AUSTRALIA

PATENTS ACT 1990

636500

PATENT REQUEST : STANDARD PATENT

I/We being the person(s) identified below as the Applicant(s), request the grant of a patent to the person(s) identified below as the Nominated Person(s), for an invention described in the accompanying standard complete specification.

Full application details follow:

[71/70] Applicant(s)/Nominated Person(s):

Boehringer Mannheim GmbH

of

Sandhofer Strasse 112–132, D–6800 Mannheim–Waldhof, Germany

[54] Invention Title:

Cloning and overexpression of glucose–6–phosphate dehydrogenase from Leuconostoc dextranicus

[72] Name(s) of actual inventor(s):

Michael JARSCH
Gunter LANG

[74] Address for service in Australia:

DAVIES & COLLISON, Patent Attorneys, 1 Little Collins Street, Melbourne, Victoria, Australia. Attorney Code: DM

Basic Convention Application(s) Details:

[31] Application Number	[33] Country	Code	[32] Date of Application
P 40 24 158.0	Germany	DE	30 July 1990

.....
a member of the firm of DAVIES & COLLISON for and on behalf of the applicant(s)

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NOTICE OF ENTITLEMENT

We, **Boehringer Mannheim GmbH**, the applicant named in the accompanying Patent Request state the following:-

The Nominated Person is entitled to the grant of the patent because the Nominated Person would, on the grant of a patent for the invention, be entitled to have the patent assigned to the Nominated Person.

The Nominated Person is entitled to claim priority from the basic application listed on the patent request because the Nominated Person made the basic application, and because that application was the first application made in a Convention country in respect of the invention.

DATED this SIXTEENTH day of JULY 1991

.....
a member of the firm of
DAVIES & COLLISON for
and on behalf of the
applicant(s)

(D&C ref: 141832!)

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(12) PATENT ABRIDGMENT (11) Document No. AU-B-80431/91
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 636500

(54) Title
CLONING AND OVEREXPRESSION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM LEUCONOSTOC DEXTRANICUS

International Patent Classification(s)
(51)[5] **C12N 015/53** **C12N 009/04** **C12Q 001/32**

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(71) Applicant(s)
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(57)

SEQ ID NO:1
TYPE OF SEQUENCE: Nucleotide with corresponding protein
LENGTH OF SEQUENCE: 1696 base pairs

FORM OF STRAND: single strand

Abstract

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TCTAGTCATT TAATCAATTT TTGACTTGTT CAACGCTTAA TATGTTTGTG AATCCCGTAC   60

TTTTCCAGAC CTTTTTGCGT TATAATGGAG AGTGAATTTA ATTATAATAT AAGGGGAACA  120

TC                                                                 122

ATG GTT TCA GAA ATC AAA ACG TTG GTA ACT TTC TTT GGC GGA ACT GGT    170
Met Val Ser Glu Ile Lys Thr Leu Val Thr Phe Phe Gly Gly Thr Gly
                  5                  10                  15

GAT TTA GCA AAG CGT AAG CTT TAC CCA TCA GTT TTC AAC CTC TAC AAA    218
Asp Leu Ala Lys Arg Lys Leu Tyr Pro Ser Val Phe Asn Leu Tyr Lys
             20                  25                  30

AAA GGA TAC TTA CAA GAA CAC TTT GCC ATT GTT GGG ACA GCA CGT CAA    266
Lys Gly Tyr Leu Gln Glu His Phe Ala Ile Val Gly Thr Ala Arg Gln
         35                  40                  45

CAA TTA AGT GAT GAC GAG TTT AAG CAA TTG GTT CGT GAT TCA ATT AAA    314
Gln Leu Ser Asp Asp Glu Phe Lys Gln Leu Val Arg Asp Ser Ile Lys
     50                  55                  60

GAC TTT ACT GAA GAT CAA GCA CAA GCC GAA GCG TTT ATT GCG CAT TTT    362
Asp Phe Thr Glu Asp Gln Ala Gln Ala Glu Ala Phe Ile Ala His Phe
 65                  70                  75                  80

TCT TAC CGT GCG CAC GAT GTC ACA GAT GCC GCT TCT TAT GGT ATC TTG    410
Ser Tyr Arg Ala His Asp Val Thr Asp Ala Ala Ser Tyr Gly Ile Leu
                 85                  90                  95
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AAG TCA GCG ATC GAA GAA GCA GCA ACC AAA TTT GAC ATT GAT GGC AAT      458
Lys Ser Ala Ile Glu Glu Ala Ala Thr Lys Phe Asp Ile Asp Gly Asn
            100                 105                 110

CGT ATT TTC TAT ATG TCA GTT GCC CCT CGT TTC TTC GGT ACA ATC GCT      506
Arg Ile Phe Tyr Met Ser Val Ala Pro Arg Phe Phe Gly Thr Ile Ala
        115                 120                 125

AAA TAT TTG AAA TCA GAA GGT TTG CTA GCT GAG ACT GGC TAC AAT CGT      554
Lys Tyr Leu Lys Ser Glu Gly Leu Leu Ala Glu Thr Gly Tyr Asn Arg
    130                 135                 140

TTG ATG ATT GAA AAG CCT TTT GGT ACA TCA TAC GCC ACC GCA GAA GAA      602
Leu Met Ile Glu Lys Pro Phe Gly Thr Ser Tyr Ala Thr Ala Glu Glu
145                 150                 155                 160

TTG CAA AGT GAT TTG GAA AAT GCA TTT GAT GAT GAC CAA CTG TTC CGT      650
Leu Gln Ser Asp Leu Glu Asn Ala Phe Asp Asp Asp Gln Leu Phe Arg
                165                 170                 175

ATT GAC CAC TAT CTT GGA AAA GAA ATG GTA CAA AAT ATT GCA GCA TTA      698
Ile Asp His Tyr Leu Gly Lys Glu Met Val Gln Asn Ile Ala Ala Leu
            180                 185                 190

CGT TTT GGT AAC CCA ATC TTT GAT GCC GCT TGG AAT AAG GAC TAT ATC      746
Arg Phe Gly Asn Pro Ile Phe Asp Ala Ala Trp Asn Lys Asp Tyr Ile
        195                 200                 205

AAA AAC GTA CAA GTA ACT TTG GCT GAA GTT CTA GGT GTT GAA GAG CGT      794
Lys Asn Val Gln Val Thr Leu Ala Glu Val Leu Gly Val Glu Glu Arg
    210                 215                 220

