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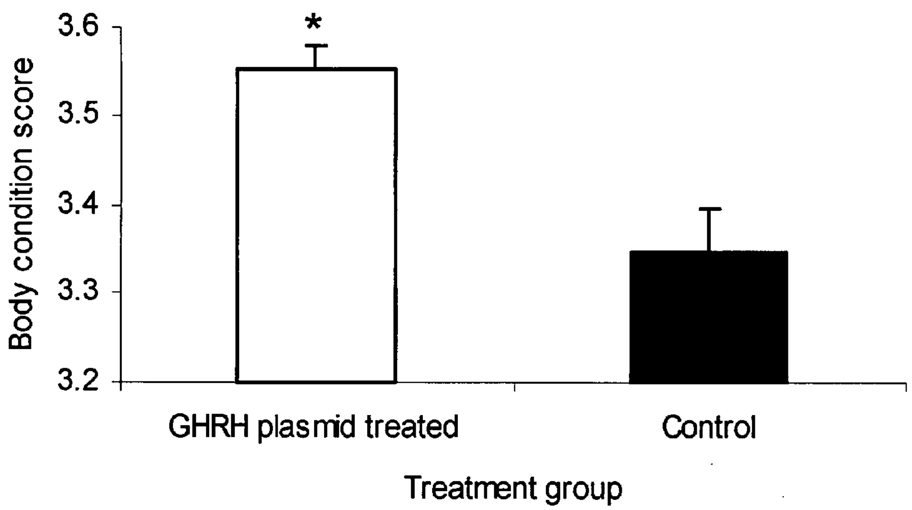

(57) Abstract: This invention discloses compositions and methods of: vaccinating a subject; enhancing the vaccination efficiency; preparing a subject prior to vaccination response; and improving the clinical outcome after infectious challenge in a subject that has been vaccinated. More specifically, the invention pertains to delivering into a tissue of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") before or concomitantly with delivering a vaccine to the subject, wherein, GHRH is expressed in vivo in the subject, wherein the subject comprises a human, pig, cow, bird, horse or any other animal species receiving a vaccine.

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GROWTH HORMONE RELEASING HORMONE ENHANCES VACCINATION RESPONSE

BACKGROUND

[0001] This application claims priority to U.S. Provisional Patent Application, Serial Number 60/590,739, entitled "GROWTH HORMONE RELEASING HORMONE ENHANCES VACCINATION RESPONSE" filed on July 23, 2004, having Ruxandra Draghia-Akli, Patricia A. Brown, Amir S. Khan and William C. Davis, listed as the inventors, the entire content of which is hereby incorporated by reference.

[0002] This invention pertains to compositions and methods of: vaccinating a subject; enhancing the vaccination efficiency; preparing a subject prior to vaccination; and improving the clinical outcome after infectious challenge in a subject that has been vaccinated. More specifically, the invention pertains to delivering into a tissue of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") before or concomitantly with delivering a vaccine to the subject, wherein, GHRH is expressed *in vivo* in the subject and the subject comprises a human, pig, cow, bird or any other animal species receiving a vaccine.

[0003] Infectious disease remains a significant problem in both humans and animals. Thus, age-appropriate antibiotic selection and evaluation of the clinical effectiveness of the specific vaccine as well as other immune-enhancing therapies are required. Substantial efforts have addressed the prevention rather than treatment of disease. The yearly market for vaccination is over $20 billion. Numerous reports indicate an interdependent relationship between the neuroendocrine and the immune systems. Hypothalamic GHRH stimulates growth hormone ("GH") secretion from the anterior pituitary gland, but recent studies have also demonstrated the immunomodulatory properties of this peptide (Siejka et al., 2004). The importance of GHRH in the modulation of immune status under physiological and pathological conditions (Marshall et al., 2001) has been described, both through stimulation of the GH/insulin-like growth factor-I ("IGF-I") axis and directly as an immune modulator (Dialynas et al., 1999; Khorram et al., 2001). GHRH is integral in the development and regulation of the immune system. Detail is still lacking, however, on exactly how GHRH mediates those effects or the impact of GHRH treatment on vaccination and pathogen challenge.

[0004] <u>**Growth Hormone Releasing Hormone ("GHRH") and Growth Hormone ("GH") Axis:**</u> To better understand utilizing GHRH plasmid-mediated supplementation as a method to enhance a specific vaccination response and to improve the clinical outcome after an infectious challenge, the mechanisms and current understanding of the GHRH/GH axis will be addressed. Although not wanting to be bound by theory, the central role of GH is controlling somatic growth in humans and other vertebrates. The physiologically relevant pathways regulating GH secretion from the pituitary are fairly well known. The GH pathway genes include: (1) ligands, such as GH and IGF-I; (2) transcription factors such as prophet of pit 1, or prop 1, and pit 1: (3) agonists and antagonists, such as GHRH and somatostatin ("SS"), respectively; and (4) receptors, such as GHRH receptor ("GHRH-R") and the GH receptor ("GH-R"). These genes are expressed in different organs and tissues, including the hypothalamus, pituitary, liver, and bone. Effective and regulated expression of the GH pathway is essential for optimal linear growth, as well as homeostasis of carbohydrate, protein, and fat metabolism. GH synthesis and secretion from the anterior pituitary is stimulated by GHRH and inhibited by somatostatin, both hypothalamic hormones. GH increases production of IGF-I, primarily in the liver, and other target organs. IGF-I and GH, in turn, feedback on the hypothalamus and pituitary to inhibit GHRH and GH release. GH elicits both direct and indirect actions on peripheral tissues, the indirect effects being mediated mainly by IGF-I.

[0005] <u>**GHRH and the Immune Function:**</u> Plasmid-mediated GHRH supplementation has been shown to have a variety of immunostimulatory effects in animals with depressed immune systems due to illness or various treatment regimens (Dorshkind and Horseman, 2001). Studies indicate that cells of the immune system produce GHRH, GH and IGF-I (Burgess et al., 1999) suggesting that immune function might be regulated by both autocrine and paracrine mechanisms. It has also been suggested that the increased morbidity in the elderly, such as respiratory disease, may be causally related to changes that occur with aging: decreased GH/IGF-I production, reduced IGF-I availability and decreased immune surveillance, especially T-cell mediated (Gelato, 1996; Krishnaraj et al., 1998). Conversely, administration of GHRH or its analogs in the elderly has resulted in profound immuno-enhancing effects, both short- and long-term after therapy. These effects include an increased number of lymphocytes, monocytes, B-cells as well as cells expressing T-cell receptor $\alpha\beta$ and T-cell receptor $\gamma\delta$ (Khorram et al., 1997). In immunocompromised patients, *e.g.*, after bone marrow transplantation, IGF-I administration can enhance lymphoid and myeloid reconstitution (Alpdogan et al., 2003). Also, studies of animal models of disease and vaccination show that *in vivo* administration of GH can effectively prime macrophages and increase the resistance to pathogens (Sakai et al., 1997).

[0006] Several studies in different animal models and human have shown that GHRH has an immune stimulatory effect, both through stimulation of the GH axis and directly as an immune-modulator (Dialynas et al., 1999; Khorram et al., 2001). GH has been known to enhance immune responses, whether directly or through the IGF-I, induced by GH. Recently, a GH secretagogue ("GHS") was found to induce the production of GH by the pituitary gland, but also determined a statistically significant increase in thymic cellularity and differentiation in old mice. When inoculated with a transplantable lymphoma cell line, EL4, the treated old mice showed statistically significant resistance to the initiation of tumors and the subsequent metastases. Generation of CTL to EL4 cells was also enhanced in the treated mice, suggesting that GHS has a considerable immune enhancing effect (Koo et al., 2001). The immune function is also modulated by IGF-I, which has two major effects on B cell development: potentiation and maturation, and as a B-cell proliferation cofactor that works together with interlukin-7. These activities were identified through the use of anti-IGF-I antibodies, antisense sequences to IGF-I, and the use of recombinant IGF-I to substitute for the activity. The treatment of mice with recombinant IGF-I confirmed these observations as it increased the number of pre-B and mature B cells in bone marrow (Jardieu et al., 1994). The mature B cell remained sensitive to IGF-I as immunoglobulin production was also stimulated by IGF-I *in vitro* and *in vivo* (Robbins et al., 1994).

[0007] In aging mammals, the GHRH-GH-IGF-I axis undergoes considerable decrement having reduced GH secretion and IGF-I production associated with a loss of skeletal muscle mass (sarcopenia), osteoporosis, arthritis, increased fat deposition and decreased lean body mass (Caroni and Schneider, 1994; Veldhuis et al., 1997). It has been demonstrated that the development of these changes can be offset by recombinant GH therapy. A therapy that would address both the increase risk of infection and the wasting would be a major step forward in the well-being and quality of life of patients.

[0008] The production of recombinant proteins in the last 2 decades provided a useful tool for the treatment of many diverse conditions. For example, GH-deficiencies in short stature children, anabolic agent in burn, sepsis, and AIDS patients. However, resistance to GH action has been reported in malnutrition and infection. Current GH therapy has several shortcomings, however, including frequent subcutaneous or intravenous injections, insulin resistance and impaired glucose tolerance (Rabinovsky et al., 1992); children are also vulnerable to premature epiphyseal closure and slippage of the capital femoral epiphysis (Liu and LeRoith, 1999). A "slow-release" form of GH (from Genentech) has been developed that only requires injections every 14 days. However, this GH product appears to perturb the normal physiological pulsatile GH profile, and is also associated with frequent side effects.

[0009] **Growth Hormone Releasing Hormone versus Growth Hormone or Growth Hormone Releasing Peptides ("GHRP"):** GH and GHRH are currently administered therapeutically as recombinant proteins. Current knowledge about the interaction between GH and its receptor suggests that the molecular heterogeneity of circulating GH may have important homeostasis implications. It has been suggested that adverse effects including insulin resistance, may result from the fact that exogenous GH elevates the basal GH serum levels and abolishes the natural GH episodic pulses. Studies have shown that continuous infusion with GHRH restores normal GH pulsatile pattern, without desensitization of GHRH receptors or depletion of GH supplies in humans, sheep or pigs (Dubreuil et al., 1990; Vance et al., 1989; Vance et al., 1985). At the same time, this system is capable of feed-back, which is totally abolished in the GH therapies. Virtually no side effects have been reported for GHRH therapies (Thorner et al., 1986a). Thus, GHRH therapy may be more physiological than GH therapy.

[0010] GHRPs are used in clinics to stimulate short term GH and IGF-I in humans. Hexarelin, a potent and well-studied GHRP, is capable of causing profound GH release in normal individuals. The GH response to hexarelin in humans becomes appreciably attenuated following long-term administration. Although this attenuation is partial and reversible, it could seriously limit the potential long-term therapeutic use of hexarelin and similar agents (Rahim and Shalet, 1998). Long-term therapy with hexarelin, in association with a vaccine, is needed to stimulate immune function. With the development of GH-releasing agents and their use in human subjects, it is clear that these agents are not specific for GH release. More recent studies in humans have demonstrated that acute increases in adrenocorticotrophic hormone (ACTH) (Ghigo et al., 1999), cortisol and prolactin (PRL) (Svensson and Bengtsson, 1999) have occurred after administration of GHRPs (hexarelin, MK-0677) (Schleim et al., 1999). The potential adverse effects of repeated episodes of transient (even minor) hyperprolactinaemia and hypercortisolaemia during long-term therapy with GHRPs and similar agents raise concern, require further study, and are undesirable in patients facing an infectious challenge (Rahim et al., 1999).

[0011] In contrast, essentially no side effects have been reported for recombinant GHRH therapies. Extracranially secreted GHRH, as mature peptide or truncated molecules (as seen with pancreatic islet cell tumors and variously located carcinoids) are often biologically active and can even produce acromegaly (Esch et al., 1982; Thorner et al., 1984). Administration of recombinant GHRH to GH-deficient children or adult humans augments IGF-I levels, increases GH secretion proportionally to the GHRH dose, yet still invokes a response to bolus doses of recombinant GHRH (Bercu and Walker, 1997). Thus, GHRH administration represents a more physiological alternative of increasing subnormal GH and IGF-I levels (Corpas et al., 1993).

[0012] Although recombinant GHRH protein therapy entrains and stimulates normal cyclical GH secretion with virtually no side effects, the short half-life of GHRH *in vivo* requires frequent (one to three times a day) intravenous, subcutaneous or intranasal (requiring 300-fold higher dose) administration. Thus, as a chronic treatment, GHRH administration is not practical.

[0013] Wild type GHRH has a relatively short half-life in the circulatory system, both in humans (Frohman et al., 1984) and in farm animals. After 60 minutes of incubation in plasma 95% of the GHRH(1-44)NH2 is degraded, while incubation of the shorter (1-40)OH form of the hormone, under similar conditions, shows only a 77% degradation of the peptide after 60 minutes of incubation (Frohman et al., 1989). Incorporation of cDNA coding for a particular protease-resistant GHRH analog in a therapeutic nucleic acid vector results in a molecule with a longer half-life in serum, increased potency, and provides greater GH release in plasmid-injected animals (Draghia-Akli et al., 1999), herein incorporated by reference. Mutagenesis *via* amino acid replacement of protease sensitive amino acids prolongs the serum half-life of the GHRH molecule. Furthermore, the enhancement of biological activity of GHRH is achieved by using super-active analogs that may increase its binding affinity to specific receptors (Draghia-Akli et al., 1999).

[0014] **Growth Hormone ("GH") and Growth Hormone Releasing Hormone ("GHRH") in Farm animals:** The administration of recombinant GH or recombinant GH has been used in subjects for many years, but not as a pathway to stimulate the response after vaccination, or to improve the clinical outcome after an infectious challenge. More specifically, recombinant GH treatment in farm animals has been shown to enhance lean tissue deposition and/or milk production, while increasing feed efficiency (Etherton et al., 1986; Klindt et al., 1998). Numerous studies have shown that recombinant GH markedly reduces the amount of carcass fat; and consequently the quality of products increases. However, chronic GH administration has practical, economical and physiological limitations that potentially mitigate its usefulness and effectiveness (Chung et al., 1985; Gopinath and Etherton, 1989b). Experimentally, recombinant GH-releasing hormone ("GHRH") has been used as a more physiological alternative. The use of GHRH in large animal species (e.g. pigs or cattle) not only enhances growth performance and milk production, but more importantly, the efficiency of production from both a practical and metabolic perspective (Dubreuil et al., 1990; Farmer et al., 1992). For example, the use of recombinant GHRH in lactating sows has beneficial effects on growth of the weanling pigs, yet optimal nutritional and hormonal conditions are needed for GHRH to exert its full potential (Farmer et al., 1996). Administration of GHRH and GH stimulate milk production, with an increase in feed to milk conversion. This therapy enhances growth primarily by increasing lean body mass (Lapierre et al., 1991; van Rooij et al., 2000) with overall improvement in feed efficiency. Hot and chilled carcass weights are increased and carcass lipid (percent of soft-tissue mass) is decrease by administration of GHRH and GH (Etherton et al., 1986).

[0015] **Transgene Delivery and *in vivo* Expression:** Although not wanting to be bound by theory, the delivery of a specific transgene to somatic tissue to correct inborn or acquired deficiencies and imbalances is possible. Such transgene-based delivery offers a number of advantages over the administration of recombinant proteins. These advantages include: the conservation of native protein structure; improved biological activity; avoidance of systemic toxicities; and avoidance of infectious and toxic impurities. Because the protein is synthesized and secreted continuously into the circulation, plasmid-mediated therapy allows for prolonged production of the protein in a therapeutic range. In contrast, the primary limitation of using recombinant protein is the limited bio-availability of protein after each administration.

[0016] In a plasmid-based expression system, a non-viral vector may comprise of a synthetic transgene delivery system in addition to the nucleic acid encoding the therapeutic genetic product. In this way, the risks associated with the use of most viral vectors can be avoided, including the expression of viral proteins that can induce immune responses against the target tissues or the viral vector and the possibility of DNA mutations or activations of oncogenes. The non-viral expression vector products generally have low toxicity due to the use of "species-specific" components for gene delivery, which minimizes the risks of plasmid-targeted immunogenicity and loss of expression. Additionally, no significant integration of plasmid sequences above the rate of spontaneous mutation into host chromosomes has been reported *in vivo* to date, so that this type of therapy should neither activate oncogenes nor inactivate tumor suppressor genes. As episomal systems residing outside the chromosomes, plasmids have defined pharmacokinetics and elimination profiles, leading to a finite duration of gene expression in target tissues. Plasmid vectors are simple to manufacture using good manufacturing practice techniques. They have a low risk to benefit ratio when compared to viral vectors, as stated on March 13-14, 2003 in a workshop sponsored by the American Society of Gene Therapy (ASGT) and the Food and Drug Administration's Center for Biologics Evaluation and Research (FDA/CBER) (Frederickson et al., 2003).

[0017] Direct plasmid DNA gene transfer is currently the basis of many emerging nucleic acid therapy strategies and does not require viral components or lipid particles (Aihara and Miyazaki, 1998; Muramatsu et al., 2001). Skeletal muscle is target tissue, because muscle fiber has a long life span and can be transduced by circular DNA plasmids that are expressed in immunocompetent hosts (Davis et al., 1993; Tripathy et al., 1996). Plasmid DNA constructs are attractive candidates for direct therapy into the subjects skeletal muscle because the constructs are well-defined entities that are biochemically stable and have been used successfully for many years (Acsadi et al., 1991; Wolff et al., 1990). The relatively low expression levels of an encoded product that are achieved after direct plasmid DNA injection are sometimes sufficient to indicate bio-activity of secreted peptides (Danko and Wolff, 1994; Tsurumi et al., 1996). Previous reports showed that human GHRH cDNA could be delivered to muscle by a plasmid in mice where it transiently stimulated GH secretion to a modest extent over a period of two weeks (Draghia-Akli et al., 1997).

[0018] **Plasmid-mediated GHRH supplementation stimulates immune function:** Preliminary studies in healthy dogs suggested that a single administration of a GHRH plasmid into skeletal muscle will ensure physiologic GHRH expression for several months (Draghia-Akli et al., 2003a). An initial study was designed to assess the ability of the plasmid-based GHRH treatment to produce beneficial effects in geriatric or cancer-afflicted companion dogs, and to assess long-term safety of the treatment regimen. A muscle-specific GHRH-expressing plasmid to 16 dogs afflicted with cancer was administered. The initial 56-day evaluation (Draghia-Akli et al., 2002a) demonstrated increased serum IGF-I concentrations, an indicator of GHRH bioactivity. A significant increase in circulating lymphocyte levels was found in treated animals. A further pilot study was conducted with severely debilitated geriatric and companion dogs with spontaneously occurring tumors. In this case, IGF-I levels were found to be elevated more than 365 days post-treatment. For the longitudinal continuation of this study, dogs that could be analyzed for at least 180 days post-treatment were included. Increases in weight, activity level and exercise tolerance in addition to improvement and maintenance of hematological parameters were observed. The overall long-term assessment of the treated dogs showed improvement in quality of life that was maintained throughout the study period. These results suggest a role for plasmid-mediated GHRH treatment in reversing the catabolic processes associated with aging and cancer anemia and/or cachexia, and suggest that the improved well-being may be associated with stimulation of immune function.

[0019] **Plasmid delivery and electroporation:** Efforts have been made to enhance the delivery of plasmid DNA to cells by physical means including electroporation, sonoporation, and pressure. Although not wanting to be bound by theory, the administration of a nucleic acid construct by electroporation involves the application of a pulsed electric field to create transient pores in the cellular membrane without causing permanent damage to the cell, which allows exogenous molecules to enter the cell (Smith and Nordstrom, 2000). By adjusting the electrical pulse generated by an electroporetic system, nucleic acid molecules can travel through passageways or pores in the cell that are created during the procedure. United States Patent 5,704,908 titled "Electroporation and iontophoresis catheter with porous balloon," issued on January 6, 1998 with Hofmann et al., listed as inventors describes an electroporation apparatus for delivering molecules to cells at a selected location within a cavity in the body of a patient. Similar pulse voltage injection devices are also described in: United States Patent 5,702,359 titled "Needle electrodes for mediated delivery of drugs and genes," issued on December 30, 1997, with Hofmann, et al., listed as inventors; United States Patent 5,439,440 titled "Electroporation system with voltage control feedback for clinical applications," issued on August 8, 1995 with Hofmann listed as inventor; PCT application WO/96/12520 titled "Electroporetic Gene and Drug Therapy by Induced Electric Fields," published on May 5, 1996 with Hofmann et al., listed as inventors; PCT application WO/96/12006 titled "Flow Through Electroporation Apparatus and Method," published on April 25, 1996 with Hofmann et al., listed as inventors; PCT application WO/95/19805 titled "Electroporation and Iontophoresis Apparatus and Method For insertion of Drugs and genes into Cells," published on July 27, 1995 with Hofmann listed as inventor; and PCT application WO/97/07826 titled "In Vivo Electroporation of Cells," published on March 6, 1997, with Nicolau et al., listed as inventors, the entire content of each of the above listed references is hereby incorporated by reference.

[0020] Recently, significant progress to enhance plasmid delivery *in vivo* and subsequently to achieve physiological levels of a secreted protein was obtained using the electroporation technique. Electroporation has been used very successfully to transfect tumor cells after injection of plasmid (Lucas et al., 2002; Matsubara et al., 2001)) or to deliver the anti-tumor drug bleomycin to cutaneous and subcutaneous tumors in humans (Gehl et al., 1998; Heller et al., 1996). Electroporation also has been extensively used in mice (Lesbordes et al., 2002; Lucas et al., 2001; Vilquin et al., 2001), rats (Terada et al., 2001; Yasui et al., 2001), and dogs (Fewell et al., 2001) to deliver therapeutic genes that encode for a variety of hormones, cytokines or enzymes. Previous studies using GHRH showed that plasmid therapy with electroporation is scalable and represents a promising approach to induce production and regulated secretion of proteins in large animals and humans (Draghia-Akli et al., 1999; Draghia-Akli et al., 2002c). Electroporation also has been extensively used in rodents and other small animals (Bettan et al., 2000; Yin and Tang, 2001). Intramuscular injection of plasmid followed by electroporation has been used successfully in ruminants for vaccination purposes (Babiuk et al., 2003; Tollefsen et al., 2003). It has been observed that the electrode configuration affects the electric field distribution, and subsequent results (Gehl et al., 1999; Miklavcic et al., 1998). Although not wanting to be bound by theory, needle electrodes give consistently better results than external caliper electrodes in a large animal model, and can be used for humans.

[0021] The ability of electroporation to enhance plasmid uptake into the skeletal muscle has been well documented. Similarly, plasmids formulated with poly-L-glutamate ("PLG") or polyvinylpyrrolidone ("PVP") were observed to have an increase in plasmid transfection, which consequently increased the expression of a desired transgene. For example, plasmids formulated with PLG or PVP were observed to increase gene expression to up to 10 fold in the skeletal muscle of mice, rats, and dogs (Fewell et al., 2001; Mumper et al., 1998). Although not wanting to be bound by theory, the anionic polymer sodium PLG enhances plasmid uptake at low plasmid concentrations and reduces any possible tissue damage caused by the procedure. PLG is a stable compound and it is resistant to relatively high temperatures (Dolnik et al., 1993). PLG has been used to increase stability of anti-cancer drugs (Li et al., 2000) and as "glue" to close wounds or to prevent bleeding from tissues during wound and tissue repair (Otani et al., 1996; Otani et al., 1998). PLG has been used to increase stability in vaccine preparations (Matsuo et al., 1994) without increasing their immunogenicity. PLG also has been used as an anti-toxin after antigen inhalation or exposure to ozone (Fryer and Jacoby, 1993).

[0022] Although not wanting to be bound by theory, PLG increases the transfection of the plasmid during the electroporation process, not only by stabilizing the plasmid DNA and facilitating the intracellular transport through the membrane pores, but also through an active mechanism. For example, positively charged surface proteins on the cells could complex the negatively charged PLG linked to plasmid DNA through protein-protein interactions. When an electric field is applied, the surface proteins reverse direction and actively internalize the DNA molecules, a process that substantially increases the transfection efficiency. Furthermore, PLG will prevent the muscle damage associated with *in vivo* plasmid delivery (Draghia-Akli et al., 2002b) and will increase plasmid stability *in vitro* prior to injection. There are studies directed to electroporation of eukaryotic cells with linear DNA (McNally et al., 1988; Neumann et al., 1982) (Toneguzzo et al., 1988) (Aratani et al., 1992; Nairn et al., 1993; Xie and Tsong, 1993; Yorifuji and Mikawa, 1990), but these examples illustrate transfection into cell suspensions, cell cultures, and the like, and such transfected cells are not present in a somatic tissue.

[0023] U.S. Patent No. 4,956,288 is directed to methods for preparing recombinant host cells containing high copy number of a foreign DNA by electroporating a population of cells in the presence of the foreign DNA, culturing the cells, and killing the cells having a low copy number of the foreign DNA.

[0024] Although not wanting to be bound by theory, a GHRH cDNA can be delivered to muscle of mice injectable myogenic expression vector where it can transiently stimulate GH secretion over a period of two weeks (Draghia-Akli et al., 1997). This injectable vector system was optimized by incorporating a powerful synthetic muscle promoter (Li et al., 1999) coupled with a novel protease-resistant GHRH molecule with a substantially longer half-life and greater GH secretory activity (pSP-HV-GHRH) (Draghia-Akli et al., 1999). Highly efficient electroporation technology was optimized to deliver the nucleic acid construct to the skeletal muscle of an animal (Draghia-Akli et al., 2002b). Using this combination of vector design and electric pulses plasmid delivery method, the inventors were able to show increased growth and favorably modified body composition in pigs (Draghia-Akli et al., 1999; Draghia-Akli et al., 2003b). The modified GHRH nucleic acid constructs increased red blood cell production in companion animals with cancer and cancer treatment-associated anemia (Draghia-Akli et al., 2002a). In pigs, available data suggested that the modified porcine HV-GHRH analog (SEQID#1) was more potent in promoting growth and positive body composition changes than the wild-type porcine GHRH (Draghia-Akli et al., 1999).

[0025] Administering novel GHRH analog proteins (U.S. Pat Nos. 5,847,066; 5846,936; 5,792,747; 5,776,901; 5,696,089; 5,486,505; 5,137,872; 5,084,442, 5,036,045; 5,023,322; 4,839,344; 4,410,512, RE33,699) or synthetic or naturally occurring peptide fragments of GHRH (U.S. Pat. Nos. 4,833,166; 4,228,158; 4,228,156; 4,226,857; 4,224,316; 4,223,021; 4,223,020; 4,223,019) for the purpose of increasing release of growth hormone have been reported. A GHRH analog containing the following mutations has been reported (U.S. Patent No. 5,846,936): Tyr at position 1 to His; Ala at position 2 to Val, Leu, or others; Asn at position 8 to Gln, Ser, or Thr; Gly at position 15 to Ala or Leu; Met at position 27 to Nle or Leu; and Ser at position 28 to Asn. The GHRH analog is the subject of United States Patent 6,551,996 titled "Super-active porcine growth hormone releasing hormone analog," issued on April 22, 2003 with Schwartz, et al., listed as inventors ("the '996 Patent"), which teaches application of a GHRH analog containing mutations that improve the ability to elicit the release of growth hormone. In addition, the '996 Patent application relates to the treatment of growth deficiencies; the improvement of growth performance; the stimulation of production of growth hormone in an animal at a greater level than that associated with normal growth; and the enhancement of growth utilizing the administration of growth hormone releasing hormone analog and is herein incorporated by reference.

[0026] In summary, enhancing vaccination response and potency and improving the clinical outcome of a subject after an infectious challenge were previously uneconomical and restricted in scope. The related art has shown that it is possible to improve these different conditions in a limited capacity utilizing recombinant protein technology, but these treatments have some significant drawbacks. It has also been taught that nucleic acid expression constructs that encode recombinant proteins are viable solutions to the problems of frequent injections and high cost of traditional recombinant therapy. There is a need in the art to expanded treatments for subjects with a disease by utilizing nucleic acid expression constructs that are delivered into a subject and express stable therapeutic proteins *in vivo*.

SUMMARY

[0027] The current invention pertains to compositions and methods of vaccinating a subject; methods for preparing a subject prior to vaccination; and methods for improving the clinical outcome after infectious challenge in a subject that has been vaccinated. Specific embodiments of the invention pertain to delivering into a tissue of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") before or concomitantly with delivering a vaccine to a subject in need of vaccination, wherein the GHRH is expressed *in vivo* in the subject and the subject comprises a human, pig, cow, bird or any other animal species receiving a vaccine.

[0028] One aspect of the current invention comprises a method of preparing a subject in need of vaccination. This method comprises delivering into a tissue of the subject a nucleic acid expression construct that encodes an *in vivo* expressed GHRH. The preferred GHRH of this invention comprises a sequence that is at least 90% identical to the encoded GHRH of SEQID#14, and the preferred nucleic acid expression constructs comprise a sequence that is at least 97% identical to mouse pAV0202 (SEQID#23); rat pAV0203 (SEQID#24); HV-GHRH pAV0224 (SEQID#25); pig-wt-GHRH pAV0225 (SEQID#26); dog pAV0235 (SEQID#27); bovine pAV0236 (SEQID#28); cat pAV0238 (SEQID#29); TI-GHRH pAV0239 (SEQID#30); ovine pAV0240 (SEQID#31); chicken pAV0241 (SEQID#32); horse pAV0249 (SEQID#33) or human pAV0226 (SEQID#34). This invention encompasses vaccines that comprise: killed microorganisms; live attenuated organisms; subunit antigens; toxoid antigens; conjugate antigens or other type of vaccine that when introduced into a subjects body produces immunity to a specific disease by causing the activation of the immune system, antibody formation, and/or creating of a T-cell and/or B-cell response. However preferred embodiments of the invention comprise vaccinations having: bovine herpesvirus-1 ("IBR"); bovine virus diarrhea ("BVD"); parainfluenza 3; respiratory syncytial virus; Leptospira canicola; Leptospira grippotyphosa, Leptospira hardjo; Leptospira icterohaemorrhagiae; Leptospira Pomona bacterinsmycoplasma hyopneumonia; mycoplasma hyopneumonia; or combinations thereof.

[0029] Generally the nucleic acid expression construct can be delivered into the subject up to 1 year before the subject is vaccinated, however, that time can vary. For example, in a preferred embodiment, the nucleic acid expression construct is delivered about 0 to about 14 days before the subject is vaccinated. One preferred method of delivering the nucleic acid expression construct into the tissue of the subject comprises tissue electroporation. Many methods of tissue electroporation have been described previously, however, a preferred tissue electroporation method comprises: penetrating the tissue in the subject with a plurality of needle electrodes, wherein the plurality of needle electrodes are arranged in a spaced relationship and the tissue of the subject comprise muscle cells; introducing the nucleic acid expression construct into the tissue between the plurality of needle electrodes in an amount in a range of about 0.01 – 5 mg; and applying an electrical pulse to the plurality of needle electrodes, wherein the electrical pulse allow the nucleic acid expression construct to traverse a muscle cell membrane. Additionally, the nucleic acid expression construct may also comprise a transfection-facilitating polypeptide or a charged polypeptide (e.g. poly-L-glutamate).

