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Canadian Intellectual Property Office

CA 2990549 A1 2017/02/09

(21) 2 990 549

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2016/07/28
(87) Date publication PCT/PCT Publication Date: 2017/02/09
(85) Entrée phase nationale/National Entry: 2017/12/21
(86) N° demande PCT/PCT Application No.: IB 2016/001183
(87) N° publication PCT/PCT Publication No.: 2017/021776
(30) Priorité/Priority: 2015/07/31 (US62/199,681)

(51) Cl.Int./Int.Cl. *C12Q 1/68* (2018.01)

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(54) Titre : PROMOTEURS POUR AMELIORER L'EXPRESSION DANS LES VIRUS DU GROUPE POX
(54) Title: PROMOTERS FOR ENHANCING EXPRESSION IN POXVIRUSES

(57) Abrégé/Abstract:
The present invention relates to one or more promoters and/or expression cassettes that can be used for enhancing expression of a heterologous gene, such as Brachury. In particular, the one or more promoters and/or expression cassettes enhance expression of heterologous genes as part of a viral vector, such as a poxvirus.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 February 2017 (09.02.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/021776 A1

(51) **International Patent Classification**:
C12Q 1/68 (2006.01)

(21) **International Application Number**:
PCT/IB2016/001183

(22) **International Filing Date**:
28 July 2016 (28.07.2016)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
62/199,681 31 July 2015 (31.07.2015) US

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2017/021776 A1

(54) **Title**: PROMOTERS FOR ENHANCING EXPRESSION IN POXVIRUSES

(57) **Abstract**: The present invention relates to one or more promoters and/or expression cassettes that can be used for enhancing expression of a heterologous gene, such as Brachury. In particular, the one or more promoters and/or expression cassettes enhance expression of heterologous genes as part of a viral vector, such as a poxvirus.

PROMOTERS FOR ENHANCING EXPRESSION IN POXVIRUSES

FIELD OF THE INVENTION

[001] The present invention relates to one or more novel promoters of protein expression in poxviruses, especially avipoxviruses such as fowlpoxvirus and canarypoxvirus. The invention further relates to polynucleotides comprising said promoter, in particular, polynucleotides comprising said promoter and a nucleic acid to be expressed; vectors comprising the same; host cells comprising the above constructs; and compositions comprising any of the above. Additionally the invention relates to one or more novel polypeptides operably linked to the one or more promoters. More particularly, the one or more novel polypeptides are related to the Brachury protein.

BACKGROUND OF THE INVENTION

[002] Recombinant poxviruses have been used as vaccines for infectious organisms and, more recently, for tumors. Mastrangelo et al. J Clin Invest. 2000;105(8):1031–1034. Two of these poxvirus groups, avipoxvirus and orthopoxvirus, have been shown to be effective at battling tumors and have been involved with potential cancer treatments. *Id.*

[003] One exemplary avipoxvirus species, fowlpox, has been shown to be a safe vehicle for human administrations as fowlpox virus enters mammalian cells and expresses proteins, but replicates abortively. Skinner et al. Expert Rev Vaccines. 2005 Feb;4(1):63-76. Additionally, the use of fowlpox virus as a vehicle for expression is being evaluated in numerous clinical trials of vaccines against cancer, malaria, tuberculosis, AIDS, and EBOLA. *Id.*

[004] Recombinant poxviruses, such as fowlpox, has been used to express a wide range of inserted genes, including several infectious disease and tumor associated genes such as p97,

HER-2/neu, p53 and ETA (Paoletti, et al., 1993). One exemplary tumor antigen that is recently being studied is Brachyury (also known as "T").

[005] Brachyury was identified in mice as a dominant short tail mutant that is also a recessive lethal; homozygous T/T embryos die in mid-gestation due to a failure of posterior mesoderm formation (Chesley, J. Exp. Zool., 70: 429-459, 1935). The murine Brachyury gene has been cloned (Herrmann et al., Nature (Lond.), 343: 617-622, 1990), as well as the homologs in other species, such as humans. The expression of the human homologue of the mouse Brachyury was detected by RT-PCR in the notochord remnant, the nucleus pulposus, of human abortuses at 14-15 weeks gestation (Edwards et al., Genome Res., 6: 226-233, 1996).

[006] Brachyury has generally proved to be a valuable marker for recognition of mesodermal differentiation (Herrmann et al., Trends Genet., 10: 280-286, 1994). For example, apart from expression in embryos themselves, Brachyury has been reported to be activated during the differentiation of certain murine EC and ES cell lines differentiating along mesodermal lineages in vitro (see, for example, Bain et al., Biochem. Biophys. Res. Commun., 223: 691-694, 1996). In humans, Brachyury has been shown to be expressed in teratocarcinomas (Gokhele et al., Cell Growth and Differentiation 11:157-62, 2000), chordomas (Vujovic et al., J. Pathol. 2: 157-65, 2006) and hemagioblastomas (Glasker et al., Cancer Res. 66: 4167-4172, 2006).

[007] More recently, it has been described that poxviruses expressing Brachyury can be effective as an active immunotherapeutic against tumors. (*See,* WO 2014/043535).

[008] There is clearly a substantial unmet medical need for improving infectious disease and cancer treatments, including active immunotherapies and vaccines. Based on the above, a

need in the art exists for improving expression of antigens, like Brachyury, used in active immunotherapies. The present invention fulfills this need.

BRIEF SUMMARY OF THE INVENTION

[009] The present invention provides one or more promoters, nucleic acids, expression cassettes, recombinant peptides, and recombinant poxviruses associated with enhancements in expression of coding sequences and antigens incorporated as part of poxviruses

[010] In one aspect of the invention, there is an expression cassette comprising:

a. a promoter comprising a nucleic sequence having at least 70% identity to any one of SEQ ID NOs: 1-10, and 77; and
b. a coding sequence operably linked to the promoter;

wherein expression of the coding sequence is controlled by the promoter.

[011] In further aspects of the present invention, the expression cassette can be incorporated as part of a vector such as a virus or plasmid, for enhanced expression of the coding sequence therein. Preferably, the expression cassette is part of a poxvirus, such as but not limited to, orthopoxvirus and avipoxvirus. More preferably, the expression cassette is an avipoxvirus such as fowlpoxvirus. In still further aspects, the expression cassette is not an expression cassette that naturally occurs in the genome of a poxvirus.

[012] In additional aspects, there are one or more promoters comprising or consisting of a) a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-10, b) a subsequence of any one of SEQ ID NOs: 1-10, and 77, or c) a derivative sequence of any one of SEQ ID NOs: 1-10, and 77, wherein the derivative has one or more substitutions, deletions, and/or insertions, and wherein the derivative sequence is active as a poxvirus promoter and/or active in a poxvirus infected cell.

[013] In still additional aspects, the present invention includes one or more nucleic acid sequences encoding one or more novel Brachyury proteins selected from the group consisting of: SEQ ID NOs: 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71. In yet further aspects, the present invention includes one more novel nucleic acid sequences selected from the group consisting of: 16-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69.

[014] In still further aspects, the present invention includes an expression cassette comprising:

a. a promoter comprising a nucleic acid sequence having at least 70% identity to any one of SEQ ID NOs: 1-10, and 77; and
b. a coding sequence operably linked to the promoter, wherein the coding sequences includes either i) a nucleic acid encoding a Brachyury protein selected from the group consisting of: SEQ ID NOs: 11, 13, 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71 or ii) comprises or consists of a nucleic acid having at least 70% identity to any one of the nucleic acids selected from the group consisting of: SEQ ID NOs12, 14-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69;

wherein expression of the coding sequence is controlled by the promoter.

[015] Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description or may be learned by practice of the invention. The objects and advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[016] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

[017] Figure 1 illustrates expression of the Brachyury protein in human dendritic cells (DCs) infected with the non-recombinant virus FPV-WT, or recombinant viruses FPV-mBN343A, FPV-mBN344A, or FPV-mBN345A . A western blot analysis was performed using rabbit monoclonal anti-Brachyury antibody as detailed in Example 1.

[018] Figure 2 illustrates expression of the Brachyury protein in human dendritic cells (DCs) infected with non-recombinant viruses MVA-WT or FPV-WT or recombinant viruses MVA-Brachyury-TRICOM, FPV-mBN249B, FPV-mBN281A clone 32, FPV-mBN281A clone 35, FPV-mBN343A, FPV-mBN344A, FPV-mBN345A, FPV-mBN354A, or FPV-mBN355A. A western blot analysis was performed using rabbit monoclonal anti-Brachyury antibody as detailed in Example 2.

[019] Figure 3 depicts the relative expression of Brachyury protein compared to GAPDH from western blot analysis of human dendritic cells (DCs) infected with non-recombinant viruses MVA-WT or FPV-WT or recombinant viruses MVA-Brachyury-TRICOM, FPV-mBN249B, FPV-mBN281A clone 32, FPV-mBN281A clone 35, FPV-mBN343A, FPV-mBN344A, FPV-mBN345A, FPV-mBN354A, or FPV-mBN355A, as detailed in Example 2.

[020] Figure 4 illustrates expression of Brachyury and TRICOM proteins in CMMT cells (a rhesus macaque mammary tumor cell line) infected with the recombinant viruses MVA-Brachyury-TRICOM, FPV-mBN249B, FPV-mBN343A, or FPV-mBN345A assessed by flow

cytometry using fluorescently labeled antibodies specific for each protein, as described in Example 3.

