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# Identification of Human Proteins Functionally Conserved with the Yeast Putative Adaptors ADA2 and GCN5

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**Transcriptional adaptor proteins are required for full function of higher eukaryotic acidic activators in the yeast *Saccharomyces cerevisiae*, suggesting that this pathway of activation is evolutionarily conserved. Consistent with this view, we have identified possible human homologs of yeast ADA2 (yADA2) and yeast GCN5 (yGCN5), components of a putative adaptor complex. While there is overall sequence similarity between the yeast and human proteins, perhaps more significant is conservation of key sequence features with other known adaptors. We show several functional similarities between the human and yeast adaptors. First, as shown for yADA2 and yGCN5, human ADA2 (hADA2) and human GCN5 (hGCN5) interacted in vivo in a yeast two-hybrid assay. Moreover, hGCN5 interacted with yADA2 in this assay, suggesting that the human proteins form similar complexes. Second, both yADA2 and hADA2 contain cryptic activation domains. Third, hGCN5 and yGCN5 had similar stabilizing effects on yADA2 in vivo. Furthermore, the region of yADA2 that interacted with yGCN5 mapped to the amino terminus of yADA2, which is highly conserved in hADA2. Most striking is the behavior of the human proteins in human cells. First, GAL4-hADA2 activated transcription in HeLa cells, and second, either hADA2 or hGCN5 augmented GAL4-VP16 activation. These data indicate that the human proteins correspond to functional homologs of the yeast adaptors, suggesting that these cofactors play a key role in transcriptional activation.**

During activation of RNA polymerase II genes, transcriptional activators, or activator complexes, bound to DNA must functionally interact with the general transcriptional machinery bound to the basal promoter. Direct physical interactions have been demonstrated between activators and basal transcription factors in vitro, such as between the acidic activation domain of herpes simplex virus VP16 (58) and TATA-binding protein (TBP) (56), TFIIB (39), or TFIIH (62). Functional and physical interactions have also been detected between activators and various transcriptional cofactors whose structure and function do not fit the profile of activators or general transcription factors (8, 26, 37, 47, 57). Cofactors of this type have been termed coactivators (48), mediators (32), or adaptors (9).

Coactivators are part of a large complex of proteins, termed TBP-associated factors (20), that are associated with TBP from human (17, 51, 57, 61, 64) *Drosophila* (20), or yeast (49) cells and form the holo-TFIID complex. While TBP alone functions in basal transcription, TBP-associated factors are required for activated transcription in vitro (20). Specific activation domains interact with specific coactivators to reconstitute activated transcription in vitro (14).

In addition to cofactors that appear to be associated with TBP in a stable complex in vivo, there are coactivators or adaptors that are not associated in complexes with TBP. Biochemical fractionation of human cell extracts has revealed specific activities which either stimulate or repress activated transcription in vitro. One positive cofactor has been cloned (PC4 [23] or P11 [33]) and has attributes of a coactivator for several types of activation domains.

Other approaches have been used to identify cofactors for specific activation domains. Direct screening of expression libraries with the activation domain of human CREB has led to the isolation of a CREB-binding protein (CBP) which has characteristics of a coactivator (16, 34). Interestingly, CBP is very similar to p300, a protein that interacts with the 12S form of adenovirus E1A (41). Recently CBP and p300 have been shown to be interchangeable in vivo for stimulation of CREB transcriptional activation (41), and both bind to E1A (5, 41).

Genetic studies of the yeast *Saccharomyces cerevisiae* have been critical in the discovery of novel classes of factors involved in eukaryotic transcription. For example, transcriptional activators that bind upstream of structural genes were identified in *S. cerevisiae*, and subsequent studies in higher eukaryotes underscored their key importance in complex processes of development and differentiation (for reviews, see references 45 and 46). Another example is the yeast SWI-SNF complex (43), which, on the basis of genetic studies, was proposed to alter chromatin structure to facilitate transcription (61). Biochemical analysis of a human homolog (31, 35) of the yeast SWI-SNF complex (18) has yielded new insight into its role in reorganization of chromatin during transcriptional activation.

In previous work, we designed a genetic selection in yeast cells to identify cofactors which functionally interact with the acidic activation domain of herpes simplex virus VP16 (10). Several genes, termed *ADA2* (10), *ADA3* (44), and *GCN5* (24, 42), were cloned. ADA2, ADA3, and GCN5 interacted in a complex in vitro (30, 42) and had transcriptional properties suggestive of adaptor function (10, 42, 44). For example, mutations in *ADA2* affected transcriptional activation in vivo and in vitro by acidic activators including VP16 and yeast GCN4 but did not affect activation by the acidic activator HAP4 (10, 44). This functional specificity was reflected in physical interactions between ADA2 and activation domains derived from VP16 and GCN4 but not HAP4 (8). Also, ADA2 was transcriptionally active as a bacterial LexA DNA-binding domain fusion (54), which suggested that ADA2 might interact with

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the basal machinery. In fact, ADA2 interacted with TBP in vitro and was required for interaction between VP16 and TBP in yeast nuclear extracts (8). These data suggest that the adaptor complex is required for physical interaction between activation domains and the basal transcription apparatus.

Here we report the discovery of human proteins (hADA2 and hGCN5) similar in sequence and function to the yeast adaptors ADA2 and GCN5 (yADA2 and yGCN5). We find that hADA2 and hGCN5 interact with each other in yeast cells similarly to the yeast adaptors. Both hADA2 and hGCN5 potentiate activation by GAL4-VP16 in HeLa cells. These and other results presented here suggest that the human proteins are functional homologs of the yeast adaptors.

## MATERIALS AND METHODS

**Screening of database and isolation of full-length hADA2 and hGCN5.** Partial sequences displaying similarity to y*ADA2* and y*GCN5* were identified by searching a database of human cDNA expressed sequence tags (ESTs), using the BLAST network service provided by the National Center for Biotechnology Information. Full-length clones were isolated by hybridization screening (7) of a randomly primed testis library (Clontech) by using one of the respective ESTs as a probe. *hADA2* was subcloned into pBLSK2− (Stratagene) as an *Eco*RI-*Xho*I fragment. *hGCN5* was subcloned into pSP72 as an *Eco*RI fragment. Full-length clones were subjected to multiple rounds of sequencing in both directions to ensure accuracy. In the case of *hADA2*, a stop codon lies three codons upstream of the initiation codon of the putative open reading frame.

**Yeast strains.** The *trp1* derivatives of strain PSY316 (10) and its *ADA2* deletion derivative were generated by transformation with pNKY1009 digested with *Eco*RI and *Bgl*II as described previously (3). *GCN5* deletion strains were generated by transforming PSY316 and PSY316Δ*ada2* with plasmid p*yGCN5*.KO (see below), digested with *Xho*I and *Bgl*II, as described previously (3).

The two-hybrid interaction assay (22) was done with strain L40 (59). The LexA-ADA2 assays were done with the *ada2 trp1* deletion derivative of PSY316. The immunoblot assay to test stabilization of LexA-yADA2 by hGCN5 or yGCN5 was done with the *ada2* deletion derivative of PSY316 and the *ada2 gcn5* deletion derivative of PSY316. β-Galactosidase (β-Gal) assays were performed as described previously (50).

