A set of deletion mutants of human RNA polymerase II-associated protein (RAP) 30, the small subunit of transcription factor IIF (TFIIF; RAP30/74), was constructed to map functional domains. Mutants were tested for accurate transcriptional activity, RAP74 binding, and TFIIB binding. Transcription assays indicate the importance of both N- and C-terminal sequences for RAP30 function. RAP74 binds to the N-terminal region of RAP30 between amino acids 1 and 98. TFIIB binds to an overlapping region of RAP30, localized to amino acids 1–176 (amino acids 27–152 comprise a minimal binding region). The C-terminal region of RAP74 (amino acids 358–517) binds directly and independently to TFIIB. Interestingly, RAP74 blocks TFIIB-RAP30 binding, both by binding TFIIB and by binding RAP30. When the TFIIF complex is intact, therefore, TFIIB-TFIIF contact is maintained through RAP74. If the TFIIB-RAP30 interaction is physiologically important, the TFIIF complex must dissociate within some transcription complexes.

A minimal pathway for assembly of pre-initiation complexes on RNA polymerase II-dependent promoters has been defined in vitro (1–5). On promoters that include a TATA box (TATA-binding protein) first binds to this recognition sequence. TFIIF, TBP, activators, and template, and the N-terminal region, which binds RNA polymerase II, affect transcriptional starts in a very similar way to these subunits may enter complexes as separate factors (7, 23, 24). Accurate initiation has been demonstrated from highly supercoiled templates using a system consisting of RNA polymerase II, TFIIB, and either TBP (25) or YY1, which is an initiator binding protein (26). One implication of these observations is that RNA polymerase II and TFIIB might minimally suffice to select transcriptional start sites. Consistent with this view, swapping Schizosaccharomyces pombe for Saccharomyces cerevisiae TFIIB and RNA polymerase II in a system otherwise comprised of S. cerevisiae factors, shifts the position of the transcriptional start to that characteristic of S. pombe (27). Also, some sua7 mutants in the S. cerevisiae gene encoding TFIIF are altered for selection of initiation sites (28), sua8 mutants, in the gene encoding the largest subunit of RNA polymerase II, affect transcriptional starts in a very similar way to these sua7 mutants (29). Interestingly, mutations in the S. cerevisiae SSU71/TFG1 gene, which encodes the homologue of the RAP74 subunit of human TFIIF, suppress abnormal start site selection in a sua7ssu71 double mutant (30). By itself, the sua71 mutant does not affect transcriptional starts, so TFIIF may suppress the sua7 mutant indirectly through another general factor. The RAP30 subunit of human TFIIF has been shown to interact physically with the N-terminal region of TFIIB (12), so the large subunit of TFIIF interacts genetically, and the small subunit interacts physically, with TFIIB.

The largest subunit of RNA polymerase II has an interesting C-terminal domain (CTD) that consists of 52 repeats of the consensus sequence YSPTSPS (31, 32). RNA polymerase II

RNA Polymerase II-associated Protein (RAP) 74 Binds Transcription Factor (TF) IIB and Blocks TFIIB-RAP30 Binding*

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*This work was initially supported by a grant from the National Institutes of Health. This work was also supported by the Michigan State University Agricultural Experiment Station, by Michigan State University, and by Verna C. Finkelstein. The costs of publication of this article were defrayed in part by the payment of page charges. This advertisement must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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enters the pre-initiation complex most efficiently in the dephosphorylated IIα state. Within the complex the CTD is multiphosphorylated on the SP serines, by a subunit of TFIIH and possibly other CTD kinases. Elongating RNA polymerase II molecules are primarily in the highly phosphorylated IIα state. So the level of CTD phosphorylation may regulate elongation, and a CTD phosphatase may be important for recycling dephosphorylated polymerase IIα to a promoter after termination.

A CTD phosphatase has recently been identified that binds to RNA polymerase II (33, 34). This phosphatase interacts with a region of polymerase distinct from the CTD and requires this contact for its dephosphorylating activity. The RAP74 subunit of TFIIH stimulates CTD phosphatase activity by binding RNA polymerase II (34). Consistent with this view, the C-terminal region of RAP74, which binds RNA polymerase II, is required for phosphatase stimulation. The C-terminal region of RAP74 is masked for phosphatase stimulation (34) just as it is for RNA polymerase II binding (35) and for TFII B binding (this paper).

