Improving Antibody Expression using Vectors Containing Insulator Elements

BACKGROUND

[0001] The current model of chromatin structure in higher eukaryotes postulates that genes are organized in "domains". Chromatin domains can consist of groups of genes that are expressed in a strictly tissue specific manner such as the human β-globin family (Grosveld et al., (1993) Baillieres Clin. Haematol. 6: 31-55), genes that are expressed ubiquitously such as the human TBP/C5 locus (Trachtulec, Z. et al., (1997) Genomics 44: 1-7), or a mixture of tissue specific and ubiquitously expressed genes such as murine γ/δ TCR/dad-1 locus, (Ortiz et al., (1997) EMBO J. 16: 5037-5045) and the human α-globin locus, (Vyas et al., (1992) Cell 69: 781-793). Chromatin domains are believed to exist in either a closed, "condensed", transcriptionally silent state or in a "de-condensed", open and transcriptionally active configuration. The establishment of an open chromatin structure characterized by DNase I sensitivity, DNA hypomethylation and histone hyperacetylation, is seen as a pre-requisite to the commencement of gene expression.

[0002] The development of stable cell lines for producing therapeutic proteins has been hampered by the negative effects of surrounding condensed chromatin on the expression of randomly integrated vector sequences. Variability in expression levels of a heterologous gene transfected into a eukaryotic cell is thought to reflect the influence of the chromatin structure and/or the presence of regulatory elements at the site of integration of the heterologous gene in the host genome, a phenomenon referred to as the "position effect".

[0003] "Insulator" is a name that has been given to a class of DNA elements that possess an ability to protect genes from these negative position effects caused by the surrounding environment. These "insulators" include such elements as boundary elements (BEs), matrix attachment regions (MARs), locus control regions (LCRs), and universal chromatin opening elements (UCOEs). When inserted into the host cell chromosome, these elements have been shown to exert an effect on gene expression of an associated heterologous gene. These elements are potentially capable of overcoming position effects, and hence are of interest for the development of stable cell lines.

[0004] Boundary elements ("BEs") define boundaries in chromatin (Bell and Felsenfeld, 1999; Udvardy, 1999) and may play a role in defining a transcriptional domain *in vivo*.

BEs lack intrinsic promoter/enhancer activity, but rather are thought to protect genes from the transcriptional influence of regulatory elements in the surrounding chromatin, thus preventing unwanted enhancer-promoter communication. An enhancer-block assay is commonly used to identify such insulator elements. In this assay, the insulator is placed between an enhancer and a promoter, and enhancer-activated transcription is measured. Boundary elements have been shown to be able to protect stably transfected reporter genes against position effects in Drosophila, yeast and in mammalian cells (Bi and Broach, 1999; Cuvier et al., 1998; Walters et al., 1999). They have also been shown to increase the proportion of transgenic mice with inducible transgene expression (Wang et al., 1997).

[0005] Matrix Attachment Regions ("MARs"), also known as Scaffold Attachment Regions or Scaffold/Matrix Attachment Regions ("S/MARs"), are DNA sequences that bind isolated nuclear scaffolds or nuclear matrices in vitro with high affinity (Hart and Laemmli, 1998). MAR sequences have been shown to interact with enhancers to increase local chromatin accessibility (Jenuwein et al., 1997). Specifically, MAR elements can enhance expression of heterologous genes in cell culture lines (Kalos and Fournier, 1995; Klehr et al., 1991; Phi-Van et al., 1990; Poljak et al., 1994), transgenic mice (Castilla et al., 1998) and plants (Allen et al., 1996). Another class of MARs has been localized near the boundary of active chromatin domains, defining them as insulators. The most characterized MAR of this type was found at the boundary of the chicken lysozyme locus. The 5'MAR was shown to shield a transgene from position effects and block enhancer action in a position-dependent manner (Stief et al. (1989) Nature 341:343-345.) It has also been shown that the matrix-associating sequence may be separated from the protection effect sequence (Phi-Van et al (1996) Biochem. 35:10735-10742).

