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(71) Demandeur/Applicant:
METAMORPHIX INTERNATIONAL, INC., US

(72) Inventeurs/Inventors:
BARKER, CHRISTOPHER A., CA;
MORSEY, MOHAMAD, US

(74) Agent: TORYS LLP

(54) Titre : TECHNIQUES IMMUNOLOGIQUES PERMETTANT DE MODULER LA MYOSTATINE CHEZ LES
VERTEBRES

(54) Title: IMMUNOLOGICAL METHODS TO MODULATE MYOSTATIN IN VERTEBRATE SUBJECTS

(57) **Abrégé/Abstract:**

Immunological compositions and methods for reducing myostatin activity in vertebrate subjects are disclosed. The compositions include myostatin peptide immunogens, myostatin multimers and/or myostatin immunoconjugates capable of eliciting an immune response in a vertebrate subject to which the compositions are administered. The methods are useful for the treatment of a wide variety of disorders.



ABSTRACT

Immunological compositions and methods for reducing myostatin activity in vertebrate subjects are disclosed. The compositions include myostatin peptide immunogens, myostatin multimers and/or myostatin immunoconjugates capable of eliciting an immune response in a vertebrate subject to which the compositions are administered. The methods are useful for the treatment of a wide variety of disorders.

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5 **IMMUNOLOGICAL METHODS TO MODULATE**
 MYOSTATIN IN VERTEBRATE SUBJECTS

Technical Field

10 The present invention relates generally to
 compositions and methods for increasing muscle
 synthesis and treating disease in vertebrate subjects.
 More particularly, the invention is directed to
 immunological compositions and methods for reducing
 myostatin activity in vertebrate subjects.

15

Background of the Invention

 Livestock producers have traditionally used
 breeding programs to select animals that yield maximum
 amounts of protein with acceptable performance as
20 measured by feed efficiency, reproductive function and
 general health. Cattle which exhibit increased muscle
 mass due to both hypertrophy and hyperplasia of muscle
 cells have been observed in a number of breeds. The
 incidence of this condition, which is referred to as
25 double-muscling, is most pronounced in Belgian Blue
 cattle. Muscle mass is increased by approximately 20%
 with a decrease in bone and fat mass in these animals
 (Shahin and Berg, *Can. J. Anim. Sci.* (1985) 65:279-
 293). Belgian Blue cattle also utilize feed
30 efficiently and give rise to a higher percentage of
 desirable cuts of meat (Casas et al., *J. Anim. Sci.*
 (1997) 75(Supp 1):149). Double-muscling in Belgian
 Blue cattle is inherited and is believed to be
 recessive since heterozygotes may be normal or have
35 only a modest increase in muscle mass.

Despite the advantages of this condition, double-muscled cattle often have undesirable traits. For example, because calves are generally 10-38% heavier than normal, dystocias are prevalent, requiring cesarean deliveries. Animals also exhibit abnormal reproduction due to poorly developed reproductive tracts and have other anatomical abnormalities such as macroglossia. Other breeds of cattle, such as the Piemontese from northern Italy, have varying degrees of double-muscling and also display many of these undesirable traits.

The double-muscling characteristic identified in some cattle breeds has now been traced to mutations in the myostatin gene (Grobet et al., *Nature Genetics* (1997) 17:71-74; Kambadur et al., *Genome Research* (1997) 7:910-915; McPherron and Lee, *Proc. Natl. Acad. Sci. USA* (1997) 94:12457-12461). This mutation appears to result mainly in an increase in the number of muscle cells (hyperplasia) rather than an increase in the size of individual muscle fibers (hypertrophy). A condition referred to as muscular hypertrophy has also been identified in the Pietrain breed of pig. This condition is not related to the myostatin gene and has been identified as a mutation in a gene responsible for calcium transport.

McPherron et al., *Nature* (1997) 387:83-90, have identified a member of the transforming growth factor- β (TGF- β) superfamily of proteins in mice, referred to as growth/differentiating factor-8 (GDF-8). GDF-8 acts as a negative regulator for skeletal muscle growth and is expressed in developing and adult skeletal muscles. Gene knockout experiments in mice have resulted in homozygous mutants which are 30% larger than wild-type mice. This increase in size is due primarily to an increase in muscle mass with individual muscles from the mutants weighing 2-3 times

more than those from wild-type mice (McPherron et al.,
Nature (1997) 387:83-90). McPherron and Lee, Proc.
Natl. Acad. Sci. USA (1997) 94:12457-12461 and Grobet
et al., Nature Genetics (1997) 17:71-74 evaluated
5 similar genomic sequences in a number of species,
including cattle, and reported that double-muscled
cattle had defects in the gene coding for a protein
highly homologous to GDF-8. This protein is now
called myostatin.

10 Thus, it appears that myostatin is produced
by muscle cells and regulates the proliferation and
differentiation of myoblasts. In Belgian Blue and
Piemontese cattle, natural defects in the gene are
believed to result either in production of an abnormal
15 protein or a reduced amount of myostatin, either of
which has the effect of increasing muscle growth.

The myostatin gene from a number of
vertebrate species, including mouse, rat, human,
baboon, cattle, pig, sheep, chicken, turkey, and
20 zebrafish has been identified and the proteins
sequenced (McPherron and Lee, Proc. Natl. Acad. Sci.
USA (1997) 94:12457-12461). The myostatin protein
sequence is highly conserved across all of these
species. Similarly, the nucleotide sequence for
25 myostatin from mouse, rat, human, baboon, cattle, pig,
sheep, chicken and turkey has been determined. See,
e.g., U.S. Patent No. 5,827,733 for the nucleotide
sequences of murine and human myostatin; International
Publication No. WO 99/02667 for the nucleotide
30 sequence of bovine myostatin; International
Publication No. WO 98/33887, for the nucleotide
sequences of rat, human, baboon, bovine, porcine,
ovine, chicken and turkey myostatin.

The nucleotide sequence of the myostatin
35 gene predicts a protein of about 376 amino acids with
a molecular weight of approximately 43 kDa. This

protein contains a secretion leader sequence and a proteolytic processing site which releases a 13 kDa peptide, containing 9 cysteine residues. Cloned myostatin expressed in Chinese hamster ovary cells yields two proteins. The first has an apparent molecular weight of about 52 kDa and the second about 15 kDa. Under nonreducing conditions, these proteins appear to be dimers with molecular weights of about 101 kDa and 25 kDa (McPherron et al., Nature (1997) 387:83-90).

Researchers have proposed delivery of mutated myostatin genes to animal subjects for the production of transgenic species having increased muscle tissue. See, e.g., International Publication No. WO 98/33887. However, such approaches pose several drawbacks. For example, because the myostatin gene becomes active during the embryonic stage, reduced myostatin production causes excessive muscle development in utero. Thus, transgenic animals which include mutated genes would likely require cesarean delivery, a serious burden to large animal producers. Additionally, public opposition to genetically engineered animals for human consumption exists and other methods of producing such animals would be desirable.

Disclosure of the Invention

The present invention is directed to immunological compositions and methods for modulating endogenous myostatin activity in a vertebrate subject. The invention is also useful for treating a number of conditions in vertebrates, including humans and other animals, such as a variety of disorders that cause degeneration or wasting of muscle. Due to the ubiquitous nature of myostatin, the compositions and

methods described herein find use in a wide variety of vertebrate subjects, as described further below.

Surprisingly, the invention achieves these results by immunological techniques. It is readily
5 known in the art that immunization against endogenous molecules, such as myostatin, is problematic because the immune system does not recognize such "self" molecules. Thus, the present invention provides a solution to a problem which would normally be
10 encountered when immunizing against an endogenous substance.

Accordingly, in one embodiment, the invention is directed to a myostatin peptide consisting of about 3 to about 100 amino acids. The
15 peptide comprises at least one epitope of myostatin. In preferred embodiments, the myostatin peptide is derived from the region of myostatin spanning amino acids 45 through 376, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36) or amino acids 235 through 376,
20 inclusive, of Figures 1A-1D (SEQ ID NOS:27-36).

In other embodiments, the myostatin peptide has at least about 75% amino acid identity to a peptide comprising an amino acid sequence selected from the group consisting of amino acids 3-18,
25 inclusive of SEQ ID NO:4; amino acids 3-15, inclusive of SEQ ID NO:6; amino acids 3-17, inclusive, of SEQ ID NO:8; amino acids 3-16, inclusive of SEQ ID NO:10; amino acids 3-22, inclusive of SEQ ID NO:12; amino acids 3-25, inclusive of SEQ ID NO:14; amino acids 3-
30 22, inclusive of SEQ ID NO:16; amino acids 3-18, inclusive of SEQ ID NO:20; and amino acids 3-18, inclusive, of SEQ ID NO:22.

In still further embodiments, the invention is directed to a myostatin peptide consisting of about
35 3 to about 200 amino acids. The peptide comprises at least one epitope of myostatin and is derived from a

region of myostatin selected from the group consisting of the region of myostatin spanning amino acids 1 through 350, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36); the region of myostatin spanning amino acids 1 through 275, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36); the region of myostatin spanning amino acids 25 through 300, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36); the region of myostatin spanning amino acids 50 through 325, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36); and the region of myostatin spanning amino acids 75 through 350, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36).

In yet further embodiments, the myostatin peptide comprises the amino acid sequence Lys-Arg-Ser-Arg-Arg-Asp (SEQ ID NO:37), the amino acid sequence Lys-Glu-Asn-Val-Glu-Lys-Glu (SEQ ID NO:38) or the amino acid sequence Ser-Leu-Lys-Asp-Asp-Asp (SEQ ID NO:39).

In yet another embodiment, the invention is directed to a myostatin multimer comprising two or more selected myostatin immunogens, wherein each of the immunogens independently comprises at least 3 amino acids defining at least one epitope of myostatin. In particularly preferred embodiments, each of the selected myostatin immunogens comprises at least one epitope of myostatin and independently consists of about 3 to about 200 amino acids, or about 3 to about 100 amino acids, or about 3 to about 30 amino acids, or about 3 to about 15 amino acids.

In other embodiments, each of the selected myostatin immunogens in the multimer independently comprise a selected myostatin peptide as described above. In particularly preferred embodiments, the multimer comprises a molecule with repeating units according to the general formula (MP-X-MP)_y, wherein MP is a myostatin peptide, X is selected from the

group consisting of a peptide linkage, an amino acid spacer group, a leukotoxin polypeptide and $[MP]_n$, where n is greater than or equal to 1, and y is greater than or equal to 1.

5 In another embodiment, the invention is directed to a myostatin immunoconjugate comprising at least one myostatin peptide or multimer, as described above, linked to an immunological carrier.

10 In still further embodiments, the invention is directed to vaccine compositions comprising the myostatin peptide, the myostatin multimer and/or the myostatin immunoconjugate, and a pharmaceutically acceptable excipient.

15 In yet other embodiments, the invention is directed to polynucleotides encoding the myostatin peptides, the myostatin multimers and the myostatin immunoconjugates above, as well as recombinant vectors comprising the polynucleotides, host cells transformed with the recombinant vectors, and methods of
20 recombinantly producing the myostatin peptides, myostatin multimers and myostatin immunoconjugates.

 In other embodiments, the invention is directed to methods of eliciting an immune response against a myostatin immunogen in a vertebrate subject
25 comprising administering the vaccine compositions or polynucleotides above to the vertebrate subject. In particularly preferred embodiments, the immune response elicited reduces endogenous myostatin activity in the vertebrate subject and results in at
30 least one of the following biological effects:

- (a) an increase in body weight;
- (b) an increase in muscle mass;
- (c) an increase in the number of muscle
cells;
- 35 (d) an increase in the size of muscle cells;
- (e) a reduction in body fat content;

- (f) an increase in muscle strength;
- (g) an increase in mammary gland tissue;
- (h) an increase in lactation;
- (i) an increase in appetite or feed uptake;

5 or

(j) an increase in the life span of the vertebrate subject.

In other embodiments, the invention is directed to methods of treating a disorder which comprises degeneration or wasting of muscle in a vertebrate subject, the method comprising administering the vaccine compositions or polynucleotides above to the subject. The invention is also directed to methods of modulating GDF11 activity in a vertebrate subject comprising administering the vaccine compositions above.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

20

Brief Description of the Figures

Figures 1A-1D show a comparison of myostatin derived from various species as follows: Mouse (SEQ ID NO:27); Rat (SEQ ID NO:28); Human (SEQ ID NO:29); Baboon (SEQ ID NO:30); Bovine (SEQ ID NO:31); Porcine (SEQ ID NO:32); Ovine (SEQ ID NO:33); Chicken (SEQ ID NO:34); Turkey (SEQ ID NO:35); and Zebrafish (SEQ ID NO:36). Amino acids are numbered to the right and left of the sequences.

Figure 2 shows the nucleotide sequence (SEQ ID NO:3) and corresponding amino acid sequence (SEQ ID NO:4) of the MYOS 1 peptide. MYOS 1 includes the proteolytic cleavage site, Arg-Ser-Arg-Arg, and the N-terminus of the active protein.

35

Figure 3 depicts the nucleotide sequence (SEQ ID NO:5) and corresponding amino acid sequence (SEQ ID NO:6) of the MYOS 3 peptide.

5 Figure 4 depicts the nucleotide sequence (SEQ ID NO:7) and corresponding amino acid sequence (SEQ ID NO:8) of the MYOS 5 peptide.

Figure 5 shows the nucleotide sequence (SEQ ID NO:9) and corresponding amino acid sequence (SEQ ID NO:10) of the MYOS 7 peptide.

10 Figure 6 shows the nucleotide sequence (SEQ ID NO:11) and corresponding amino acid sequence (SEQ ID NO:12) of the MYOS 9 peptide.

Figure 7 shows the nucleotide sequence (SEQ ID NO:13) and corresponding amino acid sequence (SEQ ID NO:14) of the MYOS 11 peptide.

Figure 8 shows the nucleotide sequence (SEQ ID NO:15) and corresponding amino acid sequence (SEQ ID NO:16) of the MYOS 13 peptide.

20 Figure 9 shows the nucleotide sequence (SEQ ID NO:17) and corresponding amino acid sequence (SEQ ID NO:18) of the MYOS 15 peptide.

Figure 10 shows the nucleotide sequence (SEQ ID NO:19) and corresponding amino acid sequence (SEQ ID NO:20) of the MYOS 17 peptide.

25 Figure 11 shows the nucleotide sequence (SEQ ID NO:21) and corresponding amino acid sequence (SEQ ID NO:22) of the MYOS 19 peptide. MYOS 19 includes the proteolytic cleavage site, Arg-Ser-Arg-Arg.

30 Figure 12 shows the approximate position of MYOS peptides 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19 within the myostatin sequence.

Figure 13 shows the nucleotide sequence (SEQ ID NO:23) and corresponding amino acid sequence (SEQ ID NO:24) for a reconstructed myostatin active region containing three sets of two amino acid linkers (Arg-

Ser) inserted in the sequence at nucleotide positions 55-60, 139-144 and 241-246 and at the C-terminus.

Figure 14 is a diagram of plasmid pCB150, encoding a leukotoxin polypeptide carrier and used to
5 create myostatin expression vectors as described in the examples.

Figures 15A-15D show the nucleotide sequence (SEQ ID NO:25) and corresponding amino acid sequence (SEQ ID NO:26) of the leukotoxin carrier polypeptide
10 present in plasmid pCB150. Myostatin oligo repeats are inserted into the *Bam*HI site present at nucleotide position 3334.

Figure 16A shows the nucleotide sequence (SEQ ID NO:1) and Figure 16B shows the predicted amino
15 acid sequence (SEQ ID NO:2) of a representative myostatin for use with the present invention. The proteolytic cleavage site is found at positions 263-266 of Figure 16B. The myostatin active region of the polypeptide spans amino acids 264-375.

Figure 17 shows a hydrophilicity profile of
20 the myostatin protein. The profile was computed using an average group length of six amino acids. The three highest points of hydrophilicity are found at amino acid positions 263-268, which span the proteolytic
25 cleavage site; positions 31-37; and positions 106-111.

Figure 18 shows the amount of weight gain in animals treated with myostatin peptide immunogens, as described in the examples.

30 Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology,
virology, recombinant DNA technology, and immunology,
35 which are within the skill of the art. Such techniques are explained fully in the literature.

See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*; *DNA Cloning*, Vols. I and II (D.N. Glover ed.); *Oligonucleotide Synthesis* (M.J. Gait ed.); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds.); B. Perbal, *A Practical Guide to Molecular Cloning*; the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., Blackwell Scientific Publications).

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

By "myostatin immunogen" is meant a polypeptide derived from a myostatin molecule which elicits an immunological response as defined below. The term includes molecules that elicit an immunological response without an associated immunological carrier, adjuvant or immunostimulant, as well as myostatin polypeptides capable of being rendered immunogenic, or more immunogenic, by way of association with a carrier molecule, adjuvant or immunostimulant, or by mutation of a native sequence, and/or by incorporation into a molecule containing multiple repeating units of at least one epitope of a myostatin molecule. The term may be used to refer to an individual macromolecule or to a homogeneous or heterogeneous population of antigenic macromolecules derived from myostatin.

For purposes of the present invention, a myostatin immunogen may be derived from any of the various known myostatin sequences, including without limitation, myostatin polypeptides derived from mouse, rat, human, baboon, cattle, pig, sheep, chicken,

turkey, and zebrafish (see, McPherron and Lee, *Proc. Natl. Acad. Sci. USA* (1997) 94:12457-12461). The myostatin protein sequence is highly conserved across all of these species (see Figures 1A-1D).

5 Additionally, the term "myostatin immunogen" includes a myostatin polypeptide molecule differing from the reference sequence by having one or more amino acid substitutions, deletions and/or additions and which has at least about 50% amino acid identity
10 to the reference molecule, more preferably about 75-85% identity and most preferably about 90-95% identity or more, to the relevant portion of the native peptide sequence in question. The amino acid sequence will have not more than about 10-20 amino acid
15 substitutions, or not more than about 5-10 amino acid substitutions, or even only 1, 2, 3 or up to 5 substitutions. Particularly preferred substitutions will generally be conservative in nature, i.e., those substitutions that take place within a family of amino
20 acids. In this regard, amino acids are generally divided into four families: (1) acidic -- aspartate and glutamate; (2) basic -- lysine, arginine, histidine; (3) non-polar -- alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine,
25 tryptophan; and (4) uncharged polar -- glycine, asparagine, glutamine, cystine, serine threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids. For example, it is reasonably predictable that an isolated
30 replacement of leucine with isoleucine or valine, or vice versa; an aspartate with a glutamate or vice versa; a threonine with a serine or vice versa; or a similar conservative replacement of an amino acid with a structurally related amino acid, will not have a
35 major effect on the activity. Proteins having substantially the same amino acid sequence as the

reference molecule, but possessing minor amino acid substitutions that do not substantially affect the immunogenicity of the protein, are therefore within the definition of a myostatin immunogen.

