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Interspersion of an Unusual GCN4 Activation Site with a Complex Transcriptional Repression Site in Ty2 Elements of *Saccharomyces cerevisiae*

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Transcription of the Ty2-917 retrotransposon of *Saccharomyces cerevisiae* is modulated by a complex set of positive and negative elements, including a negative region located within the first open reading frame, *TYA2*. The negative region includes three downstream repression sites (DRSI, DRSII, and DRSIII). In addition, the negative region includes at least two downstream activation sites (DASs). This paper concerns the characterization of DASI. A 36-bp DASI oligonucleotide acts as an autonomous transcriptional activation site and includes two sequence elements which are both required for activation. We show that these sites bind in vitro the transcriptional activation protein GCN4 and that their activity in vivo responds to the level of GCN4 in the cell. We have termed the two sites GCN4 binding sites (GBS1 and GBS2). GBS1 is a high-affinity GCN4 binding site (dissociation constant, ~25 nM at 30°C), binding GCN4 with about the affinity of a consensus UAS_{GCN4}, this though GBS1 includes two differences from the right half of the palindromic consensus site. GBS2 is more diverged from the consensus and binds GCN4 with about 20-fold-lower affinity. Nucleotides 13 to 36 of DASI overlap DRSII. Since DRSII is a transcriptional repression site, we tested whether DASI includes repression elements. We identify two sites flanking GBS2, both of which repress transcription activated by the consensus GCN4-specific upstream activation site (UAS_{GCN4}). One of these is repeated in the 12 bp immediately adjacent to DASI. Thus, in a 48-bp region of Ty2-917 are interspersed two positive and three negative transcriptional regulators. The net effect of the region must depend on the interaction of the proteins bound at these sites, which may include their competing for binding sites, and on the physiological control of the activity of these proteins.

Transcriptional control of the *Saccharomyces cerevisiae* retrotransposon Ty2-917 is unlike that of most yeast genes. The main difference is that nearly all of the *cis*-acting sites which modulate Ty2-917 transcription lie not upstream of the transcription start site, as in nearly all other yeast and indeed eukaryotic genes, but downstream within the transcribed region. In fact, these sites are not only transcribed but also are translated into the first of two Ty products, *TYA2-917*, the analog of the retroviral *gag* gene. Thus, these regulatory elements are under unusual constraints. They are constrained genetically, since the sequence of the regulatory region must encode a protein which forms the structure within which the transposon replicates itself, the virus-like particle (for a review, see reference 6). Being within the transcribed region applies special conditions on the regulatory region which regulators outside of genes do not endure. They are perturbed topologically, since RNA polymerase passes through them once per initiation event, overwinding the DNA in advance of its passing and underwinding in its wake (51). They are disturbed structurally, since the polymerase in passing unwinds the DNA strands, displacing nucleosomes (44) and, presumably, disrupting the protein-DNA complexes which form on the regulatory sites which make up the control region. Transcription can disrupt the biological function of regions not evolved to survive the insult. For example, transcription through a centromere disrupts the structure formed on the site which allows attachment of microtubules and therefore disrupts partitioning of the affected chromosome during cell division (34). Similarly, transcription through a control region would be expected to disrupt its function or at least decrease its ability to activate transcription, since at each transit of polymerase, the molecular complex formed by site-specific DNA binding proteins and their accessory factors would need to be recreated. This could drastically reduce the effective rate at which the preinitiation complex at the regulated promoter could be activated. Either topological or structural disruption by polymerase may explain why upstream activation sites (UASs) fail to function when transposed from outside to inside of the transcription unit (32, 72).

Under special circumstances genes evolve transcriptional control signals within the transcribed region. Because viruses are selected for compact genomes, many have evolved such control regions. The special requirements of the immunoglobulin genes, which must activate transcription of genes formed by combination of a variety of reiterated upstream gene segments, have led to an enhancer which is located within an intron (5, 28). Until recently one would have included polymerase III genes in this class, since they provided the canonical example of internal promoters. It has become clear recently that transcription factor TFIIIB (42) and/or TATA-binding protein (14, 45, 79) binds upstream of these genes to stimulate transcription and that binding of the factors TFIIIA and TFIIIC is required only to assemble TFIIIB onto the template (42). The small number of such genes, and their disparate structures, precludes drawing any general conclusions about how control regions cope with the disruptive effects of transcription. To the extent that these regions efficiently activate transcription, they must have evolved mechanisms which either allow them to remain undisrupted or allow reestablishment of the proper protein-DNA complex after passage of polymerase.

The transcription of genes by RNA polymerase II is most

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often regulated by modulating the rate of transcription initiation. This is probably most often accomplished by modulating the rate of formation of the transcription preinitiation complex (66). This complex is composed of several basal transcription factors and RNA polymerase II which assemble on the DNA through an ordered process (8, 52, 76). The basal machinery can be assembled in vitro and will direct accurate transcription (59). In vivo the basal machinery does not direct efficient transcription of genes but depends on a class of transcriptional activator proteins. Since the basal machinery is competent to initiate in vitro, the role of these activators could include stimulating the assembly of a limiting supply of one or more basal factors or excluding the repressive influence of chromatin (for a review, see references 7 and 31). This second role may be involved even in cases in which transcription factors stimulate the formation of the preinitiation complex. Transcription factors can overcome transcriptional repression in an in vitro chromatin assembly reaction (43, 53, 82). The role of the general repressive effect of chromatin may be to reduce transcription of genes in the absence of specific induction by transcriptional activators (31). This general effect serves to increase the induction ratio of activated systems. Mutations which alter the stoichiometry of core histones affect transcription in *S. cerevisiae*, suggesting an in vivo transcriptional control role for chromatin (11). Transcription factors might indirectly stimulate transcription by excluding nucleosomes. A possible example of this occurs in the *HIS4* gene of *Saccharomyces cerevisiae*, in which RAP1 appears to increase the accessibility of chromatin to the transcriptional activators GCN4, BAS1, and BAS2 (15).

The rate of transcription initiation can also be repressed by factors which inhibit formation of the preinitiation complex by a variety of mechanisms (30, 47, 63). These mechanisms include competition with an activator protein for its binding site, blocking activation by such a factor, sequestering an activator in an complex which makes it unavailable for activation, and directly inhibiting the formation of the preinitiation complex. Unlike repression by chromatin, these mechanisms all involve specific protein-DNA or protein-protein interactions. Even repression by chromatin can be facilitated by site-specific protein-DNA interactions. Nucleosomes may be positioned so as to preclude access to the DNA by activators at specific sites within promoters (31, 70, 81). An example of such an effect is the positioning of a short-array nucleosome adjacent to the *S. cerevisiae* a2 repressor blocks access of TFIID to the TATA box (68).

Transcription of the Ty family retrotransposon is modulated by sites both upstream and downstream of the transcriptional initiation site (13, 19–22, 26, 62, 65). Upstream are UAS and TATA sites, and downstream are sites which either stimulate or repress transcription initiation. A Ty1 class retrotransposon includes one site which is the major activator of transcription; this site is targeted by the STE12 transcriptional activator and has been termed the sterile responsive element (12, 18). The Ty2 class retrotransposon, Ty2-917, also includes a region analogous to the sterile responsive element which we have termed the Ty2 enhancer (21). This site, however, does not depend on STE12 for its activity and appears to include multiple positively acting elements (23). Ty2-917 also includes a negatively acting region which we have shown includes three downstream repression sites (DRSs); DRSI, DRSII, and DRSIII) (Fig. 1c) (22). The region also includes downstream activation sites (DASs). In this paper, we address the structure of DASI, an activation site which overlaps DRSII. We demonstrate that

