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A Double-Strand Break within a Yeast Artificial Chromosome (YAC) Containing Human DNA Can Result in YAC Loss, Deletion, or Cell Lethality

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Human chromosomal DNA contains many repeats which might provide opportunities for DNA repair. We have examined the consequences of a single double-strand break (DSB) within a 360-kb dispensable yeast artificial chromosome (YAC) containing human DNA (YAC12). An *Alu-URA3*-YZ sequence was targeted to several *Alu* sites within the YAC in strains of the yeast *Saccharomyces cerevisiae*; the strains contained a galactose-inducible HO endonuclease that cut the YAC at the YZ site. The presence of a DSB in most YACs led to deletion of the *URA3* cassette, with retention of the telomeric markers, through recombination between surrounding *Alu*s. For two YACs, the DSBs were not repaired and there was a G_2 delay associated with the persistent DSBs. The presence of persistent DSBs resulted in cell death even though the YACs were dispensable. Among the survivors of the persistent DSBs, most had lost the YAC. By a pullback procedure, cell death was observed to begin at least 6 h after induction of a break. For YACs in which the DSB was rapidly repaired, the breaks did not cause cell cycle delay or lead to cell death. These results are consistent with our previous conclusion that a persistent DSB in a plasmid (YZ-CEN) also caused lethality (C. B. Bennett, A. L. Lewis, K. K. Baldwin, and M. A. Resnick, Proc. Natl. Acad. Sci. USA 90:5613–5617, 1993). However, a break in the YZ-CEN plasmid did not induce lethality in the strain (CBY) background used in the present study. The differences in survival levels appear to be due to the rapid degradation of the plasmid in the CBY strain. We, therefore, propose that for a DSB to cause cell cycle delay and death by means other than the loss of essential genetic material, it must remain unrepaired and be long-lived.

Organisms have evolved several strategies for coping with DNA lesions, the major one being repair. In addition, most organisms appear to have systems that enhance the opportunity to repair lesions before cell division by delaying cell progression. Delay at the S or G_2/M phase of the cell cycle due to unrepaired chromosomal damage has led to the checkpoint control hypothesis, which states that cell progression is dependent on the successful completion of earlier steps in the cycle (5, 35). Many of the initial studies about checkpoint controls derived from investigations of the consequences of radiation-induced double-strand breaks (DSBs) in the yeast *Saccharomyces cerevisiae*. Following induction of DSBs, cells of *S. cerevisiae* undergo a transient arrest of the cell cycle in the G_1, S (1, 32), and G_2 (5, 8, 30, 35–37) phases. The transient cessation of cell cycle progression due to DNA lesions is under the control of several genes that are proposed to act in two pathways (1, 8, 36). The *RAD9*, *RAD17*, *RAD24*, *DUN2*, and *MEC3* genes are required for arrest in the G_2 phase of the cell cycle in response to unidentified signals generated from damaged or unreplicated DNA (5, 8, 10, 15, 30, 35, 37). The checkpoint gene, *DUN2*, is identical to *POL2*, which encodes polymerase ε, one of three essential DNA polymerases in *S. cerevisiae* (10, 14, 15). The carboxy terminus of polymerase ε, which is altered in *DUN2* mutants, is essential for checkpoint control and the ability to transcriptionally activate the damage-inducible *RNR3* gene (15). Another class of genes that includes *MEC1* (*rad3*$^+$ of *Schizosaccharomyces pombe*) and *MEC2* (*SAD1* or *RAD53*) is made up of kinases required for G_2 arrest as well as arrest in S phase in response to incomplete DNA replication (37). Recently, it was suggested that both the *RAD9* and *RAD24* gene products are also required for arrest in G_1 in response to DNA damage (32). Although many of these genes and products have been characterized (8), the signaling mechanism(s) that links DNA damage to cell cycle arrest has not been elucidated.

The efficient repair of DSBs in *S. cerevisiae* generally involves a *RAD52*-dependent recombinational repair pathway (22) involving sister chromatids or homologous chromosomes, since both G_1 haploids and diploid *rad52* cells are sensitive to the killing effects of ionizing radiation. This mechanism of repair was proposed to require an invasive 3′ end supplied by the DSB and a homologous chromosome or sister chromatid to serve as the template for the repair event (22). Recombinational repair of naturally occurring or induced DSBs is largely error-free; however, deletions, rearrangements, truncations, and aneuploidy may also result from a DSB. Another mechanism involves recombination via single-strand annealing between repeat sequences that may surround a DSB (17, 20, 25, 26). This repair is much less likely to function in the repair of genomic damage since yeast cells, unlike mammalian cells, have few long repeat sequences. This strand-annealing process can occur in the absence of *RAD52* (17, 20). Illegitimate recombination is also an infrequent repair mechanism in yeast cells, although it is commonly observed in mammalian cells (29).

Little is known about the indirect consequences of an unrepaired lesion in eukaryotes, particularly a DSB. This is due, in part, to the use of systems in which loss of essential genetic information would be lethal. It has been suggested that unrepaired DSBs can act in a dominant-lethal fashion, since one or a few radiation-induced breaks in polyploid *rad52* strains may result in lethality (6). Recently we described a system in which

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TABLE 1. Yeast strains, YACs, and plasmids

Strain, YAC, or plasmid	Relevant characteristic(s)
Strains	
YPH604	*MAT***a** *ura3-52 trp1*Δ*1 lys2-801 his3-*Δ*200 ade2-101* (*gal* mutant) with YAC12
CGY2872	*MAT*α *ura3-52 trp1*Δ*63 lys2-*Δ*202 his3-*Δ*200 ade2-1*(*oc*) *rad52*::*LEU2* (*gal*$^+$)
CGY2872 plus pGALHOT	Strain CGY2872 containing the HO-inducible plasmid
CBY plus u8, s15, s16, u17, s23, or YAC12	Diploid strains formed by crossing the YPH604 strain containing the various YACs described below and the CGY2872 plus pGALHOT strain
CBY plus YZ-CEN or CEN[No YZ]	Diploid strain CBY transformed with pGALHOT and either YZ-CEN or CEN[No YZ]
YACs	
YAC12	This YAC, which was present in strain YPH604, contains 360 kb of human DNA (19). The centromere-proximal telomere is marked by *LYS2*, and the distal telomere is marked by *HIS3*.
YACs u8, s15, s16, u17, and s23	These YACs correspond to independent integrations of the *Alu-URA3*-YZ vector into YAC12 contained in the YPH604 strain.
Plasmids	
Alu-URA3-YZ	This circular vector was targeted to *Alu* repeats within YAC12 following cutting at the *Msc*I site within the *Alu* sequence (Fig. 1).
YZ-CEN	This plasmid, which contains the selectable *URA3* marker, can be cut at the YZ sequence by HO endonuclease (2).
CEN[No YZ]	This control plasmid is identical to YZ-CEN except that it lacks the YZ sequence.
pGALHOT	This plasmid, which contains the selectable *TRP1* marker, can be induced on galactose to produce HO endonuclease (2).

the consequences of a single, persistent unrepaired DSB could be directly addressed (2). We demonstrated that a nonrepairable DSB induced by HO endonuclease at a YZ sequence in a genetically dispensable plasmid can lead to lethality. The mechanism of this indirect lethality, for which the primary DNA damage leading to lethality is not in essential genetic material, is not known. However, the cell checkpoint system plays a role, since deletion of *RAD9* partially attenuated the lethality (2).

In order to understand the cellular consequences of a single site-specific DSB, we have introduced the YZ junction into a dispensable yeast artificial chromosome (YAC) containing a unique fragment of human DNA (YAC12). Since human DNA contains many diverged repeat DNAs, such as *Alu*s and long interspersed nuclear elements (LINEs), there may be various opportunities for recombinational repair. Because the series of YACs generated contained the YZ junction at various positions within the human DNA, the consequences of context as well as chromosomal organization on the effects of a single DSB could be assessed. For most YACs, the induction of HO endonuclease had no effect on cell progression or survival. The YZ break was rapidly repaired, apparently by recombination between the surrounding *Alu* repeats. However, repair of the DSBs in two YACs was inefficient. The breaks in these YACs resulted in lethality even though the YAC was dispensable, and among the few survivors, most had lost their YAC. This indirect lethality was associated with a delay in cell progression at G_2, although a small number of divisions could follow. It appears that the DSB must persist in order for the cell to "sense" the break and thus can result in G_2 delay and eventual clonal death. These results demonstrate that an unrepaired chromosomal DSB (in a YAC) can cause indirect as well as direct lethality.

MATERIALS AND METHODS

Plasmids. The *URA3*-YZ-integrating vector was constructed by cloning a 1.1-kb *URA3 Hin*dIII fragment into a pUC-based vector (pJS113) that has a 45-bp *MAT***a** YZ junction cloned between the *Sal*I and *Bam*HI sites (2). Plasmid pBP47 (19), which contains an *Alu* with a centrally positioned unique *Msc*I site, was utilized to construct an *Alu-URA3*-YZ-integrating plasmid. The plasmid was constructed by inserting the 300-bp *Bam*HI human *Alu* fragment from pBP47 into the *URA3*-YZ vector. pGALHOT is a single-copy plasmid containing a centromere, a selectable marker (*TRP1*), and the HO endonuclease fused to the *GAL1-10* promoter (2).

