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(54) **PROCEDES DE PREPARATION DE NUCLEOTIDE INTEGRASES**

(54) **METHODS FOR PREPARING NUCLEOTIDE INTEGRASES**

(57) La présente invention concerne des procédés améliorés de fabrication de nucléotide intégrases de manipulation aisée. La présente invention concerne également une nucléotide intégrase et un procédé amélioré de production de complexes ARN-protéine destinés à la préparation in vitro de nucléotide intégrases. La préparation de nucléotide intégrase consiste à introduire, dans une cellule hôte, une molécule d'ADN à intron de groupe II qui s'exprime ensuite dans la cellule hôte de manière à former dans la cellule des particules ribonucléoprotéine à activité mucléotide intégrase. De telles particules ribonucléoprotéine comprennent un ARN à intron de groupe II excisé et une protéine codée par un intron de groupe II, tous deux codés par la molécule d'ADN introduite. On isole ensuite de la cellule la nucléotide intégrase. Dans un autre mode de réalisation, on prépare la nucléotide intégrase en combinant in vitro un ARN à intron de groupe II excisé, ou "ARN exogène", avec une protéine codée par un intron de groupe II. Dans un autre mode de réalisation, on prépare la nucléotide intégrase en combinant "l'ARN exogène" avec un complexe ARN-protéine comprenant une protéine codée par un intron de groupe II.

(57) The present invention provides new, improved and easily manipulable methods for making nucleotide integrases. The present method also relates to a nucleotide integrase and an improved method for making RNA-protein complexes for use in preparing nucleotide integrases in vitro. The nucleotide integrase is prepared by introducing a DNA molecule which comprises a group II intron DNA sequence into a host cell. The group II intron DNA sequence is then expressed in the host cell such that RNP particles having nucleotide integrase activity are formed in the cell. Such RNP particles comprise an excised group II intron RNA encoded by the introduced DNA molecule and a group II intron-encoded protein encoded by the introduced DNA molecule. Thereafter the nucleotide integrase is isolated from the cell. In another embodiment, the nucleotide integrase is prepared by combining in vitro an excised group II intron RNA, hereafter "exogenous RNA", with group II intron-encoded protein. In another embodiment, the nucleotide integrase is prepared by combining "exogenous RNA" with an RNA-protein complex which comprises a group II intron-encoded protein.

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(54) Title: METHODS FOR PREPARING NUCLEOTIDE INTEGRASES

(57) Abstract

The present invention provides new, improved and easily manipulable methods for making nucleotide integrases. The present method also relates to a nucleotide integrase and an improved method for making RNA–protein complexes for use in preparing nucleotide integrases in vitro. The nucleotide integrase is prepared by introducing a DNA molecule which comprises a group II intron DNA sequence into a host cell. The group II intron DNA sequence is then expressed in the host cell such that RNP particles having nucleotide integrase activity are formed in the cell. Such RNP particles comprise an excised group II intron RNA encoded by the introduced DNA molecule and a group II intron–encoded protein encoded by the introduced DNA molecule. Thereafter the nucleotide integrase is isolated from the cell. In another embodiment, the nucleotide integrase is prepared by combining in vitro an excised group II intron RNA, hereafter "exogenous RNA", with group II intron–encoded protein. In another embodiment, the nucleotide integrase is prepared by combining "exogenous RNA" with an RNA–protein complex which comprises a group II intron–encoded protein.

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METHODS FOR PREPARING NUCLEOTIDE INTEGRASES

BACKGROUND

Nucleotide integrases are molecular complexes that are capable of cleaving double stranded DNA substrates at specific recognition sites and of concomitantly inserting nucleic acid molecules into the DNA substrate at the cleavage site. Thus, nucleotide integrases are useful tools, particularly for genome mapping and for genetic engineering.

Structurally, nucleotide integrases are ribonucleoprotein (RNP) particles that comprise an excised, group II intron RNA and a group II intron-encoded protein, which is bound to the group II intron RNA. At present nucleotide integrases are made by two approaches. The first approach involves isolating the nucleotide integrase from source organisms; both the RNA and protein subunits of the nucleotide integrase are encoded by the DNA in such organisms. In order to obtain nucleotide integrases other than wild type, the source organisms are mutagenized. The mutagenesis is a laborious, multistep process which yields limited quantities of nucleotide integrase.

The second approach used to prepare nucleotide integrases involves combining, *in vitro*, an exogenous, excised, group II intron RNA, with an RNA-protein complex in which the group II intron-encoded protein is associated with a splicing defective group II intron RNA rather than the excised, group II intron RNA. Therefore, the RNA-protein complex lacks nucleotide integrase activity. The exogenous RNA displaces the splicing defective group II intron RNA to form a nucleotide integrase. The RNA-protein complex is obtained by isolating RNA-protein complex from source organisms. In order to obtain the RNA-protein complex or to obtain a group II intron-encoded protein other than wild type, the source organism must be mutagenized. The mutagenisis is a laborious, multistep process which yields limited quantities of the RNA-protein complex. Thus, this method also provides limited quantities of the nucleotide integrase.

Accordingly, it is desirable to have methods for preparing nucleotide integrase which are not laborious and which permit the nucleotide integrase to be readily modified from the wild type and which do not yield limited quantities of the nucleotide integrase.

SUMMARY OF THE INVENTION

The present invention provides new, improved, and easily manipulable methods for making nucleotide integrases.

In one embodiment, the nucleotide integrase is prepared by introducing a DNA molecule which comprises a group II intron DNA sequence into a host cell. The group II intron DNA sequence is then expressed in the host cell such that RNP particles having nucleotide integrase activity are formed in the cell. Such RNP particles comprise an excise introduced DNA molecule and a group II intron-encoded protein encoded by the introduced DNA molecule. Thereafter, the nucleotide integrase is isolated from the cell.

In another embodiment, the nucleotide integrase is prepared by combining *in vitro* an excised, group II intron RNA, referred to hereinafter as "exogenous RNA", with a group II intron-encoded protein. Preferably, the exogenous RNA is prepared by *in vitro* transcription of a DNA molecule which comprises the group II intron sequence. Preferably, the group II intron-encoded protein is made by introducing into a host cell a DNA molecule which comprises the open reading frame sequence of a group II intron, and then expressing the open reading frame sequence in the host cell such that the group II intron-encoded protein encoded by the open reading frame sequence is formed in the cell. Thereafter, the cell is fractionated and the protein is recovered.

In another embodiment, the nucleotide integrase is prepared by combining *in vitro* an excised, group II intron RNA, referred to hereinafter as "exogenous RNA", with an RNA-protein complex which comprises a group II intron-encoded protein. Preferably, the exogenous RNA is prepared by *in vitro* transcription of a DNA molecule which comprises the group II intron sequence. Preferably, the RNA-protein complex is made by introducing into a host cell a DNA molecule comprising a group II intron DNA sequence which encodes a splicing-defective group II intron RNA. Thereafter, the cell is fractionated and the RNA-protein complex is isolated.

The present invention also relates to a nucleotide integrase and an improved method for making RNA-protein complexes for use in preparing nucleotide integrases *in vitro*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the plasmid map of plasmid pETLtrA19.

Figure 2 shows the nucleotide sequence of the 2.8 kb HindIII fragment that is present in pETLtrA19 and that includes the Ll.HrB intron DNA sequence and portions of the nucleotide sequence of the flanking exons *ltrlBE1* and *ltrBE2*, SEQ. ID. NO. 1, the nucleotide sequence of the *ltrA*

open reading frame, SEQ. ID. NO. 2, and the amino acid sequence of the *ltrA* protein, SEQ. ID. NO. 3.

Figure 3 is the plasmid map of plasmid pETLtrA1-1.

Figure 4 is a schematic representation of the inserts in pLE12, pETLtrA19 and pETLtrA1-1.

Figure 5 is the sequence of the sense strand of the doublestranded DNA substrate, SEQ. ID. NO. 4, which was used to assess the nucleotide integrase activity of the nucleotide integrase which comprise an excised, L1.ltrB intron RNA and an *ltra* protein.

Figure 6a is a schematic depiction of the substrate which is cleaved by the nucleotide integrase comprising L1.ltrB intron RNA and the *ltra* protein, and Figure 6b shows the IBS1 and IBS2 sequences of the substrate and the cleavage sites of the doublestranded DNA substrate which is cleaved by this integrase.

DETAILED DESCRIPTION OF THE INVENTION

Nucleotide Integrases

Nucleotide integrases are enzymes that are capable of cleaving double stranded DNA substrates at specific recognition sites and of concomitantly inserting nucleic acid molecules into the DNA substrate at the cleavage site. The nucleotide integrases insert an RNA molecule into the sense strand of the cleaved DNA substrate and a cDNA molecule into the antisense strand of the cleaved DNA substrate.

Nucleotide integrases are ribonucleoprotein (RNP) particles that comprise an excised, group II intron RNA and a group II intron-encoded protein, which is bound to the group II intron RNA. "Excised group II intron RNA," as used herein, refers to the RNA that is, or that is derived from, an *in vitro* or *in vivo* transcript of the group II intron DNA and that lacks flanking exon sequences. The excised, group II intron RNA typically has six domains and a characteristic secondary and tertiary structure, which is shown in Saldahana et al. , 1993, Federation of the American Society of Experimental Biology Journal, p15-24, which is specifically incorporated herein by reference. The excised, group II intron RNA also includes at least one hybridizing region which is complementary to a recognition site on the substrate DNA. The hybridizing region has a nucleotide sequence, referred to hereinafter as the "EBS sequence", which is complementary to the sequence of the recognition site of the intended substrate DNA, referred to hereinafter as the "IBS sequence". The group II intron-encoded protein has an X domain, a reverse transcriptase domain,

and, preferably, a Zn domain. The X domain of the protein has a maturase activity. The Zn domain of the protein has Zn^{2+} finger-like motifs.

Group II intron RNA may be produced containing desired EBS sequences which hybridize to corresponding nucleotides on substrate DNA. In addition, group II intron RNA may be produced containing additional nucleotides in domain IV. In the methods of the present invention both of these group II RNA molecules are produced from an isolated DNA which is then introduced into a cell. Such isolated DNA typically is synthesized using a DNA synthesizer or is genetically-engineered, such as by *in vitro* site directed mutagenesis.

A. Preparation of the Nucleotide Integrase by Isolation from a Genetically-Engineered Cell.

In one embodiment, the nucleotide integrase is made by introducing an isolated DNA molecule which comprises a group II intron DNA sequence into a host cell. Suitable DNA molecules include, for example, viral vectors, plasmids, and linear DNA molecules. Following introduction of the DNA molecule into the host cell, the group II intron DNA sequence is expressed in the host cell such that excised RNA molecules encoded by the introduced group II intron DNA sequence and protein molecules encoded by is introduced group II intron DNA sequence are formed in the cell. The excised group II intron RNA and group II intron-encoded protein are combined within the host cell to produce the nucleotide integrase.

Preferably the introduced DNA molecule also comprises a promoter, more preferably an inducible promoter, operably linked to the group II intron DNA sequence. Preferably, the DNA molecule further comprises a sequence which encodes a tag to facilitate isolation of the nucleotide integrase such as, for example, an affinity tag and/or an epitope tag. Preferably, the tag sequences are at the 5′ or 3′ end of the open reading frame sequence. Suitable tag sequences include, for example, sequences which encode a series of histidine residues, the Herpes simplex glycoprotein D, i.e., the HSV antigen, or glutathione S-transferase. Typically, the DNA molecule also comprises nucleotide sequences that encode a replication origin and a selectable marker. Optionally, the DNA molecule comprises sequences that encode molecules that modulate expression, such as for example T7 lysozyme.

