EXHIBIT 1

(*part 2 of 2*)

P_{N25}

E

E_B

bla

dhfr

$pDS1/P_{N25},t_o1^+$

P

H

t_o

H

E

repl.

Xb

cat

F I G. 10

xXhoI,EcoRI

isolation of the

digested DNA

ligation

transformation

analysis

F I G. II

FIG. 12

F I G. 13

FIG. 14

FIG. 15

F I G. 16

FIG. 17

U.S. Patent   Nov. 8, 1994   Sheet 34 of 37   **5,362,646**

F I G. 18

F I G. 19

F I G. 20

FIG. 21

5,362,646

1

EXPRESSION CONTROL SEQUENCES

This is a continuation of application Ser. No. 07/223,597 filed Jul. 25, 1988, now abandoned.

TECHNICAL FIELD

The present invention relates to highly efficient and highly repressible expression control sequences, expression vectors which contain these expression control sequences, microorganisms transformed with these expression vectors and methods for their production by means of recombinant DNA technology. The present invention also relates to methods for the production of pro- and eukaryotic proteins using these highly repressible expression control sequences, expression vectors and transformed microorganisms.

BACKGROUND OF THE INVENTION

The level of production of a protein in a host cell is determined by three major factors: the number of copies of its structural gene within the cell, the efficiency with which the structural gene copies are transcribed and the efficiency with which the resulting messenger RNA ("mRNA") is translated. The transcription and translation efficiencies are, in turn, dependent on nucleotide sequences which are normally situated ahead of the desired structural genes or the coded sequence. These nucleotide sequences (expression control sequences) define, inter alia, the location at which the RNA polymerase binds (the promoter sequence to initiate transcription; see also EMBO J. 5, 2995–3000 [1986]) and at which the ribosomes bind and interact with the mRNA (the product of transcription) to initiate translation.

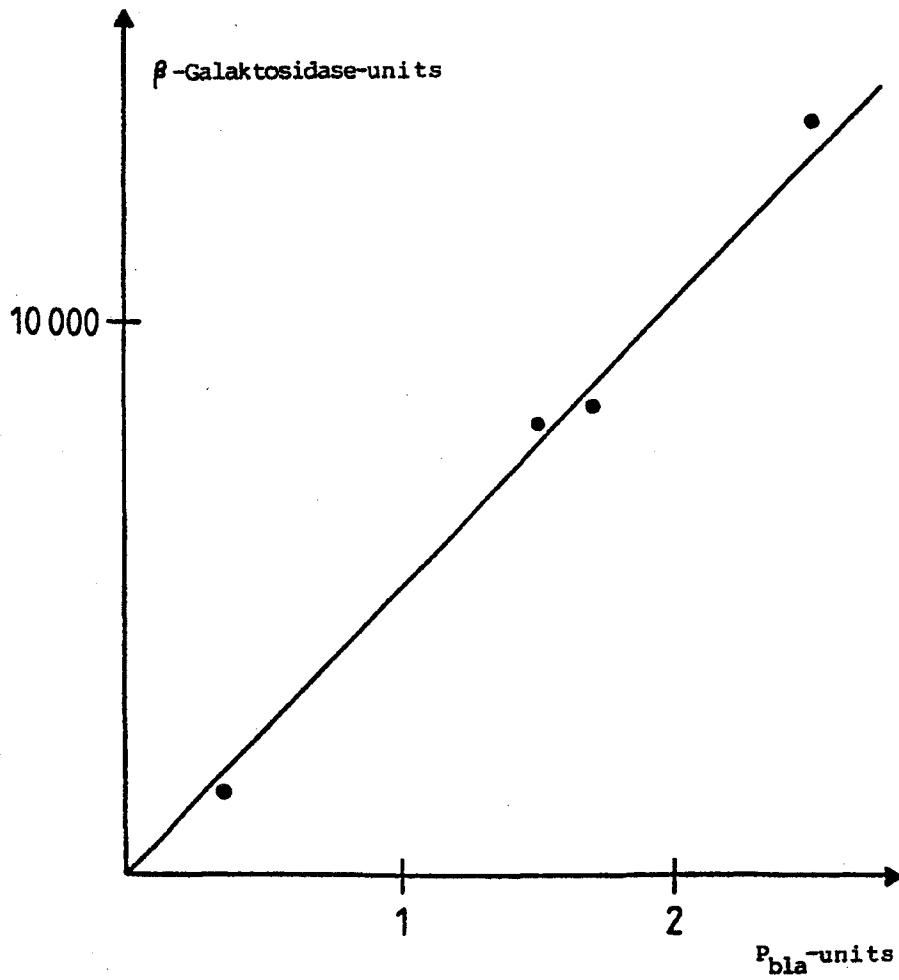Not all expression control sequences have the same efficiency. It is therefore often advantageous to separate the specific coding sequence for a desired protein from its adjacent nucleotide sequences and to link it with other expression control sequences to achieve a higher expression rate. After this has been accomplished, the newly combined DNA fragment can be inserted into a plasmid having a high copy number or a derivative of a bacteriophage to increase the structural gene copies within the cell, whereby simultaneously the yield of the desired protein can be improved.

Since the overproduction of a normally nontoxic gene product is often harmful to the host cells and lowers the stability of a specific host cell-vector system, an expression control sequence should, in addition to improving the transcription and translation efficiency of a cloned gene, be regulatable to permit the regulation of the expression during the growth of the microorganisms. Some regulatable expression control sequences can be switched off during the growth of the host cells and then can be switched on again at a desired point in time, to favour the expression of large amounts of the desired protein.

Various expression control sequences which fulfill the previously-mentioned conditions have been used for the expression of DNA sequences and genes which code for desired proteins. Such expression control sequences are known, for example, from Science 198, 1056–1063 (1977) (Itakura et al.), Proc. Natl. Acad. Sci. U.S.A. 76, 106–110 (1979) (Goeddel et al.), Nature 283, 171–174 (1980) (Emtage et al.), Science 205, 602–607 (1979) (Martial et al.), Gene 5, 59–76 (1979) (Bernard et al.), Gene 25, 167–178 (1983) (Ammann et al.), Proc. Natl. Acad. Sci. U.S.A., 80, 21–25 (1983) (de Boer et al.)

2

and from European Patent Applications Publication Nos. 41767 and 186069.

SUMMARY OF THE INVENTION

In accordance with the invention it has now been found that highly efficient and highly repressible expression control sequences can be produced by combining promoter sequences having a low signal strength and a high in vivo promoter strength with operator/-repressor systems having a high association rate (K_a). These expression control sequences are distinguished from the known expression control sequences primarily in that they are more than 1,000-fold repressible and, after induction, bring about a high RNA synthesis rate (>10 P_{bla} units) at ideal growth temperatures.

The present invention is therefore concerned with expression control sequences which are characterized by the combination of promoter sequences having a low signal strength and a high in vivo promoter strength with operator/repressor systems having a high association rate, especially those which produce a repression factor of $>1,000$.

BRIEF DESCRIPTION OF THE FIGURES

This invention can be more readily understood by reference to the accompanying Figures, in which the following abbreviations and symbols are used:

B, C, E, H, Ha, K, P, Sa, X and Xb denote cleavage sites for the restriction endonucleases BamHI, ClaI, EcoRI, HindIII, HpaI, KpnI, PstI, SalI, XhoI and XbaI, respectively.

▷◁ represents the promoters of the genes bla, lacI and neo; ▤▤▤ represents the ribosomal binding sites of the genes bla, cat, neo, lacI and lacZ, ▯▯▯ represents the terminators t_o, Tl and TE, and an arrow indicates the functional orientation of the terminators; ▭ represents the expression control sequences A1OPSA1, lacOP29 and P_{N25}; ▨▨▨ represents the operator OP in the expression control sequence OPA1OPSA1; ⇄ represents the region required for replication (repl.); ▶ represents coding regions for dihydrofolate reductase (dhfr), chloramphenicol acetyltransferase (cat), lac repressor (lacI), β-lactamase (bla), β-galactosidase (lacZ) and neomycin phosphotransferase (neo).

The Figures show the following:

FIGS. 1a–1d

Schematic representation and nucleotide sequence of the plasmid pDS1,tol+. The plasmid is schematically represented in FIG. 1a. In the nucleotide sequence (FIGS. 1b–1d) the recognition sites for the restriction endonucleases given in the schematic representation are overlined, while the regions coding for β-lactamase (bla) and dihydrofolate reductase (dhfr), respectively, are underlined.

FIGS. 2a–2d

Schematic representation and nucleotide sequence of the plasmid pDS3. The plasmid is schematically represented in FIG. 2a. In the nucleotide sequence (FIGS. 2b–2d) the recognition sites for the restriction endonucleases given in the schematic representation are overlined, while the regions coding for β-lactamase (bla) and dihydrofolate reductase (dhfr), respectively, are underlined.

FIGS. 3a–3g

Schematic representation and nucleotide sequence of the plasmid pML3/lacOP29. The plasmid is schemati-

5,362,646

3

cally represented in FIG. 3a. In the nucleotide sequence (FIGS. 3b–3g), which is given as far as it is known, the recognition sites for the restriction endonucleases given in the schematic representation are overlined, while the regions coding for β-lactamase (bla), lac-repressor (lacI) and β-galactosidase (lacZ), respectively, are underlined.

FIGS. 4a–4d

Schematic representation and nucleotide sequence of the plasmid pDMI, 1. The plasmid is schematically represented in FIG. 4a. In the nucleotide sequence (FIGS. 4b–4d) the recognition sites for the restriction endonucleases given in the schematic representation are overlined, while the regions coding for neomycin phosphotransferase (neo) and lac-repressor (lacI), respectively, are underlined.

FIGS. 5a–5b

Nucleotide sequences of the XhoI-EcoRI fragments having the expression control sequences lacOP29(a), tacOP29(b), N25OPSN25OP29(c), A1OPSA1(d), A1A1pOPSA1(f), OPUA1(g), OPUA1CON(h), A1OPSA1OP21(i) and A1OPSA1OP29(j), respectively. In these sequences the nucleotide at which RNA synthesis starts (+) and the regions at positions −10 and −35 are underlined, while lac-operator sequences are overlined.

