Manipulation of Adenovirus Early Region 1 Rescue Plasmids by Homologous Recombination in *Saccharomyces cerevisiae*.

Peter Anderson

Department of Biological Sciences

Brock University

St. Catharines, Ontario

September 2003

(Submitted in partial fulfilment of the requirements for the degree of Master of Science)

© Peter Anderson, 2003
Give us the tools, and we will finish the job.  Winston Churchill, 1941.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of Contents</td>
<td>3</td>
</tr>
<tr>
<td>List of Tables</td>
<td>7</td>
</tr>
<tr>
<td>List of Figures</td>
<td>7-11</td>
</tr>
<tr>
<td>Abstract</td>
<td>12-13</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>14</td>
</tr>
<tr>
<td>1. Introduction and Literature Review</td>
<td>15-45</td>
</tr>
<tr>
<td>1.A.1 History and Classification of the Family Adenoviridae</td>
<td>15</td>
</tr>
<tr>
<td>1.A.2 Virion Structure</td>
<td>17</td>
</tr>
<tr>
<td>1.A.3 Genome Organization</td>
<td>19</td>
</tr>
<tr>
<td>1.A.4 Adenovirus Infection and Replication Cycle</td>
<td>22</td>
</tr>
<tr>
<td>1.A.4.1 Attachment and Entry</td>
<td>24</td>
</tr>
<tr>
<td>1.A.4.2 Disruption of Host Cell Cycle Control and the Adenovirus Early Region 1</td>
<td>26</td>
</tr>
<tr>
<td>1.A.4.3 Viral DNA Replication and the Adenovirus Early Region 2</td>
<td>27</td>
</tr>
<tr>
<td>1.A.4.4 Abrogation of the Host Immune Response by the Adenovirus Early Region 3 and the Virus Associated RNAs</td>
<td>30</td>
</tr>
<tr>
<td>1.A.4.5 Early Region 4</td>
<td>32</td>
</tr>
<tr>
<td>1.A.4.6 Late Gene Expression, Virus Assembly and Release from the Cell</td>
<td>33</td>
</tr>
<tr>
<td>1.A.5 Virtues of the Adenovirus System</td>
<td>34</td>
</tr>
<tr>
<td>1.A.6 Recombinant Human Adenovirus Deletion/Substitution Virus</td>
<td>34</td>
</tr>
<tr>
<td>Rescue Systems</td>
<td></td>
</tr>
</tbody>
</table>
1.B. *S. cerevisiae In Vivo* Ligation Systems

1.C Project Objectives

2. Materials and Methods

2.A Recombinant DNA Techniques

2.A.1 Bacterial Strains

2.A.2 Propagation and Maintenance of Bacterial Cultures

2.A.3 Preparation of Competent *E. coli* DH5α

2.A.4 Transformation of *E. coli* DH5α

2.A.5 Modified Mini-preparation of Plasmid DNA (pDNA)

2.A.6 Restriction Enzyme Digestion of DNA

2.A.7 Partial Restriction Enzyme Digestion of pDNA

2.A.8 Agarose Gel Electrophoresis

2.A.9 Large Scale pDNA Isolation

2.A.10 Determination of DNA Concentration

2.A.11 Heat Inactivation of Enzymes

2.A.12 Shrimp Alkaline Phosphatase Treatment of Restriction Enzyme Digested pDNA

2.A.13 Klenow Treatment of Digested pDNA

2.A.14 Excision of Restriction Enzyme Digested pDNA Fragments from Agarose Gels

2.A.15 Ligation of Restriction Enzyme Digested pDNA Fragments

2.A.16 Phenol:Chloroform:Isoamyl Alcohol Extraction of DNA

2.A.17 Polymerase Chain Reaction
2.A.18 Propagation and Maintenance of *S. cerevisiae* Cultures

2.A.19 Replica Plating of Yeast Cultures

2.A.20 Preparation of Electrocompetent Yeast Cells

2.A.21 Electroporation of Electrocompetent Yeast Cells

2.A.22 Mini-preparation of Yeast pDNA

2.A.23 *In Vivo* Ligation Reaction Protocol

2.B) Mammalian Cell Culture Techniques

2.B.1 Ad 5 Transformed Human Embryonic Kidney Cell (293 Cell) Maintenance

2.B.2 293 Cell Passaging

2.B.3 293 Cell Freezing and Thawing

2.B.4 Transfection of 293 cells Using ExGen 500 *In Vitro* Transfection Reagent ®.

2.B.5 Visualization of GFP Expression

3. Results

3.1 Construction of the YRpXC38 Acceptor Vector

3.2 Restriction Enzyme Confirmation of YRpXC38

3.3 *S. cerevisiae* and *E. coli* Replication Origin and Selectable Marker Functionality Analysis in YRpXC38

3.4 Construction of the LtIVL and RtIVL *In Vivo* Ligation Linkers

3.5 Analysis of LtIVL and RtIVL *In Vivo* Ligation Linkers

3.6 *S. cerevisiae* JY128 Exponential Growth Inflexion Point Determination
3.7 Initial \textit{In Vivo} Ligation Experiments

3.8 Initial \textit{In Vivo} Ligation Transformant Analysis

3.9 Secondary \textit{In Vivo} Ligation Experiments

3.10 Analysis of Second \textit{In Vivo} Ligation Transformants

3.11 Sequence Analysis of Junction Regions in YRpXC-EGFP

3.12 Green Fluorescent Protein Expression Visualization

3.13 Analysis of \textit{In Vivo} Ligation Utilizing Klenow-Treated \textit{AflII} digested YRpXC38

3.14 YRpETE Construction Strategy

3.15 \textit{URA3} PCR Fragment Verification

3.16 Restriction Enzyme Confirmation of YRpETE

3.17 \textit{URA3} Selectable Marker Functionality Analysis

3.18 Knockout \textit{In Vivo} Ligation Experiments

3.19 Knockout \textit{In Vivo} Ligation Recombinant Analysis

3.20 Knockout \textit{In Vivo} Ligation Reactions Utilizing a Partially Digested Acceptor

4. Discussion

4.1 Results Relevant to General \textit{In Vivo} Ligation Research

4.2 Future Ad Research Involving Recombinational Cloning

4.3 Future Research Questions

5. Conclusions

References
List of Tables

1.1 Classification schemes for human adenoviruses 16
3.1 Predicted sizes of restriction fragments from parent plasmids YRp7 and pXC38 and both possible YRpXC38 constructs. 67
3.2 Mean and standard deviation of trp+ background transformants generated after electroporation with 0.1 μg AflIII digested YRpXC38, Klenow-treated for different durations 89

List of Figures

1.1 Schematic illustration of an adenovirus particle 18
1.2 Nucleotide sequence of the Ad5 packaging domain 20
1.3 A schematic illustration of the adenovirus type 2 genome 21
1.4 The timing of major events occurring during the adenovirus replication cycle 23
1.5 Attachment and entry of the adenoviruses to the host cell 25
1.6 Adenovirus DNA replication 29
1.7 A) Schematic representation recombinant adenovirus generation by homologous recombination.
B) Production of contaminating replication-competent wild-type virus during recombinant virus rescue. 37
1.8 A) Two-part plasmid construction using homologous recombination. 42
B) Linker-assisted plasmid construction using homologous recombination.
1.9 Diagram of DNA fragments to be utilized in the assessment of in vivo ligation utility. 45
3.1 YRpXC38 Construction Strategy

3.2 1% agarose gel showing restriction fragment banding patterns of pXC38, YRp7, and YRpXC38

3.3 A) Locations of EcoRI and NcoI sites in YRpXC38

B) 1% agarose gel showing EcoRI and NcoI restriction fragment banding patterns of YRpXC38 and pDNA isolated from ampicillin-resistant bacterial colonies generated via total plate back transformation from trp⁺ yeast transformants

3.4 A) Relative positions of linearizing restriction sites used during in vivo ligation experiments

B) PCR amplification scheme for in vivo ligation linker generation.

C) Sequences of primers used in linker amplification

3.5 2% agarose gel showing ethanol-precipitated resuspension of in vivo ligation linkers LtIVL and RtIVL

3.6 Mean and standard deviation summary of control and in vivo ligation reactions using HpaI digested YRpXC38 acceptor

3.7 A) Locations of restriction sites and predicted deletions within adenovirus sequences in YRpXC38 generated during initial in vivo ligation reactions.

B) Restriction fragment banding patterns of 1.1 kb and 2.6-2.7 kb deletion recombinants generated during in vivo ligation trials utilizing HpaI digested YRpXC38 acceptor and YRpXC38 control plasmid

3.8 Current model of the S. cerevisiae ds DNA break repair pathway
3.9 Mean and standard deviation summary of control and \textit{in vivo} ligation reactions using \textit{AfIII} digested YRpXC38 acceptor

3.10 A) 1% agarose gel showing restriction fragment banding patterns of \textit{in vivo} ligation generated recombinant and parental YRpXC38

B) Predicted \textit{AfIII}, \textit{EcoRI}, \textit{SacII}, \textit{BamHI}, and \textit{HpaI} digestion products of YRpXC38 and the predicted YRpXC-EGFP.

3.11 Expected sequences of left-end and right-end regions undergoing linker-assisted homologous recombination

3.12 Green Fluorescent Protein fluorescence in transfected 293 cell monolayers visualized by exposure to 488 nm wavelength light.

3.13 Mean and standard deviation summary of control and \textit{in vivo} ligation reactions using Klenow-treated \textit{AfIII} digested YRpXC38

3.14 1% agarose gel showing \textit{NcoI} digested \textit{in vivo} ligation recombinants generated using \textit{AfIII} digested Klenow-treated YRpXC38

3.15 YRpETE construction strategy

3.16 A) 1% agarose gel showing ethanol-precipitated resuspensions of the \textit{URA3} PCR fragment amplified from samples lacking or containing pYES2.

B) Primer sequences utilized to amplify the \textit{URA3} gene from pYES2

3.17 1% agarose gel showing restriction fragment banding patterns of pXC38 and YRpETE
3.18 A) Illustration of NcoI sites within YrpETE

B) 1% agarose gel showing restriction fragment banding patterns of YRpETE and pDNA isolated from ampicillin-resistant bacterial colonies generated via total plate back transformation from trp'/ura' yeast transformants

3.19 Mean and standard deviation summary of in vivo ligation reactions using AflII digested YRPETE acceptor

3.20 1% agarose gel showing EcoRI digested plasmid DNA of YRPXC-EGFP control, knockout in vivo ligation generated recombinant plasmid isolated by replica plating from trp' SC medium to trp'/ura' SC medium, and knockout in vivo ligation generated recombinant plasmids isolated using trp and 5-FOA selection

3.21 A) 1% agarose gel showing partial digestion series of YRPETE with SacII

B) Illustration of SacII restriction site locations in YRPETE

3.22 Mean and standard deviation summary of knockout in vivo ligation reactions using single cut SacII digested YRPETE acceptor

3.23 Mean and standard deviation summary of knockout in vivo ligation reactions using single cut SacII digested YRPXC38 acceptor

4.1 Promoters occurring in the left-end of the Ad 5 genome

4.2 General strategy for the creation of recombinational cloning vectors by two-fragment recombinational cloning

4.3 Construction strategy for generating matched genomic vectors for novel helper-cell lines
4.4 Insertion/deletion recombinational cloning strategy utilizing PCR generated-insert/homology containing linker DNA

4.5 Pathway of replication fork stalling/collapse with subsequent resetting/repair
Abstract

The manipulation of large (>10 kb) plasmid systems amplifies problems common to traditional cloning strategies. Unique or rare restriction enzyme recognition sequences are uncommon and very rarely located in opportunistic locations. Making site-specific deletions and insertions in larger plasmids consequently leads to multiple step cloning strategies that are often limited by time-consuming, low efficiency linker insertions or blunt-end cloning strategies. Manipulation of the adenovirus genome and the genomes of other viruses as bacterial plasmids are systems that typify such situations.

Recombinational cloning techniques based on homologous recombination in Saccharomyces cerevisiae that circumvent many of these common problems have been developed. However, these techniques are rarely realistic options for such large plasmid systems due to the above mentioned difficulties associated with the addition of required yeast DNA replication, partitioning and selectable marker sequences.

To determine if recombinational cloning techniques could be modified to simplify the manipulation of such a large plasmid system, a recombinational cloning system for the creation of human adenovirus E1-deletion rescue plasmids was developed.

Here we report for the first time that the 1,456 bp TRP1/ARS fragment of YRp7 is alone sufficient to foster successful recombinational cloning without additional partitioning sequences, using only slight modifications of existing protocols. In addition, we describe conditions for efficient recombinational cloning involving simultaneous deletion of large segments of DNA (>4.2 kb) and insertion of donor fragment DNA using only a single non-unique restriction site.

The discovery that recombinational cloning can foster large deletions has been used to
develop a novel recombinational cloning technique, selectable marker 'knockout' recombinational cloning, that uses deletion of a yeast selectable marker coupled with simultaneous negative and positive selection to reduce background transformants to undetectable levels.

The modification of existing protocols as described in this report facilitates the use of recombinational cloning strategies that are otherwise difficult or impractical for use with large plasmid systems. Improvement of general recombinational cloning strategies and strategies specific to the manipulation of the adenovirus genome are considered in light of data presented herein.
Acknowledgements

I would like to thank Dr. Y. Haj-Ahmad for everything that he has done for me over the last three years. I would also like to thank Dr. A. Castle, Dr. D. Inglis, and Dr. D. Bautista who always offered their assistance. Thank you to Dr. M. Bidochka for the use of his laboratory. My appreciation goes out to my lab-mates Pam Roberts and Ken Lo, and to the staff of Norgen Biotek Corp., especially Tom and Carol. Finally, thank you to Karen and my family for all your support.

My MSc education was co-sponsored by the National Science and Engineering Research Council of Canada and Norgen Biotek Corporation. I am grateful for their financial support.

All bacterial and yeast strains, plasmid DNAs, mammalian cells and equipment utilized during the course of this research were obtained from the laboratory of Dr. Y. Haj-Ahmad, the laboratory of Dr. M. Bidochka, and the Cool Climate Oenology and Viticulture Institute, Brock University, St. Catharines, Ontario.
1. Introduction and Literature Review

1.A.1 History and Classification of the Family Adenoviridae

Adenoviruses are non-enveloped, linear double-stranded DNA viruses encapsidated in an icosahedron protein shell measuring from 70 to 100 nm in diameter. These viruses cause mild self-limiting infections of the upper respiratory tract, epidemic conjunctivitis, and have been associated with a variety of additional clinical conditions—perhaps most notably, infantile gastroenteritis. The discovery of the adenoviruses dates back to 1953, when Rowe and colleagues observed spontaneous degeneration of primary human adenoid cell cultures while examining the causative agents of acute respiratory illness. Similarly, in 1954 Hilleman and Werner isolated agents responsible for cytopathic changes in human cell culture while examining an epidemic of respiratory ailments in military recruits. These agents of respiratory disease were subsequently shown to be viral in origin and were named adenoviruses after the original tissue source from which they were isolated (reviewed in Shenk, 1996).

Since the discovery of the first adenovirus, other viruses sharing similar properties have been grouped together in the family Adenoviridae. The genera in this family have been categorized based on infectivity in birds (Aviadenovirus genus), and a number of different animal species including human, simian, bovine, equine, porcine, ovine, canine, and opossum (Mastadenovirus genus). Antigenic similarities do exist between genera, however there is no known antigen common among all adenoviruses. The various human serotypes (to date, 49 human serotypes) have been distinguished on the basis of antibody cross-reactivity, hemagglutination, oncogenicity, viral protein electrophoretic mobility, and/or genomic
composition (GC content, cross-hybridization, restriction endonuclease digestion patterns). Table 1.1 summarizes one such classification scheme for the human adenoviruses.

Table 1.1: Classification schemes for human adenoviruses (reproduced from Shenk, 1996).

<table>
<thead>
<tr>
<th>Subgenus</th>
<th>Serotype</th>
<th>G+C (%)</th>
<th>Hemagglutination Pattern</th>
<th>Length of Fibers (nm)</th>
<th>Oncogenicity in Newborn Hamsters</th>
<th>Tropism Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12,18,31</td>
<td>48</td>
<td>IV</td>
<td>29-31</td>
<td>High</td>
<td>Cryptic enteric infection</td>
</tr>
<tr>
<td>B</td>
<td>3,7,11,14, 16,21,34, 35</td>
<td>51</td>
<td>I</td>
<td>9-11</td>
<td>Weak</td>
<td>Respiratory Disease, Persistent infections of the kidneys</td>
</tr>
<tr>
<td>C</td>
<td>1,2,5,6</td>
<td>58</td>
<td>II</td>
<td>12-13</td>
<td>Nil</td>
<td>Respiratory disease persists in lymphoid tissues</td>
</tr>
<tr>
<td>D</td>
<td>8,9,10,13, 15,17,19, 20,22 to 30,32,33, 36,37,38, 39,42 to 47</td>
<td>58</td>
<td>III</td>
<td>23-31</td>
<td>Nil</td>
<td>Keratoconjunctivitis</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>58</td>
<td>III</td>
<td>17</td>
<td>Nil</td>
<td>Conjunctivitis, Respiratory disease</td>
</tr>
<tr>
<td>F</td>
<td>40,41</td>
<td>52</td>
<td>IV</td>
<td>28-33</td>
<td>Nil</td>
<td>Infantile diarrhea</td>
</tr>
</tbody>
</table>

In 1962 human adenovirus research attained new levels of attention as Trentin and his colleagues demonstrated that human adenovirus type 12 was capable of inducing malignant tumours in newborn hamsters. This was the first reported instance in which a human virus was capable of being oncogenic. And though the extensive searches for adenovirus DNA in human tumours have virtually all failed to link the virus to malignant disease in humans, the use of human adenoviruses as a model system to probe the genetic regulation of oncogenesis in animal and culture systems has added substantially to our understanding of gene expression (both viral and cellular), DNA replication, cell cycle control, and cell growth regulation (reviewed in Shenk, 1996).
Undoubtedly, the single largest contribution made to modern biology by the adenovirus system was the discovery of mRNA splicing. The ability of the viral genome to give rise to a multitude of viral mRNAs generated from one larger nuclear transcript revealed the existence of introns and exons, and fostered a new appreciation of the intricacies of eukaryotic gene expression.

From its original discovery as a human pathogen, the adenoviruses may yet yield one of humankind's greatest medical achievements. The use of modified adenovirus vectors as tools for the delivery of therapeutic genes is currently under intensive exploration.