[0030] A second aspect of the current invention is a method for vaccinating a subject. This method comprises: delivering into a tissue of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH"); and then providing a vaccine to the subject in an amount effective to induce anti-vaccine antibodies in the subject. The preferred GHRH expression constructs are described above. The vaccine can be provided either concomitantly or after a period of time following delivery of the GHRH nucleic acid expression construct to the subject. In preferred embodiments, the vaccine is delivered up to a year after delivery of the GHRH nucleic acid expression construct. This invention encompasses vaccines that comprise: killed microorganisms; live attenuated organisms; subunit antigens; toxoid antigens; conjugate antigens or other type of vaccine that when introduced into a subjects body produces immunity to a specific disease by causing the activation of the immune system, antibody formation, and/or creating of a T-cell and/or B-cell response. However preferred embodiments of the invention comprise the vaccinations described above. In more preferred embodiments, the vaccine is delivered about 7 days to about 14 days after delivery of the GHRH nucleic acid expression construct. In another preferred embodiment, the GHRH nucleic acid expression construct is delivered using tissue electroporation, with a transfection-facilitating polypeptide.

[0031] A third aspect of the invention comprises a method of improving the clinical outcome, after an infectious challenge, of a vaccinated subject having arthritis. The method comprises: penetrating a muscle tissue in the subject with a plurality of needle electrodes, wherein the plurality of needle electrodes are arranged in a spaced relationship; delivering into the muscle tissue of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH"), such that an amount of expressed GHRH is effective to enhance the vaccination response; and applying an electrical pulse to the plurality of needle electrodes, wherein the electrical pulse allows the nucleic acid expression construct to traverse a muscle cell membrane. The preferred range of 0.01-5 mg of nucleic acid expression construct having a defined concentration of poly-L-glutamate polypeptide is delivered into the muscle tissue of the subject, and the nucleic acid expression construct comprises a sequence that encodes a polypeptide having an amino acid sequence that is at least 90% identical to the encoded GHRH of SEQID#14. The preferred nucleic acid expression constructs comprise sequence that is at least 97% identical to mouse pAV0202 (SEQID#23); rat pAV0203 (SEQID#24); HV-GHRH pAV0224 (SEQID#25); pig-wt-GHRH pAV0225 (SEQID#26); dog pAV0235 (SEQID#27); bovine pAV0236 (SEQID#28); cat pAV0238 (SEQID#29); TI-GHRH pAV0239 (SEQID#30); ovine pAV0240 (SEQID#31); chicken pAV0241 (SEQID#32); horse pAV0249 (SEQID#33) or human pAV0226 (SEQID#34). The preferred vaccines comprise: killed microorganisms; live attenuated organisms; subunit antigens; toxoid antigens; conjugate antigens or other type of vaccine that when introduced into a subjects body produces immunity to a specific disease by causing the activation of the immune system, antibody formation, and/or creating of a T-cell and/or B-cell response.

[0032] A fourth aspect of the current invention comprises a composition having both a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") and a vaccine. The preferred nucleic acid expression constructs comprise sequence that is at least 97% identical to mouse pAV0202 (SEQID#23); rat pAV0203 (SEQID#24); HV-GHRH pAV0224 (SEQID#25); pig-wt-GHRH pAV0225 (SEQID#26); dog pAV0235 (SEQID#27); bovine pAV0236 (SEQID#28); cat pAV0238 (SEQID#29); TI-GHRH pAV0239 (SEQID#30); ovine pAV0240 (SEQID#31); chicken pAV0241 (SEQID#32); horse pAV0249 (SEQID#33) or human pAV0226 (SEQID#34). The preferred vaccines comprise: killed microorganisms; live attenuated organisms; subunit antigens; toxoid antigens; conjugate antigens or other type of vaccine that when introduced into a subjects body produces immunity to a specific disease by causing the activation of the immune system, antibody formation, and/or creating of a T-cell and/or B-cell response.

BRIEF DESCRIPTION OF THE DRAWINGS:

[0033] **Figure 1** shows plasma levels of a secreted alkaline phosphatase ("SEAP") protein following injection of a pig with different concentrations of a SEAP expressing plasmid;

[0034] **Figure 2** shows glucose and insulin levels in control and pSP-HV-GHRH treated animals;

[0035] **Figure 3** Panel A and Panel B show the percentage of $CD2^+$ cells and $CD4^+CD45R^+$ naïve T cells at day 0 and 18 after treatment, Panel C shows the ratio of $CD45R^+/CD45R^-$ naïve T cells at 300 days post-treatment, wherein the values are presented as means ± SEM, * $P < 0.001$;

[0036] **Figure 4** shows that the $CD4^+/CD8^+$ ratio is significantly increased 14 days after vaccination with a Surround-9 way vaccine (Biocor) in cows that received the GHRH plasmid, when compared to control animals, that had a decrease in the $CD4^+/CD8^+$ ratio during the same period of time, $P < 0.05$;

[0037] **Figure 5** shows that the relative proportion of naïve T-cells is increased at 14 days after the Surround 9-way (Biocor) vaccination in animals that received the GHRH plasmid when compared to control animals, $P < 0.05$;

[0038] **Figure 6** shows the body condition scores in heifers treated with pSP-HV-GHRH versus controls at 60 to 80 DIM. Body condition scores differed between treatment groups, $P < 0.0001$;

[0039] **Figure 7** shows the weight of the animals treated with the pSP-HV-GHRH expressing plasmid compared with controls at different time points after injection;

[0040] **Figure 8** shows a restriction map of pAV0224 expression plasmid;

[0041] **Figure 9** shows a restriction map of pAV0225 expression plasmid;

[0042] **Figure 10** shows a restriction map of pAV0235 expression plasmid;

[0043] **Figure 11** shows a restriction map of pAV0236 expression plasmid;

[0044] **Figure 12** shows a restriction map of pAV0238 expression plasmid;

[0045] **Figure 13** shows a restriction map of pAV0239 expression plasmid;

[0046] **Figure 14** shows a restriction map of pAV0240 expression plasmid;

[0047] **Figure 15** shows a restriction map of pAV0241expression plasmid;

[0048] **Figure 16** shows a restriction map of pAV0249 expression plasmid; and

[0049] **Figure 17** shows a restriction map of pAV0226 expression plasmid.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0050] It will be readily apparent to one skilled in the art that various substitutions and modifications may be made in the invention disclosed herein without departing from the scope and spirit of the invention.

[0051] The term "a" or "an" as used herein in the specification may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein “another” may mean at least a second or more.

[0052] The term “analog” as used herein includes any mutant of GHRH, or synthetic or naturally occurring peptide fragments of GHRH, such as HV-GHRH (SEQID#1), pig-GHRH (SEQID#2), bovine-GHRH (SEQID#3), dog-GHRH (SEQID#4), cat-GHRH (SEQID#5), TI-GHRH (SEQID#6), ovine-GHRH (SEQID#7), chicken-GHRH (SEQID#8), horse-GHRH (SEQID#9), TV-GHRH (SEQID#11), 15/27/28-GHRH (SEQID#12), human GHRH (1-44)NH_2 (SEQID#13), human GHRH(1-40)OH (SEQID#10) forms, or any shorter form to no less than (1-29) amino acids.

[0053] The term “bodily fat proportion” as used herein is defined as the body fat mass divided by the total body weight.

[0054] The term “body condition score” (BCS) as used herein is defined as a method to evaluate the overall nutrition and management of horses or any other farm animal.

[0055] The term “cassette” as used herein is defined as one or more transgene expression vectors.

[0056] The term “cell-transfecting pulse” as used herein is defined as a transmission of a force which results in transfection of a vector, such as a linear DNA fragment, into a cell. In some embodiments, the force is from electricity, as in electroporation, or the force is from vascular pressure.

[0057] The term “coding region” as used herein refers to any portion of the DNA sequence that is transcribed into messenger RNA (mRNA) and then translated into a sequence of amino acids characteristic of a specific polypeptide.

[0058] The term "delivery" or "delivering" as used herein is defined as a means of introducing a material into a tissue, a subject, a cell or any recipient, by means of chemical or biological process, injection, mixing, electroporation, sonoporation, or combination thereof, either under or without pressure.

[0059] The term "chronically ill" as used herein is defined as patients with conditions as chronic obstructive pulmonary disease, chronic heart failure, stroke, dementia, rehabilitation after hip fracture, chronic renal failure, arthritis, rheumatoid arthritis, and multiple disorders in the elderly, with doctor visits and/or hospitalization once a month for at least two years.

[0060] The term "donor-subject" as used herein refers to any species of the animal kingdom wherein cells have been removed and maintained in a viable state for any period of time outside the subject.

[0061] The term "donor-cells" as used herein refers to any cells that have been removed and maintained in a viable state for any period of time outside the donor-subject.

[0062] The term "electroporation" as used herein refers to a method that utilized electric pulses to deliver a nucleic acid sequence into cells.

[0063] The terms "electrical pulse" and "electroporation" as used herein refer to the administration of an electrical current to a tissue or cell for the purpose of taking up a nucleic acid molecule into a cell. A skilled artisan recognizes that these terms are associated with the terms "pulsed electric field" "pulsed current device" and "pulse voltage device." A skilled artisan recognizes that the amount and duration of the electrical pulse is dependent on the tissue, size, and overall health of the recipient subject, and furthermore knows how to determine such parameters empirically.

[0064] The term "encoded GHRH" as used herein is a biologically active polypeptide of growth hormone releasing hormone.

[0065] The term "enhanced vaccination response" as used herein comprises the enhanced immunity to a specific disease by allowing a faster activation of the immune system, faster antibody formation, higher antibody titers, enhancement of a T-cell response and/or enhancement of a B-cell response.

[0066] The term "functional biological equivalent" of GHRH as used herein is a polypeptide that has a distinct amino acid sequence from a wild type GHRH polypeptide while simultaneously having similar or improved biological activity when compared to the GHRH polypeptide. The functional biological equivalent may be naturally occurring or it may be modified by an individual. A skilled artisan recognizes that the similar or improved biological activity as used herein refers to facilitating and/or releasing growth hormone or other pituitary hormones. A skilled artisan recognizes that in some embodiments the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biological activity when compared to the GHRH polypeptide. Methods known in the art to engineer such a sequence include site-directed mutagenesis.

[0067] The term "growth hormone" ("GH") as used herein is defined as a hormone that relates to growth and acts as a chemical messenger to exert its action on a target cell. In a specific embodiment, the growth hormone is released by the action of growth hormone releasing hormone.

[0068] The term "growth hormone releasing hormone" ("GHRH") as used herein is defined as a hormone that facilitates or stimulates release of growth hormone, and in a much lesser extent other pituitary hormones, such as prolactin.

[0069] The term "heterologous nucleic acid sequence" as used herein is defined as a DNA sequence comprising differing regulatory and expression elements.

[0070] The term "immunotherapy" as used herein refers to any treatment that promotes or enhances the body's immune system to build protective antibodies that will reduce the symptoms of a medical condition, prevent the development in a subject of an infectious condition and/or lessen the need for medications.

[0071] The term "modified cells" as used herein is defined as the cells from a subject that have an additional nucleic acid sequence introduced into the cell.

[0072] The term "modified-donor-cells" as used herein refers to any donor-cells that have had a GHRH-encoding nucleic acid sequence delivered.

[0073] The term "molecular switch" as used herein refers to a molecule that is delivered into a subject that can regulate transcription of a gene.

[0074] The term "nucleic acid expression construct" as used herein refers to any type of an isolated genetic construct comprising a nucleic acid coding for a RNA capable of being transcribed. The term "expression vector" can also be used interchangeably herein. In specific embodiments, the isolated nucleic acid expression construct comprises: a promoter; a nucleotide sequence of interest; and a 3′ untranslated region; wherein the promoter, the nucleotide sequence of interest, and the 3' untranslated region are operatively linked; and *in vivo* expression of the nucleotide sequence of interest is regulated by the promoter. The term "DNA fragment" as used herein refers to a substantially double stranded DNA molecule. Although the fragment may be generated by any standard molecular biology means known in the art, in some embodiments the DNA fragment or expression construct is generated by restriction digestion of a parent DNA molecule. The terms "expression vector," "expression cassette," or "expression plasmid" can also be used interchangeably. Although the parent molecule may be any standard molecular biology DNA reagent, in some embodiments the parent DNA molecule is a plasmid.

[0075] The term "operatively linked" as used herein refers to elements or structures in a nucleic acid sequence that are linked by operative ability and not physical location. The elements or structures are capable of, or characterized by accomplishing a desired operation. It is recognized by one of ordinary skill in the art that it is not necessary for elements or structures in a nucleic acid sequence to be in a tandem or adjacent order to be operatively linked.

[0076] The term "poly-L-glutamate ("PLG")" as used herein refers to a biodegradable polymer of L-glutamic acid that is suitable for use as a vector or adjuvant for DNA transfer into cells with or without electroporation.

[0077] The term "post-injection" as used herein refers to a time period following the introduction of a nucleic acid cassette that contains heterologous nucleic acid sequence encoding GHRH or a biological equivalent thereof into the cells of the subject and allowing expression of the encoded gene to occur while the modified cells are within the living organism.

[0078] The term "plasmid" as used herein refers generally to a construction comprised of extra-chromosomal genetic material, usually of a circular duplex of DNA that can replicate independently of chromosomal DNA. Plasmids, or fragments thereof, may be used as vectors. Plasmids are double-stranded DNA molecule that occur or are derived from bacteria and (rarely) other microorganisms. However, mitochondrial and chloroplast DNA, yeast killer and other cases are commonly excluded.

[0079] The term "plasmid mediated gene supplementation" as used herein refers a method to allow a subject to have prolonged exposure to a therapeutic range of a therapeutic protein by utilizing a nucleic acid-expression construct *in vivo*.

[0080] The term "pulse voltage device," or "pulse voltage injection device" as used herein relates to an apparatus that is capable of causing or causes uptake of nucleic acid molecules into the cells of an organism by emitting a localized pulse of electricity to the cells. The cell membrane then destabilizes, forming passageways or pores. Conventional devices of this type are calibrated to allow one to select or adjust the desired voltage amplitude and the duration of the pulsed voltage. The primary importance of a pulse voltage device is the capability of the device to facilitate delivery of compositions of the invention, particularly linear DNA fragments, into the cells of the organism.

[0081] The term "plasmid backbone" as used herein refers to a sequence of DNA that typically contains a bacterial origin of replication, and a bacterial antibiotic selection gene, which are necessary for the specific growth of only the bacteria that are transformed with the proper plasmid. However, there are plasmids, called mini-circles, that lack both the antibiotic resistance gene and the origin of replication (Darquet et al., 1997; Darquet et al., 1999; Soubrier et al., 1999). The use of *in vitro* amplified expression plasmid DNA (i.e. non-viral expression systems) avoids the risks associated with viral vectors. The non-viral expression systems products generally have low toxicity due to the use of "species-specific" components for gene delivery, which minimizes the risks of immunogenicity generally associated with viral vectors. One aspect of the current invention is that the plasmid backbone does not contain viral nucleotide sequences.

[0082] The term "promoter" as used herein refers to a sequence of DNA that directs the transcription of a gene. A promoter may direct the transcription of a prokaryotic or eukaryotic gene. A promoter may be "inducible", initiating transcription in response to an inducing agent or, in contrast, a promoter may be "constitutive", whereby an inducing agent does not regulate the rate of transcription. A promoter may be regulated in a tissue-specific or tissue-preferred manner, such that it is only active in transcribing the operable linked coding region in a specific tissue type or types.

[0083] The term "quality of life" or "health related quality of life" of a subject as used herein refers to those attributes valued by patients and their owners, including: their resultant comfort and well-being; the extent to which they are able to maintain reasonable physical, emotional, and intellectual function; and the degree to which they retain their ability to participate in valued activities within the family, in the workplace, and in the community.

[0084] The term "welfare" of a subject as used herein refers at a state of being or doing well, performing tasks and activities at functional levels; condition of health, happiness, and comfort; well-being; prosperity.

[0085] The term "replication element" as used herein comprises nucleic acid sequences that will lead to replication of a plasmid in a specified host. One skilled in the art of molecular biology will recognize that the replication element may include, but is not limited to a selectable marker gene promoter, a ribosomal binding site, a selectable marker gene sequence, and a origin of replication.

[0086] The term "residual linear plasmid backbone" as used herein comprises any fragment of the plasmid backbone that is left at the end of the process making the nucleic acid expression plasmid linear.

[0087] The term "recipient-subject" as used herein refers to any species of the animal kingdom wherein modified-donor-cells can be introduced from a donor-subject.

[0088] The term "regulator protein" as used herein refers to any protein that can be used to control the expression of a gene, and that is increasing the rate of transcription in response to an inducing agent.

[0089] The term "secretagogue" as used herein refers to an agent that stimulates secretion. For example, a growth hormone secretagogue is any molecule that stimulates the release of growth hormone from the pituitary when delivered into an animal. Growth hormone releasing hormone is a growth hormone secretagogue.

[0090] The terms "subject" or "animal" as used herein refers to any species of the animal kingdom. In preferred embodiments, it refers more specifically to humans and domesticated animals used for: pets (*e.g.* cats, dogs, *etc.*); work (*e.g.* horses, *etc.*); food (cows, chicken, fish, lambs, pigs, etc); and all others known in the art.

[0091] The term "tissue" as used herein refers to a collection of similar cells and the intercellular substances surrounding them. A skilled artisan recognizes that a tissue is an aggregation of similarly specialized cells for the performance of a particular function. For the scope of the present invention, the term tissue does not refer to a cell line, a suspension of cells, or a culture of cells. In a specific embodiment, the tissue is electroporated *in vivo*. In another embodiment, the tissue is not a plant tissue. A skilled artisan recognizes that there are four basic tissues in the body: 1) epithelium; 2) connective tissues, including blood, bone, and cartilage; 3) muscle tissue; and 4) nerve tissue. In a specific embodiment, the methods and compositions are directed to transfer of linear DNA into a muscle tissue by electroporation.

[0092] The term "therapeutic element" as used herein comprises nucleic acid sequences that will lead to an *in vivo* expression of an encoded gene product. One skilled in the art of molecular biology will recognize that the therapeutic element may include, but is not limited to a promoter sequence, a transgene, a poly A sequence, or a 3' or 5' UTR.

[0093] The term "transfects" as used herein refers to introduction of a nucleic acid into a eukaryotic cell. In some embodiments, the cell is not a plant tissue or a yeast cell.

[0094] The term "vector" as used herein refers to any vehicle that delivers a nucleic acid into a cell or organism. Examples include plasmid vectors, viral vectors, liposomes, or cationic lipids. The term also refers to a construction comprised of genetic material designed to direct transformation of a targeted cell by delivering a nucleic acid sequence into that cell. A vector may contain multiple genetic elements positionally and sequentially oriented with other necessary elements such that an included nucleic acid cassette can be transcribed and when necessary translated in the transfected cells. These elements are operatively linked. The term "expression vector" refers to a DNA plasmid that contains all of the information necessary to produce a recombinant protein in a heterologous cell.

[0095] The term "viral backbone" as used herein refers to a nucleic acid sequence that does not contain a promoter, a gene, and a 3' poly A signal or an untranslated region, but contain elements including, but not limited at site-specific genomic integration Rep and inverted terminal repeats ("ITRs") or the binding site for the tRNA primer for reverse transcription, or a nucleic acid sequence component that induces a viral immunogenicity response when inserted in vivo, allows integration, affects specificity and activity of tissue specific promoters, causes transcriptional silencing or poses safety risks to the subject.

[0096] The term "vascular pressure pulse" refers to a pulse of pressure from a large volume of liquid to facilitate uptake of a vector into a cell. A skilled artisan recognizes that the amount and duration of the vascular pressure pulse is dependent on the tissue, size, and overall health of the recipient animal, and furthermore knows how to determine such parameters empirically.

[0097] The term "vaccine" as used herein refers to any preparation of killed microorganisms, live attenuated organisms, subunit antigens, toxoid antigens, conjugate antigens or other type of antigenic molecule that when introduced into a subjects body produces immunity to a specific disease by causing the activation of the immune system, antibody formation, and/or creating of a T-cell and/or B-cell response. Generally vaccines against microorganisms are directed toward at least part of a virus, bacteria, parasite, mycoplasma, or other infectious agent.

[0098] Efficacy of vaccination or immunization for specific pathogens and the clinical outcome after an infectious challenge are of extraordinary importance for both human and animal medicine. One specific embodiment of the current invention is a method of enhancing the response to vaccination. The method comprises: penetrating a muscle tissue in the subject with a plurality of needle electrodes, wherein the plurality of needle electrodes are arranged in a spaced relationship; delivering into the muscle tissue of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH"), such that an amount of expressed GHRH is effective to enhance the response to a specific vaccination; and applying an electrical pulse to the plurality of needle electrodes, wherein the electrical pulse allows the nucleic acid expression construct to traverse a muscle cell membrane. A range of 0.01-5 mg of nucleic acid expression construct with a defined concentration of poly-L-glutamate polypeptide is delivered into the muscle tissue of the subject, and the nucleic acid expression construct comprises a sequence that encodes a polypeptide having an amino acid sequence that is at least 90% identical to the encoded GHRH of SEQID#14. The preferred subject comprises a human, a ruminant animal, a food animal, a horse, or a work animal. While there are many indicators of enhanced response to vaccination, a few examples comprise: increased specific antibody titer, more rapid response after an infectious challenge, an improved clinical outcome, or a combination thereof. Other specific embodiments of this invention encompass various modes of delivering into the tissue of the subject the nucleic acid expression construct (e.g. an electroporation method, a viral vector, in conjunction with a carrier, by parenteral route, or a combination thereof).

[0099] A second preferred embodiment includes the nucleic acid expression construct being delivered in a single dose, and the single dose comprising a total of about a 0.01-5 mg of nucleic acid expression construct. Generally the nucleic acid expression construct is delivered into a tissue of the subject comprising diploid cells (e.g. muscle cells).

[0100] In a third specific embodiment the nucleic acid expression construct used for transfection comprises a wt porcine-GHRH plasmid (SEQID#26). Other specific embodiments utilize other nucleic acid expression constructs (e.g. an optimized bovine GHRH plasmid, pAV0236 (SEQID#28); a TI-GHRH plasmid, pAV0239 (SEQID#30); HV-GHRH plasmid, pAV0224 (SEQID#25); ovine GHRH plasmid, pAV0240 (SEQID#31); chicken GHRH plasmid, pAV0241 (SEQID#32); dog GHRH plasmid, pAV0235 (SEQID#27); cat GHRH plasmid, pAV0238 (SEQID#29); horse GHRH plasmid, pAV0249 (SEQID#33), human GHRH plasmid, pAV0226 (SEQID#34).

[0101] In a fourth specific embodiment, the nucleic acid expression construct further comprises, a transfection-facilitating polypeptide (e.g. a charged polypeptide, or poly-L-glutamate). After delivering the nucleic acid expression construct into the tissues of the subject, expression of the encoded GHRH or functional biological equivalent thereof is initiated. The encoded GHRH comprises a biologically active polypeptide; and the encoded functional biological equivalent of GHRH is a polypeptide that has been engineered to contain a distinct amino acid sequence while simultaneously having similar or improved biologically activity when compared to the GHRH polypeptide. One embodiment of a specific encoded GHRH or functional biological equivalent thereof is of formula (SEQID#14). The animal comprises a human, a food animal, a work animal (e.g. a pig, cow, sheep, goat or chicken), or a pet (e.g. dog, cat, horse).

[0102] The current invention also pertains to methods useful for improving the clinical outcome of a patient after an infectious challenge. The general method of this invention comprises treating a subject with plasmid-mediated gene supplementation. The method comprises delivering a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") or functional biological equivalent thereof into a tissue, such as a muscle, of the subject. Specific embodiments of this invention are directed toward improving the vaccination response in treated subjects by plasmid mediated GHRH supplementation. Plasmid injection can precede or be concomitant with the specific vaccination. The subsequent *in vivo* expression of the GHRH or biological equivalent in the subject is sufficient to improve vaccination response. Thus, if an infectious challenge occurs at a later date, the clinical outcome is significantly improved. It is also possible to enhance this method by placing a plurality of electrodes in a selected tissue, then delivering nucleic acid expression construct to the selected tissue in an area that interposes the plurality of electrodes, and applying a cell-transfecting pulse (e.g. electrical) to the selected tissue in an area of the selected tissue where the nucleic acid expression construct was delivered. However, the cell-transfecting pulse need not be an electrical pulse, a different method, such as vascular pressure pulse can also be utilized. Electroporation, direct injection, gene gun, or gold particle bombardment are also used in specific embodiments to deliver the nucleic acid expression construct encoding the GHRH or biological equivalent into the subject. The subject in this invention comprises an animal (e.g. a human, a pig, a horse, a cow, a mouse, a rat, a monkey, a sheep, a goat, a dog, or a cat).

[0103] Recombinant GH replacement therapy is widely used in agriculture and clinically, with beneficial effects, but generally, the doses are supra-physiological. Such elevated doses of recombinant GH are associated with deleterious side-effects, for example, up to 30% of the recombinant GH treated subjects develop at a higher frequency insulin resistance (Gopinath and Etherton, 1989a; Gopinath and Etherton, 1989b; Verhelst et al., 1997) or accelerated bone epiphysis growth and closure in pediatric patients (Blethen and Rundle, 1996). In addition, molecular heterogeneity of circulating GH may have important implications in growth and homeostasis (Satozawa et al., 2000; Tsunekawa et al., 1999; Wada et al., 1998). Unwanted side effects result from the fact that treatment with recombinant exogenous GH protein raises basal levels of GH and abolishes the natural episodic pulses of GH. In contradistinction, no side effects have been reported for recombinant GHRH therapies. The normal levels of GHRH in the pituitary portal circulation range from about 150-to-800 pg/ml, while systemic circulating values of the hormone are up to about 100-500 pg/ml. Some patients with acromegaly caused by extracranial tumors have level that is nearly 100 times as high (*e.g.* 50 ng/ml of immunoreactive GHRH) (Thorner et al., 1984). Long-term studies using recombinant GHRH therapies (1-5 years) in children and elderly humans have shown an absence of the classical GH side-effects, such as changes in fasting glucose concentration or, in pediatric patients, the accelerated bone epiphysal growth and closure or slipping of the capital femoral epiphysis (Chevalier et al., 2000) (Duck et al., 1992; Vittone et al., 1997).

[0104] Studies in humans, sheep or pigs showed that continuous infusion with recombinant GHRH protein restores the normal GH pattern without desensitizing GHRH receptors or depleting GH supplies (Dubreuil et al., 1990). As this system is capable of a degree of feed-back which is abolished in the GH therapies, GHRH recombinant protein therapy may be more physiological than GH therapy. However, due to the short half-life of GHRH *in vivo*, frequent (one to three times per day) intravenous, subcutaneous or intranasal (requiring 300-fold higher dose) administrations are necessary (Evans et al., 1985; Thorner et al., 1986b). Thus, as a chronic therapy, recombinant GHRH protein administration is not practical. A plasmid-mediated supplementation approach, however could overcome this limitations to GHRH use. The choice of GHRH for a gene therapeutic application is favored by the fact that the gene, cDNA and native and several mutated molecules have been characterized for humans, pig, cattle and other species (Bohlen et al., 1983; Guillemin et al., 1982); the cDNA of cat, dog and horse specific GHRH have been isolated. The measurement of therapeutic efficacy is straightforward and unequivocal.

[0105] Among the non-viral techniques for gene transfer *in vivo*, the direct injection of plasmid DNA into muscle is simple, inexpensive, and safe. The inefficient DNA uptake into muscle fibers after simple direct injection had led to relatively low expression levels (Prentice et al., 1994; Wells et al., 1997) In addition, the duration of the transgene expression has been short (Wolff et al., 1990). The most successful previous clinical applications have been confined to vaccines (Danko and Wolff, 1994; Tsurumi et al., 1996). Recently, significant progress to enhance plasmid delivery *in vivo* and subsequently to achieve physiological levels of a secreted protein was obtained using the electroporation technique. Electroporation has been used very successfully to transfect tumor cells after injection of plasmid (Lucas et al., 2002; Matsubara et al., 2001) or to deliver the anti-tumor drug bleomycin to cutaneous and subcutaneous tumors in humans (Gehl et al., 1998; Heller et al., 1996). Electroporation also has been extensively used in mice (Lesbordes et al., 2002; Lucas et al., 2001; Vilquin et al., 2001), rats (Terada et al., 2001; Yasui et al., 2001), and dogs (Fewell et al., 2001) to deliver therapeutic genes that encode for a variety of hormones, cytokines or enzymes. Our previous studies using growth hormone releasing hormone (GHRH) showed that plasmid therapy with electroporation is scalable and represents a promising approach to induce production and regulated secretion of proteins in large animals and humans (Draghia-Akli et al., 1999; Draghia-Akli et al., 2002c). Electroporation also has been extensively used in rodents and other small animals (Bettan et al., 2000; Yin and Tang, 2001). It has been observed that the electrode configuration affects the electric field distribution, and subsequent results (Gehl et al., 1999; Miklavcic et al., 1998). Preliminary experiments indicated that for a large animal or humans, needle electrodes give consistently better reproducible results than external caliper electrodes.

[0106] The ability of electroporation to enhance plasmid uptake into the skeletal muscle has been well documented, as described above. In addition, plasmid formulated with PLG or polyvinylpyrrolidone ("PVP") has been observed to increase gene transfection and consequently gene expression to up to 10 fold in the skeletal muscle of mice, rats and dogs (Fewell et al., 2001; Mumper et al., 1998). Although not wanting to be bound by theory, PLG will increase the transfection of the plasmid during the electroporation process, not only by stabilizing the plasmid DNA, and facilitating the intracellular transport through the membrane pores, but also through an active mechanism. For example, positively charged surface proteins on the cells could complex the negatively charged PLG linked to plasmid DNA through protein-protein interactions. When an electric field is applied, the surface proteins reverse direction and actively internalize the DNA molecules, process that substantially increases the transfection efficiency.

[0107] Although not wanting to be bound by theory, the plasmid supplementation approach to enhance the vaccination response and to improve the clinical outcome of a subject after an infectious challenge described herein offers advantages over the limitations of directly injecting recombinant GH or GHRH protein. Expression of GHRH or novel biological equivalents of GHRH can be directed by an expression plasmid controlled by a synthetic muscle-specific promoter. Expression of such GHRH or biological equivalent thereof elicited high GH and IGF-I levels in subjects that have had the encoding sequences delivered into the cells of the subject by intramuscular injection and *in vivo* electroporation. Although *in vivo* electroporation is the preferred method of introducing the heterologous nucleic acid encoding system into the cells of the subject, other methods exist and should be known by a person skilled in the art (*e.g.* electroporation, lipofectamine, calcium phosphate, *ex vivo* transformation, direct injection, DEAE dextran, sonication loading, receptor mediated transfection, microprojectile bombardment, *etc.*). For example, it may also be possible to introduce the nucleic acid sequence that encodes the GHRH or functional biological equivalent thereof directly into the cells of the subject by first removing the cells from the body of the subject or donor, maintaining the cells in culture, then introducing the nucleic acid encoding system by a variety of methods (*e.g.* electroporation, lipofectamine, calcium phosphate, *ex vivo* transformation, direct injection, DEAE dextran, sonication loading, receptor mediated transfection, microprojectile bombardment, etc.), and finally reintroducing the modified cells into the original subject or other host subject (the *ex vivo* method). The GHRH sequence can be cloned into an adenovirus vector or an adeno-associated vector and delivered by simple intramuscular injection, or intravenously or intra-arterially. Plasmid DNA carrying the GHRH sequence can be complexed with cationic lipids or liposomes and delivered intramuscularly, intravenously or subcutaneous.

