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Identification and Cloning of *jipA* Encoding a Polypeptide That Interacts with a Homolog of Yeast Rad6, UVSJ in *Aspergillus nidulans*

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***RAD6* in yeast mediates postreplication DNA repair and is responsible for DNA-damage induced mutations. *RAD6* encodes ubiquitin-conjugating enzyme that is well conserved among eukaryotic organisms. However, the molecular targets and consequences of their ubiquitination by Rad6 have remained elusive. In *Aspergillus nidulans*, a *RAD6* homolog has been isolated and shown to be an allele of *uvsJ*. We screened a cDNA library to isolate UVSJ-interacting proteins by the yeast two-hybrid system. JIPA was identified as an interactor of UVSJ. Their interaction was confirmed *in vitro* by a GST-pull down assay. JIPA was also able to interact with mutant UVSJ proteins, UVSJ1 and the active site cysteine mutant UVSJ-C88A. The N- and the C-terminal regions of UVSJ required for the interaction with UVSH, a RAD18 homolog of yeast which physically interacts with Rad6, were not necessary for the JIPA and UVSJ interactions. About 1.4 kb *jipA* transcript was detected in Northern analysis and its amount was not significantly increased in response to DNA-damaging agents. A genomic DNA clone of the *jipA* gene was isolated from a chromosome I specific genomic library by PCR-sib selection. Sequence determination of genomic and cDNA of *jipA* revealed an ORF of 893 bp interrupted by 2 introns, encoding a putative polypeptide of 262 amino acids. JIPA has 33% amino acid sequence identity to TIP41 of *Saccharomyces cerevisiae* which negatively regulates the TOR signalling pathway.**

Key words: *Aspergillus nidulans, RAD6,* TIP41, *uvsJ,* DNA repair, TOR pathway

Defects in DNA repair contribute to aging, carcinogenesis, and genetic diseases. Mutations occur spontaneously as a consequence of DNA replication errors as well as DNA repair errors. DNA damage induced by mutagenic treatment often causes induced mutations which result from mutagenic DNA repair (Friedberg, 1988). In yeast, mutation induction is largely dependent on the *RAD6* group genes, including *RAD6*, *REV1*, *REV3*, and *REV7*. *rad6* mutants exhibit increased spontaneous mutation frequencies and lack of UV-induced mutagenesis (Lawrence and Christensen, 1976). Rad6 is a ubiquitin-conjugating enzyme (Ubc) in *Saccharomyces cerevisiae* and functions in diverse cellular processes. *Rad6* is also necessary for sporulation (Morrison *et al.*, 1988), telomere silencing (Huang *et al.*, 1997), and protein degradation based on the amino-end rule (N-end rule; Dohmen *et al.*, 1991). All cellular functions mediated by Rad6 require its Ubc activity, since substitution of the active site cysteine (Cys88) residue in Rad6 confers the *rad6* null mutant phenotypes (Sung *et al.*, 1990). However, the role of Ubc activity on a variety of cellular activities including the role in mutagenesis is still not clear. Rad6 has been to interact with Rad18; this interaction requires both the N- and C-terminal ends of Rad6 (Bailly *et al.*, 1994, 1997).

Recently, there have been several clues that possibly link the Ubc activity of Rad6 to the Rad6 role in mutation induction and gene silencing. Rad6 and Rad18 complex are able to ubiquitinate the proliferating cell nuclear antigen (PCNA), a DNA-polymerase sliding clamp involved in DNA synthesis and repair (Hoege *et al.*, 2002; Matunis, 2002). Modification of PCNA at lysine-63 residue by DNA damage-induction by Rad6-Rad18 has an effect on cell survival. Histone H2B has been identified as an ubiquitination target of Rad6 in *S. cerevisiae*, and mutation of the conserved ubiquitination site of lysine-123 confers defects in mitotic cell growth and meiosis (Robzyk *et al.*, 2000). Ubiquitinated H2B mediates methylation of histone H3 at lysine-4 which is a prerequisite for gene silencing at the telomere (Sun and Allis, 2002).

