A Multiprotein Mediator of Transcriptional Activation and Its Interaction with the C-Terminal Repeat Domain of RNA Polymerase II

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Summary
A mediator was isolated from yeast that enabled a response to the activator proteins GAL4-VP16 and GCN4 in a transcription system reconstituted with essentially homogeneous basal factors and RNA polymerase II. The mediator comprised some 20 polypeptides, including the three subunits of TFIIH and other polypeptides cross-reactive with antisera against GAL11, SUG1, SRB2, SRB4, SRB5, and SRB6 proteins. Mediator not only enabled activated transcription but also conferred 6-fold greater activity in basal transcription and 12-fold greater efficiency of phosphorylation of RNA polymerase II by the TFIIH-associated C-terminal repeat domain (CTD) kinase, indicative of mediator-CTD interaction. A holoenzyme form of RNA polymerase II was independently isolated that supported a response to activator proteins with purified basal factors. The holoenzyme proved to consist of mediator associated with core 12-subunit RNA polymerase II.

Introduction
RNA polymerase II transcription of a minimal promoter, comprising a TATA box and transcription start site, can be reconstituted from homogeneous polymerase and five highly purified yeast or rat basal factors, termed TATA-binding protein (TBP), transcription factor IIB (TFIIB) (also known as factor a/1 from yeast/rat), TFIIF (factor a/2), TFIIH (factor b/5), and TFIIJ (factor g/By), with the apparent requirement for additional proteins in the human (HeLa cell) system (Conaway and Conaway, 1993; L. Henry and R. D. K., unpublished data; J. Feaver et al., unpublished data). Activator protein binding upstream of the TATA box enhances transcription in crude systems but has not so far been shown to stimulate the reaction reconstituted from pure polymerase and basal factors. The components required for activated transcription and their modes of action therefore remain to be established.

Activator proteins may exert their effects through two types of interaction with components of the basal transcription apparatus. Direct interaction is suggested by the binding of activators to basal factors in various assays, for example, binding of the yeast–human viral activator GAL4–VP16 to TBP and TFIIB (Roberts et al., 1993; Stringer et al., 1990). Evidence for indirect interaction comes from the requirement for additional protein fractions besides the basal factors and RNA polymerase for activated transcription in vitro. One such fraction, TFIID, consists of TBP and TBP-associated factors (TAFs). Originally described in human and Drosophila systems (Pugh and Tjian, 1989; Tanese et al., 1991; Timmers and Sharp, 1991; Zhou et al., 1992), TAFs have now been identified in yeast as well (Poon and Weil, 1993). A second activity, termed mediator of transcriptional activation and originally demonstrated in yeast, confers a response to activator proteins upon a system reconstituted with TBP and is distinct from all the basal factors (Kelleher et al., 1990; Flanagan et al., 1991). Similar activities have been reported in mammalian systems, both in vitro (Kretzschmar et al., 1991; Merino et al., 1993) and in vivo (Keaveney et al., 1993).

Genetic studies have revealed several candidates for mediator proteins. The GAL11/SP113 gene product was thought to participate in transcriptional activation of a number of yeast genes, based on the phenotypes of null mutants and on the capacity of a point mutant to enhance activation by a poorly functioning GAL4 derivative in which the entire activation region was replaced by a short acidic sequence (Suzuki et al., 1988; Fassler and Winston, 1989; Himmelefbart et al., 1990; Nishizawa et al., 1990). Recently, however, GAL11 protein was shown to enhance activated transcription through its effect on the basal reaction (Sakurai et al., 1993). A suppressor of a GAL4 derivative lacking only the C-terminal activation domain has been isolated as an allele of the SUG1 gene (Swaffield et al., 1992). Crippled GAL4 proteins can also be rescued by extension of the polymerase C-terminal repeat domain (CTD), whereas the effectiveness of GAL4 is diminished by truncation of the CTD (Allison and Ingles, 1989). Such truncations may have additional phenotypes, such as temperature sensitivity and inositol auxotrophy, owing to impaired function of the INO1 activation sequence. A screen for suppressors of temperature sensitivity identified a family of genes, termed SRB5s (for suppressors of RNA polymerase II) (Nonet and Young, 1989), whose products were recently shown to reside in a large multiprotein complex (Thompson et al., 1993).

We report here on a novel mediator of transcriptional activation, functional with essentially homogeneous basal factors and RNA polymerase II. Two lines of work converged on the isolation of this protein, fractionation of mediator activity and purification of a distinctive form of RNA polymerase II that supported transcriptional activation in the apparent absence of mediator. The two approaches yielded a common set of polypeptides with possible connections to previous genetic and biochemical work.

