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# Identification of Autonomously Replicating Circular Subtelomeric Y′ Elements in *Saccharomyces cerevisiae*

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**We marked a large number of yeast telomeres within their Y′ regions by transforming strains with a fragment of Y′ DNA into which the *URA3* gene had been inserted. A few of the Ura$^+$ transformants obtained were very unstable and were found to contain autonomously replicating *URA3*-marked circular Y′ elements in high copy number. These marked extrachromosomal circles were capable of reintegrating into the chromosome at other telomeric locations. In contrast, most of the Ura$^+$ transformants obtained were quite stable mitotically and were marked at bona fide chromosomal ends. These stable transformants gave rise to mitotically unstable *URA3*-marked circular Y′ elements at a low frequency (up to 2.5%). The likelihood that such excisions and integrations represent a natural process in *Saccharomyces cerevisiae* is supported by our identification of putative Y′ circles in untransformed strains. The transfer of Y′ information among telomeres via a circular intermediate may be important for homogenizing the sequences at the ends of yeast chromosomes and for generating the frequent telomeric rearrangements that have been observed in *S. cerevisiae*.**

The ends of eucaryotic chromosomes, called telomeres, are specialized structures required for the proper replication and stable maintenance of the chromosome. Broken chromosomes are highly recombinogenic and are subject to a variety of biologically unfavorable reactions including fusion, translocation degradation, and loss (11, 17–19, 24, 26). Recent work in a number of laboratories has been directed at the elucidation of the structural and functional properties of telomeres that distinguish them from broken ends. It has been found that certain general structural features may be shared by telomeres in all eucaryotes (see reference 2 for a review), although specific details vary among different organisms.

A diagram depicting the general structure of telomeres in the yeast *Saccharomyces cerevisiae* is presented in Fig. 1. The extreme end of the yeast telomere (T in Fig. 1) is composed of short repeats of the simple sequence 5′[$C_{1\text{-}3}$A]3′ (29). Located centromere-proximal to the T region in some yeast telomeres is a 6.7-kilobase (kb) *ARS*-containing sequence, Y′ (formerly 131-Y), which may be present in up to 4 tandem copies (3, 4). The Y′ repeats appear to be highly conserved, as judged by comparison of restriction enzyme maps from different Y′ clones. However, a variant of the Y′ repeat, Y′(131S), has also been identified (3); it appears to be related to Y′ by a simple deletion of approximately 1.5 kb of contiguous sequence (Fig. 1). The function of the Y′ and Y′(131S) repeats is unknown. It is unlikely that they are required for basic telomeric function, because telomeres lacking Y′ repeats have been identified in *S. cerevisiae* (13, 29, 33). Furthermore, linear plasmids bearing only the simple $C_{1\text{-}3}$A repeats at their ends are maintained in *S. cerevisiae* (32). Nevertheless, the presence of the Y′ repeats at some telomeres may confer other, more subtle influences on chromosome maintenance and replication. Located centromere-proximal to the Y′ repeats is another repetitive element, X. This element, which is much less conserved than the Y′ repeat, also contains an *ARS* (3, 4). Walmsley et al. (33) showed that more than 100 base pairs of the simple $C_{1\text{-}3}$A repeat found at the extreme end of the telomere is also present at the junction between X and the most centromere-proximal Y′ repeat. Furthermore, there is evidence for the presence of approximately 50 base pairs of $C_{1\text{-}3}$A sequence between Y′ repeats (C. Chan and B.-K. Tye, personal communication).

In a group of studies designed to examine the mitotic and meiotic behavior of yeast telomeres, we found that regions near yeast telomeres frequently undergo rearrangement (13). By using a portion of the Y′ region as a probe in Southern blots, we found examples of rearrangements, meiotic gene conversions, and mitotic recombination events involving restriction fragments bearing Y′-homologous sequences.

We were interested in examining the recombination events that occur between chromosomal ends in a simplified system, one in which we could follow the fate of a single chromosomal end through mitosis and meiosis. To do this, we used gene-replacement techniques (28) to mark Y′ repeats with the yeast *URA3* gene. The transfer of *URA3* sequences from the originally marked Y′ segment to other locations could then be monitored by Southern analysis and standard genetic techniques. In the course of this work, we obtained direct evidence for a new mode of telomere-telomere interaction: the dispersal and potential amplification of Y′ repeats mediated through the excision and reintegration of circular Y′ elements. In this paper, we examine the evidence for and implications of a mobile Y′ element model for telomeric sequence dispersal.

## MATERIALS AND METHODS

**Enzymes and chemicals.** Restriction enzymes, *Bgl*II DNA linkers, polynucleotide kinase, DNA polymerase I, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England BioLabs, Inc., and were used as recommended by the manufacturer. [$\alpha$-$^{32}$P]dTTP and [$\gamma$-$^{32}$P]ATP were obtained from ICN Pharmaceuticals, Inc. Nitrocellulose filter paper was from Millipore Corp. The specific M13 primer d(GTAAAACGACGGCCAGT) was purchased from Collaborative Research, Inc.

**Strains and plasmids.** Yeast strains H1 (*MAT*a *MAL2*

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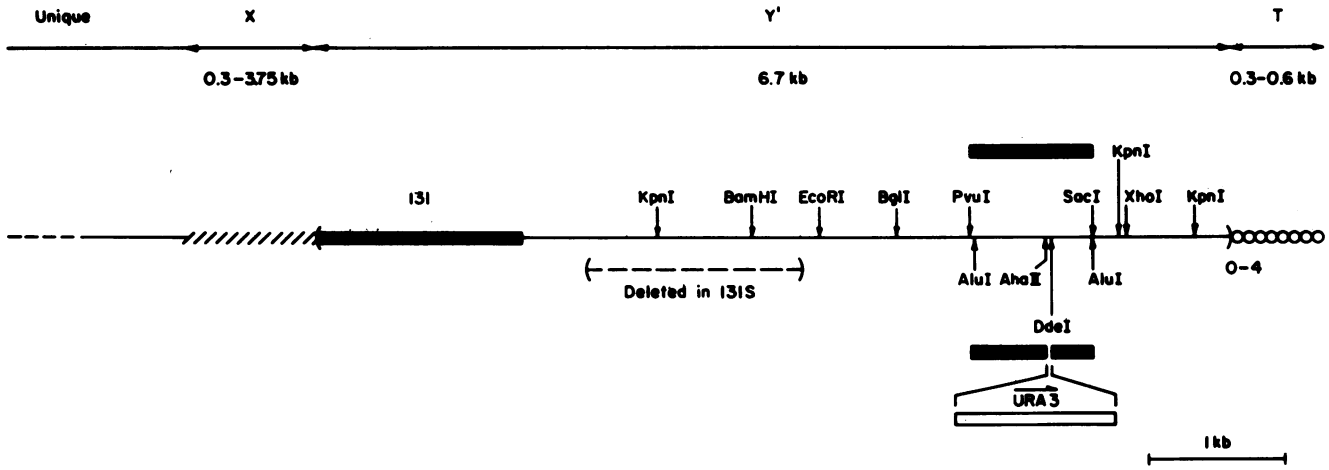


FIG. 1. Structure of the yeast telomere. The general structure presented was determined by Chan and Tye (3); the centromere is to the left. The extreme end of the telomere, T, is composed of a simple irregular repeat, $C_{1-3}A$ (29) and is heterogeneous in length. Centromere-proximal to T is the highly conserved Y' sequence (6.7 kb long) which may be present in 0 to 4 copies; it possesses an associated repetitive element, 131. Centromere-proximal to Y' is the less highly conserved sequence, X, and adjacent to that is unique chromosomal DNA. Short blocks of $C_{1-3}A$ repeats are also present at the X-Y' junction (33) and between Y' repeats (C. Chan and B.-K. Tye, personal communication). The variant of Y' denoted Y'(131S) possesses a deletion of approximately 1.5 kb, the position of which is indicated by a broken line beneath the map. Restriction enzyme sites in Y' indicated above the map are complete for those enzymes and are pertinent to the Southern analyses described in the text; those below the map are complete only for the region between the *Pvu*I and *Sac*I sites and are relevant to the construction of the plasmid described in Fig. 2a. The solid bar above the map indicates the region of DNA used as Y' probe. The interrupted solid bar beneath the map indicates the position and extent of Y' DNA in the plasmid used for the transplacement. The position and orientation of the *URA3* gene insert (open bar) is indicated.

