Efficient Homologous Recombination of Ty1 Element cDNA When Integration Is Blocked

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Integration of the yeast retrotransposon Ty1 into the genome requires the self-encoded integrase (IN) protein and specific terminal nucleotides present on full-length Ty1 cDNA. Ty1 mutants with defects in IN, the conserved termini of Ty1 cDNA, or priming plus-strand DNA synthesis, however, were still able to efficiently insert into the genome when the elements were expressed from the GAL1 promoter present on a multicopy plasmid. As with normal transposition, formation of the exceptional insertions required an RNA intermediate, Ty1 reverse transcriptase, and Ty1 protease. In contrast to Ty1 transposition, at least 70% of the chromosomal insertions consisted of complex multimeric Ty1 elements. Ty1 cDNA was transferred to the inducing plasmid as well as to the genome, and transfer required the recombination and repair gene RAD52. Furthermore, multimeric insertions occurred without altering the levels of total Ty1 RNA, virus-like particle-associated RNA or cDNA, Ty1 capsid proteins, or IN. These results suggest that Ty1 cDNA is utilized much more efficiently for homologous recombination when IN-mediated integration is blocked.

Ty1 elements are Saccharomyces cerevisiae retrotransposons that are structurally and functionally related to retroviruses (5, 22). These elements contain two long terminal repeats (LTRs) that bracket an internal coding region. The LTRs contain the retroviral subdomains U3, R, and U5, which are important for reverse transcription and integration. Ty1 elements are transcribed from LTR to LTR, forming a terminally redundant 5.7-kb transcript that contains R and U5 at the 5' end of the transcript and U3 and R at the 3' end. Ty1 RNA is reverse transcribed to form a linear cDNA that integrates into the host genome by retrovirus-like mechanisms. The LTRs are reassembled from the separated R-U5 and U3-R sequences by a series of strand transfer events that occur during reverse transcription. Ty1 RNA contains a tRNA-Met primer binding site (PBS) adjacent to U5, where minus-strand reverse transcription initiates (11), and a polyuridine tract (PPT) adjacent to U3, where plus-strand synthesis probably begins (5, 22). The dinucleotides TG and CA at the termini of the U3 and U5 segments of the LTR, respectively, are identical to those of all retroviruses and many retrotransposons (49). These dinucleotide pairs are required for Ty1 integration in a cell-free system (18).

Ty1 RNA is packaged into virus-like particles (VLPs) that are composed of Ty1 proteins (5, 22). Ty1 elements contain two overlapping genes, TYA1 and TYB1, that are equivalent to retroviral gag and pol. Ty1 element Gag (TYA1) and Gag-Pol (TYA1-TYB1) polyproteins are cleaved by a Ty1-encoded protease (PR) into mature proteins within the VLP. Among these are the mature capsid protein p54-TYA1, derived from p58-TYA1 and PR, integrase (IN; also called p90-TYB1), and reverse transcriptase (RT)/RNase H (RH), derived from p190-TYA1-TYB1 (24).

An effective way to study Ty1 transposition is to use a series of recombinant plasmids called pGTyl plasmids (3). These consist of the regulated yeast GAL1 promoter fused to a Ty1 element at its transcriptional initiation site and cloned on a multicopy shuttle plasmid. When expression of plasmid pGTyl-H3 is induced by growing cells on medium containing galactose (a process that has been called transposition induction), transposition of the plasmid-borne element and chromosomal elements occurs at a frequency of more than one event per cell. Ty1 elements have been tagged with a variety of genes that have been useful for distinguishing transposition of the plasmid-borne element from chromosomal elements (5, 22). Recently, Ty1-H3 has been marked with his3AI (13), an indicator gene composed of the yeast HIS3 gene disrupted by an artificial intron (AI [56]), which is inserted in the antisense orientation. The his3AI sequences are inserted onto Ty1 such that the intron is on the sense strand of Ty1; therefore, splicing and reverse transcription of the marked element (designated Ty1his3AI) gives rise to His+ cells. Since the AI is precisely removed from the Ty1his3AI transcript, subsequent expression of Ty1HIS3 is unaffected. One spliced Ty1HIS3 insertion and several unspliced Ty1his3AI insertions are usually present after transposition induction.

Laboratory strains contain 25 to 30 Ty1 elements and numerous single LTRs dispersed in the genome (5, 22). Chromosomal Ty elements and single LTRs can undergo homologous recombination with each other and with Ty1 cDNA produced by reverse transcription (32, 40). cDNA-mediated recombination is not restricted to Ty1 elements. Studies performed with his3AI have shown that HIS3 cDNA can serve as a donor in mitotic gene conversion events or be incorporated into the Ty1 genome to form functional pseudogenes (15, 16). The rates of these cDNA-mediated recombination events are usually very low but are stimulated by transposition induction.

Ty1 integration has been difficult to study in vivo because of a high background level of presumed nontranspositional insertion events (7, 17, 34). Here we demonstrate that Ty1 elements containing mutations that affect de novo integration are able to efficiently insert into new locations by homologous recombina-
FIG. 1. (A) Diagram of wild-type plasmid pGS99, a GALI-inducible plasmid derived from pGTV1-H3hisAI (13). The Ty1-H3 element is marked with hisAI, and the plasmid is marked with URA3. pBR322 sequences are drawn as a thin black line, yeast sequences are boxed, LTR sequences are shown as black triangles, internal Ty1 sequences are lightly shaded, hisAI sequences are darkly shaded, and the Alu is black. Restriction sites (approximate locations are shown): P, PvuII; H, HpaI; B, BstEII; Alu, AluII; Aa, AsrII; N, NcoI; Xb, XbaI. (B) Detailed diagram of Ty1-H3hisAI. The wavy arrows represent RNA transcripts, TYAI and TYBI are lightly shaded, the vertical arrows denote the location of the Ty1 mutations. Other designations are the same as described above.

MATERIALS AND METHODS

Yeast strains and media. Yeast strains yGS77 (MATα ura3-167 trp1-GB his3-Δ200 sp3-101) and yGS38 (MATα ura3-167 trp1-GB his3-Δ200 sp3-101 rad52-GB) are isogenic derivatives of strain GRF167 (3). All media were prepared as described by Sherman et al. (43) or Boeke et al. (4).

Construction of Ty1-H3 mutants. The wild-type pGTVI plasmid pGS99 was derived from plasmid pGTV1-H3his3AI by PCR mutagenesis and lacks a PstI site within the Alu (25, 26). A PCR-amplified ClaI-XbaI fragment containing the mutation was synthesized from Ty1-H3his3AI by using primers from Ty1-H3 (5'-GGCTTACATTTGATGATCAGTCAAGCAT CAG-3' [5335 to 5366; sense strand]) and the Alu (5'-CCCAG GGGATCTTCTAGGTCGACGGGCGCAGAAAAGC-3' [antisense strand; missense mutations are underlined in this and the following oligonucleotides]). The mutated fragment was subcloned into an intermediate vector, pGS101, that contains an XbaI deletion from the Alu to the 2 μm replicator (Fig. 1). The relevant XbaI fragment was subcloned into plasmid pGS101 in the proper orientation to form plasmid pGS99. The pGTV1-H3 mutants in-2600, in-2725, and in-3796 (34) were kindly provided by J. Boeke (Johns Hopkins Medical School). These pGTV1-H3 plasmids were digested with BstEII and AflIII, and the mutant restriction fragment was used to replace the equivalent wild-type fragment of plasmid pGS99.