**Plasmids.** Standard procedures were used for construction of plasmids (7).

p*yGCN5*.KO was constructed in three steps. First, the *yGCN5* gene including flanking sequences (24) was generated by PCR using yeast genomic DNA, with an *Xho*I site at the 5′ end and a *Bgl*II site at the 3′ end, and the gene was cloned into the polylinker of pSP72 (Promega) to yield pSP72-*yGCN5*. Second, pSP72-*yGCN5* was digested with *Hin*dIII, and a synthetic *Hin*dIII-*Sal*I-*Hin*dIII linker was inserted to yield pSP72-*yGCN5*S. Finally, pSP72-*yGCN5*S was cut with *Bam*HI and *Sal*I, and the *hisG-URA3-hisG* cassette (3) was ligated to yield the final plasmid, p*yGCN5*.KO.

pBTM116-NotI was generated by cloning an *Eco*RI-*Not*I-*Bgl*II-*Eco*RI-*Bam*HI linker in pBTM116 (59) digested with *Eco*RI and *Bam*HI. The *Eco*RI restriction site of pBTM116 was destroyed as a consequence of the insertion, and the *Bam*HI restriction site was regenerated. For plexA-*hGCN5* and plexA-*yGCN5*, PCR fragments of *hGCN5* or *yGCN5* (from phage subclone or yeast genomic DNA, respectively) bearing *Not*I and *Eag*I restriction sites were cut with *Eag*I and cloned into the *Not*I site of pBTM116-NotI.

To make plexA-*hADA2* or plexA-*hADA2*(1-148), full-length *hADA2* or the amino-terminal domain of *hADA2* (containing residues 1 to 148) with a 5′ *Bam*HI restriction site and a 3′ *Pst*I restriction site at its 3′ end was amplified by PCR. The fragments were digested and ligated in frame into pBTM116 cleaved with *Bam*HI and *Pst*I.

To generate plexA-*yADA2*, the amino terminus of yADA2 (amino acids 1 to 167) and its carboxyl terminus (amino acids 167 to 433) bearing a *Not*I restriction site at the 5′ end and an *Eag*I restriction site at the 3′ end were amplified by PCR. These fragments were digested with *Eag*I and inserted in pDB20-lexA (44) opened with *Not*I.

The reporter containing eight LexA DNA-binding sites upstream of the bacterial *lacZ* gene was a derivative of pSH18-34 (27). The *URA3* gene was removed as follows. pSH18-34 was digested with *Stu*I, and a *Not*I linker was inserted in its place, yielding pSH18-34-NotI. The *LEU2* gene was isolated from pSD5-*LEU2* (gift of N. Silverman) digested with *Not*I and cloned in the *Not*I site of pSH18-34-NotI.

pVP16-*yADA2* was generated by amplifying *yADA2* bearing *Bgl*II ends by PCR. The resulting fragment was digested with *Bgl*II and cloned into the *Bam*HI site of pVP16 (59). To generate pVP16-*hADA2*, the *Bam*HI-*Eco*RI fragment from plexA-*hADA2* was inserted into pVP16 opened with *Bam*HI and *Eco*RI.

The constructs for in vitro translation were made as follows. PCR fragments of *ADA3* and *yGCN5* bearing *Bam*HI restriction sites or *yADA2* bearing *Bgl*II restriction sites were cloned into pSP64 (Pharmacia) cleaved with *Bam*HI. These three genes were also cloned in a yeast expression vector and were functional, as tested by complementation in yeast strain PSY316Δ*ada2*, PSY316Δ*ada3*, or PSY316Δ*gcn5*. pSP64-NotI was generated by cloning a *Not*I linker in pSP64 digested with *Sma*I. To make pSP64-*yADA2*(1-167) and pSP64-*yADA2*(167-433), the corresponding fragments of *yADA2* were isolated by digesting plexA-*yADA2*(1-167) or plexA-*yADA2*(167-433) with *Eag*I and were cloned into the *Not*I site of pSP64-NotI.

The pGST-*yADA2* deletion mutants were made as follows. To make pGST-*yADA2*, *yADA2* bearing *Bgl*II ends was amplified by PCR, and the fragment was digested with *Bgl*II and inserted into pGEX3X (Pharmacia) opened with *Bam*HI. The deletion mutants of pGST-*yADA2* were obtained by using the two *Eco*RI sites located within *yADA2* and the third *Eco*RI site located downstream of the insert in the polylinker of pGEX3X. pGST-*yADA2* was cut with *Eco*RI and religated, generating pGST-*yADA2* N (1-175). The fragments corresponding to the middle and carboxyl-terminal domains of *yADA2* that result from this digestion were isolated and ligated in frame into pGEX5X-1 (Pharmacia) opened with *Eco*RI to render pGST-*yADA2* M (174-332) and into pGEX3X opened with *Eco*RI in the case of pGST-*yADA2* C (330-434).

**In vitro translation and coimmunoprecipitation assays.** In vitro translation assays with reticulocyte extracts were performed as instructed by the manufacturer (TNT kit; Pharmacia). Twenty microliters of the slurry of Gammabind-Sepharose beads (Pharmacia) was washed two times and resuspended in 20 μl of buffer A (10 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES; pH 7.6], 300 mM potassium acetate, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, 0.6 μM leupeptin, 2 μg of chymostatin per ml, 2 mM benzamidine). Ten microliters of reticulocyte extract containing cotranslated proteins and 2 μl of a yADA2 antiserum were added to the beads, mixed, and rotated for 3 h at 4°C. The reaction mixtures were centrifuged for 2 min at 4,000 rpm, and the supernatant was removed and washed three times with 1 ml of buffer A. Following the last wash, the beads were resuspended in 50 μl of Laemmli buffer, heated for 3 min at 85°C, and loaded onto sodium dodecyl sulfate (SDS)–8% polyacrylamide gels. The gels were dried and exposed overnight on Kodak film.

**Bacterial expression and purification of yGCN5 and preparation of antisera.** The *yGCN5* gene was amplified from genomic DNA by PCR using primers that generated a 5′ *Xho*I site and a 3′ *Hin*dIII site. After digestion, the DNA fragment was ligated into the *Xho*I and *Hin*dIII sites of pRSETA vector (Invitrogen), which contains six histidine residues fused to the N terminus. The resulting construct was transformed into *Escherichia coli* JM109 and induced with isopropylthiogalactopyranoside (IPTG) and M13/T7 phage, using the XPRESS system (Invitrogen). yGCN5 protein was isolated under denaturing conditions and purified (using the XPRESS system) on Ni-nitrilotriacetic acid-agarose (Qiagen). Purified protein fractions were concentrated by using Centricon 10 columns (Amicon) and run on an SDS-polyacrylamide gel. Gel fragments containing yGCN5 were isolated and sent to Cocalico Biologicals, Inc., for rabbit inoculation. Serum was collected following three booster injections and checked against the original recombinant protein and endogenous yGCN5. yADA2 (54) and LexA (63) antisera were described previously.

**Immunoblots.** Yeast extracts prepared as described for the β-Gal assays were analyzed on SDS–8 or 10% polyacrylamide gels and immunoprobed by using the LexA or yGCN5 antiserum and standard techniques (28). Mammalian whole-cell extracts were used for determination of GAL4-VP16 protein levels, using a GAL4 monoclonal antiserum (Santa Cruz). Immunodetection was performed with an ECL (enhanced chemiluminescence) kit (Amersham).

