Direct Interaction between the Subunit RAP30 of Transcription Factor IIF (TFIIF) and RNA Polymerase Subunit 5, Which Contributes to the Association between TFIIF and RNA Polymerase II*

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The general transcription factor IIF (TFIIF) assembled in the initiation complex, and RAP30 of TFIIF, have been shown to associate with RNA polymerase II (pol II), although it remains unclear which pol II subunit is responsible for the interaction. We examined whether TFIIF interacts with RNA polymerase II subunit 5 (RPB5), the exposed domain of which binds transcriptional regulatory factors such as hepatitis B virus X protein and a novel regulatory protein, RPB5-mediating protein. The results demonstrated that RPB5 directly binds RAP30 in vitro using purified recombinant proteins and in vivo in COS1 cells transiently expressing recombinant RAP30 and RPB5. The RAP30-binding region was mapped to the central region (amino acids aa 47–120) of RPB5, which partly overlaps the hepatitis B virus X protein-binding region. Although the middle part (aa 101–170) and the N-terminus (aa 1–100) of RAP30 independently bound RPB5, the latter was not involved in the RPB5 binding when RAP30 was present in TFIIF complex. Scanning of the middle part of RAP30 by clustered alanine substitutions and then point alanine substitutions pinpointed two residues critical for the RPB5 binding in vitro and in vivo assays. Wild type but not mutants Y124A and Q131A of RAP30 coexpressed with FLAG-RAP74 efficiently recovered endogenous RPB5 to the FLAG-RAP74-bound anti-FLAG M2 resin. The recovered endogenous RPB5 is assembled in pol II as demonstrated immunologically. Interestingly, coexpression of the central region of RPB5 and wild type RAP30 inhibited recovery of endogenous pol II to the FLAG-RAP74-bound M2 resin, strongly suggesting that the RAP30-binding region of RPB5 inhibited the association of TFIIF and pol II. The exposed domain of RPB5 interacts with RAP30 of TFIIF and is important for the association between pol II and TFIIF.

RNA polymerase II (pol II) synthesizes all messenger RNA in eukaryotes. Promoter-specific transcription initiation requires the concerted action of a complex array of factors including the general transcriptional factors TFIIID, TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH. Transcriptional activators and repressors bind distal elements of the promoter and modulate transcription by interacting with the components of preinitiation complex (1–4), and another group of proteins, cofactors or mediators, affect transcription positively or negatively by communicating with promoter-specific regulatory factors and the transcriptional machinery. All transcriptional factors and cofactors modulate positively or negatively initiation and/or elongation of mRNA synthesis. Therefore, RNA polymerase subunits may be additional targets for transcriptional regulators, because pol II is the core enzyme of the transcription machinery of gene expression. Several lines of evidence for communication between transcriptional regulators and pol II subunits have accumulated (5–14).

Pol II consists of 12 subunits, among which RPB5, RPB6, and RPB8 are commonly shared by pol I, II, and III (15). RPB1 and RPB2 of pol II are responsible for most of the catalytic activities for RNA synthesis, although the other subunits contribute to the integrity of the supramolecular complex in structure and function. We have previously found that HBx, a multifunctional viral regulator protein of hepatitis B virus, directly interacts with RPB5 (5), and both RPB5 and HBx communicate with TFIIB. The trimeric interaction of these three factors may facilitate transcription and HBx acts as coactivator in activated transcription (7, 8). Based on these observations, we proposed that RPB5 is a communicating subunit of pol II that interacts with transcriptional regulators. In support of the notion, we previously identified a novel protein, RPB5-mediating protein (RMP), which counteracts the coactivator function of HBx by competitively binding RPB5 (6). In yeast, RPB5 is an essential subunit and cannot be complemented by human RPB5. Miyao et al. (16) reported that yeast RPB5 is important for activated transcription of some genes in yeast. Recently, a backbone model of a 10-subunit yeast RNA polymerase II revealed the relative position of each subunit. RPB5 has two parts, the exposed domain (N-terminal two-thirds) and the embedded domain, the former being a component of the jaw in close...
proximity to duplex DNA downstream of the active site (17, 18). In this context, the exposed domain of RPB5 may be needed to interact with factors for transcription regulation.