5 As used herein a "myostatin immunogen" also includes a molecule derived from a native myostatin sequence, as well as recombinantly produced or chemically synthesized myostatin polypeptides including the full-length myostatin reference
10 sequence, as well as myostatin peptides which remain immunogenic, as described below. A "myostatin immunogen" thus includes molecules having the native sequence, molecules with single or multiple amino acid additions, substitutions and/or deletions, as well as
15 peptide fragments of the reference myostatin molecule, so long as the molecule retains the ability to elicit formation of antibodies that cross-react with the naturally occurring myostatin of the vertebrate species to which such an immunogen is delivered.
20 Epitopes of myostatin are also captured by the definition.

 A "myostatin peptide" is a myostatin immunogen, as described herein, which includes less than the full-length of the reference myostatin
25 molecule in question and which includes at least one epitope as defined below. Thus, a vaccine composition comprising a myostatin peptide would include a portion of the full-length molecule but not the entire myostatin molecule in question.

30 By "myostatin multimer" is meant a molecule having more than one copy of a selected myostatin immunogen, myostatin peptide or epitope, or multiple tandem repeats of a selected myostatin immunogen, myostatin peptide or epitope. The myostatin multimer
35 may correspond to a molecule with repeating units of the general formula (MP-X-MP)_y wherein MP is a

myostatin peptide, X is selected from the group consisting of a peptide linkage, an amino acid spacer group and $[MP]_n$, where n is greater than or equal to 1, y is greater than or equal to 1, and further wherein

5 "MP" may comprise any MP peptide. Y may therefore define 1-40 or more repeating units, more preferably, 1-30 repeating units and most preferably, 1-20 repeating units. Further, the selected myostatin peptide sequences may all be the same, or may

10 correspond to different derivatives, analogs, variants or epitopes of myostatin so long as they retain the ability to elicit an immune response. Additionally, if the myostatin peptides are linked either chemically or recombinantly to a carrier, myostatin peptides may

15 be linked to either the 5'-end, the 3'-end, or may flank the carrier in question. Further, the myostatin multimer may be located at sites internal to the carrier. Myostatin multimers are discussed in further detail below.

20 "Homology" refers to the percent identity between two polynucleotide or two polypeptide moieties. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 75%-85%, preferably

25 at least about 90%, and most preferably at least about 95%-98% sequence identity over a defined length of the molecules. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence.

30 Percent "identity" between two amino acid or polynucleotide sequences can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of matches between the two aligned

35 sequences, dividing by the length of the shorter sequence, and multiplying the result by 100. Readily

available computer programs can be used to aid in the analysis, such as ALIGN, Dayhoff, M.O. in *Atlas of Protein Sequence and Structure* M.O. Dayhoff ed., 5 Suppl. 3:353-358, National biomedical Research Foundation, Washington, DC, which adapts the local homology algorithm of Smith and Waterman (1981) *Advances in Appl. Math.* 2:482-489 for peptide analysis. Programs for determining nucleotide sequence identity are available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the BESTFIT, FASTA and GAP programs, which also rely on the Smith and Waterman algorithm. These programs are readily utilized with the default parameters recommended by the manufacturer and described in the Wisconsin Sequence Analysis Package referred to above. For example, percent identity of a particular nucleotide sequence to a reference sequence can be determined using the homology algorithm of Smith and Waterman with a default scoring table and a gap penalty of six nucleotide positions.

Alternatively, identity can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning, supra*; *Nucleic Acid Hybridization, supra*.

By the term "degenerate variant" is intended a polynucleotide containing changes in the nucleic

acid sequence thereof, that encodes a polypeptide having the same amino acid sequence as the polypeptide encoded by the polynucleotide from which the degenerate variant is derived.

5 An "immunological response" to an immunogen or vaccine is the development in the host of a cellular and/or antibody-mediated immune response to the immunogen or vaccine of interest. Usually, such a response includes but is not limited to one or more of
10 the following effects; the production of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or $\gamma\delta$ T cells, directed specifically to an immunogen or immunogens included in a composition or vaccine of interest. An
15 immunological response can be detected using any of several assays well known in the art, such as standard immunoassays and neutralization assays, including Western blots, dot blots and immunoaffinity assays. The presence of a cell-mediated immunological
20 responses may be determined using CTL cytotoxic cell assays, well known in the art, such as the assay described in Erickson et al. *J. Immunol.* (1993) 151:4189-4199; and Doe et al. *Eur. J. Immunol.* (1994) 24:2369-2376.

25 An "epitope" refers to any portion or region of a molecule with the ability or potential to elicit, and combine with, a myostatin-specific antibody. For the purpose of the present invention, a polypeptide epitope will usually include at least about 3 amino
30 acids, preferably at least about 5 amino acids, and most preferably at least about 10-15 amino acids to 20-30 or more amino acids, of the reference molecule. There is no critical upper limit to the length of the fragment, which could comprise nearly the full-length
35 of a protein sequence, or even a fusion protein.

comprising two or more epitopes of a protein in question.

Epitopes in polypeptide molecules can be identified using any number of epitope mapping techniques, well known in the art. See, e.g., *Epitope Mapping Protocols in Methods in Molecular Biology*, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:3998-4002; Geysen et al. (1986) *Molec. Immunol.* 23:709-715. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols*, supra. Computer programs that formulate hydropathy scales from the amino acid sequence of the protein, utilizing the hydrophobic and hydrophilic properties of each of the 20 amino acids, as described in, e.g., Kyte et al., *J. Mol. Biol.* (1982) 157:105-132; and Hopp and Woods, *Proc. Natl. Acad. Sci. USA* (1981) 78:3824-3828, can also be used to determine antigenic portions of a given molecule. For example, the technique of Hopp and Woods assigns each amino acid a numerical hydrophilicity value and then repetitively averages these values along the peptide chain. The points of highest local average hydrophilicities are indicative of antigenic portions of the molecule.

By "immunological carrier" is meant any molecule which, when associated with a myostatin immunogen of interest, imparts immunogenicity to that molecule, or enhances the immunogenicity of the molecule. Examples of suitable carriers include large, slowly metabolized macromolecules such as: proteins; polysaccharides, such as sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids such as polyglutamic acid, polylysine, and the like; amino acid copolymers; inactive virus particles; bacterial toxins such as toxoid from diphtheria, tetanus, cholera, leukotoxin molecules, and the like. Carriers are described in further detail below.

A myostatin immunogen is "linked" to a specified carrier molecule when the immunogen is chemically coupled to, or associated with the carrier, or when the immunogen is expressed from a chimeric DNA molecule which encodes the immunogen and the carrier of interest.

An "immunoconjugate" is a myostatin immunogen such as a myostatin peptide or multimer which is linked to a carrier molecule, as defined above.

The term "leukotoxin polypeptide" or "LKT polypeptide" intends a polypeptide which is derived from a protein belonging to the family of molecules characterized by the carboxy-terminus consensus amino acid sequence Gly-Gly-X-Gly-X-Asp (Highlander et al. (1989) *DNA* 8:15-28), wherein X is Lys, Asp, Val or Asn. Such proteins include, among others, leukotoxins derived from *P. haemolytica* and *Actinobacillus pleuropneumoniae*, as well as *E. coli* alpha hemolysin (Strathdee et al. (1987) *Infect. Immun.* 55:3233-3236; Lo (1990) *Can. J. Vet. Res.* 54:S33-S35; Welch (1991) *Mol. Microbiol.* 5:521-528). This family of toxins is

known as the "RTX" family of toxins (Lo (1990) *Can. J. Vet. Res.* 54:S33-S35). In addition, the term "leukotoxin polypeptide" refers to a leukotoxin polypeptide which is chemically synthesized, isolated from an organism expressing the same, or recombinantly produced. Furthermore, the term intends an immunogenic protein having an amino acid sequence substantially homologous to a contiguous amino acid sequence found in the particular native leukotoxin molecule. Thus, the term includes both full-length and partial sequences, as well as analogues. Although native full-length leukotoxins display cytotoxic activity, the term "leukotoxin" also intends molecules which remain immunogenic yet lack the cytotoxic character of native leukotoxins. The nucleotide sequences and corresponding amino acid sequences for several leukotoxins are known. See, e.g., U.S. Patent Nos. 4,957,739 and 5,055,400; Lo et al. (1985) *Infect. Immun.* 50:667-67; Lo et al. (1987) *Infect. Immun.* 55:1987-1996; Strathdee et al. (1987) *Infect. Immun.* 55:3233-3236; Highlander et al. (1989) *DNA* 8:15-28; and Welch (1991) *Mol. Microbiol.* 5:521-528. In preferred embodiments of the invention, leukotoxin chimeras are provided having a selected leukotoxin polypeptide sequence that imparts enhanced immunogenicity to one or more myostatin multimers fused thereto.

Particular examples of immunogenic leukotoxin polypeptides for use in the present invention are truncated leukotoxin molecules described in U.S. Patent Nos. 5,476,657 and 5,837,268. These truncated molecules include LKT 352, LKT 111 and LKT 114. LKT 352 is derived from the *lktA* gene present in plasmid pAA352 (ATCC Accession No. 68283). The nucleotide sequence and corresponding amino acid sequence of this gene are described in U.S. Patent

5,476,657. The gene encodes a truncated leukotoxin, having 914 amino acids and an estimated molecular weight of around 99 kDa. LKT 111 is a leukotoxin polypeptide derived from the lktA gene present in plasmid pCB111 (ATCC Accession No. 69748). The nucleotide sequence of this gene and the corresponding amino acid sequence are disclosed in U.S. Patent No. 5,837,268. The gene encodes a shortened version of leukotoxin which was developed from the recombinant leukotoxin gene present in plasmid pAA352 (ATCC Accession No. 68283) by removal of an internal DNA fragment of approximately 1300 bp in length. The LKT 111 polypeptide has an estimated molecular weight of 52 kDa (as compared to the 99 kDa LKT 352 polypeptide), but retains portions of the LKT 352 N-terminus containing T-cell epitopes which are necessary for sufficient T-cell immunogenicity, and portions of the LKT 352 C-terminus containing convenient restriction sites for use in producing fusion proteins for use in the present invention. LKT 114 is derived from the gene present in plasmid pAA114 (described in U.S. Patent No. 5,837,268) and is shown in Figures 15A-15D herein. LKT 114 differs from LKT 111 by virtue of an additional amino acid deletion from the internal portion of the molecule.

"Adjuvants" refer to agents which act in a nonspecific manner to increase an immune response to a particular antigen, thus reducing the quantity of antigen necessary in any given vaccine, and/or the frequency of injection necessary in order to generate an adequate immune response to the antigen of interest. See, e.g., A.C. Allison *J. Reticuloendothel. Soc.* (1979) 26:619-630.

"Native" proteins, polypeptides or peptides are proteins, polypeptides or peptides isolated from the source in which the proteins naturally occur.

"Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic"

5 polypeptides are those prepared by chemical synthesis.

By "polynucleotide" is meant a sequence of nucleotides including, but is not limited to, RNA such as mRNA, cDNA, genomic DNA sequences and even synthetic DNA sequences. The term also captures
10 sequences that include any of the known base analogs of DNA and RNA.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the
15 attached segment.

A DNA "coding sequence" or a "sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide in vitro or in vivo when placed under the control of
20 appropriate regulatory elements. The boundaries of the coding sequence are determined by a start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from
25 eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

The term DNA "control elements" refers
30 collectively to promoters, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in
35 a host cell. Not all of these control sequences need always be present in a recombinant vector so long as

the desired gene is capable of being transcribed and translated.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control elements operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control elements need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter and the coding sequence and the promoter can still be considered "operably linked" to the coding sequence.

A control element, such as a promoter, "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous nucleic acid molecule.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In procaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones

comprised of a population of daughter cells containing the exogenous DNA.

The term "derived from," as it is used herein, denotes an actual or theoretical source or origin of the subject molecule or immunogen. For example, an immunogen that is "derived from" a particular myostatin molecule will bear close sequence similarity with a relevant portion of the reference molecule. Thus, an immunogen that is "derived from" a particular myostatin molecule may include all of the wild-type myostatin sequence, or may be altered by insertion, deletion or substitution of amino acid residues, so long as the derived sequence provides for an immunogen that corresponds to the targeted myostatin molecule. Immunogens derived from a denoted molecule will contain at least one epitope specific to the denoted molecule.

By "vertebrate subject" is meant any member of the subphylum cordata, including, without limitation, mammals such as cattle, sheep, pigs, goats, horses, and humans; domestic animals such as dogs and cats; and birds, including domestic, wild and game birds such as cocks and hens including chickens, turkeys and other gallinaceous birds; and fish. The term does not denote a particular age or gender. Thus, both male and female adult and newborn animals, as well as fetuses and eggs, are intended to be covered.

The compositions and methods of the present invention will serve to "reduce myostatin activity." This reduction in activity may be a reduction of circulating levels of myostatin normally found in a vertebrate subject, or a reduction of circulating levels of myostatin in subjects with disorders that result in elevated circulating levels of myostatin. A reduction in myostatin activity generally results from

inactivation of circulating myostatin by antibodies generated against the myostatin peptide immunogen delivered to the subject in question. However, the reduction of activity is not limited to a particular mode of inactivation, but may be the result of decreased production or secretion of myostatin into the circulation. While not being bound by a particular theory, the myostatin peptide immunogens may elicit the production of antibodies which prevent myostatin from being cleaved to release the active portion of the protein, or prevent the protein from binding to its receptor. Alternatively, the antibodies may remove secreted myostatin from circulation or other body fluids before it reaches the active site.

The reduction in myostatin activity may manifest itself in a variety of ways. For example, reduction in myostatin activity may result in an increase in body weight, enhanced muscle mass, increased muscle strength, an alteration in the ratio of muscle to fat, an increase in fat-free muscle mass, an increase in the size and/or number of muscle cells, a reduction in body fat content, an increase in life span in a normal or diseased vertebrate, an increase in appetite or feed uptake, an enhanced quality of life, and in mammals, an increase in mammary gland tissue and lactation.

By "enhancing muscle mass" is meant that the animal administered a composition of the present invention displays an increase in muscle cell size (hypertrophy) or muscle cell numbers (hyperplasia). The increase can be in type 1 and/or type 2 muscle fibers. The term "muscle" as used herein is intended to capture analogous tissue types in fish. Methods for determining "enhanced muscle mass" are well known in the art. For example, muscle content can be

measured before and after administration of a myostatin peptide of the invention using standard techniques, such as under water weighing (see, e.g., Bhasin et al. *New Eng. J. Med.* (1996) 335:1-7) and
5 dual-energy x-ray absorptiometry (see, e.g., Bhasin et al. *Mol. Endocrinol.* (1998) 83:3155-3162). An increase in muscle size may be evidenced by weight gain of at least about 5-10%, preferably at least about 10-20% or more.

10

B. General Methods

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular formulations or process
15 parameters as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

20

Although a number of compositions and methods similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

25

Central to the instant invention is the development of immunological compositions and methods for modulating endogenous myostatin production in a vertebrate subject. Although myostatin is generally recognized as "self" and hence nonimmunogenic, the
30 compositions described herein surprisingly provide a means for producing an immunological response in a subject immunized therewith.

35

Accordingly, the invention is directed to immunogenic myostatin peptides, myostatin multimers and myostatin immunoconjugates for use in generating
an immune response in a vertebrate subject. Since the

myostatin protein is secreted, active or passive immunization of young animals serves to increase muscle mass but avoids the problems associated with other abnormalities which arise from changes induced during the embryonic period. Thus, for example, vaccination schedules can be initiated shortly after birth to achieve both hypertrophy and/or hyperplasia. Alternatively, immunization can be done at a later stage of development, (e.g., to cattle in feedlots) to improve muscle protein yield. Additionally, immunization can be done prenatally or to animals in utero, to achieve the desired results.

The compositions and techniques described herein are equally applicable to egg-laying vertebrates, such as birds and fish. In this regard, McPherron and Lee, *Proc. Natl. Acad. Sci. USA* (1997) 94:12457-12461, have identified myostatin genes in birds and fish which are highly homologous to mammalian myostatin genes. Therefore, the gene is conserved among species and is believed to serve a similar function in all species. Thus, for example, egg-laying birds and fish are immunized to create high antibody titers in maternal plasma. Since antibodies are transferred to the yolk sac of the egg, these antibodies are able to reduce myostatin during the embryonic period and cause the desired increase in size and/or numbers of muscle cells. Alternatively, immunization may be done in ovo.

Furthermore, the methods and vaccines described herein will find use for the treatment of various disorders in humans and other animals. For example, modulation of myostatin production is useful for the treatment of individuals with disorders that either primarily or incidentally cause muscle wasting such as for the treatment of paraplegics and quadriplegics, where muscle atrophy is a serious

concern. Elderly subjects may also benefit from the methods and vaccines described herein where lack of muscle strength is often a serious limitation to an active, healthy lifestyle. Additionally, the compositions of the present invention can be used to treat or prevent muscle wasting due to various cancers, anorexia, cachexia, AIDS, and like disorders.

The methods and vaccines of the present invention will find use for treating various dystrophies, such as pseudohypertrophic muscular dystrophies, facioscapulohumeral dystrophies, limb-girdle muscular dystrophies, distal muscular dystrophies, ocular myopathies, and myotonic dystrophies. These diseases include the disorders known as Becker's type muscular dystrophy, Dejerine-Landouzy muscular dystrophy, Duchenne's type muscular dystrophy, Landouzy muscular dystrophy, Emery-Dreifuss muscular dystrophy, Erb's muscular dystrophy, Fukuyama type muscular dystrophy, Gowers' muscular dystrophy, infantile neuroaxonal muscular dystrophy, Leyden-Möblus muscular dystrophy, oculopharyngeal muscular dystrophy, pelvifemoral muscular dystrophy, progressive muscular dystrophy, scapulohumeral muscular dystrophy and Simmerlin's muscular dystrophy.

Additionally, since myostatin is highly homologous to GDF11, the myostatin peptides of the present invention will also find use in modulating GDF11 activity. See, e.g., NCBI Accession No. AF092734 for the sequence of GDF11.

Immunization can be achieved by any of the methods known in the art including, but not limited to, use of peptide vaccines or DNA immunization. Such methods are described in detail below.

1. Myostatin Peptides

Myostatin peptides for use with the present invention will generally include at least about 3 amino acids to about 200 amino acids, preferably at least about 3 amino acids to about 100 amino acids, more preferably at least about 3 to about 50 amino acids, even more preferably at least about 3 amino acids to about 30 amino acids, preferably about 3 to about 15 amino acids, and most preferably at least about 5 amino acids to about 25 amino acids or 5 to about 15 amino acids, from a selected myostatin protein.

Representative myostatin proteins from 10 species from which the myostatin peptides of the present invention can be derived are shown in Figures 1A-1D. The amino acid sequence of bovine myostatin is also shown in Figure 16B. The peptide will include at least one epitope which imparts immunogenicity to the myostatin molecule.

In preferred embodiments, the myostatin peptide is derived from the region of myostatin including but not limited to the region spanning amino acids 1 through 350, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36); the region of myostatin spanning amino acids 1 through 275, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36); the region of myostatin spanning amino acids 25 through 300, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36); the region of myostatin spanning amino acids 50 through 325, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36); the region of myostatin spanning amino acids 75 through 350, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36); the region of myostatin spanning amino acids 45 through 376, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36); 100 through 376, inclusive, of Figures 1A-1D (SEQ ID NOS:27-36); the region of myostatin spanning amino acids 235 through 376, inclusive, of

Figures 1A-1D (SEQ ID NOS:27-36); or from any region believed to include an epitope of myostatin capable of eliciting an immune response in a subject to which the peptide is delivered.

