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URGENTLY REPLICATING DNA CONTAINING INSERTED DNA SEQUENCES

AT ALLOWANCE

FIGURES CLAIMS CLASS SUBCLASS

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Inventor:  George Pieczenik
For:  INSERTED DNA SEQUENCES

Enclosed are:

☐  _______ sheets of drawing.
☐  An assignment of the invention to

☐  A certified copy of a __________________ application.

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Recombinant DNA technology is a battery of techniques used to clone specific genes, construct DNA transfer vectors, and to manipulate genetic material isolated from a donor organism such that, upon transfer to a host organism, the donor genetic information may be expressed in the host. Fundamental to the practice of recombinant DNA technology is the existence of restriction endonucleases. These enzymes catalyze the hydrolysis of certain phosphodiester bonds of DNA, at specific sites as determined by a local sequence of nucleotides. Different restriction enzymes recognize different nucleotide sequences. The recognition sequences are randomly dispersed throughout the DNA of an organism. Shorter recognition sequences occur with higher frequency than longer ones. Due to the random distribution of restriction sites, the ability to cleave a DNA molecule at a desired locus, for excision of a segment of DNA or for insertion or a segment, is only possible when a restriction site is found at or near the desired locus. This limitation is a severe restriction on progress in the recombinant DNA field, since every new situation must be handled on an ad hoc basis, depending upon where the restriction sites are located in each case. Extensive mapping of restriction sites must be carried out to determine whether the desired insertions or excisions are feasible or to carry out nucleotide sequence analyses.

The present invention overcomes this major limitation by providing a general method for inserting restriction sites or other specific desired sequences into specific areas of a DNA transfer vector, virus, or other self-replicating entity. The term "autonomously replicating DNA element" is
used herein to denote generically all such entities as plasmids, episomes, viral DNAs, mitochondrial and chloroplast DNAs and other extra-chromosomal DNAs, capable of being replicated within a living cell independently of the replication of the host cell chromosome DNA. The invention provides novel transfer vectors and a method for making such vectors as desired, for the safe replication of cloned genes, for nucleotide sequence analysis of cloned DNA segments, for expression of cloned genes under control of specific desired promoters, and for construction of DNA transfer vectors that serve as a library of genes carried thereon, specifically excisable at will. Further, the invention provides a method for simplified sequence determination without prior restriction mapping, and simplifies the location and identification of structural genes on cloned nucleotide sequences.

BACKGROUND AND PRIOR ART

The present invention builds upon an extensive body of prior art including the physical and chemical nature of nucleic acids, the principles of genetics and biochemistry, and on specific chemical and enzyme catalyzed reactions. The following is a non-exhaustive list of general background references useful for explaining operating principles and defining terms generally used in the art.

1. General


2. Restriction Endonucleases:


and on specific chemical and enzyme catalyzed reactions. The following is a non-exhaustive list of general background
3. Chemical Synthesis of Oligonucleotides:

4. Restriction Site "Linkers":

5. Nucleotide Sequence Determination

6. DNA Replication:
   Kornberg, A., DNA Replication.

7. Recombinant DNA:

   Developments in recombinant DNA technology have made it possible to isolate specific genes or portions thereof from higher organisms, such as man and other mammals, and to transfer these genes or fragments to a microorganism species, such as bacteria or yeast. The transferred gene is replicated and propagated as the transformed microorganism replicates. As a result, the transformed microorganism may become endowed with the capacity to make whatever product the gene or fragment encodes, whether it be an enzyme, a hormone, an antigen or an antibody, or some portion thereof. The microorganism passes on this capability to its progeny, so that in effect, the transfer has resulted in a new strain,
having the described capability. See, for example, Ullrich, A. et al., supra, and Seeburg, P.H., et al., Nature, 270, 486 (1977). A basic fact underlying the application of this technology for practical purposes is that DNA of all living organisms, from microbes to man, is chemically similar, being composed of the same four nucleotides. The significant differences lie in the sequences of these nucleotides in the polymeric DNA molecule. The nucleotide sequences are used to specify the amino acid sequences of proteins that comprise the organism. Although most of the proteins of different organisms differ from each other, the coding relationship between nucleotide sequence and amino acid sequence is fundamentally the same for all organisms. For example, the same nucleotide sequence which codes for the amino acid sequence of a human protein in human cells, will, when transferred to a microorganism, be recognized as coding for the same amino acid sequence, and may result in synthesis by the microorganism of the same human protein.

Abbreviations used herein are given in Table 1.

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA (enzymatically synthesized from an mRNA sequence)</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxyctydine triphosphate</td>
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A - Adenine
T - Thymine
C - Cytosine
U - Uracil
ATP - Adenosine triphosphate
TTP - Thymidine triphosphate

The coding relationships between nucleotide sequence in DNA and amino acid sequence in protein are collectively known as the genetic code, shown in Table 2.
synthesized, although they may be synthesized under appropriate environmental conditions. When the protein product, coded by a given gene, is synthesized by the organism, the gene is said to be expressed. If the protein product is not made, the gene is not expressed. Normally, the expression of genes in E. coli is regulated as described generally, infra, in such manner that proteins whose function is not useful in a given environment are not synthesized and metabolic energy is conserved.

The development of capabilities for synthesizing DNA chemically (See Itakura, et al, supra) has made it possible to synthesize restriction enzyme recognition site sequences, Scheller, et al, supra. A desired oligonucleotide recognition site sequence may be chemically synthesized, attached to the ends of a DNA fragment by blunt end ligation, treated with the appropriate restriction endonuclease, to provide cohesive ends, then inserted in a transfer vector cleaved by the same restriction endonuclease, whereby the cut ends of the transfer vector DNA and of the heterologous fragment are cohesive and may be joined together with high efficiency in a ligase catalyzed reaction.

The organization of structural genes coding for individual amino acid sequences within the total genome is a matter of much current interest. It appears that the genome of virtually all organisms, with the exception of certain viruses, consists of both essential and non-essential regions. These terms are functionally defined and depend upon the growth conditions. For example, the ampicillin resistance gene is not essential for growth of bacteria in a medium lacking ampicillin; it is essential if ampicillin is in the medium. In addition to specific structural genes whose essentiality depends upon conditions, there appear to exist regions which are non-coding in the
conventional sense, whose presence is not essential. In
prior art genetic analyses, such regions have been identified
by deletion mutations in which segments of the genome are
permanently excised without loss of viability. An example
is the 52 of lambdaphage. In eucaryotes there are untranslated
regions to be found on either side of most structural
genes. In addition, current evidence indicates that the
structural genes in some instances contain internal non-
coding regions, termed introns, whose function is at present
unknown. (See Crick, F. Science 204, 264 (1979)).

The concept of essential and non-essential genes is
fundamental to the present invention. For purposes of this
application, a gene of an autonomously replicating
DNA element is essential if, under the growth conditions
employed, the function for which it codes is required for
continued replication of the element. A region which is
non-essential, with reference to a given autonomously
replicating element, is one whose function is not required,
under the growth conditions, for continued replication of
the element. It will be understood that a given region
may be defined as essential under one set of growth
conditions and non-essential under another. A case in
point is the ampicillin resistance gene previously described.
A gene which is essential under one set of conditions may
be rendered non-essential by complementation. In complemen-
tation, the function of a gene provided by one genetic element
is duplicated by providing a second gene within the cell,
coding for the same function. If the first should be defective,
bearing an insertion, deletion or base change leading to
loss of function, the growth and replication of the DNA element
is still permitted by providing a normal function from
the second gene. Therefore, by manipulation of growth
conditions and/or complementing functions, it is possible
to render virtually any gene essential or non-essential.

Nucleotide sequence analysis of DNA has proven to be a powerful technique for the elucidation of gene structure and for the prediction of amino acid sequences. The methods of Maxam and Gilbert, supra, and of Sanger, supra, provide powerful tools for rapid sequence analysis. Both methods are presently limited by the need to have detailed restriction mapping of the DNA to be sequenced. This is so because both sequencing methods employ gel electrophoresis for the separation of the oligonucleotides. The length of sequences which can be determined from a given starting point are limited by the resolution of the electrophoresis gels. In order to extend the sequence beyond the nucleotide length resolvable by current gels, it is necessary to provide, by means of restriction endonuclease cuts, new starting points for the analysis procedure. Although the sequence procedures themselves are rapid, getting sufficient information about restriction sites is relatively time consuming. Furthermore, as with other aspects of recombinant DNA technology, all experiments must be designed on an ad hoc basis, depending upon what restriction sites exist and how they are located with respect to each other, in the DNA to be sequenced. Prior to the present invention, no general method has existed for undertaking routine sequence analysis of DNA fragments.
General Description of the Invention

The present invention provides, for the first time, a general method for introducing specific restriction sites in a desired region of a genome. The technology of recombinant DNA is thereby freed of a major restraint, with consequences that are far reaching, as will be described infra.

The invention is applicable to any autonomously replicating DNA element that can be introduced into a host cell and replicated therein. Such DNA elements include plasmids, bacteriophages, and animal or plant viruses, with or without inserted heterologous sequences. The DNA element must comprise, as a condition of operability, either zero or one restriction site sequence for a given restriction enzyme. For many applications of the invention, the sequence need not be a restriction site sequence, but merely any sequence not otherwise found in that DNA. A unique sequence is defined as one which does not exist in the DNA of a given autonomously replicating element, unless inserted or generated by techniques described herein. As a practical matter, the use of restriction site sequences makes it easy to define unique sequences since the DNA will not be cut by a given restriction enzyme if its recognition sequence is missing. Most commonly, the sequence will be one of the relatively long recognition sequences such as PstI, EcoRI, or HindIII. If the DNA element bears a single restriction site, it can be deleted by techniques known in the art (Polisky, B. et al., Proc. Nat. Acad. Sci. U.S.A., 73, 3900 (1976), so that the general technique is applicable with sites of either 0 or 1 frequency.

The DNA of the autonomously replicating element is usually isolated as a double stranded circular DNA. After purification, the DNA is then randomly cleaved to give linear double stranded DNA. The procedure will yield a population of linear DNA molecules having circularly permuted sequences. It will be understood that any DNA that is directly isolatable as circularly
permuted linear sequences will be operative in the invention, although DNA molecules having natural terminal redundancies will not. In practice, it is unnecessary to achieve truly random cutting of the circular DNA. Indeed, it is unlikely that truly random cleavage can be achieved experimentally, since most DNA molecules contain areas of secondary structure which may be subject to cleavage at a lower rate than other areas of the same DNA molecule. As long as the cleavage is quasi-random, and occurs without systematic bias throughout the genome, the method of the present invention will be operative. Any method for introducing random double-strand cleavage of circular DNA, is suitable for the present invention.

After the cleavage steps, a synthetic oligonucleotide bearing a unique nucleotide sequence, is attached to both ends of the linear molecules by blunt end ligation. The molecules are then treated with the restriction endonuclease specific to the inserted sequence, to generate cohesive ends on the linear molecules. The molecules are then converted back to circles with the aid of DNA ligase under conditions promoting base pairing at the cohesive ends.

At all stages of the procedures described herein, circular and linear forms of DNA having the same molecular weight are readily separated by known fractionation techniques. In most instances, the circular form is necessary to infect or transform host cells.

The circular DNA bearing the random inserted unique sequence is transferred to a suitable host cell by appropriate means including transformation, transfection, or infection. Host cells bearing and replicating the treated DNA are then grown under defined conditions. Replication of the inserted DNA in the host and the production of progeny DNA or progeny virus, will only occur where there has been an insertion of the unique sequence in a non-essential region of the genome. The locus of the insert is therefore defined operationally.
Example 1

Random Insertion of Unique Sequences.

The system employed herein is the bacteriophage f1 and its host, Escherichia coli K38. The viral DNA of f1 phage is single stranded, however, a double stranded replicating form (RF) may be isolated from infected host cells under conditions of inhibited protein synthesis. The f1 RF is not cut by restriction enzymes PstI EcoRI or HindIII and therefore lacks the sequences 5' - CTGCAG - 3', 5' - GAATT - 3', and 5' - AAGUTT - 3', respectively. Closed circular f1 RF DNA is isolated by a modification of the method of Pieczenik, G, et al., J. Mol. Biol., 191 (1974).

Growth of E. coli K38 - Two litres of super broth (tryptone, 32.0 gm; yeast extract, 20.0 gm NaCl, 5.0 gm, 6N NaOH, 2.3 ml up to 1 litre, pH 7.4, sterilized) are distributed into 6- 1 litre flasks and sterilized. They are inoculated from an overnight growth, (6 ml). K38 is a male strain, restriction K1, modification K+, and suppressor minus, high frequency male donor C, received from Zinder (Lyons and Zinder, (ref.) 1972).

