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(54) Title: *CHLORELLA* VIRUS PROMOTERS

(57) Abstract

The present invention provides novel promoter sequences obtained from *Chlorella* virus. The invention includes gene constructs comprising a promoter sequence of the invention operably linked to a DNA sequence encoding a structural gene. The invention also provides vectors and host cells for expressing product encoded by the structural gene of a gene construct of the invention amd cells transformed with the heterologous gene operably linked to the promoter.

CHLORELLA VIRUS PROMOTERS

Field of the Invention

This invention relates to novel promoters isolated from *Chlorella* virus. The novel promoters are useful for expression of heterologous genes in host cells.

Background of the Invention

Genetic engineering allows for isolation of a structural gene from one organism and expression of that gene in a different organism. Expression of a gene includes both transcription of the nucleic acid into mRNA and translation of the mRNA into protein. In order for the structural gene to be expressed in a new organism, the gene must be linked to a regulatory sequence in the proper location to signal transcription of the gene. The regulatory sequence generally includes a promoter sequence upstream from the structural gene. A promoter sequence is a DNA sequence which directs transcription of a structural gene. The nucleic acid sequence of the structural gene is transcribed into messenger RNA (mRNA) and then translated into a sequence of amino acids characteristic of a specific polypeptide or protein. Typically, a promoter sequence is located in the 5' region of a gene, upstream from the transcriptional start site of the structural gene.

A promoter may be inducible or constitutive. In response to an inducing agent, the activity of an inducible promoter is increased, thereby increasing the rate of transcription of an operably linked coding sequence. In contrast, the rate of transcription of a gene under control of a constitutive promoter is not regulated. It is noted, however, that a constitutive promoter can be made an inducible promoter by the addition of an operator sequence. For example, the *lac* operator is added to the T7 bacteriophage promoter, changing it from a constitutive promoter to one induced by IPTG (Rosenberg, et al., U.S. Patent No. 4,952,496).

Although not under the control of an inducing agent, some constitutive promoters provide higher levels of transcription than others. High activity promoters providing of high levels of gene transcription can have significant advantage in commercial production of a gene product.

In general, the ability of a promoter to direct transcription outside of its natural host varies. Moreover, the transcription rate of a particular promoter can also vary with the particular host in which the promoter is functioning. Therefore, new promoters capable of promoting high levels of transcription in a wide variety of host cells are needed.

The *Chlorella* viruses are a group of viruses which infect certain strains of unicellular, eukaryotic, *Chlorella*-like green algae. (Van Etten, 1995, *Mol. Cells*. 5:99-106; Van Etten, et al., 1991, *Microbiol. Rev.* 55:586-620). These viruses are among the largest and most complex viruses known, generally 150-190 nm diameter polyhedrons containing greater than 300 kb of double stranded DNA. The *Chlorella* virus genome has the potential to encode several hundred gene products.

Chlorella virus methyltransferase promoters have been isolated and shown to function in prokaryotic and eukaryotic host cell systems. These methyltransferase promoters function well in some bacterial and higher plant cells. See, for example, U.S. Patent No. 5,563,328; and Mitra, et al., 1994, *Plant Molec. Biol.*, 26: 85-893 ("Mitra").

The present invention provides novel promoter sequences isolated from *Chlorella* virus that can induce a high level of gene expression in prokaryotic or eukaryotic cells, and over a wide range of temperatures, e.g., about 21°C to 37°C.

Summary of the Invention

The present invention provides novel promoter sequences isolated from *Chlorella* virus (SEQ ID NOS: 1-7). The invention includes gene constructs comprising a promoter sequence of the invention operably linked to a DNA sequence of a structural gene. The invention further provides vectors and host cells for expressing a product encoded by the structural gene of a gene construct of the invention, and cells transformed with a heterologous gene operably linked to the promoter.

The promoters of the invention include inducible *Chlorella* promoters, rendered inducible, for example, by fusion to an operator sequence. In a preferred embodiment, the *Chlorella* promoters of the invention are fused to a *lac* operator. The *Chlorella* promoters of the invention show very strong promoter

activity over a wide range of temperatures, for example, from about 21°C to about 37°C.

In one embodiment, the structural gene is a non-*Chlorella* virus DNA sequence encoding a protein for production in a host cell. According to this embodiment, the non-*Chlorella* virus DNA sequence can be any suitable structural gene which encodes a peptide, protein, hormone, enzyme, etc. Examples of suitable structural genes include glucagon like peptide 1 (GLP-1), growth hormone releasing factor (GRF), parathyroid hormone (PTH), interlinking peptides, amidation code sequences, carbonic anhydrase, beta-galactosidase, chloramphenicol acetyltransferase (CAT), glutathione acetyltransferase, and the like.

A gene construct of the invention is introduced into an appropriate host cell for expression of the gene product. Host cells are transformed directly or through a vector. In one embodiment, a suitable vector for a prokaryotic cell such as *E. coli* strains HB101 or JM109 is the plasmid pKK232-8.

The promoters of the invention are useful in a wide variety of prokaryotic and eucaryotic host cells. In one embodiment, the promoters of the invention are used to promote high levels of gene expression in plants, including tobacco, wheat, and other plants.

The invention further provides a process for producing a protein composition. According to this embodiment, a protein product is produced in a host cell transformed with a gene construct of the invention. The gene construct includes a promoter sequence of the invention operably linked to a structural gene encoding the protein to be produced in the host cell. The invention also provides methods for screening and isolating a promoter sequence having strong transcriptional properties, including truncated versions of the *Chlorella* virus promoters shown below.

The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

Brief Description of the Drawings

Figure 1 is a diagrammatic representation of the pKK232-8 plasmid map.

Figure 2 is a diagrammatic representation of gene constructs using seven *Chlorella* virus promoters linked to the heterologous DNA sequence encoding the CAT protein.

Figure 3 is a comparison of CAT activities in chloramphenicol-resistant *E. coli* transformed with the CAT gene operably linked to the promoter sequences *cvp*-10, *cvp*-13, *cvp*-15 and *cvp*-16.

Figure 4 is a comparison of promoter activities of the *Chlorella* virus promoter *cvp*-13 and the *tac* promoter transformed into *E. coli* HB101 and grown in the presence of varying concentrations of chloramphenicol.

Figure 5 is a diagrammatic representation of the pBN115-glp plasmid map.

Figure 6 is an SDS-PAGE gel showing expression of GLP-1 from pBN115-glp and pBN115-glp/tac at various temperatures.

Figure 7 is a photograph of an SDS polyacrylamide gel of proteins expressed from expression cassettes containing 3 copy GLP-1 and YX16 promoter or tac promoter, induced and uninduced, soluble and insoluble protein, at 27°C and 37°C.

Figure 8 a photograph of an SDS polyacrylamide gel of proteins expressed from expression cassettes fomed with 8 copy GLP-1 and the YX15, YX16, or tac promoter, induced and uninduced, soluble and insoluble protein, at 27°C and 37°C.

Figure 9 is a diagramatic representation of the plasmid map of pBN95lnk2[GLP(7-36)AFAM]8HAE(tac).

Detailed Description of the Invention

The present invention is directed to novel nucleic acid sequences isolated from the genome of several *Chlorella* viruses, which isolated nucleic acid sequences function as transcriptional promoters [SEQ ID NOS: 1-7]. The disclosed promoter sequences are operably linked to a structural gene to direct transcription of the structural gene in prokaryotic or eukaryotic cells. The disclosed promoter sequences provide a high level of gene expression in comparison to native or other non-native promoters, as shown in the examples below in bacterial host cells.

The disclosed promoter sequences are operably linked to a structural gene sequence to form a "gene construct" or "expression cassette". In a typical

embodiment, the structural gene sequence of a gene construct will be a heterologous sequence. As used herein, a "heterologous sequence" is a DNA sequence which is different than that to which the promoter sequence is operably linked in the *Chlorella* virus. The gene construct preferably also includes enhancers, markers, polyadenylation sequences or other regulatory nucleic acid sequences. When a secreted protein is to be produced, the coding sequence of the structural gene preferably includes a nucleic acid sequence encoding a signal peptide.

Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

The disclosed gene constructs are used to express a protein product encoded by the structural gene in host cells. The gene construct is incorporated into the host cells directly or via a vector, using known methods. The protein product remains intracellular post-expression or is secreted extracellularly when a nucleic acid sequence encoding a signal peptide is included in the gene construct.

<u>*Chlorella* Virus Promoter Sequences</u>

The following nucleic acid sequences were isolated from *Chlorella* viruses and include a promoter sequence for directing transcription of a structural gene.

cvp-1 [SEQ ID NO: 1]

CCCGGGGATC GCAGGGCATG GGCATTAAAA GAACTTTATG GAATCAAAAA
TCTTAGTGAA TTTCCACCAC AGGTATATAG TCTTCAGGAC GCTAACGATG
ATATCAACGA TTGTATCAAA GGTTATCGTT TGAGGCACTC ATATCAGGTA
GTTTCTACAC AGAAACTTGA ACAACGCCTG GGAAAAGATC CTGAGCATAG
TAACTTATAT ACTAGCAGAT GTTGTAACGA TGCTTTATAT GAATATGAAT
TAGCACAACG ACAACTACAA AAACAACTTG ATGAATTTGA CGAAGATGGG
TATGATTTTT TTCAGGCACG TATAAATACA TTAGATCCGT CGACCTGCAG
CCAAGCTT

cvp-3 [SEQ ID NO: 2]

CCCGGGGATC TAATTCAGGG TGCGAATTTC TTGAACATCA AAGGTCTGTT
GGACGTTTTG TGTGCAGCGG TTGCTGATCG CATTGAATCC ATCAATAAAC
AGATTGGGGT AAATATCAAA CCCAGTTAGT CGGACATTAG AAGGATTTGT
GAGACCACCA CATCCAACGA CACCTAATGG TGTTGTGAAT GATATATTAG
AAATGTTACT TATCATTGAT ATTTGCATAA CACCATTTCC CTTTGCTTGA
TTTCTACCTA TACTAATTGA TTGTATTGTA GTGCACGCGG CGTACTTACT
TGTATTTGCC GTCTCAGACG TGCTTGATAA TAGTGTGGAA CTCGAGTATG
ATCCGTCGAC CTGCAGCCAA GCTT

cvp-6 [SEQ ID NO: 3]

CCCGGGGATC ATCGAAAGCA ACTGCCGCAT TCGAAACTTC GACTGCCTCG
TTATAAAGGT TAGTGAAAGC CATTGTATGT TATTACTGAG TTATTTAATT
TAGCTTGCTT AAATGCTTAT CGTGTTGATA TGATAAATGA CAAATGATAC
GCTGTATCAA CATCTCAAAA GATTAATACG AAGATCCGTC GACCTGCAGC
CAAGCTT

cvp-10 [SEQ ID NO: 4]

