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ADA3: a Gene, Identified by Resistance to GAL4-VP16, with Properties Similar to and Different from Those of *ADA2*

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We describe the isolation of a yeast gene, *ADA3*, mutations in which prevent the toxicity of GAL4-VP16 in vivo. Toxicity was previously proposed to be due to the trapping of general transcription factors required at RNA polymerase II promoters (S. L. Berger, B. Piña, N. Silverman, G. A. Marcus, J. Agapite, J. L. Regier, S. J. Triezenberg, and L. Guarente, Cell 70:251–265, 1992). *trans* activation by VP16 as well as the acidic activation domain of GCN4 is reduced in the mutant. Other activation domains, such as those of GAL4 and HAP4, are only slightly affected in the mutant. This spectrum is similar to that observed for mutants with lesions in *ADA2*, a gene proposed to encode a transcriptional adaptor. The *ADA3* gene is not absolutely essential for cell growth, but gene disruption mutants grow slowly and are temperature sensitive. Strains doubly disrupted for *ada2* and *ada3* grow no more slowly than single mutants, providing further evidence that these genes function in the same pathway. Selection of initiation sites by the general transcriptional machinery in vitro is altered in the *ada3* mutant, providing a clue that ADA3 could be a novel general transcription factor involved in the response to acidic activators.

The activation of transcription in eukaryotes requires transcriptional activators, which are proteins that bind to sites distal from the TATA box, termed enhancers or upstream activation sequences (UASs) (4, 25, 44). These activators contain discrete activation domains that augment transcription initiation in the vicinity of the TATA box (8). The activation domains of one major class of activators are characterized by a high concentration of amino acids with acidic side chains. Acidic activation domains are found in many yeast activators, including GAL4, GCN4, and HAP4 (23, 34, 40). A protein from the virion of herpes simplex virus, VP16, contains an unusually potent acidic activation domain (54, 62). These acidic activators function in many eukaryotic cells, ranging from yeasts to mammals, indicating that their mechanism of action has been conserved in eukaryotes.

The transcription initiation reaction at the TATA box is mediated by general transcription factors, including the TATA box-binding protein (TBP). TBP was purified as a monomer from *Saccharomyces cerevisiae* (9) but was found as part of a multiprotein complex in higher cells (16, 52). Other general transcription factors include TFIIB and RNA polymerase II (42). Several findings have led to models on how activators might function to activate transcription at a distance. Disruption of histones in vivo can partially bypass the need for activators, suggesting that histone displacement may be part of the activation mechanism (30). Consistent with this model, the VP16 acidic activation domain counteracts the repressive effects of chromatin assembly on transcription templates in vitro (reviewed in reference 20). However, in the absence of chromatin assembly, robust transcriptional activation can still be observed in vitro, indicating the existence of a chromatin-independent mechanism of activation. In this mechanism, the activator is thought to contact some component of the general transcriptional machinery. Chromatographic studies show that the acidic activation domain of VP16 is capable of binding to TBP and also to TFIIB (36, 39, 60). How these interactions relate to transcriptional activation has not yet been clarified.

Other evidence suggests that protein-protein interactions between activation domains and the transcriptional machinery occur via intermediary factors termed adaptors or coactivators. One study showed that TBP-associated proteins were required in vitro for activation by nonacidic activator Sp1 (49). In another study, we showed that the acidic activation domain of VP16 titrated a factor needed for the activation of transcription in vitro by the yeast dA-dT activator (5). This factor, termed an adaptor, was thought to bridge the interaction between the dA-dT activator at the UAS and the general transcription factors at the TATA box. Under the conditions of these experiments, basal transcription from the TATA box was not affected. To observe these effects, we prevented GAL4-VP16 from binding to secondary GAL4-binding sites in the plasmid template by including a GAL4-specific oligonucleotide in the reaction. When we omitted the oligonucleotide, allowing GAL4-VP16 to bind to secondary binding sites in the plasmid DNA, basal transcription was also inhibited. Evidently, when GAL4-VP16 bound to the plasmid, stable complexes that sequestered the general transcription factors as well as the adaptor were established.

The challenge from the experiments described above was to isolate the putative adaptor. We developed a method to isolate mutations that might identify a gene encoding an adaptor. This method was based on the inhibition of cell growth caused by the expression of high levels of GAL4-VP16 in vivo (6). This inhibition required the integrity of both the VP16 acidic activation domain and the GAL4 DNA-binding domain, suggesting that it was due to the trapping of general transcription factors in DNA-bound complexes. If trapping of general transcription factors by GAL4-VP16 required the adaptor as a bridging factor, then mutations that inactivated the adaptor might relieve growth inhibition.

Mutations that relieved growth inhibition and that weak-

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ened the ability of GAL4-VP16 as well as yeast acidic activator GCN4 to activate transcription in vivo and in vitro have been described for gene *ADA2* (6). Strikingly, the effects of the *ada2* mutation were specific for a subset of acidic activators because the activity of yeast acidic activator HAP4 was not affected in vivo or in vitro. Basal transcription in the *ada2* mutant extract was indistinguishable from that in the wild-type extract, both in terms of levels and relative utilization of the two strong *CYC1* TATA boxes (6). From these findings, we concluded that *ADA2* was likely to encode an adaptor or part of an adaptor complex used by a subset of acidic activators.

