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Genetics

Construction of human chromosome 21-specific yeast artificial chromosomes

(flow-sorted chromosomes/genome mapping)

MARY KAY MCCORMICK*†‡, JAMES H. SHERO§, MEI CHI CHEUNG¶, YUET WAI KAN¶, PHILIP A. HIETER†§, AND STYLIANOS E. ANTONARAKIS*†‖

*Genetics Unit, Department of Pediatrics, Center for Medical Genetics, †Predoctoral Training Program in Human Genetics, and §Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205; and ¶Howard Hughes Medical Institute, University of California, San Francisco, CA 94143

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ABSTRACT **Chromosome 21-specific yeast artificial chromosomes (YACs) have been constructed by a method that performs all steps in agarose, allowing size selection by pulsed-field gel electrophoresis and the use of nanogram to microgram quantities of DNA. The DNA sources used were hybrid cell line WAV-17, containing chromosome 21 as the only human chromosome and flow-sorted chromosome 21. The transformation efficiency of ligation products was similar to that obtained in aqueous transformations and yielded YACs with sizes ranging from 100 kilobases (kb) to >1 megabase when polyamines were included in the transformation procedure. Twenty-five YACs containing human DNA have been obtained from a mouse–human hybrid, ranging in size from 200 to >1000 kb, with an average size of 410 kb. Ten of these YACs were localized to subregions of chromosome 21 by hybridization of RNA probes (corresponding to the YAC ends recovered in *Escherichia coli*) to a panel of somatic cell hybrid DNA. Twenty-one human YACs, ranging in size from 100 to 500 kb, with an average size of 150 kb, were obtained from ≈50 ng of flow-sorted chromosome 21 DNA. Three were localized to subregions of chromosome 21. YACs will aid the construction of a physical map of human chromosome 21 and the study of disorders associated with chromosome 21 such as Alzheimer disease and Down syndrome.**

The feasibility of constructing yeast artificial chromosomes (YACs) (1) containing several hundred kilobases (kb) of human DNA has been shown (2–4). Applications of this technology to the construction of libraries of human chromosomes or chromosome fragments and of the total human genome (3–5) are being attempted. The availability of such libraries would be advantageous to the progress of the human genome mapping effort due to the greater size of YAC clones over λ and cosmid clones and the possibility that segments of DNA not easily cloned in standard vectors will be represented in YAC libraries. Complete physical maps may require a combination of YAC and cosmid clones, for example, as has been demonstrated recently in the construction of a partial physical map of the *Caenorhabditis elegans* genome (6). YACs will also be useful in the analysis of human genetic diseases, since, together with pulsed-field gel electrophoresis and technologies for cloning pieces of DNA originally separated by hundreds of kilobases (7), they will aid completion of genetic and physical maps of the chromosomes and facilitate movement from cloned markers to genetically linked disease loci.

The initial success of cloning large segments of human DNA in YACs employed a strategy that used size selection on sucrose gradients, which requires several hundred micrograms of DNA, and generated an average insert size of <200 kb. We have developed a procedure that allows use of nanogram to microgram quantities of DNA, minimizes handling and shearing of high molecular weight DNA by performing all steps in low-melting-temperature agarose (LMA), and results in the recovery of YACs with an average size >400 kb. This method has been applied to the construction of human chromosome 21-specific YACs using DNA from both a somatic cell hybrid and flow-sorted chromosomes. Location of the YACs on human chromosome 21 was confirmed by using somatic cell hybrid mapping panels.

MATERIALS AND METHODS

A general strategy for the construction and characterization of YACs is outlined in Fig. 1.

Preparation of High Molecular Weight DNA. *Hybrid cell line.* WAV-17, a mouse–human hybrid containing chromosome 21 in ≈3 copies per cell, or ≈2% of the genome, as the only human chromosome (8) was grown in alpha MEM medium (GIBCO). Cells were harvested and mixed with an equal vol of 2% LMA in 0.125 M EDTA and 80-μl aliquots were distributed in blocks (1.5 × 3 mm) in a Plexiglas mold. Solidified blocks were dialyzed against NDS (0.5 M EDTA, pH 9.0/1% sodium lauryl sarcosyl/2 mg of proteinase K per ml) at 50°C overnight, followed by dialysis against TE50 (10 mM Tris·HCl, pH 7.5/50 mM EDTA), and storage at 4°C.