CCCGGGGATC GTTTCTCAGG GCGTCCGGGA GCATATTTCA GACTTGTCCA
GCCGTATGAG CATCACGTGC GCGTTCCTAG CAAGAGCGTG TACGTATATT
CTTTCGCTCT AGAAGATGCA GATTCGAGAC AACCGAATGG ATCGAATCTA
TTTGTACCCC GATATATATA GAATCTAGTC TAAACAAAAC GACCGCGGCT
CTTGCCAATA AATGTGACGC AATTAACGCA TTCGTGAATG ATGACTTGTC
CGCCCCGGTT CTTGACATTC TAAAAAAATG TGGAGTATCC TCGATCCGTC
GACCTGCAGC CAAGCTT

cvp-13 [SEQ ID NO: 5]

CCCGGGGATC TGCGTATTGC GGGACTTTTG AGCATTTTCC AGAACGGATT
GCCGGGACGT ATACTGAACC TCCAGTCCCT TTGCTCGTCG TATTTCCCAT
AATATACATA TACACTATTT TAATTATTTA CACCGGTTGT TGCTGAGTGA
TACAATGCAA ATTCCCTCCA CCGAGGAGGA TCGCGAACTG TCCAAATGTC
TTCTTTCTGC AGCTCCATAC GGAGTCGTTA GGAAACATTC ACTTAATTAT
AGGATCCGTC GACCTGCAGC CAAGCTT

cvp-15 [SEQ ID NO: 6]

CCCGGGGATC AGGCCTCGCT TATAAATATG GTATTGATGT ACTTGCCGGT
GTGATTGACT CAGATTACAG AGGAGAGTTG AAAGCAATCC TTTACAATAC
TACAGAACGT GACTATATTA TCAAAAAAGG CGATCAGCCA AGCTTCGTCG
ACCTGCGATC CGTCGACCTG CAGCCAAGCT T

cvp-16 [SEQ ID NO: 7]

CCCGGGGATC GCAAAACTCA CAGTCAACAA ACCAAAACAC GGAATGAAGA
AAGGAGAAAC TGTGATCATG TGGCAACAAG ATGGAGGTGT CATAGACTAC
ATTTACCCTC CCTCTGATCA TCGAAAGCAA CTGCCGCATT CGAAACTTCG
ACTGCCTCGT TATAAAGGTT AGTGAAAGCC ATTGTATGTT ATTACTGAGT
TATTTAATTT AGCTTGCTTA AATGCTTATC GTGTTGATAT GATAAATGAC
AAATGATACG CTGTATCAAC ATCTCAAAAG ATTAATACGA AGATCCGTCG
ACCTGCAGCC AAGCTT

The method by which these new promoter sequences were discovered is described more fully in the Examples below. Briefly, restriction DNA fragments were generated from the viral genomes of five *Chlorella* viruses, CA-4B, Al-1A, PBCV-1, SC-1A, and NC-lA. (Van Etten, 1991, *Microbiol. Rev.* 55:586-620) The restriction fragments were inserted into plasmid pKK232-8 by a shotgun cloning procedure using known methods (Sambrook, et al., 1989, *Molecular Cloning*).

The plasmid vector pKK232-8 contains a promoterless chloramphenicol acetyltransferase (CAT) gene and multiple cloning sites upstream for insertion of DNA restriction fragments. *E. coli* were then transformed with the cloned pKK232-8 and the transformants carrying promoter sequences were screened for resistance to chloramphenicol. To obtain high activity promoters, chloramphenicol-resistant transformants were further screened using increasing concentrations of chloramphenicol 0in the growth medium.

As used herein, the "strength" of a promoter refers to the level of transcription directed by the promoter. A "strong" promoter provides a greater level of transcription than a weak promoter. Thus, the phrase "strong promoter" is used interchangeably with a "high activity promoter". Strong promoters are particularly useful for commercial production of a gene product.

It is appreciated that the entire nucleic acid sequence recited for each of SEQ ID NOS: 1-7 may not be required for promoter function. Using the methods described above and in the Examples below, the disclosed promoter sequences can be further restricted, e.g. truncated or modified, and screened to refine the active promoter regions.

Preparation of Gene Constructs

According to the invention, a gene construct includes at least one structural gene coding sequence which is operably linked to a transcriptional control region. A transcriptional control region includes promoters, and other regulatory elements, such as enhancers, regulatory elements, polyadenylation sequences, transcriptional initiation regions, and transcriptional termination sequences. SEQ ID NOS: 1-7 each include a promoter sequence and may also include additional regulatory elements. Methods for operably linking a promoter sequence to a structural gene sequence are known and disclosed in, for example, Itakuri, et al., 1977, *Science* 198:1056-1063.

A structural gene of a gene construct according to the disclosure will typically encode a protein or polypeptide product. Any known or later discovered structural gene which encodes a desired product is operably linked to a promoter sequence of the invention using known methods. Examples of known structural genes suitable for use with the promoters of the invention include those nucleic acid sequences encoding: glucagon-like peptide 1 (GLP-1), growth hormone releasing factor (GRF), parathyroid hormone(PTH), carbonic anhydrase, beta-galactosidase, chloramphenicol acetyltransferase (CAT), glutathione acetyltransferase, interlinking peptides, amidation sequences, and the like structural genes.

Therefore, in one embodiment, a promoter sequence of the invention is operably linked to a DNA sequence encoding carbonic anhydrase, for example, human carbonic anhydrase. In another embodiment, a promoter sequence of the invention is linked to a DNA sequence encoding multiple copies of a desired protein. An example of a suitable multiple copy structural gene for glucagon like peptide-1 (GLP-1) is disclosed in U.S. Patent No. 5,595,887.

Gene Transformation Method

Once a gene construct is formed, it is introduced into a host cell directly or subcloned into an appropriate vector for transforming a host cell.

Methods of transforming cells are known, and the preferred method varies with the type of host cell to be transformed. As used herein, a "host cell" refers to the cell in which the structural gene of the gene construct is ultimately expressed. For prokaryotic and eukaryotic host cells, including bacterial, yeast, and animal host cells, preferred methods of transformation include methods of freeze/thaw, calcium chloride precipitation, calcium phosphate precipitation, plasmids, protoplast transformation, liposome mediated transformation, electroporation, and other known transformation methods.

For plant cells, preferred methods of transformation include *Agrobacterium*-mediated transformation, electroporation, microparticle bombardment, protoplast fusion, combinations of these and other known transformation methods.

Host Cells

The promoters of the invention are useful in a wide variety of host cells, including procaryotic and eucaryotic host cells. Suitable bacterial host cells for expression of a gene construct of the invention include *Escherichia coli*, *Bacillus subtilis* and *Streptomyces*. Plant and animal host cells, including yeast cells, such as tobacco, wheat and other plant cells, embryonic, tumor, and other animal cells, and the like, are also useful with the inventive promoters.

Suitable vector systems for carrying the gene constructs into the host cells include, for example, plasmids, viruses, phages, and yeast artificial chromosomes (YAC's). Suitable plasmids for transforming a bacterial host cell with a gene construct of the invention include pKK232-8 or pB0304, as described in the examples below.

Methods for introducing foreign genes into plants are known and can be used to insert a gene construct of the invention into a plant host, including biological and physical plant transformation protocols. See, for example, Miki, et al., 1993, "Procedure for Introducing Foreign DNA Into Plants", In: *Methods in*

Plant Molecular Biology and Biotechnology, Glick and Thompson, eds., CRC Press, Inc., Boca Raton, pages 67-88. The methods chosen vary with the host plant, and include chemical transfection methods such as calcium phosphate, microorganism-mediated gene transfer such as *Agrobacterium* (Horsch, et al., *Science* 227:1229-31, 1985), electroporation, micro-injection, and biolistic bombardment.

Expression vectors and *in vitro* culture methods for plant cell or tissue transformation and regeneration of plants are known and available. See, for example, Gruber et al., 1993, "Vectors for Plant Transformation: In: *Methods in Plant Molecular Biology and Biotechnology*, Glick and Thompson, eds., CRC Press, Inc., Boca Raton, pages 89-119.

Examples

The invention is more fully described with reference to the following examples, which are not intended to limit the invention in any way.

Example 1

Generating viral DNA fragments

Viral DNA extracted from five *Chlorella* viruses CA-4B; A1-1A; PBCV-1; SC-1A; and NC-1A was provided by Dr. Van Etten at the University of Nebraska. The extracted viral DNA (1.5 µg of each) was pooled and viral DNA fragments were generated by digestion with *Sau*3A1 (New England Biolabs, Beverly, MA) in (100 µl volume) 100 mM NaCl, 10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 µg/ml BSA. The mixture was incubated at 37°C for 120 minutes and stopped by 10mM EDTA. *Sau*3A1 fragments were precipitated from the digestion mixture by ethanol and washed with ethanol.

Example 2

Cloning fragments into pKK232-8

The *Sau*3A1 viral fragments produced as described for Example 1, were cloned into plasmid pKK232-8 at the single *Bam*HI site by a shotgun cloning procedure as described in Sambrook, et al., 1989, *Molecular Cloning*. Plasmid pKK232-8 was purchased from Pharmacia (Piscataway, NJ). The map of pKK232-8

is diagrammatically represented in Figure 1. Preparations of DNA, fill-in reaction and ligations were carried out as described in Sambrook et al., *supra* or according to the manufacturer's directions.

The *Chlorella* viral DNA fragments were ligated to 1 μg of pKK232-8 which was previously treated with *Bam*HI and calf intestinal alkaline phosphatase (CIP) to yield recombinant plasmids pCVP-1; pCVP-3; pCVP-6; pCVP-10; pCVP-13; pCVP-15; and pCVP-16. The seven *Chlorella* virus promoters operably linked to a heterologous CAT gene are diagrammatically shown in Figure 2. (Abbreviations: Sm, *Sma*I; Sa, *Sal*I H, *Hind*III; CAT, chloramphenicol acetyltransferase gene), Calf intestinal alkaline phosphatase was purchased from Promega (Madison, WI). T4 DNA ligase was purchased from BRL (Rockville, MD).

pKK232-8 is a derivative of pBR322 which contains promotorless chloramphenicol acyltransferase (CAT) gene and multiple cloning sites upstream for insertion of DNA restriction fragments. *E. coli* cells transformed with pKK232-8 are resistant to ampicillin but sensitive to chloramphenicol unless a DNA fragment containing a promoter is inserted upstream of the CAT gene to induce expression of CAT. If such a promoter is inserted, cells carrying the recombinant plasmid express CAT and thereby acquire resistance to chloramphenicol.

pKK232-8 is designed to reduce background of chloramphenicol resistance and increase the capacity of screening strong promoters. To reduce background, the CAT gene is flanked by efficient transcription terminators that block transcription into the CAT gene from other promoters present on the plasmid. Translational stop codons are introduced in all three reading frames between the multiple cloning sites and the initiation codon of the CAT gene to prevent translational readthrough from any ATG start codon that might be introduced by the cloned promoter fragment. The CAT gene also contains its own ribosome-binding signal and ATG start codon to allow efficient translation from CAT mRNA.

