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Generation of a nested series of interstitial deletions in yeast artificial chromosomes carrying human DNA

(homologous recombination/repetitive elements/factor IX gene)

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ABSTRACT **We have generated a nested series of interstitial deletions in a fragment of human X chromosome-derived DNA cloned into a yeast artificial chromosome (YAC) vector. A yeast strain carrying the YAC was transformed with a linear recombination substrate containing at one end a sequence that is uniquely represented on the YAC and at the other end a truncated long interspersed repetitive element (LINE 1, or L1). Homologous recombination between the YAC and the input DNA resulted in a nested series of interstitial deletions, the largest of which was 500 kilobases. In combination with terminal deletions that can be generated through homologous recombination, the interstitial deletions are useful for mapping and studying gene structure–function relationships.**

The ability to clone large fragments of DNA into yeast artificial chromosomes (YACs) is providing a new method for the analysis of complex genomes (1). Large fragments of DNA (as long as 1 megabase) can be introduced into YAC vectors to yield artificial chromosomes.

The open-ended size capacity of YAC cloning technology suggests that virtually any mammalian gene or gene complex can be isolated as a single contiguous segment of DNA. Because of the large insert size, methods to facilitate the mapping and functional analysis of genes within the YACs are needed. In addition, the development of methods for targeted modification and transfer of the modified YACs back into cultured cells and experimental organisms will provide powerful tools for the study of gene structure and function.

The long-range structure–function relationships of genes can be studied by introducing a large YAC that contains a gene of interest into mammalian cells and eliciting regulated gene expression. If deletions of variable portions of DNA in and around the gene (interstitial deletions) can be obtained, YACs bearing such deletions could be introduced into cells to study the effects of the modifications. Recent successes in introducing YACs into mammalian cells (2–4) provide encouragement for the feasibility of these types of studies. The ability to make interstitial deletions would also be helpful in generating restriction maps of YACs and in assigning genetic markers to precise regions within a YAC.

Homologous recombination (HR) in yeast has been used to generate terminal deletions in normal as well as artificial chromosomes in yeast (5, 6). Pavan *et al.* (6) constructed a vector that contains a yeast telomeric sequence, a yeast selectable marker, and a polylinker into which a human highly repetitive sequence (*Alu* repeat) was added. When a linearized version of this plasmid was introduced into yeast cells carrying human DNA in a YAC, the input plasmid was able to recombine with each of several homologous regions in the YAC, yielding different-size terminal deletions. The generation of these deletions requires a single crossover event between the target and the input DNA.

We have used a modification of the terminal deletion strategy to generate interstitial deletions in a YAC carrying a 650-kb fragment of human DNA. The recombination vector contains at one end a sequence that is uniquely represented in the YAC and at the other end a fragment of DNA from a repetitive element (L1 element) found in the human genome. The vector sequences located in between contain bacterial, yeast, and mammalian selectable genes. When this plasmid is introduced into yeast cells carrying human DNA, it is possible for one arm of the substrate to pair with the unique sequence and the other arm to pair with any of its homologous elements. Recombination involving a double crossover results in the loss of sequences between the unique sequence and the repetitive element. We show that a nested series of interstitial deletions with a size range of 20–500 kb can be generated by this method and that these deletions result from the expected HR events. The deletions are useful for generating maps of the YAC. Examination of the series of deletions provided insights into the mechanisms of recombination. The deletion mutants may provide excellent substrates for introducing defined deletions into mammalian chromosomes by HR.

MATERIALS AND METHODS

Yeast Strains and Propagation. A *Saccharomyces cerevisiae* AB1380 strain carrying a pYAC4-based 650-kb YAC (HYA32G5) containing the human factor IX gene (*F9*) ≈40 kb from the telomeric end of the long arm was a gift of R. Little and D. Schlessinger (Washington University). The strain bearing HYA32G5 was mated to YPH252 (7), diploids were sporulated, and tetrads were dissected. A *HIS5 his3Δ200* haploid segregant was identified (designated YPH599) to enable manipulations with *HIS3* vectors. This segregant containing the 650-kb YAC had the genotype *MAT***a** *ura3-52 lys2-801 ade2-101 trp1Δ1 his3Δ200*. Transformations were done (8) using 3 μg of *Not* I-linearized plasmid for the interstitial deletions and 6 μg of *Sal* I-linearized plasmid for the terminal deletions. Transformants were selected on minimally supplemented SD plates (9) without histidine, colony-purified, and subsequently tested for the ability to grow in the absence of tryptophan or uracil.

