A Ubiquitin-Conjugating Enzyme, RAD6, Affects the Distribution of Ty*1* Retrotransposon Integration Positions

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ABSTRACT

A galactose-inducible Ty*1* element was used to generate 59 independent Ty*1* inserts that inactivate the *CAN1* gene. As found in previous studies, the distribution of these elements shows a gradient of insertion frequency from highest to lowest between the 5′ and 3′ ends of the gene. However, 53 independent Ty*1* and Ty*2* insertions isolated by an identical procedure in an isogenic *rad6* deletion strain do not show this bias. In this strain, the Ty elements insert randomly throughout *CAN1*. These results show that the ubiquitin-conjugating enzyme, RAD6, alters the integration site preferences of Ty*1* retrotransposons.

THE insertion of retroviruses and retrotransposons into the host genome is independent of homology between the ends of the elements and the sites of the insertion. At the insertion site, a characteristic short host segment, the target sequence, is duplicated and surrounds the inserted element. It is now clear that the choice of integration site for most retroviruses or retrotransposons is not random, although little sequence specificity is displayed. Rather, integration site preferences seem to be influenced by functional or structural states of the target DNA (for review see SANDMEYER, HANSEN and CHALKER 1990).

In several studies, nonrandom association was found between positions of proviral insertions and transcriptionally active or nuclease-sensitive chromatin regions (ROHDEWOHLD *et al.* 1987; ROBINSON and GAGNON 1986; VIJAYA, STEFFEN and ROBINSON 1986; MOOSLEHNER, KARLS and HARBERS 1990; SCHERDIN, RHODES and BREINDL 1990). More extreme target site biases were demonstrated for Rous sarcoma virus, which tends to insert into a few highly preferred sites within the complete chicken genome (SHIH, STOYE and COFFIN 1988). The spleen necrosis retrovirus shows an insertion site preference for AT rich regions, although there is no specifically preferred target sequence (SHIMOTOHNO and TEMIN 1980). Drosophila gypsy-like retrotransposons insert at TAYATA, which may either represent sequence specificity, or a functional specificity for the TATA box in front of Drosophila genes (IKENAGA and SAIGO 1982; INOUYE, YUKI and SAIGO 1984; FREUND and MESELSON 1984; SNYDER *et al.* 1982; TANDA *et al.* 1988). The Dictyostelium retrotransposons, Tdd-3 and DRE, as well as the yeast Ty*3*, insert specifically into polymerase III promoters (SANDMEYER, HANSEN and CHALKER 1990). A recent study of *in vitro* murine leukemia virus integration shows that insertion occurs more frequently into nucleosomal compared with nucleosome-free regions of minichromosomes. In addition, the α2 DNA-binding protein was shown to protect its binding site from integration (PRYCIAK and VARMUS 1992).

Like other retrotransposons, the yeast Ty*1* and Ty*2* elements transpose through an RNA intermediate (BOEKE *et al.* 1985). Both Ty*1* and Ty*2* elements contain the same long terminal direct repeats of about 335 bp called deltas, which surround a central region of 5.3 kb. The central region includes the *gag-pol* (*Tya-Tyb*) gene equivalent of retroviruses. Ty*1* and Ty*2* elements differ by substitutions of 1 and 2 kb within this central region. Common laboratory strains contain about 30–35 copies of Ty*1* elements and 5–15 copies of Ty*2* elements (see BOEKE and SANDMEYER 1991 for review). The mapping and cloning of many of these elements in a variety of strains revealed a nonrandom distribution. They are found preferentially in AT rich regions (OYEN and GABRIELSEN 1983; NATSOULIS *et al.* 1989; WILKE *et al.* 1989) and are frequently associated with tRNA genes (GAFNER, DE ROBERTIS and PHILIPPSEN 1983). They also frequently exist as compound structures, indicative of repeated transpositions of one element into another (WARMINGTON *et al.* 1986).

Analyses of Ty insertions into several genes indicate a preference for insertion into the promoter or 5′ end of the coding sequence. In some instances, this specificity is due to the type of selection used. For example, insertions in the vicinity of *HIS3* (BOEKE *et al.* 1985; BOEKE, STYLES and FINK 1986) and *ADH2* (WILLIAMSON *et al.* 1983) were selected on the basis of their

activation of the target gene, and thus would be expected to occur in the promoter. However, target site biases are also observed when Ty insertions are selected on the basis of gene inactivation. Eight of 10 spontaneous Ty insertions selected on the basis of inactivation of *LYS2* at 30° fell within the 5′ noncoding region (EIBEL and PHILIPPSEN 1984; SIMCHEN *et al.* 1984). Similar results were obtained when an extensive collection of Ty insertions that inactivate *LYS2* and *URA3* was obtained by using the galactose-inducible Ty*1* element on a high copy plasmid at 22° to increase the transposition frequency. The positions of these insertions indicated that a gradient of insertion frequency, from highest to lowest, existed between the 5′ and 3′ ends of both the *LYS2* and *URA3* genes (NATSOULIS *et al.* 1989).

Likewise, we (WILKE *et al.* 1989) mapped 10 of 21 Ty insertions that inactivated *CAN1* to the 5′ noncoding region in one strain. In this study insertions were selected at 20° to increase the frequency of transposition, but without the benefit of the galactose-inducible Ty*1* element. The distribution of insertions into the *CAN1* gene was also determined in two other strains that were mutant for *rad6*. In these strains, Ty insertions into *CAN1* were obtained at 30° since the *rad6* mutation increases the transposition frequency. Unlike the 5′ biased distribution of Ty elements observed in the wild-type strain, the distribution of Ty insertions at *CAN1* in the *rad6* strains appeared to be random. These results suggest a role for *RAD6* in Ty target site selection. However, since the *RAD6* and *rad6* strains were not isogenic and were grown at different temperatures, it is not clear if the *rad6* mutation or these other factors effected the change in the distribution of insertion sites. In this paper we show that the target site distribution differences are due to the *rad6* mutation.

