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Genetics

Dhr6, a *Drosophila* homolog of the yeast DNA-repair gene *RAD6*

(ubiquitin conjugation/E2 enzyme/DNA damage/UV mutagenesis/sporulation)

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ABSTRACT **The *RAD6* gene of the yeast *Saccharomyces cerevisiae* is required for DNA repair, for DNA damage-induced mutagenesis, and for sporulation, and it encodes a ubiquitin-conjugating enzyme. We have cloned the *RAD6* homolog from *Drosophila melanogaster* and find that its encoded protein displays a very high degree of identity in amino acid sequence with the homologous RAD6 proteins from the two divergent yeasts, *S. cerevisiae* and *Schizosaccharomyces pombe*, and from human. Genetic complementation studies indicate that the *Drosophila RAD6* homolog can functionally substitute for the *S. cerevisiae RAD6* gene in its DNA-repair and UV-mutagenesis functions but cannot substitute in sporulation. The high degree of structural and functional conservation of *RAD6* in eukaryotic evolution suggests that the various protein components involved in RAD6-dependent DNA repair and mutagenesis functions have also been conserved.**

The *RAD6* gene of *Saccharomyces cerevisiae* plays a key role in a number of cellular processes. *rad6* mutants display extreme sensitivity to numerous chemical and physical DNA-damaging agents and are defective in mutation induction by these agents and in postreplication repair of UV-damaged DNA (1). In addition, *rad6* mutants do not undergo sporulation and grow poorly. *RAD6* encodes a 172-amino acid protein of 20 kDa (2) containing a globular domain that consists of approximately the first 149-amino acid residues and an extended, carboxyl-terminal tail in which 20 of the 23 amino acids are acidic (3). RAD6 is a ubiquitin-conjugating enzyme, E2 (4), that has been shown to attach multiple molecules of ubiquitin to histones H2A and H2B *in vitro* (5). The acidic-tail domain of RAD6 is important for polyubiquitination of histones (5), as well as for sporulation (3). Deletion mutation of the acidic-tail domain, however, has no effect on DNA repair or UV mutagenesis (3). The single centrally located cysteine residue at position 88 is crucial for all RAD6 functions because its substitution by either alanine or valine inactivates thioester formation with ubiquitin and produces a *rad6* null phenotype (6).

The *RAD6* homolog, *rhp6*$^+$ from the fission yeast *Schizosaccharomyces pombe*, shows a high degree of structural similarity to the *Sa. cerevisiae RAD6* gene, except that the rhp6$^+$ protein lacks the predominantly acidic carboxyl-terminal 21 residues present in the RAD6 protein (7). Like the *rad6*Δ mutation, the *rhp6*Δ mutation confers a defect in DNA repair, UV mutagenesis, and sporulation. The *RAD6* and *rhp6*$^+$ genes can functionally substitute for one another. The *RAD6* gene complements the DNA-repair, UV-mutagenesis, and sporulation defects of the *rhp6*Δ mutant, whereas the *rhp6*$^+$ gene complements the DNA-repair and UV-mutagenesis defects of *rad6*Δ but does not complement the sporulation defect. Like *rhp6*$^+$, the protein encoded by the human *RAD6* homolog ($E2_{17K}$) also does not possess the acidic-tail domain (8).

In this paper, we report the cloning of the *Dhr6* (*Drosophila* homolog of *RAD6*) gene of *Drosophila melanogaster*¶ and show that it is a structural and functional homolog of the *Sa. cerevisiae RAD6* gene. *Dhr6* encodes two transcripts of 1.3 kilobases (kb) and 2.1 kb, which differ at their 3′ end. The Dhr6 open reading frame encodes a protein of 151 amino acids of M_r 17,207, and it lacks the carboxyl-terminal acidic-tail domain.

MATERIALS AND METHODS

Yeast Strains. Yeast strains EMY7 and EMY8 are isogenic, and both are *rad6*Δ derivatives of strain 839 (*MATα ade5 his7 leu2-3 lys1 met14 pet5 ura3*) constructed by the gene-replacement method (9). Strains EMY7 and EMY8 were constructed by replacing the entire *RAD6* open reading frame of strains 839 and EMY6, respectively, by the yeast *LEU2* gene. Strain EMY6 was derived from 839 by replacing the *TRP1* gene with the yeast *URA3* gene; subsequent growth was on 5-fluoroorotic acid to select for *ura3* cells (10).

