Cell-Type-Dependent Gene Activation by Yeast Transposon Ty1 Involves Multiple Regulatory Determinants

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Ty transposable element insertion mutations of *Saccharomyces cerevisiae* can cause cell-type-dependent activation of adjacent gene expression. Several *cis*-acting regulatory regions within Ty1 that are responsible for these effects were identified. A 211-base-pair (bp) region functions as an activator. This region includes the so-called U5 domain of delta and 145 bp of adjacent epsilon sequences. Unlike activation by the intact Ty1, activation by the 211-bp Ty1 subfragment is cell-type independent. The presence of a 112-bp fragment from a more distal region of Ty1 confers cell-type specificity to the activator. The 112-bp fragment includes sequences with homology to mammalian enhancers and to a yeast α/α control site. In addition, Ty1 regions that exert negative effects on gene expression were identified. These results demonstrate that the Ty1 transcriptional control region consists of multiple components with distinct regulatory functions.

Ty transposable elements are a dispersed, repeated gene family in the yeast *Saccharomyces cerevisiae* (4). Ty elements are similar to proviral forms of vertebrate retroviruses in their structure, transcriptional properties, and gene organization (4, 5, 9, 30). As in retroviruses, the 5' and 3' boundaries of Ty elements are defined by long terminal repeats which in the case of Ty are designated delta. The central domain of Ty elements, designated epsilon, encodes gene products presumed to be involved in their transposition (2, 5, 15, 23, 30). The family of Ty elements can be divided into two broad classes of elements, Ty1 and Ty2. These two classes show considerable divergence at the DNA sequence level but are well conserved with respect to functional domains and gene products (5, 30).

Insertion of a Ty into the regulatory region of a gene may result in either increased or reduced expression of that gene (see reference 31 for a review of this and the following). Certain observations suggest that an enhancerlike mechanism is responsible for activation effects caused by Ty insertions. Like enhancers, Ty insertion mutations exhibit positional flexibility in their ability to activate gene expression. However, unlike enhancers, it seems that the orientation of Ty relative to the activated gene is important. Ty and the activated gene are oriented such that their transcription is divergent.

Transcription of both Ty and the activated gene is regulated by the mating-type locus, *MAT*. The steady-state amount of Ty RNA and adjacent gene expression are reduced ~10-fold in *MATa/MAT α* diploid cells compared with *MATa* or *MAT α* haploid cells (10, 11). Also, expression of Ty and of the adjacent gene is reduced in haploid cells that carry mutations at certain *STE* loci which are required for the haploid-specific function of mating (8, 11). This cell-type specificity of Ty gene regulation is reminiscent of the host and tissue specificity associated with many viral and cellular enhancers (see reference 16).

The *CYC7-H2* mutation was caused by insertion of a Ty1 element into the 5' noncoding region of the *CYC7* structural gene for iso-2-cytochrome *c* (11). Approximately 1.5 kilobases (kb) of Ty1 sequences proximal to the *CYC7* coding region are sufficient for the 20-fold activation and cell-type

regulation characteristic of *CYC7-H2* (12). Previous analyses of this region have revealed several relevant DNA sequence features and functional regions (Fig. 1). The *CYC7-H2* delta is abnormal in that it consists of an inverted repeat of the R to U5 region of a normal delta (14). The implication is that the U3 region of a normal delta does not contain sequences important for activation of adjacent gene expression. Two related sequences in the epsilon region designated block I and II appear to be important *cis*-acting elements. Blocks I and II have overlapping homology to the enhancer region from simian virus 40 and to a sequence from the *MAT α* intergenic region referred to here as a diploid control site (12). (The *MAT α* sequence is necessary and sufficient to cause repression of gene expression in diploid yeasts [29].) Deletion of Ty1 fragment b which includes blocks I and II caused a reduction in *CYC7-H2* expression, whereas deletion of fragment e or f had no effect (12). These observations indicated that fragments e and f contain equivalent regulatory activity and that activity is required for full activation effects of Ty1 on adjacent gene expression. Fragment d and a 28-base-pair (bp) synthetic block II sequence were subsequently tested, and each was found to provide the enhancerlike activity associated with region b (13). In the absence of other Ty sequences, fragment d caused only slight (twofold) activation of adjacent gene expression. However, fragment d does include a functional diploid control site because it caused significant repression of gene expression in diploid cells compared with haploid cells (13). Roeder et al. (25) have analyzed several Ty2 elements inserted at *HIS4* that have different effects on adjacent gene expression. The elements differ by base pair substitutions localized to delta sequences near the epsilon junction and to a single position in the vicinity of the Ty2 region with homology to the simian virus 40 enhancer. In this study, sequences throughout the 1.5-kb region of the *CYC7-H2* Ty1 were analyzed for activation and diploid control functions.