Example 3
Transformation of *E. coli* and Selection of Transformants

Transformation of *E. coli* strains HB101 and JM109 was performed as described by Sambrook, et al., 1989, *supra*. The *E. coli* strains were purchased from Promega (Madison, WI).

Transformed *E. coli* were screened for resistance to ampicillin indicating transformation with pKK232-8, and for resistance to chloramphenicol, indicating insertion of a promoter. The strength of an inserted promoter was estimated by measuring cell growth in the presence of increasing amounts of chloramphenicol.

Positive colonies were isolated on Luria Broth (LB) plates containing 30 μg/ml ampicillin and various concentrations of chloramphenicol (5, 10, 20, 30, 100 μg/ml). *E. coli* colonies which were resistant to 100 μg/ml chloramphenicol were selected and inoculated into LB medium containing 200, 400, 600, 700, 900 μg/ml chloramphenicol. Cell growth was monitored by measuring OD_{600} of cultures.

Several thousand transformants, resistant to 30 μg/ml chloramphenicol, were obtained. The number of transformants resistant to chloramphenicol dramatically decreased with increased concentration of the antibiotic. Only about 500 transformants were resistant to 100 μg/ml chloramphenicol and only 36 transformants showed resistance to 500 μg/ml chloramphenicol. Cells transformed with control pKK232-8 without an inserted promoter were not resistant to chloramphenicol above a concentration of 5 μg/ml.

The 36 transformants that showed a resistance to 500 μg/ml of chloramphenicol were further exposed to 500 μg, 700 μg, and 900 μg/ml chloramphenicol. Seven transformants showed normal growth in LB medium containing 700 μg/ml chloramphenicol and slower growth in the presence of 900 μg/ml chloramphenicol (Figure 2).

Example 4
Comparison of CAT Activity Induced by Chlorella Virus Promoters

The CAT activity expressed by the chloramphenicol-resistant *Chlorella* virus promoter transformants of Example 3 was compared. Four of the

seven chloramphenicol-resistant *E. coli* transformants, (CVP-10; CVP-13; CVP-15 and CVP-16) were grown in the presence of 600 μg/ml of chloramphenicol. Cell growth was monitored by measuring cell density (OD_{600}) at various times. As shown in Figure 3, the four transformants displayed similar cell growth, indicating that similar levels of CAT activity was expressed in each culture.

Example 5

Purification of plasmid DNA

Plasmid DNA was purified from those colonies showing the highest level of chloramphenicol resistance (700 to 900 μg/ml) using the Wizard miniprep plasmid DNA purification kit (Promega Co., Madison, WI) or Qiagen DNA purification kit (Qiagen Inc., Chatsworth, CA). Analysis of the plasmids by restriction endonuclease digestion indicated that all plasmids carried a DNA insert fragment.

The plasmids were digested with *Sma*I and *Hind*III and electrophoresed on 7.5% polyacrylamide gel or 0.8% agarose gel. The electrophoretic gel showed the seven viral promoter fragments ranged in size from 100 to 400 bp, as shown diagrammatically in Figure 2.

Example 6

Sequencing promoter fragments

The seven promoter-containing viral DNA fragments (*cvp*-1, *cvp*-3, *cvp*-6, *cvp*-10, *cvp*-13, *cvp*-15, and *cvp*-16) were excised from the pKK232-8 vector with *Sma*I and *Hind*III and subcloned into pUC19 or pBluescriptSK(+)II for sequencing. DNA sequencing was performed by the University of Nebraska Lincoln (UNL) sequencing lab using an automatic LICOR sequencer. sequences were determined in both directions using the Sanger dideoxy chain termination method.

Sequence analysis of all seven *Chlorella* virus fragments revealed *Sau*3A1 sites at each end of the *Chlorella* virus inserts and flanking the multiple cloning sites of the pKK232-8 vector. The size of the DNA sequence for the seven promoter fragments coincided with the size of the restriction fragments determined by polyacrylamide gel electrophoresis.

SEQ ID NO:	*Chlorella* Virus DNA Insert
1	cvp-1
2	cvp-3
3	cvp-6
4	cvp-10
5	cvp-13
6	cvp-15
7	cvp-16

Known *E. coli* promoter sequences contain 3 critical elements: two hexamer sequences (-35 region and -10 region) and a 16-18 bp spacing between these two regions. Using *lac, lac*UV5, *trp, tac* and PL promoter consensus sequences as references, -35 and -10 region putative sequences were assigned to each of the seven viral promoters. As shown below in Table I these hexamer sequences are either identical to, or only slightly different from, the consensus sequences of the known *E. coli* promoters. For example, the -35 sequence region of *cvp*-10 (TTGACA) or *cvp*-15(TTTACA) is identical to that found in *trp*(TTGACA) or *lac* (TATGTT).

Six of the seven isolated viral promoters of the invention have 16-18 bp spacing between the putative -35 and -10 regions. Both the *cvp*-13 viral promoter and the *lac* promoter have an identical -35 hexamer sequence (TTTACA), exactly the same spacing (18 bp) between the -35 and -10 regions, and a very similar -10 hexamer sequence (TACAAT for *cvp*-13 and TATAAT for *lac*).

Promoter consensus	-35 Position **TTGACA**	-10 Position **TATAAT**	+position SEQ.ID NO:
cvp-1	CAAAAACAAC**TTGATGA** ATTTGACGAAGATGGG**TATGA**TTTTTTTC**A**GGC		10
cvp-3	TCAGACGTGC**TTGATAA**TAG TGTGGAACTCGAG**TATGAT**CCG TCG**A**CCT		11
cvp-6	GCTTATCGTG**TTGATAT**GATAAATGACAAAT **GATACG**CTGTATC**AA**CA		12
cvp-10	GCCCCGGTTC**TTGACA**TTCTAAAAAAATGTGGAG**TATCCT**CGATCCGTCGA		13
cvp-13	ATTTTAATTA**TTTACA**CCGGTTGTTGCTGAGTGA**TACAAT**GCAAATTCCCT		14
cvp-15	AAAGCAATCC**TTTACA**ATAC TACAGAACGTGAC**TATATT**ATCAAAAAAGG		15
cvp-16	ACTGCCTCGT**TATAAA**GGTTAGTGAAAGCCATTG**TATGTT**ATTACTG**A**GTT		16
lac	CACCCCAGGC**TTTACA**CTTTATGCTTCCGGCTCG**TATGTT**GTG TGG**A**ATT		17
*lac*UV5	CACCCCAGGC**TTTACA**CTTTATGCTTCCGGCTCG**TATAAT**GTG TGG**A**ATT		18
trp	AAATGAGCTG**TTGACA**ATTAATCATCGAACTA G**TTAACT**AGTACGC**A**AGT		19
tac	AAATGAGCTG**TTGACA**ATTAATCAT CGGCTCG**TATAAT**GTG TGG**A**ATT		20
PL	TCTGGCGGTG**TTGACA**TAAATACCACT GGGGGT**GATACT**GAG CAC**A**TCA		21

"A" in bold type indicates the start site of transcription. Abbreviations: *lac*, the *lac* promoter; *lac*UV5, the *lac*UV5 promoter; *trp*, the *trp* promoter; *tac*, the *tac* promoter; *PL*, bacteriophage lamda *PL* promoter.

Example 7

Comparing promoter activity of *cvp*-13 and *tac*.

The *tac* promoter is a very strong *E. coli* promoter (de Boer, et al., 1983, *Proc. Natl. Acad. Sci.* 80:21-25) that has been widely used for gene expression in both research and industry. To compare promoter activity of viral promoter *cvp*-13 and promoter activity of *tac* in the same assay system, a promoter plasmid of *tac* was constructed using pKK232-8.

Two complementary oligomers containing the *tac* sequence,

5'-GGGAAATGAGCTGTTGACAATTAATCATGGCTCGTATAATGTGTGGAAGCTT-3'

[SEQ ID NO: 8]

and

5'-CCCTTTACTCGACAACTGTTAATTAGTAGCCGAGCATATTACACACCTTCG-3'

[SEQ ID NO: 9]

were annealed and inserted in pKK232-8 upstream of the CAT gene, between *Sma*I and *Hind*III. The resulting plasmid, pTAC-cat, was transformed into *E. coli* HB101. Cells containing either pTAC-cat or pCVP-13 were grown in the presence of 100, 300 or 600 μg/ml of chloramphenicol. Antibiotic resistance was monitored by measuring cell growth densities at various times. As shown in Figure 4, at all three levels of chloramphenicol concentration, the *cvp*-promoter showed higher promoter activity than the *tac* promoter.

Example 8

Construction of Regulated Viral Promoters

The 7 viral promoter fragments isolated from *Chlorella* viral genomes showed strong promoter activity in *E. coli*. However, their promoter activity was not regulated and, thus, could only constitutively direct protein expression. An approach for converting a constitutive promoter to an inducible promoter by fusing the promoter with the *lac* operator has been developed for a few promoters such as ribosomal RNA gene promoters (Brosius and Holy, 1984, *PNAS USA* 81:6929-6933) and the lipoprotein gene promoter (Nakamura, et al., 1982, *EMBO J.* 1:771-775). These modified hybrid promoters are repressed by the *lac* I gene product, *lac* repressor, whose specific binding to *lac* operator inhibits the start of transcription and is derepressed by an inducer such as isopropyl b- D-thiogalactoside (IPTG), which prevents the repressor protein from binding to the *lac* operator.

The *lac* operator was fused to the *Chlorella* viral promoters cvp-1, cvp-15 and cvp-16 to assess if the *Chlorella* viral promoters might be made inducible. Two complementary oligonucleotides containing the *lac* operator

sequence and suitable restriction cloning sites were chemically synthesized by Operon Technology Inc. (Alameda, CA). The sequence of these oligos shown below:

5'-TCGACCGGGTACCTCGCGAGGAATTGTGAGCGGATAACAATTCCTCTAGA-3'
[SEQ ID NO: 22]

5'-AGCTTCTAGAGGAATTGTTATCCGCTCACAATTCCTCGCGAGGTACCCGG-3'
[SEQ ID NO: 23]

After denaturation and annealing the resulting oligo duplex was inserted downstream of the viral promoters at SAII and HindIII sites on pKK232-8. The pKK232-8 clones containing a viral promoter/operator hybrid upstream of the CAT gene was transformed into *E. coli* HB101, which lacks *lac*I function. Similar levels of promoter activity as that observed for a viral promoter lacking the *lac* operator was detected by measuring resistance of the trnasformed cells to chloramphenicol. This suggested the manipulation of the viral promoters by fusing with *lac* operator did not cause reduction of promoter strength. The *lac* operator-fused promoters cvp-1, cvp-15 and cvp-16 were designed YX-1, YX-15 and YX-16, respectively.