FIG. 6

Nucleotide sequence of the EcoRI fragment having the promoter P_{N25}. In the sequence the nucleotide at which the RNA synthesis starts and the regions at positions −10 and −35 are underlined.

FIG. 7

Nucleotide sequences of the XhoI-EcoRI fragments having the expression control sequences N25*/O and N25OP29, respectively. In the sequences each nucleotide at which the RNA synthesis starts and the regions at positions −10 and −35 are underlined, while lac-operator sequences are overlined.

FIG. 8

Nucleotide sequence of the XhoI-EcoRI fragment having the promoter P_{A1}. In the sequence the nucleotide at which the RNA synthesis starts (position +1) and the regions around positions −10 and −35 are underlined.

FIG. 9

Nucleotide sequence of the SalI-EcoRI fragment containing the expression control sequence OPA1OPSA1. In the sequence the nucleotide at which the RNA synthesis starts and the regions around positions −10 and −35 are underlined, while lac-operator sequences are overlined.

FIG. 10

Schematic representation of the plasmid pDS1/P_{N25},tol⁺. Transcription from the promoter P_{N25} is in the clockwise direction.

FIG. 11

Schematic representation of the construction of the plasmid pDS3/A1OPSA1. Transcription from the expression control sequence A1OPSA1 is in the clockwise direction.

FIG. 12

Schematic representation of the construction of the plasmid pDS3/OPA1OPSA1. The nucleotide sequence of the SalI-EcoRI fragment having the expression control sequence OPA1OPSA1, from which transcription is in the clockwise direction, is given in FIG. 9.

FIG. 13

Schematic representation of the construction of the plasmid pML3/A1OPSA1. In this construction the XhoI-EcoRI fragment of the plasmid pML3/lacOP29

4

containing the expression control sequence lacOP29 was replaced by the corresponding XhoI-EcoRI fragment containing the expression control sequence A1OPSA1.

FIG. 14

Schematic representation of the construction of the plasmid pML3/OPA1OPSA1. Three DNA fragments were used for the construction of this plasmid: the SalI-EcoRI fragment from PDS3/OPA1OPSA1 containing the expression control sequence OPA1OPSA1, the EcoRI-ClaI fragment from pML3/lacOP29 containing parts of the lacZ gene and the larger ClaI-XhoI fragment from pML3/lacOP29. In this construction cleavage sites for SalI and XhoI were linked with each other, whereby both cleavage sites were destroyed.

FIG. 15

Time-dependent course of the complex formation of RNAP and promoter P_{N25} at a concentration of active RNAP of 0.42 nM and a promoter concentration of 0.02 nM.

FIG. 16

Graphical representation of the expression $-\ln[(A_o-X)/A_o]$ as a function of the reaction time at a concentration of active RNAP of 0.42 nM and a concentration of promoter P_{N25} of 0.02 nM.

FIG. 17

Graphical representation of the time-dependent course of the complex formation of RNAP and promoter P_{N25} at a promoter concentration of 0.02 nM and a concentration of active RNAP of 0.32 nM (experiment 2) and 0.15 nM (experiment 3).

FIG. 18

Graphical representation of the expression $-\ln[(A_o-X)/A_o]$ as a function of the reaction time at a promoter concentration of 0.02 nM and a concentration of active RNAP of 0.32 nM (experiment 2) and 0.15 nM (experiment 3).

FIG. 19

Determination of the complex formation rate for promoter P_{A1} with promoter P_{N25} as the internal standard: $-\ln[(B_o-Y)/B_o]$ plotted against $-\ln[(A_o-X)/A_o]$.

FIG. 20

Determination of the complex formation rate for the expression control sequence A1OPSA1 with promoter P_{A1} as the internal standard: $-\ln[(B_o-Y)/B_o]$ plotted against $-\ln[(A_o-X)/A_o]$.

FIG. 21

Determination of the factor for the conversion of β-galactosidase units into P_{bla} units: β-galactosidase units under control of the expression control sequences tacOP29, N25OP29, OPUA1 and OPUA1CON plotted against the corresponding P_{bla} units.

DESCRIPTION OF THE INVENTION

The signal strengths of promoters are specified by the K_a between promoter and RNA polymerase. The signal strengths of the promoter sequences used in this invention have K_a values of about 1×10^6 to 1.5×10^8 M⁻¹ sec⁻¹, with a preferred K_a value of 6×10^7 M⁻¹ sec⁻¹. The K_a values are determined as described in Example 3.

The in vivo promoter strength is defined by the RNA synthesis rate, which is mediated by an individual promoter sequence, and is measured in P_{bla} units (Deuschle et al., EMBO J. 5, 2987–2994 [1986]). High in vivo promoter strengths produce RNA synthesis rates of 10–100, preferably more than 20, P_{bla} units.

5,362,646

5

The K_a of operator/repressor systems describes the velocity with which a repressor binds to the operator. The operator/repressor systems used in this invention have K_a values of about 1×10^8 to 1×10^{11} M^{-1} sec^{-1}. The K_a value of $2 \times 10^9 M^{-1}$ sec^{-1} described for the lac-operator/repressor system (Biochemistry 25, 3845–3852 [1986]) is especially preferred.

Promoter sequences which can be used in this invention include natural promoter sequences and functional variants, which have been specifically altered by mutation or synthesis, and combinations of these promoter sequences. They can be obtained from gram-negative organisms such as *E. coli*, from gram-positive organisms such as *B. subtilis* and *B. stearothermophilis* and from the corresponding phages. Preferred promoter sequences are those from T-coliphages. The T7A1 promoter [the nucleotide sequence of which is shown in FIG. 8 and which is also denoted hereinafter as the A1 promoter (P_{A1})] is especially preferred.

Operator/repressor systems that can be used include all systems that are directly inducible by chemical inducers which produce, in the natural state or after corresponding variations (e.g., by mutation), a repression factor of >1,000. Directly inducible systems that are not part of this invention include systems which are inducible by SOS function (lexA/recA system) or by temperature, such as the P_L operator/repressor system.

Examples of systems which are directly regulatable by chemical induction include, e.g., the regulation units of the lactose, galactose, tryptophan and tetracycline operons, and other negatively controllable operons (i.e., operons which are regulatable by an operator/repressor action; see Miller et al. "The operon", Cold Spring Harbor Laboratory, 1980 and Hillen et al., J. Mol. Biol. 172, 185–201 [1984]).

Especially preferred operator/repressor systems are the natural lac-operator/repressor system (Miller et al., supra) and variants of the above-named operator/-repressor systems, which are specifically modified by mutation or synthesis to produce a repression factor of >1,000. The term "repression factor" describes the quotient of the in vivo promoter strengths in the presence and absence of an inducer.

The production of the expression control sequences of the invention can be carried out using recombinant DNA technology methods which are well known in the art (see, e.g., Maniatis et al., "Molecular Cloning", Cold Spring Harbor Laboratory, 1982).

The promoter sequences having a low signal strength and a high in vivo promoter strength can be fused with one or more operator/repressor system(s) to produce the desired expression control sequences. When a single operator/repressor system is used, it can be located within or outside the promoter sequence. Consequently, the operator/repressor system can be integrated into the promoter sequence, can partially replace, precede or succeed it in various positions. Preferably, an operator/repressor system is integrated into the promoter sequence. An especially preferred integration position is in the spacer region between position −12 and −29 (nomenclature as in FIG. 8).

When two operator/repressor systems are used, both can be located within or outside the promoter sequence, or one can be situated within and the other can be situated outside the promoter sequence. Preferably, one is integrated into the spacer region and the other is integrated upstream in the 5'-position, so that by repressor binding a maximal cooperativity is produced between

6

the two operator sequences of the operator/repressor systems. Through such cooperativity, a repression factor up to approximately 30,000 can be obtained.

The preferred expression control sequences of the present invention have been obtained by chemical DNA synthesis, whereby functional parts of the lac-operator sequence have been combined with functional parts the T7A1 promoter sequence. The construction of preferred expression control sequences A1OPSA1, A1OPSA1OP21, A1OPSA1OP29, A1OPSA1 and OPA1OPSA1 is described in detail in Example 2 below. The nucleotide sequences of these expression control sequences are shown in FIGS. 5 and 9, respectively, and the characterizing properties are compiled in Table 8.

The previously mentioned lac-operator sequences are negatively regulated by the lac-repressor. To obtain sufficient amounts of repressor molecules within the cell, the corresponding gene can be expressed in excess in a vector or the chromosome of a bacterium by known methods, such as by integration of the lacI^q gene (Miller et al., supra; Calos, Nature 274, 762–765 [1978].

The expression control sequences of the invention can be inserted using standard procedures into any suitable expression vector which can replicate in gram-negative and/or gram-positive bacteria. Suitable vectors can be constructed from segments of chromosomal, nonchromosomal and synthetic DNA sequences, such as various known plasmid and phage DNA's (Maniatis et al., supra). Especially suitable vectors are plasmids of the pDS family (Bujard et al., Methods in Enzymology, eds. Wu and Grossmann, Academic Press, Inc., Vol. 155, 416–433 [1987]).

The expression control sequences can also be inserted into the chromosome of gram-negative and gram-positive bacterial cells. In that case, selection agents such as antibiotics, which are generally required when working with vectors, can be dispensed with.

Suitable DNA sequences which can be expressed using the expression control sequences of the invention include those which code in vivo or in vitro for pro- or eukaryotic proteins. For example, such DNA sequences can code for enzymes, hormones, proteins having immunoregulatory, antiviral or antitumour activity, antibodies, antigens and other useful pro- or eukaryotic proteins.

