ISOLATION OF HUMAN DNA REPAIR GENES BASED ON NUCLEOTIDE SEQUENCE CONSERVATION

Marcel Koken

ISOLATION OF HUMAN DNA REPAIR GENES BASED ON NUCLEOTIDE SEQUENCE CONSERVATION

ISOLATIE VAN HUMANE DNA HERSTEL GENEN OP BASIS VAN NUCLEOTIDE SEQUENTIE CONSERVATIE

PROEFSCHRIFT

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Dans le champ de l' expérimentation le hasard ne favorise que l' esprit préparé.

Louis Pasteur

Imagination is more important than knowledge.

Albert Einstein

Voor mijn moeder die nooit ophield mij te stimuleren en te helpen, en voor Manon die voortdurend probeerde dit boekje (letterlijk) teniet te doen.

CONTENTS

AIM

The aim of the work described in this thesis was the development (and subsequent application) of a general method for the isolation of human (DNA repair) genes using probes from already cloned homologous counterparts in other organisms. After many initial problems developing and optimising this method, it was used for the isolation of two groups of genes: the *Drosophila melanogaster*[1], *Schizosaccharomyces pombe* (unpublished) and *Saccharomyces cerevisiae*[2] homologues of the TFIIH component *XPBC/ERCC3*, and an *Ss.pombe*, a *Drosophila* and two human homologues of the yeast post-replication repair gene *RAD6*. Only the characterisation of the *RAD6* homologous genes and polypeptides, implicated in the ubiquitin pathway, will be described in this thesis, Chapters II to VII. Publications related to the other part of the work conducted in the context of this PhD thesis can be found in the list of publications at page 117

GENERAL INTRODUCTION & CONCLUSIONS

General introduction to DNA repair

For all organisms it is of vital importance to secure reliability of genetic information. The genome of the cell is constantly under attack by a plethora of DNA damaging agents (e.g. the UV component of the sunlight). Therefore all living beings had to develop efficient systems to recognise and remove DNA injury. Lesions in DNA -if unrepaired- have immediate deleterious effects on transcription and replication, or after fixation into permanent mutations, they can change the coding potential of genes. This last feature implies also that correct removal of lesions from the DNA is of utmost importance for the prevention of cancer or congenital aberrations in higher organisms.

Since numerous genotoxic compounds exist, each of which can induce a wide spectrum of lesions, it is not surprising that most organisms acquired a network of partially overlapping repair pathways to recognise and remove these different adducts from their DNA. In the bacterium *Escherichia coli* several of the biochemical pathways leading to the elimination of DNA damage are rather well understood. The work described in this thesis concerns mainly two major repair mechanisms: post-replication repair (PRR) and nucleotide excision repair (NER) (For reviews on both subjects see[3-10]). Post-replication repair, a poorly understood error-prone system, is thought to permit the replication machinery to bypass lesions in the DNA strands. This, may occur either via mutagenic trans-lesion DNA synthesis or by re-initiation of DNA replication behind the lesion, in which case the single-stranded gap -opposite of the damage- is filled in using the newly synthesised complementary daughter strand as template. In both models the lesion is not removed but only tolerated, which implies that - if not repaired - it still can cause mutations. Therefore, PRR, which is also known as "daughter-strand gap repair" has to be considered mainly as a damage-tolerance process. As in contrast to the NER pathway, only very little is known about the molecular mechanism of PRR, this part of the introduction will essentially focus on the DNA excision repair system.

Nucleotide excision repair in *E.coli* is a process in which a minimum of 6 proteins participates in the elimination of a wide range of structurally diverse DNA lesions (For

review see[11]). A complex consisting of two molecules UvrA and one molecule UvrB is thought to scan the DNA for local distortions caused by the damage. After tracing DNA injury, the $UvrA_2B$ complex unwinds partially the DNA around the lesion[12]. The two UvrA proteins leave the complex, and UvrB attaches more tightly inducing a strong conformational change in the double helix around the damage. The bound UvrB molecule and the frozen DNA structure serve as tag for a third polypeptide, UvrC. The UvrBC complex makes incisions in the damaged strand; UvrC at the eighth phosphodiester bond 5', and it is unknown whether UvrC, UvrB or both are responsible for cleaving at the fifth phosphodiester bond 3' of the lesion[13]. A second DNA-helicase, UvrD, subsequently removes the damaged part from the DNA backbone, and DNA polymerase and ligase fill in and close the gap.

The *E.coli* system is relatively simple when compared to the process in eukaryotes, where in yeast and man already more than fifteen different NER genes are identified, and in part cloned (For details on the different genes and cloning methods, see below). Many of the cloned human NER genes were isolated by DNA transfection of normal genomic or cDNA into repair-deficient mutant cells. These were either laboratory-derived rodent cells or cell lines from patients with one of several rare repair disorders. Individuals with the autosomal recessive disease xeroderma pigmentosum (XP), characterised by hypersensitivity to sunlight (UV), pigmentation abnormalities and a high incidence of skin tumours in the sun-exposed areas, led Cleaver in 1968 to think that this illness could be a DNA repair disorder (For review see[14]). Cell fusion experiments showed that the XP (as well as above mentioned rodent mutant) cell lines could be divided into at least eight (and eleven) complementation groups. Seven of the XP groups are severely disturbed in the incision step of the nucleotide excision repair pathway, whereas cells of the eighth group (the variants) have problems in post-replication repair[15]. These XP variants were of special interest as the second part of this thesis describes the isolation of homologous *RAD6* genes which are implicated in post-replication repair.

Since the description of XP as a NER disorder in the late seventies, many reports appeared in the literature associating diverse diseases with one of the repair pathways. Although most of these links are still rather uncertain, some well-documented genetic instability syndromes have been found. Two of them, Cockayne's syndrome (CS)[6,16] and trichothiodystrophy (TTD)[17], were shown to represent different forms of the XP-NER syndrome (see below), which, however, unlike XP and other putative DNA repair disorders as Bloom's syndrome[18], Fanconi's anaemia[19,20], or ataxia telangiectasia[21-25], were not found to be associated with a high cancer incidence (For an extensive discussion on this subject, see[10,26]).

Cloned human DNA excision repair genes

A short description of the DNA excision repair genes isolated thus far, mostly by DNA transfections to above-mentioned mutant cell lines, is given below to illustrate the high level of evolutionary conservation which exists in the DNA excision repair pathway.

The first human NER gene isolated, *ERCC1*, was cloned[27] via the correction of the UV-sensitive, DNA excision repair-deficient, Chinese hamster mutant cell lines of rodent complementation group 1 (For review see[28]). The 32 kD protein is homologous to the yeast

NER protein RAD10, and shares at its C-terminus additional regions of similarity with parts of the prokaryotic *E.coli* UvrA and UvrC polypeptides[29]. Recently ERCC1 was shown to be complexed with the correcting activities of ERCC4, ERCC11 and XP-F cell lines[30,31]. The recent cloning of *ERCC4*[32,33] showed it to be partially homologous to yeast RAD1. Moreover, the same gene corrects by DNA transfection or microneedle injection the repair defect of the ERCC4, ERCC11 and XPF mutants, and several causative mutations from these cell lines have been characterised. In analogy with the situation in yeast, where RAD10 interacts with RAD1[34], ERCC1 forms a tight complex with ERCC4 and induces an endonucleolytic cleavage at the transition of a single-stranded to a double-stranded DNA region, only in the strand carrying the 3' single stranded end[33,35,36]. This is consistent with the idea that this complex is implicated in making the 5' incision of the NER process.

ERCC5[37], isolated by transfections of CHO complementation group 5 cells, is apparently identical to the XPG correcting factor which was cloned, in a way by accident, using a systemic lupus erythematosus autoimmune serum[38]. The gene was also isolated using PCR amplification with degenerated primers designed from the homologous *Schizosaccharomyces pombe RAD2* and *RAD13* genes[39,40]. The cDNA encodes an acidic helix-loop-helix protein partially resembling the yeast RAD2 protein. In analogy with RAD2, and the related FEN-1 (implicated in the joining of Okasaki fragments), XPG may display structure-specific ss-endonuclease activity[41-43] which, like ERCC1/ERCC4, might be required for the incision step of NER.

ERCC6[44], which corrects CHO complementation group 6, was shown to be affected in Cockayne's syndrome (CS) patients of CS-complementation group B. CS patients are characterised by a small stature, wizened appearance, sun-sensitivity, and often mental and physical retardation, but no elevated risk for cancers (For review, see[45]). The protein, of which the yeast homologue (RAD26) has recently been isolated[46], represents a putative DNA helicase implicated in preferential repair[16,47]. This process couples DNA excision repair to transcription, assuring the preferential reparation of the coding DNA strand in transcriptionally active genes[48,49].

The *XPA* gene isolated after tedious transfections of mouse DNA into XPA cells[50], encodes a Zn^{2+}finger protein which is likely to be directly involved in recognition of thymidine dimers by the excision repair system. Shown to be very well conserved during evolution[51], it was not unexpected that the yeast RAD14 NER protein was found to be its yeast homologue[52].

XPC was cloned twice. A partial cDNA was isolated by DNA transfection of an XPC cell line with a cDNA library cloned into an extrachromosomally replicating EBNA-vector[53]. The encoded hydrophilic protein is related in its C-terminal region to, but not necessarily the homologue of, yeast RAD4[54]. Two years later Masutani *et al.* cloned the gene again[55]. Using an *in vitro* repair assay, they isolated a protein fraction which complemented XPC cell extracts. After determination of the N-terminal amino acid sequence of the two proteins, p125 and p58, present in the correcting fraction, now a full length *XPC* cDNA was isolated. The second protein (p58), necessary for complete correction and forming a complex with XPC, appeared to be an ancient ubiquitin-fusion protein, HHR23B (see below). A homologue of the

protein, HHR23A was also reported, but apparently not involved in the XPC-HHR23B protein complex[55]

ERCC2 [56, and in ref.57] and *ERCC3* [58], isolated by correction of rodent complementation group 2 and 3 mutants, appear to be involved in xeroderma pigmentosum, complementation groups D (*ERCC2*) and B (*ERCC3*). Mutations in both genes are underlying also two other hereditary diseases, Trichothiodystrophy (TTD) and Cockayne's syndrome, which co-occur also in some of the XPD patients (in ref.[57]), and in the three XPB (2xCS, 1xTTD) patients described to date (ref.[59] and unpublished data). A substantial fraction of TTD patients, comprising three complementation groups, display a repair-defective phenotype[17]. They were originally described as having a problem with their sulphur metabolism, leading to sulphur-deficient brittle hair (For review see[60]). Both ERCC2 and ERCC3 (as well as ERCC6) are members of a recently defined group of DNA/RNA helicases, as they share seven consecutive amino acid domains characterising this family[61,62]. ERCC2 was shown to be the human homologue of yeast RAD3. We and others demonstrated that ERCC3 is also well conserved in evolution[1,2,63,64] leading to the isolation of a thus far unknown yeast mutant, RAD25[2]. In view of recent data this conservation is not surprising as ERCC2 (RAD3), ERCC3 (SSL2/RAD25), SSL1, TFB1, 2 and 3, CCL1 and KIN28, together with one or more as yet uncharacterised proteins, were shown to constitute the general basal transcription factor TFIIH[65,66] (For reviews see[67-69]). Apparently, these proteins (and perhaps also ERCC6) have all a primary task in transcription next to their repair functions[70]. The protein complex can explain why mutations in different proteins give very similar diseases (XPB and D), as a mutated component deregulates apparently the total complex. Moreover, it can also explain how, perhaps depending on the type of mutation, different illnesses like XP, CS and TTD can originate[10,71,72].

