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(54) Titre : ANTICORPS MONOCLONAUX CONTRE DES ANTIGENES ASSOCIES AUX TUMEURS, PROCEDES DE PREPARATION ET UTILISATION DE CEUX-CI

(54) Title: MONOCLONAL ANTIBODIES AGAINST TUMOR-ASSOCIATED ANTIGENS, PROCESSES FOR THE PREPARATION THEREOF AND THE USE THEREOF

(57) Abrégé/Abstract:
The invention relates to monoclonal antibodies against a tumor-associated antigen which is mainly derived from tumors from the group of carcinomas of the breast, ovaries and prostate, as well as adenocarcinomas of the lung, which additionally react with polymorphic epithelial mucin (PEM), to the preparation and use thereof and to the use of the epitope defined by the antibody for diagnosis and therapy.

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Abstract of the disclosure

Monoclonal antibodies against tumor-associated antigens, processes for the preparation thereof and the use thereof

The invention relates to monoclonal antibodies against a tumor-associated antigen which is mainly derived from tumors from the group of carcinomas of the breast, ovaries and prostate, as well as adenocarcinomas of the lung, which additionally react with polymorphic epithelial mucin (PEM), to the preparation and use thereof and to the use of the epitope defined by the antibody for diagnosis and therapy.

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Auslandstext

Monoclonal antibodies against tumor-associated antigens, processes for the preparation thereof and the use thereof

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The invention relates to monoclonal antibodies against a tumor-associated antigen which is mainly derived from tumors from the group of carcinomas of the breast, ovaries and prostate, as well as adenocarcinomas of the lung, which additionally react with polymorphic epithelial mucin (PEM), to the preparation and use thereof and to the use of the epitope defined by the antibody for diagnosis and therapy.

Hybridoma technology has made it possible to prepare specific monoclonal antibodies (MAbs) even against unpurified antigens. This fact has made it possible to identify a large number of tumor-associated antigens (TAAs) which occur on certain human tumors but also on normal human tissues. Examples of such TAAs are CEA (carcinoembryonic antigen), N-CAM (neural cell adhesion molecule) and PEM (polymorphic epithelial mucin).

CEA is mainly expressed on adenocarcinomas of the gastro-intestinal tract, N-CAM is located on tumors derived from neuroectoderm, and PEM occurs mainly on carcinomas of the breast and ovaries. The TAAs which have just been mentioned are high molecular weight glycoproteins which carry a large number of immunogenic epitopes for the murine immune system. Comparative immunohistochemical investigations on cryopreserved human tissues demonstrate that the specificity of an MAb which recognizes with its idiotype (V region) an epitope I on a TAA may show a different tissue binding than an MAb which recognizes an epitope II (Buchegger et al. (1984), Int. J. Cancer 33:

643 - 649).

Multifarious reasons are possible for this observation: crypticity of epitopes in certain tissues, cross-reactive epitopes on different antigens, changes in conformation of antigens on secretion from tissues into the plasma (Bosslet et al. (1988), Eur. J. Nucl. Med. 14: 523 - 528) etc. It may be concluded from this that the specificity of an MAb is not unambiguously given by the definition of the recognized antigen but is given by the exact description of the V region of the MAb in conjunction with its immunohistochemical specificity for cryopreserved human tissues and its serum specificity with circulating TAA structures in human serum or plasma.
Thus, for example, a number of MAbs against the PEM which was isolated by Shimizu from human milk, (Shimizu, M. and Yamauchi, K. (1982), J. Biochem. 91, 515 - 524) have been developed and bind to different epitopes and, accordingly, have different properties (Girling et al. (1989), 43, 1072 - 1076, Taylor-Papadimitriou et al. (1981), Int. J. Cancer, 28, 17 - 21).

Certain anti-PEM MAbs (HMFG 1,2) show a strong reaction with human carcinomas of the breast and ovaries but also react significantly with normal human tissues (Taylor-Papadimitriou et al. (1981)). Other MAbs (SM 3, Girling et al. (1989)) react more weakly and heterogeneously with carcinomas of the breast and ovaries but, on the other hand, do not bind significantly to normal human tissue.

We have succeeded, surprisingly, in producing an anti-PEM MAb which reacts <u>strongly</u> with carcinomas of the breast, ovaries and prostate, as well as adenocarcinomas of the lung, binds only weakly to normal human tissue and, in addition, is able to detect PEM very specifically in human serum or plasma. Methods for the immunochemical determination of antigen are known to the person skilled in the art (see Gosling (1990), Clin. Chem. 36/8, 1408 -

1427). In this connection a distinction is made essentially between two classes: homogeneous assays such as, for example, particle-enhanced nephelometry or turbidimetry and heterogeneous methods, also called solid-phase assays.
Solid-phase assays are designed so that the analyte antigen is immobilized out of the sample to be investigated by a trapping antibody bound to a solid phase, and the immobilized antigen is detected by a second antibody provided with a detectable labeling moiety (conjugate). Detectable labels of this type are known to the person skilled in the art, and examples are enzymes, chemiluminescent or electrochemiluminescent, radioactive or else colored labels.

The hybridoma cell line BW 835 which produces the monoclonal antibody BW 835 was deposited on Oct. 11, 1991, at DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-3300 Braunschweig, under the number DSM ACC2022.

Antibodies within the meaning of this invention also mean antibody fragments.

The preparation and the properties of this MAb (BW 835) are described below:

The MAb was generated by immunizing Balb/c mice with the MCF-7 and SW-613 breast carcinoma cell lines by methods known from the literature (EP-A2-0 141079).

The distribution of the epitope defined by the MAb BW 835 on cryopreserved human carcinomas and normal human tissues is shown in Tables Ia and b respectively, comparing with the MAb SM-3 (Girling et al. 1989). The data are based on an immunohistochemical detection with the APAAP technique (Cordell at al. (1984), J. Histochem. Cytochem. 32, 219). It can clearly be seen that MAb BW 835 reacts strongly with all 15 carcinomas of the breast from 15 tested carcinomas of the breast, whereas the MAb SM-3 detects only 11 of 15 carcinomas of the breast.

In the case of carcinoma of the ovaries, the MAb BW 835 reacts strongly with 6 of 8 tested tumors, and the MAb SM-3 reacts with a few cells in 6 of 8 carcinomas. With all the other carcinoma types tested, especially the adenocarcinomas of the lung and the carcinomas of the prostate, MAb BW 835 shows a quantitatively stronger reaction. These data show that MAb BW 835 detects more carcinomas with a quantitatively stronger reaction than the MAb SM-3 disclosed in the literature.

The binding of MAb BW 835 to normal human tissue is shown in Table Ib. The APAAP technique was employed to obtain the data in this case too. The epitope defined by MAb BW 835 is expressed significantly on the ductal epithelium of the breast and the ductal epithelium of the pancreas, and is weakly expressed on the surface epithelium of the lungs, on some nerve fibers and on the collecting tubules and the glomeruli of the kidney. All the other tested normal tissues are negative.

Because of its high selectivity, the MAb BW 835 can also be used as inducer for internal image anti-paratope MAbs. MAbs of this type might be employed as epitope vaccine for the therapy of human tumors.

The demonstration that the epitope defined by the MAb BW 835 is located on the PEM defined by MAb SM-3 was checked by a double-determinant assay.

Table I a

Binding of the MAb BW 835 to cryopreserved human tumors

MAb	Human tumor type					
	Carcinomas of the breast pos./total	Ovarian carcinomas pos./total	Prostate carcinomas pos./total	Stomach carcinomas pos./total	Colon carcinomas pos./total	Pancreas carcinomas pos./total
BW 835	15/15	6/8	4°/5	4*/6	3*/4	4*/7
SM 3	11/15	6*/8	1°/5	3*/6	1°/4	3°/7

MAb		Lung carcinomas		
	SCLC pos./total	adeno pos./total	Squamous cell pos./total	Large cell pos./total
BW 835	4*/10	11°/12	5*/11	7°/11
SM 3	2*/10	6*/12	4*/11	3*/11

* a few cells positive ° a few areas of secreted products positive

Table I b

Binding of the MAb BW 835 to cryopreserved normal human tissue

Tissue type:

Mammary gland
Positive reaction with the acinar epithelium, positive apical staining of the epithelium in the ducts and in some secreting vesicles

Ovary
Negative

Pancreas
Positive apical staining in the ducts

Liver
Negative

Spleen
Negative

Colon
Negative

Stomach
Mucosa and some mucin-containing ducts with positive reaction

Lung
Surface epithelium of the lung with weak positive reaction

Kidney
Some glomeruli weakly stained, positive apical staining of the collecting tubules

Table I b continued

<u>Brain</u>
Negative

<u>Peripheral nerve</u>
Some nerve fibers weakly stained

<u>Bone marrow</u>
Negative

<u>Peripheral blood components</u>
Lymphocytes, monocytes, granulocytes, erythrocytes, platelets are negative

The MAb BW 835 used as trap was able to trap from cell culture supernatants of the T47 cell line an antigen which was detectable by the enzyme-labeled MAb SM-3. Furthermore, in the Western blot both MAbs stain molecules which correspond to the molecular weight position of PEM.

Once the immunological specificity data for the MAb BW 835 were defined, the mRNA was isolated from 10^8 hybridoma cells which secrete the MAb BW 835, the V genes of the heavy and light chains of the MAb BW 835 were isolated by the method described by Orlandi et al. (1989), Proc. Natl. Acad. Sci. USA: 86, 3833 - 3837, and the nucleic acid sequence of the essential regions of the V gene exons were determined by the method described by Sanger et al. (1977), Proc. Natl. Acad. Sci., USA: 74, 5463 - 5467 (Figs. 1a, b).

On repeated high-dose administration of MAbs of murine origin, such as, for example, the MAb BW 835, for in vivo therapy of humans it is possible to immunize the patients. They are able to produce human anti-mouse immunoglobulin antibodies (HAMA) after about 10-14 days

(Miller et al., (1983), Blood, 62, 988; Joseph et al., (1988), European Journal of Nuclear Medicine, 14, 367). These HAMAs may have unfavorable effects on the pharmacokinetics and pharmacodynamics of the MAb and impede continuation of the treatment.

In order to reduce the immunogenicity of xenogenic antibodies as far as possible, a technique in which only the CDR loops of the V_L and V_H domains of the xenogenic antibodies are transferred to V_L and V_H domains of human antibodies has been developed (Jones, P.T., et al., (1986), Nature, 321, 522) (EP-A-87302620, G. Winter), and this process is called "humanization" and takes place at the level of the V_H and V_L genes.