GCT GGT TAC TAC GAT ACC ACT GGC GCC CTT TTG GAT ATG ATT CAA AAC      842
Ala Gly Tyr Tyr Asp Thr Thr Gly Ala Leu Leu Asp Met Ile Gln Asn
225                 230                 235                 240

CAC ACA ATG CAA ATT GTT GGT TGG TTA GCA ATG GAA AAA CCT GAA TCA      890
His Thr Met Gln Ile Val Gly Trp Leu Ala Met Glu Lys Pro Glu Ser
                245                 250                 255

TTC AAT GAT AAG GAT ATC CGT GCA GCT AAA AAC GCC GCC TTC AAT GCA      938
Phe Asn Asp Lys Asp Ile Arg Ala Ala Lys Asn Ala Ala Phe Asn Ala
            260                 265                 270

TTA AAG ATT TAT AAC GAA GAA GAA GTG AAT AAG TAC TTC GTT CGT GCA      986
Leu Lys Ile Tyr Asn Glu Glu Glu Val Asn Lys Tyr Phe Val Arg Ala
        275                 280                 285

CAA TAT GGT GCT GGT GAT ACA GCT GAT TAC AAG CCA TAT TTG GAA GAA     1034
Gln Tyr Gly Ala Gly Asp Thr Ala Asp Tyr Lys Pro Tyr Leu Glu Glu
    290                 295                 300

GCA GAT GTC CCT GCT GAC TCA AAG AAC AAC ACA TTC ATT GCT GGT GAA     1082
Ala Asp Val Pro Ala Asp Ser Lys Asn Asn Thr Phe Ile Ala Gly Glu
305                 310                 315                 320

TTG CAG TTC GAT TTG CCA CGT TGG GAA GGT GTT CCT TTC TAT GTT CGT     1130
Leu Gln Phe Asp Leu Pro Arg Trp Glu Gly Val Pro Phe Tyr Val Arg
                325                 330                 335

TCA GGT AAG CGT TTG GCT GCC AAG CAA ACA CGT GTT GAT ATT GTA TTT     1178
Ser Gly Lys Arg Leu Ala Ala Lys Gln Thr Arg Val Asp Ile Val Phe
            340                 345                 350
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AAG GCT GGC ACA TTC AAC TTT GGT TCA GAA CAA GAA GCA CAA GAA TCA   1226
Lys Ala Gly Thr Phe Asn Phe Gly Ser Glu Gln Glu Ala Gln Glu Ser
        355                 360                 365

GTA CTC TCA ATC ATC ATT GAT CCA AAG GGT GCT ATT GAA TTG AAG CTT   1274
Val Leu Ser Ile Ile Ile Asp Pro Lys Gly Ala Ile Glu Leu Lys Leu
    370                 375                 380

AAC GCT AAG TCA GTT GAA GAT GCC TTC AAC ACC CGC ACA ATC AAC TTG   1322
Asn Ala Lys Ser Val Glu Asp Ala Phe Asn Thr Arg Thr Ile Asn Leu
385                 390                 395                 400

GAT TGG GCA GTA TCT GAT GAA GAC AAG AAG AAC ACA CCA GAA CCA TAC   1370
Asp Trp Ala Val Ser Asp Glu Asp Lys Lys Asn Thr Pro Glu Pro Tyr
                405                 410                 415

GAA CGT ATG ATT CAC GAT ACA ATG AAT GGT GAC GGA TCA AAC TTT GCT   1418
Glu Arg Met Ile His Asp Thr Met Asn Gly Asp Gly Ser Asn Phe Ala
            420                 425                 430

GAT TGG AAC GGT GTA TCA ATT GCT TGG AAG TTT GTT GAC GCA ATT ACT   1466
Asp Trp Asn Gly Val Ser Ile Ala Trp Lys Phe Val Asp Ala Ile Thr
        435                 440                 445

GCC GTT TAC GAT GCA GAT AAA GCA CCA TTG GAG ACA TAT AAG TCA GGT   1514
Ala Val Tyr Asp Ala Asp Lys Ala Pro Leu Glu Thr Tyr Lys Ser Gly
    450                 455                 460

TCA ATG GGT CCT GAA GCA TCA GAC AAG CTA TTA GCT GAA AAT GGC GAT   1562
Ser Met Gly Pro Glu Ala Ser Asp Lys Leu Leu Ala Glu Asn Gly Asp
465                 470                 475                 480

GCT TGG GTA TTT AAA GGA TAAGCACATT TAAAAAGACC ATCAAACAAA         1610
Ala Trp Val Phe Lys Gly
                485
TCTTTGTTTG ACGGTCTTTT TATATTGTCT GATTTAAGAT GCGTTTGGTT TCACGGAAAA 1670

CGGCTGACAA ATTGGTGTAT TGATCC                                     1696
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CLAIMS

1. A recombinant glucose-6-phosphate dehydrogenase comprising an amino acid sequence as shown in SEQ ID NO.1.

2. A DNA comprising a nucleic acid sequence as shown in SEQ ID NO. 1 or a degenerate form thereof which codes for the glucose-6-phosphate dehydrogenase according to claim 1.

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COMPLETE SPECIFICATION

NAME OF APPLICANT(S):

Boehringer Mannheim GmbH

ADDRESS FOR SERVICE:

DAVIES & COLLISON
Patent Attorneys
1 Little Collins Street, Melbourne, 3000.

INVENTION TITLE:

Cloning and overexpression of glucose-6-phosphate dehydrogenase from Leuconostoc dextranicus

The following statement is a full description of this invention, including the best method of performing it known to me/us:-

Description

Glucose-6-phosphate dehydrogenase (G6P-DH) catalyzes the first step in the oxidative metabolism of glucose. In this process glucose-6-phosphate is oxidized to gluconic acid-6-phosphate while NAD^+ or/and $NADP^+$ is reduced as the cosubstrate. The oxidation of glucose ultimately results in the production of pentose sugars for the nucleic acid metabolism.

Glucose-6-phosphate dehydrogenase can for example be isolated from Leuconostoc mesenteroides. This enzyme can use NAD^+ as well as $NADP^+$ as cofactor, in contrast to the enzyme from yeast which is specific for $NADP^+$. The enzyme is present as a dimer consisting of two identical monomeric subunits with a molecular weight of 55000 D. Its specific activity at 25°C is 550 U/mg.

