It is possible to recruit RNA polymerase II to a target promoter and, thus, activate transcription by fusing Mediator subunits to a DNA binding domain. To investigate functional interactions within Mediator, we have tested such fusions of the lexA DNA binding domain to Med1, Med2, Gal11, Srb7, and Srb10 in wild type, med1, med2, gal11, sin4, srb8, srb10, and srb11 strains. We found that lexA-Med2 and lexA-Gal11 are strong activators that are independent of all Mediator subunits tested. lexA-Srb10 is a weak activator that depends on Srb8 and Srb11. lexA-Med1 and lexA-Srb7 are both cryptic activators that become active in the absence of Srb8, Srb10, Srb11, or Sin4. An unexpected finding was that lexA-VP16 differs from Gal4-VP16 in that it is independent of the activator binding Mediator module. Both lexA-Med1 and lexA-Srb7 are stably associated with Med4 and Med8, which suggests that they are incorporated into Mediator. Med4 and Med8 exist in two mobility forms that differ in their association with lexA-Med1 and lexA-Srb7. Within purified Mediator, Med4 is present as a phosphorylated lower mobility form. Taken together, these results suggest that assembly of Mediator is a multistep process that involves conversion of both Med4 and Med8 to their low mobility forms.

RNA polymerase II (pol II) transcribes all protein-encoding genes and some small nuclear RNA genes in eukaryotes. The yeast pol II holoenzyme (1) consists of a catalytic core enzyme of 12 subunits (2, 3) whose crystal structure has been solved (4, 5) and a regulatory Mediator complex comprising 20 subunits. All subunits of the Mediator have now been identified, and 13 of them are encoded by previously known genes. The remaining seven subunits are novel proteins named Med1, Med2, Med4, Med6, Med7, Med8, and Med11 (6–12). Transcription can be reconstituted in vitro from highly purified pol II core enzyme and five general transcription factors, which are the TATA binding protein, TFIIA, TFIIE, TFIIF, and TFIIH. These proteins are necessary and sufficient for basal transcription in vitro, but they are unable to respond to transcriptional activators (13). Mediator was originally defined as an activity in yeast nuclear extracts that is required for such regulated transcription (14, 15). In addition, Mediator enhances basal transcription, stimulates phosphorylation of the C-terminal domain of the largest pol II subunit by TFIIH, and binds to the C-terminal domain. The latter interaction is important for the integrity of the holoenzyme (1).

A partial truncation of the C-terminal domain causes a temperature-sensitive phenotype (16) that is suppressed by mutations in the SRB genes. Several of the Srb proteins are present both in Mediator and in another complex that contains the pol II core enzyme and which also has been referred to as the pol II holoenzyme (17). It differs from the holoenzyme described above in that it also contains the Srb8–11 proteins, the SWI/SNF complex, some general transcription factors, and other yet unidentified proteins (18, 19). It is likely that this reflects the different purification procedures used and that additional proteins found only in the second holoenzyme are more loosely associated with pol II in vivo. In any case, their absence from the first holoenzyme shows that they are not strictly required for regulated transcription in vitro. An attempt to further clarify the in vivo composition of the holopolymerase was recently made by Hahn and co-workers (20). They purified Mediator from yeast strains where Srb5, Srb6, or Rgr1 were epitope-tagged. This allows purification of the holopolymerase without the use of high ionic strength buffers and should, thus, better preserve its in vivo composition. Mediator was found to be present in two major forms of 1.9 and 0.55 MDa, respectively. The larger complex is identical to the holopolymerase described by Kornberg and coworkers (1, 14, 15), except that it also contains the Srb8–11 module. The smaller complex is similar to free Mediator (9) in that it lacks the Srb8–11 module, but it also lacks Nut1, Gal11, Rgr1, Sin4, Med2, Pgd1, and Rox3. Notably, several of these proteins have been proposed to form a distinct activator binding tail module (21–23), which may be more loosely associated with the rest of the holoenzyme.

The Srb8–11 module, which contains the cyclin C-dependent kinase, has been implicated in negative control of gene expression. Thus, SRB8–11 appeared in several genetic screens for genes involved in repression (24). Because TUP1 and CYC8 also appeared in the same genetic screens, it has been suggested that Srb8–11 may be involved in transmitting repressive signals from the Cyc8-Tup1 co-repressor complex to pol II. However, other Mediator subunits such as Pgd1 and Srb7 (25, 26) have also been proposed to be targets of Cyc8-Tup1. It should be emphasized that mutations in the Srb8–11 module have a complex phenotype that also includes reduced transcrip-
tion of some highly expressed genes (6, 10).

Fusions between general transcription factors and DNA binding domains can activate transcription from promoters that bind such fusion proteins. An activation-by-recruitment model, which suggests that physical recruitment of the basal RNA polymerase II machinery to a promoter may be sufficient to activate transcription, has therefore been proposed (27–29).

