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Mutational Analysis of Human RNA Polymerase II Subunit 5 (RPB5): The Residues Critical for Interactions with TFIIF Subunit RAP30 and Hepatitis B Virus X Protein

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RNA polymerase II (RNAPII) subunit 5 (RPB5) is positioned close to DNA downstream of the initiation site and is the site of interaction with several regulators. Hepatitis B virus X protein (HBx) binds the central part of RPB5 to modulate activated transcription, and TFIIF subunit RAP30 interacts with the same part of RPB5 that is critical for the association between TFIIF and RNAPII. However the residues necessary for these interactions remain unknown. Here we report systematic mutagenesis of the central part of RPB5 using two-step alanine scanning libraries to pinpoint critical residues for its binding to RAP30 in the TFIIF complex and/or to HBx, and identified these residues in both mammalian cells and in an *in vitro* binding assay. Four residues, F76, I104, T111 and S113, are critical for both TFIIF- and HBx-binding, indicating the overlapping nature of the sites of interaction. In addition, V74 and N98 are required for HBx-binding, and T56 and L58 are needed for RAP30-binding. Interestingly the residues exposed to solvent, T111 and S113, are very close to the DNA, implying that two factors may modulate the interaction between DNA and RPB5.

Key words: alanine scanning, coactivation, HBx, RAP30, RPB5, TFIIF.

Abbreviations: RPB5, RNA polymerase subunit 5; HBx, hepatitis B virus X protein; RNAPII, RNA polymerase II; RMP, RPB5-mediating protein; aa, amino acid.

Transcription is the primary regulatory step of eukaryotic gene expression and is carried out by DNA-dependent RNA Polymerase II (RNAPII) along with general transcription factors (GTFs), transcription factors, and cofactors (*1–7*). RNAPII is the ultimate target of transcription factors and cofactors acting directly or indirectly to modulate transcription. During the processes of transcription, RNAPII changes partners or complexes with which it interacts by altering its conformation. In this context, several RNA polymerase subunits have recently been reported to interact with these regulators (*5*). RNAPII consists of 12 subunits that are well conserved from yeast to human. Crystal structures and cryoelectron microscopy have solved a subset of RNAPII transcription complexes (*3*, *7–12*) that provide deep insight into the molecular basis of RNAPII transcription.

RNA polymerase II subunit 5 (RPB5) is part of the lower jaw of RNAPII , and the exposed domain of RPB5 serves in interactions with transcriptional regulators including Hepatitis B virus X protein (HBx), TFIIB, Tip120, TFIIF subunit RAP30 and RMP/URI (*3*, *8*, *13–19*). Human RPB5 consists of an exposed domain (aa 1 to 139) and an embedded domain (aa 140 to 210), which are well conserved between yeast and human (*20–22*). There is no interdomain interaction in the crystal models of RPB5, implying the flexible nature of the exposed domain relative to the embedded domain (*8*, *21*). The exposed domain seems to comprise two subdomains, the N-terminal part (aa 1 to 47), and the central part of RPB5 (aa 57 to 139), with a short loop between the subdomains. The N-terminal part is outside of the lower jaw, and the central part of RPB5 is mostly inside the jaw and close to the DNA downstream of the initiation site. The former is critical for TFIIB-binding and the latter harbors the sites of interaction with RAP30 and HBx, and probably RMP/URI (*8*, *13*, *15*, *16*, *18*, *19*).

TFIIF, a heterotetramer of RAP74 and RAP30, is a unique general transcription factor that functions in initiation, elongation, and recycling of transcription (*4*, *18*, *23*, *24*). TFIIF binds directly to RNAPII and also helps to recruit the enzyme to the promoter (*14*, *25–28*). TFIIF can induce further bending and wrapping of the promoter DNA against the mobile clamp of RNAPII during the formation of the preinitiation complex (*11*, *29–32*). The direct interaction between the central region of RAP30 and the central part of RPB5 has been reported to contribute to the association between TFIIF and RNAPII (*18*), although other interactions between TFIIF and RNAPII may occur during transcription (*11*).

HBx is a multifunctional viral regulator protein of Hepatitis B Virus that coactivates activated, but not basal, transcription *in vivo* and *in vitro* (*17*, *33*, *34*). The direct interactions of RPB5 with HBx and TFIIB are critical for the coactivating ability of HBx (*13*, *15*, *17*), which augments HBV replication positively (*35*) and modulates

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gene expression of cells. Previously we identified a novel cellular protein, RPB5-mediating protein (RMP) (*16*) or Unconventional RPB5-interacting protein (URI) (*36*), which modulates transcription as a corepressor through binding to RPB5, and counteracts transcriptional modulation by HBx (*16*).

It remains unclear whether the sites of RPB5 targeted by RAP30 and HBx are different or redundant. Here we specified the residues critical for the interactions with HBx and/or RAP30 by two-step alanine scanning with mutant libraries of RPB5. Four residues, F76, I104, T111 and S113, were found to be essential for binding to the two factors, indicating the overlapping nature of the interactions.

MATERIALS AND METHODS

Plasmid Constructions—The plasmids pNKFLAG and pNKGST derived from pSG5UTPL are FLAG-tagged and GST-tagged mammalian expression vectors, respectively, as reported. The plasmids pGENK1 and pGENKS are bacterial expression vectors for GST-fused proteins (*13*, *16*). The full-length coding region of human RPB5 (XAP4) (*1*–210) was prepared as reported (*13*). Mutagenesis was carried out by a splicing PCR method using AGCGAATTCCATGGACGACGAGGAGGAG or AGTAGATCTCTACTGCACCAG together with mutated oligonucleotide primers (sequences available upon request). An alanine-scanning method was used to construct RPB5 clustered or single alanine substitution mutants to minimize the effects of substituted amino acid residues (*15*, *37*). The positions of clustered mutants of RPB5 are shown in Fig. 1. The target sequence of 7 amino acid residues was changed to AAASAAA for practical reasons in all clustered mutants (cm). The second series of cm harboring AAASA [cm I(s)] (spanning from the 1st to 5th residue of the defining cm) and SAAA [cm II(s)] (spanning from the 4th to 7th residue of the defining cm) were constructed using the *Nhe*I site–encoding SA sequence. Then single alanine substitution mutants were constructed as follows: (i) if cm I(s) is defective, cm II(s) has no effect, first three residues will be targeted to point mutant scanning; (ii) if cm I(s) has no effect, cm II(s) is defective, last two residues will be targeted to point mutant scanning; (iii) if both cm I(s) and cm II(s) are defective, all seven residues will be targeted to point mutant scanning. In the point substitution mutants, the target residue was replaced with alanine (A) in a two-step PCR scanning. All sequences encoding full-length mutants of RPB5 were inserted into the *Eco*RI and *Bgl*II/*Bam*HI sites of pNKFLAG, pNKGST, pGENKS, and pYFLAG to construct various expression vectors. All of the constructs were sequenced by the dideoxy method using Taq sequencing kits and a DNA sequencer (370A; Applied Biosystems Inc. Co. Ltd.).