The technical procedure for humanization of an antibody is divided essentially into three sections: the cloning and nucleic acid sequence analysis of the specific V_H and V_L genes, the computer-assisted design of the synthetic oligonucleotides for the transfer of the CDR loops to the human V_H and V_L domains and the transfer of the CDR loops to human V_H and V_L domains by specific mutagenesis (Riechmann, L., et al., (1988), Nature, 332, 323; Verhoeyen, M., et al., (1988), Science, 239, 1534).

Humanization of this type can also be carried out on MAb BW 835 in order to improve its usability in vivo. This would entail the authentic CDR regions of the BW 835 V_H and V_L domains (defined by Kabat, E.A., et al. (1987) Sequences of Proteins of Immunological Interest, fourth edition, US Dept. of Health and Human Services, US Government Printing Office) or CDR regions with a few modified amino acids being transferred to human V_H and V_L domains, it being possible for a few amino acids of the framework regions located between the CDR regions to be taken over from the mouse antibody to the humanized antibody in order to minimise the change in the antigen-binding properties of the resulting MAb BW 835 in the humanized form.

The variable domains of the humMAb BW 835 are accordingly composed of the framework regions, which are authentic or modified at a few points, of the variable domains of a human MAb onto which the CDR regions which are authentic or have been modified at a few amino-acid positions of the mouse MAb BW 835 have been transplanted.

The following examples describe the steps necessary for cloning and nucleic acid sequence analysis of the V genes and for the expression of BW 835 specificity as chimeric MAb. The techniques used in Examples 1 - 12 were, unless otherwise indicated, taken from Molecular Cloning, a Laboratory Manual; Sambrook, Fritsch, Maniatis; Cold Spring Harbor Laboratory, 1982 (pages 11 - 44, 51 - 127, 133 - 134, 141, 146, 150 - 167, 170, 188 - 193, 197 - 199, 248 - 255, 270 - 294, 310 - 328, 364 - 401, 437 - 506) and from Molecular Cloning, A Laboratory Manual, Second Edition; Sambrook, Fritsch, Maniatis; Cold Spring Harbor Laboratory Press, 1989 (pages 16.2 - 16.22, 16.30 - 16.40, 16.54 - 16.55).

Example 1

The plasmid clone 54.1.24 which harbors the human $IgG_3\Delta C$ gene (Fig. 2) (DE-A1-38 25 615, Fig. 2) was cleaved with PstI. The vector resulting from this was ligated to the largest of the resulting PstI insert fragments and transformed into bacteria. The plasmid clone A which harbors a human $IgG_3\Delta C$ gene in which the H1, H2 and H3 exons have been deleted ($IgG3\Delta$) was identified by restriction analysis and nucleic acid sequence determination.

Example 2:

The plasmid clone A was cleaved with HindIII and EcoRI, the ends were filled in with Klenow polymerase, the $IgG_3\Delta$ insert was isolated and ligated into a pUC19 vector cleaved with SacI and provided with blunt ends with the aid of T_4 polymerase. A plasmid clone B in which the $IgG_3\Delta$ gene is orientated so that the HindIII cleavage site is located at the 5′ end and the EcoRI cleavage site is located at the 3′ end of the pUC19 polylinker was identified by restriction mapping and nucleic acid sequence analysis (Fig. 3).

Example 3:

The plasmid clone B was cleaved with EcoRI and HindIII, the $IgG_3\Delta$ insert was isolated and ligated into a KS+ plasmid vector (pBluescriptII KS+; Stratagene, La Jolla, CA) likewise cleaved with HindIII and EcoRI. The phasmid clone C in which the $IgG_3\Delta$ gene is flanked at the 5′ and at the 3′ end by a BamHI cleavage site was isolated (Fig. 4).

Example 4:

The phasmid clone C was cleaved with BamHI, the $IgG_3\Delta$ insert was isolated and ligated into the expression vector pABStop (Wirth et al. (1988), Gene, 73, 419 - 426) likewise cleaved with BamHI. The expression plasmid D which contains the $IgG_3\Delta$ C gene in the orientation shown in Fig. 5 was identified. In this cloning the pABStop vector loses the polyadenylation signal and SV40 stop located between the two BamHI cleavage sites.

Example 5:

The expression plasmid D was partially cleaved with BamHI, the ends were filled in with Klenow polymerase and religated. The expression plasmid E in which the BamHI cleavage site 3′ from the $IgG_3\Delta$ gene is destroyed was isolated (Fig. 6).

Example 6:

The human C kappa gene (Hieter et al. (1982), J. Biol. Chem., 257, No. 3, 1516 - 1522) was obtained as EcoRI fragment cloned in pBR 322 from Prof. P. Leder, Harvard Medical School. The pBR322 vector was cleaved with EcoRI, the EcoRI cleavage sites were filled in, the C kappa insert was isolated and ligated into a pUC19 vector cleaved with SmaI. The plasmid clone F in which the C kappa gene is flanked at the 5′ end by a HindIII after a BamHI cleavage site and at the 3′ end by an EcoRI cleavage site was isolated (Fig. 7).

Example 7:

The plasmid clone F was cleaved with HindIII and EcoRI, the C kappa insert was isolated and cloned into a HindIII/EcoRI-cleaved KS+ phasmid. The phasmid clone G in which the C kappa insert is flanked at the 5′ and at the 3′ end by a BamHI cleavage site was isolated (Fig. 8).

Example 8:

The phasmid clone G was cleaved with BamHI, the C kappa insert was isolated and cloned into a pAB stop vector cleaved with BamHI. The clone H in which the C kappa gene is orientated so that the HindIII cleavage site of the pAB stop vector is located at its 5′ end was identified by restriction mapping and nucleic acid sequence analysis (Fig. 9).

Example 9:

The clone H was partially cleaved with BamHI, the restriction ends were filled in and religated. The clone I in which the BamHI cleavage site 3′ of the C kappa gene is destroyed was identified by restriction mapping (Fig. 10).

Example 10:

The V_H and V_K genes of the MAb BW 835 were amplified using the PCR technique and specific oligonucleotides by the method of Orlandi et al. (1989) and cloned in KS+ vectors (Güssow and Seemann (1991), Methods in Enzymology, Vol. 203) which contained V_H and V_K genes with suitable restriction cleavage sites (Fig. 11 a for V_H amd b for V_K). The clones K1 and K2 which contain the V_H (K1) and V_K (K2) genes of the MAb BW 835 were isolated (Fig. 12).

Example 11:

The nucleic acid sequences of the V_H and V_K genes of the MAb BW 835 from the clones K1 and K2 were determined by the method of Sanger et al. (1977) (Figs. 1a, b). It is possible to generate mimetics based on the CDRs from this sequence by the method described by Saragovi et al. (Saragovi et al. (1991), Science 253, 792-795).

Furthermore, the V_H and V_K gene inserts were cut out of the clones K1 and K2 with the aid of the restriction endonucleases HindIII and BamHI and were cloned into the vectors D (V_H) and I (V_K) likewise cleaved with HindIII and BamHI. The expression vectors L1 and L2 which contain immunoglobulin heavy (L1) (Fig. 13) and light (L2) (Fig. 14) chain genes with the V genes of MAb BW 835 were isolated. The expression vectors L1 and L2 can be used for the expression of a chimeric MAb with the specificity of MAb BW 835.

Examples 12 and 13 are intended to explain the use of the MAb BW 835 for serodiagnosis of malignant tumors.

Example 12:

The MAb BW 835 was bound by adsorption to the walls of wells of microtiter plates (NUNC) by methods known to the person skilled in the art (Tijssen, P., "Practice and theory of enzyme immunoassay" Elsevier (1988), 297 - 328). 10 μl of sample were pipetted into each of the wells prepared in this way and each containing 100 μl of buffer (OSND, Behringwerke) and incubated at +37°C for 2 hours. After washing three times with diluted Enzygnost washing buffer (OSEW, Behringwerke), 100 μl of MAb BW 835 (1 μg/ml) which was conjugated to peroxidase by known methods were introduced into each of the individual wells. The following 2-hour incubation step at +37°C was completed by a cycle of three washes. Subsequently, for the third incubation step at room temperature, 100 μl of a buffer/substrate chromogen solution (OUVG/OUVF, Behringwerke) were pipetted into each of the wells, and the enzyme reaction was stopped after 30 minutes with 100 μl of Enzygnost stop solution (OSFA, Behringwerke). The extinction of the samples was determined as 450 nm.

Result:

The extinctions determined in this way correspond in the level thereof to the concentration of the antigen in the sample. The concentration of the antigen defined by the specific binding of MAb BW 835 in serum or plasma of patients with malignant tumors is distinctly raised by comparison with that in healthy patients (Fig. 15). This particularly applies to patients with late-stage carcinoma of the breast, but also, surprisingly, to those with early-stage carcinoma who, with other commercial tumor marker tests for detecting breast cancer-associated antigens in serum (for example CA 15-3), by comparison give false-negative findings significantly more often, and thus overall better sensitivities were found for the homologous version described.

Example 13:

It is also possible to use for the detection of PEM in serum in the double-determinant assay in combination with the MAb BW 835 other peroxidase-labeled antibodies which recognize further epitopes on the tumor-associated antigens defined by MAb BW 835. To do this, for example, a test analogous to Example 12 was carried out using the DF3 antibody disclosed in the literature (Kufe et al. (1984), Hybridoma 3, 223) in peroxidase-labeled form (CA 15-3-Test, Boehringer Mannheim) as conjugate component.

Result:

The use of, for example, DF3-POD as conjugate component to supplement the solid-phase-bound BW 835 produced distinctly higher serum values for the tumor sera compared with a normal serum pool and patients with benign diseases, which once again underlines the potential of the MAb BW 835 as specific component for a tumor marker test (Fig. 16).

WE CLAIM:

1. Monoclonal antibodies against a tumor-associated antigen, wherein the antigen derives from tumors principally from the group of carcinomas of the breast, ovaries and prostate, as well as adenocarcinomas of the lung, the antibodies andditionally react with polymorphic epithelial mucin (PEM) and wherein the antibodies bind an epitope which is bound by the monoclonal antibody which is produced by the hybridoma cell line DSM ACC2022.

2. A monoclonal antibody as claimed in claim 1, which is produced by the hybridoma cell line DSM ACC2022.

3. A specific binding partner which binds to the same epitope as the monoclonal antibody as claimed in claim 2.

4. Fusion proteins having the antigen-binding properties of a monoclonal antibody as claimed in claim 1.

5. An antibody as claimed in claim 1, wherein the non-antigen-binding parts in the amino-acid sequence correspond to a human sequence.