[0006] The chicken lysozyme 5' MAR element is able to significantly improve stable transgene expression in CHO cells, a cell line commonly used in recombinant protein production. The chicken lysozyme 5' MAR element is also able to significantly improve transient transfections, particularly when the transfected cells are contacted with butyrate. This chicken MAR element has previously been shown to enhance transcription from a heterologous promoter in heterologous cells (Phi-Van et al., 1990), and to confer position-independent hormonal and developmental regulation of the expression of the whey acidic protein gene in transgenic mice (McKnight et al., 1992).

[0007] Locus control regions ("LCRs") are cis-regulatory elements required for the initial chromatin activation of a locus and subsequent gene transcription in their native locations

(reviewed in Grosveld, 1999). The activating function of LCRs also allows the expression of a coupled transgene in the appropriate tissue in transgenic mice, irrespective of the site of integration in the host genome. While LCRs generally confer tissue-specific levels of expression on linked genes, efficient expression in nearly all tissues in transgenic mice has been reported for a truncated human T-cell receptor LCR (Ortiz et al., 1997) and a rat LAP LCR (Talbot et al., 1994). One extensively characterized LCR is that of the globin locus.

[0008] Ubiquitous chromatin opening elements ("UCOEs", also known as "ubiquitously-acting chromatin opening elements") have been reported (See WO00/05393). A simple and rapid approach to overcome position effects is to make use of chromatin elements that prevent the neighboring chromatin from affecting transgene expression.

[0009] Despite the significant amount of knowledge gained in the area of insulator elements, few studies have addressed the potential of these elements to modify or improve the expression of recombinant protein constructs for the production of biological therapeutics. The elements used to this end should improve the frequency of obtaining high-level expression clones, irrespective of the chromosomal integration site and the number of copies integrated. This effect should not be specific to a particular cell type, but rather should be observed in all cell lines commonly used in biotechnology and gene or cell therapy. Furthermore, the element should act independently of the promoter, enabling it to be used with diverse constructs. Other hurdles created by conventional methods include: low transfection efficiency; random integration results in unpredictable cell properties; many selected cell clones are not stable; developing a generic process is not feasible due to variability in the cell lines; and conventional methods are very time consuming and labor intensive.

SUMMARY OF THE INVENTION

[0010] We have developed a new approach to create cell lines with improved cellular characteristics in cell culture for the production of therapeutic antibodies. An antibody gene of interest is flanked by an element cloned from chicken DNA either at the 5' end, the 3' end or both. The element can be any one of the insulator elements discussed including Boundary Elements, Locus Control Regions, Ubiquitous chromatin opening elements, or Matrix (or Scaffold) Attachment Regions.

[0011] One embodiment of this invention is a vector comprising a chicken MAR element of approximately 1.5 Kb selected from the upstream region of the chicken lysozyme gene. Other MAR elements may be used interchangeably with this particular MAR.

[0012] The expression vectors of the invention comprise a nucleic acid encoding a heavy and/or light chain of the desired antibody to be expressed in a form suitable for expression in a host cell. The vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, and may be operatively-linked to the heterologous gene sequence to be expressed. The antibody may be a single-chain antibody, a single domain antibody, or fragments of the heavy or light chain sequences, including Fab.

[0013] Another aspect of the invention is the improved expression and production of the recombinant antibody. The insulator element's ability to prevent silencing of the transcription of the encoded antibody improves the production of the recombinant product.

[0014] Another aspect of the present invention is the improved survival of the cell due to the presence of the MAR element. Improved cell survival will also improve the amount of production of the recombinant product because more cells survive longer during the culturing and production process.

BRIEF DESCRIPTION OF THE FIGURES

[0015] Figure 1 depicts vectors encoding either the heavy chain or light chain immunoglobulin flanked by two MAR elements.

[0016] Figure 2 depicts vectors encoding either the heavy chain or light chain immunoglobulin having a single MAR element.

[0017] Figure 3 shows the increased cell survival rate of cells containing a vector encoding an antibody that contained (1) No MAR element (2) One MAR element, or (3) Two MAR elements.

[0018] Figure 4 shows the increased antibody expression level in NS0 cells containing a vector encoding an antibody that contained (1) No MAR element (2) One MAR element, or (3) Two MAR elements.