The plasmids pGST-RAP30 and His-ET-RAP74 were gifts from R.G. Roeder. The truncation mutant of RAP30 cDNA, RAP30/d4, encodes the initiation codon followed by amino acids 101 to 249, as reported (*18*).

The *Escherichia coli* histidine-tagged protein expression plasmid pLHis was derived from pET11d, by replacing the *Nde*I–*Bam*HI fragment with the annealed complementary oligonucleotides TATGAATTCCATGAAGCTTGGATC and GATCCAAGCTTCATGGAATTCATA, to introduce *Eco*RI, *Hin*dIII, and *Bam*HI digestion sites. The plasmid pLHis-HBx was constructed by inserting the coding sequence of wild type HBx into the *Eco*RI and *Bam*HI sites of pLHis as reported (*33*).

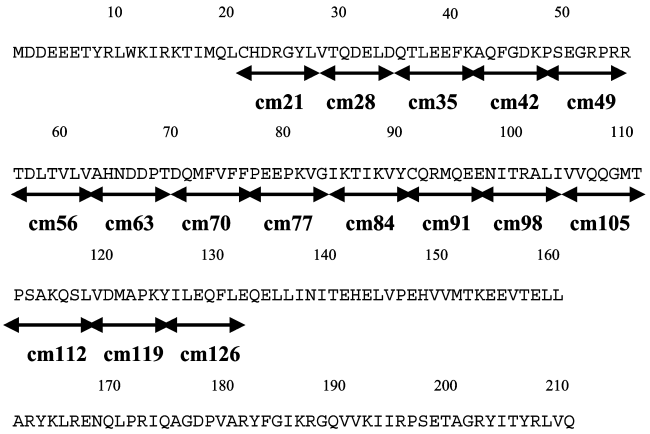

Fig. 1. **Clustered substitution mutants (cm) of human RPB5.** The amino acid sequence of human RPB5 and a mutant library of human RPB5 were constructed. The target of 7 amino acid residues was changed to AAASAAA. Names of clustered mutants (cm) are shown under two-headed arrows with numbers corresponding to the number of the first of the 7 amino acid residues substituted with AAASAAA in the human RPB5 open reading frame.

Preparation of Recombinant Proteins—GST-fused proteins were expressed in *E. coli* by induction with 0.4 mM isopropyl-D-thiogalactopyranoside at 30°C for 3 h. The cells were harvested and sonicated in PBST buffer (phosphate-buffered saline containing 1 mM DTT and 1% Triton X-100) (*16*, *18*). After centrifugation of the sonicated lysate, the supernatant was passed through DEAE Sepharose, and GST-fused proteins were recovered with glutathione-Sepharose 4B beads (Amersham Biosciences, Inc.) at room temperature for 1 h. The beads were sedimented, washed four times with an excess amount of PBST buffer, and then eluted with 10 mM of reduced glutathione in 50 mM Tris-HCl (pH 8.0). The eluted solution was dialyzed against buffer B [100 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1mM DTT], then divided into aliquots and stored at –80°C.

The His-tagged HBx protein was expressed in BL21 (DE3)/pLys using 0.7 mM isopropyl-D-thiogalactopyranoside for 3 h at 27°C. The cell pellet was harvested and washed, and resuspended in precleared buffer [50 mM sodium phosphate (pH 8.0), 500 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, and 1% Triton X-100]. The suspension was sonicated on ice and centrifuged at 12,000 × *g* for 15 min. The pellet was suspended in denaturing binding buffer (100 mM sodium phosphate, 10 mM Tris-HCl, and 8 M urea) with the pH adjusted to 8.0 using HCl. The lysate was centrifuged at 12,000 × *g* for 5 min, and the cleared lysate was subjected to affinity binding to nickel resin (Qiagen) preequilibrated with denaturing binding buffer. The resin was washed once with denaturing binding buffer then several times with denaturating buffer mixed with increasing amounts of bridge buffer (100 mM sodium phosphate, 10 mM Tris-HCl, 2 mM DTT, 1 M

