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ADA3, a Putative Transcriptional Adaptor, Consists of Two Separable Domains and Interacts with ADA2 and GCN5 in a Trimeric Complex

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Mutations in yeast *ADA2*, *ADA3*, and *GCN5* weaken the activation potential of a subset of acidic activation domains. In this report, we show that their gene products form a heterotrimeric complex in vitro, with ADA2 as the linchpin holding ADA3 and GCN5 together. Further, activation by LexA-ADA3 fusions in vivo are regulated by the levels of ADA2. Combined with a prior observation that LexA-ADA2 fusions are regulated by the levels of ADA3 (N. Silverman, J. Agapite, and L. Guarente, Proc. Natl. Acad. Sci. USA 91:11665–11668, 1994), this finding suggests that these proteins also form a complex in cells. ADA3 can be separated into two nonoverlapping domains, an amino-terminal domain and a carboxyl-terminal domain, which do not separately complement the slow-growth phenotype or transcriptional defect of a Δ*ada3* strain but together supply full complementation. The carboxyl-terminal domain of ADA3 alone suffices for heterotrimeric complex formation in vitro and activation of LexA-ADA2 in vivo. We present a model depicting the ADA complex as a coactivator in which the ADA3 amino-terminal domain mediates an interaction between activation domains and the ADA complex.

In eukaryotes, several factors that are important in the activation of transcription by RNA polymerase II are in large, heteromeric complexes. For example, the yeast SWI2/SNF2, SWI1, SWI3, SNF5, and SNF6 proteins form a large multisubunit complex, which apparently counters repression by chromatin (4, 5, 7, 23). Mutations in *SWI2* and *SNF5* result in decreased transcription and altered chromatin structure at certain promoters (19). These phenotypes can be suppressed by mutations in histone genes. In another case, the yeast SRB2, SRB4, SRB5, and SRB6 proteins form a holoenzyme complex with RNA polymerase II and certain basal transcription factors (21). The SRB proteins interact with the carboxyl-terminal domain of the largest subunit of RNA polymerase II and are important for both basal and activated transcription in vitro (33). In higher eukaryotes, the TFIID complex is composed of TATA-binding protein (TBP) and TBP-associated factors (TAFs) (9). While TBP with the other basal factors is sufficient for basal transcription, the TAFs are required for activated transcription (12, 13). Thus, the TAFs are proposed to be coactivators or adaptors required to mediate the stimulatory signal from activators to basal factors. There is also evidence that a family of factors interact with TBP in yeast cells (10, 25, 34).

In addition to the TAFs, other factors, such as the yeast *ADA2*, *ADA3*, and *GCN5* gene products, have been proposed to be coactivators (3, 22, 24). Mutations in *ADA2*, *ADA3*, and *GCN5* were isolated in a selection for mutants which confer resistance to toxicity from overexpressed GAL4-VP16. This toxicity is postulated to occur by titration of basal transcription factors away from productive transcription complexes by the strong acidic activation domain of GAL4-VP16 (2). If this titration by GAL4-VP16 requires proteins with coactivator or adaptor function, alterations in these proteins should cause resistance to toxicity. Interestingly, *GCN5* had been isolated previously as a gene encoding a transcription factor necessary for full activity of the activator GCN4 (11).

ADA2, *ADA3*, and *GCN5* mutants share several phenotypes (3, 22, 24). Strains disrupted for any of the three genes display temperature-sensitive growth as well as a severe growth defect on minimal media. Double mutants between any two of the three genes do not have a more severe slow-growth phenotype than the single mutants. Also, all three genes are required for full transcriptional activity of a similar subset of activators. GAL4-VP16 and GCN4 are dependent on ADA2, ADA3, and GCN5 activity, whereas other activators such as HAP4 and GAL4 are independent or only slightly dependent.

Because of the similar phenotypes between mutations in *ADA3*, *ADA2*, and *GCN5*, we wanted to determine whether their gene products interacted in a complex. In this study, we demonstrate that a trimeric complex is indeed formed by ADA3, ADA2, and GCN5. We further characterize ADA3 and show that it consists of two separable functional domains, both of which are required for function and one of which interacts in the adaptor complex. A model for the structure and function of the ADA complex is proposed.

MATERIALS AND METHODS

Strains and plasmids. Assays were carried out with *Saccharomyces cerevisiae* BWG1-7a (*MAT***a** *leu2-3,112 his4-519 ade1-100 ura2-52*), BWG1-7aΔ*trp1*, and derivatives disrupted for *ADA3* or *ADA2*. BWG1-7aΔ*trp1* is BWG1-7a with the *trp1* gene disrupted by using pNKY1009 (1). *ADA3* was disrupted by using the *Bam*HI-*Sal*I fragment of plasmid pΔA3-i (24). BWG1-7a Δada2 has been previously described (3).

Plasmids expressing size variants of ADA3 were constructed by first generating various *ADA3* fragments by using PCR. Oligonucleotides hybridizing to various portions of *ADA3* were synthesized and used to amplify the appropriate regions of *ADA3*. The numbers in the parentheses after each *ADA3* construct correspond to the amino acids encoded by the various *ADA3* fragments. In all constructs, six histidines were fused to the C terminus of ADA3 by encoding them on the 3′ primers except when HA follows the parentheses, in which case the 12CA5 hemagglutinin (HA) tag was fused instead. The *ADA3* plasmids carrying the *LEU2* marker were generated by cloning the *ADA3* fragments into the yeast expression plasmid pDB20L (3), while plasmids carrying the *URA3* and *TRP1* markers were generated by cloning the *ADA3* fragments into plasmids pJH1 and

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pJH2, respectively. pJH1 was generated by subcloning the *ADH1* promoter/terminator fragment from pDB20L into the *Bam*HI site of pRS316 (28), and pJH2 was generated by subcloning the same fragment into pRS314 (28). Templates used for in vitro transcription and translation of *ADA3* fragments were generated by cloning the above-specified *ADA3* fragments behind T7 promoters in the plasmid pT7plink (8) or pCITE2A (Novagen).