[021] Figure 5 depicts median expression levels of Brachyury protein in CMMT cells (a rhesus macaque mammary tumor cell line) infected with the recombinant viruses MVA-Brachyury-TRICOM, FPV-mBN249B, FPV-mBN343A, or FPV-mBN345A assessed by flow cytometry, as described in Example 3.

[022] Figure 6 depicts expression of Brachyury and TRICOM proteins in CMMT cells (a rhesus macaque mammary tumor cell line) infected with a recombinant virus FPV-mBN345B assessed by flow cytometry, as described in Example 4.

DETAILED DESCRIPTION OF THE INVENTION

[023] The invention is based on the surprising determination that the promoter sequences as set out in SEQ ID NOs:1-10, and 77 enhance expression of the tumor antigen Brachyury. Shown in Figures 1-6 and described in more detail herein, expression of Brachyury antigen is enhanced when using promoters of present invention. Expression is further enhanced when the promoters and Brachyury antigen are used as part of a recombinant poxvirus.

[024] In at least one aspect, the various embodiments of the present disclosure were created as a result of insufficient expression levels of Brachyury protein using known Vaccinia promoters, such as PrS and Vaccinia virus 40k (VV-40K). In trying to enhance Brachyury expression levels, the present inventors analyzed various vaccinia promoters and associated proteins (e.g., VV-40k, I3, etc.) and any possible homologues in FPV. The inventors realized that some FPV homologous sequences were previously discovered. *See, e.g.*, Zantinge, J Gen Virol. 1996 Apr;77 (Pt 4):603-14 and Gene FPV 088 at NCBI Reference Sequence: NP_039051.1, Afonso,C.L et al., J. Virol. 74 (8), 3815-3831 (2000).

[025] In an initial attempt to enhance Brachyury expression, a possible promoter region of Gene FPV 088 at NCBI Reference Sequence: NP_039051.1 was constructed with Brachyury by the inventors and tested yielding undesired results (*see, e.g.*, Figures 1 and 2 at mBN344A) promoter. The inventors created a subsequent promoter with an addition of nucleotides from the ORF of Gene FPV 088, which similarly yielded undesired results. (*see, e.g.*, Figures 1 and 2 at mBN354A). Further constructs were created by the present inventors with yet additional nucleotides from the ORF of Gene FPV 088, which, as described and illustrated herein, enhance expression of the tumor antigen Brachyury.

[026] Thus, in various embodiments, the present invention is directed to one or more nucleic acid sequences, one or more promoters comprising the nucleic acids sequences, one or more expression cassettes comprising the nucleic acids, one or more peptides and/or peptide sequences for enhancing/expression of coding sequences and/or nucleic acids in expression vectors such as, but not limited to, plasmids, recombinant virus and so forth. In further embodiments, the present invention is directed to one or more recombinant poxviruses comprising one or more of the nucleic acid sequences, promoters, expression cassettes, and/or peptides and/or peptide sequences described herein.

[027] Additionally, in various embodiments of the invention, there are one or more nucleic acids sequences encoding brachyury polypeptides. In one embodiment, the one or more nucleic acids encoding a brachyury polypeptide are selected from the group consisting of: SEQ ID NOs: 11, 13, 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71. The nucleotide sequences encoding the brachyury polypeptides according to the present invention are selected from the group consisting of: SEQ ID NOs: 12,

14-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69.

Definitions

[028] It must be noted that, as used herein, the singular forms "a", "an", and "the", include plural references unless the context clearly indicates otherwise. Thus, for example, reference to "an epitope" includes one or more of epitopes and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

[029] Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the present invention.

[030] Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. When used herein the term "comprising" can be substituted with the term "containing" or "including" or sometimes when used herein with the term "having". Any of the aforementioned terms (comprising, containing, including, having), though less preferred, whenever used herein in the context of an aspect or embodiment of the present invention can be substituted with the term "consisting of. When used herein "consisting of excludes any element, step, or ingredient not specified in the

claim element. When used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim.

[031] As used herein, the conjunctive term "and/or" between multiple recited elements is understood as encompassing both individual and combined options. For instance, where two elements are conjoined by "and/or", a first option refers to the applicability of the first element without the second. A second option refers to the applicability of the second element without the first. A third option refers to the applicability of the first and second elements together. Any one of these options is understood to fall within the meaning, and therefore satisfy the requirement of the term "and/or" as used herein. Concurrent applicability of more than one of the options is also understood to fall within the meaning, and therefore satisfy the requirement of the term "and/or."

[032] As used herein, the term “promoter” denotes a regulatory region of nucleic acid, usually DNA, located upstream of the sequence of a nucleic acid to be expressed, which contains specific DNA sequence elements, that are recognized and bound e.g. by protein transcription factors and polymerases responsible for synthesizing the RNA from the coding region of the gene being promoted. As promoters are typically immediately adjacent to the gene in question, positions in the promoter are designated relative to the transcriptional start site, where transcription of DNA begins for a particular gene (i.e., positions upstream are negative numbers counting back from -1, for example -100 is a position 100 base pairs upstream). Thus, the promoter sequence may comprise nucleotides until position −1. However, nucleotides from position +1 are not part of the promoter, i.e. in this regard it has to be noted that the translation initiation codon (ATG or AUG) is not part of the promoter. Thus, SEQ ID NOs: 1-10, and 77 are polynucleotides comprising promoters of the invention.

[033] As used herein, the term “enhancing” or “enhanced” when used with respect to expression levels of a coding sequence, nucleic acid, protein, and/or antigen, refers to an increase in expression of a coding sequence, nucleic acid, protein, and/or antigen when associated with and/or as part of one or more of the promoters, expression cassettes, nucleic acids, proteins, and/or vectors of the present invention relative to expression levels of a coding sequence, nucleic acids, protein, and/or antigen when associated with and/or as part of one or more of the promoters know in the art, such as PrS or VV-40k.

[034] As used herein, a nucleotide sequence having “essentially the same expression characteristics” as the nucleotide sequence set out in SEQ ID NO s: 1-10, and 77 will exhibit at least 70%, preferably at least 80%, even more preferably at least 90% of the promoter activity of SEQ ID NO s: 1-10, and 77, as measured by amount of recombinant protein produced. Whether or not a promoter sequence in question has “essentially the same expression characteristics” as any of SEQ ID NO s: 1-10, and 77 may be readily determined by one of ordinary skill in the art using the methods set forth in Examples 1-4 of the present application. The promoters according to the present invention are preferably active as poxvirus promoters, preferably avipoxvirus or active as promoters in poxvirus infected cells, preferably avipoxvirus infected cells. The avipoxvirus is preferably Fowlpox virus. “Active as pox virus promoter” means that the promoter is able to direct the expression of a gene to which it is operably linked in a pox virus after infection of cells with said virus. The cells are preferably cells that allow late and/or early and/or early/late expression of the poxvirus. “A promoter active in poxvirus infected cells” includes also the situation in which the promoter is not part of a poxvirus genome, e.g. part of a plasmid or linear polynucleotide or a non-poxvirus viral genome; in such a situation the promoter according to the present invention is active if the cell comprising the promoter also comprises a

poxvirus genome, e.g. if the cell is infected with a poxvirus. Under these circumstances the viral RNA polymerase recognizes the promoter according to the present invention and the expression of the gene/coding sequence that is linked to the promoter is activated.

[035] As used herein, the term "derived from the nucleic acid set out in SEQ ID NOs: 1-10, and 77" means that the nucleotide sequence of SEQ ID NOs: 1-10, and 77 is taken as a basis for effecting the nucleotide modifications specified, for example, at least one nucleotide addition, deletion, substitution and/or inversion. The term "derived" includes the possibility, for example, of actually modifying the physical sequence corresponding to SEQ ID NOs: 1-10, and 77 by known methods, for example, error-prone PCR. The term "derived" additionally includes the possibility of performing modifications on the sequence of SEQ ID NOs: 1-10, and 77 in silico, and then synthesizing the thus determined sequence as a physical nucleic acid. For example, the term "derived" encompasses the possibility of using any known computer program for the analysis of nucleic acid sequences with regard to, for example, hybridization stability and the possibility of any secondary nucleic acid structure in modifying the starting sequence of SEQ ID NOs: 1-10, and 77. Preferably, not more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 nucleotides have been added, deleted, substituted and/or inverted from the nucleic acids of SEQ ID NOs: 1-10, and 77. Furthermore, addition, insertion or deletion of at least one nucleotide should not result to a start codon of the nucleic acid to be expressed.

[036] As used herein, the terms "expressed", "express", "expression" and the like denote the transcription alone as well as both the transcription and translation of a sequence of interest. Thus, in referring to expression of a nucleic acid present in the form of DNA, the product resulting from this expression may be either RNA (resulting from transcription alone of the sequence to be expressed) or a polypeptide sequence (resulting from both transcription and

translation of the sequence to be expressed). The term “expression” thus also includes the possibility that both RNA and polypeptide product result from said expression and remain together in the same shared milieu. For example, this is the case when the mRNA persists following its translation into polypeptide product.

[037] As used herein, the term “expression cassette” is defined as a part of a vector or recombinant virus typically used for cloning and/or transformation. An expression cassette is typically comprised of a) one or more coding sequences (e.g., , open reading frame (ORF), genes, nucleic acids encoding a protein and/or antigen), and b) sequences controlling expression the one or more coding sequences. Additionally, an expression cassette may comprise a 3’ untranslated region that in eukaryotes usually contain a polyadenylation site.

[038] The term "recombinant" means a polynucleotide or polypeptide of semi-synthetic, or synthetic origin which either does not occur in nature or is linked to another polynucleotide in an arrangement not found in nature.

