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(54) Titre : EXPRESSION CYTOPLASMIQUE D'ANTICORPS; FRAGMENTS D'ANTICORPS ET MOLECULES DE
FUSION DE FRAGMENTS D'ANTICORPS CHEZ E. COLI

(54) Title: CYTOPLASMIC EXPRESSION OF ANTIBODIES, ANTIBODY FRAGMENTS AND ANTIBODY FRAGMENT
FUSION MOLECULES IN E. COLI

(57) **Abrégé/Abstract:**

The invention relates to the cytoplasmic expression of antibodies, antibody fragments and antibody fragment fusion molecules in E. coli. In particular, antibody fragment fusion molecules having an antibody moiety which is directed against tumors and an enzyme moiety which cleaves a nontoxic prodrug to give the toxic drug can be advantageously prepared in this way while retaining their respective functional properties.

Abstract of the disclosure

Cytoplasmic expression of antibodies, antibody fragments and antibody fragment fusion molecules in E. coli

The invention relates to the cytoplasmic expression of antibodies, antibody fragments and antibody fragment fusion molecules in E. coli. In particular, antibody fragment fusion molecules having an antibody moiety which is directed against tumors and an enzyme moiety which cleaves a nontoxic prodrug to give the toxic drug can be advantageously prepared in this way while retaining their respective functional properties.

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fragments and antibody fragment fusion molecules in
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10 The invention relates to the cytoplasmic expression of
antibodies, antibody fragments and antibody fragment
fusion molecules in E. coli. In particular, antibody
fragment fusion molecules having an antibody moiety
which is directed against tumors and an enzyme moiety
which cleaves a nontoxic prodrug to give the toxic drug
can be advantageously prepared in this way while
15 retaining their respective functional properties.

20 Previously, it has only been possible to express
antibodies or antibody fragments in E. coli, while
retaining their antigen-binding property, by using
signal sequences which control transport into the
periplasm. When expression takes place in the E. coli
periplasm, the expression yields vary in the range of a
few µg/l of culture medium (Ayala et al., Bio
Techniques 13, pp. 790 - 799, 1992). In addition,
25 refolding experiments are often required in order to
obtain functionally active antibody fragments (Fab) or
antigen-binding regions (sFv).

30 There has still been a need, therefore, to develop
improved methods for expressing functionally active
antibodies or antibody fragments. In connection with
the ADEPT concept (Bagshawe, Br. J. Cancer, vol. 60,
pp. 275 - 281, 1989), these improved methods should
also be applicable to antibody fragment-enzyme fusion
35 molecules, especially to fusion proteins containing
cytoplasmic mammalian or E. coli enzymes such as
E. coli β-glucuronidase.

The β -glucuronidase of *Escherichia coli* has been well investigated biochemically and genetically. The gene (uid A) has been cloned by Jefferson et al. (PNAS vol. 83, pp. 8447 - 8451, 1986) and employed as a reporter gene for heterologous control regions.

β -Glucuronidase (β -D-glucuronoside glucuronosohydrolase, E.C. 3.2.1.31) is an acid hydrolase which catalyzes the cleavage of β -glucuronides. As a result of the mammalian glucuronidases having been intensively investigated, a variety of substances are available for histological, spectrophotometric and fluorometric analyses. This enzyme has gained new, additional importance in its use for fusion proteins for targeted tumor therapy. In this connection, human glucuronidase is used in the form of a fusion protein which contains antibodies/antibody fragments or antigen-binding regions (Bosslet et al., Br. J. Cancer, 65, 234 - 238, 1992). As an alternative to the human enzyme, it is also possible to use the homologous *E. coli* β -glucuronidase. One of the advantages of the *E. coli* β -glucuronidase is that its catalytic activity at physiological pH is significantly higher than that of the human β -glucuronidase.

Previously, it has only been possible to express antibody fragment-enzyme fusion molecules periplasmically in *E. coli*. The enzyme moiety which is used in this context is therefore always composed of periplasmic *E. coli* enzymes such as β -lactamase (Goshorn et al., Canc. Res. 53, 2123 - 2117, 1993).

Previously, it has not been possible to express an antibody fragment-enzyme fusion molecule using a cytoplasmic *E. coli* enzyme, such as β -glucuronidase, which is in the functionally active state, that is while retaining its enzymic activity and also the antigen-binding ability of the antibody moiety. As a rule, functionally active expression of most antibodies

or antibody fragment molecules requires defined signal sequences for exporting the expressed molecules via the endoplasmic reticulum into the culture medium (animal cells and yeast) or into the periplasma (E. coli). It is only in the endoplasmic reticulum or in the periplasm that the necessary oxidative conditions pertain for forming the disulfide bridges which are important for functional activity. In addition, the secretory synthesis route is often crucial for the correct three-dimensional folding of the expressed protein.

An E. coli strain which is deficient in thioredoxin reductase, for example the strain AD 494, can form disulfide bridges in the cytoplasm and thereby enables enzymes which are naturally secretory, for example alkaline phosphatase, to be expressed intracellularly (Derman et al., Science, vol. 262, 1744 - 1747, 1993).

It has now been found that a Fab molecule, such as the Mab BW 431/26, can be expressed cytoplasmically in a functionally active state, that is while retaining its antigen-binding properties, as can a complete antibody, in an E. coli strain which is deficient in thioredoxin reductase.

Surprisingly, it has furthermore been possible to express an antibody fragment-enzyme fusion molecule composed of, for example, the cytoplasmic, non-disulfide-bridged E. coli enzyme β -glucuronidase and, for example, the Fab BW 431/26 which, for its part, requires intramolecular cystine bridges for correct folding (Fab BW 431/26-E. coli β -glucuronidase), in the cytoplasm and to isolate it from there in functional form. At the same time, the expressed molecule was soluble and no refolding experiments were necessary. This opens up novel opportunities for economically producing antibodies, antibody fragments and antibody fragment-enzyme fusion molecules for therapeutic and

diagnostic use.

The invention relates, therefore, to processes for the recombinant expression of antibodies, antibody
5 fragments or antibody fragment fusion molecules containing cytoplasmic mammalian or E. coli enzymes as fusion partners using thioredoxin reductase-deficient E. coli strains and subsequent isolation of the expression products from the cytoplasm. In this
10 context, antibody fragments are preferably Fab fragments or else antigen-binding regions (sFv, Plückthun and Skerra, Meth. Enzymol. 178, pp. 497 - 515, 1991). Finally, antibody fragment-enzyme fusion proteins are particularly preferred which contain
15 cytoplasmic E. coli enzymes as fusion partners, very particularly those containing E. coli β -glucuronidase as the fusion partner.

