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[54] **CLONED HIGH SIGNAL STRENGTH PROMOTERS**

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[58] **Field of Search** 435/29, 68, 6, 172, 435/240, 253, 254, 257, 258, 317, 91; 536/27, 28, 29

[56] **References Cited**

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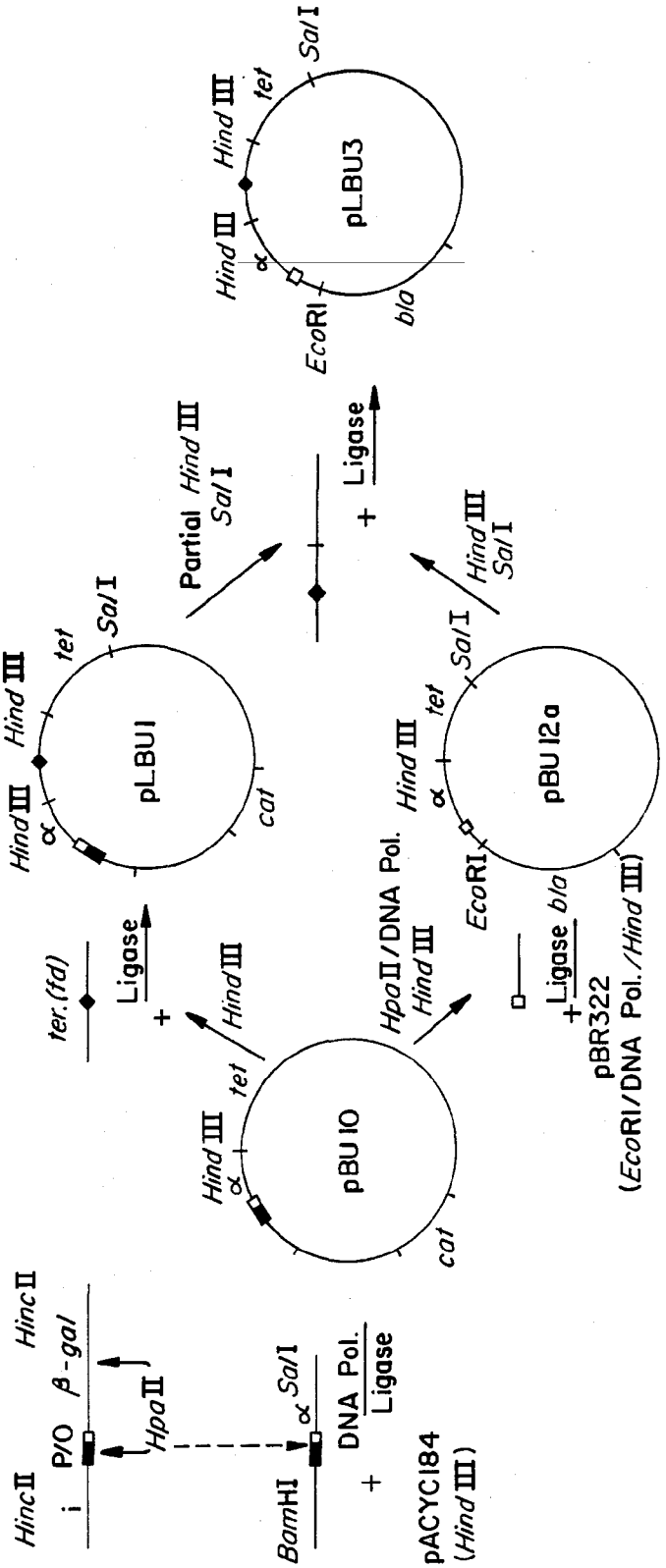
[57] **ABSTRACT**

Method for preparing high signal strength promoters and terminators and DNA compositions employing such promoters and terminators. T5 phage is cleaved to provide for DNA sequences having intact promoters. These promoters are inserted into vectors separated from a balanced terminator by a gene of interest and the terminator is desirably followed by a marker allowing for selection of transformants. High efficiencies in transcription of DNA can be achieved with the highly active T5 promoters. The promoters and terminators are used in hybrid DNA for efficient expression of structural genes and transcription to provide RNA sequences.

