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(72) Ohsuye, Kazuhiro, JP

(72) Kitano, Katsuhiko, JP

(72) Tanaka, Shoji, JP

(73) SUNTORY LIMITED, JP

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(54) **ENZYME RECOMBINANTE EXTRAITE DE LA THYROÏDE
HUMAINE**

(54) **RECOMBINANT C-TERMINAL α -AMIDATING ENZYME OF
HUMAN THYROID ORIGIN**

(57) Enzyme d' α -amidation à terminaison C provenant de la glande thyroïde humaine, qui convertit une protéine ou un peptide représentés par la formule R-X-Gly, dans laquelle Gly représente la glycine présente dans la terminaison C, X représente tout acide aminé destiné à être α -amidé, et R représente une partie résiduelle de la protéine ou du peptide, en une protéine ou peptide α -amidés représentés par la formule R-X-NH₂, dans laquelle X-NH₂ représente l'acide aminé α -amidé à terminaison C, et R représente une partie résiduelle de la protéine ou du peptide. On décrit également des dérivés biologiquement actifs qui entretiennent l'activité enzymatique, un procédé de production de ces dérivés, ainsi que des vecteurs d'expression et un transformant prokaryotique et eukaryotique pour ledit procédé.

(57) A C-terminal α -amidating enzyme of human thyroid gland origin, which converts a protein or peptide represented by the formula (I): R-X-Gly (I) wherein Gly represents glycine present at the C-terminus, X represents any amino acid to be α -amidated, and R represents a remaining portion of the protein or peptide, to an α -amidated protein or peptide represented by the formula (II): R-X-NH₂ (II) wherein X-NH₂ represent C-terminal α -amidated amino acid, and R represents a remaining portion of the protein or peptide, and biologically active derivative maintaining the enzyme activity thereof, a process for the production thereof, and expression vectors, and a prokaryotic and eukaryotic transformant for the process.

A C-terminal α -amidating enzyme of human thyroid gland origin, which converts a protein or peptide represented by the formula (I):



5 wherein Gly represents glycine present at the C-terminus, X represents any amino acid to be α -amidated, and R represents a remaining portion of the protein or peptide, to an α -amidated protein or peptide represented by the formula (II):



wherein X-NH₂ represent C-terminal α -amidated amino acid, and R represents a remaining portion of the protein or peptide, and biologically active derivative maintaining the enzyme activity thereof, a process for
15 the production thereof, and expression vectors, and a prokaryotic and eukaryotic transformant for the process.

DESCRIPTION

TITLE OF THE INVENTION

Recombinant C-Terminal α -Amidating Enzyme
of Human Thyroid Origin

BACKGROUND OF THE INVENTION

5 1. Field of the Invention

The present invention relates to recombinant C-terminal α -amidating enzymes of human thyroid origin and precursors thereof, DNAs coding for these polypeptides, plasmids containing the DNA, host cells transformed with the plasmid, and a process for the production of the enzyme using the transformed cells.

2. Description of the Related Art

10 It is generally known that, in eukaryotic cells, after translation from a messenger RNA (mRNA) some kinds of peptides or proteins are modified by an intracellular enzyme to mature to a natural-type peptide or protein (post-translational modification). But since prokaryotic hosts such as E. coli, which are widely used to produce peptides or proteins of eukaryote origin, cannot carry out a post-translational modification of an expressed peptide or protein, it is sometimes difficult to directly produce a eukaryotic peptide or protein by a recombinant DNA technique using prokaryotic host cells.

25 One post-translational modification characteristic of eukaryotic cells of peptides or proteins is a modification reaction wherein an α -position of a carboxy terminal (C-terminal) of a peptide or protein is amidated, i.e., $-\text{COOH}$ is converted to $-\text{CONH}_2$, and it is known that many physiologically active peptides or proteins have been subjected to such modification, and that, in some cases the above-mentioned modification of the peptide or protein is essential to the physiological activity thereof. For example, a conversion of the proline amide residue at the C-terminal of natural-type human calcitonin to proline residue decreases the

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physiological activity thereof to 1/1,600 of the original activity.

Because of the importance of clarifying the mechanism of α -amide formation in tissues, and the promising usefulness of the enzyme for the production of C-terminal α -amidated peptides using, for example, recombinant DNA techniques, many attempts have been made to clarify a detailed mechanism of the biosynthesis of C-terminal α -amidated peptides characteristic to eukaryotic cells. In this clarification first a structure of a precursor of an amidated peptide is clarified from a cDNA analysis of the amidated peptide, and as a result, a general biosynthesis mechanism of such amidated peptides is understood to be that in which mRNA is translated to a precursor of an amidated peptide, which is then amidated at the α -position of the C-terminal thereof by a C-terminal α -amidating enzyme. Note, in the above-mentioned reaction, the precursor of the C-terminal α -amidated peptide as a substrate for a C-terminal α -amidating enzyme is a peptide or protein represented by a general formula R-X-Gly, wherein R represents an amino acid sequence of the N-terminal side of the peptide or protein, X represents an amino acid residue which is to be α -amidated at the C-terminal thereof, and Gly represents a glycine residue.

On the other hand, in porcine pituitary, Bradburg, A.F. et al., Nature 298, 686-688, 1982, first characterized the α -amidating activity converting a synthetic substrate D-Tyr-Val-Gly to D-Tyr-Val-NH₂, and demonstrated that the C-terminal glycine in the substrate serves as a nitrogen-donor for α -amidation. Also, Eipper et al., Proc. Natl. Acad. Sci. US, 80, 5144-5148, 1983, reported that the α -amidating enzyme derived from the pituitary gland of rat requires copper cation and ascorbate, in addition to molecular oxygen, for its activity. Husain, I. et al., FEBS Lett., 152 227-281, 1983; and Kizer, J. S. et al., Proc. Natl.

Acad. Sci. US, 81, 3228-3232, 1984, also reported a C-terminal α -amidating enzyme, but did not report a purified enzyme. Recently, Murthy A.S.N. et al., J. Biol. Chem., 261, 1815-1822, 1986, partially purified a C-terminal α -amidating enzyme from the pituitary gland of cattle, and showed that several types of enzymes having different molecular weights and electric charges are present.

Recently, Mizuno et al. succeeded in isolating a C-terminal α -amidating enzyme in a homogeneous and pure form from a skin of Xenopus laevis. (see Mizuno, K. et al., Biochem. Biophys. Res. Commun. 137, 984-991, 1988, and EP 0249412 A3 corresponding to Japanese Patent Application No. 61-131089) and further succeeded in determining an entire primary amino acid sequence of the C-terminal α -amidating enzyme of a skin of Xenopus laevis origin by obtaining and sequencing cDNA; see Mizuno, K. et al., Biochem. Biophys. Res. Commun. 148, 546-552, 1987.

On the other hand, Eipper, B. et al., Mol. Endo. 1, 777-790, 1987, cloned cDNA of a C-terminal α -amidating enzyme of the bovine pituitary gland, and demonstrated that the enzyme of the bovine pituitary gland is clearly different from the above-mentioned enzyme of Xenopus laevis origin.

The above-mentioned reports demonstrate that there are many C-terminal α -amidating enzymes of eukaryote origin, and this suggests that each enzyme has a particular substrate specificity different from that of the others; namely, a particular C-terminal α -amidating enzyme is present for a particular α -amidated peptide, although this has not been fully demonstrated as yet.

On the other hand, if a C-terminal α -amidating enzyme can be produced in a large amount, it becomes possible to produce α -amidated peptides, which are industrially important, in a large amount.

Nevertheless, as described above, if various kinds of C-terminal α -amidating enzymes exist, and each enzyme has a particular substrate specificity and is responsible for a biosynthesis of a specific α -amidated peptide or protein, when producing a desired α -amidated peptide or protein, it is important to choose a most suitable C-terminal α -amidating enzyme.

Accordingly, there is a current desire to isolate novel C-terminal α -amidating enzymes other than those of Eipper et al. and Mizuno et al., to determine their structures, and to clarify their-substrate specificity, for their use for the production of particular α -amidated peptides.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides novel C-terminal α -amidating enzymes and a process for the production thereof, as well as cloned genes and expression vectors useful for the production process.

More specifically, the present invention provides a C-terminal α -amidating enzyme of human thyroid grand origin, which converts a protein or peptide represented by the formula (I):



wherein Gly represents glycine present at a C-terminus, X represents any amino acid to be α -amidated, and R represents a remaining portion of the protein or peptide, to an α -amidated protein or peptide represented by the formula (II):



wherein X-NH₂ represents C-terminal α -amidated amino acid, and R represents a remaining portion of the protein or peptide, and biologically active derivatives thereof maintain the enzyme activity.

Preferred embodiments of the enzyme and the derivative thereof are, for example, a C-terminal α -amidating enzyme or biologically active derivative thereof maintaining the enzyme activity, having a

molecular weight of not more than about 120,000 D;

5 a C-terminal α -amidating enzyme or biologically active derivative thereof maintaining the enzyme activity thereof, having a molecular weight of between about 40,000 D and about 120,000 D;

10 a C-terminal α -amidating enzyme or biologically active derivative maintaining the enzyme activity thereof comprising an amino acid sequence starting at the 37th Pro and terminating at the 378th Lys in the amino acid sequence shown in Fig. 1; a C-terminal α -amidating enzyme or biologically active derivative maintaining the enzyme activity thereof and having an amino acid sequence starting at an amino acid present between the first Met, inclusive, and the 37th Pro, inclusive, and terminating at an amino acid present between the 378th Lys, inclusive, and the 974th ser, inclusive, in Fig. 1;

20 a C-terminal α -amidating enzyme or biologically active derivative maintaining the enzyme activity thereof and having an amino acid sequence starting at an amino acid present between the first Met, inclusive, and the 37th Pro, inclusive, and terminating at an amino acid present between the 378th Lys, inclusive, and the 974th Ser, inclusive in Fig. 1, and having an additional C-terminal amino acid or amino acid sequence consisting of up to ten amino acids;

25 a C-terminal α -amidating enzyme or biologically active derivative maintaining the enzyme activity thereof and having an amino acid sequence starting at an amino acid present between the first Met, inclusive, and the 37th Pro, inclusive, and terminating at an amino acid present between the 378th Lys, inclusive, and the 866th Ser, inclusive, in Fig. 3; and

30 a C-terminal α -amidating enzyme or biologically active derivative maintaining the enzyme activity thereof and having an amino acid sequence starting at an amino acid present between the first Met,

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inclusive, and the 37th Pro, inclusive, and terminating
at an amino acid present between the 378th Lys,
inclusive, and the 866th Ser, inclusive, in Fig. 3, and
having an additional C-terminal amino acid or amino acid
5 sequence consisting of up to ten amino acids.

The present invention also provides a process for
the production of the above-mentioned C-terminal α -
amidating enzyme or biologically active derivatives
thereof, comprising the steps of:

10 (1) Culturing prokaryotic cells or eukaryotic
cells transformed with an expression vector containing a
gene coding for an enzyme or derivative thereof to
produce the enzyme or derivative thereof; and

15 (2) Recovering the enzyme or derivative
thereof from the culture.

The present invention also provides genes coding
for the above-mentioned C-terminal α -amidating enzyme or
biologically active derivative thereof.

20 The present invention further provides plasmids and
phage vectors containing the above-mentioned gene.

Moreover, the present invention provides
prokaryotic cells transfected with the plasmid or
eukaryotic cells transfected with the plasmid.

BRIEF DESCRIPTION OF THE DRAWINGS

25 Figure. 1-1 to 1-4 represent a nucleotide sequence
of a cDNA region in a phage clone λ gt10ch201, and a
corresponding amino acid sequence;

30 Figs 2-1 to 2-3 represent a nucleotide sequence of
a cDNA region in a phage clone λ gt10chT2, and a
corresponding amino acid sequence;

Figs 3-1 to 3-4 represent a nucleotide sequence of
a cDNA region in a phage clone λ gt10ch101, and a
corresponding amino acid sequence;

35 Fig. 4 represents a restriction enzyme cleavage map
of the cDNA region in the λ gt10ch201 (a), and a strategy
for the determination of a nucleotide sequence of the
cDNA region (b);

Fig. 5 represents a restriction enzyme cleavage map of the cDNA region in the λ gt10chT2(a), and a strategy for the determination of a nucleotide sequence of the cDNA region(b);

5 Fig. 6 represents a restriction enzyme cleavage map of the cDNA region in the λ gt10chT101 (a), and a strategy for the determination of a nucleotide sequence of the cDNA region(b);

10 Fig. 7 represents the relationship between cDNA regions in the λ gt10chT201, λ gt10chT2 and λ gt10chT101 as well as λ gt11chT40 and λ gt11chT28 compared with cDNA in a plasmid pXA799 derived from the Xenopus laevis cDNA library;

15 Fig. 8 represents a process for the construction of a plasmid pUC18chT201 HindIII-BglII;

Fig. 9 represents a process for the construction of a plasmid pUC19chT201 MPF;

Fig. 10 represents a process for the construction of a plasmid P_L chT201 MPF-Sph;

20 Fig. 11 represents a process for the construction of a plasmid P_L chT201 MPF-S/B;

Fig. 12 represents a process for the construction of a plasmid pUC19chT201-EcoRI 0.7;

25 Fig. 13 represents a process for the construction of a plasmid pUC18-Sal;

Fig. 14 represents a process for the construction of a plasmid P_L chT201 MPF-Sal;

Fig. 15 represents a process for the construction of a plasmid P_L chT201 MPF-Bcl;

30 Fig. 16 represents a process for the construction of a plasmid pUC19chT201 MFK;

Fig. 17 represents a process for the construction of a plasmid P_L chT201 MFK-Sph;

35 Fig. 18 represents a process for the construction of a plasmid P_L chT201 MFK-S/B;

Fig. 19 represents a process for the construction of a plasmid P_L chT201 MFK-Sal;

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Fig. 20 represents a process for the construction of a plasmid P_LchT201 MFK-Bcl;

Fig. 21 represents a process for the construction of plasmids P_LchT2 MPF-Pst and P_LchT2 MFK-Pst;

5 Fig. 22 represents a process for the construction of plasmids P_LchT2 MPF-Bcl and P_LchT2 MFK-Bcl;

Fig. 23 represents the productivity, in terms of substrate amidation ratio (%), of a C-terminal α -amidating enzyme by Escherichia coli W3100 transformed with different plasmid described above;

Fig. 24 represents a process for the construction of a plasmid pUC18-HpaII;

Fig. 25 represents a process for the construction of plasmids pKDch201 MPF-Sph, -Sal, -S/B and -Bcl;

15 Fig. 26 represents a process for the construction of a plasmid pKDchT201-PstI;

Fig. 27 represents a process for the construction of a plasmid pUC18chT2 HindIII-BglII;

Fig. 28 represents a process for the construction of a plasmid pUC18chT201-chT2;

Fig. 29 represents a process for the construction of a plasmid P_LchT2 KpnI-PstI;

Fig. 30 represents a process for the construction of a plasmid pKDchT2 PstI;

25 Fig. 31 represents a process for the construction of a plasmid pKDchT2-Bcl;

Figs. 32(a) and 32(b) represent graphs showing the productivity, in terms of substrate amidation ratio (%), of a C-terminal α -amidating enzyme by COS-1 cells transfected with different plasmids described above;

30 Figs. 33(a), 33(b) and 34 schematically shown amino acid sequences of C-terminal α -amidating enzymes derived from various plasmids.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

35 The present inventors have already succeeded in isolating two kinds of cDNAs coding for C-terminal α -amidating enzymes, from the skin of Xenopus laevis,

which were cloned in plasmids pXA 457 and pXA 799. E. coli DH1/pXA799 containing the plasmid pXA799 was deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology (FRI), 1-3, Tsukuba-shi 1-chome, Ibaraki-ken, 301 Japan, as FERM BP-1586, on December 3, 1987; (Japanese Patent Publication No. 1-104168; EP 0299790 A2). Moreover, by comparing a primary amino acid sequence encoded by cDNA for a C-terminal α -amidating enzyme of the bovine pituitary gland cloned by Eipper et al., supra, with primary amino acid sequences encoded by the above-mentioned cDNAs for Xenopus laevis C-terminal α -amidating enzymes cloned by the present inventors, it is found that the amino acid sequences of an N-terminal region, which was proven to be essential for the enzyme activity, are similar in all the C-terminal α -amidating enzymes of different origins. This suggests that, even though there are various kinds of C-terminal α -amidating enzymes in eukaryotic cells, a primary amino acid sequence of a domain essential for the enzyme activity is conserved between the species and tissues. On the basis of this concept, the present inventors attempted to clone cDNAs coding for novel C-terminal α -amidating enzymes by screening cDNA banks derived from RNAs of different origins, using as a probe the cDNA coding for a C-terminal α -amidating enzyme of Xenopus laevis origin.

In this case, considering that human calcitonin, a well known C-terminal amidated peptide, is bio-synthesized in the thyroid gland, the present inventors extracted mRNA from the human thyroid gland, prepared cDNA library, and screened the library using the above-mentioned cDNA derived from Xenopus laevis as a probe. As a result, two kinds of cDNAs coding for novel C-terminal α -amidating enzymes having primary amino acid sequences clearly different from those of the above-mentioned known C-terminal α -amidating enzymes, were

obtained. Moreover, the cDNAs thus cloned were used to transform E. coli or transfect animal cells, COS1 (ATCC CRL 1680; Cell, 23, 175-182, 1981) to express the cloned genes, and it was confirmed that the expression products exhibit the activity of a C-terminal α -amidating enzyme as determined using a synthetic substrate [^{125}I] Ac-Tyr-Phe-Gly.

According to the present invention, the C-terminal α -amidating enzyme catalyzes a conversion of a peptide or protein represented by the formula: R-X-Gly wherein X is an amino acid to be α -terminal α -amidated, Gly represents glycine and R represents the remaining part of the peptide or protein, to a peptide or protein represented by the formula: R-X-NH₂. The present C-terminal α -amidating enzyme includes, in addition to native enzymes of human thyroid gland origin, modified enzymes wherein one or more than one amino acid on an amino acid sequence of the native enzyme is replaced by one or more than one different amino acid, wherein one or more than one amino acid is added to the amino acid sequence of the native enzyme, or wherein one or more than one amino acid is deleted from the amino acid sequence of the native enzyme, while maintaining the above-mentioned enzyme activity.

Next, the present invention is explained in more detail.

(1) Isolation of cDNA coding for C-terminal α -amidating enzyme

Total RNA was prepared from human thyroid gland, and from the total RNA, poly(A) RNA was prepared using an oligo(dT) cellulose column. Next, the poly (A) RNA was used to prepared a cDNA according to a procedure of Gubler et al. (Gubler, V. and Hoffman. B. J., Gene 25, 263, 1983), and the cDNA thus obtained was used to prepare a λ gtII phage cDNA library. A plasmid pXA799 containing cDNA coding for a C-terminal α -amidating enzyme of Xenopus laevis was digested with ApaI and

DraI, and the resulting ApaI-DraI DNA fragment of about 2.8 kb was labeled with [α -³²P]dCTP by nick translation to prepare a DNA probe. Next, the above-mentioned λ gtII cDNA library was screened using the DNA probe, to obtain
5 phage clones λ gt11chT28 and λ gt11ch40. As a result of analysis of these two clones, it was found that these clones do not contain a cDNA coding for the entire C-terminal α -amidating enzyme.

Accordingly, to obtain a cDNA coding for the
10 entire C-terminal α -amidating enzyme, a phage λ gt10 cDNA library was prepared from the above-mentioned poly (A) RNA, by the same procedure as described above. The λ gt10 cDNA library was screened using the above-mentioned ApaI-PraI DNA probe derived from pXA799, to
15 obtain a phage clone λ gt10ch T2. Moreover, the cDNA library was again screened using a DNA probe derived from a cDNA region in the above-mentioned λ gt11chT28 clone, to obtain a phage clone λ gt10chT201. In addition, the cDNA library was screened using a DNA
20 probe which was a labeled SmaI-XhoI DNA fragment corresponding to a region from the 35th G to the 425th A in the λ gt10ch201, to obtain a phage clone λ gt10chl01.

(2) Analysis of cDNAs coding for C-terminal α -amidating enzyme

25 The phage DNAs obtained as described above, i.e., λ gt11chT28, λ gt11chT40, λ gt10chT201, λ gt10chT2, and λ gt10chT101, were cleaved with various restriction enzymes to prepare their restriction enzyme cleavage maps. The maps for cDNA regions in the phages
30 λ gt10chT201, λ gt10chT2, and λ gt10chT101 are shown in Figs. 4(a), 5(a), and 6(a), respectively. Next, each DNA fragment prepared by cleaving the λ gt10chT201, λ gt10chT2, and λ gt10chT101 with various restriction enzymes was subcloned in M13 phage to determine the
35 nucleotide sequence, according to a procedure of Sanger et al. (Sanger, F. et al., Proc. Natl. Acad. Sci. U.S.A. 34, 5463-5467, 1977), and an entire nucleotide sequence

of the cDNA region in the phage clone was assembled according to a strategy shown in Figs. 4(b), 5(b) or 6(b). The nucleotide sequences of the cDNA region in the phage clones λ gt10chT201, λ gt10chT2, and λ gt10chT101 are shown in Figs. 1, 2, and 3, respectively.

(a) Analysis of chT201 cDNA

The analysis of the cDNA region in the phage clone λ gt10chT201 (designated chT201 cDNA) revealed the following:

(1) The chT201 cDNA consists of 3688 base pairs and contains a long reading frame starting with the 140th nucleotide and ending at the 3061th nucleotide (according to the nucleotide number shown in Fig. 1);

(2) An amino acid sequence corresponding to the open reading frame consists of 974 amino acid residues starting with the N-terminal Met-Ala-Gly- and ending with the C-terminal -Ser-Ser-Ser;

(3) In comparison with an amino acid sequence encoded by cDNA in pXA799 derived from Xenopus laevis, the amino acid sequence encoded by the ch201 cDNA contains an additional amino acid sequence consisting of 107 amino acid residues starting with the 388th amino acid and ending at the 494th amino acid (according to the amino acid number shown in Fig. 1); and the homology between both amino acid sequences, excluding the above-mentioned additional amino acid sequence, is about 54% by DNA level, and about 65% by protein level;

(4) The amino acid sequence encoded by the chT201 cDNA contains an amino acid sequence represented by the sequence Asn-X-Ser, wherein X is any amino acid corresponding to the 411 to 413th amino acids, and the amino acid sequence represented by the sequence Asn-X-Thr, wherein X is any amino acid sequence corresponding to the 762-764th amino acids. Since these amino acid sequences are considered to be N-glycosylation sites from analyses of many other glycoprotein, there is a great possibility that the protein encoded by

the chT201 cDNA is N-glycosylated;

(5) Since the amino acid sequence encoded by the chT201 cDNA contains a hydrophobic amino acid sequence near the N-terminus thereof and the hydrophobic sequence is considered to be a signal sequence characteristic to a secretory protein, the C-terminal α -amidating enzyme is believed to be expressed as a precursor protein, which is then processed to form a mature protein; and,

(6) The amino acid sequence encoded by the chT201 cDNA contains a region starting from the 864th amino acid and ending at the 911th amino acid, in which hydrophobic amino acids continuously occur, and this region is immediately followed by basic amino acids, i.e., arginine and lysine. Since this type of structure is frequently found in a transmembrane domain of a membrane protein, there is a great possibility that the present C-terminal α -amidating enzyme is present as a membrane protein.

(b) Analysis of chT2 cDNA

The analysis of the cDNA region in a phage clone λ gt10chT2 (designated chT2 cDNA) revealed the following:

(1) The chT2 cDNA consists of 2863 base pairs wherein the first nucleotide T corresponds to the 409th nucleotide in the chT201 cDNA, and contains a long reading frame starting at the second nucleotide and ending at the 2329th nucleotide (according to the nucleotide number shown in Fig. 2;

(2) An amino acid sequence corresponding to the open reading frame consists of 776 amino acid residues starting at the N-terminal Asp-Phe-Lys- and ending at the C-terminal -Ser-Ser-Ser, wherein the N-terminal Asp-Phe-Lys- corresponds to the -Asp-Phe-Lys- sequence starting at the 91th amino acid in the amino acid sequence encoded by the chT201 cDNA;

(3) In comparison with the amino acid

sequence encoded by the chT201, the amino acid sequence encoded by the chT2 lacks an amino acid sequence corresponding to an amino acid sequence portion consisting of 107 amino acid residues starting at the 388th amino acid and ending at the 494th amino acid (according to the amino acid number shown in Fig. 1) in the amino acid sequence encoded by the chT201 cDNA. Note, the amino acid sequence consisting of the 107 amino acid residues is also absent from the amino acid sequence encoded by the cDNA in the pXA799. Moreover, the amino acid sequence encoded by the chT2 cDNA lacks an amino acid corresponding to the 897th amino acid Ala (according to the amino acid number shown in Fig. 1) in the amino acid sequence encoded by the chT201 cDNA. Namely, the amino acid sequence encoded by the chT2 cDNA is the same as the amino acid sequence encoded by the chT201 cDNA, except that the former lacks an amino acid sequence and an amino acid corresponding to the amino acid sequence portion consisting of the above-mentioned 107 amino acid residues and the 897th amino acid residue Ala of the latter.

Therefore, the chT2 cDNA was an incomplete cDNA in that the chT2 cDNA lacks a 5'-terminal region corresponding to the 5'-terminal region up to the codon for the 90th Ile (see Figs. 2 and 7).

(c) Analysis of chT101 cDNA

The analysis of the cDNA region in the phage clone λ g10chT101 (designated chT101 cDNA) revealed the following:

- (1) The chT101 cDNA consists of 3316 base pairs and contains a long reading frame starting at the 56th nucleotide and ending at the 2653th nucleotide;
- (2) An amino acid sequence corresponding to the open reading frame consists of 866 amino acid residues starting at the N-terminal Met-Ala-Gly- and ending at the C-terminal -Ser-Ser-Ser;
- (3) In comparison with the amino acid

sequence encoded by the chT201 cDNA, the amino acid
sequence encoded by the chT101 cDNA is the same as that
encoded by the chT201, except that the former lacks an
amino acid sequence corresponding to an amino acid
5 sequence portion consisting of 107 amino acid residues
starting at the 388th amino acid and ending at the 494th
amino acid, and an amino acid corresponding to the 897th
amino acid residue Ala of the latter. On the other
hand, in comparison with the amino acid sequence encoded
10 by the chT2 cDNA, the amino acid sequence encoded by the
chT2 cDNA corresponds to an amino acid sequence starting
at the 91th Asp and ending at the last amino acid Ser of
the amino acid sequence encoded by the chT101 cDNA.
Therefore, the chT101 cDNA completely supplements a
15 5'-region lacking in the chT2 cDNA.

From the above-mentioned analysis of the
chT201 cDNA, chT2 cDNA, and chT101 cDNA, it was assumed
that the C-terminal α -amidating enzymes of human thyroid
origin can be classified into two types; one represented
20 by an enzyme encoded by the chT2 cDNA or chT101 cDNA,
and another represented by an enzyme encoded by the
chT201 cDNA. It is assumed that the chT201-type enzyme
contains two N-glycosylation sites and that the
chT201-type enzyme contains one N-glycosylation site,
25 because one of the two possible N-glycosylation sites in
the ch201-type enzyme is present in the additional
region consisting of 107 amino acids not present in the
chT2-type enzyme.

Nevertheless, so far enzyme proteins
30 predicted from the chT201 cDNA and chT101 cDNA have not
been isolated from the human thyroid, and therefore, the
biosynthetic mechanism, including the processing of the
N- and/or C-terminal portion and glycosylation, of
protein corresponding to the chT201 cDNA and chT101 cDNA
35 is not clear. Accordingly, to resolve there questions,
the chT201 cDNA and chT101 cDNA were shortened to obtain
various cDNA derivatives, which were then introduced

into prokaryotic cells and eukaryotic cells to produce various modified proteins, and the modified proteins were assayed to determine their C-terminal α -amidating activity, as described hereinafter.

5 (3) Expression of C-terminal α -amidating enzyme derivatives in prokaryotic cells

Where C-terminal α -amidating enzyme derivatives are expressed in prokaryotic cells such as E. coli cells, the position of the N-terminus in the
10 cht201 cDNA, cht2 cDNA or cht101 cDNA should be chosen. Recently, Eipper et al. (Eipper, B. et al., Mol. Endo. 1, 777-790, 1987) cloned cDNA coding for a C-terminal α -amidating enzyme of bovine origin, and determined a
15 primary amino acid sequence of the enzyme. There is an approximately 92% homology between an amino acid sequence encoded by the cht201 cDNA and that encoded by the cDNA of bovine origin at the amino acid sequence level. Considering the analysis of the cDNA of bovine
20 origin by Eipper et al., and the analysis of the pXA799 and pXA457 of Xenopus laevis by the present inventors, it is assumed that a processing site of a protein encoded by the cht201 cDNA or cht101 cDNA is between the 30th Arg and the 31th Phe, or between the 36th Arg and the 37th Pro. Accordingly, the enzyme derivatives were
25 designed such that the N-terminus thereof was at the 31th Phe or the 37th Pro in the amino acid sequence shown in Figs. 1 or 3. Moreover, the enzyme derivatives were designed such that the C-termini thereof were at different sites in the amino acid sequence shown in
30 Figs. 1 or 3, although in some case the derivative has at its C-terminus an additional amino acid sequence derived from a multiple cloning site in a plasmid used to construct expression plasmid. DNAs coding for cht201-type enzyme derivatives were constructed from the
35 cht201 cDNA; and DNAs coding for cht101-type enzyme derivatives were constructed by joining a 5'-terminal side of the cht201 cDNA and a 3'-terminal side of

the chT2 cDNA rather than from the chT101 cDNA, from the viewpoint of convenience of the construction.

Figures 33(a) and 33(b) schematically represent enzyme derivatives designed for the expression in *E. coli*. The enzyme derivatives (chT201-type derivatives) shown in Fig. 33(a) start at the 31th Phe or 37th Pro and end at the 323th Ala, the 378th Lys, the 434th Asp or 857th Ile in the amino acid sequence shown in Fig. 1; although all derivatives have at their N-terminus an additional Met corresponding to a translation start codon, and at their C-terminus, some derivatives have an additional amino acid or amino acid sequence derived from a multicloning site present in a plasmid used for the construction of corresponding expression vectors. The C-terminal additional amino acid and amino acid sequence are shown outside the boxes in Fig. 33(a). The enzyme derivatives (chT101-type derivatives) shown in Fig. 33(b) start at the 31th Phe or the 37th Pro and end at the 461th Ala or the 750th Ile in the amino acid sequence shown in Fig. 3; although all derivatives have an additional amino acid Met corresponding to a translation start codon, at their N-terminus, and at their C-terminus, some derivatives have an additional amino acid or amino acid sequence derived from a multicloning site present in a plasmid used to construct corresponding expression vectors. The C-terminal additional amino acid and amino acid sequence are shown outside the boxes in Fig. 33(b).

