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A Novel Approach to Investigating Protein/Protein Interactions and Their Functions by TAP-Tagged Yeast Strains and its Application to Examine Yeast Transcription Machinery

Jung, Junho[1], Yeh-Jin Ahn[2], and Lin-Woo Kang[1]*

[1]*Department of Advanced Technology Fusion, Konkuk University, Seoul 143-701, Korea*
[2]*Division of Life Science, College of Natural Sciences, Sangmyung University, Seoul 110-743, Korea*

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Tandem affinity purification (TAP) method combined with LC-MS/MS is the most accurate and reliable way to study the interaction of proteins or proteomics in a genome-wide scale. For the first time, we used a TAP-tag as a mutagenic tool to disrupt protein interactions at the specific site. Although lots of commonly used mutational tools exist to study functions of a gene, such as deletional mutations and site-directed mutagenesis, each method has its own demerit. To test the usefulness of a TAP-tag as a mutagenic tool, we applied a TAP-tag to RNA polymerase II, which is the key enzyme of gene expression and is controlled by hundreds of transcription factors even to transcribe a gene. Our experiment is based on the hypothesis that there will be interrupted interactions between Pol II and transcription factors owing to the TAP-tag attached at the C-terminus of each subunit of Pol II, and the abnormality caused by interrupted protein interactions can be observed by measuring a cell-cycle of each yeast strain. From ten different TAP-tagged strains, Rpb7- and Rpb12-TAP-tagged strains show severe defects in growth rate and morphology. Without a heterodimer of Rpb4/Rpb7, only the ten subunits Pol II can conduct transcription normally, and there is no previously known function of Rpb7. The observed defect of the Rpb7-TAP-tagged strain shows that Rpb7 forms a complex with other proteins or compounds and the interruption of the interaction can interfere with the normal cell cycle and morphology of the cell and nucleus. This is a novel attempt to use a TAP-tag as a proteomic tool to study protein interactions.

Keywords: Tandem affinity purification (TAP), RNA polymerase II, protein/protein interactions (PPI), transcription, structural proteomics

**Corresponding author*
Phone: 82-2-450-4090; Fax: 82-2-444-6707;
E-mail: lkang@konkuk.ac.kr

During the last decade, complete genomic sequences of important model organisms have been determined, including *S. cerevisiae* (1996), *C. elegans* (1998), *D. melanogaster* (2000), *A. thaliana* (2000), and *Homo sapiens* (2001) [1, 6, 14, 16, 32]. Complete sequences of a whole genome enable us to study a target organism systematically, and the era of genomic and proteomic studies began based on those. Genomics means the comprehensive genetic analysis of a specific organism and can be understood as genome-wide mRNA expression studies [15]. Proteomics is the study of the expressed protein complement of a genome at a specific time and it is much more complicated than genomics, mostly because proteomics involves dynamics and is different from cell to cell by biochemical interactions [15]. Genomic and proteomic studies have developed fast with benefits from DNA microarrays, two-dimensional gel electrophoresis (2DE), mass spectrometry (MS), and better data mining tools of bioinformatics and computational biology. Although genomics and proteomics cover broad and various research fields, the main goal is identifying the function of every gene in the whole genome and the network of protein/protein interactions (PPI) in a genome-wide scale is essential to achieve the goal [17, 21].

The most straightforward method to identify the function of a gene is mutation and examination of its phenotype. It is based on the rationale that we can find the function of the specific domain or motif by deleting the part of gene and looking for the mutational phenotype caused from the artificial modification of the gene product. If a knock-out mutant is unavailable because of the essential function of the target gene, we need to find conditional mutants to study the function. Mutagenic tools such as knock-out or knock-down to identify the function of a gene have been used conventionally, and to find a specific functional domain within a whole gene, serial deletions of the gene and site-directed mutagenesis have been carried out. Deletional and site-directed mutagenesis methods are useful but have their

own weakness. Deletion of a part of gene can disrupt the overall folding of whole protein rather than just deleting a specific motif without interrupting a normal structure of other parts, and it is particularly true when the deleted part is located at the middle of the gene. Site-directed mutagenesis provides more precise results directly coming from mutated residues, but to cover a large interacting surface, several different residues are need to be mutated simultaneously. Site-directed mutagenesis can also influence the overall protein folding. For example, the mutation of amino acids with small side-chains like glycine or alanine into bulky charged amino acids like lysine or glutamate at the hydrophobic core can easily disrupt the overall folding of the mutated protein. If we need to mutate a gene in genomic DNA not in plasmids, it takes much more time and efforts.