In *A. nidulans* four epistasis groups of DNA repair

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mutants, UvsF, UvsB, UvsC, and UvsI groups, have been established based on the sensitivities of single and double mutants to UV or to methyl methane sulfonate (MMS) (Kafer and Mayor, 1986; Chae and Kafer, 1993). Many of the genes complementing DNA repair mutants have been cloned and characterized (for review, Kafer and May, 1998; Goldman *et al.*, 2002). Recently, a *Rad6* homolog has been cloned and shown to be an allele of *uvsJ* by the authors (Jang *et al.*, 2001). UVSJ contains the conserved Ubc active site cystein-88 residue and interacted it with UVSH, a yeast Rad18 homolog. To identify possible targets of UVSJ, we screened proteins interacting with UVSJ with the aid of the yeast two-hybrid screening system. Two positive clones were finally identified. UVSH was one of them and the other, JIPA, was further characterized in this study. JIPA exhibited moderate similarity to the yeast TIP41 which was recognized recently as a key regulator of the TOR signalling pathway of yeast (Jacinto *et al.*, 2001), providing a possible connection between the Rad6 and TOR pathways.

Materials and Methods

Strains, media, general molecular techniques, and DNA sequencing

Wild type A4 strain from Fungal Genetics Stock Center (University of Kansas Medical School, Kansas City, KS, USA) was used for Northern blots. Standard complete medium (CM), previously described (Kafer, 1977) was used for culturing *Aspergillus nidulans*. The yeast strain, L40 [*MATa his3200 trp1-901 leu2-3112 ade2 LYS2*::(4*lex*Aop-*HIS3*) *URA3*::(8*lex*Aop-*lacZ*) *GAL4*] was used for the yeast two-hybrid screening host and was purchased from Invitrogen (Groningen, The Netherlands). For yeast culture and selection of transformants, synthetic dropout minimal (SD) medium with various supplements was used. Molecular cloning techniques were followed as described by Sambrook *et al.* (1992). DNA sequences were determined by an automatic sequencer (Applied Bioscience Inc.) using a Tag DyeDeoxy terminal cycle sequencing kit.

Yeast two-hybrid screening

To facilitate screening, we used the *LexA* based yeast two-hybrid system which permits detection of the *LacZ* reporter gene expression directly on the screening plates as well as taking advantage of *Gal4* activation domain (AD) fusion-cDNA libraries of *A. nidulans* (kindly provided by Dr. K.-Y. Jahng). A bait vector, pTLexA, was constructed by inserting the *Pst*I-*Pvu*II fragment carrying the *TRP1* gene from pGBT9 (Clonetech Laboratories, Inc., USA) into the *Pst*I and Klenow filled-in *Sph*I sites of pHybLex/Zeo (Invitrogen, Groningen, The Netherlands). For a bait to screen proteins interacting with UVSJ, the open reading frame (ORF) of *uvsJ* was PCR-amplified with primers carrying the *Eco*RI or *Xho*I restriction enzyme sites at each end and ligated into the same restriction enzyme sites within pTLexA, generating pTLexA-UVSJ.

The yeast two-hybrid screening was carried out according to the instructions from Clontech Laboratories, Inc. Briefly, the *Aspergillus* two-hybrid cDNA library was transformed into yeast L40 having pTLexA-UVSJ. Positive colonies implying the existence of a UVSJ-interacting protein derived from the transformed cDNA library clone from among the transformants were directly selected on an SD plate with 2 mM of 3-amino-1,2,4-triazole (3-AT) lacking histidine. The positive colonies were subject to *lacZ* reporter assay on SD containing 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal).

Yeast transformation, plasmid isolation, and β-galactosidase assay

The lithium acetate method for preparing yeast competent cells (Ito *et al.*, 1983) was adopted to introduce the various constructs into yeast. In the plate β-galactosidase assay for a blue/white detection directly on SD medium, X-gal was added at a concentration of 40 mg/l to melted SD medium.