5 In certain embodiments, myostatin peptides are derived from one of three regions of myostatin which display the highest points of hydrophilicity in the hydrophilicity profile shown in Figure 17. The three highest points of hydrophilicity are found at
10 amino acid positions 263-268, which spans the proteolytic cleavage site; positions 31-37; and positions 106-111. Thus, in these embodiments, the myostatin peptide comprises the amino acid sequence
15 Lys-Arg-Ser-Arg-Arg-Asp (SEQ ID NO:37) which spans the proteolytic cleavage site; the amino acid sequence Lys-Glu-Asn-Val-Glu-Lys-Glu (SEQ ID NO:38) which corresponds to amino acids 31-37 of myostatin; or the amino acid sequence Ser-Leu-Lys-Asp-Asp-Asp (SEQ ID
20 NO:39) which corresponds to amino acids 106 to 111 of myostatin.

 In other embodiments, the myostatin peptide has at least about 75% amino acid identity to a peptide comprising the amino acid sequence of amino acids 3-18, inclusive of SEQ ID NO:4 (MYOS 1, shown in
25 Figure 2); amino acids 3-15, inclusive of SEQ ID NO:6 (MYOS 3, shown in Figure 3); amino acids 3-17, inclusive, of SEQ ID NO:8 (MYOS 5, shown in Figure 4); amino acids 3-16, inclusive of SEQ ID NO:10 (MYOS 7, shown in Figure 5); amino acids 3-22, inclusive of SEQ
30 ID NO:12 (MYOS 9, shown in Figure 6); amino acids 3-25, inclusive of SEQ ID NO:14 (MYOS 11, shown in Figure 7); amino acids 3-22, inclusive of SEQ ID NO:16 (MYOS 13, shown in Figure 8); amino acids 3-19, inclusive, of SEQ ID NO:18 (MYOS 15, shown in Figure
35 9); amino acids 3-18, inclusive, of SEQ ID NO:20 (MYOS 17, shown in Figure 10); or amino acids 3-18,

inclusive of SEQ ID NO:22 (MYOS 19, shown in Figure 11). The positions of the various MYOS peptides above relative to full-length myostatin are shown in Figure 12.

5 The myostatin peptide is optionally linked to an immunological carrier molecule in order to form a myostatin immunoconjugate, as described further below.

10 2. Myostatin Immunoconjugates

As explained above, myostatin is an endogenous molecule and, as such, it may be desirable to further increase the immunogenicity of the myostatin peptide (or multimers described below) by
15 linking it to a carrier to form a myostatin immunoconjugate. This is especially necessary if the myostatin immunogen will be administered to the same species from which it is derived.

Suitable carriers are generally polypeptides
20 which include antigenic regions of a protein derived from an infectious material such as a viral surface protein, or a carrier peptide sequence. These carriers serve to non-specifically stimulate T-helper cell activity and to help direct an immunogen of
25 interest to antigen presenting cells (APCs) for processing and presentation at the cell surface in association with molecules of the major histocompatibility complex (MHC).

Several carrier systems have been developed
30 for this purpose. For example, small peptide haptens are often coupled to protein carriers such as keyhole limpet hemocyanin (Bittle et al. (1982) *Nature* 298:30-33), bacterial toxins such as tetanus toxoid (Muller et al. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79:569-
35 573), ovalbumin, leukotoxin polypeptides, and sperm whale myoglobin, to produce an immune response. These

coupling reactions typically result in the incorporation of several moles of peptide hapten per mole of carrier protein.

Other suitable carriers for use with the present invention include VP6 polypeptides of rotaviruses, or functional fragments thereof, as disclosed in U.S. Patent Number 5,071,651. Also useful is a fusion product of a viral protein and one or more epitopes from myostatin, which fusion products are made by the methods disclosed in U.S. Patent No. 4,722,840. Still other suitable carriers include cells, such as lymphocytes, since presentation in this form mimics the natural mode of presentation in the subject, which gives rise to the immunized state. Alternatively, the myostatin immunogens may be coupled to erythrocytes, preferably the subject's own erythrocytes. Methods of coupling peptides to proteins or cells are known to those of skill in the art.

Delivery systems useful in the practice of the present invention may also utilize particulate carriers. For example, pre-formed particles have been used as platforms onto which immunogens can be coupled and incorporated. Systems based on proteosomes (Lowell et al. (1988) *Science* 240:800-802) and immune stimulatory complexes (Morein et al. (1984) *Nature* 308:457-460) are also known in the art.

Carrier systems using recombinantly produced chimeric proteins that self-assemble into particles may also be used with the present invention. For example, the yeast retrotransposon, Ty, encodes a series of proteins that assemble into virus like particles (Ty-VLPs; Kingsman et al. (1988) *Vaccines* 6:304-306). Thus, a gene, or fragment thereof, encoding the myostatin immunogen of interest may be inserted into the TyA gene and expressed in yeast as a

fusion protein. The fusion protein retains the capacity to self assemble into particles of uniform size. Other useful virus-like carrier systems are based on HBsAg, (Valenzuela et al. (1985) *Bio/Technol.* 3:323-326; U.S. Patent No. 4,722,840; Delpeyroux et al. (1986) *Science* 233:472-475), Hepatitis B core antigen (Clarke et al. (1988) *Vaccines* 88 (Ed. H. Ginsberg, et al.) pp. 127-131), Poliovirus (Burke et al. (1988) *Nature* 332:81-82), and Tobacco Mosaic Virus (Haynes et al. (1986) *Bio/Technol.* 4:637-641).

Especially preferred carriers include serum albumins, keyhole limpet hemocyanin, ovalbumin, sperm whale myoglobin, leukotoxin molecules as described above, and other proteins well known to those skilled in the art. One particular leukotoxin polypeptide, for use as a carrier herein, is shown in Figures 15A-15D. Myostatin is conveniently inserted into the *Bam*HI site present at nucleotide position 3334, as described further in the examples.

Protein carriers may be used in their native form or their functional group content may be modified by, for example, succinylation of lysine residues or reaction with Cys-thiolactone. A sulfhydryl group may also be incorporated into the carrier (or antigen) by, for example, reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(4-dithiopyridyl) propionate. Suitable carriers may also be modified to incorporate spacer arms (such as hexamethylene diamine or other bifunctional molecules of similar size) for attachment of peptide immunogens.

Carriers can be physically conjugated to the myostatin immunogen of interest, using standard coupling reactions. Alternatively, chimeric molecules can be prepared recombinantly for use in the present invention, such as by fusing a gene encoding a suitable polypeptide carrier to one or more copies of

a gene, or fragment thereof, encoding for a selected myostatin immunogen.

The myostatin immunogens can also be administered via a carrier virus which expresses the same. Carrier viruses which will find use herein include, but are not limited to, the vaccinia and other pox viruses, adenovirus, and herpes virus. By way of example, vaccinia virus recombinants expressing the proteins can be constructed as follows. The DNA encoding a particular protein is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the desired immunogen into the viral genome. The resulting TK-recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

3. Myostatin Multimers

Immunogenicity of the myostatin immunogens may also be significantly increased by producing immunogenic forms of the molecules that comprise multiple copies of selected epitopes. In this way, endogenous myostatin may be rendered an effective autoantigen.

Accordingly, in one aspect of the invention, vaccine compositions containing myostatin multimers are provided in either nucleic acid or peptide form for delivery to a subject. The myostatin multimer will have more than one copy of selected myostatin immunogens, peptides or epitopes, as described above, or multiple tandem repeats of a selected myostatin

immunogen, peptide or epitope. Thus, the myostatin multimers may comprise either multiple or tandem repeats of selected myostatin sequences, multiple or tandem repeats of selected myostatin epitopes, or any conceivable combination thereof. Myostatin epitopes may be identified using techniques as described in detail above.

For example, the myostatin multimer may correspond to a molecule with repeating units of the general formula (MP-X-MP)_y wherein MP is a myostatin peptide, X is selected from the group consisting of a peptide linkage, an amino acid spacer group and [MP]_n, where n is greater than or equal to 1, y is greater than or equal to 1, and further wherein "MP" may comprise any MP peptide. Thus, the myostatin multimer may contain from 2-64 or more myostatin peptides, more preferably 2- 32 or 2-16 myostatin peptides.

Further, the selected myostatin immunogen sequences may all be the same, or may correspond to different derivatives, analogs, variants or epitopes of myostatin so long as they retain the ability to elicit an immune response. Additionally, if the myostatin immunogens are linked either chemically or recombinantly to a carrier, myostatin peptides may be linked to either the 5'-end, the 3'-end, or may flank the carrier in question. Further, the myostatin multimer may be located at sites internal to the carrier.

One particular carrier for use with the present myostatin multimers is a leukotoxin polypeptide as described above. For example, myostatin oligo repeats can be conveniently inserted into the BamHI site present at nucleotide position 3334 of the leukotoxin polypeptide shown in Figures 15A-15D.

As explained above, spacer sequences may be present between the myostatin moieties. For example, Arg-Ser and Gly-Ser dimers are present in the MYOS peptides exemplified herein which provide spacers between repeating sequences of the myostatin peptides. The strategic placement of various spacer sequences between selected myostatin immunogens can be used to confer increased immunogenicity on the subject constructs. Accordingly, under the invention, a selected spacer sequence may encode a wide variety of moieties such as a single amino acid linker or a sequence of two to several amino acids. Selected spacer groups may preferably provide enzyme cleavage sites so that the expressed multimer can be processed by proteolytic enzymes in vivo (by APCs, or the like) to yield a number of peptides, each of which contain at least one T-cell epitope derived from the carrier portion, and which are preferably fused to a substantially complete myostatin peptide sequence.

The spacer groups may be constructed so that the junction region between selected myostatin moieties comprises a clearly foreign sequence to the immunized subject, thereby conferring enhanced immunogenicity upon the associated myostatin peptides. Additionally, spacer sequences may be constructed so as to provide T-cell antigenicity, such as those sequences which encode amphipathic and/or α -helical peptide sequences which are generally recognized in the art as providing immunogenic helper T-cell epitopes. The choice of particular T-cell epitopes to be provided by such spacer sequences may vary depending on the particular vertebrate species to be vaccinated. Although particular myostatin portions are exemplified which include spacer sequences, it is also an object of the invention to provide one or more myostatin multimers comprising directly adjacent

myostatin sequences (without intervening spacer sequences).

The myostatin multimeric sequence thus produced renders a highly immunogenic myostatin antigen for use in the compositions of the invention.

The myostatin peptides, immunoconjugates and multimers can be produced using the methods described below, and used for nucleic acid immunization, gene therapy, protein-based immunization methods, and the like.

4. Nucleic Acid-Based Immunization Methods

Generally, nucleic acid-based vaccines for use with the present invention will include relevant regions encoding a myostatin immunogen, with suitable control sequences and, optionally, ancillary therapeutic nucleotide sequences. The nucleic acid molecules are prepared in the form of vectors which include the necessary elements to direct transcription and translation in a recipient cell.

In order to augment an immune response in an immunized subject, the nucleic acid molecules can be administered in conjunction with ancillary substances, such as pharmacological agents, adjuvants, or in conjunction with delivery of vectors encoding biological response modifiers such as cytokines and the like. Other ancillary substances include, but are not limited to, substances to increase weight gain, muscle mass or muscle strength, such as growth hormones, growth promoting agents, beta antagonists, partitioning agents and antibiotics.

Nucleotide sequences selected for use in the present invention can be derived from known sources, for example, by isolating the same from cells or tissue containing a desired gene or nucleotide

sequence using standard techniques, or by using recombinant or synthetic techniques.

Once coding sequences for the myostatin immunogens have been prepared or isolated, such sequences can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Ligations to other sequences, e.g., ancillary molecules or carrier molecules, are performed using standard procedures, known in the art. One or more myostatin immunogen portions of the chimera can be fused 5' and/or 3' to a desired ancillary sequence or carrier molecule. Alternatively, one or more myostatin immunogen portions may be located at sites internal to the carrier molecule, or such portions can be positioned at both terminal and internal locations in the chimera.

Alternatively, DNA sequences encoding the myostatin immunogens of interest, optionally linked to carrier molecules, can be prepared synthetically rather than cloned. The DNA sequences can be designed with appropriate codons for the particular sequence. The complete sequence of the immunogen is then assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) *Nature* 292:756; Nambair et al. (1984) *Science* 223:1299; and Jay et al. (1984) *J. Biol. Chem.* 259:6311.

The coding sequence is then placed under the control of suitable control elements for expression in suitable host tissue *in vivo*. The choice of control elements will depend on the subject being treated and the type of preparation used. Thus, if the subject's endogenous transcription and translation machinery will be used to express the immunogens, control

elements compatible with the particular subject will be utilized. In this regard, several promoters for use in mammalian systems are known in the art. For example, typical promoters for mammalian cell
5 expression include the SV40 early promoter, a CMV promoter such as the CMV immediate early promoter, the mouse mammary tumor virus LTR promoter, the adenovirus major late promoter (Ad MLP), and the herpes simplex virus promoter, among others. Other nonviral
10 promoters, such as a promoter derived from the murine metallothionein gene, will also find use for mammalian expression.

Typically, transcription termination and polyadenylation sequences will also be present,
15 located 3' to the translation stop codon. Preferably, a sequence for optimization of initiation of translation, located 5' to the coding sequence, is also present. Examples of transcription terminator/polyadenylation signals include those
20 derived from SV40, as described in Sambrook et al., *supra*, as well as a bovine growth hormone terminator sequence. Introns, containing splice donor and acceptor sites, may also be designed into the constructs for use with the present invention.

25 Enhancer elements may also be used herein to increase expression levels of the constructs. Examples include the SV40 early gene enhancer (Dijkema et al. (1985) *EMBO J.* 4:761), the enhancer/promoter derived from the long terminal repeat (LTR) of the
30 Rous Sarcoma Virus (Gorman et al. (1982) *Proc. Natl. Acad. Sci. USA* 79:6777) and elements derived from human CMV (Boshart et al. (1985) *Cell* 41:521), such as elements included in the CMV intron A sequence.

Once prepared, the nucleic acid vaccine
35 compositions can be delivered to the subject using known methods. In this regard, various techniques for

immunization with antigen-encoding DNAs have been described. See, e.g., U.S. Patent No. 5,589,466 to Felgner et al.; Tang et al. (1992) *Nature* 358:152; Davis et al. (1993) *Hum. Molec. Genet.* 2:1847; Ulmer et al. (1993) *Science* 258:1745; Wang et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:4156; Eisenbraun et al. (1993) *DNA Cell Biol.* 12:791; Fynan et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:12476; Fuller et al. (1994) *AIDS Res. Human Retrovir.* 10:1433; and Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519. General methods for delivering nucleic acid molecules to cells in vitro, for the subsequent reintroduction into the host, can also be used, such as liposome-mediated gene transfer. See, e.g., Hazinski et al. (1991) *Am. J. Respir. Cell Mol. Biol.* 4:206-209; Brigham et al. (1989) *Am. J. Med. Sci.* 298:278-281; Canonico et al. (1991) *Clin. Res.* 39:219A; and Nabel et al. (1990) *Science* 249:1285-1288. Thus, the nucleic acid vaccine compositions can be delivered in either liquid or particulate form using a variety of known techniques. Typical vaccine compositions are described more fully below.

5. Protein-Based Immunization Methods

Peptide-based vaccine compositions can also be produced using a variety of methods known to those skilled in the art. In particular, myostatin immunogens can be isolated directly from native sources, using standard purification techniques. Alternatively, the immunogens can be recombinantly produced using the nucleic acid expression systems described above, and purified using known techniques. Peptide immunogens can also be synthesized, based on described amino acid sequences or amino acid sequences derived from the DNA sequence of a molecule of interest, using chemical polymer syntheses such as

solid phase peptide synthesis. Such methods are known to those skilled in the art. See, e.g., J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, IL (1984) and G. Barany and R. B. Merrifield, *The Peptides: Analysis, Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, Academic Press, New York, (1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of Peptide Synthesis*, Springer-Verlag, Berlin (1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology*, supra, Vol. 1, for classical solution synthesis.

Peptide immunogens may also be produced by cloning the coding sequences therefor into any suitable expression vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning, and host cells which they can transform, include the bacteriophage lambda (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine papilloma virus (mammalian cells). See, generally, *DNA Cloning: Vols. I & II*, supra; Sambrook et al., supra; B. Perbal, supra.

For example, the coding sequence for myostatin from a number of vertebrate species, including mouse, rat, human, baboon, cattle, pig, sheep, chicken and turkey has been determined. See, e.g., U.S. Patent No. 5,827,733 and NCBI Accession No.

U84OC5 for the nucleotide sequence of murine myostatin; U.S. Patent No. 5,827,733, International Publication No. WO 98/33887, and NCBI Accession No. AF019627 for the nucleotide sequence of human myostatin; Figure 16A herein, as well as International Publication Nos. WO 99/02667 and WO 98/33887, and NCBI Accession No. AF019620 for the nucleotide sequence of bovine myostatin; NCBI Accession No. AF019626 for the nucleotide sequence of zebrafish myostatin; International Publication No. WO 98/33887, for the nucleotide sequences of rat (see, also NCBI Accession No. AF019624), baboon (see, also NCBI Accession No. AF019619), porcine (see, also NCBI Accession No. AF019623), ovine (see, also NCBI Accession No. AF019622), chicken (see, also NCBI Accession No. AF019621) and turkey (see also NCBI Accession No. AF019625) myostatin. The myostatin sequence is highly conserved across all of these species.

Portions of these sequences encoding desired myostatin peptides, and if desired, a sequence encoding a carrier protein, can be cloned, isolated and ligated together using recombinant techniques generally known in the art. See, e.g., Sambrook et al., *supra*.

The gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator, so that the DNA sequence of interest is transcribed into RNA by a suitable transformant. The coding sequence may or may not contain a signal peptide or leader sequence. The peptide immunogens can be expressed using, for example, the *E. coli* tac promoter or the protein A gene (*spa*) promoter and signal sequence. Leader sequences can be removed by the bacterial host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397. Ancillary

sequences, such as those described above, may also be present.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the immunogen sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the particular myostatin immunogen may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it can be attached to the control sequences in the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases, it may be desirable to add sequences which cause the secretion of the immunogen

from the host organism, with subsequent cleavage of the secretory signal. It may also be desirable to produce mutants or analog of the immunogen. Mutants or analogs may be prepared by the deletion of a
 5 portion of the sequence encoding the immunogen, or if present, a portion of the sequence encoding the desired carrier molecule, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying
 10 nucleotide sequences, such as site-directed mutagenesis, and the like, are well known to those skilled in the art. See, e.g., Sambrook et al., supra; *DNA Cloning*, Vols. I and II, supra; *Nucleic Acid Hybridization*, supra; Kunkel, T.A. *Proc. Natl. Acad. Sci. USA* (1985) 82:448; Geisselsoder et al. *BioTechniques* (1987) 5:786; Zoller and Smith, *Methods Enzymol.* (1983) 100:468; Dalbie-McFarland et al. *Proc. Natl. Acad. Sci USA* (1982) 79:6409.

