

Methodology article

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Recombining overlapping BACs into a single larger BAC

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Abstract

Background: BAC clones containing entire mammalian genes including all the transcribed region and long range controlling elements are very useful for functional analysis. Sequenced BACs are available for most of the human and mouse genomes and in many cases these contain intact genes. However, large genes often span more than one BAC, and single BACs covering the entire region of interest are not available. Here we describe a system for linking two or more overlapping BACs into a single clone by homologous recombination.

Results: The method was used to link a 61-kb insert carrying the final 5 exons of the human *CFTR* gene onto a 160-kb BAC carrying the first 22 exons. Two rounds of homologous recombination were carried out in the EL350 strain of bacteria which can be induced for the Red genes. In the first round, the inserts of the two overlapping BACs were subcloned into modified BAC vectors using homologous recombination. In the second round, the BAC to be added was linearised with the very rare-cutting enzyme *I-PpoI* and electroporated into recombination efficient EL350 bacteria carrying the other BAC. Recombined BACs were identified by antibiotic selection and PCR screening and 10% of clones contained the correctly recombined 220-kb BAC.

Conclusion: The system can be used to link the inserts from any overlapping BAC or PAC clones. The original orientation of the inserts is not important and desired regions of the inserts can be selected. The size limit for the fragments recombined may be larger than the 61 kb used here and multiple BACs in a contig could be combined by alternating use of the two pBACLink vectors. This system should be of use to many investigators wishing to carry out functional analysis on large mammalian genes which are not available in single BAC clones.

Background

Sequenced BAC and PAC clones are now available for most of the human and mouse genomes and have proven extremely useful in the analysis of gene function. The large insert size of the clones allow many mammalian genes to be cloned intact with all the long range controlling elements and BAC transgenics have generally given physiological levels of tissue specific expression in transgenic mice (for example [1,2]). In addition to the sequenced

BACs, there are also databases of BAC end sequences http://www.tigr.org/tdb/humgen/bac_end_search/bac_end_intro.html which allow one to locate additional BACs covering a specific region. However, there are many large mammalian genes which are of the same order of size, or larger, than the average insert size of the BAC libraries (about 170-kb) and for these it is often difficult to find a single BAC spanning the entire gene and controlling elements. For these genes, large gene clusters and

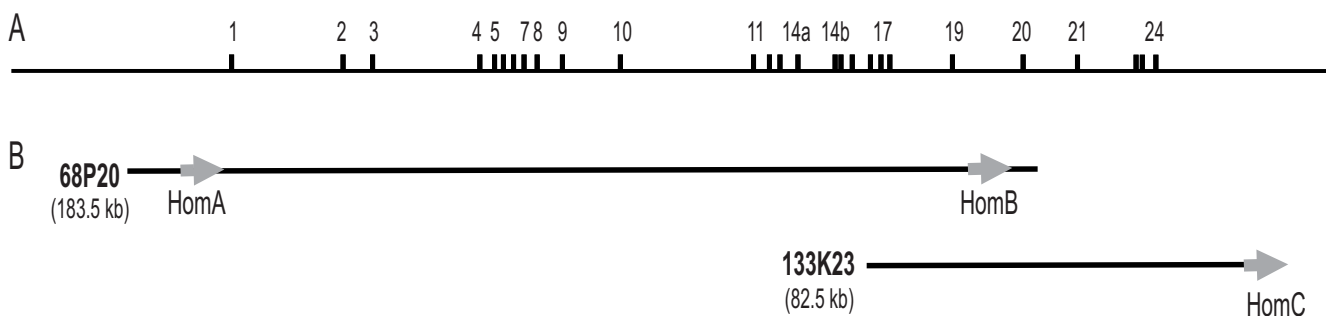


Figure 1

Organization of the *CFTR* gene and BAC clones A) Map showing the organization of the human *CFTR* gene as 27 exons spread over ~200 kb of chromosome 7q. Note that the original numbering of the exons goes up to 24, but extra exons, such as 6b, were found and numbered later. B) Map of the two overlapping BACs, 68P20 and 133K23, and the regions of homology (HomA, HomB, and HomC) which were used during the subcloning and linking steps.

other large functional regions of DNA, it will be useful to be able to link together available BAC or PAC clones into a single clone spanning the desired region.

Recently, highly efficient homologous recombination systems have been developed in *E. coli* that allow modifications of large BACs without the use of restriction enzymes and ligases, a process called recombineering [3-5]. A very efficient recombineering method is based on the Red recombination system of bacteriophage λ [6]. Modified DH10B strains have been produced with the Red recombination genes (*exo* and *bet*), and the *gam* gene, integrated into the *E. coli* chromosome under the control of the temperature sensitive λ cI857 repressor [7]. The repressor is inactivated at 42°C allowing the genes to be expressed so that recombination can take place.

Homologous recombination in *E. coli* has been used for the modification of BAC inserts to introduce reporter genes or subtle mutations for functional analysis (reviewed in [4]). It has also been used for the rapid construction of vectors for carrying out gene knockouts in mouse embryonic stem cells and to subclone desired regions from BACs. Recently, Red recombination was used to insert 22.5-kb of adjacent DNA from one BAC into another [8].