The following sections will briefly outline the virus particle and genomic organization, infectious cycle, and the other aspects of adenovirus biology relevant to the use of the human adenovirus as a gene therapy delivery vehicle. This topic has been extensively reviewed in Shenk (1996), and Fields et al. (1996).

1.A.2 Virion Structure

The virus particle is composed of protein and DNA; 87% of the viral total mass is protein, the remaining 13% being DNA. Electrophoretic analysis has revealed that the virion contains at least 11 structural proteins that are designated by Roman numerals. Seven of these structural proteins (II, III, IIIa, IV, VI, VIII, and IX) are assembled to form the viral capsid which surrounds the linear double-stranded DNA genome (Figure 1.1) (reviewed in Stewart et al., 1991).
Figure 1.1: Schematic illustration of an adenovirus particle. Virion polypeptides are indicated by Roman numerals with the exception of the terminal protein (TP) (reproduced from Stewart et al., 1991).

The viral capsid is composed of 240 hexons (trimer of polypeptide II) and 12 pentons (pentamer of polypeptide III) (Khilko et al., 1990). These penton proteins mark the vertices of the icosahedron, and from these vertices a fiber protein is projected. The fiber itself is composed of three molecules of polypeptide IV. The amino terminal of the fiber is embedded in the penton base, while the carboxy-terminus forms the terminal knob. This terminal knob region of the fiber mediates viral attachment to the host cell receptor (Van Oostrum and Burnett, 1985) and is
generally responsible of serotypic variation among related groupings of adenoviruses. The fiber/penton combination is referred to as a penton capsomere. Six whole hexon capsomeres and three penton capsomeres form one complete face of the icosahedron.

The remaining structural viral proteins stabilize the viral capsid through associations with the hexon capsomeres; polypeptide IX stabilize hexon-hexon contacts within facets, polypeptide IIIa joins hexons from adjacent facets, and polypeptides VI and III seem to play a role in connecting the core to the viral capsid (Everitt et al., 1973).

The remaining four virion proteins are located within the core. Proteins V, VII, and X are attached directly to the viral DNA, packaging and maintaining the integrity of the viral genome. The final protein associated with the virion particle is the 55 K terminal protein (TP), which is covalently attached to the 5' end of the viral DNA and plays an important role in viral DNA replication (Rekosh et al., 1977).

1.A.3 Genome Organization

The double-stranded DNA genome of the adenovirus is approximately 26 kb (Turkey Ad3) to 45 kb (Fowl AdD) in size (8.7-15 μm in length) and is divided into 100 map units by convention (human adenovirus serotype 5, Ad 5, is 35,937 bp and thus one map unit consists of 360bp) (Genbank (January 3, 2003) <url>http://www.ncbi.nlm.gov/PMGifs/Genomes/10508.html</url>. Accessed January 8, 2003).

At each termini of the viral genome lie inverted terminal repeats of 100 to 200 bp in length (serotype dependent), embedded within each are identical origins of replication. These sequences act in concert with the TP during viral DNA replication (discussed in Section 1.A.4.3). Adjacent
to the left inverted terminal repeat lies the packaging domain. These sequences have been shown to mediate viral genome encapsidation. The sequence of the packaging domain of Ad5 is located in the left 380bp (Graeble and Hearing, 1997) and is detailed in Figure 1.2.

Figure 1.2: Nucleotide sequence of the Ad5 packaging domain. Nucleotide numbers are relative to the left-end terminus. A repeats AI - AVII are circled. (Schmid and Hearing, 1997)

The packaging domain of Ad5 consists of seven functional A repeat units (termed A for their high AT content) (Schmid and Hearing, 1997).
Evolution has selected for viral genes serving similar functions to be arranged into transcriptional units; five early and one late. Organization of the adenovirus genome is detailed in Figure 1.3.

Figure 1.3: A schematic illustration of the adenovirus type 2 genome and its division into 100 map units. Early transcripts are indicated by (E), late transcripts are indicated by (L) and bold arrows. Structural polypeptides are indicated by Roman numerals, non-structural polypeptides are labeled by (K) (from Broker, 1984).
The genetic map of the adenovirus is customarily presented from left to right, the 0% position being left and the 100% position being right. The two strands are termed r-strand and l-strand (r standing for rightward transcription, l for leftward transcription) and are by convention represented with the r-strand as the upper strand, and the l-strand as the lower strand. The early transcriptional units, including early regions (E)1, 2, 3, and 4, are transcribed prior to the onset of DNA replication. The late transcription region is activated by the major late promoter (MLP), and generates five families of late mRNAs (L1 to L5) that give rise to viral structural proteins.

Transcription of E1A, E1B, pIX, and E3, and transcription from the MLP, occurs from the r-strand. E2, IVa2, and E4 are transcribed from the l-strand. Interestingly, the organization of the viral genome within the viral capsid places most of the viral transcription units into independent supercoiled domains (Figure 1.1).

1.A.4 Adenovirus Infection and Replication Cycle

The replication cycle of adenoviruses lasts between 20 and 24 hours. It is characterized by two phases: early (those events occurring prior to DNA replication), and late (those events occurring after DNA replication) phases. Figure 1.4 shows the relative timing of the major events that occur during the viral replication cycle.
Adenovirus infection commences as the infecting virus comes into contact with the host cell. The virus particle then penetrates the host cell and eventually makes its way to the nucleus. Early gene expression quickly ensues, followed by viral DNA replication. With the onset of viral DNA replication, transcription from the MLP and translation of the late proteins begins. Virus particles assembled from a precursor pool of structural virion proteins and DNA are then released as the infected cell is ruptured.
1. A. 4. 1 Attachment and Entry

Attachment of the virus to its’ host cell receptor, the coxsakievirus and adenovirus receptor (CAR) (Bergelson et al., 1997), is mediated by the knob region of the fiber protein (Devaux et al., 1987). Subsequent internalization of the virus particle is mediated by an interaction between the penton base protein and a family of heterodimeric cell surface receptors, termed integrins. The penton/αvβ3 and αvβ3 vitronectin-binding integrin (and perhaps other members of the integrin family) interaction triggers diffusion into clathrin-coated pits, followed by receptor mediated endocytosis (Wickham et al., 1993; Varga et al., 1991).

Virus particles release into the cytosol from the endosomes follows, triggered by the acidic pH of the endosome (Seth et al., 1984; Svensson et al., 1985). A series of capsid uncoating steps occurs during viral escape from the endosome. The polypeptides composing the viral capsid sequentially dissociate starting from the vertices (polypeptides IV, IIIa, III, VIII, VI, IX, and II sequentially). Transport to the nucleus is mediated by microtubule/hexon interaction (Luftig and Weiibling, 1975). The structure of the DNA-protein complex which enters the nucleus has not been elucidated, but it is known that protein V is removed prior to entry into the nucleus (Greber et al., 1993).

Viral DNA association with the nuclear matrix through the terminal protein is the final step of the infection cycle prior to the onset of early gene activation (Schaack et al., 1990). The process of adenoviral attachment and entry into the host cell is summarized in Figure 1.5.
Transcription of the early viral genes begins soon after viral DNA entry into the nucleus. The gene products of these early viral genes play critical roles in creating a cellular environment conducive to adenoviral replication.

Products of the E1 region (E1A and E1B) not only activate transcription of other early viral genes, but also antagonize cellular gene products which maintain the integrity of the cell cycle. E2 proteins mediate viral DNA replication initiation and polymerization. E3 gene products code for several proteins which antagonize the host immune response to viral infection. E4 gene products are also involved with viral DNA replication, and inhibit host cell macromolecule
biosynthesis.

Protein IX has been defined as an intermediate early gene, since it is active earlier in the infection than the late genes. Protein IX is not only a virus structural protein, but is also a transcriptional activator of several viral and cellular TATA-containing promoters. Protein IX has also been inferred in the reorganization of the nucleus which accompanies viral transcription (Rosa-Calatrava et al., 2001; and references therein).

1.A.4.2 Disruption of Host Cell Cycle Control and the Adenovirus Early Region 1

The primary actions of the E1 proteins is the induction of cell cycling in infected quiescent cells and trans-activation of viral transcription. Creating an environment conducive to viral replication begins with transcription of the first early gene, E1A. This unit produces five proteins named for the sedimentation coefficient of their mRNAs: 13S, 12S, 11S, 10S, 9S. The 13S and 12S transcripts are synthesized and translated early, while the other transcripts are transcribed late in the infection. The function of these late transcription units is still unknown (Shenk and Flint, 1991).

Transcriptional activation by the E1A proteins is accomplished primarily through competition with transcription repressor proteins (specifically the retinoblastoma susceptibility protein family of transcription repressors) for binding sites on proteins essential for transcription activation, but also stimulate transcription by binding transcription repressive DNA binding proteins and stabilizing transcription initiation complex formation. Highly conserved regions in both the E1A and E1B proteins essential for these protein-protein interactions have been
The E1B 55 K protein also functions in transporting viral mRNAs, and shutting off host cell biosynthesis (Babiss et al., 1984).

The protein-protein interactions of the early region 1 gene products not only complex with their cellular targets increasing viral gene transcription, but strongly alter cellular transcription. The early region 1 gene products induce dramatic progression of the cell cycle while preventing apoptosis, and thus foster the generation of an environment conducive to viral replication.

1.4.3 Viral DNA Replication and the Adenovirus Early Region 2

The early region 2 of human adenoviruses is comprised of two sub-regions: E2A which codes for the 72 K DNA binding protein, and E2B which codes for the 140 K DNA polymerase and the 80 K precursor to the terminal protein. Transcription of the E2 region occurs from two promoters: early and throughout infection from a promoter at 75 mu, and after DNA replication
from a promoter at 72 mu (Figure 1.3).

The 72 K DNA binding protein is required for increased efficiency of viral DNA elongation, host range determination, assembly of the viral particle, and binding of both single and double-stranded DNA (Hay and Russell, 1989).

The E2B derived DNA polymerase is responsible for both the initiation and elongation steps of viral DNA replication. In addition to forming pTP-dCMP (deoxycytidine 5'-monophosphate), the adenovirus DNA polymerase possesses a 3' to 5' exonuclease activity that may serve a proof reading function during polymerization. A cellular protein, nuclear factor II, and the viral template are the final requirements for viral DNA elongation (Hay and Russell, 1989).

The 80 K terminal protein precursor is cleaved by a virally coded 23 K protease to produce the 55 K terminal protein, though this conversion is not essential for viral DNA replication. In addition to it’s vital role in viral DNA replication, the terminal protein plays a vital role in early adenovirus infection, anchoring the viral DNA to the nuclear matrix, and mediating initiation and enhancing viral transcription (Hay and Russell, 1989).

Accumulation of a threshold level of these E2 gene products and host cell entry into S phase allows viral DNA replication to begin. Adenovirus DNA replication is a two stage process: the first being characterized by protein-primed strand displacement occurring once from either end of the viral chromosome at the ITRs. During the second stage a complement to the displaced strand is synthesized through self-circularization and consequent self-priming of the displaced strand via the ITRs. The formation of the distinctive panhandle structures allows the template to be recognised by the same replication machinery as double stranded viral DNA (Figure 1.6).
The 5' bound terminal protein (or 80 K pre-terminal protein) serves as a primer for DNA synthesis initiation and preserves the integrity of the viral chromosome terminus throughout successive rounds of DNA replication. The preterminal protein binds the template and is then complexed with the $\alpha$-phosphoryl group of dCMP at the $\beta$-OH of a serine residue in the preterminal protein by the E2-encoded polymerase. Following this coupling reaction, the preterminal protein-dCMP complex serves to prime the template DNA strand for replication (Lichy et al., 1981).

Figure 1.6: Adenovirus DNA replication (All the Virology on the WWW (February 16, 1999). [url]http://www.tulane.edu/~dmsander/WWW/335/Adenoviruses.html[/url]. Accessed September 10, 2001.).
The process of adenovirus DNA replication continues until the host cell is finally ruptured or dies (Figure 1.4).

1.A.4.4 Abrogation of the Host Immune Response by the Adenovirus Early Region 3 and the Virus Associated RNAs

Host immune response to adenovirus infection proceeds in two stages. The first stage is characterized by damage to lung tissue, the appearance of the cytokines tumor necrosis factor (TNF) and interleukins 1 and 6, and a diffuse cellular infiltration of peribronchiolar and alveolar regions. The later stage consists almost entirely of an infiltration of virus-specific cytotoxic T lymphocytes (CTL). The adenovirus has evolved several countermeasures to subvert immune system-mediated destruction of infected cells. The early region 3 gene products and the virus-associated RNAs (VA RNAs) and other early region proteins mediate these countermeasures (Ginsberg et al., 1991).

The E3 region codes for nine proteins, five of which (19 K, 14.7 K, 14.5 K, 10.4 K, and 6.7 K) are involved in protecting adenovirus infected cells from immune detection and destruction.

The 19 K E3 gene product abrogates CTL-mediated virus infected cell destruction by interfering with the type I major histocompatibility complex class I (MHC class I) antigen presentation pathway. Through interaction with the MHC class I peptide binding domain, the E3-19 K protein/MHC complex is retained in the endoplasmic reticulum. The subsequently reduced
level of antigen presentation allows infected cells to evade immune surveillance (Burgét and Kvist, 1987).

Both the E3-14.7Kd protein and the complex formed between the E3-14.5 K and E3-10.4 K proteins can prevent cytolysis by tumor necrosis factor α (TNF-α). TNF-α is a cytokine that can induce lysis of virus infected cells and is secreted by activated macrophages and lymphocytes. This E3-protein complex and the E3-6.7 K protein are required for the down-regulation of TNF-related apoptosis-inducing ligand receptors. The E3-6.7 K protein has also been shown to confer protective effects against calcium flux-induced apoptosis by operating to maintain endoplasmic reticulum calcium homeostasis (Moise et al., 2002; and references therein).

The VA RNAs work against the host immune response by providing protection against the effects of interferon. Interferon inhibits viral replication by inducing protein kinase R (PKR)-mediated inhibition of protein synthesis in infected cells. Homologous RNA sequences produced by symmetrical transcription of the viral genome can produce double stranded RNA which is subsequently bound by two molecules of protein kinase R (PKR). Autophosphorylation of PKR is required for its activity and requires two molecules of the enzyme. Phosphorylated PKR phosphorylates a key subunit required for translation initiation, eukaryotic initiation factor 2 (eIF-2), thereby inactivating it. The GC-rich, short (approximately 160 bp) VA RNA species are produced in increasing amounts as the infectious cycle progresses, but are present during the early stages of infection. The VA RNAs form short hairpin structures which allow only one PKR molecule to bind, thus inhibiting PKR autophosphorylation, and therefore decreasing both PKR-mediated eIF-2 phosphorylation, and subsequent translation inhibition (Kitajewski et al., 1986).
The E1 gene products also play a role in antagonizing the host cell immune response. E1A gene products inhibit the antiviral effects of interferon by blocking the activation of interferon response genes (Reich et al., 1988). By virtue of its anti-apoptotic effects, the E1B-19Kd gene product also combats host cell immune response by inhibiting TNFα-induced apoptosis (discussed in section 1A3.2).

1.A.4.5 Early Region 4

Recent advances in the understanding of E4 gene products at the molecular level have revealed that these proteins possess an unexpectedly diverse collection of functions which orchestrate many viral processes (Tauber and Dobner, 2001; and references therein). E4 proteins play a role in increasing viral transcription, viral DNA replication, shutting off cellular macromolecular synthesis, and contributing to the oncogenic process.

The E4-17 K protein activates the E2F transcription factor by a cooperative binding reaction. Two molecules of the 17 K protein bind cooperatively bridging the gap between a pair of E2F binding sites within the E2 promoter, thus activating E2 transcription and allowing the viral DNA replication machinery to be assembled (Raychudhuri et al., 1990).

Once DNA replication starts and enough late viral mRNA has accumulated, the transport of cellular mRNAs to the cytoplasm is blocked by an E4-34 K/E1-55 K protein complex. It is theorized that this complex localizes some nuclear factor vital to mRNA transport out of the nucleus to sites within the nucleus where viral mRNA replication is concentrated (Omelles and Shenk, 1991).
1.A.4.6 Late Gene Expression, Virus Assembly and Release from the Cell.

Adenovirus late gene expression begins with the onset of DNA replication (Figure 1.4). The primary transcript of the late coding regions is a single unit approximately 29,000 nucleotides in length. This transcript is subsequently processed through mRNA splicing to produce at least 18 distinct mRNA species. These late mRNAs have been grouped into five families, L1 to L5, based on common polyadenylation sites (Figure 1.3). All late viral mRNAs possess a 200-nucleotide “tripartite leader sequence” which is lacking in secondary structure. It has been postulated that this lack of secondary structure allows efficient translation of viral mRNAs without the need for cellular helicase (which is inactivated late in viral infection) in order to scan through the mRNA secondary structure for translation initiation sites (Huang and Schneider, 1991).

Once translation of the late viral mRNAs has taken place, trimeric hexon capsomeres are rapidly assembled with the aid of the L4-100 K protein acting as a scaffold protein (Liebowitz and Horwitz, 1975). Penton capsomeres are assembled from preformed trimeric fiber and pentameric penton base at a slower rate than the heron capsomere (Horwitz et al., 1969). After their production, the two capsomeres accumulate in the nucleus where viral assembly occurs.

Virus assembly begins with the formation of empty capsids, followed by a left-end polarized packaging of the viral DNA into the empty capsid molecule mediated by the packaging sequence and some collection of as of yet unidentified proteins. The generation of infectious virus requires that the L3-encoded viral protease process proteins VI, VII, and VIII, whose cleavages stabilize the viral particle (reviewed in Philipson, 1984).
E1B-19Kd polypeptide and the viral protease mediate disruption of the cytoskeletal system and consequent rupturing of the host cell and final release of the viral progeny (White and Cipriani, 1989; Chen et al., 1993).

1.A.5 Virtues of the Adenovirus System

The accumulation of knowledge and understanding of the replication and lytic cycle of the adenoviruses has given way to the ability to utilize the adenovirus as a general purpose mammalian expression vector, and potentially as vectors for gene therapy and vaccination. In addition to the well understood genetics of the system, the adenovirus possesses a number of amenities which make it an attractive vehicle for gene delivery. The viral particle is relatively stable, as is the genome which does not readily undergo rearrangement. Infected cells produce 1,000-10,000 infectious units per cell, and the virus remains in the cell long after maximum production allowing for easy concentration of stocks (reviewed in Graham and Provoke, 1995). And finally, several systems for manipulating the genome of the adenovirus have been developed. The following section reviews the current methods available for recombinant adenovirus rescue.

