THEORETICAL PAPERS
AND REVIEWS

Libraries of Large-Insert Genomic Clones
as a Tool for Molecular Cytogenetic Analysis of Avian Genome

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Abstract—Integration of molecular and cytegenetic levels of investigation results in complex understanding of structural and functional genome organization. Gridded libraries of large-insert genomic clones represent a powerful tool of the genome analysis. Their utilization provides coordination of data on molecular organization of nucleic acids with cytogenetic data on the chromosome structure. These libraries played an important role in sequencing of genomes of human, mouse, and other organisms as an instrument linking molecular biological and cytogenetic data via construction of contigs and their localization on the chromosomes. They also enabled analysis of orthology between the mammalian genomes. The existing avian libraries fit molecular cytogenetic analysis of the class Aves genome, and can be successfully used for the isolation and characterization of large genomic fragments. This provides utilization of these libraries not only for the chromosome mapping, but also for positional cloning and search for candidate genes for quantitative traits.

INTRODUCTION

The possibility of rapid isolation of preparative amounts of DNA corresponding to the large (more than 30 kb in size) genome fragments is the requirement of most experiments on molecular genetic and cytogenetic genome analysis. Gridded genomic libraries, i.e., those having address references for individual clones, enable isolation of large DNA fragments, containing the sequences of interest. They are widely used for genome analysis with different approaches [1].

COMPARATIVE CHARACTERIZATION
OF THE SYSTEMS FOR CLONING
OF LARGE GENOME FRAGMENTS

The first genetic system for large-scale cloning of long genomic fragments was designed by Burke in 1987 [2]. It was based on yeast artificial chromosomes (YACs), which contained the centromere, telomeres, autonomously replicating sequences, cloning site, and the selective marker genes. The system enabled cloning of nucleotide sequences up to 1000 kb in size. As the early 1990s were the years of active implementation of the Human Genome Project, these libraries were widely adopted and used for creating genomic libraries [3]. However, substantial disadvantages of such libraries, namely, high level of chimerism, genomic insert instability, along with the difficulties in obtaining preparative amounts of cloned DNA sequences, prohibited their utilization as the main tool of the genome cloning. Large number of tandem repeats typical of mammalian genome resulted in the yeast system in site-specific recombination and the loss of the inserted fragments, or in the formation of chimeric clones, i.e., clones containing DNA fragments from different parts of the donor genome [3]. Cytologically chimeric clones are identified upon in situ hybridization owing to their localization in two or more sites [1].

Bacteria appeared to be more attractive as host organisms for the genomic clones, since prokaryotic recombination systems leave less possibilities for unwanted rearrangements, and prokaryotic episomes are rather stable and can exist in supercoiled form. Plasmid vectors are not only simpler than chromosomal eukaryotic vectors (lack centromere and telomeres, which makes library construction less labor-consuming), but also substantially simplify the procedure of the cloned DNA isolation through complete degradation of chromosomal DNA.

The first genomic banks obtained with the use of *Escherichia coli* cells were based on a group of vectors, derivatives of λ phage [4]. There are phage vectors with traditional structure, which represent DNA molecules capable of only lytic degradation in vitro [4]. Cosmid vectors are formally the defective phages, i.e., plasmids of different types that contain the cos regions of λ phage and are incapable of lysis. Phagemid (phage + plasmid) vector, characterized by the ability for lytic development in vivo, is maintained in the plasmid form. Constructions of this type are widely used for producing mini-libraries and gene banks. The common disadvantage of these constructs is relatively large number of

clones (up to 10^6) required for the reliable presentation of any mammalian or avian genome fragment upon relatively small size of the insert (20 to 40 kb) [5].

