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Modification and Transfer into an Embryonal Carcinoma Cell Line of a 360-Kilobase Human-Derived Yeast Artificial Chromosome

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A neomycin resistance cassette was integrated into the human-derived insert of a 360-kilobase yeast artificial chromosome (YAC) by targeting homologous recombination to *Alu* repeat sequences. The modified YAC was transferred into an embryonal carcinoma cell line by using polyethylene glycol-mediated spheroplast fusion. A single copy of the human sequence was introduced intact and stably maintained in the absence of selection for over 40 generations. A substantial portion of the yeast genome was retained in hybrids in addition to the YAC. Hybrid cells containing the YAC retained the ability to differentiate when treated with retinoic acid. This approach provides a powerful tool for in vitro analysis because it can be used to modify any human DNA cloned as a YAC and to transfer large fragments of DNA intact into cultured mammalian cells, thereby facilitating functional studies of genes in the context of extensive flanking DNA sequences.

The ability to clone segments of DNA as large as 1 megabase in yeast cells as yeast artificial chromosomes (YACs) (3) has facilitated the mapping and analysis of complex genomes (5, 8). These large cloned segments have potential applications in the investigation of sequences involved in gene regulation, isolation of functional domains from chromosomes, and identification of mutant genes. To assay for the biological functions of genes included in YACs, it is necessary to transfer them intact from yeast cells to cultured mammalian cells and ultimately into the germ lines of mammals.

DNA from *Saccharomyces cerevisiae* (23) and *Schizosaccharomyces pombe* (1) has previously been transferred to mouse cells by polyethylene glycol (PEG)-mediated fusion of yeast spheroplasts and cultured mouse cells. To allow selection for the products of fusion in these cases, a gene cassette that confers neomycin resistance (*neo*^{r} cassette) on mammalian cells was introduced into yeast as a high-copy-number extrachromosomal plasmid (23) or by homologous recombination targeted to genomic sequences (1). The latter approach requires prior knowledge (and subclones) of the target site. Traver et al. (21) have avoided this complication by including a *neo*^{r} gene on a YAC cloning vector. However, since many existing YAC libraries have been constructed with vectors that do not contain this modification, we have devised an alternative strategy. We have previously demonstrated that human *Alu* family repetitive elements located on YAC insert DNA can serve as targets for homologous recombination during DNA-mediated transformation events (14). Since repetitive sequences will occur in virtually any large segment of mammalian DNA, they represent universal targets for modification of YACs. For example, *Alu* elements will provide recombinational target sites on average once in every 10 kilobases (kb) along a YAC.

In each of the previous cases of yeast × rodent cell fusion, the structure of the transferred material and the possible inclusion of nonselected genetic material from the yeast genome in G418-resistant hybrid cells has not been fully examined. To determine the efficacy of PEG-mediated fusion for the introduction of genes included on YACs into cultured cells and, potentially, into the germ lines of laboratory animals via embryonic stem (ES) cells, we have constructed a vector which relies on homologous recombination with *Alu* or plasmid sequences to integrate a *neo*^{r} cassette into human-derived YACs with high efficiency. PEG fusion to embryonal carcinoma cells of yeast containing a *neo*^{r} marked YAC resulted in stable retention of a single intact copy of the 360-kb human segment. Despite inclusion of the YAC and of a substantial portion of the yeast genome, yeast × rodent hybrids retained the parental cells' ability to differentiate in the presence of retinoic acid. The ease of introducing large segments of DNA by this procedure makes it a useful approach for in vitro studies of gene function and regulation.

MATERIALS AND METHODS

Yeast and mammalian cells. *S. cerevisiae* YPH252 (α *ura3-52 lys2-801*^{amber} *ade2-101*^{ochre} *trp1-1Δ1 his3-Δ200 leu2-Δ1*) and diploid strain YPH274 (19) were developed for the propagation and manipulation of YACs. Strain YPH510 contains a previously-characterized 360-kb human-derived YAC in YPH252 (14). Strain YBP296 contains the same YAC modified by insertion of plasmid pBP47 as described below. The introduction of a *neo*^{r} cassette to YPH510 did not render it resistant to G418; yeast cells failed to grow at 30 or 37°C in the presence of 500 μg of G418 per ml. The embryonal carcinoma (EC) cell line Bls-2 was kindly provided by John W. Littlefield (11) and was maintained in Dulbecco modified essential medium supplemented with 5% fetal calf serum, penicillin, and streptomycin (MEM).

Vector construction. To construct the yeast integrating plasmid pBP47 (Fig. 1), a 1.2-kb *Eco*RI-*Bam*HI restriction fragment containing the *S. cerevisiae HIS3* gene was inserted into the *Eco*RI and *Bam*HI sites of pSV2neo (20). The *Eco*RI site of the *HIS3* fragment was the result of a synthetic linker placed at −296 with respect to the first *HIS3* mRNA start (K. Struhl, personal communication); the *Bam*HI site was the result of a synthetic linker placed at the genomic *Xho*I site. The *Eco*RI site of the resulting *HIS3*/pSV2neo plasmid was ablated by linearizing at the *Eco*RI site, filling in the cohesive ends with Klenow fragment, and ligating the plasmid. A 0.3-kb *Alu* family repeat in plasmid BLUR13 (6)

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was modified by inserting an *Eco*RI linker into the *Sph*I site. The modified *Alu* fragment, BLUR13.RI, was ligated into the *Bam*HI site, yielding pBP47.