Example 9

Expression of Glucagon-like Peptide (GLP-1) from a YX-15 Expression Vector

A. Construction of a YX-15 expression vector, pBN115-glp

To subclone the viral promoters (YX-1, YX-15 and YX-16) into an expression vector (which required restriction sites Bg1II and XbaI for cloning), the promoter fragments were modified by the polymerase chain reaction (PCR) using the following oligos synthesized by the Operon Technology Inc.:

5'-GGGTTCGAAGATCTAATTCCCGGGGATC-3' [SEQ ID NO: 24] and

5'-TGCATTTCTAGAATTGTGAATTGTTATCCGCTCA-3' [SEQ ID NO: 25].

Plasmid pGEX-2T was purchased from Pharmacia Biotech Inc. (piscataway, NJ), which carries the pBR322 replication origin, *lac*Iq gene, ampicillin resistance gene, and a *tac* promoter/glutathione S-transferase (GST) fusion gene expression cassette. The *tac*/GST cassette on pGEX-2T was first deleted by digested with FspI and SmaI to remove the 1.2 kb FspI-SmaI fragment and then replaced with a DNA fragment containing BglII/XbaI/NheI/XhoI sites, which can be used as cloning sites. The resulting plasmid was designed pBN115.

GLP-1 is a 29 residue hormone peptide. A synthetic 8 copy GLP-1 gene was constructed and expressed in *E. coli*. To test GLP-1 expression under the control of YX-15, a YX-15/GLP-1 expression cassette was constructed by fusing a BglII-XbaI fragment containing YX-15 to an XbaI-XhoI fragment containing a ribosome binding site and the 8 copy GLP-1 gene. The YX-15/GLP-1 expression cassette was then inserted into pBN115 at the unique BglII and XhoI sites to generate pBN115-glp (Fig. 5). As a control expression vector, pBN115-glp/tac was constructed by replacement of YX-15 at BglII and XbaI sites on pBN115-glp with the *tac* promoter containing *lac* operator. The *tac* promoter can also be tightly regulated by IPTG.

B. **Expression of GLP-1**

Both p'BN115-glp and pBN115-glp/tac were transformed into *E. coli* host strain HMS174 (Novagen, Inc., Milwaukee, WI) in the presence of ampicillin. Expression of GLP-1 was induced with 0.1 to 2 mM IPTG at 37° C to 21° C. Cells were harvested 15 hours post induction. Total cellular protein was prepared for SDS-PAGE analysis (Fig. 6). Production of GLP-1 was evaluated by gel scanning using the Personal Color Scanner and Adobe photoshop and NIH Image Analysis computer programs. As shown in Table 1, gel scanning revealed that expression level of GLP-1 from pBN115-glp at 37° C was more than 46% of total cellular protein compared to 35% of total cellular protein from pBN115-glp/tac. One O.D.$_{600}$ of cells produced 40% more GLP-1 from pBN115-glp than from pBN115-glp/tac. The results clearly demonstrate the potential application of YX-15 as a very strong promoter for high-level recombinant protein production in *E. coli*.

Table 1

YX-15 - Directed GLP-1 Expression in *E. coli*

Induction	Reading of the intensity of the GLP-1 protein band	
	tac	YX-15
Uninduced	84 (7%)	385 (35%)
37° C	1089 (100%)	1544 (141%)
27° C	55 (5%)	1248 (114%)
21° C	ND	830 (76%)

For each treatment, total protein was extracted from 0.25 $O.D._{600}$ of cells and analyzed on a 16% PAGE gel (Fig. 6). After staining with Coomassie blue, intensity of protein bands were scanned using Personal Color Scanner and Adobe Photoshop and NIH Image Analysis computer programs. Percentage in parenthesis represents an expression level of GLP-1 relative to the expression level of GLP-1 driven by the *tac* promoter at 37° C.

C. **Induction by IPTG**

Although a basal expression level of GLP-1 in HMS174 harboring pBN115-glp was detectable in the absence of IPTG, GLP-1 expression level showed 4-fold increase after addition of IPTG. In contrast, IPTG induced 13-fold increase in GLP-1 production from pBN115-glp/tac. This suggested that GLP-1 expression from pBN115-glp does respond to IPTG induction. Regulation of GLP-1 expression from pBN115-glp, however, was less tight than that from pBN115-glp/tac.

Inducibility of IPTG at various concentrations (0,0.25, 0.5, 1.0 and 2.0 mM) were tested for GLP-1 expression at 37° C. A similar high level of GLP-1 production was observed from pBN115-glp within the IPTG concentration range of 2 mM and 0.1 mM. This suggested a concentration of IPTG as low as 0.1 mM was sufficient to induce GLP-1 overexpression.

At all tested IPTG concentrations, GLP-1 production from pBN115-glp was consistently higher than GLP-1 production from pBN115-glp/tac, suggesting higher promoter activity from YX-15 compared to *tac*.

D. **Effect of Temperature**

Cultivation temperature critically affects microbe performance. An induction temperature of 37° C is commonly used for satisfactory recombinant protein production in *E. coli*, whereas lower temperatures, in some cases, show the advantage for producing active proteins and avoiding the formation of inactive protein aggregates. However, at lower temperatures, the rates of transcription and translation, being biochemical reactions, will be greatly slowed down and, thus, the final yield of the target protein will be reduced. Therefore, the use of a promoter highly active at lower temperatures will be necessary for high-level protein production. Prior to the instant invention, such an *E. coli* promoter was not available. Commonly used promoters such as the tac promoter and the T7 promoter are very active at 37° C, but much weaker at lower temperatures, as described below by the data for the *tac* promoter.

The promoter activity of YX-15 at the temperature range of 37°C and 21°C was compared in controlling GLP-1 production. The results shown in Table 1 (above) confirm that the YX-15 promoter was able to drive a high level of GLP-1 expression at the temperature range from 37°C to 21°C. Expression levels using the YX-15 promoter at 27°C were even slightly higher than with the *tac* promoter at 37°C. The reduction in the GLP-1 expression level driven by YX-15 at 27°C and at 21°C as compared with at 37°C was 20% and 50%, respectively. In contrast, a 95% drop in expression using the *tac* was observed at 27°C as compared with at 37°C. Protein expression of the *tac* promoter at 21°C was not detected.

Example 10

Function of the Viral Promoters in Plants

To test if the isolated viral promoters function in plants, the 7 promoter fragments were inserted into the β-glucuronidase (GUS) expression vector pBI221 (Clotech Laboratories, Inc., Palo Alto, CA) upstream of the GUS gene to replace CaMV 35S promoter. The resulting plasmids were introduced into tobacco

leaves and wheat immature embryos by the method of microprojectile bombardment. The transformed explants were then assayed for GUS expression histochemically by counting the formation of blue GUS foci on the explants tested. Positive control explants were transformed with pBI221, which contains the CaMV 35S promoter.

As shown in Table 2, positive results from the GUS transient assay were observed with the promoter cvp-15, but not for the other 6 promoters.

Table 2

Vector	Range of Mean GUS Foci/Explant/Plat*	
	Tobacco	Wheat
pBI221(CaMV 35S)	2.6 - 9.5	7.6 - 17.2
pBI221-YX15	0.7 - 1.5	0.4 - 1.6

* n=3 experiments

Example 11

Low Temperature Expression of GLP Constructs with pYX16

The expression plasmid pBN115-glp (8 copy GLP-1) described above for Example 9 drives expression of the 8 copy GLP-1(7-36) expression cassette using the YX15 promoter (cvp-15 plus *lac* operator). A second plasmid containing the YX16 promoter (cvp-16 plus *lac* operator) and 3 copy GLP-1(7-36) was constructed by replacement of the Bgl II/Xbal promoter fragment in the pBN115 vector with that fragment from the YX-16 promoter. The 8 copy GLP-1 cassette was also replaced with the 3 copy GLP-1 cassette by substitution of the XbaI-XhoI fragment. This construct, pGEXlnk2 (3copy GLP)YX16 contains the same plasmid backbone as pBN115, except that the promoter is YX16 rather than YX15.

A YX16 promoter construct driving 8 copy GLP-1(7-36) expression was also made, using the pBN53 expression plasmid. The YX16 promoter was cloned into this construct as a BglII/Xbal fragment. The resulting plasmid, pBN53lnk28copyGLP YX16, is tetracycline resistant and contains the lacIq gene to

repress uninduced expression. The pBN95 plasmid is identical to the pBN53 plasmid, except that it contains the lac I gene, rather than the lacIq gene. This change results in a lower level of expression of the Lac I gene product in pBN95, and consequently, a lower level of repression of uninduced protein levels using this vector.

The expression vectors were transformed into *E.coli* cells using the methods and materials described above for Example 9. Expression of GLP-1 was induced with IPTG as described for Example 9, at temperatues of 27° C and 37°C.

Protein samples taken from cell cultures after induction of tac or YX16 promoter controlled 3 copy GLP-1(7-36) and 8 copy GLP-1(7-36) were separated on a 16% TrisTricine SDS-PAGE gel and stained with Coomassie blue.

The data, shown in Figure 7, demonstrates that the YX16 promotor functions well at 27°C, while the tac promoter is inactive at this temperature. The GLP precursor peptide is soluble at 27°C, but insoluble at 37°C.

The data, shown in Figure 8, demonstrates that both the YX15 and YX16 promoters function at 27°C, while the tac promoter is inactive at this temperature. The data demonstrates that YX16 promoter functions at low temperatures (27°C), with two indepenant target peptides.

The above specification, examples and data provide a complete description of the manufacture and use of the composition of the invention. Since many embodiments of the invention can be made without departing from the spirit and scope of the invention, the invention resides in the claims hereinafter appended.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: BIONEBRASKA, INC.

