A method of assembling large DNA fragments in a chromosome using site-specific recombinases and alternating excisionases. The method may be performed in vitro or in vivo, but larger assemblies are possible when the assembly is performed in vivo. For an in vivo assembly, the cell must be engineered to contain the desired recombinases, each in an inducible construct so that the desired recombinase can be expressed at the correct time with the correct choice of inducing agent.

8 Claims, 3 Drawing Sheets


OTHER PUBLICATIONS


* cited by examiner
Kinds of site specific recombinases

Orientation of sites and properties of recombinase can allow insertion/deletion or inversion

Fig. 1
Figure 2. Integration and Excision Scheme

Integration of Vector A

Deletion by hin/hix

Fig. 2a
Integration of Vector B

Deletion by Tn Excisionase

Fig. 2b
RECOMBINATION ASSEMBLY OF LARGE DNA FRAGMENTS

PRIOR RELATED APPLICATIONS

This patent application claims priority to U.S. Provisional Application Ser. No. 60/422,748, filed on Oct. 31, 2002, the disclosure of which is incorporated by reference in its entirety herein.

FEDERALLY SPONSORED RESEARCH STATEMENT

Not applicable.

REFERENCE TO MICROFICHE APPENDIX

Not applicable.

FIELD OF THE INVENTION

A process for recombination assembly of a series of cloned DNA fragments into a large ordered arrangement.

BACKGROUND OF THE INVENTION

With the structural analysis of DNA proceeding at a rapid pace based on the advances in DNA sequencing techniques and encouraged by potential applications of information from completely sequenced genomes of important organisms, a new chemical horizon is the synthesis of large DNA structures. This endeavor follows the trend of past chemical work on molecules of all types; first, determination of the structure, then synthesis of the structure for confirmation and producing novel structures and analogs to investigate their properties. A number of DNA analogs have been synthesized for special purposes (peptide backbone DNA, DNA with modified phosphoryl groups) and special sequence segments have been designed to interact with double helical DNA. In the case of microbial genomes, there has been discussion of preparing a minimal genome. However, general methods for constructing large precisely designed DNA segments have not been developed.

Presently, commercially available oligonucleotide synthesis can routinely produce molecules on the order of a hundred nucleotides, and through PCR amplification of known segments of a genome, defined fragments of up to 40 kilobase pairs can be prepared. Through cleavage with specific restriction enzymes and joining by ligation, designed DNA molecules (e.g., large vectors) have been made. However, this method becomes complicated as larger fragments with more restriction sites are used and each molecule to be made must have its unique route of synthesis depending on its particular arrangement of restriction endonuclease sites. The protection of certain sites by specific methylation and the recent discovery of several very rare cleaving endonucleases have extended the range of manipulations available from this basic approach.

Cloning techniques have been used to isolate and propagate large fragments on special vectors (BAC, YACs) and homologous DNA recombination has allowed the construction of known chromosomal regions of over a hundred kilobase pairs. Improved systems for direct recombination have made functional studies of genes easier through "gene knockout technology" [4]. However, the defined assembly of a novel DNA sequence of large size has not been carried out. In order to produce large designed segments that are composed of DNA sections not normally together (or not even from the same organism), new methodologies need to be developed.

Potential uses for these techniques are in areas such as the analysis of function of large interrupted coding regions that exist in many human genes, and in the construction of gene sets involved in complex metabolic processes [2]. These techniques could allow for more extensive genetic reprogramming of microbes for optimal production processes (metabolic engineering) and proposals for large scale "editing" of known genomes have been made based on engineering optimization considerations [3]. Methods for the generation of such DNA would allow the formation of optimized strains for industry and provides a way to explore global structural effects in the function of microbial genomes.

SUMMARY OF THE INVENTION

A general synthetic approach for the formation of designed unique DNA molecules of a size of hundreds of kilobase pairs has been developed. In general, the technique uses site specific recombinases to insert a vector containing a fragment of interest into a specific location on DNA in the cell. The chromosome or plasmid has been manipulated to contain a single recombinase site and a single excision site. These sites can be incorporated in to the cell DNA by any means known in the art, for example homologous recombination at the lac operon with the appropriate vectors.

