Scuola Internazionale Superiore di Studi Avanzati - Trieste

Functional and Molecular Characterization of *Saccharomyces cerevisiae* RuvB-like proteins Rvb1 and Rvb2

Thesis Submitted for the Degree of
Doctor Philosophiae

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Academic Year 2002/2003

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Abbreviations

AAA+ ATPase	ATPase associated with various cellular activities
ARR	access, repair, restore
BER	base excision repair
BIR	break-induced replication
BIR/SSA	break-induced replication associated with the single-strand annealing
CPD	cyclobutane pyrimidine dimmer
DSB	double-strand break
DSBR	double-strand break repair
EGFP	enhanced green fluorescent protein
GG-	global genome repair
HAT	histone acetyltransferase
HD	histone deacetylase
HJ	Holliday junction
HR	homologous recombination
MMS	methyl methanesulfonate
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NLS	nuclear localization signal
RNAP1	RNA polymerase I
RNAP2	RNA polymerase II
RPA	replication and repair protein A
SDSA	synthesis-dependent strand annealing
snoRNP	small nucleolar RNA-protein complex
SSA	single-strand annealing
TBP	TATA-binding interacting protein
TC-	transcription-coupled repair
6-4PP	pyrimidine (6-4) pyrimidone photoproducts

CHAPTER 1

Introduction

1 Introduction

1.1 DNA damage repair

Cellular DNA is continuously being exposed to a variety of environmental and endogenous agents that can cause its damage. These potentially lethal or mutagenic DNA lesions induce various cellular responses including cell cycle arrest, transcription alteration, and processing by different DNA repair mechanisms. The choice of the repair mechanism depends on the structural features of the lesion, and the particular phase of the cell-cycle at which it is acting.

Nucleotide excision repair (NER) is a versatile DNA repair pathway that removes a wide range of DNA lesions, including the main UV-light induced lesions, i.e., cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs), as well as lesions induced by chemicals (Friedberg, 1995). The process of NER is highly conserved in eukaryotes and consists of the following four steps: (a) recognition of the damaged DNA; (b) excision of an oligonucleotide of 24-32 residues containing the damage from DNA by dual incision of the damaged strand on each side of the lesion; (c) filling in of the resulting gap by DNA polymerase; and (d) ligation of the nick (Batty and Wood, 2000; de Laat *et al.*, 1999). In human cells, NER reaction requires at least six core protein complexes for damage recognition and dual excision (XPA, XPC-hHR23B, RPA, TFIIH, XPG and XPF-ERCC1) and other factors for DNA repair, synthesis and ligation (PCNA, RFC, DNA polymerase α or δ and DNA ligase) (Aboussekhra *et al.*, 1995; Araujo *et al.*, 2000; Mu *et al.*, 1995). NER consists of two sub-pathways termed global genome repair (GG-NER) that is transcription-independent and removes lesions from the

entire genome, and transcription-coupled repair (TC-NER) (de Laat *et al.*, 1999; Friedberg, 1995). 6-4PPs, which distort DNA more than CPDs, are removed rapidly, predominantly by GG-NER. In contrast, CPDs are repaired very slowly by GG-NER and are removed more efficiently from the transcribed strand of expressed genes by TC-NER (van Hoffen *et al.*, 1995). The elongation transcriptional machinery is though to facilitate the recognition of DNA lesions on the transcribed strand in TC-NER. However, detailed mechanisms of TC-NER still remain undefined. The importance of transcription-coupled repair is not limited only to NER. Oxidative damage, mainly processed by the base excision repair (BER), is also removed in a transcription-coupled manner (Cooper *et al.*, 1997; Le Page *et al.*, 2000). In human cells, BER proceedes via two alternative pathways, either "short-patch" DNA polymerase β-dependent pathway (Kubota *et al.*, 1996), which involves the replacement of a single nucleotide, or "long patch" PCNA-dependent pathway (Klungland and Lindahl, 1997; Matsumoto *et al.*, 1994) which involves the replacement of up to six nucleotides.

While NER and BER mostly repair lesions that affect only one of the DNA strands, other mechanisms deal with the double-strand breaks (DSBs) which can result as a consequence of ionizing irradiation and exposure to other DNA damaging agents, replication fork collapse, mechanical stress, or processing of a single-stranded nicked chromosome. One of the mechanisms is non-homologous end joining (NHEJ), a pathway exclusively dedicated to the repair of DSBs. DNA ends are joined with little or no base pairing at the junction and the end-joining products may be accompanied with insertions or deletions. In *Saccharomyces cerevisiae* NHEJ requires the end-binding factor Hdf1p-Hdf2p (human Ku70p-Ku80p) (Chen *et al.*, 2001; Feldmann *et al.*, 1996), and a specific

DNA ligase, Dnl4p, with its cofactor Lif1p (Chen *et al.*, 2001; Herrmann *et al.*, 1998; Schar *et al.*, 1997; Teo and Jackson, 1997; Wilson *et al.*, 1997; Wood *et al.*, 2001). Other proteins are needed in the end-joining reaction but not necessarily in all organisms. The Rad50p-Mre11p-Xrs2p complex is required for NHEJ in budding (Boulton and Jackson, 1998; Moore and Haber, 1996), but not in fission yeast, nor in vertebrate cells (Harfst *et al.*, 2000; Manolis *et al.*, 2001; Wilson *et al.*, 1999; Yamaguchi-Iwai *et al.*, 1999). Recently, a Lif1p-interacting factor, Lif2p (also called Nej1p) was shown to be essential for NHEJ in *S. cerevisiae*, but Lif2p orthologs in other organisms remain to be found (Frank-Vaillant and Marcand, 2001; Kegel *et al.*, 2001; Ooi *et al.*, 2001; Valencia *et al.*, 2001). NHEJ is though to be a prevalent mechanism of repair in higher eukaryotes, whereas the major mechanism of DNA DSB repair in vegetatively growing yeast cells is homologous recombination (HR). This needs a homologous sequence somewhere else in the cell genome to repair the lesion. HR is a general term that includes multiple mechanisms (the most accepted are shown in Figure 1) (Kupiec, 2000; Paques and Haber, 1999; Prado *et al.*, 2003; Symington, 2002; Sung *et al.*, 2000). Double strand break repair (DSBR) was the first model to explain the genetic relationship between gene conversion (non-reciprocal transfer of information from one DNA molecule to another) and crossover (reciprocal transfer of information between the two DNA molecules) during recombination (Szostak *et al.*, 1983). Recombination is initiated by the resection of 5' ends at the each side of the DSB, exposing long 3'-ended single-stranded tails (Sun *et al.*, 1991), which invade the homologous double-strand DNA (dsDNA) and prime DNA synthesis. After pairing of the invading single-stranded DNA (ssDNA) and the invaded dsDNA, the reaction is followed by strand exchange. This generates a DNA heteroduplex

(Goyon and Lichten, 1993; Nag and Petes, 1993), which is a key feature for gene conversion in all recombination reactions. The double-strand exchange leads to the formation of two four-stranded intermediates, termed Holliday junctions (HJs) (Collins and Newlon, 1994; Schwacha and Kleckner, 1994). The cleavage of these two HJs in either the same or the opposite orientation results in non-crossover or crossover products, respectively.

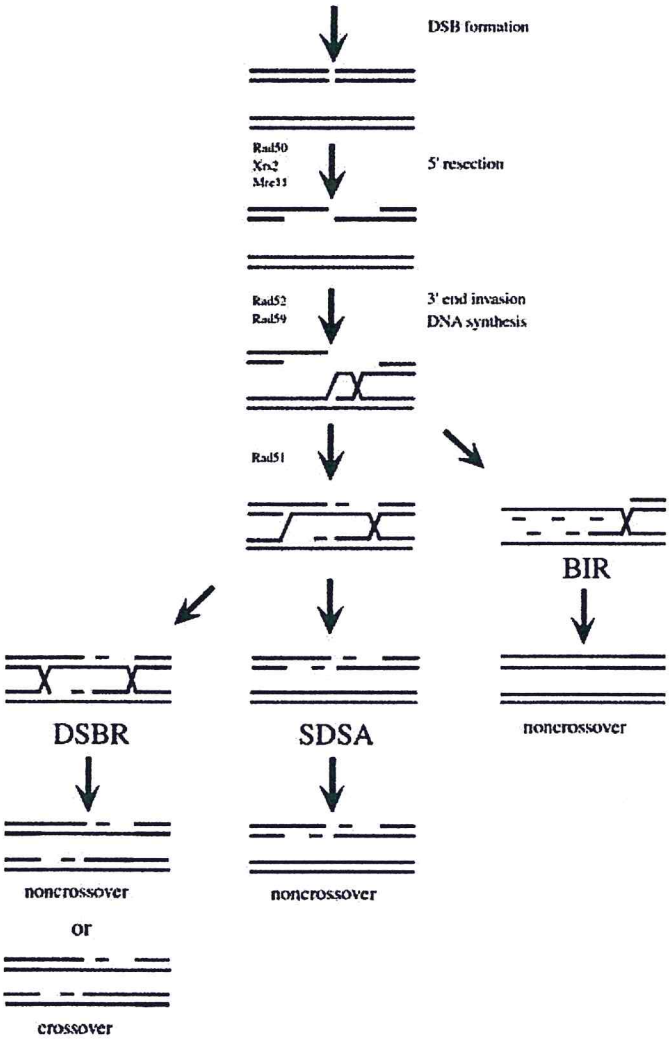

Figure 1. Mechanisms of HR. Initiation of recombination by a double-strand break (DSB) is followed by resection, invasion and DNA synthesis steps common to all mechanisms (upper part). In the double-strand

break repair (DSBR) mechanism, two cruciform or Holliday junctions (HJs) are formed, resolution of which leads to gene conversion whether or not associated with crossovers. In the synthesis-dependent strand annealing (SDSA) mechanism all outcomes are noncrossovers. In the break-induced replication (BIR) mechanism, the newly replicated DNA progresses along the rest of the invaded molecule. Each line represents a single-strand DNA molecule. From Prado et al. (2003).

However, it has been shown that many mitotic gene conversion events are not associated with crossovers (Gloor *et al.*, 1991; Johnson and Jasin, 2000; Nassif *et al.*, 1994; Virgin *et al.*, 2001). These findings led to an alternative model termed synthesis-dependent strand annealing (SDSA) (McGill *et al.*, 1989), in which, after 3' strand invasion, the newly synthesized DNA strands are released from the invaded DNA template and returned to the acceptor molecule. The third model, break-induced replication (BIR) has been proposed as an explanation for the co-conversion of markers distant up to several hundred kilo-bases in yeast (Malkova *et al.*, 1996; Voelkel-Meiman and Roeder, 1990). BIR repair occurs by invasion of the donor duplex by the broken chromosome, followed by replication to the end of the donor chromosome.

In addition to the models described above, repair of the DSBs in the DNA with repetitive homologous sequences introduces further recombination pathways whose occurrence depends on the orientation of the repeats. Direct-repeat recombination leads to gene conversion and/or to the deletion of the intervening region and one of the repeats. Deletion can result from crossover between the repeats via DSBR, or single-strand annealing (SSA) (Lin *et al.*, 1984). In SSA, upon DNA resection of 5'-ended tails, the exposed homologous sequences are annealed and the overhanging 3'-ended tails are removed (Figure 2).

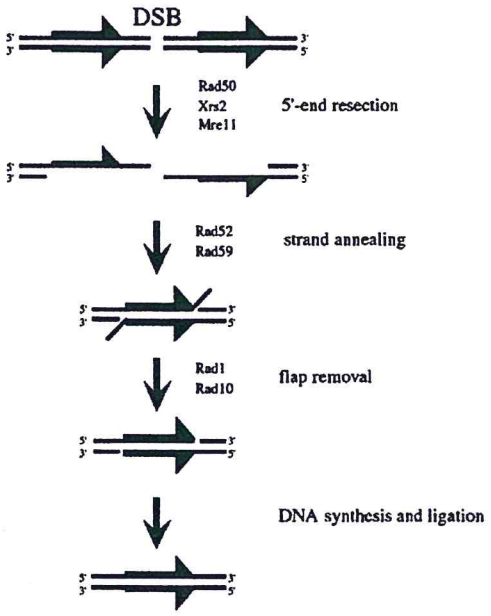

Figure 2. In the SSA model, a DSB made between direct repeats is subject to resection to generate 3' single-stranded tails. When complementary sequences are exposeed due to extensive resection, the single-stranded DNA anneals, resulting in deletion of one of the repeats and the intervening DNA. The 3' tails are endonucleolytically removed, and the nicks are ligated. The 3' ends are indicated by arrowheads. From Prado et al. (2003).

On the other hand, recombination between inverted repeats may lead to gene conversion of one of the repeats and/or inversion of intervening sequence. Inversion can be generated by reciprocal exchange via DSBR, but also by a BIR associated with an SSA event. In the later case, BIR that occurred between inverted repeats, leads to their duplication, and creation of internal direct repeats, which could be processed via SSA. Depending on the annealing of the direct repeats, the recombination event would or would not be associated with the inversion (Figure 3) (Kang and Symington, 2000; Malagon and Aguilera, 2001).

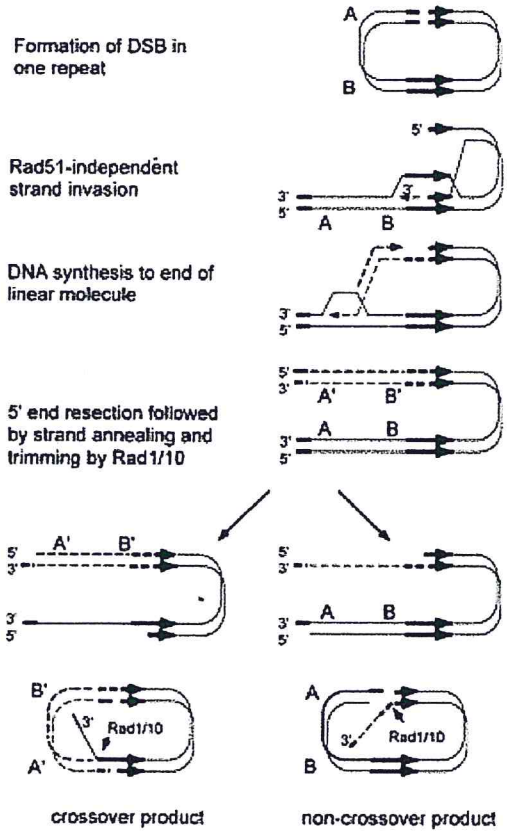

Figure 3. BIR/SSA model for repair of DSBs within inverted repeat plasmids. After formation of the DSB, ends are processed and one end invades the other repeat (drawn here as an intramolecular event, but it could also occur intermolecularly). If DNA synthesis extends to the end of the molecule, small repeats will be present at both ends of the linear molecule. Following resection of the ends, the repeats at either end of the molecule can pair with an internal repeat by SSA, generating either a noncrossover or an apparent crossover products. From Symington (2002).

Most of the genes involved in HR belong to *RAD52* epistasis group (*RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *RDH54*, *MRE11*, and *XRS2*) (Paques and Haber, 1999). Rad50p-Mre11p-Xrs2p multifunctional complex creates single-stranded 3'-tails. However, redundant nuclease activity may be involved in this step since mutation of the nuclease activity of Mre11p hardly affects the repair of DSBs induced by

the HO endonuclease (Moreau *et al.*, 1999; Moreau *et al.*, 2001). Rad52p, essential for most if not all recombination processes (Paques and Haber, 1999), and its partial homolog Rad59p, both having strand-annealing activity *in vitro*, are proposed to perform DNA invasion (Petukhova *et al.*, 1999a). Rad51p is the functional and structural homologue of bacterial DNA strand exchange protein RecA (Aboussekhra *et al.*, 1992; Shinohara *et al.*, 1992). Rad54p and Rdh54p belong to the SWI2/MOT1 family of ATP-dependent chromatin-remodelling proteins (Shinohara *et al.*, 1997) and stimulate Rad51-dependent pairing *in vitro* (Petukhova *et al.*, 1999b; Petukhova *et al.*, 2000). Finally, Rad55p and Rad57p are also proposed to participate in the strand-exchange reaction (Symington, 2002).