Flow-sorted chromosome 21. A normal diploid human lymphoblastoid cell line (GM130B) (National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Camden, NJ) was used as a source of chromosomes. Chromosome suspensions were prepared and stained with Hoechst 33342/chromomycin 3A (9). Chromosome 21 was sorted with a custom dual laser Becton Dickinson FACS IV sorter into a SW50 ultracentrifuge tube that was cushioned with 0.7% LMA in 0.125 M EDTA/10 mM Tris·HCl, pH 7.5/25 mM NaCl. Approximately 2.0 × 10^6 chromosomes were sorted and spun down at 25,000 rpm (SW50 rotor) for 30 hr. The volume of the plug was 300–400 μl. Approximately 100 μl of TE50 plus 50 mM NaCl was added to the plug and chromosomes, melted at 65°C, and resolidified. Chromosomes embedded in LMA were treated as described above for hybrid cells.

Restriction Endonuclease Digestions of High Molecular Weight DNA. *Not I complete digestion.* Blocks were dialyzed

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Abbreviations: YAC, yeast artificial chromosome; LMA, low-melting-temperature agarose; OFAGE, orthogonal-field alternation gel electrophoresis; cfu, colony-forming unit(s); Mb, megabase(s).

‡Present address: M886 Los Alamos National Laboratory, Los Alamos, NM 87545.
‖To whom reprint requests should be addressed at: Johns Hopkins University School of Medicine, CMSC 10-111, 600 North Wolfe Street, Baltimore, MD 21205.

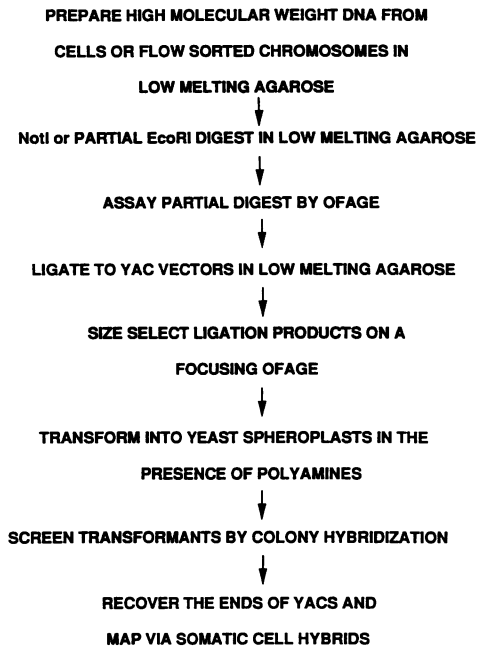

FIG. 1. A general strategy for the construction of YACs.

against 50 mM NaCl, melted at 65°C, adjusted to 1× *Not* I buffer (by addition of 0.1 vol of a 10× stock), and cooled to 37°C. Complete digestions were done with 6 units of *Not* I per μg of DNA at 37°C overnight.

EcoRI partial digestion. Blocks were dialyzed against 50 mM NaCl, melted at 65°C, adjusted to 1× restriction buffer, and cooled to 37°C. Partial digests at 37°C were done by varying the concentration of *Eco*RI from 0.0025 unit per μg to 25 units per μg of DNA. Aliquots of partial digestions were assayed by orthogonal-field alternation gel electrophoresis (OFAGE).

Ligation reactions. Digested DNA was dialyzed against 50 mM NaCl, melted at 65°C, and cooled to 42°C. Vector arms, prepared as described below, were mixed in at 65°C or 42°C in a 50- to 100-fold molar excess over insert DNA. Ligations were done overnight at 16°C with 5 μl of ligase (400,000 units/ml; NEB).

Size Selection of Ligation Products. Ligation reaction mixtures were heated at 65°C and electrophoresed in 1% LMA at 25 V for 8–18 hr in a conventional (one direction) apparatus followed by OFAGE at 270 V and a 6-sec switch time for 10–14 hr. Under these conditions, DNA fragments >200 kb were focused into a single sharp band, which was cut out of the gel.

