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(54) **COMPOSITIONS DETERGENTES CONTENANT DE LA
CELLULASE EG III TRES PURE**

(54) **DETERGENT COMPOSITIONS CONTAINING
SUBSTANTIALLY PURE EG III CELLULASE**

(57) Compositions de détergents contenant une quantité nettoyante efficace d'un tensio-actif ou d'un mélange de tensio-actifs et environ 0,01 à environ 5% en poids de cellulase EG III pure. La composition de détergents contient de préférence pas plus d'environ 5% en poids de constituants de type CBH I calculés sur la base du poids total des protéines de cellulase. Lorsqu'on les utilise en milieux de lavage aqueux les compositions de détergents confèrent des propriétés de rétention/régénération des couleurs ainsi que des propriétés d'assouplissement et de toucher améliorées aux tissus contenant du coton.

(57) Disclosed are detergent compositions containing a cleaning effective amount of a surfactant or a mixture of surfactants and from about 0.01 to about 5 weight percent of substantially pure EG III cellulase. Preferably, the detergent composition contains no more than about 5 weight percent of CBH I type components based on the total weight of cellulase proteins. When employed in aqueous wash media, the detergent compositions impart color retention/restoration properties as well as improved softening and feel properties to cotton-containing fabrics.





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(21) International Application Number: PCT/US91/07276 (22) International Filing Date: 4 October 1991 (04.10.91) (30) Priority data: 707,647 5 October 1990 (05.10.90) US (60) Parent Application or Grant (63) Related by Continuation US 707,647 (CIP) Filed on 5 October 1990 (05.10.90) (71) Applicant (for all designated States except US): GENENCOR INTERNATIONAL, INC. [US/US]; 180 Kimball Way, So. San Francisco, CA 94080 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only) : CLARKSON, Kathleen, A. [US/US]; 28th Street, No. 53, San Francisco, CA 94110 (US). WEISS, Geoffrey, L. [US/US]; 457 Buena Vista Avenue, San Francisco, CA 94117 (US). LAR-ENAS, Edmund, A. [US/US]; 301 Nevada, Moss Beach, CA 94038 (US). (74) Agent: PASSE, James, G.; Genencor International, Inc., 180 Kimball Way, So. San Francisco, CA 94080 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: DETERGENT COMPOSITIONS CONTAINING SUBSTANTIALLY PURE EG III CELLULASE		
(57) Abstract		
Disclosed are detergent compositions containing a cleaning effective amount of a surfactant or a mixture of surfactants and from about 0.01 to about 5 weight percent of substantially pure EG III cellulase. Preferably, the detergent composition contains no more than about 5 weight percent of CBH I type components based on the total weight of cellulase proteins. When employed in aqueous wash media, the detergent compositions impart color retention/restoration properties as well as improved softening and feel properties to cotton-containing fabrics.		

**DETERGENT COMPOSITIONS CONTAINING
SUBSTANTIALLY PURE EG III CELLULASE**

BACKGROUND OF THE INVENTION

1. Field of the Invention.

The present invention is directed to detergent compositions containing substantially pure EG III cellulase from Trichoderma spp. as well as to methods for employing such compositions. In particular, the detergent compositions of the present invention comprise a cleaning effective amount of one or more surfactants and substantially pure EG III cellulase.

2. State of the Art.

Cellulases are known in the art as enzymes that hydrolyze cellulose (β -1,4-glucan linkages) thereby resulting in the formation of glucose, cellobiose, cellooligosaccharides, and the like. While cellulases are produced (expressed) in fungi, bacteria and the like, cellulase produced by certain fungi and, in particular by the fungus class Trichoderma spp. (especially Trichoderma reesei), have been given the most attention because a complete cellulase system capable of degrading crystalline forms of cellulose is readily produced in large quantities via fermentation procedures.

In regard to the above, Wood et al., "Methods in Enzymology", 160, 25, pages 234 et seq. (1988), disclose that complete fungal cellulase systems comprise several different enzyme classifications including those identified as exo-cellobiohydrolases

(EC 3.2.1.91) ("CBH"), endoglucanases (EC 3.2.1.4) ("EG"), and β -glucosidases (EC 3.2.1.21) ("BG"). The fungal cellulase classifications of CBH, EG and BG can be further expanded to include multiple components within each classification. For example, multiple CBHs and EGs have been isolated from a variety of fungal sources.

The complete cellulase system comprising CBH, EG and BG components is required to efficiently convert crystalline cellulose to glucose. Isolated components are far less effective, if at all, in hydrolyzing crystalline cellulose. Moreover, a synergistic relationship is observed between the cellulase components particularly if they are of different classification.

On the other hand, cellulases and components thereof, used either singularly or in combination, are also known in the art to be useful in detergent compositions. For example, endoglucanase components of fungal cellulases have been used for the purposes of enhancing the cleaning ability of detergent compositions, for use as a softening agent, and for use in improving the feel of cotton fabrics, and the like. However, there is a problem with using the EG I and EG II components derived from Trichoderma spp. and especially Trichoderma reesei in detergent compositions. Specifically, such components have their maximal activity at acidic pHs whereas most laundry detergent compositions are formulated for use at neutral or alkaline (pH >7 to about 10) conditions. While it is disclosed in Canadian Application No. 2,093,425 that the use of one or more acidic endoglucanase components of Trichoderma reesei in detergent compositions will provide improvements in softening, color retention/restoration and feel to cotton-

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containing fabrics even when treated under alkaline conditions, the present invention is directed to the discovery that the EG III component of Trichoderma spp. provides for superior and unexpected advantages in detergent compositions as compared to the EG I and EG II components of Trichoderma reesei.

Specifically, this component has been found to possess significant enzymatic activity under alkaline conditions.

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SUMMARY OF THE INVENTION

The present invention is directed to the use of substantially pure EG III cellulase in detergent compositions to attain improvements in softening, color retention/restoration and feel. Specifically, because of the surprisingly enhanced activity of EG III cellulase under alkaline conditions, detergent compositions containing substantially pure EG III cellulase are particularly suited for use in laundry conditions where a neutral or alkaline detergent wash medium is employed.

Accordingly, in one of its composition aspects, the present invention is directed to a detergent composition comprising a cleaning effective amount of a surfactant or a mixture of surfactants and from about 0.01 to about 5 weight percent, and preferably from about 0.05 to about 2 weight percent, of a substantially pure EG III cellulase.

In one of its method aspects, the present invention is directed to a method for enhancing the softness of a cotton-containing fabric which method comprises washing the fabric in a wash medium derived from a detergent composition comprising a cleaning effective amount of a surfactant or a mixture of surfactants and from about 0.01 to about 5, and

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preferably from about 0.05 to about 2, weight percent of substantially pure EG III cellulase.

In another of its method aspects, the present invention is directed to a method for
5 retaining/restoring the color of a cotton-containing fabric which method comprises washing the fabric one or more times in a wash medium derived from a detergent composition comprising a cleaning effective amount of a
10 surfactant or a mixture of surfactants and from about 0.01 to about 5, and preferably from about 0.05 to about 2, weight percent of substantial-ly pure EG III cellulase.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates the RBB-CMC activity profile
15 over a pH range at 40°C for an EG enriched fungal cellulase composition derived from a strain of Trichoderma reesei transformed so as to be incapable of expressing CBH I and CBH II; as well as the activity profile of an enriched EG III cellulase composition
20 derived from Trichoderma reesei over a pH range at 40°C.

FIG. 2 is an isoelectrofocusing gel which, in Lane A, displays the proteins expressed by a wild type Trichoderma reesei; in Lane B displays the proteins
25 expressed by a strain of Trichoderma reesei transformed so as to be incapable of expressing EG I and EG II components; and in Lane C displays the proteins found in substantially pure EG III cellulase. The right hand margin of this figure is marked so as to identify the
30 bands attributable to CBH I, CBH II, EG I, EG II and EG III.

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FIG. 3 illustrates the fiber removal properties and hence the color restoration properties of EG III at various pH's.

FIG. 4 is the amino acid sequence obtained from two fragments of EGIII.

FIG. 5 is an outline of the construction of pΔCBHIpyr4.

FIG. 6 illustrates deletion of the T. reesei gene by integration of the larger EcoRI fragment from pΔCBHIpyr4 at the cbh1 locus on one of the T. reesei chromosomes.

FIG. 7 is an autoradiograph of DNA from T. reesei strain GC69 transformed with EcoRI digested pΔCBHIpyr4 after Southern blot analysis using a ³²P labelled pΔCBHIpyr4 as the probe. The sizes of molecular weight markers are shown in kilobase pairs to the left of the Figure.

FIG. 8 is an autoradiograph of DNA from a T. reesei strain GC69 transformed with EcoRI digested pΔCBHIpyr4 using a ³²P labelled pIntCBHI as the probe. The sizes of molecular weight markers are shown in kilobase pairs to the left of the Figure.

FIG. 9 is an isoelectric focusing gel displaying the proteins secreted by the wild type and by transformed strains of T. reesei. Specifically, in FIG. 5, Lane A of the isoelectric focusing gel employs partially purified CBHI from T. reesei; Lane B employs a wild type T. reesei; Lane C employs protein from a T. reesei strain with the cbh1 gene deleted; and Lane D

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employs protein from a T. reesei strain with the cbh1 and cbh2 genes deleted. In FIG. 9, the right hand side of the figure is marked to indicate the location of the single proteins found in one or more of the secreted proteins. Specifically, BG refers to the β -glucosidase, E1 refers to endoglucanase I, E2 refers to endoglucanase II, E3 refers to endoglucanase III, C1 refers to exo-cellobiohydrolase I and C2 refers to exo-cellobiohydrolase II.

FIG. 10A is a representation of the T. reesei cbh2 locus, cloned as a 4.1 kb EcoRI fragment on genomic DNA and FIG. 10B is a representation of the cbh2 gene deletion vector p Δ CBHII.

FIG. 11 is an autoradiograph of DNA from T. reesei strain P37P Δ CBHIPyr⁻26 transformed with EcoRI digested p Δ CBHII after Southern blot analysis using a ³²P labelled p Δ CBHII as the probe. The sizes of molecular weight markers are shown in kilobase pairs to the left of the Figure.

FIG. 12 is a diagram of the plasmid pEGIp_{pyr4}.

FIG. 13 is a diagram of the site specific alterations made in the egl1 and cbh1 genes to create convenient restriction endonuclease cleavage sites. In each case, the upper line shows the original DNA sequence, the changes introduced are shown in the middle line, and the new sequence is shown in the lower line.

FIG. 14 is a diagram of the larger EcoRI fragment which can be obtained from pCEPC1.

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FIG. 15 is an autoradiograph of DNA, from an untransformed strain of T. reesei RutC30 and from two transformants obtained by transforming T. reesei with EcoRI digested pCEPC1. The DNA was digested with PstI,
5 a Southern blot was obtained and hybridized with ³²P labelled pUC4K::cbh1. The sizes of marker DNA fragments are shown in kilobase pairs to the left of the Figure.

FIG. 16 is a diagram of the plasmid pEGII::P-1.

10 FIG. 17 is an autoradiograph of DNA from T. reesei strain P37PΔΔ67P1 transformed with HindIII and BamHI digested pEGII::P-1. A Southern blot was prepared and the DNA was hybridized with an approximately 4kb PstI fragment of radiolabelled T. reesei DNA containing the
15 egl3 gene. Lanes A, C and E contain DNA from the untransformed strain whereas, Lanes B, D and F contain DNA from the untransformed T. reesei strain. The T. reesei DNA was digested with BglII in Lanes A and B, with EcoRV in Lanes C and D and with PstI in Lanes E
20 and F. The size of marker DNA fragments are shown in kilobase pairs to the left of the Figure.

FIG. 18 is a diagram of the plasmid pΔEGI-1.

FIG. 19 is an autoradiograph of a Southern blot of DNA isolated from transformants of strain GC69 obtained
25 with HindIII digested pΔEGIp_{yr}-3. The pattern of hybridisation with the probe, radiolabelled pΔEGIp_{yr}-3, expected for an untransformed strain is shown in Lane C. Lane A shows the pattern expected for a transformant in which the egl1 gene has been disrupted
30 and Lane B shows a transformant in which pΔEGIp_{yr}-3 DNA has integrated into the genome but without disrupting

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the egl1 gene. Lane D contains pΔEGIpyr-3 digested with HindIII to provide appropriate size markers. The sizes of marker DNA fragments are shown in kilobase pairs to the right of the figure.

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DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention generally relates to detergent compositions containing substantially pure EG III cellulase as well as for methods employing such detergent compositions. When used in aqueous wash media at alkaline pH's, such compositions are particularly effective in imparting improvements in softening, color retention/restoration, and feel to the treated cotton-containing fabric.

However, prior to discussing this invention in detail, the following terms will first be defined.

The term "EG III cellulase" refers to the endoglucanase component derived from Trichoderma spp. characterized by a pH optimum of about 5.5 to 6.0, an isoelectric point (pI) of from about 7.2 to 8.0, and a molecular weight of about 23 to 28 Kdaltons. Preferably, EG III cellulase is derived from either Trichoderma reesei or from Trichoderma viride. EG III cellulase derived from Trichoderma reesei has a pH optimum of about 5.5 to 6.0, an isoelectric point (pI) of about 7.4 and a molecular weight of about 25 to 28 Kdaltons. EG III cellulase derived from Trichoderma viride has a pH optimum of about 5.5, an isoelectric point (pI) of about 7.7 and a molecular weight of about 23.5 Kdaltons.

"Substantially pure EG III cellulase" refers to a composition of cellulase proteins containing at least 40 weight percent, preferably at least 70 weight percent and most preferably at least 90 weight percent

of EG III based on the total weight of cellulase proteins.

EG III cellulase can be purified from any strain of Trichoderma spp. which produces EG III under suitable fermentation conditions. While the particular source of EG III is not critical, preferred sources are Trichoderma reesei and Trichoderma viride. A particularly preferred source of EG III from Trichoderma reesei is Cytolase 123^{*} cellulase which is commercially available from Genencor International, Inc., 180 Kimball Way, South San Francisco, CA 94080. Because of its high pI, EG III is found in a region of an isoelectrofocusing gel where high pI xylanases and other high pI components expressed by Trichoderma spp. are generally found. In fact, it has been hypothesized that the band identified as EG III in FIG. 2 was a degradation product of either EG I or II. However, gel isoelectrofocusing of EG I and EG II deleted cellulase (prepared in the manner of Canadian Application No. 2,093,425 demonstrated that this band was not attributable to a degradation product of either EG I or II. (See FIG. 2).

It is noted that EG II has been previously referred to by the nomenclature "EG III" by some authors but current nomenclature uses the term "EG II". In any event, the EG II protein is substantially different from the EG III protein in its molecular weight, pI, and pH optimum as evidenced by Table I of Example 2 presented below.

Procedures suitable for obtaining substantially pure EG III cellulase from a complete cellulase system derived from Trichoderma spp. ("whole cellulase") include those recited in the examples set forth herein below. These examples demonstrate that substantially pure EG III cellulase is readily obtained by subjecting

* Trade mark

whole cellulase to purification procedures including repeated fractionation steps utilizing different fractionation materials (columns). Additionally, the fractionation steps can be preceded by an extraction step using polyethylene glycol 8000 so as to provide for EG III cellulase fraction (about 20-50% pure EG III) which, if necessary, can be used in subsequent fractionation steps to provide for substantially pure EG III cellulase.

10 Additionally, it is contemplated that substantially pure EG III cellulase can be prepared by genetically modifying microorganisms so as to produce substantially pure EG III cellulase. For example, substantially pure EG III prepared by fractionation methods set forth in the examples below can be employed to determine the amino acid sequence of parts or all of the protein using known sequencing methods. Once the amino acid sequence of parts of the EG III cellulase is known, this information can be used to prepare synthetic DNA probes in order to clone the gene responsible for encoding this information. Once the EG III encoding gene is cloned, it could be manipulated by recognized techniques and ultimately inserted into various Trichoderma spp. strains or into other microorganisms. See, for example, Canadian Application No. 2,093,425 discloses methods for genetically engineering

30 Trichoderma reesei so that the modified microorganism is incapable of expressing one or more of the cellulase genes and, in fact, may overproduce another cellulase gene.

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methods described in this application, Trichoderma reesei could be genetically manipulated so as to produce EG III with or without other cellulase proteins. Moreover, the methods described in this application create Trichoderma reesei strains which do not produce any heterologous proteins.

Additionally, it would be possible to express the EG III-encoding gene in other microorganisms, including, but not limited to, yeast species such as Saccharomyces cerevisiae, Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis, Yarrowia lipolytica, Schanniomyces occidentalis, etc. See, for example, PCT application Publication No. WO 85/04672. In order to obtain expression in these alternative, non-Trichoderma hosts, it may be necessary to functionally combine the EG III-coding DNA sequence with promoter and terminator sequences obtained from a gene from that particular host. It may also be necessary to substitute the DNA sequence encoding a secretion signal sequence from the alternative host for the DNA sequence encoding the EG III secretion signal sequence. Production and secretion of EG III in other organisms could enable EG III to be obtained in substantially pure form.

"Cellulase proteins" refer to any and all exo-cellobiohydrolase (CBH) proteins, endoglucanase (EG) proteins and β -glucosidase (BG) proteins expressed by a wild type Trichoderma spp. or a transformed strain of Trichoderma spp. Accordingly, cellulase proteins do not include other proteins expressed by Trichoderma spp. including xylanases, proteases, amylases, etc.

"Exo-cellobiohydrolase ("CBH") components" refer to the CBH I and/or CBH II components of Trichoderma reesei and "CBH type components" refer to those fungal cellulase components which exhibit detergent activity properties similar to that of the CBH I and/or CBH II

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components of Trichoderma reesei. In this regard, when used in the absence of the EG components of Trichoderma reesei, the CBH I and CBH II components of Trichoderma reesei alone do not impart significant color retention/restoration and improved feel to the so-treated cotton-containing fabrics. Additionally, when used in combination with such EG components, the CBH I component of Trichoderma reesei can impart enhanced strength loss and incremental cleaning benefits to cotton-containing fabrics.

Accordingly, CBH I type components and CBH II type components refer to those fungal cellulase components which exhibit detergent activity properties similar to CBH I and CBH II components of Trichoderma reesei, respectively. As noted above, for CBH I type components, this includes the properties of enhancing strength loss of cotton-containing fabrics and/or imparting an incremental cleaning benefit when used in the presence of the EG components of Trichoderma reesei. In a preferred embodiment, the CBH I components also impart an incremental softening benefit when used in the presence of such EG components.

Such CBH type components may exclude components traditionally classified as exo-cellobiohydrolases using activity tests such as those used to characterize CBH I and CBH II from Trichoderma reesei. For example, using such traditional classification tests, such components are (a) competitively inhibited by cellobiose (K_i approximately 1mM); (b) hydrolyze phosphoric acid swollen cellulose and to a lesser degree highly crystalline cellulose; and (c) are unable to hydrolyze to any significant degree substituted celluloses, such as carboxymethylcellulose, etc. In contrast, it is believed that some CBH components which are characterized as exo-cellobiohydrolase components

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by such activity tests, do not possess functional properties similar to the CBH components of Trichoderma reesei. Accordingly, it is believed to be more accurate for the purposes herein not to define such
5 exo-cellobiohydrolases as CBH type components because these components do not possess similar functional properties in detergent compositions as possessed by the CBH components of Trichoderma reesei.