Disadvantages of the process for isolating G6P-DH from bacteria of the genus Leuconostoc are inter alia that the lactic acid bacteria have complex nutrient requirements and therefore grow only slowly in those nutrient media used on a large technical scale and only reach a low cell density. In addition the content of G6P-DH in the biomass is only very low when using Leuconostoc (about 1 % of the total cell protein). Thus, large fermentation dimensions are necessary in order to provide adequate amounts of G6P-DH. Moreover, it is only possible to obtain an enzyme preparation with a low specific activity because of the large amounts of foreign protein.

The most important disadvantage of the known G6P-DH from Leuconostoc bacteria is, however, their low temperature stability.

The object of the present invention was therefore to provide a glucose-6-phosphate dehydrogenase which no longer has the disadvantages of the state of the art.

The object according to the present invention is achieved by the provision of a glucose 6-phosphate dehydrogenase which contains the amino acid sequence shown in SEQ ID NO:1 and is obtainable from Leuconostoc mesenteroides, subspecies dextranicus (DSM 20187) which is denoted Leuconostoc dextranicus in the following.

In addition the present invention also provides a DNA which contains a sequence encoding the enzyme according to the present invention shown in SEQ ID NO:1 or a corresponding sequence within the scope of the degeneracy of the genetic code.

The recombinant DNA according to the present invention was isolated by screening a L. dextranicus (DSM 20187) gene bank with a suitable oligonucleotide probe which is described below in more detail.

When the recombinant DNA according to the present invention is expressed in E. coli cells it surprisingly turned out that even small fermentation volumes are sufficient to provide the desired amount of enzyme. Compared to the isolation of G6P-DH from Leuconostoc, a reduction in the fermentation volume by a factor 1:500 to 1:1000 is achieved. Moreover, G6P-DH preparations are obtained in high purity, i.e. with a specific activity

of ca. 900 U/mg, with a less extensive purification procedure. However, a surprisingly special characteristic of the recombinant enzyme according to the present invention is a substantially improved temperature stability compared to the known enzyme when isolated from E. coli. An additional advantage of the recombinant enzyme in contrast to the known enzyme from Leuconostoc is that it does not react with glucose. This well-known unspecific reaction of the Leuconostoc enzyme with glucose (Olive and Levy, Biochemistry 6 (1967), 730) has previously been a major draw-back in carrying out enzyme tests since this could lead to false results in determinations because of the presence of glucose in blood, serum or plasma. Finally the recombinant enzyme also differs from the known G6P-DH in that the K_m value for $NADP^+$ is different and the effect of activators and inhibitors (e.g. phosphate, glycerol, magnesium ions, hydrogen carbonate) is different.

The present invention also provides a recombinant vector which contains one or several copies of the recombinant DNA according to the present invention. Such a vector is intended to enable the expression of the recombinant DNA according to the present invention in foreign host organisms. The vector according to the present invention can be a vector which integrates into the chromosomal DNA of the host cell (e.g. bacteriophage lambda), it can, however, also be present extrachromosomally in the host cell (plasmid). The vector according to the present invention is preferably a plasmid.

The vector according to the present invention can be a eukaryotic as well as a prokaryotic vector, it is, however, preferably a prokaryotic vector, i.e. it is suitable for multiplication in prokaryotic host

organisms. The recombinant vector has particularly preferably an origin of replication which is active in E. coli i.e. it can be multiplied in E. coli.

In a particularly preferred embodiment the recombinant vector according to the present invention contains the nucleic acid sequence coding for the glucose-6-phosphate dehydrogenase which is under the control of a promoter sequence from Leuconostoc dextranicus which functions in E. coli and which is included in the first 122 nucleotides (upstream of the G6P-DH gene) of the nucleic acid sequence shown in SEQ ID NO:1.

In order to exhibit promoter properties it is not necessary that the DNA region has exactly this sequence of 122 nucleotides. Derived sequences or fragments of this sequence which have promoter properties are also suitable. Under a derived biologically active sequence in the sense of the invention it is therefore understood that individual nucleotides or short nucleotide sequences from the promoter sequence can be deleted, substituted or inserted and namely in such a way that the promoter activity of the sequence is preserved. A person skilled in the art does indeed know that for a promoter it is not necessary to conserve the whole sequence but rather only particular partial regions. In prokaryotic promoter sequences these are in particular the regions at -35 and at -10 with respect to the transcription start.

Thus the invention also includes a recombinant DNA which has the first 122 nucleotides of the nucleic acid sequence shown in SEQ ID NO:1 or a sequence derived therefrom with promoter properties. Surprisingly this Leuconostoc promoter also results in a good protein

expression in E. coli. Thus, this promoter can also be used for the expression of heterologous genes, i.e. genes which are different from the G6P-DH gene, in gram-negative bacteria, preferably E. coli bacteria.

The present invention in addition provides a microorganism which is transformed with a recombinant vector according to the present invention. In this connection it is preferably a gram-negative bacterium, particularly preferably an E. coli bacterium.

The recombinant DNA according to the present invention can be obtained by

(1) isolating chromosomal Leuconostoc dextranicus DNA and cleaving it with a suitable restriction enzyme,

(2) incorporating the cleaved L. dextranicus DNA into a vector, transforming a suitable organism with the vector and producing a gene bank in this way,

(3) screening the gene bank from step (2) with a nucleic acid probe which has a sequence which is specific for the glucose-6-phosphate dehydrogenase gene whereby these probes are constructed in lactic acid bacteria with respect to the codon usage and

(4) analyzing the clones of the gene bank which react positively with the probe from step (3).

The chromosomal L. dextranicus (DSM 20187) DNA can be isolated by combined polyethylene glycol/lysozyme treatment and subsequent incubation with proteinase K.

The cleavage of the isolated L. dextranicus DNA with a suitable restriction enzyme, the ligation of the cleaved DNA into a suitable cloning vector and the transformation of a suitable organism with the recombinant cloning vector for the production of a gene bank can be carried out in a manner familiar to one skilled in the area of molecular biology. The next step is the examination of the gene bank produced in this way with a nucleic acid probe which has a sequence specific for the glucose-6-phosphate dehydrogenase gene.