TFIIF is a unique general transcription factor that functions in initiation, elongation, and probably recycling of transcription (19, 20). Eukaryotic TFIIF is a heteromeric tetramer of RAP30 and RAP74 (21, 22) and communicates with transcription regulator factors (23–28). RAP30 and RAP74 have been isolated as RAPs (RNA polymerase II-associated proteins) using an affinity column containing immobilized pol II (29). The structure and function of both the RAP30 and RAP74 subunits of TFIIF have been well defined (19–20, 30–32). The N-terminus of RAP30 is proposed to associate with pol II (35, 36) and is essential for transcription initiation (33, 34). The middle part of RAP30 is a σ-homologous region, which is proposed to associate with pol II (35, 36) and is essential for transcription elongation (37). The C-terminus of RAP30 has been demonstrated to contain a cryptic DNA-binding domain, which is suggested to be homologous to the DNA template binding of prokaryotic σ factor (38, 39). RAP30 has been shown to be necessary for most, if not all, preinitiation complex formation and gene transcription (37, 40–42).

RAP30 has been shown to directly bind pol II (41), but it remains unclear which subunit of pol II is responsible for the binding with RAP30. Therefore, we examined the possibility that TFIIF interacts with RPB5. Here we show that RPB5 directly binds to the middle part of RAP30 (aa 101–170) in the presence of RAP74. The residues Tyr129 and Gin301 of RAP30 were identified as critical for the RPB5 binding, and furthermore, these mutants of RAP30 eliminated the efficient recruitment of pol II to RAP30, indicating that the direct binding of RAP30 and RPB5 is important for the recruitment of pol II to TFIIF.

MATERIALS AND METHODS

Plasmid Constructions—The plasmids pNFLAG and pNKGST, derived from pSG5UTPL, are FLAG-tagged and GST-tagged mammalian expression vectors, respectively, as reported (7, 43). The plasmids pGE3K and pG恩施 are bacterial expression vectors for GST-fused proteins as reported previously (6, 7). The full-length and truncated GST-RPB5 expression plasmids have been described (7). The plasmids pGST-RAP30 and His-ET-RAP74 were a gift from R. G. Roeder. The RAP30 cDNA of pGST-RAP30 was used as template to amplify PCR products of RAP30 with the primer set of CAGAATTCATGGC-CGAGCGCGGGGAA and GCAGATCTGTCACTCTTTTCTTC, generating a artificial EcoRI site at the 5’-end and a BamHI site at the 3’-end, respectively. The PCR product of RAP74 was amplified with the primer set of CAGAATTCATGGCGGCCGCGGGAA and GCAGATCTGTCACTCTTTTCTTC, generating an artificial EcoRI site at the 5’-end and a BamHI site at the 3’-end, respectively. The PCR products were digested and inserted into the EcoRI and BamHI sites of pNKGST, pNFLAG, pGE3K, and pGST-FLAG to construct various expression vectors. The resultant plasmids were used as template to construct a truncated version of RAP30 by a PCR-assisted method (43). The truncation mutants of RAP30 cDNA, RAP30d1, d2, d3, d4, and d5, encode the initiation codon followed by amino acids 2–100, 2–176, 177–249, 101–249, and 101–176, respectively. An alanine scanning was applied to construct clustered or single alanine substitution mutants of the middle part of RAP30 by a splicing PCR method using mutated oligonucleotide primer sets (7, 43). All of the constructs were sequenced by the dideoxy method using mutagenic oligonucleotide primer sets (7, 43). All of the constructs were sequenced by the dideoxy method using mutagenic oligonucleotide primer sets (7, 43).

Preparation of Recombinant Proteins—GST-fused proteins were expressed in E. coli by induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside at 30 °C for 3 h. The cells were harvested and sonicated in PBS buffer (phosphate-buffered saline containing 1% Triton X-100) (6, 7). After centrifugation, the extracts (supernatants) were collected and stored at −80 °C. For purification, the extracts were incubated with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) at room temperature for 1 h. The beads were precipitated, washed four times with an excess amount of PBS buffer, and then eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). The eluted proteins were divided into aliquots and stored at −80 °C.