We tried a novel approach to study the cellular function of specific PPI by using a commercially available yeast TAP-fusion library [13]. A TAP-tag consist of two affinity tags, which are calmodulin-binding peptide and protein A, and a TEV protease cleavage site is located between the two tags. Because the affinity between protein A and IgG is very high and specific, pure target protein or protein complexes can be obtained by even a purification step [28]. The usage of a TAP-tag as a mutagenic tool to interrupt PPIs has several merits compared with the previously mentioned mutagenic tools. First, because a tag is attached at the solvent exposed C-terminal end of a target protein, it has little effect on the normal protein folding. Second, in the case of yeast, predicted 4,247 ORFs are already TAP-tagged at the C-terminal end and no further bench works to manipulate the genomic DNA are necessary to produce mutants. The expression level of each tagged gene is also normal because it is expressed from its natural endogenous promoter.

As the first target to test the usefulness of a TAP-tag as a mutagenic tool, the eukaryotic transcription machinery from yeast including RNA polymerase II (Pol II) is tried. Pol II is highly conserved in eukaryotes. The sequence identity between yeast and human Pol II sequences is as high as 53% [8]. The crystal structure of yeast Pol II is also determined. The transcription machinery is one of the biggest protein complexes among all protein complexes [13]. Pol II itself consists of 12 subunits and is as big as half a megadalton (>0.5 MDa). If the holoenzyme is considered including general transcription factors such as TFIIA, TFIIB, TFIID (>0.6 MDa), TFIIE, TFIIF, TFIIH (>0.5 MDa), and mediator (>1 MDa), it is bigger than 2 MDa [9]. To transcribe a gene, Pol II needs to be coordinated by hundreds of different transcription factors. Up to now, there is no easy biochemical method available to study the structure of such a big complex. NMR has a specific molecular mass limit to determine its three-dimensional structure of target proteins, which should be less than 20 kDa owing to a technical limit, and although X-ray crystallography has no size limit for target proteins, it requires well-ordered crystals of the target protein or the target protein complex. Multiprotein complexes are not easy to purify as a native status, and their crystals are even harder to obtain. Recently, a cryo-EM method has started to show the low resolution structures of diverse composite transcription machineries, but it still needs tons of efforts and time [4, 9]. It has been heavily studied, but not answered, over which part of RNA polymerase II interacts with which transcription factors and what the function of the interaction is.

This is the first attempt to use a TAP-tag as a mutagenic tool to interrupt protein interactions at the specific location of the target protein. From the three-dimensional crystal structure of Pol II, all C-terminal ends of twelve different subunits of Pol II were determined [2]. A TAP-tag attached at the specific site of Pol II can interfere with the binding of other proteins such as transcription factors. Among ten different yeast strains, which have a TAP-tagged subunit of Pol II, Rpb7- and Rpb12-TAP-tagged strains showed the most severe defects of growth rate and morphology. Interestingly, the function of Rpb7 was previously unknown, and our results suggest that Rpb7 forms a complex with other proteins or intracellular compounds, and the interruption of the interaction can interfere with the normal cell cycle and morphology of the cell and nucleus.

Materials and Methods

TAP-tagged Yeast Strains and Expression of TAP-tagged Subunits

Ten different yeast TAP-tagged strains were generously provided by Professor Huh Won-ki at Seoul National University, and the strains are the same with those of the yeast TAP-Fusion Library (Cat. No. YSC1177) from Open Biosystems. The genotype of *Saccharomyces cerevisiae* used to construct scTAP strains was S288C (ATCC 201388: MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). The TAP-tagged subunits of Pol II in separate yeast strains included Rpb1, Rpb2, Rpb3, Rpb6, Rpb7, Rpb8, Rpb9, Rpb10, Rpb11, and Rpb12. Rpb4- and Rpb5-TAP-tagged yeast strains are unavailable from the yeast TAP-Fusion Library. The expression of TAP-tagged proteins from the library have been reported in a previous paper and also confirmed by Western blotting gel with IgG antibody in our laboratory. Cells were grown in YPD media to mid-log phase (A_{600}=0.5, or 2×10^7 cells). Cells (10^7) were washed once with water and resuspended in 30 μl of 2% SDS, and 100 μl of glass beads was added. Cells were subjected three times to 2 min of vortexing. Then, 300 μl of 1 M NaCl/2 M urea/PBS was added, followed by vortexing and centrifugation at 13,000 rpm for 1 min. The clarified supernatant was used for loading onto an SDS gel. The extracts were run on a SDS-10% polyacrylamide gel, transferred to an Immobilon-P membrane (Millipore), and probed with IgG antibodies (Sigma Cat. No. P1291). Detection was performed with horseradish-peroxide-conjugated goat-anti-mouse antibodies (Bio-Rad) and ECL (Amersham Pharmacia Biotech).