Yeast transformation. A total of 5×10^7 yeast cells from strain YPH510 were transformed by the lithium acetate procedure (10) with 5, 10, or 40 μg of pBP47 linearized with *Eco*RI. Yeast media and standard genetic techniques were as described previously (18). His^+ transformants were selected on synthetic dextrose plates supplemented with uracil, tryptophan, leucine, lysine, and limiting adenine for color selection (9). Clones were colony purified on selective media and expanded in YPD medium.

Electrophoresis and hybridization procedures. DNA for conventional Southern blot analysis and high-molecular-weight DNA for pulsed-field gel electrophoresis (PFGE) were prepared from yeast strains and cell lines by standard procedures (15, 17). Yeast chromosome-sized DNA molecules were separated across a 1% agarose gel by contour-clamped homogeneous electric field electrophoresis in 0.5× Tris-borate-EDTA buffer. Typical electrophoresis conditions were 200 V and 150 mA at 12°C, with a 60-s pulse for 14 h and then a 90-s pulse for 6 h.

DNA transfer to nylon membranes (MSI, Inc.) and hybridization with probes synthesized by random primer extension were accomplished as described previously (14). Filters were washed in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate at 55 or 65°C for 1 h. Filters were stripped by washing them with 10 mM Tris, pH 8.0–5 mM EDTA–0.5% sodium dodecyl sulfate for 30 min at 65°C and monitored for retention of probe before rehybridization.

DNA fragments used as probes included a 0.3-kb *Alu* fragment from BLUR8 (6), a 2.3-kb fragment of pSV2neo containing the neomycin resistance cassette (20), the 3-kb Bluescript plasmid (Stratagene cloning systems), a 1.2-kb *Eco*RI-*Bam*HI fragment of *HIS3* (see above), a 1.1-kb *URA3* fragment (16), a 1-kb Y′ telomere fragment (4), a 1.6-kb *Bgl*II fragment of *TRP1* which includes 0.9 kb of genomic sequences 5′ to the *trp1-Δ1* deletion (22), and a 6-kb *Ty* element (7).

Fusion of yeast cells to EC cells. Strain YBP296 yeast cells were fused to Bls-2 EC cells by a modification of the procedure described by Allshire et al. (1). Yeast cells were grown in YPD medium to stationary phase and then diluted to an optical density at 600 um of 1.0 and grown for two doublings at 30°C. Yeast cells were pelleted from 50 ml of culture, washed twice in 20 ml of 1 M sorbitol, and suspended in 20 ml of 1 M sorbitol–100 mM sodium citrate, pH 5.8–10 mM EDTA–30 mM beta-mercaptoethanol, and the cell walls were digested by adding 35 μl of a 10-mg/ml zymolyase 20T stock and incubating the mixture at 30°C until 90% of the cells were spheroplasts (approximately 20 min) as determined by lysis in 5% sodium dodecyl sulfate (18). Spheroplasts were pelleted and washed twice in 10 ml of 1 M sorbitol–10 mM Tris, pH 7.5, suspended in 10 ml of the same solution, and counted.

Bls-2 cells at 70 to 80% confluency were trypsinized, harvested, and suspended in MEM without serum (MEMss). A total of 2×10^6 cells were pelleted in a 15-ml polystyrene tube, the medium was decanted, and 2×10^7 yeast cells in 1 ml of 1 M sorbitol–10 mM Tris, pH 7.5, were layered on top and pelleted at $100 \times g$ for 5 min. The pellet was suspended in 0.2 ml of MEMss and gently mixed with 2 ml of a 40% (wt/vol) PEG solution in MEMss, pH 7.4 (PEG 1000 was obtained from Baker Chemicals). After 30, 60, 90, or 120 s, 5 ml of MEMss was added and mixed by inversion, and cells were collected by centrifugation at $100 \times g$ for 3 min. The pellets were suspended in MEM, and 5×10^5 cells were plated per 100-mm^2 plate. Twenty-four hours later, plates were washed with phosphate-buffered saline to remove dead cells and yeast cells and refed with MEM plus 500 μg of G418 per ml. Cells were refed as necessary, and resistant colonies typically appeared after 10 to 14 days. Colonies were isolated with cloning cylinders and expanded for analysis.

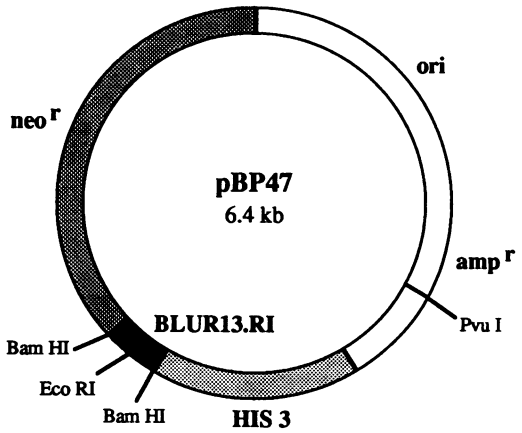

FIG. 1. Integrating plasmid pBP47. This plasmid contains a unique *Eco*RI site in a human *Alu* segment. When linearized at this site and transformed into yeast, the free ends target homologous recombination to related *Alu* sequences on human-derived YACs. Linearization at *Pvu*I will target integration to plasmid sequences in the YAC vector arm. The integrated plasmid confers a selectable His^+ phenotype on yeast and introduces a neo^r cassette that is active in mammalian cells.