Transformation. Size-selected ligation products in LMA were dialyzed against 50 mM NaCl and heated to 65°C, and 10 μl was added directly to 100 μl of yeast spheroplasts strain YPH274 (10). Spheroplasts were generated according to the procedure of Burgers and Percival (11) with the following modifications: 0.1 M sodium citrate (pH 5.8) in SCEM (1.0 M sorbitol/0.1 M sodium citrate, pH 5.8/10 mM EDTA/30 mM 2-mercaptoethanol) was replaced with 0.01 M sodium phosphate (pH 7.5). Zymolyase (20T, ICN), at 9 units per 50 ml of cells at an OD_{600} of 4.0, was used instead of lyticase. Polyamines (100× stock of 75 mM spermidine/30 mM spermine) were included in the transformation procedure at a 1× concentration beginning with addition to the DNA prior to transformation and all subsequent steps up to and including addition of PEG to the spheroplasts. Transformants were selected on medium lacking uracil and tryptophan and were visible after 2–3 days.

Characterization, Screening, and Mapping of YACs. After transformation (5–7 days), mitotic stability of transformants was determined by colony color assay (12) and the size of the YACs was determined by pulsed-field gel electrophoresis. All the transformants were picked and ordered on plates lacking uracil and tryptophan and grown for 2–3 days at 30°C followed by replica plating onto a nitrocellulose filter on a YPD (13) plate, which was then grown overnight at 30°C. Filters were removed to 3MM chromatography paper soaked in 1 M sorbitol/0.01 M sodium phosphate/10 mM EDTA/20T Zymolyase (10 μg/ml) and incubated at 37°C overnight. Filters were denatured 5 min in 0.5 M NaOH/0.6 M NaCl and neutralized in 1.0 M Tris·HCl/1.5 M NaCl twice for 5 min each, air-dried, and baked for 2 hr at 80°C. Prehybridization was for 2 hr at 37°C in 50% formamide/1× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/3× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS/1.0 mM Na_2EDTA, pH 8.0/10 mM Hepes/salmon sperm DNA (100 μg/ml), 0.05% pyrophosphate. A 2.0-kb gel-purified insert of five tandem copies of human repetitive *Alu* I sequences was labeled using a random priming kit (BRL) and added to the prehybridization mixture at 2.0×10^6 cpml. Hybridization was at 37°C for 36–48 hr. Filters were washed in 2× SSC/1% SDS at room temperature for 5 min; 0.1× SSC/1% SDS for 20 min at 65°C two times; 2× SSC at room temperature for 5 min. Exposure was overnight at −70°C. Positives were rescreened by Southern blot of pulsed-field gels probed with human genomic DNA or *Alu* I sequences labeled by random priming. Hybridization and washing were as described above. The chromosomal location of human YACs was determined by recovering the ends of the YACs as plasmids in *E. coli*, digesting the plasmid DNAs with *Hae* III (16), synthesizing RNA probes to the ends (Stratagene), and hybridizing to a somatic cell hybrid panel. Somatic cell hybrid panels contained genomic DNA from human lymphocytes, Chinese hamster ovary (CHO) fibroblasts, and mouse fibroblasts, and from mouse-human and CHO–human hybrids containing different portions of human chromosome 21 (see Fig. 3).

Vectors. Vectors pJS97 and pJS98 (J.H.S., M.K.M., S.E.A., and P.A.H., unpublished data) were used in these experiments. pJS97 was digested with either *Cla* I and *Eco*RI or with *Cla* I and *Not* I. pJS98 was digested with either *Sal* I and *Eco*RI or with *Sal* I and *Not* I. Linearized vector was treated with calf intestinal alkaline phosphatase (Boehringer Mannheim) according to the manufacturer's instructions and the arms were resuspended at a concentration of 0.5 μg/μl.

RESULTS

We have developed a protocol for the construction of YACs in which all the steps are performed in the presence of LMA, and we used this protocol to generate chromosome 21-specific YACs. Approximately 5.0 μg of size-selected ligated DNA from hybrid cell line WAV-17 generated 2100 transformants when the efficiency of transforming control plasmid DNA was 1×10^6 colony-forming units (cfu)/μg. Seventy percent of the transformants were recombinant as determined by Southern blot of pulsed-field gels, which also revealed the average size of the YACs to be 430 kb (Fig. 2 *a* and *b*). Therefore, ≈900 megabases (Mb) of the mouse genome is represented in this partial library. Approximately 2%, or 29, of the recombinants were expected to contain human DNA. Twenty-one human YACs were identified by colony hybridization using a human repetitive *Alu* I sequence as a probe. The size of the human YACs ranged from 200 to >1000 kb, with an average size of 410 kb (Fig. 2 *c* and *d*), representing between 9 and 10 Mb or ≈20% of chromosome 21. In addition, four human YACs with an average size of 120 kb were generated in an earlier experiment in which polyamines were not used. Chromosomal location for 11 of the 25