Growth Rate Measurement and Morphology of Each TAP-tagged Yeast Cell

Each yeast strain was grown for two days in 3 ml of YPD medium (20 g/l Difco peptone, 10 g/l yeast extract, 20 g/l glucose) with chloramphenicol (35 μg/ml) from a colony on a streaked plate and transferred to a 100-ml culture of YPD medium with chloramphenicol of the same concentration. Yeast cell growth was determined by measuring the OD_{600} of yeast culture initially, and the accurate growth rate was determined in triplicate by counting cells in a hemocytometer under light microscopy at every two hours for 32 h. At the mid-log phase of yeast cell culture, the shapes of yeast cells were observed using a light fluorescence microscope (Olympus BX61-32 FDIC).

Models of TAP-tagged RNA Polymerase II

For three-dimensional structure models of 12 different TAP-tagged Pol II, the crystal structures of 12 subunit Pol II (PDB ID: 1WCM) and protein A from *Staphylococcus aureus* (PDB ID: 1BDC) were downloaded from PDB. Both atomic resolution structures were read into program O from PDB. The position of Pol II was fixed and the coordinate of protein A manually moved close to the C-terminal end of each subunit to be attached. Final coordinates of 12 different TAP-tagged Pol II molecules were written into PDB files and read into program Pymol [10] for figure and movie preparations. The movies of 12 different three-dimensional TAP-tagged Pol II molecules are available at the Web site http://structure1.konkuk.ac.kr/interest.htm.

RESULTS AND DISCUSSION

Models of TAP-tagged RNA Polymerase II

Pol II consists of 12 different subunits, which are from Rpb1 to Rpb12, and is as big as about half a megadalton and basically has a spherical shape if a protruded heterodimer of Rpb4/Rpb7 is omitted. Pol II has a positively charged central cleft to bind double-strand DNA and a funnel structure at the bottom, where nucleotides are diffused to the active site and inserted into the growing 3' end of the RNA strand. Pol II can be divided into four different mobile modules of "core", "jaw-lobe", "shelf", and "clamp" [7] (Fig. 1). Among the four modules, the most mobile part is the clamp module, which can adapt open and close conformations to hold downstream double-strand DNA in the process of transcription. A TAP-tag is attached at the C-terminal end of all Pol II subunits, except Rpb4 and Rpb7, in separate strains. A normal expression level of TAP-tagged Pol II subunits from endogenous promoter was published and the expression levels of each subunit were variable, from none detected in the case where there were less than 50 molecules/cell, to 1.87E+04 protein molecules/cell [13]. We also confirmed the expression of selected TAP-tagged subunits by a Western blot (data not shown). Each TAP-tagged subunit is shown at a higher molecular mass size in the Western blot than its own molecular mass owing to the contribution of the attached TAP-tag, which is about 20 kDa. Twelve different model

