[0008] Additionally, conventional AAV virions with capsids are produced by introducing a plasmid or plasmids containing the AAV genome, rep genes, and cap genes (Grimm *et al.*, 1998). Upon introduction of these helper plasmids in *trans*, the AAV genome is "rescued" (*i.e.*, released and subsequently amplified) from the host genome, and is further encapsidated (viral capsids) to produce biologically active AAV vectors. However, such encapsidated AAV virus vectors were found to inefficiently transduce certain cell and tissue types. The capsids also induce an immune response.

[0009] Accordingly, use of adeno-associated virus (AAV) vectors for gene therapy is limited due to the single administration to patients (owing to the patient immune response), the limited range of transgene genetic material suitable for delivery in AAV vectors due to minimal viral packaging capacity (about 4.5kb) of the associated AAV capsid, as well as the slow AAV-mediated gene expression. The applications for rAAV clinical gene therapies are further encumbered by patient-to-patient variability not predicted by dose response in syngeneic mouse models or in other model species.

[0010] Recombinant capsid-free AAV vectors can be obtained as an isolated linear nucleic acid molecule comprising an expressible transgene and promoter regions flanked by two wild-type AAV inverted terminal repeat sequences (ITRs) including the Rep binding and terminal resolution sites (TRS). These recombinant AAV vectors are devoid of AAV capsid protein encoding sequences, and can be single-stranded, double-stranded or duplex with one or both ends covalently linked through the two wild-type ITR palindrome sequences (*e.g.*, WO2012/123430, U.S. Patent 9,598,703). They avoid many of the problems of AAV-mediated gene therapy in that the transgene capacity is much higher, transgene expression onset is rapid, and the patient immune system does recognize the DNA molecules as a virus to be cleared. However, constant expression of a transgene may not be desirable in all instances, and AAV canonical wild type ITRs may not be optimized for ceDNA function. Therefore, there remains an important unmet need for controllable recombinant DNA vectors as well as an improved production and/or expression properties.

SUMMARY

[0011] The disclosure described herein relates to an improved production of a non-viral capsid-free DNA vector with covalently-closed ends (referred to herein as a "closed-ended DNA vector" or a "ceDNA vector"). The ceDNA vectors produced by the methods as described herein are capsid-free, linear duplex DNA molecules formed from a continuous strand of complementary DNA with covalently-closed ends (linear, continuous and non-encapsidated structure), which comprise a 5' inverted terminal repeat (ITR) sequence and a 3' ITR sequence that are different, or asymmetrical with respect to each other.

[0012] The technology described herein relates to the production of a ceDNA vector or an AAV vector in a cell (*e.g.*, insect cell, mammalian cell) or in a cell free system with a *single Rep* protein species. In particular, the present disclosure is based, in part, on the surprising finding that either Rep78 or Rep68, alone, is sufficient for production of a ceDNA vector or an AAV vector in a cell. This is an improved and more efficient method of ceDNA vector production than described in the prior art,where AAV or ceDNA vectors are produced in cells (*e.g.*, insect cells) requiring *two* Rep proteins; for example, at least one small

Rep protein (*e.g.*, Rep52 or Rep40) and at least one large Rep protein (*e.g.*, Rep78 or Rep68). That is, the prior art describes that production of ceDNA vectors or AAV vectors is carried out using two Rep proteins, either encoded on separate nucleic acid constructs each operatively linked to a promoter, or two Rep proteins encoded on a single nucleic acid construct with two initiation sites, operatively linked to a single promoter.

[0012a] In one aspect, the present disclosure provides a DNA vector obtained from a vector polynucleotide, wherein the vector polynucleotide encodes a heterologous nucleic acid operatively positioned between a first inverted terminal repeat DNA polynucleotide sequence (ITR) and a second ITR, wherein at least one of the first ITR and the second ITR comprises a nucleotide sequence corresponding to an AAV Rep binding sequence to induce replication of the DNA vector in a cell in the presence of a single species of Rep protein, the DNA vector being obtainable from a method comprising the steps of:

a. incubating a population of cells harboring the vector polynucleotide, which is devoid of viral capsid coding sequences, in the presence of a single species of Rep protein having at least DNA binding and DNA nicking functionality, under conditions effective and for a time sufficient to induce production of the DNA vector within the cells, wherein the cells do not comprise viral capsid coding sequences, wherein no other species of Rep proteins are present, and wherein the cells are not contacted with a nucleotide sequence encoding a second Rep protein; and

b. harvesting and isolating the resultant DNA vector from the cells.

[0012b] In another aspect, the present disclosure provides a DNA vector obtained from a vector polynucleotide, wherein the vector polynucleotide encodes a heterologous nucleic acid operatively positioned between two different inverted terminal repeat sequences (ITRs), wherein at least one of the ITRs is a functional ITR comprising a functional terminal resolution site and a Rep binding site; the presence of a single species of Rep protein inducing replication of the vector polynucleotide and production of the DNA vector in a cell, the DNA vector being obtainable from a method comprising the steps of:

a. incubating a population of cells harboring the vector polynucleotide, which is devoid of viral capsid coding sequences, in the presence of a single species of Rep protein that has at least DNA binding and DNA nicking functionality under conditions effective and for time sufficient to induce production of the DNA vector within the cells, wherein the cells do not comprise any nucleic acid encoding Rep52 or Rep40 within the cells, wherein no other species of Rep are present in the cell, and wherein the cells are not contacted with a nucleotide sequence encoding a second Rep protein; and

b. harvesting and isolating the DNA vector from the cells.