The His-tagged RAP30 protein was expressed in BL21 (DE3)pLys using 0.4 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 30 °C. The cells were harvested and sonicated in native binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.8), and the His-tagged proteins were purified by incubating the sonication supernatant with nickel-nitrotriacetic acid (Invitrogen). After extensive washing, the bound proteins were eluted with imidazole elution buffer (300 mM imidazole, 20 mM sodium phosphate, 500 mM NaCl, pH 6.3). FLAG-tagged proteins were expressed in BL21 by induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside at 30 °C for 3–6 h. The cells were harvested and sonicated in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100. After centrifugation, the supernatant was stored at −80 °C. FLAG-tagged proteins were purified by incubating the sonication supernatant with anti-FLAG M2 resin (Kodak Scientific Imaging Systems) followed by several washes. The bound proteins were eluted with buffer containing FLAG peptide (0.2 mg/ml FLAG peptide, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl).

In Vitro Reconstitution of TFIIF—The reconstitution of TFIIF was carried out basically according to the method of Maldonado (44) but with one additional step for purification. Equimolar amounts of partially purified GST-RAP30 (1 mg) and His-RAP74 (2 mg) were mixed in 1.2 ml of HCl-KOH buffer (pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM EDTA) containing 4 mM urea, 0.5 mM KCl. The mixture was dialyzed against 100 volumes of buffer A with 0.5 mM KCl for 3 h at 4 °C and then dialyzed against 100 volumes of buffer A with 0.1 × KCl for another 3 h. The dialyzed solution was centrifuged at 15,000 rpm for 10 min at 4 °C, and the supernatant was collected and incubated with 60 μl of nickel resin for 20 min. After being washed with a solution containing 0.5 mM NaCl, 50 mM imidazole, and 20 mM Tris-HCl (pH 8.0) three times, the nickel resin-bound proteins were eluted with 0.5 M NaCl, 0.2 mM imidazole, 20 mM Tris-HCl, pH 8.0. The eluted proteins were then incubated with 40 μl of packed glutathione-Sepharose 4B in 1.0 ml of PBS buffer for 30 min. After extensive washings, the bound proteins were eluted with 10 mM of reduced glutathione in 50 mM Tris-HCl (pH 8.0). Ten μl of the eluate was fractionated by 12.5% SDSPAGE and stained with Coomassie Brilliant Blue.

Antibodies—Anti-RPB5, anti-RPB3, and anti-GST polyclonal antibodies were reported previously (5–7). Anti-RPB6 and anti-RPB9 antibodies were a gift from R. G. Roeder, and anti-CTD monoclonal antibody (7G5) was kindly provided by M. Vigneron. Anti-FLAG M2 antibody was purchased commercially (Kodak Science Imaging Systems).

In Vitro GST Resin Pull-down Assays—A GST resin pull-down assay was carried out as reported (6, 7). Approximately 1 μg of GST or GST-fused protein immobilized on 20 μl of glutathione-Sepharose 4B preblocked in 0.5% nonfat milk and 0.05% bovine serum albumin was incubated with 0.1 μg of FLAG-tagged proteins in 0.5 ml of modified GBT buffer for 1–2 h on a rotator at 4 °C. After being washed four times with modified GBT buffer, the GST-fused proteins were eluted, fractionated by 12.5% SDS-PAGE, and subjected to Western blot analysis using anti-FLAG monoclonal antibody (M2).

Immunoprecipitation, in Vivo Pull-down Assay, and Western Blot Analysis—Transient transfection of COS1 cells was carried out as reported previously (6, 7). The cells were harvested, washed, and sonicated in LAC buffer (10% glycerol, 20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM MgCl2, 0.1 mM dithiothreitol, 0.1 mM EDTA, 9 mM CHAPS, 0.5 mM phenylmethylsulfonyl fluoride, aprotinin and leupeptin) and then centrifuged. The total cell lysates were stored at −80 °C. Approximately 1.5 mg of protein of total cell lysates was diluted with 4 times the volume of TBST buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.05% Tween 20) and incubated with 50 μl of packed Sepharose 4B for 30 min. The supernatant was then obtained by centrifugation. Supernatants with FLAG-tagged proteins were immunoprecipitated with 20 μl of 50% anti-FLAG M2 resin, rotated for 2 h at 4 °C, and washed four times with washing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) (TBSS). For in vivo GST pull-down assay, COS1 cell lysates with GST or GST-fused protein were incubated with 20 μl of glutathione-Sepharose 4B at 4 °C. After being rotated for 2 h and washed four times with washing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1 mM EDTA), the bound proteins were eluted, fractionated by 12.5% SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to Western blot analysis with the antibody. The proteins were visualized by enhanced chemiluminescence (ECL), according to the manufacturer’s instructions (Amersham Pharmacia Biotech).
RESULTS