As almost all constituents of the general transcription machinery are very well conserved in the course of evolution[73], it is also probably that this could be a general phenomenon for DNA repair enzymes as the listing above may indicate. The work described in this thesis was initiated, when only *ERCC1* was isolated, to prove the overall conservation of repair genes and to use this conservation for the isolation of additional human DNA repair genes.

Cloning methods for DNA repair genes

The classical method for the isolation of mammalian repair genes (explained in detail in ref.[27]) uses the transfection of normal human or mouse genomic DNA into Chinese hamster or human mutant cell lines, respectively. After selection of clones resistant to the DNA damaging agent, genomic DNA is isolated for a consecutive round of transfection which reduces considerably the amount of co-incorporated irrelevant human or mouse sequences. Finally, the correcting human (or mouse) DNA is isolated from the hamster (or human) background by standard molecular biological techniques. Although this method has important pitfalls (e.g. the amount and length of intact genomic DNA which is stably taken up by the transfected cells[74]), it has thus far successfully been used for the isolation of most of the DNA excision repair genes, i.e. *ERCC1*, *ERCC2/XPD*, *ERCC3/XPB*, *ERCC4*, *ERCC5/XPG*, *ERCC6/CSB* , *XPA* and *XPC*.

A second method - often tried but thus far not very fruitful in the repair field, probably due to the low expression levels of repair proteins - consists in purification of the correcting proteins, after which the corresponding genes have to be isolated by molecular biological techniques. In this approach micro-injection of protein extracts combined with the Unscheduled DNA Synthesis assay[75] or *in vitro* repair systems are the essential screening methods[55,76-78]. This approach has led thus far to the isolation of two human repair genes: the *XPC* gene, isolated as part of a complex with HHR23B by Masutani *et al.* (see also above), and the DNA ligase I gene, which was previously thought to be implicated in the repair disorder Bloom's syndrome[79,80]. However, recent evidence contradicted this finding but showed that the gene is disturbed in a cell line derived from a unique patient, 46BR[81-83]. Moreover, recently the real Bloom's syndrome gene was isolated and appears to exhibit homology with the RecQ helicases, a subfamily of DExH box-containing DNA/RNA helicases[18]

Finally, as in the yeasts *Saccharomyces cerevisiae* (baker's yeast)[84] and *Schizosaccharomyces pombe*[85] a large number of DNA repair mutants had been isolated, it would be very convenient to utilise the many correcting yeast genes cloned for the isolation of human counterparts. Also in view of the limited number of human and hamster repair mutants (and the tedious transfection experiments) it was tempting to try to develop as first part of this thesis a general method for isolation of homologues of genes already cloned from other species. From the lessons learned in the course of this work it became obvious that, when trying to isolate similar genes in other organisms, it is essential to take the evolutionary direction which is followed into account. Descending the evolutionary ladder (e.g. from man to *Drosophila*) via low stringency hybridisations using standardised methods[86] is relatively easy, as the isolation starts from a complex genome ($3x10^6$kb) with large introns and thus many possibilities for accidental homology to the relatively simple genome of the fly ($1x10^5$kb). However, when cloning a human homologue of a yeast gene (genome size: $1x10^4$kb) the whole complexity of the human genome is encountered. Therefore, the cloning strategy had to be adapted several times, otherwise many small regions with fortuitous homology would have been isolated. The small protein domain shown in Fig.1 (Eco9), for instance, was isolated by screening *Drosophila* cDNA libraries with the total yeast *RAD1* gene.

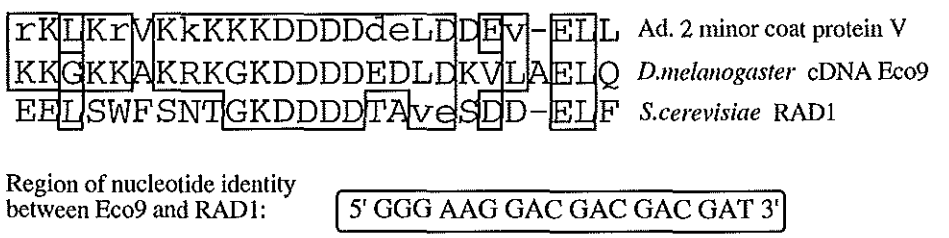

Figure 1. Representation of the domainal homology found between a Drosophila cDNA clone (Eco9), RAD1, and the adenovirus minor coat protein V.

The nucleotide sequence identity between Eco9 and *RAD1* did not exceed 18 base pairs. However, on the protein level a more extended similarity existed between these proteins. Remarkably, the same domain is also found in adenovirus 2 minor core protein V. Unfortunately, no indication exists for the function of this protein region. As we show here, small domainal homology can be very illustrative and sometimes lead to identification of protein sequence motifs. However, with the exception of several rare examples where it is the only conserved part of a gene, like for instance in the case of some of the important, but lowly conserved interleukin genes[87-89], such short sequences hardly ever represent a homologous counterpart. To avoid isolation and sequencing of many clones with fortuitous sequence homology, hereafter two flanking cDNA probes were used. In this "junction probe" strategy (see Fig. 2) it is assumed that only homology spread over a long area of DNA is of interest. When an extended area of nucleotide sequence similarity exists in the organism under investigation, and the genomic DNA is digested with several different restriction enzymes, a reasonable chance exists that the 5' as well as the 3' cDNA probes hybridise to the same genomic fragment; the junction fragment. It should be noted that instead of 5' and 3' probes also genes from distantly related species, for instance *S.cerevisiae RAD6* and *Ss.pombe rhp6*$^+$ (as done in Chapter II), can be used, at least if the two genes do not display large areas of nucleotide sequence conservation.

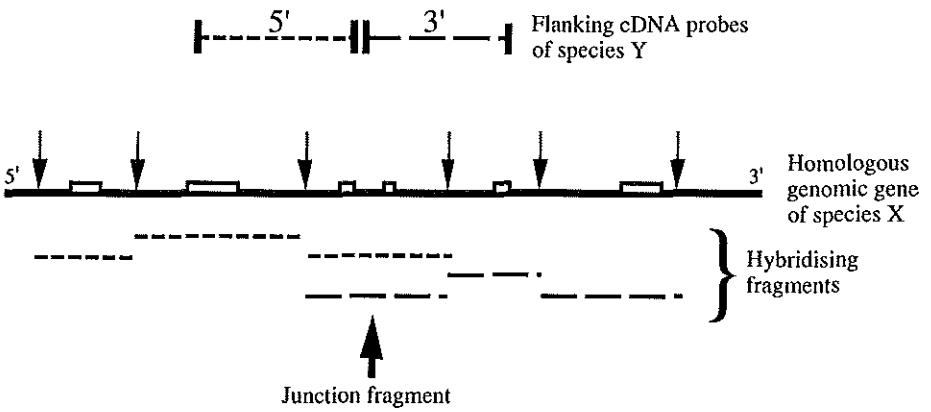

Figure 2. Junction Probe Principle. Open boxes in the genomic gene represent exons and the small arrows indicate restriction enzyme sites. The large arrow points to the junction fragment recognised by both the 5' and 3' probes.

However, also this method is not a 100% guarantee for the isolation of the correct gene. Again, in the case of the Eco9 gene, for instance, it appeared that the above described domain was repeated twice in a partial cDNA. Moreover, when digests of phage lambda clones harbouring the genomic Eco9 gene were hybridised with the conserved nucleotide stretch, the domain appeared to be repeated at least five times in the Eco9 gene. A gene with internal repetitions like Eco9 can easily lead to the isolation of sequences with fortuitous homology, if each of the junction probes contains one of the repeated domains, as this eliminates the advantage of the junction probes. Also under other circumstances repetition can lead to

problems. In the case of the RAD6 polypeptide, described below, the yeast protein contains a stretch of acidic amino acids. As the codons for these amino acids are rather uniform (GAg, GAa, GAt and GAc) repeated structures appear readily, leading in this approach to the isolation of one of the numerous proteins harbouring acidic regions[90]. Therefore, it was always tried to avoid the presence of repeated areas in the junction probes. (Each cloning attempt should be preceded by a self-comparison of the genes and proteins, a hybridisation of the 5' and 3' probes with each other, as well as an extensive computer-library screen to determine whether common or repeated motifs/protein regions are encoded by the probes).

When we developed the method it also appeared that accidentally cloned plasmid contaminations present in many lambda cDNA or genomic libraries show up by the low stringency hybridisations, due to the vector-DNA contamination of the gene probes used. This problem we circumvented by the use of PCR-generated probes or plasmid free libraries. To avoid a large part of the accidental homology one could argue not to use genomic DNA libraries for the approach but cDNA libraries. The expression levels of the repair genes isolated thus far, however, are so low that large and high complexity libraries are needed. Moreover, because a gene is not by definition conserved over its entire length, a strong need for full-length clones is obvious. Taken these facts together, genomic libraries are normally preferable over cDNA libraries when studying gene conservation in this way. However, it should be noted that especially in the higher eukaryotes the sometimes extremely large genes and distantly located small exons still may oblige to the use of cDNA libraries.

Although the above described procedure has many advantages over classical hybridisation methods, it is only applicable if several relatively extended regions of homology exist. When similarity between DNAs is too small or only found in a single small region of a gene, other methods have to be applied, which all but one have in common that brute sequencing force has to be used, as no early conclusive indications exist that the correct gene has been isolated. (In our method a double positivity with 5' and 3' probes presents good evidence for the correct gene). A quick and easy method was recently described as "computer cloning"[91]. An optimal computer search should certainly precede every attempt to clone homologous counterparts of known genes. In contrast, two brute force methods are, for instance, the enrichment for small regions of high homology using RecA protein[92] combined with low stringency hybridisations for the isolation of small domainal homology, or PCR applications with degenerated primers for the cloning of lowly conserved genes. In these cases it is worthwhile to consider other methods based on protein-sequence similarity, which is normally much higher than nucleotide homology. Antibody screening of bacterial protein expression libraries (e.g. λgt11), is very dependent on specific high affinity antisera to prevent cross-reactions. The method has been used for homology searches (for instance, RecA), but thus far not very successfully. Finally, also functional cloning, relatively quick and easy, but not yet really widely used, should perhaps be tried. The human *CDC2* and *CDC34* genes (see below), for instance, were isolated by transfection of a human cDNA library driven by an SV40-promoter[93] into a *Ss.pombe cdc2^{ts}* [94] or a *S.cerevisiae mec1* mutant[95], respectively. Several of the photoreactivating enzyme homologues were isolated by correcting a phr-defective *E.coli* mutant[96], and ref. therein and the isolation of yeast

topoisomerase II with *Drosophila* topoII was feasible with the sectoring/selection-method described by Kranz *et al.*[97].

In our case the junction-probe strategy resulted in the isolation of two groups of genes; the *Drosophila*[1], *Ss.pombe* (unpublished) and *S.cerevisiae*[2] homologues of *ERCC3*, and the *Ss.pombe*[98], *Drosophila*[99] and human[100] homologues of *RAD6/UBC2*. These latter genes are the subject of this thesis, and as RAD6 has been shown to play a role in the ubiquitin pathway, this system will be reviewed in the following part of the introduction.

General introduction to ubiquitin

"Ubiquitin is too small and too abundant to be important; you should change your research subject!" (Told to A. Haas about eleven years ago).

To state this about ubiquitin, one of the proteins most conserved in evolution, is nowadays impossible in view of the plethora of processes in which this "giant dwarf" plays a major role. Because it is impossible to cover the ubiquitin field within the limits of this introduction, the major topics will be highlighted, especially those in relation with DNA repair (For a bevy of recent reviews see[101-111]).