15 Claims, 1 Drawing Figure



CLONED HIGH SIGNAL STRENGTH PROMOTERS

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BACKGROUND OF THE INVENTION

1. Field of the Invention

Having established the feasibility of producing a wide variety of naturally occurring and synthetic polypeptides by means of hybrid DNA technology, there are continuing and extensive efforts to provide for more efficient and economic methods for producing the polypeptides. In developing a process for the commercial production of polypeptides, many factors will be involved in optimizing the economic and efficient production of the polypeptides. Included among these factors are regulatory signals, which are DNA sequences involved with the regulation of replicaton, transcription and translation.

One area of interest is at the level of transcription. Transcription involves the enzyme RNA polymerase. The RNA polymerase binds to a site called a promoter. It has been observed that promoters vary in their activity, as evidenced by the number of initiations of RNA per unit time or the strength of binding of the enzyme to the promoter site. The promoter may have one or more sequences that bind, which may or may not be contiguous. The more active promoters are referred to as strong promoters.

It was found that when introducing a strong promoter into a vector and employing the resulting plasmid for transformation, one could not select transformants based on expression of markers which allowed for selection. Therefore, cloning of the strong promoters was not feasible. It is therefore desirable that methods be provided which would allow for the screening of strong promoters and terminators and their subsequent cloning to be used in conjunction with the replication, transcription and translation of the genes for production of DNA, RNA, and polypeptides.

2. Description of the Prior Art

Promoters from bacterial and viral sources have been cloned in *E. coli*, and their signal strength in vitro has been studied using expression from distal promoterless sequences encoding β-galactosidase or other proteins (Casadaban and Cohen (1980) J. Mol. Biol 138, 179–207; West and Rodriguez (1980) Gene 9, 175–193). Attempts to clone small DNA fragments carrying the strong promoters of bacteriophage T5 have been unsuccessful (v. Gabain and Bujard (1979) PNAS USA 76, 189, 193), Fragments of T5 DNA having both a strong promoter and a strong termination signal have been cloned. (Breunig (1979) Dissertation (Universitat Heidelberg, Heidelberg, Germany)) Analysis has shown that transcriptional regions of several *E. coli* plasmids are organized in units where initiation and termination signals, are balanced. (Stuber and Bujard (1981) PNAS USA 78: 167–171) P_{25} and P_{26} promoters of the T5 bacteriophage are reported as among the most efficient RNA polymerase binding sequences. (Stuber et al (1978) Mol. gen. Genet. 166 141–149; Niemann (1981) Diplomarbeit (Universitat Heidelberg, Heidelberg, Germany)).

SUMMARY OF THE INVENTION

Methods for cloning, sequencing and using strong promoters and terminators are provided, as well as compositions resulting from the methods. By cleaving T5 phage and selecting fragments specifically binding to RNA polymerase, fragments containing promoters are isolated. A vector is constructed having a strong promoter, followed by a DNA sequence of interest, optionally followed by one or more translational stop codons in one or more reading frames, followed by a balanced terminator, followed by a marker allowing for selection of transformants. Upon introducing the resulting plasmid into a microorganism host, efficient transcription of the gene is obtained with substantially lesser expression of the marker as compared to the gene. The level of expression of the marker permits selection of transformants having the above described construct. The construct or regulatory portions thereof are used for efficient transcription of RNA or gene expression.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a flow chart of the preparation of a plasmid for cloning strong promoters and terminators.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and compositions are provided for preparing and cloning strong promoter and terminator regulatory signals and utilization of the strong regulatory sequences in the transcription and expression of genes of interest.

