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6

Gene Functional Studies Using Bacterial Artificial Chromosome (BACs)

Mingli Liu, Shanchun Guo, Monica Battle and Jonathan K. Stiles
Microbiology, Biochemistry and Immunology
Morehouse School of Medicine, Atlanta
USA

1. Introduction

1.1 Background and history of bacterial artificial chromosome (BACs)

BACs were first developed as a large insert cloning system to facilitate the construction of DNA libraries to analyze genomic structure (Shizuya, Birren et al. 1992). BACs are derived from a fertility plasmid (F-plasmid) found in the *Escherichia coli*. BACs can clone extremely large DNA molecules, ranging from 150-700kb,and averaging 350 kb. Another advantage of BACs over other cloning technologies is its stability in cell culture and ease of manipulation. Because some recombinant viruses were too large to be generated by traditional techniques, BAC technology was developed to carry out genetic and functional studies of viruses, especially herpesvirus. (Warden, Tang et al. 2011). From the time when BACs emerged a decade ago, their application have grown intensely and have benefited the research community in many fields, such as sequencing of the human genome, *in vitro* transgenesis, genomic fingerprinting, and even to vaccine development.

1.2 BACs generation

BACs can be considered as plasmid expression vectors, amplified in bacteria and composed of a small amount of bacterial DNA derived from the single copy F-plasmid (Shizuya, Birren et al. 1992). F-plasmid has genes required for prokaryotic replication, partition, and selection. Viral BACs are generated by inserting a BAC vector sequence into a viral genome (Warden, Tang et al. 2011). Direct deletion mutants and random transposon mutagenesis of viral BACs are commonly used to determine the function of viral genes (Warden, Tang et al. 2011). Mammalian DNA, human genome or mouse genome (100–350 kb) can also be cloned as BACs vectors. Currently most regions of the human genome and the genome of other species are available as BACs. These vectors are useful tools in human genome-sequencing projects (Lander, Linton et al. 2001; Venter, Adams et al. 2001) and transgenic mice studies. Using endogenous homologous recombination systems in *E. coli* (Copeland, Jenkins et al. 2001), BACs can be modified in many ways by recombination. They can be genetically engineered to express reporter genes, or to put a transgene under a specific promoter which will be more amenable in gene expression in the mammalian cells. BACs can be modified to replace any nucleotide sequence of interest (gene replacement), remove any existing DNA

sequence, or introduce new sequences without removing any of the existing sequences (gene removal or insertion). In addition BACs are also used to place nucleotide substitution through selection/counterselection strategy, and to conduct effective gap repair cloning of any target site of interest (Adamson, Jackson et al. 2011).

1.3 Advantages and disadvantages of BACs

Genes expressed from BACs mirror endogenous gene expression far more accurately than other cloning systems. The large size of BACs help to minimize site of integration effects, a phenomenon which has been defined as endogenous sequences (such as gene coding regions and distal regulatory elements) to be disrupted, and to produce potentially undesirable phenotypes (Adamson, Jackson et al. 2011) in gene cloning technology. The larger sized BAC constructs contain enhancers and locus control regions, which leads to more accurate gene expression *in vivo* (Townes, Lingrel et al. 1985; Jones, Monks et al. 1995). 1995). The human genome BACs consist of the full gene structure, including untranslated regions, exons and introns, alternative promoters and splice sites and microRNA coding sequences. RNAs such as RNA splicing or microRNAs play very important role in gene regulation (Jackson and Standard 2007). Therefore the human genome BACs will ensure full mRNA processing and splicing when genes are transcribed, and produce the full complement of protein isoforms once mRNAs are translated. BACs can be transfected and expressed in mammalian cell lines although transfection efficiency and copy numbers are low (Magin-Lachmann, Kotzamanis et al. 2004; Sparwasser and Eberl 2007).

BACs also have a number of disadvantages. A construct containing a large genomic fragment is likely to contain non-related genes that may lead to indirect, non-specific gene expression and unanticipated changes in the cell phenotype; Secondly, compared to plasmids or other gene expression vectors, the generation and screening of recombinant BAC constructs can be time-consuming and labor-intensive. Also, the oversized BAC DNA constructs are more easily sheared and degraded during manipulation before transfection; and some random recombination events may occur, for example, LoxP sites may lead to random Cre-mediated recombination (Semprini, Troup et al. 2007). Finally, repeating homologous sequences in some BACs constructs may undergo intramolecular rearrangements, which reduce the recombination efficiency and increase the rate of false-positive clones in some selection/counter-selection approaches (Narayanan 2008).

Overall, BACs have numerous advantages when compared to conventional plasmids. They protect the gene from site of integration effects and produce accurate regulation of transcription and translation. However, the large size results in technical difficulties when handling them as well as the potential non-specific gene expression. Therefore the application of BACs as a gene expression model system should be careful considered based on the pros and cons previously described.

1.4 Application of BACs: Genomic sequencing, genomic imprinting, transgenic mice, vaccine development, and gene therapy

There is increasing interest in the application of BAC technology in genomic research. High throughput determination of gains and losses of genetic material using high resolution BAC

arrays and comparative genomic hybridization (CGH) have been developed into the new tools for translational research in solid tumors and neurodegenerative disorders (Cowell and Nowak 2003; Cowell 2004; Costa, Meijer et al. 2008; Lu 2009). Among a large number of approaches for sequencing, BAC technology is becoming the most robust method for genome sequencing. The BAC-by-BAC technique uses an overlapping tilling path of large genomic fragments (150-200 kb) maintained within BACs. Every individual BAC is shotgun sequenced. Many short reads are assembled to produce the sequence of the BACs, where these large overlapping sequences of the BACs are assembled to produce the who genome sequence (Imelfort, Batley et al. 2009). BACs have also been used in mammalian genome mapping (Schalkwyk, Francis et al. 1995), genomic imprinting (Tunster, Van De Pette et al. 2011), vaccine development and gene therapy (Magin-Lachmann, Kotzamanis et al. 2004; Warden, Tang et al. 2011). Studies of the evolutionary history and functional dynamics of sex chromosomes have recently been possible using BAC libraries (Janes, Valenzuela et al. 2011). In this chapter we will review some applications of BACs in viral and non-viral gene functional studies.