A peptide sequence of G6-PDH from L. mesenteroides with a lysine residue (*) which can be pyridoxylated is known from Haghighi et al., Biochemistry 21 (1982), 6415-6420. This sequence is as follows: Phe-Leu-Leu-Lys*-Ser-Pro-Ser-Tyr-(Asp/Val)-Lys. However, it was not possible to derive an oligonucleotide probe from this sequence which can be used to find a hybridization signal in the L. dextranicus gene bank.

Bhadbhade et al., FEBS Letters 211 (1987), 243-246 discloses a peptide sequence from the active centre of the G6P-DH from L. mesenteroides with a high homology to human G6P-DH. The oligonucleotide probe mentioned in Example 2 with a length of 72 bases (SEQ ID NO:2) was produced from the multitude of oligonucleotide probes which can be constructed from this peptide sequence.

Screening the L. dextranicus DNA gene bank with this oligonucleotide in a 5' end-labelled form finally produced a positive clone which allowed the determination of the sequence of the L. dextranicus G6P-DH gene.

The DNA sequence of the G6P-DH gene from L. dextranicus was determined according to the method of Sanger. It is shown in SEQ ID NO:1.

SEQ ID NO:1 also shows the amino acid sequence of the G6P-DH from L. dextranicus which was determined from it. From this it can be seen that the amino acid sequence of the enzyme according to the present invention does not correspond to the sequence of the L. mesenteroides enzyme described in FEBS Letters 211 (1987), 243-246 in 6 out of 42 positions.

In addition the invention includes a process for the production of a G6P-DH with the amino acid sequence shown in SEQ ID NO:1 in which

(1) a suitable host organism is transformed with a DNA or a vector according to the present invention which contains one or several copies of this DNA,

(2) the transformed host organism is cultured in a suitable medium and

(3) the protein is isolated from the medium or the cells.

The expression of the recombinant protein according to the present invention in a transformed host organism, preferably in a prokaryotic host organism, particularly preferably in an E. coli cell, is in principle possible under the control of any suitable promoter. Thus, in E. coli an expression of the G6P-DH is e.g. possible under the control of heterologous promoters such as e.g. the tac promoter, mgl promoter or pfl promoter. However,

the expression is preferably carried out constitutively under the control of a Leuconostoc promoter, particularly preferably under the control of the promoter sequence shown in SEQ ID NO:1 or of a promoter sequence derived therefrom (corresponding to the first 122 nucleotides of SEQ ID NO:1). The plasmid pUC G6P-DH 1.8 which is shown in Fig. 1 is most preferred.

The commercially available E. coli strain HB 101 was chosen as a suitable E. coli host strain. When transforming E. coli HB 101 with pUC G6P-DH 1.8 it was found that the plasmid has a high stability in the cell and the expression of the G6P-DH can be carried out over several passages even without selection pressure.

It is intended to elucidate the present invention by the following examples in conjunction with SEQ ID NO:1 and 2 as well as Figure 1.

SEQ ID NO:1 shows the nucleotide sequence of the Leuconostoc DNA insertion in pUC G6P-DH 1.8 in which the first 122 bases upstream of the coding region for the L. dextranicus G6P-DH promoter and the bases 123-1580 represent the nucleotide sequence of the L. dextranicus G6P-DH gene which codes for a protein with the amino acid sequence which is also shown,

SEQ ID NO:2 shows the oligonucleotide probe for the part of the G6P-DH gene from Leuconostoc mesenteroides which codes for a region of the active centre of the G6P-DH of

L. mesenteroides which has a high homology to human G6P-DH.

Fig. 1 shows the plasmid pUC-G6P-DH 1.8.

E x a m p l e 1

Isolation of chromosomal DNA from Leuconostoc dextranicus

Genomic DNA is isolated from Leuconostoc dextranicus according to the following method:

Leuconostoc dextranicus (DSM 20187) is cultured at 30°C in APT medium (Merck No. 10454). The cells from 100 ml culture broth are centrifuged down, washed in 10 ml 20 mmol/l Tris/HCl pH 8.0 and finally resuspended in 15 ml of this buffer solution. After addition of 5 ml 24 % (w/v) polyethylene glycol 6000 and 20 mg lysozyme it is incubated for 16 h at 4°C. The cell lysis is carried out by addition of 1 ml 20 % (w/v) SDS. 2 mg protease K are added and incubated for 60 min at 37°C. The further purification of the DNA is carried out by sequential phenol and chloroform extraction, treatment with RNAse A (0.5 mg/60 min at 37°C), renewed phenol and chloroform extraction and a final ethanol precipitation.

E x a m p l e 2

Determination of the size of genomic DNA fragments which code for G6P-DH

The oligonucleotide shown in SEQ ID NO:2 is used for the hybridization.

5 µg aliquots of genomic DNA from L. dextranicus are cleaved with different restriction endonucleases (BclI, ClaI, HindIII, PstI, XbaI), electrophoretically separated on a 0.8 % agarose gel and subsequently transferred onto a nitrocellulose filter. After pre-hybridization with a solution of 6 x SSC buffer, 0.7 % skim milk, such a filter is incubated overnight in the same solution at 40°C which additionally contains the above nucleotide which is radioactively end-labelled with ^{32}P. After washing, drying and autoradiography, it can be established that a DNA fragment of ca. 3.4 kb size produced by the restriction enzyme BclI hybridizes with the oligonucleotide.

E x a m p l e 3

Cloning of a DNA fragment which codes for G6P-DH

20 µg genomic DNA from L. dextranicus is cleaved with BclI and is fractionated in a gel of low-melting agarose. DNA fragments with a size of ca. 3.4 kb +/- 0.2 kb are cut out of the gel. This gel piece is equilibrated with ligase buffer (Maniatis et al., 1982, Molecular Cloning, p 474) and liquified at 65°C. Afterwards 0.1 µg pUC18 DNA is cleaved with BamHI and 5 U T4 ligase are added, incubated for 10 min at 37°C and

then for 16 h at 15°C. The restriction endonuclease BamHI produces protruding DNA ends which are compatible with the ends produced by BclI.

Cells of E. coli HB 101 (DSM 1607) are cultured in 20 ml nutrient medium and converted into a competent state by calcium chloride treatment (Maniatis et al. 1982, Molecular Cloning, pp. 250 - 252). The ligation preparation obtained above is liquified again for 5 min at 65°C after addition of one volume portion of 50 mmol/l Tris/HCl pH 7.5 and is used for the transformation. The cells treated in this way are plated on LB agar plates with 50 µg/ml ampicillin and incubated at 37°C for one day.