Proteins which can be produced using the expression control sequences of the invention include, for example, malaria surface antigens such as the 5.1 surface antigen, the CS protein and the p190 protein of *Plasmodium falciparum*, lymphokines, interferons, insulin and insulin precursors, HIV-1 and 2 envelope and structural proteins, growth hormones and growth hormone releasing factors.

Methods for expression of the DNA sequences are well known in the art and are described in Maniatis et al., supra.

In one embodiment of the invention, a desired protein is produced by a method comprising:

(a) transforming a suitable bacterium such as *E. coli*, *Salmonella typhimurium* or *B. subtilis*, with an expression vector in which DNA which codes for a desired pro- or eukaryotic protein is operably linked to an aforementioned expression control sequence;

(b) culturing the transformed bacterium under suitable growth conditions; and

(c) isolating the desired protein from the culture.

5,362,646

7

In another embodiment of the invention, a desired protein is produced by a method comprising:

(a) inserting an aforementioned expression control sequence, which is operably linked to the coding sequence of a desired pro- or eukaryotic protein, into the chromosome of a suitable bacterium;

(b) cultivating of the thus-obtained bacterium under suitable growth conditions; and

(c) isolating the desired protein from the culture.

The selection of a suitable host organism is determined by various factors which are well known in the art. Factors to be considered include, for example, compatibility with the selected vector, toxicity of the expression product, expression characteristics, necessary biological safety precautions and costs.

Suitable host organisms include gram-negative and gram-positive bacteria, such as *E. coli, Salmonella typhimurium,* and *B. subtilis* strains. *E. coli* strain M15 is especially preferred. Other *E. coli* strains that can be used include other generally available strains such as *E. coli* 294 (ATCC No. 31446), *E. coli* RR1 (ATCC No. 31343) and *E. coli* W3110 (ATCC No. 27325).

EXAMPLES

This invention can be illustrated by the following, nonlimiting Examples.

Example 1

Description of the Plasmids Used

A. Principles

The plasmids pDS1,t_o1+ (FIG. 1), pDS3 (FIG. 2), pML3/lacOP29 (FIG. 3) and pDMI,1 (FIG. 4) were used for the production and characterization of the properties of the expression control sequences. *E. coli* cells transformed with these plasmids have been deposited under the Budapest treaty at the Deutschen Sammlung von Mikroorganismen (DSM) in Götingen and assigned the following accession Nos.: *E. coli* M15 (pDS1,t_o1+), DSM No. 3135; *E. coli* M15 (pDS5/RBSII, 3A+5A; pDMI,1), DSM No. 3517; *E. coli* M15 (pDS3), DSM No. 4198; and *E. coli* M15 (pML3/lacOP29), DSM No. 4199.

B. Plasmid pDS1,t_o1+

The part of pDS1,t_o1+ (FIG. 1) which lies between the cleavage site for the restriction endonucleases XbaI and EcoRI and the replication region and the gene for β-lactamase, which confers ampicillin resistance to the cells, is derived from plasmid pBR322 (Bolivar et al., Gene 2, 95–113 [1977]; Sutcliffe, Cold Spring Harbor Symp. Quant. Biol. 43, 77–90 [1979]). The remaining part of the plasmid carries cleavage sites for the restriction endonucleases XhoI, EcoRI and BamHI followed by the gene of the dihydrofolate reductase of the mouse cell line AT-3000 (Chang et al., Nature 275, 617–624 [1978]; Masters et al., Gene 21, 59–63 [1983]), the terminator t_o of the *E. coli* phage lambda (Schwarz et al., Nature 272, 410–414 [1978] and the promoter-free gene of chloramphenicol acetyltransferase (Marcoli et al., FEBS Letters, 110, 11–14 [1980]).

C. Plasmid pDS3

Plasmid pDS3 (FIG. 2) differs from plasmid pDS1,t_o1+ on the one hand in a region which carries, besides cleavage sites for various restriction endonucleases, the terminator T1 of the *E. coli* rrnB operon (Brosius et al., J. Mol. Biol. 148, 107–127 [1981]) and on the other hand by the absence of the cleavage site for

8

the restriction endonuclease EcoRI in the gene for chloramphenicol acetyltransferase.

D. Plasmid pML3/lacOP29

The part of pML3/lacOP29 (FIG. 3) which lies between the cleavage sites for the restriction endonucleases SalI and EcoRI and the replication region and the gene for β-lactamase is derived from plasmid pBR322 (Bolivar et al., supra; Sutcliffe, supra). The remaining part of the plasmid carries, in addition to the complete lac I gene (Farabough, Nature 274, 765–769 [1978]), which codes the lac-repressor, the terminator T_E of the *E. coli* phage T7 (Dunn et al., J. Mol. Biol., 166, 477–535 [1983]), the expression control sequence lacOP29 (see Example 2) and the promoter-free gene for β-galactosidase (Kalnins et al., EMBO J. 2, 593–597 [1983]).

E. Plasmid pDMI,1

Plasmid pDMI,1 (FIG. 4) carries the gene of neomycin phosphotransferase from the transposon Tn5 (Beck et al., Gene 19, 327–336 [1982]), which confers kanamycin resistance to the *E. coli* cells, and the lac I gene (Farabough, supra) having the promoter mutation I^q (Calos, Nature 274, 762–765 [1978]), which codes for the lac-repressor. Moreover, plasmid pDMI,1 contains a region of plasmid pACYC184 (Chang et al., J. Bacteriol. 134, 1141–1156 [1978]) which contains all information required for the replication and stable transmission to the daughter cells. Plasmid pDMI,1 is compatible with the above-described plasmids and their derivatives.

Example 2

Production and Cloning of the Expression Control Sequences

A. Principles

After the production of the expression control sequences they were integrated into suitable plasmids for the characterization of their properties.

B. Production of the Expression Control Sequences

1. Expression Control Sequences lacOP29, tacOP29, A1OPBCONA1, OPUA1, OPUA1CON, N25OPSN-25OP29, P$_{A1}$, A1OPSA1, A1pOPSA1, A1OPSA1OP21 and A1OPBA1OP29.

For the production of these expression control sequences, single-stranded DNA fragments were first synthesized chemically (Bannwarth et al., DNA 5, 413–419 [1986]). These fragments were then hybridized and ligated as described by Maniatis et al., supra. The sequences of the thus-obtained double-stranded XhoI-EcoRI fragments having the corresponding expression control sequences are given in FIGS. 5 and 8, respectively.

2. Expression Control Sequences P$_{N25}$, N25*/O and N25OP29

The expression control sequences P$_{N25}$, N25*//O and N25OP29 (FIG. 6 and FIG. 7) can be produced as described in the above paragraph.

3. The Expression Control Sequence OPA1OPSA1

The sequence of the expression control sequence OPA1OPSA1 is represented in FIG. 9. Its production is described in paragraph D.

C. Integration of Promotor P$_{N25}$ Into Plasmid pDS1, t_o1+

Promoter P$_{N25}$ was integrated into plasmid pDS1,t_o1+ (FIG. 1) as part of an EcoRI fragment (see FIG. 6) according to methods described by Maniatis et al., supra, whereby plasmid pDS1/P$_{N25}$,t_o1+ (FIG. 10)

5,362,646

9

was obtained. This plasmid was used as a source for the production of promoter P_{N25} in larger amounts.

D. Integration of the Expression Control Sequences Into Plasmid pDS3

The expression control sequences lacOP29, tacOP29, OPUA1, OPUA1CON, P_{N25}, N25*/O, N25OP29, N25OPSN25OP29, P_{Al}, A1OPSA1, A1OPSA1OP21, A1OPSA1OP29, A1OPSCONA1 and AlpOPSAL were integrated into the plasmid pDS3 (FIG. 2) using methods described by Maniatis et al., supra. One such cloning is schematically represented in FIG. 11, with the expression control sequence A1OPSA1 as an example.

The expression control sequence OPA1OPSA1 was produced (FIG. 12) by integrating a chemically synthesized DNA fragment, which contains a palindromic sequence of the lac-operator, into the HpaI cleavage site of plasmid pDS3/A1OPSA1 (FIG. 11).

The pDS3 derivatives having the corresponding expression control sequences were used both to determine the association rates between promoter and RNAP and to determine the promoter strengths of the individual expression control sequences.

E. Integration of the Expression Control Sequences into Plasmid pML3/lacOP29

Plasmid pML3/lacOP29 (FIG. 3) carries the expression control sequence lacOP29 between the cleavage sites for the restriction endonucleases XhoI and EcoRI. These plasmids were produced (FIG. 13) according to methods described in the literature (Maniatis et al., supra) in which lacOP29 was replaced in each case by one of the expression control sequences tacOP29, N25OP29, N25OPSN25OP29, A1OPSA1, A1OPSA1OP21, A1OPSA1OP29, A1OPSCONA1, AlpOPSA 1, OPUA1 and OPUA1CON, respectively.

Plasmid pML3/OPA1OPSA1 containing the expression control sequence OPA1OPSA1 was produced as described schematically in FIG. 14.

The pML3 derivatives containing the corresponding expression control sequences were used to determine the in vivo promoter strengths under repressed conditions.

Example 3

Determination of the Signal Strengths

A. Principles

For the determination of the signal strengths of the promoters and promoter/operator elements, the absolute value of the K_a between *E. coli* RNA polymerase (RNAP) and promoter P_{N25} was first determined. Then, the K_a values for the remaining signals were determined by relative measurements using promoter P_{N25} as an internal standard.

B. Determination of K_a for RNAP and Promoter P_{N25}

The K_a for RNAP and promoter P_{N25} was determined by means of filter binding experiments in which the formation of RNAP/promoter complexes as a function of the RNAP concentration and the reaction time were determined quantitatively. Since RNAP and the promoters are biological materials, the evaluation of such experiments presupposed that for both reactants the concentration of the active molecule was known. Thus, e.g., for RNAP the concentration of the active polymerase molecule had to be considered, not the protein concentration. The same was true for promoter P_{N25} and for the single-stranded fd-DNA, which was

10

used as a competitor for nonspecific binding and for the termination of association reactions.