The cultures are incubated at 37°C with constant swirling. The bacterial growth is monitored periodically by reading the O.D. at 700nm on a colorimeter. The culture is diluted at an O.D. 1.0 (or monitored O.D. of .2) for infection. This corresponds to a cell density of about $4.5 \times 10^8$ cells/ml. B) Infection with f1 Bacteriophage cells are infected at a high multiplicity of infection and after initial adsorption, protein synthesis is blocked in order to accumulate RF rather than single stranded phage. 0.1 ml of f1 virus at titre of $1.2 \times 10^{14}$/ml is added to each of the six flasks. The cultures are swirled gently at 37°C for 15 minutes at which time chloramphenicol is added directly.
or 12.5 ml of 0.75 mg/ml solution (dissolved in Ethanol/Water, 1/20 per volume) to each flask. Cultures are then shaken vigorously for another hour. The bacteria are pelleted by centrifugation at 10K RPM at 4°C for 20 minutes in a high speed centrifuge. The pellets are then washed with cold buffer (140 mM NaCl, 20 mM TrisHCl, pH 7.3). The cells are recentrifuged and resuspended in a total of 40 ml of the above solution. Approximately 5 mg of egg white lysozyme is added, the solution brought up to pH8.0, and incubated at 37°C for 30 minutes. The cells are then osmotically shocked with 80 ml of ice-water and 10 ml of 5% sodium dodecyl sulfate (SDS) (electrophoretic purity). The mixture is shaken until clear. Triton X-100 1/ (1%) can replace the SDS. This avoids getting rid of the SDS, as many enzymes are active in Triton X100. One third volume of 4M NaCl is added and shaken. The solution should be clear and viscous if proper lysis has taken place.

Chromosomal DNA removal and de-proteinization - The solution after having settled at 4°C for several hours is spun for 3 hours at 21K RPM at 4°C on a high speed centrifuge. For smaller volumes, 1 hour at 34K RPM on an ultra high speed centrifuge is effective in getting rid of most of the bacterial DNA. The supernatant is then dialyzed for three hours against 4 liters of buffer (20 mM Tris pH 8.0, 1.0 mM EDTA) to remove SDS. This is unnecessary if Triton X-100 is used.

The phenol for extraction of protein is prepared from equilibrated phenol with 10 mM Tris pH 7.8, 1.0 mM EDTA and .02 M sodium Tetraborate to bring the pH up over 7. There is an alternative procedure that does not equilibrate the phenol. One can add sodium acetate pH 5.5 to the phenol and deproteinize with the acidified phenol. The aqueous layer is separated from

1/ Trademark, Rohm and Haas, Nutly, N.I.
phenol. A DNA solution having a maximum O.D. of 15 260 nm should be made up to 3.3 ml. This is added to 4.0 gm of CsCl and 0.75 ml of 0.7 mg/ml ethidium bromide. The ethidium bromide concentration is important as it intercalates with the supercoiled RFI and shifts its density away from nicked RF and Chromosomal DNA. The tubes are centrifuged on an ultrahigh speed centrifuge at 34K RPM, 18°C, for 48-72 hours on a SW 27 or 24 hours on a sorvall vertical rotor. The lower band, which can be visualized with short or long wave ultraviolet light (long is preferable, less nicking of DNA), is the RFI-II DNA. Sometimes, on long runs RFII separates from the Chromosomal band. RFII is nicked at one unique site.

The RFI band is removed and the ethidium bromide is eluted by an isopropanol solution saturated with CsCl (20 g CsCl, 20 ml water, 40 ml isopropanol). This extraction is repeated several times. The CsCl is dialyzed from the DNA at 4°C for 12 hours with 2 liters of buffer (10 mm NaCl, 1 mm EDTA, 10 mM TRIS pH 7.8). The RFI is precipitated by adding 0.05 volumes 2M sodium acetate, pH 5.0, and 3 volumes cold 95% ethanol, -20°C overnight.

The RFI is pelleted by centrifugation in siliconized Corex tubes at 10K RPM for 30 minutes at 4°C. The precipitate is air dried and then resuspended in 0.1 tris pH 7.4, 1 M EDTA to a concentration 25.0 O.D. (260) ml or 1 µg/ml.

There are three methods to randomly introduce nicks into supercoiled RFI, or other double stranded circular DNA.

1. Let it sit on a frost free freezer for two months (this converts over 50% of RFI to RFII by introducing single-stranded breaks (nicks). The frost-free freezer cycle freezes and thaw the DNA solution.

2. DuPont Instruments, Wilmington, Del.
2. Freeze-thaw the DNA solution at least twenty times (can convert over 90% of RFI to randomly nicked RFII.

3. A more uniform method is to digest the RFI with DNase I solution of 1 mg/ml in distilled water, stored at -20°C, at an enzyme to DNA ratio of 1/500 by weight. The reaction buffer contains 10 mM tris pH 7.6, 10.0 mM MgCl₂, 10.0 mM dithiothreitol, 100 mM NaCl.

In distilled water, stored at -20°C at an enzyme to DNA ratio of 1/500. The reaction buffer contains 10 mM tris pH 7.6, 10.0 mM MgCl₂, 10.0 mM dithiothreitol, 100 mM NaCl.

The reaction is monitored over time on an agarose slab gel (1% by weight agarose 40 mM Tris Acetate, pH 7.2, 20.0 mM sodium acetate, 2.0 mM EDTA, and visualized by soaking in a 0.5 ug/ml solution of ethidium bromide by long wavelength (366 nm) ultraviolet light. The RFII moves slower than RFI. When conversion is greater than 98%, as monitored by the gel, then the DNase is removed by phenol, ether extraction, and chromatography on a sephadex G-100 column (pre-equilibrated, pre-boiled in 5 mM Tris pH 7.4 1 mM EDTA).

The void column is collected (the second 0.2 ml on a 1 ml plastic pipette column). This is desiccated and resuspended to 25.0 O.D. (260) ml in 0.01 Tris pH 7.4, 1mM EDTA. The RFII is re-run on the 1% agarose gel with RFI which migrates faster than RFI, and a HindII restriction cut of RFI and RFII (HindII, 1 unit/ug RF), 2 hours at 37°C, in 10 mM Tris pH 7.9, 6.6 mM MgCl₂, 1 mM dithiothreitol, 60 mM NaCl). The RF has one HindII restriction site and therefore the incubation generated duplex linear RFIII. RFIII moves between RFI and II on 1% agarose gel electrophoresis.

There are three ways to convert randomly nicked RFII to terminally random RFIII, generally applicable to any circular DNA.

the phenol \textsuperscript{A} by centrifugation at 3K RPM for 15 min. for the neutral phenol extraction and 9K RPM for 30 minutes for the acidic phenol extraction. The acidic phenol extraction brings down a lot of the chromosomal DNA still in solution.

The phenol extraction will give three layers. The top layer is removed, with an inverted tip Pasteur pipette, and acidified with 0.5 volumes of sodium acetate (2M) pH 5.0 and then precipitated with 2 volumes of isopropanol, at -20°C overnight, or in any dry ice-isopropanol bath for 1 hour. The nucleic acid will come out of solution as a white floc and can be spun. For deproteinization one does not need to re-acidify with sodium acetate before precipitation.) The precipitate is resuspended in 10 ml of TSE (50 mM NaCl, 5 mM EDTA, and 50 mM Tris, pH 7.8). This is re-acidified with sodium acetate and re-precipitated with cold ethanol, at -20°C. The precipitate is then washed with 75% ethanol to remove any remaining SDS (omit when using Triton X-100, in small preps). The precipitate is air dried and resuspended in 10 ml of TSE and RNA\textsuperscript{ase A} (pancreatic) added to a final concentration of 50 ug/ml. The RNA\textsuperscript{ase A} is pre-heated for 15 minutes at 80°C to kill any DNA\textsuperscript{ase activity. Incubation for RNA\textsuperscript{ase treatment is at 37°C for 30 minutes. The RNA\textsuperscript{ase A} and other nucleases are then digested with the addition of protease K up to 0.5 mg/ml final concentration for 30 minutes at 37°C. The DNA is then re-acidified with sodium acetate and re-precipitated with 2.5 volumes of cold 95% ethanol, at -20°C, for several hours. For small preparations the RNA\textsuperscript{ase treatment can be eliminated.

CsCl density centrifugation The precipitate is isolated by centrifuging at 3K RPM for 30 minutes at 4°C. This is air dried and resuspended in 5 ml of Buffer (10 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8.0). The optical absorbance is then determined at 260 and 280 nm. The ratio (260/280) should be around 2.0. If not, re-precipitate or extract with ether to eliminate
1. Freeze-thaw and monitor conversion on gel.
2. Treat RFIi with 0.2N NaOH at 37°C for several hours and monitor conversion on gel.
3. If randomly nicked RFIi is treated with DNA polymerase I and one of the deoxynucleotide triphosphates, e.g., 40μM dATP, and appropriate buffer (See DNase I buffer above), the DNA polymerase 3’ to 5’ exonuclease activity will take over and the enzyme will ninkle from the nick on the RFIi on the back to the first A residue, leaving a short single-stranded region on the circular duplex molecule.

The resulting single-stranded region will be about 3 nucleotides long on the average. The reaction can be stopped with 1 μl of 0.2M EDTA. The polymerase can be removed by extracting with phenol, and with ether, and chromatographing a sephadex G-100 column, (Pharmacia), Lyophilizing, resuspendting, and reprecipitating from ethanol (as described in detail supra. The DNA is treated with S1 nuclease under mild conditions.

Nicked RFIi polymerase-treated DNA 100 μg, is digested with 100 units of S1 nuclease in 30 mM sodium acetate pH 4.6, 50 mM NaCl, 1 mM ZnSO₄, for 5 minutes to two hours, for conversion to RFIii. The S1 nuclease is removed in the same manner as described for removal of polymerase. The DNA is now a linear DNA duplex with 1-4 nucleotides of sticky end, depending on how the S1 cut the exposed single strand. This sticky end can be filled in by adding polymerase I, the four deoxynucleoside triphosphates, 40 μM each and polymerase buffer (same as DNase I buffer, supra). The DNA polymerase is removed from the DNA as given above. The DNA RFIii is monitored on 1% agarose gel as above. It should be over 90% RFIii.

The RFIii DNA may or may not have 3’ hydroxyl and 5’ phosphate groups. The freeze-thaw methods do not guarantee this, nor does the S1 nuclease. Therefore, removing all
phosphate end groups with bacterial alkaline phosphatase and then adding 5' phosphate from gamma labeled ATP with polynucleotide kinase will guarantee that the duplex DNA has proper 5' phosphates and 3' hydroxyls.

A unique sequence of ten nucleotides is prepared for insertion into f1 DNA by chemical synthesis. The sequence to be synthesized is TTCTGACAGA. This sequence is self-complementary and contains a Pst I recognition site. The HindIII site on a fragment CCAAGCTTGG is commercially available from Collaborative Research, Beverly, Mass.

Ligation is done under conditions (as above) with a molar ratio of 10 insertion sequences to one RFIII. Ligation results in attachment of insertion sequence at both ends of the terminally random RFIII. This is repurified from the fragment by either phenol, ether, G-100 (as above) or by sucrose gradient sedimentation. After extensive dialysis and reprecipitation from ETOH, it is digested with Pst I endonuclease. Digestion conditions are 20 mM Tris (pH 7.5, 10 mM MgCl2, 50 mM (NH4)2SO4, 100 μg/ml bovine serum albumin at 30°C. Pst I endonuclease is somewhat unstable at 37°C. This digestion generates cohesive Pst I ends (4 nucleotides).

The cohesive-ended RFIII is repurified and religated at low dilution to favor intra-molecular ligation giving the closed circular RF with the insertion sequence regenerated but randomly (or quasi-randomly inserted in the RF sequence). The ring closure reaction can be monitored on an agarose gel as above.

Example 2

Screening for Single Insert Vector (SIV)

Transfection will be done as a variation of the procedure of Taketo, Hayashi, and Kuno (ref) (1972). E. coli K38 is first
made competent for transfection, by treatment with CaCl₂. An overnight culture of K38 is inoculated into 100 mls of superbroth (Example 1). Cells are grown to mid log phase. Cells are added to 1/2 volume CaCl₂ (.05M) at 0°C for 15 minutes. The cells are then concentrated at 2K RPM at 4°C for 20 minutes, the resuspended in .05 M CaCl₂ to an equivalent O.D. at 660 nM of 10.0. DNA to be transfected (RFI containing the insertion sequence) and RFI (as control) are placed in sterile siliconized tubes on ice, and 0.1 ml of CaCl₂ treated cells are added. After 10 minute incubation, the tubes placed in an incubator for at least 2.5 minutes at 37°C. 1 ml of ice cold .05M CaCl₂ is added and the mixture placed on ice. 1 ml of the mixture is then placed in molten soft Agar at 45°C. After about 15 seconds the mixture is placed on bottom agar. After plates solidify they are incubated overnight at 37°C. Plates are then screened for plaques. Formation of plaques will generally indicate transfection by an RF having the insertion sequence in a non-essential region.