Mutations in the DNA repair gene *RAD6* cause a variety of phenotypes including: extreme vulnerability to DNA damaging agents; deficiencies in sporulation, meiotic recombination and induced mutagenesis; and enhanced rates of mitotic recombination and spontaneous mutagenesis (for review see FRIEDBERG, SIEDE and COOPER 1991). Part of the increase in spontaneous mutagenesis has been shown to be due to an enhanced rate of retrotransposition of Ty elements. Mutations in *rad6* increase the rate of retrotransposition of Ty*1* and Ty*2* elements from 20 to 100 fold into the *CAN1*, *URA3* and *SUP4*-o genes (PICOLOGLOU, BROWN and LIEBMAN 1990; KANG *et al.* 1992).

RAD6 encodes an E2 ubiquitin conjugating enzyme that polyubiquitinates the core histones H2A, H2B and H3 *in vitro* (SUNG, PRAKASH and PRAKASH 1988; HASS *et al.* 1990). Attachment of the highly conserved 76-amino acid polypeptide ubiquitin to a variety of proteins targets them for degradation, stabilization or modification of structure and activity (see for review JENTSCH, SEUFERT and HAUSER 1991). An intriguing possibility is that *RAD6* may ubiquitinate chromosomal proteins, causing an alteration in chromatin structure important for the *rad6* mutant phenotypes (JENTSCH, MCGRATH and VARSHAVSKY 1987). However, while H2A is ubiquitinated in higher eucaryotes, there is no evidence for ubiquitination of H2A in yeast (SWERDLOW, SCHUSTER and FINLEY 1990). Furthermore, the C-terminal acidic domain of the RAD6 protein is required for the *in vitro* polyubiquitination of histones but not for its *in vivo* effects on DNA repair or induced mutagenesis (SUNG, PRAKASH and PRAKASH 1988). Thus, the *in vivo* target of the *RAD6* enzyme remains to be established.

Here we investigate the effect of mutations in the gene encoding the ubiquitin-conjugating enzyme, RAD6, on target site preferences of the yeast retrotransposons Ty*1* and Ty*2*.

MATERIALS AND METHODS

Media and strains: Media used to score various markers have been described (SHERMAN, FINK and LAWRENCE 1979). Nutritional markers were scored on synthetic complete glucose medium (SC), lacking an appropriate nutrient (*e.g.*, SC-Ura). Canavanine resistant mutants were isolated and scored on SC-Arg supplemented with 60 mg/liter of canavanine sulfate (SC-Arg+Can). For the galactose induction of the hybrid Ty element fused to a *GAL1* promoter on the pGTy*1*-H3 plasmid, strains were grown on SC-Ura with 2% galactose and 2% sucrose instead of glucose. Strain LP2752-4B*rad6-*Δ (also referred to as GF-299, WILKE *et al.* 1989) contains a *URA3* disruption of *rad6* and was derived from LP2752-4B (*MATα his4-260,39::*pBR313 *his4-864,1176 lys1-1 ura3-52*) (MORRISON, MILLER and PRAKASH 1988). Both strains were kindly provided by Louise Prakash. L-1249 is a *ura3* derivative of LP2752-4B*rad6-*Δ selected on 5-fluoroorotic acid containing medium (PICOLOGLOU, BROWN and LIEBMAN 1990). Strains L-1249 (*rad6-*Δ) and LP2752-4B (*RAD6*) were transformed (ITO *et al.* 1983) with pGTy*1*-H3 prior to the isolation of *can1* mutations.

Plasmids and probes: Plasmid pGTy*1*-H3, kindly supplied by J. Boeke (BOEKE *et al.* 1985), was used to increase the rate of Ty element transposition. It contains a galactose-inducible Ty*1* element on a 2-μm based vector with the *URA3* selectable marker. The 1.7-kb *Bam*HI-*Sal*I fragment containing *CAN1* (see Figure 1), isolated from plasmid pSH2 (WILKE *et al.* 1989), was used as a hybridization probe. Plasmid CEN-ACT was kindly supplied by K. Weinstock and J. Strathern, who received it from T. Dunn and D. Shortle. It is a YCp50 plasmid bearing a 3.5-kb *Bam*HI-*Eco*RI fragment that contains the entire actin gene (NG and ABELSON 1980). The 1.7-kb *Bam*HI-*Hin*dIII fragment isolated from this plasmid was used as a hybridization probe. The YCp50 based plasmid pR67, with a *Bam*HI-*Hin*dIII fragment containing *RAD6*, and plasmid pSCW218 containing a deletion of *RAD6* disrupted with *URA3*, were kindly supplied by L. Prakash (MORRISON, MILLER and PRAKASH 1988). The 2.5-kb *Bam*HI-*Hin*dIII fragment isolated from pSCW218 and containing 0.4 kb and 1.0 kb of *RAD6* flanking DNA surrounding a 1.1-kb fragment carrying *URA3* was used to disrupt the *RAD6* gene. Fragments were isolated by gel purification using DEAE-45 paper as de-