[0012c] In yet another aspect, the present disclosure provides a polynucleotide for generating a DNA vector comprising a nucleotide sequence encoding a single species of Rep protein amino acid sequence that has at least DNA binding and DNA nicking functionality operatively linked to at least one expression control sequence.

[0012d] In yet another aspect, the present disclosure provides a method of producing a DNA vector, the method comprising contacting a cell with:

(1) a nucleotide sequence encoding a single species of Rep protein that has at least DNA binding and DNA nicking functionality, linked to at least one expression control sequence, wherein the cell does not express any other species of Rep protein and is not contacted with any other species of Rep protein;
(2) a double-stranded DNA construct comprising:

an expression cassette;

a first ITR on the upstream (5'-end) of the expression cassette; and

a second ITR on the downstream (3'-end) of the expression cassette, and

(3) harvesting the DNA vector.

[0012e] In yet another aspect, the present disclosure provides a DNA vector generated by the method as described herein. In a further aspect, the present disclosure provides a pharmaceutical composition comprising the DNA vector as described herein; and optionally, an excipient.

[0012f] In yet another aspect, the present disclosure provides a kit for producing a DNA vector, the kit comprising:

an expression construct comprising at least one restriction site for insertion of at least one heterologous nucleotide sequence, or a regulatory switch, or both, the at least one restriction site operatively positioned between asymmetric inverted terminal repeat sequences (asymmetric ITRs), wherein at least one of the asymmetric ITRs comprises a functional terminal resolution site and a Rep binding site; and

a vector comprising a polynucleotide sequence that encodes a single species of Rep protein, wherein the vector is suitable for expressing the single species of Rep protein in an insect cell.

[0012g] In yet another aspect, the present disclosure provides a cell comprising: a nucleotide sequence encoding a single species of AAV Rep protein that has at least DNA binding and DNA nicking functionality, operably linked to at least one expression control sequence, wherein the cell does not express any other parvovirus Rep protein and is not contacted with any other species of Rep protein; and optionally a double-stranded DNA construct comprising an expression cassette; a first ITR on the upstream (5'-end) of the expression cassette; and a second ITR on the downstream (3'-end) of the expression cassette.

[0013] Accordingly, one example of the technology described herein relates to a nucleic acid construct for the production of DNA vectors, *e.g.*, ceDNA vectors and other recombinant parvovirus (*e.g.* adeno-associated virus) vectors in cells (*e.g.* insect cells, mammalian cells) and cell free systems, where, for example, the insect cells or cell free system comprises a first nucleotide sequence encoding a single parvoviral Rep protein, where the nucleotide sequence does not have an open reading frame (ORF) and lacks a functional initiation codon downstream of the first initiation codon and/or lacks alternative splicing sites preventing exon skipping, thereby enabling the translation of only a single parvoviral Rep protein (*e.g.*, a Rep78 or Rep 68 protein) without the translation of additional Rep proteins at the later initiation codon (*e.g.*, any one or more of Rep52 or Rep 40) in the insect cells or cell free system. That is, a nucleic acid encoding Rep78 does not also produce a Rep52 protein, and similarly, a nucleic acid encoding Rep68 does not produce a Rep40 protein. Further no other Rep protein is present or expressed in the system.

[0014] In some examples, the methods and compositions described herein to use a single Rep protein can be used in the production of any ceDNA vector, including but not limited to, a ceDNA vector comprising asymmetric ITRS as disclosed in International Patent Application PCT/US18/49996, filed on September 7, 2018 (see, e.g, Examples 1-4); a ceDNA vector for gene editing as disclosed on the International Patent Application PCT/US18/64242 filed on December 6, 2018 (see, *e.g.*, Examples 1-7), or a ceDNA vector for production of antibodies or fusion proteins, as disclosed in the International Patent Application PCT/US19/18016, filed on February 14, 2019, (*e.g.*, see Examples 1-4), all of which are incorporated by reference in their entireties herein. In some examples, it is also envisioned that the methods and compositions described herein using a single Rep protein can be used in the synthetic production of a ceDNA vector, *e.g.*, in a cell free or insect-free system of ceDNA production, as disclosed in International Application PCT/US19/14122, filed on January 18, 2019, incorporated by reference in its entirety herein, where the single Rep protein can be used for protein-assisted ligation of the ITR oligonucleotides therein.

[0015] The technology described herein relates to an improved method of production of a ceDNA vector containing at least one modified AAV inverted terminal repeat sequence (ITR) and an expressible transgene. The ceDNA vectors disclosed herein can be produced according to the described methods in eukaryotic cells, thus devoid of prokaryotic DNA modifications and bacterial endotoxin contamination in insect cells.