Plasmids. The 2.2-kb *Eco*RI fragment containing the *Drosophila melanogaster Dhr6* gene, in which the translation-initiating ATG codon is 96 bases from the artificial *Eco*RI site at the 5′ end of the fragment, was cloned downstream of the *Sa. cerevisiae RAD6* promoter in the 2-μm *URA3* plasmid pTB236 (5), generating plasmid pRR449, and downstream of the *Sa. cerevisiae* highly expressed constitutive alcohol dehydrogenase I promoter (*ADC1*) in the *TRP1* plasmid pSCW231 (11), generating pRR454.

Construction of *D. melanogaster* Genomic Library. A partial *Mbo* I digest of high-molecular-weight genomic DNA of the *D. melanogaster* cell line DM-2 was size-fractionated on a 1.5–5 M salt gradient, and the fragments with an average size of 15–20 kb were ligated to a *Bam*HI-cleaved EMBL3 vector, packaged *in vitro*, and transduced into bacterial strain LE392, as described (12). The library, consisting of 4×10^6 primary plaques (i.e., ≈400 *D. melanogaster* genome equivalents), was screened with the 550-base-pair (bp) *Eco*RI fragment from the yeast *rad6–149*-containing plasmid pR615 (3) under conditions specified (7).

Northern Blotting, Determination of Transcript Initiation Site by Primer-Extension, and Nucleotide-Sequence Analysis. Isolation of total RNA with the LiCl/urea method, preparation of poly(A)$^+$ RNA by two consecutive passages over oligo(dT) columns, and Northern (RNA) blotting protocols were all according to Maniatis *et al.* (13). For primer extension, the method described by Maniatis *et al.* (13) was followed. In brief, an 18-bp antisense synthetic oligonucleotide (dR6.4; 5′-CCACTCGTGTTGTGTTGG-3′) was an-

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M63791 and M63792).

nealed to 3 μg of *Drosophila* cell line DM-2 poly(A)$^+$ RNA in a hybridization mixture containing 30% (vol/vol) formamide. Hybridization occurred at 25, 30, and 35°C overnight; only the result of 35°C is shown. The reverse transcriptase reaction using murine reverse transcriptase and 5 mM dNTPs was done during 2 hr at 37°C. After RNase treatment, the reaction products were separated on a Hydrolink (AT Biochem, Malvern, PA) sequencing gel, next to a sequencing ladder of the genomic fragment.

Sequence analysis on double-stranded DNA was done by using the T7 polymerase modification (Pharmacia) of the dideoxynucleotide chain-termination method (14); exonuclease III-prepared deletion clones were used for sequencing one DNA strand, and derived oligonucleotides were used for the sequence of the complementary DNA strand. For separation of the fragments we used Hydrolink (AT Biochem) sequencing gels.

RESULTS

Cloning, Nucleotide-Sequence Analysis, and Transcription of the *Dhr6* Gene. Southern and immunoblot analyses using the *Sa. cerevisiae RAD6* gene and polyclonal anti-RAD6 antibodies indicated the presence of a single RAD6 homolog in *D. melanogaster*. To isolate this homolog, a *D. melanogaster* genomic λ library was constructed (12) and screened with the *Sa. cerevisiae* 0.5-kb *Eco*RI DNA fragment containing the *rad6-149* allele that encodes a protein lacking the last 23 predominantly acidic residues (3). A duplicate filter was screened with the *Sc. pombe rhp6*$^+$ probe. Fourteen plaques hybridizing with both probes were purified, and the genomic DNA inserts were partially characterized. All inserts appeared to be derived from the same *D. melanogaster* genomic region because their restriction maps overlapped and they showed cross-hybridization. The physical map of the *Dhr6* gene and flanking regions is presented in Fig. 1*A*, and the nucleotide sequence is shown in Fig. 1*B*. The *Dhr6* gene consists of three exons, and the sequences of the intron–exon boundaries are all consistent with the consensus donor mag ↓GTragt and acceptor (y)$_{\geq 11\times}$ nyAG↓g splicing signals (19). The loosely defined splicing branchpoint consensus sequence YNYTRAY (20) can be tentatively identified in both introns proximal to the splice-acceptor sites at the usual distance of 20–40 nucleotides.