Cosmids, i.e., plasmids containing λ phage DNA segment with joined sticky ends (cos sites) [6], deserve special consideration. The important feature of most of the cosmid cloning vectors is their ability of including inserts up to 45 kb in size. If circular cosmid DNA is cut at any unique site, mixed with the DNA fragments containing sticky ends, and then annealed, then long concatemers are formed. When these constructs are mixed with the proteins responsible for packing of λ phage, they are cut at cos sites, and DNA is packed into the phage head. This process enables selection of large inserts, since successful DNA packing into the phage head requires a distance of 38 to 52 kb between the cos sites. This mixture can contain the fragments without the inserts, or those with the repeated inserts, which affects the quality of the library constructed and hampers its utilization for genome analysis. Recipient cells acquire the packed cosmids as a result of infection with "false" phage particles; this process is more effective than transfection of plasmid DNA. After entering the host cell, recombinant DNA is amplified and preserved in the form of plasmid [6]. The recombinant clones obtained can be gridded, i.e., grown on the plates with precise indication of the address for every clone. Production of replicas on nitrocellulose or other filters for transfer of the clones with subsequent degradation of bacterial cell walls and purification from the proteins makes it possible to screen such libraries by use of standard dot–blot DNA–DNA hybridization [1]. The disadvantage of genomic cosmid libraries is relatively (compared to artificial yeast chromosomes) small insert size (about 45 kb), and hence, larger size of the library [about 2×10^5 clones for mammalian genomes (Human Genome Lectures, http://www.ucl.ac.uk)]. In order to combine the advantages of cosmid libraries (high stability of DNA clones, relatively low chimerism level, simplicity of construction, and convenience of DNA isolation) and the yeast libraries (large inserts and the library compactness), two cloning systems were designed on the basis of bacterial artificial chromosomes (BACs), and P1 phage artificial chromosomes (PACs). PAC vector (pCYPAC-1) was first used for the transfer of recombinant DNA in the *E. coli* cells by means of electroporation [7]. Separated by means of pulsed-field electrophoresis human genomic DNA fragments were packed into P1 bacteriophage heads. Infection of the *E. coli* strain expressing Cre recombinase with such phage particles resulted in the formation of the episome copies of recombinant phage genome. The genomic library constructed contained 15 000 clones with the mean insert size of 130 to 150 kb. Thirty-four clones were hybridized on mitotic chromosomes by use of FISH technique, and no cases of chimerism were observed. Long cultivation of the bacteria revealed no insert instability, as judged by analysis of twenty clones [7].

Artificial bacterial chromosomes (BACs) are based on F-factor (fertility factor), the low-copy plasmid, which is present in the bacterial cell in supercoiled circular form and can include the insert up to 500 kb in size. The fertility factor replication is under strict control from the side of cellular mechanisms, which remarkably diminishes recombination in episomes of this type [3]. Furthermore, genomic DNA of the donor species is practically always remains in the supercoiled form, thereby theoretically bringing the probability of unwanted exchanges between the insert fragments close to zero [8]. The first such library was constructed based on pBAC108L vector, which contained the fertility factor and cosN site [9]. The insert size varied from 10 to 300 kb, constituting, on average, 100 kb. The insert stability (relative to the character of the restriction fragment pattern preservation) was confirmed during 100 generation of bacterial cells. Screening of the clones for chimerism by use of in situ hybridization revealed only one case of rearrangement out of 28 randomly chosen transformants [9]. Further experiments with the use of this library also demonstrated low frequency of translocations [3]. It should be noted that mean sizes of the inserts cloned in artificial bacterial chromosomes are lower, compared to the yeast- or P1 bacteriophage-derived cloning system. However, disadvantages of the yeast systems mentioned above do not allow them to compete with the bacterial systems. The advantage of BAC libraries over the PAC libraries is the relative simplicity of their construction: no bacteriophages are used and transfer of donor DNA is realized through ordinary transformation. Thus, artificial bacterial chromosomes are considered to be optimal systems for cloning of large genomic sequences with the sizes of more than 50 kb, while cosmids remain effective system for cloning of the fragments lower than 50 kb in size (which have the advantages for utilization in some kinds of the genome analysis) [3].

