

such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook et al., *Molecular Cloning: A Laboratory Manual*, New York: Cold Spring Harbor Laboratory Press, (1989), is generally used for bacterial cells that contain substantial cell-wall barriers. Another method for transformation employs polyethylene glycol/DMSO, as described in Chung and Miller (Chung and Miller, *Nucleic Acids Res.* 16:3580 (1988)). Yet another method is the use of the technique termed electroporation. In addition, bacterial cells can be readily transformed using various forms of phages (i.e., transducing, temperate, lytic and lysogenic), suicide vectors for inserting DNA directly into the chromosome, and through homologous recombination using either phages, suicide vectors or linear DNA.

[0172] Bacterial cells used to produce the polypeptide of interest for purposes of this invention are cultured in suitable media in which the promoters for the nucleic acid encoding the heterologous polypeptide can be artificially induced as described generally, e.g., in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, New York: Cold Spring Harbor Laboratory Press, (1989). Examples of suitable media are given in U.S. Pat. Nos. 5,304,472 and 5,342,763.

[0173] A yeast recombinant construct can typically include one or more of the following: a promoter sequence, fusion partner sequence, leader sequence, transcription termination sequence, a selectable marker. These elements can be combined into an expression cassette, which may be maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as yeast or bacteria. The replicon may have two replication systems, thus allowing it to be maintained, for example, in yeast for expression and in a procaryotic host for cloning and amplification. Examples of such yeast-bacteria shuttle vectors include YEp24 (Botstein et al., *Gene*, 8:17-24 (1979)), pC1/1 (Brake et al., *Proc. Natl. Acad. Sci. USA*, 81:4642-4646 (1984)), and YRp17 (Stinchcomb et al., *J. Mol. Biol.*, 158:157 (1982)). In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and typically about 10 to about 150. A host containing a high copy number plasmid will preferably have at least about 10, and more preferably at least about 20.

[0174] Useful yeast promoter sequences can be derived from genes encoding enzymes in the metabolic pathway. Examples of such genes include alcohol dehydrogenase (ADH) (E.P.O. Pub. No. 284044), enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofruktokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK) (E.P.O. Pub. No. 329203). The yeast PHO5 gene, encoding acid phosphatase, also provides useful promoter sequences (Myanohara et al., *Proc. Natl. Acad. Sci. USA*, 80:1 (1983)). In addition, synthetic promoters which do not occur in nature also function as yeast promoters. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region (U.S. Pat. Nos. 4,876,197 and 4,880,734). Other examples of hybrid promoters include promoters which consist of the regulatory sequences of either the ADH2, GAL4, GAL10, or PHO5 genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as GAP or PyK (E.P.O. Pub. No. 164556). Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate tran-

scription. Examples of such promoters include, inter alia, (Cohen et al., *Proc. Natl. Acad. Sci. USA*, 77:1078 (1980); Henikoff et al., *Nature* 283:835 (1981); Hollenberg et al., *Curr. Topics Microbiol. Immunol.*, 96:119 (1981); Mercerau-Puigalon et al., *Gene*, 11:163 (1980); and Panthier et al., *Curr. Genet.*, 2:109 (1980)).

[0175] Intracellularly expressed fusion proteins provide an alternative to direct expression of the polypeptides of interest. Typically, a DNA sequence encoding the N-terminal portion of a stable protein, a fusion partner, is fused to the 5' end of heterologous structural nucleotide sequence encoding the desired polypeptide. Upon expression, this construct will provide a fusion of the two amino acid sequences. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. See, e.g., E.P.O. Pub. No. 196056. Another example is a ubiquitin fusion protein. Such a ubiquitin fusion protein preferably retains a site for a processing enzyme (e.g. ubiquitin-specific processing protease) to cleave the ubiquitin from the polypeptide of the present invention. Through this method, therefore, a mature polypeptide can be isolated [see, P.C.T. WO 88/024066].

[0176] Alternatively, polypeptides or proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion in yeast of the polypeptides. Preferably, there are processing sites encoded between the leader fragment and the polypeptide-encoding sequence fragment that can be cleaved either in vivo or in vitro. The leader sequence fragment typically encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell.

[0177] DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the yeast invertase gene (E.P.O. Pub. No. 12873; J.P.O. Pub. No. 62,096,086) and the A-factor gene (U.S. Pat. No. 4,588,684). Alternatively, leaders of non-yeast origin, such as an interferon leader, exist that also provide for secretion in yeast (E.P.O. Pub. No. 60057).

[0178] A preferred class of secretion leaders are those that employ a fragment of the yeast alpha-factor gene, which contains both a "pre" signal sequence, and a "pro" region. The types of alpha-factor fragments that can be employed include the full-length pre-pro alpha factor leader (about 83 amino acid residues) as well as truncated alpha-factor leaders (typically about 25 to about 50 amino acid residues) (U.S. Pat. Nos. 4,546,083 and 4,870,008; and E.P.O. Pub. No. 324274). Additional leaders employing an alpha-factor leader fragment that provides for secretion include hybrid alpha-factor leaders made with a pre-sequence of a first yeast, but a pro-region from a second yeast alpha factor. See, e.g., P.C.T. WO 89/02463.

[0179] Examples of transcription terminator sequence and other yeast-recognized termination sequences, such as those coding for glycolytic enzymes, are known to those of skill in the art.

[0180] Alternatively, the expression constructs can be integrated into the yeast genome with an integrating vector. Integrating vectors typically contain at least one sequence homologous to a yeast chromosome that allows the vector to integrate, and preferably contain two homologous sequences flanking the expression construct. Integrations appear to result from recombinations between homologous DNA in the vector and the yeast chromosome (Orr-Weaver et al., Meth-