Accordingly, a description of the theoretical derivation of the complex formation rates will be followed below by a detailed explanation of the production and analysis of all reactants, and that will be followed by actual experiments for the determination of K_a.

1. Theoretical Derivation of K_a

The general reaction scheme is

$$R + P \xrightarrow[\;K_d\;]{\;K_a\;} RP.$$
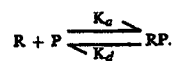
wherein

R represents the free concentration of RNAP,
P represents the free concentration of the promoters,
RP represents the concentration of the RNAP/promoter complexes,
K_a represents the association rate and
K_d represents the dissociation constant.

The following applies to the course of the reaction per unit time:

$$dP/dt = -K_a \times R \times P + K_d \times RP$$

If the half life of the RNAP/promoter complexes is large—i.e., the dissociation constant K_d is small—the term $K_d \times RP$ can be disregarded, and

$$dP/dt = -K_a \times R \times P \tag{1}$$

applies to the change in the promoter concentration per unit time.

R and P change with time, but if the RNAP is present in a large excess, its concentration during the course of the reaction can be regarded as being constant (i.e., when R is constant, then $dR/dt = 0$), and

$$K_a \times R = m \tag{2}$$

applies.

Therefore, the following equation applies to the change in the promoter concentration per unit time [see equations (1) and (2)]:

$$dP/dt = -m \times P$$

or,

$$-dP/P = m \times dt$$

Integration of this equation yields:

$$-\ln P = m \times t \tag{3}$$

Equation (3) corresponds to a reaction equation of the first order, i.e., formally to the "decomposition of free promoter fragment in RNAP/promoter complex", with the velocity constant m [1/sec]. P in this case is the amount of free promoter at time t (for t=0 seconds, P=1). This amount can be determined experimentally (see paragraph 6). Knowing A_O (total amount of promoters) and X (amount of RNAP/promoter complexes at the point in time of the reaction t), the so-called "pseudo first order" constant m can be calculated with the aid of equation (3):

$$P = (A_O - X)/A_O$$

5,362,646

11

and the result is

$$m = -\ln[(A_O - X)/A_O]/t \qquad (4)$$

Since the formation of the promoter/operator complexes is a bimolecular reaction of the second order, the constant m depends on the concentration of the RNAP. If this is known, then K_a in the equations

$$m = K_a \times R \qquad (2)$$

and

$$m = -\ln[(A_O - X)/A_O]/t \qquad (4),$$

can be calculated as follows:

$$K_a \times R = -\ln[(A_O - X)/A_O]/t$$

or,

$$K_a = -\ln[(A_O - X)/A_O]/(t \times R) \qquad (5)$$

In the derivation of K_a, it has been assumed that the reverse reaction (i.e., the decomposition of RNAP/promoter complexes), can be disregarded ($K_d \times RP = O$). Such decomposition is a reaction of the first order and is therefore dependent on the concentration of the reactants. On the other hand, the association is concentration-dependent. Accordingly, for the determination of K_a the concentrations of the reactants selected must be sufficiently high that the association process takes place in a period in which the decomposition of complexes can be disregarded. This presupposes knowledge of the stability of the RNAP/promoter complexes.

Furthermore, it has been assumed that during the course of the reaction the concentration of the free RNAP can be regarded as constant. For this purpose, the RNAP must be present in a large excess relative to the promoters. It should also be noted that the RNAP can also bind to nonspecific DNA sequences, but the number of such nonspecific binding sites can be minimized by investigating promoters on small, isolated DNA fragments. With an excess of approximately 10 RNAP molecules per promoter, the concentration of the free RNAP during the course of the reaction can be regarded as constant.

2. Production of the Probe Containing Promotor P_{N25}

Plasmid pDS1/P_{N25},t_o1^+ (FIG. 10) contains promoter P_{N25} on a 254 bp EcoRI fragment (FIG. 6). This plasmid was constructed by integrating the mentioned EcoRI fragment into the EcoRI cleavage site of plasmid pDS1, t_o1^+ (FIG. 1), which is adjacent to the XhoI cleavage site.

To purify the promoter fragment, plasmid pDS1/P_{N25},t_o1^+ was first purified (Maniatis et al., supra) and 0.5 mg of this plasmid was then cleaved with the restriction endonuclease EcoRI. After phenol extraction and ethanol precipitation (Maniatis et al., supra), the cleaved DNA was dissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.6) and, after the addition of sample buffer, subjected to electrophoresis in 6% polyacrylamide gels (Maniatis et al., supra). Subsequently, the fragments carrying promoter P_{N25} were cut out from the gel with a scalpel and electrophoresed on DEAE paper (DE 81, Whatman, England). After three-fold washing with ethanol and TE buffer, the

12

paper was dried in air and the DNA was eluted with a buffer containing 10 mM Tris HCl, 1 mM EDTA and 1.5 M NaCl, pH 7.6. After a 1:3 dilution of the resulting DNA solution with TE buffer, the DNA was precipitated with ethanol, the sediment was washed with 80% ethanol and the DNA was dissolved in TE buffer.

The concentration of this DNA solution was determined spectrophotometrically as described by Mahler et al. [J. Mol. Biol. 9, 801–811 (1964)]. A portion of the DNA stock solution was then diluted 1:30 in TE buffer, and the extinction of this dilution was measured against TE buffer as the blank. The following values were obtained:

$$\Delta E_{260} = 0.108$$

$$\Delta E_{280} = 0.058$$

$$\Delta_{260}/\Delta E_{280} = 1.86$$

Using the conversion factor of $\Delta E_{260} = 50$ μg DNA/ml and the dilution factor, a DNA concentration of 162 μg/ml was obtained for the stock solution. This value corresponded to a concentration of 0.96 pMoles DNA fragment/μl, considering the length of the promoter fragment (254 bp). The relationship of the extinctions at 260 and 280 nm permitted assessment of the purity of the DNA solution, with a high purity DNA solution having a $\Delta E_{260}/\Delta E_{180}$ value of 1.86.

The concentration of the DNA solution was also determined by comparing this solution with an RNA-free solution of plasmid pDS1, t_o1^+ of known concentration (ΔE measurement). For this purpose, 0.09 pMole portions of the DNA solution containing the promoter fragment were mixed with 0.2, 0.1, 0.5 and 0.025 pMoles of the cleaved pDS1, t_o1^+ plasmid DNA and characterized in a 6% polyacrylamide gel (Maniatis et al., supra). The pherogram obtained was evaluated densitometrically, whereby a concentration of 0.8 pMoles/μl was obtained for the promoter fragment in comparison to the cleaved pDS1, t_o1^+ plasmid DNA.

To determine the purity of the promoter fragment, 0.36 pMole of this fragment were firstly characterized in a polyacrylamide gel. After staining with ethidium bromide, no impurities could be detected. Furthermore, the fragment was radioactively labelled with ^{32}P (see below) and also characterized electrophoretically. Only the promoter fragment could be detected by autoradiography. It thus was shown that the isolated EcoRI fragment having promoter P_{N25} was not contaminated with substantial amounts of RNA or DNA and was present at a concentration of 0.88±0.08 pMoles/μl.

To determine the concentration of the active polymerase (see below), a mixture of the isolated EcoRI fragment having promoter P_{N25} and the corresponding fragment radioactively labelled with ^{32}P was used as the promoter probe. This radioactively labelled DNA was produced as follows: the DNA was first dephosphorylated with the aid of CIP (calf intestinal alkaline phosphatase) (Maniatis et al., supra) and then labelled by the incorporation of ^{32}P (γ-^{32}P ATP, Amersham, 3000 Ci/mMol) with T4-polynucleotide kinase (Maniatis et al., supra). Subsequently, the radioactively labelled EcoRI fragment having promoter P_{N25} was purified by chromatography in Sephadex G75 ® (Pharmacia, Sweden).

To determine the specific activity of this probe, the radioactivity was measured and the DNA concentra-

5,362,646

13

tion was determined electrophoretically against the pre-treated, isolated EcoRI fragment (see above). Probes having promoter P_{N25} of defined concentration and specific activity were subsequently obtained by mixing the untreated and the radioactively labelled EcoRI fragments.

3. Production and Characterization of Single-stranded M13mp8-DNA

Single-stranded M13mp8-DNA was used both as a competitor for the nonspecific binding of RNAP to DNA and to terminate association reactions. The production and characterization of this DNA is described below. E. coli JM 101 cells (Maniatis et al., supra; GIB-CO-BRL, Basle) were transformed with 0.01 pMoles of M13mp8 RFI-DNA (Pharmacia, Sweden) according to the method of Morrison [Methods in Enzymology 68, 326–331 (1979)]. The cells were then plated onto indicator plates containing isopropylthiogalactoside (IPTG) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). See Miller, "Experiments in Molecular Genetics", Cold Spring Harbor Laboratory (1972) and Messing et al. Gene 19, 269–276 (1982).

Turbid plaques containing transformed E. coli JM 101 cells were taken from the top-agar layer of these indicator plates with a Pasteur pipette, transferred into 10 ml of LB medium and incubated for 8 hours in a shaking incubator (37° C., 220 rpm). Subsequently, the cells were centrifuged and the supernatant containing M13mp8 phage was used for the re-infection of E. coli JM 101 cells.

For this purpose, E. coli JM 101 cells in 500 ml of M9 minimal medium (Miller, supra) were grown (37° C.; 220 rpm) to an $OD_{600}=2$, and 1 ml of the supernatant containing M13mp8 phage was added to the cells. After incubation at 37° C. for a further 12 hours, the cells were centrifuged. The phage were precipitated from the supernatant with 3% polyethylene glycol (PEG 6000) and 0.5M NaCl and centrifuged. The sediment was resuspended in 20 ml of TE buffer and extracted twice at 65° C. with phenol (equilibrated in 200 mM Tris HCl, pH 8) and phenol/chloroform (1:1).