A Ty1-PR active-site mutation, pr-1682, was constructed by site-directed mutagenesis (35). A pSP70 plasmid (Promega) containing the 1,932-bp Xhol-SalI fragment of Ty1-H3 (2) was digested with either BstEII plus Xhol or PvuII plus AarII. Fragments containing relevant Ty1 and plasmid sequences from each of these reactions were gel purified and then combined with the plasmid containing oligonucleotide 5'-TTCTCGATTCAGTGCTCACCGAACCCTT-3'. The mutant oligonucleotide contained a G→Ala change in the predicted active site of Ty1-PR (45) and a PvuII site to verify the presence of the mutation. The DNA mixture was heat denatured and then renatured by slow cooling to form circular chimeric molecules. T7 DNA polymerase (United States Biochemicals) and T4 DNA ligase (New England Biolabs) were used to fill in and seal, respectively, the single-stranded regions of the mutated plasmid.

The PRE-U5, PRE-U5-TIP, U3-TIP, and PPT mutations (Fig. 2) were constructed by PCR site-directed mutagenesis (25, 26). Sequences of the mutant oligonucleotides are as follows (only the coding strand is given; missense mutations are underlined, additional nucleotides are in boldface, and the deletion of a nucleotide is marked with an asterisk, and the position of the oligonucleotide in the Ty1-H3 sequence is shown within parentheses): PRE-U5 mutation (315 to 355), 5'-CAACAT TACCAGAATT*CATGTTGACGCCCTGTCCCCTGGG-3'; PRE-U5-TIP (315 to 355), 5'-CAACATTCACCAGAATT*Δ ATGTTAGCCGCGCTGCCCCTGGG-3'; U3-TIP mutation (5578 to 5617), 5'-TTATATGTTTGGGTATCCGTTGAAA TAGAAATCAACTATC-3'; and PPT mutation (polylinker adjacent to hisAI-5611), 5'-CGGATTCCGAGGCTATCGAT TCTCTCTTTTCTTTCTCCCTTTTGTGGATAGA AATCAACTATATC-3'.

The mutations possessed the following restriction site polymorphisms: PRE-U5 and PRE-U5-TIP contained EcoRI sites, U3-TIP contained a KpnI site, and the PPT mutation lacked a BglII site. Subsequent restriction enzyme analysis was used to verify whether the mutation was present. Since PCR may introduce secondary mutations, three to five independently derived pGS99 plasmids containing each of the PCR-generated mutations were tested for transposition in strain yGS37 by using a qualitative test for His+ events (see below). A representative mutant pGS99 plasmid was then chosen for further study.

Qualitative detection of His+ events. Cells were grown on SC-ura glucose plates (synthetic complete [SC] medium lacking uracil [SC-ura] with glucose as the carbon source) as 2- by 2-cm patches for 2 days at 30°C. Cells were then replica plated to SC-ura galactose plates and grown for 2 days at 20°C. After galactose induction, cells were either replica plated to YEPD or SC-ura glucose plates and incubated for various time periods at 30°C before transfer to SC-ura-his glucose plates or replica plated directly to SC-ura-his glucose plates and incubated for 3 days at 30°C.

Frequency of His+ events. Seven to nine early-stationary-phase cultures of each strain were diluted 1:100 into SC-ura galactose and grown to saturation at 20°C. The cells were then plated on SC-ura glucose plates to determine the titer and on SC-ura-his glucose plates to determine the number of histidine prototrophs.

Rate determination. About 500 cells were transferred to each of 9 or 11 liquid cultures of SC-ura galactose and grown at 20°C. Cells were titrated on SC-ura glucose plates and spread
on SC-ura-his glucose plates at 30°C to determine the number of His+ cells. The rate of formation of His+ cells was calculated by the maximum-likelihood method of Lea and Coulson (28).

**Plasmid segregation.** His+ cells were grown nonselectively to allow segregation of the relevant _URA3_-based pGS99 plasmid. Plasmid segregants selected by plating on 5-fluoro-orotic acid medium (4) were replica plated to SC-his and SC-ura plates. The frequency of plasmid-borne events was the number of Ura− His+ cosegregants divided by the total number of Ura− segregants. Note that this measurement provides the minimum number of plasmid-borne His+ events, because a cell containing both plasmid-borne and chromosomal His+ events was scored as a chromosomal event.

**Growth competition.** His+ and His− strains were grown to stationary phase and then combined to yield a 50% mixture of each strain, as judged by the optical density at 600 nm of the starting cultures. The mixed culture was diluted 100-fold with YEPD and incubated with agitation at 30°C. Samples were plated on YEPD plates after the initial dilution and after 24 and 48 h of incubation. YEPD plates were incubated for 3 days at 30°C and then replica plated to SC-his plates to determine the number of His+ and His− cells remaining in the mixed culture.

**Analysis of pGS99 plasmid derivatives containing TyHis3 insertions.** Plasmids were recovered by introducing total yeast DNA from appropriate strains into _Escherichia coli_ MC1066 (46). Plasmid DNA from several ampicillin-resistant Ura+ bacterial transformants from each transformation was analyzed by PCR (27) to verify the presence or absence of the _Al_. Restriction analysis was performed by standard techniques (30).

**DNA filter hybridization.** Total yeast DNA was prepared by using the Bio 101 genome kit as instructed by the supplier. Restriction digests of yeast DNA were electrophoretically separated on a 0.7% agarose gel and blotted to HyBond N+ nylon membrane (Amersham) and unlabeled deoxyribonucleoside triphosphates (20).

**Pulsed-field gel electrophoresis and hybridization of yeast chromosomes.** Yeast chromosomes were isolated by standard procedures (9) and separated electrophoretically by using the CHEF Mapper XA Chiller system (Bio-Rad). For general separation of yeast chromosomes, the nonalgorithm program 10 for _S. cerevisiae_ was used (Bio-Rad instruction manual and application guide). Separation of the larger yeast chromosomes (1,000 to 2,200 kb) was achieved by using a 1% (wt/vol) agarose gel in 0.5x TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA [pH 8.0]) buffer. Electrophoresis was performed for 40.3 h at 14°C, with an included angle of 120°, a voltage gradient of 6 V/cm, an initial switch time of 1 min 41.3 s, a final switch time of 3 min 48.7 s, and a linear ramping factor. Transfer of separated chromosomes to filters and DNA hybridization were carried out by standard procedures (9, 12).

**Northern (RNA) analysis.** Yeast strain _YGS37_ harboring the appropriate pGTy1 plasmid was grown overnight at 30°C in SC-ura medium with 5% (wt/vol) raffinose. The cultures were diluted threefold into 10 ml of SC-ura galactose and grown overnight at 20°C. Total RNA was isolated, separated electrophoretically, and blotted to a Hybond N (Amersham) nylon membrane as described previously (14). To detect _Tyhis3AI_ and _PYK1_ transcripts, 32P-labeled RNA probes were synthesized from plasmids pGEM-HIS3 and pGEM-PYK1, respectively, using standard in vitro transcription conditions (33). The two probes were added simultaneously.