**RPB5 Binds TFIIF through RAP30 in Vitro and in Vivo**—Since RPB5 and TFIIF seem to play roles in the initiation of transcription and both have been reported to interact with TFIIB (7, 33), we examined whether RPB5 interacts with TFIIF. The recombinant GST-RAP30 and His-RAP74 were independently expressed in E. coli, partially purified, mixed at an equimolar ratio in denaturing conditions, and dialyzed to renature the proteins (Fig. 1A, lane 1). Finally, the reconstituted TFIIF of the two factors was purified on two successive affinity columns, nickel resin and glutathione resin, as described under “Materials and Methods” (Fig. 1B, lanes 2 and 3). The two-step purification is better than the one-step purification of TFIIF (data not shown). The reconstituted TFIIF (rTFIIF) or each subunit of TFIIF (Fig. 1B) was incubated with the purified bacterial recombinant FLAG-RPB5 and then subjected to GST resin pull-down assay using glutathione resin. The reconstituted TFIIF and GST-RAP30 bound FLAG-RPB5, while GST-RAP74 and GST alone did not recover FLAG-RPB5. The result indicates the direct binding of FLAG-RPB5 and reconstituted TFIIF and indicates that RAP30 might be the subunit responsible for the binding.

Next we confirmed the binding of RPB5 and TFIIF in mammalian cells, COS1 cells. GST-RPB5 and both GST-RAP30 and FLAG-RAP74, or GST-RPB5 and either subunit of TFIIF in the FLAG-tagged form, were transiently overexpressed. All of the proteins were similarly expressed in the cells as detected by anti-FLAG M2 and anti-GST antibodies (Fig. 2, B and C). The supernatants of lysates were incubated with anti-FLAG M2 resin, washed extensively, and subjected to Western detection using anti-GST antibody (Fig. 2A). GST-RPB5 was efficiently recovered with FLAG-RAP74 together with GST-RAP30, although it was not copurified when GST-RAP30 was absent. Similar to the in vitro experiment, RPB5 directly interacted with RAP30 but not with RAP74. The result clearly indicates that RPB5 directly binds to RAP30 in the presence or absence of RAP74.

**Mapping of the RAP30-binding Region, Which Covers the HBx-binding Site in RPB5**—The RAP30-binding region of RPB5 was delineated using various truncation mutants by which we previously demonstrated the HBx and TFIIB bindings of RPB5 (7). A series of truncation mutants of RPB5 in GST form were tested for activity to bind FLAG-RAP30 (Fig. 3A). Similar amounts of the bacterial GST fusion proteins immobilized on glutathione resin

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**Fig. 1.** RPB5 binds reconstituted TFIIF and the RAP30 subunit in vitro. A, *in vitro* reconstitution of TFIIF was carried out as described under "Materials and Methods." Lanes 1–3, the mixed solution of partially purified recombinant His-RAP74 and GST-RAP30, the renatured proteins eluted from nickel resin, and the reconstituted TFIIF (*rTFIIF*) eluted from glutathione resin, respectively. B, bacterially expressed GST-RAP30, GST-RAP74, GST, and the reconstituted TFIIF were purified, fractionated by 12.5% SDS-PAGE as described under "Materials and Methods," and then visualized by Coomassie Brilliant Blue staining. The positions of molecular mass markers are indicated in kDa in A and B. C, GST resin pull-down assay. Approximately 1 µg of GST, GST-RAP30, or GST-RAP74 or 2 µg of the reconstituted TFIIF immobilized on glutathione resin was incubated with 0.1 µg of purified recombinant FLAG-RPB5 in GBT buffer for 2 h at 4 °C. After extensive washing, bound proteins were eluted, fractionated by 12.5% SDS-PAGE, and subjected to Western blot analysis with anti-FLAG M2 antibody.