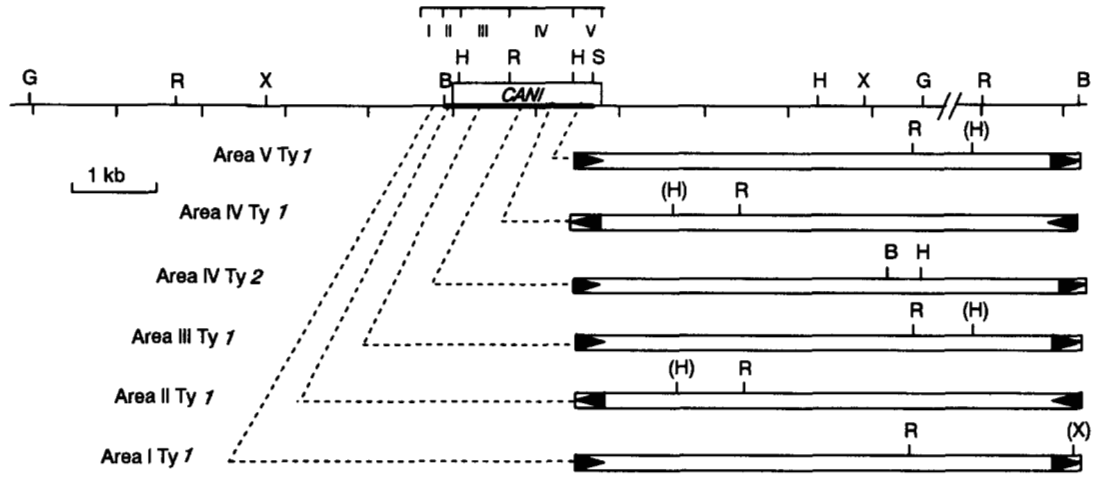

FIGURE 1.—Criteria used to localize the positions of Ty elements to one of five areas in the region of the *CAN1* gene. Insertion sites were determined by DNA blot analyses of genomic digests of *can1* mutants from the isogenic strains LP25752–4B and LP25752–4B*rad6*-Δ as described in the text. The restriction map of the *CAN1* region in these strains is shown. The bold line denotes the DNA fragment used to probe the blots; the labeled box represents the *CAN1* open reading frame. The direction of transcription of *CAN1* is from left to right. The diagonal cross-lines indicate that the length of the intervening DNA did not fit on the map. The left and right *Eco*RI fragments with homology to the probe are 4., 3 and 8.7 kb, respectively, and the *Bam*HI fragment is 11 kb. The positions of the Ty elements are indicated by dotted lines. Only one orientation of the Ty element is shown as an example in each area. At the end of each Ty element are the long terminal repeats (delta elements) indicated by small black triangles pointing in the direction of the Ty element's transcription. Only those sites that were analyzed for every insertion within a given area are shown on the respective insertion maps. The sites in parenthesis were present in some but not all inserts. Area I inserts were identified by digestion with *Eco*RI, *Xho*I and *Bam*HI; area II inserts were identified by *Eco*RI, *Hin*dIII and *Bam*HI digestions; area III–V inserts were identified by *Eco*RI and *Hin*dIII digestions. In addition, all Ty2 inserts were digested with *Bam*HI. The restriction site symbols are: R, *Eco*RI; X, *Xho*I; G, *Bgl*II; B, *Bam*HI; S, *Sal*I.

scribed by Schleicher and Schuell.

Analysis of DNA: Standard procedures were used for yeast DNA isolation (WINSTON, CHUMLEY and FINK 1983), transformation of *E. coli*, plasmid isolations, restriction analysis, gel electrophoresis and DNA blot analysis (MANIATIS, FRITSCH and SAMBROOK 1982). Probes were labeled by nick translation with the USB kit, or by the random oligonucleotide primer method (FEINBERG and VOGELSTEIN 1983). The polymerase chain reaction (PCR) (SAIKI *et al.* 1985, 1988) was used to locate the positions of Ty element insertions in the 5′ region of the *CAN1* gene. DNA was amplified from whole yeast cells, without prior extraction of genomic DNA (SATHE *et al.* 1991), for 35 cycles with 2-min extension periods in a Perkin-Elmer Cetus Thermocycler using GeneAmp reagents. A unique primer (1Can1) located 37 to 17 bp to the right of the *Bam*HI site in the 5′ end of *CAN1* (5′-GAAATAAACTTTCGATTGAC-3′) and priming synthesis in the direction of the *Bam*HI site was used in conjunction with primer U3-in (NATSOULIS *et al.* 1989). U3-in (5′-ATATTATCATATACGGTGTT-3′) is homologous to positions +53 to +72 of δ elements and primes synthesis toward the remaining 263 bp of the element.

Analysis of RNA: RNA was extracted from mid-log cultures grown in complex medium (YPD) by the method of SCHMITT, BROWN and TRUMPOWER (1990). RNA denaturation, formaldehyde-electrophoresis and blotting were according to standard procedures (MANIATIS, FRITSCH and SAMBROOK 1982). Blots were probed with the 1.7-kb *Bam*HI-*Sal*I fragment containing *CAN1* and the 1.7-kb *Bam*HI-*Hin*dIII fragment containing most of the actin gene, labeled by the random primer method described above. Following a high stringency wash, filters were scanned with the Ambis Radioactive Scanning System II (Automated Microbiology Systems, Inc., San Diego, CA). This computer-controlled imaging system directly quantitates radioactivity from the two-dimensional filter.

Gene disruption of *RAD6*: *URA3* was used to disrupt *RAD6* in selected *RAD6 can1* strains containing area I Ty*1* elements. Since the strains to be disrupted carried *URA3* on plasmid pGTy*1*-H3, it was necessary to obtain *ura3* isolates that had lost the plasmid prior to the disruption experiment. This was done by streaking cells out for single colonies on nonselective medium (YPD). Individual colonies were then checked for growth on SC-Ura. The *ura3* derivatives were then transformed (ITO *et al.* 1983) with the 2.5-kb *Bam*HI-*Hin*dIII deletion-disruption fragment isolated from pSCW218. Transformants selected on SC-Ura were picked and tested for UV sensitivity and canavanine resistance. UV-sensitive transformants were shown to be *rad6* because they failed to complement *rad6* strain YJJ49 (**a** *his3-Δ1 leu2-3, 112 trp1-289 ura3-52 rad6-Δ::LEU2*), kindly provided by L. Prakash.

