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(54) **TRANSGENE EXPRESSION IN AVIANS**

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(71) Applicant: **ALEXION PHARMACEUTICALS, INC.**, Boston, MA (US)

(72) Inventors: **Alex J. Harvey**, Athens, GA (US);
Jeffrey C. Rapp, Athens, GA (US)

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C12N 15/85 (2006.01)
C12N 7/00 (2006.01)
C07K 14/705 (2006.01)

(52) **U.S. Cl.**

CPC .. **A01K 67/0278** (2013.01); **C12N 2015/8518** (2013.01); **C12N 15/86** (2013.01); **A01K 67/0275** (2013.01); **C12N 15/8509** (2013.01); **C12N 7/00** (2013.01); **C07K 14/70521** (2013.01); **C07K 2319/30** (2013.01); **C12N 2830/008** (2013.01); **C12N 2830/15** (2013.01); **C12N 2830/90** (2013.01); **A01K 2267/01** (2013.01); **A01K 2227/30** (2013.01); **A01K 2217/052** (2013.01); **A01K 2207/15** (2013.01); **C12N 2800/24** (2013.01); **C12N 2740/11043** (2013.01); **C07K 14/56** (2013.01)

Related U.S. Application Data

(60) Continuation of application No. 15/181,987, filed on Jun. 14, 2016, now Pat. No. 10,182,561, which is a continuation of application No. 14/194,010, filed on Feb. 28, 2014, now abandoned, which is a continuation of application No. 13/747,201, filed on Jan. 22, 2013, now abandoned, which is a continuation of application No. 13/179,281, filed on Jul. 8, 2011, now Pat. No. 8,383,399, which is a division of application No. 11/978,360, filed on Oct. 29, 2007, now abandoned, which is a continuation-in-part of application No. 11/699,257, filed on Jan. 26, 2007, now Pat. No. 7,541,512, which is a continuation-in-part of application No. 11/799,253, filed on May 1, 2007, now abandoned, which is a continuation-in-part of application No. 11/210,165, filed on Aug. 23, 2005, now abandoned.

(60) Provisional application No. 60/930,491, filed on May 16, 2007, provisional application No. 60/994,203,

(57)

ABSTRACT

A transgenic avian containing in its genome an exogenous nucleotide sequence which includes a promoter component and a vector with reduced promoter interference wherein the exogenous nucleotide sequence is integrated into the genome and the avian.

Specification includes a Sequence Listing.