The construction of plexA-ADA2 and plexA-ADA3, which express LexA-ADA2 and LexA-ADA3, respectively, has been previously described (22). The pT7ADA2 and pT7GCN5 constructs used for in vitro transcription and translation of *ADA2* and *GCN5* are described by Marcus et al. (22). plexA-GCN4 (24), the *lexA* β-galactosidase reporter, pRBHis (22), and pADA2-6HisL (29), which was used to overexpress ADA2, have also been described previously. p14x2His, a *lacZ* reporter plasmid with two synthetic GCN4 binding sites upstream of a minimal *CYC1* promoter, was constructed from HIS(2)14x2 (18). The *URA3* marker was removed from HIS(2)14x2 by digestion with *Stu*I. *Not*I linkers were ligated, and a *Not*I fragment containing the *HIS4* gene was inserted.

Yeast transformations were performed by the lithium acetate method (27). For β-galactosidase assays, cells were grown in selective medium with glucose to an optical density at 600 nm of approximately 1.0, and activity was measured in glass bead extracts as described by Rose and Botstein (26). For p14x2His activity, cells were grown in minimal medium supplemented with adenine. For all other assays, cells were grown in synthetic complete medium lacking only the amino acids and nucleotide used to select for plasmids. β-Galactosidase activity was measured as nanomoles per minute per milligram of protein. Other general yeast techniques were performed as described by Guthrie and Fink (16).

In vitro transcription and translation. In vitro transcription was performed with 2.5 μg of linearized template in a 25-μl volume of 1× T7 buffer (Pharmacia), 0.5 mM rATP, rCTP, and rGTP, 0.1 mM rGTP, 100 mg of bovine serum albumin (BSA) per ml, 2 mM $MgCl_2$, 10 mM dithiothreitol, 40 U of RNasin (Promega), 500 mM $m^7G(5')ppp5'G$ (Boehringer Mannheim), and 20 U of T7 polymerase (Pharmacia). The reaction mixture was incubated at 37°C for 30 min, 1 μl of 10 mM rGTP was added, and the reaction mixture was incubated at 37°C for an additional 30 min. RNA was purified by two phenol-chloroform extractions followed by two ethanol precipitations.

In vitro translations were performed by using a rabbit reticulocyte lysate system (Promega). A 17.5-μl volume of rabbit reticulocyte lysate, 20 U of RNAsin, 0.5 μl of 1 mM amino acid mix minus methionine, 20 mCi of $[^{35}S]$methionine (1,200 Ci/mmol), and 0.3 to 1 μg of each RNA were mixed and incubated for 90 min at 30°C. Proteins were either used directly or stored at −80°C until used. The luciferase used as a negative control in Fig. 3 was supplied as a control mRNA from Promega.

Far-Western blot analysis. Plasmids for the expression of recombinant ADA3(580–702) and ADA3(452–702) were constructed by cloning the appropriate *ADA3* fragments generated by PCR (see above) into the T7 expression vector, pET21d (Novagen). Recombinant glutathione *S*-transferase (GST) was expressed from pGEX-KG (14). ADA2 and dihydrofolate reductase (DHFR) were expressed from pUH24.2ΔCAT (22).

The ADA3 expression constructs were transformed into *Escherichia coli* BL21-DE3 (30) cells, and the pGEX-KG and pUH24.2ΔCAT constructs were transformed into AG115 cells (20). Expression was induced as recommended (Novagen). The six-histidine-tagged ADA3 fragments and DHFR were purified by using a nickel column as recommended (Qiagen), while the other proteins were used as unpurified extracts.

Proteins and extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently stained with Coomassie brilliant blue or transferred to nitrocellulose. Nitrocellulose blots were denatured in 8 M urea and stepwise renatured in far-Western buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.3], 60 mM KCl, 7.5 mM $MgCl_2$, 5% glycerol, 25 mM dithiothreitol). During each renaturation step, the urea concentration was diluted twofold in the buffer until the urea concentration was below 10 mM. The blots were blocked for 1 h in far-Western buffer containing 5% BSA and then incubated overnight with $[^{35}S]$methionine-labeled in vitro-translated probe in buffer. The blots were subsequently washed three times in far-Western buffer, dried, and exposed to film.

Coimmunoprecipitations. Reactions contained 20 μl of immunoprecipitation buffer (IP buffer); (50 mM HEPES [pH 7.3], 100 mM potassium glutamate, 6 mM magnesium acetate 1 mM EGTA, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 0.5 mg of BSA per ml, 10% glycerol), 1 μl of antibody, and 5 μl of reticulocyte lysate translation product. The antibodies used were either an anti-HA monoclonal antibody (BAbCo) or an anti-ADA2 polyclonal antibody (29). Reactions were pipetted onto 10 μl of protein A-Sepharose beads which had previously been equilibrated in IP buffer. Following gentle mixing on ice, the reaction mixtures were rotated for 3 h at 4°C. The reaction mixtures were centrifuged for 15 s, and the supernatant was discarded. The beads were washed three times with 1 ml of IP buffer. If the antibody used in the immunoprecipitation was the anti-ADA2 polyclonal antibody, the immunoprecipitated complexes were separated from the beads by boiling in protein gel loading buffer. If the antibody used was the anti-HA monoclonal antibody, the immune complexes were eluted from the beads by incubation for 1 h at 15°C in 1 mg of a 12CA5 epitope peptide (BAbCo) per ml in IP buffer without BSA. The immunoprecipitated complexes were analyzed by SDS-PAGE.