Vector Construction. The starting vector for construction of the interstitial deletion vector was pRS303 (7). A 1.2-kb *Sal* I–*Xho* I fragment from PMC1neopolA (10) was cloned into the unique *Aat* II site in pRS303 to give plasmid pRSN303. A

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Abbreviations: YAC, yeast artificial chromosome; HR, homologous recombination; CHEF, contour-clamped homogeneous electric field; PFGE, pulsed-field gel electrophoresis.

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4.0-kb *Sac* I fragment of a cloned L1 element from pL1.1A (gift of B. Dombrowski, S. Mathias, A. Scott, and H. Kazazian, Johns Hopkins University) was inserted into the *Sac* I site of the polylinker in both orientations to yield pIL1a and pIL1b. The *F9* sequences were generated from a 5.4-kb *Eco*RI fragment of the plasmid pTM6 containing genomic DNA including exons 7 and 8 of the *F9* gene (11). The 3.1-kb fragment from position 120 to position 3178 (within exon 7) of the fragment was amplified by the polymerase chain reaction (12). This was blunt-end ligated into the *Xho* I and *Cla* I sites of pIL1a and pIL1b so that when the plasmids were linearized with *Not* I the 3′ end of exon 7 was exposed. The resulting interstitial deletion vectors are referred to as pF9L1a and pF9L1b (Fig. 1*a*). The terminal deletion vectors pBP110 and pBP111 (ref. 13 and Fig. 1*b*) contained the same L1 element cloned in two orientations.

Pulsed-Field Gel Electrophoresis (PFGE) and Restriction Analysis. DNA for conventional gels and for PFGE were prepared by established procedures (14, 15). Electrophoretic karyotypes were examined using a contour-clamped homogeneous electric field (CHEF) apparatus (16). The following probes were used for hybridization: a 1.2-kb *Sal* I–*Xho* I *neo* fragment from pMC1neopolA (10); a 2-kb *Eco*RI–*Bam*HI *HIS3* fragment from pBM483 (gift of M. Johnston, Washington University); a 270-bp *Bam*HI fragment isolated from Blur8 (17); a 1.6-kb *Xho* I *CEN4* fragment from YRp14/ARSI/CEN4(6.0) (18); and a 600-bp *Bam*HI *F9* fragment from pF9L1a. Radiolabeled probes were prepared by random primer extension (19) and hybridized to GeneScreen*Plus* (DuPont/NEN) or Zeta-Probe (Bio-Rad) nylon membranes.