Here we describe a method which allows one to link the inserts of two, or more, overlapping BACs or PACs into a single BAC clone using the Red homologous recombination system. We have used this system to recombine together two BACs spanning the transcribed region of the *CFTR* gene.

Results

Recombination strategy

The coding region of the *CFTR* gene is present in 27 exons spanning about 200-kb of DNA (Fig. 1A). Although various BACs containing parts of the *CFTR* gene have been described, none are available containing all the exons. Two sequenced, overlapping BACs are available as part of the human genome project. Sequence alignment shows that the first BAC, 68P20 (Ac000111), is 183.5 kb in size and contains the first 23 exons and the second, 133K23 (Ac000061), is 82.5 kb in size and contains the last 9 exons of the gene. There is a 34-kb overlap of the two clones as shown in Figure 1B.

Figure 2 shows the overall strategy for using homologous recombination to recombine the two overlapping BACs, BAC1 and BAC2. In the first step, the desired regions of the BAC inserts are subcloned, by homologous recombination, into new BAC-linking vectors. This may not include the entire region of the BAC inserts if smaller regions are desired. In addition, it is not necessary to subclone BAC1 if the region of overlap of the two BACs is adjacent to the chloramphenicol resistance gene (not the case for the *CFTR* BACs). In the second step, BAC2 is linearised at the very rare *I-PpoI* sites in the BAC-linking vector and recombined onto BAC1 using one region of homology in the overlapping region of the two BACs (HomB), and the other region in the vector (the 5' region of the chloramphenicol resistance gene, Cm^r).

Modification of the original BACs

The first step of the overall strategy shown in Figure 2 is to "subclone" the BAC inserts, by homologous recombination, into the BAC-linking vectors. For each BAC a subcloning BAC vector was constructed (Fig. 3). These subcloning vectors carry the single copy origin of

