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SPECIFICATION

CHONDROITIN SYNTHASE

TECHNICAL FIELD

The present invention relates to (a) a vector having DNA encoding chondroitin synthase, (b) a method of producing chondroitin synthase, (c) a method of producing a saccharide chain having a repeating disaccharide unit of chondroitin, and (d) a probe for hybridization of chondroitin synthase.

BACKGROUND ART

Chondroitin sulfate, which is a kind of glycosaminoglycan (GAG), exists as a proteoglycan on cell surfaces and in an extra cellular matrix. Chondroitin sulfate draws attention because Chondroitin sulfate plays an important role in neural network formation in the developing mammalian brain (Arch. Biochem. Biophys. 374, 24-34 (2000); Trends Glycosci, Glycotechnol. 12, 321-349 (2000)).

Chondroitin sulfate has a straight-chained polymer structure having a repeating disaccharide unit having a glucuronic acid residue (GlcUA) and an *N*-acetylgalactosamine residue (GalNAc). A serine residue in a core protein is covalent-bonded with chondroitin sulfate via 4-saccharide structure (GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1)

peculiar thereto (Glycoproteins, ed. Gottschalk, A. (Elsevier Science, New York), pp. 491-517 (1972); The Biochemistry of Glycoproteins and Proteoglycans, ed. Lennarz, W. J. (Plenum, New York), pp. 267-371 (1980)).

GAG is biosynthesized by sequentially transferring saccharides from UDP-sugar to a non-reducing end of a saccharide chain. It was found that (a) purification of bovine serum gave a glycosyltransferase that involves in biosynthesis of a repeating disaccharide unit of heparin/heparan sulfate, and (b) cDNA cloning revealed that a single protein of the glycosyltransferase catalyses both transferase reactions of *N*-acetylglucosamine residue (GlcNAc) and GlcUA.

On the other hand, a glycosyltransferase that involved in biosynthesis of the repeating disaccharide unit of chondroitin sulfate has not been cloned yet except the chondroitin synthase derived from a bacterium (J. Biol. Chem. 275, 24124-24129 (2000)). GlcUA transferase II (GlcAT-II) and GalNAc transferase II (GalNAcT-II) have been purified from avian cartilage (J. Biol. Chem. 272, 14399-14403 (1997)) and from bovine serum (Eur. J. Biochem. 264, 461-467 (1999)). However, cDNA cloning of those enzymes has not been performed yet because it is difficult to purify those enzymes to form homogeneity.

An object of the present invention is to provide (a) a vector having DNA encoding human chondroitin synthase,

(b) a method of producing human chondroitin synthase, (c) a method of producing a saccharide chain having a repeating disaccharide unit of chondroitin, and (d) a probe for hybridization of human chondroitin synthase.

DISCLOSURE OF INVENTION

By searching through a human cDNA database, inventors of the present invention successfully found out a candidate DNA encoding human chondroitin synthase. The inventors accomplished the present invention by actually expressing the candidate DNA and confirming that the candidate DNA encodes finding human chondroitin synthase.

The present invention provides the followings:

(1) A vector carring one of DNA (a) , (b) or (c) the DNA (b) or (c) encoding a protein having catalytic activities (α) and (β) , excluding a DNA encoding a protein at amino-acid position #1 to 802 in SEQ. ID. NO: 2):

(a) DNA encoding a protein having an amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO: 2;

(b) DNA that, in a stringent condition, hybridizes with the DNA (a), the DNA complementary with DNA (a), or DNA having part of a nucleotide sequence of the DNA (a) or the DNA complementary with the DNA (a);

(c) DNA encoding a protein having an amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO:2, wherein

one or several amino acids in the amino acid sequence are substituted, deleted, inserted, or transpositioned;

(α) catalytic activity that transferase GalNAc from UDP-GalNAc to chondroitin;

(where UDP is uridine 5'diphosphate and GalNAc is *N*-acetylgalactosamine residue), and

(β) catalytic activity that transferase GlcUA from UDP-GlcUA to chondroitin,

(where UDP is uridine 5'diphosphate and GlcUA is glucuronic acid residue).

(2) The vector as set forth (1), wherein:

the DNA (a) corresponds to nucleotide numbers 633 to 2900 in SEQ. ID. NO: 1.

(3) The vector as set forth in (1) or (2), wherein:

the proteins are soluble.

(4) The vector as set forth in any one of (1) to (3), being an expression vector.

(5) A transformant whose host is transformed by a vector having any one of DNA (a) to (c), the DNA (b) or (c) encoding a protein having catalytic activities (α) and (β):

(a) DNA encoding a protein having an amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO: 2;

(b) DNA that, in a stringent condition, hybridizes with the DNA (a), the DNA complementary with DNA (a), or DNA having part of a nucleotide sequence of the DNA (a) or the DNA complementary with the DNA (a);

(c) DNA encoding a protein having an amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO:2, wherein one or several amino acids in the amino acid sequence are substituted, deleted, inserted, or transpositioned;

(α) catalytic activity that transferase GalNAc from UDP-GalNAc to chondroitin;

(where UDP is uridine 5'diphosphate and GalNAc is *N*-acetylgalactosamine residue),

(β) catalytic activity that transferase GlcUA from UDP-GlcUA to chondroitin,

(where UDP is uridine 5'diphosphate and GlcUA is *N*-glucuronic acid residue).

(6) The transformant as set forth in (5), wherein:

the DNA (a) encodes finding from nucleotide numbers 633 to 2900 in SEQ. ID. NO: 1.

(7) The transformant as set forth in (5) or (6), wherein:

the proteins are soluble.

(8) A method for producing chondroitin synthase, the method comprising the steps of:

growing a transformant set forth in any one of (5) to (7); and

obtaining the chondroitin synthase from the trnasformant thus grown.

(9) A reagent for use in chondroitin synthesis, the reagent having an enzyme protein that has an amino acid

sequence including an amino acid sequence (A) or (B) and has catalytic activities (α) and (β):

(A) amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO: 2;

(B) amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO:2, wherein one or several amino acids in the amino acid sequence are substituted, deleted, inserted, or transpositioned.

(α) catalytic activity that transferase GalNAc from UDP-GalNAc to chondroitin,
(where UDP is uridine 5'diphosphate, and GalNAc is *N*-acetylgalactosamine residue);

(β) catalytic activity that transferase GlcUA from UDP-GlcUA to chondroitin,
(where UDP is uridine 5'diphosphate and GlcUA is *N*-glucuronic acid residue).

(10) The reagent as set forth in (9), wherein:
the enzyme protein is soluble.

(11) Method for producing a saccharide chain expressed by Formula (3), the method comprising at least the step of causing a reagent to contact with GalNAc donor and a saccharide chain expressed by Formula (1), the reagent set forth in (9) or (10):

GlcUA-GalNAc-R^1 ··· (1),

GalNAc-GlcUA-GalNAc-R^1 ··· (3),

(where GlcUA and GalNAc are as defined above, "-" indicates

a glycoside linkage, R^1 is an arbitrary group).

(12) A method for producing a saccharide chain expressed by Formula (4), the method comprising at least the step of causing a reagent to contact with GlcUA donor and a saccharide chain expressed by Formula (2), the reagent set forth (9) or (10):

GalNAc-GlcUA-R^2 ··· (2),

GlcUA-GalNAc-GlcUA-R^2 ··· (4),

(where GlcUA, GalNAc, and "-" are as defined above, R^2 is an arbitrary group).