1.A.6 Recombinant Human Adenovirus Deletion/Substitution Virus Rescue Systems

The development of recombinant adenovirus rescue systems has followed a logical and systematic path, changing and improving as discoveries in DNA manipulation technology
applicable to the creation of adenovirus vectors have been made. The first recombinant adenovirus vectors followed the development of techniques to manipulate DNA in vitro. Recombinant adenoviruses were originally generated by direct in vitro ligation of insert with viral DNA using traditional cloning methods (Ballay et al., 1985; Giraldi et al., 1990) and understandably suffered from problems associated with manipulating a large linear genome. Low virus yield and the inevitable presence of background wild type virus generated by incomplete viral DNA digestion were among such problems.

Eliminating the difficulty of dealing with a linear genome was addressed by the development of large bacterial plasmids carrying a modified Ad genome (Ghosh-Choudhury et al., 1986) which could be manipulated with traditional cloning techniques. While the use of these large bacterial plasmids made manipulation easier, the lack of unique restriction enzyme recognition sites limited the utility of these systems. The discovery that shuttle vectors carrying only a small portion of the virus genome could be easily manipulated and then fused with the viral genome by in vivo homologous recombination was utilized to overcome this hurdle. The smaller size of the shuttle vectors increased the number of unique restriction sites available and made the production of novel unique sites routine, thus allowing for the creation of larger deletions and novel recombinants. Recombination between overlapping sequences in shuttle plasmids (Ghosh-Choudhury et al., 1986) or between shuttle plasmid and digested viral DNA (Haj-Ahmad and Graham, 1986) during transfection into HEK293 helper-cells (Graham et al., 1977) generates a functional viral genome. This cell line constitutively expresses the Ad5 E1 genes, and thus can complement E1 deletions in recombinant adenoviruses by supplying the E1 gene products in trans. The co-transfection method has been utilized to generated hundreds of recombinant Ad
vectors, and protocols and techniques for the isolation of E1 and/or E3-deletion Ad vectors have been extensively reviewed in Graham and Prevec (1995).

A substantial time requirement of several months was generally required to produce pure recombinant virus when utilizing these methods due to the typically laborious in vitro construction strategies required to insert the exogenous DNA within the shuttle plasmid and the numerous plaque isolations required to purify the recombinant viral particle.

Even with numerous plaque purifications, generation of replication competent Ad (RCA) sub-populations can occur via homologous recombination between homologous virus genome and helper-cell genomic sequences (Lochmüller et al., 1994; Hehir et al., 1996). Figure 1.7 illustrates how recombinant adenovirus vectors are generated and how wild-type virus production can occur during a typical rescue experiment.
Figure 1.7: A) Schematic representation of recombinant adenovirus generation by homologous recombination. B) Production of contaminating replication-competent wild-type virus during recombinant virus rescue.

An important limitation that emerged during early *in vivo* experiments utilizing recombinant Ad vectors for transgene delivery was the loss of transgene expression occurring concurrently with the development of pathology in the organ expressing the transgene. This finding was subsequently linked to a virus-specific immune response resulting primarily from the presence of contaminating RCA in experimental preparations (Yang *et al.*, 1994; Hermens and
Given the damaging effects of RCA contamination and the virtues of the recombinant human Ad vectors for the study of basic Ad biology and their potential as gene therapy delivery systems, research efforts for several years have understandably been directed almost exclusively towards the elimination of contaminating wild-type virus in recombinant Ad vector preparations.

The first such effort involved modification of the two plasmid in vivo recombination Ad production system by making use of the packaging constraint imposed upon the viral genome by the finite size of the viral capsid (Ghosh Choudhury et al., 1987) and utilized a large genomic plasmid exceeding the viral packaging constraint (McGregory et al., 1988). Wild-type virus contamination has since been limited or eliminated using a number of novel in vitro systems (Davis et al., 1998; Mizuguchi and Kay, 1998; Anderson et al., 2000), cosmid (Miyake et al., 1996; Hisanori et al., 1998), E. coli (Chartier et al., 1996; Crouzet et al., 1997; He et al., 1998; Souza and Armentano, 1999; Youil et al., 2001) and S. cerevisiae (Ketner et al., 1994) recombinational targeting systems, chimeric Ad2/Ad5 constructs (Hehir et al., 1996), and by in vitro (Aoki et al., 1999) and in vivo (Hardy et al., 1997; Ng et al., 1999; Ng et al., 2000; ) site-specific recombinase systems. Novel E1-complementing cell lines have also been generated (Fallaux et al., 1998; Ilmer et al., 1996; Kim et al., 2001) which decrease or eliminate the degree of homology which exists between virus and cell-derived left end Ad sequences.

The need for efficient wild-type free recombinant Ad rescue has certainly been addressed. However, improvement upon the traditional method of in vitro DNA sub-cloning insertion of foreign DNA into rescue vectors (which are still required in all of the above mentioned systems) has been addressed solely by substitution for other in vitro manipulation techniques such as the
introduction of unique restriction sites (Hisanori et al., 1998; Mizuguchi et al., 1998), blunt-end ligation techniques (Miyake et al., 1996), intron-encoded nucleases (Souza and Armentano, 1999), or multiple cloning sites (Bett et al., 1994; He et al., 1998; Ng et al., 2000).

Traditional DNA sub-cloning by relying on compatible restriction enzyme sites is still a stumbling block that can lead to labourious linker insertion strategies, or subject the researcher to lengthy screening processes associated with blunt-end cloning. And the fixed locations of restriction sites within the plasmids utilized to create the above mentioned systems have dictated the position(s) of genome manipulation. Furthermore, non-essential sequences essential for the utility of the above mentioned sequences (such as site-specific recombinase recognition sequences, or residual multiple cloning site sequences) remain embedded in the rescued viral genome. The development of viral gene therapy vectors for human use calls for extremely precise deletions and additions to the viral genome to maximize efficiency and treatment efficacy. In vivo ligation strategies are available that are capable of circumventing the limitations of current DNA manipulation technology.

1.B  *S. cerevisiae In Vivo* Ligation Systems

The ability to subclone, manipulate, and transfer DNA fragments from one plasmid to another is a central activity in molecular biology. Often the availability of unique, rare, or even overabundant restriction sites within final and intermediate plasmid constructs can dictate the entire direction of experimental design. Such situations therefore lead to the undertaking of
cumbersome, multi-step \textit{in vitro} cloning experiments to remove, or add, the necessary restriction enzyme recognition sequences in order to successfully obtain desirable plasmid constructs. The double strand (ds) break repair system of \textit{S. cerevisiae} (recently reviewed in Sung \textit{et al.}, 1999; reviewed in Szostak \textit{et al.}, 1983) offers many unique opportunities for \textit{in vivo} ligation experiments which have the potential to circumvent many of these common problems in plasmid construction and manipulation.

\textit{In vivo} ligation systems in the yeast \textit{S. cerevisiae} have the same requirements as most other plasmid DNA systems in yeast. A selectable marker is required to force transformed yeast cells to maintain plasmid DNA once they become transformed. Typically the selection agent employed is some form of auxotrophy. Of equal importance to the selectable marker is the corresponding recessive and non-reverting chromosomal mutation that causes the auxotrophy. Sequences for efficient replication and mitotic segregation (2 \textmu m circle origin of replication and partitioning sequences, or chromosomal replication signal sequence, ARS, and centromere, CEN) are the other requirement of a yeast plasmid system. Usually, the yeast plasmid systems are coupled to bacterial sequences which allow efficient shuttling between yeast and bacteria (ie/ bacterial origin of replication and selectable marker). In the yeast such DNA is maintained in a circular topology, and linearized plasmid DNA lacking telomere sequences will fail to replicate and partition effectively. When linear fragments which possess overlapping homologous sequences are co-transformed into the yeast, they can undergo homologous mitotic recombination through the dominant double strand break repair pathway regenerating the circular topology of the plasmid (Mezard and Nicolas, 1994).

These principles were utilized in gene disruption using PCR products in \textit{S. cerevisiae}
(Lorenz et al., '995; Manivasakam et al., 1995) prior to the advent of current in vivo ligation protocols. Two forms of in vivo ligation strategies have been developed utilizing similar methodologies.

In both two-part plasmid construction (Oldenburg et al., 1997) and linker-mediated plasmid repair by homologous recombination (Raymond et al., 1999) an acceptor fragment (containing the necessary yeast and bacterial replication origins and selectable markers) is linearized using restriction endonucleases to provide broken ended DNA as substrate for homologous recombination. The fragment to be inserted into the acceptor backbone is termed the donor. Regions of homology between the acceptor and donor are introduced via 5' overhang regions in oligonucleotide primers utilized to PCR amplify the donor fragment (in the case of two-part plasmid construction by homologous recombination, Figure 1.8A), or by PCR amplified linker regions which possess homology to both the acceptor and donor fragments (linker-assisted homologous recombination, Figure 1.8B). Acceptor and donor, or acceptor and donor and linkers are transformed into the yeast and successful in vivo ligation manifests itself as a positive yeast transformant. Isolation of the plasmid from the transformed yeast cells and back-transformation into E. coli effectively filters out yeast transformants resulting from any integration events which might have occurred during the transformation process.
Figure 1.8: A) Two-part plasmid construction using homologous recombination. B) Linker-assisted plasmid construction using homologous recombination.

Minimum lengths of sequence homology required for successful \textit{in vivo} cloning have been defined (Hua \textit{et al.}, 1997). Also, the use of partially digested acceptor (Miletti and Leibowitz, 2000) coupled with selectable marker shuffling (Miletti and Leibowitz, 2000; Gunyuzlu \textit{et al.}, 2001), and PCR independent recombinational cloning strategies using single stranded DNA oligonucleotides in place of PCR generated linkers (DeMarini \textit{et al.}, 2001) have vastly expanded the number of \textit{in vivo} ligation protocol options available to the researcher.

\textit{In vivo} ligation techniques have been successfully adapted to simplify large plasmid manipulation (Miletti and Leibowitz, 2000). Adaptation of these techniques to adenovirus research could subvert many problems common to the various aspects of this field.
1.C Project Objectives

The manipulation of large plasmid (>10 kb) systems amplifies problems common to traditional cloning strategies. Unique or rare restriction enzyme recognition sequences are uncommon and very rarely occur in opportunistic locations. Making site-specific deletions and insertions in larger plasmids consequently leads to multiple step cloning procedures that are often limited by time consuming linker insertions or low efficiency blunt-end cloning strategies. Manipulation of the adenovirus genome and the genomes of other viruses as bacterial plasmids are systems that typify such situations.

In order to determine if recombinational cloning techniques would be capable of circumventing the difficulties common to manipulation of the larger plasmids associated with adenovirus research, we chose the human adenovirus E1-deletion rescue plasmid system, used for the production of E1-deletion recombinant adenovirus vectors, as a model system.

Bett et al. (1994) produced a generalized rescue vector containing a multiple cloning site in place of nucleotides 359 through 3522 of the adenovirus type 5 genome. Due to the large deletion generated, this plasmid became the progenitor of virtually all E1-deletion rescue systems to follow. The creation of this vector required complex partial and double digestion of multiple pDNAs with multiple enzymes and complex linker insertion strategies. Additional steps would then still have been required to make the desired insertions into the multiple cloning site. We will attempt to create a similar deletion (nucleotides 384 through 3514), while simultaneously inserting a common reporter gene encoding the Green Fluorescent Protein (Figure 1.9). This deletion leaves
the entire adenovirus packaging region (Figure 1.2) and the adenovirus protein IX promoter intact.

To this end, a YRp acceptor plasmid will be generated and the adenovirus E1 rescue vector system will be utilized to examine the location requirements of DNA ds breakage relative to the site of linker generated homology during in vivo ligation reactions, and the ability of in vivo ligation to generate the large deletions commonly required during manipulation of the adenovirus genome. We will use the Green Fluorescent Protein (GFP) gene from the AflIV/AflIII fragment of pEGFP-N1 (BD Bioscience Clonetech) as our donor fragment to be inserted into the acceptor molecule. Expression of this gene will then be used as an indication of the reliability of the recombinational cloning reaction. Homology between these two DNA sequences required for successful recombinational cloning will be generated by PCR amplification of the appropriate acceptor plasmid sequences using primers containing 5' overhang encoded homology to the donor fragment. The DNA fragments involved in the recombinational cloning reaction and the expected product are outlined in Figure 1.9.

If it is determined that in vivo ligation can foster the creation of large deletions in the acceptor plasmid during recombinational cloning, selectable marker deletion during recombinational cloning will be assessed as a method for decreasing screening workload.
Figure 1.9: Diagram of recombinational cloning reaction utilized in the assessment of homologous recombination-mediated adenovirus rescue vector manipulation.

Recombination between the donor fragment containing the Green Fluorescent Protein (GFP) gene from the AflII/AflIII fragment of pEGFP-N1 (BD Bioscience Clonetech) and the acceptor fragment containing a 22 bp truncation of the left 5,788 bp of the human adenovirus type 5 contained in the plasmid pXC38 (Bautista et al., 1991) catalyzed by PCR-generated linker regions (LtIVL and RtIVL) that supply homology between donor and acceptor fragments is depicted by crosses between the fragments. Adenovirus and pEGFP-N1 sequences are numbered according to Genbank Accession X02996 or BD Bioscience Clonetech convention, respectively. Features within the fragments are numbered in accordance with the adenovirus (italics) or pEGFP-N1 (normal type) regions that they encode, and borders between donor and acceptor sequences in the recombination product are numbered accordingly.
2. Materials and Methods

2A) Recombinant DNA Techniques

Note that recombinant DNA techniques were carried out according to Sambrook et al. (1989) unless otherwise stated.

2.A.1 Bacterial Strains

_Escherichia coli_ strain DH5α was utilized for the cloning and amplification of pEGFP-N1, and all pUC19 and pXC plasmid derivatives. _E. coli_ DH5α possesses the following genotype: F−, φ80, _slacZ, ΔM15, Δ(lacZYA-argF), U169, deoR, recA1, endA1, hsdR17(rk−, mk+), phoA, supE44, thi−1, λ, gryA96, relA1.

2.A.2 Propagation and Maintenance of Bacterial cultures

_E. coli_ was grown in sterile Luria-Bertani broth (LB; 1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, 4 N NaOH to pH 7.0) overnight at 37°C in a shaker incubator. Cells were frozen in 50% glycerol (3 ml 50% glycerol/7 ml cell culture) at -80°C for long term storage. Short term storage of _E. coli_ cultures (grown on solid (LB, 20% bacto-agar w/v) or in liquid LB media) was possible if the cultures were stored at 4°C.

2.A.3 Preparation of Competent _E. coli_ Cells

Cells from a single colony of _E. coli_, isolated from a single cell (achieved using dilution plating on LB plates) was picked and grown overnight in 2 mL LB. Incubation was at 37°C in a shaker water bath unless otherwise indicated. The other half of the same colony was inoculated
into 2 mL of LB fortified with 100 µg/mL ampicillin. If no growth occurred in the ampicillin-containing tube, 2 mL of overnight culture was then inoculated into 200 ml LB and allowed to grow to mid-log phase (O.D.₅₅₀ = 0.45-0.55). The cells were then placed on ice before being centrifuged for 10 minutes at 3,500 rpm and 4°C in a Beckman Avanti J-25 centrifuge (JA-14 head). The supernatant was removed and the cells were resuspended by gentle inversion in 25 mL of ice cold, sterile transformation buffer (75 mM CaCl₂, 5 mM Tris, pH 7.6). The cell suspension was incubated from 4-16 hours at 0°C before being centrifuged for 10 minutes at 3500 rpm and 4°C. The supernatant was discarded and the remaining cells resuspended by gentle inversion in 4 mL ice cold, sterile transformation solution and incubated at 0°C for 30 minutes. The competent cell suspension was then mixed with 50% glycerol (3 mL 50% glycerol/7 mL cell culture), partitioned into 150 µL portions and subsequently stored at -80°C.

2.A.4 Transformation of Competent E. coli

One µg of plasmid DNA suspended in TE buffer [1 mM ethylene diamine tetra-acetic acid (EDTA), 10 mM TrisHCl (pH 7.5), pH 7.0] was inoculated into 150 µL of previously frozen, competent E. coli. The mixture was subsequently incubated at 0°C for 30 minutes, with light agitation every ten minutes. The DNA-competent cell mixture was then heat-shocked at 42°C for 45 seconds, and placed back on ice for 2 minutes. Nine hundred µL of LB was then added and the mixture incubated at 37°C for 40 minutes. Seventy five to one hundred fifty µL aliquots were then plated on LB plates containing the appropriate bacterial selection agent, kanamycin for pEGFP-N1 (50 µg/mL), and ampicillin (100 µg/mL) in all other cases.
2.A.5 Modified Mini-preparation of Plasmid DNA (pDNA)

Single colonies growing on 100 µg/mL ampicillin LB plates were picked and grown individually overnight in 3 mL of ampicillin-containing liquid LB media. One and one-half mL of culture was then transferred to a sterile Eppendorf tube and centrifuged at 12,500 rpm for 10-30 seconds in a Dupont Sorvall MC12V desktop centrifuge (this model was used throughout this procedure). The media was then removed by vacuum aspiration and the cell pellets resuspended in 200 µL of sterile resuspension solution (50 mM Tris.HCl (pH 7.5), 10 mM EDTA, 10 µg/mL RNAse A) per tube by vortexing. Two hundred µL cell lysis solution (1% w/v sodium dodecyl sulphate (SDS), 0.2 M NaOH) was added to each tube and mixed by inversion for 1 minute. Two hundred µL of sterile neutralization solution (1.32 M potassium acetate (KAce), pH 4.8) was added to each tube and the tubes mixed by inversion. The contents of each tube were then centrifuged at 12500 rpm for 10 minutes at room temperature. The supernatant was then transferred to a new Eppendorf tube and two volumes (300 µL) of isopropanol was added to each tube. The tubes were then centrifuged at 12,500 rpm at room temperature for 10 minutes, the supernatant was poured off, and the tubes allowed to dry. The pDNA pellet was then resuspended in 100 µL of TE buffer.

2.A.6 Restriction Enzyme Digestion of Plasmid DNA

Procedures involving specific restriction enzymes were carried out in accordance with the manufacturers guidelines (New England Biolabs). All restriction enzyme digestions were carried out overnight (approximately 13-15 hours) at 37°C.

Typically, 5 µL of pDNA in TE buffer, 2 µL of the recommended 10 X restriction
enzyme buffer (and 2 μL of 10 X bovine serum albumen (BSA)), 1 unit/μg DNA of restriction enzyme, and double distilled water comprising the remaining 20 μL reaction mixture volume.