The thus-liberated single-stranded M13mp8-DNA was precipitated with ethanol and dissolved in 1 ml of TE buffer. The concentration of this DNA solution was determined spectrophotometrically (Mahler et al., supra), using the conversion factor 1 $OD_{260}=36$ μg of single-stranded DNA/ml.

4. Determination of the Concentration of Active Polymerase in RNAP Solutions.

For the determination of the concentration of active E. coli RNA polymerase in RNAP solutions, after incubating the RNAP in the presence of an excess of promoter fragments, the amount of RNAP/promoter complexes formed was determined with the aid of filter-binding experiments. From this amount, the concentration of bound E. coli RNA polymerase was calculated relative to the amount of promoter fragments used. The thus-determined concentration of RNAP molecules capable of binding was equivalent to the concentration of free, active RNAP.

One such concentration determination was carried out by incubating 0.12 pMole of promoter fragment (see paragraph B, 2.; specific activity; 4×10^4 cpm pMol) at 37° C. for 2 minutes in 50 μl of binding buffer (BB; 20 mM Tris HCl, pH 8.0, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, 120 mM KCl). E. coli RNAP (Pharmacia, Sweden) was diluted at 0° C. in BB containing 120 mM KCl . One hundred microliters of the

14

diluted RNAP were incubated at 37° C. for 2 minutes and then pipetted into the solution containing the promoter fragment.

The mixture was held at 37° C. for 5 minutes, and 300 μl of BB containing 0.8 μg of single-stranded M13mp8-DNA (see paragraph B, 3) which had been preincubated at 37° C. for 2 minutes were added. After incubation at 37° C. for a further 5 minutes, the mixture was filtered at 37° C. through nitrocellulose.

A nitrocellulose filter (nitrocellulose filter BA, 0.45 μm; Sartorius, Götingen) was cut into small square pieces (4×4 mm) and soaked in BB containing 40 mM KCl. One piece was transferred onto a similarly soaked glass fibre filter (GF/A, Whatman) which was on a glass frit. This glass frit was in a 37° C. water bath and was connected to a water-jet pump. The reaction mixture was filtered with a filtration velocity of 1 ml/minute. Thereafter, the filter was rinsed with 1 ml of BB containing 40 mM KCl (pre-warmed to 37° C.).

To elute the filter-bound DNA, the nitrocellulose filter was squashed into an Eppendorf test tube with a pipette tip (Eppendorf) and, after the addition of 20 μl of elution buffer (EB) (10 mM Tris HCl, mM EDTA, 0.1% SDS, pH), squeezed out with a pipette tip. The elution batch was placed on ice for 30 minutes and then centrifuged for 55 minutes (Eppendorf bench centrifuge, 12,000 rpm). The elution solution was separated and transferred to another Eppendorf test tube.

The filter was eluted further by adding 50 μl of TE buffer into the filter, shaking the mixture for 3 minutes (Eppendorf shaker), separating the rinse solution and combining the eluate with the first elution solution. After a total of three elution and rinsing steps, about 95% of the bound DNA was eluted. After removing any possibly present broken filter pieces by centrifugation, the radioactivity of the eluate was measured (210 μl).

The concentration of active polymerase in a RNAP solution is determined by adding an aliquot of the solution to 0.12 pMoles of promoter fragment. The numerical value thus obtained is compared to the value obtained when a large excess (saturating amount) of active polymerase is added to 0.12 pMoles of promoter fragment. The concentration in the solution can then be calculated using the ratio of the solution value obtained to the value produced with the saturating amount of active polymerase, taking into account any dilutions made in assaying the solution.

5. Determination of the Half Life of RNAP/promoter Complexes

As noted above, in the derivation of K_d it has been assumed that the decomposition of RNAP/promoter complexes during the experimental period can be disregarded. This assumption was checked in experiments in which the half life of RNAP/promoter complexes was determined.

To determine the half life, RNAP/P_{N25} complexes were formed by incubating 0.06 pMoles of promoter fragment (specific activity 2.4×10^6 cpm/pMol) with 1.2 pMoles of active polymerase in BB containing 120 mM KCl at 37° C. for 5 minutes. After the addition of 5 μg of single-stranded M13mp8-DNA, samples were removed from the batch, which was held at 37° C., at various times (0–180 minutes) and filtered at 37° C. through a nitrocellulose filter. The filter-bound radioactivity was measured as described above and the values obtained were plotted against the reaction time. Evaluation of this graph showed that under these experimental

5,362,646

15

conditions complexes of RNAP and promoter P_{N25} have a half life of approximately 3 hours.

6. Determination of the K_a for RNAP and Promoter P_{N25}

Kinetic measurements for the determination of K_a for RNAP and promoter P_{N25} were carried out under "pseudo first order" conditions, i.e., with a large RNAP excess. The experimental conditions and reaction times were chosen so that the reverse reaction, i.e., the decomposition of formed RNAP/promoter complexes, could be disregarded (duration of the experiment a maximum of 7 minutes with a half life of the complex of approximately 180 minutes). The binding reactions were all carried out according to a uniform scheme, whereby in three independent series of experiments both the reaction volume and the concentration of reactants were varied.

P_{N25} promoter fragments were pre-incubated in buffer before the RNAP solution, which was likewise pre-incubated in buffer and whose concentration of active polymerase was determined in experiments carried out in parallel as described above, was added and the reaction was started by mixing the batches. After selected reaction times (1–120 seconds), reaction was stopped by the addition of single-stranded M13mp8-DNA. The RNAP/promoter complexes formed were then determined quantitatively by filter-binding experiments. Three experiments for the determination of the K_a are described below.

Experiment 1

50 µl of P_{N25} promoter fragment (0.045 nM, specific activity 3×10^6 cpm/pMol) in BB containing 120 mM KCl were incubated at 37° C. for 2 minutes. RNAP was diluted stepwise (from 1:10 to 1:20) at 0° C. in BB containing 120 mM KCl. Subsequently, 100 µl of a 1:5000 dilution were pre-incubated at 37° C. for 2 minutes (0.42 nM active polymerase). The solutions with the P_{N25} promoter fragment, and the RNAP were combined and the association reaction was started by mixing the batches.

After incubation at 37° C. for 10 seconds, 300 µl of BB solution containing 0.8 µg of single-stranded M13mp8-DNA, incubated at 37° C. for 2 minutes, were added. After incubation at 37° C. for 5 minutes, the batch, as described previously, was filtered at 37° C. over nitrocellulose, the filter was rinsed with 200 µl of BB containing 40 mM KCl and the filter-bound radioactivity was determined.

Eleven additional experiments were carried out under the same conditions in which the association reactions were stopped after 1, 2, 3, 4, 5, 6, 7, 8, 15, 20, 60 and 120 seconds, by the addition of single-stranded M13mp8-DNA. The numerical values obtained from all 12 experiments were plotted against reaction time as shown in FIG. 15, where the dotted line represents the maximal radioactivity which can be filter-bound. For the determination of this value, 50 µl of P_{N25} promoter fragment were incubated at 37° C. for 3 minutes with 100 µl of BB containing 120 mM KCl and 1 pMole of active polymerase. Thereafter, the reaction was stopped and the batch was worked-up as above described above.

The maximal bindable radioactivity (A_O) corresponds to the amount of promoter fragments used (or 100% RNAP/P_{N25} complexes). The difference between A_O and the bound radioactivity (X) at time t is a relative measurement for the free promoter fragment at time t. The expression $(A_O - X)/A_O$ thus gives the amount of free promoter fragment at time t. The expression

16

$-\ln[(A_O - X)/A_O]$ was calculated for all partial experiments and plotted against the reaction time t (see FIG. 16). The slope m of the regression line corresponds to the velocity constants m of equations (3) and (4) from paragraph 1 and was calculated to be m=0.12/second. For an active polymerase concentration of 0.42 nM (determined as in paragraph B, 4), a K_a of 2.8×10^8 M^{-1} sec^{-1} was obtained using the relationship $K_a = m/R$ (equation (2) in paragraph 1).

Experiments 2 and 3

Experiments 2 and 3 were carried out in a manner analogous to Experiment 1, in which the reactants based on the reaction solutions were present in the following concentrations:

Experiment 2	Promoter fragment P_{N25}:	0.02 nM
	Active RNAP:	0.32 nM
	Reaction times	2, 4, 8, 10, 15, 20 and 60 seconds
Experiment 3	Promoter fragment P_{N25}:	0.02 nM
	Active RNAP:	0.15 nM
	Reaction times	2, 4, 8, 10, 15, 20 and 60 seconds

The numerical values were evaluated (see FIG. 17 and 18) as described for Experiment 1, whereby the following values were determined:

Experiment 2 m=0.104/sec.:

$$K_a = 3.2 \times 10^8 \ M^{-1} \ sec^{-1}$$

Experiment 3 m=0.04/sec.:

$$K_a = 2.7 \times 10^8 \ M^{-1} \ sec^{-1}$$

Averaging the results of the three experiments, the K_a for RNAP and promoter P_{N25} was found to be 2.9×10^8 M^{-1} sec^{-1}.

This value is subject to an error of approximately 15% since there are errors of measurement in the concentration determination of promoter fragments and selective RNAP and in the filter binding experiments of about 10%.

C. Determination of the Association Rates for RANP and Various Expression Control Sequences By Relative Measurements With Promotors P_{N25} and P_{AI} as Internal Standards

1. Theory

The complex formation rates for RNAP and the expression control sequences tacOP29, N25*/O, N25OP29, N25OPSN25Op29, AI, AIOPSAL, AIOPSCONA1, AIpOPSAL and OPAIOPSAl were determined by relative measurements using promoters P_{N25} and P_{AI} as internal standards. The following factors were considered in making these determinations.

Different promoters in a mixture compete according to their complex formation rates with RNAP, when RNAP is present in excess.

The change in promoter concentration per unit time of a bimolecular reaction of the second order between RNAP and promoter is defined by equation (1), $dP/dt = -K_a \times R \times P$, for formed complexes having a high half life.