Next, the unnecessary vector sequences are excised by a first excisionase, thus bringing the fragment of interest adjacent to a prior inserted fragment and leaving the initial site specific recombinase site intact. This is possible because the vector also contains a site specific excision site (e.g. a recombinase site in the reverse orientation to that found in the recipient DNA).

This process is repeated with a second vector that contains the same site specific recombinase site and a single excision site from a second excision enzyme. The first vector also included the second excision site in the opposite orientation. Thus, incorporation of the second vector into the chromosome now allows the intervening vector sequences to again be removed with the second excisionase.

Using alternating excisionases, an unlimited number of fragments can be aligned adjacent in the chromosome, BAC or YAC DNA. We have exemplified the method using alternating excisionases for simplicity, but of course, three or more excisionases may be used and this is explicitly stated to be an equivalent of using two excisionases. When fragment assembly is complete, a final excisionase can be used to excise and circularize the assembled fragment provided the final excisionase sites were appropriately placed in the chromosome (or DNA element in the cell) and the final vector.

The cre-lox system is the most commonly used site specific recombination system, but the art teaches a very large variety of site specific recombinases that are too numerous to name. Many of these recombinases can be employed as an "excisionase" in the context of this invention, merely by placing the sites in the opposite orientation (See FIG. 1). Suitable site specific recombinases include FRT, hix/hin, FlpR, xerD, shuffle, SSV1 integrase, and members of the Tn3 family, including the IS6 family of recombinases. Features of several site specific recombinases are mentioned below.

Several site specific recombinases have been used for excision of fragments from chromosomal DNA in E. coli or
other organisms. Applications have included the removal of antibiotic resistance elements after a genetic manipulation. The lambda int/xis system has been used in E. coli [5] and mammalian cells [6]. The FRT/FLP system from yeast has been used in E. coli [7] [2], Vibrio [8], and has shown capability for deletion of large segments of 100 kb in E. coli [9]. A genetically improved FLP recombinase has also been utilized [10]. A similar size deletion was also made in E. coli with the cre/lox system [11] and in Pseudomonas by the site-specific resolution system of Tn1722 [12].

The Cre/lox system is one of the most widely used systems for deletions in plant [13], yeast [14, 15], E. coli [16], and mammalian systems [17]. Another group of site specific recombinases capable of precise deletion, or excision of the transposon are those derived from the conjugative transposons. The deletion of the transposon from insertion sites placed in E. coli has been studied with the transposons Tn916 and Tn1545. Analysis of the lambda int-like enzymes [18, 19], sites [20] and in vitro mechanism [21-23] of Tn 916 or Tn1545 [24, 25] has shown how these can be used to insert and excise DNA contained within the ends of the transposon.

Recombination of a plasmid bearing a site for action of a recombinase into chromosome of a host cell has been used for manipulation in E. coli via the lambda system [6, 26] the Fpl FRT system [27], or the cre/lox system [28]. The crelox system has been used to bring large fragments (230 kb) into plant chromosomes [29]. Site specific recombinases have been used to manipulate the chromosomess of Drosophila (Fpl-frt system) [30] and a concerted effort has shown the application of a number of systems in mammalian cells; lambda int system [31], phage phiC31 integrase [32], cre/lox [33, 34], [35, 36], streptococcal plasmid beta recombinase [37], and phage R4 integrase [38].

Another group of recombinases capable of site specific deletion reactions include some of those usually involved in inversion of segments of the genome (invertase family) or resolution of dimeric structures after replication or concerted formation (resolvase family). In the Hin/hix inversion system [39] [40], inversion is preferred over deletion [41]. These recombinases are stimulated by binding of Fis at a nearby site. Mutants of Hin which can catalyze deletions at high frequency have been described [42]. These mutations are in the E-helix as with Gin and other related recombinases that have lost enhancement or specificity functions [43].

In the case of Tn5 resolvase [44, 45], a mutant resolvase, with a D102Y mutation [46] can act on inverted res sites as well as acting on a pair of sites one of which has a full res site and the other which contains only binding site I, and not binding site II or III of the full res site.

Recently the beta recombinase of pSM19035 has been studied and the minimal recognized recombination site has been defined [47], a 90 bp site with two binding site elements (site I and II) with recombination taking place in site I. [48]. Resolution also occurs on inverted sites in supercoiled plasmids [49], so an excision event can also be catalyzed.