The selection that yielded mutations in *ADA2* also identified two other genes, *ADA1* and *ADA3*. *ada1* mutations reduced the synthesis of GAL4-VP16 and reduced the activity of many yeast promoters in vivo. *ada3* mutations, however, did not reduce the synthesis of the inhibitor. In this report, we present findings suggesting that ADA3 could be a factor in the general transcriptional machinery that ultimately receives the activation signal from transcriptional activators.

MATERIALS AND METHODS

Strains and plasmids. Assays were carried out with strain BP1 (*MAT***a** *gal4::HIS4 ura3-52 leu2-2,112 his4-519 ade1* [6]) and its *ada3* mutant derivative. The *ada3* mutation was shown in crosses to confer all of the mutant phenotypes described. *ADA3* disruption was performed with PSY316 (*MAT*α *ade2-101* Δ*his3-200 leu2-3,112 lys2 ura3-53*). Plasmids expressing GAL4-VP16 and GAL4-VP16FA (FA represents the change from Phe-442→Ala) in *S. cerevisiae* (2μm origin; *ADH1* promoter) have been described elsewhere (6). Plasmid YCp88-GCN4, expressing LexA-GCN4 (*DED1* promoter), and the LexA reporter plasmid YEp21-Sc3423 (34) were generously provided by K. Struhl. Transformations were performed by the lithium acetate method (58). β-Galactosidase assays were carried out with glass bead-generated extracts as described previously (53). General yeast manipulations were performed as described by Guthrie and Fink (26) with slight modifications.

Gel retardation assays. Whole-cell extracts were prepared and gel shift assays were performed as described previously (6).

Cloning and sequencing of *ADA3*. The *ada3* single-mutant strain was transformed with a yeast genomic library on an ARS-CEN vector (a generous gift from Craig Thompson in Rick Young's laboratory), and faster-growing colonies were isolated on minimal medium with 2% glucose. Two different clones with overlapping inserts were isolated and checked for their ability to restore growth and sensitivity to GAL4-VP16 when retransformed into the *ada3* mutant strain. The smaller of the two clones (2112.3; 6.5 kb) was used for further characterization. A 2.5-kb *Hin*dIII-*Eco*RV fragment was subcloned into pRS316 (59) and used for preparing nested deletions (Erase-a-Base; Promega) and sequencing of the gene (Sequenase; United States Biochemical Corp.). A clone sufficient for complementation was obtained by subcloning a 2.2-kb *Hin*dIII fragment into the *Hin*dIII site of the above-described subclone. Sequences upstream of the *Hin*dIII site in *ADA3* were sequenced with custom-made oligonucleotides. DNA sequencing identified an open reading frame (ORF) of 702 codons. This ORF was isolated on a DNA fragment generated by a polymerase chain reaction (PCR) and subcloned into yeast expression plasmid pDB20LB (6; made by B. Turcotte). The subclone fully complemented the *ada3* mutation.

Construction of LexA fusions. A cloning vector containing the DNA-binding and dimerization domains of LexA, lexA202, was constructed by a PCR. Primers were synthesized such that lexA202 was amplified with flanking *Hin*dIII sites as well as a *Not*I site at the C terminus. This PCR product was cut with *Hin*dIII and ligated into pDB20 (21), which had been cut with *Hin*dIII and treated with calf intestinal alkaline phosphatase (CIAP). This process created a 2μm *URA3* vector expressing lexA202 from the ADH promoter followed by the ADH terminator (pADH-lexA202). The various activation domains were then amplified by a PCR with oligonucleotides that created flanking *Not*I sites in the proper reading frame. The domains amplified were as follows: GAL4, amino acids 769 to 881; GCN4, amino acids 9 to 172; and HAP4, amino acids 330 to 554. These amplified domains were digested with *Not*I and ligated into the above-described pADH-lexA202 vector, which had been cut with *Not*I and treated with CIAP. For creation of ARS-CEN expression vectors, all constructs were digested with *Bam*HI, releasing the PADH-LexA-acidic activation domain-TADH fragment. This fragment was then ligated into pRS316, which had been cut with *Bam*HI and treated with CIAP. These constructs were then transformed into the appropriate yeast strain with plasmid YEp21-Sc3423, a LexA reporter plasmid (34).

***ADA3* gene disruption.** The *Bam*HI-*Bgl*II fragment from pNYK51 (1), containing the *URA3* gene inserted between two copies of the *Escherichia coli hisG* gene, was introduced into a *Hin*dIII-*Eco*RV fragment from the genomic *ADA3* clone (subcloned in Bluescript KS+) in place of the *Bgl*II fragment that spans codons 273 to 307. The resultant construct, pΔA3-i, was integrated into the PSY316 genome as a *Sal*I-*Sac*I fragment. *URA3*$^+$ transformants were screened for resistance to GAL4-VP16 and slow growth on minimal medium. Such transformants were recovered and treated with fluoroorotic acid (1) to select for the deletion of *URA3* to yield yBPα91f.

In vitro transcription. In vitro transcription reactions were carried out with nuclear extracts as described previously (6). GAL4-VP16, GAL4-HAP4, and GCN4 proteins were prepared as described previously (6) and donated by S. Triezenberg.

Sequence analyses. DNA and predicted protein sequence analyses were performed at the Massachusetts Institute of Technology Computer Center at Whitaker College by use of the GCG package. Sequence searches of the GenBank and EMBL data banks and the PIR and TFDAA protein banks were performed by use of the algorithm of Altschul et al. (2) at the National Center for Biotechnology Information by use of the BLAST network service.

Nucleotide sequence accession number. The GenBank accession number of the *ADA3* sequence is L21189.