RESULTS

ADA3 has two functional domains. A strain deleted of *ADA3* has a severe slow-growth phenotype on minimal media (24). A wild-type copy of *ADA3* or a truncated allele of *ADA3*, lacking the first 214 codons of the 702-codon gene, fully complements the deletion (Fig. 1A and C). Expression of either the amino-terminal half of *ADA3* [*ADA3(1–346)*] or the carboxyl-terminal half of *ADA3* [*ADA3(364–702)*] does not allow complementation. However, when both nonoverlapping clones, *ADA3(1–346)* and *ADA3(364–702)*, are expressed in a Δ*ada3* strain simultaneously, growth is fully complemented. These results suggest an unusual interaction between two nonoverlapping domains of ADA3. We propose two possible models to explain this observation. First, the amino-terminal domain and the carboxyl-terminal domain of ADA3 may independently fold into functional units that do not need to interact with each other, or second, the amino-terminal domain and carboxyl-terminal domain may interact with each other without having to be covalently linked.

A Δ*ada3* strain transformed with two plasmids, one expressing a truncated amino-terminal domain, *ADA3(214–346)*, and the other expressing the full carboxyl-terminal domain, *ADA3 (364–702)*, grows as well as a wild-type strain (Fig. 1B and C). Growth is restored to an intermediate extent when the Δ*ada3* strain is transformed with plasmids expressing a truncated carboxyl-terminal domain, *ADA3(452–702)*, and the full-length amino-terminal domain, *ADA3(1–346)*. However, when both the truncated amino-terminal domain and the truncated carboxyl-terminal domain are expressed, no complementation is evident. This type of interaction can be thought of as a synthetic phenotype and suggests that both domains of ADA3 interact in the same pathway, i.e., do not have completely separate functions.

Both the amino-terminal and carboxyl-terminal domains of ADA3 are required to complement the defect in transcription of a Δ*ada3* strain. We assayed two *lacZ* reporters in wild-type and Δ*ada3* strains expressing various *ADA3* constructs. p14×2His contains *lacZ* under the control of two synthetic GCN4 binding sites upstream of a minimal *CYC1* promoter. pRBHis contains a single LexA binding site upstream of a *CYC1* minimal promoter and was transformed in combination with plexA-GCN4, which expresses a fusion consisting of residues 1 to 202 of LexA fused to residues 9 to 172 of GCN4, or plexA202, which expresses residues 1 to 202 of LexA alone. As shown in Table 1, the activity measured from the p14x2His reporter is reduce 6-fold in a Δ*ada3* strain compared with the wild type, and the activity of LexA-GCN4 is reduced 18-fold. Expressing only the amino-terminal domain or only the carboxyl-terminal domain has no effect on this transcription defect, whereas expressing both domains together restores levels to that observed in the presence of full-length ADA3. Thus, GCN4 and LexA-GCN4 seem to require both domains of ADA3 for full activity.

LexA-ADA3 activity is ADA2 dependent. When ADA2 is fused to a LexA moiety, it can activate transcription from reporters containing LexA binding sites (29; also see Table 3 controls). This activity is reduced 3.5-fold in a Δ*ada3* strain. Furthermore, LexA-ADA2 activity can be hyperstimulated when *ADA3* is overexpressed. One explanation for this hyperstimulation is that ADA2 and ADA3 interact in a heteromeric complex. *lexA-ADA2* is expressed from the strong *ADH1* promoter on a high-copy-number plasmid. Thus, there is a large excess of LexA-ADA2 compared with ADA3. If a complex of LexA-ADA2 and ADA3 is required for activity from a *lexA* operator, overexpressing *ADA3* should lead to more complexes and a hyperstimulation of activity.