MATERIALS AND METHODS

Plasmid construction. *CYC7-H2* and *CYC7⁺* plasmids used in these studies were constructed from previously described centromeric plasmids designated pNC3, pNC5, and pNC16

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FIG. 1. Location of transcriptional control regions in the *CYC7-H2* Ty1. The 1.5-kb Ty1 region is represented by the open box. The abnormal *CYC7-H2* delta structure (see text) is indicated by a diamond. Stippled boxes indicate block I and block II sequences which have overlapping homology to the simian virus 40 enhancer and to the *MATa* diploid control site. Perfect enhancer core sequences are indicated by asterisks. Diploid control site homologies are indicated by thick lines immediately below the diagram. See text (introduction and discussion) for explanation of functional designations given to different Ty1 subfragments indicated by bars below the diagram. Restriction sites defining fragment boundaries are *AccI* (Ac), *PvuII* (V), *HpaI* (Hp), *Sau3A* (Sa), *SphI* (Sp), and *BglII* (G).

(12). The *CYC7* derivative plasmid, pNC67, was described previously (13). It is identical to pNC5 except that a 90-bp *HaeIII-PstI* fragment from the *CYC7* upstream region was replaced by the indicated polylinker fragment (Fig. 2). pNC71 was constructed so that *CYC7* 5'-flanking sequences would be the same in all plasmids. The 0.8-kb *CYC7* fragment of pNC67 extending from the *PstI* site in the polylinker to the *SmaI* site in *CEN3* was inserted into the *SmaI* site at *CEN3* of pNC16. The plasmid pNC98 is a phagemid version of pNC67. It was constructed by replacing the *PvuII-PvuII lac* fragment from pUC118 with the 3.4-kb fragment from pNC67 extending from the *EcoRI* site at *TRP1* to the *XhoI* site at *CYC7* fused to the 1.1-kb fragment from pNC3 extending from the *XhoI* site at *CYC7* to the *PvuII* site in pBR322. pUC118 is a derivative of pUC18 that includes sequences from the M13 intergenic region (J. Vieira and J. Messing, personal communication). The 1.1-kb fragment from pNC3 corresponds to the *XhoI-SalI* fragment shown for pNC71 that encompasses the *CYC7* coding region (Fig. 2) (12). *BglII*, *EcoRI*, *PstI*, *SalI*, and *SphI* sites indicated by a crossed circle in pNC98 (Fig. 2) were destroyed by cleavage, end repair, and ligation. A synthetic polylinker having complementary ends for *SalI* and *PstI* was inserted into the *SalI* and *PstI* sites of pNC98 located in the 3.4-kb region derived from pNC67. The synthetic DNA fragment includes recognition sites for *SphI*, *HpaI*, *BglII*, and *EcoRI* restriction enzymes, thus expanding the cloning sites available in the pNC98 polylinker (Fig. 2).

Plasmid DNAs for construction, restriction digestion analysis, and yeast transformation were prepared from *Escherichia coli* HB101 by the rapid boiling method (3, 19). All enzymes used in constructions were purchased from New England BioLabs, Inc. (Beverly, Mass.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or U.S. Biochemicals (Cleveland, Ohio). Restriction digestions, DNA ligation,

and end repair with T4 DNA polymerase were performed by procedures described in reference 21. All construction junctions were verified by DNA sequence analysis (1).

Yeast strains and genetic procedures. *S. cerevisiae* strains used in these studies have been described previously (6, 13). They were E906-8B (*MATa cycl1-11 cyc7-67 can1 leu2-3,112 trp1-Δ1 ura3-52*), E730-4A (*MATa cycl1 cyc7-67 can1 his5 leu2 trp1*), E378-1A (*MATa cycl1-363 CYC7-H2 cry1 his1 lys2*), J14046-7A (*MATa cycl1-363 CYC7⁺ cycl10-1 met14 lys1 lys2*), KZ8-5C (*MATa cycl1-1 CYC7⁺ his4 ura1*), and B534 (*MATa cycl1-31 CYC7⁺ his1 lys2 trp2*). Media and conventional yeast genetic procedures for crosses, strain construction, and scoring nutritional markers are described by Sherman et al. (27). A LiCl procedure was used to transform yeast strain E906-8B (20).