The ~$6x10^7$ molecules of this 76 amino acid protein found in each cell of our body make it one of the most abundant polypeptides[106]. Ubiquitin has been detected in a wide variety of organisms ranging from archaebacteriae[112-114] to man, and recently even in a eubacterium, the cyanobacterium *Anabaena variabilis* [115]. Considered as the slowest evolving protein known[116], it allowed in the 1.2 billion years of evolution which separate yeast from man only three amino acid changes to occur[114].

Ubiquitin conjugation pathway

In a cell the majority of the ubiquitin molecules are not found as free protein, but conjugated to other polypeptides[106]. The linkage reaction and the proteins performing it, are apparently almost as conserved as ubiquitin itself[100,117-121](Fig.3).

Conjugation commences when the C-terminal glycine residue of a ubiquitin molecule is activated by one of the ubiquitin-activating enzymes (referred to as Uba or E1), which uses ATP to form a high energy thiol ester intermediate, that is covalently linked to an internal cysteine residue of the E1-molecule[120]. This protein-complex is able to donate the 76 amino acids protein to one of a growing family of ubiquitin-conjugating enzymes (Ubc or E2)(e.g. RAD6/UBC2). The E2-ubiquitin complex links the ubiquitin moiety via its C-terminal glycine residue to the ε-NH2 group of a lysine residue in the target protein, with or without the help of a member of a family of ubiquitin ligases (Ubr or E3). The question whether (all) the E3-proteins are only docking proteins or bind ubiquitin to themselves, and thus actually perform an enzymatic activity, remains to be resolved[122].

Originally, ubiquitination was shown to be involved in specific (extra-lysosomal) targeted degradation of the bulk of mislocalised, improperly processed, foreign or damaged

proteins[106], as well as of undamaged polypeptides which are naturally short-lived[123] or which have to be maturated. The ubiquitination finally leads to ATP-dependent degradation of the targeted proteins by the complex multicatalytic 26S protease or proteasome, and release of free or branched ubiquitin (see below), which can be re-utilised. The proteasome is the major extralysosomal proteolytic system known. Present in the cytoplasm as well as in the nucleus, it is involved in both ubiquitin dependent and independent[124] degradation (For reviews on this issue not dealt with in this introduction, see[107,110,125-131]). Note, however, that also several links of the ubiquitin system with the lysosomal system (or the yeast vacuole) have been established, and that degradation of tagged proteins (especially of the ubiquitinated surface receptors) is therefore not obligatorily executed by the proteasome[132-144]. Moreover, a protein can be degraded by different collaborating degradation systems[145-147].

[The enormous literature concerning ubiquitin[148] (and ubiquitin-system proteins[149]) as marker for autoimmune and neuro-degenerative diseases is not considered in this review, as at present it is unknown whether the antibodies or changes in ubiquitin expression-levels are cause or consequence of the disease. For reviews, see[150-156]].

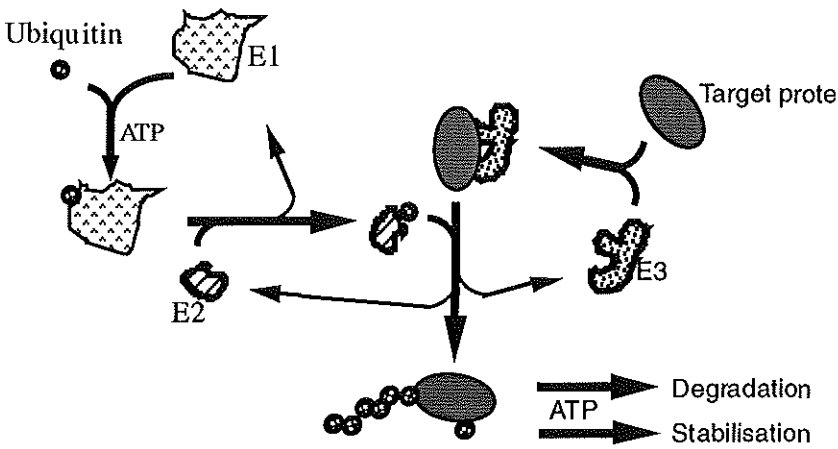

Figure 3. The ubiquitin conjugation pathway (simplified).

Involvement of ubiquitin in cellular processes

The role of ubiquitin in degradation implicates the small protein in a deluge of regulatory processes within the cell, including regulation of *gene expression* via (limited) degradation or posttranslational processing (NFkB[157], p53[158,159], c-fos[160], c-jun[161], c-mos[162-164], c-myc/c-fos[160,165]), *cell cycle control*[166-171], *DNA repair*[172,173], *recombination*[170,174], *ligand-induced degradation of cell surface receptors*[175,176], *cellular stress response*[142,177,178], *antigen processing and presentation*[179-181], *apoptosis*[182-184], *synaptic connectivity*[185-188], perhaps *subcellular compartmentalization* (import in mitochondria, uptake in synaptosomes, peroxisome biogenesis)[174,189,190], and indirect indications exist even for an implication in *nucleoside transport*[191].

Besides its degrading function the polypeptide is also involved in assuring correct protein synthesis and protein conformation, as it has been purported to be directly concerned

in the (re?)folding of (damaged?) proteins. The protein also seems to have anti-degrading functions, maybe due to its involvement in folding, as in certain circumstances ubiquitin protects against breakdown (see below).

A choice for or against degradation: linkage types

As far as what is known, the choice for or against degradation of ubiquitinated targets depends on two facts. First, whether the polypeptide is mono- or poly-ubiquitinated, and second, where in the target protein-backbone ubiquitin is attached. It was found that proteins can contain either single ubiquitin molecules (mono-ubiquitination) or tree structures of branched ubiquitin (poly-ubiquitination). These latter structures, whose formation is often dependent on the presence of an E3 enzyme, consist of ubiquitin molecules which are linked via their C-terminal glycine to specific internal lysine residues of another ubiquitin molecule. It is generally assumed that at least the lysine 48 (K-48) poly-ubiquitination leads to breakdown[192,193]. The existence of K-6, K-11, K-29 and K-63 poly-ubiquitination has also recently been described[173,194-196]. And although RAD6 can make K-6 tree structures on histone H2B in the absence of an E3 protein, the function of this linkage type remains unknown[196]. The human E2 enzyme EPF[149,197] is making K-11 linkages, which like K-29 and K-48 poly-ubiquitination, are involved in protein breakdown[198]. Finally K-63 poly-ubiquitination, performed by RAD6 in an E3-dependent manner, is apparently a poor inducer of degradation. This conjugation-type has recently been implicated in DNA repair, perhaps with a regulatory function. A yeast mutant which is unable to perform the K-63 linkage shows a phenotype which in part is comparable to that of a *rad6* deletion mutant[173](see below).

Mono-ubiquitination is normally not involved in breakdown but in the stabilising/folding functions (see below), although, in the case of the artificially-made ubiquitin-proline-β-galactosidase[199], and in the case of α-globin[200,201] it may be sufficient for degradation. In broader terms, all these results suggest that ubiquitin is a versatile signal in which different ubiquitin chain configurations are used for different functions. A single ubiquitin conjugating enzyme is apparently able to perform different linkages (for RAD6: K-6, K-48 and K-63) dependent on the target and the E3 involved[196].

Ubiquitin genes & Fusion proteins

When the first ubiquitin genes were cloned, it appeared that all organisms harboured many functional copies as well as many pseudogenes[202]. Moreover, always at least one of the genes was a poly-ubiquitin gene, harbouring a highly variable number of ubiquitin coding elements in a head-to-tail arrangement, and thus encoding a poly-ubiquitin precursor protein [e.g. 3 to 9 copies (man)[203,204], 14 (*Arabidopsis*)[205], 11 (*Caenorhabditis*), 2 to more then 40 (trypanosomatidae), 18 (*Drosophila*), 7 (maize), 6 (sunflower), 5 (yeast)[106]]. The poly-ubiquitin genes are in general inducible in the stress response (e.g. heat shock)[178,206], in contrast to the mono-ubiquitin genes. The mono-ubiquitin genes are often fusion genes as they encode the ubiquitin moiety in frame with a C-terminal extension peptide (CEP)[207]. The CEPs were found to represent two types of small very conserved ribosomal polypeptides, implicating ubiquitin in ribosome biosynthesis; the CEP80 proteins (with a variable length of

76, 80 or 81 amino acid residues) are found to be identical to ribosomal protein S27a which is part of the 40S particle, and the ribosomal CEP52 proteins which represent the L40 peptide, a constituent of the 60S or large ribosomal subunit[208-211]. This fundamental finding led to quite a few publications describing the same phenomenon in other organisms [CEP52: *Chlamydomonas*[212], *Arabidopsis*[213], *Tetrahymena* (CEP53)[214], *Dictyostelium*[215-217], *Drosophila*[218], chicken[219], and man[220], CEP80: *Neurospora*(78)[221], *Dictyostelium* (78)[217], maize(79)[222], *Arabidopsis*(80)[213], and *Drosophila*[223]], illustrating the evolutionary conservation of the C-terminal fusion partners.

Ubiquitin-like proteins

The search for C-terminal extension proteins led furthermore to the discovery of new types of ribosomal fusion proteins, like for instance the *Nicotiana tabacum* CEP72 protein[224] which is related to the CEP52 proteins.

In addition the family of ubiquitin-like sequences (Ubl) was expanded as several authors in their search for ubiquitin fusions, identified ubiquitin-like proteins with C-terminal extensions[225-228] or proteins with a C-terminal (!) ubiquitin-like extension("NEPs")[229]. The family of ubi-like protein sequences is constantly growing (Table I).

Table I: Ubiquitin-like proteins.

Ubl-CEP fusions:

1. *Caenorhabditis* CEP93[225]
2. Rat ribosomal protein S30 (also known as Fau protein or lymphokine MNSF[226-228])

NEP- Ubl fusions:

3. The Ubl moiety fused to the C-terminal end of mammalian splicing factor SF3a120 and its yeast homologue, PRP21p[229]

Normal Ubls:

4. *Xenopus* An1a and b proteins fused to a Zn^{2+}-finger protein[230]
5. 15kD interferon-induced ISG15 gene product UCRP[231-235]
6. NEDD8 protein[236,237]
7. Chinese hamster (and mouse) CHUB2 gene[238]
8. Earthworm *Eisenia fetida* Andrei Ubl[239]
9. X-chromosomal GdX protein[240]
10. BAT3 polypeptide[241]
11. Baculoviral v-ubi protein[242]
12. DNA excision repair proteins HHR23A and B[55]
13. Positive regulator subunit p18 of the SIII general transcription/elongation factor [243]
14. A whole group of non-expressed Ubl-pseudogenes in *Arabidopsis*[205]

The idea that these Ubl's can replace normal ubiquitin in its functions, is thus far only founded on the detailed analysis of UCRP. This di-ubiquitin-like protein is weakly homologous to normal ubiquitin and was shown to be conjugated to cellular proteins *in vivo*[244]. The ubiquitin-like proteins in Table I should therefore probably be divided into two different classes. A first group of functional "well" conserved (especially the C-terminal glycine residues) ubiquitin-like proteins which have similar functions as classical ubiquitin but are involved in parallel pathways (UCRP, NEDD8, Ubl-Fau, Ubl-CEP93, SF3a120, v-ubi

and although less likely, Ubl-An1a and b). And a second class of ancient "normal ubiquitin"-fusion proteins which lost the cleavage site between ubiquitin and the C-terminal extension (BAT3, GdX, SIII p18, CHUB2, SIII p18 and HHR23). As because of this most of the evolutionary pressure on the ubiquitin moiety was lost, the coding sequence slowly changed. The "stabilising function" (see below), however, was probably retained, and is apparently absolutely required for correct function of the fused partner[243].

