6) The slide glasses were immersed in 0.1% diaminobenzidine (DAB) and 0.02% H₂O₂/PBS (pH 7.5)

solutions and allowed to react at room temperature for 10 min. Then, the slide glasses were transferred into distilled water to quench the reaction. After staining with hematoxylin, washing was done with running water.

5 When either of the antibodies was used, staining of the neuralized protein in the brain tissue was observed.

10 **EXAMPLE 8 Isolation of the Murine Neuralized cDNA and Determination of Its Base Sequence and the Amino Acid Sequence Encoded by Said Gene**

1. Obtaining of DNA Fragments

A murine neonatal brain cDNA library was used as a cDNA library and PCR was performed under the following conditions.

Employing the aforementioned Primer P3 in view of the base sequence of Fragment A and the murine newborn offspring brain cDNA library as a template, single PCR was performed under the following conditions:

20

Murine new born offspring

Brain cDNA library (1 μ g/ml) 1 μ l

dNTPmix 0.6 μ l

Primer P3 1 μ l

25

3.3x PCR buffer 4.5 μ l

25 mM MgCl₂ 0.72 μ l

Distilled water	6.68 μ l
Total	14.5 μ l

Mineral oil (15 μ l) was overlaid on the
 5 aforementioned composition and it was allowed to stand
 at 94 °C for 5 min. Then, Taq polymeraseXL, 0.5 μ l,
 (Registered Trademark: available from Takara Shuzo Co.
 Ltd.) was added. The following cycle was repeated 50
 10 times to allow reaction: at 94 °C for 30 sec, at 60 °C
 for 30 sec, and then at 72 °C for 1.5 min.
 Subsequently, the extension reaction of fragment was
 carried out at 55 °C for 2 min and finally at 72 °C for
 10 min, thus completing PCR.

This PCR product was used as a template and second
 15 PCR was performed. Employing the aforementioned
 Primers P4 and T3, PCR was performed under the
 following conditions:

PCR product	2 μ l
20 dNTPmix	1 μ l
Primer P4	1 μ l
Primer T3	1 μ l
3.3x PCR buffer	7.5 μ l
25 mM MgCl ₂	1.2 μ l
25 Distilled water	11.8 μ l
Total	24.5 μ l

Mineral oil (20 μ l) was overlaid on the
aforementioned composition and it was allowed to stand
at 94 °C for 5 min. Then, Taq polymeraseXL, 0.5 μ l,
(Registered Trademark: available from Takara Shuzo Co.
5 Ltd.) was added. The following cycle was repeated 40
times to allow reaction: at 94 °C for 30 sec, at 60 °C
for 30 sec, and then at 72 °C for 1 min. Subsequently,
the extension reaction of fragment was carried out at
55 °C for 2 min and finally at 72 °C for 10 min, thus
10 completing PCR. The DNA fragment will be referred to
as "Fragment MA" hereinafter.

Fragment MA that had been obtained through the 5'-
extension reaction as described above was subjected to
electrophoresis on a minigel (0.75% agarose gel). A
15 band for Fragment MA was sliced out. Fragment MA was
recovered with a GeneClean (available from BIO 101,
Inc.) and was examined for the presence of its band on
minigel electrophoresis.

Fragment MA (1 μ l) was taken and diluted with 99
20 μ l of TE. Absorbance at 260 nm (A₂₆₀) was measured and
DNA concentrations were computed: when A₂₆₀ was 1.0,
the DNA concentration was taken as 50 μ l/ml. Fragment
MA was diluted with TE so as to give a DNA
concentration of 1 μ l/ml.

25 This diluted solution was subjected to DNA
sequencing according to the dye-terminator method,

using an autosequencer (ABI Model 373A) and the base sequence of Fragment MA was determined.

2. Obtaining of the Murine Neuralized cDNA

Sense Primer P9, 5'-GACTCCATCG GGGGCTCCTT CCC-3' (the base sequence set forth in SEQ ID NO: 17 in the Sequence Listing), which served as a 5'-primer and which corresponded to a part of Fragment MA, antisense Primer P10, 5'-CTAGGAGCTG CGGTAGGTCT TGA-3' (the base sequence set forth in SEQ ID No. 18 in the Sequence Listing), which served as a 3'-primer, were synthesized with a DNA synthesizer (ABI Model 392).

