Functional and comparative characterization of *Saccharomyces cerevisiae RVB1* and *RVB2* genes with bacterial Ruv homologues

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Received 22 September 2006; accepted 27 November 2006.
First published online 15 February 2007.

DOI:10.1111/j.1567-1364.2006.00205.x

Editor: André Groffeau

Keywords
DNA Holliday junction; homologous recombination; RuvB-like genes; UV damage.

Abstract

Expression of yeast *RuvB*-like gene analogues of bacterial RuvB is self-regulated, as episomal overexpression of *RVB1* and *RVB2* decreases the expression of their chromosomal copies by 85%. Heterozygosity for either gene correlates with lower double-strand break repair of inverted-repeat DNA and decreased survival after UV irradiation, suggesting their haploinsufficiency, while overexpression of the bacterial RuvAB complex improves UV survival in yeast. Rvb2p preferentially binds artificial DNA Holiday junctions like the bacterial RuvAB complex, whereas Rvb1p binds to duplex or cruciform DNA. As both proteins also interact with chromatin, their role in recombination and repair through chromatin remodelling, and their evolutionary relationship to the bacterial homologue, is discussed.

Introduction

To preserve DNA integrity and to prevent the deleterious consequences of genomic aberrations, cells have evolved a complex, yet highly efficient, network of DNA repair mechanisms activated by a series of cell cycle checkpoints (Paques & Haber, 1999; Lisby & Rothstein, 2005). One major mechanism is homologous recombination, the pivotal molecular intermediate of which is the Holliday junction (HJ). In Gram-negative prokaryotes, the enzymes that process HJ have been studied in detail. The RuvA-RuvB complex promotes an ATP-dependent branch migration of HJ (van Gool *et al*., 1998) and stimulates its resolution by RuvC endonuclease (Privezentzev *et al*., 2005), in addition to being essential for replication fork reversal in the occurrence of DNA replication defects (Baharoglu *et al*., 2006). *Escherichia coli ruvA*, *ruvB*, and *ruvC* genes are required for DNA repair and normal levels of cellular resistance to stress-induced mutagenesis, so their resulting mutants are thereby defective in recombination (Mezard *et al*., 1999; He *et al*., 2006). Interestingly, Gram-positive bacteria such as *Bacillus subtilis* lack RuvC (Kunst *et al*., 1997; Curtis *et al*., 2005), while its function seems to be taken over by the RecU recombination protein (Ayora *et al*., 2004). RuvB belongs to the AAA^+ ATPase superfamily, presenting a conserved amino-terminal (N) domain comprising a Walker A, Walker B and a sensor 1 amino acid motif, while the central (M) domain contains a sensor 2 motif, all of which are active sites for ATP binding and hydrolysis as well as for ATP-dependent hexamer formation (Ohnishi *et al*., 2005). Moreover, this multifunctional protein contains a third carboxyl-terminal domain (C) that, through its Arg-318 residue, is required for DNA binding. All three domains are essential for RuvAB complex formation and branch migration of HJ (Ohnishi *et al*., 2005).

In eukaryotes, the yeast *Saccharomyces cerevisiae* has been an utmost model system for the elucidation of the recombination pathways (Paques & Haber, 1999), although HJ branch migration and resolution, as well as the processing activity of RecQL1, have been detected in mammalian cell-free and nuclear extracts, respectively (Constantinou *et al*., 2001; LeRoy *et al*., 2005). A protein named RuvBL1 (RuvB-like1) was identified in human cells by a two-hybrid interaction screen with the 14-kDa subunit of DNA replication and repair factor RPA (Qiu *et al*., 1998) and, since then, RuvBL1 and RuvBL2 orthologues were discovered by database searches for closely related family members (Qiu *et al*., 1998; Kanemaki *et al*., 1999) and identified by a number of laboratories to be highly conserved in different organisms (Kurokawa *et al*., 1999). In yeast, Rvb1p and Rvb2p are nuclear proteins indispensable for cell cycle progression and RNA polymerase II transcription (Lim *et al*., 2000). They were thought to act independently as helicases of opposite polarity (Kanemaki *et al*., 1999), however little is known

about their direct or indirect function in transcription regulation of 34, or over 5%, of yeast genes (Jonsson *et al.*, 2001; Ohdate *et al.*, 2003). The Rvb1 and Rvb2 proteins are similar in sequence to bacterial RuvB, especially in the N domain containing WalkerA and WalkerB boxes (LeRoy *et al.*, 2005). In higher eukaryotes, they interact with c-Myc, β-catenin, E2F1, and ATF2, and modulate cellular transformation, signalling, apoptosis and response to stress and DNA damage (Bauer *et al.*, 2000; Cho *et al.*, 2001; Dugan *et al.*, 2002). Rvb1p-Rvb2p complex, purified from yeast, showed ATP-dependent chromatin remodeling activity *in vitro* (Jonsson *et al.*, 2001), and both proteins have also been involved in the assembling of the INO80 chromatin remodeling complex (Jonsson *et al.*, 2004). Finally, both proteins were found to be associated with the production of small nucleolar RNPs (King *et al.*, 2001).

To further characterize the eukaryotic RuvB-like proteins, we carried out a molecular study of the Rvb1 and Rvb2 yeast proteins and functionally compared them to their putative bacterial ruv gene homologues from the Gram-positive *Bacillus subtilis*. These genes have been chosen for complementation, as *B. subtilis* is a sporulating Gram-positive bacteria that is evolutionarily closer to yeast than *E. coli* (Vagner *et al.*, 1998) and with a very efficient recombination system. Our results indicate that *RVB1* and *RVB2* are self-regulated genes required for DNA repair, as the diploid strains showed Rvb1p and Rvb2p dosage-dependent phenotype when repairing DNA double-strand breaks and UV damage. Interestingly, expression of the bacterial RuvAB complex increased survival after UV irradiation, rescuing the phenotype caused by *RVB1* and *RVB2* heterozygous deletions. Finally, to study whether the RuvB-like proteins of the two yeasts bind to HJ, we developed an *in vitro* cell-free binding assay in which Rvb2p, unlike Rvb1p, preferentially bound the artificial DNA HJ.

Materials and methods

Yeast strains and media

The yeast strains used in this study are listed in Table 1. In haploid strain FAS20, as well as diploid CB89, FAY1 and DSL1-V5 strains, RVB1 or RVB2 genes were tagged with the V5His6x tag, while in haploid strain YPH258 one or the other RVB gene was tagged with the green yEGFP fluorescent tag (Waghmare *et al.*, 2003). Gene deletions and tagging were performed according to the protocols of the EUROFAN program manual (URL: http://www.mips.gsf.de/proj/eurofan/eurofan_1/60/home_requisites/guideline/sixpack.html), and were verified by PCR. YPD, YPD supplemented with kanamycin and synthetic dropout media were prepared as described (Kaiser *et al.*, 1994). *Escherichia coli* strain DH5α was used for plasmid propagation and it was grown in LB broth supplemented with ampicillin as previously described (Sambrook *et al.*, 1989).