(ii) TITLE OF THE INVENTION: CHLORELLA VIRUS PROMOTERS

(iii) NUMBER OF SEQUENCES: 25

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(E) COUNTRY: USA
(F) ZIP: 55402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: (new filing)
(B) FILING DATE: 19-MAR-1998
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: 08/821,559
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(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 358 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCCGGGGATC GCAGGGCATG GGCATTAAAA GAACTTTATG GAATCAAAAA TCTTAGTGAA    60
TTTCCACCAC AGGTATATAG TCTTCAGGAC GCTAACGATG ATATCAACGA TTGTATCAAA   120
GGTTATCGTT TGAGGCACTC ATATCAGGTA GTTTCTACAC AGAAACTTGA ACAACGCCTG   180
GGAAAAGATC CTGAGCATAG TAACTTATAT ACTAGCAGAT GTTGTAACGA TGCTTTATAT   240
GAATATGAAT TAGCACAACG ACAACTACAA AAACAACTTG ATGAATTTGA CGAAGATGGG   300
TATGATTTTT TTCAGGCACG TATAAATACA TTAGATCCGT CGACCTGCAG CCAAGCTT     358
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 374 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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TGTGCAGCGG TTGCTGATCG CATTGAATCC ATCAATAAAC AGATTGGGGT AAATATCAAA   120
CCCAGTTAGT CGGACATTAG AAGGATTTGT GAGACCACCA CATCCAACGA CACCTAATGG   180
TGTTGTGAAT GATATATTAG AAATGTTACT TATCATTGAT ATTTGCATAA CACCATTTCC   240
CTTTGCTTGA TTTCTACCTA TACTAATTGA TTGTATTGTA GTGCACGCGG CGTACTTACT   300
TGTATTTGCC GTCTCAGACG TGCTTGATAA TAGTGTGGAA CTCGAGTATG ATCCGTCGAC   360
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 207 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 317 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCGGGGATC GTTTCTCAGG GCGTCCGGGA GCATATTTCA GACTTGTCCA GCCGTATGAG 60
CATCACGTGC GCGTTCCTAG CAAGAGCGTG TACGTATATT CTTTCGCTCT AGAAGATGCA 120
GATTCGAGAC AACCGAATGG ATCGAATCTA TTTGTACCCC GATATATATA GAATCTAGTC 180
TAAACAAAAC GACCGCGGCT CTTGCCAATA AATGTGACGC AATTAACGCA TTCGTGAATG 240
ATGACTTGTC CGCCCCGGTT CTTGACATTC TAAAAAAATG TGGAGTATCC TCGATCCGTC 300
GACCTGCAGC CAAGCTT 317

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 277 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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TAATTATTTA CACCGGTTGT TGCTGAGTGA TACAATGCAA ATTCCCTCCA CCGAGGAGGA 180
TCGCGAACTG TCCAAATGTC TTCTTTCTGC AGCTCCATAC GGAGTCGTTA GGAAACATTC 240
ACTTAATTAT AGGATCCGTC GACCTGCAGC CAAGCTT 277

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCGGGGATC AGGCCTCGCT TATAAATATG GTATTGATGT ACTTGCCGGT GTGATTGACT 60
CAGATTACAG AGGAGAGTTG AAAGCAATCC TTTACAATAC TACAGAACGT GACTATATTA 120
TCAAAAAAGG CGATCAGCCA AGCTTCGTCG ACCTGCGATC CGTCGACCTG CAGCCAAGCT 180
T 181

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 316 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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TGTGATCATG TGGCAACAAG ATGGAGGTGT CATAGACTAC ATTTACCCTC CCTCTGATCA 120
TCGAAAGCAA CTGCCGCATT CGAAACTTCG ACTGCCTCGT TATAAAGGTT AGTGAAAGCC 180
ATTGTATGTT ATTACTGAGT TATTTAATTT AGCTTGCTTA AATGCTTATC GTGTTGATAT 240
GATAAATGAC AAATGATACG CTGTATCAAC ATCTCAAAAG ATTAATACGA AGATCCGTCG 300
ACCTGCAGCC AAGCTT 316

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGAAATGAG CTGTTGACAA TTAATCATGG CTCGTATAAT GTGTGGAAGC TT 52

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCTTTACTC GACAACTGTT AATTAGTAGC CGAGCATATT ACACACCTTC G 51

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAAAAACAAC TTGATGATTT GACGAAGATG GGTATGATTT TTTTCAGGC 49

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCAGACGTGC TTGATAATAG TGTGGAACTC GAGTATGATC CGTCGACCT 49

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCTTATCGTG TTGATATGAT AAATGACAAA TGATACGCTG TATCAACA 48

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCCCCGGTTC TTGACATTCT AAAAAAATGT GGAGTATCCT CGATCCGTCG A 51

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATTTTAATTA TTTACACCGG TTGTTGCTGA GTGATACAAT GCAAATTCCC T 51

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAAGCAATCC TTTACAATAC TACAGAACGT GACTATATTA TCAAAAAAGG 50

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ACTGCCTCGT TATAAAGGTT AGTGAAAGCC ATTGTATGTT ATTACTGAGT T 51

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CACCCCAGGC TTTACACTTT ATGCTTCCGG CTCGTATGTT GTGTGGAATT 50

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CACCCCAGGC TTTACACTTT ATGCTTCCGG CTCGTATAAT GTGTGGAATT 50

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAATGAGCTG TTGACAATTA ATCATCGAAC TAGTTAACTA GTACGCAAGT 50

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AAATGAGCTG TTGACAATTA ATCATCGGCT CGTATAATGT GTGGAATT 48

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCTGGCGGTG TTGACATAAA TACCACTGGG GGTGATACTG AGCACATCA 49

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TCGACCGGGT ACCTCGCGAG GAATTGTGAG CGGATAACAA TTCCTCTAGA 50

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AGCTTCTAGA GGAATTGTTA TCCGCTCACA ATTCCTCGCG AGGTACCCGG 50

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGGTTCGAAG ATCTAATTCC CGGGGATC 28

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TGCATTTCTA GAATTGTGAA TTGTTATCCG CTCA 34

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An isolated DNA molecule comprising a polynucleotide having the sequence of SEQ ID NO:1.

2. An isolated DNA molecule comprising a polynucleotide having the sequence of SEQ ID NO:2.

3. An isolated DNA molecule comprising a polynucleotide having the sequence of SEQ ID NO:3.

4. An isolated DNA molecule comprising a polynucleotide having the sequence of SEQ ID NO:4.

5. An isolated DNA molecule comprising a polynucleotide having the sequence of SEQ ID NO:5.

6. An isolated DNA molecule comprising a polynucleotide having the sequence of SEQ ID NO:6.

7. An isolated DNA molecule comprising a polynucleotide having the sequence of SEQ ID NO:7.

8. A gene construct comprising a promoter operably linked to a heterologous structural gene, said promoter comprising the nucleotide sequence set forth as SEQ ID NO: 1, 2, 3, 4, 5, 6, or 7.

9. The gene construct of claim 8, wherein said structural gene encodes carbonic anhydrase.

10. The gene construct of claim 9, wherein said carbonic anhydrase is human carbonic anhydrase.

11. The gene construct of claim 8, wherein said structural gene encodes glucagon-like peptide-1, growth hormone releasing factor, or parathyroid hormone.

12. A host cell comprising a gene construct comprising a promoter operably linked to a structural gene, said promoter comprising the nucleotide sequence of SEQ ID NO:1, 2, 3, 4, 5, 6, or 7.

13. The host cell of claim 12, wherein said host cell is a prokaryotic cell.

14. The host cell of claim 13, wherein said prokaryotic cell is an *E. coli* cell.

15. The host cell of claim 14, wherein said *E. coli* cell is selected from the group consisting of HB101, JM109, BL21 and HMS174.

16. A process for producing a protein in a host cell, comprising:

(A) transforming a host cell with a gene construct comprising a promoter operably linked to a structural gene, said promoter comprising a nucleic acid sequence set forth as SEQ ID NO: 1 ,2, 3, 4, 5, 6 or 7; and

(B) culturing the host cell under conditions whereby the protein is expressed.

17. The process of claim 16, further comprising isolating the expressed protein from the host cell.

18. A process for producing a protein, comprising incubating a host cell under conditions whereby the protein is expressed, wherein said host cell comprises a gene construct comprising a promoter operably linked to a heterologous nucleic acid sequence encoding the protein, said promoter comprising a nucleic acid sequence as set forth as SEQ ID NO: 1, 2, 3, 4, 5, 6, or 7.

19. The process of claim 18, further comprising isolating the expressed protein from the host cell.

20. An isolated, inducible promoter comprising the nucleic acid sequence set forth as SEQ ID NO: 1, 2, 3, 4, 5, 6, or 7.

21. An isolated, inducible promoter comprising the nucleic acid sequence set forth as SEQ ID NO: 1, 2, 3, 4, 5, 6, or 7, operatively linked to a *lac* operator.

22. A gene construct comprising:

a promoter comprising the nucleic acid sequence set forth as SEQ ID NO: 1, 2, 3, 4, 5, 6, or 7;

a *lac* operator; and

a structural gene,

wherein the promoter, operator, and structural gene are operatively linked.

23. A gene construct comprising:

a promoter comprising the nucleic acid sequence set forth as SEQ ID NO:15;

a *lac* operator; and

a structural gene,

wherein the promoter, operator, and structural gene are operatively linked.

24. The gene construct of claim 22, wherein the structural gene comprises a nucleic acid sequence encoding glucagon-like peptide 1, growth hormone releasing factor, parathyroid hormone, carbonic anhydrase, beta-galactosidase, chloramphenicol acetyltransferase, or glutathione acetyltransferase.

25. A host cell comprising the gene construct of claim 22.

26. The host cell of claim 25, wherein the host cell is a prokaryotic cell.

27. The host cell of claim 26, wherein the prokaryotic cell is an *E.coli* cell.

28. The host cell of claim 25, wherein the host cell is a plant cell.

29. A plant comprising the gene construct of claim 22.

30. A process for producing a protein in a host cell, comprising:

(A) transforming a host cell with a gene construct comprising a promoter comprising the nucleic acid sequence set forth as SEQ ID NO: 1, 2, 3, 4, 5, 6, or 7, a *lac* operator, and a structural gene,

wherein the promoter, operator and structural gene are operatively linked; and

(B) culturing the host cell under conditions whereby the protein is expressed.

31. The process of claim 30, wherein said transformed host cell is incubated at less than 37°C.

32. The process of claim 30, wherein said transformed host cell is incubated at a temperature in the range of 21 to 37°C.

33. The process of claim 30, wherein said transformed host cell is incubated at a temperature in the range of 21 to 30°C.

