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Stable integration and expression in mouse cells of yeast artificial chromosomes harboring human genes

(cloning/rDNA/glucose-6-phosphate dehydrogenase/transfection/mammalian DNA recombination)

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ABSTRACT **We have developed a way to fit yeast artificial chromosomes (YACs) with markers that permit the selection of stably transformed mammalian cells, and have determined the fate and expression of such YACs containing the genes for human ribosomal RNA (rDNA) or glucose-6-phosphate dehydrogenase (*G6PD*). The YACs in the yeast cell are "retrofitted" with selectable markers by homologous recombination with the *URA3* gene of one vector arm. The DNA fragment introduced contains a *LYS2* marker selective in yeast and a thymidine kinase (TK) marker selective in TK-deficient cells, bracketed by portions of the *URA3* sequence that disrupt the endogenous gene during the recombination event. Analyses of transformed L-M TK⁻ mouse cells showed that YACs containing rDNA or *G6PD* were incorporated in essentially intact form into the mammalian cell DNA. For *G6PD*, a single copy of the transfected YAC was found in each of two transformants analyzed and was fully expressed, producing the expected human isozyme as well as the heterodimer composed of the human gene product and the endogenous mouse gene product.**

To study gene expression, cloned fragments of DNA are often reintroduced into eukaryotic cells by transfection (1) or electroporation (2). Conventional vectors, however, have placed an upper limit on the size of DNA that can be studied, since inserts of no more than 40 kilobases (kb) can be accommodated, and it is now clear that many genes are larger than that. One way to circumvent this limitation has been provided by the development of yeast artificial chromosomes (YACs) as a cloning system, since DNA inserts in YACs can be as large as a megabase or more (3–7). We have designed a selective system to test the potential of transfected YACs for short- and long-term studies of gene expression.

The test genes we have used are rDNA, encoding ribosomal RNA (rRNA), and the housekeeping gene *G6PD*, encoding glucose-6-phosphate dehydrogenase (G6PD). The number of natural variants detected for G6PD, now in excess of 300, is the largest known for any enzyme (8), providing unusually favorable material for studies of natural mutational variation and possible regulatory changes.

Though the transcription units in the cases of rDNA and *G6PD* are well defined and are each contained in <20 kb, repeated searches have failed to recover the entire units in single λ or cosmid clones. In contrast, YACs of 40–50 kb contain the entire genes (9, 10), and when a YAC containing *G6PD* was transfected into mammalian cells, the normal human isozyme was transiently expressed at easily detectable levels within 48 hr (11).

Only a small number of cells take up DNA in standard assays with calcium phosphate-mediated transfections (1), and much of the DNA is either in the process of being degraded or is adventitiously stuck to cellular material. To assess the steady-state condition and the level of expression of transfected YACs more clearly, we have analyzed transformants after many generations.

Cells stably transformed with YACs could easily be obtained if a YAC vector was constructed containing genes competent for expression that permit forward selection in mammalian cells. Most of the YAC clones available thus far lack any such selectable marker, but they can be added to an existing YAC in yeast by homologous recombination from a plasmid vector. Here we describe examples of such constructions and the results of transfections with the modified YACs.

