# Role of the Ada Adaptor Complex in Gene Activation by the Glucocorticoid Receptor

ANNIKA HENRIKSSON, TOVA ALMLÖF, JACQUELINE FORD, IAIN J. McEWAN,
JAN-ÅKE GUSTAFSSON, AND ANTHONY P. H. WRIGHT*

*Department of Biosciences, Karolinska Institute, Novum, S-141 57 Huddinge, Sweden*

We have shown that the Ada adaptor complex is important for the gene activation capacity of the glucocorticoid receptor in yeast. The recently isolated human Ada2 protein also increases the potency of the receptor protein in mammalian cells. The Ada pathway is of key significance for the τ1 core transactivation domain (τ1c) of the receptor, which requires Ada for activity in vivo and in vitro. Ada2 can be precipitated from nuclear extracts by a glutathione *S*-transferase–τ1 fusion protein coupled to agarose beads, and a direct interaction between Ada2 and τ1c can be shown by using purified proteins. This interaction is strongly reduced by a mutation in τ1c that reduces transactivation activity. Mutations affecting the Ada complex do not reverse transcriptional squelching by the τ1 domain, as they do for the VP16 transactivation domain, and thus these powerful acidic activators differ in at least some important aspects of gene activation. Mutations that reduce the activity of the τ1c domain in wild-type yeast strains cause similar reductions in *ada* mutants that contain little or no Ada activity. Thus, gene activation mechanisms, in addition to the Ada pathway, are involved in the activity of the τ1c domain.

The glucocorticoid receptor (GR) belongs to the large family of ligand-inducible nuclear receptors and mediates the effects of glucocorticoid steroid hormones in mammals. Binding of hormone releases the receptor from an inactive protein complex, containing heat shock protein 90 and other heat shock proteins, thus allowing the receptor protein to interact with glucocorticoid-responsive DNA elements within glucocorticoid-regulated genes (64). Subsequently, the GR modulates the activity of the transcriptional machinery to increase or, in some cases, decrease the activity of target genes. Two regions of the human GR, τ1 (residues 77 to 262) and τ2 (residues 526 to 556), have been shown to display transactivation activity when removed from their normal receptor context (27), suggesting that they play a role in the post-DNA-binding steps of gene activation. It has also been suggested that residues close to the C terminus play a role in the transactivation activity of the receptor protein (19).

GR-mediated transactivation of the mouse mammary tumor virus (MMTV) promoter is reduced 20-fold by removal of residues 77 to 262 from the receptor, suggesting that the τ1 transactivation domain plays a key role in GR activity (27). The τ1 domain is enriched in acidic and phosphorylated amino acid residues, although mutational analysis indicates that these are not generally critical for activity when mutated individually (2, 38). A smaller fragment that represents the core activation domain (τ1c) of the τ1 domain has been localized to a 58-amino-acid segment (residues 187 to 244) (16). The τ1c domain is almost as active as the intact τ1 domain and represents the only autonomous transactivation activity within the larger fragment. Similar to other transactivation domains, purified τ1 and τ1c are poorly structured in aqueous solution but have a propensity for α-helical conformation in more hydrophobic solvents (17). Three segments with propensity for α-helix formation have been identified in the τ1c domain by nuclear

magnetic resonance spectroscopy (17). Proline substitution mutations within these segments reduce both transactivation activity and the propensity for α-helix formation, indicating that the putative helices may be important in vivo (18). Recent mutagenesis studies have highlighted the importance of hydrophobic residues that would form hydrophobic clusters on the faces of these α-helices (1).

The mechanism by which different components of τ1 structure contribute to its activity are unclear. In cell-free transcription systems, the intact GR (54) and a derivative of the rat GR (23), containing the equivalent of τ1, have been reported to increase the rate of formation of correctly assembled transcription complexes on template DNA. Excess τ1 squelches (inhibits) the expression of basal reporter genes both in vivo (60) and in vitro (40), suggesting that τ1 interacts with as yet unidentified components of the basal transcriptional machinery. In kinetic experiments, these interactions coincide with formation of the transcriptional complex and may account for at least part of the receptor's role in this process (39). However, monoclonal antibodies that recognize epitopes within τ1 are still inhibitory when added after formation of the transcription complex, and thus the τ1 domain may also function at one or more later steps in transcriptional activation. Consistent with this possibility, it is generally accepted that interactions with auxiliary proteins, which function to mediate the effects of transactivation domains, are needed in addition to interactions with the basal transcriptional machinery (26). These auxiliary proteins are generally referred to as adaptors, coactivators, or mediators. Several mammalian proteins that enhance the transactivation activity of the GR and/or interact with the receptor protein have recently been isolated (13, 21, 28, 32, 45, 56, 63). However, these studies have tended to focus on the hormone-binding domain because of its structural conservation throughout the nuclear receptor family. So far, adaptor proteins that bind to the τ1 domain of the GR have not been identified.

Genetic studies in the yeast *Saccharomyces cerevisiae* have identified a number of putative adaptor proteins (reviewed in reference 26). The apparent similarity of mechanisms regulat-

* Corresponding author. Mailing address: Department of Biosciences, Karolinska Institute, Novum, S-141 57 Huddinge, Sweden. Phone: 46 8 608 91 59. Fax: +46 8 774 55 38. E-mail: tony.wright@cbt.ki.se.

ing gene expression in mammalian and yeast cells, together with the observation that the GR functions as a transcriptional activator in yeast (50), provides an opportunity to test whether yeast adaptor proteins might be of functional significance for gene activation by the GR. From studies using such an approach, it was reported that the Swi/Snf protein complex, which functions as a modulator of chromatin structure in vivo and in vitro (reviewed in reference 46), contacted the GR via the DNA-binding domain and was important for its transactivation activity (62). Subsequently, human homologs of Swi/Snf subunits have been isolated and shown to enhance the activity of the GR in mammalian cells (42, 43).

The Ada2, Ada3/Ngg1, Gcn5/Ada4, and Ada5/Spt20 proteins represent another class of adaptor proteins in yeast that have been extensively studied (26). Mutants affecting these proteins confer resistance to squelching by high levels of a Gal4-VP16 fusion protein, and based on this phenotype, it was argued that the Ada proteins might link the transactivation domains of activator proteins to the general transcriptional machinery (5). This view has subsequently been supported by reports of direct interactions between Ada proteins and the transactivation domains of VP16 and Gcn4 as well as with the TATA-binding protein (TBP; Spt15) within the general transcriptional machinery (3, 35, 52). Many of the Ada proteins also interact with each other, and there is now strong genetic and biochemical evidence that they form a protein complex in vivo (10, 11, 12, 24, 29, 35, 36). The Ada complex functions as an adaptor for only a subset of the so-called acidic transactivation domains because several characterized acidic activators (e.g., Hap4) do not require the Ada proteins (5, 46, 57). The Ada3/Ngg1 protein has also been identified independently as a transcriptional repressor (8, 9, 37), and thus the Ada complex may have an additional role in gene repression. It was recently shown that the *Tetrahymena* homolog of the Gcn5 subunit of the complex has a histone acetyltransferase activity (10). Since histone acetylation is associated with highly expressed genes, it is possible that this activity may be an important component of the Ada adaptor's activity. It was also shown recently that homologs of the Ada2 and Gcn5 proteins exist in mammalian cells, and thus the Ada adaptor is likely to be of importance for some mammalian activator proteins (12, 61). In this study, we show that the Ada complex plays a key role in the gene activation capacity of the $\tau 1$ domain from the human GR.