Molecular and biochemical studies of the Rad52 group proteins have shown that most are required at early steps during recombinational repair. Surprisingly, yeast does not have the X-ray-sensitive mutant defective for HJ resolution. This suggests that (i) redundant activities exsist in eukaryotes, (ii) it is an essential activity, or (iii) HJ resolution is not obligatory for recombinational repair in yeast as discussed previously (Figure 1). However, some mitotic recombination events do result in reciprocal exchange, and integration of linearized plasmids into the genome is thought to occur by resolution of HJs as predicted by DSBR model (Figure 1). Branched-DNA molecules have been detected during mitotic S phase within the tandemly repeated rDNA locus, and formation of these intermediates is dependent on *RAD52*, suggesting that they correspond to recombination intermediates (Zou and Rothstein, 1997). Postreplicative, DNA replication-dependent X-shaped molecules have also been detected between sister chromatids in *Physarum polycephalum* suggesting that resolution of HJs could be

essential for chromosome segregation in eukaryotes (Benard *et al.*, 2001). In prokaryotes RuvA/RuvB and RuvC/RusA, perform branch migration and resolution of HJs, respectively. In eukaryotes, biochemical approaches have identified three HJ-resolving activities from fractioned extracts of mitotic cells. Of these, Ccelp is mitochondrial and is important for the segregation of mitochondrial genome (Symington and Kolodner, 1985). Other two are nuclear. The Mus81p-Mms4p heterodimer was recently shown to cleave four-branched DNAs. However, its preferred substrate are structures related to a stalled replication fork (Constantinou *et al.*, 2002; Doe *et al.*, 2002). Finally, a novel HJ resolvase, named Resolvase A, which co-fractionates with a branch migration activity in HeLa cells extracts, shows high specificity for HJs. Yet, Resolvase A gene identification is still under study (Constantinou *et al.*, 2002). As resolution, the branch migration activity has been detected in cell-free extracts as well (Constantinou *et al.*, 2001), nonetheless the protein(s) that perform this activity has not been identified.

1.2 RuvA and RuvB: Holliday junction processing proteins

Processing of HJs by bacterial RuvA, RuvB and RuvC proteins has been studied in detail. Biochemical studies have shown that bacterial RuvB protein (37 kDa) forms a hexamer that has 5' to 3' ATP-dependent helicase activity, and functions as a motor for branch migration of HJs (Adams and West, 1995; Stasiak *et al.*, 1994; Tsaneva *et al.*, 1993). However, it requires the HJ binding protein RuvA (22 kDa) for DNA binding and the helicase movement (Egelman, 1996; Parsons and West, 1993; Tsaneva *et al.*, 1993; Tsaneva and West, 1994). In the RuvAB-HJ complex, one RuvB hexamer is located on either side of RuvA tetramer, with the DNA passing through the center of each hexameric

ring (Parsons *et al.*, 1995) (Figure 4). In addition to their role in catalysing branch migration, the RuvAB proteins cooperate with RuvC endonuclease to promote resolution of HJ (van Gool *et al.*, 1998; Zerbib *et al.*, 1998).

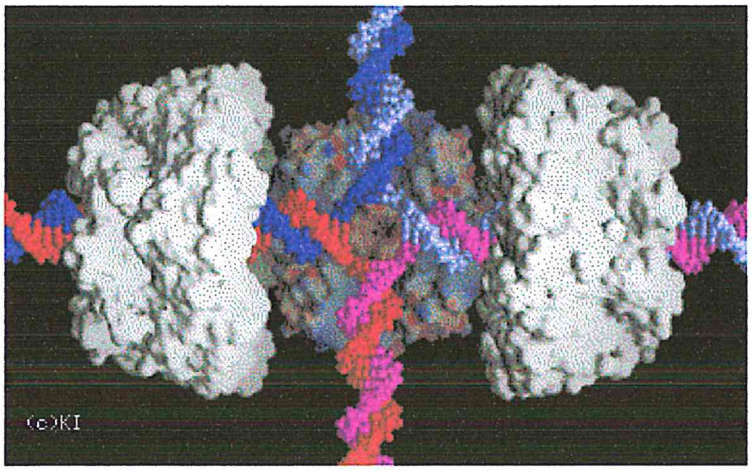

Figure 4. RuvA, RuvB and DNA complex

A simple model of a RuvA-RuvB-DNA complex in agreement with the electron microscopy results of Parsons et al. (1995). RuvA binds the Holliday junction at the central crossover point (depicted in gray) and targets two RuvB hexamers onto opposite arms of the DNA (depicted in white) where they encircle the DNA duplexes and facilitate branch migration in concert with RuvA in an ATP dependent manner. From http://www.sdsc.edu/journals/mbb/ruva.html

In *E.coli*, the *ruvA*, *ruvB*, and *ruvC* genes are required for normal levels of cellular resistance to the effects of UV- or ionizing-irradiation, or to the harmful effects of DNA-damaging agents such as mitomycin-C (Lloyd *et al.*, 1984; Mezard *et al.*, 1999; Otsuji *et al.*, 1974; Stacey and Lloyd, 1976). Their mutants are also mildly defective in recombination (Lloyd *et al.*, 1987). RuvB belongs to the class of hexameric helicases that are member of a larger family of proteins known as the AAA^+ ATPases (ATPases

associated with various cellular activities) (Neuwald *et al.*, 1999). AAA+ family members (for review see Patel and Latterich, 1998) were categorized as proteins with one, or two copies of a well conserved 230-250 amino acid cassette called the AAA module that encompasses the Walker A, and Walker B motifs, active sites for ATP (or dNTP) binding and hydrolysis.

1.3 Eukaryotic RuvB-like proteins

The first eukaryotic protein that shared similarity to RuvB, was isolated from rat liver as a 49-kDa protein that interacts with TATA-binding protein (TBP) and was named TIP49 (TBP interacting protein of 49 kDa) (Kanemaki *et al.*, 1997). Apart from Walker A and B motifs, additional sequence similarity with bacterial protein occurs in the N- and C-terminal regions that together comprise about 50% of the overall sequence (Kanemaki *et al.*, 1997). Simultaneously, in human cells the same protein was identified in a two-hybrid interaction screen with the 14-kDa subunit of the DNA replication and repair factor RPA (Qiu *et al.*, 1998). The protein was named RuvBL1 (RuvB-like). Subsequently, its yeast ortholog scRuvBL1 (*S. cerevisiae* RuvB-like protein 1), as well as RuvBL2 a closely related family member, were identified in database searches (Kanemaki *et al.*, 1999; Qiu *et al.*, 1998). From there on, these proteins were discovered independently by groups studying different processes in different organisms, and were named differently. The RuvBL1 orthologs were called Pontin52, ECP-54, NMP238, Tip49, TAP54-α, p55, p50 , TIP49a, Rvb1, and Tih1 (Bauer *et al.*, 1998; Holzmann *et al.*, 1998; Ikura *et al.*, 2000; Kanemaki *et al.*, 1999; Kikuchi *et al.*, 1999; King *et al.*, 2001; Lim *et al.*, 2000; Newman *et al.*, 2000; Salzer *et al.*, 1999; Shen *et al.*, 2000; Wood

et al., 2000). Orthologs of RuvBL2 protein have been simultaneously reported and called with a variety of different names like TIP49b, ECP51, TIP48, Reptin52, TAP54-β, p47, p50, scRuvBL2, Rvb2, and Tih2 (Bauer *et al.*, 2000; Gohshi *et al.*, 1999; Ikura *et al.*, 2000; Kanemaki *et al.*, 1999; King *et al.*, 2001; Lim *et al.*, 2000; Newman *et al.*, 2000; Qiu *et al.*, 1998; Salzer *et al.*, 1999; Shen *et al.*, 2000).

Rvb1p and Rvb2p are extremely related to each other. Their amino acid sequences share 42% of identity and 68% of similarity (Lim *et al.*, 2000; Newman *et al.*, 2000). They are also strongly conserved among the *Eukarya*, and mainly, but not all, *Archaea* that contain only one ortholog (Kurokawa *et al.*, 1999). Despite the strong similarity between two Rvb proteins, Rvb1p and Rvb2p are not redundant, since both proteins are indispensable for *S. cerevisiae* and *Drosophila melanogaster* (Bauer *et al.*, 2000; Kanemaki *et al.*, 1999; Lim *et al.*, 2000; Qiu *et al.*, 1998). Rvb1p and Rvb2p are nuclear proteins (Bauer *et al.*, 1998; Lim *et al.*, 2000; Makino *et al.*, 1998; Newman *et al.*, 2000) with helicase activity of opposite polarity (Kanemaki *et al.*, 1999; Makino *et al.*, 1999), still the later result has been disputed (Ikura *et al.*, 2000). Both proteins hydrolyse ATP, which seems to be essential for their function *in vivo* (Jonsson *et al.*, 2001; Lim *et al.*, 2000; Wood *et al.*, 2000).

Even though Rvb1 and Rvb2 proteins have been subjected to many studies, yet, little is known about their precise function. In light of the findings that they interact with TBP (Kanemaki *et al.*, 1997; Kanemaki *et al.*, 1999), and were components of the large RNA pol II holoenzyme complex (Qiu *et al.*, 1998) their role in transcription was plausible. Jónsson *et al.* (2001) demonstrated that Rvb1p and Rvb2p cooperate, directly or indirectly, in transcription regulation of over 5% of yeast genes. More recent study

identified 34 genes whose transcription is depending on Rvb2p, and showed that both Rvb proteins interact with TBP *in vivo* (Ohdate *et al.*, 2003). Furthermore, in higher eukaryotes they interact with several transcriptional activators. Both mammalian orthologs have been implicated in cell transformation by c-Myc (Wood *et al.*, 2000), whereas *Drosophila* orthologs interact with β-catenin acting antagonistically in the control of Wingless signalling through β-catenin-mediated transactivation (Bauer *et al.*, 2000). In human cells, Rvb1p was shown to bind to the E2F1 transactivation domain, and to modulate its apoptotic activities (Dugan *et al.*, 2002), while Rvb2p regulates ATF2 response to stress and DNA damage (Cho *et al.*, 2001).

On the other hand, roles of Rvb1p and Rvb2p in recombination and repair were implied by the activities of the closest homolog with a known function, eubacterial RuvB, and their interactions with Hdf1p, Msh6p, and Dmc1p proteins involved in NHEJ, mismatch repair, and HR in yeast, respectively (Ho *et al.*, 2002). In addition, the association with RPA (Qiu *et al.*, 1998) supported this hypothesis, implying a possible function in DNA replication as well. Given the similarities shared by transcription, recombination, and repair events at the DNA level it is not surprising that Rvb proteins have been found in the complexes that remodel chromatin. In yeast both proteins were found in INO80 remodelling complex that utilizes ATP for its activity (Shen *et al.*, 2000). The complex contains 5'-3' helicase activity and, moreover, *ino80* null mutant displays sensitivity to hydroxyurea, methyl methanesulfonate (MMS) and UV and ionising irradiation, indicating roles for this complex in replication and/or processing of DNA damage (Shen *et al.*, 2000). In human cells Rvb proteins were found to associate with the TIP60 histone acetylase complex which plays a role in DNA damage repair and apoptosis (Ikura *et al.*,

2000). TIP60 influences the activity of repair enzymes in chromatin including ATPase, DNA helicase, and structural DNA binding proteins. In addition, a more recent report demonstrated that Rvb1p-Rvb2p complex purified from yeast shows an ATP-dependent chromatin remodelling activity *in vitro* comparable to that of Swi/Snf complex (Jonsson *et al.*, 2001).

Finally, however intriguingly, in mouse nuclear extracts, Rvb1 and Rvb2 proteins were found to be associated with box C/D small nucleolar RNPs (snoRNPs) (Newman *et al.*, 2000; Watkins *et al.*, 2002), and the yeast Rvb2p was shown to be essential for the production of both, the box C/D and box H/ACA sno RNAs (King *et al.*, 2001).

1.4 Chromatin-remodelling complexes

In vivo, eukaryotic DNA is packaged with histones and other accessory proteins into chromatin that restricts it accessibility, thereby repressing DNA-dependent processes. A contribution to the regulation of DNA accessibility comes from intrinsic properties of nucleosomes, such as nucleosome mobility, unfolding, or partial disruption (Widom, 1998). Another contribution is made by chromatin-modifying complexes that can be classified in two major classes. The first group includes complexes that use the energy of ATP hydrolysis to perturb histone-DNA contacts. These complexes have been found to change the position of histone octamers on DNA to create nucleosome-free regions. All ATP-dependent chromatin-remodelling complexes contain a highly conserved ATPase subunit that belongs to the SWI2/SNF2 super-family of proteins (for review see Neely and Workman, 2002). The second group of complexes includes those that covalently modify the histone proteins, whether by acetylation, phosphorylation, methylation, or

ubiquitination. The most widely studied complexes in this second group are histone acetyltransferase (HAT) complexes and histone deacetylase (HD) complexes, which together regulate the level of acetylation of lysine rich residues in the N-terminal tails of histones (for review see Roth *et al.*, 2001). While both ATP-dependent remodelling, and histone-modifying complexes can bind non-specifically to DNA, a wide variety of data implies that their activities are regulated both spatially and temporally. For example, genome-wide expression studies performed with mutants of yeast Swi/Snf subunits have revealed that this complex is involved in the expression of only 6% of all yeast genes (Holstege *et al.*, 1998; Sudarsanam *et al.*, 2000). Similarly, Gcn5p-containing HAT complexes seem to be required for the expression of only 5% of yeast genes (Holstege *et al.*, 1998). Specificity for particular genes is achieved by interactions with sequence-specific transcription factors, which "target" chromatin-remodelling activities to specific locations.

Although functional analysis of the chromatin-remodelling has been focused mainly on transcription, recently, there is an increasing evidence that similar activities may assist recombination and repair (Alexiadis and Kadonaga, 2002; Fyodorov and Kadonaga, 2001; Gaillard *et al.*, 2003; Green and Almouzni, 2002; Hara and Sancar, 2002; Narlikar *et al.*, 2002; Smerdon and Lieberman, 1978; Ura *et al.*, 2001). This findings support the "access, repair, restore" (ARR) model that postulates how repair might function in the complex chromatin environment of a nucleus (Smerdon, 1991). According to the model, initial repair steps include reactions that first permit access of the repair machinery to DNA damage and later restore the canonical nucleosomal organization of the repaired DNA (Figure 5).

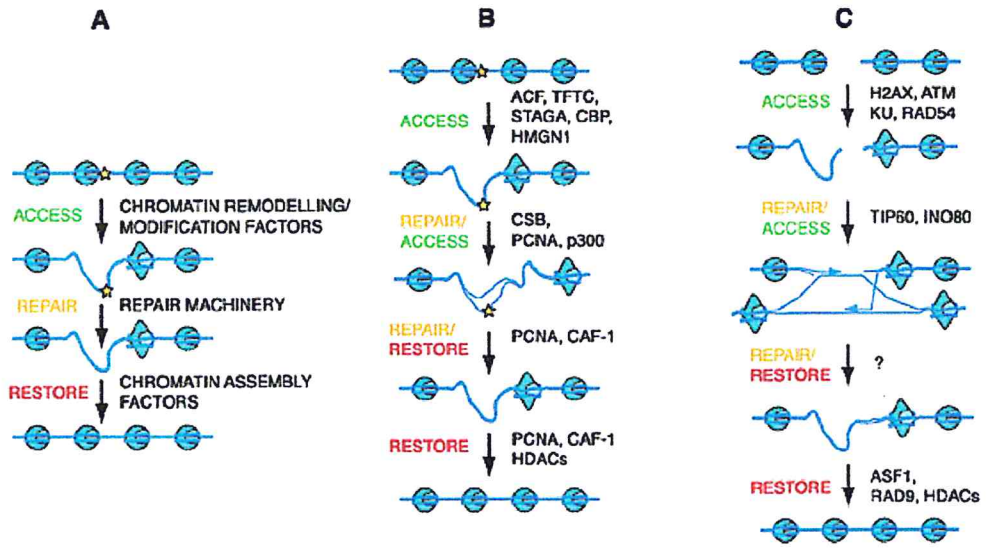

Figure 5. (A) The original three-step ARR model for NER to explain how repair can be achieved at the nucleosomal level. To overcome the inhibition by nucleosome structure (depicted in blue), the first stage in the repair process is removal or remodelling of nucleosomes to permit access to the DNA lesion (the star). Aftre repair of the DNA, the original chromatin structure must be restored to ensure that epigenetic information is maintained. (B and C) An expansion of the original model to highlight factors that may be required for alterations in chromatin structure during the different enzymatic stages that comprise NER (B) and HR (C). From Green and Almouzni (2002).

1.5 Aim and outline of this study

To better characterize eukaryotic RuvB-like proteins and to gain insight into their connection with the bacterial homolog, we carried out a molecular study of the two *S. cerevisiae* genes YDR190c and YLP235w, which code for Rvb1 and Rvb2 proteins, respectively.