Production of YACs containing the *Alu-URA3*-YZ plasmid. The Gal$^-$ strain YPH604 (Table 1), containing the 365-kb human DNA YAC12 (Fig. 1), was transformed by the *Msc*I-linearized *Alu-URA3*-YZ plasmid by a lithium acetate procedure (28). YAC12 contains approximately 30 *Alu* sequences, which are potential targets for integration of the plasmid; 19 of the *Alu*s within YAC12 have been mapped previously (Fig. 1) (18). All of the 23 isolated Ura$^+$ integrants were stable.

Strains used for examining the consequences of a DSB. The haploid isolates of YPH604 containing the integrated vector *Alu-URA3*-YZ in YAC12 were crossed with the *GAL* strain CGY2872 containing pGALHOT. The diploid strains were selected with the YAC and plasmid markers. Diploid strains that lost the resident YAC by malsegregation (CBY-CON) were retransformed with either the YZ-CEN or the CEN[No YZ] plasmid.

The *MAT* locus in these strains was left intact to allow the diploid strains to be rapidly sporulated in order to isolate *rad52* haploids. Galactose induction of HO did not produce any enhanced lethality in the control *MAT***a**/*MAT*α diploids (see Fig. 6B) or in the CBY-CON strain retransformed with YZ-CEN (see Fig. 6D) but was lethal in the haploid *rad52* CGY2872 strain under similar plating conditions (data not shown).

Molecular analysis of HO-induced YZ cutting. In order to examine the effects of HO-induced cleavage following growth in galactose, 1.7-ml aliquots containing approximately 10^8 cells were centrifuged and resuspended in 0.15 ml of 50 mM EDTA–10 mM Tris (pH 7.5) at 42°C. In addition, 0.25 ml of melted low-melting-point agarose and 1 μl of Zymolyase (5 mg of Zymolyase 100T per ml, 10 mM potassium phosphate [pH 7.5]) were added to the resuspended solution. The mixture was quickly poured into a mold at room temperature. Once solidified, the plugs were removed from the mold and incubated in 0.8 ml of 0.25 M EDTA–10 mM Tris (pH 7.5) at 37°C overnight. The next day, 0.2 ml of a proteinase K solution (10 mg of proteinase K 10 per ml, 0.5 M EDTA, 1% Sarkosyl) was added to the plugs, which were incubated at 55°C overnight (3). Chromosomes were separated by transverse-alternating-field electrophoresis (TAFE) on 1% Fastlane agarose gels (Bethesda Research Laboratories) (2). The chromosomal DNAs were transferred to nylon membranes and probed with the fluorescein-labeled (Renaissance kit; Du Pont) *Alu-URA3*-YZ plasmid probe.

HO-induced cleavage of YAC u17 or the CBY strain containing plasmid YZ-CEN was detected as previously described (2). Briefly, genomic DNA was extracted from cells (7) and separated on 0.7% agarose gels as unrestricted (u17) or *Xho*I-restricted (YZ-CEN) genomic DNA. HO-induced cleavage was visualized after Southern blotting to nylon membranes and probing with either the fluorescein-labeled *Alu-URA3*-YZ or the YZ-CEN plasmid.

Genetic analysis of marker loss following HO-induced YZ cleavage. Single-colony isolates were grown overnight in 100 ml of synthetic complete (SC [38]) medium plus 2% glucose minus Trp minus Ura (Glu − Ura) to 1×10^7 to 3×10^7 cells per ml. The cells were washed twice in SC medium minus Trp minus Ura minus sugar and resuspended in 20 ml of medium not selective for the YAC (SC

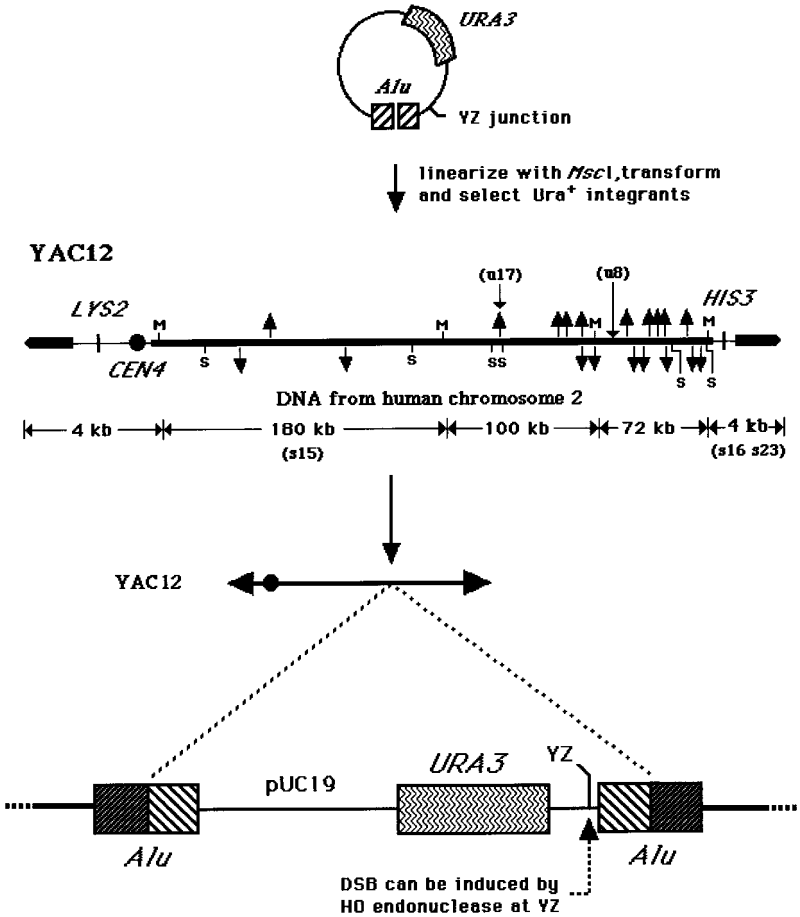

FIG. 1. Scheme for targeting the *Alu-URA3*-YZ plasmid to YAC12 and the subsequent site-specific cutting of the resulting YACs. The *Alu-URA3*-YZ-integrating vector is cleaved at the unique *Msc*I site contained within a 300-bp *Alu* sequence. The resulting sequence is transformed into a haploid strain containing the 365-kb human YAC12. The previously mapped (18, 19) restriction sites, *Mlu*I (M) and *Sac*II (S), and *Alu*s (arrows on restriction map) are indicated. Cells containing *URA3*-YZ plasmid integrations within YAC12 were mated to an isogenic Gal^+ *rad52* strain carrying the galactose-inducible HO endonuclease plasmid pGALHOT. Growth of the diploid strains in glucose represses the expression of HO endonuclease. Incubation in galactose induces the expression of HO, which can produce a site-specific DSB at the integrated YZ junction. The plasmid integration sites for the u8, u17, s15, s16, and s23 YACs (see Results) were mapped by restriction with *Mlu*I and subjecting the separated YAC fragments to Southern blotting with *URA3*-YZ as a probe and are presented below the YAC restriction map (data not shown). Not all of the ~30 *Alu* sites contained within this YAC have been mapped. The positioning of the s16 and s23 integration sites within the terminal *Mlu*I fragment identifies a previously unmapped *Alu* site(s).

medium plus 2% galactose minus Trp plus Ura [Gal + Ura]) to induce the HO endonuclease. At various times, cells were either prepared for chromosome analysis or plated onto YEPD (1% yeast extract, 2% peptone, 2% dextrose) plates and incubated for 48 h at 30°C. The genetic effects of an HO-induced DSB at the YAC YZ junction were then determined by replica plating onto SC medium or media lacking either lysine (Lys^-) or histidine (His^-) to identify the telomeric markers, uracil (Ura^-) to identify the internal marker, or tryptophan (Trp^-) to identify the HO plasmid.

Consequences of HO induction in cells plated to galactose-containing medium. Single colonies growing on Glu − Ura plates were inoculated into 10 ml of Glu − Ura. Logarithmically growing cells were obtained by growing the cells to 1×10^7 to 3×10^7 cells per ml with vigorous shaking at 30°C. Cells were washed twice and diluted in sugarless medium and plated to SC medium minus Trp containing either 2% glucose or 2% galactose and either containing or lacking Ura. Plating to Ura^- medium allows selection for the YAC containing the integrated *Alu-URA3*-YZ cassette or the YZ-CEN plasmid containing *URA3*. The plating efficiency on Glu − Ura was high (>90%). Relative plating efficiencies were determined by comparing colony formation on Gal + Ura versus that on Glu + Ura. *URA3* loss was determined by the relative plating efficiencies on Glu − Ura versus on Glu + Ura.

