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(57) Abrégé/Abstract:
The present invention provides a method to directionally clone any template DNA molecule into a single restriction site of any vector. The vector ends may be generated from any restriction enzyme cleavage. The method does not require a ligation step nor the use of carefully controlled conditions as is required with methods involving specific exonucleases alone. It has been determined that specific DNA polymerases are able to efficiently join two or more linear DNA molecules sharing ends with appropriate complementation.

ABSTRACT

The present invention provides a method to directionally clone any template DNA molecule into a single restriction site of any vector. The vector ends may be generated from any restriction enzyme cleavage. The method does not require a ligation step nor the use of carefully controlled conditions as is required with methods involving specific exonucleases alone. It has been determined that specific DNA polymerases are able to efficiently join two or more linear DNA molecules sharing ends with appropriate complementation.

TITLE: DNA JOINING METHOD

FIELD OF THE INVENTION

The present invention is in the field of recombinant DNA technology. Specifically, the present invention relates to a method of directionally joining two or more DNA molecules using a DNA polymerase, and the application of this method to the cloning of PCR products.

BACKGROUND OF THE INVENTION

The Polymerase Chain Reaction (PCR) (Mullis and Faloona 1987; Saiki, Gelfand et al. 1988) is an integral technique in scientific research. Cloning of PCR products is often an obligate step towards reaching a research objective. PCR-cloning presents numerous challenges and various techniques have been devised over the years to minimize its limitations. Cloning of PCR products generally fall into one of the following methodologies; i) traditional cloning using restriction enzymes and ligase, ii) T-vector or TA-cloning, iii) uracil DNA glycosylase (UDG)-based cloning iv) PCR-based techniques, v) in vivo recombinase methods, and vi) exonuclease-mediated cloning.

i) Traditional PCR-cloning

Cloning of PCR-amplified DNA was traditionally facilitated by incorporating restriction endonuclease (RE) sites into the PCR primers, allowing for subsequent digestion of the PCR product with the appropriate enzyme followed by insertion into a compatible vector (Sharf et al., 1990). One problem often encountered with this method is that RE's are notoriously poor cutters when their recognition sequences are close to the ends of the DNA substrate (Kaufman and Evans 1990). A second complication is that sequences within the PCR-amplified region can be lost if a second restriction site for the same RE is present unknowingly. This necessitates that the complete sequence of the PCR product be known prior to choosing which RE to use. Once the vector and DNA insert have been digested with the same RE, the two DNA molecules can be covalently joined by DNA ligase in a reaction typically taking 4-12hr. It should be noted that during any ligation reaction, it is critical that the vector and insert are present in appropriate ratios, which is often difficult to determine. The reaction products are then used to transform competent *E. coli* cells. A subtle variation on this theme called "ligation by

overlap extension" has been devised which does not require any subsequent ligation reaction, but does require two additional primers, and the entire vector sequence itself must be amplified (Shuldiner, Tanner et al. 1991).

Blunt-end cloning of PCR fragments has also been used extensively, although this technique is relatively inefficient because of the problems encountered by DNA ligase when joining together two blunt-ended DNA molecules. This technique is also complicated by the fact that *Taq* polymerase (the prototypical PCR amplifying enzyme) has a propensity for adding 3' terminal adenine residues through its terminal transferase activity (Clark 1988; Mole, Iggo et al. 1989). Approximately 50% of all PCR products generated using *Taq* polymerase contain these 3'extensions (Clark, 1988). One way around this problem is to "polish" or remove these added adenine residues with Klenow (Hemsley, Arnheim et al. 1989) or T4 DNA polymerase (Costa and Weiner 1994), adding an additional step to the protocol.

Recent discoveries of different thermostable polymerases including *Pfu* polymerase from *Pyrococcus furiosis* and $Vent_R$ polymerase from the archaebacteria *Thermococcus litoralis* do not produce 3' adenine residue extensions due to inherent 3'-5' exonuclease activities. In addition to the problems produced by the amplifying polymerase, this technique does not allow for directional cloning, meaning that the orientation of insert in the recombinant DNA cannot be predetermined.

ii) T-Vector and TA-cloning

The terminal transferase activity of *Taq* polymerase has been exploited by many researchers in a technique now commonly known as TA-cloning. The chosen vector is digested with the appropriate RE so as to yield ends with protruding thymidine residues, the natural complement to the 3'-overhanging adenosine residues found on the PCR-amplified DNA. The most significant drawback of this technique is that vectors must be specifically engineered to produce compatible ends. The only simple way of accomplishing this goal is to restrict the vector to produce blunt ends, and then treat it with *Taq* polymerase in the presence of only dTTP's. Numerous companies have developed kits based on this technique, including pCR-Script™ SK(S) kit from Stratagene, pGEM®-T from Promega, the SureClone™ ligation kit from Pharmacia and the pT7Blue T-vector kit from Novagen. The main limitation to these methods is that a vector

supplied by the manufacturer must be used and a second subcloning step is often necessary to move the cloned DNA fragment into a vector of choice. An inexpensive alternative to buying these kits is to use a T-vector pGEM®-5fZ(+) which is available for little or no cost from the American Type Culture Collection (ATCC). This vector when digested with *Xcm*I provides the T-overhangs used for TA-cloning (Kovalic, Kwak et al. 1991; Mead, Pey et al. 1991). Numerous other T-vectors have been developed independently (Cha, Bishai et al. 1993; Ichihara and Kurosawa 1993) which, after appropriate RE digestion, yield appropriate ends. Such vectors, however, required extensive manipulations to create. Some other potential problems with these kits have been reported recently (Hengen 1995). High backgrounds were observed for the pCR-Script vector when tested alone, PCRII contains a pBR322 origin of replication and thus replicate to low copy numbers, and repeated freeze-thaw cycles at -20°C can lead to instability and loss of the T-tails. All the T-vector techniques suffer from the drawback that they are non-directional and require a ligation step.

Invitrogen have improved upon traditional TA-cloning by bypassing the need for a ligation step. This method, called TOPO TA-cloning, takes advantage of a reaction catalyzed by a vaccinia virus enzyme called topoisomerase I (Shuman, 1994 and US 5,766,891). Topoisomerase can bind to double-stranded DNA and cleave the phosphodiester backbone in one of the duplex strands. The enzyme is sequence specific, cleaving primarily at the recognition sequence 5'-(C/T)CCTT↓-3' (Shuman and Prescott 1990; Shuman 1991). The enzyme is capable of re-ligating the original strand back together, or ligating two heterologous DNAs in the formation of a recombinant species (Shuman 1992; Shuman 1992). The reaction is very efficient requiring only a 5 minute benchtop incubation. The methodology also has advantages which obviates the need for ligase, does not require knowledge of the entire insert sequence and no additional nucleotides need be added to PCR primers. However, only specific plasmids engineered to contain the TOPO recognition sequence can be used. These vectors are produced by restricting the vector followed by adding specific linkers or adaptors, which is not a trivial task. Another limitation of this technique, is that the TOPO recognition sequence must be located within 10 bp from the 3'-ends of the vector, and furthermore, the insert must have a 5-OH group. The issue has been raised that internal recognition sequences

within the amplified DNA may result in complications, however these sites are simply religated and do not impose any restrictions on this technique (Shuman 1994; Stivers, Shuman et al. 1994). Under general use, the Invitrogen kit provided another potential problem (unpublished results). The traditional method for screening clones, called blue-white selection, does not produce definitive results with the Invitrogen kit. Therefore, it is necessary to assay both white and light-blue colonies to ensure the correct construct is obtained.

iii) Uracil DNA-glycosylase (UDG)-based cloning

Rashtchian et al., (1992) developed a ligase independent PCR cloning method using uracil DNA glycosylase (UDG), an enzyme whose normal cellular role is a DNA repair enzyme. The technique requires a 12-bp addition (CUACUACUACUA) to the 5'end of the PCR primers. The glycosylase selectively removes dUMP residues at the ends of the PCR products which disrupts proper base-pairing leading to single-strand 3'-overhangs (Duncan and Chambers 1984; Longo, Berninger et al. 1990). These 3'-overhangs can anneal to appropriately prepared single-strand ends of a vector. Uracil glycosylase is not active with thymine residues, the DNA counterpart of uracil residues (Duncan and Chambers, 1984), and is capable of removing dUMP residues even near the extreme ends (Varshney and van de Sande 1991). This methodology requires that the vector contain the appropriate complementary sequences, and is not amenable to use with proofreading polymerases such as *Pfu* or $Vent_R$ polymerases (Sakaguchi, Sedlak et al. 1996). The researchers must therefore use *Taq* polymerase which has a significantly increased error frequency. UDG-based cloning has been commercialized by Life Technologies with their Clone Amp^R pUC system.

A variation on UDG-cloning takes advantage of the abasic sites (AP) produced by UDG-cleavage at dUMP residues. These AP sites are substrates for AP endonucleases such as T4 endonuclease V or human AP endonuclease I. Treatment with either of these repair enzymes yields a 5-P which is suitable for subsequent ligation into the appropriate vector. One drawback of this method is the requirement for a modified base (deoxyuridine) in the primer, and success relies on two enzymes in addition to ligase treatment. A second more obscure variation of UDG-cloning involves the use of a non-base residue called 1,3-propanediol in a predetermined position within the PCR primer,

which can yield compatible 5'-ends for cloning, however, this method is much less efficient than other ligase-independent cloning methods (Kaluz and Flint 1994).

iv) PCR-Directed Cloning

PCR-specific cloning methods are often one-step procedures in which the recombinant DNA is produced during the amplification procedure itself. There are many variations on this theme, in which some are ligase-dependent and others are not. These methods are primarily used to produce site-specific mutations in cloned genes. A brief description of the current techniques follows.

Ligase-Dependent Methods

a) Stratagene have commercialized a technique for the cloning of blunt-end PCR fragments (Weiner 1993), originally described by Liu and Schwartz (1992). Their methodology requires phosphorylating the 5'end of the PCR primers. The recipient vector is linearized and treated with calf intestinal alkaline phosphatase (CIAP) and then digested with a second restriction enzyme to yield compatible ends. This is a rather convoluted technique but the resultant vector is mono-phosphorylated and allows for directional cloning. They reported a 95% success rate for directional cloning, however their technique requires an ethanol precipitation and still relies on the actions of ligase.

b) "Hetero-stagger cloning" is another ligase-dependent method which requires a total of four PCR primers (Liu 1996). One set of primers is the traditional PCR primer pair and the second set is equivalent to the first, but includes three additional 5'-GGG residues. The DNA is amplified under normal PCR conditions, the products are denatured by heat and then allowed to reanneal slowly by cooling. Reannealing results in the formation of four distinct species. Only 50% of the products are theoretically cloneable, and only 25% of the products would successfully result in directional clones. The only claimed advantage to this technique is that it allows for modern proofreading polymerases to be used during amplification.

c) A variation of the staggered re-annealing technique has also been used which requires only one primer pair (Ailenberg and Silverman 1996).

d) More recently, Gal et al., (1999) have devised a technique called "autosticky PCR" (AS-PCR) (patent application HU9801320). This technique takes advantage of the observation that abasic sites present in DNA can stall DNA polymerases. In this method,

PCR primers are designed to contain abasic sites, which stall the amplifying polymerase, resulting in 5'-overhangs thus enabling ligation into a suitably digested vector. The abasic site is produced by the incorporation of tetrahydrofuran, a stable structural analogue of 2-deoxyribose, at the desired position. This method does provide for directional-cloning, but requires non-traditional reagents and an overnight ligation is recommended.

