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Short Communication

Construction of a gorilla fosmid library and its PCR screening system

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Abstract

A gorilla fosmid library of 261,120 independent clones was constructed and characterized. The fosmid vector is similar to the cosmid in average insert size of ca. 40 kb but contains the F factor for replication, and it is more resistant to recombination. This clone library represents about 3.7 times coverage of the gorilla genome. A simple screening system by PCR was established, and we successfully found 9 clones that cover the entire Hox A gene cluster of the gorilla genome. This gorilla fosmid DNA library is a useful resource for comparative genomics of human and apes.

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Gorilla, chimpanzee, bonobo, and orangutan are great apes, and they are the closest organisms to human. Despite the clear morphological differences such as cranial shape among them, their DNA sequences differ only 1–3% [1,2]. Recently, the draft human genome sequences were reported [3,4] and they made it possible to conduct comparative genomics of human and great ape genomes.

Genetic changes responsible for human-specific characters occurred in the human lineage during the past several million years after it separated from the common ancestor of human and chimpanzee, the closest organism to human [5,6]. This is why the chimpanzee genome is now the target of human comparative genomic study [2]. However, we also need genome sequence information of the outgroup species of human and chimpanzee to identify which differences are specific to human. Therefore, we decided to construct a fosmid library of gorilla. Although BAC libraries are often used for mammalian genome studies [e.g., 7–9], fosmid libraries have some advantages. First, a fosmid library is easier to handle than a BAC library due to its small insert size (ca. 40 kb). In many comparative genomic studies, the

region of interest is not so large that one fosmid clone can cover it. Second, because of its relatively small insert size, DNA extracted from a dead animal can be used. In fact, we used a liver sample stored in ethanol in room temperature in this study.

Fosmid clones are similar in size to cosmids due to their reliance on phage packaging for their introduction into bacteria, but they contain replicons derived from the F factor for DNA replication and segregation. Because of this, they are more stable than cosmids [10,11] and are suitable for rapidly creating genomic or chromosome-specific libraries [12,13]. We report here the construction of a gorilla genome fosmid DNA library and establishment of an efficient PCR screening system.

The genomic DNA was prepared from liver tissue of a dead female gorilla (“Taiko”), donated from the Ueno Zoo (Tokyo). The liver tissue was immediately put into 99% ethanol and stored until use to prevent degradation of DNA at room temperature. The genomic DNA was extracted from 500 mg liver tissue using the QIAamp Tissue Kit (Qiagen). The pKS143 fosmid vector was prepared in a manner similar to that of the pFOS1 vector [11]. This vector was developed by one of us (A.F.) and has been used for human chromosome 21 genomic sequencing [14] as well as for chimpanzee genome study [2]. Construction of this fosmid

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vector was performed according to the procedure for double *cos* cosmids [10,15]. To generate two arms, the plasmids were successively treated with *Bam*HI, alkaline phosphatase, and *Hpa*I. DNA molecules were then fragmented and blunt-ended before the ligation with pKS143 vector arms.

Fresh cells (*Escherichia coli* XL1-Blue) were cultured in a 50-ml flask containing 10 ml medium (NZY⁺ maltose⁺ Mg²⁺) at 37°C with shaking until the OD₆₀₀ reached 0.7. This took approximately 3–5 h at 150 rpm. Five milliliters of the cell suspension was transferred to a 15-ml tube, and we performed centrifugation for harvesting cells for 15 min at 3000 rpm, 30°C. The supernatant was discarded and 5 ml of 10 mM MgSO₄ and 250 μl of the in vitro packaging reaction mixture were added. The solution was carefully mixed by tapping the tube bottom with a finger and then incubated for 30 min at 25°C. It was transferred to a 50-ml tube and suspended in 20 ml SOC, followed by a 35-min incubation at 37°C with shaking at 150 rpm. We then performed centrifugation for 15 min at 3000 rpm, 30°C, and the cells were resuspended in 6.4 ml SOC. Eight hundred microliters of the cells was spread on 22 × 22-cm LB plates containing 25 μg/ml ampicillin, and they were incubated overnight at 37°C.