## MATERIALS AND METHODS

**Strains and plasmids.** The wild-type yeast strain used throughout was PSY316 (*MAT$\alpha$ ade2-101 ura3-52 leu2-3,112 his3-$\Delta$200 lys2*), which was kindly provided by Leonard Guarente (Massachussets Institute of Technology). Strains containing disruptions of the *ADA2*, *ADA3*, and *GCN5* genes in the PSY316 background were also generously provided by Leonard Guarente. In these strains, the deleted genes are replaced by the bacterial *hisG* gene as described previously (5, 36, 46).

The intact human GR was expressed from a galactose-inducible *PGK1* promoter by using the centromeric vector pRS315-NX, described previously (33). Deletion of a *Bgl*II fragment representing the GR $\tau 1$ domain from pRS315-NX gave rise to pRS315-NX-$\Delta \tau 1$, which expresses the GR-$\Delta \tau 1$ protein, lacking GR residues 77 to 261. pRS315-lexA was constructed by inserting a *Bam*HI-*Sal*I fragment encoding the LexA DNA-binding domain (residues 1 to 87) expressed from the same promoter and followed by the *PGK1* 3' region into the equivalent sites of pRS315($\Delta$SacI). pRS315($\Delta$SacI) was produced by cleaving pRS315 (51) with *Sac*I, removing the resultant cohesive ends with T4 DNA polymerase, and recircularizing the plasmid by ligation with T4 DNA ligase. The LexA amino acids are preceded by Met Val Asn Ser Ser Ser, and there is a unique *Sac*I site directly upstream of the lexA sequence that can be used for insertion of transactivation domain-encoding fragments. This plasmid was used to construct pRS315-$\tau 1$c-lexA, expressing $\tau 1$c-LexA, by inserting a *Sac*I fragment encoding GR residues 187 to 244. The fragment was amplified by PCR using primers with the sequences 5'-GGCGAGCTCTGACCAAAGCACCTTTGACAT-3' and 5'-GGGGAGCTCGCGTCCTCATTCGAGTTTCCTTCCAA-3'. Mutated $\tau 1$c-LexA proteins were also expressed by using this expression vector. The GR-responsive *lacZ* reporter gene contains a single glucocorticoid response element

from the rat tyrosine aminotransferase promoter cloned upstream of a basal *CYC1-lacZ* promoter and is contained in the vector pLGZ-TAT, described previously (58). The LexA-responsive reporter plasmid (pLGZ-2lex) was identical to pLGZ-TAT except that the glucocorticoid response element was replaced by a fragment containing two LexA binding sites (5'-TCGAGTACTGT ATGTACATACAGTACTGCAGATCTGCAGTACTGTATGTACATACAG TAC-3'). His-tagged proteins were expressed in *Escherichia coli* by using the vector pET19bm, which was constructed from pET19b (Novagen) by replacing the *Nde*I-*Bam*HI fragment with a modified polylinker sequence (5'-CATATGG CGAGCTCCGGATCCTAATAGCTAGCTCTAGATCT-3') containing unique cloning sites for *Sac*I and *Bam*HI upstream of two in-frame termination codons. pET-lexADBD was constructed by amplifying a *Bgl*II-cleavable fragment by PCR encoding the DNA-binding domain of the *E. coli* LexA repressor protein (residues 1 to 87), followed by cleavage with *Bgl*II and insertion in the *Bam*HI site of pET19bm. The primers used were 5'-GCGAGATCTGCGAGCTCCATGAAA GCGTTAACGGCC-3' and 5'-GCGAGATCTTTATGGTTCACCGGCAGCC ACACGACC-3'. pET-$\tau 1$c-lexA and pET-$\tau 1$c(H1Ala)-lexA were constructed by inserting *Sac*I fragments obtained from pRS315-$\tau 1$c-lexA and pRS315-$\tau 1$c(H1Ala)-lexA, respectively, into *Sac*I-cleaved pET-lexADBD. pET-VP16-lexA was constructed by inserting a *Sac*I fragment encoding residues 413 to 490 of VP16 in the *Sac*I site of pET19bm. The fragment was amplified by PCR using the following primers: 5'-GCGCGAGCTCTGCCCCCCCGACCGATGTCAG CCTGGGGGACGAACTCC-3' and 5'-GCGCGACCTCATACCGTACTCGT CAATTCCAAGG-3'. Construction of the vector expressing residues 685 to 774 of the rat arylhydrocarbon receptor nuclear translocator protein (AX) fused to LexA has been described previously (49). Construction of the LexA-dependent reporter plasmid, pLexAGC-, used in vitro transcription experiments has been described elsewhere (49). pGST-$\tau 1$ was constructed by subcloning an *Eco*RI-*Xba*I (3'-blunted) fragment, taken from plasmid pEHGR770 (40), into pGEX-4T-3 (Pharmacia) cleaved with *Eco*RI and *Xho*I (3' blunted). The fragment from pEHGR770 encoded amino acids 77 to 262 from the human GR and additional amino-terminal amino acids (Asn Ser Ser Ser Val Pro Gly). The plasmid expressing the VP16–glutathione *S*-transferase (GST) fusion protein (GST-CS-VP16) was a generous gift from Stephen Johnston (University of Texas Southwestern Medical Center) (41). The plasmid expressing the GST-Ada2 protein (3) and pSB201 (5) for high-level expression of Gal4-VP16 in yeast have been described previously and were kindly provided by Shelley Berger (Wistar Institute). pKV-$\tau 1$, used for galactose-dependent, high-level expression of $\tau 1$ in yeast, has been described previously (60). Plasmids pCMVhGR$\alpha$ and pCM VhGR$\alpha$-$\Delta \tau 1$, used for expression of the intact GR and GR-$\Delta \tau 1$ proteins in mammalian cells, together with the p19LUC-TK reporter plasmid, have been described previously (2). pcDNA-hAda2, used for expression of human Ada2 (hAda2) in mammalian cells, has been described previously (12) and was generously provided by Shelley Berger. The empty pcDNA3 plasmid was obtained from the manufacturer (Invitrogen).

**Transactivation assays in yeast.** Plasmids were transformed into wild-type and mutant yeast strains by the lithium acetate (25) or spheroplast (4) procedure. Several colonies from each transformation were checked on 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside (X-Gal) plates for homogeneity of transactivation activity (7). Representative transformants were grown in minimal medium with 2% galactose for at least 18 h, to an $A_{600}$ of 0.2 to 0.3. Extracts were prepared and assayed for $\beta$-galactosidase activity as described previously (58). Protein extracts for Western blot analysis were prepared directly in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis loading buffer as described previously (30).