In the expression of the enzyme derivatives designed as above, expression vectors $P_{LchT201}$ MPF-Sph, $P_{LchT201}$ MPF-Sal, $P_{LchT201}$ MPF-S/B, $P_{LchT201}$ MPF-Bcl, $P_{LchT201}$ MFK-Sph, $P_{LchT210}$ MFK-Sal, $P_{LchT201}$ MFK-S/B, and $P_{LchT201}$ MFK-Bcl were constructed for the expression of the chT201-type derivatives, and P_{LchT2} MPF-Pst, P_{LchT2} MPF-Bcl, P_{LchT2} MFK-Pst, and P_{Lch} MFK-Bcl were constructed for the expression of the chT101 type derivatives. Note, in Figs. 33(a) and 33(b), the enzyme

derivatives are identified by the names of the expression vectors. These expression vectors contain a cDNA coding for the chT201-type protein derived from chT201 cDNA, or cDNA coding for the chT101-type protein derived from chT201 cDNA and chT2 cDNA under the control of a P_L promoter of λ phage origin. These expression vectors were constructed from the phage clones λ gt10chT201 and λ gt10chT2, commercially available plasmids pUC18 and pUC19, and a plasmid pUCP_L CI. Note, E. coli transformed with the plasmid pUCP_L CI was designated as E. coli SBM305, and deposited with the FRI under the Budapest treaty as FERM BP-2240, on January 11, 1989. A construction process for the pUCP_L CI is described in Japanese Patent Application No. 62-306867 (EP 0299790 A2).

These expression vectors can be used to transform a host such as E. coli. For example, E. coli W3110 was transformed to obtain the corresponding transformants, W3110/P_LchT201 MPF-Sph, W3110/P_LchT201 MPF-Sal, W3110/P_LchT201 MPF-S/B, W3110/P_LchT201 MPF-Bcl, W3110/P_LchT201 MFK-Sph, W3110/P_LchT201 MFK-Sal, W3110/P_LchT201 MFK-S/B, W3110/P_LchT201 MFK-Bcl, W3110/P_LchT2 MPF-Pst, W3110/P_LchT2 MPF-Bcl, W3110/P_LchT2 MFK-Pst, and W3110/P_LchT2 MFK-Bcl.

Then the transformants were separately cultured in a conventional medium to obtain cultured cells, which were sonicated to recover an expression product in a precipitated fraction.

(4) Expression of C-terminal α -amidating enzyme derivatives in eukaryotic cells

For the expression in eukaryotic cells, chT201-type enzyme derivatives starting at the first amino acid Met and ending at the 323rd Ala, the 378th Lys, the 434th Asp, the 568th Ala or the 857th Ile in the amino acid sequence shown in Fig. 1, and chT101-type enzyme derivatives starting at the first Met and ending at the 461th Ala or the 750th Ile in the

amino acid sequence in Fig. 3, (although some derivatives have an additional amino acid or amino acid sequence) were designed. These enzyme derivatives are schematically shown in Fig. 34. In this Figure, the additional amino acid and amino acid sequences derived from multicloning site present in a plasmid used for the construction of expression vectors are shown outside the boxes.

To express the above-designed enzyme derivatives in eukaryotic cells such as COS1 cells, expression plasmids pKDchT201-Sph, pKDchT201-Sal, pKDchT201-S/B, pKDchT201-Pst, pKDchT201-Bcl, pKDchT201-Pst, and pKDchT2-Bcl were constructed from the phage clones λ gt10chT201 and λ gt10chT2, commercially available plasmids pUC18 and pUC19, and a plasmid pKD. Note, *E. coli* transformed with the plasmid pKD was designated as *E. coli* SBM303 and deposited with the FRI under the Budapest treaty as FERM BP-2238, on January 11, 1989. Note, in Fig. 34, the enzyme derivatives are identified by the names of the expression vectors. These expression vectors contain a cDNA insert coding for a desired enzyme derivative under the control of the early promoter derived from SV40 virus. Further, these expression vectors were used to transfect animal cells such as COS1 cells, and the transfected cells cultured in a conventional medium. The desired enzyme derivative was then recovered from a culture medium.

(5) Assay of enzyme activity

A C-terminal α -amidating enzyme activity is assayed to confirm that a protein expressed in a transfected host is a C-terminal α -amidating enzyme, and that when a C-terminal α -amidating enzyme acts on the substrate thereof, an amidated peptide or protein is produced. For an assay of an expression of an enzyme in *E. coli*, *E. coli* cells are disrupted and the disruptant is centrifuged to obtain a precipitate containing a

major portion of the expression product, the precipitate is solubilized with 6 M guanidine hydrochloride, and the solution thus obtained is dialyzed to obtain an assay sample.

5 Enzyme activity is assayed by using a reaction wherein a substrate generally represented by R-X-Gly is converted to R-X-NH₂, for example, a reaction wherein a synthetic substrate [¹²⁵I] -Ac-Tyr-Phe-Gly is converted to [¹²⁵I] -Ac-Tyr-Phe-NH₂.
10 Namely, first a labeled substrate (labeled R-X-Gly) is subjected to a reaction with a test enzyme solution in Tris-HCl buffer, to this reaction mixture are added Tris-HCl buffer and ethyl acetate, and after mixing, the whole is centrifuged to separate an organic
15 phase and an aqueous phase. Since a major portion of the unreacted labeled substrate (labeled R-X-Gly) remains in an aqueous phase, and an amidated labeled products (labeled R-X-NH₂) transfers to an organic phase, the substrate and the product can be easily
20 separated.

 In examples of the present invention, a C-terminal α-amidating enzyme of the present invention was assayed using synthetic peptide [¹²⁵I] -Ac-Tyr-Phe-Gly as a substrate according to the following
25 procedure. [¹²⁵I] -Ac-Tyr-Phe-Gly (1 pmole, 70,000-150,000 cpm) was incubated with an enzyme preparation, in a final volume of 250 μl containing 0.2 M Tris-HCl buffer (pH 7.0), 2 μM CuSO₄, 0.25 mM ascorbic acid, 25 μg catalase (Boehringer), 0.1% Lubrol
30 (PX type, Nakarai Chemicals). The reaction mixture was kept at 37°C for 1 to 4 hours, and then 0.75 ml of 1 M Tris-HCl buffer (pH 7.0) and 2 ml of the organic phase of an ethyl acetate/water mixture was added. The two phases were mixed vigorously in a Vortex mixer, and
35 after centrifugation at 3000 rpm for 3 mins, the organic phase thus separated was transferred to another test tube. The radioactivity in the organic and aqueous

layers was measured by a gamma scintillation counter. Under the conditions described above, over 98% of the radioactivity of the authentic [^{125}I] -Ac-Tyr-Phe-Gly was retained in an aqueous phase and over 98% of the radioactivity of the authentic [^{125}I] -Ac-Tyr-Phe-NH₂ was transferred to an organic phase.

The yield of conversion is calculated from the ratio of the radioactivity in an organic phase such as an ethyl acetate phase to the total radioactivity. In this assay, one unit is defined as the enzyme activity that gives fifty percent conversion of 1 μmole substrate, such as [^{125}I] -Ac-Tyr-Phe-Gly, to [^{125}I] -Ac-Tyr-Phe-NH₂ for 1 hour.

(6) Protein derivatives exhibiting enzyme activity

The C-terminal α -amidating enzyme activity provided by culturing the above-mentioned various transformants and transfectant is shown in Figs. 23, 32(a), and 32(b). In these Figures, the enzyme activity is expressed in the conversion ratio of substrate to amidated product.

As seen from the results shown in the Figures, although E. coli transformed with P_LchT201 MPF-Sph or P_LchT201 MFK-Sph, and COS1 cells transfected with pKDch201-Sph did not provide a greater enzyme activity than that provided by the control strains, i.e., host E. coli or COS1 cells transfected with pKD, all other transformants and transfectants did provide a greater enzyme activity than the corresponding control strain.

The cDNA inserts in the vectors P_LchT201 MFK-Sph, P_LchT02 MPF-Sph, and pKDchT201-Sph which did not provide a greater enzyme activity to that of the corresponding control, terminate at the 323th Ala in the amino acid sequence in Fig. 1, but the cDNA inserts in the vectors P_LchT201 MPF-Sal, P_LchT201 MFK-Sal, and pKDchT201-Sal, which did provide greater enzyme activity than the corresponding control, terminate at the 378th Lys in the amino acid sequence shown in Fig. 1 (also

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Fig. 3). Accordingly, it can be reasonably concluded that an amino acid sequence starting at the 37th Pro and ending at the 378th Lys in the amino acid sequence shown in Figs. 1 and 3 is sufficient to provide the desired enzyme activity. Note, the amino acid sequence of this region is identical in Fig. 1 and Fig. 3.

Accordingly, the present invention includes any chT201-type enzyme derivative having an amino acid sequence which starts at any amino acid selected from the first amino acid Met to the 37th Pro and terminates at any amino acid selected from the 378th Lys to the 974th Ser, in the amino acid sequence shown in Fig. 1. More specifically, the present chT201-type enzyme derivatives include those schematically shown in Fig. 33(a) and Fig. 34, except for those provided by P_LchT201 MFK-Sph, P_LchT201 MPF-Sph and pKDT201-Sph, in addition to native enzyme starting from the first Met and ending at the 974th Ser. Nevertheless, the present chT201-type enzyme derivatives should not be limited to the above-mentioned particular derivatives. Since all tested derivatives which terminate at the 378th Lys, the 434th Asp or the 857th Ile exhibit the desired enzyme activity, it can be reasonably believed that any derivative which terminates at any amino acid between the 378th Lys to the 974th Ser will provide the desired enzyme activity. These derivatives can be easily obtained by constructing an expression vector containing a shortened cDNA insert. The shortened cDNA fragment can be easily prepared by a conventional procedure, such as exonuclease digestion.

Similarly, the present invention includes any chT101-type enzyme derivatives having an amino acid sequence which starts at any amino acid selected from the first amino acid Met to the 37th Pro and terminates at any amino acid selected from the 378th Lys to the 866th Ser, in the amino acid sequence shown in Fig. 3. More specifically, the present chT101-type enzyme

derivatives include those schematically shown in Fig. 33(b) and Fig. 34, in addition to native enzyme starting at the first Met and ending at the 866th Ser in Fig. 3. Nevertheless, the present chT101-type enzyme derivative should not be limited to the above-mentioned particular derivatives. Since all tested derivatives which terminate at 378th Lys (Same as chT201-type), the 461th Ala or the 750th Ile exhibit the desired enzyme activity, it can be reasonably believed that any derivative which terminates at any amino acid between the 379th Lys to the 866th Ser will provide the desired enzyme activity. These derivatives can be easily obtained by constructing an expression vector containing a shortened cDNA insert. The shortened cDNA fragment can be easily prepared by a conventional procedure, such as exonuclease digestion.

Moreover, the present enzyme derivatives can have an additional amino acid or amino acid sequence consisting of about up to ten amino acids, which is not present in the native sequence. In some cases, the additional amino acid or amino acid sequence is indispensably introduced by a multicloning site present in a vector used to construct an expression vector.

(7) Use of the enzyme

The present enzyme products can be used for the preparation of α -amidated peptide. In this process, a substrate peptide having a glycine residue at the C-terminal thereof is incubated with one of the present enzyme products in an aqueous reaction medium, preferably in an aqueous buffer such as Tris-HCl, at a pH of about 6 to 7, and at a temperature of about 37°C, for a time sufficient to convert a substantial amount of the starting peptide to a corresponding C-terminal α -amidated peptide. Practically, the present enzymes can be used to produce physiologically active peptides having a C-terminal α -amide from precursor peptides having glycine at the C-terminal thereof, which

precursor peptide may be produced by a recombinant DNA technique, chemical methods, or isolated from a natural source.

5 Although the present invention discloses in detail a process for the production of a C-terminal α -amidating enzyme using the cDNA in an E. coli host, and animal cells, the present cDNA can be used to express the target enzyme in another host, such as yeast cells.

Examples

10 The present invention will now be further illustrated by, but is no means limited to, the following examples.

Example 1. Preparation of poly(A) RNA from human thyroid gland

15 (1) Preparation of total RNA

First, 14 g of human thyroid grand was used to prepare total RNA, and 10 ml/2 g the tissue of PC9 (phenol: CHCl_3 : isoamylalcohol = 24: 24:10, saturated with 10 mM Tris-HCl, pH 9.0, 100 mM NaCl, 5 mM EDNA),
20 and 10 ml/2 g the tissue of NETS solution (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 10 mM EDTA, 5% SDS) were added to the thyroid gland, and cells in the thyroid gland were disrupted by a polytron. The disruptant was centrifuged at 3000 rpm for 20 minutes to obtain an
25 aqueous layer, the residual phenol layer was mixed with the NETS solution, and the mixture was centrifuged as described above to obtain an aqueous layer. The aqueous layers were combined, to the combined aqueous layer was added the same volume of CHCl_3 , and the mixture was
30 centrifuged at 3000 rpm for 20 minutes to obtain an aqueous solution; to which was added two volumes of ethanol, and the mixture then allowed to stand at -20°C overnight to form an ethanol precipitate.

The mixture was centrifuged at 3000 rpm and
35 4°C for 30 minutes to recover the precipitate, which was then washed with 80% ethanol and dried under vacuum. The dried precipitate was redissolved in 1 ml/g the

tissue of guanidine thiocyanate solution (4.2 M guanidine thiocyanate, 0.1 M sodium acetate, 5 mM EDTA, pH 5.0), 4 ml of CsCl solution (5 M CsCl, 0.1 M sodium acetate, 5 mM EDTA, pH 5.0) was put into a SW40TI centrifugation tube, and the guanidine thiocyanate solution of the precipitate was overlaid on the CsCl solution in the tube. After centrifugation at 33,000 rpm and 25°C for 15 hours, the RNA precipitate at the bottom of the tube was washed with 80% ethanol, and the precipitate was dissolved in 500 μ l of ETS solution (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS). Then to the solution was added the same volume of the PC9, and after stirring, the mixture was centrifuged at 10,000 rpm for 5 minutes to obtain an aqueous layer. To the aqueous layer was added the same volume of ethyl ether, and after stirring, the mixture was centrifuged at 10,000 rpm for one minute to obtain an aqueous solution. Then to the solution was added two volumes of ethanol, the mixture was allowed to stand at -80°C for 30 minutes to form an ethanol precipitate, and was then centrifuged at 13,000 rpm and 4°C for 15 minutes to separate the precipitate. After removing the supernatant, the precipitate was washed with 80% ethanol and dried under vacuum. The dried precipitate was then redissolved in sterile distilled water, whereby about 7 mg of total RNA was obtained.

(2) Preparation of poly (A) RNA

An oligo (dT) cellulose column was used to prepare poly(a) RNA. 0.5 g of oligo (dT) cellulose was packed to the column, which was washed with 10 ml of distilled water and 10 ml of 10 N NaOH, 5 mM EDTA, and the column was then thoroughly washed with distilled water. The column was equilibrated with 10 ml of a 1X loading buffer (20 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS), 7 mg of the total RNA in 13.5 ml of distilled water was heated at 65°C for 5 minutes, to the solution was added the same volume of 2x loading buffer,

and the mixture then applied to the column. A flow-through fraction was heated again at 65°C for 5 minutes and again applied to the column. The column was washed with 4 ml of the loading buffer and 4 ml of 20 mM Tris-HCl (pH 7.6), 0.1 M NaCl, 1 mM EDTA and 0.1% SDS. The poly(A) RNA was then eluted with 4 ml of a elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% SDS), and to the eluted fraction was added one tenth volume of 2 M sodium acetate (pH 5.0) and two volume of ethanol to recover the poly(A) RNA, whereby 144 µg of poly(A) RNA was obtained from 7 mg of the total RNA.

Example 2. Preparation of λgtII cDNA library

From 5.6 µg of the poly(A) RNA prepared from a human thyroid gland as in Example 1, a double-stranded cDNA was prepared using an Amersham cDNA synthesis kit. Next, the double-stranded cDNA was cloned to the λgtII phage, to prepare a cDNA library according to the following procedure.

To 100 µl of the double-stranded cDNA solution were added 10 µl of 10% SDS and 10 µl of 0.25 M EDTA, followed by 120 µl of PC9. After stirring, the mixture was centrifuged at 10,000 rpm for 10 minutes to obtain an aqueous solution, to the aqueous solution, were added 120 µl of 4 M ammonium acetate and 480 µl of ethanol, the mixture was allowed to stand at -80°C overnight to form a precipitate, and then centrifuged at 13,000 rpm for 20 minutes to separate the precipitate, which was then washed with 80% ethanol and dried under vacuum. The precipitate was redissolved in 100 µl of 1x TE solution (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), and an ethanol precipitation was carried out with 4 M ammonium acetate. The ethanol precipitate was dried and redissolved in 10 µl of distilled water, to the solution were added 4 µl of 5x EcoRI methylase buffer (400 mM Tris-HCl, pH 8.0, 40 mM EDTA, 8 mM DTT), 2 µl of 800 µM S-adenosylmethionine, 0.4 µl of EcoRI methylase (100: Takara Shuzo, Japan, and 3.6 µl of distilled water. The

reaction mixture was incubated at 37°C for 10 minutes, and to the reaction mixture was added the same volume of PC9, and after stirring, the mixture was centrifuged at 13,000 rpm for 10 minutes to obtain an aqueous solution. After the addition of one tenth volume of 3 M sodium acetate and two volume of ethanol, the mixture was allowed to stand at -80°C for 30 minutes to form a precipitate. The mixture was then centrifuged to separate the precipitate, which was washed with 80% ethanol and dried. To the ethanol precipitate were added 1 μ l of 10x ligation buffer (660 mM Tris-HCl, pH 7.6, 66 mM MgCl₂), 0.2 μ l of 20 mM ATP, 15 μ l of a 5'-phosphorylated EcoRI linker (12 mer, 0.1 μ g/ μ l, Takara Shuzo, Japan), and 1 μ l (5 units) of T4 DNA ligase (Takara Shuzo), and the reaction mixture was incubated at 15°C overnight. After the incubation, the mixture was heated at 65°C for 10 minutes, to inactivate the enzyme, and after the addition of 10 μ l of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and then 2 μ l of EcoRI (20 units), the reaction mixture was incubated at 37°C for 5 hours. After the incubation, the reaction mixture was treated with PC9, and adjusted to a final volume of 30 μ l by adding 1x TE. The mixture was applied to a Biogel A50*m column (0.5 x 25 cm) equilibrated with TES buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl) and eluted with a TES buffer. A main peak fraction was obtained, and to the fraction was added 0.5 μ g of phage λ gtII which had been cleaved with EcoRI and then 5'-dephosphorylated (Pharmacia), and an ethanol precipitation was carried out. To the precipitate, were added 1.5 μ l of 10x ligation buffer, 8 μ l of distilled water, 1.5 μ l of 0.1 M DTT, 1.5 μ l of 10 mM ATP, and 1.5 μ l of T4 DNA ligase (7.5 units), and the reaction mixture was incubated at 12°C overnight. Then 5 μ l of the reaction mixture was subjected to in vitro λ phage packaging, using a Giga pack gold* from Strategene, and as a result, a cDNA library of a human thyroid gland

*Trademarks

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origin consisting of about 500,000 clones was obtained.

5 Next, the cDNA library was amplified by the following procedure. E. coli VCS 257 (derived from supF; Maniatis, T. et al., Molecular Cloning, 1982, Cold Spring Harbor) was cultured in TB medium (10 g/l of Bactotrypron, 5 g/l of NaCl, pH 7.5) at 37°C overnight to prepare an inoculum. 40 ml of TB medium was inoculated with one hundredth of the inoculum, and culturing was carried out at 30°C overnight. The culture was
10 centrifuged at 3000 rpm for 10 minutes, and after discarding the supernatant, to the precipitated cells was added 16 ml of 10 mM MgSO₄. Then, the cDNA library phage was added to the cell suspension, in an amount of 500,000 pfu (plaque forming units), and the whole was
15 allowed to stand at 37°C for 20 minutes. To the culture was added 30 ml of NZY soft agar medium (10 g/l NZ amine, 5 g/l NaCl, 5 g/l yeast extract, 2 g/l MgSO₄ · 7H₂O and 10 g/l agar, pH 7.5), and the mixture was overlaid on an NZY medium containing 1.5% agar, in a petri dish (23
20 x 23 cm). After incubation at 37°C for 6 hours, 30 ml of an SM buffer (50 ml of 5.8 g/l NaCl, 2 g/l MgSO₄ · 7H₂O, 1 M Tris-HCl, pH 7.5; 2% gelatin 5 ml/l) was added to the petri dish, and the dish was allowed to stand at 4°C overnight. The SM buffer containing phages was recovered
25 as a phage stock solution. This procedure enabled an amplified cDNA library containing 4×10^{11} pfu/ml phages to be prepared.

Example 3. Isolation of λ gtII phage containing cDNA coding for novel C-terminal α -amidating enzyme

30 (1) Preparation of DNA probe

To isolate a phase clone containing a cDNA insert coding for a novel C-terminal α -amidating enzyme from the cDNA library derived from a human thyroid gland, a DNA probe was prepared by completely digesting plasmid
35 pXA 799 containing a cDNA coding for an entire Xenopus laevis C-terminal α -amidating enzyme with restriction

enzymes ApaI and DraI, and isolating an ApaI-DraI DNA fragment of about 2.8 kb containing an entire coding region, by agar gel electrophoresis. Next, the ApaI-DraI DNA fragment was radiolabeled with
5 [α -³²P]dCTP, by nick translation.

(2) Plaque hybridization

E. coli cells prepared by overnight culture and 150000 pfu of λ gtII phage were mixed with 25 ml of a soft agar NZY medium, and the mixture was overlaid on a
10 23 x 23 cm NZY agar medium in a petri dish, and incubated at 37°C overnight to form plaques. Six petri dishes were prepared. A nitrocellulose filter (22 x 22 cm; Schleicher & Schnell) was put on the agar medium for 5 minutes, and then the nitrocellulose filter was
15 put on an alkaline denaturation solution (0.1 N NaOH, 1.5 M NaCl) for 10 minutes, and thereafter, on a neutralizing buffer (0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl) for 10 minutes. Next, the nitrocellulose filter was washed with 2x SSC solution (20 x SSC = 175.3 g/l NaCl,
20 88.2 g/l trisodium citrate), dried in air, and baked at 80°C under a reduced pressure for 120 minutes.

The nitrocellulose was then put into a polymer sack, to which 60 ml of a prehybridization solution [3x SSC, 20% formamide, 50 mM Tris-HCl (pH 7.5), 0.25 mM
25 EDTA, 1x Denhardt's solution (1x Denhardt = 0.2 mg/ml each of albumin, polyvinyl pyrrolidone and Ficoll), 20 μ g/ml salmon sperm DNA] was introduced thereto, and a prehybridization was carried out at 37°C for 3 hours. After discarding the prehybridization solution,
30 2,000,000 cpm/sack of the probe and 10 ml of the above-mentioned prehybridization solution were added to the sack, to carry out hybridization at 37°C overnight. Next, the filter was twice washed in 500 ml of 3x SSC containing 0.1% sodium dodecyl sulfate (SDS), at 37°C
35 for 30 minutes, and then twice in 500 ml of 0.1x SSC containing 0.1% SDS, at 37°C for 40 minutes. The nitrocellulose was dried in air and subjected to

autoradiography.

In this procedure, two phage clones hybridized with the ApaI-DraI DNA probe containing a cDNA coding for an Xenopus laevis C-terminal α -amidating enzyme were obtained from a cDNA library comprising 90,000 phages. These clones were designated as λ gt11chT28 and λ gt11chT40. Phage DNAs of the λ gt11chT28 and λ gt11chT40 were prepared according to T. Maniatis et al., Cold Spring Harbor Molecular Cloning 1982, and digestion of the phage DNA λ gt11chT28 and λ gt11chT40 with EcoRI provided a cDNA insert of about 0.7 kb and a cDNA insert of about 2.2 kb, respectively.

For each cDNA, a restriction enzyme cleavage map was prepared, cleaved with various restriction enzymes, and the resulting DNA fragments were subcloned in M13 phage to determine the nucleotide sequence, using a Takara DNA sequencing kit (Takara Shuzo, Japan) according to the dideoxy termination method (Sanger, F. et al., Proc. Natl. Acad. Sci. U.S.A. 34, 5463-5467, 1977). As a result, it was found that the λ gt11ch28 contained a cDNA insert coding for a protein similar to a central portion of a protein encoded by the pXA799; and the λ gt11ch40 contained a cDNA insert coding for a protein similar to a C-terminal half of protein encoded by the pXA 799 (See Fig. 7). Therefore, it was assumed that both the λ gt11chT28 and λ gt11ch40 partially encode a C-terminal α -amidating enzyme of human thyroid origin.

Accordingly, to obtain a phage clone which contains a cDNA insert coding for an entire enzyme, the same cDNA library was screened. A desired clone could not be isolated, however, and therefore another cDNA library was prepared as follows.

Example 4. Preparation of λ gt10 cDNA library

4 μ g of the poly(A) RNA prepared in Example 1 was used to prepare double-stranded cDNA using a cDNA synthesis kit (Pharmacia). The resulting double-

stranded cDNA and 3 μ g of λ gt10 phage (Strategene) were ligated, using a T4 DNA ligase, at 12°C overnight, and subjected to in vitro packaging. As a result, a λ gt10 cDNA library comprising about 2×10^6 clones was obtained.

Example 5. Isolation of phage clones λ gt10chT2, λ gt10chT201 and λ gt10chT101 containing cDNA coding for desired enzyme.

(1) Preparation of DNA probes

To isolate phage clones containing a cDNA insert coding for an entire C-terminal α -amidating enzyme of human thyroid gland origin, in addition to the ApaI-DraI DNA probe prepared in Example 3 (1), an additional DNA probe was prepared by cleaving the above-prepared λ gt11chT28 with EcoRI to obtain a 0.7 kb EcoRI DNA fragment, which was then radiolabeled with [α - ^{32} P]dCTP by nick translation.

(2) Hybridization

About 280,000 clones of the λ gt10-cDNA library were plated on 23 x 23 cm petri dishes, using E. coli C600 hfl $^{-}$ as an indicator strain as described in Example 3. (2).

After an overnight culture at 37°C, the formed plaques were transferred to a nitrocellulose filter, which was then subjected to alkaline denaturation, neutralization, air drying at 80°C under a reduced pressure, and baking for 90 minutes, as described in Example 3. (2). The nitrocellulose filter was put into a polymer sack, to which 50 ml of the above-mentioned prehybridization solution additionally containing 20% formamide was introduced. The sack was incubated at 37°C for 3 hours, and after discarding the solution 10,000,000 cpm of the ApaI-DraI DNA probe and 10 ml of the prehybridization solution containing 20% formamide were introduced into the sack, which was then incubated at 37°C overnight. Next, the filter was twice washed is 500 ml of 3 x SSC solution containing 0.1% SDS, and then

twice washed with a 0.1 x SSC solution containing 0.1% SDS, at 37°C. After air drying, the filter was subjected to autoradiography, and as a results one positive clone which hybridized with the ApaI-DraI probe derived from pXA 799 was obtained, and was designated λ gt10chT2.

The same procedure as described above was repeated, except that 340,000 clones of the λ gt10 cDNA library and 10,000,000 cpm of the EcoRI DNA probe derived from λ gt11chT28 were used. The prehybridization and hybridization were carried out as described above, except that the prehybridization solution contained 50% formamide, and as a result, two positive clones hybridized with a 0.7 kb EcoRI DNA probe from λ gt11chT28 were obtained, and were designated λ gt10chT201 and λ gt10chT202.

Phage DNAs were prepared for three clones thus obtained by a conventional procedure, and to test the length of cDNA insert, each phage DNA was digested with EcoRI. As a result, the λ gt10chT2 provided two DNA fragments of about 2.4 kb and 0.4 kb, showing that the 2.8 kb cDNA had been inserted. On the other hand, the λ gt10chT201 provided three DNA fragment of about 2.2 kb, 0.8 kb, and 0.7 kb, respectively, showing that the 3.7 kb cDNA had been inserted.

(3) Determination of nucleotide sequences of cDNAs inserted in λ gt10chT2 and λ gt10chT201

The λ gt10chT201 and λ gt10chT2 phage DNAs were digested with various restriction enzymes, and the resulting fragments were separated by agar gel electrophoresis to prepare restriction enzyme cleavage maps of cDNA regions. The results are shown in Fig. 4(a) and Fig. 5(a). Next, to determine the nucleotide sequence of each cDNA insert, the DNA fragments were subcloned in M13 phage, and the nucleotide sequence was determined by using a Takara DNA sequencing kit (Takara Shuzo, Japan) and a Sequanase^(TM)

DNA sequencing kit (United States Biochemical Corporation), according to the dideoxy termination method. The strategies used for the sequencing are shown in Figs. 4(b) and 5(b). The Nucleotide sequence of cDNA in the phage clone λ gt10chT201 (abbreviated as chT201 cDNA) is shown in Fig. 1; and the nucleotide sequence of cDNA in the λ gt10chT2 (abbreviated as chT2 cDNA) is shown in Fig. 2.

As seen from Fig. 1, the chT201 cDNA has a length of about 3.7 kb, and is a full length cDNA including a 5'-terminal non-coding region, a long reading frame, and a 3'-terminal non-coding region. On the other hand, the chT2 cDNA appears to lack the 5'-terminal region of a full length cDNA. In a comparison of the nucleotide sequences of the chT201 cDNA and chT2 cDNA, the chT201 cDNA contains 321 extra nucleotides coding for 107 amino acids, which are absent from the chT2 cDNA. The other portions are roughly identical for both the chT201 cDNA and the chT2 cDNA. In a comparison of the nucleotide sequence of the cDNA in pXA 799 derived from Xenopus laevis, as shown in Fig. 7, the chT2 cDNA is similar to the cDNA in PXA 799. On the other hand, the chT201 cDNA contains 321 nucleotides coding for 107 amino acids, which are absent from the cDNA in pXA 799.

This suggests that there are two types of C-terminal α -amidating enzymes derived from human thyroid gland.

(4) Isolation of λ gtch10T101 phage clone

As discussed above, the comparison of chT201 cDNA with chT2 cDNA suggested the presence of two types of C-terminal α -amidating enzymes of human thyroid gland origin, i.e., an enzyme encoded by chT201 cDNA having 321 additional nucleotides, and an enzyme encoded by chT2 cDNA not having the 321 nucleotides.

The chT2 cDNA, however, lacks the 5-terminal portion of a postulated full length cDNA, and

accordingly, to obtain a chT2-type full length cDNA, the λ gt10 cDNA library was again screened.

5 A DNA probe was prepared by cleaving the chT201 cDNA with SmaI and XhoI to obtain a 380 bp DNA fragment comprising a 5'-noncoding region and 5'-terminal side coding region, and labeling the DNA fragment with [α -³²P]dCTP by nick translation. Plaque hybridization was carried out according to the procedure described in Example 5. (2); using 1,280,000 clones of 10 the λ gt10 cDNA library, the 380 bp DNA probe, and the prehybridization solution containing 50% formamide, to obtain one positive clone. The clone was designated as λ gt10chT101. Phage DNA of λ gt10ch101 was prepared by a conventional procedure, and the length of the cDNA 15 insert in the λ gt10chT101 (abbreviated as chT101 cDNA) was determined by cleaving the phage DNA. As a result, the cleavage provided three cDNA fragments of 0.7 kb, 0.9 kb, and 2.6 kb, showing that the λ gt10chT101 phage clone contains the 4.2 kb cDNA insert.