For cloning the Dhr6 cDNA, a λ gt11 cDNA library (21) prepared from adult *Drosophila* head RNA was screened using the *Dhr6* 295-bp *Bam*HI fragment as a probe (Fig. 1*A*). From this library, seven clones were isolated for which cDNA inserts ranged from 0.9 to 2.6 kb. The inserts of two of the longest cDNAs were subcloned and used as probes for Northern blot analysis. Two hybridizing mRNA species of 1.3 and 2.1 kb were detected in poly(A)$^+$ RNA of the *D. melanogaster* cell line DM-2 (Fig. 1*C*, lane 1). Nucleotide-sequence analysis of a 2.1-kb cDNA clone indicated that this cDNA began at position +1 (Fig. 1*B*), which coincides with the transcription initiation site, as determined by primer extension (12; Fig. 1*D*), and ended at position +2511 (Fig. 1*B*). Clearly, this cDNA is a representative of the 2.1-kb mRNA species. At the 3′ end of this cDNA, an optimal polyadenylylation signal, AATAAA, is found 16 bp 5′ of the cleavage/poly(A) addition site (YA) (22). The other 2.6-kb cDNA clone initiated within the first intron and ended at nucleotide 1765 (Fig. 1*B*), which coincides with the position where the 1.3-kb mRNA is expected to terminate as a result of alternative polyadenylylation at a suboptimal polyadenylylation signal AATTAAA that occurs in 12% of mRNAs compiled from many species (22). Downstream of this polyadenylylation signal is a putative K (K = G/T) cluster (22), which is supposed to be necessary for efficient polyadenylylation. This cDNA clone, presumably derived from a partially processed mRNA, likely represents the 1.3-kb mRNA species. This interpretation is consistent with the results of the hybridization of different 3′ Dhr6 probes (Fig. 1*C*, lanes 2 and 3).

The *Dhr6* open reading frame encodes a protein of 151 amino acids with a calculated M_r of 17,207 (Fig. 1*B*). The observed size of the Dhr6 protein on SDS/PAGE is in close agreement with the predicted molecular weight (data not shown).

Structural Conservation between *Dhr6* and Its Homolog in *Sa. cerevisiae*, *Sc. pombe*, and Human. Fig. 2*A* shows the alignment of the amino acid sequence of the RAD6 protein of *Sa. cerevisiae*, with the Dhr6 protein of *D. melanogaster*, and with the recently published sequences of the proteins encoded by the *Sc. pombe* (7) and human *RAD6* homologs (8). The *Sa. cerevisiae* RAD6 protein is 172 amino acids long and contains a highly acidic carboxyl terminus. This polyacidic region is absent in the RAD6 homologs from *Sc. pombe*, *D. melanogaster*, and human. The *Sc. pombe* and *D. melanogaster* RAD6 homologs each contain 151 amino acid residues, and the human homolog contains 152 residues. Alignment of all four sequences is continuous throughout without any gaps, and they share a high degree of identity. Fig. 2*B* presents the incidence of identical residues shared among different RAD6 homologs. The Dhr6 protein shares ≈70% identity with the RAD6 homologs from the two yeasts, and the frequency of identical residues shared between the *Drosophila* and human genes rises to 85%. An even higher degree of similarity is observed among these proteins if conservative amino acid substitutions are considered equivalent (Fig. 2*A*). Two regions of the RAD6 protein have been particularly conserved in evolution. The first 15 amino acids are identical in all the RAD6 homologs, except for one change in the *Sc. pombe* protein. The other very conserved region flanks the active-site cysteine residue at position 88, which is involved in thioester formation with ubiquitin (6).