Table 1. Genotype of the yeast strains used in this study

Yeast strain	
FAS20	MATα *ade1 ade2 ade8 trp1 leu2 lys2 ura3-52 [cir$^+$]*
YPH258	MATa *ade2 ade8::FRTX his3 trp1 leu2 lys2 ura3-52 [cir$^+$]*
FAY1	FAS20 x YPH258
DSL1-V5	MATa/α *gal2/GAL2 leu2/leu2::FRTX arg10/ARG10 ade2/ADE2 ura3-52/ura3-52 RVB1/RVB1::V5 KanMX4*
CB89	MATa/α *ade5/ade5 can1^R/CAN1^S leu2-3/leu2-3 trp1-289/trp1-289 HIS7/his7 ura3-52/ura3-52*

Plasmid construction

Plasmids pXKX, pGKG and pHKH containing the KanMX4 gene as a recyclable marker (Storici *et al.*, 1999) were used as templates to amplify DNA cassettes for deleting ADE8, RVB1 and RVB2, respectively. Plasmids pYM12 (Knop *et al.*, 1999) and pH-RuvBV5His6x (Waghmare *et al.*, 2003) were used as templates for PCR amplification of yEGFP and V5 cassettes, respectively, which then were used for C-terminal tagging of RVB1 and RVB2 at their chromosomal loci. For the construction of the plasmids carrying the *B. subtilis* ruvA and ruvB genes, these were first amplified using the genomic DNA of strain 168 as template with primers A1 and A2 for amplification of ruvA and primers B1 and B2 for amplification of ruvB (Table 2). The two-gene operon was amplified using A1 and B2 primers. The first stretch of 10 nt contains the BamHI site necessary for cloning within the polylinker of pUC18 in which the two genes and the operon were fully sequenced. The start codon (GTG) for ruvA present in the Bacillus genome was substituted with ATG (primer A1). The start codon for ruvB was chosen on the basis of similarity with its orthologue in *E. coli*. Sequencing of the *B. subtilis* ruv locus was performed by our laboratory as part of the *Bacillus* Genome Project (Kunst *et al.*, 1997) and it is reported in the SubtiList database (http://genolist.pasteur.fr/SubtiList/). The two ruv genes were cloned in-frame into SalI–NotI sites of a modified pVP16 plasmid (Clontech) containing the SV40-T nuclear localization signal (NLS) coding sequence between yeast ADH1 promoter and terminator sequences, to fuse each N-terminus of their encoded proteins with the NLS peptide. Plasmid pVT1 and pVT2 were obtained by cloning the PCR-amplified yeast RVB1 and RVB2, respectively, into the XhoI – BamHI of pVT100-U harbouring the URA3 gene. Using the pYES2.1 TOPO TA Cloning Kit (Invitrogen), the sequences encoding NLS-ruvB, RVB1 and RVB2 were fused in frame with the V5His6x epitope at their C-terminus. After PCR amplification, NLS–ruvB–V5His6x and RVB1-V5His6x were inserted into XhoI – XbaI of pVT100-U plasmid generating pVT-

Table 2. Primer sequences used in this study that are not indicated in Table 2 are available on request

Primer name	Sequence 5′–3′
A1	CGGGATCCCGATGATTGAATTTGTTAAAGGGA
A2	CGGGATCCCGTTACTTTAATAGTTTTTGTAATGCT
B1	CGGGATCCCGATGGATGAACGGCTCGTCTCAA
B2	CGGGATCCCGTCAGTCATATCGGGGAGCCT
KpnF	GGGTGAAGTGACAGAACTAACCCCTGAAG
ORF1R	TAACATGCATGCTTACAAATAATTTGCGGAAG
EllF	ATAAATAGGGCTTTGGAAGATGAGTTTGCC
ORF2R	TAACATGCATGCTTATTCCGTAGTATCCATGG
HIS1-INF	ACCTGTAGCGTTGGTCTTTC
HIS1-INR	GAAATGGTTGGTGCTCTACG
V5R	ACCGAGGAGAGGGTTAGGGAT
R2+20	GCAATTTCTGCCTTAAAGTACAAAATGC

BV5 and pVT-1V5 plasmids, respectively. The RVB2-V5His6 fragment was then subcloned into the XhoI–SacI of pVT100-U plasmid generating pVT-2V5. An 18 nt HSV epitope sequence was added to the reverse primer for amplification of the NLS – RuvA – HSV using the segment cloned in pVP16 as a template. The PCR product was inserted into the PvuII site of pVT100-U generating the pVT-AHSV plasmid. This plasmid was digested with SphI and the resulting 1.4 kb fragment was subcloned into SphI site of plasmid pVP16, generating pVP-AHSV.

Plasmid pRURA8Δwas used for recombination assays (Caputo, 2003). Plasmid DNA was extracted from *E. coli* using the Mini-preparation Kit (Promega) and restriction enzymes used were from New England Biolabs.

Sporulation and tetrad dissection

Strains heterozygous for *RVB1* or *RVB2* were transformed with plasmids pVT-1V5 or pVT-2V5, respectively. Both strains were also transformed with pVT-BV5 alone or in combination with pVP-AHSV. For sporulation, cells were grown overnight, washed once and inoculated at cell density of 5×10^6 cells mL^{-1} in 50 mL of YPA (Kaiser *et al.*, 1994) that contained 1% URA or URA LEU drop-out instead of Bacto Yeast Extract. Cells were harvested at the density of 2×10^7 cells mL^{-1}, washed twice and transferred to 50 mL of sporulation medium (1% K-acetate plus 1/5 the standard concentration of the required amino acids). The incubation continued at room temperature with shaking for up to 7 days. Before dissection, the sporulated cells were treated with lyticase at room temperature for 10 min. Well digested four-spore asci were dissected on thin YPD dissection plates using a twin-joystick Leitz electric micromanipulator mounted on a FLUOVERT SF microscope (Leitz Wetzlar, Germany). The plates were then incubated at 30 °C to form colonies.

Synchronization of yeast cells

The mating pheromone α-factor was used to synchronize YPH258 cells in G1/S phase following the protocol available at http://www.elledgelab.bwh.harvard.edu/protocols/yeast/alpha.html.

Plasmid recombination assay

Yeast cells were transformed with plasmid pRURA8Δ (Caputo, 2003) linearized by I-SceI *in vitro* and plated on SC-TRP plates, which were incubated at 30 °C. After 2 days, transformants were replica-plated on SC-URA plates, incubated at 30 °C overnight and scored for the Trp^-, Ura^+ phenotypes. Three independent experiments were performed for each strain.

UV sensitivity assay

Yeast strains were grown in appropriate liquid media at 30 °C to a density of $\sim 1 \times 10^7$ cells mL^{-1}. Approximately 200 cells per plate were plated on appropriate solid media and immediately UV irradiated using a VL-6C UV lamp at a dose of 150 J m^{-2} (measured by a VLX254 Radiometer, France). Immediately after irradiation, plates were exposed to light for 15 min and then incubated in the dark at 30 °C. After 2 days, colonies were counted and the fraction of irradiated cells that survived relative to nonirradiated cells was calculated, correcting for the CFU per cell ratio. Three independent experiments were performed for each strain.