Breeder reactions can also be started. These 10 ml of superbroth inoculated with the K38 transfected with the RFI carrying the insertion sequence. If the single plaques do not contain bacteriophage with the insertion sequence, only a few, the breeder can be used to make an RFI DNA preparation as above. This RFI DNA will be a mixed population with some DNA containing the insertion sequence. By recutting with Pst I, this RFI DNA preparation will be divided into RFI DNA and RFIII (duplex linear with Pst I ends). These are separated by sucrose gradient or CsCl ethidium bromide. The RFIII is refrigerated and used for transfection. This procedure allows one to select biochemically for the DNA molecules containing the insertion sequence and therefore to enhance for them.
Bacteriophage f1 plaques whose DNA contains the insertion sequence can be screened in several ways:

1. Filter hybridization. The phage will be replica plated with toothpicks on marked grids. Duplicate plates will be made. One set of the duplicates are transferred to Millipore filters. $^{5}$ Millipore filters (HAWP 304FB or GSWP 304 FO) are cut to petri dish size. The filters are placed on the plates for 10 minutes at 40°C. They are removed gently and floated on 1.0N NaOH, 1.5 M NaCl for 10 minutes, and then transferred to 0.2M Tris, 1.0M NaCl pH 7.4 for 30 seconds. They are washed in 6 x SSCP (0.6M NaCl, 0.75M sodium citrate, 0.065M KH$_2$PO$_4$, 0.005 M disodium EDTA for 20 seconds. They are baked in a vacuum oven (wrapped in foil) at 80°C for 2 hour. The filters then are washed for one hour in 5 x SSCP 65°C washed one hour in 5 x SSCP and D buffer (0.6MNaCl, 0.0075M sodium citrate, 0.065 KH$_2$PO$_4$, 0.005M disodium, Ficoll 400, 0.2%, polyvinyl pyrrolidone -40, 0.2% bovine serum albumin, 0.2% DNA, 50 ug/ml, SDS 0.1%) at 65°C. The labelled probe, which will be the insertion sequence made radioactive by addition of $^{32}$P using DNA kinase or polymerase I in a nick-translation reaction, will be added to the filters which are in a polyethylene bag with D buffer. The bag is sealed and placed at 65°C for 18 hours. The filters are then washed 6 X for 30 minutes in D buffer, then for 60 minutes in 1 x SSC-D buffer at 65°C, and at room temperature in 5 x SSCP. The filters are then subjected to autoradiography.

The labelled plaques contain the phage with sequences that are complementary and therefore identical to the insertion sequence. This procedure allows one to fix the denatured phage DNA on a filter and do in situ hybridization to localize the screen the bacteriophage. Positive plaques are then diluted, plated and screened again. This procedure should allow screening several hundred plaques a day.

$^{5}$ Millipore Corporation, Bedford, Mass.
Mini-RF preparation: Depending on the efficiency of ligation and transfection and if one has already transfected from a breeder RF preparation, one can screen individual rapid RF preparations as above. 10 ml cultures of single plaque infections yield enough RF1 to screen on a mini-agarose gel system (described above). The conversion of RF1 to linear RFIII is distinctive and does not require either RNAse treatment or CaCl. It requires a lysis, spin, rapid acid phenol, and ethanol precipitation. 10 colonies can be screened a day. This technique has the advantage that if any RFIII is formed from cutting with Pst I one can retransfect with the DNA to increase the chance of finding phage with inserts.

Example 3

Generation of HindIII or EcoRI Sites Adjacent to a Pst I Insert

The logic behind this screening method is as follows:
The insertion sequence is TTCTGGAGAA. It contains a Pst I Site (CTGGAC). If this site is inserted adjacent to GAA it will generate GAATTC which is an EcoRI site. The chance of this happening in this orientation is 1/64 if the insertion is random (assuming equimolar base composition). Because this can also insert near a TTC on its 3' side, TTCTGGAGAATTC, this also generated an EcoRI site. Therefore the chance of generating an EcoRI site is 1/32. This can be used to screen for the statistics of insertion. The same test can be applied to HindIII. If the insertion sequence inserts adjacent to AAGC, it generates AAGCTTCTGGAGAA which contains a HindIII site.

The procedural strategy to isolate these vectors is to take a phage stock containing the Pst I insertion fragment and grow a mixed culture. Isolate the RF1 (as above, but with 3 CsCl gradients) and restrict this RF1 preparation
with EcoR1 or HindIII exonuclease. Some or the RFI will contain these sites and will generate RFIII or duplex linears. The RFIII can be separated from the RF I on an ethidium bromide CsCl gradient. The RFIII is religated and transfected. This will generate plaques which contain EcoR1 sites or HindIII sites adjacent to the Pst I site.

Example 4
Sequencing Vector - Derived from MIRV

One half of the plaques containing the EcoR1 site or the HindIII (depending which enzyme was used to generate the duplex linear) will have the site 5' to the modified Pst I insertion sequence. The plaques in this configuration can be used as sequencing vectors for the Sanger-Coulson Dideoxyn sequencing method with the insertion sequence fragment as primer. Any EcoR1 or HindIII or Sma I fragment can be sequenced by inserting into this vector, without having to isolate restriction fragments. By inserting the fragment to be sequenced, by inserting it in the vector and transfecting, one needs to only pick plaques and isolate the single stranded DNA (as above) and prime with the insertion sequence oligonucleotide. One-half of the plaques will give sequence in the newly inserted fragment, one-half will give fl sequence. This is because one can insert the insertion sequence and generate EcoR1 or HindIII sites on either side with equal frequency. So one needs to screen by sequencing to make sure one picks the vectors containing the newly generated site on the 5' side of the insert rather than the 3' side. Once one has developed these vectors, one can keep adding insertion sites of different specificities for fl of sites that do not exist in the wild-type fl. This is done by taking the sequencing vector and putting in a HindIII and later a Sma I sequence in the manner used to put in the original modified Pst I sequence.
One selects by converting from Rfl to RfIII and back to Rfl and transfecting. The selection procedure is biochemical and genotypic. By inserting only one site per molecule, one can screen by converting the supercoiled duplex to the linear duplex. The linear duplex can be separated from the supercoiled duplex by a CsCl ethidium bromide gradient or by gel electrophoresis. The linear can be converting back to the supercoiled duplex by ligation and this can be transfected to generate phage containing this site in various locations. This is a very powerful and quick genotypic selection procedure. It does not require functional product for selection. The foregoing method can be employed for mapping non-essential regions of the DNA. This mapping technique is termed genotypic mapping.

The fl RF DNA modified to contain a Pat I site with an EcoRI site newly generated on the 3′ side of the Pat I site inserted in an area of the genome capable of accepting large DNA sequences is useful as a sequencing vector. Heterologous DNA to be sequenced is treated to attach EcoRI linker sequences to either end and inserted into the EcoRI site of the sequencing vector. The heterologous insert is precisely located with respect to a unique Pat I site. The oligonucleotide sequence that was inserted to generate the Pat I site may also be used as a primer for the sequencing reactions of the Sanger method, as described supra. The sequence of the vector itself in the region of the EcoRI site will be known in advance, in order to define the sequence boundaries of the inserted heterologous region. The length of the heterologous region which may be sequenced from a single priming site is limited only by the resolution of the gel systems used for separating the oligonucleotides.

Example 5
Nucleotide Sequence Analysis without Prior Restriction Mapping

The complete sequence of any autonomously replicating
DNA element may be determined simply by providing that the inserted sequence is distributed about the genome at a frequency comparable to the resolving power of gels used to separate oligonucleotides generated by the sequencing reaction. With currently available techniques, the limit of resolution is 300 to 400 nucleotides in length and future improvements make it likely that fragments over 1,000 nucleotides will be resolvable in the future. The frequency with which inserted fragments are distributed may be increased by providing complementation for the essential functions or providing growth conditions in which essential functions are rendered non-essential. Individual clones from the random insertion process may be used with the insert as primer to generate sequences directly in the 3' side of the inserted sequence. A family of such sequences will provide regions of overlap which will define the order of the sequences. Given a reasonably uniform distribution of insertion sites, a virus or transfer vector of having approximately 3,000 nucleotides should yield a complete sequence from the analysis of about 15 clones. Longer sequences, or more complete confirmation, can be obtained by analyzing a larger number of clones. It should be noted that the foregoing sequence technique does not require that the inserted sequence be a restriction sequence, since no restricting cutting is involved. The entire sequence may be determined without reference to a restriction site and without the necessity of restriction mapping.

Example 6

Multiple In Vitro Insertion Restriction Vector (MIRV)

A MIRV is generated by reiterations of the fundamental process for the insertion of previously nonexistent restriction sites into a transfer vector or virus DNA. A MIRV may be
constructed so as to have multiple insertions of the same restriction site or to have a multiplicity of unique restriction sites. As a consequence of predetermined growth and selection conditions, a MIRV can contain a sufficient number of restriction sites located between essential regions, to be used in effect as a library for the genes it contains. A given gene may be excised by treatment of the MIRV with a specific combination of restriction enzymes that will liberate the desired gene. In this way, each gene of the MIRV may be separately isolated for sequence analysis or for transfer to other genetic elements. Conversely, a MIRV is useful as a depository for several different genes on the same vector, each having a separate address on the transfer vector.

As examples of the above use, a polyoma virus MIRV is useful as a source for the individual genes of the virus. In this way, each gene of polyoma virus may be studied separately, without knowledge of or reference to its function. Similarly, a transfer vector MIRV may be constructed into which each of the essential regions of the polyoma may be inserted, so that all essential functions are carried on a single transfer vector, but separated from one another in non-expressed regions of the transfer vector, for enhanced safety.

Supercoiled polyoma virus DNA is prepared from cultured mouse 3T6 cells by differential salt precipitation and CsCl ethidium bromide equilibrium density centrifugation, essentially as described in Example 1. Randomly cut linear duplex DNA is prepared from supercoiled polyoma DNA by the methods described in Example 1 for converting F1 RFI DNA to RFIII DNA. Unique restriction site sequences suitable for insertion are previously determined by screening experiments to detect susceptibility
to a battery of restriction enzymes. Oligonucleotide sequences corresponding to each such sequence found with zero frequency in polyoma DNA are synthesized. Each of several sequences is introduced in turn, in a population of random cut linear double stranded DNA molecules, by iterations of the procedure described in Example 1, including the steps of attaching the oligonucleotide sequence to be inserted on the ends of random cut linear DNA', recloning the rug, transfec-
ting susceptible cells, in this case mouse 3T6 cells, isolating viable clones evidenced by the ability to form a plaque, amplifying the amount of cloned DNA by introducing a cycle of growth in susceptible cells, reisolating supercoiled circular DNA and again preparing random cut linear DNA for insertion of the next oligonucleotide. After several such cycles, insertion sites will saturate the non-essential sites of the virus, so that it will be possible to specifically and individually excise individual essential regions, for sequence analysis, structural studies and functional studies in model systems, without any prior knowledge of the functions or map locations of the essential functions. The polyoma virus MERV also serves as a source of useful sequence information, using the sequencing method of Example 4.

Example 1

**The Inserts Generated Within Genes**

The technique for generating the insertion of unique oligonucleotide sequences within specific genes simply involves manipulating the growth conditions such that the target region for receiving an insert is functionally non-essential. In practice this means either manipulation of the growth conditions by providing a particular nutrient or removing a potential inhibitor, or providing complementation...
essential for the essential function. The former technique will be effective for generating inserts in the ampicillin resistance gene of pBR322, for example. Transformed cells containing inserts in the ampicillin resistance gene will be tetracycline resistant but ampicillin sensitive. Colonies of cells growing on agar plates containing tetracycline will be transformed cells, and colonies which do not replicate onto ampicillin containing plates contain inserts in the ampicillin resistance gene.

In the case of bacteriophage fl, oligonucleotide insertion into expressed regions is accomplished by transfecting cells previously infected with wild type or mutant fl capable of complementing the target function. In general, mutant complementation is preferred in order to reduce the frequency of occurrence of plaques from cells not transfected by insert containing DNA. Such plaques will however, contain mixtures of the complementing mutant and insert containing phage. The DNA of the latter can be separated by treatment with the restriction enzyme corresponding to the inserted sequence, physically separating double stranded linear DNA thereby produced from the circular DNA of the complementing phage, the reclosing the circles with DNA ligase.