The following examples illustrate the invention.

20

Example 1

Construction of a dicistronic expression vector without signal sequences

25

A) Cloning the E. coli β -glucuronidase from E. coli RR1:

The DNA sequence encoding E. coli β -glucuronidase was
30 amplified by PCR from the E. coli strain RR1 using the primers E.c. β Gluc. for and E.c. β Gluc. back, and cloned into the vector KS by way of the Xba I and Hind III sites (Fig. 1).

35 B) Cloning of VH/CH1 and linker Mab BW 431/26 in front of E. coli β -glucuronidase:

The variable domain VH and the constant domain CH1 were amplified by PCR from an available HC-hum- β -

glucuronidase cDNA construct using the primers Fab HC for and Fab HC back, and cloned into vector KS by way of the Xba I and Eco RI sites (Fig. 2). After the DNA sequence had been verified, a DNA fragment which was
5 isolated by digesting with XbaI and NcoI was ligated into the vector from cloning step A which had been cut with XbaI and NcoI (Fig. 3).

C) Cloning the Fab BW 431/26 - E. coli β -glucuronidase
10 into pTrc 99:

An Xba I/Hind III fragment containing the corresponding heavy chain fused to E. coli β -glucuronidase was ligated to the light chain of Mab BW 431/26, which was
15 present in the expression vector pTrc99 (Amann et al., Gene 69, 301, 1988) (Fig. 4). The resulting construct has the structure: promotor (trc) - Shine Dalgarno sequence (SD) - VK/CK Mab BW 431/26 - SD - VH/CH1 Mab 431/26 - E. coli- β -gluc.-transcription termination
20 signal (Fig. 5).

Example 2

Expression in AD 494

25

Overnight cultures of AD 494, harboring pTrc dicistr. Fab-E. coli- β -gluc., described in Example 1, were diluted 1:10 and incubated at 25°C until they reached an OD₆₀₀ of 0.7. After inducing with 1 mM IPTG for
30 19 - 22 hours, the cells were incubated on ice for 1-1.5 hours. After the cells had been pelleted and resuspended in 10 ml of PBS, pH 7.2, per liter of culture volume, they were disrupted in a French press at 1000 - 1500 Psi. The disrupted cell suspension was
35 clarified at 20,000 rpm in an SS-34 rotor and the supernatant was employed for the subsequent investigations.

- 6 -

Example 3

Purification of the fusion molecule by affinity chromatography.

5

The disrupted cell suspensionsupernatant, which had been clarified by passing it through a 2 μ m filter, was purified by affinity chromatography by means of binding the fusion protein to an anti-idiotypic monoclonal antibody (BW 2064(34) (6 mg of Mab/ml of CnBr-activated Sepharose™ 4B). The fusion protein bound to the affinity column was eluted by pH shifting (pH 7.2 – pH 5.0). The eluate was then concentrated by means of ultrafiltration (Filtron Macrosep. Omega NMWL:30 KD).

15 The cell disruption suspensionsupernatant, the void volume, the concentrated eluate and the filtrate from the ultrafiltration were analyzed by SDS-PAGE. The band appearing at about 97 KD corresponds, as regards its molecular weight, to the expected fusion protein comprising the heavy chain moiety of the antibody and the E. coli glucuronidase. The band appearing at about 20 70 KD represents endogenous β -glucuronidase which has been purified concomitantly, during the affinity chromatography, due to the formation of heterotetramers (see below) between expressed heavy chain/ β -glucuronidase and endogenous β -glucuronidase.

25

Example 4

30 TSK 3000 gel chromatography

The native molecule was examined by TSK 3000 gel chromatography and its molecular weight was found to be 450 KD. Since the glucuronidase forms a tetramer in the native state, the observed molecular weight corresponds to that which is to be expected theoretically. This step is described in detail below.

35

400 ng of a fusion protein, which had been purified by

anti-idiotypic affinity, in 25 μ l were chromatographed on a TSK gel G 3000 SW XL column (TOSO HAAS, Cat. No. 3.5WxN3211, 7.8 mm $\times$ 300 mm) in an appropriate mobile phase (PBS, pH 7.2, 5 g/l maltose, 4.2 g/l arginine) at a flow rate of 0.5 ml/min. The Merck Hitachi HPLC unit (L-400 UV detector, L-6210 intelligent pump, D-2500 chromato-integrator) was operated at about 20 bar, the optical density of the eluate was determined at 280 nm, and 0.5 ml fractions were collected, using an LKB 2111 Multisac fraction collector, and subsequently analyzed in an enzyme activity specificity test (Example 5). The experiment is depicted in Fig. 6. Based on the arrowed molecular weight positions of calibration proteins, it can be concluded that the functionally active Fab-E. coli β -glucuronidase fusion protein has a molecular weight of about 450 KD.

Example 5

Demonstration of the antigen-binding properties and the enzymic activity

The ability of the Fab-E. coli β -glucuronidase fusion protein to bind specifically to the Mab 431/26-defined epitope on CEA (carcino-embryonic antigen) and simultaneously to exert the enzymic activity of the β -glucuronidase was demonstrated in an enzyme activity specificity test (EP-A-0 501 215 A2, Example J). The test determines the liberation of 4-methylumbelliferone from 4-methylumbelliferyl- β -glucuronide by the β -glucuronidase moiety of the fusion protein after the fusion protein has bound to the antigen by its Fab moiety. The fluorescence values which were determined are given as relative fluorescence units (FU) (Tab. 1). The test demonstrates that the fusion protein elicits significant liberation of methylumbelliferone in the CEA-coated plates.

PEM (polymorphic epithelial mucin)-coated plates served

as the control. In these plates, the fluorescence signal was always less than 30 FU.