Employing the aforementioned primers as a template and the murine neonatal brain cDNA library (available from CLONTECH Laboratories Inc.), PCR was performed under the following conditions:

	cdNA	1 μ l
	dNTPmix	1 μ l
	Sense primer	1 μ l
20	Antisense primer	1 μ l
	3.3x PCR buffer	7.5 μ l
	25 mM MgCl ₂	1.2 μ l
	Distilled water	11.8 μ l
	Total	24.5 μ l

25

Mineral oil (15 μ l) was overlaid on the

aforementioned composition and it was allowed to stand at 96 °C for 5 min. Then, Taq polymeraseXL, 0.5 μ l, (Registered Trademark: available from Takara Shuzo Co. Ltd.) was added. The following cycle was repeated 40
5 times to allow reaction: at 94 °C for 30 sec, at 60 °C for 30 sec, and then at 72 °C for 1.5 min. Finally, the extension reaction of fragment was carried out at 72 °C for 10 min, thus completing PCR.

This full-length cDNA was sequenced according to
10 the dye-terminator method. This gene was determined to be a murine homologous gene of the neuralized gene of *Drosophila*. This base sequence is shown in SEQ ID NO: 4 in the Sequence Listing. In addition, the amino acid sequence of the murine neuralized protein that this
15 gene encodes is shown in SEQ ID NO: 3 in the Sequence Listing.

3. Preparation of a Transformant

A murine neuralized gene comprising the aforementioned base sequence was inserted into the TA
20 cloning site of a pCRII vector (Registered Trademark, available from Invitrogen Corp.), and said vector was introduced to *E. coli* DH5 α to prepare a transformant.

INDUSTRIAL APPLICABILITY

25 The neuralized gene of *Drosophila* is one of the gene groups that function during the formation of nerve

cells. Since its deficiency causes the abnormal proliferation of immature nerve cells at a larva stage of *Drosophila*, it is believed to be the gene regulating proliferation, differentiation-inducing signals of
5 cells in the nervous system. This invention has revealed that the neuralized gene, which has traditionally been known in *Drosophila*, exists in vertebrate animals, more specifically in mammals such as human and mouse. The human neuralized gene appears
10 in brain and muscle tissues with high frequency and is a potential candidate for one of the genes that participate in the development of malignant tumors. The neuralized proteins, the genes encoding said proteins, the antisense genes of said genes, and the
15 antibodies recognizing said proteins according to this invention are powerful tools as a reagent for analyzing cerebral tumor.

配列表

SEQUENCE LISTING

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	Cys Ser Pro Ala Ser Thr Pro Thr Ser Pro Ser Ala Leu Gly Ile Arg				
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CLAIMS

1. A protein comprising an amino acid sequence having at least the amino acid sequence set forth in SEQ ID NO: 1 in the Sequence Listing.

5 2. A protein comprising an amino acid sequence having at least the amino acid sequence set forth in SEQ ID NO: 3 in the Sequence Listing.

3. A protein comprising an amino acid sequence obtained by substituting, deleting, or adding one or
10 more amino acids of the amino acid sequence set forth in SEQ ID NO: 1 in the Sequence Listing.

4. A protein comprising an amino acid sequence obtained by substituting, deleting, or adding one or
more amino acids of the amino acid sequence set forth
15 in SEQ ID NO: 3 in the Sequence Listing.

5. A polynucleotide encoding the protein according to any of claims 1 - 4.

6. A polynucleotide being a part of the polynucleotide according to claim 5 and comprising not
20 less than 12 consecutive bases.

7. A polynucleotide being an antisense polynucleotide or a part of a derivative thereof and comprising not less than 12 consecutive bases, the antisense polynucleotide comprising the base sequence
25 of an antisense strand of the polynucleotide according to claim 5.

8. The polynucleotide according to either claim 5 or claim 7, wherein the polynucleotide is chemically modified.

5 9. A method for obtaining a cDNA that is a homologous DNA comprising the base sequence set forth in SEQ ID NO: 2 or in SEQ ID NO: 4 in the Sequence Listing, the method comprising:

obtaining the cDNA from a cDNA library with the aid of a polynucleotide as a probe, the cDNA
10 capable of hybridizing to the polynucleotide used as the probe,

wherein the polynucleotide comprises a coding region of the polynucleotide according to any of claims 5 - 8.

15 10. A cDNA obtained by the method according to claim 9, being a homologous DNA comprising the base sequence set forth in SEQ ID NO: 2 or in SEQ ID NO: 4 in the Sequence Listing.

20 11. A protein comprising an amino acid sequence that the cDNA according to claim 10 encodes, and being a homologue of the protein according to claim 1 or claim 2.

12. An antibody recognizing the protein according to claim 1, 2, 3, 4 or 11.

25

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UNSCANNABLE ITEM

RECEIVED WITH THIS APPLICATION

(ITEM ON THE 10TH FLOOR ZONE 5 IN THE FILE PREPARATION SECTION)

DOCUMENT REÇU AVEC CETTE DEMANDE

NE POUVANT ÊTRE BALAYÉ

(DOCUMENT AU 10 IÈME ÉTAGE AIRE 5 DANS LA SECTION DE LA
PRÉPARATION DES DOSSIERS)