[039] One aspect of the invention includes promoters with nucleic acids having at least 70%, preferably 75%, 80%, 85%, 90% or 95% identity with SEQ ID NOs: 1-10, and 77 and having essentially the same expression characteristics as SEQ ID NOs: 1-10, and 77.

[040] "Percent (%) sequence homology or identity" with respect to nucleic acid sequences described herein is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in the reference sequence (i.e., the nucleic acid sequence from which it is derived), after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent nucleotide sequence identity or homology can be achieved in various ways that are within the

skill in the art, for example, using publically available computer software such as BLAST, ALIGN, or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximum alignment over the full length of the sequences being compared.

[041] For example, an appropriate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, (1981), Advances in Applied Mathematics 2:482- 489. This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov (1986), Nucl. Acids Res. 14(6):6745-6763. An exemplary implementation of this algorithm to determine percent identity of a sequence is provided by the Genetics Computer Group (Madison, Wis.) in the "BestFit" utility application. The default parameters for this method are described in the Wisconsin Sequence Analysis Package Program Manual, Version 8 (1995) (available from Genetics Computer Group, Madison, Wis.). A preferred method of establishing percent identity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, Calif). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence identity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using

the following default parameters: genetic code=standard; filter=none; strand=both; cutoff=60; expect=10; Matrix=BLOSUM62; Descriptions=50 sequences; sort by=HIGH SCORE; Databases=non- redundant, GenBank+EMBL+DDBJ+PDB+ GenBank CDS translations+Swiss protein+Spupdate+PIR. Details of these programs can be found at the following internet address: http:// http://blast.ncbi.nlm.nih.gov/.

Promoters and Nucleic Acid Sequences

[042] In one embodiment, the present disclosure includes a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-10, and 77.

[043] In another embodiment, the present disclosure includes a nucleic acid sequence having at least 70% identity with a nucleic acid selected from the group consisting of SEQ ID NOs: 1-10, and 77 and having essentially the same expression characteristics as a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-10, and 77. In further embodiments, there is a nucleic acid sequence having at least 75%, 80%, 85%, 90%, or 95% identity with a nucleic acid selected from the group consisting of SEQ ID NOs: 1-10, and 77 and having essentially the same expression characteristics as a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-10, and 77.

[044] In yet additional embodiment, there is a nucleic acid having a nucleotide sequence derived from the nucleic acid set out in SEQ ID NOs: 1-10, and 77, comprising at least one nucleotide addition, deletion, substitution and/or inversion as compared to the nucleotide sequence of SEQ ID NOs: 1-10, and 77 and having essentially the same expression characteristics as the nucleic acid of SEQ ID NOs: 1-10, and 77.

[045] In still an additional embodiment, there is a nucleic acid having a nucleotide sequence capable of hybridizing to a nucleic acid sequence selected from the group consisting of

SEQ ID NOs: 1-10, and 77, and having essentially the same expression characteristics as a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-10, and 77.

[046] In one aspect of the invention, there is a promoter for enhancing the expression of a coding and/or gene sequence, the promoter selected from the group consisting of:

a) a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-10, and 77 or a subsequence thereof;

b) a nucleic acid having a nucleotide sequence derived from the nucleic acid set out in (a), comprising at least one nucleotide addition, deletion, substitution and/or inversion as compared to the nucleotide sequence of (a) and having essentially the same expression characteristics as the nucleic acid of (a);

c) a nucleic acid sequence having at least 70% identity with the nucleic acid of (a) and having essentially the same expression characteristics as the nucleic acid of (a); and

d) a nucleic acid capable of hybridizing to a nucleic acid sequence of (a), (b) or (c) and having essentially the same expression characteristics as the nucleic acid of (a).

e) a nucleic acid sequence comprising SEQ ID NOs:3, 4, 5, 6, 7, 8, or 9, wherein the nucleic acid sequence includes up to 245 nucleotides.

[047] A further aspect of the invention relates to a nucleic acid capable of hybridizing to a nucleic acid sequence of (a), (b) or (c) and having essentially the same expression characteristics as the nucleic acid of (a). The term "capable of hybridizing" means hybridization in conditions in which any portion of the nucleic acid of (a), (b) or (c) is able to hybridize to another DNA sequence to allow detection and isolation of any DNA sequence having essentially the same expression characteristics as the nucleic acid of (a). The hybridization is carried out in stringent conditions; the more stringent the conditions, the more likely partially complementary

sequences are to be forced apart, i.e. higher stringency lowers the probability of hybridization. In practice, the term "stringent conditions" means hybridization conditions with high temperature (e.g. 65° C.) and/or low salt concentration (e.g. 0.1×SSC). Increasing the temperature and/or decreasing the salt concentration increases the stringency. Under stringent conditions hybridization will occur only if there is at least 70% or preferably at least 75, 80, 85, 90 or 95% identity between the sequences. Hybridization can be carried out as set out in Ausubel et al. (2002) Short Protocols in Molecular Biology Vol. 1 5th ed. Canada.

[048] Preferably, the promoter and/or nucleic acid of the invention has a length of up to and including 245 or 242 nucleotides. Also, the promoter and/or nucleic acid of the invention has a length of up to and including 232 or 215 nucleotides. More preferably, the promoter of the invention has a length of up to and including 212, 200, or a length of up to and including 195 nucleotides, or a length of up to and including 183, or a length of up to and including 170 or 167 nucleotides. In one embodiment, the nucleic acid of (b), (c) or (d), as set out above, comprises about 50-250 nucleotides, about 60-220 nucleotides, about 70-200 nucleotides, about 80-190 nucleotides, or about 90-180 nucleotides.

[049] Alternatively it is within the scope of the present invention to use a derivative of these promoters, which may be a subsequence of the sequences as defined in anyone of SEQ ID NOs: 1-10, and 77. The term "subsequence of the sequences according to anyone of SEQ ID NOs: 1-10, and 77" refers to shorter fragments of anyone of SEQ ID NO: 1 to 10, and 77 that are still active as a promoter, in particular as promoter in an orthopoxvirus, such as FPV, or in an orthopoxvirus, such as FPV, infected cells.

[050] In various embodiments, exemplary subsequences of SEQ ID NO:9 are selected from SEQ ID NOs:2-8. In one aspect, SEQ ID NOs: 4 or 5 are preferred subsequence of SEQ ID

NO: 9. In other aspects, subsequences of SEQ ID NO: 8 include SEQ ID NOs: 2-7, subsequences of SEQ ID NO:7 include SEQ ID NO:s 2-6, subsequences of SEQ ID NO:6 includes SEQ ID NOs: 2-5, subsequences of SEQ ID NO:5 include SEQ ID NOs: 2-4 subsequences of SEQ ID NO:6 includes SEQ ID NOs: 2-5, subsequences of SEQ ID NO:5 include SEQ ID NOs: 2-4, subsequences of SEQ ID NO: 4 includes SEQ ID NOs: 2-3, and subsequences of SEQ ID NO: 3 includes SEQ ID NO: 2.

[051] The derivative of the promoter comprising or consisting of a nucleotide sequence of anyone of SEQ ID NOs: 1-10, and 77 or subsequences thereof, can also be a sequence that has one or more nucleotide substitutions, deletions and/or insertions with respect to any one of the sequences of SEQ ID NOs: 1-10, and 77. The derivatives according to the present invention are still active as a promoter, in particular as orthopoxvirus and/or avipoxvirus promoter in an orthopoxvirus and/or avipoxvirus virus infected cells, more preferably as an avipoxvirus promoter in avipoxvirus infected cells. In the derivatives according to the present invention deletions, substitutions and insertions may be combined in one sequence.

[052] Preferably the derivative has a homology of at least 40%, more preferably of at least 60%, even more preferably of at least 80%, most preferably of at least 90% when compared to anyone of the sequence of SEQ ID NOs: 1-10, and 77 or subsequences thereof, as described herein.

Brachyury proteins

[053] In several aspects of the present invention, the inventors' determined that as a result of synthesizing the various promoters described herein, the inventors' created one or more recombinant Brachyury proteins. In particular, the one or more recombinant Brachyury proteins are fusion proteins resulting from the combination of one or more of the promoters of the

invention and the Brachyury protein. It is contemplated that one or more of the recombinant Brachyury proteins can be useful as a part of a vaccine, pharmaceutical or other therapeutic composition.

[054] Thus, in various aspects, the invention includes one or more synthetic and novel recombinant Brachyury proteins and/or synthetic and novel nucleic acids encoding recombinant Brachyury proteins.

[055] In one embodiment, there are one or more nucleic acids sequences having at least 70% identity to a nucleic acid encoding a Brachyury protein, wherein the Brachyury protein is selected from the group consisting of: SEQ ID NOs: 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71. In additional embodiments, there are one or more nucleic acids having at least 75%, 80%, 85%, 90%, or 95% identity to a nucleic acid encoding a Brachyury protein, wherein the Brachyury protein is selected from the group consisting of: SEQ ID NOs: 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71.

[056] In various other embodiments of the invention, there are one or more Brachyury proteins selected from the group consisting of: SEQ ID NOs: 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71. In further embodiments, there are one or more Brachyury proteins having at least 70% identity with a peptide selected from the group consisting of: SEQ ID NOs: 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71. In still further embodiments, there is a peptide having at least 75%, 80%, 85%, 90%, or 95% identity with a peptide selected from the group consisting of SEQ ID NOs: 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71.