[0016] Examples of the disclosure relate to methods and compositions to produce ceDNA vectors and AAV vectors using a single Rep protein as described herein. Other examples relate to a ceDNA vector produced by the methods and compositions as provided herein.

[0017] In one example, non-viral capsid-free DNA vectors with covalently-closed ends produced by the methods as described herein are preferably linear duplex molecules, and are obtainable from a vector polynucleotide that encodes a heterologous nucleic acid operatively positioned between two different inverted terminal repeat sequences (ITRs) (*e.g.* AAV ITRs), wherein at least one of the ITRs comprises a terminal resolution site and a replication protein binding site (RPS) (sometimes referred to as a replicative protein binding site), *e.g.* a Rep binding site, and one of the ITRs comprises a deletion, insertion, or substitution with respect to the other ITR. That is, one of the ITRs is asymmetrical relative to the other ITR. In one example, at least one of the ITRs is an AAV ITR, *e.g.* a wild type AAV ITR or modified AAV ITR. In one example, at least one of the ITRs is a modified ITR relative to the other ITR – that is, the ceDNA comprises ITRs that are asymmetric relative to each other. In one example, at least one of the ITRs is a non-functional ITR.

[0018] In some examples, a ceDNA vector produced by the methods and compositions as described herein comprises: (1) an expression cassette comprising a cis-regulatory element, a promoter and at least one transgene; or (2) a promoter operably linked to at least one transgene, and (3) two self-complementary sequences, *e.g.*, ITRs, flanking said expression cassette, wherein the ceDNA vector is not associated with a capsid protein. In some examples, the ceDNA vector comprises two self-complementary sequences found in an AAV genome, where at least one comprises an operative Rep-binding element (RBE) (also sometimes

referred to herein as "RBS") and a terminal resolution site (trs) of AAV or a functional variant of the RBE, and one or more cis-regulatory elements operatively linked to a transgene. In some examples, the ceDNA vector comprises additional components to regulate expression of the transgene, for example, regulatory switches, which are described herein in the section entitled "Regulatory Switches" for controlling and regulating the expression of the transgene, and can include a regulatory switch, *e.g.*, a kill switch to enable controlled cell death of a cell comprising a ceDNA vector.

[0019] In some examples, the two self-complementary sequences can be ITR sequences from any known parvovirus, for example a dependovirus such as AAV (*e.g.*, AAV1-AAV12). Any AAV serotype can be used, including but not limited to a modified AAV2 ITR sequence, that retains a Rep-binding site (RBS) such as 5′-GCGCGCTCGCTCGCTC-3′ (SEQ ID NO: 531) and a terminal resolution site (trs) in addition to a variable palindromic sequence allowing for hairpin secondary structure formation. In some examples, the ITR is a synthetic ITR sequence that retains a functional Rep-binding site (RBS) such as 5'-GCGCGCTCGCTCGCTC-3' (SEQ ID NO: 531) and a terminal resolution site (TRS) in addition to a variable palindromic sequence allowing for hairpin secondary structure formation. In some examples, a modified ITR sequence retains the sequence of the RBS, trs and the structure and position of a Rep binding element forming the terminal loop portion of one of the ITR hairpin secondary structure from the corresponding sequence of the wild-type AAV2 ITR.

[0020] Exemplary ITR sequences for use in the ceDNA vectors produced by the methods and compositions as described herein can be any one or more of Tables 2-10A and 10B, or SEQ ID NO: 2, 52, 101-499 and 545-547 or the partial ITR sequences shown in FIG. 26A-26B. In some examples, the ceDNA vectors produced by the methods and compositions as described herein do not have an ITR that comprises any sequence selected from SEQ ID NOs: 500-529.

[0021] In some examples, a ceDNA vector produced by the methods and compositions as described herein can comprise an ITR with a modification in the ITR corresponding to any of the modifications in ITR sequences or ITR partial sequences shown in any one or more of Tables 2, 3, 4, 5, 6, 7, 8, 9, 10A and 10B herein.

[0022] As an exemplary example, a closed-ended DNA vector produced by the methods and compositions as described herein comprises a promoter operably linked to a transgene, where the ceDNA is devoid of capsid proteins and is: (a) produced from a ceDNA-plasmid (*e.g.*, see Examples 1-2 and/or FIGS. 1A-B) that encodes a mutated right side AAV2 ITR having the same number of intramolecularly duplexed base pairs as SEQ ID NO:2 or a mutated left side AAV2 ITR having the same number of intramolecularly duplexed base pairs as SEQ ID NO:51 in its hairpin secondary configuration (preferably excluding deletion of any AAA or TTT terminal loop in this configuration compared to these reference sequences), and (b) is identified as ceDNA using the assay for the identification of ceDNA by agarose gel electrophoresis under native gel and denaturing conditions in Example 1. Examples of such modified ITR sequences are provided in Tables 2, 3, 4, 5, 6, 7, 8, 9, 10A and 10B herein.