Tab. 1

5

	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
FU, Disrupted cell suspension 1:3	8183	8149	7531	6631	4560	2509	1019	421.9
FU, void volume 1:1	6548	5231	3222	1477	525.2	214	86.19	46.29
FU, pH 5.0 eluate 1:3	7782	7571	6360	4239	1983	815.7	302	113.9
FU, pH 5.0 eluate Ultraconcent. 1:10	7904	8106	8036	7153	5802	3618	1651	665.7
FU pH 5.0 eluate Ultrafiltrate 1:1	74.65	172.7	90.23	52.30	38.84	25.79	23.51	19.39

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

- 5 1. A process for the recombinant expression of an antibody, an antibody fragment or an antibody fragment-enzyme fusion molecule containing cytoplasmic mammalian or E. coli enzymes as fusion partner, which comprises:
- 10 a) transforming a thioredoxin reductase-deficient E. coli strain with a nucleotide molecule encoding said antibody, antibody fragment or antibody fragment-enzyme fusion molecule;
- 15 b) culturing said transformed E. coli strain and expressing said antibody, antibody fragment or antibody fragment-enzyme fusion molecule; and
- c) isolating said antibody, antibody fragment or antibody fragment-enzyme fusion molecule from cytoplasm of said transformed E. coli.
- 20 2. The process as claimed in claim 1, wherein said antibody fragment is selected from a Fab fragment or an sFv fragment.
- 25 3. The process as claimed in claim 1, wherein the antibody fragment of said antibody fragment-enzyme fusion molecule is directed against tumor cells and the enzyme of said antibody fragment-enzyme fusion molecule is able to cleave non-toxic prodrugs to give toxic drugs.
- 30 4. The process as claimed in claim 3, wherein said antibody fragment is humanized and said enzyme is a human cytoplasmic enzyme.

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FIG.1

E.c. β -Gluc for: 5' AAG CTT TCA TTG TTT GCC TCC CTG CTG CGG 3'

E.c. β -Gluc back: 5' TCT AGA CCA TGG TAC GTC CTG TAC AAA CCC CA 3'