**Fig. 2.** RPB5 binds RAP30 subunit of TFIIF in vivo. A, COS1 cells were cotransfected with the mammalian expression plasmids, pNKFLAG-RAP30 or pNKFLAG-RAP74, together with pNKGST-RPB5 and pNKGST-RAP30 as indicated at the top. Cell lysates were prepared as described under “Materials and Methods.” Total lysate (~1.5 mg of protein) was immunoprecipitated with 20 µl of packed anti-FLAG M2 antibody-bound resin. After washing, the bound proteins were eluted and then fractionated by 12.5% SDS-PAGE and subjected to Western blot analysis with anti-GST antibody. The total lysates (5% of the samples) were directly subjected to Western blot analysis with anti-FLAG M2 (B) and anti-GST (C) antibodies.

(1 µg) (Fig. 3B) were incubated with FLAG-RAP30 in vitro and subjected to GST resin pull-down assay (Fig. 3C). Mutant d13 (aa 47–120), the central region of RPB5, is the minimal binding region of RAP30, since neither R83 (aa 73–120), the minimal region for the HBx-binding, nor RAD2 (aa 45–93) retained the ability to bind FLAG-RAP30. Therefore, the RAP30-binding region was mapped within aa 47–120, which covers the HBx-binding region (7).
the Middle Part, to Bind RPB5, although the Former Is Masked by RAP74 in the TFIIIF Complex—

The RPB5-binding region of RAP30 was mapped using a series of truncation mutants of RAP30 in GST-fused form both in vitro and in vivo (Fig. 4A). Bacterially expressed proteins of wild type and truncation mutants of GST-fused RAP30 were purified, fractionated by 12.5% SDS-PAGE, and visualized by Coomassie Brilliant Blue staining. The positions of molecular mass markers are indicated. A, schematic representation of various RAP30 deletion constructs. RAP30/d1, d2, d3, d4, and d5 contain the initiation codon followed by aa 2–100, 2–176, 177–249, 101–249, and 101–176, respectively. RPB5-binding ability is summarized on the right. B, bacterially expressed proteins of various GST-fused RAP30 deletion constructs were purified, fractionated by 12.5% SDS-PAGE, and visualized by Coomassie Brilliant Blue staining. The positions of the molecular mass markers are indicated. C, GST resin pull-down assay. Approximately 1 μg of GST or GST-fused full-length and truncated RAP30 immobilized on glutathione resin was incubated with 0.1 μg of bacterially expressed FLAG-RPB5 in GBT buffer for 2 h at 4 °C. Pull-down assay and Western blot analysis were carried out with anti-FLAG M2 antibody as described under “Materials and Methods.”

FIG. 3. Delineation of the RAP30-binding region in RPB5. A, schematic map of various RPB5 deletion constructs. The expression plasmids were constructed previously (7). RAP30-binding ability is summarized on the right. B, various GST-fused RPB5 truncation proteins were expressed in E. coli, purified, fractionated by 12.5% SDS-PAGE as described under “Materials and Methods,” and visualized by Coomassie Brilliant Blue staining. The positions of molecular mass markers are indicated on the left in kDa. C, GST resin pull-down assay. Approximately 1 μg of GST or GST-fused RPB5 truncation proteins immobilized on glutathione resin was incubated with 0.1 μg of bacterially expressed FLAG-RPB5 in GBT buffer. Pull-down assay and Western blot analysis were carried out with anti-FLAG M2 antibody as described in the legend to Fig. 1. Lane 9 shows 5% of the input of FLAG-RAP30 used for the pull-down assay.

FIG. 4. RPB5 binds to both the N-terminus and the middle part of RAP30 in vitro. A, schematic representation of various RAP30 deletion constructs. RAP30/d1, d2, d3, d4, and d5 contain the initiation codon followed by aa 2–100, 2–176, 177–249, 101–249, and 101–176, respectively. RPB5-binding ability is summarized on the right. B, bacterially expressed proteins of various GST-fused RAP30 deletion constructs were purified, fractionated by 12.5% SDS-PAGE, and visualized by Coomassie Brilliant Blue staining. The positions of the molecular mass markers are indicated. C, GST resin pull-down assay. Approximately 1 μg of GST or GST-fused full-length and truncated RAP30 immobilized on glutathione resin was incubated with 0.1 μg of bacterially expressed FLAG-RPB5 in GBT buffer for 2 h at 4 °C. Pull-down assay and Western blot analysis were carried out with anti-FLAG M2 antibody as described under “Materials and Methods.”

with the lysates of the COS1 cells coexpressing FLAG-RPB5 and wild type or mutant GST-RAP30. Neither GST-RAP30/d3 nor GST alone could pull down FLAG-RPB5, confirming the pull-down experiment in vitro (data not shown).