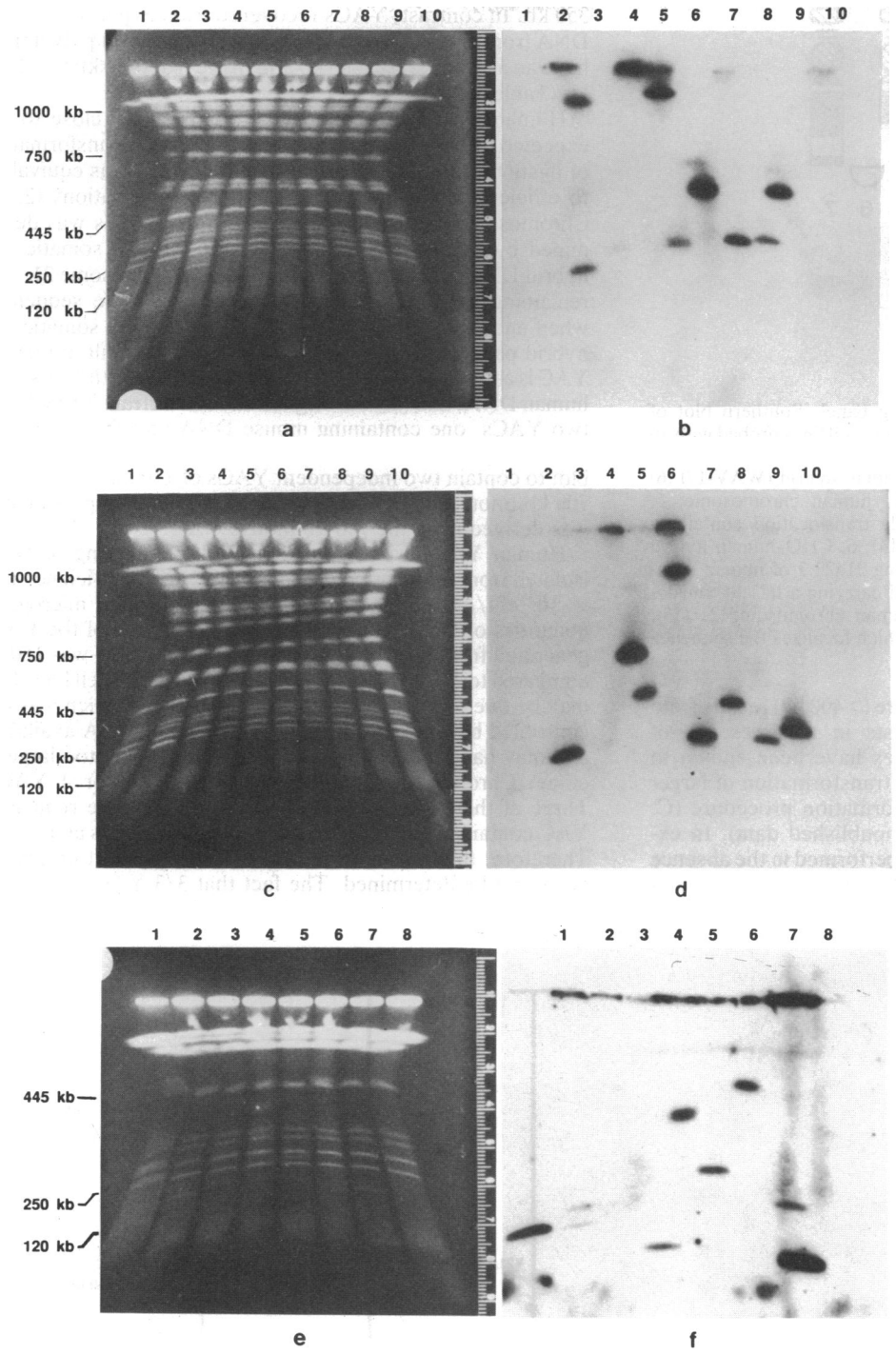

FIG. 2. Electrophoretic karyotypes and Southern blots of yeast strains containing YACs. (*a*) Ethidium bromide-stained OFAGE. Lanes: 1, DNA from a strain containing a known 120-kb human YAC; 2–10, DNA from strains containing mouse YACs generated from DNA from WAV-17. (*b*) Southern blot of *a* probed with mouse genomic DNA. (*c*) Ethidium bromide-stained OFAGE. Lanes: 1, DNA from a strain containing a known 120-kb human YAC; 2–9, DNA from strains containing YACs generated from DNA from WAV-17 that were positive by colony screening for hybridization to an *Alu* I probe; 10, DNA from a strain containing a known 280-kb mouse YAC that comigrates with an endogenous yeast chromosome (band 2). (*d*) Southern blot of *c* probed with human genomic DNA. (*e*) Ethidium bromide-stained OFAGE. Lanes: 1, DNA from a strain containing a known 120-kb human YAC; 2–7, DNA from strains containing YACs generated from flow-sorted chromosome 21; 8, DNA from a strain containing a known 280-kb mouse YAC. (*f*) Southern blot of *e* probed with human genomic DNA.

human YACs has been determined. Ten of these map on chromosome 21, 3 in the region 21pter–21q22.2, 1 in the region 21pter–21q21, 3 in the region 21q21–q22.2, and 3 in 21q22.3 (Figs. 3 and 4).

Approximately 50 ng of DNA from flow-sorted chromosome 21 was also used to construct YACs. The size of the DNA isolated from flow-sorted chromosomes was assessed by OFAGE. Approximately 5.0 ng of deproteinized DNA was electrophoresed by using a 2-min pulse at 200 V for 24 hr. Under these conditions, the 1.0-Mb *Saccharomyces cerevisiae* chromosome migrated ≈1.0 cm into the gel. After transfer of the gel and hybridization to human DNA, the majority of the signal from the flow-sorted DNA remained in the well, indicating it to be >1.0 Mb. A *Not* I or partial *Eco*RI digest of this DNA was used to construct YACs. Twenty-one recombinants were obtained as determined by Southern blot of pulsed-field gels with human genomic DNA as a probe (Fig. 2 *e* and *f*). The control transformation