Interactions between the full complement of human RNA polymerase II subunits

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Abstract **As an approach to elucidating the rules governing the assembly of human RNA polymerase II (hRPB), interactions between its subunits have been systematically analyzed. Eleven of the 12 expected hRPB subunits have previously been tested for reciprocal interactions (J. Biol. Chem. 272 (1997) 16815–16821). We now report the results obtained for the last subunit (hRPB4; Mol. Cell. Biol. 18 (1998) 1935–1945) and propose an essentially complete picture of the potential interactions occurring within hRPB. Finally, complementation experiments in yeast indicated that hRPB4 expression efficiently cured both heat and cold-sensitivity of RPB4-lacking strains, supporting the existence of conserved functional subunit interactions.**

Key words: RNA polymerase II; Subunit interactions; Complementation in yeast; *Homo sapiens*

1. Introduction

Eukaryotic RNA polymerases comprise between 10 and 17 polypeptides ranging from 220 to 7 kDa, depending on their origin [1–3]. Electron microscopy image processing and electron crystallography have been applied to yeast RNA polymerases I and II (RPB) [4–7]. These enzymes were described as fist-shaped molecules with a channel that could accommodate double-stranded DNA. In addition, functional domains have been assigned to specific subunits, based on sequence comparisons or on biochemical studies with purified polymerases or subunits [1,8–15]. In other studies, interspecies gene replacement experiments in yeast have shown that specific subunits may be functionally replaced by their human counterparts [16–19]. Such substitutions emphasize the role of conserved sequence elements and indicate that the polymerases can accommodate variations in the primary structure of their subunits. We have further investigated the ability of human RPB4 (hRBP4) to compensate for the yeast *RPB4* deletion [19].

Reconstitution of functional enzymes with recombinant subunits should ultimately establish the subunit stoichiometry and assembly pattern of the RNA polymerases and will considerably facilitate their structure-function analysis in vitro. As a preliminary step towards the reconstitution of the hRPB, the potential of each individual subunit to interact with any of the other hRPB subunits has been investigated. In an earlier study [20], the interaction network between 11 of the 12 hRPB subunits was established. Using the same glutathione-S-transferase (GST)-pulldown assay, we now analyze the interactions between the 12th subunit (hRPB4 [19]) and each of the others. A complete interaction map is proposed.

2. Materials and methods

2.1. Cloning

A cDNA fragment containing the complete coding sequence of hRPB4 was mutated to insert unique restriction sites *Nhe*I and *Spe*I, respectively, in front of the ATG and after the stop codon. This *Nhe*I-*Spe*I fragment was then inserted into the compatible *Xba*I site of the pVL1393 baculovirus transfer vector (PharMingen) and the compatible *Nhe*I site of the pVL-GST vector (see [20]). The resulting transfer vectors were recombined with linearized baculovirus DNA in Sf9 cells, according to instructions of the supplier (BaculoGold DNA, PharMingen). Two recombinant baculoviruses were recovered, directing the overexpression of either the free hRPB4 protein or the N-terminally fused GST-hRPB4 chimeric protein. These viruses were plaque-purified and the expression of the corresponding proteins was verified by Western blot analysis [19]. The other recombinant baculoviruses used in this study were described in [20].

The *Nhe*I-*Spe*I hRPB4 cDNA fragment was also inserted in the unique *Avr*II site of the pGEN yeast expression vector [16], yielding the pGENh4 recombinant plasmid.

2.2. Baculovirus infection and radiolabelling

Sf9 cells were grown in TNM-FH medium. Cells were infected with recombinant baculoviruses and, at 44 h post-infection, expressed proteins were metabolically labelled for 4 h with a mixture of ^{35}S-labelled Met and Cys, as previously described [20].

2.3. GST-pulldown assay

After co-infection of Sf9 cells with recombinant viruses expressing both the unfused and GST-fused subunits, extracts were prepared in a typical 200 μl volume. Twenty μl of each lysate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 180 μl was incubated with glutathione (GSH)-Sepharose beads, as previously described [20]. After 2 h at 4°C, the beads were washed with PBS buffer containing 0.45 M (hRPB9), 0.65 M (hRPB1, 2, 3, 4, 6, 7, 8, 10α, 10β and 11) or 1.15 M (hRPB5) NaCl and 0.1% (hRPB9) or 1% Nonidet P-40 (all the other subunits) to minimize non-specific interactions. Retained proteins were analyzed by SDS-PAGE and visualized by autoradiography.