**Efficiency of _his3AI_ splicing and detection of the _Al_.** The relative level of splicing of _his3AI_ was estimated by RT-PCR (52), using nucleic acids extracted from purified wild-type and in-2600 VLPs (17). _HIS3_-specific (47) primers upstream

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**FIG. 2.** (A) Construction of the PPT and U3-TIP mutations. The sequence of the region surrounding the Ty1-H3 PPT and U3 tip (shaded bar) is shown. The putative Ty1-H3 PPT (GGGTGTTGTA) as well as additional purines upstream of U3 are marked with dots. These purines were changed to pyrimidines to disrupt the PPT mutation: G residues were changed to C, and A residues were changed to T. To verify incorporation of the PPT mutant into plasmid pGS99, a _BglII_ site (AGATCT) was disrupted. The U3-TIP mutation converted the conserved U3 dinucleotide TG (shaded bar) to CC (box) and also created a _KpnI_ site (GGTACC). The incorporation of the U3-TIP mutation into both LTRs of _Ty1_ cDNA during the process of reverse transcription is shown below. (B) Construction of the PRE-U5-TIP mutation. The U5 tip is adjacent to the RNA PBS (shaded bar) in the _TyAI_ coding sequence. The PRE-U5-TIP mutation converted the conserved U5 dinucleotide CA (solid bar) to AA (box) in two steps. The PRE-U5 mutation positioned the U5 dinucleotide CA to create the correct substrate for further mutagenesis. The T residue adjacent to the CA was deleted (−), and a T was inserted (−) several nucleotides downstream. The alteration in the _TyAI_ coding sequence is shown by the boxed amino acid codons Met-Val-Ala-Pro. A third position change from C (shaded bar) to G (box) in a _Pro_ codon was added to create an _EcoRI_ site (GAATTC) for verification purposes. The PRE-U5-TIP mutation was created from PRE-U5 by a third position change from C to A in the _Ser_ codon to create the dinucleotide AA (boxed). The incorporation of the PRE-U5-TIP mutation into both LTRs of _Ty1_ cDNA during the process of reverse transcription is shown below. The designated mutations were subcloned into plasmid pGS99. All other designations are the same as in Fig. 1.
RESULTS

Experimental strategy. Ty1 cDNA can enter two distinct pathways that give rise to insertions in the genome: most insertions occur by the action of IN (17), while rare insertions can occur by homologous recombination with endogenous elements using cellular recombination machinery (15, 16, 32). The mechanism of Ty1 integration has been difficult to study in vivo, however, because of a high background level of presumed nontranspositional insertion events (7, 17, 34).

To determine the nature of the nontranspositional insertion events, Ty1 mutants blocked at various stages of retrotransposition were marked with the his3AI indicator gene (13) and expressed from a pGTy1 plasmid (Fig. 1). Two criteria were used to distinguish de novo transposition from nontranspositional insertion events, such as homologous recombination of Ty1 cDNA. First, studies of his3AI pseudogene formation have suggested that the plasmid-borne His+ events and at least some of the chromosomal events arise from homologous recombination of chimeric Ty1-HIS3 cDNA (15, 16). Therefore, the occurrence of His+ recombinants that cosegregate with the pGTy1 plasmid was used to monitor homologous recombination of Ty1 cDNA. Second, since mitotic recombination is dependent on the RAD52 gene (21, 39), while Ty1 transposition is not (41), we performed experiments in isogenic RAD52 (yGS37) and rad52-GB (yGS38) strains to distinguish homologous recombination from Ty1 transposition. To minimize complementation and cDNA recombination between mutant pGTy1 plasmids and chromosomal Ty elements, strains yGS37 and yGS38 also contained an spi3 null mutation. The SP73 gene is required for full-length transcription of genomic Ty elements (54) but is not required for GALI-promoted Ty1 transcription or transposition (6).

High-frequency transfer of Ty1 cDNA to new locations when de novo transposition is blocked. Four trans-acting pGTy1 mutants were analyzed (Fig. 1). Ty1-PR mutants affect VLP maturation and reverse transcription (1, 36, 57). Ty1 in-3796 contains a frameshift that creates a stop codon in the C-terminal region of IN (7, 34) and does not produce detectable levels of p60-Ty1 RT/RH, as determined by immunoblotting (42). Two Ty1-H3 IN mutants, in-2600 and in-2725 (7, 17, 34), reported previously to be partially integration defective when assayed in vivo or completely defective in a cell-free system, were also analyzed.

Three cis-acting pGTy1-H3his3AI mutants were analyzed for the ability to form His+ cells (Fig. 1 and 2). The first two mutants lacked the conserved dinucleotides TG and CA that are present at the U3 and U5 tips, respectively, of full-length double-stranded Ty1 cDNA (18). The U3 tip is defined by the junction between the PPT, the initiation site of plus-strand DNA synthesis, and the U3 segment of the Ty1 transcript. We converted the conserved terminal U3 dinucleotide TG to CC to create the U3-TIP mutant. The U5 tip is defined by the junction between the rRNA-Met PBS, the initiation site of minus-strand DNA synthesis, and the U3 segment of the Ty1 transcript. Since this junction is in the TYA1 coding sequence, no change that did not mutate TYA1 could be made. Therefore, the wild-type TYA1 coding sequence His-Gly-Ser-Ala was replaced with Met-Val-Ala-Pro by deleting a T immediately adjacent to the CA tip and adding a T several codons downstream of the PBS. Two related mutants were constructed: PRE-U5-TIP, which contains a CA→AA tip mutation and the four-codon substitution, and PRE-U5, which contains only the four-codon substitution. The third cis-acting sequence that we mutated was the PPT, which is the probable initiation site of plus-strand DNA synthesis. The Ty1 PPT is assumed to be 8 to 10 bases long and is situated within the coding region between TYA1 and the downstream U3 segment. To maximize the possibility of mutating the Ty1 PPT, we replaced all purines in the presumed Ty1 PPT region upstream of the his3AI insertion site, changing G residues to C and A residues to T.

The efficiency of His+ prototroph formation and segregation of the His+ phenotype allowed us to place the Ty1 mutants into two groups (Table 1). The pr-1682 and in-3796 mutants formed His+ prototrophs at very low levels compared with that of the wild-type Ty1 element, even when assayed in a RAD52 strain. Surprisingly, the in-2600, PRE-U5, PRE-U5-TIP, U3-TIP, and PPT mutants formed His+ cells at essentially wild-type levels. However, the His+ isolates had two properties distinguishing them from wild-type Ty1 transposition events. At least 5% of the His+ events were associated with the inducing plasmid in the mutants, whereas plasmid-borne His+ events were detected in 0.17% (0 of 594) of the wild-type isolates. The frequency of His+ cells also decreased at least 100-fold when the in-2600, U3-TIP, and PPT mutants were analyzed in a rad52-GB background. Another previously characterized IN mutant, in-2725 (7, 17, 34), had a phenotype similar to that of in-2600 (42).

The U5 tip was more complicated to analyze because these sequences are embedded in the TYA1 gene (Fig. 2). The PRE-U5 and PRE-U5-TIP mutants gave rise to comparable
numbers of His\(^+\) events in a RAD52 background, but PRE-U5-TIP gave rise to almost 20-fold-fewer His\(^+\) events than did the PRE-U5 mutant in a rad52-GB background (Table 1). Therefore, even though the TYAI mutation that was required to position the U5 tip for further mutagenesis partially inhibited Ty1 transposition, the additional alteration of the U5 tip in the PRE-U5-TIP mutation blocked transposition 20-fold more.

A low level of His\(^+\) events still occurred in the rad52-GB background (Table 1). Examination of these His\(^+\) isolates revealed that some of them were plasmid borne. This result suggests that these events were not residual Ty1 transposition events and were produced by RAD52-independent recombination.

Taken together, our results suggest that the His\(^+\) phenotype resulted from homologous recombination of Ty1HIS3 cDNA and not from de novo transposition. Recombination targets may have been one of the chromosomal Ty1 or single LTR elements or the marked Ty1\(m\)his3A1 element present on the inducing plasmid. Since the overall properties of in-2600, the tip mutants, and the PPT mutant were similar, the consequences of in-2600 transposition induction were characterized further.