[0019] Figure 5 shows the increased antibody expression level in CHO-S cells containing a vector encoding an antibody that contained (1) No MAR element (2) One MAR element, or (3) Two MAR elements.

[0020] Figure 6 shows alternative structures useful in the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0021] Terms used throughout this application are to be construed with ordinary and typical meaning to those of ordinary skill in the art. However, Applicants desire that the following terms be given the particular definition as defined below.

[0022] The term "antibody" is used in the broadest sense, and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, single-chain antibodies, single-domain antibodies and multispecific antibodies (e.g., bispecific antibodies). Antibodies (Abs) and immunoglobulins (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific target, immunoglobulins include both antibodies and other antibody-like molecules which lack target specificity. Native antibodies and immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. However, the present invention includes other constructs wherein the heavy and light chain are linked to generate a single chain antibody; the heavy and light chain are expressed from a single promoter with an internal IRES for ribosome binding; single-domain antibodies in which the functional antibody comprises only a heavy chain or light chain, such as those described in WO04081026 and WO04041865, and any other constructs that are capable of expressing a functional antibody or fragment thereof.

[0023] The terms "cell", "cell line" and "cell culture" include progeny. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological property, as screened for in the originally transformed cell, are included. The "host cells" used in the present invention generally are prokaryotic or eukaryotic hosts.

[0024] "Transformation" of a cellular organism with DNA means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration. "Transfection" of a cellular organism with DNA refers to the taking up of DNA, e.g., an expression vector, by the cell or organism whether or not any coding sequences are in fact expressed. The terms "transfected host cell" and "transformed" refer to a cell in which DNA was introduced. The cell is termed "host cell" and it may be either prokaryotic or eukaryotic. Typical prokaryotic host cells include various strains of E. coli. Typical eukaryotic host cells are mammalian, such as Chinese

hamster ovary or cells of human origin. The introduced DNA sequence may be from the same species as the host cell of a different species from the host cell, or it may be a hybrid DNA sequence, containing some foreign and some homologous DNA.

[0025] The term "vector" means a DNA construct containing a DNA sequence operably linked to a suitable control sequence capable of effecting the expression of the DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites, and sequences which control the termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once incorporated into a suitable host, the vector may replicate and function independently of the host genome, or may in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably, as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of vectors which serve equivalent function as and which are, or become, known in the art.

[0026] The expression "regulatory sequences" refers to DNA sequences necessary for the expression of the heterologous gene of interest in a particular host organism. Regulatory sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers. DNA for a presequence or secretory leader may also be used for a polypeptide to be secreted, such as an antibody; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Such regulatory sequences are described, for example, in Goeddel, Gene Expression Technology: Methods In Enzymology 185, Academic Press, San Diego, Calif. (1990). Regulatory sequences include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transfected, the level of expression of protein desired, etc.

[0027] Within a recombinant expression vector, the term "operably-linked" is intended to mean that the nucleotide sequence of interest is linked to a regulatory sequence(s) in a manner that allows for expression of the nucleotide sequence (e.g., in an in vitro transcription/translation system or in a host cell when the vector is incorporated into the host cell).

[0028] The term "variable" in the context of variable domain of antibodies, refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular target. However, the variability is not evenly distributed through the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) also known as hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely a adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the target binding site of antibodies (see Kabat et al.) As used herein, numbering of immunoglobulin amino acid residues is done according to the immunoglobulin amino acid residue numbering system of Kabat et al., (Sequences of Proteins of Immunological Interest, National Institute of Health, Bethesda, Md. 1987), unless otherwise indicated.