Complementation of the *Sa. cerevisiae rad6*Δ Mutation by the *Dhr6* Gene. The structural homology between *Dhr6* and *RAD6* suggests that *Dhr6* functions in a manner similar to *RAD6*. To examine this, we tested whether *Dhr6* can functionally substitute for *RAD6* in *Sa. cerevisiae*. The *Dhr6* gene was cloned downstream of the *RAD6* promoter in the 2-μm multicopy yeast plasmid pRR449 and downstream of the highly expressed constitutive *ADC1* promoter in plasmid pRR454. As judged by immunoblotting using anti-RAD6 antibodies, the level of Dhr6 protein in the *Sa. cerevisiae rad6*Δ mutant strain carrying the plasmid pRR449 was approximately the same as the level of RAD6 protein in *RAD6*$^+$ yeast cells, and it was a few-fold higher in *rad6*Δ cells harboring the plasmid pRR454 (data not shown). The *Dhr6* gene increases the UV resistance of the *rad6*Δ mutant strain (Fig. 3*A*). At 10 J/m^2, the *Dhr6* gene conferred increases of 2–3 fold and 4–5 fold in the slope of the UV survival curves of the *rad6*Δ strain carrying the plasmid pRR449 or pRR454, respectively; survival, however, did not reach that of the wild-type strain. The *Dhr6* gene also complemented the γ-ray sensitivity of the *rad6*Δ strain (Fig. 3*B*). The *rad6*Δ strain carrying plasmid pRR454 had nearly wild-type levels of γ-ray resistance. In addition, *Dhr6* restored wild-type levels of UV mutagenesis to the *rad6*Δ strain (Fig. 3*C*). In contrast, the sporulation defect of the *rad6*Δ/*rad6*Δ strain was not ameliorated by the *Dhr6* gene in plasmid pRR449, and only a low level of sporulation, ≈3%, occurred in *rad6*Δ/*rad6*Δ strain carrying the *ADC1 Dhr6* plasmid pRR454. This effect on sporulation is expected because the acidic domain of RAD6, which is missing in Dhr6, is required for sporulation in *Sa. cerevisiae*. The *rad6-149* mutation of *Sa. cerevisiae* lacking the entire polyacidic carboxyl terminus is defective in sporulation (3), and overproduction of the rad6-149 mutant protein permits a low level of sporulation (7).