10 "β-Glucosidase (BG) components" refer to those components of cellulase which exhibit BG activity; that is to say that such components will act from the non-reducing end of cellobiose and other soluble
15 cellooligosaccharides ("cellobiose") and give glucose as the sole product. BG components do not adsorb onto or react with cellulose polymers. Furthermore, such BG components are competitively inhibited by glucose (K_i
20 approximately 1mM). While in a strict sense, BG components are not literally cellulases because they cannot degrade cellulose, such BG components are included within the definition of the cellulase system
25 because these enzymes facilitate the overall degradation of cellulose by further degrading the inhibitory cellulose degradation products (particularly cellobiose) produced by the combined action of CBH
30 components and EG components. Without the presence of BG components, moderate or little hydrolysis of crystalline cellulose will occur. BG components are often characterized on aryl substrates such as p-nitrophenol B-D-glucoside (PNPG) and thus are often
35 called aryl-glucosidases. It should be noted that not all aryl-glucosidases are BG components, in that some do not hydrolyze cellobiose.

 In some cases, it may be desirable to incorporate BG components into the detergent compositions of this
35 invention (e.g., so as to increase the overall

hydrolysis of cellulose if the level of cellobiose becomes restrictive of such overall hydrolysis in the absence of BG components). In such cases, purified BG components can be added to the detergent composition.

5 The amount of BG component added depends upon the amount of cellobiose produced in the detergent wash which can be readily determined by the skilled artisan. However, when employed, the weight percent of BG component relative to total weight of substantially
10 pure EG III cellulase is preferably from about 0.4 to about 10 weight percent, and more preferably, from about 1 to about 5 weight percent.

Fungal cellulases can contain more than one BG component and are generally separated via ion exchange
15 chromatography and the like. In order to facilitate separation, it may be desirable to employ a cellulase composition having increased amounts of BG. Methods to increase the amount of BG components in the cellulase composition are disclosed in Canadian Application
20 No. 2,093,425 and entitled "SACCHARIFICATION OF CELLULOSE BY CLONING AND AMPLIFICATION OF THE β -GLUCOSIDASE GENE OF TRICHODERMA REESEI."

25 The term "cotton-containing fabric" refers to sewn or unsewn fabrics made of pure cotton or cotton blends including cotton woven fabrics, cotton knits, cotton denims, and the like. When cotton blends are employed, the amount of cotton in the fabric should be at least
30 about 40 percent by weight cotton; preferably, more than about 60 percent by weight cotton; and most preferably, more than about 75 percent by weight cotton. When employed as blends, the companion material employed in the fabric can include one or more
35 non-cotton fibers including synthetic fibers such as

polyamide fibers (for example, nylon 6 and nylon 66), acrylic fibers (for example, polyacrylonitrile fibers), polyester fibers (for example, polyethylene terephthalate), polyvinyl alcohol fibers (for example, Vinylon), polyvinyl chloride fibers, polyvinylidene chloride fibers, polyurethane fibers, polyurea fibers and aramid fibers. It is contemplated that regenerated cellulose, such as rayon, could be used as a substitute for cotton in cotton-containing fabrics.

10 The term "surface active agent or surfactant" refers to anionic, non-ionic and ampholytic surfactants well known for their use in detergent compositions.

15 The term "wash medium" refers to an aqueous wash solution prepared by adding a requisite amount of a detergent (surfactant) composition to water. The wash medium generally contains a cleaning effective amount of the detergent.

20 The wash medium is defined as an "acidic wash medium" if the pH of the medium is from about 4 to less than about 7. The wash medium is defined as a "neutral wash medium" if the pH of the medium is about 7. The wash medium is defined as an "alkaline wash medium" if the pH of the medium is from above 7 to about 10. Preferably, the alkaline wash medium will have a pH of
25 from above 7 to about 9 and, even more preferably, from above 7 to about 8.

30 In regard to the detergent compositions containing cellulase compositions which are CBHI deficient, CBHI enriched or EGIII enriched, it has been found that it is the amount of cellulase, and not the relative rate of hydrolysis of the specific enzymatic components to produce reducing sugars from cellulose, which imparts the desired detergent properties to cotton-containing fabrics, eg., one or more of improved color

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restoration, improved softening and improved cleaning to the detergent composition.

5 The present invention is directed to the discovery that substantially pure EG III cellulase can be used in detergent compositions to effect softening as well as effecting color retention/restoration and improved feel of cotton-containing fabrics regardless of whether such compositions are employed in an acidic, neutral or alkaline wash medium. However, because EG III
10 possesses unexpectedly high activity under alkaline conditions, detergent compositions employing substantially pure EG III cellulase provide enhanced softening, color retention/restoration and improved feel as compared to detergent compositions employing
15 other endoglucanase components derived from Trichoderma reesei.

Although the presence of EG III cellulase effects color retention/restoration, softening and improved feel, specified mixtures of EG III cellulase with other
20 cellulase components can provide some incremental benefits. Specifically, while the use of EG III cellulase will provide a cleaning and softening benefit, incremental cleaning and softening benefits are observed for cotton-containing fabrics washed with
25 a detergent composition containing EG III cellulase and CBH I type cellulase.

On the other hand, the presence of significant amounts of CBH I type components in combination with EG III cellulase may result in enhanced strength loss to
30 cotton-containing fabrics compared to EG III compositions which are either free of CBH I type components or contain reduced amounts of CBH I type components. Accordingly, when the detergent composition contains some CBH I type components, the
35 amount of CBH I type components is preferably no

greater than about 10 weight percent, more preferably no greater than about 5 weight percent, and even more preferably less than about 2 weight percent, based on the total weight of cellulase proteins.

5 In regard to the above, the total amount of substantially pure EG III cellulase generally employed in the detergent compositions of this invention is an amount sufficient to impart color retention/restoration and softness to the cotton garments. Preferably, the
10 substantially pure EG III cellulase composition is employed from about 0.01 weight percent to about 5 weight percent relative to the weight of the total detergent composition. More preferably, substantially pure EG III is employed from about 0.05 weight percent
15 to about 2 weight percent relative to the weight of the total detergent composition. In general, the amount of other cellulase proteins employed in the detergent composition is no more than about 60 weight percent relative to the total weight of cellulase proteins
20 (including EG III cellulase), preferably no more than about 30 weight percent relative to the total weight of cellulase proteins, and more preferably no more than about 10 weight percent relative to the total weight of cellulase proteins. Even more preferably, the CBH type
25 I components preferably do not exceed about 5 weight percent of the total weight of cellulase proteins (i.e., substantially pure EG III cellulase).

 The specific concentration of substantially pure EG III cellulase employed in the detergent composition
30 is selected so that upon dilution into a wash medium, the concentration of EG III cellulase will preferably range from about 0.5 to about 500 ppm, and more preferably from about 2 to about 100 ppm. The specific amount of substantially pure EG III cellulase employed
35 in the detergent composition will depend on the extent

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the detergent composition will be diluted upon addition to water to form a wash medium. These factors are readily ascertained by the skilled artisan.

At lower cellulase concentrations (i.e., concentrations of EG III cellulase of less than about 5 ppm in the wash medium), softness, color retention/restoration and improved feel achieved by use of the detergent compositions of this invention is more evident over repeated washings. At higher concentrations (i.e., concentrations of EG III cellulase of about 5 ppm and above in the wash medium), the improvements can become noticeable in a single wash.

One of the important aspects of the present invention is that by tailoring the cellulase composition to contain substantially pure EG III cellulase, it is possible to achieve the desired effects of softening, color retention/restoration while using lower concentrations of cellulase in the detergent composition. In turn, the use of lower concentrations of cellulase in the detergent compositions should lead to improved consumer safety.

The substantially pure EG III cellulase described above can be added to the detergent composition either in a liquid diluent, in granules, in emulsions, in gels, in pastes, or the like. Such forms are well known to the skilled artisan. When a solid detergent composition is employed, the cellulase composition is preferably formulated as granules. Preferably, the granules can be formulated so as to contain a cellulase protecting agent. See, for instance, Canadian Application No. 2,093,425.

entitled "GRANULES CONTAINING BOTH AN ENZYME AND AN ENZYME PROTECTING AGENT AND DETERGENT COMPOSITIONS CONTAINING SUCH GRANULES."

Likewise, the granule can be formulated so as to contain materials to reduce the rate of dissolution of the granule into the wash medium. Such materials and granules are disclosed in PCT Publication No. WO 92/06210 entitled "GRANULAR COMPOSITIONS."

10 The detergent compositions of this invention employ a surface active agent, i.e., surfactant, including anionic, non-ionic and ampholytic surfactants well known for their use in detergent compositions.

15 Suitable anionic surfactants for use in the detergent composition of this invention include linear or branched alkylbenzenesulfonates; alkyl or alkenyl ether sulfates having linear or branched alkyl groups or alkenyl groups; alkyl or alkenyl sulfates; olefinsulfonates; alkanesulfonates and the like.

20 Suitable counter ions for anionic surfactants include alkali metal ions such as sodium and potassium; alkaline earth metal ions such as calcium and magnesium; ammonium ion; and alkanolamines having 1 to 3 alkanol groups of carbon number 2 or 3.

25 Ampholytic surfactants include quaternary ammonium salt sulfonates, betaine-type ampholytic surfactants, and the like. Such ampholytic surfactants have both the positive and negative charged groups in the same molecule.

30 Nonionic surfactants generally comprise polyoxyalkylene ethers, as well as higher fatty acid alkanolamides or alkylene oxide adduct thereof, fatty acid glycerine monoesters, and the like.

35 Suitable surfactants for use in this invention are disclosed in British Patent Application No. 2 094 826

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A.

Mixtures of such surfactants can also be used.

5 The surfactant or a mixture of surfactants is generally employed in the detergent compositions of this invention in an amount from about 1 weight percent to about 95 weight percent of the total detergent composition and preferably from about 5 weight percent to about 45 weight percent of the total detergent
10 composition. Upon dilution in the wash medium, the surfactant concentration is generally about 500 ppm or more; and preferably, from about 1000 ppm to 15,000 ppm.

15 In addition to the cellulase composition and the surfactant(s), the detergent compositions of this invention can optionally contain one or more of the following components:

Hydrolases except cellulase

20 Such hydrolases include carboxylate ester hydrolase, thioester hydrolase, phosphate monoester hydrolase, and phosphate diester hydrolase which act on the ester bond; glycoside hydrolase which acts on glycosyl compounds; an enzyme that hydrolyzes N-glycosyl compounds; thioether hydrolase which acts on
25 the ether bond; and α -amino-acyl-peptide hydrolase, peptidyl-amino acid hydrolase, acyl-amino acid hydrolase, dipeptide hydrolase, and peptidyl-peptide hydrolase which act on the peptide bond. Preferable among them are carboxylate ester hydrolase, glycoside
30 hydrolase, and peptidyl-peptide hydrolase. Suitable hydrolases include (1) proteases belonging to peptidyl-peptide hydrolase such as pepsin, pepsin B, rennin, trypsin, chymotrypsin A, chymotrypsin B, elastase, enterokinase, cathepsin C, papain, chymopapain, ficin,

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thrombin, fibrinolysin, renin, subtilisin,
aspergillopeptidase A, collagenase, clostridiopeptidase
B, kallikrein, gastrisin, cathepsin D., bromelin,
keratinase, chymotrypsin C, pepsin C,
5 aspergillopeptidase B, urokinase, carboxypeptidase A
and B, and aminopeptidase; (2) glycoside hydrolases
(cellulase which is an essential ingredient is excluded
from this group) α -amylase, β -amylase, gluco amylase,
invertase, lysozyme, pectinase, chitinase, and
10 dextranase. Preferably among them are α -amylase and β -
amylase. They function in acid to neutral systems, but
one which is obtained from bacteria exhibits high
activity in an alkaline system; (3) carboxylate ester
hydrolase including carboxyl esterase, lipase, pectin
15 esterase, and chlorophyllase. Especially effective
among them is lipase.

Trade names of commercial products and producers
are as follows: "Alkalase", "Esperase", "Savinase",
"AMG", "BAN", "Fungamill", "Sweetzyme", "Thermamyl"
20 (Novo Industry, Copenhagen, Denmark); "Maksatase",
"High-alkaline protease", "Amylase THC", "Lipase" (Gist
Brocades, N.V., Delft, Holland); "Protease B-400",
"Protease B-4000", "Protease AP", "Protease AP 2100"
(Schweizerische Ferment A.G., Basel, Switzerland); "CRD
25 Protease" (Monsanto Company, St. Louis, Missouri);
"Piocase" (Piopin Corporation, Monticello, Illinois);
"Pronase P", "Pronase AS", "Pronase AF" (Kaken Chemical
Co., Ltd., Japan); "Lipidase P-2000" (Lipidas, Secran,
France); protease products (Tyler standard sieve, 100%
30 pass 16 mesh and 100% on 150 mesh) (Clington Corn
Products, Division of Standard Brands Corp., New York);
"Takamine", "Bromelain 1:10", "HT Protease 200",
"Enzyme L-W" (obtained from fungi, not from bacteria)
(Miles Chemical Company, Elkhart, Ind.); "Rhozyme P-11
35 Conc.", "Pectinol", "Lipase B", "Rhozyme PF", "Rhozyme

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J-25" (Rohm & Haas, Genencor, South San Francisco, CA);
"Ambrozyme 200" (Jack Wolf & Co., Ltd., Subsidiary of
Nopco Chemical Company, Newark, N.J.); "ATP 40", "ATP
120", "ATP 160" (Lapidas, Secran, France); "Oripase"
5 (Nagase & Co., Ltd., Japan), all of said names being trade marks.

The hydrolase other than cellulase is incorporated
into the detergent composition as much as required
according to the purpose. It should preferably be
incorporated in an amount of 0.001 to 5 weight percent,
10 and more preferably 0.02 to 3 weight percent, in terms
of purified one. This enzyme should be used in the
form of granules made of crude enzyme alone or in
combination with other components in the detergent
composition. Granules of crude enzyme are used in such
15 an amount that the purified enzyme is 0.001 to 50
weight percent in the granules. The granules are used
in an amount of 0.002 to 20 and preferably 0.1 to 10
weight percent. As with cellulases, these granules can
be formulated so as to contain an enzyme protecting
20 agent and a dissolution retardant material.

Cationic surfactants and long-chain fatty acid salts

Such cationic surfactants and long-chain fatty
acid salts include saturated or unsaturated fatty acid
salts, alkyl or alkenyl ether carboxylic acid salts, α -
25 sulfofatty acid salts or esters, amino acid-type
surfactants, phosphate ester surfactants, quaternary
ammonium salts including those having 3 to 4 alkyl
substituents and up to 1 phenyl substituted alkyl
substituents. Suitable cationic surfactants and long-
30 chain fatty acid salts are disclosed in British Patent
Application No. 2 094 826 A.

The composition may
contain from about 1 to about 20 weight percent of such
cationic surfactants and long-chain fatty acid salts.

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Builders

A. Divalent sequestering agents.

The composition may contain from about 0 to about 50 weight percent of one or more builder components selected from the group consisting of alkali metal salts and alkanolamine salts of the following compounds: phosphates, phosphonates, phosphonocarboxylates, salts of amino acids, aminopolyacetates high molecular electrolytes, non-dissociating polymers, salts of dicarboxylic acids, and aluminosilicate salts. Suitable divalent sequestering agents are disclosed in British Patent Application No. 2 094 826 A.

15 B. Alkalis or inorganic electrolytes

The composition may contain from about 1 to about 50 weight percent, preferably from about 5 to about 30 weight percent, based on the composition of one or more alkali metal salts of the following compounds as the alkalis or inorganic electrolytes: silicates, carbonates and sulfates as well as organic alkalis such as triethanolamine, diethanolamine, monoethanolamine and triisopropanolamine.

Antiredeposition agents

25 The composition may contain from about 0.1 to about 5 weight percent of one or more of the following compounds as antiredeposition agents: polyethylene glycol, polyvinyl alcohol, polyvinylpyrrolidone and carboxymethylcellulose.

30 Among them, a combination of carboxymethylcellulose or/and polyethylene glycol with the cellulase composition of the present invention provides for an especially useful dirt removing composition.

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Bleaching agents

5 The use of the cellulase of the present invention in combination with a bleaching agent such as sodium percarbonate, sodium perborate, sodium sulfate/hydrogen peroxide adduct and sodium chloride/hydrogen peroxide adduct or/and a photo-sensitive bleaching dye such as zinc or aluminum salt of sulfonated phthalocyanine further improves the deterging effects.

Bluing agents and fluorescent dyes

10 Various bluing agents and fluorescent dyes may be incorporated in the composition, if necessary. Suitable bluing agents and fluorescent dyes are disclosed in British Patent Application No. 2 094 826 A.

15

Caking inhibitors

The following caking inhibitors may be incorporated in the powdery detergent:
20 p-toluenesulfonic acid salts, xylenesulfonic acid salts, acetic acid salts, sulfosuccinic acid salts, talc, finely pulverized silica, clay, calcium silicate (such as Micro-Cell* of Johns Manville Co.), calcium carbonate and magnesium oxide.

25 Masking agents for factors inhibiting the cellulase activity

The cellulase composition of this invention are deactivated in some cases in the presence of copper, zinc, chromium, mercury, lead, manganese or silver ions or their compounds. Various metal chelating agents and metal-precipitating agents are effective against these
30 inhibitors. They include, for example, divalent metal ion sequestering agents as listed in the above item

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with reference to optional additives as well as magnesium silicate and magnesium sulfate.

5 Cellobiose, glucose and gluconolactone act sometimes as the inhibitors. It is preferred to avoid the co-presence of these saccharides with the cellulase as far as possible. In case the co-presence is unavoidable, it is necessary to avoid the direct contact of the saccharides with the cellulase by, for example, coating them.

10 Long-chain-fatty acid salts and cationic surfactants act as the inhibitors in some cases. However, the co-presence of these substances with the cellulase is allowable if the direct contact of them is prevented by some means such as tableting or coating.

15 The above-mentioned masking agents and methods may be employed, if necessary, in the present invention.

Cellulase-activators

20 The activators vary depending on variety of the cellulases. In the presence of proteins, cobalt and its salts, magnesium and its salts, and calcium and its salts, potassium and its salts, sodium and its salts or monosac-charides such as mannose and xylose, the cellulases are activated and their deterging powers are improved remarkably.

25 Antioxidants

The antioxidants include, for example, tert-butyl-hydroxytoluene, 4,4'-butylidenebis(6-tert-butyl-3-methylphenol), 2,2'-butylidenebis(6-tert-butyl-4-methylphenol), monostyrenated cresol, distyrenated
30 cresol, monostyrenated phenol, distyrenated phenol and 1,1-bis(4-hydroxy-phenyl)cyclohexane.

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Solubilizers

5 The solubilizers include, for example, lower alcohols such as ethanol, benzenesulfonate salts, lower alkylbenzenesulfonate salts such as p-toluenesulfonate salts, glycols such as propylene glycol, acetylbenzenesulfonate salts, acetamides, pyridinedicarboxylic acid amides, benzoate salts and urea.

10 The detergent composition of the present invention can be used in a broad pH range of from acidic to alkaline pH. In a preferred embodiment, the detergent composition of the present invention can be used in alkaline detergent wash media and more preferably, alkaline detergent wash media having a pH of from above 7 to no more than about 8.