Hybrid activators that contain parts of general transcription factors or holoenzyme subunits have been called non-classical activators, as opposed to classical activators with conventional activation domains (30). Several Mediator subunits, i.e. Med6, Gal11, and Sin4, have been shown to function as non-classical activators (21, 30, 31). Srb2, Srb4, Srb5, and Srb6 can activate transcription as lexA fusions but depend on the promoter context for their ability to do so (30). A lexA-Srb11 fusion could activate transcription (32), whereas a lexA-Srb10 fusion required either overexpression of Srb11 or a mutation in the kinase active site (33). Finally, we have found that lexA-Med1 is a cryptic activator that becomes active only in the absence of Srb11 or if it is overexpressed (10).

In the present investigation, we have used activation by recruitment as a tool to study functional interactions between different Mediator subunits. We find that the non-classical activators differ in their dependencies on other proteins. Thus, lexA-Med2 and lexA-Gal11 are strong activators that function in all mutants tested. lexA-Srb10 is a weak activator that is dependent on Srb11 and Srb8. lexA-Med1 and lexA-Srb7, finally, are cryptic activators that function only in the absence of either the Srb8–11 module or Sin4. The association of lexA-Med1 and lexA-Srb7 with Mediator was further investigated by co-immunoprecipitation. We found that both hybrid activators co-precipitate with Med4 and Med8, indicating that they are stably integrated into Mediator. Finally, we found that both Med4 and Med8 exist in two forms with different mobility. Only the low mobility form of Med4 associates with lexA-Med1 and lexA-Srb7. Both forms of Med8 associate with lexA-Srb7, whereas the low mobility form preferentially associates with lexA-Med1.

### EXPERIMENTAL PROCEDURES

**Yeast Strains**—All yeast strains used in this study (Table I) were W303 congenic (34) and, therefore, carry the following markers: \text{MATa ade2-1 can1-101 his3-11,15 leu2-3,112 trp1-1 ura3-1}. To generate \text{gal11-11::HIS3}, the \text{GAL11} coding sequence between two \text{Bsu36I} sites was replaced by a \text{HIS3} \\text{BamHI} fragment. To generate \text{srb8-11::LEU2}, the \text{SRB8} coding sequence between the \text{BstXI} and \text{NdeI} sites was replaced by a \text{LEU2} \text{HpaI} \text{SalI} fragment. The \text{med1::HIS3, med2::HIS3, med4::HIS3, med6::HIS3, med8::HIS3, srb11::LEU2, and srb10::HIS3} strains have been described (10, 35). Strains DY1699 and DY1702 were kindly provided by David Stillman.

**Plasmids**—The \text{pDB185}, \text{pDB181}, \text{pDB198}, and \text{pDB223} plasmids have been described (10, 36). To create \text{pDB326}, the lexA \text{HindIII-ApaI} fragment of \text{pDB198} was replaced by the \text{HindIII-ApaI} fragment of \text{pGWT} (37) containing the \text{GAL} DNA binding domain. To create the lexA-Med2 fusion, the \text{MED2} open reading frame from a baculovirus expression cassette (kindly provided by Claes Gustafsson) was cloned as an \text{EcoRI}–\text{Xhol} fragment into an \text{EcoRI}–\text{Xhol}-digested \text{pEG202}, thus producing \text{pDB212}. The whole lexA-Med2 expression cassette was then subcloned as an \text{Sp}1 fragment into a \text{pFL39} (38), thus producing \text{pDB213}. The lexA-lexA-Gal11 fusion plasmids were all made by cloning \text{BamHI}–\text{Xhol}-digested PCR products into \text{BamHI}–\text{Xhol}-digested \text{pDB223}. \text{SRB7} was amplified by using \text{AGGAGTCCCAGCGAGATTACAC} and \text{AGCTCGAGTTAGTCGTTTTTGGATT} as primers; \text{SRB10} was amplified by using the primers \text{GGATGTCGCGATGTAATGAAGCAGTA} and \text{AGCTCGAGTTAGTCGTTTTTGGATT} as primers. Both \text{SRB7} and \text{SRB10} were amplified by using the primers \text{AGGATCTCCCTAGGTCGTTCTCTGTCGCC} and \text{CGAAATTCTAGACGGCATTGCTCAAAT}. To rule out PCR artifacts in the complementation experiments, at least four different PCR clones were tested for each fusion. Plasmids \text{pGWT} and \text{pGfA}, expressing wild type \text{Gal4–VP16} (39) and the \text{Gal4–VP16–F442A} mutant (22), respectively, were kindly provided by Lawrence Myers. As 2-hybrid \text{lexA} reporter plasmids, we used \text{pSH18–34} (40) for the lexA fusions, \text{pLGSIDS} (22) for the \text{Gal4} fusions, and \text{pJK101} (41, 42) for both.