Results
In the course of resolving yeast RNA polymerase II initiation factors on hydroxylapatite (Figure 1A), we encountered an activity, termed fraction f, that behaved as an
anti-inhibitor, potentiating transcription with less than fully purified factor e (yeast TFIIB) (Sayre et al., 1992). We subsequently found that fraction f was more than an anti-inhibitor, since it supported activation in reconstituted transcription assays, apparently supplying mediator activity, and further since it stimulated basal transcription with ex-
Mediator of Transcriptional Activation

Figure 3. Multiprotein Mediator Complex Stimulates Basal Transcription and Enables Response to GAL4-VP16

(A) Specifically initiated transcripts from reconstituted reactions performed as described with core-polymerase II and with mediator fraction (60 ng) and GAL4-VP16 (30 ng) added (plus) or not (zero) as indicated. Identification of transcripts as in Figure 2A. Phosphorimager counts in GCN4/G/GAL4/G transcripts were as follows: lane 1, 298/841; lane 2, 2878/5041; lane 3, 2978/10248.

(B) Activity of SRB5-depleted mediator fraction. Mono Q fraction of mediator (IP. load) and supernatant (IP. sup.) from immunoprecipitation of the same fraction were added to a reconstituted transcription reaction containing GCN4 as described in Experimental Procedures. Immunoprecipitation was performed by incubation of Mono Q fraction 24 (100 μl) with affinity-purified anti SRB5 antibody-coupled protein essentially homogeneous preparations of all the initiation factors and RNA polymerase II (M. H. S., unpublished data; see below). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) revealed a peak of RNA polymerase II coinciding with that of fraction 24, distinct from another peak of the polymerase eluting earlier and from which the canonical 12 subunit enzyme could be derived (Sayre et al., 1992; data not shown). The occurrence of two chromatographic forms of polymerase II was confirmed by assays of both nonspecific initiation/chain elongation activity and specific (promoter-dependent) transcription activity (Figure 1B). Our resolution of mediator activity from fraction 24 began with the removal of polymerase, followed by further purification of the polymerase-depleted fraction; the properties of the mediator obtained in this way prompted us to pursue the second chromatographic form of the polymerase in fraction 24 as the basis of the stimulatory effect on basal transcription.

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Fraction 24 was depleted of polymerase by passage through a column of the 8WG16 monoclonal antibody (Thompson et al., 1989), which recognizes the CTD of the largest polymerase subunit, RPB1. The flowthrough of the column was devoid of polymerase II, as judged from specific transcription assays, but still conferred an activator response in the reconstituted system (data not shown). This mediator activity was further resolved by chromatography on TSK–heparin–5–PW and Mono Q, monitored by transcription reactions containing the GCN4 activator protein and two templates, one with GCN4-binding sites and the other with a GAL4-binding site, to reveal the levels of activated and basal transcription, respectively. Mediator activity and stimulated basal transcription activities coeluted from Mono Q (Figure 2A, GCN4/G and GAL4/G transcripts, respectively). Mediator was effective with the GAL4–VP16 activator protein as well, and quantitation revealed a 7.8-fold stimulation of basal transcription (Figure 3A, average of levels in lane 2 divided by lane 1) and a 5.5-fold activation of transcription (ratio of levels in lane 3) under the conditions used. Both effects of mediator depended on ionic strength and could be significantly greater or less than the values found in this example, as described below.

Immuno blotting showed that polypeptides reactive with antibodies against GAL11. SUG1, SRB2, SRB4, SRB5, and SRB6 coeluted from Mono Q with mediator activity, whereas the largest polymerase subunit, RPB1, did not.

A–Sepharose beads (5 μl) for 4 hr at 0°C. The beads were collected and washed, and adsorbed proteins were eluted with 100 μl of 0.1 M glycine (pH 2.6). Specifically initiated transcripts are indicated as in Figure 2A.

C) SDS–PAGE of mediator. The supernatant (sup.) of the immunoprecipitation described in (B) (50 μl), the eluate (pel.) (50 μl), holo–RNA polymerase II (h-pol) (600 ng), and core–RNA polymerase II (c-pol) (150 ng) were separated in an SDS-polyacrylamide (12%) gel, and proteins were visualized by silver staining. Molecular masses of marker proteins are indicated at the left, and RNA polymerase II subunits are indicated at the right.
(see Figure 2C; see further evidence below; data not shown). The association of SRB5 protein with mediator was further demonstrated by immunoprecipitation with anti-SRB5 antibodies. Mediator activity was fully depleted from the peak Mono Q fraction (Figure 3B). Most polypeptides in the peak fraction were immunoprecipitated as well (Figure 3C), showing that the mediator was nearly homogeneous and suggesting that its activity resided in a single large complex.