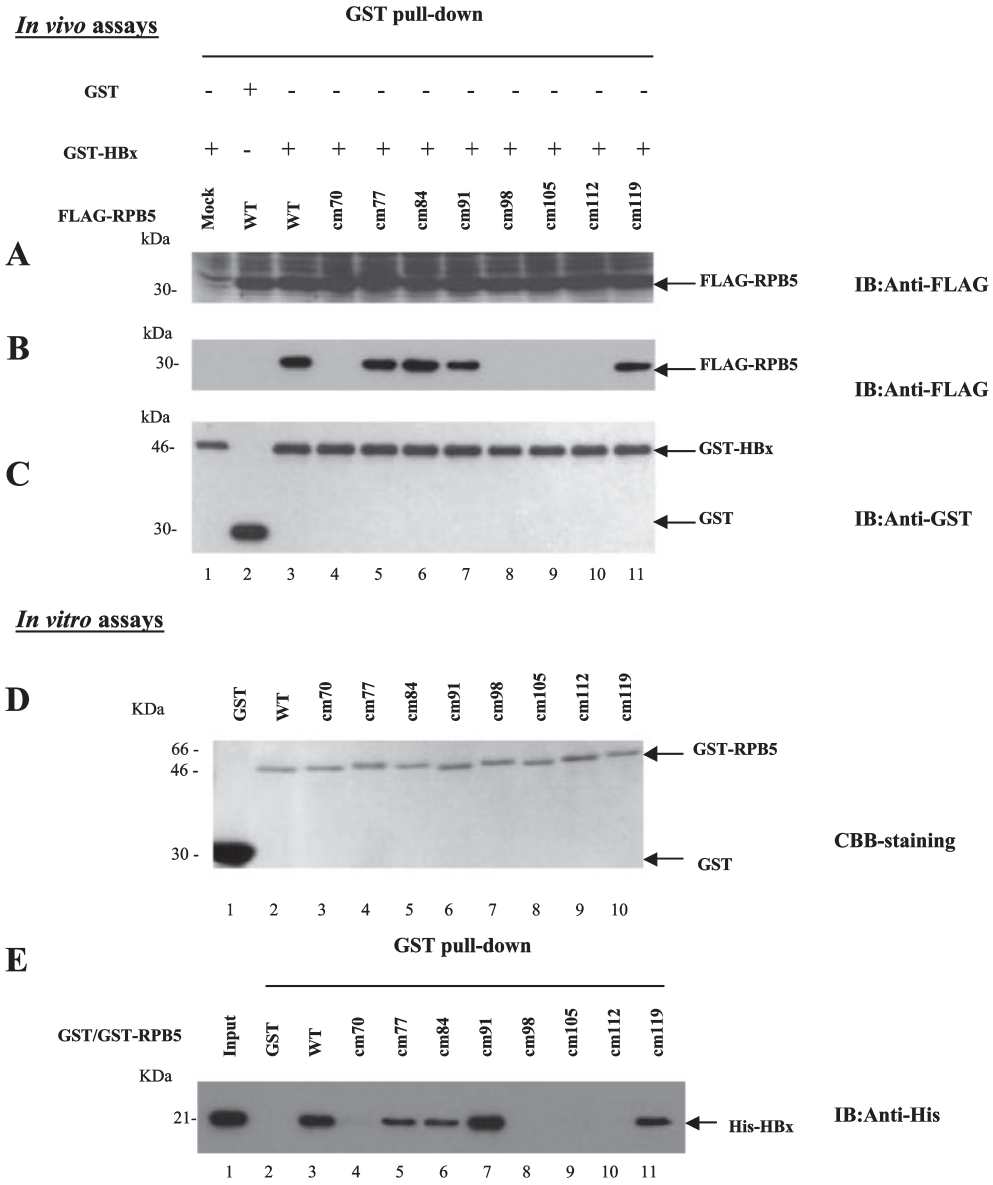

Fig. 2. **Amino acid sequences of RPB5 critical for HBx-binding as analyzed *in vivo* and *in vitro*.** (A–C) *In vivo* assays: COS1 cells were transfected with pNKGST-HBx alone in the absence of pNKFLAG-RPB5 (mock) (lane 1), or transiently cotransfected with the mammalian expression vectors pNKGST and pNKFLAG-RPB5 wild type (WT) (lane 2) or pNKGST-HBx and pNKFLAG-RPB5 wild type (WT) or different cm RPB5 as indicated at the top (lanes 3 to 11). (A) Total lysates were separated by SDS-PAGE and subjected to Western blot analysis with α-FLAG monoclonal antibody (input). (B) Cell lysates were pull-downed with glutathione resin. After washing, the bound proteins were detected with an α-FLAG monoclonal antibody. (C) The nitrocellulose membranes used for Western blot analysis with the α-FLAG antibody were reprobed with an α-GST antibody. (D and E) *In vitro* assays: (D) Purification of bacterially expressed GST, GST-RPB5 wild type (WT) or different clustered mutants as indicated. The GST (lane 1) and GST-RPB5 WT (lane 2) or mutant RPB5 (lanes 3 to 10) proteins were expressed in *E. coli* and purified. Between 0.4 and 1 μg of each purified protein was analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. (E) Interaction of RPB5 and HBx proteins *in vitro*. Approximately 1 μg of bacterial GST or bacterial recombinant GST-fused RPB5 wild type (WT) or cm proteins was immobilized on glutathione resin and incubated with 0.2 μg of His-HBx protein in GBT buffer for 2 h at 4°C. Pull-down assays and Western blot analysis were carried out with an α-His monoclonal antibody. Input (lane 1) and lanes 2 to 11 showed before and after the pull-down assay, respectively. Each experiment was repeated at least three times and the data were reproducible.

NaCl, and 2% Triton X-100) to renature the protein, and finally washed with bridge buffer. The bound His-HBx was eluted with native elution buffer [50 mM sodium phosphate (pH 8.0), 500 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, 1% Triton X-100, and 500 mM imidazole]. Eluted solutions were combined and dialyzed against buffer C [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM DTT, and 0.1% Triton X-100], and stored at –80°C in aliquots.

FLAG-tagged proteins were expressed in BL21 by induction with 0.4 mM isopropyl-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 supernatant with anti-FLAG M2 resin (Kodak Scientific Imaging Systems) and washing several times. The bound proteins were eluted with buffer containing FLAG peptide [0.2 mg/ml FLAG peptide, 50 mM Tris-HCl (pH 8.0), and 150 mM NaCl]. Eluted solutions were combined and dialyzed.

Antibodies—Anti-FLAG M2 and anti-His monoclonal antibodies were purchased from Kodak Science Imaging Systems. Anti-GST monoclonal antibody was obtained from ZYMED Laboratories.

