Distinct regions of RPB11 are required for heterodimerization with RPB3 in human and yeast RNA polymerase II

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ABSTRACT

In *Saccharomyces cerevisiae*, RNA polymerase II assembly is probably initiated by the formation of the RPB3–RPB11 heterodimer. RPB3 is encoded by a single copy gene in the yeast, mouse and human genomes. The *RPB11* gene is also unique in yeast and mouse, but in humans a gene family has been identified that potentially encodes several RPB11 proteins differing mainly in their C-terminal regions. We compared the abilities of both yeast and human proteins to heterodimerize. We show that the yeast RPB3/RPB11 heterodimer critically depends on the presence of the C-terminal region of RPB11. In contrast, the human heterodimer tolerates significant changes in RPB11 C-terminus, allowing two human RPB11 variants to heterodimerize with the same efficiency with RPB3. In keeping with this observation, the interactions between the conserved N-terminal 'α-motifs' is much more important for heterodimerization of the human subunits than for those in yeast. These data indicate that the heterodimerization interfaces have been modified during the course of evolution to allow a recent diversification of the human RPB11 subunits that remains compatible with heterodimerization with RPB3.

INTRODUCTION

The assembly of the RNA polymerase II (RNAP II) multiprotein complex is still poorly understood. It has been established in the eubacterial model that its initial step is the formation of a homodimer of the alpha subunit (1). The eukaryotic counterpart of this homodimer appears to be a heterodimer that comprises, in the case of *Saccharomyces cerevisiae*, the subunits encoded by the *RPB3* and *RPB11* genes, henceforth referred to as Sc3 and Sc11 subunits, respectively (2,3).

In the yeast genome all RNAP II subunits, including Sc3 and Sc11, are encoded by unique genes. While the mouse genome also contains single *RPB3* and *RPB11* genes, the situation is different in the human genome: in contrast to the *RPB3* gene (encoding subunit Hs3) which is unique, a family of *RPB11* genes was identified [(4), Table 1]. In the present study we shall consider only two members of this gene family, *RPB11a* and *RPB11b*. The *RPB11a* gene is the predominantly transcribed gene in all human cells and tissues tested so far. Its unique transcript encodes the Hs11a protein that is found in RNAP II from all cells analysed to date. The *RPB11b* gene yields several mRNAs resulting from alternative splicing processes, which can be detected ubiquitously in human cells as well. Whether these mRNAs are translated at all *in vivo* remains to be established (4). In this study, we shall consider only the Hs11bα isoform, although other isoforms have been described (see Table 1 and Discussion).

The structure of the yeast RNAP II has been solved at atomic resolution (2,5), and is widely considered as a representative model for all eukaryotic RNAP IIs, including the human RNAP II. This assumption is supported not only by sequence homologies but also by the observation that a number of human RNAP II subunits are indeed able to functionally replace their yeast counterparts (6,7). Nevertheless, the alpha-like subunits Hs3 and Hs11a constitute a notable exception, since they cannot substitute for their yeast homologs (4).

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Table 1. The human POLR2J gene family

Gene	Genbank accession No	Chromosomal location	cDNA	Genbank accession No
hRPB11a	NC_000007 [101 679 870–101 674 430]	7q22	hRPB11a	AA937330, X98433, X82385
hRPB11b	NC_000007 [101 872 683–101 841 531]	7q22	hRPB11bα	CR596358, CR614811, CR606303, AX405711, AJ277739
			hRPB11bβ	AJ277740, R85011
hRPB11c	NC_000007 [101 773 636–101 742 353]	7q22	hRPB11cα	AJ277741
			hRPB11cβ	AA306683
			hRPB11cγ	AF468111
hRPB11d	NC_000007 [43 799 592–43 768 503]	7p13	hRPB11dγ	BC017250, AL526460, AL554541

In an attempt to explain this lack of complementation, we performed a detailed genetic analysis of the RPB3/RPB11 heterodimerization process both in human and yeast systems. Among the various methods available, we chose the two-hybrid assay in *S.cerevisiae*, which should be optimal for assaying Sc3 and Sc11 yeast proteins. The main limitation of this method is that fusion proteins have to be used instead of the endogenous subunits. It may be stressed, however, that the yeast fusion proteins, LexA-Sc11 and VP16-Sc3, were found to be functional *in vivo* since they were able to restore viability to yeast deleted for these essential genes (strains YGVS-072 and D138-1d, respectively, see Supplementary Table 2), after chase of the balancer plasmid (data not shown). Hence, we inferred that these proteins must be able to interact in a way closely mimicking the endogenous subunits. We show here that this ability to interact extends to homologous human subunits. Our analysis, however, is based on the comparison of effects of similar mutations affecting the products of orthologous genes which are valid only if using the same interaction assay. When analysing the results, one should keep in mind that the levels of induced β-galactosidase activity represent an indirect measurement of the efficiency of the heterodimer formation *in vivo*, which cannot be formally correlated with an affinity constant.

Our results provide the first direct evidence that this initial step in the RNAP II assembly process has significantly diverged, allowing the human RPB11 C-terminal domain to be diversified without affecting the stability of the complex, while the integrity of its yeast counterpart appears to be essential. The new alleles of *RPB11* genes present in the human genome, therefore, encode proteins that potentially exhibit human-specific functions within the RNAP II complex.

MATERIALS AND METHODS

Plasmid construction and mutagenesis

Derivatives were obtained using standard molecular cloning procedures from four basic plasmid constructs (see Supplementary Table 2).

The pLex vector was modified from the 2 μm origin-containing pBTM116 yeast vector, that allows the expression of LexA-fused proteins (8), by the insertion of unique XhoI and BamHI restriction sites after the unique EcoRI cloning site.

The pVP vector was modified from pVP16, a 2 μm origin-containing yeast vector allowing the expression of VP16-fused proteins (8), by the insertion of NheI, SpeI, AvrII and XbaI restriction sites after the unique BamHI cloning site.