The evidence for SRB polypeptides in the mediator, thought on the basis of genetic studies to interact with the CTD (Nonet and Young, 1989), prompted us to investigate the effect of mediator on CTD phosphorylation by TFIIH kinase. The effect was highly stimulatory, 12-fold by the peak mediator fraction and with the same profile as that of mediator activity on Mono Q (see Figure 2B). Mediator alone exhibited no CTD kinase activity; TFIIH was absolutely required for phosphorylation. The possibility remains, however, that mediator contains a cryptic CTD kinase that is activated by TFIIH.

### Isolation of RNA Polymerase II Holoenzyme
The evidence for mediator--CTD and thus mediator--polymerase II interaction, as well as the stimulatory effect of

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Table 1. Purification of Holo--RNA Polymerase II from 2.6 kg of Yeast

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Protein (mg)</th>
<th>Activitya (10^4 x units)b</th>
<th>Specific Activity (U/mg)</th>
<th>Relative Specific Activity</th>
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<td>50^c</td>
<td>34,321^c</td>
<td>798</td>
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<td>3,617,940</td>
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</table>

* Bio-Rex 70 (0.6 M potassium acetate eluate), DEAE-Sepharose (0.55 M potassium acetate eluate), hydroxylapatite (0 M potassium phosphate eluate), Mono Q (1 M potassium acetate eluate), Sepharose CL-4B (fractions 19–24).

a Nonspecific initiation/chain elongation activity of RNA polymerase II was measured for each fraction.

b One unit is the amount of holo-polymerase II needed to incorporate 1000 cpm of ^32P into nonspecifically initiated poly(G) RNA in nonspecific initiation/chain elongation assays.

c The activities of whole-cell extract, Bio-Rex 70, and DEAE-Sepharose fractions were divided in half to represent only the activity from polymerase II, assuming half of the activity in the DEAE-Sepharose fraction was due to holo-polymerase II, based on nonspecific initiation/chain elongation assays of hydroxylapatite fractions (Figure 1B).

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Figure 4. Polypeptide Composition of Holo-RNA Polymerase II

(A) Immunoprecipitation of holo--RNA polymerase II. Sepharose CL-4B fraction 22 (0.1 ml) was incubated with anti-SRB5 antibody beads (10 μl) for 4 hr at 0°C. The antibody beads were precipitated by centrifugation (4000 × g for 1 min) and washed three times with buffer Q-1 (1 ml). Adsorbed proteins were eluted from the beads with 0.1 M glycine (pH 2.8) (0.1 ml) and neutralized. Sepharose CL-4B fraction 22 (CL4Bfr. 22) (0.1 μg) (lane 1), anti-SRB5 antibody column eluate of Sepharose CL-4B fraction 22 (α-SRB5 IP) (3 μg) (lane 2), and BWG16 monoclonal antibody column eluate of Sepharose CL-4B fraction 22 (BWG16 IP) (1 μg) (lane 3) were separated in an SDS-polyacrylamide (12%) gel, and proteins were visualized by silver staining. Molecular masses of protein standards (Bio-Rad) are given in kilodaltons at the left, and RNA polymerase II subunits are indicated at the right.

(B) SDS-PAGE of core- and holo--RNA polymerase II. Core--RNA polymerase II (c-pol II) (15 μg) and holo--RNA polymerase II (h-pol II) (50 μg) were separated in an SDS-polyacrylamide (12%) gel and stained with Coomassie brilliant blue R-250. RNA polymerase subunits are indicated at the left. Additional subunits of holo--RNA polymerase II are designated according to their apparent molecular weights at the right.
mediator on basal transcription, raised the possibility that the distinctive form of RNA polymerase II in fraction I might account for the stimulatory effect of this fraction on basal transcription. To pursue this possibility, we further purified the RNA polymerase in fraction I by chromatography on Mono Q and by filtration through Sepharose CL-4B at high ionic strength (0.8 M potassium acetate), resulting in overall enrichment from the starting extract of about 85,000-fold (Table 1). RNA polymerase II subunits comigrated on Sepharose CL-4B with a set of additional polypeptides, but were resolved from other slower migrating proteins (data not shown). Specific transcription activity coincided with reactivity toward the 8WG16 monoclonal antibody (data not shown). Evidence that the early Sepharose CL-4B fractions (19-24) contained an essentially homogeneous form of the polymerase came from immunoprecipitation with affinity-purified anti-SRB5 antibodies (Figure 4A). All polypeptides in Sepharose CL-4B fraction 22 were coimmunoprecipitated, demonstrating their association in a multiprotein assembly. We refer to this complex form of the polymerase and to the 12-subunit enzyme as holo-RNA polymerase II and core-RNA polymerase II, respectively.