20 (5) Determination of nucleotide sequence of cDNA insert in λ gt10chT101

The λ gt10chT101 phage DNA was cleaved with various restriction enzymes, and the resulting fragments were separated by agar gel electrophoresis to prepare a 25 restriction enzyme cleavage map of chT101 cDNA. The result is shown in Fig. 6(a). Next the DNA fragments were subcloned in M13 phage, and a nucleotide sequence was determined using a Sequanase^(TM) DNA sequencing kit according to the dideoxy termination method. The 30 strategy for the sequencing is shown in Fig. 6(b). The nucleotide sequence of chT101 cDNA, and a corresponding amino acid sequence, are shown in Fig. 3. Although the chT101 cDNA consists of three EcoRI fragments of 2.6 kb, 0.9 kb, and 0.9 kb, since the 0.9 kb cDNA fragment does 35 not encode a C-terminal α -amidating enzyme, the nucleotide sequence of the 0.9 kb cDNA fragment is excluded from Fig. 3. As seen from a comparison of the

nucleotide sequences shown in Figs. 2 and 3, both the chT101 cDNA and chT2 cDNA encode the same enzyme, although the chT101 cDNA encodes the entire enzyme and the chT2 cDNA encodes the same enzyme but lacking its N-terminal portion.

Figure 7 shows the relationship between pXA 799 cDNA, chT210 cDNA, chT2 cDNA, chT101 cDNA, chT40 cDNA, and chT28 cDNA.

Example 6. Construction of E.coli vector expressing chT201-type protein derivatives, P_LchT201 MPF-Sph, P_LchT201 MPF-S/B, P_LchT201 MPF-Sal and P_LchT201 MPF-Bcl

To express protein derivatives encoded within the ch201 cDNA (nucleotides 140-3061) in E. coli, expression vectors P_LchT201 MPF-Sph, P_LchT201 MPF-S/B, P_Lch201 MPF-SalI, and P_LchT201 MPF-Bcl, as well as E. coli W3110 transformed with the expression vector, were prepared as follows.

(1) Construction of pUC18chT201 HindIII-BglII (Fig. 8)

As shown in Fig. 8, the λchT201 phage DNA was cleaved with HindIII and BglII to isolate aDNA fragment of about 5 kb comprising an entire coding region of the chT201 cDNA and a part of the λgt10 phage. On the other hand, pUC18 was cleaved with HindIII and Bam HI. Both DNA fragments were ligated to construct an intermediate plasmid pUC18chT201 HindIII-BglII, which was used to transform E. coli, and the transformant was designated as E. coli SBM302, and deposited with the FRI under the Budapest Treaty as FERM BP-2237, on January 11, 1989.

(2) Construction of P_Lch201 MPF-Sph (Figs. 9 and 10)

There are two possible processing sites in an N-terminal region of a protein encoded by the chT201 cDNA, i.e., the bond between Arg(30)-Pro(31) and the bond between Arg(36)-Pro(37). To express protein derivatives starting with the Pro(37), a plasmid

pUC19chtT201 MPF was constructed.

As seen in Fig. 9, the plasmid pUC18chtT201 HindIII-BglII was cleaved with KpnI and HindIII, to isolate a KpnI-HindIII DNA fragment (E1), and a DNA
5 fragment YSO53 was synthesized having a 5'-EcoRI end, a translation start codon ATG, codons for the 37th Pro to the 43th Leu and a 3'-KpnI end, in this order. Further, a complementary DNA fragment YSO54 was synthesized. The
10 DNA fragments YSO53 and YSO54 were 5'-phosphorylated using a T4 polynucleotide kinase and ATP, and annealed at 65°C for 10 minutes to prepare the double-stranded DNA fragment shown in Fig. 9. On the other hand, pUC19 was cleaved with EcoRI and HindIII. The synthetic DNA
15 fragment, the E1 fragment, and the linearized pUC19 were ligated and used to transform E. coli W3110, to obtain a plasmid P_LchtT201 MPF-Sph and corresponding transformant E. coli W3110/P_LchtT201 MPF-Sph.

(4) Construction of P_LchtT201 MPF-S/B (Fig. 11)

As shown in Fig. 11, the plasmid pUC18chtT201
20 HindIII-BglII was cleaved with XhoI and Sau3A1 (cleaving cDNA at 1439th position) to obtain a DNA fragment E4, and the plasmid pUC19chtT201 MPF was cleaved with EcoRI and XhoI to obtain a DNA fragment E5. The plasmid
pUCP_L-CI was cleaved with EcoRI and BamHI. Next, the
25 DNA fragments E4 and E5, and the linearized pUCP_L-CI were ligated and used to transform E. coli W3110 to obtain a plasmid P_LchtT201 MPF-S/B and corresponding transformant E. coli W3110/P_LchtT201 MPF-S/B.

(5) Construction of P_LchtT201 MPF-Sal

30 To construct P_LchtT201 MPF-Sal, first plasmids pUC19chtT201-EcoRI 0.7 and pUC18-Sal were constructed.

(a) Construction of pUC19chtT201-EcoRI 0.7 (Fig. 12)

As shown in Fig. 12, the λchtT201 phage
35 DNA was cleaved with EcoRI to obtain a 0.73 kb EcoRI DNA fragment. On the other hand, pUC19 was cleaved with EcoRI. The 0.73 kb EcoRI DNA fragment and the

linearized pUC19 were ligated and used to transform E. coli JM109, to obtain a plasmid pUC19chT201-EcoRI 0.7 and corresponding transformant E. coli JM109/pUC-19chT201-EcoRI 0.7.

5 (b) Construction of pUC18-Sal (Fig. 13)

As shown in Fig. 13, the plasmid pUC19chT201-EcoRI 0.7 was cleaved with EcoRI and HinfI (cleaving cDNA at 1250 position) to obtain a DNA fragment E6. To introduce a translation stop codon TGA immediately downstream of the 378th Lys in the chT201cDNA, a DNA fragment YSO59 having an Hinf cohesive end at its 5'-end, and a DNA fragment YSO60 complementary to the YSO59 and having a SalI cohesive end at its 5'-end, were synthesized. The YSO59 and YSO60 were 5'-phosphorylated using a T4 polynucleotide kinase and ATP, and annealed at 65°C for 10 minutes. On the other hand, pUC18 was cleaved with EcoRI and SalI. Next the annealed YSO59 and YSO60, the E6 DNA fragment, and the linearized pUC18 were ligated and used to transform E. coli JM109, to obtain a plasmid pUC18-Sal and corresponding transformant E. coli JM109/pUC18-Sal.

(c) Construction of P_LchT201 MPF-Sal (Fig. 14)

As shown in Fig. 14, P_LchT201 MPF-Sph was cleaved with BstEII and then partially digested with EcoRI, to obtain an EcoRI-BstEII DNA fragment E7. On the other hand, pUC18-Sal was cleaved with SalI and BstEII to obtain a BstEII-SalI DNA fragment E8. Further, pUCP_L-CI was cleaved with EcoRI and SalI. The DNA fragments E7 and E8, and the linearized pUC18 were ligated and used to transform E. coli W3110, whereby a plasmid P_LchT201 MPF-Sal and corresponding transformant E. coli W3110/P_LchT201 were obtained.

(6) Construction of P_LchT201 MPF-Bcl (Fig. 15)

As shown in Fig. 15, P_LchT201 MPF-S/B was cleaved with XhoI (cleaving cDNA at 421 position) and BamHI to obtain an XhoI-BamHI DNA fragment E9. On the

other hand, pUC18cht201 HindIII-BglII was cleaved with XhoI and BclI (cleaving cDNA at 2707 position) to obtain an XhoI-BclI DNA fragment E10. The DNA fragments E9 and E10 were ligated and used to transformant E. coli W3110, whereby a plasmid P_Lcht201 MPF-Bcl and corresponding transformant E. coli W3110/P_Lcht201 MPF-Bcl were obtained.

Example 7. Construction of E. coli vector expressing cht201-type protein derivatives,

P_Lcht201 MFK-Sph, P_Lcht201 MFK-S/B, P_Lcht201 MFK Sal and PLcht201 MFK-Bcl

To express protein derivatives encoded within cht201 cDNA (nucleotide 140-3061) in E. coli, expression vectors P_Lcht201 MFK-Sal, P_Lcht201 MFK-S/B, P_Lcht201 MFK-Sal and P_Lcht201 MFK-Bcl, and corresponding E. coli W3110 transformants, were prepared as follows.

(1) Construction of pUC19cht201 MFK (Fig. 16)

To express protein derivatives starting with the 31th Phe, a first plasmid pUC19cht201 MFK was constructed. For this purpose, as shown in Fig. 16, a DNA fragment YS055 having a 5'-EcoRI end, a translation start codon ATG, a codon for the 31th Phe and 3'-DraI blunt end in this order, as well as a DNA fragment YS056 complementary to the YS055, were synthesized. These synthetic DNA fragments were 5'-phosphorylated using a T4 polynucleotide kinase and ATP, and annealed at 65°C for 10 minutes to prepare a double-stranded DNA fragment shown in Fig. 16. The plasmid pUC18cht201 HindIII-BglII was cleaved with PstI (cleaving cDNA at 1844 position) and DraI (cleaving cDNA at 233 position) to obtain a PstI-DraI DNA fragment E11. The synthetic DNA fragment, the DNA fragment E11, and the linearized pUC19 were ligated and used to transform E. coli JM109, whereby a plasmid pUC19cht201 MFK and corresponding transformant E. coli JM109/pUC19cht201 MFK were obtained.

(2) Construction of P_Lcht201 MFK-Sph (Fig. 17)

As shown in Fig. 17, P_Lcht201 MPF-Sph was

cleaved with SphI and XhoI to obtain an SphI-XhoI DNA fragment E12. The plasmid pUC19chT201 MFK was cleaved with EcoRI and XhoI to obtain an EcoRI-XhoI DNA fragment E13, and pUCP_L CI was cleaved with EcoRI and SphI.

Next, the DNA fragments E12 and E13, and the linearized pUCP_L CI, were ligated and used to transform E. coli W3110, whereby a plasmid P_LchT201 MFK-Sph and corresponding transformant E. coli W3110/P_LchT201 MFK-Sph were obtained.

(3) Construction of P_LchT201 MFK-S/B (Fig. 18)

As shown in Fig. 18, P_LchT201 MPF-S/B was cleaved with XhoI and BamHI to obtain an XhoI-BamHI DNA fragment E14. The plasmid pUC19chT201 MFK was cleaved with EcoRI and XhoI to obtain an EcoRI-XhoI DNA fragment E15. On the other hand, pUCP_L CI was cleaved with EcoRI and BamHI. Next, the DNA fragments E14 and E15, and the linearized pUCP_L CI, were ligated and used to transform E. coli W3110, whereby a plasmid P_LchT201 MFK-S/B and corresponding transformant E. coli W3110/P_LchT201 MFK-S/B were obtained.

(4) Construction of P_LchT201 MFK-Sal (Fig. 19)

As shown in Fig. 19, P_LchT201 MPF-Sal was cleaved with XhoI and PstI to obtain an XhoI-PstI DNA fragment E16. On the other hand, P_LchT201 MFK-S/B were cleaved with XhoI and PstI to obtain an XhoI-PstI DNA fragment E17. The DNA fragments E16 and E17 were ligated and used to transform E. coli W3110, whereby a plasmid P_LchT201 MFK-Sal and corresponding transformant E. coli W3110/P_LchT201 MFK-Sal were obtained.

(5) Construction of P_LchT201 MFK-Bcl (Fig. 20)

As shown in Fig. 20, P_LchT20 MPF-S/B was cleaved with XhoI and BamHI to obtain an XhoI-BamHI DNA fragment E18. On the other hand, pUC18chT201 HindIII-BglII was cleaved with XhoI and BclI to obtain an XhoI-BclI DNA fragment E19. Next, the DNA fragments E18 and E19 were ligated and used to transform E. coli W3110, whereby a plasmid P_LchT201 MFK-Bcl and

corresponding transformant E. coli W3110/P_LchT201 MFK-Bcl were obtained.

Example 8. Construction of E. coli vectors expressing chT101-type protein derivatives, P_LchT2 MPF-Pst, P_LchT2 MFK-Pst, P_LchT2 MPF-Bcl and P_LchT2 MFK-Bcl

Although the chT2 cDNA lacks the N-terminal coding region, the lacking region of chT2 cDNA can be supplemented with a corresponding region of chT201 cDNA, to construct a cDNA identical with the chT101 cDNA. Accordingly, to construct E. coli vectors expressing chT101-type C-terminal α -amidating enzyme derivatives, the chT2 cDNA and chT201 cDNA were used.

(1) Construction of P_LchT2 MPF-Pst and P_LchT2 MFK-Pst (Fig. 21)

As shown in Fig. 21, P_LchT201 MPF-Bcl was cleaved with XhoI and PstI to obtain an XhoI-PstI DNA fragment E20. On the other hand, pUC18chT2 HindIII-BglII constructed in Example 10. (4) (see, Fig. 27) was cleaved with XhoI, BamHI and PstI to obtain an XhoI-PstI DNA fragment E22. Next, the DNA fragments E20 and E22 were ligated and used to transform E. coli W3110, whereby a plasmid P_LchT2 MPF-Pst and corresponding transformant E. coli W3110/P_Lch2 MFK-Pst were obtained.

The same procedure as described above was repeated except that P_LchT201 MFK-Bcl was used in place of P_LchT201 MPF-Bcl to obtain a plasmid P_LchT2 MFK-Pst and corresponding transformant E. coli W3110/P_Lch MFK-Pst.

(2) Construction of P_LchT2 MPF-Bcl and P_LchT2 MFK-Bcl (Fig. 22)

As shown in Fig. 22, P_LchT201 MPF-Bcl was cleaved with XhoI and then partially digested with XbaI to obtain an XhoI-XbaI DNA fragment E23. On the other hand, the pUC18chT2 HindIII-BglI constructed in Example 10. (4) (see Fig. 27) was cleaved with XhoI and XbaI to obtain an XhoI-XbaI DNA fragment E25. Next, the

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DNA fragments E23 and E25 were ligated and used to transform E. coli W3110, whereby a plasmid P_{LchT2} MPF-Bcl and corresponding transformant E. coli W3110/ P_{LchT2} MPF-Bcl were obtained.

5 The procedure as described above was repeated except that P_{LchT2} MFK-Bcl was used to in place of P_{LchT2} MPF-Bcl, to obtain a plasmid P_{LchT2} MFK-Bcl and corresponding transformant E. coli W3110/ P_{LchT2} MFK-Bcl.

10 Example 9. C-terminal α -amidating activity
 provided by transformed E. coli

E. coli transformants prepared as above,
W3110/ P_{LchT} 201 MPF-Sph, W3110/ $P_{LchT201}$ MPF-Sal,
W3110/ $P_{LchT201}$ MPF-S/B, W3110/ $P_{LchT201}$ MPF-Bcl,
W3110/ $P_{LchT201}$ MFK-Sph, W3110/ $P_{LchT201}$ MFK-Sal,
15 W3110/ $P_{LchT201}$ MFK-S/B, W3110/ $P_{LchT201}$ MFK-Bcl,
W3110/ P_{LchT2} MPF-Pst, W3110/ P_{LchT2} MFK-Pst, W3110/ P_{LchT2}
MPF-Bcl, and W3110/ P_{LchT2} MFK-Bcl were separately
cultured in a Super broth supplemented with 50 μ g/ml
ampicillin, at 32°C overnight. As a control E. coli
20 W3110 (not transformed) was cultured in Super broth not
containing ampicillin, at 32°C overnight. Note, the
Super broth medium was prepared by dissolving 24 g of a
yeast extract, 12 g of trypton, 5 ml of glycerol, and
100 ml of a 1 M phosphate buffer (pH 7.6) in water, to a
25 total volume of 1 liter. Next, the culture was
inoculated to 5 ml of a fresh medium having the same
composition to 0.01 OD/ml at 660 nm, and culturing was
carried out at 32°C until the absorbance at 660 nm
become 0.3 OD/ml. At this time, the temperature was
30 changed from 32°C to 42°C; and culturing was continued
for further 3 hours. The cultured broth having an
absorbance of 5 OD at 660 nm was centrifuged at
13,000 rpm for 2 minutes, to collect cells, the
supernatant was discarded, the cells were suspended in
35 300 μ l of PBS(-) (0.8% NaCl, 0.02% KCl, 0.15% Na_2HPO_4
and KH_2PO_4) containing 0.1% Triton*, and the suspension
was subjected to sonication to disrupt the cells. The

*Trademark

disruptant was centrifuged at 13,000 rpm for two minutes, the supernatant was discarded, and the precipitate was suspended in 300 μ l of PBS (-). The suspension was centrifuged at 13,000 rpm for two minutes, and the supernatant was discarded. The precipitate was dissolved in 500 μ l of 6M guanidine hydrochloride (containing 10 mM Tris-HCl, pH 7.0, and 50 μ M CuSO₄) and the solution was sonicated. Next, the solutions was dialyzed against 4 M guanidine hydrochloride (containing 10 mM Tris-HCl, pH 7.0, and 50 μ M CuSO₄) for one hour, and hereafter, the dialyzate was again dialyzed against 2 M guanidine hydrochloride (containing 10 mM Tris-HCl, pH 7.0, 50 μ M CuSO₄), overnight. The dialyzate was assayed to determine the C-terminal α -amidating activity.

The assay was carried out according to Mizuno et al., B.B.R.C. 137, 984-991, 1986, as follows. Namely, 25 μ l of the sample was diluted with water to a total volume of 100 μ l, to this solution were added 25 μ l of 10 mM N-ethylmaleimide, 25 μ l of 10 mM ascorbic acid, 25 μ l of 200 μ M CuSO₄, 1.25 μ l of 20 mg/ml catalase, 25 μ l of 1% rubrol, 2 pmol of [¹²⁵I]-Ac-Tyr-Phe-Gly (170,000 cpm), and 50 μ l of 1M Tris-HCl (pH 7.0), and the reaction mixture was incubated at 37°C for 24 hours. After the reaction, to the reaction mixture were added 750 μ l of 1M Tris-HCl (pH 7.0) and ethyl acetate, and the whole was mixed and centrifuged. The ethyl acetate layer and aqueous layer were then separated, the radioactivity of both fractions counted by a γ -counter, and a ratio of radioactivity transferred to the ethyl acetate layer was calculated. Note, it has been confirmed that the C-terminal-amidated product, [¹²⁵I]-Ac-Tyr-Phe-NH₂ is selectively transferred to the ethyl acetate layer in the above-method. The result is shown in Fig. 23.

As seen from Fig. 23, W3110/P_LchT201 MPF-Sph and W3110/P_LchT201 MFK-Sph did not provide the C-terminal

amidating activity. Accordingly, it was found that a smaller protein up to the 323th Ala does not provide a sufficient enzyme activity. On the other hand, a larger protein which extends to or beyond the 378th Lys does provide the desired enzyme activity. Furthermore, the position of the N-terminus of the protein derivatives, i.e., the first Met, the 31th Pro or the 37th Pro in the sequence shown in Fig. 1, did not affect the enzyme activity.

Note, the enzyme activity provided by E. coli transformants was relatively low, which is probably due to a lack of glycosylation or an incorrect folding of protein derivative produced by E. coli.

Accordingly, the production of enzyme derivatives by animal cells was attempted as follows.

Example 10. Construction of animal cell expression plasmids pKDchT201-Sph, pKDchT201-Sal, pKDchT201-S/B, pKDch201-Pst, pKDchT201-Bcl, pKDchT2-Pst and pKDchT2-Bcl

For the expression of enzyme derivatives, plasmids pKDchT201-Sph, pKDchT201-Sal, pKDchT201-S/B, pKDchT201-Pst, and pKDchT201-Bcl (for expression of chT201-type C-terminal α -amidating enzyme derivatives), as well as pKDchT2-Pst and pKDchT2-Bcl (for expression of chT2-type C-terminal α -amidating enzyme derivatives) were constructed from chT201 cDNA, or from chT201 cDNA and chT2 cDNA. These plasmids contained cDNA insert coding for a protein starting from the first amino acid in the amino acid sequence shown in Fig. 1 (or Fig. 3), and terminating at a different amino acid in the amino acid sequence shown in Fig. 1 or that shown in Fig. 3.

These plasmids also contained an SV 40 (Simian Virus 40) early promoter, an intron derived from a rabbit β -globin gene responsible for splicing an RNA and addition of poly A, a cDNA coding for a desired enzyme derivative under the control of the SV40 early promoter, and a poly A addition signal derived from a rabbit

β -globin gene, in this order.

Note, E. coli SBM 303 containing the plasmid pKD used for construction of the above-mentioned expression plasmids was deposited with the FRI under the Budapest
5 treaty as FERM BP-2238, on January 11, 1989.

(1) Construction of pUC18-HpaII (Fig. 24)

As shown in Fig. 24, the plasmid pUC18chT201 HindIII-BglII was cleaved with HpaII to obtain a 453 bp
DNA fragment, which was then filled in using Krenow
10 enzyme in the presence of dNTPs to make the ends blunt.
The plasmid pUC18 was cleaved with SmaI. The 453 bp DNA
and the linearized pUC18 were ligated and used to
transform E. coli JM 109, whereby a plasmid pUC18-HpaII
was obtained.

15 (2) Construction of plasmids pKDchT201-Sph,
pKDchT201-Sal, pKDchT201-S/B and pKDchT201-Bcl
(Fig. 25)

The plasmid pUC18 HpaII was cleaved with KpnI
and HindIII to obtain a DNA fragment C1, and the
20 P_L chT201 MPF-Sph was cleaved with KpnI and HindIII to
obtain DNA fragment C2. Further, pKD was cleaved with
HindIII. Next the DNA fragments C1 and C2, and the
linearized plasmid pKD, were ligated and used to
transform E. coli DH1, whereby a plasmid pKDchT201-Sph
25 was obtained.

The same procedure was repeated except that
 P_L chT201 MPF-Sal was used in place of P_L chT201 MPF-Sph,
to obtain a plasmid pKDchT201-Sal.

Further, the same procedure was repeated
30 except that P_L chT201 MPF-S/B was used in place of
 P_L chT201 MPF-Sph, to obtain a plasmid pKDchT201-S/B.

Furthermore, the same procedure was repeated
except that P_L chT201 MPF-Bcl was used in place of
 P_L chT202 MPF-Sph, to obtain a plasmid pKDchT201-Bcl.

35 (3) Construction of pKDchT201-Pst (Fig. 26)

As shown in Fig. 26, the plasmid pUC18chT201
HindIII-BglII was cleaved with PstI and XhoI to obtain a

DNA fragment C6. On the other hand, pKDchT201 MPF-Sal was completely digested, with XhoI, and then partially digested with PstI, to obtain a DNA fragment C7. The DNA fragments C6 and C7 were ligated and used to transform E. coli DH1, whereby a plasmid pKDchT201-Pst was obtained.

(4) Construction of pKDchT2 HindIII-BglII
(Fig. 27)

As shown in Fig. 27, the λ gt10chT2 phage DNA was cleaved with HindIII and BglII to obtain a DNA fragment containing a chT2 cDNA coding region and a portion of the λ gt10 phage. On the other hand, pUC18 was cleaved with HindIII and BamHI. Next, the DNA fragment from the λ gt10chT2, and the linearized pUC18, were ligated and used to transform E. coli JM109, whereby a plasmid pUC18chT2 HindIII-BglII was obtained. E. coli transformed with the plasmid pUC18chT2 HindIII-BglII was designated as E. coli SBM304, and deposited with the FRI under the Budapest treaty as FERM BP-2239, on January 11, 1989.

(5) Construction of pUC18chT201-chT2 (Fig. 28)

As shown in Fig. 28, pUC18 HpaII was cleaved with HindIII and SspI to obtain a DNA fragment C8, and the plasmid pUC18chT2 HindIII-BglII was cleaved with HindIII and SspI to obtain a DNA fragment C9. Further, pUC118 was cleaved with HindIII. Next, the DNA fragments C8 and C9, and the linearized pUC118, were ligated and used to transform E. coli JM109, whereby a plasmid pUC118chT201-chT2 was obtained.

(6) Construction of P_L chT2 KpnI-PstI (Fig. 29)

As shown in Fig. 29, the plasmid pUC18chT2 HindIII-BglII was cleaved with KpnI and PstI to obtain a DNA fragment c10, and the plasmid pUCP_LCI was cleaved with KpnI. Next, the DNA fragment C10 and the linearized pUCP_LCI were ligated and used to transform E. coli JM109, whereby a plasmid P_L chT2 KpnI-PstI was obtained.

(7) Construction of pKDchT2-Pst (Fig. 30)

As shown in Fig. 30, the plasmid pUC18 HpaII was cleaved with HindIII and SspI to obtain a DNA fragment C11, and the plasmid P_LchT2 KpnI-PstI was
 5 cleaved with SspI and HindIII to obtain a DNA fragment C12. Further, the plasmid pKD was cleaved with HindIII. Next, the DNA fragments C11 and C12, and the linearized pKD, were ligated and used to transform E. coli DH1, whereby a plasmid pKDchT2-Pst was obtained.

10 (8) Construction of pKDchT2-Bcl (Fig. 31)

As shown in Fig. 31, the plasmid pUC118chT201-
 chT2 was cleaved with HindIII and BclI to obtain a DNA
 fragment C13, and the plasmid pUCP_LCI was cleaved with
 BamHI and HindIII to obtain a DNA fragment C14.
 15 Further, the plasmid pKD was cleaved with HindIII. Next, the DNA fragments C13 and C14, and the linearized pKD, were ligated and used to transform E. coli DH1, whereby a plasmid pKDchT2-Bcl was obtained.

According to the above-mentioned construction
 20 processes, five expression plasmids, pKDchT201-Sph, pKDchT201-Sal, pKDchT201-S/B, pKDchT201-Pst, and pKDchT201-Bcl derived from chT201 cDNA and coding for chT201-type protein derivatives, as well as two
 expression plasmids, pKDchT2-Pst and pKDchT2-Bcl derived
 25 from chT2 cDNA and chT201 cDNA and coding for chT101-type protein derivatives, were constructed.

Example 11. Expression of protein derivatives in COS1 cells

The Expression of C-terminal α -amidating enzymes of
 30 human thyroid origin was tested as follows.

(1) Transfection

Plasmids pKD, pKDchT201-Sph, pKDchT201-Sal,
 pKDchT201-S/B, pKDchT201-Bcl, pKDchT201-Pst, pKDchT2-Pst
 and pKDchT2-Bcl were prepared from corresponding E. coli
 35 transformants by a conventional procedure, 30 μ g of each plasmid DNA was precipitated by ethanol, and the ethanol precipitate was washed with 80% ethanol and dried under

vacuum. The dried DNA was dissolved in 720 μ l of sterile distilled water, and to this solution was added 720 μ l of an A buffer of a Cell Pfect transfection kit (Pharmacia), and the whole was thoroughly mixed and
5 allowed to stand at a room temperature for 10 minutes. Next, 1440 μ l of B buffer of the same kit was added, and the whole was thoroughly mixed and allowed to stand at a room temperature for 15 minutes, whereby a calcium phosphate-DNA complex was formed.

10 On the other hand, COS1 cells were cultured overnight in 30 medium at a concentration of 7.5×10^5 cell/10 cm petri dish, and after adding 10 ml of fresh medium per petri dish, further cultured for 4 hours. To the culture was added 960 μ l/petri dish of
15 the calcium phosphate-DNA complex, the culture was incubated for 4 hours, the supernatant was then removed by suction, and the petri dish was washed with 5 ml of a fetal calf serum-free medium. Next, 2.5 ml of a medium containing 20% glycerol was added to the dish and was
20 allowed to stand for one minute. After removing the glycerol-containing medium, the petri dish was washed with 8 ml of a medium, 10 ml of the medium was added to the petri dish, and culturing was carried out for 3 days. Usually, the medium was Dulbecco's MEM containing
25 10% fetal calf serum (Flow Lab.), and culturing was carried out at 37°C in an atmosphere of 50% CO₂.

Example 12. Determination of C-terminal α -amidating activity of culture

After the three day culture of transfected COS1
30 cells as described above, the supernatant was obtained and centrifuged at 15000 rpm for 4 minutes to obtain a supernatant, and the C-terminal α -amidating activity of the supernatant was determined by an assay. The assay was carried out according to Mizuno et al. (supra), and
35 the conversion of [¹²⁵I]-Ac-Tyr-Phe-Gly to [¹²⁵I]-Ac-Tyr-Phe-NH₄ was measured. The result is shown in Figs. 32(A) and 32(B). This result was obtained by

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reacting 100 μ l of the supernatant for 16 hours.

As seen from Figs. 32(A) and 32 (B), (1) both proteins encoded by chT201 DNA and chT2 cDNA respectively have a C-terminal α -amidating enzyme activity; and

(2) The activity is provided not only by a protein having a full length primary amino acid sequence encoded by chT201 cDNA or chT2 cDNA, but also by a shortened protein having a portion of an amino acid sequence enclosed by the chT201 cDNA or chT2 cDNA.

In practice, the expression vector such as pKDchT201-Bcl, pKDchT2-Bcl, pKDchT201-Pst, pKDcht2-Pst, pKDchT201-S/B, and pKDchT201-Sal coding for a C-terminal truncated protein derivative, when introduced to COS1 cells, exhibited a significant C-terminal α -amidating enzyme activity in a culture supernatant.

The pKDchT201-Sph, however, which encodes an amino acid sequence from the first Met to 323th Ala in the amino acid sequence shown in Fig. 1, did not exhibit the desired enzyme activity. Accordingly, it was found that a protein having an amino acid sequence which terminates at or before the 323th Ala, is insufficient, but a protein having an amino acid sequence which extends to or beyond the 378th Lys has a size sufficient to exhibit the C-terminal α -amidating enzyme activity.

According to the present invention, a large amount of C-terminal α -amidating enzyme can be produced using cloned cDNA derived from human thyroid origin. Accordingly, C-terminal amidated peptides or proteins, which are useful and important in the pharmaceutical or medical field and other industrial fields, can be economically produced.

Reference under Rule 13-2 of the Budapest treaty to deposited microorganisms:

Depository Institute: Fermentation Research Institute, Agency of Industrial Science and Technology;

2024279

Address: 1-3, Tsukuba-shi 1-chome,
Ibaraki-ken, 301 Japan;

Identification of deposited
microorganisms, deposition numbers and deposition date;

1. E. coli DH1/PXA799 FERM BP-1586
December 3, 1987.
2. E. coli SBM302 FERM BP-2237
January 11, 1989.
3. E. coli SBM303 FERM BP-2238
January 11, 1989.
4. E. coli SBM304 FERM BP-2239
January 11, 1989.
5. E. coli SBM305 FERM BP-2240
January 11, 1989.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. An enzyme which converts a protein or peptide represented by the formula (I) :



wherein Gly represents glycine present at a C-terminus, X represents any amino acid to be α -amidated, and R represents a remaining portion of the protein or peptide, to an α -amidated protein or peptide represented by the formula (II):



wherein X-NH₂ represents C-terminal α -amidated amino acid, and R represents a remaining portion of the protein or peptide, wherein said enzyme comprises at least the amino acid sequence starting with position 37 and ending at position 378 of the amino acid sequence as illustrated in Fig. 1.

2. DNA coding for the enzyme of claim 1.
3. A vector comprising the DNA of claim 2.
4. A transformant containing a vector according to claim 3.
5. A transformant according to claim 4, which is a prokaryotic cell or eukaryotic cell.
6. A process for production of an enzyme having a C-terminal α -amidating activity, comprising the steps of culturing cells transformed with an expression vector comprising a DNA according to claim 2, and recovering the enzyme from the culture.

7. A process for production of a peptide or protein represented by a formula (II):



wherein X- NH₂ represents a C-terminal α-amidated amino acid and R represents a remaining portion of the peptide or protein, comprising a step of:

treating a peptide or protein represented by the formula (I):



wherein X represents any amino acid residue to be α-amidated, Gly represents a C-terminal glycine residue, and R represents a remaining portion of the peptide or protein, with an enzyme having a C-terminal α-amidating activity according to claim 1.