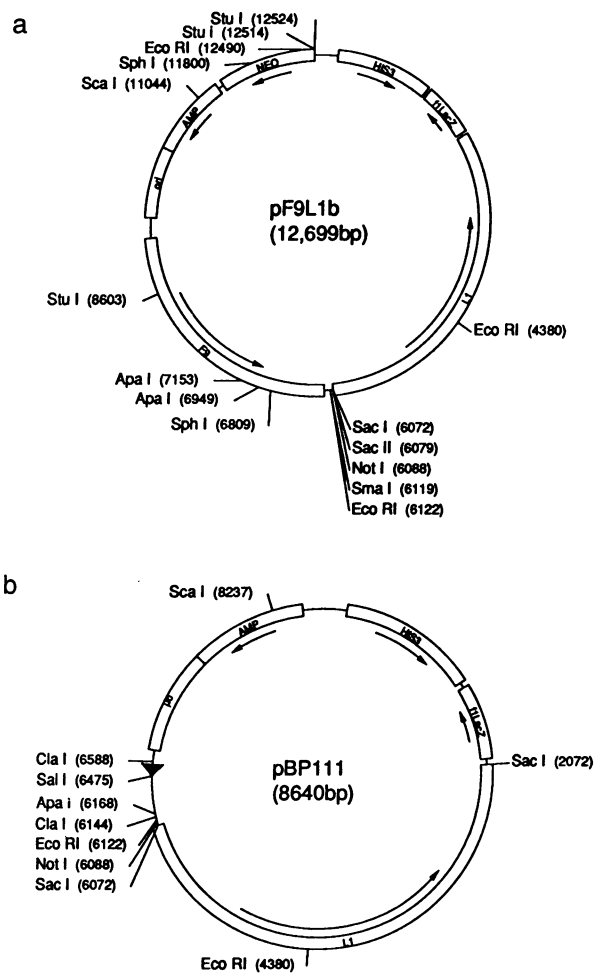

FIG. 1. Plasmids used to generate interstitial and terminal deletions. (*a*) pF9L1b. pF9L1a was generated by reversing the orientation of the L1 fragment. Boxes represent (clockwise, from top) the *HIS3* gene, *lacZ* sequences, the L1 element, the human *F9* gene, the plasmid origin of replication (ori), and the ampicillin (AMP) and neomycin (NEO)-resistance genes. bp, Base pairs. (*b*) pBP111. pBP110 was generated by reversing the orientation of the L1 fragment. Arrowhead represents yeast telomeric sequences.

RESULTS

The target YAC, HYA32G5 present in yeast strain YPH599, contained a 650-kb fragment of human DNA derived from the Xq27 region (20). This YAC is stable and contains a complete copy of the gene for clotting factor IX (*F9*), an anonymous DNA marker (*DXS102*), and part of the gene *mcf-2* (Fig. 2*a*). HR of pF9L1-series plasmids (Fig. 1*a*) with their homologous sequences in the YAC is expected to yield deletions as shown in Fig. 2*a*. His$^+$ colonies derived from transformation of

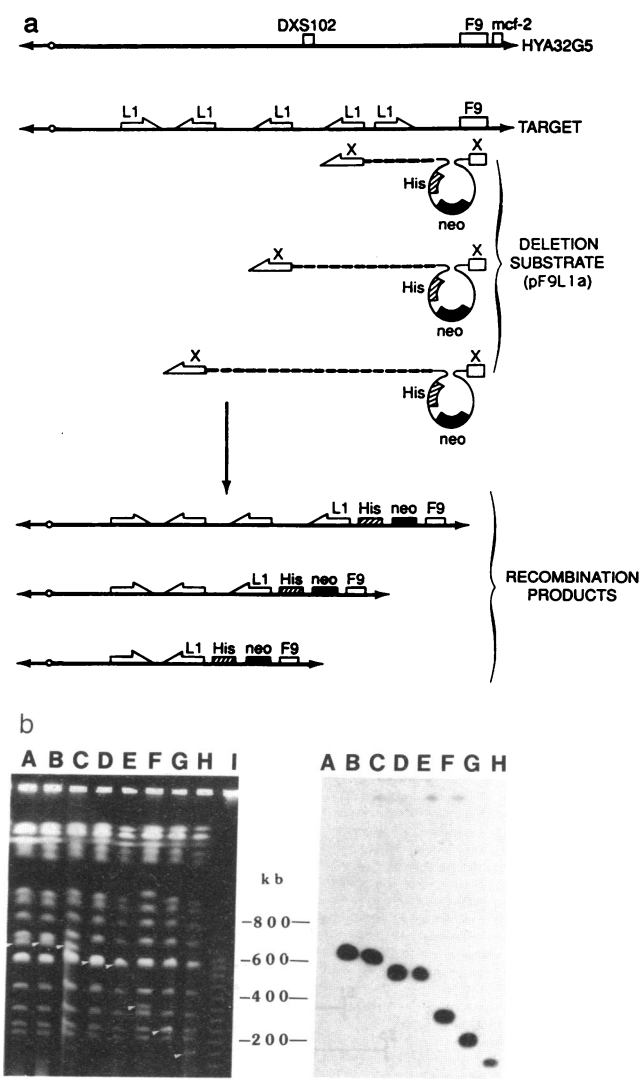

FIG. 2. Generation of interstitial deletions. (*a*) Schematic representation of the YAC HYA32G5 and the strategy to generate interstitial deletions. Different arrows in the target represent different L1 elements in different orientations. The deletion substrate is generated by digesting pF9L1a with *Not* I. (*b*) Nested series of deletions generated by HR. (*Left*) Chromosome karyotypes of different yeast strains obtained by transformation with pF9L1a and pF9L1b. Lanes: A, YPH599; B, YPH599 transformed with pF9DV; C, D, F, and G, YPH599 transformed with pF9L1b; E and H, YPH599 transformed with pF9L1a; I, size markers. Arrowheads indicate the position of the YACs. (*Right*) Autoradiograph of blot replica of the same gel, after hybridization with the *neo* probe.

YPH599 were characterized for the presence of markers on the YAC and to determine whether deletions had occurred.