RESULTS

Properties of the *ada3* mutant. We found a single allele of *ada3* among mutations that led to resistance to growth inhibition by GAL4-VP16. In these experiments, the gene encoding GAL4-VP16 was borne on a multicopy 2μm plasmid and was under the control of the *ADH1* promoter (6). Two findings indicated that the *ada3* mutant did not resist toxicity by reducing the synthesis of GAL4-VP16. First, the activity of the *ADH1* promoter, as assayed by Northern (RNA) blotting of the chromosomal *ADH1* gene, was not

affected in the mutant. Second, when challenged with GAL4-VP16 under the control of a different promoter, *CYC1* UAS2UP1, a site activated by HAP2/3/4, the mutant was resistant to toxicity.

We surmised that the *ada3-1* mutation reduced the functional activity of the VP16 activation domain. In testing whether the *ada3* mutant supported a reduced level of *trans* activation by GAL4-VP16, we took two measures to prevent the toxicity of the activator in the parental control strain. First, we moved GAL4-VP16 to a single-copy plasmid to reduce the levels of its synthesis. Second, we used a single amino acid change of Phe-442→Ala (FA) in the full-length VP16 activation domain (12, 51). The mutant protein *trans* activates to a significant degree in yeast cells but is not nearly as toxic as the wild-type protein (6, 12). The activity from a reporter driven by this activator protein was reduced about threefold in the *ada3* mutant compared with the activity in the parent. In this experiment, isogenic strain BP1 and the *ada3* mutant were transformed with reporter plasmid pLGSD5 (25), bearing the *GAL* UAS and *CYC1* TATA boxes driving the expression of *CYC1-lacZ*, as well as an ARS-CEN plasmid in which the synthesis of GAL4-VP16FA was driven by the *ADH1* promoter (6). β-Galactosidase activity was assayed by glass bead disruption of cells, and units are expressed per milligram of protein, as follows: wild type (control), 240; wild type (GAL4-VP16FA), 7,300; and *ada3* (GAL4-VP16FA), 2,700. The levels of GAL4-VP16FA present in the two strains were quantitated by gel shift analysis, and very similar levels of GAL4-VP16 and several degradation products of the fusion protein were found in the two strains (Fig. 1). We concluded, therefore, that the ability of GAL4-VP16 to activate transcription was substantially reduced by the *ada3-1* mutation.

Interestingly, the *ada3* mutant strain bore another property similar to that of the *ada2* mutant strain. Cell growth was severely limited on minimal medium in the absence of any GAL4-VP16 but was only modestly affected on rich medium. Furthermore, like the *ada2* mutant, the *ada3* mutant did not grow at 37°C on any medium. On the basis of the resistance to GAL4-VP16 and this growth phenotype, it seemed possible that *ADA2* and *ADA3* perform related functions in yeast cells. To investigate further the similarity between the *ada3* and *ada2* mutants, we determined the effects of the *ada3* mutation on several yeast promoters containing the *CYC1* TATA box-mRNA region and different UAS elements. In these reporter assays (Table 1), strong defects in the range of 10-fold were observed when we used two promoters that were activated by yeast activator GCN4. One promoter bore a fragment of *HIS4* DNA as a UAS, and the other bore two synthetic GCN4-binding sites. Promoters that were driven by HAP1 or HAP2/3/4 were also affected by the *ada3* mutation, but to a much lesser degree. The *ada2* and *ada3* mutants are similar, therefore, in that the strongest effects were observed with reporters driven by GCN4.

Cloning and sequencing of the *ADA3* gene. We used the slow-growth phenotype of the *ada3* mutant on minimal medium to clone the gene from ARS-CEN libraries. Clones that not only reversed the slow growth of the mutant but also restored sensitivity to the toxicity of GAL4-VP16 were isolated, verifying that these two phenotypes were due to the same mutation. Two of these clones were analyzed and found to bear inserts that overlapped by about 6 kb. By deleting portions of the inserts, we narrowed the complementing region to a 3-kb fragment and DNA sequencing identified an ORF of 702 codons (Fig. 2).

We obtained further evidence that the 702-codon ORF

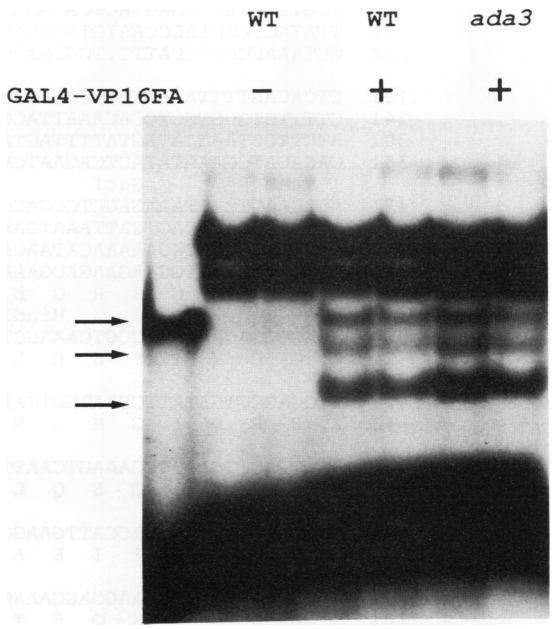

FIG. 1. Gel shift analysis of GAL4-VP16 in wild-type (WT) and *ada3* strains. Whole-cell extracts of BP1 (wild type) transformed with pDB20L (−) or with the GAL4-VP16 expression plasmid (+) and of the *ada3* mutant were made in duplicate and mixed with the GAL4-specific nucleotide 5′-CGGAGGGCTGTCAACCCG-3′ (6). Purified GAL4-VP16 (leftmost lane [5]) was used to show the mobility of the correct complex. Complexes in the control, not bearing the expression plasmid (−), were found and corresponded to cellular proteins not related to GAL4-VP16. Arrows indicate GAL4-VP16-specific complexes, the slowest one corresponding to intact GAL4-VP16. Two faster complexes corresponded to partially degraded GAL4-VP16 molecules. No difference either in the overall amounts of the complexes or in the proportions of degraded species were evident between the wild type and the *ada3* mutant.