**Subunit Composition of Holo-RNA Polymerase II**

Approximately 32 polypeptides could be detected by SDS-PAGE of holo-polymerase II and appeared to be present in roughly stoichiometric amounts. Of these polypeptides, 12 comigrated with the subunits of core-polymerase II and 3 comigrated with the subunits of yeast TFIIF (Figure 4B; data not shown). None of the additional polypeptides ( provisionally designated p130, p120, p105, p97, p68, p61, p45, p37, p35, p34, p30, p27, p22.5, p20, p19, p16, and p14) comigrated with yeast TBP, TFIIB, or subunits of TFIIE or TFIH (data not shown). These observations were supported by assays of specific transcription reconstituted with highly purified basal factors (Figure 5B). TFIIF was dispensable for transcription, whereas TBP, TFIIB, TFIIE, and TFIH were absolutely required.

Antibodies against GAL11, SRB4, SUG1, SRB5, SRB2, and SRB6 proteins reacted with p130, p97, p45, p34, p27, and p14, respectively (Figure 5A). In all cases, immunoreactivity was confined to a single polypeptide of the size expected for the gene product in question. The physical association of the immunoreactive polypeptides with the holoenzyme was confirmed by three further lines of evidence: in all cases, the immunoreactive species comigrated on Sepharose CL-4B with RNA polymerase activity and core subunits (data not shown); all immunoreactive polypeptides were coimmunoprecipitated with anti-SRB5 antibodies (Figure 5A); and affinity-purified anti-GAL11 antibodies inhibited both basal and activated transcription (data not shown), although affinity-purified anti-GAL11 antibody and preimmune sera were without effect. All immunoreactive polypeptides in holo-polymerase II corresponded with those in mediator (see Figure 2C).

None of the polypeptides in holo-polymerase II or mediator reacted with anti-TBP antibodies. Moreover, none of the polypeptides comigrated in SDS-PAGE with components of yeast TFIID (Poon and Weil, 1989), ruling out any overlap with yeast TAFs (data not shown). Finally, we investigated any relationship to a multiprotein complex containing the SWI/ADR6, SWI2/SNF2, SWI3, SNF5, and SNF6 gene products and at least four additional polypeptides, recently isolated from yeast (Cairns et al., 1994). None of the holo-polymerase II polypeptides reacted with anti-SWI3 antibodies or comigrated in SDS-PAGE with components of the yeast SWI/SNF complex (data not shown).

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**Figure 5. Identification of Holo–RNA Polymerase II Polypeptides**

(A) Immunoblot analysis of holo–RNA polymerase II. Equal volumes (5 μl) of the immunoprecipitation load (Sepharose CL-4B fraction 32), immunoprecipitation supernatant (sup.), and immunoprecipitation eluate (pellet) described in Figure 4A were submitted to electrophoresis in an SDS–polyacrylamide (12%) gel and transferred to nitrocellulose. Immunoblotting was performed as described in Figure 2C with, in addition, a 1:1000 dilution of anti-SUG1 antiserum, a 1:500 dilution of anti-SRB2 antiserum, and a 1:200 dilution of anti-SRB6 antiserum.

(B) TFIIF activity associated with holo–RNA polymerase II, revealed by reconstituted transcription assays. The complete reaction (lane 1) contained holo–RNA polymerase II (150 ng) and yeast transcription proteins as described in Experimental Procedures. The other reactions were identical except for omission of the protein indicated.
Figure 6. Comparison of Core- and Holo–RNA Polymerase II in Basal Transcription and CTD Phosphorylation

(A) Basal transcription. Specifically initiated transcripts from the reconstituted reactions as described with core–RNA polymerase II (first five lanes) or holo–RNA polymerase II (last five lanes) are shown. Amounts of polymerase, from left to right, were 0.03, 0.06, 0.12, 0.24, and 0.5 pmol (based on approximate molecular weights of 6 × 10^6 and 1.5 × 10^6 for core- and holo–polymerase II, respectively).

(B) CTD phosphorylation of core– (c-pol II) and holo–RNA polymerase II (h-pol II) by yeast TFIIH (yTFIIH). RNA polymerases (approximately 0.05 and 0.1 pmol, indicated by plus and double plus) were incubated with yeast TFIIH (15 ng) in the presence of [γ-^32P]ATP. In control reactions, polymerases (0.1 pmol) or TFIIH was incubated in the absence (zero) of TFIIH or polymerases, respectively. The extent of CTD phosphorylation was revealed by SDS–PAGE and autoradiography. The RPB1 subunit of RNA polymerase II is indicated.

