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(54) Titre : VECTEURS ET PROCEDES DESTINES A L'IMMUNISATION ET A DES PROTOCOLES THERAPEUTIQUES
(54) Title: VECTORS AND METHODS FOR IMMUNIZATION OR THERAPEUTIC PROTOCOLS

(57) Abrégé/Abstract:
The present invention shows that DNA vaccine vectors can be improved by removal of CpG-N motifs and optional addition of CpG-S motifs. In addition, for high and long-lasting levels of expression, the optimized vector should include a promoter/enhancer that is not down-regulated by the cytokines induced by the immunostimulatory CpG motifs. Vectors and methods of use for immunostimulation are provided herein. The invention also provides improved gene therapy vectors by determining the CpG-N and CpG-S motifs present in the construct, removing stimulatory CpG (CpG-S) motifs and/or inserting neutralizing CpG (CpG-N) motifs, thereby producing a nucleic acid construct providing enhanced expression of the therapeutic polypeptide. Methods of use for such vectors are also included herein.

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(54) Title: VECTORS AND METHODS FOR IMMUNIZATION OR THERAPEUTIC PROTOCOLS

(57) Abstract

The present invention shows that DNA vaccine vectors can be improved by removal of CpG–N motifs and optional addition of CpG–S motifs. In addition, for high and long–lasting levels of expression, the optimized vector should include a promoter/enhancer that is not down–regulated by the cytokines induced by the immunostimulatory CpG motifs. Vectors and methods of use for immunostimulation are provided herein. The invention also provides improved gene therapy vectors by determining the CpG–N and CpG–S motifs present in the construct, removing stimulatory CpG (CpG–S) motifs and/or inserting neutralizing CpG (CpG–N) motifs, thereby producing a nucleic acid construct providing enhanced expression of the therapeutic polypeptide. Methods of use for such vectors are also included herein.

VECTORS AND METHODS FOR IMMUNIZATION OR THERAPEUTIC PROTOCOLS

TECHNICAL FIELD

This invention relates generally to immune responses and more particularly to vectors containing immunostimulatory CpG motifs and/or a reduced number of neutralizing motifs and methods of use for immunization purposes as well as vectors containing neutralizing motifs and/or a reduced number of immunostimulatory CpG motifs and methods of use for gene therapy protocols.

BACKGROUND

Bacterial DNA, but not vertebrate DNA, has direct immunostimulatory effects on peripheral blood mononuclear cells (PBMC) *in vitro* (Messina *et al.*, *J. Immunol.* 147: 1759-1764, 1991; Tokanuga *et al.*, *JNCI.* 72: 955, 1994). These effects include proliferation of almost all (>95%) B cells and increased immunoglobulin (Ig) secretion (Krieg *et al.*, . *Nature.* 374: 546-549, 1995). In addition to its direct effects on B cells, CpG DNA also directly activates monocytes, macrophages, and dendritic cells to secrete predominantly Th 1 cytokines, including high levels of IL-12 (Klinman, D., *et al. Proc. Natl. Acad. Sci. USA.* 93: 2879-2883 (1996); Halpern *et al,* 1996; Cowdery *et al.*, *J. Immunol.* 156: 4570-4575 (1996). These cytokines stimulate natural killer (NK) cells to secrete γ-interferon (IFN-γ) and to have increased lytic activity (Klinman *et al.*, 1996, *supra*; Cowdery *et al.*, 1996, *supra*; Yamamoto *et al.*, *J. Immunol.* 148: 4072-4076 (1992); Ballas *et al.*, *J. Immunol.* 157: 1840-1845 (1996)). These stimulatory effects have been found to be due to the presence of unmethylated CpG dinucleotides in a particular sequence context (CpG-S motifs) (Krieg *et al.*, 1995, *supra*). Activation may also be triggered by addition of synthetic oligodeoxynucleotides (ODN) that contain CpG-S motifs (Tokunaga *et al.*, *Jpn. J. Cancer Res.* 79: 682-686 1988; Yi *et al.*, *J. Immunol.* 156: 558-564, 1996; Davis *et al.*, *J. Immunol.* 160: 870-876, 1998).

Unmethylated CpG dinucleotides are present at the expected frequency in bacterial DNA but are under-represented and methylated in vertebrate DNA (Bird, *Trends in Genetics.* 3: 342-347, 1987). Thus, vertebrate DNA essentially does not contain CpG stimulatory (CpG-S) motifs and it appears likely that the rapid immune activation in response to CpG-S DNA may have evolved as one component of the innate immune defense mechanisms that recognize structural patterns specific to microbial molecules.

Viruses have evolved a broad range of sophisticated strategies for avoiding host immune defenses. For example, nearly all DNA viruses and retroviruses appear to have escaped the defense mechanism of the mammalian immune system to respond to immunostimulatory CpG motifs. In most cases this has been accomplished through reducing their genomic content of CpG dinucleotides by 50-94% from that expected based on random base usage (Karlin *et al.*, *J. Virol.* 68: 2889-2897, 1994). CpG suppression is absent from bacteriophage, indicating that it is not an inevitable result of having a small genome. Statistical analysis indicates that the CpG suppression in lentiviruses is an evolutionary adaptation to replication in a eukaryotic host (Shaper *et al.*, *Nucl. Acids Res.* 18: 5793-5797, 1990).

Nearly all DNA viruses and retroviruses appear to have evolved to avoid this defense mechanism through reducing their genomic content of CpG dinucleotides by 50-94% from that expected based on random base usage. CpG suppression is absent from bacteriophage, indicating that it is not an inevitable result of having a small genome. Statistical analysis indicates that the CpG suppression in lentiviruses is an evolutionary adaptation to replication in a eukaryotic host. Adenoviruses, however, are an exception to this rule as they have the expected level of genomic CpG dinucleotides. Different groups of adenovirae can have quite different clinical characteristics. Serotype 2 and 5 adenoviruses (Subgenus C) are endemic causes of upper respiratory infections and are notable for their ability to establish persistent infections in lymphocytes. These adenoviral serotypes are frequently modified by deletion of early genes for use in gene therapy applications, where a major clinical problem has been the frequent inflammatory

immune responses to the viral particles. Serotype 12 adenovirus (subgenus A) does not establish latency, but can be oncogenic.

Despite high levels of unmethylated CpG dinucleotides, serotype 2 adenoviral DNA surprisingly is nonstimulatory and can actually inhibit activation by bacterial DNA. The arrangement and flanking bases of the CpG dinucleotides are responsible for this difference. Even though type 2 adenoviral DNA contains six times the expected frequency of CpG dinucleotides, it has CpG-S motifs at only one quarter of the frequency predicted by chance. Instead, most CpG motifs are found in clusters of direct repeats or with a C on the 5' side or a G on the 3' side. It appears that such CpG motifs are immune-neutralizing (CpG-N) in that they block the Th1-type immune activation by CpG-S motifs *in vitro*. Likewise, when CpG-N ODN and CpG-S are administered with antigen, the antigen-specific immune response is blunted compared to that with CpG-S alone. When CpG-N ODN alone is administered *in vivo* with an antigen, Th2-like antigen-specific immune responses are induced.

B cell activation by CpG-S DNA is T cell independent and antigen non-specific. However, B cell activation by low concentrations of CpG DNA has strong synergy with signals delivered through the B cell antigen receptor for both B cell proliferation and Ig secretion (Krieg *et al.*, 1995, *supra*). This strong synergy between the B cell signaling pathways triggered through the B cell antigen receptor and by CpG-S DNA promotes antigen specific immune responses. The strong direct effects (T cell independent) of CpG-S DNA on B cells, as well as the induction of cytokines which could have indirect effects on B-cells via T-help pathways, suggests utility of CpG-S DNA as a vaccine adjuvant. This could be applied either to classical antigen-based vaccines or to DNA vaccines. CpG-S ODN have potent Th-1 like adjuvant effects with protein antigens (Chu *et al.*, *J. Exp. Med.* 186: 1623-1631 1997; Lipford *et al.*, *Eur. J. Immunol.* 27: 2340-2344, 1997; Roman *et al.*, *Nature Med.* 3: 849-854, 1997; Weiner *et al.*, *Proc. Natl.*

Acad. Sci. USA. 94: 10833, 1997; Davis *et al.*, 1998, *supra*, Moldoveanu *et al.*, A Novel Adjuvant for Systemic and Mucosal Immunization with Influenza Virus. *Vaccine* (in press) 1998).

SUMMARY OF THE INVENTION

The present invention is based on the discovery that removal of neutralizing motifs (e.g., CpG-N or poly G) from a vector used for immunization purposes, results in an antigen-specific immunostimulatory effect greater than with the starting vector. Further, when neutralizing motifs (*e.g.*, CpG-N or poly G) are removed from the vector and stimulatory CpG-S motifs are inserted into the vector, the vector has even more enhanced immunostimulatory efficacy.

According to one aspect of the present invention, there is provided a method for enhancing the immunostimulatory effect of an antigen encoded by nucleic acid contained in a nucleic acid construct comprising:
(i) determining the neutralizing CpG (CpG-N) and stimulatory CpG (CpG-S) motifs present in the construct; and
(ii) removing at least one CpG-N motif, thereby producing a nucleic acid construct having enhanced immunostimulatory efficacy.

According to another aspect of the present invention, there is provided the use of a nucleic acid construct for stimulating a protective or therapeutic immune response in a subject, said nucleic acid construct comprising a nucleic acid that encodes an antigen having enhanced immunostimulatory efficacy and produced by the method described above.

According to still another aspect of the present invention, there is provided the use of a nucleic acid construct for enhancing the expression of a therapeutic

polypeptide *in vivo*, wherein the polypeptide is encoded by a nucleic acid contained in the nucleic acid construct and produced by determining the neutralizing CpG (CpG-N) and stimulatory CpG (CpG-S) motifs present in the construct, removing at least one stimulatory CpG-S motif, or inserting at least one neutralizing CpG-N motif, or a combination thereof, resulting in the nucleic acid construct providing enhanced expression of the therapeutic polypeptide.

According to yet another aspect of the present invention, there is provided the use of a nucleic acid construct for enhancing the expression of a therapeutic polypeptide *in vivo*, wherein the construct is produced by determining the neutralizing CpG (CpG-N) and stimulatory CpG (CpG-S) motifs present in the construct and removing at least one CpG-S motif, or inserting at least one CpG-N motif, or a combination thereof, resulting in enhanced expression of the therapeutic polypeptide.

In another embodiment, the invention provides a method for enhancing the immunostimulatory effect of an antigen encoded by nucleic acid contained in a nucleic acid construct including determining the CpG-N and CpG-S motifs present in the construct and removing neutralizing CpG (CPG-N) motifs and optionally inserting stimulatory CpG (CpG-S) motifs in the construct, thereby producing a nucleic acid construct having enhanced immunostimulatory efficacy. Preferably, the CpG-S motifs in the construct include a motif having the formula 5' X_1CGX_2 3' wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine.

In another embodiment, the invention provides a method for stimulating a protective or therapeutic immune response in a subject. The method includes administering to

the subject an effective amount of a nucleic acid construct produced by determining the CpG-N and CpG-S motifs present in the construct and removing neutralizing CpG (CpG-N) motifs and optionally inserting stimulatory CpG (CpG-S) motifs in the construct, thereby producing a nucleic acid construct having enhanced immunostimulatory efficacy and stimulating a protective or therapeutic immune response in the subject. Preferably, the nucleic acid construct contains a promoter that functions in eukaryotic cells and a

nucleic acid sequence that encodes an antigen to which the immune response is direct toward. Alternatively, an antigen can be admininstered simulataneously (*e.g.*, admixture) with the nucleic acid construct.

In another embodiment, the invention provides a method for enhancing the expression of a therapeutic polypeptide *in vivo* wherein the polypeptide is encoded by a nucleic acid contained in a nucleic acid construct. The method includes determining the CpG-N and CpG-S motifs present in the construct, optionally removing stimulatory CpG (CpG-S) motifs and/or inserting neutralizing CpG (CpG-N) motifs, thereby producing a nucleic acid construct providing enhanced expression of the therapeutic polypeptide.

In yet another embodiment, the invention provides a method for enhancing the expression of a therapeutic polypeptide *in vivo*. The method includes administering to a subject a nucleic acid construct, wherein the construct is produced by determining the CpG-N and CpG-S motifs present in the construct and optionally removing stimulatory CpG (CpG-S) motifs and/or inserting neutralizing CpG (CpG-N) motifs, thereby enhancing expression of the therapeutic polypeptide in the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the construction of pUK21-A1.

Figure 2 is a schematic diagram of the construction of pUK21-A2.

Figure 3 is a schematic diagram of the construction of pUK21-A.

Figure 4 is a schematic diagram of the construction of pMAS.

Figure 5 is a diagram of DNA vector pMAS. The following features are contained within pMAS. CMV promoter which drives expression of inserted genes in eukaryotic cells. BGH polyA for polyadenylation of transcribed mRNAs. ColE1 origin of

replication for high copy number growth in *E. coli*. Kanamycin resistance gene for selection in *E.coli*. Polylinker for gene cloning. Unique restriction enzyme sites *Dra*I-*Bst*RI-*Sca*I-*Ava*II-*Hpa*II for inserting immune stimulatory sequences.

Figure 6 shows the effect of adding S-ODN to plasmid DNA expressing reporter gene or antigen. ODN 1826 (10 or 100 μg) was added to DNA constructs (10 μg) encoding hepatitis B surface antigen (HBsAg) (pCMV-S, Figure 6A) or luciferase (pCMV-luc, Figure 6B) DNA prior to intramuscular (IM) injection into mice. There was an ODN dose-dependent reduction in the induction of antibodies against HBsAg (anti-HBs, end-point dilution titers at 4 wk) by the pCMV-S DNA (Figure 6A) and in the amount of luciferase expressed in relative light units per sec per mg protein (RLU/sec/mg protein at 3 days) from the pCMV-luc DNA (Figure 6B). This suggests that the lower humoral response with DNA vaccine plus ODN was due to decreased antigen expression. Each bar represents the mean of values derived from 10 animals (Figure 6A) or 10 muscles (Figure 6B) ands vertical lines represent the SEM. Numbers superimposed on the bars indicate proportion of animals responding to the DNA vaccine (Figure 6A); all muscles injected with pCMV-luc expressed luciferase (Figure 6B).

Figure 7 shows the interference of ODN with plasmid DNA depends on backbone and sequence. Luciferase activity (RLU/sec/mg protein) in mouse muscles 3 days after they were injected with 10 μg pCMV-luc DNA to which had been added no ODN (none = white bar) or 100 μg of an ODN, which had one of three backbones: phosphorothioate (S = black bars: 1628, 1826, 1911, 1982, 2001 and 2017), phosphodiester (O = pale grey bar: 2061), or a phosphorothioate- phosphodiester chimera (SOS = dark grey bars: 1585, 1844, 1972, 1980, 1981, 2018, 2021, 2022, 2023 and 2042). Three S-ODN (1911, 1982 and 2017) and two SOS-ODN (1972 and 2042) did not contain any immunostimulatory CpG motifs. One S-ODN (1628) and three SOS-ODN (1585, 1972, 1981) had poly-G ends and one SOS-ODN (2042) had a poly-G center. The (*) indicates ODN of identical sequence but different backbone: 1826 (S-ODN), 1980 (SOS-ODN) and 2061 (O-ODN).

All S-ODN (both CpG and non-CpG) resulted in decreased luciferase activity whereas SOS-ODN did not unless they had poly-G sequences.

Figure 8 shows the effect of temporal or spatial separation of plasmid DNA and S-ODN on gene expression. Luciferase activity (RLU/sec/mg protein) in mouse muscles 3 or 14 days after they were injected with 10 μg pCMV-luc DNA. Some animals also received 10 μg CpG-S ODN which was mixed with the DNA vaccine or was given at the same time but at a different site, or was given 4 days prior to or 7 days after the DNA vaccine. Only when the ODN was mixed directly with the DNA vaccine did it interfere with gene expression.

Figure 9 shows the enhancement of *in vivo* immune effects with optimized DNA vaccines. Mice were injected with 10 μg of pUK-S (black bars), pMAS-S (white bars), pMCG16-S (pale grey bars) or pMCG50-S (dark grey bars) plasmid DNA bilaterally (50 μl at 0.1 mg/ml in saline) into the TA muscle. Figure 9A shows the anti-HBs antibody response at 6 weeks (detected as described in methods). Bars represent the group means (n=5) for ELISA end-point dilution titers (performed in triplicate), and vertical lines represent the standard errors of the mean. The numbers on the bars indicate the ratio of IgG2a:IgG1 antibodies at 4 weeks, as determined in separate assays (also in triplicate) using pooled plasma. Figure 9B shows the cytotoxic T lymphocyte activity in specifically restimulated (5 d) splenocytes taken from mice 8 wk after DNA immunization. Bars represent the group means (n=3) for % specific lysis (performed in triplicate) at an effector:target (E:T) ratio of 10:1, dots represent the individual values. Non-specific lytic activity determined with non-antigen-presenting target cells, which never exceeds 10%, has been subtracted from values with HBsAg-expressing target cells to obtain % specific lysis values.

Figure 10 shows induction of a Th2-like response by a CpG-N motif and inhibition of the Th1-like response induced by a CpG-S motif. Anti-HBs antibody titers (IgG1 and IgG2a subclasses) in BALB/c mice 12 weeks after IM immunization with recombinant HBsAg,

which was given alone (none) or with 10 μg stimulatory ODN (1826), 10 μg of neutralizing ODN (1631, CGCGCGCGCGCGCGCGCGCG; 1984, TCCATGCCGTTCCTGCCGTT; or 2010 GCGGCGGGCGGCGCGCGCCC; CpG dinucleotides are underlined for clarity) or with 10 μg stimulatory ODN + 10 μg neutralizing ODN. To improve nuclease resistance for these *in vivo* experiments, all ODN were phosphorothioate-modified. Each bar represents the group mean (n=10 for none; n=15 for #1826 and n=5 for all other groups) for anti-HBs antibody titers as determined by end-point dilution ELISA assay. Black portions of bars indicate antibodies of IgG1 subclass (Th2-like) and grey portions indicate IgG2a subclass (Th1-like). The numbers above each bar indicate the IgG2a/IgG1 ratio where a ratio >1 than indicates a predominantly Th1-like response and a ratio <1 indicates a predominantly Th2-like response (a value of 0 indicates a complete absence of IgG2a antibodies).

Figure 11 shows enhancement of *in vivo* immune effects with optimized DNA vaccines. Mice were injected with 10 μg of pUK-S (black bars), pMAS-S (white bars), pMCG16-S (pale grey bars) or pMCG50-S (dark grey bars) plasmid DNA bilaterally (50 μl at 0.1 mg/ml in saline) into the TA muscle. Panel A: The anti-HBs antibody response at 6 weeks (detected as described in methods). Bars represent the group means (n=5) for ELISA end-point dilution titers (performed in triplicate), and vertical lines represent the standard errors of the mean. The numbers on the bars indicate the ratio of IgG2a:IgG1 antibodies at 4 weeks, as determined in separate assays (also in triplicate) using pooled plasma. Panel B: Cytotoxic T lymphocyte activity in specifically restimulated (5 d) splenocytes taken from mice 8 wk after DNA immunization. Bars represent the group means (n=3) for % specific lysis (performed in triplicate) at an effector:target (E:T) ratio of 10:1, dots represent the individual values. Non-specific lytic activity determined with non-antigen-presenting target cells, which never exceeds 10%, has been subtracted from values with HBsAg-expressing target cells to obtain % specific lysis values.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides vectors for immunization or therapeutic purposes based on the presence or absence of CpG dinucleotide immunomodulating motifs. For immunization purposes, immunostimulatory motifs (CpG-S) are desirable while immunoinhibitory CpG motifs (CpG-N) are undesirable, whereas for gene therapy purposes, CpG-N are desirable and CpG-S are undesirable. Plasmid DNA expression cassettes were designed using CpG-S and CpG-N motifs. In the case of DNA vaccines, removal of CpG-N motifs and addition of CpG-S motifs should allow induction of a more potent and appropriately directed immune response. The opposite approach with gene therapy vectors, namely the removal of CpG-S motifs and addition of CpG-N motifs, allows longer lasting therapeutic effects by abrogating immune responses against the expressed protein.

DNA vaccines

DNA vaccines have been found to induce potent humoral and cell-mediated immune responses. These are frequently Th1-like, especially when the DNA is administered by intramuscular injection (Davis, H.L. (1998) Gene-based Vaccines. In: Advanced Gene Delivery: From Concepts to Pharmaceutical Products (Ed. A. Rolland), Harwood Academic Publishers (in press); Donnelly *et al.*, *Life Sciences* 60:163, 1997; Donnelly *et al.*, Ann Rev. Immunol. 15:617, 1997; Sato *et al.*, *Science* 273:352, 1996). Most DNA vaccines comprise antigen-expressing plasmid DNA vectors. Since such plasmids are produced in bacteria and then purified, they usually contain several unmethylated immunostimulatory CpG-S motifs. There is now convincing evidence that the presence of such motifs is essential for the induction of immune responses with DNA vaccines (see Krieg *et al.*, *Trends Microbiology*. 6: 23-27, 1998). For example, it has been shown that removal or methylation of potent CpG-S sequences from plasmid DNA vectors reduced or abolished the *in vitro* production of Th1 cytokines (*e.g.*, IL-12, IFN-α, IFN-γ) from monocytes and the *in vivo* antibody and CTL response against an encoded antigen (β-galactosidase) (Sato *et al.*, 1996, *supra*; Klinman *et al.*, *J. Immunol.* 158: 3635-3639 (1997). Potent responses could be restored by cloning CpG-S motifs back into the vectors

(Sato *et al.*, 1996, *supra*) or by coadministering CpG-S ODN (Klinman *et al.*, 1997, *supra*). The humoral response in monkeys to a DNA vaccine can also be augmented by the addition of *E. coli* DNA (Gramzinski *et al.*, *Molec. Med.* 4: 109-119, 1998). It has also been shown that the strong Th1 cytokine pattern induced by DNA vaccines can be obtained with a protein vaccine by the coadministration of empty plasmid vectors (Leclerc *et al.*, *Cell Immunology*. 170: 97-106, 1997).

The present invention shows that DNA vaccine vectors can be improved by removal of CpG-N motifs and further improved by the addition of CpG-S motifs. In addition, for high and long-lasting levels of expression, the optimized vector should preferably include a promoter/enhancer, which is not down-regulated by the cytokines induced by the immunostimulatory CpG motifs.

It has been shown that the presence of unmethylated CpG motifs in the DNA vaccines is essential for the induction of immune responses against the antigen, which is expressed only in very small quantities (Sato *et al.*, 1996, Klinman *et al.*, 1997, *supra*). As such, the DNA vaccine provides its own adjuvant in the form of CpG DNA. Since single-stranded but not double-stranded DNA can induce immunostimulation *in vitro*, the CpG adjuvant effect of DNA vaccines *in vivo* is likely due to oligonucleotides resulting from plasmid degradation by nucleases. Only a small portion of the plasmid DNA injected into a muscle actually enters a cell and is expressed; the majority of the plasmid is degraded in the extracellular space.

The present invention provides DNA vaccine vectors further improved by removal of undesirable immunoinhibitory CpG motifs and addition of appropriate CpG immunostimulatory sequences in the appropriate number and spacing. The correct choice of immunostimulatory CpG motifs could allow one to preferentially augment humoral or CTL responses, or to preferentially induce certain cytokines.

The optimized plasmid cassettes of the invention are ready to receive genes encoding any particular antigen or group of antigens or antigenic epitopes. One of skill in the art can

create cassettes to preferentially induce certain types of immunity, and the choice of which cassette to use would depend on the disease to be immunized against.

The exact immunostimulatory CpG motif(s) to be added will depend on the ultimate purpose of the vector. If it is to be used for prophylactic vaccination, preferable motifs stimulate humoral and/or cell-mediated immunity, depending on what would be most protective for the disease in question. It the DNA vaccine is for therapeutic purposes, such as for the treatment of a chronic viral infection, then motifs which preferentially induce cell-mediated immunity and/or a particular cytokine profile is added to the cassette.

The choice of motifs also depends on the species to be immunized as different motifs are optimal in different species. Thus, there would be one set of cassettes for humans as well as cassettes for different companion and food-source animals which receive veterinary vaccination. There is a very strong correlation between certain *in vitro* immunostimulatory effects and *in vivo* adjuvant effect of specific CpG motifs. For example, the strength of the humoral response correlates very well ($r > 0.9$) with the *in vitro* induction of TNF-α, IL-6, IL-12 and B-cell proliferation. On the other hand, the strength of the cytotoxic T-cell response correlates well with *in vitro* induction of IFN-γ.

Since the entire purpose of DNA vaccines is to enhance immune responses, which necessarily includes cytokines, the preferred promoter is not down-regulated by cytokines. For example, the CMV immediate-early promoter/enhancer, which is used in almost all DNA vaccines today, is turned off by IFN-α and IFN-γ (Gribaudo *et al.*, *Virology*. 197: 303-311, 1993; Harms & Splitter, *Human Gene Ther*. 6: 1291-1297, 1995; Xiang *et al.*, *Vaccine*, 15: 896-898, 1997). Another example is the down-regulation of a hepatitis B viral promoter in the liver of HBsAg-expressing transgenic mice by IFN-γ and TNF-α (Guidotti *et al.*, *Proc. Natl. Acad. Sci. USA*. 91: 3764-3768, 1994).

Nevertheless, such viral promoters may still be used for DNA vaccines as they are very strong, they work in several cell types, and despite the possibility of promoter turn-off, the duration of expression with these promoters has been shown to be sufficient for use in DNA vaccines (Davis *et al.*, *Human Molec. Genetics.* 2: 1847-1851, 1993). The use of CpG-optimized DNA vaccine vectors could improve immune responses to antigen expressed for a limited duration, as with these viral promoters. When a strong viral promoter is desired, down-regulation of expression may be avoidable by choosing CpG-S motfis that do not induce the cytokine(s) that affect the promoter (Harms and Splitter, 1995 *supra*).

Other preferable promoters for use as described herein are eukaryotic promoters. Such promoters can be cell- or tissue-specific. Preferred cells/tissues for high antigen expression are those which can act as professional antigen presenting cells (APC) (*e.g.*, macrophages, dendritic cells), since these have been shown to be the only cell types that can induce immune responses following DNA-based immunization (Ulmer *et al.*, 1996; Corr *et al.*, *J. Exp. Med.*, 184, 1555-1560, 1996; Doe *et al.*, *Proc. Natl. Acad. Sci. USA*, 93, 8578-8583, 1996; Iwasaki *et al.*, *J. Immunol.*, 159: 11-141998). Examples of such a promoter are the mammalian MHC I or MHC II promoters.

The invention also includes the use of a promoter whose expression is up-regulated by cytokines. An example of this is the mammalian MHC I promoter that has the additional advantage of expressing in APC, which as discussed above is highly desirable. This promoter has also been shown to have enhanced expression with IFN-γ (Harms & Splitter, 1995, *supra*).

After intramuscular injection of DNA vaccines, muscle fibers may be efficiently transfected and produce a relatively large amount of antigen that may be secreted or otherwise released (*e.g.*, by cytolytic attack on the antigen-expressing muscle fibers) (Davis *et al.*, *Current Opinions Biotech.* 8: 635-640, 1997). Even though antigen-expressing muscle fibers do not appear to induce immune responses from the point of view of antigen presentation, B-cells must meet circulating antigen to be activated, it is

possible that antibody responses are augmented by antigen secreted or otherwise released from other cell types (*e.g.*, myofibers, keratinocytes). This may be particularly true for conformational B-cell epitopes, which would not be conserved by peptides presented on APC. For this purpose, expression in muscle tissue is particularly desirable since myofibers are post-mitotic and the vector will not be lost through cell-division, thus antigen expression can continue until the antigen-expressing cell is destroyed by an immune repsonse against it. Thus, when strong humoral responses are desired, other preferred promoters are strong muscle-specific promoters such as the human muscle-specific creatine kinase promoter (Bartlett *et al.*, 1996) and the rabbit ß-cardiac myosin heavy chain (full-length or truncated to 781 bp) plus the rat myosin light chain 1/3 enhancer.

In the case of DNA vaccines with muscle- or other non-APC tissue-specific promoters, it may be preferable to administer it in conjunction with a DNA vaccine encoding the same antigen but under the control of a promoter that will work strongly in APC (*e.g.*, viral promoter or tissue specific for APC). In this way, optimal immune responses can be obtained by having good antigen presentation as well as sufficient antigen load to stimulate B-cells. A hybrid construct, such as the ß-actin promoter with the CMV enhancer (Niwa et al, *Gene*. 108: 193-199, 1991) is also desirable to circumvent some of the problems of strictly viral promoters.