encoded *ADA3* in a complementation experiment involving a gene disruption generated by the clone. To generate a gene disruption, we constructed a vector containing the *hisG-URA3-hisG* cassette (1) inserted between the *Bgl*II sites at codons 273 and 307 in the ORF. This cassette generated haploid Ura$^+$ transformants that were viable but grew more slowly on minimal medium than the wild-type parental strain. These transformants were temperature sensitive,

TABLE 1. Assays with various reporters containing the *CYC1* TATA boxes and *CYC1-lacZ* and the indicated UAS inserted at the *Xho* site at −178 (6)[a]

UAS	Activator	β-Galactosidase activity in strain:		*ada3* mutant/wild type
		Wild type	*ada3* mutant	
UAS1	HAP1	680	440	0.6
UAS2UP1	HAP2/3/4	320	170	0.5
HIS4 fragment	GCN4	300	40	0.1
HIS4 oligonucleotide	GCN4	60	5	0.08

[a] The reporters used for these assays were the following: UAS1, pLGΔAluXho (24); UAS2UP1, pLG265UP1 (23); *HIS4* fragment, *HIS66* (33); and *HIS4* oligonucleotide, *HIS14X2* (33). The activator known to act at each UAS is indicated. β-Galactosidase activity was assayed in permeabilized cells, and units are expressed per unit of optical density at 600 nm.

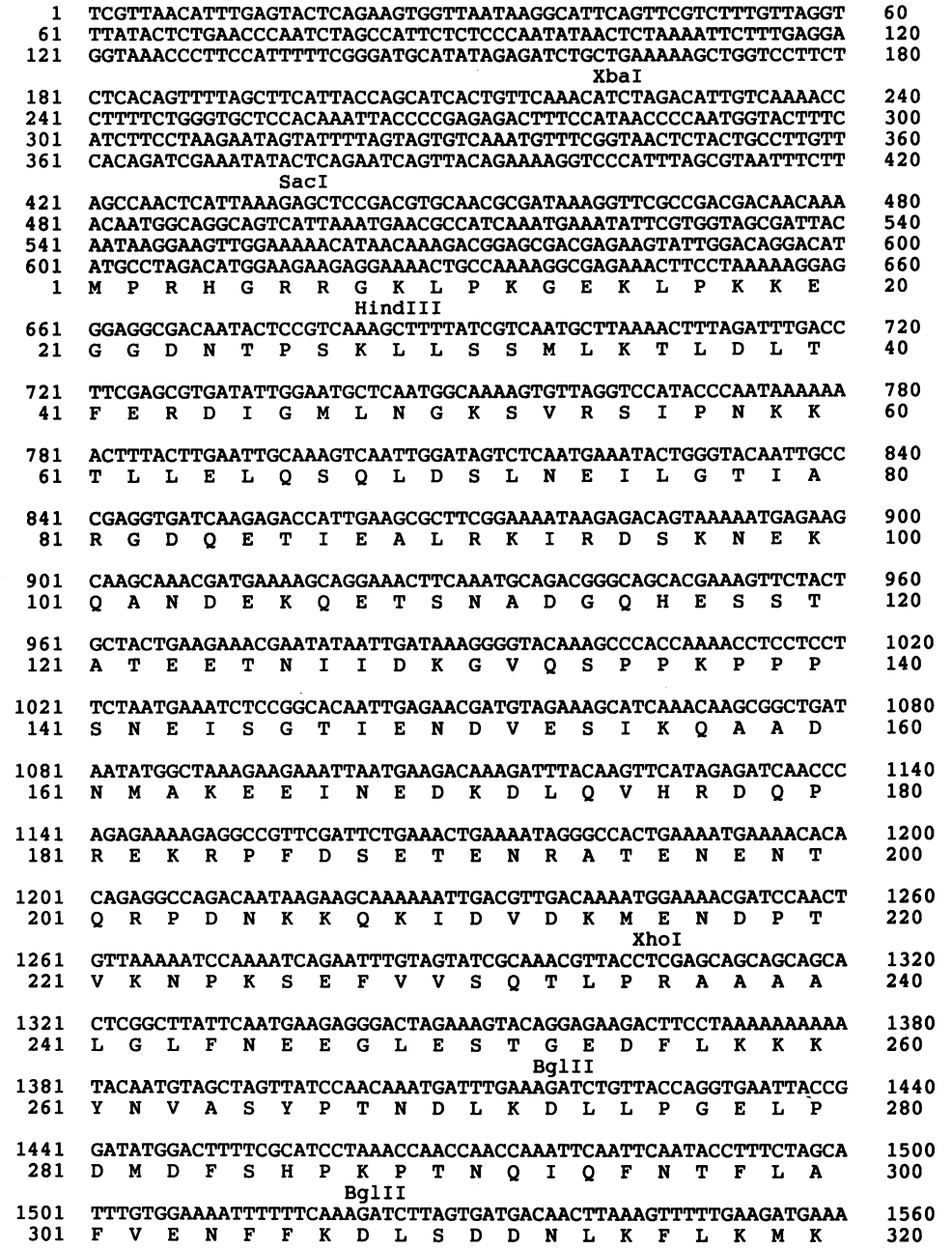

FIG. 2. Sequences of the *ADA3* gene and of the corresponding 702-codon ORF. Relevant restriction sites are shown. Extra flanking sequences revealed that the *UBC1* gene (57) lies past the 3′ end of *ADA3* and in the same orientation as *ADA3* (data not shown).