When YPH599 was transformed with an *F9* targeting vector containing 5.3 kb of homology (pF9DV; not shown) and the pF9L1 series, we obtained His^+ colonies. The average transformation efficiency of pF9DV was 175 colonies per μg of DNA, whereas the interstitial deletion vectors yielded 25 and 15 colonies per μg, respectively. From several transformations, we obtained a total of 7000 colonies from pF9DV, 750 colonies from pF9L1a, and 180 colonies from pF9L1b. A number of these colonies were isolated and tested for their ability to grow in the absence of uracil and tryptophan. Ninety-two percent (147/160) of colonies scored positive for both markers, indicating that the YAC was present in each of them.

To ascertain whether the yeast transformed with pF9L1a and pF9L1b contained modified YACs, chromosome-sized DNA was isolated in low-melt agarose and fractionated by PFGE. Representative results are shown in Fig. 2*b*. YPH599 contained a YAC of 650 kb. When this strain is modified by pF9DV, an Ω-type recombination is expected to yield a YAC that is 5.6 kb larger (Fig. 2*b*, lane B).

We examined the nature of the YACs harbored by the transformants obtained from the pF9L1 plasmids. Of a total of 147 Trp^+ His^+ Ura^+ transformants tested, 123 (84%) had a YAC that migrated to a different position than that seen in YPH599. To better understand the structure of the modified YACs, the DNA in the CHEF gels was transferred to nylon membranes and the filters were hybridized with a *neo*-specific probe. This examination was conducted on 88 clones. Of these, 9 (10%) were unchanged, 14 (16%) were larger than 650 kb, 55 (62%) were smaller, 4 (5%) did not have detectable YACs, and 6 (7%) contained two different-sized YACs. The larger YACs could have resulted from integration of the circular version of the input plasmid (either singly or in tandem) into any of its homologous sequences (L1, *F9*, or plasmid sequences). Since the majority of the derivative YACs are smaller, they must have undergone deletions. Because they carry the terminal genetic markers, *TRP1* and *URA3*, the deletions must be interstitial, and this class was analyzed further. A representative set of deletion YACs are shown in Fig. 2*b*. The deletion YACs are of different sizes. The largest of the deletion YACs is 630 kb and the smallest is 150 kb. In many cases, more than one member of each class of deletion YACs was obtained, suggesting that these molecules were the result of specific rather than random events.

Molecular Analysis of the Deletions. If the deletions are to be useful genetic tools, it is necessary that they be nested and generated by HR. We first ascertained whether a HR event occurred at the *F9* end. The input plasmid contained an *Eco*RI site in *neo* (Figs. 1*a* and 3*a*). HR of *F9* using pF9L1a and pF9L1b is expected to yield an 8.6-kb *Eco*RI band (Fig. 3*a*). In addition, digestion with *Eco*RI is expected to yield 3.6-kb and 4.8-kb internal bands, respectively. DNA from several transformants was digested with *Eco*RI and blot were hybridized with the *neo* probe (Fig. 3*b*). Thirteen of 23 deletion-bearing pF9L1a transformants and 21 of 33 pF9L1b transformants yielded bands expected from HR at the *F9* end. We continued our studies on the YACs that had undergone HR at *F9*.

We also wished to establish whether the deletions were the result of HR at the L1 end. An unambiguous determination of this feature requires that we know the restriction enzyme maps around each of the L1 elements. Such a map of the entire YAC is not available. Fortunately, much of the *F9* gene itself was sequenced and an L1 element was identified at the 5′ end of the gene. The restriction enzyme map around this region is known (ref. 11 and Fig. 3*a*). If this element is targeted by recombination, a 630-kb YAC is expected. When pF9L1b was used, we indeed obtained several independent transformants that contained a 630-kb YAC. We examined whether these were the result of targeting the L1 element at the 5′ end of the *F9* gene. HR at this site is expected to yield a 7.5-kb *Sph* I band (Fig. 3*a*). All of the independently derived