**In vitro transcription assays.** The transactivation activity of the $\tau 1$c-LexA, VP16-LexA, and AX-LexA fusion proteins in vitro was tested by adding 15, 1.5, and 60 pmol of activator protein, respectively, to nuclear extracts prepared from the wild-type and *ada2* strains as described previously (40) except that 100 ng of a LexA-responsive reporter template (pLexACG-) was used. The amounts of activator proteins were chosen to give saturated activation levels at similar points on the respective dose-response curves.

**Protein interaction assays.** Yeast nuclear extract was prepared from the *ada2* strain expressing a hemagglutinin (HA) epitope-tagged Ada2 protein by the method of Lue et al. (34). The plasmid expressing HA-Ada2 has been described previously (52) and was generously provided by Leonard Guarente. GST fusion proteins were bound to glutathione-agarose beads (Sigma) at an approximate concentration of 1 mg of protein/ml of beads. Yeast nuclear extract containing HA-Ada2 was added to the beads at an approximate concentration of 4 mg/ml in 8 bead volumes (200 $\mu$l) of Y buffer [20 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM MgCl$_2$, 5 mM EDTA, 10% (vol/vol) glycerol, 50 mM (NH$_4$)$_2$SO$_4$, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. Alternatively, purified proteins [LexA, $\tau 1$c-LexA, and $\tau 1$c(H1Ala)-LexA] were added to the beads at approximate concentrations of 20 $\mu$g/ml in 8 bead volumes (200 $\mu$l) of immunoprecipitation buffer (20 mM HEPES-KOH [pH 7.9], 10% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 1 mM DTT, 5 mM MgCl$_2$, 0.01% Nonidet P-40, 1 mg of bovine serum albumin/ml). Incubation with rotation was carried out at 4°C either for 2 h (yeast nuclear extract) or overnight (purified proteins), and the supernatants were recovered. The beads were washed four times with 20 volumes of immunoprecipitation or Y buffer, and the beads and supernatants were boiled with SDS sample buffer. The proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and

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probed with anti-LexA or 12CA5 (Boehringer Mannheim) antibody as appropriate. Blots were developed with the Amersham ECL system.

**Assay for squelching by τ1 in yeast.** The wild-type and mutant yeast strains were transformed with a plasmid (pKV-τ1) from which high levels of τ1 domain can be expressed by induction with galactose. A negative control plasmid (pKV-XE) (60) that does not express τ1 or cause squelching was also used. The resultant arrest of cell growth (60) was monitored by spotting 0.01-ml aliquots from 10-fold serial dilutions of transformant cells, made from a starting suspension at a density of $1.5 \times 10^7$ cells per ml, on minimal medium plates containing galactose.

**Transactivation assays in mammalian cells.** COS7 cells were transiently transfected with plasmids as described previously (2), with modifications as shown in Fig. 5. The amount of GR and GR-Δτ1 expression plasmids was 0.15 μg, and the amount of transfected DNA was kept constant by using the empty pcDNA3 plasmid to compensate for the different levels of the pcDNA3-hAda2 plasmid used.

## RESULTS

**The Ada adaptor complex is required for activity of the GR N-terminal transactivation domain.** To determine whether the Ada complex might function as an adaptor that mediates the gene activation capacity of the GR, we measured the transactivation activity of the intact GR and various GR derivatives (Fig. 1A) in yeast strains defective in subunits of the Ada complex. A plasmid expressing intact GR was transformed together with a GR-responsive *lacZ* reporter plasmid into mutant strains which contained deletions in the *ADA2*, *ADA3*, or *GCN5* gene and an otherwise isogenic wild-type strain. Strains expressing GR were grown in the presence of hormone (10 μM triamcinolone acetonide), exponentially growing cells were harvested, and β-galactosidase levels were measured. Figure 1B shows that the transactivation activity is substantially reduced in the mutant strains, and thus the Ada protein complex appears to play a role in gene activation via the GR.

As observed for other steroid receptors, the GR contains independent transactivation activities in different parts of the receptor protein, and since the Ada complex is required for only a subset of activation domains (5, 46, 57), we wished to determine which of the GR transactivation activities were Ada dependent. First, we measured the effect of *ada2*, *ada3*, and *gcn5* mutations on the activity of a GR derivative lacking the N-terminal, τ1 transactivation activity (Fig. 1A). Figure 1B shows that the activity of this protein is reduced to a lesser extent in the mutants. This result suggests that it is predominantly the N-terminal, τ1 transactivation domain that functions via the Ada complex, although the Ada adaptor probably plays some role in the activity of other activation domains within the receptor. To test this directly, we expressed a protein consisting of 58 GR residues, representing the functional core of the τ1 domain (τ1c), fused to the DNA-binding domain of the LexA repressor protein from *E. coli* (Fig. 1A). As shown in Fig. 1B, the expression of β-galactosidase from a LexA-dependent *lacZ* reporter gene was strongly reduced in the mutant strains. Figure 1B shows that the *ada3* deletion causes a greater reduction in activity than the *ada2* and *gcn5* deletions. It is probable that Ada3 plays roles in addition to those shared in common with Ada2 and Gcn5 (see Discussion). Taken together, these results indicate that gene activation mediated via the Ada adaptor complex is selective for the τ1c domain of the GR and that the other GR transactivation domains are less dependent on the Ada adaptor. This transactivation domain selectivity is consistent with previous results showing that even though the Hap4 transactivation domain is more active than that from Gcn4, it showed mild if any reduction in activity in *ada* mutant strains, whereas Gcn4 was reduced to 5 to 20% of the wild-type activity (5, 46, 57).

Although the differential effects of the *ada2*, *ada3*, and *gcn5* mutations on different GR constructs suggested a direct effect
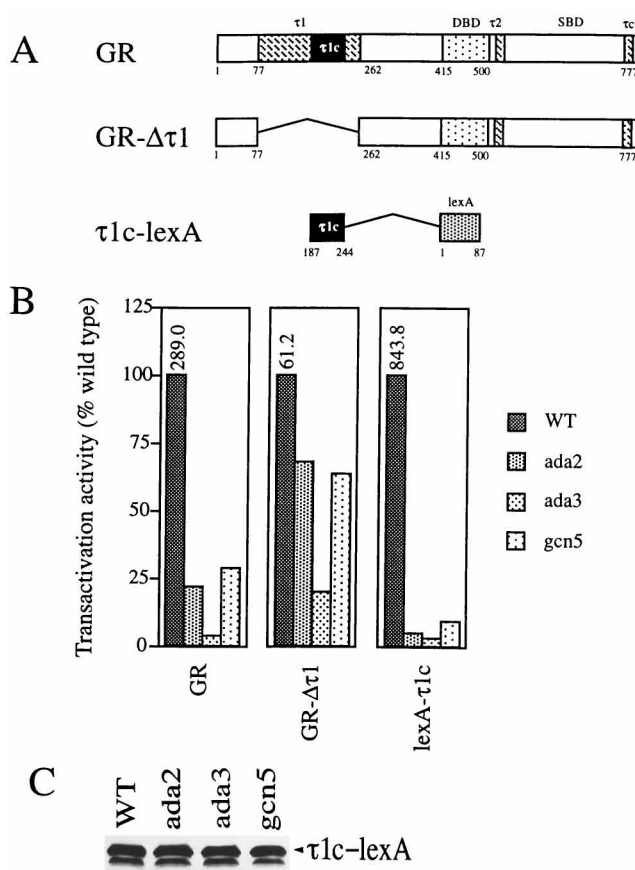