Statistical analyses: Chi-square goodness-of-fit tests were used to determine the probabilities that distributions obtained were random. A contingency chi-square test was used to evaluate the probability that distributions obtained are independent of *rad6* (HERSKOWITZ 1977).

RESULTS

Isolation of independent *can1* mutations: To determine if mutations in *RAD6* affect the distribution of Ty insertion sites, we isolated a large number of independent Ty insertion mutations in the *CAN1* gene in isogenic *RAD6* (LP2752-4B) and *rad6-Δ* (L-1249) strains. Since the fraction of the *can1* mutations that are due to Ty insertions is extremely small in *RAD6* strains (WILKE *et al.* 1989), we used the pGTy*1*-H3 plasmid (kindly supplied by J. Boeke; BOEKE *et al.* 1985), which contains a Ty*1* element fused to an

TABLE 1

Types of *can1* mutants obtained by canavanine selection of transposition induced cells

Parental strain genotype	Total *can1* mutants screened	No. single Ty1 inserts	No. single Ty2 inserts	No. not rearranged	No. other rearrangements
RAD6	121	59	0	53	9
rad6-Δ	60	48	5	2	5

inducible *GAL1* promoter, to increase the frequency of transposition. Growth on galactose causes a high level of Ty*1* transcription leading to a 20–100 fold increase in the frequency of transposition (BOEKE *et al.* 1985; BOEKE, STYLES and FINK 1986).

The *RAD6* and *rad6-Δ* transformants containing the pGTY1-H3 plasmid were streaked for single colonies on SC-Ura glucose medium at 30° to maintain the plasmid without inducing transposition. Suspensions of individual colonies were then spread on SC-Ura medium containing galactose and sucrose as the sole carbon sources to obtain independent colonies in which transposition was induced. Sucrose was included in these plates because our strains grow much better on this mixture of carbon sources and the sucrose decreases galactose induction only slightly (ALANI and KLECKNER 1987; HASHIMOTO *et al.* 1983). After 5–7 days' incubation at 22°, colonies were replica-plated to SC-Arg+Can medium and incubated at 30° for 3–7 days. Canavanine-resistant papillae, originating from different individual parent colonies, were picked, streaked for single colonies and checked for appropriate genetic markers including *can1*. All mutants resistant to 60 μg/ml of canavanine have been shown to carry mutations in the *CAN1* complementation group (WHELAN, GOCKE and MANNEY 1979).

Mapping the Ty insertion sites in *CAN1*: DNA blot analysis was used to distinguish Ty insertions from point mutations and other genomic rearrangements, to determine the transcriptional orientation of the Ty insertions, and to assign the location of each of the Ty elements to one of five areas in the region of the *CAN1* gene defined by restriction sites (see Figure 1). The results of analyses of 121 and 60 independent *can1* mutants derived respectively from the *RAD6* and *rad6-Δ* strains are summarized in Tables 1 and 2.

We have previously published extensive studies of *can1* mutants of this type isolated from the *rad6-Δ* strain used in this study, as well as from other strains. DNA blot analysis demonstrated the presence of 6-kb inserts that had Ty-characteristic patterns for *Eco*RI, *Bgl*II, *Hin*dIII, *Xho*I and *Bam*HI sites. In addition, new Ty*1*-hybridizing bands were shown to correspond to altered *CAN1*-hybridizing bands; the ends of 12 of the Ty elements in the *CAN1* locus were cloned and sequenced (WILKE *et al.* 1989; PICOLOGLOU *et al.* 1990).

TABLE 2

Location and orientation of Ty inserts in the *CAN1* gene

Parental strain genotype	Ty type and orientation[a]	Area[b] I	II	III	IV	V
RAD6	Ty*1* d	18	5	12	5	4
RAD6	Ty*1* i	0	3	6	5	1
RAD6	Ty2 d or i	0	0	0	0	0
rad6-Δ	Ty*1* d	1	1	5	8	6
rad6-Δ	Ty*1* i	0	2	9	11	5
rad6-Δ	Ty2 d	0	0	0	2	1
rad6-Δ	Ty2 i	0	0	0	2	0

[a] The direct (d) and inverse (i) notations indicate that the transcription of the Ty element is respectively in the same and opposite direction as transcription of the *CAN1* gene.

[b] Areas refer to regions in the *CAN1* gene defined by restriction sites (see Figure 1).

In the current study, mutants that retained the wild-type *Eco*RI bands of 4.3 kb and 8.7 kb were assumed to contain point mutations. Of 121 *can1* mutants isolated in the *RAD6* strain, nearly half, 53, were point mutations. In contrast, only two of 60 *can1* mutations isolated in the *rad6-Δ* strain were point mutations. All 126 mutations that gave rise to altered *Eco*RI bands were analyzed with additional enzymes; 112 of these were found to contain Ty*1* or Ty2 characteristic restriction maps; 14 others involve other rearrangements including deletions (see below).

We assigned the location of 19 Ty inserts to area I of the *CAN1* region, to the left of the *Bam*HI site (Figure 1), because they retained the wild-type 11-kb *Bam*HI fragment. Each of these inserts contained an *Eco*RI fragment of 2.7–3 kb instead of the wild-type 4.3-kb fragment. In seven of the 19 area I insertions, the wild-type 7-kb *Xho*I fragment was replaced by a 13-kb fragment, establishing the presence of a 6-kb insertion, characteristic of Ty elements. The remaining 12 area I insertions contained an approximately 5-kb *Xho*I fragment in place of the wild-type fragment. This places a new *Xho*I site 2 kb from the new *Eco*RI site at the predicted location of the right delta element (see Figure 1). Since delta elements often contain *Xho*I sites, this restriction map is diagnostic for a Ty*1* insertion. The sizes of the altered *Eco*RI fragments in these mutants indicate that all of the area I Ty*1* insertions are in the same transcriptional (direct) orientation as *can1* and are within 300 bp of the *Bam*HI site.