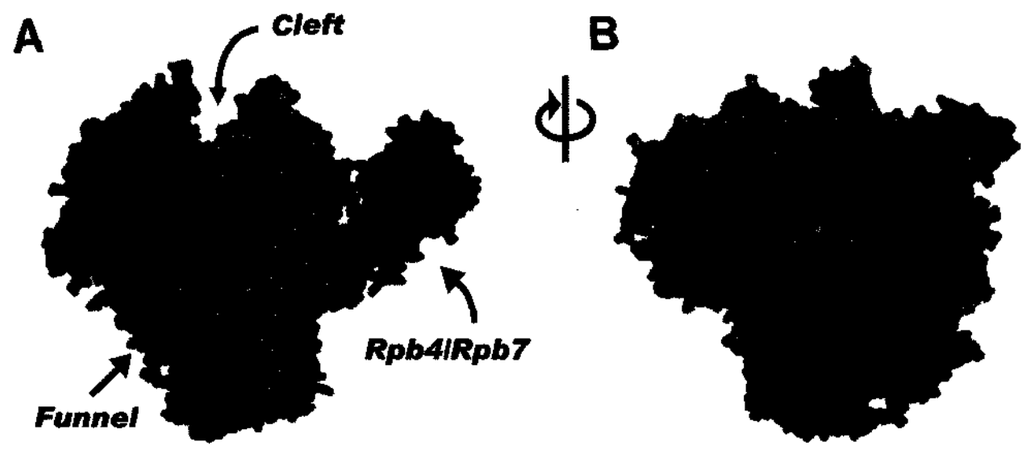

Fig. 1. Four modules of RNA polymerase II.
RNA polymerase II consists of four mobile modules of "clamp", "shelf", "jaw-lobe", and "core". The three-dimensional movie of Pol II is available at http://structure1.konkuk.ac.kr/interest.htm. **A**. Surface filling models of the core, jaw-lob, clamp, and shelf modules of RNA polymerase II shown in green, red, purple, and blue, respectively (PDB ID: 1WCM). The shelf module consists of parts of Rpb1, Rpb5, and Rpb6; the clamp module consists of parts of Rpb1 and Rpb2; the jaw-lobe module consists of parts of Rpb1, Rpb2, and Rpb9; the core module consists of parts of Rpb1, Rpb2, Rpb3, Rpb4, Rpb7, Rpb8, Rpb9, Rpb10, Rpb11, and Rpb12, which include all except the other three domains. **B**. The view in **A** rotated 90° about a vertical axis.

structures of TAP-tagged Pol II are proposed based on the crystal structure of yeast Pol II [2].

The Rpb1 subunit, 1,733 amino acids (190 kDa), is the longest peptide chain among the 12 different subunits and comprises parts of all four modules of Pol II. Rpb1 has a long extended C-terminal domain (CTD), of which the phosphorylation pattern changes during the transcription cycle, and coordinates events of nuclear mRNA biogenesis [24]. The yeast CTD contains 26 repeats of a heptapeptide sequence that is highly conserved in all eukaryotes. The phosphorylation of the second and fifth serine residues in the repeated heptapeptide sequence is precisely regulated by several site-specific kinases and phosphatases [33]. The CTD is known to be flexible rather than a rigid domain, and located just below the clamp domain close to the back side of Pol II. Its overall three-dimensional structure was not determined (Fig. 2A). The second largest subunit, Rpb2, constitutes mostly the "core" module and parts of the "jaw-lob" and "clamp" modules. Its C-terminal end exists at the left side in the lower middle of the clamp module of Pol II (Fig. 2B). Rpb3 (Fig. 2C) exists at the back side of Pol II in complex with Rpb10 (Fig. 2J), Rpb11 (Fig. 2K), and Rpb12 (Fig. 2L). The back side of Pol II is known to interact with a DNA promoter region in complex with TFIIB and TBP in the transcription initiation complex [3]. Rpb4 (Fig. 2D) and Rpb7 (Fig. 2G) exist as a heterodimer and are bound at the left back side of the ten subunits Pol II complex. The heterodimer of Rpb4 and Rpb7 functions like a wedge to hold the clamp module in close conformation to bind the downstream DNA tightly for transcription elongation, but it is not essential for transcription. Rpb5 and Rpb9 comprise the jaw domain. Rpb5 exists at the front-left side of Pol II, and its C-terminal is located at the crevice between the shelf and clamp modules. Owing to the limited space at the