[0108] Administration as used herein refers to the route of introduction of a vector or carrier of DNA into the body. Administration can be directly to a target tissue or by targeted delivery to the target tissue after systemic administration. In particular, the present invention can be used for improving the vaccination response in a subject by administration of the vector to the body in order to establishing controlled expression of any specific nucleic acid sequence within tissues at certain levels that are useful for plasmid-mediated supplementation. The preferred means for administration of vector and use of formulations for delivery are described above.

[0109] Muscle cells have the unique ability to take up DNA from the extracellular space after simple injection of DNA particles as a solution, suspension, or colloid into the muscle. Expression of DNA by this method can be sustained for several months. DNA uptake in muscle cells is further enhanced utilizing *in vivo* electroporation.

[0110] Delivery of formulated DNA vectors involves incorporating DNA into macromolecular complexes that undergo endocytosis by the target cell. Such complexes may include lipids, proteins, carbohydrates, synthetic organic compounds, or inorganic compounds. The characteristics of the complex formed with the vector (size, charge, surface characteristics, composition) determine the bioavailability of the vector within the body. Other elements of the formulation function as ligands that interact with specific receptors on the surface or interior of the cell. Other elements of the formulation function to enhance entry into the cell, release from the endosome, and entry into the nucleus.

[0111] Delivery can also be through use of DNA transporters. DNA transporters refer to molecules that bind to DNA vectors and are capable of being taken up by epidermal cells. DNA transporters contain a molecular complex capable of non-covalently binding to DNA and efficiently transporting the DNA through the cell membrane. It is preferable that the transporter also transport the DNA through the nuclear membrane. See, *e.g.*, the following applications all of which (including drawings) are hereby incorporated by reference herein: (1) Woo *et al.*, U.S. Patent No. 6,150,168 entitled: "A DNA Transporter System and Method of Use;" (2) Woo *et al.*, PCT/US93/02725, entitled "A DNA Transporter System and method of Use", filed Mar. 19, 1993; (3) Woo *et al.*, U.S. Patent No. 6,177,554 "Nucleic Acid Transporter Systems and Methods of Use;" (4) Szoka *et al.*, U.S. Patent No. 5,955,365 entitled "Self-Assembling Polynucleotide Delivery System;" and (5) Szoka *et al.*, PCT/US93/03406, entitled "Self-Assembling Polynucleotide Delivery System", filed Apr. 5, 1993.

[0112] Another method of delivery involves a DNA transporter system. The DNA transporter system consists of particles containing several elements that are independently and non-covalently bound to DNA. Each element consists of a ligand that recognizes specific receptors or other functional groups such as a protein complexed with a cationic group that binds to DNA. Examples of cations which may be used are spermine, spermine derivatives, histone, cationic peptides and/or polylysine; one element is capable of binding both to the DNA vector and to a cell surface receptor on the target cell. Examples of such elements are organic compounds which interact with the asialoglycoprotein receptor, the folate receptor, the mannose-6-phosphate receptor, or the carnitine receptor. A second element is capable of binding both to the DNA vector and to a receptor on the nuclear membrane. The nuclear ligand is capable of recognizing and transporting a transporter system through a nuclear membrane. An example of such ligand is the nuclear targeting sequence from SV40 large T antigen or histone. A third element is capable of binding to both the DNA vector and to elements which induce episomal lysis. Examples include inactivated virus particles such as adenovirus, peptides related to influenza virus hemagglutinin, or the GALA peptide described in the Skoka patent cited above.

[0113] Administration may also involve lipids. The lipids may form liposomes which are hollow spherical vesicles composed of lipids arranged in unilamellar, bilamellar, or multilamellar fashion and an internal aqueous space for entrapping water soluble compounds, such as DNA, ranging in size from 0.05 to several microns in diameter. Lipids may be useful without forming liposomes. Specific examples include the use of cationic lipids and complexes containing DOPE which interact with DNA and with the membrane of the target cell to facilitate entry of DNA into the cell.

[0114] Gene delivery can also be performed by transplanting genetically engineered cells. For example, immature muscle cells called myoblasts may be used to carry genes into the muscle fibers. Myoblast genetically engineered to express recombinant human growth hormone can secrete the growth hormone into the animal's blood. Secretion of the incorporated gene can be sustained over periods up to 3 months.

[0115] Myoblasts eventually differentiate and fuse to existing muscle tissue. Because the cell is incorporated into an existing structure, it is not just tolerated but nurtured. Myoblasts can easily be obtained by taking muscle tissue from an individual who needs plasmid-mediated supplementation and the genetically engineered cells can also be easily put back with out causing damage to the patient's muscle. Similarly, keratinocytes may be used to delivery genes to tissues. Large numbers of keratinocytes can be generated by cultivation of a small biopsy. The cultures can be prepared as stratified sheets and when grafted to humans, generate epidermis which continues to improve in histotypic quality over many years. The keratinocytes are genetically engineered while in culture by transfecting the keratinocytes with the appropriate vector. Although keratinocytes are separated from the circulation by the basement membrane dividing the epidermis from the dermis, human keratinocytes secrete into circulation the protein produced.

[0116] Delivery may also involve the use of viral vectors. For example, an adenoviral vector may be constructed by replacing the E1 and E3 regions of the virus genome with the vector elements described in this invention including promoter, 5'UTR, 3'UTR and nucleic acid cassette and introducing this recombinant genome into 293 cells which will package this gene into an infectious virus particle. Virus from this cell may then be used to infect tissue *ex vivo* or *in vivo* to introduce the vector into tissues leading to expression of the gene in the nucleic acid cassette.

VECTORS

[0117] The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell wherein, in some embodiments, it can be replicated. A nucleic acid sequence can be native to the animal, or it can be "exogenous," which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), linear DNA fragments, and artificial chromosomes (*e.g.*, YACs, BACs), although in a preferred embodiment the vector contains substantially no viral sequences. One of skill in the art would be well equipped to construct a vector through standard recombinant techniques.

[0118] The term "expression vector" refers to any type of genetic construct comprising a nucleic acid coding for a RNA capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of anti-sense molecules or ribozymes. Expression vectors can contain a variety of "control sequences," which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operatively linked coding sequence in a particular host cell. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described *infra.*

PLASMID VECTORS

[0119] In certain embodiments, a linear DNA fragment from a plasmid vector is contemplated for use to transfect a eukaryotic cell, particularly a mammalian cell. In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. In a non-limiting example, *E. coli* is often transformed using derivatives of pBR322 or pUC, a plasmid derived from an *E. coli* species. pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. Other plasmids contain genes for kanamycin or neomycin, or have a non-antibiotic selection mechanism. The pBR plasmid, or other microbial plasmid or phage should also contain, or be modified to contain, for example, promoters which can be used by the microbial organism for expression of its own proteins. A skilled artisan recognizes that any plasmid in the art may be modified for use in the methods of the present invention. In a specific embodiment, for example, a GHRH vector used for the therapeutic applications is synthetically produced and has a kanamycin resistance gene.

[0120] In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, the phage lambda GEM^{TM}-11 may be utilized in making a recombinant phage vector which can be used to transform host cells, such as, for example, *E. coli* LE392.

[0121] Further useful plasmid vectors include pIN vectors (Inouye et al., 1985); and pGEX vectors, for use in generating glutathione S-transferase soluble fusion proteins for later purification and separation or cleavage. Other suitable fusion proteins are those with β-galactosidase, ubiquitin, and the like.

[0122] Bacterial host cells, for example, *E. coli*, comprising the expression vector, are grown in any of a number of suitable media, for example, LB. The expression of the recombinant protein in certain vectors may be induced, as would be understood by those of skill in the art, by contacting a host cell with an agent specific for certain promoters, *e.g.*, by adding IPTG to the media or by switching incubation to a higher temperature. After culturing the bacteria for a further period, generally of between 2 and 24 h, the cells are collected by centrifugation and washed to remove residual media.

PROMOTERS AND ENHANCERS

[0123] A promoter is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription of a gene product are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors, to initiate the specific transcription a nucleic acid sequence. The phrases "operatively positioned," "operatively linked," "under control" and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence.

[0124] A promoter generally comprises a sequence that functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as, for example, the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation. Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. To bring a coding sequence "under the control of" a promoter, one positions the 5′ end of the transcription initiation site of the transcriptional reading frame "downstream" of (*i.e.*, 3′ of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded RNA.

[0125] The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the timidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

[0126] A promoter may be one naturally associated with a nucleic acid sequence, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant, synthetic or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant, synthetic or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other virus, or prokaryotic or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.,* containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. For example, promoters that are most commonly used in recombinant DNA construction include the β-lactamase (penicillinase), lactose and tryptophan (trp) promoter systems. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Patent Nos. 4,683,202 and 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

[0127] Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the organelle, cell type, tissue, organ, or organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression. The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

[0128] Additionally any promoter/enhancer combination (as per, for example, the Eukaryotic Promoter Data Base EPDB, http://www.epd.isb-sib.ch/) could also be used to drive expression. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

[0129] Tables 1 and 2 list non-limiting examples of elements/promoters that may be employed, in the context of the present invention, to regulate the expression of a RNA. Table 2 provides non-limiting examples of inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus.

TABLE 1 Promoter and/or Enhancer	
Promoter/Enhancer	**Relevant References**
β-Actin	(Kawamoto et al., 1988; Kawamoto et al., 1989)
Muscle Creatine Kinase (MCK)	(Horlick and Benfield, 1989; Jaynes et al., 1988)
Metallothionein (MTII)	(Inouye et al., 1994; Narum et al., 2001; Skroch et al., 1993)
Albumin	(Pinkert et al., 1987; Tronche et al., 1989)
β-Globin	(Tronche et al., 1990; Trudel and Costantini, 1987)
Insulin	(German et al., 1995; Ohlsson et al., 1991)
Rat Growth Hormone	(Larsen et al., 1986)
Troponin I (TN I)	(Lin et al., 1991; Yutzey and Konieczny, 1992)
Platelet-Derived Growth Factor	(Pech et al., 1989)
Duchenne Muscular Dystrophy	(Klamut et al., 1990; Klamut et al., 1996)
Cytomegalovirus (CMV)	(Boshart et al., 1985; Dorsch-Hasler et al., 1985)
Synthetic muscle specific promoters	(Draghia-Akli et al., 1999; Draghia-Akli et al., 2002c; Li et al., 1999)

TABLE 2 Element/Inducer	
Element	**Inducer**
MT II	Phorbol Ester (TFA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
β-Interferon	Poly(rI)x / Poly(rc)
Adenovirus 5 E2	ElA
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA)
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α-2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2κb	Interferon
HSP70	ElA, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor α	PMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone

[0130] The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Non-limiting examples of such regions include the human LIMK2 gene (Nomoto et al., 1999), the somatostatin receptor 2 gene (Kraus et al., 1998), murine epididymal retinoic acid-binding gene (Lareyre et al., 1999), human CD4 (Zhao-Emonet et al., 1998), mouse alpha2 (XI) collagen (Liu et al., 2000; Tsumaki et al., 1998), D1A dopamine receptor gene (Lee et al., 1997), insulin-like growth factor II (Dai et al., 2001; Wu et al., 1997), and human platelet endothelial cell adhesion molecule-1 (Almendro et al., 1996).

[0131] In a preferred embodiment, a synthetic muscle promoter is utilized, such as SPc5-12 (Li et al., 1999), which contains a proximal serum response element ("SRE") from skeletal α-actin, multiple MEF-2 sites, MEF-1 sites, and TEF-1 binding sites, and greatly exceeds the transcriptional potencies of natural myogenic promoters. The uniqueness of such a synthetic promoter is a significant improvement over, for instance, issued patents concerning a myogenic promoter and its use (*e.g.* U.S. Pat. No. 5,374,544) or systems for myogenic expression of a nucleic acid sequence (*e.g.* U.S. Pat. No. 5,298,422). In a preferred embodiment, the promoter utilized in the invention does not get shut off or reduced in activity significantly by endogenous cellular machinery or factors. Other elements, including *trans*-acting factor binding sites and enhancers may be used in accordance with this embodiment of the invention. In an alternative embodiment, a natural myogenic promoter is utilized, and a skilled artisan is aware how to obtain such promoter sequences from databases including the National Center for Biotechnology Information ("NCBI") GenBank database or the NCBI PubMed site. A skilled artisan is aware that these databases may be utilized to obtain sequences or relevant literature related to the present invention.

INITIATION SIGNALS AND INTERNAL RIBOSOME BINDING SITES

[0132] A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon should be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

[0133] In certain embodiments of the invention, the use of internal ribosome entry sites ("IRES") elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5′ methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Patent Nos. 5,925,565 and 5,935,819, each herein incorporated by reference).

MULTIPLE CLONING SITES

[0134] Vectors can include a MCS, which is a nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector (see, for example, (Carbonelli et al., 1999; Cocea, 1997; Levenson et al., 1998) incorporated herein by reference.) "Restriction enzyme digestion" refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. "Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

SPLICING SITES

[0135] Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic eukaryotic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression (see, for example, (Chandler et al., 1997).

TERMINATION SIGNALS

[0136] The vectors or constructs of the present invention will generally comprise at least one termination signal. A "termination signal" or "terminator" is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary *in vivo* to achieve desirable message levels.

[0137] In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript so as to expose a polyadenylation site. This signals a specialized endogenous polymerase to add a stretch of about 200 A residues ("polyA") to the 3' end of the transcript. RNA molecules modified with this polyA tail appear to more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that that terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The terminator and/or polyadenylation site elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

[0138] Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the termination sequences of genes, such as for example the bovine growth hormone terminator or viral termination sequences, such as for example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

POLYADENYLATION SIGNALS

[0139] In expression, particularly eukaryotic expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal, skeletal alpha actin 3'UTR or the human or bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

ORIGINS OF REPLICATION

[0140] In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed "ori"), which is a specific nucleic acid sequence at which replication is initiated. Alternatively an autonomously replicating sequence ("ARS") can be employed if the host cell is yeast.

SELECTABLE AND SCREENABLE MARKERS

[0141] In certain embodiments of the invention, cells containing a nucleic acid construct of the present invention may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker, for instance kanamycin.

[0142] Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colorimetric analysis, are also contemplated. Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase ("tk") or chloramphenicol acetyltransferase ("CAT") may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

MUTAGENESIS

[0143] Where employed, mutagenesis was accomplished by a variety of standard, mutagenic procedures. Mutation is the process whereby changes occur in the quantity or structure of an organism. Mutation can involve modification of the nucleotide sequence of a single gene, blocks of genes or whole chromosome. Changes in single genes may be the consequence of point mutations which involve the removal, addition or substitution of a single nucleotide base within a DNA sequence, or they may be the consequence of changes involving the insertion or deletion of large numbers of nucleotides.

[0144] Mutations can arise spontaneously as a result of events such as errors in the fidelity of DNA replication or the movement of transposable genetic elements (transposons) within the genome. They also are induced following exposure to chemical or physical mutagens. Such mutation-inducing agents include ionizing radiations, ultraviolet light and a diverse array of chemical such as alkylating agents and polycyclic aromatic hydrocarbons all of which are capable of interacting either directly or indirectly (generally following some metabolic biotransformations) with nucleic acids. The DNA lesions induced by such environmental agents may lead to modifications of base sequence when the affected DNA is replicated or repaired and thus to a mutation. Mutation also can be site-directed through the use of particular targeting methods.

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SITE-DIRECTED MUTAGENESIS

[0145] Structure-guided site-specific mutagenesis represents a powerful tool for the dissection and engineering of protein-ligand interactions (Wells, 1996, Braisted *et al.*, 1996). The technique provides for the preparation and testing of sequence variants by introducing one or more nucleotide sequence changes into a selected DNA.

[0146] Site-specific mutagenesis uses specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent, unmodified nucleotides. In this way, a primer sequence is provided with sufficient size and complexity to form a stable duplex on both sides of the deletion junction being traversed. A primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

[0147] The technique typically employs a bacteriophage vector that exists in both a single-stranded and double-stranded form. Vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double-stranded plasmids are also routinely employed in site-directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

[0148] In general, one first obtains a single-stranded vector, or melts two strands of a double-stranded vector, which includes within its sequence a DNA sequence encoding the desired protein or genetic element. An oligonucleotide primer bearing the desired mutated sequence, synthetically prepared, is then annealed with the single-stranded DNA preparation, taking into account the degree of mismatch when selecting hybridization conditions. The hybridized product is subjected to DNA polymerizing enzymes such as *E. coli* polymerase I (Klenow fragment) in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed, wherein one strand encodes the original non-mutated sequence, and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate host cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

[0149] Comprehensive information on the functional significance and information content of a given residue of protein can best be obtained by saturation mutagenesis in which all 19 amino acid substitutions are examined. The shortcoming of this approach is that the logistics of multi-residue saturation mutagenesis are daunting (Warren *et al.*, 1996, Brown *et al.*, 1996; Zeng *et al.*, 1996; Burton and Barbas, 1994; Yelton *et al.*, 1995; Jackson *et al.*, 1995; Short *et al.*, 1995; Wong *et al.*, 1996; Hilton *et al.*, 1996). Hundreds, and possibly even thousands, of site specific mutants should be studied. However, improved techniques make production and rapid screening of mutants much more straightforward. See also, U.S. Patents 5,798,208 and 5,830,650, for a description of "walk-through" mutagenesis. Other methods of site-directed mutagenesis are disclosed in U.S. Patents 5,220,007; 5,284,760; 5,354,670; 5,366,878; 5,389,514; 5,635,377; and 5,789,166.

ELECTROPORATION

[0150] In certain embodiments of the present invention, a nucleic acid is introduced into an organelle, a cell, a tissue or an organism *via* electroporation. Electroporation involves the exposure of a suspension of cells and DNA to a high-voltage electric discharge. In some variants of this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells (U.S. Patent No.5,384,253, incorporated herein by reference). Alternatively, recipient cells can be made more susceptible to transformation by mechanical wounding and other methods known in the art.

[0151] Transfection of eukaryotic cells using electroporation has been quite successful. Mouse pre-B lymphocytes have been transfected with human kappa-immunoglobulin genes (Potter et al., 1984), and rat hepatocytes have been transfected with the chloramphenicol acetyltransferase gene (Tur-Kaspa et al., 1986) in this manner.

[0152] The underlying phenomenon of electroporation is believed to be the same in all cases, but the exact mechanism responsible for the observed effects has not been elucidated. Although not wanting to be bound by theory, the overt manifestation of the electroporative effect is that cell membranes become transiently permeable to large molecules, after the cells have been exposed to electric pulses. There are conduits through cell walls, which under normal circumstances maintain a resting transmembrane potential of circa 90 mV by allowing bi-directional ionic migration.

[0153] Although not wanting to be bound by theory, electroporation makes use of the same structures, by forcing a high ionic flux through these structures and opening or enlarging the conduits. In prior art, metallic electrodes are placed in contact with tissues and predetermined voltages, proportional to the distance between the electrodes are imposed on them. The protocols used for electroporation are defined in terms of the resulting field intensities, according to the formula ***E=V/d***, where ("***E***") is the field, ("***V***") is the imposed voltage and ("***d***") is the distance between the electrodes.

[0154] The electric field intensity ***E*** has been a very important value in prior art when formulating electroporation protocols for the delivery of a drug or macromolecule into the cell of the subject. Accordingly, it is possible to calculate any electric field intensity for a variety of protocols by applying a pulse of predetermined voltage that is proportional to the distance between electrodes. However, a caveat is that an electric field can be generated in a tissue with insulated electrodes (i.e. flow of ions is not necessary to create an electric field). Although not wanting to be bound by theory, it is the current that is necessary for successful electroporation not electric field per se.

[0155] During electroporation, the heat produced is the product of the inter-electrode impedance, the square of the current, and the pulse duration. Heat is produced during electroporation in tissues and can be derived as the product of the inter-electrode current, voltage and pulse duration. The protocols currently described for electroporation are defined in terms of the resulting field intensities ***E***, which are dependent on short voltage pulses of unknown current. Accordingly, the resistance or heat generated in a tissue cannot be determined, which leads to varied success with different pulsed voltage electroporation protocols with predetermined voltages. The ability to limit heating of cells across electrodes can increase the effectiveness of any given electroporation voltage pulsing protocol. For example, prior art teaches the utilization of an array of six needle electrodes utilizing a predetermined voltage pulse across opposing electrode pairs. This situation sets up a centralized pattern during an electroporation event in an area where congruent and intersecting overlap points develop. Excessive heating of cells and tissue along electroporation path will kill the cells, and limit the effectiveness of the protocol. However, symmetrically arranged needle electrodes without opposing pairs can produce a decentralized pattern during an electroporation event in an area where no congruent electroporation overlap points can develop.

[0156] Controlling the current flow between electrodes allows one to determine the relative heating of cells. Thus, it is the current that determines the subsequent effectiveness of any given pulsing protocol and not the voltage across the electrodes. Predetermined voltages do not produce predetermined currents, and prior art does not provide a means to determine the exact dosage of current, which limits the usefulness of the technique. Thus, controlling an maintaining the current in the tissue between two electrodes under a threshold will allow one to vary the pulse conditions, reduce cell heating, create less cell death, and incorporate macromolecules into cells more efficiently when compared to predetermined voltage pulses.

[0157] Overcoming the above problem by providing a means to effectively control the dosage of electricity delivered to the cells in the inter-electrode space by precisely controlling the ionic flux that impinges on the conduits in the cell membranes. The precise dosage of electricity to tissues can be calculated as the product of the current level, the pulse length and the number of pulses delivered. Thus, a specific embodiment of the present invention can deliver the electroporative current to a volume of tissue along a plurality of paths without, causing excessive concentration of cumulative current in any one location, thereby avoiding cell death owing to overheating of the tissue.

[0158] Although not wanting to be bound by theory, the nature of the voltage pulse to be generated is determine by the nature of tissue, the size of the selected tissue and distance between electrodes. It is desirable that the voltage pulse be as homogenous as possible and of the correct amplitude. Excessive field strength results in the lysing of cells, whereas a low field strength results in reduced efficacy of electroporation. Some electroporation devices utilize the distance between electrodes to calculate the electric field strength and predetermined voltage pulses for electroporation. This reliance on knowing the distance between electrodes is a limitation to the design of electrodes.

Because the programmable current pulse controller will determine the impedance in a volume of tissue between two electrodes, the distance between electrodes is not a critical factor for determining the appropriate electrical current pulse. Therefore, an alternative embodiment of a needle electrode array design would be one that is non-symmetrical. In addition, one skilled in the art can imagine any number of suitable symmetrical and non-symmetrical needle electrode arrays that do not deviate from the spirit and scope of the invention. The depth of each individual electrode within an array and in the desired tissue could be varied with comparable results. In addition, multiple injection sites for the macromolecules could be added to the needle electrode array.

[0159] One example of an electroporation device that may be used to effectively facilitate the introduction of a macromolecule into cells of a selected tissue of a subject was described in U.S. Patent application 10/657,725 filed on 9/08/2003, titled "Constant Current Electroporation Device And Methods Of Use," with Smith et al., listed as inventors, the entirety of which is hereby incorporated by reference. The electroporation device comprises an electro-kinetic device ("EKD") whose operation is specified by software or firmware. The EKD produces a series of programmable constant-current pulse patterns between electrodes in an array based on user control and input of the pulse parameters and allows the storage and acquisition of current waveform data. The electroporation device also comprises a replaceable electrode disk having an array of needle electrodes, a central injection channel for an injection needle, and a removable guide disk.

RESTRICTION ENZYMES

[0160] In some embodiments of the present invention, a linear DNA fragment is generated by restriction enzyme digestion of a parent DNA molecule. Examples of restriction enzymes are provided below.

Name	Recognition Sequence
AatII	GACGTC
Acc65 I	GGTACC
Acc I	GTMKAC
Aci I	CCGC
Acl I	AACGTT
Afe I	AGCGCT
Afl II	CTTAAG
Afl III	ACRYGT

Age I	ACCGGT
Alu I	AGCT
Alw I	GGATC
AlwN I	CAGNNNCTG
Apa I	GGGCCC
ApaL I	GTGCAC
Apo I	RAATTY
Asc I	GGCGCGCC
Ase I	ATTAAT
Ava I	CYCGRG
Ava II	GGWCC
Avr II	CCTAGG
BamH I	GGATCC
Ban I	GGYRCC
Ban II	GRGCYC
Bbs I	GAAGAC
Bbv I	GCAGC
BbvC I	CCTCAGC
BciV I	GTATCC
Bcl I	TGATCA
Bfa I	CTAG
Bgl I	GCCNNNNNGGC
Bgl II	AGATCT
Blp I	GCTNAGC
Bmr I	ACTGGG
Bpm I	CTGGAG
BsaA I	YACGTR
BsaB I	GATNNNNATC
BsaH I	GRCGYC
Bsa I	GGTCTC
BsaJ I	CCNNGG
BsaW I	WCCGGW
BseR I	GAGGAG
Bsg I	GTGCAG
BsiE I	CGRYCG
BsiHKA I	GWGCWC
BsiW I	CGTACG
BsmA I	GTCTC
BsmB I	CGTCTC
BsmF I	GGGAC
Bsm I	GAATGC
BsoB I	CYCGRG
Bsp1286 I	GDGCHC
BspD I	ATCGAT
BspE I	TCCGGA
BspH I	TCATGA
BspM I	ACCTGC
BsrB I	CCGCTC
BsrD I	GCAATG
BsrF I	RCCGGY
BsrG I	TGTACA
Bsr I	ACTGG
BssH II	GCGCGC
BssK I	CCNGG
Bst4C I	ACNGT
BssS I	CACGAG
BstB I	TTCGAA
BstE II	GGTNACC
BstF5 I	GGATGNN
BstN I	CCWGG
BstU I	CGCG
BstY I	RGATCY
BstZ17 I	GTATAC
Bsu36 I	CCTNAGG
Btg I	CCPuPyGG
Btr I	CACGTG

Cac8 I	GCNNGC
Cla I	ATCGAT
Dde I	CTNAG
Dpn I	GATC
Dpn II	GATC
Dra I	TTTAAA
Eae I	YGGCCR
Eag I	CGGCCG
Ear I	CTCTTC
Eci I	GGCGGA
EcoO109 I	RGGNCCY
EcoR I	GAATTC
EcoR V	GATATC
Fau I	CCCGCNNNN
Fnu4H I	GCNGC
Fok I	GGATG
Fse I	GGCCGGCC
Fsp I	TGCGCA
Hae II	RGCGCY
Hae III	GGCC
Hga I	GACGC
Hha I	GCGC
Hinc II	GTYRAC
Hind III	AAGCTT
Hinf I	GANTC
HinP1 I	GCGC
Hpa I	GTTAAC
Hpa II	CCGG
Hph I	GGTGA
Kas I	GGCGCC
Kpn I	GGTACC
Mbo I	GATC
Mbo II	GAAGA
Mfe I	CAATTG
Mlu I	ACGCGT
Mnl I	CCTC
Msc I	TGGCCA
Mse I	TTAA
MspA1 I	CMGCKG
Msp I	CCGG
Nae I	GCCGGC
Nar I	GGCGCC
Nci I	CCSGG
Nco I	CCATGG
Nde I	CATATG
NgoMI V	GCCGGC
Nhe I	GCTAGC
Nla III	CATG
Nla IV	GGNNCC
Not I	GCGGCCGC
Nru I	TCGCGA
Nsi I	ATGCAT
Nsp I	RCATGY
Pac I	TTAATTAA
PaeR7 I	CTCGAG
Pci I	ACATGT
PleI	GAGTC
Pme I	GTTTAAAC
Pml I	CACGTG
PpuM I	RGGWCCY
Psi I	TTATAA
PspG I	CCWGG
PspOM I	GGGCCC
Pst I	CTGCAG
Pvu I	CGATCG
Pvu II	CAGCTG

Rsa I	GTAC
Rsr II	CGGWCCG
Sac I	GAGCTC
Sac II	CCGCGG
Sal I	GTCGAC
Sap I	GCTCTTC
Sau3A I	GATC
Sau96 I	GGNCC
Sbf I	CCTGCAGG
Sca I	AGTACT
ScrF I	CCNGG
SexA I	ACCWGGT
SfaN I	GCATC
Sfc I	CTRYAG
Sfo I	GGCGCC
SgrA I	CRCCGGYG
Sma I	CCCGGG
Sml I	CTYRAG
SnaB I	TACGTA
Spe I	ACTAGT
Sph I	GCATGC
Ssp I	AATATT
Stu I	AGGCCT
Sty I	CCWWGG
Swa I	ATTTAAAT
Taq I	TCGA
Tfi I	GAWTC
Tli I	CTCGAG
Tse I	GCWGC
Tsp45 I	GTSAC
Tsp509 I	AATT
TspR I	CAGTG
Tth111 I	GACNNNGTC
Xba I	TCTAGA
Xho I	CTCGAG
Xma I	CCCGGG
Xmn I	GAANNNNTTC

[0161] The term "restriction enzyme digestion" of DNA as used herein refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction endonucleases, and the sites for which each is specific is called a restriction site. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as established by the enzyme suppliers are used. Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1μg of plasmid or DNA fragment is used with about 1-2 units of enzyme in about 20μl of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Restriction enzymes are used to ensure plasmid integrity and correctness.