(13) A method for producing a saccharide chain selected from saccharide chains expressed by Formulas (5) and (7) respectively, the method comprising at least the step of causing a reagent to contact with GalNAc donor, GlcUA donor and a saccharide chain expressed by Formula (1), the reagent set forth in Claim (9) or (10):

GlcUA-GalNAc-R^1··· (1),

(GlcUA-GalNAc)n-GlcUA-GalNAc-R^1··· (5),

GalNAc-(GlcUA-GalNAc)n-GlcUA-GalNAc-R^1··· (7),

(where n is an integer not less than 1, GlcUA, GalNAc, and "-" are as defined above, R^1 is an arbitrary group).

(14) A method for producing a saccharide chain selected from saccharide chains expressed by Formulas (6) and (8) respectively, the method comprising at least the step of causing a reagent to contact with GalNAc donor, GlcUA donor and a saccharide chain expressed by Formula (2), the reagent

set forth in (9) or (10):

GalNAc-GlcUA-R^2 ··· (2),

(GalNAc-GlcUA)n-GalNAc-GlcUA-R^2 ··· (6),

GlcUA-(GalNAc-GlcUA)n-GalNAc-GlcUA-R^2 ··· (8),

(where n is an integer not less than 1, GlcUA, GalNAc, and "-" are as defined above, R^2 is an arbitrary group).

(15) A probe for hybridization, the probe containing a nucleotide equence from nucleotide numbers 495 to 2900 in SEQ. ID. NO: 1, or a sequence complementary with part of the nucleotide sequence.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 illustrates comparison among a putative amino acid sequence of human chondroitin synthase (Human), and amino acid sequences of homologous proteins of *C. elegans* (T25233) and *Drosophila* (AE003499). Those putative amino acid sequences were analyzed by using GENETYX-MAC (version 10) computer program. Respectively, the black boxes indicate that three of them have an identical amino acid, and the gray boxes indicate that two of them have an identical amino acid. The broken lines indicate gaps inserted for attaining highest degree of matching. Surrounded by the rectangular frames are predicted transmembrane domain. A DXD motif that was preserved is indicated by underline. Three sites predicted *N*-glycosylation sites are marked with star marks.

Figure 2 shows a genome structure of the human chondroitin synthase geneExon regions are indicated by the boxes. The black boxes indicate coding sequences, whereas the white boxes indicate 5'-and 3'-untranslated sequences. The translation initiation codon (ATG) and stop codon (TAA) are shown as well. The black horizontal line indicates introns.

Figures 3(a) and 3(b) show results of identification of reaction products from human chondroitin synthase reaction. Figure 3(a): A reaction product of GlcUA transferase collected from a Superdex peptide column was digested by chondroitinase AC-II or β-glucuronidase. The reaction product (black rectangle) that was not digested, the reaction product (black circle) that was digested by chondroitinase AC-II, and the reaction product that was digested by β-glucuronidase, were applied into the Superdex peptide column. Radioactivity of elution fractions of each (0.4ml each) was analyzed. Arrows indicate elution positions of saturated disaccharide (1, GlcUAβ1-3GalNAc), or isolated GlcUA (2, [^{14}C]GlcUA).
Figure 3(b): A reaction product of GalNAc transferase collected from a Superdex peptide column was digested by chondroitinase AC-II. The reaction product (black rectangle) that was not digested, or the reaction product (black circle) that was digested by chondroitinase AC-II, was applied into the Superdex peptide column. Radioactivity of elution fractions of each (0.4ml each) was analyzed. Arrows indicate elution positions of saturated

disaccharide (1, GlcUAβ1-3GalNAc), or isolated GalNAc (2, [^{3}H]GalNAc).

Figure 4 shows a result of Northern blot analysis (a photograph of gel-electrophoresis) of chondroitin synthase in a human tissue. Hybridization of RNAs derived from various human tissues was carried out by using probes of chondroitin synthase: Lane 1 is brain; Lane 2 is heart; Lane 3 is skeletal muscle; Lane 4 is colon; Lane 5 is thymus; Lane 6 is spleen; Lane 7 is kidney; Lane 8 is liver; Lane 9 is small intestine; Lane 10 is placenta; Lane 11 is lung, and Lane 12 is leukocyte in peripheral blood.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention is described in detail below with reference to an embodiment of the invention.

(1) Vector of the invention

A vector of the present invention is a vector carrying one of DNA (a), (b) or (c), excluding a DNA encoding a protein at amino-acid position #1 to 802 in SEQ. ID. NO: 2):

(a) DNA encoding a protein having an amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO: 2;

(b) DNA that, in a stringent condition, hybridizes with the DNA (a), the DNA complementary with DNA (a), or DNA having part of a nucleotide sequence of the DNA (a) or the DNA complementary with the DNA (a);

(c) DNA encoding a protein having an amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO:2, wherein

one or several amino acids in the amino acid sequence are substituted, deleted, inserted, or transpositioned;

the above DNA (b) or (c) encoding a protein having catalytic activities (α) and (β),

(α) catalytic activity that transferase GalNAc from UDP-GalNAc to chondroitin; and

(β) catalytic activity that transferase GlcUA from UDP-GlcUA to chondroitin.

Note that, the foregoing chondroitin is a polymer made of repeating disaccharide units of GlcUA and GalNAc. The chondroitin includes one whose non-reducing end is GlcUA and one whose non-reducing end is GalNAc. Thus, it can be said that the transfer of GalNAc is performed with respect to chondroitin having the non-reducing end of GlcUA, and the transfer of GlcUA is performed with respect to chondroitin having the non-reducing end of GalNAc.

As it will be explained in the Examples below, it was confirmed that a protein containing the amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO: 2, has enzyme activity of human chondroitin synthase. It was deduced that a transmembrane domain is included in the amino-acid sequence from amino acid numbers 1 to 46 in SEQ. ID. NO: 2. In this view, use of DNA not containing a sequence for encoding the amino acid sequence from amino acid numbers 1 to 46 is preferable in that the use of such DNA enables expression of chondroitin synthase in a soluble state. More

specifically, a preferable vector has "DNA encoding the amino-acid sequence from amino-acid numbers 47 to 802, the DNA containing no sequence for encoding the amino-acid sequence from amino acid numbers 1 to 46".

In naturally-existing proteins, besides polymorphism or mutation of the DNA that codes for the protein, other mutations, such as substitution, deletion, insertion, and transposition of the amino acid in its amino acid sequence may occur due to modification reaction of the created protein inside a cell or during purification. However, there has been known that some of the naturally-existing proteins in which such mutation occurs, have substantially equal physiology and biological activity to that of a protein in which such mutation has not occurred. The scope of the vector according to the present invention includes such a vector having the DNA that encodes a protein that is different slightly in terms of structure but is substantially similar in terms of function. The same is true for a case where such a mutations is introduced in the amino acid sequence of the protein artificially. In this case, it is possible to create a larger variety of mutant. For example, there has been known that a protein prepared by replacing, with serine, a cysteine residue in an amino acid sequence of human interleukin-2 (IL-2), has interleukin-2 activity (Science, 224, 1431 (1984)). Further, there has been known that a kind of protein has a peptide domain that

is not essential for its activity. Examples of this peptide domain may be a signal peptide contained in an extracellularly secreted protein, or a pro sequence of precursor of a protease etc. Most of such domains are removed after translation or upon conversion into an activated protein. Even though these proteins have different primary structures, functions of those proteins are equivalent finally. The foregoing DNA (b) and (c) is examples of DNA encoding such proteins.