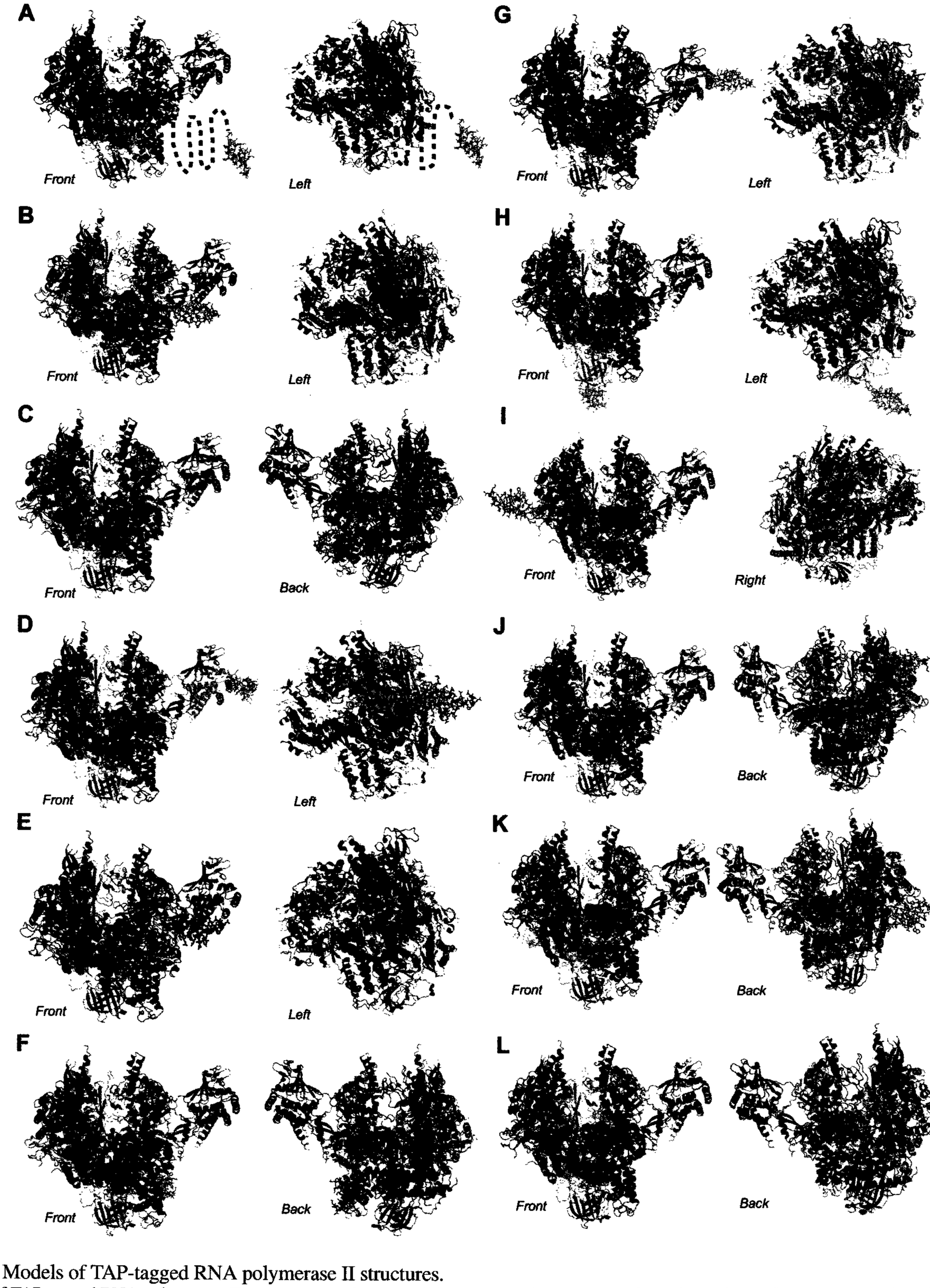

Fig. 2. Models of TAP-tagged RNA polymerase II structures.
Models of TAP-tagged RNA polymerase II structures are shown as ribbon presentations. Lower left captions of each figure represent the orientation of Pol II. Untagged subunits are shown in green; the TAP-tag molecule is shown in purple. The three-dimensional movie of each TAP-tagged Pol II is available at http://structure1.konkuk.ac.kr/interest.htm. **A**. Rpb1-TAP-tagged Pol II. Rpb1 is shown in yellow; unstructured CTD of Rpb1 is shown in dashed line. **B**. Rpb2-TAP-tagged Pol II. Rpb2 is shown in orange. **C**. Rpb3-TAP-tagged Pol II. Rpb3 is shown in red. **D**. Rpb4-TAP-tagged Pol II. Rpb4 is shown in cyan. **E**. Rpb5-TAP-tagged Pol II. Rpb5 is shown in silver. **F**. Rpb6-TAP-tagged Pol II. Rpb6 is shown in blue. **G**. Rpb7-TAP-tagged Pol II. Rpb7 is shown in black. **H**. Rpb8-TAP-tagged Pol II. Rpb8 is shown in grey. **I**. Rpb9-TAP-tagged Pol II. Rpb9 is shown in tv_blue. **J**. Rpb10-TAP-tagged Pol II. Rpb10 is shown in tv_orange. **K**. Rpb11-TAP-tagged Pol II. Rpb11 is shown in marine. **L**. Rpb12-TAP-tagged Pol II. Rpb12 is shown in tv_red.

