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 Alus. For two YACs, the DSBs were not repaired and there was a G2 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 pulldown 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 un repaired 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 G2/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 G1, S (1, 32), and G2 (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 G2 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 G2 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 G1 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 G1 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 result in lethality (6). Recently we described a system in which
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 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 G2, 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 G2 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 HindIII fragment into a pUC-based vector (pJS113) that has a 45-bp MATa YZ junction cloned between the SalI and BamHI sites (2). Plasmid pPB7 (19), which contains an Alu with a centrally positioned unique Msrl site, was utilized to construct an Alu-URA3-YZ-integrating plasmid. The plasmid was constructed by inserting the 300-bp BamHI human Alu fragment from pPB7 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 Msrl-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 Alus 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 haploid. Galactose induction of HO did not produce any enhanced lethality in the control MATa/MATa diploids (see Fig. 6D) 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 (Bethyl Research Laboratories), which were chromosomal DNAs were transferred to nylon membranes and probed with the fluorescein-labeled (Renissance 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 XhoI-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.
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 integrated YZ junction were then determined by replica plating onto SC medium or medium 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 × 10⁷ to 3 × 10⁷ 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 Glu + 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 ml 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 MluI or SacII 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 × 10⁶ 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 LSM dissection microscope (Singer Instrument Co. Ltd., Watchet, England). Cells were categorized as single (G₀), small budded (S), and large budded (G₂) (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-
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 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 MATa Rad1 haploid strain (YPH604). Restriction with MscI linearizes this vector by cutting within the Alu sequence, allowing plasmid integration at any of approximately 30 Alus in this YAC. Each of 23 independent and mitotically stable Ura+ transformants was mated with a MATa 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 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 lost (s15, s16, and s23 are examples of stable YACs and are extensively described below).

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

<table>
<thead>
<tr>
<th>YAC</th>
<th>Time in Gal (h)</th>
<th>No. of colonies</th>
<th>% Loss of URA3 and associated genetic markers after replication on media lacking supplement(s)</th>
<th>% HO plasmid loss</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Total Ura</td>
<td>Ura</td>
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<tr>
<td>u8</td>
<td>0</td>
<td>388</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>4</td>
<td>392</td>
<td>19.1</td>
<td>7.2</td>
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<td>8</td>
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<td>34.8</td>
<td>7.3</td>
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<td></td>
<td>24</td>
<td>408</td>
<td>20.3</td>
<td>6.9</td>
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<td>0</td>
<td>396</td>
<td>0</td>
<td>0</td>
</tr>
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<td>221</td>
<td>10.4</td>
<td>6.3</td>
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<td>33.5</td>
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<td></td>
<td>24</td>
<td>486</td>
<td>26.1</td>
<td>8.8</td>
</tr>
<tr>
<td>s16</td>
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<tr>
<td></td>
<td>12</td>
<td>362</td>
<td>23.5</td>
<td>21.6</td>
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<td></td>
<td>24</td>
<td>444</td>
<td>14.1</td>
<td>13.6</td>
</tr>
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</table>

* 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.

**Table footnote**: 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.

**Table footnote**: Clones that lost the HO plasmid pGALHOT did not exhibit URA3 loss.

**Table footnote**: The U17 YAC lacked the HIS3 telomere marker.

(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 loss (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 MluI restriction fragments (Fig. 1). Total DNA was restricted with MluI, 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 MluI (Fig. 2) or SacII, and the fragments were separated by TAFE and probed with an Alu-specific probe. The YACs u8, s15, s16, and s23 had identical MluI restriction patterns. For the u17 YAC, there was a simple deletion resulting in loss of the 72-kb MluI 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
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 XhoI (the YZ cassette vector lacks a XhoI 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 XhoI fragment contained the entire 4-kb cassette. It appears that the HO-induced DSB caused recombination between the surrounding Alus, 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, C, 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.
(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**

<table>
<thead>
<tr>
<th>YAC or plasmid (no. of expts)</th>
<th>Relative plating efficiency on<strong>a</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glu + Ura</td>
</tr>
<tr>
<td>u8 (7)</td>
<td>1.0</td>
</tr>
<tr>
<td>u17 (6)</td>
<td>1.0</td>
</tr>
<tr>
<td>s15 (6)</td>
<td>1.0</td>
</tr>
<tr>
<td>s16 (7)</td>
<td>1.0</td>
</tr>
<tr>
<td>s23 (3)</td>
<td>1.0</td>
</tr>
<tr>
<td>CBY with YZ-CEN (3)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**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. 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 XhoI (a unique XhoI 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 XhoI restriction of the HO-induced Ura marker 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<sup>-</sup> 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 *Ab* URA3-YZ plasmid. To determine the size of the deleted fragment, genomic DNA was extracted (7) from cells containing either the parental (Ura<sup>+</sup>) s16 YAC or the deleted (Ura<sup>-</sup>) YACs, restricted with XhoI, 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 *Ab* URA3-YZ plasmid. The XhoI sites that flank the integrated *Ab* URA3-YZ plasmid released a 9-kb fragment in the parental (intact) YAC s16. XhoI restriction of the HO-induced Ura<sup>-</sup> 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.
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 with or without Ura is added to shut off the induction of the HO endonuclease. The level 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-
Among the cells that arrested at G2, 92% progressed to viable macrocolonies (directly visible on plates) of groups of three or more cells at 12 h (Fig. 7A); they eventually arrested in G2 (i.e., large budded cells) 12 h later (Fig. 7A). However, (i.e., unbudded cells) at the time of plating, 70% were arrested from the G2 arrest. The broken molecule (identified by the 3.5-kb DNA fragment mid in the present experiments and that of the previous study) was 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) did not cause a DSB-induced lethality, the lethality induced in this strain containing the u8 YAC cannot be explained by simultaneous loss of both chromosomes III in the diploid CBY strain.

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 M4 Ta or M4 Ta tester strains. Neither cutting nor loss of chromosome III was detected on TAFE gels (as determined by XhoI to the YZ break site) persisted for only 1 or 2 h (Fig. 4B). This contrast 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. 5C) 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 G2 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 G1 (i.e., unbudded cells) at the time of plating, 70% were arrested in G2 (i.e., large budded cells) 12 h later (Fig. 7A). However, 16% of the initial G1 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 G2, 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 G1 could then lead to arrest. Among the cells initially plated in G2, 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 G2 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 G2 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 unrepaird 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 G2 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 G2 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 by G2 arrest.
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

![Graph](http://example.com/graph.png)

**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 G1 and S plus G2 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 G1 cells (unbudded), 36% were S cells (small buds), and 27% were G2 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), or contains the YZ-CEN plasmid. (A) Progression of cells that were G1 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 G2) cells. Cells either remained as initially plated (no progress) or progressed to microcolonies (3 to 4, 5 to 10, or >10 cells).
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-undefined 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 reigated 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 Alus (4). In addition, the Alus 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 Alus (which are separated by the 4-kb integrated vector) will likely lead to efficient recombination between them. In addition, if the region has many Alus, there will be additional opportunities for recombination. Alternatively, since integration during transformation can efficiently occur between diverged transforming DNA and established chromosomes (13), mosaic Alus 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 Alus. 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 RADS52 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 RADS52 gene product, since in the rad52 mutant, unlike in the Rad2 strain, breaks were detected in the s15 YAC and there was enhanced YAC loss. However, almost all deletions in both the Rad2 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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