To ensure that the G418-resistant colonies were not contaminated by yeast cells, 12 ml of medium from yeast × rodent hybrids was concentrated by centrifugation and the particulate matter was inoculated onto YPD and LB plates. No growth was apparent on either plate after 3 days at 30°C.

RESULTS

Selectable markers can be targeted to YACs by integration into human *Alu* elements. The yeast integrating plasmid, pBP47, contains a modified human *Alu* family member, BLUR13.RI, the yeast *HIS3* gene, and the neo^r gene under the regulation of the simian virus 40 early promoter (Fig. 1). Digestion of pBP47 with *Eco*RI produces a linear 6.4-kb molecule with two free ends homologous to *Alu* sequences. Free ends of DNA are highly recombinogenic in yeast cells and, since integrative transformation occurs almost exclusively by homologous recombination (13), pBP47 should target integration to *Alu* sequences on the YAC. This event will introduce the *HIS3* gene; His^+ transformants can be selected in *his3* strains. The *his3-Δ200* allele present in the yeast host strains eliminates essentially all homology to the *HIS3* marker in pBP47. Therefore, targeted integration and/or gene conversion at the genomic *HIS3* locus is totally eliminated.

pBP47 linearized in the BLUR13.RI sequence (5, 10, or 40 μg) was used to transform 5×10^7 YBP510 yeast cells. His^+ transformants were selected on yeast minimal medium lacking histidine. Fifty-two His^+ colonies were obtained from three separate experiments, and 12 lines were selected arbitrarily for analysis. One of these contained *HIS3* in a

small, high-copy-number band, indicating that the plasmid was replicating autonomously. This line was not studied further. PFGE analysis of the other 11 lines demonstrated pBP47 *HIS3* sequences associated with the YAC and not with other yeast chromosomes. The YACs in three of these lines had small deletions (<20 kb), and a fourth was 15 kb larger after transformation. The remaining seven lines contained a YAC with an electrophoretic mobility similar to that of the parental strain YAC (data not shown).

If pBP47 integrated via homologous recombination of the BLUR13.RI sequence, digestion with *Bam*HI should produce a 6.1-kb *Bam*HI fragment (Fig. 2A). In 8 of the 11 His^+ transformants analyzed, both the neo^r and *HIS3* genes hybridized to a single fragment of the expected size (Fig. 2B). Vector arms from these YACs, identified by hybridization with the *TRP1* or *URA3* probes, retained the same electrophoretic mobility as in the parental YAC. In two transformants the neo^r gene hybridized to a 9-kb *Bam*HI fragment, as did a probe against *TRP1*, while the normal *TRP1* fragment from the YAC vector arm was missing (data not shown). Since both pBP47 and the YAC vector arms contain the ampicillin resistance gene and ColE1 origin of replication, homologous recombination and integration in these clones probably occurred in the YAC vector arm that contains *TRP1*. The YAC which appeared to be 15 kb larger than the parental YAC contained both a 6.1- and a 9-kb fragment hybridizing to neo^r, consistent with two separate integration events, one targeting an *Alu* sequence and the other targeting the *TRP1* vector arm.

To determine whether pBP47 was able to target different *Alu* sequences on the YAC, the filter in Fig. 2B was rehybridized with the BLUR8 *Alu* sequence probe (Fig. 2C). The *Alu* profiles of the four His^+ transformants shown differed from each other and from the parental strain by one or two fragments, indicating that different *Alu* elements were targeted in each of these integration events. To determine more precisely the number of different integration events among the eight strains demonstrating *Alu*-specific integration, DNA from these lines was digested with *Eco*RI and hybridized with a probe to bacterial plasmid sequences. *Eco*RI has no recognition sites in pBP47 after *Alu*-mediated homologous recombination, so each integration event should produce a unique *Eco*RI fragment which hybridizes to the plasmid probe, and these fragments should be greater than 6.1 kb. Each of the eight His^+ transformants in which *Alu*-targeted transformation occurred displayed a different-sized fragment, from 6.6 to 32 kb (data not shown). This result demonstrates that multiple *Alu* sequences served as targets for homologous recombination.

Transfer of intact copies of the modified YAC to EC cells by PEG-mediated fusion. In three separate experiments, three samples of 20×10^6 YBP296 yeast cells were fused to 2×10^6 B1s-2 EC cells by PEG-mediated spheroplast fusion. Ten neo^r clones were expanded for further analysis. Hybrids YXM1 through YXM8 are independent, neo^r lines resulting from different fusions. Two additional neo^r lines, YXM1a and YXM1b, were derived from the same plate as YXM1 and may be independent lines or subclones of YXM1.