**Rate of His\(^+\) prototroph formation of in-2600.** The rate of His\(^+\) prototroph formation was determined for strains yGS37 (RAD52) and yGS38 (rad52-GB), expressing either the wild-type or in-2600 pGTy1 plasmid (Table 2). Comparable rates of His\(^+\) prototroph formation were obtained when either in-2600 or the wild-type element was galactose induced in a RAD52 background. However, the rate of His\(^+\) prototroph formation decreased more than 150-fold when in-2600 was induced in a rad52-GB background. If we assume that all of the His\(^+\) prototrophs result from cDNA recombination and that these reflect 10 to 20% of the actual number of marked Ty1 events (the splicing efficiency of his3A1 is approximately 10 to 20% [13, 19]), then 1 to 2% (rate of His\(^+\) formation of in-2600 in strain yGS37 \(\times\) correction for splicing efficiency) of all induced cells undergo recombination of marked cDNA per generation.

**Multimeric arrays of the genomic marked Ty1 insertions generated with in-2600.** To further examine the marked genomic Ty1 insertions generated by in-2600 in strain yGS37, we obtained Ura\(^-\) plasmid segregants from 19 independent His\(^+\) isolates. Total DNA from the chromosomal cDNA recombinants was digested with BstEII, which cleaves Ty1\(H3m\)his3A1 once (Fig. 1 and 3), and Southern hybridization analysis was performed with a \(^{32}\)P-labeled his3A1 probe (Table 3). Surprisingly, almost all (18 of 19) of the in-2600 recombinants displayed a common fragment of about 6.6 kb. This fragment was the size expected for a tandem Ty1 insertion if the elements were in the same orientation, separated by a single LTR, and if at least one copy contained a functional spliced HIS3 gene. Comparable results were obtained with the PRE-U5-TIP, U3-TIP, and PPT mutants.

If the marked Ty1 insertions were present in the same orientation within an array, then Southern analysis with restriction enzymes that cleave at single unique positions within the marked Ty1 element would still generate a 6.6-kb fragment (note that marked Ty1 elements containing a functional spliced HIS3 gene should be only 104 bp shorter [6.5 kb] than the starting Ty1\(H3m\)his3A1 element, and this size difference was not resolved in our analysis). DNA from the 19 His\(^+\) derivatives was analyzed with \(HpaI\) or \(AflII\) (Fig. 1), and the characteristic 6.6-kb fragment was present; three examples are shown in Fig. 3. These results indicated that some of the marked Ty1 recombinants consisted of directly repeated arrays. Recombinants generally contained multiple \(HpaI\), BstEII, and \(AflII\) fragments marked with HIS3 or his3A1, and several recombinants also contained BstEII fragments that were smaller than 5 kb, the minimum size expected for a full-length Ty1\(Hm\)his3A1/HIS3-cellular DNA junction fragment. Since two junction fragments are expected if all of the elements within an array are present as one insertion, the variability in fragment sizes is consistent with the idea that some of the arrays are large and contain rearranged marked Ty1 elements. Ty1 elements present in both orientations and deleted Ty1 elements probably contribute to the complexity of these insertions, as is observed with the Ty1 multimers inserted at HML\(\alpha\) (31, 53). Additional analyses with \(PvuI\) and \(AflII\), which cleave near the Ty1 LTRs, generating a 2.4- or 2.5-kb fragment (indicated as 2.5 kb in Fig. 3), also support the interpretation that the seven representative His\(^+\) recombinants generated from in-2600 contained multimeric arrays separated by a single

### Table 1. RAD52-dependent recombination of Ty1HIS3 cDNA

<table>
<thead>
<tr>
<th>Ty1 mutation</th>
<th>RAD52</th>
<th>rad52-GB</th>
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<tbody>
<tr>
<td></td>
<td>Frequency (10(^{-3}))</td>
<td>Plasmid fraction (%)</td>
</tr>
<tr>
<td>Wild type</td>
<td>9.4 ± 1.5</td>
<td>0/594 (&lt;0.17)</td>
</tr>
<tr>
<td>in-2600</td>
<td>6.2 ± 2</td>
<td>98/323 (30)</td>
</tr>
<tr>
<td>PRE-U5</td>
<td>5.9 ± 0.1</td>
<td>5/102 (4.7)</td>
</tr>
<tr>
<td>PRE-U5-TIP</td>
<td>7.8 ± 1.1</td>
<td>2/34 (6)</td>
</tr>
<tr>
<td>U3-TIP</td>
<td>5.0 ± 0.5</td>
<td>8/47 (17)</td>
</tr>
<tr>
<td>PPT</td>
<td>8.7 ± 0.8</td>
<td>3/50 (6)</td>
</tr>
<tr>
<td>pr-1682</td>
<td>0.01</td>
<td>1/11 (9)</td>
</tr>
<tr>
<td>in-3796</td>
<td>&lt;0.006</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Plasmids were analyzed in isogenic strains yGS37 (RAD52) and yGS38 (rad52-GB). The mean transposition frequency is the mean fraction of His\(^+\) Ura\(^-\) colonies divided by the total number of Ura\(^-\) colonies. The plasmid fraction is the number of His\(^+\) plasmid events over the total number of events; the remaining His\(^+\) events are chromosomal. Dependence on the RAD52 gene is obtained by dividing the transposition frequency obtained in strain yGS38 by that obtained in strain yGS37, ND, not determined.

### Table 2. Rate of His\(^+\) formation following induction of wild-type and in-2600 Ty1 elements

<table>
<thead>
<tr>
<th>Ty1 mutation</th>
<th>Rate of His(^+) formation</th>
<th>Fold reduction (rad52/RAD52)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(5.3 ± 0.5 \times 10^{-4})</td>
<td>(10.7 ± 1.0 \times 10^{-4})</td>
</tr>
<tr>
<td>in-2600</td>
<td>((2.0 ± 0.14) \times 10^{-4})</td>
<td>((7.0 ± 1.0) \times 10^{-4})</td>
</tr>
</tbody>
</table>

* Rates were calculated by the method of Lea and Coulson (28). RAD52 dependence was calculated as described in the footnote to Table 1.
FIG. 3. Southern hybridization analysis of three independent His\(^+\) isolates after induction of in-2600 in strain yGS37. DNA was cleaved with HpaI (H), BstEII (B), or AfII (A), separated by electrophoresis on a 0.7% agarose gel, blotted to a nylon filter, and hybridized with a \(^{32}\)P-labeled his3AI probe. Below are the fragment sizes expected from a tandem array of marked Ty1 (Ty1-Ty1) elements separated by a single LTR. Since the isolates are His\(^+\), at least one member of the array must contain Ty1HIS3. All enzymes that cleave once within the marked Ty1 sequence give rise to a 6.5- or 6.6-kb fragment (shown as 6.6 kb), depending on whether the AI is present. All other designations are the same as in Fig. 1.

LTR (42). We have not confirmed, however, that all members of an array are separated by a single LTR; some may be separated by two LTRs, and rearranged Ty1 elements within an array may not be separated by any LTRs.

To determine whether plasmid sequences other than those derived from Ty1 were transferred to the yeast genome, we examined seven of the His\(^+\) derivatives described above with a \(^{32}\)P-labeled pBR322 probe. No hybridization was detected when the DNA was analyzed after BstEII digestion (42). These results reinforce the idea that only Ty1 cDNA was transferred to the genome.