2.4. Yeast strains and genetic techniques

Yeast strains were constructed by standard genetic techniques and grown at 12, 30 or 37°C on YPD plates [21]. The following haploid *Saccharomyces cerevisiae* strains were used: SUB62 [22] containing the

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Abbreviations: RPB, RNA polymerase II; GST, glutathione-S-transferase

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wild-type *RPB4* (denoted RPB4) and MC11-1 [22] lacking this subunit (denoted rpb4$^-$). pGENh4 was introduced into MC11-1 and stable transformants bearing the plasmid (denoted rpb4$^-$+pGENh4) were recovered.

3. Results

3.1. hRPB4 is able to interact with several other hRPB subunits

To investigate the interactions between subunits of the RPB complex, the baculovirus expression system was used. This system allowed for overexpression of each of the subunits in a soluble form, whether fused to GST or not. In the case of hRPB1 and hRPB2, the two untagged subunits were co-expressed from the same recombinant baculovirus [20].

In a first series of experiments, each of the untagged subunits was assayed for its ability to interact with the GST-hRPB4 fusion, as revealed by co-retention on GSH-agarose beads (compare patterns of labelled proteins before and after GST-pulldown, in the upper and lower parts of each panel in Fig. 1). Only those experiments showing a significant interaction with GST-hRPB4 are presented. In each case, the results were compared to those of other fusion proteins previously shown to retain (positive control) or not (negative control) the untagged subunit [20]. As a second negative control, interaction with the GST moiety alone was also tested (Fig. 1). We found that both hRPB1 and 2 (Fig. 1A), hRPB3 (Fig. 1B), hRPB5 (Fig. 1C), hRPB7 (Fig. 1D), hRPB8 (Fig. 1E) and hRPB10β (Fig. 1F) were capable of interacting with GST-hRPB4. The results from these experiments and others (not shown) are summarized in Fig. 3 (upper panel).