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EXAMPLES

[0162] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

CONSTRUCTION OF DNA VECTORS AND METHODS IN ANIMAL SUBJECT

[0163] **DNA constructs:** In order to enhance the vaccination response and to improve the clinical outcome of subjects after an infectious challenge it was first necessary to design several GHRH constructs. Briefly, the plasmid vectors contained the muscle specific synthetic promoter SPc5-12 (SEQID#15)(Li et al., 1999) attached to a wild type species-specific or analog GHRH. Some wild-type GHRH sequences were cloned in our laboratory (dog, cat and horse); others (chicken, ovine, bovine, porcine, human) were synthesized according to the specialized literature. The analog GHRH sequences were generated by site directed mutagenesis as described (Draghia-Akli et al., 1999). Briefly, mammalian GHRH analog cDNA's were generated by site directed mutagenesis of GHRH cDNA (SEQID#18) (Altered Sites II *in vitro* Mutagenesis System, Promega, Madison, WI), and cloned into the BamHI/ Hind III sites of pSPc5-12, to generate the specific GHRH construct. The 3' untranslated region (3'UTR) of growth hormone was cloned downstream of GHRH cDNA. The resultant plasmids contained mammalian analog coding region for GHRH, and the resultant amino acid sequences were not naturally present in mammals. Although not wanting to be bound by theory, the enhancement of the vaccination response and the improvement of the clinical outcome of subjects after an infectious challenge are determined ultimately by the circulating levels of GHRH hormones. Several different plasmids encoded different mutated or wild type amino acid sequences of GHRH or functional biological equivalents thereof, for example:

Plasmid	**Encoded Amino Acid Sequence**

HV-GHRH(SEQID#1):

HVDAIFTNSYRKVL**A**QLSARKLLQDI**LN**RQQGE**R**N**Q**E**Q**GA-OH

Pig-GHRH(SEQID#2):

YADAIFTNSYRKVL**G**QLSARKLLQDI**MS**RQQGE**R**N**Q**E**Q**GA-OH

Bovine-GHRH (SEQID#3):

YADAIFTNSYRKVL**G**QLSARKLLQDI**MN**RQQGERN**Q**E**Q**GA-OH

Dog-GHRH (SEQID#4):

YADAIFTNSYRKVL**G**QLSARKLLQDI**MS**RQQGE**R**N**R**E**Q**GA-OH

Cat-GHRH (SEQID#5):

YADAIFTNSYRKVL**G**QLSARKLLQDI**MS**RQQGE**R**N**Q**E**Q**GA-OH

TI-GHRH(SEQID#6):

YIDAIFTNSYRKVL**A**QLSARKLLQDI**LN**RQQGE**R**N**Q**E**Q**GA-OH

Ovine-GHRH (SEQID#7):

YADAIFTNSYRKILGQLSARKLLQDIMNRQQGERNQEQGA-OH

Chicken-GHRH (SEQID#8):

HADGIFSKAYRKLLGQLSARNYLHSLMAKRVGSGLGDEAEPLS-OH

Horse-GHRH (partial)(SEQID#9):

-ADAIFTNNYRKVLGQLSARKILQDIMSR-----------OH

Human-GHRH(SEQID#10):

YADAIFTNSYRKVL**G**QLSARKLLQDI**MS**RQQGE**S**N**Q**E**R**GA-OH

TV-GHRH(SEQID#11):

YVDAIFTNSYRKVL**A**QLSARKLLQDI**LN**RQQGE**R**N**Q**E**Q**GA-OH

TA-15/27/28-**GHRH(SEQID#12):**

YADAIFTNSYRKVL**A**QLSARKLLQDI**LN**RQQGE**R**N**Q**E**Q**GA-OH

[0164] In general, the encoded GHRH or functional biological equivalent thereof is of formula:

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-X_1-X_2-DAIFTNSYRKVL-X_3-QLSARKLLQDI-X_4-X_5-RQQGE-X_6-N-X_7-E-X_8-GA-OH

(SEQID#14)

[0165] wherein: $\mathbf{X_1}$ is a D-or L-isomer of an amino acid selected from the group consisting of tyrosine ("Y"), or histidine ("H"); $\mathbf{X_2}$ is a D-or L-isomer of an amino acid selected from the group consisting of alanine ("A"), valine ("V"), or isoleucine ("I"); $\mathbf{X_3}$ is a D-or L-isomer of an amino acid selected from the group consisting of alanine ("A") or glycine ("G"); $\mathbf{X_4}$ is a D-or L-isomer of an amino acid selected from the group consisting of methionine ("M"), or leucine ("L"); $\mathbf{X_5}$ is a D-or L-isomer of an amino acid selected from the group consisting of serine ("S") or asparagines ("N"); $\mathbf{X_6}$ is a D- or L-isomer of an amino acid selected from the group consisting of arginine ("R"), or serine ("S"); $\mathbf{X_7}$ is a D- or L-isomer of an amino acid selected from the group consisting of arginine ("R"), or glutamine ("Q"); and $\mathbf{X_8}$ is a D- or L-isomer of an amino acid selected from the group consisting of arginine ("R"), or glutamine ("Q").

[0166] The plasmids described above <u>do not</u> contain polylinker, IGF-I gene, a skeletal alpha-actin promoter or a skeletal alpha actin 3' UTR /NCR. Furthermore, these plasmids were introduced by muscle injection, followed by *in vivo* electroporation, as described below.

[0167] In terms of "functional biological equivalents", it is well understood by the skilled artisan that, inherent in the definition of a "biologically functional equivalent" protein and/or polynucleotide, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule while retaining a molecule with an acceptable level of equivalent biological activity. Functional biological equivalents are thus defined herein as those proteins (and poly-nucleotides) in selected amino acids (or codons) may be substituted. A peptide comprising a functional biological equivalent of GHRH is a polypeptide that has been engineered to contain distinct amino acid sequences while simultaneously having similar or improved biologically activity when compared to GHRH. For example one biological activity of GHRH is to facilitate GH secretion in the subject.

[0168] **Optimized Plasmid Backbone.** One aspect of the current invention is the optimized plasmid backbone. The synthetic plasmids presented below contain eukaryotic sequences that are synthetically optimized for species specific mammalian transcription. An existing pSP-HV-GHRH plasmid ("pAV0125") (SEQID#22), was synthetically optimized to form a new plasmid (SEQID#25). The plasmid pAV0125 was described in U.S. Patent 6,551,996 titled "Super-active porcine growth hormone releasing hormone analog," issued on April 22, 2003 with Schwartz, et al., listed as inventors ("the Schwartz '996 Patent"), which teaches application of a GHRH analog containing mutations that improve the ability to elicit the release of growth hormone. This 3,534 bp plasmid pAV0125 (SEQID #22) contains a plasmid backbone with various component from different commercially available plasmids, for example, a synthetic promoter SPc5-12 (SEQID#15), a modified porcine GHRH sequence (SEQID#18), and a 3'end of human growth hormone (SEQID#38). Other specific examples of optimized synthetic plasmids include an optimized wt-porcine GHRH plasmid, pAV0225 (SEQID#26) Figure 8; dog GHRH plasmid, pAV0235 (SEQID#27) Figure 9; bovine GHRH plasmid, pAV0236 (SEQID#28) Figure 10; cat GHRH plasmid, pAV0238 (SEQID#29) Figure 11; a TI-GHRH plasmid, pAV0239 (SEQID#30) Figure 12; ovine GHRH plasmid, pAV0240 (SEQID#31) Figure 13; chicken GHRH plasmid, pAV0241 (SEQID#32) Figure 14; horse GHRH plasmid, pAV0249 (SEQID#33) Figure 15; human GHRH plasmid (SEQID#34). The therapeutic encoded gene for such optimized plasmids may also include optimized nucleic acid sequences that encode modified GHRH molecules or functional biological equivalents thereof.

EXAMPLE 2

PLASMID INJECTION AND ELECTROPORATION CAN BE USED TO DELIVER ANTIGENS TO LARGE ANIMALS

[0169] Young hybrid pigs of mixed gender, 3 to 6 weeks of age, with weights between 15 and 40 kg, were used (n = 6 to 7/group/experiment). Animals were group housed in pens with *ad libitum* access to 24% protein diet (Producers Cooperative Association, Bryan, TX) and water. Plasmid was obtained using a commercially available kit (Qiagen Inc., Chatsworth, CA, USA). Endotoxin levels were at less than 0.01 EU/μg, as measured by Kinetic Chromagenic LAL (Endosafe, Charleston, SC). Plasmid preparations were diluted in sterile water and formulated 1% weight/weight with poly-L-glutamate sodium salt (MW=10.5 kDa average) (Sigma, St. Louis, MO). On Day 0 of the experiment, the animals were manually restrained and a secreted alkaline phosphatase (SEAP) expressing plasmid solution was directly injected through the intact skin into the semimembranosus muscle using a 21-gauge needle, in the center of the 5 pin electrode array. All major surface blood vessels were avoided when finding an appropriate injection site. At the pre-determined time interval after plasmid injection, in our case 80 milliseconds, electroporation was initiated: 5 pulses of 52 milliseconds in length, 0.4-1 Amp electric field intensity, 1 second between pulses. Animals were maintained in accordance with NIH, USDA and Animal Welfare Act guidelines.

[0170] *Blood collection:* On days 0, 3, 7, and 10 of each experiment, animals were weighed at 8:30 AM and blood was collected by jugular vein puncture into MICROTAINER serum separator tubes. Blood was allowed to clot for 10 to 15 min at room temperature and subsequently centrifuged at 3000 *x g* for 10 min and the serum stored at -80°C until further analysis.

[0171] *Secreted embryonic alkaline phosphatase assay:* Serum samples were thawed and 50μL was assayed for SEAP activity using the Phospha-Light Chemiluminescent Reporter Assay Kit (Applied Biosystems, Bedford, MA), per manufacturer instructions. The lower limit of detection for the assay is 3pg/mL. More concentrated serum samples were diluted 1:10 in mouse serum before assaying for SEAP activity. All samples were read using LUMIstar Galaxy luminometer (BMG Labtechnologies, Offenburg, Germany).

[0172] The SEAP protein is immunogenic in pigs, and the immune-mediated clearance of the protein does occur within 10 to 14 days after plasmid delivery (Figure 1) when direct muscle injection followed by electroporation is used. Thus, the levels of SEAP expression can be studied only over a 2-week period and interpreted as a reliable measure of gene expression following intramuscular plasmid transfer. Nevertheless, the technique could be used to deliver other types of molecules that specifically enhance the immune function in a subject. As a measurable vaccination response usually occurs approximately 14 days after the vaccination, it is reasonable that a treatment with an immuno-modulator, as GHRH, could occur before the vaccination, or concomitant with the vaccination procedure.

EXAMPLE 3

GHRH ADMINISTRATION IN COWS

[0173] Thirty-two primiparous Holstein cows, 18 to 20 months of age, with an average weight of 547 ± 43kg, were treated with 2.5mg pSP-HV-GHRH once during the last trimester of gestation and designated as the treated group. Similarly, 20 pregnant heifers from the same source and of the same breed and age did not receive plasmid treatment and served as controls. Animals calved at age 23 months ± 24 days. Cows were housed in a free stall barn fitted with fans equipped with water misters for evaporative cooling and exposed to natural daylight. The herd was fed a silage-based total mixed ration *ad libitum* twice daily. Each cow was fitted with a transponder/pedometer that allowed for automatic identification upon entering the stall. At the conclusion of this experiment, all animals treated with plasmid were disposed of in such a manner that their tissues did not enter the food chain. All milk and tissues produced by treated animals were destroyed and did not enter the human food chain. Animal protocols were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

[0174] ***Intramuscular injection of plasmid DNA.*** The endotoxin-free plasmid (Qiagen Inc., Chatsworth, CA, USA) preparation of pSPc5-12-HV-GHRH was diluted in water to 5mg/mL and formulated with poly-L-glutamate 1% wt/wt. Cows were given a total quantity of 2.5mg pSP-HV-GHRH intramuscularly in the trapezius muscle using a 21G needle (Becton-Dickinson, Franklin Lacks, NJ). Two min after injection, the injected muscle was electroporated using Advisys's EKD device, and using the following conditions: 5 pulses, 1 Amp, 52 milliseconds/pulse, as described (Draghia-Akli et al., 2002b). The voltage changes with the change in resistance of the tissue during the electroporation (to maintain constant current), and it has been recorded to be between 80 and 120 V/cm. For all injections, 2cm needles were inserted through the skin into the muscle. Animals were observed immediately after injection and 24 h later for any adverse effects at the electroporation site.

[0175] ***Weight, body condition and hoof scores.*** Before treatment, heifers were weighed on the same calibrated scale (Priefert cattle squeeze-chute connected to a Weigh Tronix 915A indicator and WP233 printer, Central City Scale, Central City, NE) and randomly assigned to groups. Two independent dairy animal scientists (Texas A&M University) that were blinded to the treatment groups assessed body condition scores prior to treatment, between 60 and 80 DIM and between 100 and 120 DIM. Cows were scored by both observing and handling the backbone, loin, and rump areas (Rodenburg, 1996), with possible BCS ranging from 1 (very thin cow) to 5 (a severely over-conditioned cow). Hoof scores were measured prior to plasmid-GHRH treatment and at 60 DIM. Hoof scores included a 0 (no hoof problem) to 4 (severe hoof problem) evaluation of each foot. Each hoof was assigned an additional 2 points for abscesses eventually present (1 point), and the necessary treatments at any given time (1 point). Possible hoof scores ranged from 0 (no problem) to 24 (severe hoof problems at all 4 feet, abscesses, and intense treatment needed for each hoof).

[0176] ***Complete blood counts and immune markers.*** Whole blood from all heifers was collected in EDTA and submitted for complete blood count analysis (Texas Veterinary Medical Diagnostic Laboratory, College Station, TX) prior to treatment, and at 18 and 300 days post-treatment, and after a vaccination challenge. Hematology parameters included: erythrocyte counts, hematocrit, hemoglobin, total leukocyte count, and differential leukocyte counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), platelet count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and fibrinogen.

[0177] Immune markers were assayed on all treated cattle and controls at day 0 and 18 and on 20 treated and 10 control cows at 300 days post-treatment and after the Biocor-9 vaccination challenge. Analysis was performed by flow cytometry (FC) using monoclonal antibodies (mAb) developed in Dr. Davis's laboratory (Department of Veterinary Microbiology/ Pathology, CVM, Washington State University, Pullman, Washington). Combinations of mAbs were used in 3-color FC to determine the composition and frequency of different populations of peripheral blood mononuclear cells (PBMC) in peripheral blood and the functional status of CD4 and CD8 alpha beta ($\alpha\beta$) T cells and gamma delta ($\gamma\delta$) T cells. Two mAbs of the same specificity have been used in some combinations to increase the intensity of the fluorescent signal on CD4 and CD8 T cells. Ten mL of blood was obtained at the times indicated and processed for FC (Davis et al., 1995). The blood was lysed in Tris buffered NH_4Cl, washed in phosphate buffered saline containing acid citrate dextrose (PBS/ACD), and then distributed in 96 well conical bottom tissue culture plates containing the different combinations of mAbs. The cells were incubated for 15 min on ice, washed 3X in PBS/ACD and incubated another 15 min with different combinations of isotype specific goat anti-mouse antibodies conjugated with fluorescein, phycoerythrin, or phycoerythrin-Cy5 (Southern Biotechnology Associates, Birmingham, AL, Caltag Laboratories, Burlingame, CA). The cells were then washed 2X and fixed in PBS buffered 2% formaldehyde. The cells were kept in the refrigerator until examined. The cells were examined on a Becton Dickinson FACSort equipped with Cell Quest software. Data were analyzed on Flow Jo (Tree Star, Inc., San Carlos, CA) and FCS Express (De Novo software, Thornton, Ontario, Ca) software. Unless otherwise stated, data are presented as a percentage of the total population assayed with a particular monoclonal antibody or combination thereof (e.g. total $CD4^+$ and CD4- cells represent 100% of cells assayed; total CD2-CD3-, CD2-CD3+, CD2+CD3- or CD2+CD3+ represent 100% of cells assayed with both CD2 and CD3 antibody, etc.).

[0178] ***Biochemistry and insulin measurements.*** Serum samples were collected at 60 and 100 DIM. Serum was aliquoted for RIA and biochemical analysis and stored at -80°C prior to analysis. Biochemical analysis occurred within 48 h after serum collection (Texas Veterinary Medical Diagnostic Laboratory, College Station, TX). Serum biochemical endpoints included alanine aminotransferase, gamma glutamyltransferase, creatine phosphokinase, total bilirubin, total protein, albumin, globulin, blood urea nitrogen, creatinine, phosphorus, calcium, and glucose. Insulin and IGF-I assays were performed within 90 days after serum collection. Samples were analyzed for glucose and insulin levels by an independent laboratory (Texas Veterinary Medical Diagnostic Laboratory, College Station, TX). All samples were analyzed in the same assay. The assay variability was 3.6% for the insulin assay and 4.4% for the glucose assay. Total proteins were measured using a Bio-Rad protein assay kit on the serum samples (Bio-Rad Laboratories, Hercules, CA).

[0179] ***Radioimmunoassay for IGF-I.*** Serum IGF-I was measured using a heterologous human immunoradiometric assay kit following the manufacturer's protocol (Diagnostic System Labs, Webster, TX). The kit employs an extraction step to remove binding protein interference. All samples were run in the same assay. The intra-assay variability was 4%. Cross-reactivity of human IGF-I antibody for bovine IGF-I is 100%.

[0180] ***Surround™ 9 vaccination***: All animals enrolled in the study (GHRH-treated and controls) were vaccinated at 300 days after the study initiation, using a 9-way vaccine called Surround™ 9 (Biocor). Surround™ 9 is a multivalent vaccine containing inactivated, adjuvanted, highly antigenic strains of IBR (bovine herpesvirus-1), BVD (bovine virus diarrhea), parainfluenza 3, respiratory syncytial virus (as killed virus), and Leptospira canicola, Leptospira grippotyphosa, Leptospira hardjo, Leptospira icterohaemorrhagiae and Leptospira Pomona bacterins. All fractions are inactivated and adjuvanted to maintain maximum virus antigenicity. The Leptospira serovars are produced in media designed to assure maximum growth and antigenicity. This vaccination addressed a large array of pathogenic conditions in cows, from diarrhea, and pathogens involved in the etiology of mastitis, to respiratory disease. The vaccine is administered subcutaneously or intramuscularly in one injection of a 5 mL solution.

[0181] ***Statistical analysis.*** Data consisted of repeated measures in different time points with unequal allocation of experimental units to treatment groups (treated n = 32, controls n = 20). Additional comparisons were performed when a significant ($P <$ 0.05) treatment-x-day interaction was detected. A mixed model using SAS (analysis of simple main effects) was used to examine if there were any significant differences among the groups of each variable at different time points. Categorical data, such as morbidity and mortality, culling rate and hoof problems, were analyzed by ANOVA. Data were coded with numerical values such that ANOVA could be performed. The total hoof score for each animal was used in the analysis of this parameter. For mortality rates, an equivalent scoring system: alive = 1, dead = 0 was used. Serum IGF-I was analyzed by ANOVA for repeated measures. Values compared with Students t-test, ANOVA or linear regression are presented in the results, with $P < 0.05$ taken as the level of statistical significance.

EXAMPLE 4

CLINICAL RESPONSE OF COWS TREATED WITH GHRH PLASMID THERAPY AND VACCINATED WITH A MULTIVALENT VACCINE

[0182] **Biochemistry and CBC values:** The total white blood cell counts were similar between groups. Nevertheless, the percentage of circulating lymphocytes at 300 days was increased in GHRH-treated animals (47.4 ± 3.3% vs. controls 37.8 ± 5.3%, $P <$ 0.06). A physiological increase in hemoglobin (11.55 ± 0.15g/dL in GHRH-treated vs. 10.9 ± 0.15g/dL in controls, $P < 0.02$) and red blood cells (7.65 ± 0.1 millions/mL vs. 7.3 ± 0.2 millions/mL, $P < 0.07$) was also observed at this time point. No differences were found between the groups in other CBC or serum biochemistry panels at any time point tested. These were within the normal range of values for cattle. Glucose and insulin levels were not different between groups (Figure 2).

[0183] **Immune markers:** The total number of white blood cells, differentials, and flow cytometric (FC) profiles were similar between groups at day 0. At 18 days post-treatment, $CD2^+$ values were increased in treated animals by 14%, but there was no change in controls when compared to baseline values: 43.4 ± 1.7% vs. 37.9 ± 1.4%, $P <$ 0.004 in GHRH-treated cattle, and 37.3 ± 2.1% vs. 38.8 ± 1.8% in controls, (Figure 3A). The $CD4^+/CD8^+$ ratio increased in treated animals (day 18 – day 0 = 8 ± 0.6%, $P < 0.04$), mostly due to increase in $CD4^+$ cells (29.1 ± 0.7% at day 18 vs. 24.5 ± 0.8% at day 0). During the same period $CD4^+CD45R^+$ naïve lymphocytes increased by 53% with the GHRH treatment: day 18, 11.1 ± 0.4% vs. day 0, 7.4 ± 0.4% in GHRH-treated animals, P < 0.016, and day 18, 8 ± 0.7% vs. day 0, 7.2 ± 0.8% in control animals (Figure 3B). $CD25^+CD4^+$ cells were also significantly increased with treatment: day 18, 4.3 ± 0.3% vs. day 0, 1 ± 0.1 in GHRH-treated animals $P < 0.001$ and day 18, 3.8 ± 0.2% vs. day 0, 1.7 ± 0.2 in controls.

[0184] At 300 days post-treatment, when a more comprehensive panel was performed, the $CD45R^+$/CD45R0- naïve lymphocytes were significantly more numerous in treated animals 0.98 ± 0.08 than in controls 0.91 ± 0.08, $P < 0.05$ (Figure 3C). $CD2^{+}CD3^{+}\gamma\delta$- cells were more numerous with treatment: 68.5 ± 1.4% of all $CD2^{+}$ cells vs. 60 ± 5.6% in controls, $P < 0.02$.

[0185] Two weeks after the Surround™ 9-way vaccination (Biocor), when a more comprehensive panel was performed, the $CD4^{+}/CD8^{+}$ ratio was significantly increased in GHRH-treated animals, while it was decreased in controls: 0.23 ± 0.07 in GHRH-treated animals, versus – 0.09 ± 0.01 in controls, $P < 0.05$ (Figure 4).

[0186] Two weeks after the Surround 9-way vaccination, when the more comprehensive panel was performed, the $CD25^{-}CD4^{+}CD45R0$ naïve T-lymphocytes were significantly increased in GHRH-treated animals compared to controls, $P < 0.05$ (Figure 5).

[0187] **Mortality in treated animals:** The mortality of the heifers (involuntary cull rate) was different between GHRH-treated animals and controls. During the 360-day study, none of the treated heifers died, while 20% of control heifers had to be culled ($P <$ 0.003). The causes of death were the following: one Johne's disease, one systemic infection from hoof conditions and an infected cut, one animal with severe hoof problems complicated by rear leg paralysis, and one severe mastitis case. One treated animal was culled due to an accident. The overall involuntary cull rate prior to 120 days in milk production (DIM) was decreased by 40% with the treatment. The protection after vaccination against environmental pathogens was increased by the GHRH-plasmid mediated treatment.

[0188] **Body weights and body condition score:** Body condition scores (BCS) of heifers differed between groups at the time of stress and negative energy balance, at 60 to 80 DIM. Heifers treated with pSP-HV-GHRH showed an improvement ($P < 0.0001$, Figure 6) in BCS between 60 and 80 DIM. During the first 100 DIM, treated animals lost an average of 3.5kg (0.06% of total body weight) ($P < 0.02$, Figure 7) while control cows lost on average 26.4kg (4.6% of body weight at 60 DIM). The better BCS correlated with an increase in the serum IGF-I levels: day 100 – day 60 = 22.4 ± 4ng/mL for GHRH-treated heifers (119.7 ± 6.9 ng/mL at day 100 vs. 97.3 ± 6.6 ng/mL at day 60) vs. 8 ± 7.4ng/mL for controls (99.8 ± 3.9 ng/mL at day 100 vs. 91.8 ± 6.8 ng/mL at day 60) ($P < 0.04$).

[0189] **Morbidity:** The herd had significant hoof pathology at the beginning of the study prior to plasmid administration. Foot problems, most probably of bacterial origin (Murray et al., 1996), were also one of the principal causes of morbidity in these animals throughout the study. The digital dermatitis lesions that constituted the major hoof pathology were attenuated by the GHRH treatment. Studies have shown that as much as 29% of dairy cattle and 4% of beef cattle have gross lesions of digital dermatitis and that spirochetes are involved in more than 60% of the cases (Brown et al., 2000). The immune response to the spirochetes is of short duration (Trott et al., 2003), thus, to diminish the infection burden, a stable long-term therapy would be preferable. The combination of vaccination and GHRH treatment proved to be beneficial for the treated animals. The proportion of animals that had worsening foot problems throughout the course of the study was 40% higher for controls when compared to the treated animals: 7 out of 32 GHRH-treated animals versus 7 out of 20 controls. The overall hoof score improvement did not reach statistical significance ($P < 0.4$) due to high inter-animal variability in the control group.

[0190] In contrast to injections with porcine recombinant somatotropin (rpST) or bST, which can produce unwanted side effects (e.g. hemorrhagic ulcers, vacuolations of liver and kidney or even death of the animals (Smith et al., 1991)), the plasmid mediated GHRH gene supplementation is well tolerated having no observed side effects in the animals. Regulated tissue/fiber-type-specific hGH-containing plasmids have been used previously for the delivery and stable production of GH in livestock and GH-deficient hosts. The methods used to deliver the hGH-containing plasmids comprise transgenesis, myoblast transfer or liposome-mediated intravenous injection (Barr and Leiden, 1991; Dahler et al., 1994; Pursel et al., 1990). Nevertheless, these techniques have significant disadvantages that preclude them from being used in a large-scale operation and/or on food animals, including: 1) possible toxicity or immune response associated with liposome delivery; 2) need for extensive *ex vivo* manipulation in the transfected myoblast approach; and/or 3) risk of important side effects or inefficiency in transgenesis (Dhawan et al., 1991; Miller et al., 1989). Compared to these techniques, plasmid mediated gene supplementation and DNA injection is simple and effective, with no complication related to the delivery system or to excess expression.

[0191] The embodiments provided herein illustrate that enhanced vaccination response and improved clinical outcome results in mammals injected with a GHRH plasmid. Treated subjects display a significant improvement the subject's capability to respond to an infectious challenge. Treated subjects did not experience any side effects from the therapy, including associated pathology or death. Although not wanting to be bound by theory, the enhancement in the vaccination response indicates that ectopic expression of myogenic GHRH vectors will likely stimulate the GH axis in a more physiologically appropriate manner.

[0192] One skilled in the art readily appreciates that this invention is well adapted to carry out the objectives and obtain the ends and advantages mentioned as well as those inherent therein. Growth hormone, growth hormone releasing hormone, analogs, plasmids, vectors, pharmaceutical compositions, treatments, methods, procedures and techniques described herein are presently representative of the preferred embodiments and are intended to be exemplary and are not intended as limitations of the scope. Additionally, vaccines comprising bovine herpesvirus-1 ("IBR"), bovine virus diarrhea ("BVD"), parainfluenza 3, respiratory syncytial virus, Leptospira canicola, Leptospira grippotyphosa, Leptospira hardjo, Leptospira icterohaemorrhagiae, Leptospira Pomona bacterinsmycoplasma hyopneumonia, mycoplasma hyopneumonia, or combinations thereof are intended to be exemplary and are not intended as limitations of the scope. Thus, changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the pending claims.

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WHAT IS CLAIMED IS:

1) A method of preparing a subject in need of a vaccination comprising:
delivering into a tissue of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH"), wherein, GHRH is expressed *in vivo* in the subject.

2) The method of claim 1, wherein delivering the nucleic acid expression construct occurs up to about 1 year before the subject is vaccinated.

3) The method of claim 2, wherein delivering the nucleic acid expression construct occurs about 0 to about 14 days before the subject is vaccinated.

4) The method of claim 1, wherein delivering into the tissue of the subject the nucleic acid expression construct comprises tissue electroporation.

5) The method of claim 4, wherein tissue electroporation comprises:
(a) penetrating the tissue in the subject with a plurality of needle electrodes, wherein the plurality of needle electrodes are arranged in a spaced relationship and the tissue of the subject comprise muscle cells;
(b) introducing the nucleic acid expression construct into the tissue between the plurality of needle electrodes in an amount in a range of about 0.01 – 5 mg; and
(c) applying an electrical pulse to the plurality of needle electrodes, wherein the electrical pulse allows the nucleic acid expression construct to traverse a muscle cell membrane.

6) The method of claim 5, wherein the nucleic acid expression construct further comprises, a transfection-facilitating polypeptide, or a charged polypeptide.

7) The method of claim 6, wherein the transfection-facilitating polypeptide, or charged polypeptide comprises poly-L-glutamate.

8) The method of claim 1, wherein the nucleic acid expression construct comprises a sequence that encodes a polypeptide having an amino acid sequence of which is at least 90% identical to the encoded GHRH of SEQID#14.

9) The method of claim 1, wherein the nucleic acid expression construct comprising a sequence that is at least 97% identical to mouse pAV0202 (SEQID#23); rat pAV0203 (SEQID#24); HV-GHRH pAV0224 (SEQID#25); pig-wt-GHRH pAV0225 (SEQID#26); dog pAV0235 (SEQID#27); bovine pAV0236 (SEQID#28); cat pAV0238 (SEQID#29); TI-GHRH pAV0239 (SEQID#30); ovine pAV0240 (SEQID#31); chicken pAV0241 (SEQID#32); horse pAV0249 (SEQID#33), or human pAV0226 (SEQID#34).

10) The method of claim 1, wherein the vaccination comprises at least part of a microorganism, an infectious agent, or a toxoid.

11) The method of claim 10, wherein the microorganism comprises: a virus, a bacteria, a mycoplasma, or a parasite.

12) The method of claim 10, wherein the microorganism comprises a bovine herpesvirus-1 ("IBR"), bovine virus diarrhea ("BVD"), parainfluenza 3, respiratory syncytial virus, Leptospira canicola, Leptospira grippotyphosa, Leptospira hardjo, Leptospira icterohaemorrhagiae, Leptospira Pomona bacterinsmycoplasma hyopneumonia, mycoplasma hyopneumonia, or combination thereof.

13) The method of claim 1, wherein the subject comprises a human, a ruminant animal, a food animal, or a work animal.

14) A method for vaccinating a subject comprising:

(a) delivering into a tissue of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH"); and

(b) providing a vaccine to the subject in an amount effective to induce anti-vaccine antibodies in the subject;

wherein, GHRH is expressed *in vivo* in the subject and a vaccination response is enhanced when compared to a control subject not having a GHRH expression construct delivered.

15) The method of claim 14, wherein delivering the nucleic acid expression construct occurs concomitantly with providing the vaccine to the subject.

16) The method of claim 14, further comprising: waiting a period of time after delivering the nucleic acid expression construct encoding GHRH into the subject, but before providing the vaccine.

17) The method of claim 16 wherein the period of time is up to about 1 year.

18) The method of claim 17 wherein the period of time is in the range of about 7 days to about 14 days.

19) The method of claim 14, wherein delivering into the tissue of the subject the nucleic acid expression construct comprises tissue electroporation.

20) The method of claim 19, wherein tissue electroporation comprising:
penetrating the tissue in the subject with a plurality of needle electrodes, wherein the plurality of needle electrodes are arranged in a spaced relationship and the tissue of the subject comprise muscle cells;

introducing the nucleic acid expression construct into the tissue between the plurality of needle electrodes in an amount in a range of about 0.01 – 5 mg; and

applying an electrical pulse to the plurality of needle electrodes, wherein the electrical pulse allows the nucleic acid expression construct to traverse a muscle cell membrane.

21) The method of claim 20, wherein the nucleic acid expression construct further comprises, a transfection-facilitating polypeptide, or a charged polypeptide.

22) The method of claim 21, wherein the transfection-facilitating polypeptide, or charged polypeptide comprises poly-L-glutamate.

23) The method of claim 14, wherein the nucleic acid expression construct comprises a sequence that encodes a polypeptide having an amino acid sequence of which is at least 90% identical to the encoded GHRH of SEQID#14.