GST pull-down assay

The coding region of *uvsJ* was subcloned into the *EcoR*I/*Xho*I site of pGEX4T-1 (Amersham Pharmacia Biotech, UK) and GST-UVSJ proteins were expressed in *E. coli* BL21(DE3) with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) induction. Subsequently, cells were sonicated in ice-cold lysis buffer (200 mM Tris-Cl, pH 8.0, 0.5 M NaCl, 100 mM EDTA, 0.1% Triton X-100, 0.4 mM PMSF). Recombinant GST-UVSJ protein was incubated with glutathione coated beads (Amersham Pharmacia Biotech, UK) for 16 h at 4°C.

The ORF of *jipA* was subcloned into pcDNA3 (Invitrogen, USA). pcDNA3-JIPA was translated *in vitro* with a TnT transcription/translation system kit (Promega, USA). Briefly, 2 mg of pcDNA3-JIPA DNA was incubated with 20 mCi of [^{35}S]-methionine in TnT quick master mix for 90 min at 30°C. Equal amounts of aliquots were mixed with either GST-UVSJ or GST proteins bound onto glutathione coated beads in the binding buffer (50 mM HEPES, pH 7.6, 50 mM NaCl, 5 mM EDTA, 0.1% NP40, 10% glycerol) and incubated for 16 h at 4°C. After washing three times with lysis buffer, samples were treated with SDS-loading buffer containing 5% β-mercaptoethanol. The samples were loaded to SDS-PAGE and visualized by autoradiography.

Northern analysis

For total RNA preparation, mycelia of wild type A4 strain grown in liquid CM for 16 h at 37°C with vigorous shaking were harvested with Miracloth (Calbio-

Chem, USA). MMS was added for one hour directly into the 15 h culture. For UV-illumination, 16 h-grown mycelia were spread onto petridishes and irradiated for the desired time. The dose rate of the UV-lamp was 1.6 J/m^2/sec.

Results and Discussion

Screening and identification of UVSJ-interacting proteins

In yeast two-hybrid screening, 260 positive colonies among the total of 8×10^5 transformants selected on SD plates with 2 mM 3-AT lacking histidine were further tested for the expression of the *lacZ* reporter gene on medium containing X-gal. Two positive colonies showed a strong blue color on X-gal containing medium and were further investigated. Plasmids originated from the cDNA library were prepared from the two positive yeast colonies and transformed into *E. coli*. These cDNA were completely sequenced. One of them was the *uvsH* gene of which product was considered to interact with UVSJ (data not shown). The other cDNA contained an ORF encoding a polypeptide of 262 amino acids. This was named JIPA, UVSJ-Interacting Protein A.

GST pull-down assay for confirming* in vitro *interaction of UVSJ with JIPA

In order to test whether JIPA was able to interact with UVSJ *in vitro*, GST and GST-UVSJ fusion protein were expressed in *E. coli* strain BL21(DE3) using pGEX4T-1 and pGEX4T-UVSJ, respectively. As shown in Fig. 1, *in vitro* translated and S^{35}-labeled JIPA was pulled down with GST-UVSJ but not with GST. S^{35}-labeled JIPA was only retained on matrices to which GST-UVSJ had bound, but not GST only. Thus, the interaction of JIPA with UVSJ was demonstrated.

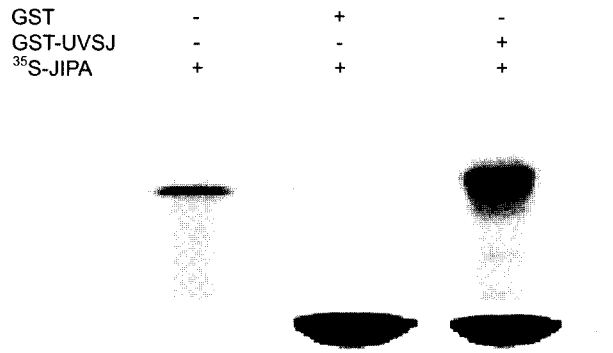

Fig. 1. Interaction of GST-UVSJ with *in vitro* translated S^{35}-JIPA. From the total of 50 µl *in vitro* translation reaction volume, 1 µl , 24 µl, and 24 µl were loaded in left, middle, and right lanes, respectively. Reaction products were resolved on a 10% SDS-polyacrylamide gel. Coomassie stained GST fusion proteins from the same gel were aligned to show protein levels added (bottom panel).