*ura3-52 ade1-100 thr4*) and HH20 (*MATα MAL2 ura3-52 leu2-113,3 ade1-100 THR4*)/*MAT*a *MAL2 ura3-52 LEU2 ade1-100 thr4*), originally SS188-5B and SS238, respectively, were kindly provided by Sue Stewart. The plasmid pSZ220 was a gift from J. Szostak.

**Construction of mp9/HH.TU-8.** The series of cloning steps involved in the construction of plasmid mp9/HH.TU-8 are shown in Fig. 2a. (1) Plasmid pSZ220 (32) and the M13 cloning vector, mp9 (21), were digested with *Pst*I, ligated together, and used to transform *Escherichia coli* JM103 (20). Clear plaques were analyzed by restriction mapping, and clone mp9/HH.TAS1 was selected for further use. (2) Plasmid mp9/HH.TAS1 was digested with *Aha*II (which cuts at one site each in vector and Y' sequences). The *Aha*II ends were rendered blunt by 3'-end filling with the Klenow fragment of DNA polymerase, and then the DNA was digested with *Pst*I (producing four fragments, each with one *Pst*I and one blunt end). This DNA was ligated to the mp9 vector that had been cut with *Pst*I and *Hin*cII in the polylinker and used to transform strain JM103. Clear plaques were analyzed (by restriction mapping), and the desired clone, mp9/HH.*Aha*II-5, was identified. (3) The *Alu*I fragment extending from nucleotide 22 to 830 of the Y' sequence reported by Horowitz and Haber (12) (see Fig. 1 for the position of these two sites in Y' DNA) was isolated from mp9/HH.TAS1. This fragment was digested with *Dde*I (which cuts the fragment in two; Fig. 1), 3'-end filled, ligated to mp9/HH.*Aha*II-5 that had been digested with *Sma*I, and used to transform JM103. Plaques were screened for the presence of the desired insert fragment in the proper orientation by nonradioactive hybridization of phage DNA to a tester clone mp9/HH.*Hin*fI 2-4 (12). Positive candidates were analyzed further by restriction analysis, and the clone mp9/HH.TASR-12 was selected for further use. (4) Plasmid mp9/HH.*URA3*-5 containing the yeast *URA3* gene *Hin*dIII fragment cloned into the *Hin*dIII site of the mp9 vector was digested with *Hin*dIII, subjected to 3'-end filling with the Klenow fragment, ligated to radioactively labeled *Bgl*II DNA linkers, and digested with *Bgl*II. The radioactive *URA3* fragment (with *Bgl*II ends) was then isolated from an acrylamide gel and ligated to mp9/HH.TASR-12 DNA digested with *Bam*HI. The ligation mix was digested with *Bam*HI (to linearize all plasmids lacking the *URA3* insert) and then used to transform JM103. Clones obtained were characterized by restriction analysis; mp9/HH.TU-8 was chosen for use in the transplacement reactions.

**Hybridizations.** DNA was transferred from agarose gels to nitrocellulose filters by the method of Southern (31). Hybridization was performed at 42°C in 50% formamide–5× SSC (0.75 M NaCl, 0.075 M sodium citrate [pH 7])–1× Denhardt solution (7)–20 mM $NaPO_4$ (pH 6.5)–10% dextran sulfate–100 μg of calf thymus DNA per ml–nick-translated probe.

**Other procedures.** Cells were grown in YEPD (yeast extract, peptone, dextrose) medium (30) or in synthetic complete medium lacking uracil (30) at 30°C. Preparation of yeast DNA was done as previously described (30). Yeast transformation was performed on cells treated with 0.1 M lithium acetate (14). *E. coli* JM103 was transformed as previously described (16). Cells were plated onto L broth plates in 0.5% top agar. Recombinant phage were analyzed by extension of the M13 primer: single-stranded DNA hybrid with radioactive nucleotides (by using the Klenow fragment of DNA polymerase I), followed by restriction analysis.

## RESULTS

**Marking yeast telomeres with *URA3*.** Figure 2a illustrates the strategy for constructing the plasmid mp9/HH.TU-8 (see above for details) that was used to transplace the *URA3* gene into yeast telomeric Y' regions. This plasmid contains a 1.2-kb *Hin*dIII fragment of the yeast *URA3* gene flanked on the left and right by 550 and 230 base pairs of the Y' sequence, respectively. The construction places the *URA3* gene immediately adjacent (telomere-proximal) to the cluster

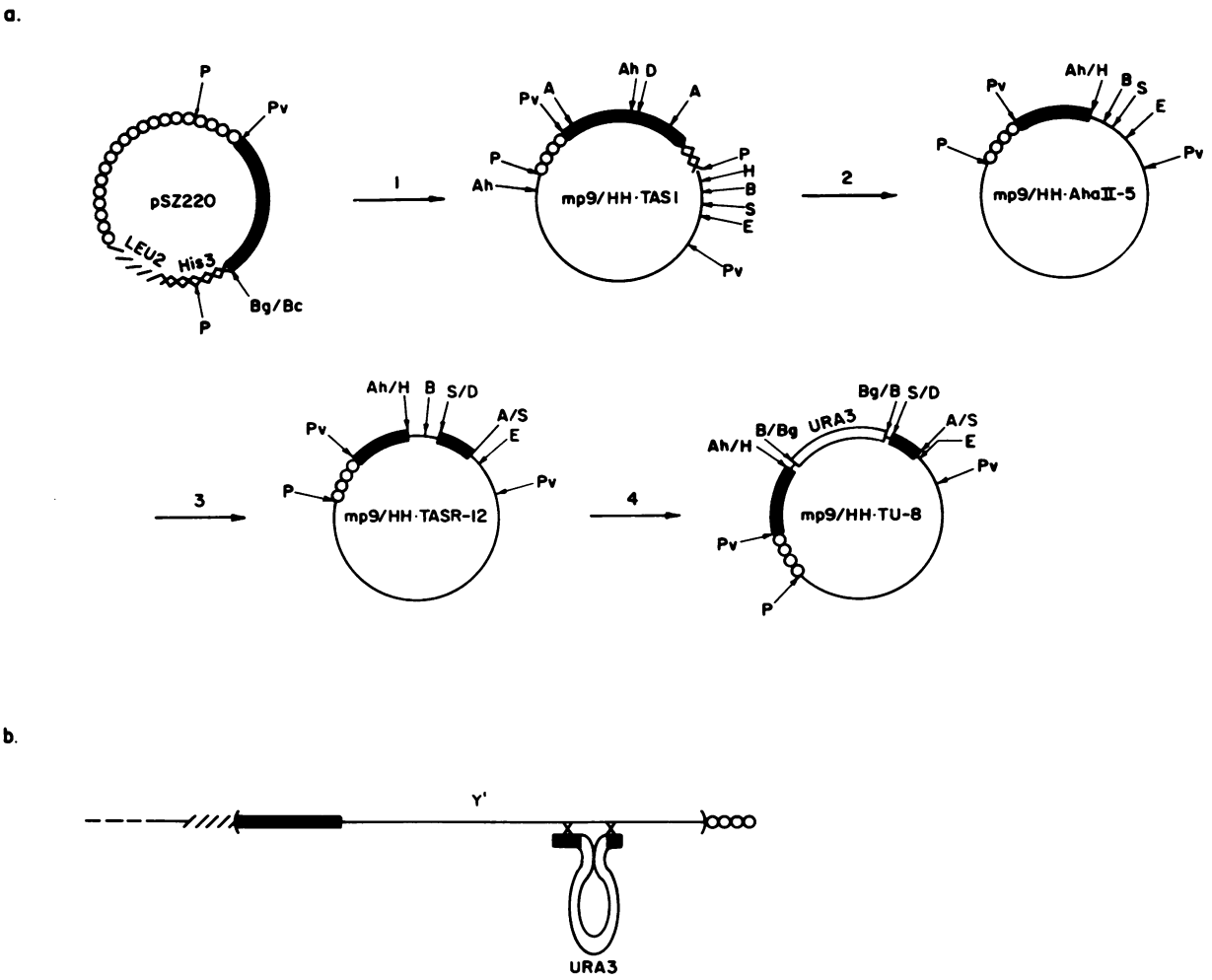


FIG. 2. Transplacement of *URA3* sequences into a telomere. (a) Construction of the plasmid mp9/HH.TU-8. The series of cloning steps are described in the text. Filled thick lines, Y' DNA; open thick lines, *URA3* DNA; thin line, mp9 vector DNA; open circles, pBR322 DNA. Restriction enzyme abbreviations: P (*Pst*I), Pv (*Pvu*I), Ah (*Aha*II), Bg (*Bgl*II), Bc (*Bcl*I), B (*Bam*HI), H (*Hin*cII), S (*Sma*I), E (*Eco*RI), D (*Dde*I), A (*Alu*I). (b) Transplacement of *URA3* sequences into a Y' repeat. Plasmid mp9/HH.TU-8 was digested with the enzymes *Pvu*I and *Eco*RI and used to transform *ura3-52* yeast strains (see the text). A simplified view of the integration of *URA3* sequences into the single Y' element of one telomere is shown. Symbols are as defined in the legend to Fig. 1.

of 12 tandem 36-base-pair repeats identified by Horowitz and Haber (12) and results in the deletion of 20 base pairs of the Y' sequence. The orientation of the *URA3* fragment is such that transcription of the gene proceeds toward the extreme end of the telomere (Fig. 1 and 2a).