Fig 1-1

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5'	GGTGTGTGCCCGCCGAGG	19
	ACGCCCCGCGGTGCCCGGGCCATGAAGTAGCGGCTGCTGGCGGCGCCGCTGCCCAACCGCC	79
	AGCCCCAGCCCCGCGCTGCGCTGCCCGGTCTCTCCCGGCGGGGTGCTATCGGCGTGGAC	139
	ATGGCTGGCCCGTCCCTAGCCTGCTAGTTCTCCTTGTTTTTCCAAGCAGCTGTTTGGCT	199
	MetAlaGlyArgValProSerLeuLeuValLeuLeuValPheProSerSerCysLeuAla	20
	TTCCGAAGCCCACTTTCTGTCTTTAAGAGGTTTAAAGAACTACCAGACCATTTTCCAAT	259
	PheArgSerProLeuSerValPheLysArgPheLysGluThrThrArgProPheSerAsn	40
	GAATGTCTTGGTACCACCAGACCCGTAGTTCCTATTGATTCATCAGATTTTGCATTGGAT	319
	GluCysLeuGlyThrThrArgProValValProIleAspSerSerAspPheAlaLeuAsp	60
	ATTCGCATGCCTGGGGTTACACCTAAACAGTCCGATACATACTTCTGCATGTCTATGCCA	379
	IleArgMetProGlyValThrProLysGlnSerAspThrTyrPheCysMetSerMetArg	80
	ATACCAGTGGATGAGGAAGCCTTCGTGATTGACTTCAAGCCTCGAGCCAGCATGGATACT	439
	IleProValAspGluGluAlaPheValIleAspPheLysProArgAlaSerMetAspThr	100
	GTCCATCACATGTTACTTTTTGGATGCAATATGCCTTCATCCACTGGAAGTTACTGGTTT	499
	ValHisHisMetLeuLeuPheGlyCysAsnMetProSerSerThrGlySerTyrTrpPhe	120
	TGTGATGAAGGAACCTGTACAGATAAAGCCAATATTCTGTATGCCTGGGCGAGAAATGCT	559
	CysAspGluGlyThrCysThrAspLysAlaAsnIleLeuTyrAlaTrpAlaArgAsnAla	140
	CCCCCTACCCGGCTCCCCAAAGGTGTTGATTGAGAGTTGGAGGAGAGACTGGAAGTAAA	619
	ProProThrArgLeuProLysGlyValGlyPheArgValGlyGlyGluThrGlySerLys	160
	TACTTTGTACTIONACAGCTACACTATGCGGATATTAGTGCTTTTAGAGATAATAACAAGGAC	679
	TyrPheValLeuGlnValHisTyrGlyAspIleSerAlaPheArgAspAsnAsnLysAsp	180
	TGTTCTGGTGTGTCTTACACCTCACACGTCTGCCACAGCCTTTAATTGCTGGCATGTAC	739
	CysSerGlyValSerLeuHisLeuThrArgLeuProGlnProLeuIleAlaGlyMetTyr	200
	CTTATGATGTCTGTTGACACTGTTATCCCAGCAGGAGAAAAAGTGGTGAATTCTGACATT	799
	LeuMetMetSerValAspThrValIleProAlaGlyGluLysValValAsnSerAspIle	220
	TCATCCCATTATAAAAAATTATCCAATGCATGTCTTTGCCTATAGAGTTCACACTCACCAT	859
	SerCysHisTyrLysAsnTyrProMetHisValPheAlaTyrArgValHisThrHisHis	240
	TTAGGTAACGTAGTAAGTGCATACAGAGTAAGAAATGGACAGTGGACACTGATTGGACGG	919
	LeuGlyLysValValSerGlyTyrArgValArgAsnGlyGlnTrpThrLeuIleGlyArg	260

Fig. 1-2

PATENT AGENT

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CAGAGCCCTCAGCTGCCACAGGCTTTCTACCCTGTGGGCATCCAGTTGATGTAAGTTT 979
GlnSerProGlnLeuProGlnAlaPheTyrProValGlyHisProValAspValSerPhe
280
GGTGACCTACTGGCTGCAAGATGTGTATTCACTGGTGAAGGAAGGACAGAAGCCACACAC 1039
GlyAspLeuLeuAlaAlaArgCysValPheThrGlyGluGlyArgThrGluAlaThrHis
300
ATTGGTGGCACGTCTAGTGATGAAATGTGCAACTTATACATTATGTATTACATGGAAGCC 1099
IleGlyGlyThrSerSerAspGluMetCysAsnLeuTyrIleMetTyrTyrMetGluAla
320
AAGCATGCAGTTTCTTTCATGACCTGTACCCAGAATGTAGCTCCAGATATGTTTCAGAACC 1159
LysHisAlaValSerPheMetThrCysThrGlnAsnValAlaProAspMetPheArgThr
340
ATACCACCAGAGGCCAACATTCCAATTCCCGTGAAGTCTGATATGTTTATGATGCATGAA 1219
IleProProGluAlaAsnIleProIleProValLysSerAspMetValMetMetHisGlu
360
CATCATAAAGAAACAGAATATAAAGATAAGATTCTTTACTACAGCAGCCAAAACGAGAA 1279
HisHisLysGluThrGluTyrLysAspLysIleProLeuLeuGlnGlnProLysArgGlu
380
GAAGAAGAAGTGTTAGACCAGGGTGATTTCTATTCACTACTTTCCAAGCTGCTAGGAGAA 1339
GluGluGluValLeuAspGlnGlyAspPheTyrSerLeuLeuSerLysLeuLeuGlyGlu
400
AGGGAAGATGTTGTTTCATGTGCACAAATATAATCCTACAGAAAAGGCAGAATCAGAGTCA 1399
ArgGluAspValValHisValHisLysTyrAsnProThrGluLysAlaGluSerGluSer
420
GACCTGGTAGCTGAGATTGCAAATGTAGTCCAAAAAAGGATCTTGGTGGATCTGATGCC 1459
AspLeuValAlaGluIleAlaAsnValValGlnLysLysAspLeuGlyArgSerAspAla
440
AGAGAGGGTGCAGAACATGAGAGGGGTAATGCTATTCTTGTCAGAGACAGAATTCACAAA 1519
ArgGluGlyAlaGluHisGluArgGlyAsnAlaIleLeuValArgAspArgIleHisLys
460
TTCCACAGACTAGTATCTACCTTGAGGCCACCAGAGAGCAGAGTTTTCTCATTACAGCAG 1579
PheHisArgLeuValSerThrLeuArgProProGluSerArgValPheSerLeuGlnGln
480
CCCCACCTGGTGAAGGCACCTGGGAACCAGAACACACAGGAGATTTCCACATGGAAGAG 1639
ProProProGlyGluGlyThrTrpGluProGluHisThrGlyAspPheHisMetGluGlu
500
GCACTGGATTGGCCTGGAGTATACTTGTTACCAGGCCAGGTTTCTGGCGTGGCTCTAGAC 1699
AlaLeuAspTrpProGlyValTyrLeuLeuProGlyGlnValSerGlyValAlaLeuAsp
520
CCTAAGAATAACCTGGTGATTTTCCACAGAGGTGACCATGTCTGGGATGGAAGTCTGTT 1759
ProLysAsnAsnLeuValIlePheHisArgGlyAspHisValTrpAspGlyAsnSerPhe
540
GACAGCAAGTTTGTTTACCAGCAAATAGGACTCGGACCAATTGAAGAAGACACTATTCTT 1819
AspSerLysPheValTyrGlnGlnIleGlyLeuGlyProIleGluGluAspThrIleLeu
560

Fig. 1-3

GTCATAGATCCAAATAATGCTGCAGTACTCCAGTCCAGTGGAAAAATCTGTTTTACTTG 1879
ValIleAspProAsnAsnAlaAlaValLeuGlnSerSerGlyLysAsnLeuPheTyrLeu
580
CCACATGGCTTGAGTATAGATAAAGATGGGAATTATTGGGTACAGACGTGGCTCTCCAT 1939
ProHisGlyLeuSerIleAspLysAspGlyAsnTyrTrpValThrAspValAlaLeuHis
600
CAGGTGTTCAAACCTGGATCCAAACAATAAAGAAGGCCCTGTATTAATCCTGGGAAGGAGC 1999
GlnValPheLysLeuAspProAsnAsnLysGluGlyProValLeuIleLeuGlyArgSer
620
ATGCAACCAGGCAGTGACCAGAATCACTTCTGTCAACCCACTGATGTGGCTGTGGATCCA 2059
MetGlnProGlySerAspGlnAsnHisPheCysGlnProThrAspValAlaValAspPro
640
GGCACTGGAGCCATTTATGTATCAGATGGTTACTGCAACAGCAGGATTGTGCAGTTTTCA 2119
GlyThrGlyAlaIleTyrValSerAspGlyTyrCysAsnSerArgIleValGlnPheSer
660
CCAAGTGGAAAGTTCATCACACAGTGGGGAGAAGAGTCTTCAGGGAGCAGTCCTCTGCCA 2179
ProSerGlyLysPheIleThrGlnTrpGlyGluGluSerSerGlySerSerProLeuPro
680
GGCCAGTTCAGTGTTCCTCACAGCTTGGCTCTTGTGCCTCTTTTGGGCCAATTATGTGTG 2239
GlyGlnPheThrValProHisSerLeuAlaLeuValProLeuLeuGlyGlnLeuCysVal
700
GCAGACCGGGAAAATGGTCCGATCCAGTGTTTTAAACTGACACCAAAGAATTTGTGAGA 2299
AlaAspArgGluAsnGlyArgIleGlnCysPheLysThrAspThrLysGluPheValArg
720
GAGATTAAGCATTATCATTTTGAAGAAATGTATTTGCAATTTTCATATATACCAGGCTTG 2359
GluIleLysHisSerSerPheGlyArgAsnValPheAlaIleSerTyrIleProGlyLeu
740
CTCTTTGCAGTGAATGGGAAGCCTCATTTTGGGGACCAAGAACCTGTACAAGGATTTGTG 2419
LeuPheAlaValAsnGlyLysProHisPheGlyAspGlnGluProValGlnGlyPheVal
760
ATGAACTTTTCCAATGGGAAATTATCGACATCTTCAAGCCAGTGGCAAGCACTTTGAT 2479
MetAsnPheSerAsnGlyGluIleIleAspIlePheLysProValArgLysHisPheAsp
780
ATGCCTCATGATATTGTTGCATCTGAAGATGGGACTGTGTACATTGGAGATGCTCATACC 2539
MetProHisAspIleValAlaSerGluAspGlyThrValTyrIleGlyAspAlaHisThr
800
AACACCGTGTGGAAGTTCACCTTGACTGAGAAATTGGAACATCGATCAGTTAAAAAGGCT 2599
AsnThrValTrpLysPheThrLeuThrGluLysLeuGluHisArgSerValLysLysAla
820
GCCATTGAGGTCCAGGAAATCAAAGAAGCCGAGGCAGTTGTTGAAACCAAAATGGAGAAC 2659
GlyIleGluValGlnGluIleLysGluAlaGluAlaValValGluThrLysMetGluAsn
840
AAACCCACCTCCTCAGAATTGCAGAAGATGCAAGAGAAACAGAACTGATCAAAGAGCCA 2719
LysProThrSerSerGluLeuGlnLysMetGlnGluLysGlnLysLeuIleLysGluPro
860

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Fig. 1-4

GGCTCGGAGTGCCTGTTGTTCTCATTACAACCCTTCTGGTTATTCCGGTGGTTGTCCTG	2779
GlySerGlyValProValValLeuIleThrThrLeuLeuValIleProValValValLeu	
880	
CTGGCCATTGCCATATTTATTCGGTGCAAAAAATCAAGGCCCTTTGGAGCAGATTCTGAA	2839
LeuAlaIleAlaIlePheIleArgTrpLysLysSerArgAlaPheGlyAlaAspSerGlu	
900	
CACAACTCGAGACGAGTTCAGGAAGAGTACTGGCAAGATTTAGAGGAAAAGGCAAGTGA	2899
HisLysLeuGluThrSerSerGlyArgValLeuGlyArgPheArgGlyLysGlySerGly	
920	
GGCTTAAACCTTGGAATTTCTTTGCAAGCCGTAAGGGCTACAGTCGAAAAGGGTTTGAC	2959
GlyLeuAsnLeuGlyAsnPhePheAlaSerArgLysGlyTyrSerArgLysGlyPheAsp	
940	
CGGCTTAGCACTGAGGGCAGTGACCAAGAGAAAGAGGATGATGGAAGTGAATCAGAAGAG	3019
ArgLeuSerThrGluGlySerAspGlnGluLysGluAspAspGlySerGluSerGluGlu	
960	
GAGTATTCAGCACCTCTGCCTCGGCTCGCACCTTCCTCCTCCTGAAAACCAAGCTTTGAT	3079
GluTyrSerAlaProLeuProAlaLeuAlaProSerSerSer***	
974	
TTAGATTGAGTAAGATTTACCCAGAATGTCAGATTCTTTCCCTTTAGCACGTTTAAAGT	3139
TCTGTGTATTTAATTGTAACCTGTACTAGTCTGTGTGGGACTGTACACACTTTATTACT	3199
TGGTTTTGGTTAAGTTGGCTTCTGTTTCTAGTTGAGGAGTTTCCTAAAAGTTCATAACAG	3259
TGCCATTGTCTTTATATGAACATAGACTAGAGAAACCGTCCTCTTTTTCCATCATAATTC	3319
TAATCTAACAATGGAAGATTTGCCCATTTACACTTTTGAGACTTTTTGGTGGATGTAAAT	3379
AACCCCATTCCTTTGCTTGAACACAGTATTTTCCCAATAGCACTTTCATTGCCAGTGTCTT	3439
TCTTTGGTGCCTTTCTGTTTCAGCATTCTTAGCCTGTGGCAGTAAAGAGAAACTTTGTGC	3499
TACATGACGACAAAAGCTGCTAAATCTCCTATTTTTTTAAATCACTAACATTATATTGCA	3559
ATGAACGAAATAAAAAAGTCTCTATTTAAATTCTTTTTTAAATTTCTTCAGTTGGTGTG	3619
TTTTTGGGATGTCTTATTTTTAGATGGTTACACTGTTAGAACACTATTTTCAGAATCTGA	3679
ATGTAATTT 3'	3688

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Fig. 2-1

5' T 1

GACTTCAAGCCTCGAGCCAGCATGGATACTGTCCATCACATGTTACTTTTTGGATGCAAT 61
AspPheLysProArgAlaSerMetAspThrValHisHisMetLeuLeuPheGlyCysAsn
20
ATGCCTTCATCCACTGGAAGTTACTGGTTTTGTGATGAAGGAACCTGTACAGATAAAGCC 121
MetProSerSerThrGlySerTyrTrpPheCysAspGluGlyThrCysThrAspLysAla
40
AATATTCTGTATGCCTGGGCGAGAAATGCTCCCCCTACCCGGCTCCCCAAAGGTGTTGGA 181
AsnIleLeuTyrAlaTrpAlaArgAsnAlaProProThrArgLeuProLysGlyValGly
60
TTCAGAGTTGCAGGAGAGACTGGAAGTAAATACTTTGTACTACAGGTACACTATGCGGAT 241
PheArgValGlyGlyGluThrGlySerLysTyrPheValLeuGlnValHisTyrGlyAsp
80
ATTAGTGCTTTTAGAGATAATAACAAGGACTGTTCTGGTGTGTCCTTACACCTCACACGT 301
IleSerAlaPheArgAspAsnAsnLysAspCysSerGlyValSerLeuHisLeuThrArg
100
CTGCCACAGCCTTTAATTGCTGGCATGTACCTTATGATGTCTGTTGACACTGTTATCCCA 361
LeuProGlnProLeuIleAlaGlyMetTyrLeuMetMetSerValAspThrValIlePro
120
GCAGGAGAAAAAGTGGTGAATTCTGACATTTTCATGCCATTATAAAAAATTATCCAATGCAT 421
AlaGlyGluLysValValAsnSerAspIleSerCysHisTyrLysAsnTyrProMetHis
140
GTCTTTGCCTATAGAGTTCACACTCACCATTTAGGTAAGGTAGTAAGTGGATACAGAGTA 481
ValPheAlaTyrArgValHisThrHisHisLeuGlyLysValValSerGlyTyrArgVal
160
AGAAATGGACAGTGGACACTGATTGGACGGCAGAGCCCTCAGCTGCCACAGGCTTTCTAC 541
ArgAsnGlyGlnTrpThrLeuIleGlyArgGlnSerProGlnLeuProGlnAlaPheTyr
180
CCTGTGGGGCATCCAGTTGATGTAAGTTTTGGTGACCTACTGGCTGCAAGATGTGTATTC 601
ProValGlyHisProValAspValSerPheGlyAspLeuLeuAlaAlaArgCysValPhe
200
ACTGGTGAAGGAAGGACAGAAGCCACACACATTGGTGGCACCTCTAGTGATGAAATGTGC 661
ThrGlyGluGlyArgThrGluAlaThrHisIleGlyGlyThrSerSerAspGluMetCys
220
AACTTATACATTATGTATTACATGGAAGCCAAGCATGCAGTTTCTTTCATGACCTGTACC 721
AsnLeuTyrIleMetTyrTyrMetGluAlaLysHisAlaValSerPheMetThrCysThr
240
CAGAATGTAGCTCCAGATATGTTTCAGAACCATACCACCAGAGGCCAACATTCCAATTCCC 781
GlnAsnValAlaProAspMetPheArgThrIleProProGluAlaAsnIleProIlePro
260
GTGAAGTCTGATATGGTTATGATGCATGAACATCATAAAGAAACAGAATATAAAGATAAG 841
ValLysSerAspMetValMetMetHisGluHisHisLysGluThrGluTyrLysAspLys
280

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Fig. 2-2

ATTCCTTTACTACAGCAGCCAAAACGAGAAGAAGAAGTGTAGACCAGGATTTCCAC 901
IleProLeuLeuGlnGlnProLysArgGluGluGluGluValLeuAspGlnAspPheHis
300
ATGGAAGAGGCACTGGATTGGCCTGGAGTATACTTGTTACCAGGCCAGGTTTCTGGCGTG 961
MetGluGluAlaLeuAspTrpProGlyValTyrLeuLeuProGlyGlnValSerGlyVal
320
GCTCTAGACCCTAAGAATAACCTGGTGATTTTCCACAGAGGTGACCATGTCTGGGATGGA 1021
AlaLeuAspProLysAsnAsnLeuValIlePheHisArgGlyAspHisValTrpAspGly
340
AACTCGTTTGACAGCAAGTTTGTGTACCAGCAAATAGGACTCGGACCAATTGAAGAAGAC 1081
AsnSerPheAspSerLysPheValTyrGlnGlnIleGlyLeuGlyProIleGluGluAsp
360
ACTATTCTTGTATAGATCCAAATAATGCTGCAGTACTCCAGTCCAGTGAAAAAATCTG 1141
ThrIleLeuValIleAspProAsnAsnAlaAlaValLeuGlnSerSerGlyLysAsnLeu
380
TTTTACTTGCCACATGGCTTGAGTATAGATAAAGATGGGAATTATTGGGTCACAGACGTG 1201
PheTyrLeuProHisGlyLeuSerIleAspLysAspGlyAsnTyrTrpValThrAspVal
400
GCTCTCCATCAGGTGTTCAAACCTGGATCCAAACAATAAAGAAGGCCCTGTATTAATCCTG 1261
AlaLeuHisGlnValPheLysLeuAspProAsnAsnLysGluGlyProValLeuIleLeu
420
GGAAGGAGCATGCAACCAGGCAGTGACCAGAATCACTTCTGTCAACCCACTGATGTGGCT 1321
GlyArgSerMetGlnProGlySerAspGlnAsnHisPheCysGlnProThrAspValAla
440
GTGGATCCAGGCACTGGAGCCATTTATGTATCAGATGGTTACTGCAACAGCAGGATTGTG 1381
ValAspProGlyThrGlyAlaIleTyrValSerAspGlyTyrCysAsnSerArgIleVal
460
CAGTTTTACCAAGTGGAAAGTTCATCACACAGTGGGGAGAAGAGTCTTCAGGGAGCAGT 1441
GlnPheSerProSerGlyLysPheIleThrGlnTrpGlyGluGluSerSerGlySerSer
480
CCTCTGCCAGGCCAGTTCAGTGTTCCTCACAGCTTGGCTCTTGTGCCTCTTTTGGGCCAA 1501
ProLeuProGlyGlnPheThrValProHisSerLeuAlaLeuValProLeuLeuGlyGln
500
TTATGTGTGGCAGACCGGGAAAATGGTCCGATCCAGTGTTTTAAACTGACACCAAAGAA 1561
LeuCysValAlaAspArgGluAsnGlyArgIleGlnCysPheLysThrAspThrLysGlu
520
TTTGTGAGAGAGATTAAAGCATTTCATCATTTCGAAGAAATGTATTTGCAATTTTCATATATA 1621
PheValArgGluIleLysHisSerSerPheGlyArgAsnValPheAlaIleSerTyrIle
540
CCAGGCTTGCTCTTTGCAGTGAATGGGAAGCCTCATTTTGGGGACCAAGAACCTGTACAA 1681
ProGlyLeuLeuPheAlaValAsnGlyLysProHisPheGlyAspGlnGluProValGln
560
GGATTTGTGATGAACTTTTCCAATGGGGAAATTATAGACATCTTCAAGCCAGTCCCAAG 1741
GlyPheValMetAsnPheSerAsnGlyGluIleIleAspIlePheLysProValArgLys
580

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Fig. 2-3

CACTTTGATATGCCTCATGATATTGTTGCATCTGAAGATGGGACTGTGTACATTGGAGAT 1801
HisPheAspMetProHisAspIleValAlaSerGluAspGlyThrValTyrIleGlyAsp
600
GCTCATACCAACACCGTGTGGAAGTTCACCTTGACTGAGAAATTGGAACATCGATCAGTT 1861
AlaHisThrAsnThrValTrpLysPheThrLeuThrGluLysLeuGluHisArgSerVal
620
AAAAAGGCTGCCATTGAGGTCCAGGAAATCAAAGAAGCCGAGGCAGTTGTTGAAACCAA 1921
LysLysAlaGlyIleGluValGlnGluIleLysGluAlaGluAlaValValGluThrLys
640
ATGGAGAACAAACCCACCTCCTCAGAATTGCAGAAGATGCAAGAGAAACAGAAACTGATC 1981
MetGluAsnLysProThrSerSerGluLeuGlnLysMetGlnGluLysGlnLysLeuIle
660
AAAGAGCCAGGCTCGGGAGTGCCTGTTGTTCTCATTACAACCCTTCTGGTTATTCCGGTG 2041
LysGluProGlySerGlyValProValValLeuIleThrThrLeuLeuValIleProVal
680
GTTGTCCTGCTGGCCATTGCCATATTTATTCCGGTGGAAAAAATCAAGGGCCTTTGGAGAT 2101
ValValLeuLeuAlaIleAlaIlePheIleArgTrpLysLysSerArgAlaPheGlyAsp
700
TCTGAACACAAACTCGAGACGAGTTCAGGAAGAGTACTGGGAAGATTTAGAGGAAAGGGA 2161
SerGluHisLysLeuGluThrSerSerGlyArgValLeuGlyArgPheArgGlyLysGly
720
AGTGGAGGCTTAAACCTTGCTAATTTCTTTGCAAGCCGTAAGGGCTACAGTCGAAAAGGG 2221
SerGlyGlyLeuAsnLeuGlyAsnPhePheAlaSerArgLysGlyTyrSerArgLysGly
740
TTTGACCGGCTTAGCACTCAGGGCAGTGACCAAGAGAAAGAGGATGATGGAAGTGAATCA 2281
PheAspArgLeuSerThrGluGlySerAspGlnGluLysGluAspAspGlySerGluSer
760
GAAGAGGAGTATTACGACCTCTGCCTCGGCTCGCACCTTCCTCCTCCTGAAAACCAAGC 2341
GluGluGluTyrSerAlaProLeuProAlaLeuAlaProSerSerSer***
776
TTTGATTTAGATTGAGTAAGATTTACCCAGAATGTCAGATTCCCTTCCCTTTAGCACGTT 2401
TAAAGTTCTGTGATTTAATTGTAAACTGTACTAGTCTGTGTGGGACTGTACACACTTTA 2461
TTTACTTCGTTTTGGTTAAGTTGGCTTCTGTTTCTAGTTGAGGAGTTTCCTAAAAGTTCA 2521
TAACAGTCCCATTGTCTTTATATGAACATAGACTAGAGAAACCGTCCTCTTTTTCCATCA 2581
TAATTCTAATCTAACAATGGAAGATTTGCCCATTTACACTTTTGAGACTTTTTGGTGGAT 2641
GTAAATAACCCCATTTCTTTGCTTGAACACAGTATTTCCCAATAGCACTTTCATTGCCAG 2701
TGTCTTTCTTTGGTGCCTTTCTGTTGAGCATTCTTAGCCTGTGGCAATAAAGAGAAACT 2761
TTGTGCTACATGACGACAAAGCTGCTAAATCTCCTATTTTTTAAATCACTAACATTAT 2821
ATTGCAATGAAGGAAATAAAAAAGTCTCTATTTAAAAA 3' 2863

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Fig. 3-1

PATENT AGENTS

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5' CAGCCCCGCGCTGCGCTGCCCCGTCTCTCCCGCGCGGTCTATCGGCGTGGAC 55
ATGGCTGGCCGCGTCCCTAGCCTGCTAGTTCTCCTTGTTTTTCCAAGCAGCTGTTTGGCT 115
MetAlaGlyArgValProSerLeuLeuValLeuLeuValPheProSerSerCysLeuAla 20
TTCCGAAGCCCACTTTCTGTCTTTAAGAGGTTTAAAGAACTACCAGACCATTTTCCAAT 175
PheArgSerProLeuSerValPheLysArgPheLysGluThrThrArgProPheSerAsn 40
GAATGTCTTGGTACCACCAGACCCGTAGTTCCTATTGATTCATCAGATTTTGCATTGGAT 235
GluCysLeuGlyThrThrArgProValValProlleAspSerSerAspPheAlaLeuAsp 60
ATTGCGATGCCTGGGGTTACACCTAAACAGTCCGATACATACTTCTGCATGTCTATGCCA 295
IleArgMetProGlyValThrProLysGlnSerAspThrTyrPheCysMetSerMetArg 80
ATACCAGTGGATGAGGAAGCCTTCGTGATTGACTTCAAGCCTCGAGCCAGCATGGATACT 355
IleProValAspGluGluAlaPheValIleAspPheLysProArgAlaSerMetAspThr 100
GTCCATCACATGTTACTTTTTGGATGCAATATGCCTTTCATCCACTGGAAGTTACTGGTTT 415
ValHisHisMetLeuLeuPheGlyCysAsnMetProSerSerThrGlySerTyrTrpPhe 120
TGTGATGAAGGAACCTGTACAGATAAAGCCAATATTCTGTATGCCTGGCCGAGAAATCCT 475
CysAspGluGlyThrCysThrAspLysAlaAsnIleLeuTyrAlaTrpAlaArgAsnAla 140
CCCCCTACCCGGCTCCCCAAAGGTGTTGGATTGAGAGTTGGAGGAGAGACTGCAAGTAAA 535
ProProThrArgLeuProLysGlyValGlyPheArgValGlyGlyGluThrGlySerLys 160
TACTTTGTAATAACAGGTACACTATGGGGATATTAGTGCTTTTAGAGATAATAACAAGGAC 595
TyrPheValLeuGlnValHisTyrGlyAspIleSerAlaPheArgAspAsnAsnLysAsp 180
TGTTCTGGTGTCTCCTTACACCTCACACGTCTGCCACAGCCTTTAATTGCTGGCATGTAC 655
CysSerGlyValSerLeuHisLeuThrArgLeuProGlnProLeuIleAlaGlyMetTyr 200
CTTATGATGTCTGTTGACACTGTTATCCCAGCAGGAGAAAAAGTGGTGAATTCTGACATT 715
LeuMetMetSerValAspThrValIleProAlaGlyGluLysValValAsnSerAspIle 220
TCATGCCATTATAAAAAATTATCCAATGCATGTCTTTGCCTATAGAGTTCACACTCACCAT 775
SerCysHisTyrLysAsnTyrProMetHisValPheAlaTyrArgValHisThrHisHis 240
TTAGGTAAGGTAGTAAGTGGATACAGAGTAAGAAATGGACAGTGGACACTGATTGGACGG 835
LeuGlyLysValValSerGlyTyrArgValArgAsnGlyGlnTrpThrLeuIleGlyArg 260
CAGAGCCCTCAGCTGCCACAGCCTTTCTACCCTGTGGGCATCCAGTTGATGTAAGTTTT 895
GlnSerProGlnLeuProGlnAlaPheTyrProValGlyHisProValAspValSerPhe 280

Fig. 3-2

GGTGACCTACTGGCTGCAAGATGTGTATTCACTGGTGAAGGAAGGACAGAAGCCACACAC 955
GlyAspLeuLeuAlaAlaArgCysValPheThrGlyGluGlyArgThrGluAlaThrHis
300
ATTGGTGGCACGTCTAGTGATGAAATGTGCAACTTATACATTATGTATTACATGGAAGCC 1015
IleGlyGlyThrSerSerAspGluMetCysAsnLeuTyrIleMetTyrTyrMetGluAla
320
AAGCATGCAGTTTCTTTCATGACCTGTACCCAGAATGTAGCTCCAGATATGTTCAGAACC 1075
LysHisAlaValSerPheMetThrCysThrGlnAsnValAlaProAspMetPheArgThr
340
ATACCACCAGAGGCCAACATTCCAATTCCTGGAAGTCTGATATGGTTATGATGCATGAA 1135
IleProProGluAlaAsnIleProIleProValLysSerAspMetValMetMetHisGlu
360
CATCATAAAGAAACAGAATATAAAGATAAGATTCCCTTACTACAGCAGCCAAAACGAGAA 1195
HisHisLysGluThrGluTyrLysAspLysIleProLeuLeuGlnGlnProLysArgGlu
380
GAAGAAGAAGTCTTAGACCAGGATTTCCACATGGAAGAGGCACTGGATTGGCCTGGAGTA 1255
GluGluGluValLeuAspGlnAspPheHisMetGluGluAlaLeuAspTrpProGlyVal
400
TACTTGTTACCAGGCCAGGTTTCTGGGTGGCTCTAGACCCTAAGAATAACCTGGTGATT 1315
TyrLeuLeuProGlyGlnValSerGlyValAlaLeuAspProLysAsnAsnLeuValIle
420
TTCCACAGAGGTGACCATGTCTGGGATGGAAACTCGTTTGACAGCAAGTTTGTGTTACCAG 1375
PheHisArgGlyAspHisValTrpAspGlyAsnSerPheAspSerLysPheValTyrGln
440
CAAATAGGACTCGGACCAATTGAAGAAGACACTATTCTTGTCATAGATCCAAATAATGCT 1435
GlnIleGlyLeuGlyProIleGluGluAspThrIleLeuValIleAspProAsnAsnAla
460
GCAGTACTCCAGTCCAGTGGAAAAAATCTGTTTTACTTCCCACATGGCTTGAGTATAGAT 1495
AlaValLeuGlnSerSerGlyLysAsnLeuPheTyrLeuProIleGlyLeuSerIleAsp
480
AAAGATGGGAATTATTGGGTCACAGACGTGGCTCTCCATCAGGTGTTCAAACCTGGATCCA 1555
LysAspGlyAsnTyrTrpValThrAspValAlaLeuHisGlnValPheLysLeuAspPro
500
AACATAAAGAAGGCCCTGTATTAATCCTGGGAAGGAGCATGCAACCAGGCAGTGACCAG 1615
AsnAsnLysGluGlyProValLeuIleLeuGlyArgSerMetGlnProGlySerAspGln
520
AATCACTTCTGTCAACCCACTGATGTGGCTGTGGATCCAGGCACTGGAGCCATTTATGTA 1675
AsnHisPheCysGlnProThrAspValAlaValAspProGlyThrGlyAlaIleTyrVal
540
TCAGATGGTTACTGCAACAGCAGGATTGTGCAGTTTTACCAAGTGGAAAGTTCATCACA 1735
SerAspGlyTyrCysAsnSerArgIleValGlnPheSerProSerGlyLysPheIleThr
560
CAGTGGGGAGAAGAGTCTTCAGGGAGCAGTCTCTGCCAGGCCAGTTCAGTGTTCCTCAC 1795
GlnTrpGlyGluGluSerSerGlySerSerProLeuProGlyGlnPheThrValProHis
580

