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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 \( \gamma \)-1 core transactivation domain (\( \gamma \)-1c) 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-\( \gamma \) fusion protein coupled to agarose beads, and a direct interaction between Ada2 and \( \gamma \)-1c can be shown by using purified proteins. This interaction is strongly reduced by a mutation in \( \gamma \)-1c that reduces transactivation activity. Mutations affecting the Ada complex do not reverse transcriptional squelching by the \( \gamma \)-1 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 \( \gamma \)-1 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 \( \gamma \)-1c 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, \( \gamma \)-1 (residues 77 to 262) and \( \gamma \)-2 (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 \( \gamma \)-1 transactivation domain plays a key role in GR activity (27). The \( \gamma \)-1 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 (\( \gamma \)-1c) of the \( \gamma \)-1 domain has been localized to a 58-amino-acid segment (residues 167 to 244) (16). The \( \gamma \)-1c domain is almost as active as the intact \( \gamma \)-1 domain and represents the only autonomous transactivation activity within the larger fragment. Similar to other transactivation domains, purified \( \gamma \)-1 and \( \gamma \)-1c are poorly structured in aqueous solution but have a propensity for \( \alpha \)-helical conformation in more hydrophobic environments (17). Three segments with propensity for \( \alpha \)-helix formation have been identified in the \( \gamma \)-1c domain by nuclear magnetic resonance spectroscopy (17). Proline substitution mutations within these segments reduce both transactivation activity and the propensity for \( \alpha \)-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 \( \alpha \)-helices (1).

The mechanism by which different components of \( \gamma \)-1 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 \( \gamma \)-1, have been reported to increase the rate of formation of correctly assembled transcription complexes on template DNA. Excess \( \gamma \)-1 squelches (inhibits) the expression of basal reporter genes both in vivo (60) and in vitro (40), suggesting that \( \gamma \)-1 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 \( \gamma \)-1 are still inhibitory when added after formation of the transcription complex, and thus the \( \gamma \)-1 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 \( \gamma \)-1 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-
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 transcriptional 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 A da2, A dA3/Ng11, Gcn5/Ada4, and A dA5/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-V P16 fusion protein, and based on this phenotype, it was argued that the A dA proteins might link the transcription domains of activator proteins to the general transcriptional machinery (5). This view has subsequently been supported by reports of direct interactions between A dA proteins and the transactivation domains of V P16 and Gcn4 as well as with the TATA-binding protein (TBP; Spt15) within the general transcriptional machinery (3, 35, 52). Many of the A dA 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 A da complex as functions as an activator for only a subset of the so-called acidic transacti-

The intact human GR was expressed from a galactoside-inducible P Gk1 pro-
motor by using the centromeric vector pRS315-NX, described previously (33). D eletion of a BglII fragment representing the GR -1 domain from pRS315-NX gave rise to pRS315-NX-11, which expresses the GR-1 protein, lacking GR residues 77 to 261. pRS315-11-lexA was constructed by inserting a BamHI-Sall fragment encoding the LexA DNA-binding domain (residues 1 to 87) expressed from the same promoter and followed by the PGK 1 region into the equivalent sites of pRS315(SacI). pRS315(SacI) was produced by cleaving pRS315 (51) with SacI, 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 SacI cloning site for the insertion of DNA fragments, placed by a fragment containing two LexA binding sites (58). The LexA-responsive reporter plasmid (pLGZ-2lex) was iden-
tified from the bacterial plasmid pLGZ-TAT, described previously (52) and was generously provided by Leonard Guarente. GST fusion proteins were bound to gluthathione-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 1 mg of protein/ml of beads. Yeast nuclear extract was prepared from the 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-indolylglucoside (X-Gal) plates for homogenous acti-

MATERIALS AND METHODS

Strains and plasmids. The wild-type yeast strain used throughout was PSY315 (MAT a ade2-101 ura3-52 leu2-3,112 his3-120 lys2), which was kindly provided by Leonard Guarente (Massachusetts Institute of Technology). Strains contain-
ing disruptions of the A da2, A dA3, and Gcn5 genes in the PSY315 background were also generously provided by Leonard G uarente. In these strains, the deleted genes are replaced by the bacterial his3 gene as described previously (5, 36, 46).