In a reaction mixture containing different promoters, the concentration of free RNAP at any point in time is the same for all promoters. Accordingly, the following relationships apply for two promoters a and b having association rates $K_{a,a}$ and $K_{a,b}$:

5,362,646

17

$$-dP_a/P_a \times 1/K_{a,a} = R \times dt$$

$$-dP_b/P_b \times 1/K_{a,b} = R \times dt$$

Since $R \times dt$ is the same for both promoters,

$$-dP_a/P_a \times 1/K_{a,a} = -dP_b/P_b \times 1/K_{a,b}$$

or,

$$-dP_a/P_a = -K_{a,a}/K_{a,b} \times dP_b/P_b$$

After integration:

$$-\ln[(A_O-x)/A_O] = -K_{a,a}/K_{a,b} \times \ln[(B_O-y)/B_O]$$

or

$$K_{a,b} = K_{a,a} \times -\ln[(B_O-y)/B_O]/-\ln[(A_O-x)/A_O] \qquad (6),$$

with A_O and B_O being the total amount of promoters a and b, respectively, bound in the RNAP excess, and with x and y being the amount of complexes from RNAP and promoter a and from RNAP and promoter b, respectively, formed in the RNAP excess.

2. Determination of Association Rates with Promoter P_{N25} as the Internal Standard

Radioactively-labelled DNA fragments having the corresponding expression control sequences (see Example 2) were incubated in parallel batches in a volume of 30 μl with different amounts of RNAP for 2 minutes at 37° C. in binding buffer. Then, 20 μl of binding buffer (37° C.) with 1 μg of single-stranded M13mp8-DNA were added to stop the association reaction. After a further 2 minutes at 37° C., the batches were filtered through a nitrocellulose filter.

The complexes retained on the filter were eluted and, after extraction with phenol, precipitated with ethanol. The DNA was taken up in 30 μl of sample buffer, and a third of the DNA was electrophoresed in 6% polyacrylamide gels with 8.3M urea and visualized via autoradiography as described by Maniatis et al., supra.

For each ratio of RNAP to promoter, the amount of bound promoter (X for P_{N25}, Y for the promoter to be measured) was determined for each promoter. For this purpose, the individual traces in the autoradiograms, which in each case corresponded to an experiment with a specific amount of RNAP, were measured densitometrically at a wavelength of 400 nm in an Elscript 400 densitometer (Hirschmann, Unterhachingen, BRD). From traces whose ratio of RNAP to promoter was greater than/equal to 1, average values for the total amounts of promoters capable of binding (A_O for P_{N25}, B_O for the promoter to be measured) were obtained.

To determine the association rates, the values for $-\ln((A_O-x)/A_O)$ and $-\ln((B_O-y)/B_O)$ were calculated and plotted against one another for each RNAP/promoter ratio. The slope of the lines m obtained corresponds to the expression $-\ln((B_O-y)/B_O)/-\ln[(A_O-x)/A_O]$ from equation (6). With the aid of this value and the association rate of promoter P_{N25}, the association rate for the measured promoter was obtained using equation (6).

To determine the association rate for promoter Al, seven parallel batches were incubated with approximately 0.02 pMole of fragment having promoter P_{N25} (~10,000 cpm) and approximately 0.02 pMole of fragment having promoter P_{Al} (~10,000 cpm), with different amounts of RNAP (0.004–0.012 pMole). The RNAP/promoter ratios, the densitometric values ob-

18

tained and the values calculated therefrom are presented in Table 1 for the individual experiments.

TABLE 1

RNAP/ Promoter Ratio	Densitometric Values[b] For		Values For	
	P_{N25}	P_{Al}	$-\ln[(A_O-x)/A_O]$	$-\ln[(B_O-y)/B_O]$
3	25,338[a]	26,844[a]		
1.5	30,331[a]	30,510[a]		
1.0	25,361[a]	22,977[a]		
0.5	23,085 (x1)	71,586 (y1)	1.97	1.05
0.3	15,729 (x2)	10,843 (y2)	0.89	0.51
0.2	13,226 (x3)	9,296 (y3)	0.67	0.41
0.1	7,203 (x4)	3,535 (y4)	0.31	0.14

[a]Values for determining the average value A_O and B_O, respectively, of promoter PN25 and PAl, respectively.
[b]400 nm reading of Elscript 400 densitometer

The values presented in Table 1 are represented graphically in FIG. 19. The value m=0.5 was obtained for the slope of the lines. Using that value and equation (6):

$$K_{a,Al} = K_{a,N25} \times m \quad (K_{a,N25} = 2.9 \times 10^8 \, M^{-1} \, sec^{-1})$$

$$K_{a,Al} = 2.9 \times 0.5 \times 10^8 \, M^{-1} \, sec^{-1})$$

$$K_{a,Al} = 1.5 \times 10^8 \, M^{-1} \, sec^{-1}$$

The following association rates were obtained for the remaining expression control sequences.
tacOP29:

$$K_a = 0.85 \times 10^8 \, M^{-1} \, sec^{-1}$$

N25*/0:

$$K_a = 2.9 \times 10^8 \, M^{-1} \, sec^{-1}$$

N25OP29:

$$K_a = 2.9 \times 10^8 \, M^{-1} \, sec^{-1}$$

N25OPSN25OP29:

$$K_a = 2.9 \times 10^8 \, M^{-1} \, sec^{-1}$$

These values are subject to an error of approximately 15%.

3. Determination of the Association Rates with Promotor P_{Al} as the Internal Standard

The association rates of the expression control sequences A1OPSA1, A1OPSCONA1, AlpOPSA 1 and OPA1OPSA1 were determined with promoter P_{Al} as the internal standard as described in paragraph C.2. To determine the association rate for element A1OPSA1, the RNAP/promoter ratios, the densitometric values obtained and the values calculated therefrom were obtained from four parallel experiments carried out with different amounts of RNAP, with the results shown in Table 2:

TABLE 2

RNAP/ Promoter Ratio	Densitrometric Values[a] For		Values For	
	P_{N25}	P_{Al}	$-\ln[(A_O-x)/A_O]$	$-\ln[(B_O-y)/B_O]$
3	61,856 (A_O)	69,018 (B_O)	0.00	0.00
0.5	41,477	24,983	1.11	0.45

5,362,646

19

TABLE 2-continued

RNAP/ Promoter Ratio	Densitrometric Values^a For P_{N25}	P_{A1}	Values For $-\ln[(A_O-x)/A_O]$	$-\ln[(B_O-y)/B_O]$
	(x_1)	(y_1)		
0.2	22,484	9,054	0.45	0.14
	(x_2)	(y_2)		
0.1	11,303	5,517	0.20	0.08
	(x_3)	(y_3)		

^{a}400 nm reading of Elscript 400 densitometer

The values presented in Table 2 are represented graphically in FIG. 20. The value m=0.4 was obtained for the slope of the lines. Using that value and equation (6):

$$K_{a, A1OPSA1}=K_{a,A1}\times m(K_{a, A1}=1.5\times10^8 \ M^{-1} sec^{-1})$$

$$K_{a, A1OPSA1}=1.5\times0.4\times10^8 \ M^{-1} sec^{-1}$$

$$K_{a, A1OPSA1}=0.6\times10^8 \ M^{-1} sec^{-1}$$

The following association rates were obtained for the remaining expression control sequences:

A1OPSCONA1:

$$K_a=1.7\times10^8 \ M^{-1} sec^{-1}$$

A1pOPSA1:

$$K_a=0.6\times10^8 \ M^{-1} sec^{-1}$$

OPA1OPSA1:

$$K_a=0.6\times10^8 \ M^{-1} sec^{-1}$$

These values are subject to an error of approximately 15%.

Example 4

Determination of the In Vivo Promoter Strengths (induced) of the Expression Control Sequences

A. Principles

The in vivo promoter strengths of the expression control sequences were determined relative to the promoter for the β-lactamase gene (P_{bla}), using that promoter as an internal standard according to the procedure of Deuschle et al., supra. For this purpose, pDS3 derivatives containing the corresponding expression control sequences were transformed into E. coli M15 cells which contained plasmid pDMI,1. Radioactively labelled RNA, synthesized in the presence of IPTG (inductor) during a specified time, was then isolated from cultures of these transformants and hybridized in separate batches against the following single-stranded DNA, probes present in excess: 1) M13mp9dhfr-DNA, 2) M13mp9bla-DNA and 3) M13mp9-DNA, as the control. After RNase treatment to degrade non-hybridized RNA the batches were filtered through a nitrocellulose filter.

Since RNA/DNA hybrids are retained on nitrocellulose, the filter-bound radioactivity was a measurement of the amount of RNA present in the individual batches, which was complimentary to the DNA probes used. After deducting the radioactivity bound in the control (single-stranded M13mp9-DNA), the ratio of radioactivity bound by single-stranded M13mp9dhfr-DNA (the corresponding RNA was synthesized under control of the expression control sequence to be measured) to radioactivity bound by single-stranded M13mp9bla-

20

DNA (this RNA was synthesized under control of P_{bla}) was determined. After a necessary correction this ratio gave the promoter strength of the measured expression control sequence in P_{bla} units.

B. Production of Single-stranded M13mp9, M13mp9dhfr and M13mp9bla Phage DNAs

To produce phage M13mp9dhfr, the BamHI-HindIII fragment of plasmid pDS1,t$_o$1$^+$, containing the dhfr gene, was integrated into the DNA of phage M13mp9 (Pharmacia Sweden; cleaved with BamHI and HindIII) according to known methods (Maniatis et al., supra). Phage M13mp9bla was produced in an analogous manner, whereby the EcoRI-PstI fragment from plasmid pDS1,t$_o$1$^+$ was integrated with parts of the bla gene in DNA of phage M13mp9 (cleaved with EcoRI and PstI). Thereafter, single-stranded M13mp9-DNA, M13mp9dhfr-DNA and M13mp9bla-DNA was produced in a manner analogous to that described in Example 3 for the production of single-stranded M13mp8-DNA.