Particularly, T5 phage promoters are isolated, cloned in conjunction with a strong terminator, and appropriate vectors developed for insertion of DNA sequences of interest, usually structual genes, to provide for high and efficient transcription and/or expression of the sequence.

The compositions of this invention are characterized as having in the downstream direction of transcription the following units: a strong T5 phage promoter; optionally a structural gene which may be a marker; a balancing terminator; and optionally a marker allowing for selection of transformants containing the construct, which marker has a relatively low level of expression in comparison with the amount of RNA polymerase initiation at the promoter. In the absence of a promoter in the construct, the construct can be used for the cloning and characterization of promoters of different strengths.

In referring to strong promoters, it is intended that the binding affinity of RNA polymerase is stronger than the commonly employed promoters such as lac and trp and at least comparable to and normally greater than the combination of lac and trp promoters. For the most part, the strongest promoters among prokaryotes are the T5 phage promoters and these will be employed as exemplary of naturally occurring or synthetic strong promoters. It is to be understood, that other prokaryotic and eukaryotic promoters, either naturally occurring or synthetic, could find application in the subject invention.

The compositions of this invention will include linear segments for insertion of DNA having the strong regulatory signal sequences (i.e., the promoter and terminator) adjacent opposite ends of the linear segment and plasmids formed by introducing a DNA sequence from a source other than the source of the promoter as a bridge between the strong regulatory signal sequences.

The termini may be blunt or staggered ended, having the same or different termini to allow for directed positioning of inserted sequences.

The compositions which are employed as already indicated have a promoter, a balanced terminator, and desirably a marker which are in the direction of transcription when the signal sequences are joined. The terminator is balanced, so that a small but useful amount of expression of the marker can occur. Normally, in most situations, a gene will bridge the promoter and terminator to provide circular DNA. The gene may be a non-structural gene or a structural gene providing RNA e.g. ribosomal or messenger, or providing a poly(amino acid).

Desirably, the gene is followed by one or a plurality of translational stop codons e.g. oop or nonsense codons, or preferably a plurality, usually up to about six, more usually from about two to five, where there is at least one stop codon in each reading frame. The stop codons aid in the efficiency of termination, both at the level of transcription and expression.

Next in the direction of transcription is the terminator sequence. The terminator sequence is balanced with the promoter in the sense that the marker is transcribed not more than about one-fourth of the times that a gene upstream from the terminator is transcribed, where both genes are under the control of the same promoter. Usually, it will be desirable that a sufficient amount of the marker is transcribed to allow for selection of transformants. In view of the fact that the marker is transcribed from a strong promoter, it will usually be sufficient that the marker is transcribed in relation to the transcription of the gene intermediate the promoter and terminator in only about 1 to 20, usually only 5 to 15 number % of the gene. The strength of the promoter is reflected in the level of expression of the marker which is transcribed from the same promoter as the gene whose expression is desired.

In addition to the DNA sequences indicated above, there will normally be other regulatory signals necessary for expression involved with the DNA sequence, such as translational start and stop sites. In addition to the foregoing regulatory signals, other regulatory signals may be included, such as additional promoters, operators, initiators, catabolite activator protein binding sites, etc. Furthermore, the promoter and terminator may be separated by more than one gene, that is, a plurality of genes, including multimers and operons.

The above DNA sequence construct will have a replicating system or be cleaved and be inserted into a vector to provide a plasmid. The vector is distinguished by having one or more DNA sequences which serve to insure stable replication of the plasmid and may also provide opportunities for high copy numbers of the plasmid in the microorganism host. The vectors may be derived from chromosomal or extrachromosomal sources. The sources include plasmids, viruses (phage), chromosomes, or the like. In addition, the vector or the essential portions thereof may be prepared synthetically.