The intact human GR was expressed from a galactoside-inducible PGk1 pro-
motor by using the centromeric vector pRS315-NX, described previously (33). D eletion of a BglII fragment representing the GR -1 domain from pRS315-NX gave rise to pRS315-NX-11, which expresses the GR-1 protein, lacking GR residues 77 to 261. pRS315-11-lexA was constructed by inserting a BamHI-Sall fragment encoding the LexA DNA-binding domain (residues 1 to 87) expressed from the same promoter and followed by the PGK 1 region into the equivalent sites of pRS315(SacI). pRS315(SacI) was produced by cleaving pRS315 (51) with SacI, 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 SacI cloning site for the insertion of DNA fragments, placed by a fragment containing two LexA binding sites (58). The LexA-responsive reporter plasmid (pLGZ-2lex) was iden-
tified from the bacterial plasmid pLGZ-TAT, described previously (52) and was generously provided by Leonard Guarente. GST fusion proteins were bound to gluthathione-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 1 mg/ml in 8 bead volumes (200 µl) of Y buffer (20 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM MgCl2, 5 mM EDTA, 10% (vol/vol) glycerol, 50 mM NH4SO4, 1 mM diethylthiotreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF)). After etra-

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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, -1 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, -1 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 -1 domain (1c), 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 -1c 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 on the activity of -1c, 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 -1c-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.

-1c-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 -1 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 -1c domain in such systems, we tested the activity of -1c in nuclear extracts prepared from wild-type and mutant strains.

FIG. 1. The Ada adaptor complex is important for gene activation mediated via the -1c 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 (1, 1c, 2, and -1c) 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-nitrophenol/β-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 -1c-LexA protein in wild-type and mutant strains.
is competent to support activation by some activators. Thus, active in both extracts, and therefore the
Conversely, the glutamine-rich AX transactivation domain was
transcription.

The r1 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 r1 domain might interact with the Ada protein complex, we used GST coprecipitation assays. The r1 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-r1 protein, most of the Ada2
protein is also found in the supernatant but a significant
portion is coprecipitated with the GST-r1 protein, and thus the r1
domain can interact with Ada2 either directly or indirectly via
another member of the Ada complex. The amount of Ada2
precipitated by GST-r1 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 in-
teract directly with the Ada2 subunit of the Ada complex (3,
52). To determine whether this is also the case for the r1
domain, purified GST or GST-Ada2 proteins were incubated with
purified r1c-LexA, r1c(H1Ala)-LexA, and LexA proteins,
followed by precipitation of the GST proteins as described
above. The r1c(H1Ala)-LexA protein contains alanine substitu-
tions of five residues near the N terminus of the r1c domain and is severely reduced in transactivation activity (1). The
purified proteins used are shown in Fig. 3B. Figure 3B also
shows coprecipitation of r1c-LexA protein together with the
GST-Ada2 protein, indicating that they can interact directly.
This interaction is specific for the r1c 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 r1c(H1Ala)-LexA protein, and thus the reduced transac-
tivation activity of this mutant can be correlated with its
reduced ability to interact with the Ada2 protein. The interac-
tion with the r1c 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 squelch-
ing by the r1 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 pheno-
type (Fig. 4A and references 5 and 36). To determine whether
ada mutants also relieve squelching resulting from high expres-
sion of the GR r1 domain (60), we expressed high levels of r1
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 r1
but not the control protein retards growth of the wild-type
strain as reported previously (60). However, none of the mu-
tants reverse the squelching phenotype; if anything, the
squelching effect of r1 is stronger in the mutant strains. Thus,
Mechanisms of GR-mediated gene activation in addition to the Ada adaptor pathway. We have shown that the Ada complex plays an important role in GR-mediated transactivation and that this is likely to involve direct interactions between the GR 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 GR that distinguish 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, GR mutations that reduce activity in wild-type strains would not reduce the activity of one of the mutants, relative to unmutated one, 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, one mutations 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 GR on its transactivation activity, we measured the activity of a range of one mutants with reduced activity in ada2, ada3, and gcn5 mutant strains. Table 1 shows the relative activities of seven one mutants, representing different segments of one, in the wild-type, ada2, ada3, and gcn5 strains. All of the mutant one domains show defects in the ada2, ada3, and gcn5 mutant...
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 $\tau$1 function, there are likely to be additional pathways that mediate $\tau$1 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-$\Delta$1 derivative was transfected (Fig. 5B). Therefore, although the hAda2-mediated enhancement of GR activity is modest, it is significant because it requires the $\tau$1 domain. Thus, as in yeast, the Ada adaptor pathway appears to play an important role in the activity of the $\tau$1 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 $\tau$1 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 hAda2 adaptor also seems to be selective for the $\tau$1 domain, since hAda2 did not enhance the activity of the GR-$\Delta$1 derivative in COS7 cells. $\tau$1c can interact directly with the Ada2 subunit of the complex, and this interaction is reduced by alanine substitution mutants in $\tau$1c 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 specifically interact with the GR $\tau$1 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