While DNA vaccine vectors may include a signal sequence to direct secretion, humoral and cell-mediated responses are possible even when the antigen is not secreted. For example, it has been found in mice immunized with hepatitis B surface antigen (HBsAg)-expressing DNA that the appearance of anti-HBs antibodies is delayed for a few weeks if the HBsAg is not secreted (Michel *et al.*, 1995). As well, antibodies are induced in rabbits following IM immunization with DNA containing the gene for cottontail rabbit papilloma virus major capsid protein (L1), which has a nuclear localization signal (Donnelly *et al.*, 1996). In these cases, the B-cells may not be fully activated until the

expressed antigen is released from transfected muscle (or other) cells upon lysis by antigen-specific CTL.

Preferably, the CpG-S motifs in the construct include a motif having the formula:

5' X_1CGX_2 3'

wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine. Exemplary CpG-S oligonucleotide motifs include GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT. Another oligonucleotide useful in the construct contains TCAACGTT. Further exemplary oligonucleotides of the invention contain GTCG(T/C)T, TGACGTT, TGTCG(T/C)T, TCCATGTCGTTCCTGTCGTT (SEQ ID NO:1), TCCTGACGTTCCTGACGTT (SEQ ID NO:2) and TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:3).

Preferably CpG-N motifs contain direct repeats of CpG dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides or a combination of any of these motifs. In addition, the neutralizing motifs of the invention may include oligos that contain a sequence motif that is a poly-G motif, which may contain at least about four Gs in a row or two G trimers, for example (Yaswen *et al., Antisense Research and Development* 3:67, 1993; Burgess *et al., PNAS* 92:4051, 1995).

In the present invention, the nucleic acid construct is preferably an expression vector. The term "expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of genetic coding sequences. Polynucleotide sequence which encode polypeptides can be operatively linked to expression control sequences.

"Operatively linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. An expression control sequence operatively linked to a coding sequence is ligated such that expression

of the coding sequence is achieved under conditions compatible with the expression control sequences. As used herein, the term "expression control sequences" refers to nucleic acid sequences that regulate the expression of a nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus expression control sequences can include appropriate promoters, enhancers, transcription terminators, a start codon (*i.e.*, ATG) in front of a protein-encoding gene, splicing signal for introns, maintenance of the correct reading frame of that gene to permit proper translation of mRNA, and stop codons.

The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

The nucleic acid construct of the invention may include any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, *etc.* may be used in the expression vector (see *e.g.*, Bitter *et al.*, 1987, Methods in Enzymology 153:516-544). When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the retrovirus long terminal repeat; the adenoviral late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted polypeptide coding sequence.

Mammalian cell systems which utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, the polypeptide coding sequence may be ligated to an adenovirus transcription/-translation control complex, *e.g.*, the late promoter and tripartite leader sequence.

Alternatively, the vaccinia virus 7.5K promoter may be used. (*e.g.*, see, Mackett *et al.*, 1982, *Proc. Natl. Acad. Sci. USA* 79: 7415-7419; Mackett *et al.*, 1984, *J. Virol.* 49: 857-864; Panicali *et al.*, 1982, *Proc. Natl. Acad. Sci. USA* 79: 4927-4931). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver, *et al.*, 1981, *Mol. Cell. Biol.* 1: 486). Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as, for example, the neo gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of the gene of interest in host cells (Cone & Mulligan, 1984, *Proc. Natl. Acad. Sci. USA* 81:6349-6353). High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothionine IIA promoter and heat shock promoters.

The polypeptide that acts as an antigen in the methods described herein refers to an immunogenic polypeptide antigen, group of antigens or peptides encoding particular epitopes. A polynucleotide encoding such antigen(s) is inserted into the nucleic acid construct as described herein. For example, a nucleic acid sequence encoding an antigenic polypeptide derived from a virus, such as Hepatitis B virus (HBV) (*e.g.*, HBV surface antigen), an antigen derived from a parasite, from a tumor, or a bacterial antigen, is cloned into the nucleic acid construct described herein. Virtually any antigen, groups of antigens, or antigenic epitopes, can be used in the construct. Other antigens, such as peptides that mimic nonpeptide antigens, such as polysaccharides, are included in the invention.

Gene transfer into eukaryotic cells can be carried out by direct (*in vivo*) or indirect (*in vitro* or *ex vivo*) means (Miller et al., A. D. *Nature*. 357: 455-460, 1992). The DNA vector can also be transferred in various forms and formulations. For example, pure plasmid DNA in an aqueous solution (also called "naked" DNA) can be delivered by

direct gene transfer. Plasmid DNA can also be formulated with cationic and neutral lipids (liposomes) (Gregoriadis et al, 1996), microencapsulated (Mathiowitz et al., 1997), or encochleated (Mannino and Gould Fogerite, 1995) for either direct or indirect delivery. The DNA sequences can also be contained within a viral (*e.g.*, adenoviral, retroviral, herpesvius, pox virus) vector, which can be used for either direct or indirect delivery.

DNA vaccines will preferably be administered by direct (*in vivo*) gene transfer. Naked DNA can be give by intramuscular (Davis et al., 1993) , intradermal (Raz et al., 1994; Condon et al., 1996; Gramzinski et al., 1998), subcutaneous, intravenous (Yokoyama et al., 1996; Liu et al., 1997), intraarterial (Nabel et al., 1993) or buccal injection (Etchart et al., 1997; Hinkula et al., 1997). Plasmid DNA may be coated onto gold particles and introduced biolistically with a "gene-gun" into the epidermis if the skin or the oral or vaginal mucosae (Fynan *et al. Proc. Natl. Acad. Sci. USA* 90:11478, 1993; Tang *et al, Nature* 356:152, 1992; Fuller, *et al., J. Med. Primatol.* 25:236, 1996; Keller *et al., Cancer Gene Ther.*, 3:186, 1996). DNA vaccine vectors may also be used in conjunction with various delivery systems. Liposomes have been used to deliver DNA vaccines by intramuscular injection (Gregoriadis *et al., FEBS Lett.*402:107, 1997) or into the respiratory system by non-invasive means such as intranasal inhalation (Fynan *et al., supra*). Other potential delivery systems include microencapsulation (Jones et al., 1998; Mathiowitz et al., 1997) or cochleates (Mannino *et al.*, 1995, Lipid matrix-based vaccines for mucosal and systemic immunization. Vaccine Designs: The Subunit and Adjuvant Approach, M.F. Powell and M.J. Newman, eds., Pleum Press, New York, 363-387), which can be used for parenteral, intranasal (*e.g.*, nasal spray) or oral (*e.g.*, liquid, gelatin capsule, solid in food) delivery. DNA vaccines can also be injected directly into tumors or directly into lymphoid tissues (*e.g.*, Peyer's patches in the gut wall). It is also possible to formulate the vector to target delivery to certain cell types, for example to APC. Targeting to APC such as dendritic cells is possible through atachment of a mannose moiety (dendritic cells have a high density of mannose receptors) or a ligand for one of the other receptors found preferentially on APC. There is no limitation as to the route that the DNA vaccine is delivered, nor the manner in which it is formulated as

long as the cells that are transfected can express antigen in such a way that an immune response is induced.

It some cases it may be desirable to carry out ex-vivo gene transfer, in which case a number a methods are possible including physical methods such as microinjection, electroportion or calcium phosphate precipitation, or facilitated transfer methods such as liposomes or dendrimers, or through the use of viral vectors. In this case, the transfected cells would be subsequently administered to the subject so that the antigen they expressed could induce an immune response.

Nucleotide sequences in the nucleic acid construct can be intentionally manipulated to produce CpG-S sequences or to reduce the number of CpG-N sequences for immunization vectors. For example, site-directed mutagenesis can be utilized to produce a desired CpG motif. Alternatively, a particular CpG motif can be synthesized and inserted into the nucleic acid construct. Further, one of skill in the art can produce double-stranded CpG oligos that have self-complementary ends that can be ligated together to form long chains or concatemers that can be ligated into a plasmid, for example. It will be apparent that the number of CpG motifs or CpG-containing oligos that can be concatenated will depend on the length of the individual oligos and can be r-eadily determined by those of skill in the art without undue experimentation. After formation of concatemers, multiple oligos can be cloned into a vector for use in the methods of the invention.

In one embodiment, the invention provides a method for stimulating a protective immune response in a subject. The method includes administering to the subject an immunomostimulatory effective amount of a nucleic acid construct produced by removing neutralizing CpG (CpG-N) motifs and optionally inserting stimulatory CpG (CpG-S) motifs, thereby producing a nucleic acid construct having enhanced immunostimulatory efficacy and stimulating a protective immune response in the subject. The construct typically further includes regulatory sequences for expression of DNA in eukaryotic cells and nucleic acid sequences encoding at least one polypeptide.

It is envisioned that methods of the present invention can be used to prevent or treat bacterial, viral, parasitic or other disease states, including tumors, in a subject. The subject can be a human or may be a non-human such as a pig, cow, sheep, horse, dog, cat, fish, chicken, for example. Generally, the terms "treating," "treatment," and the like are used herein to mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a particular infection or disease (*e.g.*, bacterial, viral or parasitic disease or cancer) or sign or symptom thereof, and/or may be therapeutic in terms of a partial or complete cure for an infection or disease and/or adverse effect attributable to the infection or disease. "Treating" as used herein covers any treatment of (*e.g.*, complete or partial), or prevention of, an infection or disease in a non-human, such as a mammal, or more particularly a human, and includes:

(a) preventing the disease from occurring in a subject that may be at risk of becoming infected by a pathogen or that may be predisposed to a disease (*e.g.*, cancer) but has not yet been diagnosed as having it;

(b) inhibiting the infection or disease, *i.e.*, arresting its development; or

(c) relieving or ameliorating the infection or disease, *i.e.*, cause regression of the infection or disease.

Delivery of polynucleotides can be achieved using a plasmid vector as described herein, that can be administered as "naked DNA" (*i.e.*, in an aqueous solution), formulated with a delivery system (*e.g.*, liposome, cochelates, microencapsulated), or coated onto gold particles. Delivery of polynucleotides can also be achieved using recombinant expression vectors such as a chimeric virus. Thus the invention includes a nucleic acid construct as described herein as a pharmaceutical composition useful for allowing transfection of some cells with the DNA vector such that antigen will be expressed and induce a protective (to prevent infection) or a therapeutic (to ameliorate symptoms attributable to infection or disease) immune response. The pharmaceutical compositions according to the invention are prepared by bringing the construct according to the present

invention into a form suitable for administration to a subject using solvents, carriers, delivery systems, excipients, and additives or auxiliaries. Frequently used solvents include sterile water and saline (buffered or not). A frequently used carrier includes gold particles, which are delivered biolistically (*i.e.*, under gas pressure). Other frequently used carriers or delivery systems include cationic liposomes, cochleates and microcapsules, which may be given as a liquid, solution, enclosed within a delivery capsule or incorporated into food.

The pharmaceutical compositions are preferably prepared and administered in dose units. Liquid dose units would be injectable solutions or nasal sprays or liquids to be instilled (*e.g.*, into the vagina) or swallowed or applied onto the skin (*e.g.*, with allergy tines, with tattoo needles or with a dermal patch). Solid dose units would be DNA-coated gold particles, creams applied to the skin or formulations incorporated into food or capsules or embedded under the skin or mucosae or pressed into the skin (*e.g.*, with allergy tines). Different doses will be required depending on the activity of the compound, form and formulation, manner of administration, and age or size of patient (*i.e.*, pediatric versus adult), purpose (prophylactic vs therapeutic). Doses will be given at appropriate intervals, separated by weeks or months, depending on the application. Under certain circumstances higher or lower, or more frequent or less frequent doses may be appropriate. The administration of a dose at a single time point may be carried out as a single administration or a multiple administration (*e.g.*, several sites with gene-gun or for intradermal injection or different routes).

Whether the pharmaceutical composition is delivered locally or systemically, it will induce systemic immune responses. By "therapeutically effective dose" is meant the quantity of a vector or construct according to the invention necessary to induce an immune response that can prevent, cure, or at least partially arrest the symptoms of the disease and its complications. Amounts effective for this will of course depend on the mode of administration, the age of the patient (pediatric versus adult) and the disease state of the patient. Animal models may be used to determine effective doses for the induction of particular immune responses and in some cases for the prevention or treatment of particular diseases.

The term "effective amount" of a nucleic acid molecule refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of a nucleic acid construct containing at least one unmethylated CpG for treating a disorder could be that amount necessary to induce an immune response of sufficient magnitude to eliminate a tumor, cancer, or bacterial, parasitic, viral or fungal infection. An effective amount for use as a vaccine could be that amount useful for priming and boosting a protective immune response in a subject. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular nucleic acid being administered (*e.g.* the number of unmethylated CpG motifs (-S or -N) or their location in the nucleic acid), the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular oligonucleotide without necessitating undue experimentation. An effective amount for use as a prophylactic vaccine is that amount useful for priming and boosting a protective immune response in a subject.

In one embodiment, the invention provides a nucleic acid construct containing CpG motifs as described herein as a pharmaceutical composition useful for inducing an immune response to a bacterial, parasitic, fungal, viral infection, or the like, or to a tumor in a subject, comprising an immunologically effective amount of nucleic acid construct of the invention in a pharmaceutically acceptable carrier. "Administering" the

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pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. By "subject" is meant any animal, preferably a mammal, most preferably a human. The term "immunogenically effective amount," as used in describing the invention, is meant to denote that amount of nucleic acid construct which is necessary to induce, in an animal, the production of a protective immune response to the bacteria, fungus, virus, tumor, or antigen in general.

In addition to the diluent or carrier, such compositions can include adjuvants or additional nucleic acid constructs that express adjuvants such as cytokines or co-stimulatory molecules. Adjuvants include CpG motifs such as those described in U.S. Patent No. 6,214,806 B1 (issued April 10, 2001).

The method of the invention also includes slow release nucleic acid delivery systems such as microencapsulation of the nucleic acid constructs or incorporation of the nucleic acid constructs into liposomes. Such particulate delivery systems may be taken up by the liver and spleen and are easily phagocytosed by macrophages. These delivery systems also allow co-entrapment of other immunomodulatory molecules, or nucleic acid constructs encoding other immunomodulatory molecules, along with the antigen-encoding nucleic acid construct, so that modulating molecules may be delivered to the site of antigen synthesis and antigen processing, allowing modulation of the immune system towards protective responses.

Many different techniques exist for the timing of the immunizations when a multiple immunization regimen is utilized. It is possible to use the antigenic preparation of the invention more than once to increase the levels and diversity of expression of the immune response of the immunized animal. Typically, if multiple immunizations are given, they will be spaced about four or more weeks apart. As discussed, subjects in which an immune response to a pathogen or cancer is desirable include humans, dogs, cattle, horses, deer, mice, goats, pigs, chickens, fish, and sheep.

Examples of infectious virus to which stimulation of a protective immune response is desirable include: *Retroviridae* (*e.g.*, human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; *Picornaviridae* (*e.g.*, polio viruses, hepatitis A virus; enteroviruses, human coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (*e.g.*, strains that cause gastroenteritis); *Togaviridae* (*e.g.*, equine encephalitis viruses, rubella viruses); *Flaviridae* (*e.g.*, dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (*e.g.*, coronaviruses); *Rhabdoviridae* (*e.g.*, vesicular stomatitis viruses, rabies viruses); *Filoviridae* (*e.g.*, ebola viruses); *Paramyxoviridae* (*e.g.*, parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (*e.g.*, influenza viruses); *Bungaviridae* (*e.g.*, Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); *Reoviridae* (*e.g.*, reoviruses, orbiviurses and rotaviruses); *Birnaviridae; Hepadnaviridae* (Hepatitis B virus); *Parvoviridae* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes viruses'); *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (*e.g.*, African swine fever virus); and unclassified viruses (*e.g.*, the etiological agents of Spongiform encephalopathies, the agent of delta hepatities (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1 = internally transmitted; class 2 = parenterally transmitted (*i.e.*, Hepatitis C); Norwalk and related viruses, and astroviruses).

Examples of infectious bacteria to which stimulation of a protective immune response is desirable include: *Helicobacter pyloris, Borellia burgdorferi, Legionella pneumophilia, Mycobacteria sps (e.g. M. tuberculosis, M. avium, M. intracellulare, M. kansaii, M. gordonae), Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis, Streptococcus bovis, Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter sp.*, *Enterococcus sp.*,

Haemophilus influenzae, *Bacillus antracis*, *corynebacterium diphtheriae*, *corynebacterium sp.*, *Erysipelothrix rhusiopathiae*, *Clostridium perfringers*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides sp.*, *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidium*, *Treponema pertenue*, *Leptospira*, and *Actinomyces israelli*.

Examples of infectious fungi to which stimulation of a protective immune response is desirable include: *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*,*Chlamydia trachomatis*, *Candida albicans*. Other infectious organisms (*i.e.*, protists) include: *Plasmodium falciparum* and *Toxoplasma gondii*.

An "immunostimulatory nucleic acid molecule" or oligonucleotide as used herein refers to a nucleic acid molecule, which contains an unmethylated cytosine, guanine dinucleotide sequence (*i.e.* "CpG DNA" or DNA containing a cytosine followed by guanosine and linked by a phosphate bond) and stimulates (*e.g.* has a mitogenic effect on, or induces or increases cytokine expression by) a vertebrate lymphocyte. An immunostimulatory nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more stable *in vivo*, while single-stranded molecules may have increased immune activity.

Unmethylated immunostimulatory CpG motifs, either within a nucleic acid construct or an oligonucleotide, directly activate lymphocytes and co-stimulate antigen-specific responses. As such, they are fundamentally different form aluminum precipitates (alum), currently the only adjuvant licensed for human use, which is thought to act largely through adsorbing the antigen thereby maintaining it available to immune cells for a longer period. Further, alum cannot be added to all types of antigens (*e.g.*, live attentuated pathogens, some multivalent vaccines), and it induces primarily Th2 type

immune responses, namely humoral immunity but rarely CTL. For many pathogens, a humoral response alone is insufficient for protection, and for some pathogens can even be detrimental.

In addition, an immunostimulatory oligonucleotide in the nucleic acid construct of the invention can be administered prior to, along with or after administration of a chemotherapy or other immunotherapy to increase the responsiveness of malignant cells to subsequent chemotherapy or immunotherapy or to speed the recovery of the bone marrow through induction of restorative cytokines such as GM-CSF. CpG nucleic acids also increase natural killer cell lytic activity and antibody dependent cellular cytotoxicity (ADCC). Induction of NK activity and ADCC may likewise be beneficial in cancer immunotherapy, alone or in conjunction with other treatments.

Gene Therapy

Plasmid or vector DNA may also be useful for certain gene therapy applications. In most such cases, an immune response against the encoded gene product would not be desirable. Thus, the optimal plasmid DNA cassette for gene therapy purposes will have all possible immunostimulatory (CpG-S) motifs removed and several immunoinhibitory (CpG-N) motifs added in. An exemplary vector for gene therapy purposes is described in the Examples.

Despite comparable levels of unmethylated CpG dinucleotides, DNA from serotype 12 adenovirus is immune stimulatory, but serotype 2 is nonstimulatory and can even inhibit activation by bacterial DNA. In type 12 genomes, the distribution of CpG-flanking bases is similar to that predicted by chance. However, in type 2 adenoviral DNA the immune stimulatory CpG-S motifs are outnumbered by a 15 to 30 fold excess of CpG dinucleotides in clusters of direct repeats or with a C on the 5' side or a G on the 3' side. Synthetic oligodeoxynucleotides containing these putative neutralizing (CpG-N) motifs block immune activation by CpG-S motifs *in vitro* and *in vivo*. Eliminating 52 of the 134 CpG-N motifs present in a DNA vaccine markedly enhanced its Th1-like function *in*

vivo, which was further increased by addition of CpG-S motifs. Thus, depending on the CpG motif, prokaryotic DNA can be either immune-stimulatory or neutralizing. These results have important implications for understanding microbial pathogenesis and molecular evolution, and for the clinical development of DNA vaccines and gene therapy vectors.

Gene therapy, like DNA-based immunization, involves introduction of new genes into cells of the body, where they will be expressed to make a desired protein. However, in contrast to DNA vaccines, an immune response against the expressed gene product is not desired for gene therapy purposes. Rather, prolonged expression of the gene product is desired to augment or replace the function of a defective gene, and thus immune responses against the gene product are definitely undesirable.

Plasmid DNA expression vectors are also used for gene therapy approaches. They may be preferable to viral vectors (*i.e.*, recombinant adenovirus or retrovirus), which themselves are immunogenic (Newman, K.D., *et al., J. Clin. Invest.*, 96:2955-2965, 1995; Zabner, J., *et al., J. Clin. Invest.*, 97:1504-1511, 1996). Immune responses directed against such vectors may interfere with successful gene transfer if the same vector is used more than once. Double-stranded DNA is poorly immunogenic (Pisetsky, D. S.*Antisense Res. Devel.* 5: 219-225, 1995; Pisetsky, D. S. *J Immunol.* 156: 421-423, 1996), and thus from this perspective, repeated use is not a problem with plasmid DNA.

Nevertheless, even when gene transfer is carried out with plasmid DNA vectors, expression of the introduced gene is often short-lived and this appears to be due to immune responses against the expressed protein (Miller, A. D. *Nature*. 357: 455-460, 1992; Lasic, D. D., and Templeton, N. S. *Advanced Drug Delivery Review*. 20: 221-266, 1996). It is not a surprise that expression of a foreign protein, as is the case with gene replacement strategies, induces immune responses. Nevertheless, it is likely that the presence of CpG-S motifs aggravates this situation. The finding that removal of CpG-S motifs from DNA vaccines can abolish their efficacy suggests that such a strategy may prove useful for creating gene therapy vectors where immune responses against the encoded protein are undesirable. Furthermore, the more recent discovery of CpG-N

motifs opens up the possibility of actually abrogating unwanted immune responses through incorporating such motifs into gene delivery vectors. In particular, the Th-2 bias of CpG-N motifs may prevent induction of cytotoxic T-cells, which are likely the primary mechanism for destruction of transfected cells.

In another embodiment, the invention provides a method for enhancing the expression of a therapeutic polypeptide *in vivo* wherein the polypeptide is contained in a nucleic acid construct. The construct is produced by removing stimulatory CpG (CpG-S) motifs and optionally inserting neutralizing CpG (CpG-N) motifs, thereby producing a nucleic acid construct providing enhanced expression of the therapeutic polypeptide. Alternatively, the invention envisions using the construct for delivery of antisense polynucleotides or ribozymes.

Typical CpG-S motifs that are removed from the construct include a motif having the formula:

$5' X_1CGX_2 3'$

wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine. Exemplary CpG-S oligonucleotide motifs include GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT. Another oligonucleotide useful in the construct contains TCAACGTT. Further exemplary oligonucleotides of the invention contain GTCG(T/C)T, TGACGTT, TGTCG(T/C)T, TCCATGTCGTTCCTGTCGTT (SEQ ID NO:1), TCCTGACGTTCCTGACGTT (SEQ ID NO:2) and TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:3). These motifs can be removed by site-directed mutagenesis, for example.

Preferably CpG-N motifs contain direct repeats of CpG dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides or a combination of any of these motifs. In addition, the neutralizing motifs of the invention may include oligos that contain a sequence motif that is a poly-G motif, which may contain at least about four Gs in a row or two G trimers, for example (Yaswen *et al.*, *Antisense Research and Development* 3:67, 1993; Burgess *et al.*, *PNAS* 92:4051, 1995).

The present invention provides gene therapy vectors and methods of use. Such therapy would achieve its therapeutic effect by introduction of a specific sense or antisense polynucleotide into cells or tissues affected by a genetic or other disease. It is also possible to introduce genetic sequences into a different cell or tissue than that affected by the disease, with the aim that the gene product will have direct or indirect impact on the diseases cells or tissues. Delivery of polynucleotides can be achieved using a plasmid vector as described herein (in "naked" or formulated form) or a recombinant expression vector (*e.g.*, a chimeric vector).

For long-term, high-yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with a heterologous cDNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, *etc.*), and a selectable marker. The selectable marker in a recombinant plasmid or vector confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler,*et al.*, 1977, *Cell* 11: 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, *Proc. Natl. Acad. Sci. USA* 48: 2026), and adenine phosphoribosyltransferase (Lowy, *et al.*, 1980, *Cell* 22: 817) genes can be employed in tk-, hgprt$^-$ or aprt$^-$ cells respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, *et al.*, 1980, *Natl. Acad. Sci. USA* 77: 3567; O'Hare, *et al.*, 1981, *Proc. Natl. Acad. Sci. USA* 78: 1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78: 2072; neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, *et al.*, 1981, *J. Mol. Biol.* 150: 1); and hygro, which confers resistance to hygromycin (Santerre, *et al.*, 1984, *Gene* 30: 147) genes. Recently, additional selectable genes have been described, namely trpB, which allows

cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, *Proc. Natl. Acad. Sci. USA* 85: 8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). When the subject is a human, a vector such as the gibbon ape leukemia virus (GaLV) can be utilized. A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated.

Therapeutic peptides or polypeptides are typically included in the vector for gene therapy. For example, immunomodulatory agents and other biological response modifiers can be administered for incorporation by a cell. The term "biological response modifiers" is meant to encompass substances which are involved in modifying the immune response. Examples of immune response modifiers include such compounds as lymphokines. Lymphokines include tumor necrosis factor, interleukins (*e.g.*, IL-2, -4, -6, -10 and -12), lymphotoxin, macrophage activating factor, migration inhibition factor, colony stimulating factor, and alpha-interferon, beta-interferon, and gamma-interferon and their subtypes. Also included are polynucleotides which encode metabolic enzymes and proteins, including Factor VIII or Factor IX. Other therapeutic polypeptides include the cystic fibrosis transmembrane conductance regulator (*e.g.*, to treat cystic fibrosis);

structural or soluble muscle proteins such as dystrophin (*e.g.*, to treat muscular dystrophies); or hormones. In addition, suicide or tumor repressor genes can be utilized in a gene therapy vector described herein.

In addition, antisense polynucleotides can be incorporated into the nucleic acid construct of the invention. Antisense polynucleotides in context of the present invention includes both short sequences of DNA known as oligonucleotides of usually 10-50 bases in length as well as longer sequences of DNA that may exceed the length of the target gene sequence itself. Antisense polynucleotides useful for the present invention are complementary to specific regions of a corresponding target mRNA. Hybridization of antisense polynucleotides to their target transcripts can be highly specific as a result of complementary base pairing.

Transcriptional regulatory sequences include a promoter region sufficient to direct the initiation of RNA synthesis. Suitable eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer *et al.*, *J. Molec. Appl. Genet.* 1: 273 (1982)); the TK promoter of Herpes virus (McKnight, *Cell* 31: 355 (1982); the SV40 early promoter (Benoist *et al.*, *Nature* 290: 304 (1981); the Rous sarcoma virus promoter (Gorman *et al.*, *Proc. Nat'l Acad. Sci. USA* 79: 6777 (1982); and the cytomegalovirus promoter (Foecking *et al.*, *Gene* 45: 101 (1980)) (See also discussion above for suitable promoters).

Alternatively, a prokaryotic promoter, such as the bacteriophage T3 RNA polymerase promoter, can be used to control fusion gene expression if the prokaryotic promoter is regulated by a eukaryotic promoter. Zhou *et al.*, *Mol. Cell. Biol.* 10: 4529 (1990); Kaufman *et al.*, *Nucl. Acids Res.* 19: 4485 (1991).

It is desirable to avoid promoters that work well in APC since that could induce an immune response. Thus, ubiquitous viral promoters, such as CMV, should be avoided. Promoters specific for the cell type requiring the gene therapy are desirable in many instances. For example, with cystic fibrosis, it would be best to have a promoter specific for the lung epithelium. In a situation where a particular cell type is used as a platform

to produce therapeutic proteins destined for another site (for either direct or indirect action), then the chosen promoter should work well in the "factory" site. Muscle is a good example for this, as it is post-mitotic, it could produce therapeutic proteins for years on end as long as there is no immune response against the protein-expressing muscle fibers . Therefore, use of strong muscle promoters as described in the previous section are particularly applicable here. Except for treating a muscle disease *per se*, use of muscle is typically only suitable where there is a secreted protein so that it can circulate and function elsewhere (*e.g.*, hormones, growth factors, clotting factors).

Administration of gene therapy vectors to a subject, either as a plasmid or as part of a viral vector can be affected by many different routes. Plasmid DNA can be "naked" or formulated with cationic and neutral lipids (liposomes), microencapsulated, or encochleated for either direct or indirect delivery. The DNA sequences can also be contained within a viral (*e.g.*, adenoviral, retroviral, herpesvius, pox virus) vector, which can be used for either direct or indirect delivery. Delivery routes include but are not limited to intramuscular, intradermal (Sato, Y. *et al., Science* 273: 352-354, 1996), intravenous, intra-arterial, intrathecal, intrahepatic, inhalation, intravaginal instillation (Bagarazzi *et al., J. Med. Primatol.* 26:27, 1997), intrarectal, intratumor or intraperitoneal.

