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Multimeric Arrays of the Yeast Retrotransposon Ty

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We have identified a novel integrated form of the yeast retrotransposon Ty consisting of multiple elements joined into large arrays. These arrays were first identified among Ty-induced α-pheromone-resistant mutants of *MAT*a cells of *Saccharomyces cerevisiae* which contain Ty insertions at *HMLα* that result in the expression of that normally silent cassette. These insertions are multimeric arrays of both the induced genetically marked Ty element and unmarked Ty elements. Structural analysis of the mutations indicated that the arrays include tandem direct repeats of Ty elements separated by only a single long terminal repeat. The Ty-*HML* junction fragments of one mutant were cloned and shown to contain a 5-base-pair duplication of the target sequence that is characteristic of a Ty transpositional insertion. In addition, the arrays include rearranged Ty elements that do not have normal long terminal repeat junctions. We have also identified multimeric Ty insertions at other chromosomal sites and as insertions that allow expression of a promoterless *his3* gene on a plasmid. The results suggest that Ty transposition includes an intermediate that can undergo recombination to produce multimers.

The yeast retrotransposon Ty1 has a life cycle similar to that of retroviruses (6, 60), except that it does not appear to have an infectious extracellular stage. The integrated element has two genes, *TYA* and *TYB*, which correspond to the *gag* and *pol* genes of retroviruses (18). These genes are bounded by long terminal repeats (LTRs) called δ elements that have the same relationship to the transcribed element as do the LTRs of retroviral proviruses. As is characteristic of retroviruses and retrotransposons, Ty integration creates a short duplication (5 base pairs in the case of Ty) of the target sequence (23, 27). Like retroviruses and other retrotransposons, the integrated form of Ty elements is generally found as a monomer. Exceptions have been reported for integration-defective viruses (30) or, in the case of Ty, as products related to self integration (68). Some yeast strains have direct repeats of Ty elements (16). We report here the characterization of Ty insertions that consist of complex multimeric arrays.

Spontaneous insertions of Ty elements have been studied at a variety of genes at which yeast mutations can be selected (6). Transposition of this element can be regulated by placing the Ty1 element under the control of an inducible yeast promoter (*GAL1*) (8). The Ty element can be genetically and physically tagged by the addition of a selectable gene (8, 10, 29, 36). The ability to induce transposition of genetically marked Ty elements has been developed into a system that uses genetically marked Ty1 elements as insertional mutagens (29).

Ty elements, like other transposons and retroviruses, can alter the expression of genes near the site of their insertion. In most cases in which a Ty element has changed the regulation of a gene, it is inserted in the 5′ regulatory region of the gene and is oriented so that the direction of transcription of the Ty element is opposite to the direction of transcription of the affected gene (6, 51). Such insertions frequently cause the altered gene to be subject to mating-type-specific regulation (repression in **a**/α cells) similar to the regulation of Ty elements themselves. Less frequently, the Ty element is inserted in the 5′ region in the same direction of transcription as the affected gene (9). The only report of a Ty insertion 3′ of a gene altering its expression involved the apparent use of the transcription terminator in the Ty LTR to suppress the defect of a *cyc1* mutation that was a deletion of the normal terminator of that gene (68).

We used Ty as an insertional mutagen to isolate yeast mutants defective in the sexual cycle. Ty transposition was induced in cells with the **a** mating type, and cells resistant to the mating pheromone α-factor were selected. Normally, **a** cells respond to α-factor by arresting in the G1 portion of the cell cycle in preparation for mating with α cells. Analysis of spontaneous mutations to α-factor resistance has defined the gene for the α-factor receptor (*STE2*) and six other genes (*STE4*, *STE5*, *STE7*, *STE11*, *STE12*, and *STE18*) involved in the sexual cycle (31, 43, 65). An additional class of α-pheromone-resistant cells can be derived from the activation of a normally silent copy of the cell type-regulatory genes. Most cells of the **a** mating type have the *MAT***a** allele at the mating type locus and a complete, but unexpressed, copy of the *MATα* gene at *HMLα*. This unexpressed gene is the donor for the sequences used to gene convert from *MAT***a** to *MATα* in homothallic yeast (60). Therefore, *MAT***a** cells can also become resistant to α-factor because of mutations that lead to the expression of the silent copy of *MATα* at *HMLα*. When the *HMLα* locus is expressed, the cells have the nonmating, α-pheromone-resistant phenotype characteristic of **a**/α diploid cells. Six transacting regulators (*SIR1*, *MAR1*, *SIR3*, *SIR4*, *NAT1*, and *ARD1*) involved in the repression of *HML* have been identified by this approach (31, 38, 44, 49, 50, 64). We have identified a novel class of mutations in which the expression of the *HMLα* locus was due to the insertion of a marked Ty element at *HML* (M. F. Mastrangelo et al., unpublished data). We report here the molecular characterization of four of these Ty insertional mutations at *HMLα*, including the demonstration that they are multimeric arrays of Ty. Evidence for the formation of such arrays at other sites is also presented.

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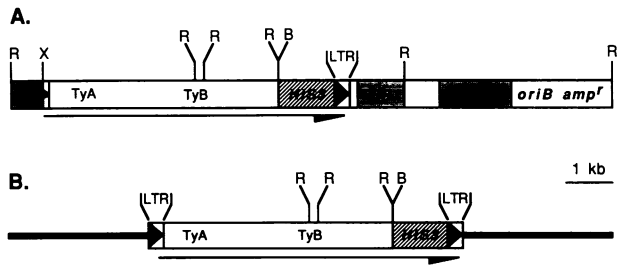

FIG. 1. (A) A linearized representation of the circular plasmid pGTy1H3*HIS3*HH4B#84 carrying a galactose-regulated, *HIS3*-marked Ty element. (B) Diagram of an insertion of a single *HIS3*-marked Ty element. The solid line represents yeast target site DNA. *Eco*RI (R), *Bam*HI (B), and *Xho*I (X) sites are indicated. The arrows indicate the direction of transcription of both the Ty element and the *HIS3* gene. LTR, δ elements; *oriB*, bacterial origin of replication; amp^r, β-lactamase gene.

MATERIALS AND METHODS

Materials. Sequenase version 2.0 and TAQuence were purchased from U.S. Biochemical Corp. (Cleveland, Ohio). The T3 and M13-20 sequencing primers were purchased from New England BioLabs, Inc. (Beverly, Mass.), and the T7 sequencing primer was purchased from Stratagene Cloning Systems (La Jolla, Calif.). BioTrace RP, a charge-modified nylon membrane, was purchased from Gelman Sciences, Inc. (Ann Arbor, Mich.). Hybond N, a neutral nylon membrane, was obtained from Amersham Corp. (Arlington Heights, Ill.). Nitrocellulose filters (BA-85) for plaque hybridizations were purchased from Schleicher & Schuell, Inc. (Keene, N.H.). *Eco*RI-digested, dephosphorylated λ ZAP vector and Gigapack Gold in vitro λ packaging extract were purchased from Stratagene. Restriction enzymes were purchased from New England BioLabs, Inc., Pharmacia-LKB Biotechnology (Piscataway, N.J.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). The 1-kilobase (kb) DNA ladder was purchased from Bethesda Research Laboratories, Inc., (Gaithersburg, Md.). [α-^{32}P]dCTP (800 or 3,000 Ci/mmol) for preparing restriction fragment probes and [α-^{35}S]dATP (>1,000 Ci/mmol) for sequencing reactions were purchased from Amersham Corp.

Strains and plasmids. The bacterial strains, XL1-Blue (15) and BB4 (59), and the bacteriophage M13 R408 (53) were obtained from Stratagene. The bacteriophage λ ZAP and the plasmid Bluescript SK(−) have been described previously (59). The starting yeast strain JSS56-11B and the selection of Ty-induced α-factor-resistant mutants have been described previously (29). MMB12, MMB21, MMD25, and MMD37 are α-factor-resistant strains in which *HML* has been activated by Ty insertion (M. F. Mastrangelo, unpublished data). The high-copy-number plasmid, pGTyH3*HIS3*, containing a *GAL1*-regulated Ty element marked with a *HIS3* gene has been described previously (29). The plasmid pGTy1H3*HIS3*HH4B#84 is closely related to pGTyH3 *HIS3*, except that the former contains a 288-base-pair (bp) *Bam*HI-*Bgl*II fragment of Rous sarcoma virus (obtained from S. Hughes) inserted into the unique *Bam*HI site 5′ to the *HIS3* gene in the vector as an additional molecular tag and has *TRP1* as the selectable marker on the plasmid (Fig. 1A). The *his3-Δ4* target plasmid, pAB100, has been described previously (8).

DNA digestions and Southern blotting. Yeast DNA was prepared by the guanidine hydrochloride method (33). Restriction fragment probes were gel purified and labeled by the random primer method (24) with [^{32}P]dCTP. DNA was digested with various restriction enzymes and electrophoresed through either 0.50 or 0.75% agarose by standard methodologies (42). Alkaline capillary DNA transfer to BioTrace RP was by the method of Reed and Mann (48). Labeled probes were hybridized in 6× SCP (1× SCP is 100 mM NaCl, 30 mM NaH_2PO_4, 1 mM Na_2EDTA [pH 6.2])–1% *N*-lauroylsarcosine–10% dextran sulfate–0.1 mg of sheared, salmon sperm DNA per ml at 65°C. Blots were washed at 65°C in 2× SCP–1% sodium dodecyl sulfate. In cases where membranes were reprobed, the label was removed with 0.4 M NaOH (42°C) for 30 min followed by two to three washes in 0.1× SCP–0.1% sodium dodecyl sulfate (65°C). *HIS3* probes were prepared with either the 913-bp *Cla*I fragment from pGTyH3*HIS3* or the 1,165-bp *Cla*I fragment from pGTy1H3*HIS3*HH4B#84. *HML*α probes were obtained from the 6.6-kb *Bam*HI fragment of *HML*α (25).

Chromosomal DNA separations. Samples of chromosomal DNA were prepared by the agarose bead technique (46). Chromosomal DNAs were separated by contour-clamped homogeneous electric field (CHEF) gel electrophoresis (18) in a model HEX-CHEF 6000 apparatus from C.B.S. Scientific Co. (Del Mar, Calif.). The gel was run at 8°C for 42 h at 200 V in 0.5× TBE (1× TBE is 90 mM Tris-borate and 2 mM EDTA) with an 83-s switch time. DNA was stained in ethidium bromide and destained in deionized water prior to being photographed. Chromosome assignments were made by comparison with the standard strain AB972 (17). The DNA was then fragmented by UV irradiation, denatured in 0.5 M NaOH–1.5 M NaCl for 45 min, and capillary blotted to a Hybond N unmodified nylon membrane in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (42). The DNA was cross-linked to the membrane by UV irradiation (308 nm; Fotodyne model 3-3000 DNA transilluminator) and was then hybridized in 6× SSC–5× Denhardt solution (42)–0.5% sodium dodecyl sulfate containing 0.02 mg of sheared denatured calf thymus DNA per ml. Half of the blot was probed with *HML*α, and the other half was probed with *HIS3* sequences.

Preparation of an *Eco*RI λ bacteriophage library. DNA from MFM1-1A, a segregant carrying the *hmlα-925* mutation from MMD25, was digested with *Eco*RI and ligated to *Eco*RI-digested, dephosphorylated λ ZAP DNA. The ligation mixture was packaged in vitro with Gigapack Gold, infected into bacterial strain BB4, and plated onto NZYM agar (42). The library was amplified once (42), and subsequent screenings were performed on platings of the once-amplified library.

Cloning Ty*HIS3*-Ty and *HML*α-Ty junction fragments. For isolation of specific sequences, 1×10^5 to 5×10^5 plaques were screened. Plaque lifts were performed by standard techniques (42) with nitrocellulose filters. The 3′ *Eco*RI junction fragments from the Ty*HIS3* elements were obtained as plaques homologous to both a *HIS3*-specific and an LTR-specific probe (the 335-bp *Bgl*II-*Hph*I 3′ LTR fragment of TyH3). The centromere-proximal *Hin*dIII-*Bam*HI fragment of the 6.6-kb *Bam*HI *HML*α clone was used to obtain the centromere-proximal *Eco*RI Ty-*HML* junction clone. The centromere-distal Ty-*HML*α *Eco*RI junction fragment was obtained by homology to a *HML*α W region probe (the 488-bp *Hha*I fragment; Fig. 2B) and a Ty ε region probe. The Bluescript SK(−) plasmids containing the *Eco*RI junction fragments were obtained in all cases by the in vivo excision method (59).

Sequencing. DNA was sequenced by the dideoxynucleotide method (54) with Sequenase version 2.0 or TAQuence

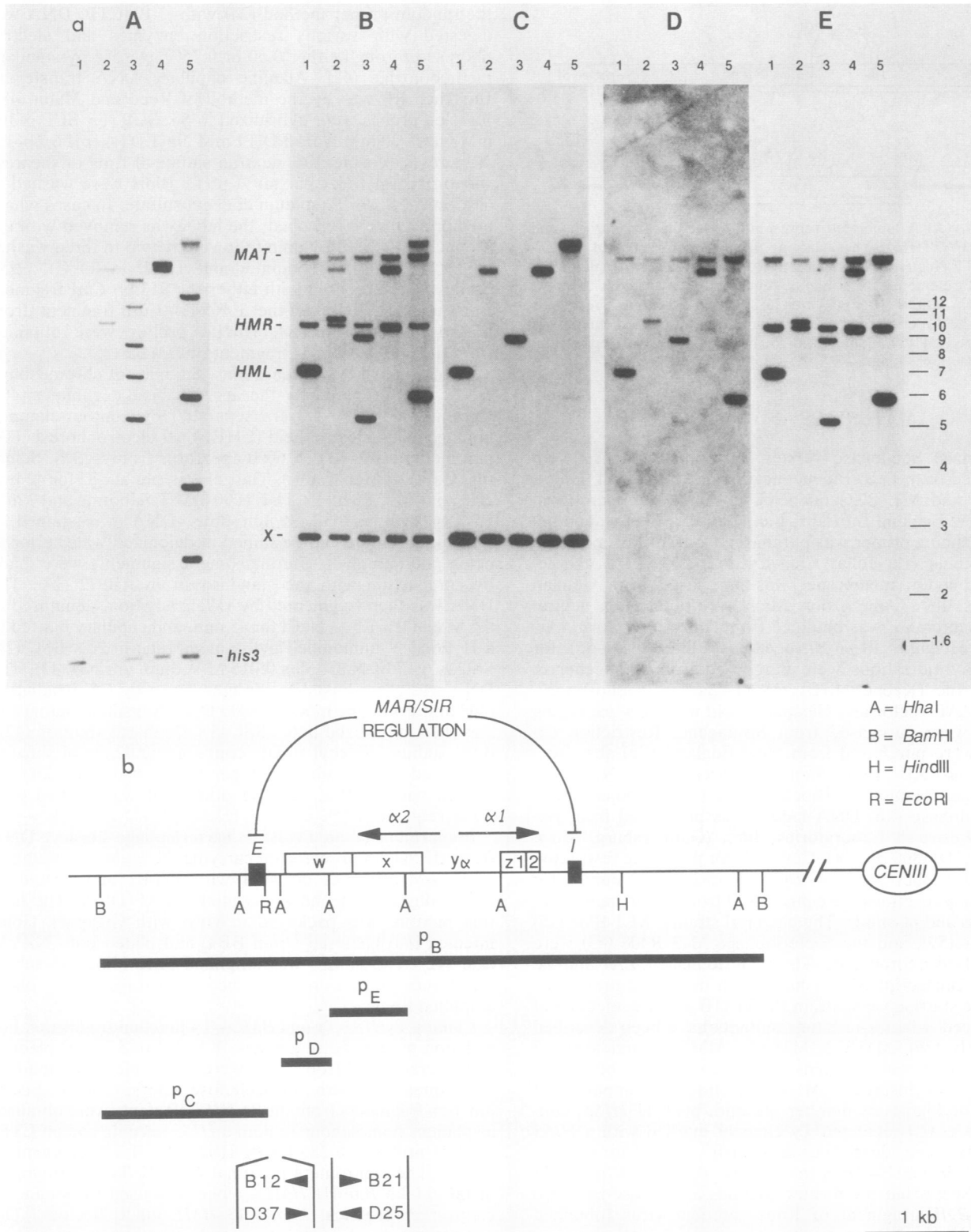

FIG. 2. Physical analysis of four *hmlα*::Ty mutants. (a) For all panels, DNA from strains JSS56-11B (lanes 1), MMB12 (lanes 2), MMB21 (lanes 3), MMD25 (lanes 4), and MMD37 (lanes 5) was digested with *Bam*HI, separated on a 0.75% agarose gel, and then blotted to a BioTrace RP membrane. (b) *HMLα* and the restriction fragment probes (p_B through p_E) used for panels B through E (shown in part a), respectively. The same filter was sequentially hybridized with *HIS3* (panel A), the complete *Bam*HI fragment of *HMLα* (panel B, probe p_B), the centromere-distal 1.7-kb *Bam*HI-*Eco*RI fragment of *HMLα*; (panel C, probe p_C), and two *Hha*I fragments from *HMLα* (panel D, probe p_D; panel E, probe p_E). The position of bands corresponding to *HMLα*, *HMR***a**, and *MAT***a** in the starting strain (JSS56-11B) are marked. An additional band that cross-hybridizes with probes p_B and p_C is labeled X. The insertion sites of the Ty arrays in the *hmlα*::Ty mutants are indicated at the bottom of the diagram. The arrowheads indicate the orientation of the *HIS3* gene on the *Bam*HI fragment containing the Ty-*HML* fusion. The W, X, Yα, Z1, and Z2 regions and the E site (E) and I site (I) have been defined previously (3, 25).

according to the recommendations of the manufacturer. Alkaline minipreps (4) were used for most of the sequencing, although in some cases, plasmids purified on isopycnic cesium trifluoroacetate gradients were used. The sequence of the unusual Ty LTR in pKWHML1-1 was obtained from a fragment extending from a *Sna*BI site near the end of TyB to a *Pst*I site at the end of *HIS3* subcloned into Bluescript SK(−). The centromere-distal Ty-*HMLα* junction was obtained from a subclone extending from a *Xho*I site present in the LTR to the *Eco*RI site near the E site. The centromere-proximal Ty-*HML* junction sequence was obtained with a synthetic oligonucleotide (5′-CGGTGAGCCTCTGGC-3′) present in the open reading frame of *HMLα2* (3) as a primer to sequence from *HMLα* into the LTR.

Analysis of His$^+$ revertants of *his3-Δ4*. His$^+$ revertants from strains containing the *his3-Δ4* target plasmid pAB100 and pGTy2-917 were generated and analyzed essentially as described previously (8, 9, 19a). At least 10 ampicillin-resistant *E. coli* HB101 transformants, which harbor putative Ty insertions in pAB100, were analyzed by colony hybridization with a Ty-specific probe. Rapid plasmid preparations were then analyzed by digestion with restriction enzymes *Bgl*II and *Eco*RI plus *Pst*I and other enzymes to classify the plasmids. Plasmids containing multimer arrays were analyzed either after recovery in bacteria or in the original His$^+$ revertant yeast strain. The DNA fragments used as hybridization probes in these experiments were derived from *HIS3* or from the Ty2-917 LTR.

Unselected transpositions. Standard procedures were used to generate unselected Ty1-H3*HIS3* transpositions (20, 29). Strain JSS56-11B containing pGTy1H3*HIS3*HH4B#84 was grown on SC-TRP plates (56) containing 2% galactose for 6 days at 20°C to induce transposition. Seventy-four independent colonies that retain the pGTy plasmid were recovered on SC-TRP containing 2% glucose. After segregation of the pGTy plasmid (Trp$^-$), 51 of the 74 colonies (69%) retained the Ty-borne *HIS3* marker. Restriction endonuclease and gel blotting of DNA from these strains was used to identify putative multimer insertion events.

RESULTS

A marked Ty element (Fig. 1) was used as an insertional mutagen in a cell with the **a** mating type, and mutants resistant to cell cycle arrest by the mating pheromone α-factor were selected. This selection has been an important means of identifying genes involved in the mating process. We repeated this selection with Ty as a mutagen to determine whether it uncovered any new loci (29; Mastrangelo et al., unpublished data). Those experiments identified a novel class of α-factor-resistant mutants that were the result of insertional activation of *HMLα*. Molecular characterization of these mutants reveals several features that are unusual for activation of a gene by a retrotransposon.

Mutagenic Ty insertions in *HMLα* contain more DNA than can be accounted for by a single insertion. Figure 1 shows a diagram of the *HIS3*-marked, galactose-inducible Ty element plasmid, pGTy1H3*HIS3*HH4B#84, used in these studies and the expected structure of a single insertion of the marked element. Figure 2a, panel B shows that the 6.6-kb *Bam*HI fragment carrying the *HMLα* locus is disrupted in four of the α-factor-resistant mutants. If there was a simple insertion of the 7.2-kb marked element, which contains a single *Bam*HI site, two novel *HMLα Bam*HI fragments that add up to 14 kb in size would be generated. One of the four mutants, MMB21, has *HML*-related bands that agree with this prediction, although subsequent analysis indicates that it is not the result of a single insertion of the element. The other three mutants, MMB12, MMD25, and MMD37, generate two novel *HML* bands that have combined sizes substantially in excess of 14 kb. This suggests that these mutations involve the insertion of additional DNA or involve more complex genomic rearrangements.

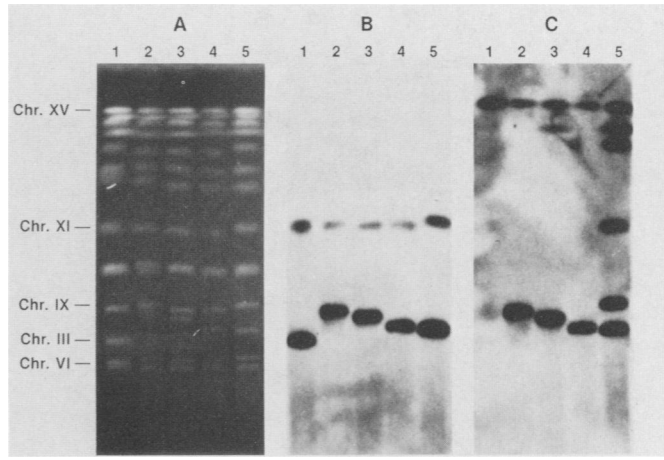

FIG. 3. Chromosomal separations of the starting strain and *hmlα*::Ty mutants. For all panels, chromosomal DNA was prepared from JSS56-11B (lanes 1), MMB12 (lanes 2), MMB21 (lanes 3), MMD25 (lanes 4), and MMD37 (lanes 5) and was then separated on a CHEF gel. The gel was stained with ethidium bromide and photographed (A) and was then transferred to a neutral nylon filter. A blot of one-half of the gel was probed with *HMLα* (B). Note that the additional chromosomal band present in JSS56-11B and all of the mutants is due to a sequence in chromosome XI which cross-hybridizes with the centromere-distal 1.7-kb *Bam*HI-*Eco*RI portion of the probe. A blot of duplicate lanes from the same CHEF gel was probed with a *HIS3* probe (C). Chromosome XV (Chr. XV), the location of *his3-Δ1* in JSS56-11B and all of the mutants, is indicated.

CHEF gel analysis. The four *hmlα*::Ty mutants were analyzed on an agarose gel system capable of separating the yeast chromosomes on the basis of molecular size. The stained gel (Fig. 3A) indicated that in each of the four mutants, the mobility of chromosome III (360 kb) decreased more than expected for a single Ty insertion. In the case of MMB21, chromosome III has gained about 90 kb and is running almost at the same mobility as chromosome IX (450 kb). When *HMLα* is used as a probe to this gel (Fig. 3B), it detects only the novel chromosome III and a homologous region of chromosome XI present in the starting strain. This suggests that there has been no reciprocal translocation between *HMLα* and any other chromosome. As expected, *HIS3* sequences are present on the altered chromosome III in each of the mutants (Fig. 3C). Mutants MMD25 and MMB12 show novel *HIS3* insertions only into chromosome III. In contrast, mutants MMB21 and MMD37 showed *HIS3* sequences in several chromosomes, as expected from genetic analysis that showed several unlinked *HIS3* genes, presumed to be independent marked Ty insertions (M. F. Mastrangelo, unpublished results).

Location, number, and orientation of the marked insertions. Probing a restriction digest of the mutants with the *HIS3* gene (Fig. 2a, panel A and Fig. 4) gives a minimum estimate of the number of marked Ty*HIS3* insertions. This physical analysis detects two and four novel bands for MMD25 and MMB12, respectively, which cosegregated with the *hmlα* mutation in subsequent crosses (data not shown).

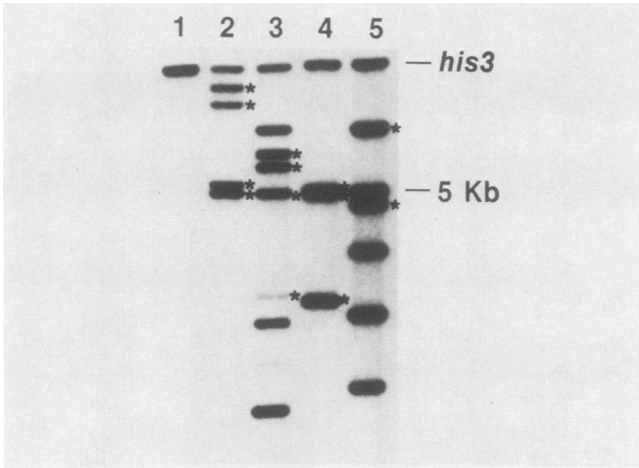

FIG. 4. *HIS3*-marked *Eco*RI junction fragments of four *hmlα*::Ty mutants. DNA of JSS56-11B, MMB12, MMB21, MMD25, and MMD37 was digested with *Eco*RI, separated on a 0.75% agarose gel, blotted to BioTrace RP membrane, and probed with *HIS3*. The 5-kb tandem junction fragment band present in all four mutants is indicated. The band corresponding to the *his3-Δ1* allele is indicated. The bands that cosegregate with the *hmlα*::Ty mutations in subsequent generations are marked with an asterisk.

Further, when the *HIS3* insertions that are not related to the activation of *HMLα* are removed by several generations of backcrosses, there remained multiple *HIS3* bands linked to the *hmlα*::Ty mutations in MMB21 and MMD37 (Fig. 4). All four of the *hmlα*::Ty mutants have an *Eco*RI band at 5 kb that is homologous to the *HIS3* probe (Fig. 3). As shown below, this band is the result of a junction fragment between two Ty elements in a tandem repeat.

To determine the site of insertion of the Ty elements in *HMLα*, we used subclones of *HML* to determine which fragments were disrupted (Fig. 2). All four mutations had insertions 3′ to *HMLα2*. Two were located near the end of the *HMLα2* transcript, and two occurred near the *Eco*RI site (Fig. 2). *Bam*HI digestion should generate a fragment that contains the junction linking the 3′ end (*HIS3*) of the marked Ty and the target sequences (Fig. 1B). The orientation of the marked Ty element relative to *HML* can be established by determining which side of *HML* remains on this Ty-*HML* junction fragment. For each of the *hmlα*::Ty mutants, there was a *Bam*HI fragment that was homologous both to *HIS3* and either a sequence centromere distal or one centromere proximal to *HML*. The orientations deduced from this analysis are indicated in Fig. 2. As noted above, the size of these *Bam*HI junction fragments is too large to result from single marked Ty insertions.

Physical analysis of a Ty multimer at *HMLα*. Because there are multiple *HIS3* bands linked to the *hmlα*::Ty mutations and because the physical analyses suggested that more DNA had been inserted in all four mutants than could be accounted for by integration of single Ty *HIS3* elements, we performed a more extensive physical characterization of one of the mutants. We chose mutant MMD25 because it showed 2+:2− His segregation, with the His^+ phenotype linked to the **a**-specific sterile phenotype caused by the activation of *HMLα*. Further, the physical analysis indicated that it had an insertion of about 40 kb of DNA, including three *HIS3* marked Ty elements.

Characterization of Ty-Ty junctions and the site of insertion in *HMLα*. DNA from a meiotic segregant carrying the mutation present in MMD25 (designated *hmlα-925*) was cloned into a lambda vector (see Materials and Methods). The restriction analysis (Fig. 2) placed the site of insertion of the Ty array in *hmlα-925* near the 3′ end of the α2 gene. The Ty-*HML* junction fragments (pKWHML left; pKWHML right) were cloned and sequenced as described in Materials and Methods. The site of insertion occurred just downstream of the α2 open reading frame (Fig. 5). Comparison of the two junction fragments confirmed that they contained the 5-bp duplication at the site of insertion that is characteristic of Ty insertions (23, 27). The centromere-distal *Eco*RI junction clone (pKWHML left) included the 3′ *Eco*RI fragment of a Ty element but no *HIS3* sequences. This supports the conclusion that the Ty array includes both marked and unmarked elements.

The presence of three distinct Ty*HIS3* insertions in *hmlα-925* was demonstrated by blot analysis with *Pvu*II, *Sal*I, and *Eco*RV (data not shown). These marked elements were cloned as *Eco*RI fragments. We obtained two different types of 5-kb *Eco*RI clones at equal frequency (the 5-kb band from MMD25 in Fig. 3 is a doublet) and a clone of the 3-kb fragment. One type of 5-kb clone (see pKWHML28-1 in Fig. 5) had the restriction pattern (*Bgl*II, *Eco*RV, *Hin*dIII, *Pst*I, *Pvu*II) expected of a Ty-Ty junction fragment between a direct repeat of a *HIS3*-marked element and another Ty. pKWHML28-1 was sequenced from the end of the *HIS3* insertion across the LTR, confirming the presence of a single complete LTR. Restriction endonuclease digestion and blotting analysis of DNA confirmed the presence of this structure in the mutant. This result indicates that at least some of the ~40 kb of DNA inserted in MMD25 consists of direct repeats of Ty.

The second type of 5-kb *Eco*RI clone is a gross rearrangement of Ty (see pKWHML27-1 in Fig. 5). We sequenced from a *Pst*I site at the 3′ end of *HIS3* across the 3′ LTR into the adjoining Ty element. In contrast to pKWHML28-1, the LTR element was incomplete and was joined to the middle of a Ty element. The sequence from the 5′ end of *HIS3* into the Ty element was also obtained. Part of the *HIS3* gene was deleted, and it was joined to a second incomplete LTR. Neither novel joint involves the ends of an LTR element, suggesting that they are not produced by the integration mechanism or that they reflect aberrant products of that process. The presence of this novel structure (and pKWHML1-1, discussed below) in the DNA from MMD25 was confirmed by blot analysis with *Pvu*II, *Sal*I, *Cla*I, and *Pst*I (data not shown).

The 3.0-kb *HIS3*-marked *Eco*RI fragment from *hmlα-925* was further characterized by restriction analysis and sequencing (see pKWHML1-1 in Fig. 5) similar to that described above. It proved to be the result of a tail-to-tail fusion of a *HIS3*-marked Ty and a second unmarked Ty. The novel joint was within the LTR elements. Neither LTR element was complete, again suggesting that it was not the product of a normal integration mechanism.

A model for the complete array of Ty elements in *hmlα-925* is shown in Figure 5. The right-side (centromere proximal) *Bam*HI junction fragment is not homologous to *HIS3* but has the size predicted for a marked element inserted at the right end of the array orientated so that *Bam*HI digestion separates *HIS3* from *HML*. This orientation of the rightmost element was confirmed by the restriction analysis and sequencing of the *Eco*RI clone of the junction fragment (pKWHML right). The *Bam*HI fragment from the left (centromere distal) end of the array has homology to *HIS3* but is about 12 kb larger than expected for a *HIS3*-marked element

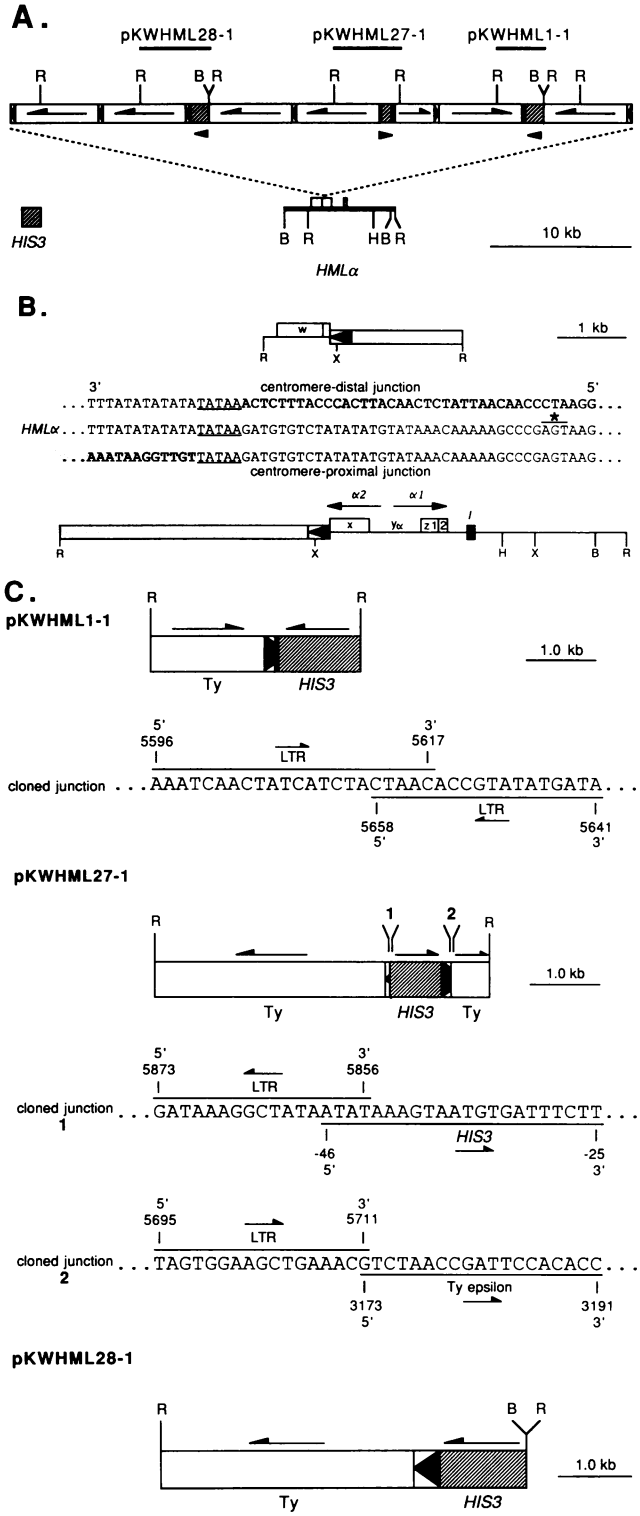

FIG. 5. (A) Model for the Ty multimer insertion of *hmlα-925*. The internal *Eco*RI fragments of the array that were cloned are indicated by the solid lines above the array. *Eco*RI sites (R) are shown for the cloned fragments only. The arrows indicate the direction of transcription of each Ty element, and the arrowheads below the array indicate the direction of transcription of each *HIS3* gene. B, *Bam*HI sites. (B) Diagrams of the centromere-distal and centromere-proximal *hmlα-925*::Ty *Eco*RI junction fragments are shown at the top and bottom, respectively. Partial DNA sequences for these junctions are shown in the center of the diagram. The sequences are shown in a 3′ to 5′ orientation. The middle of the three sequences represents the normal *HMLα* gene, and the centromere-distal and centromere-proximal junctions sequences are above and below it, respectively. Ty sequences are indicated by the shaded areas. The 5-bp target site in *HMLα* is underscored in all three sequences, and the translation termination codon of *HMLα2* is overlined and marked with an asterisk. The W, X, Yα, Z1, and Z2 regions and the I site (I) have been previously defined (3, 25). *Bam*HI (B), *Eco*RI (R), *Hin*dIII (H), and *Xho*I (X) sites are indicated. (C) Diagram of the three internal *HIS3*-marked *Eco*RI fragments cloned from *hmlα-925* (Fig. 5A). Note the presence of partial LTR sequences in both pKWHML1-1 and pKWHML27-1 and the absence of the *Bam*HI site at the 5′ end of the *HIS3* gene in pKWHML27-1. The sequence of the aberrant junctions is shown in a 5′ to 3′ orientation. The arrows represent the direction of transcription of the *HIS3* (hatched areas) or Ty elements. *Bam*HI (B) and *Eco*RI (R) sites are indicated. The underscoring or overlining indicates uninterrupted sequence identity with *HIS3* or Ty sequences, as indicated. Numbering of all three cloned junctions follows the conventions for the published sequences of *HIS3* (61) and TyH3 (7).

inserted at that position. We assume that two unmarked elements are between the marked element and the left end of the array. Consistent with that proposal, the *Eco*RI junction fragment containing the left *HML*-Ty junction (pKWHML left) is an unmarked Ty in the orientation shown. The *Eco*RI Ty*HIS3* fragments are positioned in the model based on the sizes of the *Bam*HI *HML*-Ty and *Bam*HI *HIS3* fragments, the estimate of the overall size of the array, and the assumption that the uncloned portions of the array are direct repeats of Ty elements. Alternative models require additional inversions (multiples of two) within the array.

The complete analysis of the other three *hmlα*::Ty mutations was not attempted. However, several observations demonstrate that they share features of the complex structure shown for *hmlα-925*. For MMB12, the CHEF gel suggests that about 90 kb have been added to chromosome III. If all of that DNA is in a single array it would require about 15 Ty elements. The size of the *Bam*HI fragment homologous to the left side of *HML* and *HIS3* is about 12 kb larger than expected from the site of insertion of the array, suggesting that two unmarked elements are between the last marked Ty and the end of the array. The right-side *Bam*HI Ty-*HML* junction fragment does not show homology to *HIS3* but has a size consistent with a marked element being at that end of the array and orientated so that *Bam*HI digestion separates the *HIS3* gene from *HML*. Thus, the two ends of the array in MMB12 have the same structure as MMD25 but the array is inserted at a different site. Similar arguments for the mutation in MMB21 (designated *hmlα-721*) suggest that it is an array of up to 10 Ty elements with marked elements at both ends and two marked elements internal to the array. This analysis suggests that the orientation of the Ty elements at the ends of the MMB21 array is opposite to that in MMD25 and MMB12. Likewise, the mutation in MMD37 appears to be an array of up to six Ty elements, including one *HIS3*-marked element at the right end of the array and an additional marked Ty internal.

Additional evidence that these other arrays contain rearranged Ty elements is apparent in *Eco*RI (Fig. 4) and *Cla*I digests (data not shown). The *Eco*RI digestion of MMB12 (Fig. 4) shows four novel bands homologous to *HIS3* that cosegregate with the mutation, suggesting the insertion of four marked elements. If all of the Ty elements are part of a single array of directly repeated elements, there could be at most two sizes of *HIS3* fragments; one corresponds to the

junction to the target sequence (which can differ from one mutant to another or not have homology to *HIS3* if, as in the cases of MMD25 and MMB12, the element at the end of the array is not marked with *HIS3*), and the other is the 5-kb fragment corresponding to the Ty-Ty*HIS3* junction fragment. Additional bands presumably reflect Ty elements polymorphic for *Eco*RI sites or aberrant Ty elements, like those seen in *hmlα-925*. The presence of rearranged Ty*HIS3* elements in MMB21 is further demonstrated by the lighter bands in Fig. 3. One of those bands cosegregates with the *hmlα*::Ty mutation and is presumed to be a small portion of the *HIS3* gene. The *HIS3* gene carried by the marked Ty element was inserted as a 1.2-kb *Cla*I fragment. As expected, a 1.2-kb *Cla*I fragment homologous to *HIS3* is observed in all of the mutants caused by a marked Ty. However, additional *Cla*I *HIS3* bands were observed associated with each of the *hmlα*::Ty mutations. In *hmlα-925*, there is a 4-kb *Cla*I fragment homologous to *HIS3* that corresponds to the rearranged *HIS3* gene on the pKWHML27-1 clone. The *hmlα*::Ty mutations in MMB12, MMB21, and MMD37 have additional *Cla*I bands homologous to *HIS3* of 1.1, 2.5, and 0.9 kb, respectively. These novel *HIS3 Cla*I bands presumably represent rearranged portions of the *HIS3* gene present within the Ty arrays.

Occurrence of Ty multimers at other locations in the genome. The presence of Ty multimers at *HMLα* in four of the Ty*HIS3* α-factor-resistant mutants suggested that the *HML* locus or α-pheromone treatment or both could play an important role in multimer formation. To determine whether these Ty multimers were unique to insertions at *HML*, we surveyed other strains that had marked Ty insertions. As described above, the *HML* mutants had a common 5-kb *Eco*RI fragment that contains the junction between a marked Ty element and the next member of the directly repeated array. Marked Ty-Ty junction fragments of the sizes predicted for other restriction endonucleases can also be detected. Among 36 α-factor-resistant mutants that were not the result of insertions at *HMLα*, we identified three that yielded the 5-kb *Eco*RI fragment expected of tandem arrays. Of these, two yielded the 7-kb *Bam*HI fragment indicative of direct repeats of the *HIS3*-marked Ty. We also screened 51 independent isolates containing Ty1H3*HIS3*HH4B insertions that were not selected as resistant to α-pheromone. Three of these mutants yielded the *Eco*RI (5 kb), *Pvu*II (3.1 kb), and *Sph*I (3.5 kb) fragments homologous to *HIS3* that are predicted if these marked Ty insertions are in direct repeat multimer arrays. These results suggest that Ty multimers can occur at several locations besides *HML* and can occur in the absence of α-pheromone-mediated cell-cycle arrest.

We have also observed Ty multimer insertions with a genetic selection based on activating a promoterless *his3* gene (8, 55). The plasmid pAB100 carries the *his3-Δ4* allele, which had the promoter region deleted. In strains containing a complete deletion of the chromosomal *his3* gene, over 80% of the His^+ revertants obtained at a low temperature result from Ty insertions upstream of *his3-Δ4* on the plasmid (8, 9). If a pGTy plasmid is also induced in these cells, many of the resulting His^+ revertants are caused by the induced Ty element.

To determine whether the insertion of marker gene DNA into the element affected multimer formation, we collected a large number of His^+ revertants of *his3-Δ4* that were caused by the insertion of unmarked Ty elements. The *his3-Δ4* gene also provided another genetic locus where Ty multimers might be found. In this experiment, we used unmarked

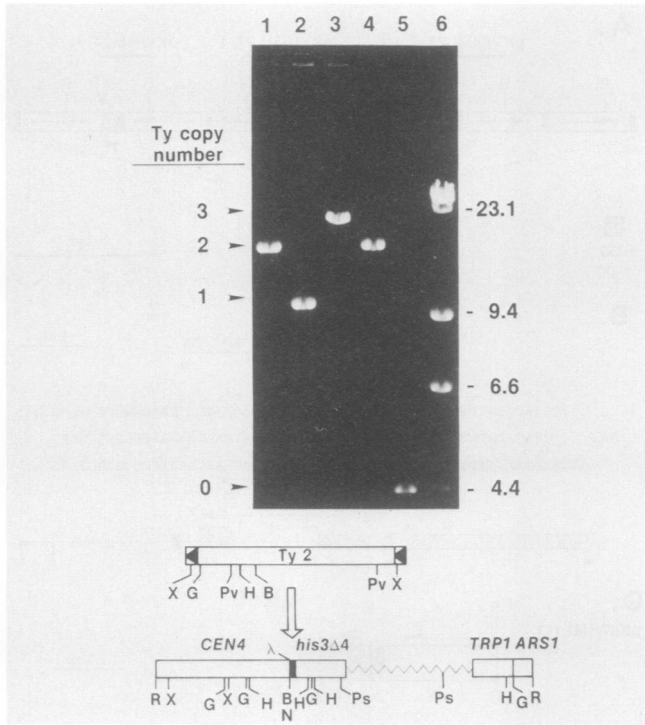

FIG. 6. Presence of multimeric Ty2 insertions at *his3-Δ4*. The target plasmid pAB100 was recovered from three His^+ revertants containing multimeric Ty2 insertions (H1827, H1875, and H1914) and from one revertant containing a single Ty insertion (H1847). Plasmid DNA from strains H1827 (lane 1), H1874 (lane 2), H1875 (lane 3), and H1914 (lane 4) and the target plasmid pAB100 (lane 5) were digested with *Eco*RI and *Pst*I, separated by electrophoresis on a 0.6% agarose gel, and stained with ethidium bromide. Ty2 elements do not contain restrictions sites for *Eco*RI or *Pst*I. Therefore, restriction fragments are generated that show the number of Ty copies inserted at *his3-Δ4*. The number of Ty elements inserted in the target plasmid is shown on the left. The promoterless *his3-Δ4* gene, prior to Ty insertion, is on a 4.3-kb *Eco*RI-*Pst*I fragment. The promoter region of *HIS3* has been replaced by a short segment of bacteriophage λ (black vertical bar). Molecular size markers (in kb) generated from *Hin*dIII-digested bacteriophage λ (lane 6) are shown on the right. B, *Bam*HI; G, *Bgl*II; H, *Hin*dIII; N, *Nco*I; Ps, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; X, *Xho*I.

GAL1-promoted Ty2-917 or Ty2-H556 elements. Ty1 and Ty2 elements are structurally and functionally related, but they contain distinct regions of divergent DNA sequence that can be used to distinguish Ty2 from Ty1 (19, 63). Among 56 plasmids recovered from His^+ revertants caused by Ty2 elements, 3 contained Ty2 multimers inserted upstream of *his3-Δ4* (Fig. 6). Restriction enzyme and Southern analyses indicated that the Ty elements were in a tandem array and were separated by a single LTR, as was observed for the multimers at *HML*. Multimers present in strains H1827 and H1914 contained two Ty elements, and the multimer in strain H1875 contained three Ty elements. The orientation of the Ty elements in these three revertants was such that Ty transcription was opposite to that of *HIS3* transcription. This is the typical arrangement for Ty elements that activate gene expression (22).

The fragmentation patterns obtained by *Eco*RI-plus-*Pst*I double digests (Fig. 6) and by *Pvu*II digestion (data not shown) were particularly instructive. Neither *Eco*RI nor *Pst*I cleaves Ty2 elements, so digestion of the revertant plasmids with these restriction enzymes should reveal the number of

Ty elements present. The normal *Eco*RI-*Pst*I fragment containing *his3-Δ4* is 4.4 kb. A monomeric Ty insertion should add about 6 kb of Ty DNA to the target fragment, while each additional insertion should add 6 kb more Ty DNA if the Ty elements are not altered. The three multimers obtained in this experiment are carried on *Eco*RI-*Pst*I fragments of 16 (pH1827 and pH1914) or 21.7 kb (pH1875), which strongly suggests that these multimers contain two or three Ty elements, respectively. Southern hybridization of *Eco*RI-plus-*Pst*I-digested total yeast DNA from the revertants with a *HIS3*-specific probe shows that the multimer arrays are present on the revertant plasmids in *S. cerevisiae* and are not created by recovery in bacteria (data not shown). To determine if the Ty junctions separating one Ty from the next contain one or two LTRs, we analyzed the revertant plasmids with *Pvu*II (data not shown). *Pvu*II cleaves Ty2 elements at positions 473 and 4370 (63). Therefore, a 1.7-kb *Pvu*II fragment should be present if the Ty-Ty junction contains one LTR and a 2.05-kb *Pvu*II fragment should be present if there are two LTRs. A 1.7-kb *Pvu*II fragment containing a single LTR was observed in each case. Digestion of pH1875 with *Pvu*II generated a doublet 1.7-kb fragment, which is expected if the multimer contains three tandem Ty2 elements. The presence of the Ty LTR in the 1.7-kb fragment was verified by Southern analysis with an LTR-specific hybridization probe. In addition, restriction patterns obtained with *Bam*HI, *Bgl*II, and *Xho*I support the interpretation that the Ty2 elements are present as simple tandem arrays in the revertant plasmids. There is no indication of rearranged Ty elements. These results suggest that Ty multimers can form at sites other than *HML* and that multimer formation is independent of α-factor treatment or the presence of a marker gene carried by the Ty element.

DISCUSSION

Retrotransposons, like retroviruses, replicate via an RNA intermediate. The life cycle of retrotransposons includes reverse transcription of the genomic RNA and the insertion of the DNA copy into the host genome to generate a template (called the provirus in retroviruses) from which the RNA copies can be transcribed. Recent experiments strongly implicate the linear DNA as the substrate for the integration reaction of retroviruses (12, 26) and Ty elements (21). The complex multimers observed for these four *hmlα*::Ty mutants and the tandem multimers we have observed elsewhere are in marked contrast to the well-characterized single insertion events reported for Ty elements at other loci (5, 62) and of retroviruses and other retrotransposons in general. Multimer formation in retroviral proviruses has been observed as a postintegrative amplification (34) or as aberrant insertion events in integration-defective mutants (30). The insertions of viral DNA in the integration-defective strains did not have the provirus-host cell DNA junctions characteristic of normal integration events. The *hmlα*::Ty structure represented in Fig. 5, while novel for retrotransposons, includes a tandem repeat of Ty elements similar to a structure reported in the original characterization of Ty elements (16). We observed this tandem structure at unselected sites and among Ty activations of *his3-Δ4*. It is not clear whether the endogenous examples of tandem repeats are the result of insertions of a multimer or the consequence of postintegrative amplification. Such tandem duplications could arise by unequal recombination between sister chromatids within the LTR regions; recombination between adjacent, directly oriented, independent Ty insertions; homologous recombination between an integrated Ty and the one LTR circular form of the Ty cDNA; or gene conversion between an LTR and a complete Ty (47, 52). The results described here suggest that multimers can be formed as a part of the transposition process.

Why multimers at *HML*? The observation that all four examples of *HML* activation involved Ty multimers suggests that there is something special about this activation event. This could reflect an unusual constraint on the mechanism by which *HML* can be activated.

Our results suggest that only a small fraction of all insertions are multimers but that only the insertion of a multimer at *HML* will result in its activation. Consistent with this view, a derivative of *hmlα-925* having only a single Ty inserted at that position does not give the α-factor-resistance phenotype (K. Weinstock, unpublished data). Therefore, *HML* activation provides a convenient trap for the identification of such Ty multimers. The observations that all four of the insertions are 3′ to the gene that is activated and that the insertions are in either orientation suggest that the normal mechanism of Ty-mediated gene activation is not operating in these cases. Repression of *HML* and *HMR* is a complex process involving the products of the *MAR* and *SIR* genes (*SIR1*, *MAR1* [*SIR2*], *SIR3*, *SIR4*, *ARD1* [35, 38, 49, 64] and *NAT1*) (44). Histone H4 (37) and RAP-1 (TUF or GRR-1) (14, 32, 58) also have roles in this silencing mechanism. Sites required for SIR regulation (designated E and I) have been defined on both sides of *HML* and *HMR* (1, 11, 14, 32, 41, 58). It has been postulated that *MAR* and *SIR* regulation acts at the E and I sites to define a chromatin domain that is transcriptionally silent. The insertions reported here do not appear to have directly inactivated the E or I sites. Experiments done with plasmids suggested a maximum size for the E-to-I domain of only about 4 kb (2) for SIR repression to function. However, it is possible that a single Ty insertion between E and I is not sufficient to disrupt the *HML* chromatin domain in a normal chromosome III. The Ty multimer insertions may have activated *HMLα* by violating a size constraint for the SIR-mediated chromatin domain normally responsible for the silencing of *HML*.

How are the multimers formed? The 5-base duplication at the ends of the *hmlα-925* mutation indicates that the insertion proceeded via the Ty integration mechanism (23, 27). The presence of both marked and unmarked elements within the arrays demonstrates that the array is not made by a simple amplification of a single insertion. In addition, the blotting analysis and characterization of the clones derived from MMD25 indicate restriction site polymorphisms among the Tys of the array, suggesting that several different Ty elements contributed to the array. The tandem direct repeats of Ty elements are readily explained as the products of homologous recombination between a one LTR circular form of Ty and a linear Ty element either before or after integration. Alternatively, they can be formed by recombination between the LTRs of linears before integration.

The rearranged Ty elements require a more complex explanation. Shoemaker et al. (57) provided a precedent for permuted retrotransposons. They demonstrated that Moloney murine leukemia virus can integrate into itself to form circular forms of the viral DNA that have inversions or deletions. Homologous recombination between such rearranged circular forms and integrated Ty elements could yield multimers with permuted structures. A Ty dimer involving a rearranged element consistent with such a mechanism has recently been characterized (66). However, the rearranged *HIS3*-marked elements observed here do not have the com-

plete LTR ends expected from such a mechanism. The pathway by which they are made may involve the Ty integration mechanism but not the normal Ty LTR ends. The tail-to-tail fusion seen in pKWHML1-1 is reminiscent of the palindromic structures formed upon transformation with plasmids linearized in regions that lack homology to *S. cerevisiae* (39, 40). Two of the aberrant Ty junctions in *hmlα-925* have very short homologies between the joined sequences (Fig. 5). These homologies could reflect base pairing in a DNA-DNA recombination intermediate or cases of aberrant priming in a reverse transcription step. The significance of these short homologies in the formation of Ty multimers is under investigation.

The proposal that a multimer is formed prior to integration requires that some portion of the reverse transcript that is the integration precursor is available for recombination with other DNA copies of the Ty genome. The portions of the Ty integration precursor that are postulated to be available for homologous recombination in this view could also be targets for the nonhomologous recombination required for the gross rearrangements seen in *hmlα-925*. The multimers formed by these pathways could still be associated with the Ty particle and its integration machinery.

The proposal that multimer formation occurs after a simple integration can be accommodated by models involving homologous recombination between the integrated element and the unintegrated DNA forms of the Ty element. In the context of this view, the size of the arrays at *HML* and the observation of arrays at novel sites among the unselected events requires that the newly integrated Ty elements are very highly favored relative to the endogenous Ty elements for the formation of these arrays. Such favored status might be conferred by nicks or single-strand gaps left at the ends of the newly integrated element. Such gaps are present in the integration intermediates of retroviral proviruses (13). Natsoulis et al. (45) observed two insertions of only one LTR (solo δ elements) among 111 Ty element-induced *ura3* or *lys2* mutations (45). That apparent frequency is higher than the formation of solo δ elements from complete Ty elements during mitotic growth (67) and could reflect a recombinagenic intermediate made upon initial insertion.

Homologous recombination is invoked in both the view that the multimers are formed prior to integration and the view that they form subsequent to the integration of a monomer. Multimers may be more frequently generated during the transposition of Ty elements in *S. cerevisiae* than during infection of higher eucaryotes by retroviruses because of the high efficiency of homologous recombination in *S. cerevisiae* and the large number of transposition intermediates formed in cells induced for high-level transcription of a plasmid-borne Ty element (28). The unique ability of *HML* activation to distinguish Ty multimers from monomers may facilitate an analysis of the mechanism by which multimers are formed.

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