The fully grown colonies are transferred onto new LB agar plates with 50 µl ampicillin onto which nitrocellulose filters are placed. After the colonies are again fully grown, the filters are lifted, the colonies are lysed as described by Grunstein and Hogness Proc. Natl. Acad. Sci. USA, 72 (1975) 3961 and hybridized with the radioactively labelled oligonucleotide probe described under 2. After autoradiography clones with recombinant, G6P-DH coding plasmids can be identified and isolated from the original plates. After isolation and characterization of the plasmid DNA of such clones it turns out that these have a size of ca. 6 kb. This means that a DNA fragment of ca. 3.4 kb size is inserted into the pUC18 DNA. Such a recombinant plasmid is chosen for the further processing.

E x a m p l e 4

Resection and expression of the gene

The recombinant plasmid obtained above can be cut up into a fragment of ca. 2.2 kb and one of ca. 3.8 kb size by cleavage with the restriction enzymes XbaI and SpeI. This 3.8 kb fragment now only contains DNA sequences from pUC18 and the nucleotide sequence of SEQ ID NO:1. Isolation and religation of the 3.8 kb fragment and subsequent transformation in E. coli HB 101 leads to a clone which expresses the G6P-DH gene. The G6P-DH gene is subcloned in this positive clone as a 1.8 kb fragment (SpeI/KpnI) in a commercial pUC18 vector cleaved with XbaI and KpnI in the polylinker region whereby the KpnI cleavage site originates from the vector portion of the positive clone from the gene bank. Thus a SpeI/BclI fragment from the Leuconostoc dextranicus genome is present. The DNA sequence of the complete subcloned 1.8 kb SpeI/BclI fragment is shown in SEQ ID NO:1.

The resulting recombinant plasmid contains the G6P-DH gene under the control of its own Leuconostoc promoter. It was denoted pUC-G6P-DH 1.8 and is shown in Fig. 1.

The expression direction of the G6P-DH gene in this case is in the opposite direction to the lac promoter (pLAC) on pUC18.

In order to determine the enzyme activity and purify the G6P-DH, such a clone is inoculated in a test tube with 5 ml LB nutrient medium containing 50 µg/ml ampicillin and is grown overnight at 37°C. An Erlenmeyer flask containing 1 l LB nutrient medium with 50 µg/ml

ampicillin is inoculated with this culture and incubated again overnight at 37°C while shaking. The cells are harvested by centrifugation.

E x a m p l e 5

Concentration and characterization of recombinant G6P-DH from E. coli

5.1 Concentration procedure

1. Lysis
   Suspend 5 kg biomass (E. coli HB101 pUC-G6P-DH 1.8) in 25 l potassium phosphate buffer 10 mmol/l, pH 7.5 containing 10^{-3} mol/l $MgCl_2$ and lyse the cells with an APV-Gaulin high pressure homogenizer at 800 bar homogenization pressure.
   Cool the resulting suspension to +4°C and centrifuge.

2. Ammonium sulphate fractionation
   Add solid ammonium sulphate to the crude extract up to a concentration of 1.9 mol/l and centrifuge down the precipitated precipitate. Precipitate the supernatant further with ammonium sulphate up to a concentration of 3.0 mol/l and centrifuge down the precipitate.

3. Heating
   Dissolve the second ammonium sulphate precipitate with 20 mmol/l potassium phosphate buffer, pH 6.0 containing 1 mmol/l EDTA and

heat for 20 min to 52°C. Centrifuge down the precipitated precipitate.

4. First crystallization
Add solid ammonium sulphate slowly to the supernatant from 3. to a concentration of 2.1 mol/l; adjust the pH with NaOH to 6.0. The G6P-DH crystallizes out overnight. The crystallization should be carried out at room temperature and while stirring gently. Centrifuge down the enzyme crystals.

5. Second crystallization
Dissolve the precipitate with 20 mmol/l potassium phosphate buffer, pH 6.0 containing 1 mmol/l EDTA and add solid ammonium sulphate to 1.9 mol/l. Adjust the pH again to 6.0 and allow the enzyme to crystallize out overnight at room temperature and while stirring gently.

6. Dialysis
Centrifuge down the enzyme crystallizate and dissolve the precipitate in a concentrated form with 10 mmol/l potassium phosphate buffer, pH 6.0 containing 1 mmol/l EDTA and dialyse for 24 hours against the same buffer.

7. Lyophilization
Lyophilize the enzyme solution without additives. This results in ca. 210 g lyophilizate with ca. 900 U/mg activity.

5.2 Characterization of recombinant G6P-DH

The G6P-DH produced by genetic engineering differs in its properties from the known enzyme from Leuconostoc.

The disadvantages of the known Leuconostoc enzyme are as follows:
The long-term stability is low. In addition the enzyme converts glucose which can lead to false results when measuring in blood, serum or plasma since glucose is always present in such samples. The lack of specificity of G6P-DH is described in Archives of Biochemistry and Biophysics 149 (1972) 102-109.

The differentiating features are:

1. K_m NADP (in 0.1 mol/l Tris pH 7.8; 25°C

rec G6P-DH	G6P-DH from Leuconostoc
3.7×10^{-5}	7.4×10^{-6} mol/l [1]
	5.7×10^{-6} mol/l [3]
	9.9×10^{-6} mol/l [2]

2. Effect of activators/inhibitors

Addition of	rel. activity (with respect to activity without additive)			
	rec.G6P-DH cosubstrate		G6P-DH from Leuconostoc cosubstrate	
	NAD^+	$NADP^+$	NAD^+	$NADP^+$
5 mmol/l phosphate	100%	80%	activation [2]	
50 mmol/l phoshate	100%	80%	107%[1]	118%[1]
30% glycerol	60%	30%	30%[1]	30%[1]
30 mmol/l Mg^{2+}	100%	100%	80%[1]	80%[1]
0.3 mol/l hydrogen carbonate	100%	100%	120%[1]	120%[1]

3. Specificity

Specificity for	rec G6P-DH with $NAD(P)^+$	G6P-DH from Leuconostoc
glucose[4]	no conversion	conversion[5]
2-deoxy-glucose-6P[4]	5 %	no conversion

4. Temperature stability

Temperature	rel. activity (with reference to 20°C)	
	rec. G6P-DH	G6P-DH from Leuconostoc dextranicus produced according to [1]
40°C	100 %	100 %
50°C	100 %	97 %
60°C	100 %	90 %
70°C	43 %	4 %

The determination of the temperature stability was carried out in 3.2 mol/l ammonium sulphate pH 6.0 for 10 minutes. (Initial activity of the enzyme 2500 U/ml).