The myostatin immunogens can be expressed in
 20 a wide variety of systems, including insect, mammalian, bacterial, viral and yeast expression systems, all well known in the art. For example, insect cell expression systems, such as baculovirus systems, are known to those of skill in the art and
 25 described in, e.g., Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555* (1987). Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, inter alia, Invitrogen, San Diego CA
 30 ("MaxBac" kit). Similarly, bacterial and mammalian cell expression systems are well known in the art and described in, e.g., Sambrook et al., supra. Yeast expression systems are also known in the art and described in, e.g., *Yeast Genetic Engineering* (Barr et
 35 al., eds., 1989) Butterworths, London.

A number of appropriate host cells for use with the above systems are also known. For example, mammalian cell lines are known in the art and include immortalized cell lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), Madin-Darby bovine kidney ("MDBK") cells, as well as others. Similarly, bacterial hosts such as *E. coli*, *Bacillus subtilis*, and *Streptococcus spp.*, will find use with the present expression constructs. Yeast hosts useful in the present invention include inter alia, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guilliermondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Insect cells for use with baculovirus expression vectors include, inter alia, *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*.

Depending on the expression system and host selected, the myostatin immunogens are produced by growing host cells transformed by an expression vector described above under conditions whereby the immunogen is expressed. The expressed immunogen is then isolated from the host cells and purified. If the expression system secretes the immunogen into growth media, the product can be purified directly from the media. If it is not secreted, it can be isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

Once obtained, the myostatin peptides, with or without associated carrier, may be formulated into vaccine compositions, such as vaccine compositions as described further below, in order to elicit antibody production in a subject vertebrate.

6. Antibody Production

The subject myostatin peptides can be used to generate antibodies for use in passive immunization methods or for immunopurification or immunodiagnostic purposes. Typically, peptides useful for producing antibodies will usually be at least about 3-5 amino acids in length, preferably 7-10 amino acids in length, and most preferably at least about 10 to 15 amino acids in length, or more.

Antibodies against the subject immunogens include polyclonal and monoclonal antibody preparations, monospecific antisera, as well as preparations including hybrid antibodies, altered antibodies, F(ab'), fragments, F(ab) fragments, F_v fragments, single domain antibodies, chimeric antibodies, humanized antibodies, and functional fragments thereof, which retain specificity for the target molecule in question. For example, an antibody can include variable regions, or fragments of variable regions, which retain specificity for the molecule in question. The remainder of the antibody can be derived from the species in which the antibody will be used. Thus, if the antibody is to be used in a human, the antibody can be "humanized" in order to reduce immunogenicity yet retain activity. For a description of chimeric antibodies, see, e.g., Winter, G. and Milstein, C. (1991) *Nature* 349:293-299; Jones, P.T. et al. (1986) *Nature* 321:522-525; Riechmann, L. et al. (1988) 332:323-327; and Carter, P. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:4285-4289. Such chimeric

antibodies may contain not only combining sites for the target molecule, but also binding sites for other proteins. In this way, bifunctional reagents can be generated with targeted specificity to both external
5 and internal antigens.

If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) is immunized with the desired antigen, or its fragment, or a mutated antigen, as described above.
10 Prior to immunization, it may be desirable to further increase the immunogenicity of a particular immunogen. This can be accomplished in any one of several ways known to those of skill in the art.

For example, immunization for the production
15 of antibodies is generally performed by mixing or emulsifying the protein in a suitable excipient, such as saline, preferably in an adjuvant such as Freund's complete adjuvant, or any of the adjuvants described below, and injecting the mixture or emulsion
20 parenterally (generally subcutaneously or intramuscularly). The animal is generally boosted 2-6 weeks later with one or more injections of the protein in saline, preferably using Freund's incomplete adjuvant, or the like. Antibodies may also be generated by in
25 vitro immunization, using methods known in the art. Polyclonal antisera is then obtained from the immunized animal and treated according to known procedures. See, e.g., Jurgens et al. (1985) *J. Chrom.* 348:363-370. If serum containing polyclonal
30 antibodies is used, the polyclonal antibodies can be purified by immunoaffinity chromatography, using known procedures.

Monoclonal antibodies are generally prepared using the method of Kohler and Milstein, *Nature* (1975)
35 256:495-96, or a modification thereof. Typically, a mouse or rat is immunized as described above.

However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) is removed and dissociated into single cells. If desired, the spleen cells may be screened (after
5 removal of nonspecifically adherent cells) by applying a cell suspension to a plate or well coated with the protein antigen. B-cells, expressing membrane-bound immunoglobulin specific for the antigen, will bind to the plate, and are not rinsed away with the rest of
10 the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium (e.g., hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas
15 are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the immunizing antigen (and which do not bind to unrelated antigens). The selected monoclonal antibody-secreting hybridomas are then cultured either
20 in vitro (e.g., in tissue culture bottles or hollow fiber reactors), or in vivo (as ascites in mice). See, e.g., M. Schreier et al., *Hybridoma Techniques* (1980); Hammerling et al., *Monoclonal Antibodies and T-cell Hybridomas* (1981); Kennett et al., *Monoclonal Antibodies* (1980); see also U.S. Patent Nos.
25 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the myostatin peptide of interest, or fragment thereof,
30 can be screened for various properties; i.e., for isotype, epitope, affinity, etc.

Functional fragments of the antibodies can also be made against the myostatin peptide of interest and can be produced by cleaving a constant region, not
35 responsible for antigen binding, from the antibody molecule, using e.g., pepsin, to produce F(ab')₂.

fragments. These fragments will contain two antigen binding sites, but lack a portion of the constant region from each of the heavy chains. Similarly, if desired, Fab fragments, comprising a single antigen binding site, can be produced, e.g., by digestion of polyclonal or monoclonal antibodies with papain. Functional fragments, including only the variable regions of the heavy and light chains, can also be produced, using standard techniques. These fragments are known as F_v.

Chimeric or humanized antibodies can also be produced using the subject immunogens. These antibodies can be designed to minimize unwanted immunological reactions attributable to heterologous constant and species-specific framework variable regions typically present in monoclonal and polyclonal antibodies. For example, if the antibodies are to be used in human subjects, chimeric antibodies can be created by replacing non-human constant regions, in either the heavy and light chains, or both, with human constant regions, using techniques generally known in the art. See, e.g., Winter, G. and Milstein, C. (1991) *Nature* 349:293-299; Jones, P.T. et al. (1986) *Nature* 321:522-525; Riechmann, L. et al. (1988) 332:323-327; and Carter, P. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:4285-4289.

7. Vaccine Compositions

Once the above molecules are produced, they are formulated into vaccine compositions for delivery to a vertebrate subject. The relevant myostatin molecule is administered alone, or mixed with a pharmaceutically acceptable vehicle or excipient. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, the vehicle may

contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccine. Suitable adjuvants are described further below. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art. See, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, 18th edition, 1990. The composition or formulation to be administered will contain a quantity of the myostatin immunogen adequate to achieve the desired immunized state in the subject being treated.

As explained above, the vaccine compositions of the present invention may include adjuvants to further increase the immunogenicity of the myostatin immunogen. Adjuvants may include for example, emulsifiers, muramyl dipeptides, avridine, aluminum hydroxide, oils, saponins and other substances known in the art. For example, compounds which may serve as emulsifiers herein include natural and synthetic emulsifying agents, as well as anionic, cationic and nonionic compounds. Among the synthetic compounds, anionic emulsifying agents include, for example, the potassium, sodium and ammonium salts of lauric and oleic acid, the calcium, magnesium and aluminum salts of fatty acids (i.e., metallic soaps), and organic sulfonates such as sodium lauryl sulfate. Synthetic cationic agents include, for example, cetyltrimethylammonium bromide, while synthetic nonionic agents are exemplified by glyceryl esters (e.g., glyceryl monostearate), polyoxyethylene glycol esters and ethers, and the sorbitan fatty acid esters (e.g., sorbitan monopalmitate) and their polyoxyethylene derivatives (e.g., polyoxyethylene sorbitan monopalmitate). Natural emulsifying agents include acacia, gelatin, lecithin and cholesterol.

Other suitable adjuvants can be formed with an oil component, such as a single oil, a mixture of oils, a water-in-oil emulsion, or an oil-in-water emulsion. The oil may be a mineral oil, a vegetable oil, or an animal oil. Mineral oil, or oil-in-water emulsions in which the oil component is mineral oil are preferred. In this regard, a "mineral oil" is defined herein as a mixture of liquid hydrocarbons obtained from petrolatum via a distillation technique; the term is synonymous with "liquid paraffin," "liquid petrolatum" and "white mineral oil." The term is also intended to include "light mineral oil," i.e., an oil which is similarly obtained by distillation of petrolatum, but which has a slightly lower specific gravity than white mineral oil. See, e.g., *Remington's Pharmaceutical Sciences, supra*. A particularly preferred oil component is the oil-in-water emulsion sold under the trade name of EMULSIGEN PLUS™ (comprising a light mineral oil as well as 0.05% formalin, and 30 mcg/mL gentamicin as preservatives), available from MVP Laboratories, Ralston, Nebraska, or the VSA-3 adjuvant which is a modified form of the EMULSIGEN PLUS™ adjuvant. Suitable animal oils include, for example, cod liver oil, halibut oil, menhaden oil, orange roughy oil and shark liver oil, all of which are available commercially. Suitable vegetable oils, include, without limitation, canola oil, almond oil, cottonseed oil, corn oil, olive oil, peanut oil, safflower oil, sesame oil, soybean oil, and the like.

Alternatively, a number of aliphatic nitrogenous bases can be used as adjuvants with the vaccine formulations. For example, known immunologic adjuvants include amines, quaternary ammonium compounds, guanidines, benzamidines and thiouroniums (Gall, D. (1966) *Immunology* 11:369-386). Specific

compounds include dimethyldioctadecylammonium bromide (DDA) (available from Kodak) and N,N-dioctadecyl-N,N-bis(2-hydroxyethyl)propanediamine ("avridine"). The use of DDA as an immunologic adjuvant has been described; see, e.g., the Kodak Laboratory Chemicals Bulletin 56(1):1-5 (1986); *Adv. Drug Deliv. Rev.* 5(3):163-187 (1990); *J. Controlled Release* 7:123-132 (1988); *Clin. Exp. Immunol.* 78(2):256-262 (1989); *J. Immunol. Methods* 97(2):159-164 (1987); *Immunology* 58(2):245-250 (1986); and *Int. Arch. Allergy Appl. Immunol.* 68(3):201-208 (1982). Avridine is also a well-known adjuvant. See, e.g., U.S. Patent No. 4,310,550 to Wolff, III et al., which describes the use of N,N-higher alkyl-N',N'-bis(2-hydroxyethyl)propane diamines in general, and avridine in particular, as vaccine adjuvants. U.S. Patent No. 5,151,267 to Babiuk, and Babiuk et al. (1986) *Virology* 159:57-66, also relate to the use of avridine as a vaccine adjuvant.

The vaccine compositions of the present invention can also include ancillary substances, such as pharmacological agents, cytokines, or other biological response modifiers. Other ancillary substances include, but are not limited to, substances to increase weight gain, muscle mass or muscle strength, such as growth hormones, growth promoting agents, beta antagonists, partitioning agents and antibiotics.

The vaccines of the present invention are normally prepared as injectables, either as liquid solutions or suspensions, or as solid forms which are suitable for solution or suspension in liquid vehicles prior to injection. The preparation may also be emulsified or the active ingredient encapsulated in liposome vehicles or other particulate carriers used.

The vaccine compositions may also be prepared in solid form. For example, solid particulate formulations can be prepared for delivery from commercially available needleless injector devices. Alternatively, solid dose implants can be provided for implantation into a subject. Controlled or sustained release formulations may also be used and are made by incorporating the myostatin immunogens into carriers or vehicles such as liposomes, nonresorbable impermeable polymers such as ethylenevinyl acetate copolymers and Hytrel® copolymers, swellable polymers such as hydrogels, or resorbable polymers such as collagen and certain polyacids or polyesters such as those used to make resorbable sutures.

Furthermore, the immunogens may be formulated into vaccine compositions in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccine composition is formulated to contain an effective amount of the myostatin immunogen, the exact amount being readily determined by one skilled in the art, wherein the amount depends on the animal to be treated, the capacity of the animal's immune system to synthesize antibodies, and the degree of immunoneutralization of myostatin

desired. For purposes of the present invention, vaccine formulations including approximately 1 μ g to about 1 mg, more generally about 5 μ g to about 200 μ g of immunogen per dose of injected solution should be adequate to raise an immunological response when administered. If a peptide-carrier chimera is used, the ratio of immunogen to carrier in the vaccine formulation will vary based on the particular carrier and immunogen selected to construct such molecules. Effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves.

The subject is immunized by administration of one of the above-described vaccine compositions in at least one dose, and preferably two or more doses. Moreover, the animal may be administered as many doses as is required to maintain a state of immunity.

Any suitable pharmaceutical delivery means may be employed to deliver the vaccine composition to the vertebrate subject. For example, conventional needle syringes, spring or compressed gas (air) injectors (U.S. Patent Nos. 1,605,763 to Smoot; 3,788,315 to Laurens; 3,853,125 to Clark et al.; 4,596,556 to Morrow et al.; and 5,062,830 to Dunlap), liquid jet injectors (U.S. Patent Nos. 2,754,818 to Scherer; 3,330,276 to Gordon; and 4,518,385 to Lindmayer et al.), and particle injectors (U.S. Patent Nos. 5,149,655 to McCabe et al. and 5,204,253 to Sanford et al.) are all appropriate for delivery of the vaccine compositions.

Preferably, the vaccine composition is administered intramuscularly, subcutaneously, intravenously, subdermally, or intradermally, to the subject. If a jet injector is used, a single jet of the liquid vaccine composition is ejected under high pressure and velocity, e.g., 1200-1400 PSI, thereby

creating an opening in the skin and penetrating to depths suitable for immunization.

Below are examples of specific embodiments for carrying out the present invention. The examples
 5 are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

C. Experimental

10

Example 1

Identification of Immunogenic Myostatin Peptides

A number of regions of the bovine myostatin molecule were identified as potentially immunogenic
 15 based on computer analysis of the full-length molecule using various computer programs. One program used formulates hydropathy scales from the amino acid sequence of the protein based on the hydrophobic and hydrophilic properties of each of the 20 amino acids.
 20 Hopp and Woods, *Proc. Natl. Acad. Sci. USA* (1981) 78:3824-3828. Figure 17 depicts a hydrophilicity profile computed using an average group length of six amino acids. The three highest points of hydrophilicity of the myostatin molecule were found at
 25 amino acid positions 263-268, which spans the proteolytic cleavage site and has the amino acid sequence Lys-Arg-Ser-Arg-Arg-Asp (SEQ ID NO:37); positions 31-37 which has the amino acid sequence Lys-Glu-Asn-Val-Glu-Lys-Glu (SEQ ID NO:38); and positions
 30 106-111 which has the amino acid sequence Ser-Leu-Lys-Asp-Asp-Asp (SEQ ID NO:39).

Analysis of the protein was also done using the program PC/Gene, Release 6.60 (Intelligenetics Inc., Geneva, Switzerland). Three-dimensional
 35 analysis of the myostatin protein was conducted using the Swiss-Pdb Viewer v2.6 (<http://expasy.hcuge.ch/>)

spdbv/mainpage.html).

From this information, a series of representative DNA oligomers were designed and constructed with a Beckman Oligo 1000M™ DNA Synthesizer using phosphoramidite chemistry. The oligomers were termed MYOS 1-20. Myos 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19 (shown in Figures 2 through 11, respectively) include portions of the coding stand of DNA while MYOS 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 include portions of the complimentary strand. The position of these peptides with reference to the full-length myostatin molecule is shown in Figure 12.

The DNA oligomers coded for peptides with 12 to 23 amino acids, flanked by 2 amino acid linkers for linkage to a carrier molecule (see further below). These peptides collectively represented the entire active portion of the protein, as well as three individual sections upstream of the proteolytic cleavage site which releases the active protein.

In particular, based on computer analysis, three portions of the active protein were selected as primary immunizing targets. The first portion was prepared by combining the oligonucleotide pair designated MYOS 1 and 2 and contained the proteolytic cleavage site and N-terminus of the active protein. MYOS 1 gave the highest antigenic determinant rating using the Hopp and Woods computer program (see Figure 17). Three-dimensional analysis of the active portion of myostatin showed that the MYOS 1 peptide is exposed on the protein surface and is therefore likely to be seen by the immune system. MYOS 1 also overlaps the proteolytic cleavage site, which releases the active portion of the protein. Blocking this site using antibodies thereto prevents cleavage of the protein and release of the active portion of the protein to prevent its effect on muscle tissue.

Two other segments of the active protein (MYOS 5 and 6 and MYOS 9 and 10) were selected because they appeared to form a loop and a helix based on a three-dimensional structural analysis. This loop structure is likely exposed on the protein surface and therefore able to be seen by the immune system. Antibodies generated to these portions of the molecule likely bind myostatin protein and remove it from circulation. The remainder of the active portion of the protein was reconstructed from the oligonucleotide pairs (MYOS 3 and 4, MYOS 7 and 8, MYOS 11 and 12, MYOS 13 and 14). Use of the entire active portion assures the proper three-dimensional structure to elicit an effective immune response. One of the regions upstream of the active portion of the protein, (MYOS 15 and 16) was selected based on computer analysis of likely antigenic epitopes. The other two upstream portions of the protein were selected to contain the proteolytic cleavage site (MYOS 19 and 20, which contain the cleavage site and amino acids immediately upstream of the cleavage site) or to be close to it (MYOS 17 and 18) so an antibody which binds to the site would interfere with the protease activity.

Based on comparisons with other known protein sequences, myostatin has areas of homology with other transforming growth factor β proteins. Bone morphogenetic protein 6 (BMP-6) has a great deal of homology to the middle and C-terminus regions of the active portion of myostatin.

Example 2

Construction of pCB150

The oligomers above were designed to be fused to the 3'-terminus of a polynucleotide encoding a 52 kDa leukotoxin (LKT) carrier protein, termed "LKT

114" herein. This polynucleotide was derived from the lktA gene present in plasmid pCB114, described in U.S. Patent No. 5,837,268. This plasmid, the nucleotide sequence of this gene and the corresponding amino acid sequence are shown in Figures 15A-15D herein and also described in U.S. Patent No. 5,837,268. The gene encodes a shortened version of leukotoxin which was developed from the recombinant leukotoxin gene present in plasmid pAA352 (ATCC Accession No. 68283 and described in U.S. Patent 5,476,657) by removal of an internal DNA fragment of approximately 1300 bp in length. The LKT 114 polypeptide has an estimated molecular weight of 52 kDa and contains convenient restriction sites for use in producing the fusion proteins of the present invention.