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Fig. 3-3

AGCTTGGCTCTTGTGCCTCTTTTGGGCCAATTATGTGTGGCAGACCGGAAAAATGGTCCG 1855
SerLeuAlaLeuValProLeuLeuGlyGlnLeuCysValAlaAspArgGluAsnGlyArg
600
ATCCAGTGTTTTAAAACTGACACCAAAGAATTTGTGAGAGAGATTAAGCATTTCATCATT 1915
IleGlnCysPheLysThrAspThrLysGluPheValArgGluIleLysHisSerSerPhe
620
GGAAGAAATGTATTTGCAATTTTCATATATACCAGGCTTGCTCTTTGCAGTGAATGGGAAG 1975
GlyArgAsnValPheAlaIleSerTyrIleProGlyLeuLeuPheAlaValAsnGlyLys
640
CCTCATTTTGGGGACCAAGAACCTGTACAAGGATTTGTGATGAACTTTTCCAATGGGGAA 2035
ProHisPheGlyAspGlnGluProValGlnGlyPheValMetAsnPheSerAsnGlyGlu
660
ATTATCGACATCTTCAAGCCAGTGGCAAGCACTTTGATATGCCTCATGATATTGTTGCA 2095
IleIleAspIlePheLysProValArgLysHisPheAspMetProHisAspIleValAla
680
TCTGAAGATGGGACTGTGTACATTGGAGATGCTCATACCAACACCGTGTGGAAGTTCACC 2155
SerGluAspGlyThrValTyrIleGlyAspAlaHisThrAsnThrValTrpLysPheThr
700
TTGACTGAGAAATTGGAACATCGATCAGTTAAAAAGGCTGCCATTGAGGTCCAGGAAATC 2215
LeuThrGluLysLeuGluHisArgSerValLysLysAlaGlyIleGluValGlnGluIle
720
AAAGAAGCCGAGGCAGTTGTTGAAACCAAAATGGAGAACAACCCACCTCCTCAGAATTG 2275
LysGluAlaGluAlaValValGluThrLysMetGluAsnLysProThrSerSerGluLeu
740
CAGAAGATGCAAGAGAAACAGAAACTGATCAAAGAGCCAGGCTCGGGAGTGCCTGTTGTT 2335
GlnLysMetGlnGluLysGlnLysLeuIleLysGluProGlySerGlyValProValVal
760
CTCATTACAACCTTCTGGTTATTCCGGTGGTTGTCTGCTGGCCATTGCCATATTTATT 2395
LeuIleThrThrLeuLeuValIleProValValValLeuLeuAlaIleAlaIlePheIle
780
CGGTGCAAAAAATCAAGGGCCTTTGGAGATTCTGAACACAACTCGAGACGAGTTCAGGA 2455
ArgTrpLysLysSerArgAlaPheGlyAspSerGluHisLysLeuGluThrSerSerGly
800
AGAGTACTGGGAAGATTTAGAGCAAAGGGAAGTGGAGGCTTAAACCTTGCTAATTTCTTT 2515
ArgValLeuGlyArgPheArgGlyLysGlySerGlyGlyLeuAsnLeuGlyAsnPhePhe
820
GCAAGCCGTAAGGGCTACAGTCGAAAAAGGTTTGACCGGCTTAGCACTGAGGGCAGTGAC 2575
AlaSerArgLysGlyTyrSerArgLysGlyPheAspArgLeuSerThrGluGlySerAsp
840
CAAGAGAAAGAGGATGATCGAAGTGAATCAGAAGAGGAGTATTCAGCACCTCTCCCTGCC 2635
GlnGluLysGluAspAspGlySerGluSerGluGluGluTyrSerAlaProLeuProAla
860
CTCGCACCTTCCTCCTCCTGAAAACCAAGCTTTGATTTAGATTGAGTAAGATTTACCCAG 2695
LeuAlaProSerSerSer***

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Fig. 3- 4

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AATGTCAGATTCCITTTCCCTTTAGCACGTTTAAAGTTCTGTGTATTTAATTGTAAACTGT 2755
ACTAGTCTGTGTGGGACTGTACACACTTTATTTACTTCGTTTTGGTTAAGTTGGCTTCTG 2815
TTTCTAGTTGAGGAGTTTCCTAAAAGTTCATAACAGTGCCATTGTCTTTATATGAACATA 2875
GACTAGAGAAACCGTCCTCTTTTTCCATCATAATTCTAATCTAACAATCGAAGATTTGCC 2935
CATTTACACTTTTGAGACTTTTTGGTGGATGTAATAACCCCATTCCTTTGCTTGAACACA 2995
GTATTTTCCCAATAGCACTTTCATTGCCAGTGTCTTTCTTTGGTGCCTTTCCGTTCAGC 3055
ATTCTTAGCCTGTGGCAGTAAAGAGAACTTTGTGCTACATGACGACAAAGCTGCTAAAT 3115
CTCCTATTTTTTTAAAATCACTAACATTATATTGCAATGAAGGAAATAAAAAAGTCTCTA 3175
TTTAAATTCTTTTTTAAATTTTCTTCAGTTGGTGTGTTTTTGGGATGTCTTATTTTTAGA 3235
TGGTTACACTGTTAGAACACTATTTTCAGAATCTGAATGTAATTTGTGTAATAAAGTGTT 3295
TTCAGAGCATTAAAAAAAAA 3' 3316

Anthony Ogilvy Renault

λgt10chT201

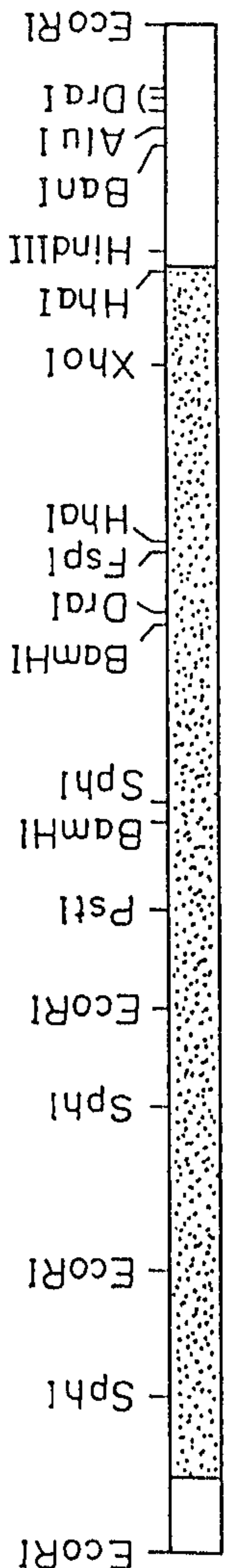


Fig. 4(a)

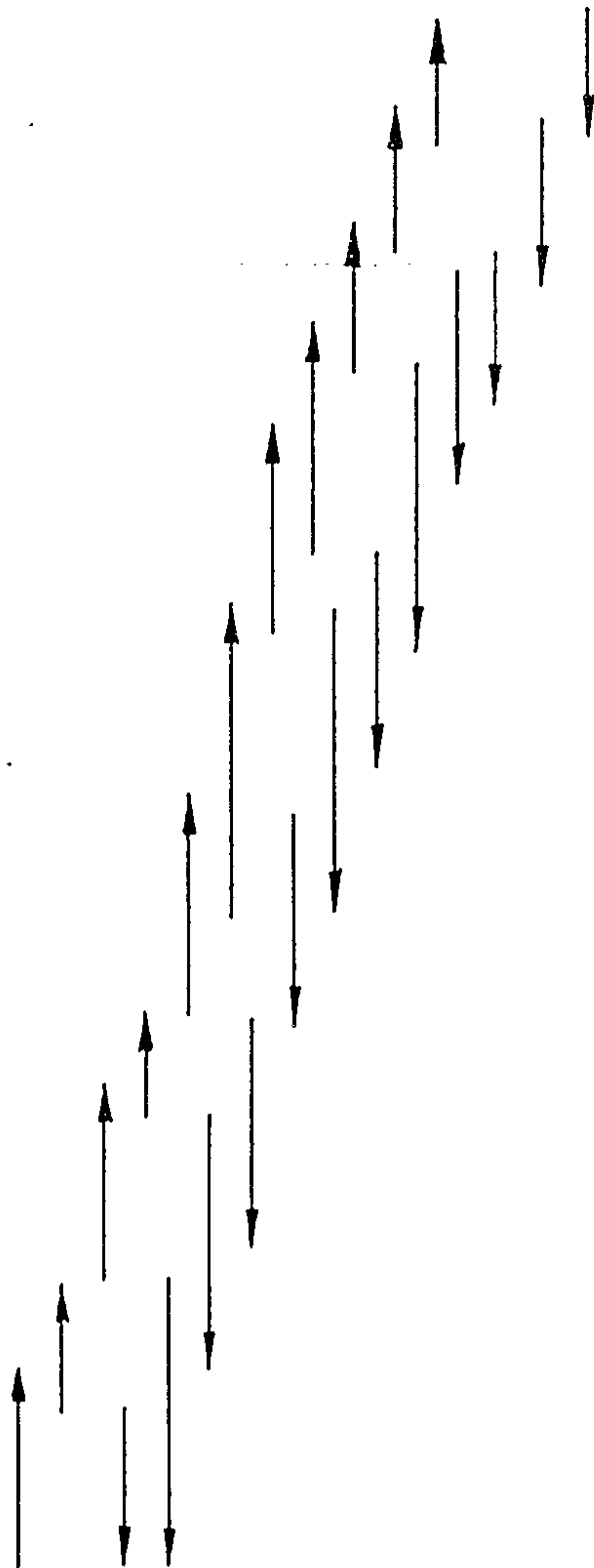
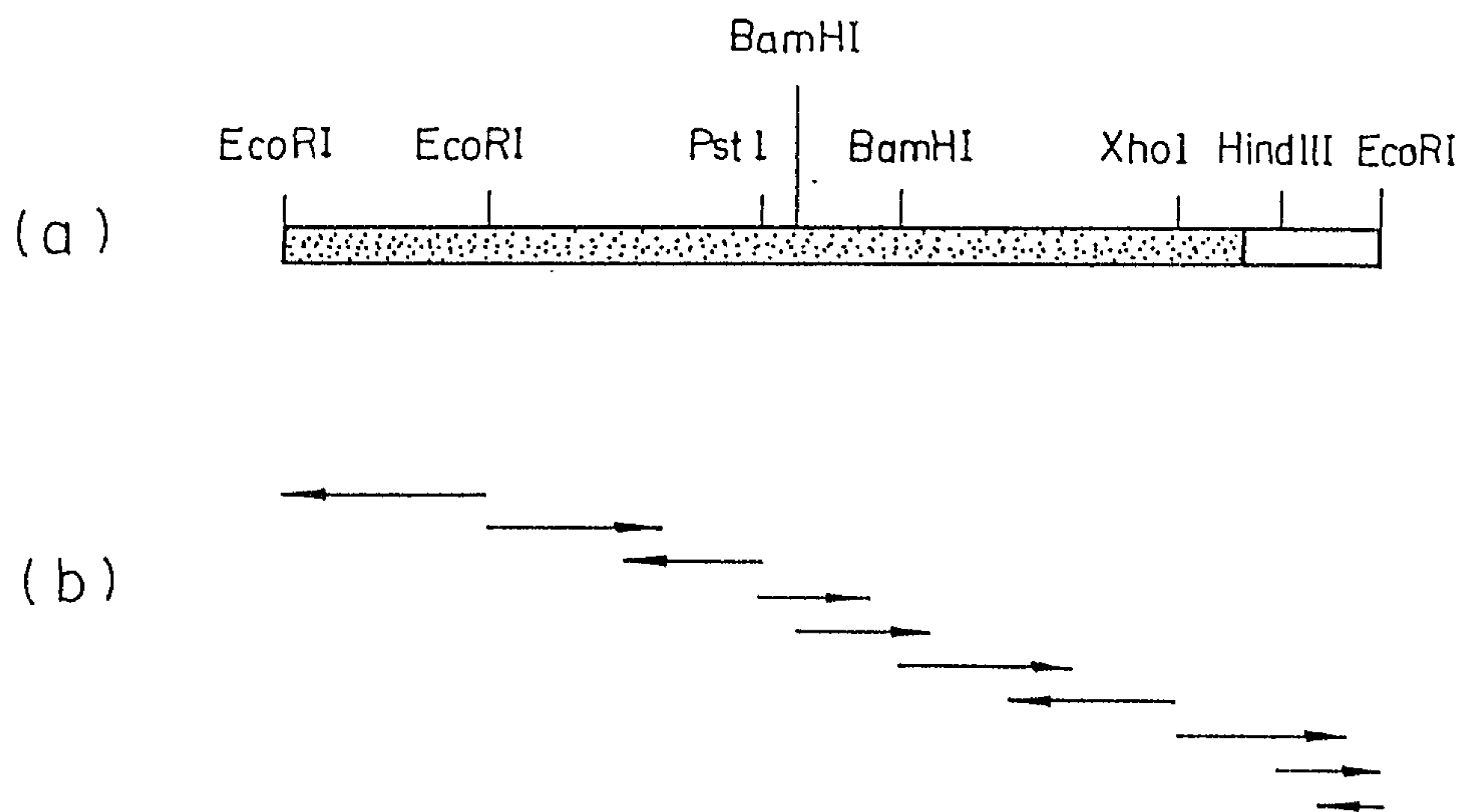


Fig. 4(b)

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Fig. 5.

λ gt10chT2



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$\lambda_{gt10chT10I}$

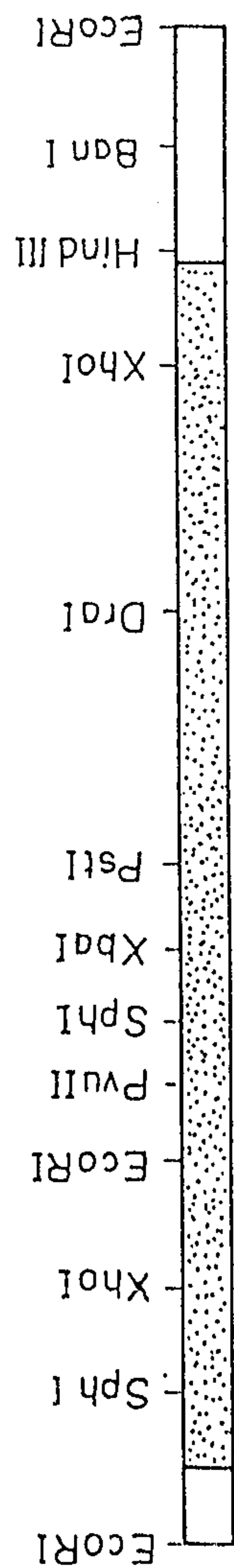


Fig. 6(a)

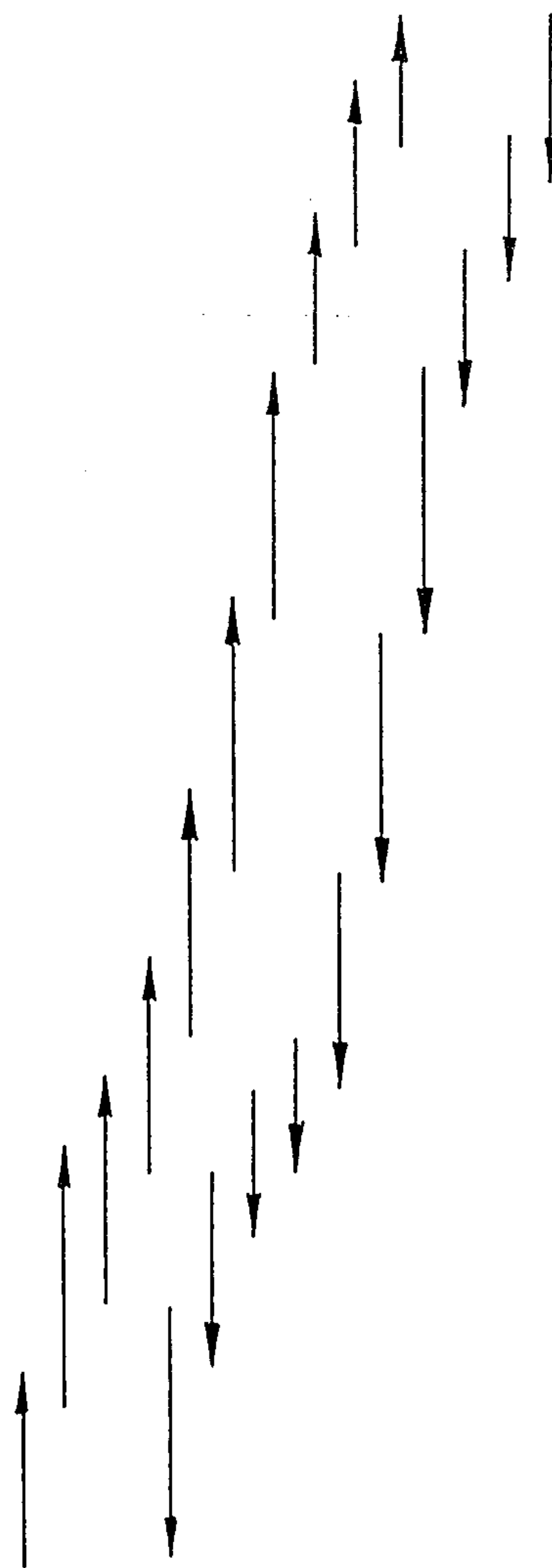


Fig. 6(b)

Fig. 7

STRUCTURE OF cDNA CLONES

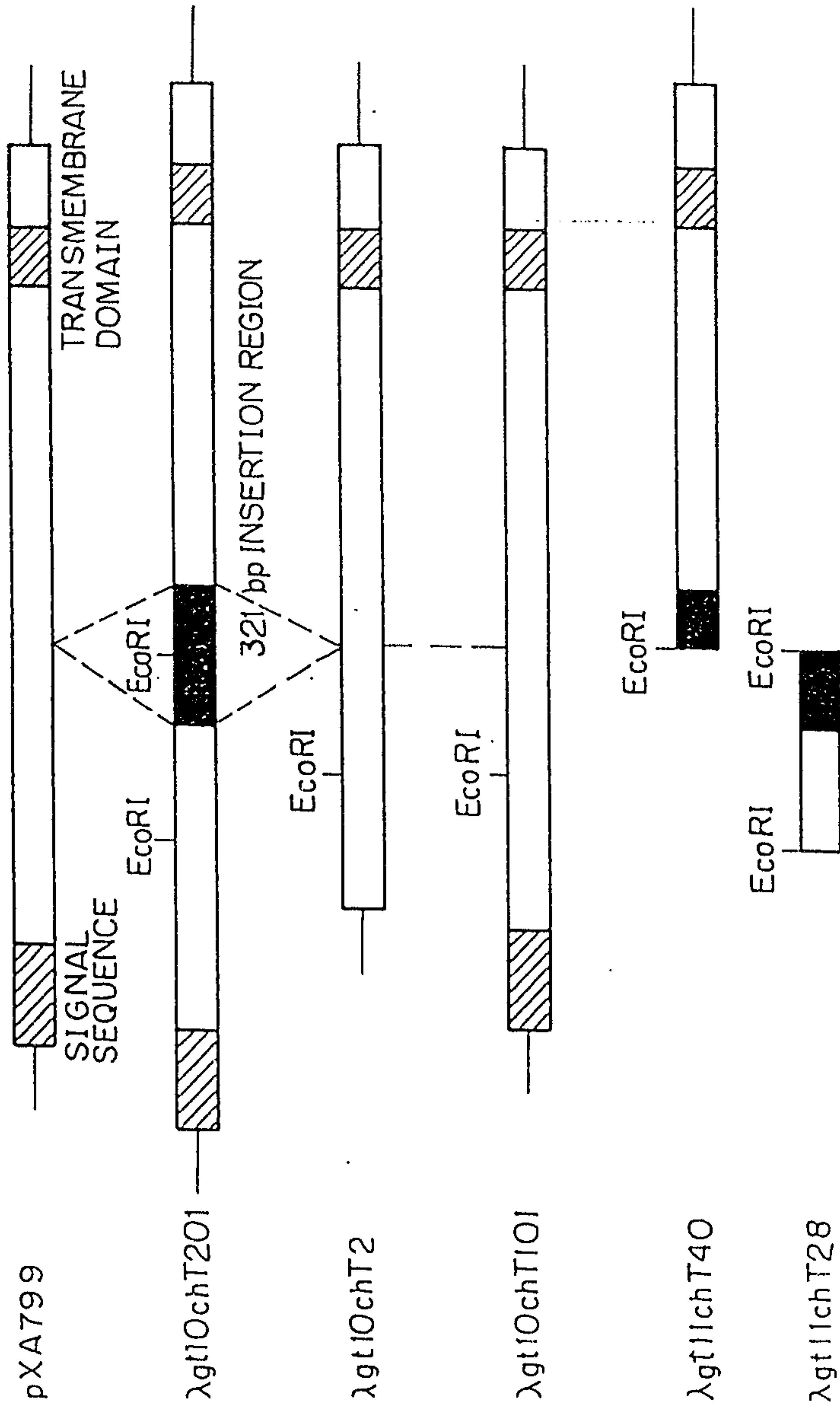
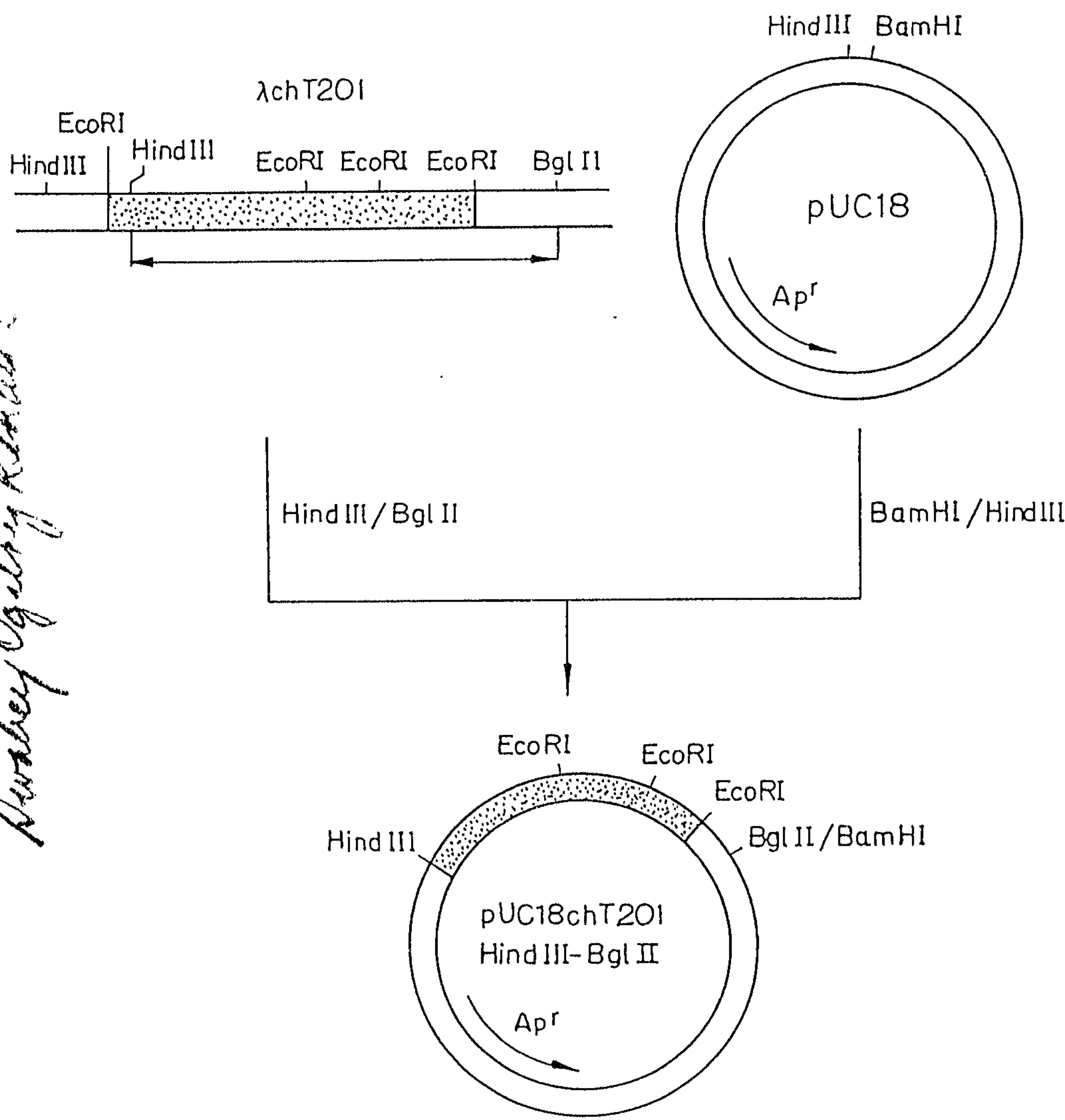


Fig. 8

pUC18chT201HindIII- Bgl II

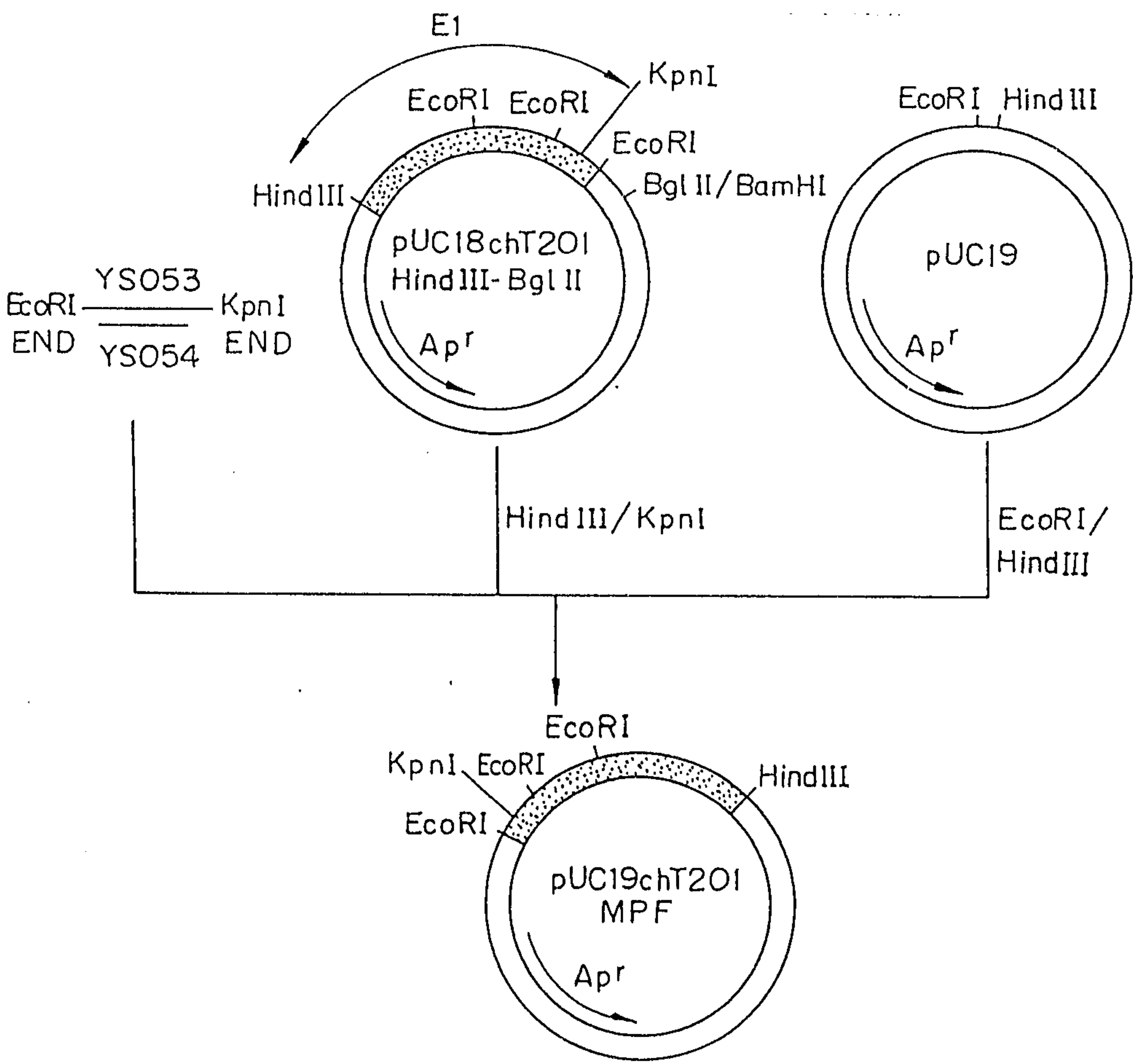


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Fig. 9

pUC19chT201MPF



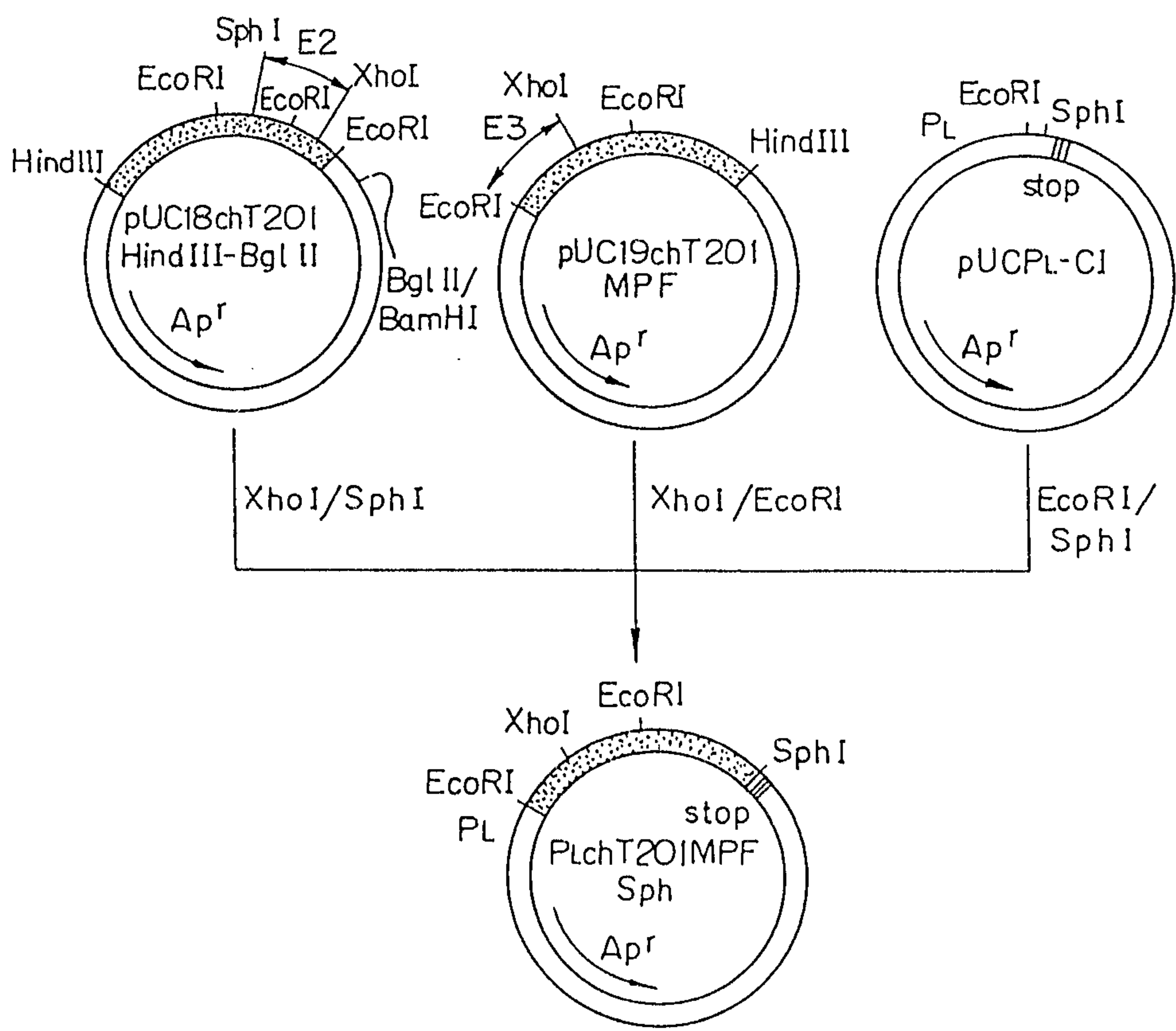
	<u>EcoRI</u>	<u>Met</u>	<u>Pro</u>	<u>Phe</u>		<u>KpnI</u>
YS053	5'	AATT	CATGCC	ATTTTCCA	ATGAATGTCTTGGTAC	3'
YS054	3'	GTACGGTAAAAG	GTTACTTACAGAAC			5'