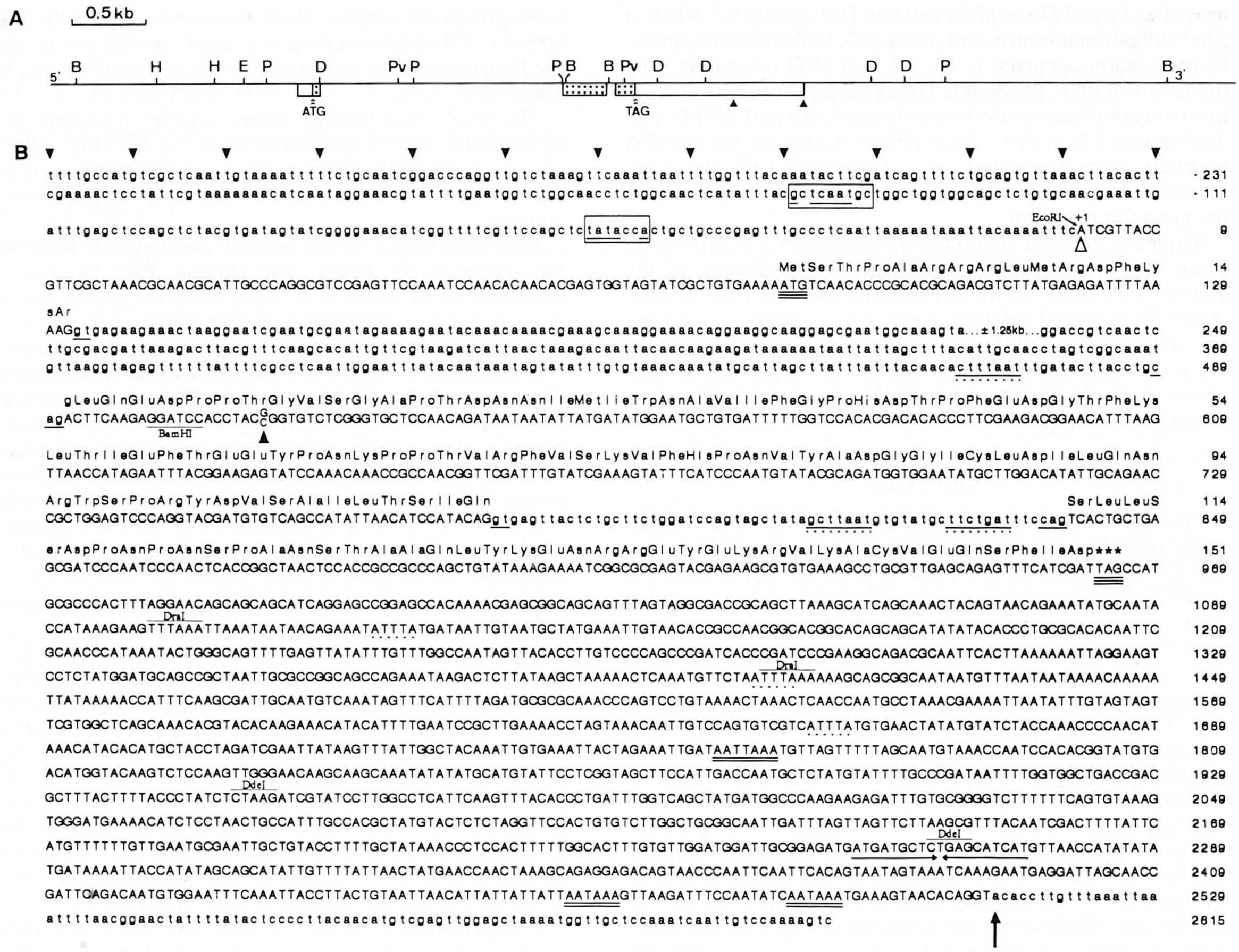

FIG. 1. Genomic organization, nucleotide sequence, and transcripts of the *Drosophila Dhr6* gene. (*A*) Partial restriction map of the three *Bam*HI fragments harboring the entire *D. melanogaster Dhr6* genomic sequence. Exons are indicated as boxes; parts with dots indicate coding region of the mRNA transcribed. Arrowheads point to the two polyadenylylation sites found. Introns and intergenic regions are indicated as single lines. B, *Bam*HI; D, *Dra* I; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I; Pv, *Pvu* II. (*B*) Nucleotide sequence of the *DHR6* gene and amino acid sequence of its encoded protein. Both the cDNA and the transcribed regions of the genome were sequenced on both strands. The cap position determined by primer extension (see *D*) is indicated at position +1 (open arrowhead). The start codon ATG at position +89 matches well with the *Drosophila* translation initiation consensus MAAMATG (15) sequence. The ATG codon as well as the stop codon TAG at position +963 are indicated by triple underlining. Presumed polyadenylylation signals are doubly underlined. The identified poly(A)-attachment site is indicated with a vertical arrow. The ATTTA sequences thought to be involved in mRNA instability are indicated by dotted lines (16). Oppositely oriented horizontal arrows denote a pronounced palindrome in the 3′-untranslated region of the mRNA. Putative TATA and CAAT sequences are boxed, and nucleotides fitting with the consensus are underlined. It is notable that these elements are located further upstream of the cap site than usually found (17). Splice-donor- and splice-acceptor sequences are singly underlined. Putative splice branch-point sequences are indicated by paired continuous and dashed lines. The filled-in arrowhead indicates a base difference found between the genomic and cDNA sequence, which probably represents a polymorphism; this difference does not cause any amino acid change. The restriction enzyme sites used for probe preparation (see *C*) are indicated. The *Eco*RI site is artificial. (*C*) Northern blot analysis of Dhr6 transcripts. Poly(A)$^+$ RNA of *in vitro*-cultured *D. melanogaster* cell line DM-2 was size fractionated on a 0.8% agarose gel. Lane 1 shows the hybridization with 5′ 148-bp *Eco*RI–*Bam*HI probe of Dhr6 cDNA, which hybridizes to both 1.3- and the 2.1-kb Dhr6 mRNA. To investigate the difference between the two transcripts, Northern blot analysis was done with different Dhr6 probes. Lanes 2 and 3 show hybridization pattern with two 3′ untranslated region-derived probes: the 306-bp *Dra* I cDNA fragment (lane 3) hybridizes to both Dhr6 RNAs, and the more 3′-located 314-bp *Dde* I cDNA fragment (lane 2) recognizes only the 2.1-kb Dhr6 transcript (see Fig. 1*B* for precise location of probes). (*D*) Determination of transcriptional start site of *Dhr6* by primer extension. Lanes 1–4 show an M13 sequence reaction with anti-sense oligonucleotide dR6.4 corresponding to nucleotide positions 54–71 (5′-CCACTCGTGTTGTGTTGG-3′) as a primer on a subclone of the 5′ *Bam*HI genomic fragment. The sequencing ladder was actually the complement of that indicated in the figure. Lane 5 shows primer extension on *Drosophila* (DM-2) poly(A)$^+$ RNA starting from oligonucleotide dR6.4. Lane 6 shows control lane with total yeast RNA as template. The deduced cap position is indicated in *B* with an open triangle and matches well with the loosely defined transcriptional start site consensus YYCAYYYYY (18).