FIG. 1. The Ada adaptor complex is important for gene activation mediated via the τ1c transactivation domain of the GR. (A) Diagram showing the domain structure of the intact human GR and GR derivatives that were tested in yeast strains containing deletions of *ADA* genes. Transactivation domains (τ1, τ1c, τ2, and τc), DNA-binding domains from the GR (DBD) and LexA (lexA), and the GR steroid-binding domain (SBD) are indicated. (B) Transactivation activities of the GR and its derivatives in *ada2*, *ada3*, and *gcn5* deletion yeast strains are shown relative to their activities in an otherwise isogenic wild-type (WT) strain. The measured β-galactosidase activity (nanomoles of *O*-nitrophenyl-β-D-galactopyranoside/milligram of protein/minute; mean of three to five experiments) for each protein in the wild-type strain is also shown. (C) Western blots showing the expression levels of the τ1c-LexA protein in wild-type and mutant strains.

on the activity of τ1c, we wished to show directly that the reductions were not due to an indirect effect of the mutations on the hybrid *GAL-PGK* promoter used to express the GR derivatives. The Western blot in Fig. 1C shows that the expression level of the τ1c-LexA fusion protein was not significantly altered in the *ada* mutant strains, even though activation of the reporter gene by this protein was reduced 10- to 20-fold.

**τ1c-dependent transcriptional activation in vitro requires the Ada complex.** The Gcn5 subunit of the Ada complex has a histone acetyltransferase activity (10), and thus it is possible that the adaptor activity triggered by the τ1 domain of the GR acts only at the level of chromatin structure modulation. However, for the transactivation domain of the VP16 protein from herpes simplex virus, the adaptor activity of the Ada complex has been demonstrated in cell-free transcription systems in vitro, in which the transcribed DNA templates would not be expected to have a classical chromatin structure (5). To determine whether the Ada complex was important for transactivation by the GR τ1c domain in such systems, we tested the activity of τ1c in nuclear extracts prepared from wild-type and
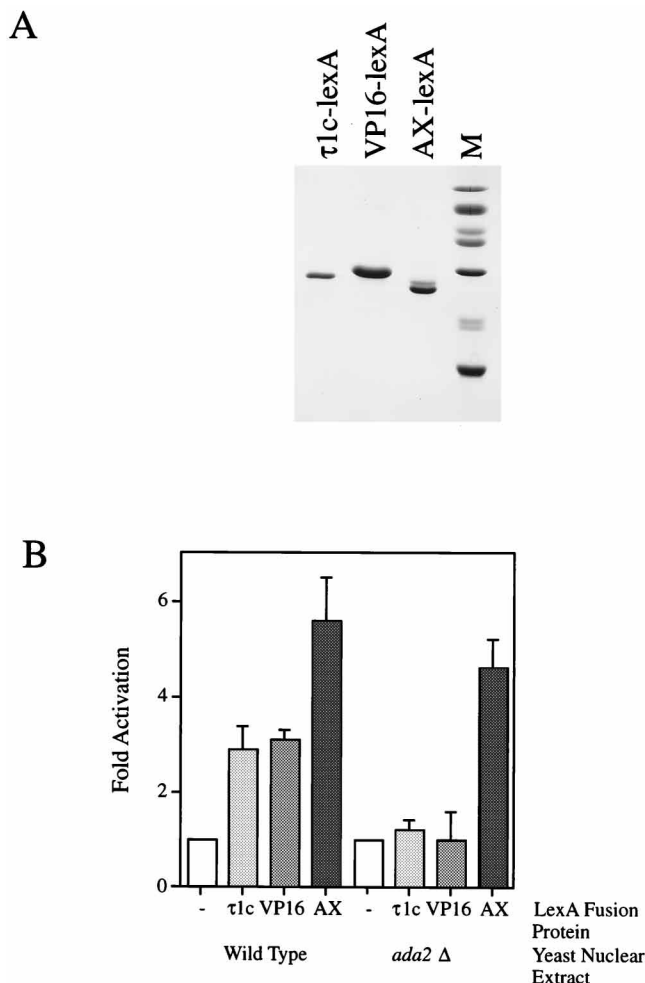
FIG. 2. The Ada adaptor complex is required for the transactivation potential of the τ1c transactivation domain in vitro. (A) Coomassie blue-stained SDS-gel showing migration of the purified τ1c-LexA, VP16-LexA, and AX-LexA proteins used for in vitro transcription experiments. The migration of molecular weight markers (90, 67, 43, 30, 20, and 14 kDa) is shown (lane M). (B) Bar chart showing the level of transactivation (mean ± standard deviation, $n = 3$) by the τ1c-LexA, VP16-LexA, and AX-LexA proteins, relative to the level of basal transcription in the absence of added activator proteins, in nuclear extracts prepared from wild-type and *ADA2*-deleted strains. RNase T$_1$-resistant transcripts were resolved by denaturing gel electrophoresis and quantitated with a bioimaging analyzer (Fuji Film). It should be noted that in these experiments, *ada2* mutant extracts consistently showed significantly reduced levels of basal transcription.

*ada2* yeast strains. A protein consisting of τ1c-LexA fused to a histidine tag, together with analogous fusion proteins containing the VP16 and AX transactivation domains as controls, was expressed in bacteria and purified to near homogeneity (Fig. 2A). These proteins were added to nuclear extracts prepared from wild-type and *ada2* mutant strains together with a LexA-responsive reporter template and nucleoside triphosphates. The levels of transcriptional activation are shown in Fig. 2B relative to the basal level of transcription measured in the absence of added activator protein. The results show that the transactivation measured for both the τ1c and VP16 proteins in the wild-type extract is lost in the *ada2* mutant extract. Conversely, the glutamine-rich AX transactivation domain was active in both extracts, and therefore the *ada2* mutant extract is competent to support activation by some activators. Thus,

mutations affecting the Ada complex have effects on the in vitro transactivation activity of the τ1c domain similar to those reported previously for the VP16 domain.