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Fig. 10

PLchT20IMPF-Sph



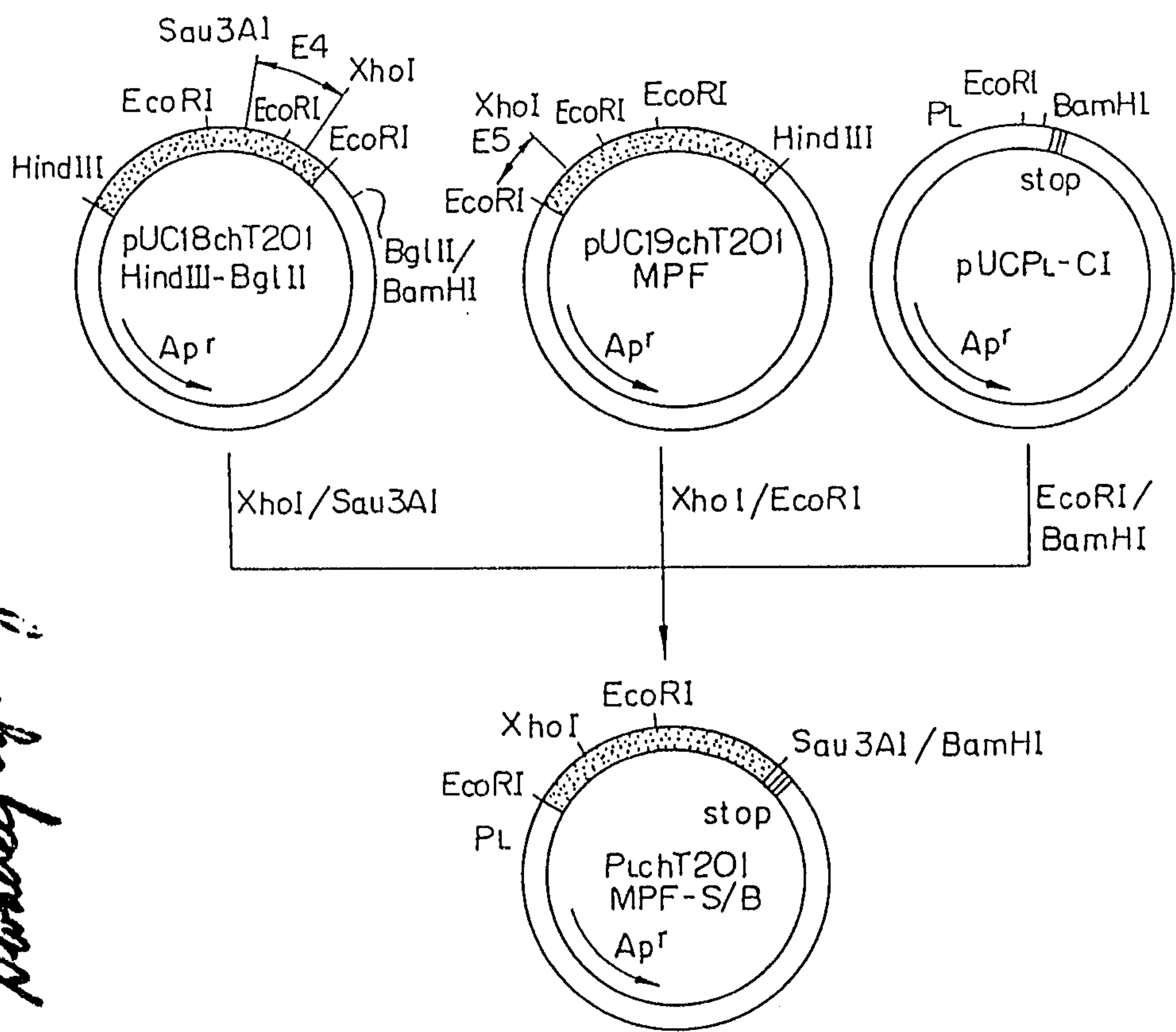
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19/44

Fig. 11

PlchT201MPF-S/B

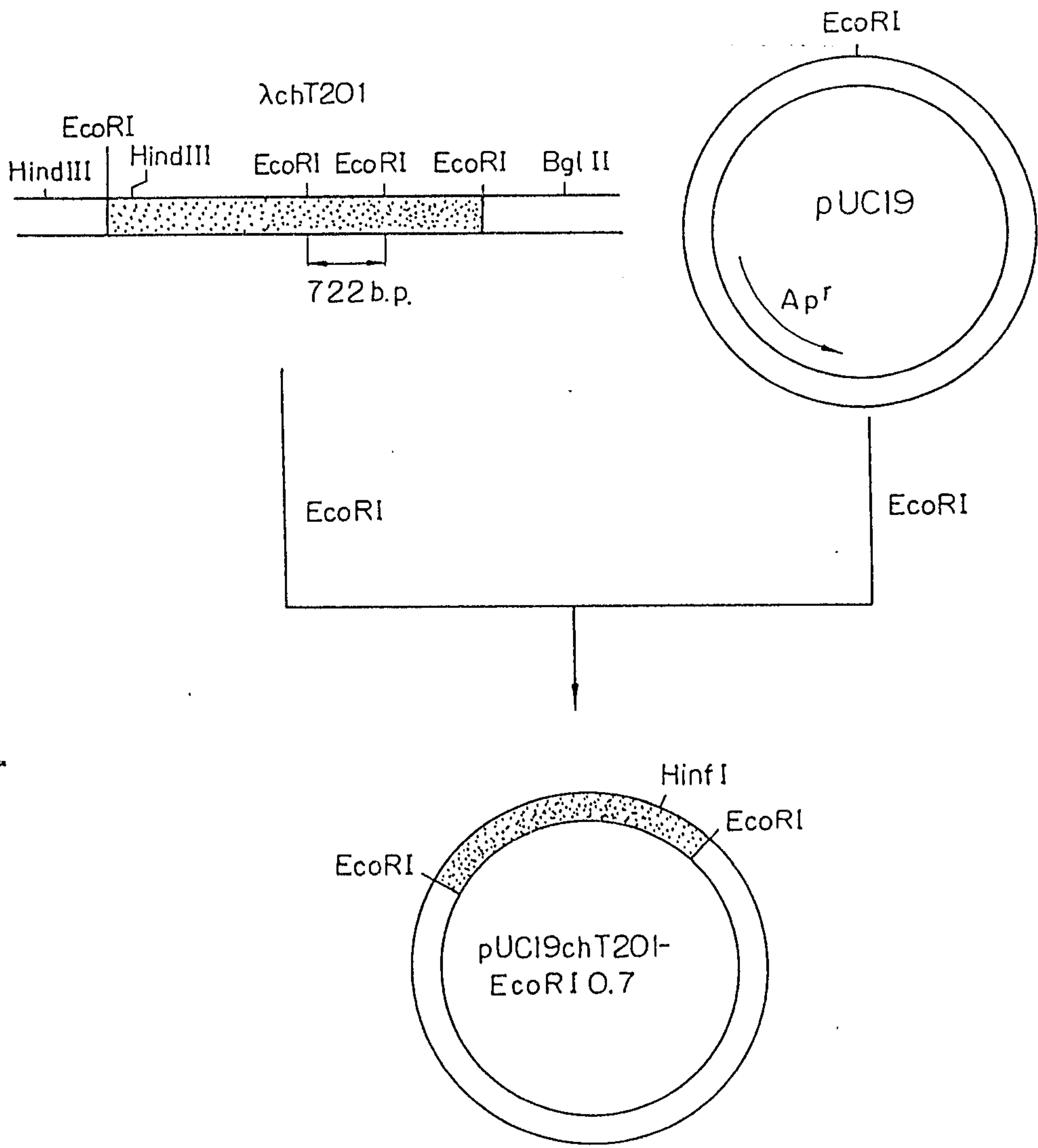


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Fig. 12

pUC19chT20I-EcoRI 0.7

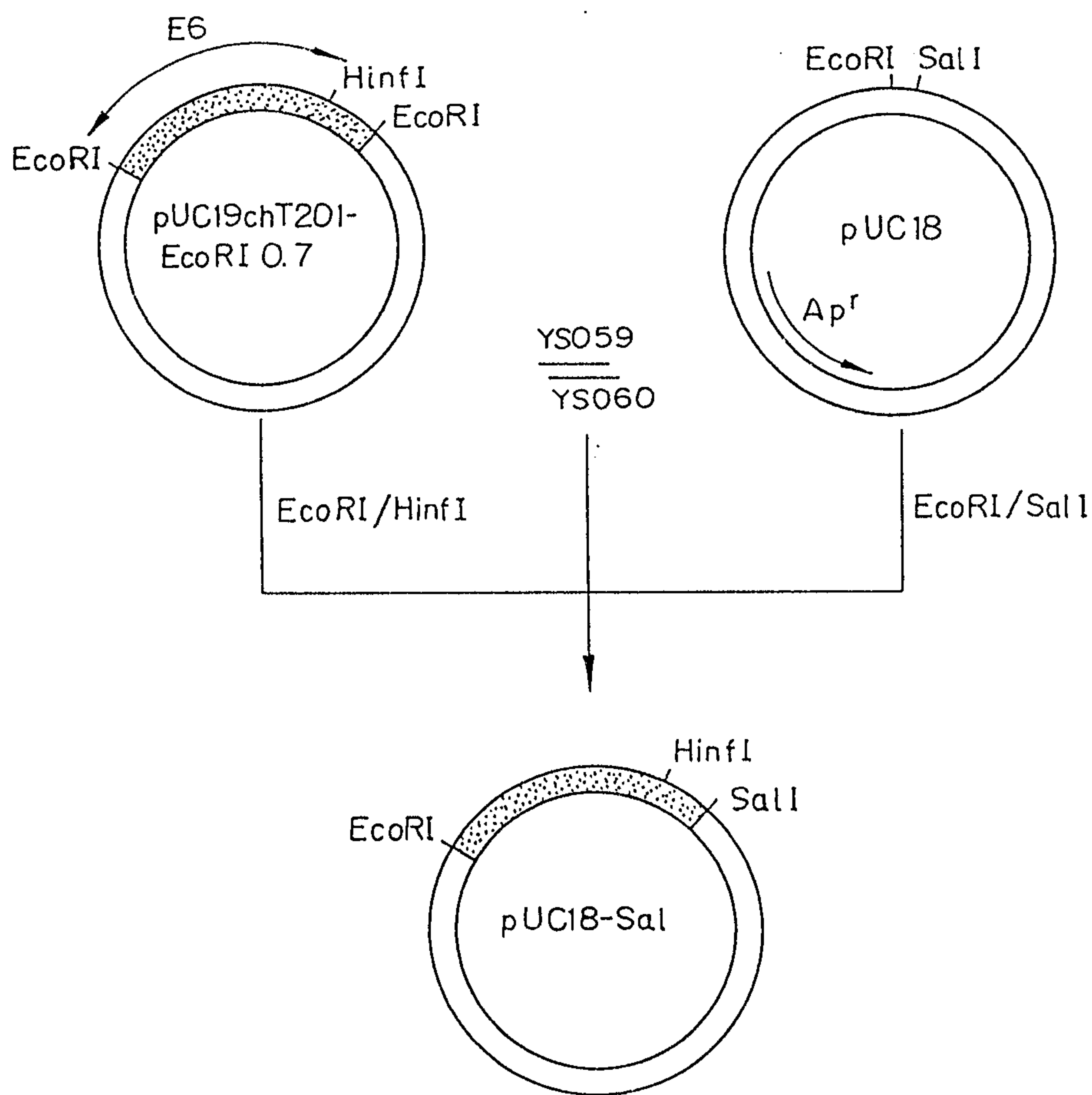


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Fig. 13

pUC18 - Sal



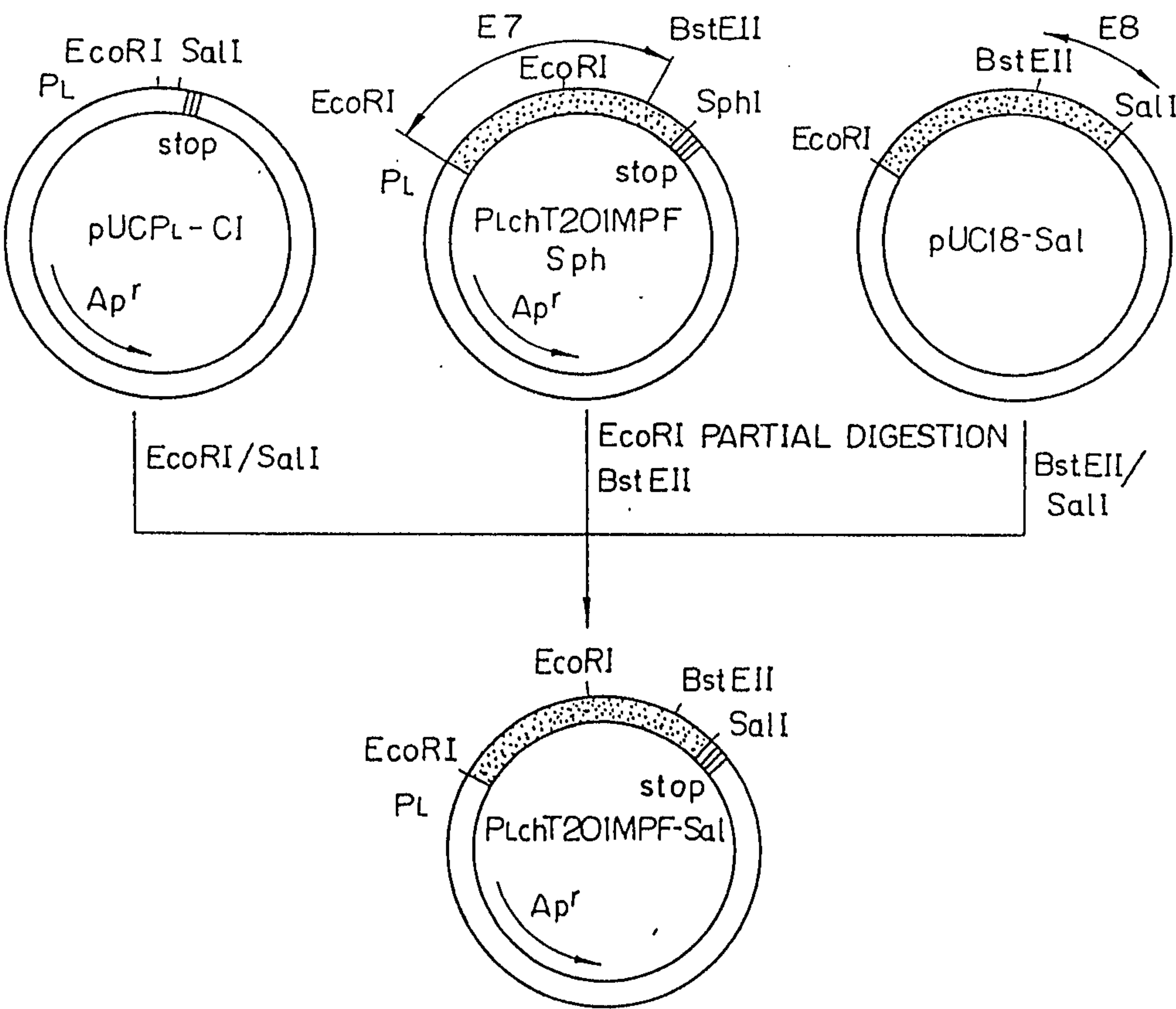
Hinf I Pro Lys stop Sal I

YS059 5' ATTCCTTTACTACAGCAGCCAAAATGAG 3'

YS060 3' GGAAATGATGTCGTCGGT TTTACTCAGCT 5'

Fig. 14

PLchT20IMPF-Sal

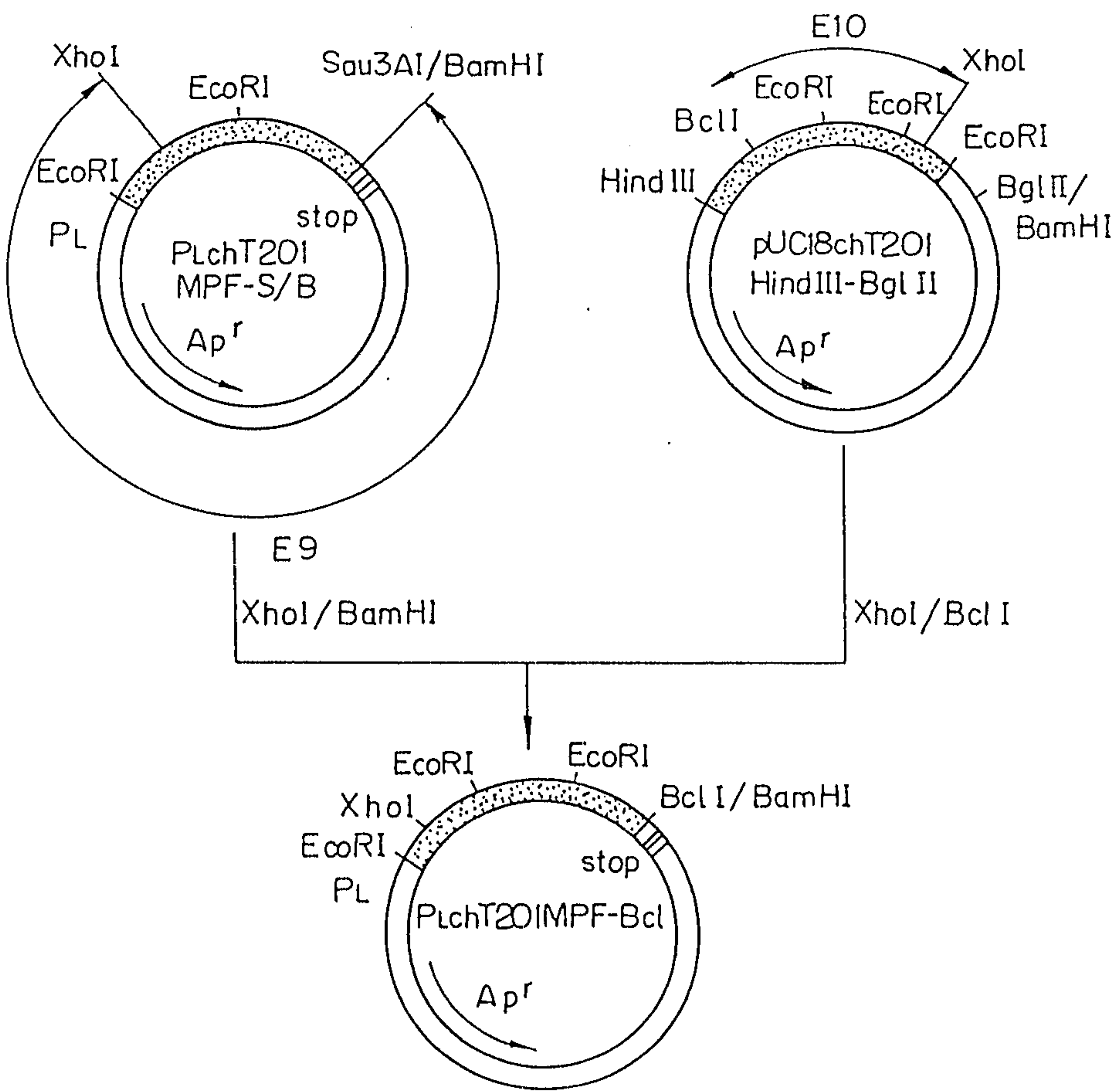


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Fig. 15

PLchT201MPF-Bcl

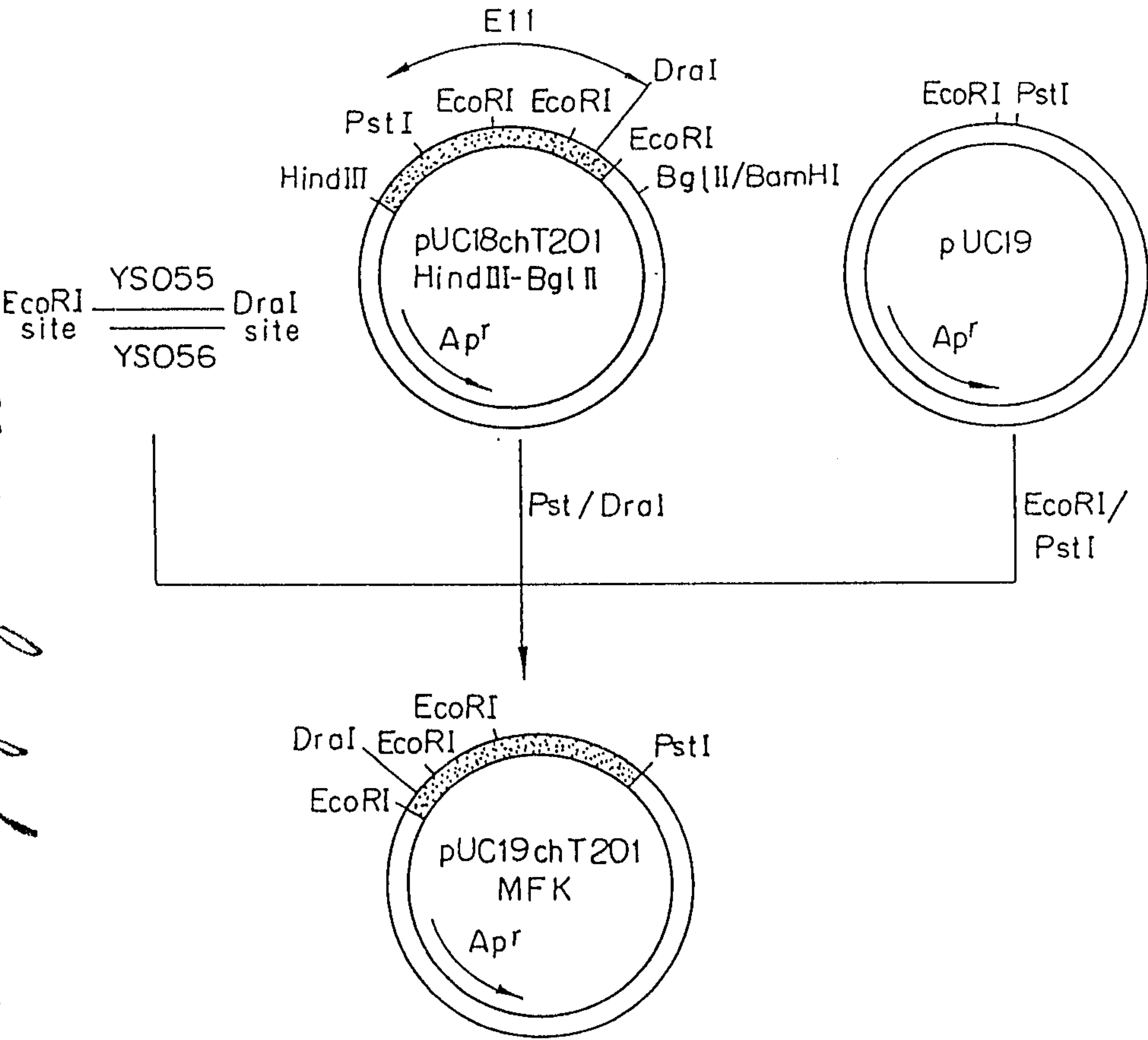


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Fig. 16

pUC19chT201MFK



YS055 5' AATTCATGTTT 3'
YS056 3' GTACAAA 5'