The "stringent condition" of the DNA (b) refers to a condition for forming a specific hybrid and no unspecific hybrid. (refer to Sambrook, J. et al., Molecular Cloning A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989) etc.). The "stringent condition" may be, for example, a condition obtained by carrying out hybridization at 42°C in a solvent containing 50% formamide, 4× SSC, 50mM HEPES (pH7.0), 10× Denhardt's solution, and a salmon sperm DNA of 100μg/ml, and then washing at room temperature with 2× SSC and a 0.1% SDS solution, and sequentially washing at 50°C with 0.1× SSC and a 0.1% SDS solution.

The "several number of amino acids" of (c) refers to an acceptable number of amino acids in which such mutation that does not cause catalytic activities (α) and (β) occurs. The catalytic activities (α) and (β) are explained later. For example, for a protein made of 800 amino-acid residue,

the acceptable number is in a range of 4 to 40, preferably 4 to 20, more preferably 4 to 10.

Note that, DNA to be carried by the vector of the present invention may have various nucleotide sequences due to degeneracy of genetic code. This is however easily understood by a person skilled in the art.

The catalytic activities (α) and (β) can be measured by a general assay method for glycosyltransferase.

More specifically, as explained in the Examples below, the catalytic activity (α) can be measured by a method using transfer reaction of GalNAc into chondroitin by using a UDP *N*-acetylgalactosamine (UDPGalNAc) as a donor, whereas the catalytic activity (β) can be measured by a method using transfer reaction of GlcUA into chondroitin by using a UDP-glucuronic acid (UDP-GlcUA) as a donor. Accordingly, by checking the presence of the transfer activities as an index, it is easy for a person skilled in the art to select at least one of substitution, deletion, insertion, and transposition of one or some of amino acid residues, the substitution, deletion, insertion, and transposition causing no substantial deterioration in the activity. Further, it is also possible to easily select DNA that codes for a protein having catalytic activities of (α) and (β), from among DNA that hybridize under the "stringent condition".

Note that chondroitin for use herein includes both (i)

one whose non-reducing terminal is GlcUA, and (ii) one whose non-reducing terminal is GalNAc.

Moreover, the protein that the DNA (b) or the DNA (c) codes for, further has the all of the following characteristics in (γ), preferably.

(γ) The following acceptors substantially receive no monosaccharide from the following donors (in the bracket).

- Galβ1-3Galβ1-4Xyl (UDP-GlcUA)
- GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1-O-Ser (UDP-GalNAc)
- α- thrombomodulin (UDP-GalNAc)
- sheep submandubular asialomucin (UDP-Gal)
- GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (UDP-Gal)

Note that, the α-thrombomodulin includes tetrasaccharide made of GlcUAβ1-3Galβ1-3Galβ1-4Xyl. Further, the sheep submandubular asialomucin contains GalNAcα1-O-Ser/Thr.

Note that, in the foregoing condition, Gal denotes a galactose residue, Xyl denotes a xylose residue, Ser denotes a serin residue, and Thr denotes a threonine residue, respectively. The remaining symbols are all the same as above.

It is preferable that a protein that is coded for by the DNA carried by the vector of the present invention is a water-soluble protein. This is because the water-soluble protein generally does not have a transmembrane domain, and the water-soluble protein expressed is soluble to an

aqueous solvent etc., thus being easy to be purified.

The DNA carried by the vector of the present invention preferably does not contain DNA encoding the amino-acid sequence from amino-acid numbers 1 to 46 in the SEQ. ID. NO: 2. Most preferable encodes finding from nucleotide numbers 633 to 2900 in the SEQ. ID. NO: 1.

Further, it is further preferable that this vector is an expression vector, since it is desirably used in the producing method of chondroitin synthase. The method will be described later.

For example, an expression vector having DNA encoding the amino-acid sequence from amino-acid numbers 47 to 802 (DNA not containing the amino-acid sequence from amino-acid numbers 1 to 46) may be prepared with the following method.

<A> Preparation of DNA combined with the vector

First, obtained is a cDNA clone (GenBank accession number AB023207) specified as "KIAA0990" in the HUGE protein database. Then, by using the cDNA clone as a template, amplification is carried out through PCR method with a 5'-primer (5'-CCCTCGAGGGGCTGCCGGTCCGGGC-3' (SEQ. ID. NO: 3)) containing a XhoI site, and 3'-primer (5'-CCCTCGAGCAATCTTAAAGGAGTCCTATGTA-3' (SEQ. ID. NO:4)) containing a XhoI site 138bp downstream from the stop codon.

The PCR method may be carried out with Pfu polymerase (Stratagene, La Jolla, California, USA) for 34 cycles of

94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 180 seconds in 5% (v/v) dimethylsulfoxide. However, the PCR method may also be carried out in a general manner

<B> Introduction of DNA fragment into vector

The vector of the present invention may be prepared by introducing, into a well-known vector, the DNA obtained in the foregoing manner.

The vector into which the DNA is introduced may be selected from appropriate expression vectors(a phage vector, a plasmid vector, or the like), which enable expression of the introduced DNA. The vector should enable expression of the foregoing DNA inside the host cells into which the vector of the present invention is transfected.

Such a host-vector system may be a combination of a mammalian cell such as a COS cell or 3LL-HK46 cell, with an expression vector for mammalian cells such as pGIR201 (Kitagawa, H., and Paulson, J. C. (1994) J. Biol. Chem. 269, 1394-1401), pEF-BOS (Mizushima, S., and Nagata, S. (1990) Nucleic Acid Res. 18, 5322), pCXN2 (Niwa, H., Yamanura, K. and Miyazaki, J. (1991) Gene 108, 193-200), pCMV-2 (Eastman Kodak), pCEV18, pME18S (Maruyama et al. Med. Immunol., 20, 27(1990)) or pSVL (Pharmacia Biotec); otherwise, a combination of a coliform (*E. Coli*) with en expression vector for prokaryotic cells, such as pTrcHis (produced by Inbitrogen Co., Ltd.), pGEX (produced by Pharmacia Biotec Inc.), pTrc99 (produced by Pharmacia Biotec Inc.), pKK233-3

(produced by Pharmacia Biotec Inc.), pEZZZ18 (produced by Pharmacia Biotec Inc.), pCH110 (produced by Pharmacia Biotec Inc.), pET (produced by Stratagene Co.), pBAD (produced by Inbitrogen Co., Ltd.), pRSET (produced by Inbitrogen Co., Ltd.), or pSE420 (produced by Inbitrogen Co., Ltd.). In addition, the host cell may be insect cells, yeast, or grass bacillus, which are used with various corresponding vectors. Among these, the combination of a mammal cell and pEF-BOS is most preferable.

Further, the vector to introduce the DNA therein may be a vector constructed to cause expression of a fusion protein made of the protein coded by the DNA and a marker peptide. This type of vector is particularly preferable in the case of purifying chondroitin synthase, which is expressed by the vector of the present invention. The marker peptide may be a protein A sequence, an insulin signal sequence, His, FLAG, CBP (calmodulin binding protein), or GST (glutathione S-transferase), for example. Fusing of such a marker peptide to a protein A sequence allows easy affinity purification, and fusing the marker peptide into an insulin signal sequence allows extra cellular secretion (into culture medium etc.) of enzyme.

In the case of any vectors, the processing may be carried out in a general way so as to allow binding of the DNA and the vector; for example, the DNA and the vector may be bonded together after treating with a restriction

endonuclease or the like, and if necessary, blunting or binding of the sticky end.