The pGEN vector, a 2 μm origin-containing plasmid exhibiting an expression cassette driven by a PGK promoter (6), was used to insert the coding sequence of interest into a unique NheI restriction site.

The pCM vector, which exhibits a centromeric replication origin and an ADH1 promoter-driven expression cassette, was used to insert the sequences of interest between unique XhoI and BamHI restriction sites [pCM185, (9)].

Each wild-type RNAP II subunit coding sequence was modified by *Taq* polymerase-mediated amplification using appropriate primers, so as to be cloned either into the NheI site of pVP or pGEN, or between the XhoI and BamHI sites of pLex or pCM (see Supplementary Table 2).

Deletion mutants were similarly obtained by *Taq* polymerase-mediated amplification, using the appropriate primers (see Supplementary Table 2).

Site directed mutagenesis of the various coding sequences was performed by the *Pfu*-mediated amplification of plasmids, using mispaired oligonucleotide primers (Stratagen).

Random mutagenesis was performed by treating pVP Sc3 and pVP Hs3 plasmids with 40 mM hydroxylamin at 37°C overnight, before transforming *Escherichia coli* (DH5α strain). The resulting libraries consisted of ∼100 000 independent clones. Individual clones were obtained after screening in yeast, and their sequences were determined after recovery by electroporation of the yeast extracts into the DH5α strain of *E.coli*.

Two hybrids assay

The L40 yeast strain [*ade2, trp1-901, leu2-3,112, his3Δ200, LYS2::(LexAop)$_4$HIS3, URA3::(LexAop)$_8$lacZ*, (8)] was transformed by electroporation in 1 M sorbitol, 50 mM HEPES, pH 7.5, at 1.6 kV, (∼4.5 s). The resulting transformants were selected on YNB minimal medium supplemented with adenine (40 μg/ml), histidine (20 μg/ml), and either leucine (60 μg/ml) or tryptophan (40 μg/ml), depending on the vector used. Independent colonies were isolated for further analysis.

The final yeast transformants were analysed for the induced β-galactosidase activity. A qualitative assay was performed using X-gal conversion as described (10). The activities were quantified after inoculation of the transformants into 15 ml YNB HA liquid medium: the cells were harvested at an OD$_{600}$ ranging from 0.6 to 1.0, resuspended in 300 μl of Z buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, pH 7.0) and broken using glass beads; the extracts were clarified by centrifugation and their ONPG cleavage activity was determined at 20°C in buffer Z, in the presence of 4 mg/ml ONPG. The activities, corrected for

protein concentrations of each sample, were expressed as $mOD_{420}.\mu g^{-1}.min^{-1}$.

Complementation assay

The YGVS072 yeast strain is deleted for the essential *RPB11* gene (see Supplementary Table 2), this deletion being compensated by the presence of a plasmid containing the RPB11 gene and a URA3 marker. Both pGEN and pCM derivatives (see Supplementary Table 2) were independently transformed in the YGVS072 strain, using a tryptophan auxotrophy selection. After transformation, the balancer plasmid was chased in the presence of 5FOA. Yeast cells, when viable, were further analysed for their growth rates and thermo- or cryo-sensitive phenotypes.

RESULTS

Sequence conservation

Comparison of the peptide sequences of Sc11 and Hs11a revealed a 45% identity between the two proteins (Figure 1A).

The RPB11 encoded subunits are, therefore, moderately conserved, since this score ranges from 31 to 73% when considering the other RNAP II subunits. The alignment of the alpha-like subunits of various RNA polymerases shows a conserved peptidic motif, the 'α-motif', boxed in Figure 1A. This motif happens to overlap with an alpha helix within the (3D) structure established for the yeast RNAP II (Figure 1C), as indicated above the sequence (Figure 1A). The human isoform Hs11bα differs from the main Hs11a isoform by the exon 4 encoded peptide, as shown by the coloured residues in Figure 1A. In addition, the Hs11bα protein is lacking Lys17 when compared to Hs11a (Figure 1A).

