MOLECULAR AND CELLULAR BIOLOGY, July 1984, p. 1393–1401
0270-7306/84/071393-09$02.00/0

Vol. 4, No. 7

Identification of Regulatory Regions Within the Ty1 Transposable Element That Regulate Iso-2-Cytochrome *c* Production in the *CYC7-H2* Yeast Mutant†

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Received 28 November 1983/Accepted 5 April 1984

The *CYC7-H2* mutation in the yeast *Saccharomyces cerevisiae* was caused by insertion of a Ty1 transposable element in front of the iso-2-cytochrome *c* structural gene, *CYC7*. The Ty1 insertion places iso-2-cytochrome *c* production under control of regulatory signals that are normally required for mating functions in yeast cells. We have investigated the regions of the Ty1 insertion that are responsible for the aberrant production of iso-2-cytochrome *c* in the *CYC7-H2* mutant. Five alterations of the *CYC7-H2* gene were obtained by specific restriction endonuclease cleavage of the cloned DNA and ligation of appropriate fragments. The *CYC7*$^+$, *CYC7-H2*, and modified *CYC7-H2* genes were each inserted into the yeast vector YIp5 and used to transform a cytochrome *c*-deficient yeast strain. Expression and regulation of each allele integrated at the *CYC7* locus have been compared in vivo by determination of the amount of iso-2-cytochrome *c* produced. These results show that distal regions of the Ty1 element are not essential for the *CYC7-H2* overproducing phenotype. In contrast, alterations in the vicinity of the proximal Ty1 junction abolish the *CYC7-H2* expression and give rise to different phenotypes.

The *CYC7-H2* mutation in the yeast *Saccharomyces cerevisiae* is a regulatory mutation that causes aberrant production of the respiratory protein iso-2-cytochrome *c*. Initial genetic analysis showed that the *CYC7-H2* mutation contains a lesion at the *CYC7* locus, which is the nuclear gene encoding iso-2-cytochrome *c* (41). The *CYC7-H2* mutation was caused by insertion of a 5.5-kilobase (kb) sequence in the 5′ noncoding region of the *CYC7* locus (15, 16). The inserted sequence is a member of the Ty1 family of dispersed repetitive elements found in yeasts (3). About 30 Ty1 elements are present in the haploid genome of most laboratory strains. These elements consist of a central segment denoted epsilon and two terminal repeat elements denoted delta. The delta elements are approximately 0.3 kb in length and occur as direct terminal repeats in Ty1 elements that have been characterized (12, 51).

DNA sequencing of the Ty1 junction region of the *CYC7-H2* mutation demonstrated that the Ty1 element is inserted 184 base pairs (bp) in front of the translated portion of the *CYC7* gene (26). This junction is 107 bp upstream from the 5′ map position of the wild-type mRNA as reported by Montgomery et al. (30). RNA blot analysis of *CYC7-H2* and *CYC7*$^+$ polyadenylated RNA showed that the sizes of mutant and wild-type RNA were the same (26). These results suggest that the inserted element is regulating the efficiency of transcription at the normal *CYC7* site.

The amount of iso-2-cytochrome *c* produced by *CYC7-H2* mutant strains is affected by the constitution of the mating type locus, *MAT*, and certain *STE* mutations. The normal function of *MAT* is to regulate cell type-specific functions including mating and sporulation (46). A number of *STE* loci have been identified by recessive mutations that prevent mating ability in *MAT***a** and *MAT*α cells (19, 27). Among these are the *ste7*, *ste11*, and *ste12* mutations which have been shown to concomitantly diminish iso-2-cytochrome *c* production in strains carrying the *CYC7-H2* mutation (15, 16). Ty1 insertion appears to have placed iso-2-cytochrome *c* production under the control of specific regulatory signals normally required for control of mating functions in yeasts.

The essential features of the *CYC7-H2* mutation are shared by other Ty1 insertion mutations in yeast cells, denoted ROAM. Regulatory mutations, including *CYP3-4* (6, 30), *cargA*$^+$*O*^{h} (10, 23), and five *ADH2*^{c} mutations (5, 53), were caused by insertion of Ty elements in the 5′ noncoding region of the corresponding structural loci which are *CYC7* (iso-2-cytochrome *c*), *CAR1* (arginase), and *ADH2* (alcohol dehydrogenase II). (The *CYP3-4* mutation is another *CYC7* allele that was independently isolated and characterized.) Each of the above mutations causes constitutive production of the respective gene products, and the amount of each is subject to the *MAT* control described for the *CYC7-H2* mutation (7, 15, 16, 37, 49, 54). It should be noted that not all Ty insertion mutations cause an overproducing phenotype. Among mutations selected for loss of function, Ty insertions have been found at the *HIS4* locus (4, 36) as well as at the *LYS2* locus (12).

We have begun a systematic investigation of the regions that are responsible for the abnormal regulation of iso-2-cytochrome *c* production in the *CYC7-H2* mutant. Five alterations of the cloned *CYC7-H2* gene were constructed. The derivative genes were integrated at the *CYC7* locus by transformation of a cytochrome *c*-deficient yeast strain, and the amount of iso-2-cytochrome *c* produced by the transformed strains was measured. Our results show that the distal two-thirds of the Ty1 element is not essential for overproduction of iso-2-cytochrome *c* in the *CYC7-H2* mutant. Alterations near the proximal Ty1 junction either restore wild-type production of iso-2-cytochrome *c* or abolish its production completely.

MATERIALS AND METHODS

Yeast strains and genetic procedures. Conventional yeast genetic procedures of crossing, sporulation, tetrad analysis,

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† University of Rochester report no. UR-3490-2371.

TABLE 1. Yeast strains

Strain no.	Integrated plasmid	Genotype
E480-1D		*MATa cyc1-363 cyc7-28 ura3-52 leu2-3,112*
E458-4B		*MATα cyc1 CYC7-H3 can1 ura3-52 his1 trp1-289 his4*
E378-1A		*MATa cyc1-363 CYC7-H2 cry1 his1 lys2*
KZ8-5C		*MATa cyc1-1 CYC7$^+$ his4 ura1*
KZ8-1D		*MATα cyc1-1 CYC7$^+$ his4 ura1*
E646-6B		*MATα ste7 cyc1 CYC7-H2 ura3-52 leu2-3,112 trp2*
E648-6B		*MATα ste12 cyc1 CYC7-H2 ura3-52 leu2-3,112 trp2 tyr1*
E480-P5	pAB57	*MATa cyc1-363 cyc7-28 CYC7-P5 ura3-52 leu2-3,112*
E480-P33	pAB45	*MATa cyc1-363 cyc7-28 CYC7-P33 ura3-52 leu2-3,112*
E480-P54	pAB50	*MATa cyc1-363 cyc7-28 CYC7-P54 ura3-52 leu2-3,112*
E480-P71	pAB51	*MATa cyc1-363 cyc7-28 CYC7-P71 ura3-52 leu2-3,112*
E480-P102	pAB52	*MATa cyc1-363 cyc7-28 CYC7-P102 ura3-52 leu2-3,112*
E480-P112	pAB56	*MATa cyc1-363 cyc7-28 CYC7-P112 ura3-52 leu2-3,112*
E480-P150	pAB58	*MATa cyc1-363 cyc7-28 CYC7-P150 ura3-52 leu2-3,112*

and scoring of nutritional markers were used for construction of strains with desired genotypes (31, 39). Complete genotypes for yeast strains used in these studies are given in Table 1.