More specifically, the DNA (PCR fragment) obtained through the foregoing method <A> is digested by XhoI, and the both ends of the fragment are partially filled with Klenow fragment (New England Biolabs, Beverly, Massachusetts), dCTP, and dTTP. Further, the pGIR201protA (J. Biol. Chem., 269, 1394-1401 (1994)) vector digested by BamHI is also partially filled with dATP and dGTP. The fragments thus obtained were then subcloned into the pGIR201protA, resulting in the fusion of the DNA encoded by the DNA created through the method <A> with the insulin signal sequence and the protein A sequence present in the vector. An NheI fragment containing this fusion protein sequence was inserted into the XbaI site of the expression vector pEF-BOS (Nucleic Acid Res., 18, 5322 (1990)), thereby obtaining an expression vector for expression of chondroitin synthase that is fused with an insulin signal sequence and a protein A sequence.

(2) Transformant of the present invention

A transformant of the present invention is a transformant where a host is transformed by the vector of the present invention (including a vector containing DNA encoding a protein of amino acid sequence from amino acid numbers 1 to 802 in SEQ ID NO. 2).

As used herein, "host" may be of any kind, provided

that it can be recombined by a vector of the present invention. Preferably, the host is the one which can make full use of the capabilities of DNA carried by a vector of the present invention or a recombinant vector into which the same DNA is recombined. Examples of the host include: animal cells; plant cells; and microorganism cells (fungus body), and COS cell (including COS-1 cell and COS-7 cell); and more specifically, a mammalian cell including 3LL-HK 46 cell; a coliform (E. coli); insect cell; yeast; and grass bacillus are included, for example. The host can be selected as appropriate in accordance with the vector of the present invention. However, in the case where the vector for use in the present invention is a vector based on pEF-BOS, for example, a cell derived from a mammal is preferably selected, and a COS cell is more preferably selected among them.

Transformation of the host by a vector of the present invention can be performed by standard methods known in the art. For example, transformation can be performed by introducing the vector into the host by (i) a method using a reagent for transfection, (ii) DEAE-dextran method, (iii) electroporation method, or (iv) other methods.

The transformant of the present invention obtained in such a manner can be used in applications such as production of chondroitin synthase, as described later.

(3) Method of Producing Chondroitin Synthase

The method of producing chondroitin synthase of the

present invention is characterized in that a transformant of the present invention is grown to obtain chondroitin synthase from the grown transformant.

As used herein, "growth" refers to a concept including proliferation of a cell as a transformant of the present invention and of a microorganism itself and growth of a creature including animal and insect into which a cell as a transformant of the present invention is introduced. Further, as used herein, "growth product" refers to a concept including a culture medium after the growth of the transformant of the present invention (supernatant of a culture solution), a cultured host cell, a secretion, and an ejection.

Conditions of the growth (culture medium, culture condition, and others) are selected as appropriate in accordance with a host to be used.

According to this production method, chondroitin synthase in various forms can be produced in accordance with a transformant to be used.

A soluble chondroitin synthase is produced by, for example, growing a transformant prepared by transformation by the expression vector having DNA encoding the amino acid sequence from amino acid numbers 47 to 802 in SEQ ID No. 2, as a vector of the present invention.

Further, an insoluble (membrane-binding) chondroitin synthase is produced by growing a transformant prepared by

transformation by the expression vector having DNA encoding the amino acid sequence from amino acid numbers 1 to 802 in SEQ ID No. 2, as a vector of the present invention.

Still further, chondroitin synthase fused with a marker peptide is produced by growing a transformant prepared by transformation by an expression vector constructed so as to express a fusion protein fused with a marker peptide.

Chondroitin synthase can be obtained from the growth product by a well-known method for protein extraction and purification, depending on a form of the produced chondroitin synthase.

For example, when chondroitin synthase is produced in a soluble form secreted in a culture medium (supernatant of a culture solution), the obtained culture medium may be directly used as chondroitin synthase. Further, when chondroitin synthase is produced in a soluble form secreted in a cytoplasm or in an insoluble (membrane-bound) form, extraction of chondroitin synthase can be performed by any one or combination of a method using a nitrogen cavitation apparatus, homogenization, glass beads mill method, sonication, osmotic shock, extraction by cell homogenization using a method such as freezing and thawing method, and surface-active agent extraction. Alternatively, the extracted product may be directly used as chondroitin synthase.

It is also possible and preferable to further purify chondroitin synthase from such culture medium and extracted product. Purification may be incomplete purification (partial purification) or complete purification, which may be selected as appropriate in accordance with (i) the intended use of chondroitin synthase, and (ii) the like.

Specifically, examples of purifying method include: any one or combination of salting-out by ammonium sulphate, sodium sulphate, or the like; centrifugal separation; dialysis; ultrafiltration; adsorption chromatography; on-exchange chromatography; hydrophobic chromatography; reverse phase chromatography; gel filtration; gel permeation chromatography; affinity chromatography; and electrophoretic migration.

For example, when chondroitin synthase is fused with protein A to produce a fusion protein, chondroitin synthase may be purified simply by affinity chromatography using a solid phase combined with IgG. Similarly, when chondroitin synthase is fused with His to produce a fusion protein, chondroitin synthase may be purified using a solid phase combined with magnetic nickel. When chondroitin synthase is fused with FLAG to produce a fusion protein, chondroitin synthase may be purified using a solid phase combined with anti-FLAG antibody. Still further, fusion with insulin signal eliminates the need for extracting operation such as cell disruption.

Production of the purified chondroitin synthase can be confirmed by analyzing its amino acid sequence, property, substrate specificity, and others.

(4) Reagent of the Present Invention

A reagent of the present invention is a chondroitin-synthesizing reagent, an enzyme protein having an amino acid sequence of (α) or (β), the enzyme protein having catalytic activities of (I) and (II):

(A) an amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO: 2;

(B) the amino acid sequence of (A) in which one or more amino acid is substituted, deleted, inserted, or transferred;

(α) catalytic activity that transferase GalNAc from UDP-GalNAc to chondroitin; and

(β) catalytic activity that transferase GlcUA from UDP-GlcUA to chondroitin.

The amino acid sequences of (A) and (B) are amino acid sequences respectively encoded by the DNA of (a) and (c), which are described in connection with the vector of the present invention. The amino acid sequences respectively encoded by the DNA of (a) and (c) are already described. (α) and (β) are already described in connection with the vector of the present invention.

An enzyme protein of the present invention is not limited to the enzyme protein from amino acid numbers 47

to 802 in SEQ. ID. NO: 2. The enzyme protein of the present invention may be, for example, an enzyme protein from amino acid numbers 1 to 802 in SEQ. ID. NO: 2.

The reagent of the present invention is a chondroitin-synthesizing reagent, which makes use of effects of an enzyme protein (chondroitin synthase) including an amino acid sequence of (A) or (B). The effects are an effect of transferring GalNAc, and an effect of transferring GlcUA.

The reagent of the present invention is used in order to synthesize chondroitin. In the present specification, to "synthesize chondroitin" is a concept that covers extending a saccharide chain of chondroitin by transferring and/or adding a saccharide to the chondroitin.

The reagent of the present invention is not limited to that of a particular form. The reagent of the present invention may be in a solution form, a frozen form, or a freeze-dried form. As long as the activities of the chondroitin synthase are not influenced, another component (e.g. a support acceptable as a reagent, or the like) may be included.