The determination of activity and specificity was carried out as described in Example 6.

1) Olive and Levy, Biochem. 6 (1967), 730
2) DeMoss, in Methods in Enzymology, Vol 1., p. 328, Acad. Press, New York, 1955
3) Levy, 626th Meeting Sheffield, p. 13 (1988)
4) Concentration 0.15 mol/l
5) Arch. Biochem. and Biophys. 149 (1972), 102-109

E x a m p l e 6

Determination of the activity of glucose-6-phosphate-dehydrogenase

G6P-DH converts glucose-6-phosphate and NAD^+ to gluconate-6-phosphate and NADH. The NADH formed is measured photometrically at 340 nm.

0.05 ml sample (G6P-DH, volume activity should if possible be between 0.3 and 0.5 U/ml) is added to 3 ml of a reagent consisting of Tris buffer (0.1 mol/l pH 7.8, 3 mmol/l $MgCl_2$), 0.1 mmol/l NAD^+, free acid and 0.15 mol/l glucose-6-phosphate at 25°C and the increase in absorbance (ΔA/min) is monitored. The volume activity is calculated as follows:

$$\text{Volume activity} = \frac{3.05}{\epsilon \times 1 \times 0.05} \times \Delta A \text{ min } [U/ml]$$

$$\epsilon_{340} = 6.3 \; [mmol^{-1} \times 1 \times cm^{-1}]$$

SEQ ID NO:1
TYPE OF SEQUENCE: Nucleotide with corresponding protein
LENGTH OF SEQUENCE: 1696 base pairs

FORM OF STRAND: single strand

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TCTAGTCATT TAATCAATTT TTGACTTGTT CAACGCTTAA TATGTTTGTG AATCCCGTAC   60

TTTTCCAGAC CTTTTTGCGT TATAATGGAG AGTGAATTTA ATTATAATAT AAGGGGAACA  120

TC                                                                 122

ATG GTT TCA GAA ATC AAA ACG TTG GTA ACT TTC TTT GGC GGA ACT GGT    170
Met Val Ser Glu Ile Lys Thr Leu Val Thr Phe Phe Gly Gly Thr Gly
                  5                  10                  15

GAT TTA GCA AAG CGT AAG CTT TAC CCA TCA GTT TTC AAC CTC TAC AAA    218
Asp Leu Ala Lys Arg Lys Leu Tyr Pro Ser Val Phe Asn Leu Tyr Lys
             20                  25                  30

AAA GGA TAC TTA CAA GAA CAC TTT GCC ATT GTT GGG ACA GCA CGT CAA    266
Lys Gly Tyr Leu Gln Glu His Phe Ala Ile Val Gly Thr Ala Arg Gln
         35                  40                  45

CAA TTA AGT GAT GAC GAG TTT AAG CAA TTG GTT CGT GAT TCA ATT AAA    314
Gln Leu Ser Asp Asp Glu Phe Lys Gln Leu Val Arg Asp Ser Ile Lys
     50                  55                  60

GAC TTT ACT GAA GAT CAA GCA CAA GCC GAA GCG TTT ATT GCG CAT TTT    362
Asp Phe Thr Glu Asp Gln Ala Gln Ala Glu Ala Phe Ile Ala His Phe
 65                  70                  75                  80

TCT TAC CGT GCG CAC GAT GTC ACA GAT GCC GCT TCT TAT GGT ATC TTG    410
Ser Tyr Arg Ala His Asp Val Thr Asp Ala Ala Ser Tyr Gly Ile Leu
                 85                  90                  95

AAG TCA GCG ATC GAA GAA GCA GCA ACC AAA TTT GAC ATT GAT GGC AAT    458
Lys Ser Ala Ile Glu Glu Ala Ala Thr Lys Phe Asp Ile Asp Gly Asn
            100                 105                 110

CGT ATT TTC TAT ATG TCA GTT GCC CCT CGT TTC TTC GGT ACA ATC GCT    506
Arg Ile Phe Tyr Met Ser Val Ala Pro Arg Phe Phe Gly Thr Ile Ala
        115                 120                 125

AAA TAT TTG AAA TCA GAA GGT TTG CTA GCT GAG ACT GGC TAC AAT CGT    554
Lys Tyr Leu Lys Ser Glu Gly Leu Leu Ala Glu Thr Gly Tyr Asn Arg
    130                 135                 140

TTG ATG ATT GAA AAG CCT TTT GGT ACA TCA TAC GCC ACC GCA GAA GAA    602
Leu Met Ile Glu Lys Pro Phe Gly Thr Ser Tyr Ala Thr Ala Glu Glu
145                 150                 155                 160

TTG CAA AGT GAT TTG GAA AAT GCA TTT GAT GAT GAC CAA CTG TTC CGT    650
Leu Gln Ser Asp Leu Glu Asn Ala Phe Asp Asp Asp Gln Leu Phe Arg
                165                 170                 175
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ATT GAC CAC TAT CTT GGA AAA GAA ATG GTA CAA AAT ATT GCA GCA TTA      698
Ile Asp His Tyr Leu Gly Lys Glu Met Val Gln Asn Ile Ala Ala Leu
            180                 185                 190

CGT TTT GGT AAC CCA ATC TTT GAT GCC GCT TGG AAT AAG GAC TAT ATC      746
Arg Phe Gly Asn Pro Ile Phe Asp Ala Ala Trp Asn Lys Asp Tyr Ile
        195                 200                 205

AAA AAC GTA CAA GTA ACT TTG GCT GAA GTT CTA GGT GTT GAA GAG CGT      794
Lys Asn Val Gln Val Thr Leu Ala Glu Val Leu Gly Val Glu Glu Arg
    210                 215                 220

GCT GGT TAC TAC GAT ACC ACT GGC GCC CTT TTG GAT ATG ATT CAA AAC      842
Ala Gly Tyr Tyr Asp Thr Thr Gly Ala Leu Leu Asp Met Ile Gln Asn
225                 230                 235                 240