**Binding of recombinant yGCN5 to GST-yADA2 fusions.** One microliter of bacterial lysate (50 mg/ml) containing overexpressed yGCN5 protein was adjusted to 150 μl with 1× binding buffer (20 mM HEPES [pH 7.8], 10 mM MgCl, 100 mM NaCl, 15% glycerol, 1 mM phenylmethylsulfonyl fluoride) and added to glutathione-Sepharose beads (Pharmacia) coupled to either glutathione *S*-transferase (GST) alone or GST-yADA2 N (1-175), GST-yADA2 M (174-332), or GST-yADA2 C (330-434). The mix was rotated for 1.5 h at 4°C. After extensive washes with binding buffer, beads were washed in the same buffer containing 0.25 M NaCl. Material remaining on beads was eluted with Laemmli buffer and analyzed by immunoblotting with the yGCN5 antiserum.

**Transient-transfection experiments in HeLa cells and chloramphenicol acetyltransferase (CAT) assays.** Plasmids for mammalian cell expression of the human adaptors were constructed in pcDNA3 (Invitrogen). *hGCN5* was cloned as an *Hin*dIII-*Eco*RI fragment from pSP72-*hGCN5*. *hADA2* was cloned as an *Apa*I-*Hin*dIII fragment from pBLSK-*hADA2*.

The GAL4 DNA-binding domain was expressed from pM1 (52). *hADA2* was cloned into pM1 as a *Bam*HI-*Pst*I fragment from plexA-*hADA2*.

*GAL4-hADA2* was cotransfected with E1bCAT, containing five tandem repeats of the GAL4 DNA-binding site (13). GAL4-VP16 was cotransfected with pBLCAT2 (40), containing five tandem repeats of the GAL4 DNA-binding site.

DEAE-dextran transfections were performed basically as described previously (7). Cells ($1.5 \times 10^5$) were plated in a 3.5-cm-diameter plate 24 h prior to transfection. Plasmids in Dulbecco modified Eagle medium–0.5 μg of DEAE-dextran per ml–50 μg of chloroquine per ml were incubated with cells for 2.75 h and then subjected to shock with Dulbecco modified Eagle medium–10% dimethyl sulfoxide for 2.5 min. Cells were harvested 48 h after transfection.

CAT assays were performed as described previously (7).


FIG. 1. (A) Amino acid sequence of the *hADA2* cDNA (hADA2) aligned with that of its yeast counterpart, *yADA2* (10). Lines between amino acids represent identity, while dots represent similarity. For this alignment, similar amino acids are grouped as follows: T, S, A, G, and C; L, I, V, and M; E, D, N, and Q; R, K, and H; F, Y, and W. The alignment was created by using the TFASTA algorithm and improved by visual inspection. Over a 423-amino-acid overlap (gaps not included), the two proteins demonstrate 31% identity and 53% similarity. (B) Alignment of the cysteine-rich regions of hADA2, yADA2, CBP (16), and p300 (21). The amino acids in either yADA2 or hADA2 showing identity to CBP and p300 are in boldface, and those conserved among all four proteins are denoted by asterisks. (C) The amino acids showing conservation between mouse Myb (mMYB) (36) and either hADA2 or yADA2 are in boldface, while residues conserved among all three proteins are denoted by asterisks.

## RESULTS

**Putative human homologs of yADA2 and yGCN5.** Acidic activators are highly conserved in function between *S. cerevisiae* and humans. We previously used a chimeric activator, GAL4-VP16, containing the yeast GAL4 DNA-binding domain fused to the herpes simplex virus VP16 acidic activation domain, in a genetic selection in *S. cerevisiae* (10) and isolated mutations in three genes, *ADA2*, *ADA3*, and *GCN5*, which reduced the function of GAL4-VP16 (10, 42, 44). Mutations in these genes also reduced activation in yeast cells of transcriptional activators from human cells, including NF-κB (11) and p53 (19a). Since mutations in *yADA2*, *yADA3*, and *yGCN5* affected human activators, it seemed possible that human homologs of these putative adaptors might exist. Therefore, we screened a database of human-expressed sequences for similar cDNAs.

A database of ESTs (1, 2) generated from multiple human tissues at Human Genome Sciences and The Institute of Genomic Research was searched for human sequence homologs of yADA2 and yGCN5, using the BLAST algorithm (4). In the case of hADA2, three independent overlapping ESTs displaying sequence conservation were identified. As none of the ESTs showing similarity to yADA2 appeared to be full length, longer clones were isolated by hybridization screening of a randomly primed human testis library. Sequence analyses of one of these longer clones revealed an open reading frame of 443 amino acids. This clone, designated *hADA2*, was used for further studies. With regard to hGCN5, several overlapping ESTs were identified as having strong sequence conservation. Again none of these clones appeared to be full-length, so hybridization screening was performed on the same human testis library to identify a full-length clone (*hGCN5*).

The amino acid sequences of yADA2 and hADA2 have 31% identity and 53% similarity (Fig. 1). It is interesting that the most conserved region is at the amino terminus, where regions of similarity between yADA2 and other proteins had been previously noted (10). At the extreme amino terminus is a conserved Cys-rich region, which is a putative Zn-binding domain; a region similar to this has also been noted in the putative human adaptors CBP and p300 (6). While the cysteines are conserved among all four proteins (Fig. 1B), the overall conservation within the Cys-rich region is actually better between yADA2 and CBP or p300 than between yADA2 and hADA2. However, the similarity between yADA2 and CBP or p300 is limited to this region, while yADA2 and hADA2 show similarity along the entire protein. For example, carboxyl to the Cys-rich region is a second region of strong similarity between yADA2 and hADA2, which is similar to a region within the *myb* proto-oncogene product (Fig. 1C) (10). Notably, conserved residues among different members of the Myb family