APPLICATION OF LARGE-INSERT LIBRARIES FOR MOLECULAR CYTOGENETIC ANALYSIS OF HUMAN AND ANIMAL GENOMES

Gridded large-insert genomic libraries were found to be the most effective instrument for the purpose of human genome mapping and sequencing. Utilization of such libraries provides integration of data on nucleic acid composition (sequencing) and the location of the sequenced clones on chromosomes (physical mapping) [10]. During five years (1995 through 2000), resource centers supporting the libraries developed for various animal and plant species and implementing projects involving these libraries have been created. In Europe, the largest resource center is RZPD (German Resource Center for Genome Projects; http://www.rzpd.de). In the New World these are GENEfinder (Laboratory of Plant Genomics and Genomic Resources; http://hbz.tamu.edu), where the libraries of microbial, plant, and animal genomes (including red jungle fowl and chicken) have been created and

stored, and BPRC (BAC/PAC Resource Center; http://bacpac.chori.org), specializing predominantly in human and mammalian genomes. Note that, unlike RZPD, the latter two centers do not expect obligatory participation of the customers in the projects financed by the states of center's affiliation. This circumstance substantially simplifies the use of the resources by Russian specialists. The centers provide researchers with the library replicas on the filters for screening (DNA hybridization). Coordinates of the positive DNA clones are reported to the resource center, where these clones are plated on the agar medium and delivered to the customers.

For the overwhelming majority of human genome regions (excluding the centromeric regions, where cloning is impossible for the reason of the great number of highly repeated DNA sequences) contigs (sets of overlapping DNA clones) containing known DNA sequence were constructed. Thus, the knowledge on any nucleotide sequence will make it possible to find large-insert DNA clone in the library (http://www.ncbi.nlm.nih.edu). For the domestic mouse, contigs of certain chromosome regions were constructed. Analogous contigs were also constructed for the chicken genome. For instance, the region of chicken chromosome 13 (GGA13) is represented by a contig comprised of 204 artificial bacterial chromosomes, covering approximately 20% of the GGA13 length [11].

Large-insert DNA clones can be assembled into a contig by use of genomic fingerprinting or fluorescence in situ hybridization on chromosome spreads (fiberFISH) [12]. The first approach enables simultaneous analysis of 190 clones (standard agarose gel for fingerprinting contains 242 lanes, 52 of which are used for molecular size markers). The capacity of the second approach is limited by the color spectrum of fluorochromes, as well as by the transmission spectrum of the optic filters, and constitutes 20 to 25 DNA samples, which can be analyzed simultaneously [12].

Genomic BAC libraries were constructed for the description of the genetic apparatus of many eukaryotic species, including human [13, 14], mouse [15], pig [16, 17], dairy cattle [18], horse [19], dog [20], and others.

In addition to genome mapping, large-insert genomic libraries is an essential tool for positional cloning of the chromosomal regions controlling quantitative traits. It is well known that most commercial traits of domestic animals have complex polygenic type of inheritance and are controlled by many genes, located in the QTLs (quantitative trait loci). Analysis of complex molecular architecture deserves special interest from the point of view of general genetics [21]. Additionally, the information on QTL region sequences can be used in cattle breeding for marker-assisted selection (MAS) [22–26].

Deep insight into the nature of QTLs requires examination of the following aspects [21]:

—the number and composition of the genes involved in the biochemical and physiological pathways of the phenotypic expression;

—the amount of mutations at QTL loci;

—the number and composition of QTLs, determining phenotypic variation of the trait at the intrapopulational, interspecific, and interspecific levels;

—epistatic interaction of the genes controlling the quantitative trait;

—possible pleiotropic effect of alleles of the genes, primarily on viability;

—molecular polymorphism of functionally different QTL alleles;

—molecular mechanisms determining phenotypic differences in quantitative traits;

—frequencies of the QTL alleles.