24) The method of claim 14, wherein the nucleic acid expression construct comprising a sequence that is at least 97% identical to mouse pAV0202 (SEQID#23); rat pAV0203 (SEQID#24); HV-GHRH pAV0224 (SEQID#25); pig-wt-GHRH pAV0225 (SEQID#26); dog pAV0235 (SEQID#27); bovine pAV0236 (SEQID#28); cat pAV0238 (SEQID#29); TI-GHRH pAV0239 (SEQID#30); ovine pAV0240 (SEQID#31); chicken pAV0241 (SEQID#32); horse pAV0249 (SEQID#33), or human pAV0226 (SEQID#34).

25) The method of claim 14, wherein the vaccine comprises at least part of a microorganism, an infectious agent, or a toxoid.

26) The method of claim 25, wherein the microorganism comprises: a virus, a bacteria, a mycoplasma, or a parasite.

27) The method of claim 25, wherein the microorganism comprises a bovine herpesvirus-1 ("IBR"), bovine virus diarrhea ("BVD"), parainfluenza 3, respiratory syncytial virus, Leptospira canicola, Leptospira grippotyphosa, Leptospira hardjo, Leptospira icterohaemorrhagiae, Leptospira Pomona bacterinsmycoplasma hyopneumonia, mycoplasma hyopneumonia, or combination thereof.

28) The method of claim 14, wherein the subject comprises a human, a ruminant animal, a food animal, or a work animal.

29) A method of improving the clinical outcome, after an infectious challenge, of a vaccinated subject having arthritis comprising:

(a) penetrating a muscle tissue in the subject with a plurality of needle electrodes, wherein the plurality of needle electrodes are arranged in a spaced relationship;

(b) delivering into the muscle tissue of the subject a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH"), such that an amount of expressed GHRH is effective to enhance the vaccination response; and

(c) applying an electrical pulse to the plurality of needle electrodes, wherein the electrical pulse allows the nucleic acid expression construct to traverse a muscle cell membrane,

wherein,

a range of 0.01-5 mg of nucleic acid expression construct with a defined concentration of poly-L-glutamate polypeptide is delivered into the muscle tissue of the subject, and the nucleic acid expression construct comprises a sequence that encodes a polypeptide having an amino acid sequence that is at least 90% identical to the encoded GHRH of SEQID#14; and the subject comprises a human, a ruminant animal, a food animal, or a work animal.

30) The method of claim 29, wherein the nucleic acid expression construct comprising a sequence that is at least 97% identical to mouse pAV0202 (SEQID#23); rat pAV0203 (SEQID#24); HV-GHRH pAV0224 (SEQID#25); pig-wt-GHRH pAV0225 (SEQID#26); dog pAV0235 (SEQID#27); bovine pAV0236 (SEQID#28); cat pAV0238 (SEQID#29); TI-GHRH pAV0239 (SEQID#30); ovine pAV0240 (SEQID#31); chicken pAV0241 (SEQID#32); horse pAV0249 (SEQID#33), or human pAV0226 (SEQID#34).

31) The method of claim 29, wherein the subject was vaccinated against a microorganism, an infectious agent, or a toxoid.

32) The method of claim 31, wherein the microorganism comprises: a virus, a bacteria, a mycoplasma, or a parasite.

33) The method of claim 31, wherein the microorganism comprises a bovine herpesvirus-1 ("IBR"), bovine virus diarrhea ("BVD"), parainfluenza 3, respiratory syncytial virus, Leptospira canicola, Leptospira grippotyphosa, Leptospira hardjo, Leptospira icterohaemorrhagiae, Leptospira Pomona bacterinsmycoplasma hyopneumonia, mycoplasma hyopneumonia, or combination thereof.

34) A composition comprising:

(a) a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH"); and

(b) a vaccine;

wherein, GHRH is expressed *in vivo* in the subject.

35) The method of claim 34, wherein the vaccine comprises at least part of a microorganism, an infectious agent, or a toxoid.

36) The method of claim 35, wherein the microorganism comprises: a virus, a bacteria, a mycoplasma, or a parasite.

37) The method of claim 35, wherein the microorganism comprises a bovine herpesvirus-1 ("IBR"), bovine virus diarrhea ("BVD"), parainfluenza 3, respiratory syncytial virus, Leptospira canicola, Leptospira grippotyphosa, Leptospira hardjo, Leptospira icterohaemorrhagiae, Leptospira Pomona bacterinsmycoplasma hyopneumonia, mycoplasma hyopneumonia, or combination thereof.

38) A method for vaccinating a subject comprising:

(a) penetrating a tissue in the subject with a plurality of needle electrodes, wherein the plurality of needle electrodes are arranged in a spaced relationship and the tissue of the subject comprise muscle cells;

(b) introducing a nucleic acid expression construct that encodes a growth-hormone-releasing-hormone ("GHRH") into the tissue between the plurality of needle electrodes in an amount in a range of about 0.01 – 5 mg, and

(c) applying an electrical pulse to the plurality of needle electrodes, wherein the electrical pulse allows the nucleic acid expression construct to traverse a muscle cell membrane;

(d) providing a vaccine to the subject in an amount effective to induce antibodies to the vaccine in the subject;

wherein the nucleic acid expression construct comprises a sequence that encodes a polypeptide having an amino acid sequence of which is at least 90% identical to the encoded GHRH of SEQID#14; and wherein the vaccine comprises at least part of a bovine herpesvirus-1 ("IBR"), bovine virus diarrhea ("BVD"), parainfluenza 3, respiratory syncytial virus, Leptospira canicola, Leptospira grippotyphosa, Leptospira hardjo, Leptospira icterohaemorrhagiae, Leptospira Pomona bacterinsmycoplasma hyopneumonia, mycoplasma hyopneumonia, or combination thereof.

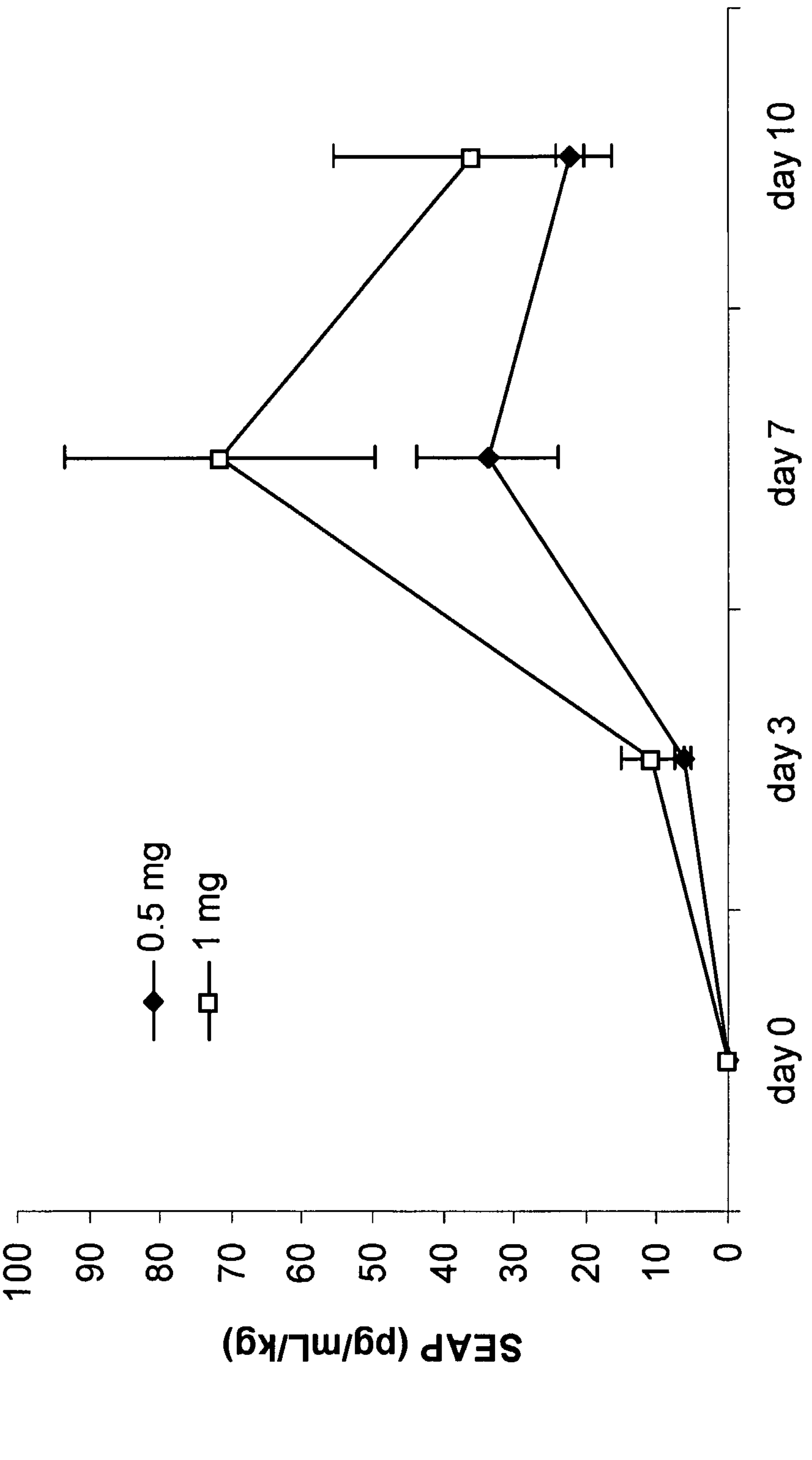

0.5 mg
1 mg
SEAP (pg/mL/kg)
100
90
80
70
60
50
40
30
20
10
0
day 0
day 3
day 7
day 10

Figure 1

		GHRH-treated	Control	*P* value
Glucose, mg/dL	60 DIM	43.9 ± 1.4	41.8 ± 4.5	0.67
	100 DIM	72.2 ± 1.4	69.3 ± 3.8	0.5
Insulin, μU/mL	60 DIM	11.6 ± 2.5	9.4 ± 3.2	0.59
	100 DIM	7.8 ± 1.5	7.4 ± 0.6	0.8

Figure 2

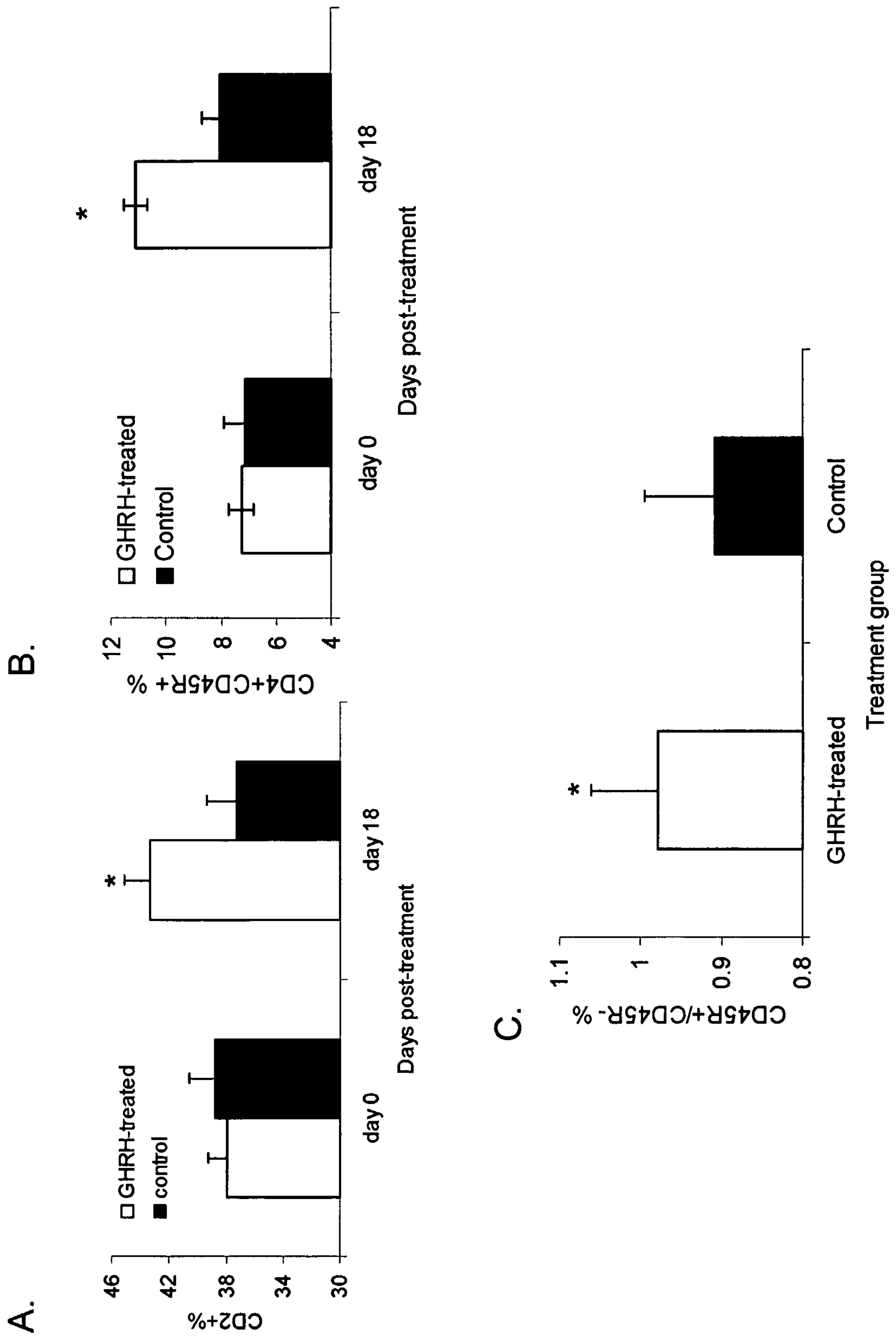

A.
CD2+%
46
42
38
34
30
GHRH-treated
control
*
day 0
day 18
Days post-treatment
B.
CD4+CD45R+ %
12
10
8
6
4
GHRH-treated
Control
*
day 0
day 18
Days post-treatment
C.
CD45R+/CD45R- %
1.1
1
0.9
0.8
*
GHRH-treated
Control
Treatment group

Figure 3

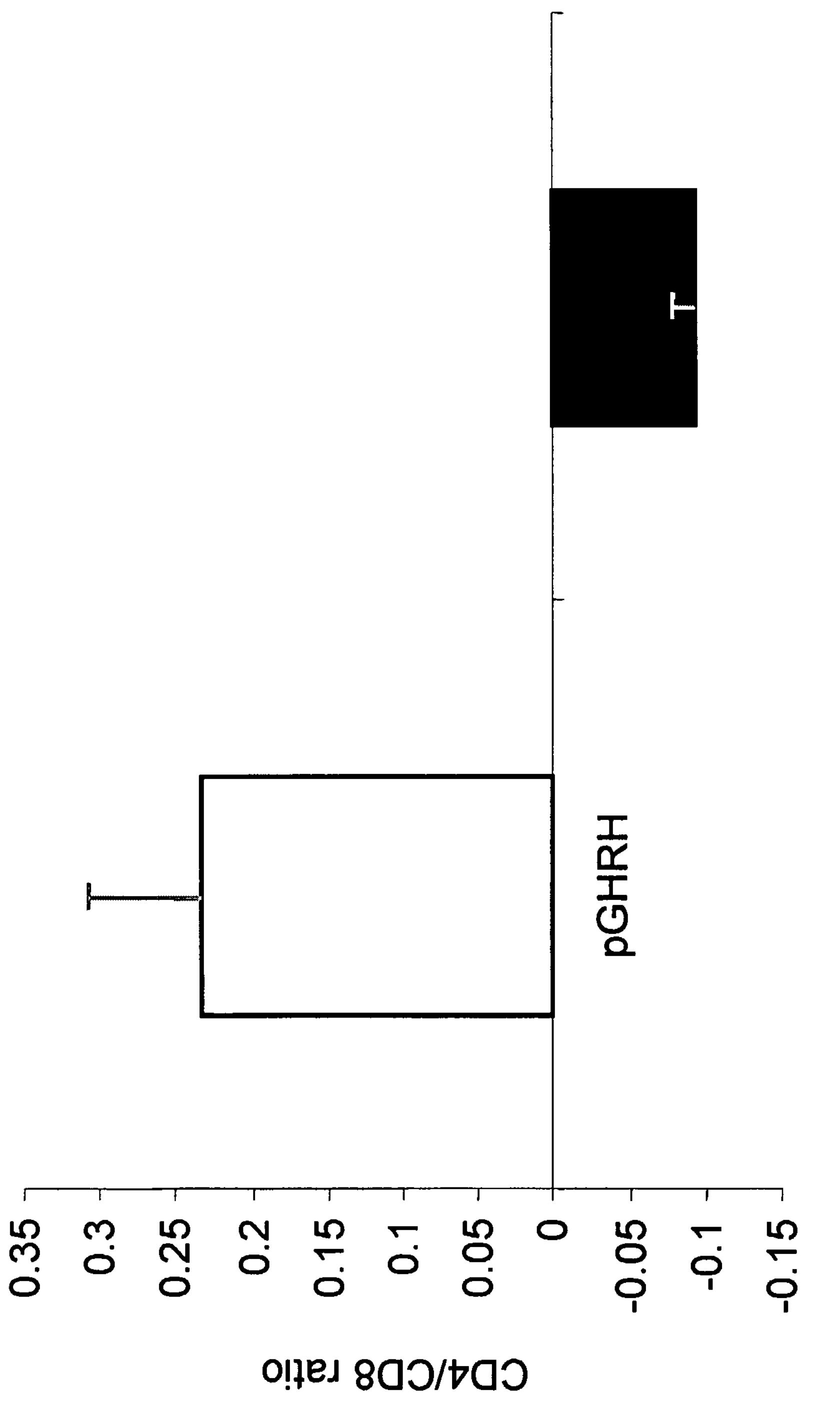


Figure 4

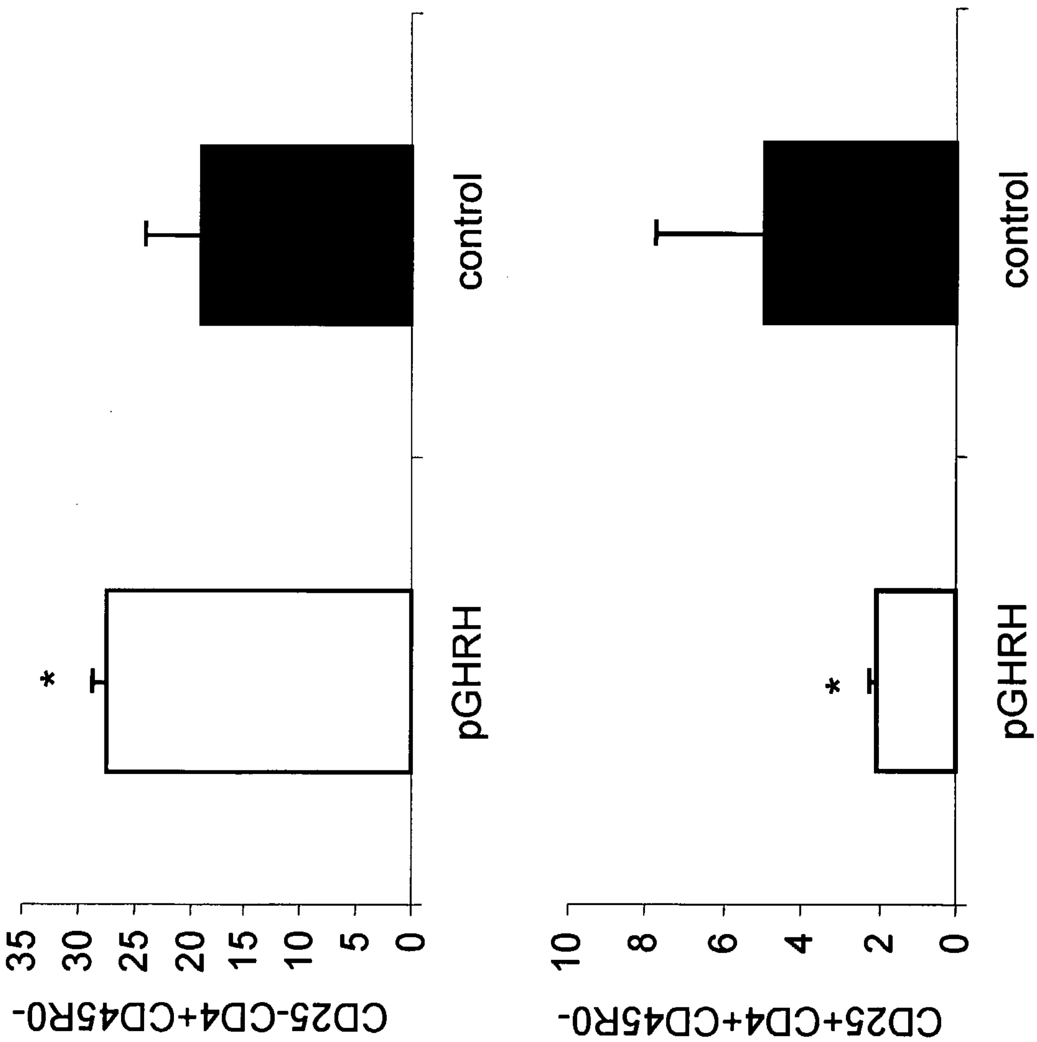

CD25-CD4+CD45R0-
35
30
25
20
15
10
5
0
*
pGHRH
control
CD25+CD4+CD45R0-
10
8
6
4
2
0
*
pGHRH
control

Figure 5

6/17

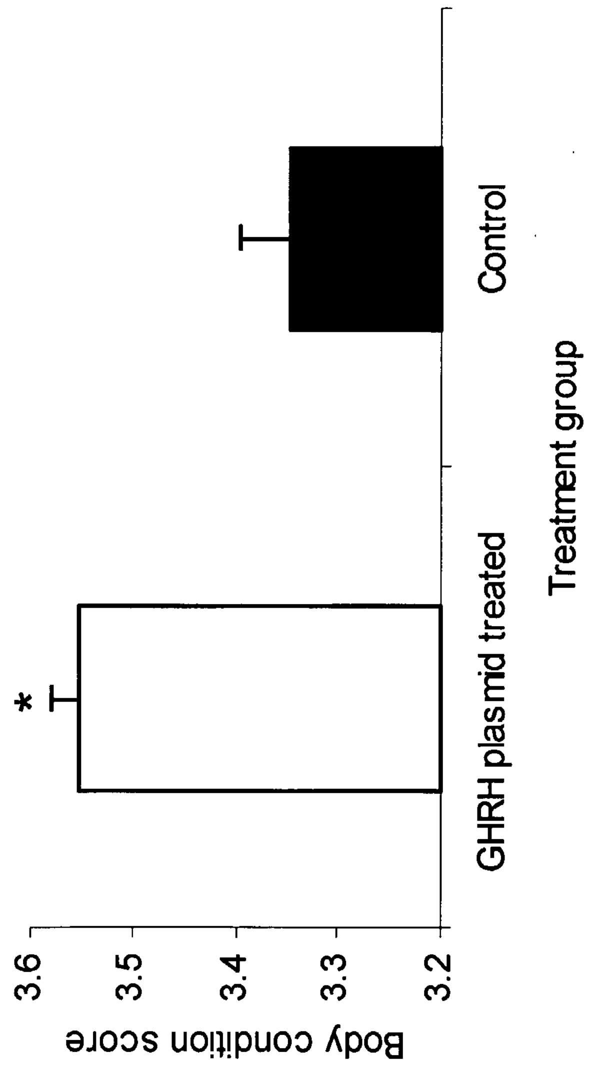
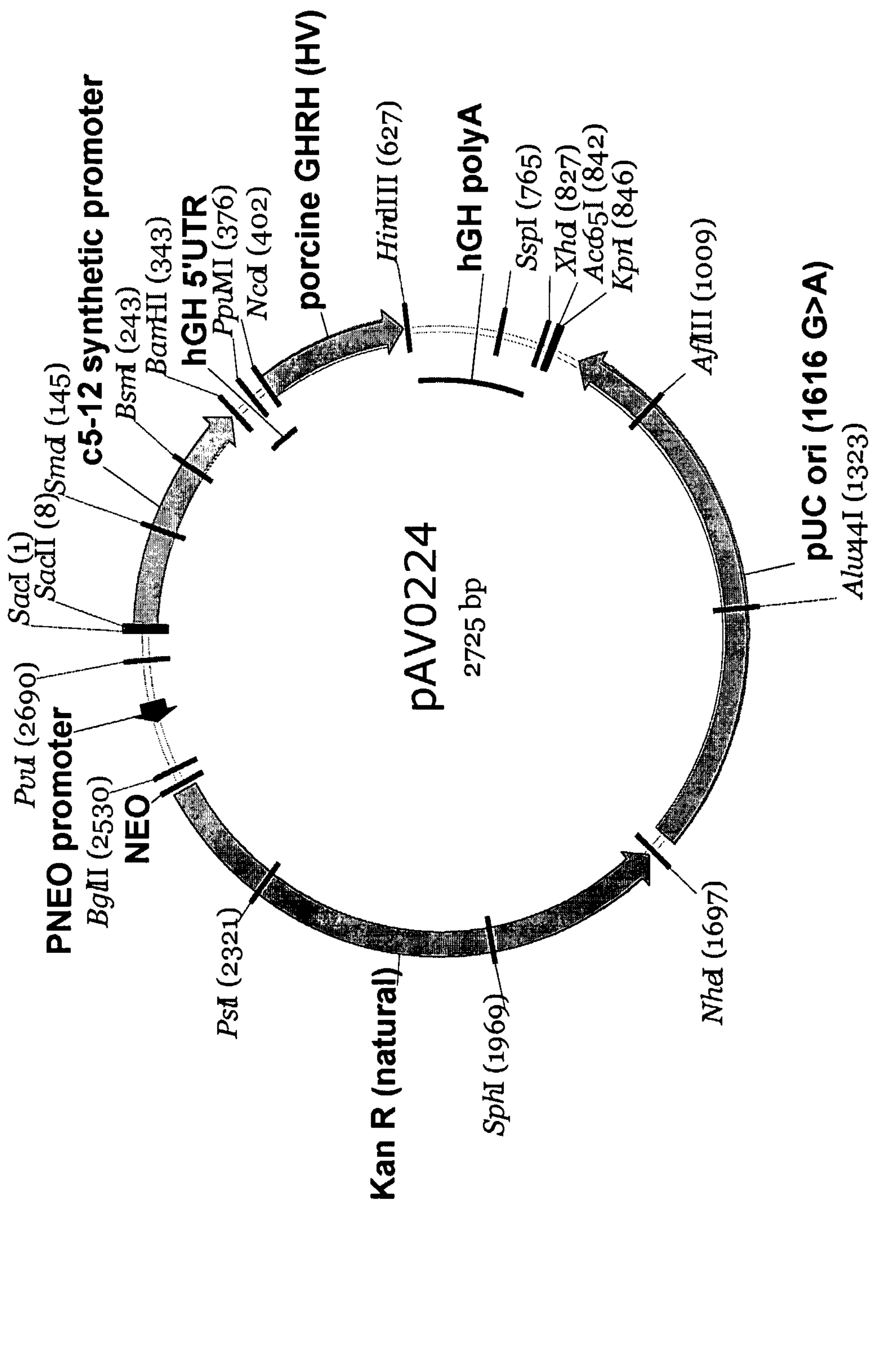

Body condition score
3.6
3.5
3.4
3.3
3.2
*
GHRH plasmid treated
Control
Treatment group

Figure 6

		GHRH-treated	Control	*P* value
	60 DPC (A)	540 ± 28.1	543 ± 42.7	0.38
Weight, kg	100 DIM (B)	536.4 ± 32.9	514.2 ± 48	***0.04***
	135 DIM (C)	544.9 ± 33.1	533.5 ± 44.8	0.17
Difference, kg	A-B	- 3.5	- 26.4	***0.02***
	A-C	4	- 9.1	0.1
Difference, %	A-B/A	- 0.5%	- 4.6%	***0.02***
	A-C/A	0.1%	- 1.4%	0.1

Figure 7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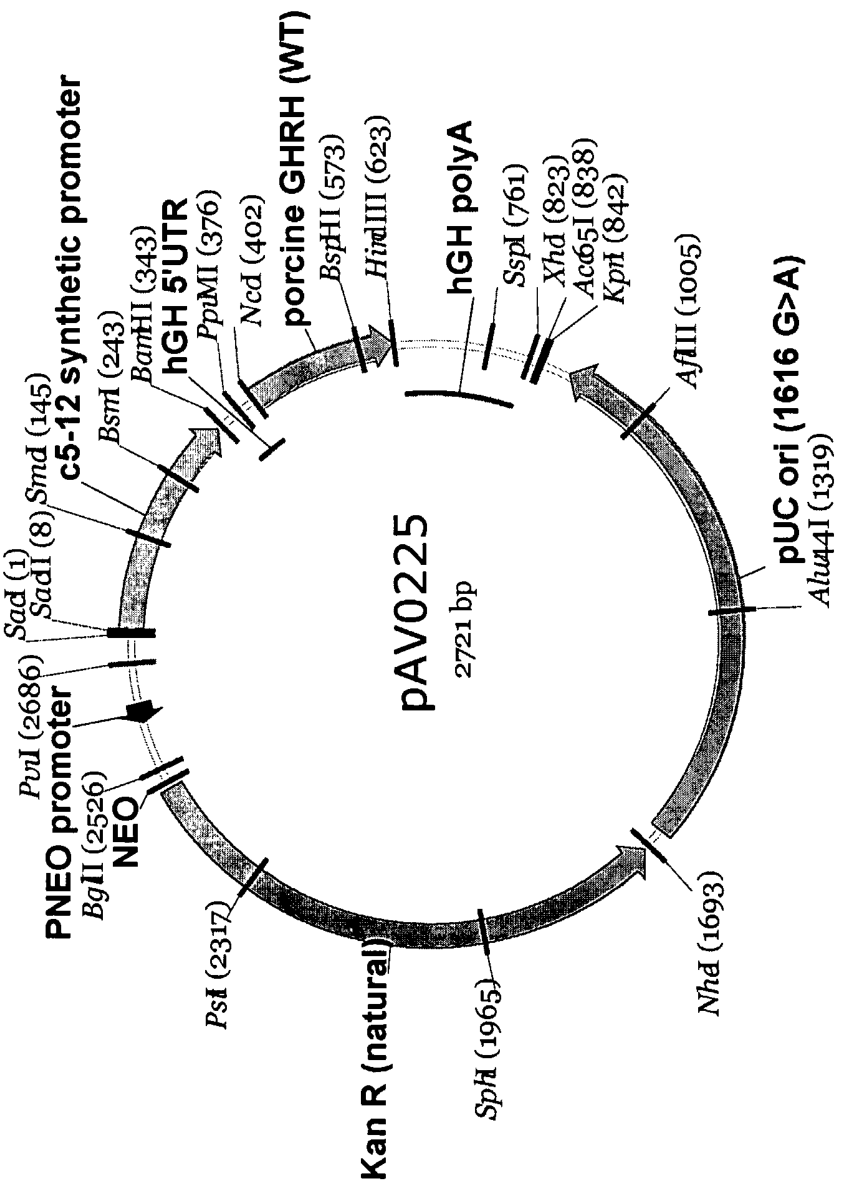
SmaI (145)
c5-12 synthetic promoter
BsmI (243)
BamHI (343)
hGH 5'UTR
PpuMI (376)
NcoI (402)
porcine GHRH (HV)
HindIII (627)
hGH polyA
SspI (765)
XhoI (827)
Acc65I (842)
KpnI (846)
AflIII (1009)
pUC ori (1616 G>A)
Alw44I (1323)
NheI (1697)
SphI (1969)
Kan R (natural)
PstI (2321)
PNEO promoter
BglII (2530)
NEO
PvuI (2690)