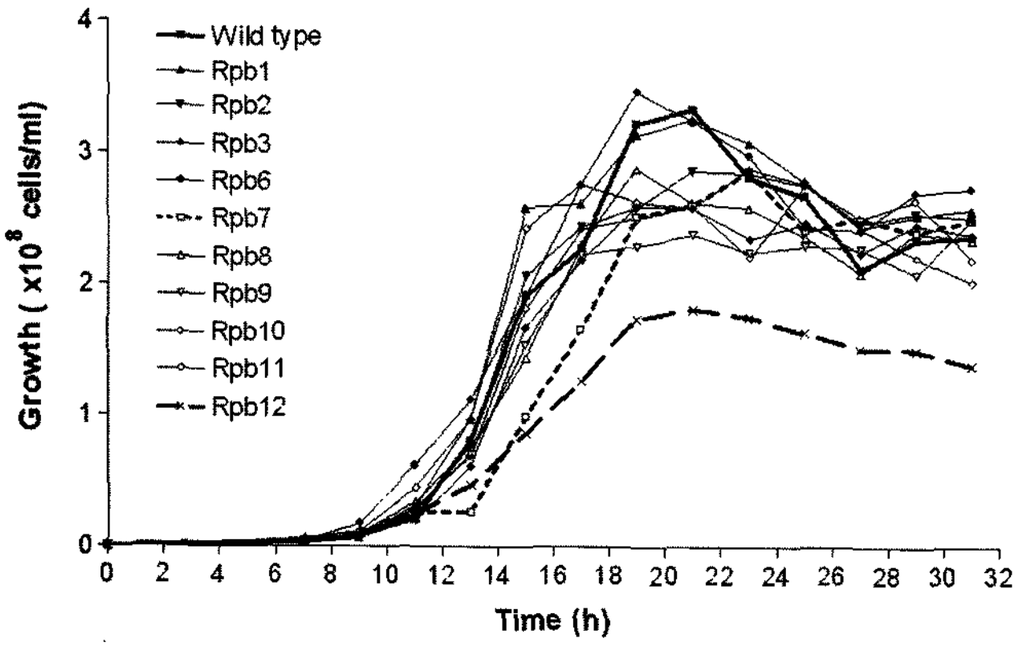

Fig. 3. Growth curve of ten different TAP-tagged yeast strains. The growth rate of each TAP-tagged yeast strain was determined in triplicate by counting cells in a hemocytometer. The legend at the left of the graph represents the type of drawn line of each strain. The growth curves of wild-type, Rpb7-TAP-tag, and Rpb12-TAP-tag strains are shown in thick lines. Rpb7 and Rpb12 TAP-tagged strains show growth defects.

C-terminal end of Rpb5, an attached tag can be positioned in the central cleft or at the left-side of the Pol II clamp module. The model of Rpb5-TAP-tagged Pol II is proposed based on the latter case (Fig. 2E). In either position, Rpb5-TAP-tag can influence the conformation change of the clamp module severely. Specifically, if the TAP-tag is located in the central cleft, the tag can completely interrupt the binding of double-strand DNA to the central cleft of Pol II. Rpb9 comprises the upper-jaw, which exists at the front-right side of Pol II (Fig. 2I). Rpb6 is located at the left side in the middle of Pol II (Fig. 2F). Rpb8 has a β-barrel structure and exists at the bottom of Pol II (Fig. 2H).

Growth Rate of Each TAP-tagged Yeast Strain

The growth rate of each yeast strain was measured to detect a possible abnormal cell cycle caused by the an attached TAP-tags on Pol II (Fig. 3). Wild-type strain showed the normal doubling time of about 90 min at the exponential phase and TAP-tagged strains of Rpb1, Rpb2, Rpb6, Rpb8, and Rpb10 showed similar growth rates as the wild type (Table 1). In the case of Rpb1, a TAP-tag is attached at the flexible CTD, which is located far away from the Pol II surface. Therefore, the position is speculated to not affect direct interactions between Pol II and transcription factors. As we expected, the Rpb1-TAP-tag strain showed almost no difference with the wild-type strain in growth rate and maximum cell density. The Rpb6-TAP-tag strain also showed similar growth statistics compared with the wild type. TAP-tagged strains of Rpb2, Rpb8, and Rpb10 showed slower growth rates, and the maximum cell density was also lower than that of wild type by about 12%. Among the 12 different subunits of Pol II, five subunits (Rpb6, Rpb5, Rpb8, Rpb10, and Rpb12) are common in all three RNA polymerases I,

Table 1. Doubling time and maximum cell density.