(i) *CYC1* and *CYC7* markers. All strains utilized in these studies are completely deficient in iso-1-cytochrome *c*. The *cyc1-1* and *cyc1-363* mutations are deletions of the iso-1-cytochrome *c* structural gene and have been described previously (34, 43, 45). Other *cyc1* alleles are not specified but cause complete absence of iso-1-cytochrome *c*. Hence, the spectroscopic procedure for cytochrome *c* determinations (described below) measures only iso-2-cytochrome *c*. The *cyc7-28* allele prevents production of iso-2-cytochrome *c*. This allele was isolated and shown to revert at a frequency of $<10^{-8}$ (K. Zaret, unpublished data). DNA sequence analysis identified the lesion as a UGA mutation corresponding to amino acid position 39 within the *CYC7* coding region (T. Cardillo, unpublished data). The combination of the *cyc1-363* and *cyc7-28* alleles causes complete deficiency in cytochrome *c*. The *CYC7-H3* mutation is a 5-kb deletion that encompasses the 5′ noncoding region of the *CYC7* locus and the closely linked *RAD23* and *ANP1* loci (29). The *CYC7-H3* deletion causes sensitivity to the compound 2-amino-1-[*p*-nitrophenyl]-1,3-propanediol (ANP). This *CYC7-H3* allele was used in genetic crosses where it was desirable to score segregation of *CYC7* alleles independently of iso-2-cytochrome *c* production. *CYC7-H3* segregants are unable to grow on complete medium (yeast extract, peptone, glucose [YPD]) containing 1 mM ANP, whereas segregants containing other *CYC7* alleles are *ANP1*$^+$ and will grow on ANP medium (29).

(ii) Sterile mutant alleles. The conditional *ste7* and *ste12* alleles were derived from strains 381-11-1-26a and 381-11-10a, respectively, which are part of the sterile mutant collection described by Hartwell (19). Crosses with these conditional mutations were made by standard procedures at the permissive temperature for mating (22°C). Segregants carrying either the *ste7* or *ste12* allele were identified by their nonmating phenotype which is observed at the nonpermissive temperature (34°C). Mating phenotypes were determined by the ability to form prototrophs with tester strains (KZ8-5C and KZ8-1D) having complementing nutritional markers. Segregants to be tested were spotted onto two sets of complete medium (YPD) plates that had been spread with one or the other tester strain. The duplicate plates were incubated at 22 or 34°C for 24 to 36 h and then replicated onto minimal medium (synthetic glucose [SD]). These plates were incubated at 30°C for 24 to 36 h and scored for the presence or absence of prototrophs.

Media. Yeast media including complete medium (YPD), synthetic minimal medium (SD), and various omission media used for growth and scoring nutritional markers are described in Sherman et al. (39). Luria broth, Luria broth plus ampicillin medium, and M9-C medium used for growth of *Escherichia coli* were prepared as described in Maniatis et al. (28).

Yeast transformation procedure. Recombinant plasmid molecules used for these experiments each contain fragments derived from the *CYC7-H2* or *CYC7*$^+$ region inserted into the vector YIp5. This vector is a derivative of pBR322 that contains the yeast *URA3*$^+$ selectable marker but does not contain sequences permitting autonomous replication in yeasts (47). Transformation with this vector occurs at a low frequency and requires homologous recombination between genomic sequences of the recipient and the yeast sequences inserted in YIp5 (20).

Yeast strain E480-1D was used as the recipient strain for all transformations described. The *ura3-52* allele of this strain permits selection of YIp5-transformed strains by requiring growth on synthetic medium lacking uracil. E480-1D was transformed with plasmid DNA by the following procedure. Spheroplasts were obtained by treatment of 10^8 cells per ml in 1 M sorbitol–10 mM Tris (pH 7.5) with 3% Glusulase (Endo-labs) for 60 to 90 min at 30°C. After transformation treatment, carried out essentially as described by Hinnen et al. (20), the polyethylene glycol suspension of spheroplasts was added directly into 100 ml of melted selection medium at 45°C and poured into four petri dishes. The selection medium used was uracil omission medium (39) prepared with 3% (wt/vol) Bacto-Agar (Difco Laboratories)–1 M sorbitol and 10 mM sodium phosphate, pH 7. Plates were incubated for 3 to 4 days at 30°C. Frequencies of transformation ranged between 0.4 and 12 Ura$^+$ transformants per μg of DNA.

Determination of cytochrome *c* content in yeast strains. Strains were grown under derepressed conditions (42), using solid medium containing 1% (wt/vol) sucrose (instead of melibiose), 1% (wt/vol) yeast extract, 2% (wt/vol) Bacto-Peptone (Difco), and 2% (wt/vol) Bacto-Agar. Intact cells were examined by the low-temperature (−196°C) spectroscopic procedure of Sherman and Slonimski (40). The amount of cytochrome *c* was estimated by comparison of the c_α band absorption intensities with those for strains having known amounts of cytochrome *c*. Values are reported relative to the amount found in standard *cyc1 CYC7*$^+$ strains which corresponds to 10 to 20 mg of iso-2-cytochrome *c* per kg (dry weight) of yeast cells (42).