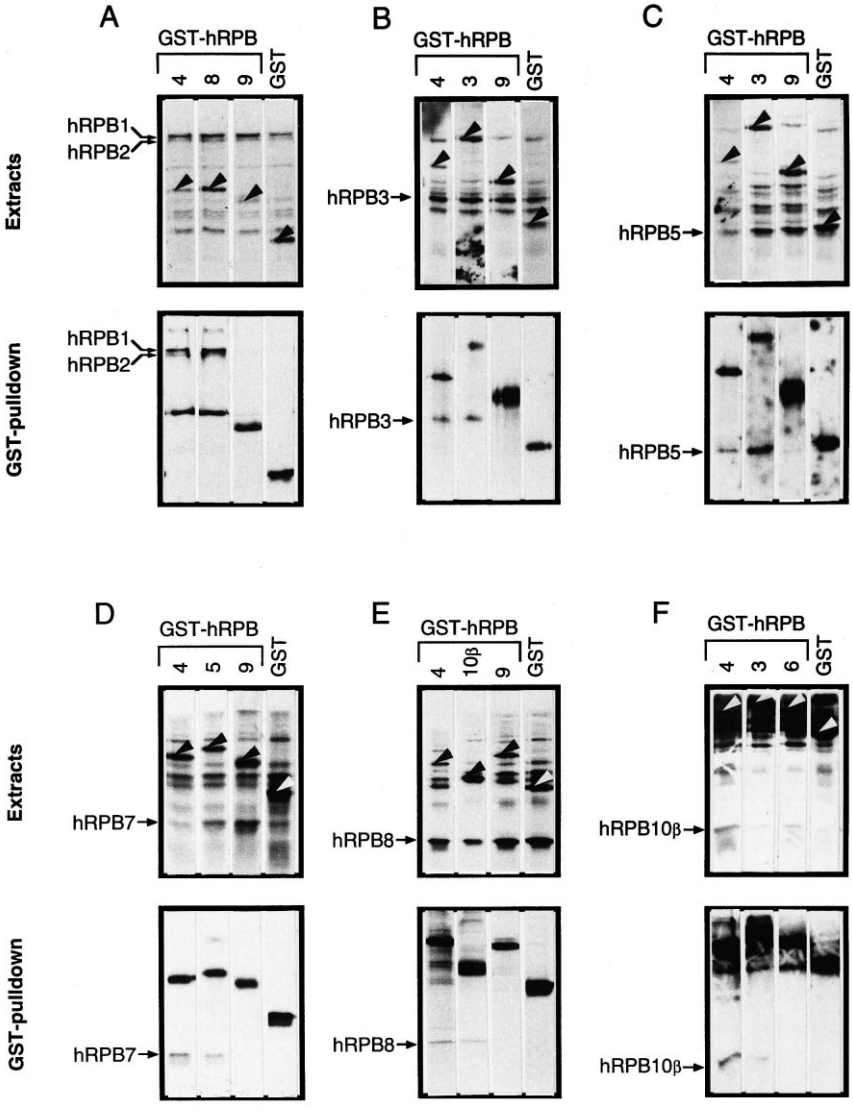

Fig. 1. Interactions between GST-hRPB4 and other RPB subunits. Sf9 cells were co-infected with two recombinant baculoviruses, one directing the expression of either a GST-fused subunit or the GST moiety alone and the other directing the expression of a non-fused subunit. In the case of hRPB1 and hRPB2, both unfused subunits were co-expressed (see text). After cell labelling, extracts were prepared and GST-pulldown assays were performed as described in Section 2. Aliquots of the total extracts (Extracts, arrowheads point to the bands corresponding to the GST-hRPB or GST polypeptides, arrows point to the positions of the non-tagged hRPB subunits) and of the GST-bound fractions (GST-pulldown, arrows point to the positions of the co-retained, non-tagged hRPB subunits) were analyzed by SDS-PAGE. In each set of experiments (A–F), the unfused subunit(s) was assayed for interaction with GST-hRPB4, in parallel with one positive and two negative control reactions (see Section 3.1).

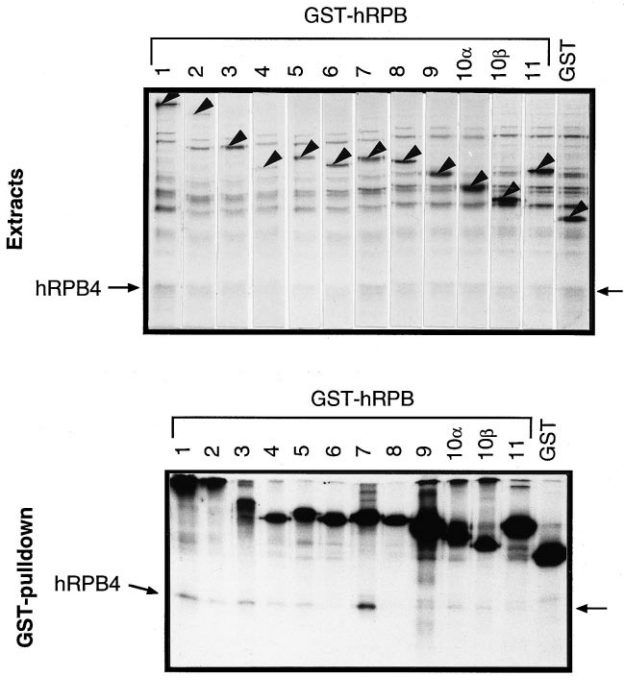

Fig. 2. Interactions between hRPB4 and the 12 GST-hRPB subunits. Sf9 cells were co-infected with two recombinant baculoviruses, one directing the expression of one of the 12 GST-fused subunits or GST alone and the other expressing the untagged hRPB4 subunit. After cell labelling, extracts were prepared and GST-pulldown assays were performed. Aliquots of the total extracts (Extracts) and of the GST-bound fractions (GST-pulldown) were analyzed by SDS-PAGE. Arrowheads were as in Fig. 1, arrows point to the position of the non-tagged hRPB4 subunit. Note the presence of a non-specific band that is retained in the GST-pulldown assay and migrates slightly slower than hRPB4.