Commitment to death following induction of a DSB. To determine the time at which *URA3*, linked to the YZ junction, was lost and when the commitment to death occurred, cells were grown and washed as described above. In these pullback experiments, cells were resuspended in SC medium minus Trp minus Ura minus sugar and aliquots of between 50 and 200 μl were delivered to plastic petri dishes. To the cell aliquots was added 20 ml of liquid SC medium plus 2% galactose with or without Ura agar medium (45°C) so that the cells were imbedded in the agar. After various periods of incubation at 30°C, a 20-ml overlay was added containing 2% agar and SC medium plus 2% glucose with or without Ura. (The results using the 2% glucose overlay did not differ from those obtained with a 4% glucose overlay.) The diffusion of glucose into the bottom layer rapidly repressed the expression of HO endonuclease (see Fig. 6).

Characterization of intact or HO-cleaved YACs by restriction digest mapping. Cells were gently lysed in agarose plugs as described above. Plugs were washed in 20 mM Tris-HCl (pH 8.0)–50 mM EDTA with 1 mM phenylmethylsulfonyl fluoride to inactivate the proteinase K. The plugs were washed twice in 2 mM Tris-HCl (pH 8.0)–5 mM EDTA and cut with restriction enzyme *Mlu*I or *Sac*II overnight (16 h). Restricted YAC fragments were separated by TAFE and detected by Southern blotting with fluorescein-labeled *Alu-URA3*-YZ-, *URA3*-YZ-, or *Alu*-specific probes.

Cell cycle analysis following induction of a DSB. Logarithmically growing cells were grown and washed as described above. Briefly, sonicated cells (2×10^7 cells per ml) were plated to Gal + Ura with a multipin pronging device. Photographs were taken of individual cells at various times following plating with an MSM dissection microscope (Singer Instrument Co. Ltd., Watchet, England). Cells were categorized as single (G_1), small budded (S), and large budded (G_2) (as described in reference 35).

RESULTS

Generation of YACs with a YZ junction. To examine the consequences of a DSB in a genetically dispensable chromosome, we developed a series of dispensable human DNA-

TABLE 2. Consequences of a HO-induced DSB in a dispensable YAC on loss of YAC genetic markers

YAC	Time in Gal (h)[a]	No. of colonies	% Loss of *URA3* and associated genetic markers after replica plating to media lacking supplements[b]:					% HO plasmid loss[c]
			Total Ura	Ura	Ura and His	Ura and Lys	Ura, His, and Lys	
u8	0	388	0	0	0	0	0	2.6
	4	392	19.1	7.2	0.2	0	11.7	11.7
	8	330	34.8	7.3	0.3	0	27.2	9.7
	12	415	21.4	8.4	0.5	0	12.5	17.6
	24	408	20.3	6.9	0.5	0	12.9	12.5
u17	0	396	0	0	—[d]	0	—	6.1
	4	221	10.4	6.3	—	4.1	—	12.6
	8	188	29.7	0	—	29.7	—	8.5
	12	310	33.5	3.9	—	29.3	—	24.2
	24	486	26.1	8.8	—	17.3	—	16.0
s16	0	436	0.92	0.23	0.23	0	0.23	3.9
	4	431	23.4	21.8	0	0	1.6	3.9
	8	353	23.8	23.2	0	0	0.57	2.5
	12	362	23.5	21.6	0.5	0	1.4	9.1
	24	444	14.1	13.6	0	0	0.45	7.0

[a] Cells were incubated for the indicated times in liquid galactose medium containing uracil, histidine, and lysine. The medium lacked tryptophan to assure retention of the HO plasmid.

[b] Colonies arising on rich glucose medium (YEPD) were replica plated to medium lacking individual supplements. YAC loss was indicated by coincident loss of the Ura, His, and Lys markers for u8 and s16. YAC loss for u17 was indicated by coincident loss of the Ura and Lys markers.

[c] Clones that lost the HO plasmid pGALHOT did not exhibit *URA3* loss.

[d] The u17 YAC lacked the *HIS3* telomere marker.

containing YACs with YZ sequences at various positions (Fig. 1). A DSB produced at the YZ sequence following induction of HO endonuclease (encoded on a selectable plasmid) would be expected to produce two YAC fragments. Among the outcomes of the DSB that would be detectable by genetic and/or physical means would be internal deletions arising between the many repeat sequences (such as *Alu*s and LINEs) present in human DNA, loss of all or the distal part of the YAC, delay in cell progression, and possibly cell death.

The YZ-containing YACs were generated with an integrating vector (*Alu-URA3*-YZ) containing a 45-bp YZ sequence, a selectable *URA3* marker, and a human *Alu* sequence. The plasmid cassette was targeted to YAC12, a 360-kb YAC which contains DNA from human chromosome 2, in a *MAT***a** Rad$^+$ haploid strain (YPH604). Restriction with *Msc*I linearizes this vector by cutting within the *Alu* sequence, allowing plasmid integration at any of approximately 30 *Alu*s in this YAC. Each of 23 independent and mitotically stable Ura$^+$ transformants was mated with a *MAT*α *rad52*::*LEU2* strain (CGY2872) carrying the HO-inducible plasmid pGALHOT (Table 1), and subsequent experiments were conducted with the diploid strains. Except for the 4-kb addition of plasmid DNA, integration of *Alu-URA3*-YZ into YAC12 did not alter the apparent size of the parental YAC in 18 of the 23 isolates (determined by probing of chromosomes separated by TAFE with the *Alu-URA3*-YZ vector [data not shown]). Two of the YACs were smaller (e.g., YAC u17 in Fig. 3B) and three of the YACs were substantially larger than the parental YAC12 (data not shown).

Physical characterization and genetic changes in YZ-containing YACs following HO induction. For 21 of 23 YACs containing the *URA3* marker, incubation of the diploid cells in galactose-containing liquid medium resulted in loss of the *URA3* gene (presented in Table 2 are results with three of the YACs). Thus, these YACs contained a YZ sequence that was accessible to the HO endonuclease. Furthermore, these results demonstrated that YZ cutting could be monitored genetically. (The remaining two YACs were not examined further.) The 21 YACs were physically examined at various times after galactose addition by Southern blotting of chromosomes following TAFE gel separation. As described below, three of these YACs (u8, u17, and u26) exhibited two persistent fragments and their YACs were subsequently lost (i.e., they were unstable), as determined by genetic and physical analysis. For the rest of the YACs, fragments were not detected following galactose induction of HO, presumably because of rapid DSB repair; these YACs were not lost (s15, s16, and s23 are examples of these stable YACs and are extensively described below).

The positions of the YZ cassettes in the u8, u17, s15, s16, and s23 YACs were localized to *Mlu*I restriction fragments (Fig. 1). Total DNA was restricted with *Mlu*I, and the large molecules were separated by TAFE and probed with the *URA3*-YZ fragment contained in the original transforming vector (data not shown).

These YACs were subsequently characterized to demonstrate that no rearrangements had occurred. Total genomic DNA was cut with either *Mlu*I (Fig. 2) or *Sac*II, and the fragments were separated by TAFE and probed with an *Alu*-specific probe. The YACs u8, s15, s16, and s23 had identical *Mlu*I restriction patterns. For the u17 YAC, there was a simple deletion resulting in loss of the 72-kb *Mlu*I fragment, which resulted in a terminally deleted 230-kb YAC (including *HIS3*; see below). The deletion which gave rise to this YAC is likely to have arisen by intrachromosomal recombination between homologous integrated pBR sequences at *HIS3* and homologous pUC sequences of the integrating vector, as has been observed for other YACs (see reference 11 and below). The 600-kb u26 YAC appeared to be due to a duplication during the integration of the YZ cassette (data not shown). Because of the undefined changes relative to the original YAC, results with this YAC are not presented here.

It was, therefore, possible to compare the consequences of a

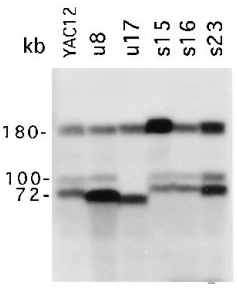

FIG. 2. Restriction digest analysis of YAC structure. Cells containing YAC12 (control), u8, u17, s15, s16, or s23 were grown under conditions of HO repression and gently lysed in agarose plugs. The plugs were then incubated with *Mlu*I, and DNA fragments were separated by TAFE. Separated chromosomes and YAC fragments were subjected to Southern blotting to nylon membranes and probed with a fluorescein-labeled *Alu* fragment isolated from the *Alu-URA3*-YZ plasmid. As shown in Fig. 1, only three large fragments are expected to hybridize with the *Alu* probe. Molecular size markers are noted at the left.

persistent DSB (in the u8 and u17 YACs) with those of a rapidly repaired DSB (a break is not detected) in a large chromosome composed of human DNA. Following induction of HO endonuclease, the u8 YAC was altered from 365 kb to 295- and 70-kb fragments (Fig. 3A and 4A). The fragments could be detected at 1 and 8 h (Fig. 4A). The break was distal to the *LYS2*-marked telomere, since a *LYS2* probe hybridized specifically with the 295-kb YAC fragment (data not shown). The 365-kb u8 band that remained at 24 h may result from uncut YACs obtained from cells that lost the pGALHOT plasmid or from inefficient cutting in liquid GAL medium (Table 2 and below). Most u8 (and u17) survivors had lost all the YAC markers (see below).