Ligase-Independent Cloning Methods (LIC)

a) The original ligase PCR-cloning method was described by Shuldiner et al., (1991). Since then, numerous adaptations of this technique have been developed. The technique described here (Temesgen and Eschrich 1996) requires three PCR primers, in which one of the primers contains an additional 24 nucleotides. This process involves two distinct PCR amplifications, thus increasing the probability of introducing PCR errors into the products. However, the linear products can be directly transformed into *E.coli* obviating the need for ligase. Competent *E. coli* strain TG2 cells are required, and it is unclear if classical strains such as JM105 or DH5α are able to be substituted. This technique does provide for directional cloning, although the success is related to the PCR parameters in the second PCR step. Any vector can be used in the technique and no restriction enzymes are needed.

b) Garces and Laborda (1995) reported a similar technique only requiring two PCR primers, one of which has a 20bp 5'-extension. The reaction occurs within a single-tube reaction, and can be adapted for use with any vector, but the efficiency is greatly affected by the PCR parameters.

v) *In Vivo* Recombination-based Cloning

PCR-cloning is traditionally completed within the test-tube environment of the laboratory, however, there are at least two reports of cloning using *in vivo* systems. The following technique was based on the observation that when yeast were co-transfected with a linear template and a gapped plasmid, homologous recombination was able to "patch" the two species together (Guthrie and Fink, 1991). PCR products have since been cloned in yeast using this method (Scharer and Iggo 1992). A similar phenomenon has been reported in *E. coli* (Oliner, Kinzler et al. 1993). This technique presumably takes advantage of endogenous exonuclease or polymerase activities encoded by the host,

but there is no speculation as to what is exactly occurring. The PCR primers are designed to contain 5'-sequences which are identical to sequences adjacent to a chosen RE site. The linearized vector and the PCR products are co-transfected into *E. coli* strain JC8679. This technique may not be suitable for use with traditional *E. coli* strains because independent reports indicate that DH5α cells cannot catalyze intramolecular gap repair, and thus might not be expected to catalyze inter-molecular recombination (Hanahan, 1985). A similar methodology described by Bubeck et al., (1993) reported successful recombination in DH5α cells but only if they were transformed by $CaCl_2$ methods. Two more commonly used techniques for bacterial transformation known as heat shock and electroporation were unsuccessfully used in the above experiment.

vi) Exonuclease-based PCR cloning

A completely different approach to the cloning of PCR fragments involves the generation of single-strand overhangs through the action of various exonucleases. All of the exo-based methods are ligase-independent and are based on the technique originally reported by Aslanidis and deJong, (1990). Numerous modifications to this technique have allowed for improvements in the method (Haun, Serventi et al. 1992; Kuijper, Wiren et al. 1992), both of which use the 3'-5' exonuclease activity of T4 DNA polymerase. PCR primers are designed to contain a 5'-extension complementary to sequences adjacent to a chosen RE site within the vector. Single-strand overhangs are generated through the exonucleolytic digestion by T4 pol and annealing of single-strand regions between the vector and insert is sufficiently stable to allow for direct bacterial transformation. These techniques require delicate control of incubation periods, as these enzymes are extremely efficient, and if one is not careful, excess DNA can be digested. Kuijper et al., (1992) also reported that there is great variation between enzyme preparations, therefore, requiring fine-tuning of temporal conditions with each new batch of enzyme. A second drawback to these specific methods is the requirement for the addition of dTTPs or dATPs in the exo reaction to stop the enzyme at the appropriate positions.

A similar method to that reported above uses a different enzyme called exonuclease III (Hsiao 1993), which was originally used for cloning in 1992 (Kaluz,

Kolble et al. 1992). Its limitation is that only blunt-ended or 5'-overhanging substrates can be efficiently cloned. Substrates with 3'-overhangs cannot be cloned by this method.

More recently, phage T7 Gene6 exonuclease has been used for PCR-cloning (Zhou and Hatahet 1995). In this technique PCR primers are designed to include internal phophorothioate bonds positioned towards the center of the primers. The 3'end of the primers are standard PCR primers, whereas the 5' ends are designed to be complementary to sequences adjacent to a certain RE site. This method produces directional clones and is ligase-independent but requires the use of non-standard PCR primers. US 5,580,759 (Yang, et al.) also discloses a method of construction of recombinant DNA by exonuclease recession.

In summary, a wide variety of methods exist for the cloning of PCR products, and each has its advantages and disadvantages. There remains a need for an optimal cloning method having the following characteristics:

- compatible with the use of any vector and any restriction enzyme;
- requires only two PCR primers comprised solely of natural bases;
- ligase independent;
- time efficient;
- provides almost exclusively directional cloning;
- only the terminal sequences of the amplified region need to be known;
- no possibility of internal digestion of the PCR product;
- any type of amplifying polymerase can be used;
- compatible with various readily available *E. coli* strains;
- transformation of bacteria can be accomplished through a variety of techniques;
- unambiguous selection; and
- adaptable to other techniques such as combinatorial cloning.

There is also a need to identify other compounds which can improve the efficiency of the joining reaction. The other compounds would preferably also stabilize the joint DNA.

SUMMARY OF THE INVENTION

DNA polymerase, such as vaccinia DNA polymerase, is able to efficiently join two or more linear DNA molecules. The method uses recombination between sequence

elements present at the end(s) of a series of DNA molecules to specify the junctions and uses specific DNA polymerases to join the molecules. The method may be used to join 2 or more linear DNA molecules precisely in a single procedure. The primary application of this new method is in the construction of recombinant DNA molecules, as specifically applied to the cloning of PCR products into any desired vector. The present method offers all of the characteristics of an optimal cloning method listed above.

In its broad aspect, the present invention provides a method of joining two or more linear DNA molecules comprising the steps of:

- obtaining one or more linear DNA molecules, each having an a and a' strand, having opposite polarities, wherein the 5' end of the a' strand of each linear DNA molecule has a sequence of nucleotides that is complementary to the 5' end of the a strand of the linear DNA molecule to which it is to be joined; and
- incubating the two or more linear DNA molecules in the presence of a DNA polymerase;

wherein the DNA polymerase has intrinsic exonuclease activity and is capable of performing the DNA joining reaction of the invention.

This method may be used to join any number of DNA molecules. The key is to ensure that the molecules are selected such that the ends of each DNA molecule have complementary regions to the molecule it is to be joined to. Internal molecules must have complementary regions on both ends. Particular applications of this technology are the combinatorial fusion of DNA cassettes and preparation of synthetic genes. The technique is also useful for reconstruction of fragmented clones.

The DNA polymerases that may be used in the method of the invention include all DNA polymerases having intrinsic exonuclease activity, specifically 3'-5' exonuclease activity, that are capable of performing the DNA joining reaction of the invention. Such a polymerase may be identified by assaying for its ability to join two linear DNA molecules, having ends with complementary nucleotide sequences, as described herein. Preferably the DNA polymerase is selected from the group consisting of vaccinia virus DNA polymerase, T4 DNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I. Most preferably, the DNA polymerase is vaccinia virus DNA polymerase. The length of the complementary nucleotide sequence on each linear DNA molecule may

be between about 5 and about 100 nucleotides, preferably between about 8 and about 50 nucleotides and, most preferably, between about 10 and 30 nucleotides.

In another of its embodiments, the present invention involves a method of constructing a recombinant DNA molecule comprising the steps of:

- obtaining a linearized vector DNA molecule and a template DNA molecule, each having a first and a second end;
- obtaining a first primer DNA molecule having a 5' end that comprises nucleotide sequences that will incorporate nucleotide sequences that are complementary to the first end of the linearized vector molecule onto the first end of the template DNA molecule and a 3' end that is designed to hybridize to a suitable location on the first end of the template DNA molecule;
- obtaining a second primer DNA molecule having a 5' end that comprises nucleotide sequences that will incorporate nucleotide sequences that are complementary to the second end of the linearized vector molecule onto the second end of the template DNA molecule and a 3' end that is designed to hybridize to a suitable location on the second end of the template DNA molecule;
- amplifying the template DNA molecule using the polymerase chain reaction with the first and second primers to generate a PCR amplified product; and
- incubating the PCR amplified product with the linearized vector DNA molecule in the presence of a DNA polymerase to generate a recombinant DNA molecule; wherein the DNA polymerase has intrinsic exonuclease activity and is capable of performing the DNA joining reaction of the invention.

The DNA polymerases that may be used in the method of the invention include all DNA polymerases having intrinsic exonuclease activity, specifically 3'-5' exonuclease activity, that are capable of performing the DNA joining reaction of the invention. Such polymerases may be identified by assaying for the ability to join two linear DNA molecules having ends with complementary nucleotide sequences, as described herein. Preferably the DNA polymerase is selected from the group consisting of vaccinia virus DNA polymerase, T4 DNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I. Most preferably, the DNA polymerase is vaccinia virus DNA polymerase.

The number of complementary nucleotides that are incorporated onto the first and second ends of the template DNA molecule may be between about 5 and about 100 nucleotides, preferably between about 8 and about 50 nucleotides and, most preferably, between about 10 and 30 nucleotides.

This method could be readily applied to the insertion of two or more DNA molecules into a vector. Each additional molecule would require two further bipartite PCR primers, each having a first portion that primes a PCR extension that is complementary to the appropriate end of the adjacent molecule and a second portion that is complementary to the appropriate end of the DNA molecule to be amplified.

The present invention provides a method to directionally clone any template DNA molecule into a single restriction site of any vector. The vector ends may be generated from any restriction enzyme cleavage. The method does not require a ligation step nor the use of carefully controlled conditions as is required with methods involving specific exonucleases alone.

The present invention is also directed to kits containing reagents for conducting the method.

These and other aspects of the present invention will be described in greater detail hereinbelow.

A DNA polymerase and a suitable single strand DNA binding protein are useful for cloning (for example, to clone PCR - amplified DNA). In one example, vaccinia DNA polymerase and gpI3L are the only proteins needed to catalyze concatemer formation *in vitro*.

BRIEF DESCRIPTION OF THE DRAWINGS

The features of the present invention will become more apparent from the following description in which reference is made to the appended drawings in which:

Figure 1 shows a schematic representation (Panel A) and the experimental results (Panel B) for the joining of two linear DNA molecules using the method of the invention.

Figure 2 shows a schematic representation (Panel A) and the experimental results (Panel B) for the joining of three linear DNA molecules using the method of the invention.

Figure 3 shows a schematic representation of the application of the method of the invention to the combinatorial fusion of DNA cassettes.

Figure 4 is an autoradiograph showing the fate of 5'- and 3'-end labels during the joining of three linear DNA molecules using the method of the invention.

Figure 5 is a schematic representation of a proposed mechanism for the reaction involved in the method of the invention.

Figure 6 is a schematic showing the application of the method of the invention to the cloning of PCR products.

Fig. 7. DNA annealing catalyzed by vaccinia DNA polymerase. pDW101 was cut with EcoRI or XhoI to produce 2.96 kbp molecules sharing 48 bp of overlapping sequence homology (Panel A.). Annealing reactions (20 µL) were then prepared containing 0.35 µg of each DNA substrate plus 0.1 µg of vaccinia virus polymerase. After incubation for the indicated times, the reaction products were fractionated using agarose gel electrophoresis and visualized by ethidium bromide staining (Panel B). Alternatively, reactions were prepared containing 0.35 µg of each DNA substrate plus the indicated quantities of DNA polymerase and incubated for 20 min at 37 (Panel C.). A 6 kbp reaction product is formed in Panels B and C.

Fig. 8. Substrate requirements. Reactions were prepared containing the indicated substrate DNAs plus or minus vaccinia DNA polymerase (0.1 µg) in 20 µL. The polymerase will not promote joint molecule formation unless two substrates are provided which share some overlapping end homology (lanes 1-6). When the DNA fragments can be differentiated by size, the reaction products comprise DNAs encoding the XhoI - EcoRI overlap region (lanes 8-13).

Fig. 9. Effect of vaccinia SSB (gpI3L) on joint molecule formation. Standard reactions (20 µL) contained 0.1 µg of vaccinia virus DNA polymerase (E) or 0.1 µg of polymerase plus 0.5 µg of vaccinia SSB (●). The reactions were incubated for the indicated time, stopped, and the yield of joint molecules determined by gel electrophoresis (inset) and densitometry. Vaccinia SSB increased the extent of the reaction and stabilized the products.