As much as 4.4 mg/kg/d of antisense polynucleotide has been administered intravenously to a patient over a course of time without signs of toxicity. Martin, 1998, "Early clinical trials with GDM91, a systemic oligodeoxynucleotide", In: Applied Oligonucleotide Technology, CA. Stein and A.M. Krieg, (Eds.), John Wiley and Sons, Inc., New York, NY). Also see Sterling, "Systemic Antisense Treatment Reported," *Genetic Engineering News* 12: 1, 28 (1992).

Delivery of polynucleotides can be achieved using a plasmid vector as described herein, that can be administered as "naked DNA" (*i.e.*, in an aqueous solution), formulated with a delivery system (*e.g.*, liposome, cochelates, microencapsulated). Delivery of polynucleotides can also be achieved using recombinant expression vectors such as a chimeric virus. Thus the invention includes a nucleic acid construct as described herein

as a pharmaceutical composition useful for allowing transfection of some cells with the DNA vector such that a therapeutic polypeptide will be expressed and have a therapeutic effect (to ameliorate symptoms attributable to infection or disease). The pharmaceutical compositions according to the invention are prepared by bringing the construct according to the present invention into a form suitable for administration to a subject using solvents, carriers, delivery systems, excipients, and additives or auxiliaries. Frequently used solvents include sterile water and saline (buffered or not). One carrier includes gold particles, which are delivered biolistically (*i.e.*, under gas pressure). Other frequently used carriers or delivery systems include cationic liposomes, cochleates and microcapsules, which may be given as a liquid solution, enclosed within a delivery capsule or incorporated into food.

An alternative formulation for the administration of gene therapy vectors involves liposomes. Liposome encapsulation provides an alternative formulation for the administration of polynucleotides and expression vectors. Liposomes are microscopic vesicles that consist of one or more lipid bilayers surrounding aqueous compartments. See, generally, Bakker-Woudenberg *et al., Eur. J. Clin. Microbiol. Infect. Dis.* 12 (Suppl. 1): S61 (1993), and Kim, *Drugs* 46: 618 (1993). Liposomes are similar in composition to cellular membranes and as a result, liposomes can be administered safely and are biodegradable. Depending on the method of preparation, liposomes may be unilamellar or multilamellar, and liposomes can vary in size with diameters ranging from 0.02 μm to greater than 10 μm. See, for example, Machy *et al.*, LIPOSOMES IN CELL BIOLOGY AND PHARMACOLOGY (John Libbey 1987), and Ostro *et al., American J. Hosp. Pharm.* 46: 1576 (1989).

After intravenous administration, conventional liposomes are preferentially phagocytosed into the reticuloendothelial system. However, the reticuloendothelial system can be circumvented by several methods including saturation with large doses of liposome particles, or selective macrophage inactivation by pharmacological means. Claassen *et al., Biochim. Biophys. Acta* 802: 428 (1984). In addition, incorporation of glycolipid- or polyethelene glycol-derivatised phospholipids into liposome membranes has been

shown to result in a significantly reduced uptake by the reticuloendothelial system. Allen *et al., Biochim. Biophys. Acta* 1068: 133 (1991); Allen *et al., Biochim. Biohys. Acta* 1150: 9 (1993). These Stealth® liposomes have an increased circulation time and an improved targeting to tumors in animals. (Woodle *et al., Proc. Amer. Assoc. Cancer Res.* 33: 2672 1992). Human clinical trials are in progress, including Phase III clinical trials against Kaposi's sarcoma. (Gregoriadis *et al., Drugs* 45: 15, 1993).

Expression vectors can be encapsulated within liposomes using standard techniques. A variety of different liposome compositions and methods for synthesis are known to those of skill in the art. See, for example, U.S. Patent No. 4,844,904, U.S. Patent No. 5,000,959, U.S. Patent No. 4,863,740, U.S. Patent No. 5,589,466, U.S. Patent No. 5,580,859, and U.S. Patent No. 4,975,282.

Liposomes can be prepared for targeting to particular cells or organs by varying phospholipid composition or by inserting receptors or ligands into the liposomes. For instance, antibodies specific to tumor associated antigens may be incorporated into liposomes, together with gene therapy vectors, to target the liposome more effectively to the tumor cells. See, for example, Zelphati *et al., Antisense Research and Development* 3: 323-338 (1993), describing the use "immunoliposomes" containing vectors for human therapy.

In general, the dosage of administered liposome-encapsulated vectors will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. Dose ranges for particular formulations can be determined by using a suitable animal model.

In addition to antisense, ribozymes can be utilized with the gene therapy vectors described herein. Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences

in an RNA molecule and cleave it (Cech, *J.Amer.Med. Assn.*, 260:3030, 1988). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

There are two basic types of ribozymes namely, *tetrahymena*-type (Hasselhoff, *Nature*, 334:585, 1988) and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences 11-18 bases in length. The longer the recognition sequence, the greater the likelihood that the sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to *tetrahymena*-type ribozymes for inactivating a specific mRNA species and 18-based recognition sequences are preferable to shorter recognition sequences.

All references cited herein are hereby incorporated by reference in their entirety. The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLE 1

Cloning of CpG Optimized Plasmid DNA Vectors

Plasmids and other reagents

The cloning vector pUK21, which contains one *Col*E1 replication region, kanamycin resistance gene and polylinker, was provided by Martin Schleef of Qiagen Inc. (Qiagen, Hilden, Germany). The expression vector pcDNA3 was purchased from Invitrogen Corp. (Carlsbad, USA). *E. coli* strain DH5α was used as the bacterial host.

Pwo DNA polymerase, T4 DNA ligase, dNTP and ATP were purchased from Boerhinger Mannheim (Mannheim, Germany). T4 DNA polymerase, large fragment of DNA polymerase I (klenow), T4 DNA polynucleotide kinase, CIP (calf intestinal alkaline phosphatase) and restriction enzymes were purchased from New England BioLabs (Beverly, USA) and GIBCO BRL (Gaithersburg, USA). General laboratory chemicals were from Sigma Chemical Corp. (St. Louis, USA).

Recombinant DNA techniques

Unless specified otherwise, all recombinant DNA methods were as described by Sambrook *et al.* (1989). Plasmid DNA was prepared with Qiagen Plasmid Kits (Qiagen Inc). DNA purification was carried out by separating DNA fragments on an agarose gel and extracting with QIAquick Gel Extraction Kit (Qiagen Inc). Double-stranded DNA sequencing was performed with ABI PRISM automatic sequencing system (Perkin Elmer Corp., Norwalk, USA). Oligonucleotides for primers were synthesized with a DNA synthesizer, model Oligo 1000, manufactured by Beckman Instrument Inc.(Palo Alto, USA). PCR was performed with the Perkin Elmer PCR system 2400.

PCR conditions

Cycling conditions for each PCR began with a 2-min denaturation at 94°C, followed by 25 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, elongation at

72°C for 45 sec (adjusted according to the size of DNA fragment), and completed with a 7-min incubation at 72°C. High-fidelity Pwo polymerase was used when fragments were created for cloning and site-directed mutagenesis.

Construction of basic expression vector

The pUK21 vector was used as the starting material to construct a basic expression vector, which was subsequently used for construction of either a CpG-optimized DNA vaccine vectors or a CpG-optimized gene therapy vectors. DNA sequences required for gene expression in eukaryotic cells were obtained by PCR using the expression vector pcDNA3 as a template.

(i) Insertion of the CMV (human cytomegalovirus) major intermediate early promoter/enhancer region

The CMV promoter (from pcDNA3 position 209 to 863) was amplified by PCR using 30 ng pcDNA3 as a template. The forward PCR primer 5' CGT GGA TAT CCG ATG TAC GGG CCA GAT AT 3' (SEQ ID NO:4) introduced an *Eco*RV site, and the reverse PCR primer 5' AGT CGC GGC CGC AAT TTC GAT AAG CCA GTA AG 3 ' (SEQ ID NO:5)introduced a *Not*I site. After digestion with *Eco*RV and *Not*I, a 0.7 kb PCR fragment containing the CMV promoter was purified and inserted into the pUK21 polylinker between *Xba*I and *Not*I sites. The *Xba*I sticky end of pUK21 was filled in with the large fragment of T4 DNA polymerase after digestion to create a blunt end. The inserted CMV promoter was confirmed by sequencing. The resulting plasmid was pUK21-A1 (Figure 1).

(ii) Insertion of the BGH polyA (bovine growth hormone polyadenylation signal)

BGH polyA (from pcDNA3 position 1018 to 1249) was amplified by PCR using pcDNA3 as template. The forward PCR primer 5' ATT CTC GAG TCT AGA CTA GAG CTC GCT GAT CAG CC 3' (SEQ ID NO:6) introduced *Xho*I and *Xba*I sites, and the reverse PCR primer 5' ATT AGG CCT TCC CCA GCA TGC CTG CTA TT 3' (SEQ ID NO:7) introduced a *Stu*I site. After digestion with *Xho*I and *Stu*I, the 0.2 kb PCR

fragment containing the BGH polyA was purified, and ligated with the 3.7 kb *Xho*I-*Stu*I fragment of pUK21-A1. The inserted BGH polyA was confirmed by sequencing. The resulting plasmid was pUK21-A2 (Figure 2).

Note: Ligation of the *Eco*RV and *Xba*I-fill-in blunt ends in the pUK21-A1 construct recreated an *Xba*I site, but this site is resistant to cleavage due to Dam methylation present in most laboratory strains of *E. coli*, such as DH5α, so the extra *Xba*I site introduced by the forward PCR primer in the pUK21-A2 construct is available as a cloning site.

CpG optimized DNA vaccine vector

The CpG-optimized DNA vaccine vectors were made from the basic expression vector (pUK21-A2) in several steps:

- Site-directed mutagenesis for removal of CpG-N motifs, with care being taken to maintain the integrity of the open reading frame. Where necessary, the mutated sequence was chosen to encode the same amino acids as the original sequence.
- Removal of unnecessary sequences (*e.g.*, f1 ori).
- Addition of suitable polylinker sequence to allow easy incorporation of CpG-motifs.
- Addition of CpG-S motifs which would be chosen to enhance a particular immune response (humoral, cell-mediated, high levels of a particular cytokine *etc.*).

The pUK21-A2 vector was used as the starting material for construction of an optimized DNA vaccine vector. Site-directed mutagenesis was carried out to mutate those CpG-N sequences that were easy to mutate. As described below, 22 point-mutations were made to change a total of 15 CpG-N motifs to alternative non-CpG sequences. For 16 of these point mutations that were in coding regions, the new sequences encoded the same amino acids as before through alternative codon usage. The mutated sequences were all in the kanamycin resistance gene or immediately adjacent regions. At present, we did not mutate any CpG-N

motifs in regions with indispensable functions such as the *Col*E1, BGH poly A or polylinker regions, or the promoter region (in this case CMV), however this should be possible.

(i) Insertion of the f1 origin of replication region

The f1 origin and two unique restriction enzyme sites (*Dra*I and *Apa*I) were introduced into pUK21-A2 for later vector construction. f1 origin (from pcDNA3 position 1313 to 1729) was amplified by PCR using pcDNA3 as template. The forward PCR primer 5' TAT AGG CCC TAT TTT AAA CGC GCC CTG TAG CGG CGC A 3' (SEQ ID NO:8) introduced *Eco*O109I and *Dra*I sites, and the reverse PCR primer 5' CTA TGG CGC CTT GGG CCC AAT TTT TGT TAA ATC AGC TC 3' (SEQ ID NO:9) introduced *Nar*I and *Apa*I site. After digestion with *Nar*I and *Eco*O109I, the 0.4 kb PCR fragment containing the f1 origin was purified and ligated with the 3.3 kb *Nar*I-*Eco*O109I fragment of pUK21-A2, resulting in pUK21-A (Figure 3).

(ii) Site-directed Mutagenesis to Remove Immunoinhibitory Sequences

Sixteen silent-mutations within the kanamycin resistance gene and another six point-mutations within a non-essential DNA region were designed in order to eliminate immunoinhibitory CpG-N sequences. At this time, mutations were not made to CpG-N motifs contained in regions of pUK21-A that had essential functions.

Site-directed mutagenesis was performed by overlap extension PCR as described by Ge *et al.* (1997). The 1.3 kb *Alw*NI-*Eco*O109I fragment of pUK21-A contained all 22 nucleotides to be mutated and was regenerated by overlap extension PCR using mutagenic primers. All the primers used for mutagenesis are listed in Table 1, and the nucleotide sequence of this *Alw*NI-*Eco*O109I fragment is listed in Table 2 (*Note*: the nucleotide numbering scheme is the same as the backbone vector pUK21).

The mutagenesis was carried out as follows: In the first round of overlap extension PCR, the pairs of primers: Mu-0F/Mu-(4+5)R Mu-(4+5)F/Mu-9R, Mu-9F/Mu-13R and Mu-13F/Mu-0R were used to introduce four point-mutations at positions 1351, 1363, 1717 and 1882. The

PCR-generated *Eco*RI/*Alw*NI-*Eco*O109I/*Xba*I fragment was inserted into the pcDNA3 polylinker between the *Eco*R I and *Xba*I sites. The mutated *Msp*I at position 1717 was used to identify the pcDNA3-insert containing the appropriate mutant DNA fragment.

In the second round of overlap extension PCR, the pcDNA3-insert from the first-round was used as a PCR template, the pairs of primers: Mu-0F/Mu-2R, Mu-2F/Mu-7R, Mu-7F/Mu-10R and Mu-10F/Mu-0R were used to introduce three point-mutations at positions 1285, 1549 and 1759. The PCR-generated *Eco*RI/*Alw*N-*Eco*O109I/*Xba*I fragment was inserted into the pcDNA3 polylinker between the *Eco*RI and *Xba*I sites. The *Sna*BI site created by mutation at position 1759 was used to identify the pcDNA3-insert containing the appropriate mutant DNA fragment.

In the third round of overlap extension PCR, the pcDNA3-insert from the second-round was used as a template, the pairs of primers: Mu-0F/Mu-3R, Mu-3F/Mu-8R, Mu-8F/Mu-14R and Mu-14F/Mu-0R were used to introduce five point-mutations at positions 1315, 1633, 1636, 1638 and 1924. The PCR-generated *Eco*RI/*Alw*NI-*Eco*O109I/*Xba*I fragment was inserted into the pcDNA3 polylinker between the *Eco*RI and *Xba*I sites. The mutated *Msp*I site at position 1636 was used to identify the pcDNA3-insert containing the appropriate DNA mutant fragment.

In the last round of overlap extension PCR, the pcDNA3-insert from the third-round was used as a template, the pairs of primers: Mu-0F/Mu-1R, Mu-1F/Mu-6R, Mu-6F/Mu-(11+12)R, Mu-(11+12)F/Mu-15R and Mu-15F/Mu-0R were used to introduce 10-point mutations at positions 1144, 1145, 1148, 1149, 1152, 1153, 1453, 1777, 1795 and 1984. After digestion with the *Eco*O109I and *Alw*NI, the PCR-generated 1.3 kb fragment was inserted into pUK21-A to replace the corresponding part, resulting in pUK21-B. All the 22 point-mutations were confirmed by sequencing, and the PCR-generated *Alw*NI-*Eco*O109I fragment was free from PCR errors.

(iii) Replacement of the f1 origin with unique restriction enzyme sites

Oligonucleotides 5' AAA TTC GAA AGT ACT GGA CCT GTT AAC A 3' (SEQ ID NO:10) and its complementary strand 5' CGT GTT AAC AGG TCC AGT ACT TTC GAA TTT 3' (SEQ ID NO:11) were synthesized, and 5'-phosphorylated. Annealing of these two phosphorylated oligos resulted in 28 base pair double-stranded DNA containing three unique restriction enzyme sites (*Sca*I, *Ava*II, *Hpa*I), one sticky end and one blunt end. Replacing the 0.4 kb *Nar*I-*Dra*I fragment of pUK21-B with this double-stranded DNA fragment resulted in the universal vector pMAS for DNA vaccine development (Figures 4 and 5).

(iv) Insertion of immunostimulatory motifs into the vector pMAS

The vector is now ready for cloning CpG-S motifs. The exact motif which would be added to the vector would depend on its ultimate application, including the species it is to be used in and whether a strong humoral and/or a cell-mediated response was preferred. The following description gives an example of how a varying number of a given motif could be added to the vector.

Insertion of murine-specific CpG-S motifs was carried out by first synthesizing the oligonucleotide 5' GAC TCC ATG ACG TTC CTG ACG TTT CCA TGA CGT TCC TGA CGT TG 3' (SEQ ID NO:12) which contains four CpG-S motifs (underlined), and its complementary sequence 5' GTC CAA CGT CAG GAA CGT CAT GGA AAC GTC AGG AAC GTC ATG GA 3' (SEQ ID NO:13). This sequence is based on the CpG-S motifs contained in oligo #1826, which has potent stimulatory effects on murine cells *in vitro* and is a potent adjuvant for protein vaccines *in vivo*. After 5'-phosphorylation, annealing was performed to create a 44 bp double-stranded DNA fragment with *Ava*II-cut sticky ends. Self-ligation of this 44 bp DNA fragment resulted in a mixture of larger DNA fragments containing different copy numbers of the stimulatory motif. These DNA fragments with different numbers of mouse CpG-S motifs were inserted into the *Ava*II site of pMAS, which was first dephosphorylated with CIP to prevent self-ligation. The resulting recombinant plasmids maintained one *Ava*II site due to the design of the synthetic oligonucleotide sequence allowing the cloning process to be repeated until the desired number of CpG-S

motifs were inserted. Sixteen and 50 mouse CpG-S motifs were inserted into the *Ava*II site of pMAS, creating pMCG-16 and pMCG-50 respectively. The DNA fragment containing 50 CpG-S motifs was excised from pMCG-50, and inserted into *Hpa*I-*Ava*II-*Sca*I-*Dra*I linker of pMCG-50, creating pMCG-100. The same procedure was followed to create pMCG-200 (Table 3).

Two different sequences containing human-specific CpG-S motifs were cloned in different numbers into pMAS to create two series of vectors, pHCG and pHIS, following the same strategies as described above.

The pHCG series of vectors contain multiple copies of the following sequence 5' GAC TTC GTG TCG TTC TTC TGT CGT CTT TAG CGC TTC TCC TGC GTG CGT CCC TTG 3' (SEQ ID NO:14) (CpG-S motifs are underlined). This sequence incorporates various CpG-S motifs that had previously been found to have potent stimulatory effects on human cells *in vitro*. The vector pHCG-30, pHCG-50, pHCG-100 and pHCG-200 contain 30, 50, 100 and 200 human CpG-S motifs respectively (Table 3).

The pHIS series of vectors contain multiple copies of the following sequence: 5' GAC TCG TCG TTT TGT CGT TTT GTC GTT TCG TCG TTT TGT CGT TTT GTC GTT G 3' (SEQ ID NO:15) (CpG-S motifs are underlined). This sequence is based on the CpG-S motifs in oligo #2006, which has potent stimulatory effects on human cells *in vitro* The vector pHIS-40, pHIS-64, pHIS-128 and pHIS-192 contain 40, 64, 128 and 192 human CpG motifs respectively (Table 3).

(v) Cloning of the hepatitis B surface antigen gene

To create a DNA vaccine, the S gene (subtype *ayw*) encoding the hepatitis B surface antigen (HBsAg) was amplified by PCR and cloned into the polylinker of pUK21-A2 using the *Eco*RV and *Pst* I restriction enzyme sites. The S gene was analyzed by sequencing, and then subcloned into the same restriction enzyme sites of the pMCG and pHCG series of vectors (Table 4).

The S gene (subtype adw2) encoding the hepatitis B surface antigen (HBsAg) was cloned into the pHIS series of vectors following the same strategy as described above (Table 4).

CpG optimized gene therapy vector

The optimized gene therapy vectors were constructed from the basic expression vector (pUK21-A2) in several steps.

(i) Site-directed mutagenesis for removal of CpG immunostimulatory sequences within pUK21-A2

Only point-mutations, which would not interfere with the replication and function of the expression vector, pUK21-A2, were designed. Seventy-five point-mutations, including 55 nucleotides within non-essential regions and 20 silent-mutations within the kanamycin resistance gene, were carried out following the same strategy as described previously in *(ii) Site-directed mutagenesis to remove immunoinhibitory sequences*. The point mutations eliminated 64 CpG stimulatory motifs resulting in the vector pGT (Table 5).

ii) Insertion of unique restriction enzyme sites into pGT

Oligonucleotides 5' GCC CTA GTA CTG TTA ACT TTA AAG GGC CC 3' (SEQ ID NO:16) and its complementary strand 5' GGC GGG CCC TTT AAA GTT AAC AGT ACT AG 3' (SEQ ID NO:17) were synthesized, and 5'-phosphorylated. Annealing of these two phosphorylated oligos resulted in a 26 bp double-stranded DNA fragment containing four unique restriction enzyme sites (*Sca*I, *Hpa*I, *Dra*I and *Apa*I) and two *Eco*O109 I-cut sticky ends. Insertion of this 26 bp DNA fragment into pGT created the vector pGTU.

iii) Insertion of immunoinhibitory motifs into the vector pGTU

Human CpG-N motifs were cloned into the pGTU following the same strategies as described previously in *(iv) Insertion of immunostimulatory motifs into the vector pMAS*. The oligonucleotide 5' GCC CTG GCG GGG ATA AGG CGG GGA TTT GGC GGG GGA TAA GGC GGG GAA 3' (SEQ ID NO:18) and its complementary strand 5' GGC CCC CGC CTT

ATC CCC GCC AAA TCC CCG CCT TAT CCC CGC CAG 3' (SEQ ID NO:19) (four CpG motifs are underlined) were synthesized and phosphorylated. Annealing of these two oligonucleotides created a double-stranded DNA fragment, which was self-ligated first and then cloned into the *Eco*O109I site of the vector pGTU. The recombinant plasmids will be screened by restriction enzyme digestion and the vectors with the desired number of CpG inhibitory motifs will be sequenced and tested.

Immunization of Mice and Assay of Immune Responses

Female BALB/c mice aged 6-8 weeks (Charles River, Montreal) were immunized with DNA vaccines of HBsAg-encoding DNA (see vectors described above) by intramuscular injection into the tibialis anterior (TA) muscle. The plasmid DNA was produced in *E. coli* and purified using Qiagen endotoxin-free DNA purification mega columns (Qiagen GmbH, Chatsworth, CA). DNA was precipitated and redissolved in endotoxin-free PBS (Sigma, St. Louis MO) at a concentration of 0.01, 0.1 or 1 mg/ml. Total doses of 1, 10 or 100 μg were delivered by injection of 50 μl bilaterally into the TA muscles, as previously described (Davis *et al.*, 1993b).

In some cases, 10 or 100 μg of CpG ODN was added to the DNA vaccine (pCMV-S, Davis *et al.*, 1993b). The sequences and backbones of the ODN used are outlined in Table 6.

Mice were bled via the retro-orbital plexus at various times after immunization and recovered plasma was assayed for presence of anti-HBs antibodies (total IgG or IgG1 and IgG2a isotypes) by end-point dilution ELISA assay, as previously described (Davis *et al.*, 1993a).

For assay of CTL activity, mice were killed and their spleens removed. Splenocytes were restimulated *in vitro* with HBsAg-expressing cells and CTL activity was evaluated by chromium release assay as previously described (Davis *et al.*, 1998).

EXAMPLE 2

1. *In vitro* Effects of CpG-N Motifs

Nearly all DNA viruses and retroviruses have 50-94% fewer CpG dinucleotides than would

be expected based on random base usage. This would appear to be an evolutionary adaptation to avoid the vertebrate defense mechanisms related to recognition of CpG-S motifs. CpG suppression is absent from bacteriophage, indicating that it is not an inevitable result of having a small genome. Statistical analysis indicates that the CpG suppression in lentiviruses is an evolutionary adaptation to replication in a eukaryotic host. Adenoviruses, however, are an exception to this rule as they have the expected level of genomic CpG dinucleotides. Different groups of adenovirae can have quite different clinical characteristics.

Unlike the genome of almost all DNA viruses and retroviruses, some adenoviral genomes do not show suppression of CpG dinucleotides (Karlin *et al.*, 1994; Sun *et al.*, 1997). Analysis of different adenoviral genomes (types 2, 5, 12, and 40) reveals surprising variability among each other and compared to human and *E. coli* in the flanking bases around CpG dinucleotides (Table 7).

Adenoviral strains 2 and 5 belong to the same family but strain 12 is quite distinct from them. Purified type 12 adenoviral DNA induced cytokine secretion from human PBMC to a degree similar to that seen with bacterial DNA (EC DNA) (Table 8). In contrast, DNA from types 2 and 5 adenoviruses induced little or no production of cytokines (Tables 3, 4). Remarkably, not only did type 2 and 5 adenoviral DNA fail to induce TNF-α or IFN-γ secretion, it actively inhibited the induction of this secretion by EC DNA (Table 9). In contrast, type 12 adenoviral DNA had no discernible inhibitory effects. These data suggested that type 2 and 5 adenoviral DNA contains sequence motifs that inhibit the cytokine responses to the stimulatory motifs present.

The bases flanking CpG motifs determine whether a CpG dinucleotide will cause immune stimulation, and may also determine the type of cytokines secreted. The fact that type 2 and 5 adenoviral DNA was not only nonstimulatory but actually inhibitory of CpG DNA, suggested that certain nonstimulatory CpG motifs may even be able to block the stimulatory motifs and that the inhibitory motifs should be over-represented in the genomes of adenovirus type 2 and 5 compared to type 12 (or to human DNA). By analysis of these genomes, it was possible to identify sequences that could block the effects of known CpG-S sequences on *in vitro* B cell proliferation (Table 10) and cytokine secretion (Table 11).

Sequences which were found to be immunoinhibitory by *in vitro* assay were chosen to be mutated (wherever easily possible) from the backbone of the DNA vaccine vector.

2. CpG-S ODN cannot be used as an Adjuvant for DNA Vaccines

It has previously been shown that CpG-S ODN is a potent vaccine adjuvant when given with HBsAg protein (Davis *et al.*, 1998). Antibodies against HBsAg (anti-HBs) were augmented many times over those obtained with HBsAg alone or even HBsAg with alum as adjuvant. In addition, the humoral response was more strongly Th1, as indicated by a greater proportion of IgG2a than IgG1 isotypes of antibodies in immunized BALB/c mice. The strong Th1 effect of the CpG-S motifs was further demonstrated by the greatly enhanced cytotoxic T-cell activity. One of the most potent CpG-S ODN in mice was 1826, a 20-mer with 2 CpG-dinucleotides and made with a synthetic phosphorothioate backbone (see Table 6 for sequence).

In contrast to the success with protein antigens, attempts to augment immune responses induced by a HBsAg-expressing DNA vaccine by the addition of CpG-S ODN 1826 failed. Surprisingly, the immune responses decreased with the addition of CpG-S ODN in a dose-dependent manner (Figure 6a). Addition of ODN #1826 to a luciferase reporter gene construct (pCMV-luc, Davis *et al.*, 1993b) resulted in a dose-dependent decrease in luciferase expression (Figure 6b). This indicates that the negative effects of the CpG-S ODN on the DNA vaccine were due to reduced gene expression rather than an effect on the immune response against the gene product.

ODN #1826 used in the above studies is an ODN with a phosphorothioate backbone (S-ODN) and it is possible that the synthetic sulfur-containing backbone interfered with the ability of the plasmid DNA to transfect target cells. Zhao *et al.* (1994) investigated the effect of the backbone on binding, uptake and degradation of ODN by mouse splenocytes and found that S-ODN had the highest affinity for ODN-binding sites on the cell membrane and could competitively inhibit binding of ODN made with a natural phosphodiester backbone (O-ODN). A similar blocking of binding might be taking place when S-ODN is mixed with plasmid DNA, which contains a natural phosphodiester backbone like O-ODN. Furthermore,

it was shown that the affinity of ODN made with a phophorothioate-phosphodiester chimeric backbone (SOS-ODN) for ODN-binding sites was lower than that of S-ODN (Zhao *et al.*, 1994). Thus, we evaluated the effect of adding SOS-ODN 1980, which has the identical sequence to S-ODN 1826, to pCMV-luc DNA and found that even at a 100 μg dose, this did not alter the expression of the luciferase reporter gene (Figure 7). While ODN with a chimeric backbone (SOS-ODN) do not adversely affect the level of gene expression (except when certain sequences such as a poly G are present) (Figure 7), this is not useful since SOS-ODN are apparently also not sufficiently nuclease-resistant to exert a strong CpG adjuvant effect (Table 12). Administering the CpG S-ODN at a different time or site than the plasmid DNA does not interfere with gene expression either (Figure 8), however nor do these approaches augment responses to DNA vaccines by administering the CpG S-ODN at a different time or site than the plasmid DNA (Table 12). Thus it appears that the immune system must see the antigen and the CpG-S motif at the same time and the same place to augment antigen-specific responses. Thus, at least for the present, it appears necessary to clone CpG motifs into DNA vaccine vectors in order to take advantage of their adjuvant effect.