Interaction of Holo–RNA Polymerase II Polypeptides with the CTD

SRB proteins are thought to interact with the CTD of polymerase II on the basis of genetic evidence, as already mentioned. Two further observations also point to an association of most or all polypeptides of holo–polymerase II, except for those of the core enzyme with the CTD. First, immunooaffinity chromatography of fraction I on an 8WG16 (anti-CTD) monoclonal antibody column yielded only core–polymerase II in the bound fraction (see Figure 4A). A straightforward inference is that holo–polymerase II polypeptides competed with the monoclonal antibody for binding to the CTD. Second, the level of phosphorylation of holo–polymerase II by TFIIH kinase was about 40-fold greater than that for core–polymerase II (Figure 6B). Inasmuch as holo–polymerase II exhibited no intrinsic CTD kinase activity, one or more holo–polymerase II polypeptides apparently modulate the conformation of the CTD or otherwise enhance its function as a kinase substrate.

Activities of Holo–RNA Polymerase II in Basal and Activated Transcription

Although the two peaks of RNA polymerase II resolved on hydroxylapatite exhibited comparable activities in non-specific initiation/chain elongation, the polymerase in fraction I was at least 10 fold more effective in specific (promoter-dependent) transcription than was the core enzyme in the other peak (see Figure 1B). This difference between the two forms of the polymerase persisted during further fractionation: apparently homogeneous holo–polymerase II was indistinguishable from core–polymerase II in non-specific transcription activity but had a 5.5-fold greater specific activity in promoter dependent transcription reconstituted with essentially pure basal factors (Figure 6A; data not shown). Evidently the additional polypeptides present in holo–polymerase II have no effect on non-specific initiation/chain elongation but stimulate promoter-dependent transcription.

The two forms of polymerase II also differed in their response to transcriptional activators (Figure 7A). Specific transcription reconstituted with pure core–polymerase II and essentially homogeneous basal factors was unaffected by either of two activator proteins tested, GAL4–VP16 and GCN4. By contrast, transcription with holo–polymerase II was enhanced by both activators. We conclude that the additional polypeptides present in holo–polymerase II enable transcriptional activation.

Both basal and activated transcription depended on the ionic strength of the reaction (Figures 7B and 7C). Basal transcription with holo–polymerase II was optimal at a potassium acetate concentration between 150 and 180 mM, and activated transcription was optimal at 180 mM. The extent of activation (the level of activated transcription divided by that of basal) was 8.3-fold at 180 mM and rose to 30-fold at 210 mM, at which the basal reaction was much diminished. Core–polymerase II in the absence of mediator remained unresponsive to activators at any ionic strength or under any other conditions tested.

Holo–RNA Polymerase II as the Sum of Mediator and Core Polymerase

The findings presented here show that holo–polymerase II possesses all functional properties of the core polymerase II plus those conferred by mediator. When compared directly, on a roughly equimolar basis, holo–polymerase II was indistinguishable in basal and activated transcription from the sum of core polymerase and mediator (see Figure 2A). Comparison of holo–polymerase II with immunoprecipitated mediator by SDS–PAGE showed that the polypeptides of holoenzyme were the sum of those of the core polymerase and mediator (see Figure 3C). We conclude that the functional differences between holo– and core–polymerase II are attributable to mediator, which contains all but the 12 core polypeptides and which likely interacts with the CTD.

The abundance of mediator and of holo–polymerase II point to their generality. Mediator appeared to be almost entirely associated with polymerase in the form of holo–polymerase II, since immunoblot analysis with antibodies against SRB5 showed copurification with holo–polymerase II throughout the isolation procedure, with little significant loss in any side fractions (data not shown). Moreover, holo–polymerase II is a major form of the polymerase, since it was comparable in abundance to the core enzyme resolved on hydroxylapatite and since immunoblot analysis with anti CTD antibodies revealed no RNA polymerase...
comprising essentially homogeneous basal factors and RNA polymerase II. Previous studies have employed one or more impure components, leaving the true requirements for and the basis of the activation process open to question. Second, it demonstrates lack of an activator response in the absence of mediator, arguing against simple, direct mechanisms whereby activator proteins contact basal factors or polymerase and thereby exert their effects. Contacts between activators and basal factors documented in previously published work (Roberts et al., 1993; Stringer et al., 1990) may play a role, but they are evidently not sufficient for activation. Third, it demonstrates the resolution of mediator to near homogeneity as a complex of some 20 polypeptides. The existence of a discrete mediator is thereby conclusively demonstrated, and its structure and mechanism are open to study. Fourth, it shows that mediator stimulates basal as well as potentiating activated transcription. It remains to be seen whether these effects are distinct or whether they have a common basis. Fifth, it identifies nine mediator polypeptides as subunits of TFIIA and the GAL11, SUG1, SRB2, SRB4, SRB5, and SRB6 gene products. Disparate lines of genetic evidence are thus related through a common biochemical entity to fundamental aspects of transcription. Sixth, it presents evidence for mediator—CTD interaction, especially the profound effect on CTD phosphorylation. Mediator—CTD interaction is nicely consistent with phenotypes of CTD truncations, such as growth defects and impaired function of activation sequences (Nonet and Young, 1988; Allison et al., 1988; Nonet et al., 1987; Bartolomaei et al., 1988). Roles of mediator in both basal and activated transcription may account for these phenotypes. Seventh, it provides, through the purification to homogeneity of an RNA polymerase II holoenzyme, support for the notion of a mediator—RNA polymerase II complex consisting of mediator plus polymerase and possessing all functional attributes conferred by mediator.