Preparation of DNA. Yeast DNA was isolated according to

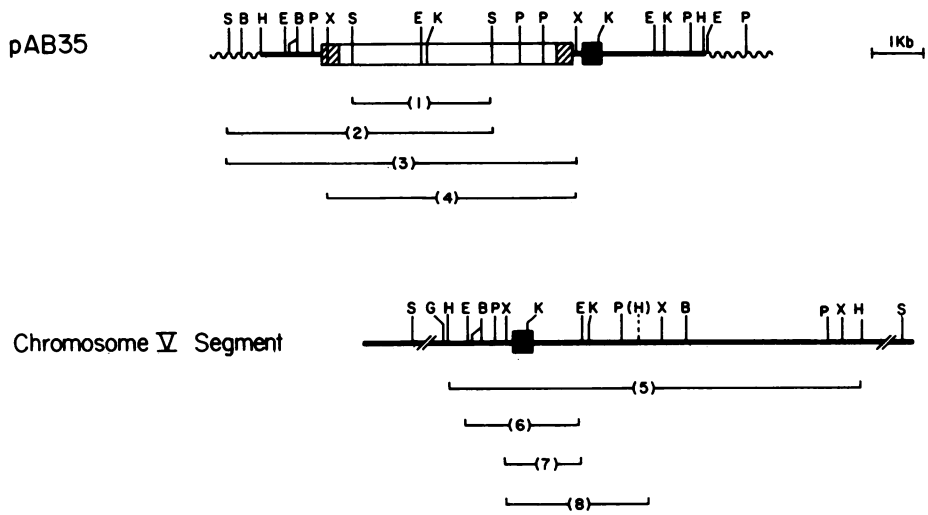

FIG. 1. Restriction sites in the cloned region of the *CYC7-H2* gene and in the *CYC7*$^+$ region of chromosome V. The plasmid designated pAB35 is composed of the 8.9-kb *CYC7-H2 Hin*dIII fragment inserted into pBR322. pAB45 (not shown) contains the same *CYC7-H2 Hin*dIII fragment inserted into YIp5. YIp5 contains the 1,1-kb, dC-tailed, *URA3*$^+$ fragment joined to dG-tailed, *Ava*I-cleaved pBR322 (47). The orientation of the *CYC7-H2 Hin*dIII fragment in pAB45 is opposite to that in pAB35 with respect to the flanking pBR322 sequences. Cleavage sites were determined with the following restriction enzymes: *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Pst*I (P), *Sal*I (S), and *Xho*I (X). The solid box in the *CYC7*$^+$ region of chromosome V and in the *CYC7-H2* plasmids represents the translated portion of the *CYC7* locus. The epsilon and delta sequences of the Ty1 element in the *CYC7-H2* plasmids are represented by open boxes and hatched boxes, respectively. A portion of vector sequences flanking the *CYC7-H2* fragment in each plasmid is indicated by wavy lines. Various fragments containing the *CYC7*$^+$ gene were used as hybridization probes in these studies. These fragments, indicated at the bottom, are the 2.2-kb *Eco*RI fragment (6), the 1.5-kb *Xho*I-*Eco*RI fragment (7), and the 2.7-kb *Xho*I fragment (8).

the "DNA miniprep" procedure of Roeder and Fink (36). In some instances the DNA was further purified by equilibrium density centrifugation in ethidium bromide-cesium chloride gradients. Isolates of *E. coli* strain HB101 carrying recombinant plasmids were grown as described by Norgard et al. (32). Plasmid DNA was purified by ethidium bromide-cesium chloride density gradient centrifugation as described by Clewell and Helinski (8).

Restriction endonuclease digestions and analyses of plasmid DNA. Treatment of DNA with the various restriction endonuclease enzymes was carried out under conditions recommended by the vendor (Bethesda Research Laboratories or New England BioLabs). The products of endonuclease digestion were separated by electrophoresis for approximately 16 h at 1 V/cm on 0.7 to 1% agarose gels (20 by 13 by 0.3 cm) containing TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.9). Fractionated DNA was visualized by fluorescence after staining gels with ethidium bromide at 1 μg per ml of TAE. Fragments obtained by digestion of lambda DNA with *Hin*dIII endonuclease or ϕX174 replicative form DNA with *Hae*III endonuclease were used as standard size markers.

Hybridization analysis of yeast genomic DNA. Total yeast DNA was digested with the specified restriction enzymes, and the fragments were separated by electrophoresis on 0.7% agarose gels as described above. DNA fragments were transferred to nitrocellulose sheets by the procedure of Southern (44). Several different restriction fragments encompassing the *CYC7*$^+$ gene (fragments 6, 7, and 8 indicated in Fig. 1) were isolated from plasmid DNA and used as DNA probes for various experiments. These fragments were labeled with ^{32}P by the "nick translation" procedure of Rigby et al. (35). Hybridization of DNA probes to the nitrocellulose sheets was carried out in 2× SSC (1× SSC is 150 mM sodium chloride plus 15 mM sodium citrate, pH 7)–1% (wt/vol) sodium dodecyl sulfate–100 μg of sonicated calf thymus DNA per ml at 70°C for 18 h. The filters were then washed in 2× SSC–1% (wt/vol) sodium dodecyl sulfate with successive changes at temperatures and times as follows: 5 min at 45°C; 60 min at 45°C; and twice for 10 min at room temperature. The filters were dried under vacuum at 70°C and autoradiographed at −70°C with the use of Dupont Cronex intensifying screens and Kodak XAR X-ray film.

Construction of *CYC7-H2* derivative genes. Two deletions of the *CYC7-H2* region were made with plasmid pAB35, which contains the 8.9-kb *CYC7-H2 Hin*dIII fragment inserted into pBR322 (Fig. 1) (15). pAB35 was partially digested with *Sal*I endonuclease. The mixture of resulting fragments was treated with T4 DNA ligase (New England Biolabs) at dilute DNA concentration (0.1 μg/25 μl) under conditions recommended by the vendor. The mixture was used to transform *E. coli* strain HB101 by the $CaCl_2$-thymidine procedure as described in Davis et al. (9). Two ampicillin-resistant clones were chosen for further study. One contained a 10.3-kb plasmid molecule, and the other contained an 8.0-kb plasmid. The size of *Eco*RI and *Hin*dIII restriction fragments indicated that these plasmids have deletions corresponding to *Sal*I fragments designated 1 and 2, respectively, in Fig. 1. By standard procedures, the 5.3-kb *Hin*dIII-*Bam*HI fragment from the larger plasmid and the 3.7-kb *Hin*dIII-*Sal*I fragment from the smaller plasmid were transferred to the yeast vector YIp5 (47). Analysis with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, and *Xho*I restriction endonucleases verified the described constructions in the resulting plasmids, designated pAB50 and pAB51, respectively.