Transfer of YAC sequences to the G418-resistant lines was assessed initially by Southern blot analysis using the cloned human BLUR8 *Alu* family element as a probe (Fig. 3). Thirty to thirty-five *Alu*-containing fragments were identified in yeast strain YBP296 after digestion with *Pst*I, providing a diagnostic *Alu* profile for the presence of this YAC. The *Alu* probe did not hybridize to DNA from yeast cells lacking a YAC or from murine B1s-2 EC cells under the hybridization and wash conditions employed. The *Alu* profiles of the G418-resistant cell lines YXM1, YXM1a, YXM1b, YXM2, and YXM3 each contained all of the fragments seen in YBP296. The relative intensities of the bands in these hybrids were the same as those in YBP296, as was expected if no major rearrangements or segmental

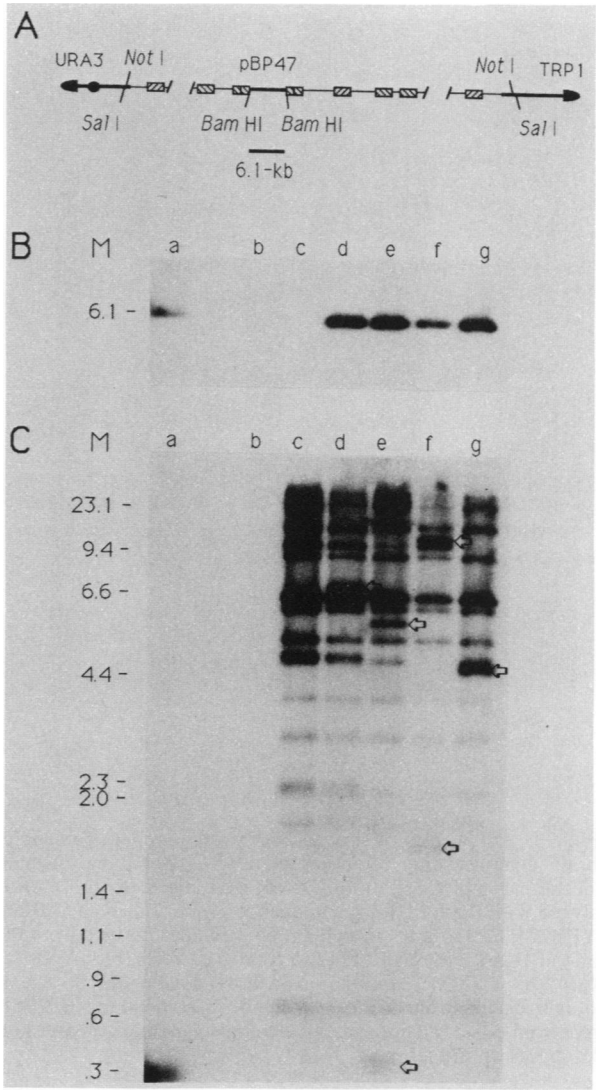

FIG. 2. Integration of pBP47 occurs via homologous recombination with *Alu* repeats on a human-derived YAC. (A) Expected result of *Alu*-mediated integration. Digestion of pBP47 with *Bam*HI cuts adjacent to the BLUR8 *Alu* segment. If recombination occurs via the *Alu* segments, a 6.1-kb linear fragment containing the *HIS3* and neo^r genes and plasmid sequences will be produced, and the electrophoretic mobility of an *Alu*-containing restriction fragment of the parental YAC will be altered. (B) Transformed yeast cells containing the 6.1-kb fragment predicted for *Alu*-mediated homologous recombination. Results of hybridization of the neo^r probe to *Bam*HI digests of pBP47 (lane a), yeast strain YPH274 (lacking a YAC) (lane b), YPH510 (containing the 360-kb human YAC) (lane c), and the His^+ transformants of YPH510:YBP296 (lane d), YBP297 (lane e), YBP298 (lane f), and YBP299 (lane g). (C) *Alu* profiles of His^+ transformants demonstrating disruption of parental *Alu*-containing fragments by targeted insertion. Arrows indicate new fragments in transformed yeast cells that hybridized to the BLUR8 probe. Lanes are the same as in panel B. Positions of molecular weight markers (M) are shown in kilobases on the left.