15 Aside from the above ingredients, perfumes, buffers, preservatives, dyes and the like can be used, if desired, with the detergent compositions of this invention. Such components are conventionally employed in amounts heretofore used in the art.

20 When a detergent base used in the present invention is in the form of a powder, it may be one which is prepared by any known preparation methods including a spray-drying method and a granulation method. The detergent base obtained particularly by the spray-drying method and/or spray-drying granulation method are preferred. The detergent base obtained by the spray-drying method is not restricted with respect to preparation conditions. The detergent base obtained by the spray-drying method is hollow granules which are
25 obtained by spraying an aqueous slurry of heat-resistant ingredients, such as surface active agents and builders, into a hot space. The granules have a size of from 50 to 2000 micrometers. After the spray-drying, perfumes, enzymes, bleaching agents, inorganic
30 alkaline builders may be added. With a highly dense,

granular detergent base obtained such as by the spray-drying-granulation method, various ingredients may also be added after the preparation of the base.

5 When the detergent base is a liquid, it may be either a homogeneous solution or an inhomogeneous dispersion. For removing the decomposition of carboxymethylcellulose by the cellulase in the detergent, it is desirable that carboxymethylcellulose is granulated or coated before the incorporation in the
10 composition.

The detergent compositions of this invention are used in industrial and household uses at temperatures and liquor ratios conventionally employed in these environments.

15 In addition to their use in laundry detergents, substantially pure EG III cellulase described herein can additionally be used in a pre-washing step in the appropriate solution at an intermediate pH where sufficient activity exists to provide desired
20 improvements in color retention/restoration, softening and feel. When the substantially pure EG III cellulase is employed in a pre-soak (e.g., pre-wash) composition, either as a liquid, spray, gel or paste composition, the substantially pure EG III cellulase is generally
25 employed from about 0.01 to about 20 weight percent based on the total weight of the pre-soak composition. In such compositions, a surfactant may optionally be employed and when employed, is generally present at a concentration of from about 0.01 to about 20 weight
30 percent based on the total weight of the pre-soak. The remainder of the composition comprises conventional components used in the pre-soak, i.e., diluent, buffers, other enzymes (proteases), and the like at their conventional concentrations. Accordingly, such
35 pre-soak compositions comprise from about 0 to about 20

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weight percent of a surfactant and from about 0.01 to about 20 weight percent of substantially pure EG III cellulase.

5 Also, it is contemplated that the substantially pure EG III cellulase described herein can be used in home use as a stand alone composition suitable for restoring color to faded fabrics (see, for example, U.S. Patent No. 4,738,682 as well as used in a spot-remover.

10

Additionally, it is further contemplated that the high activity under neutral to alkaline conditions for EG III cellulase would be beneficial in textile processes for treating cotton-containing fabrics (see Canadian Application Nos. 2,093,424 and 2,093,423 as well as in silage and/or composting processes.

20 The following examples are offered to illustrate the present invention and should not be construed in any way as limiting the scope of this invention.

EXAMPLES

Example 1 demonstrates the isolation of the components other than EG III from Cytolase 123 Cellulase (a complete fungal cellulase composition obtained from Trichoderma reesei and available from Genencor International, Inc., South San Francisco, CA) via purification procedures.

Example 1

Purification of Cytolase 123 Cellulase into Cellulase Components

30

CYTOLASE 123 cellulase was fractionated in the following manner. The normal distribution of cellulase components in this cellulase system is as follows:

5	CBH I	45-55 weight percent
	CBH II	13-15 weight percent
	EG I	11-13 weight percent
	EG II	8-10 weight percent
	EG III	1-4 weight percent
	BG	0.5-1 weight percent.

The fractionation was done using columns containing the following resins: Sephadex* G-25 gel filtration resin from Sigma Chemical Company (St. Louis, Mo), QA Trisacryl* M anion exchange resin and SP Trisacryl M cation exchange resin from IBF Biotechnics (Savage, Md). CYTOLASE 123 cellulase, 0.5g, was desalted using a column of 3 liters of Sephadex G-25 gel filtration resin with 10 mM sodium phosphate buffer at pH 6.8. The desalted solution, was then loaded onto a column of 20 ml of QA Trisacryl M anion exchange resin. The fraction bound on this column contained CBH I and EG I. These components were separated by gradient elution using an aqueous gradient containing from 0 to about 500 mM sodium chloride. The fraction not bound on this column contained CBH II and EG II. These fractions were desalted using a column of Sephadex G-25 gel filtration resin equilibrated with 10 mM sodium citrate, pH 3.3. This solution, 200 ml, was then loaded onto a column of 20 ml of SP Trisacryl M cation exchange resin. CBH II and EG II were eluted separately using an aqueous gradient containing from 0 to about 200 mM sodium chloride.

Example 2

30 Purification of EG III from Cytolase 123 Cellulase

Example 1 above demonstrated the isolation of several components from Cytolase 123 Cellulase. However, because EG III is present in very small

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quantities in Cytolase 123 Cellulase, the following procedures were employed to isolate this component.

A. Large Scale Extraction of EG III Cellulase Enzyme

One hundred liters of cell free cellulase filtrate were heated to about 30°C. The heated material was made about 4% wt/vol PEG 8000 (polyethylene glycol, MW of about 8000) and about 10% wt/vol anhydrous sodium sulfate. The mixture formed a two phase liquid mixture. The phases were separated using an SA-1 disk stack centrifuge. The phases were analyzed using silver staining isoelectric focusing gels. Fractionation and enrichment were obtained for EG III and xylanase. The recovered composition contained about 20 to 50 weight percent of EG III.

Regarding the above procedure, use of a polyethylene glycol having a molecular weight substantially less than about 8000 gave inadequate separation; whereas, use of polyethylene glycol having a molecular weight substantially greater than about 8000 resulted in the exclusion of desired enzymes in the recovered composition. With regard to the amount of sodium sulfate, sodium sulfate levels substantially greater than about 10% wt/vol caused precipitation problems; whereas, sodium sulfate levels substantially less than about 10% wt/vol gave poor separation or the solution remained in a single phase.

B. Purification of EG III Via Fractionation

The purification of EG III is conducted by fractionation from a complete fungal cellulase composition (CYTOLASE 123 cellulase, commercially available from Genencor International, South San Francisco, CA) which is produced by wild type Trichoderma reesei. Specifically, the fractionation is

4

done using columns containing the following resins:
Sephadex G-25 gel filtration resin from Sigma Chemical
Company (St. Louis, Mo), QA Trisacryl M anion exchange
resin and SP Trisacryl M cation exchange resin from IBF
5 Biotechnics (Savage, Md). CYTOLASE 123 cellulase,
0.5g, is desalted using a column of 3 liters of
Sephadex G-25 gel filtration resin with 10 mM sodium
phosphate buffer at pH 6.8. The desalted solution, is
10 then loaded onto a column of 20 ml of QA Trisacryl M
anion exchange resin. The fraction bound on this
column contained CBH I and EG I. The fraction not bound
on this column contains CBH II, EG II and EG III.
These fractions are desalted using a column of Sephadex
G-25 gel filtration resin equilibrated with 10 mM
15 sodium citrate, pH 4.5. This solution, 200 ml, is then
loaded onto a column of 20 ml of SP Trisacryl M cation
exchange resin. The EG III was eluted with 100 mL of
an aqueous solution of 200 mM sodium chloride.

In order to enhance the efficiency of the
20 isolation of EG III, it may be desirable to employ
Trichoderma reesei genetically modified so as to
overexpress EG III and/or to be incapable of producing
one or more of EG I, EG II, CBH I and/or CBH II
components. This will necessarily lead to more
25 efficient isolation of EG III by, for example,
fractionation and/or PEG extraction as described above.
Production of some of these strains of Trichoderma
reesei are disclosed in Canadian Application No. 2,093,425.

30 Likewise, it may be desirable for the EG III
compositions described above to be further purified.
For example, EG III protein isolated in procedures A or
B above can be further purified by utilizing material
obtained from procedure A in procedure B or vice versa.
35 One particular method for further purifying EG III is

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by further fractionation of an EG III sample obtained in part b) of this Example 2. The further fraction was done on a FPLC system using a Mono-S^{*}-HR 5/5 column (available from Pharmacia LKB Biotechnology, Piscataway, NJ). The FPLC system consists of a liquid chromatography controller, 2 pumps, a dual path monitor, a fraction collector and a chart recorder (all of which are available from Pharmacia LKB Biotechnology, Piscataway, NJ). The fractionation was conducted by desalting 5 ml of the EG III sample prepared in part b) of this Example 2 with a 20 ml Sephadex G-25 column which had been previously equilibrated with 10 mM sodium citrate pH 4. The column was then eluted with 0-200 mM aqueous gradient of NaCl at a rate of 0.5 ml/minute with samples collected in 1 ml fractions. EG III was recovered in fractions 10 and 11 and was determined to be greater than 90% pure by gel electrophoresis. EG III of this purity is suitable for determining the N-terminal amino acid sequence by known techniques.

Substantially pure EG III has the following characteristics which are compared to the other endoglucanases isolated from Trichoderma reesei.

TABLE I

	MW	pI	pH optimum ¹
EG I	~47-49 kD	4.7	~5
EG II	~35 kD	5.5	~5
EG III	~25-28 kD	7.4	~5.5-6.0

1. pH optimum determined by RBB-CMC activity as per Example 3 below.

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As can be seen from the above table, EG III has both a higher pH optimum and a higher pI as compared to the other endoglucanase components of Trichoderma reesei. In Example 3 below, it is seen that EG III
5 also retains significant RBB-CMC activity under alkaline pHs.

Likewise, EG III cellulase from other strains of Trichoderma spp. can be purified. For example, EG III cellulase derived from Trichoderma viride has been
10 described by Voragen et al., Methods in Enzymology, 160:243-249. This reference describes the EG III cellulase as having a molecular weight of about 23.5 Kdaltons, a pH optimum of 5.5, and a pI of 7.7.

Example 3

15 Activity of Cellulase Compositions Over a pH Range

The following procedure was employed to determine the pH profiles of two different cellulase compositions. The first cellulase composition was a
20 CBH I and II deleted cellulase composition prepared from Trichoderma reesei genetically modified in a manner similar to that described above so as to be unable to produce CBH I and CBH II components. Insofar as this cellulase composition does not contain CBH I
25 and CBH II which generally comprise from about 58 to 70 percent of a cellulase composition derived from Trichoderma reesei, this cellulase composition is necessarily enriched in EG components. Since EG III is the most minor of the endoglucanase components of
30 Trichoderma reesei, this composition predominates in EG I and EG II components.

The second cellulase composition was an approximately 20-40% pure fraction of EG III isolated from a cellulase composition derived from Trichoderma

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reesei via purification methods similar to part b) of Example 2.

5 The activity of these cellulase compositions was determined at 40°C and the determinations were made using the following procedures.

10 Add 5 to 20 µl of an appropriate enzyme solution at a concentration sufficient to provide the requisite amount of enzyme in the final solution. Add 250 µl of 2 weight percent RBB-CMC (Remazol Brilliant Blue R-Carboxymethyl-cellulose -- commercially available from MegaZyme, 6 Altona Place, North Rocks, N.S.W. 2151, Australia) in 0.05M citrate/phosphate buffer at pH 4, 5, 5.5, 6, 6.5, 7, 7.5 and 8.

15 Vortex and incubate at 40°C for 30 minutes. Chill in an ice bath for 5 to 10 minutes. Add 1000 µl of methyl cellosolve containing 0.3M sodium acetate and 0.02M zinc acetate. Vortex and let sit for 5-10 minutes. Centrifuge and pour supernatant into cuvetts.

20 Relative enzyme activity was determined by measuring the optical density (OD) of the solution in each cuvet at 590 nm. Higher levels of optical density correspond to higher levels of enzyme activity.

25 The results of this analysis are set forth in FIG. 1 which illustrates the relative activity of the CBH I and II deleted cellulase composition compared to the EG III cellulase composition. From this figure, it is seen that the cellulase composition deleted in CBH I and CBH II possesses optimum cellulolytic activity against RBB-CMC at near pH 5.5 and has some activity at 30 alkaline pHs, i.e., at pHs from above 7 to 8. On the other hand, the cellulase composition enriched in EG III possesses optimum cellulolytic activity at about pH 5.5 - 6 and possesses significant activity at alkaline pHs.

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Example 4Isoelectrofocusing Gels

The purpose of this example is to illustrate isoelectrofocusing gels of different EG III cellulase compositions. Specifically, cellulase produced by a wild type Trichoderma reesei; cellulase derived from a strain of Trichoderma reesei transformed so as to be incapable of expressing EG I and EG II cellulase proteins; and substantially pure EG III cellulase were analyzed on isoelectrofocusing gels.

Samples of these cellulases were analyzed by isoelectrofocusing using a Pharmacia IEF system (FBE-3000, Pharmacia Inc., Piscataway, NJ) and pH 3-10 precast gels (Servalyt Precote, available from Serva, Carl-Berg, Germany) according to the manufacturer's instructions. The gels were stained with EphortecTM stain (Serva Blue W, available from Serva Fine Biochemicals, Westbury, NY 11590) stain to visualize the protein bands. The resulting gel is set forth in FIG. 2 wherein Lane A of FIG. 2 illustrates the isoelectrofocusing gel of cellulase derived from a wild strain Trichoderma reesei; Lane B illustrates the isoelectrofocusing gel of cellulase derived from a strain of Trichoderma reesei so as to be incapable of expressing EG I and II; and Lane C illustrates the isoelectrofocusing gel of substantially pure EG III cellulase. In this figure, the margin adjacent to Lane A is marked to identify the bands corresponding to cellulase proteins so as to permit identification of the proteins.

From the above figure, it is seen that because of EG III's high pI, this protein is found in a region usually associated with other high pI components such as high pI xylanases, high pI β -glucosidases, etc. Moreover, this figure demonstrates that EG III is not a

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degradation product of either EG I or EG II proteins because, in Lane B of this figure, these proteins are not present while the EG III protein is.

Example 5

5 Color Restoration

The ability of EG III cellulase to restore color in cotton-containing fabrics was analyzed in the following experiment. Specifically, reduced color clarity in a worn cotton fabric arises from the accumulation on the fabric of surface fibers over a period of time. These fibers give rise to a faded and matted appearance for the fabric and accordingly, the removal of these fibers is a necessary prerequisite to restoring the original sharp color to the fabric. In view of the above, this experiment determines the ability of EG III cellulase to restore color by measuring the ability of the EG III cellulase to remove surface fibers.

In this experiment, two different compositions were compared for the ability to remove fiber. Specifically, the first composition contains substantially pure EG III cellulase (prepared in a manner similar to that set forth in Example 2) whereas the second composition contains no EG III cellulase or any other cellulase composition.

In this example, an appropriate amount of cellulase (if employed) was added to separate solutions of 400 ml of a 20 mM citrate/phosphate buffer containing 0.5 ml of a non-ionic surfactant. Samples were prepared and titrated so as to provide for samples at pH 6, pH 7, pH 8 and pH 9. Each of the resulting solution was then added to a separate launderometer canister. Into these canisters were added a quantity

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of marbles to facilitate fiber removal as well as a 7 inch x 5 inch cotton fabric (100% woven cotton, available as Style No. 439W from Test Fabrics, Inc., 200 Blackford Ave., Middlesex, NJ 08846). The canister was then closed and the canister lowered into the launderometer bath which was maintained at 43°C. The canister was then rotated in the bath at a speed of at least about 40 revolutions per minute (rpms) for about 1 hour. Afterwards, the cloth is removed, rinsed well and dried in a standard drier.

The so treated fabrics were then analyzed for fiber removal by evaluation in a panel test. In particular, the fabrics (unmarked) were rated for levels of fiber by 6 individuals. The fabrics were visually evaluated for surface fibers and rated on a 1 to 7 scale. The scale has seven standards to allow for meaningful comparisons. The standards are:

	Rating	Standard ^a
	7	Fabric not treated with cellulase
20	6	Fabric treated ^b with 8 ppm cellulase
	5	Fabric treated with 16 ppm cellulase
	4	Fabric treated with 20 ppm cellulase
	3	Fabric treated with 40 ppm cellulase
	2	Fabric treated with 50 ppm cellulase
25	1	Fabric treated with 100 ppm cellulase

a In all of the standards, the fabric was a 100% cotton sheeting standardized test fabric (Style No. 439W) available from Test Fabrics, Inc., 200 Blackford Ave., Middlesex, NJ 08846

30 b For all samples treated with the same cellulase composition. Cellulase concentrations are in total protein. The launderometer treatment conditions are the same as set forth in Example 16 above.

35 The fabric to be rated was provided a rating which most closely matched one of the standards. After

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complete analysis of the fabrics, the values assigned to each fabric by all of the individuals were added and an average value generated. In these results, lower numbers correspond to improved fiber removal.

5 The results of this analysis are set forth in FIG. 3 which illustrates that at acidic, neutral and alkaline pH's, substantially pure EG III cellulase provides for fiber removal.

Example 6

10 Peptide Sequencing of EGIII

 The EG III component was precipitated by the addition of 0.9 ml of acetone to 0.1 ml of protein solution (at a concentration of 1 mg/ml) and incubation at -20°C for 10 minutes. The protein was collected by
15 centrifugation and the pellet dried and resuspended in 0.01 ml of 8 M urea in 88% formic acid and 0.01 ml of cyanogen bromide (200 mg/ml) in 88% formic acid. The mixture was incubated at room temperature for four hours.

20 Individual peptides were purified on a HPLC (high pressure liquid chromatography) column. A Synchropak* RP-4 column was equilibrated in milliQ* water with 0.05% TEA (triethylamine) and 0.05% TFA. The sample was loaded onto the HPLC column and elution was carried out
25 with 100% acetonitrile and 0.05% TEA and 0.05% TFA, with a gradient of 1% per minute. The amino-terminal regions of isolated peptides were sequenced by the method of Edman using a fully automated apparatus. The amino acid sequence obtained from two fragments of the
30 EG III component are shown in FIG. 4

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Example 7Selection for pyr4 derivatives of Trichoderma reesei

The pyr4 gene encodes orotidine-5'-monophosphate decarboxylase, an enzyme required for the biosynthesis of uridine. The toxic inhibitor 5-fluoroorotic acid (FOA) is incorporated into uridine by wild-type cells and thus poisons the cells. However, cells defective in the pyr4 gene are resistant to this inhibitor but require uridine for growth. It is, therefore, possible to select for pyr4 derivative strains using FOA. In practice, spores of T. reesei strain RL-P37 (Sheir-Neiss, G. and Montenecourt, B.S., Appl. Microbiol. Biotechnol. 20, p. 46-53 (1984)) were spread on the surface of a solidified medium containing 2 mg/ml uridine and 1.2 mg/ml FOA. Spontaneous FOA-resistant colonies appeared within three to four days and it was possible to subsequently identify those FOA-resistant derivatives which required uridine for growth. In order to identify those derivatives which specifically had a defective pyr4 gene, protoplasts were generated and transformed with a plasmid containing a wild-type pyr4 gene (see Examples 9 and 10). Following transformation, protoplasts were plated on medium lacking uridine. Subsequent growth of transformed colonies demonstrated complementation of a defective pyr4 gene by the plasmid-borne pyr4 gene. In this way, strain GC69 was identified as a pyr4 derivative of strain RL-P37.