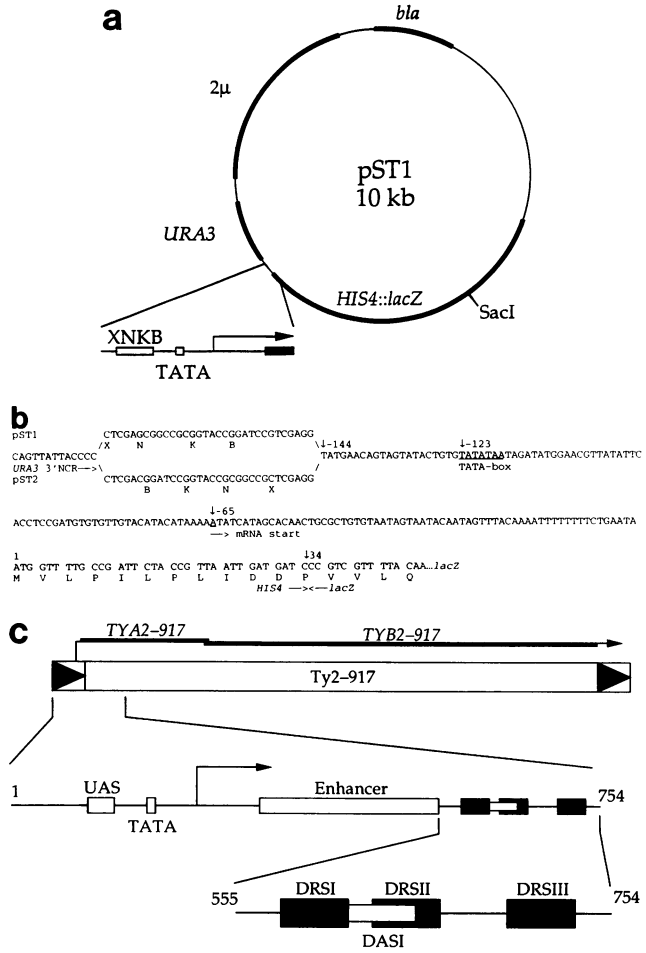

FIG. 1. Structure of plasmids pST1 and pST2. (a) Map of plasmid pST1. The regions corresponding to the *URA3* gene and segment of 2μm circle DNA of *S. cerevisiae*, the plasmid *bla* gene and the *HIS4::lacZ* fusion gene are indicated by thick arcs. The promoter region of the *HIS4::lacZ* gene is shown in detail with the transcription start site indicated by the arrow and the positions of the TATA box and polylinker (XNKB corresponding to *Xho*I, *Not*I, *Kpn*I, and *Bam*HI sites) indicated. (b) The sequence of the promoter region of plasmids pST1 and pST2. The sequence given extends from the 3′ noncoding region of *URA3* (*URA3* 3′NCR) to the *HIS4::lacZ* fusion junction. The sequences of the two different polylinkers are shown. (C) The arrangement of control sites in Ty2-917. All activation sites are shown as white boxes, and all repression sites are shown as grey boxes. The transcribed region is indicated by an arrow at the top of the figure. On this line are displayed the regions corresponding to the two encoded genes, *TYA2-917* and *TYB2-917*.

activation by DASI is proportional to the in vivo concentration of the transcriptional activator GCN4. GCN4 activates a battery of genes involved in biosynthesis of amino acids. The 281-amino-acid protein is a member of the basic zipper (bZIP) family (78) and binds as a dimer to a palindromic site 5′-RRTGACTCATTT-3′ termed UAS_{GCN4} (2, 35). The genes controlled by GCN4 generally contain several of these sites; activation by two copies of synthetic UAS_{GCN4} leads to greater than additive increase in expression suggestive of cooperative interaction between bound dimers (37). The canonical UAS_{GCN4} binding site was defined by comparing multiple sites present upstream of GCN4-controlled genes

(2, 36), by targeted mutagenesis of the canonical site (35), or by selection of random oligonucleotides capable of binding GCN4 (54, 60). Changes outside of the inner 6 bp of the binding site (TGACTC) have little effect on affinity, as measured by half-maximal binding concentration, though alterations to this core can decrease affinity up to 50-fold (2).

The purified GCN4 bZIP domain (GCN4-bZ) binds DASI in vitro to two GCN4 binding sites (GBS; GBS1 and GBS2), both of which are also required for activation in vivo. These data are consistent with a model whereby GCN4 activates transcription directly by binding to the GBSs, though an indirect model involving a second DNA binding domain whose expression is regulated by GCN4 cannot be excluded. We also show that the DASI/DRSII region includes three transcriptional repression sites. Two of these sites flank and partially overlap GBS2; one of these sites is repeated precisely two helical turns downstream. The effect of this region on transcriptional initiation results from the interaction of multiple proteins, some of which may compete for occupation of the DNA, as well as from physiological control of the activity of these proteins.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* strains used were PF387-1D (*MATα his4-38 trp1-289 ura3-52*), H1534 (*MATα ura3-52*), F98 (*MATα ura3-52 gcd1-101*), F113 (*MATa ino1 ura3-52*), and F212 (*MATa gcn4Δ1 ino1 ura3-52*). H1534, F98, F113, and F212 are isogenic other than the markers noted and were gifts of A. Hinnebusch. The *gcn4Δ1* marker is a in vitro-constructed deletion between a *Kpn*I site in codon 150 and an *Mlu*I site 272 bp downstream of the gene; the deletion removes the C-terminal 47% of the gene, including the bZIP DNA binding and dimerization domain (40, 78). Strains were transformed by the method of Ito et al. (41). Transformants were grown in liquid yeast nitrogen base minimal medium (Difco) containing 2% glucose. Two plasmids have been used in various transformation experiments as controls. pFN8x-n is a 2μm-*URA3*-based plasmid bearing a fusion of the *HIS4* gene to the *Escherichia coli lacZ* gene (58). Expression of β-galactosidase by this construct is induced in response to GCN4. pLGΔ312 is an identical 2μm-*URA3* vector bearing a *CYC1*::*lacZ* fusion; expression of β-galactosidase by this construct is unrelated to GCN4.

DNA oligonucleotides. Oligodeoxyribonucleotides were synthesized on a Biosearch Cyclone DNA synthesizer by phosphoramidite chemistry and purified away from failure sequences by retention on Oligo-pak columns (Milligen) by following the manufacturers specifications.

Plasmid constructions. All of the plasmids used in this study were derived from pST1 and pST2, 2μm-based shuttle plasmids carrying the *URA3* gene for selection in *S. cerevisiae* and *bla* for selection in *E. coli* (pPST1 is diagrammed in Fig. 1a). The plasmids include a *his4*::*lacZ* translational fusion (between codon 12 of *HIS4* and 8 of *lacZ*). Transcription of this gene is directed by a 5′ truncated *HIS4* promoter including the 144 bp upstream of the *HIS4* initiation codon; this truncation removes the binding sites for all known transcriptional activators, retaining only the TATA box (123 bp upstream of the gene) and start site (65 bp upstream) (3, 58). The construction of this plasmid has been previously described (22). Both pST1 and pST2 have polylinkers immediately upstream of the *HIS4* promoter. The pST1 polylinker includes *Xho*I, *Not*I, *Kpn*I, and *Bam*HI sites, in that order, with the *Bam*HI site adjoining the *HIS4* promoter; pST2 has a similar polylinker inserted in the opposite orientation (the sequence of the polylinker and promoter region is given in Fig. 1b).

Wild-type and mutant forms of the DASI oligonucleotide (Table 1) were inserted into the *Xho*I site of pST1. Each oligonucleotide has a *Xho*I half-site at one end and a *Sal*I half-site at the other; immediately interior to the *Sal*I half-site is a complete *Sal*I site. Insertion of the oligonucleotide into a *Xho*I site creates an insertion of the 36-bp DASI site flanked on one side by a *Xho*I site (one insertion junction) and on the other by a *Sal*I site (from the oligonucleotide) and a *Xho*I-*Sal*II composite site (the other insertion junction). Insertions in either orientation were distinguished first by cleavage by *Sal*I or *Xho*I along with *Apa*I which cleaves 510 bp upstream of the insertion point. An example of each orientation was identified by sequencing candidate clones. Mutant forms of the DASI oligonucleotide were synthesized and inserted in the same manner as for the wild type. These oligonucleotides include six linker-scanning mutations (SCN1 to SCN6), replacing each successive 6 bp of the DASI with either an *Sph*I or an *Nar*I restriction site as shown in Table 1. In addition, two mutants which insert or delete 6 bp between the GCN4 binding sites, DEL(3-4) and INS(4-3), were constructed by combining SCN3 and SCN4 in the two possible ways, resulting in the sites listed in Table 1. The wild-type and mutant DASI oligonucleotides were reiterated after insertion into pST1 by ligating together *Sal*I-*Sac*I and *Xho*I-*Sac*I fragments of the plasmid such that the region between *Sal*I and *Xho*I are present twice in the resulting plasmid. Repeated rounds of such ligations on successively larger oligonucleotide arrays created insertions of up to 48 copies. The number of inserts was determined both by the size of the arrays present in the two parental plasmids and by sizing fragments (*Apa*I-*Sal*I or *Apa*I-*Xho*I) by agarose electrophoresis.

An oligonucleotide corresponding to the putative negative region, ATTATCAA, repeated overlapping the upstream end of GBS2 and 6 bp downstream of GBS2 (upstream referring to toward the RNA initiation point) was synthesized. The sequence of the oligonucleotide is given in Table 1; the oligonucleotide was inserted into the *Bam*HI site of pST2. Insertions in either orientation were identified. The plasmid with the sequence ATTATCAA in the top strand of the promoter is termed pTAT-A; the opposite orientation is pTAT-R. A UAS_{GCN4} (Table 1) was inserted into the *Xho*I site of pST2 to create pST2-GCN4. The wild-type and mutant DASI and the ATTATCAA site were placed upstream of UAS_{GCN4} by joining either the unique *Sal*I site of the DASI plasmid or the unique *Xho*I site of either pTAT-A or pTAT-R to the unique *Xho*I site upstream of the UAS_{GCN4} oligonucleotide.