### RESULTS

**lexA-Med1, lexA-Med2, and lexA-Srb7 Can Functionally Replace the Corresponding Wild Type Proteins**—We have previously shown that the \text{Med1} protein becomes a cryptic activator when it is fused to the \text{lexA} DNA binding domain (10). To test how general a phenomenon this is and to study functional interactions between different Mediator subunits, we have now made lexA fusions to \text{Med2}, \text{Gal11}, \text{Srb7}, and \text{Srb10}. As a first step in characterizing these new fusion proteins, we attempted to determine if they could complement deletions of the corresponding wild type genes. This is an important question be-
cause non-classical activators are thought to function by being incorporated into the holoenzyme in place of the wild type protein, thereby recruiting pol II to the target promoter. However, this has to the best of our knowledge never been proven experimentally. Complementation of a deletion mutation would provide strong evidence that a fusion protein can replace the wild type protein within the holoenzyme. We found that lexA-Med1, lexA-Med2, and lexA-Srb7 are all able to complement deletions of the corresponding genes (Fig. 1). In contrast, lexA-Gal11 and lexA-Srb10 failed to do so. It should be noted that whereas complementation provides evidence of functional replacement, lack of complementation does not necessarily mean that a fusion protein cannot be incorporated into the holoenzyme. It may also reflect steric hindrances that prevent the incorporated protein from functioning properly. We conclude that complementation supports the notion that lexA-Med1, lexA-Med2, and lexA-Srb7 are incorporated into the pol II holoenzyme, whereas the presence of lexA-Gal11 and lexA-Srb10 within the holoenzyme must be ascertained by other means.

lexA-Med2 and lexA-Gal11 Are Strong Activators That Are Independent of All Mediator Subunits Tested—We proceeded to test the ability of lexA-Med1, lexA-Med2, lexA-Gal11, lexA-Srb7, and lexA-Srb10 to activate transcription both in wild type cells and in med1, med2, gal11, sin4, srb8, srb10, and srb11 deletion strains (Table II). We found that the lexA fusions fall into three distinct groups with respect to how they respond to mutations in other Mediator subunits. lexA-Med2 and lexA-Gal11 comprise the first group and behave similarly to the classical activator lexA-VP16, which we included as a control in these experiments. Thus, lexA-Med2 and lexA-Gal11 are strong activators comparable in strength to lexA-VP16 both in the wild type and in all mutant strains tested. One difference between the two activators is seen in the gal11 strain, where the activity of lexA-Gal11 is enhanced, whereas the activity of lexA-Med2 is unaffected. Conceivably, this could be due to increased incorporation of lexA-Gal11 into the Mediator in the absence of wild type Gal11 protein. In conclusion, our results show that both lexA-Med2 and lexA-Gal11 can function as activators in the absence of all other Mediator subunits tested. It should be noted that our finding that lexA-VP16 is independent of Med2, Gal11, and Sin4 was unexpected and is further discussed below.

lexA-Srb10 Is a Weak Activator That Requires Srb11 and Srb8—A second kind of result was obtained with the lexA-Srb10 fusion. We found that it is a weak activator in all strains except in the srb11 mutant, where it is completely inactive, and in the srb8 mutant, where its activity is reduced (Table II). These findings suggest that the integrity of the Srb8–11 module is important for the ability of lexA-Srb10 to recruit the holopolymerase. This may reflect the fact that cyclin-dependent kinases such as Srb10 usually depend on their cyclins (in this case Srb11) both for activity and for interaction with the correct target protein (47, 48). Accordingly, the ability of Srb10 to activate by recruitment would suggest that it has at least one target within the pol II holoenzyme. The rather low level of activity for lexA-Srb10 compared with other fusions may result from a more loose association between the Srb8–11 module and Mediator. Alternatively, the inherent repressing activity of the Srb8–11 module interferes with the ability of lexA-Srb10 to activate transcription.

lexA-Med1 and lexA-Srb7 Are Cryptic Activators That Become Active in srb8, srb10, srb11, and sin4 Mutants—A third type of result was obtained with lexA-Med1 and lexA-Srb7. We have previously shown that lexA-Med1 is a cryptic activator that has little or no activity in wild type cells but becomes active in srb11 cells (10). Here we show that deletions of SRB8, SRB10, or SIN4 have a similar effect (Table II). In contrast, deletions of MED1, GAL11, or MED2 have no effect on lexA-Med1, which remains inactive in these strains. We conclude that the cryptic activity of lexA-Med1 can be unmasked in at least three ways, by mutations in the cyclin C-dependent protein kinase complex (Srb8–11), by mutations in Sin4, and by overexpression of lexA-Med1 itself (10). This suggests a possible functional link between the cyclin C-dependent protein kinase and Sin4.

Surprisingly, we found that lexA-Srb7 also is a cryptic activator that behaves similarly to lexA-Med1. Thus, it has no activity in the wild type, whereas deletions of SRB8, SRB10, SRB11, and SIN4 cause it to become active (Table II). However, lexA-Srb7 differs from lexA-Med1 in three ways. First, its activity when unmasked by the srb8, srb10, or srb11 deletions is about 8-fold lower than that of lexA-Med1. Second, the sin4 deletion has a much stronger effect on lexA-Srb7 compared with the srb8, srb10, and srb11 deletions (Table II). Third and most important, we found that a deletion of MED1 activates lexA-Srb7. This suggests that Med1 may be involved in transmitting the negative effect that the Srb8–11 module exerts on lexA-Srb7 (see “Discussion”). The fact that a similar effect was not observed for lexA-Med1 is most likely due to the ability of lexA-Med1 itself to complement the med1 deletion, thus making it phenotypically wild type.