Whole-cell and nuclear extracts preparation

To follow Rvb1p and Rvb2p expression and localization after UV damage, cells were grown in 20 mL liquid YPD to the density of $\sim 1 \times 10^7$ cells mL^{-1}, washed once, resuspended in PBS and UV irradiated with the same dose of 150 J m^{-2}. After irradiation, cells were harvested and incubated in 20 mL liquid YPD medium at 30 °C. One milliliter samples of the culture were taken after 5, 15, 30, 60, 120, 300, and 480 min of incubation for protein isolation and purification. Whole protein extracts were prepared according to the protocol available at http://www.fhcrc.org/science/labs/hahn/methods/biochem_meth/yeast_glass_bead_SDS_html. Nuclei, and nuclear extract isolation was performed as described (Ausubel *et al.*, 1994). Nuclear extracts were stored in nuclear extract buffer (NEB; 20 mM Tris-Cl pH 7.5, 0.1 mM EDTA, 10% glycerol, 100 mM KCl, 1 mM DTT, 1 mM PMSF, 1 × protein inhibitor mixture). Chromatin fractionation was performed as described (Mendez & Stillman, 2000) and all protein preparations were resolved on 10% SDS-PAGE (Sambrook *et al.*, 1989).

Protein – DNA binding assay

The synthetic Holliday junction X26 was prepared by annealing four 60-mer oligonucleotides (Constantinou

et al., 2001), while the annealing of 5′-biotinylated X26-2 oligonucleotide and X26-1, X26-3 and X26-4 was performed as described previously (Elborough & West, 1990). The quadruplex product was then purified by electroelution through 2% agarose gel using TaKaRa RECOCHIP (TaKaRa). A protein-DNA binding assay based on magnetic separation (Gabrielsen *et al.*, 1989) was adapted for our purpose as follows: 0.75 μg of purified biotinylated X26 were bound to 40 μL of Streptavidin Magnetic Particles (Roche) and pulled down by the Magnetic Particle Separator (Roche) under conditions recommended by the manufacturer. The beads were then equilibrated by washing 3 × with binding buffer (BB) (NEB supplemented with 1 mM $MgCl_2$, 0.1% Triton-X), then mixed with 250 μL nuclear extracts obtained from $\sim 5 \times 10^8$ cells, and supplemented with 1 mM $MgCl_2$, 0.1% Triton-X, and 80 mM ammonium acetate. The mixture was incubated at room temperature with rocking for 15 min, then pelleted and washed three times with BB. Proteins were eluted by pulling down the beads in the BB with an increasing NaCl concentration of up to 175, 300, 400 600 mM and, finally, 1 M. After each round of pelleting and resuspending, the supernatant, containing the proteins released from X26, was collected, desalted on Sepharose G25 columns, dried and resuspended in SDS sample buffer.

In parallel, the same binding procedure was performed with all nuclear extracts using, as binding substrate, 1.5 μg of duplex DNA prepared by annealing 5′-biotinylated X26-2 with its reverse complement.

Immunoblots and quantitative analysis of protein

The HSV and V5 epitope-tagged proteins were detected with mouse monoclonal anti-HSV (Novagen), and mouse monoclonal anti-V5 (Invitrogen) immunoglobulin G (IgG), respectively, and horseradish peroxidase (HRP) conjugated sheep anti-mouse IgG (Amersham Pharmacia Biotech). Rad53p and Rvb2p, or their V5-tagged variant, were detected with rabbit polyclonal anti-Rad53p and anti-Rvb2p antibodies, respectively, and HRP-goat anti-rabbit IgG (DAKO). Orc2p was detected with the goat anti-Orc2 (yC-19) IgG, and HRP-donkey anti-goat IgG (Santa Cruz Biotechnology). All Western blot hybridizations were performed as described (Sambrook *et al.*, 1989). The enhanced chemoluminescence Western blotting detection reagent (ECL™, Amersham) was used to visualize the protein bands by exposing the hybridization membrane to X-film (Kodak, Rochester, NY). For protein quantification, the intensity of each band was scanned with an UltroScan XL laser-scanner densitometer (Pharmacia LKB). The protein content of whole-cell extracts preparations was determined by the Bio-Rad Protein Assay Kit (Bio-Rad), using BSA as standard.

RNA preparation and analysis

Total RNA was extracted by the RNA Mini-preparation Kit (Promega). The first strand synthesis was performed using AMV Reverse Transcriptase (Promega). The amount of cDNA was quantified by absorbance at 260 nm, and 0.5 μg of cDNA was used to perform PCR amplification (28–30 cycles) using Taq Polymerase (Promega). For the cell cycle regulation experiment, *RVB1*, *RVB2* and *HIS1* fragments were amplified using the primers listed in Table 2.

In the self-regulation experiment, endogenous expression of RVB1 was followed using primers KpnF and V5R specific to the V5 epitope, while the RVB2 endogenous expression was followed by primers EIIF and R2+20 specific to the terminator sequence immediately after the stop codon, which is not present in plasmid pVT-2V5. PCR products were subjected to electrophoresis in 1% agarose gel and quantified after EtBr staining by UltroScan XL laser densitometry.

Fluorescent microscopy

Strains that carry yEGFP-tagged Rvb1p or Rvb2p were grown in liquid YPD media to a density of $\sim 1 \times 10^7$ cells mL^{-1}. Cells were washed once with water, spotted on a glass slide, air-dried and covered with 5 μL of mounting medium (Vector Laboratories). The yEGFP-tagged proteins were visualized using an Axiovert 100 M confocal microscope (Carl Zeiss Jena).

Results

Rvb1 and Rvb2 are constitutively expressed chromatin-associated proteins

Both human *RVB* genes have been shown to be expressed in all mammalian cells, with increased expression in testis (Makino *et al.*, 2000) and *RVB1* mRNA was found to be expressed uniformly throughout the cell cycle (Qiu *et al.*, 1998). To determine whether the expression of *RVB1* and *RVB2* genes is regulated during the cell cycle in yeast, YPH258 cells, in which RVB1 or RVB2 were tagged with yEGFP at their chromosomal loci, were synchronized in G1/S phase using α-factor. After release from α-factor, samples were taken each 15 min for a 120 min period, covering more than one cell cycle. We used RT-PCR to examine mRNA levels, and fluorescent microscopy to follow Rvb1- and Rvb2-yEGFP proteins throughout the cell cycle. The *RVB1* and *RVB2* mRNA was detected at constant levels during the cell cycle (when normalized to the levels of *HIS1* mRNA; Fig. 1a). In agreement with mRNA levels, both proteins were present and homogeneously distributed in the nucleus at all time points according to fluorescence microscopy analysis (data not shown).