**The τ1 domain interacts directly with the Ada complex.** One mechanism by which adaptor proteins have been suggested to function is by contacting both activator proteins and the general transcriptional machinery, thus linking them together physically. To determine whether the GR τ1 domain might interact with the Ada protein complex, we used GST coprecipitation assays. The τ1 domain and the VP16 transactivation domain (as a positive control) were expressed in *E. coli* as fusion proteins with GST and purified (Fig. 3A). The fusion proteins were coupled to glutathione-agarose beads and then incubated with nuclear extract in which the Ada2 protein was tagged with an HA epitope. Interacting proteins were then pelleted together with the GST fusion proteins by centrifugation. The presence of Ada2 in the pellet and supernatant fractions was then determined by Western blotting using an antibody against the HA epitope. The results (Fig. 3A) show that the GST protein alone does not interact with the Ada complex since HA-Ada2 protein is found only in the supernatant fraction. In the case of the GST-τ1 protein, most of the Ada2 protein is also found in the supernatant but a significant portion is coprecipitated with the GST-τ1 protein, and thus the τ1 domain can interact with Ada2 either directly or indirectly via another member of the Ada complex. The amount of Ada2 precipitated by GST-τ1 is similar to the amount interacting with VP16, which has been shown to interact with the Ada complex previously (3, 52).

The VP16 transactivation domain has been reported to interact directly with the Ada2 subunit of the Ada complex (3, 52). To determine whether this is also the case for the τ1 domain, purified GST or GST-Ada2 proteins were incubated with purified τ1c-LexA, τ1c(H1Ala)-LexA, and LexA proteins, followed by precipitation of the GST proteins as described above. The τ1c(H1Ala)-LexA protein contains alanine substitutions of five residues near the N terminus of the τ1c domain and is severely reduced in transactivation activity (1). The purified proteins used are shown in Fig. 3B. Figure 3B also shows coprecipitation of τ1c-LexA protein together with the GST-Ada2 protein, indicating that they can interact directly. This interaction is specific for the τ1c part of the fusion protein because the LexA protein alone does not interact with the GST-Ada2 protein. Interestingly, the interaction is not seen with the τ1c(H1Ala)-LexA protein, and thus the reduced transactivation activity of this mutant can be correlated with its reduced ability to interact with the Ada2 protein. The interaction with the τ1c domain is specific for the Ada2 part of the GST-Ada2 protein, since GST alone does not interact with any of the LexA proteins.

**Mutations affecting the Ada complex do not relieve squelching by the τ1 domain.** As discussed in the introduction, the *ADA* genes were originally discovered because *ada* mutants relieved squelching, caused by a highly expressed Gal4-VP16 protein, thus overcoming the associated growth arrest phenotype (Fig. 4A and references 5 and 36). To determine whether *ada* mutants also relieve squelching resulting from high expression of the GR τ1 domain (60), we expressed high levels of τ1 from the galactose-inducible *GAL-PGK* promoter in the wild-type and mutant strains. As a negative control, a fragment containing the DNA-binding domain of the GR was expressed from the same vector. Figure 4B shows that expression of τ1 but not the control protein retards growth of the wild-type strain as reported previously (60). However, none of the mutants reverse the squelching phenotype; if anything, the squelching effect of τ1 is stronger in the mutant strains. Thus,

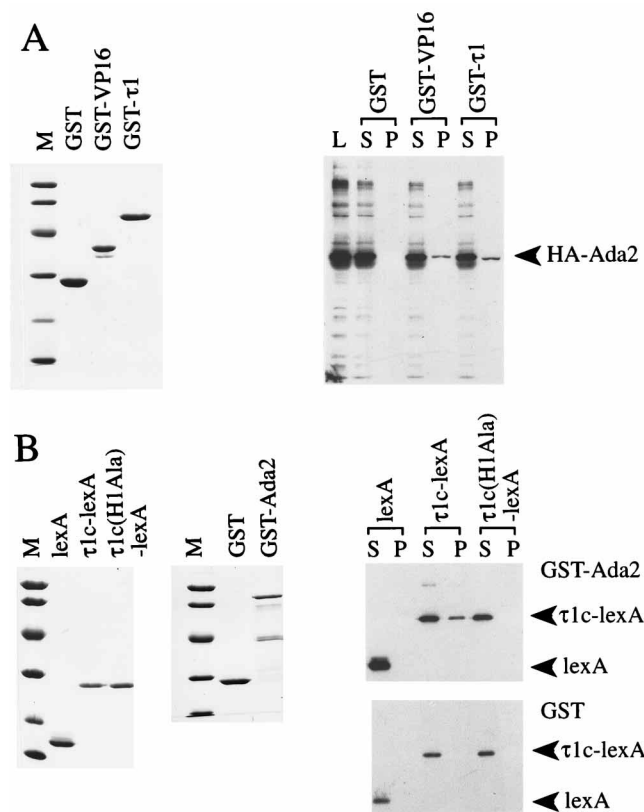FIG. 3. The GR τ1 domain interacts physically with the Ada2 subunit of the Ada adaptor complex. (A) τ1 interacts directly or indirectly with Ada2 in nuclear extracts. A Coomassie blue-stained SDS-gel (left panel) shows the purified GST fusion proteins used. The migration of molecular weight markers (90, 67, 43, 30, 20, and 14 kDa) is shown (lane M). The right panel is a Western blot showing coprecipitation of Ada2 with GST-τ1 and GST-VP16 (positive control) fusion proteins after incubation with nuclear extracts prepared from a strain in which the endogenous Ada2 protein was replaced with an Ada2 protein containing an HA epitope tag (52). HA-Ada2 was detected in the load (lane L) before precipitation and in the supernatant (lanes S) and pellet (lanes P) fractions after precipitation of the GST proteins, using monoclonal antibody 12CA5, which recognizes the HA epitope. Equal volumes of the supernatant and pellet fractions were loaded, but the total supernatant volume was eightfold greater than that for the pellet. (B) τ1c interacts directly with Ada2. Coomassie blue-stained SDS-gels (left and middle panels) showing the purified proteins used. The migration of molecular weight markers (90, 67, 43, 30, 20, and 14 kDa) is shown (lane M). The 14-kDa marker migrated with the gel front in the gel shown in the middle panel. The right panels are Western blots showing coprecipitation of the purified τ1c-LexA fusion protein but not the LexA and τ1c(H1Ala)-LexA proteins with purified GST-Ada2 protein. The H1(Ala) mutation, in which F191, I193, L194, L197, and F199 are replaced by alanine, reduces the activity of τ1c domain to less than 10% of the wild-type level (1). The supernatant (lanes S) and pellet (lanes P) fractions obtained after precipitation of the GST-Ada2 protein are shown. The LexA fusion proteins were detected by using an antibody raised against the LexA moiety of the proteins. The lower right panel shows that none of the LexA fusion proteins interact with purified GST.

we conclude that although both the VP16 and τ1 transactivation domains function via the Ada adaptor complex, there are also important differences in their mechanisms of transactivation.