Figure 8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c5-12 synthetic promoter
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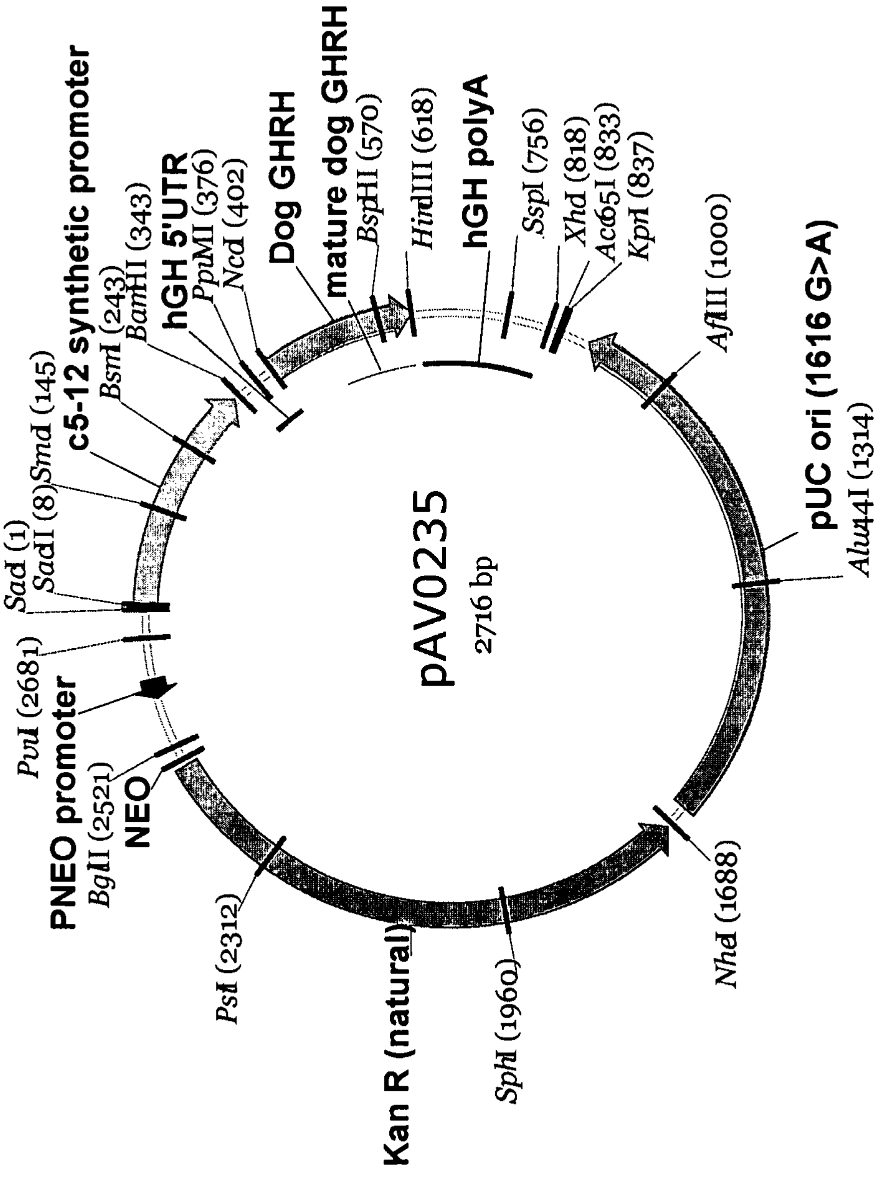
hGH 5'UTR
PpuMI (376)
NcoI (402)
porcine GHRH (WT)
BspHI (573)
HindIII (623)
hGH polyA
SspI (761)
XhoI (823)
Acc65I (838)
KpnI (842)
AflIII (1005)
pUC ori (1616 G>A)
Alw44I (1319)
NheI (1693)
SphI (1965)
Kan R (natural)
PstI (2317)
PNEO promoter
BglII (2526)
NEO
PvuI (2686)

Figure 9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PpuMI (376)
NcoI (402)
Dog GHRH
mature dog GHRH
BspHI (570)
HindIII (618)
hGH polyA
SspI (756)
XhoI (818)
Acc65I (833)
KpnI (837)
AflIII (1000)
pUC ori (1616 G>A)
Alw44I (1314)
NheI (1688)
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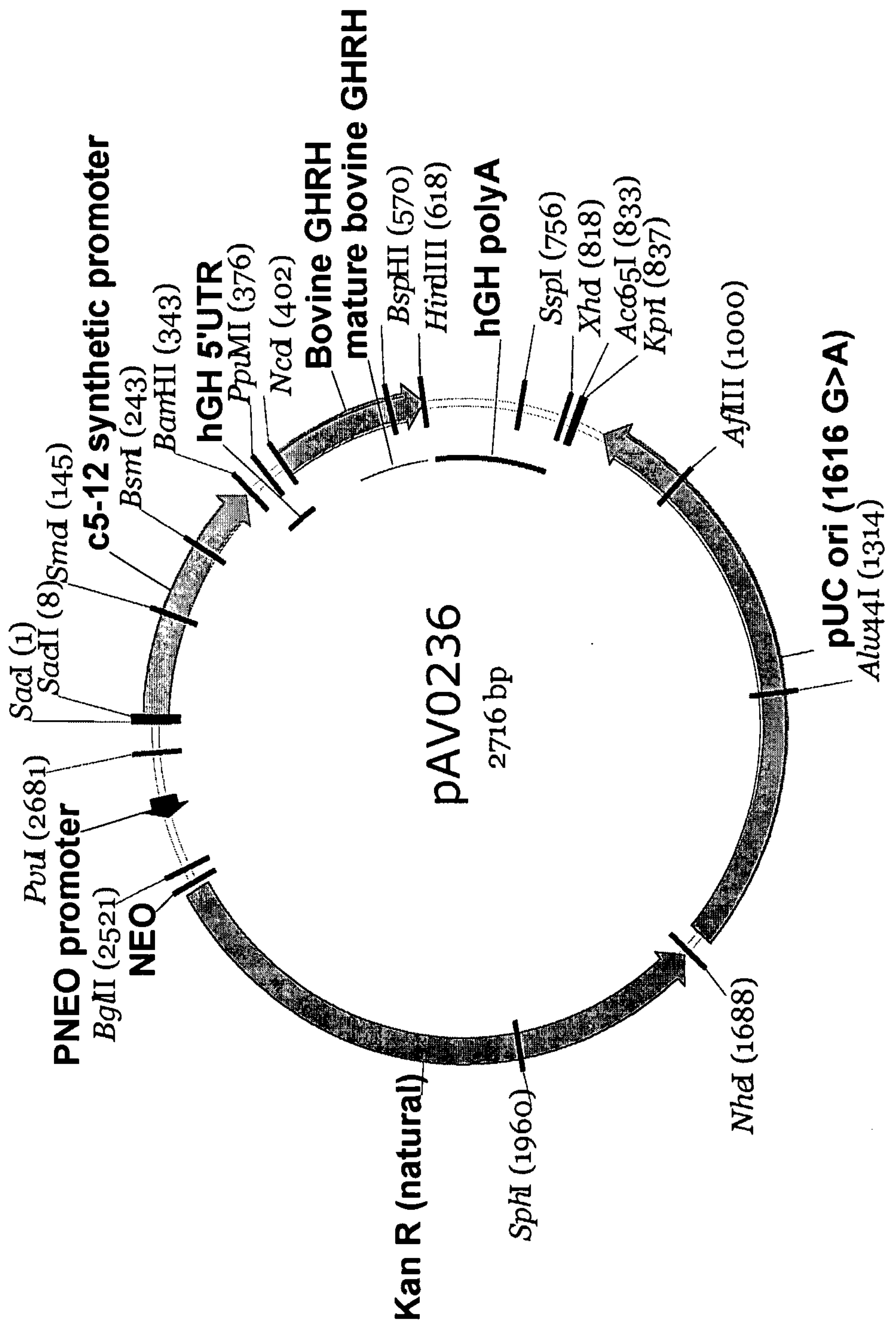
NEO
BglII (2521)
PNEO promoter
PvuI (2681)

Figure 10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PpuMI (376)
NcoI (402)
Bovine GHRH
mature bovine GHRH
BspHI (570)
HindIII (618)
hGH polyA
SspI (756)
XhoI (818)
Acc65I (833)
KpnI (837)
AflIII (1000)
pUC ori (1616 G>A)
Alw44I (1314)
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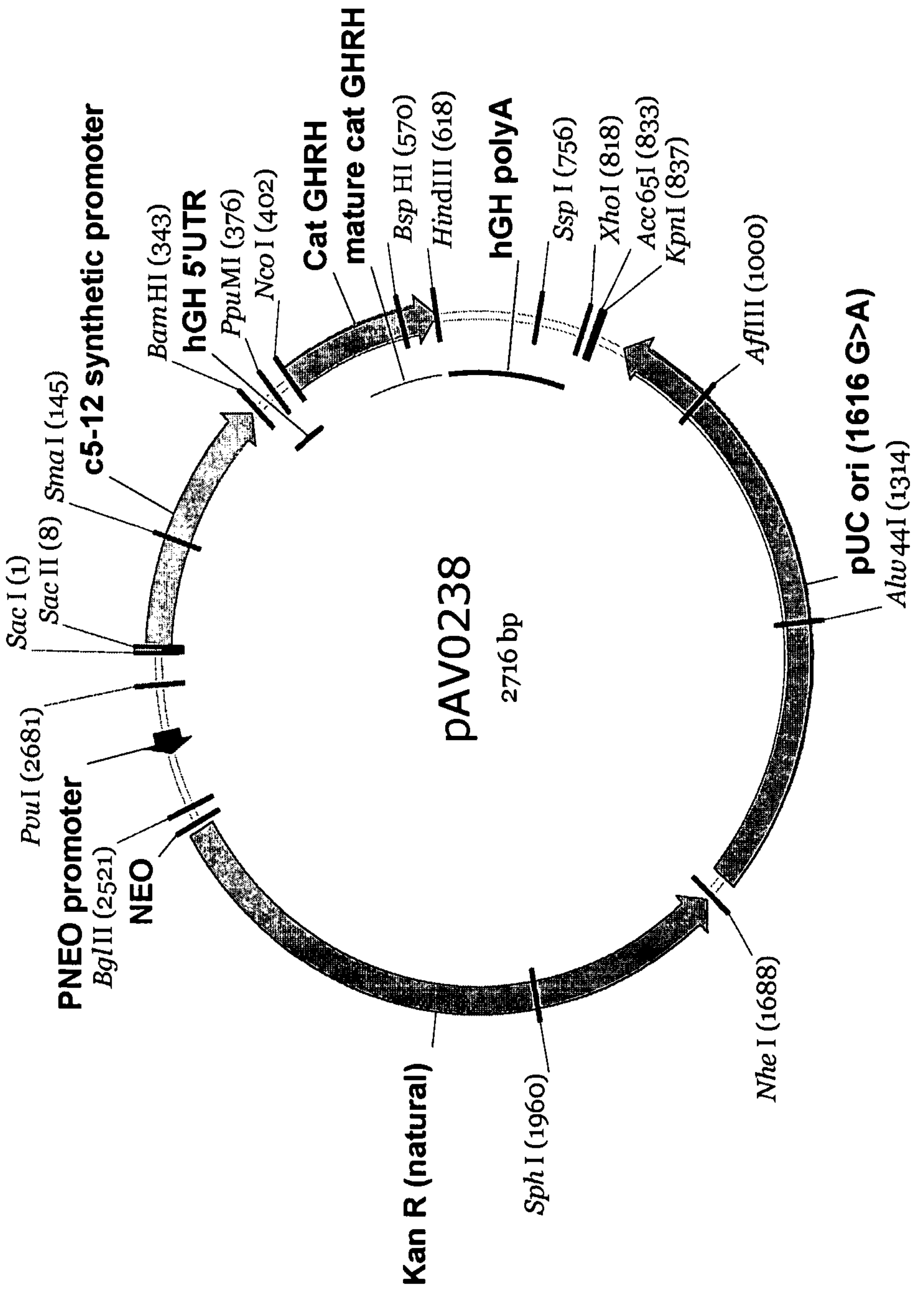
SphI (1960)
Kan R (natural)
BglII (2521)
NEO
PNEO promoter
PvuI (2681)

Figure 11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Sma I (145)
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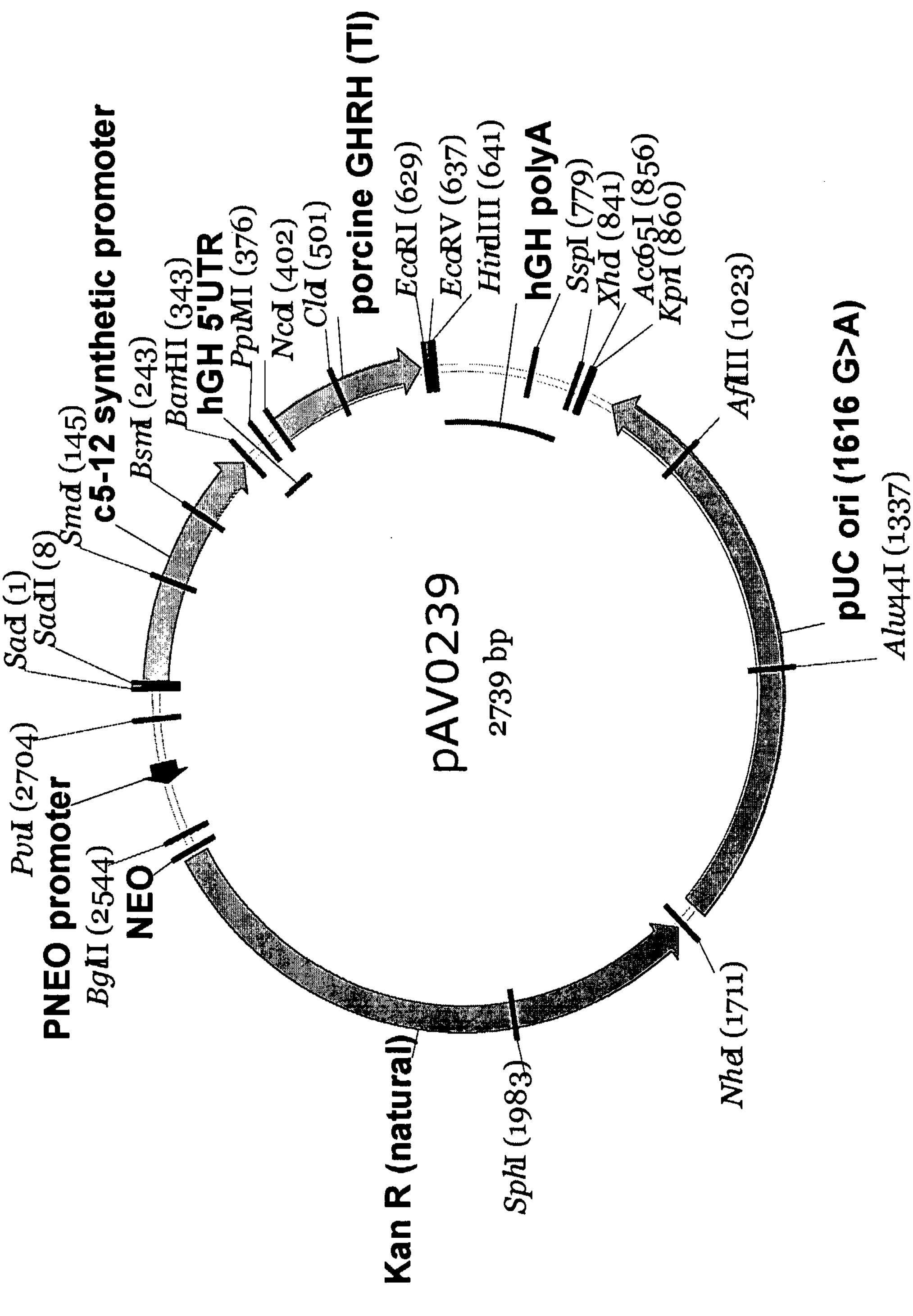
hGH 5'UTR
Ppu MI (376)
Nco I (402)
Cat GHRH
mature cat GHRH
Bsp HI (570)
Hind III (618)
hGH polyA
Ssp I (756)
Xho I (818)
Acc 65I (833)
Kpn I (837)
Afl III (1000)
pUC ori (1616 G>A)
Alw 44I (1314)
Nhe I (1688)
Sph I (1960)
Kan R (natural)
NEO
Bgl II (2521)
PNEO promoter
Pvu I (2681)

Figure 12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BamHI (343)
hGH 5'UTR
PpuMI (376)
NcoI (402)
ClaI (501)
porcine GHRH (TI)
EcoRI (629)
EcoRV (637)
HindIII (641)
hGH polyA
SspI (779)
XhoI (841)
Acc65I (856)
KpnI (860)
AflIII (1023)
pUC ori (1616 G>A)
Alw44I (1337)
NheI (1711)
SphI (1983)
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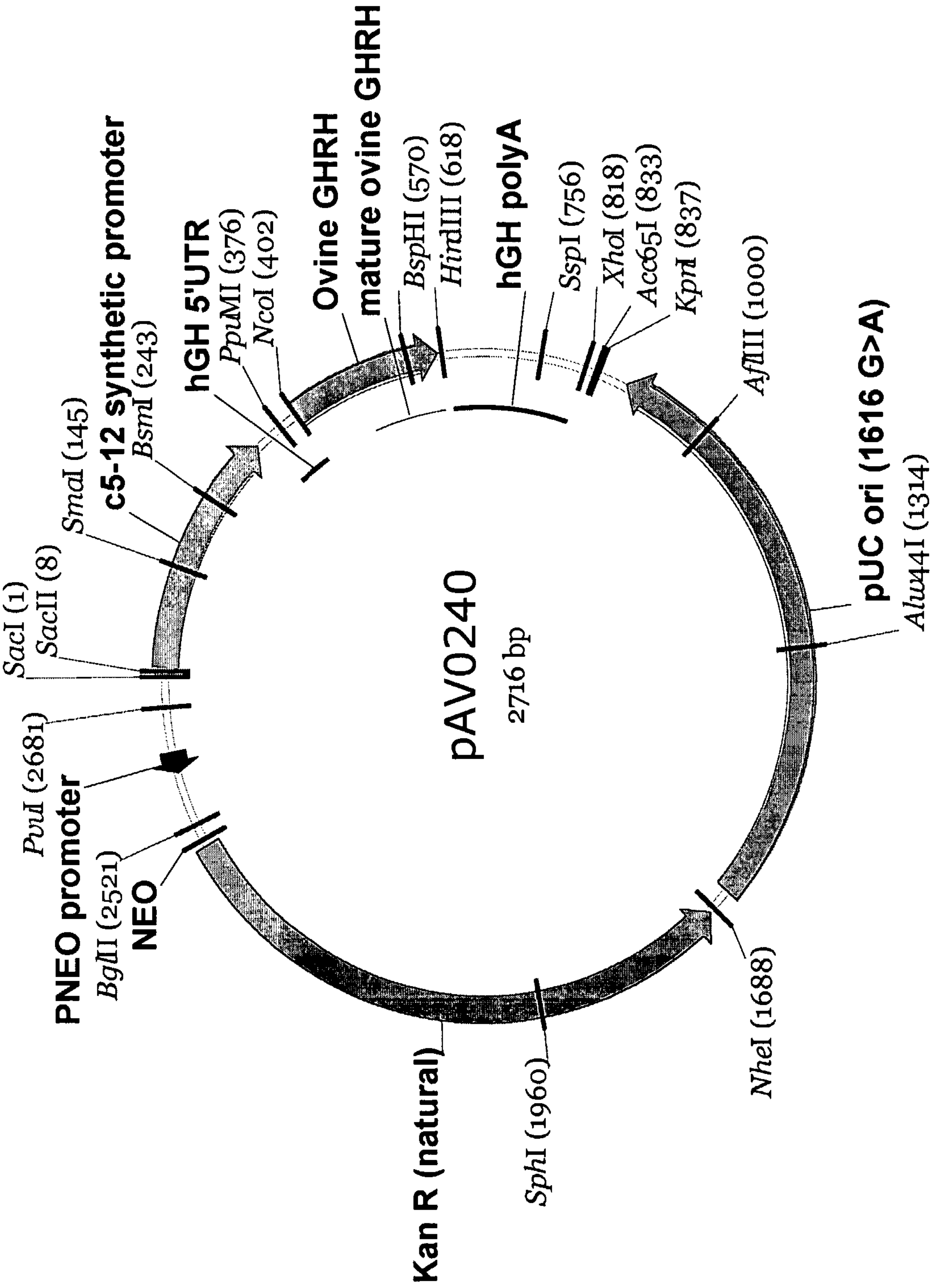
BglII (2544)
PNEO promoter
PvuI (2704)

Figure 13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PpuMI (376)
NcoI (402)
Ovine GHRH
mature ovine GHRH
BspHI (570)
HindIII (618)
hGH polyA
SspI (756)
XhoI (818)
Acc65I (833)
KpnI (837)
AflIII (1000)
pUC ori (1616 G>A)
Alw44I (1314)
NheI (1688)
SphI (1960)
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BglII (2521)
PNEO promoter
PvuI (2681)

Figure 14

PCT/US05/25747

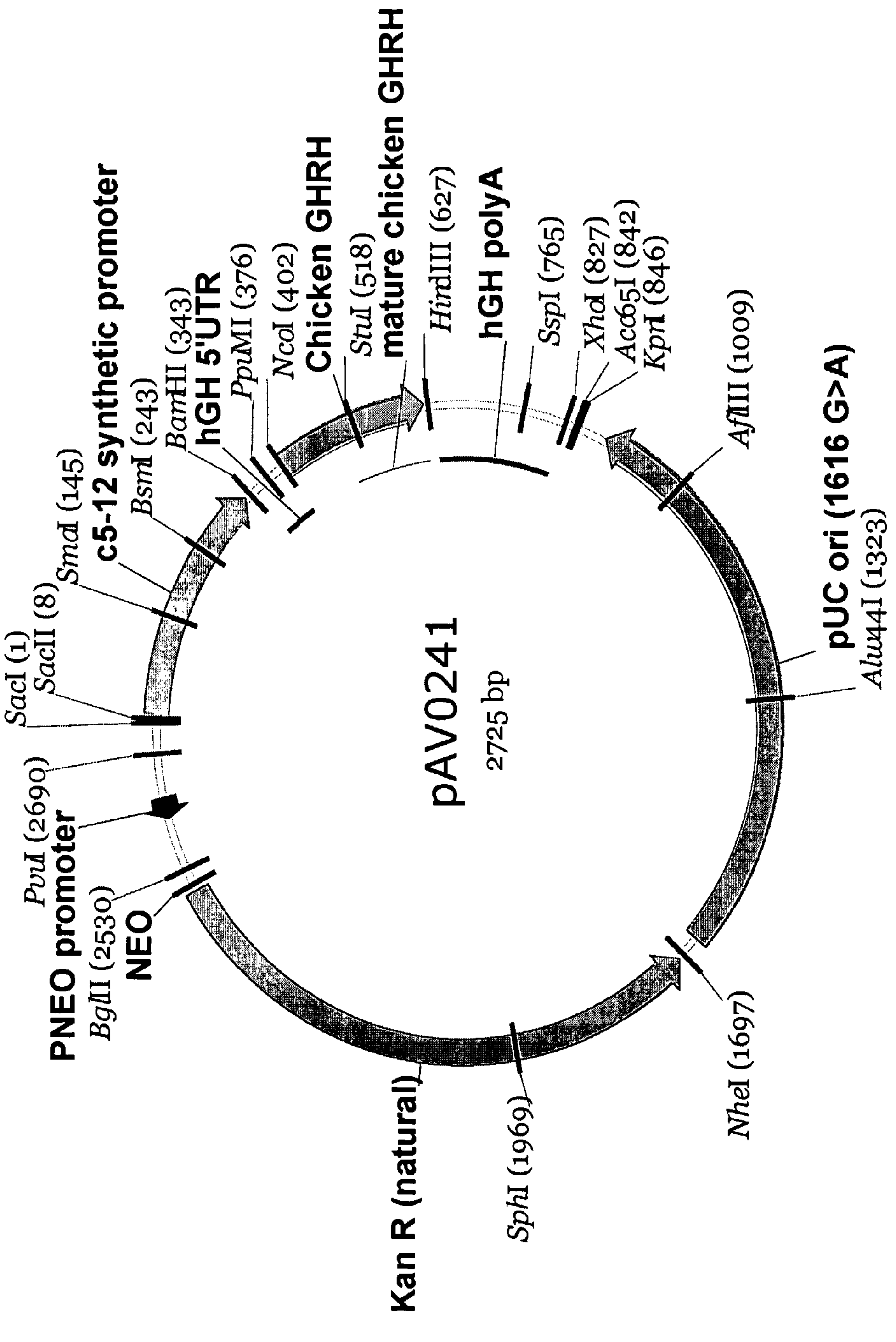


Figure 15

PCT/US05/25747

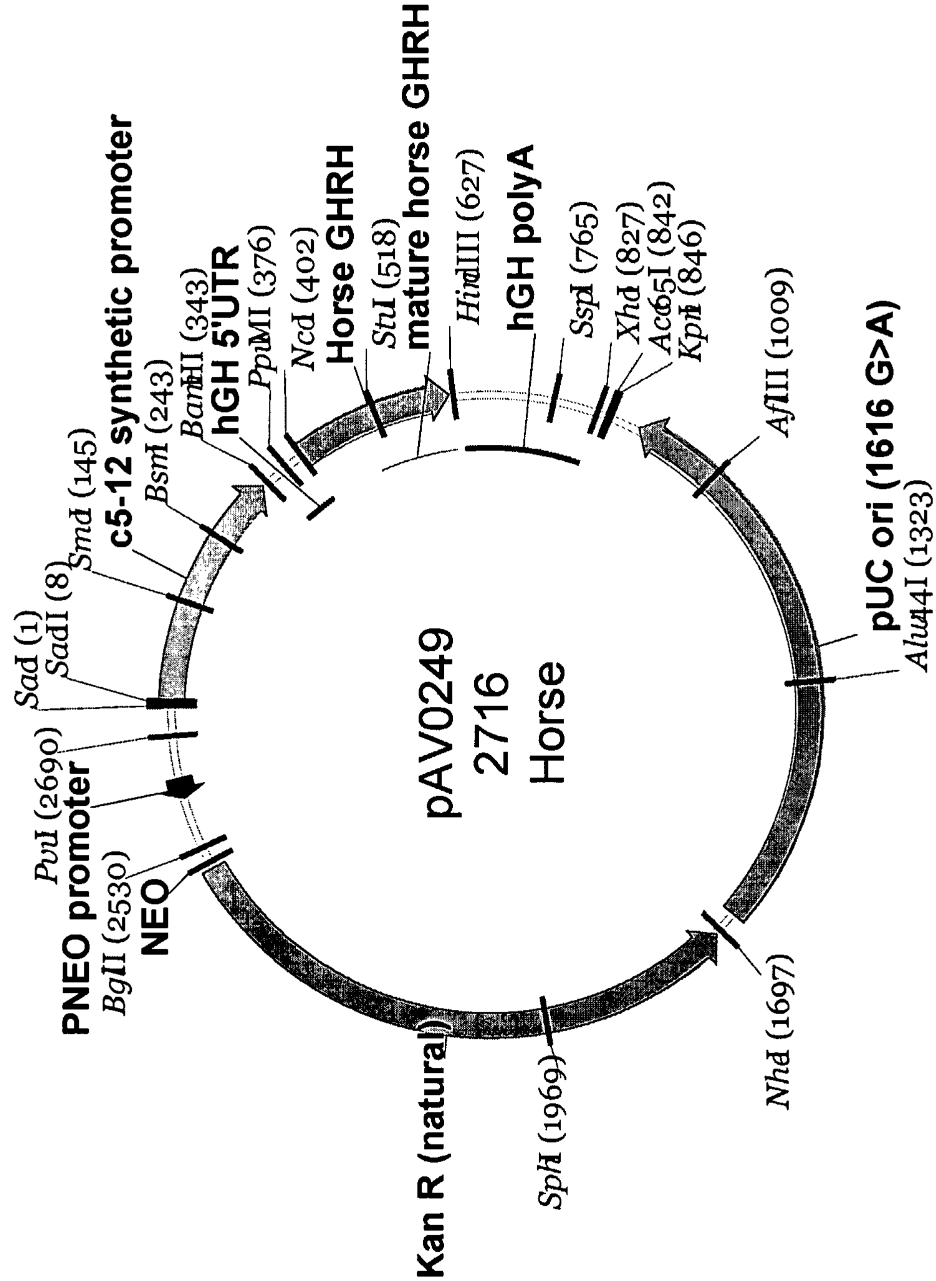
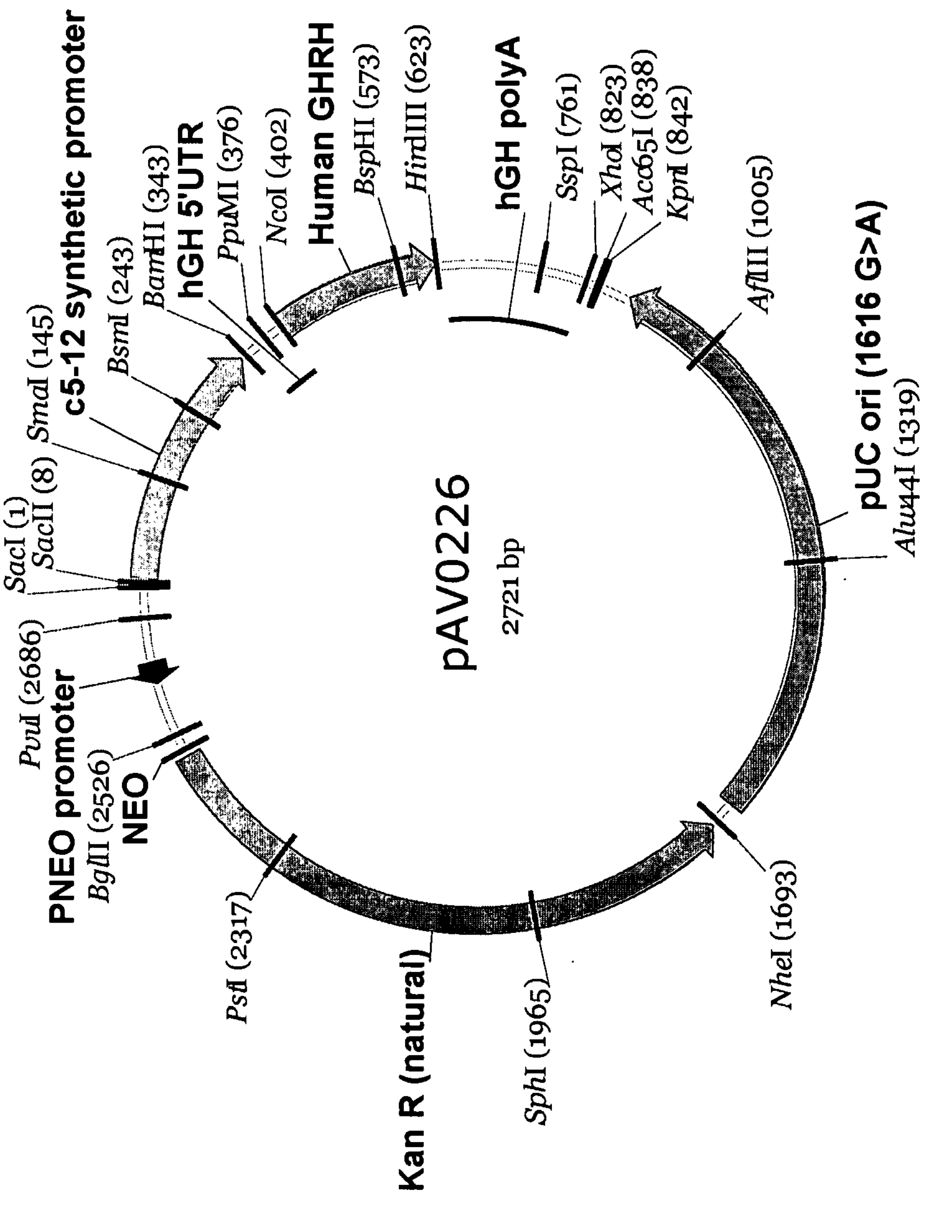


Figure 16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hGH 5'UTR
PpuMI (376)
NcoI (402)
Human GHRH
BspHI (573)
HindIII (623)
hGH polyA
SspI (761)
XhoI (823)
Acc65I (838)
KpnI (842)
AflIII (1005)
pUC ori (1616 G>A)
Alw44I (1319)
NheI (1693)
SphI (1965)
Kan R (natural)
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NEO
BglII (2526)
PNEO promoter
PvuI (2686)

Figure 17

SEQUENCE LISTING

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<170> PatentIn version 3.1

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1               5                   10                  15

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            20                  25                  30

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<213>  Artificial Sequence

<220>
<223>  This is an amino acid sequence for Cat-GHRH

<400>  5

Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly Gln
1               5                   10                  15

Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Arg Gln Gln Gly
            20                  25                  30

Glu Arg Asn Gln Glu Gln Gly Ala
        35                  40

<210>  6
<211>  40
<212>  PRT
<213>  artificial sequence

<220>
<223>  This is a TI- growth hormone releasing hormone ("GHRH") analog.