failing to grow at 37°C. Furthermore, the disrupted strain was resistant to GAL4-VP16. The disrupted strain was crossed with a strain bearing the original *ada3* mutation or with the isogenic *ADA3* parental strain. The diploid with the genotype Disruption/*ada3* grew very slowly on minimal medium, while the diploid with the genotype Disruption/+ grew at the same rate as +/+. Also, the original *ada3* mutant strain was crossed with a strain in which *URA3* had been integrated at the *ADA3* locus via a plasmid bearing the entire *ADA3* gene. Tetrads from the diploid were dissected, and in all five tetrads, Ura^+ and slow growth segregated in opposition. Therefore, the cloned gene corresponds to the same complementation group as the original *ada3* mutation. The viability of the disrupted strain suggests that *ADA3* is not required for growth.

A data base search indicated that the 702-codon ORF did not correspond to any known yeast gene. Furthermore, it was not an obvious homolog of any gene from any other source. Analysis of the *ADA3* sequence revealed three regions of possible interest. The first is a 15-amino-acid hydrophobic region (residues 292 to 306) that contains five Phe residues and that bears significant similarity to numerous proteins in the data base, including human immunodeficiency virus (HIV) Gag (Fig. 3A). The second is a 16-amino-

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1561  TATATTATACCCGATAGCTTGCAATTTGACAAAACTTATGACCCTGAGGTAAACCCGTTT   1620
 321  Y  I  I  P  D  S  L  Q  F  D  K  T  Y  D  P  E  V  N  P  F    340

1621  ATTATACCGAAACTAGGCCCTTTATACACAGATGTTTGGTTCAAAGATGAAAACGACAAA   1680
 341  I  I  P  K  L  G  P  L  Y  T  D  V  W  F  K  D  E  N  D  K    360

1681  AATTCTGCCTATAAAAAACCTTCACCATATTCAAACGATGCATCTACTATACTACCAAAA   1740
 361  N  S  A  Y  K  K  P  S  P  Y  S  N  D  A  S  T  I  L  P  K    380

1741  AAAAGTGCCAACGAACTTGATGATAATGCTTTGGAATCGGGCAGTATATCGTGTGGGCCC   1800
 381  K  S  A  N  E  L  D  D  N  A  L  E  S  G  S  I  S  C  G  P    400

1801  TTATTATCTAGGCTGTTGAGTGCTGTATTAAAAGATGACAATGACAAATCAGAATTGCAA   1860
 401  L  L  S  R  L  L  S  A  V  L  K  D  D  N  D  K  S  E  L  Q    420

1861  TCTTCTAAAATAATACGGGATGGCGGACtACCGAGAACCGGAGGGGAAGACGATATACAA   1920
 421  S  S  K  I  I  R  D  G  G  L  P  R  T  G  G  E  D  D  I  Q    440

1921  TCGTTTAGAAACAACAACAATGATACTGTAGACATGACATTATCTCAGGAGAACGGGCCC   1980
 441  S  F  R  N  N  N  N  D  T  V  D  M  T  L  S  Q  E  N  G  P    460

1981  AGTGTTCAAACACCTGACAATGACATTGATGAGGAAGCATCTTTCCAAGCAAAACTGGCA   2040
 461  S  V  Q  T  P  D  N  D  I  D  E  E  A  S  F  Q  A  K  L  A    480

2041  GAAAATAAAGGCAGTAATGGCGGTACTACAAGTACGTTACCCCAACAAATTGGGTGGATA   2100
 481  E  N  K  G  S  N  G  G  T  T  S  T  L  P  Q  Q  I  G  W  I    500

2101  ACAAATGGAATTAACCTGGACTATCCAACATTCGAAGAACGATTGAAAAGAGAACTAATG   2160
 501  T  N  G  I  N  L  D  Y  P  T  F  E  E  R  L  K  R  E  L  M    520

2161  TATGTAGGGATATACATGAATTTGCCTAAGGATGAGAACAACCCTAACTCAGATGACCCC   2220
 521  Y  V  G  I  Y  M  N  L  P  K  D  E  N  N  P  N  S  D  D  P    540

2221  GATTGGGTCACCGGTAGAGAAGACGACGAAATAAGCGCAGAGTTAAGAGAATTGCAAGGC   2280
 541  D  W  V  T  G  R  E  D  D  E  I  S  A  E  L  R  E  L  Q  G    560

2281  ACTTTAAAACAAGTGACCAAGAAAAACCAAAAGAGGAAAGCCCAATTGATTCCACTAGTG   2340
 561  T  L  K  Q  V  T  K  K  N  Q  K  R  K  A  Q  L  I  P  L  V    580

2341  GAAAGACAACTAGCATGGCAGGAATACTCATCTATTTTAGAAGATTTAGACAAACAAATC   2400
 581  E  R  Q  L  A  W  Q  E  Y  S  S  I  L  E  D  L  D  K  Q  I    600