DNA from each strain containing an area I Ty*1*

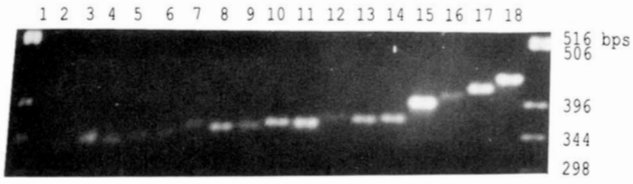

FIGURE 2.—PCR amplicons showing positions of area I Ty element insertions. Genomic DNA was amplified using primers 1Can1 and U3-in, which amplify DNA between the 5′ end of *CAN1* and a directly oriented δ element upstream. Amplicons were analyzed on a 2% agarose gel with *Hin*fI-pBR322 markers. Lane 1 was from the single area I Ty*1* insertion obtained in the *rad6*-Δ strain. Lanes 2–18 were from area I Ty*1* insertions in the *RAD6* strain. An additional *RAD6* strain containing an area I Ty*1* insertion identical in size to that shown in lane 13 is not included in this gel.

insertion was amplified by PCR. Synthesis was primed by the primer pair 1Can1 and U3-in. While this primer pair did not direct the synthesis of an amplified product from the parental *can1* strain, unique amplification products ranging in size from about 335–435 bp were generated from the strains containing area I Ty elements. The ability of U3-in to prime the synthesis of specific amplification products in these insertions verifies the presence of a δ element in the predicted orientation within 15–115 bp of the *Bam*HI site. The 19 area I Ty elements represent at least nine distinct insertion sites, since they gave rise to amplicons of different sizes when analyzed together on the same gel (see Figure 2).

Area II inserts all caused a 6-kb increase in the size of the *Bam*HI hybridizing fragment, but did not alter the sizes of the 1.3-kb and 3-kb *Hin*dIII fragments. All of the area II inserts contained an *Eco*RI site, which defined the orientation of the Ty*1* elements. Six were in the direct orientation while five were in the inverse orientation.

Area III and IV inserts caused alterations in the 1.3-kb but not the 3-kb *Hin*dIII fragments. The 1.3-kb fragment either became a single 7.3-kb fragment or became two fragments, the sum of which was 7.3-kb. Area III and IV Ty elements were distinguished by their effect on the *Eco*RI fragments. Area III elements caused an alteration in the 4.3-kb but not in the 8.7-kb *Eco*RI fragments, while the reverse was true of area IV elements. The sizes of the altered *Eco*RI bands defined the orientation of the Ty*1* elements and indicated that four area IV insertions were probably Ty*2* elements since they did not contain any *Eco*RI sites. The orientation of these Ty*2* elements was determined from the sizes of the *Hin*dIII and *Bam*HI fragments. There were 32 area III and IV Ty elements in the direct orientation and 33 in the inverse orientation.

Area V inserts caused alterations in the 3-kb but not the 1.3-kb *Hin*dIII fragment. The 3-kb fragment either became a single 9-kb fragment or became two fragments, the sum of which was 9-kb. The 8.7-kb but not the 4.3-kb *Eco*RI fragment was also altered in these inserts, and the sizes of the altered bands established that 10 Ty*1* elements had inserted in the direct orientation while six Ty*1* elements were in the inverse orientation. In addition, one Ty*2* element was found to be in the direct orientation on the basis of the absence of an *Eco*RI site and the sizes of the *Hin*dIII and *Bam*HI bands.

Comparison of the distribution of insertion sites in *rad6*-Δ and *RAD6* strains: The distribution of Ty insertions at *CAN1* is shown in Figure 3 and Table 3. In the *RAD6* strain the 5′ noncoding region and the 5′ end of the gene are preferred targets compared with the rest of the gene. This biased distribution is not found in the *rad6*-Δ strain. It should be noted that the distribution bias is even greater than apparent in Figure 3 since the number of Ty insertions per kb shown for area I was not corrected for the fact that only directly oriented Ty elements in area I cause canavanine resistance. A chi-square test of the data in Table 3 indicates that the distribution of Ty insertions in the *RAD6* strain is not random ($P < 0.0001$), while the distribution in the *rad6*-Δ strain is random ($P > 0.7$). Also, a contingency chi-square test comparing the *RAD6* and *rad6*-Δ distributions indicates that they are not the same ($P < 0.001$).

***RAD6* or *rad6*-Δ alleles do not affect the phenotypes of Ty insertions at *CAN1*:** As described above, area I is a preferred target in the *RAD6* strain, but not in the isogenic *rad6*-Δ strain. This might mean that mutations in *RAD6* alter the frequency of Ty*1* element insertion into certain regions of the *CAN1* gene. However, this altered distribution would also result if Ty*1* insertions in the 5′-noncoding region of *CAN1* were lethal, or were less likely to cause a canavanine-resistant phenotype, in the presence of *rad6*-Δ. We eliminated these possibilities by showing that six *RAD6 can1* mutants carrying independent area I Ty*1* elements all remained viable and resistant to canavanine after we disrupted their *RAD6* genes. The Ty*1* elements in these six mutants were found at various locations in area I and are pictured in Figure 2 lanes 5, 6, 13, 15 and 16.