METHODS

Retrofitting YACs with Selectable Markers. The vector designed for the retrofitting of YACs incorporates portions of several plasmids to provide markers in the right configuration to make them selectable in yeasts and in mammalian cells. First, from the plasmid TK321 (12), a 2040-base-pair (bp) *Pvu* II–*Pvu* II DNA fragment containing the herpes simplex virus thymidine kinase (TK) gene was liberated by digestion with *Pvu* II. The fragment was purified by gel electrophoresis and inserted into YIp333 (13), a plasmid containing the yeast *LYS2* gene bracketed by two *Hin*dIII sites. YIp333 has an *Eco*RI site between the *LYS2* gene and one of the *Hin*dIII sites. The plasmid was digested with *Eco*RI, the ends were made flush by filling in nucleotides with the Klenow fragment of DNA polymerase I, and the product was dephosphorylated. This generated an intermediate plasmid that contained the *LYS2* and *TK* genes fused into the *Eco*RI site of YIp333. The *Hin*dIII–*Hin*dIII fragment containing *LYS2* and *TK* genes was then repurified by gel electrophoresis. After the ends were filled in, this fragment was ligated into the single *Stu* I site of plasmid pUC-URA3 [a construct made by ligating a *Hin*dIII–*Hin*dIII fragment of plasmid YEp24 (14), which contains the *URA3* gene, into pUC18 (15)]. The *Stu* I site is in the middle of the yeast *URA3* gene of the plasmid, so that the net result of ligation is an 11-kb bacterial plasmid, TKLU2, containing the *LYS2* and *TK* genes inserted between the halves of a split *URA3* gene in pUC-URA3.

The TKLU2 vector can be used to add selectable markers to YACs because the yeast *URA3* gene is also found in pYAC4 (3), and homologous recombination can occur when a *Hin*dIII fragment of the vector is transformed into a cell containing pYAC4 or a YAC (see *Results* and Fig. 1). The two

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Abbreviations: YAC, yeast artificial chromosome; G6PD, glucose-6-phosphate dehydrogenase; TK, thymidine kinase.

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interstitial genes can thereby be rescued from the inviable plasmid fragment by transfer to the YAC. One of the two, *LYS2*, can complement a deficiency in the yeast strain AB1380 (3), which is the standard host for the YACs studied here.

Selection of Stable Transformants. Transfection was by the calcium phosphate coprecipitation method, as in ref. 11, but with total yeast DNA from the YAC-containing strain (rather than with the purified YAC and carrier human DNA). DNA of high molecular weight was extracted (16) from the strain containing the retrofitted XY206 and was dialyzed against 1 mM EDTA/10 mM Tris·HCl, pH 7.4, for 48 hr at 4°C with two changes of buffer. To 15 μg of high molecular weight DNA (containing about 0.1 μg of YAC DNA) in 130 μl of buffer was added 20 μl of 2 M $CaCl_2$. Then 150 μl of 280 mM NaCl/50 mM Hepes, 1.5 mM Na_2HPO_4, pH 7.1 (2× HBS) was added slowly. The precipitate was allowed to form for 20 min at room temperature and then added to a 25-cm^2 flask containing 300,000 L-M TK^- (37) cells pregrown overnight in fresh medium containing 10% fetal bovine serum. Three hours later the cells were washed with serum-free medium and then treated immediately for 1.5 min at 37°C with 1.5 ml of 15% (vol/vol) glycerol in 2× HBS. The medium was removed and fresh complete medium was added. After 48 hr the cells were split into selective hypoxanthine/aminopterin/thymidine (HAT) medium (17) and grown with medium replaced every 3 days until clones of stable transformants had grown up (15–20 days). Clones were picked with a sterile cotton swab, added to a flask containing HAT medium, and grown under selective pressure until confluent. Cells were then collected and DNA was extracted for standard Southern analysis (18, 19).

Test of G6PD Activity. Cultures of stable transformants and control cells were lysed for the extraction of protein, and about 1 μg of total protein was applied to each lane on Cellogel for electrophoresis and subsequent enzyme assay (20).

RESULTS

Retrofitting YACs with Selectable Markers. The plasmid we have constructed (see *Methods*), TKLU2, contains selectable genes in a context that permits homologous recombination into one arm of the pYAC4 vector. Resultant recombinant YACs (Fig. 1) contain both the *LYS2* gene (selectable in the yeast strain being used) and the *TK* gene (selectable in mammalian TK^- cells), inserted between moieties of a split *URA3* gene. After transformation of Lys^-Ura^+ yeast cells and plating on medium lacking lysine, cells in which the recombination had taken place produced colonies that were Lys^+. The double selection helps to avoid recombinants in which the fragment has entered the chromosomal *URA* locus.