(5) Method of Producing Saccharide Chain

All methods of producing a saccharide chain according to the present invention, which use a reagent of the present invention, can be categorized into the following four types in accordance with substrates of saccharide donor and

receptor used.

<1> Method of producing a saccharide chain expressed by the following formula (3), including at least the step of bringing a reagent of the present invention into contact with GalNAc donor and a saccharide chain expressed by the following formula (1)

GlcUA-GalNAc-R^1 (1)

GalNAc-GlcUA-GalNAc-R^1 (3)

<2> Method of producing a saccharide chain expressed by the following formula (4), including at least the step of bringing a reagent of the present invention into contact with GlcUA donor and a saccharide chain expressed by the following formula (2)

GalNAc-GlcUA-R^2 (2)

GlcUA-GalNAc-GlcUA-R^2 (4)

<3> Method of producing a saccharide chain selected from the following formulas (5)and(7), including at least the step of bringing a reagent of the present invention into contact with GalNAc donor, GlcUA donor, and a saccharide chain expressed by the following formula (1)

GlcUA-GalNAc-R^1 (1)

(GlcUA-GalNAc)n-GlcUA-GalNAc-R^1 (5)

GalNAc-(GlcUA-GalNAc)n-GlcUA-GalNAc-R^1 (7)

<4> Method of producing a saccharide chain selected from the following formulas (6)and(8), including at least the step of bringing a reagent of the present invention into

contact with GalNAc donor, GlcUA donor, and a saccharide chain expressed by the following formula (2)

GalNAc-GlcUA-R^2 (2)

(GalNAc-GlcUA)n-GalNAc-GlcUA-R^2 (6)

GlcUA-(GalNAc-GlcUA)n-GalNAc-GlcUA-R^2 (8)

As GlcUA donor, nucleotide hypophosphoric acid-GalNAc is preferable, and UDP-GalNAc is especially preferable.

As GlcUA donor, nucleotide hypophosphoric acid-GlcUA is preferable, and UDP-GlcUA is especially preferable.

The way of contacting is not especially limited provided that respective molecules of the chondroitin synthase, donor, and receptor (saccharide chain) are brought into contact with one another to generate enzyme reaction, the chondroitin synthase, donor, and receptor being included in a reagent of the present invention. For example, these three types of molecules may bring into contact with one another in a solution in which they are dissolved. For continuous enzyme reactions, chondroitin synthase can be used in the form of immobilized enzyme coupled with a suitable solid phase (beads, etc.), and a membrane-type reactor using a membrane such as ultrafilter membrane and dialysis membrane may be used. As in the method described in WO 00/27437, enzyme reaction can be generated by coupling a receptor with a solid phase. Still further, a bioreactor for reproducing (synthesizing) a donor may be used together.

In the above <3> and <4>, GalNAc donor and GlcUA donor do not always need to be simultaneously brought into contact with a reagent of the present invention and the saccharide chain shown by the formula (1) or (2), and these donors may be alternately brought into contact with a reagent of the present invention and the saccharide chain shown by the formula (1) or (2).

Although conditions for enzyme reaction are not limited provided that chondroitin synthase acts, enzyme reaction at about neutral pH is preferable, and enzyme reaction in a buffer having buffer action at about neutral pH is more preferable. Further, although a temperature during enzyme reaction is not especially limited provided that activity of chondroitin synthase is held, about 30 to 40°C (e.g. 37°C) is exemplified. When there is a substance for increasing activity of chondroitin synthase, that substance may be added. For example, it is preferable that Mn^{2+} or other substance exists together. A reaction time can be determined as appropriate by a person skilled in the art in accordance with a reagent used of the present invention, the amount of donors and receptors, and other reaction conditions. Isolation of choidroitin from a reaction product, and the like process can be performed by the well known method.

Chondroitin sulfate can be produced by using a reagent of the present invention (chondroitin synthase) together

with sulfotransferase.

For example, in the above method of producing a saccharide chain (method of producing chondroitin), it is possible to produce chondroitin sulfate by causing sulfate donor (3'-phosphoadenosine 5'-phosphosulfate (PAPS), or the like) to exist together with sulfotransferase to simultaneously perform generation of chondroitin and transfer of sulfuric acid. In the same manner as described above, sulfotransferase may be used as immobilized enzyme combined with a suitable solid phase (beads, etc.), and a membrane-type reactor using a membrane such as ultrafilter membrane and dialysis membrane may be used for continuous reactions. At this moment, a bioreactor for reproducing (synthesizing) a sulfate donor may be used together.

Also, chondroitin can be produced by directly generating chondroitin in a host transformed by a vector of the present invention (transformant of the present invention).

Further, chondroitin sulfate can be directly produced in a host by introducing a vector of the present invention and cDNA encoding sulfotransferase into a host, and causing chondroitin synthase and sulfotransferase to simultaneously express in the host (transformant of the present invention including cDNA encoding sulfotransferase).

Sulfotransferase (or cDNA encoding sulfotransferase)

used herein may be an enzyme that transferase sulfuric acid to chondroitin (or cDNA encoding sulfotransferase) and can be selected as appropriate from the well known enzymes in accordance with the type of a desired sulfuric acid.

Further, two or more types of sulfotransferases each having a different transfer position of sulfuric acid (or cDNAs encoding the sulfotransferases) may be used together.

As an example of sulfotransferase, chondroitin6-O-sulfotransferase (J. Biol. Chem., 275(28), 21075-21080 (2000)) can be given, but the present invention is not limited to this, and other enzyme can be also used.

(6) Probe of the Present Invention

A probe of the present invention is a probe for hybridization having a nucleotide sequence from nucleotide numbers 495 to 2900, more preferably 633 to 2900, in SEQ ID No: 1, or having a complementary sequence partially in the same nucleotide sequence.

A probe of the present invention can be obtained by generating oligonucleotide having a nucleotide sequence from nucleotide numbers 495 to 2900, more preferably 633 to 2900, in SEQ ID No: 1, or having a complementary sequence partially in the same nucleotide sequence and labeling this oligonucleotide with a marker suitable for hybridization (e.g. radio isotope).

The length of oligonucleotide is selected as appropriate depending on conditions of hybridization using

a probe of the present invention.

A probe of the present invention is expected to be a useful tool for examining biological functions of chondroitin sulfate. This is because chondroitin sulfate widely expresses and plays an important role in many tissues, especially in a brain. This probe is considered to be useful for assessment of a connection between gene and disease.

[EXAMPLES]

The present invention is more specifically described below with reference to examples.

[Example 1]

(1) In Silico Cloning of Novel Human Glycosyltransferase cDNA

Screening of HUGE protein database at Kazusa DNA Research Institute (in Chiba Prefecture; http://www.kazusa.or.jp/huge/) was conducted by the keywords "one transmembrane domain" and "galactosyltransferase family". As a result of this, one clone (KIAA0990; GenBankTM accession number AB023207) was identified. An analysis of a nucleotide sequence of this clone revealed that this clone includes (i) a 5'-untranslated region of 494bp, (ii) a single open reading frame of 2406bp coding for a protein of 802 amino acids with three potential *N*-glycosylation sites (marked with asterisks in Fig. 1), and (iii) a 3'-untranslated region of about 1.7 kb with a presumptive polyadenylation signal. The nucleotide sequence

and an amino acid sequence deduced from the same are shown in SEQ. ID. NO: 1, whereas only the amino acid sequence is shown in SEQ. ID. NO: 2.