Plasmid mp9/HH.TU-8 was digested with the restriction enzymes *Pvu*I and *Eco*RI, yielding two fragments containing mp9 vector sequences and one fragment containing the *URA3* gene flanked by Y' sequences. The digested DNA was used to transform two *ura3* strains, the haploid H1 and the diploid HH20, to uracil prototrophy (28). Both of these strains harbor a Ty1 transposable element insertion in the *URA3* gene on chromosome V (*ura3-52*) and are unable to grow in the absence of uracil (27). Transformants were selected on media lacking uracil. A diagram depicting the transplacement event is shown in Fig. 2b. Ten haploid and 75 diploid Ura$^+$ transformant colonies were obtained.

To characterize the chromosomal environment of the inserted *URA3* gene, DNA was prepared from a number of the transformants and subjected to analysis by restriction enzyme digestion and Southern hybridization. Comparison with published restriction maps of Y' regions (3) along with the finding that the new *URA3*-homologous bands also hybridize with a Y' probe indicated that *URA3* sequences are associated with Y' regions in the telomeres of the transformants. Figure 3a is an autoradiogram of a Southern blot (probed with *URA3* sequences) illustrating representative restriction enzyme digests of DNA from several of the transformants. Lanes 1 to 4 contain DNA from the untransformed strain, H1; the bands obtained derived from the copy of *URA3* (*ura3-52*) on chromosome V. (The same pattern of bands was obtained upon digestion of HH20 DNA.) Restriction enzyme digests performed on the DNA of several Ura$^+$ transformants are shown in lanes 5 to 24. These lanes contain the *ura3-52* bands that were seen in lanes 1 to 4, as well as an additional band derived from the telomeric copy of *URA3*. The limited number of distinct fragment lengths observed is a consequence of the small number of potential environments for the *URA3* marker: transplacements may occur in Y' or Y'(131S) elements flanked on one, both, or neither side by additional Y' [or Y'(131S)] elements.

For example, restriction analysis indicated that the telomere marked in transformant HH20/TU-1 (Fig. 3a, lanes 5 to 8) contains a single Y' repeat (Fig. 3b). Digestion of

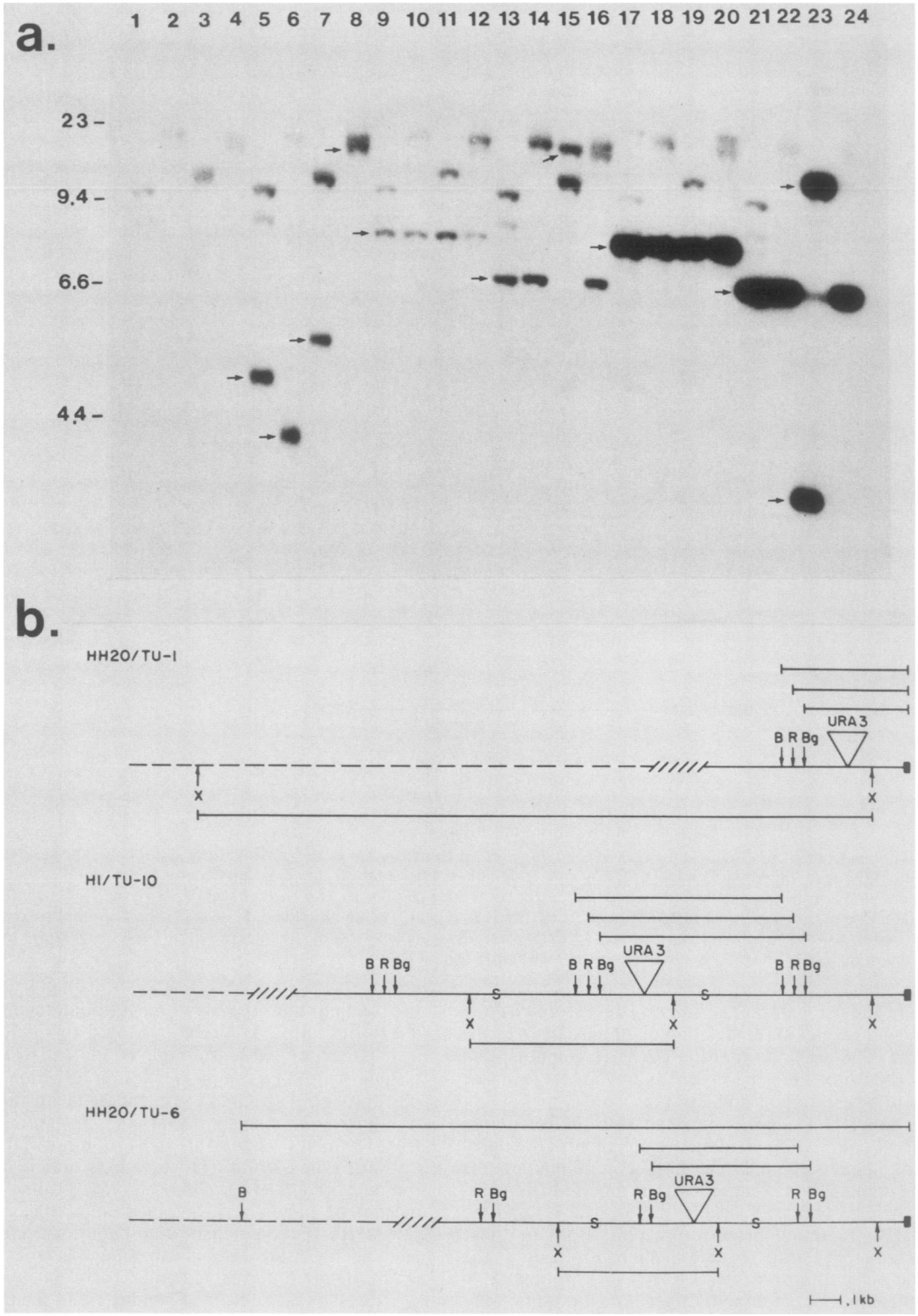


FIG. 3. Southern analysis of marked telomeres. (a) Southern blot. DNA from the indicated strains was digested with various restriction enzymes, subjected to electrophoresis in a 0.8% agarose gel, transferred to nitrocellulose filter paper, and probed with *URA3* sequences. Lanes 1 to 4, H1 (parent) DNA; lanes 5 to 8, HH20/TU-1; lanes 9 to 12, H1/TU-10; lanes 13 to 16, HH20/TU-6; lanes 17 to 20, H1/TU-2; lanes 21 to 24, H1/TU-9. DNA was digested with *Eco*RI (lanes 1, 5, 9, 13, 17, and 21), *Bgl*I (lanes 2, 6, 10, 14, 18, and 22), *Bam*HI (lanes 3, 7, 11, 15, 19, and 23), or *Xho*I (lanes 4, 8, 12, 16, 20, and 24). The digestion reactions in lanes 1 to 4 are presented as a reference for bands derived from *ura3-52* sequences on chromosome V. Bands derived from *URA3* sequences located in Y′ or Y′(131S) regions are indicated with arrows (see the text for details). The increased intensities of bands in lanes 17 to 24 can be judged by comparison of the intensities of the *ura3-52* bands with those in the other lanes. (b) Diagram indicating the different locations of the *URA3* insertion in three transformants. Broken line, unique