The comparison between the Sc3 protein and its Hs3 homolog yielded a 42% identity score (Figure 1B). The RPB3-encoded subunits are, therefore, also moderately conserved. The first N-terminal alpha helix of this polypeptide was also noticed to exhibit some homology with the eubacterial alpha subunit (data not shown). While Sc3 and Hs3 are homologous until residue Q268 in Hs3, Hs3 lacks a C-terminal peptide present in Sc3 (268–318). The 3D structure of this peptide could not be resolved (Figure 1C) (2,5).
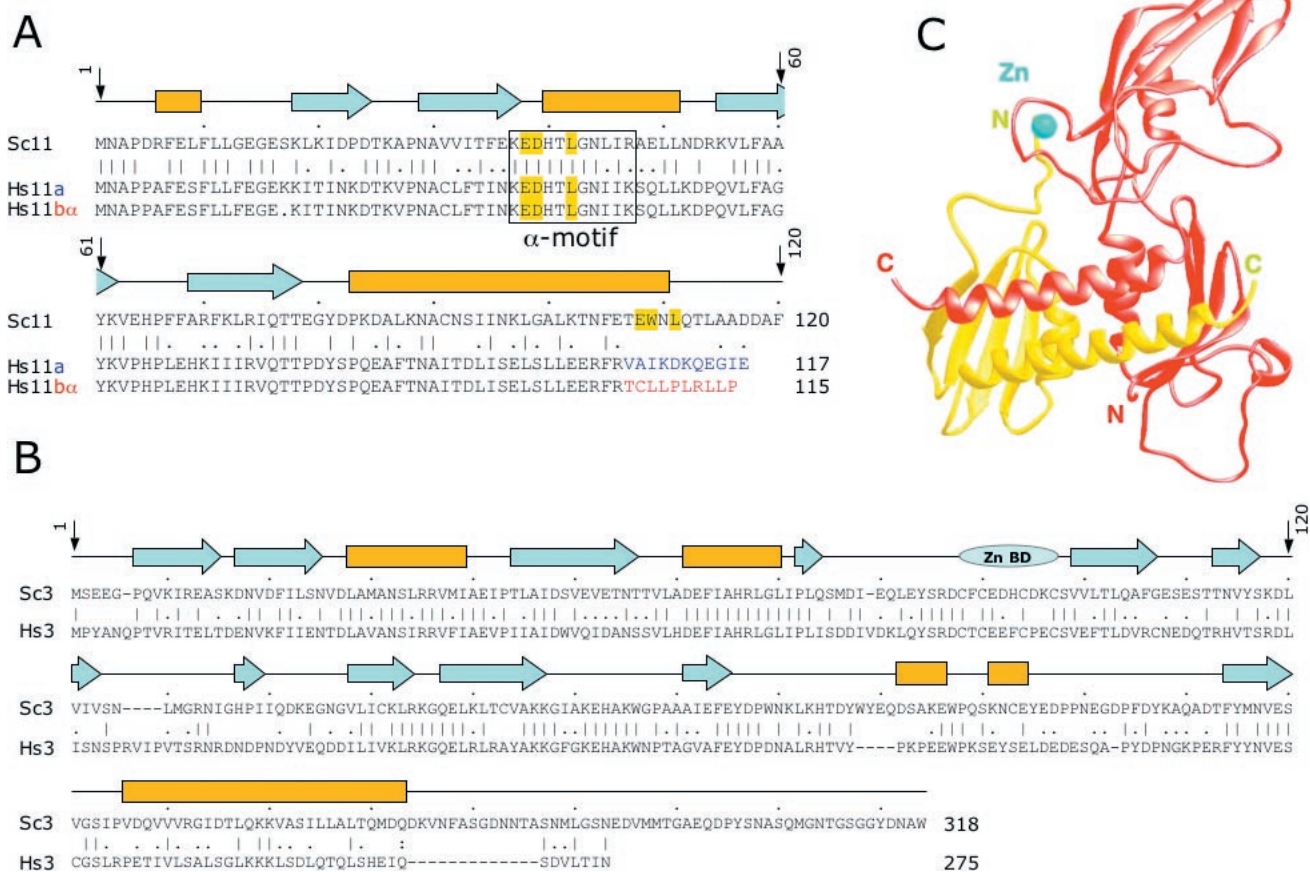

Figure 1. Structure of yeast and human RPB11 and RPB3 subunits. (**A**) Alignment of the peptidic sequences of Sc11, Hs11a and Hs11bα. The homologies between the yeast and human polypeptides are shown, vertical bars and dots indicating the identical and the similar residues, respectively. Conserved element (α-motif) initially noticed by comparing the alpha subunits of procaryotes is boxed (18,19). The residues highlighted in yellow were subjected to site directed mutagenesis (see text). The secondary structure elements previously described [1ENO PDB structure, (5), see (C)] are schematically shown above the Sc11 sequence with the alpha helixes and the beta sheets represented as yellow boxes and blue arrows, respectively. (**B**) Alignment of the peptidic sequences of Sc3 and Hs3. The main features are pointed as in panel 1A. Similarly are the structure elements shown above the Sc3 sequence. The position of a zinc binding domain (Zn BD) is shown. (**C**) 3D view of the yeast Sc3/Sc11 heterodimer (5). The red and yellow ribbons represent the Sc3 and Sc11 polypeptides, respectively, with their corresponding the N- and C-terminal ends positioned. The Zn atom (in blue) coordinated by four Cys residues of the Zn BD of Sc3 is also shown.

The 3D structure of the yeast Sc3/Sc11 heterodimer, as resolved within the 10-subunit complex, suggests that the two proteins mainly heterodimerize via their two N-terminal alpha helices, as well as their respective C-terminal alpha helices (Figure 1C). The structure of the human heterodimer has not been resolved so far.

Interaction analysis

Hs3 and Sc11 do not interact. Sc11/Sc3, Hs11a/Hs3 and Hs11bα/Hs3 heterodimers were readily detectable using the two-hybrid system (Figure 2, lines 1, 12 and 20). The β-galactosidase activities measured in yeast extracts expressing these combinations were very close, suggesting that the three heterodimers exhibit similar stabilities within these cells. Indeed, the overexpressed fused proteins were detectable at similar levels as judged by western blot (data not shown).

By contrast, while formation of both Hs11a/Sc3 and Hs11bα/Sc3 heterodimers was readily detectable (Figure 2, lines 9 and 17), the association of Sc11 and Hs3 could not be revealed (line 4), as there is no production of any detectable β-galacosidase activity in the cells. Since both Sc11 and Hs3 are able to interact with other proteins, this can be explained only if these proteins do not interact.

These results account for the lack of Sc3 complementation by Hs3 in yeast, but do not explain the lack of Sc11 complementation by Hs11a protein [(4), and data not shown].

Two point mutants of Sc3 were isolated from a mutant library which dramatically affect both Hs11a/Sc3 and Hs11bα/Sc3 heterodimer formation (Figure 2, lines 10, 11, 18 and 19). In sharp contrast, those mutations do not affect Sc11/Sc3 heterodimer formation (Figure 2, lines 2 and 3). One mutant (L33F Q267Stop) demonstrates that the C-terminal end of Sc3 is not required for Sc11/Sc3 interaction (Figure 2, line 3). The two substitutions present in the other mutant (G130E E208K) map are quite far from the interaction surface, in agreement with the lack of effect on the Sc11/Sc3 heterodimer formation (Figure 2, line 2). However, since Hs11bα protein was shown to be able to functionally replace Sc11

in vivo (4), this indicates that the same residues of Sc3 may not be identically involved in the interactions between this subunit and the orthologous Sc11 and Hs11bα proteins.

Similarly, two point mutants of Hs3 were isolated that affect the Hs3/Hs11 heterodimerisation to various extents. The A40V substitution revealed the importance of the region corresponding to the first alpha helix, although it seemed to affect less the Hs11a/Hs3 interaction than the Hs11bα/Hs3 one (Figure 2, compare lines 13 and 21), while other substitutions such as E91K have little effect (Figure 2, lines 14 and 22). Deletion of the Hs3 C-terminal helix (Q217Stop) abolished the interaction in both cases (Figure 2, lines 15 and 23). This observation is compatible with the 3D model established for the yeast RNAP II, pointing towards the major interaction surface between the Sc3 and Sc11 C-terminal helices (Figure 1C).