The clone was acquired from Kazusa DNA Research Institute. Northern blot analysis showed that the mRNA corresponding to the clone was about 5.0 kb in length in various human tissues (see Example 2), suggesting that the cDNA was approximately full-length. The deduced amino acid sequence corresponded to a 91,728-Da polypeptide. A predicted translation initiation site conformed to the Kozak consensus sequence for initiation (Nucleic Acids Res. 12, 857-872 (1984)), and an in-frame stop codon existed upstream of an initiation ATG codon allocated thereto.

A Kyte-Doolittle hydropathy analysis (J. Mol. Biol. 157, 105-132 (1982)) revealed one prominent hydrophobic segment of 17 amino acid residues in the NH_2-terminal region, predicting that the protein has a type II transmembrane topology which is typical in many Golgi localized glycosyltransferases having been cloned until today (see Fig. 1).

Database searches revealed that the amino acid sequence was, at its amino terminal, slightly homologous to human core 1 UDP-Gal:GalNAcα-Rβ1,3-Gal transferase (GenBankTM accession number AF155582), while the amino acid sequence was, at the carboxyl terminal, slightly homologous to a human UDP-Gal:GlcNAcβ-Rβ1,4-Gal transferase II

(GenBankTM accession number AB024434). Glycosyltransferases being homologous to the amino acid are characterized in that the connection patterns of saccharide chains are often preserved, even though different members are specific to different donors or receptors (Biochim. Biophys. Acta 1254, 35-53 (1999)).

Thus, the features of the amino acid sequence to be encoded suggested a possibility that the identified gene product could have activities of both the β1,3-GlcUA transferase (GlcAT-II) and β1,4-GalNAc transferase (GalNAcT-II). Furthermore, a homologue of the identified human gene was found in the Caenorhabditis elegans genome or Drosophila genome. Fig. 1 illustrates that to what extent the protein sequences respectively derived from human, C. elegans, and Drosophila are homologous to each other. The human protein sequence shares 36 homologies with the sequence of the C. elegans, and 42 homologies with the sequence of the Drosophila. These three proteins all include DDD at the amino terminal and DVD at the carboxyl terminal (cf. Fig. 1), thereby being considered as a conserved DXD motif which is found in most glycosyltransferases (Proc. Natl. Acad. Sci. U.S.A. 95, 7945-7950 (1998)).

In addition to the above, a data base search of the Human Genome Project identified a genome sequence (accession number NT010274.3) identical with the above-

mentioned cDNA sequence. Comparison of the cDNA and genome sequences revealed the genomic structure and chromosomal localization of the gene. The gene spans over 40 kb, and the coding region thereof was divided into three discrete exons as shown in Fig. 2. The intron/exon junctions followed the GT/AG rule and were flanked by conserved sequences. This gene is located on the human chromosome number 15.

(2) Construction of a Plasmid Including DNA Encoding a Novel Soluble Glycosyltransferase

A cDNA of a Glycosyltransferase that lacked 46 amino acid residues at its *N*-terminal from this novel glycosyltransferase was amplified by PCR. Specifically, with a KIAA0990 cDNA as a template, amplification was carried out using (a) a 5'-primer (5'-CCCTCGAGGGGCTGCCGGTCCGGGC-3' (SEQ. ID. NO: 3)) including an XhoI site and (b) a 3'-primer (5'-CCCTCGAGCAATCTTAAAGGAGTCCTATGTA-3' (SEQ. ID. NO: 4)) including a XhoI site located 138 bp downstream from the stop codon. The PCR was carried out with Pfu polymerase (Stratagene Co., La Jolla, California) for 34 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 180 seconds in 5(v/v) dimethyl sulfoxide. The PCR-product was then digested with the XhoI. Then, at both terminals of its fragment, the PCP product was partially filled with Klenow Fragment (New England Biolabs Inc., Beverly, Massachusetts), a dCTP, and a dTTP. A pGIR201protA (J. Biol.

Chem. 269, 1394-1401 (1994)) vector digested with BamHI was also partially filled with a dATP and a dGTP. The obtained fragment was subcloned into the pGIR201protA, so that the novel glycosyltransferase was fused with the insulin signal sequence and the protein A sequence which were carried in the vector. An NheI fragment including the above-mentioned fusion protein sequence was inserted into the XbaI site of the expression vector pEF-BOS (Nucleic Acids Res. 18, 5322 (1990)), whereby an expression plasmid was obtained.

This expression plasmid encodes a protein in which the first 46 amino acids of the glycosyltransferase is replaced with a cleavable insulin signal sequence and a protein A IgG-binding domain. In other words, the expression plasmid encodes a soluble chondroitin synthase fused with a cleavable insulin signal sequence and a protein A.

(3) Expression of a novel soluble Glycosyltransferase, and Enzymatic Assay thereof

By using FuGENE (Trademark) 6 (Roche Molecular Biochemicals Co., Tokyo), an expression plasmid (6.7μg) was transfected into a COS-1 cell on a 100mm plate, in accordance with a manual of the manufacture. On the second day from the transfection, 1ml of an incubation liquid was collected and incubated together with 10μL of IgG-Sepharose (Amersham Pharmacia Biotech) at 40C for one hour. Beads of the IgG-Sepharose was collected by centrifugation, and then washed with an assay buffer. After that, the beads

were resuspended in an assay buffer of the same kind as the assay buffer. The beads were used for assaying GalNAc transferase, GlcUA transferase, and Gal transferase. That is, a fused protein occurred in the incubation liquid was absorbed by IgG-Sepharose so as to remove glycosyltransferases in the incubation liquid. Then, by using the enzyme bound beads as an enzyme source, glycosyltransferase activity of the fused protein that was bound to the beads was assayed with various receptor substrates and donor substrates.

As a receptor for GalNAc transferase, a polymer (167 μg) of chondroitin, α- thrombomodulin(1 nmol), or GlcUAβ1-3Galβ1-3Galβ1-4Xy1β1-0-Ser (1 nmol) was used. Moreover, as a receptor for GlcUA transferase, the polymer (167 μg) of chondroitin or Galβ1-3Galβ1-4Xy1β (1 nmol) was used. As a receptor of Gal transferase, sheep submandibular asialomucin (300 μg) or GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (1 nmol) was used. Assay of GalNAc transferase was carried out with a mixture of, in 30 μL in total, 10 μL of the resuspended beads, the receptor substrate, 8.57 μM UDP-[^{3}H]GalNAc (3.60×10^5 dpm), 50 mM MES buffer, pH 6.5, 10mM MnCl2, and 171 μM of sodium salt of ATP (J. Biochem. 117, 1083-1087 (1995)).