Yeast strain	Number of replications/2 h	Maximum cell density ($\times 10^8$ cells/ml)
WT	2.2	3.3
Rpb1-TAP	2.2	3.3
Rpb2-TAP	2.1	2.9
Rpb3-TAP	1.8	2.6
Rpb6-TAP	2.2	3.5
Rpb7-TAP	1.8	2.9
Rpb8-TAP	2.0	2.9
Rpb9-TAP	1.9	2.4
Rpb10-TAP	2.0	2.9
Rpb11-TAP	1.9	2.8
Rpb12-TAP	1.8	1.8

II, and III. Accordingly, the phenotypes of Rpb6-, Rpb5-, Rpb8-, Rpb10-, and Rpb12-TAP-tagged strains can originate from either ofthe three RNA polymerases or all of them. Specifically, Rpb12-TAP-tag showed severe defects in both growth rate and maximum cell density, being 18% and 45% lower than that of wild type, respectively. The Rpb5-TAP-tagged strain was not available from the 4,247 TAP-fusion library of "Open Biosystems", possibly due to a lethal mutation. The location of the C-terminal end of Rpb5 has a limited space, and the attached tag can cause an improper conformation change of clamp module, or prohibit double-strand DNA binding to Pol II directly.

The back side of Pol II is especially interesting because Pol II is known to interact with a mediator, which is a multiprotein complex that functions as a transcriptional coactivator [18], on the back side of Pol II [9]. The back side of Pol II consists of four different subunits of Rpb3, Rpb10, Rpb11, and Rpb12. Among the four subunits, Rpb3- and Rpb12-TAP-tagged strains showed defects in growth rate. In the three-dimensional structure of Pol II, the C-terminal ends of Rpb3 and Rpb12 are located at the left back side of Pol II and those of Rpb11 and Rpb12 are located at the right back side of Pol II. The defective growth in Rpb3- and Rpb12-TAP-tagged strains suggests that the left back side of Pol II has close contacts with transcription factors, including mediator, and it agrees with the current model structure of transcription initiation complex [3].

The Rpb7-TAP-tagged strain also showed severe defect in growth rate, and it is interesting because Rpb7 has previously no known function. Although binding of a heterodimer of Rpb4/Rpb7 to ten subunits Pol II can secure the close conformation of the clamp module during transcription elongation, the heterodimer is dispensable for transcription. Our data show that the Rpb7-TAP-tagged strain has severe defects in the cell cycle, and this supports that Rpb7 has interacting proteins and the disruption of interactions can lead to cell growth inhibition.

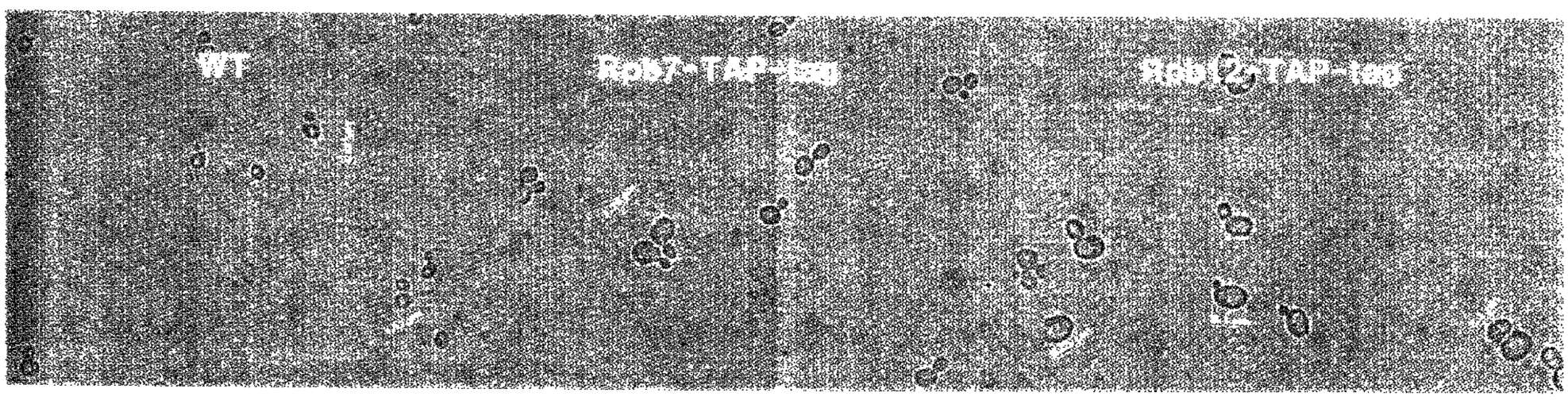

Fig. 4. Shapes of wild-type, Rpb7-TAP-tagged, and Rpb12-TAP-tagged yeast cells.
Red caption shows the measured size of yeast cells. Among ten different yeast strains, Rpb7 and Rpb12 TAP-tagged strains showed the most severe change of morphology.