The role of the C-terminal domain of the *RPB11*-encoded subunits

In order to elucidate the contributions of the various structural elements, we undertook a systematic deletion analysis of *RPB11*-encoded subunits as depicted in Figure 3. Hs11a and Hs11bα coding sequences were deleted for the residues corresponding to exon 4, thus retaining residues 1–106 and 1–105, respectively. Sc11 was deleted similarly (1–106). The complete C-terminal helix was further deleted in mutants, retaining residues 1–79, 1–79, 1–78 of Sc11, Hs11a and Hs11bα, respectively. In addition, each C-terminal helix was assayed on its own (80–120, 80–117, 79–115, for Sc11, Hs11a and Hs11bα, respectively). Finally, chimeric coding sequences were assayed, in which the very C-terminal residues (from 107 to the end) were interchanged between human and yeast proteins as depicted (Figure 3, bottom).

Deletion of the complete C-terminal helix domain (as in mutants 1–79 or 1–78) abolished the production of any β-galactosidase activity (Figure 3, lines 3, 7, 11, 20, 24 and 28), suggesting that this domain is indispensible for the
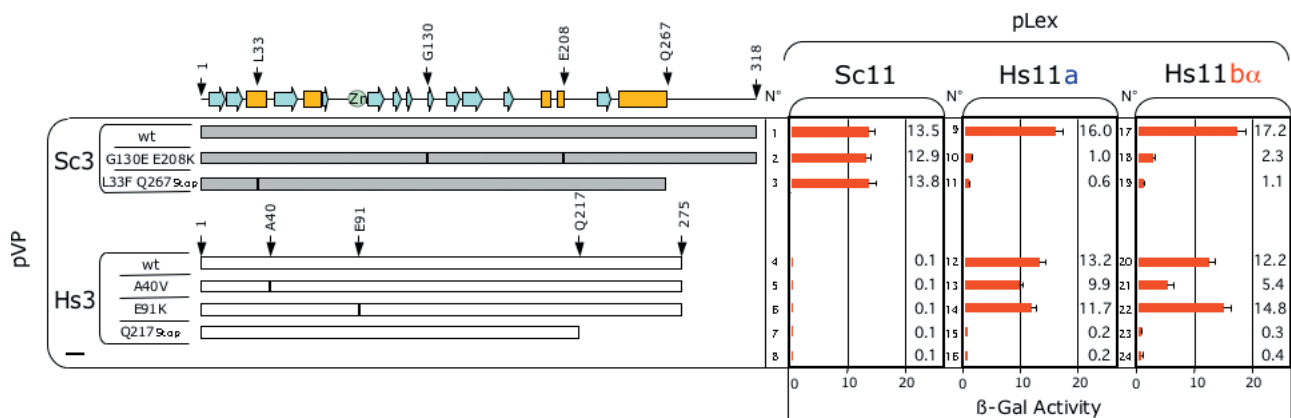

Figure 2. Interactions between human and yeast RPB3 mutants, and the human and yeast RPB11 subunits. The peptidic sequence of Sc3 is schematically depicted as grey bars with the secondary structure elements represented above as in Figure 1. Two mutants are presented with the point mutations indicated by vertical bars and their name indicated on the left. The peptidic sequence of Hs3 is depicted as an opened bar together with three point mutants, as described above. The origin of the coding sequences inserted into the pLex and pVP vectors are indicated on the top and on the left, respectively. Both recombinant vectors were transformed in the L40 yeast strain. The resulting β-galactosidase activity was measured using ONPG (red bars, see Materials and Methods) in independent yeast colonies (right panel). The β-galactosidase activities generated by transforming the cells with the pLex (lines 8, 16 and 24) and the pVP recombinants (not shown) separately were systematically examined and found to be <10% of the highest value in each panel.
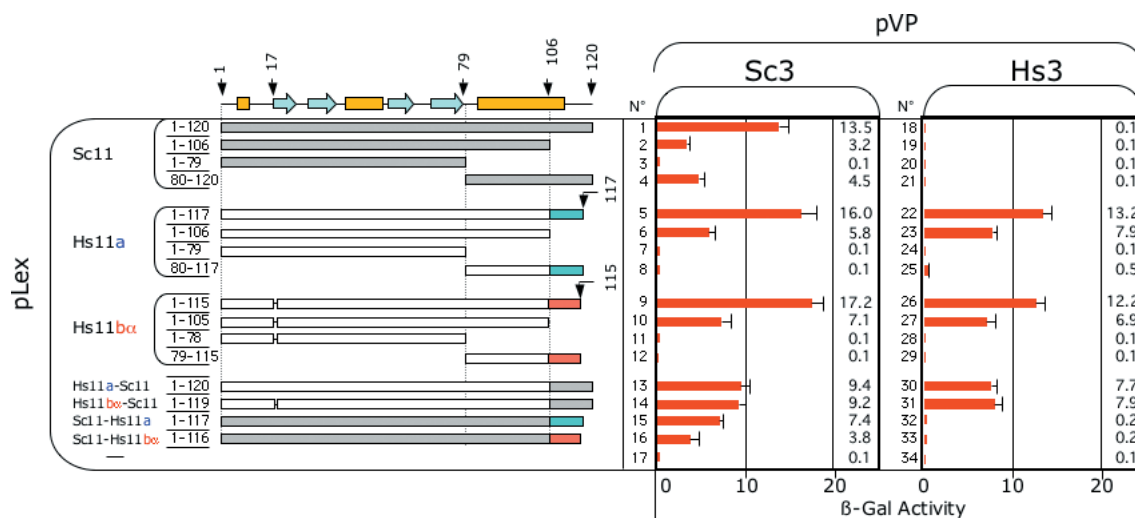
Figure 3. Interactions between the human and yeast RPB3 subunits, and deletion and recombinant mutants of human and yeast RPB11 subunits. The peptidic sequence of Sc11 and deletion mutants are schematically depicted as grey bars with the secondary structure elements represented above (see Figure 1). The sequences of Hs11a and Hs11bα and deletion mutants are shown below as open bars, except for the peptides that are encoded by the exons 4, shown in blue and red, respectively. The Lys 17 is missing in the h11bα protein, as figured by the small gap in the corresponding bars. Four chimeric coding sequences are presented below: the yeast and human portions are shown with a code as above. The nature of the coding sequences inserted into the pLex and pVP vectors are indicated on the left and on the top, respectively. Both recombinant vectors were transformed in the L40 yeast strain. The resulting β-galactosidase activity was measured using ONPG (red bars, see Materials and Methods) in independent yeast colonies (right panel). The β-galactosidase activities generated by transforming the cells with the pLex (lines 17 and 34) and the pVP recombinants (not shown) separately were systematically examined and found to be <10% of the highest value in each panel.