[0023] The technology described herein further relates to production of a ceDNA vector that can be used to deliver and encode one or more transgenes in a target cell, for example, where the ceDNA vector comprises a multicistronic sequence, or where the transgene and its native genomic context (*e.g.*, transgene, introns and endogenous untranslated regions) are together incorporated into the ceDNA vector. The transgenes can be protein encoding transcripts, non-coding transcripts, or both. The ceDNA vector produced by the methods and compositions as described herein can comprise multiple coding sequences, and a non-canonical translation initiation site or more than one promoter to express protein encoding transcripts, non-coding transcripts, or both. The transgene can comprise a sequence encoding more than one proteins, or can be a sequence of a non-coding transcript. The expression cassette can comprise, *e.g.*, more than 4000 nucleotides, 5000 nucleotides, 10,000 nucleotides or 20,000 nucleotides, or 30,000 nucleotides, or 40,000 nucleotides or 50,000 nucleotides, or any range between about 4000-10,000 nucleotides or 10,000-50,000 nucleotides, or more than 50,000 nucleotides. The ceDNA vectors produced by the methods and compositions as described herein do not have the size limitations of encapsidated AAV vectors, thus enable delivery of a large-size expression cassette to provide efficient expression of transgenes. In some examples, the ceDNA vector produced by the methods and compositions as described herein is devoid of prokaryote-specific methylation.

[0024] The expression cassette of a ceDNA vector produced by the methods and compositions as described herein can also comprise an internal ribosome entry site (IRES) and/or a 2A element. The cis-regulatory elements include, but are not limited to, a promoter, a riboswitch, an insulator, a mir-regulatable element, a post-transcriptional regulatory element, a tissue- and cell type-specific promoter and an enhancer. In some examples the ITR can act as the promoter for the transgene. In some examples, the ceDNA vector comprises additional components to regulate expression of the transgene. For example, the additional regulatory component can be a regulator switch as disclosed herein, including but not limited to a kill switch, which can kill the ceDNA infected cell, if necessary, and other inducible and/or repressible elements.

[0025] The technology described herein further provides novel methods of efficiently producing a ceDNA vector or other AAV vector that can selectively express one or more transgenes. A ceDNA vector produced by the methods and compositions as described herein has the capacity to be taken up into host cells, as well as to be transported into the nucleus in the absence of the AAV capsid. In addition, the ceDNA vectors produced by the methods and compositions as described herein described herein lack a capsid and thus avoid the immune response that can arise in response to capsid-containing vectors.

[0026] In one example, the capsid free non-viral DNA vector (ceDNA vector) is obtained from a plasmid (referred to herein as a "ceDNA-plasmid") comprising a polynucleotide expression construct template comprising in this order: a first 5' inverted terminal repeat (*e.g.* AAV ITR); an expression cassette; and a 3' ITR (*e.g.* AAV ITR), where at least one of the 5' and 3' ITR is a modified ITR, or where when both the 5' and 3' ITRs are modified, they have different modifications from one another and are not the same

sequence. In such an example, the ceDNA vector is obtained by the process as exemplified in the Examples and shown in FIG. 4A-4D herein, where only a single Rep protein is required for the production.

[0027] A ceDNA vector is obtainable by a number of means that would be known to the ordinarily skilled artisan after reading this disclosure. For example, a polynucleotide expression construct template used for generating the ceDNA vectors of the present disclosure can be a ceDNA-plasmid (*e.g.* see Table 12 or FIG. 10B), a ceDNA-bacmid, and/or a ceDNA-baculovirus. In one example, the ceDNA-plasmid comprises a restriction cloning site (*e.g.* SEQ ID NO: 7) operably positioned between the ITRs where an expression cassette comprising *e.g.*, a promoter operatively linked to a transgene, *e.g.*, a reporter gene and/or a therapeutic gene) can be inserted. In some examples, ceDNA vectors are produced from a polynucleotide template (*e.g.*, ceDNA-plasmid, ceDNA-bacmid, ceDNA-baculovirus) containing an ITR modified as compared to the corresponding flanking AAV3 ITR or wild-type AAV2 ITR sequence, where the modification is any one or more of deletion, insertion, and/or substitution.

[0028] According to some examples, the disclosure provides a method for producing a ceDNA vector in an insect cell (*e.g.*, Sf9, Sf21, Trichoplusia ni cells, and High Five cells) or mammalian cell (*e.g.*, HEK293, Huh-7, HeLa, HepG2, HeplA, 911, CHO, COS, MeWo, NIH3T3, A549, HT1080, monocytes, and mature and immature dendritic cells); the method comprising culturing an insect cell or mammalian cell comprising a first nucleotide sequence encoding a single parvoviral Rep protein, where the first nucleotide sequence lacks a functional initiation codon downstream of the first initiation codon and lacks alternative splicing sites preventing exon skipping, thereby enabling the translation of only a single Rep protein (*e.g.*, a Rep78) without the translation of additional Rep proteins at the later initiation codon (*e.g.*, any one or more of Rep52 or Rep40) or a spliced variant of the full-length (*e.g.*, Rep68) in the cell.