Induction of HO endonuclease in cells containing the 230-kb u17 YAC resulted in the appearance of an approximately 6-kb fragment (Fig. 3B and C). A shift in the size of the larger fragment from the parental YAC was not resolved in these experiments (Fig. 3B). The 6-kb fragment first appeared at 2 h and persisted until 8 h after galactose addition (Fig. 3C).

The remaining YACs (18 of 21) exhibited rapid loss of the *URA3* marker on galactose (data not shown). Under the conditions of TAFE analysis, the 17 YACs that were comparable in size to YAC12 (~365 kb) and the one that was smaller (~200 kb) did not reveal any fragments following galactose induction (s16 is an example and is presented in Fig. 3D). These YACs were genetically stable in that there was no loss of the *HIS3* or *LYS2* telomeric markers (as an example, see s16 in Table 2). Therefore, the HO-induced DSB that resulted in loss of the *URA3* marker must be short-lived and repaired quickly in these YACs.

Of the stable YACs, the s15 and s16 YACs were examined more extensively. No changes were observed in the s16 YAC in cells isolated at hourly intervals up to 10 h (data not shown) or at longer intervals of up to 24 h after induction (Fig. 3D). This, as well as the other stable YACs, was shown genetically to lose the *URA3* marker. Comparable physical data, which are not presented, were obtained with s15. Cutting at the YZ junction could be demonstrated in the s15 YAC when it was present in a haploid *rad52* strain, in which the amount of repair of the YZ break was reduced, supporting the view that the breaks in the diploid are normally repaired rapidly (unpublished data).

A persistent DSB leads to YAC loss. A DSB in a YAC could have several genetic consequences in addition to loss of *URA3* next to the YZ junction. To distinguish these consequences, cells that had been growing in liquid Gal + Ura medium (i.e., without selection) were plated to nonselective YEPD medium. The subsequent colonies were replica plated to medium diagnostic for the YAC markers. The maximum frequencies of loss of the *URA3* marker for the s15 (26.3%), s16 (23.8%), and s23 (14.6%) YACs were similar to those observed for the u8 (34.8%) and u17 (33.5%) YACs, in which a DSB was persistent (s16, u8, and u17 are described more extensively in Table 2). Thus, on the basis of genetic evidence, the efficiencies of induction of a DSB were comparable for those YACs that could and those that could not repair the HO-induced DSB.

For the u8 and u17 YACs, the majority of the colonies that were Ura$^-$ lacked the entire YAC (Table 2). As shown in Table 2, the maximum loss (~30%) was observed at 8 h after suspending cells in galactose medium for both u8 and u17. Therefore, a persistent DSB resulted in a high rate of YAC loss among the surviving u8 and u17 cells. For those colonies in which the telomere markers were retained, the YACs generally had large internal (u8) and terminal (u17) deletions (unpublished data). The number of colonies that lacked either the *URA3* marker or all three YAC markers was much higher for cells plated directly to galactose plates than for cells plated at various times from the galactose liquid medium to glucose plates. This may reflect the dispersion of dividing cells in liquid versus clonal growth on a galactose-containing plate.

The rapid repair of a DSB results in a small deletion. Among the isolates that did not exhibit persistent DSBs, YAC loss was infrequent (as an example, see s16 in Table 2). These results are consistent with the observation that the Ura$^-$ YACs revealed little change in size after induction of HO. Among 15 Ura$^-$ s16 YAC isolates, there were no apparent changes in size when compared with that of the control s16 YAC (Fig. 5A). However, on the basis of restriction with *Xba*I (the YZ cassette vector lacks a *Xba*I site) and probing with the *Alu-URA3*-YZ plasmid, all of the s16 Ura$^-$ YACs examined (11 YACs in Fig. 5, plus an additional 3 YACs not shown) had experienced a 4-kb deletion (Fig. 5B). The original 9-kb *Xba*I fragment contained the entire 4-kb cassette. It appears that the HO-induced DSB caused recombination between the surrounding *Alu*s, thereby deleting *URA3* and leaving behind a single *Alu* sequence contained in the remaining 5-kb deletion fragment.

Lethality can result from a persistent unrepaired DSB. Previously we showed that a single, nonrepairable DSB in a dispensable plasmid could lead to loss of viability (see reference 2, in which the strain background was different from that in the present experiments). We, therefore, investigated whether a persistent DSB in YACs u8 and u17 could also result in indirect lethality when there was no selection for any of the YAC markers (Table 3). Strains containing these YACs as well as the strains with s15, s16, and s23, which exhibited rapid repair of the induced DSB, were grown to log phase and plated to glucose or galactose media selective for the HO plasmid and either containing uracil (no selection for the YACs) or lacking uracil (selection for the *URA3* marker next to YZ). On the basis of the small number of colonies arising on Gal − Ura medium, loss of the *URA3* marker was very efficient (<0.1% survival on the medium lacking uracil) for both unstable and stable YACs. (We note that there was a much smaller number of Ura$^+$ colonies when cells were plated to Gal − Ura than when cells were grown in liquid Gal + Ura and plated to nonselective glucose media as described above, possibly reflecting, as mentioned above, the ability of cells in liquid to divide and disperse.) This genetic evidence suggests that DSB induction resulting in subsequent loss of the *URA3* marker was very efficient on solid plates.

The average level of survival on Gal + Ura solid medium was greatly reduced for strains with the u8 (19%) and u17