In a second set of experiments, the ability of the untagged hRPB4 polypeptide to interact with each of the GST-fused subunits was examined (Fig. 2). As revealed in the upper panel, the non-fused hRPB4 subunit was relatively poorly expressed. After GST-pulldown, the bound material was analyzed (Fig. 2, lower panel). The hRPB4 subunit was most efficiently retained on the GST-hRPB7 fusion. A slightly weaker hRPB4 signal was observed with GST-hRPB1 and 3. An equally strong signal was detected in association with GST-hRPB2, if corrected for the reduced level of GST-hRPB2 expression. Finally, a weak interaction was revealed with the GST-hRPB4, 5, 10α and 10β subunits. No detectable hRPB4 signal was found with GST-RPB6, 8 and 11. In the case of GST-hRPB9 and 11, the band at the expected position of hRPB4 was not reproducibly observed and was very weak considering the amount of GST fusion protein. Using specific anti-RPB4 antibodies, we confirmed that these signals did not correspond to hRPB4 (data not shown). We conclude that no interaction occurs between subunits hRPB4 and GST-RPB6, 8, 9 and 11 (Fig. 3, lower panel). The results obtained from the two sets of experiments are summarized in Figs. 3 and 4.

3.2. *hRPB4 efficiently replaces its homolog in yeast*

Complementation assays in yeast have been used to establish structural and functional conservation of RNA polymerase subunits [16–19]. Deletion of *RPB4* in *S. cerevisiae* results in a characteristic heat and cold-sensitivity [23,24]. It has previously been reported that GAL1 promoter driven hRPB4 expression is able to partially complement growth at 34°C of a yeast WY-4 *rpb4* null mutant [19]. We confirmed this observation by showing that hRPB4 expressed under the control of a PGK promoter (rpb4$^-$+pGENh4) gives rise to an even better complementation, supporting growth at 37°C on a complete medium. The growth efficiency of the complemented strain was however lower than that of wild-type (Fig. 5A) or MC11-1 strains complemented with the recently discovered *Schizosaccharomyces pombe* Rpb4 (G.V.S. and G.M. Baranova, unpublished data). In addition, our results indicate that interspecific complementation also occurred at a low temperature: expression from the pGENh4 vector clearly restored growth at 12°C (Fig. 5B). Together, these results show that hRPB4 can functionally replace its yeast counterpart in vivo, indicating that the human subunit is capable of establishing the essential contacts with the yeast RPB subunits required for proper polymerase activity.

4. Discussion

Various interaction assays, such as the two-hybrid system, were tried using complete hRPB subunit cDNAs, but very few contacts were detected. This is consistent with the data recently obtained in yeast that show that partial peptides interact when the complete protein did not [15]. We have therefore investigated the interaction potential of hRPB4 with each of the subunits of hRPB using a GST-pulldown assay in recombinant baculovirus-infected Sf9 cells. Very similar patterns were found, regardless of which subunit was GST-tagged (Fig. 3), further strengthening the relevance of the observed interactions. The present results, together with previously determined interactions [20], have been integrated into

hRPB	[1	2]	3	4	5	6	7	8	9	10α	10β	11
GST-hRPB4	++		++	+	+	-	+++	+	-	-	++	-

GST-hRPB	1	2	3	4	5	6	7	8	9	10α	10β	11
hRPB4	++	++	++	+	+	-	+++	-	-	+	+	-

Fig. 3. Summary of the interactions between hRPB4 and the 12 RPB subunits. The intensities of the interactions were deduced from experiments including and similar to those shown in Figs. 1 and 2. Based on comparisons with both positive and negative controls, the interactions were qualified as 'strong' (+++), 'significant' (++) or 'weak' (+). The untagged hRPB1 and 2 subunits are in brackets to indicate that they were co-expressed.

an overview of the potential interaction network (Fig. 4). In agreement with previous findings [19], hRPB4 establishes strong contacts with hRPB7, in keeping with the ability of their yeast homologs to form an independent subcomplex [25–27]. A mutation altering the largest subunit (RpoB1 [26]) was shown to facilitate the release of the RPB4-RPB7 heterodimer from the RPB, most likely by affecting either the RPB1-RPB4 or RPB1-RPB7 interactions or both. As summarized in Fig. 4, both hRPB4 and 7 exhibit a number of additional, though weaker, contacts with other hRPB subunits. This correlates well with the central localization inferred from the three-dimensional structure analysis of yeast polymerase [28]. No pairwise interaction with hRPB9 has been detected, although we have obtained higher order complexes that include this subunit (unpublished results). This suggests that this subunit interacts with more than a single subunit.