Assay of GlcUA transferase I (GlcAT-I), which is necessary for synthesis of tetrasaccharide for the linkage region, was carried out with a mixture of, in 30 μL in total,

10μL of the resuspended beads, 1nmol Galβ1-3Galβ1-4Xyl, 14.3μM UDP-[^{14}C]GlcUA (1.46 × 10^5 dpm), 50mM MES buffer, pH6.5, and 2mM $MnCl_2$ (FEBS lett. 459, 415-420 (1999)).In assay of GlcAT-II, 10μL of the resuspended beads, 167μg of the polymer of chondroitin, 14.3μM UDP-[^{14}C]GlcUA (1.46 × 10^5 dpm), 50mM sodium acetic acid buffer, pH 5.6, and 10mM MnCl2 were included in 30μL in total (Glycobiology 7, 905-911(1997)). Assay of Gal transferase was carried out with a mixture of, in 30μL in total, 10μL of the resuspended beads, the receptor substrate, 60μM UDP-[^{3}H]Gal (5.30 × 10^5 dpm), 50mM MES buffer, pH 6.5, 10mM $MnCl_2$, and 171 μM of sodium salt of ATP. Reaction mixtures were incubated at 370C for one hour. Products that had been radiolabeled was separated from UDP-[^{3}H]GalNAc, UDP-[^{14}C]GlcUA, or UDP-[^{3}H]Gal, by gel filtration using a syringe column packed with Sephadex G-25 (super fine), a superdex peptide column, or a Pasteur pipette column containing Dowex 1-X8 (PO42-type, 100-400 mesh, Bio-Rad Laboratories, Tokyo) (J. Biochem. 117, 1083-1087 (1995); J. Biol. Chem. 273, 6615-6618 (1998); FEBS Lett. 459, 415-420 (1999); Glycobiology 7, 905-911 (1997); Glycobiology 7, 531-537 (1997)). The thus collected labeled products were quantified by liquid scintillation spectroscopy.

Note that the substrates and the like were obtained as follows. UDP-[U-^{14}C]GlcUA (285.2 mCi/mmol), UDP-[^{3}H]GalNAc (10 Ci/mmol) and UDP-[^{3}H]Gal (15 Ci/mmol) were

purchased from NEN Life Science Products Inc. Unlabeled UDP-GlcUA, UDP-GalNAc and UDP-Gal were obtained from Sigma. Chondroitin (a derivative prepared by chemically desulfurizing chondroitin sulfuric acid A derived from whale cartilage) was purchased from Sekikagaku Corp. (Tokyo). Homogeneity purified Hepatopancreas β-glucuronidase (EC3.2.1.31) (Comp. Biochem. Physiol. 86B, 565-569 (1987)) derived from Amlullaria (freshwater apple snail) was provided from Tokyo Internal Organ Co. Ltd. (Tokyo).

Galβ1-3Galβ1-4Xyl was kindly provided from Dr. Nancy B. Schwartz (University of Chicago). The purified α-thrombomodulin (Biochem. Biophys. Res. Coummn. 171, 729-737 (1990)) was provided from Daiichi Pharmaceutical Co. Ltd (Tokyo) and included the tetrasaccharide (GlcUAβ1-3Galβ1-3Galβ1-4Xyl) (J. Biol. Chem. 273, 33728-33734 (1998)) for the linkage region. *N*-acetyl chondroitin (GlcUAβ1-3GalNAc) and GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc was kindly provided from Dr. K. Yoshida (Seikagaku Corp.). Linkage tetrasaccharide-serine (GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1-O-Ser) (Liebigs Ann. 1239-1257 (1996)) was kindly provided from Dr. T. Ogawa (Physical and Chemical Research Institute, Saitama Prefecture).

Sheep submandibular asialomucin was obtained by treating sheep submandibular mucin with sialidase derived

from Arthrobacter ureafaciens (Nacalai Tesque Inc. Kyoto), the sheep submandubular mucin having been prepared according to methods of Tettamanti and Pigman (Arch. Biochem. Biophys. 124, 45-50 (1968)). Superdex (Trademark) peptide HR10/30 column was supplied from Amersham Pharmacia Biotech (Uppsala, Sweden).

Results were shown in Table 1. Activities were detected when the polymer of chondroitin was used as the receptor and UDP-GlcUA or UDP-GlNAc was used as the donor. On the other hand, no activity was detected when the other receptor substrate was used and one of UDP-GlcUA, UDP-GalNAc and UDP-Gal was used as the donor. Such activities included activities of (a) GlcAT-I (which relates to initiation of biosynthesis of chondroitin sulfate), (b) GalNAc transferase I, (c) core 1 UDP-Gal:GalNAc α-R β 1,3-Gal transferase, and (d) UDP-Gal:GlcNAc β-R β 1,4-Gal transferase. Glycosyltransferase activity was not detected in an affinity purification product that was a sample prepared as a control by transfecting pEF-BOS. Those results clearly show that expressed proteins were GlcUA/GalNAc transferases having a high specificity for the polymer of chondroitin.

As described above, chondroitin (the polymer of chondroitin) includes one whose non-reducing terminal is GlcUA and one whose non-reducing terminal is GalNAc. It can be said that the transfer of GalNAc is for the chondroitin

whose non-reducing terminal is GlcUA, and the transfer of GlcUA is for the chondroitin whose non-reducing terminal is GalNAc.

TABLE 1 RECEPTOR SPECIFICITY

RECEPTOR (DONOR)	ACTIVITY 3) (pmol/ml medium/time)
Chondroitin (UDP-GlcUA)	5.2
Galβ1-3Galβ1-4Xyl (UDP-GlcUA)	ND
Chondroitin (UDP-GalNAc)	1.4
GlcUAβ1-3Galβ1-3Galβ1-4Xylβ-O-Ser(UDP-GalNAc)	ND
α- thrombomodulin (UDP-GalNAc)	ND
Sheep submandubular asialomucin (UDP-Gal)	ND
GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc(UDP-Gal)	ND

ND: Not Detected (<0.1 pmol/ml medium/time)

1) α- thrombomodulin included tetrasaccharide linkage GlcUAβ1-3Galβ1-3Galβ1-4Xyl (J. Biol. Chem. 273, 33728-33734 (1998)).

2) Sheep submandibular asialomucin had a large number of GalNAc α 1-O-Ser/Thr residues.

3) Each value is an average of the measures taken in two independents experiments.

(4) Identification of Enzymatic Reaction Products

Isolation of products of GalNAc transferase reaction or GlcUA transferase reaction, in which the polymer of chondroitin was used as the receptor, was carried out by

using gel filtration using a superdex peptide column that had been equilibrated with 0.25m NH_4HCO_3/7-propanol. With radioactivity peak pooled, the radioactivity peak containing each enzymatic reaction was evaporated to dryness. The thus isolated products (about 120μg) of GalNAc transferase reaction was digested, at 37°C for one night, in a reaction liquid of 30μL by using 100 mIU of Chondroitinase AC-II (EC4.2.2.5) (Seikagaku Corp. (Tokyo)) derived from Arthrobacter aurescens, the reaction liquid containing 50mM sodium acetic acid buffer, at pH 6.0. Degree of digestion thereof was evaluated. The thus isolated products (about 180μg) of GlcUA transferase reaction was digested for one night at 37°C in 30μL of 50 mM sodium acetic acid buffer, at pH6.0, containing 100 mIU of chondroitinase AC-II, or in 30μL of 0.05M sodium citric acid buffer, at pH4.5, containing 22mIU of β-glucuronidase. Digestion products of each enzyme were analyzed by using the same superdex peptide column.

An analysis result of the products of the GlcUA transferase reaction is shown in Figure 3(a). The labeled products were completely digested by β-glucuronidase or chondroitinase AC-II. Peaks were observed at positions of free [^{14}C]GlcUA or free [^{14}C]GlcUAβ1-3GalNAc. This result suggests that the GlcUA residue is transferred to GalNAc that existed at the non-reducing terminal of the polymer of chondroitin, and caused the GlcUA residue to form β1-3

bonding with GalNAc.

An analysis result of the products of the GalNAc transferase reaction is shown in Figure 3(b). The labeled products were completely digested by chondroitinase AC-II. A Peak was observed at a position of free [^{3}H]GalNAc. This result suggests that the GalNAc residue is transferred to GlcUA that existed at the non-reducing terminal of the polymer of chondroitin, and caused the GalNAc residue to form β1-4 bonding with GlcUA. To sum up the results, it was found that the proteins thus identified were chondroitin synthase that had both the activities of GlcAT-II and GalNAcT-II.