We studied transcriptional regulation of *RVB* genes, determined nuclear localization of

the Rvb proteins, and their binding specificity for quadruplex DNA. Furthermore, the roles of both proteins in DNA recombination, and UV damage repair were assessed in *RVB1* and *RVB2* heterozygous diploid strains, as well as the effect of bacterial RuvAB complex on survival after UV irradiation.

On a further approach, *RVB1* and *RVB2* genes were randomly mutagenized, and the sensitivity of the yeast strains carrying mutant alleles to the different types of damage was tested. In addition, we performed bioinformatic analysis of both Rvb proteins and their bacterial homolog, and proposed a partial three-dimensional model for N- and C-terminal regions of Rvb proteins.

CHAPTER 2

Molecular characterization of *Saccharomyces cerevisiae* RuvB-like proteins Rvb1 and Rvb2

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Submitted to J. Biol. Chem.

2.1 Abstract

Based on sequence homology, two highly conserved eukaryotic proteins, Rvb1 and Rvb2, were proposed to be yeast analogues of the bacterial RuvB, which is involved in the processing of Holliday junctions together with RuvA and RuvC. Here we present data on transcriptional regulation of *RVB1* and *RVB2* genes, and the evidence for their novel roles in DNA recombination, and UV damage repair. The expression of yeast RuvB-like genes is not cell-cycle regulated. Nevertheless, both genes are self-regulated, since the episomal over-expression of *RVB1*, and *RVB2* decreases expression of chromosomal *RVB1*, and *RVB2* genes, respectively, by 85%. Diploid strains heterozygous for either gene show significantly lower DNA double-strand break repair in inverted-repeat substrate, and decreased survival after UV irradiation. Still, the expression, and localisation of Rvb1 and Rvb2 proteins is unchanged after the damage. Interestingly, over-expression of bacterial RuvAB complex improves survival of yeast strains after UV damage. Rvb2p preferentially binds artificial Holiday junction, in our cell-free protein-DNA binding assay similarly to bacterial RuvAB complex, whereas Rvb1p tightly binds to either duplex or cruciform DNA. Taking in consideration that both proteins also bind to chromatin, a role in recombination and repair through chromatin remodelling, as well as their relation to bacterial homolog is discussed.

2.2 Introduction

To preserve the DNA integrity, and to prevent the deleterious consequences of genetic degeneration, cells have evolved a network of DNA repair mechanisms and cell cycle

checkpoints. In vegetatively growing yeast cells, the major mechanism of DNA repair is homologous recombination, the central molecular intermediate of which is the Holliday junction (HJ) (Paques and Haber, 1999). In prokaryotes, enzymes that process HJ have been studied in detail. RuvA-RuvB complex drives branch migration of HJ (Tsaneva *et al.*, 1993; Tsaneva and West, 1994), and stimulates its resolution by RuvC endonuclease (van Gool *et al.*, 1998; Zerbib *et al.*, 1998). In *E. coli*, the *ruvA*, *ruvB*, and *ruvC* genes are required for normal levels of cellular resistance to the effects of UV- or ionising-irradiation, and mitomycin-C (Lloyd *et al.*, 1984; Mezard *et al.*, 1999; Otsuji *et al.*, 1974; Stacey and Lloyd, 1976). Furthermore, their mutants are also mildly defective in recombination (Lloyd *et al.*, 1987). RuvB belongs to the class of hexameric helicases that are member of a larger family of proteins known as the AAA^+ class (Neuwald *et al.*, 1999), which among other amino acid sequence motifs contains Walker A and Walker B boxes that are active sites for ATP (or dNTP) binding and hydrolysis. Although in eukaryotes, branch migration and resolution activities have been detected in cell-free extracts (Constantinou *et al.*, 2001), the proteins that perform these activities have not been identified. The first eukaryotic protein that shared similarity to bacterial RuvB, was isolated from rat liver as a 49-kDa protein that interacts with TATA-binding protein (TBP) and was named TIP49 (TBP interacting protein of 49 kDa) (Kanemaki *et al.*, 1997). Apart from Walker A and B motifs, it showed additional sequence similarity with bacterial protein in the N- and C-terminal regions that together comprise about 50% of the overall sequence (Kanemaki *et al.*, 1997). Simultaneously, in human cells, the same protein was identified in a two-hybrid interaction screen with the 14-kDa subunit of DNA replication and repair factor RPA (Qiu *et al.*, 1998). The protein was named RuvBL1

(RuvB-like1). Subsequently, its yeast *Saccharomyces cerevisiae* ortholog scRuvBL1, as well as RuvBL2 as a closely related family member were identified in database searches (Kanemaki *et al.*, 1999; Qiu *et al.*, 1998). From there on, RuvBL1 and RuvBL2 were discovered independently by number of groups that studied different processes in different organisms, and were named Pontin52, ECP-54, NMP238, Tip49, TAP54-α, p55, p50, TIP49a, Rvb1, or Tih1 and Reptin52, ECP51, TIP48, TAP54-β, p50, p47, TIP49b, Rvb2, or Tih2, respectively, (Bauer *et al.*, 1998; Gohshi *et al.*, 1999; Holzmann *et al.*, 1998; Ikura *et al.*, 2000; Kanemaki *et al.*, 1999; Kikuchi *et al.*, 1999; Lim *et al.*, 2000; Newman *et al.*, 2000; Salzer *et al.*, 1999; Shen *et al.*, 2000; Wood *et al.*, 2000). Rvb1p and Rvb2p are highly conserved (Kurokawa *et al.*, 1999) nuclear proteins (Bauer *et al.*, 1998; Lim *et al.*, 2000; Makino *et al.*, 1998; Newman *et al.*, 2000) that are indispensable for yeast (Kanemaki *et al.*, 1999; Lim *et al.*, 2000; Qiu *et al.*, 1998), and for *Drosophila melanogaster* (Bauer *et al.*, 2000). They were reported to act independently as helicases of opposite polarity(Kanemaki *et al.*, 1999; Makino *et al.*, 1999) although this result has been disputed (Ikura *et al.*, 2000).

Even though these two proteins have been studied heavily, little is known about their precise function. It was demonstrated that transcription of 34 genes depends on Rvb2p (Ohdate *et al.*, 2003), and that Rvb1p and Rvb2p cooperate, directly or indirectly, in transcription regulation of over 5% of yeast genes (Jònsson *et al.*, 2001). In higher eukaryotes they interact with c-Myc, β-catenin, E2F1, and ATF2 and modulate cellular transformation, signaling, apoptosis, and response to stress and DNA damage (Bauer *et al.*, 2000; Cho *et al.*, 2001; Dugan *et al.*, 2002; Wood *et al.*, 2000). Rvb1p-Rvb2p complex, purified from yeast, showed ATP-dependent chromatin remodelling activity *in*

vitro (Jonsson *et al.*, 2001), and both proteins have also been found in other complexes that remodel chromatin such as INO80 in yeast (Shen *et al.*, 2000) and TIP60 in human cells (Ikura *et al.*, 2000). Finally, both proteins were found to be associated with the production of small nucleolar RNPs (King *et al.*, 2001; Newman *et al.*, 2000; Watkins *et al.*, 2002).

To better characterize eukaryotic RuvB-like proteins, and to compare them to their bacterial homolog, we carried out a molecular studies of two yeast proteins. Accordingly, we refer to them as Rvb1 and Rvb2, adopting the names from the *S. cerevisiae* Genome Database. Our results indicate that *RVB1* and *RVB2* are self-regulated genes, required for DNA repair, since the diploid strains showed Rvb1p and Rvb2p dosage-dependent phenotype when repairing DNA double-strand breaks, and damage caused by UV irradiation. 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 two yeast's RuvB-like proteins bind to HJ, we developed an *in vitro* cell-free binding assay in which Rvb2p, unlike Rvb1p, preferentially bound the artificial HJ.

2.3 Materials and Methods

Yeast strains and media

Yeast strains used in this study are haploids FAS20 MATα *ade1 ade2 ade8 trp1 leu2 lys2 ura3-52 (cir$^+$)*, YPH258 MATa *ade2 ade8::FRTX his3 trp1 leu2 lys2 ura3-52 (cir$^+$)*, and its derivates in which *RVB1* or *RVB2* genes were tagged with yEGFP fluorescent tag, and

diploids DUPOT-SL1V5 MATa/α *gal2/GAL2 leu2/leu2::FRTX arg10/ARG10 ade2/ADE2 ura3-52/ura3-52 RVB1/RVB1::V5 KanMX4* (Waghmare *et al.*, 2003), CB89 MATa/α *ade5/ade5 can1^{R}/CAN1^{S} leu2-3/leu2-3 trp1-289/trp1-289 HIS7/his7 ura3-52/ura3-52*, and FAY1 that was constructed by mating YPH258 with FAS20, as well as its derivates in which one or the other *RVB* gene was tagged with V5His6x tag. Gene deletions and tagging were performed according to the protocols of the EUROFAN program manual (URL http://www.mips.biochem.mpg.de/proj/eurofan/eurofan_1/b0/home_requisites/guideline/exp-transformation.html), and were verified by PCR. YPD, YPD supplemented with kanamycine, and synthetic dropout media were prepared as described (Kaiser *et al.*, 1994). *E. coli* strain DH5α was used for plasmid propagation. *E. coli* cultures were grown in LB broth supplemented with ampicilin as previously described (Sambrook *et al.*, 1989).

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 cassettes for deleting *ADE8*, *RVB1* and *RVB2*, respectively. Plasmids pYM12[6] (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 were used for C-terminal tagging of *RVB1* and *RVB2* at their chromosomal loci. The pVP16* plasmid containing SV40-T nuclear localization signal (NLS) sequence between *ADH1* promoter and terminator sequences, and *LEU2* gene was kindly provided by Michela Visintin (SISSA, Italy). Applying a

PCR-based strategy, *B. subtilis ruvA* and *ruvB* genes were cloned into *Sal*I-*Not*I sites of pVP16* plasmid in order to fuse each in frame with NLS at their N termini. Plasmid pVT1 and pVT2 were constructed by the cloning of the PCR amplified *RVB1* and *RVB2*, respectively, into the *Xho*I-*Bam*HI of pVT100-U which contains *URA3* gene. Using the pYES2.1 TOPO TA Cloning Kit (Invitrogen), NLS-*ruvB*, *RVB1* and *RVB2* were fused in frame with the V5His6x epitope at their C termini. After PCR amplification NLS- *ruvB* - V5His6x and *RVB1*-V5His6x were inserted into *Xho*I-*Xba*I of pVT100-U plasmid generating pVT-BV5 and pVT-1V5 plasmids, respectively. *RVB2*-V5His6 fragment was subcloned into the *Xho*I-*Sac*I of pVT100-U plasmid generating pVT-2V5. An 18nt 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. PCR product was inserted into the *Pvu*II site of pVT100-U generating the pVT-AHSV plasmid. This plasmid was digested with *Sph*I and the 1.4kb fragment was subcloned into *Sph*I site of pVP16* plasmid generating pVP-AHSV. The pRURA8Δ plasmid was used in recombination assay (Caputo, 2003). Plasmid DNA was extracted from *E. coli* using the Mini-preparation Kit (Promega). Restriction enzymes used were obtained 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 over-night in appropriate liquid media, washed once and inoculated at cell density of 5 x 10^6 cells/ml in

50 ml of YPA (1% Bacto Yeast Extract, 1% Bacto Peptone, 1% KAc), or similar media that contained 1% URA or URA LEU drop-out instead of Bacto Yeast Extract. Cells were harvested at the density of 2 x 10^7 cells/ml, washed twice and transferred to 50 ml of sporulation medium (1% KAc plus 1/5 the standard concentration of the required amino acids). The incubation continued at room temperature with shaking for 10 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 plates using a twin-joystic 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 URL http//www.bcm.tmc.edu/elledge/links/techno/alpha.htm.

Recombination assay

Yeast cells were transformed with the *I-Sce*I in vitro linearized pRURA8Δ plasmid (Caputo, 2003) and plated on SC-TRP plates, which were incubated at 30°C. After two days, transformants were replica-plated on SC-URA plates and incubated at 30°C over-night. 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 ~1 x 10^7 cells/ml. ~200 cells per plate were plated on appropriate solid media and immediately UV irradiated using UV-Lamp VL-6C at a dose of 150 J/m^2 (measured by a Radiometer VLX254, France). After irradiation plates were exposed to light for 15 min and then incubated in the dark at 30°C. Simultaneously, the same procedure was performed for each strain excluding the UV irradiation step. After 2 days, colonies were counted and the fraction of irradiated cells that survived relative to non-irradiated cells was calculated. Three independent experiments were performed for each strain.

To follow Rvb1p and Rvb2p expression and localization after UV damage, cells were grown in 20 ml liquid YPD to the density of ~1 x 10^7 cells/ml, then washed once, resuspended in PBS, and UV irradiated at the dose of 150 J/m^2. After irradiation cells were harvested, and incubated in 20 ml liquid YPD medium at 30°C. 1 ml of culture was taken after 5, 15, 30, 60, 120, 300, and 480 min of incubation.

Total cell extracts and nuclear extracts preparation, and nuclear fractionation

Whole protein extracts were prepared according to the protocol available at URL http//www.pmci.unimelb.edu.au/core_facilites/manual/mb460.asp. Nuclei, and nuclear extract isolation were performed as previously 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, 1x protein inhibitor mixture). Chromatin fractionation was performed as described previously (Mendez and

Stillman, 2000). All protein preparations were resolved by 10% SDS-PAGE as described (Sambrook *et al.*, 1989).

Protein-DNA binding assay

Synthetic Holliday junction X26 was prepared by annealing four 60-mer oligonucleotides (Constantinou *et al.*, 2001). The annealing of 5'-biotinylated X26-2 oligonucleotide and X26-1, X26-3, and X26-4 was performed as described previously (Elborough and West, 1990). The quadruplex product was then purified by electroelution through 2% agarose gel using TaKaRa RECOCHIP (TaKaRa). Protein-DNA binding assay based on magnetic separation (Gabrielsen *et al.*, 1989) was adapted for our purpose as following. 0.75 μg of purified biotinylated X26 was bound to 40μl of Streptavidin Magnetic Particles (Roche) and pulled-down by the Magnetic Particle Separator (Roche) under conditions recommended by the manufacturer. The beads were equilibrated by washing 3x with binding buffer (BB) (NEB supplemented with 1 mM $MgCl_2$, 0.1% Triton-X) and then mixed with 250μl nuclear extracts that was obtained from ~ 5 x 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 rocking for 15 min, then pelleted, and washed 3x with BB. Proteins were eluted by resuspending and pulling-down the beads in the binding buffer with increasing NaCl concentration up to 175 mM, 300 mM, 400 mM, 600 mM and finally 1 M. After each round of pelleting and resuspending, the supernatant with 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 a binding substrate 1.5 μg of duplex DNA prepared by annealing 5'- biotinylated X26-2 and its reverse complement.

Immunoblots and quantitative analysis of protein

The HSV, and V5 epitope-tagged proteins were detected with the mouse monoclonal anti-HSV (Novagen), and the 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 its V5-tagged variant, were detected with the rabbit polyclonal anti-Rad53p (kindly provided by D.F. Stern, Yale University School of Medicine, New Haven, USA), and anti-Rvb2p antibodies (kindly provided by M.J. Fournier, University of Massachusetts, Amherst, USA), 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 (both from Santa Cruz Biotechnology). Western blot hybridization was 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, HY). For protein quantification, the intensity of each band was scanned with an UltroScan XL (Pharmacia LKB, Uppsala, Sweden). The protein content of the 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 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 the 5 μg of cDNA was used to perform PCR amplification (28–30 cycles) using Taq Polymerase (Promega). In cell cycle regulation experiment, *RVB1*, *RVB2*, and *HIS1* fragments were amplified using primers KpnF/ORF1R (5'-GGGTGAAGTGACAGAACTAACCCCTGAAG/5'-TAACATGCATGCTTACAAATAATTTGCGGAAG), EIIF/ORF2R (5'-ATAAATAGGGCTTTGGAAGATGAGTTTGCC/5'-TAACATGCATGCTTATTCCGTAGTATCCATGG), and HIS1-INF/HIS1-INR (5'-ACCTGTAGCGTTGGTCTTTC/5'-GAAATGGTTGGTGCTCTACG), respectively. In self-regulation experiment, endogenous expression of *RVB1* was followed by using KpnF and V5R (5'-ACCGAGGAGAGGGTTAGGGAT), reverse primer specific for V5 epitope, while the *RVB2* endogenous expression was followed by using EIIF and R2+20 (5'-GCAATTTCTGCCTTAAAGTACAAAATGC), reverse primer specific for the terminator sequence immediately after stop codon, which is not present in the pVT-2V5 plasmid. PCR products were electrophorised in 1% agarose gel and analyzed after EtBr staining by UltroScan XL.