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**TABLE 1. Transactivation activities of $\tau$1c mutants in wild-type and ada mutant strains**

<table>
<thead>
<tr>
<th>$\tau$1c mutant</th>
<th>Wild type</th>
<th>ada2</th>
<th>ada3</th>
<th>gcn5</th>
</tr>
</thead>
<tbody>
<tr>
<td>F191D</td>
<td>100 (843)</td>
<td>100 (42)</td>
<td>100 (21)</td>
<td>100 (88)</td>
</tr>
<tr>
<td>L197E</td>
<td>42</td>
<td>31</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>W213A</td>
<td>30</td>
<td>22</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>H2(A)la$^c$</td>
<td>12</td>
<td>21</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>L194A/L224/V/L225F</td>
<td>4</td>
<td>10</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>F235L/L236V</td>
<td>5</td>
<td>8</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>13</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>

*$^a$The results for the mutant $\tau$1c-LexA mutant proteins were calculated from mean values obtained from two to three independent experiments (individual values for the mutant proteins varied by $\pm$35% from the mean). The actual $\beta$-galactosidase levels (units) are shown in parentheses for unmutated $\tau$1c.

*$^b$Values from Fig. 1B, $\tau$1c-LexA.

*$^c$L217, L218, I219, L224, and L225 are replaced by alanine.

**FIG. 5. Functional interaction between the GR and hAda2 in mammalian cells.** (A) Transactivation activity of the GR, with or without 1 $\mu$M 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 ($+\text{Dex}$) 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-$\Delta$1 protein.

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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 +1 domain, was required for chromatin derepression of the MMTV long terminal repeat (14).

Since transcriptional activation by the +1c 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 transcriptional 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 sp3 and sp15 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 sp15 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 +1 domain, since the other transcriptional 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) transcriptional domains require the Ada adaptor, the transcriptional domains from Hap4 (5, 46, 57) and probably E1A (12) do not. There are apparent similarities of structure and function between +1c and the VP16 transcriptional 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 +1c domain, like VP16, requires the Ada complex for activity and interacts with it at a similar level. As for VP16, this involves interactions between +1c and the Ada2 subunit of the complex. However, in spite of these similarities, the mechanism of +1c-mediated gene activation shows important differences compared to that of VP16. Notably, the ada mutations did not relieve the growth arrest phenotype associated with +1 squelching as they do for Gal4-VP16-mediated squelching. The most likely explanation for these observations is that +1 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 transcriptional domains in addition to the distinction between the Ada-dependent and Ada-independent activator classes.

To investigate further the existence of Ada-independent transcriptional 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 +1c mutants, with the activity of the respective single mutants (i.e., wild-type +1c in mutant yeast strains and +1c mutants in the wild-type yeast strain). In all cases, the mutant +1c domains caused additional defects in strains already defective for the Ada pathway. Thus, while the Ada adaptor is clearly important for +1c activity, failure of the Ada pathway is not sufficient to account for all of the defects caused by a range of mutations in +1c. One reason for studying a range of mutant +1c mutant alleles was to determine whether any of the structural elements that we had identified previously in +1c (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 transcriptional 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). Notwithstanding these considerations, it should be noted that +1c 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 +1-mediated gene activation. Interestingly, such an alternative pathway has recently been reported for the Ada-independent yeast activator, Gcn4 (53).

Different types of alternative gene activation pathways that might be employed by the +1 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 +1. It is also possible that the Swi/Snf complex (see the introduction) or other analogous complexes (55) interact with the +1 domain and contribute to its function. There might
also be alternative pathways, in addition to the A da 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 Sp3 protein, which is homologous to human TAF 18, plays a role in GR-mediated transactivation in yeast (31). Furthermore, our recent results suggest that TBP 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 TBP-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 II complex) 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-μm plasmid-based vector nor addition of excess yeast TFIIB to TBP-squelched in vitro transcription reactions alleviates squelching by TBP in yeast (59).

Although the bromodomain of GCN5 and the putative zinc-finger binding domain of A da2 have been noted in proteins from higher eukaryotes, it has been uncertain whether adaptors similar to the A da complex might exist in mammalian cells. The recent identification of human homologs of A da2 and GCN5 (12) suggests that mammalian cells are likely to have an A da2 protein. 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 A da2 (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 A da complexes of heterogeneous composition. Nonetheless, our observation that the transactivation activity of the GR is enhanced by cotransfected plasmids encoding A da2 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 A da complex is important for the transactivation activity of the GR -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 -1 domain and adds an important new dimension to the transactivation domain structure-function studies that are being performed. Similarly, the demonstration that the A da 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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