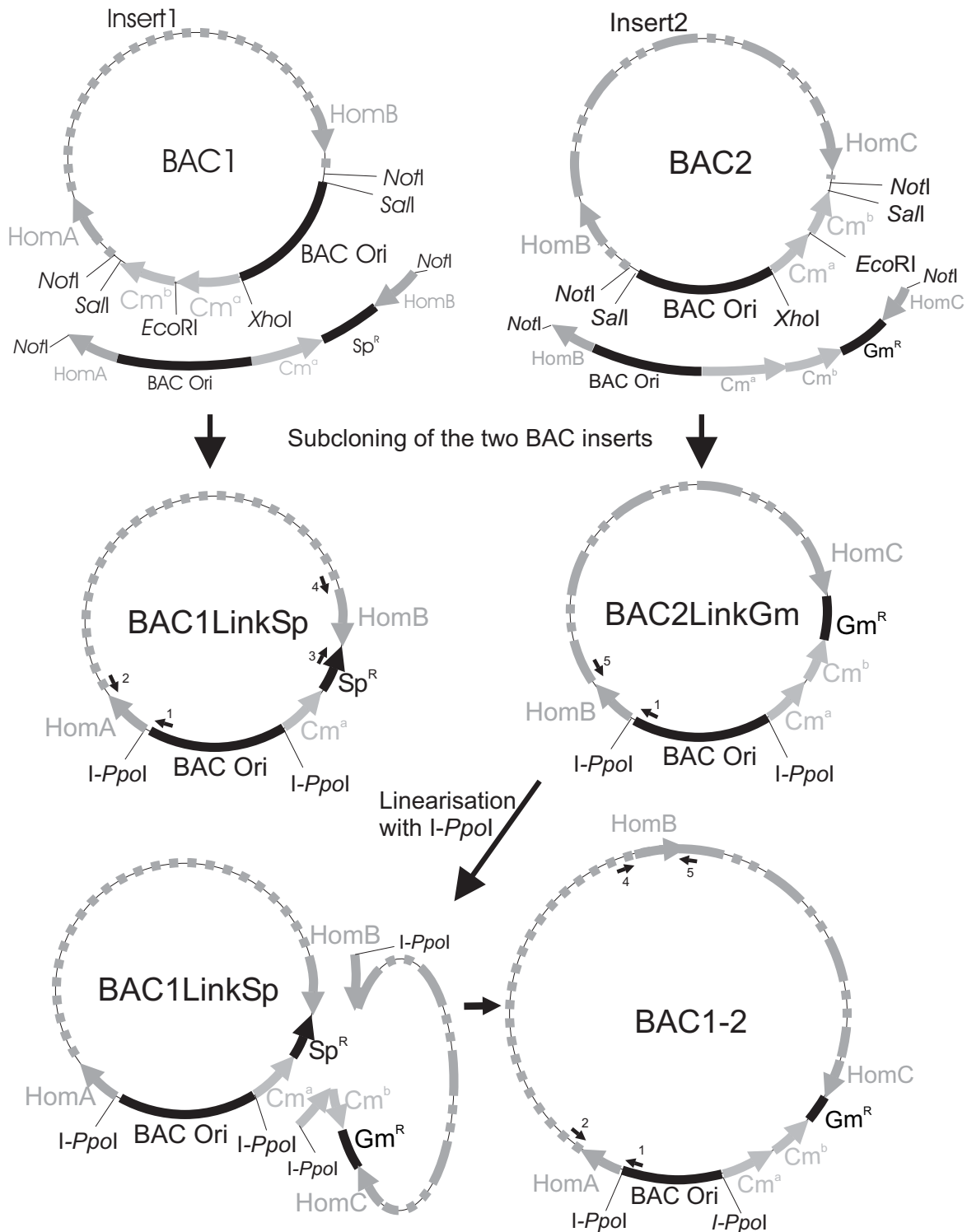


Figure 2

Strategy for subcloning the two BAC inserts and linking the BACs together BAC1 is recombined with pBACLink-SpAB linearized with *NotI* to give BAC1LinkSp. BAC2 is recombined with pBACLinkGmBC linearized with *NotI* to give BAC2LinkGm. BAC2LinkGm is linearized with *I-Ppol* and transfected into EL350 bacteria carrying BAC1LinkSp. Correctly recombined clones should be gentamycin resistant, chloramphenicol resistant and spectinomycin sensitive. The primers used to check each homologous recombination event are indicated as arrows labeled 1 to 5.

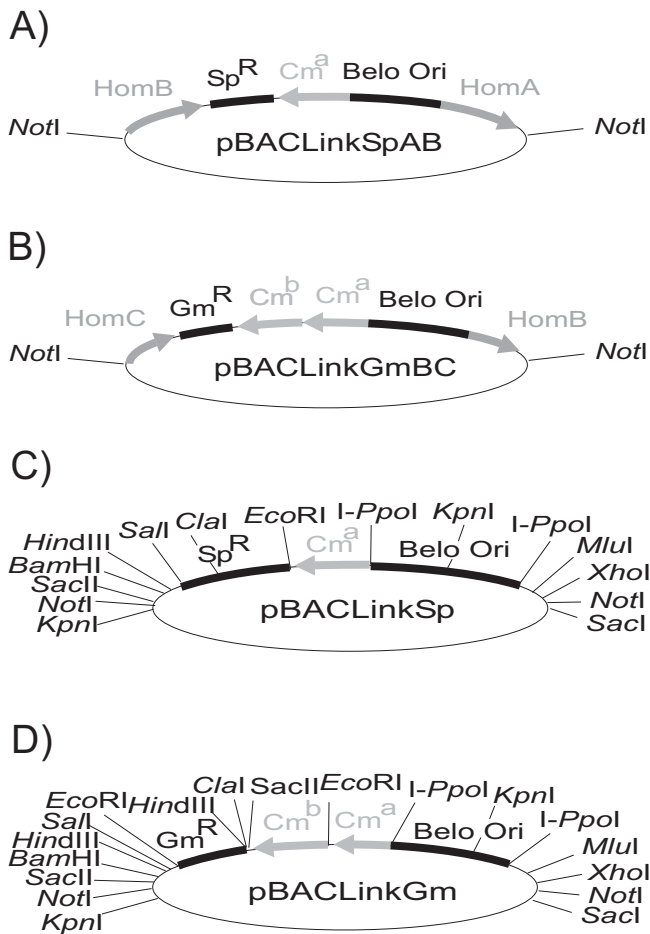


Figure 3
Diagrams of the pBACLink vectors Diagrams of pBACLinkSpAB (A), pBACLinkGmBC (B) and maps of pBACLinkSp (C) and pBACLinkGm (D) showing useful restriction sites. The vector region between the *NotI* sites is pBluescript including a high-copy origin and ampicillin resistance gene. HomA, HomB and HomC are the regions of homology with the BAC inserts. Sp^R and Gm^R are spectinomycin and gentamycin resistance genes. Cm^a and Cm^b are the 5' and 3' ends of the chloramphenicol resistance gene, and together make a functional gene. Belo Ori is the mini F plasmid origin from pBeloBAC11.

replication from pBeloBAC11 (Belo Ori), a selectable marker (either spectinomycin resistance or gentamycin resistance), a region of homology to the other subcloning BAC vector (the 5' end of the chloramphenicol resistance gene, Cm^a), and two regions of homology to the BAC insert (HomA, HomB or HomC). In addition, there is a high copy origin of replication from pBluescript to allow easy manipulation of the vectors as high-copy plasmids

before subcloning the BAC inserts which are then maintained at single copy.

For the modification of BAC1 (68P20, 183.5 kb insert), the subcloning vector pBACLinkSpAB was constructed (Fig. 3A). This carries two regions of homology HomA and HomB located at the beginning of the BAC insert and in the overlapping region between BACs 1 and 2 (Fig. 1B). This retrofitting plasmid was linearised with *NotI*, and the 10.8-kb fragment with HomA and HomB at its ends was purified and electroporated into induced EL350 bacteria containing BAC1 (Fig. 2). Clones were selected for spectinomycin resistance and also for chloramphenicol sensitivity as the vector of BAC1 is replaced by the vector from pBACLinkSpAB by homologous recombination to give BAC1LinkSp. 500 ng of the purified linear retrofitting construct was electroporated and 8 Sp^R, Cm^S colonies were obtained. Pulsed-field gel electrophoresis analysis after digestion with *I-PpoI* showed that 4 of them had the correctly retrofitted BAC1LinkSp with the 160-kb insert between HomA and HomB (one shown in Fig. 4A). One clone was further analysed by PCR using primers Belo2658 and CFTR1402 (labeled 1 and 2 in Figure 2) which amplify across HomA and primers Sp2 and CFTR20819 (labeled 3 and 4 in Figure 2) which amplify across HomB only after correct homologous recombination. These positive PCR results confirmed that the correct homologous recombinations had occurred. If the orientation of BAC1 had been such that the overlapping region was adjacent to the chloramphenicol resistance gene, this subcloning step would have been unnecessary.