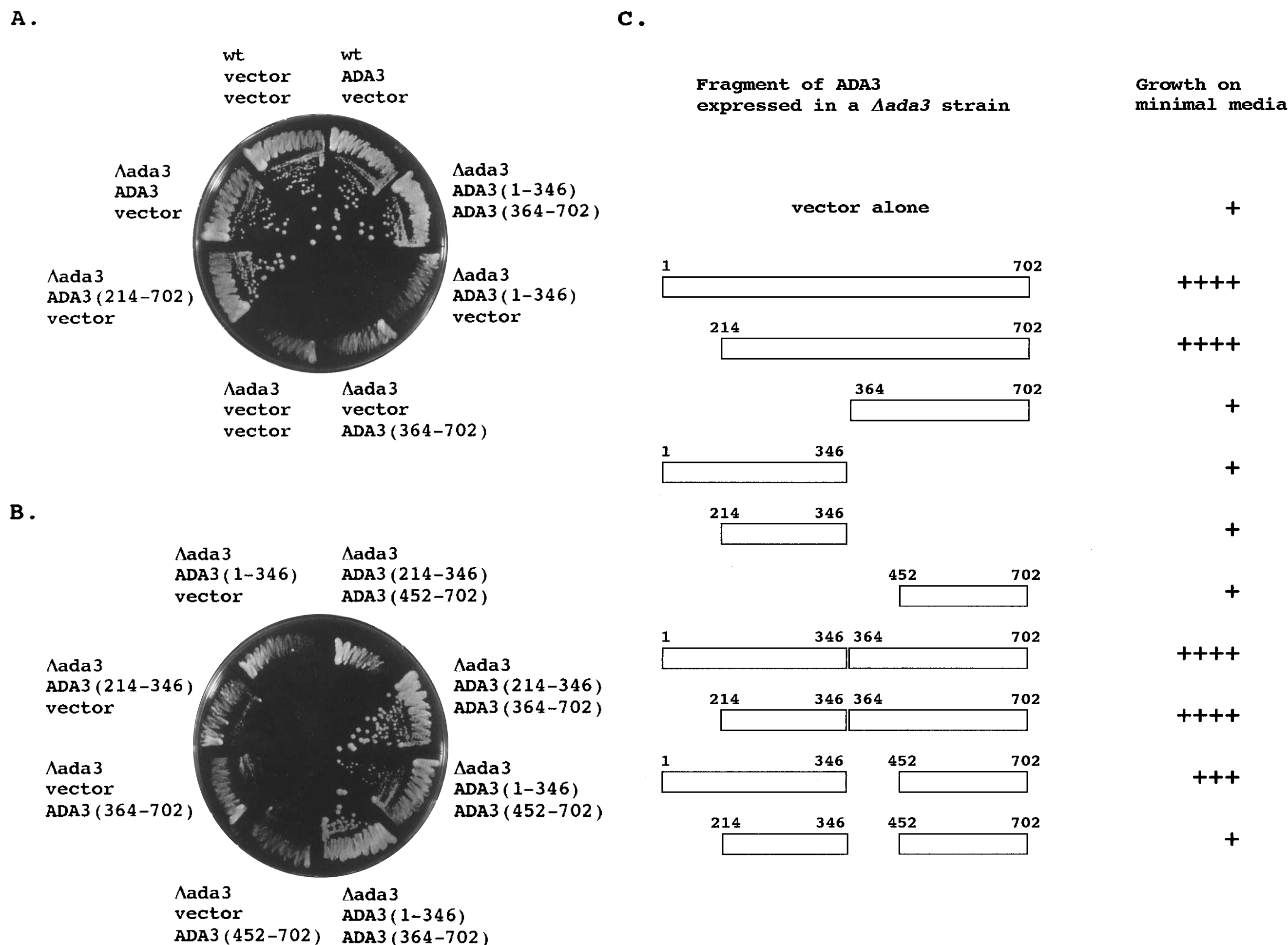

FIG. 1. Complementation of the slow-growth phenotype on minimal media of a Δ*ada3* strain by various ADA3 fragments. Strain BWG1-7a (wild type [wt]) or 1-7aΔada3 was transformed with combinations of plasmids and restreaked onto plates containing no amino acid or nucleoside supplements except histidine and adenine. Each restreak is labeled first with the strain, followed by the fragment of ADA3 expressed from pJH1, followed by the fragment of ADA3 expressed from DB20L. Vector refers to either pJH1 or DB20L with no insert. ADA3 with no parentheses refers to the full-length protein. (A) Complementation tests of a Δ*ada3* strain expressing the 1–346 domain of ADA3, the 364–702 domain, and both domains together. Wild-type cells and Δ*ada3* cells transformed with vectors, ADA3, and ADA3(214–702) are shown in comparison. (B) Complementation tests of a Δ*ada3* strain expressing the following domains of ADA3: the amino-terminal domains (1 to 346 and 214 to 346), the carboxyl-terminal domains (364 to 702 and 452 to 702), and each combination of an amino- and a carboxyl-terminal domain. (C) Data from panels A and B in schematic form. ++++, wild-type growth; +, growth of a Δ*ada3* strain with vector alone. Full-length ADA3 extends from residues 1 to 702.

The dependence of LexA-ADA2 upon ADA3 for activity from LexA sites prompted us to determine whether LexA-ADA3 can also activate transcription and, if so, whether this activation requires ADA2. As shown in Table 2, LexA-ADA3 activates transcription to a similar level as LexA-ADA2. This activation is dependent upon ADA2 to the same extent as LexA-ADA2 activation is dependent upon ADA3. Finally, LexA-ADA3 activity can be hyperstimulated by overexpressing ADA2 in a manner identical to LexA-ADA2 activity being hyperstimulated by excess ADA3. This mutual dependence that each ADA has for the other in terms of LexA activity is consistent with a model in which ADA2 and ADA3 form a heteromeric complex. Combined with the results described later that ADA3 interacts with ADA2 in vitro, this finding provides supporting evidence that a ADA3-ADA2 heteromeric complex exists in vivo.

The carboxyl-terminal domain of ADA3 alone can activate LexA-ADA2. We wanted to determine whether the amino-terminal domain, the carboxyl-terminal domain, or both together were required for the stimulation of LexA-ADA2 activity by ADA3. As shown in Table 3, overexpressing the amino-terminal domain of ADA3 alone has no effect on LexA-ADA2 activation in both wild-type and Δ*ada3* strains. However, overexpressing the carboxyl-terminal domain of ADA3 alone stimulates LexA-ADA2 activity in wild-type and Δ*ada3* strains seven- and fourfold, respectively. In wild-type cells, this stimulation is even greater than that by full-length ADA3. In Δ*ada3* cells, stimulation by the carboxyl-terminal domain is observed but is not as great as with full-length ADA3. These data support the model that the carboxyl-terminal domain is the region of ADA3 responsible for complexing with ADA2 in vivo.

The carboxyl-terminal domain of ADA3 interacts with ADA2 via far-Western analysis. To demonstrate an interaction between the ADA3 carboxyl-terminal domain and ADA2 biochemically, far-Western experiments were performed. In Fig. 2A, purified recombinant DHFR, ADA3(452–702), and ADA3(580–702), as well as an extract from *E. coli* expressing GST, were run on denaturing gels and either stained with Coomassie blue or transferred to nitrocellulose. The nitrocel-

TABLE 1. Effects of expressing ADA3 amino- and carboxyl-terminal domains on GCN4 and LexA-GCN4 activation

Plasmid 1[a]	Plasmid 2[b]	Activity[c]			
		p14x2His[d] reporter		pRbHis reporter with plexA-GCN4[e]	
		Wild type	Δ*ada3*	Wild type	Δ*ada3*
Vector	Vector	74	12	5,300	290
ADA3(1–346)	Vector	86	10	5,000	330
Vector	*ADA3 (364–702)*	84	16	3,800	270
ADA3(1–346)	*ADA3 (364–702)*	107	80	3,300	3,100
Vector	*ADA3*	ND	82	4,200	2,100

[a] For p14x2His assays, the vector was pRS316. *ADA3(1–346)* refers to an *ADH1* promoter terminator cassette expressing *ADA3(1–346)* cloned into pRS316. For pRbHis assays, the vector was pDB20L. *ADA3(1–346)* refers to *ADA3(1–346)* cloned behind the *ADH1* promoter of pDB20L.