efficiency was 1 × 10^6 cfu/μg of plasmid DNA. The size of the YACs ranged from 100 to 500 kb, with an average size of 150 kb, representing ≈3.0 Mb of chromosome 21. Chromosome location for 4 of the 21 YACs was determined by using a somatic cell hybrid panel. Three were localized on chromosome 21 and two were subregionally mapped, 1 in the region 21q21–q22.2 and 1 in 21q22.3.

DISCUSSION

We have developed an alternative protocol to construct and recover YACs several hundred kilobases in size and we used this protocol to generate a partial library of the mouse genome and of human chromosome 21. The average size of

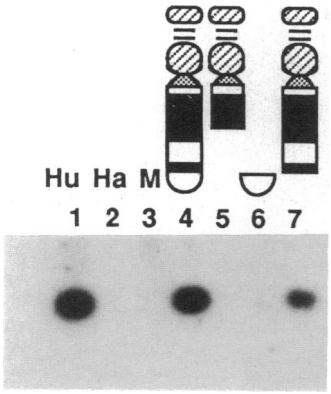

FIG. 3. Somatic cell hybrid mapping panel. Southern blot of *Bam*HI/*Eco*RI-digested genomic DNA from cell lines probed with an RNA probe synthesized to the end of human YAC Y21.31. Lanes: 1, human; 2, CHO; 3, mouse; 4, mouse–human hybrid (WAV-17) (6) containing chromosome 21 as the only human chromosome; 5, mouse–human hybrid carrying a t(1;21) translocation containing 21pter–q21 of human chromosome 21 (14); 6, CHO–human hybrid carrying a t(8;21) translocation containing 21q22.3 of human chromosome 21 (15); 7, CHO–human hybrid carrying a t(21;8) translocation containing 21pter–21q22.2 of human chromosome 21 (15). Y21.31 is present in lanes 1, 4, and 7, which localizes the sequence in the region 21q21–q22.2.

the YACs from WAV-17 reported here (>400 kb) results from transformation of yeast spheroplasts in the presence of agarose and polyamines. Polyamines have been shown to reduce an extreme size bias against transformation of larger YACs when included in this transformation procedure (C. Connelly, P.A.H., and M.K.M., unpublished data). In experiments with DNA from WAV-17 performed in the absence of polyamines, the average size of the recovered YACs was ≈150 kb and the range of sizes was narrow, between 100 and 350 kb. In contrast, YACs recovered from experiments with DNA from WAV-17 performed in the presence of polyamines have an average size >400 kb and range from 100 kb to >1000 kb (Table 1).