EXAMPLE 3

CpG-optimized DNA Vaccines

Eliminating 52 of 134 CpG-N motifs from a DNA vaccine markedly enhanced its Th1-like function *in vivo* and immune responses were further augmented by the addition of CpG-S motifs to the DNA vaccine vectors (Figure 9).

Titers of antibodies were increased by the removal of CpG-N motifs. With the addition of 16 or 50 CpG-S motifs, humoral responses became increasingly more Th1, with an ever greater proportion of IgG2a antibodies. The anti-HBs titer was higher with 16 than 50 CpG-S motifs, perhaps because the strong cytokine response with the greater number of motifs inhibited antigen expression that was driven by the CMV promoter. Viral promoters such as that from CMV are known to be down-regulated by cytokines such as the IFNs (Gribaudo *et al.*, 1993; Harms & Splitter, 1995; Xiang *et al.*, 1997).

CTL responses were likewise improved by removal of CpG-N motifs, and then more so by the addition of CpG-S motifs to the DNA vaccines.

EXAMPLE 4

CpG-Optimized Gene Therapy Vectors

Oligodeoxynucleotides (ODN) and DNA Phosphodiester ODN were purchased from Operon Technologies (Alameda, CA) and nuclease resistant phosphorothioate ODN were purchased from Oligos *Etc.* (Wilsonville, OR) or Hybridon Specialty Products (Milford, MA). All ODN had undetectable endotoxin levels (less than 1 ng/mg) by Limulus assay (Whittaker Bioproducts, Walkersville, MD). *E. coli* (strain B) DNA was purchased from Sigma (St. Louis, MO), purified by repeated extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and/or Triton X114 extraction and ethanol precipitation and made single stranded by boiling for 10 min followed by cooling on ice for 5 min. Highly purified type 2, 5, and 12 adenoviral DNA was prepared from viral preparations using standard techniques and processed in the same manner as the *E. coli* DNA. Plasmids for DNA vaccination were purified using two rounds of passage over Endo-free columns (Qiagen, Hilden, Germany).

Cell Cultures and ELISA assays for cytokines. ELISA assays were performed using standard techniques and commercially available reagents as previously described (Klinman, D., *et al.*, *Proc. Natl. Acad. Sci. USA*, 93, 2879-2883 (1996); Yi *et al.*, *J. Immunol.*, 157, 5394-5402 (1996)). Standard deviations of the triplicate wells were <10%.

Construction of optimized DNA vectors. The starting material was pUK21-A2, an expression vector containing the immediate early promoter of human cytomegalovirus (CMV IE), the bovine growth hormone (BGH) polyadenylation signal, and the kanamycin resistance gene (Wu and Davis, unpublished). To avoid disrupting the plasmid origin of replication, mutagenesis designed to eliminate CpG-N motifs was restricted to the kanamycin resistance gene and non-essential DNA sequences following the gene. A total of 22 point mutations were introduced to alter 15 CpG-N motifs (a "motif" refers to a hexamer containing one or more CpG dinucleotides) containing 19 CpG dinucleotides, 12 of which were eliminated and 7 of which were transformed into CpG-S motifs. Site-directed mutagenesis was performed by overlap extension PCR as described by Ge *et al.* (Prosch, S., *et al.*, *Biol. Chem.*, 377, 195-

201 (1996)). The 1.3 kb *Alw*N I-*Eco*O109 I fragment of pUK21-A2, which contained all 22 nucleotides to be mutated, was used as the template for PCR. The 1.3 kb fragment was regenerated by four rounds of overlap extension PCR using appropriate mutagenic primers, and substituted for the original *Alw*N I-*Eco*O109 I fragment, resulting in pUK21-B2. All the mutations were confirmed by sequencing.

Another 37 CpG-N motifs were removed by replacing the f1 origin with a multiple cloning site. Oligonucleotides 5' GCCCTATTTTAAATTCGAAAGTACTGGACCTGTTAACA 3' (SEQ ID NO:20) and its complementary strand 5' CGTGTTAACAGGTCCAGTACTTTCGAATTTAAAATAG 3' (SEQ ID NO:21) were synthesized, and 5'-phosphorylated. Annealing of these two phosphorylated-oligos resulted in a 35 bp double-stranded DNA fragment containing four unique restriction enzyme sites (*Dra* I, *Sca* I, *Ava* II, *Hpa* I) and two sticky ends. Replacing the 0.6 kb *Nar* I-*Eco*O109 I fragment of pUK21-B2, with this double-stranded DNA fragment resulted in the master vector pMAS.

Next, different numbers of CpG-S motifs were inserted into the vector by allowing self-ligation of a 20bp DNA fragment with the sequence 5' GACTCCATGACGTTCCTGACGTTTCCATGACGTTCCTGACGTTG 3' (SEQ ID NO:12) with a complementary strand and inserting different numbers of copies into the *Ava* II site of pMAS. Recombinant clones were screened and the two vectors were chosen for further testing with 16 and 50 CpG-S motifs, and named pMCG16 and pMCG50 respectively.

To create a DNA vaccine, the S gene encoding *ay* subtype of hepatitis B surface antigen (HBsAg) was amplified by PCR and cloned into the *Eco*RV - *Pst*I sites of the vectors, resulting in pUK-S, pMAS-S, pMCG16-S, and pMCG50-S respectively. Vector sequences were confirmed by sequencing and have been deposited in GenBank under accession numbers AFO53406 (pUK-S), AFO53407 (pMAS-S), AFO53408 (pMCG16-S), and AFO53409 (pMCG50-S).

Immunization of mice against HBsAg: Immunization of 6-8 wk old female BALB/c mice (Charles River, Montreal, QC) was by injection into the tibialis anterior muscle (TA) of 1 μg

recombinant HBsAg or 10 μg HBsAg-expressing DNA vaccine (Chace, J.H., *et al.*, *Immunopath*, **In press** (1997)). Assay for antibodies against HBsAg (anti-HBs) was by end point dilution and for cytotoxic T lymphocytes (CTL) was by chromium release assay as described previously[19]. Both the protein (± ODN) and DNA vaccines were resuspended in saline for injection.

EXAMPLE 5

Type 12 adenoviral DNA is immune stimulatory, but types 2 and 5 adenoviral DNA are immune neutralizing. To investigate possible functional differences in the immune effects of various prokaryotic DNAs, we determined their ability to induce cytokine secretion from human PBMC. In contrast to bacterial DNA and genomic DNA from type 12 adenovirus, DNA from types 2 and 5 adenovirus failed to induce cytokine production (Table 8). In fact, despite their similar frequency of CpG dinucleotides, type 2 or 5 adenoviral DNA severely reduced the cytokine expression induced by co-administered immunostimulatory *E. coli* genomic DNA (Table 9). This indicates that type 2 and 5 adenoviral DNA does not simply lack CpG-S motifs, but contains sequences that actively suppress those in *E. coli* DNA.

Identification of putative immune neutralizing CpG-N motifs in type 2 and 5 adenoviral genomes. To identify possible non-random skewing of the bases flanking the CpG dinucleotides in the various adenoviral genomes, we examined their frequency of all 4096 hexamers. The six most common hexamers in the type 2 adenoviral genome are shown in Table 7, along with their frequency in the Type 12 and *E. coli* genomes. Remarkably, all of these over-represented hexamers contain either direct repeats of CpG dinucleotides, or CpGs that are preceded by a C and/or followed by a G. These CpG-N motifs are approximately three to six fold more common in the immune inhibitory type 2 and 5 adenoviral genomes than in those of immune-stimulatory type 12 adenoviral, *E. coli* or non-stimulatory human genomic DNAs (Table 7). This hexamer analysis further revealed that the frequency of hexamers containing CpG-S motifs (*e.g.*, GACGTT or AACGTT) in the type 2 adenoviral genome is as low as that in the human genome: only 1/3 to 1/6 of that in *E. coli* and type 12 adenoviral DNA (Table 7).

Effect of CpG-N motifs on the immune stimulatory effects of CpG-S motifs. To determine whether these over-represented CpG-N motifs could explain the neutralizing properties of type 2 and 5 adenoviral DNA, we tested the *in vitro* immune effects of synthetic oligodeoxynucleotides bearing a CpG-S motif, one or more CpG-N motifs, or combinations of both. An ODN containing a single CpG-S motif induces spleen cell production of IL-6, IL-12, and IFN-γ (ODN 1619, Table 13). However, when the 3' end of this ODN was modified by substituting either repeating CpG dinucleotides or a CpG dinucleotide preceded by a C, the level of cytokine production was reduced by approximately 50% (ODN 1952 and 1953, Table 13). ODN consisting exclusively of these neutralizing CpG (CpG-N) motifs induced little or no cytokine production (Table 14). Indeed, addition of ODN containing one or more CpG-N motifs to spleen cells along with the CpG-S ODN 1619 caused a substantial decrease in the induction of IL-12 expression indicating that the neutralizing effects can be exerted in *trans* (**Table 14**).

To determine whether the *in vivo* immune activation by ODN containing CpG-S motifs would be reversed by CpG-N motifs, we immunized mice with recombinant hepatitis B surface antigen (HBsAg), with or without nuclease resistant phosphorothioate-modified ODN containing various types of CpG motifs. As expected, a CpG-S ODN promoted a high titer of antibodies against HBsAg (anti-HBs antibodies) which were predominantly of the IgG2a subclass, indicating a Th1-type immune response (Figure 10; ODN 1826). The various CpG-N ODN induced either little or no production of anti-HBs antibodies (ODN 1631, 1984, and 2010) (Figure 10). Mice immunized with combinations of CpG-S and CpG-N ODN had a reduced level of anti-HBs antibodies compared to mice immunized with CpG-S ODN alone, but these were still predominantly IgG2a (Figure 10).

Enhanced DNA vaccination by deletion of plasmid CpG-N motifs. DNA vaccines can be highly effective inducers of Th1-like immune responses (Raz, E., *et al.*, *Proc. Natl. Sci. Acad. USA*, 93, 5141-5145 (1996); Donnelly , J.J., *et al.*, *Ann. Rev. Immunol.*, 15, 617-648 (1997)). Based on the *in vivo* and *in vitro* effects of CpG-N motifs, we hypothesized that their presence within a DNA vaccine would decrease its immunostimulatory effects. The starting vector, pUK21-A2, contained 254 CpG dinucleotides, of which 134 were within

CpG-N motifs. In order to test the hypothesis that these CpG-N motifs adversely affected the efficacy of this vector for DNA-based vaccination, the number of CpG-N motifs was reduced, either by mutation or deletion. Since mutations in the plasmid origin of replication interfere with replication of the plasmid, we restricted our initial mutations to the kanamycin resistance gene and a nonessential flanking region. We were able to eliminate 19 CpG dinucleotides contained within 15 of the 20 CpG-N motifs in these regions without changing the protein sequence. The F1 origin of replication containing 37 CpG-N motifs and only 17 other CpG dinucleotides was then deleted, creating the vector pMAS. This vector was further modified by the introduction of 16 or 50 CpG-S motifs, yielding vectors pMCG16 and pMCG50 respectively. The S gene for HBsAg was then cloned into these vectors downstream from the CMV promoter, to make pUK-S, pMAS-S, pMCG16-S, and pMCG50-S respectively.

When tested for their ability to induce cytokine (IL-6 and IL-12) secretion from cultured spleen cells, we found that the pMAS-S, pMCG16-S and pMCG50-S vectors had significantly enhanced immune stimulatory activity compared to pUK-S. When used as a DNA vaccine, the anti-HBs response at 4 and 6 weeks was substantially stronger with DNA vaccines from which CpG-N motifs had been deleted, and even more so when 16 CpG-S motifs had been inserted. The vector with 50 CpG-S motifs, however, was less effective at inducing antibody production than that with 16 motifs. (Figure 11A). Removal of CpG-N motifs and addition of CpG-S motifs resulted in a more than three-fold increase in the proportion of IgG2a relative to IgG1 anti-HBs antibodies, indicating an enhanced Th-1 response. This accentuated Th1 response also was demonstrated by the striking progressive increases in CTL responses induced by vectors from which CpG-N motifs were deleted and/or CpG-S motifs added (Figure 11B).

The discovery of immune activating CpG-S motifs in bacterial DNA has led to the realization that aside from encoding genetic information, DNA can also function as a signal transducing molecule. Our present results demonstrate that genomic DNA from type 12 adenovirus is immune stimulatory, compatible with its relatively high content of CpG-S motifs. In contrast, genomic DNA from type 2 and 5 adenoviruses is not stimulatory, but rather is immune neutralizing and blocks the cytokine induction of bacterial DNA (**Tables 8 and 9**). To

identify possible differences in the CpG motifs present in these different adenoviral genomes, analyzed the genomic frequency of all hexamer sequences was analyzed. This analysis demonstrated that only the type 2 and 5 adenoviral genomes had a dramatic overrepresentation of CpG motifs containing direct repeats of CpG dinucleotides and/or CpGs preceded by a C and/or followed by a G (Table 7). Synthetic ODN containing such putative immune neutralizing (CpG-N) motifs not only did not induce cytokine production *in vitro*, but also inhibited the ability of an immune stimulatory CpG-S motif to induce cytokine expression (Tables 13, 14). These studies reveal that there are immune neutralizing CpG-N as well as stimulatory CpG-S motifs and that there is a surprisingly complex role for the bases flanking CpG dinucleotides in determining these immune effects. In general, CpG-N motifs oppose CpG-S motifs in *cis* or *trans*. The mechanism through which CpG-N motifs work is not yet clear, but does not appear to involve competition for cell uptake or binding to a CpG-S-specific binding protein. Further studies are underway to determine the molecular mechanisms through which CpG-N and CpG-S motifs exert their respective immune effects.

The hexamers that contain CpG-N motifs are from 15 to 30 times more common in type 2 and 5 adenoviral genomes than those that contain immune stimulatory CpG-S motifs. However, in type 12 adenoviral genomes the frequencies of hexamers containing CpG-N and CpG-S motifs do not differ substantially from chance. These data suggest that the immune neutralizing effects of types 2 and 5 adenoviral DNA are not merely a result of their propagation in eukaryotic cells, but rather are due to the overall excess of CpG-N compared to CpG-S motifs. It is tempting to speculate that the marked over-representation of CpG-N motifs in the genomes of types 2 and 5 adenovirus may contribute to the biologic properties, such as persistent infection of lymphocytes, which distinguish them from type 12 adenovirus. The presence of large numbers of CpG-N motifs within these adenoviral genomes may have played an important role in the evolution of this virus by enabling it to avoid triggering CpG-induced immune defenses. It will be interesting to determine the general distribution of CpG-N and CpG-S motifs in different families of microbial and viral genomes, and to explore their possible roles in disease pathogenesis.

CpG-N motifs are also over-represented in the human genome, where their hexamers are approximately two to five-fold more common than CpG-S motifs. While this skewing is far less marked than that in adenoviral DNA, it would still be expected to reduce or eliminate any immune stimulatory effect from the unmethylated CpGs present in CpG islands within vertebrate DNA. We and others have found that even when predominantly or completely unmethylated, vertebrate DNA is still not immune stimulatory (A. Krieg and P. Jones, unpublished data) (Sun, S., *et al.*, *J. Immunol.*, 159:3119-3125 (1997)) which is in keeping with its predominance of CpG-N motifs (Table 7). Given the overall level of CpG suppression in the human genome, the molecular mechanisms responsible for the skewing of the frequency of CpG-N to CpG-S motifs are unclear. Such a distortion from the expected random patterns would seem to require the existence of pathways that preferentially mutate the flanking bases of CpG-S motifs in vertebrate genomes, but do not affect CpG-N motifs. Indeed, statistical analyses of vertebrate genomes have provided evidence that CpGs flanked by A or T (as in CpG-S motifs) mutate at a faster rate than CpGs flanked by C or G (Bains, W., *et al.*, *Mutation Res.*, 267:43-54 (1992)).

Based on our *in vitro* experiments we hypothesized that the presence of CpG-N motifs in DNA vaccines interferes with the induction of the desired immune response. Indeed, the present study demonstrates that elimination of CpG-N motifs from a DNA vaccine leads to improved induction of antibodies. By removing 52 of the CpG-N motifs from a DNA vaccine (45 were deleted and 7 turned into CpG-S motifs) the serologic response was more than doubled; by then adding an additional 16 CpG-S motifs, the response was enhanced nearly 10 fold (Figure 11A). Likewise, CTL responses were improved by removing CpG-N motifs and even more so by adding 16 or 50 CpG-S motifs (Figure 11B). These increased responses are especially notable in view of the fact that the total number of CpG dinucleotides in the mutated vaccines is considerably below the original number.

The finding that the vector with 50 CpG-S motifs was inferior to that with 16 motifs for induction of humoral immunity was unexpected, and may be secondary to CpG-induced production of type I interferons, and subsequent reduction in the amount of antigen expressed. The decreased antibody response induced by pMCG50-S seems unlikely to be

explained by vector instability since this vector gave the best CTL responses (Figure 11B). Although the pMCG50-S vector was slightly larger than pMCG16-S, the 10 μg dose still contained 93% as many plasmid copies as it did pMCG16-S, so lower copy number is unlikely to account for the reduced antibody levels. The current generation of DNA vaccines are quite effective in mice, but much less effective in primates (Davis, H.L., *et al.*, *Proc. Natl. Acad. Sci. USA*, 93:7213-7218 (1996); Letvin, N.L., *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:9378-9383 (1997); Fuller, D.H., *et al.*, *J. Med. Primatol.*, 25:236-241 (1996); Lu, S., *et al.*, *J. Virol.*, 70:3978-3991 (1996); Liu, M.A., *et al.*, *Vaccine*, 15:909-919 (1997); Prince, A.M., *et al.*, *Vaccine*, 15:9196-919 (1997); Gramzinski, R.A., *et al.*, *Molec. Med.*, 4:109-119 (1998)). Our present results indicate that attaining the full clinical potential of DNA vaccines will require using engineered vectors in which CpG-N motifs have been deleted, and CpG-S motifs added.

On the other hand, the field of gene therapy may benefit from the discovery of CpG-N motifs through their insertion into gene transfer vectors to prevent or reduce the induction of host immune responses. Most of the CpG-N motifs in the adenoviral genome are in the left hand (5') side, which is generally partially or totally deleted for the preparation of gene therapy vectors, especially with the "gutless" vectors (Kochanek, S., *et al.*, *Proc. Natl. Acad. Sci. USA*, 93:5731-5736 (1996)). This could lead to an enhanced CpG-S effect. Since nucleic acids produced in viral vectors are unmethylated, they may produce inflammatory effects if they contain a relative excess of CpG-S over CpG-N motifs and are delivered at an effective concentration (about 1 μg/ml). Gene therapy studies with adenoviral vectors have used doses up to 10 infectious units (IU)/ml (which contains 0.4 μg of DNA/ml based on the genome size of 36 kb). Given that approximately 99% of adenoviral particles are noninfectious, this corresponds to a DNA dose of approximately 40 μg/ml, which is well within the range at which CpG DNA causes *in vivo* immune stimulatory effects; just 10 μg/mouse induces IFN-γ production acts as an adjuvant for immunization (Davis, H.L., *et al.*, *J. Immunol.*, 160:870-876 (1998); Chu, R.S., *et al.*, *J. Exp.Med.*, 186:1623-1631 (1997); Lipford, G.B., *et al.*, *Eur. J. Immunol.*, 27:2340-2344 (1997); Weiner, G.J., *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:10833 (1997); Moldoveanu, Z., *et al.*, *Vaccine*, In press (1998)), and causes acute pulmonary inflammation when delivered into mouse airways (Schwartz, D., *et al.*, *J. Clin. Invest.*,

100:68-73 (1997)). Multiple mechanisms besides the presence of CpG-S DNA are doubtless responsible for the inflammatory responses that have limited the therapeutic development of adenoviral vectors (Newman, K.D., *et al., J. Clin. Invest.*, 96:2955-2965 (1995); Zabner, J., *et al.*, *J. Clin. Invest.*, 97:1504-1511 (1996)). Nonetheless, our present results suggest that consideration be given to the maintenance or insertion of CpG-N motifs in adenoviral vectors, and to the engineering of backbones and inserts so that CpG-S motifs are mutated in order to reduce immune activation.

In recent years, it has become clear that effective gene expression need not require a viral delivery system. The use of plasmids for gene delivery (with or without lipids or other formulations) avoids some of the problems of viral vectors. On the other hand, much larger doses of DNA are typically required, since delivery is far less efficient than with a targeted system such as a virus. For example, effective gene expression in mice typically may require 500-1000 μg DNA/mouse (Philip, R., *et al.*, *J. Biol. Chem.*, 268:16087-16090 (1993); Wang, C., *et al.*, *J. Clin. Invest.*, 95:1710-1715 (1995)). A recent human clinical trial using lipid/DNA complexes and naked DNA for delivery of CFTR to the nasal epithelium of patients with cystic fibrosis used doses of 1.25 mg of plasmid/nostril (Zabner, J., *et al., J. Clin. Invest.*, 100:1529-1537 (1997)). The successful application of naked DNA expression vectors for gene therapy will depend on the safety of repeatedly delivering high doses of DNA. Since the plasmids used for gene therapy typically contain several hundred unmethylated CpG dinucleotides, many of which are in CpG-S motifs, some immune activation may be expected to occur. Indeed, mice given repeated doses of just 10 μg of plasmid DNA daily develop elevated lymphocyte levels and several humans who received intranasal plasmid DNA had elevated serum IL-6 levels (Philip, R., *et al., J. Biol. Chem.*, 268:16087-16090 (1993)). Furthermore, delivery of 4 mg of a gene therapy plasmid to cystic fibrosis patients in a recent clinical trial caused acute onset of symptoms compatible with immune activation, including fever, chills, and pulmonary congestion. Another reason to avoid the presence of CpG-S motifs in gene therapy vectors is that the cytokines that are produced due to the immune stimulation may reduce plasmid vector expression, especially when this is driven by viral promoters (Raz, E., *et al., Proc. Natl. Acad. Sci. USA*, 93:5141-5145 (1996)).

It is, therefore, highly desirable to develop improved gene delivery systems with reduced immune activation. It is not possible to simply methylate the CpG-S dinucleotides in gene therapy plasmids, since methylation of promoters abolishes or severely reduces their activity. The only promoter resistant to methylation-induced silencing is the MMTV promoter, which contains no essential CpGs, but is fairly weak. In any case, even when the promoter is unmethylated , expression is still greatly reduced if the coding sequences are methylated. In fact, even the strong CMV IE promoter is completely inactivated by CpG methylation. Deletion of all CpGs from an expression plasmid is not feasible since many of these are located in the origin of replication (approximately 1.2 Kb long) where even single base changes can dramatically reduce plasmid replication. For these reasons, we propose that addition of CpG-N motifs, and/or mutation or conversion of CpG-S to CpG-N motifs may lead to the generation of less immune stimulatory vectors for gene therapy. Studies to investigate this possibility are under way.

Table 1.

Primers used for site-directed mutagenesis.

Mutated nucleotides are underlined. Restriction enzyme sites for cloning are indicated in bold.

Forward primers:

Mu-0F		5' GTC**TCTAGACAGCCACTG**GTAACAGGATT 3' (845)
Mu-1F	(1144)	5' GTCGTTGTGTCGTCAAGTCAGCGTAATGC 3' (1172)
Mu-2F	(1285)	5' TCGTTTCTGTAATGAAGGAG 3' (1304)
Mu-3F	(1315)	5' AAGGCAGTTCCATAGGATGG 3' (1334)
Mu-(4+5)F	(1348)	5' TCGATCTGCGATTCCAACTCGTCCAACATCAATAC 3' (1382)
Mu-6F	(1453)	5' TGGTGAGAATGGCAAAAGTT 3' (1472)
Mu-7F	(1548)	5' CATTATTCATTCGTGATTGCG 3' (1568)
Mu-8F	(1633)	5' ACGTCTCAGGAACACTGCCAGCGC 3' (1656)
Mu-9F	(1717)	5' AGGGATCGCAGTGGTGAGTA 3' (1736)
Mu-10F	(1759)	5' TATAAAATGCTTGATGGTCGG 3' (1779)

Mu-(11+12)F	(1777)	5' GGGAAGAGGCATAAATTCTGTCAGCCAGTTTAGTC 3' (1811)
Mu-13F	(1882)	5' TGGCTTCCCATACAAGCGAT 3' (1901)
Mu-14F	(1924)	5' TACATTATCGCGAGCCCATT 3' (1943)
Mu-15F	(1984)	5' TGGCCTCGACGTTTCCCGT 3' (2002)

Reverse primers:

Mu-0R		5' ATCGAATTCAGGGCCTCGTGATACGCCTA 3' (2160)
Mu-1R	(1163)	5' TGACTTGACGACACAACGACAGCTCATGACCAAAATCCC 3' (1125)
Mu-2R	(1304)	5' CTCCTTCATTACAGAAACGACTTTTTCAAAAATATGGTA 3' (1266)
Mu-3R	(1334)	5' CCATCCTATGGAACTGCCTTGGTGAGTTTTCTCCTTC 3' (1298)
Mu-(4+5)R	(1367)	5' GAGTTGGAATCGCAGATCGATACCAGGATCTTGC 3' (1334)
Mu-6R	(1472)	5' AACTTTTGCCATTCTCACCAGATTCAGTCGTCACTCA 3' (1436)
Mu-7R	(1568)	5' CGCAATCACGAATGAATAATGGTTTGGTTGATGCGAGTG 3' (1530)
Mu-8R	(1652)	5' TGGCAGTGTTCCTGAGACGTTTGCATTCGATTCCTGTT 3' (1615)
Mu-9R	(1736)	5' TACTCACCACTGCGATCCCTGGAAAAACAGCATTCCAG 3' (1736)
Mu-10R	(1779)	5' CCGACCATCAAGCATTTTATACGTACTCCTGATGATGCA 3' (1741)
Mu-(11+12)	(1796)	5' CAGAATTTATGCCTCTTCCCACCATCAAGCATTTTATAC 3' (1758)
Mu-13R	(1901)	5'ATCGCTTGTATGGGAAGCCAGATGCGCCAGAGTTGTTT 3' (1882)
Mu-14R	(1943)	5' AATGGGCTCGCGATAATGTAGGGCAATCAGGTGCGAC 3' (1907)
Mu-15R	(2002)	5'ACGGGAAACGTCGAGGCCACGATTAAATTCCAACATGG 5' (1965)

(SEQ ID NO:23-50, respectively)

Table 2 *Nucleotide and amino acid sequences of the AlwNI-coO109I fragment.*

kan(wt) 2180	AAGGGCCTCG	TGATACGCCT	ATTTTTATAG	GTTAATGTCA	TGGGGGGGGG	GGGGAAAGCC
kan(wt) 2120	ACGTTGTGTC	TCAAAATCTC	TGATGTTACA	TTGCACAAGA	TAAAAATATA	TCATCATGAA
kan(wt) 2060 kan(mu) ORF	CAATAAAACT	GTCTGCTTAC	ATAAACAGTA	ATACAAGGGG	TGTTATGAGC M S	CATATTCAAC H I Q
kan(wt) 2000 kan(mu) ORF	GGGAAACGTC R E T S	GAGGCCGCGA R P R	TTAAATTCCA L N S	ACATGGATGC N M D A	TGATTTATAT D L Y	GGGTATAAAT G Y K
kan(wt) 1940 kan(mu) ORF	GGGCTCGCGA W A R D	TAATGTCGGG N V G	CAATCAGGTG Q S G	CGACAATCTA A T I Y	TCGCTTGTAT R L Y	GGGAAGCCCG G K P
kan(wt) 1880 kan(mu) ORF	ATGCGCCAGA D A P E	GTTGTTTCTG L F L	AAACATGGCA K H G	AAGGTAGCGT K G S V	TGCCAATGAT A N D	GTTACAGATG V T D
kan(wt) 1820 kan(mu) ORF	AGATGGTCAG E M V R	ACTAAACTGG L N W	CTGACGGAAT L T E	TTATGCCTCT F M P L	TCCGACCATC P T I	AAGCATTTTA K H F
kan(wt) 1760 kan(mu) ORF	TCCGTACTCC I R T P	TGATGATGCA D D A	TGGTTACTCA W L L	CCACTGCGAT T T A I	CCCCGGAAAA P G K	ACAGCATTCC T A F
kan(wt) 1700 kan(mu) ORF	AGGTATTAGA Q V L E	AGAATATCCT E Y P	GATTCAGGTG D S G	AAAATATTGT E N I V	TGATGCGCTG D A L	GCAGTGTTCC A V F
kan(wt) 1640 kan(mu) ORF	TGCGCCGGTT L R R L	GCATTCGATT H S I	CCTGTTTGTA P V C	ATTGTCCTTT N C P F	TAACAGCGAT N S D	CGCGTATTTC R V F
kan(wt) 1580 kan(mu) ORF	GTCTCGCTCA R L A Q	GGCGCAATCA A Q S	CGAATGAATA R M N	ACGGTTTGGT N G L V	TGATGCGAGT D A S	GATTTTGATG D F D
kan(wt) 1520 kan(mu) ORF	ACGAGCGTAA D E R N	TGGCTGGCCT G W P	GTTGAACAAG V E Q	TCTGGAAAGA V W K E	AATGCATAAA M H K	CTTTTGCCAT L L P
kan(wt) 1460 kan(mu) ORF	TCTCACCGGA F S P D	TTCAGTCGTC S V V	ACTCATGGTG T H G	ATTTCTCACT D F S L	TGATAACCTT D N L	ATTTTTGACG I F D
kan(wt) 1400 kan(mu) ORF	AGCGGAAATT E G K L	AATAGGTTGT I G C	ATTGATGTTG I D V	GACGAGTCGG G R V G	AATCGCAGAC I A D	CGATACCAGG R Y Q
kan(wt) 1340 kan(mu) ORF	ATCTTGCCAT D L A I	CCTATGGAAC L W N	TGCCTCGGTG C L G	AGTTTTCTCC E F S P	TTCATTACAG S L Q	AAACGGCTTT K R L
kan(wt) 1280 kan(mu) ORF	TTCAAAAATA F Q K Y	TGGTATTGAT G I D	AATCCTGATA N P D	TGAATAAATT M N K L	GCAGTTTCAT Q F H	TTGATGCTCG L M L
kan(wt) 1220 kan(mu) ORF	ATGAGTTTTT D E F F	CTAATCAGAA	TTGGTTAATT	GGTTGTAACA	CTGGCAGAGC	ATTACGCTGA
kan(wt) 1160 Kan(mu)	CTTGACGGGA AC	CGGCGCAAGC AA AC	TCATGACCAA	AATCCCTTAA	CGTGAGTTTT	CGTTCCACTG
kan(wt) 1100	AGCGTCAGAC	CCCGTAGAAA	AGATCAAAGG	ATCTTCTTGA	GATCCTTTTT	TTCTGCGCGT
kan(wt) 1040	AATCTGCTGC	TTGCAAACAA	AAAAACCACC	GCTACCAGCG	GTGGTTTGTT	TGCCGGATCA
kan(wt) 980	AGAGCTACCA	ACTCTTTTTC	CGAAGGTAAC	TGGCTTCAGC	AGAGCGCAGA	TACCAAATAC
kan(wt) 920	TGTTCTTCTA	GTGTAGCCGT	AGTTAGGCCA	CCACTTCAAG	AACTCTGTAG	CACCGCCTAC
kan(wt) 860	ATACCTCGCT	CTGCTAATCC	TGTTACCAGT	GGCTGCTGCC		

Note: Mutated nucleotides are underlined. The AlwNI and EcoO109I sites are indicated in bold type. The nucleotide numbering of pMS1 is the same as the backbone vector pUK21.