Southern blot analysis following BstEII digestion was also performed with 17 His\(^+\) isolates that were recovered after galactose-induced expression of the wild-type pGS99 plasmid in strain yGS37. In contrast to the in-2600 mutant, the hybridization patterns of the wild-type Ty1-H3mhis3AI element were relatively simple (Fig. 4). Only one of 17 His\(^+\) derivatives contained a 6.6-kyBstEII fragment (Fig. 4, lane 5). Further analysis demonstrated that this isolate contained a marked Ty1 multimer (42). Although the presence of the 6.6-kyBstEII fragment was characteristic of a marked Ty1 multimer, it was probably derived from a de novo transposition event. Complex multicentric Ty transposition events have been observed at HML\(\alpha\) and a promoterless his3-\(\Delta\)A gene after transposition induction of a wild-type Ty element (53).

**Linkage of marked Ty1 insertions.** To determine the approximate chromosomal location and molecular linkage of the marked Ty1 elements that resulted from the induction of in-2600, we analyzed a chromosomal marker strain (Fig. 5, lanes 1), six representative independent His\(^+\) recombinants (lanes 2 to 7), and the parental strain yGS37 (lanes 8) by pulsed-field gel electrophoresis. Two pulse regimens were used: one that resolved chromosomes between 200 and 2,200 kb (Fig. 5A and B) and another that resolved chromosomes between 1,000 and 2,000 kb (Fig. 5C and D). Most of the insertions occurred in larger yeast chromosomes, as expected if the recombination targets (solo LTRs and Ty1 elements) are distributed throughout the genome (5, 22). When Southern analysis of the separated chromosomes was performed, a single chromosome hybridized with the his3AI probe in each of five recombinants, although one recombinant (Fig. 5, lanes 5) probably contains an insertion in each of two chromosomes. In the six recombinants, the chromosome containing the Ty1 marker migrated more slowly, suggesting that the altered chromosomes were at least 75 kb larger than the wild-type chromosome. These results also suggest that the Ty1HIS3/mhis3AI copies detected by the original Southern hybridization analysis were inserted into single chromosomes (Fig. 3 and Table 3). Southern hybridization analysis performed after digestion with NcoI, an enzyme that does not cleave Ty1-H3mhis3AI, displayed a single fragment in all cases (42). This hybridization pattern suggests that the marked elements within an array were contiguous.

**Multicentric insertions present in a rad52 background.** The frequency of His\(^+\) prototrophs decreased at least 100-fold when in-2600, PRE-U5-TIP, U3-TIP, or the PPT mutant was induced in strain yGS38 (Table 1). When DNA from these

*The frequency of tandem marked insertions in the genome was determined by Southern analysis using a \(^{32}\)P-labeled his3AI probe. The frequency of tandem marked insertions is the number of strains with marked tandem insertions divided by the total number of His\(^+\) strains analyzed.
His\(^+\) derivatives was cleaved by BssEII and analyzed by Southern hybridization using his3AI as the probe, multimers were detected with in-2600, U3-TIP, and PPT but not PRE-U5-TIP (Table 3). Multimers appeared more frequently in a rad52-GB background when Ty1 transposition was blocked by a mutation in IN (13 of 19) than when transposition was blocked by altering the U3 or U5 tip (2 of 22) or the PPT region (1 of 9). This result suggests that IN may interfere with multimer formation in the absence of a functional Rad52 protein.

Growth competition between strains containing Ty1HIS3 multimers and the parental strain yGS37. Previous studies performed with in-2600 and in-2725 indicate that these mutants do not efficiently transfer a Ty1 marker gene into the genome (7, 17, 34). The difference between our results and those of previous studies became clear when we tagged in-2600 with his3AI and repeated the transposition assays under conditions similar to those used previously. When we did not impose His\(^+\) selection immediately after transposition induction of in-2600 but instead allowed the cells to grow nonselectively in YEPD or SC-ura glucose prior to plating on SC-ura-his, the frequency of His\(^+\) prototrophs was much lower. To determine whether His\(^+\) cells derived from inducing in-2600 were lost during nonselective growth in YEPD, we performed growth competition experiments with each of five independent His\(^+\) isolates containing a multimeric insertion and the His\(^-\) parental strain yGS37. A significant reduction in the number of His\(^+\) cells was observed after 24 and 48 h of competitive growth (Table 4). No such reduction was detected when cells containing monomeric Ty1HIS3 insertions derived from in-2600 or wild-type Ty1-H3mbis3AI induction were competed with strain yGS37.

The reduction in the number of cells containing marked Ty1 multimers was due either to genetic instability or to a growth disadvantage of cells containing multimers. To distinguish between these two possibilities, we repeated the experiment without mixing the strains. If the His\(^+\) phenotype was genetically unstable, His\(^-\) cells should rapidly give rise to His\(^+\) cells. One of the multimeric His\(^+\) strains gave rise to 2 His\(^+\) derivatives out of 74 tested after 48 h of nonselective growth. The other four multimeric strains did not yield any His\(^+\) derivatives (42). Therefore, strains containing Ty1 multimers were probably lost from the population because they were at a growth disadvantage.

### Plasmid insertions

A number of plasmid-borne insertions containing HIS3 were detected after galactose induction of the in-2600, PRE-U5-TIP, U3-TIP, or PPT mutant in strain yGS37 (Table 1). To determine the organization of the marked Ty1 element(s) present on the pGTy1 plasmid, plasmid DNA from 31 independent His\(^+\) isolates were recovered in E. coli (42). Several plasmids were analyzed by PCR and restriction enzyme

<table>
<thead>
<tr>
<th>Insertion type</th>
<th>% His(^+) cells remaining</th>
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<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Multimeric</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>56 (39/70)</td>
</tr>
<tr>
<td>2</td>
<td>58 (32/55)</td>
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<tr>
<td>3</td>
<td>27 (16/59)</td>
</tr>
<tr>
<td>4</td>
<td>22 (13/60)</td>
</tr>
<tr>
<td>5</td>
<td>50 (31/63)</td>
</tr>
<tr>
<td>Monomeric</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>74 (37/50)</td>
</tr>
<tr>
<td>7</td>
<td>63 (34/54)</td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>25 (16/63)</td>
</tr>
<tr>
<td>9</td>
<td>34 (23/68)</td>
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</tbody>
</table>

* Stationary-phase cells of each of the His\(^+\) derivatives were mixed with an approximately equal number of His\(^-\) cells of the parental strain yGS37. The mixtures were diluted 100-fold in YEPD and grown with agitation at 30°C. The percentage of His\(^+\) cells was checked immediately after dilution (0 h) and after 24 and 48 h. Insertions: 1 to 5, mixtures of strain yGS37 and five independent in-2600 derivatives containing a Ty1HIS3/mbis3AI multimer; 6 and 7, mixtures of strain yGS37 with two independent in-2600 derivatives containing a Ty1HIS3 monomer; 8 and 9, mixtures of strain yGS37 with two wild-type derivatives containing a Ty1HIS3 monomer.
mapping from each His\(^+\) isolate, since both his3AI and HIS3 derivatives were recovered. Plasmids recovered from about 77% (24 of 31) of the His\(^+\) recombinants contained the AI or had the AI precisely removed; no other plasmid rearrangements were observed. Plasmids recovered from about 23% (7 of 31) of the His\(^+\) recombinants contained the AI or had acquired multimeric Ty1 elements, with one member of the array containing a functional HIS3 gene and the rest containing his3AI. In general, the organization of the marked multimeric Ty1 element(s) present on the pGTyl plasmid was similar to that of the chromosomal recombinants. The arrays were randomly arranged, separated by single LTRs, but contained only two or three marked Ty1 elements.