[0029] The term "single-domain antibody" refers to a single immunoglobulin variable domain (V_H, V_{HH} or V_L) polypeptide that specifically binds antigen. While the antigen binding unit of a naturally-occurring antibody (e.g., in humans and most other mammals) is generally known to be comprised of a pair of V regions (V_L/V_H), camelid species express a large proportion of fully functional, highly specific antibodies that are devoid of light chain sequences. The camelid heavy chain antibodies are found as homodimers of a single heavy chain, dimerized via their constant regions. The variable domains of these camelid heavy chain antibodies are referred to as V_{HH} domains and retain the ability, when isolated as fragments of the V_H chain, to bind antigen with high specificity ((Hamers-Casterman et al., 1993, Nature 363: 446-448; Gahroudi et al., 1997, FEBS Lett. 414: 521-526). Antigen binding single V_H domains have also been identified from, for example, a library of murine V_H genes amplified from genomic DNA from the spleens of immunized

mice and expressed in E. coli (Ward et al., 1989, Nature 341: 544-546). Ward et al. named the isolated single V_H domains "dAbs," for "domain antibodies."

INSULATOR ELEMENTS

[0030] The present invention involves compositions and methods that can modulate the efficiency of cell transfection, the level of expression of a recombinant protein and the survival of the cell using insulator elements (e.g., MAR elements, Bes, LCRs, and UCOEs). In accordance with the invention, one embodiment comprises a MAR element used in eukaryotic cell transfection methods. For example, a MAR element suitable for use in the present invention includes chicken lysozyme MAR element, which is shown in SEQ ID NO: 1, or a fragment thereof. Additional insulator elements to be used in accordance with the invention may be identified, isolated, and cloned using a variety of techniques well known to those of ordinary skill in the art.

VECTORS

[0031] The invention provides isolated nucleic acid encoding an antibody or fragment thereof as disclosed herein, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the antibody. For recombinant production of the antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the antibody is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody variant).

[0032] Many basic vectors are readily available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

[0033] Figure 1 depicts one embodiment of a vector useful in the present invention. The vector comprises two MAR elements, one located upstream of the promoter and one located downstream of the gene. Figure 2 depicts the same vectors, but with a single MAR element located upstream of the promoter on each vector. Although not depicted in Figure 2, the MAR element may also be located downstream. A skilled artisan would recognize that the antibodies or their fragments could be independently expressed from separate promoters or that the secretion signal need not be the viral sequence depicted, but could be any prokaryotic or eukaryotic signal sequence suitable for the secretion of the

antibody fragments from the chosen host cell. It should also be recognized that the heavy chain and light chain depicted in these figures may also be expressed from a single vector under the control of individual expression cassettes or as one expression cassette with an internal ribosome site (IRES).

[0034] The vectors in Figures 1 and 2 also provide different selection markers, e.g., gpt and neo. These vectors may also contain, e.g., a His tag or a myc tag for easy purification, as well as detection. The following describe various components that may be incorporated into the vector in addition to the MAR element and the antibody gene of interest. These are meant to illustrate possible embodiments and not meant to be an exhaustive description.

A. Signal Sequence Component

[0035] The antibody may be expressed as a fusion polypeptide fused with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native antibody signal sequence, the signal sequence may be substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. Or in the case of the vector of Figure 1, the signal sequence depicted is a viral signal sequence from gene III. For yeast secretion the native signal sequence may be substituted by, e.g., the yeast invertase leader, α-factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or a signal described in e.g., WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available. The DNA for such precursor region is ligated in reading frame to DNA encoding the antibody variant.

B. Origin of Replication Component

[0036] Vectors usually contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322

is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

C. Selection Gene Component

[0037] Vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

[0038] One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

[0039] Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the antibody nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

[0040] For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contain methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity.

[0041] Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding antibody, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. (U.S. Pat. No. 4,965,199).

[0042] A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid Yrp7 (Stinchcomb et al., Nature 282: 39 (1979)). The trp1 gene provides a selection marker for a variant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, Genetics 85: 12 (1977). The presence of the

trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

D. Promoter Component

[0043] Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the nucleic acid to be expressed. Regulatable gene expression promoters are well known in the art, and include, by way of non-limiting example, any promoter that modulates expression of a gene encoding a desired protein by binding an exogenous molecule, such as the CRE/LOX system, the TET system, the NFkappaB/UV light system, the Leu3p/isopropylmalate system, and the GLVPc/GAL4 system (See e.g., Sauer, 1998, Methods 14(4): 381-92).