The plasmids may then be used for transformation of an appropriate microorganism host. Methods of introducing DNA into an appropriate host are well known. Illustrative of such methods, but not exhaustive of such methods, are transformation e.g. calcium shock, transfection, and conjugation. Descriptions of these methods may be found in Genetic Engineering, ed. Setlaw and Hollaender, Vol. 1, Plenum Press, New York and London, 1979; Molecular Cloning of Recombinant DNA, ed. Scott and Werner, Vol. 13, Academic Press, Inc. New York, 1973, and references cited therein.

In order to allow for flexibility in preparing the construct and self-replicating sequence or plasmid containing the construct restriction sites should be present to allow for unique insertions and isolation of the various elements. The restriction sites may be naturally present, introduced by linkers, result by partial sequential nucleotide removal from a chain using an exonuclease, or the like. Desirably, the restriction sites will provide for different ends to permit only the proper orientation of the inserted fragment.

A wide variety of structural genes are of interest for production of proteins, including but not limited to proteins of physiological interest, proteins as chemicals, and enzymes which may be of direct interest or of interest in transforming another product, which may be proteinaceous or non-proteinaceous. The proteins may be prepared as a single unit or as individual subunits and then joined together in appropriate ways. Furthermore, as appropriate, the protein products may be modified by glycosylation, acylation with aliphatic acids, e.g. lipid acids, phosphorolation, sulfonation or the like. The different classes of proteins which may be prepared include protamines, histones, albumins globulins, scleroproteins, phosphoproteins, mucoproteins, chromoproteins, lipoproteins, nucleoproteins, and the remaining proteins which are unclassified.

The following is a representative list of proteins of interest.

Insulin; growth hormone; interferon e.g. leukocyte, lumphoblastoid, T-immune and fibroblast; β-lipotropin; β-endorphin; dynorphin; histocompatability proteins; immunoglobulins e.g. IgA, IgD, IgE, IgG and IgM and fragments thereof; hemoglobin, somatomedins; lymphokines; growth factors e.g. epidermal, fibroblast, platelet-derived, multiplication stimulating and nerve; hematoporetic-stimulating factors e.g. erythropoietin, colony-stimulating, erythroid potentating activity or burst-promoting activity and lymphopoietins; albumin and prealbumin;
Prealbumin
Albumin
α_1-Lipoprotein
α_1-Acid glycoprotein
α_1-Antitrypsin
α_1-Glycoprotein
Transcortin
4.6S-Postalbumin
Tryptophan-poor
α_1-glycoprotein
α_1-X-Glycoprotein
Thyroxin-binding globulin
Inter-α-trypsin-inhibitor
Gc-globulin:
(Gc 1-1),
(Gc 2-1),
(Gc 2-2),
Haptoglobin:
(Hp 1-1),
(Hp 2-1),
(Hp 2-2),
Ceruloplasmin
Cholinesterase
α_2-Lipoprotein(s)
α_2-Macroglobulin
α_2-HS-Glycoprotein

Zn-α_2-glycoprotein
α_2-Neuramino-glycoprotein
Erythropoietin
β-lipoprotein
Transferrin
Hemopexin
Fibrinogen
Plasminogen
α_2-glycoprotein I
α_2-glycoprotein II
Immunoglobulin G
(IgG) or γG-globulin
Mol. formula:
$\gamma_2\kappa_2$ or $\gamma_2\lambda_2$
Immunoglobulin A (IgA) or γA-globulin
Mol. formula:
$(\alpha_2\kappa_2)^n$ or $(\alpha_2\lambda_2)^n$
Immunoglobulin M (IgM) or γM-globulin
Mol. formula:
$(\mu_2\kappa_2)^5$ or $(\mu_2\lambda_2)^5$
Immunoglobulin D (IgD) or γD-Globulin (γD)
Mol. formula:
$(\delta_2\kappa_2)$ or $(\delta_2\lambda_2)$
Immunoglobulin E (IgE) or γE-Globulin (γE)
Mol. formula:
$(\epsilon_2\kappa_2)$ or $(\epsilon_2\lambda_2)$
Free light chains
Complement factors:
C'1
C'1q
C'1r
C'1s
C'2
C'3
β_1A
α_2D
C'4
C'5
C'6
C'7
C'8
C'9.

