1124–1125 *Nucleic Acids Research, 1998, Vol. 26, No. 4*

Functional complementation by electroporation of human BACs into mammalian fibroblast cells

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Received November 17, 1997; Revised and Accepted January 7, 1998

ABSTRACT

Bacterial Artificial Chromosomes (BACs) have been used to complement a metabolic defect and to transfer a drug resistance marker into mammalian cells by electroporation. The selectable markers are stable and the recipient cells have BAC DNA integrated into the chromosomes as shown by fluorescent *in situ* hybridization, PCR and Southern hybridization.

Effective strategies for cloning human genes include positional cloning as well as functional complementation by direct selection of human cells transfected with cDNA expression libraries. Often positional cloning results in a region of interest spanning 1 Mb or more, from which candidate expressed sequence tags (ESTs) or cDNAs are screened by sequence and mutation analysis. If a suitable selection for phenotypic correction is available, functional complementation can yield a cDNA. However, large, low-abundance cDNAs have been difficult to clone by this method. Bacterial Artificial Chromosomes (BACs) were developed as genomic cloning vectors containing inserts that range up to 300 kb (1). Provided with a complementation assay, a BAC library with adequate coverage of the genome and a gene of average size, the identification of an individual BAC might reduce the region of interest to 100–200 kb. We have tested the feasibility of functional complementation with human BACs by electroporation of BAC DNA into fibroblast cells. A BAC carrying the human adenosine phosphoribosyltransferase (*aprt*) gene was electroporated into *aprt*-deleted mouse A9 fibroblasts, allowing metabolic selection. A different BAC which had been retrofitted with a selectable marker conferring hygromycin resistance was electroporated into human fibroblasts, allowing selection by drug resistance. In each case we determined that cells with a stable selectable marker could be identified and that the selectable marker was integrated into the fibroblast chromosomes. A9 cells (2) are deleted in a region of chromosome 8 which is syntenic to human chromosome 16q, where the *aprt* gene is located (3). Consequently, A9 cells are sensitive to AAT medium, which blocks *de novo* purine biosynthesis. The introduction of a human *aprt* gene on a BAC should complement the mouse *aprt* deficiency by restoring the purine salvage pathway, allowing for the selection of corrected colonies in AAT medium (4,5). A BAC carrying the human *aprt* gene was isolated from the Research Genetics Human BAC Library using primers designed for genomic *aprt* (6,7). The BAC DNA was purified on Qiagen Tip-500 columns and the genomic insert was determined to be 110 kb by pulse-field gel electrophoresis (PFGE) after restriction with *Not*I which excises the insert. Electroporation was with cells harvested at 80% confluency from D-MEM with 15% bovine calf serum, using the BioRad Gene Pulser (300 V at 960 µF with a 0.4 cm gap). Cells were harvested and washed with HBSS (Gibco BRL), held on ice for 10 min in 0.85 ml cytomix (8) containing 5 µg BAC DNA, electroporated, returned to ice for several minutes and re-plated. After a change of medium on day two, selecting agents (3.75×10^{-3} M adenine, 4×10^{-5} M aminopterin, 8×10^{-4} M thymidine) and/or 5×10^{-5} M azaserine were added on day three. Colonies were counted after 3–4 weeks of continuous selection. Complemented colonies grew under continued selection with AAT medium, indicating that they arose from the successful introduction and stable maintenance of the *aprt* gene into the mouse genome. Approximately 30 colonies were obtained per electroporation. The human *aprt* gene was detected only in corrected A9 cells by PCR using human-specific primers (*aprt*-J1: 5′-CAGGACAGAGGGTGGTCGTC; *aprt*-J2: 5′-CACTCCAGGACCTCAGCCTG). Since stable maintenance suggested recombination of BAC DNA into the chromosome, fluorescent *in situ* hybridization (FISH) was performed on corrected A9 clonal cell lines, using the entire 110 kb BAC as a probe. Figure 1B shows that BAC 64A15 had integrated. The site of integration was different in each of two clonal cell lines inspected. The strength of the signal suggests a reiterated integrate. A control FISH to a normal human cell line gave a signal at the tip of chromosome 16q, as expected (not shown). The same BAC failed to hybridize to mouse A9 cells, deleted in the *aprt* region (Fig. 1A), or a normal mouse cell line (not shown). The presence of the human *aprt* gene was also shown by Southern (9) hybridization. The probe detected an 11 kb *Ssp*I fragment which was identical in size to a fragment detected in total human DNA. The A9 cell line also gave no signal without electroporation with BAC 64A15, using the human *aprt* PCR product as a probe. The lower size limit for FISH detection is ~10 kb; the fact that a single, large signal was detected suggests that the BAC integrated as a unit rather than fragmenting.