2. Viral gene functional studies

2.1 Many human and animal herpesviruses genomes have been cloned as BACs

Human herpesviruses are the second leading cause of human viral disease. Therefore the utilization of human herpesvirus BACs to study viral gene function (Warden, Tang et al. 2011) has become more and more common. The herpesviruses are a family of DNA viruses which contain large and complex genomes. Genetic control and management of recombinant viruses have been notoriously difficult. The development of herpesvirus BACs have facilitated generation of recombinant viruses and subsequent studies of the biology and pathogenesis of herpesviruses (Knipe, Batterson et al. 1981; Zhou and Roizman 2005). Table 1 shows the human herpesviruses which have been cloned as BACs, including Herpes simples virus type 1 [(HSV-1 or human herpesvirus (HHV-1)], varicella-zoster virus (VZV or HHV-3), human cytomegalovirus (HCMV or HHV-5), Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) (Feederle, Bartlett et al. 2010; Warden, Tang et al. 2011). In general, BAC clones are relatively easy to make for alpha- and beta herpes viruses than gamma herpes viruses. This is due to the fact that DNA can only persistently stay in bacterial cells when it has a prokaryotic replicon. When a BACs flanked by specific Herpesvirus genomic sequences were introduced into infected cells to trigger homologous recombination. The great efficiency was achieved in alpha- and beta herpes viruses because lytic cellular systems are available, but was difficult for gamma herpesviruses (Delecluse, Hilsendegen et al. 1998; Delecluse, Kost et al. 2001; Zhou, Zhang et al. 2002; Kanda, Yajima et al. 2004; Chen, Li et al. 2007). In addition to BAC-based human herpesvirus studies, BAC-based other animal herpesviruses are also currently available. These include murine cytomegalovirus 68 (MHV-68), murine gammaherpesvirus (mCMV), rhesus cytomegalovirus (rhCMV), rhesus rhadinovirus (RRV), pseudorabies virus (PrV), herpesvirus saimiri (HVS), Marek's disease virus (MDV), bovine herpesvirus type 1 (BHV-1), equine herpesvirus type 1 (EHV-1), feline herpesvirus (FHV-1), guinea pig cytomegalovirus (GPCMV), Koi herpesvirus (KHV) and turkery herpesvirus (HVT) (Feederle, Bartlett et al. 2010; Warden, Tang et al. 2011).

type	synonym	subfamily	biological function and application	reference
HHV-1	Herpes simplex virus-1 (HSV-1)	α (Alpha)	Generates a replication-proficient but packaging-deficient HSV-1 genome (152-kb HSV-1) for genetic manipulation as research tools or vectors in gene therapy.	(Saeki, Ichikawa et al. 1998; Stavropoulos and Strathdee 1998; Horsburgh, Hubinette et al. 1999)
HHV-2	Herpes simplex virus-2 (HSV-2)	α	Generates a recombinant HSV-2 BAC with the deletion of the HSV-2 glycoprotein D (gD), elicites an HSV-2 specific antibody response, serves as the basis for novel HSV-2 vaccine production.	(Meseda, Schmeisser et al. 2004)
HHV-3 Varicella zoster virus (VZV)		α	Human embryonic lung cells transfected with VZV BAC DNA show cytopathic effect, and viruses can spread to neighboring cells.	(Nagaike, Mori et al. 2004)
			Luciferase VZV BAC generates recombinant VZV variants, eases subsequent viral growth kinetic analysis both *in vitro* MeWo cells and SCID-hu mice *in vivo*.	(Zhang, Rowe et al. 2007)
			The mini-F transposition technique optimizes, repairs or restructures BACs, facilitates the development of gene therapy or vaccine vectors.	(Wussow, Fickenscher et al. 2009)
HHV-4 Epstein-Barr virus (EBV), lymphocryptovirus (LCV)		γ (Gamma)	Genetic analysis of all EBV functions, generation of attenuated EBV strains for vaccine design, development of viral vectors for human gene therapy.	(Delecluse, Hilsendegen et al. 1998; Kanda, Yajima et al. 2004)
			Generates a self-recombining BAC containing 172-kb of the EBV genome; provides proof that EBNA-3B is not essential for EBV-mediated B-cell growth transformation *in vitro*.	(Chen, Divisconte et al. 2005)