Iso-2-cytochrome *c* determinations. Cells were grown under derepressing conditions in YPS medium (1% [wt/vol] yeast extract [Difco Laboratories, Detroit, Mich.], 2% [wt/vol] Bacto-peptone [Difco], 1% [wt/vol] sucrose). For routine determinations cells were grown as narrow lines on solid medium. For experiments in which both cytochrome *c* and plasmid copy number were to be determined (see below), 10 ml of cells in liquid culture were collected on GF/C glass microfiber filters (Whatman, Inc., Clifton, N.J.). Intact cells were examined at -196°C for cytochrome *c* content with a wavelength prism spectroscopy (28). The absorption intensities of the c_{α} bands were visually compared with the c_{α} band intensities of strains with known amounts of iso-2-cytochrome *c*. Standard strains used for reference were KZ8-5C (*CYC7⁺*), J14046-7A (*CYC7⁺ cycl10*), and E378-1A (*CYC7-H2*) which produce 1, 4, and 22 units of iso-2-cytochrome *c*, respectively. In the range of 0.5 to 30 units, values for cytochrome *c* content estimated by the visual method are within about 15% of the values determined by a quantitative extraction method (see Table 2 of reference 13).

Yeast DNA preparation and Southern hybridization analysis. Cells were grown in liquid YPS medium to 3×10^8 to 5×10^8 cells per ml. Total DNA was prepared by procedures described in reference 27. A ^{32}P -labeled hybridization probe was prepared from a 1.1-kb *CYC7⁺* fragment which was isolated from replicative-form DNA of the M13 recombinant designated M18C7-7 (13). Nick translation, Southern transfer, and hybridization were performed by procedures and conditions described in reference 21. The relative amount of plasmid DNA was determined from the ratio of plasmid to genomic band intensities on autoradiograms by previously described procedures (13).

Yeast RNA preparation and primer extension analysis. Cells were grown in liquid YPS medium to 1×10^7 to 2×10^7 cells per ml. Total RNA preparation and primer extension analyses were performed by the procedures described in reference 6. A 20-base oligonucleotide with the sequence 5'-CCCGTACTTTCTTTAGCCAT was synthesized on an Applied Biosystems model 380A synthesizer and used as an end-labeled primer. The oligonucleotide is complementary to the *CYC7* coding region at positions +1 to +20. The same end-labeled primer was used with pNC98 single-stranded DNA serving as template to generate a dideoxy sequence ladder (22).

RESULTS

Ty1 fragment deletions in *CYC7-H2*. Regulatory regions important for Ty1 activation of adjacent *CYC7* expression

region a extending from the *PvuII* site toward delta sequences caused gradual reductions in *CYC7* expression. Deletion of the *PvuII*-*AluI* fragment in this region (pNC74) resulted in production of *CYC7*⁺ (pNC5) amounts of iso-2-cytochrome *c*. A more extensive deletion (*PvuII*-*HaeII*) extending to the delta-epsilon junction (pNC75) resulted in less than pNC5 amounts of iso-2-cytochrome *c*. The largest deletion extended into delta sequences (*PvuII*-*AccI*) and resulted in amounts of iso-2-cytochrome *c* that were barely detectable (pNC76). Deletion of the central Ty1 fragment b (pNC73) significantly decreased overproduction of iso-2-cytochrome *c* (12, 13). A similar decrease in iso-2-cytochrome *c* production was obtained for deletion of the distal fragment c (pNC72). An unexpected result was that deletion of fragments b and c together (pNC77) caused little if any deviation from production of *CYC7-H2* plasmid (pNC71) amounts of iso-2-cytochrome *c*. A deletion of the *PvuII*-*AluI* fragment from the remaining Ty1 sequences (pNC78) abolished the activating effects of region a and resulted in

FIG. 3. Structure and expression of *CYC7*⁺, *CYC7-H2*, and *CYC7-H2* deletion plasmids. Ty1 sequences shown by open, hatched, or stippled boxes delineate different regions designated a, b, and c, respectively. Delta sequences are indicated by a diamond. *CYC7* coding sequences are shown by the solid box. The direction and extent of the *CYC7* transcription unit is shown by the arrow. Restriction fragment deletions are indicated by thick lines below the diagram. Unique restriction sites in the diagrammed region are *Bam*HI (B), *Bgl*II (G), *Hae*II (Ha), *Hpa*I (Hp), *Pvu*II (V), and *Sph*I (Sp). Only those *Acc*I (Ac), *Alu*I (A), and *Sau*3A (Sa) sites that delimit specific restriction fragments analyzed here are shown. Amounts of iso-2-cytochrome *c* were estimated by low-temperature spectroscopic examination of intact cells. Average amounts were calculated from determinations on 12 independent transformation isolates for each plasmid. Numbers in parentheses are standard deviations. Normalized expression values are from measurements made with one representative transformed strain for each plasmid. Amount is cytochrome *c* content estimated from one to four cultures of the representative strain. The average value is given when more than one determination was made. Gene dosage is the ratio of *CYC7* plasmid copy to single *CYC7* gene copy. When more than one determination was made, the average is given and the range of values is indicated. Amount per gene is amount/gene dosage.