In Vitro GST Resin Pull-Down Assays—Approximately 1 μg of GST or GST-fused protein immobilized on 20 μl of glutathione-Sepharose 4B preblocked in 1% bovine serum albumin was incubated with 0.2 μg of FLAG-tagged proteins or 0.5 μg of His-tagged proteins in 0.5 ml of GBT buffer [10% glycerol, 50 mM Hepes-NaOH (pH 8.0), 170 mM KCl, 7.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 1% Triton X-100] for 3 h on a rotator at 4°C. After being

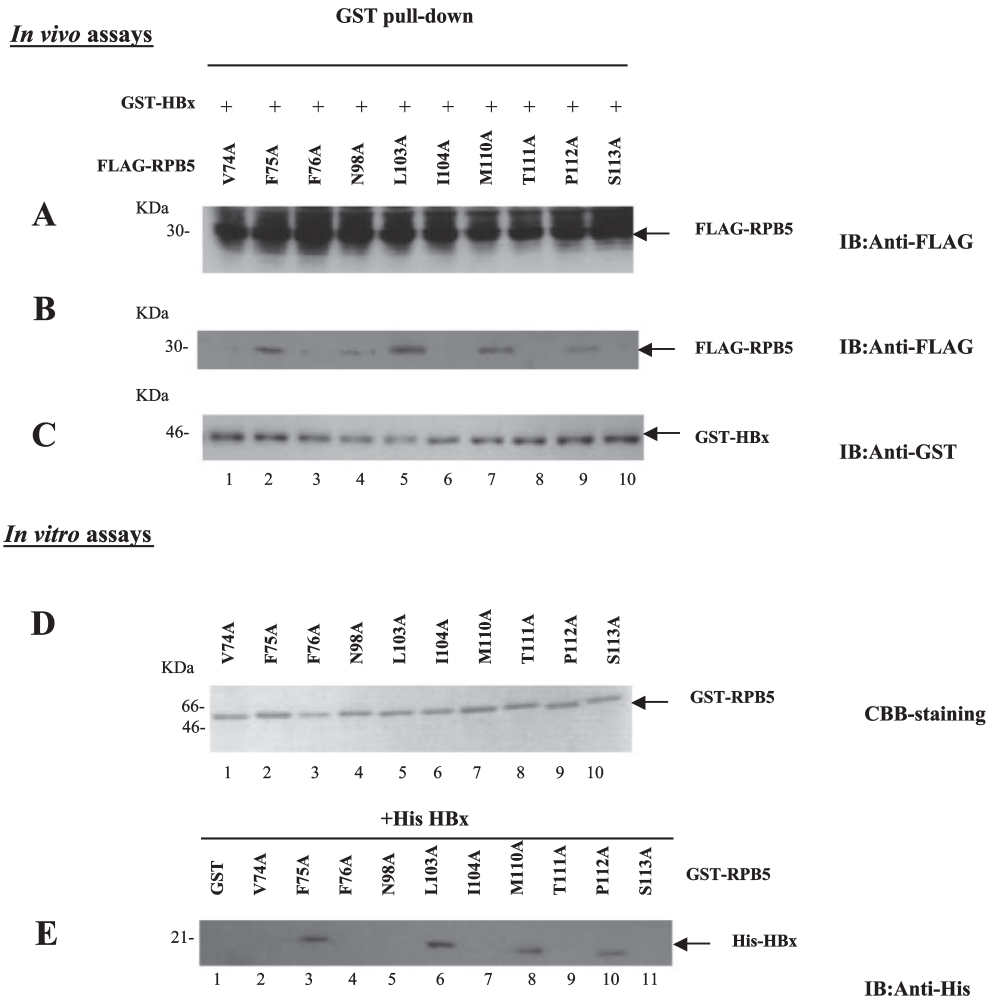

Fig. 3. **Amino acid residues of RPB5 critical for the HBx-binding as analyzed *in vivo* and *in vitro*.** The experiments were carried out as in Fig. 2 except that pNKFLAG-RPB5 harboring a single amino acid substitution (pm) as indicated at the top was used. (A–C) *In vivo* assays: (A) Western blot analysis of total lysates with an α-FLAG monoclonal antibody. (B) Western blot analysis of recovered proteins bound to glutathione resin with the α-FLAG monoclonal antibody. (C) The nitrocellulose membranes used for Western blot analysis with the α-FLAG antibody were reprobed with an α-GST antibody. (D and E) *In vitro* assays: (D) Purified bacterially expressed GST-RPB5 pm proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. (E) Recovered His-HBx protein bound to GST or GST-RPB5 pm protein detected with an α-His monoclonal antibody. Each experiment was repeated at least three times and the data were reproducible.

washed four times with GBT buffer, the bound proteins were eluted, fractionated by 12% SDS-PAGE, and subjected to Western blot analysis using anti-FLAG monoclonal antibody (M2) or anti-His monoclonal antibody.

Preparation of Cell Extract, Coprecipitation with Glutathione-Sepharose 4B Resin, and Western Blot Analysis—The transient transfection of COS1 cells was carried out by standard $CaCl_2$-mediated transfection. The cells were harvested, washed, and sonicated in lysis buffer [10% glycerol, 20 mM HEPES (pH 7.9), 50 mM KCl, 0.4 M NaCl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA, 9 mM CHAPS, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin and leupeptin], and then centrifuged. The total cell lysates were stored at –80°C. Total cell lysates were diluted with 4 times the volume of buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] and incubated with 50 μl of glutathione-Sepharose 4B beads for 3 h on a rotator at 4°C. After extensive washing, the bound proteins were eluted, fractionated by 15% SDS-PAGE, transferred onto nitrocellulose membranes, and subjected to Western blot analysis with the anti-FLAG M2 antibody. The proteins were visualized by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Biosciences). The nitrocellulose membranes used for Western blot analysis with anti-FLAG monoclonal antibody were reprobed with anti-GST monoclonal antibody (Zymed Laboratories Inc.) according to the manufacturer's instructions (Amersham Biosciences).

Cell Culture and Transient Transfection—COS1 cells (a monkey kidney cell line) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogene Co) supplemented with 5% fetal calf serum (FCS; CELLect R GOLD) and 20 mg/ml antibiotics (ampicillin and kanamycin; Meigi Co. Ltd) and maintained in an incubator with 5% CO_2 at 37°C.

RESULTS

Amino Acid Sequences of the Central Part of RPB5 Necessary for HBx-Binding In Vivo and In Vitro—The HBx-binding region has been mapped within the central part of RPB5 using various versions of truncation mutants (*15*). To specify the amino acid sequence(s) and amino acid residue(s) critical for HBx-binding, we applied a two-step alanine scanning method using clustered substitution mutants (cm) and point substitution mutants (pm). A cm library of human RPB5 covering aa 21 to 132 was constructed by systematically substituting 7 amino acid residues in a row with AAASAAA as shown in Fig. 1.