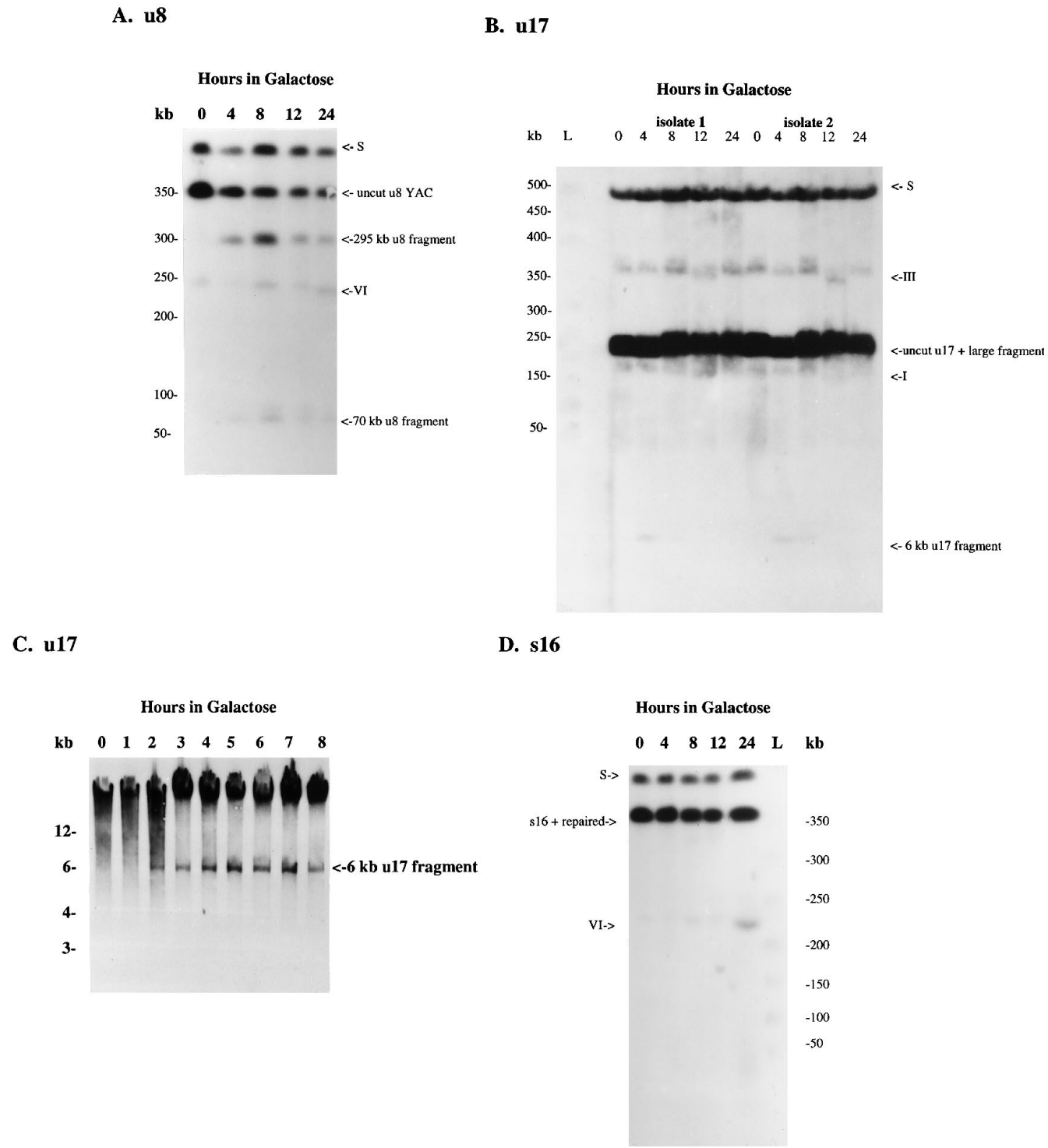

FIG. 3. Site-specific cutting of YACs containing a YZ sequence. Cells containing the YACs u8 (A), u17 (two isolates) (B), u17 (C), and s16 (D) were transferred to liquid Gal medium and collected at the indicated times, and the sizes of YACs or YAC fragments were determined. Molecular size markers are noted beside the gels. (The results with the s15 and s23 YACs were identical to those with s16 in that cutting was not detected.) In panels A, B, and D, chromosomes were released from cells by gentle lysis and separated on TAFE gels (see Materials and Methods). In panel C, genomic DNA from cells containing YAC u17 was extracted by a rapid glass bead and phenol method (7) and separated on a 0.7% agarose gel. The gels were blotted to nylon membranes by Southern hybridization and probed with the fluorescein-labeled *Alu-URA3*-YZ plasmid, per the manufacturer's instructions (Renaissance kit; Dupont). (A) Arrows indicate HO-induced fragments of 295 and 70 kb from YAC u8. Intact YAC (u8) was observed at 365 kb. Cross-hybridization of the probe to yeast chromosome VI was observed at 240 kb. (B) The lowest arrow indicates an HO-induced 6-kb fragment from YAC u17. The large u17 fragment plus uncut u17 were observed at ~225 to 230 kb. Cross-hybridization of the probe to yeast chromosomes III and I was observed at 360 and 190 kb, respectively. (C) The arrow indicates the HO-induced 6-kb fragment from u17 separated in a 0.7% agarose gel. The upper band (>12 kb) represents sheared u17 DNA and u17 large fragment DNA. (D) Uncut YAC s16 plus repaired s16 (with small deletions) were observed at 360 to 365 kb. Cross-hybridization of the probe to yeast chromosome VI material was observed at 230 kb. For panels A, B, and D the material at approximately 500 to 550 kb corresponds to the region of chromosomal DNA stacking (S band) that exhibits nonspecific cross-hybridization with the probe. Lane L in panels B and D corresponds to a 50-kb DNA ladder that cross-hybridized with the *Alu-URA3*-YZ probe.

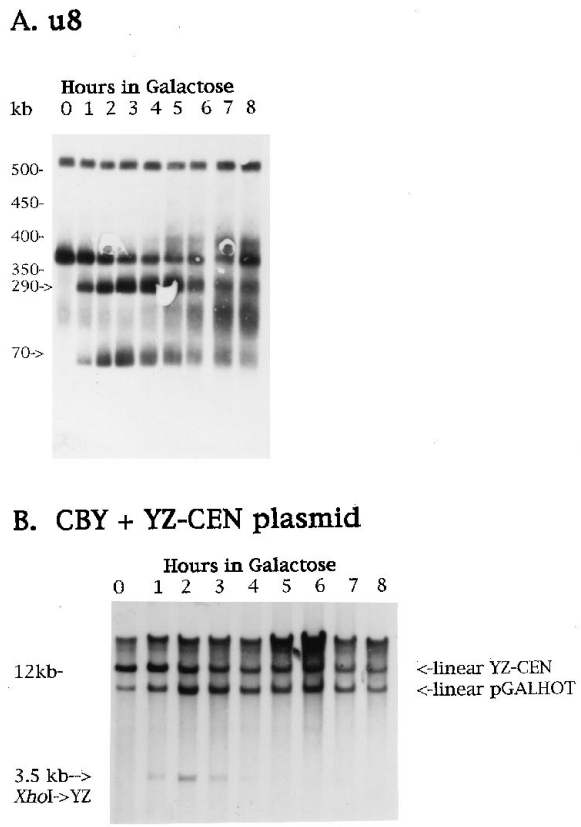

FIG. 4. Persistence of a site-specific DSB in the u8 YAC (A) and the YZ-CEN plasmid (B). The CBY diploid strain containing either the YAC or the plasmid was incubated in Gal + Ura medium. For the strain containing u8, cells were collected and gently lysed in agarose and chromosomes were separated and characterized as described in the legend to Fig. 3. Cutting is demonstrated for the u8 YAC (A) by the appearance of the 295- and 70-kb bands. Cells with the YZ-CEN plasmid (B) were collected at the indicated times and frozen, and total DNA was released by a glass bead extraction procedure (7). The DNA was restricted with *Xho*I (a unique *Xho*I site is positioned 3.5 kb from the YZ junction on YZ-CEN), electrophoresed in a 0.7% agarose gel, and blotted to a nylon membrane. The YZ-CEN plasmid was visualized with fluorescein-labeled YZ-CEN as the probe. A 3.5-kb *Xho*I→YZ fragment (arrow) resulted from site-specific, HO-induced cutting at the YZ junction. The 3.5-kb *Xho*I→YZ fragment was not observed in galactose-induced cells containing the CEN[No YZ] control plasmid (data not shown). The sizes of the DNA fragments (noted at left) were determined by appropriate standards and staining with ethidium bromide.

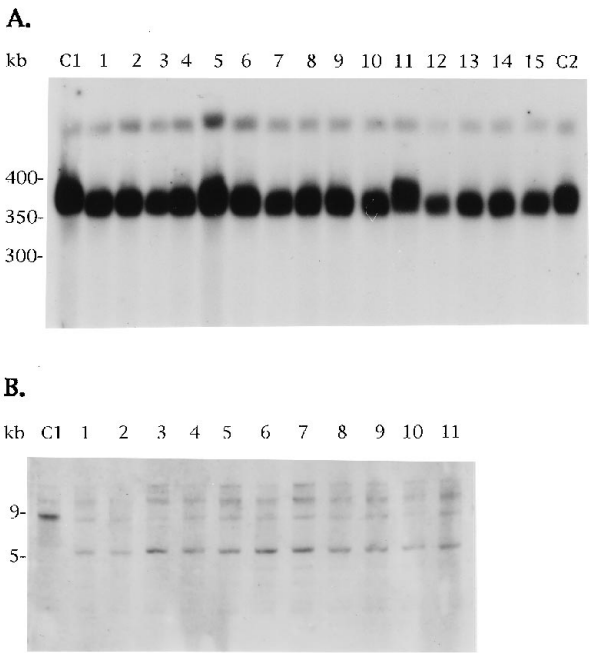

FIG. 5. HO-induced small deletions within the s16 YAC. Cells containing the s16 YAC were plated to Gal + Ura. Colonies that were Ura$^-$ but retained the telomere markers (*HIS3* and *LYS2*) were identified by replica plating. Cells were grown in SC medium plus glucose minus His minus Lys and subsequently prepared for TAFE separation of chromosomes. The sizes of YACs from 15 independent isolates (panel A, lanes 1 to 15) were determined by probing with fluorescein-labeled *Alu URA3*-YZ plasmid. To determine the size of the deleted fragment, genomic DNA was extracted (7) from cells containing either the parental (Ura$^+$) s16 YAC or the deleted (Ura$^-$) YACs, restricted with *Xba*I, and separated on 0.7% agarose gels (panel B, lanes 1 to 11). These isolates correspond to the isolates in panel A. The DNAs were blotted to nylon membranes and probed with fluorescein-labeled *Alu URA3*-YZ plasmid. The *Xba*I sites that flank the integrated *Alu URA3*-YZ plasmid released a 9-kb fragment in the parental (intact) YAC s16. *Xba*I restriction of the HO-induced Ura$^-$ YACs resulted in the appearance of a new 5-kb deletion fragment not evident in the DNAs extracted from cells containing the intact YAC. Controls C1 and C2 correspond to the parental s16 YAC. The sizes of the DNA fragments (noted at left) were determined by appropriate standards and staining with ethidium bromide. The bands of less intensity in panel B correspond to undefined cross-hybridizing material.

(18%) YACs compared with the level of survival for the s15 (58%), s16 (75%), or s23 (79%) YAC-containing strains. (There is a 20 to 40% reduction in plating efficiency in this strain background when cells are plated to galactose- compared with glucose-containing medium, as with previous results [2].) YAC loss was frequent among the u8 (81%) and u17 (98%) colonies arising on Gal + Ura plates. (As noted above, these frequencies are considerably higher than when cells are plated to YEPD at various times after induction in liquid galactose medium.) Much lower frequencies of YAC loss were observed for s15 (12.8%), s16 (1.1%), and s23 (0.25%) colonies. In cells grown on Glu + Ura plates, all YACs were stable: only 4 of 3,661 single colonies had lost their YACs (pooled colonies from s15, s16, s23, u17, and u8). Thus, a persistent DSB may lead to either lethality or loss of a dispensable chromosome in survivors.