Determination of JIPA and UVSJ interacting regions

To identify the interacting regions between JIPA and UVSJ, deletion mutant proteins of UVSJ and JIPA were constructed and cloned into pTLexA and pGAD424 vectors (Clonetech Laboratories, Inc., USA), respectively for the interaction assay based on the yeast two-hybrid system. Deletions on the C-terminal 31 amino acids and the N-terminal 13 amino acids of UVSJ did not affect the interactions with full-sized JIPA (Fig. 2). In yeast Rad6, a homolog of UVSJ, the indicated N-terminal and C-terminal regions were both required for the interaction with Rad18. The same was true for UVSJ in interacting with UVSH (Chae, S.-K, unpublished data). In contrast, the N-terminal 127 amino acids and the C-terminal 128 amino

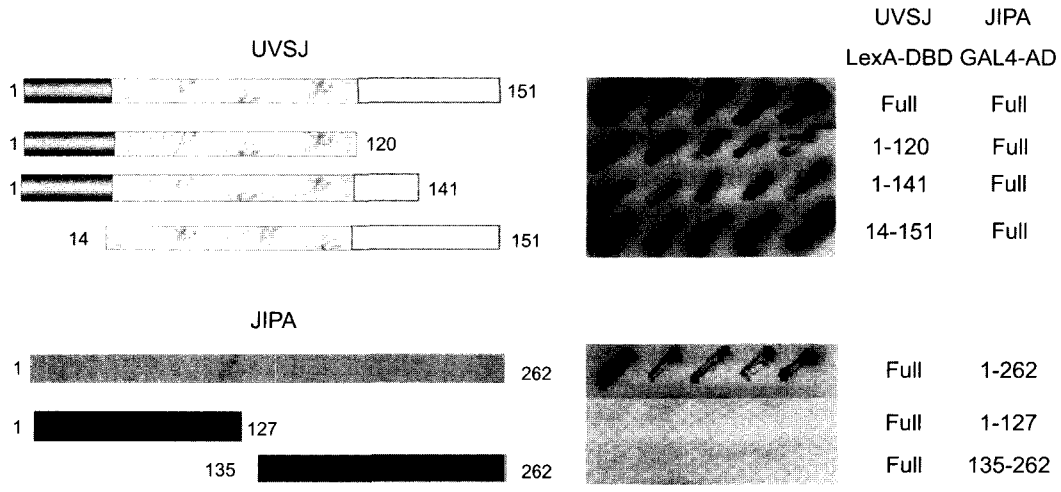

Fig. 2. Interacting regions of UVSJ with JIPA. The yeast two-hybrid system was used to map the interacting regions of both proteins. The constructs producing various kinds of truncated UVSJ and JIPA fused to LexA DBD and GAL4 AD, respectively, were made based on PCR with cognate primers having *Eco*RI or *Xho*I restriction enzyme site at the ends. Two pairs of constructs indicated in the figure were cotransformed into L40, then transformants were subject to blue/white test on X-gal containing SD medium. Blue color indicates protein interaction between two proteins expressed within a yeast cell.

acids of JIPA truncates were not sufficient to interact with UVSJ (Fig. 2).

In order to test whether UVSJ1-[H58P] mutant protein as well as the Ubc active-site mutant UVSJ-[C88A] protein were able to interact with JIPA, both mutant proteins were expressed as a LexA DBD fusion protein in yeast. Cotransformants of yeast expressing both LexA DBD-UVSJ1 (or -UVSJ [C88A]) mutant protein and GAL4 AD-JIPA were able to transactivate the *lacZ* and *HIS3*