[057] In another embodiment, there are one or more nucleic acids sequences encoding a Brachyury protein, the nucleic acid sequence selected from the group consisting of: SEQ ID NOs: 16-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69.

[058] In other embodiments, there are one or more nucleic acid sequences having at least 70% identity with a nucleic acid selected from the group consisting of SEQ ID NOs: 16-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69 and having essentially the same expression characteristics as a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 16-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69. In further embodiments, there is a nucleic acid sequence having at least 75%, 80%, 85%, 90%, or 95% identity with a nucleic acid selected from the group consisting of SEQ ID NOs: 16-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69 and having essentially the same expression characteristics as a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 16-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69.

[059] In a preferred embodiment, there are one or more nucleic acid sequences selected from the group consisting of SEQ ID NOs: 16-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69.

Expression Cassettes

[060] According to a further embodiment, the present invention refers to an expression cassette comprising one or more of the promoters and/or Brachyury recombinant proteins and/or nucleic acids according to the present invention.

[061] In one embodiment, there is an expression cassette comprising a promoter having at least 70% identity with a nucleic acid selected from the group consisting of SEQ ID NOs: 1-10 and a nucleic acid encoding a Brachyury peptide selected from the group consisting of: SEQ ID NOs: 11, 13, 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71. In an another embodiment, there is an expression cassette comprising a promoter selected from the group consisting of SEQ ID NOs: 1-10, and 77 and/or a nucleic acid encoding a Brachyury peptide, wherein the nucleic acid is selected from the group consisting of: SEQ ID NOs: 12, 14-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69.

[062] In a preferred embodiment, there is an expression cassette comprising a nucleic acid sequence at least 70% homologous to a nucleic acid sequence selected from the group consisting of: SEQ ID NOs: 72-76. In further embodiments, there is an expression cassette comprising a nucleic acid sequence having at least 75%, 80%, 85%, 90%, or 95% identity with a nucleic acid selected from the group consisting of SEQ ID NOs:72-76.

[063] In a more preferred embodiment, there is an expression cassette comprising SEQ ID NO: 72.

[064] In a more preferred embodiment, there is an expression cassette comprising SEQ ID NO: 73.

[065] In a more preferred embodiment, there is an expression cassette comprising SEQ ID NO: 74.

Recombinant Poxviruses

[066] According to a further embodiment the nucleic acids, promoters, recombinant proteins, and/or expression cassettes according to the present invention may be part of a vector.

The term “vector ” refers to any vectors known to the person skilled in the art. A vector can be a plasmid vector such as pBR322 or a vector of the pUC series. More preferably the vector is a recombinant virus. In the context of the present invention the term “virus” or “recombinant virus” refers to an infectious virus comprising a viral genome. In this case the nucleic acids, promoters, recombinant proteins, and/or expression cassettes of the present invention are part of the viral genome of the respective recombinant virus. The recombinant viral genome is packaged and the obtained recombinant viruses can be used for the infection of cells and cell lines, in particular for the infection of living animals including humans. Typical recombinant viruses that may be used according to the present invention are adenoviral vectors, retroviral vectors or vectors on the basis of the adeno associated virus 2 (AAV2). Most preferred are poxviral vectors.

[067] In several embodiments, the nucleic acids, promoters, polypeptides, and expression cassettes according to the present invention are preferably active as poxviral promoters or active as promoters in poxvirus infected cells. The poxvirus is preferably an avipoxvirus or an orthopoxvirus. More preferably, the poxvirus is an avipoxvirus.

[068] The term "avipoxvirus" refers to any avipoxvirus, such as Fowlpoxvirus, Canarypoxvirus, Uncopoxvirus, Mynahpoxvirus, Pigeonpoxvirus, Psittacinepoxvirus, Quailpoxvirus, Peacockpoxvirus, Penguinpoxvirus, Sparrowpoxvirus, Starlingpoxvirus and Turkeypoxvirus. Preferred avipoxviruses are Canarypoxvirus and Fowlpoxvirus.

[069] An example of a canarypox virus is strain Rentschler. A plaque purified Canarypox strain termed ALVAC (U.S. Pat. No. 5,766,598) was deposited under the terms of the Budapest treaty with the American Type Culture Collection (ATCC), accession number VR-

2547. Another Canarypox strain is the commercial canarypox vaccine strain designated LF2 CEP 524 24 10 75, available from Institute Merieux, Inc.

[070] Examples of a Fowlpox virus are strains FP-1, FP-5, TROVAC (U.S. Pat. No. 5,766,598), and POXVAC-TC (U.S. Patent 7,410,644). FP-1 is a Duvette strain modified to be used as a vaccine in one-day old chickens. The strain is a commercial fowlpox virus vaccine strain designated O DCEP 25/CEP67/2309 October 1980 and is available from Institute Merieux, Inc. FP-5 is a commercial fowlpox virus vaccine strain of chicken embryo origin available from American Scientific Laboratories (Division of Schering Corp.) Madison, Wis., United States Veterinary License No. 165, serial No. 30321.

[071] In certain embodiments, the recombinant FPV comprises a nucleic acid sequence selected from the group consisting of: SEQ ID NOs: 1-10, and 77. In various additional embodiments, the recombinant FPV comprises an expression cassette comprising a nucleic acid sequence selected from SEQ ID NOs: 72-76. In a more preferred embodiment, the recombinant FPV comprises an expression cassette comprising a nucleic acid sequence selected from SEQ ID NOs: 72, 73, and 74.

[072] In other embodiments, the recombinant FPV comprises a nucleic acid encoding a brachyury antigen selected from the group consisting of: SEQ ID NOs: 11, 13, 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71. In further embodiments, the recombinant FPV comprises a Brachyury peptide selected from the group consisting of: SEQ ID NOs: 11, 13, 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71. In further embodiments, the recombinant FPV comprises a nucleic acid selected from the group consisting of: SEQ ID NOs: 12, 14-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69.

In more preferred embodiments, , the recombinant FPV comprises a Brachyury peptide selected from the group consisting of: SEQ ID NOs: 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71 and/or a nucleic acid selected from the group consisting of: SEQ ID NOs: 16-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69.

[073] In the various other embodiments of the present disclosure, the recombinant poxvirus is an orthopoxvirus such as, but not limited to, a vaccinia virus, a Modified Vaccinia Virus Ankara (MVA), or MVA-BN.

[074] Examples of vaccinia virus strains are the strains Temple of Heaven, Copenhagen, Paris, Budapest, Dairen, Gam, MRIVP, Per, Tashkent, TBK, Tom, Bern, Patwadangar, BIEM, B-15, Lister, EM-63, New York City Board of Health, Elstree, Ikeda and WR. A preferred vaccinia virus (VV) strain is the Wyeth (DRYVAX) strain (U.S. Patent 7,410,644). Another preferred VV strain is MVA (Sutter, G. et al. [1994], Vaccine 12: 1032-40). Another preferred VV strain is MVA-BN.

[075] Examples of MVA virus strains that are useful in the practice of the present invention and that have been deposited in compliance with the requirements of the Budapest Treaty are strains MVA 572, deposited at the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom, with the deposition number ECACC 94012707 on January 27, 1994, and MVA 575, deposited under ECACC 00120707 on December 7, 2000. MVA-BN, deposited on Aug. 30, 2000 at the European Collection of Cell Cultures (ECACC) under number V00083008, and its derivatives, are additional exemplary strains.

[076] Although MVA-BN is preferred for its higher safety (less replication competent), all MVAs are suitable for this invention. According to an embodiment of the present invention, the MVA strain is MVA-BN and its derivatives. A definition of MVA-BN and its derivatives is given in PCT/EP01/13628 which is incorporated by reference herein.

[077] In one embodiment, the invention encompasses recombinant orthopoxviruses, preferably a vaccinia virus (VV), a Wyeth strain, ACAM 1000, ACAM 2000, MVA, or MVA-BN for cancer therapy. Recombinant orthopoxviruses are generated by insertion of heterologous sequences into an orthopoxvirus.

~~[078]~~ In certain embodiments, the MVA is MVA-BN, deposited on Aug. 30, 2000, at the European Collection of Cell Cultures (ECACC) under number V00083008, and described in International PCT publication WO2002042480 (see also e.g. U.S. Pat. Nos. 6,761,893 and 6,913,752), which are incorporated by reference herein.

[079] In certain embodiments, a recombinant MVA is a derivative of MVA-BN. Such "derivatives" include viruses exhibiting essentially the same replication characteristics as the deposited strain (ECACC No. V00083008), but exhibiting differences in one or more parts of its genome. Viruses having the same "replication characteristics" as the deposited virus are viruses that replicate with similar amplification ratios as the deposited strain in CEF cells and the cell lines, HeLa, HaCat and 143B; and that show similar replication. characteristics *in vivo*, as determined, for example, in the AGR129 transgenic mouse model.

[080] In certain embodiments, the recombinant orthopoxvirus comprises a nucleic acid selected from the group consisting of: SEQ ID NOs: 1-10, and 77. In various additional embodiments, the recombinant orthopoxvirus comprises an expression cassette comprising a nucleic acid sequence selected from SEQ ID NOs: 72-76. In a more preferred embodiment, the

recombinant orthopoxvirus comprises an expression cassette comprising a nucleic acid sequence selected from SEQ ID NOs: 72, 73, and 74.