BAC2 (133K23, 82.5-kb insert) was subcloned in a similar way using the same HomB region as for BAC1 and HomC at the other end of the insert, both cloned into the plasmid pBACLinkGmBC (Fig. 3B). The subcloning plasmid was again digested with *NotI* and 500 ng of the purified 9.3-kb fragment were electroporated into induced EL350 bacteria containing BAC2. The modified BAC2LinkGm should have both the gentamycin and the chloramphenicol resistance genes. 163 Gm^R colonies were obtained and 6 were further analysed. DNA was digested with *I-PpoI* and run on a pulsed-field gel which showed that 2 had the modified BAC2LinkGm with the expected 61-kb insert (one shown in Fig. 4A). PCR analysis with primers Belo2658 and CFTR22748 (labeled 1 and 5 in Figure 2) which are located on either side of HomB confirmed correct recombination across HomB. This subcloning step is necessary to have the insert of BAC2 in a vector which has unique sites (in this case *I-PpoI*) positioned adjacent to the two regions of homology to be used for the next recombination step. The two BACs were now ready to be combined together by a second round of homologous recombination.

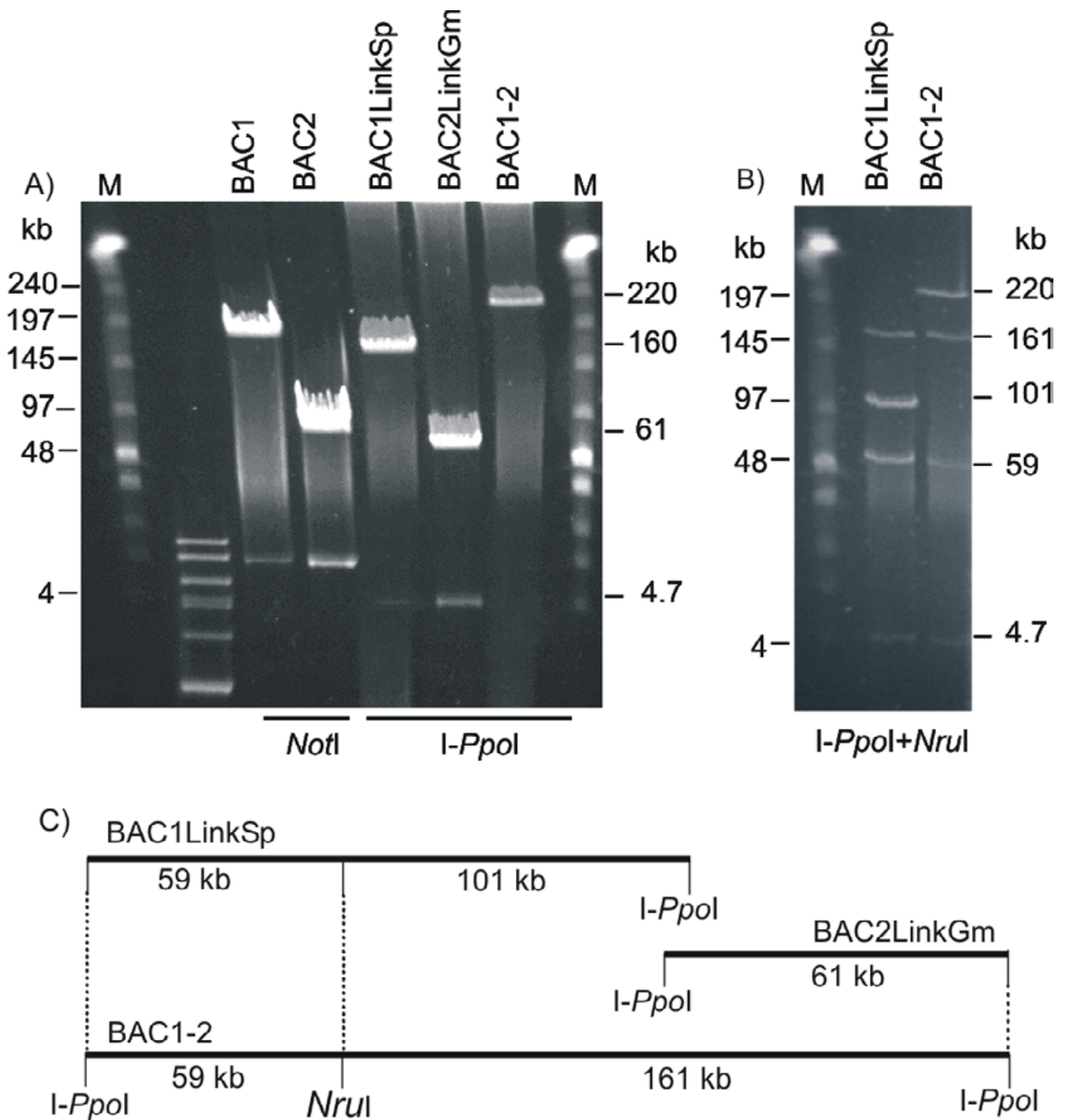


Figure 4
Pulsed field gels showing the various steps of the recombination process A) BAC1 and BAC2 cut with *NotI* showing the expected inserts and the 6.8-kb vector fragments. BAC1LinkSp, BAC2LinkGm and BAC1-2 cut with *I-Ppol* showing the 4.7-kb vector fragments and the expected inserts. B) BAC1LinkSp and BAC1-2 cut with *I-Ppol* and *NruI*. The expected fragments of 4.7, 59, 101 and 161 kb are shown. The 160-kb fragment of BAC1LinkSp and the 220-kb fragment of BAC1-2 are due to partial digestion with *NruI*. C) Map of the subcloned BAC1LinkSp and BAC2LinkGm and the combined BAC1-2 showing the expected fragments for *NruI* and *I-Ppol* double digestion. The sizes of the markers (M) are indicated to the left of each panel and the sizes of the BAC fragments are indicated to the right of each panel in parts A) and B).

pBACLink Vectors

The cloning steps used to make the subcloning vectors used in this work were more complex than necessary due to some of the restriction sites used not being unique. We therefore constructed two vectors pBACLinkSp and pBACLinkGm for use by other investigators (Fig. 3C,3D). Plasmid pBACLinkSp has unique *MluI* and *XhoI* sites for cloning HomA and unique *BamHI* and *Sall* sites for cloning HomB. Plasmid pBACLinkGm has unique *MluI* and *XhoI* sites for cloning HomB and *BamHI* and *Sall* sites for cloning HomC. For each BAC which needs to be subcloned, it is necessary to clone the two regions of homology into these sites. Alternating use of these vectors should allow multiple regions to be recombined together.

Combination of the two BACs