heterodimerization process. In fact, the Sc11 C-terminal domain is capable of interacting with Sc3 by itself (80–120) to a significant extent (Figure 3, lines 4). Similarly, the (80–117) domain of Hs11a is able to interact with Hs3, although very weakly (Figure 3, line 25). In contrast, however, the (79–115) domain of Hs11bα shows no activity (Figure 3, line 29). None of these human deletion mutants yielded any detectable activity in the presence of the Sc3 ortholog (Figure 3, lines 7, 8, 11 and 12). There is, therefore, a clear species-specific restriction in the heterodimerization related to this C-terminal domain.

We then investigated the contribution of the sequences corresponding to Hs11a and Hs11bα exon 4 or their equivalent in Sc11. When assayed in the presence of Hs3, the deletion of this element in Hs11a and Hs11bα only moderately affects the interaction between type 3 and 11 subunits (Figure 3, lines 22, 23 and 26, 27). In contrast, the same deletion of the Sc11 in the presence of Sc3 severely decreased this interaction (Figure 3, lines 1 and 2).

All chimeras, in which the human or yeast sequences corresponding to exon 4 had been interchanged, showed reduced interaction abilities when compared to the wild-type proteins (Figure 3, compare lines 13–16 with 1, 5 and 9, or lines 30 and 31 with 22 and 26).

In the presence of Sc3, the Sc11-Hs11bα chimera yielded an activity similar to that of the (1–106) deletion (Figure 3, compare lines 2 and 16), suggesting that the sequences corresponding to human *RPB11b* exon 4 do not significantly contribute to the interaction, whereas the 107–117 residues of Hs11a enhanced the observed activity (compare lines 2 and 15). Similarly, the fusion of the 107–120 residues of Sc11 partially restored the effect of the deletion of the human C-terminal deletion (compare lines 6 and 10 with 13 and 14).

In contrast, in the presence of Hs3, these C-terminal Sc11 residues were unable to replace, even partially, their human counterparts (Figure 3, compare lines 23 and 27 with lines 30 and 31).

Thus, it appears that the end of the C-terminal alpha helix of Sc11 is more critical to the heterodimerization process in yeast than the corresponding human domain.

The role of the α-motif of the *RPB11*-encoded subunits

In order to assess the contribution of the so-called 'α-motif' to subunit heterodimerization, the conserved sequence was examined in the context of the available 3D structure: three residues (E, D and L, highlighted in yellow in Figure 1A) were suspected to critically contribute to the interactions. They were substituted by Ala residues and the effect of the resulting mutations on heterodimerization is presented in Figure 4.

Contrary to our expectations, alteration of these conserved residues of Sc11 had no detectable effect on the heterodimerization with Sc3 (Figure 4, compare lines 1–3). In sharp contrast, the mutation of the same residues of Hs11a and Hs11bα strongly reduced the β-galactosidase activity in the presence of Sc3, although the double mutant (ED) of Hs11bα had only a 2-fold effect (compare lines 4–9). In the human heterodimer, however, the ED mutation of Hs11a and Hs11bα resulted in a 3-fold reduced activity. The additional L42A mutation did not affect further the β-galactosidase activity in the case of Hs11a, while the same mutation severely reduced the activity in the case of Hs11bα, as in the presence of Sc3 (Figure 4, compare lines 14–19).