[0029] According to some other examples, the disclosure provides a method for producing a ceDNA vector in an insect cell (*e.g.*, Sf9, Sf21, Trichoplusia ni cells, and High Five cells) or mammalian cell (*e.g.*, HEK293, Huh-7, HeLa, HepG2, HeplA, 911, CHO, COS, MeWo, NIH3T3, A549, HT1080, monocytes, and mature and immature dendritic cells); the method comprising culturing an insect cell or mammalian cell comprising a first nucleotide sequence encoding a signle parvoviral Rep protein, wherein the first nucleotide sequence lacks a functional initiation codon downstream of the first inititation codon and contains a deletion of a carboxy terminal spliced sequence (*e.g.*, any portion or full-length of a c-terminal intron/skipped exon), thereby enabling the translation of only a signle Rep protein (*e.g.*, a Rep68) without the translation of additional Rep proteins at the later initiation codon (*e.g.*, any one or more of Rep52 or Rep40) or the full-length Rep72 protein in the cell.

[0030] According to some other examples, the disclosure provides a method for producting a ceDNA vector in an insect cell (*e.g.*, Sf9, Sf21, Trichoplusia ni cells, High Five cells) or mammalian cell (*e.g.*, HEK293, Huh-7, HeLa, HepG2, HeplA, 911, CHO, COS, MeWo, NIH3T3, A549, HT1080, monocytes, and mature and immature dendritic cells); the method comprising culturing an insect cell or mammalian cell comprising a first nucleotide sequence encoding one or two Rep protein (*e.g.*, a Rep 78 and/or Rep68

protein), wherein the first nucleotide sequence lacks a functional initiation codon downstream of the first initiation codon and intact alternative splicing sites, thereby enabling the translation of a Rep78 and/or Rep68 protein only, without the translation of additional Rep proteins at the later initiation codon (*e.g.*, any one or more of Rep52 or Rep40).

[0031] The cell described in the methods above can further comprise a second nucleotide sequence comprising at least one AAV inverted terminal repeat (ITR) sequence flanking a heterologous sequence under conditions such that when the first sequence is expressed to produce Rep78 and/or Rep68, a ceDNA is produced by the Rep78 and/or Rep68 protein, without the presence of Rep52 or Rep40. The ceDNA vector then can be recovered from the cell. According to some examples, the nucleotide sequence comprising at least one AAV is part of an expression construct. According to some examples, the heterologous sequence comprises a therapeutic nucleic acid. According to some examples, the therapeutic nucleic acid is part of an expression construct. According to some examples, the cell further comprises a nucleic acid that serves as a marker. According to some examples, the nucleic acid that serves as a marker is part of an expression construct.

[0032] In a permissive host cell, in the presence of *e.g.*, a *single* Rep protein, the polynucleotide template having at least one modified ITR replicates to produce ceDNA vectors. ceDNA vector production undergoes two steps: (i) the single Rep proteins results in an excision ("rescue") step of template from the template backbone (*e.g.* ceDNA-plasmid, ceDNA-bacmid, ceDNA-baculovirus genome *etc*.), and (ii) the single Rep protein mediates replication of the excised ceDNA vector. The single Rep protein required for the exision and replication steps (i) and (ii) can be any Rep protein described herein. Rep proteins and Rep binding sites of the various AAV serotypes are well known to those of ordinary skill in the art.

[0033] One of ordinary skill understands to choose a Rep protein from a serotype that binds to and replicates the nucleic acid sequence based upon at least one functional ITR. For example, if the replication competent ITR is from AAV serotype 2, the corresponding Rep would be from an AAV serotype that works with that serotype such as AAV2 ITR with AAV2 or AAV4 Rep but not AAV5 Rep, which does not. Upon replication (*i.e.*, after step (ii)), the covalently-closed ended ceDNA vector continues to accumulate in permissive cells and ceDNA vector is preferably sufficiently stable over time in the presence of the single Rep protein under standard replication conditions, *e.g.* to accumulate in an amount that is at least 1 pg/cell, preferably at least 2 pg/cell, preferably at least 3 pg/cell, more preferably at least 4 pg/cell, even more preferably at least 5 pg/cell.

[0034] Accordingly, one example of the disclosure relates to a process comprising the steps of: a) incubating a population of host cells (*e.g.* insect cells) harboring the polynucleotide expression construct template (*e.g.*, a ceDNA-plasmid, a ceDNA-bacmid, and/or a ceDNA-baculovirus), which is devoid of viral capsid coding sequences, in the presence of a *single* Rep protein under conditions effective and for a time sufficient to induce production of the ceDNA vector within the host cells, and wherein the host cells do not comprise viral capsid coding sequences; and b) harvesting and isolating the ceDNA vector from the host

cells. The presence of a single Rep protein induces replication of the vector polynucleotide with a modified ITR to produce the ceDNA vector in a host cell. However, no viral particles (*e.g.* AAV virions) are expressed. Thus, there is no virion-enforced size limitation. It is envisioned that if the nucleic acid sequence encoding the Rep protein encodes a large Rep protein, *e.g.*, a Rep78 or Rep 68 protein, that the initiation codon for the smaller Rep proteins is modified such that only the large Rep protein is expressed in the cell.