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Fig. 17

PLchT20IMFK-Sph

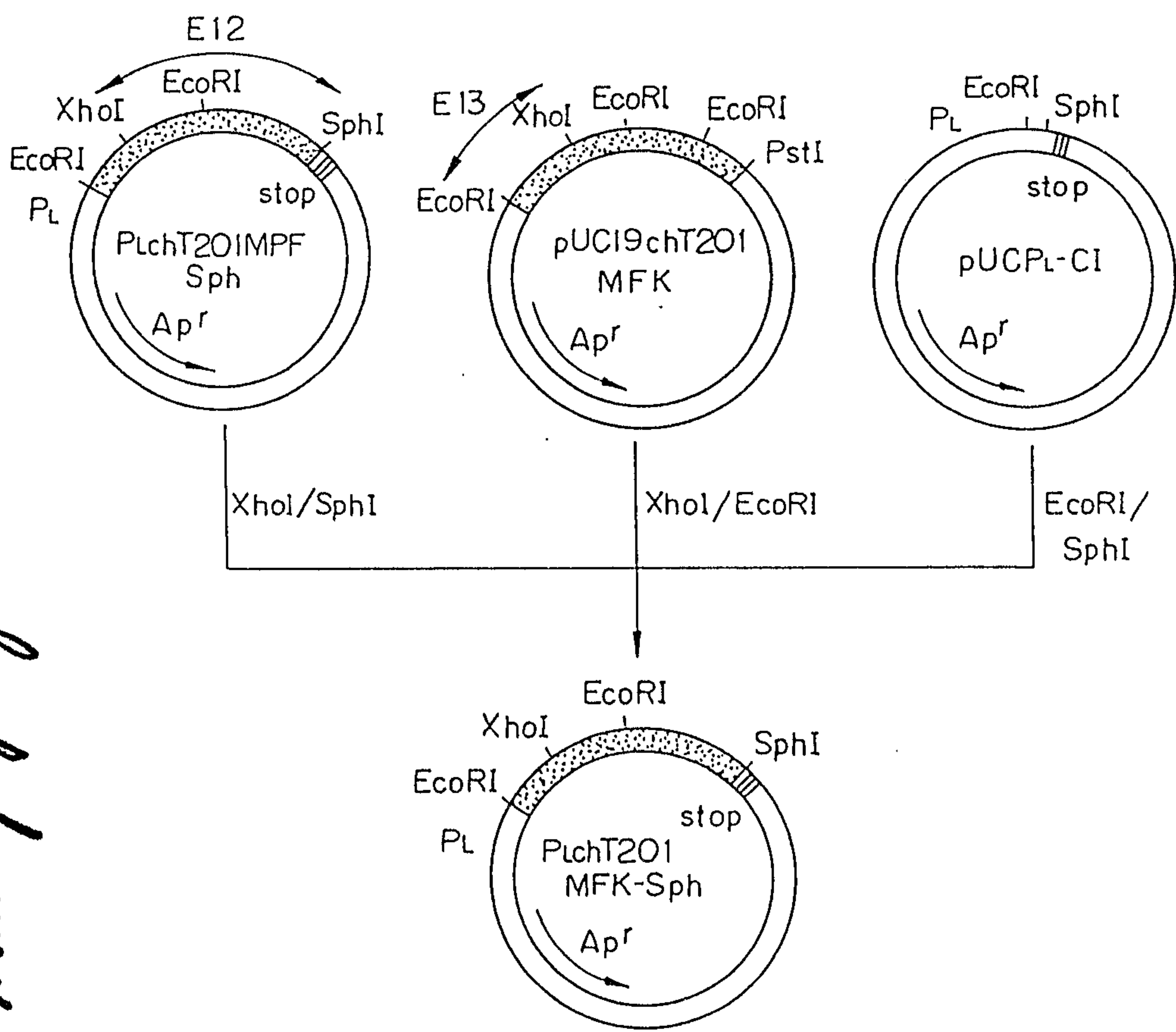


Fig. 18

PLchT201MFK- S/B

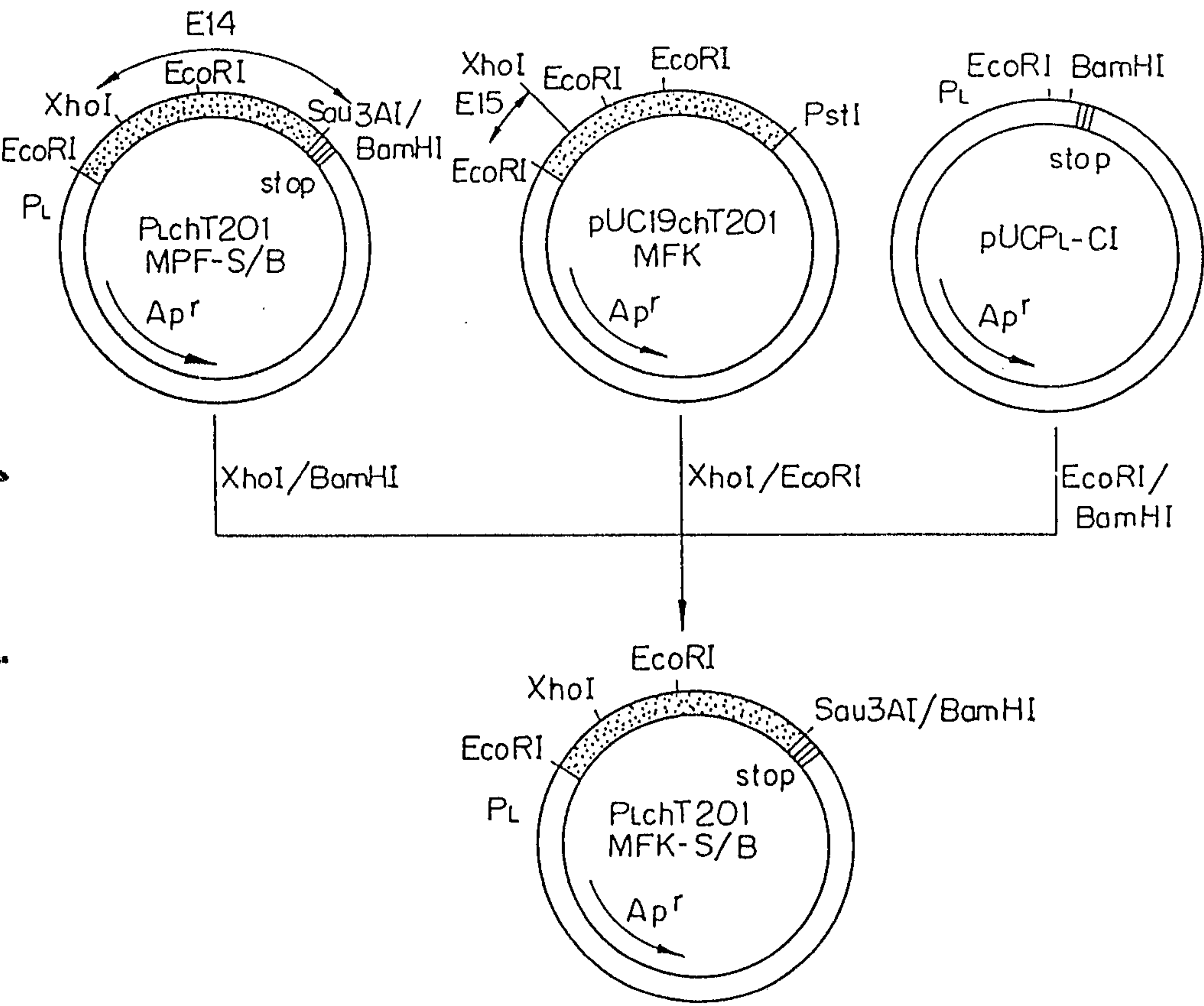


Fig. 19

PLchT2O1MFK-Sal

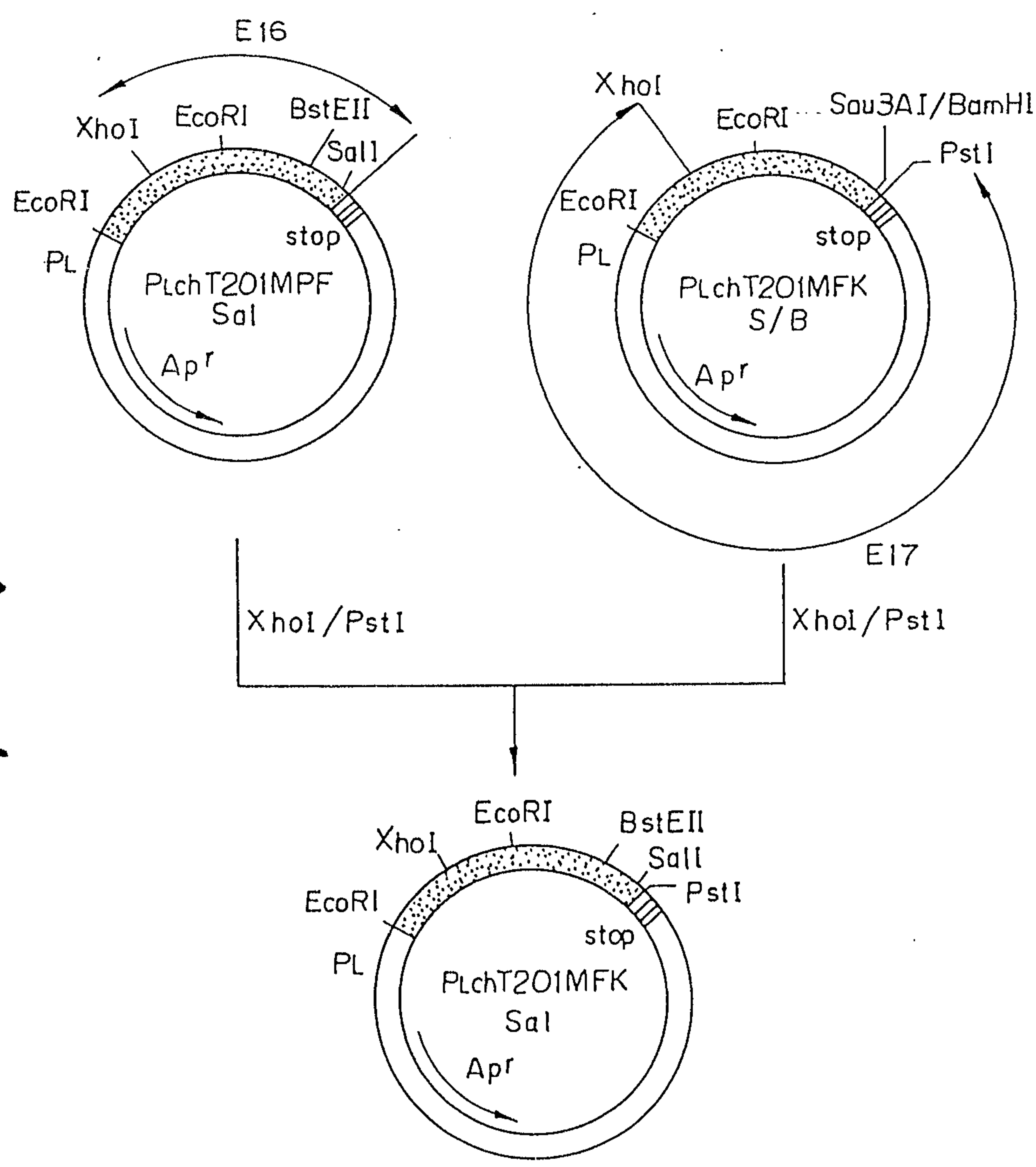
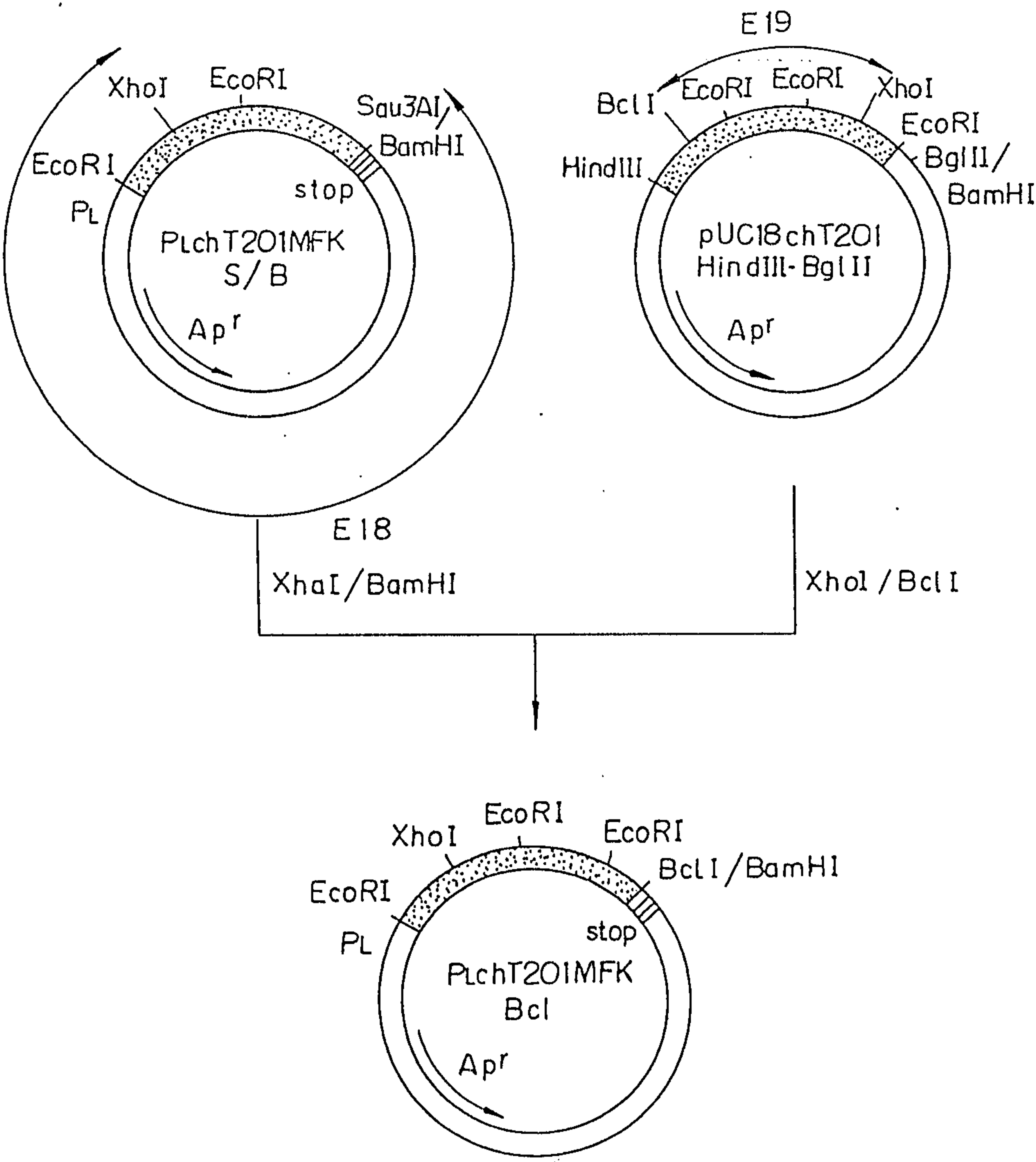


Fig. 20

PLchT20IMFK-Bcl

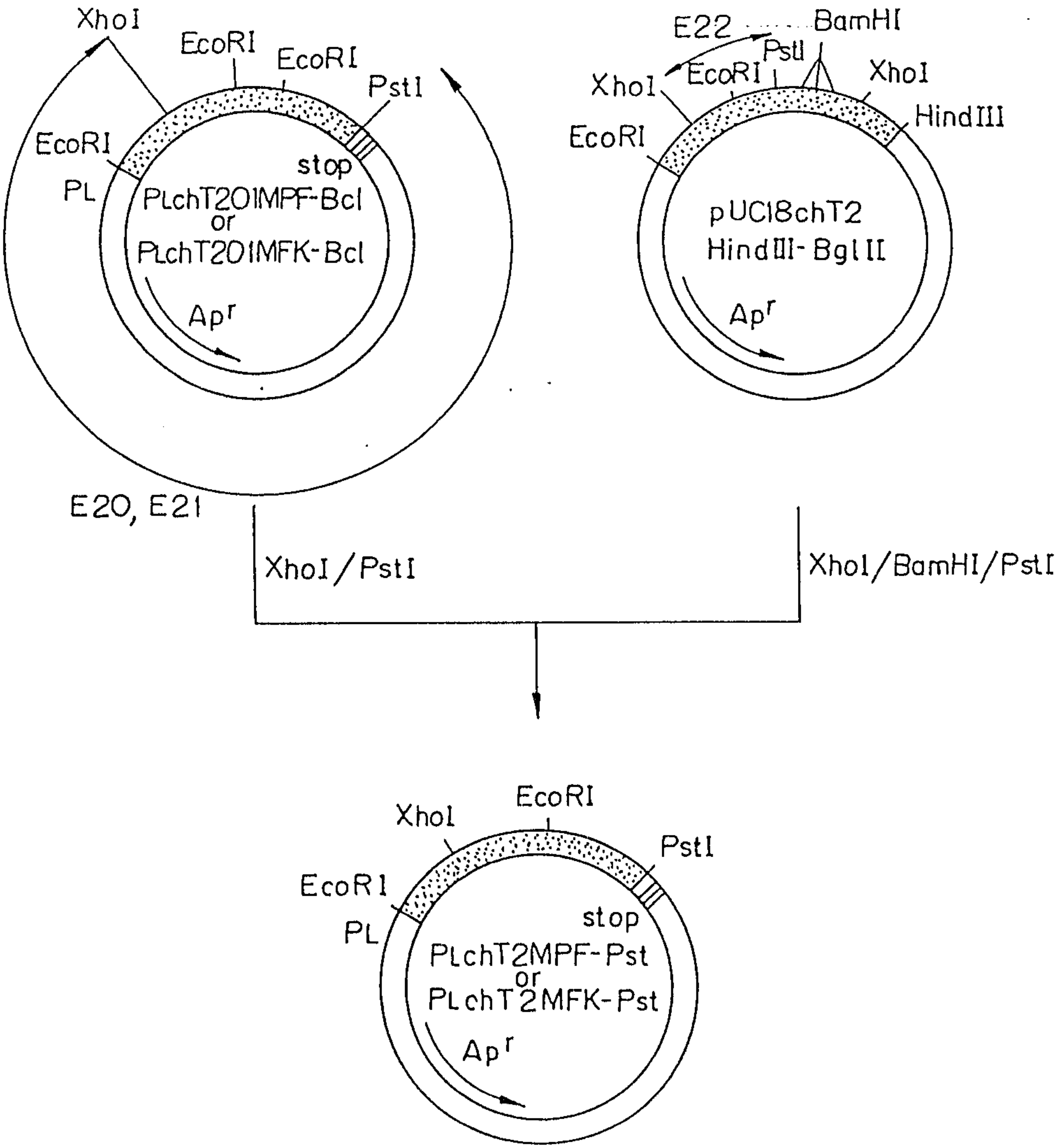


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Fig. 21

PLchT2MPF-Pst AND PLchT2MFK-Pst

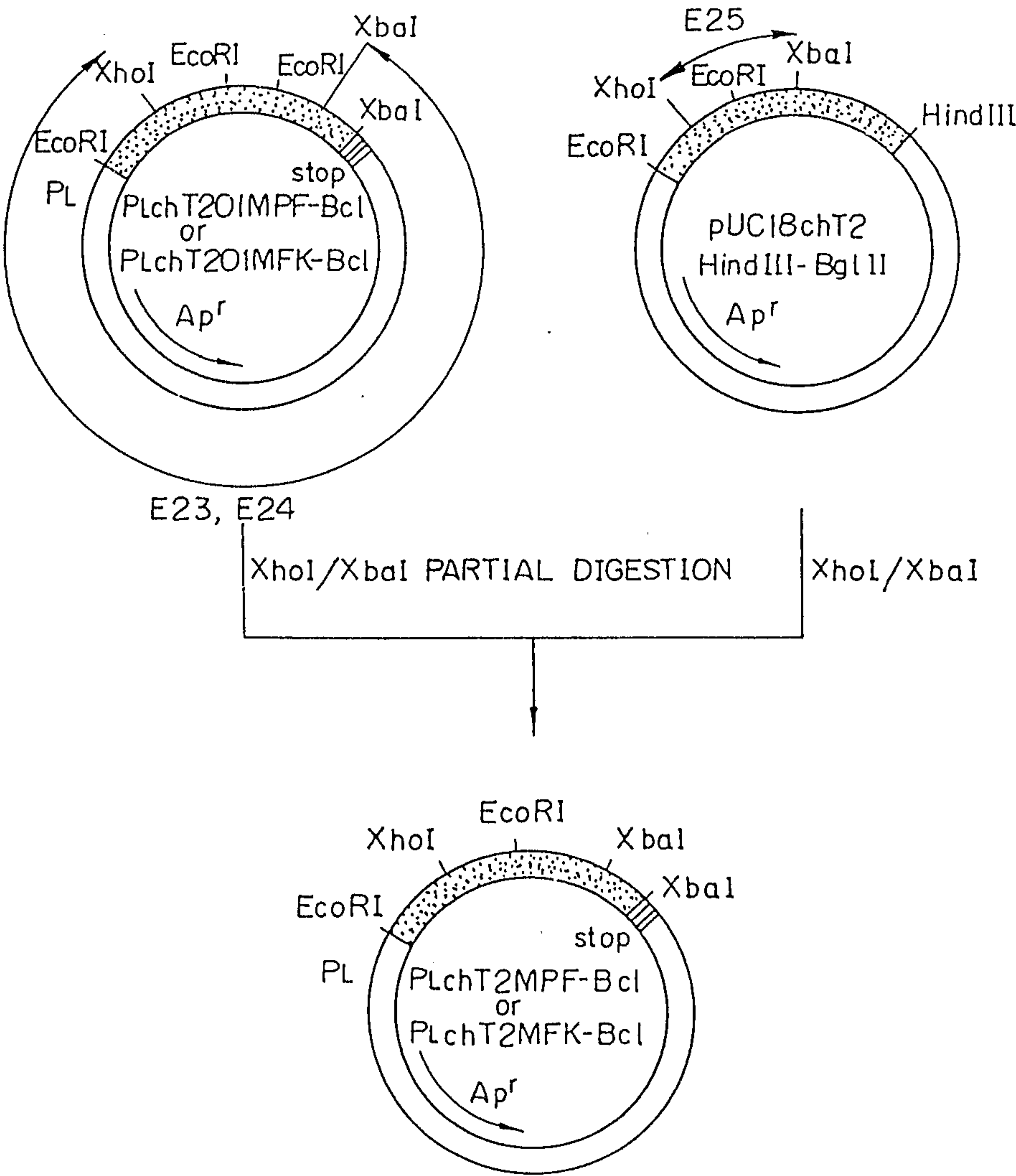


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Fig. 22

PLchT2MPF-Bcl AND PLchT2MFK-Bcl



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Fig. 23

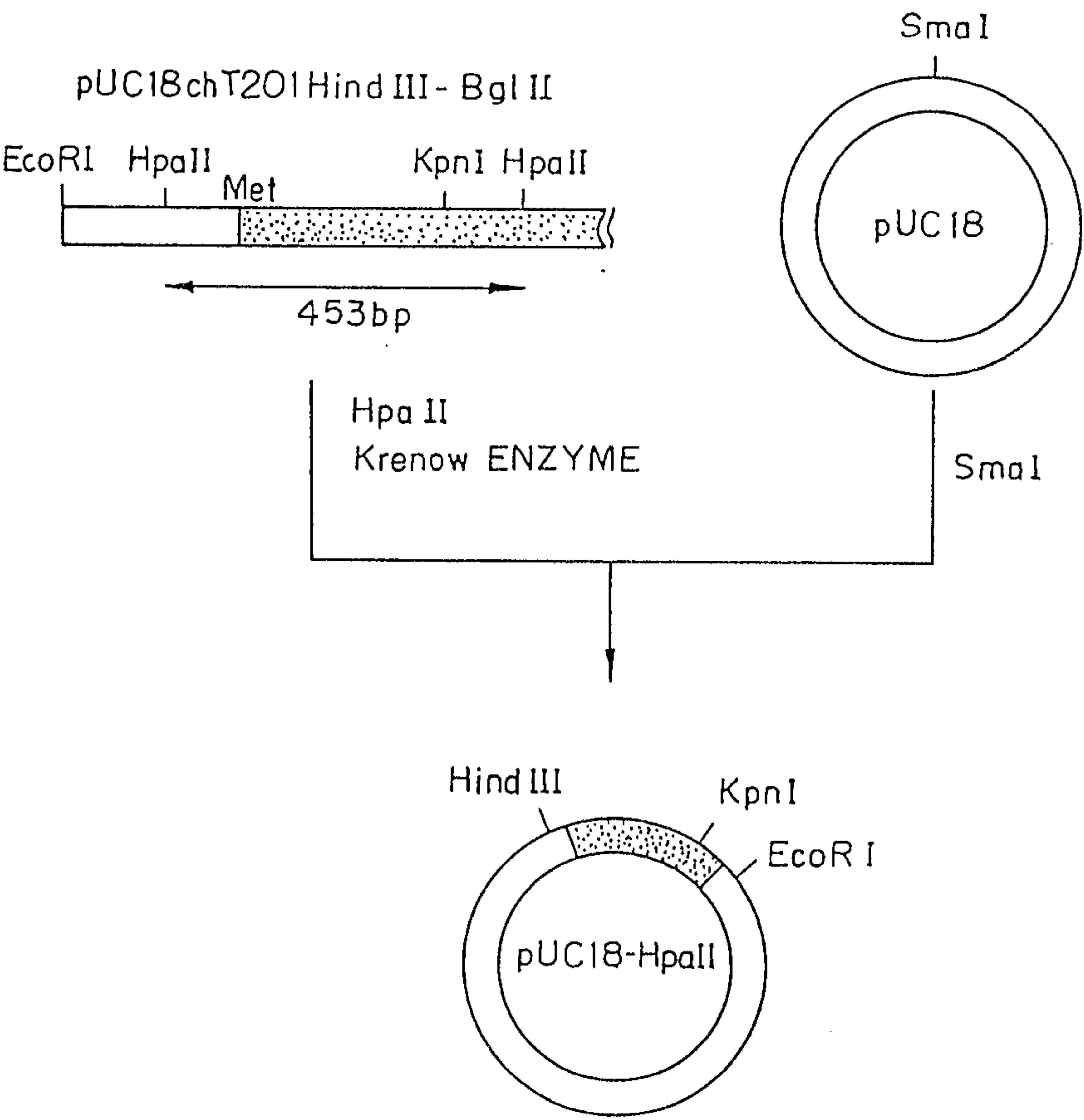
EXPRESSION OF C-TERMINAL α -AMIDATING
ENZYME DERIVATIVES IN E. COLI

E. COLI STRAINS	CONVERSION RATIO (%)
W3110	9.0
W3110/PLchT201MPF-Sph	9.1
W3110/PLchT201MFK-Sph	9.1
W3110/PLchT201MPF-Sal	10.2
W3110/PLchT201MFK-Sal	11.4
W3110/PLchT201MPF-S/B	11.2
W3110/PLchT201MFK-S/B	12.0
W3110/PLchT201MPF-Bcl	10.5
W3110/PLchT201MFK-Bcl	11.0
W3110/PLchT2MPF-Pst	10.7
W3110/PLchT2MFK-Pst	10.7
W3110/PLchT2MPF-Bcl	9.7
W3110/PLchT2MFK-Bcl	10.1

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Fig. 24

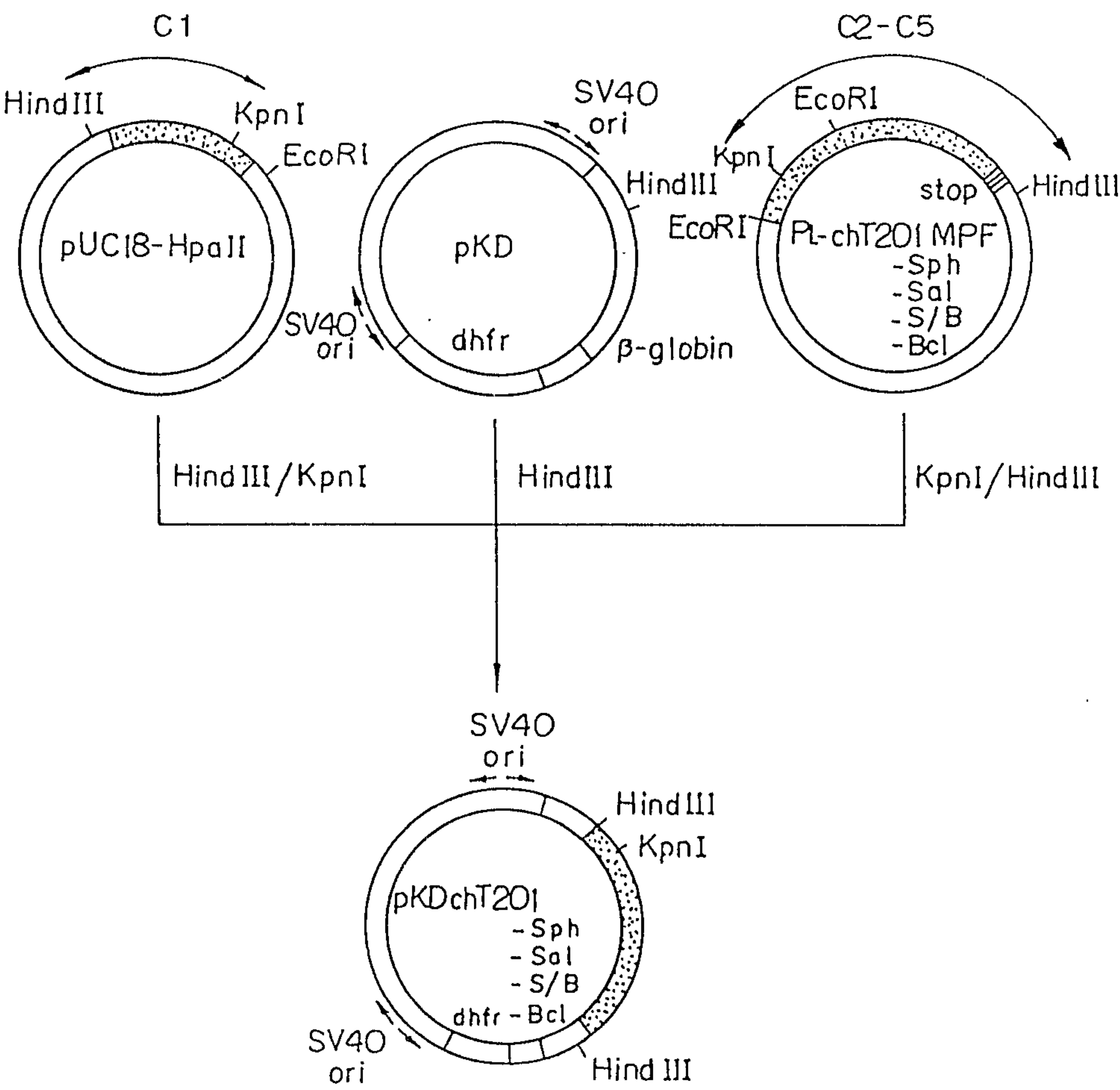
pUC18-Hpa II



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Fig. 25

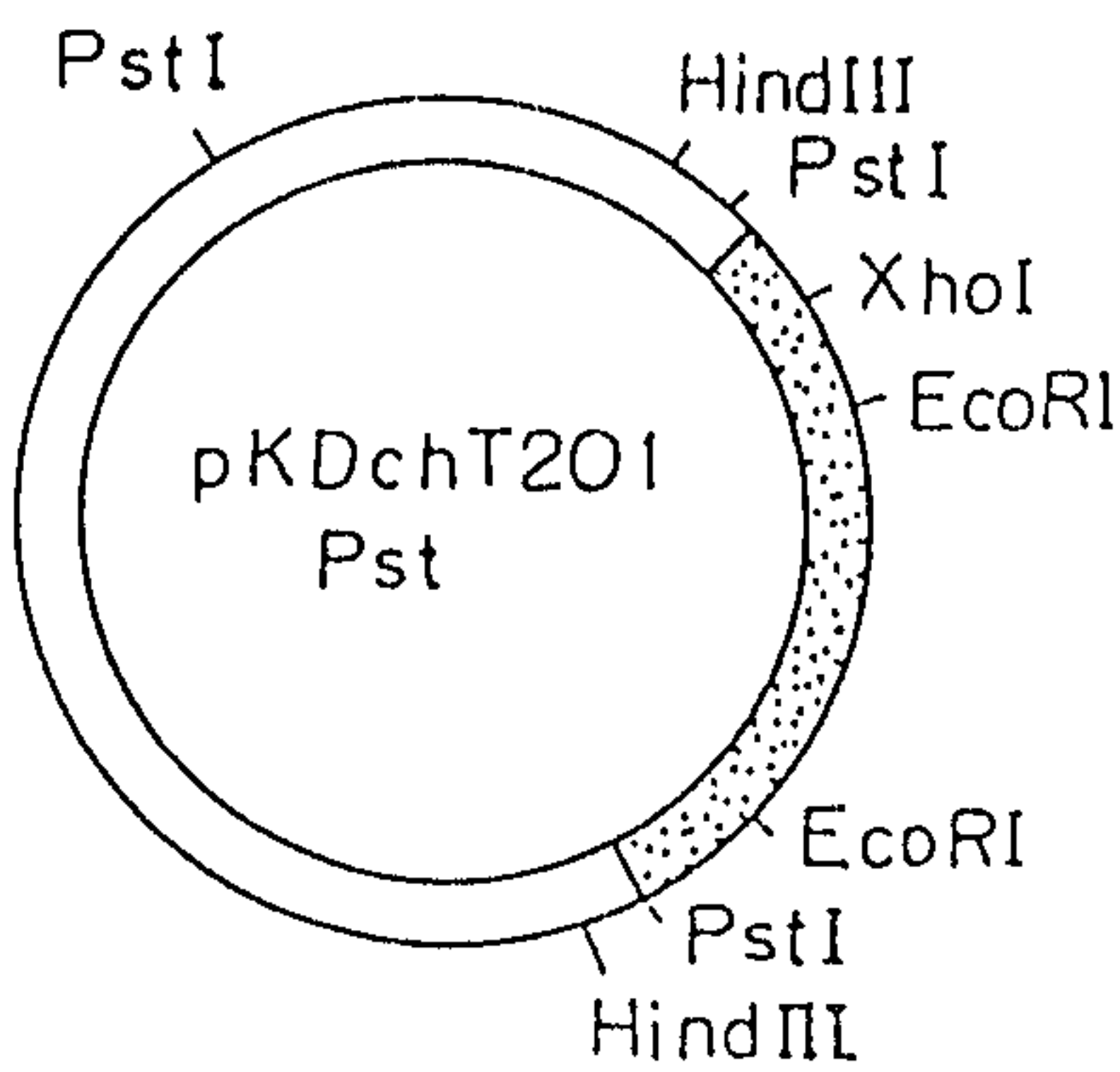
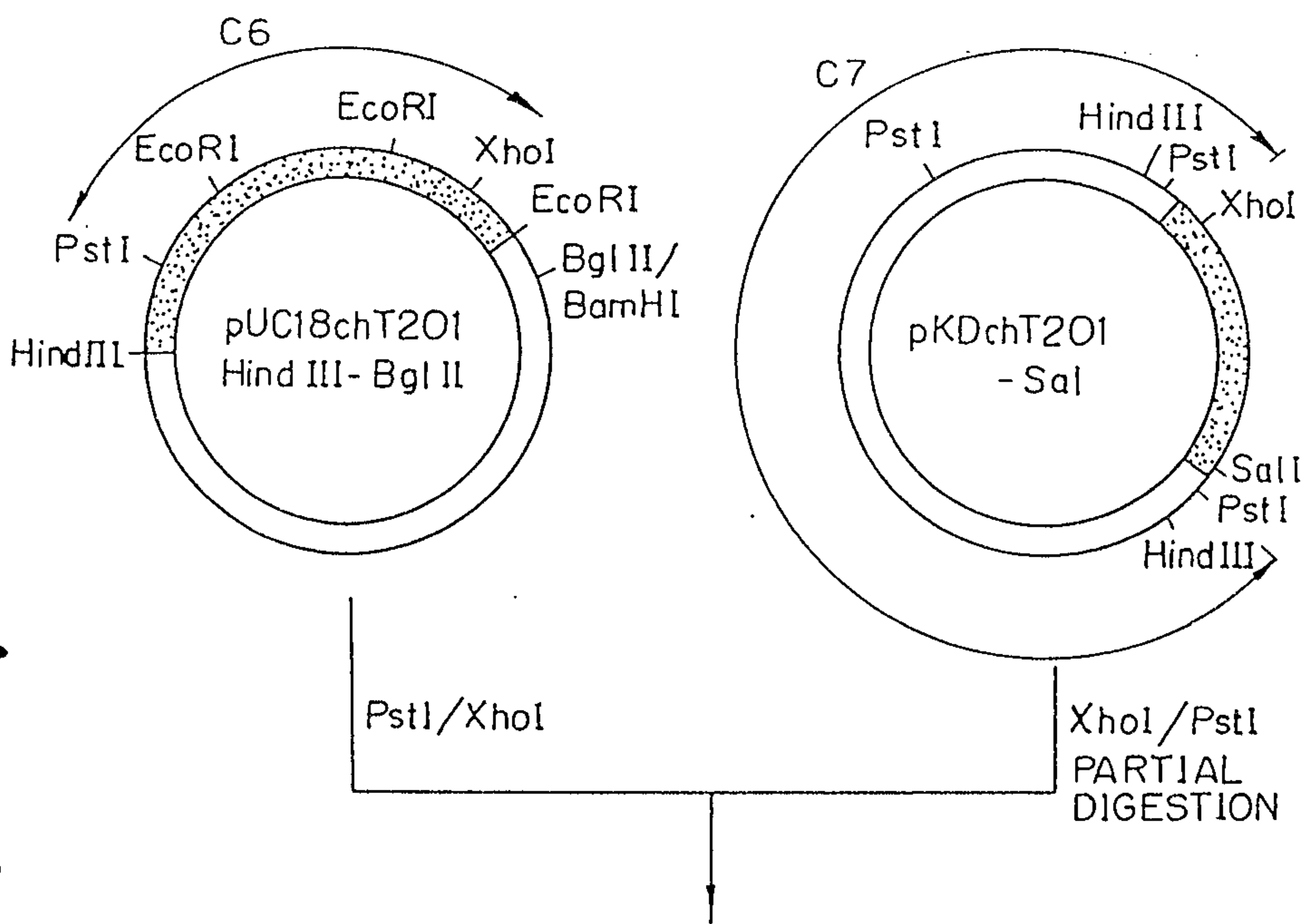
pKDchT201-Sph,-Sal,-S/B,-Bcl