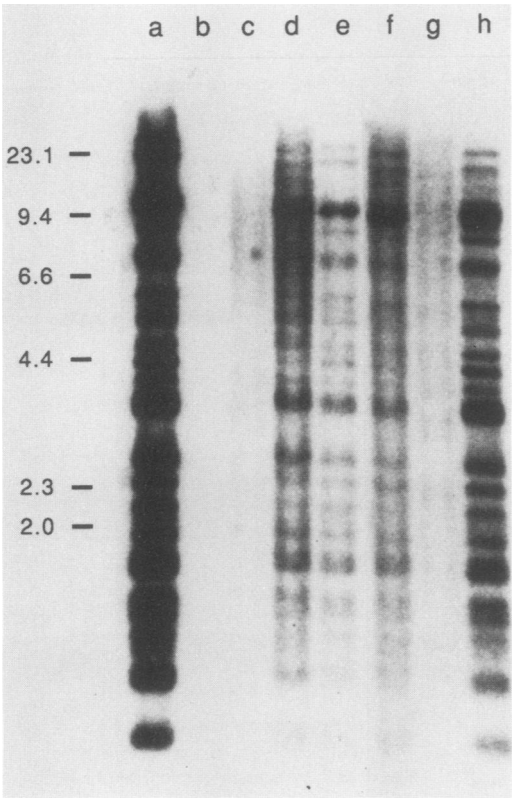

FIG. 3. *Alu* profiles of several *neo*^{r} yeast × rodent somatic cell hybrids demonstrating retention of all *Alu*-containing fragments from the parental yeast strain. DNAs were digested with *Pst*I and analyzed with the BLUR8 *Alu* sequence probe. Lanes: a, YBP296; b, YPH274; c, B1s-2; d through h, neo^r cell lines derived from PEG fusion of B1s-2 with YBP296 (d, YXM1a; e, YXM1b; f, YXM2; g, YXM4; h, YXM1). Multiple exposures demonstrated that the weakly hybridizing bands in lane g do not correspond to YBP296 but derive from B1s-2. Positions of molecular weight markers are shown in kilobases on the left.

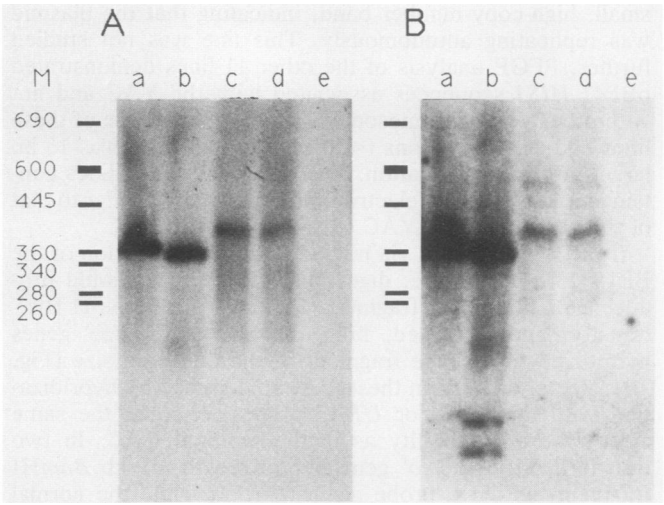

FIG. 4. The complete 350-kb human DNA fragment cloned in YBP296 is retained in hybrid cells. (A) Hybridization of the BLUR8 probe to products of *Not*I digestion (lanes b through e) separated by PFGE. Lanes: a, YBP296 uncut; b, YBP296; c, YXM1; d, YXM1b; e, B1s-2. (B) Hybridization of the same filter with a plasmid probe detecting YAC vector sequences removed from the ends of the YAC in YBP296 (lane b) (Fig. 2A) and linked to flanking *Not*I fragments of 450 and 600 kb in hybrids (lanes c and d). *S. cerevisiae* chromosomes visualized after ethidium bromide staining were used as molecular weight markers (shown in kilobases on the left).

amplifications of the YAC occurred during transfer. DNA from these lines also hybridized to probes specific for the YAC arms (see below), indicating that segments of DNA very close to the ends of the YAC were maintained in the yeast × EC hybrid lines.

The five remaining G418-resistant lines, YXM4 through YXM8, were analyzed variously with probes for *Alu* family elements, plasmid sequences, Y′ sequences, and *Ty1* sequences. They showed no hybridization, indicating that no corresponding human or yeast sequences were retained in these lines.

Further evidence for the structural integrity of YACs introduced by PEG-mediated fusion was obtained by PFGE analysis of large restriction fragments. *Not*I sites are located on both arms of the YAC, but no sites are present in the insert DNA (Fig. 2A). DNA from yeast and mouse cells digested with *Not*I was compared after hybridization with the BLUR8 probe (Fig. 4A). A single *Not*I fragment of 350 kb hybridized to the BLUR8 probe in DNA from YBP296. The same fragment was present in the G418-resistant, *Alu*-positive hybrid cell lines. (The decreased electrophoretic mobility of the *Alu* band in the *neo*^{r} lines is due to the fact that these lanes contain approximately 200-fold more DNA than the yeast lanes. Similar mobilities of *Not*I fragments from yeast and hybrids were seen when DNA from YBP296 was mixed with B1s-2 DNA before PFGE.)

The structure of the YAC vector arms in hybrid lines was analyzed by hybridization of *Not*I-digested DNA with a plasmid probe (Fig. 4B). As expected, the 350-kb *Not*I human insert in the YAC from yeast strain YBP296 hybridized to this probe because of the presence of the integrated pBP47 plasmid. Both of the vector arms, which are separated from the YAC insert by digestion with *Not*I, also hybridized to the plasmid probe. The hybrid lines contained the 350-kb fragment plus two bands of higher molecular weight that hybridized to the plasmid probe, as was expected if the vector sequences were ligated to flanking genomic DNA sequences.

Determination of copy number. The copy number of YACs present per cell was estimated on Southern blots of DNA from YXM1 and YXM1b. DNA from these lines was hybridized with the *neo*^{r} gene probe, which is present at a single site in these YACs (see above), and hybridization intensities were compared with B1s-2 DNA mixed with 0 to 20 copies of pBP47 DNA (Fig. 5). The relative intensity of the 6.1-kb *Bam*HI fragment in each sample was determined with a scanning densitometer. The results indicate that a single copy of the YAC is present in these cells.