6. A hybridoma cell line which produces a monoclonal antibody as claimed in claim 1.

7. A hybridoma cell line as claimed in claim 6 having the deposition number DSM ACC2022.

8. A nucleic acid having the nucleotide sequence according to Fig. 1a or 1b.

9. A polypeptide having the amino acid sequence according to Fig. 1a or 1b.

10. A diagnostic aid containing a monoclonal antibody as claimed in claim 1.

11. An antibody as claimed in claim 1 for use in an in vitro diagnostic aid.

12. The use as claimed in claim 11 for detecting tumor-associated antigens in biological samples.

13. An antibody as claimed in claim 1 for use in an in vivo diagnostic aid.

14. An antibody as claimed in claim 1 for use as a therapeutic agent.

Fig.: 1a.

BW 835 VH

```
10                  30                  50
L  Q  S  L  R  A  L  V  Q  P  G  G  S  M  K  L  S  C  V  A
CTGCAGAGTCTGAGAGCCTTGGTGCAACCTGGAGGATCCATGAAACTCTCCTGTGTTGCC

     70                  90                  110
S  G  F  T  F  S  N  Y  W  M  N  W  V  R  Q  S  P  E  K  G
TCTGGATTCACTTTCAGTAACTACTGGATGAACTGGGTCCGCCAGTCTCCAGAGAAGGGG

     130                 150                 170
L  E  W  V  A  E  I  R  L  K  S  N  N  Y  A  T  H  Y  A  E
CTTGAGTGGGTTGCTGAAATTAGATTGAAATCTAATAATTATGCAACACATTATGCGGAG

     190                 210                 230
S  V  K  G  R  F  T  I  S  R  D  D  S  K  S  S  V  Y  L  Q
TCTGTGAAAGGGAGGTTCACCATCTCAAGAGATGATTCCAAAAGTAGTGTCTACCTGCAA

     250                 270                 290
M  N  N  L  R  A  E  D  T  G  I  Y  Y  C  I  R  E  T  V  F
ATGAACAACTTAAGAGCTGAAGACACTGGCATTTATTACTGTATCAGGGAGACGGTTTTT

     310                 330
Y  Y  Y  A  M  D  Y  W  G  Q  G  T  T  V  T
TATTACTATGCTATGGACTACTGGGGCCAAGGGACCACGGTCACC
```

Fig.: 1b.