[0044] Examples of suitable promoter sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

[0045] Antibody transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters--provided such promoters are compatible with the host cell systems.

[0046] The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. Alternatively, a

thymidine kinase promoter from herpes simplex virus may be used or the Rous sarcoma virus long terminal repeat may be used as the promoter.

E. Enhancer Element Component

[0047] Transcription of a DNA encoding the antibody of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature 297: 17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the antibody-encoding sequence, but is preferably located at a site 5' from the promoter.

F. Transcription Termination Component

[0048] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) may also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. See e.g.,WO94/11026.

SELECTION AND TRANSFORMATION OF HOST CELLS

[0049] Suitable host cells for cloning or expressing the DNA in the vectors herein are prokaryotic, yeast, or higher eukaryotic cells. Suitable prokaryotes for this purpose include both Gram-negative and Gram-positive organisms, for example, Enterobacteria such as *E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, Serratia,* and *Shigella,* as well as Bacilli, Pseudomonas, and Streptomyces. One preferred *E. coli* cloning host is E. coli 294 (ATCC 31,446), although other strains such as E. coli B, E. coli X1776 (ATCC 31,537), and E. coli W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

[0050] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors. *Saccharomyces*

cerevisiae is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Pichia pastoris*, Kluyveromyces; Candida; Trichoderma; *Neurospora crassa*; and filamentous fungi such as e.g., Neurospora, Penicillium, Tolypocladium, and Aspergillus hosts, such as *A. nidulans* and *A. niger*.

[0051] Suitable host cells for the expression of glycosylated antibodies are derived from multicellular organisms. In principal, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells, Luckow et al., Bio/Technology 6, 47-55 (1988); Miller et al., Genetic Engineering, Setlow et al. eds. Vol. 8, pp. 277-279 (Plenam publishing 1986); Mseda et al., Nature 315, 592-594 (1985). Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as Spodoptera frugiperda (caterpillar), Aedes (mosquito), Drosophila melanogaster (fruitfly), and Bombyx mori have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of Autographa californica NPV and the Bm-5 strain of Bombyx mori NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of Spodoptera frugiperda cells. Moreover, plant cells cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco and also be utilized as hosts.

[0052] Vertebrate cells, and propagation of vertebrate cells, in culture (tissue culture) have become a routine procedure. See Tissue Culture, Academic Press, Kruse and Patterson, eds. (1973). Examples of useful mammalian host cell lines are monkey kidney; human embryonic kidney line; baby hamster kidney cells; Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77: 4216 (1980)); mouse sertoli cells; human cervical carcinoma cells (HELA); canine kidney cells; human lung cells; human liver cells; mouse mammary tumor; and NS0 cells.

[0053] Host cells are transformed with the above-described vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

[0054] The host cells used to produce the antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium (MEM, Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's

Medium (DMEM, Sigma) are suitable for culturing host cells. In addition, any of the media described in Ham et al., Meth. Enzymol. 58: 44 (1979), Barnes et al., Anal. Biochem. 102: 255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,560,655; 5,122,469; 5,712,163; or 6,048,728 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as X-chlorides, where X is sodium, calcium, magnesium; and phosphates), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN.TM. drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

EXAMPLES

EXAMPLE 1: Isolation of Chicken MAR (1.46kb) from Chicken Genomic DNA

[0055] Two primers were synthesized based on the chicken genomic sequence of the chicken lysozyme MAR.

MAR-F 5' – GCTGCTggatccTGCCGCCTTCTTTGATATTC– 3' (SEQ ID NO: 2), and

MAR-R 5' – GTAAGATGAAGAGTGCTGTGC – 3' (SEQ ID NO: 3).

The PCR conditions used were 94°C for 30s, 50°C for 30s, and 72°C for 2.5min for 5 cycles; followed by 94°C for 30s, 55°C for 30s, and 72°C for 2.5min for 25cycles (using 50ng of chicken genomic DNA as template).

