The importance and efficiency of the recombineering-based methods of BAC Clones for the different uses of functional genomic studies

William A. Ramos*, Kirk W. Beisel.
Department of Biomedical Sciences Creighton University 2500 California Plaza Omaha, NE 68178

Abstract

The natural and homologous formations of genetic combinations, termed recombination, on the phage-based bacterium Escherichia coli have recently been developed. Recombination creates feasible systems or methods, thus, allowing DNA to be sub cloned or modified into plasmids, BACs (Bacterial Artificial Chromosome), or PACs (P1-derived Artificial Chromosome) all of which is devoid of the need for restriction enzymes or DNA ligases. Therefore, homologous recombination is the new form of chromosome engineering, which promises great efficiency because it copiously decreases the time needed to create transgenic or knockout/knockin models by conventional ways. Here, we describe the manipulative construction of one of the genes, Kcnq4, derived from two species mice and humans, and the application of a mini-lambda that integrates for excellent BAC engineering. We intend to generate a transgenic model by using recombineering-based methods by isolating large constructs of genomic DNA-free BAC Clones from the KCNQ4 gene then undergoing screening methods to identify the appropriate BAC clone to use. Once we finish identifying the BAC clones, we proceed to the generation of a mini-lambda that possibly provides the Red recombination functions and can be easily introduced by electroporation into any Escherichia coli strain. As a result, with the assistance from the preceding recombination methods, our goal of finding the molecular relationship of the inner and outer hair cells with inherited types of hearing impairments, such as Dominant Progressive Hearing Loss, would become a reality.

Introduction

Modern day science is completing the gaps and refining the flaws initiated by older day science for further identification and analyses of the general sequence of the human genome. Thus, in the next few years this sequence will be completed and interpreted. However, modern day science and its scientists, from an array of various fields, face numerous challenges such as determining how each of these genes functions independently and dependently of other genes, and understanding the broad developmental system or program of a human. Therefore, it is essential to derive the unknown answers from these challenges by creating model organisms and studying these model organisms, such as the mouse, for future references.

The ability to introduce and ponder practically any manipulated or mutated segment of genomic DNA into a mouse genome is an excellent approach for clarifying

questions regarding gene function. Thus, null and legitimate mutations can be introduced into any gene of the mouse genome using homologous-recombination-based methods and gene-targeting technology methods. The reason being is that the mouse and human genomes are extremely similar in many aspects of successful gene functions. In addition, during the past two decades transgenic and knockout/knockin technologies have indicated that the mouse genome allows gene function studies which are not possible in many other organisms.

Unfortunately, there is a major limitation of promising any accurate results with classical transgenic technologies, which are the generation of these transgenic and knockout/knockin models, because they require the creation of complicated targeting and selection constructs, such as restriction enzymes and DNA ligases, to modify the DNA. Traditional genetic engineering technology, which consisted of the usage of numerous restriction enzymes and DNA ligases to cut and rejoin the DNA fragments from the mutation, becomes inaccurate when its encountered with cloning vectors and its specific target site contains hundreds of kb of DNA. The reason being, even rare restriction-enzyme sites occur frequently on large DNA molecules, such as BACs and the bacterial chromosome, making the discovery of these unique sites extremely unfeasible. Therefore, once BAC cloning technology becomes available in Escherichia coli form, in the classical approach, modifying the available BAC clones befalls as the primary concern for further experimentation.

In the past years, highly efficient phage-based homologous recombination systems have been developed, thus easily abridging the generation of transgenic or Knockout/Knockin constructs because it simplifies the possibility of engineering large segments of DNA, such as the segments found on BACS that duplicate at less frequency in Escherichia coli. The usage of phage-based homologous recombination for genetic and chromosome engineering has been termed recombineering. Furthermore, recombineering in Escherichia coli offers numerous advantages over the previous choice of yeast; manipulating recombinant DNA produced in yeast form was extremely arduous and tedious because it had to be transferred to Escherichia coli form for subsequent procedures (Copeland et al 2001). In contrast, Escherichia coli permits the DNA produced in its form to be completed directly without the need of subsequent procedures for transferring the DNA. Most importantly, recombineering facilitates and encourages the creation of more refined and accurate transgenic mouse models of human diseases, in our case hearing impairments.

Efficient recombineering on the Escherichia coli is feasible by the use of phage-encoded proteins, suitable enough are those encoded by the Red genes of the lambda bacteriophage, which permits linear double-strand DNA fragments to be inserted into DNA cloned on BACs (Liu et al 2003). Red recombination entails three genes: exo, bet, and gam, even though two (exo and bet) are required for recombination. Exo gene encodes a 5'-3' exonuclease that produces 3' overhangs from introduced double-stranded DNA targeting cassettes (Warming et al 2005). The bet gene encodes a pairing protein that binds to the 3'overhangs and mediates its annealing and homologous recombination with complementary DNA present on the BAC (Warming et al 2005). Thus, using homologous recombination mediated by the lambda phage Red proteins we can engineer, more proficiently, large DNA fragments cloned into BACs. Specifically, by transferring the BAC clones into bacterial cells that shelter a defective lambda prophage. Introducing

the mini-λ phage into a BAC recombinant allows the BAC recombinant to inherit all of the Red recombination functions. Thus, the mini-λ provides a tractable system to express transiently the phage recombination genes at high levels (Court et at 2003).