BW 835 VK

```
        10                  30                  50
Q  L  T  Q  S  P  P  S  V  P  V  T  P  G  E  S  V  S  I  S
CAGCTGACCCAGTCTCCACCCTCTGTACCTGTCACTCCTGGAGAGTCAGTATCCATCTCC

        70                  90                  110
C  R  S  S  Q  S  L  L  H  G  D  G  N  T  Y  L  Y  W  F  L
TGCAGGTCTAGTCAGAGTCTCCTGCATGGTGATGGCAACACTTACTTGTATTGGTTCCTG

        130                 150                 170
Q  R  P  G  Q  S  P  R  L  L  I  Y  R  M  S  N  L  A  S  G
CAGAGGCCAGGCCAGTCTCCTCGGCTCCTGATATATCGGATGTCCAACCTTGCCTCAGGA

        190                 210                 230
V  P  D  R  F  S  G  S  G  S  G  T  A  F  T  L  R  I  S  R
GTCCCAGACAGGTTCAGTGGCAGTGGGTCAGGAACTGCTTTCACACTGAGAATCAGTAGA

        250                 270                 290
V  E  A  E  D  V  G  V  Y  Y  C  M  Q  H  L  E  Y  P  F  T
GTGGAGGCTGAGGATGTGGGTGTTTATTACTGTATGCAACATCTAGAATATCCTTTCACG

        310
F  G  G  G  K  V  E  I
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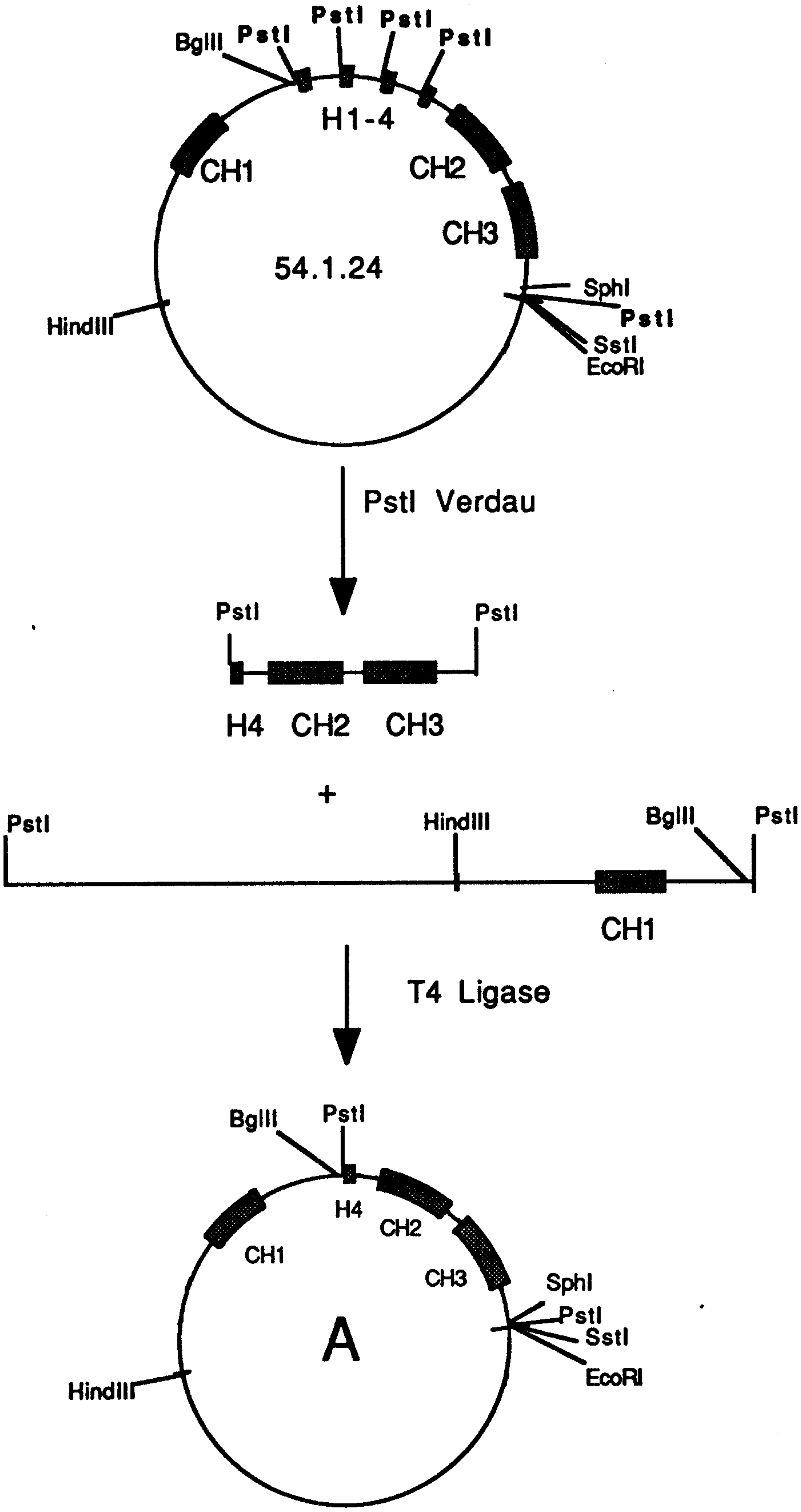
Fig. 2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CH2
CH3
54.1.24
HindIII
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PstI
SstI
EcoRI
PstI Verdau
PstI
PstI
H4
CH2
CH3
+
PstI
HindIII
BglII
PstI
CH1
T4 Ligase
BglII
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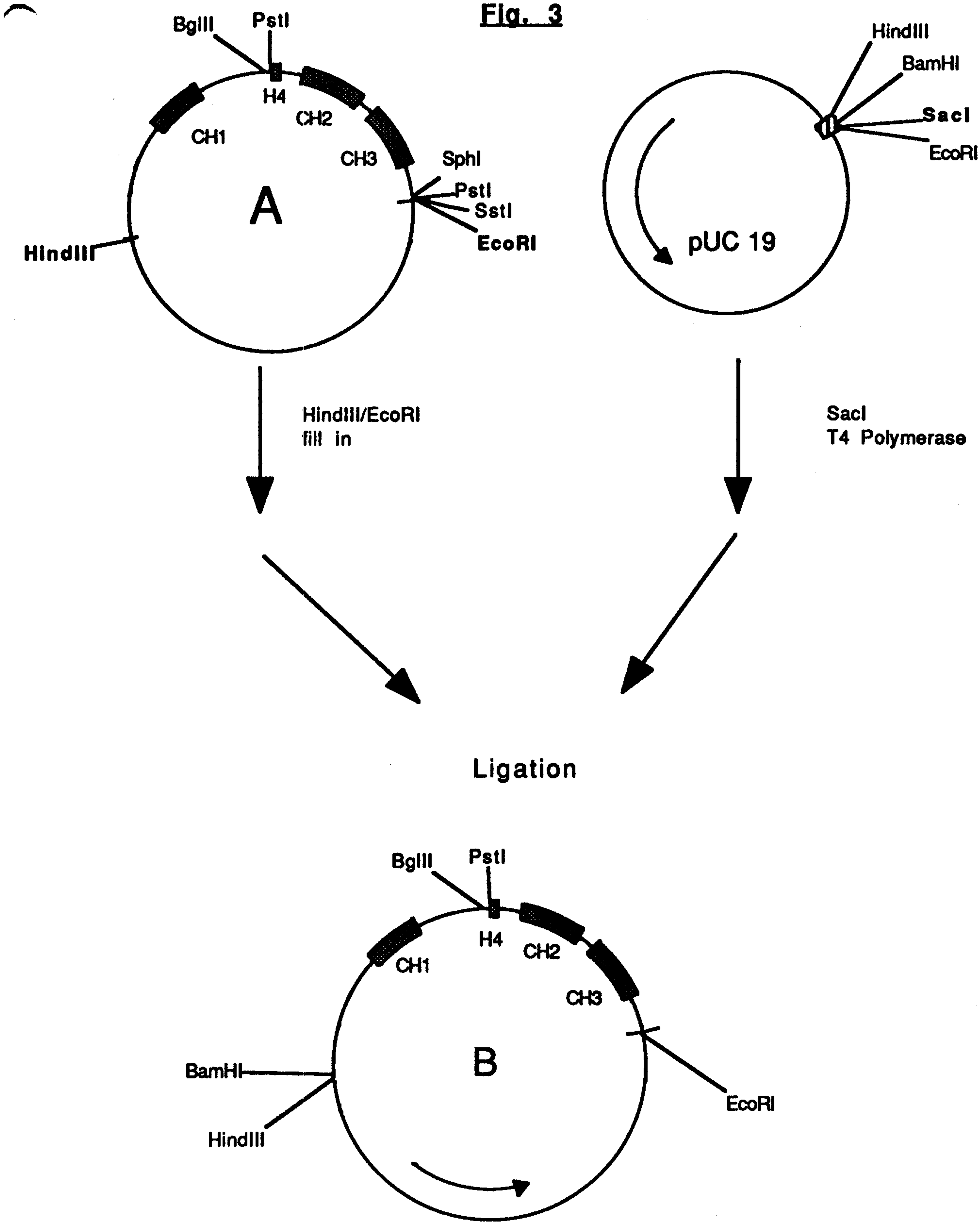
SstI
EcoRI
A
HindIII

Fig. 3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SacI
EcoRI
pUC 19