It should be noted that almost all of these positions require detailed sequence information, which can be obtained via positional cloning of QTLs. At present, several cases of successful positional cloning in human and mouse [27–29], as well as one case in dairy cattle [30], have been reported. In the latter study, the region having the highest effect on milk fat content was cloned. Subsequent reduction of the QTL localization interval by use of co-segregation analysis of the milk fat content trait and microsatellite alleles demonstrated that the candidate gene for this trait was located within the 3-cM chromosomal region flanked by BULGE13 and BULGE09 microsatellites. Screening of the dairy cattle genomic library using these markers as the hybridization probes resulted in the identification of long-insert DNA clones representing border sequences of this region. Using the method of "chromosome walk" (clones were end sequenced and their sequences were used for the creation of the DNA probes for the series of subsequent screenings) along with the data on the gene composition of the orthologous region of human chromosome, contig, consisting of 50 artificial bacterial chromosomes and giving the threefold coverage of the chromosomal region of interest, was constructed. Contig sequencing resulted in the discovery of the *DGAT1* gene (code for acyl-CoA diacylglycerol acyltransferase), which judged by its physiological activity, could be a candidate gene for this QTL. Sequence analysis of this gene in cattle breeds with alternative expression of the milk fat content trait enabled identification of the K232A point mutation, which significantly affected the expression of the trait in question [30]. Note that exactly long-insert genomic libraries serve as the basis for positional cloning. Special importance of genomic libraries for analysis of avian genomes, caused by rather limited capacity of classical cytogenetic analysis of these genomes owing to the presence of morphologically unidentifiable microchromosomes, accounting for about one third of avian genome, should be also emphasized [1].

AVIAN GENOMIC LIBRARIES

The first genomic library for the only member of Aves (which can be compared to the mammals relative to the level of the genome investigation), chicken *Gallus gallus* was constructed based on yeast artificial chromosomes [31]. Partly digested with the *Eco*RI restriction endonuclease genomic fragments were separated by use of pulsed-field electrophoresis and cloned into the pCGS966 YAC vector. One part of the library included 16 000 clones with the average insert size of 634 kb, which corresponded to 8.5 equivalents of the chicken haploid genome. The second part comprised 20 000 clones, among which 20% contained inserts of about 450 kb; the average insert size was not evaluated. The total number of clones was 36 000, which according to the authors' estimates, provided tenfold genome coverage [31]. By the late 1990s, the disadvantages of the yeast cloning systems became evident from the human genome investigations. For this reason, this library remained practically unused, although chimerism and clone stability in it were not estimated.

The first chicken genomic library based on bacterial artificial chromosome was constructed in two steps utilizing pBeloBAC11 vector [32]. First, using standard approach [9], 1440 clones with the average insert size of 180 kb were obtained. The rest of the library (2976 clones) was constructed using pulsed-field electrophoretic separation of donor DNA fragments, which provided generation of inserts with the average size of 490 kb. The latter condition was the reason for modification of the *E. coli* transformation technique to that utilizing strain DH10B. Thus, in the library, the insert size varied from 25 to 725 kb, with an average of 390 kb, which was unusually high for BAC libraries [3]. Based on the total number of clones in the library and on the average insert size, the genome coverage was evaluated as constituting 0.8 of chicken haploid genome, which was apparently insufficient for the effective library utilization. Within the framework of the European ChickMap project, a collection of random large-insert DNA clones was obtained and used as the probes for in situ hybridization of chicken mitotic chromosomes [33, 34].

Later, gridded chicken cosmid library RZPD-125, based on the genomic DNA from chicken Rhode Island breed from the collection of the Department of Selection, Munich Technical University, was constructed and characterized [1]. Genomic DNA was partly digested with *Sau*3A restriction endonuclease, and the fragment sizes were controlled by electrophoretic fractionation in agarose gels. Restriction was stopped at the fragment size reaching approximately 50 kb. Then, genomic fragments were cloned into sCos1 vector (Stratagene), using two different bacterial strains as the host cells, DH5αMCR (panels 1–51), and DL735 (panels 52–288). Note that the first bacterial system is more resistant towards not precisely accurate robot manipulations, used in the Resource Centers for colony replating. Unlike humans, robot cannot see the irregularities of