The ability of these cm RPB5 proteins to bind HBx was examined with lysates of mammalian cells transiently

Table 1. **Summary of RPB5-binding abilities.**

Mutated human RPB5 residue	HBx	RAP30 (TFIIF)	Mutated human RPB5 residue	HBx	RAP30 (TFIIF)
T56A	++[a]	–	I99A	++	++
D57A	++	++	T100A	++	++
L58A	++	–	R101A	++	++
D70A	++	++	L103A	++	++
Q71A	++	++	I104A	–	–
M72A	++	++	V105A	++	++
F73A	++	++	M110A	++	++
V74A	–	++	T111A	–	–
F75A	++	++	P112A	++	++
F76A	–	–	S113A	–	–
N98A	–	++			

[a](++): binding positive; (–): binding negative.

coexpressing GST-HBx and each FLAG-RPB5 cm protein *in vivo*. COS1 cells were transfected with the mammalian expression vectors and the cell lysates were pull-downed with glutathione resin. GST-HBx recovered FLAG-RPB5 while GST alone did not (Fig. 2B, lanes 2 and 3). The specific interaction of two proteins was also detected with a different combination of tagged proteins (data not shown). Under these conditions, four cm proteins of RPB5 (cm 70, cm 98, cm 105, and cm 112) were not recovered with HBx (Fig. 2B lanes 4, 8, 9, and 10, respectively), although the other three cm proteins were recovered similar to the wild type RPB5, and cm 91 was recovered, but inefficiently (Fig. 2B, compared lane 3, and 7 with lanes 5, 6, and 11, respectively). This result was not due to different expression levels of proteins and/or the efficiency of GST pull-down since the recovery of GST-fused proteins was similar (Fig. 2C) and the expression levels of FLAG-RPB5 wild type (WT) and clustered mutants were similar (Fig. 2A). Each experiment was repeated at least three times and the data are reproducible.

To confirm the binding properties of cm mutants to HBx *in vivo*, we performed an *in vitro* pull-down assay. Bacterially expressed wild type and cm GST-RPB5 were bound to glutathione resin (Fig. 2D) and subjected to binding assays with purified His-tagged HBx (as described in "MATERIALS AND METHODS"). To purify the HBx protein expressed *in vitro*, the denatured followed by renaturation method is better than the native method (see details of purification in "MATERIALS AND METHODS"). The bound HBx was subjected to Western blot analysis with anti-His antibody. Consistent with the result *in vivo*, we observed that the four cm RPB5 (cm 70, cm 98, cm 105, and cm 112) were unable to interact with HBx (Fig. 2E lanes 4, 8, 9, and 10, respectively). The other cm mutants retained the ability to interact with HBx, although the amount of protein recovered was not the same for each mutant (Fig. 2E lanes 5, 6, 7, 11 and data not shown). Four sequences of RPB5 (cm70, cm98, cm105 and cm112) critical for HBx-binding *in vivo* and *in vitro* are in two different areas within the central part of RPB5 (aa 53 to 136).

Interaction between HBx and Alanine Point Mutant (pm) RPB5 In Vivo and In Vitro—To identify residues critical for binding HBx within the four cm mutants, we constructed a second series of cm mutants harboring AAASA and SAAA in the target sequence using the *Nhe*I site–encoding SA sequence and analyzed them for protein–protein interactions (data not shown). Then single alanine substitution mutants covering the sequence(s) were constructed as explained in "MATERIALS AND METHODS." Similar experiments with the pm mutant series of RPB5 were conducted *in vivo* and *in vitro*. We failed to detect any interaction of HBx with RPB5 having V74A, I104A, T111A, or S113A in both the *in vivo* and *in vitro* assays (Fig. 3B, lanes 1, 6, 8, and 10, respectively; Fig. 3E, lanes 2, 7, 9, and 11, respectively). RPB5 harboring F76A or N98A are severely affected *in vivo* and completely defective *in vitro* in their ability to interact with HBx (Fig. 3B, lanes 3 and 4, respectively; Fig. 3E, lanes 4 and 5, respectively). On the other hand, none of the other residues listed in Table 1 impaired the HBx interaction (Fig. 3B, lanes 2, 5, 7, and 9; Fig. 3E, lanes 3, 6, 8, and 10; and data not shown). Taken together, the results of the experiments with coexpressed proteins in COS1 cells are consistent with those with purified bacterial proteins *in vitro*, indicating that the RPB5 residues critical for HBx interaction are V74, F76, N98, I104, T111, and S113.

Interaction of RPB5 and (RAP30) TFIIF Proteins In Vivo and In Vitro Analyzed with cm and pm Mutants of RPB5—Previously we reported a direct interaction between RPB5 and subunit RAP30 of TFIIF (*18*); however, the residues within RPB5 responsible for this interaction remain unknown. In this study, we addressed the question of which sequences in the central part of RPB5 are required for the interaction between RAP30 in the TFIIF complex and RPB5. To this end, COS1 cells were cotransfected with mammalian plasmids expressing two subunits of TFIIF: GST-RAP30 and FLAG-RAP74 together with wild type or one of the cm mutants of FLAG-RPB5. The cell lysates were subjected to GST pull-down with glutathione resin, and the recovered FLAG-tagged RPB5 and RAP74 were detected immunologically using an anti-FLAG monoclonal antibody. All the clustered mutants of RPB5 were expressed equally upon transfection into COS1 cells, and GST-RAP30 in each combination of cotransfection was similarly recovered by GST pull-down (Fig. 4, A and C). GST-RAP30, but not GST alone, recovered FLAG-RPB5 and FLAG-RAP74 (Fig. 4B, lanes 2 and 3). The five cm mutants (cm 56, cm 70, cm 98, cm 105, and cm 112) were impaired in their interaction with RAP30 in the TFIIF complex (Fig. 4B,