Plasmid DNA Vectors

Davis *et al.* (1998)

Table 3

Plasmids containing immunostimulatory CpG motifs

Plasmid	Backbone	No CpG motifs	Species-specificity and ODN equivalence of CpG-S Insert
pMCG-16	pMAS	16	mouse-specific CpG motif #1826 [1]
pMCG-50	pMAS	50	
pMCG-100	pMAS	100	
pMCG-200	pMAS	200	
pHCG-30	pMAS	30	human-specific CpG motif – no ODN equivalent [2]
pHCG-50	pMAS	50	
pHCG-100	pMAS	100	
pHCG-200	pMAS	200	
pHIS-40	pMAS	40	human-specific CpG motif #2006 [3]
pHIS-64	pMAS	64	
pHIS-128	pMAS	128	
pHIS-192	pMAS	192	

1 sequence of 1826 is TCCATGACGTTCCTGACGTT

2 sequence used as source of CpG motifs is GACTTCGTGTCGTTCTTCTGTCGTCTTTAGCGCTTCTCCTGCGTGCGTCCCTTG

3 sequence of 2006 is TCGTCGTTTTGTCGTTTTGTCGTT

Table 4

Plasmids encoding hepatitis B surface antigen (derived from ayw or adw subtypes of HBV)

Plasmid	Backbone	Insert
pUK-S	pUK21-A2	HBV-S (ayw)
pUKAX-S	pUK21-AX*	HBV-S (ayw)
pMAS-S	pMAS	HBV-S (ayw)
pMCG16-S	pMCG-16	HBV-S (ayw)
pMCG50-S	pMCG-50	HBV-S (ayw)
pMCG100-S	pMCG-100	HBV-S (ayw)
pMCG200-S	pMCG-200	HBV-S (ayw)
pHCG30-S	pHCG-30	HBV-S (ayw)
pHCG50-S	pHCG-50	HBV-S (ayw)
pHCG100-S	pHCG-100	HBV-S (ayw)
pHCG200-S	pHCG-200	HBV-S (ayw)
pHIS20-S(ad)	pHIS-20	HBV-S(adw2)
pHIS36-S(ad)	pHIS-36	HBV-S(adw2)
pHIS72-S(ad)	pHIS-72	HBV-S(adw2)
pHIS108-S(ad)	PHIS-108	HBV-S(adw2)

*pUK21-AX was created by deleting f1 origin from pUK21-A

Table 5 *Sequence comparison of pUKA2 and pGT.*
75 point-mutations (indicated with *) in pUK21-A2 results in the gene therapy vector (pGT).

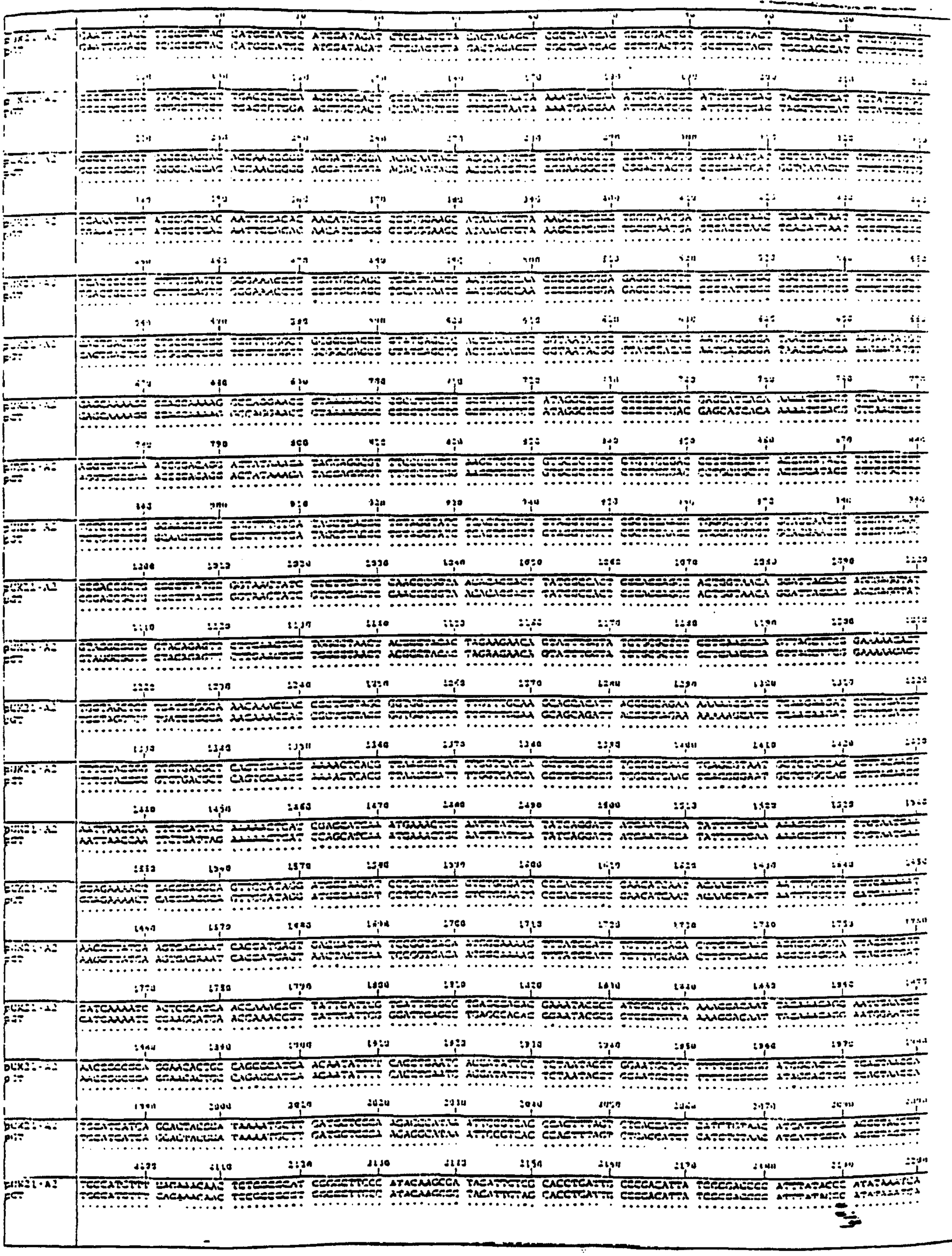


Table 6 *ODN used with plasmid DNA*

Backbone	ODN code number	Sequence
S-ODN	1826	TCCATGACGTTCCTGACGTT
	1628	GGGGTCAACGTTGAGGGGGG
	1911	TCCAGGACTTTCCTCAGGTT
	1982	TCCAGGACTTCTCTCAGGTT
	2017	CCCCCCCCCCCCCCCCCCCC
O-ODN	2061	TCCATGACGTTCCTGACGTT
	2001	GGCGGCGGCGGCGGCGGCGG
SOS-ODN	1980	TCCATGACGTTCCTGACGTT
	1585	GGGGTCAACGTTGAGGGGGG
	1844	TCTCCCAGCGTGCGCCATAT
	1972	GGGGTCTGTGCTTTTGGGGGG
	2042	TCAGGGGTGGGGGGAACCTT
	1981	GGGGTTGACGTTTTGGGGGG
	2018	TCTAGCGTTTTTAGCGTTCC
	2021	TCGTCGTTGTCGTTGTCGTT
	2022	TCGTCGTTTTGTCGTTTTGTCGTT
	2023	TCGTCGTTGTCGTTTTGTCGTT

Note: (SEQ ID NO:51-67, respectively)

SOS-ODN had two S-linkages at the 5' end, five S-linkages at the 3' end, and O-linkages in between.

Three ODN used in this study were of the same murine-specific immunostimulatory sequence in three different backbones (1826, 2061 and 1980).

All ODN were synthesized by Hybridon (Milford, MA) or Operon (Alameda, CA). ODN were ethanol precipitated and resuspended in saline prior to use alone or as an additive to the plasmid DNA solution.

Table 7 Genomic frequencies of selected hexamers

	Genomic frequency (X 10^{-3})			
hexamer:	Adenovirus Type 2	Adenovirus Type 12	E. coli	Human
GCGCGC	1.614	0.498	0.462	0.153
GCGGCG	1.530	0.469	0.745	0.285
GGCGGC	1.419	0.440	0.674	0.388
CGCGCG	1.336	0.322	0.379	0.106
GCCGCC	1.280	0.410	0.466	0.377
CGCCGC	1.252	0.410	0.623	0.274
GACGTT	0.083	0.234	0.263	0.068
AACGTT (CpG-S)	0.056	0.205	0.347	0.056

The frequencies of hexamers in adenoviral and *E. coli* genomes were kindly provided by J. Han (University of Alabama, Birmingham), who also determined those for the human genome[52]. The hexamer frequencies in type 5 adenovirus are essentially identical to those in type 2, and are therefore not shown. The last two hexamers are CpG-S motifs shown for comparison and are the most stimulatory of all tested CpG-S motifs.

Note that the expected frequency of a randomly selected hexamer is $1/4096 = 0.244 \times 10^{-3}$.

Table 8 Genomic DNA from type 12 but not type 2 adenovirus stimulates cytokine secretion from human PBMC

	Experiment 1[1]		Experiment 2[1]	
	TNF-α	IL-6	TNF-α	IL-6
Cells	27	800	30	800
EC 3 μg/ml	235	26,500	563	34,000
CT 10 μg/ml	0	1,400	0	2,800
Adv 2; 3 μg/ml	15.6	900	30	1,900
Adv 12; 3 μg/ml	86	11,300	120	11,250

[1]PBMC were obtained from normal human donors and cultured at $1X10^5$ cells/200 μl in RPMI with 10% autologous serum for 4 hr (TNF-α assay) or 24 hr (IL-6 assay). The level of cytokine present in culture supernatants was determined by ELISA (pg/ml).

Adv = adenovirus serotype

Table 9

Adenoviral type 5 DNA suppresses the cytokine response to EC DNA by human PBMC

DNA Source	IL-6 (pg/ml)[1]	IFN- (pg/ml)[1]	TNF- (pg/ml)[1]
EC DNA (50 μg/ml)	>3000	700	700
EC DNA (5 μg/ml)	>3000	400	675
EC DNA (0.5 μg/ml)	>3000	200	350
EC DNA (0.05 μg/ml)	3000	ND	100
Adenoviral DNA (50 μg/ml)	2500	0	0
Adenoviral DNA (5 μg/ml)	1500	0	0
EC:Adeno DNA (50:50 μg/ml)	2000	35	675
EC:Adeno DNA (5:5 μg/ml)	1500	40	ND

1 Represents the level of cytokine production above that in wells cultured with cells alone without any DNA . Levels of cytokines were determined by ELISA using Quantikine kits from R&D Systems.

ND = not done

Table 10

Inhibitory CpG motifs can block B cell proliferation induced by a stimulatory CpG motif

Oligonucleotide added	cpm
medium	194
1668 (TCCATGACGTTCCTGATGCT)	34,669
1668 + 1735 (GCGTTTTTTTTTGCG)	24,452
1720 (TCCATGAGCTTCCTGATGCT)	601
1720 + 1735	1109

Splenic B cells from a DBA/2 mouse were cultured at 5 X 10^4 cells/100 μl well in 96 well microtiter plates in RPMI as previously described (Krieg, *et al.*, 1995) with or without the indicated phosphorothioate modified oligonucleotides at a concentration of 60 ng/ml for 48 hr. The cells were then pulsed with ^{3}H thymidine, harvested, and the cpm determined by scintillation counting. The stimulatory CpG oligo 1668 was slightly but significantly inhibited by the inhibitory motifs in oligo 1735. The non CpG oligo 1720 is included as a negative control. (SEQ ID NO:68-70, respectively).

Table 11

Inhibitory effects of "bad" CpG motifs on the "good" Oligo 1619

Note:

The sequence of oligo 1619 is TCCATGTCGTTCCTGATGCT

1949 has only 1 GCG at the 3' end, which has essentially no inhibitory activity

Oligonucleotide added	IL-12 in pg/ml
medium	0
1619 alone	6
1619 + 1949 (TCCATGTCGTTCCTGATGCG)	16
1619 + 1952 (TCCATGTCGTTCCGCGCGCG)	0
1619 + 1953 (TCCATGTCGTTCCTGCCGCT)	0
1619 + 1955 (GCGGCGGGCGGCGCGCGCCC)	0

Human PBMC were cultured in 96 well microtiter plates at 10^5/200µl for 24 hr in RPMI containing 10% autologous serum. Supernatants were collected at the end of the culture and tested for IL-12 by ELISA. All wells except the control (medium) contained 60 µg/ml of the stimulatory CpG oligodeoxynucleotide 1619; stimulatory (1949) and inhibitory (all other sequences have a strong inhibitory motif) oligos were added to the indicated wells at the same concentration at the beginning of culture. All oligos have unmodified backbones.

Table 12

Effect of CpG-S ODN adjuvant on anti-HBs response in mice immunized with HBsAg-expressing DNA vaccine (pCMV-S): comparison of mixed formulation with temporal or spatial separation of plasmid DNA and ODN

CpG ODN (100 µg)		Site and Time Relative to DNA vaccine (pCMV-S. 10 µg)	Anti-HBs Titer at 12 wk
Sequence	Backbone		
None	-	----	6 379 ± 2 126
1826O	O-ODN	Mixed together (same time, same muscle)	4 395 ± 1 390

Table 13 Identification of neutralizing CpG motifs which reduce the induction of cytokine secretion by a CpG-S motif in the same ODN (*cis*-neutralization)

ODN	sequence 5'-3'[1]	ODN-induced cytokine expression[2]		
		IL-6[2]	IL-12	IFN-γ
None		<5	206	898
1619	TCCATGTCGTTCCTGATGCT	1405	3130	4628
1952	.....GCGCGCG	559	1615	2135
1953	.....CC...	577	1854	2000

[1]Dots in the sequence of ODN 1952 and 1953 indicate identity to ODN 1619; CpG dinucleotides are underlined for clarity. ODN without CpG-N or CpG-S motifs had little or no effect on cytokine production. The data shown are representative of 4 experiments.

[2]All cytokines are given in pg/ml; measured by ELISA on supernatants from DBA/2 spleen cells cultured in 96 well plates at 2 X 10^7 cells/ml for 24 hr with the indicated ODN at 30 µg/ml. Std. dev. of the triplicate wells was <7%. None of the ODN induced significant amounts of IL-5.

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Table 14 Inhibition of CpG-induced cytokine secretion by ODN containing CpG-N motifs

ODN	sequence 5'-3'	IL-12 secretion[1]	CpG-S-induced IL-12 secretion[2]
none		268	5453
1895	GCGCGCGCGCGCGCGCGCGC	123	2719
1896	CCGGCCGGCCGGCCGGCCGG	292	2740
1955	GCGGCGGGCGGCGCGCGCCC	270	2539
2037	TCCATGCCGTTCCTGCCGTT	423	2847

[1]BALB/c spleen cells were cultured in 96 well plates at 2 X 10^7 cells/ml with the indicated ODN for 24 hr and then the supernatants were assayed for IL-12 by ELISA (pg/ml).

[2]Cells were set up the same as in [1] except that IL-12 secretion was induced by the addition of the CpG ODN 1619 (TCCATGTCGTTCCTGATGCT) at 30 μg/ml. The data shown are representative of 5 experiments.

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A number of embodiments of the present invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:OTTAWA CIVIC HOSPITAL LOEB RESEARCH INSTITUTE -AND-

UNIVERSITY OF IOWA RESEARCH FOUNDATION -AND- QIAGEN GMBH

(ii) TITLE OF INVENTION: VECTORS AND METHODS FOR IMMUNIZATION OR THERAPEUTIC PROTOCOLS

(iii) NUMBER OF SEQUENCES: 84

(iv) CORRESPONDENCE ADDRESS:

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(F) ZIP: K1P 5Y6

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: ASCII (text)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: CA 2,301,575

(B) FILING DATE: 20-MAY-1998

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/047,209

(B) FILING DATE: 20-MAY-1997

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(A) APPLICATION NUMBER: US 60/047,233

(B) FILING DATE: 20-MAY-1997

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(2) INFORMATION FOR SEQ ID NO.: 1:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 1:

TCCATGTCGT TCCTGTCGTT 20

(2) INFORMATION FOR SEQ ID NO.: 2:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 19

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 2:

TCCTGACGTT CCTGACGTT 19

(2) INFORMATION FOR SEQ ID NO.: 3:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 24

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (0)...(0)

(C) OTHER INFORMATION: Has a phosphorothioate backbone.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 3:

TCGTCGTTTT GTCGTTTTGT CGTT 24

(2) INFORMATION FOR SEQ ID NO.: 4:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 30

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 4:

CCGTGGATAT CCGATGTACG GGCCAGATAT 30

(2) INFORMATION FOR SEQ ID NO.: 5:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 32

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 5:

AGTCGCGGCC GCAATTTCGA TAAGCCAGTA AG 32

(2) INFORMATION FOR SEQ ID NO.: 6:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 35

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 6:

ATTCTCGAGT CTAGACTAGA GCTCGCTGAT CAGCC 35

(2) INFORMATION FOR SEQ ID NO.: 7:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 29

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 7:

ATTAGGCCTT CCCCAGCATG CCTGCTATT 29

(2) INFORMATION FOR SEQ ID NO.: 8:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 37

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 8:

TATAGGCCCT ATTTTAAACG CGCCCTGTAG CGGCGCA 37

(2) INFORMATION FOR SEQ ID NO.: 9:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 38

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 9:

CTATGGCGCC TTGGGCCCAA TTTTTGTTAA ATCAGCTC 38

(2) INFORMATION FOR SEQ ID NO.: 10:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 28

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 10:

AAATTCGAAA GTACTGGACC TGTTAACA 28

(2) INFORMATION FOR SEQ ID NO.: 11:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 30

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 11:

CGTGTTAACA GGTCCAGTAC TTTCGAATTT 30

(2) INFORMATION FOR SEQ ID NO.: 12:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 44

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 12:

GACTCCATGA CGTTCCTGAC GTTTCCATGA CGTTCCTGAC GTTG 44

(2) INFORMATION FOR SEQ ID NO.: 13:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 44

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 13:

GTCCAACGTC AGGAACGTCA TGGAAACGTC AGGAACGTCA TGGA 44

(2) INFORMATION FOR SEQ ID NO.: 14:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 54

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 14:

GACTTCGTGT CGTTCTTCTG TCGTCTTTAG CGCTTCTCCT GCGTGCGTCC CTTG 54

(2) INFORMATION FOR SEQ ID NO.: 15:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 52

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 15:

GACTCGTCGT TTTGTCGTTT TGTCGTTTCG TCGTTTTGTC GTTTTGTCGT TG 52

(2) INFORMATION FOR SEQ ID NO.: 16:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 29

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 16:

GCCCTAGTAC TGTTAACTTT AAAGGGCCC 29

(2) INFORMATION FOR SEQ ID NO.: 17:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 29

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 17:

GGCGGGCCCT TTAAAGTTAA CAGTACTAG 29

(2) INFORMATION FOR SEQ ID NO.: 18:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 48

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 18:

GCCCTGGCGG GGATAAGGCG GGGATTTGGC GGGGGATAAG GCGGGGAA 48

(2) INFORMATION FOR SEQ ID NO.: 19:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 45

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 19:

GGCCCCCGCC TTATCCCCGC CAAATCCCCG CCTTATCCCC GCCAG 45

(2) INFORMATION FOR SEQ ID NO.: 20:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 38

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 20:

GCCCTATTTT AAATTCGAAA GTACTGGACC TGTTAACA 38

(2) INFORMATION FOR SEQ ID NO.: 21:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 37

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 21:

CGTGTTAACA GGTCCAGTAC TTTCGAATTT AAAATAG 37

(2) INFORMATION FOR SEQ ID NO.: 22:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 22:

CGCGCGCGCG CGCGCGCGCG 20

(2) INFORMATION FOR SEQ ID NO.: 23:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 29

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 23:

GTCTCTAGAC AGCCACTGGT AACAGGATT 29

(2) INFORMATION FOR SEQ ID NO.: 24:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 29

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 24:

GTCGTTGTGT CGTCAAGTCA GCGTAATGC 29

(2) INFORMATION FOR SEQ ID NO.: 25:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 25:

TCGTTTCTGT AATGAAGGAG 20

(2) INFORMATION FOR SEQ ID NO.: 26:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 26:

AAGGCAGTTC CATAGGATGG 20

(2) INFORMATION FOR SEQ ID NO.: 27:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 35

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 27:

TCGATCTGCG ATTCCAACTC GTCCAACATC AATAC 35

(2) INFORMATION FOR SEQ ID NO.: 28:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 28:

TGGTGAGAAT GGCAAAAGTT 20

(2) INFORMATION FOR SEQ ID NO.: 29:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 21

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 29:

CATTATTCAT TCGTGATTGC G 21

(2) INFORMATION FOR SEQ ID NO.: 30:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 24

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 30:

ACGTCTCAGG AACACTGCCA GCGC 24

(2) INFORMATION FOR SEQ ID NO.: 31:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 31:

AGGGATCGCA GTGGTGAGTA 20

(2) INFORMATION FOR SEQ ID NO.: 32:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 21

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 32:

TATAAAATGC TTGATGGTCG G 21

(2) INFORMATION FOR SEQ ID NO.: 33:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 35

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 33:

GGGAAGAGGC ATAAATTCTG TCAGCCAGTT TAGTC 35

(2) INFORMATION FOR SEQ ID NO.: 34:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 34:

TGGCTTCCCA TACAAGCGAT 20

(2) INFORMATION FOR SEQ ID NO.: 35:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 35:

TACATTATCG CGAGCCCATT 20

(2) INFORMATION FOR SEQ ID NO.: 36:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 19

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 36:

TGGCCTCGAC GTTTCCCGT 19

(2) INFORMATION FOR SEQ ID NO.: 37:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 29

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 37:

ATCGAATTCA GGGCCTCGTG ATACGCCTA 29

(2) INFORMATION FOR SEQ ID NO.: 38:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 39

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 38:

TGACTTGACG ACACAACGAC AGCTCATGAC CAAAATCCC 39

(2) INFORMATION FOR SEQ ID NO.: 39:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 39

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 39:

CTCCTTCATT ACAGAAACGA CTTTTTCAAA AATATGGTA 39

(2) INFORMATION FOR SEQ ID NO.: 40:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 37

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 40:

CCATCCTATG GAACTGCCTT GGTGAGTTTT CTCCTTC 37

(2) INFORMATION FOR SEQ ID NO.: 41:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 34

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 41:

GAGTTGGAAT CGCAGATCGA TACCAGGATC TTGC 34

(2) INFORMATION FOR SEQ ID NO.: 42:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 37

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 42:

AACTTTTGCC ATTCTCACCA GATTCAGTCG TCACTCA 37

(2) INFORMATION FOR SEQ ID NO.: 43:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 39

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 43:

CGCAATCACG AATGAATAAT GGTTTGGTTG ATGCGAGTG 39

(2) INFORMATION FOR SEQ ID NO.: 44:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 38

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 44:

TGGCAGTGTT CCTGAGACGT TTGCATTCGA TTCCTGTT 38

(2) INFORMATION FOR SEQ ID NO.: 45:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 38

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 45:

TACTCACCAC TGCGATCCCT GGAAAAACAG CATTCCAG 38

(2) INFORMATION FOR SEQ ID NO.: 46:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 39

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 46:

CCGACCATCA AGCATTTTAT ACGTACTCCT GATGATGCA 39

(2) INFORMATION FOR SEQ ID NO.: 47:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 39

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 47:

CAGAATTTAT GCCTCTTCCC ACCATCAAGC ATTTTATAC 39

(2) INFORMATION FOR SEQ ID NO.: 48:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 38

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 48:

ATCGCTTGTA TGGGAAGCCA GATGCGCCAG AGTTGTTT 38

(2) INFORMATION FOR SEQ ID NO.: 49:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 37

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 49:

AATGGGCTCG CGATAATGTA GGGCAATCAG GTGCGAC 37

(2) INFORMATION FOR SEQ ID NO.: 50:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 38

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 50:

ACGGGAAACG TCGAGGCCAC GATTAAATTC CAACATGG 38

(2) INFORMATION FOR SEQ ID NO.: 51:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (0)...(0)

(C) OTHER INFORMATION: Has a phosphorothioate backbone.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 51:

TCCATGACGT TCCTGACGTT 20

(2) INFORMATION FOR SEQ ID NO.: 52:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (0)...(0)

(C) OTHER INFORMATION: Has a phosphorothioate backbone.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 52:

GGGGTCAACG TTGAGGGGGG 20

(2) INFORMATION FOR SEQ ID NO.: 53:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 53:

TCCAGGACTT TCCTCAGGTT 20

(2) INFORMATION FOR SEQ ID NO.: 54:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 54:

TCCAGGACTT CTCTCAGGTT 20

(2) INFORMATION FOR SEQ ID NO.: 55:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 55:

CCCCCCCCCC CCCCCCCCCC

(2) INFORMATION FOR SEQ ID NO.: 56:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (0)...(0)

(C) OTHER INFORMATION: Has phosphodiester backbone.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 56:

TCCATGACGT TCCTGACGTT

(2) INFORMATION FOR SEQ ID NO.: 57:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 57:

GGCGGCGGCG GCGGCGGCGG 20

(2) INFORMATION FOR SEQ ID NO.: 58:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (0)...(0)

(C) OTHER INFORMATION: Backbone is phosphorothioate-phosphodiester chimera.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 58:

TCCATGACGT TCCTGACGTT 20

(2) INFORMATION FOR SEQ ID NO.: 59:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (0)...(0)

(C) OTHER INFORMATION: Has SOS-ODN backbone with two S-linkages at the 5' end, five S-linkages at the 3' end, and O-linkages in between.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 59:

GGGGTCAACG TTGAGGGGGG 20

(2) INFORMATION FOR SEQ ID NO.: 60:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 60:

TCTCCCAGCG TGCGCCATAT 20

(2) INFORMATION FOR SEQ ID NO.: 61:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 21