Fluorescent microscopy

Strains that carry yEGFP-tagged Rvb1p or Rvb2p were grown in liquid YPD media to a density of ~1 x 10^7 cells/ml. Cells were washed once with water, spotted on a glass slide,

air-dried and covered with 5 µl of mounting medium (Vector Laboratories, Burlinghame, CA, USA). The yEGFP-tagged proteins were visualized using an Axiovert 100M confocal microscope (Carl Zeiss Jena, Jena, Germany).

Primers

All primer sequences used in this study that are not indicated in Materials and Methods are available on request.

2.4 Results

2.4.1 Rvb1 and Rvb2 are Constitutively Expressed Chromatin-associated Proteins

Both *RVB* genes have been shown to be expressed in all mammalian cells, with increased expression in testis (Makino *et al.*, 2000). The human *RVB1* mRNA has been found to be ubiquitously expressed throughout the cell cycle (Bauer *et al.*, 1998; Qiu *et al.*, 1998). However, it was also reported that serum stimulation of rat fibroblasts induces both, *RVB1*, and *RVB2* mRNA levels 4- to 5-fold within 3 hours after serum addition to quiescent cells (Wood *et al.*, 2000). 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 α-factor release, samples were taken each 15 min for a 120 min period, covering more than one cell cycle, since the replication time for YPH258 strain is ~75 min. 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) (Figure 1A). In concordance with mRNA levels, both proteins were present, and homogeneously distributed in the nucleus at all time points (data not shown).

Although the expression of *RVB1* and *RVB2* was not cell cycle regulated, we observed self-regulation of expression for both genes. Exponentially growing diploid strain FAY1, transformed with 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 to the non-transformed FAY1 strain (Figure 1B and 1C). To determine whether the ectopic over-expression 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 Figure 1B and 1C, the amounts of endogenously expressed *RVB1*-V5 mRNA, and the Rvb1-V5 protein were decreased in the pVT1 transformed strain when compared to the non-transformed strain. Normalization of the Western blot data to the levels of Rad53p revealed that the episomal over-expression of Rvb1p and Rvb2p decreased by 85% expression of chromosomal *RVB1*, and *RVB2* genes, respectively (Figure 1D). The same self-repression for both *RVB* genes was observed in the haploid YPH258 strain (data not shown). On the other hand, over-expression of Rvb1p increased a little Rvb2p expression, and *vice-versa* (Figure 1C and D). The trend of increase for either Rvb protein, when the other one was over-expressed, was not observed for their mRNAs (Figure 1B). Since it is known that Rvb1p and Rvb2p interact with each other (Kanemaki *et al.*, 1999; Lim *et al.*, 2000), apparently in stoichiometric amounts (Jonsson *et al.*, 2001;

Shen *et al.*, 2000), this behavior could indicate that the increase at the protein level represents stabilization of the partner 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 occurs in nuclear matrix preparations from human and rat cells (Holzmann *et al.*, 1998). To determine whether yeast Rvb1 and Rvb2 proteins are associated with chromatin we performed nuclear fractionation. Nuclei were prepared from a culture of the DUPOT-SL1V5 cells in which *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 and 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 get released from the chromatin.

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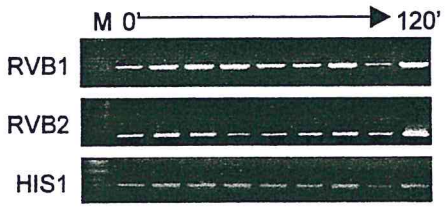

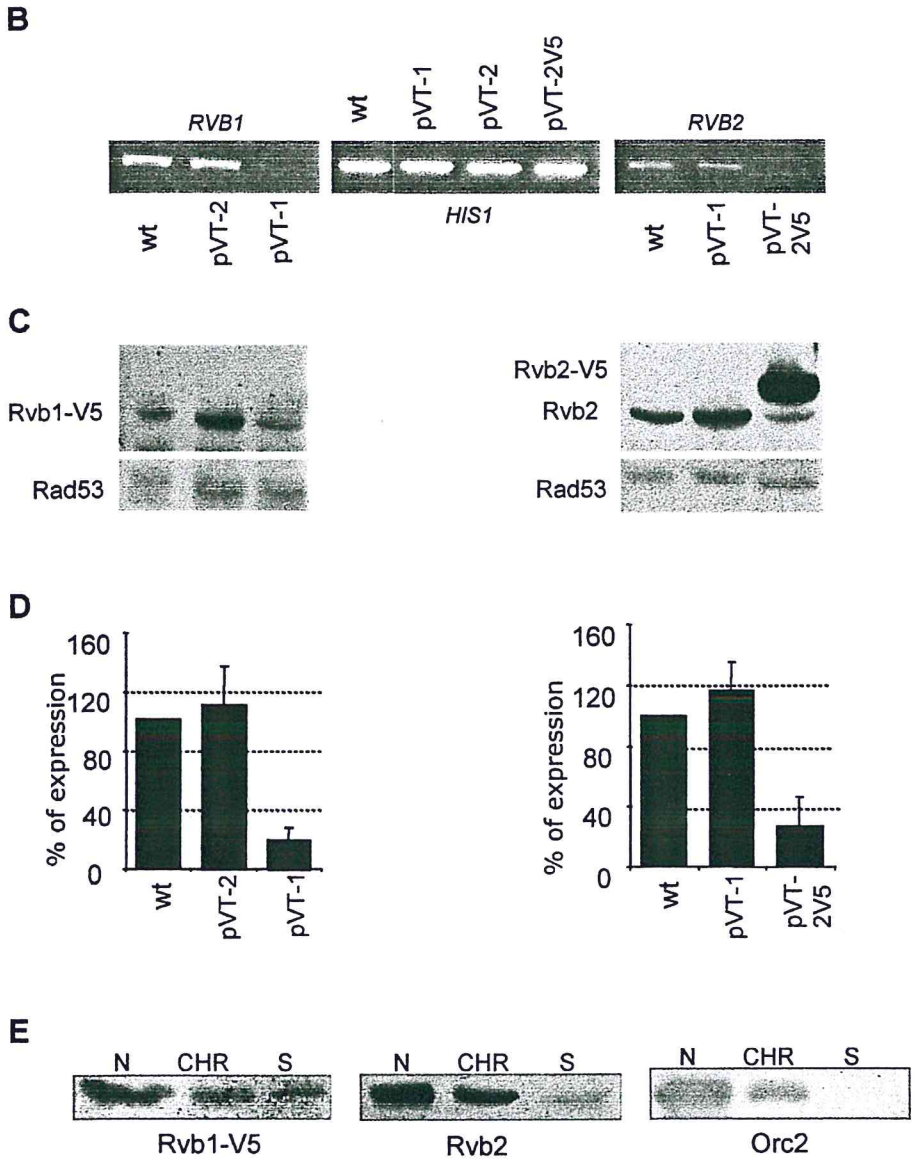

Figure 1. Rvb1p and Rvb2p are chromatin-associated nuclear proteins whose expression is self regulated. A, *RVB1*, and *RVB2* mRNA expression through the cell cycle. Levels of *RVB1* and *RVB2* mRNA in YPH258 cells was examined by RT-PCR following the indicated times of release from α-factor block. As a constitutively expressed gene, *HIS1* was used as expression, and internal reference for the amount of PCR product loaded. B, and C, Expression of *RVB1* and *RVB2* is self-regulated. RT-PCR (B), and Western blot (C) analysis of endogenous *RVB1* and *RVB2* mRNA and Rvb1 and Rvb2 protein levels in wild type FAY1 strain, in which *RVB1* was V5-tagged at chromosomal loci, and its transformant strain FAY1/pVT1, which over-expressed Rvb1p, or FAY1/pVT2 and FAY1/pVT-2V5, which over-expressed Rvb2p or its V5 tagged variant, respectively. 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. Amount of Rad53 protein was used as an internal reference for the amount of protein loaded. E, Rvb1p and Rvb2p bind to chromatin. Western blot of DUPOT-SL1V5 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.

2.4.2 *RVB1* and *RVB2* are required 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 the association with RPA, a protein involved in DNA replication and repair (Qiu *et al.*, 1998). Since, *RVB1* and *RVB2* genes are essential for yeast viability (Kanemaki *et al.*, 1999; Lim *et al.*, 2000; Qiu *et al.*, 1998), their possible haploinsufficiency effect on DNA double-strand break (DSB), and UV damage repair was studied in diploid strains heterozygous for one, or the other gene. The deletion of either *RVB1*, or *RVB2* did not influence growth of the heterozygous strains in YPD, or synthetic-complete medium, since 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 Figure 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 *RVB1* gene did not

influence expression of *RVB2*, since the amount of Rvb2p in FAY1Δ1 was comparable to that in the wild-type strain (Figure 2A).

Homologous recombination is an error-free pathway that uses homologous sequence present in a cell to repair DNA damage. To investigate whether the two yeast RuvB-like proteins participate in DNA DSB repair by HR we used pRURA8Δ plasmid (Caputo, 2003) as a repair 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 whose unique restriction site 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 all transformants that repaired 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 (Kang and Symington, 2000; Malagon and 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 significant decrease in the ability to repair DNA DSBs by inversion in comparison to the wild-type FAY1 strain (Figure 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 (Figure 2B). In addition, CB89 mutants showed larger decrease in this ability than FAY1 mutant strains (Figure 2B). However, no synergistic effect was observed for CB89Δ1Δ2 mutant whose ability was comparable to that of CB89Δ1 and CB89Δ2, suggesting that both genes operate in the same pathway.

Interestingly, CB89Δ2 and CB89Δ1Δ2 mutants displayed strong, while CB89Δ1 only weak flocculation phenotype in both, YPD and synthetic-complete medium (Figure 2D). Over-expression of *RVB2* in the CB89Δ2 transformed with the pVT-2V5 plasmid, moderated flocculation (Figure 2D). This pointed out *RVB2* as responsible for the phenotype. The flocculation phenotype was background dependent, since it was not noticed for any of FAY1 mutant strains. This indicates that CB89 strain carries an additional mutation that, 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 to that of FAY1 mutants, may depend on the same mutation that is causing flocculation phenotype.

Strains heterozygous for *RVB1* and *RVB2* genes also showed decreased ability to repair damage caused by UV irradiation (Figure 2B). To express the ability of the wild-type FAY1, and CB89 strains, as well as their *RVB1* and *RVB2* heterozygous mutants, to repair UV damage after irradiation, their survival data were normalized by assigning the value of 100% repair ability to the wild-type strains.

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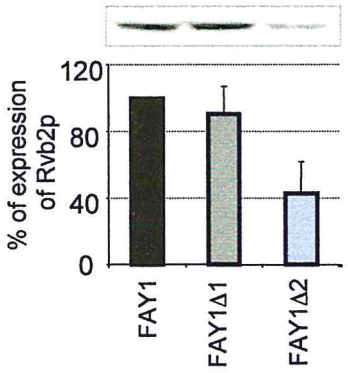
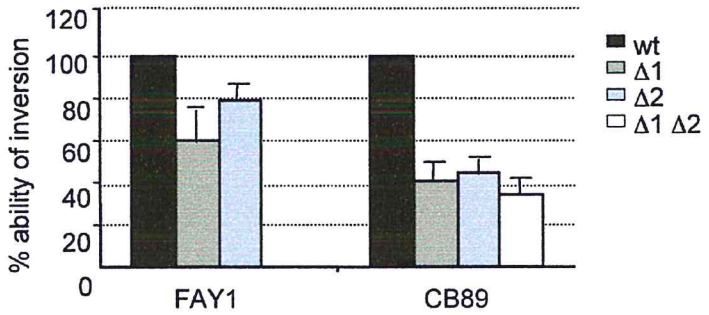
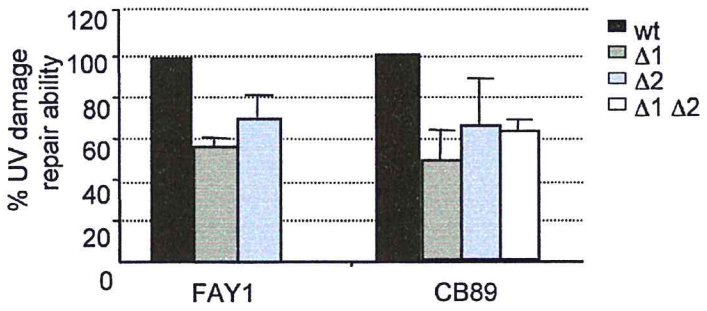

% of expression of Rvb2p
120
80
40
0
FAY1
FAY1Δ1
FAY1Δ2

B


% ability of inversion
120
100
80
60
40
20
0
FAY1
CB89
wt
Δ1
Δ2
Δ1 Δ2

C


% UV damage repair ability
120
100
80
60
40
20
0
FAY1
CB89
wt
Δ1
Δ2
Δ1 Δ2

D

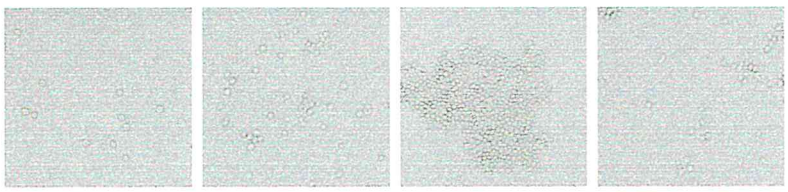

CB89
CB89Δ1
CB89Δ2
CB89Δ2/pVT-2V5

Figure 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. The 5 μg of total protein extract was loaded for each strain. Data obtained from four independent Western blottting 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 inversion (CO+BIR-SSA), and UV damage. Values are expressed as % of the wild type strains ability to repair damage. See Materials and Methods for further details. D, Deletion of one copy of *RVB2* in CB89 causes flocculation phenotype. Strains indicated, were grown in YPD until mid-log phase, and then photographed.

The deletion of *RVB1* resulted in ~50% decrease, and the deletion of *RVB2* caused milder, but still significant decrease in UV damage repair ability in both diploid strains (Figure 2C). Comparable repair ability between strain heterozygous for both (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, this data indicate that both Rvb proteins are required, and act together in DNA DSB repair by inversion, and UV damage repair in yeast.

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

To study the behavior 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 V5 epitope at its chromosomal locus. No change in expression was observed for either protein at 2, 5, or 8 hours after irradiation (Figure 3A). Non-inducibility is also expounding haploinsufficiency, indicating that the lack of Rvb1p and Rvb2p is indeed limiting factor for the repair.

A number of proteins involved in DNA damage signaling and repair, including those in MRE11 complex (Maser *et al.*, 1997), γ-H2AX (Paull *et al.*, 2000), and BRCA1 (Scully *et al.*, 1997b) in human or Rad52p (Lisby *et al.*, 2001) in yeast, relocalize and form subnuclear foci in 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 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, and comparable to that of non-irradiated cells even after two hours (Figure 3B).

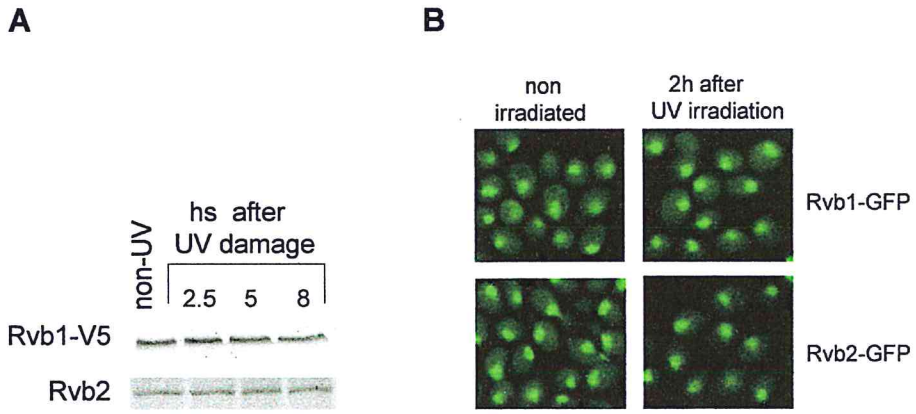


Figure 3. Expression and localization of Rvb1p and Rvb2p is not changed after UV irradiation. A, Western blot of lysates made from FAY1 cells, in which *RVB1* was V5 tagged at chromosomal locus, at 2.5, 5, and 8 hours after UV irradiation, or from non-irradiated cells. 5 μg of proteins was loaded for each time point. B, Fluorescent microscopy of FAY1 cells in which *RVB1*, or *RVB2* were yEGFP-tagged at their chromosomal loci, two hours after UV irradiation, and in non-irradiated cells.