With the identification of the ubiquitin-like molecules the complexity of the system is increasing even further. If normal ubiquitin is already implicated in the plethora of processes specified in this introduction, what will be the function of these ubiquitin homologues and why did they evolve? What will be the function of recently cloned E1-like proteins[245-248] or of the different virus-encoded proteins: the ubiquitin(-like) proteins of baculovirus (v-ubi[242,249,250]), bovine viral diarrhoea virus[251,252], and Finkel-Biskis-Reilly murine sarcoma virus[226,227], and the E2 protein UBCv1 (related to RAD6) of African swine fever virus[253-255]? Do these viruses use the ubiquitin system for their benefit in a similar way as Human Papilloma Virus 16 and 18, whose E6 protein interacts with a cellular E3 protein, E6-AP, and forces it to recognise p53, leading to the poly-ubiquitination and degradation of this anti-oncogene[159,171,256,257]? Or do they try to escape the attacks by the cell's degradation systems by titrating the cellular ubiquitin with non-conjugatable ubiquitin homologues or by mimicking their proper ubiquitination with their own E2's[249]?

Ubiquitin specific proteases

Although ubiquitin carboxyl-terminal hydrolases or ubiquitin specific proteases (UBP's) were known to exist, and to be implicated in the production of mono-ubiquitin from the poly-ubiquitin precursors, the isolation and cloning of these hydrolases was also accelerated due to the identification of the ubiquitin-CEP fusions.

Some of them were shown to remove small peptides or single amino acids from the C-terminal end of ubiquitin[258,259], and to be necessary for the maturation of the last ubiquitin moiety of a poly-ubiquitin protein. This last ubiquitin-copy of a poly-ubiquitin gene often contains some additional C-terminal amino acids, probably to prevent the non-branched poly-ubiquitin molecules from participating in the linkage reactions. A second class is implicated in the production of single ubiquitin-moieties from the poly-ubiquitin precursors, or in the maturation of the C-terminal fusion proteins[260,261]. Finally, the third group of ubiquitin lyases, to which the human oncogene product Tre-2 or its yeast homologues DOA4[262] or UBP5[263] belong, releases and/or degrades poly-ubiquitin trees. Proteins from the last group are implicated in the rescue of faulty-targetted proteins or to recuperate free ubiquitin for reutilization after degradation of the tagged proteins[264-266]. Note, however, that degradation of tree-structures is not absolutely necessary as they also can be re-used directly. Moreover, free tree-structures can made by certain E2 enzymes independent of the presence of a target protein[267-269].

With the identification of the UBPs the description of the ubiquitin system components is complete: single ubiquitin can be made from the fusion or poly-ubiquitin gene products; E1, E2 and E3 proteins can do their work; the proteasome degrades the targetted proteins; and finally the poly-ubiquitin trees can be recuperated to yield again free ubiquitin.

In the next paragraphs the implications of ubiquitin in degradation and in anti-proteolysis, protein structure and folding will be briefly discussed. The chapter finishes with a summary of our current knowledge on the role of ubiquitin in DNA repair and chromatin structure, which is of course obligatorily linked to one of the E2-enzymes, RAD6.

Ubiquitin in protein degradation

All living cells have to regulate the content and composition of their resident proteins, but the mechanisms by which this is done are not well known. Intracellular protein degradation is important in determining steady state and fluctuations of protein concentrations as well as for the generation of protein fragments that act as hormones, antigens, or other effectors. Breakdown can be regulated by innate properties of the protein substrate (e.g. PEST[270]- or KFERQ[271]-sequences), or by chemical modifications (e.g. ubiquitin) which mark them for breakdown, in other words which confer metabolic instability. The initial event leading to degradation may or may not involve 1) proteolysis, 2) non-proteolytic (covalent) modifications (e.g. oxidation of methionines, ubiquitin conjugation, AANDENYALAA-tagging[272] [i.e. A COOH-terminal peptide-sequence, thus far only detected in *E.coli*, which is linked to a protein while it is being translated from an erroneous mRNA which does not encode a stopcodon. This tagged incomplete protein is subsequently degraded by tag-specific proteases. The process involves a new RNA type (with both a transfer and messenger function (tmRNA)) and a switch of the translation machinery from the defective mRNA to the tmRNA. It represents a magnificent quality control mechanism for defective mRNAs], 3) denaturation or unfolding of the protein, or 4) sequestration in cytoplasmic or nuclear "organelles". These processes, however, have to be selective as an enormous heterogeneity in degradation rates exists for the different proteins in the cell. Ubiquitination is one of the ways to achieve such a selectivity. Although the number of natural degradation-targets of the ubiquitin machinery[273] starts to grow (see Table II, pg. 21), the issue of what determines the specificity of the ubiquitin ligation system i.e. the degradation signals for commitment of certain proteins to degradation is not yet resolved.

Ubiquitin degradation signals

The only general prerequisite for degradation of a protein via ubiquitination is the obligatory presence of a lysine residue to whose ε-amino group the ubiquitin moiety will be finally attached[310]. The other additional structural features of a substrate which are recognised by the ligation system are for the moment not well known, and rather non-uniform[310](see below). To complicate the situation it even appeared that proteins which do not contain any degradation-signals themselves can be degraded by their interaction with other polypeptides or subunits which only serve as (undegradable) tag for the ubiquitin machinery (trans-recognition)[199,280,286,311].

N-rule system and RAD6

Varshavsky and co-workers identified the first of the ubiquitin-system degradation-signals; the presence of a free alpha-amino group (For extensive reviews and detailed explanations, see[199,312]).

Table II: Identified natural substrates of the ubiquitin-degradation system.

1. Plant photoreceptor chromoprotein: phytochrome[274]
2. Bovine photoreceptor G protein transducin[275]
3. Sindbis virus RNA polymerase (*in vitro*)[276]
4. Encephalomyocarditis Virus-3C Protease[277,278]
5. *c-mos* proto-oncogene product[162-164]
6. *c-jun* proto-oncogene product[161]
7. *c-fos* proto-oncogene product[160]
8. *c-cbl* proto-oncogene product[279]
9. p53[158,165,257,280]
10. *N-myc*, *c-myc*, *c-fos* and *E1A* product (*in vitro*)[165]
11. p105-NF-κB (activation and processing via partial degradation)[157]
12. NF-κB inhibitor IκBα[281-283]
13. Yeast Gcn4 transcriptional activator[284]
14. Yeast MAT-alpha-2 Repressor[123,285]
15. Yeast ABC-transporter Ste6[137]
16. Yeast Sec61 endoplasmic reticulum membrane protein[190]
17. Yeast multidrug transporter Pdr5[136]
18. Yeast G protein-coupled pheromone plasma membrane receptor Ste2p[141]
19. GPA1, α subunit of yeast G protein (pheromone-dependent signal transduction)[286]
20. Regulatory subunits of cAMP-dependent protein kinase in Aplysia[287]
21. Class I (EGFR[288]), III (PDGFR, CSF-1-R, c-kit) and IV (FGFR) receptor tyrosine kinases[289,290]
22. High-affinity IgE receptor (FcεRI), β and γ chains[291]
23. T cell receptor ζ and CD3ε[292]
24. Rat uterine estrogen receptor[293]
25. Growth hormone receptor[294,295]
26. Yeast kinetochore protein, Cbf2p/Ndc10p[296]
27. Cyclins A and B[167,169]
28. Mammalian cyclin-dependent kinase inhibitor p27[168]
29. Yeast cyclin-dependent kinase inhibitor Sic1[297]
30. *Ss.pombe* mitotic activating tyrosine phosphatase CDC25[171]
31. Oat ribulose-1,5-biphosphate carboxylase[298]
32. Yeast fructose-1,6-biphosphatase[299]
33. CFTR[145,300]
34. Yeast uracil permease[301,302]
35. Yeast gap junction protein connexin 43[138]
36. Soluble rabbit hexokinase type I[303]
37. Calmodulin[304-307]
38. Rat microsomal protein P-450_{2B1}[308]
39. O6-methylguanine-DNA methyltransferase[309]
40. Human α-globin[200,201]

As shown in Table III and summary Figure 4, the *in vivo* half life of a protein is (at least in part) related to the identity of its free amino-terminal residue, which serves as a primary degradation signal[199,313]. In eukaryotes, the identity of the first amino acid of a protein is recognised by a specific E3 protein and identification of so-called destabilising residues (N-degrons) results in breakdown by the ubiquitin pathway.

To elucidate this so-called N-rule system, N-terminal ubiquitin fusions to

βgalactosidase were used. Fusion of ubiquitin to the N-terminal amino acid of a protein leads to a paradoxal effect in that it optimises the folding of the protein to which it is fused, and protects it against degradation (See below "Ubiquitin in protein structure, folding and anti-proteolysis" and Koken *et al.*[314], for an extensive review). Moreover, all eukaryotic cells contain highly specific ubiquitin specific proteases (see above) which are able to remove the N-terminal ubiquitin moiety, leaving the C-terminally fused protein unharmed. By introducing modifications at the N-terminal amino acid of βgalactosidase in these ubiquitin-βgalactosidase fusions, Bachmair *et al.* were able to produce *in vivo/in vitro* the free N-terminal end of their choice[313]. The half-lives of the different N-terminally modified βgalactosidase forms were determined in eukaryotic and prokaryotic cells (See Table III). The table shows the half-lives of proteins which contain the indicated aminoterminal amino acids as determined in the specified organisms[199], as well as a compilation of results obtained in the cited articles concerning initiator-methionine-removal and N-terminal acetylation[315-318]

Table III: The N-end rule, methylation and acetylation

Residue X in Ub-X-βgal	$t_{1/2}$ *E.coli*	$t_{1/2}$ Yeast *in vivo*	Methionine Removal Acetylation	$t_{1/2}$ Reticulo-cytes *in vitro*	Methionine Removal Acetylation
Arg	2 min	2 min	M-X----	1.0 hr	M-X-----
Lys	2 min	3 min	M-X----	1.3 hr	M-X-----
Phe	2 min	3 min	M-X----	1.1 hr	AcM-X-----
Leu	2 min	3 min	M-X----	5.5 hr	AcM-X-60-
Trp	2 min	3 min	M-X----	2.8 hr	AcM-X-75-
Tyr	2 min	10 min	M-X----	2.8 hr	AcM-X-95-
His	>10 hr	3 min	M-X----	3.5 hr	AcM-X-70-
Ile	>10 hr	30 min	M-X----	20.0 hr	AcM-X-40-
Asp	>10 hr	3 min	AcM-X---	1.1 hr	AcM-X-100
Glu	>10 hr	30 min	M-X----	1.0 hr	AcM-X-100
Asn	>10 hr	3 min	AcM-X---	1.4 hr	AcM-X-100
Gln	>10 hr	10 min	AcM-X---	0.8 hr	AcM-X-100
Cys	>10 hr	>20 hr	X---	12.0 hr	Ac-X-100
Ala	>10 hr	>20 hr	Ac-X---	4.4 hr	Ac-X-95-
Ser	>10 hr	>20 hr	Ac-X---	1.9 hr	Ac-X-100
Thr	>10 hr	>20 hr	Ac-X---	7.2 hr	Ac-X-50-
Gly	>10 hr	>20 hr	Ac-X---	30.0 hr	Ac-X-20-
Val	>10 hr	>20 hr	X---	100.0 hr	*-X-----
Pro***	?	>20 hr	X---	>20.0 hr	80-X-----
Met	>10 hr	>20 hr	M-X---	30.0 hr	/AcM-X-40- AcM-X-70-

***Val-His (not-Acetylated) and Val-Asp (50% Acetylated) show both 100% removal of Met; however, Val-Pro undergoes 0% Met removal and is not acetylated!**

Columns 2,3 and 5 give the half-lifes of N-terminally modified (see. column 1) βgalactosidase forms in different organisms. **Columns 4** (mutants of the plant protein, thaumatin, expressed in yeast)[317] **and 6** (human βglobin mutants, in reticulocyte extracts)[315] indicate whether in naturally occurring polypeptides the N-terminally encoded methionine residue will be removed when it is followed by the amino acids indicated in the first column. The "M" indicates that the methionine is retained in 100% of the polypeptides; 80 in the case of proline indicates the removal in 80% of the cases. "Ac" marks that cotranslational amino-terminal acetylation occurs, and the number behind the X indicates in which percentage an acetyl group is added dependent on the identity of the N-terminal amino acid.
*** Proline is a special case, see the end of this paragraph.