Fig. 10. Trimer formation catalyzed by vaccinia virus DNA polymerase. Three substrates were prepared as indicated in the upper panel, and the middle substrate ("B")

labeled using polynucleotide kinase or Klenow polymerase. Reactions were prepared as indicated, incubated at 37°C for 20 min, and then fractionated using agarose gel electrophoresis. The reaction products were first visualized with ethidium bromide, and then by autoradiography. No joint molecules were formed in reactions containing polymerase plus only a single DNA substrate (lanes 1 and 4). Only a 5' label survived incubation with the polymerase and was incorporated into dimer and trimer molecules (lane 3).

Fig. 11. Cloning of PCR-amplified DNA using vaccinia virus DNA polymerase. An 800 bp PCR-amplified DNA fragment ("A") was incubated with or without NotI-linearized pDW101 ("B") and vaccinia virus DNA polymerase, as indicated. The reaction products were separated by electrophoresis and visualized with ethidium bromide. Joint molecules were seen only in lane 6. Most of these joint molecules migrated at a position expected of linear dimers (lane 6), but a small portion of the reaction product migrated at a position typical of nicked circular molecules (arrowed). Adding 5 μM dNTPs blocked the reaction completely (lane 7). Table 1 summarizes the effect of transforming E. coli with these reaction products.

Fig. 12. Effect of homology length on joint molecule formation. PCR amplified DNAs were prepared containing 4, 6, 8, ..., 18 bp of end sequence identical to sequences found flanking the NotI site in a pBluescript vector. A mixture of PCR amplified DNA plus NotI-cut vector was incubated with 0.1 μg of vaccinia DNA polymerase and 0.5 μg of gpI3L for 10 min and then separated by agarose gel electrophoresis (Panel B, inset). The yield of linear-duplex (E) and circular (●) joint molecules in each reaction was determined by densitometry (Panel B). Omitting the polymerase yielded no joint molecules (C). The reaction products (plus joint molecules prepared in two additional experiments) were used to transform bacterial SURE cells and the yield of recombinants determined as in Table I (Panel A).

Fig. 13 Map of DNA to be recombined.

Fig. 14 In vitro recombination showed on 0.8 % agarose gel. After the joining reaction, 1 ul of reaction solution was used for transformation. The rest of solution was deproteined and electrophoresized.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS:

The term "complementary" as used herein, refers to nucleotide sequences in a single stranded molecule of DNA that are sufficiently complementary to a strand of nucleotide sequences in another DNA molecule to specifically (non-randomly) hybridize to it with consequent hydrogen bonding.

The term "linear DNA molecule" as used herein refers to a double stranded (duplex) nucleic acid molecule comprising two strands of opposite polarity (sense and antisense) of substantially complementary polynucleotides held together by one or more hydrogen bonds between each of the complementary bases present in a base pair of the molecule.

The term "recombinant DNA molecule" as used herein refers to a hybrid DNA molecule comprising at least two nucleotide sequences not normally found together in nature.

The term "nucleotide" as used herein refers to a monomeric unit of DNA consisting of a sugar moiety (pentose), a phosphate group and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of pentose) and that combination of base and sugar is a nucleoside. When the nucleoside contains a phosphate group bonded to the 3' or 5' position of the pentose it is referred to as a nucleotide. A sequence of operatively linked nucleotides is typically referred to herein as a "nucleotide sequence", and their grammatical equivalents, and is represented by a formula whose left to right orientation is in the conventional direction of 5'-terminus to 3'-terminus.

The present invention provides a method to join two or more linear DNA molecules using specific DNA polymerases. The method relies on the ability of specific DNA polymerases to facilitate the recombination of linear DNA molecules having ends that share complementary nucleotides.

The present invention therefore provides a method of joining two or more linear DNA molecules comprising the steps of:

- obtaining one or more linear DNA molecules, each having an a and a' strand having opposite polarities, wherein the 5' end of the a' strand of each linear DNA

molecule has a sequence of nucleotides that is complementary to the 5' end of the a strand of the linear DNA molecule to which it is to be joined; and

- incubating the two or more linear DNA molecules in the presence of a DNA polymerase;

wherein the DNA polymerase has intrinsic exonuclease activity and is capable of performing the DNA joining reaction of the invention. A single strand DNA binding protein may optionally be used in the methods of the invention. For example, one may incubate the two or more linear DNA molecules in the presence of a DNA polymerase and a single strand DNA binding protein.

DNA polymerases that work in this method are those having intrinsic exonuclease activity, specifically 3'-5' exonuclease activity, that are capable of performing the DNA joining reaction of the invention. Such polymerases may be identified by assaying for the ability to join two linear DNA molecules having ends with complementary nucleotide sequences as described in Experiment 2 herein (Experimental Section). Purified vaccinia DNA polymerase was used in the examples presented herein. The reaction has also been shown to work with T4 DNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I. The DNA polymerases of the invention are either commercially available or may be prepared using standard recombinant DNA technology. For example, vaccinia DNA polymerase may be purified from vaccinia-infected BSC-40 cells as described in McDonald and Traktman (1994) and Willer, Mann *et al.* (1999). Alternatively, vaccinia DNA polymerase may be purified from cells infected with wild-type vaccinia virus. We assay polymerases to measure their ability to join two linear DNA molecules having ends with complementary nucleotide sequences These include but are not limited to, those encoded by bacteriophage (e.g. T4, T7), bacteria (e.g. *E. coli*), fungi (e.g. *S. cerevisiae*) and viruses (e.g. poxviruses, herpes viruses, adenoviruses, African swine fever virus and other Iridoviruses, and Bacculoviruses).

The degree of complementation between the ends of the linear DNA molecules can vary from between about 5 and about 100 nucleotides, preferably between about 8 and about 50 nucleotides and, most preferably, between about 12 and 30 nucleotides. The linear DNA molecules that may be joined using the method of the invention may be obtained from genomic DNA of prokaryotic or eukaryotic genomes, as well as from

various vectors, including plasmids, cosmids, phage, BACs and the like, using restriction enzyme cleavage. Alternatively, the linear DNA molecule may be synthesized by chemical techniques, for example, using automated synthesis and the like. The DNA molecule may also be derived from RNA species, such as mRNA, tRNA and rRNA, from any species and may first be converted to cDNA by reverse transcriptase and the amplified as described in Sambrook *et al.* (1989).

In its most general application, the present invention provides a method to join two or more linear DNA molecules. Figure 1 shows a schematic of this reaction for the joining of two linear DNA molecules (Panel A). This illustrates how two substrates sharing complementary sequences on the left and right ends can be incubated in the presence of a specific DNA polymerase to form a dimer. Panel B of Figure 1 shows the results from an experiment where a 1:1 mixture of two substrates, sharing 30 bp of complementary sequence at the left and right ends, was incubated with varying quantities of vaccinia DNA polymerase and the products separated by size using an agarose gel. No reaction is observed when the polymerase is omitted (lane 2) and increasing yields of products were seen when increasing amounts of polymerase were added (lanes 3-9).

Figure 2, Panel A, shows the joining of three linear DNA molecules to produce a trimer. In this case the "middle" molecule must have sequences of DNA on each end that are complementary to the ends of the two flanking molecules. The results from an actual experiment where the middle molecule had about 20 bp of complementary DNA sequences with the two flanking molecules on each end are shown in Panel B of Figure 2. The results in Panel B of Figure 2, also illustrate an important control (lane 5). The polymerase will not form concatemers when provided with just one of the three substrates, since the ends of identical molecules do not share the appropriate type of complementation. This specificity means that one can direct exactly how two or more molecules are fused, simply by controlling a nearly infinite array of terminal sequence overlaps.

Another application of the present invention is in the combinatorial fusion of DNA cassettes as illustrated in Figure 3. In this example, three different pools of DNA molecules are prepared. Within a pool of molecules, the central portion of each molecule varies in sequence while the ends are identical. For example, as shown in Figure 3, A1-

A5 all have the same about 5 to about 100, preferably about 8 to about 50, more preferably about 10 to about 30, nucleotide sequences at the right end, but the remaining portion of the molecules vary in sequence. The left ends of the molecules in the "B" pool are compatible with the right ends of molecules in the "A" pool. Similarly, the left ends of the molecules in the "C" pool are compatible with the right ends of molecules in the "B" pool. By compatible, it is meant that they share appropriate complementary sequences ranging from about 5 to about 100, preferably about 8 to about 50, more preferably about 10 to about 30, nucleotides. Incubation of the mixture of molecules from pools A, B and C with a DNA polymerase useful in the method of the invention will create trimers composed of one of each of the molecules selected from the A, B, and C pools. The resulting pool of trimers should express a random assortment of "A", "B" and "C" variants. The number of "pools" and molecules within each pool could be varied to potentially produce a random assortment of millions of different DNA molecules. The combinatorial fusion of DNA cassettes using the method of the invention may be applied to the construction of DNA sequences encoding novel proteins through the combinatorial fusion of DNA molecules encoding different protein structural domains. These libraries of proteins may be screened for novel binding specificities.

To gain insights into the mechanism underlying this reaction the ends of the "middle" DNA molecule in the reaction involving three linear DNA substrates were ^{32}P end-labelled and the fate of the label when joint molecules are formed was examined. Although joint molecules were formed regardless of where the label was located, it was determined that 5' ^{32}P-end labels are quantitatively retained in the concatemer while 3' ^{32}P-end labels are removed (Figure 4). While not wishing to be limited by theory, the simplest explanation for how this reaction works is that the vaccinia viruses have evolved a peculiar form of DNA polymerase which can synapse double stranded molecules bearing terminal complementary sequences, while using the intrinsic 3'-5' exonuclease activity to remove a small number of bases displaced during the annealing reaction, as shown in Figure 5. The exposed single stranded ends are then capable of base-pairing to reform a DNA duplex. The result is a joint molecule that is held together by "sticky ends" and the reaction is driven by the exonucleolytic degradation of small numbers of bases from the 3' ends of both molecules. Such sticky ends are larger than, but otherwise

much like, those formed when complementary ends of restricted DNAs basepair in solution.

Several additional reaction features were investigated. Perhaps most important is the fact that vaccinia polymerase does not seem to care what type of ends are present on the two recombining molecules. Molecules bearing 5' overhanging, 3' overhanging or blunt ends, in any pairwise combination, were still substrates as long as the two molecules shared appropriate sequence complementation.

In another of its embodiments, the present invention provides a method of cloning any double stranded PCR product into any vector. Therefore there is provided, a method of constructing a recombinant DNA molecule comprising the steps of:

- obtaining a linearized vector DNA molecule and a template DNA molecule, each having a first and a second end;
- obtaining a first primer DNA molecule having a 5' end that comprises nucleotide sequences that will incorporate nucleotide sequences that are complementary to the first end of the linearized vector molecule onto the first end of the template DNA molecule and a 3' end that is designed to hybridize to a suitable location on the first end of the template DNA molecule;
- obtaining a second primer DNA molecule having a 5' end that comprises nucleotide sequences that will incorporate nucleotide sequences that are complementary to the second end of the linearized vector molecule onto the second end of the template DNA molecule and a 3' end that is designed to hybridize to a suitable location on the second end of the template DNA molecule;
- amplifying the template DNA molecule using the polymerase chain reaction with the first and second primers to provide a PCR amplified product; and
- incubating the PCR amplified product with the linearized vector DNA molecule in the presence of a DNA polymerase to generate a recombinant DNA molecule; wherein the DNA polymerase has intrinsic exonuclease activity and is capable of performing the DNA joining reaction of the invention. A single strand DNA binding protein may optionally be used in the methods of the invention. For example, one may incubate the PCR amplified product with the linearized vector DNA molecule in the presence of a DNA polymerase and a single strand DNA binding protein.