Complementation assays in yeast ([19] and present work) have shown that growth defects resulting from the absence of RPB4 can be readily corrected by overexpression of hRPB4. The reason for the slight difference in complementation efficiency at 37°C in the two sets of experiments is not clear, but could be attributed to the distinct genetic background of the two yeast strains. From the above-mentioned observations, it is likely that at least part of this complementation implies interactions between hRPB4 and yeast RPB7. However, such interactions were found to be much weaker than the natural RPB4/7 interactions in yeast [19]. In addition, the RPB4 subunit is absolutely required for RPB activity both in vivo and in vitro at an elevated temperature [24,29]. Interactions of hRPB4 with other subunits, such as those identified in the present work, may therefore contribute to the efficient phenotype rescue.

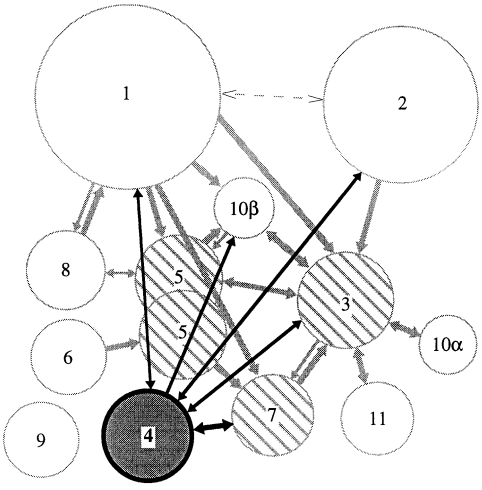

Fig. 4. Major pairwise contacts between the 12 known hRPB subunits. The hRPB is schematically represented with subunits to approximate scale. Contacts that were identified by the binary GST-pulldown assay (Figs. 1–3 and results in [20]) are represented by arrows pointing towards the non-tagged subunit retained in this assay. Only the most prominent interactions have been outlined: unilateral and reciprocal interactions are represented by single and double-headed arrows, respectively. Contact strength is depicted by the thickness of the arrows. The dashed double-headed arrow between the two largest subunits is speculative. Hatched subunits exhibit a significant dimerization ability (see [20]) and only hRPB5 is represented in two copies, in agreement with its established stoichiometry. The interactions concerning hRPB4 are highlighted in dark.

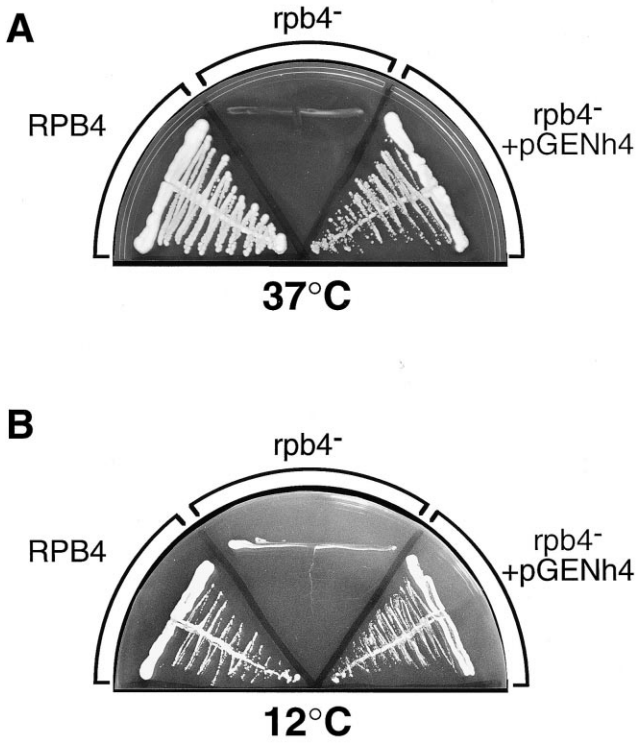

Fig. 5. Interspecific complementation of both heat and cold-sensitivity of rpb4$^-$ yeast strain by hRPB4. Cultures were grown (A) at a high (3 days at 37°C) or (B) a low temperature (10 days at 12°C). The following yeast strains were streaked: SUB62, which harbours the wild-type *RPB4* gene (RPB4), MC11-1, which lacks a functional *RPB4* gene (rpb4$^-$), and transformed MC11-1/pGENh4 (rpb4$^-$+pGENh4).

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