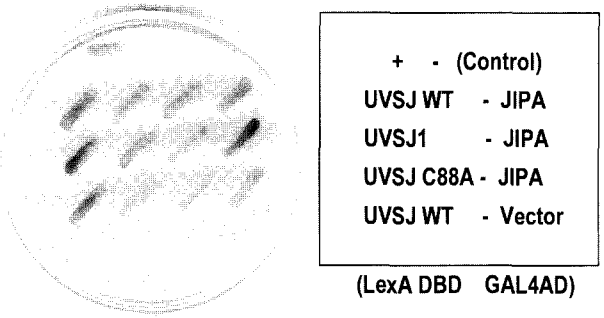

Fig. 3. Interaction assay between JIPA and UVSJ mutant proteins. The plasmids expressing UVSJ1 (pTLexA-UVSJ1) or the Ubc active site cystein UVSJ-[C88A] mutant proteins (pTLexA-UVSJ-[C88A]) fused with LexA DBD were made by inserting *Eco*RI-*Xho*I fragments of UVSJ1 and UVSJ-[C88A] ORF into pTLexA. These plasmids were cotransformed with pGAL4AD-JIPA expressing JIPA fused with GAL4 AD or pGAL4AD vector as a negative control into L40, then *lacZ* reporter was assayed on SD containing X-gal.

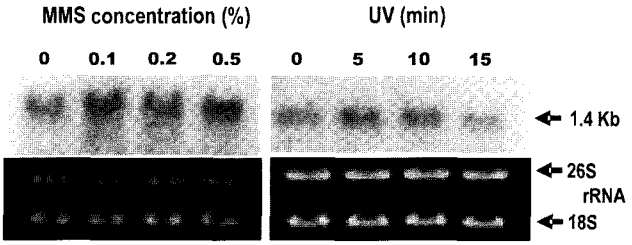

Fig. 4. Northern analysis of *jipA*. Total RNA from MMS or UV-treated mycelia was transferred onto nylon membrane and hybridized with P^{32}-labeled *jipA* cDNA as a probe. The same gel picture of rRNA is shown as a control (bottom panel).

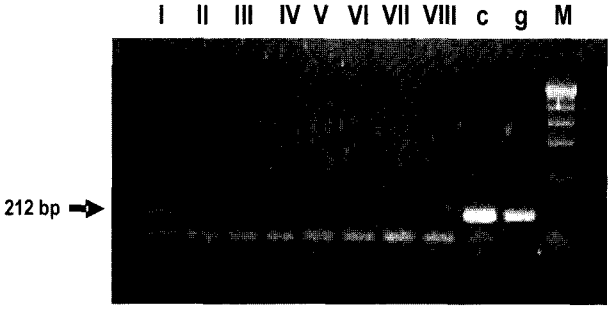

Fig. 5. PCR products from the chromosome specific DNA pools as templates with primers specific for *jipA*. The expected 212 bp *jipA* PCR product was seen in chromosome I specific genomic DNA pool (left lane). I - VIII indicate DNA pools from chromosome I - VIII specific libraries. c, isolated *jipA* cDNA as a template; g, isolated genomic DNA from A4 wild type as a template; M, size marker.