type	synonym	subfamily	biological function and application	reference
HHV-5 Cytomegalovirus (HCMV)		β (Beta)	A new approach for construction of HCMV mutants.	(Borst, Hahn et al. 1999)
			IE-2 (UL122) is required for successful HCMV infection and indicates that virus lacking IE-2 arrests early in the replication cycle.	(Marchini, Liu et al. 2001)
			UL45 is dispensable for growth of HCMV in human fibroblasts and human endothelial cells	(Hahn, Khan et al. 2002)
			A total of 252 ORFs with the potential to encode proteins have been identified in two laboratory strains (AD169 and Towne) and four clinical isolates Toledo, FIX, PH, and TR	(Murphy, Yu et al. 2003)
			HCMV strain TB40/E is available as a BAC clone suitable for genetic engineering.	(Sinzger, Hahn et al. 2008)
			Develops a "gene capture" method to rescue a large deletion mutant (15kb) of HCMV.	(Dulal, Zhang et al. 2009)
HHV-6	Roseolovirus, Herpes lymphotropic virus	β	Develops a single-step production of viral BACs by introducing the 160-kb human herpes virus 6A genome into BACs by digesting the viral DNA replicative intermediates with the SfiI enzyme that cleaves the viral genome in a single site	(Borenstein and Frenkel 2009)
HHV-7	Roseolovirus	β		Not available
HHV-8	KSHV	γ	BAC KSHV can be efficiently shuttled between bacteria and mammalian cells, such as BCBL-1, 293, HeLa and E6E7-immortalized human endothelial cells.	(Delecluse, Kost et al. 2001; Zhou, Zhang et al. 2002; Liu 2010)

Table 1. List of available BACs for human HSV

2.2 vGPCR-mediated angiogenesis through activation of p38 and STAT3 in KSHV infected cells using KSHV BACs

The molecular mechanism whereby viral G protein-coupled receptor (vGPCR) signaling regulates vascular endothelial growth factor (VEGF) expression in Kaposi sarcoma (KS) formation remains somewhat undefined. mECK36 cells, generated by transfection of mice bone marrow endothelial cells with KSHV bacterial artificial chromosome (KSHVBac36), have been reported to be angiogenic, tumorigenic, and suitable for demonstrating a nonredundant role for vGPCR in KSHV-mediated tumorigenesis (Mutlu, Cavallin et al. 2007). In our previous report (Liu 2010), we utilized mECK36, the cells composed of wild-type KSHVBac36 or the cells without vGPCR, namely vGPCR-null KSHVBac36 mutant, to dissect the molecular mechanisms of VEGF secretion induced by vGPCR in the context of KSHV infection. The mice bone marrow endothelial cells (mEC) were obtained from Balb/C An Ncr-nu mice (NCI, Bethesda, MD) bone marrow. Mice femurs were flushed twice with phosphate-buffered saline (PBS), and the elutes were incubated in Dulbecco's modified Eagle's medium (DMEM) media plus 30% fetal borine serum (FBS) (Gemini Bioproducts, Calabasas, CA), endothelial growth factor (EGF) 0.2 mg/mL (Sigma, St. Louis, MO), endothelial cell growth factor supplement (ECGS) 0.2 mg/mL (Sigma), heparin 1.2 mg/L (Sigma), insulin transferrine selenium (Invitrogen, Carlsbad, CA), penicilin-streptomicin 1% (Invitrogen), and BME vitamin (VWR Scientific, Rochester, NY). KSHVBac36 was constructed by inserting a full-length recombinant KSHV genome into a bacterial artificial chromosome, KSHVBac36 was transfected into mEC cells to generate mECK36 cells using lipofectamine 2000 (Invitrogen) and selected with hygromycin-B. The cells were then grown in the absence of hygromycin to negatively select cells and therefore generate mECK36-KSHV-Null cells, which lost the KSHV episome (KSHV episome was measured by GFP marker). Next, KSHVBac36 construct was retransfected into mECK36-KSHV-Null cells to generate BBac36. Finally, the genotypic markers of vGPCR were knocked out from KSHVBac36 by transposon mutagenesis to generate ORF74/vGPCR deletion mutant and stably transfected into mECK36-KSHV-Null cells to create BΔvGPCR cells in the presence of hygromycin selection. We found (Liu 2010) that vGPCR activates VEGF transcription via p38 MAPK and STAT3 in mECK36 and mECK36-derived cell models. In addition, we also found that in cells containing KSHV genome, STAT3 is tyrosine-phosphorylated and translocated into the nucleus, transactivating the target VEGF gene by binding to the specific DNA element TT (N4–5) AA in a vGPCR-dependent manner. Moreover, treatment of mECK36-derived cells with AG490 or a dominant negative STAT3 DNA vector showed strong inhibitory effects on vGPCR-induced VEGF promoter activity. In addition, vGPCR can up-regulate STAT3 mRNA levels. Together, our findings show that vGPCR plays a nonredundant role in STAT3 activation in KSHV infected cells, and this activation plays an important role in the connection of the viral oncogene vGPCR and VEGF up-regulation. Our results indicate that vGPCR has a broad signaling activating capacity in the context of KSHV infection and suggest that the STAT3 pathway could be a good target for preventing KSHV-mediated angiogenesis in KS.

2.3 Genetic determinants of virus tropism genes using BACs

Many cell types, including endothelial cells (ECs), myeloid lineage cells, and smooth muscle cells are permissive cells for HCMV persistent replication and latency (Jarvis and Nelson 2007). During acute infection of CMV in immune-compromised patients, a number of cell types, such as ECs, various leukocytes, epithelial cells, hepatocytes, smooth muscle cells, and fibroblasts,

can be infected because of uncontrolled replication of viruses (Howell, Miller et al. 1979; Myerson, Hackman et al. 1984; Gnann, Ahlmen et al. 1988; Wiley and Nelson 1988; Dankner, McCutchan et al. 1990; Sinzger, Grefte et al. 1995; Read, Zhang et al. 1999; Bissinger, Sinzger et al. 2002). ECs appear to play a critical role in the process of HCMV persistent active infection and maintenance within the host, which is controlled by genetic determinants. Previous studies observed that HCMV strains differed in their ability to infect ECs, which are called EC tropism (MacCormac and Grundy 1999; Sinzger, Schmidt et al. 1999; Kahl, Siegel-Axel et al. 2000). The research on EC tropism has been strengthened by the availability of genetically stable CMV BACs and subsequent mutagenesis of these BACs (Brune, Menard et al. 2001; Scrivano, Sinzger et al. 2011). The switch of cell tropism in different cell types after alternate replication might direct infection from one cell type to the other.