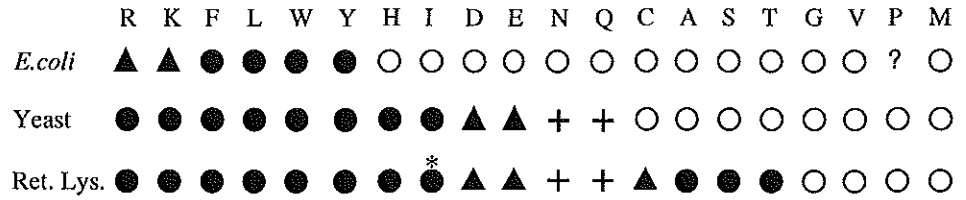

Figure 4. Summary of Table III. Comparison of eukaryotic and bacterial N-rules. Open circles, stabilising residues, filled circles, triangles, and crosses denote, respectively, primary, secondary, and tertiary destabilising residues in the different N-rules (see Fig. 5). The question mark indicates the unknown status of proline in the bacterium, and the asterisk indicates that isoleucine is a borderline destabilising residue in ret. lysates, and a weak one in yeast.

Table III and Figure 4 show that approximately the same N-terminal amino acids cause short half lives in prokaryotes and eukaryotes. The Varshavsky group assembled existing isolated data[319,320] and filled in some missing links to obtain the recognition/modification mechanism (Fig. 5), which precedes the actual degradation. They showed that principally the same mechanisms are used in pro- and eukaryotes, but that only in the latter organisms, ubiquitin is involved in the subsequent degradation which follows upon the N-end recognition[312].

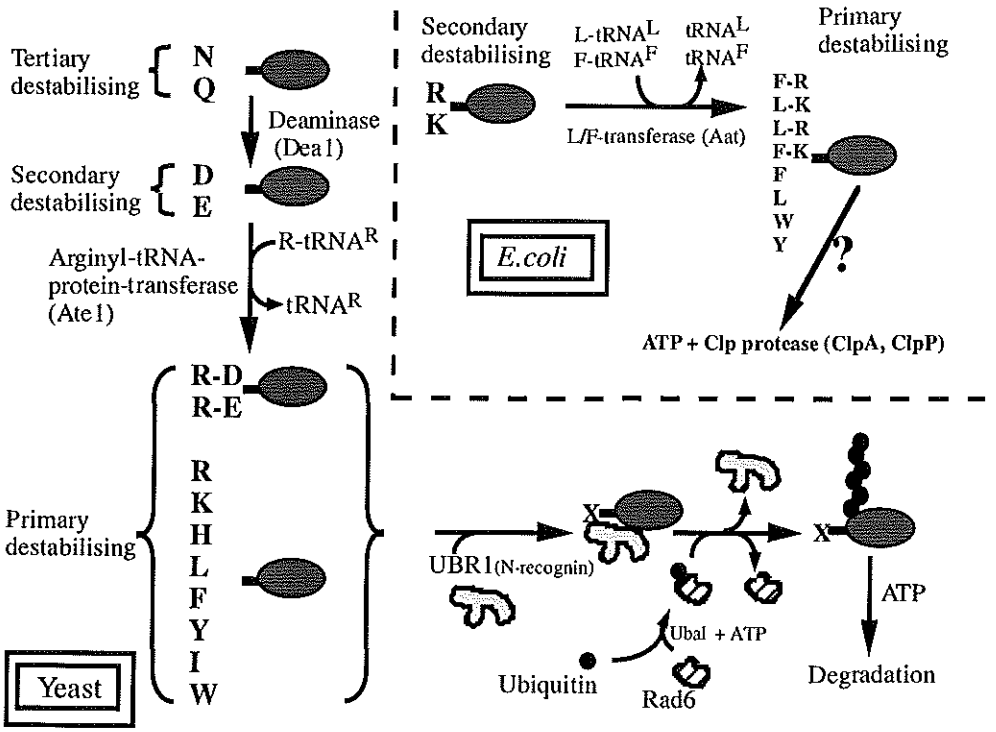

Figure 5. Schematic representation of the N-rule degradation-pathway in pro- and eukaryotes.

In short, proteins become prone to the N-end rule degradation machinery when the mRNA-encoded amino-terminal methionine is removed. When the second amino acid is a so-called stabilising residue, the protein is not a target for the N-rule pathway, and no degradation occurs. This type of proteins is normally highly stable, and their degradation is apparently independent of the ubiquitin pathway[312]. The destabilising residues can be divided into tertiary, secondary and primary destabilising amino acids. Via deamination tertiary residues are transformed into secondary. Through addition of a primary destabilising arginine residue the secondary destabilising residues are transformed into primary which are recognised by the ubiquitin machinery. The addition is done arginyl-tRNA protein transferase which explained old data of tRNA dependence of the ubiquitin system[319,320]. In eukaryotes, the recently identified 225 kD E3-protein UBR1 recognises these residues[321]. Together with the ubiquitin-conjugating protein RAD6 (UBC2) the poly-ubiquitination is undertaken[322,323] which finally leads to degradation by the proteasome.

However, thus far, only two physiological substrates for the N-rule degradation system have been identified, the Sindbis virus RNA polymerase[276] and GPA1, the α subunit of a heterotrimeric G protein involved in pheromone-dependent signal transduction in yeast[286]. So, apparently, although the elucidation of the N-rule pathway represents an elegant piece of work, it has probably only limited *in vivo* significance, as a free amino-terminal residue is not at all a predominant recognition marker of native proteins. Most proteins have blocked, acetylated amino termini (see Table III), which prevent N-rule mediated degradation. [Note, however, that acetylated short-lived proteins are still targets for ubiquitin-mediated degradation which probably implicates yet unidentified E2 and E3 proteins[324]. This pathway curiously necessitates the presence of elongation factor EF-1α[325]!] Moreover, the rules which govern methionine-removal (see Table III), show that proteins apparently only allow the removal of their first methionine if the second amino acid in chain is a so-called stabilising one. Otherwise, the initiator methionine is not removed and often even acetylated. [The removal of the methionine residue is largely dictated by the substrate specificities of methionine aminopeptidase, an enzyme present in both eukaryotes and bacteria which cleaves off the amino-terminal Met residue.]

So, it is likely that this pathway will only be used for proteins which have lost their N-terminal residues, exposing now destabilising residues, or for protein fragments, generated by endopeptidases. It can also not be excluded (and indications exists[326,412]) that other methionine aminopeptidases (governed by other methionine-removal rules) exist which could compete with above-mentioned protein, and remove methionine from destabilising second amino acids. Finally, as in the case of GPA1, signalling to the N-rule pathway could occur in *trans*. GPA1 does not contain a destabilising N-terminal amino acid (Met^1-Gly^2), neither is an N-degron produced by an internal cleavage. It seems that the protein is forced into N-rule degradation via its interaction with the protein Sst2[286], which, however, does not bear a

destabilising N-terminus (Met[1]-Val[2]) either[327]. From the above data it is not clear which factors determine the N-rule mediated degradation of the GPA1 protein.

Ubiquitin Fusion Degradation

When resolving the N-rule, an N-terminal proline was found to be a stabilising residue (Table III). However, the ubiquitin-proline-βgal fusion protein (used to produce proline-βgal) was found to be extremely short-lived ($t_{1/2}$=7 min)[312]! As explained in the introduction to the N-rule, the fusion of ubiquitin to the N-terminal amino acid of a polypeptide normally stabilises the C-terminal fusion partner. However, an N-terminal proline, or ubiquitin-fusions in which the C-terminal glycine-76 residue of ubiquitin is modified or absent e.g. Ub$^{\underline{Val76}}$-V-βgal[198] leads to a short half-life. The removal of the ubiquitin-moiety, which is supposed to occur almost co-translationally, is in this type of fusions extremely slow or even absent (if the C-terminal glycine is absent). This "proline discrepancy" led (again) the Varshavsky group to decorticate the phenomenon, leading to yet another degradation pathway, the UFD (Ubiquitin Fusion Degradation). In this pathway the "non-removable" N-terminal ubiquitin is recognised as degradation signal. The targeting of a ubiquitin fusion by the UFD pathway results in the poly-ubiquitination (K48 or K29-poly-ubiquitination, dependent on the C-terminal partner) of the fusion's "non-removable" ubiquitin moiety, a step required for the subsequent proteasomal degradation. Thus far five genes were isolated from this pathway, but details are not yet known[198]. Moreover, like long time for the N-rule pathway, substrates are still unrecognised, although some of the "non-removable" ubiquitin-like fusion proteins (see "Ubiquitin-like proteins") are good candidates.

"2nd Codon rule" and Destruction box

Another breakdown signal different from the free N-terminals of the N-rule pathway or the non-removable N-terminal ubiquitin was detected in the *c-mos* proto-oncogene product. This protein which is implicated in cell cycle control is degraded by the ubiquitin pathway. The second (proline-2) and third (serine-3) N-terminal amino acids were shown to determine the half-life of this polypeptide[162-164]. As shown above, proline was found as a stabilising residue in the N-rule. And as said before, the proteins containing such residues are normally stable, and not dependent on ubiquitin for their degradation[312]. c-mos, however, is unstable when its third residue (Ser-3) is in an unphosphorylated state. This situation favours the recognition of proline-2 by an E3 protein. This interaction leads then to quick degradation of the unphosphorylated protein via the ubiquitin pathway. However, if serine-3 is phosphorylated, proline-2 is not recognised anymore, and no ubiquitin tree can be added to lysine-34 of c-mos.

The fourth ubiquitin-degradation signal known is the so-called destruction box found in A- and B-type cyclins, which are quickly degraded at the end of mitosis. In the highly

variable 100 to 150 N-terminal residues of these proteins a small conserved island was identified, the destruction box (consensus: Arg-X-X-Leu-X-X-Ile/(Leu)-X-(Asn), followed by dispersed lysine residues), which is recognised by the ubiquitin system[167,328], leading to the addition of a ubiquitin tree elsewhere in the cyclin molecule. The degradation is triggered upon phosphorylation of an uncharacterised E3 protein by CDC2 kinase during mitosis[329]. However, degradation of cyclin is not as simple as this, as it is not only dependent on the ubiquitin system, but under certain circumstances also upon calpain[330].

Finally, using a random peptide approach, Sadis *et al.*[326] selected small sequences capable of destabilising the βgalactosidase reporter in yeast. The authors detect three classes of degradation signals: Class I, the N-rule re-invented, dependent on RAD6/UBC2 and UBR1. Class II signals form amphipathic alpha helices often containing hydrophobic residues, and their degradation is dependent on UBC4, 5, 6 and 7. Matα2 could represent an example of this type of signal. Class III signals consist of obligatorily-short tracts (4-5 amino acids) of hydrophobic residues such as Leu or Ile. Degradation involves UBC4 and 5.

As can be seen from all these degradation signals, they are for the moment rather non-uniform, and many others have probably to be elucidated before the general recognition mechanism (if existing?) will be understood.