Our holo—RNA polymerase II may correspond to the high molecular mass SRB—RNA polymerase II complex of Thompson et al. (1993), since both preparations were derived from the previously described (Sayre et al., 1992) hydroxylapatite fraction f. If the holoenzyme and the SRB—polymerase complex are indeed related, then our studies extend previous work on the complex along the lines enumerated above. There are, however, a number of important differences between our findings and those of Thompson et al. (1993). First, the SRB—polymerase complex contained TBP, and Thompson et al. suggested that the SRB proteins are assembled with TBP, which, in turn, interacts with the polymerase CTD. By contrast, we have shown that holo—polymerase II contains no TBP and no TAFs. Poon and Weil (1993) recently described yeast homologs of mammalian TAFs, and, in collaboration with Weil, we have found that yeast TAFs enable transcriptional activation in the absence of mediator (P. A. Weil and S. B., unpublished data). It remains to be determined whether the combination of mediator and TAFs will allow even higher levels of activated transcription to be achieved.

A second important difference between our findings and those of Thompson et al. (1993) concerns the abundance of transcriptional events in the Mediator of RNA Polymerase II Transcriptional Activation

Figure 7. Comparison of Core— and Holo—RNA Polymerase II in Activated Transcription

(A) Specifically initiated transcripts from reconstituted reactions performed as described with approximately 300 ng of core—polymerase II (c-pol II) or holo—polymerase II (h-pol II), with GAL4—VP16 (30 ng) added (plus) or not (zero) and at the potassium acetate concentrations indicated. Identification of transcripts as in Figure 2A.

(B) Quantitation of GAL4—G— (activated; solid line) and GCN4—G— (basal; broken line) transcripts obtained with the holo—polymerase II in (A).

(C) Ratio of activated to basal transcription (fold activation) for transcripts in (A).

II other than the core and holoenzymes (data not shown). Inasmuch as the procedures and conditions employed prior to the hydroxylapatite step may have caused some disruption of the holoenzyme, it may even be the predominant form of RNA polymerase II in vivo.

Discussion

The novelty of this work lies in the following. First, it demonstrates transcriptional activation in a fully defined system,
of holo-RNA polymerase II. Thompson et al. found that the SRB–polymerase complex accounted for about 2% of the total RNA polymerase II in yeast. It seemed the complex might be active at only a minority of polymerase II promoters. We find, however, that at least half of the RNA polymerase II can be recovered as holoenzyme, which transforms our view of its significance. Most, if not all, RNA polymerase II promoters are transcribed by the holoenzyme in vivo.

The reported requirement for SRB2 and SRB5 proteins for basal transcription (Koleske et al., 1992; Thompson et al., 1993), despite the absence of these proteins from our fully defined transcription system, may be explained by the stimulatory effect of mediator on basal transcription. Evidence that SRB2 and SRB5 proteins are required for activated transcription (Koleske et al., 1992; Thompson et al., 1993) may be similarly attributed to the presence of these proteins in mediator. The question of whether SHB2 and SHB5 are directly involved in either basal or activated transcription can only be resolved by further studies, for example, with modulator or holoenzyme derived from SRB2 and SRB5 deletion strains.

There is better evidence for direct roles of other mediator polypeptides in transcription, especially GAL11 and SUG1 proteins, identified by immunoblotting, and TFII F. GAL11 protein was previously reported to influence basal transcription in vitro (Sakurai et al., 1993). SUG1 protein has recently been shown to bind several activators, including GAL4–VP16 (Swaffield et al., submitted). Conceivably, SUG1 represents a point of contact between activators and mediator. Additional contacts between activators and basal factors (Roberts et al., 1993; Stringer et al., 1990) may also be important, but that with mediator, possibly owing to SUG1, is crucial for the activation process. The surprising finding of TFII F in the mediator complex raises the further possibility of a distinctive form of this basal factor that contributes to both heightened basal and activated transcription.

The identification and functional roles of some 10 additional polypeptides of mediator remain to be established. These polypeptides may be among the products of more than two dozen SHB and SP1 genes isolated but not yet assigned functions or otherwise further characterized. Some of the additional mediator polypeptides may also be involved in transcriptional activation, while others doubtless participate in diverse aspects of the modulation and regulation of transcription.