The DNA molecule comprising the group II intron sequence is introduced into the host cell by conventional methods, such as, by cloning the DNA molecule into a vector and by introducing the vector into the host

cell by conventional methods, such as electroporation or by $CaCl_2$-mediated transformation procedures. The method used to introduce the DNA molecule is related to the particular host cell used. Suitable host cells are those which are capable of expressing the group II intron DNA sequence. Suitable host cells include, for example, heterologous or homologous bacterial cells, yeast cells, mammalian cells, and plant cells. In those instances where the host cell genome and the group II intron DNA sequence use different genetic codes, it is preferred that the group II intron DNA sequence be modified to comprise codons that correspond to the genetic code of the host cell. The group II intron DNA sequence, typically, is modified by using a DNA synthesizer or by *in vitro* site directed mutagenesis to prepare a group II intron DNA sequence with different codons. Alternatively, to resolve the differences in the genetic code of the intron and the host cell, DNA sequences that encode the TRNA molecules which correspond to the genetic code of the group II intron are introduced into the host cell. Optionally, DNA molecules which comprise sequences that encode factors that assist in RNA or protein folding, or that inhibit RNA or protein degradation are also introduced into the cell.

The DNA sequences of the introduced DNA molecules are then expressed in the host cell to provide a transformed host cell. As used herein the term "transformed cell" means a host cell that has been genetically engineered to contain additional DNA, and is not limited to cells which are cancerous. Then the RNP particles having nucleotide integrase activity are isolated from the transformed host cells.

Preferably, the nucleotide integrase is isolated by lysing the transformed cells, such as by mechanically and/or enzymatically disrupting the cell membranes of the transformed cell. Then the cell lysate is fractionated into an insoluble fraction and soluble fraction. Preferably, an RNP particle preparation is isolated from the soluble fraction. RNP particle preparations include the RNP particles having nucleotide integrase activity as well as ribosomes, mRNA and tRNA molecules. Suitable methods for isolating RNP particle preparations include, for example, centrifugation of the soluble fraction through a sucrose cushion. The RNP particles, preferably, are further purified from the RNP particle preparation or from the soluble fraction by, for example, separation on a sucrose gradient, or a gel filtration column, or by other types of chromatography. For example, in those instances where the protein component of the desired RNP particle has been engineered to include a tag such as a series of histidine residues, the RNP particle may be further

purified from the RNP particle preparation by affinity chromatography on a matrix which recognizes and binds to the tag. For example, NiNTA Superflow from Qiagen, Chatsworth CA, is suitable for isolating RNP particles in which the group II intron-encoded protein has a His_6 tag.

B. Preparation of the Nucleotide Integrase by Combining Exogenous RNA with a Group II Intron-Encoded Protein to Form a Reconstituted RNP Particle

In another embodiment, the nucleotide integrase is formed by combining an isolated exogenous RNA with an isolated group II intron-encoded protein *in vitro* to provide a reconstituted RNP particle. Preferably the exogenous RNA is made by *in vitro* transcription of the group II intron DNA. Alternatively, the exogenous RNA is made by *in vitro* transcription of the group II intron DNA and the DNA of all, or portions, of the flanking exons to produce an unprocessed transcript which contains the group II intron RNA and the RNA encoded by the flanking exons or portions thereof. Then the exogenous RNA is spliced from the unprocessed transcript.

The purified group II intron-encoded protein is prepared by introducing into a host cell an isolated DNA molecule. The introduced DNA molecule comprises the DNA sequence of the open reading frame (ORF) sequence of the group II intron operably linked to a promoter, preferably an inducible promoter. Alternatively, 3S the introduced DNA molecule comprises (1) the ORF sequence and (2) at least some portion of the DNA sequence of the group II intron which lies outside of the ORF sequence and (3) a promoter which is oriented in the DNA molecule to control expression of the ORF sequence. Preferably, the introduced DNA molecule also comprises a sequence at the 5′ or 3′ end of the group II intron ORF which, when expressed in the host cell, provides an affinity tag or epitope on the N-terminus or C-terminus of the group II intronencoded protein. Tagging the protein in this manner facilitates isolation of the expressed protein. Thus, the DNA molecule may comprise at the 5′ or 3′ end of the ORF, for example, a sequence which encode a series of histidine residues, or the HSV antigen, or glutathione-S-transferase. These DNA molecules may also comprise at the 5′ or 3′ end of the ORF a sequence that encodes thioredoxin or any other molecule which enhances distribution of the protein encoded by the ORF into the soluble fraction of the host cell. Typically, the DNA molecule also comprises nucleotide sequences that encode a replication origin and a selectable marker.

Conventional methods are used to introduce these DNA molecules into any host cell which is capable of expressing the group II intron ORF sequence. For example, the $CaCl_2$-mediated transformation procedure as described by Sambrook et al. in "Molecular Coning A Laboratory Manual", pages 1-82, 1989, can be used to introduce the DNA molecules into E. coli cells. Suitable host cells include, for example, heterologous or homologous bacterial cells, yeast cells, mammalian cells, and plant cells. In those instances where the host cells either lack or have limiting amounts of the tRNA molecules for one or more of the codons which are present in the ORF, it is preferred that a DNA molecule encoding the rare tRNA molecules also be introduced into the host cell to increase the yield of the protein. Alternatively, the DNA sequence of the ORF is modified to match the preferred codon usage of the host cell.

The ORF sequence is then expressed in the host, preferably by adding a molecule which induces expression, to provide a transformed host cell. Then the transformed cell is lysed, and preferably fractionated into a soluble fraction and an insoluble fraction. Then the group II intron-encoded protein is isolated, preferably, from the soluble fraction. Methods of isolating the protein from the soluble fraction include, for example, chromatographic methods such as gel filtration chromatography, ion exchange chromatography, and affinity chromatography, which is particularly useful for isolating tagged protein molecules.

Following purification of the group II intron-encoded protein, the protein is incubated with the exogenous RNA, preferably in a buffer, to allow formation of the nucleotide integrase. Optionally, the protein and RNA are denatured prior to the incubation using guanidinium hydrochloride or urea. Then, during incubation, the denaturant is removed in the presence of cosolvents like salts and metal ions to allow proper folding of the protein and RNA in the nucleotide integrase.

C. Preparation of the Nucleotide Integrase by Combining Exogenous RNA with an RNA-Protein Complex.

Alternatively, the nucleotide integrase is prepared by combining the exogenous RNA with an RNA-protein complex that has been isolated from an organism that has been genetically engineered to produce an RNA-protein complex in which the desired group II intron-encoded protein molecules are associated with RNA molecules that include a splicing defective, group II intron RNA but which lack the excised group II RNA. Preferably, the

exogenous RNA is prepared by *in vitro* transcription of a DNA molecule which comprises the group II intron sequence.

Preferably, the RNA-protein complex is made by introducing into a host cell an isolated DNA molecule which comprises a group II intron sequence operably linked to a promoter, preferably an inducible promoter. The group II intron sequence encodes a splicing defective group II intron RNA. Typically, the DNA molecule also comprises nucleotide sequences that encode a replication origin and a selectable marker. Then the group II intron DNA sequence is expressed in the host cell. The group II intron encodes functional group II intron-encoded protein and a splicing-defective group II intron RNA. Thus, the RNA-protein complex made in this manner lack excised, group II RNA molecules that encode the group II intron-encoded protein. The RNA-protein complexes do, however, contain the functional group II intronencoded protein associated with RNA molecules that comprise the mutant, unspliced form of the group II intron RNA as well as other RNA molecules.

The resulting RNA-protein complex is isolated from the host cell and then incubated with the exogenous RNA, preferably in a buffer, to form the nucleotide integrase. During the incubation the group II intron-encoded protein becomes disassociated from the RNA molecules which are present in the RNA-protein complex and combines with the exogenous RNA to form the nucleotide integrase.

These methods enable production of increased quantities of nucleotide integrases. Conventional methods produce approximately 0.1 to 1 μg of nucleotide integrase per liter of cultured cells. In the present invention, at least 3 to 10 mg of nucleotide integrase is produced per liter of cultured cells. These methods also offer the further advantage of permitting the sequences of the RNA component and the protein component of the nucleotide integrase to be readily modified.

The following examples of methods for preparing a group II intron-encoded protein and for preparing nucleotide integrases are included for purposes of illustration and are not intended to limit the scope of the invention.

Preparing Nucleotide Integrases In Vivo

Example 1

A nucleotide integrase comprising an excised RNA which is encoded by the L1.ltrB intron of a lactococcal cojugative element prSO1 of Lactococcus lactis and the protein encoded by the ORF of the L1.ltrB intron were

prepared by transforming cells of the BLR(DE3) strain of the bacterium *Escherichia coli*, which has the recA genotype, with the plasmid pETRLtrA19. Plasmid pETLtrA19, which is schematically depicted in Figure 1, comprises the DNA sequence for the group II intron Ll.ltrB from *Lactococcus lactis*, shown as a thick line, positioned between portions of the flanking exons *ltrBE1* and *ltrBE2*, shown as open boxes. pETLtrA19 also comprises the DNA sequence for the T7 RNA polymerase promoter and the T7 transcription terminator. The sequences are oriented in the plasmid in such a manner that the ORF sequence, SEQ. ID. NO. 2, within the Ll.ltrB intron is under the control of the T7 RNA polymerase promoter. The ORF of the Ll.ltrB intron, shown as an arrow box, encodes the protein *ltra*. The sequence of the Ll.ltrB intron and the flanking exon sequences present in pETLtrA19 are shown in Figure 2 and SEQ. ID. NO. 1. Vertical lines in Figure 2 denote the junctions between the intron and the flanking sequences. The amino acid sequence of the *ltra* protein, SEQ. ID. NO. 4 is shown under the ORF sequence, SEQ. ID. NO. 2, in Figure 2. The exon binding sites are encoded by sequences from and including nucleotides 457 go and including 463 (EBS1) from and including nucleotides 401 to and including nucleotides 406 (EBS2a), and from and including nucleotides 367 to and including 367-372 (EBS2b). Domain IV is encoded by nucleotide 705 to 2572.

pETLtrA19 was prepared first by digesting pLE12, which was obtained from Dr. Gary Dunny from the University of Minnesota, with *Hin*dIII and isolating the restriction fragments on a 1% agarose gel. A 2.8 kb *Hin*dIII fragment which contains the Ll.ltrB intron together with portions of the flanking exons ltrBEl and ltrBE2 was recovered from the agarose gel and the single-stranded overhangs were filled in with the Klenow fragment of DNA polymerase I obtained from Gibco BRL, Gaithersburg, MD. The resulting fragment was ligated into plasmid pET-11a that had been digested with *Xba*I and treated with Klenow fragment. pET-11a was obtained from Novagen, Madison, WI.

pETLtrA19 was introduced into the *E. coli* cells using the conventional $CaCl_2$-mediated transformation procedure of Sambrook et al. as described in "Molecular Coning A Laboratory Manual", pages 1-82, 1989. Single transformed colonies were selected on plates containing Luria-Bertani (LB) medium supplemented with ampicillin to select the plasmid and with tetracycline to select the BLR strain. One or more colonies were inoculated into 2 ml of LB medium supplemented with ampicillin and grown overnight at 37°C with shaking. 1 ml of this culture was inoculated into 100 ml LB medium supplemented with ampicillin and grown at 37°C with

shaking at 200 rpm until OD_{595} of the culture reached 0.4. Then isopropylbeta-D-thiogalactoside was added to the culture to a final concentration of 1 mM and incubation was continued for 3 hours. Then the entire culture was harvested by centrifugation at 2,200 x g, 4°C, for 5 minutes. The bacterial pellet was washed with 150 mM NaCl and finally resuspended in 1/20 volume of the original culture in 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol (Buffer A). Bacteria were frozen at -70°C.