Morphology Changes of Rpb7 TAP-tagged and Rpb12 TAP-tagged Strains

Among ten different yeast strains, Rpb7- and Rpb12-TAP-tagged strains showed abnormal morphology as well as defective growth rates (Fig. 4). The Rpb7-TAP-tagged strain showed 18% times slower growth rate than that of the wild type, and its cell shape was an elongation form rather than a sphere from. Its longest axial length is almost 1.6 times longer than the wild type. The nucleus is also bigger than the wild type. The Rpb12-TAP-tagged strain showed an 18% slower growth rate, and the maximum cell density at culture was about 50% lower than that of the wild type. The cell of the Rpb12-TAP-tagged strain is 80% bigger than the wild-type cell and its nucleus covers most of the inner cellular space.

We tried to use TAP-tagged yeast strains as a proteomic tool to study the function of PPI at a specific site. Based on the crystal structure of Pol II, we already know each TAP-tagged site in each strain, and the attached tag on Pol II can disrupt the interactions between Pol II and transcription factors. We confirmed the expression of TAP-tags by Western blots with IgG antibodies and measured the growth rate of each strain to identify defects in the cell cycle caused from interrupted interactions. As we expected, various differences in growth rate among different strains were observed, and especially the Rpb7- and Rpb12-TAP-tagged strains showed severe defects in growth rate as well as morphological changes. This supports that the attached TAP-tag interrupted the important protein interactions at the defined location. The causes of defects in cell cycle and morphology can be a single or multiple interfered interactions at the same site, and we are in the middle of characterizing the direct cause of the disrupted cell cycle by using other proteomic analysis tools such as real-time PCR. cDNA libraries were constructed from each strain, and the expression levels of transcription factors, already known to bind to the specific location of Pol II, will be studied (Table 2). In our study, we showed that the TAP-tagged yeast strains can be successful

Table 2. Rpb7 interacting proteins from the Yeast Proteomic Database.

Standard name	Systematic name	Description	Experimental system	References
AOS1	YPR180W	Heterodimer with *Uba2p*, activate *Smt3p*	Affinity capture-MS	[12]
SPT4	YGR063C	Mediates both activation and inhibition of transcription elongation	Affinity capture-MS	[5]
SPT5	YML010W	Mediates both activation and inhibition of transcription elongation	Affinity capture-MS	[23]
TFG1	YGR186W	Subunit of TFIIF	Affinity capture-MS	[5]
TFG2	YGR005C	Subunit of TFIIF	Affinity capture-Western	[11]
TAF14	YPL129W	TATA-binding protein-associated factor	Affinity capture-MS	[29]
NRD1	YNL251C	RNA-binding protein that interacts with CTD of Pol II	Two-hybrid	[25]
RSP5	YER125W	Ubiquitin-protein ligase	Affinity capture-Western	[20]
SET2	YJL168C	Histone methyltransferase	Affinity capture-MS	[30]
RGR1	YLR071C	Mediator subunit	Co-purification	[22]
IWR1	YDL115C	Unknown function	Affinity capture-MS	[19]
SAT4	YCR008W	Ser/Thr protein kinase	Biochemical activity	[27]
SRB4	YER022W	Mediator subunit	Co-purification	[31]
BUR6	YER159C	Subunit of a heterodimeric NC2 transcription regulator complex with *Ncb2p*	Synthetic rescue	[26]

proteomic tools to study PPIs and the functional role of the interactions at the cell level. The eukaryotic transcription machinery is one of the best candidates to apply this method. Pol II is a big molecule composed of 12 different subunits and interacts with hundreds of transcription factors to transcribe even a single gene. The C-terminal ends of the 12 subunits are already TAP-tagged in different yeast strains. This novel proteomic tool can be applied to other big complexes such as a mediator, chromatin remodeling complex (RSC), ribosome, proteasome, exosome, and kinetochore [19]. This method is easy to use with commercially available yeast TAP-fusion libraries, and can be extremely powerful to examine the function of protein interactions at a specific location if combined with the three-dimensional structure of the target protein complex. However, PPI is not limited only in the C-terminal region of an existing subunit in complexes, and only limited numbers among total PPIs on the target complex can be studied by this method.

Acknowledgments

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