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CCGCGGGGTATTGAATGCCGCCTCCGCAGCGACCCCTCCATTAAGCTATTGTTACTGTCATCTGAACGATACGTTGAAGA  80
CGTGGCAAAGACACGAGGGGAAACCGCGAATCGCTTCCAGACCTGGCTATTCCGGCTATAGAGGAGAGACAACATGGCGT 160
                ▽
CCAATAGCCGCACGGCTTCTGTACCTATCGCGGGGAACACGGCCGCAATGTCGCCTACCTCGCAGCGGCCAGACACCATT 240
                                                M  S  P  T  S  Q  R  P  D  T  I
ACTGTCAAGGGCTTCAAGATTTCAACCCAAAAGCTTCCAATTCTCAAGGCAGGGCCCATTGAAGCAATGGCGAAAAAGCT 320
T  V  K  G  F  K  I  S  T  Q  K  L  P  I  L  K  A  G  P  I  E  A  M  A  K  K  L
TGGTATCGCCCCACCAGAAATGATCTTTGGGGATAACTTTGTTTCTATTGAACATGAGAAGAGTGGATGGAGCATTCACT 400
 G  I  A  P  P  E  M  I  F  G  D  N  F  V  S  I  E  H  E  K  S  G  W  S  I  H  F
TCAACGCTTTGGACGCCTTAGACCGCGTTGATAAAACGGGAGAATCAATGCTTGAGGTAGCACATTCAAAAGAATGGCAA 480
  N  A  L  D  A  L  D  R  V  D  K  T  G  E  S  M  L  E  V  A  H  S  K  E  W  Q
AAAAGCAGgtgcgttcgcgcatttggttgtgaccggttcgggacggctgatatattgacagAGAGACAACTCATGAAGGT 560
K  S  R                                                       E  T  T  H  E  G
ATCAAAGATGTCATCAAACCATTTGACTGGTCCTACAGTACAGATTACAAGGGCACTGTATTGTCCATTCAAGGCCCCGA 640
I  K  D  V  I  K  P  F  D  W  S  Y  S  T  D  Y  K  G  T  V  L  S  I  Q  G  P  D
CTTCGAGGAGACCTCAAAGCCCATACCCATTGAACTGCTGAAACGCCCTGACCCAATACTTTTCTTCGATGAAGTAATAT 720
 F  E  E  T  S  K  P  I  P  I  E  L  L  K  R  P  D  P  I  L  F  F  D  E  V  I  L
TGTACGAAGATGAACTCGCCGATAATGGTATTACTATGCTGTCTTGTAAAATTCGCGTTATGCAGGACAGACTTCTTCTT 800
  Y  E  D  E  L  A  D  N  G  I  T  M  L  S  C  K  I  R  V  M  Q  D  R  L  L  L
CTCTCGCGGTTCTTCTTGAGATTAGACAACGTTCTTTTCCGACTTCGGGACACCAGGGTATATGTCGATTTCGAGAAATC 880
L  S  R  F  F  L  R  L  D  N  V  L  F  R  L  R  D  T  R  V  Y  V  D  F  E  K  S
AGAAGTTATAAGGGAGTATCAGTCCAAAGAGTGTGATTACGGCATCGTCAGACAGgtgggttcgctgtgagagccaagga 960
 E  V  I  R  E  Y  Q  S  K  E  C  D  Y  G  I  V  R  Q
attccaaggctaactcatcaacatagAAGTTAGCAAGCGCACGGGATGATATACCCGCTATCATGAGGGACCCTAACAGG 1040
                          K  L  A  S  A  R  D  D  I  P  A  I  M  R  D  P  N  R
CTCTCCGAACTTCTTCCCCTTGTTGACCGACGACTAGAACGCGTGGTTCTCGACGGATAGATTGAGTAGCAGAGACTGTT 1120
L  S  E  L  L  P  L  V  D  R  R  L  E  R  V  V  L  D  G  *
CGTACGAGAGAATATTGTTTGGCATAGAGgtgcgagcaagttcctcgaagatcttgatagaccgagggaactatatccag 1200
acgcatatccgcgacctgctcactagcatccagTTCGAACCGCCAGCCGGGCGTCTATCAACTCAGCACAGCGTTTCAGG 1280
CCTGTGAGAGCATTTCTGCGAGCCATGCACGCAGCGCGGTGATATATCAAATGGCCATCGCAACATAAAACAAGCATATG 1360
                ▽
CAATCAAGTGGAATTCTTGACTCACACTTGTAGCGCATTTATCATGTAAAAGCTCATACTTTGAGGTAGCAGGCCTCACA 1440
GTAGACTTGGATCTGCCAATCAGAGCATGTATTACTTAAACCGATGCGTCAAACAGTGCAAACGGTCGAC           1510
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Fig. 6. Nucleotide and deduced amino acid sequences of the *jipA* gene of *A. nidulans*. The sequences of three introns are shown in lowercase letters. The stop codon is indicated by an asterisk. The starting- and end-point of cDNA sequence derived from two-hybrid screening is indicated with inverted triangles (▽). The nucleotide sequences corresponding to the PCR primers used PCR-sib selection are indicated by underlines. *Sac*II and *Sal*I restriction enzyme sites at both ends are italicized. The sequence shown above has been assigned a GenBank accession number AY238937.

reporters, indicating protein interactions between these proteins, respectively (Fig. 3).