BAC2LinkGm was linearised with *I-PpoI* and electroporated into induced EL350 bacteria containing BAC1LinkSp (Fig. 2). After recombination in the HomB and Cm^a regions, the Sp^R gene on BAC1LinkSp should be replaced by the Gm^R and Cm^R genes from BAC2LinkGm. Therefore, the clones were selected for gentamycin resistance and also for being spectinomycin sensitive. 1.5 µg of digested BAC2LinkGm were electroporated and most of the gentamycin resistant clones were spectinomycin sensitive. Thirty two gentamycin resistant and spectinomycin sensitive clones were subjected to PCR with primers CFTR20819 and CFTR22748 (primers 4 and 5 in Figure 2 located in the CFTR regions on either side of HomB) and 4 were found to be positive. Pulsed-field gel analysis after *I-PpoI* digestion showed that 3 consisted of the combined BAC1 and BAC2 (BAC1-2) with a 220-kb insert (one shown in Fig. 4A). Figure 4A shows the unmodified BAC1 and BAC2, both cut with *NotI* showing the 6.8-kb vector fragments and the inserts. It also shows the subcloned BAC1LinkSp and BAC2LinkGm, and the combined BAC1-2 all cut with *I-PpoI* showing the 4.7-kb vector fragments and the expected insert fragments. Further digests were carried out with *NruI* and *I-PpoI* on BAC1LinkSp and BAC1-2 (Fig. 4B,4C). Both BACs show the expected 4.7-kb vector fragment and the 59-kb fragment with exons 1 to 4. BAC1-2 also gives the expected 161-kb fragment containing exons 4 to 27 (*NruI* cuts within exon 4). BAC1LinkSp also shows a 160-kb fragment, and BAC1-2 a 220-kb fragment, which are due to partial digestion with *NruI*.

Discussion

We describe a method for linking any two overlapping BACs into a single clone. Starting with two overlapping BAC clones, two vectors are constructed to subclone the BAC inserts so that they carry appropriate regions of homology, restriction sites and selectable markers for the linking step. The vectors pBACLinkSp and pBACLinkGm have been designed with ease of cloning in mind and have unique restriction sites for cloning the homology regions.

We used regions of homology ranging from 1.2 to 1.7 kb in size and the homology regions were amplified by PCR (using a high-fidelity polymerase so as not to introduce mutations) and cloned using restriction sites introduced on the primers. These homology regions are larger than necessary for efficient homologous recombination but they are a convenient size for gel purification and cloning. Other investigators have used homology regions down to 40 or 50 bp in size to achieve efficient homologous recombination using RecET or Red recombination [6,9,10]. Such small regions can be easily cloned into the restriction sites using overlapping oligonucleotides. However, lower frequencies of clones and more aberrant recombinants have been observed with such small regions of homology [11].