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 61:

GGGGTCTGTG CTTTTGGGGG G 21

(2) INFORMATION FOR SEQ ID NO.: 62:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 62:

TCAGGGGTGG GGGGAACCTT 20

(2) INFORMATION FOR SEQ ID NO.: 63:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 63:

GGGGTTGACG TTTTGGGGGG 20

(2) INFORMATION FOR SEQ ID NO.: 64:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 64:

TCTAGCGTTT TTAGCGTTCC 20

(2) INFORMATION FOR SEQ ID NO.: 65:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 65:

TCGTCGTTGT CGTTGTCGTT 20

(2) INFORMATION FOR SEQ ID NO.: 66:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 24

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (0)...(0)

(C) OTHER INFORMATION: Backbone is a phosphorothioate-phosphodiester chimera.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 66:

TCGTCGTTTT GTCGTTTTGT CGTT 24

(2) INFORMATION FOR SEQ ID NO.: 67:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 22

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 67:

TCGTCGTTGT CGTTTTGTCG TT 22

(2) INFORMATION FOR SEQ ID NO.: 68:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(ix) FEATURE

(A) NAME/KEY: misc_feature

(B) LOCATION: (0)...(0)

(C) OTHER INFORMATION: Has a phosphodiester backbone.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 68:

TCCATGACGT TCCTGATGCT

(2) INFORMATION FOR SEQ ID NO.: 69:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 15

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 69:

GCGTTTTTTT TTGCG

(2) INFORMATION FOR SEQ ID NO.: 70:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 70:

TCCATGAGCT TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO.: 71:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 71:

TCCATGTCGT TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO.: 72:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 72:

TCCATGTCGT TCCTGATGCG 20

(2) INFORMATION FOR SEQ ID NO.: 73:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 73:

TCCATGTCGT TCCGCGCGCG 20

(2) INFORMATION FOR SEQ ID NO.: 74:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 74:

TCCATGTCGT TCCTGCCGCT 20

(2) INFORMATION FOR SEQ ID NO.: 75:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 75:

GCGGCGGGCG GCGCGCGCCC 20

(2) INFORMATION FOR SEQ ID NO.: 76:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 76:

GCGCGCGCGC GCGCGCGCGC

(2) INFORMATION FOR SEQ ID NO.: 77:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 77:

CCGGCCGGCC GGCCGGCCGG

(2) INFORMATION FOR SEQ ID NO.: 78:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 78:

TCCATGCCGT TCCTGCCGTT 20

(2) INFORMATION FOR SEQ ID NO.: 79:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 20

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: synthetic oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 79:

TCCATGACGT TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO.: 80:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 1360

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: plasmid DNA wild-type Kanamycin resistance gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 80:

AAGGGCCTCG TGATACGCCT ATTTTTATAG GTTAATGTCA TGGGGGGGGG GGGGAAAGCC 60

ACGTTGTGTC TCAAAATCTC TGATGTTACA TTGCACAAGA TAAAAATATA TCATCATGAA 120

CAATAAAACT GTCTGCTTAC ATAAACAGTA ATACAAGGGG TGTTATGAGC CATATTCAAC 180

GGGAAACGTC GAGGCCGCGA TTAAATTCCA ACATGGATGC TGATTTATAT GGGTATAAAT 240

GGGCTCGCGA TAATGTCGGG CAATCAGGTG CGACAATCTA TCGCTTGTAT GGGAAGCCCG 300

ATGCGCCAGA GTTGTTTCTG AAACATGGCA AAGGTAGCGT TGCCAATGAT GTTACAGATG 360

AGATGGTCAG ACTAAACTGG CTGACGGAAT TTATGCCTCT TCCGACCATC AAGCATTTTA 420

TCCGTACTCC TGATGATGCA TGGTTACTCA CCACTGCGAT CCCCGGAAAA ACAGCATTCC 480

AGGTATTAGA AGAATATCCT GATTCAGGTG AAAATATTGT TGATGCGCTG GCAGTGTTCC 540

TGCGCCGGTT GCATTCGATT CCTGTTTGTA ATTGTCCTTT TAACAGCGAT CGCGTATTTC 600

GTCTCGCTCA GGCGCAATCA CGAATGAATA ACGGTTTGGT TGATGCGAGT GATTTTGATG 660

ACGAGCGTAA TGGCTGGCCT GTTGAACAAG TCTGGAAAGA AATGCATAAA CTTTTGCCAT 720

TCTCACCGGA TTCAGTCGTC ACTCATGGTG ATTTCTCACT TGATAACCTT ATTTTTGACG 780

AGGGGAAATT AATAGGTTGT ATTGATGTTG GACGAGTCGG AATCGCAGAC CGATACCAGG 840

ATCTTGCCAT CCTATGGAAC TGCCTCGGTG AGTTTTCTCC TTCATTACAG AAACGGCTTT 900

TTCAAAAATA TGGTATTGAT AATCCTGATA TGAATAAATT GCAGTTTCAT TTGATGCTCG 960

ATGAGTTTTT CTAATCAGAA TTGGTTAATT GGTTGTAACA CTGGCAGAGC ATTACGCTGA 1020

CTTGACGGGA CGGCGCAAGC TCATGACCAA AATCCCTTAA CGTGAGTTTT CGTTCCACTG 1080

AGCGTCAGAC CCCGTAGAAA AGATCAAAGG ATCTTCTTGA GATCCTTTTT TTCTGCGCGT 1140

AATCTGCTGC TTGCAAACAA AAAAACCACC GCTACCAGCG GTGGTTTGTT TGCCGGATCA 1200

AGAGCTACCA ACTCTTTTTC CGAAGGTAAC TGGCTTCAGC AGAGCGCAGA TACCAAATAC 1260

TGTTCTTCTA GTGTAGCCGT AGTTAGGCCA CCACTTCAAG AACTCTGTAG CACCGCCTAC 1320

ATACCTCGCT CTGCTAATCC TGTTACCAGT GGCTGCTGCC 1360

(2) INFORMATION FOR SEQ ID NO.: 81:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 1360

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: plasmid DNA mutant Kanamycin resistance gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 81:

AAGGGCCTCG TGATACGCCT ATTTTTATAG GTTAATGTCA TGGGGGGGGG GGGGAAAGCC 60

ACGTTGTGTC TCAAAATCTC TGATGTTACA TTGCACAAGA TAAAAATATA TCATCATGAA 120

CAATAAAACT GTCTGCTTAC ATAAACAGTA ATACAAGGGG TGTTATGAGC CATATTCAAC 180

GGGAAACGTC GAGGCCACGA TTAAATTCCA ACATGGATGC TGATTTATAT GGGTATAAAT 240

GGGCTCGCGA TAATGTAGGG CAATCAGGTG CGACAATCTA TCGCTTGTAT GGGAAGCCAG 300

ATGCGCCAGA GTTGTTTCTG AAACATGGCA AAGGTAGCGT TGCCAATGAT GTTACAGATG 360

AGATGGTCAG ACTAAACTGG CTGACAGAAT TTATGCCTCT TCCCACCATC AAGCATTTTA 420

TACGTACTCC TGATGATGCA TGGTTACTCA CCACTGCGAT CCCTGGAAAA ACAGCATTCC 480

AGGTATTAGA AGAATATCCT GATTCAGGTG AAAATATTGT TGATGCGCTG GCAGTGTTCC 540

TGAGACGTTT GCATTCGATT CCTGTTTGTA ATTGTCCTTT TAACAGCGAT CGCGTATTTC 600

GTCTCGCTCA GGCGCAATCA CGAATGAATA ATGGTTTGGT TGATGCGAGT GATTTTGATG 660

ACGAGCGTAA TGGCTGGCCT GTTGAACAAG TCTGGAAAGA AATGCATAAA CTTTTGCCAT 720

TCTCACCAGA TTCAGTCGTC ACTCATGGTG ATTTCTCACT TGATAACCTT ATTTTTGACG 780

AGGGGAAATT AATAGGTTGT ATTGATGTTG GACGAGTTGG AATCGCAGAT CGATACCAGG 840

ATCTTGCCAT CCTATGGAAC TGCCTTGGTG AGTTTTCTCC TTCATTACAG AAACGACTTT 900

TTCAAAAATA TGGTATTGAT AATCCTGATA TGAATAAATT GCAGTTTCAT TTGATGCTCG 960

ATGAGTTTTT CTAATCAGAA TTGGTTAATT GGTTGTAACA CTGGCAGAGC ATTACGCTGA 1020

CTTGACGACA CAACGACAGC TCATGACCAA AATCCCTTAA CGTGAGTTTT CGTTCCACTG 1080

AGCGTCAGAC CCCGTAGAAA AGATCAAAGG ATCTTCTTGA GATCCTTTTT TTCTGCGCGT 1140

AATCTGCTGC TTGCAAACAA AAAAACCACC GCTACCAGCG GTGGTTTGTT TGCCGGATCA 1200

AGAGCTACCA ACTCTTTTTC CGAAGGTAAC TGGCTTCAGC AGAGCGCAGA TACCAAATAC 1260

TGTTCTTCTA GTGTAGCCGT AGTTAGGCCA CCACTTCAAG AACTCTGTAG CACCGCCTAC 1320

ATACCTCGCT CTGCTAATCC TGTTACCAGT GGCTGCTGCC 1360

(2) INFORMATION FOR SEQ ID NO.: 82:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 269

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: polypeptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: mutant Kanamycin resistance gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 82:

Met Ser His Ile Gln Arg Glu Thr Ser Arg Pro Arg Leu Asn Ser Asn
1 5 10 15

Met Asp Ala Asp Leu Tyr Gly Tyr Lys Trp Ala Arg Asp Asn Val Gly
20 25 30

Gln Ser Gly Ala Thr Ile Tyr Arg Leu Tyr Gly Lys Pro Asp Ala Pro
35 40 45

Glu Leu Phe Leu Lys His Gly Lys Gly Ser Val Ala Asn Asp Val Thr
50 55 60

Asp Glu Met Val Arg Leu Asn Trp Leu Thr Glu Phe Met Pro Leu Pro
65 70 75 80

Thr Ile Lys His Phe Ile Arg Thr Pro Asp Asp Ala Trp Leu Leu Thr
85 90 95

Thr Ala Ile Pro Gly Lys Thr Ala Phe Gln Val Leu Glu Glu Tyr Pro
100 105 110

Asp Ser Gly Glu Asn Ile Val Asp Ala Leu Ala Val Phe Leu Arg Arg
115 120 125

Leu His Ser Ile Pro Val Cys Asn Cys Pro Phe Asn Ser Asp Arg Val
130 135 140

Phe Arg Leu Ala Gln Ala Gln Ser Arg Met Asn Asn Gly Leu Val Asp
145 150 155 160

Ala Ser Asp Phe Asp Asp Glu Arg Asn Gly Trp Pro Val Glu Gln Val
165 170 175

Trp Lys Glu Met His Lys Leu Leu Pro Phe Ser Pro Asp Ser Val Val
180 185 190

Thr His Gly Asp Phe Ser Leu Asp Asn Leu Ile Phe Asp Glu Gly Lys
195 200 205

Leu Ile Gly Cys Ile Asp Val Gly Arg Val Gly Ile Ala Asp Arg Tyr
210 215 220

Gln Asp Leu Ala Ile Leu Trp Asn Cys Leu Gly Glu Phe Ser Pro Ser
225 230 235 240

Leu Gln Lys Arg Leu Phe Gln Lys Tyr Gly Ile Asp Asn Pro Asp Met
245 250 255

Asn Lys Leu Gln Phe His Leu Met Leu Asp Glu Phe Phe
260 265

(2) INFORMATION FOR SEQ ID NO.: 83:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 3987

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: plasmid pUK21-A2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 83:

GAATTCGAGC TCCCGGGTAC CATGGCATGC ATCGATAGAT CTCGAGTCTA GACTAGAGCT 60

CGCTGATCAG CCTCGACTGT GCCTTCTAGT TGCCAGCCAT CTGTTGTTTG CCCCTCCCCC 120

GTGCCTTCCT TGACCCTGGA AGGTGCCACT CCCACTGTCC TTTCCTAATA AAATGAGGAA 180

ATTGCATCGC ATTGTCTGAG TAGGTGTCAT TCTATTCTGG GGGGTGGGGT GGGGCAGGAC 240

AGCAAGGGGG AGGATTGGGA AGACAATAGC AGGCATGCTG GGGAAGGCCT CGGACTAGTG 300

GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCTCAC AATTCCACAC 360

AACATACGAG CCGCGGAAGC ATAAAGTGTA AAGCCTGGGG TGCCTAATGA GTGAGCTAAC 420

TCACATTAAT TGCGTTGCGC TCACTGCCCG CTTTCCAGTC GGGAAACCTG TCGTGCCAGC 480

TGCATTAATG AATCGGCCAA CGCGCGGGGA GAGGCGGTTT GCGTATTGGG CGCTCTTCCG 540

CTTCCTCGCT CACTGACTCG CTGCGCTCGG TCGTTCGGCT GCGGCGAGCG GTATCAGCTC 600

ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA TAACGCAGGA AAGAACATGT 660

GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC 720

ATAGGCTCCG CCCCCCTGAC GAGCATCACA AAAATCGACG CTCAAGTCAG AGGTGGCGAA 780

ACCCGACAGG ACTATAAAGA TACCAGGCGT TTCCCCCTGG AAGCTCCCTC GTGCGCTCTC 840

CTGTTCCGAC CCTGCCGCTT ACCGGATACC TGTCCGCCTT TCTCCCTTCG GGAAGCGTGG 900

CGCTTTCTCA TAGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC 960

TGGGCTGTGT GCACGAACCC CCCGTTCAGC CCGACCGCTG CGCCTTATCC GGTAACTATC 1020

GTCTTGAGTC CAACCCGGTA AGACACGACT TATCGCCACT GGCAGCAGCC ACTGGTAACA 1080

GGATTAGCAG AGCGAGGTAT GTAGGCGGTG CTACAGAGTT CTTGAAGTGG TGGCCTAACT 1140

ACGGCTACAC TAGAAGAACA GTATTTGGTA TCTGCGCTCT GCTGAAGCCA GTTACCTTCG 1200

GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAAACCAC CGCTGGTAGC GGTGGTTTTT 1260

TTGTTTGCAA GCAGCAGATT ACGCGCAGAA AAAAAGGATC TCAAGAAGAT CCTTTGATCT 1320

TTTCTACGGG GTCTGACGCT CAGTGGAACG AAAACTCACG TTAAGGGATT TTGGTCATGA 1380

GCTTGCGCCG TCCCGTCAAG TCAGCGTAAT GCTCTGCCAG TGTTACAACC AATTAACCAA 1440

TTCTGATTAG AAAAACTCAT CGAGCATCAA ATGAAACTGC AATTTATTCA TATCAGGATT 1500

ATCAATACCA TATTTTTGAA AAAGCCGTTT CTGTAATGAA GGAGAAAACT CACCGAGGCA 1560

GTTCCATAGG ATGGCAAGAT CCTGGTATCG GTCTGCGATT CCGACTCGTC CAACATCAAT 1620

ACAACCTATT AATTTCCCCT CGTCAAAAAT AAGGTTATCA AGTGAGAAAT CACCATGAGT 1680

GACGACTGAA TCCGGTGAGA ATGGCAAAAG TTTATGCATT TCTTTCCAGA CTTGTTCAAC 1740

AGGCCAGCCA TTACGCTCGT CATCAAAATC ACTCGCATCA ACCAAACCGT TATTCATTCG 1800

TGATTGCGCC TGAGCGAGAC GAAATACGCG ATCGCTGTTA AAAGGACAAT TACAAACAGG 1860

AATCGAATGC AACCGGCGCA GGAACACTGC CAGCGCATCA ACAATATTTT CACCTGAATC 1920

AGGATATTCT TCTAATACCT GGAATGCTGT TTTTCCGGGG ATCGCAGTGG TGAGTAACCA 1980

TGCATCATCA GGAGTACGGA TAAAATGCTT GATGGTCGGA AGAGGCATAA ATTCCGTCAG 2040

CCAGTTTAGT CTGACCATCT CATCTGTAAC ATCATTGGCA ACGCTACCTT TGCCATGTTT 2100

CAGAAACAAC TCTGGCGCAT CGGGCTTCCC ATACAAGCGA TAGATTGTCG CACCTGATTG 2160

CCCGACATTA TCGCGAGCCC ATTTATACCC ATATAAATCA GCATCCATGT TGGAATTTAA 2220

TCGCGGCCTC GACGTTTCCC GTTGAATATG GCTCATAACA CCCCTTGTAT TACTGTTTAT 2280

GTAAGCAGAC AGTTTTATTG TTCATGATGA TATATTTTTA TCTTGTGCAA TGTAACATCA 2340

GAGATTTTGA GACACAACGT GGCTTTCCCC CCCCCCCCCA TGACATTAAC CTATAAAAAT 2400

AGGCGTATCA CGAGGCCCTT TCGTCTCGCG CGTTTCGGTG ATGACGGTGA AAACCTCTGA 2460

CACATGCAGC TCCCGGAGAC GGTCACAGCT TGTCTGTAAG CGGATGCCGG GAGCAGACAA 2520

GCCCGTCAGG GCGCGTCAGC GGGTGTTGGC GGGTGTCGGG GCTGGCTTAA CTATGCGGCA 2580

TCAGAGCAGA TTGTACTGAG AGTGCACCAT AAAATTGTAA ACGTTAATAT TTTGTTAAAA 2640

TTCGCGTTAA ATTTTTGTTA AATCAGCTCA TTTTTTAACC AATAGACCGA AATCGGCAAA 2700

ATCCCTTATA AATCAAAAGA ATAGCCCGAG ATAGAGTTGA GTGTTGTTCC AGTTTGGAAC 2760

AAGAGTCCAC TATTAAAGAA CGTGGACTCC AACGTCAAAG GGCGAAAAAC CGTCTATCAG 2820

GGCGATGGCC CACCCCGATT TAGAGCTTGA CGGGGAAAGC CGGCGAACGT GGCGAGAAAG 2880

GAAGGGAAGA AAGCGAAAGG AGCGGGCGCT AAGGCGCTGG CAAGTGTAGC GGTCACGCTG 2940

CGCGTAACCA CCACACCCGC CGCGCTTAAT GCGCCGCTAC AGGGCGCGTA CTATGGTTGC 3000

TTTGACGTAT GCGGTGTGAA ATACCGCACA GATGCGTAAG GAGAAAATAC CGCATCAGGC 3060

GCCATTCGCC ATTCAGGCTG CGCAACTGTT GGGAAGGGCG ATCGGTGCGG GCCTCTTCGC 3120

TATTACGCCA GCTGGCGAAA GGGGGATGTG CTGCAAGGCG ATTAAGTTGG GTAACGCCAG 3180

GGTTTTCCCA GTCACGACGT TGTAAAACGA CGGCCAGTGA ATTGTAATAC GACTCACTAT 3240

AGGGCGAATT GGGGATCGAT CCACTAGTTC TAGATCCGAT GTACGGGCCA GATATACGCG 3300

TTGACATTGA TTATTGACTA GTTATTAATA GTAATCAATT ACGGGGTCAT TAGTTCATAG 3360

CCCATATATG GAGTTCCGCG TTACATAACT TACGGTAAAT GGCCCGCCTG GCTGACCGCC 3420

CAACGACCCC CGCCCATTGA CGTCAATAAT GACGTATGTT CCCATAGTAA CGCCAATAGG 3480

GACTTTCCAT TGACGTCAAT GGGTGGAGTA TTTACGGTAA ACTGCCCACT TGGCAGTACA 3540

TCAAGTGTAT CATATGCCAA GTACGCCCCC TATTGACGTC AATGACGGTA AATGGCCCGC 3600

CTGGCATTAT GCCCAGTACA TGACCTTATG GGACTTTCCT ACTTGGCAGT ACATCTACGT 3660

ATTAGTCATC GCTATTACCA TGGTGATGCG GTTTTGGCAG TACATCAATG GGCGTGGATA 3720

GCGGTTTGAC TCACGGGGAT TTCCAAGTCT CCACCCCATT GACGTCAATG GGAGTTTGTT 3780

TTGGCACCAA AATCAACGGG ACTTTCCAAA ATGTCGTAAC AACTCCGCCC CATTGACGCA 3840

AATGGGCGGT AGGCGTGTAC GGTGGGAGGT CTATATAAGC AGAGCTCTCT GGCTAACTAG 3900

AGAACCCACT GCTTACTGGC TTATCGAAAT TGCGGCCGCC ACGGCGATAT CGGATCCATA 3960

TGACGTCGAC GCGTCTGCAG AAGCTTC 3987

(2) INFORMATION FOR SEQ ID NO.: 84:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 3987

(B) TYPE: nucleic acid

(C) STRANDEDNESS:

(D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Artificial Sequence

(ix) FEATURE

(C) OTHER INFORMATION: plasmid pGT

(xi) SEQUENCE DESCRIPTION: SEQ ID NO.: 84:

GAATTCGAGC TCCCGGGTAC CATGGCATGC ATCGATAGAT CTCGAGTCTA GACTAGAGCT 60

CGCTGATCAG CCTCGACTGT GCCTTCTAGT TGCCAGCCAT CTGTTGTTTG CCCCTCCCCC 120

GTGCCTTCCT TGACCCTGGA AGGTGCCACT CCCACTGTCC TTTCCTAATA AAATGAGGAA 180

ATTGCATCGC ATTGTCTGAG TAGGTGTCAT TCTATTCTGG GGGGTGGGGT GGGGCAGGAC 240

AGCAAGGGGG AGGATTGGGA AGACAATAGC AGGCATGCTG GGGAAGGCCT CGGACTAGTG 300

CCGGAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCTCAC AATTCCACAC 360

AACATCCGGG CCGCGGAAGC ATAAAGTGTA AAGCCTGGGG TGCCTAATGA GTGAGCTAAC 420

TCACATTAAT TCCGTTCCGC TCACTGCCCG CTTTCCAGTC GGGAAACCTG CCGTGCCAGC 480

TGCATTAATG AATCGGCCAA CGCGCGGGGA GAGCCGGTTT CCGTATTGGC CGCTCTTCCG 540

CTTCCTCGCT CACTGACTCG CTGCGCTCGG TCGTTCGGCT GCGGCGAGCG GTATCAGCTC 600

ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA TAACGCAGGA AAGAACATGT 660

GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC 720

ATAGGCTCCG CCCCCCTGAC GAGCATCACA AAAATCGACG CTCAAGTCAG AGGTGGCGAA 780

ACCCGACAGG ACTATAAAGA TACCAGGCGT TTCCCCCTGG AAGCTCCCTC GTGCGCTCTC 840

CTGTTCCGAC CCTGCCGCTT ACCGGATACC TGTCCGCCTT TCTCCCTTCG GGAAGCGTGG 900

CGCTTTCTCA TAGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC 960

TGGGCTGTGT GCACGAACCC CCCGTTCAGC CCGACCGCTG CGCCTTATCC GGTAACTATC 1020

GTCTTGAGTC CAACCCGGTA AGACACGACT TATCGCCACT GGCAGCAGCC ACTGGTAACA 1080

GGATTAGCAG AGCGAGGTAT GTAGGCGGTG CTACAGAGTT CTTGAAGTGG TGGCCTAACT 1140

ACGGCTACAC TAGAAGAACA GTATTTGGTA TCTGCGCTCT GCTGAAGCCA GTTACCTTCG 1200

GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAAACCAC CGCTGGTAGC GGTGGTTTTT 1260

TTGTTTGCAA GCAGCAGATT ACGCGCAGAA AAAAAGGATC TCAAGAAGAT CCTTTGATCT 1320

TTTCTACGGG GTCTGACGCT CAGTGGAACG AAAACTCACG TTAAGGGATT TTGGTCATGA 1380

GCTTGCGCCG TCCCGTCAAG TCACCGGAAT GCTCTGCCAG TGTTACAACC AATTAACCAA 1440

TTCTGATTAG AAAAACTCAT CCAGCATCAA ATGAAACTGC AATTTATTCA TATCAGGATT 1500

ATCAATACCA TATTTTTGAA AAAGCCGTTT CTGTAATGAA GGAGAAAACT CACCGAGGCA 1560

GTTCCATAGG ATGGCAAGAT CCTGGTATCG GTCTGCAATT CCGACTCGGC CAACATCAAT 1620

ACAACCTATT AATTTCCCCT CATCAAAAAT AAGGTTATCA AGTGAGAAAT CACCATGAGT 1680

AACTACTGAA TCCGGTGAGA ATGGCAAAAG TTTATGCATT TCTTTCCAGA CTTGTTCAAC 1740

AGGCCAGCCA TTACGCTCAT CATCAAAATC GGAAGCATCA ACCAAACCGT TATTCATTCG 1800

GGATTGAGCC TGAGCCAGAC GGAATACGCG GTCGCTGTTA AAAGGACAAT TACAAACAGG 1860

AATGGAATGC AACCGGCGGA GGAACACTGC CAGAGCATCA ACAATATTTT CACCTGAATC 1920

AGGATATTCT TCTAATACCT GGAATGCTGT TTTTCCGGGG ATAGCAGTGG TGAGTAACCA 1980

TGCATCATCA GGAGTACGGA TAAAATGCTT GATGGTCGGA AGAGGCATAA ATTCCGTCAG 2040

CCAGTTTAGT CTGACCATCT CATCTGTAAC ATCATTGGCA ACGCTACCTT TGCCATGTTT 2100

CAGAAACAAC TCCGGCGCGT CGGGCTTCCC ATACAAGCGG TAGATTGTAG CACCTGATTG 2160

CCCGACATTA TCGCGAGCCC ATTTATACCC ATATAAATCA GCATCCATGT TGGAATTTAA 2220

TCGCGGCCTG GAGGTTTCCC GTTGAATATG GCTCATAACA CCCCTTGTAT TACTGTTTAT 2280

GTAAGCAGAC AGTTTTATTG TTCATGATGA TATATTTTTA TCTTGTGCAA TGTAACATCA 2340

GAGATTTTGA GACACACCGG GGCTTTCCCC CCCCCCCCCA TGACATTAAC CTATAAAAAT 2400

AGCCGTATCC CGAGGCCCTT CCGTCTCGCG CGTTCCGGTG ATGCCGGTGA AAACCTCTGA 2460

CACATGCAGC TCCCGGAGAC GGTCACAGCT TGTCTGTAAG CGGATGCCGG GAGCAGACAA 2520

GCCCGTCAGG GCGCGTCAGC GGGTGTTGGC GGGTGTCGGG GCTGGCTTAA CTATGCGGCA 2580

TCAGAGCAGA TTGTACTGAG AGTGCACCAT AAAATTGTAA CCGTTAATAT TTTGTTAAAA 2640

TTCGCGTTAA ATTTTTGTTA AATCAGCTCA TTTTTTAACC AATAGACCGA AATCGGCAAA 2700

ATCCCTTATA AATCAAAAGA ATAGCCCGAG ATAGAGTTGA GTGTTGTTCC AGTTTGGAAC 2760

AAGAGTCCAC TATTAAAGAC CGTGGACTCC ACCGTCAAAG GCCGAAAAAC CGTCTATCAG 2820

GCCGATGGCC CACCCCGATT TAGAGCTTGA CGGGGAAAGC CGGCGCGCGT GCCGAGAAAG 2880

GAAGGGAAGA AACCGAAAGG AGCGGCCGCT AAGCCGCTGG CAAGTGTAGC GGTCCCGCTG 2940

CGCGTAACCA CCACACCCGC CGCGCTTAAT CCGCCGCTAC AGGGCGCGTA CTATGGTTGC 3000

TTTGCCGTAT GCGGTGTGAA ATACCGCACA GATCCGTAAG GAGAAAATAC CGCATCAGCC 3060

GCCATCCGCC ATTCAGGCTC CGCAACTGTT GGGAAGGCCG ATCGGTGCGG GCCTCTCCGC 3120

TATTCCGCCA GCTGCCGAAA GGGGGATGTG CTGCAAGCCG ATTAAGTTGG GTACCGCCAG 3180

GGTTTTCCCA GTCACGGCGG TGTAAACCGA CGGCCAGTGA ATTGTAATCC GACTCACTAT 3240

AGGCCGAATT GGGGACCGAT CCACTAGTTC TAGATCCGAT GTACGGGCCA GATATACGCG 3300

TTGACATTGA TTATTGACTA GTTATTAATA GTAATCAATT ACGGGGTCAT TAGTTCATAG 3360

CCCATATATG GAGTTCCGCG TTACATAACT TACGGTAAAT GGCCCGCCTG GCTGACCGCC 3420

CAACGACCCC CGCCCATTGA CGTCAATAAT GACGTATGTT CCCATAGTAA CGCCAATAGG 3480

GACTTTCCAT TGACGTCAAT GGGTGGAGTA TTTACGGTAA ACTGCCCACT TGGCAGTACA 3540

TCAAGTGTAT CATATGCCAA GTACGCCCCC TATTGACGTC AATGACGGTA AATGGCCCGC 3600

CTGGCATTAT GCCCAGTACA TGACCTTATG GGACTTTCCT ACTTGGCAGT ACATCTACGT 3660

ATTAGTCATC GCTATTACCA TGGTGATGCG GTTTTGGCAG TACATCAATG GGCGTGGATA 3720

GCGGTTTGAC TCACGGGGAT TTCCAAGTCT CCACCCCATT GACGTCAATG GGAGTTTGTT 3780

TTGGCACCAA AATCAACGGG ACTTTCCAAA ATGTCGTAAC AACTCCGCCC CATTGACGCA 3840

AATGGGCGGT AGGCGTGTAC GGTGGGAGGT CTATATAAGC AGAGCTCTCT GGCTAACTAG 3900

AGAACCCACT GCTTACTGGC TTATCGAAAT TGCGGCCGCC ACGGCGATAT CGGATCCATA 3960

TGACGTCGAC GCGTCTGCAG AAGCTTC 3987

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CLAIMS:

1. A method for enhancing the immunostimulatory effect of an antigen encoded by nucleic acid contained in a nucleic acid construct comprising:

(i) determining the neutralizing CpG (CpG-N) and stimulatory CpG (CpG-S) motifs present in the construct; and

(ii) removing at least one CpG-N motif,

thereby producing a nucleic acid construct having enhanced immunostimulatory efficacy.