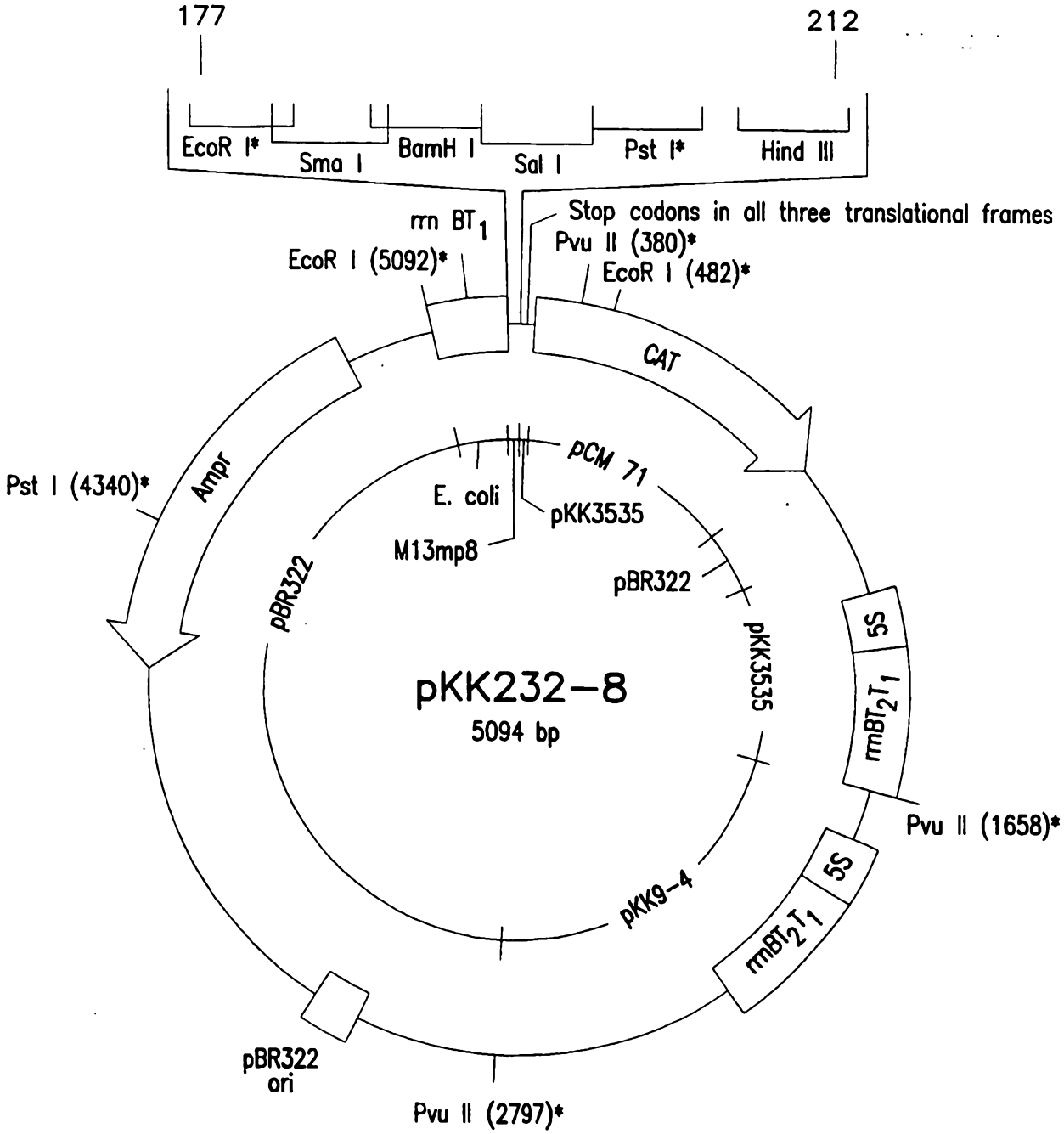
34. The inducible promoter of claim 21, wherein said promoter operates in a range of 21 to 37°C.

35. The inducible promoter of claim 21, wherein said promoter operates at a temperature below 37°C.

DATED: 3 October, 2001
PHILLIPS ORMONDE & FITZPATRICK
Attorneys for:

BIONEBRASKA, INC

FIG. 1



*Site not unique

FIG. 2

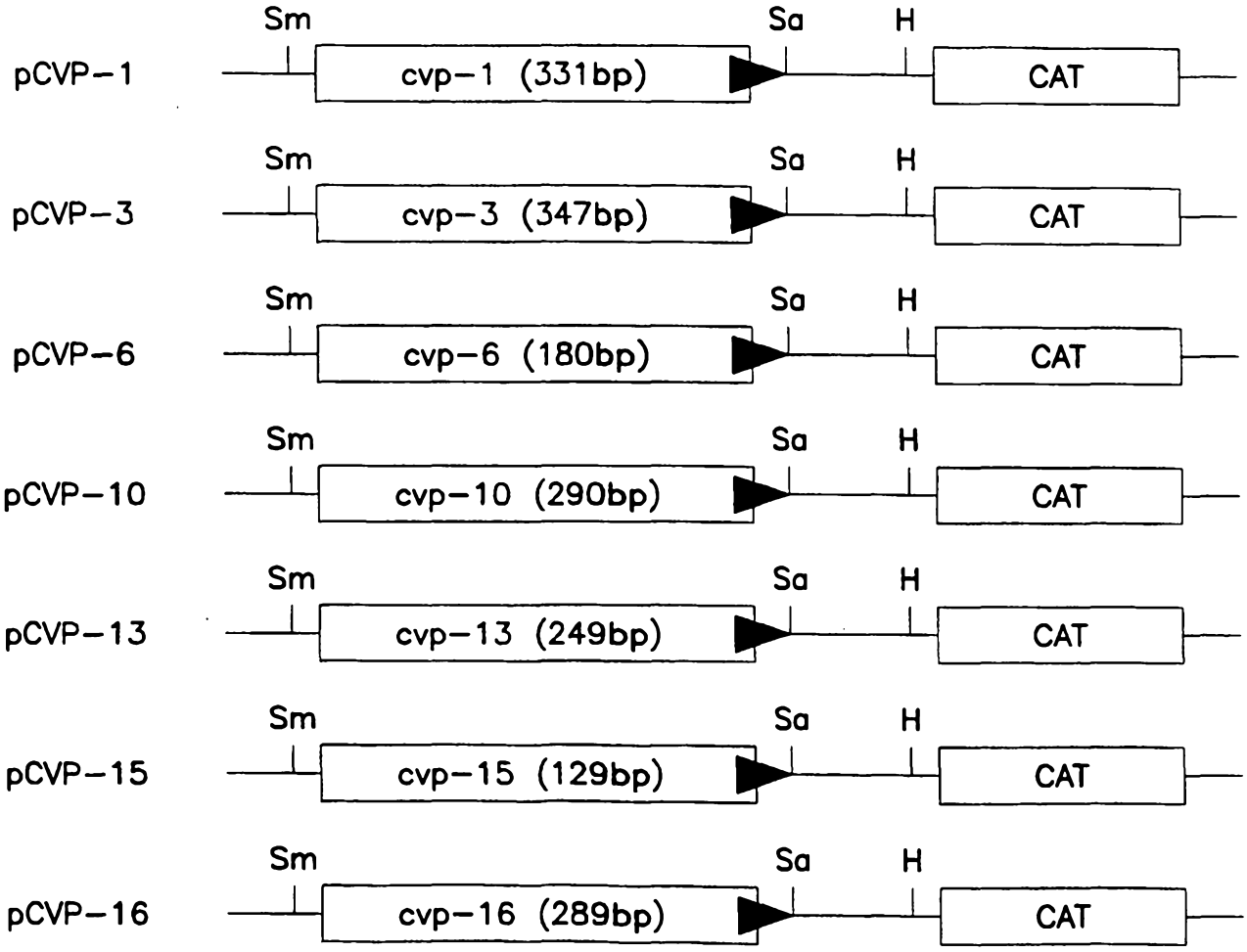
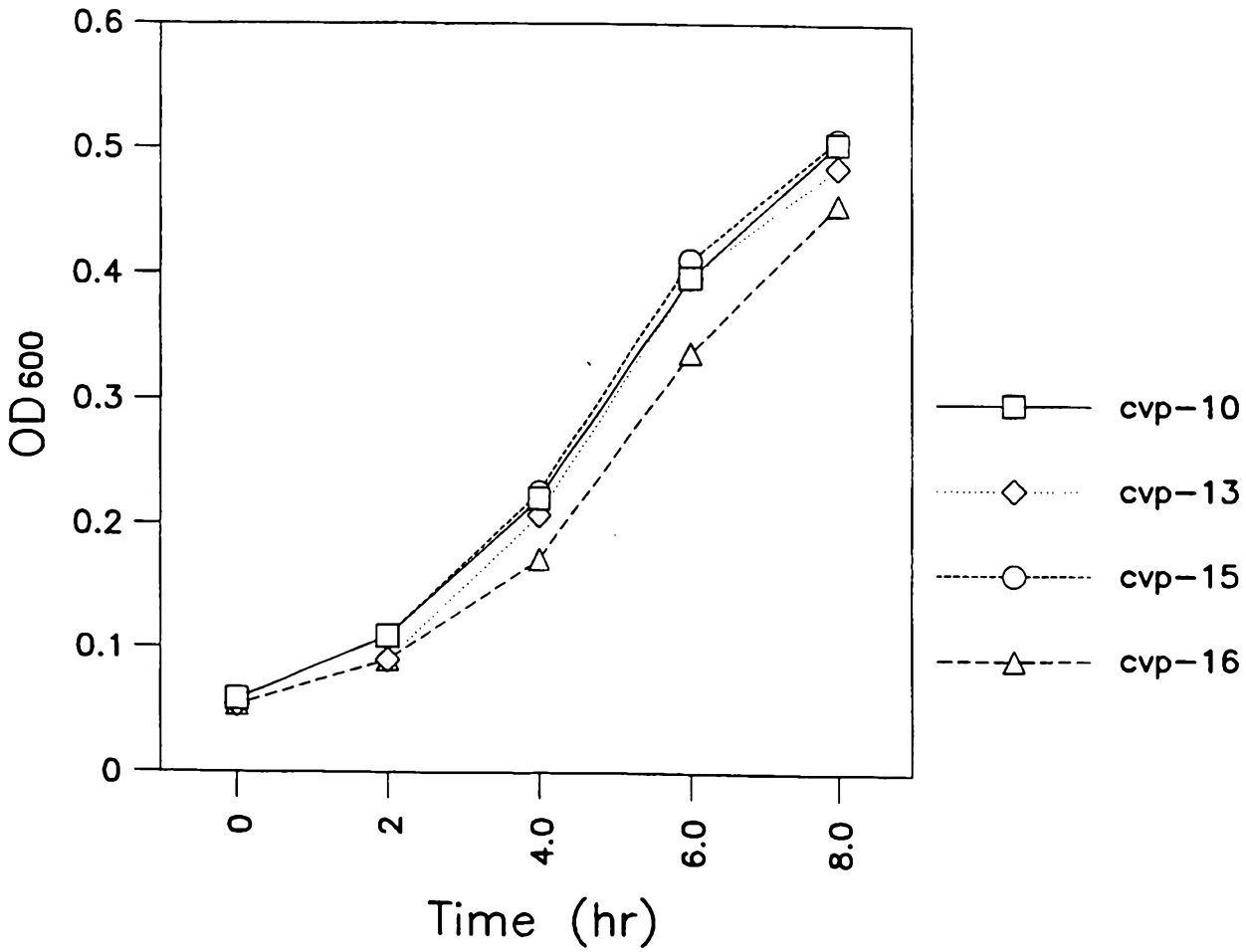