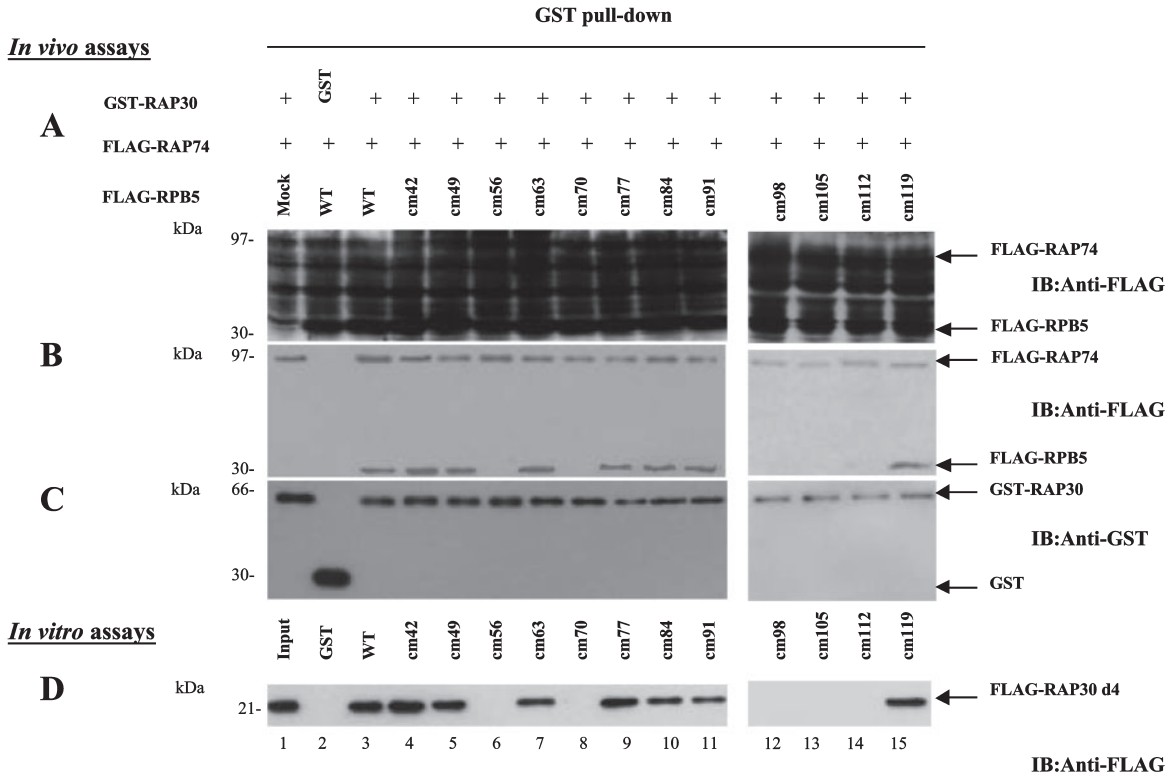

Fig. 4. **Amino acid sequences of RPB5 critical for RAP30-binding in the TFIIF complex as analyzed *in vivo* and *in vitro*.** (A–C) *In vivo* assays: Experiments were conducted in COS1 cells as described in Fig. 2, A–C, except that COS1 was transiently cotransfected with the mammalian expression vectors pNKGST-RAP30 and pNKFLAG-RAP74 in the absence of pNKFLAG-RPB5 (mock) (lane 1), or together with pNKFLAG-RPB5 WT or cm as indicated. (D) *In vitro* assays: Recovered bacterially expressed FLAG-RAP30 d4 (see text in detail) bound to GST or GST-RPB5 WT or cm protein prebound to glutathione resin was detected by Western blot analysis with an α-FLAG antibody. Each experiment was repeated at least three times and the data were reproducible.

lanes 6, 8, 12, 13 and 14, respectively), although similar amounts of RAP74 were recovered in the presence or absence of wild type or mutant FLAG-RPB5. In contrast, the other clustered mutants of RPB5 did show an ability to bind TFIIF similar to that of the wild type RPB5 (Fig. 4B, lanes 3, 4, 5, 7, 9, 10, 11, 15 and data not shown). Interestingly, all five of these cm mutants, except cm 56, are not only defective in TFIIF-binding but also HBx-binding (Fig. 2), indicating that overlapping sequences are involved in the bindings to the two factors.

As previously reported, the N-terminus and the middle part of subunit RAP30 can interact independently with RPB5, although the former is masked by RAP74 in the TFIIF complex (*18*). Therefore, the middle part of RAP30, the construct RAP30 d4 (aa 101 to 249), was tested for binding. It could bind RPB5 regardless of the presence or absence of RAP74, a binding consistent with a previous report (data not shown). Based on this observation, next we used RAP30 d4 (aa 101 to 249) for the pull-down assay *in vitro* to evaluate its ability to bind bacterially expressed and purified wild type or cm RPB5. As shown in Fig. 4D, all proteins except cm 56, cm 70, cm 98, cm 105, and cm 112, were recovered with GST-RPB5 bounded resin. Although we observed that cm 63, cm 84, cm 91 showed weaker binding ability (Fig. 4D, compared lanes 3, 4, 5, 9 and 15 with lanes 7, 10, and 11).

Using the same method as described in "MATERIALS AND METHODS" to pinpoint critical residues of RPB5 for RAP30-binding, we further constructed mutants of RPB5 with point alanine substitutions and examined their ability to interact with RAP30 (subunit of TFIIF) both *in vivo* and *in vitro*. The six pm mutants of RPB5 (T56A, L58A, F76A, I104A, T111A, and S113A) were clearly defective in both RAP30-binding in the TFIIF complex *in vivo* and in RAP30-binding using RAP30 d4 (which binds RPB5 regardless of the presence or absence of RAP74) *in vitro* (Fig. 5B, lanes 1, 3, 5, 7, 9, and 11, respectively; Fig. 5D, lanes 2, 4, 6, 8, 10, and 12, respectively). In contrast, other pm mutants of RPB5 listed in Table 1 were able to bind (Fig. 5B, lanes 2, 4, 6, 8 and 10; Fig. 5D, lanes 3, 5, 7, 9, and 11; and data not shown). Interestingly, among them, in the *in vivo* experiment, two pm mutants, D57A and P112A, exhibited much weaker ability to bind RAP30; however, *in vitro*, pm mutant L103A exhibited weaker interaction, suggesting that the GST pull-down assay with two purified proteins *in vitro* may be not the same as the GST pull-down of total cell lysate proteins *in vivo*.