Reaction mixtures which were to be used for “Gene Clean®” band excision from agarose gels (procedure to be outlined in Materials and Methods section 2A.14) had a final volume or 30 μL; 15 μL of pDNA in TE buffer, 3 μL of the recommended 10 X restriction enzyme buffer with 3 μL of 10 X BSA, 1 unit/μg DNA of restriction enzyme, and double distilled water comprising the remaining reaction mixture volume.

2.A.7 Partial Restriction Enzyme Digestion of pDNA

Partial restriction enzyme digestion was carried out according to the method of Parker et al., 1977. Typically, μg amounts of plasmid DNA were matched with equal unit values of the appropriate restriction enzyme, buffer (and BSA if necessary) in the presence of varying amounts of ethidium bromide (EtBr)(0.1 to 10 μg) in 100 μL reaction volumes. Reactions were incubated at 37°C and 10 μL aliquots were taken at multiples of equal time intervals (usually 15 s or 30s). The reactions were stopped by the addition of 5 μL of loading buffer to each aliquot. Agarose gel electrophoresis and visualization with EtBr was then utilized to visualize the optimal time interval for which to incubate the reaction. A full 100 μL reaction was then performed and stopped by the addition of 50 μL of loading buffer. The desired partially digested fragment was then purified from the agarose gel after electrophoresis and visualization with EtBr.

2.A.8 Agarose Gel Electrophoresis

DNA fragments resulting from restriction enzyme digestion of pDNA were separated and
visualized using agarose gel electrophoresis. Agarose gels were made by dissolving 0.9% (w/v) agarose in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) by heating in a microwave oven. The gel was allowed to solidify in a taped gel tray with an appropriately sized gel comb inserted into the gel. The gel comb used was dictated by the volume of the restriction enzyme digestion reaction mixture and by the purpose for the DNA fragment separation. If the pDNA was digested for visualization of fragment size (only 20 µL total volume) a gel comb creating approximately 25 µL wells was used. If the pDNA was digested for band excision and band recovery by “Gene Cleaning” (30 µL total volume) a gel comb creating approximately 55 µL wells was used.)

Once the gel had solidified, the tape and gel comb were removed and the gel placed in an appropriately sized gel box containing EtBr (0.15 µg/mL) TAE buffer. Loading buffer (0.05% bromophenol blue, 20% glycerol, 2% SDS) was mixed with (1/6 ratio of buffer/ reaction mixture) the reaction mixture prior to loading into the wells. Five µL of Norgen N1 DNA ladder was also added to one of the wells allowing estimation of pDNA fragment size. Plasmid DNA fragments were separated at 1-10 V/cm, viewed under UV light and then photographed using a Bio-Rad Gel-Doc 1000 and Multi Analyst Software.

2.A.9 Large Scale pDNA Isolation

Frozen cells (or cells remaining from modified mini- preparation of pDNA) were inoculated into 2 mL of ampicillin LB broth and grown overnight in a 37°C shaker bath. One mL of overnight culture was transferred to 500 mL of ampicillin LB broth and allowed to grow at 37°C for 14-15 hours in a shaker- incubator. The culture was then centrifuged at 5,000 rpm for
5-10 minutes at 4°C in an IEC PR-6000 centrifuge. The supernatant was poured off and the cells resuspended in 25 mL of resuspension solution and allowed to sit for 35 minutes. The cell resuspension was then transferred to centrifuging bottles along with 25 mL of lysis solution (10% SDS w/v, 0.2M NaOH) and the mixture was mixed by gentle inversion for 5 minutes. Twenty-five mL of neutralization solution (2M KAc, pH 4.8) was added, and mixed in by inversion. The bottles were then centrifuged at 12,000 rpm for 16 minutes at 4°C in a Beckman Avanti J-25 centrifuge (JA-14 head). This centrifuge was used for all 12,000 rpm centrifugations. The supernatant was then filtered through a sterile coffee filter.

To facilitate banding in cesium chloride gradients, 25mL 3M sodium acetate (3M NaAc, pH 7) and 150mL of ice-cold absolute ethanol was added to the supernatant, and the mixture was then centrifuged at 12000 rpm for 15 minutes at 4°C. The supernatant was removed by vacuum aspiration, and the pellet resuspended in 16 mL of TE buffer.

Plasmid DNA resuspended in TE buffer was then examined for correct restriction fragment banding patterns by agarose gel electrophoresis to verify the presence of the desired plasmid prior to cesium chloride (CsCl) banding.

The density of the resuspended pDNA was adjusted to 1.57-1.59 g/mL using CsCl. Density was determined by solution weight. Five hundred μL (0.15 μg/mL) EtBr/10 mL pDNA/CsCl mixture was added and the density adjusted back to 1.57-1.59 g/mL. (Note: Once concentrated EtBr solution had been added, the plasmid DNA solution container was covered in aluminum foil at all times to prevent exposure to light.) The mixture was centrifuged at 60,000 rpm for 22 hours at 0°C in a Beckman Optima XL-100K ultracentrifuge using an NVT-65 rotor.

After centrifugation, the upper relaxed circular and linear pDNA band was allowed to leak
out of the centrifuge tube by creating a small hole in the centrifuge tube with a 16 gauge needle, just below the nicked pDNA band. The supercoiled pDNA band was then withdrawn from the centrifuge tube using a 16 gauge needle and syringe.

EtBr extraction from the isolated supercoiled pDNA was facilitated by repeated mixing of equal volumes of DNA suspension with CsCl saturated isoamyl alcohol. Once the isoamyl alcohol had been added and mixed with the pDNA solution by inversion, the solution was centrifuged at 500 rpm for 2 minutes in an International Equipment Company Centra-8R centrifuge prior to removal of the EtBr containing isoamyl alcohol layer removal. This process was repeated until no EtBr remained in solution.

The pDNA solution was transferred into boiled, clip-sealed dialysis tubing, and incubated in 2L sterile, 4°C SSC buffer (0.15 M NaCl, 0.015 M sodium citrate) to facilitate removal of any remaining isoamyl alcohol and CsCl. Dialysis was carried out in the buffer for two hours before the buffer was changed. The pDNA solution was then dialysed for two additional 12 hour exchanges in SSC buffer.

After dialysis against SSC buffer, purified pDNA was dispensed into sterile Eppendorf tubes for storage at 4°C.

2.A.10 Determination of pDNA Concentration

The concentration of pDNA was determined spectrophotometrically. Plasmid DNA was diluted in TE buffer. The ATI UNICAM UV/Vis spectrometer was then blanked using TE buffer and the absorbance values of the diluted pDNA solutions were measured at 280 nm and 260 nm wavelength light using Vision software version 3.00. The following formula,
\[ [\text{pDNA}] = \frac{(A_{260})(\text{dilution factor})(50)}{1000} \]

was then applied.

Only pDNA solutions yielding \( A_{260}/A_{280} \) values of greater than 1.6 were utilized for yeast transformation experiments.

2.A.11 Heat Inactivation of Enzymes

It was at times necessary to inactivate certain restriction enzymes (or shrimp alkaline phosphatase) prior to use of the reaction mixture in ligations or transformations. Heating reaction mixtures to 65°C for 20 minutes inactivated all enzymes utilized during the course of these experiments.

2.12 Shrimp Alkaline Phosphatase Treatment of Restriction Enzyme Digested pDNA

In order to suppress recircularization of restriction enzyme digested pDNA upon ligation, shrimp alkaline phosphatase (SAP) was used to remove the 5' phosphate groups from the pDNA fragments. Reactions were carried out in accordance with the manufacturers guidelines (Boehringer- Mannheim). A typical SAP reaction mixture was as follows: 25 μL heat inactivated restriction enzyme digestion reaction mixture, 2 μL SAP, 5 μL 10 X SAP buffer, 18 μL double distilled water.

SAP reaction mixtures were allowed to incubate for 45 minutes at 37°C, before heat inactivation as described in the Materials and Methods section 3A.10.
2.A.13 Klenow Treatment of Digested pDNA

In order to create blunt-end DNA fragments from restriction enzyme digested pDNA, the Klenow fragment of *E. coli* DNA polymerase I (NEB ©) was utilized to fill in 5' overhangs generated by cohesive end generating restriction enzymes. Typically, 1 µg of digested plasmid DNA was incubated with 2.5 units of Klenow fragment in the presence of 200 µM dNTP and the appropriate volume of 10X EcoPol reaction buffer for 15 minutes at room temperature. Reactions were stopped by phenol:chloroform:isoamyl alcohol extraction.

2.A.14 Excision of Restriction Enzyme Digested pDNA Fragments from Agarose Gels

Individual bands of pDNA fragments were excised and purified directly from agarose gels using the Bio 101 Gene Clean Kit. The kit removes salts, RNA, proteins, and EtBr from DNA. A sterile, sharp razor was used to cut the appropriate band from the agarose gel under UV light. The band was placed into a previously weighed Eppendorf tube and the tube weighed again. Three volumes NaI/weight of agarose gel (mL/g) were then added to the tube and the tube was incubated at 45°C for 5 minutes. Five µL of vortexed glassmilk was then added to the mixture. The mixture was then incubated at room temperature and mixed every minute for 5 minutes. The mixture was then centrifuged for 5 seconds at 12,500 rpm in a Dupont Sorvall MC12V desktop centrifuge (this centrifuge was used throughout this procedure) and the supernatant removed by aspiration. The pellet was resuspended by pipetting in 300 µL of New Wash, and then centrifuged for 5 seconds at 12500 rpms. This was repeated three times before the pellet was resuspended in 25 µL of double distilled water. The resuspension was then incubated at 55°C for 5 minutes before centrifuging at 12,500 rpm for 30 seconds. Twenty µL of
supernatant containing the purified pDNA fragment was removed and placed into a sterile Eppendorf tube. The remaining 3-5 uL of supernatant was subjected to agarose gel electrophoresis in order to verify that the correct fragment had been isolated.

2.A.15 Ligation of Restriction Enzyme Digested pDNA Fragments

Bacteriophage T4 DNA ligase was used to catalyse ligation of separate DNA fragments. Reaction mixtures were set up according to the manufacturers guidelines (MBI Fermentas). A typical reaction mixture contained the following reactants; 15 µL heat-inactivated restriction enzyme digestion mixture containing the smaller insert pDNA fragment, 5 µL heat-inactivated restriction enzyme digestion mixture containing the larger pDNA fragment, 4 µL 10 X T4 DNA ligase reaction buffer, 1 µL T4 DNA ligase, 15 µL double distilled water.

Ligations of restriction enzyme digested pDNA fragments were carried out in an ice box at 4°C overnight (14-15 hours). The reaction mixture was then utilized to transform E. coli DH5α.

2.A.16 Phenol:Chloroform:Isoamyl Alcohol Extraction of DNA

An equal volumes of 25:24:1 (v:v:v) phenol:chloroform:isoamyl alcohol (PCI) capped with 8-hydroxy-quinoline and the solution containing the DNA of interest were mixed in an Eppendorf tube followed by vigorous vortexing until an emulsion had formed. The tube was then centrifuged at 12,500 rpm for 5 minutes at room temperature. In cases when it was necessary to do so, the PCI extraction was repeated until the white protein precipitate separating the aqueous
and organic phases was no longer present. The aqueous layer containing DNA was then removed and transferred to a sterile Eppendorf tube with an equal volume of water saturated chloroform. The tube was vortexed for one minute and centrifuged at 12,500 rpm for one minute. The aqueous layer was then removed. The sodium acetate concentration of this solution was adjusted so that a final concentration of 0.3 M would be reached with 3 M sodium acetate (pH 7.0), and 2 volumes of ice cold ethanol were added. The solution was stored at -20°C for one hour before centrifugation at 12,500 rpm for 10 minutes. The supernatant was then poured off and all remnants aspirated taking care not to disrupt the pellet. The pellet was then washed with 80% ethanol and centrifuged at 12,500 rpm for 10 minutes. The supernatant was then poured off and all remnants aspirated taking care not to disrupt the pellet. The pellet was dried at 37°C for 5 minutes before resuspension in an appropriate volume of TE buffer.

2.A.17 Polymerase Chain Reaction

All polymerase chain reactions were carried out in 50 μL reaction volumes in DiaMed Lab Supply Inc. PCR tubes in an ERICOMP singleblock Easycycler thermocycler. Typical reactions contained 5 μL 10X PCR reaction buffer without MgCl₂ (MBI Fermentas), 0.01-1 μg template DNA, 15 pmoles forward and reverse primers (generated by SigmaGenoSys Inc.), 200 μM dNTP mixture (New England Biolabs), 2 mM MgCl₂ (MBI Fermentas), and 2.5 units of Taq polymerase (New England Biolabs). All reactions entailed a 5 minute initial denaturation step at 94°C, followed by 35 rounds of 1 minute denaturation at 94°C, 1 minute annealing at 47°C, and 1 minute extension at 72°C, followed by a final 5 minute extension at 72°C.
2.A.18 Propagation and Maintenance of *S. cerevisiae* Cultures

The haploid *Saccharomyces cerevisiae* laboratory strain of JY128 was utilized during the course of this research. *S. cerevisiae* JY128 carries the following auxotrophic and genetic markers: Matα, ura3-52, lys2-801, ade2-101, trp1-Δ63, his3Δ200, leu2Δ1, gln3, KAN^R^.

Yeast were allowed to grow to stationary phase in sterile YPD broth (1% bacto- yeast extract w/v, 2% bacto- peptone w/v, 2% dextrose w/v) overnight at 30 °C in a shaker incubator. Culture stocks were stored on solid YPD plates (YPD, 2% bacto- agar w/v) or in liquid YPD media at 4 °C. For long term storage of yeast cells, stationary phase liquid cultures were adjusted to 15% glycerol and frozen at -80°C.

During transformation experiments, synthetic medium (0.67 % Bacto-yeast nitrogen base without amino acids, 2% dextrose, 1 M sorbitol, 20 mg/L Adenine sulfate, 20 mg/L Uracil, 20 mg/L L-Tryptophan, 20 mg/L L-Histidine-HCl, 20 mg/L L-Methionine, 30 mg/L L-Leucine, 30 mg/L L-Lysine-HCl, 100 mg/L L-Glutamic acid, in the case of solid medium 2% w/v bacto-agar) was utilized. Selective medium was made by omission of the amino acid coinciding with the auxotrophy being complemented. Plates utilized to select for loss of the *URA3* marker were made according to Boeke *et al.*, 1984. Briefly, a 2g/L concentrate of 5-fluoro-orotic acid was filter sterilized, and mixed with a 2-fold sterile concentrate of uracil fortified selective media. Plates were then poured from the mixture and allowed to solidify at room temperature overnight.

2.A.19 Replica Plating of Yeast Cultures

Yeast colonies growing at suitable sizes and densities (less than 150 colonies per plate) were transferred between selective media plates using sterile velvet sheets reinforced by a circular
wooden block (of slightly lesser diameter than that of the plates). For plates with larger colony densities, selective media plates bearing two different auxotrophies were paired, and sectioned identically. Sterile toothpicks were used to transfer the colony under investigation first to the plate with the untested auxotrophy, then to the selective plate for which complementation had previously been verified. The plates were then incubated for 3-5 days before examination.

2.A.20 Preparation of Electrocompetent Yeast Cells

Electrocompetent yeast cells were prepared according to a modified version of the protocol described in Becker and Guarente, 1991.

One hundred mL of YPD in a 500-millilitre flask was inoculated with a suitable aliquot from a stationary phase, yeast culture to give an O.D.\textsubscript{600} of 1.4 - 1.5 the next morning. It was determined through growth curve analysis that the inflection point of the logarithmic growth phase occurred within this O.D. range for \textit{S. cerevisiae} strain JY128. The culture was grown overnight at 30°C with vigorous shaking.

The next morning the culture was centrifuged at 3,000 rpm at 4°C for 5 minutes. The supernatant was poured off, and the cell pellet was resuspended in 100 ml of sterile, ice-cold dH\textsubscript{2}O by shaking the centrifuge bottles. The cell resuspension was then pelleted again, and resuspended in 100 ml of ice-cold dH\textsubscript{2}O and pelleted for a third time. Once again the supernatant was discarded, the cell pellet was resuspended in 50 ml of sterile, ice-cold 1 M sorbitol, and the cell resuspension was again centrifuged. The supernatant was poured off and the cell pellet was resuspended in 0.1 ml of ice-cold 1 M sorbitol with a 200 \( \mu \)l micropipette. All centrifugation steps were carried out in an International Equipment Company Centra-8R centrifuge.
2.A.21 Electroporation of Electrocompetent Yeast Cells

Electroporation of electrocompetent yeast cells was carried out according to the method of Becker and Guarente, 1991.

Fifty μl aliquots of electrocompetent yeast cells were aliquoted into ice-chilled, sterile Eppendorf tubes containing no more than 5 ul total of DNA. The sample was mixed using a 200 μl micropipettor and then transferred to a 0.2-cm Bio Rad electroporation cuvette. The mixture was tapped to the bottom of the electroporation chamber, and allowed to incubate on ice for approximately 5 minutes. The sample was then pulsed at 1.5 kV, 25 mF, 200 Ω in a Bio Rad Gene Pulser II Electroporation System with Pulse Controller ®. Immediately after the pulse, one ml of ice-cold, sterile 1 M sorbitol was added to the sample using a sterile micropipette. Aliquots of the electroporated cell suspension and 10-fold, 100-fold, and 1000-fold dilutions were then plated by spreading on selective plates containing 1 M sorbitol.

2.A.22 Mini- Preparation of Yeast pDNA

Single colony positive yeast transformants were inoculated into 2 mL of selective liquid medium and grown overnight to stationary phase in a 30 °C shaker- incubator. One and one-half mL of the overnight culture was transferred to a sterile Eppendorf tube and centrifuged at 12,500 rpm at room temperature for 5 seconds in a Dupont Sorvall MC12V desktop centrifuge (this centrifuge was used throughout this procedure). The supernatant was then poured off, and the pellet disrupted by brief vortexing. Alternatively, entire plates of transformants were isolated simultaneously by plate washing with 10 mL of selective liquid medium. The cell suspension was
then repeatedly centrifuged and poured off until the entire resuspension had been pelleted. The pellet was then disrupted by brief vortexing. To this yeast cell resuspension, 100 μL of breaking buffer (1% SDS, 0.5% Triton-X 100, 100 mM NaCl, 10 mM Tris.HCl), 100 μL 25:24:1 (v:v:v) phenol:chloroform:isoamyl alcohol, and 0.2 g of acid washed glass beads (Sigma Aldrich Co.) (prepared by one hour soaking in nitric acid, followed by extensive washing with distilled water and overnight drying at 80°C) was added, followed by vigorous vortexing for at least 2 minutes. The tube was then centrifuged at 12,500 rpm for 5 minutes at room temperature. The aqueous layer containing yeast pDNA was then removed and transferred to a sterile Eppendorf tube with an equal volume of water saturated chloroform. The tube was vortexed for one minute and centrifuged at 12,500 rpm for one minute. The aqueous layer was then removed. The sodium acetate concentration of this solution was adjusted to 0.3 M with 3 M sodium acetate (pH 7.0), and 2 volumes of ice cold ethanol were added. The DNA precipitation was stored at -20°C for one hour, before centrifugation at 12,500 rpm for 10 minutes. The supernatant was then poured off and all remnants aspirated taking care not to disrupt the pellet. The pellet was then washed with 80% ethanol and centrifuged at 12,500 rpm for 10 minutes. Again, the supernatant was then poured off and all remnants aspirated taking care not to disrupt the pellet. The pellet was dried at 37°C for 5 minutes before resuspension in 10-30 μL of TE buffer. One half of this resuspension was subsequently utilized to transform competent *E. coli*.