2.4.4 Over-expression of a bacterial RuvAB complex improves survival of the yeast strains after UV irradiation

The Rvb1 and Rvb2 proteins are similar in sequence to bacterial RuvB, especially in the regions that correspond to those containing WalkerA and WalkerB boxes (Kanemaki *et al.*, 1997). Given that in bacteria, *ruvA*, *ruvB* and *ruvC* genes are required for cellular resistance to UV damage (Mezard *et al.*, 1999; Otsuji *et al.*, 1974), we investigated whether the expression of the bacterial genes can restore normal UV response in *RVB1* and *RVB2* heterozygous strains. *Bacillus subtilis ruv* genes have been chosen for complementation, since *B. subtilis* is, as Gram + bacteria that can sporulate, evolutionary closer to yeast (Sonenshein *et al.*, 1993). In addition, similarly to yeast, it has very efficient recombination system (Vagner *et al.*, 1998). Wild-type FAY1 and its heterozygous strains, FAY1Δ1 and FAY1Δ2 were transformed either with plasmid pVT-1V5, pVT-2V5, pVT-BV5, pVP-AHSV, empty vector pVT100-U, or co-transformed 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). UV-irradiated wild-type FAY1 cells co-expressing bacterial RuvA and RuvB showed higher UV resistance than the FAY1 cells carrying empty vector (Figure 4). Moreover, bacterial RuvAB complex increased survival of the FAY1Δ1 and FAY1Δ2 strains to the levels of the FAY1Δ1 and FAY1Δ2 over-expressing *RVB1* and *RVB2*, respectively (Figure 4). On the other hand, over-expression of the RuvA alone did not have any effect, while over-expression of the RuvB alone slightly improved ability of *RVB1* heterozygous strain to repair UV damage (Figure 4).

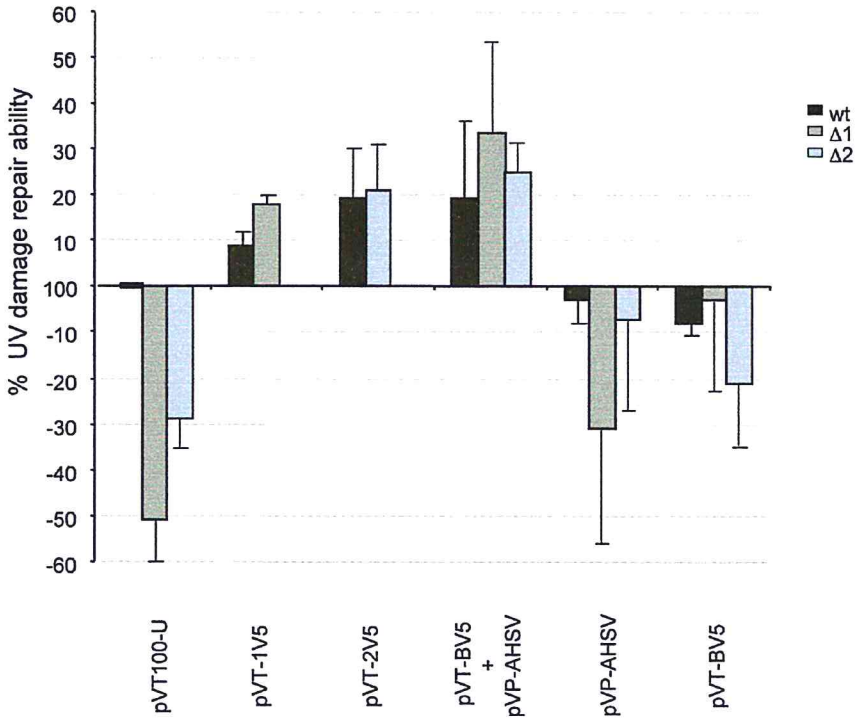


Figure 4. Effect of NLS-RuvA-HSV and NLS-RuvB-V5 on UV irradiated FAY1 and its *RVB1* and *RVB2* heterozygous strains. Cells were transformed with the plasmids indicated, and assayed for colony formation before and after UV irradiation as described in Materials and Methods. Value of 100% UV damage repair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

Encouraged by the improved UV survival caused by the RuvAB complex, we investigated whether the expression of bacterial genes could functionally complement *rvb1* and *rvb2* deletions. FAY1Δ1 and FAY1Δ2 strains were transformed with pVT-BV5 alone, or in combination with pVP-AHSV. The 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,

transformation with control plasmids, pVT-1V5 and pVT-2V5, rescued *rvb1* and *rvb2* deletions respectively, resulting in a 4:0 segregation for viability following meiosis. For all tetrads tested, two spores from the tetrad amplified the wild type band, and the other two the deletion band after the PCR. Thus the yeast *RVB1*, or *RVB2* genes cannot be complemented either by bacterial *ruvB* alone or in its combination with *ruvA*.

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

Bacterial RuvB binds to HJ by forming a complex with HJ binding protein RuvA (West, 1997). Human TIP60 complex, which contains both Rvb orthologs, shows affinity for the structural DNA that mimics cruciform (Ikura *et al.*, 2000). To study the ability of the two yeast's RuvB-like proteins to bind HJ, we developed an *in vitro* cell-free binding assay in which Rvb2p was, unlike Rvb1p, preferentially bound to synthetic HJ. Since the majority of Rvb1 and Rvb2 proteins were bound to the chromatin (Figure 1A), we over-expressed 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 to 300 mM) (Figure 5). Bacterial RuvB, which is targeted to the HJ by RuvA, elutes together with it (Figure 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 (Figure 5). Its binding 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 to its structure (Figure 5).

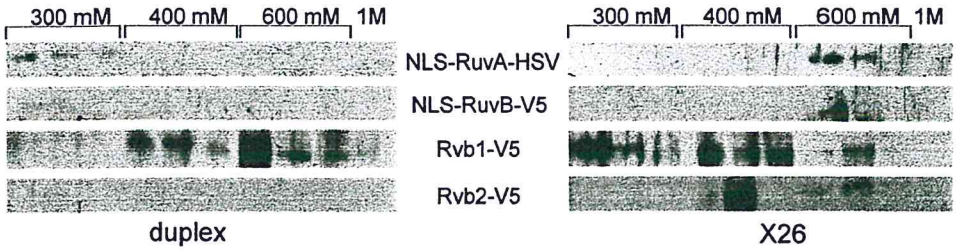


Figure 5. Binding specificity of Rvb1p and Rvb2p to duplex or to cruciform (X26) DNA compared to bacterial RuvAB complex. Western blot of fractions eluted in cell-free protein-DNA binding assay, performed as described in Materials and Methods, with nuclear extracts from FAY1 cells co-expressing NLS-RuvA-HSV and NLS-RuvB-V5, or over-expressing either Rvb1-V5 or Rvb2-V5. Salt concentrations in the fractions are indicated.

2.5 Discussion

2.5.1 Regulation of *RVB1* and *RVB2* expression

The expression of *RVB1* and *RVB2* in yeast is not cell-cycle regulated (Figure 1A, and data not shown). Similarly, no cell cycle regulation 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 three hours after serum stimulation of

quiescent rat cells, even though, progression through the cell-cycle does not change level of expression after the initial induction (Wood *et al.*, 2000). The discrepancy could be explained by the state of the cells used for experiment. While no cell cycle regulation is observed for the growing (log phase) cells, the cells that are stimulated to divide show cell-cycle regulation. This suggests that *RVB* genes are required for actively dividing cells, while they are repressed during stationary phase. It is likely that both genes are expressed to the maximum levels during the cell cycle, since the heterozygous deletion caused ~50% decrease in expression of deleted gene (Figure 2A). In addition, non-inducibility observed after UV irradiation, or DNA DSB induction supports this hypothesis. Still, little is known about mechanism that regulates *RVB1* and *RVB2* expression. It was reported that in human, expression of both genes partially depends on c-Myc expression, since c-*myc* null cells have 3- to 4-fold reduction in both mRNAs (Wood *et al.*, 2000). Interestingly, we observed that the expression of both *RVB* genes is self-repressed. 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. The high level of expression of exogenous XPB coincides with low expression of endogenous XPB, and *vice versa*, that keeps the total cellular content of XPB constant (Hoogstraten *et al.*, 2002).

2.5.2 Functional comparison to bacterial homolog

Certain genes reveal a phenotype when member of a homologous 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 haloinsufficiency effect on recombination and UV damage repair. However, it is important to notice that the growth rate of heterozygous strains was comparable to that of the wild-type, indicating that the effect is not reflecting impaired growth. The correspondence between the Rvb1p and Rvb2p involvement in recombination, and UV damage repair, and bacterial RuvB functions is striking. Interestingly, expression of RuvAB complex in yeast increased its UV resistance. It is known that the expression of bacterial genes that are involved in DNA repair and recombination, such as RecA, RuvC and RusA, can stimulate similar processes in eukaryotes (Doe *et al.*, 2000; Reiss *et al.*, 1996; Shalev *et al.*, 1999). The stimulating effect observed for the RuvAB complex on the UV damage repair (Figure 4), might result from its branch migration activity, that could correct 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, since it has been reported that even mammalian *RVB1* and *RVB2* orthologs cannot complement the deletion of either of the two yeast genes (Lim *et al.*, 2000; Qiu *et al.*, 1998), although they share 70% of sequence identity. The failure in complementation may reflect a requirement for additional partners, which cannot be recognized by "foreign" homologs, or the involvement of yeast proteins in other, non-overlapping pathways essential for the cell. Even though RuvAB complex did rescue UV sensitivity of *RVB1*, and *RVB2* heterozygous strains, this, however, does not prove that eukaryotic and bacterial proteins function equally during UV damage response. The possibility that yeast proteins may be

involved in UV damage repair through transcriptional regulation of genes involved in 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, like *APEX/Ref1*, *BRCA1*, *MSH2* (Menssen and 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 (Ikura *et al.*, 2000; Jonsson *et al.*, 2001; Shen *et al.*, 2000). On the other hand, chromatin remodeling could by itself facilitate DNA repair (Frit *et al.*, 2002; Gaillard *et al.*, 2003). Taken together, one could speculate that Rvb1p and Rvb2p function similarly as their bacterial homolog, and that they acquired additional functions as an adaptation to the chromatin environment as hypothesised previously (Shen *et al.*, 2000).

2.5.3 Rvb2p binds to cruciform DNA

In all previous studies, Rvb1p and Rvb2p, failed to interact with, or branch migrate HJ (Ikura *et al.*, 2000; Qiu *et al.*, 1998). 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 eukaryotic proteins as well need additional protein(s)-link to bind HJ. It was reported that human TIP60 complex, which among other proteins contains Rvb1p and Rvb2p, binds to 3- and 4-way junctions (Ikura *et al.*, 2000). The TIP60 complex could be anchored the to structured DNA through Rvb2p interaction with the "link". Yet, further experiments will be required to determine factor(s) responsible for Rvb2p binding to the cruciform. Although Rvb1p and Rvb2p interact and form complex, it is important to emphasize that exclusively Rvb2p

specifically bound HJ. In addition to their antagonistic role in transcriptional activation (Bauer *et al.*, 2000; Cho *et al.*, 2001; Wood *et al.*, 2000), differential binding to the structural DNA could be one of the crucial differences between two Rvb proteins, which, despite being so similar are still not redundant.

2.5.4 Rvb1p, Rvb2p and chromatin remodelling

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 in consideration chromatin structure that creates a barrier, which should be overcome during both processes. Recently, an increasing number of evidence demonstrate the requirement for chromatin remodelling complexes in recombination and DNA repair, in addition to their already well-established role in transcription (Alexiadis and Kadonaga, 2002; Bird *et al.*, 2002; Frit *et al.*, 2002; Fyodorov and Kadonaga, 2001; Gaillard *et al.*, 2003; Green and Almouzni, 2002; Hara and Sancar, 2002; Narlikar *et al.*, 2002). Consistent with the idea of Rvb proteins as chromatin remodellers, is the observation that Rvb1p/Rvb2p complex, isolated from yeast, shows an ATP-dependent chromatin remodelling activity *in vitro*, which is comparable to that of Swi2/Snf1 complex (Jonsson *et al.*, 2001). Furthermore, both Rvb proteins are also found in yeast Ino80 (Shen *et al.*, 2000), and in human TIP60 (Ikura *et al.*, 2000) chromatin remodelling complexes. In addition, it is worth noting that deletion of *RVB2* in combination with an additional mutation caused flocculation phenotype (Figure 2D), since it is known that the expression of *FLO1*, a

dominant and best-characterized flocculation gene, is regulated by antagonistic chromatin remodelling activities of the Tup1-Ssn6, and Swi-Snf complexes (Fleming and Pennings, 2001). However, it was reported that Rvb2 ts mutant, represses expression of *DIA3* (Ohdate *et al.*, 2003), whose protein product regulates expression of another flocculation gene *FLO11* (Palecek *et al.*, 2000). Still, the requirement for an additional mutation suggests necessity for complex formation, favouring former scenario.

2.5.5 Rvb1p-Rvb2p as a multifunctional complex

Human Rvb orthologs have been isolated in RNA polymerase II (RNAP2) holoenzyme complex (Qiu *et al.*, 1998). RNAP2 holo-enzyme in human cells contains DNA repair factors like BRCA1 (Scully *et al.*, 1997a), 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 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 the 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 behaviour of TFIIH in transcription and repair has been explained by the distinct complex composition for 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 that show 6:1 stoichiometry compared with other polypeptides in their complexes (Jonsson *et al.*, 2001; Shen *et al.*, 2000), could form a hetero-hexamer (or a hetero-dodecamer) core of a larger complex (Jonsson *et al.*, 2001). The ability of the complex to recognize DNA secondary structure could facilitate progression of RNAP2, as well as DNA repair by using ATP as the 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. Nevertheless, to define the mechanism(s) by which Rvb1p and Rvb2p influence diverse cellular processes further studies will be required.

We demonstrated the involvement of Rvb1p and Rvb2p in recombination, and UV damage repair processes since *RVB1* and *RVB2* heterozygous strains showed a decrease in the ability to repair either type of damage in yeast. Mutational analysis performed to date demonstrated the significance of Walker A and B motifs for Rvb1p and Rvb2p function(s) (Jònsson *et al.*, 2001; King *et al.*, 2001; Lim *et al.*, 2000; Wood *et al.*, 2000). To characterize amino acid residues important for the repair activities of two Rvb proteins we carried out PCR-mediated random mutagenesis, and identified and mapped some thermo-sensitive mutations that cause UV, and MMS sensitivity. In addition, bioinformatic analysis of Rvb1, Rvb2 and RuvB protein sequences revealed differences between the two Rvb proteins, as well as their similarities with the bacterial homolog. Based on this similarity, we propose a partial three-dimensional model of Rvb2p.

CHAPTER 3

Mutational analysis of *Saccharomyces cerevisiae* Rvb1p and Rvb2p homologs of bacterial RuvB reveals their involvement in UV and MMS sensitivity

Slobodanka Radovic, Viviana A. Rapisarda and Carlo V. Bruschi

Submitted to DNA Repair

3.1 Abstract

Rvb1p and Rvb2p are two highly conserved eukaryotic proteins, related to the helicase subset of the AAA+ family of ATPases. They display significant sequence similarity with bacterial RuvB. Apart from conserved Walker A and B fingerprint motifs, additional sequence similarity with bacterial protein occurs in the N- and C-terminal regions of the Rvb proteins. Rvb1p, and Rvb2p have been studied heavily, yet their precise function is still not known. Until now, mutational analysis demonstrated the significance of Walker A and B motifs for Rvb1p and Rvb2p function(s). To characterize other functionally important residues, we carried out PCR-mediated random mutagenesis, and mapped several thermo-sensitive mutations that cause UV, and methyl methanesulfonate (MMS) sensitivity. Bioinformatic studies showed that the mutations are located outside the RuvB fingerprint motifs, mostly inside the Rvb-specific domain between Walker A and B boxes, which in addition displays structural difference among the two Rvb proteins. Since our studies also revealed remarkable similarity between secondary structure of the N- and C-terminal regions of eukaryotic proteins and that of the bacterial RuvB, we propose a partial three-dimensional model of Rvb based on this resemblance.