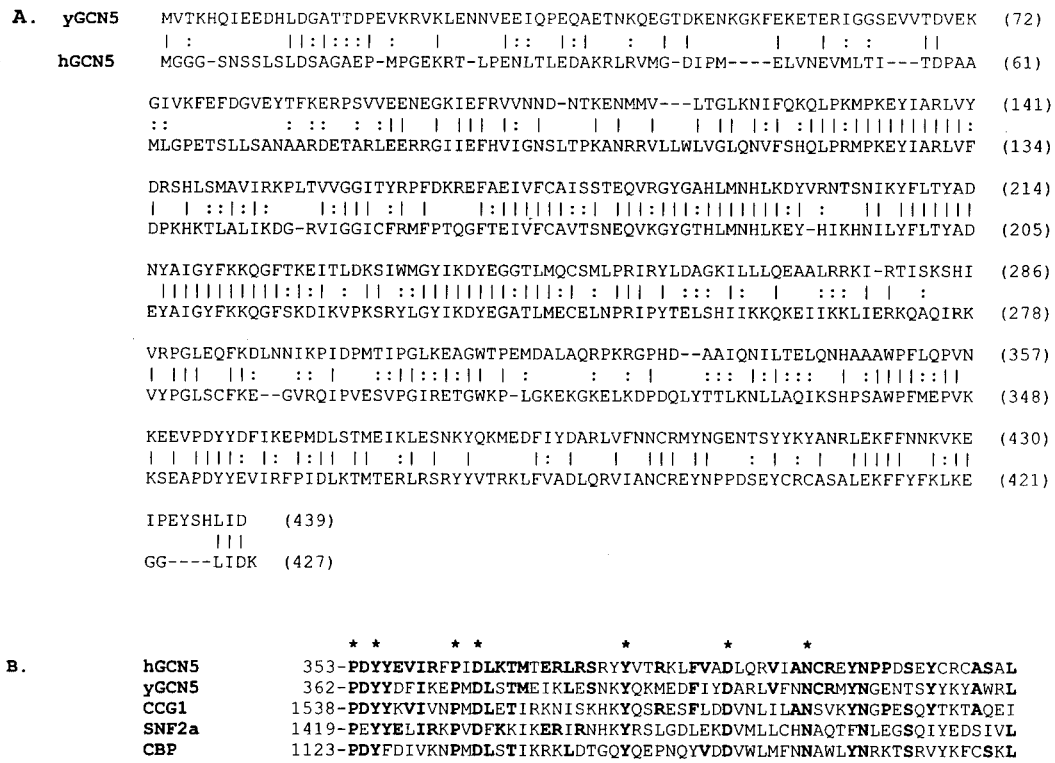


FIG. 2. (A) Amino acid sequence of the *hGCN5* cDNA (hGCN5) aligned with that of its yeast counterpart, *yGCN5* (24). Over a 419-amino-acid overlap (gaps not included), the two proteins demonstrate 43% identity and 62.5% similarity. Amino acids are grouped as in Fig. 1A. (B) The bromo domain regions of hGCN5, yGCN5 (24), CCG1 (29, 51, 53), SNF2α (15), and CBP (16) are aligned. Amino acids showing identity to the corresponding residues in hGCN5 are in boldface. Amino acids conserved among all five proteins are designated with asterisks.

are also found in both yADA2 (10) and hADA2. While the similarity between hADA2 and yADA2 is less marked through the remainder of the proteins, it is striking that throughout the proteins there is clear sequence conservation and that their overall sizes are similar.

yGCN5 and hGCN5 are 43% identical and 62.5% similar (Fig. 2A). In the central part of the protein the identity is particularly high: 60% identity and 76% similarity between residues 94 and 260 relative to the yeast protein. The carboxyl-terminal similarity is not as high as in the central region, but residues previously noted to be conserved between the bromo domain of yGCN5 and other transcription factors (24) are also conserved in hGCN5 (Fig. 2B). The amino terminus is more diverged between the yeast and human proteins, especially upstream of amino acid 94 in yGCN5. Two independent cDNA clones of *hGCN5* were sequenced in this upstream diverged region and were found to be identical (data not shown), suggesting that the dissimilarity in the amino termini between yGCN5 and hGCN5 is not a cloning artifact.

Functional homology of hADA2 and hGCN5 to their yeast counterparts was tested by complementation of growth deficiencies on minimal media of *ada2*, *gcn5*, or *ada2 gcn5* deletion strains (10, 42, 44). Neither low- nor high-copy-number plasmids carrying the genes complemented the growth defects (data not shown). Quantitative tests of transcriptional activation by GCN4 in the deletion strains (10, 42) also showed a failure of the human genes to complement (data not shown). Because the amino termini of yGCN5 and hGCN5 are divergent (Fig. 2A), the amino terminus of hGCN5 upstream of amino acid 82 was replaced with the equivalent region of yGCN5. The chimera did not complement the *gcn5* deletion strain for growth (data not shown). Moreover, a fusion protein in which the bromo domain of yGCN5 (24) replaced that of hGCN5 also failed to complement (data not shown). These results were not due to lack of expression of the human or chimeric human-yeast adaptors, since Western blot (immunoblot) analysis showed that the proteins were stable in yeast cells (data not shown).

**hGCN5 interacts with hADA2 and yADA2.** yADA2 and yGCN5 interact in vivo (42) in a yeast two-hybrid interaction system (22). To determine whether hADA2 and hGCN5 interact, we fused hGCN5 to the bacterial LexA DNA-binding domain (12) and hADA2 to the herpes simplex virus VP16 activation domain and tested their interaction after yeast cotransformation. LexA-hGCN5 interacted with hADA2-VP16, as shown by its ability to activate expression of the *lacZ* gene reporter (Fig. 3A, row 2). Neither hADA2 or VP16 by itself activated the reporter when cotransformed with *lexA-hGCN5* (rows 3 and 4). The extent of activation induced by interaction of the human proteins was quantitatively similar to that induced by interaction of LexA-yGCN5 and yADA2-VP16 (row 5). Thus, as found for the yeast proteins, hGCN5 and hADA2 interacted in vivo.

Next, interactions were tested across species. While LexA-hGCN5 and yADA2-VP16 interacted in the assay (Fig. 3A, row 8), LexA-yGCN5 did not interact with hADA2-VP16 (row 10). These interactions between hGCN5 and hADA2 or yADA2, as well as other evidence described below, suggest that the human genes are functional homologs of the yeast genes. As a control, we used immunoblot analysis to examine LexA-GCN5 proteins in the presence of the various VP16 fusions. LexA-hGCN5 (Fig. 3B, top) and LexA-yGCN5 (Fig.

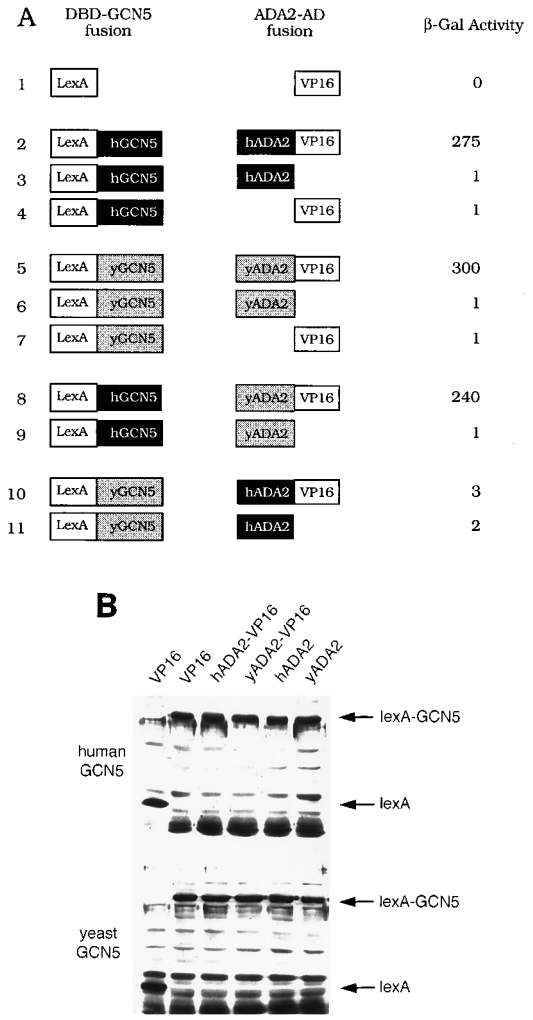


FIG. 3. (A) Two-hybrid interaction assay between ADA2 and GCN5. yGCN5 or hGCN5 fused to the bacterial LexA DNA-binding domain was cotransformed into yeast cells with yADA2 or hADA2 fused to the VP16 activation domain (AD). As controls, the LexA-GCN5 fusions were tested with either ADA2 or VP16. The LexA DNA-binding domain was cotransformed with the VP16 activation domain as an additional negative control. The reporter contained eight LexA DNA-binding sites upstream of the bacterial *lacZ* gene. β-Gal activity was determined as units per milligram of protein and represents an average of at least four independent transformants; the error varied between 5 and 16%, with a standard error average of 8%. (B) Immunoblot analysis of LexA-GCN5. The cell extracts used for the β-Gal assay in panel A were tested for the level of LexA fusions to either hGCN5 (top) or yGCN5 (bottom) or LexA alone (lane 1, top and bottom), using a LexA antiserum. Along the top are listed the two-hybrid activation domain components in the assay in panel A. Arrows on the right indicate the LexA or LexA-hGCN5 proteins. Bands in all samples that are not indicated by arrows are likely to be antigenically related cellular proteins.