The resulting PCR fragment was used to generate a second PCR fragment using two oligo nucleotide primers:

MAR-F 5' – TAGCAAggatccTGCAGCTGTTTACGGC – 3' (SEQ ID NO: 4), and

MAR-R 5' – TAGCATctcgagGCAGCTGTAACTCCACTG – 3'(SEQ ID NO: 5),

The PCR conditions used for this second round were: 94°C for 30s, 55°C for 30s, and 72 ° C for 1.5min for 30 cycles using 1ul of first round PCR as template. This second PCR fragment was then cleaved at BamHI (5') and XhoI (3') and ligated into the commercially available vector pcDNA3.1.

[0056] The second round PCR fragment was gel purified and digested with BamHI and XhoI, followed by ligation into a pcDNA3.1(+) vector (from Invitrogen,

catalog@invitrogen.com). The resulting construct was sequenced using vector sequences T7 and BGH as primers. The verified sequence was identical to that of SEQ ID NO 1.

EXAMPLE 2: Cloning of MAR fragment (1.46kb) into vectors

[0057] The MAR fragment isolated in Example 1 was cloned into a vector containing the heavy chain of an anti-IgE antibody and a vector containing the corresponding light chain. The MAR was inserted upstream of a CMV promoter in the same orientation as the antibody gene. (See Figure 2)

[0058] The MAR fragment isolated in Example 1 was also cloned into a vector containing the heavy chain of an anti-IgE antibody and a vector containing the corresponding light chain. In this case, the MAR was inserted upstream of a CMV promoter in the same orientation as the antibody gene and downstream of the antibody gene in the opposite orientation. (See Figure 1)

EXAMPLE 3: Transfection of an NS0 cell line

[0059] Three sets of vectors were linearized: (1) one set with a light chain vector and a heavy chain vector but no MAR element; (2) one set with a light chain vector and a heavy chain vector each containing one MAR element as depicted in Figure 2; and (3) one set with a light chain vector and a heavy chain vector each containing two MAR elements as depicted in Figure 1.

[0060] NS0 host cells were grown to a density of $1x10^6$ cells/ml in typical growth medium, keeping the cells in exponential growth by providing fresh medium until the day before transfection. A volume of cells equaling 40 x 10^6 cells was then washed with 15 ml D15 + 2% FBS and resuspend in 0.8ml of D15 + 2% FBS.

[0061] Transfection was performed by adding 10ug of linearized light chain DNA and 10ug of linearized heavy chain DNA of each set to the cell suspension (total DNA volume was less than 50ul) and the cells were held on ice for 15min. Cells were transferred into pre-chilled cuvette (0.4cm) and an electric pulse (200 volts, 960uF) was applied. The cuvette was immediately returned to the ice after the electric pulse and held for 15min.

[0062] Cells were recovered in medium + 1%FBS at density of 0.3 X 10^6cells/ml and grown for 48hrs. The recovered cells were then plated in medium + 2% FBS + 0.1ug/ml HXM + 150ug/ml G418 at density of 1x 10^5 cells/ml in 96 well plates and grown for two weeks. Following growth period, the supernatant was collected from each well, and an ELISA was performed to test for the level of expression.

[0063] The results in Figure 3 show not only that the MAR element increased cell survival, but that two MAR elements improve cell survival over a single MAR. The level of antibody production was also greatly improved as depicted in Figure 4. Here, one MAR element increased the level of production from less than 0.2 ug/ml with no MAR element present, to 9ug/ml with one MAR element present and 12ug/ml with two MAR elements present. Thus, the level of expression was increased 45 fold with one MAR and 60 fold with two MAR elements.

EXAMPLE 4: Transfection of CHO-S cells using Lipofectamine 2000

[0064] A similar experiment as that described in Example 3 was carried out in a different cell line to show the versatility of the invention. Three sets of vectors were linearized: (1) one set with a light chain vector and a heavy chain vector but no MAR element; (2) one set with a light chain vector and a heavy chain vector each containing one MAR element as depicted in Figure 2; and (3) one set with a light chain vector and a heavy chain vector each containing two MAR elements as depicted in Figure 1.

[0065] A suspension cell line CHO-S was converted back to adhering cells in DMEM + 10% FBS medium before transfection. Prior to transfection, the cells were aliquoted at 1.3 x 10^6 cells/ml in a six well plate (2ml/well). Transfection was carried out according to the manufacturer's instructions (Invitrogen, Lipofectamine 2000 Transfection System).