The homologous recombination reactions to subclone the BAC inserts were efficient. In the first example, with a 160-kb insert and both positive and negative selection for the recombination event, 4 out of 8 clones obtained contained the correctly subcloned insert. In the second example, with a 61-kb insert, and only positive selection, 2 out of 6 clones analysed were correct. The presence of homologous BAC vector DNA on both the original BAC and the subcloning vector might have interfered with the desired homologous recombination event. However, in our experience, this is not a problem and homologous recombination preferentially takes place within the terminal regions of homology. In no cases were rearrangements observed within the vector inserts – the incorrect clones either contained the input vector or the BAC DNA did not digest and so could not be identified.

The second step of the procedure involves recombination of one BAC onto another and the fragments of DNA involved are very much larger. In this case, a 61-kb fragment was homologously recombined onto a 160-kb BAC. PCR primers flanking the overlapping region of homology (HomB) were used to allow efficient screening of a number of clones and 3 out of 32 clones were identified as correctly recombined. The 4th clone identified by PCR actually contained the transfected BAC2LinkGm and in no cases were rearrangements observed in the combined BAC insert. This efficiency of 10% of clones having the correct recombination event is quite high and indicates that the reaction will probably work adequately efficiently with even larger fragments. The 61-kb fragment was not gel purified, instead the mixture of *I-PpoI* digested DNA was transfected into the EL350 cells. This simplifies the procedure and increases the probability of keeping the DNA intact during the transfection steps. This may be the limiting factor with using much larger DNA as the degree of breakage will increase with size.

The recombination step requires digestion of one BAC clone with *I-PpoI* which should not cut in the insert region. *I-PpoI* has a 15-bp recognition site which occurs in only 12 human sequences in Genbank (excluding the 28S ribosomal repeating unit). However, as *I-PpoI* tolerates some degeneracy in its cleavage site [12], it is difficult to predict how often cleavage within a BAC insert will be observed in practice.

Zhang and Huang recently described a similar method for inserting DNA from an overlapping BAC [8]. In contrast to their method, ours allows the linking of very large fragments and the BACLink vectors are designed for ease of use. The introduction of large fragments is made possible by the use of single copy plasmids for the subcloning vectors so that entire BAC inserts can be subcloned and maintained stably. We have demonstrated the introduction of a 61-kb fragment but there is no reason to think that this is the maximum size possible. In contrast, Zhang and Huang used pBR322 as the subcloning vector which may limit the size of fragments used and they subcloned a 22.5-kb fragment. In addition, Zhang and Huang did not include a selectable marker on the subcloning vector, but introduced it separately into the BAC insert requiring an extra recombination step and the subsequent removal of the selectable marker from the insert by *frt* recombination [8].

Another method, described by Majia and Larin, allows linking of two unrelated BACs using *loxP*/Cre recombination [13]. However, this method does not give precise recombination of overlapping BACs but rather leaves a *loxP* site and some vector sequence between the two BAC inserts.

It should be noted that our system can be applied to large inserts cloned into any BAC or PAC vector as the inserts are subcloned by homologous recombination into the pBACLink vectors before the inserts are recombined. It is not necessary to introduce the entire BAC insert as any particular region can be subcloned into the pBACLink vectors.

In many cases, only one of the original clones needs to be subcloned in the initial step. If the insert of BAC1 is oriented in the BAC vector such that the overlap of the two BACs is adjacent to the chloramphenicol resistance gene, then it is not necessary to subclone BAC1. BAC2 can be subcloned with either pBACLinkSp or pBACLinkGm and then recombined directly onto the unmodified BAC1.

Finally, if it is required to link more than two BACs then one can add more clones by alternating the use of pBACLinkSp and pBACLinkGm.

Conclusions

The method described in this paper should be of general utility to researchers who wish to carry out functional analysis on intact genes. Many genes, such as the *CFTR* gene, are not readily available in single BAC clones, but are available in overlapping, fully sequenced clones as part of the human genome project. These can now be linked together into a single clone using fragments at least 60 kb in size and repeated use of alternating BACLink vectors allow multiple BACs (or PACs) in a contig to be recombined.

Methods

Construction of plasmids

Initially, pBluescript (Stratagene) was modified by digesting it with *KpnI* and *SacI* and ligating separately with three pairs of complementary synthetic oligonucleotides to give plasmids pBIS1, pBIS2 and pBIS3 with new polylinker regions (For BIS1; 5'CGCGGCCGCGGATCCTCTGGTTCGACCTCTCTTAAGG TAGCCTCGAGAGACGAGCT and 5'CGTCTCTCGAG-GCTACCTTAAGAGAGGTCGACCAGAGGATCCGCG-GCCGCGGTAC. For BIS2; 5'CGTTCGACTCTCTTAAGGTAGCATCGATTCCACCGCG-GACCTCTCGAGCGGCCGCGAGCT and 5'CGCG-GCCGCTCGAGAGGTCCGCGGTGGAATCGATGCTACCT TAAGAGAGTCGACGGTAC. For BIs3; 5'CGGATC-CGACAAAGCTTGTCGACTCTGGAATTCTGTCCTCGAG-GAGCT and 5'CCTCGAGGACAGAATTCCAGAGTCGACAAGCTTTGT CGGATCCGCGGTAC).