The typical model for tropism is the difference in cell tropism of virus released from EC and fibroblasts. Supernatants from infected human foreskin fibroblasts (HFF) showed a higher ability to infect EC than EC-derived supernatants (Scrivano, Sinzger et al. 2011). Scrivano et al (Scrivano, Sinzger et al. 2011) using mutagenesis of the BAC-cloned HCMV strain TB40/E (TB40-BAC4) found that ECs release a virus progeny of unEC-tropic (not EC-tropic), and retain a progeny of highly EC-tropic; while HFF release both EC-tropic and non EC-tropic virus progeny, HFF progeny is composed of both EC-tropic and non EC-tropic virus populations. The biochemical basis for this phenomenon is due to a different level of gH/gL/pUL(128,130,131A) complex in virions (Scrivano, Sinzger et al. 2011). The CMV EC tropism has been characterized by a "genomic tropism island" composed of three open reading frames (ORFs): UL128, UL130, and UL131A. The region of these genes is important for EC tropism (Hahn, Revello et al. 2004; Scrivano, Sinzger et al. 2011). EC-tropic population most likely is a population with a high gH/gL/pUL(128,130,131A) content. UL128, UL130, and UL131A are required for replication of HCMV in HUVECs (Hahn, Revello et al. 2004). EC tropism for HCMV is highly dependent on the roles of pUL128, pUL130, and pUL131A (Jarvis and Nelson 2007) in virions. EC-tropism produced by an EC-tropic progeny released by HFF, can be depleted with antibodies directed against pUL131A. They propose that the difference in cell tropism of virus released from EC and fibroblasts is caused by a sorting process. EC strongly and specifically retain EC-tropic viruses through the gH/gL/pUL(128,130,131A) complex. Thus, the levels of gH/gL/pUL(128,130,131A) complexes could define whether a particle is EC-tropic or not. A disulfide-linked complex between gH/gL glycoproteins is required for viral entry and fusion. The gH/gL exists in two distinct forms, one composed of pUL128, pUL130, and pUL131A. The pUL128 and pUL130 proteins are linked with gH/gL; pUL131A is required for infection of ECs. The second distinct form is composed of gO alone; the gO protein is linked with gH/gL and is required for replication in fibroblasts. The gH/gL/pUL128/pUL130/pUL131A unit in virions is mandatory for access into ECs which are pH-dependent. Whereas the gH/gL/gO unit in virions are mandatory for access into fibroblasts which are also pH-independent (Jarvis and Nelson 2007). Recently, results from Wang et al showed (Wang, Yu et al. 2007) that HCMV progenies derived from epithelial cells and fibroblasts are also different. It seems the propensity of cells to release viruses plays a crucial role in the establishment of infection and transfer of viruses to new hosts or the fetus.

In addition to HCMV, EBV also works as a cell type-tropic virus. Hutt-Fletcher et al (Hutt-Fletcher 2007) has established the paradigm that epithelial cells produce a EBV virus

progeny with high levels of gH/gL/gp42 complexes, facilitating B-cell infection. B-cells in turn, generate virus progeny with low levels of gH/gL/gp42 complexes which efficiently infect epithelial cells, but not B cells. To some extent, this relative switch of cell tropism after alternate replication in epithelial and B-cells directs infection from one cell type to the other.

2.4 Study of the immune response against the EBV using EBV BACs

There are three recombinant wild-type EBVs that have been generated so far (Delecluse, Hilsendegen et al. 1998; Kanda, Yajima et al. 2004; Chen, Divisconte et al. 2005). They were generated by the insertion of the prokaryotic F-plasmid (F-factor) in two B95.8 or one Akata strains. Although the insertion sites differ in these three EBV BACs, at the site of the B95.8 deletion (Delecluse, Hilsendegen et al. 1998), or at the major internal repeat region of the B95.5 strain (Chen, Divisconte et al. 2005), or at BXLF1 open reading frame (ORF) in Akata strain (Kanda, Yajima et al. 2004), the insertion site of the F-plasmid does not affect the phenotype of the virus.

2.4.1 EBV infection

EBV is tightly related to the development of many human cancers. Chen et al (Chen, Divisconte et al. 2005) has developed a BAC-GFP-EBV (containing 172-kb of the EBV genome) system to monitor early cellular and viral events associated with EBV infection. BAC-GFP-EBV was transfected into the HEK 293T epithelial cell line (Halder, Murakami et al. 2009). Then the progeny virus produced by a chemical was used to immortalize human primary B-cell which can be easily monitored by green fluorescence and proliferation. The results showed a dramatic increase in Ki-67, CD40, and CD23 signals. The viral genes express a pattern of an early burst of lytic gene expression. This up-regulation of lytic gene expression prior to latent genes during early infection strongly suggests that the resulting progeny virus is capable of infecting new primary B-cells (Halder, Murakami et al. 2009). This process may be critical for establishment of latency prior to cellular transformation (Halder, Murakami et al. 2009).