Clearly, the α-motif seems to be differentially involved in subunit heterodimerization, depending on its yeast or human
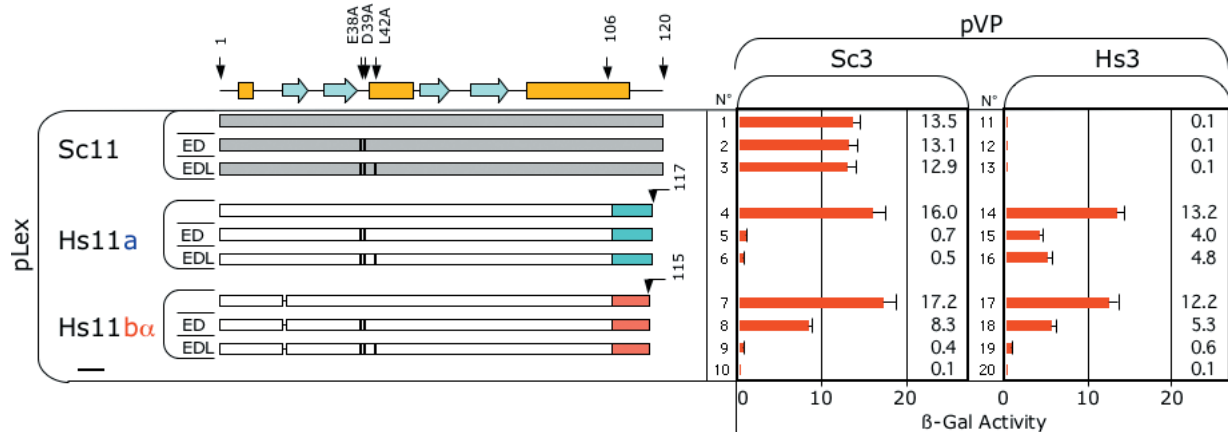
Figure 4. Interactions between point mutants, affecting the α-motifs of human and yeast RPB11 subunits, and the human and yeast RPB3 subunits. The peptidic sequence of Sc11 and corresponding point mutants are schematically depicted as grey bars with the secondary structure elements represented as above (Figure 1). The sequences of Hs11a and Hs11bα are shown below as in Figure 3 together with two point mutants. The positions of the point mutations are indicated by vertical bars. The origin of the coding sequences inserted into the pLex and pVP vectors are indicated on the left and on the top, respectively. Both recombinant vectors were transformed in the L40 yeast strain. The resulting β-galactosidase activity was measured using ONPG (red bars, see Materials and Methods) in independent yeast colonies (right panel). The β-galactosidase activities generated by transforming the cells with the pLex (lines 10 and 20) and the pVP recombinants (not shown) separately were systematically examined and found to be <10% of the highest value in each panel.
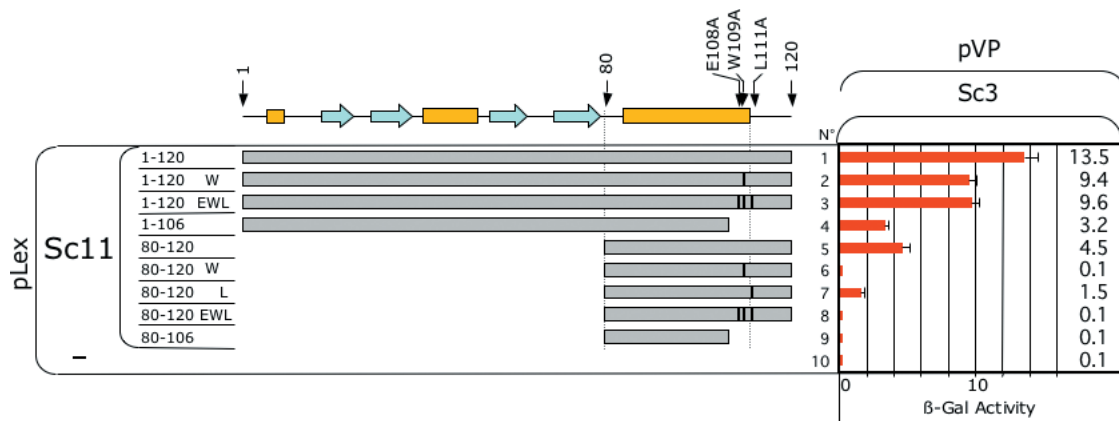

Figure 5. Interactions between Sc3 and point mutants affecting the C-terminal domain of Sc11. The peptidic sequence of Sc11 is schematically depicted as grey bars with the secondary structure elements, represented as above (Figure 1), as well as three point mutations (vertical bars). The tests were performed and described as in Figure 2. The β-galactosidase activities generated by transforming the cells with the pLex (lines 10) and the pVP recombinants (not shown) separately were systematically examined and found to be <10% of the highest value in each panel.

origin. The requirement of this element actually appears to be a specific property of the human RPB11-encoded subunits.

Sc11 C-terminal domain residues critical for heterodimerization

The end of the C-terminal alpha helix of Sc11 appeared to play an important role for the interaction with Sc3 (Figure 3). The comparison of Sc11 with its ortholog from *Schizosaccharomyces pombe*, which was shown to be able to functionally replace Sc11 (11), indicated a conservation for the EW and L residues at positions 108–111 (highlighted in Figure 1A). We, therefore, decided to elucidate their respective contributions to the Sc3/Sc11 interaction by studying the effect of substituting them by Ala residues, either within the context of the complete protein, or with the isolated C-terminal domain (80–120) (Figure 5).

Both the triple (E18A,W109A,L111A) and single (W109A) mutations of the full-length Sc11 proteins only slightly affected the efficiency of interaction with Sc3, as measured by β-galactosidase activity (Figure 5, compare lines 1–3). In contrast, the deletion of all residues beyond position 107 reduced the activity to 25% of the wild-type activity (Figure 5, line 4, as in Figure 3, line 2).