To produce a lysate the bacteria were thawed and frozen at -70°C. three times. Then 4 volumes of 500 mM KC1, 50 mMCaC12, 25 mM Tris, pH 7.5, and 5 mM DTT (HKCTD) were added to the lysate and the mixture was sonicated until no longer viscous, i.e. for 5 seconds or longer. The lysate was fractionated into a soluble fraction and insoluble fraction by centrifugation at 14,000 x g, 4°C, for 15 minutes. Then 5 ml of the resulting supernatant, i.e., the soluble fraction, were loaded onto a sucrose cushion of 1.85 M sucrose in HKCTD and centrifuged for 17 hours at 4°C, 50,0000 rpm in a Ti 50 rotor from Beckman. The pellet which contains the RNP particles was washed with 1 ml water and then dissolved in 25 μl 10 mM Tris, pH 8.0, 1 mM DTT on ice. Insoluble material was removed by centrifugation at 1,500 x g, 4°C, for 5 minutes. The yield of RNP particles prepared according to this method comprise the excised L1.ltrB intron RNA and the *ltra* protein.

Example 2

A nucleotide integrase comprising the ltra protein and the excised L1.ltrB intron RNA was prepared as described in example 1 except the plasmid pETLtrA19 was used to transform cells of the BL21(D3) strain of E. coli.

Example 3

A nucleotide integrase was prepared by transforming cells of the E. coli strains BLR(DE3) with pETLtrA19 as described in Example 1 except that the transformed E. Coli were grown in Super-Broth (SOB) medium and shaken at 300 rpm during the 3 hour incubation.

Example 4

A nucleotide integrase was prepared by transforming cells of the E. coli strain BL21(DE3) with pETLtrA19 as described above in Example 2 except the cells were also transformed with plasmid pOM62 which is based on the

plasmid pACYC184 and has an approximately 150 bp insert of the *argU*(*dnaY*) gene at the EcoRI site. The *argU* gene encodes the tRNA for the rare arginine codons AGA and AGG. The *ltrA* gene contains 17 of the rare arginine codons. The transformed cells were grown in SOB medium as described in Example 3 and fractionated into a soluble fraction and an insoluble fraction as described in Example 1.

Preparing a Group II Intron-Encoded Protein Having a Purification Tag on the C Terminus.

Example 5

To facilitate purification of the protein, the ltra ORF was tagged at the C-terminus with a His_6 affinity tag and an epitope derived from the Herpes simplex virus glycoprotein D. The plasmid adding the tags was made in two steps by using PCR. In the first step, a fragment containing exon 1 and the ltra ORF was amplified using primers LtrAex1.Xba having the sequence 5' TCACCTCATCTAGACATTTTCTCC 3', SEQ. ID. NO. 5 which introduces an *Xba* I site in exon 1 of ltrB, and ltrA expr3 5'CGTTCGTAAAGCTAGCCTTGTGTTTATG 3', SEQ. ID. NO. 6, which substitutes a CGA (arginine) codon for the stop codon and introduces an *Nhe* I site at the 3' end of the *LtrA* ORF. The PCR product was cut with XbaI and *Nhe* I, and the restriction fragments gel purified and cloned into pET-27b(+), cut with *Xba* I and *Nhe I* obtained from Novagen, Madison, WI. The resulting plasmid pIntermediate-C fuses the 3' end of the *ltra* ORF to an HSV tag and His_6 purification tag, both of which are present on the vector pET-27b(+). In a second step, intron sequences 3' to the ORF and exon 2 were amplified using pLE12 as a substrate and the 5' primer LtrAConZnl, having the sequence 5'CACAAGTGATCATTTACGAACG 3', SEQ. ID. No. 7 and the 3' primer LtrAex2, which has the sequence 5'TTGGGATCCTCATAAGCTTT GCCGC 3', SEQ. ID. NO. 8. The PCR product was cut with *Bcl1 and BamH1*, the resulting fragment filled in, gel-purified and cloned into pIntermediate-C, which had been cleaved with *Bpu*1021 and filled in. The resulting plasmid is designated pC-hisLtrA19.

Cells of the BLR(DE3) strain of *E. coli* were transformed as described in example 1 with pIntermediate-C and cultured at 37° C for 3 hours in SOB medium as described in example 3. The cells were also fractionated into a soluble fraction, which contains RNP particles, and an insoluble fraction as described in example 1.

EXAMPLE 6

To facilitate purification of the protein, the ltra ORF was tagged at the N-terminus with a His_6 affinity tag and the epitope tag XPRESS™ which was obtained from Invitrogen, San Diego, CA. The plasmid adding the tags was made in two steps by using PCR. In the first step, a fragment was made in two steps by using PCR mutagenesis. In the first step, the *ltrA* ORF and 3′ exon were amplified and *BamHI* sites were appended to both the 5′ an 3′ end of the *ltrA* ORF using pLE12 as a substrate and the following pair: 5′ primer N-LtrA 5′, having the sequence 5′CAAAGGATCCGATGAAACCAACAATGGCAA 3′, SEQ. ID. NO. 9; and the 3′ primer LtrAex2, SEQ. ID. NO. 8. The PCR product was cut with *BamHI* and the resulting restriction fragment was gel purified and cloned into the *BamHI* site of plasmid pRSETB obtained from Invitrogen, San Diego, CA. The resulting plasmid pIntermediate-N fuses the N-terminus of the *ltrA* ORF to a His_6 purification tag, and adds an XPRESS™ epitope tag from the vector. In a second step, the 5′ exon and L1.ltrB intron sequences 5′ to the ORF were amplified using pLE12 as a substrate and the 5′ primer NdeLTR5, having the sequence 5′AGTGGCTTCCATATGCTTGGTCATCACCTCATC 3′, SEQ. ID. No. 10 and 3′ primer NdeLTR3′, which has the sequence 5′ GGTAGAACCATATGAAATTCCTCCTCCCTAATCAATTTT 3′, SEQ. ID. NO. 11. The PCR product was cut with *Nde* I, filled in, the fragment gel purified and cloned into pIntermediate-N, which had also been cut with *Nde* I. Plasmids were screened for the orientation of the insert, and those oriented such that the 5′ exon was proximal to the T7 promoter were used to transform the host cells. The resulting plasmid pFinal-N expresses a message under the control of the T7 polymerase promoter which comprises the E1 and E2 portions of the exons *ltrBE1* and *ltrBE2*, and the *ltrA* ORF fused at the 5′ end with an His_6 purification tag and the XPRESS™ epitope tag.

Cells of the BLR(DE3) strain of *E. coli* were transformed as described in example 1 with pIntermediate-N and cultured at 37°C for 3 hours in SOB medium as described in example 3. The cells were also fractionated into a soluble fraction, which contains RNP particles, and an insoluble fraction as described in example 1.

EXAMPLE 7

Plasmid pETLtrA1-1 was used to prepare a partially-purified preparation of the *ltra* protein, which is encoded by the ORF of the L1.trB intron. Plasmid pETLtrA1-1 is a derivative of pETLtrA19 and lacks exon 1 and the intron sequences upstream of the *ltrA* ORF. Accordingly, the *ltrA* ORF is directly downstream of the phage T7 promoter following the Shine-

Dalgarno sequence in the plasmid. The plasmid map of pETLtrA1-1 is shown in Figure 3.

PETLtrA1-1 was made by using the polymerase chain reaction to amplify the *ltrA* ORF using the 5' primer LtrAexpr 5' AAAACCTCCATATG AAACCAACAATG 3', SEQ. ID. NO. 12, which introduces an *Nde*I site and 3' primer LtrAex2, SEQ. ID. NO. 8. The PCR product was cut with *Nde*I and *Bam*HI, gel purified on a 1% agarose gel, and cloned into pET20-11a. The inserts of pLE12, pETLtrA19 and pETLtrA1-1, each of which contain the *ltrA* ORF is depicted in Figure 4.

PETLtrA-1 was introduced into cells of the *E. coli* strain BLR(DE3) as described in Example 1 and the transformed cells grown for 3 hours in SOB medium at 37°C as described in Example 3. Thereafter, the cells were lysed and the resulting lysate fractionated into a soluble fraction and insoluble fraction by low speed centrifugation as described in Example 1.

Preparing a Nucleotide

Example 8

A nucleotide-integrase is prepared *in vitro* by combining an exogenous RNA comprising an excised L1.ltrB intron RNA with a purified LtrA protein. The purified LtrA is obtained by subjecting the partially-purified ltra protein of example 7 to standard chromatographic methods. The exogenous RNA is prepared by cloning the L1.ltrB intron together with its flanking exons into a plasmid downstream of a T7 promoter, linearizing the plasmid downstream of the exon 2 using a restriction enzyme, and transcribing the intron with T7 RNA polymerase. The *in vitro* transcript is incubated for one hour at 37° C in 500 mM NH_4Cl and 50 mM $MgCl_2$, 10 mM DTT, 2 units RNase inhibitor, to increase or produce excised intron RNA. The exogenous RNA and purified ltra protein are then incubated in a buffer to form the nucleotide integrase. The nucleotide integrase is then isolated from the reaction mixture.

Comparative Example A

RNP particles were prepared as described in Example 1 from cells of the BLR(DE3) strain of E. coli that had been transformed with plasmid pET11a, which lacks a group II intron. Accordingly, these RNP particles do not comprise excised, group II RNA or group II intron-encoded proteins and therefore, do not have nucleotide integrase activity.

Comparative Example B

RNP particles were prepared as described in Example 1 from cells of the BLR(DE3) strain of coli that had been transformed with plasmid pETLtrA19FS, which comprises the sequence of an ltra ORF having a frame shift 372 base pairs downstream from the initiation codon of the ltra ORF. frame. Accordingly, these RNP particles contain a truncated ltra protein, i.e. an ltra protein lacking the Zn domain and, therefore, do not have nucleotide integrase activity.

Characterization of the RNP particles of Examples 1 and 2.

A portion of the RNP particle preparation of examples 1 and 2 and comparative examples A and B were subjected to SDS gel electrophoresis. Staining of the resulting gel with Coomasie Blue permitted visualization of the proteins in each of the fractions. A band of approximately 70 kDa, which corresponds to the predicted molecular weight of the *ltra* protein was seen in the lanes containing aliquots of the RNP particles of Examples 1 and 2. This band was absent from the lanes containing the RNP particles prepared from comparative examples A and B. On the basis of the staining intensity of the 70 kDa band, the quantity of *ltra* protein in 10 OD_{260}, units of RNP particles was estimated to be approximately 3 μg. These results indicate that RNP particles containing the group II intron-encoded protein *ltra* can be prepared by expression of the group II intron Ll.ltrB in a heterologous host cell.

The reverse transcriptase activities of the RNP particles of examples 1 and 2 and the RNP particles of comparative examples A and B were assayed by incubating each of the RNP particle preparations with a poly(ra) template and oligo (dT18) as a primer. The RNP particles of examples 1 and 2 exhibited reverse transcriptase activity, while the RNP particles of comparative examples A and B exhibited no reverse transcriptase activity. These results indicate that the methods described in examples 1 and 2 are useful for preparing RNP particles that have reverse transcriptase activity. The reverse transcriptase activity that is present in nucleotide integrases allows incorporation of a CDNA molecule into the cleavage site of the double stranded DNA which is cut by the nucleotide integrase.