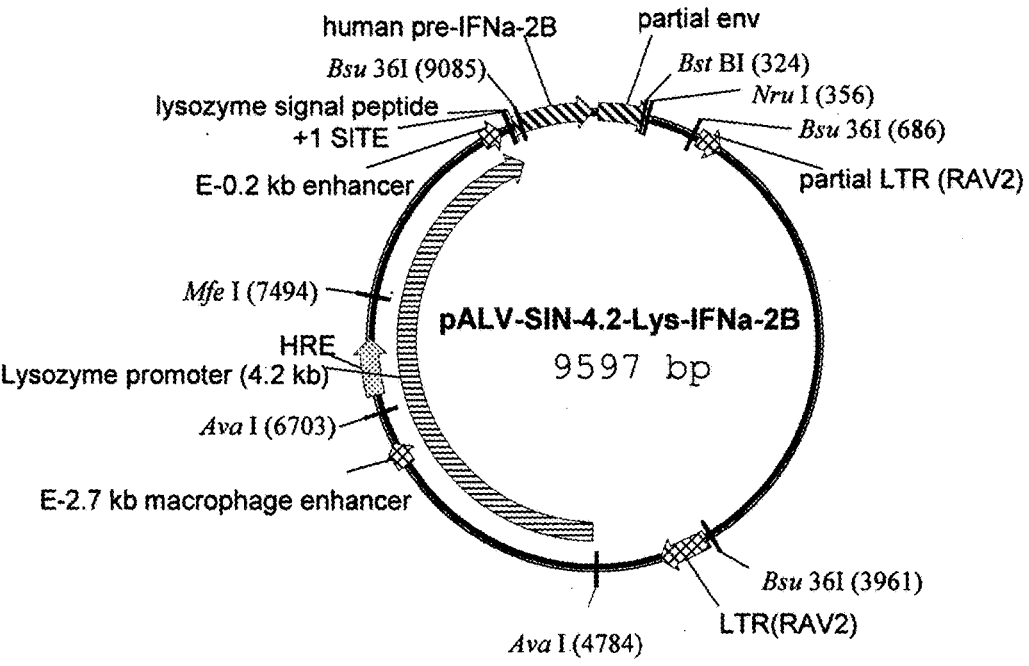


FIG. 1

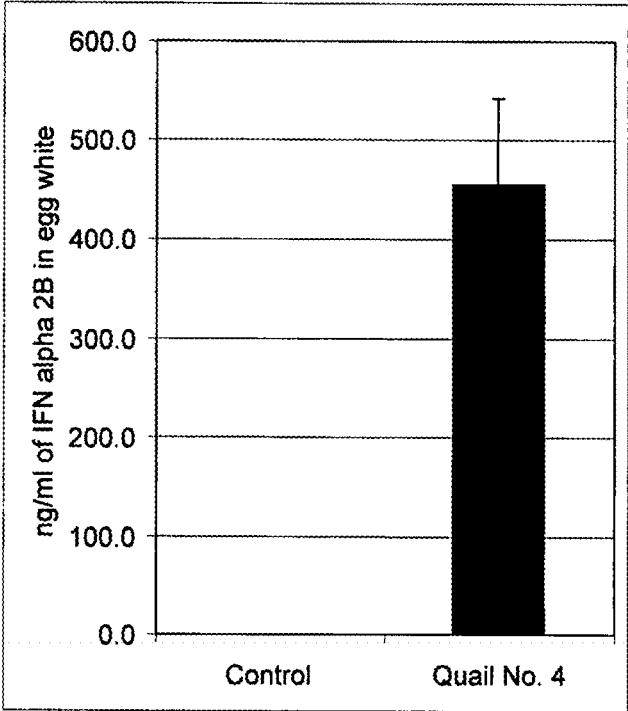


FIG. 2

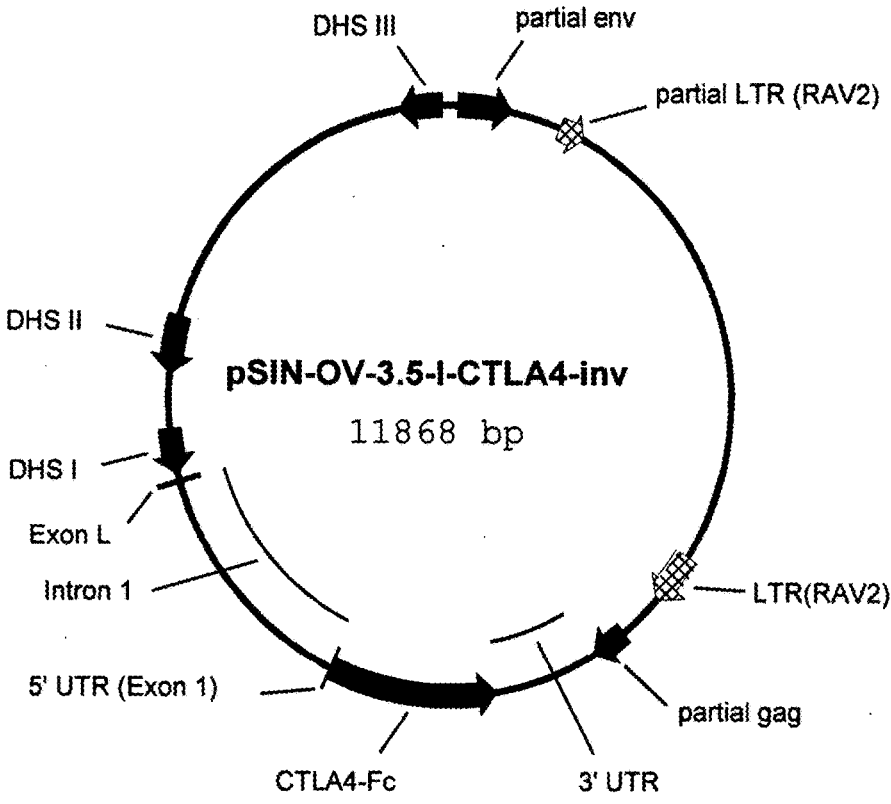


FIG. 3

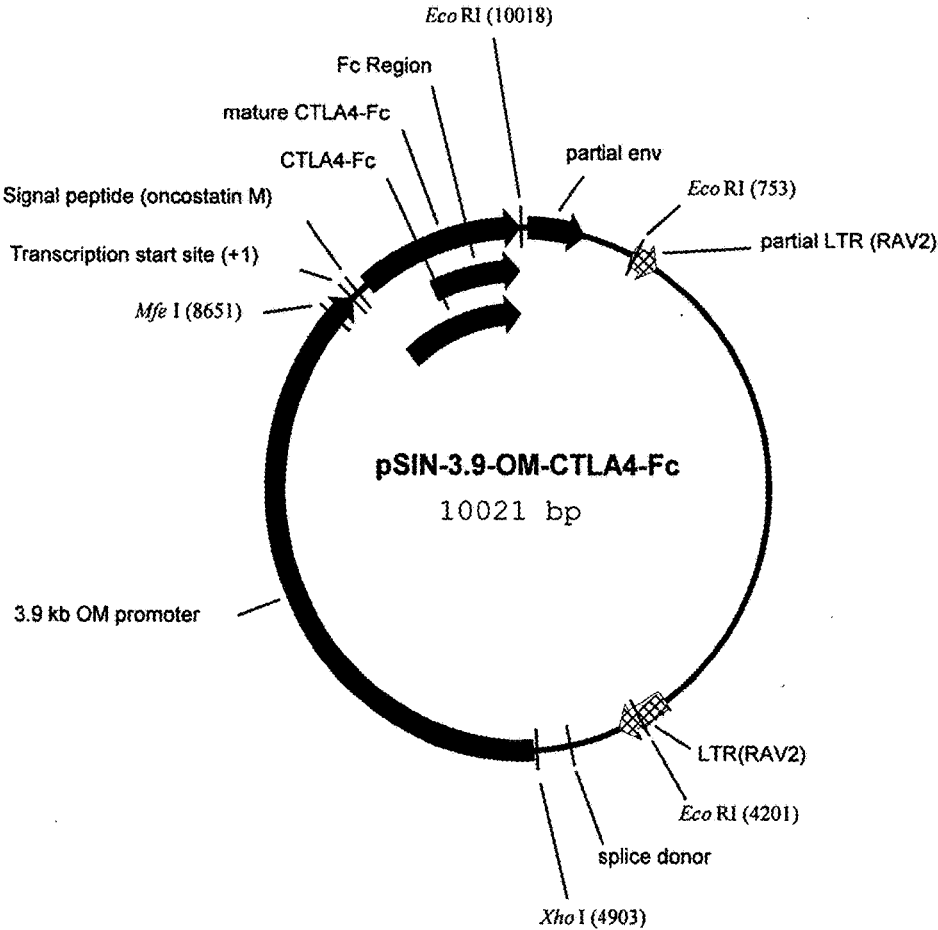


FIG. 4

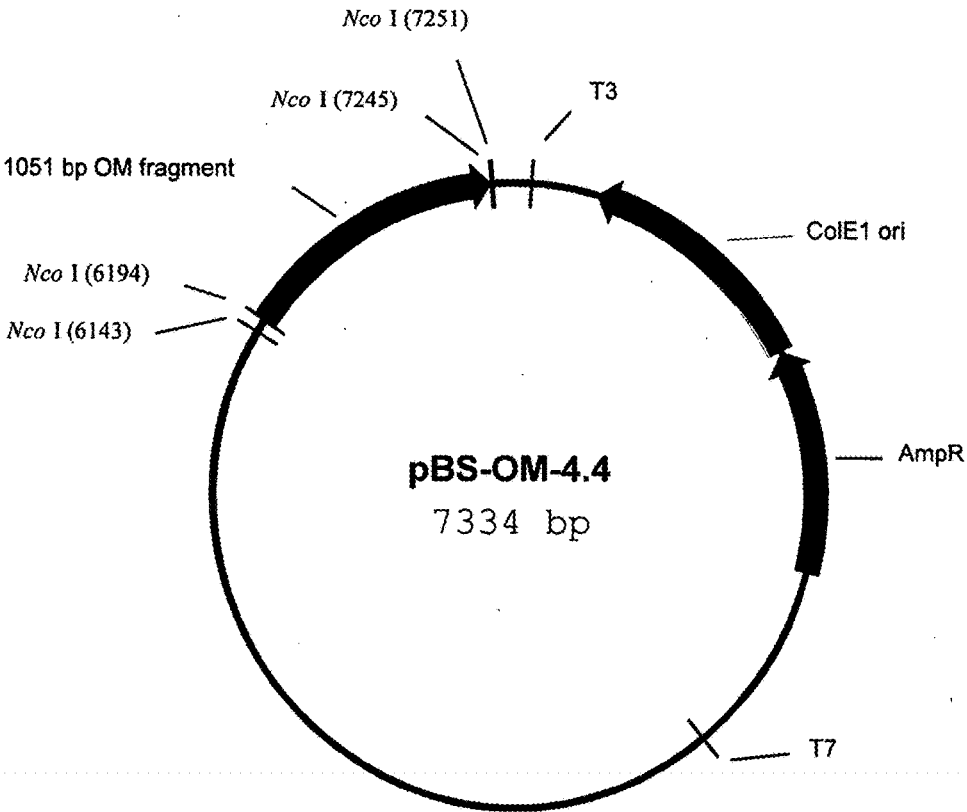


FIG. 5

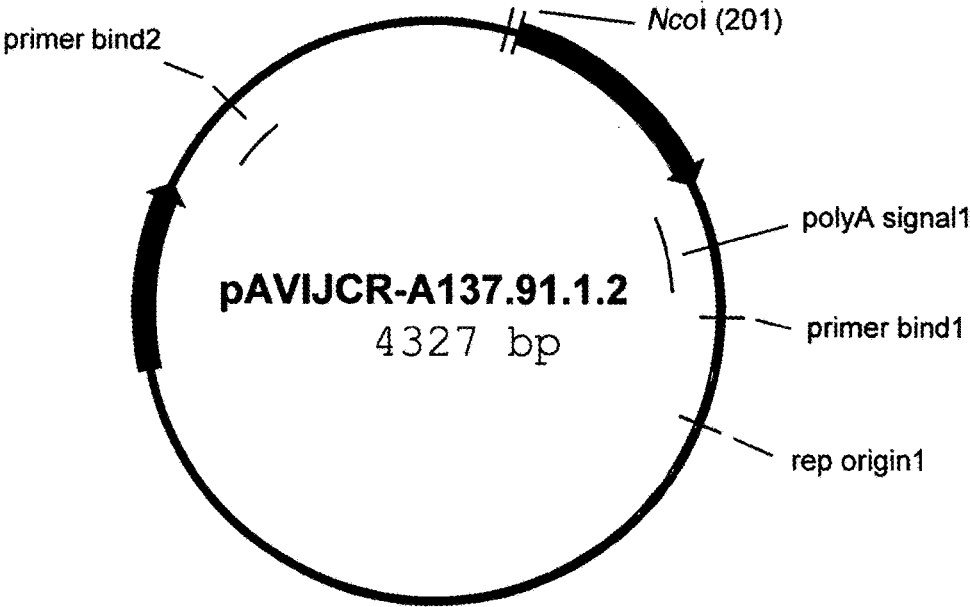


FIG. 6

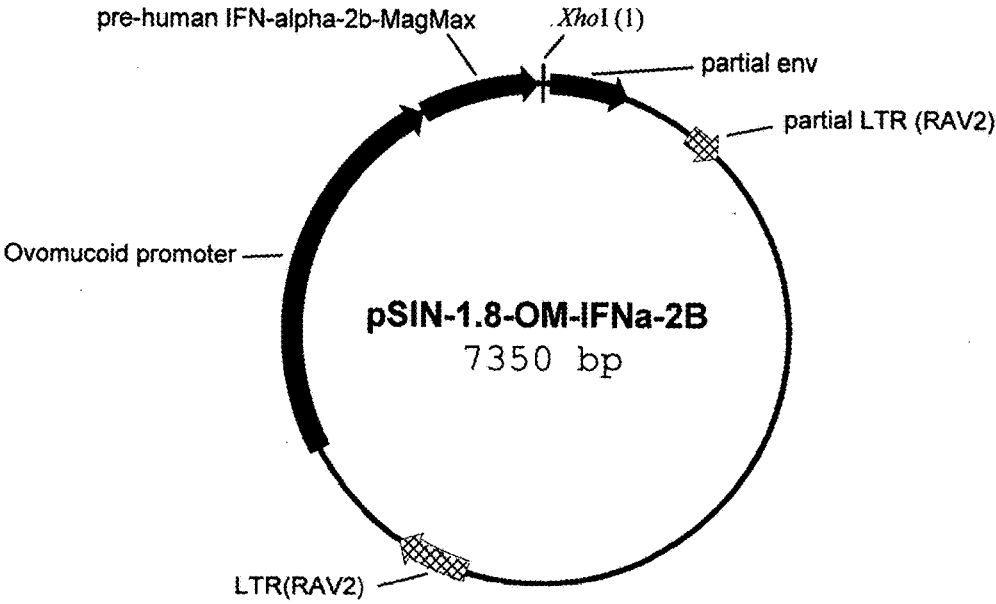


FIG. 7

ctgcagccca	ggcagcacac	tagagcagag	aatctagtt	agcagcaacc	actggcagac	60
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gaaaggactg	ggaagaagag	agtgcaataa	cgaagctgaa	gctaggagga	aggcaaggag	180
aactgaagct	gactagggaa	aagggggatt	aaaggtttaa	gtgtctattc	catagtttgc	240
tggtttgttt	tttgtcaatt	cctgaatcag	taatttttat	gttaattagc	aaaaaattac	300
aaacactccc	caagtcagga	ctgttaccta	caacagaagc	tcagatcagc	tgagccttag	360
tctttttggc	cctccctagg	gaatgctgta	tgtgtctctc	tctccaggcc	tgctcaaaat	420
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aacacagctg	tgcccaatcc	tgcccaagga	ctcctagtat	gacctgggct	gtgccttgct	720
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ctgaagaaca	aggtgtcagt	gaactctag	ctccatagat	aggactgca	tcacatgtca	2160
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ggtggactat	gaactcacat	ccaaaggagc	ttgacctgat	acctgatatt	cttcaaacag	2940
gggaaacaac	acaatcccac	aaaatagctc	agagagaaac	catcactgat	ggctacagca	3000
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tcacaaaaca	aagctctgca	agtcaaatgc	atacaaaact	gttctgtagg	tctggaatca	3840

FIG. 8a

ggacactatg	tggaagtcaa	atagagaagc	tttaaaaaaa	cctttgggat	cattctcatc	3900
ttatatttgc	agpacgatac	tatgacagtg	ataactgaca	taactgcac	aatttccctg	3960
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caggtctgga	caggtccgtg	gtccatgtat	gataaaaagag	atgaagggaa	ggagaatgga	4080
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ttgtacagca	gttgttctta	catcttgctc	ctcctgaggt	gcactctagca	tcgatcactg	4500
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FIG. 8b

gaattacatg	cgtgtccttc	tcttagacta	catgacccca	tataaattac	attaacttacc	7740
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ctcttgtttt	tacaaatgaa	agagaggaca	aataacattt	ctctatcacc	gacctgaaac	9660
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tagcttttta	cactactata	ttattaatat	ctgttaattc	cagtcttgca	ttccacattt	9840
gcaaaaactg	ttgaaattcg	tatctgaaag	ctgaatactc	ttgctttaca	ggaatacttg	9900
cagtgtgtga	aggaactgta	tagaggagc	ttggaacctc	tcaactttca	aacagctgca	9960
gatcaagcca	gagagctcat	caattcctgg	gtgaaaagtc	agacaaatgg	taaggtagaa	10020
catgctttgt	acatagtgag	agttggttca	ccctaatact	gagaacttgg	atatagctca	10080
gccagcgtgc	tttgcgttca	agcttaccag	agctgttgta	tgctgttaa	gcagggcata	10140
cagtcagtag	gctctgaaa	aatcttaaca	gacaaaggcc	aatggaaaaat	cggagttaag	10200
ggatggtagg	gtataaatgc	atagaaaagag	gtaccacaat	tttgattttt	gcctaatgac	10260
ctctctgctg	ggttctctca	ttttctact	tcattctctc	tctcctcaga	gcattccttt	10320
ccctcatgct	tgaacacacag	atgaaagact	gtgaattcta	actgagatga	aaacatccac	10380
aaccacacaa	cctctggtgt	ggagtcacat	tctgtgaagg	caaaaactag	gccacgtaat	10440
ctatgcgtgc	aagctacgag	taagctatgt	gtgtgacagg	acaatgtgag	gaacatacta	10500
tgtgcacaag	gactgcagaa	taaacaggag	caaagttttt	gaagaaaaca	gagtaaaatc	10560
ctgttttctc	ctttgtttac	attctttaca	tatatctcaa	atttctctt	tggttagaag	10620
caagtaatat	ttatgtttct	tggtactgtt	tgggttgaag	accattctgg	gataagagaa	10680
atccagtggt	ttcttccct	aatcataaaa	tgtcaggttt	agtttttttg	taacacagaa	10740
atctcttcat	cttttatctt	ttgttgtgat	tcttgataga	gagagaaaaca	agacttactg	10800
acaatagcag	caagaaaatc	aatcttgtaa	gaacaagatt	gcaattgcaa	aaacaaacca	10860
atgtccttgc	ccctacatcc	tcttccccat	aaattctaca	ttctctatct	accttgtgct	10920
tgccaacatg	ataacgttaa	actctctttt	cctattcatt	cttaaaggaa	ttatcagaaa	10980
tgctcttcag	ccaagctccg	tggattctca	aactgcaatg	gttctgggta	atgccattgt	11040
cttcaaagga	ctgtgggaga	aagcatttaa	ggatgaagac	acacaagcaa	tgcttttcag	11100
agtgactgag	gtatatgggc	ataccttaga	gatgtaatct	agaatttatg	aagagagtag	11160
acatgtttgt	atatgaacac	tgcattagcg	tatctgctca	tttgtctgca	tctctttcag	11220
acactgtgtt	aaaagcaggg	aattttcctt	atgtctctct	cgtcacataa	ttctgacat	11280
tgcaaaagctc	ctgagaaata	acttcagatt	ccacttttcc	taggaaggct	tctggatgag	11340
aactaatcat	cttaactgta	actagacatt	tctgcatcca	agaataatct	ttgttaaac	11400
tatattctct	ctctcttttt	ttttttttt	tggttctcca	gcaagaaagc	aaacctgtgc	11460
agatgatgta	ccagattggg	ttatttagag	tggcatcaat	ggcttctgag	aaaatgaaga	11520

FIG. 8c

tectggagct	tccatttgcc	agtgggacaa	tgagcatggt	ggtgctggtg	cctgatgaag	11580
tctcaggcct	tgagcaggta	tgcccctaga	agttggcttc	agaatattaa	aaacacatgg	11640
aaatttagct	gttgtaaacg	tcttttcaac	acagttatcc	taaaacattt	aaccagcaca	11700
aatttcacat	tgattcaata	tgtgattggt	gcataagaag	gtagatttgt	cccactgggt	11760
cctgcaatag	cccactgctga	gcacggcttg	ctgaaagaac	tgcttagag	ggtgaaaagt	11820
ttgacacagc	agacaagatg	attctcacct	aagcagctgt	tactgtagtg	gcttgaactc	11880
taaaggtcct	gtatctccat	tectgtgcac	tgaggagctt	cttgaaaagt	tcataataag	11940
tttactagtt	ctaactatta	tctcatttgg	tgccactcaa	tgtgctttgt	tcacgtcttc	12000
ataaattaat	ctatctaaaa	atgggatgtg	gttaaagcaa	tttcagaaat	aacatgtaca	12060
taatgtacaa	ttattgatat	gaacagaaca	caggcatagc	atattgtaat	taggaggact	12120
gtagttattt	tgaataggaa	acacaatgta	ataaatgaga	attcattgaa	atgttagtat	12180
gctaactcaa	tctaaattat	aaagataaag	aggcatttaa	tcacagctag	atttccatca	12240
cttgtgacag	acaggcatat	gaatgattat	gtacagctct	aggaaaaaaa	gtatgtagga	12300
aaactagtag	atthttgatta	gaaagtctga	aaatgaggtg	ccttgatcaa	agagaatacg	12360
tgtgtttgag	aaaaaaaaaag	tttgataga	ggtggtaga	gagaatata	tgaatgggtg	12420
tttctacaaa	ctgccatggc	cagatttgtg	taagagacat	tcagtaagta	ggcaaggaaa	12480
gaaatattac	taggtacaaa	gcaacatcag	taataccaaa	agaaaccaat	tattccagat	12540
gccaatctcg	taatagggtt	aagagatttc	cacccctcta	gtggtcacca	gtgcaaccag	12600
faactttgag	aaattacatt	tctttttttt	aaatggcaga	tatagctttg	aactgagtga	12660
tcataactg	gtactgtgta	atagatgaag	acatacttga	cgactaaact	tctgattttt	12720
aaaaactcaa	attctcttga	aagatcagtt	cccagcttag	taacagctga	tagtttaagt	12780
atcagtaatt	ggctaccatt	aacaactggc	tcctgagagg	tcttaaatgt	agagacagct	12840
ttaaactcaa	aagcacagag	tgatthttag	aatagatttc	ccaagcaag	aaaataaaca	12900
gggaggagct	ttaagggagt	agccatctca	ttattattat	tatttaaaga	aatggcagca	12960
agcctacaaa	agaaaaataa	gacagagcag	agaagaaga	gtcatggtat	gcttttctat	13020
cttagcaaaa	ttaatctcta	catgcctagg	aaaaagccat	gacaagagca	atcagttcaa	13080
aagggttatg	caaaaaacca	cataatagta	actagtactg	catgcccagg	aaggaagtta	13140
tgtgccatt	ccatggatct	cattctcatt	tccttgcagc	ttgagagtat	aatcaacttt	13200
gaaaaactga	ctgaatggac	cagttctaat	gttatggaag	agaggaagat	caaagtgtac	13260
ttactctgca	tgaagatgga	ggaaaaatac	aacctcacat	ctgtcttaat	ggctatgggc	13320
attactgacg	tgtttagctc	ttcagccaat	ctgctcggca	tctcctcagc	agagagcctg	13380
aagatatctc	aagctgtcca	tgacgacat	gcagaaatca	atgaagcag	cagagaggtg	13440
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catcatttcc	tcttctgtat	caagcacatc	gcaaccacag	ccgttctctt	ctttggcaga	13560
tgtgtttccc	cttaaaaaaga	agaaagctga	aaaactctgt	ccctccaac	aagaccaga	13620
gcactgtagt	atcaggggta	aaatgaaaag	tatgttatct	gctgcatcca	gacttcataa	13680
aagctggagc	ttaatctaga	aaaaaatca	gaagaaatt	acactgtgag	aacaggtgca	13740
attcactttt	cctttacaca	gagtaatact	ggtaactcat	ggatgaaggc	ttaagggaa	13800
gaaattggag	tcacagtagt	gagtcactac	actgaaaaat	gcaacctgat	acatcagcag	13860
aaggtttatg	ggggaaaaat	gcagccttcc	aattaagcca	gatatactga	tgaccaagct	13920
gctccagaat	tagtcactca	aaatctctca	gattaaatta	tcaactgtca	ccaaccattc	13980
ctatgctgac	aaggcaattg	ctgttctctt	gtgttctctg	tactacaagg	ctcttctctg	14040
cttctctaaa	atgcattata	aaaactctat	aattcacatt	tctcctctaa	cttgactca	14100
atcatggtat	gttggcaaat	atggtatatt	actattcaaa	tgttttctct	tgtaccata	14160
tgtaatgggt	cttgtgaatg	tgctcttttg	ttcctttaat	cataataaaa	acatgtttaa	14220
gcaaacactt	ttcacttgta	gtatthtgaag	tacagcaagg	ttgtgtagca	gggaaagaat	14280
gacatgcaga	ggaataagta	tgacacacaca	ggctagcagc	gactgtagaa	caagtactag	14340
tgggtgagaa	gttgaacaag	agtcccttac	aagcaactta	atctaataag	ctagtgtctt	14400
acatcagcta	aaagagcata	gtgagggatg	aaattggttc	tcctttctaa	gcataccctg	14460
ggacaactca	tctggagcag	tgtgtccaat	ctgcccgtgc	cctgatctctg	gctgggggtga	14520
tgggacagac	cttggctgcc	actgagacat	ctgagacact	gagatctgtc	tcaactcaga	14580
tttaccacaag	aacagctcat	tgccaacaga	acaaaatctc	aaacttatgg	ctagtgtatga	14640
cagcagctcag	ttgtcccac	tgtgaccac	caaggctggc	atgctggaat	gagcaggctt	14700
tggtggcatg	tagttactgg	acagcaccac	tgacatgggc	aggggaaaaa	ctgagcatgg	14760
tgtaaatcac	tgctcaaaag	ccacttctct	gtgctctgac	catgctgaa	agctcttcta	14820
caggagctgg	gtttgttcaa	gaaagcttct	gtttctccca	tctgcttctt	gtaccttcac	14880
agggacagag	ttagaagggt	acagccatgg	ctggaagggg	ctgactttca	aatgtgccta	14940
atthtctctt	ggttgtctgt	gcagctgcag	aagaaggggt	tcagaagcca	agagctttga	15000
gataaggatg	cttaacctat	gttgaagaca	tttgtgctga	cacctcaggc	cccagatag	15060
gacaactgct	ggattgtggc	taaccacta	gctacagaac	ctaatttata	ttaccagatt	15120
aggaagagca	aaagaacatg	tatttataac	aggaggtctt	ctgtgcttct	ctactaaaag	15180
gtgctgtgaa	ggagcccaca	gtgcagcagt	gtatgagccc	tgaagaggc	cgagcacac	15240
gaagaccctt	gttaggagca	gcacacagag	gggcaggagg	gctgggggaa	ctgcccacca	15300
tggggacctg	tgtgaagcag	tgactcctg	aggggtggac	tgcgtgggaa	aggaaaagaa	15360