lexA-VP16 Is Independent of the Activator Binding Module—Our finding that the classical activator lexA-VP16 is independent of all Mediator subunits tested, including Med2, Pgd1, Gal11, and Sin4 (Table II), was unexpected for two reasons. First, in vitro experiments suggest that Gal11 is required for activation by Gal4-VP16 (21). Second, Myers et al. (22) find that the in vivo activity of Gal4-VP16 is reduced 8-fold in med2 cells. It has, therefore, been suggested that the VP16-activating domain is dependent on the activator binding tail module of the Mediator, which includes Med2, Gal11, and Sin4. Because different strains and plasmids were used in the two experiments, we obtained the plasmids used by Myers et al. (22) and tested them in our own strains. We could confirm that a significant difference exists between Gal4-VP16 and lexA-VP16, with the former but not the latter dependent on Med2. In fact, we see an even larger effect than Myers et al. (22), with a more than 100-fold reduction of Gal4-VP16 activity in the med2 strain using the Gal4 binding reporter pLGSD5 (Table III). We also tested the effect of sin4 and gal11. We found a 14-fold reduction in Gal4-VP16 activity in the sin4 strain and a 3-fold reduction in the gal11 strain. Neither deletion has a significant effect on lexA-VP16 (Table II). To rule out that the observed differences are due to different reporters being used with lexA-VP16 and
Gal4-VP16, we proceeded to test the activators with a reporter (pJK101) that has both Gal4 and lexA binding sites (41, 42). The results were similar, with Gal4-VP16 but not lexA-VP16 dependent on Med2, Sin4, and, to some extent, also on Gal11 (Table III). We conclude that Gal4-VP16 is dependent on Med2, Sin4, and Gal11, whereas lexA-VP16 shows no such dependencies. This suggests that the dependence of Gal4-VP16 on the activator binding module may be mediated by the Gal4 DNA binding domain rather than by the VP16 activation domain. The Dependence of Gal4-VP16 on the Activator Binding Module Is Strongly Affected by the Gal4-VP16 Copy Number—One further difference in our experiments was that lexA-VP16 is expressed from a single copy plasmid, whereas Gal4-VP16 is expressed from a high copy number plasmid. We therefore proceeded to express Gal4-VP16 from the single copy plasmid pDB326 and test it with both the pLGSD5 and pJK101 reporters (Table III). We found that a reduced copy number for Gal4-VP16 strongly affects both its activity in the wild type strain and its dependence on subunits within the activator binding module. Furthermore, quite different effects were seen for different subunits. Thus, the activity in the wild type strain and in the gal11 mutant both drop 20–40-fold as compared with Gal4-VP16 on a high copy number plasmid (Table III). In contrast, the residual activity in the med2 mutant (11 and 64 units, respectively, for the 2 reporters) is unaffected by the Gal4-VP16 copy number. A third kind of result was seen in the sin4 mutant, where the activity actually increases 2–3-fold as compared with Gal4-VP16 on a high copy number plasmid (Table III). These effects are statistically significant; moreover, the same pattern was seen with both reporters. In a final experiment, we decided to determine if the copy number effects could be reproduced by reducing the level of Gal4-VP16 activity through other means. For this, we used the attenuated activation domain mutant Gal4-VP16-P442A (22). This mutant, when expressed from a high copy number plasmid, has a 2–4-fold reduced activity in wild type cells (Table III). We saw a similar 2–4-fold drop in activity also in the med2, gal11, and sin4 mutants. This is consistent with the findings of Myers et al. (22) regarding med2 and sin4, although the effects on both Gal4-VP16 and Gal4-VP16-P442A activity were smaller in that case. We conclude that reducing the level of Gal4-VP16 activity through an attenuating mutation does not affect its dependence on Med2, Gal11, or Sin4.

Effects on the ADH1 Promoter in Mediator Mutants—We have previously noted that the amount of lexA-Med1 protein that can be detected in Western blots increases 3-fold in strains lacking Srb11 (10). This raised the question if deletions of other Mediator subunits would have a similar effect. We therefore transformed the deletion strains with a reporter where the lacZ gene is expressed from the same promoter (ADH1) that was used to express the lexA fusion proteins. We found that all Mediator deletions tested cause an increase in lacZ expression ranging from 2-fold in the gal11 strains to 9-fold in the sin4 strain (Table II). A med2 srb11 double deletion did not produce a stronger effect than either deletion alone (see below). We conclude that expression from the ADH1 promoter is increased in strains where the integrity of the Mediator is disturbed. This effect is too small to explain the 20–80-fold increase in lexA-Med1 or lexA-Srb7 activity that is seen in some of the mutants. Furthermore, the effects on the ADH1 promoter is seen also in the med2 mutant that has no effect on either lexA-Med1 or lexA-Srb7 (Table II). Nevertheless, this effect should be kept in mind when interpreting the lexA-Med1 and lexA-Srb7 results. Med2 Is Required for Activation by lexA-Med1 and lexA-Srb7 in srb11 Cells—Although lexA-Med1 fails to activate transcription when expressed from a single copy plasmid in wild type cells, overexpression from a high copy number plasmid causes it to become active (10). The activity achieved in this case is comparable with that seen with a single copy lexA-Med1 plasmid in srb11 mutant cells. Interestingly, this activity is reduced more than 10-fold in a med2 strain that, together with our previous results that Med2 is absent from Mediator in cells lacking Med1, indicate an interaction between these two proteins (10). To determine if this requirement also exists when lexA-Med1 is activated by an srb11 mutation, we assayed its activity in a med2 srb11 double mutant strain. We found that
the lexA-Med1 fusion is inactive (1.7 ± 0.3 units) in the double mutant strain. This is probably not caused by a failure to express lexA-Med1, since we found that expression of the β-galactosidase gene from the ADH1 promoter used to express lexA-Med1 produces a level of activity (24 ± 4 units) in the med2 srb11 double mutant that is comparable with that seen in the srb11 and med2 single mutants (Table II). Instead, it suggests that Med2 is required for transcriptional activation by the lexA-Med1 cryptic activator irrespective of how it is activated. We next proceeded to determine the ability of lexA-Srb7 to activate transcription in the med2 srb11 strain. Just as for lexA-Med1, we found that it is inactive (1.5 ± 0.4 units) in the double mutant strain. It should be noted that Med2 is unlikely to be required for incorporation of Srb7 into the Mediator since SBR7 is an essential gene, whereas MED2 is not. Moreover, lexA-Srb7 co-immunoprecipitates with other Mediator subunits in med2 cells (see below), which reinforces the notion that its incorporation into the Mediator is independent of Med2. Therefore, the epistasis of med2 over srb11 in this case must reflect a partial functional dependence of lexA-Srb7 on Med2.