Plasmid pCB150, containing the coding sequence for LKT 114, into which the MYOS oligonucleotides were cloned, was prepared as follows. The leukotoxin gene was isolated as described in U.S. Patent Nos. 5,476,657 and 5,837,268. In particular, to isolate the leukotoxin gene, gene libraries of *P. haemolytica* A1 (strain B122) were constructed using standard techniques. See, Lo et al., *Infect. Immun.*, *supra*; *DNA CLONING*: Vols. I and II, *supra*; and Sambrook et al., *supra*. A genomic library was constructed in the plasmid vector pUC13 and a DNA library constructed in the bacteriophage lambda gt11. The resulting clones were used to transform *E. coli* and individual colonies were pooled and screened for reaction with serum from a calf which had survived a *P. haemolytica* infection and that had been boosted with a concentrated culture supernatant of *P. haemolytica* to increase anti-leukotoxin antibody levels. Positive colonies were screened for their ability to produce leukotoxin by incubating cell lysates with bovine neutrophils and subsequently

measuring release of lactate dehydrogenase from the latter.

Several positive colonies were identified and these recombinants were analyzed by restriction endonuclease mapping. One clone appeared to be identical to a leukotoxin gene cloned previously. See, Lo et al., *Infect. Immun.*, *supra*. To confirm this, smaller fragments were re-cloned and the restriction maps compared. It was determined that approximately 4 kilobase pairs of DNA had been cloned. Progressively larger clones were isolated by carrying out a chromosome walk (5' to 3' direction) in order to isolate full-length recombinants which were approximately 8 kb in length. The final construct was termed pAA114. This construct contained the entire leukotoxin gene sequence.

lktA, a *MaeI* restriction endonuclease fragment from pAA114 which contained the entire leukotoxin gene, was treated with the Klenow fragment of DNA polymerase I plus nucleotide triphosphates and ligated into the *SmaI* site of the cloning vector pUC13. This plasmid was named pAA179. From this, two expression constructs were made in the *ptac*-based vector pGH432:*lacI* digested with *SmaI*. One, pAA342, consisted of the 5'-*AbaIII* fragment of the *lktA* gene while the other, pAA345, contained the entire *MaeI* fragment described above. The clone pAA342 expressed a truncated leukotoxin peptide at high levels while pAA345 expressed full length leukotoxin at very low levels. Therefore, the 3' end of the *lktA* gene (*StyI* *BamHI* fragment from pAA345) was ligated to *StyI* *BamHI*-digested pAA342, yielding the plasmid pAA352. The *P. haemolytica* leukotoxin produced from the pAA352 construct is hereinafter referred to as LKT 352.

Plasmid pAA352 was then used to prepare a shortened version of the recombinant leukotoxin

polypeptide. The shortened LKT gene was produced by deleting an internal DNA fragment of approximately 1300 bp in length from the recombinant LKT gene as follows. The plasmid pCB113, (ATCC Accession No. 69749 and described in U.S. Patent No. 5,837,268) which includes the LKT-352 polypeptide, was digested with the restriction enzyme *Bst*B1 (New England Biolabs). The resultant linearized plasmid was then digested with mung-bean nuclease (Pharmacia) to remove the single stranded protruding termini produced by the *Bst*B1 digestion. The blunted DNA was then digested with the restriction enzyme *Nae*I (New England Biolabs), and the digested DNA was loaded onto a 1% agarose gel where the DNA fragments were separated by electrophoresis. A large DNA fragment of approximately 6190 bp was isolated and purified from the agarose gel using a Gene Clean kit (Bio 101), and the purified fragment was allowed to ligate to itself using bacteriophage T4 DNA ligase (Pharmacia). The resulting ligation mix was used to transform competent *E. coli* JM105 cells, and positive clones were identified by their ability to produce an aggregate protein having an appropriate molecular weight. The recombinant plasmid thus formed was designated pCB114, (described in U.S. Patent No. 5,837,268), and produces a shortened leukotoxin polypeptide termed "LKT 114".

Plasmid pCB114 was then used to produce plasmid pSLKT-30. Plasmid pSLKT-30 was made by cloning the leukotoxin-encoding fragment from pCB114 by PCR into plasmid pAA352 (ATCC Accession No. 68283 and described in U.S. Patent 5,476,657). In doing so, mutations were introduced near the C-terminus, resulting in two amino acid changes to the native leukotoxin molecule. Thus, a PCR fragment of the affected area was cloned back into plasmid pSLKT-30. Specifically, a fragment from pSLKT-30 was created by

PCR using LKT6 (SEQ ID NO:40) as the upstream PCR primer, and LKT13 (SEQ ID NO:41) as the downstream PCR primer:

LKT6: TTA GAG AGT TAT GCC GAA CGC (SEQ ID NO:40);

5 LKT13: GAT GCC ATC GCT AGC TAG CTA GGA TCC CCT AGC
AAA TTC AAG AGA AGA TAA ACT TTG ATC CAA CAT
TGA (SEQ ID NO:41).

The fragment contained the desired change and the *Nsi*I and *Nco*I restriction sites. The isolated fragment was
10 digested using the restriction enzymes *Nsi*I and *Nco*I, as was the plasmid pSLKT-30. The *Nsi*I/*Nco*I fragment was removed from the plasmid and replaced with the PCR fragment, resulting in the mutation back to the original sequence. The plasmid was termed pCB150. A
15 diagram of plasmid pCB150 is shown in Figure 14. The nucleic acid sequence of LKT 114 from plasmid pCB150 is shown in Figures 15A-15D.

Example 3

20 Construction of LKT-Myostatin Peptide Multimer Fusions

Multiple copies of each oligomer pair described in Example 1 were used to prepare tandem repeats of coding sequences for myostatin peptide multimers joined to the LKT 114 gene. The entire
25 active portion of the protein was also reconstructed and fused to LKT 114 for use as an immunizing agent.

Representative LKT-myostatin peptide fusions were constructed as follows. Oligonucleotide pairs from Example 1 were annealed and ligated into the
30 vector pUC19 (Pharmacia) which had been digested with the restriction endonuclease *Hinc*II. The ligated DNA was used to transform *E. coli* strain TOP10F' (Invitrogen). Transformants containing the oligonucleotide inserts were identified by PCR and
35 restriction endonuclease mapping.

The oligonucleotide pairs were designed to be linked together by ligating the *Bam*HI site at the front end of one oligonucleotide pair to the *Bgl*II site at the back end of a second copy of the oligonucleotide pair. The restriction sites at the point of ligation were disabled leaving a single *Bam*HI site at the front end of the repeat and a single *Bgl*II site at the back end of the repeat. Tandem repeats of each oligonucleotide pair were constructed by digesting the oligonucleotide-containing plasmid with the restriction endonucleases *Bam*HI and *Bgl*II to release the inserted oligonucleotide fragment. This fragment was then ligated back into the oligonucleotide-containing plasmid, which had been digested with the restriction endonuclease *Bgl*II. The ligated DNA was used to transform *E. coli* strain TOP10F'. Transformants containing repeats of the oligonucleotide inserts were identified by PCR and restriction endonuclease mapping. This process was repeated until pUC19 plasmids containing at least four repeating copies and up to 8 copies of each oligonucleotide pair in the correct orientation were produced.

In addition to being linked to themselves, some of the oligonucleotide pairs were also designed to link to each other to recreate the active region of the myostatin protein as closely as possible. This was done by ligating *Bam*HI, *Bgl*II cut oligonucleotide pair MYOS 3/4 in to the *Bgl*II site behind oligonucleotide pair MYOS 1/2 in the pUC19 vector. This was followed by ligating in oligonucleotide pair MYOS 5/6 cut with *Bst*BI and *Bgl*II into the vector containing the reconstructed myostatin active region cut with *Bst*BI and *Bgl*II. Oligonucleotide pair MYOS 7/8 was cut with *Bam*HI and *Bgl*II and ligated into the vector containing the reconstructed myostatin active

region at the *Bgl*III site. Oligonucleotide pair MYOS 9/10 containing vector was cut with *Eco*RI and the *Eco*RI fragment from the pUC19 myostatin reconstruction was ligated in. Oligonucleotide pair MYOS 11/12 was cut with *Bam*HI and *Bgl*III and ligated into the vector containing the reconstructed myostatin active region at the *Bgl*III site. This was followed by ligating in oligonucleotide pair MYOS 13/14 cut with *Bam*I and *Bgl*III into the vector containing the reconstructed myostatin active region cut with *Bam*I and *Bgl*III. This completed the reconstruction of the coding sequence for the myostatin active region, which contained three sets of two amino acid linkers inserted into the myostatin active region sequence at positions 55-60, 139-144 and 241-246 and at the C-terminus (see Figure 13).

The multiple copies of each oligonucleotide pair and the myostatin active region reconstruction were then released from the pUC19 plasmid by digestion with the restriction endonucleases *Bam*HI and *Bgl*III. These DNA fragments were then ligated into the plasmid pCB150. Plasmid pCB150 was digested with the restriction endonuclease *Bam*HI. The ligated DNA was used to transform *E. coli* strain TOP10F'. Transformants containing the oligonucleotide inserts were identified by PCR and restriction endonuclease mapping. The recombinant plasmids were designated pJS121, pJS122, pJS123, pJS124, pJS125, pJS126, pJS127, pJS128, pJS129, pJS130, and pCB317.

The plasmid pJS121 contains 6 repeating copies of oligonucleotide pair MYOS 1/2 fused to LKT 114. The plasmid pJS122 contains 8 repeating copies of oligonucleotide pair MYOS 3/4 fused to LKT 114. The plasmid pJS123 contains 8 repeating copies of oligonucleotide pair MYOS 5/6 fused to LKT 114. The plasmid pJS124 contains 8 repeating copies of

oligonucleotide pair MYOS 7/8 fused to LKT 114. The
 plasmid pJS125 contains 6 repeating copies of
 oligonucleotide pair MYOS 9/10 fused to LKT 114. The
 plasmid pJS126 contains 4 repeating copies of
 5 oligonucleotide pair MYOS 11/12 fused to LKT 114. The
 plasmid pJS127 contains 6 repeating copies of
 oligonucleotide pair MYOS 13/14 fused to LKT 114. The
 plasmid pJS128 contains 4 repeating copies of
 oligonucleotide pair MYOS 15/16 fused to LKT 114. The
 10 plasmid pJS129 contains 8 repeating copies of
 oligonucleotide pair MYOS 17/18 fused to LKT 114. The
 plasmid pJS130 contains 4 repeating copies of
 oligonucleotide pair MYOS 19/20 fused to LKT 114. The
 plasmid pCB317 contains a single copy of the myostatin
 15 active region reconstruction fused to LKT 114.

Example 4

Purification of LKT-Myostatin Peptide Fusions

The recombinant LKT-myostatin peptide fusion
 20 proteins from above were expressed as inclusion bodies
 and purified using the following procedure. A loop of
 cells from each frozen stock was inoculated into 10 ml
 of TB broth in a 50 ml Erlenmeyer flask. The TB broth
 was supplemented with 100 µg/ml of ampicillin and
 25 incubated at 37°C for 12-16 hours on an Innova 4000™
 shaker at 250 rpm. The culture was used to inoculate
 one liter of TB broth in a 4L Erlenmeyer flask. The
 TB broth was supplemented with 100 µg/ml of ampicillin
 and incubated at 37°C for approximately 3 hours on an
 30 Innova 4000™ shaker at 250 rpm. 1 ml of a 1M IPTG
 (isoprpyl-B,D-thiogalactopyranoside) solution was then
 added to the culture to induce recombinant protein
 production. The culture was then incubated for a
 further two hours. The cells were harvested by
 35 centrifugation for 10 min at 6000 rpm in 3X 500 ml
 polypropylene bottles using a JA 10 rotor in an Avanti™

J25 centrifuge. The cell pellet was resuspended in 40 ml of 25% sucrose, 50mM Tris-hydrochloride, pH 8.0 and frozen at -70°C for 15 min. The frozen cells were thawed at room temperature and mixed with 10 ml of
5 Lysozyme (Sigma, 10 mg/ml in 250mM Tris-hydrochloride, pH 8.0). After incubation for 15 min on ice, 300 ml of lysis buffer (2% Triton X100™, 50mM EDTA, 100mM Tris-hydrochloride, pH 8.0) was added and mixed by shaking. The lysed cell suspension was then sonicated
10 for 4X 30 second bursts at full power with a large probe on a Misonix™ sonicator. The solution was split into 2X 250 ml centrifuge bottles and centrifuged for 25 min at 10000 rpm in a JA 14 rotor. The inclusion body pellets were washed by resuspending in 100 ml of
15 double-distilled water and centrifuging to collect the inclusion bodies. This washing procedure was repeated once more and the final inclusion body pellet was suspended in 10 ml of double-distilled water and stored at -20°C until needed.

20 All of the isolated fusion proteins were tested by SDS-PAGE to determine their identity by molecular weight, concentration and purity, by comparing the proteins to known standards. 10 µl aliquots of each fusion protein were solubilized with
25 10 µl of 8M Urea and 2 µl of the solubilized protein was then mixed with 100 µl of 1X SDS-PAGE loading buffer. The loading buffer samples were heated to 94°C for 5 min and run on a 10% polyacrylamide gel. Recombinant LKT 114 from pCB150 was also run as a
30 control.

Example 5

In Vivo Biologic Effect of LKT-Myostatin

Peptide Fusion Proteins

35 To test the ability of the fusion proteins comprising multiple copies of various peptides of

myostatin fused to a carrier protein to manifest a biologic effect *in vivo*, the following vaccination trial was preformed. Recombinant LKT-myostatin peptide fusion proteins were prepared as described above. Vaccines for each were prepared by solubilizing each of the fusion proteins in a final concentration of 6M Urea (used for the first injection) or 4M Guanidine-HCl (used for all subsequent injections). To 2.5 ml (used for the first two injections) or 1.5 ml (used for the last injection) aliquots of VSA-3 adjuvant (a modified Emulsigen Plus adjuvant) 1250 µg of each solubilized protein was added and mixed by 5X5 sec bursts with a Misonix sonicator with a microtip probe at a power setting of 5. To these mixtures, 50 µl of a 1% Thimerosal solution and PBS pH 7.4 (Phosphate Buffer Saline) to a final volume of 5 ml were added and the mixtures sonicated again. A volume of 200 µl was used for each injection. Each injection contained 50 µg of fusion protein. This initial injection (day 0) was given at 3-4 weeks of age with subsequent injections at days 28 and 56.

Fourteen treatment groups each contained 15 CD1 Swiss mice. The treatment groups were as follows (see Table 1): Group 1 no vaccination control, Group 2 adjuvant only control, Group 3 pCB150 carrier protein control, Groups 4 to 13 pJS121 to pJS130 test proteins, Group 14 pCB317 test protein. The mice were weighed weekly to determine weight gain over the course of the 98 day experiment. The results of this trial are summarized in Table 2 and Figure 18. Guanidine-HCl was used as the solubilizing agent of choice as it appeared to provide improved protein stability over Urea in the vaccine formulation. The concentration of VSA-3 was reduced from 50% to 30% in

the vaccine formulation in an effort to reduce injection site reactions.

Table 1		
Treatment Group	Myos Oligo	Plasmid
1	-	-
2	-	-
3	-	pCB150
4	1	pJS121
5	3	pSJ122
6	5	pSJ123
7	7	pJS124
8	9	pJS125
9	11	pJS126
10	13	pJS127
11	15	pJS128
12	17	pJS129
13	19	pJS130
14	reconstruction	pCB317

Table 2			
Treatment Group	Mean Group Weight Day 0 ± SEM	Mean Group Weight Day 98 ± SEM	Mean Group Weight Gain Through Day 84 ± SEM
1 Control	16.67 ± 0.32	29.33 ± 0.71	12.67 ± 0.65
2 Control	16.11 ± 0.25	29.09 ± 0.79	12.99 ± 0.72
3 Control	16.05 ± 0.34	29.33 ± 0.70	13.27 ± 0.61
4 Test	16.39 ± 0.37	30.02 ± 0.60	13.63 ± 0.60
5 Test	15.52 ± 0.35	30.48 ± 0.84	14.96 ± 0.72
6 Test	15.78 ± 0.33	30.84 ± 0.99	15.06 ± 0.90
7 Test	15.72 ± 0.27	30.36 ± 0.76	14.64 ± 0.65
8 Test	15.46 ± 0.25	29.42 ± 0.84	13.96 ± 0.79
9 Test	15.32 ± 0.32	29.48 ± 0.54	14.16 ± 0.50
10 Test	16.44 ± 0.31	31.27 ± 0.92	14.85 ± 0.92
11 Test	16.30 ± 0.41	31.02 ± 0.70	14.72 ± 0.75
12 Test	15.54 ± 0.28	30.73 ± 0.71	15.19 ± 0.69
13 Test	15.57 ± 0.30	31.04 ± 0.96	15.47 ± 0.99
14 Test	15.51 ± 0.20	29.25 ± 0.62	13.73 ± 0.56

Example 6

Statistical Analysis of Trial Results

Statistical analysis of the trial results was performed using a statistical software package (Statistix Version 1.0). In this trial, all control groups had very similar mean total weights, while several test groups had elevated mean total weights. A one-way ANOVA on the weight gain over the 98 days of the experiment was performed. An LSD comparison of means test indicated that treatment group 13 was significantly different from any of the control groups. Test groups 12 and 6 were significantly different from two of the three control groups. The treatment groups were also analyzed by grouping them

into controls (groups 1-3) and test group (groups 4-14). A one-way ANOVA on the weight gains from these two groups was performed. An LSD comparison of means test indicated that the group that received a test
5 treatment was significantly different from the control group.

Deposits of Strains Useful in Practicing the Invention

A deposit of biologically pure cultures of
10 the following strains was made with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. The deposits were made
15 under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for a period of
20 thirty (30) years from the date of deposit and at least five (5) years after the most recent request for the furnishing of a sample of the deposit by the depository. The organisms will be made available by the ATCC under the terms of the Budapest Treaty, which
25 assures permanent and unrestricted availability of the cultures to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 U.S.C. §122 and the Commissioner's rules pursuant thereto (including 37 C.F.R. §1.12).
30 Upon the granting of a patent, all restrictions on the availability to the public of the deposited cultures will be irrevocably removed.

These deposits are provided merely as convenience to those of skill in the art, and are not
35 an admission that a deposit is required under 35 U.S.C. §112. The nucleic acid sequences of these

plasmids, as well as the amino acid sequences of the polypeptides encoded thereby are controlling in the event of any conflict with the description herein. A license may
5 be required to make, use, or sell the deposited materials, and no such license is hereby granted.