PCR + Cloning into KS

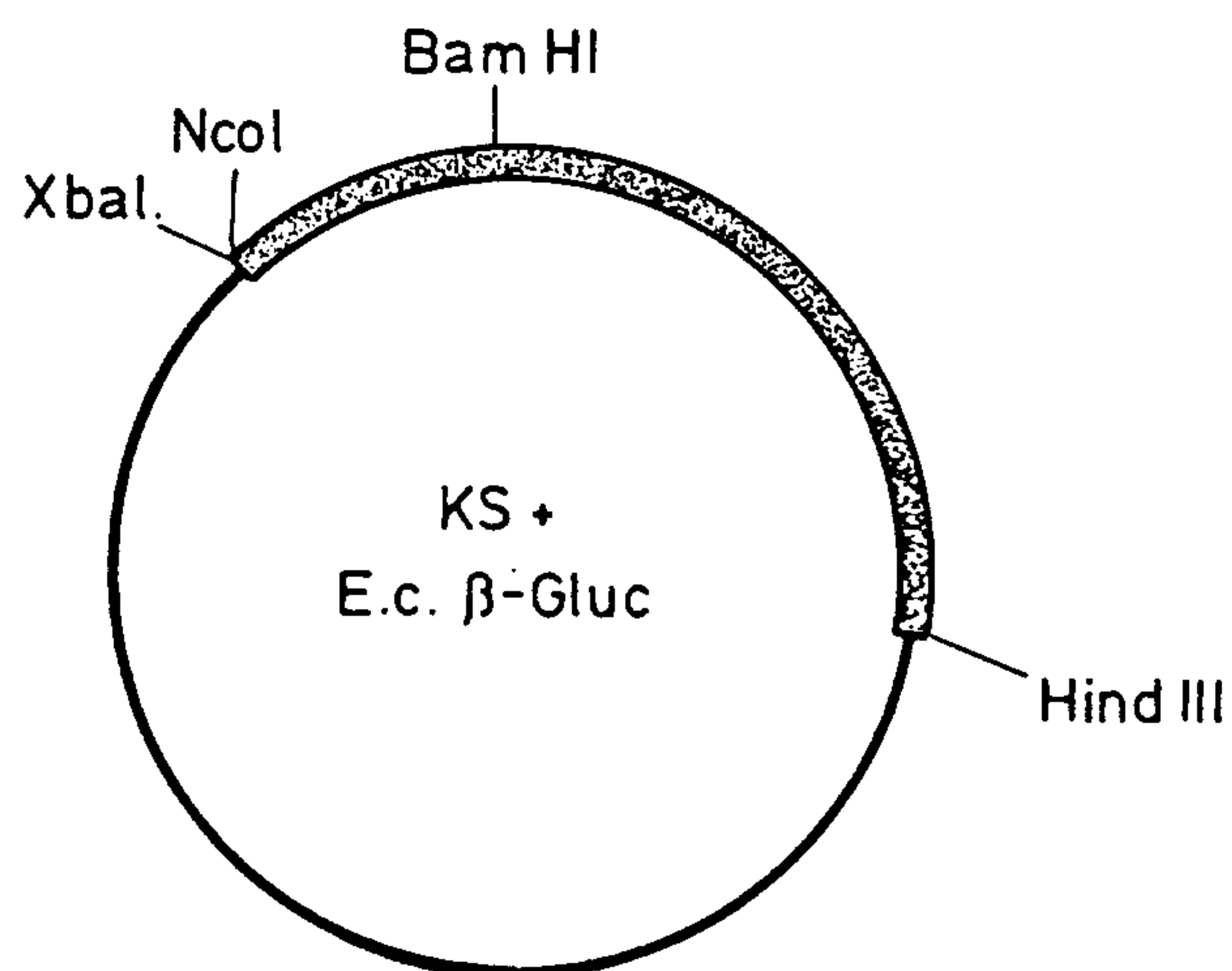


FIG. 2

Fab HC for: 5' GAA TTC CAT GGA ACC AGA ACC AGA ACC GAG CTC AAC TCT N2
 CTT GTC CAC CTT GGT GTT 3' link
 C H 1

Fab HC back: 5' TCT AGA TAA CGA GGG CAA AAA ATG GAG GTC CAA CTG CAG N2b