2401  GACCAGGCTTATGTCAAACGTATTCGCGTACCCAAGAAAAGAAAGAAGCATCATACAGCA   2460
 601  D  Q  A  Y  V  K  R  I  R  V  P  K  K  R  K  K  H  H  T  A    620

2461  GCTTCAAATAATGTGAACACAGGAACCACGTCTCAAATAGCACAACAAAAGGCTGCAAAC   2520
 621  A  S  N  N  V  N  T  G  T  T  S  Q  I  A  Q  Q  K  A  A  N    640

2521  TCAAGTTTAAAATCCCTTCTGGATAAAAGGCAAAGGTGGATTAATAAGATTGGTCCATTG   2580
 641  S  S  L  K  S  L  L  D  K  R  Q  R  W  I  N  K  I  G  P  L    660

2581  TTTGATAAACCTGAAATTATGAAGAGAATCCCCAATGAAAGCGTATTCAAGGACATGGAC   2640
 661  F  D  K  P  E  I  M  K  R  I  P  N  E  S  V  F  K  D  M  D    680

2641  CAAGAAGAAGATGAGGATGAAGCCGATGTATTTGCACAAAACACTAATAAGGACGTGGAA   2700
 681  Q  E  E  D  E  D  E  A  D  V  F  A  Q  N  T  N  K  D  V  E    700

2701  CTAAATTAAAATATACGAACTCTAAGGAAAAATACGTAGCATAATAACGCATATAACTAA   2760
 701  L  N  *   702

2761  CAATTTGGATAACTATCCATAAAAAAAAAAAAAATAGTCACATAATACGAACATTAATATC   2820
2821  CTTTTTTGTTACATTTTCTTCCATCTGAAATATTTGGAGGGGCAAAAACAATGAGATGAC   2880
2881  AGTTGAATGCATGAACTTCCGATCGCGGGCGTATAGGTAAAGTCAATTGAAGCAAGTGTG   2940
2941  ACGACATAAGTATCGTAATTTAGTGGTTGGATACATTAAAAAAAACAAGTGGTATATATA   3000
3001  TAAGTAGTAGTAGTAAGAAGTAAGCGATGTCTAGGGCTAAGAGAATTATGAAAGAAATCC   3060
                                                          EcoRV
3061  AAGCTGTGAAGGATGATCCTGCAGCTCACATTACTCTTGAATTTGTGAGTGAATCTGATA   3120
3121  TC  3122
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FIG. 2—*Continued.*

acid region of ADA3 (residues 487 to 502) that is 75% identical to residues 293 to 307 of HIV Gag (Fig. 3B). A third ADA3 region (residues 157 to 170) also shows high homology to HIV Gag (Fig. 3B). All three regions are homologous to the p24 product from HIV Gag, which constitutes the major core protein of HIV (63). Why ADA3 bears three regions with similarity to p24 from HIV is a question for further experimentation on the functions of these proteins. The genomic insert also contained two known yeast genes, the *UBC1* gene (57) to the downstream side and the *ARG82* gene (ARGRIII) (15) to the upstream side. *ARG82* and therefore *ADA3* map to chromosome 4R (26).

A.

		abcdefghijkl
ADA3	295	FLAFVENFFKDL
KEX1	173	FMDFLENYFKIF
HAP1	481	IALFIEKFFKLI
LAC9	386	ISAYIDAYFKHY
SIV-gag	295	FQSYVDRFYKSL
HIV-gag	301	FRDYVDRFYKTL
VPS15	300	FYTFIYDYFRNL
FIPV-22	92	FYSFVDNFYIVS
gltC	291	FYEFVIQFFSKL
ΦDNApol	313	FGAFIDHFFSKR
LpS1	287	FNEFVNRFFSNC
Smg p25A	143	FLVFVANFDEND

B.

ADA3	487	GGTTSTLPQQIGWITN
HIV-gag	239	AGTTSTLQEQIGWMTN
ADA3	157	QAADNMAKEEINED
HIV-gag	197	QAAMQMLKETINEE

FIG. 3. Sequence motifs in ADA3. (A) A 12-amino-acid stretch (residues 295 to 306) from ADA3 is compared with similar sequences in several other proteins. The yeast proteins were KEX1 (14), VPS15 (31), HAP1 (47), and LAC9 (55). The higher-eukaryote proteins were LpS1 (sea urchin [64]) and Smg p25A (bovine [41]). The eukaryotic virus proteins were FIPV-22 (feline infectious peritonitis virus [13]), simian immunodeficiency virus Gag (11), and HIV Gag (38). The bacterial proteins were GltC (from *Bacillus subtilis* [7]) and bacteriophage PRD1 DNA polymerase (ϕDNApol [37]). Conserved residues are shown in boldface type. Note the abundance of conserved aromatic residues (positions *a*, *d*, *h*, and *i*) as well as the conservation of hydrophobic, nonaromatic residues at positions *e* and, to a minor extent, *l*. Positions *f* and *j* are occupied in most cases by charged or polar residues. (B) Two other stretches of ADA3 (residues 487 to 502 and 157 to 170) show strong homology to HIV Gag (38). Identical residues are shown in boldface type; several substitutions are conservative changes (data not shown).