Both lexA-Med1 and lexA-Srb7 Are Stably Associated with Med4 and Med8 in Wild Type and Mutant Cells—To find out more about the mechanism(s) of action of the two cryptic activators, lexA-Med1 and lexA-Srb7, we decided to investigate to what extent they are associated with other subunits within the Mediator. Extracts were prepared from wild type cells expressing either lexA-Med1 or lexA-Srb7 and precipitated with monoclonal antibodies specific for the lexA DNA binding domain. Precipitated proteins were separated by SDS-PAGE and analyzed by Western blots using antisera against Med4 and Med8. As a control, we also included extracts from strains expressing lexA-VP16. Our assumption was that lexA-VP16 would function as a classical activator by forming transient interactions with the Mediator. Consistent with this, we found that although lexA-VP16 itself was easily detected in the precipitate, there was no evidence of Med4 or Med8 being co-precipitated with lexA-VP16 in any of the strains (data not shown). We conclude that any interactions that occur between lexA-VP16 and Mediator are either too transient or too weak to be detected in our co-immunoprecipitation experiments.

In contrast, we found that both Med4 and Med8 co-precipitate with lexA-Med1 in wild type cells (Fig. 2). Similarly, Med4 and Med8 also co-precipitate with lexA-Srb7 (Fig. 3). This suggests that both cryptic activators are more stably associated with Mediator than lexA-VP16 and is consistent with the notion that these non-classical activators function by being incorporated into the holoenzyme, thereby taking the place of the wild type protein. The association of lexA-Med1 and lexA-Srb7 with Med4 and Med8 is independent of all the other Mediator subunits tested, since it was seen also in the med1, med2, gal11, sin4, srb8, srb10, and srb11 strains (Figs. 2 and 3). This suggests that none of these subunits is required for assembly of the middle and head regions of the Mediator, where Med1, Srb7, Med4, and Med8 are found.

Two Distinct Forms of Med4 and Med8 Differ in Their Associations with lexA-Med1 and lexA-Srb7—Interestingly, we found that both Med4 and Med8 give rise to doublet bands in the Western blots. This is most clearly seen in the blots with whole cell extracts (Figs. 2 and 3). It further seems that the upper (low mobility) band is slightly more abundant for Med4, whereas the lower (high mobility) band is somewhat more pronounced for Med8. In the case of Med4, others have already noted the presence of two distinct bands in protein gels. In fact, the two bands were named Med4 and Med5 before sequencing revealed that they are that same polypeptide (9). It is noteworthy that the two forms of Med4 and Med8 differ in their degree of association with lexA-Med1 and lexA-Srb7. Thus, the two fusion proteins co-precipitate mainly with the low mobility form of Med4 (Figs. 2 and 3). For Med8, the picture is more complex. Thus, lexA-Med1 associates preferentially with the low mobility form of Med8 (Fig. 2), whereas lexA-Srb7 associates with both forms of Med8 (Fig. 3). These findings suggest that assembly of Mediator is affected by the modification status of association with lexA-Med1 and lexA-Srb7. Therefore, it is likely that these non-classical activators function by being incorporated into the Mediator rather than acting as classical activators by forming transient interactions with the Mediator.
of Med4 and Med8. Furthermore, the fact that the high mobility form of Med8 associates with lexA-Srb7 but only weakly or not at all with lexA-Med1 further suggests that assembly of the Mediator could be a multistep process (see “Discussion”).