These were then used to construct the subcloning vectors. The *Cm^a* homology region (which allows homologous recombination between the BACs, see Figure 2) was obtained from pBeloBAC11 [14] as a 1.1-kb *XhoI*-*EcoRI* fragment and ligated into *XhoI*/*EcoRI* cut pBIS3, giving pBIS3^{Cm^a}. Then, the *Sp^R* gene was taken from pLP3S [15] as a 2.2-kb *Sall*-*EcoRI* fragment and cloned into *Sall*/*EcoRI* cut pBIS3^{Cm^a} giving pBIS3^{Cm^a}Sp. At the same time the single copy origin from pBeloBAC11 [14] was excised as a 4.6-kb *Sall*-*XhoI* fragment and ligated to pBIS2 digested with *Sall* and dephosphorylated giving pBIS2Belo. Homology region HomA was amplified from BAC 68P20 by PCR using primers HomAL 5'CCACCCGCGCAA-GAGCCTCTGTCCACCACA and HomAR 5'CAAACCTCGA-GATTGCACATGGGCACTAACA (12202 – 13962 of BAC 68P20). HomA was cloned using the restriction sites present in the PCR primers into *SacII*/*XhoI* cut pBIS2Belo to give pBIS2BeloHomA. PCR to amplify Homology regions A, B and C were carried out with Platinum^R *Pfx* DNA polymerase (Invitrogen) to minimize introduction of mutations. Homology region HomB was amplified from BAC 68P20 by PCR using primers HomBL ATTT-GGATCCACTGGTTCAGTCAGGTTGGG and HomBR

ATTGTGCGACAACCTCAGACCCCGTTCACAC (21266 – 22558 of BAC 133K23). HomB was cloned using the restriction sites present in the PCR primers into *Bam*HI/*Sal*I cut pBIS3Cm^aSp to give pBIS3Cm^aSpHomB. Finally, pBIS2BeloHomA was digested with *Sal*I and *Sac*I and the 6.3-kb fragment was cloned into the *Xho*I/*Sac*I cut pBIS3Cm^aSpHomB giving pCm^aSpHomBBeloHomA. This is the subcloning vector called pBACLinkSpAB in the rest of the text.

To make the other subcloning vector, the total Cm^R gene was excised as a 1.7-kb *Sal*I-*Xho*I fragment from pBeloBAC11 [14] and cloned into *Xho*I digested and dephosphorylated pBIS2Belo giving pBI2BeloCm. The Cm^R gene was then excised from pBIS2BeloCm by *Xho*I and *Cla*I digestion and cloned into *Xho*I/*Cla*I cut pBluescript to give pBISCm. The Gm^R cassette was excised from pBSL141 [16] as a 0.8-kb *Mlu*I fragment and after generation of blunt ends with Klenow polymerase, it was cloned into *Eco*RV digested and dephosphorylated pBISCm to give pBISCmGm. This last plasmid was digested with *Eco*RI and the 1.4-kb fragment carrying the Cm^b region and the Gm^R gene was cloned into *Eco*RI digested and dephosphorylated pBIS3Cm^a giving pBIS3CmGm (an intact functioning Cm^R gene is recreated in this cloning step). Homology region HomC was amplified from BAC 133K23 by PCR using primers HomCL 5'ATTGGATCCAGGCCTGTAGTCCCACCTA and HomCR 5'ATTGTGCGACAGGCCAACCATGCAAATAAG (81389 – 82432 of BAC 133K23). HomC was cloned into *Bam*HI/*Sal*I cut pBIS3CmGm to give pBIS3CmGmHomC. Homology region HomB was reamplified using primers HomBL 5'ATTTCGCGGACTGGTTCAGTCAGGTTGGG and HomBR 5'ATTTCTCGAGAACCTCAGACCCCGTTCACAC and cloned into *Sac*II/*Xho*I cut pBIS2Belo giving pBIS2BeloHomB. pBIS2BeloHomB was then digested with *Sal*I and *Sac*I and the 5.8-kb fragment was cloned into *Xho*I/*Sac*I cut pBIS1 resulting in pBIS1BeloHomB. Finally, pBIS3CmGmHomC and pBIS1BeloHomB were combined together by cloning the 3.5-kb *Bam*HI/*Xho*I fragment from pBIS3CmGmHomC into *Bam*HI/*Sal*I cut pBIS1BeloHomB giving pCmGmHomCBeloHomB. This is referred to as pBACLinkGmBC in the rest of the text.

The modifying vectors pCm^aSpHomBBeloHomA (pBACLinkSpAB) and pCmGmHomCBeloHomB (pBACLinkGmBC) cannot be directly modified by other investigators for use with other BACs. This is because the *Sac*II site used to clone HomA and HomB respectively is not unique in the final vectors. New versions of the vectors which have unique sites for insertion of HomA, HomB and HomC were therefore made.

To make pBACLinkGm, pBIS1BeloHomB was digested with *Cla*I and *Xho*I and ligated to a pair of complementary

synthetic oligonucleotides (CGAACGCGTCTGC and TCGAGCAGAACGCGTT) to give pBIS1BeloMluI. A 2.5-kb *Bam*HI-*Xho*I fragment with the Gm^R and Cm^R genes was excised from pBIS3CmGm and ligated to pBIS1BeloMluI cut with *Bam*HI and *Sal*I resulting in pBACLinkGm.