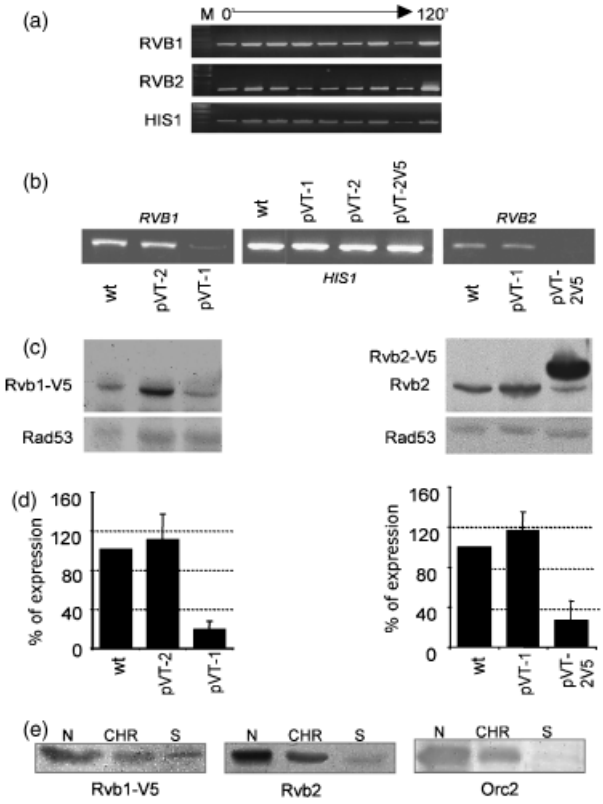

Fig. 1. Expression analysis of *RVB1* and *RVB2*. (a) *RVB1* and *RVB2* mRNA expression through the cell cycle. Levels of *RVB1* and *RVB2* mRNA in YPH258 cells was examined by RT-PCR following the indicated times of release from the α-factor block. *HIS1* expression was used as the internal control reference for constitutively expressed genes and the amount of PCR product loaded. (b) RT-PCR of endogenous *RVB1* and *RVB2* mRNA and (c) Western blot analysis of Rvb1 and Rvb2 protein levels in wild-type FAY1 strain harbouring a V5 chromosomally tagged RVB1 gene, strain FAY1/pVT1, which overexpressed Rvb1p, or FAY1/pVT2 and FAY1/pVT-2V5, which overexpressed Rvb2p or its V5 tagged variant, respectively. The amount of Rad53 protein was used as an internal control reference for the amount of protein loaded. (d) quantification of the Western blot data as in (c), obtained from four independent experiments. Endogenous expression of Rvb1-V5 and Rvb2 proteins is represented relative to endogenous expression of each Rvbp in the wild-type strain. (e) Rvb1p and Rvb2p bind to chromatin. Western blot of DSL1-V5 nuclear extracts prepared and fractionated as described in 'Materials and methods'. Orc2p, as chromatin associated protein was used as a control. N, whole nuclear extract; CHR, chromatin-enriched pellet; S, nuclear soluble proteins.

Although expression of *RVB1* and *RVB2* was not cell-cycle regulated, we observed a self-regulation of the expression for both genes. Exponentially growing diploid strain FAY1, transformed with high copy-number plasmid pVT2-V5, which carried the *RVB2*-V5 tagged variant, showed decreased expression of the endogenous *RVB2* at the protein and the mRNA level, when compared with the nontransformed FAY1 strain (Fig. 1b and c). To determine whether the ectopic overexpression of the *RVB1* had the same effect on the endogenous *RVB1* expression, the RVB1 gene was tagged with the V5 epitope at its chromosomal locus and the cells were transformed with the plasmid pVT1, which carried wild-type *RVB1*. As shown in Fig. 1b and c, the amounts of endogenously expressed *RVB1*-V5 mRNA and the Rvb1-V5 protein were decreased in the pVT1 transformed strain when compared with the nontransformed strain. Normalization of the Western blot data to the levels of Rad53p revealed that the episomal overexpression of Rvb1p and Rvb2p decreased by up to 85% the expression of chromosomal *RVB1* and *RVB2* genes, respectively (Fig. 1d). The same self-repression for both *RVB* genes was observed in the haploid YPH258 strain (data not shown). On the other hand, overexpression of Rvb1p increased a little Rvb2p, and vice-versa (Fig. 1c and d). This last behaviour for either Rvb protein was consistent with what was observed for their mRNAs (Fig. 1b). As it is known that Rvb1p and Rvb2p interact with each other (Kanemaki *et al.*, 1999), apparently in stoichiometric proportions (Jonsson *et al.*, 2001), these data could indicate that the increase at the protein level represents the result of an independent regulation of the two gene products in the complex.

When preparing nuclear extract (NE), we observed that some of the Rvb1 and Rvb2 proteins were always present in the nuclear pellets after the nuclei were broken, despite the use of high salt concentration buffers. It was also reported previously that Rvb1 and Rvb2 are part of the INO80 chromatin-remodeling complex (Jonsson *et al.*, 2001, 2004). To determine whether yeast Rvb1 and Rvb2 proteins are associated with chromatin itself, we performed nuclear fractionation. Nuclei were prepared from a culture of the DSL1-V5 cells in which the *RVB1* gene was tagged with V5 epitope at its chromosomal locus (Waghmare *et al.*, 2003), and then lysed and fractionated. After fractionation, the majority of the Rvb1-V5p and Rvb2p, ∼70% and ∼85% respectively, were found in the chromatin-enriched fraction (CHR), together with Orc2p that is known to be chromatin-bound (Mendez & Stillman, 2000), Fig. 1e. However, small amounts of the proteins was present in the nuclear soluble fraction (S), suggesting that these proteins can also form complexes that are not strictly bound to the DNA, and/or that at some point during the cell cycle they are released from the chromatin.

Haploinsufficiency of RVB1 and RVB2 for DNA double-strand break and UV damage repair

A role of *RVB1* and *RVB2* genes in recombination and DNA damage repair was implied by the activities of their closest homolog with a known function, the eubacterial RuvB, and its association with RPA, a protein involved in DNA replication and repair (Qiu *et al.*, 1998). As *RVB1* and *RVB2* genes