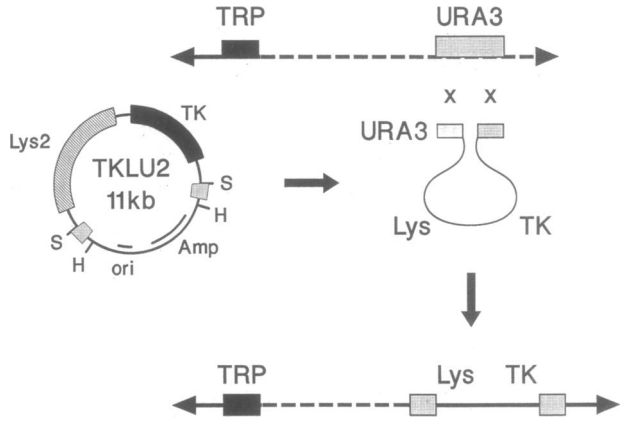

FIG. 1. Retrofitting a YAC. The homologous recombination event is diagrammed. A fragment generated from plasmid TKLU2 by digestion with *Hin*dIII (H), containing the *LYS2* and *TK* genes between two fragments (S–H) of the *URA3* gene (see *Methods*), is recombined into the *URA3* gene of the YAC vector arm.

The vector was used in experiments to modify two different YACs. One, yB3B3, was a 70-kb YAC containing about 1.5 repeat units of human rDNA (9). The other, XY206, was a 40-kb YAC containing the complete DNA required to produce enzymatically active G6PD in mammalian cells (10, 11). In two separate experiments with XY206, 1 of 12 and 3 of 14 colonies that had become Lys^+ had also become Ura^-; with yB3B3, 9 of 17 and 2 of 15 colonies similarly satisfied the selective criteria.

When DNAs from these transformants were fractionated by pulsed-field gel electrophoresis and analyzed by the Southern procedure, a number of them showed complex patterns indicating that YAC DNA had been incorporated into yeast chromosomes; but three each of those made with XY206 and yB3B3 contained a YAC of the expected size that hybridized as expected either with a probe specific for rDNA (C_{XH}, a 275-bp *Xba* I–*Hin*dIII fragment indicated at the top of Fig. 2) or *TK* (the 2040-bp *Pvu* II fragment from TK321; see *Methods*). XY206-11 and yB3B3-1 were chosen for further studies.

Stable Transformants with Retrofitted yB3B3 and XY206. DNA from a yeast strain contained a retrofitted YAC was transfected into L-M TK^- cells and stable transformants were obtained. Sample results are shown in Fig. 2 for DNA from the yeast strain containing YAC yB3B3-1. The schematic at the top of the figure shows some features of the YAC, which has an insert including all four *Eco*RI fragments of the rDNA repeat unit and a second copy of the region upstream of the initiation site for transcription.

For Fig. 2*A*, as a control, cells of the yeast strain containing the YAC were incorporated into an agarose plug, lysed, and digested with *Pst* I before being applied to the gel for electrophoresis (lane 1). DNA extracted from L-M TK^- cells (lanes 2 and 3) and from three independently isolated transformants (lanes 4–6) was also digested with *Pst* I and electrophoresed. The DNA was then transferred to a nylon membrane and hybridized with labeled probe "a" (a 561-bp *Ssp* I–*Pst* I fragment internal to the left vector arm of the YAC; see schematic). The YAC, but not the untransfected mouse cells, showed the characteristic signal from the 1.6-kb *Pst* I–*Pst* I fragment internal to the vector arm. (A 4.1-kb band was also seen; it was the product of incomplete digestion of the *Pst* I–telomere fragment of the YAC and thus hybridized to both probes a and b; see Fig. 2*B*). This region of the YAC was apparently largely preserved in the three transformants.