Sm
Sa
H
pCVP-1
cvp-1 (331bp)
CAT
pCVP-3
cvp-3 (347bp)
pCVP-6
cvp-6 (180bp)
pCVP-10
cvp-10 (290bp)
pCVP-13
cvp-13 (249bp)
pCVP-15
cvp-15 (129bp)
pCVP-16
cvp-16 (289bp)

FIG. 3


0.6
0.5
0.4
0.3
0.2
0.1
0
OD600
0
2
4.0
6.0
8.0
Time (hr)
cvp-10
cvp-13
cvp-15
cvp-16

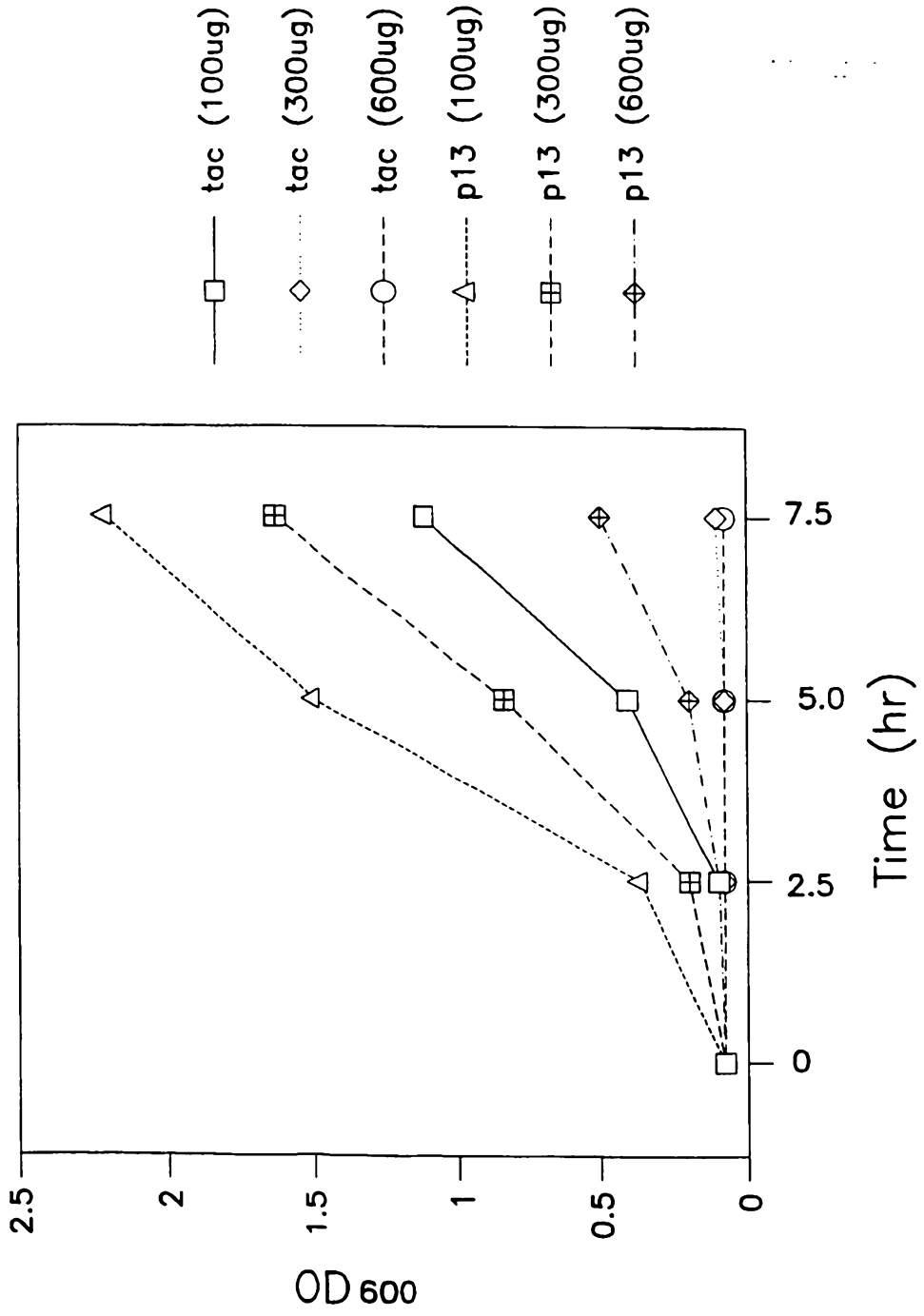

tac (100ug)
tac (300ug)
tac (600ug)
p13 (100ug)
p13 (300ug)
p13 (600ug)
2.5
2
1.5
1
0.5
0
OD 600
7.5
5.0
2.5
0
Time (hr)

FIG. 4

FIG. 5

Plasmid map of the pBN115–glp expression vector. The construction of the vector plasmid is described in the text. Symdols: ori, pBR322 replication origin. 1ac Iq, lac I repression gene. Ap^R, ampicillin resistance gene. 8cGLP, 8 copy GLP–1 gene. YX–15, the YX–15 promoter.

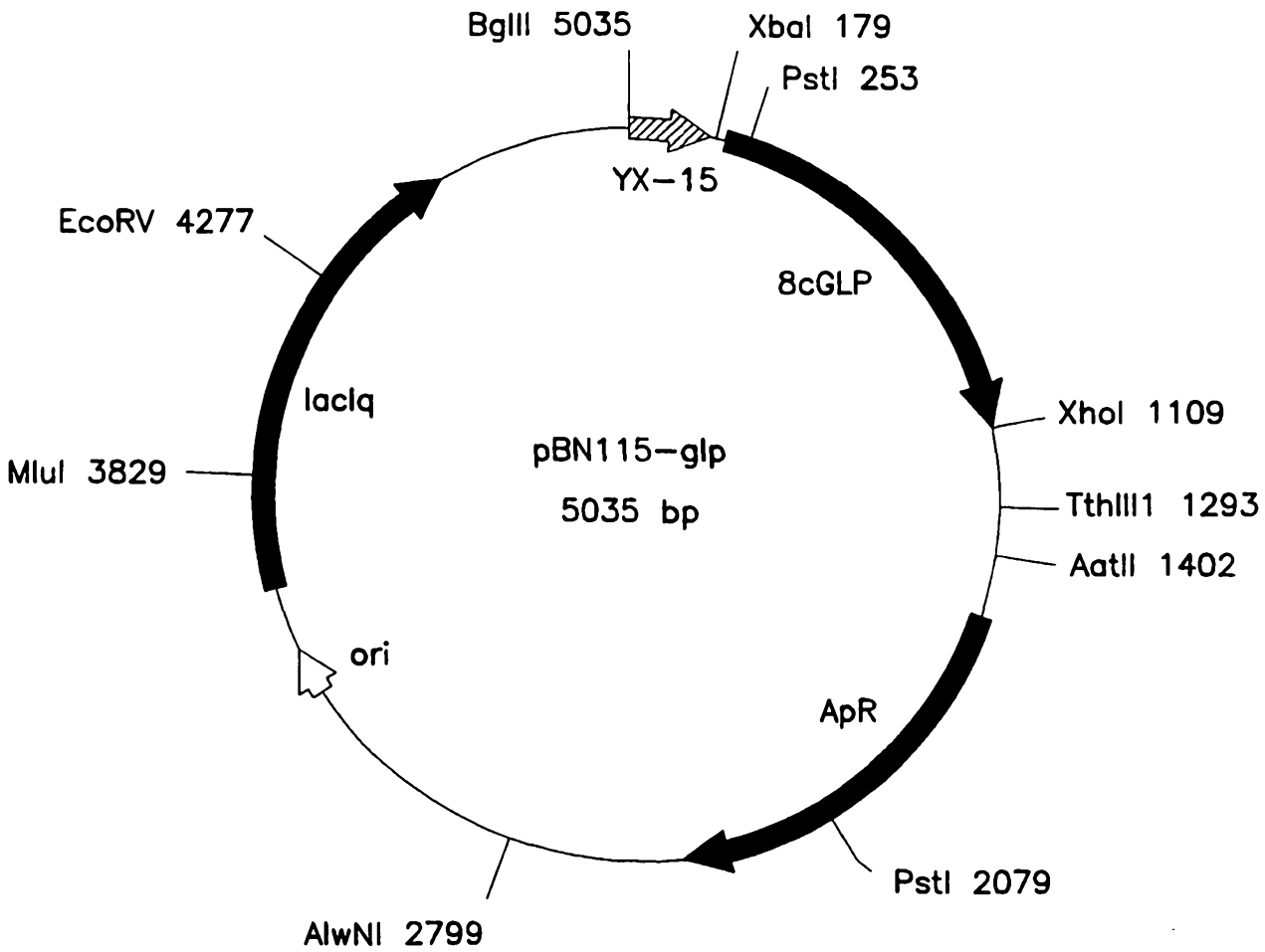


FIG. 6

Comparison of expression of GLP-1 from pBN115-glp and pBN115-glp/tac at various temperatures. E. coli HMS174 containing pBN115-glp (lanes 1-4) and pBN115-glp/tac (lanes 5-8) was grown in the absence (lanes 1 and 5) and presence (lanes 2-4 and 6-8) of 1 mM IPTG, at 37°C (lanes 2 and 6), 27°C (lane 3 and 7), or 21°C (lane 4 and 8) for 15 hours. Cells were then harvested by centrifugation and sonicated. Total cell lysates from a 0.1 OD 600 unit of cells were analyzed on a 16% SDS-polyacrylimade gel in 100 mM Tris, 100 mM Tricine, and 0.1% SDS (pH 8.3). The gel was stained with Coomassie blue. Arrow identifies the GLP-1 polyprotein bands. The relative amounts of the GLP-1 protein are summarized in Table 1. Lane M represents molecular weight standards in kilodaltons.