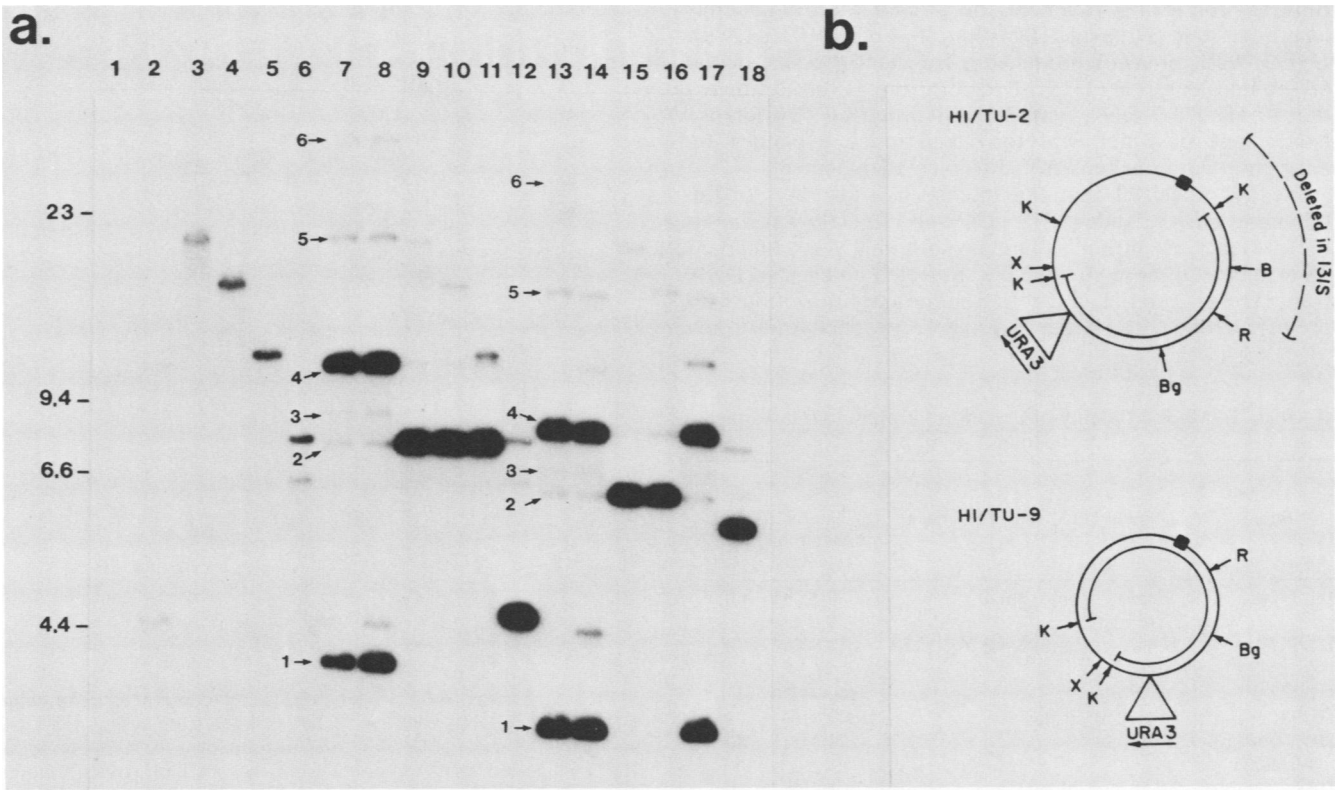


FIG. 4. Some strains contain *URA3*-marked circular Y′ [or Y′(131S)] elements. (a) Southern blot characterizing the autonomously replicating elements in H1/TU-2 and H1/TU-9 (0.5% gel probed with *URA3*). Lanes 1 to 6, H1 DNA; lanes 7 to 12, H1/TU-2; lanes 13 to 18, H1/TU-9. DNA was subjected to digestion with the indicated restriction enzymes. Lanes 1, 7, and 13, No enzyme; lanes 2, 8, and 14, *Pvu*II; lanes 3, 9, and 15, *Bgl*I; lanes 4, 10, and 16, *Xho*I; lanes 5, 11, and 17, *Bam*HI; lanes 6, 12, and 18, *Kpn*I. The numbered bands correspond as follows: 1, supercoiled monomer circle; 2, monomer linear; 3, supercoiled dimer circle; 4, relaxed monomer circle; 5, dimer linear; 6, relaxed dimer circle. (b) Circular maps of the H1/TU-2 and H1/TU-9 monomer circles. The region of DNA deleted in the H1/TU-9 circle is indicated in the map of H1/TU-2. The source of the *URA3*-homologous *Kpn*I fragment obtained in panel a is indicated for each element. The circular species in H1/TU-2 and H1/TU-9 contain sequences that hybridize with a synthetic dAdC.dGdT probe (data not shown); the position of these sequences, indicated by a solid box, was predicted from the finding that tandem Y′ repeats are separated by $C_{1\text{-}3}A$ repeats (C. Chan and B.-K. Tye, personal communication). K, *Kpn*I. Other abbreviations are as in the legend to Fig. 3b.

HH20/TU-1 DNA with the enzyme *Eco*RI, *Bgl*I, or *Bam*HI produced a band of the size predicted for a fragment extending from the relevant restriction site in Y′ to the end of the chromosome. Digestion with *Xho*I (which permitted examination of sequences centromere-proximal to the *URA3* insertion) produced a very large band. The next closest *Xho*I site is most likely in unique chromosomal sequence. Restriction analysis of transformant H1/TU-10 (Fig. 3a, lanes 9 to 12) indicated that the telomere is marked in a Y′ repeat that is flanked on each side by at least one additional Y′ repeat (Fig. 3b). Digestion with any one of the four enzymes used (which each cut once in Y′) produced the same unit-length Y′::*URA3* fragment (7.9 kb). Restriction analysis of transformant HH20/TU-6 (Fig. 3a, lanes 13 to 16) indicated that it contains a telomere marked by *URA3* in a Y′(131S) repeat flanked on the centromere-distal side by at least one Y′(131S) element and on the centromere-proximal side by at least one Y′(131S) or Y′ element (Fig. 3b). Thus, digestion with *Eco*RI, *Bgl*I, or *Xho*I produced the unit-length 6.7-kb Y′(131S)::*URA3* fragment. Digestion with *Bam*HI produced a very large fragment, because the Y′(131S) variant lacks a *Bam*HI site (3); the fragment most likely extends from a *Bam*HI site in X or unique sequences to the end of the chromosome. The deduced structures of the marked telomeres in these three transformants, which have also been confirmed by *Hin*dIII and *Kpn*I digests (data not shown), are summarized in Fig. 3b.

**Presence of *URA3*-marked circular Y′ and Y′(131S) elements.** We noticed several interesting features of transformants H1/TU-2 (Fig. 3a, lanes 17 to 20) and H1/TU-9 (lanes 21 to 24). First, the *URA3*-containing telomere bands were much darker than in the other transformants. This was not a result of more DNA having been loaded in these lanes; the *URA3* bands from chromosome V were of comparable (or reduced) intensity compared with those shown in lanes 1 to 16. Digestion of H1/TU-2 DNA with *Eco*RI, *Bgl*I, *Xho*I, or

sequence; hatch marks, X region; solid line, Y′ [or Y′(131S)] repeats; solid box, T region. For H1/TU-10 and HH20/TU-6, at least one additional repeat flanked the marked repeat on either side; thus, the diagram depicts three Y′ or Y′(131S) elements for these marked telomeres as the simplest case. The bars above and below the map for each transformant indicate the source of the telomeric *URA3*-homologous bands in the Southern blot shown in panel a. Abbreviations: B, *Bam*HI; Bg, *Bgl*I; R, *Eco*RI; X, *Xho*I.

*Bam*HI produced a fragment the size of the 7.9-kb unit-length Y′::*URA3* repeat. Digestion of H1/TU-9 DNA with *Eco*RI, *Bgl*I, or *Xho*I produced a fragment the size of the 6.7-kb unit-length Y′(131S)::*URA3* repeat. However, digestion of H1/TU-9 DNA with *Bam*HI produced two intense bands, one of which was too small to be explained by restriction data for known Y′ [or Y′(131S)] elements.

We hypothesized that the intense hybridization to the telomeric *URA3* bands in H1/TU-2 and H1/TU-9 was due to the presence of multiple extrachromosomal *URA3*-marked Y′ elements in these strains. We subjected undigested DNA from H1/TU-2 and H1/TU-9 to Southern hybridization with a *URA3* probe (Fig. 4a, lanes 7 and 13, respectively). Each strain contained two very dark bands and several light bands, all of which also hybridized to a Y′ probe. The fastest-migrating bands in both transformants were present at positions in the gel corresponding to DNA elements much smaller than the intact chromosomes; the set of bands in H1/TU-9 were smaller than those in H1/TU-2. Thus, there appear to be small, freely replicating *URA3*-marked, Y′-homologous DNA elements in these two strains that are not found in the other Ura$^+$ transformants. We presume that the *ARS*-containing sequence within Y′ which permits the autonomous replication of pBR322 plasmids in yeast (4) is also capable of supporting the autonomous replication of these extrachromosomal Y′ elements. On the basis of this information, we formulated and tested three predictions regarding these extrachromosomal elements. (i) They are unstable and easily lost from the cell; (ii) they are capable of transforming a *ura3* strain of yeast to uracil prototrophy; and (iii) they are circular.