YACs are stably maintained in yeast × mouse hybrid cell lines. Two phenotypes of cell growth were observed among the hybrid clones. The lines described above grew with doubling times similar to that of the rapidly dividing parental B1s-2 strain. A second class of hybrid lines persisted and demonstrated a high frequency of mitotic figures in the presence of G418 long after control cells were killed by the drug, but failed to expand in numbers. These lines were eventually lost before sufficient numbers of cells were accumulated for analysis. Allshire et al. (1) described a similar phenomenon for *S. pombe* × rodent cell hybrids and suggested that the slowly accumulating cells contained uninte-

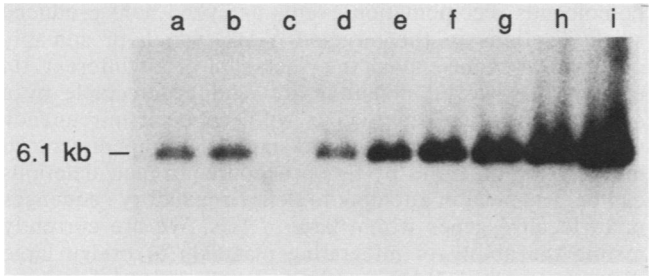

FIG. 5. A single copy of the transferred YAC is present in hybrid cells. DNA (5 μg) from hybrid lines YXM1 and YXM1b (lanes a and b) was run in parallel with 5 μg of DNA from B1s-2 mixed with an equivalent of 0, 1, 2, 3, 5, 10, or 20 copies of pBP47 per genome (lanes c through i, respectively). The filter was hybridized with the *neo*^{r} probe, and intensities of hybridization were compared by using a scanning densitometer.

grated DNA that included the *neo*^{r} gene and was mitotically unstable. Cells with a normal growth pattern contained the *neo*^{r} gene from an *S. pombe* chromosome integrated in a mouse chromosome.

To assess the stability of the *neo*^{r} marker in lines exhibiting normal growth, cells were cloned by limiting dilution and multiple clones were expanded to approximately 2×10^6 cells in the absence of G418 selection. Each of the lines obtained was cloned and expanded again. In all, the original clones underwent >40 population doublings in the absence of G418. At this point, two samples of 10^5 cells from each clone were grown for 4 days in the presence or absence of 500 μg of G418 per ml and counted to determine whether they retained the ability to replicate in the presence of the drug. No difference in cell number was noted between cultures derived from the marker-selected and nonselected samples of the 12 different subclones analyzed.

Taken together, the presence of junction fragments, the low copy number, and the stability of the YACs in the absence of selection suggest that YACs are maintained because of integration in a mouse chromosome. The integration occurred at sites very close to the end of the YAC.

Yeast × rodent EC cell hybrids retain the ability to differentiate. The B1s-2 EC cell line grows as a rapidly proliferating population of undifferentiated cells. In the presence of retinoic acid, the cells stop dividing and differentiate to form broad, flat cells that express several products characteristic of parietal endoderm (11). To determine whether the ability to differentiate was impaired after fusion and introduction of YACs, cells from hybrid lines YXM1 through -4 and unfused B1s-2 controls were maintained in medium containing 3 μM retinoic acid for 5 days. All cells from both control and hybrid lines differentiated with no discernible differences in the time course or final morphology of the cultures.

Endogenous yeast chromosomal DNA is retained in hybrid cell lines. Analysis of yeast × rodent hybrid DNA with the *URA3* and *TRP1* probes revealed the presence of the homologous segments from the YAC vector (Fig. 6). Additional restriction fragments corresponding to the endogenous yeast *ura3* and *trp1* loci were also seen in subsets of the hybrids (Fig. 6A and B). Analysis of the five *neo*^{r} lines with these probes revealed that the endogenous segments, which reside on different yeast chromosomes, were retained independently from each other and from the YAC. While all of the hybrid lines retained the *URA3* and *TRP1* restriction fragments present on the vector arms, YXM1 retained only the endogenous *ura3* locus, YXM1a and YXM1b retained both