**Med4 Is Phosphorylated When Present in Purified Mediator**—Multiple bands in protein gels are frequently caused by differential phosphorylation. We therefore proceeded to determine if Med4 and Med8 are phosphorylated in vivo. For this we used a yeast strain in which the Mediator subunit Rgr1 has been tandem affinity purification-tagged (45), thus allowing rapid isolation of the holopolymerase by tandem affinity purification (46). The purified holopolymerase was treated with calf intestine phosphatase, after which the subunits were separated by SDS-PAGE and probed in a Western blot with antisera against Med4 and Med8. As shown in Fig. 4, we found that Med4 from purified holopolymerase preferentially exists as a low mobility form that is converted into a higher mobility form upon phosphatase treatment. We conclude that Med4 is phosphorylated when present in Mediator and that this is a possible reason for the two mobility forms seen in Figs. 2 and 3. In contrast, we saw no evidence that Med8 from purified holopolymerase is phosphorylated (Fig. 4).

**Synthetic Interactions between med2, gal11, and sin4**—Deletions of **MED2**, **GAL11**, and **SIN4** have been reported to produce similar phenotypes, although some differences were observed for growth on galactose (22). The differences that we saw between the congenic **med2**, **gal11**, and **sin4** strains used in our lexA experiments, therefore, prompted us to examine their growth phenotypes. We found that all three strains have a general growth defect that is most severe in the **med2** strain and least severe in the **gal11** strain. All three strains also have a reduced ability to grow on galactose and gluconeogenic carbon sources such as acetate. However, in this case the effect was most pronounced in the **med2** strain and least pronounced in the **sin4** strain. Finally, all three strains are temperature-sensitive at 38 °C, although the **gal11** strain can grow on synthetic media at 38 °C. This may reflect the fact that the **gal11** strain is generally healthier than the **med2** and **sin4** strains (Fig. 5). We proceeded to test the **sin4** disruption for genetic interactions with the **med2** and **gal11** disruptions using tetrad analysis. We found that **gal11 sin4** spores grow more slowly than spores lacking either **sin4** or **gal11** alone (Fig. 6), indicating a synthetic interaction between the two disruptions. A similar interaction was seen between **sin4** and **med2** (data not shown).

**Partial Suppression of med2, gal11, and sin4 by Overexpression of PDC1**—The fact that **med2** cells are temperature-sensitive makes it possible to screen for high copy number suppressors. To this end we transformed a **med2** strain with a high copy number yeast genomic library. The transformed cells were plated at 35 °C, a temperature at which the **med2** strain still can grow but is unable to form colonies after transformation using the lithium acetate procedure. Plasmids were rescued

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**Fig. 4. Med4 is phosphorylated in purified holopolymerase.** Holopolymerase was purified from cells in which its Rgr1 subunit has been tandem affinity purification-tagged (45) as previously described (46). The purified holopolymerase was incubated for 1 h at 37 °C in the presence or absence of calf intestine phosphatase (**CIP**) and then analyzed by Western blots as described under “Experimental Procedures” using antisera against Med4 and Med8 (9).

**Fig. 5. Phenotypic differences between med2, gal11, and sin4 strains.** Wild type and mutant strains (Table I) were patched onto rich glucose medium (**YPD**) and then replicated to rich glucose medium, synthetic glucose medium (**SGlu**), synthetic acetate medium (**SAc**), or synthetic galactose medium containing 20 μg/ml of ethidium bromide (**SGal EB**). The plates were incubated at 30 °C unless otherwise stated. **wt**, wild type.

**Fig. 6. Synthetic interaction between sin4 and gal11 disruptions.** A diploid obtained by crossing strains H966 and DY1699 (Table I) was sporulated, and tetrads were dissected onto rich glucose medium (**YPD**). After colonies had formed, the plates were replicated to synthetic media lacking either leucine or histidine to identify cells carrying the **sin4** and **gal11** disruptions, respectively.
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from colonies formed and tested by re-transformation. We found 14 plasmids that did not contain MED2 itself (Table IV). Seven contained the PDC1 gene encoding pyruvate decarboxylase (49), and four contained PDC2, which encodes a transcriptional activator of PDC1 (50). The remaining three plasmids contained ZDS1 (Zillion Different Screens), a gene that is frequently found in suppressor screens, regardless of the mutant phenotype (51, 52). We conclude that overexpression of PDC1 either directly or through its activator PDC2 can suppress the inability of med2 cells to form colonies at 35 °C. Two other med2 phenotypes, failure to grow on galactose and temperature sensitivity at 38 °C, were not suppressed by these plasmids.

We proceeded to test if other mediator mutants also are unable to form colonies at 35 °C. We found that gal11 and sin4 deletion mutants display a similar phenotype, although less severe. In contrast, there was little or no effect on the plating efficiency in the med1 and srb11 strains. We further found that all three suppressor genes recovered in the med2 screen can suppress the reduced plating efficiency of the gal11 strain, with PDC1 being most efficient and ZDS1 the least efficient (Table IV). In the case of sin4, only PDC1 could clearly suppress the reduced plating efficiency, but the underlying phenotype is weaker in this case, making detection of suppression more difficult. We conclude that med2, gal11, and sin4 strains are similar in that their reduced plating efficiency is suppressed by overexpression of pyruvate decarboxylase, a finding that suggests a similar underlying defect in all three strains.

## DISCUSSION

We have used activation by recruitment as a tool to study functional interactions between different Mediator subunits. Full-length Med1, Med2, Gal11, Srb7, and Srb10 proteins were fused to the lexA DNA binding domain and tested for their ability to activate transcription in wild type, med1, med2, gal11, sin4, srb8, srb10, and srb11 strains. As a positive control, we also studied the effect of these mutations on a lexA-VP16 hybrid activator. Several conclusions can be drawn from our results (Table II).