For Fig. 2*B*, probe b, the 1.5-kb *Pst* I–*Hin*dIII fragment of the vector arm, was added to the mixture and hybridization was repeated. The control untransformed cells again gave no signal, as expected; and the YAC now showed the expected signals from the 2.5-kb fragment that runs from the distal *Pst* I site through the telomere of the YAC. In the transformants, however, probe b hybridized only to a larger fragment (5 kb). The 5-kb fragment probably contains the pBR322 sequence of the YAC vector arm integrated into mammalian DNA (see *Discussion*).

When the same samples were probed with the C_{XH} rDNA fragment (Fig. 2*C* and ref. 9), two bands were observed. One 3-kb fragment arose from a cross-hybridizing DNA fragment found in untransfected mouse cells; the other, a 1.8-kb fragment, was not found in mouse cells but was observed in the transfecting YAC (lane 1) as well as in the transformants.

Because human rDNA is not expressed in mouse cells (21, 22), we could not assess whether the human rDNA had intact coding capacity. However, such a test could be carried out with the retrofitted XY206-11, containing the *G6PD* gene. In

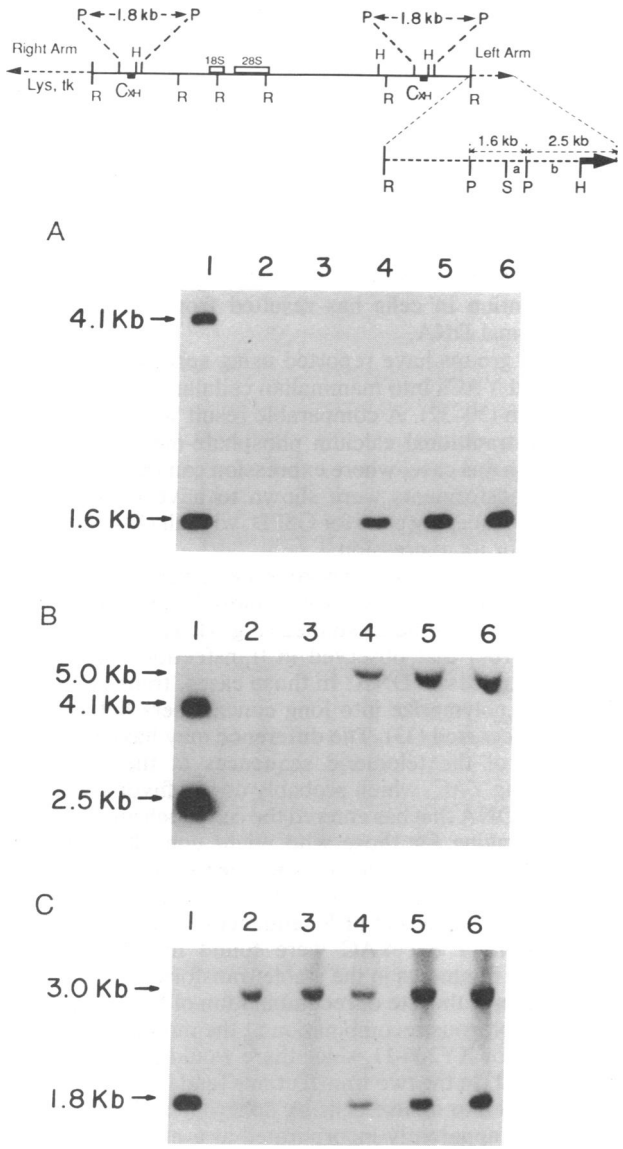

FIG. 2. Southern analysis of DNAs from L-M TK$^-$ cells stably transformed with retrofitted yB3B3-1. The schematic of yB3B3-1 at the top indicates the content of rDNA with its 18S and 28S rDNA units, characteristic *Eco*RI (R) sites, and the nontranscribed C_{XH} sequence used as a probe in *C*. Also indicated are the *Pst* I (P), *Ssp* I (S), and *Hin*dIII (H) sites in the left arm of the vector used to generate probes for *A* and *B*. Cells from a control of the retrofitted YAC DNA (lane 1), from L-M TK$^-$ mouse cells (lanes 2 and 3), and from cultures of each of three stably transformed mouse cell clones (lanes 4–6) were embedded in small blocks of agarose. DNA in the blocks was digested with *Pst* I and then fractionated by gel electrophoresis in 0.8% agarose, transferred to a nylon membrane, and hybridized with probes labeled using hexamer primers (18, 19). The filter was successively hybridized with a *Pst* I–*Ssp* I fragment (probe a) (*A*) and a *Pst* I–*Hin*dIII fragment (probe b) (*B*) of the vector arm. *C* shows the hybridization with the C_{XH} fragment as probe.

this case, stable transformants were again observed among mammalian cells transfected with a retrofitted YAC. Two stably transformed clones were selected for further analysis.

Fig. 3 shows the zymogram of extracts prepared from the stable transformants compared with human cells and untransfected mouse cells. The analysis was facilitated by the difference in mobility of the enzyme from the two species, and the human enzyme was clearly present in addition to the mouse enzyme in the transfected cells. The mobility of the human enzyme in the transfected cells (lanes 2 and 3) was

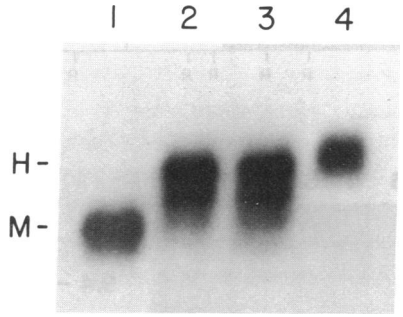