**Mechanisms of τ1-mediated gene activation in addition to the Ada adaptor pathway.** We have shown that the Ada complex plays an important role in τ1-mediated transactivation and that this is likely to involve direct interactions between the τ1 domain and the Ada2 subunit of the complex. However, the results in Fig. 4 suggest that there may be other, Ada-independent mechanisms of transactivation employed by τ1 that dis-
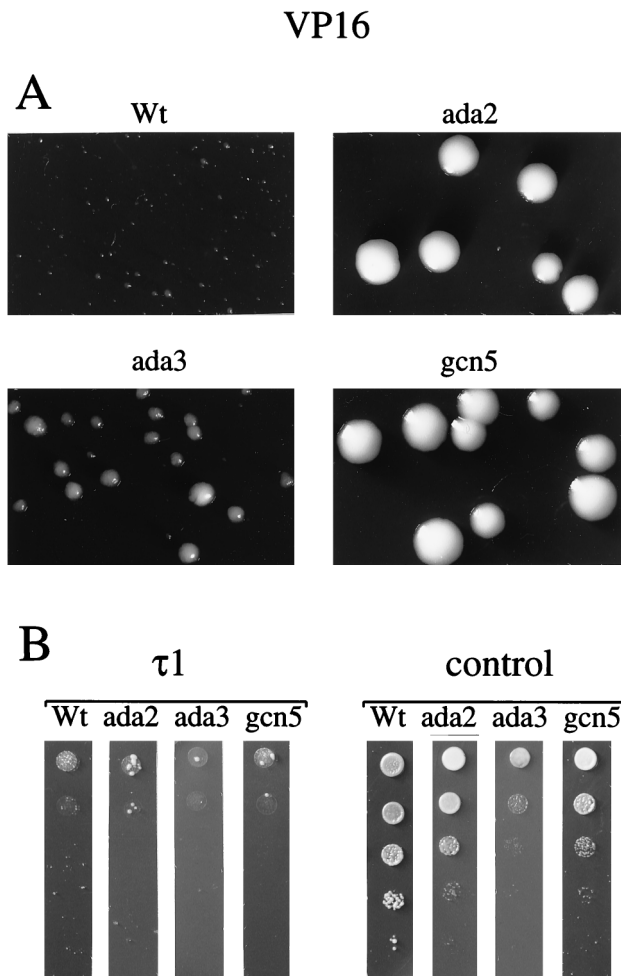


FIG. 4. Deletion of *ADA* genes does not alleviate transcriptional squelching by the GR τ1 domain. (A) Growth of colonies after transformation of wild-type (Wt) *ada2*, *ada3*, and *gcn5* yeast strains with pSB201, expressing high levels of a Gal4-VP16 fusion protein. Squelching of cell growth in the wild-type strain is alleviated in each of the mutant strains. (B) Dilution series showing the growth of wild-type (Wt), *ada2*, *ada3*, and *gcn5* yeast strains expressing high levels of the GR τ1 domain (τ1) or the GR DNA-binding domain (control) proteins. τ1 but not the control protein inhibits growth due to transcriptional squelching, and this phenotype is not reversed in the mutant strains (see text for more details).

tinguish it from VP16. Importantly for our approach to this question, previously published genetic and biochemical evidence suggests that deletion of single *ADA* genes is sufficient to destroy the adaptor activity of the complex (see Discussion). Thus, τ1 mutations that reduce activity in wild-type strains would not reduce the activity of τ1c in *ada* mutants, relative to unmutated τ1c, if the defect that they cause is restricted to the Ada pathway because the Ada complex is already destroyed by deletions in the *ADA* genes. Conversely, τ1c mutants that also cause activity reductions in *ada* mutants would contain defects in transactivation mechanisms that are independent of the Ada pathway. Thus, to determine whether the Ada pathway alone can account for the contribution of different regions of the τ1c domain to its transactivation activity, we measured the activity of a range of τ1c mutants with reduced activity in *ada2*, *ada3*, and *gcn5* mutant strains. Table 1 shows the relative activities of seven τ1c mutants, representing different segments of τ1c, in the wild-type, *ada2*, *ada3*, and *gcn5* strains. All of the mutant τ1c domains show defects in the *ada2*, *ada3*, and *gcn5* mutant

TABLE 1. Transactivation activities of τ1c mutants in wild-type and *ada* mutant strains

| τ1c mutant | Transactivation activity (% of unmutated τ1c activity)[a] | | | |
| --- | --- | --- | --- | --- |
| | Wild type | *ada2* | *ada3* | *gcn5* |
| τ1c (unmutated)[b] | 100 (843) | 100 (42) | 100 (21) | 100 (88) |
| F191D | 49 | 25 | 13 | 26 |
| I193D | 42 | 31 | 11 | 19 |
| L197E | 30 | 22 | 12 | 16 |
| W213A | 12 | 21 | 8 | 14 |
| H2(Ala)[c] | 4 | 10 | 8 | 7 |
| L194A/L224V/L225F | 5 | 8 | 7 | 4 |
| F235L/L236V | 11 | 13 | 10 | 8 |

[a] The results for the mutant τ1c-LexA mutant proteins were calculated from mean values obtained from two to three independent experiments (individual values for the mutant proteins varied by ≤35% from the mean). The actual β-galactosidase levels (units) are shown in parentheses for unmutated τ1c.
[b] Values from Fig. 1B, τ1c-LexA.
[c] L217, L218, I219, L224, and L225 are replaced by alanine.

strains that are generally similar in extent to those seen in the wild-type strain. Thus, we conclude that although the Ada interaction is important for τ1 function, there are likely to be additional pathways that mediate τ1 function independently of the Ada pathway.

**The human homolog of Ada2 enhances transactivation by the GR in mammalian cells.** During the course of these experiments, we noted the appearance of human partial cDNA sequences in the database with homology to subunits from the yeast Ada complex, giving confidence that the mechanism that we have characterized in yeast might have a direct parallel in human cells. Recently full-length cDNAs encoding human homologs of Ada2 and Gcn5 have been published (12, 61), and thus we have been able to test directly whether subunits of a putative hAda adaptor might play a functional role during GR-mediated transcriptional activation in mammalian cells. Figure 5A shows the results of experiments in which plasmids expressing the GR and hAda2 were cotransfected together with a GR-responsive luciferase reporter gene into COS7 cells. The level of hormone-dependent transactivation by the GR was significantly enhanced at the highest level of cotransfected hAda2 expression plasmid. Conversely, no significant enhancement was seen in a control experiment in which a plasmid encoding the GR-Δτ1 derivative was transfected (Fig. 5B). Therefore, although the hAda2-mediated enhancement of GR activity is modest, it is significant because it requires the τ1 domain. Thus, as in yeast, the Ada adaptor pathway appears to play an important role in the activity of by the τ1 domain during GR-mediated gene activation in mammalian cells.