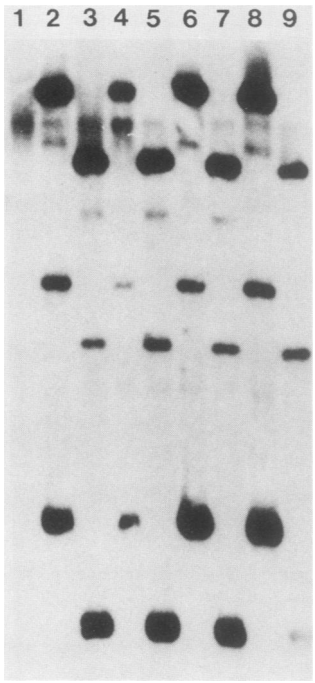


FIG. 5. Strains H1/TU-2 and H1/TU-9 contain transformable *URA3*-marked DNA elements. DNA isolated from strains H1/TU-2 and H1/TU-9 was pooled and used to transform the haploid strain, H1. Undigested DNA prepared from the six Ura$^+$ transformants obtained (H4 1 to 6) was subjected to agarose gel electrophoresis and Southern analysis with a *URA3* probe. Uncut DNA is from the indicated strains. Lane 1, H1; lane 2, H1/TU-2; lane 3, H1/TU-9; lanes 4 to 9, H4 1 to 6 (retransformants).

TABLE 1. Mitotic stability of uracil prototrophy in transformed strains[a]

| Strain | Site of URA3-marked element | No. of Ura$^-$ colonies/ total no. of colonies | % Ura$^+$ loss |
|---|---|---|---|
| H1/TU-5 | Single chromosomal Y′ repeat | 0/1,295 | 0 |
| H1/TU-1 | Single chromosomal Y′(131S) repeat | 0/830 | 0 |
| H1/TU-2 | Y′ circles | 340/442 | 77 |
| H1/TU-9 | Y′(131S) circles | 642/813 | 79 |
| H4 3 (retransformant) | Y′ circles | 274/343 | 80 |
| H3S 1 (stable subclone of H1/TU-2) | Minimum of two chromosomal Y′ repeats; the most centromere-distal repeat is marked | 3/613 | 0.5 |
| H3S 2 (stable subclone of H1/TU-2) | Minimum of three chromosomal Y′ repeats; the two most centromere-distal repeats are marked | 0/590 | 0 |
| H3S 5 (stable subclone of H1/TU-2) | Minimum of two chromosomal Y′ repeats; the most centromere-proximal repeat is marked | 0/639 | 0 |
| HH20/TU-1 | Single chromosomal Y′ repeat | 2/1,000 | 0.2 |
| HH20/TU-8 | Minimum of two chromosomal Y′ repeats; the most centromere-distal is marked | 4/716 | 0.6 |
| HH20/TU-9 | Single chromosomal Y′(131S) repeat | 6/763 | 0.8 |
| HH20/TU-6 | Minimum of three chromosomal Y′(131S) repeats; the central repeat is marked | 22/1,216 | 1.8 |
| HH20/TU-16 | Minimum of three chromosomal Y′(131S) repeats; the central repeat is marked | 27/1,060 | 2.6 |
| HH20/TU-16, segregant 1 | Same as HH20/TU-16 | 5/627 | 0.8 |
| HH20/TU-16, segregant 2 | Same as HH20/TU-16 | 11/1,217 | 0.9 |

[a] Strains were picked from plates lacking uracil and put into 5 ml of YEPD broth. Cultures were grown overnight, and then 1 ml of the culture was used to inoculate 50 ml of YEPD broth. The 50-ml cultures were grown overnight, and then dilutions were spread onto YEPD agar plates. The colonies that grew up were then tested for uracil prototrophy after being replica plated onto agar plates lacking uracil. The first eight strains listed are haploid strains in the H1 background; HH20/TU-16 segregants 1 and 2 are Ura$^+$ haploid segregants of the diploid transformant HH20/TU-16. All other strains are transformants of the diploid HH20.

First, we predicted that these elements would readily be lost from cells when selection for uracil prototrophy was removed. This was indeed the case. We compared the mitotic stability of several different transformants (Table 1). The strains were grown for about 50 generations in YEPD broth, plated on YEPD agar, and then replica plated onto agar plates lacking uracil. The percentage of cells that had become Ura$^-$ was then calculated. H1/TU-5 had a stable *URA3* insertion into the single Y′ repeat present at one particular telomere. Of 1,295 colonies grown on YEPD, none had become Ura$^-$. In contrast, approximately 80% of H1/TU-2 or H1/TU-9 colonies grown on YEPD became Ura$^-$. The 20% that remained Ura$^+$ are described below.