A deletion corresponding to *Sal*I-*Xho*I fragment 3 (Fig. 1) was made in pAB51. It should be noted that the *Xho*I site, which defines the proximal endpoint of this deletion, lies in the normal *CYC7* sequence at a position 140 bp upstream from the coding region. pAB51 was digested sequentially with *Xho*I and *Sal*I endonucleases. The resulting mixture was treated with T4 DNA ligase under dilute DNA condi-

tions as before and used to transform *E. coli* strain HB101 to ampicillin resistance. Plasmid DNA from one of the clones was designated pAB58. pAB58 was characterized on the basis of size of *Pst*I, *Hin*dIII, and *Eco*RI restriction fragments. As expected, pAB58 was not cleaved by either *Xho*I or *Sal*I endonuclease. (The fusion of the complementary ends resulting from digestion with this enzyme pair does not preserve the recognition sequence for either.)

A deletion and inversion of *Xho*I fragment 4 (Fig. 1) was made in the plasmid designated pAB45 that contains the 8.9-kb *CYC7-H2* *Hin*dIII fragment inserted in YIp5. The proximal endpoint of both rearrangements is at the *Xho*I site 140 bp upstream from the *CYC7* coding region. pAB45 was digested to completion with *Xho*I endonuclease, and a portion of the mixture was treated with T4 DNA ligase at dilute DNA concentration. The plasmid designated pAB52 was recovered after transformation of *E. coli* strain HB101. pAB52 was shown to lack *Xho*I fragment 4 by restriction cleavage site analysis with *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I, and *Xho*I endonucleases. Another portion of the pAB45-*Xho*I restriction mixture was treated with T4 DNA ligase at high DNA concentration and then used to transform *E. coli* strain HB101. Approximately 3,000 transformed colonies were screened by colony hybridization (18), using a lambda phage containing the *CYC7-H2* clone as the radioactively labeled DNA probe. Plasmid DNA from two colonies which gave a strong positive signal was subjected to *Xho*I and *Eco*RI restriction endonuclease analysis. The size of fragments established that both clones contained a recombinant plasmid with an inversion of *Xho*I fragment 4. One of these, designated pAB56, was further characterized by *Bam*HI, *Bgl*II, *Eco*RI, *Pst*I, and *Xho*I restriction endonuclease analyses.

A 7.5-kb *Hin*dIII fragment from pAB25 (29) that encompasses the *CYC7*$^+$ region of chromosome V (fragment 5, Fig. 1) was transferred to the *Hin*dIII site of YIp5. The resulting 13.1-kb plasmid, designated pAB57, was characterized with *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, and *Xho*I restriction endonucleases. The pAB57 plasmid was used in these studies to provide a reference for iso-2-cytochrome *c* expression in transformed strains. Some strains do not have the *Hin*dIII site shown in parentheses in Fig. 1. The *CYC7*$^+$ fragment in pAB57 is derived from one such strain and therefore contains an additional 4 kb of yeast sequence 3′ to the *CYC7* coding region. The chromosome V region of strain E480-1D is also missing this *Hin*dIII site.

RESULTS

Identification of transformed strains with one copy of the *CYC7*$^+$, *CYC7-H2*, or *CYC7-H2* derivative plasmid integrated at the *CYC7* locus. To characterize the regions responsible for overproduction of iso-2-cytochrome *c* in the *CYC7-H2* mutant, a DNA fragment containing the *CYC7-H2* gene was altered by producing a number of deletions and rearrangements. Plasmids containing the wild-type *CYC7*$^+$ gene and the mutant *CYC7-H2* gene were compared with plasmids with the modified *CYC7-H2* fragments by transformation of a cytochrome *c*-deficient strain and examination of the phenotypes of resulting strains containing single and multiple copies of the plasmids integrated at the normal chromosomal site.

Yeast strain E480-1D was transformed with the *CYC7*$^+$ control plasmid pAB57, the *CYC7-H2* plasmid pAB45, and the *CYC7-H2* derivative plasmids pAB50, pAB51, pAB52, pAB56, and pAB58. The *CYC7* regions of these plasmids are compared in Fig. 2. Transformed strains were selected on the basis of the vector *URA3*$^+$ marker and not a cytochrome *c* phenotype. Independent colonies from transformation with each plasmid were picked, grown on complete medium (YPD), and then tested for ability to grow on medium lacking uracil. More than 95% of the colonies showed a Ura$^+$ phenotype after nonselective growth, indicating that the plasmid had become stably integrated in the genome of these transformed strains. Each of the Ura$^+$ colonies was purified by subcloning on uracil omission medium, and the subclones were tested for iso-2-cytochrome *c* production. Each isolate produced a discrete amount of iso-2-cytochrome *c*, but the amounts were not the same for all strains that were derived from transformation with a given plasmid. Several factors contributed to these variations. We have found in these studies and others have reported (33, 48; T. Petes and H. Klein, personal communication cited in reference 21) that tandem integration of multiple plasmid molecules is frequently observed in transformations. Strains producing amounts of iso-2-cytochrome *c* greater than the amount characteristic of a particular plasmid reflect gene dosage due to such tandem insertions. Rearrangements or alterations of plasmid sequences were also found to occur in the transformation process. Therefore, comparison of gene expression of *CYC7-H2* and *CYC7-H2* derivatives required the identification of transformed strains in which one complete copy of the plasmid is integrated at the *CYC7* locus.

Representative transformed strains from the different groups were genetically analyzed to identify integrations that occurred at the *CYC7* locus. Whereas pAB57 and pAB58 contain YIp5 inserts homologous with only the *CYC7* region, the other plasmids contain sequences which would permit homologous recombination with either *CYC7* sequences or Ty1 and delta sequences dispersed throughout the genome. *CYC7* and other site integrations were distinguished by the standard methods of tetrad or random-spore analysis. Transformed strains (*ANP1*$^+$ *cyc7-28 ura3-52 CYC7-P URA3-P*) were each crossed to tester strain E458-4B (*CYC7-H3 ura3-52*). Resulting diploid strains were isolated by prototroph selection and were sporulated. The Anp and Ura phenotypes of the meiotic segregants were used to determine whether or not the integrated plasmid (*URA3*$^+$) was tightly linked to the recipient *CYC7* locus (*ANP1*$^+$). Strains which were shown genetically to have plasmid molecules integrated at the *CYC7* locus were further analyzed by restriction endonuclease and hybridization analyses of genomic DNA to identify transformants that carry one intact copy of each plasmid. Strains E480-P5, E480-P33, E480-P54, E480-P71, E480-P102, and E480-P150 were identified as containing one copy of the respective pAB57, pAB45, pAB50, pAB51, pAB52, and pAB58 molecules integrated at the *CYC7* locus. pAB56-transformed strain E480-P112 appears to contain two copies of pAB56 at the *CYC7* locus. The amount of iso-2-cytochrome *c* produced by each strain together with the corresponding genetic analysis and genomic restriction fragment sizes are summarized in Table 2. These data support the conclusion that this group of transformed strains represents the expression of the integrated structures schematically shown in Fig. 2.