FIG. 8d

```
agcaaacaga cctgtgatga actgtcacac agactgcaga gtgacagagg agggcacgag 15420
gcagtgcgcc cactgcaggg agtggcgctc cttcctcaca gcagcgctaa cagcttgcca 15480
ccaatattca gtagtctgtg gtgatacttt ttccagtttc accacacagc atttcgcttg 15540
ttctacttgt tttagctttc cccctccaca agataacaca tactttgcca gtcagtccct 15600
aagaccttaa cttaacagtt agcaaacagg atcttgcaaa agaaggaaga taacatgaca 15660
ccaccttcac tgggtataa atagtcaaa tactttcctt cactttcccg taaattagtt 15720
gattgcaggt caggagataa caggggaact tactgcaaga gagaaaatga tgtttaatat 15780
tgtcttggac tttctggtgg tctgggcatg aaaatggggt actcaaaaac ctcgggacgt 15840
ttatTTTTca cctgatttat tcccaaactg cactatttct aggccattgg agttcttata 15900
aattaaatta tactttggct ctctgctatc tcactccctt tcattctcag catcactttc 15960
agcacaatta caggagaaga cttagactca gagctttagg actcatcata agaggctttc 16020
attgctctgt caccacacc catatagatc t 16051
```

FIG. 8e

AATTGAGGAGCTTTGTTCCCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCGCAGC
GTCAATGACGCTGACGGTACAGGCCAGACAATTATTGTCTGGTATAGTGCAGCAGCAGA
ACAATTTGCTGAGGGCTATTGAGGCGCAACAGCATCTGTTGCAACTCACAGTCTGGGGC
ATCAAGCAGCTCCAGGCAAGAATCCTGGCTGTGGAAAGATACCTAAAGGATCAACAGCT
CCTGGTAC

FIG. 9a

AATGTGGGGAGGGCAAGGCTTGCGAATCGGGTTGTAACGGGCAAGGCTTGACTGAGGGG
ACAATAGCATGTTTAGGCCGAAAAGCGGGGCTTCGGTTGTACGCGGTTAGGAGTCCCCTC
AGGATATAGTAGTTTCGCTTTTGCATAGGGAGGGGAAAT

FIG. 9b

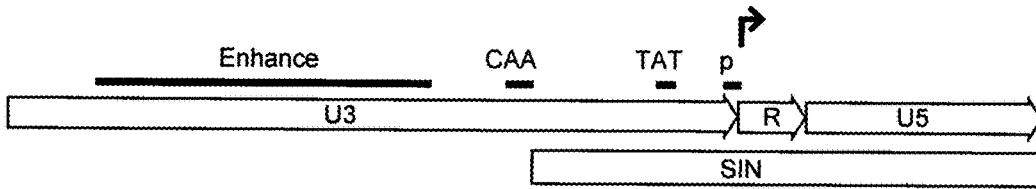


FIG. 10 a

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AATGTAGTCTTATGCAATACTCTTGTAGTCTTGCAACATGCTTATGTAACGATGAGTTAG 60
CAACATGCCTTATAAGGAGAGAAAAAGCACCGTGCATGCCGATTGGTGGGAGTAAGGTGG 120
TATGATCGTGGTATGATCGTGCCTTGTTAGGAAGGCAACAGACGGGTCTAACACGGATTG 180
GACGAACCACTGAATTCCGCATTGCAGAGATATTGTATTTAAGTGCCTAGCTCGATACAA 240
TAAACGCCATTTGACCATTACCACATTGGTGTGCACCTGGGTTGATGGCCGGACCGTTG 300
ATTCCTGRCGACTACGAGCACATGCATGAAGCAGAAGGCTTCATT 346
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FIG. 10 b

TRANSGENE EXPRESSION IN AVIANS

RELATED APPLICATION INFORMATION

[0001] This application claims the benefit of U.S. provisional application Nos. 60/930,491, filed May 16, 2007 and 60/994,203, filed Sep. 18, 2007 and is a continuation-in-part of U.S. patent application Ser. No. 11/699,257, filed Jan. 26, 2007 and is also a continuation-in-part of U.S. patent application Ser. No. 11/799,253, filed May 1, 2007 which is a continuation-in-part of U.S. patent application Ser. No. 11/210,165, filed Aug. 23, 2005 which claims the benefit of U.S. provisional application No. 60/640,203, filed Dec. 29, 2004. The disclosures of each of these three U.S. patent applications and two provisional applications are incorporated in their entirety herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the use of promoters which function in cells of a transgenic avian (e.g., oviduct cells) such as a transgenic chicken and vectors which contain such promoters. More specifically, the invention relates to recombinant nucleic acids and expression vectors, transfected cells and transgenic animals, for example, transgenic avians such as transgenic chickens, that contain vectors with gene expression controlling regions operably linked to coding sequences.

BACKGROUND

[0003] The field of transgenics was initially developed to understand the action of a single gene in the context of the whole animal and the phenomena of gene activation, expression and interaction. Transgenics technology has also been used to produce models for various diseases in humans and other animals and is among the most powerful tools available for the study of genetics, and the understanding of genetic mechanisms and function. From an economic perspective, the use of transgenic technology to convert animals into "protein factories" for the production of specific proteins or other substances of pharmaceutical interest (Gordon et al., 1987, *Biotechnology* 5: 1183-1187; Wilmut et al., 1990, *Theriogenology* 33: 113-123) offers significant advantages over more conventional methods of protein production by gene expression.

[0004] One system useful for expressing foreign proteins is the avian reproductive system. The production of an avian egg begins with formation of a large yolk in the ovary of the hen. The unfertilized oocyte or ovum is positioned on top of the yolk sac. After ovulation, the ovum passes into the infundibulum of the oviduct where it is fertilized, if sperm are present, and then moves into the magnum of the oviduct, which is lined with tubular gland cells. These cells secrete the egg-white proteins, including ovalbumin, lysozyme, ovomucoid, conalbumin and ovomucin, into the lumen of the magnum where they are deposited onto the avian embryo and yolk. In the past exogenous protein production has been performed in the avian reproductive system specifically targeting the avian oviduct.

[0005] Advantages of targeting the avian oviduct for exogenous protein expression can include proper folding and post-translation modification of the target protein, the ease of product recovery, and a shorter developmental period of birds such as chickens compared to other animal species.

[0006] Directing expression of a heterologous gene product in the oviduct of a transgenic avian can be significantly advantageous over ubiquitous expression in the bird. That is, the consequences of ubiquitous expression of a bioactive gene product in a host animal may be undesirable. For example, in certain instances the ubiquitous presence of the recombinant protein may be harmful to the development of the avian which can kill the bird. Additionally, the bird's health may be negatively effected leading to reduced levels of protein production.

[0007] By weight, approximately 60% of an avian egg is composed of albumen which is composed of four major protein components; ovalbumin, ovomucoid, lysozyme and ovotransferrin with ovalbumin and ovomucoid being present in the greatest quantities.

[0008] The ovalbumin promoter, ovomucoid promoter and lysozyme promoter have been successfully employed for the production of heterologous (exogenous) protein in the oviduct of transgenic avians in the past. See, for example, U.S. Pat. No. 6,875,588, issued Apr. 5, 2005; U.S. Pat. No. 7,176,300, issued Feb. 13, 2007; U.S. Pat. No. 7,199,279, issued Apr. 3, 2007; and US patent publication No. 2006/0130170, published Jun. 15, 2006 (the disclosures of each of these three issued patents and one published patent application are incorporated in their entirety herein by reference) which discloses the production of exogenous protein in the avian oviduct facilitated by various avian promoters which are primarily or exclusively expressed in the oviduct. Though expression levels in avians using the promoters and fragments of the promoters disclosed in these issued patents and published application have been at useful levels, the yields have typically been well below 0.1 mg/ml of egg white.

[0009] What is needed is a system that will provide for high level expression of an exogenous coding sequence in the cells of a transgenic avian, in particular, in the oviduct cells (e.g., tubular gland cells) of a transgenic avian.

SUMMARY OF THE INVENTION

[0010] The present invention meets this need and more. After years of exogenous protein production in transgenic avian oviduct tissue with modest yield the inventors of the present invention have discovered that such production levels can be boosted by about 10 fold to about 100 fold and more by employing new compositions and methods as disclosed herein.

[0011] In one aspect, the invention is directed to transgenic avians (e.g., chicken, turkey, quail) containing in their genome an exogenous nucleotide sequence which includes a promoter component and a SIN vector. Typically, the promoter component is linked to a coding sequence exogenous to the avian, i.e., the coding sequence is not normally or naturally present in the avian. Typically, the exogenous nucleotide sequence is integrated into the genome of the avian. In one particularly useful embodiment, the promoter component functions or expresses primarily in the oviduct (e.g., tubular gland cells) of an avian. For example, the promoter component may be an oviduct specific promoter. For example, the promoter component may be one of an avian ovomucoid promoter component, an avian ovalbumin promoter component, an avian lysozyme promoter component and an avian ovoinhibitor promoter component (i.e., conalbumin promoter component).

[0012] SIN vectors have been shown by the inventors to be particularly useful for increasing the quantity of exogenous protein produced in the avian oviduct. This effect can be further enhanced when the SIN vector is also an SC negative vector (i.e., a vector not containing a selectable marker cassette with a functional promoter).

[0013] The invention also includes methods of making the transgenic avians of the invention and methods of producing an exogenous protein using transgenic avians of the invention. In one embodiment, the transgenic avian has a nucleotide sequence in its genome comprising a vector which is at least one of a SIN vector and an SC negative vector. Typically, the nucleotide sequence includes a promoter component linked to an exogenous coding sequence.

[0014] In one useful embodiment, the exogenous coding sequence is expressed in avian oviduct cells and is secreted from the oviduct cells. For example, the exogenous coding sequence may be expressed in tubular gland cells. In one embodiment, the exogenous protein is deposited in a hard shell egg laid by the transgenic avian. In one embodiment, the exogenous protein is a human protein. In one embodiment, the exogenous protein is a therapeutic protein, e.g., a cytokine.

[0015] In one embodiment, the transgenic avian contains an exogenous nucleotide sequence in its genome which has a SC negative vector and a promoter component linked to an exogenous coding sequence encoding an exogenous protein. In one embodiment, the SC negative vector is also a SIN vector.

[0016] In one aspect, avian leukosis virus vector (ALV), a murine leukemia virus (MLV) retroviral vector, moloney murine leukemia virus (MMLV) and a lentiviral vector can be used in accordance with the invention.

[0017] The invention includes chimeric transgenic avians and fully transgenic germline avians which can be obtained from germline chimeras as is understood by a practitioner of skill in the art of poultry breeding.

[0018] The invention also includes gene expression controlling regions or promoters having a nucleotide, sequence (i.e., DNA sequence) similar or identical to the following sequences numbered 1 to 8. In a particularly useful embodiment of the invention, the fragments are listed top to bottom in the 5' to 3' linear order in which they are present on a single DNA molecule. For example, the 3' end of the 3.5 kb OV fragment of sequence 1 would be covalently linked to the 5' end of the 5' UTR-5' portion and the 3' end of the 5' UTR-5' portion would be covalently linked to the 5' end of 5' UTR-3' portion. However, the invention is not limited to any particular order of the fragments and intervening nucleotide sequences may be present between the fragments.