The nucleotide sequence of the region of the oligonucleotide insertions of each plasmid was determined with a double-stranded dideoxynucleotide sequencing protocol and Sequenase enzyme (United States Biochemicals).

A plasmid was constructed to overproduce GCN4. The translation of *GCN4* is regulated in response to starvation of any of several amino acids (38). Normally translation of *GCN4* is repressed by multiple upstream open reading frames. An overproducing allele which lacked these upstream open reading frames had been constructed (57). The plasmid carrying this allele is p238. The *Sal*I-*Pvu*II fragment encompassing *GCN4* from p238 was ligated into *Sal*I-*Sma*I-digested pRS414, a *TRP1 CEN6 ARSH4* shuttle plasmid (69) to create the overproducing plasmid pRS414-GCN4.

Assay of β-galactosidase. Transformants of plasmids bear-

TABLE 1. Sequences of site oligonucleotides

Site	Sequence[a]
DASI (wild type)	tcgagTATGATGACGTGTTCACATTATCAAACGTCACCTGCgtcgacg cATACTACTGCACAAGTGTAATAGTTTGCAGTGGACCcagctgcagct /XhoI SalI SalI/
DASI (SCN1)	tcgagGCATGCGACGTGTTCACATTATCAAACGTCACCTGCgtcgacg cCGTACGCTGCACAAGTGTAATAGTTTGCAGTGGACGcagctgcagct
DASI (SCN2)	tcgagTATGATGCATGCTTCACATTATCAAACGTCACCTGCgtcgacg cATACTACGTACGAAGTGTAATAGTTTGCAGTGGACGcagctgcagct
DASI (SCN3)	tcgagTATGATGACGTGGCATGCTTATCAAACGTCACCTGCgtcgacg cATACTACTGCACCGTACGAATAGTTTGCAGTGGACGcagctgcagct
DASI (SCN4)	tcgagTATGATGACGTGTTCACAGCATGCAACGTCACCTGCgtcgacg cATACTACTGCACAAGTGTCGTACGTTGCAGTGGACGcagctgcagct
DASI (SCN5)	tcgagTATGATGACGTGTTCACATTATCAGCATGCACCTGCgtcgacg cATACTACTGCACAAGTGTAATAGTCGTACGTGGACGcagctgcagct
DASI (SCN6)	tcgagTATGATGACGTGTTCACATTATCAAACGTCGGCGCCgtcgacg cATACTACTGCACAAGTGTAATAGTTTGCAGCCGCGGcagctgcagct
DASI [DEL(3-4)]	tcgagTATGATGACGTGGCATGCAACGTCACCTGCgtcgacg cATACTACTGCACCGTACGTTGCAGTGGACGcagctgcagct
DASI [INS(4-3)]	tcgagTATGATGACGTGTTCACAGCATGCTTATCAAACGTCACCTGCgtcgacg cATACTACTGCACAAGTGTCGTACGAATAGTTTGCAGTGGACGcagctgcagct
UAS_{GCN4}	tcgagCTGACTCAGTTTTTgtcgacg cGACTGAGTCAAAAAcagctgcagct /XhoI SalI SalI/
ATTATCAA	gatccATTATCAAAagatcta gTAATAGTTTtctagatctag /BamHI BglII BglII/
DASI-GS (for mobility shift)	tttTATGATGACGTGTTCACATTATCAAACGTCA ATACTACTGCACAAGTGTAATAGTTTGCAGTGGACGttt
UAS_{GCN4}-GS (for mobility shift)	tcgaGATGACTCATTTTTTTTgtcgacg CTACTGAGTAAAAAAAAcagctgcagct

[a] Sequences corresponding to the sites are capitalized. Other sequences added for cloning or labeling purposes are in lowercase. Restriction sites introduced as part of linker-scanning, deletion, or insertion mutations are in boldface.

ing each deleted element were assayed for the level of β-galactosidase expression. Cells grown to mid-log phase (optical density at 600 nm, ≈0.9) were harvested by centrifugation, washed with distilled water, resuspended in 200 μl of breaking buffer (20% glycerol, 0.1 M Tris-HCl, 1 mM dithiothreitol, 2.5 mM phenylmethylsulfonyl fluoride [pH 8.0]), and permeabilized by addition of 20 μl of chloroform and 20 μl of sodium dodecyl sulfate (0.1% [wt/vol]). Multiple transformants were assayed in triplicate as previously described (56). Units are given in nanomoles of orthonitrophenyl-β-D-galactoside (Sigma) cleaved per minute per milligram of protein. All standard errors were below 10%.

Gel mobility shift assays. These assays were performed with a purified preparation of the basic region-leucine zipper (bZIP) DNA binding-dimerization domain of GCN4 (a generous gift of John Shuman, University of Alabama). This protein, which we will refer to as GCN4-bZ, includes the C-terminal 54 residues of GCN4. The assays were performed essentially as previously described (4) with the following alterations. Double-stranded oligonucleotides corresponding to the wild-type and various mutant forms of the DASI site and a consensus GCN4 binding site were synthesized with 5′ extensions and labelled by filling in these extensions with the Klenow fragment of DNA polymerase I and [α-^{32}P]deoxynucleotides (300 Ci/mmol; Amersham); the sequences of the oligonucleotides are given in Table 1. For the SCN mutants, the same oligonucleotides were used for mobility shift analysis as were used in constructing mutant oligonucleotide insertions. Various amounts of GCN4-bZ were incubated for 15 min at 30°C with 1 ng of radiolabelled oligonucleotide in 20 μl of binding buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-NaOH [pH 7.9], 60 mM KCl, 1 mM dithiothreitol, 12% [vol/vol] glycerol, 4 mM Tris-HCl [pH 7.9], 1 mM EDTA [pH 8.0], 10 mM $MgCl_2$). The products of the reactions were separated by electrophoresis in a 4% polyacrylamide gel (1:80, bis-acrylamide) in high-ionic-strengy buffer (4) at 2.5 V/cm in a 4°C room. The products were visualized by autoradiography. The dissociation constant (K_d) for interaction between GCN4-bZ and the oligonucleotide was determined by estimating by eye the concentration of protein required to bind half of the target oligonucleotide. K_d is defined as the ratio of the two reaction rate constants for formation (k_f) and dissociation (k_r), i.e., $K_d = k_f/k_r$. We have not measured the two rate constants; however, as first shown for the *lac* repressor (64), the equilibrium dissociation constant is equal to the concentration which binds half of the DNA present in the reaction mixture, provided that the concentration of DNA is much less than the concentration of protein (9); in our case, the concentration of DNA is less than 10^{-13} M.

DNase protection assays. A portion of Ty2-917 encompassing the GBS1 and GBS2 sites was cloned into pUC18. A fragment from the *Bgl*II site inserted at position 555 to the *Kpn*I site at the *lacZ* fusion junction (at position 754) was cloned into *Bam*HI-*Kpn*I-digested pUC18. The clone was cleaved at polylinker sites flanking the insert, *Xba*I upstream of the *Bam*HI-*Bgl*II end and *Eco*RI downstream of the *Kpn*I end. The fragments were labelled at either end by filling in the 5′ extensions with Klenow fragment of DNA polymerase I and [α-^{32}P]dATP. End-labelled fragment (1 to 2 ng) was incubated at 30°C for 15 min in 50 μl of binding buffer (50 mM Tris-HCl [pH 8.0], 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 15% [vol/vol] glycerol, 1 mM dithiothreitol) with 1 to 300 ng of GCN4-bZ. Single-stranded nicks were introduced

with a commercial kit, Footprinting System AP-1 (Promega) by following manufacturers specifications; the DNA was exposed to 0.1 U of DNase I for 1 to 1.5 min. Deproteinized DNase digestion products were separated by electrophoresis in a gel of 6% polyacrylamide (1:20, bis-acrylamide)–50% urea.

RESULTS

An oligonucleotide encompassing putative GCN4 binding sites activates transcription. The Ty2-917 retrotransposon includes multiple regions which modulate transcription. Most of these sites are downstream of the transcription start site, within the coding region of the *TYA2-917* gene (21, 22) (Fig. 1c diagrams the transcriptional control sites identified in Ty2-917). We have previously identified two regions which appear to have opposite effects on transcription. An enhancer region lies within the first 315 bp of the transcribed region, to nucleotide 555 of the element. More recently we have mapped in detail the structure of a 200-bp region downstream of the enhancer which represses transcription. Using Ty2-917::*lacZ* fusions and introducing linker-scanning mutations and deletions which remove various portions of this region, we mapped three distinct DRSs, DRSI, DRSII, and DRSIII (22). Independent of any other Ty2-917 sequences, both oligonucleotides encompassing the putative DRSII or DRSIII sites repress transcription stimulated by the transcriptional activator GCN4 when inserted into a model promoter construct utilizing the *HIS4* TATA box and initiator site; this effect suggests that the DRSs repress transcription initiation (22). Interspersed with these sites are at least two positively acting sites (22). One of these, DASI, is the subject of this paper. DASI was originally defined as lying immediately upstream of or overlapping DRSII.