**Experimental Procedures**

**Protein Purification**

Fractionation of whole-cell extract by chromatography on Bio-Rex 70 (Bio-Rad), DEAE–Sephadex (Pharmacia), and Bio-Gel HTP hydroxylapatite (Bio-Rad) was as described (Sayre et al., 1992) with the following modifications. Active dry yeast (Pichia man's yeast, 5 kg) was washed in cold distilled water, and the hydrated cells (14 kg) were suspended in 7 liters of 3 x HEPES-tyrosine buffer (0.45 M HEPES–KOH [pH 7.6], 0.15 M potassium acetate, 30 mM glycylglycine, 3 mM EDTA, 3 mM dithiothreitol [DTT], and protease inhibitors [Flanagan et al., 1990]). The cells were broken with glass beads by continuous flow (7 l/hr) through a Dyno-Mill (Glenn Mills). The whole-cell extract (300 g) was applied to a 4 liter Bio-Rex 70 column, and the 0.6 M potassium acetate eluate (14 g of protein) was dialyzed and applied to a 0.5 liter DEAE–Sephacel column. The 0.5 M potassium acetate eluate (DE550, 1.5 g) was fractionated on hydroxylapatite, and the fractions (13 ml) were assayed for mediator and RNA polymerase II activities. Mediator activity was assayed at 50 mM potassium phosphate, while RNA polymerase II activity was in two peaks, one at 40 mM and the other at 90 mM potassium phosphate. The latter peak (fraction 1, 94 mg) was pooled, dialyzed against buffer Q-0.1 (buffer Q contained 20 mM Tris acetate [pH 7.9], 20% glycerol, 0.2 mM EDTA, 1 mM DTT, and protease inhibitors, with the molality of potassium acetate indicated after the hyphen) to the conductivity of buffer Q-0.1, applied at 2 ml/min to a Mono Q HR16/16 column (Pharmacia) equilibrated with buffer Q-0.1, and adsorbed proteins were eluted with 80 ml each of buffers Q-0.6, Q-1, and Q-1.5. RNA polymerase II activity was recovered in the 1 M potassium acetate eluate, and the peak fraction (0.4 mg in 2 ml) was applied at 4 ml/hr to a Sepharose CL-4B column (1.5 x 5 cm; Pharmacia) equilibrated with buffer Q-0.8. Fractions (0.8 ml) were analyzed (10 μl) in an SDS–polyacrylamide gel with silver staining. Holo–RNA polymerase II eluted between 0.5 and 0.6 of the column bed volume.

To separate the mediator and RNA polymerase II activities in fraction 1, RNA polymerase II was removed by adsorption on BWG16 antibody coupled to protein A–Sepharose (Thompson et al., 1989). Fraction 1 (17 mg) was incubated at 0°C for 4 hr with 4 ml of BWG16 beads. Following centrifugation, the absorbed RNA polymerase II was recovered from BWG16 beads as described (Edwards et al., 1990), while the supernatant (16 mg) was dialyzed against buffer H-0.1 (buffer H contained 20 mM potassium phosphate [pH 7.4], 10% glycerol, 0.2 mM EDTA, 1 mM DTT, protease inhibitors, and the molality of potassium acetate indicated after the hyphen) to the conductivity of H-0.1 and applied at 0.2 ml/min to a TSK–HR1000–PW HPLC column (75 x 7.5 mm; Supelco) equilibrated with buffer H-0.1. Adsorbed proteins were eluted with a linear gradient (56 ml) from buffers H-0.3 to H-0.7. Fractions (1 ml) were assayed (5 μl) for mediator activity, revealing a single peak at 0.5 M potassium acetate. Peak fractions were pooled (26.4 mg), dialyzed against buffer Q-0.1 to the conductivity of buffer Q-0.1, and applied at 0.5 ml/min to Mono Q HR15/5 column (Pharmacia) equilibrated with buffer Q-0.1. Adsorbed proteins were eluted with a linear gradient (20 ml) from buffers Q-0.4 to Q-0.8. Fractions (0.8 ml) were assayed (5 μl) for mediator activity, revealing a single peak at 0.62 M potassium acetate.

**Non-specific Transcription Assay**

Hydroxylapatite fractions (0.1 mg) were assayed in 100 μl of 70 mM Tris–HCl (pH 8.0), 3 mM manganese chloride, 40 mM ammonium sulfate, 5 mM DTT, 0.5 mM GTP, 5 μg of poly–r-ribocytosine (Sigma), and 1 μCi of [α-32P]GTP, for the presence and absence of a-amanitin (10 μg/ml). Reactions were performed for 20 min at room temperature and were stopped by spotting onto DE81 paper. The paper was washed three times in 200 ml of 5% (w/v) ribasic sodium phosphate for 10 min, rinsed in 95% ethanol, air dried, and counted by liquid scintillation. The α-amanitin incorporation in the presence of α-amanitin was subtracted from that in its absence to obtain the value due to RNA polymerase II.