[0035] According to some examples, the disclosure provides an insect cell (*e.g.*, Sf9, Sf21, Trichoplusia ni cells, and High Five cells) or mammalian cell (*e.g.*, HEK293, Huh-7, HeLa, HepG2, HeplA, 911, CHO, COS, MeWo, NIH3T3, A549, HT1080, monocytes, and mature and immature dendritic cells); the insect cell or mammalian cell-line comprising a first nucleotide sequence encoding a single parvoviral Rep protein, where the first nucleotide sequence lacks a functional initiation codon downstream of the first initiation codon and lacks alternative splicing sites preventing exon skipping, thereby enabling the translation of only a single Rep protein (*e.g.*, a Rep78) without the translation of additional Rep proteins at the later initiation codon (*e.g.*, any one or more of Rep52 or Rep40) or a spliced variant of the full-length (*e.g.*, Rep68) in the cell.

[0036] According to some other examples, the disclosure provides an insect cell (*e.g.*, Sf9, Sf21, Trichoplusia ni cells, and High Five cells) or mammalian cell (*e.g.*, HEK293, Huh-7, HeLa, HepG2, HeplA, 911, CHO, COS, MeWo, NIH3T3, A549, HT1080, monocytes, and mature and immature dendritic cells); the insect cell or mammalian cell comprising a first nucleotide sequence encoding a signle parvoviral Rep protein, wherein the first nucleotide sequence lacks a functional initiation codon downstream of the first inititation codon and contains a deletion of a carboxy terminal spliced sequence (*e.g.*, any portion or full-length of a c-terminal intron/skipped exon), thereby enabling the translation of only a signle Rep protein (*e.g.*, a Rep68) without the translation of additional Rep proteins at the later initiation codon (*e.g.*, any one or more of Rep52 or Rep40) or the full-length Rep72 protein in the cell.

[0037] According to some other examples, the disclosure provides an insect cell (*e.g.*, Sf9, Sf21, Trichoplusia ni cells, High Five cells) or mammalian cell (*e.g.*, HEK293, Huh-7, HeLa, HepG2, HeplA, 911, CHO, COS, MeWo, NIH3T3, A549, HT1080, monocytes, and mature and immature dendritic cells); the insect cell or mammalian cell-line comprising a first nucleotide sequence encoding one or two Rep protein (*e.g.*, a Rep 78 and/or Rep68 protein), wherein the first nucleotide sequence lacks a functional initiation codon downstream of the first initiation codon and intact alternative splicing sites, thereby enabling the translation of a Rep78 and/or Rep68 protein only, without the translation of additional Rep proteins at the later initiation codon (e.g., any one or more of Rep52 or Rep40)

[0038] The cell described above can further comprise a second nucleotide sequence comprising at least one AAV inverted terminal repeat (ITR) sequence flanking a heterologous sequence under conditions such that when the first sequence is expressed to produce Rep78 and/or Rep68, a ceDNA is produced by the Rep78 and/or Rep68 protein, without the presence of Rep52 or Rep40.The ceDNA vector then can be recovered from the cell. According to some examples, the nucleotide sequence comprising at least one AAV is part of

an expression construct. According to some examples, the heterologous sequence comprises a therapeutic nucleic acid. According to some examples, the therapeutic nucleic acid is part of an expression construct. According to some examples, the cell further comprises a nucleic acid that serves as a marker. According to some examples, the nucleic acid that serves as a marker is part of an expression construct.

[0039] According to some examples, the disclosure provides a cell free system comprising a first nucleotide sequence encoding a single parvoviral Rep protein, where the nucleotide sequence lacks a functional initiation codon downstream of the first initiation codon and/or lacks alternative splicing sites preventing exon skipping, thereby enabling the translation of only a single parvoviral Rep protein (*e.g.*, a Rep78 or Rep 68 protein) without the translation of additional Rep proteins at the later initiation codon (*e.g.*, any one or more of Rep52 or Rep 40) in the cell free system. According to some examples, a nucleic acid encoding Rep78 does not also produce a Rep52 or Rep40 protein. According to some examples, a nucleic acid encoding Rep68 does not produce a Rep52 or Rep40 protein. According to some examples, the insect cell, the mammalian cell or the cell free system does not express any other Rep protein.

[0040] A ceDNA vector produced according to the methods as described herein using a single Rep protein, is isolated from the host cells, and its presence can be confirmed by digesting DNA isolated from the host cell with a restriction enzyme having a single recognition site on the ceDNA vector and analyzing the digested DNA material on denaturing and non-denaturing gels to confirm the presence of characteristic bands of linear and continuous DNA as compared to linear and non-continuous DNA.

CLAIMS

What is claimed is:

1. A DNA vector obtained from a vector polynucleotide, wherein the vector polynucleotide encodes a heterologous nucleic acid operatively positioned between a first inverted terminal repeat DNA polynucleotide sequence (ITR) and a second ITR, wherein at least one of the first ITR and the second ITR comprises a nucleotide sequence corresponding to an AAV Rep binding sequence to induce replication of the DNA vector in a cell in the presence of a single species of Rep protein, the DNA vector being obtainable from a method comprising the steps of:

a. incubating a population of cells harboring the vector polynucleotide, which is devoid of viral capsid coding sequences, in the presence of a single species of Rep protein having at least DNA binding and DNA nicking functionality, under conditions effective and for a time sufficient to induce production of the DNA vector within the cells, wherein the cells do not comprise viral capsid coding sequences, wherein no other species of Rep proteins are present, and wherein the cells are not contacted with a nucleotide sequence encoding a second Rep protein; and

b. harvesting and isolating the resultant DNA vector from the cells.