In general, two complementing strains having their mutations in two separate genes will be the minimum required to permit the insertion of oligonucleotide sequences throughout the genome, with the exception of true cis-acting genes such as the replication origin. In general, any technique for providing conditions of non-essentiality for a given region will enable the insertion of oligonucleotide segments into that region. Such methods are well known to those of ordinary skill in the art.
The combination of techniques for insertion into essential and non-essential regions, as defined herein, makes possible construction of a MIRV comprising a library of subfunctions. Such sub-functions include ribosome binding sites, promoters, attenuators, splicing sequences, sequences coding for precursor portions of proteins and functional domains within proteins, i.e., active sites, or antigenic determinants. The subfunctions are individually isolatable and may then be used as building blocks for construction of vectors having desired properties.

Example 8

Sequencing large inserts in a single sequencing vector

The technique for random insertion of restriction sites in a circular DNA, such as mitochondrial DNA, can be exploited to provide the sequence of the entire circular DNA using a sequencing vector as described in Example 3. As with previously described sequencing techniques herein, knowledge of the restriction map of mitochondrial DNA is unnecessary.

In the following embodiment of the invention, a large segment of DNA is inserted at an insertion site on a sequencing vector or expression vector as described in Example 1. It is anticipated that structural restraints will prevent the acceptance of large inserts at all sites where oligonucleotide inserts could exist. Therefore it will be necessary to select for those vectors which permit insertion of large sequences. The location of the areas capable of accepting long insert sequences will provide a map of the structural axes of or regions where large discontinuities interfere with viability. Vectors stable to large inserts must be separately selected for sequencing such large inserts.

The selection process provides a structural map of the vector.
The circular mitochondrial DNA is converted to randomly terminal duplex linear DNA essentially as described in Example 1. The modified Pat I site described in Example 1 is then blunt-end ligated to the ends of the linear molecules, treated with Pat I endonuclease and inserted into the modified Pat I containing f1 sequencing vector described in Example 3. E. coli K38 cells are transfected and DNA preparations are made from individual plaque isolates. The oligonucleotide sequence is then used to prime the synthesis of complementary oligonucleotide strands as described by Sanger et al. supra. In this method, the replication of the sequencing vector depends only on f1 bacteriophage functions. All functions of the DNA to be sequenced, mitochondrial DNA in the present example, are operationally non-essential, since the only essential genes are those required for replication of the vector. Consequently insertions of the Pat I oligonucleotide sequence in the mitochondrial DNA will be randomly distributed. Each sequence segment, determined by priming from an individual plaque isolate, will be different. By picking a sufficient number of plaques, the entire sequence is determined. The average number of plaque isolates needed to determine the sequence is equal the total sequence length divided by the resolution of the gel plus the square root of that value times three. Given a resolution of 200 nucleotides per gel, a sequence 20,000 nucleotides in length should be 99% covered with overlapping sequences by picking 100 plaques plus 30 (3 times the square route of 100). The sequence is confirmed by internal consistency of the overlapping sequences and
may be further confirmed by restriction maps.

Concluding remarks

It can be seen from the foregoing general description of the invention and from the specific examples illustrating applications thereof, that the invention has manifold and far reaching consequences. The invention basically provides novel treated DNA molecules and a method of treating DNA molecules to obtain the product molecules themselves. The fundamental product of the invention is a population linear DNA molecule comprising permutations of the same nucleotide sequence and having a unique oligonucleotide sequence attached at both ends. The attached sequence is unique in the sense that it is found nowhere else in the linear molecule. Subsequent manipulations, selection techniques, growth conditions, cleavage methods, and sequencing methods all contribute to providing end uses for such modified DNA molecules that vastly extend the scope and power of recombinant DNA technology by providing restriction site sequences either in essential or non-essential regions, as desired, and by providing improved and simplified nucleotide sequencing techniques.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.
WHAT IS CLAIMED IS:

1. A population of linear DNA molecules of an autonomously replicating DNA element, comprising circular permutations of the same nucleotide sequence, to the ends of which have been covalently attached an oligonucleotide of unique sequence.

2. An autonomously replicating, transferable DNA comprising a unique nucleotide sequence inserted in a non-essential region thereof.

3. An autonomously replicating, transferable DNA comprising a unique oligonucleotide sequence inserted in a structural gene thereof.

4. A DNA according to claim 2 or 3 having more than one inserted oligonucleotide sequence.

5. A method of inserting a unique oligonucleotide sequence into a non-essential region of an autonomously replicating DNA element, comprising,

   isolating double stranded circular molecules of the DNA, each having the same nucleotide sequence,
   cleaving the circular DNA at random with respect to nucleotide sequence, producing a population of linear, double stranded DNA molecules comprising circular permutations of the same nucleotide sequence,
   joining an oligonucleotide of unique sequence to the ends of the linear DNA molecules, then
   rejoining the ends to form circular double stranded DNA molecules having the oligonucleotide of unique sequence inserted at random with respect to the nucleotide sequence of each circular DNA molecule,
transferring the circular DNA having a unique insert sequence to a host organism under conditions permitting replication of the DNA, and selecting for progeny of the circular DNA having a unique insert sequence, said progeny bearing said insert in a non-essential region of the DNA.

6. A method of making a population of linear DNA molecules having the same nucleotide sequence circularly permuted and having a unique oligonucleotide sequence covalently attached at the ends thereof comprising:
   a) isolating circular DNA of an autonomously replicating DNA element,
   b) cutting the circular DNA at random, thereby generating a population of linear DNA molecules having the same nucleotide sequence circularly permuted, and
   c) covalently attaching a unique oligonucleotide sequence to the ends of the linear DNA molecules of step b).

7. A method of making a modified autonomously replicating DNA element wherein the modification comprises a unique oligonucleotide sequence inserted randomly with respect to the nucleotide sequence comprising the steps of:
   a) isolating and purifying circular DNA of an autonomously replicating DNA element,
   b) cutting the circular DNA at random, thereby generating a population of linear DNA molecules having the same nucleotide sequence circularly permuted,
   c) covalently attaching a unique oligonucleotide sequence to the ends of the linear DNA molecules of step b),
   d) covalently attaching the ends of the linear DNA molecules intramolecularly, whereby circular DNA containing a unique oligonucleotide sequence inserted randomly with respect to nucleotide sequence is made.
COMBINED DECLARATION AND POWER OF ATTORNEY
IN ORIGINAL APPLICATION

As a below named inventor, I hereby declare that:
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INSERTED DNA SEQUENCES
described and claimed in the attached specification, that I understand the content of the attached specification,
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Inventor's signature
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**PAM III APPLICATION FILE DATA CODING SHEET**

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**Format No. 3**

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**RCD** Attorney's Name: 2
- **Reg. No.**: 1
- **No. 2**: 3
- **No. 4**: 5
- **No. 6**: 7

**Format No. 4**

**APPLICANT(S) NAME AND ADDRESS**

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Applicant: PIECZNENIK, G.
Serial No.: 080,668
Filed: October 1, 1979
For: INSERTED DNA SEQUENCES

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REVOCATION AND SUBSTITUTE POWER OF ATTORNEY

Sir,

I, the undersigned, having filed an Application for Letters Patent in the above-identified case hereby revoke all powers of attorney previously given and hereby appoint Lorance L. Greenlee, Registration No. 27,894; Herbert B. Keil, Registration No. 18,967; John F. Witherspoon, Registration No. 20,844; John W. Schneller, Registration No. 26,031; Barry E. Bretschneider, Registration No. 28,055; Michael P. Bucklo, Registration No. 26,444 and Matthew C. Thompson, Registration No. 17,423; the address of all being KEIL & WITHERSPOON, 1101 Connecticut Avenue, N. W., Washington, D. C. 20036 (202-659-0100) to prosecute this application and to transmit all business in the Patent and
Trademark Office connected therewith and with the resulting patent.

Date: Jan. 29, 1977

George Pieniek

GEORGE PIECZENIK
This is in response to the communication re the Power of Attorney filed ______________.

1. ☒ The power of attorney to you in this application has been revoked by the applicant.

2. ☐ In view of the above in this application of the death of ______________, his power of attorney is terminated.

3. ☒ The power of attorney to you in this application has been accepted by the Commissioner of Patents & Trademarks.

4. ☐ The assignee in this application has intervened and appointed an attorney of his own selection. Further correspondence will be held with said attorney. (Rule 36, Rules of Practice.)

5. ☐ The revocation of the power of attorney to ______________ has been entered and said attorney has been notified. Further correspondence will be addressed to you.

6. ☐ On ______________, the applicant appointed ______________ as additional attorney in this application. Further correspondence will continue to be addressed to you as specified in the new power of attorney.

7. ☐ On ______________, the applicant appointed ______________ as additional attorney in this application. Further correspondence will be addressed to said attorney. MPEP 403.02

8. ☐ The associate power of attorney to you in this application has been revoked by the attorney of record.

Keil & Witherspoon
1100 Conn. Ave., N.W.
Washington, D.C. 20036

Laura L. Stutz
For Director, Opposition
Group 170

RETAIN THIS COPY IN THE APPLICATION FILE
6. A DNA according to claim 5 comprising heterologous DNA inserted at said unique restriction site.

7. A DNA according to claim 3 wherein the unique oligonucleotide sequence comprises a unique restriction site, wherein heterologous DNA is inserted such that said heterologous DNA is expressible.

8. A DNA according to claim 7 wherein said heterologous DNA is rendered expressible by addition of 0, 1 or 2 nucleotides to said unique oligonucleotide sequence, whereby any coding sequence present in said heterologous DNA is in reading frame phase with said structural gene.

9. A DNA according to claim 2 wherein the unique oligonucleotide sequence comprises a unique restriction site having inserted therein heterologous DNA such that said heterologous DNA is not expressible.

10. An autonomously replicating DNA element bearing a unique oligonucleotide sequence inserted in a non-essential region thereof, made according to the method of claim 5.

11. An autonomously replicating DNA element according to claim 13 wherein the unique oligonucleotide sequence comprises a unique restriction site.

12. A DNA element according to claim 14 comprising heterologous DNA inserted at said unique restriction site.

13. A DNA element according to claim 15 wherein the inserted heterologous DNA is not expressed.
17. A DNA element according to claim 14 comprising a plurality of inserted restriction sites.

18. The method of claim 5 wherein the unique oligonucleotide sequence is inserted into a structural gene of the autonomously replicating DNA element by providing growth conditions for a host organism such that the structural gene for receiving the insert is functionally non-essential, whereby the circular DNA having a unique insert sequence in said structural gene is transferred to said host organism under conditions permitting replication of the DNA.

19. A DNA element made according to the method of claim 18, bearing a unique oligonucleotide sequence inserted in a structural gene thereof.

20. A DNA element according to claim 19 wherein the unique oligonucleotide sequence comprises a unique restriction site.

21. A DNA element according to claim 20 comprising heterologous DNA inserted at said unique restriction site.

22. A DNA element according to claim 21 wherein the inserted heterologous DNA is expressible.

23. A population of linear DNA molecules having the same nucleotide sequence circularly permuted and having a unique oligonucleotide sequence covalently attached at the ends thereof, made according to the method of claim 6.

24. A modified autonomously replicating DNA element comprising a unique oligonucleotide sequence inserted randomly
with respect to the nucleotide sequence, made according to
the method of claim 7.--

--25. A gene library comprising a multiple insert
restriction vector having structural genes between the
inserts.--

Subst B

--26. A population of linear DNA molecules comprising
the same nucleotide sequence circularly permuted and a
unique oligonucleotide sequence covalently attached at the
ends thereof.--

--27. The DNA molecules of claim 26 comprising the
nucleotide sequence of DNA selected from the group consisting
of a virus, a plasmid, or mitochondrial DNA.--

Subst B 3

--28. A sequencing vector comprising an autonomously
replicating DNA element having an insert comprising a unique
restriction site adjacent to a unique nucleotide sequence,
and on the 5' side thereof.--

--29. A sequencing vector according to claim 28,
comprising a MIRV having a plurality of unique restriction
sites adjacent to unique nucleotide sequences.--

--30. A sequencing vector according to claim 28,
comprising additionally a population of linear DNA molecules
comprising circular permutations of the same nucleotide
sequence, inserted in said unique restriction site.--

--31. A sequencing vector according to claim 28,
comprising additionally a nucleotide sequence inserted at
one or said unique restriction sites.--
--32. The method of genotypically mapping an autonomously replicating DNA element comprising the steps of:

(a) providing a population of linear DNA molecules of said DNA element, comprising circular permutations of the same nucleotide sequence,

(b) attaching an unique oligonucleotide sequence to the ends of the linear DNA molecules of step (a),

(c) rejoining the ends of the linear molecules of step (b), thereby forming a population of circular autonomously replicating DNA molecules having an unique oligonucleotide sequence inserted randomly with respect to the nucleotide sequence,

(d) transforming or transfecting a population of host cells with the population of circular DNA molecules resulting from step (c),

(e) isolating clones of the DNA molecules of step (c), said clones having the unique oligonucleotide sequence inserted in a non-essential region of the autonomously replicating DNA element, under preselected growth conditions, and

(f) measuring the relative positions of the unique oligonucleotide sequence inserts of individual clones, with respect to the nucleotide sequence of the DNA element, thereby mapping the nonessential regions of its genome, under the preselected growth conditions, without reference to or knowledge of the function of any gene therein, said mapping being genotypic.--
REMARKS

The amendment to page 34 of the Specification is requested to correct a typographical error and to make the sentence beginning on line 19 logical and consistent with the sentence immediately following it beginning on line 23. The requested amendment does not introduce new matter since the amended sentence is a general statement and the following sentence beginning line 23 is an explanatory example. The amendment merely makes the general statement consistent with the example. The remaining amendments to the Specification are requested in order to correct typographical errors.