growing colonies and at replating is guided exclusively by the colony coordinates. The advantage of the second system is the absence of the TN100 insertion sequence, which can be transferred into cosmids, destroying the integrity of the genomic fragments [1]. Analysis of 68 clones showed that upon replating on panels 1–51 (host strain DH5αMCR) 98% of the colonies were preserved; on panels 52–288 (host strain DL735) 95% of the clones were preserved, which was quite sufficient in both cases [1]. The library contains 110 000 clones with an average insert size of about 40 kb, and the range of 20 to 48 kb [1]. The insert size was evaluated by restriction analysis of 68 clones. It was demonstrated that the genomic insert was preserved in all of the clones examined, which was a high index for libraries of this type. In further investigations, analysis of more than 100 clones revealed only one case of the insertion absence. Thus, stability of the cosmids can be estimated as 99%. Based on the average insert size and the total number of clones, it was established that the coverage of the library is four genome equivalents. The probability of recovering any specific chicken sequence (P) in this library is estimated as higher than 0.99 (if the number of clones = $\ln(1 - P)/\ln(1 -$ average insert size) [35]), which is consistent with the data on the presence of at least one hybridization signal upon the use of 22 DNA probes for the screening [1]. The library was screened using coding sequences as the probes, and for each probe at least one positive hybridization signal was detected. The average positive signal number was six with the range from one (gene *INS*) to ten (genes *AVR1–AVR7*, *ETS1*, and *GDF8*). Thirty-one cosmid clones from the RZPD-125 were physically mapped on mitotic chromosomes with the help of fluorescence in situ hybridization (FISH). No chimerism was detected [1]. It can be thereby concluded that the presence of avian-specific GC-repeats did not disturb stability of chicken genomic sequences in the *E. coli* cells.

The gridding scheme of this library provides precise identification of the positive clones at the presence of double hybridization signals corresponding to one clone, which makes unnecessary the rescreening procedure, required in other gridding schemes. Multiple copy number of the cosmids provides high density of DNA on the filters, which reduces the exposition time of the X-ray films to 2 h. The use of DH5αMCR and DL735 strains provides DNA isolation with standard kits for plasmid DNA isolation (for example, QIAGEN). From the one hand, the great number of clones (110 000) results in some difficulties of the library utilization, since it increases the replica size. On the other hand, this clone number may be useful for screening with combined DNA probes, representing different DNA regions.

The other three gridded genomic libraries, 031-JF256-BI, 032-JF256-RI, and 033-JF256-H3, were constructed using genomic DNA from the UCD001 inbred line of red jungle fowl, which demonstrates less than 1% of coding region sequence differences from

Avian genomic BAC-libraries from the resource centers GENEfinder (http://hbz.tamu.edu), BPRC (http://bacpac.chori.org), and AGI (http://genome.arizona.edu)

Species/breed/(strain)/individual	Average insert size (kb)	Number of clones	Genome coverage	Vector	Cloning site	Library code (website)
Red jungle fowl/inbred line/no. 256	150	38400	5.2×	pBeloBAC11	*Bam*HI	031-JF256-BI (hbz.tamu.edu)
	152	38400	5.3×	pECBAC1	*Eco*RI	032-JF256-RI (hbz.tamu.edu)
	171	38400	6.0×	pECBAC1	*Hin*dIII	033-JF256-H3 (hbz.tamu.edu)
	195	73700	12.0×	pTARBAC2.1	*Eco*RI	CHORI-261 (bacpac.chori.org)
Chicken/White Leghorn	130	49920	5.4×	pECBAC1	*Hin*dIII	020-CHK-H3 (hbz.tamu.edu)
Common turkey/inbred line Nicholas Turkey Breeding Farms	190	71000	11.1×	pTARBAC2.1	*Eco*RI	CHORI-260 (bacpac.chori.org)
Zebra finch	134	147456	10.2×	pCUGIBAC1	*Hin*dIII	TG_Ba (www.genome.arizona.edu)