[b] For p14x2His assays, the vector was pDB20L. *ADA3* and *ADA3(364–702)* were cloned behind the *ADH1* promoter of pDB20L. For pRBHis assays, the vector was pRS314. *ADH1* promoter terminator cassettes expressing *ADA3* and *ADA3(364–702)* were cloned into pRS314. See Materials and Methods for definition of units.

[c] β-Galactosidase measurements were carried out in quadruplicate, with standard errors, in most cases, less than 20% of the mean. ND, not determined.

[d] p14x2His-*lacZ* expression is regulated by two 14-mer GCN4 binding sites upstream of a minimal *CYC1* promoter.

[e] pRbHis-*lacZ* expression is regulated by a single *lexA* operator site upstream of a minimal *CYC1* promoter. plexA-GCN4 expresses a fusion consisting of residues 1 to 202 of LexA fused to residues 9 to 172 of GCN4. A control plasmid expressing only residues 1 to 202 of LexA gives 5 to 15 U of activity in wild-type and Δ*ada3* strains.

TABLE 2. Effects of deleting or overexpressing *ADA2* on LexA-ADA3 activity

Strain	LexA-ADA3 activity[a]	
	Vector	*ADA2*
Wild type	140	440
Δ*ada2*	32	ND

[a] A control plasmid, plex202 (21), expressing only residues 1 to 202 of LexA, gives 5 to 15 U of activity in wild-type and Δ*ada2* cells. β-Galactosidase measurements represent averages of assays performed on four independent transformants. Standard errors were less than 20% of the mean. Vector refers to pDB20L. *ADA2* refers to a construct in which *ADA2* was cloned behind the *ADH1* promoter of pDB20L. ND, not determined. See Materials and Methods for definition of units.

TABLE 3. Effects of overexpressing ADA3 amino-terminal or carboxyl-terminal fragments on LexA-ADA2 activity

Strain	LexA-ADA2 activity[a]			
	Vector	*ADA3*	*ADA3(1–346)*	*ADA3(364–702)*
Wild type	110	370	100	700
Δ*ada3*	31	380	24	130

[a] A control plasmid, plex202 (21), expressing only residues 1 to 202 of LexA gives 5 to 15 U of activity in wild-type and Δ*ada3* cells. β-Galactosidase measurements were performed as described in the footnote to Table 2. Standard errors were less than 20% of the mean. The vector used was pDB20L. *ADA3*, *ADA3(1–346)*, and *ADA3(364–702)* refer to constructs in which the appropriate gene or gene fragment was cloned behind the *ADH1* promoter of pDB20L. See Materials and Methods for definition of units.

lulose blot was subsequently washed under renaturing conditions and probed with in vitro-translated [^{35}S]methionine-labeled ADA2. As seen in Fig. 2A, only the lane containing ADA3(452–702) shows any radiolabeling. Thus, ADA2 does not interact with GST, DHFR, or the carboxyl-terminal 122 amino acids of ADA3 by this method. The 37-kDa band in the lane marked ADA3(452–702) is full-length ADA3(452–702). The lower-molecular-weight bands observed are most likely degradation products from the amino-terminal end (see the legend to Fig. 2). These results indicate that a region larger than the carboxyl-terminal 122 amino acids yet smaller than the carboxyl-terminal 252 amino acids of ADA3 is sufficient to bind to ADA2.

The converse experiment is shown in Fig. 2B. In this case, purified DHFR and *E. coli* extracts expressing GST, vector, or ADA2 were run on gels and either stained with Coomassie blue or transferred to nitrocellulose, renatured, and probed with in vitro-translated [^{35}S]methionine-labeled ADA3(452–702). It is clear that only the lane in which recombinant ADA2 is present shows any radiolabeling. The major radiolabeled band runs at a position identical to that of recombinant ADA2. The faster-migrating and fainter band corresponds to a degraded form of ADA2 (data not shown).

The carboxyl-terminal domain of ADA3 can coimmunoprecipitate ADA2 in vitro. In a further attempt to demonstrate the interaction between the ADA3 carboxyl-terminal domain and ADA2, coimmunoprecipitation experiments with in vitro-translated products were performed (Fig. 3). Two forms of ADA3(452–702), one tagged with an HA epitope and one lacking the epitope, were cotranslated with either ADA2 or a negative control protein, luciferase. The translation products were immunoprecipitated with a monoclonal antibody to the HA epitope. Lanes 1 to 4 show the cotranslation products, and lanes 5 to 8 show the immunoprecipitation products. As seen in lane 7, ADA2 clearly coprecipitates with ADA3, whereas luciferase does not (lanes 3 and 6). Neither ADA2 nor luciferase is recognized nonspecifically by the HA antibody (lanes 4 and 5), and neither ADA2 nor ADA3(452–702) is precipitated when ADA3 is untagged (lanes 1 and 8). This result in combination with the far-Western results argues strongly for a specific interaction between ADA2 and the carboxyl-terminal domain of ADA3.