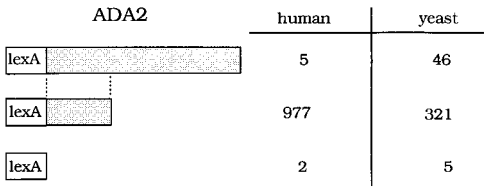


FIG. 4. β-Gal activity of LexA-ADA2 fusions. yADA2 or hADA2 was fused to the LexA DNA-binding domain. In both cases, either full-length ADA2 or the amino-terminal region of ADA2 was included in the LexA fusion. The amino-terminal truncation of hADA2 contained amino acids 1 to 147, and that of yADA2 contained amino acids 1 to 166. The LexA DNA-binding domain was used as a control. The reporter contained eight LexA DNA-binding sites upstream of the bacterial *lacZ* gene. β-Gal activity was determined as units per milligram of protein and represents an average of at least two independent transformants; the error varied between 0 and 26%, with a standard error average of 9%. The amounts of LexA fusion protein in all samples were comparable, as determined by immunoblotting with a LexA antiserum (data not shown).

3B, bottom) were equally stable in all conditions tested. Thus, the differences observed in β-Gal activity were not due to alterations in levels of proteins but rather were due to interactions between proteins.

**hADA2 and yADA2 have functional similarities.** yADA2 activates transcription in yeast cells as a bacterial LexA DNA-binding domain fusion (54), suggesting that yADA2 interacts with the basal machinery. We therefore wished to determine whether hADA2 was similarly active in yeast cells as a LexA DNA-binding domain fusion. To this end, either full-length hADA2, yADA2, or amino-terminal truncations were fused to LexA and tested for activity in yeast cells (Fig. 4). We focused on the amino termini because of the strong sequence conservation between hADA2 and yADA2 in this region. As seen before, the LexA fusion to full-length yADA2 was a weak activator. In contrast, the full-length hADA2 fusion was inactive. Surprisingly, both the yeast and human amino-terminally truncated fusions of ADA2 had strong activity. These results suggest that the amino termini of both yADA2 and hADA2 contain cryptic activation domains. Furthermore, these activation domains appear to be masked in both the human and yeast full-length proteins, although in the human protein this inhibition by the carboxyl terminus was more pronounced.

**Interaction of yADA2 with yGCN5 and yADA3.** It has previously been shown that yADA2 interacts with both yGCN5 and yADA3 in a putative adaptor complex in vitro (30, 42). Since the amino termini of yADA2 and hADA2 show a high degree of similarity in sequence and function, it is possible that interactions with other components in the complex occur through the amino terminus. Therefore, full-length yADA2 or a truncated protein (amino acids 1 to 166 or 167 to 434) was translated in vitro to check stability (Fig. 5A) and then cotranslated with either yGCN5 or yADA3, and complexes were immunoprecipitated with a yADA2 antiserum. As previously seen (30, 42), full-length yADA2 immunoprecipitated yGCN5 (Fig. 5B) or yADA3 (Fig. 5C). The 1–166 region of yADA2 immunoprecipitated yGCN5 but not yADA3, while the 167–434 region of yADA2 immunoprecipitated yADA3 but not yGCN5. Thus, yADA2 appears to have distinct and separable domains of interaction with other components of the adaptor complex. yGCN5 interacts with the amino terminus of yADA2, which is highly conserved in sequence with hADA2.

Since yGCN5 coimmunoprecipitates with the amino terminus of yADA2, we wished to test whether this interaction was direct. GST fusions (55) were constructed with full-length yADA2 or subdomains of yADA2, composed of either the amino terminus (amino acids 1 to 176), the middle region (amino acids 176 to 333), or the carboxyl terminus (amino acids 333 to 434) (Fig. 6A). The GST fusions were expressed in bacteria and immobilized on glutathione-Sepharose beads. Equal amounts of all GST-yADA2 fusions (Fig. 6B) were incubated with purified yGCN5 protein, and bound protein was immunoblotted with a yGCN5 antiserum (Fig. 6C). Purified yGCN5 specifically interacted with both full-length yADA2 and the amino terminus but not with either the carboxyl terminus or the middle of yADA2. These results confirm the result of the immunoprecipitation assay, that yGCN5 interacts

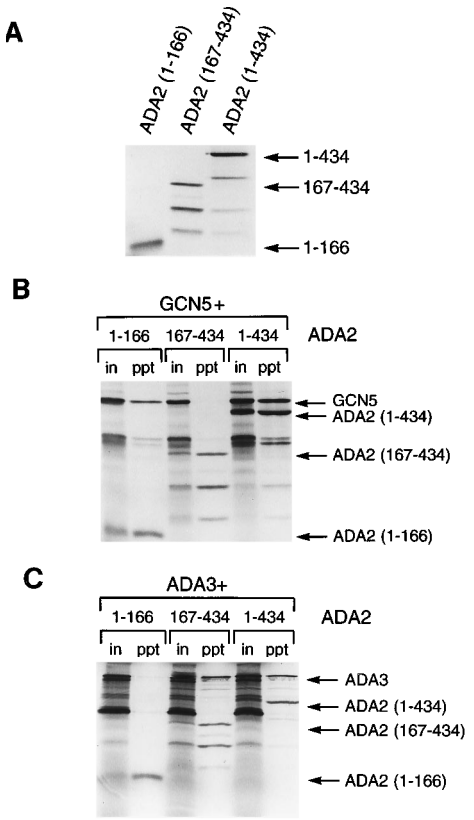


FIG. 5. Coimmunoprecipitation analysis of yADA2 with yGCN5 or yADA3. (A) In vitro translation of yADA2 peptides. Full-length yADA2 (amino acids 1 to 434), the yADA2 amino terminus (amino acids 1 to 166), or the yADA2 carboxyl terminus (amino acids 167 to 434) was translated in vitro and precipitated with a yADA2 antiserum. $^{35}$S-labeled proteins were visualized by autoradiography after electrophoresis on an SDS–12% polyacrylamide gel. Arrows on the right indicate the yADA2 peptides. (B) The amino terminus of yADA2 interacts with yGCN5. yGCN5 was cotranslated with each of the yADA2 peptides shown in panel A. Cotranslated proteins were immunoprecipitated with a yADA2 antiserum, and $^{35}$S-labeled proteins were visualized by autoradiography after electrophoresis on an SDS–10% polyacrylamide gel. In each case, input (in) was 50% of the material used in the immunoprecipitation reaction (ppt). Arrows on the right indicate the yADA2 peptides and yGCN5. (C) The carboxyl terminus of yADA2 interacts with yADA3. yADA3 was cotranslated with each of the yADA2 peptides shown in panel A. Cotranslated proteins were immunoprecipitated with a yADA2 antiserum, and $^{35}$S-labeled proteins were visualized by autoradiography after electrophoresis on an SDS–10% polyacrylamide gel. In each case, input (in) was 50% of the material used in the immunoprecipitation reaction (ppt). Arrows on the right side indicate the yADA2 peptides and yADA3.