## DISCUSSION

In this study, we have shown that the Ada adaptor complex is critical for the activity of the GR τ1c transactivation domain in yeast. Furthermore, the hAda2 protein potentiates the GR response in COS7 cells, suggesting that the Ada pathway is also important for GR-mediated transactivation in mammalian cells. The hAda adaptor also seems to be selective for the τ1 domain, since hAda2 did not enhance the activity of the GR-Δτ1 derivative in COS7 cells. τ1c can interact directly with the Ada2 subunit of the complex, and this interaction is reduced by alanine substitution mutants in τ1c that also reduce its transactivation activity. Thus, the Ada complex fulfills the requirements of an adaptor protein involved in GR-mediated gene regulation. This represents the first report of a protein that can
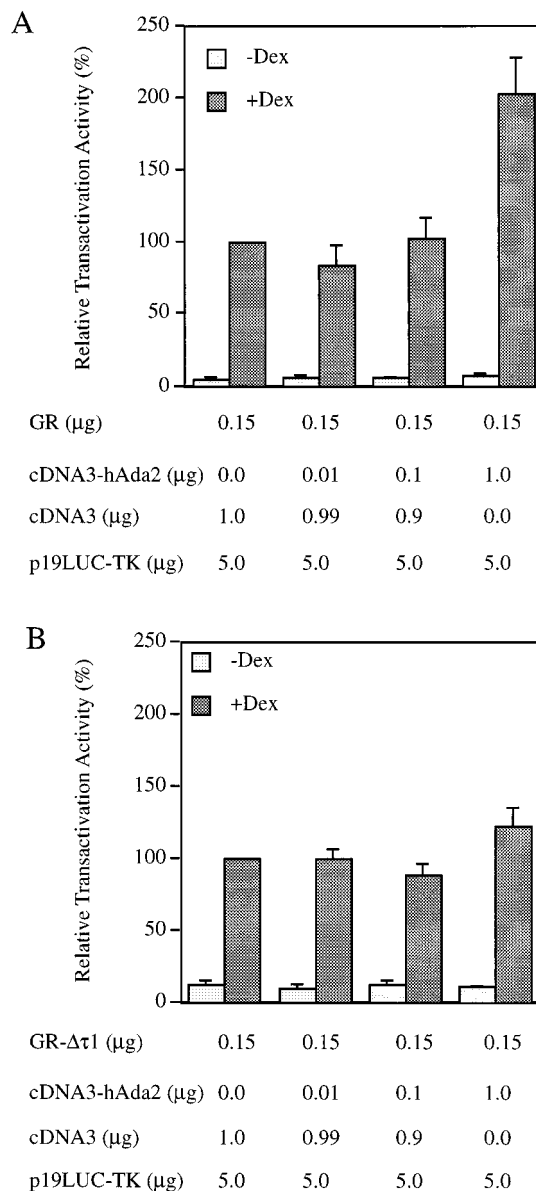
FIG. 5. Functional interaction between the GR and hAda2 in mammalian cells. (A) Transactivation activity of the GR, with or without 1 μM dexamethasone (Dex), after cotransfection with expression plasmids for the GR and hAda2. The activity of the luciferase reporter gene in each condition is expressed relative to the activity of the GR (+Dex) in the absence of cotransfected plasmid encoding hAda2. The results represent mean values (± standard deviation) obtained from three independent transfections. (B) Experiment identical to that in panel A except that the plasmid expressing GR was replaced by the same expression vector expressing the GR-Δτ1 protein.

specifically interact with the GR τ1 domain and mediate its transactivation activity.

The recent observation that the Gcn5 subunit of the Ada complex contains histone acetyltransferase A activity (10) provides a possible mechanism by which the Ada adaptor might function. Many studies show that acetylation of core histones is associated with highly expressed genes, and the discovery that an adaptor complex containing an acetyltransferase activity can be recruited to promoters by activator proteins suggests that acetylation may be a cause rather than a consequence of gene activation (10). Extensive studies of the MMTV long

terminal repeat and rat tyrosine aminotransferase gene promoter have shown that the GR plays an important role in chromatin structure modulation, allowing promoter access to a range of other activator proteins in a glucocorticoid-dependent manner (15, 47). It is tempting to speculate that recruitment of the Ada adaptor complex by the GR might contribute to these rearrangements of chromatin structure. However, at present little is known about the mechanism of GR-mediated chromatin structure modulation and the receptor domains involved, although one recent study did report that the N-terminal half of the GR, which contains the τ1 domain, was required for chromatin derepression of the MMTV long terminal repeat (14).

Since transcriptional activation by the τ1c domain in vitro occurs in the absence of a classical chromatin structure but is still dependent on the Ada complex, it is likely that the Ada complex plays a role in later steps of gene activation. The postulated role of the Ada complex in connecting the transactivation domains of activator proteins to the general transcriptional machinery via TBP (3) is consistent with previous studies on gene activation by the GR and provides a complementary alternative to a putative role in chromatin structure modulation. The GR has been shown to facilitate formation of transcription complexes in vitro (23, 39, 54), and it is conceivable that the Ada complex plays a role in this process. Support for the view that the Ada complex exerts its effect at the level of TBP in vivo has come from the independent isolation of the ADA5 gene (35) as SPT20 (48), a member of the TBP class of SPT genes. This group of genes includes SPT15 (encoding TBP) and SPT3, which binds TBP and potentiates its activity at some promoters. Mutations in the other members of the group (SPT7, SPT8, and SPT20) cause phenotypes similar to those seen for spt3 and spt15 mutants (48). The physiological link with TBP is further strengthened by the observation that deletion of SPT7 also causes an Ada phenotype and furthermore that some spt15 mutant alleles result in partial relief of squelching by Gal4-VP16 (35). Thus, together with our findings, this observation suggests that gene activation by the GR in vivo involves an Ada/Spt-dependent mechanism that potentiates the activity of TBP, perhaps by stabilizing its interaction with DNA.

The Ada pathway is mainly associated with the τ1 domain, since the other transactivation activities within the GR are reduced to a lesser extent in ada mutant strains. This selectivity is consistent with previous studies on yeast and viral activator proteins, which show that while the Gcn4, VP16 (5, 46), and Bel-1 (6) transactivation domains require the Ada adaptor, the transactivation domains from Hap4 (5, 46, 57) and probably E1A (12) do not. There are apparent similarities of structure and function between τ1c and the VP16 transactivation domains. Both are strong activators, enriched in acidic residues and with a propensity to adopt an α-helical conformation under appropriate conditions (17, 20, 44). In both cases, hydrophobic amino acids are critically important for activity (1, 48). In this study, we have shown that the τ1c domain, like VP16, requires the Ada complex for activity and interacts with it at a similar level. As for VP16, this involves interactions between τ1c and the Ada2 subunit of the complex. However, in spite of these similarities, the mechanism of τ1c-mediated gene activation shows important differences compared to that of VP16. Notably, the ada mutations did not relieve the growth arrest phenotype associated with τ1 squelching as they do for Gal4-VP16-mediated squelching. The most likely explanation for these observations is that τ1 squelching affects an Ada-independent gene activation pathway that is relatively insensitive to excess levels of Gal4-VP16 protein. Thus, there are likely to be

mechanistically distinct subclasses of Ada-dependent transactivation domains in addition to the distinction between the Ada-dependent and Ada-independent activator classes.