The effect of the same mutations was strikingly different in the isolated C-terminal domain (80–120), where both the triple and the single (W109A) mutations completely abolished the activity (lines 8 and 6), while the L111A mutation was still able to interact with Sc3, although with a reduced efficiency (line 7). The deletion of residues 107–120 abolished the activity (lines 9). Therefore, residue W109 appears to be chiefly involved in the Sc3/Sc11 interaction, whereas the E108 and L111 residues only marginally contribute to dimer formation.
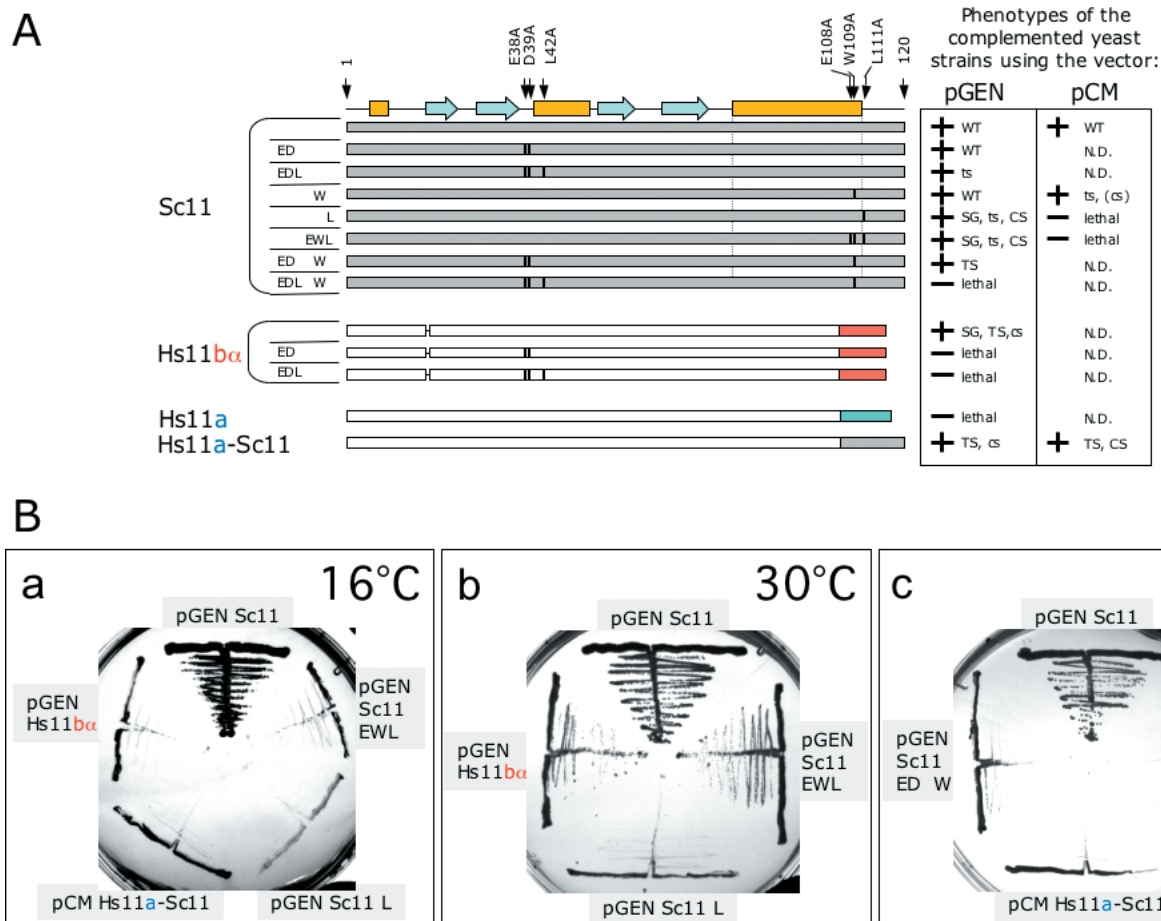
Figure 6. *In vivo* effects of the RPB11 mutations. (**A**) The peptidic sequence of Sc11 and corresponding point mutants are schematically depicted as grey bars with the secondary structure elements (Figure 1). The sequences of Hs11a and Hs11bα are positioned below as in Figure 3. The mutated residues are shown by vertical bars. These coding sequences were inserted in two expression vectors, pGEN and pCM183, resulting in plasmids that were transformed in YGVS-072 yeast strains. The resulting complemented strains exhibited phenotypes that are described in the table on the right. WT, SG, ts, TS, cs, CS stand for wild-type, slow growth, mild thermosensitivity, strong thermosensitivity, mild cryosensitivity, strong cryosensitivity, respectively. N.D. indicates that the experiments has not been done. (**B**) Growth of complemented yeast strains. RPB11 deleted yeast cells were complemented and the resulting strains were streaked on rich medium at various temperatures, as indicated on the top right of each panel. The plasmids used for the complementation are indicated next to each streaked yeast strain.

Effect of the mutations on cell growth

Having established the contribution of the distinct elements of Sc11 to its interaction with Sc3, we investigated their importance *in vivo* by performing complementation assays with either 2 μm or centromeric expression vectors (pGEN and pCM, respectively). The viable complemented strains were then assayed for temperature-sensitive phenotypes as described in Figure 6A. The most striking phenotypes obtained are shown in Figure 6B.

Mutations E38A, D39A of the Sc11 'α-motif' had essentially no effect on yeast growth, whereas the triple alteration (E38A,D39A,L42A) generated a very slight thermosensitivity when expressed from the pGEN vector (Figure 6A). Mutation W109A of the Sc11 C-terminal alpha helix had no effect in the pGEN expression system, but showed a moderate temperature sensitivity when expressed from the pCM vector. In contrast, mutation L111A induced a very severe phenotype when expressed from the pGEN vector, while it was lethal when expressed from pCM (Figure 6A). The triple mutation E108A,W109A, L111A produced a very similar phenotype, although slightly weaker than the single mutation L111A

(Figure 6B, a and b). The combination of the E38A,D39A and W109A mutations expressed from the pGEN elicited a strong thermosensitivity, while the additional L42A mutation generated a lethal phenotype, thus strongly supporting the implication of these residues in an essential process *in vivo* (Figure 6A).

In contrast, both double (E38A,D39A) and triple (E38A, D39A,L42A) mutations of the 'α-motif' in Hs11bα abolished viability of yeast cells, where this protein replaced Sc11.

Although cells harboring Hs11a in place of Sc11 were not viable, the Hs11a-Sc11 chimeric subunit restored viability (albeit with a strong thermosensitive phenotype), whether expressed from either pGEN or pCM (Figure 6B, c).

DISCUSSION

A genetic assay to score protein interactions in yeast

We present a comparative analysis of the cross-heterodimerization between yeast and human *RPB3* and *RPB11* encoded subunits. This interaction is likely to represent

the initial assembly step of RNAP II *in vivo* (12,13). Although the 3D structure of the yeast RNAP II has been established (2,5), the functional importance of every potential contact cannot be directly inferred from the model but requires experimental validation. On the other hand, contacts critically involved during subunit assembly may not be required in the final structure.

Hs3 subunit does not interact with Sc11 in yeast

As expected from partners within well characterized RNAP II molecules, both human Hs3/Hs11a and yeast Sc3/Sc11 heterodimers were readily detectable using this two-hybrid analysis, while under the same conditions Hs3/Sc11 heterodimers could not be revealed. The incapacity of these fusion proteins to interact is probably not related to some abnormal behaviour, since both of them were able to interact with other proteins. This lack of interaction actually accounts for the inability of Hs3 to substitute for Sc3 in yeast (data not shown).