Since the N-terminus of RAP30 has been reported to be essential for the complex formation with RAP74 (33, 34), we evaluated the RPB5 binding of RAP30 in the presence of RAP74 in vitro and in vivo. At first, the purified bacterial wild type and mutant RAP30 in GST-fused form were examined for binding to FLAG-RAP74. The N-terminal RAP30 (d1, aa 1–100) as well as the full-length RAP30 could pull-down FLAG-RAP74, but no binding was observed with d3 and d5 mutants of
RAP30, confirming that the N-terminal region is critical for the RAP74-binding (Fig. 5A, lanes 1 and 4). Next we examined the effect of RAP74 on the RPB5-binding of RAP30 in vitro. As shown in Fig. 5B, the N-terminal RAP30 (d1) could not recruit FLAG-RPB5 in the presence of FLAG-RAP74, while RAP30/d1 could bind to FLAG-RPB5 in the absence of RAP74. In contrast, the middle part of RAP30 (d5) could bind FLAG-RPB5 regardless of the presence of FLAG-RAP74 (Fig. 5B, lanes 3 and 4). Since the full-size RAP30 bound FLAG-RPB5 even in the presence of FLAG-RAP74 (Fig. 5B, lanes 5 and 6), it is strongly suggested that the middle part of RAP30 is responsible for interacting with RPB5 in the TFIIF complex. The N-terminal RAP30 (d1) predominantly bound to FLAG-RPB5 when equal amounts of FLAG-RAP74 and FLAG-RPB5 were present.

To prove a similar role for the middle part of RAP30 in the RPB5 binding in TFIIF, lysates of COS1 cells coexpressing FLAG-RAP74 and wild type or truncated RAP30 in GST-fused form were subjected to a GST resin pull-down assay. The result confirmed that FLAG-RAP74 binds to the N-terminus (d1) but not to other regions (d3 and d5) of RAP30 (Fig. 5C). Then the lysates of COS1 cells coexpressing FLAG-RAP74, FLAG-RPB5, and wild type or d1 mutant of RAP30 in GST-fused form were subjected to GST resin pull-down assay. RAP30/d1 recruited FLAG-RPB5 in the absence of FLAG-RAP74, but no recruitment was observed when FLAG-RAP74 was present (Fig. 5D, lanes 1 and 2). In contrast, the full-length RAP30 recruited FLAG-RPB5 even in the presence of FLAG-RAP74 (Fig. 5D, lanes 3 and 4). This result is consistent with the result in vitro and indicates that the middle part of RAP30 is the RPB5-binding region in the TFIIF complex.

Amino Acid Residues of RAP30 Important for the RPB5 Binding—To evaluate the relevance of the direct binding between the middle part of RAP30 of the TFIIF complex and RPB5, we tried to identify the amino acid residue(s) that may be critical for the interaction between RPB5 and RAP30. Clustered alanine substitutions covering 3–7 amino acid residues in a row were introduced to the middle part of RAP30 (aa 101–176). A primary screening was carried out to search for defective mutants by GST resin pull-down assay in vitro using FLAG-RPB5 and mutants of RAP30/d4 (deleting the N-terminus of RAP30) in GST-fused form. All but two clustered mutants, cm124 (aa 124–126) and cm131 (aa 130–132), retained the ability to bind RPB5 (data not shown). Next, a point alanine substitution was introduced to the six single-amino acid residues covering two cm mutants defective in RPB5 binding.

Effects of the six single alanine-substitution mutations in the middle part of RAP30 on the RPB5 binding in TFIIF complex were evaluated in GST resin pull-down in vitro and in mammalian cells. TFIIF was reconstituted with His-RAP74 and wild type or mutated GST-RAP30 in vitro and purified to examine the ability to bind FLAG-RPB5. The single mutations in the middle part of RAP30 have no inhibitory effect on the TFIIF complex formation, the same as the clustered mutants. Only two mutants of RAP30 with point mutations at aa 124 and 131 were defective in RPB5-binding, although the wild type and the other mutant proteins retained the binding ability (data not shown). Next, the lysates of COS1 cells transiently coexpressing GST-RPB5, FLAG-RAP74, and wild type or mutated RAP30 in GST-fused form were prepared and subjected to coimmunoprecipitation with anti-FLAG M2 antibody bound to
resin. These recombinant proteins were well expressed in the COS1 cells as detected by Western blotting with either anti-FLAG M2 or anti-GST antibody (Fig. 6A). As shown in Fig. 6B, the two point mutations, m124 and m131, eliminated the RPB5 binding but had no effect on the TFIIF complex formation in vivo (Fig. 6C). These results indicate that two residues, Tyr124 and Gln131, are critical for the direct binding of RAP30 in the TFIIF complex to RPB5 in vivo and in vitro.