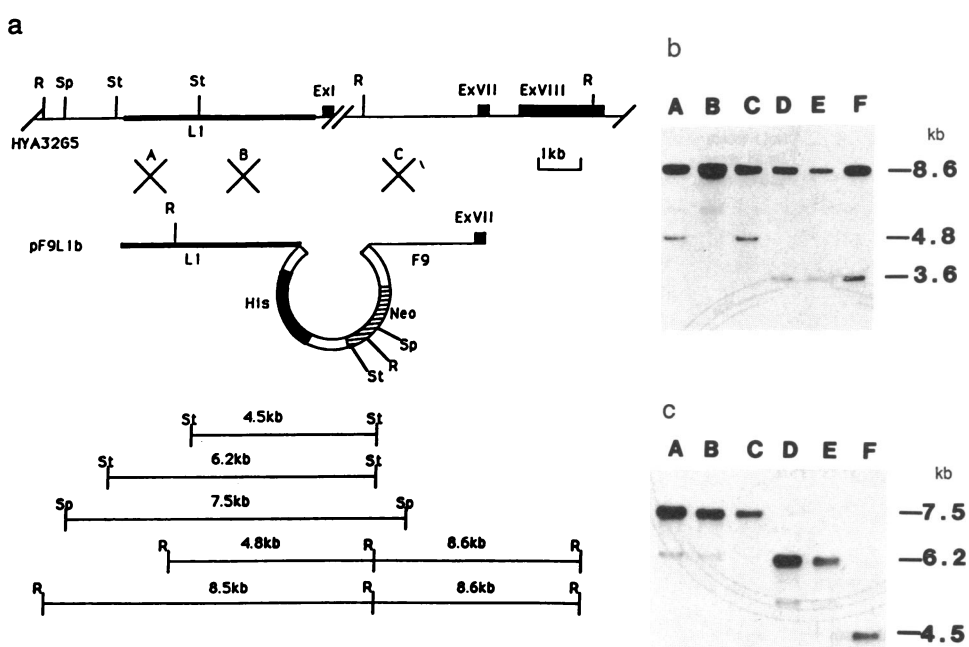

FIG. 3. HR between the YAC and the input plasmids. (*a*) Restriction enzyme map of *F9* and the expected products of recombination. Exons (Ex) are identified by Roman numerals. Sites A–C are discussed in the text. R, *Eco*RI; Sp, *Sph* I; St, *Stu* I. (*b*) Southern blot of *Eco*RI-digested DNA from YACs that have undergone deletions. Hybridization probe was *neo*. Lanes A–C (derived from pF9L1b): A, L1b-3 (630 kb); B, L1b-7 (630 kb); C, L1b-8 (280 kb). Lanes D–F (derived from pF9L1a): D, L1a-7 (590 kb); E, L1a-71 (150 kb); F, L1a-70 (280 kb). (*c*) Southern blots of *Sph* I-digested (lanes A–C) or *Stu* I-digested (lanes D–F) DNA from the 630-kb class derived from pF9L1b plasmid transformations. Probe was *HIS3*. Lanes: A and D, L1b-3; B and E, L1b-7; C and F, L1b-23. The fainter bands are the result of a slight contamination of the probe with plasmid sequences.

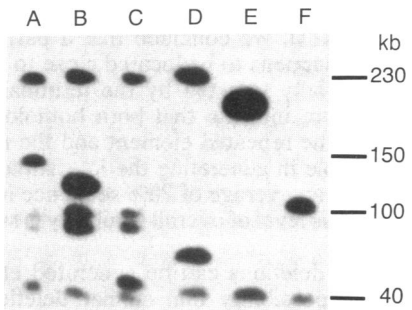

FIG. 4. *Sal* I digestion patterns of a nested series of deletions. *Sal* I digested DNA was fractionated by CHEF electrophoresis, and the blot was hybridized using total human DNA as the probe. Lanes: A, YPH599; B, 630-kb YAC; C, 590-kb YAC; D, 380-kb YAC; E, 280-kb YAC; F, 150-kb YAC.

cell lines of this class that were tested had such a band (Fig. 3*c*, lanes A–C).

The L1 sequence present in the deletion plasmids and the target L1 at the 5′ end of the *F9* gene have at least two differences in the restriction enzyme sites (Fig. 3*a*). The L1 in the input plasmid has an *Eco*RI site that is not shared by the target. The target contains a *Stu* I site that is not present in the plasmid-borne L1. Therefore, the site of crossover can be localized with respect to these restriction enzyme-site differences. Possible positions of crossovers with respect to the *Eco*RI site are represented as A and B in Fig. 3*a*. If a crossover occurred at A, it would yield a 4.8-kb *Eco*RI fragment. If crossover occurred at site B, it would result in an *Eco*RI band of 8.4 kb. Analysis of three of the cell lines from the 630-kb class revealed that two of them resulted from crossover at A (e.g., Fig. 3*b*, lane A) and one resulted from crossover at site B (lane B). Crossover to the left of the *Stu* I site on the target will yield a 6.2-kb fragment, and a 4.5-kb band will result from crossover to the right of the *Stu* I site. Results shown in Fig. 3*c* indicate that two members of the 630-kb class have undergone a crossover to the left of the *Stu* I site (lanes D and E) and one to the right of the *Stu* I site (lane F). Combining these results, we can conclude that the YAC in L1b-7 (Fig. 3*b*, lane B, and Fig. 3*c*, lane E) resulted from a crossover in the interval defined by the *Stu* I and *Eco*RI polymorphisms. These results demonstrate that the 20-kb deletions are the result of HR involving *F9* at one end and an L1 element at the other. It is therefore reasonable to extrapolate that most of the deletions have resulted from similar events involving other L1 elements.