Respectfully submitted,
KEIL & WITHERSPOON

By Lorance L. Greenlee
Reg. No. 27,894

LLG:ch
1101 Connecticut Ave., N.W.
Washington, D.C. 20036
202/659-0100

March 16, 1980
This is a communication from the examiner in charge of your application.

COMMISSIONER OF PATENTS AND TRADEMARKS

The examiner has concluded that the following claims are pending in the application:

1. Claims 1-32

Of the above, claims are withdrawn from consideration.

2. Claims have been cancelled.

3. Claims are allowed.

4. Claims are rejected.

5. Claims are objected to.

6. Claims 1-32 are subject to restriction or election requirement.

7. The formal drawings filed on are acceptable.

8. The drawing correction request filed on has been [stamped] approved, [stamped] disapproved.

9. Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has been received, not been received, been filed in parent application, serial no. filed on.

10. Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11: 452 O.G. 213.

11. Other

EXAMINER'S ACTION

A.E. TANENHOLTS

ART UNIT 172

DATE MAILED: 25 AUG 1981

KEIL & WITHERSTAFF
1100 CONN AVE., N.W.
WASHINGTON, D.C. 20036

ATTORNEY DOCKET NO. (080,668)

10/01/79 GEORGE PIECZENIK

PTOL-324 (rev. 7-79)
This application contains claims to more than one invention and restriction under rule 142 is required between

I. Claims 1 to 4, 8 to 17 and 19 to 31 are drawn to replicating DNA elements classified in 435-317

II. Claims 5 to 7 and 18 are directed to methods that modify replicating DNA classified in 435-172 and

III. Claim 32 for a method mapping a replicating DNA element classified in 435-6

The three invention grouped above as I, II and III are distinct each from the other because they have acquired a separate status in the art, involve divergent searches and can support separate patents. Furthermore, for example the replicating DNA element of Group I does not have to be replicated initially and does not require an isolation step as in the method of Group II since it can be chemically synthesized. Additionally the DNA element of Group I could be used as a source of nucleotide variants instead of in the method of Group III.

Applicant is advised that his response to be complete must include a provisional election of one of the inventions identified above as I, II and III even though they traverse the requirement.

Further action on the merits is deferred pending the determination of the question of division.
In re application of: 
George PIECZEKNIK 
Serial No. 80,668 
Filed: October 1, 1979 
For: INSERTED DNA SEQUENCES

RESPONSE

Hon. Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

Applicant hereby responds to the Examiner's restriction requirement as between claims of Group:

I. Claims 1-4, 8-17 and 19-31;
II. Claims 5-7 and 18;
III. Claim 32

The restriction requirement is traversed on the following grounds:

1. The groups suggested for restriction do not constitute separate and distinct inventions.

As evidence, it is noted that claims 19-31, listed in Group I, are in fact dependent upon claims considered to be part of Group II.

2. The suggested categories of search do not appear to correctly reflect the nature of the invention.

Applicant respectfully suggests that the entire invention is more appropriately categorized in Class 435-41, "A microorganism, tissue cell culture or enzyme using process to synthesize a desired chemical compound or composition". The products of the present invention are chemical compounds or
compositions, i.e., DNA, which are produced by enzyme or microbial action or a combination of both. The action of a microorganism is significant with respect to certain species of the invention since it is the microorganism and its interaction with the growth environment which effectively defines where the unique nucleotide sequences of the invention can be inserted. For the foregoing reasons, Applicant requests reconsideration of the restriction requirement.

Applicant provisionally elects the claims of Group I for further prosecution.

Respectfully submitted,
KEIL & WITHERSPOON

By
Lorance L. Greenlee
Reg. No. 21,594

LLG:ch
1101 Connecticut Ave., N.W.
Washington, D.C. 20036
202/659-0100

September 24, 1980
This is a communication from the examiner in charge of your application.

A shortened statutory period for response to this action is set to expire [ ] months from the date of this letter. Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I - THE FOLLOWING ATTACHMENTS ARE PART OF THIS ACTION:

1. Notice of References Cited by Examiner, PTO-892
2. Notice of Informal Patent Drawing, PTO-946
3. Notice of References Cited by Applicant, PTO-1449
4. Notice of Informal Patent Application, Form PTO-152

Part II - SUMMARY OF ACTION

1. Claims 1-32 are pending in the application.
   Of the above, claims 5-7, 18, 32 are withdrawn from consideration.

2. Claims [ ] have been cancelled.

3. Claims [ ] are allowed.

4. Claims 1-4, 6-17, 19-31 are rejected.

5. Claims [ ] are objected to.

6. Claims [ ] are subject to restriction or election requirement.

7. The formal drawings filed on [ ] are acceptable.

8. The drawing correction request filed on [ ] has been [ ] approved, [ ] disapproved.

9. Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has [ ] been received, [ ] not been received. [ ] been filed in parent application, serial no. [ ] filed on [ ].

10. Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11, 453 O.G. 213.

11. [ ] Other

EXAMINER'S ACTION
NOTICE OF REFEREES CITED

U.S. PATENT DOCUMENTS

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FOREIGN PATENT DOCUMENTS

| DOCUMENT NO. | DATE | COUNTRY | NAME        | CLASS | SUB-CLASS |

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

| T            | Mivanie et al, Natural vol 263 pp 744-748 (1976) |

EXAMINER

DATE 12-22-80

* A copy of this reference is not being furnished with this office action.

(See Manual of Patent Operations Procedure, section 707.00 (a))
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### Other References (Including Author, Title, Date, Pertinent Pages, Etc.)

- **Wax et al., Molecular Cloning of Recombinant DNA**
- **Miami Winter Symposium Vol. 13, edited by Scott, pp. 1-244 (1977)**

* A copy of this reference is not being furnished with this office action. (See Manual of Patent Examining Procedure, section 707.05 (a).)
EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, attorney, agent) representing applicant:

(1) Tamar Greenlee
(2) George Pnungni

Date of interview: 3/6/81

Type: ☐ Telephone ☑ Personal (copy is given to applicant).

Exhibit shown or demonstration conducted: ☐ Yes ☑ No.

Agreement ☐ was reached with respect to some or all of the claims in question. ☑ was not reached.

Claims discussed: 1-4

Identification of prior art discussed:

Description of the general nature of what was agreed to if an agreement was reached, or any other comments:

Discussed fundamental principle of invention and how it differs from the restricted site cleavage of Celere 21. Will invent unique sequence language in claims so as to differentiate DNA molecule from those of prior art.

(A full necessary description and any available copy of amendments that the examiner agreed would render the claims allowable, or where no copy of the amendments is available, a summary thereof, is attached.)

☐ It is not necessary for applicant to supplement the information on this form or to submit a separate record of the substance of the interview.

APPLICANTS, ATTORNEYS AND AGENTS ARE REMINDED OF THEIR RESPONSIBILITY TO SUPPLEMENT THIS RECORD WITH AN INDICATION OF THE Substance OF THE INTERVIEW AS REQUIRED BY 37 CFR 1.133(b) AND SECTION 713.04 OF THE MANUAL OF PATENT EXAMINING PROCEDURE. (See reverse side for text of Section 713.04.)

ORIGINAL FOR INSERTION IN RIGHT HAND FLAP OF FILE WRAPPER
AMENDMENT

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

In response to the Official Action mailed January 19, 1981, please amend the above-identified application as follows:

IN THE SPECIFICATION:

Page 10, line 5, after "b2" insert--region--
line 9, delete "terms" and substitute therefore--

Further on page 10, note that the marginal line
numbers are incorrect and should be changed to 11, 21 and 31,
respectively.

IN THE CLAIMS:

Please amend the claims as follows:

B1 (amended). A population of linear DNA molecules
of an autonomously replicating DNA element, comprising circular
permutations of the same nucleotide sequence, to the ends or
which have been covalently attached an oligonucleotide of
unique sequence, being an oligonucleotide sequence which does
not otherwise exist in the DNA element.
and knowledge of their relative location on the vector. The claimed invention does not depend upon the use of restriction sites (although restriction sites and restriction endonucleases may be used in conjunction therewith), but instead rests upon an entirely different conceptual framework and methodology.

An essential feature of all recombinant DNA technology to date is the use of restriction sites existing at defined loci of a vector, or autonomously replicating DNA element. Where only one such site occurs in the vector, cleavage by a restriction endonuclease yields a linear molecule. Since every molecule so cleaved is cleaved at the same site, the linear molecules all have the same nucleotide sequence in the same order with the same starting point and stopping point. In contrast, the present invention and compositions derived therefrom make use of random cleavage by enzymatic or non-enzymatic means, does not use restriction endonucleases, and yields a linear DNA in which the molecules have the same sequence in the same order, but the start and stop points vary from one molecule to the next due to random cutting of the circle. The resulting population of linear molecules, therefore, has the same sequence circularly permuted, a term known and understood in the art.

An oligonucleotide of unique sequence (one not otherwise found in the vector) may be attached at the ends of the randomly-cut linear molecules so that, upon ring closure, a population of circular molecules results, having the unique sequence inserted randomly. The cited art does not teach or suggest such random insertions, since the art has heretofore depended upon restriction endonucleases to make single cuts at defined sites. Because of this fundamental difference at the
outset, it can be seen that the present invention takes a
direction away from teachings of the prior art. It follows that
none of the subsequent products, manipulations and methods of
use of the present invention are taught or suggested by the
cited prior art.

In the cited prior art, the use of a given vector
was limited by the location of pre-existing restriction sites.
The present invention is not so limited. The invention, in one
embodiment, allows the insertion of a restriction site sequence
such that it can be located at virtually any desired locus.
Means are disclosed for selecting a vector having a unique
nucleotide sequence, which could in principle be a restriction
site sequence, located within a non-essential region of the
vector or, if desired, within a given structural gene of the
vector. Thus, the invention provides novel and unobvious vectors
(autonomously replicating transferable DNA elements) having an
insertion within a non-essential region or within an essential
region, as desired. As more and more such sites are mapped,
it is possible to construct a variety of vectors, or vectors with
multiple insertions, for a variety of end uses, as disclosed in
the application.

To summarize, the present invention deals with the
manipulation or structurally different entities than those
contemplated in the cited prior art. The use of circularly
permuted linear DNA and the use of randomly inserted nucleotide
sequences in a vector are novel and unobvious departures from
the prior art whose consequences lead and teach away from the
teachings of the prior art. Further, the uses to which the
present invention's techniques may be put are not subject to the
restrictions imposed by the limitations of the prior art. There are many such uses, as detailed in the Specification, and these uses considerably expand the scope of utility of recombinant DNA vector over that previously available.

It is noted herein that some of the claims currently pending in the case, more particularly, claims 8, 19, and claims dependent therefrom, are themselves dependent upon claims withdrawn from consideration due to the Examiner's restriction requirement. The non-elected claims are methods of making the claimed products. Applicant respectfully requests reconsideration of the restriction request as it relates to the products and methods of making the same, pursuant to MPEP § 806.05(E).

It is believed that the above amendments to the Specification and claims now place this case in condition for allowance by the Examiner, and reconsideration is respectfully requested.

Respectfully submitted,
KEIL & WITHERSPOON

[Signature]

By

LLG:ch
1101 Connecticut Ave., N.W.
Washington, D.C. 20036
202/659-0100

April 20, 1981
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
George PIECZENIK
Serial No. 80,668
Filed: October 1, 1979
For: INSERTED DNA SEQUENCES

GROUP ART UNIT: 1756
Examiner: A.E. Tanenholz

PRIOR ART STATEMENT

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

The following articles are considered to be relevant to the background and prior art of the subject of the present invention. Copies are submitted herewith.