***trans* activation by different activation domains in the *ada3* mutant.** To determine whether the effect on GCN4 represented a defect in the function of the activation domain, we constructed vectors to provide residues 1 to 202 of LexA as a DNA-binding domain for *trans*-activation assays (see Materials and Methods). Note that this LexA fragment contains sequences needed for dimerization as well as DNA binding. Fusions of the GCN4 activation domain as well as the activation domains of GAL4 and HAP4 were constructed and assayed for the *trans* activation of a *lacZ* reporter driven by a LexA-binding site (Fig. 4). Assays were carried out with the wild type and the isogenic *ada3* deletion strain as well as an isogenic *ada2* deletion strain for comparison. The activity of LexA-GCN4 was dramatically reduced in the *ada3* mutant (about 9-fold), the activity of LexA-HAP4 was reduced to a lesser degree (about 2.5-fold), and the activity of LexA-GAL4 was only slightly reduced (about 30%). This pattern was qualitatively similar to that obtained with the *ada2* deletion, which greatly reduced the activity of the GCN4 activation domain and actually slightly increased the activities of the GAL4 and HAP4 activation domains. Other data show that the levels of LexA-GCN4 fusion proteins are not reduced in the *ada3* mutant (data not shown). The similarities of the patterns of effects of the *ada3* mutant and the *ada2* mutant are further evidence that these genes function in the same pathway. The differential effects on the different LexA fusions are an indication that *ADA3* activity may relate to the function of activation domains, rather than some broader aspect of transcription.

FIG. 4. *trans* activation by different acidic activation domains in the *ada3* mutant. LexA fusions bore the DNA-binding and dimerization domains of the bacterial repressor and were constructed as described in Materials and Methods. All strains were isogenic and were either wild type (WT) (*ADA2 ADA3*) or bore a deletion in *ADA2* or *ADA3*. *trans* activation of a *lacZ* reporter driven by a single LexA site was determined by assaying β-galactosidase activities in cell extracts prepared by glass bead lysis of cells. Activities were normalized to the protein concentration in each extract.

Construction of strains doubly disrupted for *ada2* and *ada3*. To obtain further evidence that *ADA2* and *ADA3* may function in the same pathway, we constructed strains with double deletions of these genes. If the genes functioned together, then the slow growth of the single-deletion strains should be no slower than that of the double mutant. If, however, the genes affected different aspects of transcription, then the double-deletion strain should show a more severe phenotype, perhaps lethality. Strain BWG 1-7A (*Δada2::hisG-URA3-hisG*) was crossed with PSY316 (*Δada3*). Fourteen tetrads were dissected, and all spores were viable. Thirteen of 14 tetrads segregated 2 Ura^+/2 Ura^-. To show that double mutants were recovered, we jettisoned the *URA3* gene from segregants in two tetrads by plating them on fluoroorotic acid. The *ADA* genotypes of all four segregants of both tetrads were then determined by transformation with a plasmid that bore *ADA2* (or the related *URA3* vector) or *ADA3* (or the related *LEU2* vector), both *ADA* plasmids, or both vectors. Transformants were tested for the wild-type phenotypes of rapid growth on minimal medium plates or growth at 37°C. Segregants that grew like the wild type when transformed with the vectors alone were deemed *ADA2 ADA3*, segregants that were rescued by *ADA2* or *ADA3* plasmids were deemed *Δada2* or *Δada3*, and segregants that required both *ADA* plasmids for rescue were deemed *Δada2 Δada3*. By this analysis, one tetrad was found nonparental ditype (2 *ADA2 ADA3*/2*Δada2 Δada3*) and one tetrad was found tetratype (*ADA2 ADA3*/*Δada2 ADA3*/*ADA2 Δada3*/*Δada2 Δada3*). This experiment clearly shows that *Δada2 Δada3* double mutants grow no more slowly than single mutants.

Effects of the *ada3* mutation on basal transcription in vitro. In vitro, basal transcription as well as activated transcription can be measured because the level of basal transcription is significant. Previous studies of the *ada2* mutant in vitro indicated no alteration in basal transcription compared with that of the wild type, consistent with the model that ADA2 is a molecule needed for transcriptional activation. We wished to carry out a similar analysis of basal transcription in the *ada3* mutant. To proceed, we used the *CYC1* promoter in pLGSD5 (25), which contains several TATA boxes. The −52 TATA box drives initiation at the major start site at +43 in vitro (29). The −106 TATA box drives initiation at an additional start site in vitro, the +10 site (29). Strikingly,

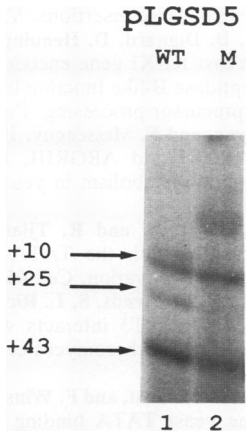

FIG. 5. In vitro transcription in PSY316 (wild-type [WT]) and Δ*ada3* (yBPα91f [M]) extracts. Basal transcription was carried out with the pLGSD5 template, which contains two different functional TATA boxes. Note the increased initiation rate at the +10 site in the mutant relative to the wild type and the decreased rates at sites +25 and +43 (arrows). Additional transcripts were seen in the vicinity of −10 in the mutant.

initiation at the +25 and +43 sites was reduced in the *ada3* mutant extract, but initiation at the +10 site was slightly elevated in the mutant extract (Fig. 5). Moreover, new initiation sites were observed in the vicinity of −10 in the mutant extract. These novel sites were totally absent in the wild-type extract and were not observed in the *ada2* mutant extract (6). These findings show that basal transcription is qualitatively altered in the *ada3* mutant, suggesting that the mutation affects the general transcriptional machinery. This alteration displays a clear pattern in that transcripts that are distal to the TATA boxes are underrepresented and transcripts that are proximal, including novel transcripts at −10, are overrepresented.