Human YACs were obtained from WAV-17 close to the expected frequency of ≈2%. The efficiency of transformation of ligation products, 2×10^2–1×10^3 cfu/μg, was equivalent to efficiencies reported for aqueous transformations (2, 3). Chromosome location for 11 of 25 human YACs was determined by hybridizing recovered YAC ends to somatic cell hybrid DNAs. Ten of these localized on chromosome 21. The remaining YAC hybridized to mouse repetitive sequences when ends were recovered and used to probe a somatic cell hybrid panel. Possible explanations for this result are (*i*) the YAC is a false positive; (*ii*) the YAC contained both mouse and human DNA; and (*iii*) the original strain was transformed with two YACs, one containing mouse DNA and the other containing human DNA. The strain was later shown by Southern blot to contain two independent YACs of 150 kb (human) and 700 kb (mouse). The end recovered and mapped presumably was derived from the 700-kb mouse YAC.

Human YACs were also recovered from 50 ng of DNA isolated from flow-sorted chromosome 21 at an efficiency (4.2×10^2 cfu/μg) within the range obtained when microgram quantities of DNA were used. The average size of the YACs generated from flow-sorted chromosome 21 DNA was 150 kb compared to 410 kb for YACs generated from the cell line. This may be due to an incomplete *Not* I digestion, which was not controlled because of the small quantities of DNA available, and may have resulted in many fragments being too large to clone. Chromosome location was attempted for 4 YACs. Three of them localized on chromosome 21. The remaining YAC contained highly repetitive human sequences at its ends. Therefore, the human chromosome from which it originated could not be determined. The fact that 3/3 YACs for which unique probes were obtained mapped to chromosome 21