<400>  6

Tyr Ile Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Ala Gln
1               5                   10                  15
```

```
Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Leu Asn Arg Gln Gln Gly
            20                  25                  30


Glu Arg Asn Gln Glu Gln Gly Ala
        35                  40


<210>  7
<211>  40
<212>  PRT
<213>  artificial sequence

<220>
<223>  This is an amino acid sequence for Ovine-GHRH

<400>  7

Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Ile Leu Gly Gln
1               5                   10                  15


Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Asn Arg Gln Gln Gly
            20                  25                  30


Glu Arg Asn Gln Glu Gln Gly Ala
        35                  40


<210>  8
<211>  43
<212>  PRT
<213>  artificial sequence

<220>
<223>  This is an amino acid sequence for Chicken-GHRH

<400>  8

His Ala Asp Gly Ile Phe Ser Lys Ala Tyr Arg Lys Leu Leu Gly Gln
1               5                   10                  15


Leu Ser Ala Arg Asn Tyr Leu His Ser Leu Met Ala Lys Arg Val Gly
            20                  25                  30


Ser Gly Leu Gly Asp Glu Ala Glu Pro Leu Ser
        35                  40


<210>  9
<211>  40
<212>  PRT
<213>  artificial sequence

<220>
<223>  This is an amino acid sequence for Horse GHRH.

<220>
<221>  misc_feature
<222>  (1)..(1)
<223>  "X" can be any AA sequence


<220>
<221>  misc_feature
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```
<222>  (30)..(40)
<223>  "X" can be any AA sequence


<400>  9

Xaa Ala Asp Ala Ile Phe Thr Asn Asn Tyr Arg Lys Val Leu Gly Gln
1               5                   10                  15


Leu Ser Ala Arg Lys Ile Leu Gln Asp Ile Met Ser Arg Xaa Xaa Xaa
            20                  25                  30


Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
        35                  40


<210>  10
<211>  40
<212>  PRT
<213>  artificial sequence

<220>
<223>  This is an amino acid sequence for human (1-40)-GHRH

<400>  10

Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly Gln
1               5                   10                  15


Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Arg Gln Gln Gly
            20                  25                  30


Glu Ser Asn Gln Glu Arg Gly Ala
        35                  40


<210>  11
<211>  40
<212>  PRT
<213>  artificial sequence

<220>
<223>  This is a TV-growth hormone releasing hormone ("GHRH") analog.

<400>  11

Tyr Val Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Ala Gln
1               5                   10                  15


Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Leu Asn Arg Gln Gln Gly
            20                  25                  30


Glu Arg Asn Gln Glu Gln Gly Ala
        35                  40


<210>  12
<211>  40
<212>  PRT
<213>  artificial sequence

<220>
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<223> This is a TA-growth hormone releasing hormone ("GHRH") analog.

<400> 12

Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Ala Gln
1 5 10 15

Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Leu Asn Arg Gln Gln Gly
20 25 30

Glu Arg Asn Gln Glu Gln Gly Ala
35 40

<210> 13
<211> 44
<212> PRT
<213> artificial sequence

<220>
<223> This is a human (1-44) growth hormone releasing hormone ("GHRH").

<400> 13

Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly Gln
1 5 10 15

Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Arg Gln Gln Gly
20 25 30

Glu Ser Asn Gln Glu Arg Gly Ala Arg Ala Arg Leu
35 40

<210> 14
<211> 40
<212> PRT
<213> artificial sequence

<220>
<223> This is the artificial sequence for GHRH (1-40)OH.

<220>
<221> MISC_FEATURE
<222> (1)..(1)
<223> Xaa at position 1 may be tyrosine, or histidine

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> Xaa at position 2 may be alanine, valine, or isoleucine.

<220>
<221> MISC_FEATURE
<222> (15)..(15)
<223> Xaa at position 15 may be alanine, valine, or isoleucine.

<220>
<221> MISC_FEATURE
<222> (27)..(27)
<223> Xaa at position 27 may be methionine, or leucine.

<220>
<221> MISC_FEATURE
<222> (28)..(28)
<223> Xaa at position 28 may be serine or asparagine.

<220>
<221> MISC_FEATURE
<222> (34)..(34)
<223> Xaa at position 34 may be arginine or serine.

<220>
<221> MISC_FEATURE
<222> (36)..(36)
<223> Xaa at position 36 may be arginine or glutamine.

<220>
<221> MISC_FEATURE
<222> (38)..(38)
<223> Xaa at position 38 may be arginine or glutamine

<400> 14

```
Xaa Xaa Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Xaa Gln
1               5                   10                  15

Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Xaa Xaa Arg Gln Gln Gly
            20                  25                  30

Glu Xaa Asn Xaa Glu Xaa Gly Ala
        35                  40
```

<210> 15
<211> 323
<212> DNA
<213> artificial sequence

<220>
<223> This is a nucleic acid sequence of a eukaryotic promoter c5-12.

<400> 15

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cggccgtccg ccctcggcac catcctcacg acacccaaat atggcgacgg gtgaggaatg     60