[0019] 1. 3.5 kb OV fragment (includes DHS I, II & III)

[0020] 5' UTR-5' portion (from Exon L)

[0021] 5' UTR-3' portion (from Exon 1);

[0022] 2. 3.5 kb OV fragment (includes DHS I, II & III)

[0023] 5' UTR-5' portion (from Exon L)

[0024] Intron A

[0025] 5' UTR-3' portion (from Exon 1)

[0026] 3' UTR;

[0027] 3. 3.5 kb OV fragment (includes DHS I, II & III)

[0028] 5' UTR-5' portion (from Exon L)

[0029] Intron A

[0030] 5' UTR-3' portion (from Exon 1);

[0031] 4. 3.5 kb OV fragment (includes DHS I, II & III)

[0032] 5' UTR-5' portion (from Exon L)

[0033] 5' UTR-3' portion (from Exon 1)

[0034] 3' UTR;

[0035] 5. 3.5 kb OV fragment (includes DHS I, II & III)

[0036] 5' UTR-5' portion (from Exon L)

[0037] Intron A

[0038] 5' UTR-3' portion (from Exon 1)

[0039] 3' UTR/DHS A (bp 13576 to 15163 of SEQ ID NO: 22)

[0040] 6. 3.5 kb OV fragment (includes DHS I, II & III)

[0041] 5' UTR-5' portion (from Exon L)

[0042] 5' UTR-3' portion (from Exon 1)

[0043] 3' UTR/DHS A (bp 13576 to 15163 of SEQ ID NO: 22)

[0044] 7. 3.5 kb OV fragment (includes DHS I, II & III)

[0045] 5' UTR-5' portion (from Exon L)

[0046] Intron A

[0047] 5' UTR-3' portion (from Exon 1)

[0048] partial 3' UTR

[0049] RRE (Rev response element) FIG. 9a

[0050] 8. ALV CTE (FIG. 9b) inserted 5' of 3.5 kb OV fragment

[0051] 3.5 kb OV fragment (includes DHS I, II & III)

[0052] 5' UTR-5' portion (from Exon L)

[0053] Intron A

[0054] 5' UTR-3' portion (from Exon 1)

[0055] partial 3' UTR;

[0056] Coordinates of some of the elements for specific ovalbumin constructs disclosed herein (e.g., constructs 1 to 8 described above) are shown in the 16051 bp ovalbumin DNA segment of SEQ ID NO: 22 as follows:

[0057] 3.5 kb OV fragment (includes DHS I, II & III): Start: 3199 End: 6659 of FIG. 8 (SEQ ID NO: 22);

[0058] 1.4 kb OV fragment (includes DHS I & II): Start: 5209 End: 6659 of FIG. 8 (SEQ ID NO: 22);

[0059] 3.8 kb OV fragment: Start: 2863 End: 6659 of FIG. 8 (SEQ ID NO: 22);

[0060] 5.2 kb OV fragment: Start: 1463 End: 6659 of FIG. 8 (SEQ ID NO: 22);

[0061] 5' UTR-5' portion (from Exon L): Start: 6659 End: 6705 of FIG. 8 (SEQ ID NO: 22);

[0062] 5' UTR-3' portion (from Exon 1): Start: 8295 End: 8311 of FIG. 8 (SEQ ID NO: 22);

[0063] 3' UTR: Start: 13576 End: 14209 of FIG. 8 (SEQ ID NO: 22);

[0064] partial 3' UTR: Start 13576 End: 13996 of FIG. 8 (SEQ ID NO: 22);

[0065] Intron A: Start: 6706 End: 8294 of FIG. 8 (SEQ ID NO: 22);

[0066] Intron E: Start: 10010 End: 10968 of FIG. 8 (SEQ ID NO: 22);

[0067] Exon L: Start: 6659 End: 6705 of FIG. 8 (SEQ ID NO: 22);

[0068] Exon 1: Start: 8295 End: 8478 of FIG. 8 (SEQ ID NO: 22);

[0069] Exon 2: Start: 8731 End: 8781 of FIG. 8 (SEQ ID NO: 22);

[0070] Exon 3: Start: 9363 End: 9491 of FIG. 8 (SEQ ID NO: 22);

[0071] Exon 4: Start: 9892 End: 10009 of FIG. 8 (SEQ ID NO: 22);

[0072] Exon 5: Start: 10968 End: 11110 of FIG. 8 (SEQ ID NO: 22);

[0073] Exon 6: Start: 11442 End: 11597 of FIG. 8 (SEQ ID NO: 22);

[0074] Exon 7: Start: 13180 End: 13575 of FIG. 8 (SEQ ID NO: 22);

[0075] +1 SITE: Start: 6659 End: 6659 of FIG. 8 (SEQ ID NO: 22);

[0076] ATG: Start: 8312 End: 8312 of FIG. 8 (SEQ ID NO: 22);

[0077] Poly A: Start: 14204 End: 14209 of FIG. 8 (SEQ ID NO: 22);

[0078] TATA: Start: 6627 End: 6632 of FIG. 8 (SEQ ID NO: 22);

[0079] DHS A: Start: 13858 End: 15163 of FIG. 8 (SEQ ID NO: 22);

[0080] DHS IV: Start: 459 End: 859 of FIG. 8 (SEQ ID NO: 22);

[0081] DHS III: Start: 3253 End: 3559 of FIG. 8 (SEQ ID NO: 22);

[0082] DHS II: Start: 5629 End: 6009 of FIG. 8 (SEQ ID NO: 22); and

[0083] DHS I: Start: 6359 End: 6659 of FIG. 8 (SEQ ID NO: 22).

[0084] Promoter constructs are also contemplated that have a nucleotide sequence 80% identical and 85% identical and 90% identical and 91% identical and 92% identical and 93% identical and 94% identical and 95% identical and 96% identical and 97% identical and 98% identical and 99% identical to each of the promoter constructs disclosed herein, such as those described above (i.e., 1 to 8 above).

[0085] The invention also contemplates promoter constructs which correspond to promoter constructs 1 through 8 above in which the 3.5 kb OV fragment is replaced with the 3.8 kb OV fragment. The invention also contemplates promoter constructs which correspond to promoter constructs 1 through 8 in which the 3.5 kb OV fragment is replaced with the 5.2 kb OV fragment.

[0086] Promoter constructs are also contemplated for each of the above specified recombinant promoters (i.e., 1 to 8) in which DHS III is omitted from the construct.

[0087] Promoter constructs are contemplated corresponding to each of constructs 2, 3, 5, 7 and 8 above in which Intron A is replaced with Intron E which may lead to increased levels of exogenous protein production. Intron A and E have DNA sequences that induce alignment of histones in surrounding DNA regions. Such alignment can provide for transcriptional regulation of the OV gene. Without wishing to be bound to any particular theory or mechanism of operation, substitution of Intron E with Intron A may provide a preferential spacing of histones that result from use of Intron E (i.e., the periodicity for Intron A is 202 bp +/-5 bp, for Intron E is 196 bp +/-5 bp). For example, it is believed that the packaging of DNA by histones leads to topological alteration of DNA the manipulation of which can lead to preferential alignment of binding sites for proteins responsible for the transcription regulation (e.g., transcription factors) leading to an enhanced level of transcription.

[0088] Also included in the invention are vector constructs, and other constructs and nucleotide sequences disclosed herein, having a nucleotide sequence 80% identical and 85% identical and 90% identical and 91% identical and 92% identical and 93% identical and 94% identical and 95% identical and 96% identical and 97% identical and 98% identical and 99% identical to each vector construct and other constructs and nucleotide sequences disclosed herein.

[0089] Any useful combination of features described herein is included within the scope of the present invention

provided that the features included in any such combination are not mutually inconsistent as will be apparent from the context, this specification, and the knowledge of one of ordinary skill in the art

[0090] Additional objects and aspects of the present invention will become more apparent upon review of the detailed description set forth below when taken in conjunction with the accompanying figures, which are briefly described as follows.

BRIEF DESCRIPTION OF THE FIGURES

[0091] FIG. 1 shows a circular map of the pALV-SIN-4.2-Lys-IFNa-2B vector. The sequence of pALV-SIN-4.2-Lys-IFNa-2B is shown in SEQ ID NO: 1.

[0092] FIG. 2 is a bar graph illustrating expression levels of IFNa in the egg white of a transgenic quail. G0 quail was produced by injection of pALV-SIN-4.0-Lys-IFNa-2B retroviral vector transduction particles into Japanese quail embryos.

[0093] FIG. 3 shows a circular map of the pSIN-OV-3.5-I-CTLA4-inv vector. The nucleotide sequence of pSIN-OV-3.5-I-CTLA4-inv is shown in SEQ ID NO: 19.

[0094] FIG. 4 shows a circular map of the pSIN-3.9-OM-CTLA4-Fc vector. The nucleotide sequence of pSIN-3.9-OM-CTLA4-Fc is shown in SEQ ID NO: 20.

[0095] FIG. 5 shows a circular map of the pBS-OM-4.4 vector. The nucleotide sequence of pBS-OM-4.4 is shown in SEQ ID NO: 23.

[0096] FIG. 6 shows a circular map of the pAVIJCR-A137.91.1.2 vector. The nucleotide sequence of pAVIJCR-A137.91.1.2 is shown in SEQ ID NO: 24.

[0097] FIG. 7 shows a circular map of the pSIN-1.8-OM-IFNa-2B plasmid vector. The nucleotide sequence of pSIN-1.8-OM-IFNa-2B is shown in SEQ ID NO: 21.

[0098] FIG. 8a-e (SEQ ID NO: 22) shows a segment of a chicken ovalbumin gene.

[0099] FIG. 9a (SEQ ID NO: 25) shows the RRE (rev responsive element) sequence of a lenti virus. FIG. 9b (SEQ ID NO: 26) shows the ALV CTE (constitutive transport element) sequence.

[0100] FIG. 10a shows a diagram of the segment deleted from an exemplary retroviral LTR (ALV) to make a SIN vector. FIG. 10b (SEQ ID NO: 29) shows the sequence of the LTR shown in 10a. The underlined sequence is the deleted sequence.

DETAILED DESCRIPTION

Definitions

[0101] The term "animal" is used herein to include all vertebrate animals, including avians and may include humans. It also includes an individual animal in all stages of development, including embryonic and fetal stages.

[0102] The term "antibody" as used herein refers to polyclonal and monoclonal antibodies and functional fragments thereof. An antibody includes modified or derivatised antibody variants that retain the ability to specifically bind an epitope. Antibodies are capable of selectively binding to a target antigen or epitope. Antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized and other chimeric antibodies, single chain antibodies (scFvs), Fab fragments, F(ab')₂ fragments and disulfide-linked Fvs (sdFv) fragments.

[0103] The term “avian” as used herein refers to any species, subspecies or strain of organism of the taxonomic class avia, such as, but not limited to, such organisms as chicken, turkey, duck, goose, quail, pheasants, parrots, finches, hawks, crows and ratites including ostrich, emu and cassowary. The term includes the various known strains of *Gallus gallus*, or chickens, (for example, White Leghorn, Brown Leghorn, Barred-Rock, Sussex, New Hampshire, Rhode Island, Ausstralorp, Minorca, Amrox, California Gray, Italian Partridge-colored), as well as strains of turkeys, pheasants, quails, duck, ostriches and other poultry commonly bred in commercial quantities.

[0104] The phrase “based on” or “derived from” as in a retroviral vector being based on or derived from a particular retrovirus or based on a nucleotide sequence of a particular retrovirus mean that the genome of the retroviral vector contains a substantial portion of the nucleotide sequence of the genome of the particular retrovirus. The substantial portion may be a particular gene or nucleotide sequence such as the nucleotide sequence encoding the gag, pol and/or env proteins or other structural or functional nucleotide sequence of the virus genome such as sequences encoding the LTRs or may be substantially the complete retrovirus genome, for example, most (e.g., more than 60% or more than 70% or more than 80% or more than 90%) or all of the retrovirus genome, as will be apparent from the context in the specification as the knowledge of one skilled in the art. Examples of retroviral vectors that are based on or derived from a retrovirus are the NL retroviral vectors (e.g., NLB) which are based on the ALV retrovirus as disclosed in Cosset et al, *Journal of Virology* (1991) vol 65, p 3388-3394.

[0105] The term “coding sequence” and “coding region” as used herein refer to nucleotide sequences and nucleic acid sequences, including both RNA and DNA, that encode genetic information for the synthesis of an RNA, a protein, or any portion of an RNA or protein. Nucleotide sequences that are not naturally part of a particular organism’s genome are referred to as “foreign nucleotide sequences,” “heterologous nucleotide sequences” or “exogenous nucleotide sequences”. “Heterologous proteins” are proteins encoded by foreign, heterologous or exogenous nucleotide sequences and therefore are often not naturally expressed in the cell. A nucleotide sequence that has been isolated and then reintroduced into the same type (e.g., same species) of organism is not considered to be a naturally occurring part of a particular organism’s genome and is therefore considered exogenous or heterologous.

[0106] The term “construct” as used herein refers to a linear or circular nucleotide sequence such as DNA that has been assembled from more than one segments of nucleotide sequence which have been isolated from a natural source or have been chemically synthesized, or combinations thereof.

[0107] The term “complementary” as used herein refers to two nucleic acid molecules that can form specific interactions with one another. In the specific interactions, an adenine base within one strand of a nucleic acid can form two hydrogen bonds with thymine within a second nucleic acid strand when the two nucleic acid strands are in opposing polarities. Also in the specific interactions, a guanine base within one strand of a nucleic acid can form three hydrogen bonds with cytosine within a second nucleic acid strand when the two nucleic acid strands are in opposing polarities. Complementary nucleic acids as referred to herein, may further comprise modified bases wherein a

modified adenine may form hydrogen bonds with a thymine or modified thymine, and a modified cytosine may form hydrogen bonds with a guanine or a modified guanine.