In this method, all that is required is that the PCR primers be extended to incorporate some nucleotides that duplicate the base sequence flanking the chosen cloning site in the vector. The suggested approach is illustrated in Figure 6. A set of two bipartite primers is designed. The 3' end of the first primer molecule is designed to hybridize with the first end of the template DNA molecule, and the 5' end of the first primer molecule has a sequence of from about 5 to about 100, preferably about 8 to about 50, more preferably about 10 to about 30, nucleotides that are designed to incorporate sequences in the final PCR product that are complementary to the first end of the vector DNA molecule. The 3' end of the second primer is designed to hybridize with the second end of the template DNA molecule, and the 5' end of the second primer molecule has a sequence of from about 5 to about 100, preferably about 8 to about 50, more preferably about 10 to about 30, nucleotides that are designed to incorporate sequences in the final PCR product that are complementary to the second end of the vector DNA molecule. The two primers are then annealed to the template DNA molecule which is then PCR amplified using standard conditions to generate a PCR amplified product. The vector is prepared by treating it with the appropriate restriction enzyme to cut it at the chosen insert site using standard conditions.

The term primer as used herein is meant to describe a bipartite primer or a primer having a first and second portion. A first portion of the primer is designed to be complementary to the appropriate end of a template DNA molecule and a second portion of the primer is designed to be complementary to nucleotide sequences on one side of the chosen restriction site of the vector. The bipartite oligonucleotide primers of the present invention may be broadly defined as single stranded oligonucleotides that are complementary to DNA molecules of interest and will allow the DNA molecules to be incorporated into a vector. Bipartite primers will generally have a minimum length of about 18 nucleotides and a maximum length of about 200 nucleotides, preferably about from 25 nucleotides to about 100 nucleotides, more preferably from about 30 nucleotides and about 40 nucleotides. This method could be readily applied to the insertion of two or more DNA molecules into a vector. Each additional molecule would require two further bipartite PCR primers, each having a first portion that primes a PCR extension that is

complementary to the appropriate end of the adjacent molecule and a second portion that is complementary to the appropriate end of the DNA molecule to be amplified.

The PCR amplified product and the linearized vector are then incubated in a suitable reaction buffer in the presence of a DNA polymerase having intrinsic exonuclease activity, specifically 3'-5' exonuclease activity, that is capable of performing the DNA joining reaction of the invention, for about 5 to about 60 minutes, preferably from about 10 to about 40 minutes, most preferably from about 15 to about 30 minutes. Purified vaccinia DNA polymerase was used in the examples presented herein. The reaction has also been shown to work with T4 DNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I. The reaction buffer may be any buffer that is used in DNA annealing reactions. The temperature may be in the range of from about 35-40 °C, more preferably about 37 °C.

The DNA polymerases that work in this method may be identified by assaying for the ability to join two linear DNA molecules having ends with appropriate complementary sequences as describe in Example 2 herein (Experimental Section). The DNA polymerases of the invention are either commercially available or may be prepared using standard recombinant DNA technology. For example, vaccinia DNA polymerase may be purified from vaccinia-infected BSC-40 cells as described in McDonald and Traktman (1994) and Willer, Mann *et al.* (1999). Alternatively, vaccinia DNA polymerase may be purified from cells infected with wild-type vaccinia virus. We assay polymerases to measure their abilityto join two linear DNA molecules having ends with complementary nucleotide sequences. These include, but are not limited to, those encoded by bacteriophage (e.g. T4, T7), bacteria (e.g. *E. coli*), fungi (e.g. *S. cerevisiae*) and viruses (e.g. poxviruses, herpes viruses, adenoviruses, African swine fever virus and other Iridoviruses, and Bacculoviruses).

The method of the invention may be used to PCR clone any variety or number of target DNA molecules. The only limitation on size is the capacity of the vector molecule to carry the insert in transformation and replication in the host cell. Any vector capable of replicating in a prokaryotic or eukaryotic cell is usable with the present invention, such as plasmids, cosmids, phage, BACs and the like. The choice of vector depends on the functional properties desired, for example, protein expression, and the host cell to be

transformed. Preferably, the vector has a known sequence of about 5 to about 100, preferably about 8 to about 50, most preferably about 10 to about 30 nucleotides, on either side of the chosen restriction enzyme site. The DNA molecules that may be incorporated into a recombinant DNA molecule using the method of the invention may be obtained from genomic DNA of prokaryotic or eukaryotic genomes, as well as from various vectors, including plasmids, cosmids, phage, BACs and the like, using restriction enzyme cleavage. Alternatively, the DNA molecules may also be synthesized by automated synthesis and the like. The nucleic acid may also be derived from RNA species, such as mRNA, tRNA and rRNA, from any species and may first be converted to cDNA by reverse transcriptase and the amplified as described in Sambrook *et al.* (1989).

The reaction mixture obtained from the incubation of DNA polymerase with the linearized vector and the PCR amplified product may be used directly to transform any host cell using standard transformation procedures. Therefore the present invention also provides a method to produce a recombinant DNA product comprising the steps of:

- obtaining a linearized vector DNA molecule and a template DNA molecule, each having a first and a second end;
- obtaining a first primer DNA molecule having a 5' end that comprises nucleotide sequences that will incorporate nucleotide sequences that are complementary to the first end of the linearized vector molecule onto the first end of the template DNA molecule and a 3' end that is designed to hybridize to a suitable location on the first end of the template DNA molecule;
- obtaining a second primer DNA molecule having a 5' end that comprises nucleotide sequences that will incorporate nucleotide sequences that are complementary to the second end of the linearized vector molecule onto the second end of the template DNA molecule and a 3' end that is designed to hybridize to a suitable location on the second end of the template DNA molecule;
- amplifying the template DNA molecule using the polymerase chain reaction with the first and second primers to provide a PCR amplified product;
- incubating the PCR amplified product with the linearized vector DNA molecule in the presence of a DNA polymerase to generate a recombinant DNA molecule;
- transforming the recombinant DNA molecule into a host cell; and

- isolating the recombinant DNA product;

wherein the DNA polymerase has intrinsic exonuclease activity and is capable of performing the DNA joining reaction of the invention. A single strand DNA binding protein may optionally be used in the methods of the invention. For example, one may incubate the PCR amplified product with the linearized vector DNA molecule in the presence of a DNA polymerase and a single strand DNA binding protein to generate a recombinant DNA molecule

Any variety of cell that is transformable may serve as a host cell, such as *E. coli* SURE, JM105, DH5α, HB101, XL1-blue and the like. Other bacterial hosts may include *Bacillus* or *Pseudomonas* species and the like. By way of example, eukaryotic host cells may include *Saccharomyces* species. One of the advantages of the present invention is that the method is selective for the correct orientation of the insert DNA. Therefore, host cells that have been transformed by a recombinant vector will have the insert DNA in the correct orientation in that vector.

The present invention also provides kits suitable for directionally cloning PCR products into a linearized vector. The kit may comprise, in separate containers, an aliquot of a DNA polymerase having intrinsic exonuclease activity that is capable of performing the DNA joining reaction of the invention and an aliquot of reaction buffer. An aliquot refers to an amount of the component sufficient to perform at least one program of cloning. The DNA polymerase may be provided as a solution of known concentration, in a buffer optionally comprising a suitable stabilizer, or may be provided as a predetermined aliquot of a freeze-dried product for dissolution in a suitable buffer. The kit may also comprise reagents required to perform a positive control reaction. Such reagents may include, in separate containers, an aliquot of linearized vector, an aliquot of insert DNA with first and second ends having appropriate complementary sequences, an aliquot of DNA polymerase having intrinsic exonuclease activity that is capable of performing the DNA joining reaction of the invention and an aliquot of reaction buffer.

In one preferred variation of the invention, DNA polymerase may be combined with a single strand DNA binding protein. For example, vaccinia virus DNA polymerase may be used in cloning and other methods of the invention along with vaccinia virus single strand DNA binding protein. A skilled person would be able to readily identify

other suitable DNA binding proteins that are usefully combined this DNA polymerase in methods of the invention.

The invention shows that vaccinia virus DNA polymerase can catalyze a Mg^{+2}-dependent reaction which assembles non-covalently linked recombinant concatemers. The reaction seems to depend upon exonuclease processing of the DNA ends sharing as little as 10-12 bases of properly oriented overlapping sequence homology (Fig. 12). This exonuclease is presumably the 3'-5' exonuclease as judged by the fact that 3'-end labels are lost from recombinant molecules (Fig. 10) and the reaction is inhibited by dNTP's. The efficiency of the reaction is enhanced significantly by adding gpI3L, which also stabilizes the newly formed joint molecules (Fig. 9). The compound gpI3L has only limited and irreproducible strand annealing properties, which suggests that adding vaccinia SSB probably does not increase the yield of joint molecules by stimulating annealing within protein-coated aggregates. Instead, we suspect that its presence counters the reaction-limiting effects of the "end dilution" which accompanies reactant consumption. These newly assembled concatemers were joined by imperfect non-covalent joints which are readily converted into stable recombinants by E. coli repair systems (Table 1 and Fig. 12). The reaction is sufficiently simple and efficient that it is suitable as a method of cloning DNAs, such as PCR-amplified DNAs.

Together, vaccinia DNA polymerase and gpI3L generate concatemers, and the single-strand annealing reactions we observe in vitro are a well-established way of promoting the "pop-out" (Ball, 1987) and other repeat-associated deletion events affecting replicating poxviruses (Aguado, Selmes and Smith, 1992; Shchelkunov and Totmenin, 1995). These types of non-conservative, single-strand annealing reactions are also expected to generate large quantities of hybrid DNA (Fisher et al., 1991) and, if these annealing reactions were to involve sequences duplicated within virus terminal-inverted repeats, the origin of mirror image deletions (McFadden and Dales, 1979) and the cause of telomeric repeat instability (Pickup et al., 1982) also becomes clearer.

Besides providing insights into viral recombination, concatemer forming, and mutational processes, strand annealing reactions might also have important implications for our understanding of poxviral replication. It has long been proposed that replication originates within the viral telomeres, but no viral or cellular primase has been identified

that initiates viral replication (Du and Traktman, 1996; Moyer and Graves, 1981). Nor is it understood how transfected DNAs can replicate in poxvirus-infected cells without any regard to DNA sequence (DeLange and McFadden, 1986). These problems might be resolved if poxviruses are eventually shown to use the types of strand-annealing reactions characterized here to prime DNA replication. For example, were the virus replicative machinery to take one randomly broken and transfected molecule and anneal it to a second transfected molecule, the 3' end might serve as an origin for non-specific plasmid replication reactions.

Two particular features of this reaction provide a way of further investigating the biological relevance of this process. The first concerns the fact that in vitro strand joining reactions are very sensitive to changes in dNTP concentrations. Although the physiological concentration of dNTPs is not known with precision in vaccinia-infected cells [and probably varies during the course of an infection (Howell et al., 1993)], one rough estimate suggests that it lies in the 5-to-15 μM range (Hendricks and Mathews, 1998). A small decrease in intracellular dNTP concentrations favours single-strand annealing reactions over DNA replication. We showed this by inhibiting the activity of vaccinia virus ribonucleotide reductase with hydroxyurea. These preliminary experiments detect dramatic increases in the amount of recombination relative to the amount of replication under such conditions. A second, and much more specific feature of the reaction concerns the effect of sequence length on strand joining efficiency. For example, twelve to 14 bp of properly oriented homologous sequence are useful for optimal strand joining in vitro and subsequent transformant production in E. coli (Fig. 12). Using vaccinia-infected cells, tranfections using linearized luciferase-reporter plasmids suggest that this number lies remarkably close to the minimum amount of sequence homology required for recombinant formation in vivo – about 14 bp.

The present invention will be further illustrated in the following examples. However, it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

TABLE 1

Transformation of E. coli with recombinant joint molecules.