In this paper we address the nature of DASI. Deletion analysis had mapped DASI to nucleotides 615 to 650, overlapping DRSII (nucleotides 627 to 662) (22). To determine if DASI can directly regulate transcription initiation, a DASI oligonucleotide was inserted upstream of a UAS-less promoter driving expression of a *his4*::*lacZ* reporter gene. The plasmid carrying this fusion gene, pST1, includes a *HIS4* promoter in which the binding sites for the four known activation proteins (GCN4, BAS1, BAS2, and RAP1) have been deleted and replaced with a polylinker. The DASI oligonucleotide was inserted into this polylinker in either orientation in from 1 to 48 head-to-tail copies (pST1-G1A to pST1-G48A and pST1-G1R to pST1-G48R). As plotted in Fig. 2, even one copy of the DASI oligonucleotide stimulated significant transcription of the *his4*::*lacZ* gene, 20-fold above the naive promoter, and its effect was independent of orientation. Two copies stimulated 10-fold more transcription, again independent of orientation. This effect is synergistic, fivefold above the level predicted if the sites acted independently. The increase in activation when four to eight copies are present is nearly linear, an increase of about 200 U per copy. Activation saturates with 10 or more copies. The way that activation increases with number of copies of a site is reminiscent of the effect of reiteration of the UAS_{GAL}, the binding site of the GAL4 activator (10, 29, 48, 61). These data show that DASI acts as a canonical transcriptional activator.

Transcriptional activation by DASI requires two separate sites. In order to determine what sequences are required for activation by DASI, we constructed a series of the six possible 6-bp linker-scanning mutations of the DASI oligonucleotide. These were inserted into the pST1 expression

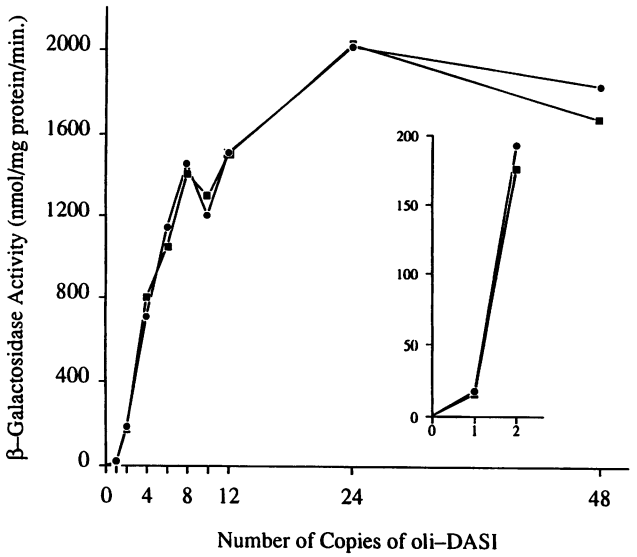

FIG. 2. Effects of reiterating the DASI oligonucleotide. The graph shows the β-galactosidase activity of 387-1D (*MATα his4-38 trp1-289 ura3-52*) transformed with constructs containing increasing numbers of DASI oligonucleotides. Uncorrected activities are given; the vector lacking any insert expresses 1 to 2 U of β-galactosidase (data not shown). The inset shows in more detail the effect of one or two copies. The oligonucleotides are oriented such that the top-strand sequence of DASI in Ty2-917 is in the top strand of the promoter (●) or in the bottom strand of the promoter (■).

reporter construct in one, two, and four copies and assayed for their effect on expression. Table 2 shows the results of this experiment; all constructs are in one orientation, though reorienting the site had no effect (data not shown). Three linker-scanning mutations had a severe effect, reducing activation by the fourfold-reiterated DASI oligonucleotide 22- to 28-fold. These include mutations affecting bp 1 to 6 (SCN1), 7 to 12 (SCN2), and 25 to 30 (SCN5). The other three linker-scanning mutations reduced activation no more than threefold. These data show that at least two sites are necessary for the DASI oligonucleotide to activate transcription and that neither is sufficient.

Inspection of the sequence of DASI shows that SCN2 and SCN5 target the two copies of a sequence present as an

TABLE 2. Both GBS1 and GBS2 are required for transcriptional activation

DASI oligonucleotide[a]	β-Galactosidase activity (U[b])		
	1 copy[c]	2 copies	4 copies
Wild type	18	190	700
SCN1	3.2	7.6	25
SCN2	1.5	4.5	32
SCN3	7.9	80	280
SCN4	13	110	370
SCN5	5.3	22	29
SCN6	20	160	400
DEL(3-4)	25	160	640
INS(4-3)	55	410	1,600

[a] See Table 1 for oligonucleotide sequences.
[b] Units of activity are nanomoles of substrate (*o*-nitrophenyl-β-D-galactoside) cleaved per minute per milligram of protein.
[c] Number of reiterated copies of each DASI oligonucleotide inserted into the polylinker upstream of the *HIS4* TATA box.

inverted repeat, 5'-TGACGT-3'. These sequences are reminiscent of the recognition site of the GCN4 transcriptional activator, 5'-RRTGACTCATTT-3' (2, 35). The sites include an additional G (5'-TGACGT-3') which is characteristic of the recognition site of the ATF family of activators whose canonical site is 5'-GTGACGT$^{C}_{A}$A-3' (49). Both GCN4 and ATF-1 are members of a superfamily of related activators. In fact, GCN4 binds to the ATF-1-related expanded binding site (67). The GCN4 binding site is a palindrome of the sequence 5'-RRTGAC-3' (67). Both of the two motifs in DASI have perfect left half-sites, but the right half-site is diverged from consensus. Each diverge by one more change—in each case the dinucleotide CA in the consensus GCN4 binding site (RRTGACTCATT) is changed. For the SCN2 site it is changed to GT (GATGACGTGTT; the number of thymidines required at the 3' end is not well defined). For the SCN5 site the CA is omitted (GGTG ACG∧TTT; the caret indicates the site of the omission). We note that no experiment has been performed to determine what nucleotides of the expanded site are required for GCN4 binding, so we cannot predict the relative affinity of these two sites for GCN4 protein. On the basis of the similarity to the canonical site and considering the evidence to be presented below, we term these two sites GBS1 and GBS2.

One way to explain the requirement for each GBS is that cooperativity derived from physical contact between GCN4 dimers stimulates binding. If the proteins must interact with each other, that interaction might require the binding sites to be located on the same face of the DNA. Such stereospecificity has been evidenced in other systems (73). In fact, the centers of GBS1 and GBS2 are spaced about two helical turns apart, so the pair of GCN4 dimers might bind to the same face of the DNA. To test for stereospecificity, we generated two mutations in which six nucleotides from the region between GBS1 and GBS2, about one-half helical turn, were deleted, DEL(3-4), or a 6-bp *Sph*I site was inserted, INS(4-3) (described in Materials and Methods). These mutations should have little intrinsic effect on activation, since the two linker-scanning mutations of the region (SCN3 and SCN4) have little effect on expression. If the bound proteins interact, the deletion or insertion should cause a significant decrease in activation; the lack of an effect would imply that no interaction occurs. As shown in Table 2, the 6-bp deletion had little effect on activation, and the 6-bp insertion actually caused a twofold increase in activation. These results are not those expected if the stereospecific alignment of sites were required for a cooperative interaction between bound proteins. As we will show, the region between GBS1 and GBS2 includes a transcription repression sequence, ATTATCAA (see Table 5). Since DEL(3-4) and INS(4-3) disrupt this site, the results obtained might be explained by the combined effects of eliminating both a repression site (increasing activation) and a cooperative interaction of activators (decreasing activation). Lacking a convincing reduction in activation, these experiments cannot be interpreted to either eliminate or validate the hypothesis that GCN4 molecules bound to GBS1 and GBS2 interact.