[0108] The term “cytokine” as used herein refers to any secreted amino acid sequence that affects the functions of cells and is a molecule that modulates interactions between cells in the immune, inflammatory or hematopoietic responses. A cytokine includes, but is not limited to, monokines and lymphokines regardless of which cells produce them. For instance, a monokine is generally referred to as being produced and secreted by a mononuclear cell, such as a macrophage and/or monocyte. Many other cells however also produce monokines, such as natural killer cells, fibroblasts, basophils, neutrophils, endothelial cells, brain astrocytes, bone marrow stromal cells, epidermal keratinocytes and B-lymphocytes. Lymphokines are generally referred to as being produced by lymphocyte cells. Examples of cytokines include, but are not limited to, Interleukin-1 (IL-1), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Tumor Necrosis Factor-alpha (TNF-alpha) and Tumor Necrosis Factor beta (TNF-beta).

[0109] The term “expressed” or “expression” as used herein refers to the transcription from a gene to give an RNA nucleic acid molecule at least complementary in part to a region of one of the two nucleic acid strands of the gene. The term “expressed” or “expression” as used herein can also refer to the translation of RNA to produce a protein or peptide.

[0110] The term “expression vector” as used herein refers to a nucleic acid vector that comprises a gene expression controlling region, such as a promoter or promoter component, operably linked to a nucleotide sequence coding at least one polypeptide.

[0111] The term “fragment” as used herein can refer to, for example, an at least about 10, 20, 50, 75, 100, 150, 200, 250, 300, 500, 1000, 2000, 5000, 6,000, 8,000, 10,000, 20,000, 30,000, 40,000, 50,000 or 60,000 nucleotide long portion of a nucleic acid that has been constructed artificially (e.g., by chemical synthesis) or by cleaving a natural product into multiple pieces, using restriction endonucleases or mechanical shearing, or enzymatically, for example, by PCR or any other polymerizing technique known in the art, or expressed in a host cell by recombinant nucleic acid technology known to one of skill in the art. The term “fragment” as used herein may also refer to, for example, an at least about 5, 10, 20, 30, 40, 50, 75, 100, 150, 200, 250, 300, 400, 500, 1000, 2000, 5000, 6,000, 8,000 or 10,000 amino acid portion of an amino acid sequence, which portion is cleaved from a naturally occurring amino acid sequence by proteolytic cleavage by at least one protease, or is a portion of the naturally occurring amino acid sequence synthesized by chemical methods or using recombinant DNA technology (e.g., expressed from a portion of the nucleotide sequence encoding the naturally occurring amino acid sequence) known to one of skill in the art. “Fragment” may also refer to a portion, for example, of about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80% about 90% about 95% or about 99% of a particular nucleotide sequence or amino acid sequence.

[0112] “Functional portion” or “functional fragment” are used interchangeably and as used herein means a portion or fragment of a whole capable of performing, in whole or in part, a function of the whole. For example, a biologically functional portion of a molecule means a portion of the

molecule that performs a biological function of the whole or intact molecule. For example, a functional portion of a gene expression controlling region is a fragment or portion of the specified gene expression controlling region that, in whole or in part, regulates or controls gene expression (e.g., facilitates either in whole or in part) in a biological system (e.g., a promoter). Functional portions may be of any useful size. For example, a functional fragment may range in size from about 20 bases in length to a length equal to the entire length of the specified sequence minus one nucleotide. In another example, a functional fragment may range in size from about 50 bases in length to a length equal to the entire length of the specified sequence minus one nucleotide. In another example, a functional fragment may range in size from about 50 bases in length to about 20 kb in length. In another example, a functional fragment may range in size from about 500 bases in length to about 20 kb in length. In another example, a functional fragment may range in size from about 1 kb in length to about 20 kb in length. In another example, a functional fragment may range in size from about 0.1 kb in length to about 10 kb in length. In another example, a functional fragment may range in size from about 20 bases kb in length to about 10 kb in length.

[0113] The term “gene expression controlling region” as used herein refers to nucleotide sequences that are associated with a coding sequence and which regulate, in whole or in part, expression of the coding sequence, for example, regulate, in whole or in part, the transcription of the coding sequence. Gene expression controlling regions may be isolated from a naturally occurring source or may be chemically synthesized and can be incorporated into a nucleic acid vector to enable regulated transcription in appropriate cells. The “gene expression controlling regions” may precede, but is not limited to preceding, the region of a nucleic acid sequence that is in the region 5' of the end of a coding sequence that may be transcribed into mRNA.

[0114] The terms “heterologous”, “exogenous” and “foreign” are used interchangeably herein and in general refer to a biomolecule such as a nucleic acid or a protein that is not normally found in a certain organism or in a certain cell, tissue or other component contained in or produced by an organism. For example, a protein that is heterologous or exogenous to an egg is a protein that is not normally found in the egg. As used herein, the terms “heterologous”, “exogenous” and “foreign” with reference to nucleic acids, such as DNA and RNA, are used interchangeably and refer to nucleic acid that does not occur naturally as part of a chromosome, a genome or cell in which it is present or which is found in a location(s) and/or in amounts that differ from the location(s) and/or amounts in which it occurs in nature. It can be nucleic acid that is not endogenous to the genome, chromosome or cell and has been exogenously introduced into the genome, chromosome or cell. Examples of heterologous DNA include, but are not limited to, a DNA comprising a gene expression control region and DNA that encodes a product or products, for example, RNA or protein product. Examples of heterologous DNA include, but are not limited to, gene expression controlling regions or promoters disclosed herein once isolated from the avian and as used thereafter, e.g., after re-introduction into an avian genome.

[0115] The term “isolated nucleic acid” as used herein covers, for example, (a) a DNA which has the sequence of part of a naturally occurring genomic molecule but is not flanked by at least one of the sequences that flank that part

of the molecule in the genome of the species in which it naturally occurs; (b) a nucleic acid which has been incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting vector or genomic DNA is not identical to naturally occurring DNA from which the nucleic acid was obtained; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), ligase chain reaction (LCR) or chemical synthesis, or a restriction fragment; (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein, and (e) a recombinant nucleotide sequence that is part of a hybrid sequence that is not naturally occurring. Isolated nucleic acid molecules of the present invention can include, for example, natural allelic variants as well as nucleic acid molecules modified by nucleotide deletions, insertions, inversions, or substitutions.

[0116] The term “nucleic acid” as used herein refers to any linear or sequential array of nucleotides and nucleosides, for example cDNA, genomic DNA, mRNA, tRNA, oligonucleotides, oligonucleosides and derivatives thereof. For ease of discussion, non-naturally occurring nucleic acids may be referred to herein as constructs. Nucleic acids can include bacterial plasmid vectors including expression, cloning, cosmid and transformation vectors such as, animal viral vectors such as, but not limited to, modified adenovirus, influenza virus, polio virus, pox virus, retroviruses such as avian leukosis virus (ALV) retroviral vector, a murine leukemia virus (MLV) retroviral vector, and a lentivirus vector, and the like and fragments thereof. In addition, the nucleic acid can be an LTR of an avian leukosis virus (ALV) retroviral vector, a murine leukemia virus (MLV) retroviral vector, or a lentivirus vector and fragments thereof. Nucleic acids can also include NL vectors such as NLB, NLD and NLA and fragments thereof and synthetic oligonucleotides such as chemically synthesized DNA or RNA. Nucleic acids can include modified or derivatised nucleotides and nucleosides such as, but not limited to, halogenated nucleotides such as, but not only, 5-bromouracil, and derivatised nucleotides such as biotin-labeled nucleotides.

[0117] The term “vector” and “nucleic acid vector” as used herein refers to a natural or synthetic single or double stranded plasmid or viral nucleic acid molecule that can be transfected or transformed into cells and replicate independently of, or within, the host cell genome. A circular double stranded vector can be linearized by treatment with an appropriate restriction enzyme based on the nucleotide sequence of the vector. A nucleic acid can be inserted into a vector by cutting the vector with restriction enzymes and ligating the desired pieces together.

[0118] The term “operably linked” refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Gene expression controlling regions or promoters (e.g., promoter components) operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The controlling sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered “operably linked” to the coding sequence.

[0119] The term “oviduct specific promoter” as used herein refers to promoters and promoter components which are functional, i.e., provide for transcription of a coding sequence, to a large extent, for example, primarily (i.e., more than 50% of the transcription product produced in the animal by a particular promoter type being produced in oviduct cells) or exclusively in oviduct cells of a bird. Examples of oviduct specific promoters include, ovalbumin promoter, ovomucoid promoter, ovoinhibitor promoter, lysozyme promoter and ovotransferrin promoter and functional portions of these promoters, e.g., promoter components.

[0120] The terms “percent sequence identity” “percent identity” as used in, for example, “% identical” and “percent sequence homology” “percent homology”, as used in, for example, “% homology” and “percent sequence similarity” each refer to the degree of sequence matching between two nucleic acid sequences or two amino acid sequences as determined using the algorithm of Karlin & Atschul (1990) Proc. Natl. Acad. Sci. 87: 2264-2268, modified as in Karlin & Atschul (1993) Proc. Natl. Acad. Sci. 90: 5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Atschul et al. (1990) T. Mol. Biol. Q15: 403-410. BLAST nucleotide searches are performed with the NBLAST program, score=100, wordlength=12, to obtain nucleotide sequences homologous to a nucleic acid molecule of the invention. BLAST protein searches are performed with the XBLAST program, score=50, wordlength=3, to obtain amino acid sequences homologous to a reference amino acid sequence. To obtain gapped alignments for comparison purposes, Gapped BLAST is utilized as described in Atschul et al. (1997) Nucl. Acids Res. 25: 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g. XBLAST and NBLAST) are used. Other algorithms, programs and default settings may also be suitable such as, but not only, the GCG-Sequence Analysis Package of the U.K. Human Genome Mapping Project Resource Centre that includes programs for nucleotide or amino acid sequence comparisons.

[0121] The terms “polynucleotide,” “oligonucleotide”, “nucleotide sequence” and “nucleic acid sequence” can be used interchangeably herein and include, but are not limited to, coding sequences, i.e., polynucleotide(s) or nucleic acid sequence(s) which are transcribed and translated into polypeptide in vitro or in vivo when placed under the control of appropriate regulatory or control sequences; controlling sequences, e.g., translational start and stop codons, promoter sequences, ribosome binding sites, polyadenylation signals, transcription factor binding sites, transcription termination sequences, upstream and downstream regulatory domains, enhancers, silencers, DNA sequences to which a transcription factor(s) binds and alters the activity of a gene’s promoter either positively (induction) or negatively (repression) and the like. No limitation as to length or to synthetic origin are suggested by the terms described herein.

[0122] As used herein the terms “polypeptide” and “protein” refer to a polymer of amino acids of three or more amino acids in a serial array, linked through peptide bonds. The term “polypeptide” includes proteins, protein fragments, protein analogues, oligopeptides and the like. The term “polypeptides” includes polypeptides as defined above that are encoded by nucleic acids, produced through recombinant technology (e.g., isolated from a transgenic bird), or synthesized. The term “polypeptides” further contemplates

polypeptides as defined above that include chemically modified amino acids or amino acids covalently or noncovalently linked to labeling ligands.

[0123] The term “promoter” as used herein refers to a DNA sequence useful to initiate transcription initiation by an RNA polymerase in an avian cell. A “promoter component” is a DNA sequence that can, by itself or, in combination with other DNA sequences effect or facilitate transcription. Specific promoter components such as ovalbumin promoter components, ovomucoid promoter components and lysozyme promoter components and other promoters and promoter components disclosed and claimed herein do not describe a specific promoter sequence. Rather, they encompass any sequence or sequence fragment of the respective promoter that is useful to effect or facilitate transcription of a coding sequence. For example, an ovomucoid promoter component includes, without limitation, the about 1.8 kb, the about 3.9 kb and the about 10 kb ovomucoid promoters disclosed in U.S. Ser. No. 11/649,543, published May 17 2007, which is incorporated in its entirety herein by reference. “Promoter components” can also encompass rearranged gene expression controlling regions which function to initiate RNA transcription and hybrid DNA molecules composed of naturally occurring DNA sequences and/or synthetic DNA sequences which function to initiate RNA transcription.

[0124] The terms “recombinant nucleic acid” and “recombinant DNA” as used herein refer to combinations of at least two nucleic acid sequences that are not naturally found in a eukaryotic or prokaryotic cell. The nucleic acid sequences may include, but are not limited to, nucleic acid vectors, gene expression regulatory elements, origins of replication, suitable gene sequences that when expressed confer antibiotic resistance, protein-encoding sequences and the like. The term “recombinant polypeptide” is meant to include a polypeptide produced by recombinant DNA techniques such that it is distinct from a naturally occurring polypeptide either in its location, purity or structure. Generally, such a recombinant polypeptide will be present in a cell in an amount different from that normally observed in nature.

[0125] As used herein, the term “regulatory sequences” includes promoters, enhancers, and other elements that may control gene expression.

[0126] An “SC negative vector” is a vector that does not contain a selectable or screenable cassette marker having a functional promoter. The promoter may be deleted in whole or in part or may be inactivated by a nucleotide sequence insertion. Screenable cassettes include, without limitation, DNA sequences for antibiotic resistance markers such as neomycin resistance and DNA sequences for other selectable markers such as GFP or lacZ.

[0127] A “SIN vector” is a self-inactivating vector. In particular, a SIN vector is a retroviral vector having an altered genome such that upon integration into genomic DNA of the target cell (e.g., avian embryo cells) the 5' LTR of the integrated retroviral vector will not function as a promoter. For example, a portion or all of the nucleotide sequence of the retroviral vector that results in the U3 region of the 5' LTR of the retroviral vector once integrated may be deleted or altered in order to reduce or eliminate promoter activity of the 5' LTR. In certain examples, deletion of the CAAT box and/or the TAATA box from U3 of the 5' LTR can result in a SIN vector, as is understood in the art.