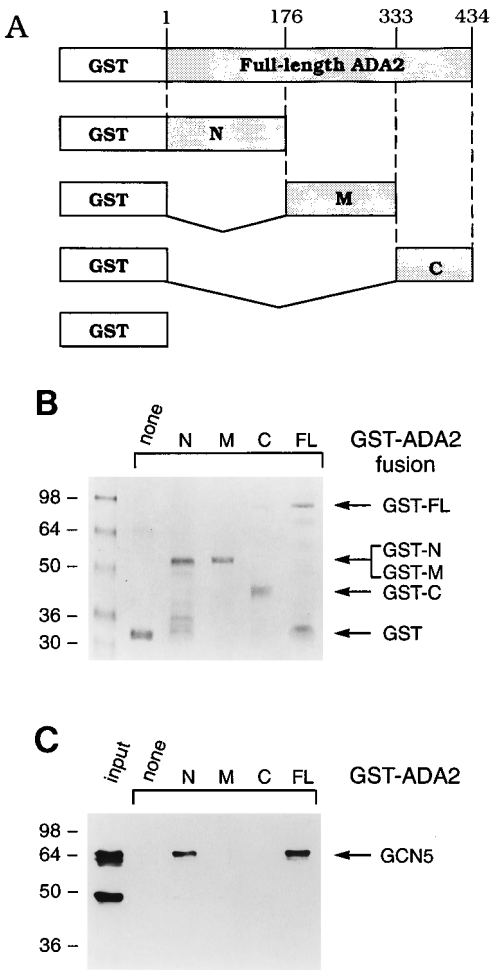


FIG. 6. Binding of purified yGCN5 to GST-yADA2. (A) Schematic of GST fusions to yADA2 and its deletion derivatives. Full-length yADA2 comprises amino acids 1 to 434. Structures of deleted versions of yADA2, 1-176 (N), 176-333 (M), and 333-434 (C), are shown below. (B) Coomassie blue staining of GST-activation domain fusion proteins. Each lane represents a sample of bead preparation that was equal to the amount used for the binding assay in panel C. Lanes: 1, protein size markers; 2, GST alone; 3, GST-yADA2(1-176) (N); 4, GST-yADA2(333-434) (C); 5, GST-yADA2 (full length) (FL). (C) Immunoblot analysis of purified yGCN5 binding to GST fusions to yADA2 (full-length and deletion derivatives). Purified yGCN5 was incubated with the GST fusions or GST alone, as shown in panel B. Material remaining on beads after washing was immunoprobed with a yGCN5 antiserum. Lane input represents 70% of the total material used in the GST fusion binding assay. Positions of protein size standards are shown on the left in kilodaltons.

with the evolutionarily conserved amino terminus of yADA2, and further show that yADA2 and yGCN5 interact directly.

**Both yGCN5 and hGCN5 stabilize LexA-yADA2 in vivo.** As shown above, yGCN5 coimmunoprecipitated with the amino terminus of yADA2 and interacted directly with this region of yADA2. To determine the relevance of these interactions in vivo, we tested whether yGCN5 affects the stability of yADA2. Therefore, the levels of various LexA-yADA2 fusion proteins were examined in the absence or presence of endogenous levels of yGCN5 (Fig. 7A). The protein amount of LexA-yADA2(1-434), containing full-length yADA2, was dramatically affected by yGCN5. The level of LexA-yADA2(1-166) was similarly affected by yGCN5, whereas LexA-yADA2(167-434) was not affected by yGCN5. Longer exposure of the immunoblot revealed the same amount of LexA-yADA2(167-434) in the presence or absence of yGCN5 (not shown). As a control, the amount of LexA protein was not altered by yGCN5, showing that yGCN5 did not affect the level of expression of the proteins and that yGCN5 specifically stabilized yADA2. Thus, yGCN5 functionally interacted with the amino terminus of yADA2 both in vitro and in vivo.

Since yGCN5 stabilized LexA-yADA2, and since hGCN5 interacted with yADA2 in the two-hybrid test, we wished to examine the ability of hGCN5 to stabilize yADA2. The level of protein of LexA-yADA2 (amino terminus) was assayed in the presence of yGCN5, hGCN5, or no GCN5. As shown for yGCN5 (Fig. 7A and B), hGCN5 strongly stabilized LexA-yADA2 (Fig. 7B). The amount of LexA protein was constant in every case. Thus, hGCN5 and yGCN5 had similar abilities to stabilize yADA2, which suggests that hGCN5, like yGCN5,

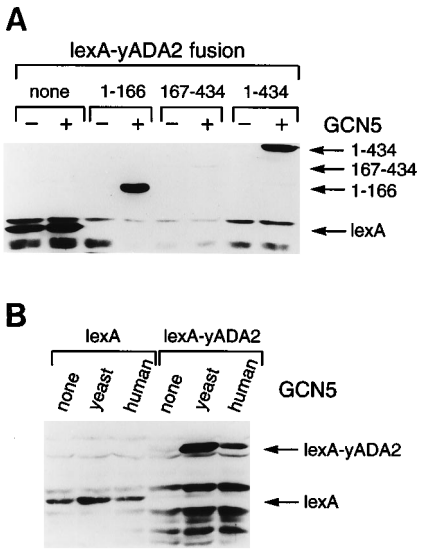


FIG. 7. yGCN5 or hGCN5 stabilizes LexA-yADA2 protein. (A) Immunoblot of LexA-yADA2 fusion proteins. LexA fusions to either full-length yADA2 (1-434), the amino terminus (1-166), or the carboxyl terminus (167-434) were cotransformed into yeast cells in the presence or absence of endogenous *yGCN5*. Whole cell extracts were analyzed by electrophoresis on SDS–10% polyacrylamide gels and immunoblotted with a LexA antiserum. Arrows indicate the LexA-yADA2 peptides. LexA alone was included as a control. (B) Immunoblot of LexA-yADA2 in the presence of yGCN5 or hGCN5. LexA-yADA2(1-166) or LexA alone was cotransformed with *yGCN5*, *hGCN5*, or vector alone. Whole-cell extracts were analyzed by electrophoresis on SDS–10% polyacrylamide gels and immunoblotted with a LexA antiserum. Arrows indicate the LexA-yADA2 peptide and LexA alone as a control. *yGCN5* or *hGCN5* was expressed from the yeast *ADH1* promoter in a low-copy-number plasmid.

interacts with the amino terminus of yADA2. This result suggests conservation in function of yGCN5 and hGCN5.

**Putative human adaptors are transcriptionally active in HeLa cells.** yADA2 fused to the LexA DNA-binding domain activated transcription in yeast cells; therefore, we wished to determine whether hADA2 has similar properties in human cells. To test this, *hADA2* was genetically fused to the yeast GAL4 DNA-binding domain and transfected into HeLa cells. GAL4-hADA2 activation of a CAT reporter containing five GAL4 DNA-binding sites was assayed. GAL4-hADA2 activated transcription approximately 20-fold over the GAL4 DNA-binding domain negative control (Fig. 8A). This activity of GAL4-hADA2 in human cells paralleled the behavior of LexA-yADA2 in yeast cells.