These textbooks are also referenced as general prior art. Copies are not included.


3. Kornberg, A., DNA Replication

The following testimony is considered to be relevant to the background and prior art of the subject of the present invention. A copy is not included, but will be submitted on request.


Respectfully submitted,

KEIL & WITHERSPOON

By: [Signature]

Lorance L. Greenlee
Reg. No. 27,894

1101 Connecticut Avenue, N.W.
Washington, D.C. 20036

(202) 659-0100

April 20, 1981
Keil & Witherspoon
1100 Conn. Ave., N.W.
Washington, D.C. 20036

cali

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

☐ This application has been examined. ☑ Responsive to communication filed on 4/20-8. This action is made final.

A shortened statutory period for response to this action is set to expire 5 months from the date of this letter. Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I  THE FOLLOWING ATTACHMENTS ARE PART OF THIS ACTION:
1. ☑ Notice of References Cited by Examiner, PTO-892
2. ☑ Notice of Informal Patent Drawing, PTO-948
3. ☑ Notice of References Cited by Applicant, PTO-1449
4. □ Notice of Informal Patent Application, Form PTO-152

Part II  SUMMARY OF ACTION
1. ☑ Claims 1-32. The above claims 5-7, 18, 32 are pending in the application.
2. ☐ Claims ____________________________________________________________ have been withdrawn.
3. ☐ Claims ____________________________________________________________ are allowed.
4. ☑ Claims 1-4, 8-17, 19-31 are rejected.
5. ☐ Claims ____________________________________________________________ are objected to.
6. ☐ Claims ____________________________________________________________ are subject to restriction or election requirement.
7. ☑ The formal drawings filed on 07/15/81 are acceptable.
8. ☑ The drawing correction request filed on 07/15/81 has been approved. ☐ disapproved.
9. ☐ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has been received. ☐ Not been received. ☑ been filed in parent application, serial no. ________ filed on ________.
10. ☐ Since this application answers to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
11. ☐ Other

PTOL-326 (rev. 7-79)
Applicant should submit a copy of his testimony in Congressional Record, 95th Congress No. 24, pp. S23-34U (1977).

The restriction requirement as set out in paper No. 5 is deemed sound and is adhered to, repeated and made final.

Accordingly claims 5 to 7, 18 and 32 remain withdrawn from further consideration under Rule 142(u) as being for non-elected invention, the requirement having been traversed in paper Nos. 6 and 9.

Claims 3, 4, 8 to 17 and 19 to 25 are rejected as indefinite under 35 USC 112 2nd paragraph. The recitation of "unique sequence", "unique oligonucleotide sequence" and "unique restriction site" are nebulous and unclear comparative expressions. Similarly the recitation in claim 25 of "a multitude insert restriction vector is not clear and complete. Claims and not the specification should define the invention.

Claims 1 to 4, 8 to 17 and 19 to 31 are rejected unpativeable under the 1st paragraph of 35 USC 112. The disclosure is not enabling to support the breadth of claims. Only one specific and unique kind of vector, namely F1 RF phage, has been shown to be susceptible to the instant unique "random cleavage" process.

Claims 2 to 4, 8 to 17, 19 to 22, 24 and 28 to 31 are rejected as unpatentable over each of Cohen et al, Itakura et al, Polisky et al, Marions et al, Ullrich et al, Higuchi et al and Wu et al under 35 USC 102 or 103. The references disclose hybrid plasmids that read on or substantially read on the claimed autonomously replicating DNA element. Applicant contends that the process of the references do not randomly insert nucleotide sequences in a vector or yield linear DNA that has been circularly
permitted. However, the claims are not so limited. For example, claims 2, 13 and 28 merely read on a hybrid plasmid containing foreign DNA while the composition of claim 26 does not even have to be a vector. Thus arguments that applicant's invention rests upon a conceptual framework and methodology different from that of the references and results in a population of linear DNA having random insertions are patently irrelevant. With regard to claims such as 29, reiterations of the process to produce multiple inserts of the foreign DNA in the vectors of the references would only involve an arbitrary matter of choice.

Claims 1 to 4, 6 to 17 and 19 to 31 are rejected as unpatentable over each of Heffron et al and Maniatis et al under 35USC 102 or 103. Heffron et al and Maniatis et al disclose the same or substantially the same linear DNA molecules or autonomous replicating DNA element having circular permutations of the same nucleotide sequence as recited in the claims.

A.T. Tanenholtz: cal
703-557-3611
06/25/81

[Signature]
**NOTICE OF REFERENCES CITED**

**U.S. PATENT DOCUMENTS**

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**OTHER REFERENCES** (Including Author, Title, Date, Pertinent Pages, Etc.)


**EXAMINER**

[Signature]

[Date: 6/19/87]

* A copy of this reference is not being furnished with this office action.
(See Manual of Patent Examining Procedure, section 707.06 (a).)
**NOTICE OF REFERENCES CITED**

**U.S. PATENT DOCUMENTS**

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**OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)**

- R: Stabun et al., J. Am. Chem. Soc. 97, 73227 (1975)
- S: Scheller et al., Science 196, 177 (1972)

**EXAMINER**

* A copy of this reference is not being furnished with this office action. (See Manual of Patent Examining Procedure, section 707.06 (a.).)
**NOTICE OF REFERENCES CITED**

### U.S. Patent Documents

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### Foreign Patent Documents

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### Other References (Including Author, Title, Date, Pertinent Pages, Etc.)

| S | Ulbrich et al., Science 196, 1313 (1977)    |
| U | Burwell et al., Nature, 279, 43 (1979)      |

**EXAMINER**

[Signature]

**DATE**

4/4/83

*A copy of this reference is not being furnished with this office action. (See Manual of Patent Examining Procedure, section 707.05 (a).)*
**NOTICE OF REFERENCES CITED**

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**EXAMINER**

Date: 6/19/81

* A copy of this reference is not being furnished with this office action. (See Manual of Patent Examining Procedure, section 707.05 (a).)
EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, applicant's representative, PTO personnel):

(1) Lawrence J. O'Keefe

(2)

(3)

(4)

Date of Interview: 9-28-81

Type: □ Telephonic  ☑ Personal (copy is given to □ applicant  ☑ applicant's representative).

Exhibit shown or demonstration conducted: □ Yes ☑ No. If yes, brief description:

Agreement ☑ was reached with respect to some or all of the claims in question, □ was not reached.

Claims Amended: 1-4, 8, 17, 19, 31

Identification of prior art discussed: All used in rejection

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: will remedy unique sequence language of claim 3, 17, 20 amended to read claim 2 et al. All references except Heffron et al. and possible Mr. {Maniaci} should be removed by amending claims 1 to read randomly chosen - Maniaci signer not taken permission of

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

Unless the paragraphs below have been checked to indicate the contrary, A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW (e.g., items 1-7 on the reverse side of this form). If a response to the last Office action has already been filed, then applicant is given one month from this interview date to provide a statement of the substance of the interview.

□ It is not necessary for and not to provide a separate report of the substance of the interview.

□ Since the examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action.

81-3679 PTOL-413 (rev. 1-81)

 Examiner's Signature

ORI GINAL FOR INSERTION IN RIGHT HAND FLAP OF FILE WRAPPER
AMENDMENT

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

In response to the Official Action mailed July 8, 1981, kindly enter the following amendments.

IN THE CLAIMS:

Please amend the claims as follows:

Claim 1, line 2, after "element," insert--randomly cleaved--

Claim 2, line 2, after "thereof", insert--at a site not previously susceptible of restriction endonuclease cleavage--

Claim 3, line 2, after "sequence", insert--being an oligonucleotide sequence which does not otherwise exist in the DNA--

Claim 3, line 3, after "thereof", insert--at a site not previously susceptible of restriction endonuclease cleavage--
Claims 1-4, 8-17 and 19-31 are rejected under 35 USC §112, on the ground that support is lacking for application of the random cleavage process to any vector other than FI RF phage. The techniques for generating random cleavage in DNA are old in the art. For example, the enzyme DNase I hydrolyzes internal phosphodiester bonds in double-stranded DNA at random, without regard to the source of DNA. (See enclosed description of the action of DNase I in Biological Chemistry, Second Edition, Mahler, H.R. and Cordes, E.H., 1971. The italicized cleavage referred to therein is diagrammed at the top of page 195, yielding 5' phosphate-terminated oligonucleotides). The Specification, at page 21, line 25 through page 22, line 4 discloses several generally applicable methods for randomly cleaving a circular DNA. Such methods are not limited to FI RF DNA. No particular sequence specificity, such as the location or existence of certain restriction sites, is required for the operation of the invention or to define the products thereof. Accordingly, claims 1 and 26 have been amended to explicitly recite the random cleavage which yields the resulting circular permutations of the same nucleotide sequence.

Claims 2-4, 8-17 and 19-22, 24, and 28-31 are rejected under "35 USC §102 or 103", citing Cohen et al, Itaskwa et al, Polisky et al, Marions et al, Ullrich et al, Higuchi et al, and Wu et al. The rejection is based upon the contention that the language of the rejected claims is broad enough to read upon any hybrid plasmid containing foreign DNA. A distinguishing feature of the present invention is the ability to generate hybrid plasmids without resorting to use of restriction endonucleases. As detailed in the Specification,
the insertion of heterologous DNA into a transfer vector could only be carried out in the prior art at sites susceptible to restriction endonuclease cleavage. The present invention permits insertion of a heterologous DNA segment at virtually any desired site.

The distinction between the method of the present invention and the prior art has structural consequences for the resulting hybrid vector. In the prior art process, the first step is cleavage of the vector with a restriction endonuclease, at a site susceptible of restriction endonuclease cleavage. Such a site has a specific oligonucleotide recognition sequence. (For example, cleaves only at the sequence GAATTC, between the G and A residues). After cleavage, one strand of the DNA is always terminated by a G at one end and an A at the other. Anything inserted at an EcoRI cleavage site will be bounded on one side by a G, and on the other by an A, assuming no special precautions are taken to remove these moieties. In contrast, the random cleavage step of the present invention does not consistently yield the same end groups.

To use the language of Cohen et al, the prior art method yields a cleaved vector having "predetermined ends" which, after insertion of heterologous DNA, remain as a footprint of the site on the vector which was previously susceptible of restriction endonuclease cleavage. The random cleavage process of the present invention yields no such predetermined ends and leaves no such footprint after insertion of heterologous DNA.

Claims 2, 3 and 26 have been amended, in the light of the rejection and the foregoing considerations, to
distinguish the present invention, whose insertions leave no footprints, by reciting that the insertion site be one "not previously susceptible of restriction endonuclease cleavage". Support for the term "susceptible of cleavage" is found at page 7 of the Specification and the discussion relating to restriction sites. Since the structural state of the vector prior to cleavage determines whether a footprint will be left after insertion, the claim is couched in terms of that prior state to distinguish the possibility, unique to the present invention, of inserting a restriction site where none had previously existed. The foregoing structural limitations are believed to distinguish the vectors of the present invention from those of the prior art.

Claims 1-4, 8-17 and 19-31 were rejected under 35 USC §102 or 103, citing Heffron et al and Maniatis et al. The two references disclose substantially different subject matter and are therefore discussed separately.

Heffron et al discloses a method of mutagenesis and phenotypic mapping of a plasmid which employs random cleavage of the plasmid followed by insertion of a restriction site sequence. If the insertion disrupts a gene, a change in the functional properties of the organism, attributable to the action of that gene, may be observed. The physical location of the insertion producing such change may then be deduced by restriction mapping. Heffron et al discloses the mapping of the plasmid RSF 1050 by insertion of an octamer bearing the EcoRI restriction site sequence. RSF 1050 had a single pre-existing EcoRI site, so that insertion of the octamer resulted in an altered plasmid having two such sites. The location
of the new EcoRI site, relative to the pre-existing site. was inferred from analysis of the molecular size of fragments generated by EcoRI endonuclease digestion, as shown in Figure 2 of the reference. Therefore, the reference does not disclose the structures claimed herein. Said structures have inserted, or attached to the ends (depending on whether circular or linear structures are being claimed), a unique nucleotide sequence, being an oligonucleotide sequence not otherwise found in the DNA. Furthermore, Heffron et al does not teach or suggest insertion of a unique nucleotide sequence to one of ordinary skill in the art. The entire thrust of Heffron et al is in a direction contrary to the present invention despite the superficial similarity. Thus, Heffron et al is concerned with conventional phenotypic mapping, based upon mutogenesis and analysis of the mutant phenotype by measurement of the specific known function of a given gene. The present invention is based upon a concept of genotypic mapping, which requires no prior knowledge of the existence or function of any genes, but instead classifies regions of the genome according to whether they be essential or non-essential for replication under specific conditions. The concept of genotypic mapping is a unique contribution of the Applicant. The concept advances the art and frees it from conceptual restraints which have imposed a barrier to appreciating the broad applicability of the techniques of the present invention. For example, Heffron et al makes no mention of the insertion of oligonucleotide sequences other than restriction site sequences. Heffron et al makes no suggestion for the insertion of a unique oligonucleotide sequence of
any sort, and in fact teaches away therefrom by demonstrating
its mutagenesis technique by insertion of a sequence which is
not unique in the plasmid. For these reasons, the claims are
believed to show patentable differences over Heffron et al.