FIG. 3. Stable transformants express the human *G6PD* gene. For this zymogram, total cellular protein from recipient mouse cells (lane 1), two clonal cell lines (Gd1 and Gd2) stably transformed with *G6PD*-containing XY206-11 DNA (lanes 2 and 3), and HeLa cells (lane 4) was fractionated by gel electrophoresis and the histological assay for G6PD was then carried out on the gel. Human (H) and mouse (M) enzyme species in the transformants are indicated.

comparable to that from HeLa cells (lane 4), because both those cells and those of the patient from whose DNA XY206 was constructed (23) produce A-type variants of G6PD (24), with a faster mobility than normal.

G6PD is a dimeric enzyme that can form heterologous dimers from monomers of different species (24), and it is therefore expected that in addition to the enzyme bands specific for human and mouse, one will see a band of intermediate mobility corresponding to the human–mouse hybrid enzyme (lanes 2 and 3). Apparently, the human species is expressed at a relatively high level, so that most of the mouse monomer is incorporated into heterodimers. It remains to be seen whether the expression of the YAC is, for example, position-dependent, or whether the transformants contain higher numbers of the human than of the endogenous mouse gene. [It is notable that in transient expression assays with either cDNA clones (25) or YACs (11), no heterodimeric species were seen. This has been attributed to the fact that the human dimer dominates in the small number of transfected cells, but only the mouse dimeric enzyme is formed in the bulk of the cells, which have not taken up any transforming DNA.]

Direct Southern analyses showed signals from the integrated XY206-11 YACs that were, by visual inspection, more similar to those from cells containing one X chromosome than to those with two or four X chromosomes (Fig. 4). In that experiment 10-μg samples of DNA from the recipient mouse cells, from the hamster–human hybrid cell from which XY206 was derived (23), and from controls were digested with *Eco*RI, electrophoresed in agarose gels, transferred to a nylon membrane, and hybridized to the probe of the complete *G6PD* cDNA (Fig. 4 *Left*). As expected from the structure of the *G6PD* gene (refs. 26 and 27; schematic at top of Fig. 4), the YAC (lane 1) showed the 3.5-, 5-, and 11.7-kb *Eco*RI fragments characteristic of the *G6PD* gene structure. The 4X, 2X, and 1X cells (lanes 2, 3, and 4) showed the same three bands with the intensities predicted by the relative dosage of specific DNA. Control DNA from mouse showed three bands from the cross-hybridizing mouse gene (lane 7), and the control from the hamster–human hybrid cell (lane 8) showed all three human-specific bands (though with a weak signal from the largest of the three), and an additional band attributed to the hamster-specific *G6PD* DNA. The DNAs from the two transformants (Gd1 and Gd2, lanes 5 and 6) clearly contained both the three bands arising from mouse DNA and the three bands characteristic of human DNA, with levels comparable to that observed in DNA from the male (1X) cells in lane 4.