GCN5 binds to the ADA3-ADA2 complex. As mentioned earlier, a third gene, *GCN5*, was also isolated from the adaptor screen. We wanted to determine whether GCN5 might also bind to the ADA2-ADA3 complex. Thus, the three proteins, ADA3(214–702), ADA2, and GCN5, were cotranslated in vitro. As described above, two forms of ADA3 were used, one tagged with the HA epitope and one untagged (Fig. 4, lanes 3 and 4). When immunoprecipitated with a monoclonal antibody directed against the HA epitope, ADA2 and GCN5 were coprecipitated with tagged ADA3, while none of the proteins were precipitated when ADA3 was untagged (lanes 1 and 2). Furthermore, antibody directed against ADA2 could immunoprecipitate both GCN5 and both forms of ADA3 (lanes 5 and 7). Preimmune serum failed to precipitate any of the three proteins (lane 6). Thus, GCN5 binds to either ADA2 or ADA3 or both.

Both the ADA3 carboxyl-terminal domain and GCN5 bind to ADA2 to form a trimeric complex. Figure 4 demonstrates an interaction among ADA2, ADA3(214–702), and GCN5. However, it does not address the question of which proteins make direct contact or whether all three comprise a single complex. To address this issue, all three proteins, ADA3(452–702)HA, ADA2, and GCN5, as well as each combination of two proteins were cotranslated. ADA3(452–702)HA was also translated alone as a negative control. As shown in Fig. 5, lane 7, when all three proteins are cotranslated, all three are precipitated with an anti-HA epitope monoclonal antibody. When ADA2 and ADA3(452–702)HA are cotranslated, ADA2 is coimmunopre-

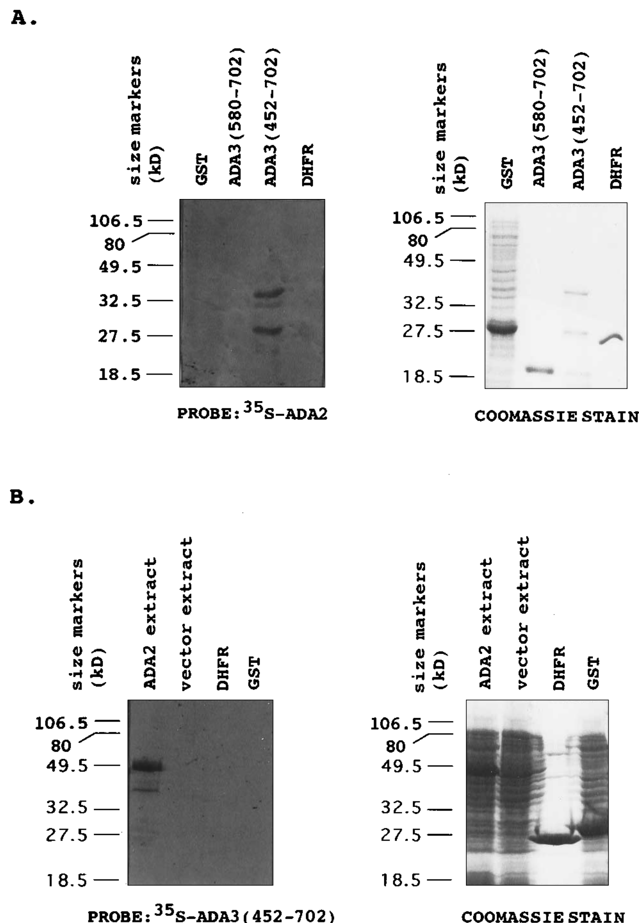

FIG. 2. Far-Western experiments of ADA2 and ADA3. (A) An extract from *E. coli* expressing GST as well as purified recombinant ADA3(580–702), ADA3(452–702), and DHFR were subjected to SDS-PAGE and either stained with Coomassie blue or transferred to nitrocellulose, denatured, stepwise renatured, and probed with [^{35}S]methionine-labeled ADA2. The 37-kDa band in the lane labeled ADA3(452–702) represents full-length ADA3(452–702). Smaller bands result from amino-terminal degradation, since the fragment is expressed with a carboxyl-terminal six-histidine fusion and purified from a nickel column. For the gel transferred to nitrocellulose and probed with radiolabeled ADA2, extract from 5×10^7 cells expressing GST and 5 μg each of ADA3(580–702), ADA3(452–702), and DHFR were loaded. For the Coomassie blue-stained gel, extract from 10^7 cells expressing GST, 1 μg of ADA3(580–702), 400 ng of ADA3(452–702), and 0.5 μg of DHFR were loaded. (B) *E. coli* extracts expressing ADA2, vector (pUH24.2ΔCAT), GST, and purified recombinant DHFR were subjected to SDS-PAGE. As above, the gels were either stained or transferred to nitrocellulose. The nitrocellulose blot was probed with ^{35}S-labeled ADA3(452–702). The 50-kDa band represents ADA2, and the smaller band is a degradation product of ADA2 (data not shown). Extracts from 2×10^8 cells expressing ADA2, vector, or GST and 20 μg of purified DHFR were loaded on both gels.

cipitated with ADA3. However, when GCN5 is cotranslated with ADA3(452–702)HA in the absence of ADA2, only ADA3 is precipitated. Further, GCN5 is not precipitated with the amino-terminal domain, ADA3(1–346) (data not shown). This result indicates that there is no direct interaction between ADA3 and GCN5. These findings suggest that ADA2, ADA3, and GCN5 form a trimeric complex, with ADA2 serving as a linchpin. Consistent with this view, it has been shown that ADA2 and GCN5 can form a complex in the absence of ADA3 (22).