Maniatis et al discloses the cloning of chromosomal
DNA fragments to prepare a gene library. High molecular weight
eucaryotic DNA was cleaved by a method designed to give random
cleavage, the fragments thus produced were ligated to EcoRI
linker oligonucleotides, and these DNA fragments were then
inserted to a cloning vector at an EcoRI site, using standard,
prior art method of insertion. In Maniatis et al, it is the
inserted DNA that is randomly cleaved, whereas in the present
invention, it is the vector that is randomly cleaved. The
linear cleavage product in Maniatis does not constitute
"circular permutations of the same nucleotide sequence" as do
the linear products of the present invention. The insertion
techniques employed in Maniatis et al are conventional and in
no way suggest the insertion techniques of the present inven-
tion. Therefore, the instant claims do not read on the
linear or circular DNA products disclosed by Maniatis et al,
and are not suggested thereby.

In view of the foregoing discussion, the claims as
amended should now be in condition for allowance. A copy of
Applicant's testimony in the Congressional Record is supplied
herewith, in compliance with the Examiner's request.

Respectfully submitted.

KEIL & WITHERSPOON

By Lorance L. Greenlee
Reg. No. 27,894

LLG:ch
1101 Connecticut Ave., N.W.
Washington, D.C. 20036
202/659-0100

236
This is a communication from the examiner in charge of your application.

COMMISSIONER OF PATENTS AND TRADEMARKS

□ This application has been examined.  ❑ Responsive to communication filed on 10-5-81  ❑ This action is made final.

A shortened statutory period for response to this action is set to expire ___ months from the date of this letter. Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:
1. □ Notice of References Cited by Examiner, PTO-892
2. □ Notice of Informal Patent Drawing, PTO-848
3. □ Notice of References Cited by Applicant, PTO-1449
4. □ Notice of Informal Patent Application, Form PTO-152

Part II SUMMARY OF ACTION
1. ❑ Claims 1-32

Of the above, claims 5-7, 18 + 32 are withdrawn from consideration.

2. □ Claims ____________________ have been cancelled.
3. □ Claims ____________________ are allowed.

4. ❑ Claims 1-4, 8-17 + 19-32 are rejected.

5. □ Claims ____________________ are objected to.

6. □ Claims ____________________ are subject to rejection or objection requirement.

7. □ The formal drawings filed on ____________________ are acceptable.
8. □ The drawing correction request filed on ____________________ has been approved. ❑ disapproved.

9. □ Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has been received. □ not been received. □ been filed in parent application, serial no. ____________________ filed on ____________________

10. □ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.

11. □ Other

PTOL-326 (rev. 7-79)

248

EXAMINER'S ACTION
EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, applicant's representative, PTO personnel):

(1) Clarence Copley
(2) George Piscopink
(3) Alvin E. Tanenhold

Date of interview: 3/15/82

Type: ☐ Telephone ☑ Personal (copy is given to ☐ applicant ☑ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☑ No. If yes, brief description:

Agreement ☐ was reached with respect to some or all of the claims in question. ☒ was not reached.

Claims discussed: 1

Identification of prior art discussed: Heffner et al.

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: Applicant contends that the Heffner et al. invention requires a sequence that results in the reactivity of the cysteine residues that are present in the human and rat sequences.

Unless the paragraphs below have been checked to indicate to the contrary, A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCORPORATE THE SUBSTANCE OF THE INTERVIEW (see MPEP 713.04). If a response to the last Office action has already been filed, then applicant is given one month from the interview date to provide a statement of the substance of the interview.

☐ It is not necessary for applicant to provide a separate record of the substance of the interview.

☐ Since the examiner's interview summary shows (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action.

Original for insertion in right hand flap of file wrapper.
In re application of:
George PIECZENIK
Serial No. 80,668
Filed: October 1, 1979
for: INSERTED DNA SEQUENCES

REQUEST FOR EXTENSION OF TIME

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Applicant, through his undersigned attorney, hereby respectfully requests an extension of one (1) month in which to respond to the outstanding Official Action mailed December 29, 1981. If granted, the response will be due April 29, 1982.

The reason for this request is as follows. Applicant and his undersigned attorney held an extensive interview with the Examiner on March 18, 1982. Due to the nature of the results of that interview and the Examiner's rejections, this additional time is required for the attorney to consult with his client and prepare a more comprehensive and thorough response to the outstanding rejections. This is a first request for extension, and is not made for the purpose of delay.

Respectfully submitted,
KEIL & WITHERSPOON

By
Lorance L. Greenlee
Reg. No. 27,894

1101 Connecticut Ave., N.W.
Washington, D.C. 20231
202/659-0100
March 29, 1982

APR 5 1982
RUTH W. STEIN
In response to the final rejection of December 29, 1981, kindly enter the following amendments:

IN THE CLAIMS:

Claim 1 (thrice amended). A population of linear DNA molecules of an autonomously replicating DNA element, randomly cleaved, comprising circular permutations of the same nucleotide sequence, to the ends of which have been covalently attached an oligonucleotide of unique sequence, being an oligonucleotide of which its sequence [which] does not otherwise exist in said [the] DNA element.

Claim 2 (thrice amended). An autonomously replicating, transferable DNA comprising a unique nucleotide sequence, being an oligonucleotide of which its sequence [which] does not otherwise exist in said transferable [the] DNA, inserted in a non-essential region thereof at a site not previously susceptible of restriction endonuclease cleavage.

Claim 3 (twice amended). An autonomously replicating, transferable DNA comprising a unique nucleotide [oligo-
nucleotide] sequence, being an oligonucleotide of which its sequence [which] does not otherwise exist in [the] said transferrable DNA inserted in a structural gene thereof at a site not previously susceptible of restriction endonuclease cleavage.

Kindly withdraw claim 25 without prejudice to further prosecution.

Claim 26 (thrice amended). A population of linear DNA molecules, randomly cleaved, comprising the same nucleotide sequence circularly permuted and a unique oligonucleotide sequence, being an oligonucleotide [sequence] of which its sequence does not otherwise exist in the DNA molecules, covalently attached at the ends thereof at a site not previously susceptible of restriction endonuclease cleavage.

Claim 28 (twice amended). A sequencing vector comprising an autonomously replicating DNA element having an insert comprising a unique restriction site, being a restriction site of which its sequence does not otherwise exist in the DNA element, adjacent to a unique nucleotide sequence [.] of which its [being a nucleotide] sequence [which] does not otherwise exist in the DNA element, said unique restriction site being located on the 5' side of said unique nucleotide sequence.

REMARKS

The Specification, at page 12, lines 15-17, defines "unique sequence" as follows: "A unique sequence is defined as one which does not otherwise exist in the DNA of a given autonomously replicating element, unless inserted or generated by techniques described herein." This means, quite simply, that in comparing the sequence to be inserted with all
sequences of the same length in the vector, no exactly comparable sequence can be found in the vector. Clearly, the longer the sequence to be inserted or attached to the ends of randomly cut vector DNA, the more likely it is that the sequence to be inserted is unique.

The Examiner has reiterated his rejection over Heffron et al, stating: "Contrary to applicant's contention the randomly inserted restriction sites of the reference are oligonucleotide sequences not otherwise found in the DNA." The Examiner's statement is respectfully contested. The disagreement is believed to arise over differing interpretations of the term "unique oligonucleotide sequence" used in the Specification. There are two fundamental differences between the mutation process described by Heffron et al and the process of making the vectors disclosed herein. First, the sequence inserted in or ligated to an autonomously replicating DNA element according to the present invention is unique, in the formal sense that no such sequence existed in the DNA element prior to said insertion or ligation. Second, the insertion is not confined to sites within expressed regions of the DNA element. Heffron et al, by contrast, expressly discloses insertion of a sequence, namely an EcoRI site, which previously did exist within the vector at another locus. The purpose and intent of inserting a pre-existing (non-unique) sequence was to permit restriction mapping, to measure the distance between the old site and the new, using restriction endonuclease cuts and gel electrophoresis to measure the lengths of resulting fragments.

Heffron et al further requires an extra step in the construction process not required in the process of constructing Applicant's claimed DNA elements. The pre-existing EcoRI
site in Heffron et al must first be blocked by methylation (using EcoRI methylase) to prevent internal cleavage at the old EcoRI site, in a later step. (See, e.g., Figure 1, page 6013). The Heffron et al process would appear to be limited to use of blockable restriction sites. Of course the blocking step demands the pre-existence of the sequence to be blocked in the vector. The present invention requires no such blocking step, and is not so limited.

Finally, the purpose, intent and utility of Heffron et al is quite different from the present invention. Heffron et al is a mutagenesis technique. Its purpose is to create mutations within expressible (and phenotypically detectible) regions of the vector. Applicant, on the contrary, can place his insertions outside expressible regions. In fact, a fundamental aspect of Applicant's invention is insertion of unique sequences in non-essential, phenotypically silent regions, thereby permitting the genotypic characterization ("mapping") of such regions. For these reasons, Applicant reasserts the essential distinction between his invention and the Heffron et al reference.

In order to clarify the foregoing differences between the reference and the claimed invention, the claims have been further modified. For example, amended claim 1 now provides that "an oligonucleotide of unique sequence" is "an oligonucleotide of which its sequence does not otherwise exist in said DNA element" (emphasis added). The claim therefore states unambiguously that the sequence of the added oligonucleotide differs from, and is not otherwise found in, the DNA element to which it is added. The amendment is believed to provide the requisite degree of clarity to enable the distinctions with Heffron et al to be evident.
It is considered that the foregoing considerations apply also to the rejection over Maniatis et al. Thus, the fact that the fragments of inserted foreign DNA described therein "must necessarily" have many restriction sites is irrelevant, since the insertions are not made randomly into vectors but at pre-existing insertion sites. Furthermore, as with Hefron et al, the DNA to be inserted by Maniatis et al is first treated with EcoRI methylase to block internal EcoRI sites and prevent internal cleavage before insertion into a pre-existing EcoRI site on the vector. In summary, in Maniatis et al, the vector is not randomly cleaved, a DNA methylation step is required to generate the desired insertion, and restriction sites are required for insertion, all in contrast to Applicant's invention.

The Examiner's contention that claim 25 as presently drafted reads on inherently present restriction sites as disclosed by Maniatis et al is deemed well founded. Accordingly, claim 25 is withdrawn without prejudice from the instant case.

In view of the proposed amendments and foregoing discussion thereof, the remaining claims as amended are considered to be in condition for allowance, which is hereby respectfully requested.

Respectfully submitted,
KEIL & WITHERSPOON

[signature]

Lorance L. Greenlee
Reg. No. 27,894

LtG:ch
1101 Connecticut Ave., N.W.
Washington, D.C. 20036
202/659-0100
April 29, 1982
Below is a communication from the EXAMINER in charge of this application.

COMMISSIONER OF PATENTS, AND TRADEMARKS

ADVISORY ACTION

THE PERIOD FOR RESPONSE IS EXTENDED TO RUN 6 MONTHS FROM THE DATE OF THE FINAL REJECTION.

1. ☐ The proposed amendments to the claim and/or specification will not be entered and the final rejection stands because:
   a. ☐ There is no convincing showing under Rule 116(b).
   b. ☐ They raise new issues that would require further consideration and/or search.
   c. ☐ They raise the issue of new matter.
   d. ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal.
   e. ☐ They present additional claims without cancelling a corresponding number of finally rejected claims.

2. ☐ Newly proposed or amended claims ____________________________ would be allowed if submitted in a separately filed amendment cancelling the non-allowable claims.

3. ☐ Upon the filing of an appeal, the proposed amendment ☐ will be ☐ will not be entered and the status of the claims in this application would be as follows.
   a. ☐ Claims ____________________________ would be allowable.
   b. ☐ Claims ____________________________ would not be allowable.

   However:
   (1) ☐ The rejection of claims ____________________________ on references is deemed to be overcome by applicant's response.
   (2) ☐ The rejection of claims ____________________________ on non-reference grounds only is deemed to be overcome by applicant's response.

4. ☐ The affidavit, exhibit or request for reconsideration has been entered but does not overcome the rejection.

5. ☐ The affidavit or exhibit will not be admitted because applicant has not shown good and sufficient reasons why it was not earlier presented.