 GAG AGC 3' S D V H

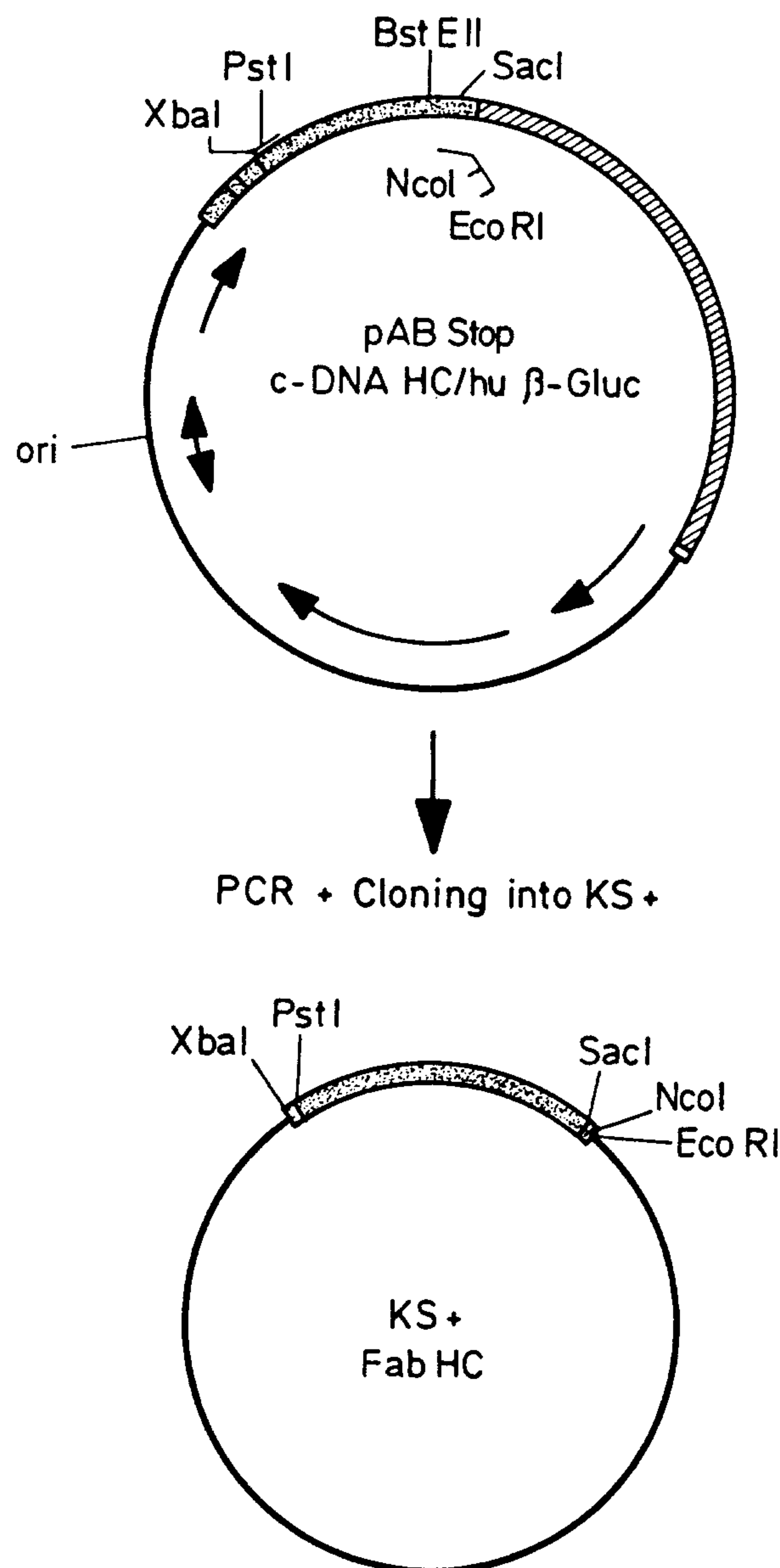


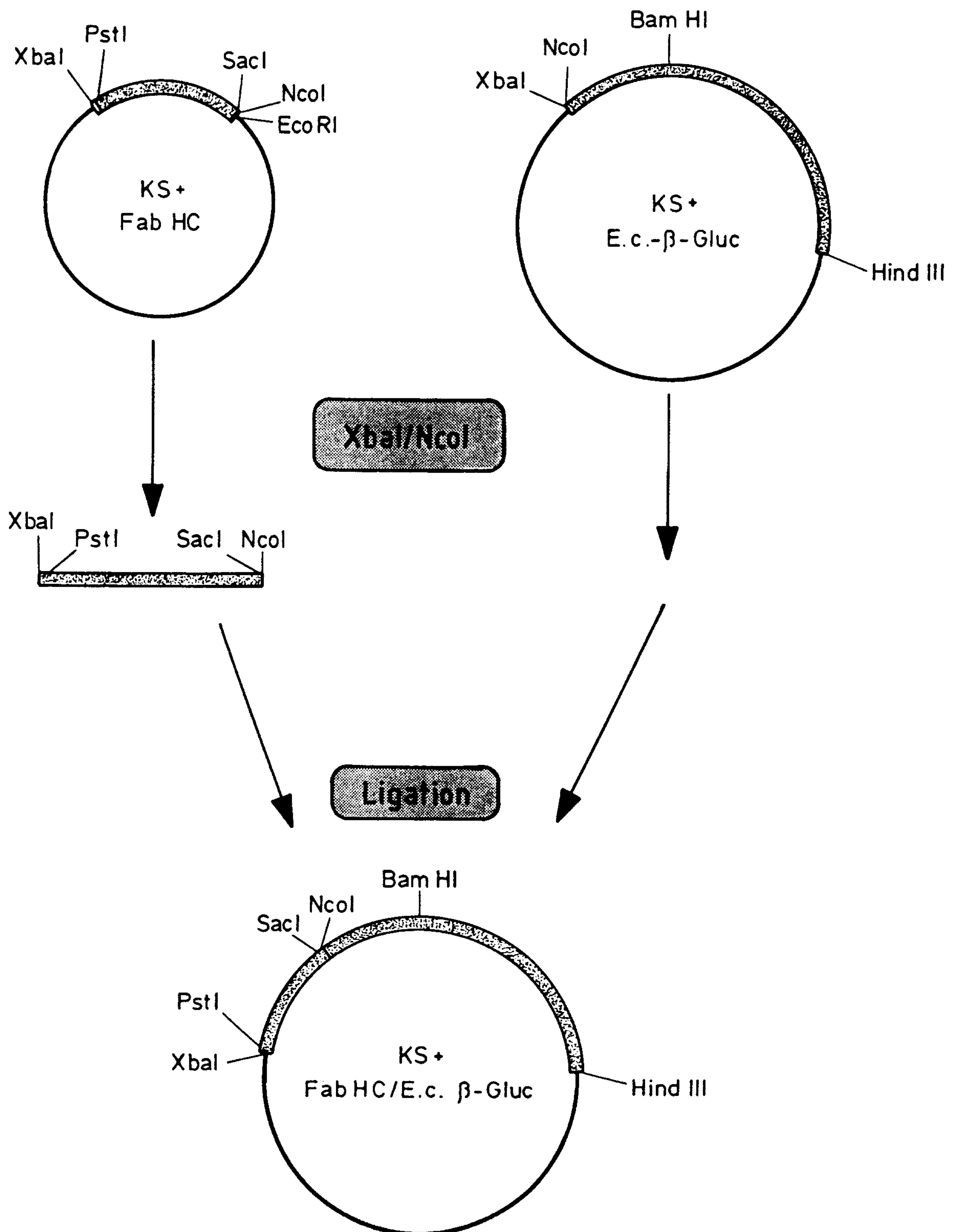
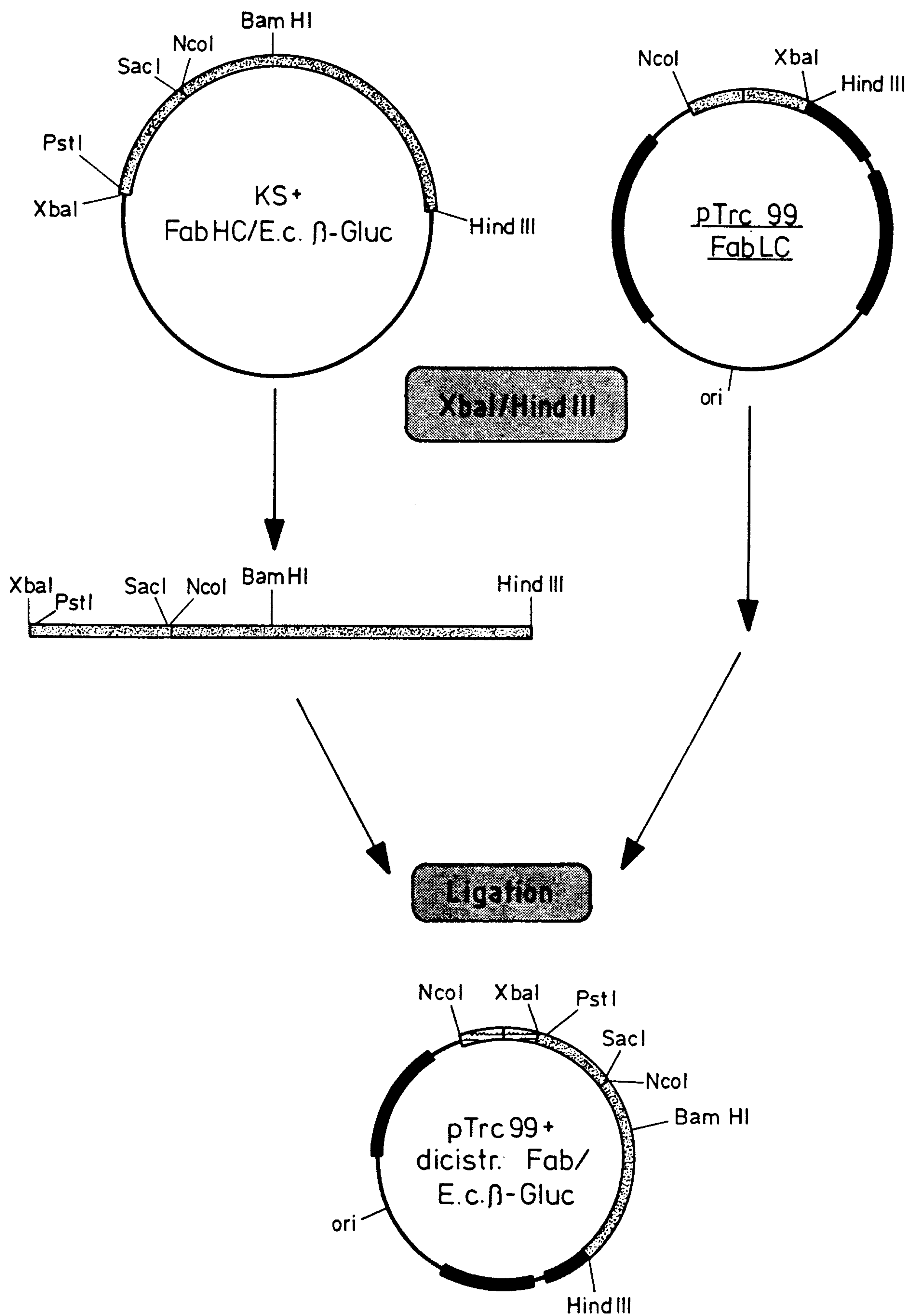
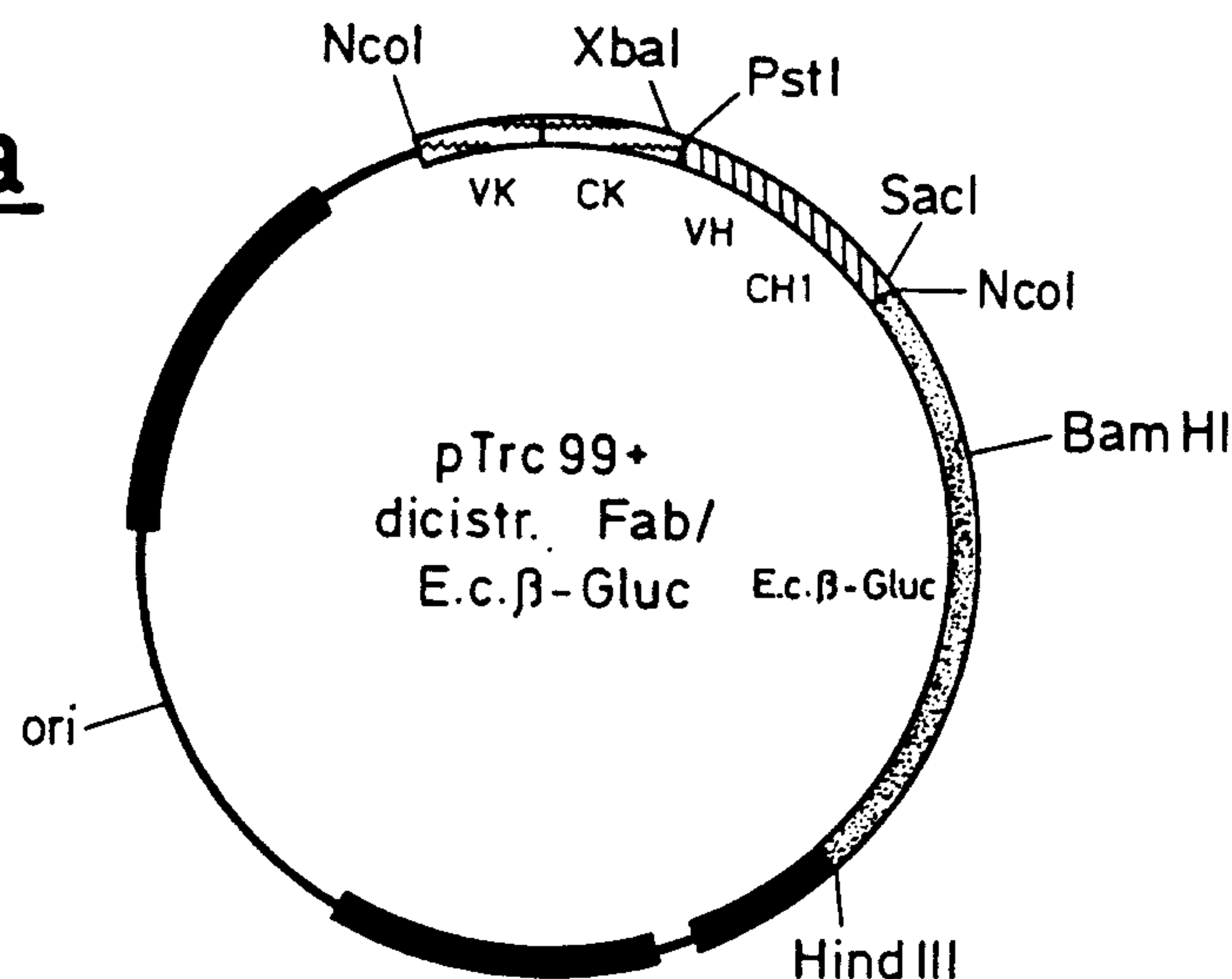
FIG. 3