3.2 Introduction

Rvb1p and Rvb2p are two highly conserved eukaryotic proteins (Kurokawa *et al.*, 1999), related to the helicase subset of the AAA+ family of ATPases (ATPases associated with various cellular activities) (Neuwald *et al.*, 1999). AAA+ family members were categorized as proteins with one, or two copies of a well conserved 230-250 amino acid

cassette called the AAA module that encompasses the Walker A, and Walker B motifs, active sites for ATP (or dNTP) binding and hydrolysis. The only helicase with which Rvb1p and Rvb2p display significant sequence similarity is bacterial RuvB. Apart from Walker A and B motifs, additional sequence similarity with bacterial protein occurs in the N- and C-terminal regions that together comprise about 50% of the overall sequence (Kanemaki *et al.*, 1997). RuvB is a helicase that, together with RuvA, promotes branch migration of Holliday junction (Iwasaki *et al.*, 1992; Tsaneva *et al.*, 1992) and facilitates its resolution by the RuvC endonuclease (van Gool *et al.*, 1998; Zerbib *et al.*, 1998).

First, Rvb1p was discovered as interactor of TATA-binding protein in rat, and was named TIP49a (Kanemaki *et al.*, 1997). In human, the same protein was identified in a two-hybrid interaction screen with the hsRPA3 and called RuvBL1 (RuvB-like 1) (Qiu *et al.*, 1998). Its yeast *Saccharomyces cerevisiae* ortholog, as well as *RVB2* (RuvBL2), a closely related family member, were subsequently identified in database searches (Kanemaki *et al.*, 1999; Qiu *et al.*, 1998). Later, both proteins were found in human cells in a large nuclear protein complex (named ECP-51 and ECP-54) (Salzer *et al.*, 1999), and were shown to be essential interactors of β-catenin (named pontin52 and reptin52) (Bauer *et al.*, 1998; Bauer *et al.*, 2000) or c-Myc (Wood *et al.*, 2000).

Rvb1p, and Rvb2p have been studied heavily, yet their precise function is still not known, although both of them are indispensable for yeast (Kanemaki *et al.*, 1999; Qiu *et al.*, 1998), and for *Drosophila* (Bauer *et al.*, 2000). They were demonstrated to be involved, directly or indirectly, in transcriptional regulation of yeast genes (Jonsson *et al.*, 2001; Lim *et al.*, 2000; Ohdate *et al.*, 2003), and to interact with several transcription activators in higher eukaryotes, modulating the cellular transformation, signaling,

apoptosis, and response to stress and DNA damage (Bauer *et al.*, 2000; Cho *et al.*, 2001; Dugan *et al.*, 2002; Wood *et al.*, 2000). We also demonstrated involvement of Rvb1p and Rvb2p in double-strand break and UV damage repair since *RVB1* and *RVB2* heterozygous strains showed decreased ability in repair both types of damage in yeast. The two proteins were co-precipitated immunologically with yeast INO80 (Shen *et al.*, 2000), and human TIP60 chromatin remodelling complexes (Ikura *et al.*, 2000). Moreover, Rvb1p-Rvb2p complex, purified from yeast, showed ATP-dependent chromatin remodelling activity *in vitro* (Jonsson *et al.*, 2001). Finally, Rvb1 and Rvb2 proteins were found to be associated with the production of small nucleolar RNPs (snoRNPs) (King *et al.*, 2001; Newman *et al.*, 2000; Watkins *et al.*, 2002).

Until now, mutational analysis demonstrated the significance of Walker A and B motifs for Rvb1p and Rvb2p function(s) (Jonsson *et al.*, 2001; King *et al.*, 2001; Lim *et al.*, 2000; Wood *et al.*, 2000). To characterize other functionally important domains and amino acid residues, we carried out PCR-mediated random mutagenesis, and mapped some thermo-sensitive mutations that cause UV and MMS sensitivity. Bioinformatic studies showed that the mutations are located outside of the RuvB fingerprint motifs, mostly inside the Rvb-specific domain between Walker A and B boxes, which in addition displays structural difference among two Rvb proteins. Since the studies also revealed remarkable similarity between the secondary structure of the N- and C-terminal regions of eukaryotic proteins and that of the bacterial RuvB, we propose a partial three-dimensional model of Rvb2p based on this resemblance.

3.3 Materials and Methods

Strains and media

Yeast strains used in this study are diploid FAY1 a/α *ADE1/ade1 ade2/ade2 ade8/ade8::FRTX his3/HIS3 leu2/leu2 lys2/lys2 trp1/trp1 ura3-52/ura3-52 [cir$^+$]*, and its derivate strains FAY1Δ1, and FAY1Δ2 heterozygous for *RVB1* and *RVB2*, respectively. Haploids FS1 and FS2 were obtained as described in sporulation and tetrad dissection section. Gene deletions were performed according to the protocols in the EUROFAN program manual (URL http://www.mips.biochem.mpg.de/proj/eurofan/eurofan_1/b0/home_requisites/guideline/exp-transformation.html), and were verified by PCR. The YPD, YPD supplemented with kanamycine, synthetic dropout, and 5'-FOA media were prepared as described (Kaiser *et al.*, 1994). For the preparation of MMS containing plates, MMS was added to YPD to achieve required concentrations. *E. coli* strain DH5α was used for plasmid propagation. *E. coli* cultures were grown in LB broth supplemented with ampicilin as previously described (Sambrook *et al.*, 1989).

Plasmids and primers

Primers used are listed in Table 1. Those not indicated are available on request. Plasmids pGKG and pHKH containing the *KanMX4* gene as a recyclable marker (Storici *et al.*, 1999) were used as templates to amplify cassettes for deleting *RVB1* and *RVB2* respectively. Plasmids pVT-1V5 and pVT-2V5 were constructed using a pYES2.1 TOPO TA Cloning Kit (Invitrogen), in which *RVB1* and *RVB2* ORFs were fused in frame with the V5His6x epitope at their C termini. After PCR amplification, *RVB1*-V5His6x was

inserted into *Xho*I-*Xba*I of pVT100-U plasmid, which contains *URA3* marker gene, generating pVT-1V5 plasmids. Similarly, *RVB2*-V5His6 fragment was subcloned into the *Xho*I-*Sac*I of pVT100-U plasmid generating pVT-2V5. *RVB1* or *RVB2* ORF, and 500 bp of up- (P), and 300 bp of downstream (T) sequence of each gene were amplified separately by PCR from FAY1 genomic DNA using primers P1F, P1R, ORF1F, ORF1HSVR, T1F and T1R, or P2F, P2RNEW, ORF2F, ORF2HSVR, T2F and T2R (Figure 1). Herculase polymerase (Stratagene) was used for amplification under conditions recommended by the manufacturer. *RVB1*-HSV, and *RVB2*-HSV ORFs, and their up- and downstream sequences were sequentially cloned into pYAC3Δ plasmid (pYAC3 whose *URA3* gene was truncated at its 5'), to generate pY1 and pY2, respectively. Both plasmids were sequenced. Sequencing of pY2 plasmid revealed eight silent, G306A, C381T, A543G, C711T, G816A, T972C, A1053G, T1074C, and one mis-sense mutation, T949C, which caused amino acid substitution of non-conserved phenylalanine at position 317 to leucine, in the *RVB2* DNA sequence in respect to that reported in *Saccharomyces cerevisiae* Genome Database. Nevertheless, the plasmid successfully complemented *RVB2* deletion without showing any particular phenotype. No discrepancy in sequence was detected for *RVB*1 fragment in pY1, which also successfully complemented *RVB1* deletion.

Table1. Primers list

primer	sequence 5'-3'
P1F	TATATGTCGACAGGCCTATATGACAGTCTAG
P1R	TATATCTCGAGATTTACTCAATTATTTTAAC
ORF1F	TACCGCTCGAGATGGTCGCTATCAGTGAAG
ORF1HSVR	TAACATGCATGCATTATCCTCGGGGTCTTCCGGGGCGAGTTC TGGCTGGCTTCAAATAATTTGCGGAAGTTTG
T1F	TATCTCGAGACATGCATGCCAGTGAAGAAAGCCTAAAGC

T1R	TATATGTCGACATGGTAGCACAGCCATTACC
KPNF	GGGTGAAGTGACAGAACTAACCCCTGAAG
P2F	TATATGTCGACTTATCCCTAGTCAGTCGCTG
P2RNEW	TATCTCGAGGCTGACAATTTTTTTTATTTCCTTTCAAAGTAACTTGTGC
ORF2F	TACCGCTCGAGATGTCGATTCAAACTAGTG
ORF2HSVR	TAACATGCATGCATTATCCTCGGGGTCTTCCGGGGCGAGTTCTGGCTGGCTTTCCGTAGTATCC ATGGCATCAGGG
T2F	TATCTCGAGACATGCATGCGCATTTTGTACTTTAAGGCAG
T2R	TATATGTCGACGAGTCAAAAGTTTAAAAAAA
EIIR	AACTTCCTCCTCTTGTGCTCTTATAGATAAAA

Sporulation and tetrad dissection

FAY1Δ1 and FAY1Δ2 cells transformed with pVT1-V5 and pVT2-V5 respectively, were grown over night in SC-URA liquid media, washed once, and inoculated in 50 ml of YPA (1% Bacto Yeast Extract, 1% Bacto Peptone, 1% KAc), to a density of 5 x 10^6 cells/ml. Cells were harvested at a density of 2 x 10^7 cells/ml, washed twice, and transferred in 50 ml of SPM (1% KAc plus 1/5 the standard concentration of required amino acids), and then incubated with shaking for 10 days at room temperature. The sporulated cells were treated with lyticase for 10 min on ice. Well-digested four-spore asci were dissected on thin YPD plates using a twin-joystic electric micromanipulator mounted on a FLUOVERT SF microscope (Leitz Wetzlar, Germany). The plates were incubated for 2 days at 30°C to form colonies. Chromosomal deletion of *RVB1* or *RVB2* in spores was confirmed by PCR. The resulting strain in which the chromosomal deletion of *RVB1* was complemented by the plasmid pVT-1V5 was named FS1, while the strain in which the chromosomal deletion of *RVB2* was complemented by the plasmid pVT-2V5 was named FS2.

PCR-mediated random mutagenesis

The scheme of PCR-mediated random mutagenesis is shown in Figure 1. Using pY1 or pY2 as templates, *RVB1* (from position +423 to +1392 with respect to ATG codon), or *RVB2* (from position +1 to +1132 with respect to ATG codon) were mutagenised by Random PCR Mutagenesis Kit (Clontech), using KPNF/ORF1HSVR or ORF2F/EIIR primers, respectively. After PCR, *RVB1* products were digested by *Kpn*I and *Sph*I, and the resulting 870 bp fragment was subcloned into pY1 to replace corresponding region in *RVB1*, generating a pY1-based *RVB1* mutant plasmid library. Similarly, *RVB2* mutagenized PCR products were digested by *Xho*I and *Bst*EII, and 1024 bp fragment was subcloned into pY2, to generate a pY2-based *RVB2* mutant plasmid library.

Plasmid shuffling

The pY1- and pY2- mutant plasmid libraries were introduced into haploid strains FS1 and FS2, respectively. Transformants were plated on 5-FOA plates and incubated for 2 days at 26°C to counter select against the pVT-1V5 or pVT-2V5 plasmids. At this point, the absence of V5-tagged Rvb proteins in several 5-FOA selected clones was confirmed by Western blotting. A total of 900 5-FOA^R colonies for each transformation were isolated and streaked on SC-TRP plates. The plates were incubated for 2-3 days at permissive (26°C) and non-permissive (34°C) temperature.

UV and MMS sensitivity assay

Thermo-sensitive mutants were grown over-night in liquid SC-TRP media at 26°C. For UV sensitivity assay, cultures were diluted ranging from $4x10^6$ to 10^3 and spoted on SC-TRP plates. Plates were immediately UV irradiated using the UV-Lamp VL-6C at a dose of 150 J/m^2 (measured by Radiometer VLX254, France). After irradiation plates were exposed to light for 15 min and then incubated in the dark at 26°C for 2 days. Simultaneously, the same procedure, excluding the UV irradiation step, was performed for the control plates. For MMS sensitivity assay, over-night cultures were diluted as in UV sensitivity assay, and spoted on YPD and YPD+0.01 or 0.02% MMS plates, and then incubated at 26°C for 2 days.

Plasmid rescue and sequencing

The genomic DNA was extracted from UV sensitive mutants by using Wizard Genomic Purification Kit (Promega). The mutagenized *RVB1*, or *RVB2* fragments were PCR amplified from extracted DNA by Expand High Fidelity polymerase (Roch), using KPNF/ORF1HSVR, or ORF2F/EIIR primers, respectively. Following amplification, 0.5 U of Taq polymerase (Promega), and additional 10 mM dATP (Invitrogen) were added into the reaction mix, which was then incubated for 15 min at 72°C to enable cloning into pGEM-T plasmid using pGEM®-T- Easy Vector System I (Promega). The mutation sites were determined by DNA sequencing (.BMR Padova).

Protein analysis

Whole protein extracts were prepared according to the protocol available at URL http://www.pmci.unimelb.edu.au/core_facilites/manual/mb460.asp. 10% SDS-PAGE and Western blot analysis were performed as previously described (Sambrook *et al.*, 1989). The HSV, and V5 epitope-tagged proteins were detected with the mouse monoclonal anti-HSV (Novagen), and anti-V5 (Invitrogen) immunoglobulins G (IgG), respectively, and horseradish peroxidase (HRP) conjugated sheep anti-mouse IgG (Amersham Pharmacia Biotech).

Bioinformatic analysis

Multiple sequence alignments were performed by ClustalW (Thompson *et al.*, 1994) using opening, and end gap penalty 20, and all the other parameters as default. The sequences were obtained from Swiss-prot database, and their accession numbers are: Q03940 for Rvb1, Q12464 for Rvb2, and Q56214 for *T. thermophilus* RuvB. Sequence consensus motifs were searched at PROSITE (Falquet *et al.*, 2002) database at URL http://www.expasy.ch/prosite/. Protein domain analysis was performed by ProDom (Servant *et al.*, 2002) at URL http://prodes.toulouse.inra.fr/prodom/current/html/form.php. Secondary structure predictions were performed by PHD (Rost and Sander, 1993, 1994a), PSIPRED (McGuffin et al., 2000), PHWWW (Ouali and King, 2000), and SCRATCH (Baldi *et al.*, 1999; Pollastri *et al.*, 2002) at Predict Protein server http://cubic.bioc.columbia.edu/pp/, and Jpred (Cuff *et al.*, 1998; Cuff and Barton, 2000) at URL

http://www.compbio.dundee.ac.uk/~www-jpred/, using automatic PSIBLAST (Altschul *et al.*, 1997) alignments as queries. A secondary structure consensus was obtained by comparing all prediction data. Solvent accessibility was predicted by Jnet at Jpred server, and PHD_{acc} (Rost and Sander, 1994b) at Predict Protein server. Search for putative nuclear localisation signals was done using PSORT (URL http://psort.nibb.ac.jp/) and PredictNLS, at Predict Protein server. Three-dimensional model was constructed using Swiss Model (Guex and Peitsch, 1997; Schwede *et al.*, 2003) at URL http://www.expasy.org/swissmod/SWISS-MODEL.html. To visualize the results we used RasMol v2.6 (Sayle and Milner-White, 1995) at URL http://www.umass.edu/microbio/rasmol/.

3.4 Results and Discussion

3.4.1 Isolation of *RVB1* and *RVB2* UV- and MMS-sensitive mutants

Error-prone PCR was used to randomly introduce a single point mutation in the segments of *RVB1* and *RVB2* encompassing their C-terminal and N-terminal regions, respectively (see Materials and Methods and Figure 1). In both cases, mutagenesis covered the sequence between Walker A and Walker B boxes in each protein. In fact, we were particularly interested in novel mutant phenotypes, other than those, already known, deriving from modifications of Walker A and Walker B motifs. Mutagenized fragments were cloned into centromeric plasmids, and the resulting pY1- and pY2-mutant plasmid libraries were transformed into strains FS1 (*RVB1* null mutant), and FS2 (*RVB2* null mutant), respectively. The clones were counter-selected on 5-FOA against the plasmids

already present in the FS1 and FS2 strains that carried the *URA3* selectable marker as well as wild-type *RVB1* or *RVB2* genes, and then tested for their thermo-sensitivity. In total, ~40 out of 900 clones, for either transformation, grew very poorly or did not grow at the non-permissive temperature (34°C). Since we previously demonstrated that *RVB1* and *RVB2* are involved in UV damage repair, these putative thermo-sensitive mutants were further tested for survival after UV irradiation. In addition, we investigated their sensitivity to MMS.