production of iso-2-cytochrome *c* similar to that of the *CYC7*⁺ plasmid (pNC5). This result was similar to that with pNC74 in which the only deletion in Ty1 was the *Pvu*II-*Alu*I fragment. In summary, the 270 bp of Ty1 proximal to *CYC7* functioned as an activator of adjacent *CYC7* expression. However, when these sequences were present with either region b or c but not both, inhibition of activation resulted.

Ty1 fragment insertions at *CYC7*. To test the effect regions a, b, and c have on *CYC7* expression in the absence of other Ty1 sequences, we inserted the corresponding restriction fragments immediately upstream of *CYC7* in the polylinker sites of either pNC98 or pNC67 (Fig. 2). In both plasmids the polylinker replaces 90 bp of *CYC7* sequences required for function of the normal transcriptional control elements at *CYC7*. Results of Wright and Zitomer (32, 33) indicated that the normal *CYC7* transcription control region consists of both positive and negative sites. The *CYC7* sequences that were replaced in our construction include the entire positive control region and one of the three elements required for function of the negative control site. We found that *CYC7*⁺ amounts of expression were observed for a replacement of this 90 bp with polylinker fragments (pNC5, Fig. 3; pNC67 and pNC98, Fig. 4). The same result was reported for deletions that inactivate both the positive and negative control sites (32). To minimize differences in *CYC7* sequences flanking Ty1 in *CYC7-H2* compared with Ty1 frag-

ments in these constructions, we placed the cloning region (position -200) close to the Ty1 insertion junction (position -184) in the *CYC7-H2* mutation.

The various Ty fragment insertions were compared for their effects on *CYC7* expression in haploid yeasts (Fig. 4). The insertion of fragment b in either orientation upstream of the *CYC7* coding region had a negative effect on *CYC7* expression and resulted in less than *CYC7*⁺ amounts of iso-2-cytochrome *c* (pNC101 and pNC102). Fragment c insertion in either orientation similarly had a negative effect (pNC103 and pNC104). Insertion of fragment a in the normal orientation resulted in approximately fivefold overproduction of iso-2-cytochrome *c* (pNC105 and pNC69) compared with the corresponding control plasmids (pNC98 and pNC67). The fragment was a slightly better (1.6-fold) activator in the normal orientation (pNC105, pNC69) compared with the inverted orientation (pNC106). Fragment d by itself caused only a twofold activation of *CYC7* expression (pNC68). Fragment d was joined directly to fragment a to determine whether it would cause *CYC7* expression as found when d was placed directly between a and c (13) or inhibition as found when a and b were joined in pNC72. Fragment d did not inhibit or enhance the ability of fragment a to activate *CYC7* expression (pNC70).

RNA characterization. 5'-end-mapping experiments were performed to determine whether the normal *CYC7* mRNA

Cell-type specificity of Ty1 activator. Ty1 activation of *CYC7* expression is observed in a or α haploid cell types but is repressed ~ 10 -fold in the a/ α diploid cell type (11). To determine whether activation of *CYC7* by region a alone is cell type dependent, we compared plasmid constructions for production of iso-2-cytochrome *c* in haploid (*MATa*) and diploid (*MATa/MAT α*) cells. Diploid cells were constructed by crossing *MAT α* tester strain E730-4A to representative *MATa* transformed strains. Amounts of iso-2-cytochrome *c* and relative gene dosage were determined as before (Fig. 4). Essentially the same normalized amount of iso-2-cytochrome *c* was observed in haploid and diploid cells for the control plasmids (pNC67 and pNC98) and the corresponding constructions in which fragment a is inserted upstream of *CYC7* (pNC69, pNC105). This result indicated that region a includes *cis*-acting sequences that activate