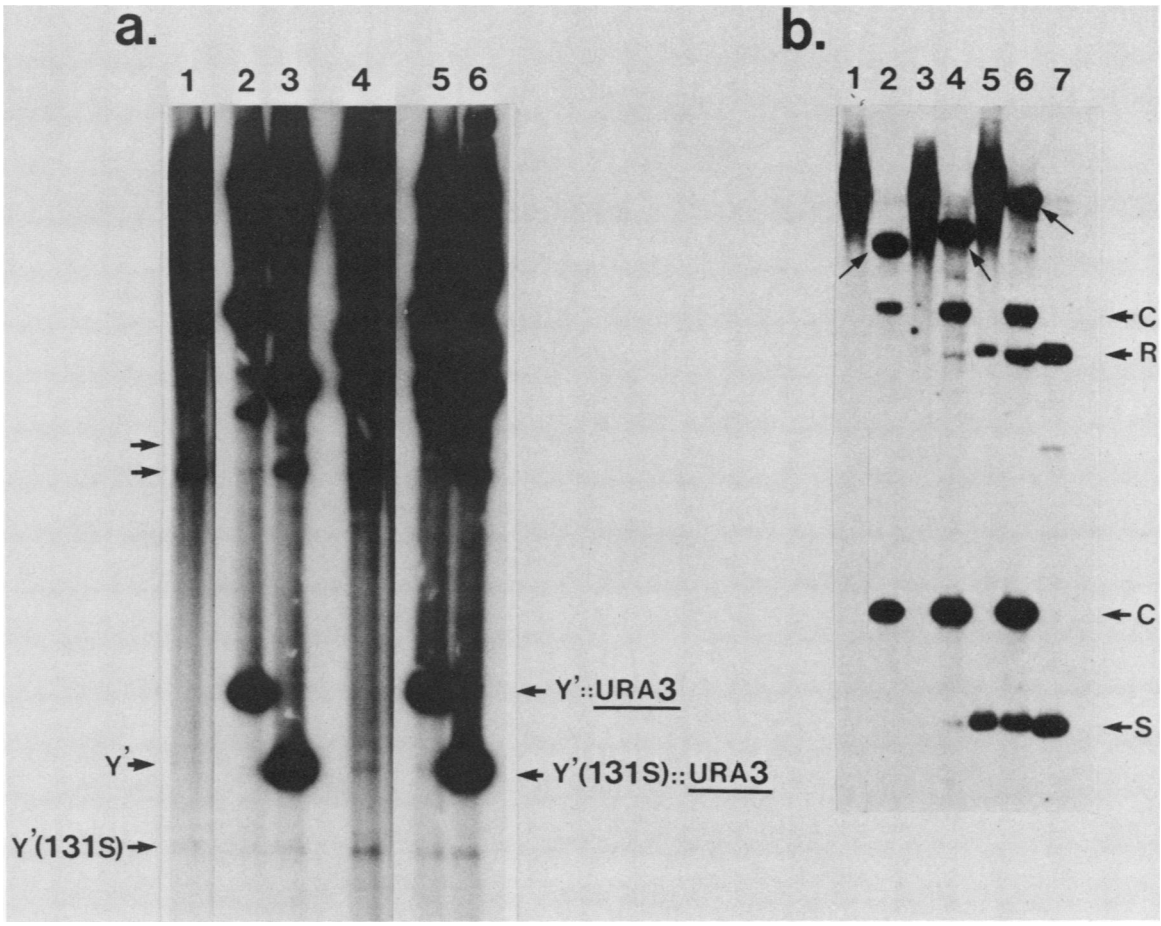


FIG. 6. Production of naturally occurring and *URA3*-marked Y′ and Y′(131S) circles. (a) Autoradiogram of a Southern blot probed with the isolated *Pvu*I-*Sac*I fragment of Y′ sequence. Lanes 1 and 4, H1 DNA; lanes 2 and 5, H1/TU-2 DNA; lanes 3 and 6, H1/TU-9 DNA. Lanes 1 to 3, No enzyme added; lanes 4 to 6, *Pvu*II digested. The low-intensity bands predicted to be Y′(131S) and Y′ monomer supercoiled circular forms (based on comparison of migration distances of these species with those of H1/TU-2 and H1/TU-9) are indicated by the lower pair of arrows to the left of the figure. The light band predicted to correspond to the Y′ monomer supercoiled circle migrated at the same position as did the Y′(131S)::*URA3* monomer supercoiled circle of H1/TU-9. The upper pair of arrows to the left of the figure indicate species which may correspond to other forms of the elements. (b) Autoradiogram of a Southern blot probed with *URA3* sequences. The low-level excision of Y′(131S)::*URA3* circles from the marked telomeres of two strains is illustrated. Lanes 1, 3, and 5, uncut DNA; lanes 2, 4, and 6, *Pvu*II-digested DNA from strains H1/TU-1, HH20/TU-6, and HH20/TU-16, respectively; lane 7, uncut DNA from strain H1/TU-9. No circles are evident in H1/TU-1 DNA. The bands corresponding to the low-level Y′(131S)::*URA3* supercoiled and relaxed monomer circles produced in strains HH20/TU-6 and HH20/TU-16 are indicated as S and R, respectively. As predicted, these bands were insensitive to digestion with *Pvu*II. C, *URA3*-homologous *Pvu*II bands derived from *ura3-52*. Light arrows indicate the chromosomally derived *Pvu*II bands of the *URA3*-marked telomeres.

Thus, the *URA3*-marked Y′ elements present in H1/TU-2 or H1/TU-9 were easily lost from the cell in the absence of selection for Ura$^+$.

Our second prediction was that we would be able to transform a *ura3* strain to Ura$^+$ with total DNA isolated from the strains H1/TU-2 and H1/TU-9 (which harbor the autonomously replicating Ura$^+$ elements). DNA isolated from the two strains was combined and used to transform the same haploid strain, H1, as that used in the initial experiments. Six Ura$^+$ transformants were obtained. Southern hybridization of undigested DNA from each of these (with a *URA3* probe) showed that three contained bands identical in size to H1/TU-2 and three contained bands identical in size to H1/TU-9 (Fig. 5). The finding that the complete set of H1/TU-2 or H1/TU-9 bands was present in each new transformant, but that there was no mixing of H1/TU-2 and H1/TU-9 species, suggests that the bands in a set are structurally related and most likely reflect the various replicative or structural forms of the element (see below). These six strains also exhibited the same frequency of Ura$^+$ loss (80%) as was observed for the original H1/TU-2 and H1/TU-9 transformants (Table 1).

**Structure of the autonomously replicating marked Y′ elements.** We confirmed that the freely replicating elements occur primarily as monomer circles, although a detectable quantity of head-to-tail dimer circles is also present. Several lines of evidence suggest the circular nature of the free elements present in H1/TU-2 and H1/TU-9 DNA. We found that these species have variable migration patterns relative to standard DNA markers, depending on the percentage of agarose in the gel (for H1/TU-2; c.f. lane 7, Fig. 4a, with lane 8, Fig. 7a). Six bands are present in uncut H1/TU-2 or H1/TU-9 DNA; these are numbered 1 to 6 in Fig. 4a. We believe that the two dark bands, 1 and 4, represent

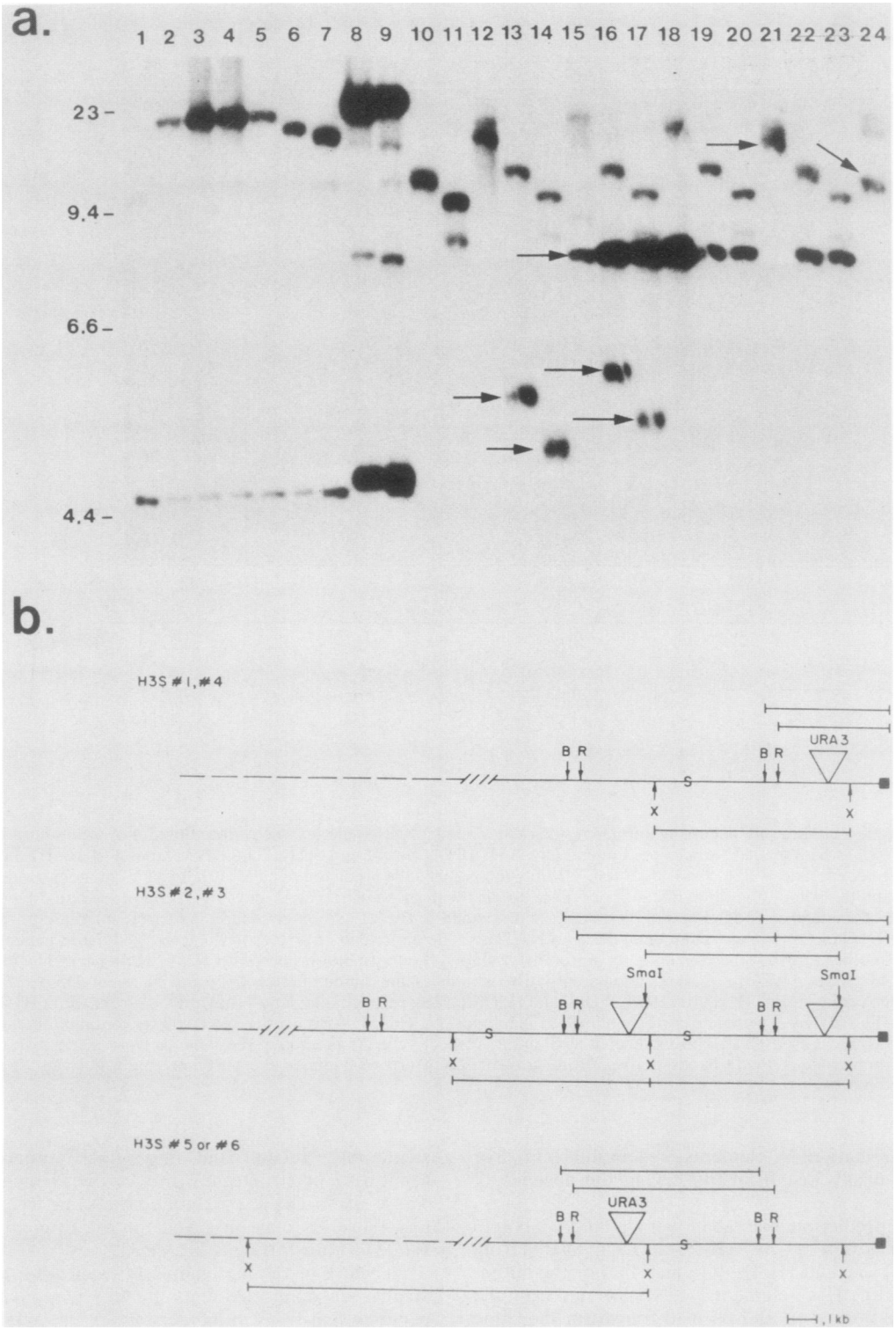


FIG. 7. Y′::*URA3* circles can reintegrate at telomeres. (a) Autoradiogram of a Southern blot of a 0.8% agarose gel probed with *URA3* sequences. Six stable Ura$^+$ subclones of H1/TU-2 (H3S 1 to 6) were analyzed by digestion with a variety of restriction enzymes. Lanes 1 to 7 and 9, *Pvu*II; lane 8, no enzyme added; lanes 10, 13, 16, 19, and 22, *Bam*HI; lanes 11, 14, 17, 20, and 23, *Eco*RI; lanes 12, 15, 18, 21, and 24, *Xho*I. DNA was from strains H1 (lanes 1 and 10 to 12), H3S 1 (lanes 2 and 13 to 15), H3S 2 (lanes 3 and 16 to 18); H3S 3 (lane 4), H3S 4 (lane 5), H3S 5 (lanes 6 and 19 to 21), H3S 6 (lanes 7 and 22 to 24), and H1/TU-2 (lanes 8 and 9). The *Pvu*II digests in lanes 2 to 7 show that *URA3* sequences in the stable subclones were present in different telomeric locations (compare lanes 2 through 7 with the pattern obtained with Y′::*URA3* circles, lane 9). Stable Ura$^-$ subclone pairs H3S 1 and 4 and H3S 2 and 3 were indistinguishable in *Bam*HI, *Eco*RI, and *Xho*I digests. In lanes 10 to 24, bands derived from integration of the Y′::*URA3* circles are indicated with arrows. The increased intensity of the *Pvu*II band and the presence of two bands in *Bam*HI and *Eco*RI digests of H3S 2 (and H3S 3; data not shown) were the result of the integration of a dimer Y′::*URA3* circle in a Y′ telomeric region (see the text). (b) Maps of stable Ura$^+$ subclones of H1/TU-2. For H3S 1