Consistent with functional interaction between TFII B and TFII F, TFII B suppresses stimulation of CTD phosphatase activity by RAP74 (34).

In this report, the region of RAP30 that binds RAP74 and the regions of RAP30 and RAP74 that bind TF II B were mapped. The region of RAP30 that binds RAP74 overlaps with the regions of RAP30 and RAP74 that bind TF II B. RAP30 binding to RAP74 both block TF II B-RAP30 region of RAP30 that binds TF II B. RAP74 binding to TF II B, the region of RAP30 that binds RAP74 overlaps with the regions of RAP30 and RAP74 that bind TF II B were mapped.

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TRANSCRIPTIONAL ACTIVITIES OF RAP30 DELETION MUTANTS.

Interactions between TFIIF subunits and TFIIB—Because of

genic and physical interactions between TFIIF and TFIIB (12, 30), RAP30 and RAP74 were tested for binding to TFIIB

immobilized on agarose beads (Fig. 3). In Fig. 3A, TFIIB is

shown to bind to both RAP30 (lane 2) and RAP74 (lane 4). This

result confirms the previous report of a direct interaction be-

between TFIIB and RAP30 (12) and demonstrates for the first
time that TFIIB interacts directly with RAP74.

To demonstrate the specificity of TFIIB-TFIIF interactions and to indicate their functional importance, the regions of

RAP30 and RAP74 required to bind TFIIB were mapped. In

Fig. 3, B and C, RAP30 deletion mutants were tested for binding

interaction. Binding was assayed using two methods that may
differ in sensitivity. For the experiment shown in Fig. 3B, TFIIB was covalently immobilized on agarose beads and used as an affinity adsorbent for RAP30 mutants. RAP30 and RAP30-(1–176) bound most tightly to TFIIB (lanes 1 and 6). RAP30-(1–152) also bound but much less efficiently (lane 7). None of the other deletion mutants was observed to bind. Apparently, primary sequence distributed over a significant portion of RAP30 contributes to TFIIB binding. In Fig. 3C, an ELISA 10 plate test was used to measure TFIIB-RAP30 binding. Microtiter plate wells were coated with RAP30 or a RAP30 mutant. A control ELISA experiment developed with anti-RAP30 antiserum demonstrated that immobilization of RAP30 mutants was of comparable efficiency (data not shown). After blocking, TFIIB was incubated with RAP30 mutants bound to

TFIIB-TFIIF Interactions

An extract derived from human HeLa cells was depleted of TFIIB by

immunoprecipitation with anti-RAP30 antibodies. This extract was supplemented with RAP74 (3.4 pmol) and the indicated amounts of RAP30 or RAP30 mutant, and accurate transcription was determined from the adenovirus major late promoter. Transcripts were quantitated using a phosphorimager. Data are shown only for those mutants for which stimulation of accurate transcription was detected. ●, 1–249; ○, 1–127; ×, 1–176; ■, 27–249.

Sequences within the N-terminal region and central region of RAP74 affect masking of the C-terminal domain for RNA polymerase II binding (35) and CTD phosphatase stimulation (34). Since RAP74-(358–517) bound most tightly (lane 4). Full-length RAP74, RAP74-(87–517), and -(137–356) bound with somewhat lower affinity (lanes 1, 2, and 6). RAP74-(207–517) and -(407–517) bound much more weakly. RAP74 mutants from which the C-terminal region was deleted were not observed to bind TFIIB (data not shown). The TFIIB binding site on RAP74, therefore, appears to be located within the C-terminal region between amino acids 358 and 517.

Sequences within the N-terminal region and central region of RAP74 affect masking of the C-terminal domain for RNA polymerase II binding (35) and CTD phosphatase stimulation (34). Since RAP74-(358–517) binds more tightly to TFIIB than full-length RAP74, or RAP74-(87–517), and -(207–517), and -(407–517) bind much more weakly. RAP74 mutants from which the C-terminal domain was deleted also showed reduced affinity. RAP74-(51–249) and -(110–249) were further reduced in affinity, and RAP74-(1–98) was not observed to bind. Since RAP74-(27–152) binds tightly to TFIIB by this analysis, most TFIIB-RAP30 contacts appear to involve sequence within this region. Since RAP30-(1–118) and -(110–249) bind TFIIB weakly, amino acids between 27–118 and 110–176 (or 110–152) appear to contribute to this interaction. The ELISA binding assay was done in SB 0.1, and the affinity bead assay involved a washing step with SB 0.25, so a difference in salt concentration may account for slightly different but consistent results using these two procedures.