Iso-2-cytochrome *c* production in transformed strains. The amount of cytochrome *c* produced by the selected transformed strains provides a direct measure of expression for the plasmid *CYC7*$^+$, *CYC7-H2*, and *CYC7-H2* derivative genes. The relative amounts of iso-2-cytochrome *c* produced by each of the transformed strains selected for further study are compared in Table 2 and Fig. 2. Strain E480-P5 contains

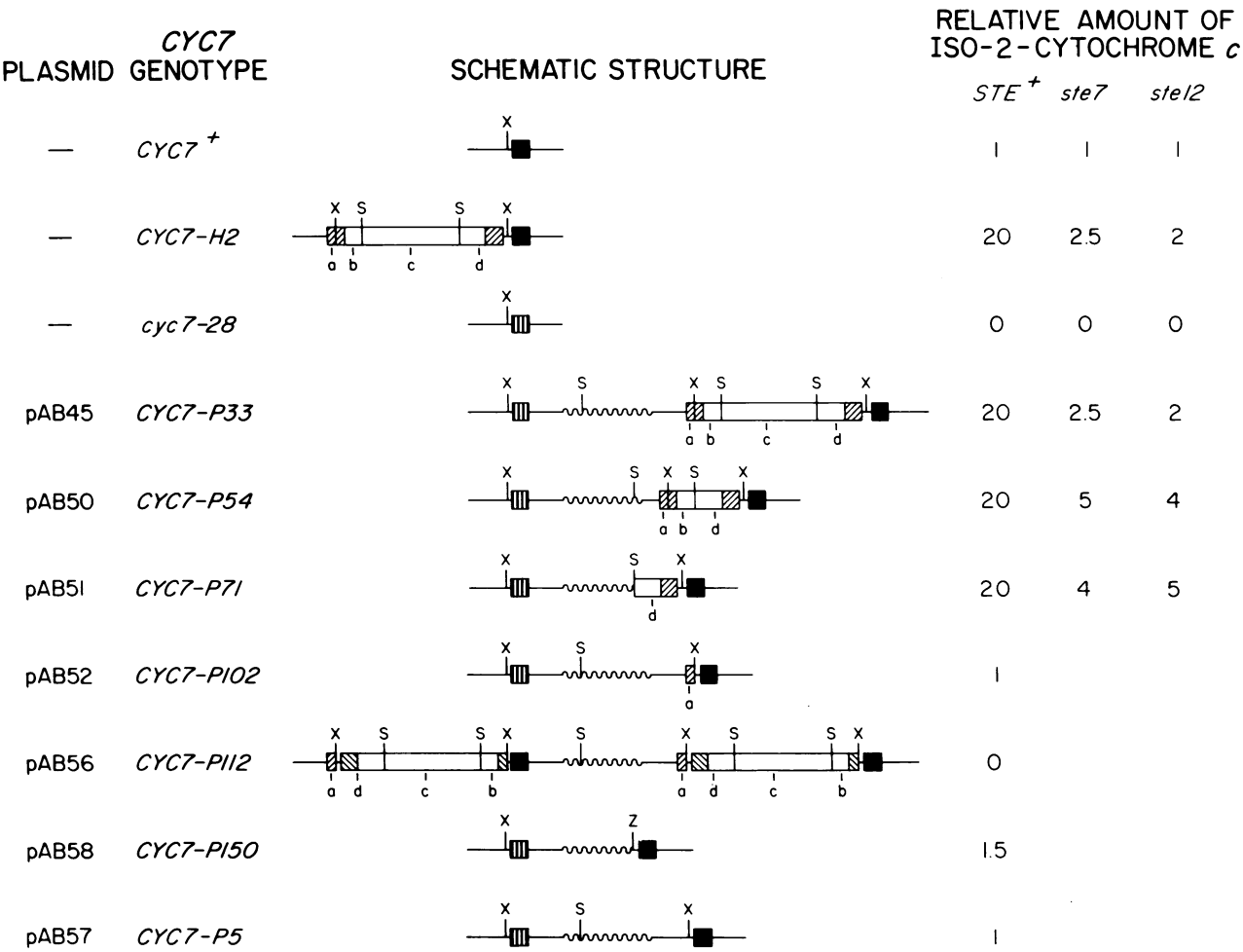

FIG. 2. Expression and regulation in the control and transformed strains carrying integrated copies of *CYC7*$^+$, *CYC7-H2*, and *CYC7-H2* derivative plasmids. Schematic structures show the *CYC7* region in control and transformed strains. The solid box represents the coding region of the normal *CYC7* region. The striped box represents the defective *cyc7-28* coding region in the recipient strain and transformed strains. The epsilon and delta sequences of the Ty1 element are represented by an open box and a hatched box, respectively. Flanking yeast sequences from the *CYC7* regions are represented by thin lines, and vector sequences are represented by wavy lines. Restriction endonuclease sites are specified as in the legend to Fig. 1. Diagrams are not drawn to scale. The relative amounts of iso-2-cytochrome *c* are taken from Table 2 and Fig. 3.

the *CYC7*$^+$ plasmid, pAB57, and produces amounts of cytochrome *c* comparable to wild-type *CYC7*$^+$ strains. Strain E480-P33 contains pAB45 which carries the complete *CYC7-H2* gene. This strain produces an amount of iso-2-cytochrome *c* that is comparable to amounts produced by bona fide *CYC7-H2* strains. We conclude that vector sequences do not interfere with the plasmid *CYC7*$^+$ and *CYC7-H2* gene expression. *CYC7-H2* amounts of iso-2-cytochrome *c* are produced by strains E480-P54 and E480-P71 which contain the *CYC7-H2* derivative plasmids pAB50 and pAB51, respectively. These results show that the Ty1 segment labeled d (Fig. 2) is sufficient for the *CYC7-H2* overproducing phenotype. Strains E480-P102 and E480-P150 carry the *CYC7-H2* derivative plasmids pAB52 and pAB58, respectively. These two modifications abolish overproduction of iso-2-cytochrome *c* characteristic of the *CYC7-H2* phenotype but do not prevent production of *CYC7*$^+$ amounts. In the case of pAB58, we have confirmed that the *CYC7*$^+$ expression is not due to a gene conversion event that could restore a functional coding region adjacent to the normal *CYC7* control region. A strain containing a *CYC7* allele with a 0.4-kb deletion was transformed with pAB58. Restriction site analysis of genomic DNA from three independent transformed strains producing *CYC7*$^+$ amounts of iso-2-cytochrome *c* confirmed the presence of the deletion allele and one copy of the integrated pAB58 plasmid in all three strains. This observation confirms that the pAB58 structure produces *CYC7*$^+$ amounts of iso-2-cytochrome *c*. Strain E480-P112 carries two copies of pAB56, the *CYC7-H2* derivative in which *CYC7-H2 Xho*I fragment 4 (Fig. 1) has been inverted. In contrast to deletion of segments b to d (Fig. 2, E480-P102), the effect of b–d inversion (Fig. 2, E480-P112) is to prevent iso-2-cytochrome *c* production. Because two deletion derivatives (pAB52 and pAB58) with a proximal endpoint at the same *Xho*I site do not share this phenotype, we conclude that the arrangement of sequences in the *CYC7-H2* inversion derivative are specifically preventing iso-2-cytochrome *c* production.