Since the yeast adaptors were required for full function of GAL4-VP16 in yeast cells, we tested the effect of the human adaptors on activation by GAL4-VP16 in HeLa cells. The human genes, *hADA2* and *hGCN5*, were cloned into mammalian expression vectors and cotransfected with variable amounts of GAL4-VP16 and a CAT reporter containing GAL4 DNA-binding sites. Either hADA2 or hGCN5 potentiated GAL4-VP16 activity (Fig. 8B). The strongest effect of the adaptors (approximately 10-fold) was seen at an intermediate GAL4-VP16 concentration (25 ng), and the effect of the adaptors was lower (3- to 5-fold) when the GAL4-VP16 concentration was reduced (5 ng; Fig. 8B). The human adaptors affected GAL4-VP16 activity similarly at an even smaller (1 ng) or larger (100 ng) amount of transfected GAL4-VP16 (data not shown). The adaptors did not alter the intracellular concentration of GAL4-VP16 (Fig. 8C). Thus, consistent with a role as adaptors, and as previously observed for the yeast putative adaptors, the human proteins increased transcriptional activation by GAL4-VP16.

We previously observed specificity of the effects of yADA2 on activity of different activation domains in yeast cells (8, 10). To determine possible specificity of hADA2 action in HeLa cells, we compared the effect of hADA2 on GAL4-E1A (38) with its effect on GAL4-VP16. The adenovirus E1A activation domain forms a Zn finger (19, 60) and thus apparently is dissimilar in structure from VP16, suggesting possible dissimilarity in function with regard to the human adaptors. As before, hADA2 potentiated GAL4-VP16 activity, while similar amounts of hADA2 had nearly no effect on GAL4-E1A activation (Fig. 8C). Thus, hADA2 did not affect either the plasmid reporter construct or the DNA-binding domain used in these experiments, and moreover, hADA2 had a specific effect on the VP16 activation domain.

## DISCUSSION

The process of transcriptional activation is strongly conserved in higher eukaryotes. The transcriptional activation domain derived from herpes simplex virus VP16 has potent activity in yeast cells which is dependent on the yeast putative adaptor proteins ADA2, GCN5, and ADA3 (10, 42, 44). Dependence on adaptors in yeast cells has also been demonstrated for NF-κB (11) and for p53 (19a), two human transcriptional activators containing acidic activation domains. The dependence of mammalian activators on yeast adaptors suggests evolutionary conservation of adaptor function and prompted us to search for human adaptor homologs.

We have identified human proteins containing strong sequence similarity to the yeast putative adaptors ADA2 and GCN5. Both human proteins display strong conservation of short sequences that were recognized previously to be of potential importance in the yeast proteins. For example, yADA2 (10) and hADA2 have cysteine-rich regions, which may be Zn-binding domains, at their amino termini. Both ADA2 proteins also have a region strongly resembling a sequence in the *myb* oncogene product. yGCN5 (24) and hGCN5 have a highly conserved region, termed a bromo domain, at their carboxyl termini. These regions have been identified in other transcription factors and also in factors in the coactivator/adaptor class (see, for example, references 16 and 24). The functional importance of each of these regions has been established for the yeast proteins. The yADA2 Myb region (12a) and yGCN5 bromo domain (42) are required for full complementation in deletion strains, and the yADA2 putative Zn-binding domain is required for binding to yGCN5 in vitro (12a). Therefore, regions of strong sequence similarity between the yeast and human adaptors are necessary for function.

The region of strong conservation between yADA2 and hADA2 is required in yADA2 for interaction with yGCN5, another factor that is conserved between *S. cerevisiae* and humans. In vitro mapping of these interactions shows that the strongly conserved amino-terminal region of yADA2 interacts directly with yGCN5 and that the carboxyl-terminal region of yADA2 interacts with yADA3. Thus, there is a correlation between sequence similarity in the amino termini of the putative ADA2 homologs and evolutionary conservation of the interacting partner, GCN5. We previously showed that the VP16 activation domain also interacts directly with the conserved amino terminus of yADA2 (8). Since VP16 is a higher eukaryotic activator, we predict the interaction to be conserved with hADA2. Detailed characterization of in vitro interactions of the human adaptors is in progress.