Reaction	Number of colonies White	Blue	Percent white	Transformants per µg (x 10^{-5})
PCR-amplified insert				
(-) polymerase	2	0	100	N/A
(+) polymerase	1	0	100	N/A
NotI restricted vector				
(-) polymerase	8	1,190	0.7	4.0
(+) polymerase	0	27	0.0	0.4
PCR-amplified insert plus NotI restricted vector				
(-) polymerase	12	1,030	1.2	2.3
(+) polymerase	1,020	210	83	2.7
(+) polymerase, (+) dNTP	7	220	3.1	0.5

Joint molecules were prepared in reactions containing the indicated reaction components. E. coli SURE cells were electroporated with 1 µL of reaction products. The cells (100 µL or its equivalent) were plated and colonies were counted next day. Cell competency was estimated as 6 x 10^{10} transformants per µg using unrestricted pDW101.

EXPERIMENTAL EXAMPLES

Materials and Methods

Linear substrates were prepared by restriction enzyme digestion of plasmid pBDW/KS (+), followed by phenol extraction and ethanol precipitation. pBDW/KS is a derivative of pBluescript KS (+) which contains natural deletions at positions 618, 619 and 621. Substrates used for cloning experiments were purified by agarose electrophoresis and extracted from the agarose using a Geneclean II kit (New England Biolabs). [α - ^{32}P] dCTP and [γ -^{32}P] dATP were purchased from NEN/Mandel Scientific. End labeled substrates were generated by treatment with shrimp alkaline phosphatase (United States Biochemical, Cleveland), followed by incubation with either T4 polynucleotide kinase and [γ -^{32}P] dATP (3000 Ci/mmol) or Klenow enzyme and [α - ^{32}P] dCTP (3000 Ci/mmol). Unincorporated radionucleotides were removed by G-25 spin columns (Pharmacia) and radioactivity was quantitated by Cherenkov (Schneider, 1971). *Hind*III-digested lambda phage DNA and *Hae*III-digested ΦX174 DNA markers were purchased from New England Biolabs. Luciferase assay kit was purchased from Promega.

Cell and Virus Culture

BSC-40 cells were kindly provided by Dr. E. Niles (SUNY, Buffalo) and grown at 37 °C in DMEM (Gibco BRL) supplemented with 1% nonessential amino acids and 5% fetal calf serum in a 5% CO_2 environment. Vaccinia virus (strain WR) was purchased from the ATCC.

Linear substrates were prepared by restriction of plasmid pDW101, followed by phenol extraction and ethanol precipitation. [pDW101 derives from pBluescript (KS+) (Stratagene) through a spontaneous deletion of nucleotides 618, 619 and 621.] In some experiments the restricted DNAs were further gel purified and recovered using a Geneclean II kit (NEB). Labeled substrates were prepared by treating restricted DNA with shrimp alkaline phosphatase (USB), followed by incubation with T4 polynucleotide kinase and [γ-^{32}P] ATP (NEN) or Klenow polymerase and [α-^{32}P] dCTP (NEN).

Recombinant vaccinia virus single-strand DNA binding protein (SSB) was prepared as described (Tseng et al., 1999).

Cell and virus culture

BSC-40 cells were grown at 37°C in Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 1% nonessential amino acids and 5% fetal calf serum. Vaccinia virus (strain WR) was purchased from the ATCC.

DNA polymerase purification

A five-step protocol was used to purify vaccinia DNA polymerase from BSC-40 cells co-infected with vTMPOL and VTF7.5 recombinant viruses (McDonald and Traktman, 1994; Willer et al., 1999). Active fractions were identified using polymerase assays and protein concentrations determined using a dye-binding assay (Biorad).

Concatemer formation assays

"Standard" assays contained 30 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 70 mM NaCl, 1.8 mM dithiothreitol, 88 μg/mL acetylated BSA, 350 ng of each linear substrate, and varying quantities (usually 0.1 μg) of vaccinia polymerase in 20 μL. "Optimized" assays were identical except that 25 μg/mL of vaccinia gpI3L was also added. Reactions were incubated at 37°C for 20 min, deproteinized, and the products were separated using a 0.8% agarose gel (Zhang and Evans, 1993). Ethidium-strained gels were photographed using Polaroid film and the DNA was quantitated by densitometry.

Ligation-independent cloning

The polymerase chain reaction, two primers (5'-ACTAGTTCTAGAGCGGCCAGAAACAGGCATCTTACGCGTG-3' and 5'-TCCACCGCGGTGGCGGCCACGGAAACGCCTTGGT-3') and cloned Shope fibroma virus DNA (Delange et al., 1984) were used to amplify an ~800 bp DNA fragment flanked by two, 18 nt sequences, also found flanking the NotI site in a pBluescript polylinker (underlined above). NotI-digested pDW101 (0.6 μg) and the PCR-amplified insert (0.3 μg) were incubated with 0.15 μg of polymerase as described above. Competent E. coli cells were transformed by electroporation [SURE (Promega) and DH5α] or heat shock (JM105) using 1 μL of unpurified reaction mix. The cells were plated on Luria broth agar supplemented with 100 μg/mL ampicillin, 40 μg/mL 5-bromo-4-choro-3-indolyl-β-D-galactoside, 0.5 mM isopropylthio-β-galactoside, and 50 μg/mL thymine, and incubated overnight at 37°C. DNA was isolated from transformed colonies and sequenced as described previously (Willer, McFadden and Evans, 1999). For the experiments shown in Fig. 12, seven additional primer pairs were synthesized in which

the number of nucleotides identical to sequences flanking the NotI site were reduced in steps of two, starting from the 5' ends of each of the above primers. For example, the two shortest primer pairs, which retained 4 nt of homology, were (5'-GGCCAGAAACAGGCATCTTACGCGTG-3' and 5'-GGCCACGGAAACGCCTTGGT-3').

Experiment 1: Vaccinia DNA Polymerase Purification

Purification of vaccinia DNA polymerase from vaccinia-infected BSC-40 cells has been described (McDonald and Traktman 1994; Willer, Mann *et al.* 1999). Briefly, a crude lysate from 60 150-cm^2 dishes of BSC40 cells co-infected with vTMPOL and VTF7.5 was subjected to purification through DEAE cellulose, phosphocellulose, ceramic hydroxyapatite and HiTrap heparin columns. Active fractions eluting at each chromatographic step were determined by DNA polymerase assays (Willer, Mann *et al.* 1999). Protein purity was determined by silver staining of denaturing polyacrylamide gels. Protein concentrations were assayed by using a dye-binding assay (Biorad) and a bovine serum albumin standard.

Experiment 2: Assay for Correct DNA Polymerase Activity

We measure the intrinsic exonuclease activity of DNA polymerases, such as, those encoded by bacteriophage (e.g. T4, T7), bacteria (e.g. *E. coli*), fungi (e.g. *S. cerevisiae*) and viruses (e.g. poxviruses, herpes viruses, adenoviruses, African swine fever virus and other Iridoviruses, and Baculoviruses). The polymerases are assayed to measure their activity in the DNA joining reaction by incubating a reaction mixture comprising 30 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 70 mM NaCl, 1.8 mM dithiothreitol, 88 µg/mL acetylated BSA, 350 ng of each of a first and second linear DNA substrate and the polymerase to be assayed. The first and second linear DNA substrate must have complementary nucleotide sequences on their right and left ends respectively and may be obtained by a variety of methods including chemical synthesis and derivation of nucleic acid fragments from native nucleic acid sequences existing as genes, or parts of genes, in a genome, plasmid, or other vector, such as by restriction endonuclease digest (see, for e.g., preparation of linear substrates described in the Materials and Methods section above). The polymerase has the required activity if the formation of dimers is detected

on an agarose gel stained with ethidium bromide. A preferred polymerase will allow the formation of more that about 1% of dimeric products.

Experiment 3: DNA Annealing Assay

DNA annealing assays (20 µL) contained 30 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 70 mM NaCl, 1.8 mM dithiothreitol, 88 µg/mL acetylated BSA, 350 ng of each linear substrate, 0.1 µg of purified vaccinia DNA polymerase. Reactions were incubated at 37 °C for 20 min, deproteinized and the reaction products were separated through a 0.8% agarose gel as described previously (Zhang and Evans 1993). Gels were photographed under UV illumination using Polaroid 665 film. DNA quantitation was determined by desitometry.

Example 4: PCR Step

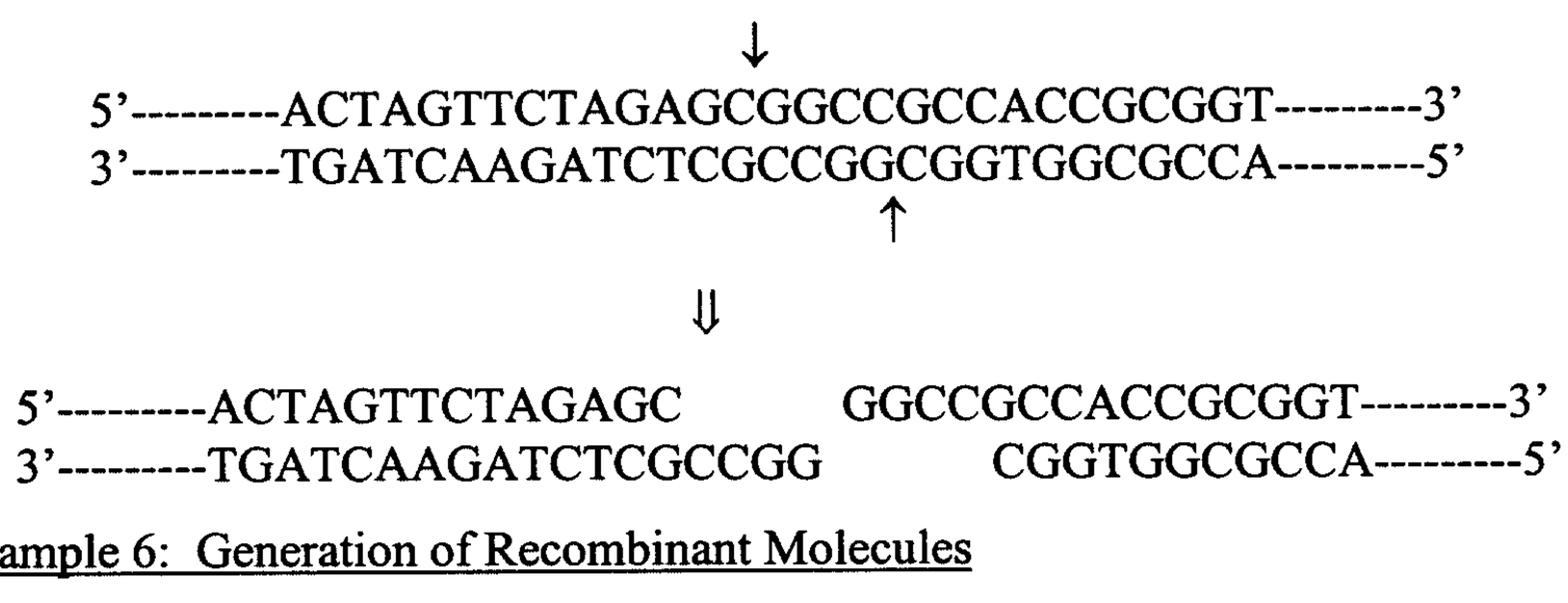
A 788 bp region from a BamHI clonederived from Shope Fibroma Virus (Wills, Delange *et al.* 1983; Delange, Macaulay *et al.* 1984) chosen at random was amplified using the PCR primers shown below. Capital letters represent the sequence complementary to the vector (pBDW/KS) and small letters represent the sequence complementary to viral DNA sequence. PCR primers were designed to include complementary sequences from the vector adjacent to the recognition site for the restriction enzyme NotI.