Overproduction of GCN4 protein leads to increased activation by GBS1 and GBS2. An in vivo test for interaction of GCN4 at the GBSs is to assess the effect changes in in vivo concentration of GCN4 have on the level of activation. GCN4 is the activator responsible for inducing the expression of a battery of genes of amino acid biosynthesis (2, 39). It is expressed at a low constitutive level; starvation for any of several amino acids increases the translation of the *GCN4* mRNA, thereby increasing steady-state levels of GCN4 protein (for a review, see reference 38). Maintaining constitutively low-level translation of *GCN4* mRNA depends upon several negatively acting gene products; a mutation in one of these, *GCD1*, also greatly increases *GCN4* translation. A third way to increase GCN4 expression is to introduce an allele of *GCN4* which is insensitive to *GCD1*-dependent repression of translation. If GCN4 binding is responsible for activation by GBS1 or GBS2, increasing the concentration of GCN4 should increase activation just as overproduction causes increased activation of the genes under general amino acid control.

TABLE 3. GCN4 stimulation of DASI-dependent activation requires GBS1 and GBS2

Plasmid[a]	β-Galactosidase activity (U[b])	
	H1534 (*GCD1*)[c]	F98 (*gcd1*)[c]
pST1	4	3
pST1-DASI	240	2,300
pST1-DASI(SCN2)	14	15
pST1-DASI(SCN5)	35	130
pST1-DASI[DEL(3-4)]	82	2,000
pST1-DASI[INS(4-3)]	290	2,700
pFN8x-n (*HIS4*::*lacZ*)	700	5,600
pLGΔ312 (*CYC1*::*lacZ*)	270	160

[a] All DASI oligonucleotides are present in two copies.
[b] See footnote *a* to Table 2.
[c] Strains into which the plasmids were introduced by transformation.

Isogenic *GCD1*$^{+}$ (H1534) and *gcd1* (F98) strains, which produce low and high amounts of GCN4, respectively, were tested for their effects on the wild-type and mutant forms of DASI. Overproduction of GCN4 in a *gcd1* background caused a 10-fold increase in activation by two tandem copies of the wild-type DASI oligonucleotide (Table 3, compare columns 1 and 2). Activation of a control *his4*::*lacZ* fusion was increased eightfold as expected, and activation of the heterologous *CYC1*::*lacZ* fusion actually decreased slightly. The amount of activation of the SCN2 and SCN5 linker-scanning mutations was very low in either background, but expression of the 6-bp deletion, DEL(3-4), and insertion, INS(4-3), was similar to that of the wild type. Starvation for histidine, and thus derepression of *GCN4*, can be induced in a *HIS*$^{+}$ strain by exposing it to 3-aminotriazole, a competitive inhibitor of the *HIS3* gene product. Treating the H1534 transformants with 3-aminotriazole caused a sevenfold increase in activation by two tandem copies of the DASI oligonucleotide, caused a twofold increase in activation of the *his4*::*lacZ* fusion, and caused no effect on the *CYC1*::*lacZ* fusion (data not shown). These data are consistent with the DASI oligonucleotide being the target of GCN4, as is the *HIS4* promoter. Alternatively, it is possible that the oligonucleotide is targeted by a distinct factor whose expression is also controlled by starvation through the *GCD1* product. To confirm that the effect is related to concentration of GCN4, we transformed a *GCD1 GCN4* strain, PF387-1D, with both the *URA3*-based vectors carrying the promoter activation reporter constructs and a *TRP1*-based plasmid carrying a constitutively overproducing allele of *GCN4*, pRS414-GCN4 (see Materials and Methods). In this case activation by two tandem copies of the DASI oligonucleotide increased threefold, as did activation of the *his4*::*lacZ* fusion (data not shown). The simplest explanation of these data is that GCN4 binds to GBS1 and GBS2 to activate transcription of adjacent promoters.

The GBSs are recognized by GCN4 in vitro. Though the genetic evidence strongly supports the idea that DASI is targeted by GCN4 to activate transcription, it is still conceivable that the effect of GCN4 is indirect, for example, they might be targeted by a distinct factor whose expression is induced by GCN4. One argument in favor of this conclusion is that the putative GCN4 binding sites (GBS1 and GBS2) are diverged from the canonical GCN4 site and are more similar to GCN4 half-sites. We cannot conclude that the sites are targeted by GCN4 without in vitro evidence. We therefore attempted to determine if GBS1 and GBS2 could bind GCN4 by a gel mobility shift assay (25, 27). We obtained a preparation of a truncated form of GCN4, corresponding to the 54 C-terminal residues of GCN4, which includes the entire bZIP DNA binding domain of GCN4 (1, 46, 74, 78). A similar 60-amino-acid peptide binds to DNA specifically, as judged by both gel mobility shift and footprinting assays (74).

The GCN4 bZIP region (GCN4-bZ) bound to an oligonucleotide encompassing DASI (Fig. 3a). In these experiments, GCN4-bZ is always in excess over the target oligonucleotide. When the concentration of GCN4-bZ is much greater than the dissociation constant (K_d), essentially all of the target is bound (Fig. 3a, lane 8). This indicates that all of the oligonucleotide present are capable of binding to GCN4-bZ, and therefore, for the purposes of calculating dissociation constant (see Materials and Methods), half-maximal binding is defined as binding half of the DNA in the reaction mixture. At low concentrations of GCN4-bZ a single complex with DASI was observed; at high concentrations, a second, more slowly migrating complex occurred. This is consistent with GCN4-bZ binding at low concentrations to one GBS and at higher concentrations binding to both sites simultaneously. Half-maximal binding of the first molecule of GCN4-bZ occurs at between 2 and 5 ng of protein at 30°C, corresponding to a dissociation constant, K_d, of between 15 and 38 nM for the 6,547-Da protein. The fact that the concentration giving half-maximal binding equals the equilibrium dissociation constant was first determined for the *lac* repressor protein (64) and has been used routinely since, particularly in gel mobility shift experiments (9). Since half-maximal binding of GCN4-bZ to GBS2 (visualized as the formation of a second, more slowly migrating complex) didn't occur with even the highest amount (300 ng) of protein, the apparent K_d of GCN4-bZ for GBS2 appears to be greater than 2 μM. For purposes of comparison we measured binding of GCN4-bZ to the canonical GCN4 binding site. Binding to this oligonucleotide results in a single complex with an apparent K_d also between 15 and 38 nM (Fig. 3a, lanes 9 to 16). This result suggests that GBS1 and the consensus GCN4 binding site are roughly equivalent in affinity. Excess unlabelled oligonucleotide of either site specifically inhibited binding to both oligonucleotides (data not shown). The mobility-shifted species were sensitive to mutations which eliminate the GBS1 and GBS2 motifs (Fig. 3b). An oligonucleotide retaining only GBS1 (the SCN5 oligonucleotide [Fig. 3b, lanes 1 to 8]) binds with a K_d roughly equivalent to the high-affinity complex on the wild-type oligonucleotide. Thus, the K_d for binding to GBS1 is about ≤38 nM. The SCN2 mutation mutates GBS1 and eliminates this high-affinity binding; however, the SCN2 oligonucleotide still shows two shifted species (Fig. 3b, lanes 9 to 16). The higher-mobility species binds with a K_d of between 304 and 760 nM, and a second, lower-affinity species has a K_d of greater than 2 mM. The SCN2/SCN5 double mutant oligonucleotide retains only the higher affinity of these complexes (K_d, ≤760 nM) (Fig. 3c, lanes 1 to 8). We conclude that GBS2 corresponds to the complex with a K_d of ≥2 mM and that the SCN2 mutation both eliminates GBS1 and creates a lower-affinity binding site (K_d, ≤760 nM). Inspection of the sequence of GBS2