CAC ACA ATG CAA ATT GTT GGT TGG TTA GCA ATG GAA AAA CCT GAA TCA      890
His Thr Met Gln Ile Val Gly Trp Leu Ala Met Glu Lys Pro Glu Ser
                245                 250                 255

TTC AAT GAT AAG GAT ATC CGT GCA GCT AAA AAC GCC GCC TTC AAT GCA      938
Phe Asn Asp Lys Asp Ile Arg Ala Ala Lys Asn Ala Ala Phe Asn Ala
            260                 265                 270

TTA AAG ATT TAT AAC GAA GAA GAA GTG AAT AAG TAC TTC GTT CGT GCA      986
Leu Lys Ile Tyr Asn Glu Glu Glu Val Asn Lys Tyr Phe Val Arg Ala
        275                 280                 285

CAA TAT GGT GCT GGT GAT ACA GCT GAT TAC AAG CCA TAT TTG GAA GAA     1034
Gln Tyr Gly Ala Gly Asp Thr Ala Asp Tyr Lys Pro Tyr Leu Glu Glu
    290                 295                 300

GCA GAT GTC CCT GCT GAC TCA AAG AAC AAC ACA TTC ATT GCT GGT GAA     1082
Ala Asp Val Pro Ala Asp Ser Lys Asn Asn Thr Phe Ile Ala Gly Glu
305                 310                 315                 320

TTG CAG TTC GAT TTG CCA CGT TGG GAA GGT GTT CCT TTC TAT GTT CGT     1130
Leu Gln Phe Asp Leu Pro Arg Trp Glu Gly Val Pro Phe Tyr Val Arg
                325                 330                 335

TCA GGT AAG CGT TTG GCT GCC AAG CAA ACA CGT GTT GAT ATT GTA TTT     1178
Ser Gly Lys Arg Leu Ala Ala Lys Gln Thr Arg Val Asp Ile Val Phe
            340                 345                 350

AAG GCT GGC ACA TTC AAC TTT GGT TCA GAA CAA GAA GCA CAA GAA TCA     1226
Lys Ala Gly Thr Phe Asn Phe Gly Ser Glu Gln Glu Ala Gln Glu Ser
        355                 360                 365

GTA CTC TCA ATC ATC ATT GAT CCA AAG GGT GCT ATT GAA TTG AAG CTT     1274
Val Leu Ser Ile Ile Ile Asp Pro Lys Gly Ala Ile Glu Leu Lys Leu
    370                 375                 380

AAC GCT AAG TCA GTT GAA GAT GCC TTC AAC ACC CGC ACA ATC AAC TTG     1322
Asn Ala Lys Ser Val Glu Asp Ala Phe Asn Thr Arg Thr Ile Asn Leu
385                 390                 395                 400
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GAT TGG GCA GTA TCT GAT GAA GAC AAG AAG AAC ACA CCA GAA CCA TAC   1370
Asp Trp Ala Val Ser Asp Glu Asp Lys Lys Asn Thr Pro Glu Pro Tyr
                405                 410                 415

GAA CGT ATG ATT CAC GAT ACA ATG AAT GGT GAC GGA TCA AAC TTT GCT   1418
Glu Arg Met Ile His Asp Thr Met Asn Gly Asp Gly Ser Asn Phe Ala
            420                 425                 430

GAT TGG AAC GGT GTA TCA ATT GCT TGG AAG TTT GTT GAC GCA ATT ACT   1466
Asp Trp Asn Gly Val Ser Ile Ala Trp Lys Phe Val Asp Ala Ile Thr
        435                 440                 445

GCC GTT TAC GAT GCA GAT AAA GCA CCA TTG GAG ACA TAT AAG TCA GGT   1514
Ala Val Tyr Asp Ala Asp Lys Ala Pro Leu Glu Thr Tyr Lys Ser Gly
    450                 455                 460

TCA ATG GGT CCT GAA GCA TCA GAC AAG CTA TTA GCT GAA AAT GGC GAT   1562
Ser Met Gly Pro Glu Ala Ser Asp Lys Leu Leu Ala Glu Asn Gly Asp
465                 470                 475                 480

GCT TGG GTA TTT AAA GGA TAAGCACATT TAAAAAGACC ATCAAACAAA         1610
Ala Trp Val Phe Lys Gly
                485

TCTTTGTTTG ACGGTCTTTT TATATTGTCT GATTTAAGAT GCGTTTGGTT TCACGGAAAA 1670

CGGCTGACAA ATTGGTGTAT TGATCC                                      1696
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SEQ ID NO:2
TYPE OF SEQUENCE: Nucleotide sequence
LENGTH OF SEQUENCE: 72 base pairs

FORM OF STRAND: single strand

RTTTTGRACC ATTTCTTTWC CTAAATAATG ATCAATWCKA AATAATTGRT TATCATCAAA 60

RGCGTTTTCA AA 72

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A recombinant glucose-6-phosphate dehydrogenase comprising an amino acid sequence as shown in SEQ ID NO.1.

2. A DNA comprising a nucleic acid sequence as shown in SEQ ID NO. 1 or a degenerate form thereof which codes for the glucose-6-phosphate dehydrogenase according to claim 1.

3. A recombinant vector comprising one or more copies of the DNA according to claim 2.

4. Recombinant vector as claimed in claim 3, w h e r e i n it is a prokaryotic vector.

5. Recombinant vector as claimed in claim 4, w h e r e i n it contains an origin of replication which is active in E. coli.

6. Recombinant vector as claimed in one of the claims 3 to 5, w h e r e i n the nucleic acid sequence coding for the glucose-6-phosphate dehydrogenase is under the control of a Leuconostoc promoter.

7. Recombinant vector as claimed in claim 6, w h e r e i n the promoter is the native promoter of the glucose-6-phosphate dehydrogenase gene and the first 122 bases of the nucleic acid sequence shown in SEQ ID NO:1 contain a sequence derived therefrom with promoter properties or contain a fragment of this region with promoter properties.

8. Plasmid pUC-G6P-DH 1.8 as hereinbefore defined.

9. The DNA according to claim 2 further comprising a native promoter of the glucose-6-phosphate dehydrogenase gene and the first 122 bases of the nucleic acid sequence shown in SEQ ID NO. 1 or degenerate, derivative or fragment forms thereof with promoter properties.

10. Microorganism, w h e r e i n it is transformed with a DNA as claimed in claim 2 or with a recombinant vector as claimed in one of the claims 3 to 8.