Example 8

30

Preparation of CBHI Deletion Vector

A cbh1 gene encoding the CBHI protein was cloned from the genomic DNA of T. reesei strain RL-P37 by

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hybridization with an oligonucleotide probe designed on the basis of the published sequence for this gene using known probe synthesis methods (Shoemaker et al., 1983b). The cbh1 gene resides on a 6.5 kb PstI fragment and was inserted into PstI cut pUC4K (purchased from Pharmacia Inc., Piscataway, NJ) replacing the Kan^r gene of this vector using techniques known in the art, which techniques are set forth in Maniatis et al., Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press. The resulting plasmid, pUC4K::cbh1 was then cut with HindIII and the larger fragment of about 6 kb was isolated and religated to give pUC4K::cbh1ΔH/H (see FIG. 5). This procedure removes the entire cbh1 coding sequence and approximately 1.2 kb upstream and 1.5 kb downstream of flanking sequences. Approximately, 1 kb of flanking DNA from either end of the original PstI fragment remains.

The T. reesei pyr4 gene was cloned as a 6.5 kb HindIII fragment of genomic DNA in pUC18 to form pTpyr2 (Smith et al., Current Genetics, Vol. 19, pp. 27-33, 1991) following the methods of Maniatis et al., supra. The plasmid pUC4K::cbh1ΔH/H was cut with HindIII and the ends were dephosphorylated with calf intestinal alkaline phosphatase. This end dephosphorylated DNA was ligated with the 6.5 kb HindIII fragment containing the T. reesei pyr4 gene to give pΔCBH1pyr4. FIG. 5 illustrates the construction of this plasmid.

Example 9

Isolation of Protoplasts

30 Mycelium was obtained by inoculating 100 ml of YEG (0.5% yeast extract, 2% glucose) in a 500 ml flask with about 5×10^7 T. reesei GC69 spores (the pyr4

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derivative strain). The flask was then incubated at 37°C with shaking for about 16 hours. The mycelium was harvested by centrifugation at 2,750 x g. The harvested mycelium was further washed in a 1.2 M sorbitol solution and resuspended in 40 ml of a solution containing 5 mg/ml Novozym^R 234 solution (which is the tradename for a multicomponent enzyme system containing 1,3-alpha-glucanase, 1,3-beta-glucanase, laminarinase, xylanase, chitinase and protease from Novo Biolabs, Danbury, CT); 5 mg/ml MgSO₄.7H₂O; 0.5 mg/ml bovine serum albumin; 1.2 M sorbitol. The protoplasts were removed from the cellular debris by filtration through Miracloth* (Calbiochem Corp, La Jolla, CA) and collected by centrifugation at 2,000 x g. The protoplasts were washed three times in 1.2 M sorbitol and once in 1.2 M sorbitol, 50 mM CaCl₂, centrifuged and resuspended at a density of approximately 2 x 10⁸ protoplasts per ml of 1.2 M sorbitol, 50 mM CaCl₂.

20

Example 10Transformation of Fungal Protoplasts with pΔCBHIpyr4

200 μl of the protoplast suspension prepared in Example 9 was added to 20 μl of EcoRI digested pΔCBHIpyr4 (prepared in Example 8) in TE buffer (10 mM Tris, pH 7.4; 1 mM EDTA) and 50 μl of a polyethylene glycol (PEG) solution containing 25% PEG 4000, 0.6 M KCl and 50 mM CaCl₂. This mixture was incubated on ice for 20 minutes. After this incubation period 2.0 ml of the above-identified PEG solution was added thereto, the solution was further mixed and incubated at room temperature for 5 minutes. After this second incubation, 4.0 ml of a solution containing 1.2 M sorbitol and 50 mM CaCl₂ was added thereto and this

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solution was further mixed. The protoplast solution was then immediately added to molten aliquots of Vogel's Medium N (3 grams sodium citrate, 5 grams KH_2PO_4 , 2 grams NH_4NO_3 , 0.2 grams $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 gram $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 μg α -biotin, 5 mg citric acid, 5 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg $\text{Fe}(\text{NH}_4)_2 \cdot 6\text{H}_2\text{O}$, 0.25 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 50 μg $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ per liter) containing an additional 1% glucose, 1.2 M sorbitol and 1% agarose. The protoplast/medium mixture was then poured onto a solid medium containing the same Vogel's medium as stated above. No uridine was present in the medium and therefore only transformed colonies were able to grow as a result of complementation of the pyr4 mutation of strain GC69 by the wild type pyr4 gene insert in p Δ CBHIpyr4. These colonies were subsequently transferred and purified on a solid Vogel's medium N containing as an additive, 1% glucose and stable transformants were chosen for further analysis.

At this stage stable transformants were distinguished from unstable transformants by their faster growth rate and formation of circular colonies with a smooth, rather than ragged outline on solid culture medium lacking uridine. In some cases a further test of stability was made by growing the transformants on solid non-selective medium (i.e. containing uridine), harvesting spores from this medium and determining the percentage of these spores which will subsequently germinate and grow on selective medium lacking uridine.

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Example 11Analysis of the Transformants

DNA was isolated from the transformants obtained in Example 10 after they were grown in liquid Vogel's medium N containing 1% glucose. These transformant DNA samples were further cut with a PstI restriction enzyme and subjected to agarose gel electrophoresis. The gel was then blotted onto a Nytran membrane filter and hybridized with a ³²P labelled pΔCBHIpyr4 probe. The probe was selected to identify the native cbh1 gene as a 6.5 kb PstI fragment, the native pyr4 gene and any DNA sequences derived from the transforming DNA fragment.

The radioactive bands from the hybridization were visualized by autoradiography. The autoradiograph is seen in FIG. 7. Five samples were run as described above, hence samples A, B, C, D, and E. Lane E is the untransformed strain GC69 and was used as a control in the present analysis. Lanes A-D represent transformants obtained by the methods described above. The numbers on the side of the autoradiograph represent the sizes of molecular weight markers. As can be seen from this autoradiograph, lane D does not contain the 6.5 kb CBHI band, indicating that this gene has been totally deleted in the transformant by integration of the DNA fragment at the cbh1 gene. The cbh1 deleted strain is called P37PΔCBHI. Figure 6 outlines the deletion of the T. reesei cbh1 gene by integration through a double cross-over event of the larger EcoRI fragment from pΔCBHIpyr4 at the cbh1 locus on one of the T. reesei chromosomes. The other transformants analyzed appear identical to the untransformed control strain.

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Example 12Analysis of the Transformants with pIntCBHI

The same procedure was used in this example as in Example 11, except that the probe used was changed to a ³²P labelled pIntCBHI probe. This probe is a pUC-type plasmid containing a 2 kb BglII fragment from the cbh1 locus within the region that was deleted in pUC4K::cbh1ΔH/H. Two samples were run in this example including a control, sample A, which is the untransformed strain GC69 and the transformant P37PΔCBHI, sample B. As can be seen in FIG. 8, sample A contained the cbh1 gene, as indicated by the band at 6.5 kb; however the transformant, sample B, does not contain this 6.5 kb band and therefore does not contain the cbh1 gene and does not contain any sequences derived from the pUC plasmid.

Example 13Protein Secretion by Strain P37PΔCBHI

Spores from the produced P37PΔCBHI strain were inoculated into 50 ml of a Trichoderma basal medium containing 1% glucose, 0.14% (NH₄)₂SO₄, 0.2% KH₂PO₄, 0.03% MgSO₄, 0.03% urea, 0.75% bactotryptone, 0.05% Tween 80, 0.000016% CuSO₄.5H₂O, 0.001% FeSO₄.7H₂O, 0.000128% ZnSO₄.7H₂O, 0.0000054% Na₂MoO₄.2H₂O, 0.0000007% MnCl₄.4H₂O). The medium was incubated with shaking in a 250 ml flask at 37°C for about 48 hours. The resulting mycelium was collected by filtering through Miracloth (Calbiochem Corp.) and washed two or three times with 17 mM potassium phosphate. The mycelium was finally suspended in 17 mM potassium phosphate with 1 mM sophorose and further incubated for 24 hours at 30°C with shaking. The supernatant was then collected from these cultures and the mycelium was discarded. Samples

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of the culture supernatant were analyzed by isoelectric focusing using a Pharmacia Phastgel* system and pH 3-9 precast gels according to the manufacturer's instructions. The gel was stained with silver stain to visualize the protein bands. The band corresponding to the cbh1 protein was absent from the sample derived from the strain P37PACBHI, as shown in FIG. 9. This isoelectric focusing gel shows various proteins in different supernatant cultures of T. reesei. Lane A is partially purified CBHI; Lane B is the supernatant from an untransformed T. reesei culture; Lane C is the supernatant from strain P37PACBHI produced according to the methods of the present invention. The position of various cellulase components are labelled CBHI, CBHII, EGI, EGII, and EGIII. Since CBHI constitutes 50% of the total extracellular protein, it is the major secreted protein and hence is the darkest band on the gel. This isoelectric focusing gel clearly shows depletion of the CBHI protein in the P37PACBHI strain.

20

Example 14

Preparation of pPACBHII

The cbh2 gene of T. reesei, encoding the CBHII protein, has been cloned as a 4.1 kb EcoRI fragment of genomic DNA which is shown diagrammatically in FIG. 10A (Chen et al., 1987, Biotechnology, 5:274-278). This 4.1 kb fragment was inserted between the EcoRI sites of pUC4XL. The latter plasmid is a pUC derivative (constructed by R.M. Berka, Genencor International Inc.) which contains a multiple cloning site with a symmetrical pattern of restriction endonuclease sites arranged in the order shown here: EcoRI, BamHI, SacI, SmaI, HindIII, XhoI, BglII, ClaI, BglII, XhoI, HindIII, SmaI, SacI, BamHI, EcoRI. Using methods known in the

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art, a plasmid, pPACBHII (FIG. 10B), has been constructed in which a 1.7 kb central region of this gene between a HindIII site (at 74 bp 3' of the CBHII translation initiation site) and a ClaI site (at 265 bp 3' of the last codon of CBHII) has been removed and replaced by a 1.6 kb HindIII-ClaI DNA fragment containing the T. reesei pyr4 gene.

The T. reesei pyr4 gene was excised from pTpyr2 (see Example 8) on a 1.6 kb NheI-SphI fragment and inserted between the SphI and XbaI sites of pUC219 (see Example 22) to create p219M (Smith et al., 1991, Curr. Genet 19 p. 27-33). The pyr4 gene was then removed as a HindIII-ClaI fragment having seven bp of DNA at one end and six bp of DNA at the other end derived from the pUC219 multiple cloning site and inserted into the HindIII and ClaI sites of the cbh2 gene to form the plasmid pPACBHII (see FIG. 10B).

Digestion of this plasmid with EcoRI will liberate a fragment having 0.7 kb of flanking DNA from the cbh2 locus at one end, 1.7 kb of flanking DNA from the cbh2 locus at the other end and the T. reesei pyr4 gene in the middle.

Example 15

Deletion of the cbh2 gene in T. reesei strain GC69

Protoplasts of strain GC69 will be generated and transformed with EcoRI digested pPACBHII according to the methods outlined in Examples 8 and 9. DNA from the transformants will be digested with EcoRI and Asp718, and subjected to agarose gel electrophoresis. The DNA from the gel will be blotted to a membrane filter and hybridized with ³²P labelled pPACBHII according to the

methods in Example 17. Transformants will be identified which have a single copy of the EcoRI fragment from p Δ CBHII integrated precisely at the cbh2 locus. The transformants will also be grown in shaker
5 flasks as in Example 13 and the protein in the culture supernatants examined by isoelectric focusing. In this manner T. reesei GC69 transformants which do not produce the CBHII protein will be generated.

Example 16

10 Generation of a pyr4⁻ Derivative of P37P Δ CBHI

Spores of the transformant (P37P Δ CBHI) which was deleted for the cbh1 gene were spread onto medium containing FOA. A pyr4⁻ derivative of this transformant was subsequently obtained using the methods of Example
15 7. This pyr4⁻ strain was designated P37P Δ CBHIPyr⁻26.

Example 17

Deletion of the cbh2 gene in a strain previously deleted for cbh1

Protoplasts of strain P37P Δ CBHIPyr⁻26 were
20 generated and transformed with EcoRI digested p Δ CBHII according to the methods outlined in Examples 8 and 9.

Purified stable transformants were cultured in shaker flasks as in Example 13 and the protein in the culture supernatants was examined by isoelectric
25 focusing. One transformant (designated P37P $\Delta\Delta$ CBH67) was identified which did not produce any CBHII protein. Lane D of FIG. 9 shows the supernatant from a transformant deleted for both the cbh1 and cbh2 genes produced according to the methods of the present
30 invention.

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DNA was extracted from strain P37P Δ CBH67, digested with EcoRI and Asp718, and subjected to agarose gel electrophoresis. The DNA from this gel was blotted to a membrane filter and hybridized with ³²p labelled p Δ CBHII (FIG. 11). Lane A of FIG. 11 shows the hybridization pattern observed for DNA from an untransformed T. reesei strain. The 4.1 kb EcoRI fragment containing the wild-type cbh2 gene was observed. Lane B shows the hybridization pattern observed for strain P37P Δ CBH67. The single 4.1 kb band has been eliminated and replaced by two bands of approximately 0.9 and 3.1 kb. This is the expected pattern if a single copy of the EcoRI fragment from p Δ CBHII had integrated precisely at the cbh2 locus.

The same DNA samples were also digested with EcoRI and Southern blot analysis was performed as above. In this Example, the probe was ³²P labelled pIntCBHII. This plasmid contains a portion of the cbh2 gene coding sequence from within that segment of the cbh2 gene which was deleted in plasmid p Δ CBHII. No hybridization was seen with DNA from strain P37P Δ CBH67 showing that the cbh2 gene was deleted and that no sequences derived from the pUC plasmid were present in this strain.

25

Example 18

Construction of pEGIp_{pyr4}

The T. reesei egl1 gene, which encodes EGI, has been cloned as a 4.2 kb HindIII fragment of genomic DNA from strain RL-P37 by hybridization with oligonucleotides synthesized according to the published sequence (Penttila et al., 1986, Gene 45:253-263; van Arsdell et al., 1987, Bio/Technology 5:60-64). A 3.6

kb HindIII-BamHI fragment was taken from this clone and ligated with a 1.6 kb HindIII-BamHI fragment containing the T. reesei pyr4 gene obtained from pTpyr2 (see Example 8) and pUC218 (identical to pUC219, see Example 22, but with the multiple cloning site in the opposite orientation) cut with HindIII to give the plasmid pEGIpyr4 (FIG. 12). Digestion of pEGIpyr4 with HindIII would liberate a fragment of DNA containing only T. reesei genomic DNA (the eql1 and pyr4 genes) except for 24 bp of sequenced, synthetic DNA between the two genes and 6 bp of sequenced, synthetic DNA at one end (see FIG. 12).

Example 19

Transformants of Trichoderma reesei Containing the plasmid pEGIpyr4

A pyr4 defective derivative of T. reesei strain RutC30 (Sheir-Neiss and Montenecourt, (1984), Appl. Microbiol. Biotechnol. 20:46-53) was obtained by the method outlined in Example 7. Protoplasts of this strain were transformed with undigested pEGIpyr4 and stable transformants were purified.

Five of these transformants (designated EP2, EP4, EP5, EP6, EP11), as well as untransformed RutC30 were inoculated into 50 ml of YEG medium (yeast extract, 5 g/l; glucose, 20 g/l) in 250 ml shake flasks and cultured with shaking for two days at 28°C. The resulting mycelium was washed with sterile water and added to 50 ml of TSF medium (0.05M citrate-phosphate buffer, pH 5.0; Avicel microcrystalline cellulose, 10 g/l; KH_2PO_4 , 2.0 g/l; $(\text{NH}_4)_2\text{SO}_4$, 1.4 g/l; proteose peptone, 1.0 g/l; Urea, 0.3 g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g/l; CaCl_2 , 0.3 g/l; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 mg/l; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.6 mg/l;

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ZnSO₄, 1.4 mg/l; CoCl₂, 2.0 mg/l; 0.1% Tween 80). These cultures were incubated with shaking for a further four days at 28°C. Samples of the supernatant were taken from these cultures and assays designed to measure the total amount of protein and of endoglucanase activity were performed as described below.

The endoglucanase assay relied on the release of soluble, dyed oligosaccharides from Remazol Brilliant Blue-carboxymethylcellulose (RBB-CMC, obtained from MegaZyme, North Rocks, NSW, Australia). The substrate was prepared by adding 2 g of dry RBB-CMC to 80 ml of just boiled deionized water with vigorous stirring. When cooled to room temperature, 5 ml of 2 M sodium acetate buffer (pH 4.8) was added and the pH adjusted to 4.5. The volume was finally adjusted to 100 ml with deionized water and sodium azide added to a final concentration of 0.02%. Aliquots of T. reesei control culture, pEGIp_{pyr4} transformant culture supernatant or 0.1 M sodium acetate as a blank (10-20 µl) were placed in tubes, 250 µl of substrate was added and the tubes were incubated for 30 minutes at 37°C. The tubes were placed on ice for 10 minutes and 1 ml of cold precipitant (3.3% sodium acetate, 0.4% zinc acetate, pH 5 with HCl, 76% ethanol) was then added. The tubes were vortexed and allowed to sit for five minutes before centrifuging for three minutes at approximately 13,000 x g. The optical density was measured spectrophotometrically at a wavelength of 590-600 nm.

The protein assay used was the BCA (bicinchoninic acid) assay using reagents obtained from Pierce, Rockford, Illinois, USA. The standard was bovine serum albumin (BSA). BCA reagent was made by mixing 1 part of reagent B with 50 parts of reagent A. One ml of the

5 BCA reagent was mixed with 50 μ l of appropriately diluted BSA or test culture supernatant. Incubation was for 30 minutes at 37°C and the optical density was finally measured spectrophotometrically at a wavelength of 562 nm.

10 The results of the assays described above are shown in Table 1. It is clear that some of the transformants produced increased amounts of endoglucanase activity compared to untransformed strain RutC30. It is thought that the endoglucanases and exo-cellobiohydrolases produced by untransformed T. reesei constitute approximately 20 and 70 percent respectively of the total amount of protein secreted. Therefore a transformant such as EP5, which produces approximately
15 four-fold more endoglucanase than strain RutC30, would be expected to secrete approximately equal amounts of endoglucanase-type and exo-cellobiohydrolase-type proteins.

20 The transformants described in this Example were obtained using intact pEGIp_{yr4} and will contain DNA sequences integrated in the genome which were derived from the pUC plasmid. Prior to transformation it would be possible to digest pEGIp_{yr4} with HindIII and isolate the larger DNA fragment containing only T. reesei DNA.
25 Transformation of T. reesei with this isolated fragment of DNA would allow isolation of transformants which overproduced EGI and contained no heterologous DNA sequences except for the two short pieces of synthetic DNA shown in FIG. 12. It would also be possible to use
30 pEGIp_{yr4} to transform a strain which was deleted for either the cbh1 gene, or the cbh2 gene, or for both genes. In this way a strain could be constructed which

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would over-produce EGI and produce either a limited range of, or no, exo-cellobiohydrolases.

The methods of Example 19 could be used to produce T. reesei strains which would over-produce any of the other cellulase components, xylanase components or other proteins normally produced by T. reesei.