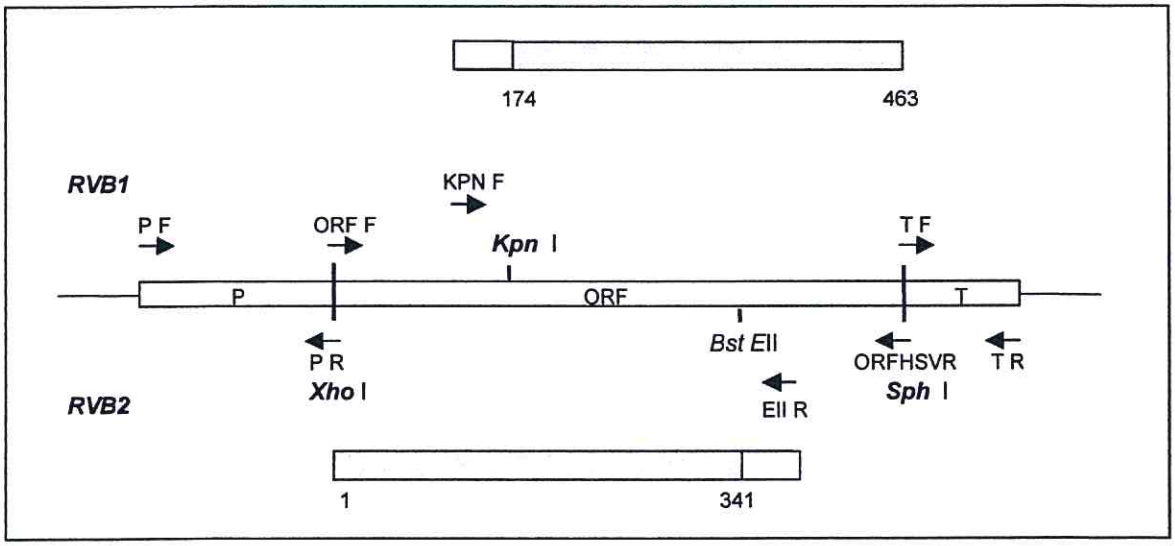
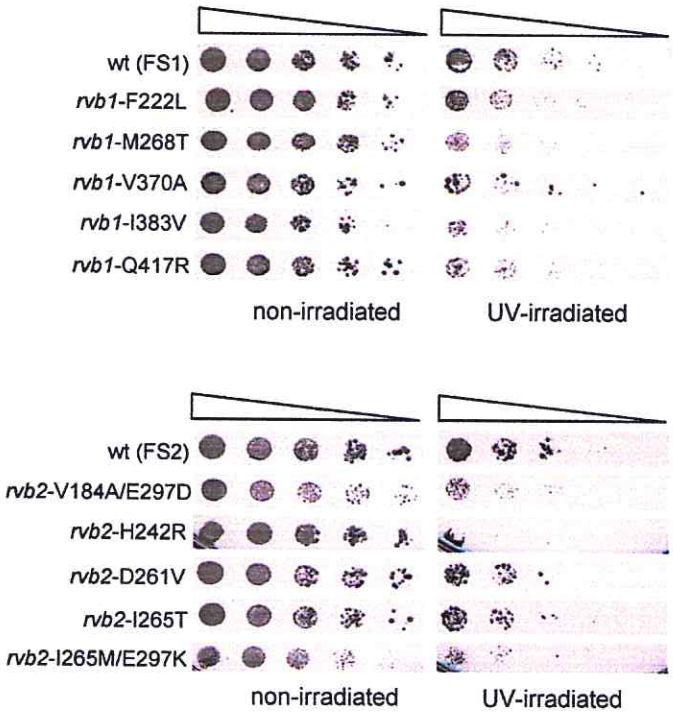


Figure 1. Strategy of cloning and mutagenesis. A general scheme for the *RVB1* or *RVB2* gene is represented in the center of the figure. The position of the primers used to amplify ORF and 500 bp up- and 300 bp downstream sequences of each *RVB* gene. For cloning purposes, the sequence of *Xho*I was added at 5' of the PR and ORFF primers, and that of *Sph*I at TF and ORFHSVR primers. An 18nt HSV epitope sequence was added to each ORF reverse primer to C-terminally tag *RVB1*, or *RVB2*. Primers KPNF and EIIR, positioned ~100 bp upstream of *Kpn*I site in *RVB1*, and downstream of *Bst*EII site in *RVB2*, respectively, were used in combination with ORF1HSVR or ORFF for PCR-mediated random mutagenesis. The amino acids sequences encoded by the PCR-mutagenized DNA fragments are shown in the upper and the lower part of the figure. Of these, the portions from position 174 to 463 in *RVB1*, and 1 to 341 in *RVB2* that were included into the gene products are shown in gray.

Finally, five isolated mutants (F222L, M268T, V370A, I383V, Q417R) from *RVB1* and five of *RVB2* (V184A/E297D, H242R, D261V, I265T and I265M/E297K) showed UV, and MMS sensitivity (Figure 2). One of them, $Rvb2^{H242R}$, was extremely sensitive to UV, but it exhibited low sensitivity to MMS, being susceptible only to high doses (0.02%) (Figure 2B).

A



B

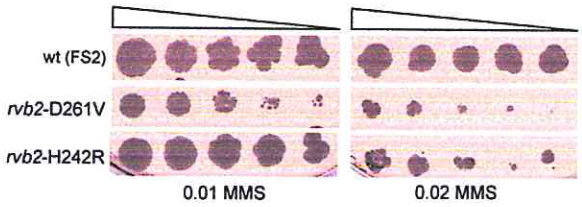


Figure 2. Isolated ts mutations causing UV and MMS sensitivity. A, Serial dilutions of *rvb1*- (top) and *rvb2*-mutants (bottom), and their isogenic wild-type control FS1 and FS2 strains, respectively, plated on TRP medium. One set (right panels) was exposed to ultraviolet irradiation. B, Serial dilutions of *rvb2*-mutant strains D261V and H242R and their isogenic wild-type control (FS2) plated on medium containing the indicated concentrations of MMS.

It is important to note that the amount of the proteins detected in whole cell extracts of all mutants by Western blotting, after four hours of incubation at either permissive, or non-permissive temperature, were comparable to that of the wild-type (data not shown). This suggests that the mutant phenotype was due to loss of function, rather than protein degradation of the protein. These results confirm that Rvb1p and Rvb2p are indeed required for proper UV and MMS damage repair. Differential sensitivity of the $Rvb2^{H242R}$ mutant to UV and MMS damage implies that particular amino acids of the protein are specifically required for the two repair processes.

3.4.2 Secondary structure analysis

Since there is little information about the structure of eukaryotic RuvB-like proteins we used bioinformatics to design a one-dimensional map of Rvb1p and Rvb2p (Figure 3). The multiple sequence alignments of each Rvb protein generated by PHD, or Jpred programs using Psiblast algorithm, were used as inputs. Analyses performed with the complete protein sequences did not give any helpful information for the region between Walker A and Walker B motifs, which is absent in bacterial RuvB, since the secondary structure predictions obtained by different approaches were not matching. To further refine secondary structure prediction the sequences from ^{107}G to ^{302}E for Rvb1p, and from ^{103}G to ^{287}E for Rvb2p, referred to as intra-domains, were considered as unique entities,

and were queried independently. As revealed by the analysis, the secondary structure of the two Rvb proteins is much more conserved than their primary sequence, which shows 42% of identity, and 68% of similarity (Lim *et al.*, 2000; Newman *et al.*, 2000). Indeed, at the secondary structure level, Rvb1 and Rvb2 proteins are almost identical. The only disparity occurs at the end of the intra-domain, where the α-helix present in Rvb1p (from ^{264}D to ^{273}L) is replaced by a β-strand in Rvb2p (from ^{254}F to ^{258}F) (Figure 3). Interestingly, this disparity coincides with the evolutionary acquisition of a distinctive sequence, conserved in all eukaryotic Rvb1p. It is known that two Rvb proteins act antagonistically in transcriptional activation (Bauer *et al.*, 2000; Wood *et al.*, 2000), and that only Rvb2p, and not Rvb1p, interact with the transcriptional activator ATF2 (Cho *et al.*, 2001). We also found that Rvb1p and Rvb2p differentially interact with structural DNAs. These differential actions might be caused by this modest difference inside the intra-domain, which could, in addition, render both of the proteins essential. It is worth mentioning that *Archaea* contain only one Rvb ortholog in which the Rvb1-specific sequence is absent. Phylogenetic studies suggested that archaeal *RVB* gene belongs to the *RVB2* ancestor but not to the *RVB1*, and that *RVB1* evolved from *RVB2* (Kurokawa *et al.*, 1999).

A further difference between Rvb1p and Rvb2p arose during the search for putative nuclear localization signals (NLS). The two short four-residue patterns characteristic of the SV40 large T antigen nuclear targeting signal were found only in Rvb1p (^{237}HKKK and ^{273}KPKK) (Figure 3). The NLS patterns are positioned in the non-conserved region of the intra-domain. Since both Rvbs are nuclear proteins (Bauer *et al.*, 1998; Lim *et al.*, 2000; Makino *et al.*, 1998; Newman *et al.*, 2000), and the two proteins form a complex

(Kanemaki *et al.*, 1999; Lim *et al.*, 2000; Shen *et al.*, 2000), it is plausible that Rvb2p enters the nucleus co-transported by Rvb1p. This hypothesis warrants further experiments in order to be confirmed.

3.4.3 Rvb1p and Rvb2p sequence alignment with bacterial RuvB

When discovered in human cells, Rvb1p and Rvb2p were named RuvB-like1 and RuvB-like2 (Qiu *et al.*, 1998), respectively, since the only protein with which they displayed significant sequence similarity was the bacterial RuvB (Kanemaki *et al.*, 1997). Bacterial protein belongs to the AAA+ protein family lying outside the classical helicase families (Caruthers and McKay, 2002). RuvB structural analysis and sequence conservation identify five fingerprint motifs, which we found to be also well conserved in Rvb1 and Rvb2 proteins (Figure 3). Walker A motif binds triphosphates and presents the γ-phosphate group for cleavage, while Walker B motif binds a divalent metal ion and likely activates the water nucleophile for ATP cleavage. Sensor 1 and Sensor 2 motifs are proposed to be critical, additionally to Walker motifs, for the nucleotide-driven conformational changes of the protein structure. While Sensor 1 may help to distinguish between nucleotide diphosphate and nucleotide triphosphate-bound states of the enzyme, Sensor 2 likely distinguishes between nucleotide-bound and unbound states in addition to enforcing a strained ATP-bound conformational state to prevent non-productive binding (Putnam *et al.*, 2001). Finally, the fifth motif, Arginine finger, facilitates ATP hydrolysis (Putnam *et al.*, 2001).

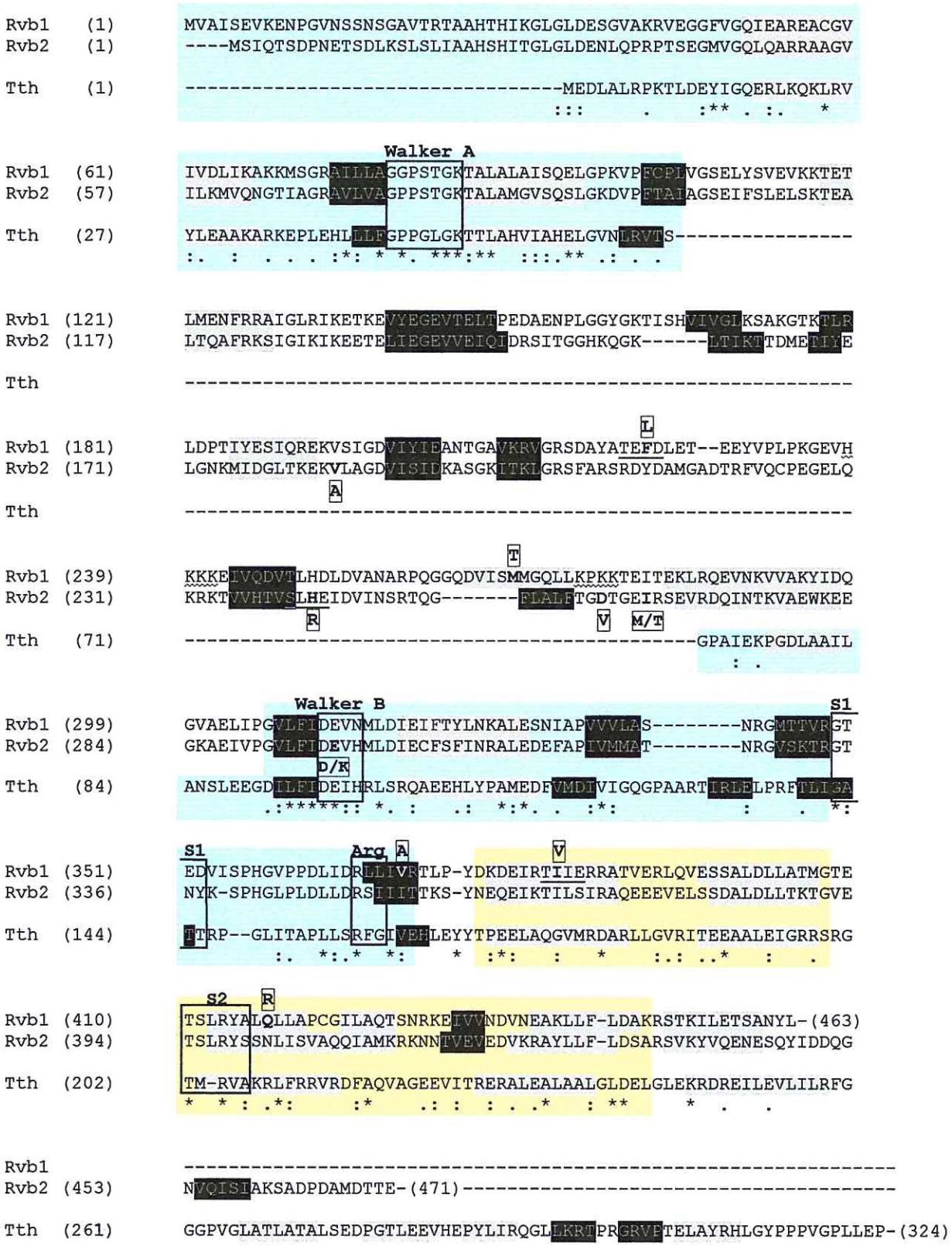

Rvb1 (1) MVAISEVKENPGVNSSNSGAVTRTAAHTHIKGLGLDESGVAKRVEGGFVGQIEAREACGV
Rvb2 (1) ----MSIQTSDPNETSDLKSLSLIAAHSHITGLGLDENLQPRPTSEGMVGQLQARRAAGV
Tth (1) ----------MEDLALRPKTLDEYIGQERLKQKLRV
Walker A
Rvb1 (61) IVDLIKAKKMSGRAILLAGGPSTGKTALALAISQELGPKVPFCPIVGSELYSVEVKKTET
Rvb2 (57) ILKMVQNGTIAGRAVLVAGPPSTGKTALAMGVSQSLGKDVPFTAIAGSEIFSLELSKTEA
Tth (27) YLEAAKARKEPLEHLLLFGPPGLGKTTLAHVIAHELGVNLRVTS----------
Rvb1 (121) LMENFRRAIGLRIKETKEVYEGEVTELTPEDAENPLGGYGKTISHVIVGIKSAKGTKTLR
Rvb2 (117) LTQAFRKSIGIKIKEETELIEGEVVEIQIDRSITGGHKQGK------LTIKTTDMETIYE
Tth ----------
Rvb1 (181) LDPTIYESIQREKVSIGDVIYIEANTGAVKRVGRSDAYATEFDLET--EEYVPLPKGEVH
Rvb2 (171) LGNKMIDGLTKEKVLAGDVISIDKASGKITKLGRSFARSRDYDAMGADTRFVQCPEGELQ
Tth ----------
Rvb1 (239) KKKEIVQDVTLHDLDVANARPQGGQDVISMMGQLLKPKKTEITEKLRQEVNKVVAKYIDQ
Rvb2 (231) KRKTVVHTVSLHEIDVINSRTQG-------FLALFTGDTGEIRSEVRDQINTKVAEWKEE
Tth (71) ----------GPAIEKPGDLAAIL
Walker B
S1
Rvb1 (299) GVAELIPGVLFIDEVNMLDIEIFTYLNKALESNIAPVVVLAS--------NRGMTTVRGT
Rvb2 (284) GKAEIVPGVLFIDEVHMLDIECFSFINRALEDEFAPIVMMAT--------NRGVSKTRGT
D/K
Tth (84) ANSLEEGDILFIDEIHRLSRQAEEHLYPAMEDFVMDIVIGQGPAARTIRLELPRFTLIGA
S1
Arg A
V
Rvb1 (351) EDVISPHGVPPDLIDRLLIVFTLP-YDKDEIRTIIERRATVERLQVESSALDLLATMGTE
Rvb2 (336) NYK-SPHGLPLDLLDRSIIITTKS-YNEQEIKTILSIRAQEEEVELSSDALDLLTKTGVE
Tth (144) TTRP--GLITAPLLSRFGIVEHLEYYTPEELAQGVMRDARLLGVRITEEAALEIGRRSRG
S2
R
Rvb1 (410) TSLRYALQLLAPCGILAQTSNRKEIVVNDVNEAKLLF-LDAKRSTKILETSANYL-(463)
Rvb2 (394) TSLRYSSNLISVAQQIAMKRKNNTVEVEDVKRAYLLF-LDSARSVKYVQENESQYIDDQG
Tth (202) TM-RVAKRLFRRVRDFAQVAGEEVITRERALEALAALGLDELGLEKRDREILEVLILRFG
Rvb1 ----------
Rvb2 (453) NVQISIAKSADPDAMDTTE-(471)----------
Tth (261) GGPVGLATLATALSEDPGTLEEVHEPYLIRQGLLKRTPRGRVPTELAYRHLGYPPPVGPLLEP-(324)

Figure 3. Protein sequence alignment of Rvb1 and Rvb2 against *T. thermophilus* RuvB. The alignment of Rvb1, Rvb2, and *T. thermophilus* RuvB was performed by Clustal W 1.8 program forcing the long gap in the Tth sequence (after ^{70}Ser). Identical amino acids are marked with *; similar amino acids with : or ., and gaps are displayed as dashes. The fingerprint motifs Walker A, Walker B, Sensor 1 (S1), Sensor 2 (S2), and Arg finger are indicated by squares. The secondary structure prediction for Rvb1 and Rvb2 was performed as described in Materials and Methods. Structure of the crystallized *T. thermophilus* RuvB (PDB: 1hqc) was taken from Yamada *et al.* (2001). α-helices and β-strands are boxed in gray, and in black, respectively. The mutated amino acids, causing UV sensitivity are marked bold-faced in squares above or below their wild-type correspondent, in Rvb1 or Rvb2, respectively. Predicted NLS motifs in Rvb1 are wavy underlined, and the putative phosphorylation motifs containing a mutation are underlined. Numbers in parenthesis indicate the position of the first amino acid of each line, and the last amino acid of each sequence.