C. Determination of In Vivo Promotor Strengths (induced)

The determination of the promoter strengths was carried out as follows according to the procedure of Deuschle et al., supra:

1) Production of In Vivo Labelled ^{3}H-RNA

E. coli M15 cells containing plasmid pDMI,1, and a pDS3 derivative containing one of the various expression control sequences (see Example 2), were stored at $-20°$ C. in 20% glycerol. 10 ml of LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin were inoculated with one of these stock cultures and grown at 37° C. overnight in a shaking incubator (180 rpm). 0.1 ml of this overnight culture was diluted in 25 ml of M9 minimal medium (Miller, supra), containing 5% casein hydrolysate, 0.1% bactotryptone, 0.05% yeast extract, 0.05% NaCl, 0.5% glycerol, 1 mM IPTG, 100 μg/ml ampicillin and 25 μg/ml kanamycin, which had been pre-warmed to 37° C. The cells were grown at 37° C. in a shaking incubator (250 rpm) to an optical density of $OD_{600}=0.6$.

To 10 ml of this culture were added 0.5 mCi of 5,6-^{3}H-uridine (40–60 mCi/mmol, 1 mCi/ml aqueous solution; Amersham, Braunschweig, FRG). After 45 seconds the culture was cooled rapidly to 0° C. with liquid nitrogen and the cells were centrifuged and re-suspended in TES buffer (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl , 1% SDS). After incubation at 95° C. for 3 minutes, the resulting mixture of lysed cells was centrifuged as described by Glisin et al. (Biochemistry 13, 2633–2637 [1974]) (CsCl gradient centrifugation, 150,000× g, 16 hours, 20° C.).

After removing the supernatant the centrifuge test tubes were cut 0.8 cm above the bottom with a heated scalpel. The RNA in the bottom part of the test tube was dissolved with 2×80 μl of TE buffer containing 0.2% SDS and precipitated with ethanol in the presence of 3M sodium acetate. The precipitate was washed with 80% ethanol, dried in a vacuum and dissolved in hybridization buffer (see below). As a rule, 200–300 μg of RNA having a specific activity of 1–3×10^5 cpm/μg RNA were obtained from 10 ml of culture.

2. Hybridization of the RNA to An Excess of Single-stranded DNA

All hybridizations were carried out for 2 hours at 42° C. in 20 μl of hybridization buffer (50% formamide, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA).

5,362,646

21

In a typical experiment, 10 μl of the in vivo ^{3}H-RNA ($\sim 5 \times 10^5$ cpm) were mixed with 10 μl of single-stranded M13mp9-DNA (0.2 pMoles, control), M13mp9dhfr-DNA (0.2 pMoles) and M13mp9bla-DNA (0.2 pMoles), respectively, incubated at 65° C. for 3 minutes and then held at 42° C. for 2 hours.

3) Quantification of the Hybridized RNA

The hybridization batches were diluted ten-fold with 2×SSC buffer and filtered through a nitrocellulose filter (0.45 μm BA85 filter, minifold system, Scheicher and Schüll, FRG). The capacity of the filter used for single-stranded M13mp9-DNA was approximately 6 pMole/cm^2. The filter was washed with 2 ml of 2×SSC buffer, baked at 80° C. for 30 minutes in a vacuum and then incubated for 1 hour at 42° C. in 100 ml of 2×SSC buffer containing 50 μg/ml of RNase A. Subsequently, the filter was washed three times with 100 ml of 2×SSC buffer during 10 minutes at 42° C. After drying the filter, the radioactivity retained was counted in a scintillation liquid ("universal liquid scintillator", NEN). The ratio of dhfr- to bla-specific RNA was calculated taking into account the number of uridines within the various RNAs. Since the single-stranded DNA inserts specific for dhfr and bla code respectively for 169 and 148 uridines (148/169=0.87), the in vivo promoter strength S of a desired expression control sequence in P$_{bla}$ units is obtained using the formula:

$$S = 0.87 \times (cpm_{dhfr} - cpm_{control})/(cpm_{bla} - cpm_{control}).$$

D. In Vivo Promoter Strengths of the Expression Control Sequences

Determination of the in vivo promoter strength for each of the various expression control sequences was carried out at least three times as described in paragraph C, whereby the radioactively labelled RNA synthesized was determined in each case twice. For the expression control sequence A1OPSA1, the following values in the determination of the synthesized RNA were obtained:

Measurement No.	M13mp9-DNA	Single stranded M13mp9dhrf-DNA	M13mP9bla-DNA
1	38	33,581	839
2	22	36,099	831

Using the foregoing data, the following values were obtained for the in vivo promoter strengths:

$$S_1 = 0.87 \times (33,581 - 38)/(839 - 38) = 36.4 \ P_{bla} \text{ units.}$$

$$S_2 = 0.87 \times (36,099 - 22)/(831 - 22) = 38.8 \ P_{bla} \text{ units.}$$

An in vivo promoter strength of 37.6 P$_{bla}$ units was calculated as the average value.

Averaged over a total of three determinations, a promoter strength of 38.1±3.4 P$_{bla}$ units was calculated for the expression control sequence A1OPSA1. The promoter strengths determined as described previously for all tested expression control sequences are presented in Table 3.

TABLE 3

Expression Control Sequence	In Vivo Promoter Strength Induced [Pbla units]
lacOP29	5.5 ± 1.0
tacOP29	17.6 ± 1.8
N25	26.2 ± 2.0

22

TABLE 3-continued

Expression Control Sequence	In Vivo Promoter Strength Induced [Pbla units]
N25*/O	8.0 ± 1.5
N25OP29	7.7 ± 1.3
N25OPSN25OP29	9.3 ± 1.2
A1	66.1 ± 2.5
A1OPSA1	38.1 ± 3.4
A1OPSCONA1	16.8 ± 2.0
A1POPSA1	25.1 ± 1.3
OPA1OPSA1	38.1 ± 3.4
A1OPSA1OP21	32.9 ± 5.0
A1OPSA1OP29	31.5 ± 3.0

Example 5

Determination of the Repression Factor of the Various Expression Control Sequences

A. Principles

The repression factors, i.e., the ratio of the in vivo promoter strengths under induced and repressed conditions for the individual expression control sequences, were determined as follows:

a) Expression Control Sequences Having a Repression Factor of Less Than 100

The expression control sequences tacOP29, N25*/O, N25OP29, OPUA1 and OPUA1CON initiated transcription of sufficient amounts of RNA in vivo under repressed conditions (excess of repressor, no inducer) so that they could be determined directly. The repression factors were then calculated using the values determined in Example 4 for the in vivo promoter strengths (induced).

b) Expression Control Sequences Having a Repression Factor of Greater Than 100

The in vivo promoter strengths for the expression control sequences lacOP29, N25OPSA25OP29, A1OPSA1, A1OPSA1OP21, A1OPSA1OP29, A1OPSCONA1, A1pOPSA1 and OPA1OPSA1 were determined indirectly under repressed conditions. For this purpose, for the individual elements the amount of β-galactosidase which was produced in a suitable system under repressed conditions was first determined by means of an enzymatic test. The values obtained were then converted into P$_{bla}$ units by means of a correction factor. The repression factors were then calculated using the values determined in Example 4.

Direct Determination of the In Vivo Promoter Strengths (P$_{bla}$ units) of the Expression Control Sequences Under Repressed Conditions

The in vivo promoter strengths under repressed conditions were determined as described in Example 4, but without the addition of IPTG. The values obtained are compiled in Table 4:

TABLE 4

Expression Control Sequence	Promoter Strengths Repressed [P$_{bla}$ units]
tacOP29	0.36 ± 0.02
N25OP29	1.5 ± 0.1
N25*/O	1.5 ± 0.1
OPUA1	2.7 ± 0.5
OPUA1CON	1.7 ± 0.6

C. Indirect Determination of the In Vivo Promoter Strengths of the Expression Control Sequences Under Repressed Conditions

1. Determination of β-Galactosidase Units

5,362,646

23   24

The pML3 derivatives having the expression control sequences lacOP29, tacOP29, N25OP29, N25OPSN-25OP29, A1OPSA1, A1OPSA1OP21, A1OPSA1OP29, A1OPSCONA1, AlpOPSA1, OPA1OPSA1, OPUA1 and OPUA1CON were transformed into *E. coli* M15 cells as described by Maniatis et al., supra. The transformed *E. coli* M15 cells obtained having the corresponding plasmids were then grown to the logarithmic phase, and the amount of β-galactosidase was determined according to the procedure of Miller ("Experiments in Molecular Genetics", Cold Spring Harbor, N.Y., 1972). One such determination is described below as an example for the expression control sequence N25OP29.

10 ml of supplemented minimal medium (Miller, supra) were inoculated with 0.05 ml of an overnight culture of transformed *E. coli* M15 cells containing plasmid pML3/N25OP29 and incubated at 37° C. in a shaking incubator (200 rpm). After attaining an OD_{600} (optical density at a wavelength of 600 nm) of 0.55, the culture was placed on ice for 20 minutes, after which the optical density was again determined. A value of $OD_{600}=0.607$ was obtained. 0.1 ml of the culture were then diluted with Z buffer (Miller, supra) to a final volume of 1 ml. This sample was treated as follows, together with a control (1 ml of Z buffer):

Addition of 60 μl of chloroform and 30 μl of 0.1% SDS (cell rupture);

mixing of the sample for 10 seconds by means of a vortex;

incubation of the sample at 28° C. for 5 minutes;

addition of 200 μl of ONPG (Miller, supra);

mixing of the sample by means of a vortex;

incubation of the sample at 28° C. until a yellow color was visible;

addition of 250 μl of 2M Na_2CO_3 (the time between the addition of ONPG and Na_2CO_3 amounted to 1.5 minutes);

mixing the sample by means of a vortex;

centrifugation of the cell fragments (Eppendorf bench centrifuge, 13000 rmp 5 minutes); and

determination of the extinction at 420 nm against the control.