Well-separated colonies were picked and transferred into individual wells of 384 microtiter plates containing 60 μl/well LB supplemented with 7.5% glycerol and 25 μg/ml ampicillin using a Flexys colony picker (PBA Technology) and then incubated overnight at 37°C. The plates were stored at –80°C.

All 384-well plates containing gorilla fosmid clones were replicated using MicroGrid (BioRobotics) and incubated overnight at 37°C. Each 384-well plate was reversed (using a U-bottom polypropylene plate (Iwaki) instead of the 384-well plate cover) and centrifuged for 1 min at 1000 rpm, 4°C. These combined cells were carefully mixed and purified using an automatic plasmid isolation system PI-100 (Kurabo).

A total of 261,120 independent clones were isolated and arrayed to 680 × 384-well microtiter plates containing LB–7.5% glycerol plus ampicillin. The plates were incubated overnight at 37°C, replicated, and stored at –80°C. To evaluate the average insert size in the library, 100 clones were randomly selected from the gorilla fosmid library. Fosmid clone DNA was then completely digested using *Not*I, and the insert size was estimated by pulsed-field gel electrophoresis. The insert size was more or less constant at ca. 40 kb, as expected (data not shown). Therefore, the total size of this gorilla fosmid library is estimated to be 10,445 Mb (= 261,120 × 40 kb). The size of the gorilla genome was estimated to be 102% of the human genome [16], which was estimated to be 2800 Mb [3]. Our gorilla fosmid library thus represents about 3.66 (10,445/(2800 × 1.02)) coverage of the gorilla genome.

We named this fosmid clone library GGFP, after “*Gorilla gorilla* genomic library constructed by using fosmid vector pKS143.” This is the first gorilla fosmid library and

it will become a useful resource for human and ape genomic studies. This successful construction of a fosmid library also paves the way for constructing clone libraries for endangered species, whose DNA is not easy to obtain.

We introduced a two-step PCR screening system for our GGFP library. The first step is to use 384 fosmid clones in one plate as the unit. DNA is extracted from the pool of all 384 clones and used as template. Several 96-well plate PCRs are necessary for this step (Fig. 1A). The PCR is performed for 35 cycles consisting of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min.

It may be possible to pool further DNA of 10 or more plates in a future modification. When a plate with positive signal is found, the search is restricted to that plate, and the second step is to use DNA pooled from clones located in all 24 columns of the same row for the PCR template. At the same time, DNA of this plate is pooled from clones located in all 16 rows of the same column for the PCR template. A total of only 40 PCR reactions are necessary for this second step (Fig. 1B). We can then identify positive clones efficiently. This kind of hierarchical screening system is also used by various clone libraries [e.g., 10,17]. One advantage of our system is that the unit of pooled DNA is always a clear physical entity (plate, row, and column), and they are easy to handle.

The Hox A gene cluster is one of four Hox clusters in mammals and is known to establish the proper body patterning along the anterior–posterior axis [18]. It is of general interest to investigate the gorilla Hox A cluster as a comparative target with the human HOX A cluster. We thus screened the Hox A gene cluster region to test the quality of this library. Five PCR primer pairs used in this study are listed in Table 1. These primer sequences were based on the human BAC clone sequence (DDBJ/EMBL/GenBank International Nucleotide Sequence Database Accession No. AC004080) containing the HOX A gene cluster genes located at human chromosome 7. Those primers were first tested to obtain amplicons from the gorilla genomic DNA used as template.

Table 1 shows the summary of the PCR screening results for the five primer pairs. Because we were originally interested in obtaining any gorilla fosmid clones that cover the Hox A region, the number of screened plates (hence clones) varies for each primer pair. We calculated “the number of found clones/genome,” where 1 genome corresponds to 2856 Mb or 71,400 fosmid clones. The average number of positive clones found per gorilla genome was 0.89. This value is close to the expected number of clones detectable per genome, namely 1.0. This shows that our gorilla fosmid library GGFP well represents the 3.7× coverage of the gorilla genome.