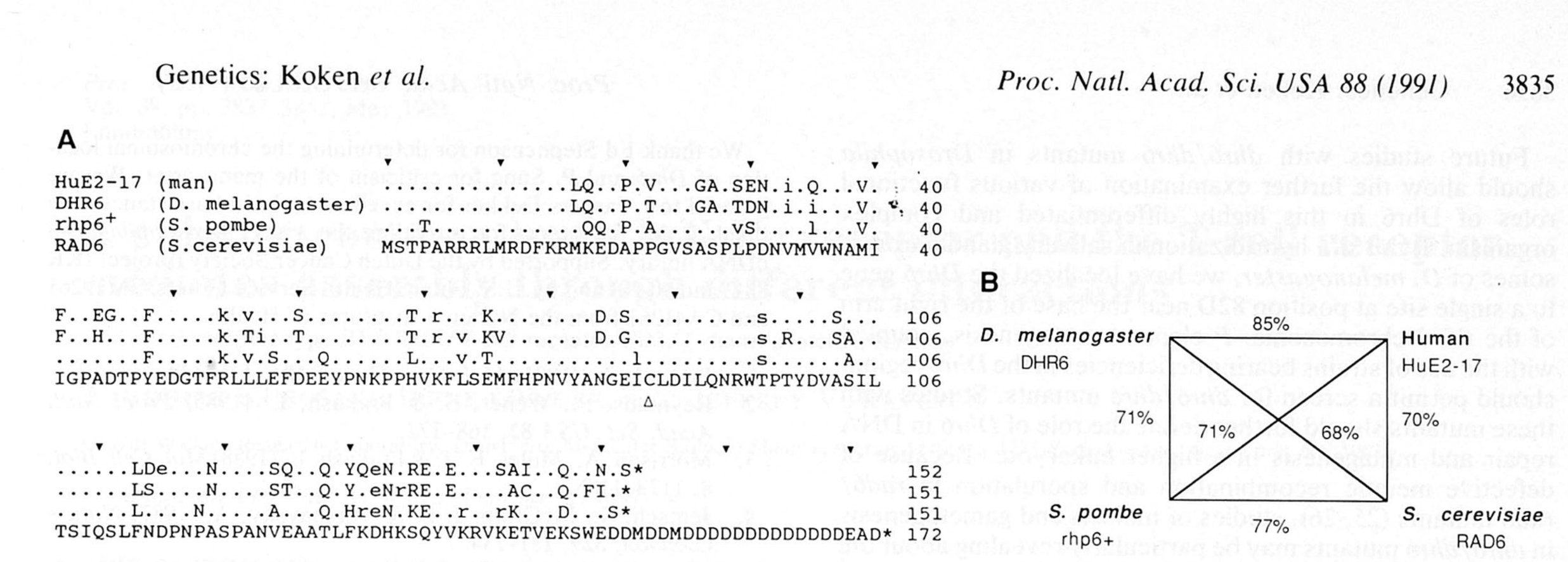

FIG. 2. (*A*) Comparison of amino acid sequences of RAD6 homologs from *Sa. cerevisiae*, *Sc. pombe*, *D. melanogaster*, and human. The 172-amino acid-long RAD6 protein is aligned with the 151 amino acids of Dhr6 and rhp6$^+$ proteins and the 152 amino acids of the human homolog. The position of Cys-88, involved in thioester formation with ubiquitin, is indicated by an open triangle. Sequences are completely colinear, except for the acidic tail in *Sa. cerevisiae* RAD6. Dots indicate identity, whereas small letters indicate conservative changes in *Sc. pombe*, *D. melanogaster*, and human proteins compared with the *Sa. cerevisiae* protein. Similar amino acids: R = K, E = D, I = V = L, S = T. (*B*) Percent identical amino acid residues shared among RAD6 homologs from *Sa. cerevisiae*, *Sc. pombe*, *D. melanogaster*, and *Homo sapiens*. Only the residues present in both homologs were considered; thus, comparison of Dhr6 and rhp6$^+$ proteins with each other and with RAD6 and HuE2–17 proteins included 151 residues, and the comparison of HuE2–17 with RAD6 protein included 152 residues.