[081] In other embodiments, the recombinant orthopoxvirus comprises a nucleic acid encoding a brachyury antigen selected from the group consisting of: SEQ ID NOs: 11, 13, 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71. In further embodiments, the recombinant orthopoxvirus comprises a nucleic acid selected from the group consisting of: SEQ ID NOs: 12, 14-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69. In more preferred embodiments, the recombinant orthopoxvirus comprises a Brachyury peptide selected from the group consisting of: SEQ ID NOs: 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71 and/or a nucleic acid selected from the group consisting of: SEQ ID NOs: 16-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69.

[082] Methods are known to the person skilled in the art how the expression cassette or the promoter according to the present invention can be inserted into a viral genome, in particular into the genome of a poxvirus, most preferably into the genome of an orthopoxvirus and/or FPV. For example, the expression cassette or the promoter or derivative thereof according to the present invention may be inserted into the genome of a poxvirus by homologous recombination. To this end a nucleic acid is transfected into a permissive cell line such as CEF or BHK cells, wherein the nucleic acid comprises the expression cassette or the promoter or derivative thereof according to the present invention flanked by nucleotide stretches that are homologous to the region of the poxviral genome in which the expression cassette or the promoter or derivative thereof according to the present invention is to be inserted. The cells are infected by the poxvirus

and in the infected cells homologous recombination occurs between the nucleic acid and the viral genome. Alternatively, it is also possible to first infect the cells with a poxvirus and then to transfect the nucleic acid into the infected cells. Again recombination occurs in the cells. The recombinant poxvirus is then selected by methods known in the prior art. The construction of recombinant poxvirus is not restricted to this particular method. Instead, any suitable method known to the person skilled in the art may be used to this end.

[083] The expression cassette or the promoter according to the present invention may be introduced into any suitable part of the virus or viral vector, in particular into a viral, genome. In case of an orthopoxvirus and an avipoxvirus, the insertion may be made into non-essential parts of the viral genome or into an intergenic region of the viral genome. The term "intergenic region" refers preferably to those parts of the viral genome located between two adjacent genes that do not comprise coding sequences. If the virus is an orthopoxvirus and an avipoxvirus the insertion may also be made into a deletion site of the viral genome. The term "deletion site" refers to those parts of the viral genome that are deleted with respect to the genome of a naturally occurring orthopoxvirus or avipoxvirus. However, the insertion sites are not restricted to these preferred insertion sites in the orthopoxvirus and an avipoxvirus genome, since it is within the scope of the present invention that the expression cassette may be inserted anywhere in the viral genome as long as it is possible to obtain recombinants that can be amplified and propagated in at least one cell culture system, such as Chicken Embryo Fibroblasts (CEF cells).

[084] The promoter according to the present invention may be used to express a gene that is already part of the vector, e.g. the genome of an orthopoxvirus and/or an avipoxvirus. Such a gene may be a gene that is naturally part of the viral genome or a foreign gene that has already been inserted into the vector. In these cases the promoter according to the present

invention is inserted upstream of the gene in the vector, the expression of which is to be controlled by the promoter.

Vaccines and/or Compositions

[085] According to a further embodiment the invention concerns the vector according to the present invention as vaccine or medicament. In more general term the invention relates to a vaccine or pharmaceutical composition comprising an expression cassette, a DNA or a vector according to the present invention. Methods are known to the person skilled in the art how the vaccine or pharmaceutical composition can be administered to the animal or human body. In case of DNA and recombinant plasmid vectors the DNA and the vector can simply be administered by injection. If the vaccine or composition is a recombinant virus such as an orthopoxvirus or an avipoxvirus , in particular a recombinant MVA or recombinant FPV, it may also be administered to the animal or human body according to the knowledge of the person skilled in the art, e.g. by intra venous, intra muscular, intra nasal, intra dermal or subcutaneous administration. Further details on the amount of virus administered are given below.

[086] The pharmaceutical composition or the vaccine may generally include one or more pharmaceutical acceptable and/or approved carriers, additives, antibiotics, preservatives, adjuvants, diluents and/or stabilizers in addition to the promoter, expression cassette or vector according to the present invention. Such auxiliary substances can be water, saline, glycerol, ethanol, wetting or emulsifying agents, pH buffering substances, or the like. Suitable carriers are typically large, slowly metabolized molecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates, or the like.

[087] For the preparation of pharmaceutical compositions or vaccines, the DNA, expression cassette or vector according to the present invention, in particular a recombinant

orthopoxvirus or avipoxvirus such as recombinant MVA or recombinant FPV is converted into a physiologically acceptable form. For MVA and FPV, this can be done based on the experience in the preparation of poxvirus vaccines used for vaccination against smallpox (as described by Stickl, H. et al. [1974] Dtsch. med. Wschr. 99, 2386-2392). For example, the purified virus is stored at −80° C. with a titre of 5×10^8 TCID50/ml formulated in about 10 mM Tris, 140 mM NaCl pH 7.4. For the preparation of vaccine shots, e.g., 10^1-10^9 particles of the recombinant virus according to the present invention are lyophilized in phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule. Alternatively, the vaccine shots can be produced by stepwise freeze-drying of the virus in a formulation. This formulation can contain additional additives such as mannitol, dextran, sugar, glycine, lactose or polyvinylpyrrolidone or other additives such as antioxidants or inert gas, stabilizers or recombinant proteins (e.g. human serum albumin) suitable for in vivo administration. A typical virus containing formulation suitable for freeze-drying comprises 10 mM Tris-buffer, 140 mM NaCl, 18.9 g/l Dextran (MW 36000-40000), 45 g/l Sucrose, 0.108 g/l L-glutamic acid mono potassium salt monohydrate pH 7.4. The glass ampoule is then sealed and can be stored between 4° C. and room temperature for several months. However, as long as no need exists the ampoule is stored preferably at temperatures below −20° C.

[088] For vaccination or therapy the lyophilisate or the freeze-dried product can be dissolved in 0.1 to 0.5 ml of an aqueous solution, preferably water, physiological saline or Tris buffer, and administered either systemically or locally, i.e. by parenteral, intramuscular or any other path of administration know to the skilled practitioner. The mode of administration, the dose and the number of administrations can be optimized by those skilled in the art in a known manner.

[089] In one or more embodiments, the invention further concerns a method for introducing a coding sequence into a target cell, such as human cell for therapeutic purposes, comprising the introduction of the nucleic acids, promoters, recombinant proteins, and/or expression cassettes according to the present invention into the target cell. Exemplary human target cells can include antigen presenting cells (APCs) such as dendritic cells, macrophages and other non APC'S such as fibroblasts, tumor cells, and so forth.

[090] The invention further relates to a method for producing a peptide, protein and/or virus comprising the infection of a host cell with a recombinant virus according to the present invention, followed by the cultivation of the infected host cell under suitable conditions, and further followed by the isolation and/or enrichment of the peptide and/or protein and/or viruses produced by said host cell. If it is intended to produce, i.e. amplify the virus according to the present invention the cell has to be a cell in which the virus is able to replicate. For poxviruses, in particular MVA, suitable cells are CEF (chicken embryonic fibroblast) or BHK (baby hamster kidney) cells. For avipoxviruses, such as fowlpoxvirus, suitable cells include CEF or CED (chicken embryonic dermal) cells. If it is intended to produce a peptide/protein encoded by the recombinant virus according to the present invention the cell may be any cell that can be infected by the recombinant virus vector and that allows the expression of the virus encoded proteins/peptides.

[091] The invention further relates to a method for producing a peptide, protein and/or virus comprising the transfection of a cell with the expression cassette, a nucleic acid, promoter, recombinant protein, and/or expression cassette DNA according to the present invention, followed by the infection of the cell with a poxvirus. The infected host cell is cultivated under suitable conditions. A further step comprises the isolation and/or enrichment of the peptide

and/or protein and/or viruses produced by said host cell. The step of infecting the cells with a poxvirus may be made before or after the step of transfection of the cells.

[092] The invention further relates to cells comprising a nucleic acid, promoter, recombinant protein, and/or expression cassette according to the present invention. In particular the invention relates to cells infected with the recombinant virus according to the present invention.

Examples

[093] The following examples further illustrate the present invention. It will be understood by a person of skill in the art that the provided examples in no way may be interpreted as limiting the applicability of the technology.

Example 1: Expression of Brachyury in Human DCs infected with recombinant fowlpox viruses

[094] To identify expression of Brachyury protein, human dendritic cells (DC) were infected with a positive control virus, recombinant MVA comprising Brachyury and TRICOM, at a multiplicity of infection (MOI) of 2.5. Human DCs were also infected with a negative control non-recombinant fowlpox (FPV-WT) or recombinant fowlpox virus strains comprising of a Brachyury expression cassette and TRICOM in accordance with the present disclosure. Each FPV strain, (listed in more detail in Table 1) including FPV-WT, FPV-mBN343A, FPV-mBN344A, and FPV-mBN345A were used to infect DCs with an MOI of 20. FPVBrachyury expression was detected via western blot analysis performed with a rabbit monoclonal anti-Brachyury antibody. The housekeeping protein glyceraldehyde 3-phoshate dehyrdrogenase (GAPDH) was also detected via western blot analysis as a loading control.

Table 1. Recombinant fowlpox virus strains comprising a Brachyury expression cassette and TRICOM

Recombinant FPV strain	Expression Cassette
FPV-mBN343A	SEQ ID NO: 74
FPV-mBN344A	SEQ ID NO: 75
FPV-mBN345A	SEQ ID NO: 72
FPV-mBN354A	SEQ ID NO: 76
FPV-mBN355A	SEQ ID NO: 73

[095] Results are shown in Figure 1. Expression of Brachyury was detected with the FPV-mBN343A and FPVmBN345 recombinant FPVs, but was not detected using the FPV-mBN344A recombinant FPV. Brachyury expression was also detected with MVA-Brachyury-TRICOM. Similar loading of samples was demonstrated by GAPDH expression.