Characterizing the Distribution and Yield of the ltra Protein

A portion of the insoluble fraction and soluble fraction of the lysates from the cells transformed and cultured according to the methods described in examples 1, 2, 3, and 4 were subjected to SDS polyacrylamide

gel electrophoresis. Following electrophoresis, the SDS gels were stained with Coomassie blue to compare the yield of the *ltra* protein and the distribution of the 70 kDa *ltra* protein prepared by the methods of examples 1, 2, 3, and 4. The results of this assay demonstrated that more of the ltra protein was found in the soluble fraction when the transformed BLR (DE3) cells were grown in SOB medium and shaken at 300 rpm than when the transformed BLR cells were grown in LB medium and shaken at 200 rpm, These results also indicated that the total amount of *ltra* protein produced by the transformed BLR cells, that is the amount of LtrA in both the soluble and insoluble fractions, increased when a plasmid comprising the Ll.ltrB intron and a plasmid comprising *argU(dnaY)* gene were both introduced into the host cells.

Characterization of the Group II Intron-Encoded Protein Prepared According to the Methods of Examples 5 and 6.

A portion of the insoluble fraction and soluble fractions of the lysates from the cells transformed and cultured according to the methods described in examples 5 and 6 and in comparative examples A and B were subjected to electrophoresis on duplicate SDS-polyacrylamide gels. one of the gels was stained with Coomasie blueand the proteins on the duplicate were transferred to nitrocellulose paper by Western blotting. A primary antibody to the HSV antigen or the and an alkaline phosphatase-labeled anti-mouse IgG secondary antibody were used in an enzyme-linked immunoassay to identify proteins carrying the HSV epitope or the XPRESS™. The results of these assays showed that the anti-HSV antibody and the anti-XPRESS™ antibody bound to a protein having a molecular weight of approximately 70 kDa, which is the molecular weight of the ltra protein. The HSV tagged ltra protein and the xpress™ tagged ltra protein were found in the soluble and insoluble fractions from cells transformed with pIntermediateC and bIntermediateN but not in the soluble fractions and insoluble fractions of cells transformed with pet 27b(+) and PRSETB. Thus, the methods of examples 5 and 6 are useful for preparing a tagged group II intron encoded protein. These assays also demonstrated that the amount of the tagged group II intron-encoded protein present in the soluble fraction, from which the RNP particles are derived, increases when the transformed and induced cells are incubated at 28°C as compared to 37°C. Alternative studies showed that incubation times of 30 minutes to 3 hours resulted in production of the tagged protein, but these incubation times resulted in production of less of the protein and are therefore less preferred.

Using the RNP Particles to Cleave Double-Stranded DNA and to Insert Nucleotide Sequences into the Cleavage Site.

Nucleotide integrases are useful for cleaving one or both strands of a double-stranded DNA substrate, catalyzing the attachment of the excised, group II intron RNA molecule to one of the strands of the substrate DNA and catalyzing the formation of a CDNA molecule on the other strand of the cleaved double-stranded DNA substrate. Thus, the nucleotide integrases are useful analytical tools for determining the location of a defined sequence in a double-stranded DNA substrate. Moreover, the simultaneous insertion of the nucleic acid molecule into the first strand of DNA permits tagging of the cleavage site of the first strand with a radiolabeled molecule. In addition, the automatic attachment of an RNA molecule onto one strand of the DNA substrate permits identification of the cleavage site through hybridization studies that use a probe that is complementary to the attached RNA molecule. An attached RNA molecule that is tagged with a molecule such as biotin also enables the cleaved DNA to be affinity purified. Moreover, the cleavage of one or both strands of the double stranded DNA and the concomitant insertion of a nucleotide sequence into the cleavage site permits incorporation of new genetic information or a genetic marker into the cleavage site, as well as disruption of the cleaved gene. Thus, the nucleotide integrases are also useful for rendering the substrate DNA nonfunctional or for changing the characteristics of the RNA and protein encoded by the substrate DNA. while nucleotide integrases can be used to cleave doublestranded DNA substrates at a wide range of temperatures, good results are obtained at a reaction temperature of from about 30° C to about 42° C, preferably from about 30° to about 37° C. A suitable reaction medium contains a monovalent cation such as Na^+ or K^+, and a divalent cation, preferably a magnesium or manganese ion, more preferably a magnesium ion, at a concentration that is less than 100 mM and greater than 1 mM. Preferably the divalent cation is at a concentration of about 5 to about 20 mM. The preferred pH for the medium is from about 6.0-8.5, more preferably about 7.5-8.0.

Cleavage of 3' and 5' end labeled double stranded DNA

0.025 $O.D._{260}$ of the RNP particles of Example 1 and comparative examples A and B were incubated for 20 minutes with 150,000 cpm of each of a 5' and 3' end-labeled DNA substrate that comprises the exon 1 and exon 2 junction of the *ltrB* gene. The sequence of the 129 base pair substrate, which comprises the 70 base pair exon 1 and exon 2 junction of the *ltrB*

gene, plus sequences of the plasmid is depicted in Figure 5 and SEQ. ID. NO. 4. To verify cleavage, the products were isolated on a 6% polyacrylamide gel.

The substrate which is cleaved by the nucleotide integrase comprising the excised Ll.trB intron RNA and the *ltra* protein is schematically depicted in Figure 6(a). In addition, the IBS1 and IBS2 sequence of the substrate is shown in figure 6(b). As shown in Figure 6, the IBS1 and IBS2 sequences which are complementary to the EBS sequences of the Lltr.B intron RNA are present in exon 1 of the ltrb gene. As depicted in Figure 6, the RNP particles prepared according to the method of example 1 cleaved the sense strand of the substrate at position 0, which is the exon 1 and exon 2 junction, and the antisense strand at +9. When the RNP particles of prepared according to the method of example 1 were treated with either RNase A/Tl to degrade the RNA in the particles, or with proteinase K to degrade the protein component of the particles prior to incubation of the particles with the substrate, no cleavage of the substrate was observed. These results indicate that both the RNA component and the protein component of the nucleotide integrase are needed to cleave both strands of the substrate DNA.

Cleaving Both Strands of Double-Stranded DNA and Inserting the Intron RNA of the Nucleotide Integrase into the Cleavage Site.

0.025 $O.D._{260}$ units of the RNP particle preparation of example 1 were reacted with 125 fmoles (150,000 cpm) of the 129 base pair internally-labeled DNA substrate for 20 minutes. To verify cleavage, the products were glyoxalated and analyzed in a 1% agarose gel.

A dark band of radiolabel of approximately 1.0 kb RNA and a lighter bands of approximately 0.8, 1.1, 1.4, 1.5, 1.6, 1.9, 2.5, 3.2 were observed on the gel. Pretreatment of the reaction products with RNase prior to isolation on the agarose gel resulted in the complete disappearance of these bands. These results indicate that Ll.trB intron RNA was attached to the DNA substrate during reaction of the substrate with the RNP particles of example 1. On the basis of the size of Ll.trB intron, it is believed that the band at 2.5 kb represents the integration of the full length group II intron RNA into the cleavage site of the sense strand. The presence of smaller radiolabeled products on the gel is believed to be due to degradation of the integrated intron RNA by RNases which may be present in the RNP particle preparation. The finding that the RNADNA products

withstand denaturation with glyoxal indicates a covalent linkage between the intron RNA and the DNA substrate.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Lambowitz Dr., Alan M
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Saldanha Dr., Roland
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(ii) TITLE OF INVENTION: Method for Preparing Nucleotide Integrase

(iii) NUMBER OF SEQUENCES: 12

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 34,829
(C) REFERENCE/DOCKET NUMBER: 24671/00103

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2761 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

AAGCTTAGAG AAAAATAATG CGGTGCTTGG TCATCACCTC ATCCAATCAT TTTCTCCTGA 60

TGACAATCTA ACTCCTGAAC AAATTCATGA AATAGGTCGT CAAACCATAT TAGAATTTAC 120

AGGTGGCGAA TATGAATTTG TGATTGCAAC CCACGTCGAT CGTGAACACA TCCATAACGT 180

GCGCCCAGAT AGGGTGTTAA GTCAAGTAGT TTAAGGTACT ACTCTGTAAG ATAACACAGA 240

AAACAGCCAA CCTAACCGAA AAGCGAAAGC TGATACGGGA ACAGAGCACG GTTGGAAAGC 300

GATGAGTTAC CTAAAGACAA TCGGGTACGA CTGAGTCGCA ATGTTAATCA GATATAAGGT 360

ATAAGTTGTG TTTACTGAAC GCAAGTTTCT AATTTCGGTT ATGTGTCGAT AGAGGAAAGT 420

GTCTGAAACC TCTAGTACAA AGAAAGGTAA GTTATGGTTG TGGACTTATC TGTTATCACC 480

ACATTTGTAC AATCTGTAGG AGAACCTATG GGAACGAAAC GAAAGCGATG CCGAGAATCT 540

GAATTTACCA AGACTTAACA CTAACTGGGG ATACCCTAAA CAAGAATGCC TAATAGAAAG 600

GAGGAAAAAG GCTATAGCAC TAGAGCTTGA AAATCTTGCA AGGGTACGGA GTACTCGTAG 660

TATTCTGAGA AGGGTAACGC CCTTTACATG GCAAAGGGGT ACAGTTATTG TGTACTAAAA 720

TTAAAAATTG ATTAGGGAGG AAAACCTCAA AATGAAACCA ACAATGGCAA TTTTAGAAAG 780

AATCAGTAAA AATTCACAAG AAAATATAGA CGAAGTTTTT ACAAGACTTT ATCGTTATCT 840

TTTACGTCCA GATATTTATT ACGTGGCGTA TCAAAATTTA TATTCCAATA AAGGAGCTTC 900

CACAAAAGGA ATATTAGATG ATACAGCGGA TGGCTTTAGT GAAGAAAAAA TAAAAAAGAT 960

TATTCAATCT TTAAAAGACG GAACTTACTA TCCTCAACCT GTACGAAGAA TGTATATTGC 1020

AAAAAAGAAT TCTAAAAAGA TGAGACCTTT AGGAATTCCA ACTTTCACAG ATAAATTGAT 1080

CCAAGAAGCT GTGAGAATAA TTCTTGAATC TATCTATGAA CCGGTATTCG AAGATGTGTC 1140

TCACGGTTTT AGACCTCAAC GAAGCTGTCA CACAGCTTTG AAAACAATCA AAAGAGAGTT 1200

TGGCGGCGCA AGATGGTTTG TGGAGGGAGA TATAAAAGGC TGCTTCGATA ATATAGACCA 1260

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TAAGTTTGTT TTACAACTCA AAATGAAGTT TGACCGAGAA AGTCCAGAAA GAATAACACC 1500

TGAATATCGG GAACTTCACA ATGAGATAAA AAGAATTTCT CACCGTCTCA AGAAGTTGGA 1560

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ACTCCCCTGT ACCTCACAGA CAAATAAAGT ATTGAAATAC GTCCGGTATG CGGACGACTT 1680

CATTATCTCT GTTAAAGGAA GCAAAGAGGA CTGTCAATGG ATAAAAGAAC AATTAAAACT 1740

TTTTATTCAT AACAAGCTAA AAATGGAATT GAGTGAAGAA AAAACACTCA TCACACATAG 1800

CAGTCAACCC GCTCGTTTTC TGGGATATGA TATACGAGTA AGGAGAAGTG GAACGATAAA 1860

ACGATCTGGT AAAGTCAAAA AGAGAACACT CAATGGGAGT GTAGAACTCC TTATTCCTCT 1920

TCAAGACAAA ATTCGTCAAT TTATTTTTGA CAAGAAAATA GCTATCCAAA AGAAAGATAG 1980

CTCATGGTTT CCAGTTCACA GGAAATATCT TATTCGTTCA ACAGACTTAG AAATCATCAC 2040

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CCAGCTCAAT TATTTTGCTT ATCTTATGGA ATACAGCTGT CTAAAAACGA TAGCCTCCAA 2160

ACATAAGGGA ACACTTTCAA AAACCATTTC CATGTTTAAA GATGGAAGTG GTTCGTGGGG 2220

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TAAATCCCCT TATCAATTTA CGGATGAGAT AAGTCAAGCT CCTGTATTGT ATGGCTATGC 2340