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Fig. 26

pKDchT201 Pst I

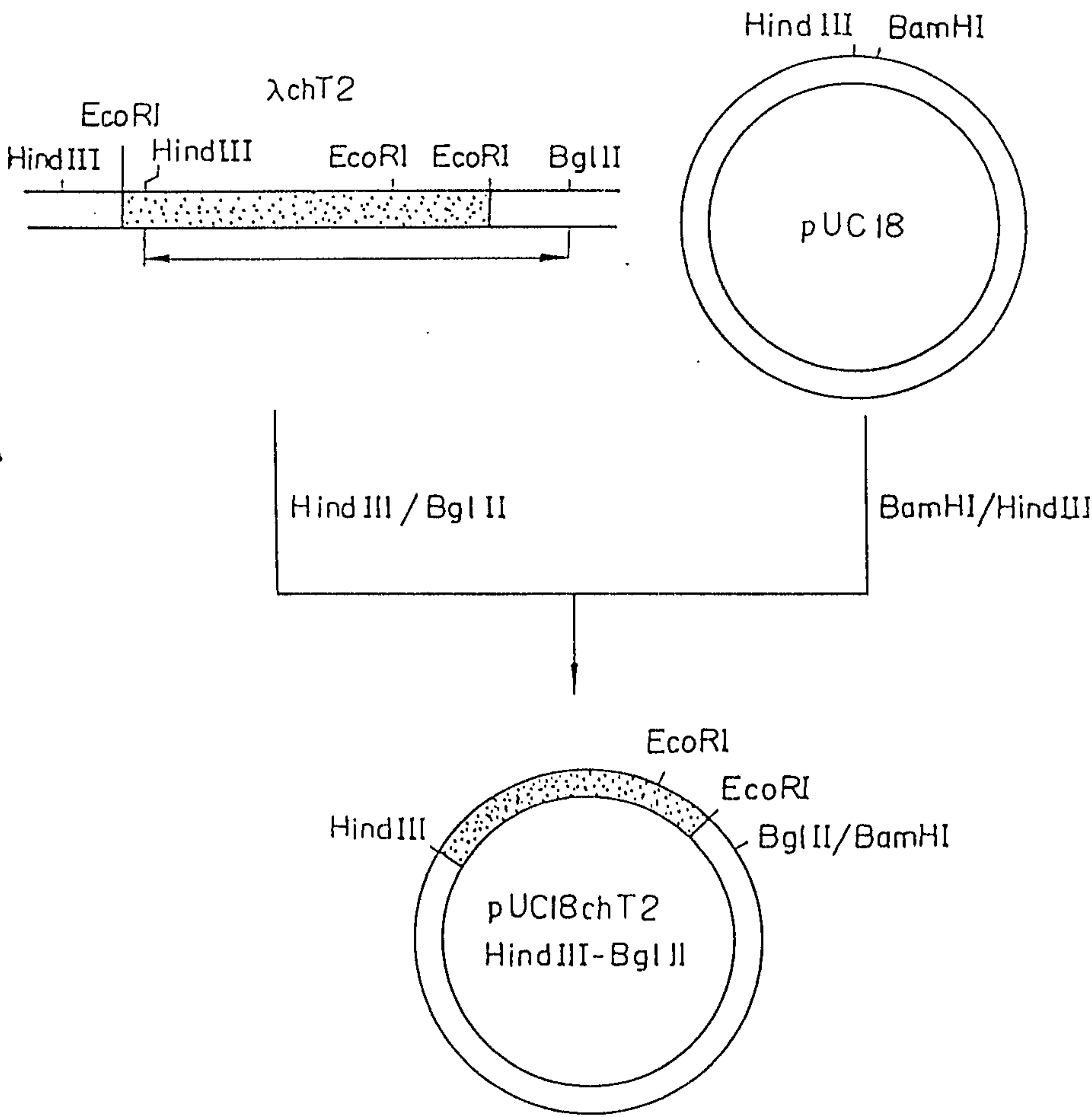


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Fig. 27

pUC18chT2Hind III- Bgl II

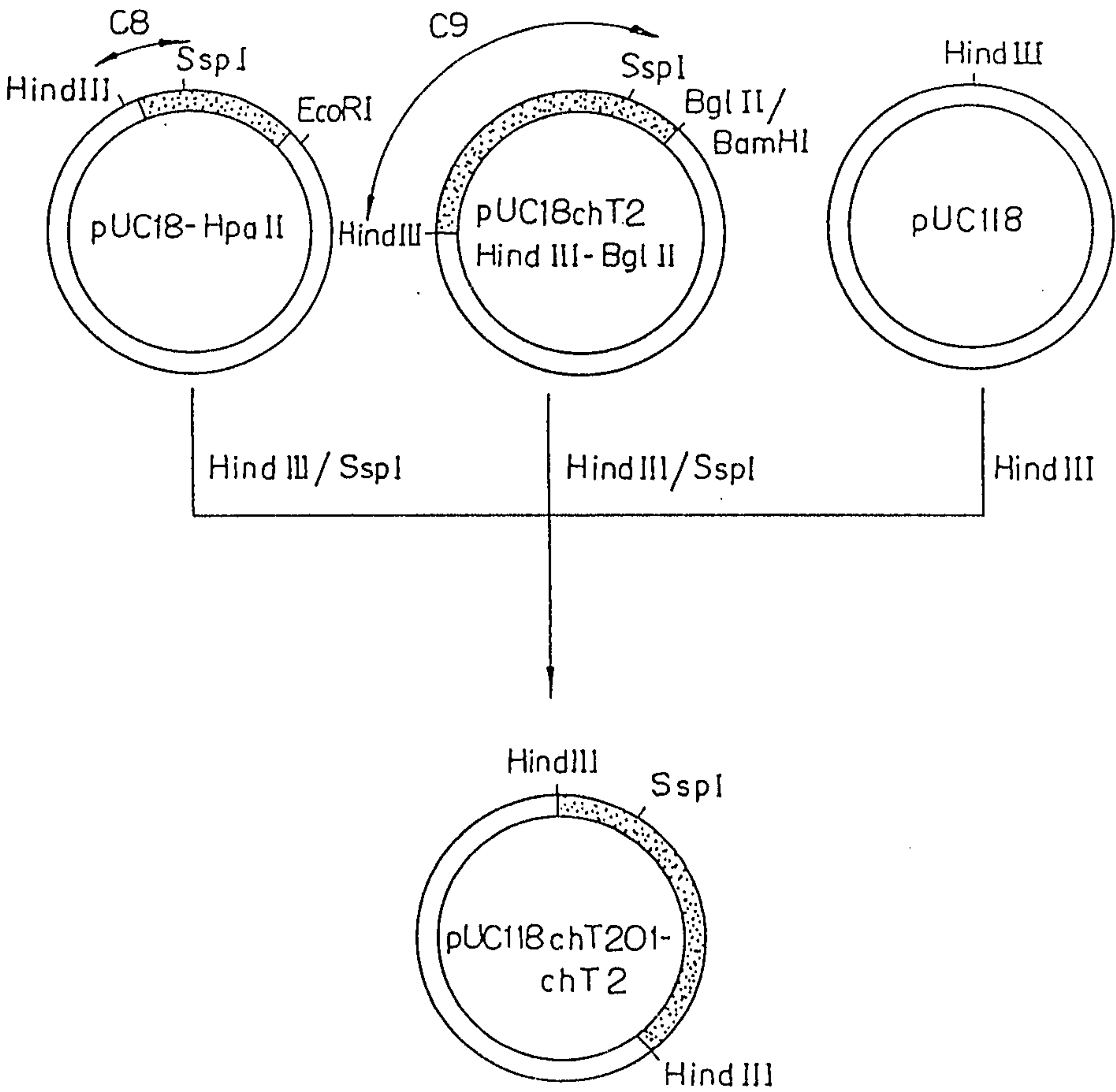


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Fig. 28

pUC118chT201- chT 2

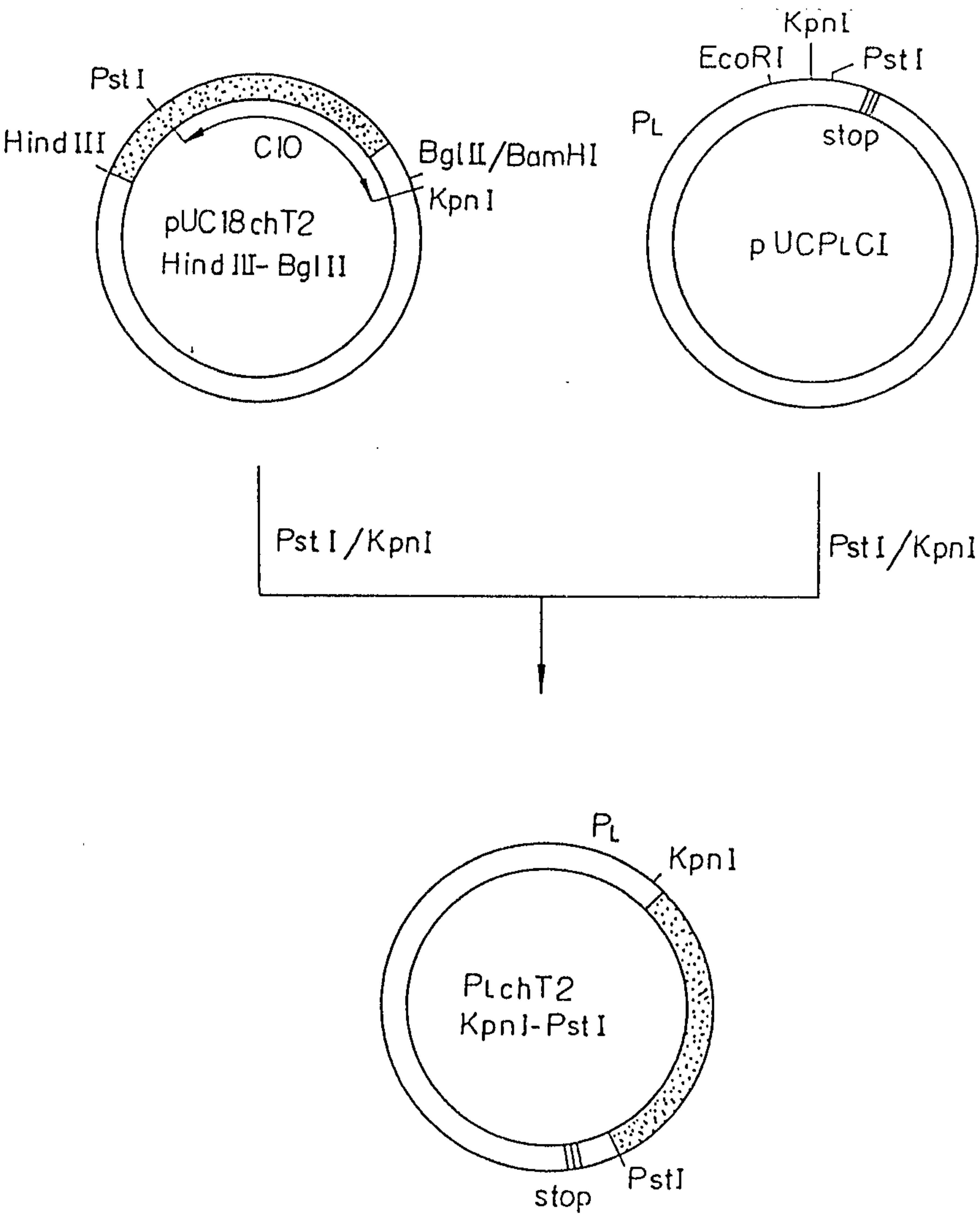


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Fig. 29

PLchT2Kpn I-Pst I

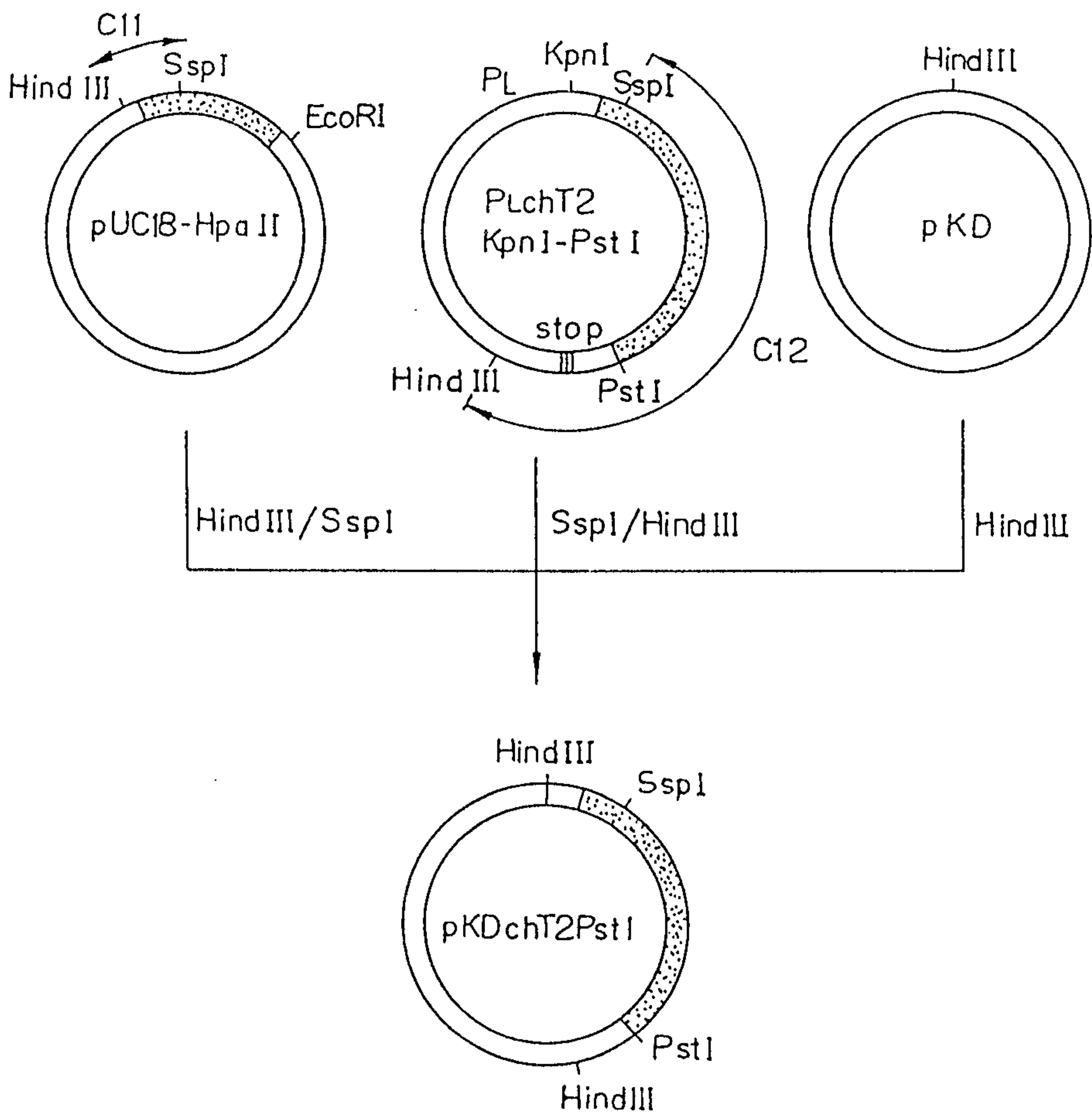


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Fig. 30

pKDchT2Pst I

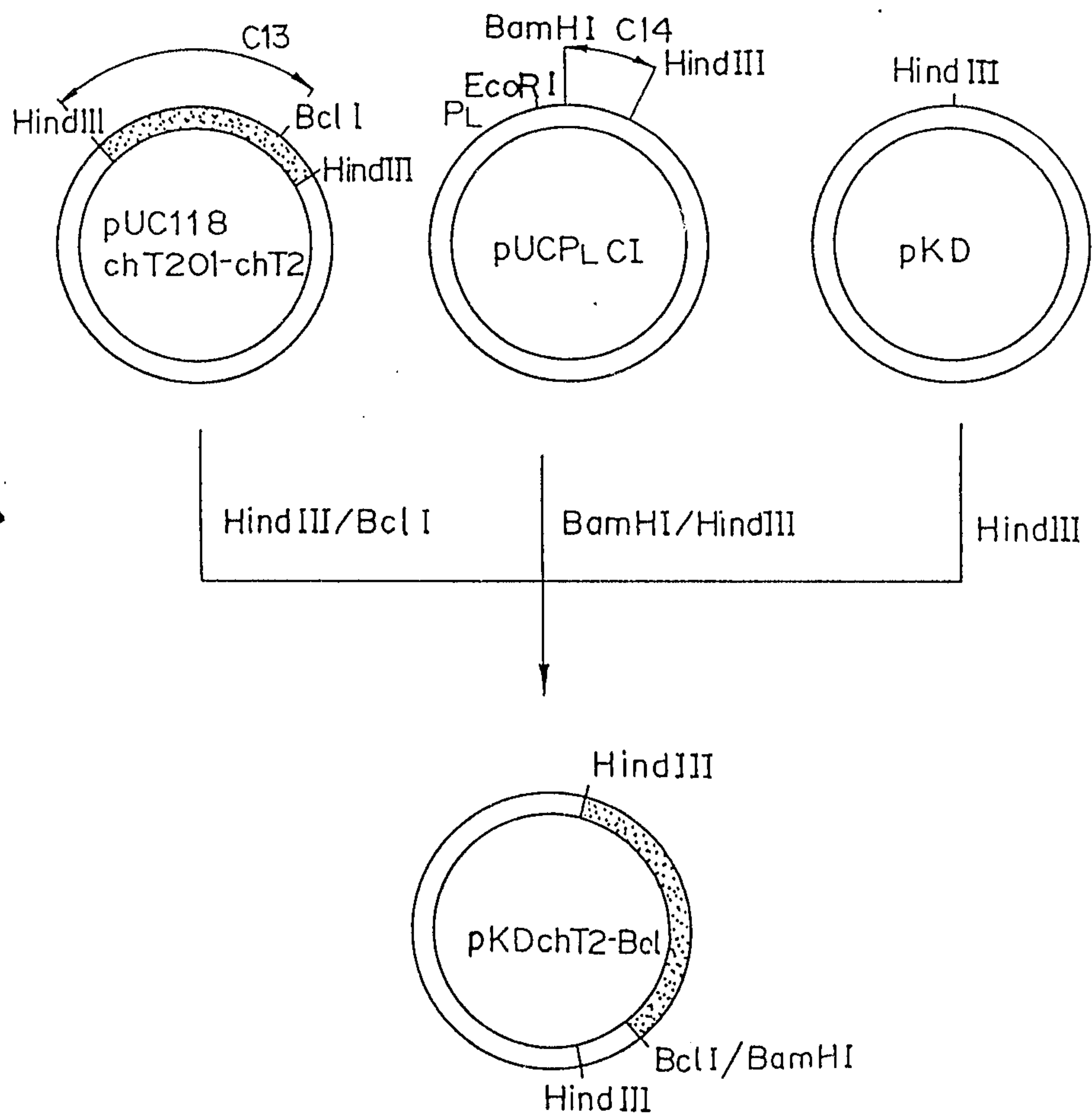


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Fig. 3 I

pKDchT2 Bcl

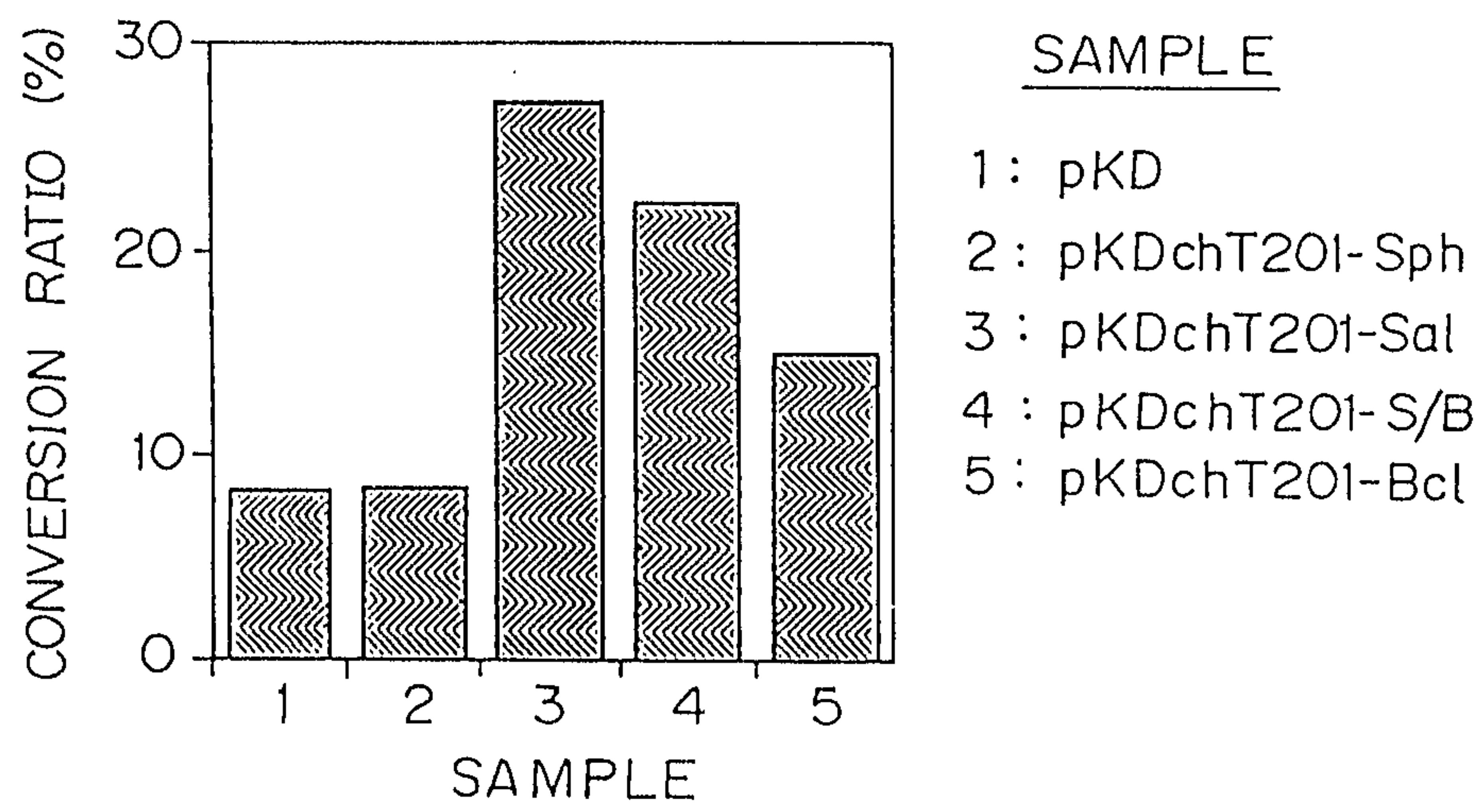


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Fig. 32(a)

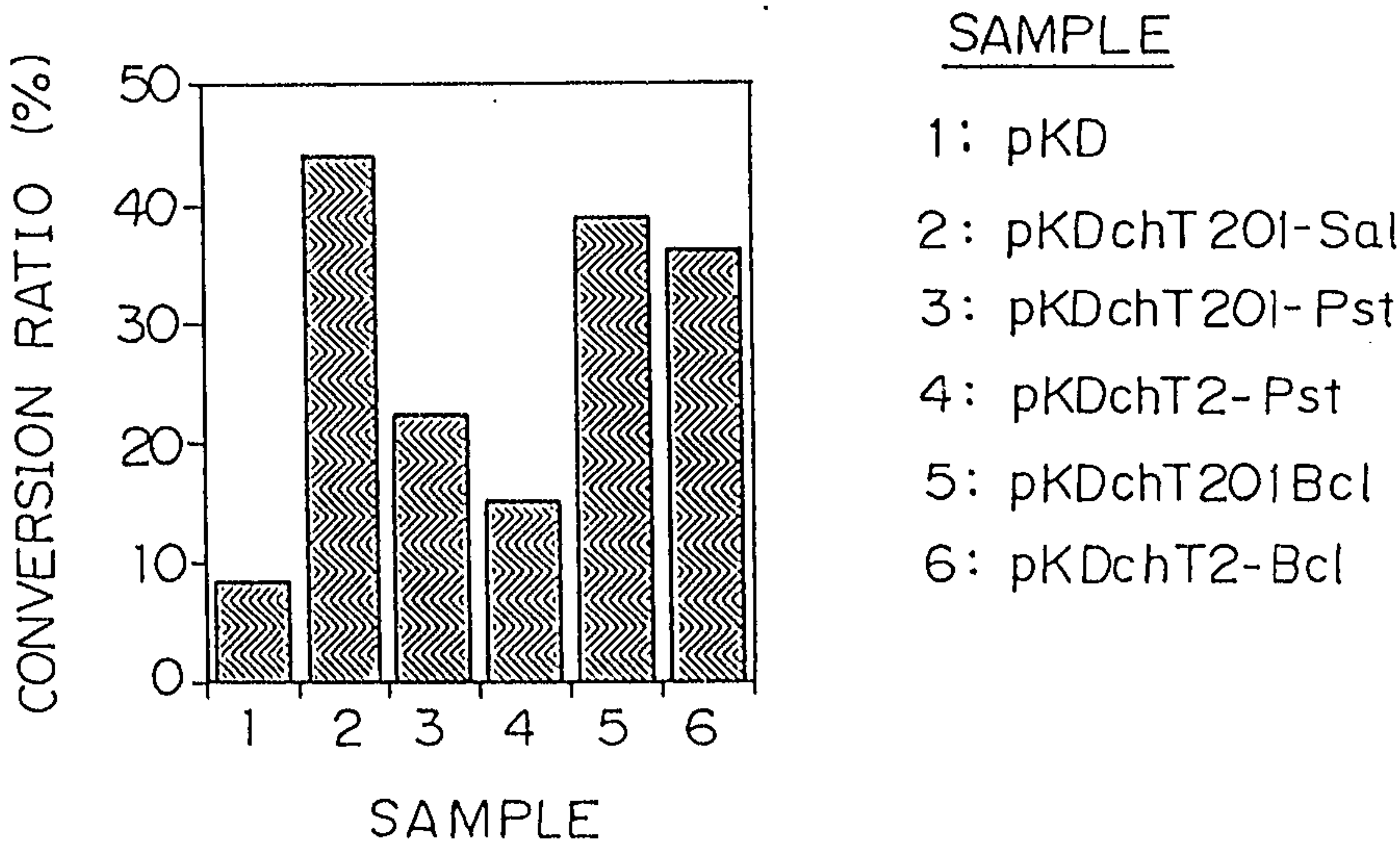
EXPRESSION OF C-TERMINAL α -AMIDATING
ENZYME DERIVATIVES IN COS1 CELLS



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Fig. 32(b)

EXPRESSION OF C-TERMINAL α -AMIDATING
ENZYME DERIVATIVES IN COS1 CELLS



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Amberley Ogilvy Renault

Fig. 33(a)

AMINO ACID SEQUENCE OF
C-TERMINAL α-AMIDATING ENZYME DERIVATIVES

PLchT20IMFK-Sph	31	323	MetPheLys	Ala
PLchT20IMPF-Sph			MetProPhe	Ala
		37		
PLchT20IMFK-Sal		378		Lys
PLchT20IMPF-Sal				Lys
PLchT20IMFK-S/B		434	Asp	Pro-Leu-Glu-Ser-Thr-Cys-Arg-His-Ala
PLchT20IMPF-S/B			Asp	CCTCTAGAGTCGACCTGCAGGCATGCT
PLchT20IMFK-Bcl		857	Ile	Leu
PLchT20IMPF-Bcl			Ile	CTC

□ : AMINO ACID SEQUENCE OF chT20I cDNA

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Fig. 33(b)

AMINO ACID SEQUENCE OF
C-TERMINAL α -AMIDATING ENZYME DERIVATIVES

PLchT2MFK-Pst	31 MetPheLys	461 Ala	Gly-Met-Leu-Asn GGCATGCTTAAT
PLchT2MPF-Pst	37 MetProPhe	Ala	Gly-Met-Leu-Asn GGCATGGTTAAT
PLchT2MFK-Bcl	31 MetPheLys	750 Ile	Leu CTC
PLchT2MPF-Bcl	37 MetProPhe	Ile	Leu CTC

: AMINO ACID SEQUENCE OF
chT2O1 cDNA + chT2 cDNA

Fig. 34

AMINO ACID SEQUENCE OF C-TERMINAL
α - AMIDATING ENZYME DERIVATIVES

pKDchT20I-Sph	1	Met	323	Ala
pKDchT20I-Sal	1	Met	378	Lys
pKDchT20I-S/B	1	Met	434	Asp
pKDchT20I-Pst	1	Met	568	Ala
pKDchT20I-Bcl	1	Met	857	Ile
pKDchT2-Pst	1	Met	461	Ala
pKDchT2-Bcl	1	Met	750	Ile

Pro-Leu-Glu-Ser-Thr-Cys-Arg-His-Ala
CCTCTAGAGTCGACCTGCAGGCATGCT

Gly-Met-Leu-Asn
GGCATGCTTAAT

Gly-Met-Leu-Asn
GGCATGCTTAAT

Leu
CTC

Leu
CTC