BAC 183L15 (from chromosome 3p) was retrofitted by linearizing with *Bam*HI at a unique site in the vector multi-cloning site, then ligating the BAC with pREP4 (Invitrogen) vector similarly cut with *Bam*HI. The BAC 183L15 60 kb genomic insert from chromosome 3p25 does not contain a *Bam*HI site. PFGE of BAC 183L15-Hygro DNA showed that the size of the genomic insert after retrofitting, remained unchanged after extensive growth

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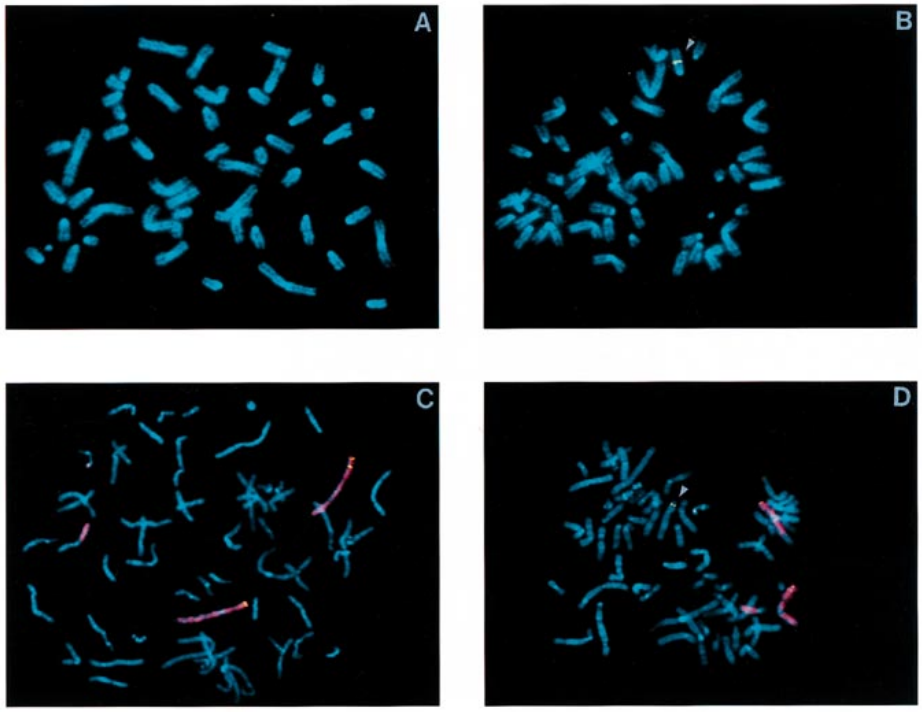

Figure 1. FISH analysis. Wet mount slides were prepared for FISH analysis (11). BAC DNA was nick translated with digoxygenin-11-dUTP (Boehringer Mannheim). A biotin-labeled chromosome 3 paint (Cambio) was prepared according to the manufacturer. Following denaturation of chromosome preparations, probes were combined and hybridized overnight at 37°C. Detection of 64A15 APRT was with anti-digoxygenin FITC (Oncor); dual detection of 183L15 Hygro BAC and chromosome 3 paint was with anti-digoxygenin FITC and avidin Texas Red (Oncor). Arrows indicate integrated BACs. (**A**) A9 cells prior to electroporation. (**B**) A9 cells electroporated with BAC 64A15. (**C**) PD 224 cells prior to electroporation. (**D**) PD 224 cells electroporated with BAC 183L15-Hygro.

despite the 10-fold greater yield, presumably due to an increased copy number in the presence of the colE1 origin.

PD224 fibroblasts [immortalized human fibroblasts, Fanconi anemia, type A (10)] were electroporated with BAC 183L15-Hygro and selected with 100 µg/ml hygromycin. The electroporation yielded three to five times more selected colonies than the electroporated A9 cells with AAT selection. To test whether the compact, supercoiled form of the BACs improved electroporation, λ terminase (Epicentre Technologies) was used to linearize BAC183L15-Hygro at the *cosN* site prior to electroporation. There was a 30–40% reduction in drug-resistant colonies, suggesting that the supercoiled form of the BAC is preferable for electroporation. Co-electroporation of intact BACs with small plasmids carrying antibiotic resistance markers at varying ratios decreased the efficiency of transfer of BAC DNA. The presence of BAC183L15-Hygro in PD224 fibroblasts was detected by PCR of genomic DNA, using primers spanning the junctions of the BAC vector and the genomic insert (M13 reverse primer: 5′-CACAGGAAACAGCTATGACC; 183R-2: 5′-TTA-CAAAAGTGTGGGAGCCC). The junction between the BAC vector and the pREP4 vector also was detected by PCR. This implies that at least 17.7 kb of contiguous DNA (10.2 kb of pREP4 vector plus 7.5 kb of pBeloBAC-11 vector) had integrated into the genome. Attempts to find episomal BAC DNA after long term selection were unsuccessful using Hirt lysates (9). Integration of BAC 183L15-Hygro was also demonstrated by FISH analysis (Fig. 1). PD224 cells show aneuploidy and rearrangement, including translocation of 3p (Fig. 1C); however, before electroporation the signals for the modified BAC were associated only with chromosome 3, as defined by a chromosome 3 paint. After electroporation a signal was seen on a chromosome other than 3, when probed with BAC 183L15-Hygro (Fig. 1D). The unmodified BAC 183L15 gave the same signal (not shown).

Southern hybridization was used to show the presence of pREP4-BAC 183L15 in genomic DNA of PD224 cells after electroporation (not shown), using a 3 kb restriction fragment containing BAC vector DNA as a probe. No signal was detected in non-electroporated cells. Our results demonstrate that human BACs may be used for stable long-term complementation in mammalian fibroblasts as an aid to gene localization, in agreement with recent results by others (12).

ACKNOWLEDGEMENT

This research supported by a grant from the National Institute of Health (1P01HL48546).

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