2.4.2 EBV transformation

EBV is associated with a number of human malignancies. There is increasing research interest in the molecular functions of these EBV gene products in transformation and evasion from host immune surveillance systems (Izumi 2001). BAC technology made the study on the molecular function of EBV transforming genes feasible because some latent genes such as EBNA1 cannot be maintained in latently infected B cells using traditional cosmid technology (Izumi 2001; Feederle, Bartlett et al. 2010). EBNA1 was found to function as a transactivator of other latent proteins, and was required for replication of the viral genome (Altmann, Pich et al. 2006). When 71kb of EBV DNA genome was amplified in *E.coli* and transfected into primary B-lymphocyes, Altmann et al (Altmann, Pich et al. 2006) identified that EBV DNA is sufficient to immortalize primary human B lymphocytes. Kempkes et al (Kempkes, Pich et al. 1995; Izumi 2001) also identified EBNA3a as a transforming gene, which contributes primarily to the initiation of cell proliferation (Kempkes, Pich et al. 1995; Izumi 2001). Two genes BALF1 and BHRF1 which encode homologous cellular antiapoptotic viral Bcl-2 proteins (vBcl-2), were suggested to interfere with the cell apoptosis program to counteract cell death, which protects the virus from apoptosis in its host cell during virus synthesis (Altmann and Hammerschmidt 2005).

2.4.3 Immune evasion

Several viral proteins have been found to block immune recognition of viral proteins as antigens during lytic replication, such as BGLF5, BZLF2, BILF1 and BNLF2a (Ressing, van Leeuwen et al. 2005; Rowe, Glaunsinger et al. 2007; Zuo, Thomas et al. 2008; Croft, Shannon-Lowe et al. 2009; Zuo, Currin et al. 2009; Zuo, Quinn et al. 2011). The direct contribution of BNFL2a in immune evasion was evidenced using an EBV BAC which initially disrupted the BNLF2a gene of the B95.8 strain by insertional mutagenesis (Croft, Shannon-Lowe et al. 2009). BNLF2 inhibits transporter associated with antigen (TAP). It encodes a 60 amino acid protein which prevents both peptide- and ATP-binding to TAP complex (Hislop, Ressing et al. 2007). Consequently, when co-expressed with target-antigens, cells expressing BNLF2a show decreased levels of surface human leukocyte antigen (HLA)-class I and are resistant to CD8+ cytotoxic T cell killing (Hislop, Ressing et al. 2007). Croft et al (Croft, Shannon-Lowe et al. 2009) created a targeting plasmid with BNLF2a gene which was replaced by tetracycline resistant cassette. This plasmid was then flanked by FLP recombinase target (FRT) sites. This vector was homologously recombined with the EBV BAC, and designated as ΔDBNLF2a, which had the tetracycline gene removed by FLP recombinase. ΔDBNLF2a BACs were then stably transduced into 293 cells, virus replication induced by transfection of a plasmid encoding the EBV lytic switch protein BZLF1 (Feederle, Kost et al. 2000). Compared to wild-type EBV BAC, this recombinant virus induces a strong MHC I T cell response against viral lytic genes than the wild type viruses (Feederle, Bartlett et al. 2010). Overall, these results indicate that BNLF2 prevents the immediate early and early proteins from being efficiently processed and presented to CD8 + T cells during lytic cycle replication. Contrary to BNLF2a in early evasion mechanism in the lytic cycle of EBV, other mechanism seems to operate later during immune evasion (Croft, Shannon-Lowe et al. 2009). Such stage-specific expression of immune evasion genes are a feature of several herpesviruses, such as CMV (Croft, Shannon-Lowe et al. 2009). Taken together, BNLF2a acts in concert with other immune-evasion genes encoded by EBV T-cell surveillance (Croft, Shannon-Lowe et al. 2009).

3. Non-viral gene functional studies

3.1 Translational research by the analysis of entire cancer genomes using BAC arrays

The development of high-resolution microarray-based comparative genomic hybridization (aCGH) using cDNA of BACs makes it possible for translational research to analyze the entire cancer genome in a single experiment. Well-designed aCGH studies will increase our understanding of the genetic basis of cancer, help to identify novel predictive and prognostic biomarkers for cancer, and molecular therapeutic targets in cancer. Compared to oligonucleotide arrays, BAC arrays have some specific features. BACs have been widely used in aCGH studies (Pinkel and Albertson 2005; Lockwood, Chari et al. 2006; Ylstra, van den Ijssel et al. 2006). The vast majority of array CGH data available today has been generated using BAC CGH arrays. BACs probes vary in length from 150 to 200 kb (Pinkel and Albertson 2005). The probe of genome-wide BAC arrays range from 2,400 to 32, 000 unique elements in tiling path array, where each BAC overlaps with its contiguous BACs. The resolution (the distance between each DNA target represented on the array) of each BAC array is defined by the number of unique probes it contains (Tan, Lambros et al. 2007). BAC tiling path arrays provide a resolution of up to 50 kb (Tan, Lambros et al. 2007). The development of a whole-genome BAC tiling path approach has improved resolution of CGH

by using overlapping clones (Ishkanian, Malloff et al. 2004; Lockwood, Chari et al. 2006). These platforms provide sufficiently strong signals to detect single-copy change, and are able to accurately define the boundaries of genomic aberrations, which can possibly be utilized in archival formalin-fixed, paraffin-embedded (FFPE) tissue (Johnson, Hamoudi et al. 2006; Little, Vuononvirta et al. 2006).

High amounts of high-quality BAC DNA are needed to obtain good array performance (Ylstra, van den Ijssel et al. 2006). BACs DNA yield is generally low when isolated from *E. coli* (Pinkel and Albertson 2005). Because of the low yields of DNA from isolated BAC clones, DNA amplification is required to generate sufficient quantities of adequately pure BAC DNA for the assay. Therefore a tiling path array is costly and highly labor intensive. In addition, as BAC probes are representative of the human genome, they also contain repetitive sequences, which can result in nonspecific hybridization (Tan, Lambros et al. 2007).