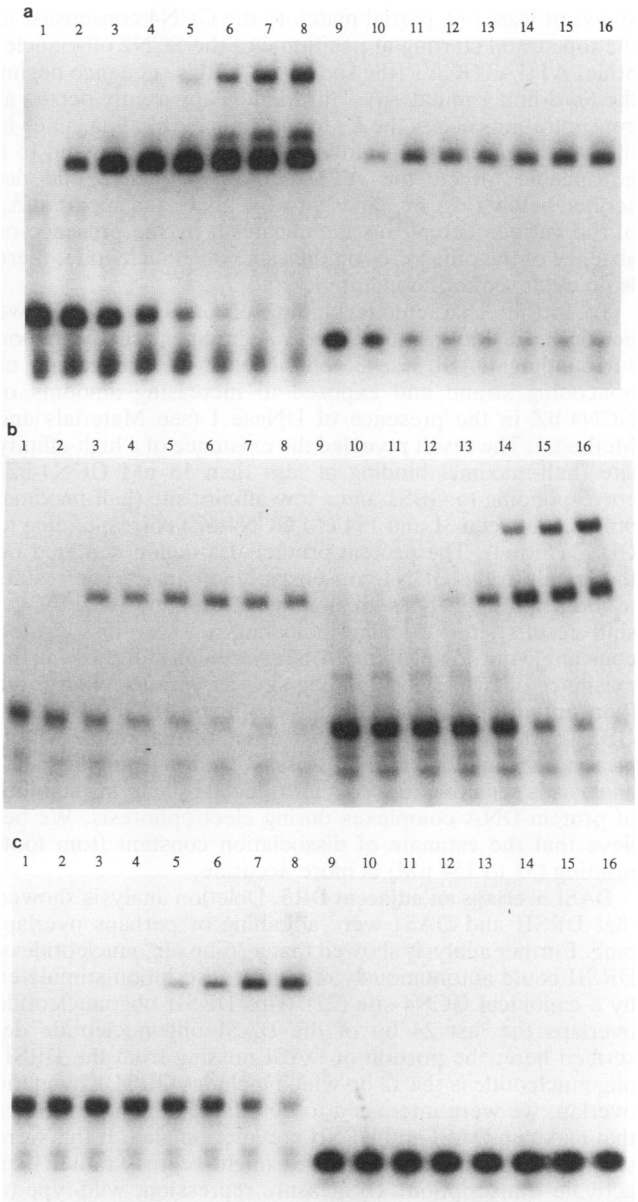

FIG. 3. Gel mobility shift analysis of GCN4-bZ. (a) Binding of GCN4-bZ to the DASI-GS (lanes 1 to 8) or UAS_{GCN4}-GS (lanes 9 to 16) oligonucleotides was analyzed as described in Materials and Methods. Bands at the bottom of the gel correspond to unbound DNA. (b) Binding of GCN4-bZ to an oligonucleotide retaining only GBS1 [DASI (SCN5), lanes 1 to 8] or only GBS2 [DASI (SCN2), lanes 9 to 16]. (c) Binding of GCN4-bZ to an oligonucleotide lacking both GBS1 and GBS2 [DASI(SCN2/SCN5), lanes 1 to 8] or to an unrelated oligonucleotide (ATTATCAA [Table 1], lanes 9 to 16). Each panel demonstrates the effects of increasing amounts of GCN4-bZ as follows: lanes 1 and 9, no protein; lanes 2 and 10, 2 ng; lanes 3 and 11, 5 ng; lanes 4 and 12, 10 ng; lanes 5 and 13, 40 ng; lanes 6 and 14, 100 ng; lanes 7 and 15, 200 ng; lanes 8 and 16, 300 ng. Each increment of 1 ng of protein in the 20-μl reaction corresponds to an increment in concentration of 7.6 nM.

shows at least one partial match to the GCN4 consensus: in the top strand starting at position 2 of the SCN2 oligonucleotide, ATGATGCAT (the sixth base of this sequence begins the *Sph*I linker mutation). This binding apparently occurs at concentrations below the K_d for nonspecific binding, since in the same concentration range GCN4-bZ does not bind to a nonspecific target, the ATTATCAA oligonucleotide described below (Fig. 3c, lanes 9 to 16). Since the apparent K_d of the various complexes is unchanged by the presence or absence of the other sites on the same oligonucleotide, there is no evidence for cooperativity.

To further characterize binding of GCN4 to DASI, we performed a footprinting assay. A fragment of the region surrounding DASI was 3′ end-labelled on the coding or noncoding strand and exposed to increasing amounts of GCN4-bZ in the presence of DNase I (see Materials and Methods). The result revealed the existence of a high-affinity site (half-maximal binding at less than 15 nM GCN4-bZ) corresponding to GBS1 and a low-affinity site (half-maximal binding between 31 and 154 nM GCN4-bZ) corresponding to GBS2 (Fig. 4). The protein protected a region centered on the match to the GCN4 consensus binding site. The results of footprinting analysis are somewhat different from the gel shift results, in particular indicating a lower dissociation constant (31 to 154 nM) for GBS2. Although still showing the existence of two GCN4 binding sites of various affinity, the footprint results suggest that the affinity of GCN4-bZ for GBS1 and GBS2 are within an order of magnitude. Footprinting is far more rapid an assay, and gel mobility shift analysis is sensitive to errors introduced by varying stability of protein-DNA complexes during electrophoresis. We believe that the estimate of dissociation constant from footprinting (31 to 154 nM) is more accurate.

DASI overlaps an adjacent DRS. Deletion analysis showed that DRSII and DASI were adjoining or perhaps overlapping. Further analysis showed that a 36-bp oligonucleotide of DRSII could autonomously repress transcription stimulated by a canonical GCN4 site (22). This DRSII oligonucleotide overlaps the last 24 bp of the DASI oligonucleotide described here; the portion of DASI missing from the DRSII oligonucleotide is the 12 bp which includes GBS1. Given the overlap, we were interested to determine whether the sites that make up DASI and DRSII are intermingled. If this were so, then some mutants of DASI might repress rather than activate transcription. To measure repression, wild-type or mutant DASI oligonucleotides were inserted upstream of a UAS_{GCN4} within the polylinker region of the plasmid pST2 as described in Materials and Methods. The naive pST2 UAS-less promoter initiated little transcription (Table 4). Addition of a UAS_{GCN4} caused a 24-fold increase in transcription in a wild-type background and an 85-fold increase in a *gcd1* strain derepressed for GCN4 expression. Addition of the DASI oligonucleotide adjacent to UAS_{GCN4} increased transcription in either background. The increase in the *gcd1* background was less than expected. Reiteration of UAS_{GCN4} leads to very high levels of expression (data not shown) comparable to the increase caused by reiteration of the DASI oligonucleotide (see line 1 of Table 2). It is not immediately clear why combining UAS_{GCN4} and DASI results in so little synergy in the *gcd1* strain. The presence of DRSII-derived repression sites might explain this effect if their presence partially blocks GCN4 binding to DASI or interferes with contacts between GCN4 and other proteins. To attempt to determine whether DASI includes such repression sites, six linker-scanning mutant DASI sites were cloned in single copies upstream of UAS_{GCN4}. The strongest

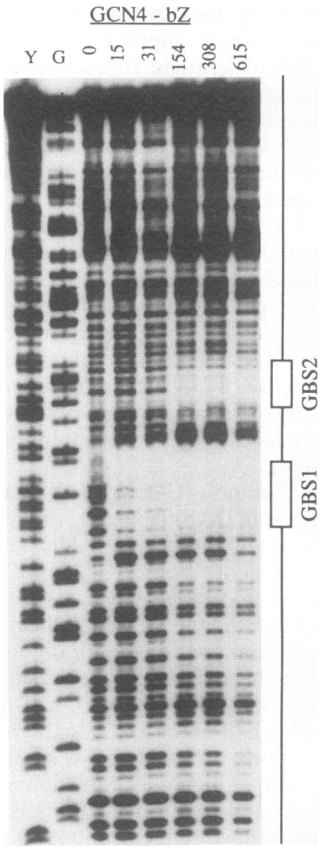

FIG. 4. Footprinting of GBS1 and GBS2 by GCN4-bZ. Footprint analysis was performed on a fragment encompassing the DASI/DRSII region 3′ end labelled at either end. Shown are the results from footprinting on the noncoding strand (the bottom strand in Fig. 5). The first two lanes are sequence markers created by Maxam-Gilbert sequencing of the labelled fragment (55); Y, cleavage at cystosine and thymine; G, cleavage at guanine. Increasing GCN4-bZ was added to the final concentrations (nanomolar) shown. The position of GBS1 and GBS2 are indicated by the labelled boxes beside the sequence.

evidence that DASI includes negatively acting sites was provided by the effect of the SCN1, SCN2, and SCN5 mutants in the *gcd1* strain. Inserting a GBS1 mutant oligonucleotide (SCN2) repressed GCN4-activated transcription 44-fold. The SCN5 mutant DASI oligonucleotide, targeting GBS2, caused 3.3-fold repression. The other linker-scanning mutant oligonucleotides had little or no effect.