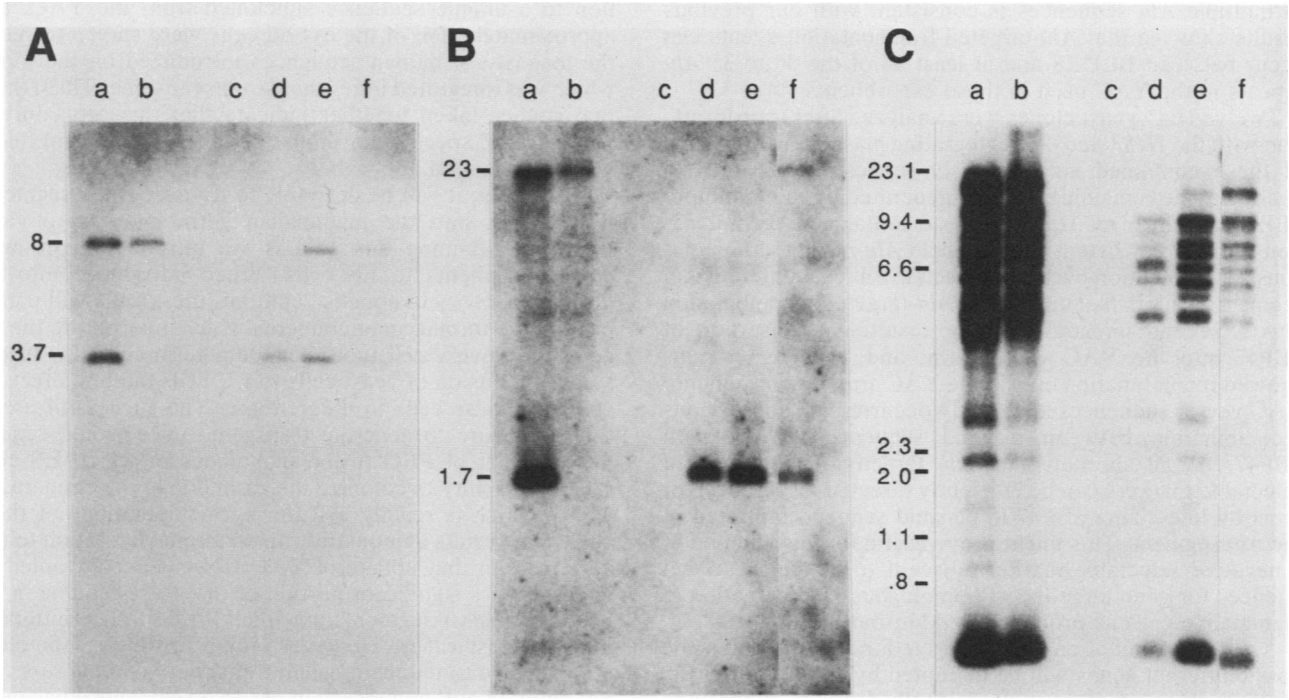

FIG. 6. Hybrids contain significant portions of the yeast genome in addition to the YAC. DNAs from yeast cells and hybrids were digested with *Pst*I for restriction analysis, and the filter was sequentially hybridized with *URA3* (A), *TRP1* (B), or *Ty* (C). YBP296 demonstrated 3.7- and 1.7-kb *URA3* and *TRP1* bands in the YAC vector arms in addition to endogenous *ura3-52* (8 kb) and *trp1-Δ1* (23 kb) fragments. Lanes: a, YBP296; b, YPH274; c, B1s-2; d, YXM3; e, YXM1; f, YXM1b. Positions of molecular weight markers and sizes of relevant fragments are indicated in kilobases on the left.

ura3 and *trp1*, YXM2 retained only *trp1*, and YXM3 retained neither locus. To determine the extent of yeast-derived information present in the hybrids, DNA from several lines was hybridized with a probe which recognizes *Ty* elements, retrotransposons found in approximately 35 copies throughout the genome of YBP296. Between 11 and 16 of the 21 resolved parental *Ty*-containing restriction fragments were retained in the various hybrids analyzed (Fig. 6C). This result demonstrates that these hybrids contain yeast DNA from multiple chromosomes in addition to the YAC.

DISCUSSION

Large segments of mammalian DNA cloned as YACs can be manipulated in ways analogous to those used for genetic analysis in yeast. A method is presented here for targeting the insertion of mammalian selectable markers to human-derived YACs. This procedure is based on homologous recombination with repetitive elements dispersed along YACs. To facilitate these manipulations, the YAC was propagated in a yeast strain with four nonreverting auxotrophic markers (*ura3*, *trp1*, *his3*, and *leu2*), only two of which (*ura3* and *trp1*) are complemented by the presence of the YAC. Furthermore, the *HIS3* yeast selectable marker used in the *Alu* integration approach described here is entirely deleted from the yeast genome, precluding recombination at the *his3-Δ200* locus and thereby ensuring targeted recombination to the YAC. Although many YAC libraries have been constructed for yeast which do not utilize these additional auxotrophies, individual YACs can be readily transferred to this background by mating and tetrad dissection or by direct transformation. Introducing modifications by targeting to repetitive sequences can be used with virtually any YAC and requires no prior knowledge about or subcloning of YAC sequences. The targeting of integration to multiple *Alu* sequences is consistent with our previous results showing that *Alu*-targeted fragmentation events can occur between BLUR8 and at least 18 of the 30 to 35 *Alu* repeats in the YAC used in these experiments (14).

Twelve His^+ yeast clones were analyzed after transformation with the *HIS3*/neo^r *Alu*-integrating plasmid, pBP47. One of these contained autonomously replicating plasmid sequences. The remaining 11 were generated by integration of pBP47 into the YAC. Eight of these contained a recombination product consistent with a single *Alu*-mediated homologous recombination event, and each involved a different *Alu* segment. A ninth had undergone *Alu*-targeted recombination plus a second integration event resulting in insertion of pBP47 into the YAC vector arm, and two His^+ strains showed recombination only in the YAC arm. Integration into YAC vector sequences apparently occurred via homologous recombination between plasmid sequences shared with pBP47. As an alternative to *Alu* targeting, integration of selectable markers can be efficiently directed to YAC vector arms by linearizing pBP47 in plasmid sequences instead of the *Alu* segment. This might prove useful for introduction of a negative selectable marker adjacent to human DNA sequences for gene targeting of homologous recombination in mammalian cells as proposed by Mansour et al. (12).