3.2 Measurement of neuroblastoma DNA copy number aberrations (CNAs)

BAC technique has increasing been applied in detecting structural changes in chromosomes, such as copy number aberrations (CANs) and rearrangement. Mosse et al (Mosse, Greshock et al. 2005) generated 4135 BAC clones spanning the human genome at about 1.0 Mb resolution as targets for array-based comparative genomic hybridization (aCGH) experiments (Greshock, Naylor et al. 2004). They measured the relevance of neuroblastoma DNA copy number changes (CNAs) in forty-two human neuroblastoma cell lines. They found that all cell lines exhibited CNAs ranging from 2% to 41% of the genome. Chromosome 17 showed the highest frequency of CANs. The most frequent region of gain with high-level amplification localized to 2p24.22-2p24.3 detected in 81% of cell lines (Mosse, Greshock et al. 2005). Potential oncogenes such as MYCN, NAG and DDX1 were located in these regions. The less frequent region of gain localized to 17q23.2, 17q23.3-17q24.1, 17q24.1-17q24.2, 17q25.2-17q25.3 was detected in about 70% of the cell lines. Potential oncogene BIRC5 was localized in these regions. Although gain of 17q material was common, this low level gain of chromosomal material was rather complicated (Mosse, Greshock et al. 2005). The most frequent hemizygous deletion localized to a 4.0 Mb region at 1p36.23-36.32, was detected in 60% of the cell lines. Potential tumor suppressor genes TP73, CHD5, RPL22 and HKR3 were also localized. A 10.4 Mb region at 11q23.3-11q25 was detected in 36% of the cell lines, and the potential tumor suppressor gene CHEK1 was found there as well (Mosse, Greshock et al. 2005). Overall, the array CGH could be reliable in examining DNA copy number aberrations including single copy gain or loss. Compared to the data with standard techniques, data from array CGH correlates well with known aberrations detected by standard techniques. Therefore, array CGH can be applied to identify novel regions of genomic imbalance.

3.3 Fluorescence in situ hybridization (FISH) analysis of pathological archives with BACs

It is now known that there are extensive somatic changes, including multiple point mutations (Wood, Parsons et al. 2007; Velculescu 2008), copy number alterations (Weir, Woo et al. 2007; Kubo, Kuroda et al. 2009), and further complex rearrangements (Campbell, Stephens et al. 2008) tumors. But when and where these genetic changes occur during human cancer development remains unclear. Human archival tissue blocks contain

specimens of human tumors in various stages of development, which are precious in the post-human-genome-sequencing era. Based on their findings and other's work, Sugimura et al (Sugimura, Mori et al. 2010) stated that the intensive application BAC clones as probes for FISH that have exact 'addresses' in the whole genome will become a useful diagnostic tools for pathologists. Thousands of BAC clones are commercially available, and any of them can be used as FISH probes. Sugimura et al tested 100 BAC probes containing different kinase loci in a gastric, colorectal, and lung cancer detection sets (20 cases for each organ) by using tissue microarray (TMA)-FISH technology (Sugimura, Mori et al. 2010). Sugimura et al found that unexpected kinase loci were amplified in a significant proportion of human common solid tumors (Sugimura, Mori et al. 2010). Combinatory chemistry has generated many drugs by targeting kinase genes or their products. Thus, amplification of specific regions on certain kinase genes are amenable to pharmacological intervention which could result in the target specific therapy. Therefore it is reasonable to believe that the FISH-BACs diagnostic system combined with particular kinase probes may provide the practical basis of individual cancer therapy.

3.4 Interferon-γ locus regulation with BACs

To investigate the regulatory properties of conserved non-coding sequence (CNS) element of interferon-γ (Ifng) gene *in vivo*, Hatton et al (Hatton, Harrington et al. 2006) developed a BAC-based transgenic reporter system to express Ifng gene expression. They introduced a Thy1.1 reporter into exon 1 of Ifng and placed this reporter into a BAC containing approximately 60 kb upstream of exon 1 of ifng and approximately 100 kb downstream of exon 4 of ifng sequences. The CNS-22 region of BAC was then flanked with loxP sites (Hatton, Harrington et al. 2006; Wilson and Schoenborn 2006). Because activation of the large BAC transgenic allele unlikely perturbs endogenous alleles (Valjent, Bertran-Gonzalez et al. 2009), potential confounding effects of altered IFNg production were possibly eliminated (Hatton, Harrington et al. 2006). Hatton et al chose Thy1.1 (CD90.1) as a reporter because of its low immunogenicity and easy detection in the context of CD90.2 allotype of the C57BL/6 background. The recombined BACs (Ifng-Thy1.1 BAC) containing the Thy1.1 reporter and floxed CNS-22 were microinjected into fertilized C57BL/6 oocytes (Hatton, Harrington et al. 2006; Wilson and Schoenborn 2006). As a result, the Ifng-Thy1.1 BAC-in transgene completely mirrored endogenous Ifng gene expression; and conditional deletion of the CNS-22 element from the single copy transgene by Cre recombinase resulted in almost complete loss of Thy1.1 expression in Th1 cells, CD8+ T cells, and NK cells irrespective of activation through the T cell receptor (TCR)-dependent or TCR-independent pathways. Thus, CNS-22 is considered to be critically involved in Ifng gene expression, irrespective of adaptive or innate immune cell lineage (Hatton, Harrington et al. 2006; Wilson and Schoenborn 2006). CNS-22 functions as an enhancer both *in vitro* and *in vivo*, which will shed new light on ifng regulation and open up avenues for future investigation.