	<u>Strain</u>	<u>Deposit Date</u>	<u>ATCC No.</u>
	pAA352 in <i>E. coli</i> W1485	March 30, 1990	68283
10	pCB113 in <i>E. coli</i> JM105	February 1, 1995	69749

Thus, immunogenic myostatin peptides, multimers and immunoconjugates are disclosed, as are methods of making and using the same. Although
15 preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

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DEMANDES OU BREVETS VOLUMINEUX

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JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

CLAIMS:

1. Use of a composition comprising a myostatin immunoconjugate for the preparation of a medicament for treating a mammal having a disorder that results in muscle wasting, wherein the immunoconjugate comprises a carrier fused to a myostatin multimer, wherein the myostatin multimer has the general formula (MP-X-MP)_y, wherein each MP is independently selected from the group consisting of:

- a) amino acids 3-15 of SEQ ID NO:6;
- b) amino acids 3-17 of SEQ ID NO:8;
- c) amino acids 3-16 of SEQ ID NO:10;
- d) amino acids 3-22 of SEQ ID NO:16;
- e) amino acids 3-18 of SEQ ID NO:18;
- f) amino acids 3-18 of SEQ ID NO:20; and
- g) amino acids 3-18 of SEQ ID NO:22,

wherein X is selected from the group consisting of a peptide linkage, an amino acid spacer group, and [MP]_n, where n is greater than or equal to 1, and y is greater than or equal to 1.

2. Use of a composition comprising a myostatin immunoconjugate for treating a mammal having a disorder that results in muscle wasting, wherein the immunoconjugate comprises a carrier fused to a myostatin multimer, wherein the myostatin multimer has the general formula (MP-X-MP)_y, wherein each MP is independently selected from the group consisting of:

- a) amino acids 3-15 of SEQ ID NO:6;
- b) amino acids 3-17 of SEQ ID NO:8;
- c) amino acids 3-16 of SEQ ID NO:10;
- d) amino acids 3-22 of SEQ ID NO:16;
- e) amino acids 3-18 of SEQ ID NO:18;
- f) amino acids 3-18 of SEQ ID NO:20; and
- g) amino acids 3-18 of SEQ ID NO:22,

wherein X is selected from the group consisting of a peptide linkage, an amino acid spacer group, and $[MP]_n$, where n is greater than or equal to 1, and y is greater than or equal to 1.

3. The use of claim 1 or 2, wherein the disorder is selected from the group consisting of muscle atrophy, anorexia, cachexia, facioscapulohumoral dystrophy, ocular myopathy, and myotonic dystrophy.
4. The use of claim 1 or 2, wherein the disorder is a muscular dystrophy.
5. The use of claim 4, wherein the muscular dystrophy is Becker's or Duchennes's.
6. The use of claim 4, wherein the muscular dystrophy is selected from the group consisting of Becker's, pseudohypertrophic, limb-girdle, Dejerine-Landouzy, Duchennes's, Landouzy, Emery-Dreifuss, Erb's, Fukuyama, Gowers', infantile neuroaxonal, Leyden-Mobius, oculopharyngeal, pelvifemoral, progressive, scapulohumeral, distal, and Simmerlin's.

7. The use of claims 1 or 2, wherein the carrier is selected from the group consisting of keyhole limpet hemocyanin, tetanus toxoid, ovalbumin, a leukotoxin (LKT) polypeptide, and sperm whale myoglobin.
8. The use of claim 7, wherein the carrier is a leukotoxin (LKT) polypeptide.
9. The use of any of claims 1 - 4, wherein the myostatin multimer comprises at least two copies of amino acids 3-15 of SEQ ID NO:6.
10. The use of claims 1 or 2, wherein the myostatin multimer comprises eight copies of amino acids 3-15 of SEQ ID NO:6.
11. The use of claims 1 or 2, wherein the myostatin multimer comprises at least two copies of amino acids 3-17 of SEQ ID NO:8.
12. The use of claims 1 or 2, wherein the myostatin multimer comprises eight copies of amino acids 3-17 of SEQ ID NO:8.
13. The use of claims 1 or 2, wherein the myostatin multimer comprises at least two copies of amino acids 3-16 of SEQ ID NO:10.
14. The use of claims 1 or 2, wherein the myostatin multimer comprises at least eight copies of amino acids 3-16 of SEQ ID NO:10.
15. The use of claims 1 or 2, wherein the myostatin multimer comprises at least two copies of amino acids 3-22 of SEQ ID NO:16.
16. The use of claims 1 or 2, wherein the myostatin multimer comprises at least six copies of amino acids 3-22 of SEQ ID NO:16.
17. The use of claims 1 or 2, wherein the myostatin multimer comprises at least two copies of amino acids 3-18 of SEQ ID NO:18.

18. The use of claims 1 or 2, wherein the myostatin multimer comprises at least four copies of amino acids 3-18 of SEQ ID NO:18.
19. The use of claims 1 or 2, wherein the myostatin multimer comprises at least two copies of amino acids 3-18 of SEQ ID NO:20.
20. The use of claims 1 or 2, wherein the myostatin multimer comprises at least eight copies of amino acids 3-18 of SEQ ID NO:20.
21. The use of claims 1 or 2, wherein the myostatin multimer comprises at least two copies of amino acids 3-18 of SEQ ID NO:22.
22. The use of claims 1 or 2, wherein the myostatin multimer comprises at least four copies of amino acids 3-18 of SEQ ID NO:22 fused to a LKT polypeptide.
23. The use of claims 1 or 2, wherein the amino acid spacer group includes at least one helper T-cell epitope.
24. The use of claims 1 or 2, wherein the immunoconjugate further comprises an adjuvant.
25. The use of claims 1 or 2, wherein the mammal is human.
26. A vaccine composition comprising a myostatin immunoconjugate comprising a myostatin multimer comprising the general formula (MP-X-MP)_y, wherein MP is a myostatin polypeptide selected from the group consisting of:
 - a) amino acids 3-15 of SEQ ID NO:6;
 - b) amino acids 3-17 of SEQ ID NO:8;
 - c) amino acids 3-16 of SEQ ID NO:10;
 - d) amino acids 3-22 of SEQ ID NO:16;

- e) amino acids 3-18 of SEQ ID NO:18;
- f) amino acids 3-18 of SEQ ID NO:20; and
- g) amino acids 3-18 of SEQ ID NO:22,

wherein X is selected from the group consisting of a peptide linkage, an amino acid spacer group, and $[MP]_n$, where n is greater than or equal to 1, and y is greater than or equal to 1; and a pharmaceutically acceptable excipient.

- 27. The composition of claim 26, further comprising a carrier.
- 28. The composition of claim 27, wherein the carrier is selected from the group consisting of keyhole limpet hemocyanin, tetanus toxoid, ovalbumin, a leukotoxin (LKT) polypeptide, and sperm whale myoglobin.
- 29. The composition of claim 27, wherein the carrier is a leukotoxin (LKT) polypeptide.
- 30. The composition of claim 26, wherein X comprises an amino acid spacer group including at least one helper T-cell epitope.
- 31. The composition of claim 26, wherein the multimer comprises at least two copies of amino acids 3-15 of SEQ ID NO:6.
- 32. The composition of claim 26, wherein the myostatin multimer comprises eight copies of amino acids 3-15 of SEQ ID NO:6.
- 33. The composition of claim 26, wherein the myostatin multimer comprises at least two copies of amino acids 3-17 of SEQ ID NO:8.
- 34. The composition of claim 26, wherein the myostatin multimer comprises eight copies of amino acids 3-17 of SEQ ID NO:8.

35. The composition of claim 26, wherein the myostatin multimer comprises at least two copies of amino acids 3-16 of SEQ ID NO:10.
36. The composition of claim 26, wherein the myostatin multimer comprises at least eight copies of amino acids 3-16 of SEQ ID NO:10.
37. The composition of claim 26, wherein the myostatin multimer comprises at least two copies of amino acids 3-22 of SEQ ID NO:16.
38. The composition of claim 26, wherein the myostatin multimer comprises at least six copies of amino acids 3-22 of SEQ ID NO:16.
39. The composition of claim 26, wherein the myostatin multimer comprises at least two copies of amino acids 3-18 of SEQ ID NO:18.
40. The composition of claim 26, wherein the myostatin multimer comprises at least four copies of amino acids 3-18 of SEQ ID NO:18.
41. The composition of claim 26, wherein the myostatin multimer comprises at least two copies of amino acids 3-18 of SEQ ID NO:20.
42. The composition of claim 26, wherein the myostatin multimer comprises at least eight copies of amino acids 3-18 of SEQ ID NO:20.
43. The composition of claim 26, wherein the myostatin multimer comprises at least two copies of amino acids 3-18 of SEQ ID NO:22.
44. The composition of claim 26, wherein the myostatin multimer comprises at least four copies of amino acids 3-18 of SEQ ID NO:22 fused to a LKT polypeptide.
45. The composition of any of claims 26 - 44, further comprising an adjuvant.

1 / 17

1 MMQKLQMYVY 10	11 IYLFMLIAAG 20	21 PV——DLNEGSER 30 Mouse
1 M I QKPQMYVY 10	11 IYLFVLI AAG 20	21 PV——DLNEDSER 30 Rat
1 -M QKLQ L CVY 9	10 IYLFMLI VAG 19	20 PV——DLNENSEQ 29 Human
1 -M QKLQ L CVY 9	10 IYLFMLI VAG 19	20 PV——DLNENSEQ 29 Baboon
1 -M QKLQ I SVY 9	10 IYLFMLI VAG 19	20 PV——DLNENSEQ 29 Bovine
1 -M QKLQ I YVY 9	10 IYLFMLI VAG 19	20 PV——DLNENSEQ 29 Porcine
1 -M QKLQ I FVY 9	10 IYLFMLLVAG 19	20 PV——DLNENSEQ 29 Ovine
1 -M QKLA VYVY 9	10 IYLFMQI AVD 19	20 PV——ALDGSSQP 29 Chicken
1 -M Q I LA VYVY 9	10 IYLFMQ I LVH 19	20 PV——ALDGSSQP 29 Turkey
1 -MH FTQ——VL 7	8 ISLS VL I ACG 17	18 PVGYGDITAHQQP 30 Zebrafish
31 EENVEKEGLC 40	41 NACA WRQNTR 50	51 YS RI E AIKIQ 60 Mouse
31 EANVEKEGLC 40	41 NACA WRQNTR 50	51 YS RI E AIKIQ 60 Rat
30 KENVEKEGLC 39	40 NACT WRQNTK 49	50 SS RI E AIKIQ 59 Human
30 KENVEKEGLC 39	40 NACT WRQNTK 49	50 SS RI E AIKIQ 59 Baboon
30 KENVEKEGLC 39	40 NACL WRE NTT 49	50 SS RLE AIKIQ 59 Bovine
30 KENVEKEGLC 39	40 NACMWRQNTK 49	50 SS RLE AIKIQ 59 Porcine
30 KENVEKKGLC 39	40 NACL WRQNNK 49	50 SS RLE AIKIQ 59 Ovine
30 TENAEKDGLC 39	40 NACT WRQNTK 49	50 SS RI E AIKIQ 59 Chicken
30 TENAEKDGLC 39	40 NACT WRQNTK 49	50 SS RI E AIKIQ 59 Turkey
31 STAT EES ELC 40	41 S TCE FRQHSK 50	51 LM RLHAISQ 60 Zebrafish
61 ILSKLRLETA 70	71 PNISKDA IRQ 80	81 LLPRAPPLRE 90 Mouse
61 ILSKLRLETA 70	71 PNISKDA IRQ 80	81 LLPRAPPLRE 90 Rat
60 ILSKLRLETA 69	70 PNISKDV IRQ 79	80 LLPKAPPLRE 89 Human
60 ILSKLRLETA 69	70 PNISKDA IRQ 79	80 LLPKAPPLRE 89 Baboon
60 ILSKLRLETA 69	70 PNISKDA IRQ 79	80 LLPRAPPLLE 89 Bovine
60 ILSKLRLETA 69	70 PNISKDA IRQ 79	80 LLPRAPPLRE 89 Porcine
60 ILSKLRLETA 69	70 PNISKDA IRQ 79	80 LLPRAPPLRE 89 Ovine
60 ILSKLRLEQA 69	70 PNISRDV IKQ 79	80 LLPRAPPLQE 89 Chicken
60 ILSKLRLEQA 69	70 PNISRDV IKQ 79	80 LLPRAPPLQE 89 Turkey
61 ILSKLRLEQA 70	71 PNISRDVVKQ 80	81 LLPRAPPLQQ 90 Zebrafish
91 LI DQYDVQRD 100	101 DS SDGSLEDD 110	111 DYH ATTET I I 120 Mouse
91 LI DQYDVQRD 100	101 DS SDGSLEDD 110	111 DYH ATTET I I 120 Rat
90 LI DQYDVQRD 99	100 DS SDGSLEDD 109	110 DYH ATTET I I 119 Human
90 LI DQYDVQRD 99	100 DS SDGSLEDD 109	110 DYH ATTET I I 119 Baboon
90 LI DQFDVQRD 99	100 AS SDGSLEDD 109	110 DYH ARTETV I I 119 Bovine
90 LI DQYDVQRD 99	100 DS SDGSLEDD 109	110 DYH ATTET I I 119 Porcine
90 LI DQYDVQRD 99	100 DS SDGSLEDD 109	110 DYH VTTETV I I 119 Ovine
90 LI DQYDVQRD 99	100 DS SDGSLEDD 109	110 DYH ATTET I I 119 Chicken
90 LI DQYDVQRD 99	100 DS SDGSLEDD 109	110 DYH ATTET I I 119 Turkey
91 LLDQYDVLGD 100	101 DSKDGAVEED 110	111 DEH ATTET IM 120 Zebrafish

FIG. 1A

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121 TMPTESDFLM 130	131 QADGKPKCCF 140	141 FKFSSKIQYN 150 Mouse
121 TMPTESDFLM 130	131 QADGKPKCCF 140	141 FKFSSKIQYN 150 Rat
120 TMPTESDFLM 129	130 QVDGKPKCCF 139	140 FKFSSKIQYN 149 Human
120 TMPTESDFLM 129	130 QVDGKPKCCF 139	140 FKFSSKIQYN 149 Baboon
120 TMPTESDLL T 129	130 QVEGKPKCCF 139	140 FKFSSKIQYN 149 Bovine
120 TMPTESDLLM 129	130 QVEGKPKCCF 139	140 FKFSSKIQYN 149 Porcine
120 TMPTESDLL A 129	130 EVQEKPKCCF 139	140 FKFSSKIQHN 149 Ovine
120 TMPTESDFL V 129	130 QMEGKPKCCF 139	140 FKFSSKIQYN 149 Chicken
120 TMPTESDFL V 129	130 QMEGKPKCCF 139	140 FKFSSKIQYN 149 Turkey
121 TMATEPDPI V 130	131 QVDRKPKCCF 140	141 FS FSPKIQAN 150 Zebrafish
151 KVVKAQLW IY 160	161 LRP VKTPTTV 170	171 FVQILRLIKP 180 Mouse
151 KVVKAQLW IY 160	161 LRAVKTPTTV 170	171 FVQILRLIKP 180 Rat
150 KVVKAQLW IY 159	160 LRP VETPTTV 169	170 FVQILRLIKP 179 Human
150 KVVKAQLW IY 159	160 LRP VETPTTV 169	170 FVQILRLIKP 179 Baboon
150 KLVKAQLW IY 159	160 LRP VKTPATV 169	170 FVQILRLIKP 179 Bovine
150 KVVKAQLW IY 159	160 LRP VKTPTTV 169	170 FVQILRLIKP 179 Porcine
150 KVVKAQLW IY 159	160 LRP VKTPTTV 169	170 FVQILRLIKP 179 Ovine
150 KVVKAQLW IY 159	160 LRQVQKPTTV 169	170 FVQILRLIKP 179 Chicken
150 KVVKAQLW IY 159	160 LRQVQKPTTV 169	170 FVQILRLIKP 179 Turkey
151 R I VRAQLWVH 160	161 LRP AEEATTV 169	170 FLQISRLM-P 179 Zebrafish
181 MKDGTRYTGI 190	191 RSLKLDMSPG 200	201 TGIWQSIDVK 210 Mouse
181 MKDGTRYTGI 190	191 RSLKLDMSPG 200	201 TGIWQSIDVK 210 Rat
180 MKDGTRYTGI 189	190 RSLKLDMNPG 199	200 TGIWQSIDVK 209 Human
180 MKDGTRYTGI 189	190 RSLKLDMNPG 199	200 TGIWQSIDVK 209 Baboon
180 MKDGTRYTGI 189	190 RSLKLDMNPG 199	200 TGIWQSIDVK 209 Bovine
180 MKDGTRYTGI 189	190 RSLKLDMNPG 199	200 TGIWQSIDVK 209 Porcine
180 MKDGTRYTGI 189	190 RSLKLDMNPG 199	200 TGIWQSIDVK 209 Ovine
180 MKDGTRYTGI 189	190 RSLKLDMNPG 199	200 TGIWQSIDVK 209 Chicken
180 MKDGTRYTGI 189	190 RSLKLDMNPG 199	200 TGIWQSIDVK 209 Turkey
180 V KDGRHR-I 188	189 RSLK IDV NAG 198	199 VTSWQSIDVK 208 Zebrafish
211 TVLQNLWLKQP 220	221 ESNLGEIKA 230	231 LD ENGHDLAV 240 Mouse
211 TVLQNLWLKQP 220	221 ESNLGEIKA 230	231 LD ENGHDLAV 240 Rat
210 TVLQNLWLKQP 219	220 ESNLGEIKA 229	230 LD ENGHDLAV 239 Human
210 TVLQNLWLKQP 219	220 ESNLGEIKA 229	230 LD ENGHDLAV 239 Baboon
210 TVLQNLWLKQP 219	220 ESNLGEIKA 229	230 LD ENGHDLAV 239 Bovine
210 TVLQNLWLKQP 219	220 ESNLGEIKA 229	230 LD ENGHDLAV 239 Porcine
210 TVLQNLWLKQP 219	220 ESNLGEIKA 229	230 LD ENGHDLAV 239 Ovine
210 TVLQNLWLKQP 219	220 ESNLGEIKA 229	230 FD ETGRDLAV 239 Chicken
210 TVLQNLWLKQP 219	220 ESNLGEIKA 229	230 FD ENGRDLAV 239 Turkey
209 QVLTWVWLKQP 218	219 ETNRGEINA 228	229 YDAKGNDLAV 238 Zebrafish