The DASI linker-scanning mutant oligonucleotides can be combined via the inserted restriction site to create a set of internal deletions of DASI. These deletion oligonucleotides were inserted upstream of UAS_{GCN4}. Any deletion which removed all or part of GBS1 was highly repressing in combination with UAS_{GCN4} (data not shown). Deletions affecting the 5′ half of DASI, and thus removing GBS2, had a paradoxical effect on expression. Rather than reducing or eliminating expression, as might have been expected for mutants removing this activation site, expression increased to levels far above the undeleted DASI control. Two such deletions are shown in Table 4. An oligonucleotide retaining the region upstream of GBS2, DEL(5-6), induced 3.6-fold-more expression (690 versus 190 U) in the *gcd1* strain than did the wild-type oligonucleotide. An oligonucleotide retain-

TABLE 4. DASI includes sites capable of repressing UAS_{GCN4}

Plasmid[a]	β-Galactosidase activity (U[b])	
	H1534 (*GCD1*)[c]	F98 (*gcd1*)[c]
pST2 (no insert)	1.0	1.3
UAS_{GCN4}	24	110
DASI-UAS_{GCN4}	130	190
SCN1-UAS_{GCN4}	19	2.5
SCN2-UAS_{GCN4}	0.2	2.4
SCN3-UAS_{GCN4}	38	130
SCN4-UAS_{GCN4}	98	200
SCN5-UAS_{GCN4}	16	34
SCN6-UAS_{GCN4}	130	180
SCN2/SCN5-UAS_{GCN4}	0.3	1.2
DEL(5-6)-UAS_{GCN4}	150	690
DEL(4-6)-UAS_{GCN4}	330	2600

[a] All plasmids are based on pST2 and carry insertions of a UAS_{GCN4} oligonucleotide or UAS_{GCN4} and wild-type or mutant DASI oligonucleotides as indicated. All oligonucleotides are present in one copy.
[b] See footnote *a* to Table 2.
[c] Strains into which the plasmids were introduced by transformation.

ing only the 5′ half of DASI, DEL(4-6), stimulated 13.7-fold-more expression (2,600 versus 190 U). The fact that linker-scanning mutant oligonucleotides lacking GBS1 or GBS2 repressed UAS_{GCN4} activation and that deletion of the 3′ half of DASI led to greatly increased activation of expression suggests that the 3′ half of DASI includes one or more repressing sites.

One deletion in particular was particularly informative. DEL(1-5), a deletion which retains only the last 6 bp of DASI, caused 27-fold repression of UAS_{GCN4} in a *GCD1* strain and at least a 220-fold repression in a *gcd1* (compare lines 1 and 2 in Table 5). This 6-bp sequence must encode a strong transcriptional repression site; in fact, in the *gcd1* background we were unable to measure any expression over background, suggesting that UAS_{GCN4} may be completely silenced by this repression site. Removal of this site in DEL(5-6), described above, would account for the increase in expression seen. DEL(4-6) removes the 6 bp upstream of GBS2. We noted that this 6 bp targets a sequence, 5′-ATTATCAA-3′, which is present in two copies in DRSII, spaced 21 bp, or two helical turns, apart; the distal repeat is in the first 12 bp past DASI. We hypothesized that this site also constituted a transcriptional repression element of DRSII and that its removal accounted for the increase in expression in DEL(4-6) compared with that of DEL(5-6). We inserted one copy of this site upstream of UAS_{GCN4}; in one orientation ($ATTATCAA_{1R}$) the site caused 2.9-fold repression in *GCD1* and 5.5-fold repression in *gcd1*, while in the opposite orientation ($ATTATCAA_{1A}$) it had little effect (Table 5). Tandem duplication of this oligonucleotide had little effect on the first orientation but revealed that the other orientation ($ATTATCAA_{1A}$) also repressed from 2.9-fold (*GCD1*) to 4.6-fold (*gcd1*). Thus, DASI includes two repression elements which are parts of DRSII: one strongly and one weakly repressing site.

It is odd that though deletions of one or both of these repression sites caused significant increases in DASI-stimulated expression, neither of the linker-scanning mutations of the sites cause any increase in expression. In particular, why does DEL(5-6) and not SCN6 increase expression, although both eliminate the strong repression site, even though DEL(5-6) also eliminates the activator GBS2? The answer may be that DEL(5-6) also affects the weak repression site, altering the last A in ATTATCAA. The expression of DEL(5-6) intermediate between SCN6 and DEL(4-6) may reflect the partial inactivation of ATTATCAA. That conclusion conforms with the fact that neither SCN4 nor SCN6 increases activation by DASI, since it would appear to take a mutation of both repression sites to increase expression. If the two sites are redundant, acting in parallel to repress GBS1 and GBS2, then neither SCN4 not SCN6 would have any effect, as observed.

These sequence elements are intermingled with the GBSs of DASI as shown in Fig. 5. We presume that the net effect of DASI on transcription depends on the interplay between these positively and negatively acting elements and upon the physiological control of the expression or activity of the proteins. The nature of the proteins which bind to the negative elements, or their physiological control, is not known but is under investigation.

DISCUSSION

An unusual GCN4 activation site is intermingled with a complex transcriptional repression site. Transcriptional regulation of the retrotransposon Ty2-917 is complex, involving multiple positive and negative elements both upstream and downstream of the start site of transcription. Here we describe the detailed analysis of a cluster of sites which are located within the *TYA2* coding frame 375 bp downstream of the transcriptional initiation site. This region corresponds to two sites which have opposite effects on transcription, DASI and DRSII. Both of these sites regulate transcriptional

TABLE 5. Two sites flanking GBS2 can repress UAS_{GCN4}

Plasmid[a]	H1534 (*GCD1*)[b]		F98 (*gcd1*)[b]	
	β-Galactosidase activity (U[c])	Repression (fold)[d]	β-Galactosidase activity (U[c])	Repression (fold)[d]
UAS_{GCN4}	24		110	
DEL(1-5)-UAS_{GCN4}	0.9	27	≤0.5	≥220
$ATTATCAA_{1A}$-UAS_{GCN4}	25	0.96	75	1.5
$ATTATCAA_{2A}$-UAS_{GCN4}	10	2.4	24	4.6
$ATTATCAA_{1R}$-UAS_{GCN4}	8.2	2.9	20	5.5
$ATTATCAA_{2R}$-UAS_{GCN4}	9.7	2.5	11	10

[a] All plasmids based on pST2. They carry UAS_{GCN4} with or without the oligonucleotides corresponding to the indicated putative negative elements derived from DASI. The ATTATCAA oligonucleotide is present in either of two orientations; see Materials and Methods for description.
[b] Strains into which plasmids were introduced by transformation.
[c] See footnote *a* to Table 2.
[d] Repression ratio expressed as the ratio of the expression of the UAS_{GCN4} plasmid to the indicated plasmid.

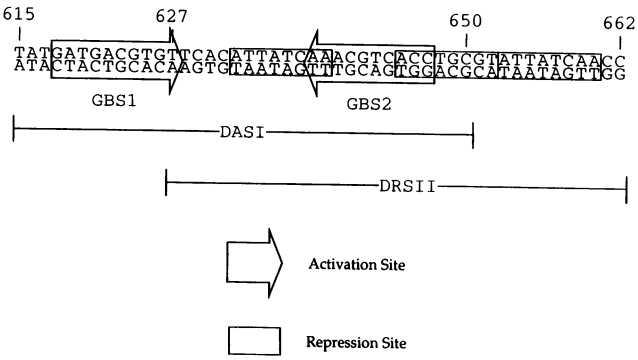

FIG. 5. Interspersion of activation and repression sites within overlapping DASI and DRSII. The sequence of the 48 bp corresponding to DASI and DRSII is given with the region of each site indicated below the sequence. The two GCN4 binding sites (GBS1 and GBS2) are indicated by the open arrows, with the direction of the arrow indicating the orientation of the imperfectly palindromic sites. The three repression sites are indicated by boxes. The repression sites are referred to in the text by the sequence of the top strand of each; the site 5′-ACCTGCGT-3′ corresponds to DASI [DEL(1-5)].

initiation independent of any other Ty2-917 sequences. Here we show that both of DASI and DRSII are complex, consisting of two and three distinct elements, respectively.

Within DASI is an inverted repeat of GBSs (GBS1 and GBS2). These two sites both bind the transcriptional activation protein GCN4. GBS1 is a high-affinity site for GCN4, binding with an apparent dissociation constant, K_d, of 15 to 38 nM at 30°C (as measured with a purified recombinant protein, GCN4-bZ, consisting of the GCN4 bZIP domain, the C-terminal 54 amino acids of GCN4). GBS1 has an affinity about that of the consensus UAS_{GCN4} (K_d, between 15 and 38 nM). GBS2 is a lower-affinity site, $K_d \geq 300$ nM. Transcriptional activation by DASI depends on both GBS1 and GBS2, and the level of activation responds to the level of GCN4 in the cell. The two sites are also not equivalent in vivo, since elimination of GBS1 has about a 10-fold-greater effect on activation than elimination of GBS2; the larger effect of GBS1 probably relates directly to its higher affinity for GCN4. The fact that the two sites are synergistic suggested that the two GCN4 dimers bound to the two sites might physically interact. Physical interaction has been inferred from evidence of stereospecificity in the placement of sites (73), since two proteins can interact if their binding sites are on the same face of the DNA helix but not if they are on opposite faces. We tested stereospecificity of the GBSs by inserting or deleting 6 bp, ca. one-half helical turn, between them. Neither mutation reduced transcriptional activation by DASI; in fact, the 6-bp insertion caused a twofold increase in activation. This conclusion suggests that GBS-bound GCN4 dimers do not interact; however, drawing a clear conclusion is not possible, since the mutations used to change spacing of the GBSs also perturb a transcriptional repression region located adjacent to GBS2, the sequence ATTATCAA. In vitro DNA binding assays with the DNA binding and dimerization domain of GCN4 (GCN4-bZ) confirm that binding to the two sites is independent. One can compare the binding of GCN4-bZ to the wild-type DASI oligonucleotide (Fig. 3a, lanes 1 to 8) to those lacking one or the other GBS or both (Fig. 3b, lanes 9 to 16, GBS1; Fig. 3b, lanes 1 to 8, GBS2; Fig. 3c, lanes 1 to 8, both) to look for effects on affinity caused by cooperative interactions between bound proteins. The apparent K_d for the various sites, GBS1, GBS2, and an artifactual site created by the SCN2 mutation, does not change when second binding sites are present. These data also suggest that there is no cooperativity in binding of GCN4-bZ to DASI.