**Specific Transcription Assay**

Reconstituted transcription was performed as described (Sayre et al., 1992) with the following modifications. Reactions contained 100 ng each of two templates, pQ(GCN4)7OG, with two copies of a consensus GCN4-binding sequence upstream of the yeast CYC7 promoter region, to a 0.4 kb G-less sequence in pSP73 (Flanagan et al., 1991), and pJ44/u, with a consensus (GAU4-binding sequence upstream of the yeast CYC7 promoter region, fused to a 0.32 kb G-less sequence in pUC18 (gift from M. Woomer and J. Jaehning). Transcription proteins were yeast TBP (60 ng), TFIIB (30 ng), TFIIE (40 ng), GAL4–VP16 (30 ng), and G4CN (30 ng) isolated from Escherichia coli expression strains as described (J. Feaver et al., unpublished data; Flanagan et al., 1990; Chasman et al., 1989; O’Neil et al., 1991) and yeast TFIIF (40 ng), TFIH (30 ng), and core–RNA polymerase II purified to near homogeneity by yeast as described (Edwards et al., 1990; Hawey et al., 1992; Feaver et al., 1993).
CTD Phosphorylation
Core– or holo–RNA polymerase II (60 and 150 ng, respectively; approxi-
mately 0.1 pmol), 1F1H (10 ng), and 0.3 μCi of γ-32P-ATP were incub-
ated for 30 min at room temperature in 15 μl of 20 mM HEPES–KOH
(pH 7.6), 7.5 mM magnesium acetate, 2 mM DTT, 1 μM ATP, and 100
mM potassium acetate; treated with 7 μl of 0.5 × SDS gel-loading buffer;
and analyzed in an SDS–polyacrylamide gel. Incorporation of 32P into
the RPB1 was quantified on a phosphorimager (Molecular Dynamics),
calibrated by liquid scintillation counting.

Acknowledgments
Correspondence should be addressed to R. D. K. We thank D. Bush-
nell, B. Cairns, J. Feavor, P. Flanagan, A. Grant, J. LaPointe, and J.
Svejstrup for purified transcription proteins; A. Koleskis, C. Thompson,
and R. Young for anti-SRB antibodies; H. Sakuragi and T. Fukasawa
for anti-GAL1 antibody; J. Sfratiwhland and S. Johnson for anti-SU901
antibody; P. A. Wiel for yeast TAFs; and K. Leither for helpful advice.
S. B. is a recipient of a European Molecular Biology Organization
long-term postdoctoral fellowship. M. H. S. was a recipient of an Ameri-
can Cancer Society (California Division) senior postdoctoral fellow-
ship. This research was supported by National Institutes of Health
grant GM-36929 to R. D. K.

Received February 14, 1994.

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Note Added in Proof

While this paper was under review, related findings concerning yeast RNA polymerase II were reported by A. J. Koleske and R. A. Young (Nature 358, 466–469). In addressing the issue of activated transcription, Koleske and Young refer to our previous work (Flanagan et al. [1991]. Nature 350, 430–436, Flanagan et al. [1992]. Proc. Natl. Acad. Sci. USA 89, 7669–7676) done with crude forms of the basal factors. We caution that the presence of inhibitors of transcription in those crude preparations makes it difficult to draw definitive conclusions from comparison with that work. In this paper, we contrast the response to activators by RNA polymerase II holoenzyme to the lack of response by core (12-subunit) RNA polymerase II through a direct comparison of the two enzymes in a fully defined system, reconstituted with essentially homogeneous basal transcription factors. We resolve RNA polymerase II holoenzyme into core polymerase and mediator and show that mediator contains some 20 subunits, including only one basal transcription factor, TFIIF. By contrast, Koleske and Young describe a holoenzyme containing three basal factors, TFIIB, TFIIF, and TAFIIH. In our experience, TFIIB and TAFIIH do not comigrate with RNA polymerase II throughout purification, and a pure stoichiometric complex of RNA polymerase II with these additional basal factors is not obtained.

We do not know the basis for this discrepancy. Much evidence for the formation of stable complexes in vitro between RNA polymerase II and various basal transcription factors has been reported (e.g., Serizawa et al. [1984]. In Transcription: Mechanisms and Regulation, Conaway and Conaway, eds. [New York: Raven Press], pp. 27–43). Whether such complexes are recruited to promoters in vivo remains to be determined.