TABLE 1

Secreted Endoglucanase Activity of
T. reesei Transformants

	A	B	
	ENDOGLUCANASE ACTIVITY	PROTEIN	
<u>STRAIN</u>	<u>(O.D. AT 590 nm)</u>	<u>(mg/ml)</u>	<u>A/B</u>
RutC30	0.32	4.1	0.078
15 EP2	0.70	3.7	0.189
EP4	0.76	3.65	0.208
EP5	1.24	4.1	0.302
EP6	0.52	2.93	0.177
EP11	0.99	4.11	0.241

The above results are presented for the purpose of demonstrating the overproduction of the EGI component relative to total protein and not for the purpose of demonstrating the extent of overproduction. In this regard, the extent of overproduction is expected to vary with each experiment.

Example 20

Construction of pCEPC1

A plasmid, pCEPC1, was constructed in which the coding sequence for EGI was functionally fused to the promoter from the cbh1 gene. This was achieved using

in vitro, site-specific mutagenesis to alter the DNA sequence of the cbh1 and egl1 genes in order to create convenient restriction endonuclease cleavage sites just 5' (upstream) of their respective translation initiation sites. DNA sequence analysis was performed to verify the expected sequence at the junction between the two DNA segments. The specific alterations made are shown in FIG. 13.

The DNA fragments which were combined to form pCEPC1 were inserted between the EcoRI sites of pUC4K and were as follows (see FIG. 14):

A) A 2.1 kb fragment from the 5' flanking region of the cbh1 locus. This includes the promoter region and extends to the engineered BclI site and so contains no cbh1 coding sequence.

B) A 1.9 kb fragment of genomic DNA from the egl1 locus starting at the 5' end with the engineered BamHI site and extending through the coding region and including approximately 0.5 kb beyond the translation stop codon. At the 3' end of the fragment is 18 bp derived from the pUC218 multiple cloning site and a 15 bp synthetic oligonucleotide used to link this fragment with the fragment below.

C) A fragment of DNA from the 3' flanking region of the cbh1 locus, extending from a position approximately 1 kb downstream to approximately 2.5 kb downstream of the cbh1 translation stop codon.

D) Inserted into an NheI site in fragment (C) was a 3.1 kb NheI-SphI fragment of DNA containing the T. reesei pyr4 gene obtained from pTpyr2 (Example 8) and having 24 bp of DNA at one end derived from the pUC18 multiple cloning site.

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The plasmid, pCEPC1 was designed so that the EGI coding sequence would be integrated at the cbh1 locus, replacing the coding sequence for CBHI without introducing any foreign DNA into the host strain.

5 Digestion of this plasmid with EcoRI liberates a fragment which includes the cbh1 promoter region, the egl1 coding sequence and transcription termination region, the T. reesei pyr4 gene and a segment of DNA from the 3' (downstream) flanking region of the cbh1

10 locus (see Fig. 13).

Example 21

Transformants containing pCEPC1 DNA

A pyr4 defective strain of T. reesei RutC30 (Sheir-Neiss, supra) was obtained by the method

15 outlined in Example 7. This strain was transformed with pCEPC1 which had been digested with EcoRI. Stable transformants were selected and subsequently cultured in shaker flasks for cellulase production as described in Example 19. In order to visualize the cellulase

20 proteins, isoelectric focusing gel electrophoresis was performed on samples from these cultures using the method described in Example 13. Of a total of 23 transformants analysed in this manner 12 were found to produce no CBHI protein, which is the expected result

25 of integration of the CEPC1 DNA at the cbh1 locus. Southern blot analysis was used to confirm that integration had indeed occurred at the cbh1 locus in some of these transformants and that no sequences derived from the bacterial plasmid vector (pUC4K) were

30 present (see FIG. 15). For this analysis the DNA from the transformants was digested with PstI before being subjected to electrophoresis and blotting to a membrane filter. The resulting Southern blot was probed with

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radiolabelled plasmid pUC4K::cbh1 (see Example 8). The probe hybridised to the cbh1 gene on a 6.5 kb fragment of DNA from the untransformed control culture (FIG. 15, lane A). Integration of the CEPC1 fragment of DNA at the cbh1 locus would be expected to result in the loss of this 6.5 kb band and the appearance of three other bands corresponding to approximately 1.0 kb, 2.0 kb and 3.5 kb DNA fragments. This is exactly the pattern observed for the transformant shown in FIG. 15, lane C. Also shown in FIG. 15, lane B is an example of a transformant in which multiple copies of pCEPC1 have integrated at sites in the genome other than the cbh1 locus.

Endoglucanase activity assays were performed on samples of culture supernatant from the untransformed culture and the transformants exactly as described in Example 19 except that the samples were diluted 50 fold prior to the assay so that the protein concentration in the samples was between approximately 0.03 and 0.07 mg/ml. The results of assays performed with the untransformed control culture and four different transformants (designated CEPC1-101, CEPC1-103, CEPC1-105 and CEPC1-112) are shown in Table 2. Transformants CEPC1-103 and CEPC1-112 are examples in which integration of the CEPC1 fragment had led to loss of CBHI production.

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Table 2
Secreted endoglucanase activity of T. reesei
transformants

	A	B	A/B
5	ENDOGLUCANASE		
	ACTIVITY		
	<u>STRAIN</u>	<u>(O.D. at 590 nm)</u>	<u>PROTEIN</u> <u>(mg/ml)</u>
	RutC300	0.037	2.38
	CEPC1-101	0.082	2.72
10	CEPC1-103	0.099	1.93
	CEPC1-105	0.033	2.07
	CEPC1-112	0.093	1.72
			0.016
			0.030
			0.051
			0.016
			0.054

15 The above results are presented for the purpose of demonstrating the overproduction of the EGI component relative to total protein and not for the purpose of demonstrating the extent of overproduction. In this regard, the extent of overproduction is expected to vary with each experiment.

20 It would be possible to construct plasmids similar to pCEPC1 but with any other T. reesei gene replacing the egl1 gene. In this way, overexpression of other genes and simultaneous deletion of the cbh1 gene could be achieved.

25 It would also be possible to transform pyr4 derivative strains of T. reesei which had previously been deleted for other genes, eg. for cbh2, with pCEPC1 to construct transformants which would, for example, produce no exo-cellobiohydrolases and overexpress
 30 endoglucanases.

Using constructions similar to pCEPC1, but in which DNA from another locus of T. reesei was substituted for the DNA from the cbh1 locus, it would

be possible to insert genes under the control of another promoter at another locus in the T. reesei genome.

Example 22

5

Construction of pEGII::P-1

The egl3 gene, encoding EGII (previously referred to as EGIII by others), has been cloned from T. reesei and the DNA sequence published (Saloheimo et al., 1988, Gene 63:11-21). We have obtained the gene from strain
10 RL-P37 as an approximately 4 kb PstI-XhoI fragment of genomic DNA inserted between the PstI and XhoI sites of pUC219. The latter vector, pUC219, is derived from pUC119 (described in Wilson et al., 1989, Gene 77:69-78) by expanding the multiple cloning site to include
15 restriction sites for BglII, ClaI and XhoI. Using methods known in the art the T. reesei pyr4 gene, present on a 2.7 kb SalI fragment of genomic DNA, was inserted into a SalI site within the EGII coding sequence to create plasmid pEGII::P-1 (FIG. 16). This
20 resulted in disruption of the EGII coding sequence but without deletion of any sequences. The plasmid, pEGII::P-1 can be digested with HindIII and BamHI to yield a linear fragment of DNA derived exclusively from T. reesei except for 5 bp on one end and 16 bp on the
25 other end, both of which are derived from the multiple cloning site of pUC219.

Example 23

Transformation of T. reesei GC69 with pEGII::P-1 to create a strain unable to produce EGII

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T. reesei strain GC69 will be transformed with pEGII::P-1 which had been previously digested with HindIII and BamHI and stable transformants will be

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selected. Total DNA will be isolated from the transformants and Southern blot analysis used to identify those transformants in which the fragment of DNA containing the pyr4 and egl3 genes had integrated at the egl3 locus and consequently disrupted the EGII coding sequence. The transformants will be unable to produce EGII. It would also be possible to use pEGII::P-1 to transform a strain which was deleted for either or all of the cbh1, cbh2, or egl1 genes. In this way a strain could be constructed which would only produce certain cellulase components and no EGII component.

Example 24

15 Transformation of T. reesei with pEGII::P-1
 to create a strain unable to produce CBHI,
 CBHII and EGII

A pyr4 deficient derivative of strain P37PΔΔCBH67 (from Example 17) was obtained by the method outlined in Example 7. This strain P37PΔΔ67P⁻¹ was transformed with pEGII::P-1 which had been previously digested with HindIII and BamHI and stable transformants were selected. Total DNA was isolated from transformants and Southern blot analysis used to identify strains in which the fragment of DNA containing the pyr4 and egl3 genes had integrated at the egl3 locus and consequently disrupted the EGII coding sequence. The Southern blot illustrated in FIG. 17 was probed with an approximately 4 kb PstI fragment of T. reesei DNA containing the egl3 gene which had been cloned into the PstI site of pUC18 and subsequently re-isolated. When the DNA isolated from strain P37PΔΔ67P⁻¹ was digested with PstI for Southern blot analysis the egl3 locus was subsequently visualized as a single 4 kb band on the autoradiograph (FIG. 17, lane E). However, for a transformant disrupted for the egl3 gene this band was lost and was

replaced by two new bands as expected (FIG. 17, Lane F). If the DNA was digested with EcoRV or BglII the size of the band corresponding to the egl3 gene increased in size by approximately 2.7 kb (the size of the inserted pyr4 fragment) between the untransformed P37P $\Delta\Delta$ 67P⁻¹ strain (Lanes A and C) and the transformant disrupted for egl3 (FIG. 17, Lanes B and D). The transformant containing the disrupted egl3 gene illustrated in FIG. 17 (Lanes B, D and F) was named A22. The transformant identified in FIG. 17 is unable to produce CBHI, CBHII or EGII.

Example 25

Construction of pP Δ EGI-1

The egl1 gene of T. reesei strain RL-P37 was obtained, as described in Example 18, as a 4.2 kb HindIII fragment of genomic DNA. This fragment was inserted at the HindIII site of pUC100 (a derivative of pUC18; Yanisch-Perron et al., 1985, Gene 33:103-119, with an oligonucleotide inserted into the multiple cloning site adding restriction sites for BglII, ClaI and XhoI). Using methodology known in the art an approximately 1 kb EcoRV fragment extending from a position close to the middle of the EGI coding sequence to a position beyond the 3' end of the coding sequence was removed and replaced by a 3.5 kb ScaI fragment of T. reesei DNA containing the pyr4 gene. The resulting plasmid was called pP Δ EGI-1 (see FIG. 18).

The plasmid pP Δ EGI-1 can be digested with HindIII to release a DNA fragment comprising only T. reesei genomic DNA having a segment of the egl1 gene at

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either end and the pyr4 gene replacing part of the EGI coding sequence, in the center.

Transformation of a suitable T. reesei pyr4 deficient strain with the p Δ EGI-1 digested with
5 HindIII will lead to integration of this DNA fragment at the egl1 locus in some proportion of the transformants. In this manner a strain unable to produce EGI will be obtained.

Example 26

10 Construction of p Δ EGIp_{pyr}-3 and Transformation of a pyr4 deficient strain of T. reesei

The expectation that the EGI gene could be inactivated using the method outlined in Example 25 is strengthened by this experiment. In this case a
15 plasmid, p Δ EGIp_{pyr}-3, was constructed which was similar to p Δ EGI-1 except that the Aspergillus niger pyr4 gene replaced the T. reesei pyr4 gene as selectable marker. In this case the egl1 gene was again present as a 4.2 kb HindIII fragment inserted at the HindIII site of
20 pUC100. The same internal 1 kb EcoRV fragment was removed as during the construction of p Δ EGI-1 (see Example 25) but in this case it was replaced by a 2.2 kb fragment containing the cloned A. niger pyrG gene (Wilson et al., 1988, Nucl. Acids Res. 16 p.2339).
25 Transformation of a pyr4 deficient strain of T. reesei (strain GC69) with p Δ EGIp_{pyr}-3, after it had been digested with HindIII to release the fragment containing the pyrG gene with flanking regions from the egl1 locus at either end, led to transformants in which
30 the egl1 gene was disrupted. These transformants were recognized by Southern blot analysis of transformant DNA digested with HindIII and probed with radiolabelled p Δ EGIp_{pyr}-3. In the untransformed strain of T. reesei

the egl1 gene was present on a 4.2 kb HindIII fragment of DNA and this pattern of hybridization is represented by Fig. 19, lane C. However, following deletion of the egl1 gene by integration of the desired fragment from pΔEGIp_{yr}-3 this 4.2 kb fragment disappeared and was replaced by a fragment approximately 1.2 kb larger in size, FIG. 19, lane A. Also shown in FIG. 19, lane B is an example of a transformant in which integration of a single copy of pΔEGIp_{yr}-3 has occurred at a site in the genome other than the egl1 locus.

Example 27

Transformation of T.reesei with pΔEGI-1 to create a strain unable to produce CBHI, CBHII, EGI and EGII

A pyr4 deficient derivative of strain A22 (from Example 24) will be obtained by the method outlined in Example 7. This strain will be transformed with pΔEGI-1 which had been previously digested with HindIII to release a DNA fragment comprising only T. reesei genomic DNA having a segment of the egl1 gene at either end with part of the EGI coding sequence replaced by the pyr4 gene.

Stable pyr4⁺ transformants will be selected and total DNA isolated from the transformants. The DNA will be probed with ³²P labelled pΔEGI-1 after Southern blot analysis in order to identify transformants in which the fragment of DNA containing the pyr4 gene and egl1 sequences has integrated at the egl1 locus and consequently disrupted the EGI coding sequence. The transformants identified will be unable to produce CBHI, CBHII, EGI and EGII.

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WHAT IS CLAIMED IS:

- 1 1. A detergent composition comprising a cleaning
2 effective amount of a surfactant or a mixture of surfactants and
3 from about 0.01 to about 5 weight percent of substantially pure
4 EG III cellulase having a molecular weight of about 23 to 28 K
5 daltons and an isoelectric point (PI) of from about 7.2 to 8.0.
- 1 2. A detergent composition according to Claim 1 wherein
2 said substantially pure EG III cellulase comprises from about
3 0.05 to about 2 weight percent of said detergent composition.
- 1 3. A detergent composition according to Claim 1 wherein
2 said detergent composition is free of all CBH I type components.
- 1 4. A detergent composition according to Claim 3 wherein
2 said detergent composition is free of all CBH type components.
- 1 5. A method for enhancing the softness of a cotton-
2 containing fabric which method comprises washing the fabric in
3 a wash medium derived from a detergent composition comprising a
4 cleaning effective amount of a surfactant or a mixture of
5 surfactants and from about 0.01 to about 5 weight percent of
6 substantially pure EG III cellulase having a molecular weight of
7 about 23 to 28 K daltons and an isoelectric point (PI) of from
8 about 7.2 to 8.0, the said wash medium having a pH in the
 range of about 4 to about 10.
- 1 6. A method according to Claim 5 wherein said
2 substantially pure EG III cellulase comprises from about 0.05 to
3 about 2 weight percent of said detergent composition.

1 7. A method according to Claim 5 wherein said detergent
2 composition is free of all CBH I type components.

1 8. A method according to Claim 7 wherein said detergent
2 composition is free of all CBH type components.

1 9. A method for retaining/restoring the color of a
2 cotton-containing fabric which method comprises washing the
3 fabric one or more times in a wash medium derived from a
4 detergent composition comprising a cleaning effective amount of
5 a surfactant or a mixture of surfactants and from about 0.01 to
6 about 5 weight percent of substantially pure EG III cellulase
7 having a molecular weight of about 23 to 28 K daltons and an
8 isoelectric point (PI) of from about 7.2 to 8.0, said wash
medium having a pH in the range of about 4 to 10.

1 10. A method according to Claim 9 wherein said
2 substantially pure EG III cellulase comprises from about 0.05 to
3 about 2 weight percent of said detergent composition.

1 11. A method according to Claim 9 wherein said detergent
2 composition is free of all CBH I type components.

1 12. A method according to Claim 11 wherein said detergent
2 composition is free of all CBH type components.