Neither hADA2 nor hGCN5 complemented growth defects

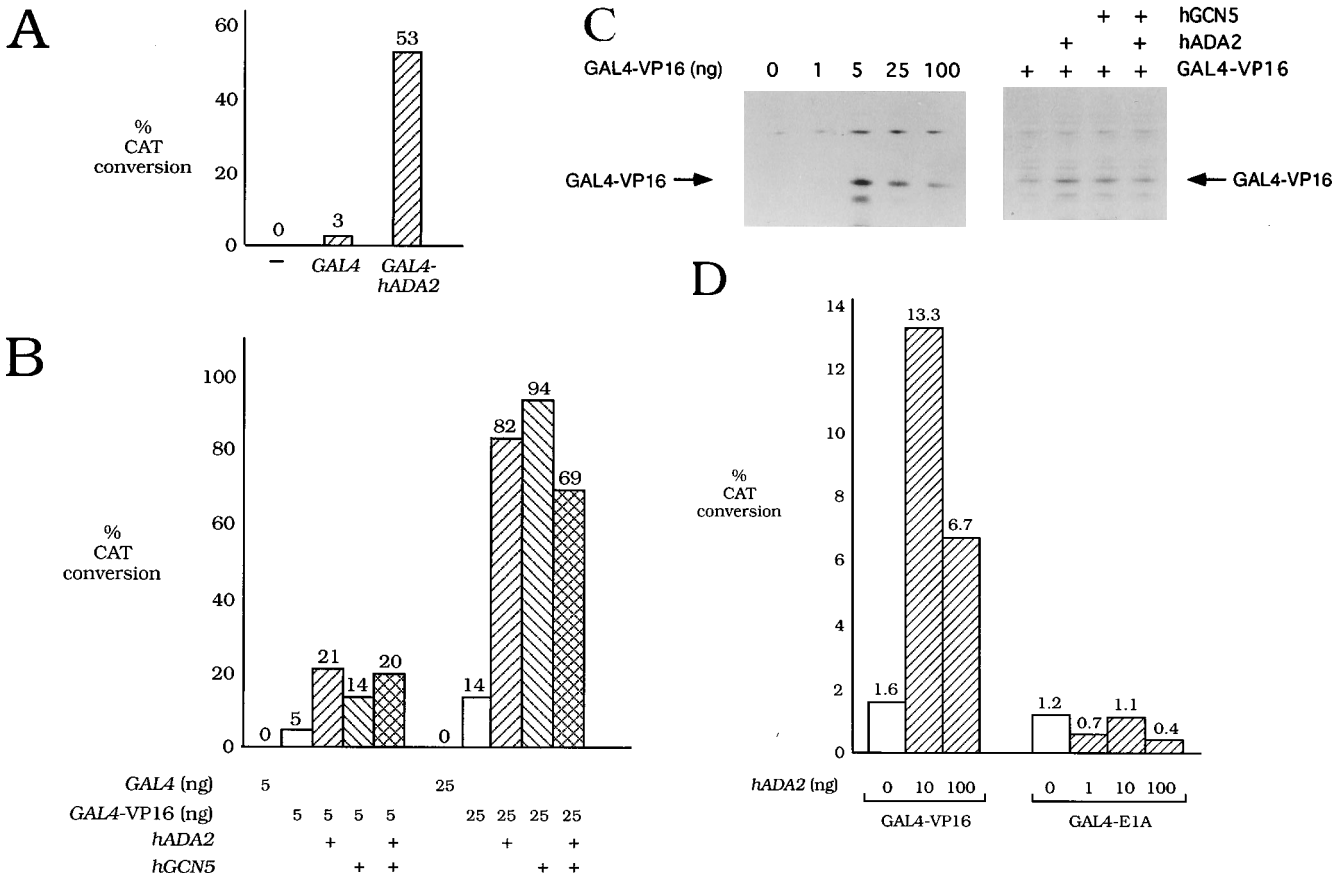


FIG. 8. hADA2 and hGCN5 are transcriptionally active in HeLa cells. (A) Transcriptional activity of GAL4-hADA2. Cells were transfected with either vector (pcDNA3) alone (−), vector expressing the GAL4 DNA-binding domain (*GAL4*), or vector expressing GAL4-hADA2 (*GAL4-hADA2*). The reporter contained five copies of the GAL4 DNA-binding site upstream of the basal adenovirus E1b promoter and the CAT gene. One microgram of reporter was cotransfected with 2 μg of vector, *GAL4*, or *GAL4-hADA2*. The activity is expressed as percent conversion of chloramphenicol to acetylated forms. Activation by GAL4-hADA2 varied between 5- and 16-fold in three independent experiments. (B) Effect of hADA2 or hGCN5 on GAL4-VP16 activation. Cells were transfected with either 5 or 25 ng of GAL4 or GAL4-VP16, as indicated. In addition, either 1 μg of *hADA2* plasmid, 1 μg of *hGCN5* plasmid, or 1 μg of each plasmid was cotransfected. The transfections included 1 μg of reporter, containing five copies of the GAL4 DNA-binding site upstream of the basal herpes simplex virus *tk* promoter, and the CAT gene. Activity is expressed as in panel A. Potentiation of GAL4-VP16 activity by hADA2 ranged between two- and eightfold in seven independent experiments. The effect of hGCN5 varied from two- to sevenfold in six independent experiments. (C) Effect of hADA2 or hGCN5 on protein level of GAL4-VP16 in HeLa cells. Extracts from cells transfected with various amounts of GAL4-VP16 (left panel) or GAL4-VP16 and human adaptors (right panel) were immunoblotted with a GAL4 antiserum. (Left) Zero, 1, 5, 25, or 100 ng of GAL4-VP16 was transfected, as indicated; (right) 5 ng of GAL4-VP16 was transfected with 1 μg of *hADA2*, 1 μg of *hGCN5*, or 1 μg of each. (D) Comparison of the effects of hADA2 on GAL4-E1A and GAL-VP16 activity. Cells were transfected with either 1 ng of GAL4-VP16 or 100 ng of GAL4-E1A. (The amount of DNA used was different for each GAL4 fusion in order to obtain equivalent levels of activation.) In addition, either 1, 10, or 100 ng of *hADA2* was cotransfected, as indicated. All transfections included 100 ng of reporter as described for panel B. Activity is expressed as in panel A. The difference in effect of hADA2 on GAL4-VP16 and GAL4-E1A was observed in three independent experiments.

in yeast strains with deletions in *yADA2* or *yGCN5*, despite high protein sequence similarity, especially between hGCN5 and yGCN5. However, we detected functional similarity between the human and yeast proteins. First, as shown for yADA2 and yGCN5 (42), hADA2 and hGCN5 interacted in vivo in a two-hybrid system, suggesting that the human proteins, like the yeast proteins, are in a complex and thus are likely to be involved in a single pathway. Moreover, hGCN5 interacted with yADA2 in the same assay. The ability of hGCN5 to substitute for yGCN5 in binding to yADA2 is of particular importance because it suggests conservation in function between human and yeast adaptor complexes.

Second, both yADA2 and hADA2 contain cryptic activation domains in their amino termini that were active as LexA-ADA2 fusions. These activation domains were similarly masked in the full-length proteins in both species. Since yADA3 interacts with yADA2 in its carboxyl terminus, this interaction may dampen the activity of the amino-terminal cryptic activation domain of LexA-yADA2. However, there is not yet evidence of a human homolog of yADA3, nor have we detected interactions between ADA3 and hADA2 in the yeast context. Alternatively, a second yeast protein may interact in an analogous fashion with the carboxyl termini of both yADA2 and hADA2 to mask their cryptic activation domains. Another possibility is an intramolecular inhibition of the activation domain by the carboxyl terminus of each protein.

The presence of a cryptic activation domain in hADA2 that correlates in behavior with a similar domain in yADA2 suggests that both proteins act as transcription factors. These amino-terminal activation domains of yADA2 and hADA2 might function by stimulating activity of the basal transcriptional machinery through protein-protein interactions with basal factors, other adaptors in the complex, and/or yeast TBP-associated factors (49). We have detected physical interaction between endogenous yADA2 in nuclear extracts and TBP (8), suggesting that the adaptor complex may communicate with

the basal machinery through initial contacts with TBP. The fact that LexA-hADA2 activates transcription in yeast cells implies that at least some of these interactions with the basal machinery are conserved. However, since hADA2 does not complement the *yADA2* deletion, clearly not all interactions of hADA2 are conserved in *S. cerevisiae.*

The ability of hGCN5 to stabilize yADA2 to an extent comparable to that of stabilization by yGCN5 suggests physical contact between hGCN5 and yADA2, although these data do not rule out an indirect stabilizing effect. The interactions detected between hGCN5 and yADA2 are not sufficient for complementation of a *yGCN5* deletion strain, which indicates other essential domains within yGCN5 that are not conserved between *S. cerevisiae* and humans. This is not surprising, since certain transcription factors, such as human TBP, which shares a remarkably high conservation of sequence with the yeast protein, also failed to complement function in yeast cells (17, 25).

The human proteins in HeLa cells have apparent transcriptional adaptor activity. First, as shown for LexA-yADA2 in yeast cells, GAL4-hADA2 activated transcription. As discussed above, a transcriptional adaptor would link upstream activators and the basal machinery by protein-protein interactions. The region that contacts the basal machinery would be revealed as a cryptic activation domain in a DNA-binding domain fusion.

Second, either hADA2 or hGCN5 potentiated GAL4-VP16 transcriptional activation in HeLa cells. Direct comparison of the effects of hADA2 on GAL4-VP16 and GAL4-E1A revealed specificity of hADA2 for the VP16 activation domain. Thus, the behavior of the human proteins fits the predicted profile of transcriptional adaptors from the previous yeast data, since yADA2 showed specificity in its effects on activators in that system as well (10). Overall, our results support the hypothesis that the human genes are functional homologs of the putative yeast adaptors.

Transfection of both hADA2 and hGCN5 did not further increase GAL4-VP16 activity over the effect of either protein separately. Possible explanations are that the human proteins interact with other factors that limit the activity of a putative human adaptor complex or that one of the human proteins stabilizes the other. It is also possible that the proteins are in separate complexes in vivo and that yet other homologs of these proteins exist in human cells.

Discovery of human adaptors is of importance for several reasons. First, the apparent conservation of the adaptor pathway suggests a fundamental role of this class of cofactors in transcriptional activation. Second, analysis of regions of sequence conserved over the evolutionary interval from *S. cerevisiae* to humans gives valuable clues as to essential regions of the genes. These observations can be followed by mutagenesis and functional studies, as we have begun to do here. Third, isolation of novel human proteins which appear to participate in the transcription complex may provide tools which can be used to identify other, previously unknown proteins involved in gene regulation. Since the human adaptors do not complement the corresponding yeast mutants, the complexes must differ in significant ways, and hence use of the human adaptors may be essential in isolation of other components of the human complex. Finally, it appears that key human genes such as p53 act as activators in yeast cells through interaction with yeast adaptors. It is therefore not unreasonable to suppose that in human cells p53 (and by extension other human acidic activators) acts through hADA2. If this is so, mutations in human adaptors could play a critical role in the genesis of human disease.

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