The converse experiment was also performed on *rad6*-Δ *can1* area II–V mutants. Two mutants from each area were made *RAD6* by transformation with plasmid pR67 (following the loss of the pGTy*1*-H3 plasmid). These transformants became UV resistant and remained canavanine resistant.

Comparison of *CAN1* mRNA levels in *rad6*-Δ and *RAD6* strains: RNA blot analysis was used to determine the levels of the *CAN1* transcript in the isogenic *RAD6* (LP2752-4B) and *rad6*-Δ (L-1249) strains. The amounts of the 2.3-kb *CAN1* mRNA were determined using a computer assisted radioisotope scanning system with the 1.4-kb actin mRNA as the loading con-

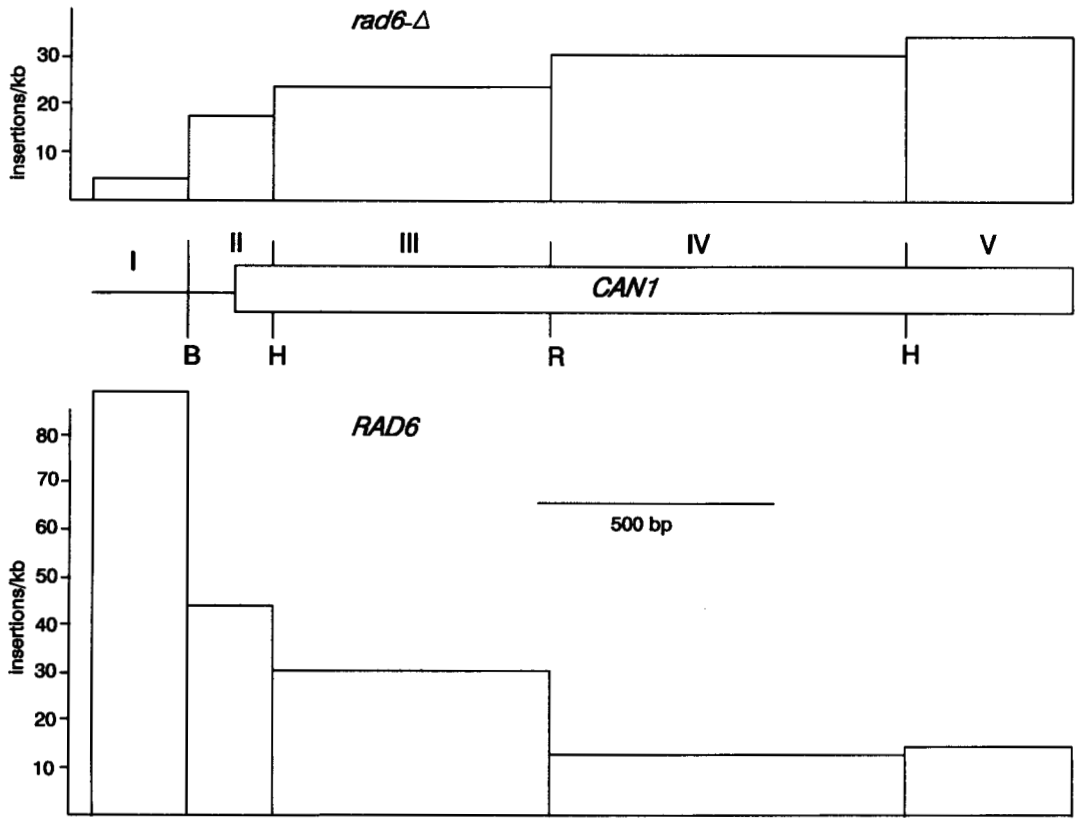

FIGURE 3.—Distribution of Ty insertions at the *CAN1* gene in isogenic *RAD6* and *rad6*-Δ strains. The bar graphs show the number of insertions per kb. The *CAN1* open reading frame starting at position 0 and ending at +1770 is boxed. Area I extends from −300 to the *Bam*HI site at −97. Area II is from −97 to the *Hin*dIII site at +83. Area III is from +83 to the *Eco*RI site at +673. Area IV is from +673 to the *Hin*dIII site at +1426. Area V is from +1426 to the position of the UAG stop codon at +1770. From our PCR, DNA blot and sequencing data in this and our previous study (WILKE *et al.* 1989), we estimate that none of the area I insertions are located further to the left than −300 bp; likewise, all area V insertions fall to the left or at most only slightly to the right of the UAG.

TABLE 3

Statistical analysis of the distribution of insertions in the *CAN1* gene

Area	% Expected if random[a]	*RAD6* Expected[b]	*RAD6* Observed	*rad6*-Δ Expected[b]	*rad6*-Δ Observed
I	5.2	3.07	18	2.76	1
II	9.1	5.37	8	4.82	3
III	30.0	17.70	18	15.90	14
IV	38.2	22.54	10	20.25	23
V	17.5	10.32	5	9.27	12
Total	100.0	59	59	53	53

[a] Percentages expected were based on the number of base pairs in each area. However we found that only directly oriented Ty insertions to left of the transcription start sites (at −90 to −72 bp, HOFFMAN 1985) cause canavanine resistance, while insertions in the rest of the gene in either orientation cause canavanine resistance. To correct this, the contribution of the area I distance was halved.

[b] The number of Ty insertions expected in each area assuming a random distribution.

trol. The normalized levels of *CAN1* mRNA were estimated to be similar in the *rad6*-Δ and *RAD6* strains, respectively, 0.8 and 1.0. This result clearly shows that there is no increase in the *CAN1* transcript due to the *rad6*-Δ mutation.