CCGGAATACT CTTGAAAACA GGTTAAAAGC TAAATGTTGT GAATTATGTG GAACATCTGA 2400

TGAAAATACT TCCTATGAAA TTCACCATGT CAATAAGGTC AAAAATCTTA AAGGCAAAGA 2460

AAAATGGGAA ATGGCAATGA TAGCGAAACA ACGTAAAACT CTTGTTGTAT GCTTTCATTG 2520

TCATCGTCAC GTGATTCATA AACACAAGTG AATTTTTACG AACGAACAAT AACAGAGCCG 2580

TATACTCCGA GAGGGGTACG TACGGTTCCC GAAGAGGGTG GTGCAAACCA GTCACAGTAA 2640

TGTGAACAAG GCGGTACCTC CCTACTTCAC CATATCATTT TTAATTCTAC GAATCTTTAT 2700

ACTGGCAAAC AATTTGACTG GAAAGTCATT CCTAAAGAGA AAACAAAAAG CGGCAAAGCT 2760

T 2761

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1800 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1800

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

ATG AAA CCA ACA ATG GCA ATT TTA GAA AGA ATC AGT AAA AAT TCA CAA 48
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1 5 10 15

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CCA GAT ATT TAT TAC GTG GCG TAT CAA AAT TTA TAT TCC AAT AAA GGA      144
Pro Asp Ile Tyr Tyr Val Ala Tyr Gln Asn Leu Tyr Ser Asn Lys Gly
            35                  40                  45

GCT TCC ACA AAA GGA ATA TTA GAT GAT ACA GCG GAT GGC TTT AGT GAA      192
Ala Ser Thr Lys Gly Ile Leu Asp Asp Thr Ala Asp Gly Phe Ser Glu
        50                  55                  60

GAA AAA ATA AAA AAG ATT ATT CAA TCT TTA AAA GAC GGA ACT TAC TAT      240
Glu Lys Ile Lys Lys Ile Ile Gln Ser Leu Lys Asp Gly Thr Tyr Tyr
    65                  70                  75                  80

CCT CAA CCT GTA CGA AGA ATG TAT ATT GCA AAA AAG AAT TCT AAA AAG      288
Pro Gln Pro Val Arg Arg Met Tyr Ile Ala Lys Lys Asn Ser Lys Lys
                85                  90                  95

ATG AGA CCT TTA GGA ATT CCA ACT TTC ACA GAT AAA TTG ATC CAA GAA      336
Met Arg Pro Leu Gly Ile Pro Thr Phe Thr Asp Lys Leu Ile Gln Glu
            100                 105                 110

GCT GTG AGA ATA ATT CTT GAA TCT ATC TAT GAA CCG GTA TTC GAA GAT      384
Ala Val Arg Ile Ile Leu Glu Ser Ile Tyr Glu Pro Val Phe Glu Asp
        115                 120                 125

GTG TCT CAC GGT TTT AGA CCT CAA CGA AGC TGT CAC ACA GCT TTG AAA      432
Val Ser His Gly Phe Arg Pro Gln Arg Ser Cys His Thr Ala Leu Lys
    130                 135                 140

ACA ATC AAA AGA GAG TTT GGC GGC GCA AGA TGG TTT GTG GAG GGA GAT      480
Thr Ile Lys Arg Glu Phe Gly Gly Ala Arg Trp Phe Val Glu Gly Asp
145                 150                 155                 160

ATA AAA GGC TGC TTC GAT AAT ATA GAC CAC GTT ACA CTC ATT GGA CTC      528
Ile Lys Gly Cys Phe Asp Asn Ile Asp His Val Thr Leu Ile Gly Leu
                165                 170                 175

ATC AAT CTT AAA ATC AAA GAT ATG AAA ATG AGC CAA TTG ATT TAT AAA      576
Ile Asn Leu Lys Ile Lys Asp Met Lys Met Ser Gln Leu Ile Tyr Lys
            180                 185                 190

TTT CTA AAA GCA GGT TAT CTG GAA AAC TGG CAG TAT CAC AAA ACT TAC      624
Phe Leu Lys Ala Gly Tyr Leu Glu Asn Trp Gln Tyr His Lys Thr Tyr
        195                 200                 205

AGC GGA ACA CCT CAA GGT GGA ATT CTA TCT CCT CTT TTG GCC AAC ATC      672
Ser Gly Thr Pro Gln Gly Gly Ile Leu Ser Pro Leu Leu Ala Asn Ile
    210                 215                 220

TAT CTT CAT GAA TTG GAT AAG TTT GTT TTA CAA CTC AAA ATG AAG TTT      720
Tyr Leu His Glu Leu Asp Lys Phe Val Leu Gln Leu Lys Met Lys Phe
225                 230                 235                 240

GAC CGA GAA AGT CCA GAA AGA ATA ACA CCT GAA TAT CGG GAA CTT CAC      768
Asp Arg Glu Ser Pro Glu Arg Ile Thr Pro Glu Tyr Arg Glu Leu His
                245                 250                 255
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AAT GAG ATA AAA AGA ATT TCT CAC CGT CTC AAG AAG TTG GAG GGT GAA      816
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            260                 265                 270

GAA AAA GCT AAA GTT CTT TTA GAA TAT CAA GAA AAA CGT AAA AGA TTA      864
Glu Lys Ala Lys Val Leu Leu Glu Tyr Gln Glu Lys Arg Lys Arg Leu
        275                 280                 285

CCC ACA CTC CCC TGT ACC TCA CAG ACA AAT AAA GTA TTG AAA TAC GTC      912
Pro Thr Leu Pro Cys Thr Ser Gln Thr Asn Lys Val Leu Lys Tyr Val
    290                 295                 300

CGG TAT GCG GAC GAC TTC ATT ATC TCT GTT AAA GGA AGC AAA GAG GAC      960
Arg Tyr Ala Asp Asp Phe Ile Ile Ser Val Lys Gly Ser Lys Glu Asp
305                 310                 315                 320

TGT CAA TGG ATA AAA GAA CAA TTA AAA CTT TTT ATT CAT AAC AAG CTA     1008
Cys Gln Trp Ile Lys Glu Gln Leu Lys Leu Phe Ile His Asn Lys Leu
                325                 330                 335

AAA ATG GAA TTG AGT GAA GAA AAA ACA CTC ATC ACA CAT AGC AGT CAA     1056
Lys Met Glu Leu Ser Glu Glu Lys Thr Leu Ile Thr His Ser Ser Gln
            340                 345                 350

CCC GCT CGT TTT CTG GGA TAT GAT ATA CGA GTA AGG AGA AGT GGA ACG     1104
Pro Ala Arg Phe Leu Gly Tyr Asp Ile Arg Val Arg Arg Ser Gly Thr
        355                 360                 365

ATA AAA CGA TCT GGT AAA GTC AAA AAG AGA ACA CTC AAT GGG AGT GTA     1152
Ile Lys Arg Ser Gly Lys Val Lys Lys Arg Thr Leu Asn Gly Ser Val
    370                 375                 380

GAA CTC CTT ATT CCT CTT CAA GAC AAA ATT CGT CAA TTT ATT TTT GAC     1200
Glu Leu Leu Ile Pro Leu Gln Asp Lys Ile Arg Gln Phe Ile Phe Asp
385                 390                 395                 400

AAG AAA ATA GCT ATC CAA AAG AAA GAT AGC TCA TGG TTT CCA GTT CAC     1248
Lys Lys Ile Ala Ile Gln Lys Lys Asp Ser Ser Trp Phe Pro Val His
                405                 410                 415

AGG AAA TAT CTT ATT CGT TCA ACA GAC TTA GAA ATC ATC ACA ATT TAT     1296
Arg Lys Tyr Leu Ile Arg Ser Thr Asp Leu Glu Ile Ile Thr Ile Tyr
            420                 425                 430

AAT TCT GAA TTA AGA GGG ATT TGT AAT TAC TAC GGT CTA GCA AGT AAT     1344
Asn Ser Glu Leu Arg Gly Ile Cys Asn Tyr Tyr Gly Leu Ala Ser Asn
        435                 440                 445

TTT AAC CAG CTC AAT TAT TTT GCT TAT CTT ATG GAA TAC AGC TGT CTA     1392
Phe Asn Gln Leu Asn Tyr Phe Ala Tyr Leu Met Glu Tyr Ser Cys Leu
    450                 455                 460

AAA ACG ATA GCC TCC AAA CAT AAG GGA ACA CTT TCA AAA ACC ATT TCC     1440
Lys Thr Ile Ala Ser Lys His Lys Gly Thr Leu Ser Lys Thr Ile Ser
465                 470                 475                 480

ATG TTT AAA GAT GGA AGT GGT TCG TGG GGC ATC CCG TAT GAG ATA AAG     1488
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Met Phe Lys Asp Gly Ser Gly Ser Trp Gly Ile Pro Tyr Glu Ile Lys
                485                 490                 495

CAA GGT AAG CAG CGC CGT TAT TTT GCA AAT TTT AGT GAA TGT AAA TCC      1536
Gln Gly Lys Gln Arg Arg Tyr Phe Ala Asn Phe Ser Glu Cys Lys Ser
            500                 505                 510

CCT TAT CAA TTT ACG GAT GAG ATA AGT CAA GCT CCT GTA TTG TAT GGC      1584
Pro Tyr Gln Phe Thr Asp Glu Ile Ser Gln Ala Pro Val Leu Tyr Gly
        515                 520                 525

TAT GCC CGG AAT ACT CTT GAA AAC AGG TTA AAA GCT AAA TGT TGT GAA      1632
Tyr Ala Arg Asn Thr Leu Glu Asn Arg Leu Lys Ala Lys Cys Cys Glu
    530                 535                 540

TTA TGT GGA ACA TCT GAT GAA AAT ACT TCC TAT GAA ATT CAC CAT GTC      1680
Leu Cys Gly Thr Ser Asp Glu Asn Thr Ser Tyr Glu Ile His His Val
545                 550                 555                 560

AAT AAG GTC AAA AAT CTT AAA GGC AAA GAA AAA TGG GAA ATG GCA ATG      1728
Asn Lys Val Lys Asn Leu Lys Gly Lys Glu Lys Trp Glu Met Ala Met
                565                 570                 575

ATA GCG AAA CAA CGT AAA ACT CTT GTT GTA TGC TTT CAT TGT CAT CGT      1776
Ile Ala Lys Gln Arg Lys Thr Leu Val Val Cys Phe His Cys His Arg
            580                 585                 590

CAC GTG ATT CAT AAA CAC AAG TGA                                     1800
His Val Ile His Lys His Lys  *
        595                 600
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 600 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

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Met Lys Pro Thr Met Ala Ile Leu Glu Arg Ile Ser Lys Asn Ser Gln
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Glu Asn Ile Asp Glu Val Phe Thr Arg Leu Tyr Arg Tyr Leu Leu Arg
            20                  25                  30

Pro Asp Ile Tyr Tyr Val Ala Tyr Gln Asn Leu Tyr Ser Asn Lys Gly
        35                  40                  45

Ala Ser Thr Lys Gly Ile Leu Asp Asp Thr Ala Asp Gly Phe Ser Glu
    50                  55                  60

Glu Lys Ile Lys Lys Ile Ile Gln Ser Leu Lys Asp Gly Thr Tyr Tyr
65                  70                  75                  80
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Pro Gln Pro Val Arg Arg Met Tyr Ile Ala Lys Lys Asn Ser Lys Lys
85 90 95