Regulation of *CYC7-H2* derivatives. Transformed strains E480-P33, E480-P54, and E480-P71 which carry the plasmid alleles *CYC7-P33*, *CYC7-P54*, and *CYC7-P71*, respectively, produce *CYC7-H2* amounts of iso-2-cytochrome *c* (Table 2; Fig. 2). The question remains as to whether the observed production of iso-2-cytochrome *c* in the transformed strains

TABLE 2. Units of cytochrome *c* in transformed strains containing plasmids integrated at the *CYC7* locus

Yeast strain	*CYC7* allele	Plasmid	Copy no.	Units of cytochrome *c*[a]	No. of tetrads[b]			Genomic DNA analysis		
					PD	NPD	T	Enzyme(s)	Fragment size(s) (kb)[c]	Probe[d]
KZ8-5C	*CYC7*$^+$			1						
E378-1A	*CYC7-H2*			20						
E480-1D	*cyc7-28*			0				*Eco*RI	2.2	6
								*Hin*dIII	7.5	6, 7
								*Pst*I	2.3, 1.2	6
								*Sal*I	16.5	7
								*Bgl*II/*Sal*I	14.0	8
								*Kpn*I/*Pst*I	1.4, 1.2, 0.6	6
E480-P5	*CYC7-P5*	pAB57	1	1	9	0	1	*Sal*I/*Bgl*II	18.3, 8.5	7
E480-P33	*CYC7-P33*	pAB45	1	20	9	0	0	*Hin*dIII	14.3, 3.3	7
								*Eco*RI	5.0, 2.5, 2.2	6
								*Pst*I	3.0, 2.3, 1.8, 1.2	6
E480-P54	*CYC7-P54*	pAB50	1	20	9	0	0	*Hin*dIII	14.2, 3.3	6
								*Sal*I	14.0, 12.5	7
E480-P71	*CYC7-P71*	pAB51	1	20	9	0	0	*Hin*dIII	14.5, 3.5	7
E480-P102	*CYC7-P102*	pAB52	1	1	11	0	0	*Hin*dIII	7.4, 3.4	6
								*Sal*I	19.5, 8.8	7
								*Kpn*I/*Pst*I	2.0, 1.4, 1.2, 0.7, 0.6	6
E480-P112	*CYC7-P112*	pAB56	2	0	8	0	0	*Hin*dIII	13.7, 9.0	7
								*Pst*I	6.0, 1.8, 1.2	6
								*Eco*RI	4.3, 3.2	6
E480-P150	*CYC7-P150*	pAB58	1	1.5	8	0	0	*Hin*dIII	12.0, 3.5	6

[a] One unit corresponds to the normal *CYC7*$^+$ amount.

[b] Transformed strains (*ANP1*$^+$ *cyc7-28 ura3-52 CYC7-P URA3-P*) were crossed to tester strain E458-4B (*CYC7-H3 ura3-52*). Resulting diploid strains were sporulated, and meoitic segregants were scored for Anp and Ura phenotypes. The segregant phenotypes associated with each tetrad type are as follows: parental ditype (PD), 2 Anp$^+$ Ura$^+$:2 Anp$^-$ Ura$^-$; tetratype (T), 1 Anp$^+$ Ura$^+$:1 Anp$^+$ Ura$^-$:1 Anp$^-$ Ura$^+$:1 Anp$^-$ Ura$^-$; nonparental ditype (NPD), 2Anp$^+$ Ura$^-$:2 Anp$^-$ Ura$^+$.

[c] Observed fragment sizes are consistent with sizes predicted by using values derived from Fig. 1 for the integrated plasmids as illustrated in Fig. 2.

[d] Numbers refer to restriction fragments encompassing the *CYC7*$^+$ gene as indicated in Fig. 1.

is sensitive to the genetic constitutions that affect production in bona fide *CYC7-H2* strains. One level of control is exhibited by certain of the unlinked *STE* loci. The amount of iso-2-cytochrome *c* produced in *ste7 CYC7-H2* and *ste12 CYC7-H2* strains is much lower than the amount produced in *STE*$^+$ *CYC7-H2* strains (15, 16). The response of the modified *CYC7-H2* alleles *CYC7-P54* and *CYC7-P71* to the *ste7* and *ste12* mutations was compared with that of the control *CYC7-P33* allele and with the unmodified *CYC7-H2* allele. Strains E480-P33 (*MAT***a** *CYC7-P33 STE*$^+$), E480-P54 (*MAT***a** *CYC7-P54 STE*$^+$), and E480-P71 (*MAT***a** *CYC7-P71 STE*$^+$) were crossed to strains E646-6B (*MAT*α *CYC7-H2 ste7*) and E648-6B (*MAT*α *CYC7-H2 ste12*). Each of these diploid strains was sporulated and subjected to tetrad analysis. The amount of iso-2-cytochrome *c* in a number of segregants having the various possible combinations of *CYC7* and *STE* genotypes from each pedigree was determined. The data are presented in Fig. 3. Note that this method of comparison randomizes genetic background which causes fluctuations in cytochrome *c* production. It also provides internal controls for comparisons with *CYC7-H2 STE*$^+$ and *CYC7-H2 ste*$^-$ strains in the same genetic background. The *CYC7-P33* allele responds to the *ste7* and *ste12* mutations identically to the *CYC7-H2* allele segregating in the two respective pedigrees. The amount of iso-2-cytochrome *c* in *CYC7-P54 ste7*, *CYC7-P54 ste12*, *CYC7-P71 ste7*, and *CYC7-P71 ste12* segregants is significantly reduced compared with *CYC7-P54 STE*$^+$ and *CYC7-P71 STE*$^+$ segregants in the respective pedigrees. We conclude that Ty1 segment d is sufficient for the regulation by *STE* loci observed for the *CYC7-H2* mutant.