Ubiquitin in anti-proteolysis, protein structure and folding

As explained above (see Ubiquitin genes & Fusion proteins) many ubiquitin genes encode fusion-proteins between ubiquitin and C-terminal extension peptides[208,210]. This finding implicated ubiquitin in ribosome biogenesis, and apparently not in its normal degradation function. The C-terminal moiety is clipped off by a ubiquitin carboxyl-terminal hydrolase and the ribosomal protein interacts normally with other ribosome subunits. This co-translation of ubiquitin with the ribosomal proteins has led to the suggestion that ubiquitin may function as a chaperonin for the associated protein[210] or may target the C-terminal protein to a specific cellular site[208]. These ideas came from the finding that the C-terminal moieties of the fusion genes could correct the yeast mutants, but only if placed on a multicopy plasmid. So, apparently the ubiquitin-part was not absolutely necessary, but facilitated integration in the ribosome, stabilised the C-terminal peptide (by protecting its N-terminus?) and/or assured its correct folding. Subsequent experiments with artificial fusions in *E.coli*, yeast and man showed that a "protective effect" could be mediated through fusion with a "removable" N-terminal ubiquitin molecule (otherwise, see UFD pathway). This so-called ubiquitin-fusion technology is now widely used in biotechnology as it often gives high amounts of correctly folded proteins (For review see[314] and ref. therein). It is now generally thought that "removable" N-terminal fusion with ubiquitin causes more efficient translation of the fusion protein, better folding and perhaps N-end protection.

[Ubiquitin is used more and more in basic methodology (e.g. half-live determination,

protein overproduction, protein-protein interactions) as may become clear from the following references[314,331-334]]

A second indication that ubiquitin also has non-degrading functions came from the finding that several cell surface receptors sometimes contain covalently linked mono- or poly-ubiquitin. [i.e. PDGFR(β chain)[175,176,289,335], c-kit proto-oncogene product[290], growth hormone receptor[294,295], 65kD-TNF-receptor[336], High-affinity immunoglobulin E receptor (FcεRI)(β and γ chains)[291], T cell antigen receptor ζ and CD3ε[292], and finally the lymphocyte homing receptor gp90MEL-14[337-341]] In the case of the PDGFR (and most of the other receptor tyrosine kinases[289]), the T cell/CD3 and IgE receptors poly-ubiquitination is ligand/activation-dependent and leads to classical breakdown of the receptors as well as their ligands. However, the Mel-14 receptor is only mono-ubiquitinated, and apparently not degraded. It is hypothesised that the structural alteration conferred by the ubiquitination could change signal transducing properties and affect associations of the modified molecules with other effectors.

Also in the case of actin [342], histone H2A (= protein A24, the first ubiquitinated protein identified!), histone H2B[266], ganglioside binding proteins[343], α-spectrin[344], the yeast ubiquitin-conjugating enzyme UBC4[345], Leukemia derived growth factor[346,347], and several plant viral coat proteins[348,349], the core protein is mono-ubiquitinated, apparently without causing its degradation. [Note that also several ubiquitin-conjugating enzymes (UBC4, CDC34, $E2_{EPF}$, ASFV[254] and probably also RAD6/HHR6 (ref.[350] and this thesis, Chapter 5) are found to be mono- or poly-ubiquitinated. The function of this is thus far unknown.]

Finally, there are indications that ubiquitin is implicated in "non-degrading" processes like insertion of proteins in mitochondrial membranes[351], peroxisome biogenesis[189], as chaperonin after heat shock, as suppressor of platelet function[352], as differentiating[346,353] or suppressive[228,354] lymphokine, or in transport into or out of the endoplasmic reticulum. In this last case the authors explain their data as ER-degradation. However, alternative explanations, such as folding, protection, addition of ubiquitin to surface receptors, etc. are as likely[190].

Thus, although many of the ubiquitin functions can be explained by degradation, the examples mentioned above suggest that the protein has a spectrum of functions which exceeds that of only degradation. A possible other example of such a non-degrading function may be its implication in DNA repair, as discussed below.

DNA repair and ubiquitination

In this section the two major examples of an involvement of the ubiquitin system in DNA repair will be discussed, i.e. RAD6 and the ubiquitin K63R-mutant, both apparently involved in the post-replication repair pathway. Several other indications exist that ubiquitin or ubiquitin-like proteins are involved in DNA repair. However, these will be discussed only briefly as only limited knowledge on the proteins and processes involved exists.

HHR23B, DOA4 and p53

The XPC-correcting protein complex harbours, for instance, a ubiquitin-like fusion protein, HHR23B, in which the ubiquitin(-like) portion is absolutely necessary for the function of the complex (For review see[91]). A second link between the repair protein, HHR23B

and the ubiquitin system is the presence of the highly conserved ~50 amino acids UBA domain, a structure of unknown function that is found in several ubiquitin hydrolases, E2 and E3 proteins, as well as in several protein kinases[355,356].

Another example of involvement of the ubiquitin pathway in repair processes is the yeast *DOA4* gene which is related to the human *tre-2* or mouse *Unp* oncogene[262,263]. This gene encodes a de-ubiquitinating enzyme involved in the recycling of ubiquitin late in the proteolytic pathway. In contrast to for instance the YUH1, UBP1, 2 and 3 hydrolases, DOA4 is rather essential as deletion of the gene results in poor growth and a severe inhibition of general proteolysis. The deletion mutant is very sensitive to UV and γ-irradiation, sporulation is almost absent, and the degradation of both N-rule and UFD-target proteins is inhibited (10-20 fold).

Yet another aspect of the role of ubiquitin in DNA repair is its involvement in the degradation of "checkpoint protein" p53. The p53 tumour suppressor protein co-ordinates multiple responses to DNA injury. DNA damage causes an increase of functional p53 in the cell. Increase in p53 activity leads to cell cycle arrest which allows the cell to repair its DNA injury. When damage is beyond repair, p53 activates the apoptosis pathway and the cell dies. Proper regulation of this crucial protein is of utmost importance, ... and ubiquitin is part of the regulatory processes affecting p53[158,159,165,257,280,357].

RAD6 mutant, gene and protein

When our initial attempts to isolate *Drosophila* DNA excision repair genes with probes from their possible yeast counterparts turned out negative, probably due too low conservation (*RAD1*, *RAD2* and *RAD7*) or too much domainal similarity (*RAD3*), our interest was raised by the yeast gene *RAD6*, the putative "master-gene" of yeast post-replication repair (For extensive reviews on the mutants, the gene and protein, see[4,5,358-361])(For RAD6 3D structure see[362]). As explained in the first chapter, patients of the so-called XP-variant group have a defect in this post-replication repair pathway, and a human *RAD6* gene could represent a candidate gene for this repair disorder. However, at that moment, we were very well aware of the fact that *RAD6*-deficient mutants display a very severe and pleiotropic phenotype (see Table IV), in contrast to the human XP-variants which in general present only very mild XP-features. (Note that in preliminary experiments thus far no obvious changes of *HHR6A* or *B* RNA/protein expression were found in several XP variant cell lines studied, data not shown.)

The 172 amino acids yeast RAD6 protein which corrects the *rad6* mutant phenotype was at the time of its isolation devoid of any recognisable domains and showed as a single peculiarity a long acidic amino acid sequence at its C-terminus[366,367]. The "acidic tail" was subsequently shown to be indispensable for sporulation, but not necessary for induced mutagenesis and DNA repair[368]. The yeast protein's expression is induced by DNA damage and during meiosis, but remains constant during the mitotic cell cycle[369,370]. In this it resembles several other proteins implicated in the ubiquitin or RAD6 pathways[369,371,372]. Both induction phenomena were, however not conserved in the RAD6 (or RAD23) homologues of higher eukaryotes (see Chapter V and ref.[91]), pointing to differences in regulation between lower and higher organisms. In rats the mRNA is induced upon fasting and decreases upon insulin treatment, providing a first example of hormonal regulation of the

ubiquitin system[373]. All lower eukaryotes seem to contain a single *RAD6* homologous gene. In mammals and *Arabidopsis thaliana*, however, gene duplications and triplications occurred, respectively[100,374]. The highly similar "twin" genes are mostly co-expressed in the tissues tested, and it is unknown if the individual gene copies have distinct functions, although the characterisation of *mHR6B* knockout mice (Chapter VII) starts shedding light on this phenomenon.

Table IV: Phenotype of the yeast *RAD6* deletion mutant and yeast UbK63R mutant

	Mutant=>	*rad6Δ*	*UbK63R*
DNA repair and mutagenesis			
Sensitivity to:	UV, 4NQO	+++	+++
	Crosslinking agents (eg.8MOP + UV)	+++	nd
	Alkylating agents (eg.MMS)	+++	+++
	X/γ-irradiation	+++	wt
Mutagenesis:	Spontaneous	elevated[363]	wt
	Induced by damaging agents	deficient	deficient
Excision of dimers		normal	nd
Post-replication repair		deficient*	nd
Recombination			
Mitotic (spontaneous/induced)		increased	nd
Meiotic		defective	nd
Retrotransposition of Ty elements		increased[363-365]	nd
Cell Growth			
Cell cycle		S-phase prolonged	wt
Growth rate		slow	almost wt
Sporulation		defective	wt
-N-rule degradation pathway		defective	wt

*Defect in reappearance of high molecular weight DNA after replication of damaged templates
wt=wild type levels, nd= not done

RAD6 and histones

While we were executing our initial cloning attempts in *Ss.pombe*, Jentsch and co-workers cloned the already known *S.cerevisiae RAD6* gene in their search for the ubiquitin-conjugating enzymes of this fungus. The protein was shown to add *in vitro* specifically a single ubiquitin-moiety to the C-terminal lysine-119 of histone H2A or lysine-120 of histone H2B[172,375], but not to several other highly basic control proteins. This ubiquitin-conjugation activity was shown to be necessary for all RAD6 functions known, because a mutation of the ubiquitin-acceptor cysteine residue into a valine, alanine or serine residue leads to a RAD6-deficient phenotype[376,377] (see Table IV). In the presence of the yeast E3 ubiquitin-ligase, UBR1, with which RAD6 interacts through its highly conserved N-terminus (Chapter IV), the histones can *in vitro* even be poly-ubiquitinated. This shows that RAD6, like for instance CDC34[95,166] (an E2 protein implicated in G1-S phase cell cycle transition) is a bifunctional

enzyme competent in both E3-independent and E3-dependent conjugation reactions[195](see for this bifunctionality also[197]). This poly-ubiquitination of histones is dependent on the acidic tail of yeast RAD6. Therefore sporulation, which is tail-dependent, needs apparently poly-ubiquitination, whereas DNA repair and mutagenesis involve only mono-ubiqitination. Although these ideas are generally accepted, the function of histone mono- and poly-ubiquitination by RAD6 *in vivo* and its implication in DNA repair remain a subject of debate.

--- Eukaryotic DNA is organised in nucleosomes: a stretch of ~146 base pairs of DNA is wound around a histone octamer which consists of two subunits of histones H2A(14kD), H2B(14kD), H3(15.3kD) and H4(11.2kD) [$(H2A:H2B)_2H3_2H4_2$)]. The nucleosomes are connected by 50-100 base pair stretches of DNA to which, in (higher[413]) eukaryotes, a molecule of histone H1(22kD) binds which stabilises the higher order chromatin structure resulting in the compact "30nm" fibers. The degree of local packing has to be tightly regulated, as it has been shown that the chromatin is highly condensed in regions containing quiescent genes and more accessible in regions of transcriptional activity. It is now generally admitted that this regulation probably takes place through a variety of non-permanent post-translational modifications; methylation, acetylation, phosphorylation, poly(ADP)ribosylation and ubiquitination of the flexible N- or C-terminal domains of the different nucleosome components. However, although extensive, often contradictory, literature exists on this subject, no really clear relationship between a specific modification and its implication in transcription, replication, DNA repair, or spermiogenesis has been demonstrated, with the exception of lysine-acetylation and phosphorylation. (For an extensive review on the subject of histones and their modifications, see[378].)