Example 2: Expression of Brachyury in Human DCs infected with recombinant fowlpox viruses

[096] Expression-levels of Brachyury protein were also compared between additional recombinant FPV strains expressing Brachyury with different promoters. Human dendritic cells (DC) were infected with an MOI of 5 of the positive control recombinant MVA comprising Brachyury and TRICOM, and the negative control non-recombinant strain MVA-WT. Human DCs were also infected with a recombinant FPVs comprising of a Brachyury expression cassette and TRICOM in accordance with the present disclosure (e.g, FPV-mBN343A, FPV-mBN344A, FPV-mBN345A, FPV-mBN354A, FPV-mBN355A, See Table 1). Human DCs were additionally infected with a recombinant Fowlpoxvirus (FPV) comprising a Brachyury expression cassette having either a Vaccinia Virus (VV)-40k promoter or a PrS promoter. The non-recombinant FPV-WT strain served as a negative control. All of the FPVs were used at an MOI of 40. Brachyury expression was detected via western blot analysis performed with a rabbit monoclonal anti-Brachyury antibody. GAPDH was also detected as a loading control.

[097] Results are shown in Figure 2. Expression of Brachyury was detected with the FPV-mBN343A and FPV-mBN345A recombinant FPVs, but was not detected using the FPV-mBN344A and FPV-mBN354A recombinant FPV. More particularly, expression of Brachyury was detected at lower levels for the recombinant FPVs having the VV-40k or PrS promoters driving Brachyury (i.e., FVP-mBN281A, FVP-mBN249B). No Brachyury expression was detected in the negative controls (uninfected DCs, MVA-WT, or FPV-WT). Similar loading of samples was demonstrated by GAPDH expression.

[098] The Brachyury expression levels from the Western blot shown in Example 2 were normalized relative to expression of the housekeeping gene GAPDH, which is expected to be expressed at equivalent levels between cells. The intensity of each Brachyury and GAPDH band was measured and a ratio was calculated between the intensity of the Brachyury and GAPDH bands within the same sample.

[099] Results are shown in Figure 3. Among the FPV constructs, the highest expression of Brachyury relative to GAPDH was detected in DCs infected with FPV-mBN355A. Moderate relative Brachyury expression was detected in DCs infected with FPV-mBN343A or FPV-mBN345A. The lowest relative Brachyury expression was detected from the recombinant FPVs having the VV-40k or PrS promoters driving Brachyury (i.e., FVP-mBN281A, FVP-mBN249B). Highest relative expression of Brachyury was observed from infection with the positive control virus MVA-Brachyury-TRICOM; no Brachyury expression was detected in the negative control samples. Therefore, among the recombinant FPV strains, superior expression of Brachyury was induced by vectors driving Brachyury expression from the FPV-mBN355, FPV-mBN345, or FPV-mBN344 promoters.

Example 3: Expression of Brachyury and TRICOM in CMMT cells infected with recombinant fowlpox viruses

[0100] Expression of the Brachyury and TRICOM proteins was also assessed by flow cytometry using fluorescently labeled antibodies specific for each protein. CMMT cells (a rhesus macaque mammary tumor cell line) were infected with the positive control recombinant MVA comprising Brachyury and TRICOM, or with a recombinant FPVs comprising of a Brachyury expression cassette and TRICOM in accordance with the present disclosure (e.g, FPV-mBN343A, FPV-mBN345A), or with the recombinant FPV having the PrS promoter driving Brachyury (FVP-mBN249B). Cells were infected with an MOI below 1, so that a mixture of uninfected and infected cells were analyzed. Uninfected CMMT cells served as a negative control.

[0101] FACS samples were acquired on the BD LSRII or Fortessa and analyzed using BD FACSDIVA software (BD Bioscience, San Jose, CA) or FlowJo (TreeStar Inc., Ashland, OR).

[0102] Results are shown in Figure 4. Histograms of the signals detected for Brachyury and the three TRICOM proteins (CD80, CD54, and CD58) were plotted, and gates were drawn where positive signal was detected (black lines). Among the FPV constructs, the highest expression of Brachyury (shown by the biggest shift in signal along the x-axis) was detected in CMMT cells infected with FPV-mBN343A or FPV-mBN345A. The lowest Brachyury expression was detected from the recombinant FPV with the PrS promoter driving Brachyury (i.e., FVP-mBN281A, FVP-mBN249B). Similar expression levels of the TRICOM proteins were detected among the FPV constructs. Expression of Brachyury and the TRICOM proteins were also observed from infection with the positive control virus MVA-Brachyury-TRICOM.

[0103] To quantify the expression level of Brachyury in infected cells the median fluorescence intensity (MFI) was calculated for the Brachyury-positive cells gated in Figure 4.

[0104] Results are shown in Figure 5. Among the FPV constructs, the highest Brachyury MFI was detected in CMMT cells infected with FPV-mBN343A or FPV-mBN345A. The lowest Brachyury MFI was detected from the recombinant FPV with the PrS promoter driving Brachyury (FVP-mBN249B). The highest Brachyury MFI of all tested constructs was in cells infected with the positive control virus MVA-Brachyury-TRICOM. Therefore, the median expression level of Brachyury in infected CMMT cells was higher from vectors driving Brachyury expression from the FPV-mBN345 or FPV-mBN344 promoters than from the PrS promoter.

Example 4: Expression of Brachyury from FPV-mBN345B

[0105] The drug selection cassette used to initially generate the recombinant FPV-mBN345A strain was removed to generate a recombinant vector suitable for clinical development. This was accomplished by passaging the virus on chicken embryonic fibroblast (CEF) cells without drug selection, plaque purifying individual clones, and identifying clones lacking the selection cassette by PCR and DNA sequencing. This resulted in generation of FPV-mBN345B which comprises of a Brachyury expression cassette driven by the I3+15aa promoter and TRICOM, but does not contain the drug selection cassette.

[0106] Expression of Brachyury and the TRICOM proteins from FPV-mBN345B was confirmed in CMMT cells by infecting them with FPV-BN345B at an MOI of 0.625 to infect a subset of cells, or of 40 to infect all cells.

[0107] Results are shown in Figure 6. Histograms of the signals detected for Brachyury and the three TRICOM proteins (CD80, CD54, and CD58) were plotted with a red lines for

samples infected with an MOI of 0.625 and blue lines for samples infected with an MOI of 40. Uninfected cells served as a negative control (black lines). At the MOI of 0.625, expression of Brachyury and the three TRICOM proteins was detected as a peak shifted to the right of the taller peak from uninfected cells. At the MOI of 40, expression of Brachyury and TRICOM was detected in all cells as a peak shifted to the right of the uninfected cells.

[0108] Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

CLAIMS

We claim:

1. An expression cassette for expression of a coding sequence, the expression cassette comprising:

a. a promoter comprising a nucleic sequence having at least 70% identity to any one of SEQ ID NOs: 1-10, and 77; and

b. a coding sequence operably linked to the promoter;

wherein expression of the coding sequence is controlled by the promoter.

2. The expression cassette of claim 1, wherein the promoter comprises a nucleic acid sequence having at least 75%, 80%, 85%, or 90% identity to any one of SEQ ID NOs: 1-10, and 77.

3. The expression cassette of claims 1-2, wherein the promoter is selected from a nucleic acid sequence capable of hybridizing to anyone of SEQ ID NOs:1-10, and 77.

4. The expression cassette of claim 1-3, wherein the expression cassette is not an expression cassette that naturally occurs in the genome of a poxvirus.

5. The expression cassette of claims 1-4, wherein the promoter comprises a nucleic acid sequence having at least 70% identity to any one of SEQ ID NO:4 and SEQ ID NO:5.

6. The expression cassette of claims 1-4, wherein the promoter comprises a nucleic acid sequences selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:5.

7. The expression cassette of claims 1-4, wherein the promoter is SEQ ID NO:9, or a subsequence thereof.

8. The expression cassette of claim 7, wherein the subsequence is selected from SEQ ID NOs: 4 and 5.

9. The expression cassette of claims 1-8, wherein the coding sequence encodes for at least one: (a) therapeutic protein or peptide, antigen, antigenic epitope, antisense RNA, tumor-associated antigen or epitope, or ribozyme.

10. The expression cassette of claims 1-8, wherein the coding sequence encodes for at least one tumor-associated antigen (TAA).

11. The expression cassette of claims 1-10, wherein the coding sequence encodes for at least one TAA selected from CEA, MUC-1, PAP, PSA, HER-2, survivin, tyrosine related protein 1 (tyrp1), tyrosine related protein 2 (tyrp2), Brachyury antigen, or combinations thereof.

12. The expression cassette of claims 1-11, wherein the coding sequence encodes for a brachury antigen.

13. The expression cassette of claim 12, wherein the brachyury antigen is Brachury L254V.

14. The expression cassette of claim 12, wherein the coding sequence comprises a nucleic acid sequence encoding a brachyury antigen selected from the group consisting of: SEQ ID NO: 11 and SEQ ID NO:13.

15. The expression cassette of claim 12, wherein the coding sequence is selected from the group consisting of: SEQ ID NO: 12, SEQ ID NO:14, and SEQ ID NO:15.