2.A.23 *In Vivo* Ligation Reaction Protocol

All *in vivo* ligation experiments were carried out according to previously published protocols (Raymond *et al.*, 1999).
Typically, acceptor fragment was prepared as follows: restriction enzyme linearized acceptor plasmid was PCI extracted twice, followed by one extraction with water saturated chloroform. DNA in the aqueous layer was then precipitated and resuspended in 5-10 μL of TE buffer. When a partial digest was required to linearize the acceptor, the acceptor was digested as described in section 2.A.7 and isolated from agarose gels as described in section 2.A.14. In both cases, the fragment concentration was judged by comparing band density against DNA standards of known concentration after agarose gel electrophoresis and visualization with EtBr under UV light. The resuspended linearized acceptor was then adjusted to a concentration of 0.1 μg/μL with TE buffer.

The donor fragment was prepared by isolating the 1,697bp AflII/AflIII fragment of pEGFP-N1 (BD Biosciences Clonetech®) from agarose gels as described in section 2.A.14. Typically, it was necessary to pool several isolations to achieve the desired final concentration of 1 μg/μL. Several isolations were pooled in one Eppendorf tube and the DNA precipitated. The resuspended linearized acceptor was then adjusted to a concentration of 1 μg/μL with TE buffer.

The polymerase chain reaction protocol used to amplify linker DNAs (named RtIVL and LtIVL) containing homology to both the donor and acceptor fragments is detailed in section 2.A.17. Isolation and analysis of linker DNA after PCR amplification are detailed in Results sections 3.4 and 3.5. Each linker was resuspended at a final concentration of 1 μg/μL.

During in vivo ligation reactions 0.1 μg of acceptor fragment (1 μL of 0.1 μg/μL resuspension), 1 μg of donor fragment (1 μL of 1 μg/μL resuspension), and 1 μg of RtIVL and 1 μg LtIVL linker DNA (1 μL of each 1 μg/μL resuspension) were mixed and cooled on ice in Eppendorf tubes prior to the addition of electrocompetent yeast cells (preparation detailed in
section 2.A.20), electroporation and plating (detailed in section 2.A.21).

All in vivo ligation experiments were performed in triplicate. The total volume of each electroporation was plated as three equal portions on separate selective media plates. Transformants growing on the selective plates were counted and analysed 5-7 days later.

2.B) Mammalian Cell Culture Techniques

Note that unless otherwise stated, all mammalian cell culture techniques were performed as indicated in Graham and Prevec (1995).

2.B.1 Ad 5 Transformed Human Embryonic Kidney Cell (293 Cell) Maintenance

Human Embryonic Kidney 293 cells (Microbix Inc., ATCC CRL 1573) were maintained as monolayer cultures at 37°C in a humid 5% CO₂ atmosphere in a Napco 5200 CO₂ incubator. Medium utilized was MEM (Gibco BRL) supplemented with 0.225 % (w/v) sodium bicarbonate (GibcoBRL ), 0.292 microg/mL L-Glutamine, 5% v/v heat-inactivated fetal bovine serum (FBS) and 5% heat-inactivated horse serum (HS) (CanSera International, Inc.), and fortified with 1% Antibiotic-Antimycotic (10,000 units/mL penicillin G sodium, 25 µg/mL amphotericin B, and 10,000 units/mL streptomycin sulphate, Gibco BRL).

All tissue culture dishes and tubes with the exception of 6-well plates (Costar) were obtained from Sarstedt.

All cell manipulations were carried out in a Baker Company, Inc. SterilGARD Hood after 20 minute UV sterilization periods. Aseptic technique was utilized in all cell manipulations. Cells
were observed using a TELAVAL 3 microscope (Carl Zeiss Jena).

2.B.2 293 Cell Passaging

Nearly confluent 293 cell monolayers were passaged by using sterile saline citrate (15 mM sodium citrate, 135 mM potassium chloride) to facilitate cell lifting from culture dishes. Used medium was aspirated from the plate, and the cells washed once with 5 mL saline citrate. The wash was then aspirated and 3 mL saline citrate was applied to the monolayer. The cells were then allowed to incubate at 37°C until cell rounding and lifting was observed. The cells were dislodged by gentle tapping of the culture dish. Seven mL of new medium was then added to the cell suspension and the cells were separated by gentle tituration. Five mL of the cell suspension was then added to a new culture dish, and was adjusted to a final volume of 20 mL with additional culture medium. The cells were then allowed to reattach overnight at 37°C.

2.B.3 293 Cell Freezing and Thawing

Frozen 293 cell cultures were maintained in liquid nitrogen storage. Used medium was aspirated from the plates containing nearly confluent 293 cell monolayers, and the cells washed once with 5 mL saline citrate. The wash was then aspirated and 3 mL saline citrate was applied to the monolayer. The cells were then allowed to incubate at 37°C until cell rounding and lifting were observed. The cells were dislodged by gentle tapping of the culture dish. Seven mL of culture medium was then added to the cell suspension and the total cell suspension transferred to a 12 mL conical tube. The cells were then pelleted at room temperature by centrifugation at 800
rpm for 5 minutes in an International Equipment Company Centra-8R centrifuge. The medium was then poured off and the cells resuspended in 4 mL freezing medium (90% FBS, 10% dimethyl sulphoxide) by gentle inversion. The cell suspension was then aliquoted into 2 mL cryotubes and incubated at -80°C overnight. The cells were then transferred to liquid nitrogen storage.

Frozen cell cultures were thawed by incubation in a 37°C water bath and added to a culture dish containing pre-warmed 37°C medium. The cells were allowed to attach for 6-12 hours before the medium was changed to remove unattached cells.

2.B.4 Transfection of 293 cells Using ExGen 500 In Vitro Transfection Reagent ®.

Transfection using this procedure was carried out according to the supplier's recommendations (MBI Fermentas). Briefly, 293 cells were plated in 6-well culture dishes one day prior to transfection so that cells would be 50-80% confluent at the time of transfection. The medium in the 6-well plates was changed 3 hours prior to transfection. For each transfection, 1-5 μg mixtures of pDNA were diluted in 270 μl sterile 150 mM NaCl. The ExGen 500 transfection reagent (3.3 μL per 1 μg pDNA) was added to the pDNA solutions and immediately vortexed. The ExGen reagent was then allowed to complex with the pDNA for 10 minutes. The transfection solution was then mixed by gently pipetting before being added to one well of a 6-well plate. The plates were agitated gently before being incubated at 37°C.
2.B.5 Visualization of Green Fluorescent Protein Expression

Forty eight hours after transfection, medium was aspirated from transfected 293 cell monolayers prior to Green Fluorescent Protein (GFP) expression visualization. GFP expression was visualized using a Leitz Diaplan Microscope outfitted with a fluorescein filter set (excitation wavelength of 488 nm) and photographed using a Wild Leitz Wild MPS 46 Photo Autoformat system. Photographs were taken with Kodak Gold 400 speed film. The film was manually exposed for between 1 and 6 seconds.
3. Results

3.1 Construction of the YRpXC38 Acceptor Vector

For any \textit{in vivo} ligation reaction to be successful, an acceptor vector with the appropriate backbone sequences for replication and selection in \textit{S. cerevisiae} must be present in the reaction. In order to create an acceptor plasmid with the appropriate yeast replication and selectable marker sequences the 1,456 bp \textit{TRP}1/\textit{ARS EcoRI} fragment of YRp7 (Tschumper and Carbon, 1980) was inserted into the unique \textit{EcoRI} site of the bacterial cloning vector pXC38 (Bautista \textit{et al.}, 1991) as indicated in Figure 3.1. The \textit{TRP}1 gene of \textit{S. cerevisiae} encodes N-(5'-phosphoribosyl)-anthranilate isomerase, a vital enzyme in the tryptophan amino acid synthesis pathway (Jones and Fink, 1982). The \textit{ARS} sequence of the 1,456 bp \textit{TRP}1/\textit{ARS EcoRI} fragment of YRp7 encodes a \textit{S. cerevisiae} chromosomal origin of replication (Tschumper and Carbon, 1980). pXC38 contains the appropriate bacterial origin of replication and selectable marker for plasmid maintenance and propagation in \textit{E. coli} and contains a 22 bp truncation of the left 5,788 bp of the adenovirus type 5 genome which encodes one of the inverted terminal repeats, the packaging domain, the early region 1, and the protein IX gene.
Figure 3.1: Construction Strategy for YRpXC38. The 1,456 bp TRP1/ARS EcoRI positioning values for the restriction sites used to verify YRpXC38 conformation are given in brackets and are relative to the unique EcoRI digestion site of pXC38.

3.2 Restriction Enzyme Confirmation of YRpXC38 construction

Transformation of competent *E. coli* DH5α with overnight ligation reactions of purified 1,456 bp TRP1/ARS EcoRI fragment from YRp7 and shrimp alkaline phosphatase treated *EcoRI* digested pXC38 generated ampicillin-resistant bacterial colonies. *EcoRI* screening of pDNA isolated from forty-two such ampicillin-resistant
bacterial colonies revealed that only one contained a pXC38 clone harboring the 1,456 bp TRP1/ARS fragment (as illustrated in lane 7 of Figure 3.2). By convention, this plasmid was termed YRpXC38. In order to determine the orientation of the TRP1/ARS insert in the YRpXC38 plasmid restriction fragment banding pattern analysis was performed (Figure 3.2). The predicted sizes of restriction fragments from the parent plasmids YRp7 and pXC38 and both possible YRpXC38 conformations are listed in Table 3.1.

Table 3.1: Predicted sizes of restriction fragments from parent plasmids YRp7 and pXC38 and both possible YRpXC38 constructs. ND indicates no predicted digestion products.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Restriction Enzyme</th>
<th>KpnI</th>
<th>EcoRI</th>
<th>BglII</th>
<th>HindIII</th>
<th>XbaI</th>
</tr>
</thead>
<tbody>
<tr>
<td>YRp7</td>
<td>ND</td>
<td>4,361</td>
<td>5,817</td>
<td>5171</td>
<td>5,817</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,456</td>
<td></td>
<td></td>
<td></td>
<td>646</td>
</tr>
<tr>
<td>pXC38</td>
<td>9,553</td>
<td>9,553</td>
<td>9,553</td>
<td>9,553</td>
<td>9,553</td>
<td></td>
</tr>
<tr>
<td>YRpXC38 (A)</td>
<td>1,1007</td>
<td>9,553</td>
<td>6,764</td>
<td>7,525</td>
<td>9,419</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,454</td>
<td>4,243</td>
<td>3,482</td>
<td>1,588</td>
<td></td>
</tr>
<tr>
<td>YRpXC38 (B)</td>
<td>1,1007</td>
<td>9,553</td>
<td>7,012</td>
<td>7,299</td>
<td>8,335</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,454</td>
<td>3,995</td>
<td>3,708</td>
<td>2,672</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.2: 1% agarose gel showing restriction fragment banding patterns of parental plasmids pXC38 (C1) and YRp7 (C2), and the YRpXC38 construct (Y) generated by insertion of the TRP1/ARS EcoRI fragment of YRp7 into the unique EcoRI site of pXC38. Each plasmid was digested with restriction endonucleases KpnI, EcoRI, BglII, HindIII, or XbaI. Marker lane is indicated by M, and sizes of marker bands are indicated (in Kb). The 1,456 bp TRP1/ARS fragment is indicated by an arrow.
Comparison of the values for predicted restriction fragment sizes for YRpXC38 conformation (Å) from Table 3.1 with the banding patterns observed in Figure 3.2 demonstrated that the TRP1/ARS fragment was inserted such that the distance between the two XbaI recognition sequences in YRpXC38 was minimized. This conformation is illustrated in Figure 3.1.

3.3 Analysis of S. cerevisiae and E. coli Replication Origin and Selectable Markers in YRpXC38.

YRpXC38 isolated from ampicillin-resistant E. coli DH5α was banded by CsCl2 density gradient centrifugation and was utilized to transform S. cerevisiae strain JY128 by electroporation. Electroporation experiments generated an average of 5.6 x 10^4 trp+ transformants/µg pDNA, and thus verified the functionality of the ARS and TRP1 sequences in YRpXC38.

Transformation of competent E. coli DH5α with total plate isolation of trp+ S. cerevisiae transformant pDNA generated an average of 50 +/- 20 ampicillin-resistant E. coli colonies per transformation. Restriction endonuclease digestion of pDNA isolated from the ampicillin-resistant E. coli transformants yielded identical banding patterns to that of digested YRpXC38 (Figure 3.3).
Figure 3.3: A) Locations of EcoRI and NcoI sites in YRpXC38. B) 1% agarose gel showing EcoRI and NcoI restriction fragment banding patterns of YRpXC38 (C) and pDNA isolated from ampicillin-resistant bacterial colonies (1, and 2) generated via total plate back transformation with pDNA isolated from trp+ yeast transformants. Marker lane is indicated by M, and sizes of marker bands are indicated (in Kb).

The ampicillin-resistant E. coli generated using isolated pDNA from the positive yeast trp+ transformants coupled with the identical banding patterns of the digested YRpXC38 to banding patterns of pDNA of all isolates examined. EcoRI digestion
patterns observed in Figure 3.3 are consistent with those observed in Figure 3.1 and described in Table 3.1, and the observed NcoI fragments in Figure 3.3B are consistent with those predicted for such a digestion (7,759 bp, 1,785 bp, and 1,463 bp fragments generated via three recognition sites at YRpXC38 positions 2269, 3732, and 5517). Taken together these data offered substantial proof that positive trp+ yeast transformants indeed resulted from transformation of the yeast by YRpXC38.

3.4 Construction of the LtIVL and RtIVL In Vivo Ligation Linkers

Linker DNA providing homology between the donor and acceptor fragments was generated via PCR amplification of acceptor sequences in YRpXC38 with primers containing 5' overhangs with homology to donor fragment sequences (Gunyuzla et al., 2001). Linker LtIVL contained homology to bases 152 through 384 of the Ad5 genome fused to the first forty bases of the CMV promoter driving transcription of the GFP coding sequence in pEGFP-N1 (Figure 1.9). Linker RtIVL contained homology to bases 1570 to 1610 of the pEGFP-N1 plasmid (this region encompasses 32 bases of the 3' end of the GFP mRNA plus an extra eight bases of the pEGFP plasmid) fused to bases 3514 through 3757 of the Ad5 genome (Figure 1.9). The primer sequences and the regions amplified in the PCR reaction are detailed in Figure 3.4.
Figure 3.4: A) Relative positions of linearizing restriction sites used during in vivo ligation experiments. B) PCR amplification scheme for in vivo ligation linker generation. The dark horizontal lines represent adenovirus sequences within the plasmid. Hatched lines represent template sequences for linker amplification. Adenovirus sequence and pEGFP-N1 nucleotide position values are labeled in normal and bold, respectively. C) Sequences of primers used in linker amplification (homology to pEGFP-N1 and Ad5 sequences indicated by bold and normal type, respectively).
3.5 Analysis of LtIVL and RtIVL *In Vivo* Ligation Linkers

Following linker PCR amplification, the size and concentration of the amplified linkers were determined by agarose gel electrophoresis. The expected sizes of the LtIVL and RtIVL linkers are 273 bp and 285 bp, respectively (Section 3.4). Both LtIVL and RtIVL migrated as expected and appear to be of the proper size, as seen in Figure 3.5.

![Figure 3.5: 2% agarose gel showing *in vivo* ligation linkers LtIVL and RtIVL after ethanol-precipitation and resuspension in TE buffer. Marker lane is indicated by M, and sizes of marker fragments are given (in bp).](image)

Linker amplification yields were typically between 6-10 µg per reaction.
3.6  *S. cerevisiae* JY128 Exponential Growth Inflexion Point Determination

Three 100 mL sterile YPD culture flasks were inoculated sequentially with 20 µL of stationary phase *S. cerevisiae* JY128 broth cultures at 15 minute intervals. Measurements OD$_{600}$ were taken at one hour intervals after the initial inoculation and were used to determine the OD$_{600}$ at which this *S. cerevisiae* strain grows at its maximum rate. The inflection point of the exponential growth phase occurred at approximately OD$_{600}$ =1.49. Yeast cells were consequently harvested between OD$_{600}$=1.4 and 1.45 for preparation of all electrocompetent cells.

3.7  Initial *In Vivo* Ligation Experiments

It has previously been demonstrated that use of blunt-end restriction sites significantly reduces numbers of background transformants generated by resealing of cohesive-end linearized acceptor by *S. cerevisiae* during *in vivo* ligation (Raymond *et al.*, 1999). Consequently, in an effort to decrease subsequent screening workload blunt-end *HpaI*-digested YRpXC38 acceptor (Figure 3.4) was the first acceptor substrate utilized during *in vivo* ligation reactions.

Co-introduction of *HpaI*-linearized YRpXC38 acceptor, 1,657 bp *AfIII/AfIIII* donor fragment, and LtIVL and RtIVL PCR amplified linkers into *S. cerevisiae* by electroporation resulted in an average of 172 (+/-22) trp$^+$ colonies (see Figure 3.4 for YRpXC38 *HpaI* site location and Figure 1.9 for predicted recombination reaction). However, as indicated by Figure 3.6, control reactions lacking either donor fragment
(A+L) or linker DNA (A+D) or both (A) were not significantly different from in vivo ligation reactions containing HpaI-linearized acceptor, donor, and linker DNA (A+L+D).