RPB5 Is an Important Subunit of RNA Polymerase II for the Association with TFIIF through Binding to RAP30—The direct interaction of RPB5 and TFIIF was demonstrated in vitro and in vivo; however, all experiments were carried out with a free RPB5 subunit instead of the assembled form of RPB5 in pol II. Since the middle part of RAP30 has been proposed to be the pol II-interacting region (35, 36), RPB5 may be the subunit of pol II responsible for the interaction with TFIIF. To test this possibility, GST-RAP30 and FLAG-RAP74 were transiently overexpressed in COS1 cells, and immunoprecipitation was carried out with anti-FLAG M2 antibody-bound resin. The GST-RAP30 efficiently recruited endogenous RPB5 (Fig. 7A, lane 1), although GST alone could not recover endogenous RPB5 (data not shown). The mutant RAP30, m124, or m131 in GST-fused form could not recover endogenous RPB5, but mutant m132 recruited endogenous RPB5 as the wild type RAP30 did. The result indicates that the binding of RAP30 to RPB5 is critical for the association of TFIIF and pol II, since the two mutants defective in the binding to free RPB5 have no ability to recruit endogenous RPB5. In contrast, wild type RAP30 and mutant m132 could recruit endogenous RPB5. To further confirm the relevance of the direct binding of RPB5 and RAP30 to the recruitment of TFIIF to pol II, FLAG-RAP74 and GST-RAP30 were transiently overexpressed in COS1 cells in the presence of GST-RPB5/d13, which covers the RAP30-binding region but lacks the embedded domain into RPB1 and RPB2 (see “Discussion”). FLAG-RAP74 recruited GST-RAP30 but could not recruit endogenous RPB5 or the other subunit of pol II when GST-RPB5/d13 was present. In contrast, FLAG-RAP74 efficiently pulled down all of the examined subunits of pol II when the RAP30-binding region of RPB5, RPB5/d13, was absent. The endogenous RPB5 recruited to TFIIF might be assembled in pol II, since the molar ratios of the pol II subunits in the recruited fractions of wild type and m132 mutant of RAP30 in TFIIF are similar to those detected in the unfractionated sample (Fig. 7B, lanes 1, 5, and 6). m124 and m131 mutants of RAP30 were defective in the recruiting of pol II subunits (Fig. 7B, lanes 3 and 4). Taken together, these results clearly indicate that the direct binding of RPB5 and RAP30 in TFIIF is critical for the recruitment of TFIIF to pol II and that two residues, Tyr124 and Gln131, of RAP30 are vital for the recruitment.

**DISCUSSION**

Although basal transcription in vitro requires TATA binding protein (TBP) and TFIIB in addition to pol II when the template is negatively supercoiled (45) and TFIIB is able to bind RNA polymerase II (46), the complex formation in the absence of TFIIF seems to be rather weak, since TBP and TFIIB are not able to recruit pol II into the preinitiation complex as detected by electrophoretic mobility shift assay. The addition of TFIIF or recombinant RAP30 alone was demonstrated to recruit the pol II to the complex in the assay, indicating that RAP30 is necessary for the recruitment of pol II in preinitiation complex (40, 42). The middle part of RAP30 has been assessed to be important for recruitment of pol II by the fact that pol II blocked the phosphorylation of serine residues located in the middle part of RAP30 by protein kinase (35). However, the subunit of pol II responsible for interacting with TFIIF remains unknown.

In this report, we showed that RPB5 binds RAP30 but not RAP74 and associates to TFIIF through the binding to RAP30.