The Sc3/Hs11bα heterodimerization obeys rules different from Sc3/Sc11

The Hs3/Hs11bα heterodimer appears to be as stable in yeast cells as the Hs3/Hs11a heterodimer. This observation indicates that Hs11bα has the potential to integrate a bonafide RNAP II complex in a human cell, as suggested by the abilities for this subunit, both to substitute for Sc11 in yeast cells [(4), Figure 6] and to interact with Sc3 with an efficiency similar to that of Sc11 (Figure 2).

Surprisingly, however, we identified residues in Sc3 that happened to be critical for an efficient interaction with Hs11bα and Hs11a, while they had no effect on Sc3/Sc11 heterodimerization (Figure 2). The Sc3/Hs11bα and the Sc3/Sc11 interactions, although both viable in yeast, seem therefore to differ at the molecular level. This is further substantiated by the observation that mutations affecting the 'α-motif' of Sc11 had no detectable effect on the Sc3/Sc11 interaction, although the same mutations of Hs11bα severely impaired Sc3/Hs11bα heterodimerization (Figure 4). These residues within the 'α-motif' of both Hs11a and Hs11bα human proteins happened to be important for all the interactions tested.

This observation is further confirmed by the lack of a significant phenotype for the Sc11 mutants *in vivo*, although the same mutations completely abolish the viability of Hs11bα (Figure 6).

Specific functions are associated with the C-terminal domain of RPB11 encoded subunits

Although Hs11a cannot substitute for Sc11 in yeast [(4), Figure 6], it interacts with Sc3 just as well as Hs11bα, which does complement. The failure of Hs11a to do so is, therefore, probably related to the most C-terminal domain of the protein, in keeping with the fact that the substitution of this domain by the yeast equivalent (as in Hs11a-Sc11 chimera, see Figures 3 and 6) yields a protein which is able to interact with Sc3 and is also viable in yeast. Critical functions, which are not related to the ability of the subunits to interact with Sc3, must therefore be linked to the C-terminal domain in Sc11 and its human counterparts.

Deletion of the last 14 residues of C-terminal of Sc11 impairs more effectively its interaction with Sc3 than the equivalent deletion of the human proteins Hs11a and Hs11bα affects their interactions with Hs3 (Figure 3). In addition, the C-terminal alpha-helix of Sc11 interacts more efficiently with Sc3 than with Hs11a and Hs3 (Figure 3). This Sc11 domain, therefore, appears to be much more involved in the heterodimerization process, when compared to its human counterparts. Indeed, the human heterodimers do not seem to rely as much on this contact, since the deletion of the sequences corresponding to exon 4 only moderately affects the interaction with Hs3 (Figure 3).

A detailed analysis (Figure 5) of the importance of W109 and L111 of Sc11 in the heterodimerization indicated that their respective contributions are in good agreement with the established model for the complete RNAP II (2,5): W109 (whose side chain points toward Sc3) was critical, whereas L111 (whose side chain protrudes from the complex) was dispensable, when assayed on the isolated domain. In marked contrast, when assayed on cell growth, the contribution of W109 was only marginal, while that of L111 turned out to be essential (Figure 6A). An attractive explanation could be the interaction of this Sc11 domain with an essential factor external to the RNAP II complex. That W109 contributes to the stability of the RNAP II complex *in vivo* may be inferred from the observation that the W109A mutation, which does not yield a strong phenotype by itself, produces very severe phenotypes when combined with the 'α-motif' mutations (Figure 6A). This might be due to the cumulative effects of separate substitutions at the interface between Sc3 and Sc11, in agreement with the established RNAP II model (2,5).

CONCLUSIONS

We demonstrate that the Sc3/Sc11 and Hs3/Hs11 heterodimers are stabilized in distinct ways. Although some degree of conservation has been preserved, the same residues within the conserved α-motif are differentially involved in the yeast and human systems (Figure 7). The Sc3/Sc11 heterodimer
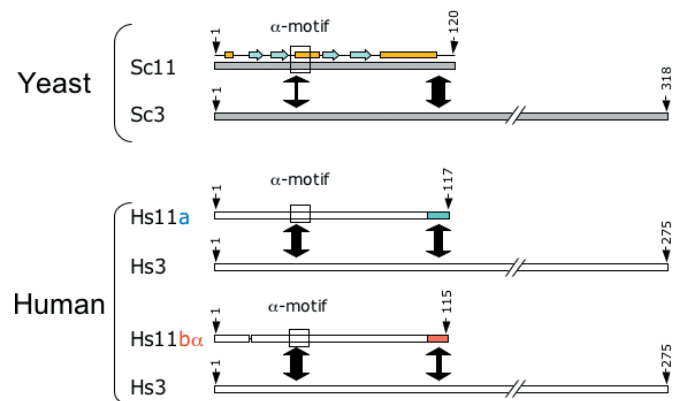

Figure 7. Summary of the identified contacts in the yeast and human subunits and their contributions to the stability of the heterodimers. The subunits sequences are schematically depicted, as in the previous figures. The contributions of the homologous domains of Sc11, Hs11a and Hs11bα in the heterodimerization with Sc3 and Hs3 subunits are represented by double-headed arrows with thicknesses referring to the importance of the contacts.

strongly depends on a specific C-terminal molecular motif, in contrast to the Hs3/Hs11 heterodimers. This possibly allowed the mammalian genomes to produce RPB11-encoded proteins that exhibit various C-terminal domains, some of which being restricted to human, without exceedingly affecting the heterodimerization process. It is, therefore, possible that human-specific relationships have been established between transcription and other cellular processes, during the course of evolution. Indeed, several proteins were described to be able to interact with the Hs11a (14,15) or with another variant, Hs11bγ (16), the physiological significances of some interactions being substantiated (17). It will be of utmost interest to identify the functions associated with this C-terminal domain both in yeast and human proteins. The identification of all the proteins able to interact with those domains in both systems should be a first step toward this goal.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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