Figure 3.6: Mean and standard deviation summary of in vivo ligation reactions using HpaI digested YRpXC38 acceptor. L, D, and A represent 1μg of each linker (LtIVL and RtILV), 1μg of 1,697 bp AflII/AflIII pEGFP-N1 donor fragment, and 0.1μg HpaI digested YRpXC38 acceptor, respectively. Control reactions were carried out simultaneously by omitting the donor (A+L), the linkers (A+D), or both (A).
Literature values indicate that a successful \textit{in vivo} ligation reaction is typically marked by a large increase in positive transformant generation with 80-95\% being of the desired conformation (Raymond \textit{et al.}, 1999; Hua \textit{et al.}, 1997). Comparison of \textit{in vivo} ligation reaction (A+L+D) and control reaction (A+L, A+D) efficiencies by t-test analysis revealed that \textit{in vivo} ligation reactions did not differ from controls.

3.8 Initial \textit{In Vivo} Ligation Transformant Analysis

In order to assess the structure of the recombinant plasmids generated by initial \textit{in vivo} ligation experiments, back transformations to competent \textit{E. coli} were performed using pDNA isolated from total plate isolations of trp\textsuperscript{+} positive yeast transformants generated during these experiments. Forty ampicillin-resistant colonies were produced. Restriction fragment banding patterns of pDNA isolated from these colonies revealed that initial \textit{in vivo} ligation experiments did not generate the plasmid of the conformation predicted in Figure 1.9, but did generate two other populations of recombinant plasmids. These plasmids were determined to be acceptor molecules containing deletions of either 1.1 kb or 2.6-2.7 kb. These deletions were mapped by comparing restriction enzyme fragment banding patterns of the recombinant plasmids to those of the YRpXC38 acceptor (Figure 3.7).
Figure 3.7: A) Locations of restriction sites and predicted deletions within adenovirus sequences in YRpXC38 generated during initial in vivo ligation reactions. B) Restriction fragment banding patterns of 1.1-1.2 kb and 2.6-2.7 kb deletion recombinants (E) generated during in vivo ligation experiments utilizing HpaI digested YRpXC38 acceptor and YRpXC38 control plasmid (C). Note that SacII digestion was incomplete.
Ninety-five percent (38 of 40) of the recombinant plasmids isolated from ampicillin-resistant \textit{E. coli} possessed an approximately 1.1 kb deletion. This deletion was flanked by \textit{SacII} (414) and \textit{KpnI} (2115) as demonstrated by the retention of those sites in the recombinant plasmid (Figure 3.7B, lanes 3 and 5, respectively). Figures 3.4 and 3.7A show the location of these sites in YRpXC38. The approximate size of the deletion was determined by comparing the \textit{EcoRI}/\textit{KpnI} 2,115 bp fragment (Figure 3.7B, lanes 6) and the 1 kb \textit{EcoRI}/\textit{KpnI} fragment released from the recombinant (Figure 3.7B, lane 7).

The remaining 2 recombinant plasmids analyzed possessed a 2.6-2.7 kb deletion flanked by \textit{SacII} (414) and extending beyond \textit{BglII} (3391), but not beyond \textit{AflIII} (3596). Figure 3.7B demonstrates the recombinant and YRpXC38 possess the same number of \textit{SacII} sites (Figure 3.7, lanes 8 and 9) and \textit{AflIII} sites (Figure 3.7B, lanes 12 and 13), however \textit{BglII} (3391) is absent in the recombinant (Figure 3.7B, lanes 10 and 11). The approximate size of the deletion was determined by comparing the 3,476 bp \textit{SacII} fragment from YRpXC38 (Figure 3.7B, lane 9) with the approximately 800 bp \textit{SacII} fragment from the recombinant (Figure 3.7B, lane 8).

3.9 Second \textit{In Vivo} Ligation Experiment

Published results indicate that the ratio of the desired \textit{in vivo} ligation product to other recombinants should be much higher than ratios observed during initial \textit{in vivo} ligation experiments (Raymond \textit{et al.}, 1999; Hua \textit{et al.}, 1997). One possible reason for the lack of successful \textit{in vivo} homologous recombination could have been the distance between the left and right regions of linker-generated homology and the \textit{HpaI} site utilized.
to linearize the acceptor. *Hpa*I digestion of the acceptor generated 1,197 bp and 1,936 bp extension between the left and right regions of linker-generated homology and the ds break, respectively (Figure 3.4). It is likely that such a distance was too great for the yeast recombination machinery to successfully mediate homologous recombination. Ds DNA break repair utilizes 3' single stranded (ss) DNA tails arising through ds break processing (Figure 3.8) as substrate for homologous recombination reactions. The maximum size of such ss DNA tails observed to this point has been approximately 1kb (reviewed in Sung *et al.*, 1999).
Figure 3.8: Current model of double strand DNA break repair in *S. cerevisiae*. Repair is initiated by the processing of the free ds DNA end by a three enzyme complex, Rad50/MreII/Xrs2, producing a ss DNA molecule with a free 3' end. The ss DNA is then complexed by Rad51, stimulated by the ss DNA binding factor RPA, to form a ss DNA/protein filament. The Rad51/ssDNA complex invades homologous ds DNA duplex substrate with which the ds break is repaired. Resolution of recombination intermediates results in an unbroken DNA molecule.

A unique *AflII* site occurs at nucleotide position 3596 in the acceptor (Figure 3.4). This site falls 225 bp within the rightward region of linker-generated homology.
Given the size of the linker regions and the location of such a ds DNA break, it was hypothesized that linearization of YRpXC38 at this site would provide an acceptor which would serve as sufficient substrate to promote homologous recombination. To test this hypothesis, initial *in vivo* ligation experiments were repeated using *AflIII* digested acceptor.

Co-transformation with *AflIII*-linearized acceptor, donor fragment, and *LtiVL* and *RtiVL* PCR amplified linkers into *S. cerevisiae* resulted in an average of 11,000 (+/- 1,300) trp*+* colonies, approximately 1.5-fold more colonies than control reactions lacking either donor (A+L) or linker DNA (A+D) and approximately 2.2-fold more colonies than control reactions lacking both donor and linker DNA (A) (Figure 3.9).
Figure 3.9: Mean and standard deviation summary of *in vivo* ligation reactions using *AflIII* digested YRpXC38 acceptor. L, D, and A represent 1 μg of each linker (LtILV and RtILV), 1 μg of 1,697 bp *AflIII/AflIII* pEGFP-N1 donor fragment, and 0.1 μg *AflIII* digested YRpXC38 acceptor, respectively. Control reactions were carried out simultaneously by omitting the donor (A+L), the linkers (A+D), or both (A).

Control reactions lacking donor (A+L) or linker DNA (A+D) or both (A) did not differ significantly from one another by Student's t-test analysis. However, a significant difference between control (A+L, A+D) and *in vivo* ligation (A+L+D) reactions was observed (P=0.05).
Analysis of Second *In Vivo* Ligation Transformants

In order to assess the structure of the recombinant plasmids generated by secondary *in vivo* ligation trials, back transformations of competent *E. coli* were performed using pDNA isolated from total plate isolations of trp⁺ yeast transformants. Seventy ampicillin-resistant colonies were produced. *EcoR*I restriction fragment banding patterns of pDNA isolated from 67 of these colonies were identical to that of the acceptor. The remaining three plasmids possessed banding patterns predicted for successful *in vivo* ligation recombinants (Figure 1.9 and Figure 3:10B). Restriction fragment banding patterns of the recombinant plasmid is compared to that of the YRpXC38 parental plasmid in Figure 3.10.
Figure 3.10: A) 1% agarose gel showing restriction fragment banding patterns of *in vivo* ligation generated recombinant (R) pDNA and parental YRpXC38 (C) digested with *AflII, SacII, BamHI, HpaI*, or *EcoRI*. B) Predicted *AflII, EcoRI, SacII, BamHI*, and *HpaI* digestion products of YRpXC38 and the predicted YRpXC-EGFP. ND refers to no predicted digestion products.
Comparison of the predicted banding patterns with those observed in Figure 3:10 demonstrates that in vivo ligation utilizing AflII digested YRpXC38 generated the desired recombinant and that no detectable errors resulting from the in vivo ligation reaction could be detected using restriction enzyme screening. AflII, BamHI, and Hpal digests of the recombinant plasmid produced 9.5 kb fragments, the expected size of the recombinant plasmid. The recombinant plasmid contained one more SacII site than the YRpXC38 parent molecule and contained the same unique AflII site. When compared, these digests indicated that the leftward and rightward regions undergoing homologous recombination retained restriction sites within these regions. Comparison of the EcoRI digests demonstrated an extra EcoRI site present in the recombinant. Comparison of the BamHI digests demonstrated the presence of a unique BamHI site in the recombinant that was not present in the parent plasmid. Taken together these digests revealed that the donor fragment was complete within the acceptor backbone (as predicted by Figure 1.9).

3.11 Sequence Analysis of Junction Regions in YRpXC-EGFP

Because of the error rate associated with Taq DNA polymerase (2.85 x 10^-4 base insertions, NEB ® 2002-2003 Catalog and Technical Reference) the linker regions incorporated into the final recombinant plasmid were the only sequences amplified by PCR and thus contained the greatest risk of possessing single base errors. In order to verify the integrity of the left-end and right-end junctions undergoing linker-assisted homologous recombination, dideoxy sequencing reactions were performed and results provided by Norgen Biotek Corp. The expected sequences of the recombinants are displayed in Figure 3.11.
Figure 3.11: Expected sequences of left-end and right-end regions undergoing linker-assisted homologous recombination. Regular type indicates expected acceptor fragment-derived sequences, bold type indicates expected donor fragment-derived sequences.

Position 29 in the right-end junction region was the only sequence abnormality observed and is underlined.

Of the three recombinants isolated, one recombinant (isolate 4.4) possessed a single thymine base deletion at position 29 of the CMV promoter in the left-end region of linker-assisted homologous recombination. Recombinant 7.1 contained no detectable sequence abnormalities and was subsequently termed YRpXC-GFP. YRpXC-GFP was subsequently used for all mammalian cell transfection experiments.
3.12 Green Fluorescent Protein Expression in 293 Cells

In order to confirm that homologous recombination events during \textit{in vivo} ligation had not altered reporter gene or other vital expression sequences, YRpXC-EGFP was transfected into HEK 293 cells. Green Fluorescent Protein fluorescence was observed from transfected 293 cell monolayers exposed to 488 nm wavelength light at 48 hours after transfection (Figure 3.12).

![Figure 3.12: Green Fluorescent Protein fluorescence in YRpXC-EGFP transfected 293 cell monolayers visualized by exposure to 488 nm wavelength light.](image)

It was not possible to quantify transfection efficiency due to the techniques required to examine GFP fluorescence. Expression of the GFP from YRpXC-EGFP after transfection into 293 cells verifies that \textit{in vivo} ligation reactions did not alter the function of the GFP coding or expression sequences.
3.13 Analysis of *In Vivo* Ligation Utilizing Klenow-Treated *AflIII* digested YRpXC38.

It was demonstrated that the use of acceptor plasmid linearized with cohesive-end generating restriction enzymes during *in vivo* ligation reactions allowed the regeneration of the acceptor plasmid during *in vivo* ligation reactions (96% of back-transformed ampicillin-resistant bacterial colonies harbored YRpXC38 acceptor). It has previously been demonstrated that the use of blunt-end restriction sites significantly reduces background yeast transformant numbers during *in vivo* ligation by preventing acceptor plasmid reformation by ligation of cohesive ends, thus making more substrate available for processing by the ds DNA break repair machinery (Raymond *et al.*, 1999).

Klenow treatment of the cohesive end digested plasmid vector prior to subsequent ligation and bacterial transformation has been demonstrated to vastly reduce transformation efficiency (New England Biolabs © Inc. Catalog and Technical Reference, 2002-2003). It was hypothesized that sufficient Klenow treatment of the cohesive ends would decrease acceptor plasmid reformation and resultant background yeast transformant numbers during *in vivo* ligation as well, effectively mimicking the use of blunt end digested acceptor. The observation that digestion of the acceptor with *AflIII* (which moved the site of ds DNA breakage within the rightward region of linker generated homology) provided substrate sufficient to participate in the predicted homologous recombination reaction (Figure 1.9) prompted the generation of blunt-end digested acceptor from *AflIII* digested acceptor using the Klenow fragment of *E. coli* DNA polymerase I.
The duration of Klenow fill-in of *AflII* 5' overhang fill-in necessary to mimic blunt-end YRpXC38 acceptor digestion (similar to *HpaI* restriction of the YRpXC38 acceptor) was examined. Even after a 40 minute Klenow treatment (15 minute incubation recommended by the manufacturer, NEB©), electroporation of *S. cerevisiae* JY128 with equivalent amounts (0.1 μg) of Klenow-treated *AflII* digested YRpXC38 generated 1.6-fold more trp<sup>+</sup> transformants than did electroporation with the *HpaI* digested YRpXC38 acceptor. (Table 3.2).

Table 3.2: Mean and standard deviation of trp<sup>+</sup> transformants generated after electroporation with 0.1 μg *AflII* digested YRpXC38 which was Klenow treated for different durations.

<table>
<thead>
<tr>
<th>Klenow reaction duration (min)</th>
<th>Mean +/- SD trp&lt;sup&gt;+&lt;/sup&gt; transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>(HpaI digestion</em></td>
<td>153 +/- 7.5</td>
</tr>
<tr>
<td>10</td>
<td>504 +/- 30</td>
</tr>
<tr>
<td>20</td>
<td>412 +/- 28</td>
</tr>
<tr>
<td>25</td>
<td>230 +/- 45</td>
</tr>
<tr>
<td>30</td>
<td>250 +/- 11</td>
</tr>
<tr>
<td>35</td>
<td>246 +/- 21</td>
</tr>
<tr>
<td>40</td>
<td>241 +/- 19</td>
</tr>
</tbody>
</table>

Klenow treatment vastly decreased the numbers of trp<sup>+</sup> transformants (from Figure 3.9, 5100 +/- 400 trp<sup>+</sup> transformants when *AflII* digested YRpXC38 is used to
transform *S. cerevisiae* JY128) Klenow treatment of any duration could not decrease trp\(^+\) transformant numbers to values attained when using *HpaI* digested vector. Perhaps a small population of molecules retained cohesive ends despite the treatment. A 40 minute Klenow incubation was utilized for subsequent experiments.

Co-transformation of *AflIII* digested Klenow-treated YRpXC38 acceptor, donor fragment, and LtIVL and RtIVL PCR amplified linkers into *S. cerevisiae* by electroporation resulted in an average of 450 (+/-30) trp\(^+\) colonies, approximately 2-fold more colonies than control reactions lacking donor fragment (A+L) or control reactions lacking both donor and linker DNA (A) and approximately 1.7-fold more colonies than control reactions lacking linker DNA (A+D) (Figure 3.13). Control (A+L, A+D) and experimental (A+L+D) groups differed significantly by t-test analysis (P=0.05).
Figure 3.13: Mean and standard deviation summary of knockout *in vivo* ligation reactions using *Afl*III digested Klenow-treated YRpXC38 acceptor. L, D, and A represent 1µg of each linker (LtIVL and RtILV), 1µg of 1,697 bp *Afl*III/*Afl*III pEGFP-N1 donor fragment, and 0.1µg *Afl*III digested Klenow-treated YRpXC38 acceptor, respectively. Control reactions were carried out simultaneously by omitting the donor (A+L), the linkers (A+D), or both (A).
Total plate isolations of plasmid DNA from trp+ transformants generated during these in vivo ligation experiments were back-transformed into competent E. coli. Plasmid DNA from 11 ampicillin-resistant colonies was analyzed by NcoI digestion (Figure 3.14).

Figure 3.14: 1% agarose gel showing 12 NcoI digested in vivo ligation recombinants generated using AflII digested Klenow-treated YRpXC38 (Lanes 1-11). YRpXC-GFP control NcoI digest (C) and marker DNA (M) were included.

Nine of the eleven isolated colonies possessed the expected conformation. YRpXC-GFP (9,482 bp) possesses four NcoI restriction sites at positions 741, 1058, 2207, and 3992. These sites generate NcoI RE fragments 317 bp, 1,149 bp, 1,785 bp, and 6,231 bp in length. Since earlier recombinant plasmid sequencing and GFP expression analysis had demonstrated the fidelity of in vivo plasmid construction, no further characterization of recombinant plasmids was performed.
As was the case when \textit{HpaI} digested acceptor was used (blunt end digested acceptor) a deletion recombinant resulting from intramolecular recombination within the acceptor was observed (lane 1) but was not characterized further.

Isolate 3 was subsequently determined to be supercoiled DNA of pUC19 (MBI Fermentas Inc.), which possesses no \textit{NcoI} sites. Back transformation into \textit{E. coli} was utilized and it is possible that pUC19 cross contamination from other experiments in the laboratory could have occurred.

3.14 YRpETE Construction Strategy

Klenow treatment of cohesive end digested acceptor is capable of effectively decreasing regeneration of acceptor plasmid during \textit{in vivo} ligation, however the reaction products must be isolated and analyzed at the pDNA level in order to determine which transformant yeast will harbor the recombinant plasmid of interest. This represents a significant investment in both time and effort. In order to circumvent this pDNA analysis a system to enable identifying yeast colonies harboring exclusively recombinant plasmid by examination of yeast colony phenotype was developed.

It was demonstrated that \textit{in vivo} ligation reactions are capable of deleting large segments of DNA (over 3.1 kb during secondary \textit{in vivo} ligation reactions) from acceptor molecules. If the acceptor DNA sequences being deleted during an \textit{in vivo} ligation reaction encoded some form of yeast selectable marker, deletion of that DNA would manifest itself as an observable change in \textit{S. cerevisiae} phenotype. Such a change in phenotype could then be used to distinguish between transformants harboring recombinant or parental plasmid.
In order to assess the ability of such a selectable marker "knockout" in vivo ligation strategy to decrease screening workload a second yeast selectable marker, *URA3*, was inserted into a region of the acceptor that would be deleted during a successful in vivo ligation reaction. Briefly, the *URA3* gene from pYES2 (BD Biosciences Clonetech, kindly provided by Dr. D. Inglis, Cool Climate Oenology and Viticulture Institute, Brock University) was PCR amplified with primers possessing *KpnI* recognition sequences encoded by 5' overhangs. The *URA3* fragment was then *KpnI* digested and ligated into the unique *KpnI* site occurring within the adenovirus E1 region sequences in the acceptor molecule. The resulting plasmid was termed YRpETE. The construction strategy of this molecule is detailed in Figure 3.15.
Figure 3.15: YRpETE construction strategy. The URA3 gene of pYES2 (BD Biosciences Clonetech) was PCR amplified with primers possessing KpnI recognition sequences encoded in 5' overhangs. The PCR fragment was then KpnI digested and ligated into the unique KpnI site within the E1 region of the left-end Ad sequences in the acceptor molecule.
The $URA3$ gene encodes the orotidine-5'-phosphate decarboxylase, which is an essential enzyme in the uracil synthesis biochemical pathway. The $URA3$ gene product converts the pyrimidine analog 5-fluoro-orotic acid (5-FOA) into a toxic product. Cells possessing an active $URA3$ gene, either chromosomally or episomally, are unable to grow on media supplemented with this chemical. Thus $URA3$ selection in $S.~cerevisiae$ can be either a positive or negative selection system (Boeke et al., 1984).