FIG.4

431/26 hum dicistr. Fab/E.c.β-Glucuronidase in pTrc 99

FIG.5a



NcoI

1 CC ATG GAC ATC CAG ATG ACC CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT GAC AGA
1▶Met Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg N5

60 GTG ACC ATC ACC TGT AGT ACC AGC TCG AGT GTA AGT TAC ATG CAC TGG TAC CAG CAG AAG
20▶Val Thr Ile Thr Cys Ser Thr Ser Ser Ser Val Ser Tyr Met His Trp Tyr Gln Gln Lys

120 CCA GGT AAG GCT CCA AAG CTG CTG ATC TAC AGC ACA TCC AAC CTG GCT TCT GGT GTG CCA VK
40▶Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro

180 AGC AGA TTC AGC GGT AGC GGT AGC GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC CTC CAG
60▶Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln

240 CCA GAG GAC ATC GCC ACC TAC TAC TGC CAT CAG TGG AGT AGT TAT CCC ACG TTC GGC CAA
80▶Pro Glu Asp Ile Ala Thr Tyr Tyr Cys His Gln Trp Ser Ser Tyr Pro Thr Phe Gly Gln

300 GGG ACC AAG GTG GAA ATC AAA CGT ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC CCG CCA
100▶Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro

360 TCT GAT GAG CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC TTC TAT
120▶Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr

420 CCC AGA GAG GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC TCC CAG CK
140▶Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln

480 GAG AGT GTC ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC CTG ACG
160▶Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr

540 CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC TGC GAA GTC ACC CAT CAG GGC
180▶Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly

600 CTG AGC TCG CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG TGT TAG TCTAGATAACGAGGGCAAAA XbaI
200▶Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys ...

PstI

664 AA ATG GAG GTC CAA CTG CAG GAG AGC GGT CCA GGT CTT GTG AGA CCT AGC CAG ACC CTG
1▶Met Glu Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg Pro Ser Gln Thr Leu

FIG.5b

723 AGC CTG ACC TGC ACC GTG TCT GGC TTC ACC ATC AGC AGT GGT TAT AGC TGG CAC TGG GTG
20 Ser Leu Thr Cys Thr Val Ser Gly Phe Thr Ile Ser Ser Gly Tyr Ser Trp His Trp Val

783 AGA CAG CCA CCT GGA CGA GGT CTT GAG TGG ATT GGA TAC ATA CAG TAC AGT GGT ATC ACT
40 Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp Ile Gly Tyr Ile Gln Tyr Ser Gly Ile Thr

VH

843 AAC TAC AAC CCC TCT CTC AAA AGT AGA GTG ACA ATG CTG GTA GAC ACC AGC AAG AAC CAG
60 Asn Tyr Asn Pro Ser Leu Lys Ser Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn Gln

903 TTC AGC CTG AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC GCG GTC TAT TAT TGT GCA AGA
80 Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys Ala Arg

963 GAA GAC TAT GAT TAC CAC TGG TAC TTC GAT GTC TGG GGT CAA GGC AGC CTC GTC ACA GTC
100 Glu Asp Tyr Asp Tyr His Trp Tyr Phe Asp Val Trp Gly Gln Gly Ser Leu Val Thr Val

1023 ACA GTC TCC TCA GCT TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCG CCC TGC TCC AGG
120 Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg

1083 AGC ACC TCT GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC AAG GAC TAC TTC CCC GAA CCG
140 Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro

CH1

1143 GTG ACG GTG TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG GCT GTC
160 Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val

1203 CTA CAG TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC TCC AGC AGC TTG
180 Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu

1263 GGC ACC CAG ACC TAC ACC TGC AAC GTG AAT CAC AAG CCC AGC AAC ACC AAG GTG GAC AAG
200 Gly Thr Gln Thr Tyr Thr Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys
link

SacI NcoI
1323 AGA GTT GAG CTC GGT TCT GGT TCT GGT TCC ATG GTA CGT CCT GTA GAA ACC CCA ACC CGT
220 Arg Val Glu Leu Gly Ser Gly Ser Gly Ser Met Val Arg Pro Val Glu Thr Pro Thr Arg

1383 GAA ATC AAA AAA CTC GAC GGC CTG TGG GCA TTC AGT CTG GAT CCG GAA AAC TGT GGA ATT
240 Glu Ile Lys Lys Leu Asp Gly Leu Trp Ala Phe Ser Leu Asp Arg Glu Asn Cys Gly Ile

1443 GAT CAG CGT TGG TGG GAA AGC GCG TTA CAA GAA AGC CCG GCA ATT GCT GTG CCA GGC AGT
260 Asp Gln Arg Trp Trp Glu Ser Ala Leu Gln Glu Ser Arg Ala Ile Ala Val Pro Gly Ser