2. The DNA vector of claim 1, wherein the single species of Rep protein further has helicase, ligase, and ATPase functionality;
the single species of Rep protein is an AAV Rep protein;
the single species of Rep protein is selected from any of: an AAV1, an AAV2, an AAV3, an AAV4, an AAV5, an AAV6, an AAV7, an AAV8, an AAV9, an AAV10, an AAV11, or an AAV12 Rep protein;
the single species of Rep protein is an AAV2 Rep 68 protein; and/or
the single species of Rep protein is an AAV2 Rep 78 protein.

3. The DNA vector of claim 2, wherein if the Rep protein is a Rep 78 protein, the Rep 78 protein is encoded by a mutant Rep78 nucleotide sequence
and wherein:
the mutant Rep78 nucleotide sequence does not have a functional translational initiation codon for Rep 52; the mutant Rep 78 nucleotide sequence encodes a mutant Rep 78 protein which comprises a mutation at amino acid position 225 of SEQ ID NO: 588; and/or

the mutant Rep 78 nucleotide sequence encodes a mutant Rep78 protein which comprises a sequence having at least 95% sequence identity to SEQ ID NO: 588 and has at least DNA binding and DNA nicking functionality.

4. The DNA vector of claim 1, wherein the ITR is a parvovirus ITR.

5. The DNA vector of claim 1, wherein the DNA vector is a non-viral capsid-free double-stranded DNA vector with covalently closed ends (ceDNA vector).

6. The DNA vector of claim 13, wherein the presence of the ceDNA vector isolated from the cells can be confirmed by digesting DNA isolated from the cells with a restriction enzyme having a single recognition site on the DNA vector, and analyzing the digested DNA material on a non-denaturing gel to confirm the presence of characteristic bands of linear and continuous DNA as compared to linear and non-continuous DNA.

7. A DNA vector obtained from a vector polynucleotide, wherein the vector polynucleotide encodes a heterologous nucleic acid operatively positioned between two different inverted terminal repeat sequences (ITRs), wherein at least one of the ITRs is a functional ITR comprising a functional terminal resolution site and a Rep binding site; the presence of a single species of Rep protein inducing replication of the vector polynucleotide and production of the DNA vector in a cell, the DNA vector being obtainable from a method comprising the steps of:

a. incubating a population of cells harboring the vector polynucleotide, which is devoid of viral capsid coding sequences, in the presence of a single species of Rep protein that has at least DNA binding and DNA nicking functionality under conditions effective and for time sufficient to induce production of the DNA vector within the cells, wherein the cells do not comprise any nucleic acid encoding Rep52 or Rep40 within the cells, wherein no other species of Rep are present in the cell, and wherein the cells are not contacted with a nucleotide sequence encoding a second Rep protein; and

b. harvesting and isolating the DNA vector from the cells.

8. A polynucleotide for generating a DNA vector comprising a nucleotide sequence encoding a single species of Rep protein amino acid sequence that has at least DNA binding and DNA nicking functionality operatively linked to at least one expression control sequence.

9. The polynucleotide of claim 17, wherein the Rep protein has helicase, ligase, and ATPase functionality;

the Rep protein is an AAV Rep protein;

the Rep protein is selected from any of: an AAV1, an AAV2, an AAV3, an AAV4, an AAV5, an AAV6, an AAV7, an AAV8, an AAV9, an AAV10, an AAV11, or an AAV12 Rep protein;
the Rep protein is an AAV2 Rep protein; and/or
the Rep protein is an AAV2 Rep 78 protein.

10. The polynucleotide of claim 10, wherein if the Rep protein is a Rep 78 protein the Rep 78 protein is encoded by a mutant Rep78 nucleotide sequence
and wherein:
the mutant Rep78 nucleotide sequence does not have a functional initiation codon for Rep 52; the mutant Rep 78 nucleotide sequence encodes a mutant Rep 78 protein which comprises a mutation at amino acid position 225 of SEQ ID NO: 588; and/or
the mutant Rep 78 nucleotide sequence encodes a mutant Rep78 protein which comprises a sequence having at least 95% sequence identity to SEQ ID NO: 588 and has at least DNA binding and DNA nicking functionality.

11. The polynucleotide of claim 8, wherein the DNA vector is a non-viral capsid-free double stranded DNA vector with covalently closed ends (ceDNA vector).

12. The polynucleotide of claim 11, wherein presence of the ceDNA vector isolated from the cells can be confirmed by digesting DNA isolated from the cells with a restriction enzyme having a single recognition site on the DNA vector and analyzing the digested DNA material on a non-denaturing gel to confirm the presence of characteristic bands of linear and continuous DNA as compared to linear and non-continuous DNA.