A set of RAP74 mutants was also tested for binding to im-

mobilized TFIIB (Fig. 3D). RAP74-(358–517) bound most tightly (lane 4). Full-length RAP74, RAP74-(87–517), and -(137–356) bound with somewhat lower affinity (lanes 1, 2, and 6). RAP74-(207–517) and -(407–517) bound much more weakly. RAP74 mutants from which the C-terminal region was deleted were not observed to bind TFIIB (data not shown). The TFIIB binding site on RAP74, therefore, appears to be located within the C-terminal region between amino acids 358 and 517.

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RAP74 Blocks TFIIB-RAP30 Binding—Since both TFIIB and RAP74 interact independently with overlapping regions of RAP30, RAP74 might stimulate or antagonize TFIIB-RAP30 binding. In Fig. 4A, an ELISA test for interactions between TFIIB, RAP30, and RAP74 was done in which one protein was immobilized in the well of the microtiter plate, another was added to the well as a binding partner, to be detected with region more accessible for protein-protein interactions.
antibody, and the third was added as a potential competitor or facilitator of the binding interaction. Using this protocol, RAP74 was shown to inhibit TFIIB binding to RAP30 (filled circles). The same conclusion was obtained using a Ni²⁺-affinity bead procedure in which histidine-tagged RAP30 was used to retain TFIIB in the presence or absence of RAP74 (Fig. 4B). So RAP74 can block the TFIIB-RAP30 interaction, but in a similar test, RAP30 was not observed to block the interaction between TFIIB and RAP74 (Fig. 4A; (×-×)). This was perhaps the expected result because the N-terminal region of RAP74 binds to RAP30 (35, 44), and the C-terminal region of RAP74 binds to TFIIB (Fig. 3D) and thereby maps between amino acids 358 and 517 (Fig. 3D) and the region of RAP74 that binds RAP30 maps between amino acids 1 and 172 (35, 44). E, calf thymus RNA polymerase II (RNAP II) does not appear to block TFIIB-RAP74 interactions. The ELISA competition assay was done as indicated in the key.

Fig. 4. RAP74 blocks TFIIB-RAP30 binding by two mechanisms. A, RAP74 blocks formation of a TFIIB-RAP30 complex (filled circles). TFIIB does not block formation of a TFIIF complex (×-×). TFIIB or RAP74 (500 ng) was immobilized in the wells of a microtiter plate. A mixture of a mobile protein (3.5 pmol) and increasing quantities of a potential binding competitor (or facilitator) were added. Detection of bound protein was with anti-RAP30 or anti-RAP74 antiserum. B, RAP74 blocks formation of the TFIIB-RAP30 complex. A Ni²⁺-affinity bead procedure was used. Histidine-tagged RAP30 or RAP30-(1–176) (300 pmol) was bound to TFIIB (600 pmol) in the presence or absence of RAP74 (600 pmol). In the presence of RAP74, TFIIB did not bind to the Ni²⁺ beads. A Western blot is shown developed with anti-TFIIB antiserum. C, TFIIB does not block formation of a TFIIF complex. Histidine-tagged RAP30 or RAP30-(1–176) retained RAP74 on Ni²⁺ beads in both the presence and absence of TFIIB. A Western blot is shown developed with anti-RAP74 antiserum. D, RAP74 blocks formation of the TFIIB-RAP30 complex both by binding to TFIIB (left panel) and by binding to RAP30 (right panel). RAP74 or RAP74 mutants (competitor) were tested in the ELISA competition assay as in A. The region of RAP74 that binds TFIIB maps between amino acids 358 and 517 (Fig. 3D) and the region of RAP74 that binds RAP30 maps between amino acids 1 and 172 (35, 44). E, calf thymus RNA polymerase II (RNAP II) does not appear to block TFIIB-RAP74 interactions. The ELISA competition assay was done as indicated in the key.
region of RAP74. For the TFIIB-RAP30 interaction to be maintained within a transcription complex, TFIIF would have to dissociate into RAP30 and RAP74 subunits.