gtggggagtt atttttagag cggtgaggaa ggtgggcagg cagcaggtgt tggcgctcta    120

aaaataactc ccgggagtta tttttagagc ggaggaatgg tggacaccca aatatggcga    180

cggttcctca cccgtcgcca tatttgggtg tccgccctcg gccggggccg cattcctggg    240

ggccgggcgg tgctcccgcc cgcctcgata aaaggctccg gggccggcgg cggcccacga    300

gctacccgga ggagcgggag gcg                                            323
```

<210> 16
<211> 190
<212> DNA
<213> artificial sequence

<220>

<223> Nucleic acid sequence of a human growth hormone poly A tail.

<400> 16
gggtggcatc cctgtgaccc ctccccagtg cctctcctgg ccctggaagt tgccactcca 60

gtgcccacca gccttgtcct aataaaatta agttgcatca ttttgtctga ctaggtgtcc 120

ttctataata ttatggggtg gaggggggtg gtatggagca aggggcaagt tgggaagaca 180

acctgtaggg 190

<210> 17
<211> 795
<212> DNA
<213> artificial sequence

<220>
<223> Nucleic acid sequence for antibiotic resistance gene kanamycin.

<400> 17
atgattgaac aagatggatt gcacgcaggt tctccggccg cttgggtgga gaggctattc 60

ggctatgact gggcacaaca gacaatcggc tgctctgatg ccgccgtgtt ccggctgtca 120

gcgcaggggc gcccggttct ttttgtcaag accgacctgt ccggtgccct gaatgaactg 180

caggacgagg cagcgcggct atcgtggctg gccacgacgg gcgttccttg cgcagctgtg 240

ctcgacgttg tcactgaagc gggaagggac tggctgctat tgggcgaagt gccggggcag 300

gatctcctgt catctcacct tgctcctgcc gagaaagtat ccatcatggc tgatgcaatg 360

cggcggctgc atacgcttga tccggctacc tgcccattcg accaccaagc gaaacatcgc 420

atcgagcgag cacgtactcg gatggaagcc ggtcttgtcg atcaggatga tctggacgaa 480

gagcatcagg ggctcgcgcc agccgaactg ttcgccaggc tcaaggcgcg catgcccgac 540

ggcgaggatc tcgtcgtgac tcatggcgat gcctgcttgc cgaatatcat ggtggaaaat 600

ggccgctttt ctggattcat cgactgtggc cggctgggtg tggcggaccg ctatcaggac 660

atagcgttgg ctacccgtga tattgctgaa gagcttggcg gcgaatgggc tgaccgcttc 720

ctcgtgcttt acggtatcgc cgctcccgat tcgcagcgca tcgccttcta tcgccttctt 780

gacgagttct tctga 795

<210> 18
<211> 219
<212> DNA
<213> artificial sequence

<220>
<223> Sequence for an analog porcine GHRH sequence.

<400> 18
atggtgctct gggtgttctt ctttgtgatc ctcaccctca gcaacagctc ccactgctcc 60

ccacctcccc ctttgaccct caggatgcgg cggcacgtag atgccatctt caccaacagc 120

taccggaagg tgctggccca gctgtccgcc cgcaagctgc tccaggacat cctgaacagg 180

cagcagggag agaggaacca agagcaagga gcataatga 219

<210> 19
<211> 246
<212> DNA
<213> artificial sequence

<220>
<223> Sequence for an analog mouse GHRH sequence.

<400> 19
gccatggtgc tctgggtgct ctttgtgatc ctcatcctca ccagcggcag ccactgcagc 60

ctgcctccca gccctccctt caggatgcag aggcacgtgg acgccatctt caccaccaac 120

tacaggaagc tgctgagcca gctgtacgcc aggaaggtga tccaggacat catgaacaag 180

cagggcgaga ggatccagga gcagagggcc aggctgagct gataagcttg cgatgagttc 240

ttctaa 246

<210> 20
<211> 234
<212> DNA
<213> artificial sequence

<220>
<223> Sequence for an analog rat GHRH sequence.

<400> 20
gccatggccc tgtgggtgtt cttcgtgctg ctgaccctga ccagcggaag ccactgcagc 60

ctgcctccca gccctccctt cagggtgcgc cggcacgccg acgccatctt caccagcagc 120

tacaggagga tcctgggcca gctgtacgct aggaagctcc tgcacgagat catgaacagg 180

cagcagggcg agaggaacca ggagcagagg agcaggttca actgataagc ttgc 234

<210> 21
<211> 29
<212> DNA
<213> artificial sequence

<220>
<223> Nucleic acid sequence of a prokaryotic PNEO promoter.

<400> 21
accttaccag agggcgcccc agctggcaa 29

<210> 22
<211> 3534
<212> DNA
<213> artificial sequence

<220>
<223> Plasmid vector having an analog GHRH sequence.

<400> 22
gttgtaaaac gacggccagt gaattgtaat acgactcact atagggcgaa ttggagctcc 60

accgcggtgg cggccgtccg ccctcggcac catcctcacg acacccaaat atggcgacgg 120

gtgaggaatg gtggggagtt atttttagag cggtgaggaa ggtgggcagg cagcaggtgt 180

tggcgctcta aaaataactc ccgggagtta tttttagagc ggaggaatgg tggacaccca 240

aatatggcga cggttcctca cccgtcgcca tatttgggtg tccgccctcg gccggggccg 300

cattcctggg ggccgggcgg tgctcccgcc cgcctcgata aaaggctccg gggccggcgg 360
cggcccacga gctacccgga ggagcgggag gcgccaagct ctagaactag tggatcccaa 420
ggcccaactc cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct 480
ctgggtgttc ttctttgtga tcctcaccct cagcaacagc tcccactgct ccccacctcc 540
ccctttgacc ctcaggatgc ggcggcacgt agatgccatc ttcaccaaca gctaccggaa 600
ggtgctggcc cagctgtccg cccgcaagct gctccaggac atcctgaaca ggcagcaggg 660
agagaggaac caagagcaag gagcataatg actgcaggaa ttcgatatca agcttatcgg 720
ggtggcatcc ctgtgacccc tccccagtgc ctctcctggc cctggaagtt gccactccag 780
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ccgctttcca gtcgggaaac ctgtcgtgcc agctgcatta atgaatcggc caacgcgcgg 1620
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accgtaaaaa ggccgcgttg ctggcgtttt tccataggct ccgcccccct gacgagcatc 1860
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cgtttccccc tggaagctcc ctcgtgcgct ctcctgttcc gaccctgccg cttaccggat 1980
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gcaaacaaac caccgctggt agcggtggtt tttttgtttg caagcagcag attacgcgca 2400

gaaaaaaagg atctcaagaa gatcctttga tcttttctac ggggtctgac gctcagaaga 2460

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gcacgaggaa gcggtcagcc cattcgccgc caagctcttc agcaatatca cgggtagcca 2580

acgctatgtc ctgatagcgg tccgccacac ccagccggcc acagtcgatg aatccagaaa 2640

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agggcgcccc agctggcaat tccggttcgc ttgctgtcca taaaaccgcc cagtctagca 3420

actgttggga agggcgatcg gtgcgggcct cttcgctatt acgccagctg gcgaaagggg 3480

gatgtgctgc aaggcgatta agttgggtaa cgccagggtt ttcccagtca cgac 3534

<210> 23
<211> 2722
<212> DNA
<213> artificial sequence

<220>
<223> Plasmid vector having a codon optimized mouse GHRH sequence

<400> 23
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gggtgaggaa tggtggggag ttatttttag agcggtgagg aaggtgggca ggcagcaggt 120

gttggcgctc taaaaataac tcccgggagt tatttttaga gcggaggaat ggtggacacc 180

caaatatggc gacggttcct cacccgtcgc catatttggg tgtccgccct cggccggggc 240

cgcattcctg ggggccgggc ggtgctcccg cccgcctcga taaaaggctc cggggccggc 300

ggcggcccac gagctacccg gaggagcggg aggcgccaag cggatcccaa ggcccaactc 360

cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct ctgggtgctc 420

tttgtgatcc tcatcctcac cagcggcagc cactgcagcc tgcctcccag ccctcccttc 480

aggatgcaga ggcacgtgga cgccatcttc accaccaact acaggaagct gctgagccag 540

ctgtacgcca ggaaggtgat ccaggacatc atgaacaagc agggcgagag gatccaggag 600
cagagggcca ggctgagctg ataagcttat cggggtggca tccctgtgac ccctccccag 660
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taagttgcat cattttgtct gactaggtgt ccttctataa tattatgggg tggagggggg 780
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tgatcagatc ttgatcccct gcgccatcag atccttggcg gcaagaaagc catccagttt 2580

actttgcagg gcttcccaac cttaccagag ggcgccccag ctggcaattc cggttcgctt 2640

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cactataggg cgaattggag ct 2722

<210> 24
<211> 2725
<212> DNA
<213> artificial sequence

<220>
<223> Plasmid vector having a codon optimized rat GHRH sequence

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gttggcgctc taaaaataac tcccgggagt tatttttaga gcggaggaat ggtggacacc 180

caaatatggc gacggttcct cacccgtcgc catatttggg tgtccgccct cggccggggc 240

cgcattcctg ggggccgggc ggtgctcccg cccgcctcga taaaaggctc cggggccggc 300

ggcggcccac gagctacccg gaggagcggg aggcgccaag cggatcccaa ggcccaactc 360

cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggccct gtgggtgttc 420

ttcgtgctgc tgaccctgac cagcggaagc cactgcagcc tgcctcccag ccctcccttc 480

agggtgcgcc ggcacgccga cgccatcttc accagcagct acaggaggat cctgggccag 540

ctgtacgcta ggaagctcct gcacgagatc atgaacaggc agcagggcga gaggaaccag 600

gagcagagga gcaggttcaa ctgataagct tatcggggtg gcatccctgt gacccctccc 660

cagtgcctct cctggccctg gaagttgcca ctccagtgcc caccagcctt gtcctaataa 720

aattaagttg catcattttg tctgactagg tgtccttcta taatattatg gggtggaggg 780

gggtggtatg gagcaagggg caagttggga agacaacctg tagggctcga gggggggccc 840

ggtaccagct tttgttccct ttagtgaggg ttaatttcga gcttggtctt ccgcttcctc 900

gctcactgac tcgctgcgct cggtcgttcg gctgcggcga gcggtatcag ctcactcaaa 960

ggcggtaata cggttatcca cagaatcagg ggataacgca ggaaagaaca tgtgagcaaa 1020

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gaccctgccg cttaccggat acctgtccgc ctttctccct tcgggaagcg tggcgctttc 1260

tcatagctca cgctgtaggt atctcagttc ggtgtaggtc gttcgctcca agctgggctg 1320

tgtgcacgaa ccccccgttc agcccgaccg ctgcgcctta tccggtaact atcgtcttga 1380

gtccaacccg gtaagacacg acttatcgcc actggcagca gccactggta acaggattag 1440

cagagcgagg tatgtaggcg gtgctacaga gttcttgaag tggtggccta actacggcta 1500

cactagaaga acagtatttg gtatctgcgc tctgctgaag ccagttacct tcggaaaaag 1560 agttggtagc tcttgatccg gcaaacaaac caccgctggt agcggtggtt tttttgtttg 1620

caagcagcag attacgcgca gaaaaaaagg atctcaagaa gatcctttga tcttttctac 1680

ggggtctgac gctcagctag cgctcagaag aactcgtcaa gaaggcgata gaaggcgatg 1740

cgctgcgaat cgggagcggc gataccgtaa agcacgagga agcggtcagc ccattcgccg 1800

ccaagctctt cagcaatatc acgggtagcc aacgctatgt cctgatagcg gtccgccaca 1860

cccagccggc cacagtcgat gaatccagaa aagcggccat tttccaccat gatattcggc 1920

aagcaggcat cgccatgagt cacgacgaga tcctcgccgt cgggcatgcg cgccttgagc 1980

ctggcgaaca gttcggctgg cgcgagcccc tgatgctctt cgtccagatc atcctgatcg 2040

acaagaccgg cttccatccg agtacgtgct cgctcgatgc gatgtttcgc ttggtggtcg 2100

aatgggcagg tagccggatc aagcgtatgc agccgccgca ttgcatcagc catgatggat 2160

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agcagccagt cccttcccgc ttcagtgaca acgtcgagca cagctgcgca aggaacgccc 2280

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aggtcggtct tgacaaaaag aaccgggcgc ccctgcgctg acagccggaa cacggcggca 2400

tcagagcagc cgattgtctg ttgtgcccag tcatagccga atagcctctc cacccaagcg 2460

gccggagaac ctgcgtgcaa tccatcttgt tcaatcatgc gaaacgatcc tcatcctgtc 2520

tcttgatcag atcttgatcc cctgcgccat cagatccttg gcggcaagaa agccatccag 2580

tttactttgc agggcttccc aaccttacca gagggcgccc cagctggcaa ttccggttcg 2640

cttgctgtcc ataaaaccgc ccagtctagc aactgttggg aagggcgatc gtgtaatacg 2700

actcactata gggcgaattg gagct 2725

<210> 25
<211> 2725
<212> DNA
<213> artificial sequence

<220>
<223> This is the codon optimized HV-GHRH expression plasmid.

<400> 25
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gggtgaggaa tggtggggag ttatttttag agcggtgagg aaggtgggca ggcagcaggt 120

gttggcgctc taaaaataac tcccgggagt tatttttaga gcggaggaat ggtggacacc 180

caaatatggc gacggttcct cacccgtcgc catatttggg tgtccgccct cggccggggc 240

cgcattcctg ggggccgggc ggtgctcccg cccgcctcga taaaaggctc cggggccggc 300

ggcggcccac gagctacccg gaggagcggg aggcgccaag cggatcccaa ggcccaactc 360

cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct ctgggtgttc 420

ttctttgtga tcctcaccct cagcaacagc tcccactgct ccccacctcc ccctttgacc 480

ctcaggatgc ggcggcacgt agatgccatc ttcaccaaca gctaccggaa ggtgctggcc 540

cagctgtccg cccgcaagct gctccaggac atcctgaaca ggcagcaggg agagaggaac 600
caagagcaag gagcataatg acatcaagct tatcggggtg gcatccctgt gacccctccc 660
cagtgcctct cctggccctg gaagttgcca ctccagtgcc caccagcctt gtcctaataa 720
aattaagttg catcattttg tctgactagg tgtccttcta taatattatg ggtggagggg 780
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ggtaccagct tttgttccct ttagtgaggg ttaatttcga gcttggtctt ccgcttcctc 900
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gtccaacccg gtaagacacg acttatcgcc actggcagca gccactggta acaggattag 1440
cagagcgagg tatgtaggcg gtgctacaga gttcttgaag tggtggccta actacggcta 1500
cactagaaga acagtatttg gtatctgcgc tctgctgaag ccagttacct tcggaaaaag 1560
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ggggtctgac gctcagctag cgctcagaag aactcgtcaa gaaggcgata gaaggcgatg 1740
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ccaagctctt cagcaatatc acgggtagcc aacgctatgt cctgatagcg gtccgccaca 1860
cccagccggc cacagtcgat gaatccagaa aagcggccat tttccaccat gatattcggc 1920
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aatgggcagg tagccggatc aagcgtatgc agccgccgca ttgcatcagc catgatggat 2160
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gtcgtggcca gccacgatag ccgcgctgcc tcgtcctgca gttcattcag ggcaccggac 2340
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gccggagaac ctgcgtgcaa tccatcttgt tcaatcatgc gaaacgatcc tcatcctgtc 2520
tcttgatcag atcttgatcc cctgcgccat cagatccttg gcggcaagaa agccatccag 2580

tttactttgc agggcttccc aaccttacca gagggcgccc cagctggcaa ttccggttcg 2640

cttgctgtcc ataaaaccgc ccagtctagc aactgttggg aagggcgatc gtgtaatacg 2700

actcactata gggcgaattg gagct 2725

<210> 26
<211> 2721
<212> DNA
<213> artificial sequence

<220>
<223> This is the codon optimized pig-GHRH expression plasmid.

<400> 26
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gggtgaggaa tggtggggag ttatttttag agcggtgagg aaggtgggca ggcagcaggt 120

gttggcgctc taaaaataac tcccgggagt tatttttaga gcggaggaat ggtggacacc 180

caaatatggc gacggttcct cacccgtcgc catatttggg tgtccgccct cggccggggc 240

cgcattcctg gggggccgggc ggtgctcccg cccgcctcga taaaaggctc cggggccggc 300

ggcggcccac gagctacccg gaggagcggg aggcgccaag cggatcccaa ggcccaactc 360

cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct ctgggtgttc 420

ttctttgtga tcctcaccct cagcaacagc tcccactgct ccccacctcc ccctttgacc 480

ctcaggatgc ggcggtatgc agatgccatc ttcaccaaca gctaccggaa ggtgctgggc 540

cagctgtccg cccgcaagct gctccaggac atcatgagca ggcagcaggg agagaggaac 600

caagagcaag gagcataatg aaagcttatc ggggtggcat ccctgtgacc cctccccagt 660

gcctctcctg gccctggaag ttgccactcc agtgcccacc agccttgtcc taataaaatt 720

aagttgcatc attttgtctg actaggtgtc cttctataat attatggggt ggaggggggt 780

ggtatggagc aaggggcaag ttgggaagac aacctgtagg gctcgagggg gggcccggta 840

ccagcttttg ttccctttag tgagggttaa tttcgagctt ggtcttccgc ttcctcgctc 900

actgactcgc tgcgctcggt cgttcggctg cggcgagcgg tatcagctca ctcaaaggcg 960

gtaatacggt tatccacaga atcaggggat aacgcaggaa agaacatgtg agcaaaaggc 1020

cagcaaaagg ccaggaaccg taaaaaggcc gcgttgctgg cgtttttcca taggctccgc 1080

ccccctgacg agcatcacaa aaatcgacgc tcaagtcaga ggtggcgaaa cccgacagga 1140

ctataaagat accaggcgtt tccccctgga agctccctcg tgcgctctcc tgttccgacc 1200

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cacgaacccc ccgttcagcc cgaccgctgc gccttatccg gtaactatcg tcttgagtcc 1380

aacccggtaa gacacgactt atcgccactg gcagcagcca ctggtaacag gattagcaga 1440

gcgaggtatg taggcggtgc tacagagttc ttgaagtggt ggcctaacta cggctacact 1500

agaagaacag tatttggtat ctgcgctctg ctgaagccag ttaccttcgg aaaaagagtt 1560 ggtagctctt gatccggcaa acaaaccacc gctggtagcg gtggtttttt tgtttacaag 1620

cagcagatta cgcgcagaaa aaaaggatct caagaagatc ctttgatctt ttctacgggg 1680

tctgacgctc agctagcgct cagaagaact cgtcaagaag gcgatagaag gcgatgcgct 1740

gcgaatcggg agcggcgata ccgtaaagca cgaggaagcg gtcagcccat tcgccgccaa 1800

gctcttcagc aatatcacgg gtagccaacg ctatgtcctg atagcggtcc gccacaccca 1860

gccggccaca gtcgatgaat ccagaaaagc ggccattttc caccatgata ttcggcaagc 1920

aggcatcgcc atgagtcacg acgagatcct cgccgtcggg catgcgcgcc ttgagcctgg 1980

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gccagtccct tcccgcttca gtgacaacgt cgagcacagc tgcgcaagga acgcccgtcg 2280

tggccagcca cgatagccgc gctgcctcgt cctgcagttc attcagggca ccggacaggt 2340

cggtcttgac aaaaagaacc gggcgcccct gcgctgacag ccggaacacg gcggcatcag 2400

agcagccgat tgtctgttgt gcccagtcat agccgaatag cctctccacc caagcggccg 2460

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gatcagatct tgatcccctg cgccatcaga tccttggcgg caagaaagcc atccagttta 2580

ctttgcaggg cttcccaacc ttaccagagg gcgccccagc tggcaattcc ggttcgcttg 2640

ctgtccataa aaccgcccag tctagcaact gttgggaagg gcgatcgtgt aatacgactc 2700

actatagggc gaattggagc t 2721

<210> 27
<211> 2716
<212> DNA
<213> artificial sequence

<220>
<223> This is the codon optimized dog-GHRH expression plasmid.

<400> 27
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gggtgaggaa tggtggggag ttatttttag agcggtgagg aaggtgggca ggcagcaggt 120

gttggcgctc taaaaataac tcccgggagt tatttttaga gcggaggaat ggtggacacc 180

caaatatggc gacggttcct cacccgtcgc catatttggg tgtccgccct cggccggggc 240

cgcattcctg ggggccgggc ggtgctcccg cccgcctcga taaaaggctc cggggccggc 300

ggcggcccac gagctacccg gaggagcggg aggcgccaag cggatcccaa ggcccaactc 360

cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct ctgggtgttc 420

ttcctggtga tcctcaccct cagcagtggt tcccactctt ccccgccatc cctgcccatc 480

agaatccctc ggtatgcaga cgccatcttc accaacagct accggaaggt gctgggccag 540

ctgtccgccc gcaagctcct scaggacatc atgagccggc agcagggaga gagaaaccgg 600
gagcaaggag catagtaagc ttatcggggt ggcatccctg tgacccctcc ccagtgcctc 660
tcctggccct ggaagttgcc actccagtgc ccaccagcct tgtcctaata aaattaagtt 720
gcatcatttt gtctgactag gtgtccttct ataatattat ggggtggagg ggggtggtat 780
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ttttgttccc tttagtgagg gttaatttcg agcttggtct tccgcttcct cgctcactga 900
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acgctgtagg tatctcagtt cggtgtaggt cgttcgctcc aagctgggct gtgtgcacga 1320
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aacagtattt ggtatctgcg ctctgctgaa gccagttacc ttcggaaaaa gagttggtag 1560
ctcttgatcc ggcaaacaaa ccaccgctgg tagcggtggt ttttttgttt acaagcagca 1620
gattacgcgc agaaaaaaag gatctcaaga agatcctttg atcttttcta cggggtctga 1680
cgctcagcta gcgctcagaa gaactcgtca agaaggcgat agaaggcgat gcgctgcgaa 1740
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ccacagtcga tgaatccaga aaagcggcca ttttccacca tgatattcgg caagcaggca 1920
tcgccatgag tcacgacgag atcctcgccg tcgggcatgc gcgccttgag cctggcgaac 1980
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agccacgata gccgcgctgc ctcgtcctgc agttcattca gggcaccgga caggtcggtc 2340
ttgacaaaaa gaaccgggcg cccctgcgct gacagccgga acacggcggc atcagagcag 2400
ccgattgtct gttgtgccca gtcatagccg aatagcctct ccacccaagc ggccggagaa 2460
cctgcgtgca atccatcttg ttcaatcatg cgaaacgatc ctcatcctgt ctcttgatca 2520
gatcttgatc ccctgcgcca tcagatcctt ggcggcaaga aagccatcca gtttactttg 2580

PCT/US05/25747

cagggcttcc caaccttacc agagggcgcc ccagctggca attccggttc gcttgctgtc 2640

cataaaaccg cccagtctag caactgttgg gaagggcgat cgtgtaatac gactcactat 2700

agggcgaatt ggagct 2716

<210> 28
<211> 2716
<212> DNA
<213> artificial sequence

<220>
<223> This is the codon optimized bovine-GHRH expression plasmid.

<400> 28
ccaccgcggt ggcggccgtc cgccctcggc accatcctca cgacacccaa atatggcgac 60

gggtgaggaa tggtggggag ttatttttag agcggtgagg aaggtgggca ggcagcaggt 120

gttggcgctc taaaaataac tcccgggagt tatttttaga gcggaggaat ggtggacacc 180

caaatatggc gacggttcct cacccgtcgc catatttggg tgtccgccct cggccggggc 240

cgcattcctg ggggccgggc ggtgctcccg cccgcctcga taaaaggctc cggggccggc 300

ggcggcccac gagctacccg gaggagcggg aggcgccaag cggatcccaa ggcccaactc 360

cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct gtgggtgttc 420

ttcctggtga ccctgaccct gagcagcggc tcccacggct ccctgccctc ccagcctctg 480

cgcatccctc gctacgccga cgccatcttc accaacagct accgcaaggt gctcggccag 540

ctcagcgccc gcaagctcct gcaggacatc atgaaccggc agcagggcga gcgcaaccag 600

gagcagggag cctgataagc ttatcggggt ggcatccctg tgacccctcc ccagtgcctc 660

tcctggccct ggaagttgcc actccagtgc ccaccagcct tgtcctaata aaattaagtt 720

gcatcatttt gtctgactag gtgtccttct ataatattat ggggtggagg ggggtggtat 780

ggagcaaggg gcaagttggg aagacaacct gtagggctcg aggggggccc cggtaccagc 840

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ctcgctgcgc tcggtcgttc ggctgcggcg agcggtatca gctcactcaa aggcggtaat 960

acggttatcc acagaatcag gggataacgc aggaaagaac atgtgagcaa aaggccagca 1020

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tgacgagcat cacaaaaatc gacgctcaag tcagaggtgg cgaaacccga caggactata 1140

aagataccag gcgtttcccc ctggaagctc cctcgtgcgc tctcctgttc cgaccctgcc 1200

gcttaccgga tacctgtccg cctttctccc ttcgggaagc gtggcgcttt ctcatagctc 1260

acgctgtagg tatctcagtt cggtgtaggt cgttcgctcc aagctgggct gtgtgcacga 1320

accccccgtt cagcccgacc gctgcgcctt atccggtaac tatcgtcttg agtccaaccc 1380

ggtaagacac gacttatcgc cactggcagc agccactggt aacaggatta gcagagcgag 1440

gtatgtaggc ggtgctacag agttcttgaa gtggtggcct aactacggct acactagaag 1500

aacagtattt ggtatctgcg ctctgctgaa gccagttacc ttcggaaaaa gagttggtag 1560 ctcttgatcc ggcaaacaaa ccaccgctgg tagcggtggt ttttttgttt acaagcagca 1620

gattacgcgc agaaaaaaag gatctcaaga agatcctttg atcttttcta cggggtctga 1680

cgctcagcta gcgctcagaa gaactcgtca agaaggcgat agaaggcgat gcgctgcgaa 1740

tcgggagcgg cgataccgta aagcacgagg aagcggtcag cccattcgcc gccaagctct 1800

tcagcaatat cacgggtagc caacgctatg tcctgatagc ggtccgccac acccagccgg 1860

ccacagtcga tgaatccaga aaagcggcca ttttccacca tgatattcgg caagcaggca 1920

tcgccatgag tcacgacgag atcctcgccg tcgggcatgc gcgccttgag cctggcgaac 1980

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gtagccggat caagcgtatg cagccgccgc attgcatcag ccatgatgga tactttctcg 2160

gcaggagcaa ggtgagatga caggagatcc tgccccggca cttcgcccaa tagcagccag 2220

tcccttcccg cttcagtgac aacgtcgagc acagctgcgc aaggaacgcc cgtcgtggcc 2280

agccacgata gccgcgctgc ctcgtcctgc agttcattca gggcaccgga caggtcggtc 2340

ttgacaaaaa gaaccgggcg cccctgcgct gacagccgga acacggcggc atcagagcag 2400

ccgattgtct gttgtgccca gtcatagccg aatagcctct ccacccaagc ggccggagaa 2460

cctgcgtgca atccatcttg ttcaatcatg cgaaacgatc ctcatcctgt ctcttgatca 2520

gatcttgatc ccctgcgcca tcagatcctt ggcggcaaga aagccatcca gtttactttg 2580

cagggcttcc caaccttacc agagggcgcc ccagctggca attccggttc gcttgctgtc 2640

cataaaaccg cccagtctag caactgttgg gaagggcgat cgtgtaatac gactcactat 2700

agggcgaatt ggagct 2716

<210> 29
<211> 2716
<212> DNA
<213> artificial sequence

<220>
<223> This is the codon optimized cat-GHRH expression plasmid.

<400> 29
ccaccgcggt ggcggccgtc cgccctcggc accatcctca cgacacccaa atatggcgac 60

gggtgaggaa tggtggggag ttatttttag agcggtgagg aaggtgggca ggcagcaggt 120

gttggcgctc taaaaataac tcccgggagt tatttttaga gcggaggaat ggtggacacc 180

caaatatggc gacggttcct cacccgtcgc catatttggg tgtccgccct cggccggggc 240

cgcattcctg ggggccgggc ggtgctcccg cccgcctcga taaaaggctc cggggccggc 300

ggcggcccac gagctacccg gaggagcggg aggcgccaag cggatcccaa ggcccaactc 360

cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct ctgggtgttc 420

ttcctggtga tcctcacccs ssacagtggc tcccactgct ccccgccatc cctgcccctc 480

agaatgcctc ggtatgcaga tgccatcttc accaacagct accggaaggt gctgggtcag 540

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gagcaaggag cataataagc ttatcggggt ggcatccctg tgacccctcc ccagtgcctc    660
tcctggccct ggaagttgcc actccagtgc ccaccagcct tgtcctaata aaattaagtt    720
gcatcatttt gtctgactag gtgtccttct ataatattat ggggtggagg ggggtggtat    780
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gattacgcgc agaaaaaaag gatctcaaga agatcctttg atcttttcta cggggtctga   1680
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tcagcaatat cacgggtagc caacgctatg tcctgatagc ggtccgccac acccagccgg   1860
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ccgattgtct gttgtgccca gtcatagccg aatagcctct ccacccaagc ggccggagaa   2460
cctgcgtgca atccatcttg ttcaatcatg cgaaacgatc ctcatcctgt ctcttgatca   2520
gatcttgatc ccctgcgcca tcagatcctt ggcggcaaga aagccatcca gtttactttg   2580
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cagggcttcc caaccttacc agagggcgcc ccagctggca attccggttc gcttgctgtc 2640

cataaaaccg cccagtctag caactgttgg gaagggcgat cgtgtaatac gactcactat 2700

agggcgaatt ggagct 2716

<210> 30
<211> 2739
<212> DNA
<213> artificial sequence

<220>
<223> This is the codon optimized TI-GHRH expression plasmid.

<400> 30
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gggtgaggaa tggtggggag ttatttttag agcggtgagg aaggtgggca ggcagcaggt 120

gttggcgctc taaaaataac tcccgggagt tatttttaga gcggaggaat ggtggacacc 180

caaatatggc gacggttcct cacccgtcgc catatttggg tgtccgccct cggccggggc 240

cgcattcctg ggggccgggc ggtgctcccg cccgcctcga taaaaggctc cggggccggc 300

ggcggcccac gagctacccg gaggagcggg aggcgccaag cggatcccaa ggcccaactc 360

cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct ctgggtgttc 420

ttctttgtga tcctcaccct cagcaacagc tcccactgct ccccacctcc ccctttgacc 480

ctcaggatgc ggcggtatat cgatgccatc ttcaccaaca gctaccggaa ggtgctggcc 540

cagctgtccg cccgcaagct gctccaggac atcctgaaca ggcagcaggg agagaggaac 600

caagagcaag gagcataatg actgcaggaa ttcgatatca agcttatcgg ggtggcatcc 660

ctgtgacccc tccccagtgc ctctcctggc cctggaagtt gccactccag tgcccaccag 720

ccttgtccta ataaaattaa gttgcatcat tttgtctgac taggtgtcct tctataatat 780

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tcgagggggg gcccggtacc agcttttgtt ccctttagtg agggttaatt tcgagcttgg 900

tcttccgctt cctcgctcac tgactcgctg cgctcggtcg ttcggctgcg gcgagcggta 960

tcagctcact caaaggcggt aatacggtta tccacagaat caggggataa cgcaggaaag 1020

aacatgtgag caaaaggcca gcaaaaggcc aggaaccgta aaaaggccgc gttgctggcg 1080

tttttccata ggctccgccc ccctgacgag catcacaaaa atcgacgctc aagtcagagg 1140

tggcgaaacc cgacaggact ataaagatac caggcgtttc cccctggaag ctccctcgtg 1200

cgctctcctg ttccgaccct gccgcttacc ggatacctgt ccgcctttct cccttcggga 1260

agcgtggcgc tttctcatag ctcacgctgt aggtatctca gttcggtgta ggtcgttcgc 1320

tccaagctgg gctgtgtgca cgaacccccc gttcagcccg accgctgcgc cttatccggt 1380

aactatcgtc ttgagtccaa cccggtaaga cacgacttat cgccactggc agcagccact 1440

ggtaacagga ttagcagagc gaggtatgta ggcggtgcta cagagttctt gaagtggtgg 1500

cctaactacg gctacactag aagaacagta tttggtatct gcgctctgct gaagccagtt 1560

PCT/US05/25747

accttcggaa aaagagttgg tagctcttga tccggcaaac aaaccaccgc tggtagcggt 1620

ggtttttttg tttacaagca gcagattacg cgcagaaaaa aaggatctca agaagatcct 1680

ttgatctttt ctacggggtc tgacgctcag ctagcgctca gaagaactcg tcaagaaggc 1740

gatagaaggc gatgcgctgc gaatcgggag cggcgatacc gtaaagcacg aggaagcggt 1800

cagcccattc gccgccaagc tcttcagcaa tatcacgggt agccaacgct atgtcctgat 1860

agcggtccgc cacacccagc cggccacagt cgatgaatcc agaaaagcgg ccattttcca 1920

ccatgatatt cggcaagcag gcatcgccat gagtcacgac gagatcctcg ccgtcgggca 1980

tgcgcgcctt gagcctggcg aacagttcgg ctggcgcgag cccctgatgc tcttcgtcca 2040

gatcatcctg atcgacaaga ccggcttcca tccgagtacg tgctcgctcg atgcgatgtt 2100

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ggaacacggc ggcatcagag cagccgattg tctgttgtgc ccagtcatag ccgaatagcc 2460

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gcaattccgg ttcgcttgct gtccataaaa ccgcccagtc tagcaactgt tgggaagggc 2700

gatcgtgtaa tacgactcac tatagggcga attggagct 2739

<210> 31
<211> 2716
<212> DNA
<213> artificial sequence

<220>
<223> This is the codon optimized ovine-GHRH expression plasmid.

<400> 31
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gggtgaggaa tggtggggag ttatttttag agcggtgagg aaggtgggca ggcagcaggt 120

gttggcgctc taaaaataac tcccgggagt tatttttaga gcggaggaat ggtggacacc 180

caaatatggc gacggttcct cacccgtcgc catatttggg tgtccgccct cggccggggc 240

cgcattcctg ggggccgggc ggtgctcccg cccgcctcga taaaaggctc cggggccggc 300

ggcggcccac gagctacccg gaggagcggg aggcgccaag cggatcccaa ggcccaactc 360

cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct gtgggtgttc 420

ttcctggtga ccctgaccct gagcagcgga agccacggca gcctgcccag ccagcccctg 480

aggatcccta ggtacgccga cgccatcttc accaacagct acaggaagat cctgggccag 540 ctgagcgcta ggaagctcct gcaggacatc atgaacaggc agcagggcga gaggaaccag 600

gagcagggcg cctgataagc ttatcggggt ggcatccctg tgacccctcc ccagtgcctc 660

tcctggccct ggaagttgcc actccagtgc ccaccagcct tgtcctaata aaattaagtt 720

gcatcatttt gtctgactag gtgtccttct ataatattat ggggtggagg ggggtggtat 780

ggagcaaggg gcaagttggg aagacaacct gtagggctcg agggggggcc cggtaccagc 840

ttttgttccc tttagtgagg gttaatttcg agcttggtct tccgcttcct cgctcactga 900

ctcgctgcgc tcggtcgttc ggctgcggcg agcggtatca gctcactcaa aggcggtaat 960

acggttatcc acagaatcag gggataacgc aggaaagaac atgtgagcaa aaggccagca 1020

aaaggccagg aaccgtaaaa aggccgcgtt gctggcgttt ttccataggc tccgcccccc 1080

tgacgagcat cacaaaaatc gacgctcaag tcagaggtgg cgaaacccga caggactata 1140

aagataccag gcgtttcccc ctggaagctc cctcgtgcgc tctcctgttc cgaccctgcc 1200

gcttaccgga tacctgtccg cctttctccc ttcgggaagc gtggcgcttt ctcatagctc 1260

acgctgtagg tatctcagtt cggtgtaggt cgttcgctcc aagctgggct gtgtgcacga 1320

accccccgtt cagcccgacc gctgcgcctt atccggtaac tatcgtcttg agtccaaccc 1380

ggtaagacac gacttatcgc cactggcagc agccactggt aacaggatta gcagagcgag 1440

gtatgtaggc ggtgctacag agttcttgaa gtggtggcct aactacggct acactagaag 1500

aacagtattt ggtatctgcg ctctgctgaa gccagttacc ttcggaaaaa gagttggtag 1560

ctcttgatcc ggcaaacaaa ccaccgctgg tagcggtggt ttttttgttt acaagcagca 1620

gattacgcgc agaaaaaaag gatctcaaga agatcctttg atcttttcta cggggtctga 1680

cgctcagcta gcgctcagaa gaactcgtca agaaggcgat agaaggcgat gcgctgcgaa 1740

tcgggagcgg cgataccgta aagcacgagg aagcggtcag cccattcgcc gccaagctct 1800

tcagcaatat cacgggtagc caacgctatg tcctgatagc ggtccgccac acccagccgg 1860

ccacagtcga tgaatccaga aaagcggcca ttttccacca tgatattcgg caagcaggca 1920

tcgccatgag tcacgacgag atcctcgccg tcgggcatgc gcgccttgag cctggcgaac 1980

agttcggctg gcgcgagccc ctgatgctct tcgtccagat catcctgatc gacaagaccg 2040

gcttccatcc gagtacgtgc tcgctcgatg cgatgtttcg cttggtggtc gaatgggcag 2100

gtagccggat caagcgtatg cagccgccgc attgcatcag ccatgatgga tactttctcg 2160

gcaggagcaa ggtgagatga caggagatcc tgccccggca cttcgcccaa tagcagccag 2220

tcccttcccg cttcagtgac aacgtcgagc acagctgcgc aaggaacgcc cgtcgtggcc 2280

agccacgata gccgcgctgc ctcgtcctgc agttcattca gggcaccgga caggtcggtc 2340

ttgacaaaaa gaaccgggcg cccctgcgct gacagccgga acacggcggc atcagagcag 2400

ccgattgtct gttgtgccca gtcatagccg aatagcctct ccacccaagc ggccggagaa 2460

cctgcgtgca atccatcttg ttcaatcatg cgaaacgatc ctcatcctgt ctcttgatca 2520

gatcttgatc ccctgcgcca tcagatcctt ggcggcaaga aagccatcca gtttactttg 2580 caggcttcc caaccttacc agagggcgcc ccagctggca attccggttc gcttgctgtc 2640

cataaaaccg cccagtctag caactgttgg gaagggcgat cgtgtaatac gactcactat 2700

agggcgaatt ggagct 2716

<210> 32
<211> 2725
<212> DNA
<213> artificial sequence

<220>
<223> This is the codon optimized chicken-GHRH expression plasmid.

<400> 32
ccaccgcggt ggcggccgtc cgccctcggc accatcctca cgacacccaa atatggcgac 60

gggtgaggaa tggtggggag ttatttttag agcggtgagg aaggtgggca ggcagcaggt 120

gttggcgctc taaaaataac tcccgggagt tatttttaga gcggaggaat ggtggacacc 180

caaatatggc gacggttcct cacccgtcgc catatttggg tgtccgccct cggccggggc 240

cgcattcctg ggggccgggc ggtgctcccg cccgcctcga taaaaggctc cggggccggc 300

ggcggcccac gagctacccg gaggagcggg aggcgccaag cggatcccaa ggcccaactc 360

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tttgtgctgc tgaccctgac ctccggaagc cactgcagcc tgccacccag cccacccttc 480

cgcgtcaggc gccacgccga cggcatcttc agcaaggcct accgcaagct cctgggccag 540

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gacgaggccg agcccctgag ctgataagct tatcggggtg gcatccctgt gacccctccc 660

cagtgcctct cctggccctg gaagttgcca ctccagtgcc caccagcctt gtcctaataa 720

aattaagttg catcattttg tctgactagg tgtccttcta taatattatg gggtggaggg 780

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aggactataa agataccagg cgtttccccc tggaagctcc ctcgtgcgct ctcctgttcc 1200

gaccctgccg cttaccggat acctgtccgc ctttctccct tcgggaagcg tggcgctttc 1260

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tgtgcacgaa ccccccgttc agcccgaccg ctgcgcctta tccggtaact atcgtcttga 1380

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cagagcgagg tatgtaggcg gtgctacaga gttcttgaag tggtggccta actacggcta 1500

cactagaaga acagtatttg gtatctgcgc tctgctgaag ccagttacct tcggaaaaag 1560 agttggtagc tcttgatccg gcaaacaaac caccgctggt agcggtggtt tttttgttta 1620

caagcagcag attacgcgca gaaaaaaagg atctcaagaa gatcctttga tcttttctac 1680

ggggtctgac gctcagctag cgctcagaag aactcgtcaa gaaggcgata gaaggcgatg 1740

cgctgcgaat cgggagcggc gataccgtaa agcacgagga agcggtcagc ccattcgccg 1800

ccaagctctt cagcaatatc acgggtagcc aacgctatgt cctgatagcg gtccgccaca 1860

cccagccggc cacagtcgat gaatccagaa aagcggccat tttccaccat gatattcggc 1920

aagcaggcat cgccatgagt cacgacgaga tcctcgccgt cgggcatgcg cgccttgagc 1980

ctggcgaaca gttcggctgg cgcgagcccc tgatgctctt cgtccagatc atcctgatcg 2040

acaagaccgg cttccatccg agtacgtgct cgctcgatgc gatgtttcgc ttggtggtcg 2100

aatgggcagg tagccggatc aagcgtatgc agccgccgca ttgcatcagc catgatggat 2160

actttctcgg caggagcaag gtgagatgac aggagatcct gccccggcac ttcgcccaat 2220

agcagccagt cccttcccgc ttcagtgaca acgtcgagca cagctgcgca aggaacgccc 2280

gtcgtggcca gccacgatag ccgcgctgcc tcgtcctgca gttcattcag ggcaccggac 2340

aggtcggtct tgacaaaaag aaccgggcgc ccctgcgctg acagccggaa cacggcggca 2400

tcagagcagc cgattgtctg ttgtgcccag tcatagccga atagcctctc cacccaagcg 2460

gccggagaac ctgcgtgcaa tccatcttgt tcaatcatgc gaaacgatcc tcatcctgtc 2520

tcttgatcag atcttgatcc cctgcgccat cagatccttg gcggcaagaa agccatccag 2580

tttactttgc agggcttccc aaccttacca gagggcgccc cagctggcaa ttccggttcg 2640

cttgctgtcc ataaaaccgc ccagtctagc aactgttggg aagggcgatc gtgtaatacg 2700

actcactata gggcgaattg gagct 2725

<210> 33
<211> 2700
<212> DNA
<213> artificial sequence

<220>
<223> This is the optimized plasmid for Horse GHRH.

<400> 33
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gggtgaggaa tggtggggag ttatttttag agcggtgagg aaggtgggca ggcagcaggt 120

gttggcgctc taaaaataac tcccgggagt tatttttaga gcggaggaat ggtggacacc 180

caaatatggc gacggttcct cacccgtcgc catatttggg tgtccgccct cggccggggc 240

cgcattcctg gggggccgggc ggtgctcccg cccgcctcga taaaaggctc cggggccggc 300

ggcggcccac gagctacccg gaggagcggg aggcgccaag cggatcccaa ggcccaactc 360

cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct ctgggtgttc 420

ttctttgtga tcctcaccct cagcaacagc tcccactgct ccccacctcc ccctttgacc 480

ctcaggatgc ggcggtatgc agatgccatc ttcaccaaca gctaccggaa ggtgctgggc 540

cagctgtccg cccgcaagct gctgcaggac atcatgagca ggcagcaggg agagagcaac 600
caagagcgag gagcataatg aaagcttatc ggggtggcat ccctgtgacc cctccccagt 660
gcctctcctg gccctggaag ttgccactcc agtgcccacc agccttgtcc taataaaatt 720
aagttgcatc attttgtctg actaggtgtc cttctataat attatggggt ggaggggggt 780
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cacgaacccc ccgttcagcc cgaccgctgc gccttatccg gtaactatcg tcttgagtcc 1380
aacccggtaa gacacgactt atcgccactg gcagcagcca ctggtaacag gattagcaga 1440
gcgaggtatg taggcggtgc tacagagttc ttgaagtggt ggcctaacta cggctacact 1500
agaagaacag tatttggtat ctgcgctctg ctgaagccag ttaccttcgg aaaaagagtt 1560
ggtagctctt gatccggcaa acaaaccacc gctggtagcg gtggtttttt tgtttacaag 1620
cagcagatta cgcgcagaaa aaaaggatct caagaagatc ctttgatctt ttctacgggg 1680
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gagaacctgc gtgcaatcca tcttgttcaa tcatgcgaaa cgatcctcat cctgtctctt 2520
gatcagatct tgatcccctg cgccatcaga tccttggcgg caagaaagcc atccagttta 2580

ctttgcaggg cttcccaacc ttaccagagg gcgccccagc tggcaattcc ggttcgcttg 2640

ctgtccataa aaccgcccag tctagcaact gttgggaagg gcgatcgtgt aatacgactc 2700

<210> 34
<211> 2721
<212> DNA
<213> artificial sequence

<220>
<223> This is the codon optimized Human-GHRH expression plasmid.

<400> 34
ccaccgcggt ggcggccgtc cgccctcggc accatcctca cgacacccaa atatggcgac 60

gggtgaggaa tggtggggag ttatttttag agcggtgagg aaggtgggca ggcagcaggt 120

gttggcgctc taaaaataac tcccgggagt tatttttaga gcggaggaat ggtggacacc 180

caaatatggc gacggttcct cacccgtcgc catatttggg tgtccgccct cggccggggc 240

cgcattcctg ggggccgggc ggtgctcccg cccgcctcga taaaaggctc cggggccggc 300

ggcggcccac gagctacccg gaggagcggg aggcgccaag cggatcccaa ggcccaactc 360

cccgaaccac tcagggtcct gtggacagct cacctagctg ccatggtgct ctgggtgttc 420

ttctttgtga tcctcaccct cagcaacagc tcccactgct ccccacctcc cccttgacc 480

ctcaggatgc ggcggtatgc agatgccatc ttcaccaaca gctaccggaa ggtgctgggc 540

cagctgtccg cccgcaagct gctgcaggac atcatgagca ggcagcaggg agagagcaac 600

caagagcgag gagcataatg aaagcttatc ggggtggcat ccctgtgacc cctccccagt 660

gcctctcctg gccctggaag ttgccactcc agtgcccacc agccttgtcc taataaaatt 720

aagttgcatc attttgtctg actaggtgtc cttctataat attatggggt ggaggggggt 780

ggtatggagc aaggggcaag ttgggaagac aacctgtagg gctcgagggg gggcccggta 840

ccagcttttg ttccctttag tgagggttaa tttcgagctt ggtcttccgc ttcctcgctc 900

actgactcgc tgcgctcggt cgttcggctg cggcgagcgg tatcagctca ctcaaaggcg 960

gtaatacggt tatccacaga atcaggggat aacgcaggaa agaacatgtg agcaaaaggc 1020

cagcaaaagg ccaggaaccg taaaaaggcc gcgttgctgg cgtttttcca taggctccgc 1080

ccccctgacg agcatcacaa aaatcgacgc tcaagtcaga ggtggcgaaa cccgacagga 1140

ctataaagat accaggcgtt tccccctgga agctccctcg tgcgctctcc tgttccgacc 1200

ctgccgctta ccggatacct gtccgccttt ctcccttcgg gaagcgtggc gctttctcat 1260

agctcacgct gtaggtatct cagttcggtg taggtcgttc gctccaagct gggctgtgtg 1320

cacgaacccc ccgttcagcc cgaccgctgc gccttatccg gtaactatcg tcttgagtcc 1380

aacccggtaa gacacgactt atcgccactg gcagcagcca ctggtaacag gattagcaga 1440

gcgaggtatg taggcggtgc tacagagttc ttgaagtggt ggcctaacta cggctacact 1500

agaagaacag tatttggtat ctgcgctctg ctgaagccag ttaccttcgg aaaaagagtt 1560

ggtagctctt gatccggcaa acaaaccacc gctggtagcg gtggtttttt tgtttacaag 1620 cagcagatta cgcgcagaaa aaaaggatct caagaagatc ctttgatctt ttctacgggg 1680

tctgacgctc agctagcgct cagaagaact cgtcaagaag gcgatagaag gcgatgcgct 1740

gcgaatcggg agcggcgata ccgtaaagca cgaggaagcg gtcagcccat tcgccgccaa 1800

gctcttcagc aatatcacgg gtagccaacg ctatgtcctg atagcggtcc gccacaccca 1860

gccggccaca gtcgatgaat ccagaaaagc ggccattttc caccatgata ttcggcaagc 1920

aggcatcgcc atgagtcacg acgagatcct cgccgtcggg catgcgcgcc ttgagcctgg 1980

cgaacagttc ggctggcgcg agcccctgat gctcttcgtc cagatcatcc tgatcgacaa 2040

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ggcaggtagc cggatcaagc gtatgcagcc gccgcattgc atcagccatg atggatactt 2160

tctcggcagg agcaaggtga gatgacagga gatcctgccc cggcacttcg cccaatagca 2220

gccagtccct tcccgcttca gtgacaacgt cgagcacagc tgcgcaagga acgcccgtcg 2280

tggccagcca cgatagccgc gctgcctcgt cctgcagttc attcagggca ccggacaggt 2340

cggtcttgac aaaaagaacc gggcgcccct gcgctgacag ccggaacacg gcggcatcag 2400

agcagccgat tgtctgttgt gcccagtcat agccgaatag cctctccacc caagcggccg 2460

gagaacctgc gtgcaatcca tcttgttcaa tcatgcgaaa cgatcctcat cctgtctctt 2520

gatcagatct tgatcccctg cgccatcaga tccttggcgg caagaaagcc atccagttta 2580

ctttgcaggg cttcccaacc ttaccagagg gcgccccagc tggcaattcc ggttcgcttg 2640

ctgtccataa aaccgcccag tctagcaact gttgggaagg gcgatcgtgt aatacgactc 2700

actatagggc gaattggagc t 2721

<210> 35
<211> 225
<212> DNA
<213> artificial sequence

<220>
<223> Sequence for an analog bovine GHRH sequence.

<400> 35
gccatggtgc tgtgggtgtt cttcctggtg accctgaccc tgagcagcgg ctcccacggc 60

tccctgccct cccagcctct gcgcatccct cgctacgccg acgccatctt caccaacagc 120

taccgcaagg tgctcggcca gctcagcgcc cgcaagctcc tgcaggacat catgaaccgg 180

cagcagggcg agcgcaacca ggagcaggga gcctgataag cttgc 225

<210> 36
<211> 225
<212> DNA
<213> artificial sequence

<220>
<223> Sequence for an analog ovine GHRH sequence.

<400> 36
gccatggtgc tgtgggtgtt cttcctggtg accctgaccc tgagcagcgg aagccacggc 60

agcctgccca gccagcccct gaggatccct aggtacgccg acgccatctt caccaacagc 120

tacaggaaga tcctgggcca gctgagcgct aggaagctcc tgcaggacat catgaacagg 180

cagcagggcg agaggaacca ggagcagggc gcctgataag cttgc 225

<210> 37
<211> 246
<212> DNA
<213> artificial sequence

<220>
<223> Sequence for an analog chicken GHRH sequence.

<400> 37
gccatggtgc tctgggtgct ctttgtgatc ctcatcctca ccagcggcag ccactgcagc 60

ctgcctccca gccctccctt caggatgcag aggcacgtgg acgccatctt caccaccaac 120

tacaggaagc tgctgagcca gctgtacgcc aggaaggtga tccaggacat catgaacaag 180

cagggcgaga ggatccagga gcagagggcc aggctgagct gataagcttg cgatgagttc 240

ttctaa 246

<210> 38
<211> 190
<212> DNA
<213> artificial sequence

<220>
<223> Nucleic acid sequence of human growth hormone poly A tail.

<400> 38
gggtggcatc cctgtgaccc ctccccagtg cctctcctgg ccctggaagt tgccactcca 60

gtgcccacca gccttgtcct aataaaatta agttgcatca ttttgtctga ctaggtgtcc 120

ttctataata ttatggggtg gaggggggtg gtatggagca aggggcaagt tgggaagaca 180

acctgtaggg 190

<210> 39
<211> 55
<212> DNA
<213> artificial sequence

<220>
<223> Nucleic acid sequence of human growth hormone 5' UTR

<400> 39
caaggcccaa ctccccgaac cactcagggt cctgtggaca gctcacctag ctgcc 55

<210> 40
<211> 782
<212> DNA
<213> artificial sequence

<220>
<223> Nucleic acid sequence of a plasmid pUC-18 origin of replication

<400> 40
tcttccgctt cctcgctcac tgactcgctg cgctcggtcg ttcggctgcg gcgagcggta 60

tcagctcact caaaggcggt aatacggtta tccacagaat caggggataa cgcaggaaag 120

aacatgtgag caaaaggcca gcaaaaggcc aggaaccgta aaaaggccgc gttgctggcg 180

tttttccata ggctccgccc ccctgacgag catcacaaaa atcgacgctc aagtcagagg 240

tggcgaaacc cgacaggact ataaagatac caggcgtttc cccctggaag ctccctcgtg 300

cgctctcctg ttccgaccct gccgcttacc ggatacctgt ccgcctttct cccttcggga 360

agcgtggcgc tttctcatag ctcacgctgt aggtatctca gttcggtgta ggtcgttcgc 420

tccaagctgg gctgtgtgca cgaacccccc gttcagcccg accgctgcgc cttatccggt 480

aactatcgtc ttgagtccaa cccggtaaga cacgacttat cgccactggc agcagccact 540

ggtaacagga ttagcagagc gaggtatgta ggcggtgcta cagagttctt gaagtggtgg 600

cctaactacg gctacactag aaggacagta tttggtatct gcgctctgct gaagccagtt 660

accttcggaa aaagagttgg tagctcttga tccggcaaac aaaccaccgc tggtagcggt 720

ggtttttttg tttgcaagca gcagattacg cgcagaaaaa aaggatctca agaagatcct 780

tt 782

<210> 41
<211> 5
<212> DNA
<213> artificial sequence

<220>
<223> This is a NEO ribosomal binding site

<400> 41
tcctc 5