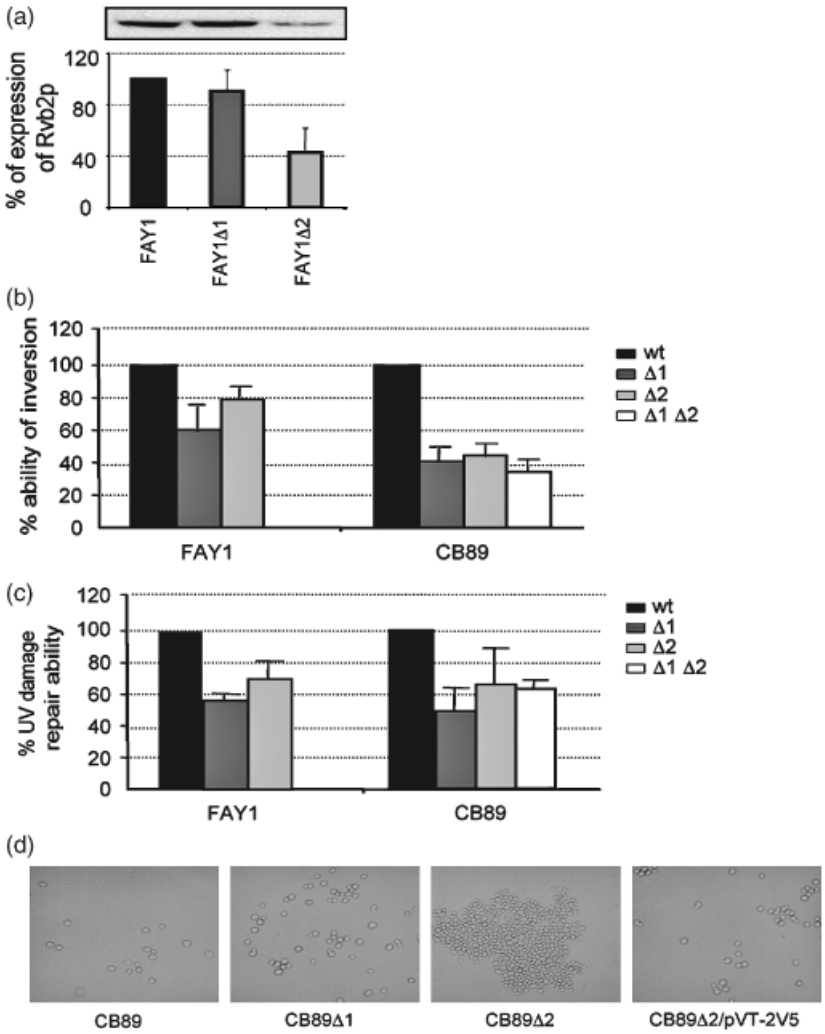

Fig. 2. *RVB1*, and *RVB2* haploinsufficiency effect on DNA DSB and UV damage repair, and flocculation. (a) Western blot showing amounts of Rvb2p in wild-type FAY1, and its *RVB1*, or *RVB2* heterozygous strains. Five micrograms of total protein extract were loaded for each strain. Data obtained from four independent Western blotting experiments were quantified, and the expression of the Rvb2p in the mutants is represented as the % of its wt expression. (b and c) Impaired ability of *RVB1* and/or *RVB2* heterozygous strains to repair DSB, by DNA inversion (CO+BIR-SSA), and to repair UV damage. Values are expressed as % of the wild-type strain ability to repair the damage. (d) Deletion of one copy of RVB2 in CB89 causes a flocculation phenotype. The strains indicated were grown in YPD until mid-log phase, and then photographed.

are essential for yeast viability (Kanemaki *et al.*, 1999; He *et al.*, 2006), we studied their potential haploinsufficiency effect on DNA double-strand break (DSB) and UV damage repair in diploid strains heterozygous for one or the other gene. The deletion of either *RVB1* or *RVB2* did not influence the growth of the heterozygous strains in YPD, or synthetic-complete medium, as their growth rates were comparable to that of the wild-type strain (data not shown). To confirm that the deletion of one *RVB* copy results in a decrease of Rvb protein amount, we followed the expression of Rvb2p in the wild-type FAY1, and in its mutant, FAY1Δ1 and FAY1Δ2 strains, heterozygous for *RVB1* and *RVB2*, respectively. As shown in Fig. 2a, the amount of the Rvb2p present in the FAY1Δ2 strain was half of that present in the FAY1. It is also important to notice that the deletion of the *RVB1* gene did not influence the expression of *RVB2*, as the amount of Rvb2p in FAY1Δ1 was comparable to that in the wild-type strain (Fig. 2a).

To investigate whether the two yeast RuvB-like proteins participate in DNA DSB repair by homologous recombination (HR), we used plasmid pRURA8Δ (Caputo, 2003) as a DNA substrate. This centromeric plasmid contains two truncated *URA3* copies (one truncated at its 5′, and the other at its 3′) in indirect orientation. The plasmid was cut *in vitro* by I-Sce I endonuclease, the unique restriction site of which is present in the 5′Δ*URA3*, inside the homologous region shared by two truncated copies. FAY1, FAY1Δ1 and FAY1Δ2 strains were transformed with the linearized plasmid, and plated on SC-TRP plates to select for the transformants that repaired the induced DNA DSB. Recombination repair might lead to gene conversion, or to inversion of the intervening sequence between the two inverted repeats. Inversion could be generated either by reciprocal exchange via classical crossing-over, or by break-induced replication event associated with single-strand annealing (BIR-SSA) process (Malagon & Aguilera, 2001). In any case, inversion

would produce a functional *URA3* copy. Transformants from SC-TRP plates were replica-plated on SC-URA plates, and the ratio between SC-URA/SC-TRP growing colonies represented the fraction of DSB repair by inversion. The inversion ability of each strain was calculated by normalizing inversion data, assigning the 100% of the ability to the wild-type strain. FAY1Δ1 and FAY1Δ2 strains showed an appreciable decrease in the ability to repair DNA DSBs by inversion in comparison to the wild-type FAY1 strain (Fig. 2b). To confirm that this behaviour was not strain-dependent, the same experiment was repeated with another diploid strain CB89, and its *RVB1* and *RVB2* heterozygous mutants, CB89Δ1 and CB89Δ2, respectively. For this strain we also created a mutant heterozygous for both genes, CB89Δ1Δ2. Indeed, heterozygous strains again showed haploinsufficiency in the ability to repair DNA DSB by inversion in comparison to the wild-type CB89 strain (Fig. 2b). In addition, CB89 mutants showed larger decreases in this ability than FAY1 mutant strains (Fig. 2b). No synergistic effect was observed for CB89Δ1Δ2 mutant whose ability was comparable to that of CB89Δ1 and CB89Δ2, indicating that both genes operate in the same pathway. Strains heterozygous for *RVB1* and *RVB2* genes also showed decreased ability to repair damage caused by UV irradiation (Fig. 2c, in which the UV survival data were normalized by assigning the value of 100% repair ability to the wild-type strains). The deletion of *RVB1* resulted in ~50% decrease, and the deletion of *RVB2* caused a milder, but still significant, decrease in the UV damage repair ability in both diploid strains (Fig. 2c). Comparable repair ability between the strain that is heterozygous for both genes (CB89Δ1Δ2) and those heterozygous for only one gene (CB89Δ1 and CB89Δ2) indicated that *RVB1* and *RVB2* genes are epistatic for UV damage repair as well. Taken together, these data indicate that both Rvb proteins are required, and act together in DNA DSB repair by inversion, and UV damage repair in yeast.