HindIII/EcoRI
fill in
SacI
T4 Polymerase
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EcoRI

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Fig.4

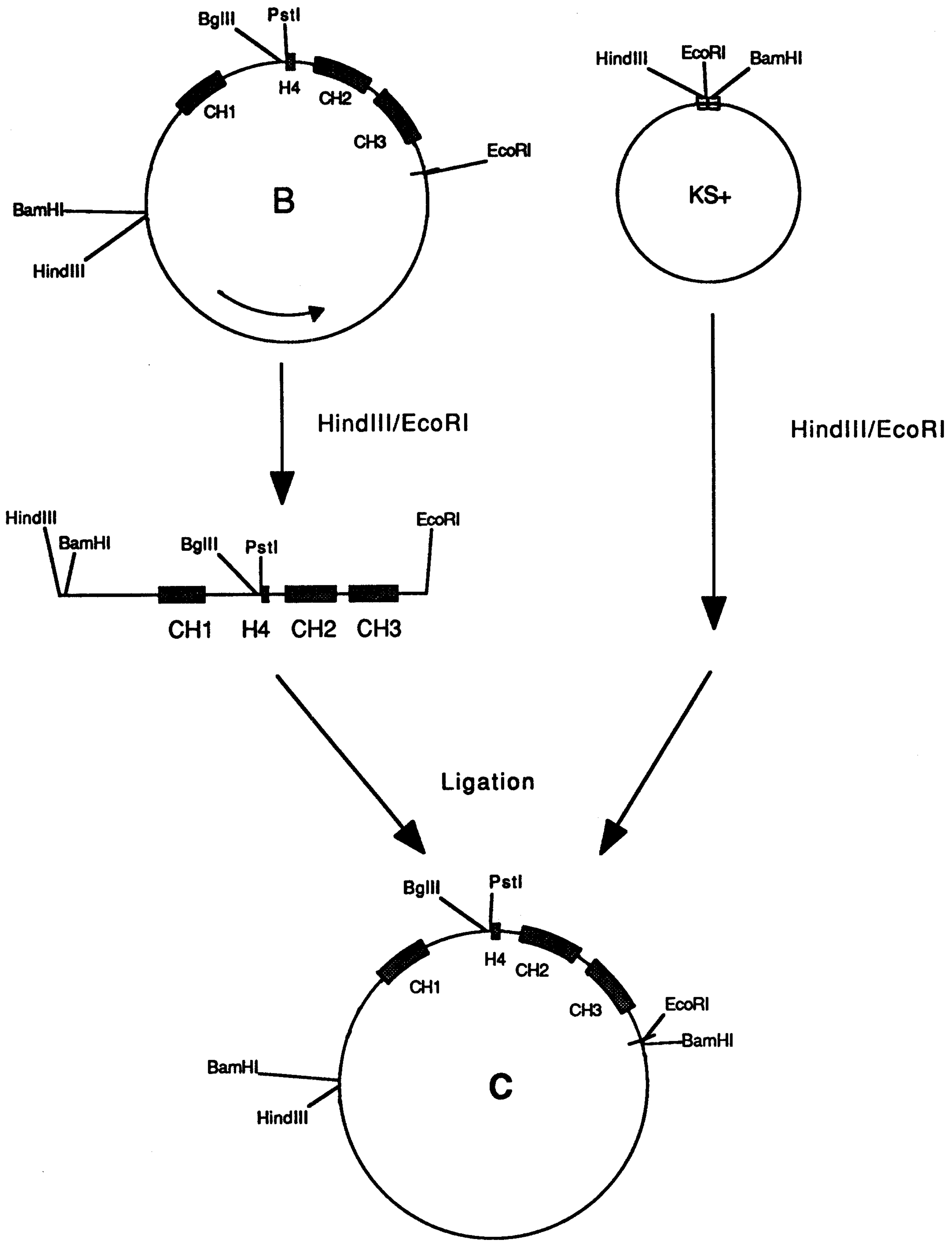
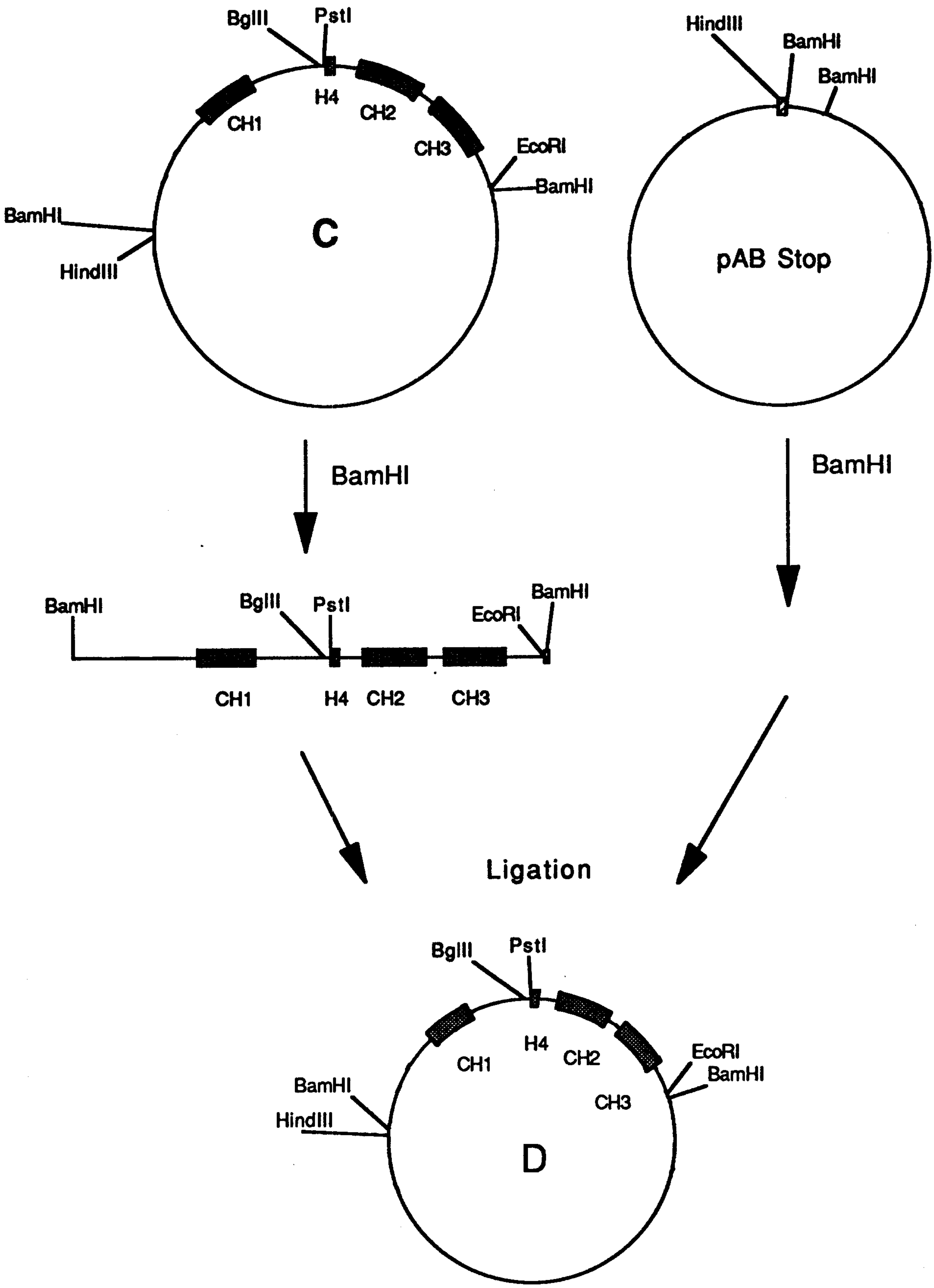


Fig. 5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BamHI
BamHI
BamHI
BglII
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EcoRI
BamHI
CH1
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Fig. 6

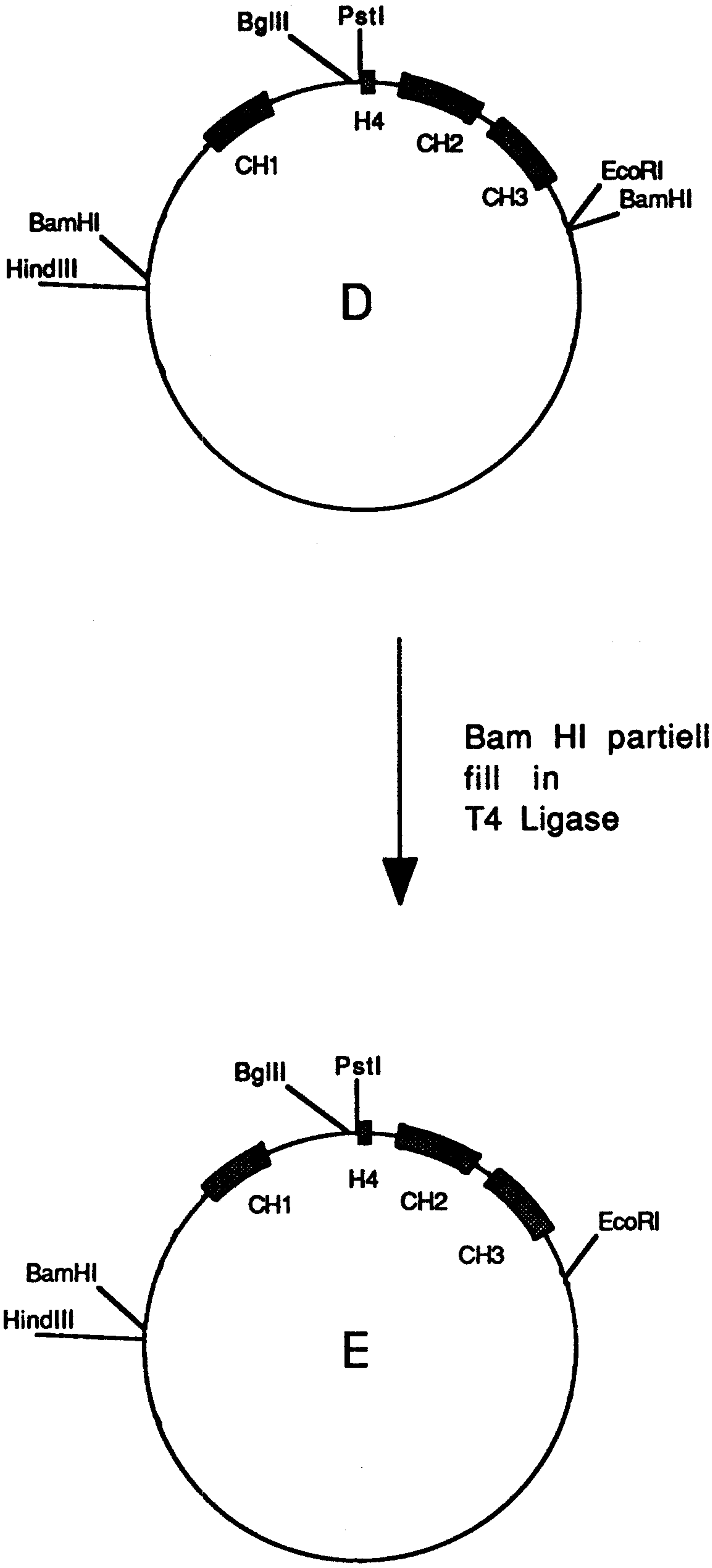


Fig. 7

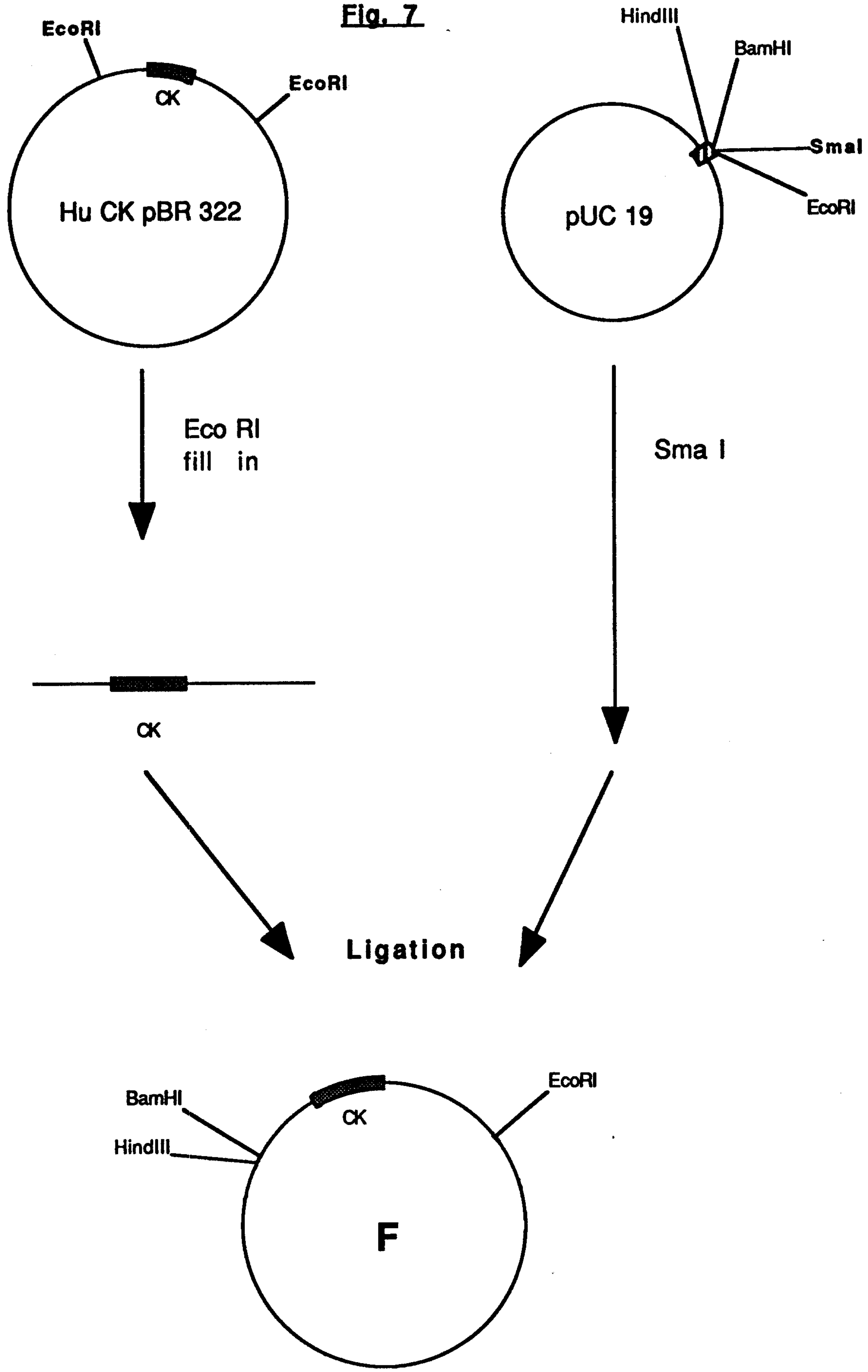

EcoRI
CK
EcoRI
Hu CK pBR 322
HindIII
BamHI
SmaI
EcoRI
pUC 19
Eco RI
fill in
Sma I
CK
Ligation
BamHI
HindIII
CK
EcoRI
F

Fig. 8

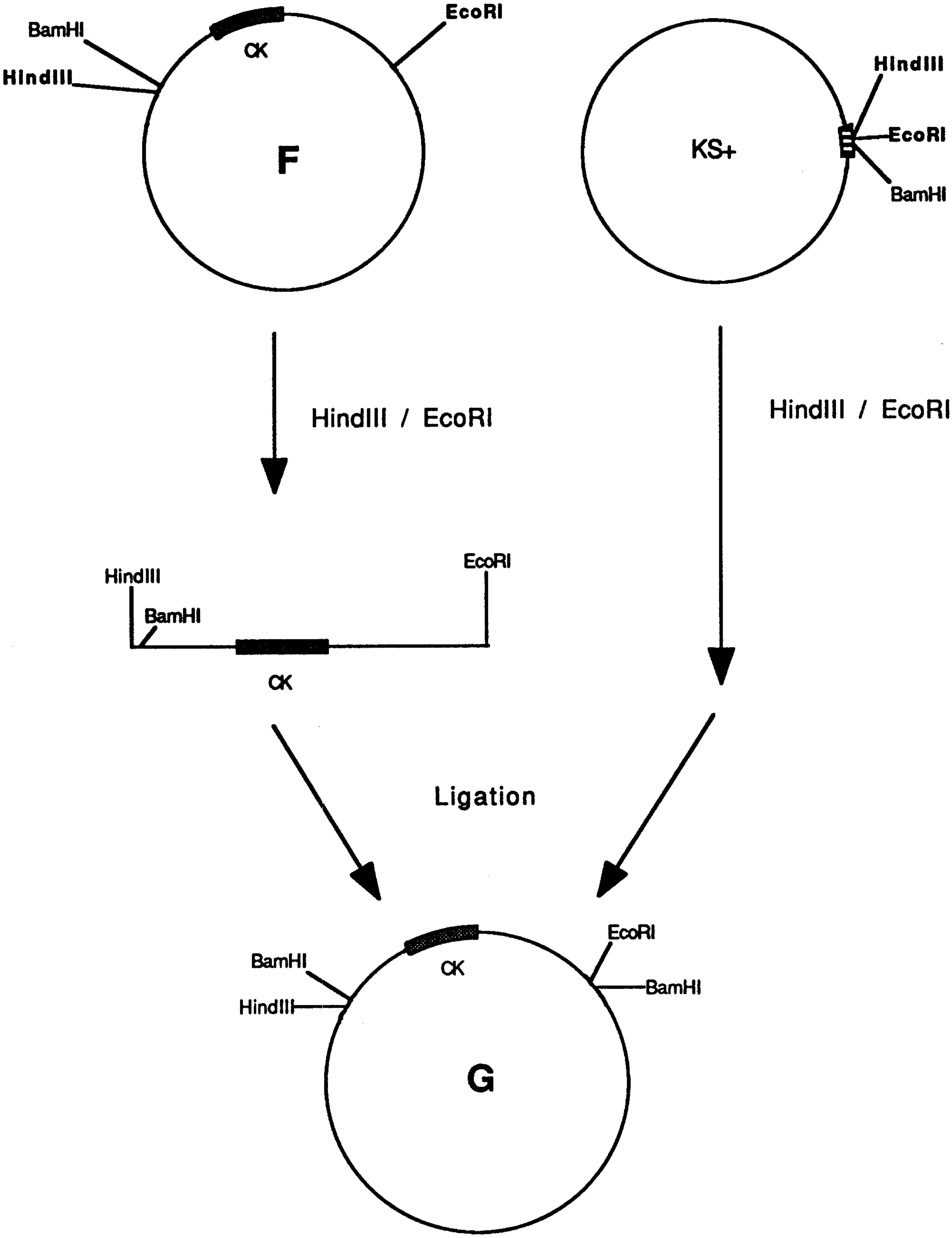