**Does mutating Ty1-IN elevate the level of multimer formation and cDNA recombination?** To address this question, we analyzed both chromosomal and plasmid recombination frequencies obtained with in-2600 and the wild-type Ty1-H3mhis3AI element (Tables 1 and 3). If we assume that Ty1-IN is not required for multimer formation or cDNA recombination, then the maximum frequency of marked chromosomal multimers for in-2600 should be the same as the maximum frequency of marked multimeric Ty1 insertions for the wild type; the maximum wild-type frequency of multimer formation is about \(5.6 \times 10^{-4}\) (frequency of wild-type chromosomal His\(^+\) events \([9.4 \times 10^{-3}]\times\) fraction of wild-type insertions that are multimers \([6.0 \times 10^{-6}]\)). However, chromosomal His\(^+\) events arose with a 7.3-fold-higher frequency of 4.1 \(\times 10^{-3}\) (frequency of in-2600 His\(^+\) formation \([6.2 \times 10^{-3}]\times\) fraction of in-2600 chromosomal insertions that are multimers \([9.5] \times\) fraction of in-2600 chromosomal recombinants \([0.7]\)) for in-2600 than for the wild type. Therefore, this comparison suggests that Ty1 multimer formation and cDNA recombination with chromosomal targets are elevated when integration is blocked.

The frequency of plasmid-borne His\(^+\) recombinants was also examined to address the efficiency of homologous recombination of Ty1 cDNA when IN is defective (Table 1). If we assume that Ty1-IN is not required for cDNA recombination with the pGTyl plasmid, then the maximum frequency of plasmid-borne His\(^+\) events for in-2600 should be the same as the maximum frequency of plasmid-borne His\(^+\) events for the wild type; the wild-type frequency of His\(^+\) plasmid events is about 1.6 \(\times 10^{-3}\) (frequency of wild-type His\(^+\) events \([4.1 \times 10^{-3}]\times\) fraction of wild-type plasmid recombinants \([1.7] \times\) fraction of in-2600 chromosomal recombinants \([0.7]\)). However, plasmid-borne His\(^+\) events arose with at least a 100-fold-higher frequency of 1.9 \(\times 10^{-3}\) (frequency of in-2600 His\(^+\) events \([6.2 \times 10^{-3}]\times\) fraction of in-2600 plasmid recombinants \([0.3]\)) for in-2600 than for the wild type. Furthermore, we probably underestimated the number of plasmid events, because cells containing both plasmid and chromosomal events were scored as a chromosomal event. These results suggest that recombination of Ty1 cDNA with plasmid targets is also elevated in an in-2600 mutant.

**Levels of total Ty1mhis3AI RNA, VLP-associated nucleic acids, and Ty1 proteins when integration is blocked.** We examined several steps in the Ty1 life cycle to determine the basis for the higher incidence of cDNA recombination and multimer formation when de novo integration is blocked. Increased Ty1 RNA levels or reverse transcription of the functional spliced HIS3 gene would cause higher levels of His\(^+\) formation that would probably be independent of Ty1-IN. To determine whether Ty1-H3mhis3AI RNA levels were altered in the in-2600 mutant, we performed Northern analysis using the PYK1 transcript as a loading control. The same level of total marked Ty1 RNA was observed when either the wild-type pGTyl plasmid or in-2600 was galactose induced in strain yGS37 (Fig. 6). An increase in the level of Ty1-H3mhis3AI splicing or reverse transcription of Ty1-H3HIS3 nucleic acid spanning the splice junction was analyzed by RT-PCR. HIS3 sequences were amplified by using total nucleic acid extracted from wild-type or in-2600 VLPs and oligonucleotide primers flanking the Al. The ratios between spliced and unspliced PCR products in wild-type Ty1 and in-2600 were similar, as determined by densitometry (42). Furthermore, it has been shown that the in-2600 mutant has normal levels of RT activity, Ty1 cDNA, and TYB1 proteins (7, 17, 34). The RT-PCR analysis reported above also supports the finding that VLPs isolated from in-2600 contain wild-type levels of Ty1 cDNA.

We reasoned that a change in the stability of the mutant in-2600 protein may allow greater access of homologous recombination proteins to Ty1 cDNA. Therefore, the persistence of VLP-associated proteins produced by the wild-type Ty1 element and in-2600 was examined by following the level of the IN protein p90-TYB1 and TYA1-encoded capsid proteins immunologically (Fig. 7). The levels of these Ty1 proteins are indicative of the presence of VLPs, even under conditions in which VLP maturation is severely inhibited (1, 24, 36, 57). To determine the persistence of these Ty1 proteins over long time periods, cells containing either the wild-type pGTyl or in-2600 plasmid were galactose induced for 24 h and transferred to SC-ura glucose medium for 4 h to repress GAL1-promoted Ty1 expression, and then the protein synthesis inhibitor cycloheximide was added. Total protein was extracted from cells withdrawn at the time of glucose addition (Fig. 7, lanes 1 and 5) and at various times up to 88 h after glucose addition (Fig. 7, lanes 2 to 4 and 6 to 8). Samples containing equal amounts of total protein were separated by electrophoresis and immunoblotted. The resulting filter was reacted first with an IN antiserum (Fig. 7A), which recognizes IN and its precursors, and then with a VLP antiserum (Fig. 7B), which recognizes the capsid proteins p58-TYA1 and p54-TYA1. Both the mutant in-2600 protein (Fig. 7A, lanes 1 [time zero; glucose addition], 2 [16 h after glucose addition], 3 [40 h], and 4 [88 h]) and the wild-type IN (Fig. 7A, lanes 5 [time zero], 6 [16 h], 7
were induced for the parental wild-type strain yGS37 expressing in-2600 or the wild-type pGTyl plasmid. After the strains were induced for transposition by growth on galactose, GAL1-promoted Ty1 expression was repressed by addition of glucose, and cellular protein synthesis was inhibited by addition of cycloheximide 4 h later. Total protein was isolated from in-2600 (lanes 1 to 4) and the parental wild-type strain (lanes 5 to 8) for immunoblot analysis at the time of glucose addition (lanes 1 and 5) and after subsequent incubation for 16 h (lanes 2 and 6), 40 h (lanes 3 and 7), and 88 h (lanes 4 and 8). Proteins were separated by electrophoresis on a 10% (wt/vol) polyacrylamide gel, transferred to an Immobilon-P membrane, and cross-reacted with antibodies to IN (A; antiserum B2). This filter was allowed to decay and then cross-reacted with antibodies to TYAI capsid proteins (B; VLP antisem). The positions of IN precursors, mature IN (p90-TYA1), and the capsid proteins p58-TYA1 and p54-TYA1 are shown (1, 24, 36, 57). The capsid proteins p58-TYA1 and p54-TYA1 were not resolved under the gel electrophoresis and immunoblotting conditions used here because of the amount of protein loaded per lane (refer to Materials and Methods). When less protein from in-2600 and the wild type was analyzed with the VLP antisem, however, approximately equal amounts of p58-TYA1 and p54-TYA1 were observed at each time point for both strains (19). The minor bands observed between IN and the IN precursors are probably caused by cellular proteolysis because they are present in immunoblots prepared from a Ty1-PR mutant (24).