2. The method of claim 1 further comprising at step (ii), inserting at least one CpG-S motif in the construct.

3. The method of claim 1 or 2, wherein the CpG-N motif is removed by site-specific mutagenesis.

4. The method of any one of claims 1 to 3, wherein the CpG-N motif is selected from the group consisting of clusters of direct repeats of CpG dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides and a combination thereof.

5. The method of any one of claims 1 to 4, wherein the nucleic acid construct is an expression vector.

6. The method of claim 5, wherein the vector is a plasmid.

7. The method of claim 6, wherein the vector is a viral vector.

8. The method of any one of claims 2 to 7, wherein the at least one CpG-S motif in the construct comprises a motif having the formula:

5' X_1CGX_2 3'

wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine.

9. The method of claim 8, wherein the motif is selected from the group consisting of GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT.

10. The method of claim 8, wherein the motif comprises TCAACGTT.

11. The method of claim 8, wherein the motif comprises GTCG(T/C)T or TGACGTT.

12. The method of claim 8, wherein the motif comprises TGTCG(T/C)T.

13. The method of claim 8, wherein the motif comprises TCCATGTCGTTCCTGTCGTT.

14. The method of claim 8, wherein the motif comprises TCCTGACGTTCCTGACGTT.

15. The method of claim 8, wherein the motif comprises TCGTCGTTTTGTCGTTTTGTCGTT.

16. The method of any one of claims 1 to 15, wherein the antigen is a viral antigen.

17. The method of claim 16, wherein the viral antigen is from Hepatitis B virus (HBV).

18. The method of claim 17, wherein the viral antigen is HBV surface antigen.

19. The method of any one of claims 1 to 15, wherein the antigen is a bacterial antigen.

20. The method of any one of claims 1 to 15, wherein the antigen is derived from a parasite.

21. The method of any one of claims 1 to 20, wherein the nucleic acid construct further comprises a regulatory sequence for expression of DNA in eukaryotic cells and a nucleic acid sequence encoding at least one antigenic polypeptide.

22. The method of claim 21, wherein the regulatory sequence is a promoter.

23. The method of claim 22, wherein the promoter is insensitive to cytokine regulation.

24. The method of claim 22, wherein the promoter is cytokine sensitive.

25. The method of claim 22, wherein the promoter is a non-viral promoter.

26. The method of claim 22, wherein the promoter is a viral promoter.

27. The method of claim 22, wherein the promoter is a tissue- or cell-specific promoter.

28. The method of claim 27, wherein the cell-specific promoter is operative in antigen-presenting cells.

29. The method of claim 28, wherein the promoter is a mammalian MHC I promoter.

30. The method of claim 26, wherein the promoter is a CMV promoter.

31. The use of a nucleic acid construct for stimulating a protective or therapeutic immune response in a subject, said nucleic acid construct comprising a nucleic acid that encodes an antigen having enhanced immunostimulatory efficacy and produced by the method defined in any one of claims 1 to 29.

32. The use of claim 31, wherein the nucleic acid construct further comprises a regulatory sequence for expression of DNA in eukaryotic cells and a nucleic acid sequence encoding at least one antigenic polypeptide.

33. The use of claim 31, wherein the construct is an expression vector.

34. The use of claim 32, wherein the regulatory sequence is a promoter.

35. The use of claim 34, wherein the promoter is insensitive to cytokine regulation.

36. The use of claim 34, wherein the promoter is cytokine sensitive.

37. The use of claim 34, wherein the promoter is a non-viral promoter.

38. The use of claim 34, wherein the promoter is a viral promoter.

39. The use of claim 34, wherein the promoter is a tissue-specific promoter.

40. The use of claim 34, wherein the promoter is a cell-specific promoter.

41. The use of claim 40, wherein the cell-specific promoter is operative in antigen-presenting cells.

42. The use of claim 41, wherein the promoter is a mammalian MHC I promoter.

43. The use of claim 38, wherein the promoter is a CMV promoter.

44. The use of any one of claims 31 to 43, wherein the antigen is a viral antigen.

45. The use of claim 44, wherein the viral antigen is from Hepatitis B virus (HBV).

46. The use of any one of claims 31 to 43, wherein the antigen is a bacterial antigen.

47. The use of any one of claims 31 to 46, wherein the at least one CpG-N motif is selected from the group consisting of clusters of direct repeats of CpG dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides and a combination thereof.

48. The use of claim 33, wherein the vector is a plasmid.

49. The use of claim 33, wherein the vector is a viral vector.

50. The use of any one of claims 31 to 49, wherein the at least one CpG-S motif in the construct comprises a motif having the formula:

$$5' \; X_1CGX_2 \; 3'$$

wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine.

51. The use of claim 50, wherein the motif is selected from the group consisting of GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT.

52. The use of claim 50, wherein the motif comprises TCAACGTT.

53. The use of claim 50, wherein the motif comprises GTCG(T/C)T or TGACGTT.

54. The use of claim 50, wherein the motif comprises TGTCG(T/C)T.

55. The use of claim 50, wherein the motif comprises TCCATGTCGTTCCTGTCGTT.

56. The use of claim 50, wherein the motif comprises TCCTGACGTTCCTGACGTT.

57. The use of claim 50, wherein the motif comprises TCGTCGTTTTGTCGTTTTGTCGTT.

58. The use of any one of claims 31 to 57, wherein the antigen is derived from a parasite.

59. The use of claim 30, wherein the antigen is adapted for simultaneous administration with the nucleic acid construct.

60. The use of a nucleic acid construct for enhancing the expression of a therapeutic polypeptide *in vivo*, wherein the polypeptide is encoded by a nucleic acid contained in the nucleic acid construct and produced by determining the neutralizing CpG (CpG-N) and stimulatory CpG (CpG-S) motifs

present in the construct, removing at least one stimulatory CpG-S motif, or inserting at least one neutralizing CpG-N motif, or a combination thereof, resulting in the nucleic acid construct providing enhanced expression of the therapeutic polypeptide.

61. The use of claim 60, wherein the at least one CpG-S motif is removed by site-specific mutagenesis.

62. The use of claim 60 or 61, wherein the at least one CpG-N motif is selected from the group consisting of clusters of direct repeats of CpG dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides and a combination thereof.

63. The use of any one of claims 60 to 62, wherein the nucleic acid construct is an expression vector.

64. The use of claim 63, wherein the vector is a plasmid.

65. The use of claim 63, wherein the vector is a viral vector.

66. The use of any one of claims 60 to 65, wherein the at least one CpG-S motif in the construct comprises a motif having the formula:

5' X_1CGX_2 3'

wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine.

67. The use of claim 66, wherein the motif is selected from the group consisting of GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT.

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68. The use of claim 66, wherein the motif contains TCAACGTT.

69. The use of claim 66, wherein the motif contains GTCG(T/C)T or TGACGTT.

70. The use of claim 66, wherein the motif contains TGTCG(T/C)T.

71. The use of claim 66, wherein the motif contains TCCATGTCGTTCCTGTCGTT.

72. The use of claim 66, wherein the motif contains TCCTGACGTTCCTGACGTT.

73. The use of claim 66, wherein the motif contains TCGTCGTTTTGTCGTTTTGTCGTT.

74. The use of any one of claims 60 to 73, wherein the therapeutic polypeptide is selected from the group consisting of growth factors, toxins, tumor suppressors, cytokines, apoptotic proteins, interferons, hormones, clotting factors, ligands and receptors.

75. The use of any one of claims 60 to 74, wherein the nucleic acid construct further comprises a regulatory sequence for expression of DNA in eukaryotic cells and a nucleic acid sequence encoding at least one therapeutic polypeptide.

76. The use of claim 75, wherein the regulatory sequence is a promoter.

77. The use of claim 76, wherein the promoter is insensitive to cytokine regulation.

78. The use of claim 76, wherein the promoter is a non-viral promoter.

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79. The use of claim 76, wherein the promoter is a viral promoter.

80. The use of claim 79, wherein the promoter is a CMV promoter.

81. The use of claim 76, wherein the promoter is a tissue- or cell-specific promoter.

82. The use of claim 81, wherein the tissue is muscle.

83. The use of claim 81, wherein the cell is a non-immune system cell.

84. The use of any one of claims 60 to 83, wherein therapeutic nucleic acid sequence is an antisense nucleic acid sequence.

85. The use of a nucleic acid construct for enhancing the expression of a therapeutic polypeptide *in vivo*, wherein the construct is produced by determining the neutralizing CpG (CpG-N) and stimulatory CpG (CpG-S) motifs present in the construct and removing at least one CpG-S motif, or inserting at least one CpG-N motif, or a combination thereof, resulting in enhanced expression of the therapeutic polypeptide.

86. The use of claim 85, wherein the nucleic acid construct further comprises a regulatory sequence for expression of DNA in eukaryotic cells and a nucleic acid sequence encoding at least one therapeutic polypeptide.

87. The use of claim 86, wherein the regulatory sequence is a promoter.

88. The use of claim 87, wherein the promoter is insensitive to cytokine regulation.

89. The use of claim 87, wherein the promoter is a non-viral promoter.

90. The use of claim 87, wherein the promoter is a viral promoter.

91. The use of claim 90, wherein the promoter is a CMV promoter.

92. The use of claim 87, wherein the promoter is a tissue- or cell-specific promoter.

93. The use of claim 92, wherein the tissue is muscle.

94. The use of claim 92, wherein the cell is a non-immune system cell.

95. The use of any one of claims 85 to 94, wherein the CpG-S motifs are removed by site-specific mutagenesis.

96. The use of any one of claims 85 to 95, wherein the at least one CpG-N motif is selected from the group consisting of clusters of direct repeats of CpG dinucleotides, CCG trinucleotides, CGG trinucleotides, CCGG tetranucleotides, CGCG tetranucleotides and a combination thereof.

97. The use of any one of claims 85 to 96, wherein the nucleic acid construct is an expression vector.

98. The use of claim 97, wherein the vector is a plasmid.

99. The use of claim 97, wherein the vector is a viral vector.

100. The use of any one of claims 85 to 99, wherein the at least one CpG-S motif comprises a motif having the formula:

$$5' \; X_1CGX_2 \; 3'$$

wherein at least one nucleotide separates consecutive CpGs, X_1 is adenine, guanine, or thymine and X_2 is cytosine, thymine, or adenine.

101. The use of claim 100, wherein the motif is selected from the group consisting of GACGTT, AGCGTT, AACGCT, GTCGTT and AACGAT.

102. The use of claim 100, wherein the motif contains TCAACGTT.

103. The use of claim 100, wherein the motif contains GTCG(T/C)T or TGACGTT.

104. The use of claim 100, wherein the motif contains TGTCG(T/C)T.

105. The use of claim 100, wherein the motif contains TCCATGTCGTTCCTGTCGTT.

106. The use of claim 100, wherein the motif contains TCCTGACGTTCCTGACGTT.

107. The use of claim 100, wherein the motif contains TCGTCGTTTTGTCGTTTTGTCGTT.

108. The use of any one of claims 85 to 107, wherein the therapeutic polypeptide is selected from the group consisting of growth factors, toxins, tumor suppressors, cytokines, apoptotic proteins, interferons, hormones, clotting factors, ligands and receptors.

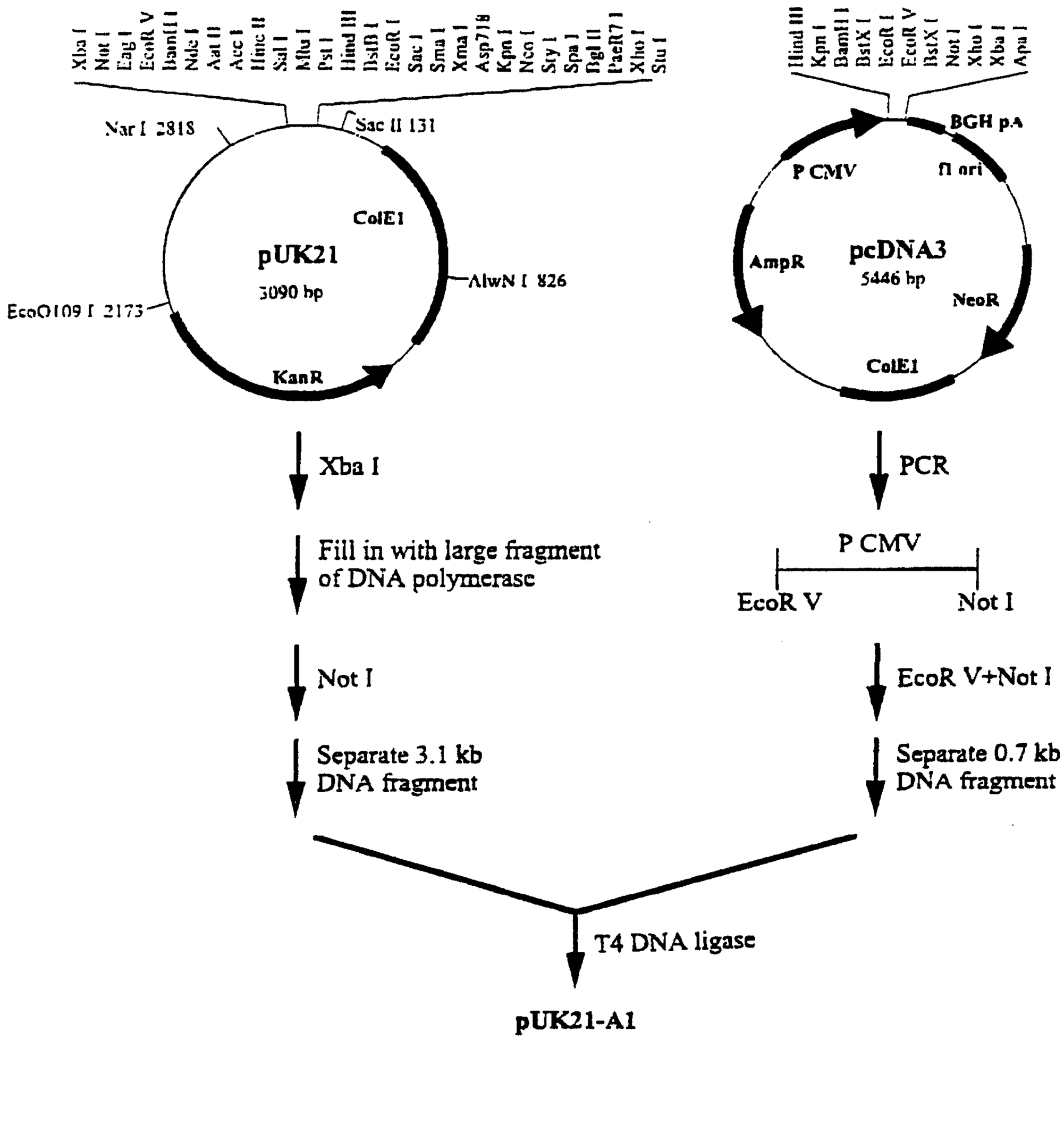

Xba I
Not I
Eag I
EcoR V
BamH I
Nde I
Aat II
Acc I
Hinc II
Sal I
Mlu I
Pst I
Hind III
BstB I
EcoR I
Sac I
Sma I
Xma I
Asp718
Kpn I
Nco I
Sty I
Spe I
Bgl II
PaeR7 I
Xho I
Stu I
Nar I 2818
Sac II 131
ColE1
pUK21
3090 bp
AlwN I 826
EcoO109 I 2173
KanR
Xba I
Fill in with large fragment
of DNA polymerase
Not I
Separate 3.1 kb
DNA fragment
Hind III
Kpn I
BamH I
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Xba I
Apa I
BGH pA
P CMV
f1 ori
AmpR
pcDNA3
5446 bp
NeoR
ColE1
PCR
P CMV
EcoR V
Not I
EcoR V+Not I
Separate 0.7 kb
DNA fragment
T4 DNA ligase
pUK21-A1

Figure 1

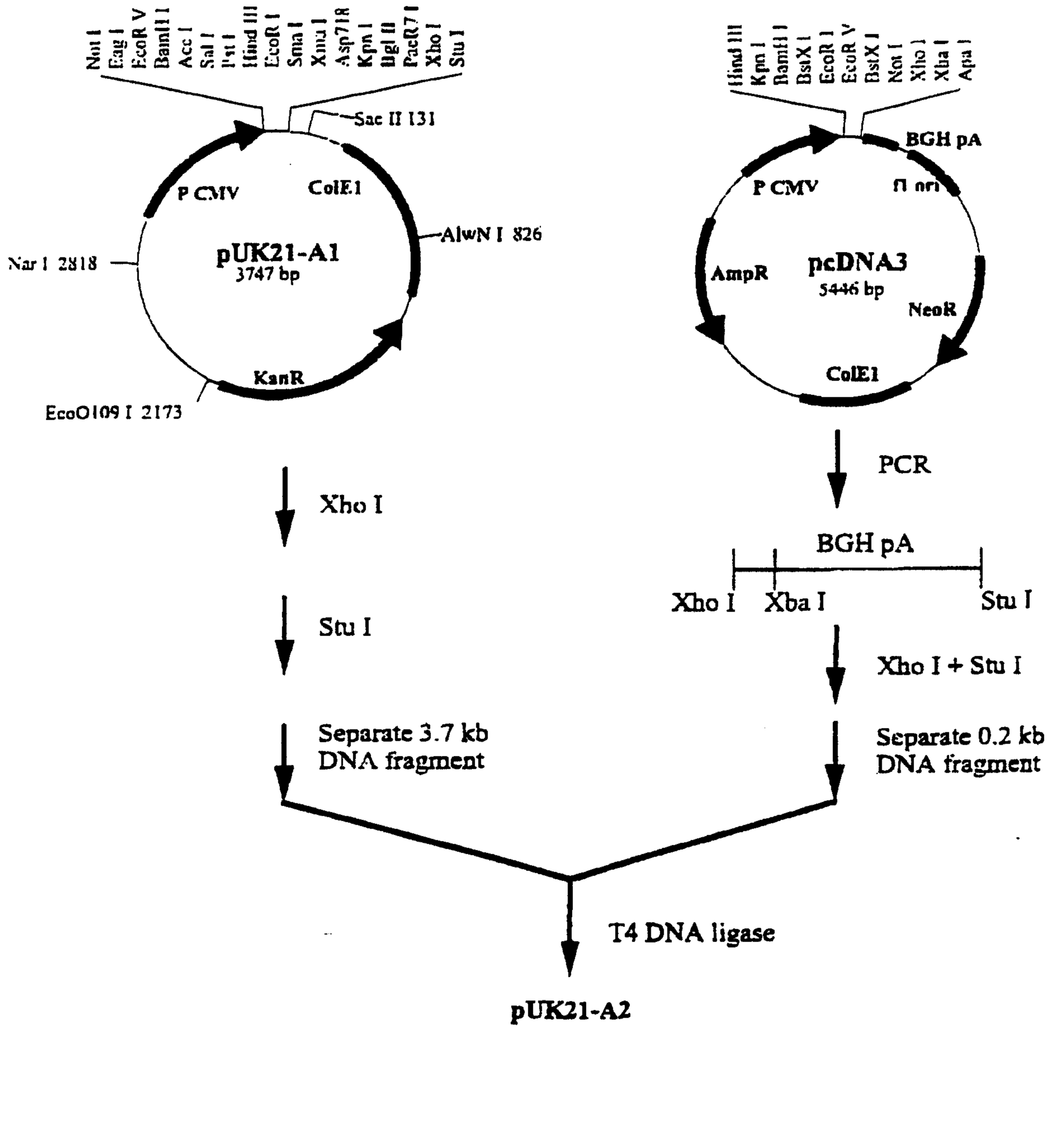

Not I
Eag I
EcoR V
BamH I
Acc I
Sal I
Pst I
Hind III
EcoR I
Sma I
Xma I
Asp718
Kpn I
Bgl II
PacR7 I
Xho I
Stu I
Sac II 131
P CMV
ColE1
AlwN I 826
Nar I 2818
pUK21-A1
3747 bp
KanR
EcoO109 I 2173
Xho I
Stu I
Separate 3.7 kb
DNA fragment
Hind III
Kpn I
BamH I
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Xba I
Apa I
BGH pA
P CMV
f1 ori
AmpR
pcDNA3
5446 bp
NeoR
ColE1
PCR
BGH pA
Xho I
Xba I
Stu I
Xho I + Stu I
Separate 0.2 kb
DNA fragment
T4 DNA ligase
pUK21-A2

Figure 2

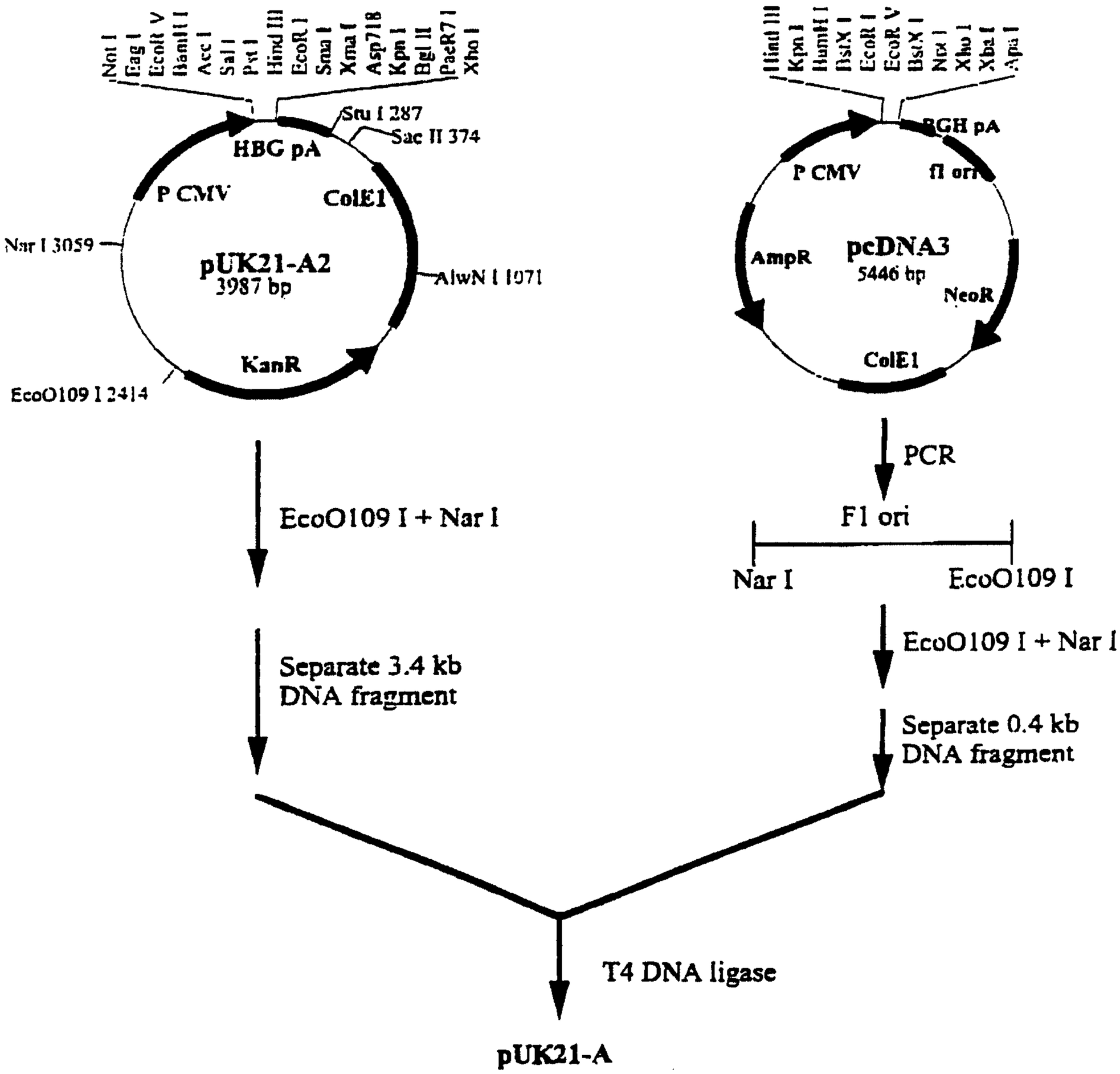
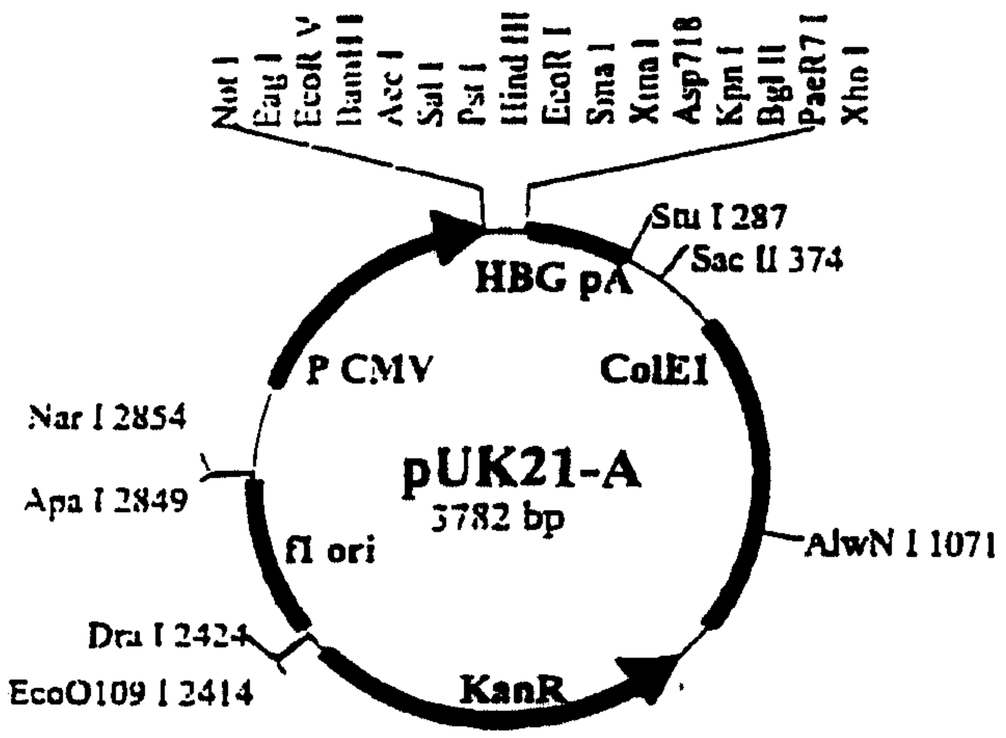

Not I
Bag I
EcoR V
BamH I
Acc I
Sal I
Pst I
Hind III
EcoR I
Sma I
Xma I
Asp718
Kpn I
Bgl II
PacR7 I
Xho I
Stu I 287
Sac II 374
HBG pA
P CMV
ColE1
Nar I 3059
pUK21-A2
3987 bp
AlwN I 1071
KanR
EcoO109 I 2414
EcoO109 I + Nar I
Separate 3.4 kb
DNA fragment
Hind III
Kpn I
BamH I
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Xba I
Apa I
BGH pA
P CMV
f1 ori
AmpR
pcDNA3
5446 bp
NeoR
ColE1
PCR
F1 ori
Nar I
EcoO109 I
EcoO109 I + Nar I
Separate 0.4 kb
DNA fragment
T4 DNA ligase
pUK21-A

Figure 3



22 point mutations within kanR

pUK21-B

Nar I + Dra I

Separate 3.4 kb
DNA fragment

Synthetic oligonucleotides

5'-phosphorylation

Annealing

5' AAATTCGAAAGTACTGGACCTGTTAACA 3'
3' TTTAAGCTTTCATGACCTGGACAATTGTGC 5'