FIG. 1B

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241 TFPGPGEDGL 250	251 NPFLEVKVTD 260	261 TPKRSRRDFG 270 Mouse
241 TFPGPGEDGL 250	251 NPFLEVKVTD 260	261 TPKRSRRDFG 270 Rat
240 TFPGPGEDGL 249	250 NPFLEVKVTD 259	260 TPKRSRRDFG 269 Human
240 TFPGPGEDGL 249	250 NPFLEVKVTD 259	260 TPKRSRRDFG 269 Baboon
240 TFPEPGEDGL 249	250 TPFLEVKVTD 259	260 TPKRSRRDFG 269 Bovine
240 TFPGPGEDGL 249	250 NPFLEVKVTD 259	260 TPKRSRRDFG 269 Porcine
240 TFPEPGEEGL 249	250 NPFLEVKVTD 259	260 TPKRSRRDFG 269 Ovine
240 TFPGPGEDGL 249	250 NPFLEVRVTD 259	260 TPKRSRRDFG 269 Chicken
240 TFPGPGEDGL 249	250 NPFLEVRVTD 259	260 TPKRSRRDFG 269 Turkey
239 TSTETGEDGL 248	249 LPFMEVKI SE 258	259 GPKRIRRDG 268 Zebrafish
271 LDCDEHSTES 280	281 RCCRYPLTVD 290	291 FEAFGWDWII 300 Mouse
271 LDCDEHSTES 280	281 RCCRYPLTVD 290	291 FEAFGWDWII 300 Rat
270 LDCDEHSTES 279	280 RCCRYPLTVD 289	290 FEAFGWDWII 299 Human
270 LDCDEHSTES 279	280 RCCRYPLTVD 289	290 FEAFGWDWII 299 Baboon
270 LDCDEHSTES 279	280 RCCRYPLTVD 289	290 FEAFGWDWII 299 Bovine
270 LDCDEHSTES 279	280 RCCRYPLTVD 289	290 FEAFGWDWII 299 Porcine
270 LDCDEHSTES 279	280 RCCRYPLTVD 289	290 FEAFGWDWII 299 Ovine
270 LDCDEHSTES 279	280 RCCRYPLTVD 289	290 FEAFGWDWII 299 Chicken
270 LDCDEHSTES 279	280 RCCRYPLTVD 289	290 FEAFGWDWII 299 Turkey
269 LDCDENSSSES 278	279 RCCRYPLTVD 288	289 FEDFGWDWII 298 Zebrafish
301 APKRYKANYC 310	311 SGECE FV FLQ 320	321 KYPHTHLVHQ 330 Mouse
301 APKRYKANYC 310	311 SGECE FV FLQ 320	321 KYPHTHLVHQ 330 Rat
300 APKRYKANYC 309	310 SGECE FV FLQ 319	320 KYPHTHLVHQ 329 Human
300 APKRYKANYC 309	310 SGECE FV FLQ 319	320 KYPHTHLVHQ 329 Baboon
300 APKRYKANYC 309	310 SGECE FV FLQ 319	320 KYPHTHLVHQ 329 Bovine
300 APKRYKANYC 309	310 SGECE FV FLQ 319	320 KYPHTHLVHQ 329 Porcine
300 APKRYKANYC 309	310 SGECE FL FLQ 319	320 KYPHTHLVHQ 329 Ovine
300 APKRYKANYC 309	310 SGECE FV FLQ 319	320 KYPHTHLVHQ 329 Chicken
300 APKRYKANYC 309	310 SGECE FV FLQ 319	320 KYPHTHLVHQ 329 Turkey
299 APKRYKANYC 308	309 SGECDYMYLQ 318	319 KYPHTHLVNK 328 Zebrafish
331 ANPRGSAGPC 340	341 CTPTKMSPIN 350	351 MLYFNGKEQI 360 Mouse
331 ANPRGSAGPC 340	341 CTPTKMSPIN 350	351 MLYFNGKEQI 360 Rat
330 ANPRGSAGPC 339	340 CTPTKMSPIN 349	350 MLYFNGKEQI 359 Human
330 ANPRGSAGPC 339	340 CTPTKMSPIN 349	350 MLYFNGKEQI 359 Baboon
330 ANPRGSAGPC 339	340 CTPTKMSPIN 349	350 MLYFNGKEQI 359 Bovine
330 ANPRGSAGPC 339	340 CTPTKMSPIN 349	350 MLYFNGKEQI 359 Porcine
330 ANPKGSAGPC 339	340 CTPTKMSPIN 349	350 MLYFNGKEQI 359 Ovine
330 ANPRGSAGPC 339	340 CTPTKMSPIN 349	350 MLYFNGKEQI 359 Chicken
330 ANPRGSAGPC 339	340 CTPTKMSPIN 349	350 MLYFNGKEQI 359 Turkey
329 ASPRGTAGPC 338	339 CTPTKMSPIN 248	349 MLYFNGKEQI 359 Zebrafish

FIG. 1C

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361 IYGKIPAMVV 370	371 DRCGCS 376 Mouse
361 IYGKIPAMVV 370	371 DRCGCS 376 Rat
360 IYGKIPAMVV 369	370 DRCGCS 375 Human
360 IYGKIPAMVV 369	370 DRCGCS 375 Baboon
360 IYGKIPAMVV 369	370 DRCGCS 375 Bovine
360 IYGKIPAMVV 369	370 DRCGCS 375 Porcine
360 IYGKIPAMVV 369	370 DRCGCS 375 Ovine
360 IYGKIPAMVV 369	370 DRCGCS 375 Chicken
360 IYGKIPAMVV 369	370 DRCGCS 375 Turkey
359 IYGKIP SMVV 368	369 DRCGCS 374 Zebrafish

FIG. 1D

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			10			20			30			40		
			↓			↓			↓			↓		
GGA	TCC	CGT	TCT	CGT	CGC	GAC	TTT	GGT	CTG	GAC	TGC	GAC	GAA	CAT
Gly	Ser	Arg	Ser	Arg	Arg	Asp	Phe	Gly	Leu	Asp	Cys	Asp	Glu	His
		50						60						
		↓						↓						
TCT	ACC	GAA	AGA	TCT										
Ser	Thr	Glu	Arg	Ser										

FIG. 2

			10			20			30			40		
			↓			↓			↓			↓		
GGA	TCC	TCT	CGT	TGC	TGT	CGC	TAT	CCG	CTG	ACC	GTT	GAC	TTC	GAA
Gly	Ser	Ser	Arg	Cys	Cys	Arg	Tyr	Pro	Leu	Thr	Val	Asp	Phe	Glu
		50												
		↓												
AGA	TCT													
Arg	Ser													

FIG. 3

			10			20			30			40		
			↓			↓			↓			↓		
GGA	TCC	TTC	GAA	GCT	TTT	GGT	TGG	GAC	TGG	ATC	ATT	GCA	CCG	AAA
Gly	Ser	Phe	Glu	Ala	Phe	Gly	Trp	Asp	Trp	Ile	Ile	Ala	Pro	Lys
		50												
		↓												
CGT	TAT	AGA	TCT											
Arg	Tyr	Arg	Ser											

FIG. 4

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10 20 30 40
 GGA TCC AAA CGT TAT AAA GCT AAC TAT TGC TCT GGT GAA TGC GAA
 Gly Ser Lys Arg Tyr Lys Ala Asn Tyr Cys Ser Gly Glu Cys Glu

50
 TTC AGA TCT
 Phe Arg Ser

FIG. 5

10 20 30 40
 GGA TCC GAA TTC GTT TTC CTG CAG AAA TAT CCG CAT ACC CAT CTG
 Gly Ser Glu Phe Val Phe Leu Gln Lys Tyr Pro His Thr His Leu

50 60 70
 GTT CAT CAG GCT AAC CCG CGT AGA TCT
 Val His Gln Ala Asn Pro Arg Arg Ser

FIG. 6

10 20 30 40
 GGA TCC GCT GGT CCG TGC TGT TAT CCG ACC AAA ATG TCT CCG ATC
 Gly Ser Ala Gly Pro Cys Cys Tyr Pro Thr Lys MET Ser Pro Ile

50 60 70 80
 AAC ATG CTG TAT TTC AAC GGT GAA TGC CAG AGA TCT
 Asn MET Leu Tyr Phe Asn Gly Glu Cys Gln Arg Ser

FIG. 7

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			10			20			30			40			
GGA	TCC	GAA	TGC	CAG	ATC	ATT	TAT	TGC	AAA	ATC	CCG	GCT	ATG	GTT	
Gly	Ser	Glu	Cys	Gln	Ile	Ile	Tyr	Cys	Lys	Ile	Pro	Ala	~~MET~~	Val	

	50			60			70								
GTA	GAC	CGT	TGC	GGT	TGT	TCT	AGA	TCT							
Val	Asp	Arg	Cys	Gly	Cys	Ser	Arg	Ser							

FIG. 8

			10			20			30			40			
GGA	TCC	GAA	CAG	AAA	GAA	AAC	GTT	GAA	AAA	GAA	GGT	CTG	TGC	AAC	
Gly	Ser	Glu	Gln	Lys	Glu	Asn	Val	Glu	Lys	Glu	Gly	Leu	Cys	Asn	

	50			60											
GCT	TGC	CTG	TGG	AGA	TCT										
Ala	Cys	Leu	Trp	Arg	Ser										

FIG. 9

			10			20			30			40			
GGA	TCC	CAT	GAC	CTG	GCT	GTT	ACC	TTC	CCG	GAA	CCG	GGT	GAA	GAC	
Gly	Ser	His	Asp	Leu	Ala	Val	Thr	Phe	Pro	Glu	Pro	Gly	Glu	Asp	

	50			60											
GGT	CTG	ACC	AGA	TCT											
Gly	Leu	Thr	Arg	Ser											

FIG. 10

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			10				20				30				40	
			↓				↓				↓				↓	
GGA	TCC	ACC	CCG	TTC	CTG	GAA	GTT	AAA	GTT	ACC	GAC	ACT	CCG	AAA		
Gly	Ser	Thr	Pro	Phe	Leu	Glu	Val	Lys	Val	Thr	Asp	Thr	Pro	Lys		
			50												60	
			↓												↓	
CGT	TCT	CGT	AGA	TCT												
Arg	Ser	Arg	Arg	Ser												

FIG. 11

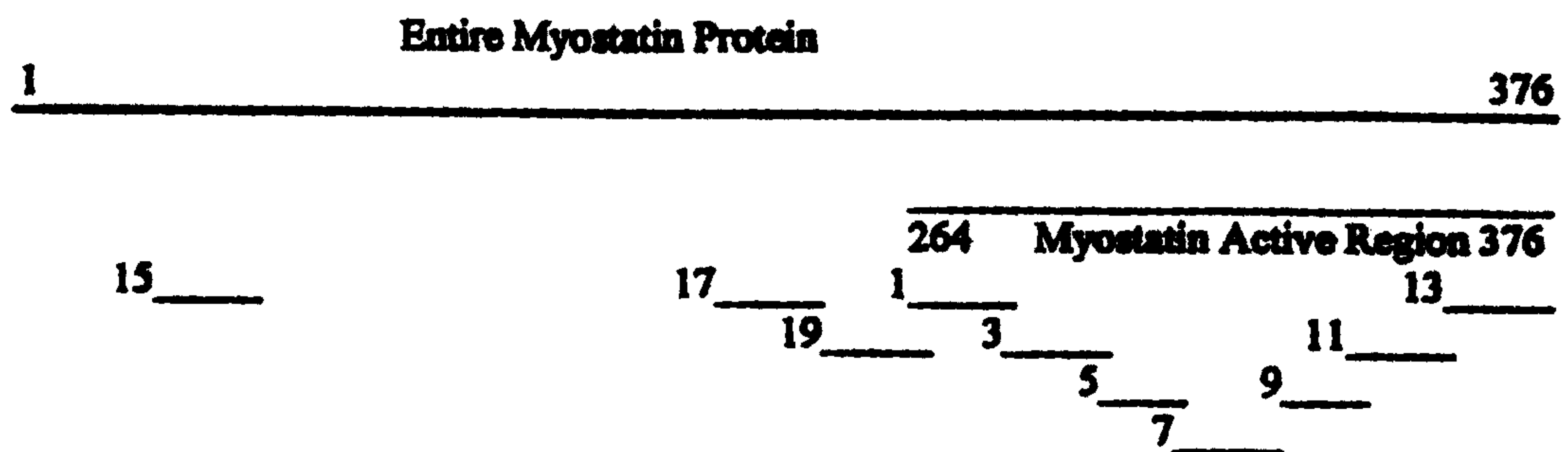


FIG. 12

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			10				20			30			40		
GGA	TCC	CGT	TCT	CGT	CGC	GAC	TTT	GGT	CTG	GAC	TGC	GAC	GAA	CAT	
Gly	Ser	Arg	Ser	Arg	Arg	Asp	Phe	Gly	Leu	Asp	Cys	Asp	Glu	His	
		50			60			70			80			90	
TCT	ACC	GAA	AGA	TCC	TCT	CGT	TGC	TGT	CGC	TAT	CCG	CTG	ACC	GTT	
Ser	Thr	Glu	Arg	Ser	Ser	Arg	Cys	Cys	Arg	Tyr	Pro	Leu	Thr	Val	
			100			110			120			130			
GAC	TTC	GAA	GCT	TTT	GGT	TGG	GAC	TGG	ATC	ATT	GCA	CCG	AAA	CGT	
Asp	Phe	Glu	Ala	Phe	Gly	Trp	Asp	Trp	Ile	Ile	Ala	Pro	Lys	Arg	
	140			150			160			170			180		
TAT	AGA	TCC	AAA	CGT	TAT	AAA	GCT	AAC	TAT	TGC	TCT	GGT	GAA	TGC	
Tyr	Arg	Ser	Lys	Arg	Tyr	Lys	Ala	Asn	Tyr	Cys	Ser	Gly	Glu	Cys	
		190			200			210			220				
GAA	TTC	GTT	TTC	CTG	CAG	AAA	TAT	CCG	CAT	ACC	CAT	CTG	GTT	CAT	
Glu	Phe	Val	Phe	Leu	Gln	Lys	Tyr	Pro	His	Thr	His	Leu	Val	His	
	230			240			250			260			270		
CAG	GCT	AAC	CCG	CGT	AGA	TCC	GCT	GGT	CCG	TGC	TGT	TAT	CCG	ACC	
Gln	Ala	Asn	Pro	Arg	Arg	Ser	Ala	Gly	Pro	Cys	Cys	Tyr	Pro	Thr	
		280			290			300			310				
AAA	ATG	TCT	CCG	ATC	AAC	ATG	CTG	TAT	TTC	AAC	GGT	GAA	TGC	CAG	
Lys	~~MET~~	Ser	Pro	Ile	Asn	~~MET~~	Leu	Tyr	Phe	Asn	Gly	Glu	Cys	Gln	
	320			330			340			350			360		
ATC	ATT	TAT	TGC	AAA	ATC	CCG	GCT	ATG	GTT	GTA	GAC	CGT	TGC	GGT	
Ile	Ile	Tyr	Cys	Lys	Ile	Pro	Ala	~~MET~~	Val	Val	Asp	Arg	Cys	Gly	
		370													
TGT	TCT	AGA	TCT												
Cys	Ser	Arg	Ser												

FIG. 13

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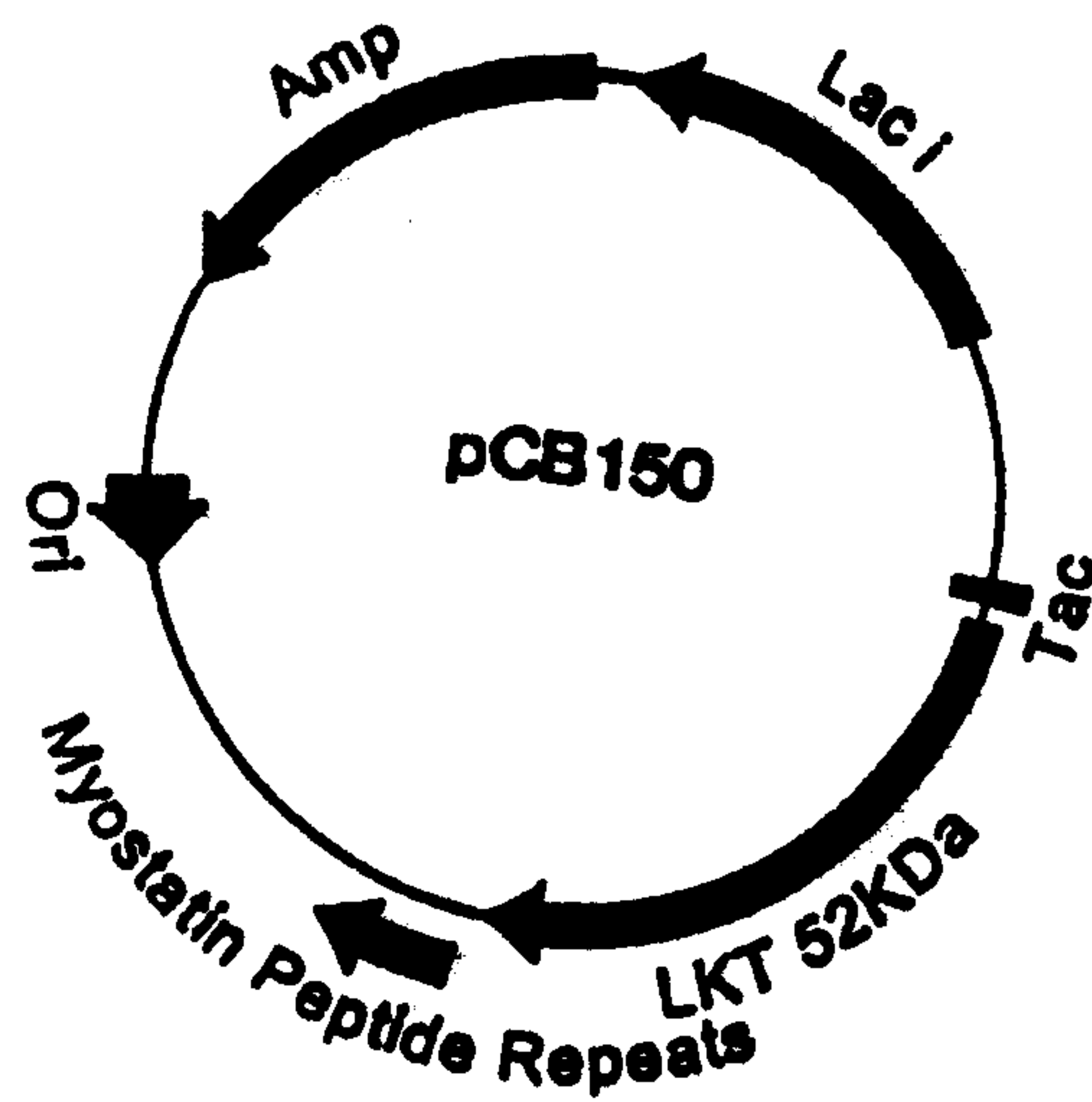


FIG. 14

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1870	1880	1890	1900	1910
ATG	GCT	ACT	GTT	ATA
GAT	CTA	AGC	TTC	CCA
AAA	ACT	GGG	GCA	AAA
<u>MET</u>	Ala	Thr	Val	Ile
Asp	Leu	Ser	Phe	Pro
Lys	Thr	Gly	Ala	Lys

1920	1930	1940	1950
AAA	ATT	ATC	CTC
TAT	ATT	CCC	CAA
AAT	TAC	CAA	TAT
GAT	ACT	GAA	
Lys	Ile	Ile	Leu
Tyr	Ile	Pro	Gln
Asn	Tyr	Gln	Tyr
Asp	Thr	Glu	

1960	1970	1980	1990	2000
CAA	GGT	AAT	GGT	TTA
CAG	GAT	TTA	GTC	AAA
GCG	GCC	GAA	GAG	TTG
Gln	Gly	Asn	Gly	Leu
Gln	Asp	Leu	Val	Lys
Ala	Ala	Glu	Glu	Leu