Delay between the appearance of a DSB and indirect lethality. Since it was possible to determine genetically the time at which a DSB was induced (measured by loss of *URA3*), we reasoned that it might be feasible to relate this to the time by which the presence of a DSB results in lethality. Presented in Fig. 6A is a pullback strategy designed to determine the time of

TABLE 3. Relative plating efficiencies of cells containing either a YAC or a CEN plasmid with a YZ sequence after induction of HO endonuclease

YAC or plasmid (no. of expts)	Relative plating efficiency on[a]:			
	Glu + Ura	Glu − Ura	Gal + Ura	Gal − Ura
u8 (7)	1.0	1.04	0.19 ± 0.07	8.8×10^{-4}
u17 (6)	1.0	0.96	0.18 ± 0.10	2.3×10^{-4}
s15 (6)	1.0	0.94	0.58 ± 0.13	6.9×10^{-4}
s16 (7)	1.0	0.99	0.75 ± 0.15	$<1.7 \times 10^{-4}$
s23 (3)	1.0	0.93	0.79 ± 0.23	$<4.3 \times 10^{-4}$
CBY with YZ-CEN (3)	1.0	0.96	1.40 ± 0.10	1.4×10^{-3}

[a] Strains were plated to synthetic media lacking tryptophan in order to maintain selection for the HO-inducible plasmid pGALHOT and either containing or lacking uracil. Relative plating efficiencies are normalized to the colony counts obtained on Glu + Ura. For data with Gal + Ura, error measurements are ± 1 standard deviation of the mean.

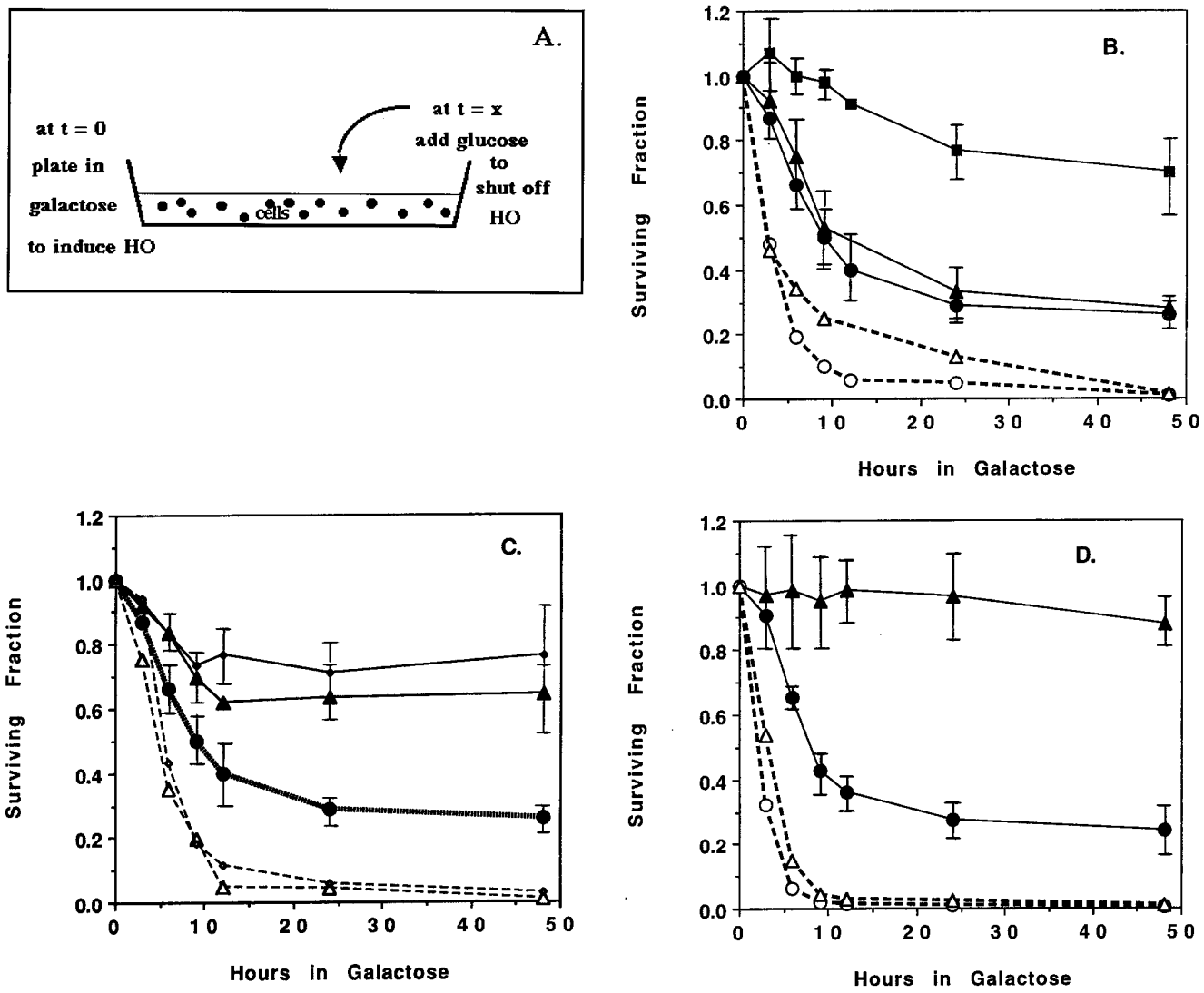

FIG. 6. Commitment to death following induction of a site-specific DSB within a dispensable plasmid or YAC by the described pullback (galactose-to-glucose) procedure. (A) Pullback scheme. At time zero (t = 0), cells are plated within agar that contains galactose with or without Ura to induce the HO endonuclease. At various times (t = x), overlays containing glucose with and without Ura are added to shut off the induction of the HO endonuclease. (B) Cells containing u8 (● and ○), u17 (▲ and △), and controls lacking a YAC YZ target sequence (CBY-CON and CBY plus YAC12) (■) were imbedded in Gal + Ura (closed symbols) or Gal − Ura (open symbols) and overlaid with the corresponding glucose medium with or without Ura. Both the CBY-CON control strain, which lacks a YAC, and the strain CBY-YAC12, which lacks a YZ junction in YAC12, exhibited similarly high levels of survival (70 and 77%, respectively) after 72 h in Gal + Ura. All strains contained pGALHOT. Error bars are ± 1 standard deviation of the mean and are presented for the Glu + Ura results. Each curve represents two to eight experiments. (C) Cells of strains s15 (▲ and △), s16 (◆ and ◇), and u8 (●, data from panel B) were imbedded and overlaid as described above for panel B. All strains contain pGALHOT. Error bars are ± 1 standard deviation of the mean and are presented for the Glu + Ura results. In some cases the error bars are contained within the datum points. Each curve represents two to eight experiments. (D) Cells of strains u8 (● and ○, data from panel B) and CBY with YZ-CEN (▲ and △) were imbedded and overlaid as described above. All strains contained pGALHOT. Error bars are ± 1 standard deviation of the mean. Each curve represents two experiments. Loss of the *URA3* marker within YACs u8, u17, s15, s16, and s23 was determined for colonies arising within Gal + Ura plates (at 12 and 24 h) by picking colonies with a Pasteur pipette and growing them in YEPD in microtiter dishes and then by replica pronging the colonies to Glu − Ura medium. Among the pooled colonies examined, all but one (191 of 192) were Ura$^-$.

commitment to loss of the *URA3* marker versus the loss of cellular survival. Cells are plated within galactose-containing medium which contains uracil (YAC dispensable) or lacks uracil (to determine if a YAC has been cut and has lost the *URA3* marker). At various times after plating, an agar overlay that contains glucose is added (to repress the induction of HO, which is turned over rapidly) with or without uracil. The time at which the lethal effects of a DSB are evident could be assessed by plating to Gal + Ura and overlaying with Glu + Ura. Plating to Gal − Ura followed by overlaying with Glu − Ura provided the opportunity to measure the time at which the *URA3* marker was lost, thereby making the YAC essential for growth.

The levels of survival of strains u8 and u17 decreased as the length of time between plating and the addition of the glucose overlay was increased (Fig. 6B). (Presented are the average survival levels obtained in eight and three experiments, respectively.) There were two components in the kinetics of killing: a rapid decrease in the survival level occurring over the first 3 to 9 h followed by a gradual decrease over the next 15 h. There was little additional lethality over the next 48 h (data not shown). The indirect lethality was delayed several hours rela-

tive to loss of the *URA3* marker. For example, 50% of the cells lost the *URA3* marker by 3 h; however, survival was 90%. It took an additional 6 h to reduce survival to 50%. This suggests that the indirect lethal consequences of a DSB in dispensable DNA may be rescued by growth in glucose soon after the break is made.

Isogenic control strains that either lacked a YAC-YZ target (i.e., the CBY-CON strain, cured of the resident u8 YAC-YZ) or contained a YAC12 without the YZ cassette (CBY-YAC12) did not show enhanced lethality in Gal + Ura (Fig. 6B). Furthermore, for those strains containing YACs in which a DSB was repaired rapidly (s15 and s16), there was no decrease in the level of survival because of induction of HO (Fig. 6C). It appears that DSB-induced lethality depends on both persistence of the break and growth conditions following DSB induction.

We also examined the effects of induced HO endonuclease at the *MAT* locus. Induced cutting and repair was evident, since approximately 82% of u8, u17, s15, or s16 colonies arising on Gal + Ura contained cells that could mate to either *MAT***a** or *MAT*α tester strains. Neither cutting nor loss of chromosome III was detected on TAFE gels (as determined by ethidium bromide staining [data not shown]). Since induction of pGALHOT in a diploid lacking the u8 YAC (Fig. 6B) or containing the YZ-CEN plasmid (Fig. 6D) (discussed below) did not cause lethality, the lethality induced in the strain containing the u8 YAC cannot be explained by simultaneous loss of both chromosomes III in the diploid CBY strain.