3.15 $URA3$ PCR Fragment Verification

Following PCR amplification of the $URA3$ selectable marker, ethanol precipitation, and resuspension in TE buffer, the size and concentration of the amplified product was determined by agarose gel electrophoresis (Figure 3.16). The URA3 fragment was approximately 1.1 kb (expected size 1,123 bp).
Figure 3.16: A) 1% agarose gel showing ethanol-precipitated resuspensions of the \textit{URA3} PCR fragment amplified from samples lacking (C) or containing pYES2 (\textit{URA3}).

Marker lane is labeled M. B) Primer sequences utilized to amplify the \textit{URA3} gene from pYES2.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>URA3F</strong></td>
<td>5'—GGG \textbf{GGTACC}GCTTTCAATTCAATTCAATC-3'</td>
</tr>
<tr>
<td><strong>URA3R</strong></td>
<td>5'—GGG \textbf{GGTACC}GGGTAATAACTGATATAATTA-3'</td>
</tr>
</tbody>
</table>

Primers URA3F and URA3R correspond to positions 3840 – 3821 and 2734 – 2754 of the vector pYES2, respectively, and include 9 base overhangs containing \textit{KpnI} sites (underlined).
3.16 Restriction Enzyme Confirmation of YRpETE

Transformation of competent *E. coli* DH5α with overnight ligation reactions of *KpnI* digested PCR-generated *URA3* fragment from pYES2 and shrimp alkaline phosphate treated *KpnI* digested YRpXC38 generated ampicillin-resistant bacterial colonies. *NdeI* screening of pDNA isolated from forty-six such ampicillin-resistant bacterial colonies revealed that only two contained YRpXC38 clones harboring the 1,111 bp *URA3* fragment (as illustrated in lane 7 of Figure 3.17). In order to determine the orientation of the *URA3* insert within the new constructs, restriction fragment banding pattern analysis was performed (Figure 3.17). Relative positions of the restriction sites used to analyze YRpETE can be seen in Figure 3.15.
Figure 3.17: 1% agarose gel showing restriction fragment banding patterns of the parental plasmid YRpXC38 (C), and the YRpETE construct (E) digested with restriction endonucleases *AflII*, *EcoRI*, *KpnI*, or *NheI/HpaI*. Marker lane is labeled M and marker fragment sizes are indicated (in Kb).

The presence of a 1.1 Kb insert was verified by comparison of YRpXC38 and YRpETE *KpnI* digests (Figure 3.17, lanes 6 and 7, respectively). Comparison of the
AflII and EcoRI fragments of YRpXC38 (lanes 2 and 4, respectively) and YRpETE (lanes 3 and 5, respectively) revealed that the insert was not duplicated. The orientation of the URA3 fragment was determined by NdeI/HpaI digest. The 0.6 Kb fragment generated by this double digest confirms that the URA3 fragment was inserted in the orientation that minimizes the distance between the unique HpaI (1637) site of YRpXC38 and the NdeI site occurring at position 140 of the URA3 PCR fragment. The exact location of the second NdeI site in YRpXC38 is not known. It is located within the bacterial origin of replication or the ampicillin-resistance gene. Both recombinants isolated possessed the same restriction fragment banding patterns. The first isolate examined was termed YRpETE and was used for all subsequent experimentation.

3.17 Analysis of URA3 Selectable Marker Functionality

YRpETE isolated from ampicillin-resistant E. coli DH5α was banded by CsCl density gradient centrifugation and utilized to transform S. cerevisiae strain JY128 by electroporation. Electroporation experiments generated an average of $7.1 \times 10^3$ trp+/ura+ transformants/μg pDNA when plated on trp−/ura− SC medium, verifying the functionality of the URA3 gene in YRpETE. YRpETE electroporation experiments plated on trp− SC plates fortified with 5-FOA generated no colonies, further verifying the functionality of the URA3 gene.

Transformation of competent E. coli DH5α with total plate isolations of trp+/ura+ S. cerevisiae transformant plasmid DNA generated ampicillin-resistant E. coli colonies. Restriction endonuclease digestion of plasmid DNA isolated from the ampicillin-resistant
E. coli transformants yielded identical banding patterns to that of digested YRpETE (Figure 3.18).

Figure 3.18: A) Illustration of NcoI sites within YRpETE. B) 1% agarose gel showing EcoRI and NcoI restriction fragment banding patterns of YRpETE (C) and pDNA isolated from ampicillin-resistant bacterial colonies (A and B) generated via total plate back transformation from trp⁺/ura⁺ yeast transformants. Marker lane is labeled M and marker fragment sizes are indicated (in Kb).
The ampicillin-resistant *E. coli* generated using isolated pDNA from the positive yeast trp⁺/ura⁺ transformants coupled with the identical banding patterns of the digested YRpETE (the EcoRI digest is identical to that described in Section 3.16, the digest NcoI is identical to that described in Section 3.3 except that the *URA3* insert contains one NcoI site creating 8,039bp and 806bp fragments from the 7,759bp NcoI fragment YRpXC38) to those of all isolates examined provided sufficient proof that positive yeast transformants indeed resulted from transformation of the yeast by YRpETE.

3.18 Knockout *In Vivo* Ligation Experiments

In order to assess the utility of the knockout *in vivo* ligation system, *in vivo* ligation experiments were carried out using *AflIII* digested YRpETE as the acceptor. Cotransformation of *AflIII*-linearized acceptor, donor fragment, and LtIVL and RtIVL PCR amplified linkers into *S. cerevisiae* and plating on trp⁻ SC selective plates resulted in an average of 10,200 (+/-500) trp⁺ colonies, approximately 1.3-fold more colonies than control reactions lacking either donor (A+L) or linker DNA (A+D) and approximately 1.4-fold more colonies than control reactions lacking both donor and linker DNA (A) (Figure 3.19).
Figure 3.19: Mean and standard deviation summary of *in vivo* ligation reactions using *AflII* digested YRpETE acceptor. L, D, and A represent 1 μg of each linker (LtIVL and RtIVL), 1 μg of 1,697 bp *AflII/AflIII* pEGFP-N1 donor fragment, and 0.1 μg *AflII* digested YRpETE acceptor, respectively. Control reactions were carried out simultaneously by omitting the donor (A+L), the linkers (A+D), or both (A).

Control reactions (A+L, A+D, A) did not differ significantly from one another by t-test analysis. However, a significant difference between control (A+L, A+D) and *in vivo* ligation (A+L+D) reactions was observed (P=0.05).
Duplicate experiments were carried out and plated on trp\(^-\) SC selective plates supplemented with 5-FOA. An average of 200 \(+/-\) 11 trp\(^+\) transformants were observed per \textit{in vivo} ligation reaction, while no transformants were observed for any control reactions.

3.19 Knockout \textit{In Vivo} Ligation Recombinant Analysis

Forty trp\(^+\) yeast transformants generated by the knockout \textit{in vivo} ligation experiments were transferred to trp\(^-\)/ura\(^-\) SC medium plates. Of these forty replica plated isolates, only one was incapable of growth on the trp\(^-\)/ura\(^-\) SC medium plates, implying that it had lost the \textit{URA3} marker and had undergone a successful \textit{in vivo} homologous recombination reaction. Plasmid DNA was isolated from this transformant and from thirty transformants growing on trp\(^-\) SC medium supplemented with 5-FOA. Similarly, resistance to 5-FOA implied that these yeast transformants had lost a functioning \textit{URA3} marker and had undergone a successful \textit{in vivo} homologous recombination reaction. This DNA was utilized to back-transform competent \textit{E. coli}. Plasmid DNA isolated from ampicillin-resistant colonies generated by each back-transformation was subsequently analyzed by \textit{EcoRI} digestion (Figure 3.20).
Figure 3.20: 1% agarose gel showing *EcoRI* digested plasmid DNA of YRpXC-EGFP control (C), knockout *in vivo* ligation generated recombinant plasmid isolated by replica plating from trp* SC medium to trp*/ura* SC medium (RP), and knockout *in vivo* ligation generated recombinant plasmids isolated using trp and 5-FOA selection (1-8). M indicates marker lane and marker fragments sizes are given (in Kb).

Whether isolated by replica plating from trp* SC medium to trp*/ura* SC medium or by selection on trp* SC medium supplemented with 5-FOA selection, all yeast transformants that possessed a ura* phenotype had undergone a successful *in vivo* homologous recombination reaction and showed identical *EcoRI* banding patterns to that of YRpXC-GFP generated by *in vivo* ligation reactions using AflII digested YRpXC38 as substrate. Since earlier recombinant plasmid sequencing and GFP expression analysis had demonstrated the fidelity of *in vivo* plasmid construction, no further characterization of recombinant plasmids was performed.
Knockout *In Vivo* Ligation Reactions Using a Partially Digested Acceptor

Given the previously observed low frequency of recombinant plasmid generation (4%) when using a unique cohesive-end generating restriction enzyme to linearize the acceptor, it would be expected that using a partially digested acceptor with three cohesive-end recognition sites would generate far too much background to be considered a feasible option. However when manipulating the large plasmids associated with adenovirus research, very often the use of non-unique restriction sites may be the only option in manipulating a particular region of the genome. Knockout *in vivo* ligation has been shown to be an extremely effective screening tool, and as such may be able to overcome the limitations of working with non-unique restriction sites.

In order to further examine the utility of the knockout *in vivo* ligation system, knockout *in vivo* ligation reactions utilizing a partial SacII digestion of the YRpETE acceptor were carried out. Figure 3.4 depicts these sites in YRpXC38 at positions 414, 3708, and 5708. Insertion of the *URA3* marker changes the positions of these sites to 414, 5004, and 6658, relative to *EcoRI*(1) in YRpETE. Since SacII sites occur on both sides of the *URA3* gene in YRpETE, purified single cut acceptor was utilized to prevent false positives that might be generated by recombinants and ligation products deleting the *URA3* selectable marker (Figure 3.21).
Figure 3.21: A) 1% agarose gel showing partial digestion series of YRpETE with SacII. Lanes 1 through 9 contain digests incubated for increasing 15 second intervals. Arrow indicates single cut fragment. B) Illustration of SacII restriction site locations in YRpETE.

Single cut SacII digested YRpETE was isolated and used as acceptor in knockout in vivo ligation reactions, and the results summarized in Figure 3.22.
<table>
<thead>
<tr>
<th></th>
<th>5.1</th>
<th>4.9</th>
<th>5.2</th>
<th>5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>0.6</td>
<td>0.9</td>
<td>1.0</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

trp+ colonies (in 1000s) per electroporation

Figure 3.22: Mean and standard deviation summary of knockout *in vivo* ligation reactions using single cut SacII digested YRpETE acceptor. L, D, and A represent 1μg of each linker (LtIVL and RtILV), 1μg of 1,697 bp *AflIII/AflIII* pEGFP-N1 donor fragment, and 0.1μg single cut SacII digested YRpETE acceptor, respectively. Control reactions were carried out simultaneously by omitting the donor (A+L), the linkers (A+D), or both (A).

Control reactions lacking donor (A+L) or linker DNA (A+D) or both (A) did not differ significantly from one another or from knockout *in vivo* ligation reactions single cut SacII digested YRpETE acceptor (A+L+D) by t-test analysis. Because no
significant difference was observed between experimental and control reactions replica plating analysis from trp$^{-}$ SC plates onto trp$^{-}$/ura$^{-}$ SC media plates was not performed. Negative selection using trp$^{-}$ SC 5-FOA supplemented fortified plates yielded an average of 50 +/- 9 trp$^{+}$ 5-FOA resistant yeast colonies per in vivo ligation reaction, while no colonies were observed as a result of control reactions. Plasmid DNA was isolated from twenty trp$^{+}$ 5-FOA resistant transformants and back-transformed into competent E. coli. Plasmid DNA from 20 ampicillin-resistant E. coli colonies produced EcoRI restriction fragment banding patterns identical to those observed in Figure 3.19.

In order to compare screening workloads accompanying knockout and traditional in vivo ligation, identical in vivo ligation reactions were carried out using single cut SacII partially digested YRpXC38 acceptor (Figure 3.23).
Figure 3.23: Mean and standard deviation summary of knockout *in vivo* ligation reactions using single cut *SacII* digested YRpXC38 acceptor. L, D, and A represent 1µg of each linker (LtIVL and RtILV), 1µg of 1,697 bp *AfIII/AfIII* pEGFP-N1 donor fragment, and 0.1µg single cut *SacII* digested YRpXC38 acceptor, respectively. Control reactions were carried out simultaneously by omitting the donor (A+L), the linkers (A+D), or both (A).

Plasmid DNA was isolated from *trp*+ yeast transformants generated during *in vivo* ligation reactions using single cut *SacII* partially digested YRpXC38 acceptor.
Restriction enzyme screening of plasmid DNA from one hundred ampicillin-resistant *E. coli* produced by back-transformation with the isolated plasmid DNA yielded only YRpXC38 parental plasmid and no recombinant plasmid.

Reaction efficiencies (trp+ yeast transformants) for knockout *in vivo* ligation reactions using partially digested single cut acceptor were two-fold lower than those obtained utilizing the *AflII* digested acceptor. The decrease in efficiency was most likely due to the three populations of acceptor utilized in the single cut *SacII* digested acceptor *in vivo* ligation reactions which would have decreased the abundance of acceptor suitable for homologous recombination. In addition, the method chosen to purify the acceptor from agarose gels could have sheared a small percentage of the acceptor molecules or rendered the DNA incapable of undergoing recombination in some other fashion.
4. Discussion

4.1 Results Relevant to General In Vivo Ligation Research

Given the ability of in vivo ligation to foster both deletions and insertions, the term recombinational cloning will be used interchangeably with the term in vivo ligation throughout the remainder of this thesis.

The main purpose of this study was to develop and evaluate protocols that would facilitate the large plasmid manipulations common to adenovirus manipulation by recombinational cloning. Successful secondary in vivo ligation experiments revealed high numbers of background yeast transformants (3/70 recombinant plasmids) that imposed a lengthy screening process in order to isolate the desired recombinant plasmid. Presumably the high frequency of acceptor plasmid regeneration reflects a tendency of S. cerevisiae to preferentially repair ds DNA breaks with cohesive ends before attempting plasmid repair through homologous recombination. Klenow treatment of linearized acceptor was found to drastically reduce the regeneration of the acceptor plasmid and decrease screening workload (3/70 recombinants as compared to 9/11 for untreated and Klenow treated acceptor, respectively). When unique restriction sites for acceptor linearization were not available, knockout recombinational cloning was shown to provide zero background screening (0/100 recombinant plasmids generated by recombinational cloning isolated from back transformed E. coli as compared to 20/20 using knockout recombinational cloning when partially digested acceptor was used in both cases). No recombinants other than those containing the predicted conformation were observed.
during knockout recombinational cloning, however only a proportion of the total number of recombinants were analyzed for each reaction and some other recombinants may have been generated and simply not observed.

*In vivo* ligation is still a relatively novel technique, and as a result the mechanisms underlying its success are not fully understood. Analysis of the failed *in vivo* ligation attempts and subsequent success bring the processes involved during *in vivo* ligation closer to being fully understood.

Initial *in vivo* ligation attempts utilizing *Hpa*I restricted acceptor (Figure 3.4) and *in vivo* ligation trials utilizing *Afl*III digested Klenow-treated acceptor (Figure 3.4) generated recombinants of unexpected structure. Recombinants generated were of two distinct populations during initial *in vivo* ligation trials (bearing a small 1.1 kb deletion flanked by *Sac*II (414) and *Kpn*I (2115), or bearing a larger 2.6-2.7 kb deletion flanked by *Sac*II (414) and *Afl*III (3596)), and only one recombinant was observed in trials utilizing *Afl*III-digested Klenow-treated acceptor.

Recombinants generated during initial *in vivo* ligation trials can be explained by examining regions of microhomology within the Ad5 left-end sequences. The E1A and E1B promoters drive early gene function, and the sequence homology reflecting that similarity in function is evident when those promoter sequences are examined (Figure 4.1).
<table>
<thead>
<tr>
<th>Gene (nt)</th>
<th>“tata” box</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1A (431-498)</td>
<td>gcgtttattattatagtcagctgacgtgtagtg tatttata cccggtgagttcctcaagaggccac</td>
</tr>
<tr>
<td>E1B (1641-1701)</td>
<td>tggcgttttaaatggggcggggcttaaaggg tatataa tgcgccgtgctaatctttgttaca</td>
</tr>
<tr>
<td>IX (3520-3584)</td>
<td>tgtgtggcgctgcttaagggtggaaagaa tatataa ggtgggggtcttatgtagttttgtatc</td>
</tr>
</tbody>
</table>

Figure 4.1: Promoters occurring in the left-end of the Ad5 genome. *AflIII* site indicated by bold type. Nucleotide positions are listed as defined by Genbank Accession X02996.

Homology between these promoter regions is not precise, but it is plainly observable even without performing sequence alignment. The TATA boxes are surrounded on either side by stretches of G/T rich runs of more than 20 bp (Figure 4.1). Mezard *et al.* (1994) demonstrated that minimal sequence homology of 2-21 bases is necessary for illegitimate recombination. Yeast recombination machinery is very efficient, and will not tolerate broken DNA (Mezard *et al.*, 1994; Mezard and Nicolas, 1994; reviewed in Sung *et al.*, 1999). In the absence of homology with which to correct ds DNA breaks, illegitimate recombination is used to correct the damaged DNA (Mezard and Nicolas, 1994). The generation of two distinct populations of deletion recombinants resulting from intramolecular illegitimate recombination within the acceptor during initial *in vivo* ligation experiments is likely due to the probability that the recombination machinery would most likely repair the gap with regions of microhomology that are proximal rather than distal to the ds DNA break. The smaller deletion is likely generated by recombination between the E1A and E1B promoter regions. This recombination event may have been favored (and thus more prevalent in 38 out of 40 recombinant plasmids.
analyzed) because the E1B promoter region more closely flanks the ds DNA break site ($HpaI$ (1637) site) than do the protein IX promoter regions involved in creating the less prevalent larger deletion product (Figures 3.4 and 4.1). The E1A promoter would be predicted to be involved in any such intramolecular illegitimate recombination reaction since it is the only region of microhomology to the E1B or protein IX promoters on its side of the ds DNA break (Figures 3.4 and 4.1). These results echo previous results obtained by Mezard and Nicholas (1994) in the efficiency of recombinant generation by illegitimate recombination ($LiCl$ transformation was used with comparable efficiency to that observed for electroporation in this report, perhaps owing to the much larger size of plasmids utilized herein), and in the generations of distinct populations of recombinants generated by intramolecular recombination in the absence of sufficient homology between broken ends of DNA fragments at the site of the ds DNA break.