In additional Southern analyses (data not shown) the DNAs were digested with several enzymes, including *Kpn* I,

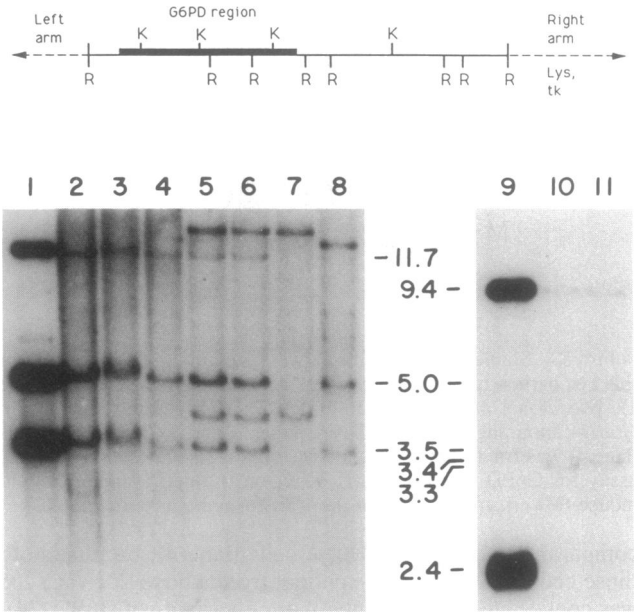

FIG. 4. Southern blot analysis of YAC DNA in two mouse cell clones stably transformed with XY206-11. At the top is a schematic of XY206-11. The restriction sites for *Eco*RI (R) and *Kpn* I (K) are indicated, as well as the transcription unit. Below, the DNA samples used were digested with *Eco*RI, electrophoresed in a 1% agarose gel, and then transferred to a nylon membrane (18) and probed with labeled (19) *G6PD* cDNA. The samples included 10 μg of DNA extracted from each of the two stable transformants (Gd1 and Gd2, lanes 5 and 6) or from controls of the DNA from the yeast cells containing the YAC DNA (lane 1), human DNA from cells containing four (4X), two (XX), or one (XY) X chromosome (lanes 2–4); mouse DNA (lane 7); or the hamster–human somatic cell hybrid from which XY206 was derived (lane 8). For lanes 9–11, the nylon filter was deprobed and rehybridized with pBR sequences specific for the right vector arm of the YAC. Results are shown for the three lanes that showed a signal: the YAC (lane 9) and the Gd1 and Gd2 transformants (lanes 10 and 11). Sizes are given in kilobases.

and probed with various sequence probes from across the *G6PD* region. Because the entire YAC insert has already been mapped, the probes used were known to test for the entire insert, and the results confirmed that the entire human insert DNA of XY206 was transferred and retained in the stable transformants.

These results were extended to begin to assess the state of the YAC DNA in the transformants. In particular, a fragment of pBR322 DNA present in the right arm of the YAC vector (3) was used as a probe against the same filter in the experiment of Fig. 4. Results are shown at the right for the lanes containing the DNA from the yeast strain (lane 9) and L-M TK$^-$ cell transformants Gd1 and Gd2 (lanes 10 and 11). In the YAC the right arm of the vector detected two intense *Eco*RI bands, as expected. One (9.4 kb) extends from the cloning site to an *Eco*RI recognition site in the *LYS2* gene of the vector; the other continues from that site through the telomere sequence (2.4 kb). Both transformants showed a clear signal from the fragment internal to the vector arm (9.4 kb), indicating that the YAC had maintained its structure through that point. The other portion of the vector, however, was found in a restriction fragment larger than 2.4 kb: about 3.3 kb in Gd1 and perhaps slightly larger (about 3.4 kb) in Gd2. Other fainter bands were also seen in Gd2. In additional experiments, a probe from the left arm of the vector, which hybridized to a 6-kb DNA fragment from the YAC-containing strain, hybridized to a shorter (4 kb) DNA in Gd1 and still recognized a 6-kb species in Gd2. Taken together, the data show that much, though not all, of the vector arm sequences was retained in two distinct stable transformants.