For the construction of pBACLinkSp, pBIS2BeloHomA was modified between *Cla*I and *Xho*I using the same pair of oligonucleotides as for pBACLinkGm giving pBIS2BeloMluI. The 4.6 kb *Sal*I/*Sac*I fragment from pBIS2BeloMluI was then ligated to *Xho*I/*Sac*I digested pCm^aSp to give pBACLinkSp.

Homologous recombinations

To make electrocompetent EL350 bacteria [7], 1 L of LB media was inoculated with 1 ml of saturated culture grown overnight from the previous day at 30°C. The culture was grown at 30°C with shaking until an OD₆₀₀ of 0.6–0.8 was reached (8–9 hours). The culture was cooled on ice for 30 minutes and then two lots of 500 ml were spun down at 4000 × g for 10 minutes at 4°C. The bacteria pellets were resuspended in 100 ml of ice-cold 10% glycerol and spun down as before. Bacteria from each 500 ml lot were resuspended in 25 ml of 10% glycerol, pooled and transferred into a single 50 ml centrifuge tube and spun down as before. The bacteria were washed twice by resuspending in 50 ml of ice-cold 10% glycerol followed by centrifugation as above. Finally, the bacteria were resuspended in 2 ml of ice-cold 10% glycerol, divided into 40 µl aliquots in 1.5 ml microcentrifuge tubes, quick frozen in a dry-ice/ethanol bath for 5 minutes and stored at -70°C or used directly. Each 40 µl aliquot was used in a single electroporation.

The CFTR BACs 68P20 and 133K23 were introduced into EL350 bacteria as follows. About 500 ng of BAC DNA was put in the tube with the electrocompetent EL350 bacteria and left on ice for 10 minutes. The DNA-bacteria mix was then transferred into a 0.1 cm cuvette (BioRad) and electroporated at 1.8 kV, 25 µF and 200 Ω using a Gene Pulser II electroporator (BioRad). One ml of LB was added to the electroporated bacteria which were then incubated at 30°C for 1.5 hour with shaking. They were then spun down, plated in total on a plate with 12.5 µg/ml chloramphenicol and incubated for 24 hours at 30°C.

To make electrocompetent EL350 bacteria containing a BAC and induced for Red recombination, the same protocol was followed as above except that the 1:1000 diluted culture was grown in the presence of the appropriate antibiotic (12.5 µg/ml chloramphenicol for 68P20 and 133K23 and 50 µg/ml spectinomycin for BAC1LinkSp) at 30°C until an OD₆₀₀ of 0.6–0.8 was reached. Two lots of 500 ml were then incubated in a 42°C water bath with shaking

for exactly 15 minutes and immediately placed into an ice bath slurry, shaken by hand and left for 30 minutes to cool before proceeding with the 10% glycerol washes as above.

For recombination with pBACLinkSpAB and pBACLinkG-mBC, *NotI* linearized DNA was gel purified using the Qiaquick Gel Extraction Kit (Qiagen). After electroporation as described above, the bacteria were spread on plates containing 50 µg/ml of spectinomycin or 5 µg/ml gentamycin respectively. For BAC linking, about 1.5 µg of *I-PpoI* digested BAC DNA (not purified) was electroporated and the bacteria were spread on plates containing 5 µg/ml gentamycin.

PCR assays for checking recombination events

The following primers were used to check that correct homologous recombinations had occurred as described in the text and figures.

Belo2658; 5'TTTGTCACAGGGTTAAGGGC (2658-2638 of pBeloBAC11 Acc. U51113)

BELO6951; 5'GGTTATGTGGACAAAATACCTGG (6951-6974 of pBeloBAC11)

Sp1; 5'TTTGCAACTGCGGGTCAAGG

Sp2; 5'GCCCTACACAAATTGGGAGA

CFTR14102; 5'ATTAGAAAGGCAGGGGTGC (14102-14083 of BAC 68P20)

CFTR20819; 5'CTTGTCACATGATCCGAAA (20819-20838 of BAC 133K23)

CFTR22748; 5'GACAGCATTTGCCATGTTG (22748-22729 of BAC 133K23)

PCR was carried out with bacteria picked directly from colonies with a toothpick into 50 µl reactions. PCR was carried out with the *Taq* PCR Master Mix Kit (Qiagen).

BAC DNA preparation and pulsed-field electrophoresis

Small amounts of BAC DNA were prepared from 1.5 ml of saturated culture using a standard alkali miniprep protocol [17]. Larger amounts of BAC DNA were prepared from 500 ml of saturated culture using the Qiagen Plasmid Maxi Kit with the protocol for low copy plasmids.

BAC DNA was analysed by restriction enzyme digestion followed by separation by pulsed-field gel electrophoresis. 1% agarose gels were cast in 0.5 × TBE. Electrophoresis was conducted in 0.5 × TBE at 14°C, 5.9 V/cm, with a fixed angle of 120°, usually with switching times from 5

sec to 15 sec for 18 hours using a Chef-DRIII and a Mini Chiller model 1000 with a variable speed pump (BioRad). Gels were stained for 30 minutes with ethidium bromide and subjected to UV light for visualization of the DNA.

Author's Contributions

GK carried out all the experiments. CH supervised the research.

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