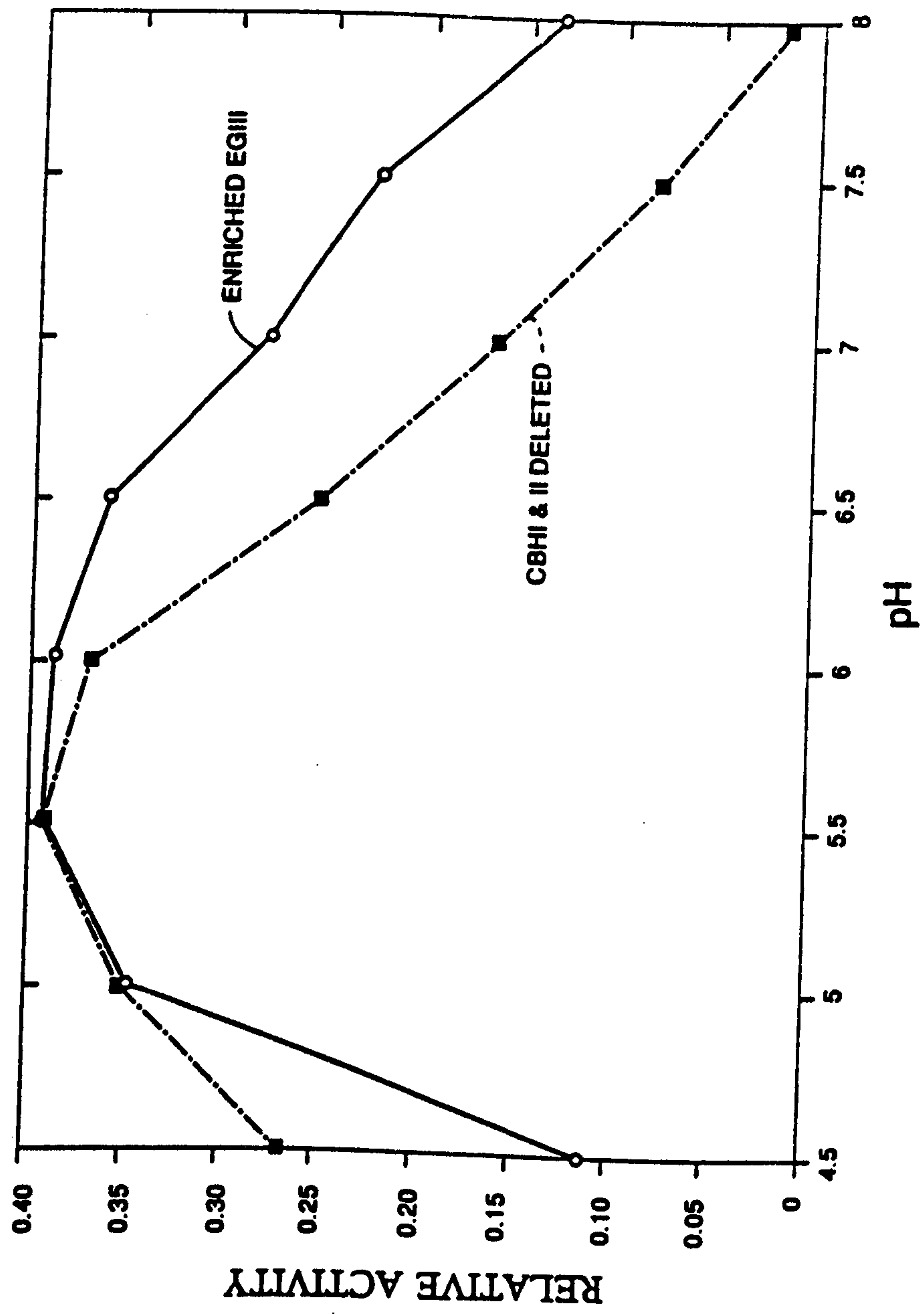


FIG. 1

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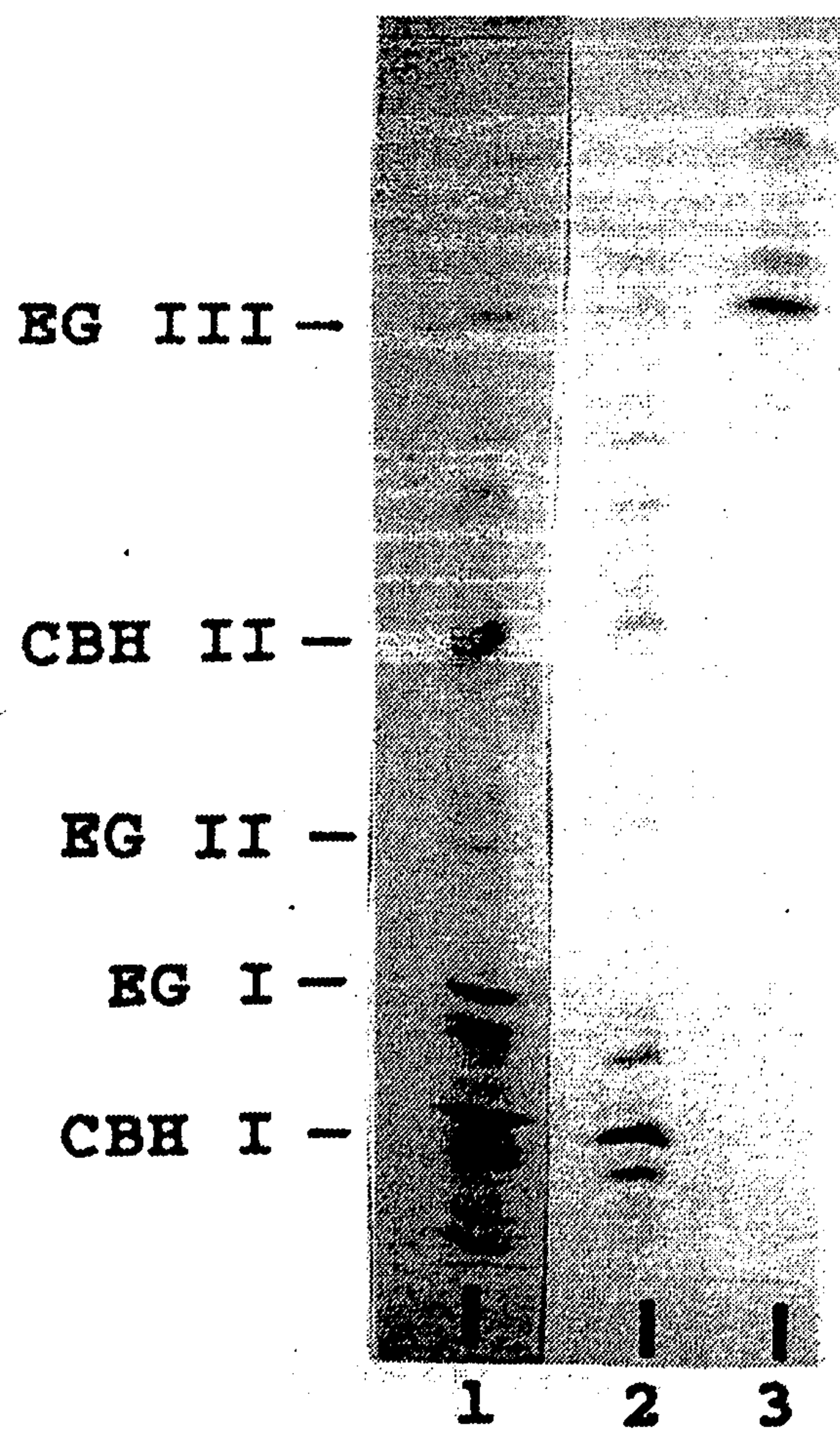
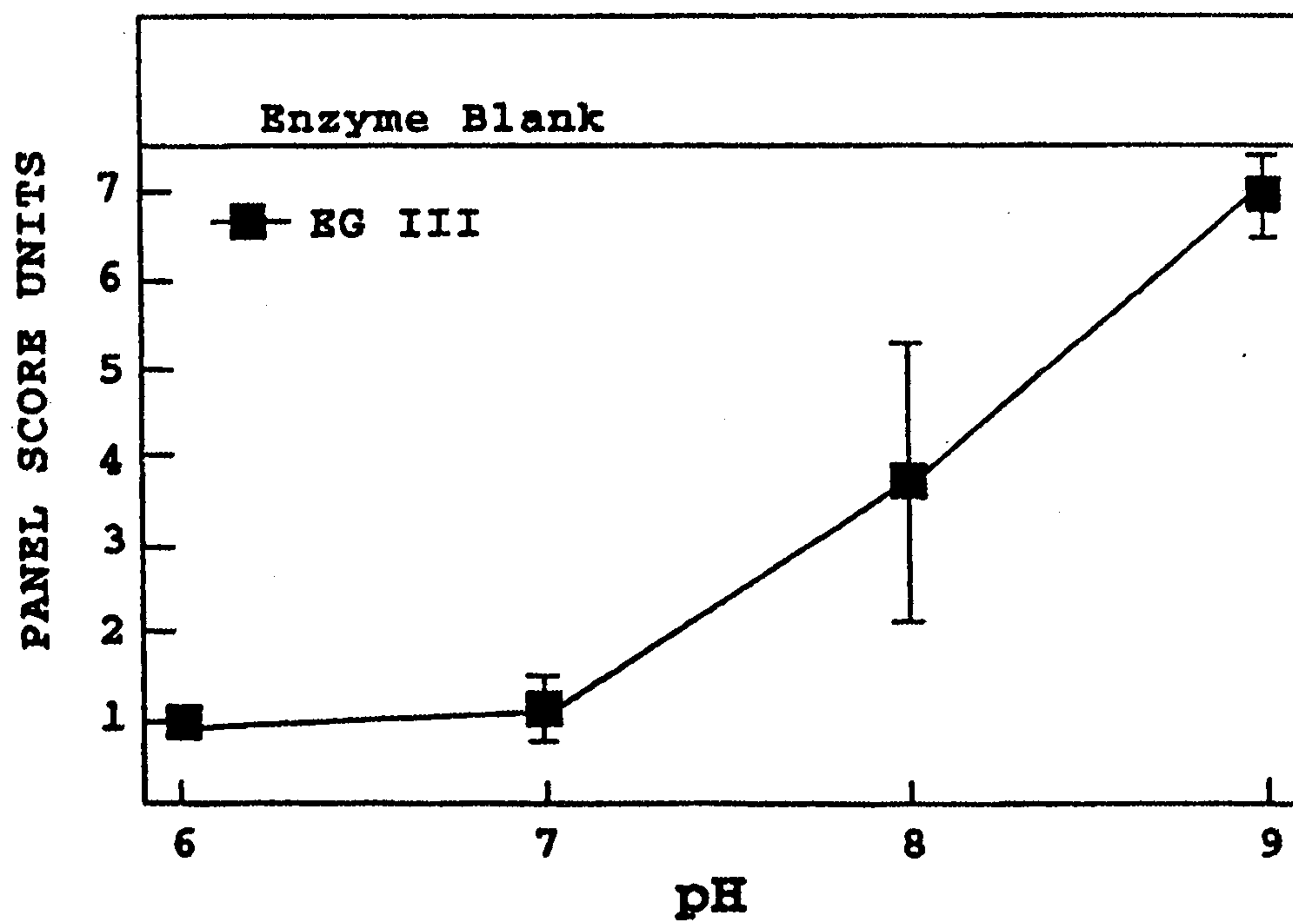


FIG. 2

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**FIG. 3**

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- 1) IWLKYG DGPIGSSQG XNVGGQ
- 2) PTTASW SYSGSNIRANVAYDLFTAAN

FIG. 4

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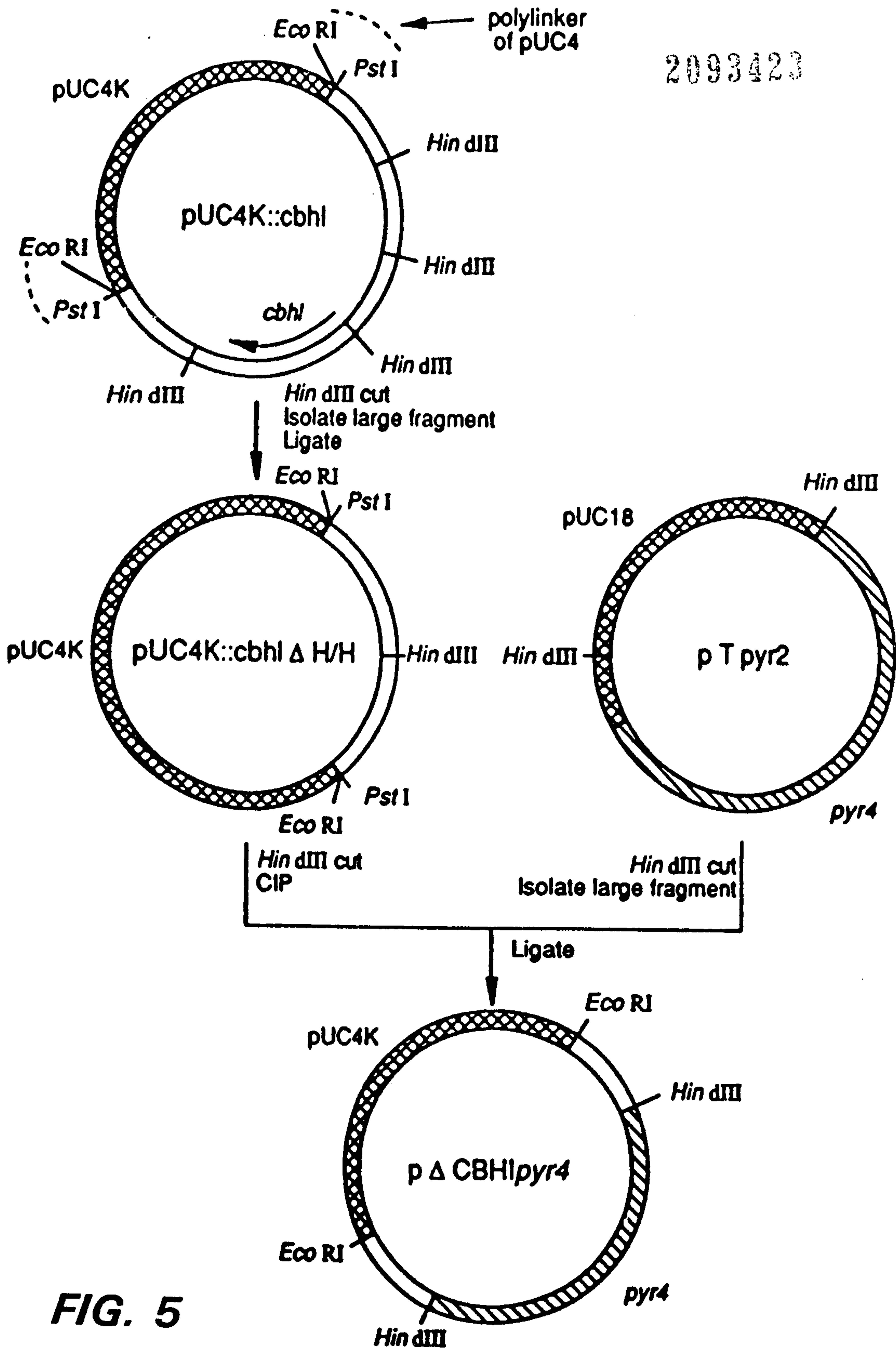
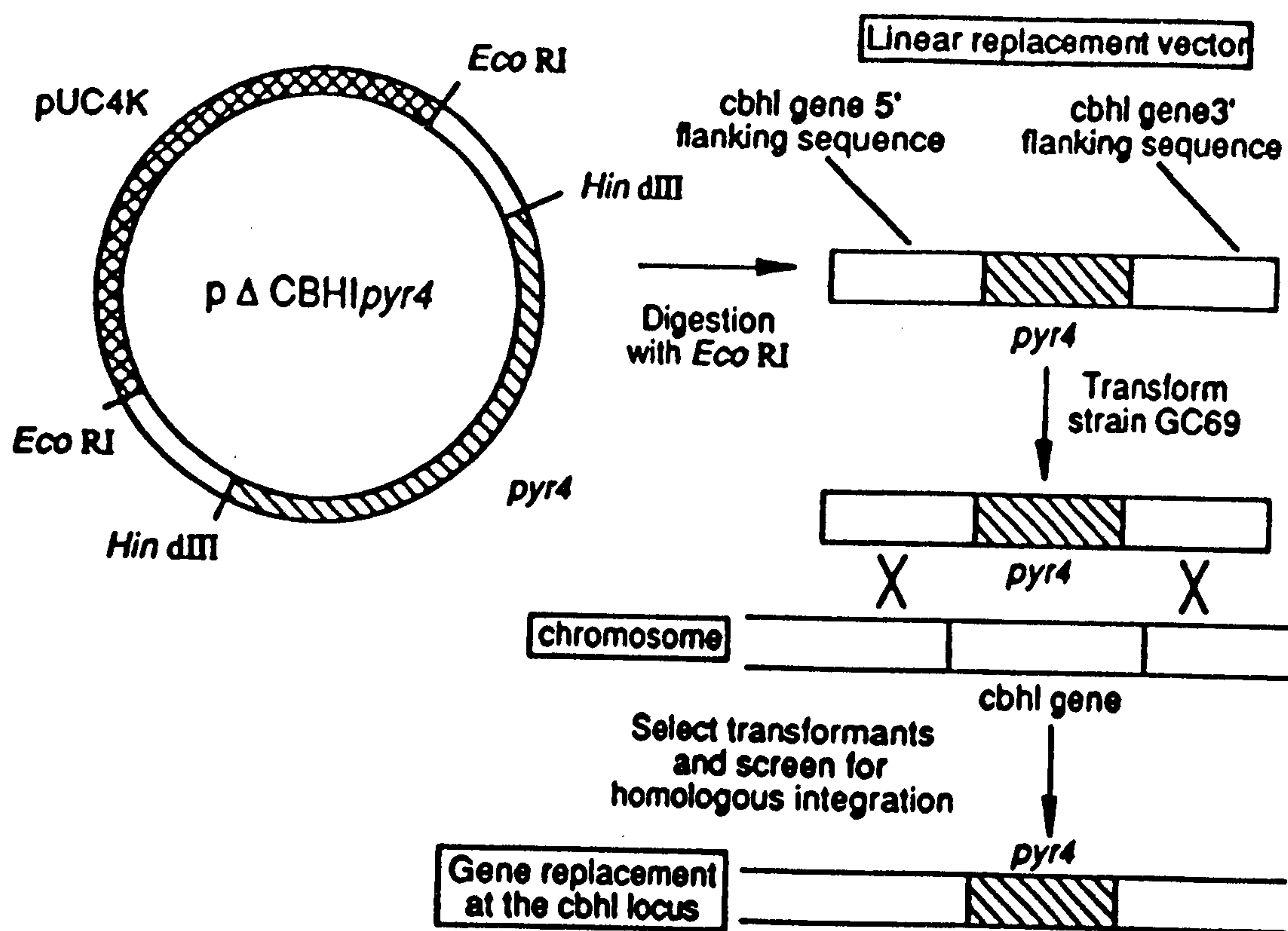


FIG. 5

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**FIG. 6**

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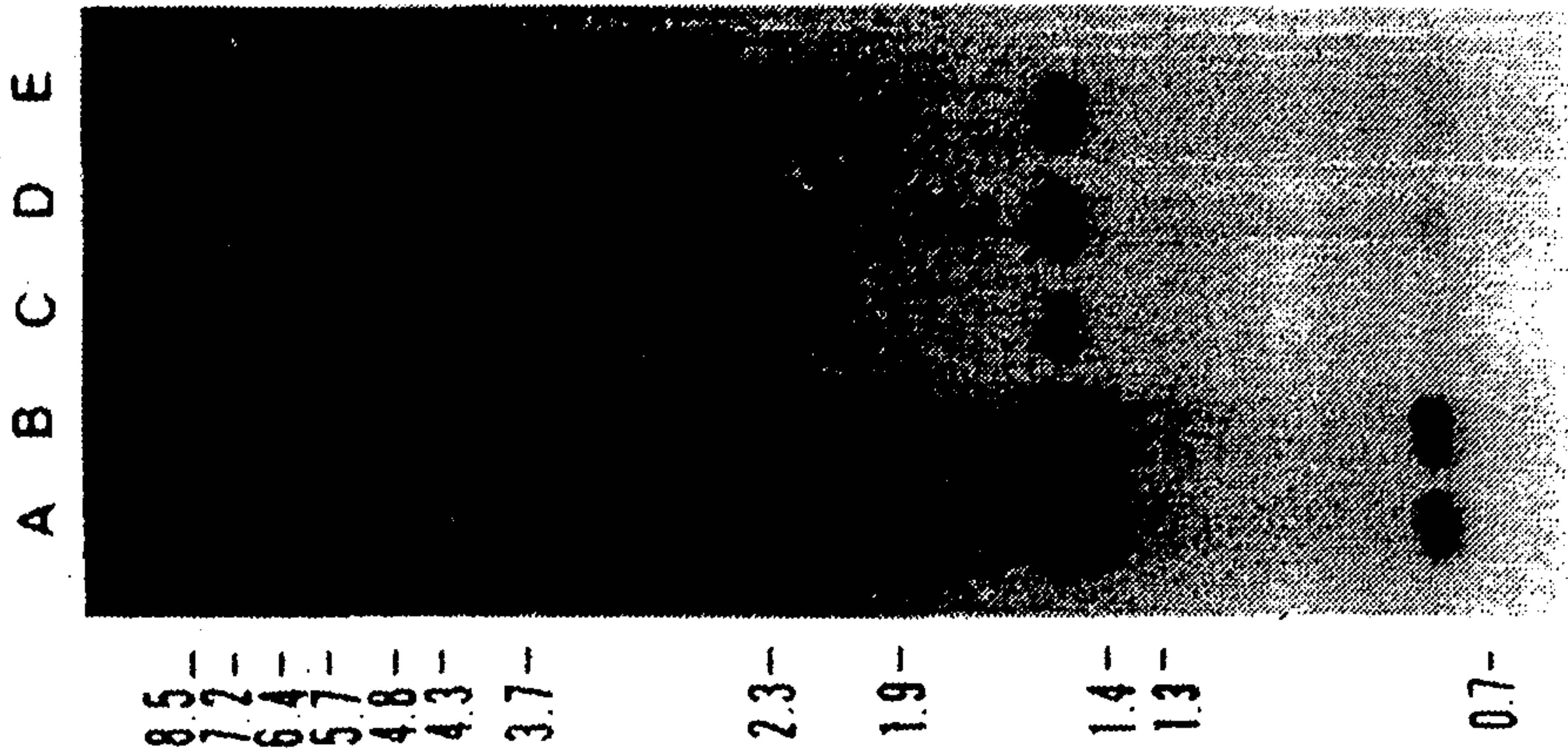