DISCUSSION

The retrofitting of YAC clones (XY206, containing *G6PD*, and yB3B3, containing the rDNA repeat unit) with the *TK* gene shows that the technique works easily, generating constructs that can be selected to yield stable transformants. Although the transfecting YACs contain telomeres, on which the mammalian telomerase would be expected to function (28), and may contain replication origins (29), they lack centromeric sequences, and the restriction patterns observed with DNA from stable transformants imply that as expected, their retention in cells has resulted from integration into chromosomal DNA.

Several groups have reported using spheroplast fusion to introduced YACs into mammalian cellular DNA in a largely intact form (30–32). A comparable result was obtained with the more traditional calcium phosphate-mediated transfection, and in this case, where expression can be unequivocally tested, transformants were shown to have maintained the entire housekeeping gene for G6PD, with all of the sequences required for its expression.

From comparison with appropriate controls, one or a very few copies of *G6PD* DNA were stably incorporated in each of the two transformants studied (Fig. 4). This result is rather different from that observed in transfections of cells with linear fragments of DNA. In those cases, transfecting DNA tended to polymerize into long concatamers before recombination occurred (33). The difference may have arisen from an effect of the telomeric sequences at the ends of the transfecting YAC, which probably do not favor concatamerization of DNA that has entered the cell. Such an effect would be encouraging for those who might consider YACs as a vector for gene-replacement experiments. However, the process of recombination is not likely to be straightforward in all cases, since portions of pBR sequences arising from the right vector arm of the YAC were found in different *Eco*RI restriction fragments in the stable transformant Gd2 (Fig. 4).

Concerning the site of recombination of transfected DNA, quasihomologous recombination at the mouse G6PD gene is ruled out for XY206-11, since there would then be no mouse G6PD at all. In the two transfectants [and in reports of YACs introduced into mouse cells by spheroplast fusion (31, 32)], DNA was apparently incorporated at a number of different sites. One YAC arm had been incorporated into a chromosomal DNA site that yielded a larger restriction fragment; and in addition, one of the two showed additional bands, suggestive of additional, poorly defined recombination events. It was not possible to test for incorporated yeast telomeric sequences, since the yeast telomeric probe (34) cross-hybridized extensively with related mammalian telomeric sequence. The presence of telomere sequences could possibly be seen by sequencing DNA products amplified by the polymerase chain reaction across the junction of YAC and mouse DNA (35).

In the case of rDNA, the first three isolated transformants all produced a probe-containing *Pst* I restriction fragment of comparable size; but this was probably fortuitous, and the clones might even be siblings, since the 18S, 5.8S, and 28S sequences are the only portions of the rDNA conserved between mouse and man, and recombination of the YAC repeatedly into any single locus seems relatively unlikely.

Homologous recombination of human DNA would, however, certainly be more favored in the transfection of human cells. In experiments with mouse embryonic stem cells as recipients, some favoring of homologous over nonhomologous recombination has been observed for larger as compared with smaller tracts of cloned DNAs (36). In comparable experiments, YACs may promote homologous recombination to an even greater extent. Tests of this notion should be possible with recipient cells that have a variant enzyme

activity with different electrophoretic mobility or a defined difference in gene sequence (24). The degree to which the endogenous enzyme was replaced could be assessed by polymerase chain reaction analyses of critical sequence tracts.

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