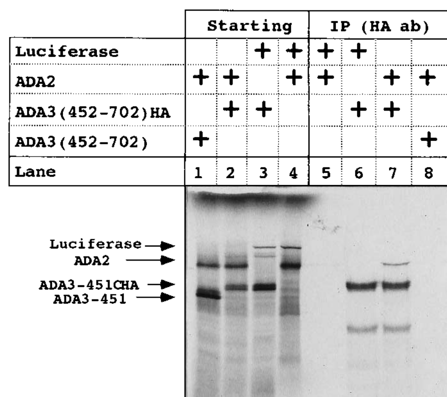

FIG. 3. Coimmunoprecipitation experiments of ADA2 with the C-terminal domain of ADA3. Combinations of HA epitope-tagged or untagged ADA3(452–702), ADA2, and luciferase were cotranslated in an in vitro reticulocyte lysate system. Lanes 1 to 4 show SDS-PAGE analysis of the cotranslated products. The cotranslated products were immunoprecipitated (IP) with antibody (ab) directed against the HA epitope and analyzed by SDS-PAGE (lanes 5 to 8). The lower-molecular-weight bands in lanes 6 and 7 seem to be a degradation product of ADA3(452–702)HA.

DISCUSSION

In this report, we demonstrate the formation of an ADA2-ADA3-GCN5 complex in vitro and begin a structural dissection of this complex. It is now emerging that several factors that are generally important in transcription, such as TFIID (9), the SRBs (21), and the SWI/SNF complex, are heteromeric complexes (23). Also, the similarity in phenotypes of mutations in several *SPT* genes suggests that their products might exist in a complex (31).

Two domains in ADA3. Two nonoverlapping segments of the *ADA3* gene, one amino terminal and the other carboxyl terminal, work together to complement defects in a Δ*ada3* strain. Expression of both domains of the protein restores wild-type

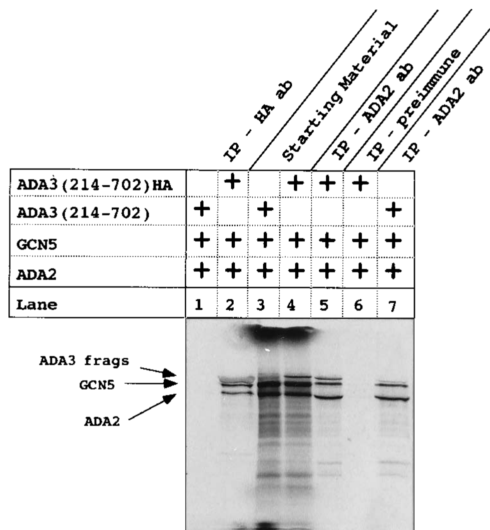

FIG. 4. Coimmunoprecipitation experiments of ADA2, ADA3, and GCN5. ADA2, GCN5, and either HA epitope-tagged or untagged ADA3(214–702) were cotranslated (lanes 3 and 4). Lanes 1 and 2 show anti-HA antibody (ab)-immunoprecipitated (IP) products; lanes 5 and 7 show complexes immunoprecipitated with anti-ADA2 antibody, and lane 6 shows products immunoprecipitated by preimmune serum.

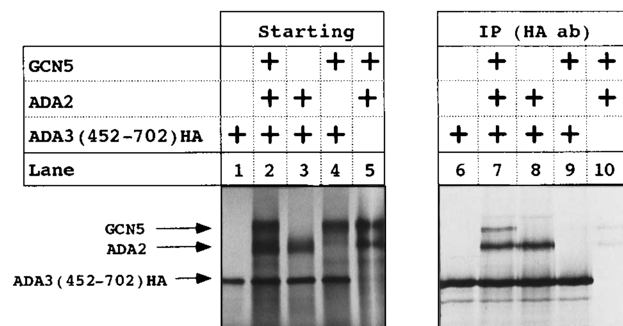

FIG. 5. ADA3-GCN5 coimmunoprecipitation experiments. Combinations of two of the three proteins in the ADA complex were cotranslated along with controls. Lanes 1 to 5 show cotranslation products; lanes 6 to 10 show products immunoprecipitated (IP) with anti-HA antibody (ab).

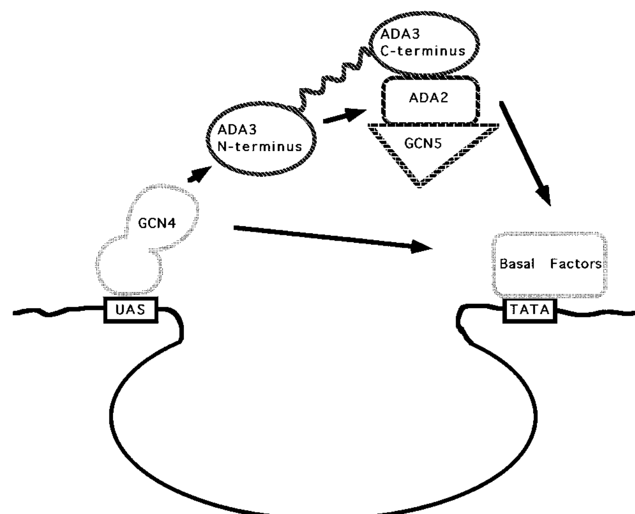

FIG. 6. Model for the role of the *ADA* genes in transcriptional activation. The dark black line represents DNA. GCN4 is depicted binding to its upstream activation sequence (UAS), and basal transcription factors are shown binding to the TATA region of the promoter. We envision two parallel activation pathways between activators and basal factors that may act in synergy to activate transcription, an ADA-dependent pathway and an ADA-independent, perhaps direct, pathway. The ADA3 carboxyl-terminal domain–ADA2–GCN5 complex is required for the ADA-dependent pathway. ADA2 serves as a linchpin-type molecule binding to both ADA3 and GCN5 at the same time. The amino-terminal domain is shown between GCN4 and the carboxyl-terminal complex since it is required for GCN4-dependent transcription but not for LexA-ADA2-dependent transcription.

growth to the mutant strain, while expression of one or the other has no effect. Do these two domains have unrelated functions, or do they work together in the same pathway of transcriptional activation? Two findings suggest that they function in the same pathway. First, full activity of the GCN4 activation domain, as assayed from a GCN4-responsive reporter as well as from a *lexA* reporter in the presence of LexA-GCN4, requires both domains of ADA3. Expression of one domain or the other is without effect in this assay. Second, synthetic effects are observed when shortened versions of the amino- and carboxyl-terminal domains are expressed. Each of these shortened domains is functional in a strain expressing the unshortened version of the other. However, when the two shortened domains are expressed in the same strain, they do not provide function. This synthetic interaction implies that the two domains of ADA3 function in the same pathway, possibly at the same step of the pathway (15). For example, the combination of two weakened interactions might destabilize a multicomponent complex, whereas a weakening of either single interaction might not.