DISCUSSION

The protein encoded by the *Dhr6* gene of *Drosophila* shares a high degree of homology with the RAD6, rhp6$^+$, and $E2_{17k}$ proteins of *Sa. cerevisiae*, *Sc. pombe*, and human, respectively. However, the acidic carboxyl-terminal domain present in the *Sa. cerevisiae* RAD6 protein is absent in the *Sc. pombe*, *Drosophila*, and human homologs. Two regions, one flanking the Cys-88 residue, and the other consisting of the amino-terminal 15 residues, have been in particular highly conserved among these homologs. Because the Cys-88 residue is involved in thioester formation with ubiquitin (6), the region flanking this cysteine residue is likely involved in interactions with the ubiquitin-activating enzyme (E1). The highly conserved amino terminus in these homologs is very basic and shows similarity to nuclear-localization signal sequences. However, mutational studies with *RAD6* suggest that this is not the role of this sequence (J. Watkins, S.P., and L.P., unpublished observations). Because the high degree of conservation of the amino terminus among the various RAD6 homologs does not extend to other ubiquitin-conjugating enzymes (23, 24), this sequence may be involved in specific interactions with protein components of the DNA-repair and mutagenesis machinery, rather than in interactions with the E1 enzyme.

Genetic studies in *Sa. cerevisiae* with the *Dhr6* gene reported here clearly demonstrate conservation of *RAD6* function in higher eukaryotes. The *Dhr6* gene complemented the UV and γ-ray sensitivity and defective UV mutagenesis of *rad6*Δ mutant strains. However, whereas UV mutagenesis was restored to wild-type levels, UV survival was complemented to a lesser degree. As expected, *Dhr6* did not complement the sporulation defect of the *rad6*Δ/*rad6*Δ strain because the RAD6 acidic-tail sequence required for sporulation in *Sa. cerevisiae* is absent in the Dhr6 protein. In *Drosophila* and other eukaryotes (7, 8), a different protein may perform the role of the RAD6 acidic domain.