A potential limitation of *Alu*-targeted transformation is the possibility that genes will be disrupted by integration of the plasmid. *Alu* elements are occasionally found in introns and occur in the 3′-untranslated regions of 1 to 3% of mRNAs. In most cases, insertion at either of these types of sites is unlikely to affect gene expression. An additional concern comes from the observation that three of eight *Alu*-mediated homologous recombination events analyzed here produced small deletions in the targeted YAC, which presumably occurred when the ends of the plasmid targeted different *Alu* elements. However, deletions are readily detectable by a simple screen, and they occur with sufficient infrequency that they do not represent a significant impediment to modification of YACs by this procedure. In fact, deletions can be beneficial in attempts to define regulatory sequences or to localize genes within large YACs. We are currently testing the ability of integrating plasmids to bridge large distances across a YAC in order to create defined deletions.

The ability to transfer genetic material from yeast cells to cultured cells by PEG fusion was first demonstrated for *S. cerevisiae* by Ward et al. (23) and subsequently for *S. pombe* by Allshire et al. (1). Recently, a 75-kb YAC made with a neo^r cassette in one vector arm was reported to have been transferred by this procedure (21). In none of these cases was the fate of the transferred material analyzed thoroughly. This report demonstrates the stable uptake and maintenance of a very large segment of human DNA in cultured cells. PFGE analysis and assessment of *Alu* profiles demonstrated the structural integrity of the YAC in hybrid cells derived from three independent neo^r clones. Integration was inferred by stability of the YAC for over 40 population doublings in the absence of selection, by the low copy number (which would be unlikely to support the neo^r phenotype if the YAC was present as a randomly replicating and segregating episome), and by linkage of the sequences near the ends of the YAC vectors to flanking sequences. Stable uptake of YACs has also been accomplished recently by PEG-mediated fusion of yeast cells with mouse L cells (V. Pachnis, L. Pevny, R. Rothstein, and F. Constantini, Proc. Natl. Acad. Sci. USA, in press) with essentially identical results. Products of this fusion were selected on the basis of a neo^r cassette which was introduced by targeting homologous recombination to a unique sequence subcloned from the YAC, and approximately half of the hybrid cells were shown to retain the majority of human sequences introduced on the YAC, which was integrated into a mouse chromosome. Thus, these two studies taken together indicate that this procedure is neither YAC specific nor mouse cell line specific and that it will be of general applicability.

Ultimately, it will be desirable to transfer DNA segments from YACs into the mammalian germ line. An obvious means of attaining this goal is via introduction of large cloned fragments to ES cells. When introduced into the blastocoel cavity of a mouse embryo, these cells will participate in the formation of chimeras which incorporate the ES genome in every cell type, including germ cells (2). In this study, the fusion of yeast cells to EC cells did not affect the ability of these cells to differentiate. The successful use of this procedure to generate transgenic mice would require development of PEG fusion techniques in which ES cells retain the ability to colonize the germ line of the chimera, an ability which is readily lost during manipulations of these cells. Our results demonstrate an additional limitation to this approach, in that uptake of YAC DNA is accompanied by inclusion of a significant portion of the yeast genome in the host cell. Yeast segments are likely to be transcriptionally inert in most cases. However, some fortuitous homology recognizable to the mammalian transcription apparatus cannot be ruled out, and insertion of yeast segments adjacent to a mammalian promoter could lead to gene expression. More importantly, each event in which a segment of yeast DNA is inserted into the mouse genome is a potential insertion mutation which would be carried into the animal.

A procedure for passing pulsed-field gel-purified YAC DNA through a microinjection needle without shearing has been developed recently (J. Bennett and J. Gearhart, in preparation). Isolated YACs into which a *neo*^{r} cassette has been integrated by targeted transformation with the pBP47 plasmid could thus be introduced into ES cells by microinjection. While the copy number of YACs per injection would be low, G418 selection would permit efficient recovery of modified ES cells. Both microinjection and G418 selection have been used in ES cells without eliminating the ability to colonize the germ line (24). While PEG fusion provides a convenient means of introducing YACs into cultured cells for genetic analysis, a more precise method may prove advantageous for modification of the mouse germ line.

There is enormous potential for the application of YAC technology to studies of gene regulation. With a cloning capacity of at least 1 megabase, almost any mammalian gene or gene complex can be encompassed in a single YAC. The incorporation of large amounts of adjacent sequences guarantees the inclusion of all *cis* elements important in gene regulation and may include sufficient information to confer higher orders of regulation, e.g., those affecting chromatin conformation in the vicinity of the gene. It should also be possible to identify a gene from among YACs spanning a known region of the genome on the basis of the ability of the YAC to complement recessive mutations or to produce dominant ones. Homologous recombination frequencies in mammalian cells might be enhanced by the large targeting segments provided by YACs and provide an alternative means of searching for genes by deleting large segments of a chromosome. To realize this potential, it is necessary to develop means by which gene functions can be tested in biologically active systems. The YAC modification and PEG-mediated fusion procedures described here provide tools that should prove useful in realizing these goals.

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