FIG-7

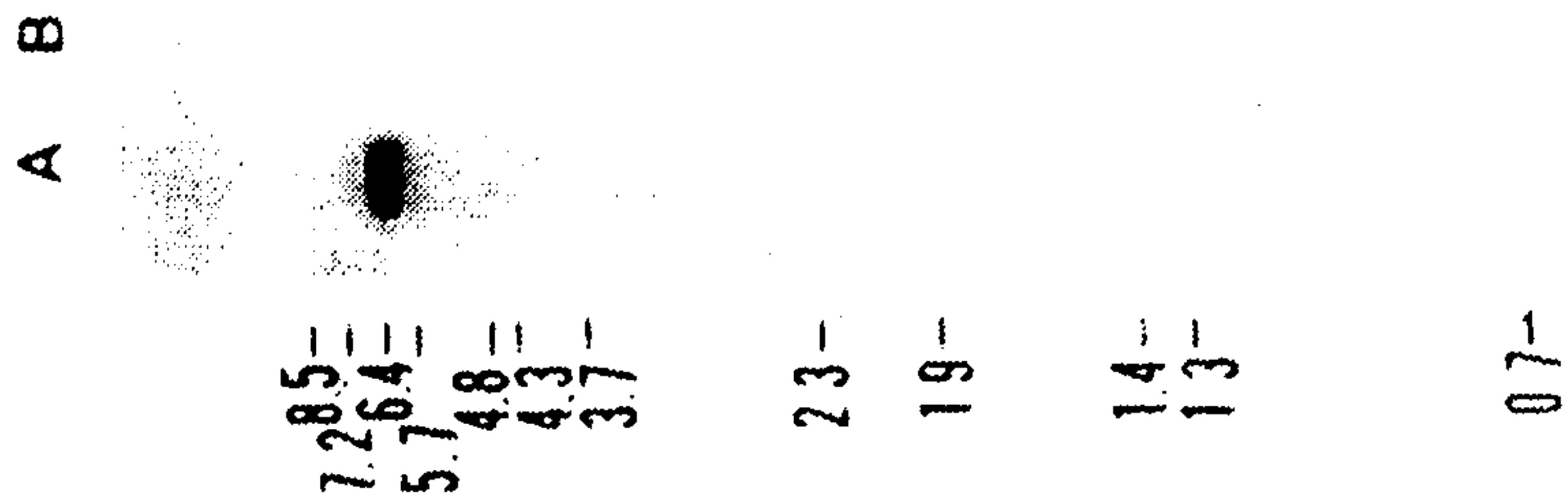


FIG-8

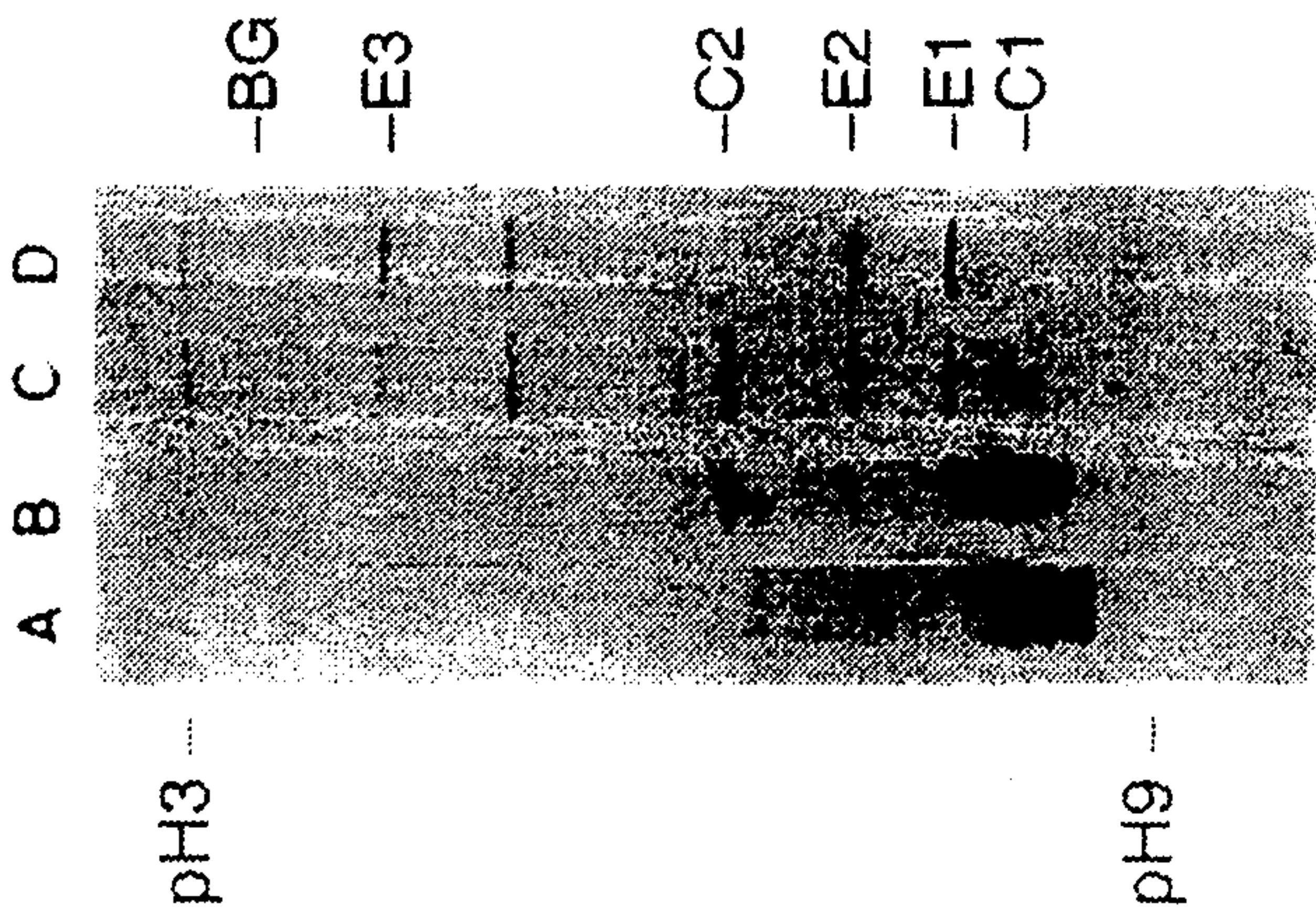


FIG-9

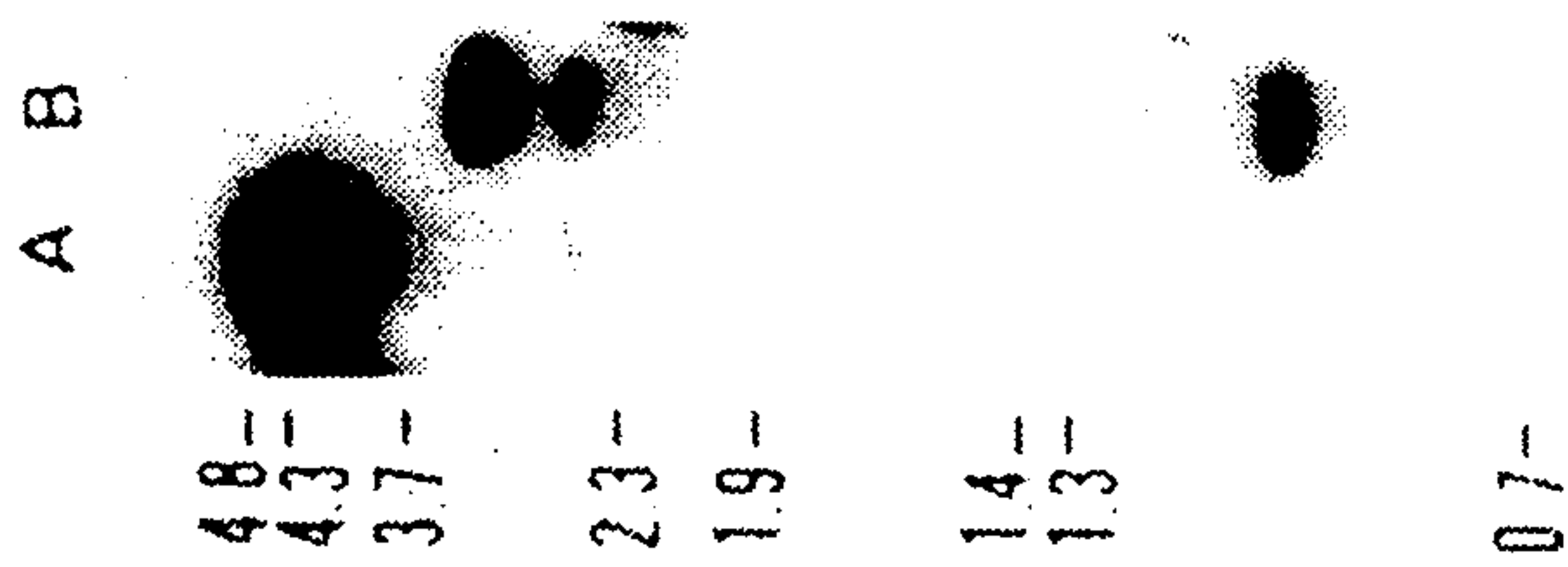


FIG-11

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FIG. 10A

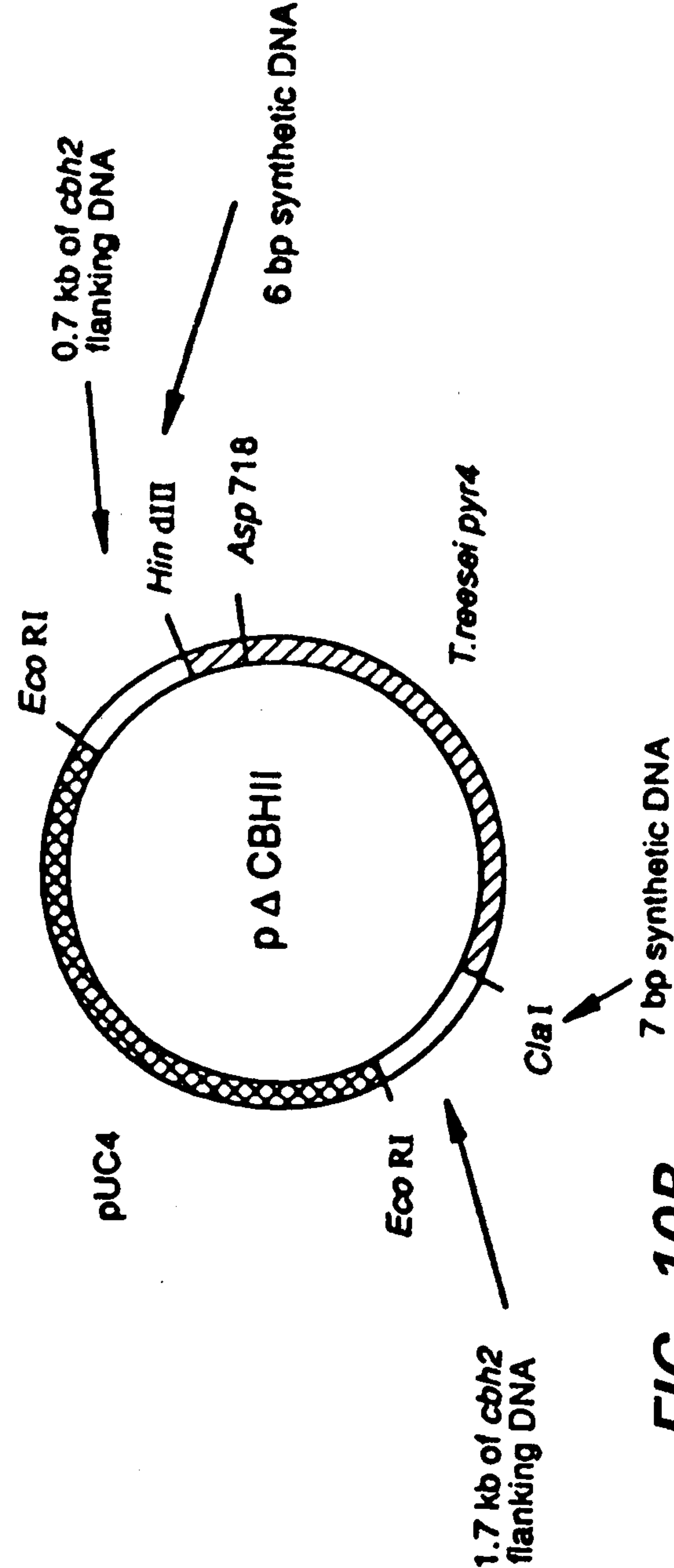


FIG. 10B

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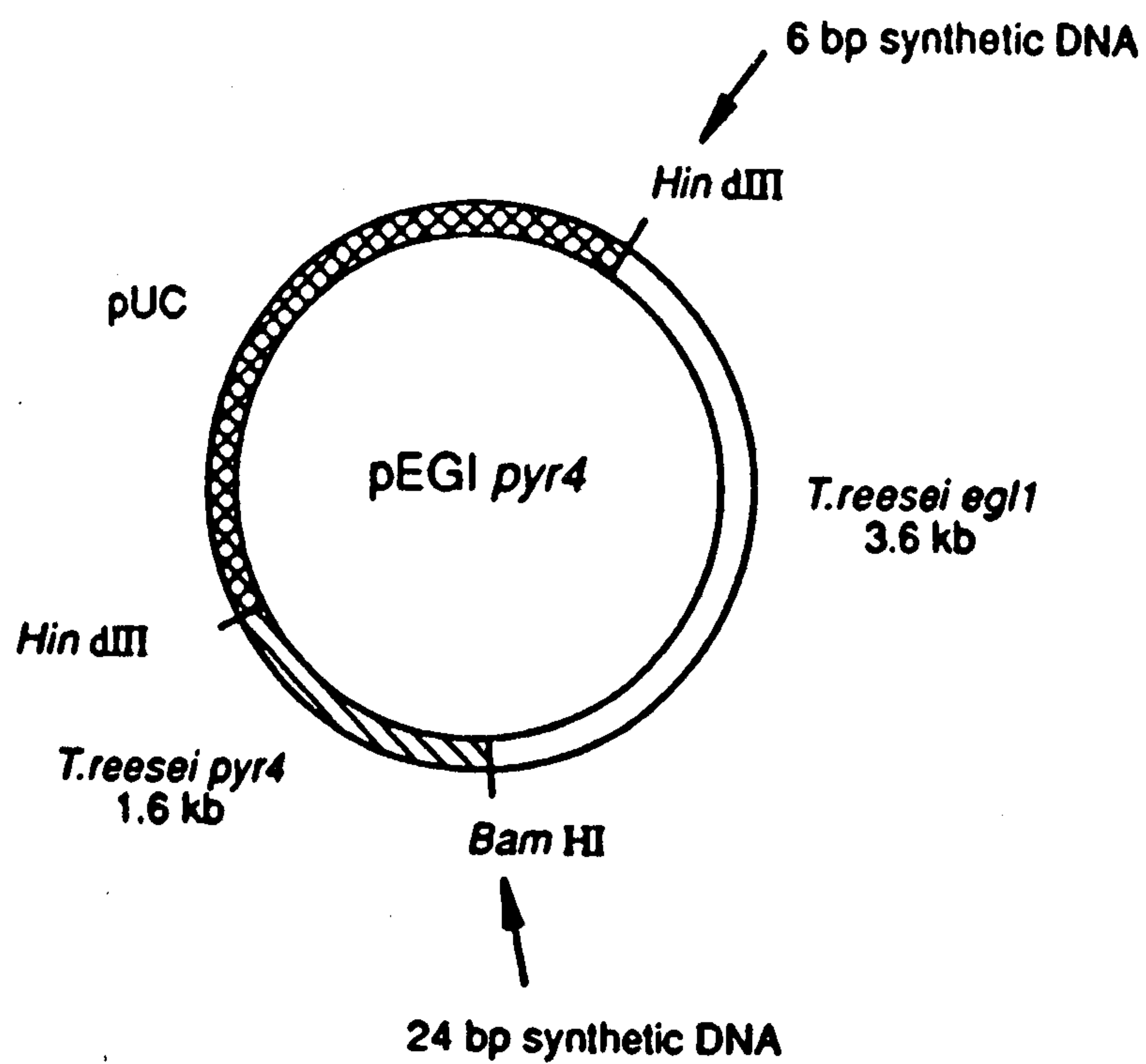