supercoiled and relaxed monomer circles, respectively; light band 2 is the monomer linear. Similarly, light bands 3 and 6 are supercoiled and relaxed dimer circles, respectively; light band 5 is the dimer linear. These assignments were supported by digestion of H1/TU-2 or H1/TU-9 DNA with the enzyme *Bgl*I (which cuts once within the Y′ [or Y′(131S)] repeat) in the presence of ethidium bromide (data not shown). This procedure, which relaxes supercoiled plasmids (25), resulted in an increased intensity of bands 4 and 6 with a concomitant decrease in the intensity of bands 1 and 3. The intensity of bands 2 and 5 (presumed to be the monomer and dimer linears, respectively) did not change. Furthermore, complete digestion of H1/TU-2 or H1/TU-9 DNA with *Bgl*I resulted in the production of only one band—the band 3 monomer linear (see below).

We performed a number of restriction enzyme digests to analyze the structures of the free elements in H1/TU-2 and H1/TU-9. These digests are shown in Fig. 4a alongside the undigested DNA samples. Digestion with the enzyme *Pvu*II (for which no sites exist in any of the Y′ elements characterized thus far) did not alter any of the bands obtained from the extrachromosomal *URA3*-containing Y′ or Y′(131S) elements (c.f. lanes 7 and 8 and lanes 13 and 14, Fig. 4a), although additional bands derived from the copy of *URA3* on chromosome V were apparent. Digestion of H1/TU-2 DNA with any enzyme that cuts only once within the Y′::*URA3* element (e.g., *Bgl*I, *Xho*I, and *Bam*HI) produced a single band migrating at the expected position for the 7.9-kb Y′::*URA3* repeat (lanes 9 to 11). This is the same position as light band 2 (monomer linear) present in undigested DNA. The finding that digestion with these enzymes resulted in decreased mobility of dark band 1 identified in undigested DNA strongly suggests that this species is a supercoiled circle. The fact that a single unit-length linear element was produced from all the extrachromosomal elements suggests that the species presumed to be dimers are in a head-to-tail configuration. Similar results were found upon digestion of H1/TU-9 DNA with a variety of enzymes, except that in this case the fragments produced were those expected from Y′(131S)::*URA3* circles. The Y′(131S) element is smaller than the intact Y′ by about 1.5 kb, and thus many of the fragments produced were correspondingly smaller. However, the deletion also removes the sites for several restriction enzymes (*Bam*HI, *Kpn*I, *Hin*dIII) (3). Because H1/TU-9 circles lack a site for *Bam*HI, the bands produced upon digestion of the DNA with this enzyme were indistinguishable from those of undigested DNA. This accounts for the inexplicably small *Bam*HI fragment described above. In fact, the fastest-migrating band in Fig. 3a, lane 23, was the supercoiled monomer Y′(131S)::*URA3* circle and not the result of a digestion with *Bam*HI. Similarly, the absence of one of the three *Kpn*I sites in the Y′(131S)::*URA3* circle of H1/TU-9 resulted in production of a larger band than that obtained upon digestion of H1/TU-2 DNA with this enzyme (c.f. lanes 12 and 18, Fig. 4a).

We determined that the extrachromosomal species in H1/TU-2 and H1/TU-9 hybridize to a synthetic probe, dAdC.dGdT (data not shown). Walmsley et al. (33, 34) showed that this probe hybridizes to the $C_{1\text{-}3}A$ sequences present at the extreme ends of telomeres and at the X-Y′ junction. Furthermore, Chan and Tye determined that there are at least 50 base pairs of $C_{1\text{-}3}A$ sequence between tandem Y′ repeats and have suggested that these sequences be included in the designated Y′ repeat unit (personal communication). Thus, the extrachromosomal species we identified possess all of the sequences of the Y′ [or Y′(131S)] repeat unit. The dAdC.dGdT-hybridizable sequences we detected are probably located at the position where the two ends of the repeat unit join to form a circle. The inferred structures of the H1/TU-2 and H1/TU-9 monomer circles are illustrated in Fig. 4b.

**Presence of naturally occurring extrachromosomal Y′ elements.** We attempted to identify unmarked Y′ or Y′(131S) circles in a number of strains to determine whether these species are naturally present in the yeast cell. Uncut DNA was run on a gel and subjected to Southern hybridization with a Y′-specific probe (Fig. 6a). Two light bands were observed which migrated at the positions expected for the Y′(131S) and Y′ monomer circles. As expected, these bands were not affected by digestion with the enzyme *Pvu*II (which does not cut within the Y′ repeat). The very low abundance of the putative circles was not unexpected, because our studies with Y′::*URA3* circles indicated that these elements are readily lost from the cell in the absence of selection for $Ura^+$.

**Infrequent excision of circles from marked telomeres.** The identification of putative unmarked Y′ and Y′(131S) circles in untransformed yeast strains suggested to us that circular elements may be excised at a low rate from chromosomal Y′ regions. To examine this possibility, we probed a Southern blot of undigested DNA from a number of transformants marked at bona fide telomeres and identified two transformants, HH20/TU-6 and HH20/TU-16, that contain detectable quantities of Y′(131S)::*URA3* circles. Uncut DNA from these two strains (which are marked at different telomeres) is shown in Fig. 6b, lanes 3 and 5, and the bands corresponding to the supercoiled (S) and relaxed (R) marked circles are indicated. As was expected, these bands were not affected by digestion of the DNA with *Pvu*II (lanes 4 and 6), as no sites for this enzyme are present in Y′. Analysis of these strains by digestion with other restriction enzymes indicated that the *URA3*-marked chromosomal Y′ element in each is, in fact, the 131S variant and it is flanked on either side by additional Y′(131S) elements (see Fig. 3a, for an analysis of HH20/TU-6). This suggested to us that free circles might be generated via excision (probably a single crossover event) of a Y′(131S)::*URA3* element from the telomere. This hypothesis was further supported by subcloning experiments which revealed that ~2.5% of HH20/TU-16 and ~1.8% of HH20/TU-6 cells grown in rich media become $Ura^-$, presumably through loss of the circle in the absence of selection (Table 1). Haploid segregants of HH20/TU-16 also become

and 4, there was at least one Y′ repeat centromere-proximal to the marked one; thus, two repeats are indicated as the simplest case. For H3S 2 and 3, there was at least one Y′ repeat centromere-proximal to the internal marked element; thus, three repeats are indicated as the simplest case. For H3S 5 and 6, there was at least one Y′ repeat centromere-distal to the marked repeat; thus, two repeats are indicated. For these two transformants, the position of the *Xho*I site in unique telomeric sequences was different, yielding the differently sized bands in panel a. Bars above and below the maps indicate the source of bands in the Southern blot shown in panel a. The source of the 7.9-kb unit-length Y′::*URA3* fragment obtained in *Sma*I digests of H3S 2 and 3 (see the text) is indicated. Symbols and abbreviations are as described in the legend to Fig. 3b.

Ura$^-$ in subcloning experiments, although the frequency was somewhat reduced (Table 1). No extrachromosomal elements were evident in uncut DNA from strain H1/TU-1, shown in Fig. 5b, lane 1 for comparison. Accordingly, no Ura$^-$ colonies were produced in over 800 subclones of H1/TU-1 grown on YEPD.