Met Arg Pro Leu Gly Ile Pro Thr Phe Thr Asp Lys Leu Ile Gln Glu
100 105 110

Ala Val Arg Ile Ile Leu Glu Ser Ile Tyr Glu Pro Val Phe Glu Asp
115 120 125

Val Ser His Gly Phe Arg Pro Gln Arg Ser Cys His Thr Ala Leu Lys
130 135 140

Thr Ile Lys Arg Glu Phe Gly Gly Ala Arg Trp Phe Val Glu Gly Asp
145 150 155 160

Ile Lys Gly Cys Phe Asp Asn Ile Asp His Val Thr Leu Ile Gly Leu
165 170 175

Ile Asn Leu Lys Ile Lys Asp Met Lys Met Ser Gln Leu Ile Tyr Lys
180 185 190

Phe Leu Lys Ala Gly Tyr Leu Glu Asn Trp Gln Tyr His Lys Thr Tyr
195 200 205

Ser Gly Thr Pro Gln Gly Gly Ile Leu Ser Pro Leu Leu Ala Asn Ile
210 215 220

Tyr Leu His Glu Leu Asp Lys Phe Val Leu Gln Leu Lys Met Lys Phe
225 230 235 240

Asp Arg Glu Ser Pro Glu Arg Ile Thr Pro Glu Tyr Arg Glu Leu His
245 250 255

Asn Glu Ile Lys Arg Ile Ser His Arg Leu Lys Lys Leu Glu Gly Glu
260 265 270

Glu Lys Ala Lys Val Leu Leu Glu Tyr Gln Glu Lys Arg Lys Arg Leu
275 280 285

Pro Thr Leu Pro Cys Thr Ser Gln Thr Asn Lys Val Leu Lys Tyr Val
290 295 300

Arg Tyr Ala Asp Asp Phe Ile Ile Ser Val Lys Gly Ser Lys Glu Asp
305 310 315 320

Cys Gln Trp Ile Lys Glu Gln Leu Lys Leu Phe Ile His Asn Lys Leu
325 330 335

Lys Met Glu Leu Ser Glu Glu Lys Thr Leu Ile Thr His Ser Ser Gln
340 345 350

Pro Ala Arg Phe Leu Gly Tyr Asp Ile Arg Val Arg Arg Ser Gly Thr
355 360 365

Ile Lys Arg Ser Gly Lys Val Lys Lys Arg Thr Leu Asn Gly Ser Val
370 375 380

Glu Leu Leu Ile Pro Leu Gln Asp Lys Ile Arg Gln Phe Ile Phe Asp

```
385                 390                 395                 400

Lys Lys Ile Ala Ile Gln Lys Lys Asp Ser Ser Trp Phe Pro Val His
                405                 410                 415

Arg Lys Tyr Leu Ile Arg Ser Thr Asp Leu Glu Ile Ile Thr Ile Tyr
            420                 425                 430

Asn Ser Glu Leu Arg Gly Ile Cys Asn Tyr Tyr Gly Leu Ala Ser Asn
        435                 440                 445

Phe Asn Gln Leu Asn Tyr Phe Ala Tyr Leu Met Glu Tyr Ser Cys Leu
    450                 455                 460

Lys Thr Ile Ala Ser Lys His Lys Gly Thr Leu Ser Lys Thr Ile Ser
465                 470                 475                 480

Met Phe Lys Asp Gly Ser Gly Ser Trp Gly Ile Pro Tyr Glu Ile Lys
                485                 490                 495

Gln Gly Lys Gln Arg Arg Tyr Phe Ala Asn Phe Ser Glu Cys Lys Ser
            500                 505                 510

Pro Tyr Gln Phe Thr Asp Glu Ile Ser Gln Ala Pro Val Leu Tyr Gly
        515                 520                 525

Tyr Ala Arg Asn Thr Leu Glu Asn Arg Leu Lys Ala Lys Cys Cys Glu
    530                 535                 540

Leu Cys Gly Thr Ser Asp Glu Asn Thr Ser Tyr Glu Ile His His Val
545                 550                 555                 560

Asn Lys Val Lys Asn Leu Lys Gly Lys Glu Lys Trp Glu Met Ala Met
                565                 570                 575

Ile Ala Lys Gln Arg Lys Thr Leu Val Val Cys Phe His Cys His Arg
            580                 585                 590

His Val Ile His Lys His Lys  *
        595                 600
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 129 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

CGCTCTAGAA CTAGTGGATC CTTGCAACCC ACGTCGATCG TGAACACATC CATAACCATA 60

TCATTTTTAA TTCTACGAAT CTTTATACTG GGAATTCGAT ATCAAGCTTA TCGATACCGT 120

CGACCTCGA 129

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

TCTACCTCAT CTAGACATTT TCTCC 25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

CGTTCGTAAA GCTAGCCTTG TGTTTATG 28

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

CACAAAGTGA TCATTTAACG AACG 24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

TTGGGATCCT CATAAGCTTT GCCGC 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

CAAAGGATCC GATGAAACCA ACAATGGCAA 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

AGTGGCTTCC ATATGCTTGG TCATCACCTC ATC 33

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

GGTAGAACCA TATGAAATTC CTCCTCCCTA ATCAATTTT 39

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

AAAACCTCCA TATGAAACCA ACAATG 26

What is Claimed is:

1. A method for preparing a nucleotide integrase which cleaves a double-stranded DNA substrate, said method comprising the following steps:

(a) providing a DNA molecule comprising a group II intron DNA sequence, wherein the group II intron DNA sequence encodes a group II intron RNA and comprises an open reading frame sequence which encodes a group II intron-encoded protein;

(b) introducing the DNA molecule into a host cell;

(c) expressing the group II intron DNA sequence in the host cell, to provide an excised group II intron RNA and a group II intron-encoded protein molecule, wherein the protein and the RNA combine in the host cell to form the nucleotide integrase;

(d) obtaining the nucleotide integrase of step (c) from the host cell.

2. The method of claim 1 wherein the DNA molecule further comprises a promoter operably linked to the group II intron DNA sequence.

3. The method of claim 2 wherein the promoter is an inducible promoter.

4. The method of claim 2 wherein the DNA molecule further comprises a nucleotide sequence which encodes a tag for facilitating isolation of the nucleotide integrase from the host cell; and

wherein the method further comprises expressing the nucleotide sequence which encodes the tag in the host cell to provide a tagged group II intron-encoded protein; and

wherein step (d) involves employing the tag to recover the nucleotide integrase.

5. The method of claim 4 wherein the sequence which encodes the tag is located at the 51 end or the 3′ end of the open reading frame sequence of the group II intron DNA sequence.

6. The method of claim 2 further comprising the steps of:

introducing a DNA sequence encoding at TRNA which corresponds to the genetic code of the group II intron DNA sequence into the host cell before step (b) and

expressing the tRNA-encoding DNA sequence in the host cell.

7. The method of claim 1 wherein the DNA molecule is prepared by the following steps of:

preparing a synthetic group II intron DNA sequence; wherein the group II intron DNA sequence comprises a sequence of nucleotides that bind to the recognition site of the substrate DNA and

incorporating the synthetic group II intron DNA sequence into the DNA molecule.

8. The method of claim 1 wherein the group II intron DNA sequence comprises the DNA sequence of the Ll.ltrB intron and the RNP particles comprise an excised Ll.ltrB intron RNA and an ltra protein.

9. The method of claim 1 wherein the group II intron DNA sequence comprises a modified DNA sequence of the Ll.ltrB intron and the RNP particles comprise a modified excised Ll.ltrB intron RNA and an *ltra* protein molecule.

10. The method of claim 1 wherein the group II intron DNA sequence comprises a modified DNA sequence of the Ll.ltrB intron and the RNP particles comprise a modified excised Ll.ltrB intron RNA and a modified ltra protein molecule.

11. The method of claim 1 wherein the host cell is *E. coli*.

12. The method of claim 8 wherein the host cell is *E. coli*.

13. A method of preparing a nucleotide integrase in vitro comprising the steps of:

(a) providing an isolated, excised, group II intron RNA;

(b) providing an isolated group II intron-encoded protein; and

(c) incubating the excised, group II intron RNA with the group II intron-encoded protein for a sufficient time to form a nucleotide integrase comprising the excised, group II intron RNA bound to the group II, intron-encoded protein.

14. The method of claim 13 wherein the group II intronencoded protein is produced by a process comprising the steps of:

(a) providing a DNA molecule comprising an open reading frame sequence of a group II intron, said open reading frame sequence being operably linked to a promoter;

(b) introducing the DNA molecule of step (a) into a host cell;

(c) expressing the open reading frame sequence in the host cell to provide the group II intron-encoded protein; and (d) isolating the group II intron-encoded protein from the host cell.

15. The method of claim 13 wherein the DNA molecule further comprises a sequence which encodes a tag that facilitates isolation of the group II intron-encoded protein from the host cell; and

wherein the method further comprises expressing the nucleotide sequence which encodes the tag in the host cell to provide a tagged group II intron-encoded protein; and

wherein step (d) involves obtaining a tagged nucleotide integrase from the host cell.

16. The method of claim 15 wherein the sequence which encodes the tag is located at a position selected from the 51 end and the 31 end of the open reading frame sequence.

17. The method of claim 13 wherein the open reading frame sequence encodes the ltra protein and wherein the excised, group II RNA is elected from the group consisting of an unmodified, excised L1.ltrB intron RNA and a modified, excised L1.ltrB intron RNA.

18. A method of preparing a nucleotide integrase in vitro comprising the steps of:

(a) providing an exogenous RNA which comprises an excised group II intron RNA;

(b) providing an RNA-protein complex, wherein the RNA-protein complex comprises a protein having an amino acid sequence encoded by a group II intron and RNA that is free of excised, group II intron RNA molecules having a sequence that encodes said protein; said RNA-protein complex being prepared by the following steps:

(i) providing an isolated DNA molecule comprising a group II intron DNA sequence, wherein said group II intron DNA sequence

encodes a group II intron-encoded protein and a splicing defective group II RNA;

(ii) introducing the DNA molecule into a host cell;

(iii) expressing the mutated group II intron DNA sequence in the host cell, wherein an RNA-protein complex comprising the group II intron-encoded protein and the splicing-defective group II RNA are formed in the cell

(iv) obtaining the RNA-protein complex of step (iii) from the host cell; and

(c) incubating the exogenous RNA of step (a) with the RNP particle preparation for a sufficient time to form a nucleotide integrase comprising the excised group II RNA and the protein having an amino acid sequence encoded by a group II intron.

19. A nucleotide integrase prepared according to a method selected from the group consisting of the method of claim 1, the method of claim 13 and the method of claim 18.

20. An isolated nucleotide integrase comprising an excised Ll.ltrB intron RNA and an ltra protein molecule.

Fig. 1.

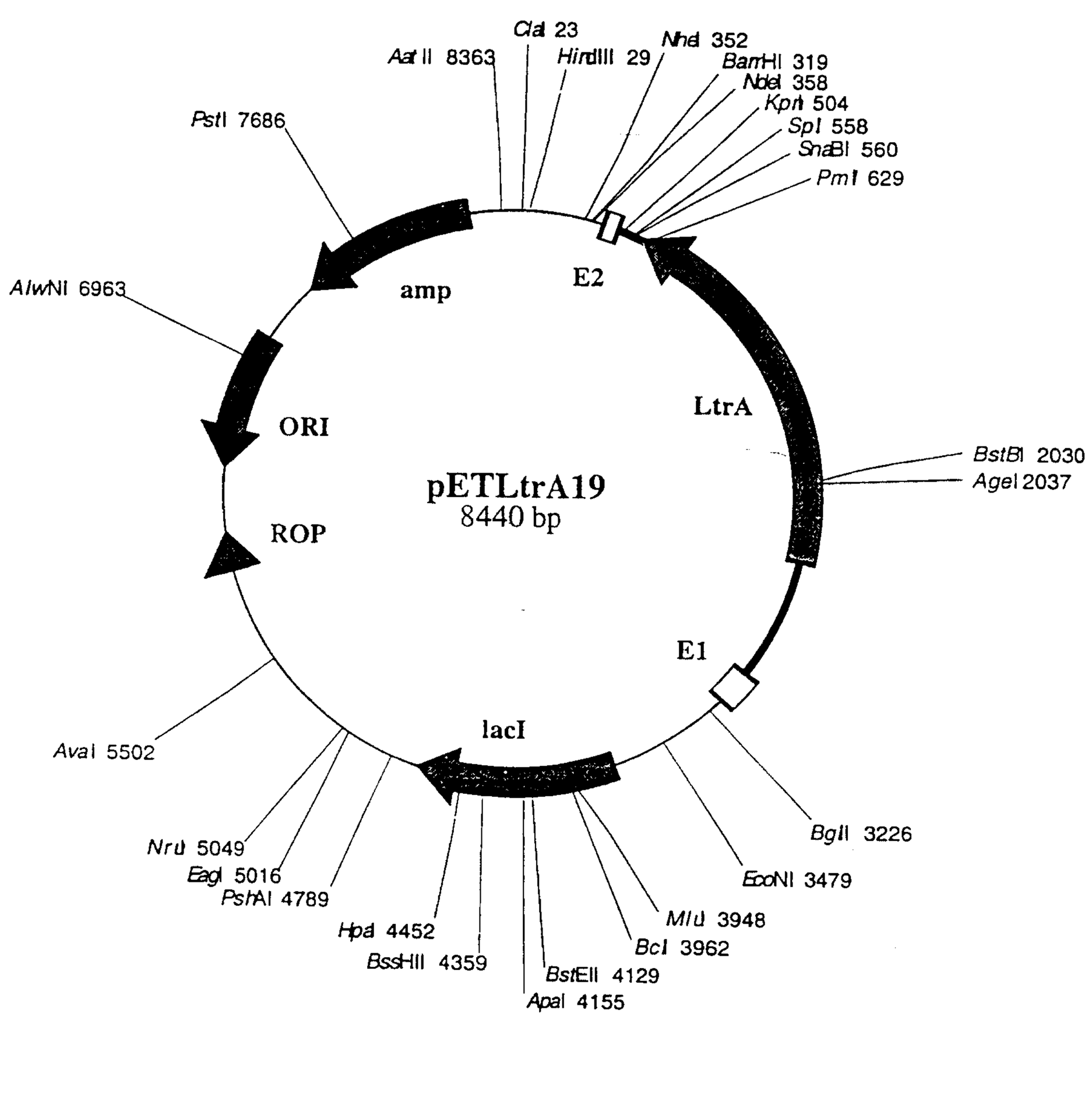

ClaI 23
NheI 352
AatII 8363
HindIII 29
BamHI 319
NdeI 358
KpnI 504
PstI 7686
SpI 558
SnaBI 560
PmI 629
AlwNI 6963
amp
E2
LtrA
ORI
pETLtrA19
8440 bp
BstBI 2030
AgeI 2037
ROP
E1
lacI
AvaI 5502
BglII 3226
NruI 5049
EagI 5016
PshAI 4789
EcoNI 3479
MluI 3948
HpaI 4452
BclI 3962
BssHII 4359
BstEII 4129
ApaI 4155

Fig. 2