6. ☐ The application having been examined under the special accelerated examining procedure (M.P.R. 706.05), the proposed amendment has not been considered since it does not prima facie place the application in condition for allowance or in better condition for appeal.

25
This is an ADVISORY ACTION.

THE PERIOD FOR RESPONSE IS EXTENDED TO RUN 6 MONTHS FROM THE DATE OF THE FINAL REJECTION. 855 O.G. 1109.

Applicant's response has been considered under the requirements and/or conditions of 37 CFR 1.116 which reads as follows:

a) After final rejection or action 37 CFR 1.113 amendments may be made cancelling claim(s) or complying with any requirement of 37 CFR 1.13 which has been made. Amendments presenting rejected claim(s) in better form for consideration on appeal may be admitted. The admission of, or refusal to admit, any relative thereto, shall not operate to relieve the application or patent under reexamination from its condition as subject to appeal or to save the application from abandonment under 37 CFR 1.135.

b) If amendments touching the merits of the application or patent under reexamination are presented after final rejection, or after appeal has been taken, or when such amendment might not otherwise be proper, they may be admitted upon a showing of good and sufficient reasons why they are necessary and were not earlier presented.
c) No amendment can be made as a matter of right in appealed cases. After decision on appeal, amendments can only be made as provided in 37 CFR 1.198, or to carry into effect a recommendation under 37 CFR 1.196.

Consequently, Applicant's response to the FINAL rejection, filed April 30, 1982, has been considered with the following effect, but it is not deemed to place the application in condition for allowance.

Upon the filing of an appeal, the proposed amendment will be entered and the status of the claim(s) in this application would be as follows:

Allowed claims: 1-9, 8-12 and 28-31
Rejected claims: 13-17, 19-24 and 26
The rejection of claim 27 on references is deemed to be overcome by applicant's response.

ABTANENHOLTZ:hm
703-557-3611
6/1/82

[Signature]
ALVIN E. TANENHOLTZ
PRIMARY EXAMINER
In re Application of
George PIECZEK
Serial No. 80,668
Filed: October 1, 1979
For: INSERTED DNA SEQUENCES

AMENDMENT

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Kindly cancel claims 13-17, 19-24 and 26-27.

REMARKS

In accord with the Advisory Action mailed
June 4, 1982, the rejected claims have been cancelled.
Only allowed claims remain pending. Applicant respectfully
requests the case to proceed to issue.

Respectfully submitted,
KEIL & WYTHERSPOON

1101 Connecticut Avenue, N.W.
Washington, D.C. 20231
202/659-0100

June 29, 1982
This is a communication from the examiner in charge of your application.

COMMISSIONER OF PATENTS AND TRADEMARKS

1. [ ] THIS IS AN ATTACHMENT TO THE NOTICE OF ALLOWANCE AND BASE ISSUE FEE DUE

2. [X] All of the claims are allowable. PROSECUTION ON THE MERITS IS CLOSED in this application (in view of the attached Examiner's Amendment).

a. [X] Applicant's communication filed 2/14/82
b. [ ] Interview summary and attached EXAMINER INTERVIEW SUMMARY RECORD
c. [X] Examiner's Amendment to the record below. Should the changes and/or additions below be unacceptable to applicant, an appropriate amendment to the record may be proposed as provided by 37 C.F.R. 1.312. To ensure consideration of such an amendment, it MUST be submitted before or with the remittance of the Base Issue Fee.

d. [ ] An Examiner's Amendment will follow

e. [X] The allowed claims are 1-4, 8-12 and 20-31

3. [ ] PROSECUTION ON THE MERITS REMAINS CLOSED. Should the changes and/or additions below be unacceptable to applicant, an appropriate amendment to the record may be proposed as provided by 37 C.F.R. 1.312. To ensure consideration of such an amendment, it MUST be submitted before or with the remittance of the Base Issue Fee.

EXAMINER'S AMENDMENT TO THE RECORD

A. [ ] Note statement of reasons for allowance contained below. Any comments considered necessary by applicant regarding the statement of reasons for allowance will be presented to the examiner for the purpose of completing the investigation of such application.

B. [ ] Note attached NOTICE OF ERRORS CITED, PTO — 860, which is part of this communication. The listed references are considered to be pertinent to the invention, but the claims are deemed to be patentable thereunder.

C. [ ] Note attached LIST OF PRIOR ART CITED BY EXAMINER, PTO-144B, which is part of this communication and serves as an acknowledgment of receipt of applicants prior art statement. The references which were considered have been indicated on the form by the examiner, and the claims are deemed patentable thereunder.

D. [ ] The formal drawings filed on ___________ are acceptable

E. [X] The drawing correction request filed on ___________ has been approved. [ ] disapproved

F. [ ] Acknowledgment is made of the claim for priority under 35 U.S.C. 119. The certified copy has been received.

[ ] not been received. [ ] been filed in parent application, serial no.

G. [X] Note amendment to Specification. Claims and/or Drawing contained below.

261
Since no petition under 37 CFR 1.144 has been filed and the period for response to the final action of December 29, 1981 has expired, non elected claims 5 to 7, 18 and 32 have been canceled.

The following has been added to the specification.

ABSTRACT

-Autonomously replicating DNA containing a unique nucleotide sequence, being an oligonucleotide of which its sequence does not otherwise exist in said DNA, inserted in a non-essential region thereof at a site not previously susceptible of restriction endonuclease cleavage-

ALVIN E. REINHOLTZ
703-557-3611
7/14/82
**NOTICE OF ALLOWANCE AND BASE ISSUE FEE DUE**

The application identified by docket number has been examined and found allowable for issuance of Letters Patent. **PROSECUTION ON THE MERITS IS CLOSED.**

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First Name: **GEORGE**  
Last Name: **DZULNIK**

**TITLE OF INVENTION:** Autonomously Replicating DNA Containing Inserted DNA Sequences

**BASE FEE COMPUTATION**

- **BASE FEE DUE**
- **ATTY’S DOCKET NO.**
- **CLASS - SUBCLASS**
- **BATCH NO.**

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The complete issue fee is one hundred dollars ($100) plus two dollars ($2) for each sheet of drawing, plus ten dollars ($10) for each printed page of specification (excluding claims) or portion thereof. Inasmuch as the final number of printed pages cannot be determined in advance of printing, an initial BASE ISSUE FEE (consisting of the fee for printing the first page of specification ($10) plus the fee of $2) for each sheet of drawing, added to the fee of $100, MUST BE PAID WITHIN THREE MONTHS FROM THE DATE OF THIS NOTICE, or the application shall be regarded as ABANDONED. The Base Issue Fee will not be accepted from anyone other than the applicant or a registered attorney or agent of the assignee or other party to the business as directed by the records of the Patent and Trademark Office.

In order to minimize delays in the issuance of a patent based on this application, this Notice may have been mailed prior to completion of final processing by the Examinining Group. It is recognized that the nature and/or extent of the remaining revision or processing requirements may cause slight delays in the printing of the patent. In addition, if prosecution is to be reopened, this Notice of Allowance will be reopened and the appropriate Office action will follow in due course. If the base issue fee has already been paid and prosecution is reopened, the applicant may request a refund or request that the fee be credited to a Deposit Account. However, applicant may wait until the application is either granted, allowable or held abandoned. If allowed, upon receipt of a new Notice of Allowance, applicant may request that the previously submitted base issue fee be applied. If abandoned, applicant may request refund or credit to a Deposit Account.

In the case of each patent being issued without an assignee, the complete post office address of the inventor(s) will be printed in the patent heading and in the Official Gazette. If the inventor’s address is different from the address which appears in the application, please file in the information in the space provided or PTOL-85b enclosed. If there are address changes for more than two inventors, enter the additional addresses on the reverse side of the PTOL-85b.

The appropriate spaces in the ASSIGNMENT DATA section of PTOL-85b must be completed in all cases. If it is desired to have the patent issue to an assignee, an assignment must have been previously submitted to the Parent and Trademark Office or must be submitted herewith as required by 37 C.F.R. 3.34. Where there is an assignee, the assignee's name and address must be provided on the PTOL-85b to insure its inclusion in the printed patent.

Advisory written for 10 or more printed copies of the printed patent can be made by completing the information in section of PTOL-85b and submitting payment therewith. If use of a Deposit Account is being authorized for payment, PTOL-85b should also be forwarded. The order must be for at least 10 copies and must accompany the issue fee. The copies ordered will be sent only to the address specified in section 1 of PTOL-85b.

If an additional fee is due, a Notice of Balance of Issue Fee Due will be mailed together with the patentee's copy of the patent. Payment must be made within three months from the date shown on said Notice since FAILURE TO PAY THIS BALANCE WITHIN THE SPECIFIED PERIOD WILL RESULT IN LATE FEE TO THE PATENT.

[ ] Note attached communication from Examiner.

[ ] This notice is issued view of applicant's communication filed

**IMPORTANT**

**ATTENTION IS DIRECTED TO 37 C.F.R. 3.34**

**THE PATENT WILL ISSUE TO APPLICANT UNLESS AN ASSIGNEE IS SHOWN IN ITEM 3 ON FORM PTOL-85b, ATTACHED**
**BASE ISSUE FEE TRANSMITTAL**

This form is provided in lieu of a formal transmittal and should be used for transmitting the Base Issue Fee. The sections 1A through 4 below must be completed as appropriate.

**MAILING INSTRUCTIONS**

All further correspondence including the Issue Fee Receipt, the Notice of Balance of Issue Fee Due if applicable, and all changes will be mailed to the address entered in section 4 at the left below, unless you direct otherwise by completing the appropriate lines and boxes below.

1. I hereby certify that the correspondence is being sent to:

   **Name:** KEIL & WITHERSPoon  
   **Address:** 1100 CONN. AVE., N.W.  
   **City:** WASHINGTON, D.C. 20005

   **Signature of party in interest of record:**  
   **Date:** August 11, 1982

   **Register No.:** 27,894  
   **Series:** 8-118

   **Signature of party in interest of record:**  
   **Date:** Aug 11, 1982

   **Register No.:** 27,894  
   **Series:** 8-118

2. The COMMISSIONER OF PATENTS AND TRADEMARKS is requested to apply the Base Issue Fee to the application identified below:

   **Signature of party in interest of record:**  
   **Date:**  

   **Register No.:** 27,894  
   **Series:** 8-118

   **Signature of party in interest of record:**  
   **Date:**

   **Register No.:** 27,894  
   **Series:** 8-118

   **Note:** The Base Issue Fee will not be accepted from anyone other than the applicant.

**APPLICATION DATA**

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**TITLE OF INVENTION**

AUTONOMOUSLY REPLICATING DNA CONTAINING INSERTED DNA SEQUENCES

**BASE FEE COMPUTATION**

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</table>

1. Further correspondence to be mailed to the following:

2. For printing on the patent front page, list below the names of not more than 3 registered patent attorneys or agents OR, alternatively, the name of a firm having as a member a registered attorney or agent. If no name is listed below, no name will be printed.

   1. KEIL & WITHERSPoon

   2. 

   3. 

3. ASSIGNMENT DATA (print or type)

   A. 

   (1) **This application is NOT assigned.**

   (2) Assignment previously submitted to the Patent and Trademark Office.

   (3) Assignment submitted herewith.

   B. For Printing On The Patent: Unless an assignee is identified below, no assignee data will appear on the patent. Inclusion of assignee data below is only appropriate when an assignment has been previously submitted to the PTO or is submitted herewith. Completion of this form is NOT a substitute for filing of an assignment as required by 37 C.F.R. 1.324(a).

   **NAME OF ASSIGNEE:**

   **ADDRESS:** (City & State or Country)

   **STATE OF INCORPORATION, IF ASSIGNEE IS A CORPORATION:**

4. The following fees are enclosed:

   - Base fee
   - Advanced order
   - Assignment recording

   The following fees should be charged to deposit acc. no. 11-0345

   - Base fee
   - Advanced order
   - Assignment recording

   - Return of issue fee, if any

   Number of advanced order copies requested: 10

   (must be for 10 or more copies)

**TRANSMIT THIS FORM WITH FEE**

264
NOTIFICATION BALANCE OF ISSUE FEE DUE

010 Pages x $10 per page = $10 (paid with Base Issue Fee) = $ 90

PAYMENT DUE:

PAYMENT OF THIS BALANCE MUST BE MADE WITHIN THREE (3) MONTHS FROM THE DATE OF THIS NOTICE.

Failure to pay this balance within the time specified will result in lapse of the patent. To expedite processing, please use the attached revised PTO-1031C form to transmit the fee. Where use of a Deposit Account is being authorized, both parts C and D of this form should be transmitted.

By direction of the Commissioner.