Interestingly, CB89Δ2 and CB89Δ1Δ2 mutants displayed a strong, while CB89Δ1 only displayed a weak, flocculation phenotype in both YPD and synthetic-complete medium (Fig. 2d). The overexpression of RVB2 in the CB89Δ2 transformed with the pVT-2V5 plasmid, and produced moderated flocculation (Fig. 2d). This pointed out RVB2 as responsible for the phenotype. The flocculation phenotype was background-dependent, as it was not noticed for any of FAY1 mutant strains. This indicates that CB89 strain carries an additional mutation which, in combination with the decreased amount of Rvb2p, is causing flocculation. At the moment we can only note that further decrease in ability to repair DNA DSB by inversion in the CB89 mutants, compared with that of FAY1 mutants, could coincide with the same mutation that is causing the flocculation phenotype.

Expression and localization of Rvb1p and Rvb2p is not changed in response to UV damage

To study the behaviour of Rvb1p and Rvb2p during DNA damage response we followed the expression of both Rvb proteins after UV irradiation in the FAY1 cells, in which RVB1 was tagged with the V5 epitope at its chromosomal locus. No change in expression was observed for either protein at 2, 5 or 8 h after irradiation (Fig. 3a). This lack of inducibility would support the haploinsufficiency phenotype, indicating that the decrease of Rvb1p and Rvb2p is indeed a limiting factor for repair. A number of proteins involved in DNA damage signalling and repair, including those in MRE11 complex (Maser *et al.*, 1997), γ-H2AX (Paull *et al.*, 2000) and BRCA1 (Scully et al., 1997a) in human or Rad52p (Lisby *et al.*, 2001) in yeast, re-localize and form subnuclear foci during the cell's response to DNA damage or replication blocks. To investigate whether Rvb1p and Rvb2p change their nuclear distribution in response to UV irradiation, the localization of both Rvb-yEGFP tagged variants in FAY1 cells was followed by fluorescent

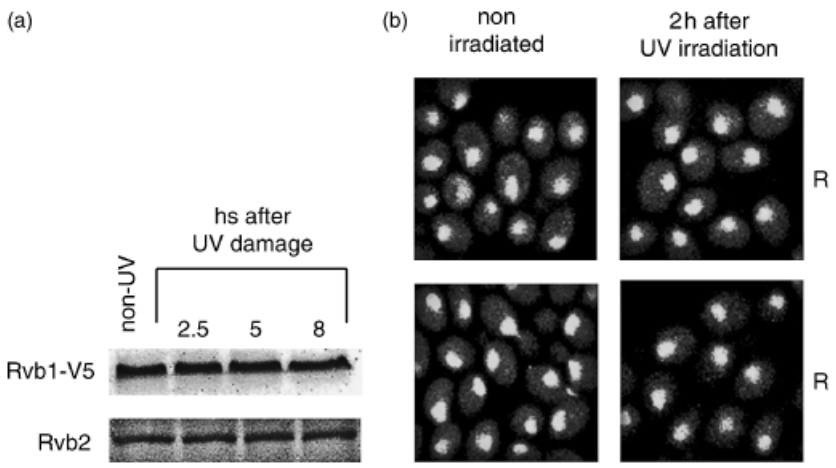

Fig. 3. Expression and localization of Rvb1p and Rvb2p after UV irradiation. (a) Western blot of lysates made from FAY1 cells (in which *RVB1* was tagged with V5 at its chromosomal locus), at 2.5, 5 and 8 h after UV irradiation, or from nonirradiated cells. Five μg of proteins were loaded for each time point. (b) Fluorescent microscopy of FAY1 cells (in which RVB1, or RVB2 were tagged with yEGFP at their chromosomal loci), 2 h after UV irradiation, and in nonirradiated cells.

microscopy at 5, 15, 30, 60 and 120 min after exposure to UV irradiation. The nuclear distribution of Rvb1- and Rvb2-yEGFP proteins was homogenous, comparable to that of nonirradiated cells even after 2 h (Fig. 3b) and remained the same at all observation times (data not shown).

Overexpression of bacterial RuvAB complex improves survival of the yeast strains after UV irradiation

Because *ruvA*, *ruvB* and *ruvC* genes are required in bacteria for cellular resistance to UV damage (Mezard *et al.*, 1999), we investigated whether the expression of the *B. subtilis* bacterial genes could restore normal cellular UV response in *RVB1* and *RVB2* heterozygous deletant strains. Wild-type FAY1 and its heterozygous strains, FAY1Δ1 and FAY1Δ2, were transformed either with vector pVT100-U or with plasmid pVT-1V5, pVT-2V5, pVT-BV5 or pVP-AHSV, or cotransformed with pVT-BV5 and pVP-AHSV plasmids. The nuclear localization of the NLS-RuvA-HSV and NLS-RuvB-V5 proteins was confirmed by Western blotting performed on nuclear protein extracts (data not shown). Interestingly, UV-irradiated wild-type FAY1 cells co-expressing bacterial RuvA or RuvB showed higher UV resistance than the FAY1 cells carrying the vector alone (Fig. 4). Moreover, the bacterial RuvAB complex increased the survival of the FAY1Δ1 and FAY1Δ2 strains to the levels of the FAY1Δ1 and FAY1Δ2 overexpressing *RVB1* and *RVB2*, respectively (Fig. 4). On the other hand, overexpression of the RuvA alone did not have any effect, while overexpression of the RuvB alone slightly improved the ability of *RVB1* heterozygous deletant strain to repair UV damage.

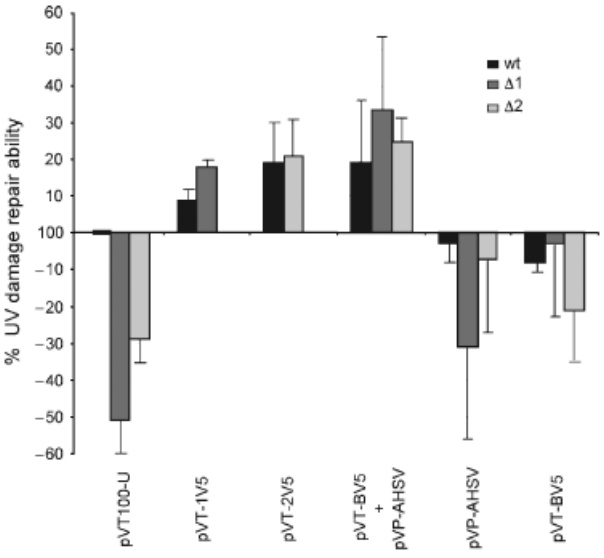

Fig. 4. Effect of NLS-RuvA-HSV and NLS-RuvB-V5 on UV irradiated FAY1 and its *RVB1* and *RVB2* heterozygous deletant strains. Cells were transformed with the plasmids indicated, and assayed for colony formation before and after UV irradiation as described in 'Materials and methods'. The 100% of UV damage repair ability is assigned to the wild-type strain transformed with empty vector pVT100-U.