DISCUSSION

Wild-type *S. cerevisiae* cells contain two iso-cytochromes *c* that perform similar functions but differ in primary structure. Iso-2-cytochrome *c* is the minor species, comprising only 5% of the total cytochrome *c* complement in normal yeast strains. Overproduction of iso-2-cytochrome *c* has been associated with mutations that are *cis* dominant and have extended alterations in the 5′ region adjacent to the *CYC7* structural gene. Proximal endpoints of these alterations were localized to a region between 184 and 285 bp upstream from the translated portion of the *CYC7* region (26, 30). In addition to mutations in the control region of the *CYC7* locus, recessive mutations at four unlinked loci are also found to cause overproduction of iso-2-cytochrome *c* (6, 7, 26). Presumably, a negative regulatory region at the *CYC7* locus interacts with components of a control network involving these loci. RNA blot analysis of the two classes of overproducing mutants has shown there is an increased amount of normal-sized *CYC7* mRNA (26, 30). Hence, the increased production of iso-2-cytochrome *c* is caused either by the presence of abnormal sequences at the 5′ regulatory region or by mutation of components involved in regulation of *CYC7* transcription.

The DNA alteration of the *CYC7-H2* mutation was characterized by restriction endonuclease analysis of the cloned segment, by heteroduplex analysis of *CYC7*$^+$ and *CYC7-H2* segments, and by DNA sequence of the pertinent regions (15, 16; B. Kosiba, unpublished data). The results demonstrated that a Ty1 element was inserted at a site 184 bp in front of the translated portion of the gene. This junction is upstream from the normal iso-2-cytochrome *c* mRNA start site which was reported by Montgomery et al. to be at position −77 with respect to the ATG initiation codon (30). These results together with the results from RNA blot analysis indicate that sequences within the Ty1 element are affecting the efficiency of normal iso-2-cytochrome *c* transcription. In vitro modification of the cloned *CYC7-H2* DNA was undertaken to identify regions of the Ty1 element

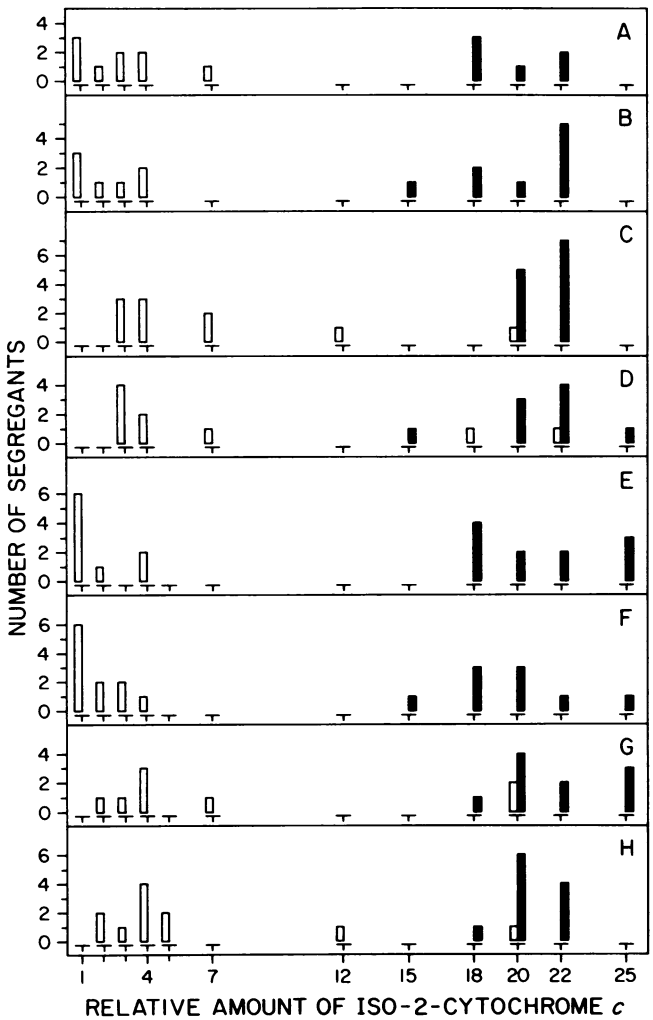

FIG. 3. Concentrations of iso-2-cytochrome *c* in *ste*$^-$ and *STE*$^+$ segregants. Low-temperature (−196°C) spectroscopic examination of intact cells was used to estimate the relative amount of iso-2-cytochrome *c* in the following: (A) *CYC7-H2* (solid bars) and *ste7 CYC7-H2* (open bars) segregants from diploid strain E659 (E646-6B × E480-P33) (*CYC7-H2* and *ste7 CYC7-H2* segregants from diploid strains E660 and E661 [see C and D, respectively] gave similar results); (B) *CYC7-P33* (solid bars) and *ste7 CYC7-P33* (open bars) segregants from E659 (E646-6B × E480-P33); (C) *CYC7-P54* (solid bars) and *ste7 CYC7-P54* (open bars) segregants from E660 (E646-6B × E480-P54); (D) *CYC7-P71* (solid bars) and *ste7 CYC7-P71* (open bars) segregants from E661 (E646-6B × E480-P71); (E) *CYC7-H2* (solid bars) and *ste12 CYC7-H2* (open bars) segregants from diploid strain E662 (E648-6B × E480-P33) (*CYC7-H2* and *ste12 CYC7-H2* segregants from diploid strains E663 and E664 [see G and H, respectively] gave similar results); (F) *CYC7-P33* (solid bars) and *ste12 CYC7-P33* (open bars) segregants from E662 (E648-6B × E480-P33); (G) *CYC7-P54* (solid bars) and *ste12 CYC7-P54* (open bars) segregants from E663 (E648-6B × E480-P54); (H) *CYC7-P71* (solid bars) and *ste12 CYC7-P71* (open bars) segregants from E664 (E648-6B × E480-P71). The absorption intensities of the c_α bands of segregants were visually compared with the c_α band intensities of strains with known amounts of iso-2-cytochrome *c*. The values of the intensities were assigned to discrete units indicated by thick lines under the abscissa. Strains were grown at 34°C, the temperature preventing conjugation of the *ste* segregants. These results indicate that *ste7* and *ste12* regulation of the modified alleles is identical to that of the *CYC7-H2* allele.

responsible for this effect. These studies rely on the ability to determine in vivo expression of the *CYC7-H2* derivative genes by transformation of a cytochrome *c*-deficient yeast strain. Control studies with the cloned *CYC7*$^+$ and *CYC7-H2* alleles demonstrate that this approach is valid when one copy of the recombinant plasmid is integrated at the recipient *CYC7* locus. A comparison of expression observed for *CYC7*$^+$, *CYC7-H2*, and *CYC7-H2* derivative alleles in transformed strains that satisfy the above conditions is summarized in Fig. 2.