Constructing a Restriction Map of the YAC. If the deletion series are indeed nested, it should be possible to use them to construct a restriction enzyme map of the YAC that should correspond to a map generated by conventional methods. We digested the DNA of the series of deletion YACs with *Sal* I, fractionated by PFGE, and hybridized with total human DNA. The patterns are shown in Fig. 4. Lane A is the 650-kb YAC showing bands corresponding to 230, 150, 100, and 90 kb and an unresolved doublet at about 40 kb. A 20-kb deletion proximal to *F9* resulted in the disappearance of the 150-kb band and replacement by a 130-kb band (lane B). A 60-kb deletion resulted in a corresponding reduction in the size of this band (lane C). Based upon this kind of analysis, it was possible to deduce that the YAC contained *Sal* I sites approximately 30, 260, 360, 450, and 610 kb from the centromere. This information corresponds to the *Sal* I map of HYA32G5 (R. Little and D. Schlessinger, personal communication).

Choice of L1 Elements Targeted by HR. The L1 elements that are targeted by the interstitial deletion vectors are shown in Fig. 5. We observed that the elements were not targeted at an equal frequency. This nonrandom distribution in the choice of L1 elements for targeting might be based upon the homology of the L1 elements in the target to the input L1 or it could be a function of the distance from the anchor site at *F9*. To distinguish between these possibilities, we used a terminal deletion vector (refs. 5, 6, and 13; Fig. 1*b*) containing the same L1 element as that in the pF9L1 series. His^+ colonies were generated with a frequency of 19 colonies per μg with pBP110 and 26 colonies per μg with pBP111. One hundred colonies derived from each transformation were tested for phenotype. For pBP110 and pBP111 derived transformants, 90% and 89%, respectively, were found to be His^+ Trp^+ Ura^-, confirming that the targeted YAC was present. Thirty-two independent transformants from pBP110 and 24 independent transformants from pBP111 were further characterized by PFGE and Southern analysis: 30 of 32 and 23 of 24 were found to contain recombination products of several different size classes (Fig. 6; results were verified by probing with *CEN4*, data not shown). The data in Fig. 5 summarize

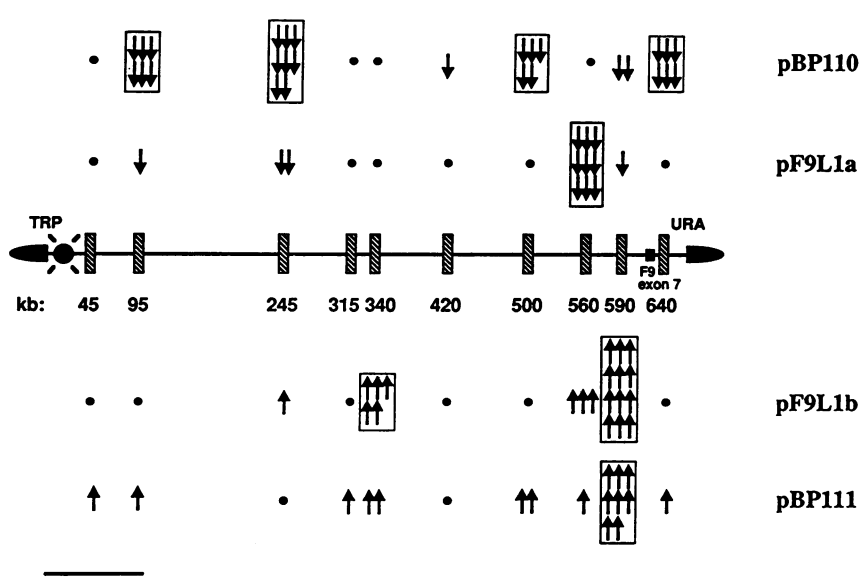

FIG. 5. L1 elements targeted by HR. L1 elements are indicated by hatched bars on the YAC. The location of exon 7 in *F9* is indicated by a black square. Each arrow represents a targeting event at that site. A dot indicates no targeting of that site by the vector.