Methods

Isolation of BAC DNA from the gene *Kcnq4*

Isolation of large constructs of genomic BAC clones was performed using a protocol from the Qiagen kit. It instructed as follows:

(1)Isolate a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for ~8 h at 37°C with vigorous shaking (~300 rpm). (2)Dilute 0.5–1.0 ml of the starter culture into 500 ml selective LB medium (1/500 to1/1000 dilution). (3)Grow at 37°C for 12–16 h with vigorous shaking (~300 rpm). (4)Harvest the bacterial cells by centrifugation at 6000 x *g* for 15 min at 4°C. (5)Resuspend the bacterial pellet in 20 ml Buffer P1. (6) Add 20 ml Buffer P2, mix gently but thoroughly by inverting 4–6 times, and incubate at room temperature for 5 min. (7) Add 20 ml chilled Buffer P3, mix immediately but gently by inverting 4–6 times, and incubate on ice for 10 min. Centrifuge at ≥20,000 x *g* for 30 min at 4°C. (8) Remove supernatant containing BAC/PAC/P1/cosmid DNA promptly. (9) Filter the lysate through a folded filter prewetted with distilled water. (10) Precipitate DNA by adding 0.6 volumes (approximately 36 ml) room-temperature isopropanol to the cleared lysate. Mix and centrifuge immediately at ≥15,000 x *g* for 30 min at 4°C. Carefully decant the supernatant. (11) Wash DNA pellet with 5 ml room-temperature 70% ethanol, and centrifuge at ≥15,000 x *g* for 15 min. Carefully decant the supernatant without disturbing the pellet. Place the tube containing the DNA pellet upside down on a paper towel and allow the DNA to air-dry for 2–3 min. Carefully remove any additional liquid visible on the tube opening and carefully redissolve the DNA in 9.5 ml Buffer EX, until the DNA is completely dissolved. (12) Add 200 µl ATP-Dependent Exonuclease and 300 µl ATP solution to the dissolved DNA, mix gently but thoroughly, and incubate in a water bath or heating block at 37°C for 60 min. (14) Equilibrate a QIAGEN-tip 500 by applying 10 ml Buffer QBT, and allow the column to empty by gravity flow. Add 10 ml Buffer QS to the DNA sample from step 12, apply the whole sample to the QIAGEN-tip, and allow it to enter the resin by gravity flow. (15) Wash the QIAGEN-tip with 2 x 30 ml Buffer QC. (16) Elute DNA with 15 ml Buffer QF, prewarmed to 65°C. (17) Precipitate DNA by adding 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥15,000 x *g* for 30 min at 4°C. Carefully decant the supernatant. (18) Wash DNA pellet with 5 ml room-temperature 70% ethanol, and centrifuge at ≥15,000 x *g* for 15 min. Carefully decant the supernatant without disturbing the

pellet. (19) Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5).

After the experiment, I dissolved the BAC pellet in 600 μg of TE buffer; to confirm its presence and determine its quantitative result, I ran the solution on an agarose gel (0.75%).

Engineering of BAC DNA via mini-λ. Purification of mini-λ.

The mini-λ cells were plated on an LB agar plate containing the selected antibiotic, tetracycline, and incubated overnight at 32 degrees Celsius. Isolated colonies were selected for purification of mini-λ DNA. The purification of mini-l DNA was performed using a protocol from a Qiagen kit (Court et al 2003). It instructed as follows: (1) Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for approximately 8 h at 37°C with vigorous shaking (approx. 300 rpm). (2) Dilute the starter culture 1/500 to 1/1000 into 3 ml selective LB medium. Grow at 37°C for 12–16 h with vigorous shaking (approx. 300 rpm). (3) Harvest the bacterial cells by centrifugation at 6000 x *g* for 15 min at 4°C. (4) Resuspend the bacterial pellet in 0.3 ml of Buffer P1. (5) Add 0.3 ml of Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4–6 times, and incubate at room temperature (15–25°C) for 5 min. (6) Add 0.3 ml of chilled Buffer P3, mix immediately and thoroughly by vigorously inverting 4–6 times, and incubate on ice for 5 min. (7) Centrifuge at maximum speed in a microcentrifuge for 10 min. Remove supernatant containing plasmid DNA promptly. (8) Equilibrate a QIAGEN-tip 20 by applying 1 ml Buffer QBT, and allow the column to empty by gravity flow. (9) Apply the supernatant from step 7 to the QIAGEN-tip 20 and allow it to enter the resin by gravity flow. (10) Wash the QIAGEN-tip 20 with 2 x 2 ml Buffer QC. (11) Elute DNA with 0.8 ml Buffer QF. (12) Precipitate DNA by adding 0.7 volumes (0.56 ml per 0.8 ml of elution volume) of room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥10,000 rpm for 30 min in a microcentrifuge. Carefully decant the supernatant. (13) Wash DNA pellet with 1 ml of 70% ethanol and centrifuge at 10,000 rpm for 10 min. Carefully decant the supernatant without disturbing the pellet. (14) Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10mM Tris·Cl, pH 8.5).

After the experiment, I dissolved the mini-λ pellet in 300 μg of H_2O; to confirm its presence and determine its quantitative result, I ran the solution on an agarose gel (0.75%).

Results

Isolation of BAC Clones dissolved in 600 microliters of TE Buffer. Digestion of BAC Clone recombinant with premeditated restriction enzymes was successful and accurate. Growth of mini-lambda colonies on a LB agar plate with the tetracycline antibiotic was successful. The usage of the bacteriophage lambda recombination system Red on the BAC Clone recombinant indicated the possibility of using this system for

manipulation of BACS by homologous recombination is feasible. Thus, mini-λ can modify BACs, which is isolated from the gene *Kcnq4* that is susceptible to the hearing impairment Dominant Progressive Hearing Loss, with high-targeting efficiency to generate minor or major changes without the use of any selectable markers.

Conclusion

The preceding experiments show optimistic promises for our long-term objective of finding the molecular relationship of the inner and outer hair cells with inherited types of hearing impairments, such as Dominant Progressive Hearing Loss.

References

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