First, we found that the Mediator subunits included in our study fall into three distinct groups with respect to their functional interactions. The first group comprises Med2 and Gal11, both of which are part of the proposed activator binding module (21, 22). They resemble the classical activator lexA-VP16 in that their lexA fusions are strong activators that do not depend on any other Mediator subunits tested. lexA-Gal11 has been proposed to recruit pol II by replacing the Gal11 wild type protein within the holoenzyme (29). Our finding that lexA-Med2 can complement a med2 deletion (Fig. 1) makes a strong argument for this activation by recruitment model in the case of lexA-Med2. Several observations suggest that the activator binding module may function primarily to enhance the expression of strongly expressed genes. Thus, a limited number of genes, all of which are strongly expressed, depend on Med2 for full expression (22). Similarly, Gal11 is required for full expression of several strongly expressed genes, including the GAL genes, which also depend on Med2. Interestingly, we found that med2, gal11, and sin4 mutants are partly suppressed by overexpression of PDC1. This is a highly expressed gene that is important for rapid growth on glucose and that is known to be Med2-dependent (22). Its ability to suppress mutations in the activator binding module when overexpressed reinforces the notion that the latter is required for high level expression of a limited set of genes.

Our finding that the classical activator lexA-VP16 is independent of the activator binding module is surprising given the fact that Gal4-VP16 does exhibit such a dependence (Ref. 22 and Table III). It suggests that the dependence could be mediated by the Gal4 part of Gal4-VP16 rather than by the VP16 part. However, we cannot rule out that other factors such as different expression levels for the two hybrid proteins contribute to the observed differences. When we expressed Gal4-VP16 from a single copy plasmid, its dependencies on the activator binding module subunits were reduced and in one case (Sin4) even reversed to a negative effect (Table III). We were unable to do the opposite experiment, i.e. express lexA-VP16 from a high copy number plasmid, since this plasmid was deleterious. This may simply reflect the fact that lexA-VP16 is a much stronger activator than Gal4-VP16 (cf. pDB198 and pDB326 in Table III). High level expression of strong activators is known to be deleterious, probably due to squelching (53). An alternative interpretation of our results would, therefore, be that lexA-VP16 is independent of the activator binding module because it is a very strong activator, whereas the weaker activator Gal4-VP16 needs the module for full activity. However, this is not consistent with the fact that Gal4-VP16 is less dependent on the activator binding module when expressed in single copy nor does it fit with the finding that the attenuated Gal4-VP16-F442A mutant is as dependent on the activator binding module as is wild type Gal4-VP16 (Ref. 9 and Table III). We conclude that the most likely explanation for the observed differences between lexA-VP16 and Gal4-VP16 is that the dependence of the latter on the activator binding module is mediated by its Gal4 part, which is absent in lexA-VP16.

The second kind of result obtained with our fusion proteins is represented by the cyclin C-dependent protein kinase Srb10. We found that lexA-Srb10 is a weak activator that is dependent on Srb11 (cyclin C) and Srb8 for its activity. Deletions of the SRR8-11 genes have identical phenotypes and show no synergism with each other (32, 35). This indicates that the four proteins function closely together and that they all are needed for the in vivo function of the kinase. It has been shown for other cyclin-dependent kinases that the kinase depends on its cyclin both for induction of its catalytic activity and for targeting to the correct substrate, which is mediated by direct interactions between the substrate and the cyclin (47, 48). It is, therefore, likely that this is the case also for Srb10. It is not clear what the in vivo substrate(s) of Srb10 are, although it is one of several kinases that can phosphorylate the C-terminal domain of RNA polymerase II. Our finding that lexA-Srb10 is dependent on Srb11 may suggest that at least one substrate is present within Mediator and that Srb11 is required for the interaction of Srb10 with this substrate. This would also be

### TABLE IV

Effects of med2 high copy number suppressors in different Mediator mutants

| Insert | Wild type | med2 | gal11 | sin4 | srb11 | med1 |
|--------|-----------|------|-------|------|-------|------|
| None   | +++       | -    | +     | +    | +++   | ++   |
| PDC1   | +++       | +    | ++    | +    | +++   | ++   |
| PDC2   | +++       | +    | +     | +    | ++    | +++  |
| ZDS1   | +++       | +    | +     | +    | +++   | ++   |
consistent with a previous observation that a lexA-Srb10 fusion became a stronger activator when Srb11 was overexpressed (32).

A third kind of result was obtained for lexA-Med1 and lexA-Srb7. We have previously shown that lexA-Med1 is a cryptic activator that lacks activity in the wild type but becomes active in the absence of Srb11 (10). We have now extended this finding by demonstrating that loss of Srb8, Srb10, or Sin4 also activates lexA-Med1. This was an expected result for Srb8 and Srb10 since we know that the Srb8–11 module is a functional unit. The fact that loss of Sin4 activates lexA-Med1 is more surprising, since Sin4 is thought to be part of the activator binding module that also includes Med2, Gal11, and Pgl1 (21, 22). It should be noted, however, that Sin4 resembles Srb8–11 in that it also appears to play a role in transcriptional repression. Thus, Sin4 and ROX3 were the only bona fide Mediator subunit genes that were recovered in the same genetic screens for relief of repression as Srb8–11 (24).