***URA3*-marked circles can integrate into telomeres.** As described above, we found that approximately 20% of H1/TU-2 subclones grown on nonselective media remained Ura$^+$. We questioned whether these cells had retained the free Y′::*URA3* circles or possessed some new (and possibly more stable) configuration of *URA3* sequences. Subcloning experiments revealed that the *URA3* sequences in these strains were indeed more stable: depending on the strain, only 0 to 0.5% of subclones became Ura$^-$ (Table 1). Figure 7a shows *Pvu*II digests of six stable subclones probed with *URA3*. None of the subclones possessed the bands corresponding to the free circles of H1/TU-2 (compare lanes 1 to 6 with lane 8). Rather, each possessed a single large (~20-kb) band indicative of a chromosomal location for the *URA3* sequences. The bands present in stable subclones 2 and 3 appeared darker than those in the other stable subclones. We analyzed the six subclones in greater detail with a variety of restriction enzymes to better characterize the new chromosomal location of *URA3* sequences. From this analysis, we were able to determine that *URA3* sequences had been integrated into at least four distinct locations for the six subclones (see Fig. 7a, lanes 9 to 24 and Fig. 7b for an analysis of the four distinct subclones).

Insertion was most likely mediated by a single crossover event in which the entire circle became integrated. For stable subclones 2 and 4, *URA3* sequences were present in the most telomeric Y′ repeat, and there was at least one Y′ repeat centromere-proximal to the marked one. For subclones 5 and 6, the *URA3* sequences were present in a Y′ element which was flanked by at least one additional Y′ repeat on the telomere-proximal side. Furthermore, these two subclones represented different sites of insertion based on the finding that the *URA3*-containing *Xho*I and *Pvu*II fragments from the two strains were of different lengths. Stable subclones 2 and 3 each appeared to have two sites of *URA3* insertion: one in a most telomeric Y′ element and one in a Y′ element flanked on the telomere-proximal side by another Y′ element. These double insertions were most likely the result of integration of a dimer circle: digestion with *Sma*I, which cut once within the cloned *URA3* fragment but not within Y′, produced a fragment the length of a Y′::*URA3* repeat unit, indicating that the marked Y′ repeats were adjacent (data not shown; see Fig. 7b for diagram). Thus, the greater intensity of hybridization to the single *Pvu*II band observed in subclones 2 and 3 was due to the presence of two copies of *URA3* sequences in this fragment. These findings indicate that integration of both free monomer and dimer Y′ circles into various chromosomal locations was a facile reaction. It is likely that all of the stable Ura$^+$ colonies obtained in our subcloning experiments (20% of the total) represented integration events.

## DISCUSSION

We marked a variety of telomeres of the yeast *S. cerevisiae* with the yeast *URA3* gene and, by doing so, identified two transformants marked in circular Y′ or Y′(131S) elements. The identification of these species prompted additional experiments, which revealed the presence of a novel mechanism for the transfer of telomeric sequences from one site to another. This process involves the excision of a circular Y′ [or Y′(131S)] element from a telomere followed by the integration of the circle at potentially new Y′-containing sites.

The excision reaction is most clearly demonstrated by the presence of a low level of Y′(131S)::*URA3* circles in DNA from HH20/TU-16 cells. We conclude that these circles were derived from the chromosomal Y′(131S)::*URA3* element, because subcloning experiments with HH20/TU-16 showed that 2.6% of cells grown in rich media become Ura$^-$. These cells presumably underwent excision of circles which were subsequently lost in the absence of selection. Subcloning experiments revealed that a number of other transformants marked at telomeres that have one or multiple Y′ [or Y′(131S)] repeats also become Ura$^-$ at various frequencies (Table 1). Excision of circles from a telomere with multiple repeats probably occurs by a single crossover event anywhere in the homologous sequences of two repeats present at that telomere. One or more repeats may be looped out. In contrast, excision of circles from telomeres having only one repeat most likely occurs via a recombination event between $C_{1\text{-}3}A$ sequences present at the X-Y′ junction and at T. However, we cannot rule out the possibility that some of the marked circles we observed in strains HH20/TU-6 and HH20/TU-16 were generated by recombination (either gene conversions or double crossover events) between the marked chromosomal Y′(131S) repeat and naturally occurring Y′(131S) circles. Likewise, some of the Ura$^+$ losses observed in subcloning experiments could be due to these types of events, as well as to unequal sister chromatid exchange or gene conversion between telomeres.

Southern blots of uncut DNA from a number of strains (Fig. 6a) revealed the presence of Y′-homologous bands that migrate in positions expected for unmarked Y′(131S) and Y′ monomer circles. The very low abundance of these species, which we believe are products of excision from the chromosome, was not unexpected. In comparison, we found that excision of the Y′::*URA3* circles is a relatively rare event and that marked circles are readily lost from the cell in the absence of selection. It is only by marking Y′ elements with a selectable marker that we were able to enrich for cells harboring the Y′(131S)::*URA3* or Y′(131S)::*URA3* circles in strains H1/TU-2 and H1/TU-9, respectively. We believe that the *URA3*-marked circles in these two strains were produced by a transplacement that occurred directly on a Y′ [or Y′(131S)] circle naturally present in the strain. However, we cannot rule out the possibility that the marked circles may have been excised from a chromosomal site early in the lifetime of the transformant colony.

Although the marked circles were readily lost from strains in the absence of selection for Ura$^+$, 20% of the cells underwent a reaction whereby *URA3* sequences were stably integrated into the chromosome. The insertion probably occur by a simple crossover event between a chromosomal Y′ element and its circular counterpart: in the six cases we examined, the *URA3*-containing Y′ element was flanked on at least one side by another Y′ element in the telomere. Furthermore, we saw two cases of integration involving a marked dimer circle. The finding that two of six stable integrants involved integration of a dimer circle is interesting because dimers represent such a small fraction of the extrachromosomal Y′ elements present (compare the intensity of bands 1 and 3 in Fig. 4a). Three explanations are apparent. First, by subcloning the cells in rich media, we could have enriched for dimer circles which, by virtue of having two *ARS* elements instead of one, were more stable than the monomers. Integration would then occur from a

population of circles with an altered (increased) ratio of dimers to monomers. Second, the greater amount of Y′ DNA in the dimer circle may have rendered it a better substrate for homologous recombination with the Y′ repeats in the chromosome. Third, a gene conversion event associated with integration of the marked monomer circle could have converted the resident chromosomal Y′ element to contain *URA3* sequences as well.

Dunn et al. (9) recently described a number of experiments in which linear plasmids were capable of picking up Y′ elements from intact chromosomal ends. The process was found to be *RAD52*-dependent, prompting the authors to suggest that the addition of Y′ elements to linear plasmids occurs via a conversion event, presumably leaving the chromosome unaltered. It was proposed that analogous conversion reactions occur between natural chromosomes, leading to the dispersal of Y′ elements from one progenitor copy to other chromosomal ends. Unequal crossing over and conversion events would then generate tandem arrays of Y′ elements.

We describe here another mechanism for the dispersal of Y′ elements. Our experiments suggest that telomeres are capable of contracting and expanding in units of Y′ via the excision and integration of Y′ circles. The circular Y′ elements act as a mobile source of Y′ information, capable of integrating at numerous different telomeric locations. The potential multicopy nature of circles, as well as the capacity for dimer integration, may provide the cell with the ability to increase total Y′ information. The facile excision and integration of circular Y′ elements may be partly responsible for the high degree of rearrangement observed at yeast telomeres (13) and for the homogenization of sequences at different chromosomal ends. We have found that digestion of DNA from different strains with certain restriction enzymes produces considerably different patterns of telomere bands (13). Because these differences often involve many ends, we concluded that there must exist a mechanism for maintaining telomeric sequence homogeneity within a strain. The excision and integration of circular Y′ elements may promote the dispersal of telomeric sequences throughout the genome and thus may facilitate this homogenization process. We are now conducting experiments to examine these possibilities in greater detail and to identify factors which may influence the rates of circle excision and integration in the yeast cell.

The excision of circular elements from the chromosome appears to be a general process in *S. cerevisiae*. Circular monomers and head-to-tail multimers of the repeat unit of the genes coding for rRNA (rDNA) have been observed in approximately 3 to 5 copies per cell in several species of yeast (5, 6, 8, 10, 15, 22). These circles may be derived by excision from the multiple tandem array of rDNA repeats present in the chromosome and, by analogy to the experiments we describe here, may integrate into the chromosome as well. Indeed, it has been suggested that integration and excision of circular rDNA molecules play a role in the regulation of rDNA gene dosage (15, 22). However, no direct evidence for either excision or integration exists, nor has it been shown that these elements replicate autonomously.

Circular transposable elements (Ty1) have also been identified in *S. cerevisiae* (1, 23). These elements may be produced by recombination between the direct repeats (delta sequences) flanking the element in the chromosome (23). Estimates of the abundance of Ty1 circles range from 0.003 to 0.004 (1) to 0.02 to 2 copies per cell (23); the putative Y′ and Y′(131S) circles we identified were present at a level similar to the lower estimates for Ty1 circles.

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