BamHI
HindIII
CK
EcoRI
F
KS+
HindIII
EcoRI
BamHI
HindIII / EcoRI
HindIII / EcoRI
HindIII
BamHI
EcoRI
CK
Ligation
BamHI
HindIII
CK
EcoRI
BamHI
G

Fig. 9

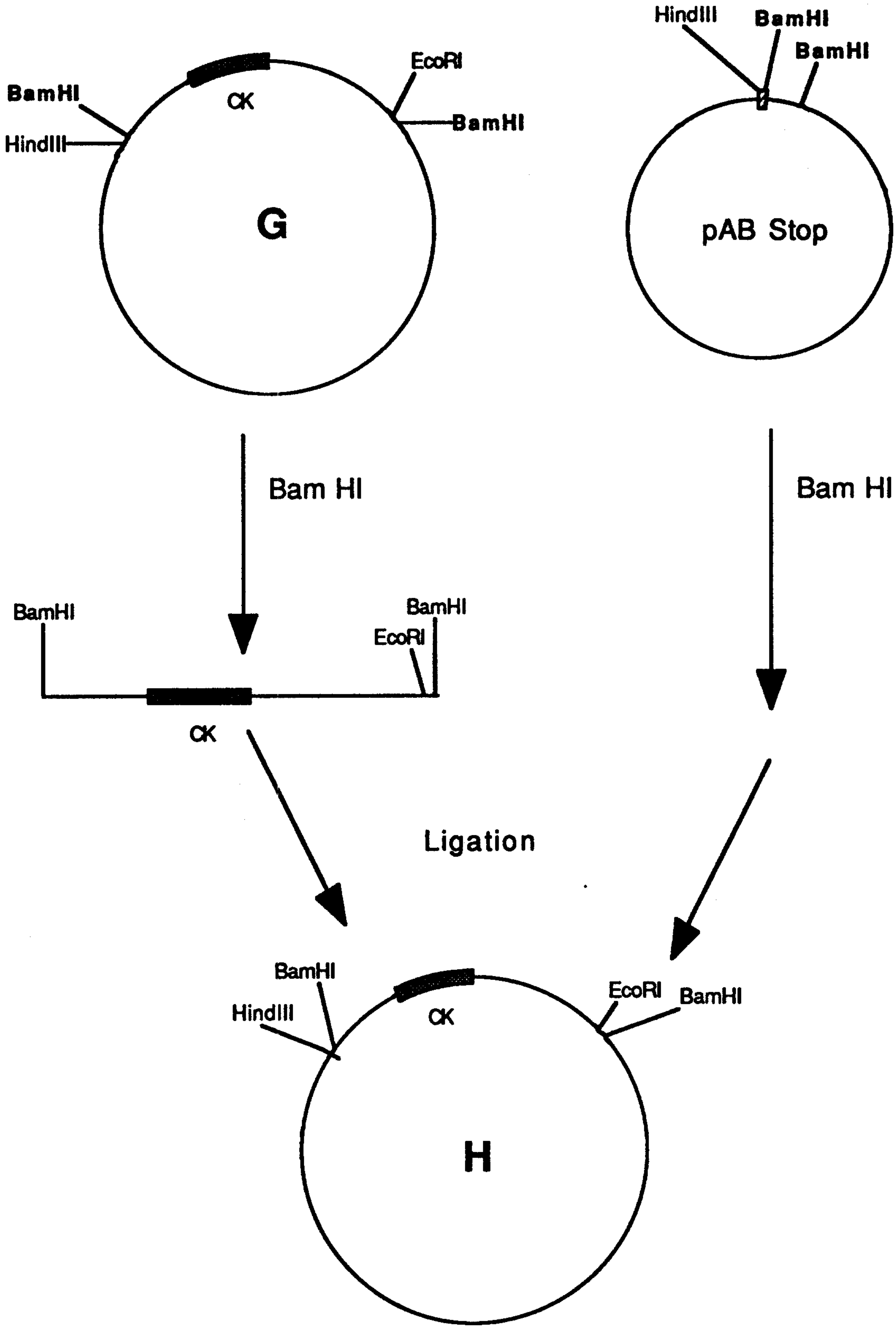

BamHI
HindIII
CK
EcoRI
BamHI
G
HindIII
BamHI
BamHI
pAB Stop
Bam HI
Bam HI
BamHI
BamHI
EcoRI
CK
Ligation
BamHI
HindIII
CK
EcoRI
BamHI
H

Fig. 10

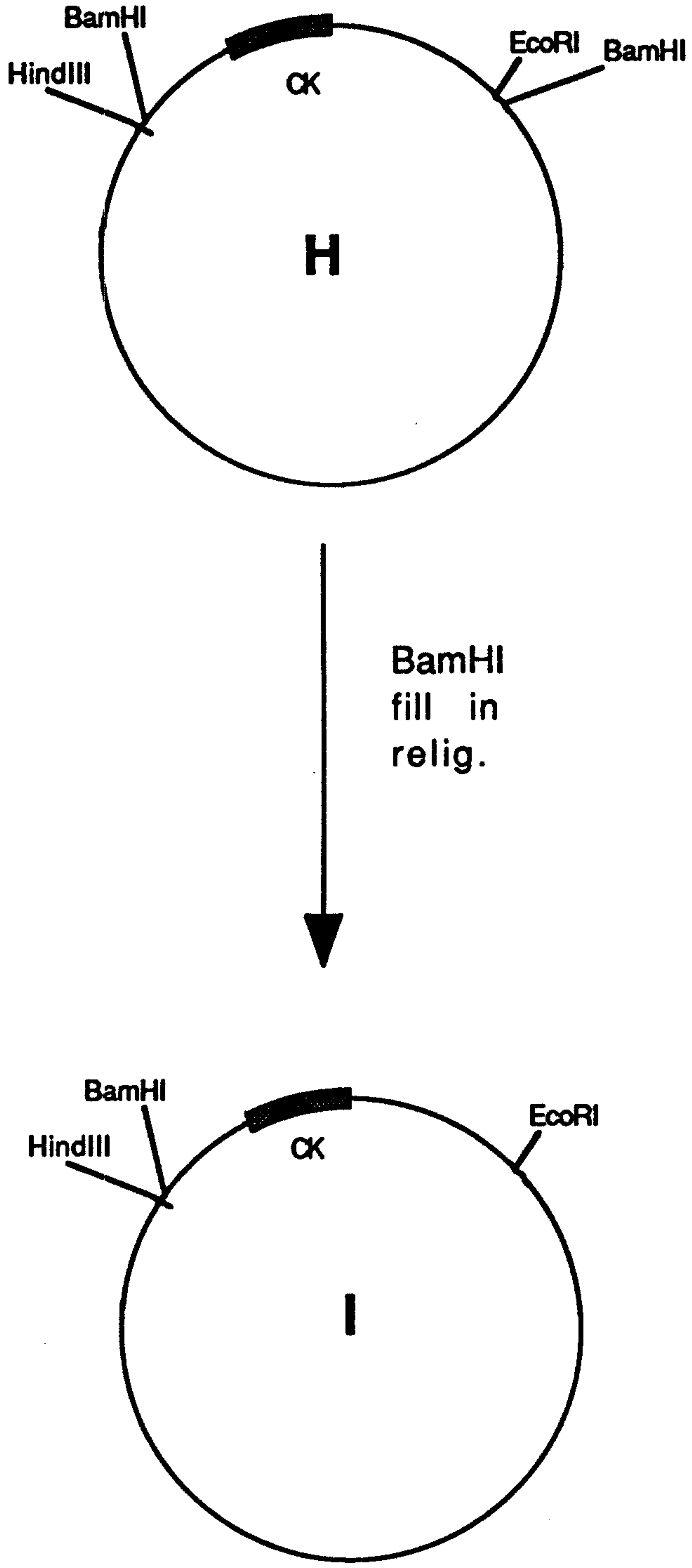
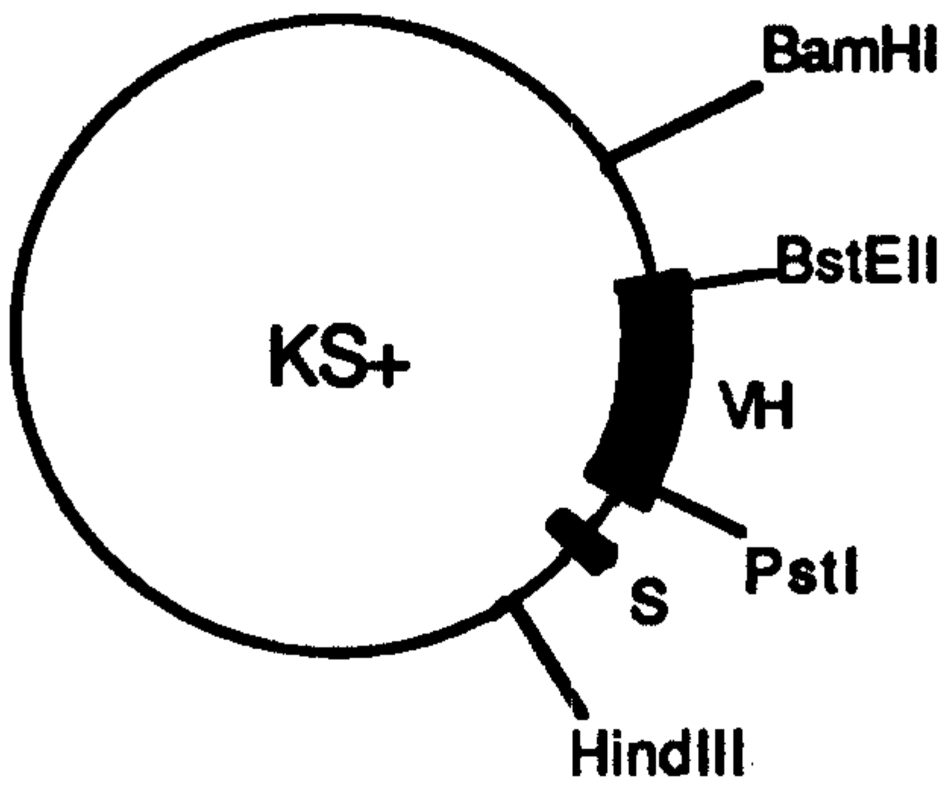

BamHI
HindIII
CK
EcoRI
BamHI
H
BamHI
fill in
relig.
BamHI
HindIII
CK
EcoRI
I

Fig. 11

a


KS+
BamHI
BstEII
VH
PstI
S
HindIII

b

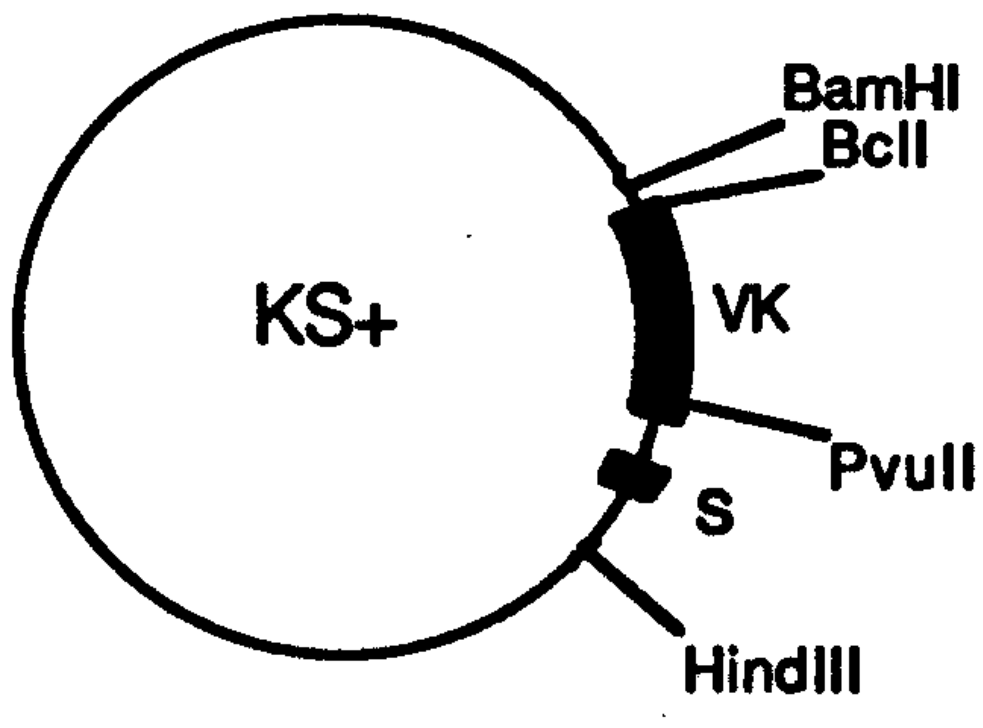
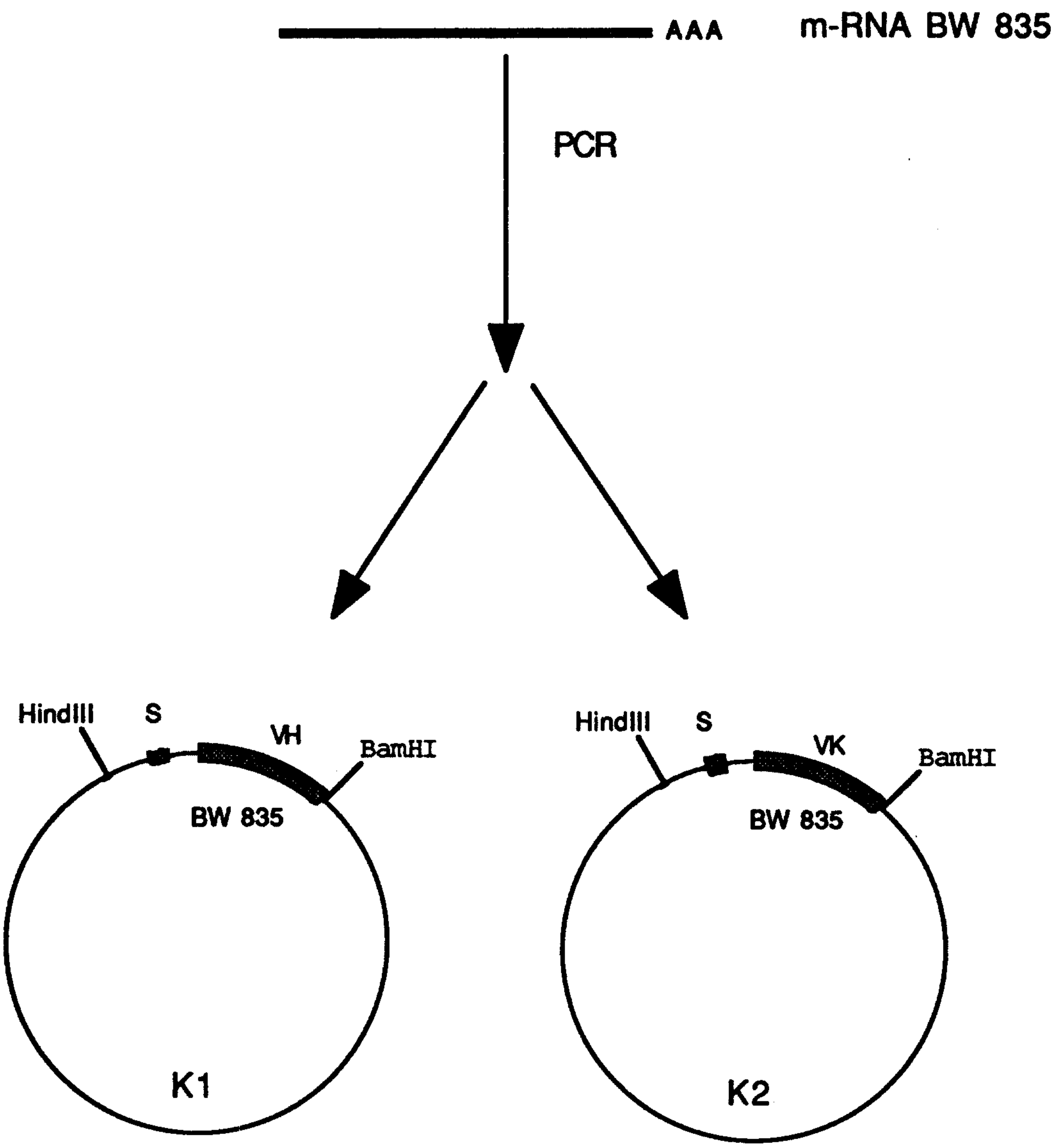

KS+
BamHI
BclI
VK
PvuII
S
HindIII

Fig. 12


AAA
m-RNA BW 835
PCR
HindIII
S
VH
BamHI