1503 TTT AAC GAT CAG TTC GCC GAT GCA GAT ATT CGT AAT TAT GCG GGC AAC GTC TGG TAT CAG
280 Phe Asn Asp Gln Phe Ala Asp Ala Asp Ile Arg Asn Tyr Ala Gly Asn Val Trp Tyr Gln

E.c. β -Gluc

1563 CCG GAA GTC TTT ATA CCG AAA GGT TGG GCA GGC CAG CGT ATC GTG CTG CGT TTC GAT GCG
300 Arg Glu Val Phe Ile Pro Lys Gly Trp Ala Gly Gln Arg Ile Val Leu Arg Phe Asp Ala

1623 GTC ACT CAT TAC GGC AAA GTG TGG GTC AAT AAT CAG GAA GTG ATG GAG CAT CAG GGC GGC
320 Val Thr His Tyr Gly Lys Val Trp Val Asn Asn Gln Glu Val Met Glu His Gln Gly Gly

1683 TAT ACG CCA TTT GAA GCC GAT GTC ACG CCG TAT GTT ATT GGC GGG AAA AGT GTA CGT ATC
340 Tyr Thr Pro Phe Glu Ala Asp Val Thr Pro Tyr Val Ile Ala Gly Lys Ser Val Arg Ile

1743 ACC GTT TGT GTG AAC AAC GAA CTG AAC TGG CAG ACT ATC CCG CCG GGA ATG GTG ATT ACC
360 Thr Val Cys Val Asn Asn Glu Leu Asn Trp Gln Thr Ile Pro Pro Gly Met Val Ile Thr

BamHI
1803 GAC GAA AAC GGC AAG AAA AAG CAG TCT TAC TTC CAT AAT TTC TTT AAC TAT GCC GCG ATC
380 Asp Glu Asn Gly Lys Lys Lys Gln Ser Tyr Phe His Asn Phe Phe Asn Tyr Ala Gly Ile

1863 CAT CCG AGC GTA ATG CTC TAC ACC ACG CCG AAC ACC TGG GTG GAC GAT ATC ACC GTG GTG
400 His Arg Ser Val Met Leu Tyr Thr Thr Pro Asn Thr Trp Val Asp Asp Ile Thr Val Val

1923 ACG CAT GTC GCG CAA GAC TGT AAC CAC GCG TCT GTT GAC TGG CAG GTG GTG GCC AAT GGT
420 Thr His Val Ala Gln Asp Cys Asn His Ala Ser Val Asp Trp Gln Val Val Ala Asn Gly

FIG.5c

1983 GAT GTC AGC GTT GAA CTG CGT GAT GCG GAT CAA CAG GTG GTT GCA ACT GGA CAA GGC ACT
 440▶ Asp Val Ser Val Glu Leu Arg Asp Ala Asp Gln Gln Val Val Ala Thr Gly Gln Gly Thr

2043 AGC GCG ACT TTG CAA GTG GTG AAT CCG CAC CTC TGG CAA CCG GGT GAA GGT TAT CTC TAT
 460▶ Ser Gly Thr Leu Gln Val Val Asn Pro His Leu Trp Gln Pro Gly Glu Gly Tyr Leu Tyr

2103 GAA CTG TGC GTC ACA GCC AAA AGC CAG ACA GAG TGT GAT ATC TAC CCG CTT CGC GTC GGC
 480▶ Glu Leu Cys Val Thr Ala Lys Ser Gln Thr Glu Cys Asp Ile Tyr Pro Leu Arg Val Gly

2163 ATC CCG TCA GTG GCA GTG AAG GGC GAA CAG TTC CTG ATT AAC CAC AAA CCG TTC TAC TTT
 500▶ Ile Arg Ser Val Ala Val Lys Gly Glu Gln Phe Leu Ile Asn His Lys Pro Phe Tyr Phe

2223 ACT GGC TTT GGT CGT CAT GAA GAT GCG GAC TTA CGT GGC AAA GGA TTC GAT AAC GTG CTG
 520▶ Thr Gly Phe Gly Arg His Glu Asp Ala Asp Leu Arg Gly Lys Gly Phe Asp Asn Val Leu

2283 ATG GTG CAC GAC CAC GCA TTA ATG GAC TGG ATT GGG GCC AAC TCC TAC CGT ACC TCG CAT
 540▶ Met Val His Asp His Ala Leu Met Asp Trp Ile Gly Ala Asn Ser Tyr Arg Thr Ser His

2343 TAC CCT TAC GCT GAA GAG ATG CTC GAC TGG GCA GAT GAA CAT GGC ATC GTG GTG ATT GAT
 560▶ Tyr Pro Tyr Ala Glu Glu Met Leu Asp Trp Ala Asp Glu His Gly Ile Val Val Ile Asp

2403 GAA ACT GCT GCT GTC GGC TTT AAC CTC TCT TTA GGC ATT GGT TTC GAA GCG GGC AAC AAG
 580▶ Glu Thr Ala Ala Val Gly Phe Asn Leu Ser Leu Gly Ile Gly Phe Glu Ala Gly Asn Lys

2463 CCG AAA GAA CTG TAC AGC GAA GAG GCA GTC AAC GGG GAA ACT CAG CAA GCG CAC TTA CAG
 600▶ Pro Lys Glu Leu Tyr Ser Glu Glu Ala Val Asn Gly Glu Thr Gln Gln Ala His Leu Gln

2523 GCG ATT AAA GAG CTG ATA GCG CGT GAC AAA AAC CAC CCA AGC GTG GTG ATG TGG AGT ATT
 620▶ Ala Ile Lys Glu Leu Ile Ala Arg Asp Lys Asn His Pro Ser Val Val Met Trp Ser Ile

2583 GCC AAC GAA CCG GAT ACC CGT CCG CAA GGT GCA CCG GAA TAT TTC GCG CCA CTG GCG GAA
 640▶ Ala Asn Glu Pro Asp Thr Arg Pro Gln Gly Ala Arg Glu Tyr Phe Ala Pro Leu Ala Glu

2643 GCA ACG CGT AAA CTC GAC CCG ACG CGT CCG ATC ACC TGC GTC AAT GTA ATG TTC TGC GAC
 660▶ Ala Thr Arg Lys Leu Asp Pro Thr Arg Pro Ile Thr Cys Val Asn Val Met Phe Cys Asp

2703 GCT CAC ACC GAT ACC ATC AGC GAT CTC TTT GAT GTG CTG TGC CTG AAC CGT TAT TAC GGA
 680▶ Ala His Thr Asp Thr Ile Ser Asp Leu Phe Asp Val Leu Cys Leu Asn Arg Tyr Tyr Gly