Northern analysis of jipA

To estimate *jipA* transcript size, Northern analysis was carried out with *jipA* cDNA isolated from the two-hybrid screening as a probe. The 1.4 Kb transcript was detected in Northern analysis (Fig. 4). To test whether *jipA* transcripts are induced similar to that for *uvsJ* after treatment with DNA damaging agents, RNAs prepared from MMS and UV-treated mycelia of the wild type were subjected to Northern analysis. The amount of *jipA* transcripts was not increased significantly, if any, after treatment with DNA-damaging agents (Fig. 4). In general, transcripts of genes involved in DNA repair processes of *Aspergillus,* including *uvsJ* have been shown to be induced significantly after treatment with DNA damaging agents (Chae, S.-K, unpublished data.) Thus, *jipA* might not be involved in DNA repair processes.

Isolation, localization, and DNA sequence determination of jipA genomic clones

To clone the *jipA* gene from the genomic DNA library, DNA was prepared from eight separate pools of *E. coli* of which each contained *A. nidulans* chromosome I to VIII specific genomic DNA libraries. As a template of DNA from each pool, PCR reactions with two *jipA* specific primers (primer sequences in Fig. 6) were carried out. An expected size of 212 bp *jipA* specific DNA band was only generated from the chromosome I specific genomic DNA pool (Fig. 5). A total of 462 individual cosmid clones in the chromosome I specific library were sub-pooled without overlap. Similarly, DNA was extracted from each sub-pooled *E. coli* and subjected to PCR reaction. Again, a certain pool exhibiting the desired band after PCR reaction was further divided into sub-sub-pools, and subjected to another PCR to select a *jipA* containing pool. Finally, this PCR-sib selection was able to identify 3 individual colonies, W9H02, W17O06, and W10H02 which showed

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A. nidulans     MS---------------------------------PTS-------------------- 5
S. cerevisiae   MSKRNTPPLRSSGINTIQINAAREMHAQTVRARRMPMPTSGITTPSVQPTAAPATPPRHI 60
D. melanogaster ------------------------------------------------------------
H. sapiens      ------------------------------------------------------------

A. nidulans     -QRPD-------------------------TITVKGFKISTQKLPILKAG---------- 29
S. cerevisiae   CNNPNNPQCLHCGSVIIPSPRATLPLEDNPSISINDWTISSRKKPILNSQEL-------- 112
D. melanogaster ----------------MEECFVPRLPVDSEFIQFHDWAIKYEKSHILKSSCQLGTAKCCP 44
H. sapiens      ------------------MMIHGFQSSHRDFCFGPWKLTASKTHIMKS----------- 30
                                     :  .  : :.  *  *:::

A. nidulans     --------PIEAMAKKLGIAPPEMIFGDNFVSIEHEKSGWSIHFNALDALDRVDKTGESM 81
S. cerevisiae   --------DIWENEKLKGLTLPEMIFGNNYIRIENSKQHWSIEFNALDALKEVQLQ-DSG 163
D. melanogaster KDSADRCDLCHYQHSLQLPHLPDMVFHKNRLVLQHK-DGATLEFCPMDALALVDNG-KQP 102
H. sapiens      ------ADVEKLADELHMPSLPEMMFGDNVLRIQHG-SGFGIEFNATDALRCVNNY-QGM 82
                           .      *:*:* .* : :::  .   :.* . ***  *:   .

A. nidulans     LEVAHSKEWQKS--RETTHEGIK----------DVIKPFDWSYSTDYKGTVLS-IQGPD 127
S. cerevisiae   IRVAYSNDWINSKKRQNSTNGAQRFTNDVNDDSLNIIHKYDWTYTTRYKGTESS-PESKF 222
D. melanogaster LEVACAQEWRETRNE-QTMEEKFK-------------PFDWTFTSTYQGTMN---EKVR 144
H. sapiens      LKVACAEEWQESRTEGEHSKEVIK-------------PYDWTYTTDYKGTLLGESLKLK 128
                :.** :::* ::  .    :                  :**:::: *:**