Acetylation is found to affect 5-10% of the N-terminal flexible domains of the core histones. These core histones are mainly present in transcriptionally active regions of the chromatin. Acetylation is thought to neutralise the net positive charge of the basic histone proteins, and in that way it would contribute to opening up the chromatin.

Serine/threonine-phosphorylation of histones H1 and H3 is thought to counter-act acetylation thus favouring chromatin-condensation. H1 is moderately phosphorylated during S phase, but throughout G2 phosphorylation increases to reach a hyperphosphorylated state of all H1s at metaphase. Immediately upon nuclear division H1s are dephosphorylated to S-phase levels. Just before metaphase histone H3 is also phosphorylated.

Histones can be methylated irreversibly on lysine residues, a modification of which the function is not known at present[379]. Poly(ADP)ribosylation is thought to cause local chromatin decondensation and is almost exclusively found upon introduction of DNA strand breaks, and thus probably important for DNA repair[380,381].

Finally, mono-ubiquitination of the C-terminal flexible domains of histones was shown to occur principally on histones H2A and H2B. 5-15% of histones H2A in higher eukaryotes and ~2% of H2B are mono-ubiquitinated *in vivo*. Note, however, that these percentages vary enormously from cell to cell and organism to organism[382,383]. Ubiquitination is supposed to open up the chromatin, as it introduces a major structural perturbation due to the size of the 76 amino acids protein. However, no such structural changes are detected at present (by for instance DNAse I footprinting)[384,385]. During the cell cycle uH2A and uH2B are present throughout S-phase and G2-phase up to prophase. From prophase to metaphase histones are

deubiquitinated, but immediately re-ubiquitinated in anaphase. The modification is important as for instance in the E1-ts mutant cell line, ts85, it was shown that with reduced ubiquitination cells arrest close to the S/G2 boundary of the cell cycle, accompanied by a loss of uH2A. Mono-ubiquitinated histones are very stable and ubiquitin is thus apparently not involved in breakdown of these molecules. Some reports show an association of especially uH2B with active DNA sequences[387-391](and a higher affinity of uH2A for AT-rich DNA[386]), which is contradicted by others[392,393]. Thus although mono-ubiquitinated histones exist, and although they seem important, their precise function is still completely unknown. ---

As outlined above RAD6 is able to mono- and poly-ubiquitinate histones *in vitro*. However, the implication of RAD6 in the ubiquitination of histones *in vivo* remains a point of debate, as may become clear from the following arguments.

First, it seems thus far impossible to detect ubiquitinated histones in the yeast *S.cerevisiae*. This organism apparently contains very few, if any, ubiquitinated histones (less than the detection limit of 0.1% of all histones). The C-terminal amino acids of H2A (and H2B), which in man harbour the unique ubiquitin-attachment site (Lysine-119/120), are very well conserved in evolution and shown to be essential for yeast viability. Swerdlow and co-workers wanted to test whether this same lysine residue is also used for ubiquitination in yeast. Therefore, the two normal H2A histone genes were replaced by a gene copy mutated in the (for yeast putative) ubiquitination site. This caused no detectable phenotypic change in growth (solid/liquid medium, different temperatures, heat-killing, osmotic killing, use of alternative carbon sources), sporulation and 254nm UV radiation sensitivity[383]. Therefore, *S.cerevisiae* which contains mainly uncondensed/active chromatin, does either not need ubiquitination or ubiquitinates only a very small, undetectable, proportion of its histones at another site in the molecule.

Second, histones are a general target protein used for *in vitro* testing of ubiquitin-conjugating enzymes and many of these enzymes are able to add, mostly without high specificity, a single or multiple ubiquitin moieties to these basic molecules[166,254,375,394-398].

In the case of rabbit $E2_{14kD}$, the rabbit homologue of yeast RAD6, Haas *et al.* have shown that *in vitro* this protein can weakly mono-ubiquitinate histones, but reaction kinetics and constants let these authors to consider the reaction as a-specific in an *in vivo* situation[375]. No specific poly-ubiquitination of histones can be detected with the rabbit protein[375,397]. It was however shown that poly-ubiquitination of histones can be performed by the yeast protein *in vitro*, and that it is dependent on the acidic tail[350]. Without "acidic tail" yeast RAD6 can only mono-ubiquitinate histones. So the tail is important for poly-ubiquitination of histones and sporulation, but can be missed for mono-ubiquitination of histones (with questionable specificity!) and thus for DNA repair and DNA mutagenesis. However, the *Caenorhabditis elegans* (which contains also an acidic tail sequence) and the Arabidopsis RAD6 homologues are apparently completely unable to ubiquitinate histones H2A or H2B *in vitro*[399,400]. So, taken these results together, the proposition that histones are *in vivo* targets for the RAD6 or its homologues is unlikely. It is more plausible that RAD6, like many other E2's, ubiquitinates histones with low affinity and that these are not its real targets. The phenomenon of histone-poly-ubiquitination by RAD6 alone (without E3-protein), an activity

which is only displayed by the yeast protein, probably represents "an artefact" as the acidic tail could cause a higher but a-specific affinity of RAD6 for the basic histones.

RAD6 targets

What are the real targets for RAD6, especially in DNA repair and during late spermatogenesis? Histones would be ideal targets due to their requirement for DNA repair, meiosis or more broadly spermatogenesis. However, due to above-mentioned results it seems at least unlikely that histones present one of the RAD6 targets in a normal cell. Whether they are targetted in special cell types, like for instance spermatids, in the presence of a specific E3 protein (which might target RAD6 or increase its affinity) remains to be established. (Preliminary experiments studying ubiquitination in *mHR6B* knockout mice could indicate that histones in spermatids might still be a target for HHR6B (W.M. Baarends & H. Roest, pers. comm.)).

Also the interaction of RAD6 with the ssDNA-binding protein RAD18 is in favour of a function of RAD6 in close contact with the DNA[402,403]. The Prakash group provided indications that RAD18 can transport RAD6 to the DNA, where the protein then could perform its function. They also showed that the interaction site between RAD6 and RAD18 is well conserved in evolution because the interaction of yeast RAD18 is also possible with *Ss.pombe rhp6+* and human HHR6A and 6B proteins[402]. This is rather remarkable as the RAD18 protein, in contrast to RAD6, is not very well conserved in evolution[401]. Since only very short regions of amino acid sequence similarity exist, it is possible that the interaction surface between the two proteins is probably not a linear sequence but a three dimensional one.

However, although the *rad6* phenotype and these last findings still favour a function of RAD6 nearby the DNA, the only three "real" RAD6 targets identified thus far (all recognised with the help of a specific E3-protein[405]) are not strictly DNA-associated:

1) RAD6 is the E2 protein involved in degradation of N-rule targets[322,323] (see above). It interacts specifically via its very conserved N-terminus (see Chapter 4) with the yeast UBR1-encoded E3 protein[322,406]. In reticulocyte lysates the reaction is independent of the presence of its acidic tail[323]. However, *in vivo*, in yeast, the acidic tail is important for N-rule degradation[407,408]. Yeast RAD6 has apparently three ways of recognising targets: alone, tail dependent, unassisted-C-terminus independent, and E3-assisted C-terminus independent[408] [. The first mode of action is apparently lost in higher eukaryotes, as the tail is absent from these homologues (see Chapters II, III and IV)]. Thus far the only N-rule target protein for which it has been proven that it involves RAD6, is GPA1, the α subunit of a yeast G protein implicated in pheromone-dependent signal transduction[286].

2) Gcn4 is a yeast transcriptional activator of the bZIP family involved in regulation of the biosynthesis of amino acids and purines. Its normal rapid degradation, dependent upon CDC34 and RAD6, is inhibited under starvation conditions[284].

3) The p27 mammalian cell cycle protein is an inhibitor of cyclin-dependent kinases. Both *in vivo* and *in vitro*, the protein is degraded by the ubiquitin-proteasome pathway. The human ubiquitin-conjugating enzymes RAD6 and UBC3 were specifically involved in its ubiquitination[168]. It is for the moment unknown whether Gcn4 or p27 are N-rule targets.

Note that measurement of reaction kinetics on total reticulocyte proteins favour the implication of RAD6/E2-14K in E3-assisted poly-ubiquitination, although mono-ubiquitination was also observed but relatively non-specific[375]. This poly-ubiquitination, however, does not always seem to occur via the normal lysine-48 of ubiquitin (which is used by RAD6 and UBR1 in the N-rule degradation pathway)[195]. Recently, it was shown that RAD6 is also capable of forming K-6 (to histones H2B in the absence of an E3 protein) and K-63 (made by RAD6 in an E3-dependent manner) linkages, both apparently not involved in degradation[196]. The K-63 poly-ubiquitination of RAD6 is the most interesting for DNA repair. Haas and co-workers constructed a yeast in which they replaced the normal four ubiquitin genes by a ubiquitin mutant encoding an arginine instead of lysine-63 (strain UbK63R)[173], which prevents the addition of other ubiquitin moieties at that position of the molecule. The strain grows at wild type levels (see Table III) and degrades a set of short-lived N-rule proteins. However, the strain which is shown to be epistatic with a *rad6Δ* mutant is highly sensitive to DNA damaging agents and has a deficiency in DNA damage-induced mutagenesis. Also the most abundant, but rather restricted family of multiubiquitin-protein conjugates found in wild type *S.cerevisiae* cells is completely absent. So, apparently, this mutant ubiquitin is involved in the DNA mutagenesis pathway. The *rad6* phenotype is much more pleiotropic, which suggests that for sporulation, resistance to γ-rays and N-rule degradation, the RAD6 protein uses another type of lysine linkage or mono-ubiquitinates its targets. In the RAD6Δ/UbK63R double mutant a relative high UV resistance is observed when compared to the single *rad6Δ* mutant which indicates that UbK63R is a partial suppressor of RAD6. This suggests that the K-63 ubiquitination persists in the absence of *RAD6,* and that other ubiquitin conjugating proteins (making K-63 linkages) participate in other RAD6-independent repair pathways which work more efficiently in the absence of K-63.

So, in conclusion, as already indicated above, all the data suggest that ubiquitin is a very versatile signal, as different ubiquitin chain configurations can be used to perform different functions. A single ubiquitin conjugating enzyme, like for instance RAD6, is able to perform different linkages (i.e. K-6, K-48 and K-63) dependent on the type of target/process and the E3 protein involved. The type of linkage as well as the E2 and E3 protein involved, determines whether a protein can be degraded, deactivated, correctly folded, protected against degradation, stabilised, ... leading to a complex pleiotropic phenotype and implications in many different functions ...

Ubiquitin: small, but very powerful!

In the next chapters the isolation and characterisation of *RAD6*-homologous genes and proteins is described from the distantly related yeast *Schizosaccharomyces pombe*[98], the fly *Drosophila melanogaster*[99] and a duplicated locus from man[100,409,410]. (Note that the *HHR6B* gene was isolated three times independently[100,117,191,411].) In the course of this work, also RAD6 proteins from *Arabidopsis thaliana* and wheat[374,400], *Caenorhabditis elegans*[399], rat[373]

and rabbit[404] were isolated; the most important data of these articles have been included in this introduction.

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Chapter II

The rhp6^{+} gene of Schizosaccharomyces pombe: a structural and functional homologue of the RAD6 gene from the distantly related yeast Saccharomyces cerevisiae.