```
      10         20         30         40         50         60         70         80         90
aagcttAGAG AAAAATAATG CGGTGCTTGG TCATCACCTC ATCCAATCAT TTTCTCCTGA TGACAATCTA ACTCCTGAAC AAATTCATGA

     100        110        120        130        140        150        160        170        180
AATAGGTCGT CAAACCATAT TAGAATTTAC AGGTGGCGAA TATGAATTTG TGATTGCAAC CCACGTCGAT CGTGAACACA TCCATAACGT

     190        200        210        220        230        240        250        260        270
GCGCCCAGAT AGGGTGTTAA GTCAAGTAGT TTAAGGTACT ACTCTGTAAG ATAACACAGA AAACAGCCAA CCTAACCGAA AAGCGAAAGC

     280        290        300        310        320        330        340        350        360
TGATACGGGA ACAGAGCACG GTTGGAAAGC GATGAGTTAC CTAAAGACAA TCGGCTACGA CTGAGTCGCA ATGTTAATCA GATATAAGGT

     370        380        390        400        410        420        430        440        450
ATAAGTTGTG TTTACTGAAC GCAAGTTTCT AATTTCGGTT ATGTGTCGAT AGAGGAAAGT GTCTGAAACC TCTAGTACAA AGAAAGGTAA

     460        470        480        490        500        510        520        530        540
GTTATGGTTG TGGACTTATC TGTTATCACC ACATTTGTAC AATCTGTAGG AGAACCTATG GGAACGAAAC GAAAGCGATG CCGAGAATCT

     550        560        570        580        590        600        610        620        630
GAATTTACCA AGACTTAACA CTAACTGGGG ATACCCTAAA CAAGAATGCC TAATAGAAAG GAGGAAAAAG GCTATAGCAC TAGAGCTTGA

     640        650        660        670        680        690        700        710        720
AAATCTTGCA AGGGTACGGA GTACTCGTAG TAGTCTGAGA AGGGTAACGC CCTTTACATG GCAAAGGGGT ACAGTTATTG TGTACTAAAA

     730        740        750        760        770        780        790        800        810
TTAAAAATTG ATTAGGGAGG AAAACCTCAA AATGAAACCA ACAATGGCAA TTTTAGAAAG AATCAGTAAA AATTCACAAG AAAATATAGA
                                 M  K  P  T  M  A  I  L  E  R  I  S  K  N  S  Q  E  N  I  D

     820        830        840        850        860        870        880        890        900
CGAAGTTTTT ACAAGACTTT ATCGTTATCT TTTACGTCCA GATATTTATT ACGTGGCGTA TCAAAATTTA TATTCCAATA AAGGAGCTTC
  E  V  F  T  R  L  Y  R  Y  L  L  P  D  I  Y  Y  V  A  Y  Q  N  L  Y  S  N  K  G  A  S

     910        920        930        940        950        960        970        980        990
CACAAAAGGA ATATTAGATG ATACAGCGGA TGGCTTTAGT GAAGAAAAAA TAAAAAAGAT TATTCAATCT TTAAAAGACG GAACTTACTA
  T  K  G  I  L  D  D  T  A  D  G  F  S  E  E  K  I  K  K  I  I  Q  S  L  K  D  G  T  Y  Y

    1000       1010       1020       1030       1040       1050       1060       1070       1080
TCCTCAACCT GTACGAAGAA TGTATATTGC AAAAAAGAAT TCTAAAAAGA TGAGACCTTT AGGAATTCCA ACTTTCACAG ATAAATTGAT
  P  Q  P  V  R  R  M  Y  I  A  K  K  N  S  K  K  M  R  P  L  G  I  P  T  F  T  D  K  L  I

    1090       1100       1110       1120       1130       1140       1150       1160       1170
CCAAGAAGCT GTGAGAATAA TTCTTGAATC TATCTATGAA CCGGTATTCG AAGATGTGTC TCACGGTTTT AGACCTCAAC GAAGCTGTCA
  Q  E  A  V  R  I  I  L  E  S  I  Y  E  P  V  F  E  D  V  S  H  G  F  R  P  Q  R  S  C  H

    1180       1190       1200       1210       1220       1230       1240       1250       1260
CACAGCTTTG AAAACAATCA AAAGAGAGTT TGGCGGCGCA AGATGGTTTG TGGAGGGAGA TATAAAAGGC TGCTTCGATA ATATAGACCA
  T  A  L  K  T  I  K  R  E  F  G  G  A  R  W  F  V  E  G  D  I  K  G  C  F  D  N  I  D  H

    1270       1280       1290       1300       1310       1320       1330       1340       1350
CGTTACACTC ATTGGACTCA TCAATCTTAA AATCAAAGAT ATGAAAATGA GCCAATTGAT TTATAAATTT CTAAAAGCAG GTTATCTGGA
  V  T  L  I  G  L  I  N  L  K  I  K  D  M  K  M  S  Q  L  I  Y  K  F  L  K  A  G  Y  L  E

    1360       1370       1380       1390       1400       1410       1420       1430       1440
AAACTGGCAG TATCACAAAA CTTACAGCGG AACACCTCAA GGTGGAATTC TATCTCCTCT TTTGGCCAAC ATCTATCTTC ATGAATTGGA
  N  W  Q  Y  H  K  T  Y  S  G  T  P  Q  G  G  I  L  S  P  L  L  A  N  I  Y  L  H  E  L  D

    1450       1460       1470       1480       1490       1500       1510       1520       1530
TAAGTTTGTT TTACAACTCA AAATGAAGTT TGACCGAGAA AGTCCAGAAA GAATAACACC TGAATATCGG GAACTTCACA ATGAGATAAA
  K  F  V  L  Q  L  K  M  K  F  D  R  E  S  P  E  R  I  T  P  E  Y  R  E  L  H  D  E  I  K
```

Fig. 2
(Cont.)

```
      1540        1550        1560        1570        1580        1590        1600        1610        1620
AAGAATTTCT CACCGTCTCA AGAAGTTGGA GGGTGAAGAA AAAGCTAAAG TTCTTTTAGA ATATCAAGAA AAACGTAAAA GATTACCCAC
 R  I  S    H  R  L  K    K  L  E    G  E  E    K  A  K  V    L  L  E    Y  Q  E    K  R  K  R    L  P  T

      1630        1640        1650        1660        1670        1680        1690        1700        1710
ACTCCCCTGT ACCTCACAGA CAAATAAAGT ATTGAAATAC GTCCGGTATG CGGACGACTT CATTATCTCT GTTAAAGGAA GCAAAGAGGA
 L  P  C    T  S  Q  T    N  K  V    L  K  Y    V  R  Y  A    D  D  F    I  I  S    V  K  G  S    K  E  D

      1720        1730        1740        1750        1760        1770        1780        1790        1800
CTGTCAATGG ATAAAAGAAC AATTAAAACT TTTTATTCAT AACAAGCTAA AAATGGAATT GAGTGAAGAA AAAACACTCA TCACACATAG
 C  Q  W    I  K  E  Q    L  K  L    F  I  H    N  K  L  K    M  E  L    S  E  E    K  T  L  I    T  H  S

      1810        1820        1830        1840        1850        1860        1870        1880        1890
CAGTCAACCC GCTCGTTTTC TGGGATATGA TATACGAGTA AGGAGAAGTG GAACGATAAA ACGATCTGGT AAAGTCAAAA AGAGAACACT
 S  Q  P    A  R  F  L    G  Y  D    I  R  V    R  R  S  G    T  I  K    R  S  G    K  V  K  K    R  T  L

      1900        1910        1920        1930        1940        1950        1960        1970        1980
CAATGGGAGT GTAGAACTCC TTATTCCTCT TCAAGACAAA ATTCGTCAAT TTATTTTTGA CAAGAAAATA GCTATCCAAA AGAAAGATAG
 N  G  S    V  E  L  L    I  P  L    Q  D  K    I  R  Q  F    I  F  D    K  K  I    A  I  Q  K    K  D  S

      1990        2000        2010        2020        2030        2040        2050        2060        2070
CTCATGGTTT CCAGTTCACA GGAAATATCT TATTCGTTCA ACAGACTTAG AAATCATCAC AATTTATAAT TCTGAATTAA GAGGGATTTG
 S  W  F    P  V  H  R    K  Y  L    I  R  S    T  D  L  E    I  I  T    I  Y  N    S  E  L  R    G  I  C