A. nidulans     FEETSKPIPIELLKRPDPILFFDEVILYEDELADNGITMLSCKIRVMQDRLLLLSRFFLR 187
S. cerevisiae   RLDNDQKLPLDKLAVHDKILFYDDMILFEDELADNGISILNVKIRVMNERLLLLSRFFLR 282
D. melanogaster SETTNQTLNKFKLMQRENIIFYHDLTLFEDELHDHGISVMSVRIRVMPSGFFILLRHFLR 204
H. sapiens      VVPTTDHIDTEKLKAREQIKFFEEVLLFEDELHDHGVSSLSVKIRVMPSSFFLLLRFFLR 188
                  . . :    *   : * *:.:: *:**** *:*:: :. :**** . :::* *.***

A. nidulans     LDNVLFRLRDTRVYVDFEKSEVIREYQSKECDYG-IVRQKLASARDDIPAIMRDPNRLSE 246
S. cerevisiae   VDDVLVRVYDTRIYVEFDENVVIRESKEFEGKYQDVLAKHRLSQSHDPKAALRDSNWVAQ 342
D. melanogaster VDHVLIRMHDTRFHHEIENDFILKEYIHREAPCT--------ELQNC-VAFWTNPDEMQE 255
H. sapiens      IDGVLIRMNDTRLYHEADKTYMLREYTSRESKIS--------SLMHVPPSLFTEPNEISQ 240
                :* **.*: ***.: : :: :::*    *            . .   :  :.: : :

A. nidulans     LLPLVDRRLERVVLDG---------------- 262
S. cerevisiae   NTPMIKRQCEIIQF------------------ 356
D. melanogaster FVPVKSKQLHKLFFK----------------- 270
H. sapiens      YLPIKEAVCEKLIFPERIDPNPADSQKSTQVE 272
                  *: .  . : :
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Fig. 7. Comparison of the amino acid sequences of *A. nidulans* JIPA with homologs found in other eukaryotes. Alignment was produced using CLUSTAL W program with amino acid sequences of JIPA homologs from *S. cerevisiae* (NP_015365), *D. melanogaster* (AAF45376), *H. sapiens* (CAA16171). Identical and similar amino acids are marked with asterisks and dots, respectively.

the desired 212 bp *jipA* PCR bands. All PCR DNA fragments from these colonies demonstrated the same sizes of DNA fragments after digestion with *Ssp*I restriction enzyme similarly found for the PCR fragments from cDNA of *jipA* (data not shown). The W10H02 was further analyzed to locate and sub-clone the *jipA* gene.

Cosmid DNA from W10H02 was digested with various restriction enzymes and subjected to Southern analysis with *jipA* cDNA as the probe. The 2.7 Kb *Sal*I DNA fragment hybridizing the probe was subcloned into pBluescript SK vector and the DNA sequence of both strands was determined. DNA sequence of the 1.5 Kb *Sac*II and *Sal*I DNA fragment within the 2.7 Kb *Sal*I fragment (deposited in the GenBank database under the accession number AY238937) is shown in Fig. 6. *jipA* contains two introns of 53 bp and 51 bp within the coding region and one intron of 84 bp in 3'-untranslational region (3'-UT). The deduced 262 JIPA amino acid sequence was used to search protein databases. Homologous proteins of JIPA were found among eukaryotic organisms, from lower eukaryotes including budding and fission yeasts to human (Fig. 7). TIP41 (or SDF1 and YPR040Wp) of *S. cerevisiae* showed 33% amino acid identity to JIPA. Homozygous *sdf1 (tip41)* null mutants constructed through the *Saccharomyces* genome deletion project did not sporulate and showed resistance to benomyl. Homozygous and haploid *sdf1* null mutants were also sensitive to thiabendazol. Recently, TIP41 was identified as a TAP42-interacting protein involved in the regulation of Sit4 in yeast (Jacinto *et al.*, 2001). Sit4 is the type 2A-related phosphatase required for G1/S transition of mitotic cell cycles and is regulated negatively by the rapamycin-sensitive TOR kinases (for review Abraham, 2002). TOR kinases promote the association of Sit4 with the inhibitor TAP42. Dephosphorylated TIP41 by Sit4 phosphatase was able to bind to TAP42, thus inhibiting TAP42-SIT4 interaction. It will be very interesting to see whether ubiquitination of TIP41 by Rad6 is a part of TOR signalling regulation.

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