Encouraged by the improved UV survival caused by the RuvAB complex, we asked whether the expression of bacterial genes could functionally complement *rvb1Δ* and *rvb2Δ* deletions. FAY1Δ1 and FAY1Δ2 strains were transformed with pVT-BV5 alone, or in combination with pVP-AHSV. Cells were induced to sporulate, and subsequent dissection of 20 tetrads resulted in 2 : 2 segregation for viability for all FAY1Δ1 and FAY1Δ2 transformants. Furthermore, PCR of the chromosomal *RVB1* or *RVB2* loci in viable spores coming from the FAY1Δ1 or FAY1Δ2 transformants, respectively, amplified only the wild-type band (data not shown). On the other hand, control transformation with plasmids, pVT-1V5 and pVT-2V5, rescued *rvb1Δ* and *rvb2Δ* deletions respectively, resulting in a 4 : 0 segregation for viability following meiosis. For all tetrads tested, in two spores it was possible to amplify by PCR the wild-type band, and in the other two, the deletion band. Thus the yeast *RVB1* or *RVB2* genes cannot be functionally complemented either by bacterial *ruvB* alone or in its combination with *ruvA*.

Rvb2p, unlike Rvb1p, preferentially binds to Holliday junction-like structure

Bacterial RuvB binds to HJ by forming a complex with the HJ-binding protein RuvA (West, 1997). The human TIP60 complex, which contains both Rvb orthologs, shows affinity for the structural DNA that mimics cruciform (Ohdate *et al.*, 2003). To study the ability of the two RuvB-like proteins of yeast to bind HJ, we developed an *in vitro* cell-free binding assay in which Rvb2p was, unlike Rvb1p, preferentially bound to synthetic HJ. Because the majority of Rvb1 and Rvb2 proteins were bound to the chromatin (Fig. 1a), we overexpressed each protein by transforming FAY1 cells with the pVT-1V5 or pVT-2V5 plasmids. Exponentially growing cells were used to isolate nuclear extracts, and the binding to duplex DNA or to cruciform X26 substrate was performed as described in 'Materials and methods'. Bound proteins were eluted by increasing salt concentration, and the presence of Rvb1-V5 and Rvb2-V5 proteins in eluted fractions was analyzed by Western blotting. Simultaneously, the same binding assays were performed with the nuclear extract coming from the strain that was co-expressing bacterial NLS-RuvA-HSV and NLS-RuvB-V5 proteins. The interaction of the bacterial proteins with both DNA substrates was used as a measure of binding specificity. In our assay, as expected, bacterial RuvA bound more specifically to X26 than to the duplex DNA, being eluted at higher salt concentrations (600 mM compared with 300 mM) (Fig. 5). Bacterial RuvB, which is targeted to the

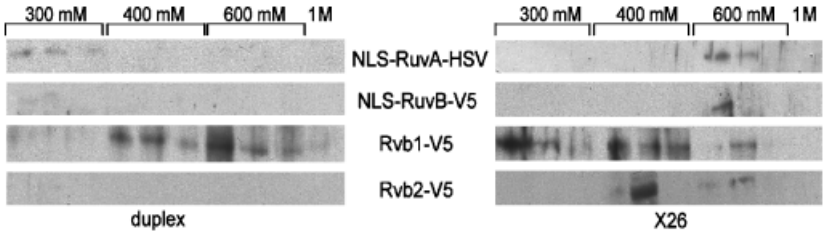

Fig. 5. Binding specificity of Rvb1p and Rvb2p to duplex or to cruciform (X26) DNA compared with bacterial RuvAB complex. Western blot of fractions eluted in cell-free protein–DNA binding assay, performed as described in 'Materials and methods', with nuclear extracts from FAY1 cells coexpressing NLS-RuvA-HSV and NLS-RuvB-V5, or overexpressing either Rvb1-V5 or Rvb2-V5. The salt concentrations of the various fractions are indicated.

HJ by RuvA, elutes together with it (Fig. 5). A similar elution dynamics was observed for Rvb2p. However, the yeast protein eluted in two peaks, one at 400 mM, which contained the majority of the protein, and the other at 600 mM (Fig. 5). The binding of this protein to duplex DNA was detected only in the fractions eluted with low salt concentrations (below 300 mM, data not shown). On the other hand, Rvb1p was tightly binding to duplex and to X26 substrates, indicating that it binds to the DNA regardless of its structure (Fig. 5).

Discussion

Regulation of RVB1 and RVB2 expression

The expression of *RVB1* and *RVB2* in yeast is not cell-cycle regulated (Fig. 1a), similarly to what has been observed for human *RVB1* ortholog after release from an M phase block by nocodazole (Qiu *et al*., 1998). However, it was reported that both genes are induced within 3 h of serum stimulation of quiescent rat cells, even though the progression through the cell cycle does not change the level of expression after the initial induction (Jonsson *et al*., 2001). This discrepancy could be explained by the state of the cells used in the experiment. While no cell cycle regulation is observed for the growing (log phase) cells, the cells that are stimulated to resume division show cell cycle regulation. This suggests that *RVB* genes are required for actively dividing cells, while they are repressed during the stationary phase. It is likely that both genes are expressed at their constant level during the cell cycle, as the heterozygous deletion caused a ~50% decrease in expression of deleted gene (Fig. 2a). In addition, lack of inducibility observed after UV irradiation or DNA DSB formation supports this hypothesis. Still, little is known about the mechanism that regulates *RVB1* and *RVB2* expression. It was reported that, in humans, the expression of both genes partially depends on c-Myc expression, as c-*myc* null cells have three- to four-fold reduction in both mRNAs (Jonsson *et al*., 2001). Interestingly, we observed that the expression of both *RVB* genes is negatively self-regulated and a similar regulation of expression was observed for the human *Xeroderma pigmentosum* group B 3′–5′ helicase (XPB), which is one of the core subunits of the TFIIH complex (Hoogstraten *et al*., 2002).

Haploinsufficiency and functional comparison to bacterial homologues

Certain aberrant phenotypes become evident when one gene of a homozygous pair is deleted. This phenomenon, caused by insufficient concentration of the gene product in the cell, is known as haploinsufficiency (Giaever *et al*., 1999). *RVB1* and *RVB2* heterozygous strains showed a haploinsufficiency effect on recombination and UV damage repair. However, the growth rate of heterozygous strains was comparable to that of the wild type, indicating that the effect is not reflecting on impaired growth. The correspondence between the Rvb1p and Rvb2p involvement in recombination and UV damage repair in yeast and bacterial RuvB functions is striking; in particular, the yeast increases its UV resistance due to expression of the bacterial RuvAB complex. This is consistent with the knowledge that the expression of bacterial genes involved in DNA repair and recombination, such as RecA, RuvC and RusA, can stimulate similar processes in eukaryotes (Shalev *et al*., 1999; Doe *et al*., 2000). This effect (Fig. 4) might result from branch migration activity that could unwind irregular structures in DNA, such as cruciforms and hairpins, to facilitate DNA repair. The complex could also target stalled and regressed replication forks caused by UV irradiation, and mediate their resolution by recruiting RuvC-like yeast endonuclease. However, RuvB alone, or in a complex with RuvA, failed to fully complement either *RVB1* or *RVB2* deletion. These later results are not surprising, as it has been reported that even mammalian RVB1 and RVB2 orthologues cannot complement the deletion of either of the two yeast genes (Qiu *et al*., 1998; Lim *et al*., 2000), although they share 70% of sequence identity. This failure in complementation may reflect a requirement for additional partners, which cannot be recognized by 'foreign' homologues, or the involvement of yeast proteins in other, nonoverlapping, pathways essential for the cell. Although the bacterial RuvAB complex is able to abolish the UV sensitivity of *RVB1* and *RVB2* heterozygous strains, this,