3.5 Studies of Kras-mediated pancreatic tumorigenesis with BACs

Activation of Kras gene by mutation plays a critical role in human pancreatic cancer (Almoguera, Shibata et al. 1988; Shibata, Almoguera et al. 1990). Although the known capability of oncogenic Kras to function as a key initiator of pancreatic malignancy, the mechanism(s) of Kras-caused initiating events are still unclear. This is an important reason

why prognosis for patients with malignant pancreatic tumors have not entirely improved over the past twenty years (Jemal, Siegel et al. 2006). Moore et al have discovered that the zebrafish develop pancreatic cancer after exposure to chemical mutagens (Moore, Rush et al. 2006). The studies have also shown that mammalian and zebrafish pancreas are significantly similar in anatomy and histology (Wallace and Pack 2003; Chen, Li et al. 2007). Therefore, the Zebrafish has emerged as an experimental model for study of human pancreatic cancer biology (Davison, Woo Park et al. 2008; Park, Davison et al. 2008). Another benefits of working with zebrafish model is their translucency, which greatly improves the visualization of fluorescent trangenes in both embryos and adult zebrafish (Davison, Woo Park et al. 2008). Park et al (Park, Davison et al. 2008) discovered that oncogenic Kras causes pancreatic cell expansion and malignant transformation in the zebrafish exocrine pancreas by utilizing eGFP-Kras BAC transgenes (160kb) under the regulation of Ptf1a regulatory elements. Ptf1a induces differentiation, growth and proliferation of pancreatic progenitor cells (Park, Davison et al. 2008). Briefly, they expressed either extended green fluorescent protein (eGFP) alone or eGFP fused to oncogenic Kras in developing zebrafish pancreas and continuously detected the expression of fluorescent transgenes transcutaneously during all stages of development including the adult zebrafish. They first generated polymerase chain reaction (PCR) products encoding the eGFP and eGFP-Kras transgenes flanked by sequences homologous to the CH211-142 BAC that spans the Pfta1 gene locus. Homologous recombination leads to accurate replacement of the Ptf1a coding sequences with the eGFP and eGFP-Kras transgene (Davison, Woo Park et al. 2008). Their results demonstrate that oncogenic Kras-expressed pancreatic progenitor cells fail to undergo characteristic exocrine differentiation although their initial specification and migration are observed to be normal (Davison, Woo Park et al. 2008; Park, Davison et al. 2008). Blocks of differentiation leads to abnormal accumulation of the undifferentiated progenitor cells, correlates with the formation of invasive pancreatic cancer. Besides similarity in anatomy and histology, Zebrafish pancreatic tumors share several activated signaling pathways with the human pancreatic tumors, including activation of ERK and AKTby phosphorylation, as well as abnormal Hedgehog pathway activation which was justified by the up-regulation of ptc1 mRNA and gli1 mRNA (Park, Davison et al. 2008). These findings provide a unique view of the tumor-initiating effects of oncogenic Kras in a living vertebrate organism, but more important it suggest that BACs transgene targeting other oncogenes or tumor suppressor genes in zebrafish pancreatic cancer may improve our understanding of the human disease.

3.6 Studies on striatal signaling pathways in central nervous system (CNS) with BACs

To understand the role of molecular signaling pathways involved in behavioral responses, it is necessary to delineate the molecular events that take place in neurons. This task has been hampered by the complexity of neuronal system. There are hundreds of distinct neuronal populations and these populations are very difficult to distinguish (Valjent, Bertran-Gonzalez et al. 2009). The development of BAC transgenic mice expressing various reporters, epitope tagged-proteins or Cre recombinase driven by specific promoters, greatly facilitates the research in this field. Generally speaking, transgene expression is influenced by copy numbers and site of insertions (positional effects). Large BAC transgenes (BACs contain large fragments 150-200kb of mouse genome) have usually a low copy number, are less likely influenced by positional effects, and are able to recapitulate the regulation of endogenous genes much better than shorter transgenes (Yang, Model et al. 1997). Over the past few years, the use of BAC EGFP transgenic mice have generated significant

development in the analysis of striatal physiology and physiopathology (Valjent, Bertran-Gonzalez et al. 2009). The drd1a-EGFP (EGFP reporter is driven by dopamine D1 receptor-D1R promoter), drd2-EGFP (EGFP reporter is driven by dopamine D2 receptor-D2R promoter) and chrm4-EGFP (EGFP reporter is driven by cholinergic receptor, muscarinic 4-CHRM4 receptor promoter) BAC transgenic mice have been extensively utilized to investigate the physiological features of striatonigral and striatopallidal medium spiny projection neurons (MSNs) (Lobo, Karsten et al. 2006; Kreitzer and Malenka 2007; Cepeda, Andre et al. 2008; Gertler, Chan et al. 2008). Among these major findings are as follows: 1)D1R-expressing MSNs are less excitable than D2R–MSNs (Lobo, Karsten et al. 2006; Kreitzer and Malenka 2007; Cepeda, Andre et al. 2008; Gertler, Chan et al. 2008) due to different morphology (Gertler, Chan et al. 2008), and some presynaptic factors (Kreitzer and Malenka 2007; Cepeda, Andre et al. 2008). Corticostriatal synapses are activated by repetitive stimulation; in contrast, thalamostriatal synapses are inhibited by repetitive stimulation (Ding, Peterson et al. 2008); 2) D1R-expressing MSNs collaterals are functionally connected primarily with other D1R–MSNs, whereas D2R-expressing neurons collaterals are connected with both D2R– and D1R–MSNs (Taverna, Ilijic et al. 2008). D2R–MSNs synapse with GABAA receptors are stronger (Taverna, Ilijic et al. 2008) and generate greater GABAA receptor-mediated tonic currents (Ade, Janssen et al. 2008) than D1R–MSNs (Janssen, Ade et al. 2009); 3)The single back-propagating action potentials invade more distal dendritic regions in D2R–than in D1R–MSNs, due to a difference in voltage-dependent Na+channels and Kv4 K+ channels (Day, Wokosin et al. 2008); 4) In the dopamine-depleted striatum, the corticostriatal connections are decreased in D2R neurons (Day, Wang et al. 2006), whereas dendritic excitability is increased in this region (Day, Wokosin et al. 2008; Taverna, Ilijic et al. 2008).