[0128] A “SIN/SC negative vector” is a vector, i.e., a retroviral vector, that is both a SIN vector and a SC negative vector.

[0129] The term “sense strand” as used herein refers to a single stranded DNA, molecule from a genomic DNA that may be transcribed into RNA and translated into the natural polypeptide product of the gene. The term “antisense strand” as used herein refers to the single strand DNA molecule of a genomic DNA that is complementary with the sense strand of the gene.

[0130] A “therapeutic protein” or “pharmaceutical protein” is a substance that, in whole or in part, makes up a drug. In particular, “therapeutic proteins” and “pharmaceutical proteins” include an amino acid sequence which in whole or in part makes up a drug.

[0131] The terms “transcription regulatory sequences” and “gene expression control regions” and “promoter components” as used herein refer to nucleotide sequences that are associated with a nucleic acid sequence and which regulate the transcriptional expression of a coding sequence. Exemplary transcription regulatory sequences include enhancer elements, hormone response elements, steroid response elements, negative regulatory elements, and the like. The “transcription regulatory sequences” may be isolated and incorporated into a vector nucleic acid to enable regulated transcription in appropriate cells of portions of the vector DNA. The “transcription regulatory sequence” may precede, but is not limited to, the region of a nucleic acid sequence that is in the region 5' of the end of a protein coding sequence that may be transcribed into mRNA. Transcriptional regulatory sequences may also be located within a protein coding region, in regions of a gene that are identified as “intron” regions, or may be in regions of nucleic acid sequence that are in the region of nucleic acid.

[0132] The terms “transformation” and “transfection” as used herein refer to the process of inserting a nucleic acid into a host. Many techniques are well known to those skilled in the art to facilitate transformation or transfection of a nucleic acid into a prokaryotic or eukaryotic organism. These methods involve a variety of techniques, such as treating the cells with high concentrations of salt such as, but not only a calcium or magnesium salt, an electric field, detergent, or liposome mediated transfection, to render the host cell competent for the uptake of the nucleic acid molecules.

[0133] As used herein, a “transgenic animal” is any non-human animal, such as an avian species, including the chicken, in which one or more of the cells of the avian may contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques known in the art (see, for example, US patent publication No. 2007/0243165, published Oct. 18, 2007, the disclosure of which is incorporated in its entirety herein by reference) including those disclosed herein. The nucleic acid is introduced into an animal, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or in vitro fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic animal, the transgene can cause cells to express a recombinant form of the target protein or

polypeptide. The terms “chimeric animal” or “mosaic animal” are used herein to refer to animals in which a transgene is found, or in which the recombinant nucleotide sequence is expressed in some but not all cells of the animal. A germ-line chimeric animal contains a transgene in its germ cells and can give rise to a transgenic animal in which most or all cells of the offspring animal will contain the transgene.

[0134] As used herein, the term “transgene” means a nucleic acid sequence (encoding, for example, a human protein) that is partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal’s genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene according to the present invention can include a vector of the invention (e.g., SIN vector) which contains sequences useful for exogenous protein production in an avian (e.g., in an avian oviduct).

[0135] Techniques useful for isolating and characterizing the nucleic acids and proteins of the present invention are well known to those of skill in the art and standard molecular biology and biochemical manuals may be consulted to select suitable protocols for use without undue experimentation. See, for example, Sambrook et al, 1989, “Molecular Cloning: A Laboratory Manual”, 2nd ed., Cold Spring Harbor, the content of which is herein incorporated by reference in its entirety.

Abbreviations:

[0136] Abbreviations used herein may include the following: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to an RNA; nt, nucleotide(s); kb, 1000 base pairs; µg, microgram; ml, milliliter; ng, nanogram.

Description:

[0137] SIN vectors designed and used in accordance with the invention can reduce or eliminate promoter interference of promoters of interest which are employed in transgenic avians. In a particularly useful embodiment, the promoters (i.e., promoter components) of interest preferentially express their gene product in oviduct cells or oviduct tissue, e.g., oviduct specific promoters. Examples of such promoters (e.g., promoter components) include but are not limited to, functional portions of the ovalbumin, lysozyme, conalbumin (i.e., ovotransferrin), ovomucoid, ovomucin, and/or ovo-inhibitor gene expression controlling regions or promoter regions. In one embodiment, the promoter of interest is a combination or a fusion of one or more promoters or a fusion of a fragment of one or more promoters such as ovalbumin, lysozyme, conalbumin (i.e., ovotransferrin), ovomucoid, ovomucin, and/or ovo-inhibitor promoters with another promoter or promoter fragment such as a viral promoter (e.g., an LTR promoter).

[0138] SIN vectors have been shown to be particularly useful with oviduct specific promoters. Without wishing to limit the invention to any particular theory or mechanism of operation it is believed that oviduct specific promoters can be particularly susceptible to influences of a retroviral LTR

promoter. As a result, SIN vectors are particularly useful when employed in combination with avian oviduct specific promoters.

[0139] In one particularly useful embodiment, a SIN vector is produced in which an interfering promoter (e.g., an LTR promoter) that can at least partially inhibit transcription of a coding sequence operably linked to an oviduct specific promoter of the invention is inactivated, for example, by a deletion, insertion or transposition of all or part of the interfering promoter sequence. For example, the vector pALV-SIN-4.2-Lys-IFNa-2B, shown in FIG. 1, the 3' RAV2 LTR has a deletion in the enhancer such that when the retroviral region integrates, the 5' LTR is inactivated, as is understood in the art. For a detailed diagrammatic of an LTR deletion, see FIG. 10.

[0140] In one useful embodiment of the invention, a SIN vector is employed that is also an SC negative vector to produce a SIN/SC negative vector. The combination of SC negative vector and SIN vector can result in a vector with a substantially reduced amount of promoter interference compared to a vector that is only a SIN vector or only a SC negative vector. For example, pALV-SIN-4.2-Lys-IFNa-2B as well as other SIN vectors disclosed in the Examples also lacks an antibiotic resistance marker making it both a SC negative vector and a SIN vector.

[0141] SIN vectors, SC negative vectors and SIN/SC negative vectors are contemplated for use in accordance with the invention in any useful avian such as chicken, quail and turkey to produce chimeras including germ-line chimeras and progeny birds produced using breeding techniques such as those known to practitioners of ordinary skill in the art. In addition, it is contemplated that an SC negative retroviral vector (which is a non-SIN vector) will also enhance or increase the quantity of exogenous protein produced in a transgenic avian relative to a transgenic avian produced with essentially the same retroviral vector that is not a SC negative vector.

[0142] Without wishing to limit the invention to any particular theory or mechanism of operation it is believed that the lack of a selectable marker cassette decreases the presence of promoter elements such as enhancers which would otherwise be in cis and in close proximity to the promoter employed for exogenous protein production in avian oviduct cells (e.g., oviduct specific promoters). This close proximity may allow for interference by the transcription regulating elements of the marker gene with the promoter of interest, i.e., the promoter employed for exogenous protein production. However, the invention contemplates that marker gene coding sequences, for example, and without limitation, neomycin resistance coding sequence and beta lactamase coding sequence, may be operably linked to a promoter (i.e., second promoter) which does not interfere with the promoter employed for exogenous protein production in avian oviduct cells (i.e., first promoter). For example, it is contemplated that if the marker promoter and the promoter of interest are the same or similar promoters, interference by the selectable cassette will be minimized or eliminated. For example, a second ovalbumin promoter operably linked to a marker gene coding sequence may not interfere with a first ovalbumin promoter employed for exogenous protein production in avian oviduct cells.

[0143] The invention contemplates the employment of any useful oviduct specific promoter, and oviduct specific promoter fragments, in vectors of the invention for exogenous

protein expression in avians. For example, promoters and useful (e.g., functional) fragments of promoters (e.g., promoter components) disclosed in US patent publication No. 2005/0176047, filed Jan. 31, 2005, the disclosure of which is incorporated in its entirety herein by reference, and US patent publication No. 2007/0124829, filed Jan. 26, 2007, the disclosure of which is incorporated in its entirety herein by reference, and US patent publication No. 2006/0130170, filed Dec. 11, 2003, the disclosure of which is incorporated in its entirety herein by reference, are contemplated for use in conjunction with SIN vectors and SC negative vectors and SIN/SC negative vectors in accordance with the invention.

[0144] The invention also contemplates other promoters and transcriptionally functional portions thereof (e.g., promoter components) for use as promoters of interest in accordance with the invention such as a cytomegalovirus (CMV) promoter, a rous-sarcoma virus (RSV) promoter, a β -actin promoter (e.g., a chicken β -actin promoter) a murine leukemia virus (MLV) promoter, a mouse mammary tumor virus (MMTV) promoter.

[0145] The invention also includes various ovalbumin promoter components which are contemplated for use in producing exogenous proteins in transgenic avians. Each of the promoters disclosed herein are contemplated for use in vectors in accordance with the invention.

[0146] Examples of vectors of the invention which contain recombinant ovalbumin DNA are shown below. The fragments are listed top to bottom in the 5' to 3' linear order in which they are present on a single DNA molecule. For example, the 3' end of the 3.5 kb OV fragment of sequence 1 would be covalently linked to the 5' end of the 5' UTR-5' portion and the 3' end of the 5' UTR-5' portion would be covalently linked to the 5' end of 5' UTR-3' portion.

- [0147]** 1. pSIN-OV-3.5-CSI
- [0148]** 3.5 kb OV fragment (includes DHS I, II & III)
- [0149]** 5' UTR-5' portion (from Exon L)
- [0150]** 5' UTR-3' portion (from Exon L)
- [0151]** 2. pSIN-OV-3.5-Int-CSI-inv
- [0152]** 3.5 kb OV fragment (includes DHS I, II & III)
- [0153]** 5' UTR-5' portion (from Exon L)
- [0154]** Intron A
- [0155]** 5' UTR-3' portion (from Exon L)
- [0156]** 3' UTR
- [0157]** 3. pSIN-OV-3.5-Int-CSI
- [0158]** 3.5 kb OV fragment (includes DHS I, II & III)
- [0159]** 5' UTR-5' portion (from Exon L)
- [0160]** Intron A
- [0161]** 5' UTR-3' portion (from Exon L)
- [0162]** 4. pSIN-OV-3.5-CSI -UTR-inv
- [0163]** 3.5 kb OV fragment (includes DHS I, II & III)
- [0164]** 5' UTR-5' portion (from Exon L)
- [0165]** 5' UTR-3' portion (from Exon L)
- [0166]** 3' UTR
- [0167]** 5. pSIN-OV-3.5-Int-CSI-LUTR-inv
- [0168]** 3.5 kb OV fragment (includes DHS I, II & III)
- [0169]** 5' UTR-5' portion (from Exon L)
- [0170]** Intron A
- [0171]** 5' UTR-3' portion (from Exon L)
- [0172]** 3' UTR/DHS A (bp 13576 to 15163 of FIG. 8);
- [0173]** 6. pSIN-OV-3.5-CSI-LUTR-inv
- [0174]** 3.5 kb OV fragment (includes DHS I, II & III)
- [0175]** 5' UTR-5' portion (from Exon L)
- [0176]** 5' UTR-3' portion (from Exon L)
- [0177]** 3' UTR/DHS A (bp 13576 to 15163 of FIG. 8);

[0178] 7. pSIN-OV-3.5-Int-CSI -RRE
[0179] 3.5 kb OV fragment (includes DHS I, II & III)
[0180] 5' UTR-5' portion (from Exon L)
[0181] Intron A
[0182] 5' UTR-3' portion (from Exon 1)
[0183] partial 3' UTR
[0184] RRE (Rev response element) FIG. 9a
[0185] Construct 7 includes RRE to allow transport of the unspliced RNA genome to the cytoplasm and thus may enhance packaging of intact retroviral RNA. RRE is only active in presence of the Rev protein. Rev activity is provided in the form of DNA encoding the Rev, RNA encoding the Rev, and/or the Rev protein, which is well known in the art and commercially available (e.g., Invitrogen, Inc.), during the transient transfection of retroviral components. Thus the intron will be present in the transgene contained in the genome of the transgenic bird produced by the virus particles (the rev protein is not present in the cells of the transgenic bird). As a result the RNA should be spliced in the oviduct cells of a laying hen resulting in an enhanced level of protein expression compared to a same transgenic bird having the same transgene without the intron.
[0186] 8. pSIN-CTE-OV-3.5-Int-CSI
[0187] ALV CTE (FIG. 9b) inserted 5' of 3.5 kb OV fragment
[0188] 3.5 kb OV fragment (includes DHS I, II & III)
[0189] 5' UTR-5' portion (from Exon L)
[0190] Intron A
[0191] 5' UTR-3' portion (from Exon 1)
[0192] partial 3' UTR
[0193] Coordinates for some of the elements for the above eight vectors are described elsewhere in the application. For example, coordinates of sequences from the ovalbumin nucleotide sequence are described in the Summary section above. CSI means a coding sequence of interest, i.e., nucleotide sequence encoding the protein desired to be expressed in a transgenic avian oviduct.
[0194] SIN vectors, SIN/SC negative vectors and SC negative vectors for use in accordance with the invention include vectors such as Avian Leukemia/Leukosis Viruses (ALV), for example, and without limitation, RAV-0, RAV-1, RAV-2; Avian Sarcoma Viruses (ASV); Avian Sarcoma/Acute Leukemia Viruses (ASLV) including, without limitation, Rous Sarcoma Virus (RSV); Fujinami Sarcoma Viruses (FSV); Avian Myeloblastosis Viruses (AMV); Avian Erythroblastosis Viruses (AEV); Avian Myelocytomatosis Viruses (MCV), for example, and without limitation, MC29; Reticuloendotheliosis Viruses (REV), for example, and without limitation, Spleen Necrosis Virus (SNV). The invention also contemplates other useful retroviral vector, including, without limitation, retroviral vectors based upon Murine Leukemia Viruses (MLV); Molony Murine Sarcoma Viruses (MMSV); Moloney Murine Leukemia Viruses (MMLV); and lentiviruses (e.g., human immunodeficiency virus (HIV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV) and simian immunodeficiency virus (SIV) which are altered to be SIN vectors, SIN/SC negative vectors or SC negative vectors as is understood by a practitioner of ordinary skill in the art.
[0195] In one very specific embodiment, a portion of the 5' LTR of a modified ALV vector disclosed in Cosset et al, J of Virology (1991) vol 65, no. 6, p 3388-3394, the disclosure of which is incorporated in its entirety herein by reference, is deleted to produce a SIN vector. In particular,

nucleotides 1 to 173 were deleted from the ALV based vector LTR sequence shown in SEQ ID NO: 29. Specific deletions from 5' LTR sequences useful to produce SIN vectors from other vectors which can be used in avian transgenesis can be determined by a practitioner of ordinary skill in the art.