Important protein hormones include:

Peptide and Protein Hormones

Parathyroid hormone: (parathormone)
Thyrocalcitonin
Insulin
Glucagon
Relaxin
Erythropoietin
Melanotropin: (Melanocyte-stimulating hormone; intermedin)
Somatotropin: (growth hormone)
Corticotropin: (adrenocorticotropic hormone)
Thyrotropin
Follicle-stimulating hormone
Leuteinizing hormone: (interstitial cell-simulating hormone)
Luteomammotropic hormone: (Luteotropin, prolactin)
Gonadotropin: (chorionic gonadotropin).

Tissue Hormones

Secretin
Gastrin
Angiotensin I and II
Bradykinin
Human placental lactogen

Peptide Hormones from the Neurohypophysis

Oxytocin
Vasopressin
Releasing factors (RF): CRF, LRF, TRF, Somatotropin-RF, GRF, FSHRF, PIF, MIF.

In addition to various non-enzymatic proteins of physiological interest, enzymes can also be produced as an end product or for intracellular transformation of a substrate present in the host or substrate introduced extracellularly, or for enzymatic transformation in vitro.

In accordance with the I.U.B. classification, the enzymes fall into varying categories such as 1. oxidoreductases; 2. transferases; 3. hydrolases; 4. lyases; 5. isomerases; 6. ligases. Enzymes of particular interest will be hydrolases and oxidoreductases for use in commercial processing, for example, hydrolases for hydrolysing polysaccharides, lipids and polypeptides; oxidoreductases for oxidation of alcohols and aldehydes, epoxidation, and the like.

The microorganism host may be bacteria, such as Escherichia, Bacillus, Aerobacter, Klebsiella, Proteus, Pseudomonas, Streptococcus, Staphylococcus, Clostridium, Mycobacterium, Streptomyces and Actinomyces; Fungi e.g. Gymnomycota, Dimastygomycota, Eumycota, Zygomycetes, Ascomycetes and Basidomycetes, such as, Candida, Aspergillus, Rhizobus, Microsporum, and Fonsecaea; Protozoa e.g. Mastigophora, Sarcodina, Sporozoa and Celiophora, such as, Trypanosoma, Codosiga, Protospongra and Entameba, and Alga e.g. Dinoflagellates, Euglenoids, and Diatoms.

Higher cells, e.g., mammalian, may also be employed as hosts, where viral, e.g., bovine papilloma virus or other DNA sequence is available which has plasmid-like activity.

Depending upon the nature of the host, a wide variety of vectors may be employed. The vector will have an intact replicon and be capable of replication in the host. In addition, replicons can be developed which may have one or more other regulatory signals. Regulatory signals can include temperature sensitive replicons, runaway-replication sequences, temperature sensitive operators, and the like. Various additional DNA sequences may be present providing for restriction sites, markers, termination sequences, or the like. Desirably, the vector should be of a substantially different size from the construct to allow for excision of the construct and ease of separation of the construct from the vector by molecular weight separation techniques e.g. electrophoresis and density gradient centrifugation.

The subject invention also provides for a technique for screening the strength of promoters and terminators, thus allowing for the determination of the use of a promoter or terminator in a particular application, where it may be desirable to have promoters or terminators or combinations thereof of varying strength. In screening promoters and/or terminators one employs a promoter or terminator of known activity. A DNA construct is made having the sequence described previously, where appropriate restriction sites are provided for introducing the various elements. The construct provides in the direction of transcription the promoter, a first gene marker, optionally termination codons, such as nonsense codons and oop terminator, the terminator, and a second gene marker. This DNA construct is inserted into an appropriate vector. Where the promoter and

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