4. Conclusions and overall perspectives

Studies on BACs have demonstrated their importance in many research fields, from microbiology, virology, to human genetics, neuroscience, and proteomics (Narayanan 2008; Adamson, Jackson et al. 2011). The power to clone and handle large sized intact genome with high fidelity by BAC has enabled scientists to design and perform both mechanistic and functional studies in an ever expanding field.

5. Acknowledgements

This review was supported by the National Institutes of Health grant numbers NIH-FIC (1T90-HG004151-01) for postdoctoral training in Genomics and Hemoglobinopathies, NIH/FIC/NINDS R21 and NIH-RCMI (RR033062).

6. Glossary

aCGH: array-based comparative genomic hybridization
BACs: bacterial artificial chromosome
BHV-1: bovine herpesvirus Type 1
CGH: comparative genomic hybridization
CHRM4: cholinergic receptor, muscarinic 4- receptor
CMV: cytomegalovirus
CNAs: copy number changes
CNS: central nervous system

D1R: dopamine D1 receptor
D2R: dopamine D2 receptor
ECs: endothelial cells
eGFP: extended green fluorescent protein
EHV-1: Equine herpesvirus Type 1
FFPE: archival formalin-fixed, paraffin-embedded
FHV-1: Feline herpesvirus
FISH: fluorescence in situ hybridization
F-plasmid: a fertility plasmid
GPCMV: Guinea pig cytomegalovirus
HCMV or HHV-5: human cytomegalovirus
HFF: human foreskin fibroblasts
HLA: human leukocyte antigen
HSV-1: Herpes simples virus type 1
HSV-2: Herpes simples virus type 2
HUVECs: human umbilical vein endothelial cells
HVS: herpesvirus saimiri
HVT: Turkery herpesvirus
HPV: herpesviruses
Ifng: interferon-γ
KHV: Koi herpesvirus
KSHV or HHV-8: Kaposi's sarcoma-associated herpesvirus,
LCV: lymphocryptovirus
MDV: Marek's disease virus
mECK36 cells: mouse bone marrow endothelial cells generated by transfection of with KSHVBac36
MSNs: medium spiny projection neurons
VEGF: vascular endothelial growth factor
vGPCR: viral G protein-coupled receptor
VZV or HHV-3: varicella-zoster virus,
KSHVBac36: KSHV bacterial artificial chromosome,
mCMV: murine gammaherpesvirus
MHV-68: Murine cytomegalovirus 68
ORFs: open reading frames
PCR: polymerase chain reaction
PrV: pseudorabies virus
rhCMV: Rhesus cytomegalovirus
RRV: rhesus rhadinovirus
TMA: tissue microarray

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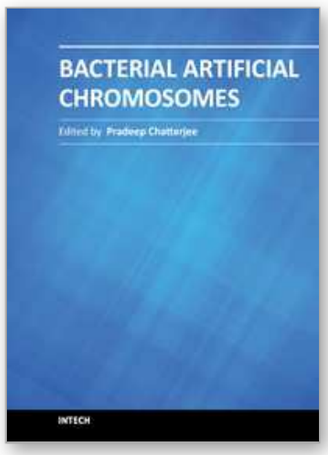
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Bacterial Artificial Chromosomes

Edited by Dr Pradeep Chatterjee

ISBN 978-953-307-725-3
Hard cover, 148 pages
Publisher InTech
Published online 25, November, 2011
Published in print edition November, 2011

This book focuses on the numerous applications of Bacterial Artificial Chromosomes (BACs) in a variety of studies. The topics reviewed range from using BAC libraries as resources for marsupial and monotreme gene mapping and comparative genomic studies, to using BACs as vehicles for maintaining the large infectious DNA genomes of viruses. The large size of the insert DNA in BACs and the ease of engineering mutations in that DNA within the bacterial host, allowed manipulating the BAC-viral DNA of Varicella-Zoster Virus. Other reviews include the maintenance and suitable expression of foreign genes from a Baculovirus genome, including protein complexes, from the BAC-viral DNA and generating vaccines from BAC-viral DNA genomes of Marek's disease virus. Production of multi-purpose BAC clones in the novel Bacillus subtilis host is described, along with chapters that illustrate the use of BAC transgenic animals to address important issues of gene regulation in vertebrates, such as functionally identifying novel cis-acting distal gene regulatory sequences.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Mingli Liu, Shanchun Guo, Monica Battle and Jonathan K. Stiles (2011). Gene Functional Studies Using Bacterial Artificial Chromosome (BACs), Bacterial Artificial Chromosomes, Dr Pradeep Chatterjee (Ed.), ISBN: 978-953-307-725-3, InTech, Available from: http://www.intechopen.com/books/bacterial-artificial-chromosomes/gene-functional-studies-using-bacterial-artificial-chromosome-bacs-

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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