The results of footprinting to the wild-type DASI site offer an independent assay of affinity for GBS1 and GBS2. The K_d of GCN4-bZ for GBS1 is consistent with the gel shift results: $K_d < 15$ nM. However, footprinting indicated at least 10-fold-higher affinity of GCN4-bZ for GBS2, a K_d of between 31 and 154 nM rather than ≥2 mM. Since the footprinting experiment requires the complex of GCN4-bZ with its site to be stable only for the period of DNase digestion rather than during a prolonged gel electrophoresis, the lower K_d is probably more accurate. The complex with GBS2 could be lost during electrophoresis, so conclusions about cooperativity from these experiments are probably unwarranted. Such conclusions would require that footprinting experiments be performed on templates retaining one or the other or both GBSs. The GBSs are cooperative in vivo, since mutations in either site nearly abolish DASI activation of transcription. The synergy between the two sites in vivo could reflect cooperative DNA binding, though it could also derive from interaction of both GCN4 dimers with another target, presumably the basal transcriptional machinery. By analogy with experiments done with other activators, GCN4 could contact the basal transcription factors TFIID and TFIIB, as does GAL4 (50, 71), or it could contact one or more transcriptional coactivators, as do several activators (16, 24, 75). As proposed by Ptashne (61) and Herbomel (33), cooperativity can be "promiscuous," resulting from multiple independent contacts with the transcriptional machinery.

DRSII (nucleotides 627 to 662 of Ty2-917) overlaps the distal 24 bp of DASI (nucleotides 615 to 650). We had previously shown that a DRSII oligonucleotide represses transcription activated by a UAS_{GCN4} (22). Since this transcriptional repression site overlaps DASI, we were interested in whether DASI also includes transcriptional repression sites which are elements of DRSII. We were able to show that DASI includes two repression sites of the overlapping DRSII. One is located immediately upstream of GBS2, and the other is located immediately downstream of GBS2 (Fig. 5). Initially we hypothesized that the DASI oligonucleotide included one or more repression sites, because when the GBSs were eliminated by mutation, the oligonucleotide repressed rather than activated (Table 4, line 10). Interestingly, none of the linker-scanning mutations had the phenotype expected if they eliminated a repression site. Those falling outside of GBS1 and GBS2 either decreased transcription slightly or had no effect. However, an 8-bp sequence targeted by SCN6 (ACCTGCGT) repressed UAS_{GCN4} strongly (up to 220-fold), and a second sequence targeted by SCN4 (ATTATCAA) repressed UAS_{GCN4} less strongly (up to 10-fold). This presents a conundrum. Why do linker-scanning mutations targeting these repression sites not cause transcription stimulated by DASI to increase? We can imagine two general models to explain this behavior. First, the SCN4 and SCN5 mutations affect GBS2 as well as the repression sites. It could be that the mutations simultaneously eliminate a repression site (tending to increase expression) and mutate an activation site (tending to decrease expression). Depending on the relative strengths of these effects, transcription could increase or decrease slightly or be unaffected. Second, the two sites could be redundant. Repression would not be eliminated by removing one site but would require removing both. Thus, neither

SCN4 nor SCN5 would eliminate repression. This is seemingly contradicted by the great difference in effect of the two sites, as much as a 20-fold difference in repression of the model UAS_{GCN4} promoter. However, repression of the model promoter might be mechanistically different from that occurring in DASI, even though the same activator (GCN4) is targeted. In DASI the sites overlap: ATTATCAA overlapping GBS1 by 2 bp, and ACCTGCGT overlapping GBS1 by 3 bp. It may be that the sites repress in DASI at least partly by binding proteins which occlude GCN4 binding to GBS2. In the model promoter the ATTATCAA sequence was introduced in isolation 26 or 32 bp upstream of a UAS_{GCN4}, and the ACCTGCGT sequence was introduced 8 bp upstream. In neither case do the sites overlap, and yet they repress strongly. The latter sequence may repress more efficiently because the site is much closer to the UAS_{GCN4}, or it could be an intrinsic feature of the site. An additional argument against the sites repressing by occluding the neighboring site comes from experiments in which the UAS_{GCN4} was replaced by a consensus UAS_{GAL4}; in this case neither site represses GAL4-activated transcription (data not shown). Since even the ACCTGCGT sequence located less than one helical turn from the UAS_{GAL} does not repress it, a nonspecific occlusion mechanism seems unlikely in the model promoter.

It is not clear, however, that occlusion does not play a role in determining the net effect of the multiple sites. Since GBS2 overlaps the two repression sites, it is unlikely that both GBS2 and the repression sites could be occupied simultaneously. Footprint analysis shows that in vitro virtually the entire region of DASI is protected from DNase I digestion by saturating amounts of GCN4-bZ. If this reflects the in vivo situation, binding of GCN4 would appear to occlude the repression sites making recognition by the repressing proteins impossible; conversely, binding of the repressing proteins might make recognition of GBS2 by GCN4 impossible. Activation by this region would depend on the interplay of the mutually exclusive regulatory proteins.

The DASI/DRSII region is densely packed with regulatory sites. The 36-bp DASI includes two 10-bp activation sites and two 8-bp repression sites. In addition one of these repression sites, ATTATCAA, is repeated again 21 bp downstream, in the portion of DRSII immediately downstream of DASI. As diagrammed in Fig. 5, 39 of the 48 bp in the DASI/DRSII region are part of a regulatory element, and because of overlap of sites, 44 bp of regulatory information are present. The impression of density of information in the region is increased if one considers that the same region also encodes 16 amino acids of *TYA2*.

Structural complexity, the most obvious feature of the DASI/DRSII region, is recapitulated in the larger context of the negative region. DRSII is only one of three independent repression sites. Together with DASI these four sites account for 120 bp of the 200-bp negative region. In addition our evidence suggests that a second DAS exists in the 36 bp between DRSII and DRSIII, though it has not been precisely mapped (22). In addition, preliminary data suggest that DRSIII, like DRSII, includes both activation and repression elements, though the latter are stronger and give the site its net repressive effect (77). Despite the large number of sites present, when the negative region is inserted upstream of a naive, UAS-less promoter, or a promoter activated by a UAS_{GCN4}, it has no effect on transcription. The region does have an effect on Ty2-917 transcription, causing about a 10-fold repression of the Ty2-917 enhancer when both are present in their normal downstream position. How is it that a collection of sites, all of which appear to have a significant effect on transcription in isolation, add up to a region which has no effect or a modest effect on transcription? One answer is that the system may have evolved so that the effect of the many positive and negative elements nearly balance out under normal growth conditions. This creates a highly buffered system in which loss of any one transcription regulatory site has little effect on overall expression. For an element which appears to be evolved for survival this situation results in a very stable expression, unaffected by subtle changes in physiology or by the effects of the high mutation rate of the element which could eliminate any single regulatory site during each transposition event. Such a system has evolved to respond very differently than do the various anabolic or catabolic systems in which small changes in physiology must result in large swings in transcription, for example, the switch from glucose to galactose as a carbon source resulting in a 1,000-fold increase in transcription of the *GAL1*, *-7*, and *-10* genes. Such systems are lightly buffered and many are rather simple in the molecular architecture of their promoters. The existence of this highly buffered system in Ty elements makes the analysis of their *cis*-acting control sites difficult. It also has made the elements fruitful targets for isolation of mutations which have a global effect on gene expression (for a review, see reference 6). Searches for suppressors of Ty-mediated gene expression have identified many genes which encode elements of the basal transcription machinery (17), structural elements of chromatin (11), or highly pleiotrophic effectors of gene expression, many of which also affect chromatin structure (for a review, see reference 80). In a system as buffered as is Ty transcription, suppressors appear to modify expression only by changing the basic ground rules of transcription rather than by eliminating one or another transcription factor. However, the result has been that knowledge of the factors regulating Ty transcription has been largely lacking. We hope that by focusing on individual transcriptional control sites we may begin to identify the individual factors which modulate Ty transcription and to understand the interplay between these factors at the DNA.

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