[40 h], and 8 [88 h]) showed similar decay kinetics after pGTyl expression was repressed and protein synthesis was inhibited. When the level of capsid proteins was examined, comparable amounts of these proteins were observed at each time point for both in-2600 and the wild type (Fig. 7B). The level of protein in the samples did not permit a clear separation of the p58-TYA1 precursor from the mature p54-TYA1 capsid protein. When less total protein was immunoblotted and cross-reacted with the VLP antisem, however, approximately equal amounts of p58-TYA1 and p54-TYA1 were observed at each time point for both in-2600 and the wild type (19). These results suggest that the increased accessibility of Ty1 cDNA to homologous recombination machinery in in-2600 cannot be accounted for by a change in stability of IN or the VLP capsid proteins.

DISCUSSION

Most of the information concerning the mechanism of Ty1 integration has come from studies performed in vitro with purified Ty1 VLPs (17, 18). Ty1 integration has been difficult to study in vivo, however, because of a high background level of presumed nontranspositional insertion events (7, 17, 34), thus challenging the idea that IN is the only protein needed for transpositional integration in vivo. In this study, we developed an assay that distinguishes de novo transposition from cDNA recombination and used this assay to determine whether Ty1 mutants blocked at various steps in the retrotransposition process undergo cDNA recombination.

Surprisingly, Ty1 mutants defective in integration or in priming plus-strand DNA synthesis gave rise to essentially wild-type levels of integration events, as monitored by the his3A1 indicator gene. Unlike the case for de novo Ty1 transposition, however, these insertions consisted of multimeric Ty1 element arrays, required the recombination and repair gene RAD52 for their high incidence, and were found on the pGTyl expression plasmid as well as in the genome. The apparent increase in cDNA recombinaction took place without altering the levels of total marked Ty1 RNA, VLP-associated RNA and DNA, or TYAI and IN protein turnover. These results suggest that Ty1 cDNA is more efficiently utilized for homologous recombination with endogenous Ty elements or LTRs when IN-mediated integration is blocked.

Ty1 mutants that are defective in PR or RT/RH yielded very few cDNA recombinants. Ty1-PR mutants have multiple defects, including accumulation of unprocessed TYAI and TYB1 proteins and immature VLPs (1, 36, 57). Perhaps pr-1062 is defective for homologous recombination of Ty1 cDNA as well as transposition because VLPs from PR mutants contain very little Ty1 RNA that can be reverse transcribed (57). The inability of in-3796 to yield high levels of cDNA recombination reinforces the hypothesis that Ty1 RT/RH is absolutely required for producing Ty1 cDNA; no other cellular activity can substitute. Similar results have been obtained by Melamed et al. (32) using a defective Ty1-RT/RH mutant and a targeted cDNA gene conversion system.

We performed most of our analyses with the previously characterized in-2600 mutant (7, 17, 34). This mutant has wild-type levels of Ty1-IN antigen and RT activity but has been reported to be partially transposition defective in vivo and integration defective in vitro. However, when we marked in-2600 with his3A1, essentially wild-type rates of His+ cells were observed. Almost all of the chromosomal Ty1HIS3/ his3A1 insertions were present in multimeric arrays. Pulsed-field gel electrophoresis from a representative number of chromosomal His+ derivatives suggested that the altered chromosomes were at least 75 kb larger than those in the parental strain yGS37. Our results clearly demonstrate that His+ derivatives containing chromosomal Ty1 multimers do not compete as well with the wild-type parent as strains containing monomeric insertions, even though the multimers are genetically stable. Perhaps some feature of the multimeric array, such as its size, organization, or location within a chromosome, altered the competitiveness of these strains. We have not, however, eliminated the possibility that the structure of an array contributes to its electrophoretic mobility, nor have we determined the minimal number or arrangement of Ty1 elements that must be present within a multimer to alter strain competitiveness.

We repeated the transposition assays under conditions similar to those described previously (7, 17, 34) with in-2600 and found that the severity of the transposition defect, as determined by the number of His+ events, is dependent on the incubation time on nonselective plates after galactose induction. The number of recoverable His+ cells decreased dramatically as the incubation time on nonselective plates increased. Therefore, cells containing multimeric arrays were not detected in earlier studies because cells containing the arrays were overgrown either by cells that did not contain marked insertions or by rare cells containing monomeric insertions. The fact that we can directly select for cDNA-mediated events by using his3A1 in RAD52 and rad52 strains allowed us to detect His+ cells containing multimeric insertions, even though there was growth competition and a large excess of His- cells, and to distinguish de novo transposition events from homologous recombination of Ty1 cDNA. Note that plasmid-borne events also went undetected in the other studies.
because the mutant elements were not marked with a retro-
transposition indicator gene, such as his3AI. Taken together, these
results show that Ty1-IN is absolutely required for transpo-
sational integration in vivo. It also follows from our studies
that other negative modulators of de novo Ty1 transpo-
sition, such as increased temperatures (7, 32, 38), may
enhance the level of cDNA recombination.

To learn more about the possible interactions between
IN-dependent integration and cellular homologous recombi-
nation pathways, we constructed mutants in which Ty1-IN
remained active but the sites of IN action were mutated. The
U3-TIP and PRE-U5-TIP mutations removed the conserved
terminal dinucleotides TG and CA, respectively, that are
required for Ty1 integration in vitro (18). In general, the tip
mutants exhibit phenotypes similar to those of in-2600. Our
results extend the results obtained from the integration assays
performed in vitro (17, 18), by showing that a change in one of
the tips is sufficient to block IN activity on a given cDNA and
that a defective IN is not required to enhance cDNA recom-
bination.

We also analyzed a Ty1 mutant that lacks the putative PPT.
Such a mutant should be able to produce minus-strand Ty1
cDNA but not full-length double-stranded Ty1 cDNA. The
PPT is the site where plus-strand DNA synthesis is usually
primed by Ty1 RNA molecules produced by RH action on the
RNA/minus-strand DNA hybrid. In the PPT mutant, we
expected that normal plus-strand priming would not take place
but that other plus-strand priming sites could be used (37).
Interestingly, apparent full-length Ty1 multimers were still
formed by the PPT mutant, even though incompletely reverse
transcribed single-stranded cDNA should be recombogenic
in yeast cells (44, 51). Therefore, partially reverse transcribed
Ty1 cDNA molecules may be able to recombine to form
full-length elements. Understanding the molecular basis of this
result will require characterizing the reverse transcription
products present in VLPs from the PPT mutant. Melamed et
al. (32) also found that blocking minus-strand synthesis by
mutating the PBS abolishes targeted gene conversion by Ty1
cDNA. It will be interesting to determine whether blocking
other steps in the process of Ty1 retrotransposition, such as
development of Ty1 RNA present in the RNA-DNA duplex by
RH, also leads to high levels of cDNA recombination and
multimer formation.

Since the chromosomal insertions described in this work are
not selected at a specific locus, we have assumed that preex-
isting LTR or Ty1 sequences served as targets for homologous
recombination. Two characteristics of the His+ events support
this idea. First, pulsed-field gel analyses show that multiple
chromosomes served as the target for the multimeric inser-
tions. This result would be expected if Ty1 cDNA recombines
with Ty elements or LTRs dispersed in the genome. Second,
the plasmids containing tandem marked Ty1 elements are
readily explained as products of homologous recombination
between Ty1 cDNA and the element on the pGTy1 plasmid.
Since both plasmid and chromosomal events contained Ty
multimers that were dependent on RAD52 for their increased
incidence, the randomly targeted chromosomal insertions
should also be the products of homologous recombination with
genomic Ty sequences. The differences between chromosomal
and plasmid insertions in terms of their frequency, size, and
structural complexity may be due to stricter selection for
shorter insertions in the plasmid.