[0196] In one particularly useful embodiment, the invention is drawn to the production of therapeutic proteins which may be produced in the oviduct of a transgenic avian, such as a chicken, in accordance with the invention. Exemplary proteins for production in accordance with the invention include, without limitation, erythropoietin, GM-CSF, interferon β , fusion protein, CTLA4-Fc fusion protein, growth hormones, cytokines, structural proteins, interferon, lysozyme, β -casein, albumin, α -1 antitrypsin, antithrombin III, collagen, factors VIII, IX, X (and the like), fibrinogen, lactoferrin, protein C, tissue-type plasminogen activator (tPA), somatotropin, and chymotrypsin, immunoglobulins, antibodies, immunotoxins, factor VIII, b-domain deleted factor VIII, factor VIIa, factor IX, anticoagulants; hirudin, alteplase, tpa, reteplase, tpa, tpa-3 of 5 domains deleted, insulin, insulin lispro, insulin aspart, insulin glargine, long-acting insulin analogs, glucagons, tsh, follitropin-beta, fsh, pdgh, inf-beta, inf-alpha 1, ifn-alpha 2, inf-beta, inf-beta 1b, ifn-beta 1a, ifn-gamma, ifn-gamma 1b, il-2, il-11, hbsag, ospa, domase-alpha dnase, beta glucocerebrosidase, tnf-alpha, il-2-diphtheria toxin fusion protein, tnfr-igg fragment fusion protein laronidase, dnaases, alefacept, tositumomab, murine mab, alemtuzumab, rasburicase, agalsidase beta, teriparatide, parathyroid hormone derivatives, adalimumab (lgg1), anakinra, nesiritide, human b-type natriuretic peptide (hbnp), colony stimulating factors, pegvisomant, human growth hormone receptor antagonist, recombinant activated protein c, omalizumab, immunoglobulin e (ige) blocker, lbratumab tiuxetan, ACTH, glucagon, somatostatin, somatotropin, thymosin, parathyroid hormone, pigmentary hormones, somatomedin, luteinizing hormone, chorionic gonadotropin, hypothalamic releasing factors, etanercept, antidiuretic hormones, prolactin and thyroid stimulating hormone, an immunoglobulin polypeptide, immunoglobulin polypeptide D region, immunoglobulin polypeptide J region, immunoglobulin polypeptide C region, immunoglobulin light chain, immunoglobulin heavy chain, an immunoglobulin heavy chain variable region, an immunoglobulin light chain variable region and a linker peptide. Production of each of these, and other, proteins is contemplated using methods, vectors and promoters of the invention.

[0197] The present invention is further illustrated by the following examples, which are provided by way of illustration and should not be construed as limiting. The contents of all references, published patents and patents cited throughout the present application are hereby incorporated by reference in their entireties.

Example 1

Production of pALV-SIN-4.2-Lys-IFNa-2B

[0198] The vector pALV-SIN-4.2-Lys-IFNa-2B (shown in FIG. 1) was constructed and is shown in FIG. 1. The sequence of pALV-SIN-4.2-Lys-IFNa-2B is shown in SEQ ID NO: 1. The 4.2 Kb lysozyme promoter spans from nucleotides 4810 to 9008 of SEQ ID NO: 1. The lysozyme signal peptide coding sequence spans from nucleotides 9037 to 9090 of SEQ ID NO: 1. The interferon alpha 2b coding

sequence spans from nucleotides 9091 to 9585 of SEQ ID NO: 1. Other components of the sequence include LTRs spanning from nucleotides 4000 to 4345 and from nucleotides 725 to 897 of SEQ ID NO: 1.

[0199] pALV-SIN-4.2-Lys-IFNa-2B can be constructed by a variety of methods which are apparent to a practitioner of skill in the art. However, the method believed to be the most useful for making the vector is as follows: A 3427 bp region of pNLB-CMV-IFN-alpha2B (disclosed in U.S. patent application Ser. No. 11/167,052, filed Jun. 24, 2005, the disclosure of which is incorporated in its entirety herein by reference) is PCR amplified using primers ATGCGCGCAT-TGGTAATTGATCGGCTGG (Primer ALV-SIN-1, SEQ ID NO: 2) and ATATGCGGCCGCGGTACCGC-CCGGGCATCGATATCAAGCTTACGGTTCAC T AAC-GAGCTCTGCTTATATAGACCTCCCA (Primer ALV-SIN-2, SEQ ID NO: 3). The product is digested with BssHII and Not I resulting in a 3428 bp fragment which can be isolated by gel purification. A 1436 bp region of pNLB-CMV-IFN-alpha2B is PCR amplified with primers ATATGCGGC-CGCGTTCGACGGCCGCGCCAGATCTGCTGAGCCG-GTCGCTACCA TTACCAGT (Primer ALV-SIN-3, SEQ ID NO: 4) and ATACGCGTATTCCTAACGATCACGTCG (Primer ALV-SIN-4, SEQ ID NO: 5).

[0200] The resulting product is digested with Not I and Mlu I yielding a 1438 bp fragment which is isolated by gel purification. A Bluescript II SK vector containing a BssHII stuffer fragment is digested with BssHII resulting in a linearized Bluescript vector of 2788 bp which is gel purified and then ligated to the 3428 bp and 1438 bp PCR products to yield JCR.A108.49.5.24.

[0201] JCR.A108.49.5.24 is digested with Hind III and the resulting 6823 bp fragment is circularized by ligation to yield JCR.A108.76.1.1.

[0202] A 1175 bp region of JCR.A108.76.1.1 is PCR amplified with primers CTGAAGTGTAAGGAATGTAAG (Primer ALV-SIN-5, SEQ ID NO: 6) and GCGCGTCT-CATCCCCCTCCCTATGCAAAAAG (Primer ALV-SIN-6, SEQ ID NO: 7) and the resulting fragment is digested with Blp I and Esp3I producing a 1030 bp fragment which is isolated by gel purification. A 660 bp region of JCR.A108.76.1.1 is PCR amplified with primers GGGCGTCTCA-GGGACGGATTGGACGAACCACTGAATT (Primer ALV-SIN-7, SEQ ID NO: 8) and TTAGTGCTTTACG-GCACCTC (Primer ALV-SIN-8, SEQ ID NO: 9) and digested with Esp3I and DraIII resulting in a 596 bp fragment which is isolated by gel purification. JCR.A108.76.1.1 is digested with DraIII and Blp I and the 5024 bp linear vector is ligated to the 1030 and 596 bp PCR fragments to produce pALV-SIN.

[0203] pALV-SIN is digested with BamHI and the 4795 bp linear vector is isolated by gel purification. A 4815 bp region of JCR.115.93.1.2 (disclosed in US patent application No. 2007/0124829, filed Jan. 26, 2007,) is PCR amplified with primers GACGGATCCGATACCGTCCCTATTTTTGT-GTTTGCTTC (Primer ALV-SIN-9, SEQ ID NO: 10) and TAACGGATCCTAGACTTTTACTCCTTAGA (Primer ALV-SIN-10, SEQ ID NO: 11) and is digested with BamHI. The resulting 4802 fragment is ligated to the 4795 bp linear pALV-SIN to produce pALV-SIN-4.0-Lys-IFNa-2B.

Example 2

Production of Transgenic Quail Using pALV-SIN-4.2-Lys-IFNa-2B

[0204] Transduction particles of the vector pALV-SIN-4.2-Lys-IFNa-2B were produced in fibroblast cells as disclosed in US patent publication No. 2007/0077650, published Apr. 5, 2007, entitled: Rapid Production of High Titer Virus, the disclosure of which is incorporated in its entirety herein by reference.

[0205] Fertilized Japanese quail eggs were windowed essentially according to the Speksnijder procedure disclosed in U.S. Pat. No. 5,897,998, the disclosure of which is incorporated in its entirety herein by reference. Eighty eggs were injected in the subgerminal cavity with about 7 microliters (approximately 7×10^4 viral particles total) of pALV-SIN-4.2-Lys-IFNa-2B transducing particles per egg. Since no selectable marker is used in pALV-SIN-4.2-Lys-IFNa-2B, the concentration of viral particles is estimated based upon past results for viral particle production where a selectable cassette or marker was used in the vector which allowed for particle quantification. Sixteen chicks hatched about 18 days after injection and human IFN levels were measured by IFN ELISA from serum samples collected from chicks 12 weeks after hatch. None were positive for the IFN protein in the serum.

[0206] In order to identify G0 quail which contained the interferon alpha 2 coding sequence containing transgene in their genome, DNA was extracted from blood of the birds and the DNA samples were subjected to Taqman® analysis on a 7700 Sequence Detector (Perkin Elmer).

[0207] Eggs from eight G0 quail were tested for the presence of the IFN protein in the egg white by ELISA. Quail No. 4 was found to have significant levels of IFN in egg white from her eggs. FIG. 2 shows a bar graph illustrating expression levels of IFN in the egg white of Quail No. 4. Quail No. 4 expressed IFN-alpha-2 at 0.45 µg/ml of egg white, which is a high level of expression for a G0 avian. There was no interferon alpha 2 detected in the blood of Quail No. 4 which is particularly significant. For example, in certain instances the recombinant protein may be harmful to the development or health of the avian when present in the blood which can kill the bird or can lead to reduced levels of protein production.

Example 3

Production of Transgenic Quail Using pALV-SIN-6.5-Lys-IFNa-2B

[0208] The 4.2 kb lysozyme promoter of vector pALV-SIN-4.2-Lys-IFNa-2B is removed and replaced with a 6.5 kb lysozyme promoter corresponding to about nucleotides 5363 to 11863 of SEQ ID NO: 12, using standard methodologies known to practitioners of skill in the art, resulting in pALV-SIN-6.5-Lys-IFNa-2B. Transduction particles of the new vector pALV-SIN-6.5-Lys-IFNa-2B are produced as disclosed in US patent publication No. 2007/0077650, published Apr. 5, 2007.

[0209] Fertilized chicken eggs or Japanese quail eggs are windowed and about 7×10^4 pALV-SIN-6.5-Lys-IFNa-2B transducing particles are injected into the subgerminal cavity of each egg. Eggs hatch 21 or 18 days after injection and chimeric birds are identified that contain the active transgene

in their genome, as described in Example 2. Fully transgenic G1 birds which contain the transgene in their genome are produced from chimeras using methods known in the art, i.e., crossing male chimeras with non-transgenic females.

Example 4

Production of Vector pSIN-OV-3.5-I-CTLA4-Fc-Inv

[0210] This vector includes the ovalbumin Dnase hypersensitive sites (DHS) I, II and III, the first exon (exon L), the first intron and the CTLA4-Fc fusion protein coding sequence inserted in frame with the ATG of second exon (exon 1) and with the 3' untranslated region (UTR). The expression cassette is inserted in the inverse orientation into an avian leukosis virus (ALV) vector, which was made self-inactivating (SIN) by deletion of nucleotides 1 to 173 of the ALV LTR sequence shown in SEQ ID NO: 29.

[0211] The vector was constructed as follows: pNLB-3.9-OM-CTLA4-Fc, disclosed in Example 20 of US patent publication No. 2007/0113299, published May 17, 2007, the disclosure of which is incorporated in its entirety herein by reference, was cut with Nae I and Not I. The Not I site was filled in by Klenow reaction. The resulting 8125 bp fragment was gel purified, religated, producing pOM-3.9-CTLA4-dSacl.

[0212] pOM-3.9-CTLA4-dSacl was cut with EcoRI and Kpn I and the 8115 bp fragment gel purified. The 3' UTR of the chicken ovalbumin gene was PCR'd from BAC 26, disclosed in US patent publication No. 2006/0130170, published Jun. 15, 2006, with the primers 5'-GCGGAAT-TCAAAGAAGAAAGCTGAAAAAC-3' (SEQ ID NO: 13) and 5'-GCGGGTACCTTCAAATACTACAAGTGAAA-3' (SEQ ID NO: 14). The 3' UTR PCR was cut with Eco RI and Kpn I and the 684 bp fragment gel purified. The 8115 bp fragment of pOM-3.9-CTLA4-dSacl was ligated to the 684 bp fragment of 3' UTR PCR, producing pOM-3.9-CTLA4-OV3'UTR.

[0213] The 3.5 kb OV promoter region, exon L, first intron and the UTR of exon 1 was PCR amplified with BAC26 as a template and with primers 5'-GGCCTCGAGTCAAGT-TCTGAGTAGGTTTTAGTG-3' (SEQ ID NO: 15) and 5'-GCGCGTCTCTGTCTAGAGCAAACAGCAGAACA-GTGAAAATG-3' (SEQ ID NO: 16). The PCR product was cut with Xho I and Esp3I and the 5094 bp product was gel purified.

[0214] A 5' portion of the CTLA4-Fc gene was PCR amplified using pOM-3.9-CTLA4 as a template and primers 5'-GCGCGTCTCAAGACAACCTCAGAGTTCAC-CATGGGTGTACTGCTCACACAG-3' (SEQ ID NO: 17) and 5'-GGCCCCGGGAGTTTTGTGTCAGAAGATTTGGG-3' (SEQ ID NO: 18). The PCR product was cut with Esp3I and Sad and the 384 bp product gel purified.