13. A method of producing a DNA vector, the method comprising contacting a cell with:
(1) a nucleotide sequence encoding a single species of Rep protein that has at least DNA binding and DNA nicking functionality, linked to at least one expression control sequence, wherein the cell does not express any other species of Rep protein and is not contacted with any other species of Rep protein;
(2) a double-stranded DNA construct comprising:
an expression cassette;
a first ITR on the upstream (5’-end) of the expression cassette; and
a second ITR on the downstream (3’-end) of the expression cassette, and
(3) harvesting the DNA vector.

14. The method of claim 13, wherein the Rep protein is an AAV Rep protein;

the Rep protein is selected from any of: an AAV1, an AAV2, an AAV3, an AAV4, an AAV5, an AAV6, an AAV7, an AAV8, an AAV9, an AAV10, an AAV11, or an AAV12 Rep protein;
the Rep protein is an AAV2 Rep 68 protein; and/or
the Rep protein is an AAV2 Rep 78 protein.

15. The method of claim 14, wherein if the Rep protein is a Rep 78 protein, the Rep 78 protein is encoded by a mutant Rep78 nucleotide sequence
and wherein:
the mutant Rep78 nucleotide sequence does not have a functional initiation codon for Rep 52;
the mutant Rep 78 nucleotide sequence encodes a mutant Rep 78 protein which comprises a mutation at amino acid position 225 of SEQ ID NO: 588; and/or
the mutant Rep 78 nucleotide sequence encodes a mutant Rep78 protein which comprises a sequence having at least 95% sequence identity to SEQ ID NO: 588.

16. The method of claim 13, wherein the double-stranded DNA construct is a bacmid, a plasmid, a minicircle, or a linear double-stranded DNA molecule.

17. The method of claim 13, wherein the first ITR upstream of the expression cassette is a wild-type ITR or a modified ITR;
the second ITR downstream of the expression cassette is a wild-type ITR or a modified ITR;
the first ITR upstream of the expression cassette and the second ITR downstream of the expression cassette are symmetrical or substantially symmetrical, or are asymmetrical relative to each other;
the ITRs are replication-competent;
the ITRs are AAV ITRs
wherein the wild-type ITR comprises SEQ ID NO: 51;
wherein the modified ITR comprises SEQ ID NO: 2;
wherein the modified ITR comprises SEQ ID NO: 52;
wherein the wild-type ITR comprises SEQ ID NO: 1; and/or
the ITR sequences are selected from any of those listed in Tables 2, 4A, 4B and 5 of International Patent Application PCT/US18/65242.

18. The method of claim 13, wherein said expression cassette further comprises an exogenous sequence.

19. The method of claim 13, wherein the DNA vector has a linear and continuous structure.

20. A DNA vector generated by the method of claim 13.

21. A pharmaceutical composition comprising the DNA vector of claim 20; and optionally, an excipient.

22. A kit for producing a DNA vector, the kit comprising:

an expression construct comprising at least one restriction site for insertion of at least one heterologous nucleotide sequence, or a regulatory switch, or both, the at least one restriction site operatively positioned between asymmetric inverted terminal repeat sequences (asymmetric ITRs), wherein at least one of the asymmetric ITRs comprises a functional terminal resolution site and a Rep binding site; and

a vector comprising a polynucleotide sequence that encodes a single species of Rep protein, wherein the vector is suitable for expressing the single species of Rep protein in an insect cell.

23. The kit of claim 64, further comprising a population of insect cells which is devoid of viral capsid coding sequences, that in the presence of a single species of Rep protein can induce production of the DNA vector.

24. A cell comprising: a nucleotide sequence encoding a single species of AAV Rep protein that has at least DNA binding and DNA nicking functionality, operably linked to at least one expression control sequence, wherein the cell does not express any other parvovirus Rep protein and is not contacted with any other species of Rep protein; and optionally a double-stranded DNA construct comprising an expression cassette; a first ITR on the upstream (5'-end) of the expression cassette; and a second ITR on the downstream (3'-end) of the expression cassette.

25. The cell of claim 24, wherein the cell is an insect cell or a mammalian cell.

26. The cell of claim 24, wherein the nucleotide sequence encoding a single species of AAV Rep protein encodes Rep78 or Rep68.

27. The cell of claim 26, wherein the nucleotide sequence does not have a functional initiation codon for Rep52 or Rep40.

28. The cell of claim 27, wherein the nucleotide sequence encodes Rep78 protein or Rep68 protein;

the nucleotide sequence encodes a mutant Rep78 protein which comprises a mutation at amino acid position 225 of SEQ ID NO: 588;
the nucleotide sequence is full length and contains intact alternative splicing sites in the carboxy terminal end, resulting in production of both Rep78 and Rep68;
the nucleotide sequence comprises a deletion of a carboxy terminal intron sequence, resulting in production of Rep68 only; and/or
the nucleotide sequence encodes a Rep78 protein which comprises a sequence having at least 95% sequence identity to SEQ ID NO: 588 and has at least DNA binding and DNA nicking functionality.

29. The cell of claim 27, wherein amino acid 225 (methionine) of SEQ ID NO: 588 is mutated to a glycine (Gly) or threonine (Thr).

30. The cell of claim 27, wherein the nucleotide sequence further comprises one or more modifications in alternative splicing sites in the carboxy terminus, preventing a splicing event leading to production of Rep68, thereby enabling production of Rep78 only.