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~~example, 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~~example, 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~~example, 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~~one aspect, the present disclosure provides a method of producing a non-viral capsid-free double-stranded DNA vector with covalently closed ends (ceDNA vector), the method comprising contacting a cell with:

(1) nucleic acid~~nucleotide~~ sequence encoding a single species of replication (Rep) protein that has at least DNA binding and DNA nicking functionality, wherein the single species of Rep protein is an AAV2 Rep78 protein or an AAV2 Rep68 protein, and is 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 ceDNA vector.

[0012e] In yet another aspect, the present disclosure provides a non-viral capsid-free double-stranded DNA vector with covalently closed ends (ceDNA 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 non-viral capsid-free double-stranded DNA vector with covalently closed ends (ceDNA vector), the kit comprising:

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

a vector comprising a nucleic acid~~polynucleotide~~ sequence that encodes a single species of Rep protein, wherein the single species of Rep protein is an AAV2 Rep78 protein or an AAV2 Rep68 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 nucleic acid~~nucleotide~~ sequence encoding a single species of AAV replication (Rep) protein that has at least DNA binding and DNA nicking functionality, ~~operably~~ linked to at least one expression control sequence, wherein the single species of Rep protein is an AAV2 Rep78 protein or an AAV2 Rep68 protein, 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.

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.~~

1~~3~~. A method of producing a non-viral capsid-free double-stranded DNA vector with covalently closed ends (ceDNA vector), the method comprising contacting a cell with:
(1) a nucleic acid~~nucleotide~~ sequence encoding a single species of replication (Rep) protein that has at least DNA binding and DNA nicking functionality, wherein the single species of Rep protein is an AAV2 Rep78 protein or an AAV2 Rep68 protein, and is 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 ceDNA vector.

~~14~~2. The method of claim 1~~3~~, 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~~

3. The method of claim 13, wherein the Rep protein is an AAV2 Rep 78 protein.

~~15~~4. The method of claim ~~14~~1, ~~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 nucleic acid~~nucleotide~~ sequence does not have a functional initiation codon for Rep 52;
the mutant Rep-78 nucleic acid~~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 nucleic acid~~nucleotide~~ sequence encodes a mutant Rep78 protein which comprises a sequence having at least 95% sequence identity to SEQ ID NO: 588.

5. The method of claim 1, wherein:
the mutant Rep68 nucleic acid sequence does not have a functional initiation codon for Rep52;
the mutant Rep68 nucleic acid sequence encodes a mutant Rep68 protein which comprises a mutation at amino acid position 225 of SEQ ID NO: 579; and/or
the mutant Rep68 nucleic acid sequence encodes a mutant Rep68 protein which comprises a sequence having at least 95% sequence identity to SEQ ID NO: 579.

~~1~~6. The method of claim 1~~3~~, wherein the double-stranded DNA construct is a bacmid, a plasmid, a minicircle, or a linear double-stranded DNA molecule.

~~1~~7. The method of claim 1~~3~~, 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 symmetric~~al~~ or substantially symmetric~~al~~, or are asymmetric~~al~~ relative to each other;
the first ITR and the second ITR~~ITRs~~ are replication-competent;
one or both of the first ITR and the second ITR is an ~~the ITRs are~~ AAV ITR~~s~~;
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
one or both of the first ITR and the second ~~the~~ ITR comprise a sequence~~s are~~ selected from the group consisting of: any of those listed in Tables 2, 4A, 4B and 5 of International Patent Application PCT/US18/64242~~PCT/US18/65242~~.

~~1~~8. The method of claim 1~~3~~, wherein said expression cassette further comprises an exogenous sequence.

~~1~~9. The method of claim 1~~3~~, wherein the ceDNA vector has a linear and continuous structure.

~~20~~10. A non-viral capsid-free double-stranded DNA vector with covalently closed ends (ceDNA vector) generated by the method of claim 1~~3~~.

~~21~~11. A pharmaceutical composition comprising the ceDNA vector of claim 1~~2~~0; and optionally, an excipient.

~~22~~12. A kit for producing a non-viral capsid-free double-stranded DNA vector with covalently closed ends (ceDNA vector), the kit comprising:

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

a vector comprising a nucleic acid~~polynucleotide~~ sequence that encodes a single species of Rep protein, wherein the single species of Rep protein is an AAV2 Rep78 protein or an AAV2 Rep68 protein, wherein the vector is suitable for expressing the single species of Rep protein in an insect cell.

~~23~~13. The kit of claim ~~64~~12, 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~~14. A cell comprising: a nucleic acid~~nucleotide~~ sequence encoding a single species of AAV replication (Rep) protein that has at least DNA binding and DNA nicking functionality, ~~operably~~ linked to at least one expression control sequence, wherein the single species of Rep protein is an AAV2 Rep78 protein or an AAV2 Rep68 protein, 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~~15. The cell of claim ~~24~~14, wherein the cell is an insect cell or a mammalian cell.

~~26~~16. The cell of claim ~~24~~14, wherein the nucleic acid~~nucleotide~~ sequence encoding a single species of AAV Rep protein encodes ~~Rep78 or~~ Rep68.

17. The cell of claim 14, wherein the nucleic acid sequence encoding a single species of AAV Rep protein encodes Rep78.

~~27~~18. The cell of claim ~~26~~14, wherein the nucleic acid~~nucleotide~~ sequence does not have a functional initiation codon for Rep52 or Rep40.

~~28~~19. The cell of claim ~~27~~14, wherein ~~the nucleotide sequence encodes Rep78 protein or Rep68 protein;~~
the nucleic acid~~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 nucleic acid~~nucleotide~~ sequence comprises a deletion of a carboxy terminal intron sequence, resulting in production of Rep68 only; and/or
the nucleic acid~~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~~20. The cell of claim ~~27~~19, wherein amino acid 225 (methionine) of SEQ ID NO: 588 is mutated to a glycine (Gly) or threonine (Thr).

~~30~~21. The cell of claim ~~27~~14, wherein the nucleic acid~~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.