T4 DNA ligase

pMAS

Figure 4

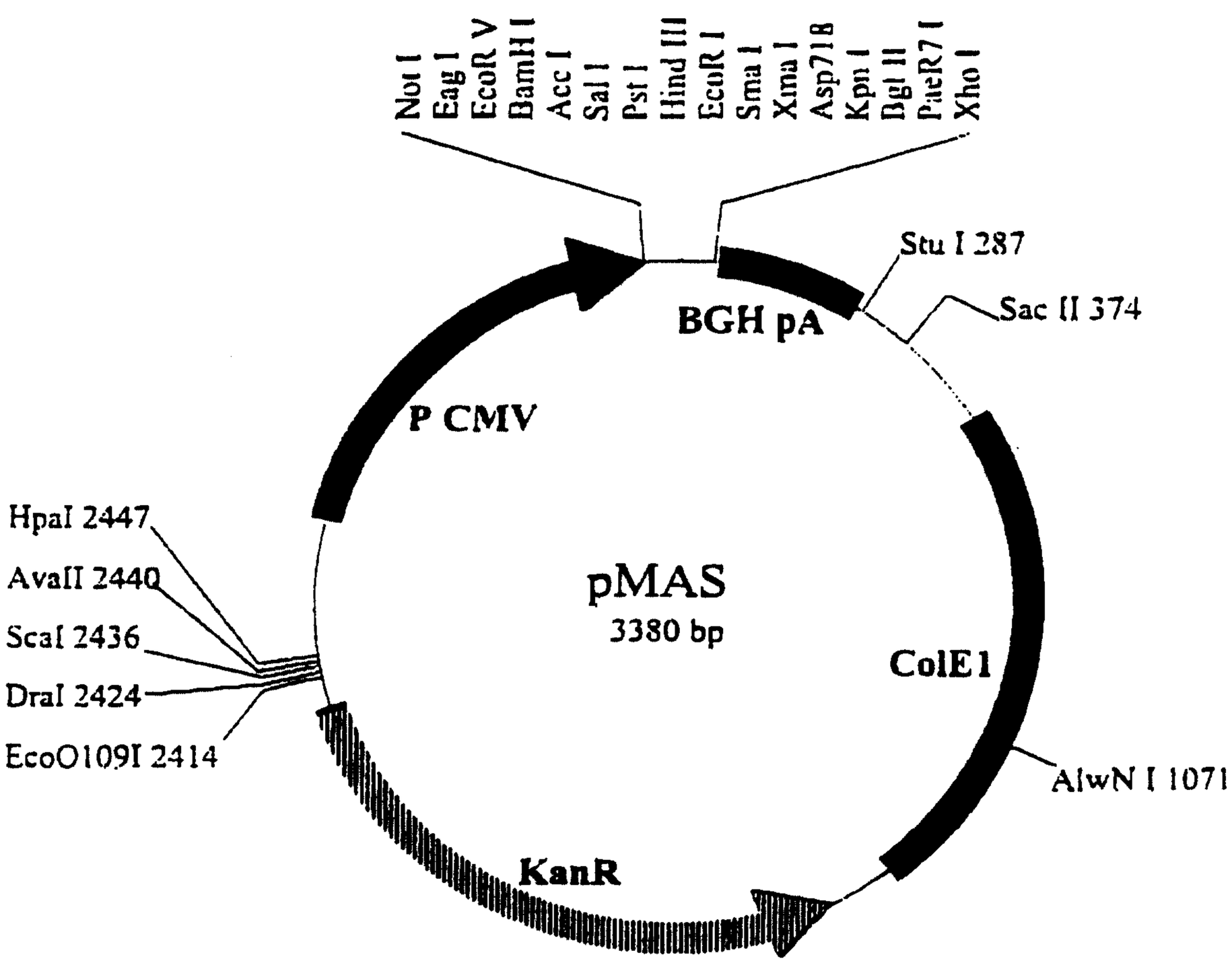

Not I
Eag I
EcoR V
BamH I
Acc I
Sal I
Pst I
Hind III
EcoR I
Sma I
Xma I
Asp718
Kpn I
Bgl II
PteR7 I
Xho I
Stu I 287
Sac II 374
BGH pA
P CMV
HpaI 2447
AvaII 2440
ScaI 2436
DraI 2424
EcoO109I 2414
pMAS
3380 bp
ColE1
AlwN I 1071
KanR

Figure 5

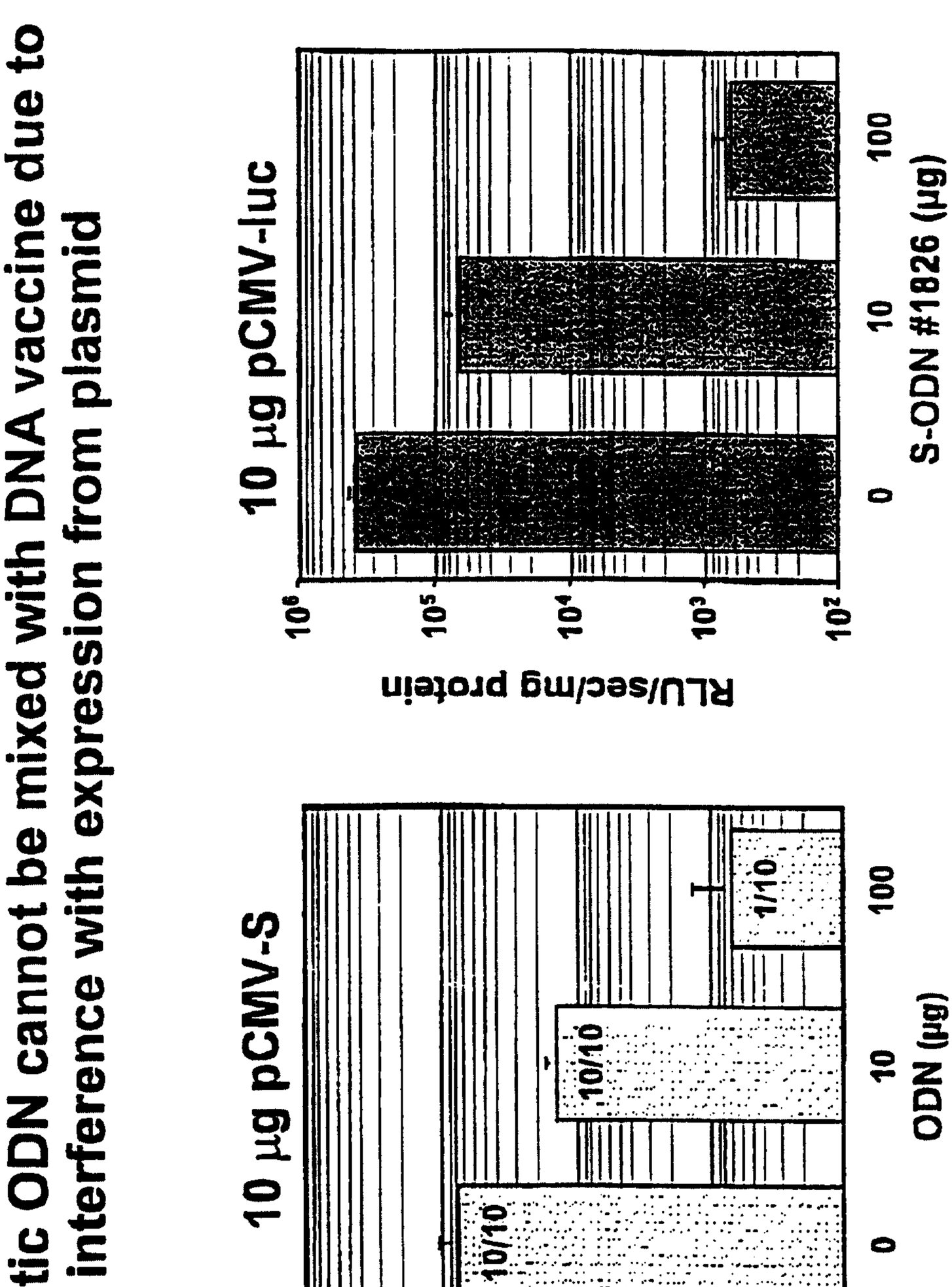

Synthetic ODN cannot be mixed with DNA vaccine due to interference with expression from plasmid
10 µg pCMV-S
10/10
1/10
Anti-HBs (total IgG) titers
ODN (µg)
RLU/sec/mg protein
10 µg pCMV-luc
S-ODN #1826 (µg)

Figure 6

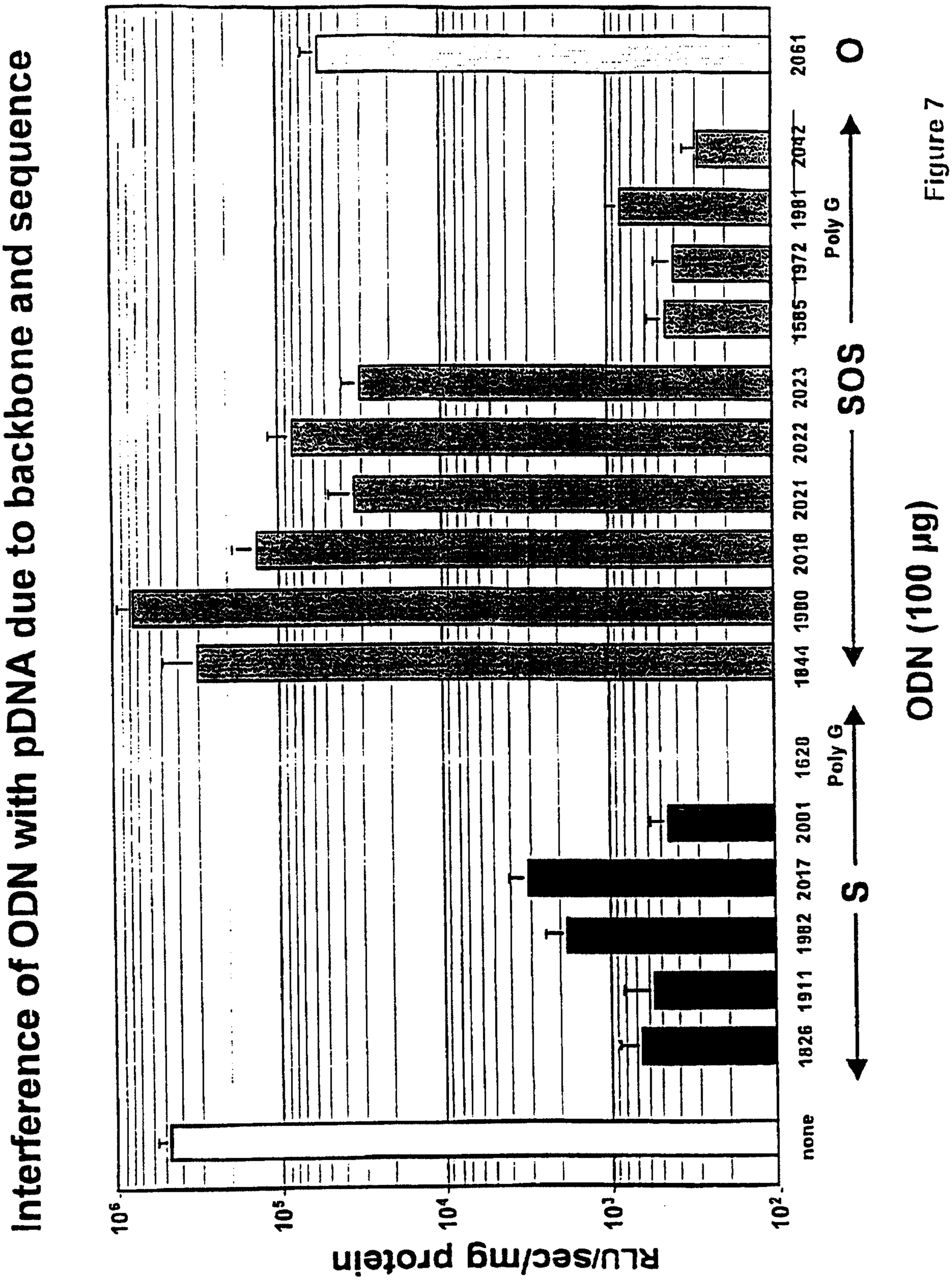

Interference of ODN with pDNA due to backbone and sequence
RLU/sec/mg protein
10^6
10^5
10^4
10^3
10^2
ODN (100 µg)
none
S
1826 1911 1982 2017 2001 1620
Poly G
SOS
1841 1900 2018 2021
Poly G
O
1585 1972 1981 2042 2061
Poly G

Figure 7

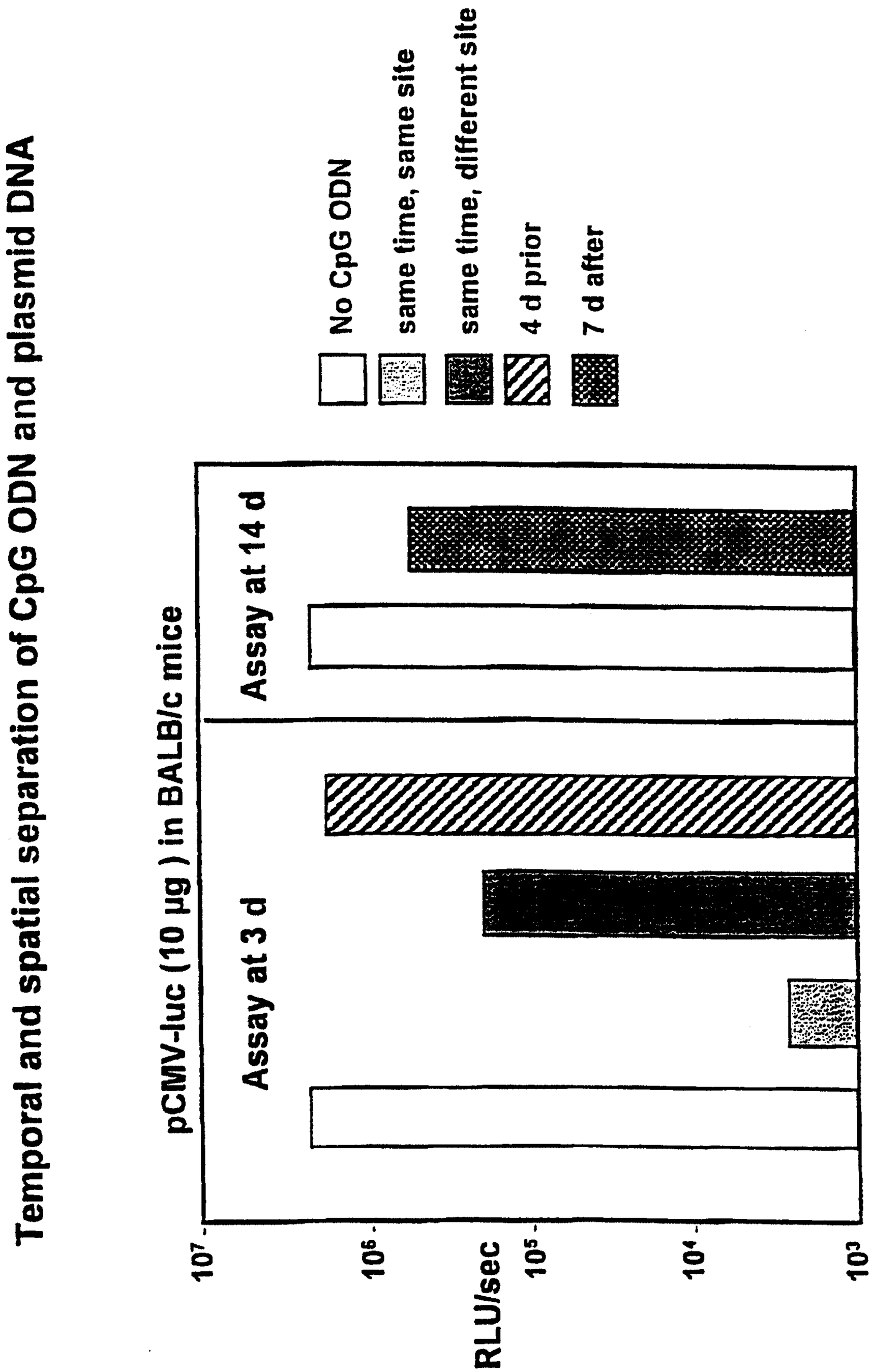

Temporal and spatial separation of CpG ODN and plasmid DNA
pCMV-luc (10 µg) in BALB/c mice
Assay at 3 d
Assay at 14 d
RLU/sec
10⁷
10⁶
10⁵
10⁴
10³
No CpG ODN
same time, same site
same time, different site
4 d prior
7 d after

Figure 8

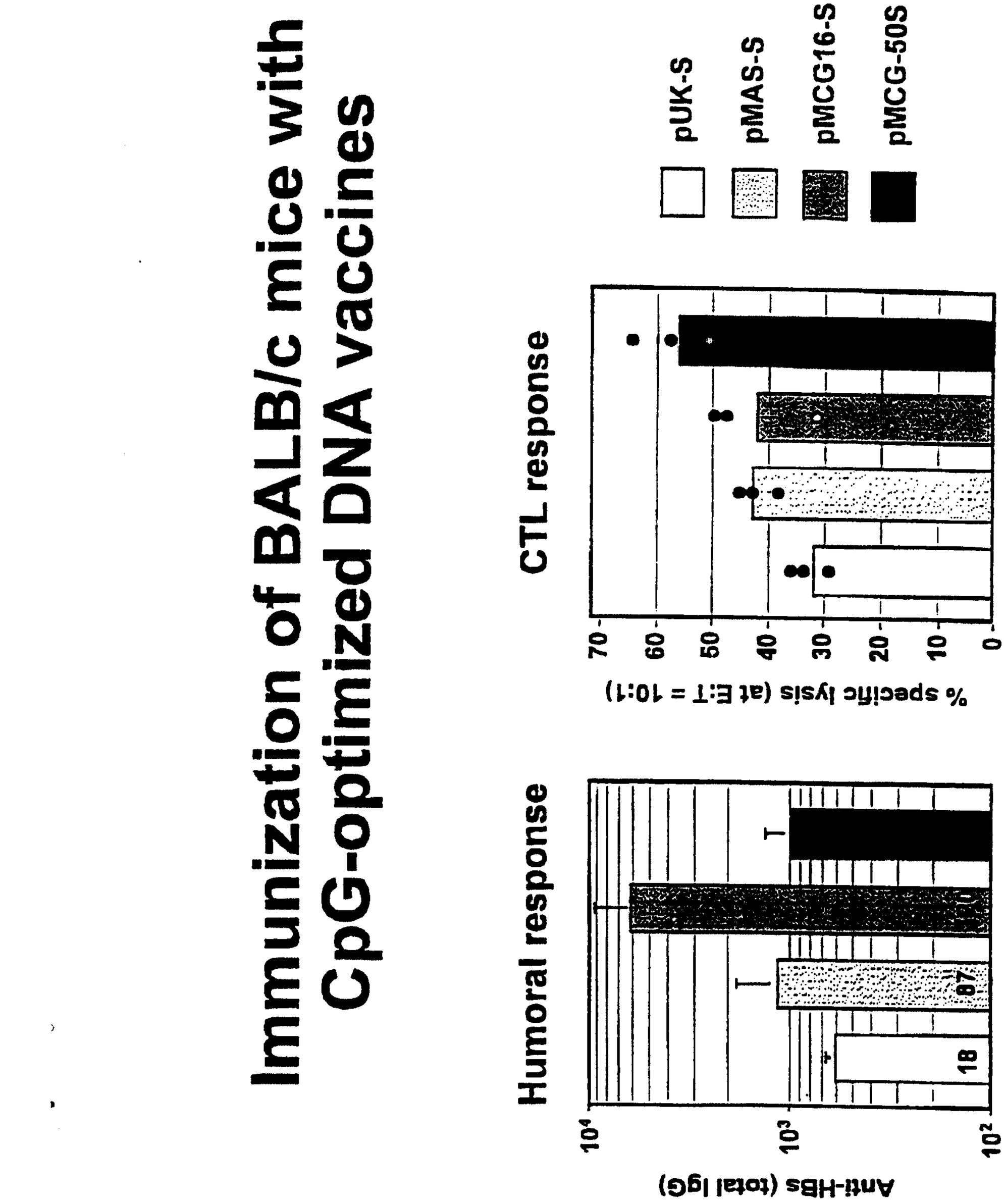

Immunization of BALB/c mice with CpG-optimized DNA vaccines
CTL response
% specific lysis (at E:T = 10:1)
70
60
50
40
30
20
10
0
pUK-S
pMAS-S
pMCG16-S
pMCG-50S
Humoral response
Anti-HBs (total IgG)
10^4
10^3
10^2
87
18

Figure 9

Figure 10

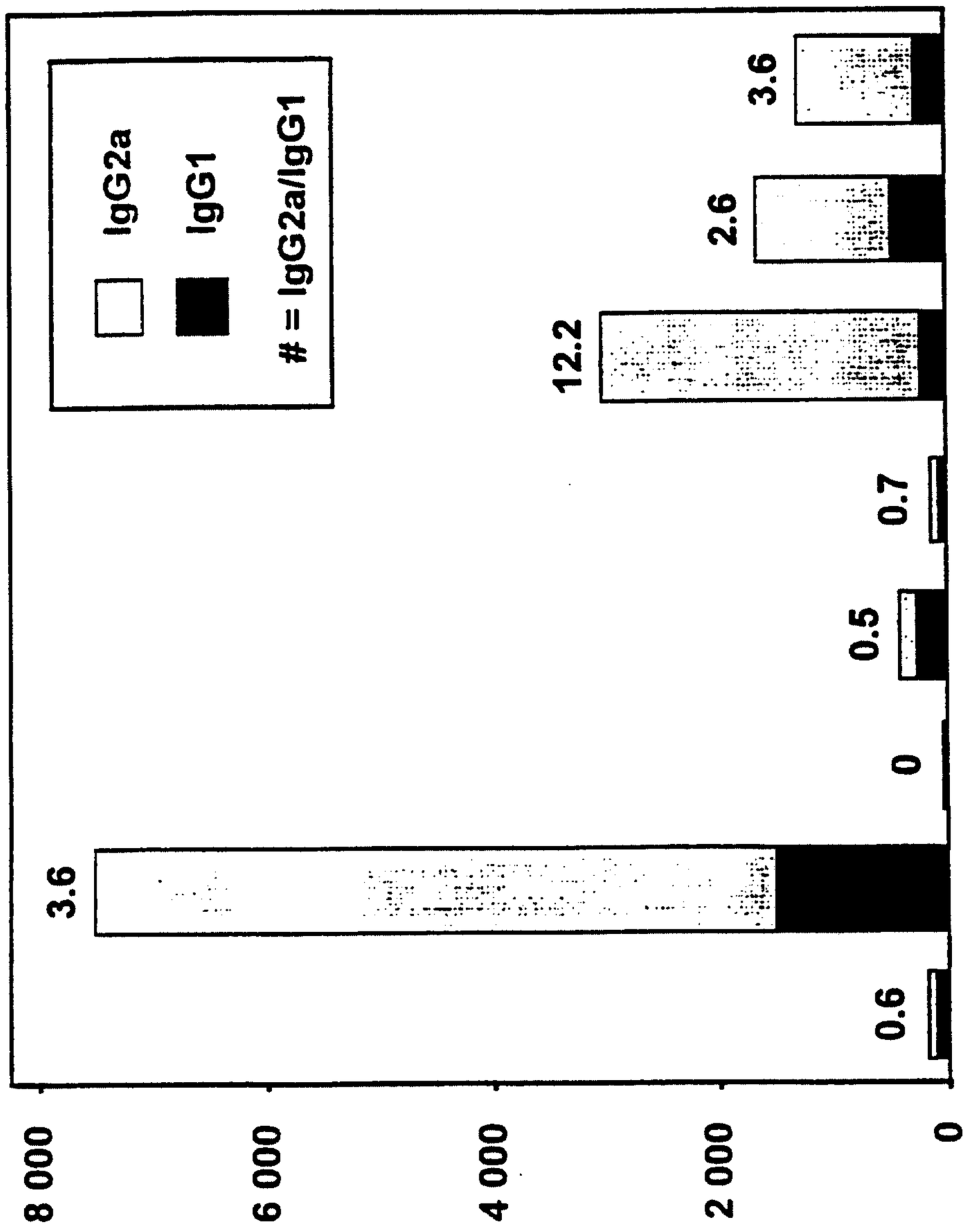
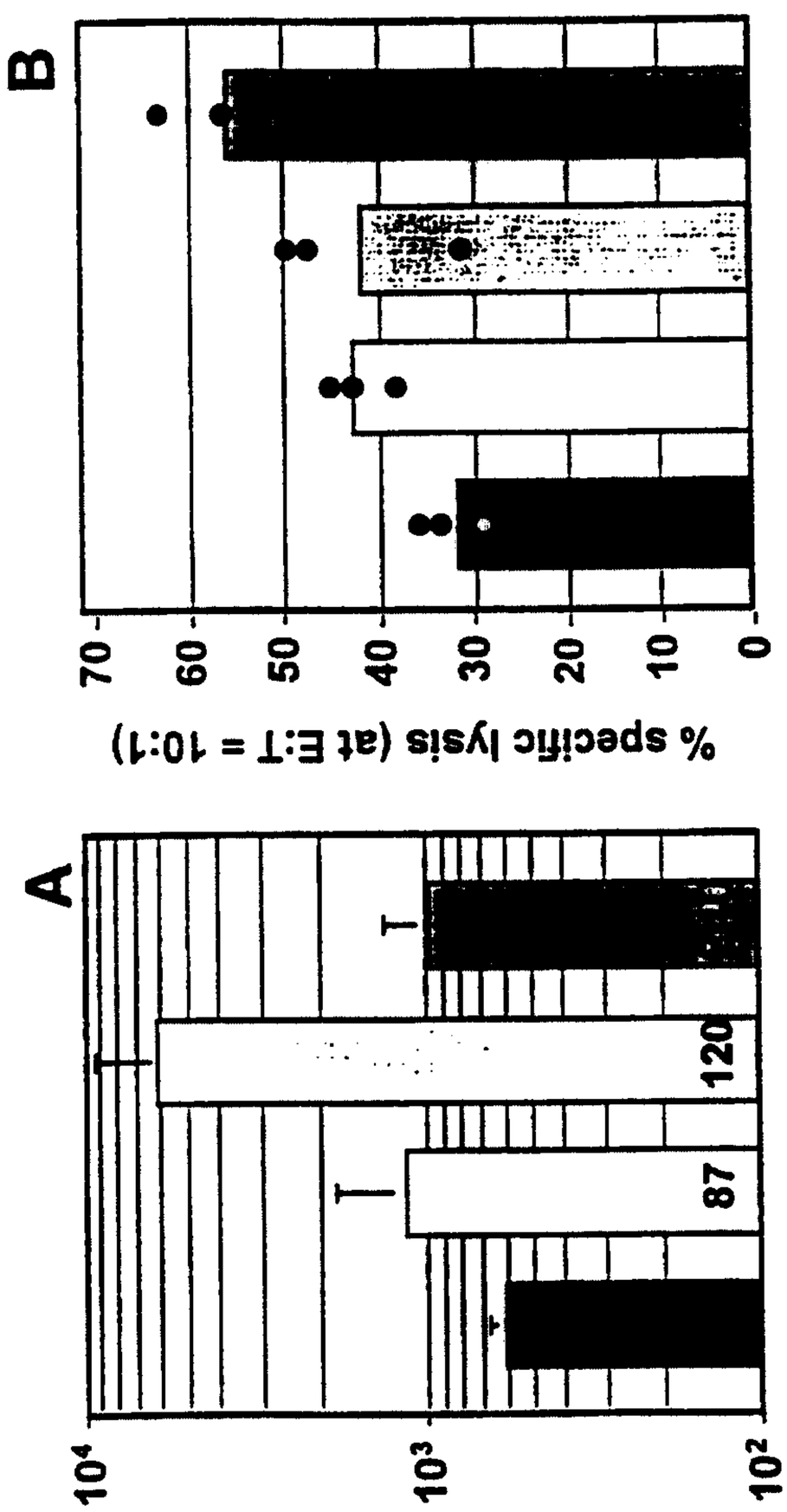

IgG2a
IgG1
= IgG2a/IgG1
12.2
2.6
3.6
3.6
0.6
0
0.5
0.7
1631 1984 2010
1826 1826 1826
none 1826 1631 1984 2010
8 000
6 000
4 000
2 000
0
Anti-HBs ELISA titer

Figure 11


B
70
60
50
40
30
20
10
0
% specific lysis (at E:T = 10:1)
A
T
120
87
4
10^4
10^3
10^2
Anti-HBs (total IgG)