The crystal structures of *Thermus thermophilus* (Yamada *et al.*, 2001) and *Thermotoga maritima* RuvB (Putnam *et al.*, 2001) revealed that the monomer is made up of three sequential domains. The N-terminal ATPase domain, domain I, folds in characteristic Rossmann fold (Rossmann and Argos, 1981) and contains Walker A, Walker B, Sensor 1 and Arginine finger motifs. It is followed by α-helical domain II that contains Sensor 2 motif (Figure 3 and 4). Interestingly, the predicted three-dimensional structure of the N- and C-terminal parts of either Rvb protein (depicted in pale blue and yellow in Figure 3), resembles domain I, and the hexamerization domain II of the bacterial protein (Figure 4) if we exclude their intra-domain. The only inconsistency between RuvB and Rvb structures was observed in the RuvB region (^{123}G to ^{138}F) which is not conserved in either Rvb, or in other AAA+ class proteins (Guenther *et al.*, 1997; Lenzen *et al.*, 1998). RuvB-specific sequence forms β-hairpin 1 that protrudes from the first domain (Iwasaki et al., 2000), and is required for interaction with RuvA (Han *et al.*, 2001). On the other hand,

the RuvB domain III, which possesses a DNA-binding fold similar to that observed in many transcriptional regulators and in non-specific DNA-binding proteins such as histone H5 (revieved by Gajiwala and Burley, 2000) lacks in Rvb proteins. Nevertheless, eukaryotic proteins have a ~200 amino acid residue insertion between Walker A and Walker B motifs (intra-domain), which was suggested to have a location and function similar to domain III of the bacterial protein (Putnam *et al.*, 2001).

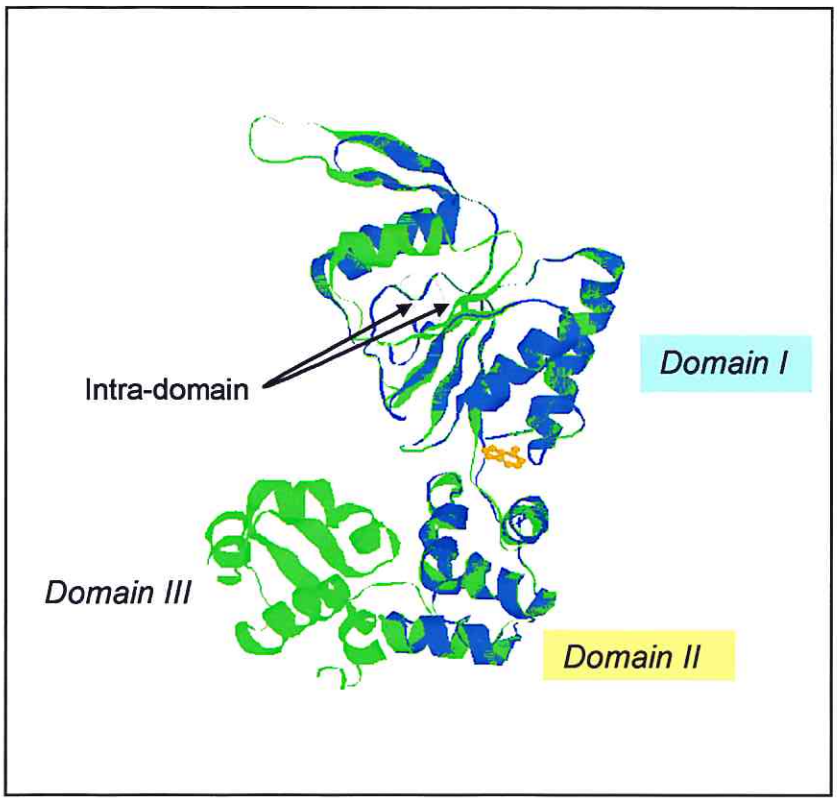


Figure 4. Rvb2p three-dimensional model based on *T. thermophilus* RuvB crystal structure (1hqcA). Ribbon diagram representing superposition of the Rvb2p domains (pale blue and yellow in Figure 3) in dark blue, and RuvB in green. The total energy of the Rvb2p model is –4245.9 kJ/mol. The arrows are indicating the omitted Rvb2p intra-domain. The adenine molecule is shown in orange. Domain I, II, and III of RuvB are indicated.

3.4.4 Mutations in the structural context

As mentioned above, sequence analysis of Rvb1p and Rvb2p indicates the presence of a number of motifs that are important for ATP processing. In yeast, ATP binding and hydrolysis by Rvb1p and Rvb2p are required for cell viability (Jonsson *et al.*, 2001; King *et al.*, 2001; Lim *et al.*, 2000). Surprisingly, two Rvb2 mutants selected carried mutations within the Walker B motif. It is interesting to consider that in both cases ^{297}E was substituted either by D or K, and that in addition both mutants carried an extra mutation in the sequence, V184A or I265M, respectively (Figure 2A). It is questionable which of the two mutations is responsible for the sensitivity phenotypes in either case. It was reported that the E297D mutation caused mild growth inhibition at 37°C (King et al., 2001). On the other hand, we observed that ^{265}I substitution with threonine caused UV and MMS sensitivity by itself (Figure 2A). However, the sensitivity of the double mutant I265M/E297K seems to be greater, suggesting that these two mutations have a synergistic effect (Figure 2A). All the other mutations in Rvb1p or Rvb2p were located outside the fingerprint motifs. Nevertheless, in two Rvb1 mutants, Rvb1^{V370A} and Rvb1^{Q417R} amino acid substitutions occurred nearby the Arginine finger motif inside the predicted β-strand, and the Sensor 2 motif inside the predicted α-helix, respectively (Figure 3). These mutations could possibly impair the motif functions by altering the structure around it. Furthermore, the ^{417}Q, that is predicted to be part of the completely buried α-helix, is exchanged with the basic arginine. Some of the mutations were located in the region distinctive for the two Rvb proteins. In Rvb2 the replacement of the acidic residue ^{261}D, which we predicted to be exposed to the solvent with the hydrophobic valine caused the

sensitivity phenotypes, as well as the replacement of the ^{265}I, a hydrophobic residue predicted to be buried, with the polar threonine, Moreover, in the same region of Rvb1^{M268T} mutant, the hydrophobic methionine, conserved in all eukaryotic Rvb1p, excluding plants, was substituted by the polar threonine, suggesting the importance of this part of the proteins for UV and MMS damage repair. Interestingly, some of the mutations were situated in predicted phosphorylation motifs (^{220}TEFD and ^{382}TIIE, in Rvb1 and ^{240}SLHE in Rvb2). Still, none of them altered conserved amino acids in the T/SXXE/D target motif for Casein kinase II (PS00006). Even though there are no reports on Rvb1p and Rvb2p modifications, this does not exclude the possibility that they exist in phosphorylated form. If this is true, further experiments should be performed to analyze the influence of the mutations on the phosphorylation status of the proteins.

3.4.5 Intra-domain of Rvb1 and Rvb2 proteins

The domain between Walker A and Walker B motifs appears to be unique to Rvb proteins. However, despite of the high E values, the intra-domain shares some sequence similarity with several proteins, as revealed by the Blastp search in *S. cerevisiae* Genome Database, which we used to avoid redundant matches with orthologs. On the other hand, one should consider that the search was performed in one complete genome only. It is remarkable that two Rvb intra-domains, though highly conserved in their primary and secondary structure (Figure 3), retrieved two non-overlapping sets of proteins. The set obtained using the Rvb1p intra-domain as input sequence was larger and contained RNA polymerase II transcription factors like Iws1 and Taf6 (Krogan *et al.*, 2002; Tansey and Herr, 1997). The latter protein was found to be involved in chromatin modification,

histone acetylation, and establishment and/or maintenance of chromatin architecture as a component of the SAGA complex (Grant *et al.*, 1998; Sterner and Berger, 2000). Moreover, Sir4 a histone-binding protein and structural constituent of chromatin involved in silencing and DNA double-strand break repair by non-homologous end joining (Gartenberg, 2000; Hegde and Klein, 2000; Luo *et al.*, 2002; Moretti and Shore, 2001) was identified, as well as Hmi1 involved in maintenance of the mitochondrial genome (Sedman *et al.*, 2000). This indicates that the intra-domain is responsible for Rvb1p involvement in DNA metabolism. It is also significant that search for domains in ProDom database revealed that Rvb1p region (from ^{263}Q to ^{373}L), which covers the last part of the intra-domain, corresponds to the domain found in the topoisomerase 2 family (PD000742), further supporting this hypothesis. In contrast to Rvb1p, the Rvb2p intra-domain showed similarity to a small number of proteins, most of which have unknown function. The ProDom search did not detect domains other than those related to Rvb orthologs.

3.5 Conclusions

In this paper we have described the production and isolation of thermo-sensitive *RVB1* and *RVB2* mutant alleles that cause UV and MMS sensitivity. In most of the cases subtle changes in the amino acid sequence were responsible for the phenotypes. Mutations were mainly located in the domain between Walker A and B motifs, specific for the two Rvb proteins, implying that in addition to Walker motifs, the intra-domain plays an important role in protein function. Moreover, bioinformatic analysis pointed out the involvement of intra-domain in the difference between these two highly conserved proteins. Sequence-

structure alignments also revealed conservation of the secondary structure of Rvb1p and Rvb2p with respect to their bacterial homolog. Based on this observation, we proposed a three-dimensional model for the N- and C-terminal regions of Rvb2p, using *T. thermophilus* RuvB domain I and II as templates. Taken together, the results presented in this report may provide a valuable starting point for further genetic and biochemical studies required to elucidate the role of Rvb1p and Rvb2p in nucleic acid metabolism.

CHAPTER 4

Summary and Conclusions

4 Summary and Conclusions

This thesis presents data obtained from genetic, biochemical and bioinformatic studies of *S. cerevisiae RVB1* and *RVB2* genes and their protein products. Both *RVB* genes are found to be constitutively expressed throughout the cell-cycle in haploid and diploid cells. However, their expression is self-regulated, given that the episomal over-expression of *RVB1* and *RVB2* decreases by ~85% expression of chromosomal *RVB1* and *RVB2* genes, respectively. Rvb proteins are localized in the nucleus throughout the cell-cycle. After nuclear fractionation experiment ~70% and ~85% of Rvb1p and Rvb2p were found in the chromatin-enriched fraction. Both proteins also interact with the DNA, however, in our cell-free protein-DNA binding assay, Rvb2p preferentially binds to artificial HJ, whereas Rvb1p binds to either duplex or cruciform DNA equally.

It has been suggested that Rvb1p and Rvb2p act as transcription factors (Jonsson *et al.*, 2001; Lim *et al.*, 2000; Ohdate *et al.*, 2003). Nonetheless, as both proteins have been proposed to be yeast analogues of bacterial RuvB based on sequence homology, we investigated their involvement in recombination and UV damage repair. Interestingly, diploid strains heterozygous for either gene show significantly lower repair of the DNA DSB in inverted repeat substrates, as well as a decreased survival after UV irradiation. However, neither DSB nor UV damage had any effect on the expression or localization of either Rvb protein. Bacterial *ruvB* alone, or in combination with *ruvA* does not complement functionally *rvb1* or *rvb2* deletions. However, over-expression of the bacterial RuvAB complex increased survival of the *RVB* heterozygous, and wild-type strains after UV irradiation.

Mutational analysis of both *RVB* genes confirmed their involvement in UV damage repair, revealing, in addition, their requirement for normal levels of cellular resistance to the harmful effects of MMS. In most of the cases, subtle changes in amino acid sequence were responsible for the sensitive phenotypes. Mutations were mainly situated in the domain between Walker A and B motifs, specific for two Rvb proteins, confirming that, besides Walker motifs, intra-domain plays important role in proteins functions as well.

Rvb1p and Rvb2p are highly related (Lim *et al.*, 2000; Newman *et al.*, 2000). Bioinformatic analysis performed in this study showed that they are even more similar in their secondary structure than in their primary sequence. The only disparity occurs at the end of the intra-domain, where the α-helix present in Rvb1p is replaced by a β-strand in Rvb2p, highlighting this part as responsible for the differences between two proteins. To determine the degree of similarity of eukaryotic RuvB-like proteins and their bacterial homolog, we compared sequences of the two Rvb proteins and *T. thermophilus* RuvB. These sequence-structure alignments revealed high conservation in the secondary structure between eukaryotic and bacterial proteins. Based on this observation, we proposed a three-dimensional model for the N- and C-terminal regions of Rvb2p, using *T. thermophilus* RuvB domain I, and II as templates.

It is worth mentioning that the human *RVB1* gene is mapped to 3q21, region with frequent rearrangements in different types of leukemia and solid tumors (Makino *et al.*, 2000). In addition, both Rvb proteins interact and modulate activities of several transcription factors, among others c-Myc, mediating oncogenic transformation (Cho *et al.*, 2001; Dugan *et al.*, 2002; Wood *et al.*, 2000). The analysis performed may provide valuable starting point for further genetic and biochemical studies required to better

understand the essential role of both Rvb proteins in cell growth. This could shed light on their involvement in various cellular processes, leading to a possible development of pharmacological agents that interfere with oncogenic transformation through the inhibition of Rvb1p and Rvb2p.

CHAPTER 5

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5 References

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Acknowledgements

This Thesis was carried out in the Microbiology group at the International Center for Genetic Engineering and Biotechnology.

I would like to express my sincere gratitude to:

Prof. Carlo V. Bruschi, my supervisor, for welcoming me in his laboratory, for his guidance and advice.

Kresimir Gjuracic, who has contributed a lot to my understanding of molecular biology for his patience and friendship.

Viviana Rapisarda for introducing me to the "world" of the proteins, but also for all "mamma advice" that she gave.

Valentina Caputo Galarse for being a generous and a very, very, very special friend.

All the other people who have been part of the Microbiology group in these years, Sanjeev Waghmare, Lucia Andreoli, Paolo Floreano, Valentina Tosato, Yuhui Zang, Laura Ciarloni, Mauro Di Giusto, Max Garre' and Monica Sturni for providing a friendly environment.

Agnieszka Chmiel for general philosophical discussions and "wild" life experience.

Patrizia for the "last minute" medium preparations, Carlo Gregori for his help in photographic job, and to all the staff members of the ICGEB.

Everyone I forgot to mention.

My parents for all their support.

Goran for his great patience.