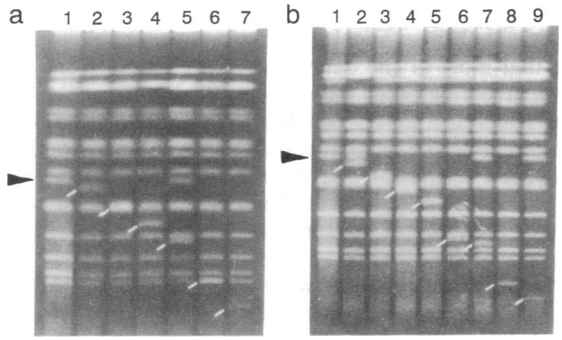

FIG. 6. Terminal deletion series. (*a*) Representatives of deletions obtained with pBP110. Lane 1, YPH599 (arrowhead indicates position of parental YAC, 650 kb); lanes 2–7, independent transformants of YPH599 with pBP110 (white angled lines indicate positions of the derivative YACs, 645, 595, 505, 425, 250, and 100 kb, respectively). (*b*) Representatives of deletions obtained with pBP111. Lane 1, YPH599 (arrowhead indicates position of parental YAC, 650 kb); lanes 2–9, independent transformants of YPH599 with pBP111 (white angled lines indicate positions of the derivative YACs, 645, 595, 565, 505, 345, 320, 100, and 50 kb, respectively).

the positions of the L1 elements targeted by different vectors and the number of times each class was recovered. Although some of the L1 elements are targeted by both classes of vectors, others were targeted by only one class of vectors.

DISCUSSION

We have obtained a nested series of interstitial deletions in human DNA cloned into YACs. These deletions resulted from HR. The use of an L1 element (10,000–50,000 copies per genome) permitted us to divide the 650-kb YAC into 50-kb segments. Use of other repetitive sequences will permit generation of deletions at any desired interval.

Fingerprints of progressively larger deletions (21) enabled us to establish that they were indeed a nested series (results not shown). These deletions have many uses. They can be used to construct restriction enzyme maps. Indeed, the *Sal* I maps constructed by us and by R. Little and D. Schlessinger (personal communication) correspond to each other. Since the deletion endpoints are close to vector sequences, it would be possible to rescue the unique DNA sequences at the sites of deletions. Such unique sequences could serve as sequence-tagged sites used as landmarks in human gene mapping. It is possible to introduce YACs (2, 3) into mammalian cells. The combination of defined interstitial deletions and YAC transfer would permit study of putative regulatory elements that have the capacity to act at long distances.

Examination of the interstitial deletions provides some understanding of the recombination processes. Most recombination vectors target contiguous DNA sequences. In our experiments, homologous pairing between sequences as distant as 500 kb is required to yield recombination products. It is of interest that several of the L1 elements are targeted by the pair of vectors in which the L1 is in opposite orientation. This implies that several of the L1 elements have undergone internal rearrangements so that they could pair with the vector L1 in either orientation. The observation of one such internal rearrangement (22) lends credence to this view. By examining our results summarized in Fig. 5, we can see that the interstitial deletions are preferentially targeting L1 elements located relatively close to *F9*. In one orientation (pF9L1a and pBP110) we can determine that this is not due solely to a particularly high degree of sequence homology between the target L1 and the vector L1, because the terminal deletions targeted L1 elements in a fairly random manner along the entire YAC. For the opposite orientation (pF9L1b and pBP111), we conclude that a particularly recombinogenic L1 happens to be located close to *F9*, since it was also preferentially targeted by the terminal deletions. These data therefore indicate that both homology and the distance between the repeated element and the unique (*F9*) sequence play a role in generating the interstitial deletions. L1 elements share an average of 70% sequence identity (23, 24). Apparently, this level of overall similarity is sufficient for HR in yeast.

That interstitial deletions can be generated efficiently in YACs raises the possibility that similar deletions can be generated in mammalian cells. The ability to generate such large deletions would not only have implications for gene mapping but could also prove to be a valuable genetic tool.

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