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**FIG. 6. Amino acid residues of RAP30 critical for the RPB5-binding in TFIIF form.** A, COS1 cells were transfected with the mammalian expression plasmids, pNKFLAG-RAP74, pNKGST-RPB5, and pNKGST-RAP30, or the RAP30 mutants with a single amino acid substitution as indicated at the top. Total lysates (60 µg) were fractionated and detected by Western blot analysis. The positions of the molecular mass markers are indicated on the left. B, the total lysate of each sample (1.5 mg of protein) shown in A was immunoprecipitated with 20 µl of packed anti-FLAG M2 antibody-bound resin. After washing, the bound proteins were eluted, fractionated, and subjected to Western blot analysis. C, the RAP30 mutants are intact for the RAP74-binding. COS1 cells were transfected with the mammalian expression plasmids, pNKFLAG-RAP74 and pNKGST-RAP30, or the RAP30 mutant with a single amino acid substitution. The total lysate (1.5 mg of protein) was immunoprecipitated with anti-FLAG M2 antibody-bound resin. The bound proteins were eluted, fractionated, and subjected to Western blot analysis. Anti-FLAG M2 (upper panels) or anti-GST (lower panels) antibody was used in (A–C) for Western blot detection.
Although both the N-terminus and the middle part of RAP30 independently bind RPB5 in the absence of RAP74, the middle part but not the N-terminus of RAP30 can interact with RPB5 in the presence of RAP74 in vivo and in vitro. This result is consistent with the previous reports of the interaction between RAP74 and the N-terminus of RAP30 in the TFIIF complex (35, 34). This phenomenon seems to be similar to the finding that RAP74 bound RAP30 and blocked TFIIB from binding to endogenous pol II. COS1 cells were transfected with pNKFLAG-RAP74 and pNKGST-RAP30 or the RAP30 mutant as indicated at the top. Immunoprecipitation was carried out with anti-FLAG M2 antibody-bound resin. The bound proteins were eluted, fractionated, and subjected to Western blot with anti-GST (upper panel) and anti-RPB5 (lower panel) antibodies. Lane 5 shows 3% input of the total lysate. B, TFIIF mutants defective in association with endogenous pol II. COS1 cells were transfected with pNKFLAG-RAP74 and pNKGST-RAP30 or the RAP30 mutant in the presence or the absence of pNKGST-RPB5/d13. The total cell lysate (∼2.5 mg of protein) of each sample was immunoprecipitated with 20 μl of packed anti-FLAG M2 antibody-bound resin. After washing, the bound proteins were eluted, fractionated, and subjected to Western blot analysis with antibodies against RPB1, RPB3, RPB5, and RPB9 as indicated on the right. Lane 6 shows 3% of the total cell lysate used for lane 1. C, the total lysates (60 μg) for use in B were subjected to Western blot analysis with anti-FLAG M2 (upper panel) and anti-GST (lower panel) antibodies.

Our results raise several important questions. First, the close location of the TFIIIB-binding region and the RAP30-binding region of RPB5 may imply that RAP30 and TFIIIB can interact with each other on the platform of RPB5. The crystal model of human RPB5 is quite close to that of yeast (by Insight II, data not shown) except for a longer loop of yeast RPB5 around aa 67–75 (in aa number of yeast RPB5). Within the exposed domain, the RAP30 binding region (aa 47–120) is immediately downstream of TFIIIB-binding regions (aa 21–47) and overlaps partly the HBx-binding region. RPB5 and duplex DNA downstream of the active site are very close in the crystal model, which is consistent with the report that RPB5 is in the region from −5 to +15 of the promoter site in preinitiation complex (47). Although a crystal model of RAP30 or TFIIIF is not available at present, the TFIIIF complex is supposed to make contact with promoter DNA in the region from −61 to +34 of DNA template (48). The direct binding of RAP30 and RPB5 described in this paper seems to be consistent with the previous findings and may occur between pol II and TFIIIF complex.
a crystal model of the full-length TFIIB complexed with pol II has not been reported. A linker between the N-terminal domain and the core domain of TFIIB may make the TFIIB molecule flexible. Second, the mutations of RAP30 may affect basal or activated transcription in vitro. In addition, HBx may affect the binding between RBPS and RAP30 or TFIIIF, since HBx and Masutomi for the construction of pNKGST, pNKFLAG, and pGENK plasmids; and to F. Momoshima, M. Yasukawa, and K. Kuwabara for technical assistance. We also thank Dr. K. Ikeda for encouraging discussions.

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