Using this screening method, a total of nine positive clones were found, and they completely cover the gorilla Hox A gene cluster region (Fig. 2). These nine positive clones are GGFP-562J15, GGFP-367A20, GGFP-347D05,

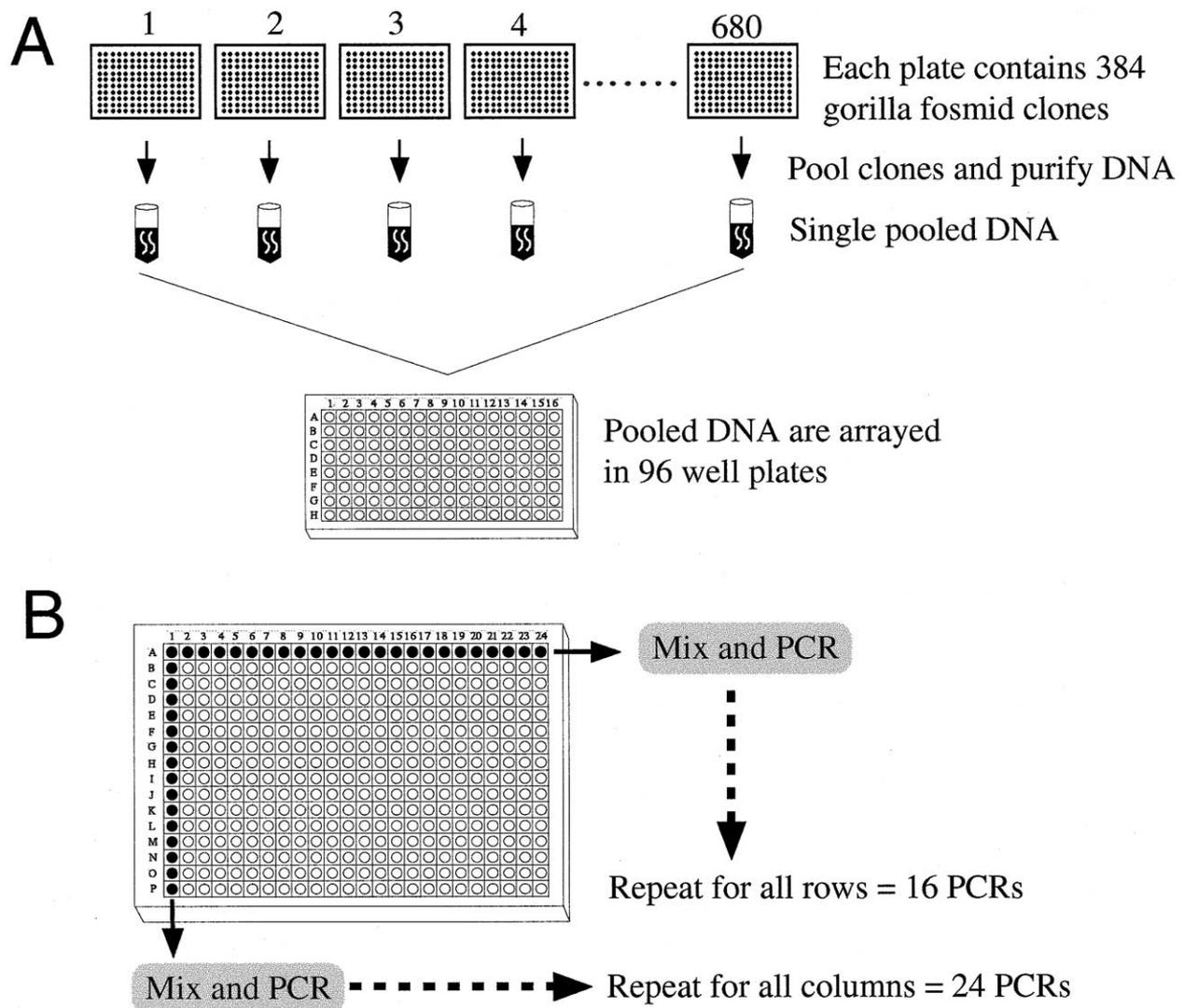


Fig. 1. A schematic illustration of our two-step PCR screening system. (A) The first step. The plates containing 384 independent fosmid clones were pooled and purified, and then PCR's were performed in 96-well plates using target primers. (B) The second step. After a plate containing a positive clone(s) is found, row-by-row and column-by-column screenings follow by pooling clones from the same row and same column, respectively.