of course, does not prove that eukaryotic and bacterial proteins function equally during UV damage response. The possibility that the Rvb yeast proteins may be involved in UV damage repair through transcriptional regulation of genes of the response is not excluded. In human cells for example, Rvb1p and Rvb2p interact with c-Myc, whose putative targets are also genes related to DNA repair, such as *APEX/Ref1*, *BRCA1*, *MSH2* (Menssen & Hermeking, 2002) and *NBS1* (Chiang *et al.*, 2003). The mechanism by which Rvb proteins would perform this activation may relate to its role in chromatin remodeling complexes (Jonsson *et al.*, 2001). On the other hand, chromatin remodeling could by itself facilitate DNA repair (Frit *et al.*, 2002; Gaillard *et al.*, 2003). One could hypothesize that Rvb1p and Rvb2p function similarly to their bacterial homologues, but that they acquired additional functions adapting to the need of remodeling the chromatin environment through recruitment of factors such as Aar5p (Jonsson *et al.*, 2004).

Rvb2p binds to cruciform DNA

In all previous studies, Rvb1p and Rvb2p failed to interact with, or branch migrate, HJ (Qiu *et al.*, 1998; Ikura *et al.*, 2000). However, each time, the pure proteins were used for the reactions. Given that RuvB requires RuvA for its binding and helicase activities under physiological conditions (West, 1997), it is probable that they need to interact with additional link-proteins to bind HJ. It was reported that human TIP60 complex, which contains, among other proteins, hRvb1p and hRvb2p, binds to three- and four-way junctions (Ohdate *et al.*, 2003). The TIP60 complex could be anchored to the secondary structures of DNA through Rvb2p interaction with the 'link'. Further experiments will be required to determine the factor(s) responsible for Rvb2p binding to the DNA cruciform structure. Although Rvb1p and Rvb2p interact and form a complex, it is important to emphasize that exclusively Rvb2p bound specifically to HJ. In addition to their antagonistic role in transcriptional activation (Bauer *et al.*, 2000; Cho *et al.*, 2001), differential binding to the DNA structures could be one of the crucial differences between the two Rvb proteins which, despite being so similar, are not redundant.

Rvb1p, Rvb2p-chromatin remodeling in DNA repair and recombination

Our results indicate that *RVB1* and *RVB2* are required for recombination and UV damage repair. Both genes are also implicated in transcription on other grounds (Jonsson *et al.*, 2001; Ohdate *et al.*, 2003). However, involvement in transcription does not exclude their possible roles in recombination and repair processes, especially if we take into consideration the chromatin structure as a barrier that must be overcome for both processes to occur. Indeed, an increasing amount of evidence has recently demonstrated the requirement for chromatin remodeling complexes in recombination and DNA repair, in addition to their already well-established role in transcription (Fyodorov & Kadonaga, 2001; Alexiadis & Kadonaga, 2002; Bird *et al.*, 2002; Green & Almouzni, 2002; Narlikar *et al.*, 2002). The Rvb1p/Rvb2p complex, isolated from yeast, shows an ATP-dependent chromatin remodeling activity *in vitro*, which is comparable to that of Swi2/Snf1 complex (Jonsson *et al.*, 2001); both Rvb proteins are also found in the yeast Ino80 (Jonsson *et al.*, 2004) chromatin remodeling complex. On this subject, it is worth noting that deletion of *RVB2* in combination with an additional mutation caused a flocculation phenotype (Fig. 2d), as it is known that the expression of *FLO1*, a dominant and best-characterized flocculation gene, is regulated by antagonistic chromatin remodeling activities of the Tup1-Ssn6, and Swi-Snf complexes (Fleming & Pennings, 2001). Moreover, it was reported that a Rvb2 *ts* mutant represses the expression of *DIA3* (Ohdate *et al.*, 2003), whose protein product regulates expression of another flocculation gene *FLO11* (Palecek *et al.*, 2000). The requirement of an additional mutation of the strain suggests the possibility of complex formation. Transcription of rec/repair genes, or changes in chromatic accessibility to them, could be the indirect method of action of *RVB1* and *RVB2* in recombination and repair of DNA damage.

Rvb1p – Rvb2p as a multifunctional complex

Human Rvb orthologues have been isolated in the RNA polymerase II (RNAP2) holo-enzyme complex (Qiu *et al.*, 1998). RNAP2 holo-enzyme in human cells contains DNA repair factors such as BRCA1 (Scully et al., 1997b), hRad52 (Liu *et al.*, 2002), Ku70, DNA-PKcs, RPA, RFC and hRad51 (Maldonado *et al.*, 1996). It was proposed that, in this way, RNAP2 forms a completely assembled, ready-to act multifunctional complex that roams the nuclear space in search for either promoters or DNA damage, and the presence of Rvb1p-Rvb2p ATPase could be useful for its functions. On the other hand, Rvb1p and Rvb2p could act similarly to a transcriptional cofactor with the most obvious analogy, TFIIH. This multi-protein complex, of which two subunits have intrinsic helicase activity with opposite polarity, functions both as a general transcription factor for RNAP1 and RNAP2 and in nucleotide excision repair (Drapkin *et al.*, 1994; Sung *et al.*, 1996). The differential behavior of TFIIH in transcription and repair has been explained by the distinct complex composition in each process (Svejstrup *et al.*, 1995), specific modification of its subunits (van Oosterwijk *et al.*, 1998), and the ability to randomly get access to sites where it becomes transiently engaged in one of its transactions by free diffusion

(Hoogstraten *et al*., 2002). As already proposed, Rvb1p and Rvb2p, showing a 6 : 1 stoichiometry, compared with other polypeptides in their complexes (Jonsson *et al*., 2001), could form a hetero-hexamer (or a hetero-dodecamer) core of a larger complex (Jonsson *et al*., 2004). The ability of the complex to recognize DNA secondary structures could facilitate progression of RNAP2, as well as DNA repair using ATP as an energy source to overcome structural barriers. In addition, the core Rvb1p-Rvb2p ATPase could associate with different proteins, and form several different complexes that would be involved in transcription and/or preservation of genomic integrity.

The definition of the intriguing mechanism(s) by which yeast Rvb1p and Rvb2p influence cellular processes as diverse as transcription and recombinational DNA repair warrants further investigation.

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