DISCUSSION

In this report, we describe a gene, *ADA3*, mutations in which relieved the toxicity of GAL4-VP16 (6) in yeast cells. In the *ada3* mutant strain, the ability of the activation domains of VP16, GCN4, GAL4, or HAP4 to *trans* activate was reduced. In *ada3* mutant extracts, GAL4-VP16 and GAL4-HAP4 were defective. Furthermore, the effect of the *ada3* mutation was due to defects in activation domains, because a LexA fusion to the acidic activation domain of GCN4 was severely defective in *trans* activation in the mutant strain, while a fusion to the GAL4 activation domain was only slightly affected in the mutant strain.

The specificity of the *ada3* mutation is similar to that of another mutation to emerge from this selection, *ada2*. Mutations in *ADA2* also reduced activation by GAL4-VP16 and GCN4 in vivo but not by HAP4 (6). In vitro, GAL4-VP16 was defective, while GAL4-HAP4 was not. On the basis of the specificity of the *ada2* mutation as well as the results of an earlier study (5), we concluded that *ADA2* could encode an adaptor (or part of an adaptor complex) that is used by some but not all acidic activation domains (6). Here we show that the HAP4 and GAL4 activation domains, when fused to LexA, are fully active in the *ada2* mutant. We postulate that *ADA3* functions in the same pathway as *ADA2*.

The growth phenotype of a strain bearing a deletion of *ADA3* parallels that of a strain with a deletion of *ADA2* (6), very slow growth on minimal medium and temperature-sensitive growth on any medium, further suggesting that the gene products participate in the same pathway. Moreover, strains in which both *ADA2* and *ADA3* have been deleted grow no more slowly than single mutants. If the genes function in two parallel pathways in transcription, we might expect to observe a synthetic phenotype in the double null mutant. Thus, we infer that *ADA2* and *ADA3* are likely to function in the same pathway.

However, we do not believe that the *ADA2* and *ADA3* gene products mediate the same biochemical function, for example, as two subunits of an adaptor complex. While the *ada2* mutant was indistinguishable from the wild type in the basal transcription reaction in vitro (6), the *ada3* mutant showed alterations in basal transcription. Specifically, the relative utilization of mRNA initiation sites was skewed in the mutant. The initiation sites at +26 and +43, which are both directed by the −52 TATA box (29), were underutilized in the *ada3* mutant in vitro. In contrast, the utilization of the +10 site, which is directed by the −106 TATA box, was equal to or greater than that in the wild type. We speculate that the small defects in the function of the GAL4 activation domain in the *ada3* mutant, not observed in the *ada2* mutant, may be due to a defect in initiation site selection in the *ada3* mutant.

One hypothesis for explaining the defect in initiation in the *ada3* mutant is that the TBP in the mutant extract cannot utilize the TATA box at −52 but is fully capable of utilizing the −106 TATA box. In this model, *ADA3* could be a TBP-associated protein; such proteins have been described for mammalian cells (16). We do not believe that the *ada3* mutation reduces the amount of TBP because the level of TBP mRNA is not reduced by the mutation (6). Alternatively, the *ada3* mutation could result in an alteration of another component of the transcriptional machinery, such as TFIIB or RNA polymerase II. Indeed, TFIIB mutations have been shown to alter mRNA initiation sites in *S. cerevisiae* (48).

It remains to be demonstrated that ADA3 acts directly as a novel component of the general transcriptional machinery. *ADA3* does not encode any previously described general transcription factor, such as TBP, TFIIB, TFIIA, TFIIE, TFIIF, or an RNA polymerase subunit, since the sequence of ADA3 is not similar to that of any of these factors (3, 10, 18, 22, 27, 28, 35, 43, 45, 46, 50, 56, 61, 65). Furthermore, unlike disruptions of genes encoding other yeast general transcription factors, such as TBP, TFIIB, TFIIA, or RNA polymerase subunits (18, 48, 50, 65), disruption of the *ADA3* gene is not lethal to *S. cerevisiae*. Therefore, ADA3 is mechanistically distinct from any previously described general transcription factor.

The alteration of initiation site selection by the *ada3* mutation is somewhat reminiscent of the effects of mutations in *SPT* genes, which encode a wide variety of factors, ranging from TBP itself to histones. One *SPT* gene product, SPT3, has been shown to bind to TBP (17). Furthermore, mutations in certain *SPT* genes, such as *SPT13* (*GAL11*) (19), can also affect activation by some activators (32). However, despite these apparent similarities, there are differences between the *ada3* and *spt* mutations. For example, while *spt* mutations affect initiation in promoters bearing TY1 or delta element insertions, effects on initiation events at natural yeast promoters are not typically observed. Also, no *SPT* genes with a null phenotype of temperature-sensitive growth have been reported. We believe that the selection for resistance to GAL4-VP16 yields mutations that reduce the

stability of a DNA-bound complex between the activator and the general transcription factors. Whether genes so identified will overlap with *SPT* genes is not yet clear, but no overlap is currently evident.

We speculate that ADA3 could be the component in the general transcriptional machinery that is contacted by ADA2. Activation domains that use the ADA2 adaptor would therefore also use ADA3. This model predicts several protein-protein interactions (VP16-ADA2, ADA2-ADA3, and ADA3-TBP, ADA3-TFIIB, or ADA3-RNA polymerase II) and does not rule out other interactions occurring directly between the acidic activation surface and TBP and/or TFIIB.

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