DISCUSSION

The major Ty1 activator is located in region a which includes 70 bp of delta sequences (U5 domain) and 145 bp of adjacent epsilon sequences (Fig. 1). Our deletion analysis suggested that both delta and epsilon sequences were necessary for activator function of this region. Similarly, Roeder et al. (25) have identified delta sequences in the corresponding regions of Ty2 that are important for activation of adjacent gene expression. The observation that progressive

FIG. 5. Autoradiogram showing *CYC7* RNA primer extension products. Amounts of total RNA used for samples shown are as follows: lanes 5 to 8, 50 μ g; lane 9, 20 μ g; lane 10, 50 μ g; lane 11, 100 μ g; lane 12, 30 μ g; lane 13, 20 μ g; lane 14, 15 μ g; lane 15, 30 μ g; and lane 16, 100 μ g. Dideoxy sequence ladders are shown in lanes 1 to 4 and 17 to 20 for size reference. The 5' map positions of major and minor RNAs are indicated by large or small arrows, respectively. The position numbers are with respect to the ATG codon.

deletions in region a caused more severe reductions in adjacent gene expression suggests that region a is composed of several *cis*-acting elements which together exert a positive regulatory effect on gene expression.

A second region analyzed (d) showed activator function under some conditions but not others. Region d, which includes block II sequences, is a functional enhancer when placed between Ty1 regions a and c (13). In the absence of other Ty sequences, one copy of region d does not significantly activate adjacent gene expression. However, two to four tandem copies of the block II region, in either orientation, cause as much or more activation of adjacent gene expression as does region a (Company and Errede, manuscript in preparation). Our interpretation of these results is that both regions a and d contain activator elements but that the two regions differ in the details of their action. Other results (below) indicate that the two regions also differ in their responsiveness to cell-type regulation.

Identification of two different fragments (b and c) that directly inhibit *CYC7* expression was unexpected (Fig. 1). Assignment of an inhibitory function to these fragments was based on comparisons with other Ty1 fragments inserted at the same site adjacent to *CYC7* (Fig. 4). An extrapolation from the function of isolated fragments in an artificial context to their function in the normal Ty1 context is speculative but useful in formulating models for the complex interactions observed. When the activator element was present with one but not both of the negative regulatory regions (b and c), repression of gene expression was observed. When region b and c were both present the negative effect each one has on the activator was masked. One model is that regions b and c include both activator and inhibitory regions. We have not identified the postulated negative regulatory sequences in regions b and c. The block II element is one activator

identified within region b. The block II enhancer may act by preventing effects of the postulated negative elements. Further analysis of the relationship of these regions to each other and identification of the interacting regulatory factors will be necessary to fully understand Ty1 regulation of adjacent genes.

Transcription of Ty, Ty-controlled genes, and other haploid-specific genes such as *MAT α 1*, *HO*, and *STE5* is repressed in diploid cells. Closely related sequences have been identified in Ty1 and in the regulatory regions of *MAT α* , *STE5*, and *HO* (12, 24, 29). Some of these sites have been shown to be sufficient for repression of adjacent gene expression in diploid yeasts (13, 24, 29). Although one of the diploid control site homologies in the *CYC7-H2* Ty1 is present in region a (Fig. 1), activation of *CYC7* expression by region a was not significantly repressed in *a/a* diploid cells. Region d includes two diploid control site homologies (Fig. 1). Fragment d by itself and in association with the activator region a caused significant repression of *CYC7* expression in *a/a* diploid cells.

Ty effects on adjacent gene expression are mimicked by an ~330-bp sequence composed of regions a and d. Depending on the yeast cell type, one or the other region appears to be the predominant controlling regulatory element. In haploid cells the activator element (region a) is the controlling element and results in increased amounts of gene expression. In diploid cells the cell-type specificity element (region d) is the controlling element and represses activation of gene expression.

The data presented here show that Ty1 cell-type control of adjacent gene expression is accomplished through the action of distinct positive and negative regulatory elements. The indicated multicomponent structure for a regulatory region is not unique to Ty1. For example, two distinct enhancer

elements have been identified within the enhancer region of the polyomavirus and Moloney murine sarcoma virus (7, 18). Negative regulatory elements have been identified in the enhancer region of the human T-cell lymphotropic virus type III (26). The beta interferon enhancer consists of a constitutive transcriptional activator and a negative regulatory element. In this system, enhancer activity is observed when inhibition by the negative element is relieved upon induction (17). Because a multicomponent structure is indicated for transcriptional control regions in wide-ranging systems, this may be a general strategy for achieving complex controls associated with cell-type-specific gene expression in eucaryotic organisms.

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