FIG. 12

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cbh1

AAACCCAATAGTCAACCGCGGACTGGCAT ATG TAT CGG

G T A

AAACCCAATAGTGATCAGCGGACTGGCAT ATG TAT CGGBclI

First 3 codons

egl1

TAGTCCTTCTTGTTGTCCCAA ATG GCG CCC

GGA

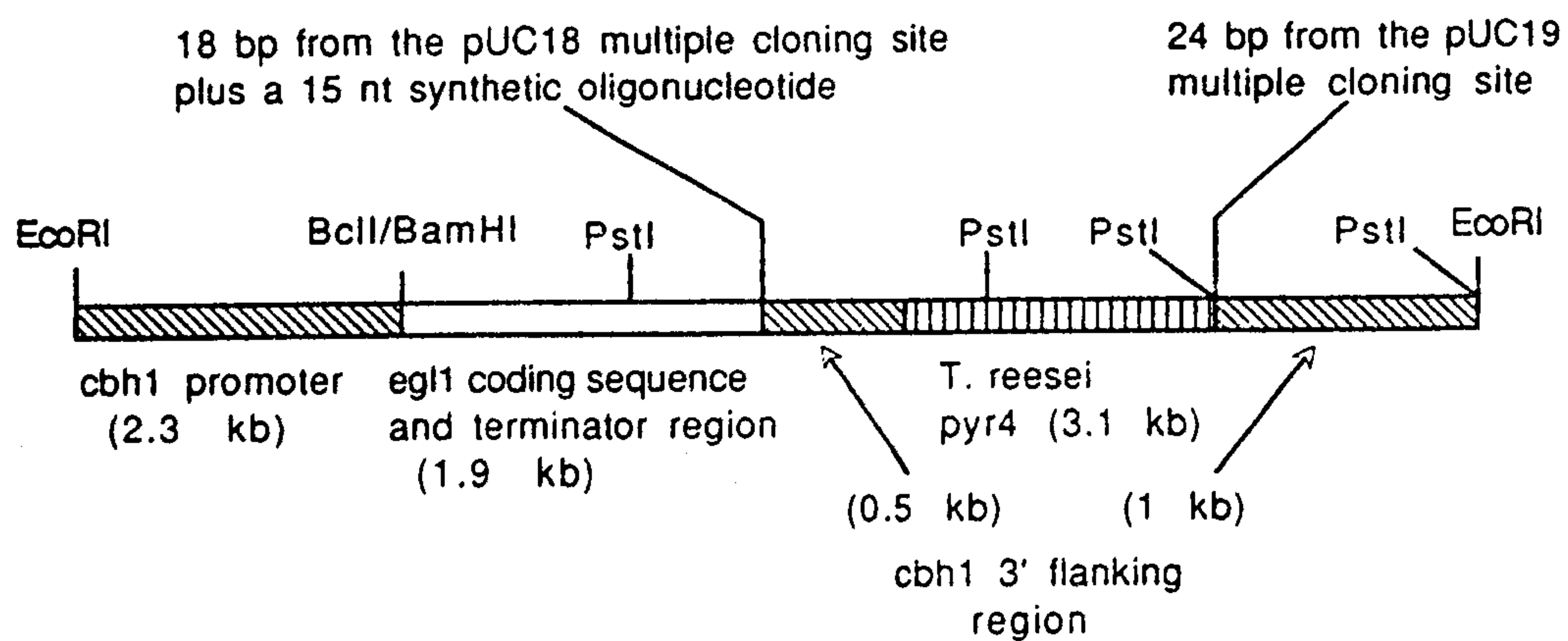
TAGTCCTTCTTGGATCCCCAA ATG GCG CCCBamHI

First 3 codons

FIG. 13

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LINEAR FRAGMENT OF DNA OBTAINED FROM pCEPC1 BY EcoRI DIGESTION

**FIG. 14**

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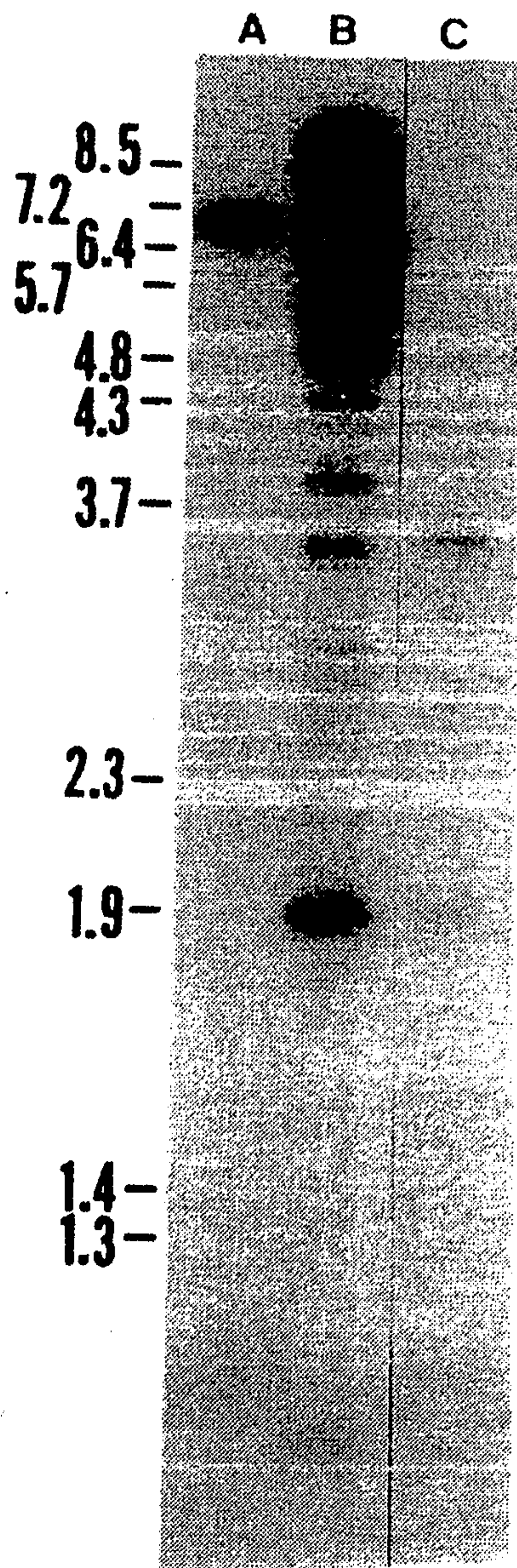


FIG. 15

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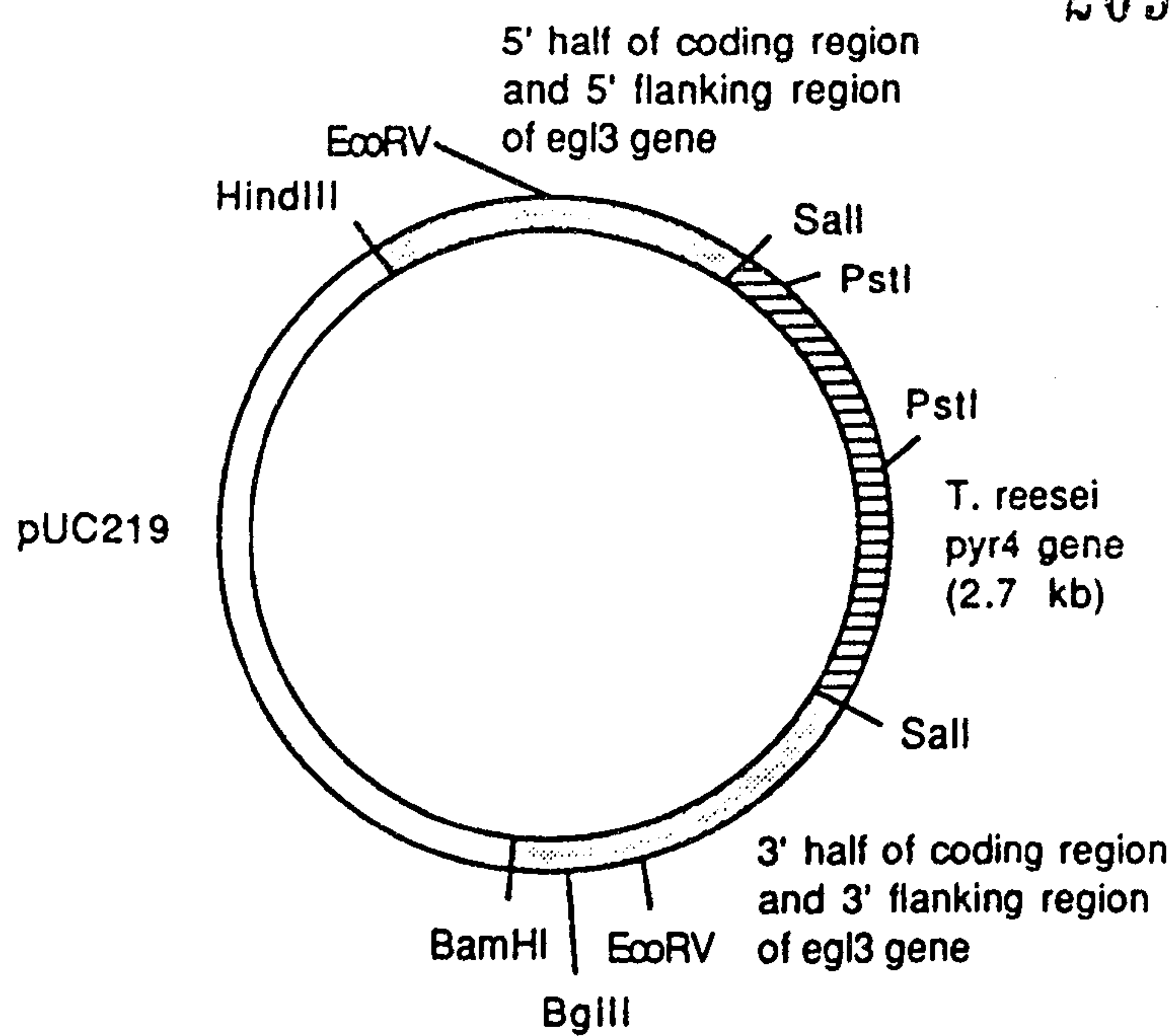


DIAGRAM OF pEGII::P-1

FIG. 16

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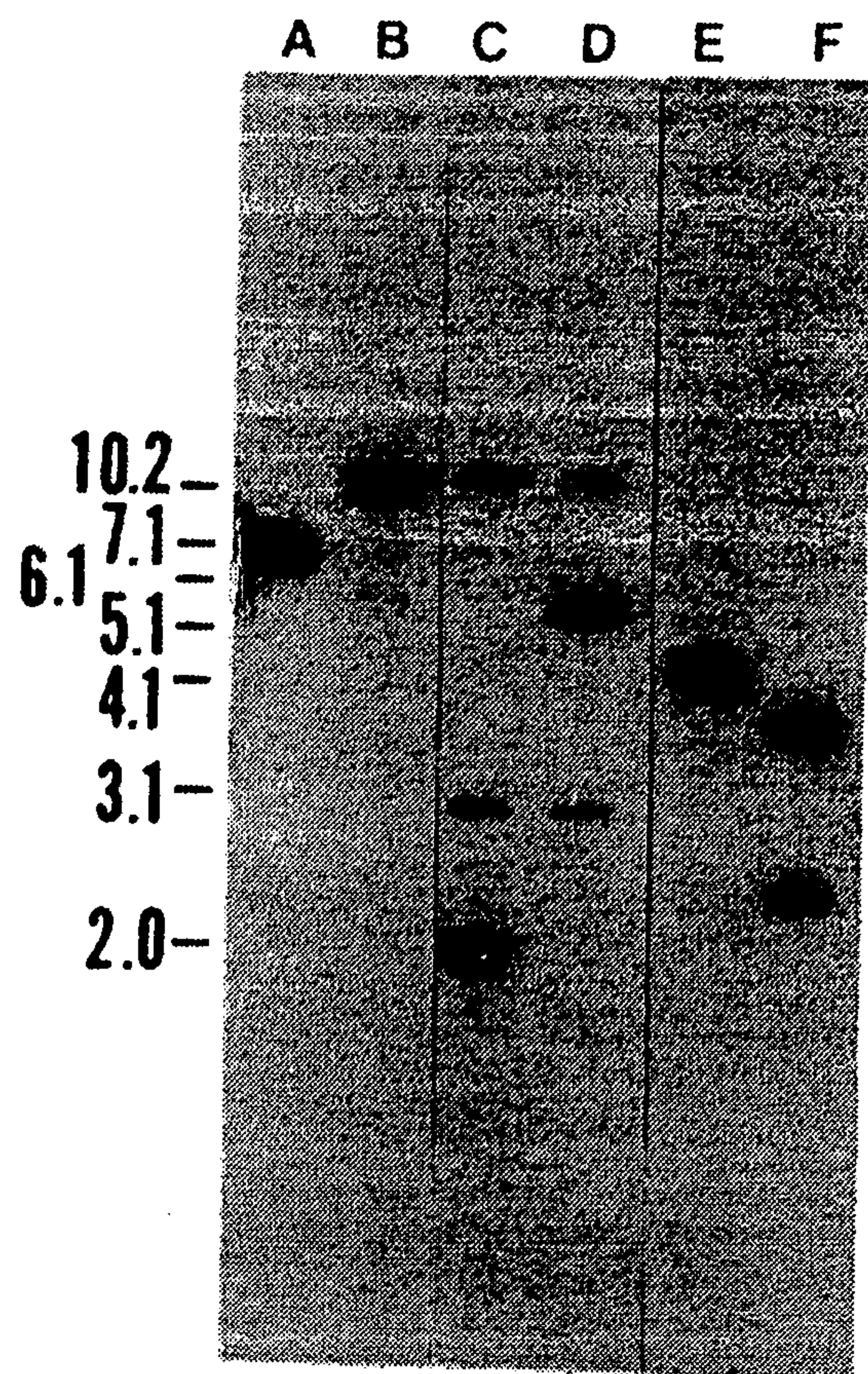


FIG. 17

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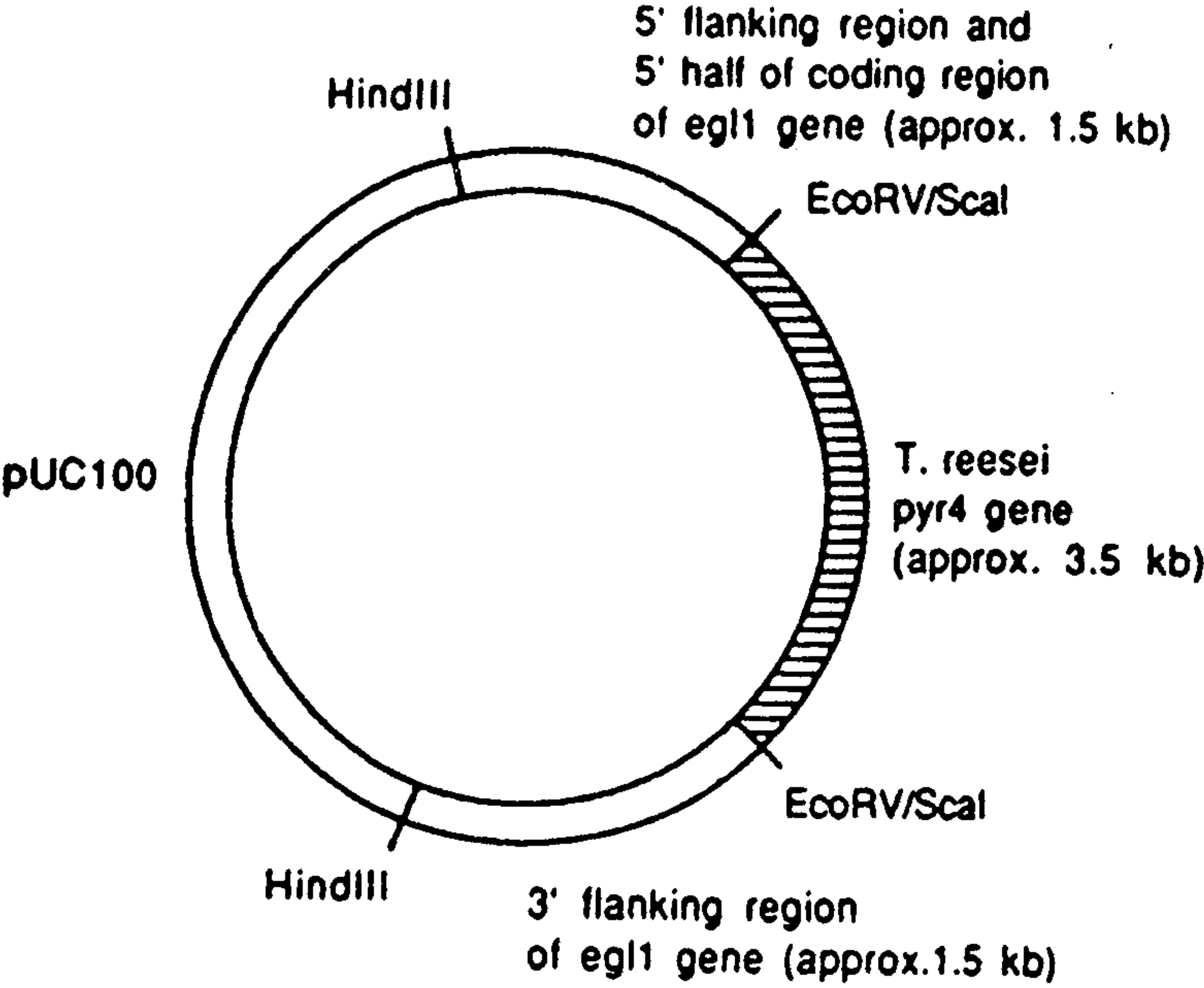


FIG. 18

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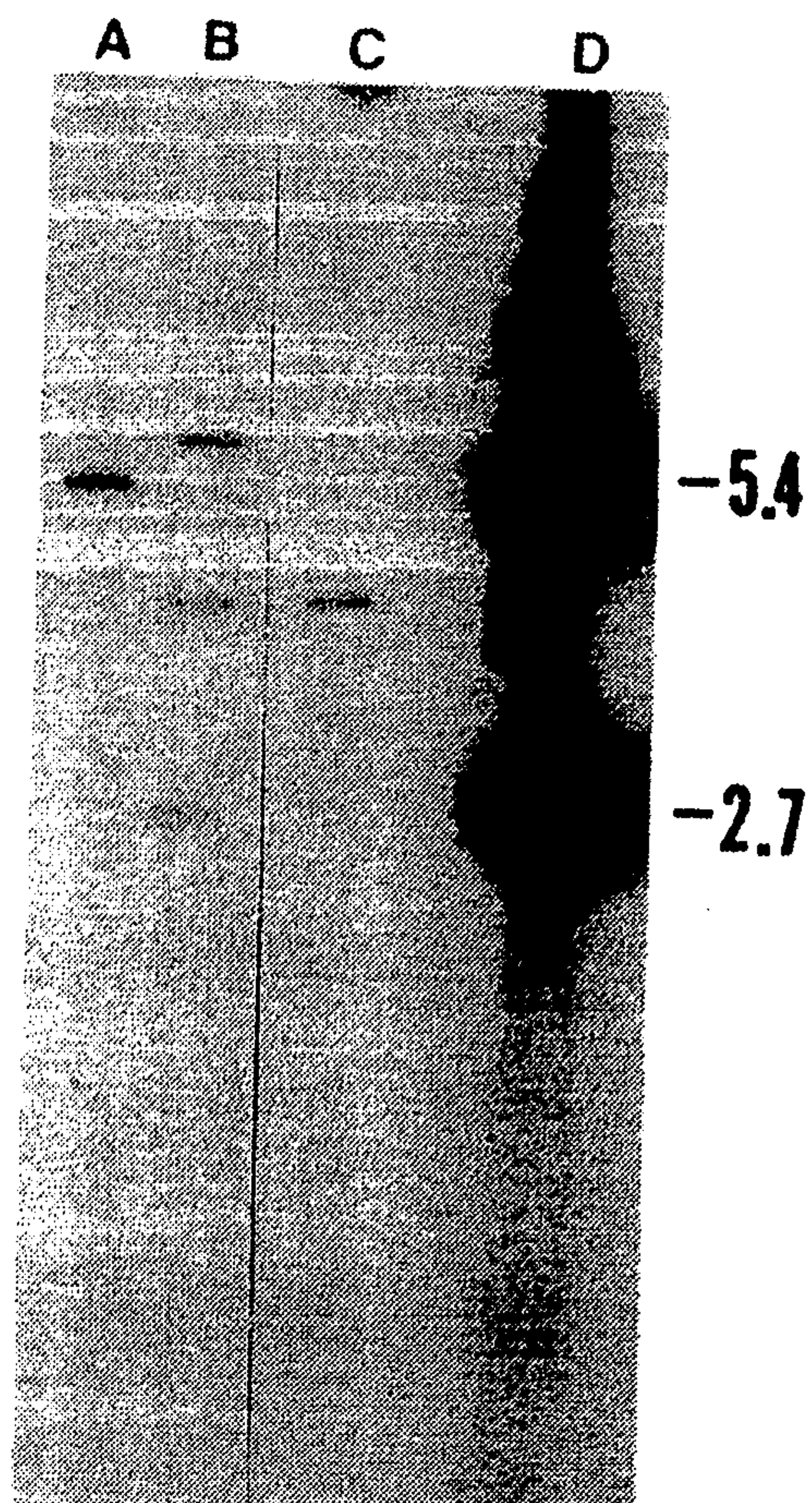


FIG_19

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