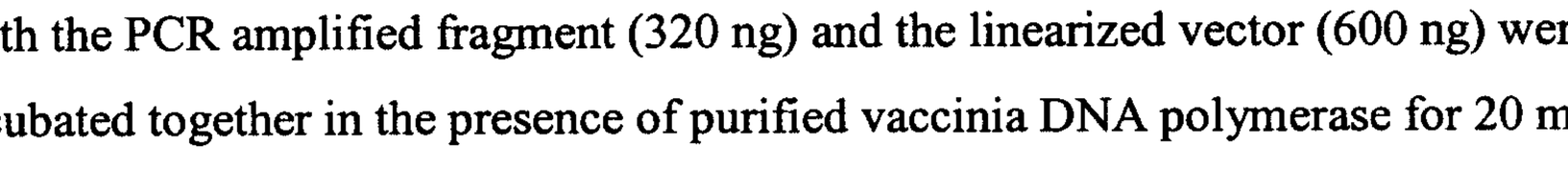
Primer #1: 5' TTCTAGAGCGGCCagaaacaggcatcttacgcgtg 3'

Primer #2: 5' TCCACCGCGGTGGCGGCCacggaaacgccttggt 3'

Example 5: Vector Preparation

The vector "pBDW/KS" was digested with NotI as depicted below:





Example 6: Generation of Recombinant Molecules

Both the PCR amplified fragment (320 ng) and the linearized vector (600 ng) were incubated together in the presence of purified vaccinia DNA polymerase for 20 minutes

as per the annealing assay described in the Materials and Methods. Reaction products were visualized by agarose electrophoresis.

Example 7: Transformation of Competent Cells

An aliquot (2 μL) of the reaction mixture from Example 3 was used to transform three different strains of *E. coli* (SURE, JM105 and DH5α) using standard procedures (SURE and DH5α, by electroporation and JM105, by heat shock). Appropriate dilutions were plated on LB+T plates containing 100 μg/mL ampicillan, 400 μg/mL X-gal and 0.5 mM IPTG and incubated overnight at 37 °C. The number and type of transformations are summarized in Table 2. White (W) colonies indicate transformation with potentially the correct construct. Blue (B) colonies indicate transformation with the vector substrate alone.

TABLE 2

	SURE			**DH5α**			**JM105**		
Rxn*	**W**	**B**	**%W**	**W**	**B**	**%W**	**W**	**B**	**%W**
-vPOL	6	515	1.2	0	413	0	6	450	1.3
+vPOL	550	122	81.8	114	26	81.4	136	4	97.1

* -vPOL indicates the reaction was performed without vaccinia polymerase while +vPOL indicates that the reaction was performed with vaccinia polymerase

The percentage of recombinants formed after incubation with the viral polymerase was very high and this value would be expected to increase significantly if the vector is dephosphorylated prior to the assay. A representative number of these white colonies and some blues were further analyzed by DNA sequencing. Remarkably, 13/13 of the white colonies tested had undergone the correct recombination event to produce the desired product. This result showcases one important quality of this cloning method; i.e. the insert DNA is only inserted in the *correct* orientation. Further analysis of the thirteen clones identified 5 base pair substitutions, however, three of these mutations were in the area generated by primer #2. The approximate overall error frequency for this reaction was:

$$\frac{\text{5 mutations within PCR'd region}}{\text{13 x 788 bp}} = 4.9 \times 10^{-4}$$

This error is well below the known error frequency for PCR reactions. Further analysis has indicated that the PCR primer itself is causing some of the mutations and that it is not an artifact of the cloning method. This reaction is extremely efficient and has the advantage of directing the insert into the correct orientation with 100% accuracy.

Example 8: Vaccinia DNA polymerase catalyzes concatemer formation

The examples below show that during poxvirus infection, both viral genomes and transfected DNAs are converted into high-molecular-weight concatemers by the replicative machinery. However, aside from the fact that concatemer formation conincides with viral replication, the mechanism and protein(s) catalyzing the reaction are unknown. We showed that vaccinia virus DNA polymerase can catalyze single strand annealing reactions in vitro, converting linear duplex substrates into linear or circular concatemers, in a manner directed by sequences located at the DNA ends. The reaction used 12 bp of shared sequence and was stimulated by vaccinia single-strand DNA binding protein (gpI3L). Varying the structures at the cleaved ends of the molecules had no effect on efficiency. These duplex joining reactions are dependent upon nucleolytic processing of the molecules by the 3'-to-5' proofreading exonuclease, as judged by the fact that only a 5'-^{32}P end label is retained in the joint molecules and the reaction is inhibited by dNTPs. The resulting concatemers are joined only through non-covalent bonds, but can be processed into stable molecules in E. coli if the homologies permit formation of circular molecules.

To show that vaccinia DNA polymerase can catalyze strand joining reactions, we prepared two linear duplex substrates sharing a small amount of overlapping sequence homology at the ends of the molecules. This was accomplished by cutting pDW101 with EcoRI and XhoI (Fig. 7A), leaving 33 bp of properly oriented sequences. DNA polymerase was then incubated with these DNAs and assayed for concatemer-forming activity using gel electrophoresis. We observed that the polymerase converted 45% of the input DNA into dimeric products over a 10 min period, with a concomitant loss of monomeric molecules (Fig. 7B). The yield of dimer declined slightly thereafter, stabilizing with about 35% of the molecules converted to product. The reaction kinetics suggested that there might be a short delay prior to the appearance of dimers, which was

not caused by a delay in reaching thermal equilibrium. The reaction yield was also dependent upon protein concentration (Fig. 7C).

Example 9: Duplex annealing selectively pairs homologous ends

This pairing reaction required two different DNA substrates sharing some small amount of overlapping end homology. Controls showed that the reaction cannot produce joint molecules when the only available substrates were a pool of identical duplexes (Fig. 8, lanes 1-6). To determine which ends of the two substrates were being joined, we further digested EcoRI and XhoI cut molecules with ScaI, producing DNA fragments that can be differentiated by size (Fig. 8, inset). Vaccinia DNA polymerase selectively joined the two molecules sharing a segment of DNA bounded by the XhoI and EcoRI sites, as judged by the appearance of recombinants 4.2, 4.8, and 3.0 kbp in length (Fig. 8, lanes 9, 11, and 13, respectively). Such product sizes are consistent with these molecules being composed of the 3.0+1.2, 3.0 + 1.8, and 1.2 + 1.8 kbp DNA fragments encoding the XhoI-EcoRI interval.

The restriction enzymes used to prepare the interacting ends shown in Figures 1 and 2 leave 5'-overhanging ends with 4 nt overhangs. To test whether ends also affected reaction efficiency, various substrates were prepared by cutting pDW101 with different restriction enzymes. All possible combinations of molecules bearing 5'-overhanging, blunt, or 5'-recessed ends were then tested to see whether they were still substrates. We found that vaccinia polymerase has no end preference. There was also no obvious correlation between the yield of duplex product and the length of sequence overlap, when the length of shared end homology ranged from 18 to 84 bp.

Example 10: Other reactions

We also examined the effect of adding $MgCl_2$, spermidine, or vaccinia virus SSB on the reaction efficiency, using KpnI- and NotI-cut substrates sharing 84 bp of overlapping homology. Magnesium had a reaction optimum of ~10 mM. However, 20 mM $MgCl_2$ inhibited the reaction, as did spermidine concentrations exceeding 1 mM. We also examined the effects of adding recombinant vaccinia SSB (Tseng et al., 1999), since many studies, including the aforementioned research involving lambda β-protein, have demonstrated the stimulatory effects of this class of protein on recombination and repair reactions. The principle replicative high-affinity single-strand DNA binding

protein (SSB) is thought to be the I3L gene product (Rochester and Traktman, 1998; Tseng et al., 1999). Vaccinia SSB enhanced the yield of joint molecules 2-3-fold when added at concentrations between 25 and 100 μg/mL (Fig. 9). This appeared to be the result of a combination of effects caused by gpI3L prolonging the joining reaction while also stabilizing the joint molecules once formed. The net result was that over 80% of the substrate DNAs could be converted to dimers under these optimized reaction conditions.

Example 11: Duplex joining reactions require 3'-5' exonuclease activity

The DNA-joining reaction can also recombine multiple substrates into higher-order concatemers and this can be exploited to provide further insights into the reaction mechanism. We cut pDW101 with restriction enzymes so that the "middle" substrate (Fig. 10, molecule "B") shared sequences in common with either end of two additional molecules ("A" and "C"). Alone, the middle duplex was not a substrate for the polymerase catalyzed reaction (Fig. 10, lanes 1 and 4). However, when the three substrates were incubated together with vaccinia polymerase in standard assays, they were rapidly converted into a mixture of dimers and trimers (Fig. 10, lanes 3 and 6). A small amount of the DNA (~2%) was also converted into higher-order multimers whose structure is uncertain. Two-dimensional gel electrophoresis suggested that they may be branched molecules.

For the middle molecule to have been incorporated into a linear trimer, both ends of the molecule must have been subjected to enzymatic processing. To study any modifications which might have been introduced into the ends of these molecules, we labeled the HindIII-restricted substrate and monitored the ^{32}P-label using autoradiography. The ^{32}P-labels were incorporated into the middle substrates using T4 polynucleotide kinase or Klenow polymerase, to see whether 3' or 5' end-labels suffered different fates. After electrophoresis and ethidium staining, the gel was fixed and autoradiographed to locate the label. It was clear, from inspection of the ethidium-stained gel, that duplex "B" was efficiently incorporated into concatemers regardless of whether it had been labeled on the 3' or on the 5' end (Fig. 10, lanes 3 and 6). However, whereas most of the 5' label was incorporated into trimeric concatemers (Fig. 10 lane 3), none of the 3'-end label was retained in these molecules (Fig. 10, lane 6) or in the unreacted polymerase-treated substrate (Fig. 10, lane 4). These data, plus the $MgCl_2$

requirement, suggests that the polymerase 3'-5' exonuclease plays a key role in joint molecule production in vitro. Parenthetically, the stability of the 5' end-label confirms reports that vaccinia polymerase does not encode a 5'-3' exonuclease (Challberg and Englund, 1979).

If this reaction is dependent upon the activity of the 3'-5' exonuclease, one would predict that dNTPs should inhibit end-joining by inhibiting the nuclease. Experiments confirmed this prediction. Adding 1 μM (total) of all four dNTPs reduced the yield about 50%, while little if any joint molecules were formed in reactions containing >5 μM dNTPs. Dideoxyribonucleotides were also inhibitory, but only at much higher concentrations (>50 μM).

Example 12: Joint molecules are readily processed into stable recombinants

When the concatemers formed by vaccinia polymerase were fractionated on alkaline agarose gels, and visualized by autoradiography, they were found to be joined non-covalently. Moreover, there appeared to be gaps or other strand discontinuities because T4 DNA ligase could not ligate the junctions. The reaction products can be further processed into stable recombinants by other enzymes. We transfected reaction products into E. coli and looked for the recovery of stable transformants. The stability of the joints formed by vaccinia polymerase, coupled with the simplicity of the reaction, showed that this method provides a practical alternative to more traditional E. coli-based cloning strategies.