Indirect lethality by a DSB requires that the break be persistent. We compared the effects of a DSB in a YAC with those resulting from a DSB in a small plasmid, since we previously reported that a persistent DSB in a plasmid could lead to cell death (2). The effects of the breaks in the plasmid were examined in the CBY strain background, which is different from that of the NR85 strain that was previously used (2). The strain containing the u8 YAC was cured of the YAC and then transformed with plasmid YZ-CEN (2). Surprisingly, a DSB in this dispensable plasmid was not lethal in this strain (Table 3 and Fig. 6D), unlike its effect in the NR85 strain. There was a considerable difference between the persistence of the cut plasmid in the present experiments and that of the previous study. The broken molecule (identified by the 3.5-kb DNA fragment from *Xho*I to the YZ break site) persisted for only 1 or 2 h (Fig. 4B). This contrasts with its persistence in the NR85 strain (see Fig. 3A in reference 2) and the persistence of the broken u8 (Fig. 4A) or u17 (Fig. 3C) YAC. It appears that a DSB must persist in order for it to lead to indirect lethality. The rapid degradation of the plasmid in the CBY genetic background would preclude its having a lethal effect.

Cells with a persistent DSB exhibit G_2 arrest. Since unrepaired DNA damage can block cell progression (6, 32, 36), we examined the consequences of a single DSB in the dispensable YACs on cell division. Long doubling times or extended cell cycle arrest might contribute to the observed indirect lethality.

Individual cells containing the u8, u17, s15, and s16 YACs or YZ-CEN were monitored microscopically after plating to Gal + Ura. Of the cells containing the u8 YAC that were in G_1 (i.e., unbudded cells) at the time of plating, 70% were arrested in G_2 (i.e., large budded cells) 12 h later (Fig. 7A). However, 16% of the initial G_1 cells did not arrest and divided to form groups of three or more cells at 12 h (Fig. 7A); they eventually progressed to viable macrocolonies (directly visible on plates) by 72 h. Among the cells that arrested at G_2, 92% progressed to microcolonies (approximately 50 cells or less) by 72 h but did not exhibit further growth. These microcolonies contained cells with elongated shapes similar to those described previously (reference 2 and unpublished data).

The u8 cells that were budded at the time of plating also exhibited arrest; however, the arrest was not seen until the subsequent cell cycle. Presumably, these budded cells could repair single DSBs by sister chromatid recombination, and breaks appearing in the next G_1 could then lead to arrest. Among the cells initially plated in G_2, 48% accumulated as groups of three or four cells at 12 h after plating to Gal + Ura and 33% exhibited additional divisions which eventually gave rise to macrocolonies (Fig. 7B). Similar results were obtained with the strain containing the u17 YAC (Fig. 7).

As shown in Fig. 7, the G_2 delay observed with u8 and u17 was not seen with strains carrying s15 (or s16 YAC [data not shown]) or the YZ-CEN plasmid. Therefore, the persistent DSBs were interpreted differently from the nonpersistent DSBs. Thus, the persistence and/or the context of the DSB may be important in determining G_2 arrest and the likelihood of indirect lethality.

DISCUSSION

Because of limited genetic and molecular accessibility, there have been few opportunities to study the consequences of specific DNA lesions in human DNA. Using genetic and physical approaches, we have examined the effects of a site-specific DSB at different positions in human DNA in order to understand the potential for DSBs to induce chromosomal changes and to address the consequences of an unrepaired DSB in a large, genetically dispensable chromosome.

A DSB in most YACs was rapidly repaired through recombination with repeat sequences immediately surrounding the break, resulting in small deletions. Lack of repair usually resulted in the complete loss of the YAC among the survivors, but large internal and terminal deletions were also observed (>90% for u8 [unpublished data]). These results complement our previous studies in which we showed that random, radiation-induced lesions in diverged yeast chromosomes or in human YACs could result in loss of these molecules (23, 24). More importantly, an unrepaired DSB could delay cell progression and lead to cell death.

Indirect lethality resulting from an unrepaired DSB. The consequences of a DSB in the YACs or the YZ-CEN plasmids are diagrammed in Fig. 8. For two YACs (u8 and u17), the break usually prevented cells from producing viable colonies. Previously we had demonstrated that a single DSB in a dispensable plasmid could cause cell death (2). With these dispensable YACs, it was, therefore, possible to investigate factors that contribute to DSB-induced indirect lethality when the break is present in a chromosome-like molecule.

There were common consequences of the persistent DSBs in the u8 and u17 YACs. Presumably, the persistence of these breaks generated a long-lived signal(s) that was detected by the cell, resulting in a long G_2 arrest (Fig. 8). Many of these cells would subsequently divide slowly, leading to microcolonies that grew no further. For example, 92% of the u8 cells that were arrested in G_2 (at 12 h after plating to galactose) eventually progressed and arrested as microcolonies by 72 h (unpublished data). Among the survivors, most had lost their YAC. Surprisingly, the time at which cells began to lose viability in pullback experiments was much later than the time at which cutting was detected (by physical and genetic means) for both YACs. While the HO endonuclease is shut off shortly after glucose addition, the DSBs accumulated up to that point would be expected to persist. We, therefore, propose that there is a time-dependent component to the indirect lethality caused

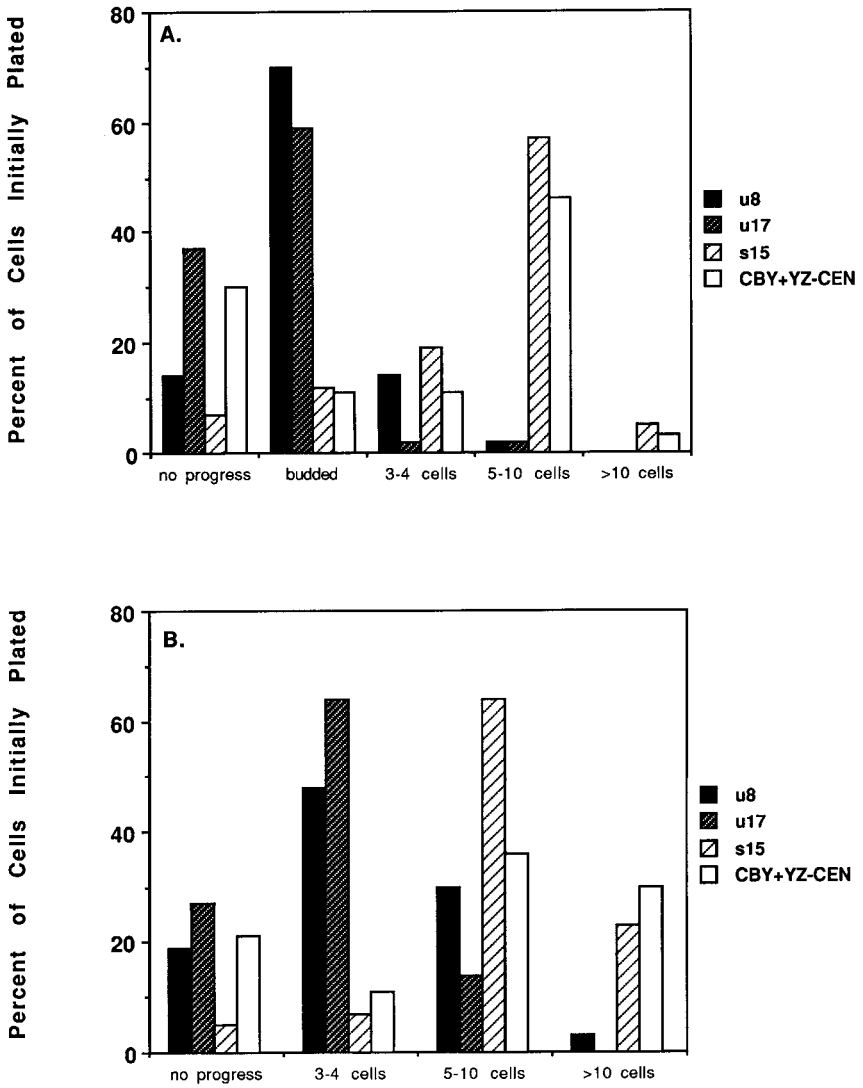

FIG. 7. Effect of a DSB on cell progression. Cells were grown as described in Materials and Methods and plated to Gal + Ura with a multipin pronging device. Cell progression of more than 110 individual cells (for both the G_1 and S plus G_2 categories) was monitored by capturing images of individual cells after 12 h of growth at 30°C with a Singer MSM dissecting microscope. At the time of plating, the strains had similar cell distributions; approximately 37% were G_1 cells (unbudded), 36% were S cells (small buds), and 27% were G_2 cells (containing a large bud). Presented are the results with strains containing YACs in which a DSB in a YAC is persistent and leads to cell death (u8 and u17), is repaired rapidly (s15), or contains the YZ-CEN plasmid. (A) Progression of cells that were G_1 at the time of plating. Cells either remained as initially plated (no progress), progressed to budded cells, or underwent divisions leading to microcolonies (3 to 4, 5 to 10, or >10 cells). (B) Progression of budded (S plus G_2) cells. Cells either remained as initially plated (no progress) or progressed to microcolonies (3 to 4, 5 to 10, or >10 cells).

by a persistent DSB. When cells were transferred from glucose to galactose, there was a delay in cell growth and also a reduced rate of growth. Thus, in addition to a checkpoint delay, there was a delay because of changing carbon sources. The combination of these two growth delays may have been important to the cellular changes that eventually led to cell death.