BW 835
K1
HindIII
S
VK
BamHI
BW 835
K2

2080260

Fig. 13

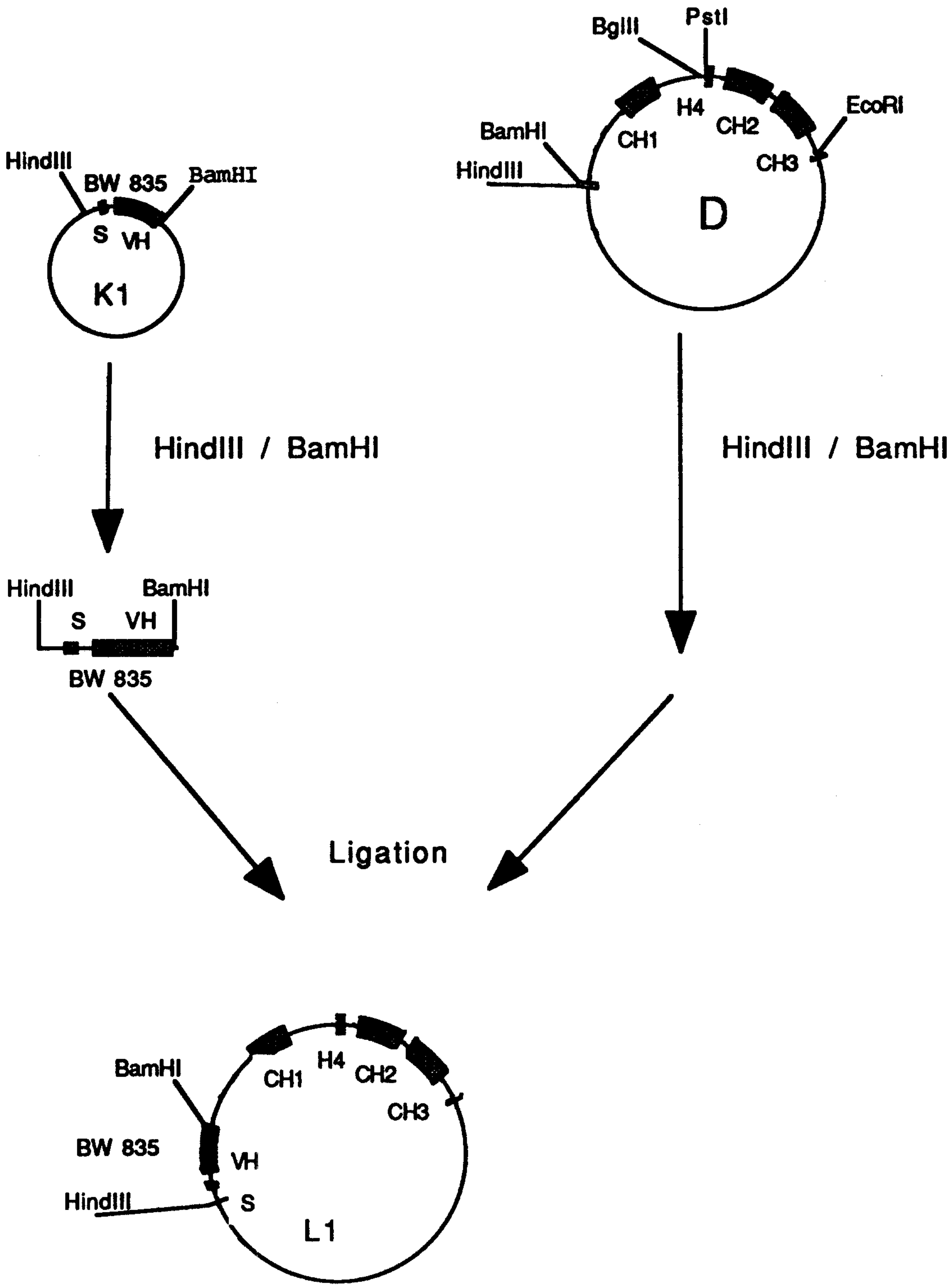


Fig. 14

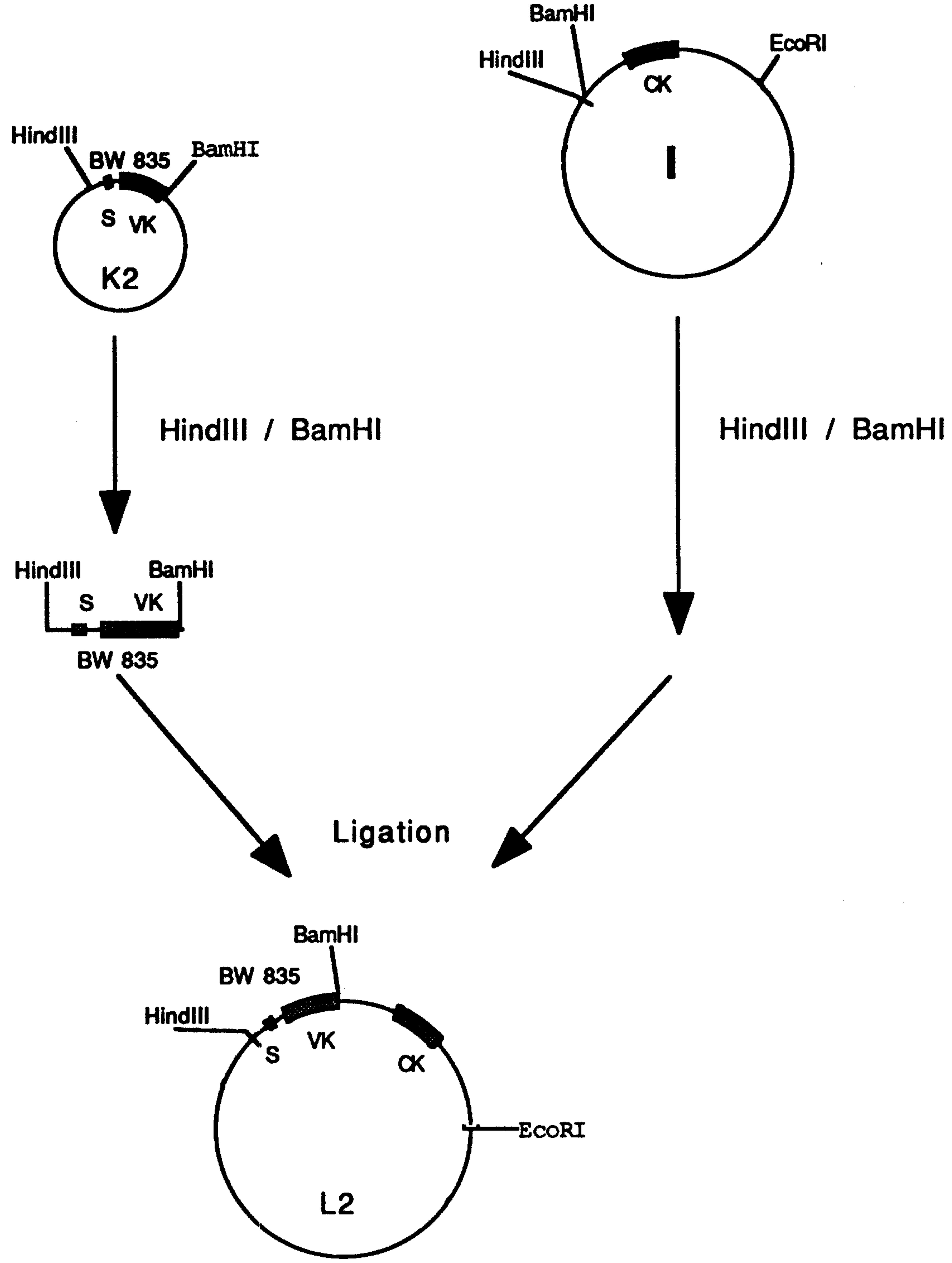
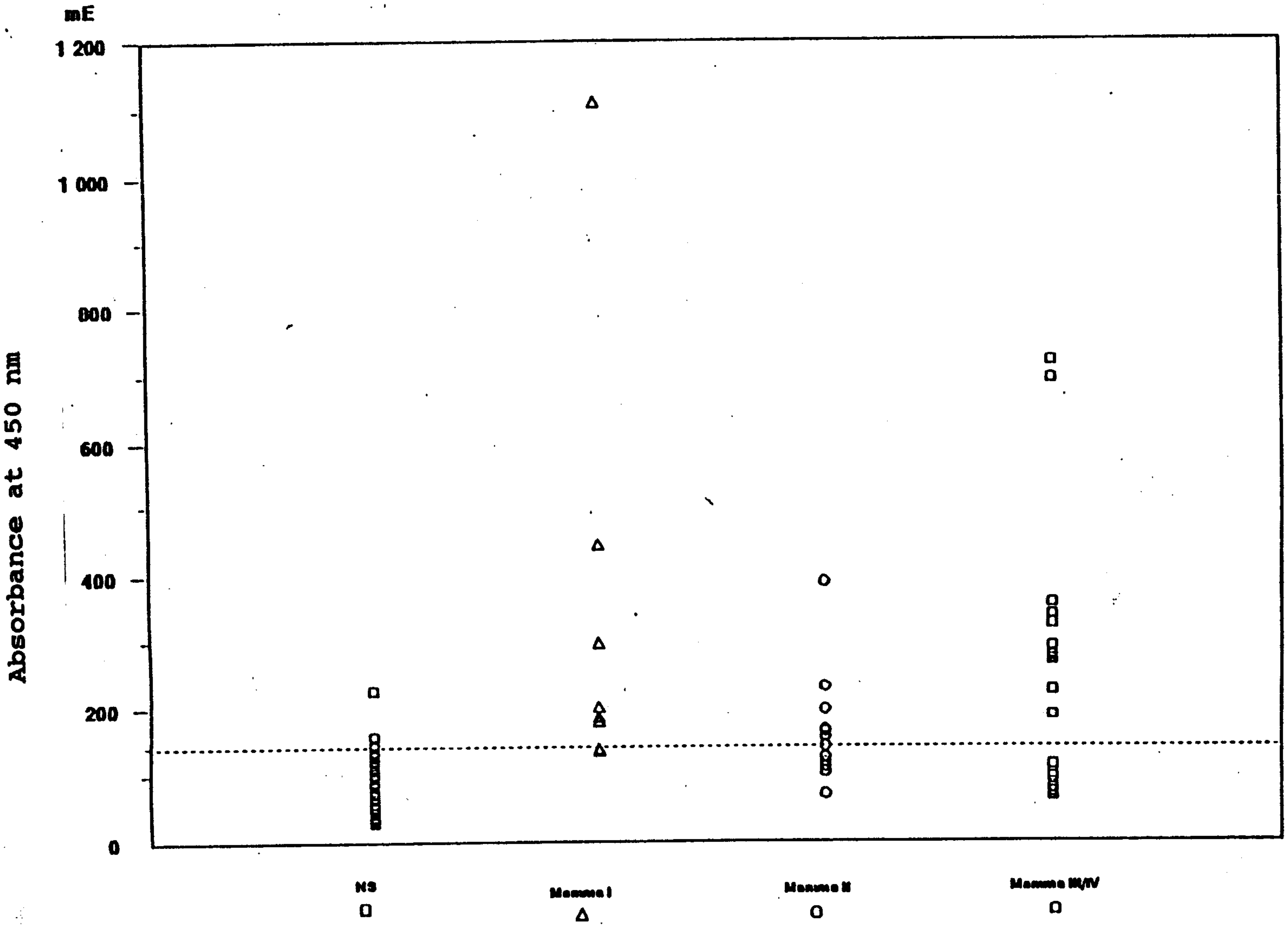

HindIII
BW 835
BamHI
S
VK
K2
BamHI
HindIII
EcoRI
CK
I
HindIII / BamHI
HindIII / BamHI
HindIII
BamHI
S
VK
BW 835
Ligation
BamHI
BW 835
HindIII
S
VK
CK
EcoRI
L2

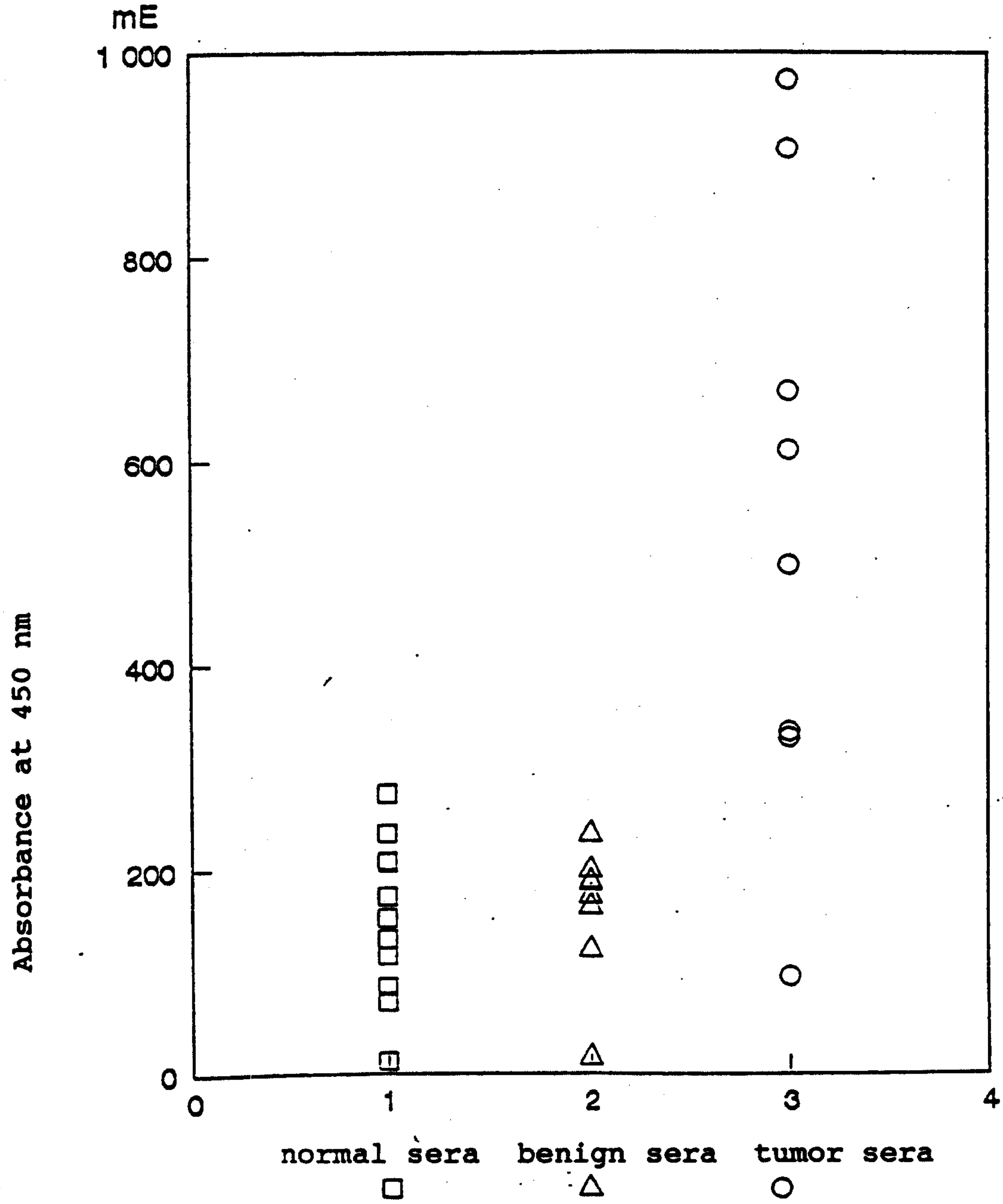
Fig. 15



normal sera
Mamma I - IV ≙ different tumor stages of malignant breast cancer

The dotted line represents the determined cut-off value which was defined as the 90th percentile based on the normal serum pool.

Fig. 16


mE
1 000
800
600
400
200
0
Absorbance at 450 nm
0
1
2
3
4
normal sera
benign sera
tumor sera