Position of Residues Critical for the Interaction of Human RPB5 with HBx/RAP30—Collectively, in the central part of human RPB5, we identified six residues critical for the interaction with HBx and six residues indispensable for the interaction with RAP30. Interestingly, among them, four residues (hF76, hI104, hT111, and hS113) are required for the interactions with both HBx and RAP30 in the TFIIF complex, and are conserved the among human and yeast proteins (Table 1). The

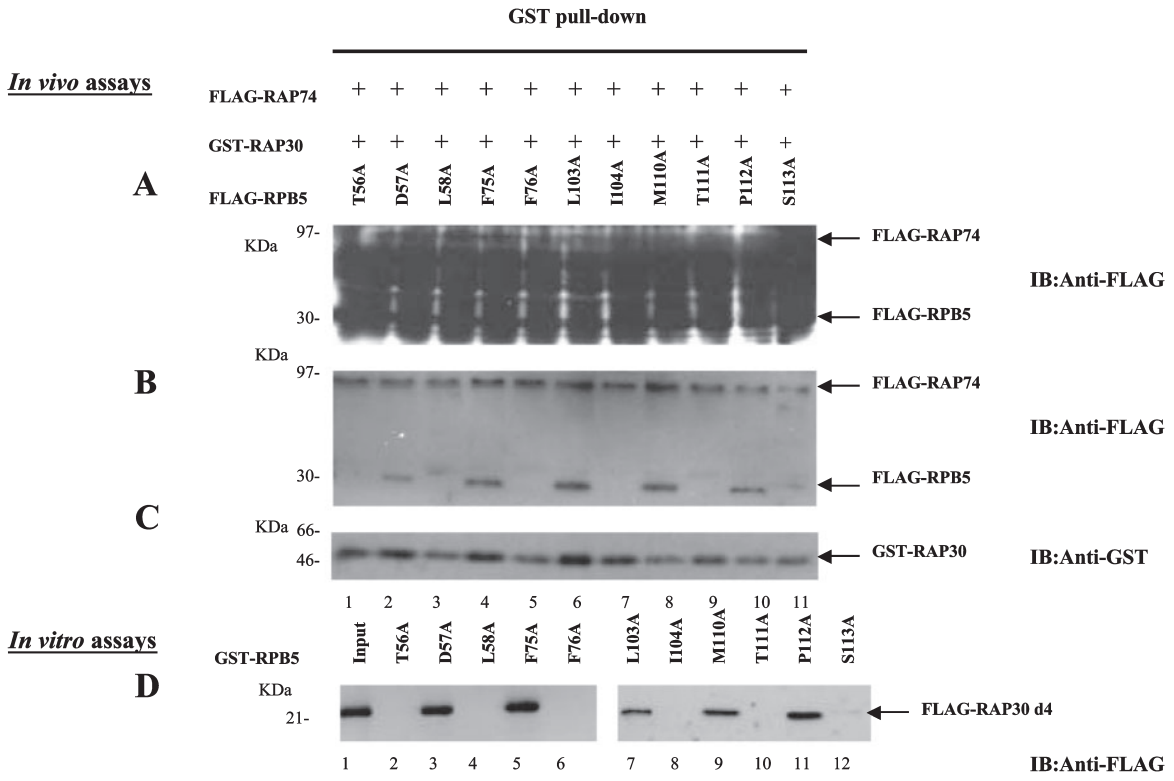

Fig. 5. **Amino acid residues of RPB5 critical for the RAP30-binding in the TFIIF complex as analyzed *in vivo* and *in vitro*.** (A–C) *In vivo* assays: The experiments were conducted in COS1 cells as described in Fig. 4 except that pNKFLAG-RPB5 pm as indicated at the *top* was used. (D) *In vitro* assays: bacterially expressed GST-RPB5 proteins with a single amino acid substitution as indicated were used as described in Fig. 4 and Western blot analysis was carried out with an α-FLAG monoclonal antibody. Each experiment was repeated at least three times and the data were reproducible.

newly identified critical amino acid residues of hRPB5 and the corresponding conserved residues in yRPB5 are shown in Fig. 6. Among the four conserved residues, yF82 (hF76) is juxtaposed in a β-sheet [designated A2 (21)] in parallel with a β-sheet (A3) harboring yI109 (hI104), which is close to yF82 (hF76). Amino acids yT117 (hT111) and yS119 (hS113) are at the top of the exposed helix or in the loop region (Fig. 6), and are the nearest neighbors of yP118 (hP112), which has been proposed to be one of the residues closest to the DNA. The two residues specific for RAP30-binding, hT56 and hL58, are in the loop between the N-terminal part and the central part of RPB5, and are not conserved among human and yeast. The two residues specific for HBx interaction, yV80 (hV74) and yN104 (hN98), are in A3 and in a loop between a helix (α5) and A3.

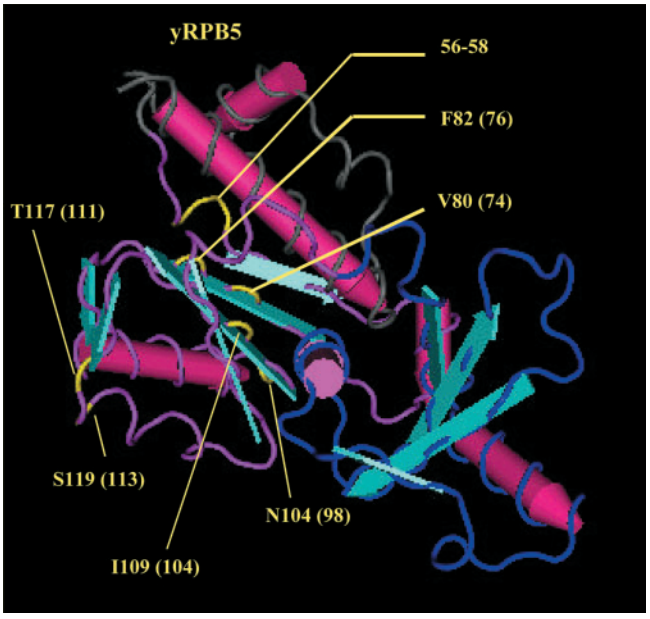

Fig. 6. **Position of the newly identified residues in the crystal model of RPB5.** The corresponding conserved amino acid residues predicted in *Saccharomyces cerevisie* RPB5 are shown in yellow (except residues 56 to 58 belonging to a non-conserved region), and the amino acid residues of human RPB5 essential for binding HBx and the RAP30 subunit of TFIIF: threonine 56 (T56), leucine 58 (L58), valine 74 (V74), phenylalanine 76 (F76), asparagine 98 (N98), isoleucine 104 (I104), threonine 111 (T111), and serine 113 (S113), are shown in parentheses.