A DSB in the YZ-CEN plasmid was previously shown to cause indirect lethality in strain NR85. The broken plasmid was long-lived, and there was extensive growth delay on galactose. In the CBY strain utilized in this study, there was no cell cycle delay in response to a DSB in the YZ-CEN plasmid (Fig. 7) and there was no lethal effect (Fig. 6D). Contrary to results with the NR85 strain, the plasmid was rapidly degraded in the CBY strain (Fig. 8). As a result, there would be little opportunity for the break to be detected and generate a signal, resulting in cell cycle delay. Thus, studies addressing the consequences of a plasmid-based DSB need to take into account the stability of the plasmid DNA following induction of the break. Because YACs are much larger, a broken (albeit partially degraded) molecule may persist much longer.

Recently, experiments similar to ours in which site-specific DSBs were induced near telomeres in dispensable yeast chromosomes (27) and in a lambda DNA-containing YAC in the strain background LS20 (31) were described. These elegant experiments clearly showed that a DSB led to loss of a telomere and usually resulted in chromosome loss. The nonrepairable DSB resulted in a transient *RAD9*-dependent delay in cell progression with about 20 to 30% of cells delayed in the two- to four-cell stage 10 h after plating to galactose. On the basis of pedigree analysis, the broken chromosome did not result in indirect lethality (27, 39), although it could persist for up to 10 generations (27). These results suggest that a persistent DSB

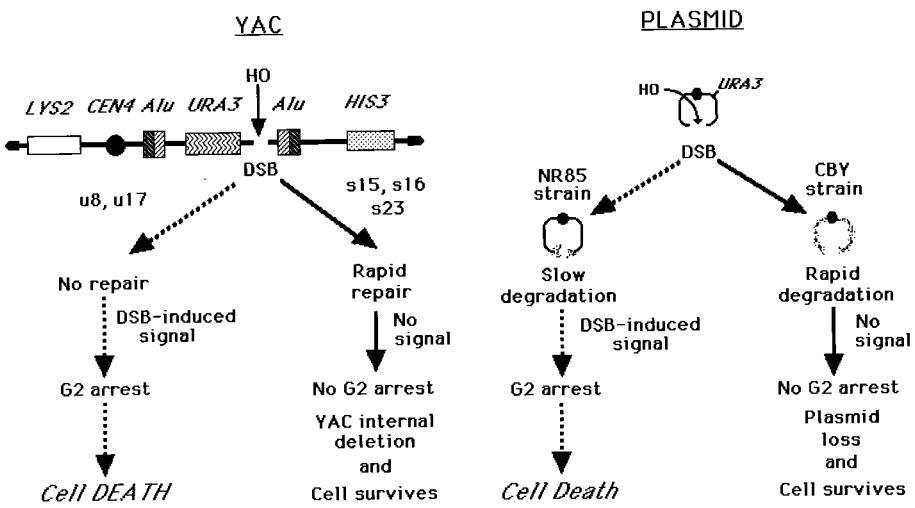

FIG. 8. Possible consequences of an HO-induced, site-specific DSB within a YAC that contains human DNA or within a plasmid (YZ-CEN). DSBs that are persistent, either because of lack of repair (i.e., YACs u8 and u17 in the CBY strain) or because of lack of rapid plasmid degradation (YZ-CEN in the NR85 strain), signal the cell to undergo G_2 arrest. Eventually, many of the cells divide a few generations and then die. DSBs that are rapidly repaired, as with the s15, s16, and s23 YACs, or that lead to the rapid degradation of the broken plasmid (YZ-CEN in the CBY strain) do not signal arrest and do not lead to lethality.

per se is not sufficient to induce indirect lethality. Recent results with the same lambda DNA-containing VS8 YAC (31) transformed into strain NR85 showed that an HO-induced DSB in this YAC was lethal (2a). A DSB in this YAC in the original LS20 strain did not cause lethality. The high survival level that we observed with LS20 was correlated with delayed induction of the DSB in the YAC, as determined genetically under our pullback conditions (unpublished data). Thus, genetic background and delay in the appearance of the break may be important factors for DSB-induced indirect lethality.

The nature of the signal(s) and the cellular targets responsible for indirect lethality are unknown. At this point, the components appear to involve at least a long-lived DSB and extended delay in cell growth. It is likely that *RAD9* and other genes (such as *RAD17*, *RAD24*, *DUN2*, and *MEC3*) (5, 8, 10, 15, 30, 37) involved in checkpoint controls mediate the DSB effect, since the checkpoint genes would increase the time over which a DSB has the opportunity to cause indirect lethality. Several cellular targets that include DNA repair systems, the segregation apparatus, or even some as-yet-unidentified mechanism of apoptosis could be involved. The dependency of both radiation-induced cell cycle arrest and apoptotic lethality on p53 in mammalian cells (9, 12) bears a striking similarity to the indirect lethality described in this work. Possibly, extended expression of certain kinases might lead to some permanent change in cell development or the segregational apparatus. Processing of the DSB lesion may also contribute to indirect lethality. Recently, Ku proteins that bind specifically to the ends of broken DNAs have been isolated from humans and *Drosophila melanogaster* (16, 21). Similar proteins in yeast cells have been identified (33) and may determine the relative persistence of a DSB by protecting the ends from extensive nucleolytic attack or the likelihood that a DSB will lead to subsequent cellular changes.

Deletions and loss of YACs. DSBs in mammalian cells frequently lead to chromosome aberrations and rearrangements. Our system may provide insight into how such changes may occur in human DNAs. Regardless of the alteration, the induction of the site-specific DSB resulted in loss of the *URA3* marker immediately adjacent to YZ in nearly all of the cells plated to Gal − Ura medium (>99%) (Table 3), indicating that for the 21 YACs examined, simple ligation or end-joining is an infrequent repair event. Since HO is rapidly shut off after switching carbon sources (Gal to Glu), uncut or precisely religated YACs would result in the production of Ura^+ colonies within Gal + Ura plates after glucose was added. However, the majority of colonies rescued in pullback experiments were Ura^-. Therefore, it is unlikely that YACs undergo repetitive cycles of YZ cleavage followed by precise ligation.

There were four categories of genetically and physically detectable changes associated with loss of the *URA3* marker: small internal deletions, loss of the entire YAC, and large internal and terminal deletions (unpublished data). We suggest that the outcome is related to the nature and position of the integrated *Alu*-targeted plasmid. The BLUR13 *Alu* used for targeting has considerably diverged from the consensus sequence for human *Alu*s (4). In addition, the *Alu*s within YAC12 are clustered, with the centromere-distal region being *Alu* rich (Fig. 1). Thus, if the site of integration is an *Alu* with a high level of homology to BLUR13, the induction of a DSB between the *Alu*s (which are separated by the 4-kb integrated vector) will likely lead to efficient recombination between them. In addition, if the region has many *Alu*s, there will be additional opportunities for recombination. Alternatively, since integration during transformation can efficiently occur between diverged transforming DNA and established chromosomes (13), mosaic *Alu*s may be created surrounding a YZ junction and these may be less likely to undergo recombination in the mitotic cell, which might account for the results with the u8 YAC.

For most YACs, repair of the DSB was rapid and had no effect on cell cycle arrest or lethality (Fig. 8). Since these DSBs were not long-lived, presumably they failed to generate the signal(s) required for cell cycle arrest and lethality. The rapid repair of these breaks is likely to occur by single-strand annealing between the directly repeated *Alu*s. This nonconservative recombination process is proposed to involve 5′-to-3′ exonuclease digestion, leaving long single-strand regions on both sides of the break (34). The regions of homology flanking the DSB could anneal, and the nonhomologous sequences would be removed. This efficient mechanism of repair can occur over long distances with relatively short regions of homology. The

role of the *RAD52* gene (which is required for many types of conservative recombination) in this process is not clear (17, 20, 25, 26). In our experiments there appears to be some dependency on the *RAD52* gene product, since in the *rad52* mutant, unlike in the Rad^+ strain, breaks were detected in the s15 YAC and there was enhanced YAC loss. However, almost all deletions in both the Rad^+ and the *rad52* strains were small (unpublished data).

Implications. A DSB in dispensable human DNA within yeast cells can have several outcomes: cell cycle delay, recombination, deletion, YAC loss, and decreased viability. The YAC system that we developed will be useful for identifying the mechanisms by which DNA lesions can have an indirect effect on cell viability as well as for characterizing the genetic controls. The information gained with the human YACs may be useful for understanding how DNA lesions lead to biological consequences in mammalian cells.

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