RAP74 could block TFIIB-RAP30 binding by two mechanisms. 1) RAP74 could bind TFIIB to block the RAP30 binding site on TFIIB. 2) RAP74 could bind RAP30 to block the TFIIB binding site on RAP30. Both mechanisms contribute to blocking the TFIIB-RAP30 interaction (Fig. 4D). RAP74 mutants containing the TFIIB binding region (RAP74 amino acids 358–517) but missing the RAP30 binding region (RAP74 amino acids 1–172) block TFIIB-RAP30 binding (left panel). There is a close correlation between the RAP74 mutants that bind TFIIB most tightly and the ability of these mutants to compete the TFIIB-RAP30 interaction (compare Figs. 3D and 4D (left panel)). Also, RAP74 mutants containing the RAP30 binding region but missing the TFIIB binding region block TFIIB-RAP30 binding (right panel).

Since the TFIIB-RAP74 interaction takes precedence over the TFIIB-RAP30 interaction, one question that arises is whether the TFIIB-RAP74 interaction is maintained in higher order complexes containing RNA polymerase II. Although this question is difficult to address directly, we have tested the effect of RNA polymerase II on binding of TFIIB and RAP74 (Fig. 4E). Since both TFIIB and RNA polymerase II bind to overlapping regions within the C-terminal domain of RAP74 (amino acids 358–517 for TFIIB binding versus 363–444 for polymerase binding), these proteins might compete or cooperate for binding. RNA polymerase II does not appear to strongly compete or facilitate the TFIIB-RAP74 interaction. When RAP74 was immobilized and TFIIB was added as the binding partner, addition of RNA polymerase II was not observed to affect formation of complexes containing TFIIB and RAP74 (filled circles). When TFIIB was immobilized and RAP74 added as the binding partner, addition of RNA polymerase II caused a moderate reduction in the retention signal (open circles). Of course, RNA polymerase II is expected to interact with both TFIIB and RAP74, and polymerase might interfere with binding between anti-RAP74 antibody and RAP74. The slight apparent inhibition of binding that is observed, in the experiment in which TFIIB was immobilized (open circles), could be attributable to antibody interference or distribution of TFIIB-polymerase and RAP74-polymerase complexes. It may be difficult to exchange TFIIB and RAP74 bound to separate polymerase molecules into a RNA polymerase II-TFIIB-RAP74 complex, since this requires dissociation of either TFIIB or RAP74 from polymerase. Consistent with this idea, when RNA polymerase II was immobilized and competition for binding was between RAP74 and TFIIB, a smaller reduction in the retention signal was observed. This was true whether detection was for TFIIB (×××) or RAP74 (filled squares). Of course TFIIB and RAP74 could bind independently to polymerase without maintaining the TFIIB-RAP74 interaction. Additional experiments will be required to demonstrate TFIIB-RAP74 contacts within the pre-initiation complex.

**DISCUSSION**

There is mounting evidence for functional interaction between TFIIF and TFIIB. These factors cooperate to bring RNA polymerase II into the pre-initiation complex (3–6). Transcriptional start sites are selected by a complex that includes TFIIF, TFIIB, and RNA polymerase II, and genetic experiments implicate each of these factors in this process (27–30). TFIIB and TFIIF also can cooperate to control dephosphorylation of the CTD (34).

RAP30 appears to consist of three functional regions. The N-terminal region binds RAP74 (43, 44), the central region binds RNA polymerase II (45), and the C-terminal region binds DNA (41, 42). In the current work, we have mapped the functional domains of human RAP30 that are required for accurate transcription, that bind RAP74, and that bind TFIIB. Tight TFIIB-RAP30 binding requires primary sequence distributed over an extensive region of RAP30 between amino acids 1 and 176 (Fig. 3B and C).