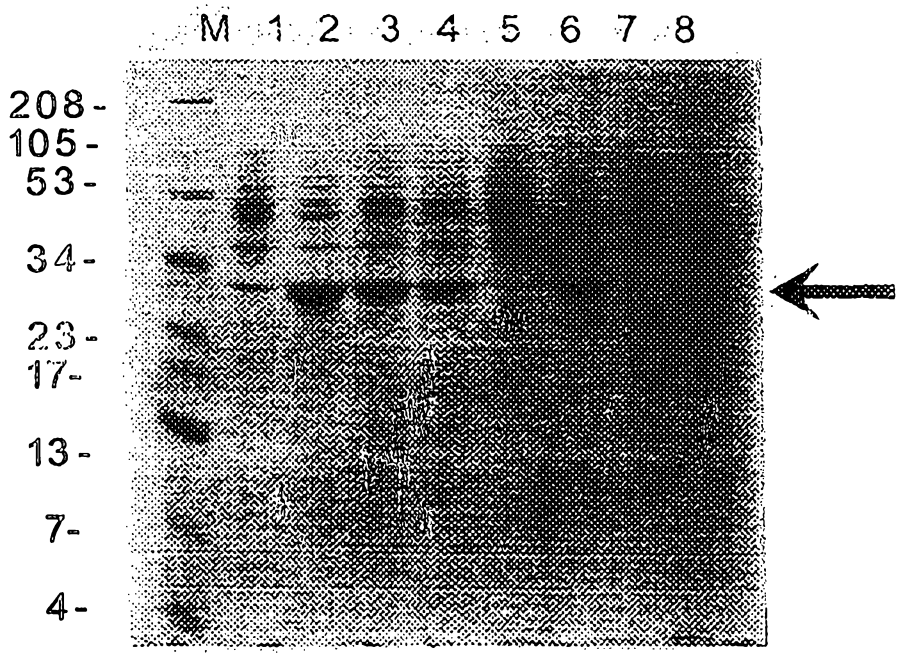


FIG. 7

Tac and YX16 expression of 3 copy GLP(7-36) protein

Lane 1: Novex MW marker
Lane 2: pGEXlnk2 (3 copy GLP) YX16 uninduced total protein, 37 C
Lane 3: pGEXlnk2 (3 copy GLP) YX16 induced total protein, 37 C
Lane 4: pGEXlnk2 (3 copy GLP) YX16 induced total protein, 27 C
Lane 5: pGEXlnk2 (3 copy GLP) YX16 induced soluble protein, 27 C
Lane 6: pBN95lnk2 (3 copy GLP) Tac @ uninduced total protein, 37 C
Lane 7: pBN95lnk2 (3 copy GLP) Tac @ induced total protein, 37 C
Lane 8: pBN95lnk2 (3 copy GLP) Tac @ induced total protein, 27 C
Lane 9: pBN95lnk2 (3 copy GLP) Tac @ induced soluble protein, 27 C

@ Full description of this plasmid is pBN95lnk2[GLP(7-36)AFAM]8HAE (tac). The fusion protein is identical in the 3 copy GLP pGEX plasmid from lanes 2-5.

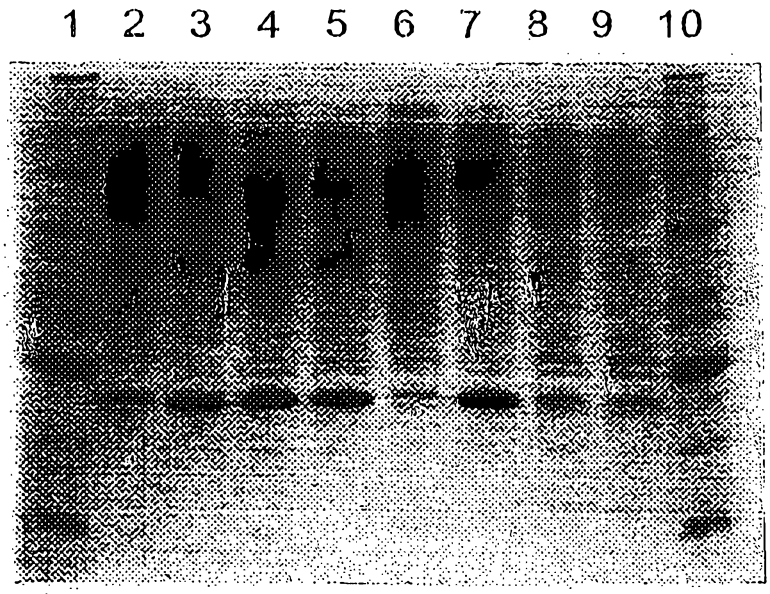


FIG. 8

Tac, YX15 and YX16 expression of 8 copy GLP(7-36) protein

Lane 1: pBN95Ink2[GLP(7-36)AFAM]8HAE (tac) Induced total protein, 37C
Lane 2: pBN95Ink2[GLP(7-36)AFAM]8HAE (tac) Induced total protein, 27C
Lane 3: pBN95Ink2[GLP(7-36)AFAM]8HAE (tac) Induced soluble protein, 27C
Lane 4: pBN53Ink2[GLP(7-36)AFAM]8HAE (YX16) Induced total protein, 37C
Lane 5: pBN53Ink2[GLP(7-36)AFAM]8HAE(YX16) Induced total protein, 27C
Lane 6: pBN53Ink2[GLP(7-36)AFAM]8HAE(YX16) Induced soluble protein, 27C
Lane 7: pBN115-glp (8 copy GLP) (YX15) Induced total protein, 37C
Lane 8: pBN115-glp (8 copy GLP) (YX15) Induced total protein, 27C
Lane 9: pBN115-glp (8 copy GLP) (YX15) Induced soluble protein, 27C
Lane 10: Novex MW marker

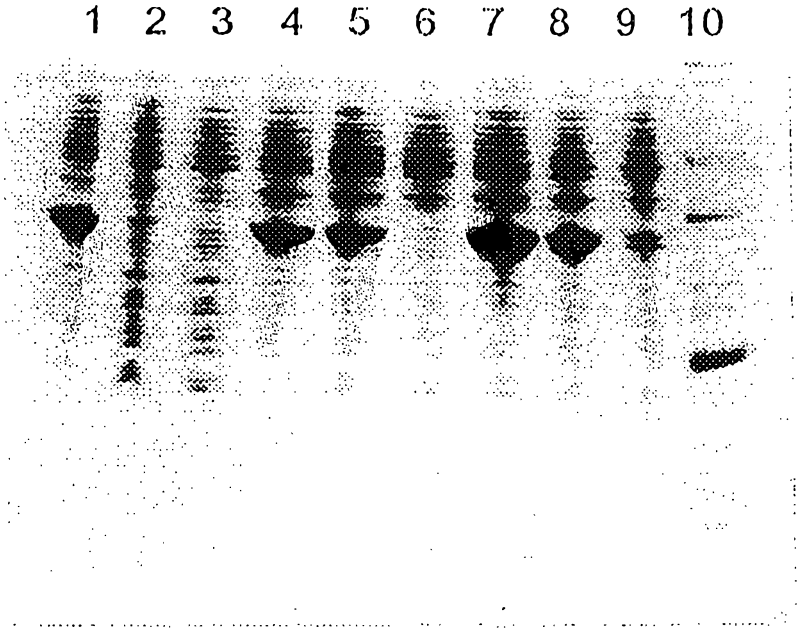
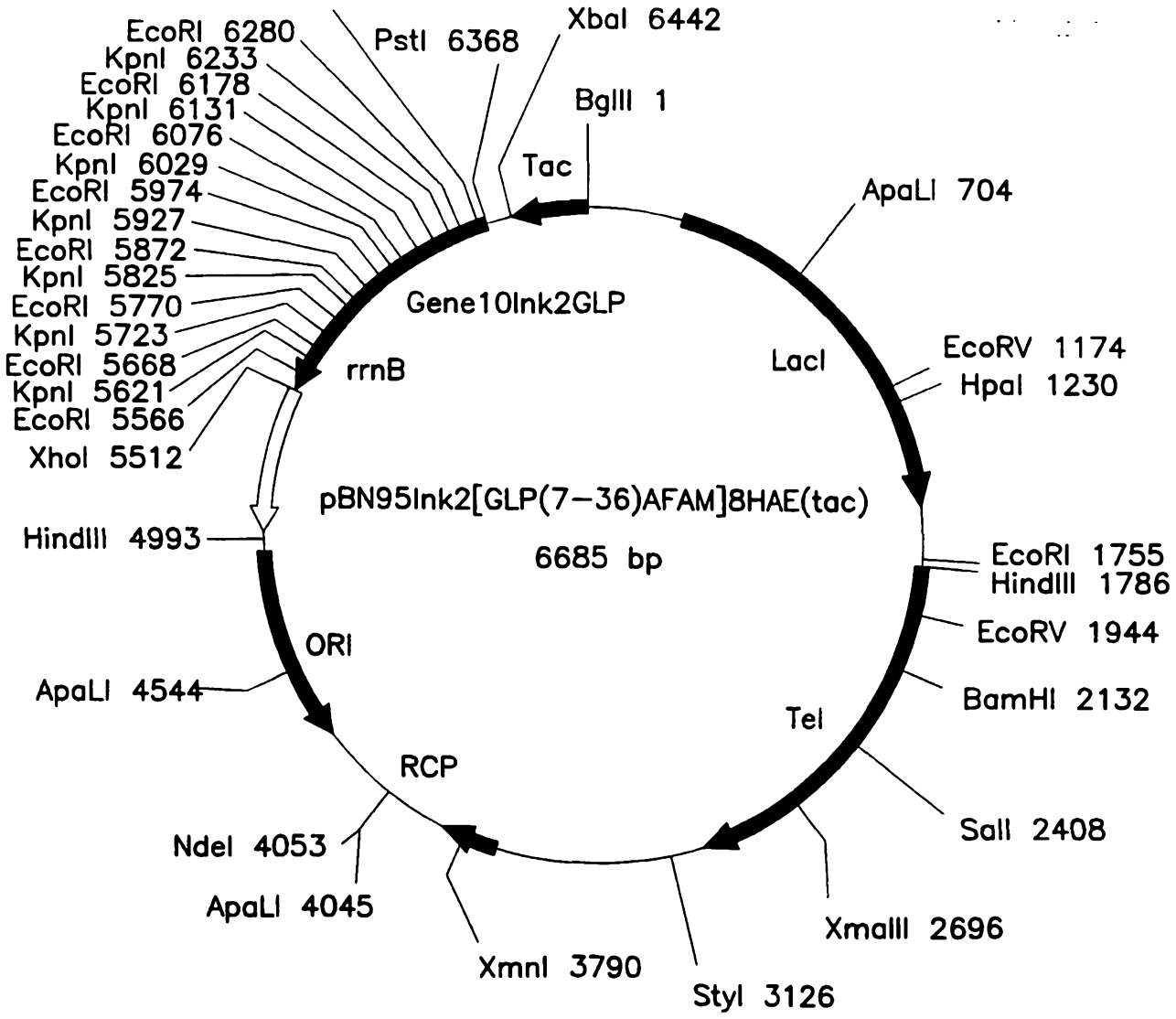


FIG. 9



Plasmid name: pBN95link2[GLP(7-36)AFAM]8HAE(tac)
Plasmid size: 6685 bp
Constructed by:
Construction date: 6/13/97
Comments/References: