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Transfer RNA Genes Are Genomic Targets for *de Novo* Transposition of the Yeast Retrotransposon Ty*3*

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ABSTRACT

Insertions of the yeast element Ty*3* resulting from induced retrotransposition were characterized in order to identify the genomic targets of transposition. The DNA sequences of the junctions between Ty*3* and flanking DNA were determined for two insertions of an unmarked element. Each insertion was at position −17 from the 5′ end of a tRNA-coding sequence. Ninety-one independent insertions of a marked Ty*3* element were studied by Southern blot analysis. Pairs of independent insertions into seven genomic loci accounted for 14 of these insertions. The DNA sequence flanking the insertion site was determined for at least one member of each pair of integrated elements. In each case, insertion was at position −16 or −17 relative to the 5′ end of one of seven different tRNA genes. This proportion of genomic loci used twice for Ty*3* integration is consistent with that predicted by a Poisson distribution for a number of genomic targets roughly equivalent to the estimated number of yeast tRNA genes. In addition, insertions upstream of the same tRNA gene in one case were at different positions, but in all cases were in the same orientation. Thus, genomic insertions of Ty*3* in a particular orientation are apparently specified by the target, while the actual position of the insertion relative to the tRNA-coding sequence can vary slightly.

INSERTION site selection is a critical step in the life cycles of retrotransposons and retroviruses about which relatively little is known. Insertions of these elements are flanked by direct repeats generated by cleavage and repair of the host genome (reviewed in VARMUS and BROWN 1989; BOEKE 1989; BINGHAM and ZACHAR 1989). Mutations in the retroviral or retrotransposon integrase block the integration step *in vivo* and *in vitro* (SCHWARTZBERG, COLICELLI and GOFF 1984; DONEHOWER and VARMUS 1984; PANGANIBAN and TEMIN 1984; DONEHOWER 1988; BOEKE *et al.* 1988; EICHINGER and BOEKE 1988; BROWN *et al.* 1989; reviewed by GRANDGENETT and MUMM 1990). Integrase is required to produce the recessed ends of the replicated retroviral DNA (BROWN *et al.*, 1989; ROTH, SCHWARTZBERG and GOFF 1989) and may also cleave the genomic target. The position and orientation of integrated retrotransposons and retroviruses have been shown to influence transcription from the inserted sequences and to determine the extent of *cis*-acting effects of the insertion (reviewed in VARMUS and BROWN 1989; BOEKE 1989; BINGHAM and ZACHAR 1989). The diversity of effects generated from different insertions suggests that the spectrum of potential insertion sites may be quite broad. Nevertheless, experiments from a number of laboratories show that insertion is not a random process.

Preferred insertion sites have been observed for animal retroviruses and retrotransposons, including Drosophila gypsy-like elements and Saccharomyces Ty elements. Twenty-four insertions of avian leukosis virus, isolated from lymphomas and mapped to c-*myc*, all occurred within 150 basepairs (bp) of one of five DNase I-hypersensitive sites located within a 3 kilobasepair (kbp) region upstream of the c-*myc*-coding sequence (ROBINSON and GAGNON 1986). A selected integration of Moloney murine leukemia virus (MoMLV) also mapped near a DNase I-hypersensitive site in the mouse gene for α_1-collagen (ROHDEWOHLD *et al.* 1987). A study by VIJAYA, STEFFEN and ROBINSON (1986) that characterized several selected and unselected insertions recovered from MoMLV-induced thymomas showed that they occurred within 500 bp of DNase I-hypersensitive sites. Analysis of insertion libraries of turkey embryo fibroblasts infected with Rous sarcoma virus conducted by SHIH, STOYE and COFFIN (1988) failed to reveal a consensus sequence, but did identify a small number of sites that are used at a frequency estimated at one million times the expected frequency for random insertion. These authors suggested that a subpopulation of highly used sites might have a common structural feature, such as proximity to nuclear matrix attachment sites. In contrast to the results of these *in vivo* experiments, examination by BROWN *et al.* (1987) of *in vitro* integration sites of MoMLV into naked lambda DNA showed a relatively random distribution of insertion positions and orientations.

Selected and unselected insertions of elements and retroviruses have been examined for evidence of con-

sensus sequences flanking sites of insertion. Several selected insertions and ten unselected insertions of the Drosophila element, 17.6, are flanked by a direct repeat of the sequence ATAT, which is presumed to occur once prior to insertion at that site (INOUYE, YUKI, and SAIGO 1984). The sequence ATAT has also been reported flanking insertions of the Drosophila retrotransposon 297 (IKENAGA and SAIGO 1982). The more recently described *Drosophila ananassae* element, tom, is flanked by direct repeats of TATAT in six characterized insertions (TANDA *et al.* 1988). In a study of twelve insertions of the human element, THE 1, DEKA *et al.* (1988) deduced the consensus sequence, G/CAYAC, flanking the site of integration. Although this consensus is not strictly conserved as the sequence flanking insertions of the above Drosophila elements, its presence suggests that insertion may occur in a sequence-specific manner. However, the occurrence of a specific flanking sequence appears to distinguish these elements from other retrotransposons and retroviruses.

Examination of a large number of Ty*1* insertions that were selected for on the basis of activation of a promoterless *HIS3* gene (BOEKE, STYLES and FINK 1986), or inactivation of *URA3*, *LYS2* or *CAN1* genes (EIBEL and PHILLIPSEN 1984; SIMCHEN *et al.*, 1984; NATSOULIS *et al.* 1989; WILKE *et al.* 1989) did not reveal a strong consensus at the insertion site. At the *URA3*, *LYS2* and *CAN1* loci, where insertion anywhere within the coding sequence would have inactivated the gene, insertions appeared to occur more frequently at the 5′ end of each gene, although this was shown to be strain dependent in the case of *CAN1* (WILKE *et al.* 1989). Hotspots for insertion were identified at all four loci; nevertheless, no strong, target-site consensus is evidenced, and overall there is no apparent bias in the orientations of insertions at *URA3* and *LYS2*. In addition to *de novo* insertion at these genes transcribed by polymerase II, a number of Ty*1* elements have been characterized in genomic regions that also contain tRNA genes (EIGEL and FELDMANN 1982; TSCHUMPER and CARBON 1982; GAFNER, DE ROBERTIS and PHILIPPSEN 1983; NELBOCK, STUCKA and FELDMANN 1985; HAUBER *et al.* 1988). In a study of spontaneous mutations in the *SUP4-o* tRNA gene, GIROUX *et al.* (1988) observed that Ty*1* insertion accounted for 6% of the mutations analyzed. Ty*4*, a recently described yeast retrotransposon, is also found in regions of the genome containing tRNA genes (STUCKA, LOCHMULLER, and FELDMANN 1989). A similar association of repetitive elements with tRNA genes has been observed in the cellular slime mold *Dictyostelium discoideum*. MARSCHALEK *et al.* (1989) analyzed insertions of two classes of elements, DRE and Tdd, and found them to be positioned at fixed distances upstream and downstream, respectively, of tRNA genes. The basis for the insertion preference of these elements is not understood.

If we consider the results from these different systems together, they suggest that there are hotspots for genomic insertion, but that, with the possible exception of the Drosophila gypsy-like and THE 1 elements, hotspots are not specified by DNA sequence alone. Furthermore, a number of the 17.6, 297, and tom insertion sites correspond to the TATA box consensus, and it is therefore not clear that sequence is the sole target determinant for these elements. The occurrence of insertion hotspots *in vivo*, but not *in vitro* (albeit in a phage DNA target), the absence of distinctive consensus sequences, and the association of a number of insertions with the 5′ ends of genes or DNase I-hypersensitive sites are all consistent with a role for DNA or chromatin conformation in targeting the integration of retrotransposons and retroviruses.

The yeast retrotransposon Ty*3* has organizational and sequence similarity to animal retroviruses, and has a distinctive, nonrandom distribution in the genome. Insertions of this element have been described at positions −17 and −16 upstream of tRNA-coding sequence. The complete element is 5.4 kbp in length and is composed of an internal domain flanked by long terminal (direct) repeats (LTRs) of the 340-bp sigma element (CLARK *et al.* 1988; HANSEN, CHALKER and SANDMEYER 1988). A large number of sigma element insertion sites have been characterized by ourselves and other laboratories (DEL REY, DONAHUE and FINK 1982; SANDMEYER and OLSON 1982; BRODEUR, SANDMEYER and OLSON 1983; GENBAUFFE, CHISHOLM and COOPER 1984; NELBOCK, STUCKA and FELDMANN 1985; VAN ARSDELL, STETLER and THORNER 1987; HAUBER *et al.* 1988; SANDMEYER *et al.* 1988). These constitute a collection of more than 30 sigma elements, and all occur within 1 or 2 bases of position −17, relative to the 5′ end of mature tRNA-coding sequences. Sigma insertions also display an orientation bias; about two-thirds are oriented so that sigma-promoted transcription would be divergent to that of the tRNA gene. However, despite the apparent specificity of these insertions, there is no obvious consensus sequence immediately flanking the sigma elements. The "target" of insertion could therefore consist of some relatively subtle consensus at the insertion site, conserved sequences within the tRNA gene, or a DNA structure or DNA-protein complex present in the region.

A transpositionally active Ty*3* element, Ty*3*-1, has been described (HANSEN, CHALKER and SANDMEYER 1988; HANSEN and SANDMEYER 1990). Ty*3* is transcribed into a polyadenylated RNA approximately 5.2 kb in length that is analogous to the retroviral genome. The RNA encodes two open reading frames, *TYA3* and *TYB3*. The former encodes a protein with

a sequence motif found in the retroviral nucleocapsid protein and the latter encodes domains with homology to retroviral protease, reverse transcriptase, RNaseH and integrase, in that order. Induction of Ty*3* transcription under control of the *GAL1–10* upstream activating sequence (UAS) (JOHNSTON and DAVIS 1984) was shown to result in transposition of the unmarked Ty*3*-1 element carried on a donor plasmid in a strain containing no endogenous Ty*3* elements. This showed that Ty*3* elements are capable of transposition, but did not address the question of whether these *de novo* transpositions are position specific.

The defined nature of Ty*3* insertion could offer an opportunity to measure the relative contributions of primary structure *vs.* more complex parameters, such as DNA conformation or transcriptional activity, to the targeting of retroviruses and retrotransposons. We analyzed a large number of Ty*3* genomic insertions to determine whether induced transposition is position specific, and, if so, whether the class of potential targets is composed of tRNA genes, polymerase III-transcribed genes, or a subset of these loci. Evidence is presented here that the primary genomic target of *de novo* Ty*3* transposition is the population of tRNA genes.

MATERIALS AND METHODS

Construction of recombinant plasmids: Standard recombinant DNA techniques were used (AUSUBEL *et al.* 1989) unless otherwise noted. Transposition of two genetically marked, Ty*3* donor plasmids, pEGTy*3*-H and pEGTy*3*-HN, was monitored. These plasmids were derived from pEGTy*3*-1 (HANSEN, CHALKER and SANDMEYER 1988), that contains a Ty*3* element under transcriptional control of the *GAL1–10* UAS, yeast 2-μm episome sequences, the yeast *URA3* gene, and bacterial ori and *amp*^{r} sequences. In pEGTy*3*-H, the Ty*3* element is genetically marked with an insertion of the yeast *HIS3* gene and in pEGTy*3*-HN, it is marked with the bacterial *neo*^{r} gene in addition to *HIS3* (see Figure 1). A *Bam*HI site was created in pEGTy*3*-1 starting at nucleotide (nt) position 4882 of the Ty*3* element by oligonucleotide mutagenesis (KUNKEL 1985). This site was cleaved and the resulting ends were made blunt by polymerization using the Klenow fragment of DNA polymerase I. The *Pst*I and *Bam*HI ends of an 880-bp fragment from plasmid pDG201 (a gift from D. GARFINKEL, Frederick Cancer Research Facility) containing the *HIS3* gene were made blunt by treatment with S1 nuclease and Klenow polymerase, respectively. This fragment was ligated to the blunt ends of the vector in a reaction catalyzed by T4 DNA ligase. The resulting plasmid, pEGTy*3*-H, contains the *HIS3* gene oriented so that its direction of transcription is the same as the direction of Ty*3* transcription.

To facilitate the eventual cloning of integrated Ty*3* elements, a 1060-bp *Bam*HI fragment containing the bacterial *neo*^{r} gene from pGH54 (JOYCE and GRINDLEY 1984) (a gift from J. BOEKE, Johns Hopkins University) was inserted into the pEGTy*3*-H *Bgl*II site at nt position 4064 of Ty*3* and oriented so that *neo*^{r} transcription is in the same direction as Ty*3* transcription. This plasmid was designated pEGTy*3*-HN.

Plasmid pGTy*3*-T1 is a bacterial plasmid designed for the integrative transformation used to construct the helper yeast strain (see below). pGTy*3*-T1 carries the *Hin*cII/*Eco*RI fragment, which contains the galactose-inducible Ty*3* element from pEGTy*3*-1, in the *Hin*cII/*Eco*RI sites of pIBI21, and the 857 bp *Eco*RI/*Bgl*II fragment from YRP7 (STRUHL *et al.* 1979), which contains the *TRP1* gene, cloned into the *Bgl*II/*Eco*RI sites within the yeast DNA just downstream of the Ty*3*-1 element.

In order to overexpress Ty*3* in a strain with a *URA3* genetic background, a high-copy plasmid containing a galactose-inducible Ty*3* element and marked with the yeast *TRP1* gene was constructed. Plasmid pGTy*3*-T1 was converted to an autonomously replicating plasmid by the following steps. First, the pIBI21 sequence between the *Hin*dIII and *Eco*RI restriction sites was replaced with the corresponding pIBI20 sequence. The resulting plasmid, pGTy*3*-T2, was digested with *Eco*RI and ligated to a 2.2-kb *Eco*RI fragment containing the yeast 2-μm episome, creating the plasmid pEGTy*3*-T2 (J. KIRCHNER, personal communication).

Construction of the helper strain: Yeast strain manipulations were according to standard practice (SHERMAN, FINK and HICKS 1986). Transposition studies involving genetically marked, defective Ty*3* elements were carried out in a strain that contains a stably integrated, galactose-inducible Ty*3*-1 element. This strain was created by integrative transformation of strain yVB110 (*MAT***a** *trp1-Δ901 ura3-52 his3-Δ200 ade2-101 lys2-1 leu1-12 can1-100*), which lacks all endogenous Ty*3* elements (HANSEN, CHALKER and SANDMEYER 1988), with linearized plasmid pGTy*3*-T1. pGTy*3*-T1 was cleaved within the sigma repeats of Ty*3* by partial digestion with *Xho*I in order to direct integration of the plasmid into an endogenous sigma element. The DNA was transformed into yVB110 by a modification of the procedure of ITO *et al.* (1983), and transformants were selected on synthetic, minus-tryptophan medium. DNA from Trp$^+$ colonies was screened by Southern hybridization analysis to detect recombination involving Ty*3* and sigma sequences. The Ty*3*-specific probe was a 2934-bp *Bgl*II fragment spanning positions 1130 to 4064 of the Ty*3*-1 internal domain. The sigma-specific probe was an *Eco*RI/*Hin*dII fragment from pSBS6 (CLARK *et al.* 1988) containing a nearly complete sigma element. A strain containing an integrated Ty*3* element was identified and designated yDLC221 (Figure 1A). This strain was used in the transposition studies described below. The hybridization pattern of genomic DNA from yDLC221 was consistent with integration by homologous recombination between the ends of the extrachromosomal DNA and an endogenous, isolated sigma element (data not shown).

Analysis of Ty*3* transposition: Identification of cells that had undergone transposition of unmarked Ty*3* elements was as previously described (HANSEN, CHALKER and SANDMEYER 1988). Briefly, strain yVB110 transformed with pEGTy*3*-1 was grown at 30° on synthetic, minus-uracil, galactose-containing medium, in order to maintain the donor plasmid and to induce high levels of Ty*3* transcription. After 10 days, colonies were streaked for single-cell isolates on YPD (1% yeast extract, 2% Bacto-peptone and 2% glucose), and then transferred to synthetic medium containing 5-fluoro-orotic acid (5-FOA) at a concentration of 1 g/liter (BOEKE, LACROUTE and FINK 1984) to select for cells that had lost the *URA3*-containing donor plasmid. Cells that had acquired integrated Ty*3* elements were identified by colony hybridization with a Ty*3*-specific probe.

In experiments involving marked, donor Ty*3* elements, cells that had undergone transposition were identified by growth on selective medium. Strain yDLC221 was transformed with plasmid pEGTy*3*-HN and 128 colonies were

selected on synthetic, minus-uracil medium. In order to induce Ty*3* transcription, cells representing each of the original transformed colonies were streaked onto synthetic minus-uracil medium, containing galactose and grown for 5 days at 30°. Isolates were grown on YPD to allow for loss of the donor plasmid, and were then transferred by replica-plating to synthetic medium, containing 5-FOA, and lacking histidine. This step selected for cells that had lost the *URA3* plasmid and acquired a genomic copy of the *HIS3* gene (Figure 2). These colonies, each originating from an independent transformant, were restreaked on medium lacking histidine and 122 clonally pure strains were obtained. The strains were cultured in 10 ml of YPD. Genomic DNA was isolated from each culture by the method of BOEKE *et al.* (1985), and digested separately with *Eco*RI, *Bam*HI, and *Sal*I (see Figures 3 and 4). There are no recognition sites for *Eco*RI and *Bam*HI in Ty*3*-1; there is one site for *Sal*I at nt position 3133. The digested DNA was fractionated by electrophoresis in 0.8% agarose gels in TBE buffer (2.5 mM EDTA, 45 mM borate, 133 mM Tris-HCl, pH 8.3) and transferred to nitrocellulose by the method of SOUTHERN (1975). DNA bound to the nitrocellulose was hybridized with Ty*3*-, *HIS3*-, sigma-, 5S-, or U6-specific probes labeled by the random primer method (FEINBERG and VOGELSTEIN 1983, 1984). The Ty*3*- and sigma-specific probes are described above. The *HIS3* probe, an 1149 bp *Bam*HI fragment extending from position −230 throughout the coding region, was from plasmid pBM457, a gift from M. JOHNSTON, Washington University, St. Louis. The 5S-specific probe, plasmid pBB111R, was a gift from E. P. GEIDUSCHEK, University of California, San Diego and D. RIGGS, University of California, Irvine (BRAUN *et al.* 1989). The U6 probe, an *Eco*RI/*Pst*I fragment containing U6 sequences from +6 to +629, was taken from plasmid pNH6, a gift from D. BROW, University of Wisconsin, Madison and C. Guthrie, University of California, San Francisco (BROW and GUTHRIE 1988). Hybridization and wash conditions were as previously described (CLARK *et al.* 1988). Filters were exposed overnight to Kodak XAR-5 film in the presence of a Cronex Quanta III Intensifying Screen at −70°. The molecular weights of Ty*3*-hybridizing fragments were determined relative to size markers of *Hin*dIII-digested, lambda DNA, end-labeled with ^{32}P, with the use of a linear regression program written by G. W. HATFIELD, University of California, Irvine, for the MacIntosh personal computer.

Cloning of Ty*3* integrants: Integrated Ty*3* elements and flanking regions were cloned from several strains that had undergone transposition. Examples of wild-type (unmarked) Ty*3* and Ty*3*-HN insertions were examined. Genomic DNA from cells that had undergone transposition of unmarked Ty*3* elements was digested with *Eco*RI and fractionated by electrophoresis in 0.8% agarose gels. The size of *Eco*RI genomic fragments containing integrated Ty*3* elements was determined by Southern hybridization analysis as described above. Fragments containing Ty*3* elements were cloned into the *Eco*RI site of pIBI20 and transformed into *Escherichia coli* HB101 (*F-hsdS20*[r_B^-, m_B^-] *recA13 leuB6 ara-14 proA2 lacY1 galK2 rpsL20* [Sm^r] *xyl-5 mtl-1 supE44*). The ampicillin-resistant transformants were transferred to nitrocellulose and screened for insertions containing Ty*3* sequences by colony hybridization using a Ty*3*-specific probe.

Integrated, *HIS3*-, *neo*^{r}-marked Ty*3* elements were cloned as described above. However, in this case, ampicillin-resistant transformants were further screened for Ty*3*-containing plasmids by their ability to grow on L-plates containing 25 or 50 μg/ml kanamycin. In cases where the cloning efficiency was limited by the large size of the *Eco*RI restriction fragments containing Ty*3* insertions, genomic DNA was digested with *Pst*I, *Bgl*II or a combination of *Eco*RI and *Sal*I (which cuts once in the Ty*3* element) or *Bam*HI and *Sal*I. In these cases, digested genomic DNA was fractionated by electrophoresis and DNA of the appropriate length was ligated into the compatible sites of pIBI20 or pIBI21. For these integrants, ampicillin-resistant transformants were transferred to nitrocellulose and screened for clones containing Ty*3* insertions by hybridization with a Ty*3*-specific probe.

Four integration sites also were cloned from strain yDLC221 before transposition occurred, using one of two methods. Two were cloned as genomic DNA fragments. Genomic DNA was digested with *Eco*RI and separated by electrophoresis. Fractions containing DNA fragments of the appropriate size were inserted into the *Eco*RI site of pIBI20. Clones containing preintegration target sites were identified by colony hybridization using probes to genomic DNA flanking cloned sites of Ty*3* insertions. Two sites were cloned using the polymerase chain reaction method (PCR) (SAIKI *et al.* 1988). Oligonucleotide primers were designed containing both sequences flanking Ty*3* insertion sites and restriction enzyme recognition sites. Fragments amplified by PCR were digested with the appropriate restriction enzymes and isolated by electroelution from 7% acrylamide:bis gels (30:1). Isolated fragments were inserted into compatible restriction sites in pIBI20 and transformed into *E. coli*. Plasmids containing an amplified genomic fragment were identified by nucleotide sequence analysis using the M13 universal primer.

Nucleotide sequence analysis: The nucleotide sequences in the regions of Ty*3* insertion were determined for cloned integrants and preintegration target-sites using the dideoxy-chain termination method (SANGER, NICKLEN and COULSON 1977). All reactions utilized the Sequenase enzyme (U.S. Biochemical Corp.) and [α-^{35}S]dATP (1000 Ci/mmol; Amersham Corp.). Fragments containing complete Ty*3* elements were subcloned as *Eco*RI/*Sal*I restriction fragments to separate 5′ and 3′ sigma elements. Oligonucleotides complementary to sigma sequence were used to prime polymerization on double-stranded plasmid templates (HALTINER, KEMPE and TJIAN 1985).

Analysis of *ura3* mutations after galactose induction of Ty*3* transposition: Strain yPKSUP2U is an isogenic derivative of yVB110 in which the *URA3* gene has been substituted for the *SUP2* tRNA^{Tyr} gene (P. KINSEY, personal communication). In this strain, only six nucleotides of the 3′ end of the mature tRNA-coding sequence remain. This strain was transformed with the plasmid pEGTy*3*-T2. Transformants were selected on synthetic medium lacking tryptophan, inoculated into 40 independent 2 ml cultures of synthetic, galactose-containing medium, minus tryptophan, and grown at 30° to a cell density of 2–4 × 10^7 cells/ml (3 days). Cells were collected by low-speed centrifugation and resuspended in 200 μl of synthetic medium. Half of this cell suspension was plated onto medium containing 5-FOA. Genomic DNA was isolated from 27 of the 5-FOA-resistant colonies originating from individual galactose-grown cultures. This DNA was digested with *Eco*RI and analyzed by Southern hybridization with a *URA*3-specific probe as described above.

RESULTS

Specificity of insertion of unmarked Ty*3* elements: Ty*3* and sigma elements are found associated with tRNA genes. Nevertheless, these elements do not undergo high frequency transposition, and it was not

known whether Ty*3*-1 would display this specificity when undergoing induced transposition. Yeast cells transformed with plasmid pEGTy*3*-1, which contains a galactose-inducible Ty*3* element, produce high levels of Ty*3* RNA when grown on medium containing galactose. Ty*3* transposition occurred in 3–6% of transformed cells upon induction of Ty*3* transcription (HANSEN, CHALKER and SANDMEYER 1988). In order to determine whether this transposition was into tRNA gene-flanking sequences, two Ty*3* elements that had transposed independently in strain yVB110 were cloned, and the sequences of the Ty*3*-genomic junctions were determined. In both cases, the Ty*3* elements had integrated into the 5′-flanking sequence of a tRNA gene. DNA sequence analysis showed that one element had integrated at position -17 relative to the 5′ end of a $tRNA^{Val}$(AAC)-coding sequence, while the other had inserted at position −17 relative to a $tRNA^{Asp}$(GUC)-coding sequence. These two Ty*3* insertions are flanked by the 5-bp repeats (in the tRNA noncoding strand) 5′ TATTC 3′ and 5′ AAACT 3′, respectively. Both insertions are in the orientation that is predominant among sigma elements (SANDMEYER *et al.* 1988). In this orientation, Ty*3* transcription is divergent to that of the tRNA gene.

Selection for Ty*3* transposition: Previous studies have relied on hybridization analysis to identify cells in which Ty*3* transposition occurred. This type of assay is relatively insensitive and labor intensive. A system was therefore developed in which Ty*3* integration can be selected. The galactose-inducible Ty*3* element was tagged with the yeast *HIS3* gene so that, in a *his3* genetic background, genomic integration can be detected by the acquisition of the *HIS3* marker. A similar approach has been used to study the transposition of the yeast retrotransposons, Ty*1* and Ty*2* (BOEKE, XU and FINK 1988; GARFINKEL *et al.* 1988).

Because *TYB3*, the second Ty*3* open reading frame, is continuous with the downstream sigma (HANSEN and SANDMEYER 1990), it is difficult to interrupt the Ty*3* internal domain without destroying the transposition function of the Ty*3* element. To circumvent this problem, a fully competent, galactose-inducible, Ty*3* element was integrated into the genome of yVB110 (shown schematically in Figure 1A) in order to provide in *trans* the proteins required for transposition of a marked, defective element. When the resulting strain, yDLC221, is grown on galactose as a carbon source, high levels of Ty*3* transcripts are observed (data not shown).

In order to determine whether this integrated Ty*3* could complement transposition of a marked element, yVB110 cells, transformed with pEGTy*3*-H (Figure 1B), and yDLC221 cells, transformed separately with plasmids pEGTy*3*-H and pEGTy*3*-HN (Figure 1C), were subjected to the transposition selection. Transformants were grown on minus-uracil medium, containing glucose (uninduced) or galactose (induced), patched onto YPD plates, then assayed for the presence of His^+, 5-FOA-resistant cells. The results of this assay are shown in Figure 2. yVB110 transformants grown on glucose- or galactose-containing medium and yDLC221 transformants maintained on glucose-containing medium showed little or no reversion to a His^+ phenotype. However, yDLC221 transformants grown on galactose-containing medium showed a high frequency of reversion, indicating that transposition of the marked Ty*3* element is dependent upon its own transcription and complementation by the integrated, helper element. Cells containing the doubly marked element in plasmid pEGTy*3*-HN showed a significantly lower reversion frequency after maintenance on galactose compared to cells containing the element marked with *HIS3* alone. It is not known if this difference is caused by decreased levels of full-length Ty*3* transcripts, inefficient packaging, or poor replication of the transcript from the doubly marked element.

Analysis of multiple independent Ty*3* integrations: A large number of independent Ty*3* genomic integrations were studied by Southern blot analysis. Helper strain yDLC221 was transformed with pEGTy*3*-HN, containing the doubly marked Ty*3* element. After induction of 128 independent transformants by growth on galactose-containing medium, cells were patched onto YPD medium, then transferred to histidine-minus medium, containing 5-FOA, in order to select cells that retained the marked Ty*3* in the absence of the plasmid *URA3* gene. A total of 122 His^+ revertants were isolated. In order to ensure that each isolate had undergone independent transposition event(s) only a single derivative of each of the original 122 independent transformants was analyzed.

Southern hybridization analysis was performed in order to determine the nature of each event resulting in the His^+ phenotype. Genomic DNA samples isolated from His^+ cells were digested with *Eco*RI, for which there are no recognition sites within Ty*3*. These digests were fractionated and analyzed on Southern blots by hybridization to Ty*3*-, *HIS3*-, and sigma-specific probes. Three types of events can be postulated to result in the His^+ phenotype of 5-FOA-resistant cells: (1) transposition of the *HIS3*-marked element and loss of the plasmid; (2) gene conversion or other homologous recombination events between the integrated helper Ty*3* or an endogenous sigma and the plasmid-borne, marked Ty*3*, together with loss of the *URA3* sequences; and (3) mutations inactivating the *URA3* gene carried by the plasmid. A representative sample of the analysis is shown in Figure 3. Panel A shows the Ty*3*-specific hybridization pattern for a selection of 17 independent His^+ revertants (lanes 2–18). In almost every case, there were one or more

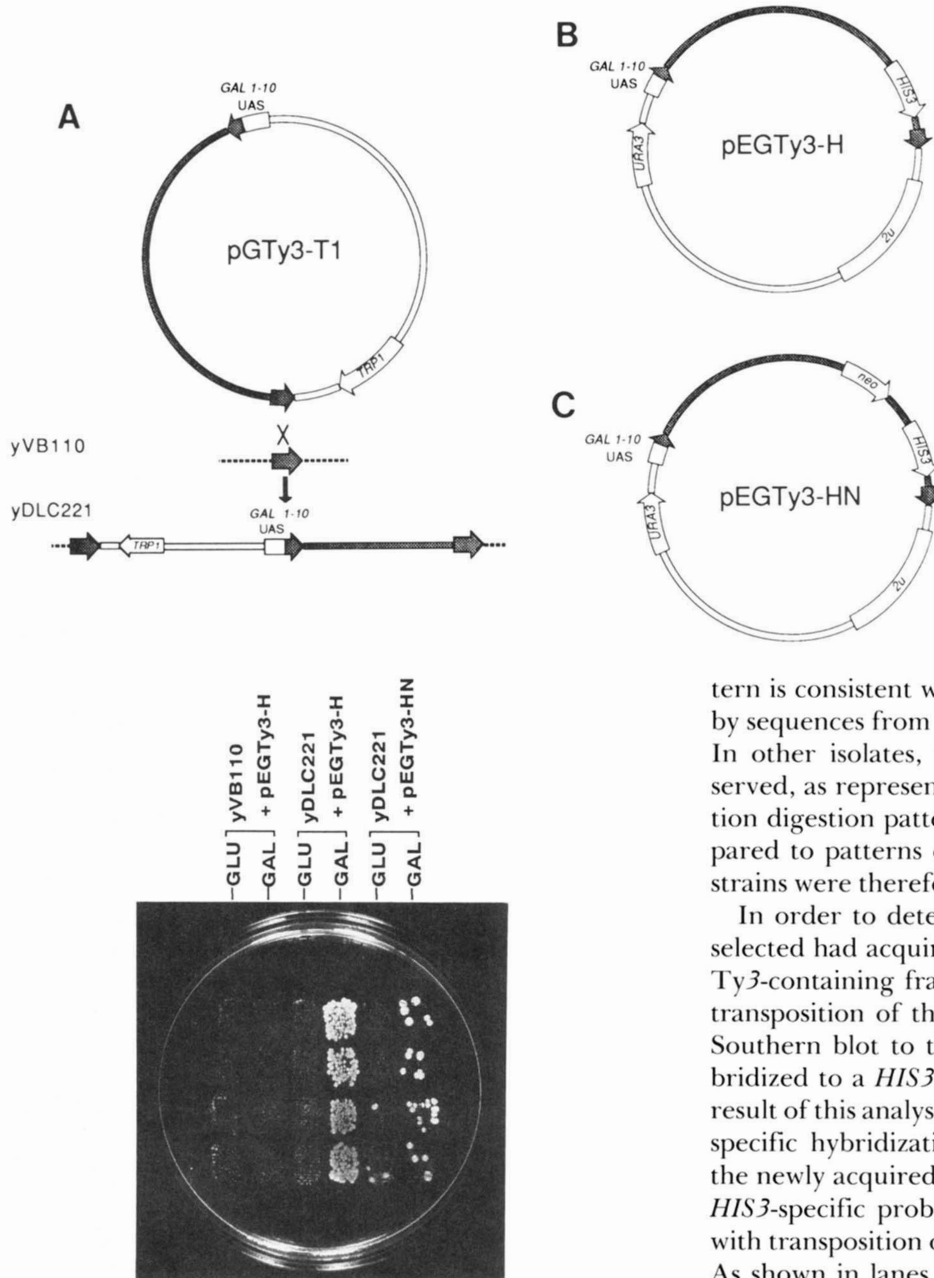

FIGURE 1.—Construction of helper strain and structures of donor plasmids. (A) The helper strain, yDLC221, and marked, donor plasmids, (B) pEGTy*3*-H and (C) pEGTy*3*-HN, were constructed as described in the MATERIALS AND METHODS. A schematic diagram depicting the integration of the helper-Ty*3* element is shown beside the donor plasmids: shaded arrows and shaded boxes, sigma elements and Ty*3* internal domains, respectively; open arrows, selectable markers *TRP1*, *URA3*, *HIS3* and *neo*^{r}, as labeled in the diagram; open end of arrows, *GAL1–10* UAS; wide open boxes, yeast 2-μm episome; narrow open boxes, *E. coli amp*^{r}, and ori sequences; and dashed lines, genomic DNA flanking the endogenous sigma element and integrated, helper Ty*3*.

FIGURE 2.—Selection for Ty*3* transposition. After growth of yVB110 cells transformed with pEGTy*3*-H and yDLC221 cells transformed with either pEGTy*3*-H or pEGTy*3*-HN on glucose (GLU)- or galactose (GAL)-containing medium, cells were passaged on YPD, and then replica-plated onto synthetic medium containing 5-FOA and lacking histidine. Growth of His$^+$, 5-FOA-resistant cells is shown above. Each patch represents cells originating from an individual colony grown on either glucose- or galactose-containing medium.

Ty*3*-hybridizing fragments in addition to the single Ty*3*-hybridizing fragment originally present in the helper strain (lane 1). In the pattern of hybridization of three isolates shown in this panel (lanes 6, 7 and 17), the 11-kbp fragment containing the helper Ty*3* is not visualized, but in each case a larger fragment (13 kbp, lanes 6 and 7; 12 kbp, lane 17) which hybridized to the Ty*3*-specific probe is apparent. This pattern is consistent with replacement of the helper Ty*3* by sequences from the longer, donor Ty*3* (see below). In other isolates, multiple Ty*3* insertions were observed, as represented in lane 18. These gave restriction digestion patterns that could not simply be compared to patterns of single Ty*3* insertions and these strains were therefore excluded from further analysis.

In order to determine whether the clones we had selected had acquired genomic copies of *HIS3* on the Ty*3*-containing fragment, as would be predicted for transposition of the marked donor Ty*3*, a duplicate Southern blot to the one shown in panel A was hybridized to a *HIS3*-specific probe. Panel B shows the result of this analysis. A comparison of Ty*3*- and *HIS3*-specific hybridization patterns showed that most of the newly acquired Ty*3* elements hybridized with the *HIS3*-specific probe. These patterns were consistent with transposition of the marked, donor Ty*3* element. As shown in lanes 6, 7 and 17, in which the 11-kbp fragment containing the endogenous helper Ty*3* was not apparent, the new, larger fragment that hybridized to the Ty*3*-specific probe also hybridized to the *HIS3*-specific probe. Transposition into the fragment containing the helper Ty*3* DNA would have increased the size of that fragment by approximately 7 kbp, rather than the 1–2-kbp increase observed. Therefore, these results are consistent with a double crossover or gene conversion event substituting all or part of the *HIS3*-, *neo*^{r}-containing internal domain for the helper Ty*3* sequences, but are not consistent with *de novo* transposition.

Some of the strains we selected showed evidence of having retained plasmid sequences. Hybridization with the *HIS3*- and sigma-specific probes shown in panels B and C (lane 10) revealed a fragment that is

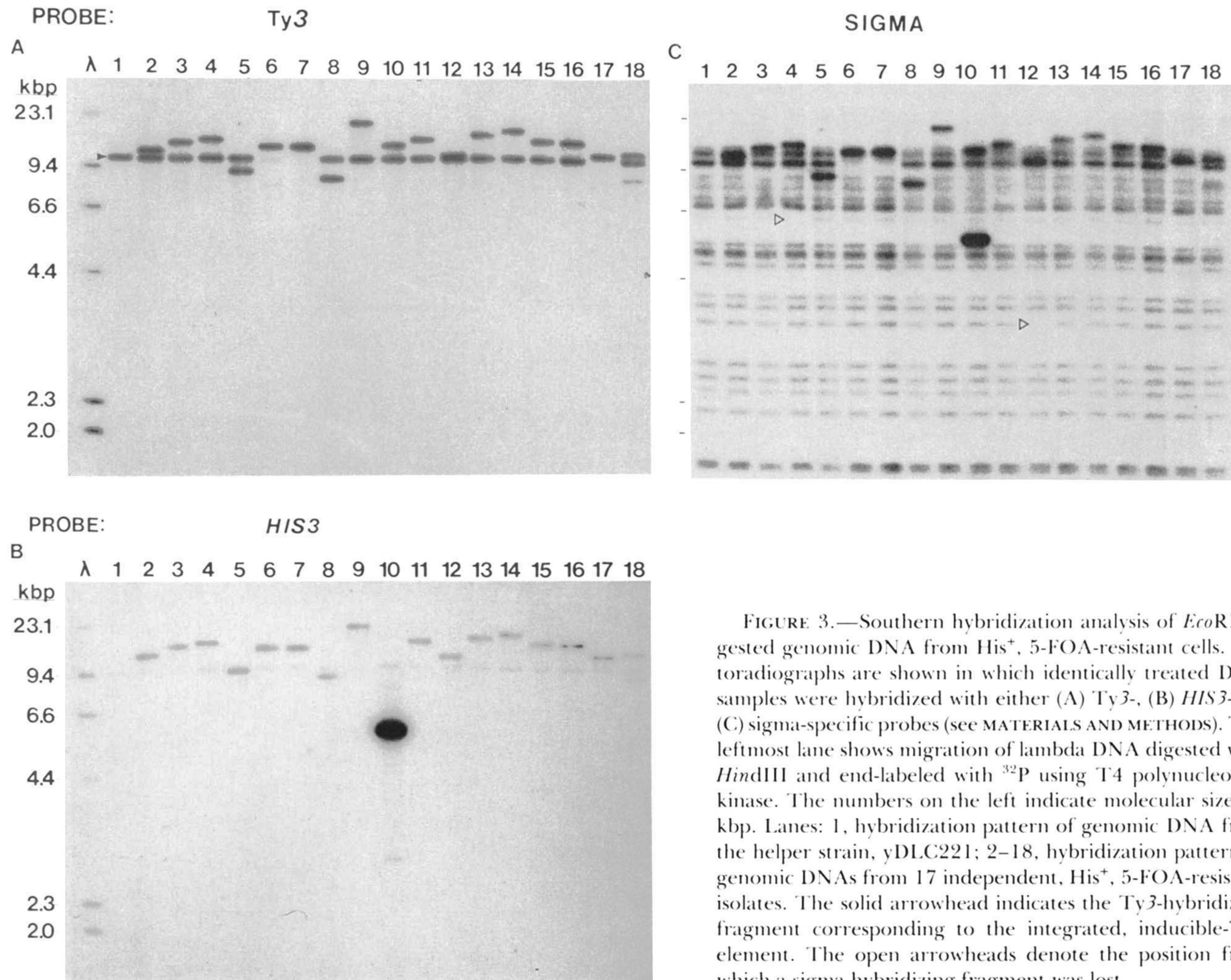

FIGURE 3.—Southern hybridization analysis of *Eco*RI-digested genomic DNA from His$^+$, 5-FOA-resistant cells. Autoradiographs are shown in which identically treated DNA samples were hybridized with either (A) Ty*3*-, (B) *HIS3*-, or (C) sigma-specific probes (see MATERIALS AND METHODS). The leftmost lane shows migration of lambda DNA digested with *Hin*dIII and end-labeled with ^{32}P using T4 polynucleotide kinase. The numbers on the left indicate molecular sizes in kbp. Lanes: 1, hybridization pattern of genomic DNA from the helper strain, yDLC221; 2–18, hybridization pattern of genomic DNAs from 17 independent, His$^+$, 5-FOA-resistant isolates. The solid arrowhead indicates the Ty*3*-hybridizing fragment corresponding to the integrated, inducible-Ty*3* element. The open arrowheads denote the position from which a sigma-hybridizing fragment was lost.

highly represented. The intensity of hybridization indicated that this fragment was likely to be of plasmid origin. The small size of the hybridizing fragment, lack of hybridization with a Ty*3*-specific probe, and 5-FOA resistant phenotype of the strain were consistent with deletion of both Ty*3* and *URA3* sequences from the plasmid. The revertant from which DNA was isolated for the analysis shown in lane 10 also appeared to have undergone a genomic insertion of an unmarked Ty*3* element, because it acquired a unique Ty*3*-hybridizing fragment that did not hybridize to the *HIS3*-specific probe. Data were consistent with retention of extrachromosomal plasmid sequences in nine of the 122 His$^+$ revertants; however, the alterations in these plasmids allowing for maintenance on 5-FOA-containing medium appeared quite heterogeneous.

Screening a third, identical blot with a sigma-specific probe (panel C) allowed determination of whether any changes in fragments containing sigma elements accompanied acquisition of the Ty*3* element and His$^+$ phenotype. That the Ty*3* insertions represented in lanes 4 and 12 are associated with fragments previously containing sigma elements was indicated by the loss of 6.4 and 3.8 kbp sigma-hybridizing fragments present in the helper strain and the appearance of 14- and 11-kbp Ty*3*-hybridizing fragments, respectively. Such a change is consistent with insertion upstream of a tRNA gene associated with a sigma element, homologous recombination of the marked Ty*3* into an endogenous sigma element, or Ty*3* integration upstream of a tRNA gene which is found coincidentally on an *Eco*RI restriction fragment containing a tRNA associated with a sigma element. These three possibilities were not distinguished by Southern blot analysis. Hybridization patterns generated from four of the 122 His$^+$ revertants were consistent with Ty*3* insertions into sigma-containing *Eco*RI restriction fragments. Some alterations in sigma-containing fragments would have escaped detection due to the number of fragments that hybridize to the sigma-specific probe. Nevertheless, most sigma-containing fragments were resolved, so this analysis should provide a reasonable estimate of the number

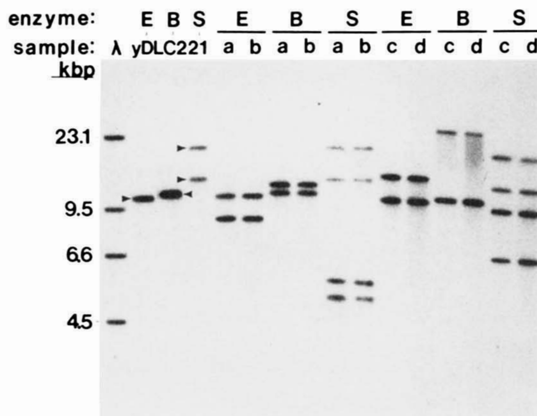

FIGURE 4.—Comparison of the Ty*3*-hybridization patterns of genomic DNA from cells containing *de novo* insertions of Ty*3* elements at the same genomic location. Southern hybridization analysis of DNA from four independent, His$^+$, 5-FOA-resistant isolates is shown. The leftmost lane shows migration of ^{32}P-labeled, lambda DNA digested with *Hin*dIII. The molecular sizes are indicated in kbp. The next three lanes show the Ty*3*-hybridization to genomic DNA isolated from the helper strain, yDLC221, digested with *Eco*RI (E), *Bam*HI (B), or *Sal*I (S) restriction enzymes. Arrowheads indicate genomic fragments containing the integrated, inducible Ty*3* element. Samples a, b, c and d represent DNA from cells that were independently induced for Ty*3* transposition. Genomic DNA from samples a and b or samples c and d were digested with the indicated enzyme and analyzed side-by-side to directly compare the Ty*3*-hybridization patterns.

of events that involved fragments containing sigma elements. Because an estimated 10% of tRNA genes are associated with sigma elements, this number of insertions into sigma-containing fragments would be consistent with tRNA gene-specific transposition. This kind of event was documented for one genomic locus (see below).

In order to determine whether multiple, independent Ty*3* insertions occurred at any genomic loci, DNA from each revertant was digested with *Bam*HI and *Sal*I in addition to *Eco*RI. Digests were fractionated by electrophoresis, transferred to nitrocellulose, and the Ty*3*-hybridization pattern for each was analyzed. *Bam*HI, like *Eco*RI, does not cleave within the Ty*3* element, so a single characteristic fragment was observed for each insertion. *Sal*I cleaves once internally within the element, so two distinct, Ty*3*-hybridizing fragments were produced for each unique insertion. DNA samples from integrants that had similar *Eco*RI, *Bam*HI and *Sal*I restriction patterns were rescreened side-by-side in order to identify subtle differences in the sizes of Ty*3*-containing fragments. Figure 4 shows a representative autoradiogram from this analysis. The first three lanes to the right of the *Hin*dIII-digested, lambda DNA size markers show the hybridization pattern generated from the Ty*3* element present in the helper strain, after digestion with each of the three enzymes. Samples a and b and samples c and d show identical patterns of Ty*3* hybridization after digestion with each of the three enzymes, indicating

TABLE 1

Ty*3* genomic integration

Insertions per locus	Observed[a]	Expected[b]	χ^2
0	276	279.6	0.046
1	77	70.7	0.561
2	7	8.9	0.406
>2	0	0.8	0.802
			1.815
Excluded events:			
Multiple integrations	9		
Homologous recombination[c]	13		
Plasmid mutations	9		

[a] The zero class for the observed was determined by subtracting the number of genomic sites used from the estimated 360 targets. The four integrants that were associated with changes in sigma-hybridization patterns are included in the observed sample of 91 insertions.

[b] Expected values are those predicted for a Poisson distribution of 91 independent integrations assuming a target size equal to 360, the estimated number of tRNA genes in the haploid genome.

[c] Nine of these events represented gene conversion or double crossover events within the integrated Ty*3* sequence. The other four events represented homologous recombination events associated with integrated vector sequences at the helper locus as identified by the hybridization pattern of *Eco*RI-digested DNA with a probe that recognized plasmid sequences (data not shown).

that Ty*3* insertion was likely to have occurred into the same genomic location for both members in each of these pairs. Of the 122 His$^+$ revertants, six genomic loci appeared to have served as targets for two independent Ty*3* insertions. In each of these cases, identical patterns of Ty*3* hybridization were observed for each member of a pair after digestion with *Sal*I, suggesting that the independent integrations into a given fragment were close to the same position and in the same relative orientation. For a seventh pair, the hybridizing fragments produced with each of the three enzymes from one member were shifted in mobility by a distance corresponding to 1.5 kbp relative to the fragments generated from the other member of the pair. Further analysis showed that this pair of integrations was into a single locus, but that one of the Ty*3* elements had sustained an internal deletion.

The data for the 122 revertants are summarized in Table 1. A total of 31 revertants resulted from either homologous recombination events, plasmid mutations, or multiple Ty*3* insertions. The remaining 91 of the 122 revertants represented single integrations of the marked-Ty*3* element. Of these 91, fourteen represent members of seven pairs of independent integrations into seven different loci. The remaining 77 insertions occurred into unique genomic sites. Assuming that each of the estimated 360 tRNA genes present in the haploid genome (SCHWEIZER, MACKECHNIE and HALVORSON 1969; FELDMAN 1976; GUTHRIE and ABELSON 1982) is equally available as a target for Ty*3* integration, a Poisson distribution predicts that, in a survey of 91 independent events, approximately 71 loci would be used once, and ap-

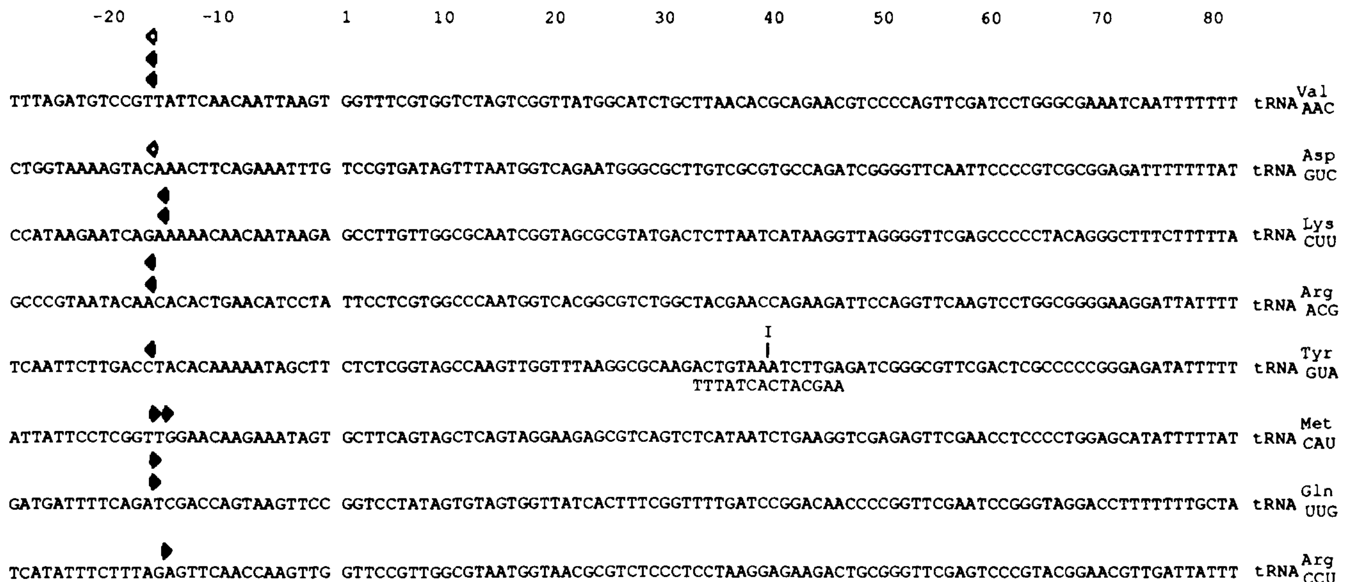

FIGURE 5.—Nucleotide sequences of tRNA genes and 5′-flanking regions used as target sites for *de novo* Ty*3* integration. Arrowheads denote positions of Ty*3* insertion and point in the direction of Ty*3* transcription. Open and solid arrowheads represent integrations of Ty*3*-1 and Ty*3*-HN elements, respectively. The identity of each tRNA was inferred from the nucleotide sequence of the anticodon (as indicated) and confirmed by comparison to published sequences (BAKER *et al.* 1982; DRABKIN and RAJBHANDARY 1985; NELBOCK, STUCKA and FELDMANN 1985; GOODMAN, OLSON and HALL 1977; OLAH and FELDMANN 1980; WEISSENBACH, MARTIN, and DIRHEIMER 1975; TSCHUMPER and CARBON 1982; GAFNER, DE ROBERTIS and PHILIPPSEN 1983). To facilitate the alignment of each tRNA-coding sequence, the sequence of the 14-bp intron contained within the $tRNA^{Tyr}$(GUA) gene is shown beneath the coding sequence and its position within the gene is indicated above (I). The $tRNA^{Met}$(CAU) gene identified contains a T to C base substitution at position +63. This mutation is located outside essential promoter elements necessary for tRNA transcription, but is within the predicted T-loop stem and probably does not allow proper secondary-structure formation of the tRNA produced.

proximately nine loci would be used twice. The number of loci expected to be used more than twice is less than one. The χ^2 values determined from these integrations are given in Table 1. The probability of a χ^2 of the calculated value, if the number of targets is 360 and usage is random, is 42%. Therefore, the number of unique and repeated insertions observed for the 91 independent events is consistent with that predicted if the number of target sites is approximately equal to the estimated number of tRNA genes. These data suggest that the target size is similar to the number of tRNA genes, and that if all of the insertions are into tRNA genes, that there is no highly preferred subset of targets.

Sequence analysis of genomic DNA flanking integrated Ty*3* elements: To examine whether Ty*3* insertions into the same fragment were in fact upstream of the same tRNA gene (as assumed in the preceding statistical analysis) and in order to elucidate other characteristics of the insertion process, the sequences at the Ty*3*-genomic junction for each of five pairs of insertions and a single member from each of the remaining two pairs were determined. Each of the twelve insertions of the marked Ty*3* element characterized by DNA sequence analysis was at position −16 or −17, relative to the 5′ end of a tRNA-coding region (Figure 5). Genes encoding $tRNA^{Val}$(AAC), $tRNA^{Lys}$(CUU), $tRNA^{Arg}$(ACG), $tRNA^{Arg}$(CCU), $tRNA^{Tyr}$(GUA), $tRNA^{Met}$(CAU) and $tRNA^{Gln}$(UUG) were identified flanking the insertions. For the $tRNA^{Val}$(AAC), $tRNA^{Lys}$(CUU), $tRNA^{Arg}$(ACG) and $tRNA^{Gln}$(UUG) genes, insertions into the same locus occurred at the same nucleotide positions in the upstream sequence, positions −17, −16, −17 and −17, respectively. In addition, the $tRNA^{Val}$(AAC) gene used as a target for insertions of the marked Ty*3* was also used as a target for one of the characterized insertions of the unmarked Ty*3*. The insertion of the unmarked element was also at position −17 upstream of the $tRNA^{Val}$(AAC) gene. Interestingly, the two insertions upstream of the $tRNA^{Met}$(CAU) gene occurred at positions one nucleotide apart, −16 and −17. Therefore, at least for this particular gene, the site of Ty*3* integration is not absolutely constrained. Only single clones representing each of the insertion pairs flanking the $tRNA^{Tyr}$(GUA) gene and the $tRNA^{Arg}$(CCU) gene were isolated. These insertions occurred at positions −17 and −16, respectively.

At four of the seven genomic integration sites analyzed above, the Ty*3* insertion occurred in the orientation previously observed most commonly, so that the tRNA gene was proximal to the 5′ end of the element. Interestingly, although both orientations of Ty*3* with respect to the tRNA gene were observed among these cloned insertions, all Ty*3* integrations into any particular genomic locus were in the same

orientation, including the three insertions at the tRNA^{Val}(AAC) gene and the two pairs of insertions in the less common orientation.

To ensure that insertions occurred via transposition, rather than recombination into a preexisting sigma, clones containing four of the insertion sites were isolated from the parental strain yDLC221 before induced transposition. The nucleotide sequences in the regions of Ty*3* insertion at the tRNA^{Val}(AAC), tRNA^{Lys}(CUU), tRNA^{Arg}(ACG) and tRNA^{Tyr}(GUA) genes indicated that integration had occurred by a transposition mechanism as no sigma elements were present at the exact position of insertion. In addition, for at least the tRNA^{Val}(AAC) and tRNA^{Lys}(CUU) genes, only a single copy of the 5-bp duplication flanking the ends of the integrated element existed prior to insertion. The sequences at the tRNA-distal, genomic-Ty*3* junction upstream of the tRNA^{Arg}(ACG) and tRNA^{Tyr}(GUA) genes were not determined. The sequence upstream of the tRNA^{Arg}(ACG) gene showed a sigma element at position oriented so that transcription of the sigma element and the tRNA gene are in the same direction. Insertion of the two marked Ty*3* elements upstream of this gene occurred at position −17, two nucleotides within the endogenous sigma sequence and in the opposite orientation.

Screen for insertions into fragments containing genes for 5S or U6: All characterized Ty*3* and sigma insertions were upstream of tRNA genes. The absence of a target consensus at the integration sites suggested that insertion is directed by features common to tRNA genes or even to genes transcribed by polymerase III. If this is the case, then 5S and U6 genes also might be targets for Ty*3* transposition; promoter sequences or transcription complexes might contribute to the formation of the Ty*3* integration target. The genes for 5S rRNA are located within the tandem rDNA repeat present in approximately 140 copies on chromosome *XII* (SCHWEIZER, MACKECHNIE, and HALVORSON 1969; BELL *et al.* 1977; PETES 1979). If 5S genes are equivalent to tRNA genes as genomic targets for Ty*3* integration, then several insertions into these genes would be expected in a sample of 91 Ty*3* insertions. The U6 gene encodes a small nuclear RNA and is present in a single copy within the yeast genome (BROW and GUTHRIE 1988). Although U6 genes are transcribed by polymerase III, their promoter sequences and transcription factor requirements may differ from those of the 5S and tRNA genes (BROW and GUTHRIE 1988; KUNKEL *et al.* 1986; REDDY *et al.* 1987; KROL *et al.* 1987; REDDY 1988).

Southern blots of *Eco*RI- or *Bam*HI-digested genomic DNA isolated from the 122 His$^+$ revertants were hybridized separately with 5S- and U6-specific probes (data not shown). No evidence of Ty*3* integration adjacent to members of either of these gene classes was observed. Therefore, if chromosomal 5S genes are capable of serving as targets for Ty*3* integrations, they are used at a much lower frequency than are tRNA genes. Because U6 is a single-copy gene, the absence of a U6 target in a sample of 91 integrations argues only that U6 is not a highly preferred target.

Analysis of *ura3* mutations after galactose induction of Ty*3* transposition: In order to test whether Ty*3* could also show high frequency integration into a gene transcribed by polymerase II, strain yVB110 was modified by substituting the *URA3* gene for the *SUP2* tRNA^{Tyr} gene by integrative transformation. Yeast strains have been identified in which the *SUP2* gene is associated with a sigma element (SANDMEYER and OLSON, 1982). Thus, there is no long-range regional exclusion of Ty*3* integration at this locus. This strain was used to investigate the ability of Ty*3* to integrate into a *URA3* target. The yPKSUP2U cells transformed with plasmid pEGTy*3*-T2 were grown in 40 separate, 2 ml cultures of synthetic medium containing galactose as a carbon source and lacking tryptophan. Under similar conditions in previous experiments, 3 to 6% of galactose-grown cells showed evidence of having undergone Ty*3* transposition (HANSEN, CHALKER and SANDMEYER 1988). After three days at 30°, cells in stationary phase were plated onto 5-FOA-containing medium in order to identify cells lacking a functional *URA3* gene. No increase in the frequency of 5-FOA resistant colonies was observed in cells overexpressing Ty*3*, compared to identically grown, nontransformed cells (data not shown). Of the 40 independent galactose-grown cultures, 30 gave rise to at least one 5-FOA-resistant colony with an average number of seven resistant colonies per 3×10^7 cells plated. If 1% of cells had undergone random transposition, then approximately 20 insertions into the 1-kbp *URA3* gene ($1/1.4 \times 10^4$ of the yeast genome) would be expected to result per 3×10^7 cells plated. Genomic DNA was isolated from 27 of the 5-FOA-resistant colonies, each representing an independent culture. Southern hybridization analysis with a *URA3*-specific probe showed no evidence of Ty*3* integration associated with the 5-FOA resistant phenotype (data not shown). In addition, because none of the genomic DNA samples showed a change in the mobility of the fragment containing the *URA3* gene, our results suggest that high level expression of Ty*3* does not greatly stimulate Ty*1* insertion.

DISCUSSION

This study was undertaken to characterize *de novo* transposition of Ty*3* associated with high levels of Ty*3* transcripts. Because the entire Ty*3* internal domain is occupied by essential coding sequences, we first char-

acterized transpositions of unmarked Ty3 elements identified by hybridization screening. Subsequently, genetically marked Ty3 elements carried on high copy donor plasmids were constructed that were shown to be complemented for transposition by expression of a single integrated, galactose-inducible Ty3 element.

This study showed that newly transposed Ty3 elements are associated with tRNA genes. Because previously characterized sigma and Ty3 elements were stably existing in the genomes of the strains from which they were isolated, it was possible that the association that we had observed with tRNA genes reflected the relative stability of these insertions, rather than specificity of integration. In order to examine a large number of events, transposition was separately induced in 122 colonies independently transformed with the marked, Ty3 donor plasmid. Sequence analysis of insertion junctions of a total of fourteen transposed, wild-type and marked Ty3 elements showed position specificity as was previously observed for naturally occurring Ty3 and sigma elements. Although we have not excluded the possibility that insertions that are not associated with tRNA genes are highly unstable, the results of these experiments do suggest that a high proportion of Ty3 insertions are into the 5′-flanking sequence of tRNA genes and that insertion itself is position specific. In addition, these experiments showed that the specificity of Ty3 transposition is not compromised by *trans* complementation or by high levels of Ty3 RNA and proteins resulting from overexpression under the *GAL1–10* UAS.

Colonies surviving the transposition induction regimen and selected on 5-FOA-containing, minus-histidine medium were classified as strains that had undergone loss of *URA3*, Ty3-related recombination events or Ty3 transposition by Southern blot analysis. Nine strains contained mutations in donor plasmids that allowed plasmid maintenance in the presence of 5-FOA. Nine other strains displayed patterns indicating the occurrence of multiple, Ty3-related genomic rearrangements. Homologous recombination events occurred in thirteen cases. Four involved integration of the donor vector and probable deletion of *URA3* sequences, while the remainder were consistent with the pattern expected for gene conversion of the helper by the marked, donor Ty3 element. Of the 91 integration events that had Southern blot patterns consistent with Ty3 transposition events, four integrations were accompanied by a detectable change in a sigma-hybridizing fragment and so potentially involved a sigma-associated tRNA gene target. Fourteen of the 91 integration events were contributed by two independent insertions at each of seven tRNA genes.

Because of the presence of a single, helper Ty3 element and approximately 30 sigma elements in the genome of the transformed strain, it is possible to ask whether local homology increases the usage of particular target sites. There were four events that showed hybridization patterns consistent with either Ty3 recombination with the genome mediated by sigma homology or Ty3 transposition into a fragment containing a sigma element. If sigma-associated tRNA genes were average targets, close to nine integrations into sigma-containing fragments should have been observed. Our data suggest, therefore, that homology of several hundred nucleotides at potential targets, eg. sigma-associated tRNA genes, does not necessarily cause them to be favored as targets. In addition, a sigma element was identified by sequence analysis in the upstream flank of a $tRNA^{Arg}$(ACG) gene that was used in two independent integrations. These insertions were in the orientation opposite that of the endogenous sigma, arguing against an effect of homology in target selection.

We conducted an analysis of genomic loci that were the targets of multiple insertion events in order to estimate the number of targets in the yeast genome using a Poisson distribution. Hybridization analyses have been used to estimate that there are between 320 and 400 tRNA genes and that they are dispersed in the yeast genome (SCHWEIZER, MACKECHNIE and HALVORSON 1969; FELDMANN 1976; reviewed in GUTHRIE and ABELSON 1982). The probability of observing two insertions in the same site in a study of 91 events with a target size of 360 was calculated using the formula $P(2) = e^{-m} \times m^2/2!$, where $m = 91/360$–the probability that a given tRNA gene would be sampled (Table 1). Among 91 integrations, assuming 360 equivalent targets, the probability of recovering the observed number of seven double insertions is therefore 42%. Using the Poisson distribution, a target size near 500 would actually have provided the best fit to the number of double insertions we recovered. In order to independently test this prediction, the number of unique and repeated insertions expected for target sizes ranging from 300 to 600 was estimated using the Monte Carlo method and a random number generating program, written in C language for the MacIntosh II computer (M. SUNDSTROM, Fort Worth, Texas, personal communication). For a target size of 500, a compilation of 200 trials of 91 samples each gave an average of 75.5 unique and 7.1 double selections. This independent estimation is consistent with the Poisson distribution estimate of a target size of about 500, slightly larger than the estimated number of tRNA genes. Therefore, the distribution of unique and repeated insertions, together with the previous analysis of sequences flanking Ty3 and sigma element insertions, and the current sequence analysis of fourteen *de novo* Ty3 insertions, argues that tRNA genes are the primary targets of

Ty*3* transposition. A fraction of insertions into other genomic sites cannot be ruled out by our study. However, these results do make some additional predictions about what constitutes a target, by clarifying what is not a target.

Although the coincidence of the number of tRNA genes and the number of targets estimated from the Poisson distribution must be taken as approximate, two points deserve special emphasis. First, the estimated number of target sites for insertion is not grossly larger than the estimated number of tRNA genes. In particular, no insertions into fragments containing other polymerase III-transcribed genes, such as 5S genes, were observed out of the 91 insertions studied.

Second, because the estimated number of Ty*3* targets is not much smaller than the number of tRNA genes, there is no evidence that there are tRNA genes that are hotspots for Ty*3* insertion. For instance, sigma-associated tRNA genes did not appear to be preferred. Our data also suggest that there is not a set of tRNA genes that are much better targets for Ty*3* insertion by virtue of distinctively higher–or lower–transcriptional activity.

In an effort to directly test whether a polymerase II-transcribed gene, *URA3*, could function as a target for Ty*3* integration, Ty*3* was expressed at high levels in a *URA3* genetic background. No evidence was obtained for Ty*3*-mediated inactivation of *URA3*. These results contrast with those from a similar experiment in which the yeast retrotransposon Ty1 was induced from the *GAL1* promoter on a high copy plasmid; in that experiment, 82 out of 83 5-FOA-resistant strains isolated, showed evidence of Ty*1* insertion into the *URA*3 gene (NATSOULIS *et al.* 1989). Our analysis does not definitively exclude the possibility of Ty*3* insertion into genomic targets transcribed by polymerase II. It is possible that the frequency of Ty*3* integration into *URA3* was below the level of detectability or that Ty*3* insertion at *URA3* could be into an upstream region that does not disrupt function. Nevertheless, it is clear that Ty*3* insertions capable of *URA3* disruption occurred much less frequently than other mutations; therefore, Ty*3* clearly differs from Ty*1* in its ability to disrupt this locus.

Sequence analysis of multiple Ty*3* insertions at five loci allowed us to address additional questions about the nature of the tRNA gene target: are all Ty*3* insertions at a given tRNA gene at the identical base, and are all insertions at a given tRNA gene in the same orientation? The sequences of a total of ten insertions at five loci showed that at four genes, the pairs of insertions occurred at the same nucleotide position, but that at one gene, one insertion occurred at position −16, and one occurred at the more common position −17. Although the sample is limited, the observation of insertions at different positions upstream from the same tRNA gene suggests that insertion is not absolutely spaced from conserved nucleotides within the tRNA gene.

Southern hybridization analysis supported by the DNA sequence suggested that some feature of the tRNA gene target causes the observed 1:2 bias in the orientation of Ty*3* insertions. The overall orientation of Ty*3* insertions characterized by DNA sequence analysis in this study is consistent with the bias that was observed previously. Out of fourteen insertions analyzed, nine were in the common orientation with divergent transcription of Ty*3* and the tRNA gene, and the remaining five were in the minor orientation with a common direction of transcription. If this bias in the orientation results from some intrinsic asymmetry in the Ty*3* integration apparatus, then Ty*3* insertions would be distributed in orientation independent of the target tRNA gene. On the other hand, if orientation is determined by the target, then insertions in the minor orientation should be clustered at particular tRNA genes. Although only two insertions of marked-Ty*3* elements were recovered for each of seven genes, in every case, *de novo* insertions into the same target were in the same orientation with respect to the tRNA gene. DNA sequence further refined the analysis. Two loci were each the targets of two insertions in the minor orientation. The random probability of two insertions at the same locus occurring in the minor orientation would be 0.3×0.3 or 0.09. The probability that this would occur twice is 0.008. Thus, circumstantial evidence suggests that the orientation of Ty*3* insertions is determined by the target tRNA gene. However, at one locus, two insertions occurred in the orientation opposite that of a preexisting sigma element. This suggests that selection of the orientation of the element is not absolute or may change with flanking sequence, but, nevertheless, is influenced by features of the target.

The results of this study are consistent with a complex target for Ty*3* insertion. Insertion is in the vicinity of transcription initiation and is not absolutely linked at a defined distance to an apparent consensus sequence. One candidate for the target is the transcription complex. There is evidence that global gene organization affects Ty*3* insertion as well. Although 5S genes were not genomic targets for Ty*3* insertion, recent experiments show that a 5S gene can function as a target when located on a plasmid, rather than within the rDNA repeat. In addition, orientation appears to be influenced by the genomic locus, but insertions recovered from plasmids show that this is not strictly a function of the tRNA gene sequence (unpublished data).

The existence of a relatively limited number of integration sites for Ty*3* should facilitate definition of

the target requirements of this retrotransposon. Furthermore, understanding the interaction of the Ty3 transposition machinery with tRNA genes may provide information about the structure and organization of these genes within the yeast genome. Mutational analysis of a *SUP2* tRNA^{Tyr} gene, present on a target plasmid, is underway to identify the features of tRNA genes that make them targets of Ty3 transposition.

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