A value of $\Delta E_{420}=0.737$ was obtained. According to the formula described by Miller, supra:

$$\beta\text{-Galactosidase units} = 1000 \times \Delta E_{420}/OD_{600} \times V \times t$$

wherein ΔE_{420} is the E_{420} measured value of the reaction batch against the control (0.737), OD_{600} is the cell density of the culture sample used (0.607), t is the reaction time (1.5 minutes) and V is the volume of culture used (0.1 ml),

8,094 β-galactosidase units were calculated. Averaged over 8 experiments 8,160$\pm$450 β-galactosidase units were obtained.

The values obtained for all measured expression control sequences are compiled in Table 5, whereby at least 4 measurements were carried out for each expression control sequence.

TABLE 5

Expression Control Sequence	β-Galactosidase Units
lacOP29	30 ± 5
tacOP29	1510 ± 170
N25OP29	8160 ± 450

TABLE 5-continued

Expression Control Sequence	β-Galactosidase Units
N25OPSN25OP29	99 ± 6
A1OPSA1	110 ± 2
A1OPSCONA1	220 ± 12
A1POPSA1	58 ± 3
OPA1OPSA1	56 ± 1
OPUA1	13700 ± 3000
OPUA1CON	8500 ± 2000
A1OPSA1OP29	23 ± 3
A1OPSA1OP21	15 ± 3

2. Determination of the Factor For the Conversion of β-Galactosidase Units Into P_{bla} Units

The in vitro promoter strengths under repressed conditions for the expression control sequences tacOP29, N25OP29, OPUA1 and OPUA1CON could be determined directly (P_{bla} units, Table 4) and indirectly (β-galactosidase units, Table 5). The values obtained are plotted against each other in FIG. 21. The slope of the regression line is a measurement for the conversion of β-galactosidase units into P_{bla} units. A value of 5,000 β-galactosidase units/P_{bla} unit was obtained.

3. Conversion of β-Galactosidase Units in P_{bla} Units

The P_{bla} units (Table 6) were calculated for the corresponding expression control sequences from the measured β-galactosidase units (Table 5) using the conversion factor 5,000 β-galactosidase units/P_{bla} unit.

TABLE 6

Expression Control Sequence	β-Galactosidase Units	P_{bla} Units
lacOP29	30 ± 5	0.006 ± 0.001
N25OPSN25OP29	99 ± 6	0.02 ± 0.003
A1OPSA1	110 ± 2	0.022 ± 0.001
A1OPSCONA1	220 ± 12	0.044 ± 0.003
A1POPSA1	58 ± 3	0.012 ± 0.001
OPA1OPSA1	56 ± 1	0.011 ± 0.001
A1OPSA1OP29	23 ± 3	0.0046 ± 0.0006
A1OPSA1OP21	15 ± 3	0.003 ± 0.0006

D. Calculation of the Repression Factors

The values listed in Tables 3, 4 and 6 were used to calculate the repression factors (P_{bla} units, induced/P_{bla} units, repressed) for the individual expression control sequences (Table 7).

TABLE 7

Expression Control Sequence	In Vivo Promoter Strengths		Repression Factor
	P_{bla} Units Induced	P_{bla} Units Repressed	
lacOP29	5.5 ± 1.0	0.006 ± 0.001	920 ± 230
tacOP29	17.6 ± 1.8	0.36 ± 0.02[a]	49 ± 6
N25°/O	8.0 ± 1.5	1.5 ± 0.1[a]	5.3 ± 2
N25OP29	7.7 ± 1.3	1.5 ± 0.1[a]	5.1 ± 2
N25OPSN25OP29	9.3 ± 1.2	0.02 ± 0.003	465 ± 92
A1OpSA1	38.1 ± 3.4	0.022 ± 0.001	1730 ± 170
A1OPSCONA1	16.8 ± 2.0	0.044 ± 0.003	380 ± 50
A1POPSA1	2.51 ± 1.3	0.012 ± 0.001	2090 ± 240
OPA1OPSA1	38.1 ± 3.4	0.011 ± 0.001	3460 ± 460
A1OPSA1OP29	3.15 ± 3.0	0.0046 ± 0.0006	6850 ± 610
A1OPSA1OP21	32.9 ± 5.0	0.003 ± 0.0006	10970 ± 2000

[a]direct determination of the in vivo promoter strength

E. Compilation of the Characteristic Properties of the Individual Expression Control Sequences

The values for the in vitro association rate, the in vivo promoter strength and the repression factor obtained for the individual expression control sequences are shown in Table 8.

5,362,646

25   26

TABLE 8

Expression Control Sequence	In Vitro Complex Formation Rate (K_a) [$10^8 M^{-1} sec^{-1}$] (Signal strength)	In Vivo Promoter Strengths		Repression Factor
		Repressed [P_{blu}-units]	Induced [P_{blu} units]	
lacOP29	0.02[a]	0.006 ± 0.001	5.5 ± 1.0	920 ± 230
tacOP29	0.85	0.36 ± 0.02	17.6 ± 1.8	49 ± 6
N25	2.9	—	26.2 ± 2.0	—
N25*/0	2.9	1.5 ± 0.1	8.0 ± 1.5	5.3 ± 2
N25OP29	2.9	1.5 ± 0.1	7.7 ± 1.3	5.1 ± 2
N25PSN25OP29	2.9	0.2 ± 0.003	9.3 ± 1.2	465 ± 92
A1	1.5	—	66.1 ± 2.5	—
A10PSA1	0.6	0.022 ± 0.001	38.1 ± 3.4	1730 ± 170
A10PSC0NA1	1.7	0.044 ± 0.003	16.8 ± 2.0	380 ± 50
A1pOPSA1	0.6	0.012 ± 0.001	25.1 ± 1.3	2090 ± 240
APA1OPSA1	0.6	0.011 ± 0.001	38.1 ± 3.4	3460 ± 460
A10PSA10P29	N.D.	0.0046 ± 0.0006	31.5 ± 3.0	6850 ± 610
A10PSA10P21	N.D.	0.003 ± 0.006	32.9 ± 5.0	10970 ± 2000

[a]Literature value from McClure et al. ("Promoters, Structure and Function", Hrsg. Rodriguez and Chamberlin, Praeger, pages 111-120 [1982])
N.D. = Not determined

What is claimed is:

1. A DNA expression control sequence comprising a T-coliphage promoter sequence having a low signal strength in the induced state and a high in vivo promoter strength combined with a lac-operator sequence from a lac-operator/repressor system wherein said system has a high association rate prior to said combination with said promoter.

2. The expression control sequence of claim 1 which contains a T7A1 promoter and a lac-operator sequence.

3. The expression control sequence of claim 2 which is A1OPSA1 and contains the nucleotide sequence

```
CTCGAGAAAA TTTATCAAAA AGAGTGTTGA CTTGTGAGCG GATAACAATG
ATACTTAGAT TCATCGAGAG GGACACGGCG AATTC.
```

4. The expression control sequence of claim 2 which is A1POPSA1 and contains the nucleotide sequence

```
CTCGAGAAAA TTTATCAAAA AGAGTGTTGA CTTGTGAGCG CTCACAATTG
ATACTTAGAT TCATCGAGAG GGACACGGCG AATTC.
```

5. The expression control sequence of claim 2 which is OPA1OPSA1 and contains the nucleotide sequence

```
GTCGACGTTG ATCCCCTAGA AATTGTGAGC GCTCACAATT TCTAGGGATT
TAACGGTACC GAGCTTGTGG CAGTTTAAGG CGGGCGTCCT GCCCGCCACC
CTCCGGGCCG TTGCTTCGCA ACGTTCAAAT CCGCTCCCGG CGGATTTGTC
CTACTCAGGA GAGCGTTCAC CGACAAACAA CAGATAAAAC GAAAGGCCCA
GTCTTTCGAC TGAGCCTTTC GTTTTATTTG ATGCCTCAAG CTCGGTACCT
CGAGAAAATT TATCAAAAAG AGTGTTGACT TGTGAGCGGA TAACAATGAT
ACTTAGATTC ATCGAGAGGG ACACGGCGAA TTC.
```

6. The expression control sequence of claim 2 which is A1OPSA1OP21 and contains the nucleotide sequence

```
CTCGAGAAAA TTTATCAAAA AGAGTGTTGA CTTGTGAGCG GATAACAATG
ATACTTAGAT TCAATTGTGA GCGGATAACA ATTTCACACA GAATTC.
```

7. The expression control sequence of claim 2 which is A1OPSA1OP29 and contains the nucleotide sequence

```
CTCGAGAAAA TTTATCAAAA AGAGTGTTGA CTTGTGAGCG GATAACAATG
ATACTTAGAT TCAAATTGTG AGCGGATAAC AATTTGAATT C.
```

8. A DNA expression vector which can replicate in E. coli containing 1) an expression control sequence comprising a T-coliphage promoter sequence having a low signal strength in the induced state and a high in vivo promoter strength combined with a lac-operator sequence from a lac-operator/repressor system wherein said system has a high association rate prior to said combination with said promoter; and 2) a sequence which codes for the lac-repressor polypeptide of the lac-operator/repressor system.

9. A non-naturally occurring E. coli bacterium which contains a 1) an expression control sequence comprising a T-coliphage promoter sequence having a low signal strength in the induced state and a high in vivo promoter strength combined with a lac-operator sequence from a lac-operator/repressor system wherein said system has a high association rate prior to said combination with said promoter; and 2) a sequence which codes for the lac-repressor polypeptide of the lac-operator/-repressor system.

10. A non-naturally occurring E. Coli bacterium, the chromosome of which has be modified to contain 1) a heterologous DNA expression control sequence comprising a T-coliphage promoter sequence having a low signal strength in the induced state and a high in vivo promoter strength combined with a lac-operator sequence from a lac-operator/repressor system wherein said system has a high association rate prior to combination with said promoter; and 2) a sequence which codes for the lac-repressor polypeptide of the lac-operator/-repressor system.

* * * * *