11. Microorganism as claimed in claim 10, w h e r e i n it is a gram-negative bacterium.

12. Microorganism as claimed in claim 11, w h e r e i n it is an E, coli bacterium.

13. Process for the isolation of a DNA as claimed in claim 2, w h e r e i n
    (1) Leuconostoc dextranicus (DSM 20187) DNA is isolated and cleaved with a suitable restriction enzyme,
    (2) the cleaved L. dextranicus DNA is incorporated into a vector, a suitable host organism is transformed with the vector and a gene bank is produced in this way,
    (3) the gene bank from (2) is screened with a nucleic acid probe which has a sequence specific for the glucose-6-phosphate dehydrogenase gene and
    (4) the clones of the gene bank which react positively with the probe (3) are analyzed.

14. Process as claimed in claim 13, w h e r e i n a probe is used which is 50 to 80 nucleotides long.

15. Process as claimed in claim 13 or 14, w h e r e i n E. coli is used as the host organism.

16. Process for the isolation of a protein as claimed in claim 1, w h e r e i n
    (1) a suitable host organism is transformed with a DNA as claimed in claim 2 or with a vector as claimed in one of the claims 3 to 8,
    (2) the transformed host organism is cultured in a suitable medium and
    (3) the protein is concentrated from the medium or the cells.

17. Process as claimed in claim 16, w h e r e i n a prokaryotic host organism is used.

18. Process as claimed in claim 17, w h e r e i n E. coli is used as the host organism.

19. Process for the enzymatic determination of the content of glucose-6-phosphate in a sample solution in which glucose-6-phosphate dehydrogenase is used as the determination enzyme, w h e r e i n a recombinant glucose-6-phosphate dehydrogenase as claimed in claim 1 is used.

20. Reagent for the enzymatic determination of the content of glucose-6-phosphate in a sample solution in which glucose-6-phosphate dehydrogenase is used as the determination enzyme, w h e r e i n it contains a recombinant glucose-6-phosphate dehydrogenase as claimed in claim 1.

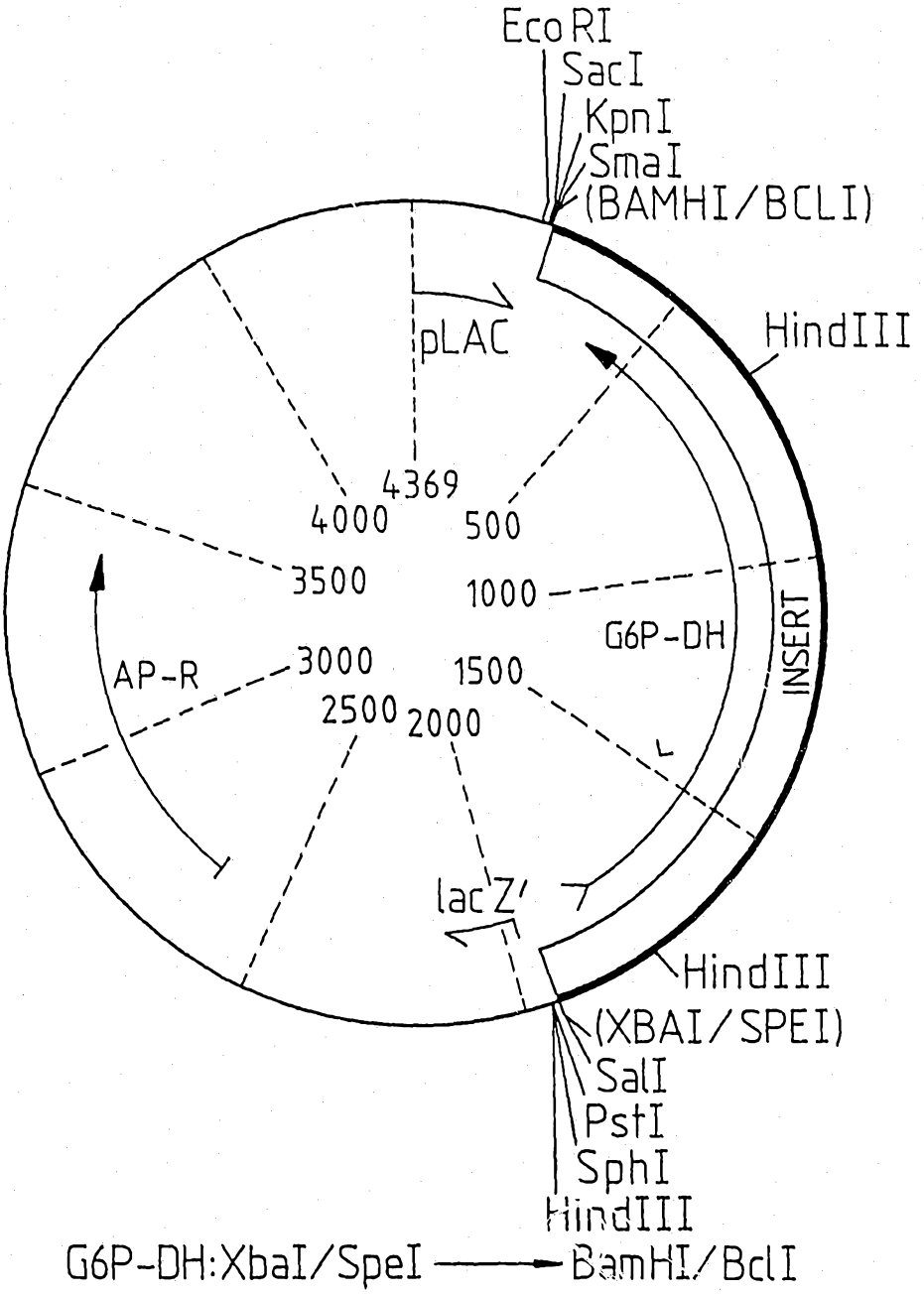
21. A product or process of any one of claims 1 to 20, substantially as hereinbefore described with reference to the drawings and/or Examples.

~~22. The steps, features, compositions and compounds disclosed herein or referred to or indicated in the specification and/or claims of this application, individually or collectively, and any and all combinations of any two or more of said steps or features.~~

DATED this SIXTEENTH day of JULY 1991

Boehringer Mannheim GmbH

by DAVIES & COLLISON
Patent Attorneys for the applicant(s)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SphI
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