16. The expression cassette of claims 1-11, wherein the coding sequence comprises a nucleic acid encoding for a brachury antigen selected from the group consisting of: SEQ ID NOs: 11, 13, 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63 66-67, 70, and 71.

17. The expression cassette of claims 1-11, wherein the coding sequence comprises a nucleic acid encoding for a brachury antigen selected from the group consisting of: SEQ ID NOs: 13, 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42, and 43.

18. The expression cassette of claims 1-11, wherein the coding sequence comprises a nucleic acid encoding for a brachury antigen selected from the group consisting of: SEQ ID NOs: 26-27, 30-31, 38-39, 42, and 43.

19. The expression cassette of claims 1-11, wherein the coding sequence comprises a nucleic acid having at least 70% identity to nucleic acid selected from the group consisting of: SEQ ID NOs: 24-25, 28-29, 36-37, 40, and 41.

20. The expression cassette of claims 1-11, wherein the coding sequence comprises a nucleic acid having at least 70% identity to SEQ ID NOs: 25 or 29, or a subsequence thereof.

21. The expression cassette of claims 1-11, wherein the coding sequence comprises a nucleic acid having at least 70% sequence identity to a nucleic acid selected from the group consisting of: 12, 14-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69.

22. An expression cassette comprising a nucleic acid selected from the group consisting of: SEQ ID NOs: 72-76.

23. An expression cassette comprising a nucleic acid selected from the group consisting of: SEQ ID NO:72, SEQ ID NO: 73, and SEQ ID NO:74.

24. A promoter for enhancing expression of a coding sequence, the promoter comprising or consisting of a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-10, and 77, or a derivative sequence of any one of SEQ ID NOs: 1-10, and 77, wherein the derivative has one or more substitutions, deletions, and/or insertions, and wherein the derivative sequence is active as a poxvirus promoter and/or active in a poxvirus infected cell.

25. A promoter for enhancing expression of a coding sequence, the promoter comprising or consisting of a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-10, and 77, or a subsequence thereof.

26. A promoter for enhancing expression of a coding sequence, the promoter comprising or consisting of a nucleic acid sequence having at least 70% identity to SEQ ID NO:9,or a subsequence thereof.

27. The promoter of claim 26, wherein the subsequence is selected from the group consisting of SEQ ID NOs: 3-8.

28. A synthetic nucleic acid having at least 70% identity to a nucleic acid encoding for a brachury antigen selected from the group consisting: SEQ ID NOs: 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71, or a subsequence thereof.

29. A synthetic nucleic acid selected from the group consisting of: 16-17, 20-21, 24-25, 28-29, 32-33, 36-37, 40-41, 44-45, 48-49, 52-53, 56-57, 60-61, 64-65, 68, and 69, or a subsequence thereof.

30. A synthetic protein selected from the group consisting of: 18-19, 22-23, 26-27, 30-31, 34-35, 38-39, 42-43, 46-47, 50-51, 54-55, 58-59, 62-63, 66-67, 70, and 71, or a subsequence thereof.

31. A vector comprising the expression cassette of claims 1-23, the promoter of claims 24-27, the nucleic acid of claims 28-29, and/or the protein of claim 30.

32. The vector of claim 31, wherein the vector is a plasmid.

33. The vector of claim 31, wherein the vector is a recombinant virus.

34. The vector of claim 33, wherein the recombinant virus is a poxvirus.

35. The vector of claim 34, wherein the poxvirus is an avipoxvirus.

36. The vector of claim 35, wherein the avipoxvirus is a fowlpox virus.

37. The vector of claims 31-36, wherein the expression cassette is inserted into a deletion site of the poxvirus genome.

38. The vector of claims 31-35, wherein the expression cassette is inserted into an intergenic region of the poxviral genome.

39. The vector of claims 33-34, wherein the vector is an orthopoxvirus.

40. The vector of claim 39, wherein the orthopoxvirus is selected from a vaccinia virus, a modified Ankara Virus (MVA), or MVA-BN.

41. A vaccine, medicament, or pharmaceutical composition comprising: a) an expression cassette according to any one of claims 1-23, b) a promoter of claims 24-27, c) a nucleic acid of claims 28-29, d) a protein of claim 30, or e) a vector according to any one of claims 31-40.

42. A cell comprising: a) an expression cassette according to any one of claims 1-23, b) a promoter of claims 24-27, c) a nucleic acid of claims 28-29, d) a protein of claim 30, or e) a vector according to any one of claims 31-40.

43. A method for introducing a coding sequence into a target cell comprising introducing into the target cell a) an expression cassette according to any one of claims 1-23, b) a promoter of claims 24-27, c) a nucleic acid of claims 28-29, or d) a vector according to any one of claims 31-40.

44. A method for producing a peptide, protein, or virus comprising infection of a host cells with a a) an expression cassette according to any one of claims 1-23, b) a promoter of claims 24-27, c) a nucleic acid of claims 28-29, or d) a vector according to any one of claims 31-40.

45. A method for expressing a nucleic acid comprising:

a. providing an expression vector or virus comprising a) an expression cassette according to any one of claims 1-23, b) a promoter of claims 24-27, c) a nucleic acid of claims 28-29; and
b. subjecting the expression vector or virus to conditions conducive to expression of the expression cassette, the promoter, or the nucleic acid.

46. Use of a) an expression cassette according to any one of claims 1-23, b) a promoter of claims 24-27, c) a nucleic acid of claims 28-29, d) a protein of claim 30, or e) a vector according to any one of claims 31-40 in the preparation of a medicament preferably a vaccine.

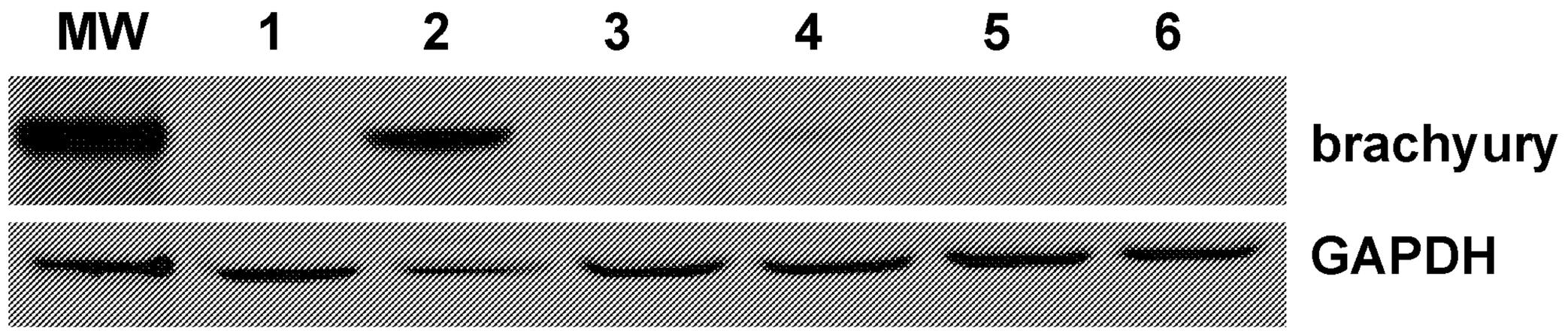
47. Use of a) an expression cassette according to any one of claims 1-23, b) a promoter of claims 24-27, c) a nucleic acid of claims 28-29, d) a protein of claim 30, or e) a vector according to any one of claims 31-40 for expressing a nucleic acid.

48. An a) an expression cassette according to any one of claims 1-23, b) a promoter of claims 24-27, c) a nucleic acid of claims 28-29, d) a protein of claim 30, or e) a vector according to any one of claims 31-40 for use as a medicament preferably a vaccine.

49. An a) an expression cassette according to any one of claims 1-23, b) a promoter of claims 24-27, c) a nucleic acid of claims 28-29, or d) a vector according to any one of claims 31-40 for use in a method for introducing a coding sequence into a target cell

Figure 1

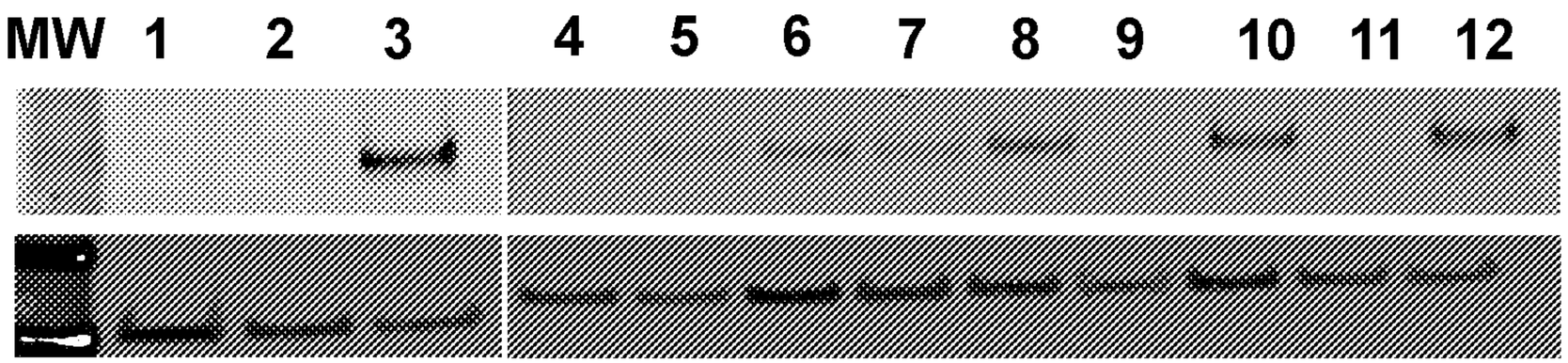
Expression of Brachyury in human DCs



1. Uninfected DCs
2. MVA-Brachyury-TRICOM
3. FPV-WT
4. FPV-mBN343A
5. FPV-mBN344A
6. FPV-mBN345A

Figure 2

Expression of Brachyury in human DCs



1 Uninfected DCs
2 MVA-WT
3 MVA-Brachyury-TRICOM
4 FPV-WT
5 FPV-mBN249B
6 FPV-mBN281A, cl 32
7 FPV-mBN281A, cl 35
8 FPV-mBN343A
9 FPV-mBN344A
10 FPV-mBN345A
11 FPV-mBN354A
12 FPV-mBN355A