Comparison of *CYC7-H2* and *CYC7-H2* derivative gene expression. The pAB45-, pAB50-, and pAB51-transformed strains which carry the *CYC7-P33*, *CYC7-P54*, and *CYC7-P71* alleles, respectively, each produce *CYC7-H2* amounts of iso-2-cytochrome *c*. The 20-fold overproduction of iso-2-cytochrome *c* in the *CYC7-H2* mutant as well as in this group of transformed strains is diminished by the *ste7* and *ste12* mutations. These observations show that the 1.8-kb Ty1 segment d contains sequences sufficient for overproduction and *STE* regulation of Ty1-mediated gene expression.

Three other derivative genes involve rearrangements at the −140 *Xho*I site located in the normal *CYC7* sequence. The pAB58-transformed strain carrying the *CYC7-P150* allele produces *CYC7*$^+$ amounts of iso-2-cytochrome *c*. This result indicates that sequences upstream from position −140 are not required for production of wild-type amounts of iso-2-cytochrome *c*. The structure of the integrated pAB58, *CYC7-P150*, can be viewed as a 6.6-kb insertion that includes 5.1 kb of YIp5 vector and 1.5 kb of *cyc7-28* sequences (Fig. 2). The production of wild-type amounts of iso-2-cytochrome *c* in pAB58-transformed strains argues against the possibility that overproduction in the *CYC7-H2* mutant is merely a consequence of an insertion that displaces regions of negative control. The modification carried by plasmid pAB52 can be viewed as a replacement of *CYC7* sequence from −140 to −184 by *CYC7-H2* segment a (Fig. 2). Segment a consists of one-third of the Ty1 distal delta element. pAB52-transformed strain E480-P102 also produces wild-type amounts of iso-2-cytochrome *c*, showing that this portion of a delta element is not sufficient to cause overproduction of the adjacent structural gene. The pAB56 construction inverts the *Xho*I fragment that includes Ty1 segments b, c, and d along with 44 bp of *CYC7* flanking DNA (Fig. 2). The *CYC7-P112* allele resulting from pAB56 integration abolishes *CYC7* gene expression. Because this phenotype is not observed for the two deletions that have the same *Xho*I endpoint, it is possible the specific DNA sequence at the junction of the pAB56 inversion is inhibiting iso-2-cytochrome *c* production. It is also possible that the orientation of Ty1 segment d or its position with respect to intervening segment c–b in the pAB56 construction or both are responsible for the inhibition.

Relationship to Ty DNA transcription. Elder et al. (14) have identified and characterized abundant RNA homologous to Ty1 DNA. The major population is approximately 5.7 kb in size and is regulated by the mating type locus identically to the regulation of iso-2-cytochrome *c* in the *CYC7-H2* mutant. More recently, it has been shown that Ty1 transcripts are also regulated by the *STE7* locus (11). The 5′ and 3′ ends of the major Ty1 transcripts have been mapped (13). Ty1 transcription initiates in one delta repeat at a position 92 to 95 bp from the epsilon junction and terminates in the opposite delta approximately 20 to 40 bp from flanking DNA sequences. The orientation of the Ty1 element in the *CYC7-H2* mutation is such that the delta element proximal to the *CYC7* region corresponds to the "promoter" delta. If the

Ty1 element in the *CYC7-H2* mutation is transcribed, the polarity would be opposite to that of *CYC7* transcription. Our results show that the promoter end of Ty1 is important for overproduction of iso-2-cytochrome *c* in the *CYC7-H2* mutant. The overproduction is abolished by modifications of the *CYC7-H2* Ty1 that delete the proximal delta, as in pAB52, or that change the orientation and position of the Ty1 promoter region with respect to the *CYC7* structural sequences, as in pAB56.

Relationship to other Ty insertion mutations. ROAM mutations designated *CYP3-4* (6, 7, 30), *cargA*$^+$*O*^{h} (10, 11, 23), and *ADH2*^{c} (5, 49, 52–54) were caused by insertion of Ty elements in the 5′ noncoding region of the corresponding structural loci. Each of these mutations causes production of the respective gene products that respond to signals normally controlling mating type functions in yeast cells. Characterization of DNA cloned from this group of mutants has shown that the orientation of the Ty elements is the same as that in the *CYC7-H2* mutant. In each case the junctions of the Ty insertions were located within a region between 125 and 600 bp upstream from the corresponding coding sequences. In all of the above mutations, the steady-state mRNA for the corresponding gene products is increased or made constitutively, but the 5′ map position or size or both are not affected (11, 26, 30, 52). These results show that the Ty1 elements are not providing a new transcription initiation site but are regulating transcription from various upstream positions. This effect is similar in certain respects to the action of enhancer sequences of the simian virus 40 promoter (1, 2, 17, 50). However, unlike enhancer sequences, the orientation of the Ty1 sequences inserted at the 5′ end of affected genes appears to be crucial.

Although the majority of the ROAM mutations characterized to date involve insertions belonging to class 1 elements (Ty1), the *ADH2-3*^{c} mutation was caused by insertion of a class 2 element (Ty*2*) (49, 51, 52). Kingsman et al. (24) have shown that the two classes of Ty elements differ by large substitutions within the epsilon segment of the Ty elements. Our studies have shown that Ty1 segment d is sufficient for the overproduction of iso-2-cytochrome *c* in the *CYC7-H2* mutant. Homology shared by Ty1 and Ty2 elements in region d is limited to the delta segment and approximately 200 bp of the adjacent epsilon region. We predict that required sequences for regulation of adjacent structural genes would be limited to this region of the Ty elements.

ACKNOWLEDGMENTS

We thank Ronald Swanstrom, Patricia Pukkila, and Kerry Bloom for critical reading of the manuscript. We thank Valerie Williamson and Kenneth Zaret for valuable discussions in the early phases of this work. We also acknowledge the technical assistance of W. Yarnell and S. Consaul.

This investigation was supported in part by U.S. Public Health Service research grants R01 GM30619 and R01 GM12702 from the National Institutes of Health. Support was also provided by an institutional grant from the Research Council at the University of North Carolina and by the U.S. Department of Energy contract DE-AC02-76EV03490 at the University of Rochester Department of Radiation Biology and Biophysics.

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