The Ty1 cDNA recombinants observed in this study show
certain differences from and similarities to Ty1 cDNA-medi-
ated gene conversion of a specific target Ty1 element (32). In
contrast to our results, the homologous recombination events

that Melamed et al. (32) observed between Ty1Neo cDNA and a
Ty1URA3 element inserted at the LYS2 locus were appar-
ently simple replacement events; no multimeric insertions were
observed. There are major differences in the cDNA recombi-
nation assays that may explain the absence of multimeric
insertions at the his2:Ty1URA3 locus. Melamed et al. (32)
performed their experiments with a wild-type pGTy1 element
at 32°C, which is inhibitory for Ty1 transposition (38). This
temperature block has not been strictly defined but is corre-
lated with a temperature-sensitive RT activity (23). The cDNA
intermediates formed at higher temperature undergo homol-
ogous recombination but may not be able to efficiently form
multimers. Another difference is that gene conversion events
are required in the selection for G418-resistant Ura+ recom-
binants. Multimers may not stably form under those condi-
tions. Finally, the LYS2 region of the genome could be a cold
spot for multimer formation.

There are marked similarities between Ty1 multimers
formed when de novo integration is blocked and multimers
formed as a result of transposition events (31, 53). Some of
the Ty1-induced mutants resistant to α-pheromone are caused by
Ty1 transposition events at HMLα, where multimeric Ty1
transposition events activate HMLα expression. Multimeric
Ty2 element transposition events have also been shown to
activate the plasmid-borne his3-A4 allele. Although the Ty
integration events that give rise to the multimers at HMLα and
his3-A4 are different from the multimeric genomic and plasmid
cDNA recombinants described here, the structures of the
arrays are remarkably similar. The arrays can be very large,
can contain both directly repeated as well as rearranged Ty1
elements, and are usually separated by single LTR, and are gen-
etically stable.

The his3AI indicator gene has been used to detect the pro-
duction of functional pseudogenes and cDNA-mediated
gene conversion events (15, 16, 16a). The His+ events that are
recovered when just his3AI is expressed from the GAL1
promoter are structurally similar to those formed when IN-
mediated integration was blocked. This similarity is best illus-
trated by the plasmid-borne His+ events. In both studies, these
events result from homologous recombination between HIS3
cDNA and plasmid his3AI sequences. No other plasmid rearran-
gements are detected in this simple replacement event. Two
differences were observed between plasmid events obtained by
blocking IN-mediated integration and plasmid events obtained
with the HIS3 pseudogene system. First, multimeric insertions
were observed in about 25% of the plasmid-borne His+ events
when IN-mediated integration was blocked, whereas only
intron loss is observed among the plasmid events from the
HIS3 pseudogene system. This difference may be due to
homology created by the Ty1 sequences present on the pGTy1
plasmid. Second, the level of plasmid-borne His+ events
obtained with in-2600 decreased almost 100-fold in a rad52
mutant, whereas the level of plasmid events obtained with the
HIS3 pseudogene system decreases only 2-fold in a rad52
mutant (16). The reason for this marker effect is unknown, but
dramatically different reductions in gene conversion rates of
between 3- and 300-fold have been observed at different loci
in rad52 null mutants (39). Several chromosomal pseudogenes
that contain HIS3 sequences embedded in repeated Ty1 ele-
ments and are dependent on Ty1 RT/RH for their formation
have been obtained. It has not been determined whether these
chromosomal pseudogenes insert via IN or by homologous
recombination. Recently, chromosomal HIS3 pseudogenes
have also been obtained in yeast strains by using a human L1
RT (16a).

The results presented here and elsewhere (15, 16, 32) allow
us to propose a model for Ty1 cDNA recombination and multimer formation in *S. cerevisiae*. The product of Ty1 reverse transcription is a genome-length cDNA that can enter two distinct recombinational pathways. If Ty1 retrotransposition is functioning properly, Ty1 cDNA undergoes transpositional integration using Ty1 machinery. Ty1-IN may be associated with the cDNA termini in such a way that blocks access to homologous recombination proteins. If Ty1-IN and the cDNA tips cannot interact correctly, then Ty1 cDNA is utilized for homologous recombination. Aberrant association between IN and the cDNA termini can be enhanced by a variety of genetic or environmental alterations, including mutations in IN or the cDNA tips, incomplete reverse transcription, or possibly elevated temperatures (32, 42). In addition to the results presented in this work, two other key observations support our model. First, genetic, biochemical, and biophysical studies suggest that Ty1 VLPs are loosely organized structures that are permeable to a variety of agents (8, 15, 16, 55). Therefore, the permeability of VLPS may also contribute to homologous recombination of Ty1 cDNA. Second, linear DNA is highly recombinogenic in yeast cells and sometimes undergoes homologous recombination with chromosomal sequences to form simple tandem repeats when introduced during transformation (reference 39 and references therein). Further tests of this model will come from assessing the role that other recombination genes have on Ty1 cDNA recombination and by characterizing Ty1 IN mutants that do not permit transpositional integration or cDNA recombination.

How multimers form during normal retrotransposition, and the reasons for the apparent ubiquity of these rearrangements when integration is blocked or when pseudogenes are formed, is not clear (15, 53). The presence of rearranged elements within the multimeric Ty1 recombinants studied here and at *HMLa* suggests that multimer formation is more complicated than amplification of a single insertion or concatemerization of linear DNA. Multimers may form by homologous recombination using cellular machinery or by aberrant strand transfer during reverse transcription. However, our results cannot distinguish between the role that RAD52 may play in multimer formation from its role in recombination of Ty1 cDNA. Explanations for homologous recombination or aberrant reverse transcription require that portions of the Ty1 integration precursors be available to each other and to cellular homologous recombination proteins (see above). Ty1 VLPs may also be permeable enough to permit interactions between cDNAs, although attempts to demonstrate the existence of multimeric Ty1 DNA in wild-type or *in-2600* VLPs have been unsuccessful (19). Alternatively, homologous recombination of monomeric Ty1 cDNA or, to a lesser extent, IN-mediated integration might be an initiating event for multimer formation. This view requires that a newly recombined or integrated Ty element be preferred over the endogenous elements in the genome as a target for further recombination with unintegrated Ty1 cDNA. The high level of multimer formation observed may be caused by transient nicks or gaps that result from the initial recombination-integration event, since these lesions are recombinogenic in yeast cells (39). In fact, mitotic recombination between the incoming Ty1 LTRs or with Ty1 sequences flanking de novo integration sites apparently increases during or soon after Ty1 integration (48).

Chromosomes in *S. cerevisiae*, as in many other organisms, contain subtelomeric elements located adjacent to the G-rich repeats (10). The X (Ty5) and Y' families of subtelomeric repeats have certain structural features characteristic of transposons, but it is not known whether these elements are capable of transpositional movement (10, 50, 58). Recently, Lundblad and Blackburn (29) observed that expansion of Y' elements partially rescues the lethality of an est1 mutation. Since est1 mutants are defective in telomere replication, the accumulation of Y' element arrays and associated G1-3T repeats on many yeast chromosomes restores telomere function. Interestingly, the Y' element arrays and multimeric Ty1 insertions observed when integration is blocked share the following properties: the arrays can be large, contain both full-length and rearranged elements, and form by a RAD52-dependent pathway. These striking similarities suggest that Y' expansion can occur through an RNA intermediate.

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