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CA 02990549

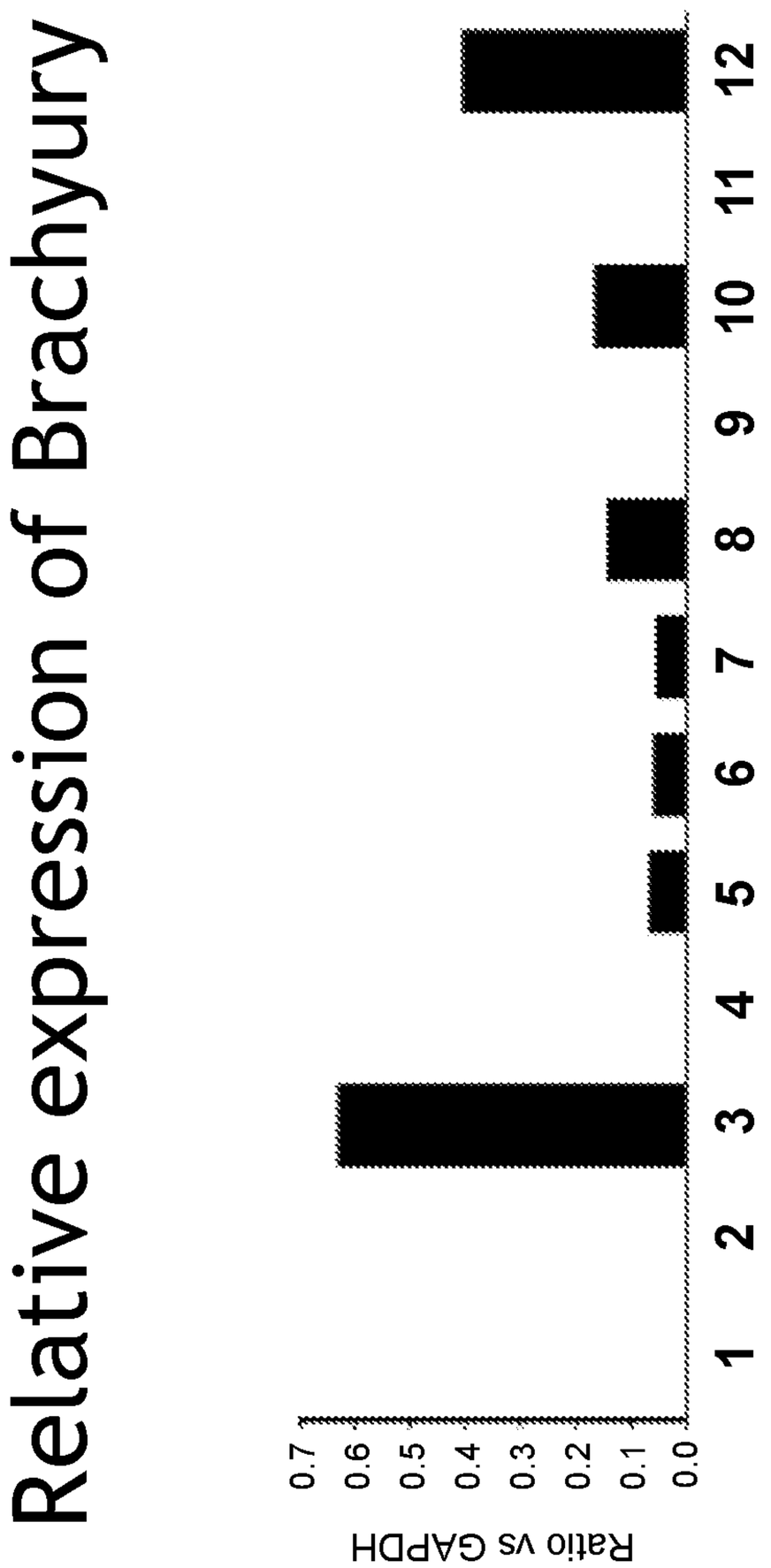
Figure 3
Relative expression of Brachyury
Ratio vs GAPDH
0
.1
.2
.3
.4
.5
.6
.7
1 2 3 4 5 6 7 8 9 10 11 12
1 Uninfected DCs
2 MVA-WT
3 MVA-Brachyury-TRICOM
4 FPV-WT
5 FPV-mBN249B
6 FPV-mBN281A, cl 32
7 FPV-mBN281A, cl 35
8 FPV-mBN343A
9 FPV-mBN344A
10 FPV-mBN345A
11 FPV-mBN354A
12 FPV-mBN355A

Figure 4

Expression of Brachyury and TRICOM in CMMT cells

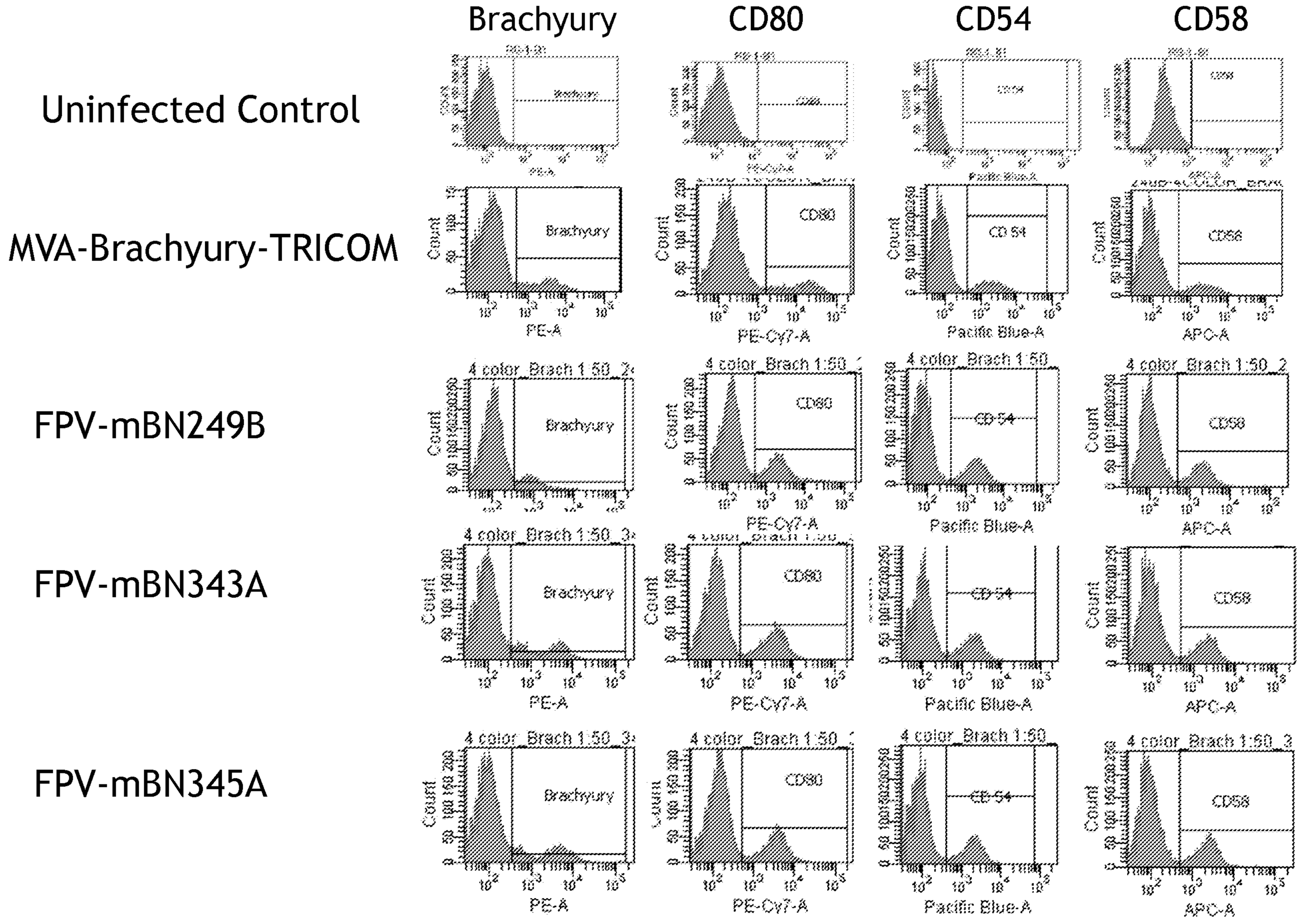

Figure 5

Brachyury Expression Level in CMMT cells

Median fluorescence intensity (MFI)

	Brachyury
Uninfected control	584
MVA240B	3783
FPV249B	788
FPV-mBN343A#37	1732
FPV-mBN345A#37	2391

Figure 6 Expression of Brachyury and TRICOM from FPV-mBN345B