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, rhpp6+ from the fission yeast Schizosaccharomyces pombe, shows a high degree of structural similarity to the S. cerevisiae RAD6 gene, except that the rhpp6+ protein lacks the predominately acidic carboxyl-terminal 21 residues present in the RAD6 protein (7). Like the rad6Δ mutation, the rhpp6+ mutation confers a defect in DNA repair, UV mutagenesis, and sporulation. The RAD6 and rhpp6+ genes can functionally substitute for one another. The RAD6 gene complements the DNA-repair, UV-mutagenesis, and sporulation defects of the rhpp6+ mutant, whereas the rhpp6+ gene complements the DNA-repair and UV-mutagenesis defects of rad6Δ but does not complement the sporulation defect. Like rhpp6+, the protein encoded by the human RAD6 homolog (E217K) 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 S. 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 Mr 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 rad6Δ derivatives of strain 839 (MATa 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 EcoRI fragment containing the Drosophila melanogaster Dhr6 gene, in which the translation-initiating ATG codon is 96 bases from the artificial EcoRI 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 (ADCl) in the TRP1 plasmid pSCW231 (11), generating pRR454.

Construction of D. melanogaster Genomic Library. A partial MboI 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 BamHI-cleaved EMBL3 vector, packaged in vitro, and transfected into bacterial strain LE392, as described (12). The library, consisting of 4 × 109 primary plaques (i.e., ≈400 D. melanogaster genome equivalents), was screened with the 550-base-pair (bp) EcoRI fragment from the yeast rad6Δ149-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'-CCACTCGTGTGGTGGTTGG-3') was an-

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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 library was constructed (12) and screened with the Sa. cerevisiae 0.5-kb EcoRI DNA fragment containing the rad5-149 allele that encodes a protein lacking the last 23 predominantly acidic residues (3). A duplicate filter was screened with the Sc. pombe rpm6+ 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. 1A, and the nucleotide sequence is shown in Fig. 1B. The Dhr6 gene consists of three exons, and the sequences of the intron–exon boundaries are all consistent with the consensus donor mag 5’ GTAG and acceptor (y)11 t 3’ AG splice 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 λgt11 cDNA library (21) prepared from adult Drosophila head RNA was screened using the Dhr6 295-bp BamH1 fragment as a probe (Fig. 1A). 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. 1C, lane 1). Nucleotide-sequence analysis of a 2.1-kb cDNA clone indicated that this cDNA began at position +1 (Fig. 1B), which coincides with the transcription initiation site, as determined by primer extension (12; Fig. 1D), and ended at position +2511 (Fig. 1B). Clearly, this cDNA is a representative of the 2.1-kb mRNA species. At the 3’ end of this cDNA, an optimal polyadenylation 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. 1B), which coincides with the position where the 1.3-kb mRNA is expected to terminate as a result of alternative polyadenylation at a suboptimal polyadenylation signal ATTAAA that occurs in 12% of mRNAs compiled from many species (22). Downstream of this polyadenylation signal is a putative K (K = G/T) cluster (22), which is supposed to be necessary for efficient polyadenylation. 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. 1C, lanes 2 and 3).

The Dhr6 open reading frame encodes a protein of 151 amino acids with a calculated M, of 17,207 (Fig. 1B). 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. 2A 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. 2B 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. 2A). 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 rad6Δ 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 rad6Δ 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 rad6Δ cells harboring the plasmid pRR454 (data not shown). The Dhr6 gene increases the UV resistance of the rad6Δ mutant strain (Fig. 3A). At 10 J/m², the Dhr6 gene conferred increases of 2–3 fold and 4–5 fold in the slope of the UV survival curves of the rad6Δ 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 rad6Δ strain (Fig. 3B). The rad6Δ strain carrying plasmid pRR454 had nearly wild-type levels of γ-ray sensitivity. In addition, Dhr6 restored wild-type levels of UV mutagenesis to the rad6Δ strain (Fig. 3C). In contrast, the sporulation defect of the rad6Δ/rad6Δ strain was not ameliorated by the Dhr6 gene in plasmid pRR449, and only a low level of sporulation, ~3%, occurred in rad6Δ/rad6Δ 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* gene. (A) Partial restriction map of the three BamHI 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 polyadenylation sites found. Introns and intergenic regions are indicated as single lines. B. *BamHI*; D. *DraI*; E. *EcoRI*; H. *HindIII*; P. *PstI*; Pv. *PvuII*. (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 were shown (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 polyadenylation 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 proposed palindromic 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 *EcoRI* site is artificial. (C) Northern blot analysis of Dhr6 transcripts. Poly(A)^ypsy^RNA of *in vitro*-transcribed *D. melanogaster* cell line DM-2 was size fractionated on a 0.8% agarose gel. Lane 1 shows the hybridization with 5'-148-bp *EcoRI*-BamHI 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 *DraI* 1.2-kDNA fragment (lane 3) hybridizes to both Dhr6 RNAs, and the more 3'-located 314-bp *DraI* 1.2-kDNA fragment (lane 2) recognizes only the 2.1-kb Dhr6 transcript (see Fig. 1B for precise location of probes). (D) Determination of transcription start site. Fig. 1C shows an M13 sequence reaction with anti-sense oligonucleotide dR6.4 corresponding to nucleotide positions 54-71 (5'-CCACTCGTGTGTTGGTGGTG) as a primer on a subclone of the 5' *BamHI* 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)^ypsy^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).
**DISCUSSION**

The protein encoded by the *Dhr6* gene of *Drosophila* shares a high degree of homology with the RAD6, *rhp6*, and E217K proteins of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *D. melanogaster*, and human proteins, respectively. However, the acidic carboxyl-terminal domain present in the *Saccharomyces cerevisiae* RAD6 protein is absent in the *Schizosaccharomyces pombe*, *Drosophila melanogaster*, 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 *Saccharomyces 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 *rad6Δ/rad6Δ* strain because the RAD6 acidic-tail sequence required for sporulation in *Saccharomyces 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.
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 rad6Δ 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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