[Example 2]

A commercially-available human 12-lane multiple tissue Northern blot (Clontech) membrane was used for analysis. To each lane, 1 μg of a polyadenylated RNA was applied. The membrane was probed with a gel-purified and radiolabeled (>1 x 10^9 cpm/μg) 0.84 kb chondroitin-synthase-specific fragment corresponding to nucleotides 631-1469 of the KIAA0990 cDNA (GenBankTM accession number AB023207).

As a result, a single band of up to 5.0 kb was demonstrated for all human tissues, at least in this analysis(Fig.4). The degree of the expression of the chondroitin synthase gene which is prevalent in human tissues varied with the types of human tissues. Notably,

a particularly strong expression of the mRNA was observed in the placenta. The expressions observed in the spleen, lung, and peripheral blood leukocytes were also strong but not as much as that of the placenta. This result corresponds to an observation that chondroitin sulfate proteoglycans are distributed at the surfaces of many cells and in the extracellular matrix of almost all tissues.

INDUSTRIAL APPLICABILITY

Provided are (a) a vector having DNA encoding human chondroitin synthase, (b) a method of producing human chondroitin synthase, (c) a method of producing a saccharide chain having a repeating disaccharide unit of chondroitin, and (d) a probe for hybridization of human chondroitin synthase.

CLAIMS

1 A vector carring one of DNA (a) , (b) or (c), the DNA (b) or (c) encoding a protein having catalytic activities (α) and (β) , excluding a DNA encoding a protein at amino-acid position #1 to 802 in SEQ. ID. NO: 2):

(a) DNA encoding a protein having an amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO: 2;

(b) DNA that, in a stringent condition, hybridizes with the DNA (a), the DNA complementary with DNA (a), or DNA having part of a nucleotide sequence of the DNA (a) or the DNA complementary with the DNA (a);

(c) DNA encoding a protein having an amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO:2, wherein one or several amino acids in the amino acid sequence are substituted, deleted, inserted, or transpositioned;

(α) catalytic activity that transferase GalNAc from UDP-GalNAc to chondroitin;

(where UDP is uridine 5'diphosphate, and GalNAc is N-acetylgalactosamine residue), and

(β) catalytic activity that transferase GlcUA from UDP-GlcUA to chondroitin,

(where UDP is uridine 5'diphosphate and GlcUA is N-glucuronic acid residue).

2. The vector as set forth in Claim 1, wherein:

the DNA (a) encodes finding from nucleotide numbers 633 to 2900 in SEQ. ID. NO: 1.

3. The vector as set forth in Claim 1 or 2, wherein the proteins are soluble.

4. The vector as set forth in any one of Claims 1 to 3, being an expression vector.

5. A transformant whose host is transformed by a vector having any one of DNA (a) to (c), the DNA (b) or (c) encoding a protein having catalytic activities (α) and (β):

(a) DNA encoding a protein having an amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO: 2;

(b) DNA that, in a stringent condition, hybridizes with the DNA (a), the DNA complementary with DNA (a), or DNA having part of a nucleotide sequence of the DNA (a) or the DNA complementary with the DNA (a);

(c) DNA encoding a protein having an amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO:2, wherein one or several amino acids in the amino acid sequence are substituted, deleted, inserted, or transpositioned;

(α) catalytic activity that transferase GalNAc from UDP-GalNAc to chondroitin;

(where UDP is uridine 5'diphosphate, and GalNAc is N-acetylgalactosamine residue),

(β) catalytic activity that transferase from UDP-GlcUA to chondroitin,

(where UDP is uridine 5'diphosphate and GlcUA is N-glucuronic acid residue).

6. The transformant as set forth in Claim 5, wherein:

the DNA (a) encodes finding from nucleotide numbers 633 to 2900 in SEQ. ID. NO: 1.

7. The transformant as set forth in Claim 5 or 6, wherein:

the proteins are soluble.

8. A method for producing chondroitin synthase, the method comprising the steps of:

growing a transformant set forth in any one of Claims 5 to 7; and

obtaining the chondroitin synthase from the trnasformant thus grown.

9. A reagent for use in chondroitin synthesis, the reagent having an enzyme protein that has an amino acid sequence including an amino acid sequence (A) or (B) and has catalytic activities (α) and (β)

(A) amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO: 2;

(B) amino acid sequence from amino acid numbers 47 to 802 in SEQ. ID. NO:2, wherein one or several amino acids in the amino acid sequence are substituted, deleted, inserted, or transpositioned.

(α) catalytic activity that transferase GalNAc from UDP-GalNAc to chondroitin,

(where UDP is uridine 5'diphosphate, and GalNAc is N-acetylgalactosamine residue);

(β) catalytic activity that transferase GlcUA from UDP-GlcUA to chondroitin,

(where UDP is uridine 5'diphosphate and GlcUA is glucuronic acid residue).

10. The reagent as set forth in Claim 9, wherein the enzyme protein is soluble.

11. A method for producing a saccharide chain expressed by Formula (3), the method comprising at least the step of causing a reagent to contact with GalNAc donor and a saccharide chain expressed by Formula (1), the reagent set forth in Claim 9 or 10:

GlcUA-GalNAc-R^1 ··· (1),

GalNAc-GlcUA-GalNAc-R^1 ··· (3),

(where GlcUA and GalNAc are as defined above, "-" indicates a glycoside linkage, R^1 is an arbitrary group).

12. A method for producing a saccharide chain expressed by Formula (4), the method comprising at least the step of causing a reagent to contact with GlcUA donor and a saccharide chain expressed by Formula (2), the reagent set forth in Claim 9 or 10:

GalNAc-GlcUA-R^2 ··· (2),

GlcUA-GalNAc-GlcUA-R^2 ··· (4),

(where GlcUA, GalNAc, and "-" are as defined above, R^2 is an arbitrary group).

13. A method for producing a saccharide chain selected from saccharide chains expressed by Formulas (5) and (7) respectively, the method comprising at least the step of causing a reagent to contact with GalNAc donor, GlcUA donor and a saccharide chain expressed by Formula (1), the reagent set forth in Claim 9 or 10:

GlcUA-GalNAc-R^1··· (1),

(GlcUA-GalNAc)n-GlcUA-GalNAc-R^1··· (5),

GalNAc-(GlcUA-GalNAc)n-GlcUA-GalNAc-R^1··· (7),

(where n is an integer not less than 1, GlcUA, GalNAc, and "-" are as defined above, R^1 is an arbitrary group).

14. A method for producing a saccharide chain selected from saccharide chains expressed by Formulas (6) and (8) respectively, the method comprising at least the step of causing a reagent to contact with GalNAc donor, GlcUA donor

and a saccharide chain expressed by Formula (2), the reagent set forth in Claim 9 or 10:

GalNAc-GlcUA-R^2 ··· (2),

(GalNAc-GlcUA)n-GalNAc-GlcUA-R^2 ··· (6),

GlcUA-(GalNAc-GlcUA)n-GalNAc-GlcUA-R^2 ··· (8),

(where n is an integer not less than 1, GlcUA, GalNAc, and "-" are as defined above, R^2 is an arbitrary group).

15. A probe for hybridization, the probe containing a nucleotide sequence from nucleotide numbers 495 to 2900 in SEQ. ID. NO: 1, or a sequence complementary with part of the nucleotide sequence.