DISCUSSION

Here, we report six residues (V74, F76, N98, I104, T111 and S113) critical for HBx-binding, and six residues (T56, L58, F76, I104, T111 and S113) critical for RAP30-binding by RPB5. Interestingly, four of these residues are redundant for RAP30- and HBx-binding, strongly implying that the two factors compete with each other for RPB5-binding. In this context, HBx may inhibit or delay a step in transcription in which the direct interaction between RPB5 and TFIIF is involved. TFIIF has several contact sites for RNAPII during initiation and elongation

of transcription, and the initiation step before the interaction of RPB5 and TFIIF may be critical for activated transcription. Another possibility is that the central region of the exposed domain covering aa 72 to 120 may be an important surface for unidentified factors (as discussed below).

The central part of RPB5 (aa 53 to 136), proximal to DNA downstream of the promoter (see Fig. 6), acts in communication with several transcriptional regulators, the TFIIF subunit RAP30, HBx and a novel corepressor RMP/URI (*8*, *13*, *15*, *16*, *18*, *20*, *21*, *36*, *38*). We previously identified HBx as a transcriptional coactivator through its direct interaction with the central part of RPB5 (*15*, *17*, *33*, *34*). The putative role of human RPB5 in activated transcription is consistent with the reported role of yeast RPB5 in the activated transcription of some promoters (*38*), although it remains uncertain how RPB5 is involved in activated transcription but not in basal transcription. By two-step scanning with clustered and point alanine substitution(s) libraries, we dissected the central part of hRPB5 to address whether the sequence and residues needed for RAP30-binding and HBx-binding are redundant. In this paper, the interaction of RPB5 with HBx and TFIIF was examined *in vitro* and *in vivo* with RPB5 and its partner co-expressed transiently in cells. The interaction of RPB5 in RNAPII with these factors could be elucidated since ectopically expressed RPB5 can be efficiently incorporated into RNAPII (data not shown).

As RPB5 is well conserved between yeast and human (*8*, *10*, *13*, *15*, *18*, *21*, *22*, *38*, *39*), and the topology of the preinitiation complex is remarkably similar in the two species as analyzed by cross-link experiments and cryoelectron microscopy (*11*, *12*, *32*), the relevance of the positions of the conserved residues in human RPB5 were evaluated on basis of the yeast crystal model (Fig. 6). The results suggest that the rigid property of the four-stranded β-sheet may be important for the structural integrity of the central part of RPB5 (aa 53 to 144 of yRPB5 or aa 53 to 139 of hRPB5). All residues except T117 and S119 are non-charged and hydrophobic in the four-stranded mixed β-sheet. As all the RPB5 residues critical for binding RAP30 and/or HBx are conserved between yeast and human except aa 56 to 58, we next addressed the biological role of the six conserved residues in yRPB5 by introducing point mutations in yeast in place of the wild type counterpart. The yeast *rpb5* series of alanine/glycine substituted point mutant alleles was generated by two-step PCR mutagenesis. The mutations had no influence on cell viability but affected yeast cell growth, especially at suboptimal temperatures (ts or/and cs phenotype) (unpublished data). These results clearly support the notion that the conserved residues of RPB5 required for RAP30-binding as well as HBx-binding play an important role in cell growth in yeast. Interestingly, the two charged residues, T117 and S119 of yRPB5 (T111 and S113 of hRPB5), are exposed to the solvent, and are the nearest neighbors of the proline (yP118) that has been reported to be closest to the DNA (*8*). We identified the DNA-binding properties of hRPB5, and showed that T111 and S113, but not P112, are critical or important for DNA-binding (Zhang S. and Murakami S. et al, in a separate manuscript). DNA has been reported to wrap around the yeast and human RNAPII complex (*9*, *29*, *30*, *32*), and TFIIF forms multiple DNA contacts, spanning the DNA from upstream to downstream of the promoter as detected in cross-linking experiments (*11*, *23*, *30*, *32*). In that context, it is possible that both TFIIF and HBx may modulate the interactions of RNAPII and DNA by preventing RPB5 from accessing DNA downstream of the initiation site. In this respect, it remains to be addressed whether RPB5 is involved in recognition of a novel downstream promoter element, MTE (motif ten element), that is conserved from *Drosophila* to human, as recently reported (*40*).

We further defined the sequences of HBx that are important for RPB5-binding (Le T. and Murakami S. *et al.*, unpublished data). All sequences are in the coactivation domain of HBx, but, interestingly, they are much narrower than those necessary for the coactivation function of HBx as analyzed with the clustered mutant library of HBx (*35*). The results imply that multiple partners are necessary for the coactivation or transactivation function of HBx, in addition to RPB5 as reviewed (*17*).

RMP/URI (*16*, *36*) is another factor that requires the central part of RPB5 for interaction. Three out of the four residues critical for the binding of both RAP30 and HBx seem also to be indispensable for the binding of RMP/URI (in preparation). Gstaiger, et al. clearly demonstrated that RMP/URI belongs to a prefoldin (PFD) family whose members are able to assemble into molecular chaperone complexes (*36*), and that yeast and human RMP/URIs both seem to be targets of nutrient signaling controlled by mTOR kinase in the cytoplasm (*19*, *36*). Therefore, one possibility is that the role of RPB5 in activated transcription is not due to the function of RPB5 assembled in RNAPII, and that HBx may induce the dissociation of the cytoplasmic RPB5 and RMP/URI complex and facilitate the assembly of nuclear RPB5 in RNAPII as HBx can inhibit the binding between RPB5 and RMP/URI (unpublished data). Recently, we reported the nuclear role of RMP/URI in a complex with DMAP1, DNA methyltransferase 1-interacting protein, and showed that this complex exhibits corepressor activity (*19*). In this context, it seems possible that HBx releases the corepressor complex RMP and DMAP1, and facilitates activated transcription. The role of RPB5 in activated transcription remains to be elucidated.

Taken together, the central part of RPB5 proximal to DNA downstream of the promoter can be a target of several transcriptional regulators that may interfere with the interaction between the exposed domain of RPB5 and DNA.

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