Analysis of sequence (46, 47) and deletion mutants (35) indicates that RAP74 is also divided into three functional regions. The RAP74 primary sequence can be divided into an N-terminal basic region, a highly charged central region with overall negative charge, and a C-terminal basic region. The N-terminal region binds to RAP30 (35, 44). The central region appears to be a largely unstructured hinge that controls accessibility of the C-terminal region. The C-terminal region of RAP74 binds directly to TFIIB (Fig. 3D) and to RNA polymerase II (35). Polymerase-RAP74 contact stimulates a CTD phosphatase that must bind to the "body" of polymerase to access the CTD "tail" (34). Deletion from the N terminus of RAP74 initially increases masking of TFIIB binding (Fig. 3D), RNA polymerase II binding (35), and stimulation of CTD phosphatase activity (34). Further deletion from the N terminus and within the central region unmask TFIIB binding, polymerase binding, and stimulation of CTD phosphatase activity. The central region is also the site of phosphorylation by casein kinase II, and phosphorylation by this and/or other kinases appears to stimulate polymerase binding by TFIIF (48).

Although TFIIB can bind directly to RAP30, RAP74 blocks TFIIB-RAP30 binding through two mechanisms. By binding the N-terminal region of RAP30, the N-terminal region of RAP74 blocks TFIIB-RAP30 binding (Fig. 4D, right panel). Also, by binding TFIIB, the C-terminal region of RAP74 blocks TFIIB-RAP30 binding (Fig. 4D, left panel). The structure of the TATA box-TBP-TFIIB complex strongly indicates that interaction between TFIIB and TFIIF might be through the N-terminal region of TFIIB (17). TFIIB-TFIIF contact, therefore, is most likely maintained through the N-terminal region of TFIIB and the C-terminal region of RAP74.

The strongest contacts between TFIIB and RAP74 map between RAP74 amino acids 358–517 (Fig. 3D). Although RAP74 blocks TFIIB-RAP30 binding, RAP74 can bind to RAP30 and TFIIB simultaneously (Fig. 4A). This result is consistent with the mapping of RAP74 functional domains, since the N-terminal region binds RAP30 and the C-terminal region binds TFIIB. Although RNA polymerase II and TFIIB bind to overlapping regions within the C-terminal domain of RAP74, RNA polymerase II does not appear to strongly compete TFIIB-RAP74 binding (Fig. 4E). That TFIIB and RAP74 might interact in complexes containing RNA polymerase II is also indicated by the genetic interaction between yeast SUA7 and SSU71 (30) and by TFIIB-mediated suppression of RAP74 stimulation of CTD phosphatase activity (34). Interaction between RAP74 and TFIIB, therefore, may be maintained in complexes with RNA polymerase II.

The SUA7 gene in yeast encodes TFIIB, and the RAP74 homologue in yeast is encoded by SSU71/TFG1. sua7 mutants that confer cold sensitivity and altered transcriptional start site selection, and are suppressed by sua71 mutants, map to a particular region of TFIIB near the N terminus (11). From our work, we would have predicted that the sua71 mutations that suppress sua7 might map within the C-terminal region of SSU71 where human TFIIB and RAP74 physically interact. The sua71 G363D and G363R alleles, however, correspond to

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2 S. M. Fang and Z. F. Burton, unpublished data.

3 M. Hampsey, personal communication.
the Lys-111 position of human RAP74 (30, 35), within the RAP30 binding region. So both TFIIF subunits may cooperate with TFIIB in the process of start site selection.

Although the experiments shown here demonstrate specific protein-protein contacts between general transcription factors TFIIB and TFIIF, the importance of RAP74 blocking TFIIB-RAP30 interaction is not clear. TFIIB is expected to interact with TFIIF through the RAP74 subunit, although in the absence of RAP74, a TFIIB-RAP30 complex could be maintained. Such alternate structures are most easily understood if TFIIF does not invariably function as an intact unit, but rather, under some circumstances, as two independent factors, RAP30 and RAP74. Chang et al. (23) demonstrated that accurate initiation could be reconstituted in a TFIIF-depleted extract by addition of RAP30 alone. In those experiments, elongation to the run-off position required further addition of RAP74. Thus, RAP30 and RAP74 functions appear to be partially separable in initiation and elongation, at least in an extract transcription system. TFIIB-RAP30 interactions, therefore, might be maintained within some promoter-bound complexes to support initiation but not elongation. Addition of RAP74 would displace the TFIIB-RAP30 interaction, and elongation would begin. Regulated interaction between TFIIB, RAP30, and RAP74, therefore, might allow for distinct pathways of pre-initiation complex assembly on different promoters and for separate timing of RAP30 and RAP74 function in initiation and elongation.

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