The rare occurrence of the recombinant generated during Klenow-assisted $in$ $vivo$ ligation trials could be explained by assuming that a minute population of cells existed in each reaction that took up acceptor, but failed to take up linker and/or donor DNA. In such a case illegitimate recombination would be the only option by which to repair the acceptor. Additionally, since no regeneration of the acceptor was observed during these $in$ $vivo$ ligation reactions, the forty-minute Klenow treatment was sufficient to diminish the presence of cohesive-end acceptor to undetectable levels.

Only after the ds DNA break was repositioned so that the regions of linker generated homology overlapped with the site of the ds DNA break ($AfiI$ site at position 3596, and $SacII$ at position 414, Figure 3.4), was successful homologous recombination
observed and the desired recombinant obtained. The success of these reactions is most easily explained by examining the current model of the ds DNA break repair pathway of *S. cerevisiae* (Figure 3.8). This model dictates that homology to be utilized for repair must occur at (or near) the site of the ds DNA break (Sung *et al.*, 1999). When viewed in the context of the above mentioned pathway, the failure of initial *in vivo* ligation experiments to produce the desired recombinant may have been due to the lack of available homology within the acceptor near the site of the ds DNA break. The ss DNA tails generated during ds DNA break repair have been documented to be up to 1kb in length (Figure 3.8; reviewed in Sung *et al.*, 1999). However the distance between the ds DNA break and the regions of homology supplied by the linkers during initial *in vivo* ligation experiments was greater than 1.2 kb in either direction. Two fragment *in vivo* ligation reactions have in fact been successfully attempted with distances of 340 bp and 580 bp separating the regions of PCR generated homology from the ds DNA break (Oldenburg *et al.*, 1997). It is likely that distances separating the regions of linker generated homology during initial *in vivo* ligation reactions were simply too great for the yeast recombination machinery to overcome.

Once conditions conducive to successful *in vivo* ligation were provided, recombinants of the predicted conformation were observed much more frequently than were other recombinants. It is likely that once initial recombination events had occurred and incorporated the regions of linker homology, sufficient homology was present to complete the *in vivo* ligation reaction. This deduction is consistent with previous observations that repair is shunted into illegitimate recombination pathways only when homologous recombination fails (Mezard *et al.*, 1994; reviewed in Sung *et al.*, 1999).
Once conditions for successful in vivo ligation were provided the desired recombinant plasmid was observed in only 4 % (3/70) of ampicillin resistant bacterial colonies analyzed during in vivo ligation reactions using AflIII digested YRpXC38. If the assumption is made that the numbers of bacterial transformants harboring the recombinant plasmid of interest are proportional the to numbers of yeast transformants harboring the same plasmid, expanding this percentage using the average total number of trp\(^+\) yeast transformants generated during these in vivo ligation reactions (11000 positive transformants, from Figure 3.9) yields an expected number of 471 recombinants of the desired conformation. An average of only 200 recombinants was achieved during knockout in vivo ligation reactions plated on trp\(^-\)/5-FOA supplemented SC medium when the same restriction site (AflII) was utilized to linearize the acceptor. Since no yeast colonies growing on trp\(^-\)/5-FOA supplemented SC medium were observed to harbor plasmid other than the desired recombinant, it will be assumed that all 200 of this average carry the plasmid of interest (though this could only be determined by direct examination of all yeast colonies’ pDNA). Despite the 1.1 kb increase in size of the YRpETE acceptor, no significant difference in transformation efficiency was observed between the in vivo ligation and knockout in vivo ligation experiments (by Student’s t-test). Since no detectable difference in transformation efficiency was detected, a comparison of these two values (471 and 200 yeast transformants carrying the desired plasmid recombinant) indicates a decrease in efficiency as the size of the deletion increased. However since the total numbers yeast colonies harboring the desired recombinant plasmid generated must be implied from measured data and were not directly measured, no definitive statements about the effect of increasing deletion size can be made from this report.
The restriction sites necessary for incorporation of both the yeast selectable markers and ARS sequences were present in positions convenient for the construction of plasmids described herein. This may not be the case for other cloning vector systems. The TRP1/ARS and URA3 fragments mentioned in this report are sufficiently short that they can be amplified by PCR and subsequently utilized for two fragment recombinational cloning reactions (Figure 4.2). Furthermore, if such a strategy were to be undertaken, integration events and PCR and recombination-induced mutations disrupting the proper function of these markers or replication sequences would be effectively filtered out by the selective pressure of plating on the appropriate yeast selective medium and recovery of the recombinant plasmid by total plate back-transformation into *E. coli.*
Figure 4.2: General strategy for the creation of recombinational cloning vectors by two-fragment recombinational cloning.

In hindsight, a similar strategy could have been implemented to insert the TRP1/ARS fragment into pXC38, and the URA3 fragments into the YRpXC38 plasmid. In fact, I have successfully attempted such a strategy during the production of general-purpose knockout recombinational cloning vectors (unpublished data).

Given the advantages of recombinational cloning over traditional in vitro DNA manipulation techniques it may be only a matter of time before all cloning vectors
possess the necessary yeast replication and selectable marker sequences to facilitate both traditional and recombinational cloning.

4.2 Future Ad Research Involving Recombinational Cloning.

Because the adenovirus system presents a unique set of challenges in successfully manipulating the genome, other facets of Ad research presenting similar challenges to rescue vector manipulation could benefit from the use of in vivo ligation strategies.

One of the major hurdles of Ad mediated gene therapy has been a mounted immune response against both the vector itself and the transgene product (Yang et al., 1994; Dai et al., 1995; van Ginkel et al., 1997). The discovery of an immune response directed against first-generation Ad vectors has spawned the development of increasingly crippled vectors: second-generation vectors with additional deletions in E2, E3, and E4 regions, and third-generation "gutless" Ad vectors lacking all viral genes. Unfortunately, these vectors continue to suffer from various weaknesses such as residual low-level viral gene transcription and low-level replication competent adenovirus contamination, respectively (reviewed in Benihoud et al., 1999). Research into novel second generation Ad vectors is particularly hampered by the current time investment involved in creating novel recombinants, since the trade-off between cost and benefit of each combination of viral genes must be analyzed and helper-cell lines expressing viral genes deleted from matched viral genomic vector systems must be developed for each combination. The use of in vivo ligation to generate large site-specific deletions would vastly simplify matched
genomic vector construction strategies by enabling the researcher to extend adenovirus genome deletions to exact nucleotide positions, thus preventing homologous recombination between helper-cell encoded viral genes and the viral genome (Figure 4.3).

Figure 4.3: Construction strategy for generating matched genomic vectors for novel helper-cell lines. Virus genomic deletions must extend beyond the boundaries of virus sequences integrated into the cell-line genome to prevent homologous recombination and rescue of the deleted virus sequences into the viral genome.
Likewise, modification and control of Ad vector tropism by genetic engineering of the capsid (Dmitriev et al., 2002; recently reviewed in Nicklin and Baker, 2002 and Barnett et al., 2002) is limited only by the speed at which novel recombinants can be generated. These targeted Ad vectors have shown great promise for not only gene therapy, but for controlling immune responses involved in transplantation rejection (Bilbao et al., 2002) and for cancer therapy (Suzuki et al., 2001).

If only small insertions must be made to an acceptor, as may be the case when adding short nucleotide sequences encoding targeting peptides to coding regions for capsid proteins, the site-specific mutagenesis strategy offered by Kitagawa and Abdulle (2002) would be the ideal alternative to a tedious in vitro insertion strategy. Overlapping regions of homology encoding insert sequences created entirely by PCR would allow targeted, in-frame insertions at virtually any position within the genome where a restriction site is available to linearize the acceptor (Figure 4.4).

The advantage of this system over traditional linker insertion strategies lies in the fact that oligonucleotides can only be generated up to a maximum length of 110 bases (www.genosys.com). This grossly limits such strategies due to lack of convenient restriction sites proximal to the region to be altered. PCR/recombination-based strategies would allow the use of more convenient, but distal restriction sites, because PCR can easily generate products of greater than 1kb for recombinational cloning.

Such strategies may require the addition of a proof-reading enzyme. In this study a sequence error was detected in one of the three in vivo ligation generated recombinants
within a region of linker generated homology and similar errors would be more abundant in sequences derived from overlapping linker regions.

Figure 4.4: Insertion/deletion recombinational cloning strategy utilizing PCR generated insert donor DNA (white block).

As a consequence of tropism modified Ad vectors becoming able to infect a growing number of cell types, the need for the transgene expression to be tightly regulated will be of the highest priority. Immune response directed against transgene products in unintended cell types, in particular professional antigen presenting cells, can be abrogated through the use of tissue-specific promoters (Weeratna et al., 2001). Such
tissue-specific (Larochelle et al., 2002; Ribault et al., 2001) and tumor-specific (Qiao et al., 2002) promoters have been used successfully in this capacity.

With the discovery of novel tissue-specific (Asnagli et al., 2002; Wolfe et al., 2002) and tumor-specific (Cao et al., 1998) promoters, the development of novel second and third generation Ad vectors, and novel tropism modification strategies becoming available, improved construction strategies for generating Ad vectors will be required. *In vivo* ligation and knockout *in vivo* ligation have been shown to be extremely effective Ad genome manipulation tools.

Yeast artificial chromosome (YAC) (Ketner et al., 1994) and *E. coli* plasmid-based recombinational cloning systems (Chartier et al., 1996; Crouzet et al., 1997; He et al., 1998; Souza and Armentano, 1999; Youil et al., 2001) for the creation of Ad vectors offer the ability to manipulate the entire viral genome by relying on the high rates of homologous recombination in *E. coli* and *S. cerevisiae*. Recombinant full-length viral genomes are generated by recombination between small targeting vectors containing homology to the region of the viral genome to be altered and large bacterial plasmids or YACs containing the viral genome. In addition, these recombinant vectors can then be restriction enzyme digested releasing full-length recombinant viral genomes. Virus rescue efficiency by transfection of permissive cell-lines with these full-length recombinant genomes is far more efficient than virus rescue by plasmid co-transfection and subsequent *in vivo* recombination (Ketner et al., 1994; Chartier et al., 1996; Crouzet et al., 1997; Kojima et al., 1998). While these methods improve virus rescue efficiency, they have yet to become widely utilized. The production of the recombinational targeting
vectors used to alter the viral genome is hampered by restriction enzyme recognition site availability. Consequently, these methods currently offer no improvement over traditional \textit{in vitro} manipulation of the viral genome.

Using \textit{in vivo} ligation and/or knockout \textit{in vivo} ligation to generate plasmids for recombinationally targeted cloning for use with these above mentioned systems would allow unprecedented ease of Ad genome manipulation in regions of the genome with previously poor accessibility due to a lack of available, unique restriction sites. This report demonstrates that insertions and deletions can be made using \textit{in vivo} ligation strategies described herein without the need for unique or multiple restriction sites. Such a system would offer the potential to produce many novel recombinant Ad vectors with minimal construction time.

The final hurdle to overcome in Ad-mediated gene delivery is the abrogation of the immune response to Ad gene therapy vectors and their transgene products. Progress in this field is limited only by the ability to successfully generate novel recombinants. The \textit{in vivo} ligation systems described herein may offer the potential to bring Ad-mediated gene replacement therapy, cancer therapy, and vaccination out of the realm of science fiction and into the realm of routine medical procedure.

4.3 Future Research Questions

This report has demonstrated the importance of the position of the ds DNA break relative to the position of linker-supplied homology during \textit{in vivo} ligation mediated
insertion and deletion reactions. However, new questions have been generated by this discovery. To what extent does the size of the deletion to be made depend on the amount of the linker-provided homology? Also, to what extent (if any) can the position of the ds DNA break within the acceptor extend beyond the regions of linker-supplied homology? In Section 3.8 it was demonstrated that a 1,197 bp region separating the DS DNA break and the leftward site of linker-supplied homology inhibited successful in vivo ligation.

As previously mentioned, two fragment in vivo ligation reactions have been successfully attempted with distances of 340 bp and 580 bp separating the regions of PCR-generated homology from the ds DNA break (Oldenburg et al., 1997). As mentioned herein, it is likely that a limiting factor defining acceptable distances from the PCR-generated homology to the site of acceptor linearization is the size of the ss DNA tails produced during ds DNA break end processing. These two reports could be used as a starting point to define the upper limits of distances separating ds DNA break from PCR-generated homology which still produce successful recombinational cloning.

Finally, it has always been assumed that in vivo ligation operates through the ds DNA break/repair pathway (Raymond et al., 1999). However, data presented herein suggest that it is likely that episomal DNA replication and subsequent repair is the mechanism underlying successful in vivo ligation.

Current hypotheses regarding replication fork faltering and repair in yeast rely heavily on the concept that replication forks can collapse, while homologous recombination can be utilized by the cell to repair these faltering replication forks.
(Kuzminov, 2001, and references therein). Repair of ds DNA breaks by homologous recombination is believed to be initiated by faltering DNA replication events as follows:

i) As a replication fork encounters an unrepaired DNA lesion in the template DNA, it collapses, generating a ds end. (Figure 4.5, A to B to C).

ii) Repair enzymes are recruited and the double-strand end is processed to produce a 3' ssDNA overhang, which is subsequently complexed with ssDNA binding proteins (Figure 4.5, C).

iii) This complex mediates invasion or the ssDNA end into homologous DNA sequences to be used as repair substrate, and forms a recombination intermediate resembling a replication fork (Figure 4.5, D).

iv) Subsequent resolution of the Holliday junction behind the reassembled fork structure (Figure 4.5, D to A) restores template DNA integrity allowing replication to continue by "excising" template DNA containing the break.
Figure 4.5: Pathway of replication fork stalling/collapse with subsequent resetting/repair. DNA duplexes are shown as double lines. (A) A replication fork. (B) The replication fork approaching a ds DNA break. (C) The replication fork collapses at the interruption and the ds end and is enzymatically degraded to produce a 3’ overhang. (D) Invasion of homologous DNA and resolution of the Holliday junction to restore replication fork structure. Newly replicated DNA is indicated by hatched lines.

According to this view of coupled DNA replication and repair, recombination induced by a block in replication generated by a ds break would only proceed if sufficient homology at or near the site of the ds DNA break were present. One can envision the \textit{in vivo} ligation reactions performed in this report as a series of replication fork falterings and reassembly. The ds DNA break would lead to replication fork recession and strand
invasion within the regions of homology provided by the appropriate linker and subsequent repair by homologous recombination. This process would repeat itself incorporating the donor fragment and the second linker region into the growing DNA duplex. The only way to regenerate the circular topology of the plasmid would then be for the final linker mediated homologous recombination to occur.

The most compelling evidence that DNA repair necessary for recombinational cloning initiation by replication rather than by the ds break repair pathway comes from the observation that initial *in vivo* ligation reactions produced no detectable recombinant plasmid. Donor and linker fragments were available as substrate for the ds DNA break repair pathway at much higher concentrations than the acceptor during initial *in vivo* ligation reactions, and yet these reactions failed to produce the desired recombinant plasmid. It was only after repositioning of the ds DNA break during secondary *in vivo* ligation reactions that successful recombinational cloning was detected. Furthermore, it has been demonstrated that intact donor plasmid can be used as substrate for recombinational cloning (Gunyuzlu et al., 2001). These data demonstrate that the position of acceptor linearization is a key to successful recombinational cloning and provides some basis for the argument that recombinational cloning is indeed initiated by acceptor fragment replication rather than by the ds DNA break repair pathway, as was originally assumed (Oldenburg *et al.*, 1997; Raymond *et al.*, 1999). Undoubtedly, both pathways ultimately contribute to the final outcome. The question of which system, ds DNA breakage repair or DNA replication leading to subsequent DNA repair initiates *in vivo* ligation should be definitively addressed.
5. Conclusions

The primary goal of this research was to analyze the ability of \textit{in vivo} ligation to manipulate the adenovirus system in an attempt to circumvent many of the problems associated with \textit{in vitro} manipulation by traditional cloning techniques. By mimicking a deletion that involved a cumbersome multi-step construction strategy, we have demonstrated that \textit{in vivo} ligation can hasten production of rescue vectors by creating precise insertions and deletions in a single step without the need for multiple or unique restriction enzyme recognition sites.

Traditionally, recombinational cloning systems have utilized YEp or YCp yeast plasmid systems. Insertion of these large sequences in addition to the necessary yeast selectable markers into traditional cloning vectors involves time-consuming multi-step cloning procedures. The discovery that the 1,456 bp \textit{TRP1/ARS} fragment of YRp7, which is small enough to be readily amplified by PCR, is sufficient for efficient \textit{in vivo} ligation may open up the advantages of recombinational cloning to systems that have previously not examined the possibility of utilizing recombinational cloning.

It has been generally assumed that the amount of PCR-generated homology was the most important aspect of any \textit{in vivo} ligation reaction (Oldenburg et al., 1997; Hua et al., 1997). Herein we demonstrated that not only the amount and location of PCR-generated homology, but the location of the ds break relative to that homology, and the size of deletions being carried out should be viewed as essential elements to be considered in each \textit{in vivo} ligation reaction.
Modification of existing recombinational cloning protocols as described herein facilitates the use of recombinational cloning strategies that are otherwise impractical for use with larger plasmid systems. Klenow fill-in reactions of cohesive ends in digested acceptor DNA and yeast selectable marker deletion have been determined to vastly decrease screening workload for recombinant plasmids. The zero background screening system utilizing \textit{URA3} gene deletion coupled with simultaneous 5-FOA positive selection and negative selection has been termed 'knockout' \textit{in vivo} ligation.

The ability of \textit{in vivo} ligation to produce site-specific recombination without the need for unique or multiple restriction enzyme recognition sites represents a substantial improvement over current cloning techniques. The ability of knockout recombinational cloning to generate precise insertions and deletions in a short time while providing zero background screening could be essential in the development of systems requiring precise DNA manipulation.
References