Other genomic rearrangements: The restriction maps of 14 of the 181 independent *can1* mutations isolated in this study are not consistent with either the creation of a point mutation or the insertion of a single Ty*1* or Ty*2* element (Table 4, Figure 4). Three of these mutations contain unusual insertions. One, obtained in the *rad6*-Δ strain (Figure 4a) contains an insert of 5 kb in area IV. This insert has no *Xho*I sites and is divided into 1-kb and 4-kb pieces by a single *Eco*RI site. The second insertion was about 300 bp and was found in area III of the *RAD6* strain (Figure 4b). Although this insertion does not contain an *Xho*I

TABLE 4

Characteristics of other rearrangements

Parental strain genotype	Type of rearrangement	No. found
RAD6	Complete deletion of *CAN1*	5
RAD6	Partial deletion of *CAN1* to telomere	1
RAD6	Delta size insert in area III	1
RAD6	Rearrangement in area V	2
rad6-Δ	Partial deletion of *CAN1* to telomere	1
rad6-Δ	Insertion of 5 kb in area IV	1
rad6-Δ	Ty1 multimer in area III	1
rad6-Δ	Rearrangement in area IV and V	1
rad6-Δ	Rearrangement in area I	1

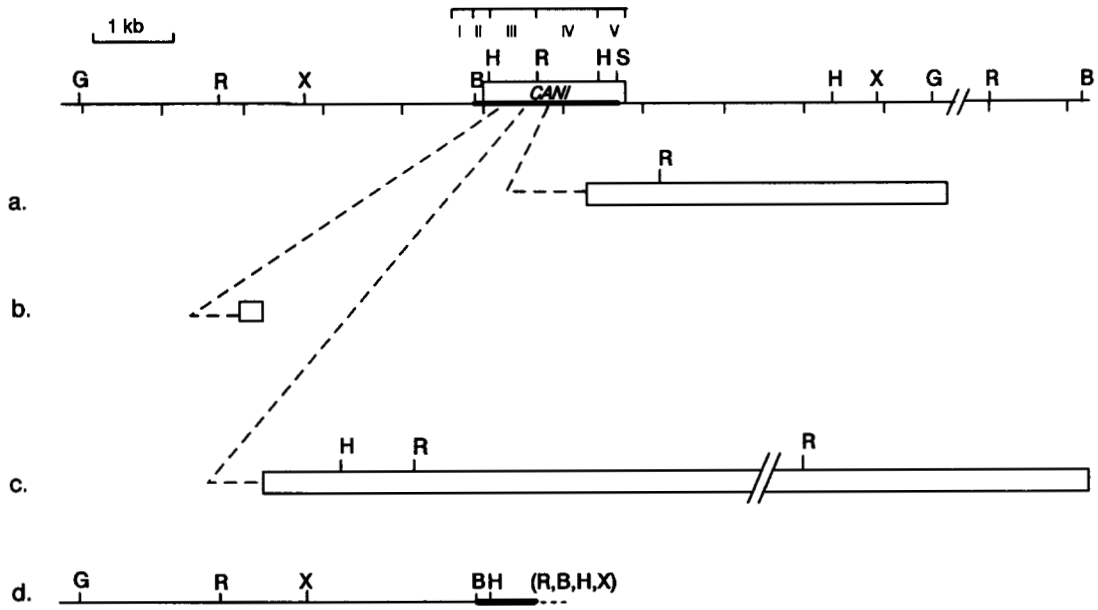

FIGURE 4.—Restriction maps of other *can1* rearrangements. Maps were determined by DNA blot analyses of genomic digests as described in the text. Above is pictured the restriction map of the *CAN1* region in the parental canavanine-sensitive strains. The bold line denotes the DNA fragment used to probe the blots; the labeled box represents the *CAN1* open reading frame. See Figure 1 legend for definition of restriction site symbols and for sizes of wild-type *Eco*RI and *Bam*HI hybridizing fragments. (a–c) Restriction maps of unusual insertions in *can1* mutants: (a) a 5-kb insert; (b) a delta-size insert; (c) a Ty multimer. The positions of the insertions are indicated by the dotted lines. Diagonal cross-lines indicate that the length of the intervening DNA did not fit on the map. (d) Restriction map of two *can1* partial deletions proposed to extend from the middle of *can1* to the telomere. The bold line denotes the DNA region homologous to the probe; the dashed line indicates the variable length telomere. Sites in parentheses all mapped at the same position and are proposed to mark the end of the chromosome.

site, its size suggests that it might be a solo delta element. The third unusual insertion (Figure 4c) was found in area III of the *rad6*-Δ strain. Here, the 8.7-kb *Eco*RI fragment was unaltered, while the 4.3-kb *Eco*RI piece was replaced by 6.3-kb and 4.0-kb bands. This is consistent with the insertion of a Ty*1* element in the inverse orientation slightly to the left of the *can1* *Eco*RI site. Also consistent with this interpretation is the finding that the 3-kb *Hin*dIII fragment was unaltered, while the 1.3-kb *Hin*dIII fragment was replaced by two fragments. However, the sizes of these two fragments did not add up to 7.3 kb as expected for the insertion of a single Ty*1* element. Rather, bands of 1.8 kb and greater than 20 kb were observed. These results can be explained by the insertion of several Ty*1* elements that do not contain *Hin*dIII sites, in tandem with the leftmost Ty*1* element with the characteristic but optional *Hin*dIII site. Ty*1* multimers were also suggested by the increase in size of the *Bam*HI fragment from 11 to greater than 25 kb. Ty multimers of this type have been reported previously in a mutant hunt involving gal-Ty (WEINSTOCK *et al.* 1990).