[0066] The results depicted in Figure 5 show an increased level of production of antibody for each MAR element incorporated into the vector.

[0067] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

WE CLAIM:

1. A vector comprising at least one insulator element and a polynucleotide sequence encoding an immunoglobulin heavy chain and/or an immunoglobulin light chain.
2. The vector of claim 1, wherein the polynucleotide sequence encodes a variable region of the immunoglobulin heavy chain and/or a variable region of an immunoglobulin light chain.
3. The vector of claim 1, wherein the polynucleotide sequence encodes a single domain antibody.
4. The vector of any one of claims 1-3, wherein the vector comprises two insulator elements.
5. The vector of claim 4, wherein the polynucleotide sequence encodes the heavy or light chain flanked by the two insulator elements as depicted in Figure 1.
6. The vector of any one of claims 1-5, wherein the insulator element is a boundary element, a MAR element, a locus-control region, or a ubiquitous-acting chromatin opening element.
7. The vector of any one of claims 1-6, further comprising an additional functional component selected from the group consisting of a signal peptide, an immune enhancer, a toxin, and a biologically active enzyme.
8. The vector of any one claims 1-7, wherein the insulator element is a MAR element, such as a chicken lysozyme MAR.
9. The vector of any one of claims 1-8, wherein the insulator element is SEQ ID NO 1.
10. A method for increasing the level of immunoglobulin expression in a cell comprising incorporating a vector comprising a polynucleotide sequence encoding an immunoglobulin heavy chain and/or light chain and at least one insulator element.
11. The method of claim 10, wherein the polynucleotide sequence encodes a variable region of the immunoglobulin heavy chain and/or a variable region of an immunoglobulin light chain.
12. The method of claim 10, wherein the polynucleotide encodes a single domain antibody.
13. The method of any one of claims 10-12, wherein the vector comprises two insulator elements.

14. The method of claim 13, wherein the polynucleotide sequence encodes the heavy or light chain flanked by the two insulator elements as depicted in Figure 1.
15. The method of any one of claims 10-14, wherein the insulator element is a boundary element, a MAR element, a locus-control region, or a ubiquitous-acting chromatin opening element.
16. The method of any one of claims 10-15, further comprising an additional functional component selected from the group consisting of a signal peptide, an immune enhancer, a toxin, and a biologically active enzyme.
17. The method of any one claims 10-16, wherein the insulator element is a MAR element, such as a chicken lysozyme MAR.
18. The vector of any one of claims 10-17, wherein the insulator element is SEQ ID NO 1.
19. The method of any one of claims 10-18, wherein the cell is a mammalian cell.
20. The method of any one of claims 10-19, wherein the level of immunoglobulin expression is increased at least 45 fold.
21. A method of increasing the survival of a cell expressing a recombinant protein comprising incorporating a vector comprising at least one insulator element.
22. The method of claim 21, wherein the recombinant protein is an immunoglobulin.
23. The method of claim 21, wherein the vector encodes a variable region of the immunoglobulin heavy chain and/or a variable region of an immunoglobulin light chain.
24. The method of claim 21, wherein the recombinant protein is a single domain antibody.
25. The method of any one of claims 21-24, wherein the vector comprises two insulator elements.
26. The method of claim 25, wherein the polynucleotide sequence encodes the heavy or light chain flanked by the two insulator elements as depicted in Figure 1.
27. The method of any one of claims 21-26, wherein the insulator element is a boundary element, a MAR element, a locus-control region, or a ubiquitous-acting chromatin opening element.
28. The method of any one of claims 21-27, further comprising an additional functional component selected from the group consisting of a signal peptide, an immune enhancer, a toxin, and a biologically active enzyme.

29. The method of any one claims 21-28, wherein the insulator element is a MAR element, such as a chicken lysozyme MAR.

30. The vector of any one of claims 21-29, wherein the insulator element is SEQ ID NO 1.

31. The method of any one of claims 21-30, wherein the cell is a mammalian cell.