The end-joining reaction was assembled containing NotI-restricted pDW101 plus a Taq-polymerase amplified fragment encoding 788 bp of Shope fibroma virus DNA. The primers added 18 bp of DNA sequence, homologous to sequences flanking the NotI site, to each end of the PCR-amplified viral DNA. After these molecules were incubated together with vaccinia polymerase in standard assays, some of the products were characterized using agarose gels (Fig. 11), while the rest of the DNA (3 x 1 μL) was used to transform three strains of E. coli (SURE, DH5α, and JM105). Agarose gels showed that the majority of molecules formed under these conditions were the linear-dimers seen previously (Fig. 11, lane 5). However, because both ends of the PCR-amplified insert shared homology with the vector, a small portion of the reaction products were expected to, and did, migrate at positions characteristic of nicked-circular and higher concatemer

forms (Fig. 11, lane 6). None of these reactions occurred in the presence of 5 μM dNTP (Fig. 11, lane 7). These joint molecules efficiently transformed all three E. coli strains without further treatment. Table 1 shows data acquired using multiply-recombination deficient SURE cells, although all three strains gave similar results (0.5 - 2 x 10^5 transformants per μg). It was clear that the yield of stable transformants was greatly dependent upon adding vaccinia DNA polymerase to the reaction mix. Recombinant (white) colonies were the most abundant product (83% of transformants in this particular experiment) even though we did not dephosphorylate the NotI-cut vector. We purified 13 putative recombinant plasmids and observed that all 13 of the plasmids recovered from SURE cells were monomers incorporating a single DNA insert. DNA sequencing showed that all 13 molecules also encoded the insert in the correct orientation and at the expected position. There were 3 base substitutions located within one of the primer-binding sites and 2 other mutations within Taq polymerase-amplified DNA. The fusion points were those expected to be formed through annealing of homologous ends. We concluded that the concatemers assembled by vaccinia virus polymerase can be processed into stable recombinants with a fidelity comparable to traditional cloning methods.

Example 13: The minimal sequence overlap

To determine the minimal amount of homology required for production of joint molecules, we synthesized seven additional primer pairs and then used the set of eight oligonucleotide pairs to again PCR amplify a 788 bp fragment of SFV DNA. These 14 new PCR primers were similar in structure to the primers described above, except that the amount of sequence homologous to nucleotides flanking the NotI site in a NotI-cut vector now ranged from 4 to 18 bp. These substrates were incubated with vector DNA in optimized assays containing both vaccinia DNA polymerase and gpI3L (which increased the ability to detect otherwise faint circular reaction products) and the yield of both linear and circular joint molecules were quantitated using densitometry. These and other experiments showed that as little as 10 bp of sequence homology between substrates still permitted strand joining by vaccinia polymerase, with a reaction optimum of about 14 bp (Fig. 12, panel B). These reaction products were also used to transform SURE cells, in triplicate, as there was some variation in the absolute efficiency of white transformant production from experiment to experiment. In all of the experiments, the percentage of

white (recombinant) bacterial transformants closely paralleled the yield of joint molecules as detected by gel electrophoresis (Fig. 12A).

Example 14 - Cloning a recombinant plasmid DNA with vaccinia virus DNA polymerase

The purified vaccinia virus DNA polymerase (v pol) is able to catalyze linear DNA containing more than 12 nucleotide homology at ends into contatemers. It has been proved that the joining reaction is carried out by its 3'-5' exonuclease activity. The in vitro recombination is enhanced in presence of vaccinia virus single strand DNA binding protein (v SSB). We successfully utilized the properties of this enzyme to clone a recombinant DNA pRP406-SFV (Figure 13, 14).

We compared our cloning strategy with the traditional clone method. The invention simpler and quicker (Table 3).

Vector: a linear plasmid, pRP406 with Pac I and Bst Ellsites on the both ends respectively (~4kb).

Insert: PCR-SFV(~800 bp), a PCR-amplified Shope fibroma virus DNA containing 16 overlapping sequences which are the same as the vector ends (Fig. 13).

Table 3 Cloning strategy and result

	Joining by v DNA pol	Traditional method with ligase
PCR – amplified insert	With >12 nt homology with linear vector at both ends 300 ng, 600 fmol	Cleaved with Bst *EII* and Pac I for generating the sticky ends 150 ng, 300 fmol
Linear vector	250 ng, 100 fmol	100 ng, 40 fmol
Reaction condition	v pol 100 ng, v SSB 500 ng in 20 ul 37 °C 10 min	T4 DNA ligase 5 U in 10 ul 20 °C 2 hr
Transfor-mation	1 of 20^{th} of reaction solution into E.coli SURE	1 of 10^{th} of reaction solution into E.coli SURE
Recombi-nant frequency	4.8×10^4 / ug vector	9.2×10^4 / ug vector

While the present application has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

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DETAILED LEGENDS FOR FIGURES

Figure 1. DNA joining catalyzed by vaccinia DNA polymerase. Panel A shows a schematic of the reaction and illustrates how the two substrates share 30 bp of complementary sequence at the left and right ends. A ~1:1 mixture of these two molecules was incubated with varying quantities of vaccinia DNA polymerase for 20 min and the products separated by size using an agarose gel. The gel was stained with ethidium bromide and photographed (Panel B). (A negative image is shown for clarity.) Note the conversion of the two substrates into a molecule twice the length of the two original molecules (lanes 5-9). No reaction is observed when the polymerase is omitted (lane 2) and increasing yields of products were seen when increasing amounts of polymerase were added (lanes 3-9).

Figure 2. Trimer formation. Panel A shows a schematic of the reaction. Note that joining three linear molecules is essentially the same reaction needed to join an insert DNA to the two flanking arms of a vector molecule. Panel B shows the reaction products analyzed on an ethidium-stained agarose gel. No joint molecules are formed when only one of the three substrates are provided to the polymerase. This shows that the reaction requires correct complementary sequences at the ends of the reacting molecules.

Figure 3. Combinatorial fusion of DNA cassettes. In this simple scheme three different pools of DNA duplexes are prepared. Within a pool of molecules, the central portion of each molecule varies in sequence while the ends are identical. For example, A1-A5 all have the same ~20 base sequence at the right end but the middle portions of these molecules vary in sequence. The left ends of molecules in the "B" pool are compatible with the right ends of molecules in the "A" pool. Similarily, the left ends of molecules in the "C" pool are compatible with the right ends of molecules in the "B" pool. Reaction with vaccinia polymerase will create trimers composed of one each of molecules selected from the "A", "B", and "C" pools. The resulting pool of trimers should express a random assortment of "A", "B", and "C" variants.

Figure 4. Autoradiograph showing the fate of 5'- and 3'-end labels. The "middle" of three substrates (see Fig. 2, panel A) was radioactively labelled on either the two 5' (lanes 2-4) or the two 3' (lanes 5-7) ends. The DNA was then incubated with or without DNA polymerase. After gel electrophoresis the gel was dried and then autoradiographed

to detect incorporated label. Only 5' end labels survived the reaction, the 3' end-labels were removed. Although not shown here, dimers and trimers were formed with equal efficiency in lanes 4 and 7 as judged by ethidium bromide staining prior to autoradiography.

Figure 5. Proposed mechanism of polymerase-mediated strand joining. "a" is simply some DNA sequence, "a'" is the Watson-Crick complement or antisense of "a". It is suggested that a synaptic step joins the two DNA duplexes and the polymerase 3'-5' exonuclease then stabilizes the joint by degrading excess nucleotides. The order in which these two steps occurs is unclear at present.

Figure 6. Proposed cloning method. The PCR primers encode 10-20 additional nucleotides complementary to sequences flanking the vector insertion site.

We claim:

1. A method of joining two or more linear DNA molecules comprising the steps of:
   - obtaining one or more linear DNA molecules, each having an a and a' strand having opposite polarities, wherein the 5' end of the a' strand of each linear DNA molecule has a sequence of nucleotides that is complementary to the 5' end of the a strand of the linear DNA molecule to which it is to be joined; and
   - incubating the two or more DNA molecules in the presence of a DNA polymerase;

   wherein the DNA polymerase has intrinsic exonuclease activity and joins the DNA molecules.

2. The method according to claim 1, wherein the DNA polymerase has 3'-5'-exonuclease activity.

3. The method according to claim 1 or 2, wherein the DNA polymerase comprises a polymerase selected from the group consisting of vaccinia virus DNA polymerase, T4 DNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I.

4. The method according to claim 3, wherein the DNA polymerase comprises vaccinia virus DNA polymerase.

5. The method according to any of claims 1 to 4, wherein the length of DNA sequence on the 5'-end of the a' strand of each linear DNA molecule that is complementary to the 5'-end of the a strand of the linear DNA molecule to which it is to be joined is between about 5 and about 100 nucleotides.

6. The method according to claim 5, wherein the length of DNA sequence on the 5'-end of the a' strand of each linear DNA molecule that is complementary to the 5'-end of the a strand of the linear DNA molecule to which it is to be joined is between about 8 and about 50 nucleotides.

7. The method according to claim 6, wherein the length of DNA sequence on the 5'-end of the a' strand of each linear DNA molecule that is complementary to the 5'-end of the a strand of the linear DNA molecule to which it is to be joined, is between about 10 and about 30 nucleotides.

8. A method of contructing a recombinant molecule comprising the steps of:

- obtaining a linearized vector DNA molecule and a template DNA molecule, each having a first and a second end;
- obtaining a first primer DNA molecule having a 5' end that comprises nucleotide sequences that will incorporate nucleotide sequences that are complementary to the first end of the linearized vector molecule onto the first end of the template DNA molecule and a 3' end that hybridizes to a suitable location on the first end of the template DNA molecule;
- obtaining a second primer DNA molecule having a 5' end that comprises nucleotide sequences that will incorporate nucleotide sequences that are complementary to the second end of the linearized vector molecule onto the second end of the template DNA molecule and a 3' end that hybridizes to a suitable location on the second end of the template DNA molecule;
- amplifying the template DNA molecule using the polymerase chain reaction with the first and second primers to provide a PCR amplified product; and
- incubating the PCR amplified product with the linearized vector DNA molecule in the presence of a DNA polymerase to generate a recombinant DNA molecule; wherein the DNA polymerase has intrinsic exonuclease activity and joins the DNA molecules.

9. The method according to claim 8, wherein wherein the DNA polymerase has 3'-5' exonuclease activity.

10. The method according to claim 8 or 9, wherein the DNA polymerase comprises a polymerase selected from the group consisting of vaccinia virus DNA

polymerase, T4 DNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I.

11. The method according to claim 10, wherein the DNA polymerase comprises vaccinia virus DNA polymerase.

12. The method according to any of claims 8 to 11, wherein the number of nucleotides on the 5' ends of the first and second primer DNA molecules, that will incorporate nucleotide sequences that are complementary to the first and second ends of the linearized vector molecule onto the template DNA molecule, is between about 5 and about 100 nucleotides.

13. The method according to claim 12, wherein the number of nucleotides on the 5' ends of the first and second primer DNA molecules, that will incorporate nucleotide sequences that are complementary to the first and second ends of the linearized vector molecule onto the template DNA molecule, is between about 8 and about 50 nucleotides.

14. The method according to claim 13, wherein the number of nucleotides on the 5' ends of the first and second primer DNA molecules, that will incorporate nucleotide sequences that are complementary to the first and second ends of the linearized vector molecule onto the template DNA molecule, is between about 10 and about 30 nucleotides.

15. The method according to any of claims 8 to 14, wherein the vector is capable of replicating in a prokaryotic or eukaryotic host cell.

16. The method according to any of claims 8 to 15, wherein the vector comprises a vector selected from the group consisting of plasmids, cosmids, phage and BACs.

17. The method according to claim 16, wherein the vector comprises a plasmid.

18. The method according to claim 17, wherein the vector comprises pBDW/KS.

19. The method according to claim any of claims 8 to 18, wherein the selected DNA molecule comprises prokaryote genomic DNA or eukaryote genomic DNA.

20. The method according to any of claims 8 to 19, wherein the selected DNA molecule is obtained by synthetic methodology.

21. A method of producing a recombinant DNA product comprising the steps of:

- obtaining a linearized vector DNA molecule and a template DNA molecule, each having a first and a second end;
- obtaining a first primer DNA molecule having a 5' end that comprises nucleotide sequences that will incorporate nucleotide sequences that are complementary to the first end of the linearized vector molecule onto the first end of the template DNA molecule and a 3' end that hybridizes to a suitable location on the first end of the template DNA molecule;
- obtaining a second primer DNA molecule having a 5' end that comprises nucleotide sequences that will incorporate nucleotide sequences that are complementary to the second end of the linearized vector molecule onto the second end of the template DNA molecule and a 3' end that hybridizes to a suitable location on the second end of the template DNA molecule;
- amplifying the template DNA molecule using the polymerase chain reaction with the first and second primers to provide a PCR amplified product;
- incubating the PCR amplified product with the linearized vector DNA molecule in the presence of a DNA polymerase to generate a recombinant DNA molecule;
- transforming the recombinant DNA molecule into a host cell; and
- isolating the recombinant DNA product;

wherein the DNA polymerase has intrinsic exonuclease activity and joins the DNA molecules.