Function of the carboxyl-terminal domain—assembly of a heterotrimeric complex. Our in vitro experiments show that ADA2, ADA3, and GCN5 form a trimeric complex. This complex was first demonstrated by immunoprecipitation of cotranslated ADA2, GCN5, and an HA epitope-tagged version of ADA3 containing both the amino- and carboxyl-terminal domains. Precipitation of this translation mix with either a monoclonal antibody to HA or an antiserum to ADA2 brings down all three proteins.

What region of ADA3 is required for formation of this complex? The carboxyl-terminal domain is clearly sufficient for assembly of the trimeric complex, as demonstrated by the following assays. First, this domain binds to ADA2 when the latter has been transferred to nitrocellulose in a far-Western experiment. Second, ADA2 binds to the carboxyl-terminal domain of ADA3 in the converse far-Western experiment. Third, HA antibody precipitates the three proteins in a cotranslation of ADA2, GCN5, and the HA-tagged carboxyl-terminal domain of ADA3. The region of ADA3 that is functional in these assays, residues 452 to 702, is partially active in the complementation experiments in vivo (Fig. 1B).

Architecture of the ADA complex. What are the binary protein-protein contacts that hold ADA2, ADA3, and GCN5 together? In an important experiment, when ADA2 is omitted from the translation mix, the HA-tagged ADA3 carboxyl-terminal domain is unable to precipitate GCN5. This finding indicates that there is no direct contact between ADA3 and GCN5 and that GCN5 is recruited to the complex by ADA2. This model proposes that ADA2 is the linchpin in the complex, binding to both ADA3 and GCN5 (Fig. 6). An alternative explanation for our findings is that the conformation of ADA3 is altered when it binds to ADA2, allowing it to make direct contact with GCN5. In a separate analysis of GCN5, however, we found that it can bind directly to ADA2 (22). Therefore, we conclude that the simplest model from our data is that ADA2 is the linchpin in the trimeric complex.

Function of the amino-terminal domain of ADA3. The amino-terminal domain of ADA3 is not required for formation of the ternary complex but clearly is required for function in vivo. Neither slow growth nor the ability of the GCN4 activation domain to function is rescued by the ADA3 amino-terminal domain in a Δ*ada3* strain. What is the role of this domain in transcriptional activation? One clue is provided by the activation properties of a LexA-ADA2 fusion. This fusion is highly dependent on ADA3 for activation at the LexA site. In a Δ*ada3* strain, activation is reduced 3.5-fold, and in a strain with *ADA3* on a 2 μm plasmid, activation is increased 4-fold. Interestingly, the activity of LexA-ADA2 is increased sevenfold in a strain with the carboxyl-terminal domain of ADA3 on a 2 μm plasmid. Further, the carboxyl-terminal domain partially restores activation by LexA-ADA2 in a Δ*ada3* strain. We surmise from these findings that the requirement of the LexA-ADA2 fusion for ADA3 can be met by the carboxyl-terminal domain of ADA3. If activation by LexA-ADA2 reflects the normal activity of the ADA complex when tethered to an activation domain, we can surmise that the amino-terminal domain of ADA3 is not required for this activity.

Thus, there are several possible explanations for the requirement of the amino-terminal domain of ADA3 for activation by LexA-GCN4. In the first model, this domain is required for the interaction between the acidic activation domain of GCN4

(and other activators that use this adaptor) and the ADA complex, as depicted in Fig. 6. Alternatively, the amino-terminal domain may be required to recruit one or more additional subunits to the ADA complex, which are themselves necessary for interaction with activation domains. Our genetic selection for GAL4-VP16 mutants turned up at least one additional gene which we are studying to determine if it is related to *ADA2*, *ADA3*, and *GCN5*.

Model of the ADA complex. Figure 6 shows two parallel pathways, one direct and the other *ADA* dependent, that connect activators to basal factors. Because LexA-GCN4 still has a low level of activity in the absence of the *ADA* genes, we envision that an *ADA*-independent pathway may act in concert with an *ADA*-dependent pathway for activation. However, at this point, it is unclear whether the final target of the ADA complex is the basal factors or some other target such as nucleosomes. We favor the idea that the ADAs interact with basal factors since the transcriptional defect of a strain mutated for *ADA2* has been demonstrated in in vitro transcription experiments in which the template is added as naked DNA (3).

The finding that ADA2, ADA3, and GCN5 comprise a single complex will allow us to relate structural domains that reside in different subunits to a common function. ADA2 contains a Cys-rich domain that is conserved in the mammalian factor CBP (3). The latter protein has been proposed to be a coactivator because it binds to the transcription factor CREB and potentiates its activity (6). GCN5 contains a bromo domain, which is found in several factors in other important transcription complexes, such as TAF250, SWI2, SPT7, and others (17, 32). The bromo domain has been shown to be important in GCN5 function (22) and may exemplify some common function that all of these complexes share.

The *ADA* genes were isolated from *S. cerevisiae* and are required for activator-dependent transcription for a subset of activators including GCN4 and GAL4-VP16. We show in this report that at least three of the ADA proteins form a heteromeric complex. Combined with the observation that the ADA complex binds to activators (29), we believe that we have identified a complex recruited by activators to help transcriptional activation.

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