Surprisingly, we found that lexA-Srb7 also is a cryptic activator. Although its activity is lower than for lexA-Med1, the pattern of activation is almost identical. Thus, lexA-Srb7 is inactive in wild type cells but becomes active in the absence of Srb8, Srb10, Srb11, or Sin4. It differs from lexA-Med1 in that it is activated also in the absence of Med1. This difference is, however, most likely due to the ability of lexA-Med1 itself to complement the med1 deletion. We conclude that the two cryptic activators are activated under the same conditions, which suggests a common underlying mechanism. This is notable since Srb11 is an essential protein, whereas Med1 is not. Loss of Med1 instead causes a mild phenotype very similar to that of srb10 or srb11 (10). Similar to srb10 and srb11 mutations, med1 mutations can also partially suppress the constitutive glucose repression phenotype of snf1 cells. These similarities suggest a possible link between Med1 and the Srb8–11 complex (10). Interestingly, the latter has been proposed to be involved in repression mediated by the general co-repressor Tup1, perhaps serving as one of its downstream targets (54). Srb7 is known to interact with Tup1 (26), thus providing a possible functional link between Srb8–11, Med1, and Srb7.

It is conceivable that the ability of lexA-Med1 and lexA-Srb7 to activate transcription in the absence of Srb8–11 reflects the mechanism by which the latter complex inhibits gene expression in wild type cells. According to this model, Med1 and/or Srb7 would provide a signal or activity that can stimulate transcription but which is normally inhibited by the Srb8–11 complex. Signals that cause activation of transcription would unravel this cryptic activator function perhaps by modulating the kinase activity of Srb10. It has been proposed that Tup1 inhibits gene expression by binding to Srb7, thus preventing it from interacting with Med6. The latter interaction is thought to be essential for activation of transcription (26). If this notion is correct, lexA-Srb7 may well activate transcription through the same mechanism.

In an attempt to learn more about the two cryptic activators, we investigated to what extent they are associated with other Mediator proteins in wild type and mutant cells. We found that lexA-Med1 and lexA-Srb7 are stably associated with Med4 and Med8 both in wild type cells and in the med1, med2, gal11, sin4, srb8, srb10, and srb11 strains (Figs. 1 and 2). In contrast, this was not the case for the classical activator lexA-VP16. This suggests that lexA-Med1 and lexA-Srb7 both activate transcription by recruitment, i.e. by stably integrating into the Mediator and, thus, recruiting it to the target promoter. This is also consistent with our finding that both fusion proteins can complement deletions of the corresponding wild type genes. Our finding that lexA-Srb7, but not lexA-Med1, associates with wild type Med1 provides further support for the notion that lexA-Med1 replaces wild type Med1 within Mediator. The fact that the association of lexA-Med1 and lexA-Srb7 with both Med4 and Med8 remained unaffected in all the gene disruption strains tested may seem surprising. However, none of these disruptions affect essential core subunits of Mediator.

Of particular interest is our finding that both Med4 and Med8 exist in two different mobility forms and that these forms differ in their ability to associate with lexA-Med1 and lexA-Srb7 (Figs. 2 and 3). Thus, only the low mobility form of Med4 associates with lexA-Med1 and lexA-Srb7. Of the two Med8 species, the low mobility form associates preferentially with lexA-Med1, whereas both forms associate with lexA-Srb7. The latter finding is noteworthy since it suggests that assembly of Mediator could be a multistep process. According to this interpretation, a core complex containing Srb7, Med8, and Med4 (as well as other subunits from the head and middle domains) would be formed first. The fact that only the low mobility form of Med4 co-precipitates with lexA-Srb7 suggests that Med4 has to be converted to this form to be included in the complex or, alternatively, that conversion of Med4 to its high mobility form causes it to dissociate from Srb7 and Med8. In either case, modification of Med4 would modulate assembly or disassembly of Mediator. In a second step, the core complex would bind Med1 and perhaps also other subunits such as the activator binding tail domain. However, this would happen only after Med8 has been converted to its low mobility form, since only the latter co-precipitates with lexA-Med1. As with Med4, one may argue that disassembly is the regulated step in which case conversion of Med8 to its high mobility form would trigger dissociation of Med1. The nature of the modifications remains to be determined, but our experiments with purified holopolymerase show that Med4 is phosphorylated when present in this complex and that the phosphorylation converts Med4 into a lower mobility form (Fig. 4). Differential phosphorylation is, therefore, a possible explanation for the two forms of Med4. In contrast, we saw no evidence that Med8 is phosphorylated in the purified holopolymerase. It is, therefore, possible that the two forms of Med8 could reflect some other kind of modification. In this context, it is interesting to note that a mouse Med8 homolog recently was shown to be an Elongin BC-interacting protein that can assemble with Cul2 and Rbx1 to reconstitute a ubiquitin ligase (55). In conclusion, our results suggest that assembly of Mediator is a multistep process that involves conversion of both Med4 and Med8 to their low mobility forms.

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