2010	2020	2030	2040
GGG	ATT	GAG	GTA
CAA	AGA	GAA	GAA
CGC	AAT	AAT	ATT
GCA	ACA	GCT	
Gly	Ile	Glu	Val
Gln	Arg	Glu	Glu
Arg	Asn	Asn	Ile
Ala	Thr	Ala	

2050	2060	2070	2080	2090
CAA	ACC	AGT	TTA	GGC
ACG	ATT	CAA	ACC	GCT
ATT	GGC	TTA	ACT	GAG
Gln	Thr	Ser	Leu	Gly
Thr	Ile	Gln	Thr	Ala
Ile	Gly	Leu	Thr	Glu

2100	2110	2120	2130
CGT	GGC	ATT	GTG
TTA	TCC	GCT	CCA
CAA	ATT	GAT	AAA
TTG	CTA	CAG	
Arg	Gly	Ile	Val
Leu	Ser	Ala	Pro
Gln	Ile	Asp	Lys
Leu	Leu	Gln	

2140	2150	2160	2170	2180
AAA	ACT	AAA	GCA	GGC
CAA	GCA	TTA	GGT	TCT
GCC	GAA	AGC	ATT	GTA
Lys	Thr	Lys	Ala	Gly
Gln	Ala	Leu	Gly	Ser
Ala	Glu	Ser	Ile	Val

2190	2200	2210	2220
CAA	AAT	GCA	AAT
AAA	GCC	AAA	ACT
GTA	TTA	TCT	GGC
ATT	CAA	TCT	
Gln	Asn	Ala	Asn
Lys	Ala	Lys	Thr
Val	Leu	Ser	Gly
Ile	Gln	Ser	

2230	2240	2250	2260	2270
ATT	TTA	GGC	TCA	GTA
TTG	GCT	GGA	ATG	GAT
TTA	GAT	GAG	GCC	TTA
Ile	Leu	Gly	Ser	Val
Leu	Ala	Gly	<u>MET</u>	Asp
Leu	Asp	Glu	Ala	Leu

FIG. 15A

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2280	2290	2300	2310
CAG AAT AAC AGC AAC CAA CAT GCT CTT GCT AAA GCT GGC TTG GAG			
Gln Asn Asn Ser Asn Gln His Ala Leu Ala Lys Ala Gly Leu Glu			

2320	2330	2340	2350	2360
CTA ACA AAT TCA TTA ATT GAA AAT ATT GCT AAT TCA GTA AAA ACA				
Leu Thr Asn Ser Leu Ile Glu Asn Ile Ala Asn Ser Val Lys Thr				

2370	2380	2390	2400
CTT GAC GAA TTT GGT GAG CAA ATT AGT CAA TTT GGT TCA AAA CTA			
Leu Asp Glu Phe Gly Glu Gln Ile Ser Gln Phe Gly Ser Lys Leu			

2410	2420	2430	2440	2450
CAA AAT ATC AAA GGC TTA GGG ACT TTA GGA GAC AAA CTC AAA AAT				
Gln Asn Ile Lys Gly Leu Gly Thr Leu Gly Asp Lys Leu Lys Asn				

2460	2470	2480	2490
ATC GGT GGA CTT GAT AAA GCT GGC CTT GGT TTA GAT GTT ATC TCA			
Ile Gly Gly Leu Asp Lys Ala Gly Leu Gly Leu Asp Val Ile Ser			

2500	2510	2520	2530	2540
GGG CTA TTA TCG GGC GCA ACC GCT GCA CTT GTA CTT GCA GAT AAA				
Gly Leu Leu Ser Gly Ala Thr Ala Ala Leu Val Leu Ala Asp Lys				

2550	2560	2570	2580
AAT GCT TCA ACA GCT AAA AAA GTG GGT GCG GGT TTT GAA TTG GCA			
Asn Ala Ser Thr Ala Lys Lys Val Gly Ala Gly Phe Glu Leu Ala			

2590	2600	2610	2620	2630
AAC CAA GTT GTT GGT AAT ATT ACC AAA GCC GTT TCT TCT TAC ATT				
Asn Gln Val Val Gly Asn Ile Thr Lys Ala Val Ser Ser Tyr Ile				

2640	2650	2660	2670
TTA GCC CAA CGT GTT GCA GCA GGT TTA TCT TCA ACT GGG CCT GTG			
Leu Ala Gln Arg Val Ala Ala Gly Leu Ser Ser Thr Gly Pro Val			

FIG. 15B

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2680	2690	2700	2710	2720
GCT	GCT	TTA	ATT	GCT
Ala	Ala	Leu	Ile	Ala
Ser	Thr	Val	Ser	Leu
Ala	Ile	Ser	Pro	Leu
2730	2740	2750	2760	
GCA	TTT	GCC	GGT	ATT
Ala	Phe	Ala	Gly	Ile
Ala	Asp	Lys	Phe	Asn
His	Ala	Lys	Ser	Leu
2770	2780	2790	2800	2810
GAG	AGT	TAT	GCC	GAA
Glu	Ser	Tyr	Ala	Glu
Arg	Phe	Lys	Lys	Leu
Gly	Tyr	Asp	Gly	Asp
2820	2830	2840	2850	
AAT	TTA	TTA	GCA	GAA
Asn	Leu	Leu	Ala	Glu
Tyr	Gln	Arg	Gly	Thr
Gly	Thr	Ile	Asp	Ala
2860	2870	2880	2890	2900
TCG	GTT	ACT	GCA	ATT
Ser	Val	Thr	Ala	Ile
Asn	Thr	Ala	Leu	Ala
Ala	Ile	Ala	Gly	Gly
2910	2920	2930	2940	
GTG	TCT	GCT	GCT	GCA
Val	Ser	Ala	Ala	Ala
Asp	Leu	Thr	Phe	Glu
Lys	Val	Lys	His	
2950	2960	2970	2980	2990
AAT	CTT	GTC	ATC	ACG
Asn	Leu	Val	Ile	Thr
Asn	Ser	Lys	Lys	Glu
Lys	Val	Thr	Ile	Gln
3000	3010	3020	3030	
AAC	TGG	TTC	CGA	GAG
Asn	Trp	Phe	Arg	Glu
Ala	Asp	Phe	Ala	Lys
Glu	Val	Pro	Asn	Tyr
3040	3050	3060	3070	3080
AAA	GCA	ACT	AAA	GAT
Lys	Ala	Thr	Lys	Asp
Glu	Lys	Ile	Glu	Glu
Ile	Ile	Gly	Gln	Asn

FIG. 15C

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3090	3100	3110	3120	
GGC GAG CGG ATC ACC TCA AAG CAA GTT GAT GAT CTT ATC GCA AAA				
Gly Glu Arg Ile Thr Ser Lys Gln Val Asp Asp Leu Ile Ala Lys				
3130	3140	3150	3160	3170
GGT AAC GGC AAA ATT ACC CAA GAT GAG CTA TCA AAA GTT GTT GAT				
Gly Asn Gly Lys Ile Thr Gln Asp Glu Leu Ser Lys Val Val Asp				
3180	3190	3200	3210	
AAC TAT GAA TTG CTC AAA CAT AGC AAA AAT GTG ACA AAC AGC TTA				
Asn Tyr Glu Leu Leu Lys His Ser Lys Asn Val Thr Asn Ser Leu				
3220	3230	3240	3250	3260
GAT AAG TTA ATC TCA TCT GTA AGT GCA TTT ACC TCG TCT AAT GAT				
Asp Lys Leu Ile Ser Ser Val Ser Ala Phe Thr Ser Ser Asn Asp				
3270	3280	3290	3300	
TCG AGA AAT GTA TTA GTG GCT CCA ACT TCA ATG TTG GAT CAA AGT				
Ser Arg Asn Val Leu Val Ala Pro Thr Ser ~~MEI~~ Leu Asp Gln Ser				
3310	3320	3330	3340	
TTA TCT TCT CTT CAA TTT GCT AGG ~~GGA TCC~~ TAG				
Leu Ser Ser Leu Gln Phe Ala Arg Gly Ser ---				

FIG. 15D

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1  ATGCAAAAAC TGCAAATCTC TGTATTATATT TACCTATTTA CGCTGATTGT
51  TGCTGGCCCA GTGGATCTGA ATGAGAACAG CGAGCAGAAG GAAAATGTGG
101 AAAAAGAGGG GCTGTGTAAT GCATGTTTGT GGAGGGAAAA CACTACATCC
151 TCAAGACTAG AAGCCATAAA AATCCAAATC CTCAGTAAAC TTCGCCTGGA
201 AACAGCTCCT AACATCAGCA AAGATGCTAT CAGACAACTT TTGCCCAAGG
251 CTCCTCCACT CCTGGAACGT ATTGATCAGT TCGATGTCCA GAGAGATGCC
301 AGCAGTGACG GCTCCTTGGA AGACGATGAC TACCACGCCA GGACGGAAAC
351 GGTCATTACC ATGCCACGG AGTCTGATCT TCTAACGCAA GTGGAAGGAA
401 AACCCAAATG TTGCTTCTTT AAATTTAGCT CTAAGATACA ATACAATAAA
451 CTAGTAAAGG CCCAACTGTG GATATATCTG AGGCCTGTCA AGACTCCTGC
501 GACAGTGTTT GTGCAAATCC TGAGACTCAT CAAACCCATG AAAGACGGTA
551 CAAGGTATAC TGAATCCGA TCTCTGAAAC TTGACATGAA CCCAGGCACT
601 GGTATTTGGC AGAGCATTGA TGTGAAGACA GTGTTGCAGA ACTGGCTCAA
651 ACAACCTGAA TCCAACCTAG GCATTGAAAT CAAAGCTTTA GATGAGAATG
701 GCCATGATCT TGCTGTAACC TTCCCAGAAC CAGGAGAAGA TGGACTGACT
751 CCTTTTTTAG AAGTCAAGGT AACAGACACA CCAAAAAGAT CTAGGAGAGA
801 TTTTGGGCTT GATTGTGATG AACACTCCAC AGAATCTCGA TGCTGTCGCT
851 ACCCCCTCAC GGTGGATTTT GAAGCTTTTG GATGGGATTG GATTATTGCA
901 CCTAAAAGAT ATAAGGCCAA TTACTGCTCT GGAGAATGTG AATTGTATT
951 TTTGCAAAAG TATCCTCATA CCCATCTTGT GCACCAAGCA AACCCAGAG
1001 GTTCAGCCGG CCCCTGCTGT ACTCCTACAA AGATGTCTCC AATTAATATG
1051 CTATATTTTA ATGGCGAAGG ACAAATAATA TACGGAAGA TTCCAGCCAT
1101 GGTAGTAGAT CGCTGTGGGT GCTCATGA

```

FIG. 16A

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1  MQKLQISVYI YLFTLIVAGP VDLNENSEQK ENVEKEGLCN ACLNRENTTS
51  SRLRAIKIQI LSKLRLETAP NISKDAIRQI LPKAPLLEL IDQFDVORDA
101 SSDGSLEDDD YHARTETVIT MPTESDLLTQ VEGKPKCCFP KFSSKIQYNK
151 LVKAQLNIYL RPVKTPATVF VOILRLIKPM KDGTRYTGIR SLKLDMPGT
201 GIWQSIDVKE VLQNLKQPE SNLGIBIKAL DENGHD LAVT FPEPGEDGLT
251 PFLEVKVTDI PKRSRRDPGL DCDEHSTESR CCRYPLTVDF EAFGWDWIIA
301 PKRYKANYCS GECEVFLQK YPHTHLVHQA NPRGSAGPCC TPTKMSPINM
351 LYFNGBGQII YGKIPAMVVD RCGCS

```

FIG. 16B

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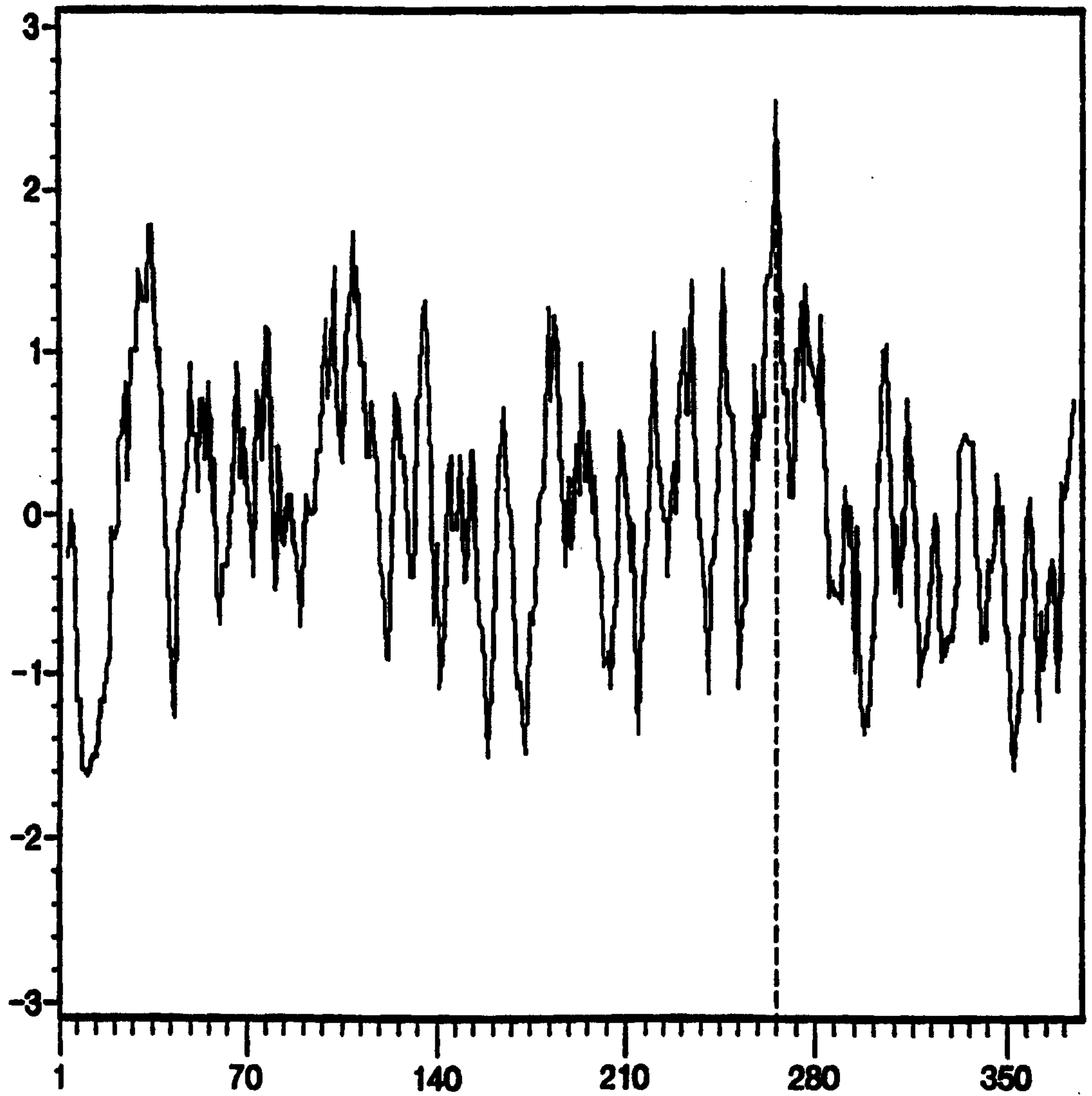


FIG. 17

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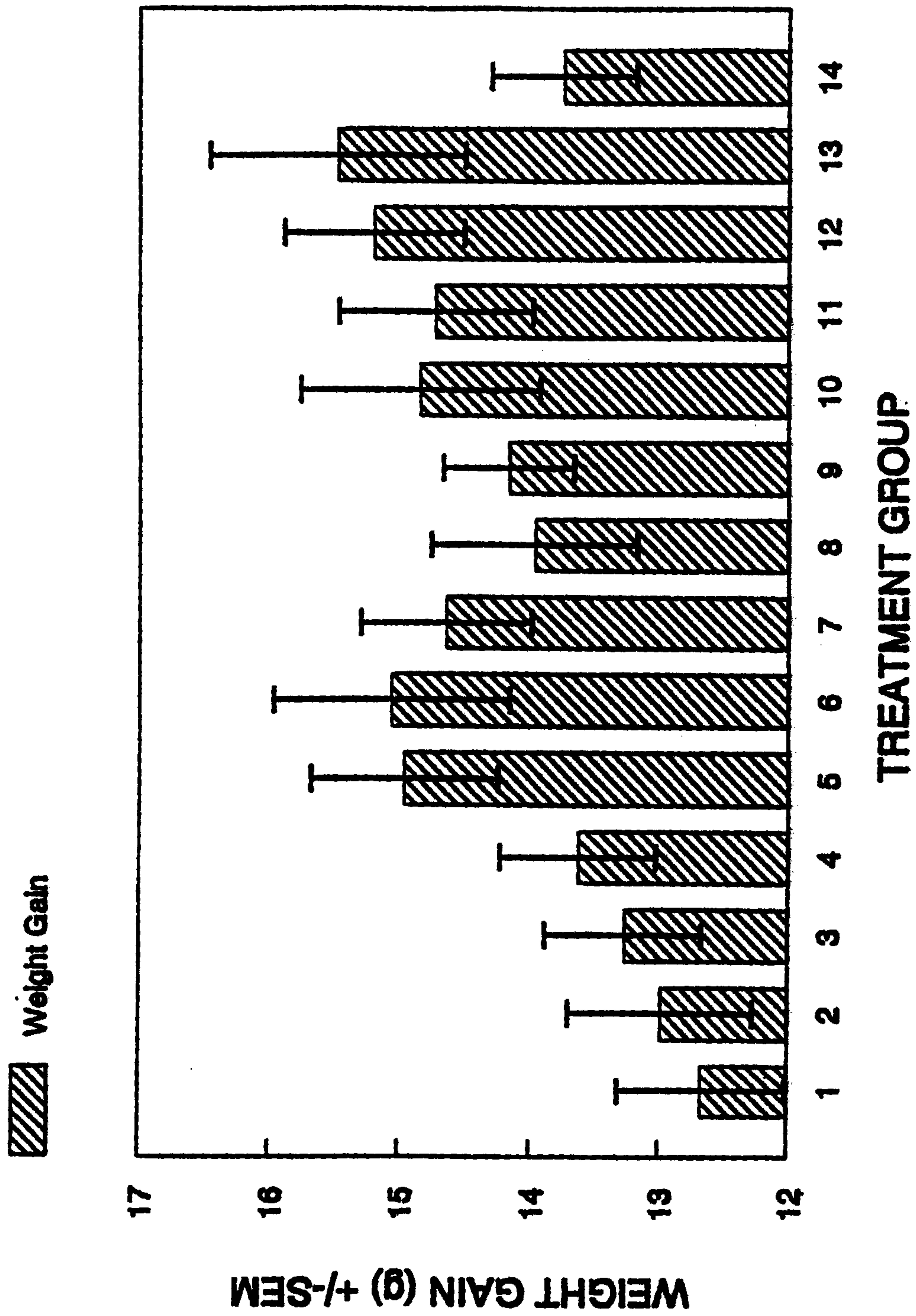


FIG. 18