[0215] pOM-3.9-CTLA4-OV3'UTR was cut with Sac I and Xho I, the 4473 bp product gel purified and ligated to the 5094 bp OV PCR fragment and 384 bp CTLA4-Fc fragment, producing pOV-3.5-I-CTLA4.

[0216] pALV-SIN, disclosed, for example, in Example 10 of parent case US patent publication No. 2007/0124829, published May 31, 2007, was cut with Mfe I and Xho I, filled in with Klenow and the 4911 bp fragment gel purified.

[0217] pOV-3.5-I-CTLA4 was cut with XhoI and BamHI, filled in with Klenow and the 6957 bp fragment gel purified. This fragment was ligated into the 4911 bp fragment of pAVI-SIN such that the CTLA4-Fc gene and flanking

expression elements are in the opposite orientation of the ALV long terminal repeats, producing pSIN-OV-3.5-I-CTLA4-inv. See FIG. 3 and SEQ ID NO: 19. Such opposite orientation may be preferred if the coding sequence of interest (i.e., CSI) in the transgene contains one or more introns or splice sites.

Example 5

Production Of Transgenic Quail Using SIN-OV-3.5-I-CTLA4-inv

[0218] Retroviral particles containing the pSIN-OV-3.5-I-CTLA4-inv vector (FIG. 3) and pseudotyped with the VSV envelope protein were produced as described in US patent publication No.2007/0077650, published Apr. 5, 2007. Virus particles were harvested at 48 hours post-transfection, concentrated and on the same day, approximately 4 microliters of the virus suspension containing about 1×10^5 particles was injected into the subgerminal cavity of stage X quail eggs. Eggs were resealed and hatched.

[0219] ALV has a CTE element in the 3' end of its genome that allows transport of unspliced retroviral RNA to the cytoplasm. In pSIN-OV-3.5-I-CTLA4-inv, due to the inverse orientation of the OV promoter relative to the LTRs, the CTE is upstream of the OV promoter such that the CTE element is only in RNAs derived from the 5' LTR promoter and not in RNAs transcribed by the OV promoter. Therefore, any RNA transcribed by the OV promoter should be spliced prior to being transported into the cytoplasm.

[0220] Egg whites from chimeric quail were assayed using an ELISA for CTLA4-Fc. One quail was found to have CTLA4-Fc in her egg white at approximately 16 $\mu\text{g/ml}$. The transgenesis level in these quail is estimated at about 5% or less. Thus the level in a G1 should be substantially greater. It is expected that similar levels would be seen in a chicken and other avians, as the quail and chicken ovalbumin genes, as well as ovalbumin genes of other avians, are very similar.

Example 6

Construction of pSIN-3.9-OM-CTLA4-Fc

[0221] The 4907 bp Mfe I/Xho I fragment of pALV-SIN (disclosed, for example, in US patent publication No. 2007/0124829, published May 31, 2007) was ligated to the 5115 XhoI/EcoRI fragment of pOM-3.9-CTLA4 (shown in FIG. 15 of US patent publication No. 2007/0113299, published May 17, 2007), producing pSIN-3.9-OM-CTLA4-Fc Shown in FIG. 4 and SEQ ID NO: 20,

Example 7

Production Of Transgenic Chickens Using pSIN-3.9-OM-CTLA4-Fc

[0222] Retroviral particles pseudotyped with the VSV envelope protein and containing the pSIN-3.9-OM-CTLA4-Fc (FIG. 4) vector were produced as described in US patent publication No. 2007/0077650, published Apr. 5, 2007. Virus was harvested at 48 hours post-transfection, concentrated and on the same day approximately 7 microliters injected into the subgerminal cavity of stage X eggs. Eggs were resealed and incubated until hatch.

[0223] Egg white from hens was assayed using an ELISA for CTLA4-Fc. One hen was found to have CTLA4-Fc in

her egg white at approximately 0.37 µg/ml. The transgenesis level in these hens is estimated at 5% or less. Thus the levels in a G1 should be substantially greater.

[0224] Any useful coding sequence may be inserted in place of the CTLA4-Fc coding sequence for production of the corresponding product.

Example 8

Construction of pSIN-1.8-OM-IFNa-2B

[0225] The 1051 bp Nco I-Nco I fragment from pBS-OM-4.4 (FIG. 5 SEQ ID NO: 23) was inserted into the Nco I site of pAVIJCR-A137.91.1.2 (FIG. 6 SEQ ID NO: 24), thereby inserting the 1 kb ovomucoid promoter in front of an IFN coding sequence and SV40 polyadenylation signal and producing p1kb-OM-IFNMM. A 1816 bp Cla I-Sac I fragment of plkb-OM-IFNMM was inserted into the 6245 bp Cla I-Sac I fragment of pBS-OM-4.4, thereby fusing the 4.4 kb ovomucoid fragment with the IFN coding sequence and producing p4.4OM-IFNMM. The 8511 bp BamH I-Sal I fragment of pBS-OMUP-10 was ligated to the 5148 bp BamH I-Sal I fragment of p4.4OM-IFN, thereby placing the 10 kb ovomucoid promoter in front of the IFN coding sequence, producing p10-OM-IFN.

[0226] Region 2487-4889 of p10.0-OM-IFN was PCR amplified with primers 5'-GGCGTCGACGGATCCGT-TAACCTAGAACTAGTGGATCTCTGCCCTTGTGCTGAC-3' (SEQ ID NO: 27) and 5'-GGCCTCGAGCCTA-GACTTTTTACTCCTTAGA-3' (SEQ ID NO: 28). The PCR product was digested with Sal I and Xho I and the 2435 bp isolated. pALV-SIN (disclosed, for example, in US patent publication No. 2007/0124829, published May 31, 2007) was digested with Xho I and the 4915 bp fragment isolated and ligated to the 2435 bp fragment, producing pSIN-1.8-OM-IFNa-2B, shown in FIG. 7 and SEQ ID NO: 21.

Example 9

Production Of Transgenic Chickens Using pSIN-1.8-OM-IFNa-2B

[0227] Retroviral particles having the pSIN-1.8-OM-IFNa-2B transgene and pseudotyped with the VSV envelope protein were produced as described in US patent publication No. 2007/0077650, published Apr. 5, 2007. Virus was harvested at 48 hours post-transfection, concentrated and, on

the same day, approximately 7 microliters injected into the subgerminal cavity of stage X eggs. Eggs were resealed and incubated until hatch.

[0228] Egg whites from hens were assayed using an ELISA for IFNa-2B. Hens were found to have IFNa-2B in egg white at levels that ranged from 1.5 to 865.0 ng/ml with IFNa-2B levels at least about 600 fold lower in the serum. The transgenesis level in these hens is estimated at 5% or less.

[0229] Five G0 sperm positive roosters were bred to non-transgenic hens. Of 1251 offspring, 30 carried the pSIN-1.8-OM-IFNa-2B transgene. Six of the 30 hens expressed human IFN-α-2B at 34.1 to 165.6 µg/ml of egg white. Each of the six hens had a single copy of the transgene. Serum levels of human IFN-α-2B were 0.3 to 9.2 ng/ml which, on average, was 30,000 fold lower than egg white levels.

Example 10

Production of Transgenic Chickens Using Lentivirus Vectors and Moloney Murine Leukemia Virus

[0230] The invention specifically contemplates the employment of other retroviral vectors that are useful in avian transgenesis to be used in accordance with the present invention. Such vectors can be employed to produce transgenic avians, for example, in the same way as ALV-SIN vectors have been used in Examples 1 to 9 above. For example, Moloney Murine Leukemia Virus (MMLV) and Lentiviral Vectors can be used in accordance with the invention, each, for example, by deleting one or more of the CAAT box; the TAATA box; and enhancer contained in the U3 region of the upstream LTR of each virus to produce a SIN vector. Alternatively, or in addition (i.e., in conjunction with a SIN vector) no transcriptionally active markers or selectable cassettes are included in each of the retroviral vectors.

[0231] Although preferred embodiments of the invention have been described using specific terms, devices, and methods, such description is for illustrative purposes only. The words used are words of description rather than of limitation. It is to be understood that changes and variations may be made by those of ordinary skill in the art without departing from the spirit or the scope of the present invention, which is set forth in the following claims. In addition, it should be understood that aspects of the various embodiments may be interchanged both in whole or in part.

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taactcata	ggtagatata	ggtagctcca	gcttcacctg	gcagtgggtg	gtcagttctg	6720
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<210> SEQ ID NO 24

<211> LENGTH: 4327

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
pAVIJCR-A137.91.1.2 vector polynucleotide

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caaaaggggg tgggaggaag ttaaaagaag aggcaggtgc aagagagctt gcagtcccgc	180
tgtgtgtaag aactggcac catggctttg accttgcct tactggtggc tctcctggtg	240
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agcaggagga ccctgatgct gctggctcag atgaggagaa tcagcctggt tagctgctg	360
aaggataggc acgattttgg ctttctcaa gaggagtgtg gcaaccagtt tcagaaggct	420
gagaccatcc ctgtgctgca cgagatgac cagcagatct ttaacctggt tagcaccaag	480
gatagcagcg ctgcttggga tgagaccctg ctggataagt tttacaccga gctgtaccag	540
cagctgaaag atctggaggc ttgcgtgatc cagggcgtgg gcgtgaccga gaccctctg	600
atgaaggagg atagcatcct ggctgtgagg aagtacttcc agaggatcac cctgtacctg	660
aaggagaaga agtacagccc ctgcgcttgg gaagtctga gggctgagat catgaggagc	720
tttagcctga gcaccaacct gcaagagagc ttgaggtcta aggagtaaaa agtctagagt	780
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<210> SEQ ID NO 25
<211> LENGTH: 244
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
RRE (rev responsive element) polynucleotide

```

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<400> SEQUENCE: 25
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aatttgctga gggctattga ggcgcaacag catctgttgc aactcacagt ctggggcatc 180
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gtac 244

```

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<210> SEQ ID NO 26
<211> LENGTH: 158
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
ALV CTE (constitutive transport element) polynucleotide

```

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<400> SEQUENCE: 26
aatgtgggga gggcaaggct tgcgaatcgg gttgtaacgg gcaaggcttg actgagggga 60
caatagcatg tttaggcga aagcggggct tgggtgtac gcggttagga gtcccctcag 120
gatatagtag tttcgctttt gcatagggag ggggaaat 158

```

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<210> SEQ ID NO 27
<211> LENGTH: 55
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
p10.0-OM-IFN-1 primer

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```

<400> SEQUENCE: 27
ggcgtcgacg gatccgtaa ccctagaact agtggatctc tgccccttctg ctgac 55

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<210> SEQ ID NO 28
<211> LENGTH: 31
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 28

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<210> SEQ ID NO 29
<211> LENGTH: 346
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      ALV vector 5' LTR polynucleotide

<400> SEQUENCE: 29

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tatgatcgtg gtatgatcgt gccttgtagg gaaggcaaca gacgggtcta acacggattg     180
gacgaaccac tgaattccgc attgcagaga tattgtattt aagtcctag ctcgatacaa     240
taaacgccat ttgaccattc accacattgg tgtgcacctg gggtgatggc cggaccgttg     300
attcctgrc  gactacgagc acatgcatga agcagaaggc ttcatt                       346

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1-25. (canceled)

26. A method of using a transgenic chicken to produce an exogenous protein comprising:

providing a transgenic chicken,
 wherein the transgenic chicken contains in its genome an exogenous nucleotide sequence comprising (i) an ovalbumin promoter operably linked to an exogenous coding region encoding an exogenous protein and (ii) a self-inactivating avian leukosis viral (ALV) vector comprising a 5' long terminal repeat that comprises a nucleic acid at least 90% identical to nucleotides 174-346 SEQ ID NO:29,

wherein the transgenic chicken makes the exogenous protein in a hard shell egg laid by the chicken.

27. A method of using a transgenic chicken to produce an exogenous protein comprising:

providing a transgenic chicken,
 wherein the transgenic chicken contains in its genome an exogenous nucleotide sequence comprising (i) an oviduct specific promoter operably linked to an exogenous coding region encoding an exogenous protein and (ii) a self-inactivating avian leukosis viral (ALV) vector comprising a 5' long terminal repeat that comprises a nucleic acid at least 90% identical to nucleotides 174-346 SEQ ID NO:29,

wherein the transgenic chicken makes the exogenous protein in a hard shell egg laid by the chicken,

wherein the oviduct specific promoter comprises a nucleic acid at least 90% identical to a nucleic acid molecule

that comprises from 5' to 3' the ovalbumin Dnase hypersensitive site (DHS) III, 5' UTR-3' portion of Exon 1, and 3' UTR;

wherein

the 5' UTR-3' portion from Exon 1 is the nucleotide sequence 8295 through 8311 of SEQ ID NO: 22;

the 3' UTR is the nucleotide sequence 13576 through 14209 of SEQ ID NO: 22; and

the DHS III is the nucleotide sequence 3253 through 3559 of SEQ ID NO: 22.

28. The method of claim **26** wherein the 5' long terminal repeat that comprises a nucleic acid at least 95% identical to nucleotides 174-346 SEQ ID NO:29.

29. The method of claim **27** wherein the 5' long terminal repeat that comprises a nucleic acid at least 95% identical to nucleotides 174-346 SEQ ID NO:29.

30. The method of claim **26** wherein the exogenous nucleotide further comprises a nucleotide sequence for antibiotic resistance that is not operably linked to a functional promoter and the self-inactivating ALV vector is also a SC negative vector.

31. The method of claim **27** wherein the exogenous nucleotide further comprises a nucleotide sequence for antibiotic resistance that is not operably linked to a functional promoter and the self-inactivating ALV vector is also a SC negative vector.

* * * * *