22. A kit for direct cloning of PCR amplified products comprising, in separate containers, an aliquot of DNA polymerase and an aliquot of reaction buffer, wherein the DNA polymerase has intrinsic exonuclease activity and is capable of performing the DNA joining reaction of the invention

23. The kit of claim 22, further comprising reagents to perform a positive control reaction.

24. The kit of claim 22 or 23, wherein the reagents to perform a positive control comprise an aliquot of linearized vector, an aliquot of insert DNA with first and second ends having appropriate complementary sequences, an aliquot of DNA polymerase having intrinsic exonuclease activity that is capable of performing the DNA joining reaction of the invention and an aliquot of reaction buffer.

25. A DNA molecule prepared according to the method of any of claims 1 to 21.

26. The use of a DNA polymerase molecule having exonuclease activity and capable of joining DNA molecules, for cloning DNA molecules.

27. The use of claim 26, wherein the DNA molecules are produced by PCR.

28. The use of claim 26 or 27, wherein the DNA polymerase comprises a polymerase selected from the group consisting of vaccinia virus DNA polymerase, T4 DNA polymerase and the Klenow fragment of *E. coli* DNA polymerase I.

29. A composition for cloning DNA molecules, comprising a carrier and DNA polymerase having exonuclease activity and capable of joining linear DNA molecules.

UNSCANNABLE ITEM (OWP)

RECEIVED WITH THIS APPLICATION

(ITEM ON THE 10TH FLOOR ZONE 5 IN THE FILE PREPARATION SECTION)

DOCUMENT REÇU AVEC CETTE DEMANDE

NE POUVANT ÊTRE BALAYÉ

(DOCUMENT AU 10 IÈME ÉTAGE AIRE 5 DANS LA SECTION DE LA

PRÉPARATION DES DOSSIERS)

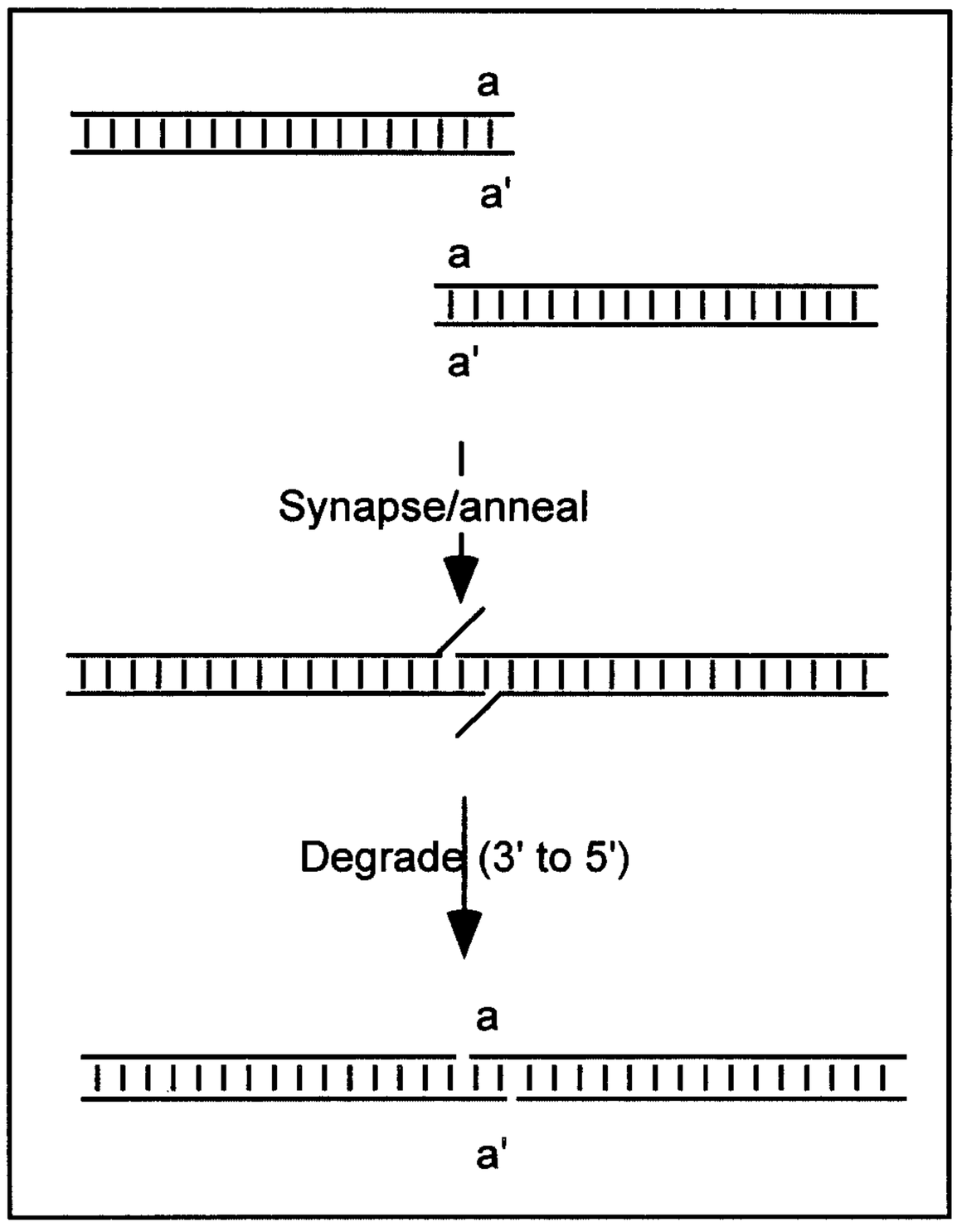

FIGURE 5

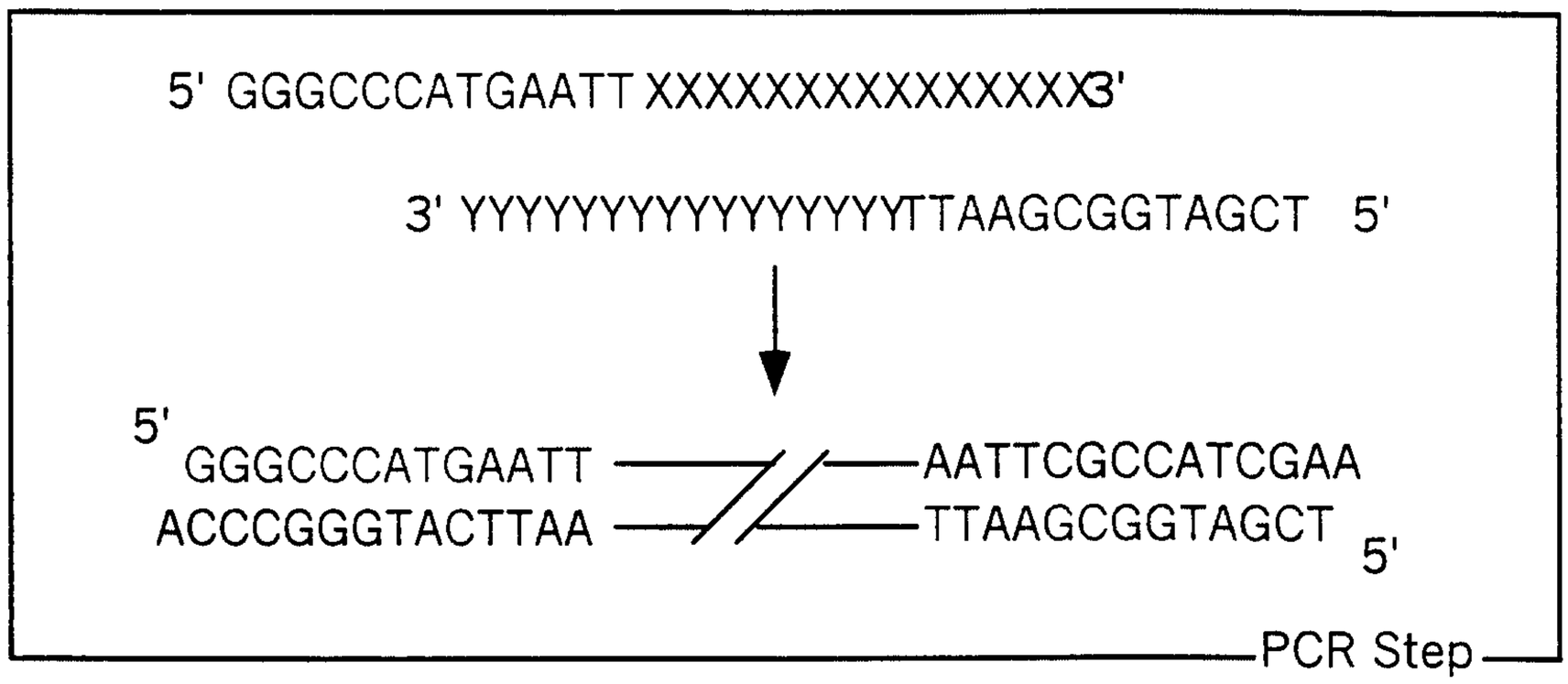
5' GGGCCCATGAATT XXXXXXXXXXXXXXX 3'
3' YYYYYYYYYYYYYYYYTTAAGCGGTAGCT 5'
5'
GGGCCCATGAATT
ACCCGGGTACTTAA
AATTCGCCATCGAA
TTAAGCGGTAGCT
5'
PCR Step
5'
-----GGGCCCATG AATTCGCCATCGA----
-----CCCGGGTACTTAA GCGGTAGCT----
5'
-----GGGCCCATG
-----CCCGGGTACTTAA
AATTCGCCATCGA----
GCGGTAGCT----
Vector preparation (e.g. EcoRI restriction)
5'
GGGCCCATGAATT
ACCCGGGTACTTAA
AATTCGCCATCGAA
TTAAGCGGTAGCT
5'
plus
-----GGGCCCATG
-----CCCGGGTACTTAA
AATTCGCCATCGA----
GCGGTAGCT----
polymerase
----- GGGCCCATGAATT
-----CCCGGGTACTTAA
AATTCGCCATCGA----
TTAAGCGGTAGCT----
Fusion step

FIGURE 6

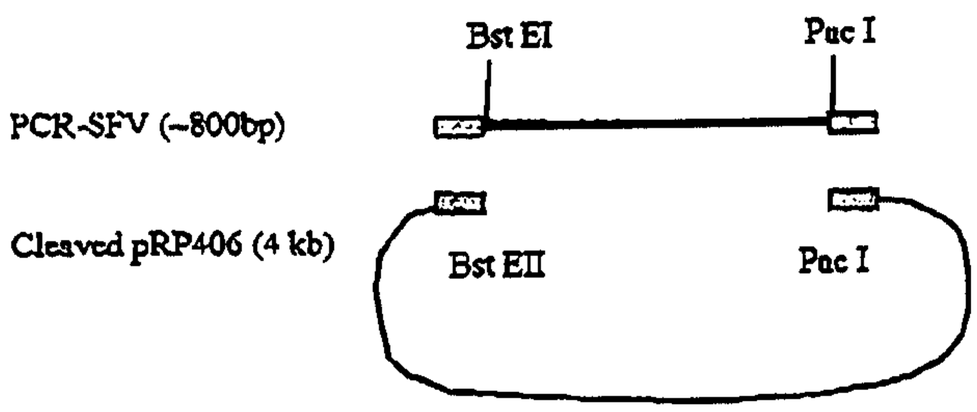

Fig. 13