To investigate further the existence of Ada-independent transactivation mechanisms, we used the classical genetic approach of comparing the activities of double mutants, in which each of the ada deletion mutants was combined with a range of τ1c mutants, with the activity of the respective single mutants (i.e., wild-type τ1c in mutant yeast strains and τ1c mutants in the wild-type yeast strain). In all cases, the mutant τ1c domains caused additional defects in strains already defective for the Ada pathway. Thus, while the Ada adaptor is clearly important for τ1c activity, failure of the Ada pathway is not sufficient to account for all of the defects caused by a range of mutations in τ1c. One reason for studying a range of mutant τ1c mutant alleles was to determine whether any of the structural elements that we had identified previously in τ1c (17) might represent segments exclusively involved in the Ada pathway. The results suggest that this is not the case, since mutations representing all these elements appear to have defects in Ada-independent pathways.

The interpretation of our double-mutant analysis is uncomplicated if single ada deletion mutants contain little or no residual Ada activity as we have claimed. However, our results show that the ada3 deletion causes a more severe phenotype than the ada2 and gcn5 deletions, and so it could be argued that, at least, the ada2 and gcn5 strains contain residual Ada activity. This is unlikely to be the case for several reasons. (i) Double ada (e.g., ada2 gcn5) mutants do not have more severe Ada phenotypes than the most defective of the single mutants (24, 35, 36, 46) as would be expected if the single mutants still contained significant Ada activity. (ii) Deletion of GCN5 causes a drastic reduction in the level of Ada2 protein, and thus single deletions affect multiple Ada subunits at the protein level (12). (iii) Ada2 interacts with both Ada3 and Gcn5 but Ada3 and Gcn5 do not interact in the absence of Ada2, suggesting that assembly of the remaining Ada subunits is severely compromised in ada2 mutants (11, 29). (iv) Ada2 is a contact site for transactivation domains, and in ada2 mutants, the coupling of Gal4-VP16 to TBP is no longer detectable (3). It is more likely that the Ada3 protein has functions in addition to its role in the Ada complex and that defects in these functions directly or indirectly cause the lower levels of activator activity in ada3 strains. The Ada complex-independent role of Ada3 could be associated with its role in gene repression (8, 9, 37). The recently characterized Ada5 protein also has a broader spectrum of activity than the other Ada proteins (35). Not withstanding these considerations, it should be noted that τ1c mutants do have reduced activity even in the ada3 deletion strain that shows the strongest Ada phenotype. Thus taken together with the squelching results, our double-mutant analysis provides strong support for the existence of Ada-independent pathways of τ1-mediated gene activation. Interestingly, such an alternative pathway has recently been reported for the Ada-dependent yeast activator, Gcn4 (53).

Different types of alternative gene activation pathways that might be employed by the τ1 domain can be envisioned. The fact that Gcn5 is not essential for viability and that it is required for the activation of only a subset of genes while histone acetylation has been generally associated with gene activation has led to speculation that there may be alternative pathways of histone acetylation that augment Gcn5 activity (10). Such activities could be linked to other adaptor pathways that are utilized by τ1. It is also possible that the Swi/Snf complex (see the introduction) or other analogous complexes (55) interact with the τ1 domain and contribute to its function. There might

also be alternative pathways, in addition to the Ada pathway, that target TBP. The best-characterized candidate in this case is the TFIID complex, which is composed of TBP and a number of additional subunits, termed TBP-associated factors (TAFs). Interestingly, it was recently reported that the yeast Spt3 protein, which is homologous to human $TAF_{II}18$, plays a role in GR-mediated transactivation in yeast (31). Furthermore, our recent results suggests that $\tau$1 can activate transcription in HeLa nuclear extracts via direct interactions with the TFIID protein complex (22). However, when considering pathways that impact at the level of TBP, it should be noted that excess TBP is not able to overcome $\tau$1 mediated squelching in vitro (40), suggesting a role for at least one non-TBP-associated mechanism. Alternative mechanisms could be routed via the RNA holopolymerase II complex. This complex contains RNA polymerase II, a number of general transcription factors, and a range of putative adaptor proteins (e.g., Gal11, Sin4, and Rgr1). Consistent with this type of pathway, squelching by Gcn4 can be overcome by high-level expression of TFIIB (a component of the holopolymerase), and two unlinked mutants which also relieve GCN4 squelching have been identified, possibly identifying a new adaptor pathway (53). However, it is significant that neither increased gene dosage of SUA7 (yeast TFIIB) effected by using a 2-$\mu$m plasmid-based vector nor addition of excess yeast TFIIB to $\tau$1-squelched in vitro transcription reactions alleviates squelching by $\tau$1 in yeast (59).

Although the bromodomain of Gcn5 and the putative zinc-binding domain of Ada2 have been noted in proteins from higher eukaryotes, it has been uncertain whether adaptors similar to the Ada complex might exist in mammalian cells. The recent identification of human homologs of Ada2 and Gcn5 (12) suggests that mammalian cells are likely to have an Ada adaptor. Since this initial report, two further cDNA clones that represent variants of Gcn5 have been reported (61). The deduced protein sequence of the first is identical to the initially reported human Gcn5 sequence except that it contains a 49-amino-acid extension at the N terminus. The second, P/CAF, has a 352-amino-acid residue P/CAF-specific domain followed by a 480-residue domain that is highly homologous to Gcn5 throughout its length. The P/CAF-specific domain interacts with the CREB-binding protein (CBP)/p300 family of mammalian adaptor proteins. Interestingly, CBP has a domain that is homologous to a region of Ada2 (11) and has recently been shown to interact with the GR (32). It was suggested that this interaction might contribute to the anti-inflammatory role of glucocorticoids by reducing the pool of CBP needed for AP-1-dependent activation of inflammatory response genes, such as the collagenase gene. A recent report suggests that CBP might also function as an adaptor for the GR (13). The identification of several Gcn5 homologs in human cells suggests a more complex situation in humans than in yeast, with the possibility of multiple Ada complexes of heterogeneous composition. Nonetheless, our observation that the transactivation activity of the GR is enhanced by cotransfected plasmids encoding hAda2 suggests that this class of proteins also plays an important role for gene activation by the GR in mammalian cells.

In conclusion, our results indicate that the Ada complex is important for the transactivation activity of the GR $\tau$1 domain, although other adaptor pathways are also likely to be important. This represents the first identification of an adaptor protein that can contact the $\tau$1 domain and adds an important new dimension to the transactivation domain structure-function studies that are being performed. Similarly, the demonstration that the Ada complex is important for the GR provides new possibilities for genetic screens to identify the as yet unidentified pathways involved in gene activation by the GR.

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