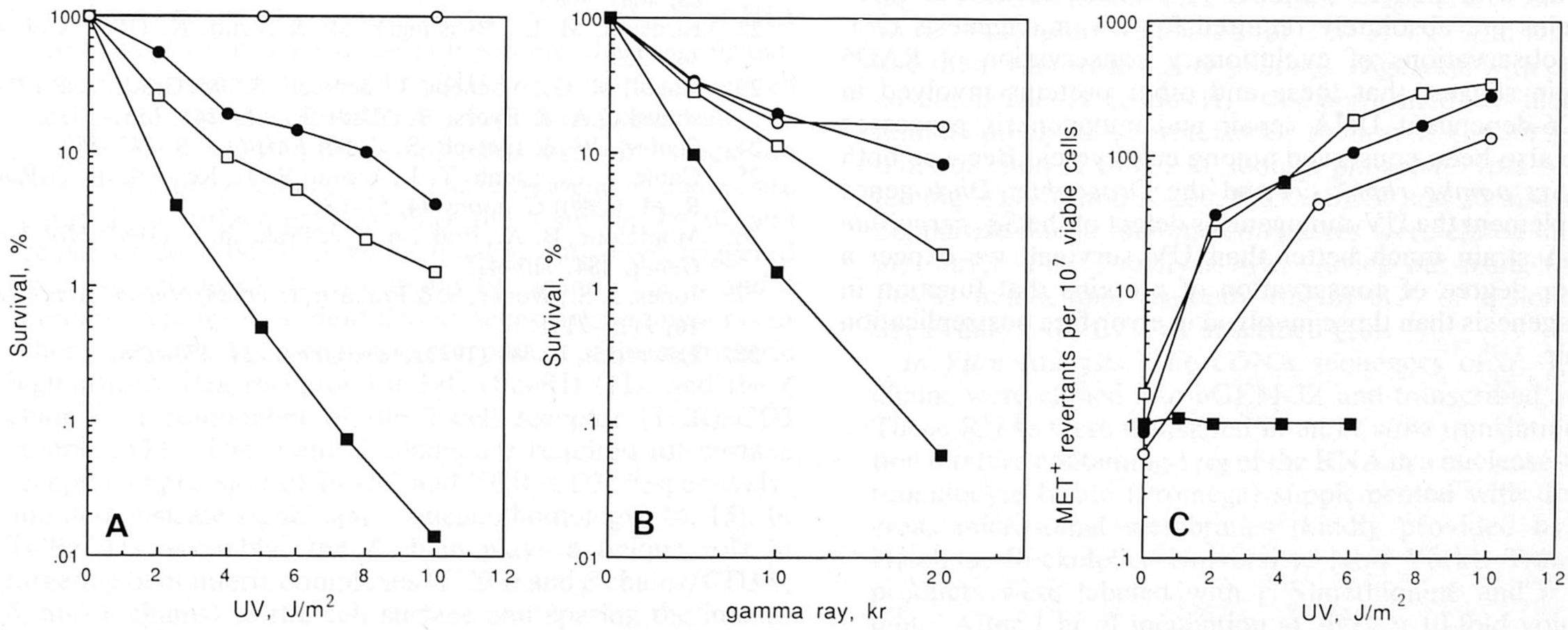

FIG. 3. Complementation of the radiation sensitivity and UV immutability of the *Sa. cerevisiae rad6*Δ mutation by the *D. melanogaster Dhr6* gene. Survival after UV (*A*) or γ-ray irradiation (*B*), and UV-induced reversion of *met14* (*C*) in *Sa. cerevisiae rad6*Δ strains carrying the *Dhr6* gene on various plasmids. UV survival and mutagenesis experiments were done at least three times, and separate experiments gave very similar results. Cells grown in synthetic complete medium lacking uracil or tryptophan for maintaining selection of the plasmid were harvested in mid-exponential phase, plated on appropriate media, and irradiated with UV light at a dose rate of 1 J/m^2 per sec or, for γ-ray irradiation, irradiated with a ^{60}Co source at a dose rate of 9 kilorads (kr) (1 rad = 0.01 Gy) per min. UV-irradiated plates were incubated in the dark to avoid photoreactivation. ■, EMY7 (*rad6*Δ) + pTB236 (2 μm vector); □, EMY7 + pRR449 (*Dhr6* gene on 2 μm plasmid); ●, EMY8 (*rad6*Δ) + pRR454 (*Dhr6* gene fused to *ADC1* promoter); ○, EMY7 + pR67 (*RAD6* gene in *CEN* plasmid).

Future studies with *dhr6/dhr6* mutants in *Drosophila* should allow the further examination of various functional roles of Dhr6 in this highly differentiated and complex organism. By *in situ* hybridization in salivary gland chromosomes of *D. melanogaster*, we have localized the *Dhr6* gene to a single site at position 82D near the base of the right arm of the third chromosome. *P* element mutagenesis, coupled with the use of strains bearing deficiencies in the *Dhr6* region, should permit a screen for *dhr6/dhr6* mutants. Studies with these mutants should further define the role of *Dhr6* in DNA repair and mutagenesis in a higher eukaryote. Because of defective meiotic recombination and sporulation in *rad6/rad6* mutants (25, 26), studies of meiosis and gametogenesis in *dhr6/dhr6* mutants may be particularly revealing about the role of *Dhr6* in these processes. In higher eukaryotes, during spermatogenesis chromatin-bound histones are replaced by protamines. The requirement of the acidic domain of RAD6 both for sporulation (3) and for attachment of multiple molecules of ubiquitin to histones (5) suggests that during sporulation, RAD6 could effect the degradation of histones by the ubiquitin-specific ATP-dependent protease complex. Even though Dhr6 does not possess the acidic-tail domain, it could still function in this capacity in association with a highly acidic protein. Because considerably more information is available on *Drosophila* spermatogenesis than yeast sporulation, studies on the possible involvement of *Dhr6* in turnover of histones during spermatogenesis might be particularly informative.

These studies demonstrate that the structure and function of RAD6 has been conserved to a remarkable degree among eukaryotes. This conservation very likely reflects the evolutionary constraints on RAD6 protein due to its interactions with protein factors functioning in the ubiquitin conjugation pathway and with proteins involved in DNA repair and mutagenesis. In *Sa. cerevisiae, RAD6* functions with *RAD18* in postreplication repair, as both the *rad6* and *rad18* mutants are defective in this process (1). Because RAD6 protein has no DNA-binding capacity (P. Sung, personal communication), interaction with RAD18 may target the RAD6 protein to the damage sites in DNA. The *RAD18*-encoded protein contains three potential zinc-finger domains that could be involved in binding to damaged DNA (27). RAD6 may also interact with the REV1 and REV3 proteins because all three proteins are absolutely required for UV mutagenesis (28). Our observations of evolutionary conservation of RAD6 protein suggest that these and other proteins involved in RAD6-dependent DNA repair and mutagenesis processes have also been conserved among eukaryotes. Because both the *Sc. pombe rhp6*$^+$ (7) and the *Drosophila Dhr6* genes complement the UV-mutagenesis defect of the *Sa. cerevisiae rad6*Δ strain much better than UV survival, we expect a higher degree of conservation of proteins that function in mutagenesis than those involved in error-free postreplication repair.

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