We have exemplified the system using FRT as the site specific recombinase, hin/hix as the first excisionase, TnE (L/R)/Tn excisionase as the second excisionase, and cre/lox as the final excisionase. The method is illustrated in FIGS. 2A and 2B.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1. Kinds of Site Specific Recombinases

FIGS. 2A and 2B. Integration and Excision Scheme.
The following vectors were employed in the test reactions:

<table>
<thead>
<tr>
<th>Vector</th>
<th>Induction System</th>
<th>Citation</th>
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All references cited herein are incorporated by reference in their entirety for all purposes.

What is claimed is:

1. A method for the assembly of large DNA fragments, comprising:
   a) manipulating a replicon to comprise in order a first excision site, a first fragment, a first excision site and a first recombinase site;
   b) manipulating a first vector to comprise in order the first excisionase site from step a), undesired vector sequences, the first excision site from step a), a second fragment, and a second excision site;
   c) inserting the first vector into the replicon using a first recombinase so that the two first site specific excision sites are oriented in an appropriate orientation for excision with undesired vector sequences therebetween;
   d) treating the replicon with a first excisionase to completely remove the undesired vector sequences including both first excisionase sites and bring the second fragment directly adjacent to the first fragment;
   e) manipulating a second vector to comprise in order the first excisionase site from step a), undesired vector sequences, the second excision site from step b), a third fragment, and the first excision site from step a);
   f) inserting the second vector into the replicon using the first excisionase so that the two second site specific excision sites are oriented in an appropriate orientation for excision with undesired vector sequences therebetween;
   g) treating the replicon with a second excisionase to completely remove the undesired vector sequences including both second excisionase sites and bring the third fragment directly adjacent to the second fragment;
   h) repeating steps b-g using at least the first and second excisionases to make an assembled DNA, wherein the final vector also comprises the final excisionase site 5’ to all other sequences and in an appropriate orientation for excision; and
   i) excising and circularizing the assembled DNA with a final excisionase.

2. The method of claim 1, which is performed in vivo.

3. The method of claim 1, which is performed in vitro.

4. The method of claim 1, wherein the final excisionase site is lox and the final excisionase is cre, the first recombinase is FR1, the first excisionase site is hix and the first excisionase is hin, the second excisionase site is TNE (L/R) and the second excisionase is Tn excisionase.

5. The method of claim 1, wherein the assembled DNA fragment (exclusive of the original replicon) is greater than 100, 150, 200, 250, 300, 500, 1000, 1500, 2000, 3000, or 5000 kb.
6. A method for the assembly of large DNA fragments in vivo, comprising:
   a) manipulating a replicon to comprise in order a first fragment, a first excision site and a first recombinase site;
   b) manipulating a first vector to comprise in order the first recombinase site from step a), undesired vector sequences, the first excision site from step a), a second fragment, and a second excision site;
   c) inserting the first vector into the replicon in vivo using a first recombinase so that the two first site specific excision sites are oriented in an appropriate orientation for excision with undesired vector sequences therebetween;
   d) treating the replicon with a first excisionase in vivo to completely remove the undesired vector sequences including both first excisionase sites and bring the second fragment directly adjacent to the first fragment;
   e) manipulating a second vector to comprise in order the first recombinase site from step a), undesired vector sequences, the second excision site from step b), a third fragment, and the first excision site from step a);
   f) inserting the second vector into the replicon in vivo using the first recombinase so that the two second site specific excision sites are oriented in an appropriate orientation for excision with undesired vector sequences therebetween;
   g) treating the replicon with a second excisionase in vivo to completely remove the undesired vector sequences including both second excisionase sites and bring the third fragment directly adjacent to the second fragment; and
   h) optionally repeating steps b-g using at least the first and second excisionases in vivo to make an assembled DNA.

7. The method of claim 6, wherein the first recombinase is FRT, the first excisionase site is hix and the first excisionase is hin, the second excisionase site is TNE (L/R) and the second excisionase is Tn excisionase.

8. The method of claim 6, wherein the assembled DNA fragment (exclusive of the original replicon) is greater than 100, 150, 200, 250, 500, 1000, 1500, 2000, 3000, or 5000 kb.