Five mutations, obtained in the *RAD6* strain, have been characterized as complete deletions of *can1* since all homology to the *can1* probe was missing. Two other mutations, one obtained in the *RAD6* strain and the other in the *rad6*-Δ strain, contain deletions of the right portion of the gene (Figure 4d). In these deletions "new" *Bam*HI, *Hin*dIII and *Xho*I sites each map at the approximate position of the *Eco*RI site in *can1*. Some of the bands produced by these "new sites" are unusually broad, suggestive of some variations in fragment length. This is what would be expected if the deletions extended to the end of the chromosome V right arm, fusing the variable length telomere to the middle of the *can1* gene. In this case, the "new sites" would simply denote the end of the chromosome. The four remaining *can1* mutations contain less well characterized rearrangements consistent with partial deletions covering respectively areas I, V and IV–V (Table 4).

DISCUSSION

The target site distribution of Ty*1* and Ty2 elements that inactivate the pol II genes *LYS2*, *HIS4*, *URA3* and *CAN1* have been previously analyzed in a *RAD6* genetic background. In each case, an integration site preference for the 5′ region of the gene was demonstrated and hot spots for transposition were observed (EIBEL and PHILIPPSEN 1984; FINK *et al.* 1980; SIMCHEN *et al.* 1984; NATSOULIS *et al.* 1989; WILKE *et al.* 1989).

In the present study 59 independent Ty*1* inserts that inactivate *CAN1* were isolated using a galactose-inducible Ty*1* element. As in the previous studies, we

found that the distribution of these elements shows a gradient of insertion frequency from highest to lowest between the 5′ and 3′ ends of the gene. However, 53 independent Ty*1* and Ty*2* insertions isolated by an identical procedure in an isogenic *rad6* mutant strain do not show this bias. In this strain, Ty*1* elements insert randomly throughout *CAN1*.

Several trivial explanations of these results have been eliminated. For example, it was possible that Ty insertions into the 5′-noncoding sequence of *CAN1* were not obtained in the *rad6*-Δ strain because such insertions do not cause a canavanine-resistant phenotype, or are lethal, in a *rad6*-Δ background. This possibility was disproved since Ty insertions into the 5′ region of *CAN1* in the *RAD6* strain remained viable and canavanine resistant when the *RAD6* gene was disrupted. Likewise, Ty insertions into the coding region of *CAN1* that were obtained in the *rad6*-Δ strain remained viable and canavanine resistant when transformed with a plasmid containing the *RAD6* gene. Finally, the positions of Ty insertions within the *CAN1* gene (areas I–V) do not affect colony size on canavanine medium in either the *rad6*-Δ or *RAD6* strains. Thus, the difference in the distribution of Ty target sites is not due to a bias in the selection of canavanine resistant mutants.

RAD6 also affects the target site distribution of Ty*1* elements that inactivate a plasmid borne pol III gene, the *SUP4*-o tRNA suppressor (KANG *et al.* 1992). In a *RAD6* strain, 23/26 insertions (88.5%) were at a single hot spot within the tRNA gene. In contrast, only 33/53 insertions (62.3%) occurred at this site in the isogenic *rad6* strain, with the remaining insertions occurring at numerous other positions.

These results demonstrate that the stimulation of Ty*1* transposition frequencies in *rad6* strains varies for different target sites. One hypothesis to explain this is that in *RAD6* strains the chromatin conformation of certain chromosomal regions, such as the 3′ ends of genes, inhibits the insertion of Ty elements. In contrast, the conformation of other regions such as hots spots and the 5′ ends of genes leaves them accessible to transposition. In *rad6* strains the conformation of the inaccessible chromatin is altered such that all regions become more equally available for transposition. In this way *rad6* would cause an increase in the rate of transposition particularly into chromosomal regions that were inhospitable to the insertion of Ty elements in the presence of *RAD6*. This proposed change in chromatin structure does not appear to be accompanied by increased transcription of the *CAN1* gene, since *CAN1* message levels are not increased by the *rad6* mutation.

The orientation of Ty elements located downstream of the *CAN1* transcription start sites at −90 to −72 bp (HOFFMAN 1985) is random ($P > 0.6$), indicating that Ty element insertion occurs equally in either orientation. However, all 29 inserts that occur, upstream of transcription start sites were in the same transcriptional orientation as *CAN1* (current study and WILKE *et al.* 1989). We propose that Ty elements do insert in the inverse orientation in this region of the gene, but that such insertions do not inactivate *CAN1* transcription because this orientation places the internal Ty element enhancer near the *CAN1* transcription start site. Indeed, Ty elements in this inverse orientation have been found to stimulate transcription of nearby downstream genes (ROEDER, ROSE and PEARLMAN 1985; ERREDE *et al.* 1985).

In addition to Ty insertions and point mutations, a variety of other rearrangements were detected among the *can1* mutants. One interesting type of rearrangement was obtained from both the *rad6* and *RAD6* strains. These rearrangements were missing the downstream half of the *CAN1* gene and appeared to contain a restriction site for every enzyme tested at about the position of the deletion breakpoint. This, together with the fact that these sites gave rise to unusually broad bands, indicative of some variations in fragment length, suggested that the *can1* deletion might extend to the end of chromosome V. The variability in fragment length would then be expected since telomeres are known to be of heterogeneous lengths (LOUIS and HABER 1990). The location of the *CAN1* gene within 55 kb of the telomere (LINK and OLSON 1991) is consistent with this hypothesis. Such rearrangements predict that the 3′ end of the *CAN1* gene is closest to the telomere.

The RAD6 protein is highly conserved among eukaryotes. Human homologs have about 70% identity to the yeast enzyme and can complement the DNA repair and mutagenesis phenotypes of *Saccharomyces cerevisiae rad6*-Δ mutations (KOKEN *et al.* 1991). Since retrotransposons such as Ty elements are also common in eukaryotes, the results presented here may have relevance to cellular mechanisms used to control target site selection of retrotransposons and retroviruses in other organisms, including humans.

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