      2080        2090        2100        2110        2120        2130        2140        2150        2160
TAATTACTAC GGTCTAGCAA GTAATTTTAA CCAGCTCAAT TATTTTGCTT ATCTTATGGA ATACAGCTGT CTAAAAACGA TAGCCTCCAA
 N  Y  Y    G  L  A  S    N  F  N    Q  L  N    Y  F  A  Y    L  M  E    Y  S  C    L  K  T  I    A  S  K

      2170        2180        2190        2200        2210        2220        2230        2240        2250
ACATAAGGGA ACACTTTCAA AAACCATTTC CATGTTTAAA GATGGAAGTG GTTCGTGGGG CATCCCGTAT GAGATAAAGC AAGGTAAGCA
 H  K  G    T  L  S  K    T  I  S    M  F  K    D  G  S  G    S  W  G    I  P  Y    E  I  K  Q    G  K  Q

      2260        2270        2280        2290        2300        2310        2320        2330        2340
GCGCCGTTAT TTTGCAAATT TTAGTGAATG TAAATCCCCT TATCAATTTA CGGATGAGAT AAGTCAAGCT CCTGTATTGT ATGGCTATGC
 R  R  Y    F  A  N  F    S  E  C    K  S  P    Y  Q  F  T    D  E  I    S  Q  A    P  V  L  Y    G  Y  A

      2350        2360        2370        2380        2390        2400        2410        2420        2430
CCGGAATACT CTTGAAAACA GGTTAAAAGC TAAATGTTGT GAATTATGTG GAACATCTGA TGAAAATACT TCCTATGAAA TTCACCATGT
 R  N  T    L  E  N  R    L  K  A    K  C  C    E  L  C  G    T  S  D    E  N  T    S  Y  E  I    H  H  V

      2440        2450        2460        2470        2480        2490        2500        2510        2520
CAATAAGGTC AAAAATCTTA AAGGCAAAGA AAAATGGGAA ATGGCAATGA TAGCGAAACA ACGTAAAACT CTTGTTGTAT GCTTTCATTG
 N  K  V    K  N  L  K    G  K  E    K  W  E    M  A  M  I    A  K  Q    R  K  T    L  V  V  C    F  H  C

      2530        2540        2550        2560        2570        2580        2590        2600        2610
TCATCGTCAC GTGATTCATA AACACAAGTG AATTTTTACG AACGAACAAT AACAGAGCCG TATACTCCGA GAGGGGTACG TACGGTTCCC
 H  R  H    V  I  H  K    H  K  *

      2620        2630        2640        2650        2660        2670        2680        2690        2700
GAAGAGGGTG GTGCAAACCA GTCACAGTAA TGTGAACAAG GCGGTACCTC CCTACTTCAC CATATCATTT TTAATTCTAC GAATCTTTAT

      2710        2720        2730        2740        2750        2760        2770
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Fig. 3

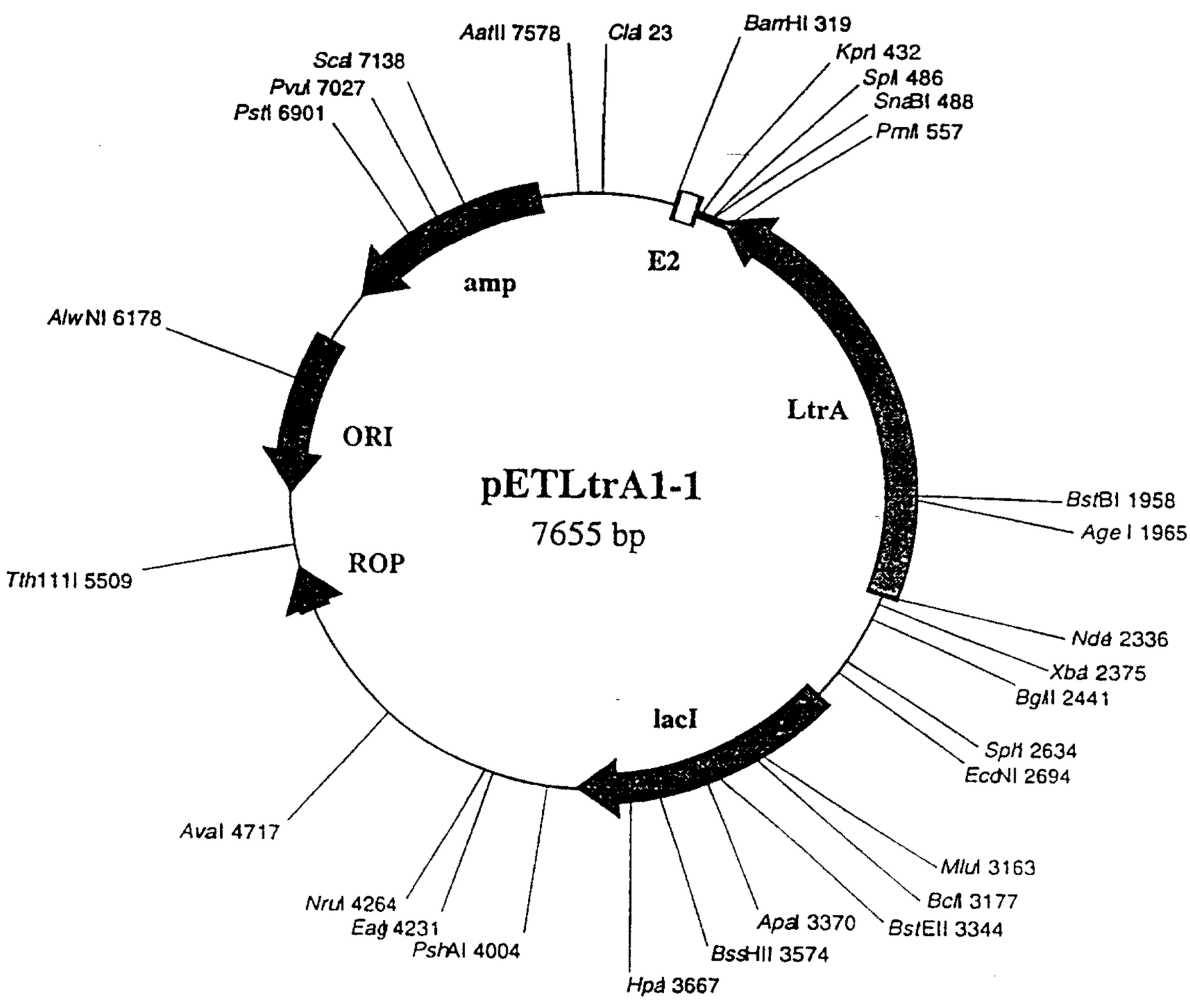

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BamHI 319
KpnI 432
SpeI 486
SnaBI 488
PmlI 557
ScaI 7138
PvuI 7027
PstI 6901
amp
E2
AlwNI 6178
ORI
LtrA
pETLtrA1-1
7655 bp
BstBI 1958
AgeI 1965
ROP
Tth111I 5509
NdeI 2336
XbaI 2375
BglII 2441
lacI
SphI 2634
EcoNI 2694
MluI 3163
BclI 3177
ApaI 3370
BstEII 3344
BssHII 3574
AvaI 4717
NruI 4264
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PshAI 4004
HpaI 3667

Fig. 4

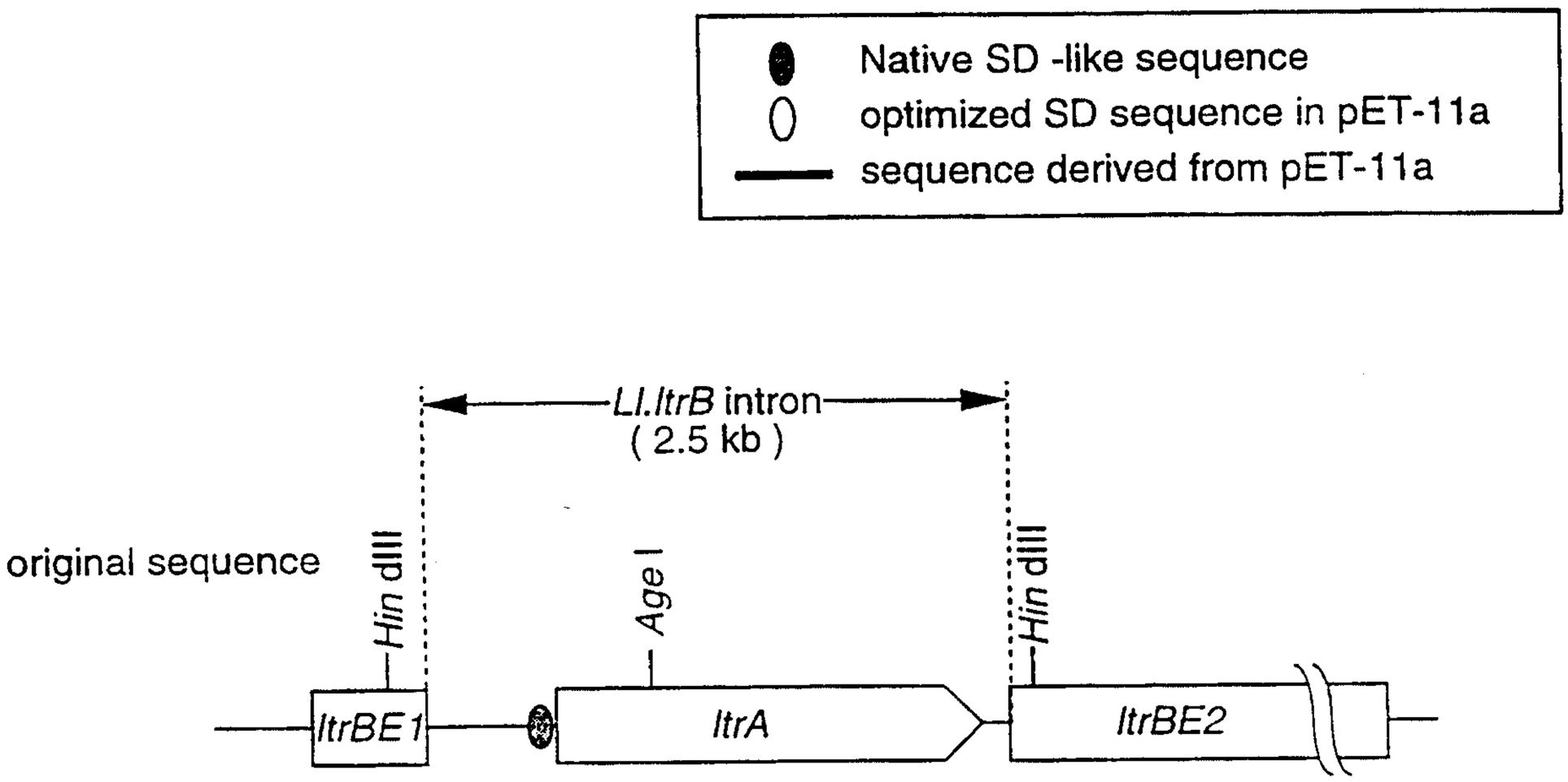

Native SD -like sequence
optimized SD sequence in pET-11a
sequence derived from pET-11a
LI.ltrB intron
(2.5 kb)
original sequence
Hin dIII
Age I
Hin dIII
ltrBE1
ltrA
ltrBE2
pETLtrA19
pT7
TT7
1
ltrA
pETLtrA1-1
pT7
TT7
ltrA

Fig. 5

```
         10          20          30          40          50          60
cgctctagaa ctagtggatc cTTGCAACCC ACGTCGATCG TGAACACATC CATAACCATA

         70          80          90         100         110         120
TCATTTTTAA TTCTACGAAT CTTTATACTG Ggaattcgat atcaagctta tcgataccgt

        130
cgacctcga
```

Fig. 6

DNA Substrate