2763 TGG TAT GTC CAA AGC GGC GAT TTG GAA ACG GCA GAG AAG GTA CTG GAA AAA GAA CTT CTG
 700▶ Trp Tyr Val Gln Ser Gly Asp Leu Glu Thr Ala Glu Lys Val Leu Glu Lys Glu Leu Leu

2823 GCC TGG CAG GAG AAA CTG CAT CAG CCG ATT ATC ATC ACC GAA TAC GGC GTG GAT ACG TTA
 720▶ Ala Trp Gln Glu Lys Leu His Gln Pro Ile Ile Ile Thr Glu Tyr Gly Val Asp Thr Leu

2883 GCC GGG CTG CAC TCA ATG TAC ACC GAC ATG TGG AGT GAA GAG TAT CAG TGT GCA TGG CTG
 740▶ Ala Gly Leu His Ser Met Tyr Thr Asp Met Trp Ser Glu Glu Tyr Gln Cys Ala Trp Leu

2943 GAT ATG TAT CAC CGC GTC TTT GAT CGC GTC AGC GCC GTC GTC GGT GAA CAG GTA TGG AAT
 760▶ Asp Met Tyr His Arg Val Phe Asp Arg Val Ser Ala Val Val Gly Glu Gln Val Trp Asn

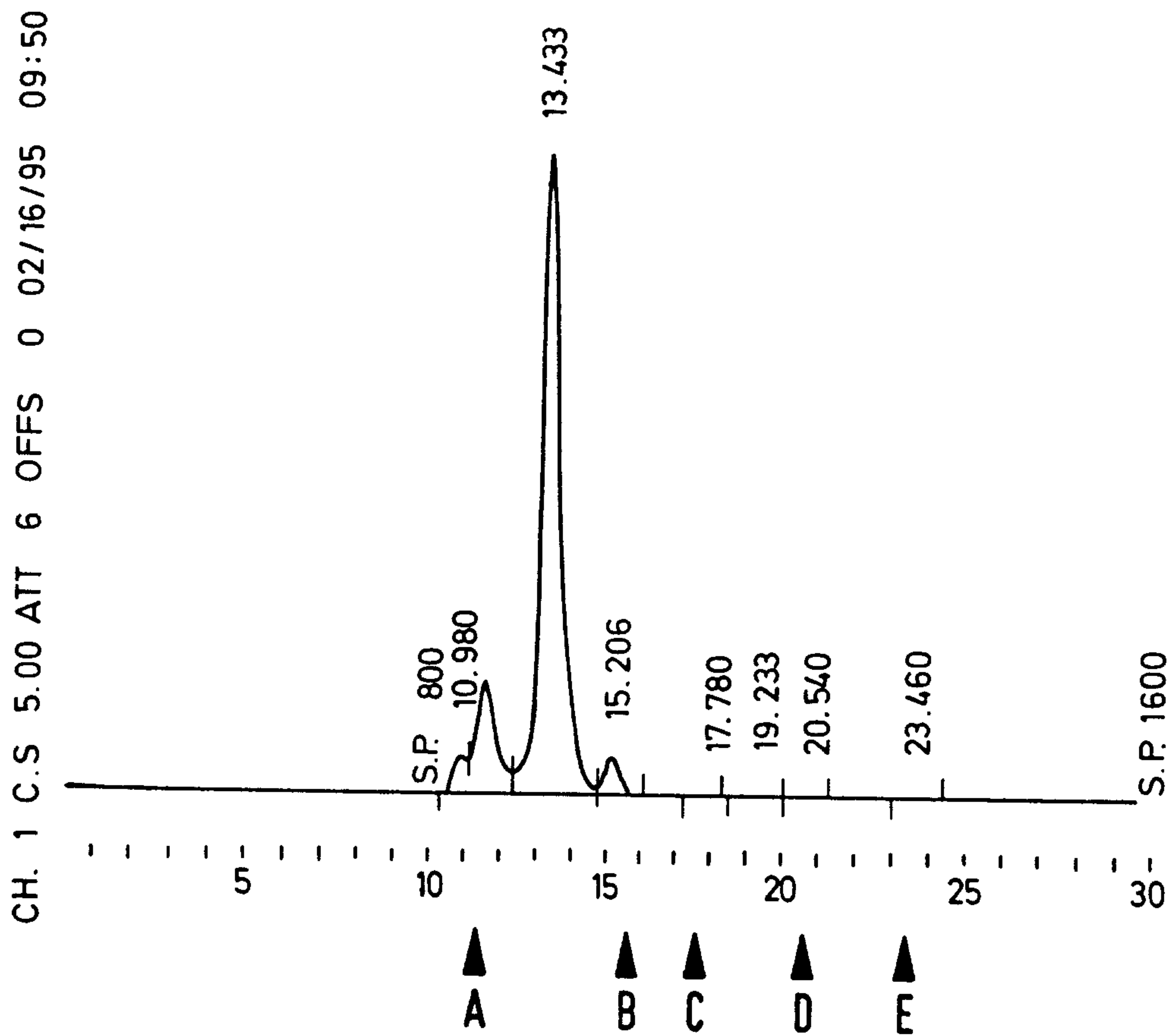
3003 TTC GCC GAT TTT GCG ACC TCG CAA GGC ATA TTG CCG GTT GGC GGT AAC AAG AAA GGG ATC
 780▶ Phe Ala Asp Phe Ala Thr Ser Gln Gly Ile Leu Arg Val Gly Gly Asn Lys Lys Gly Ile

3063 TTC ACT CCG GAC CGC AAA CCG AAG TCG GCG GCT TTT CTG CTG CAA AAA CCG TGG ACT GGC
 800▶ Phe Thr Arg Asp Arg Lys Pro Lys Ser Ala Ala Phe Leu Leu Gln Lys Arg Trp Thr Gly

HindIII
 3123 ATG AAC TTC GGT GAA AAA CCG CAG CAG GGA GGC AAA CAA TGA AGCTT
 820▶ Met Asn Phe Gly Glu Lys Pro Gln Gln Gly Gly Lys Gln ...

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FIG.6



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D-2500 METHOD: TSK 3000 A TAG: 150 CH: 1

TSK - 3000 Gel chromatography. Retention time of the fusion protein, 13.4 min.

A: Thyroglobulin 670.000 D

B: Gamma - Globulin 158.000 D

C: Ovalbumin 44.000 D

D: Myoglobin 17.000 D

E: Vitamin B-12 1.350 D