chicken (http://hbz.tamu.edu; [36]). The main characteristics of these libraries are presented in the table. To reduce the heterozygosity level, genomic DNA for the libraries construction was isolated from one individual (no. 256). At the same time, this circumstance gives the chance that specific features of this individual can influence the nucleic acids composition in the library. The total number of BAC-clones in the libraries is 115 000 with the average insert size of 150 kb (ranging from 45 to 290 kb, as judged by 944 clones analyzed). The libraries provide the coverage of 15.2 haploid genome equivalents. The number of clones without the inserts in the libraries constitutes 4.8, 3.4, and 2.6%, respectively, which is higher than in the RZPD-125 library (approximately, 1%). On the other hand, compared to RZPD-125, the above libraries have the advantages of larger insert, higher genome coverage, and substantially smaller size of each library (almost threefold).

Preliminary screening of these libraries with 30 gene fragments demonstrated the possibility of using them for genome mapping. A total of 60 large-insert genomic clones were detected, which contained the sequences of interest, as judged by Southern blot-hybridization, PCR testing, or OVERGO hybridization [36, 37].

In addition to the three *G. gallus* genomic BAC-libraries described, a library based on the chicken (White Leghorn breed) genomic DNA fragments partly digested with the *Hin*dIII restriction endonuclease was constructed in the resource center GENEfinder (http://hbz.tamu.edu) [38]. The library was successfully used for the construction of contigs and regional chromosome sequencing [11].

Chicken BAC-libraries are widely used for mapping of its genome, as well as for establishing synteny with the genomes of other species, including, primarily, human. For instance, orthology of chromosome GGA15 regions and the regions of HAS12q24 and HSA22q11–q12 was established [39]. In addition, an association between GGA5 and HSA19 [40], as well as between GGA10 and HSA15 [41] was demonstrated. A comparative map of microchromosome GGA24 and HSA11 was constructed [42]. Conserved synteny of some mammalian and avian genomes, which can be traced back to the early stages of the vertebrate evolution, and involving the genes for melanocortin on chicken chromosome 2 and human chromosome 18, was demonstrated [43]. The regions of orthology for a number of chicken and human chromosomes were verified and extended [44–48]. The first physical map of chicken genome, comprised of 2331 BAC-contigs, containing from 2 to more than 200 individual clones, was generated [49].

In recent years, avian genomic resources were enlarged with the new BAC libraries (table). In the resource center BPRC one more red jungle fowl library, CHORI-261, as well as the library of common turkey (*Meleagris gallopavo*), CHORI-260 (http://bacpac.chori.org; [50]) were constructed. In the first case DNA from the same jungle fowl individual (no. 256), which served as a basis for the three GENEfinder libraries, was utilized. This fact enabled a substantial enhancement of the total genome coverage: genomic equivalent of the four red jungle fowl BAC libraries constituted more than 28 (average insert size, 167 kb, and total number of clones, 188 900). The CHORI-260 library was successfully screened for the integration of the chicken linkage map and the physical map of BAC contigs [37, 51]. Four red jungle fowl libraries and one chicken library served as a basis for the creation of total physical map and sequencing of chicken genome [52, 53]. In BAC/EST Resource Center of the Arizona Institute of Genomics

(AGI; http://www.genome.arizona.edu) BAC library of zebra finch *Taeniopygia guttata*, family Fringillidae, was generated. Additionally, in the BPRC Resource Center BAC library of the Californian condor (*Gymnogyps californianus*), family Accipitridae, was constructed (M. Nefedov, personal communication, 2003). In the same center fosmid library of red jungle fowl, CHORI-261, comprising 999 936 recombinant clones with the average insert size of 45 kb, was constructed using DNA from the same individual no. 256 (http://bacpac.chori.org/library.php?id = 201, http://poultry.mph.msu.edu/newsltrs/news034g.htlm; M. Nefedov, personal communication, 2003).

In conclusion, gridded large-insert genomic libraries (based on either cosmids, or artificial bacterial chromosomes) proved themselves in the experiments with mammals, and can be successfully applied for genome analysis, and possibly, for positional cloning of quantitative trait loci in Aves.

ACKNOWLEDGMENTS

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