GGFP-175G7, GGFP-452O13, GGFP-012E07, GGFP-210K06, GGFP-470N12, and GGFP-473C20. The tiling map of positive clones shown in Fig. 2 was constructed using fosmid-end sequencing of each clone. The end se-

quencing procedures were the same as published [2]. The seven clones that constitute a minimum tiling path are now being used for Hox A region nucleotide sequence determination (Kitano et al., manuscript in preparation).

Table 1
PCR screening result of the Hox A gene cluster from the gorilla fosmid library GGFP

Primer pair (forward/reverse)	No. positive clones	No. screened plates (No. screened clones)	No. found clones/genome
(1) GATATAGCGAAAGGCCGAGTC/CAGAAACTCAGTGTGGTACTT	1	672 (258,048)	0.28
(2) TTTATTCTTAGCACATGGCT/TTCTCTTCTCCTGCTCCGTT	2	384 (147,456)	0.97
(3) GAGCTGACTGTAAC TACTC/AATTCGCCTTTGTCTCCTGCGG	3	672 (258,048)	0.83
(4) TTAAC TAGTGGTTTTCAGTTG/ACGTGGTCTCCCATCCCTCGG	4	576 (221,184)	1.29
(5) ATCGCAGTTTTCTGTCTGGG/GAGCCACCTCAGGAATGTA	3	384 (147,456)	1.45
Total	13 ^a	2688 (1,032,192)	0.89

^a Some fosmid clones were found more than once by using different PCR primers (see Fig. 2).

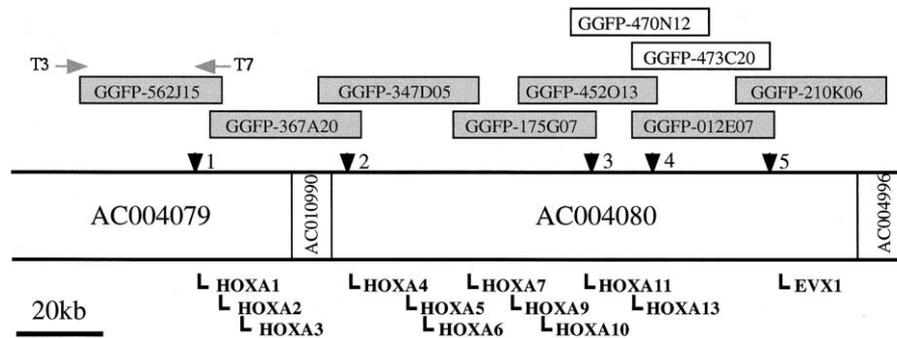


Fig. 2. The genomic map of the nine gorilla fosmid clones that cover the Hox A cluster region. The information on the human BAC clone was from the Ensembl genome server (http://www.ensembl.org/Homo_sapiens/). The position of each clone was determined by fosmid-end sequencing using the T3 and T7 primer sets. The clone names are represented by library name, plate number, and well position. For example, GGFP-562J15 stands for GGFP library plate 562, well J15. The seven shaded fosmid clones constitute the minimum tiling path. The triangles with numbers indicate primer pairs used for screening listed in Table 1.

We additionally screened four genes to test the genome-wide coverage of this gorilla fosmid library. The names of these genes and their chromosomal locations in the human genome are the ABO blood group gene at chromosome 9 (two clones using one PCR primer pair), the Rh blood group gene at chromosome 1 (nine clones using two PCR primer pairs), the alcohol dehydrogenase gene at chromosome 4 (three clones using two PCR primer pairs), and HERV at various chromosomes (seven clones using two PCR primer pairs). We found multiple positive clones in all cases. This is more evidence that our fosmid library GGFP covers the gorilla genome quite well.

We hope this newly constructed gorilla fosmid library will become a useful resource for human and ape comparative genomic studies. Our library GGFP is available for PCR screening upon request.

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