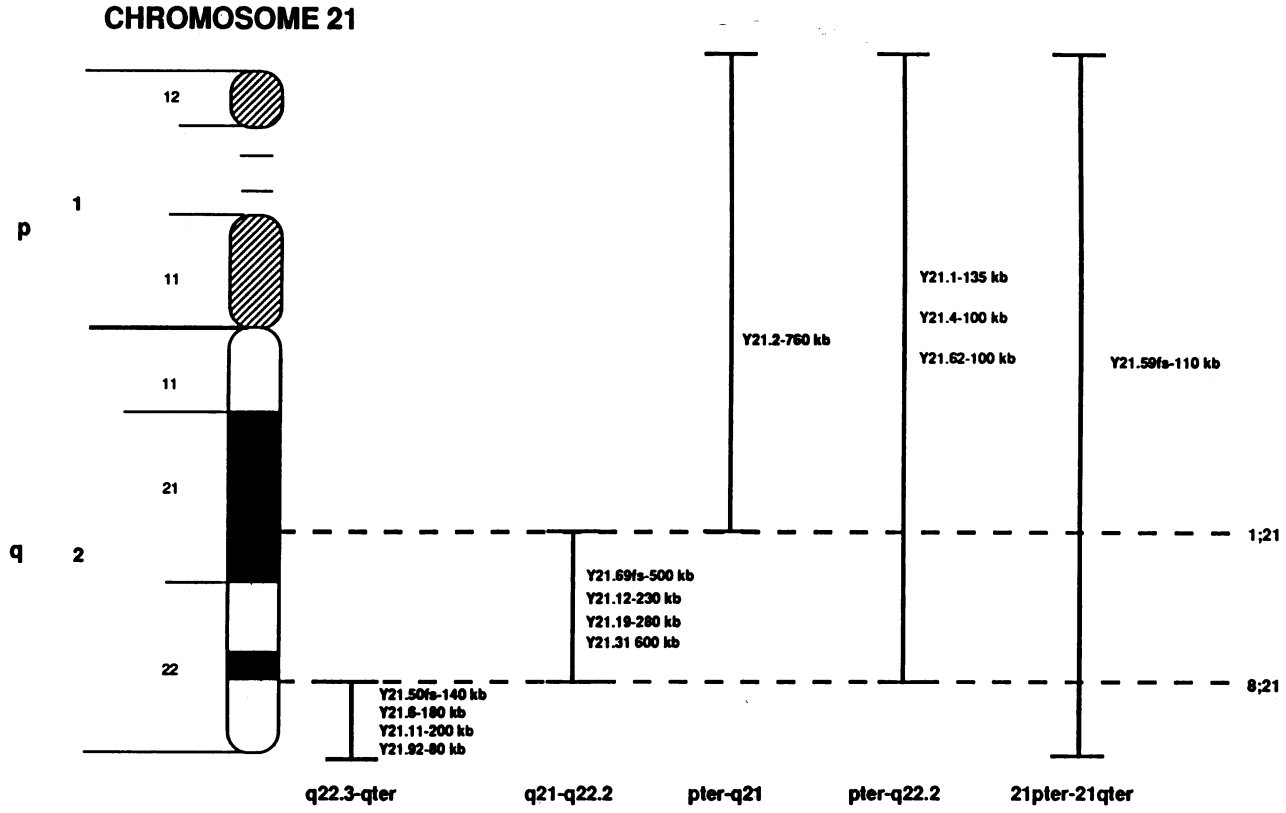

FIG. 4. Regional mapping of YACs on chromosome 21. The regions of chromosome 21 were defined by the hybrid cell lines described in Fig. 3. Placement of a YAC in a region was based on the presence or absence of hybridization of a YAC to each of the cell lines.

Table 1. Summary of YAC data from hybrid cells and flow-sorted chromosome 21

DNA source	Transformation efficiency, cfu/μg	Average size, kb	Size range, kb	No. of human YACs	Mapping data	
					Total mapped	Chromosome 21
WAV-17	200–1000	430	250 to >1000	25	11	10
Flow-sorted chromosome 21	≈400	150	100 to 500	21	4	3

indicates that flow-sorted chromosome DNA is an effective DNA source for constructing chromosome-specific YAC libraries.

The demonstrated feasibility of using flow-sorted chromosomes as an alternative to hybrid cell lines for a DNA source in the construction of YAC libraries is significant. Since a high percentage of flow-sorted material will correspond to the chromosome of interest compared to using DNA from a cell line, fewer transformants need to be generated and screened to obtain enough clones to represent a complete library. For example, with average sized YACs of ≈400 kb, a chromosome 21 library would be represented with a 3-fold redundancy by ≈330 YACs. If only 75% of the YACs are chromosome 21 specific, this number could be generated from ≈1.0 μg of DNA from flow-sorted chromosome 21. At least 0.1 μg of flow-sorted chromosome 21 can be obtained per week. In comparison, using WAV-17 as a source of DNA, ≈80 μg of size-selected DNA would be required to generate a minimum of 16,000 YACs of which ≈2%, or 320, would contain human chromosome 21 DNA.

The advantage of having a chromosome-specific library in YACs is somewhat dependent on the ability to manipulate YACs once they are obtained. The vectors used here were designed to allow recovery of both ends of a YAC as plasmids in *E. coli* and to allow the synthesis of RNA probes corresponding to each end of a YAC (J.H.S., M.K.M., S.E.A., and P.A.H., unpublished data). These features facilitate rapid determination of the chromosome location of a YAC and will simplify the construction of a physical map of the chromosome. YACs can also be manipulated by techniques developed for yeast genetics. For example, physical mapping of sequences on a YAC can be accomplished through chromosome fragmentation (17, 18) and integrating vectors can be used to add, exchange, or disrupt sequences on a YAC, which has potential uses in the screening, characterization, and mapping of YACs (18, 19).

Our interest in generating a YAC library of human chromosome 21 is 2-fold. As it is the smallest human chromosome, representing ≈1.5% of the genome, it is a logical choice for an initial attempt at construction of a physical map. In addition, at least two genetic disorders, a type of familial Alzheimer disease (20) and Down syndrome (21), are associated with human chromosome 21. The availability of a physical map of the chromosome may contribute to the identification of the gene(s) involved in these disorders. Chromosome 21 YACs may now be used as a source of new probes for the continued molecular characterization of the area of chromosome 21 that may be important in contributing to Down syndrome when triplicated (22, 23). The contribution of candidate genes involved in the phenotype of Down syndrome can potentially be assessed by using YACs to create transomic mice (24). The difficulty of cloning and characterizing such candidate genes will be reduced by the availability of a chromosome 21 YAC library.

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