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The *rhp6*$^+$ gene of *Schizosaccharomyces pombe*: a structural and functional homolog of the *RAD6* gene from the distantly related yeast *Saccharomyces cerevisiae*

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The *RAD6* gene of *Saccharomyces cerevisiae* encodes a ubiquitin conjugating enyzme and is required for DNA repair, DNA-damage-induced mutagenesis and sporulation. Here, we show that *RAD6* and the *rhp6*$^+$ gene from the distantly related yeast *Schizosaccharomyces pombe* share a high degree of structural and functional homology. The predominantly acidic carboxyl-terminal 21 amino acids present in the RAD6 protein are absent in the *rhp6*$^+$-encoded protein; otherwise, the two proteins are very similar, with 77% identical residues. Like *rad6*, null mutations of the *rhp6*$^+$ gene confer a defect in DNA repair, UV mutagenesis and sporulation, and the *RAD6* and *rhp6*$^+$ genes can functionally substitute for one another. These observations suggest that functional interactions between RAD6 (rhp6$^+$) protein and other components of the DNA repair complex have been conserved among eukaryotes.

Key words: DNA repair/E2 enzyme/RAD6 gene/*rhp6*$^+$ gene/*Schizosaccharomyces pombe*

Introduction

The *RAD6* gene of *Saccharomyces cerevisiae* is involved in a variety of cellular processes. *rad6* mutants are highly sensitive to numerous DNA damaging agents, including UV, γ-rays and alkylating agents (Cox and Parry, 1968; Game and Mortimer, 1974; Prakash, 1974) and are defective in mutation induction by these agents (Prakash, 1974; Lawrence and Christensen, 1976; McKee and Lawrence, 1979). *rad6* mutants are defective in post-replication repair of UV damage: DNA strand discontinuities left during DNA replication in the newly synthesized DNA strand across from the non-coding UV lesion remain unrepaired in *rad6* mutants (Prakash, 1981). *rad6* mutants are also defective in sporulation (Game *et al.*, 1980; Montelone *et al.*, 1981), and they grow poorly and have poor plating efficiency.

The *RAD6*-encoded protein (M_r 19.7 kd) possesses a highly acidic carboxyl terminus in which 20 of the 23 residues are acidic (Reynolds *et al.*, 1985). The polyacidic sequence of RAD6 protein forms a disordered linear structure that is appended to the globular domain constituted by the first 149 residues (Morrison *et al.*, 1988). RAD6 protein is a ubiquitin-conjugating enzyme (E2) (Jentsch *et al.*, 1987) that mediates the attachment of multiple molecules of ubiquitin to histones H2A and H2B *in vitro* (Sung *et al.*, 1988). Multiple ubiquitination of histones may effect an open chromatin configuration, or it may mark histones for degradation by the ATP-dependent proteolytic system (Hershko *et al.*, 1984a,b; Hershko and Ciechanover, 1986). The acidic domain of RAD6 is required for the multiple ubiquitination of histones (Sung *et al.*, 1988). *rad6* mutants bearing a deletion of the acidic sequence fail to sporulate, but the DNA repair and UV mutagenesis functions are not affected (Morrison *et al.*, 1988). Mutation of the sole cysteine residue (Cys-88) in RAD6 to alanine or valine abrogates its E2 activity, and these mutants resemble *rad6* null mutants in being defective in DNA repair, UV mutagenesis and sporulation (Sung *et al.*, 1990), suggesting that RAD6 mediates all of its cellular functions via its role as an E2 enzyme.

Because of the central role of *RAD6* in DNA repair and in DNA-damage-induced mutagenesis, we have become interested in determining whether RAD6 is conserved among eukaryotes. A high degree of conservation of RAD6 would also suggest a parallel evolutionary conservation of proteins with which RAD6 might interact in its various cellular roles. In this paper, we report our studies with the RAD6 homolog from the evolutionarily divergent fission yeast *Schizosaccharomyces pombe*. Phylogenetic studies with 5S ribosomal RNAs indicate that *S.pombe* is evolutionarily closer to *Homo sapiens* than to *S.cerevisiae* (Huysmans *et al.*, 1983). *S.pombe* also resembles the higher eukaryotes in the control of the mitotic cell cycle (Russell and Nurse, 1986; Russell *et al.*, 1989), in the presence of introns in many of its genes and in the sequence requirements for the splicing of introns (Käufer *et al.*, 1985; Russell and Nurse, 1986). Therefore, a comparison of the structure and function of RAD6 from these two divergent yeast species should provide a good measure of evolutionary conservation of RAD6 among eukaryotes.

Our studies indicate a remarkable similarity in the amino acid sequences of the proteins encoded by the *S.cerevisiae RAD6* gene and by its homolog in *S.pombe*, *rhp6*$^+$ (rad homolog in *S.pombe*-6). The major difference between the two proteins is that the rhp6$^+$ protein lacks 21 carboxyl-terminal acidic residues present in RAD6. Like *rad6* mutations, null mutations of *rhp6*$^+$ confer a defect in DNA repair, mutagenesis and sporulation. We also show that the *RAD6* and *rhp6*$^+$ genes can functionally substitute for one another.

Results

Cloning of rhp6$^+$*, the S.pombe homolog of RAD6*

Southern blots of *S.pombe* genomic DNA were probed with the *S.cerevisiae* 0.5 kb *Eco*RI DNA fragment containing the *rad6-149* allele (Morrison *et al.*, 1988), in which the last 23 codons of the *RAD6* gene from nucleotide positions +448 to +516 (Reynolds *et al.*, 1985) are deleted. A single

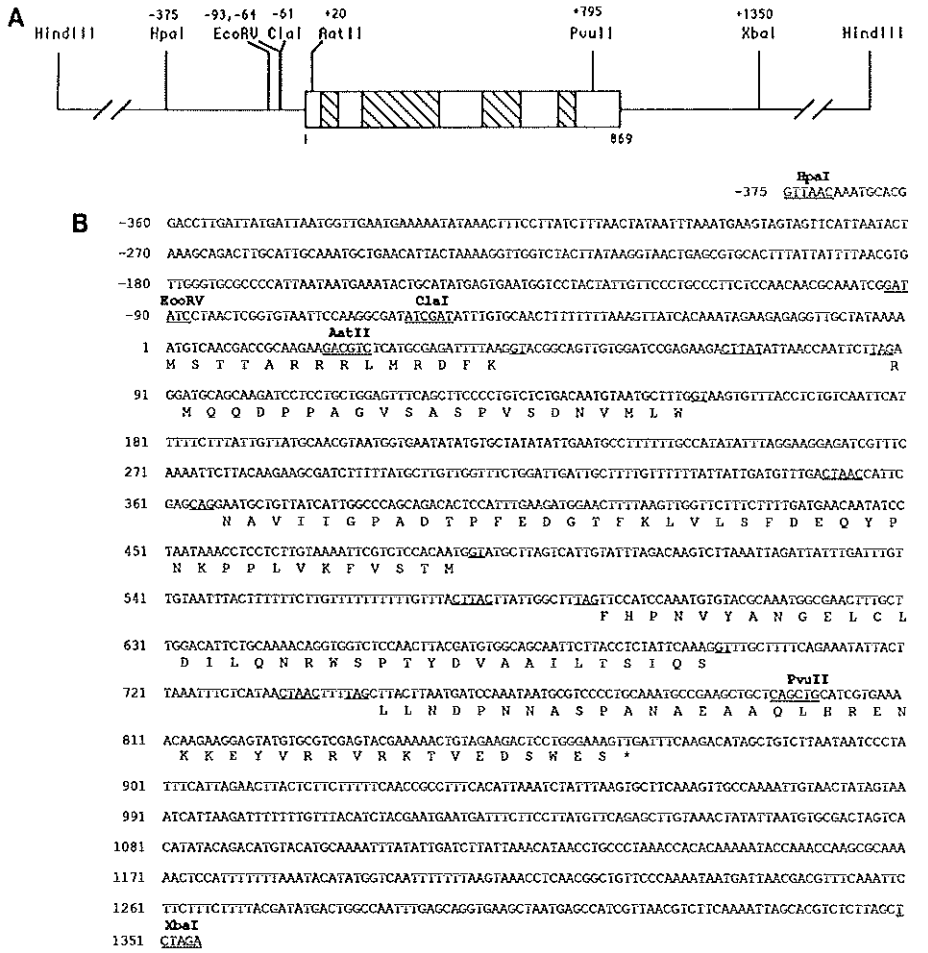

Fig. 1. Restriction map and nucleotide sequence of the *rhp6*$^+$ gene of *S.pombe*. (A) Partial restriction map of the 3.2 kb *Hin*dIII DNA segment originally isolated from the bacteriophage λ vector EMBL-3 harboring the *S.pombe* genomic sequence hybridizing to the *S.cerevisiae rad6-149* probe. Sequence analysis of the genomic region and of the cDNA synthesized by PCR predicted the exons (open boxes) and introns (hatched boxes). Numbers above the restriction sites refer to nucleotide position relative to the first ATG codon within the rhp6$^+$ ORF. (B) Nucleotide sequence of the *rhp6*$^+$ gene and amino acid sequence of its encoded protein. The first nucleotide of the first ATG codon in the rhp6$^+$ ORF is indicated at position 1. An asterisk marks the position of the termination TGA codon. Splice sequences in the introns are underlined. Restriction sites are marked by a wavy underline.

hybridizing band was detected in *Pvu*II, *Hin*dIII, *Pst*I or *Eco*RI digests of *S.pombe* genomic DNA when hybridization was carried out at 55°C in 1 M NaCl followed by two 5 min washes in 3 × SSC at 55°C and two 5 min washes in 1 × SSC at 55°C (results not shown). To isolate the *S.pombe RAD6* homolog, an *S.pombe* partial *Mbo*I genomic library was constructed in the λ vector EMBL-3 (Frischauf *et al.*, 1983) and screened with the *rad6-149* probe, using the hybridization conditions described above. Characterization of the DNA inserts in all 15 cross-hybridizing plaques obtained indicated that they originated from the same region of the *S.pombe* genome. The restriction map of the 3.2 kb *Hin*dIII fragment containing the *RAD6* homolog from *S.pombe rhp6*$^+$, is given in Figure 1(A).

Nucleotide sequence of the rhp6$^+$ *gene*

The *rhp6*$^+$ gene encodes a polyadenylated transcript of ~0.8 kb. The nucleotide sequence of *rhp6*$^+$ and its flanking regions is shown in Figure 1(B). The *rhp6*$^+$ open reading frame (ORF) is interrupted by the presence of four introns, all of which contain the consensus splicing signal sequences (Mertins and Gallwitz, 1987; Gatermann *et al.*, 1989). The 5′ splice site 5′-GTANGN-3′ is present in all the introns except for intron 4, which contains a T instead of an A at the third position. The branch sites have the conserved sequence 5′-CTPuAPy-3′, and this sequence is present 3−13 nt from the 3′ splice site PyAG. Nucleotide sequence analysis of *rhp6*$^+$ cDNA obtained by the polymerase chain reaction (PCR) (see Materials and

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MSTPARRRLMKDFKRMKEDAPFGVSASPLPDNVMVMNAMIIGPADIPYED 50  RAD6
MSTTARRRLMKDFKRMQQDPPAGVSASPVSDNVMLMNAVIIGPADIPFED 50  rhp6+

GIFRLLLEFDEEYPNKPPHVKFLSEMFHPNVYANGEICLDILQNRWTPTY 100 RAD6
GIFKLVLSFDEQYPNKPPLVRFVSTMFHPNVYANGELCLDILQNKWSPTY 100 rhp6+

DVASILTSIQSLFNDPNPASPANVEAATLFKDHFSQYVKRVKETVEKSWE 150 RAD6
DVAAILTSIQSLLNDPNNASPANAEAAQLHRENKEYVKRVRKIVEDSWE 149  rhp6+

DDMDDMDDDDDDDDDDDDDEAD                              172 RAD6

S                                                   151 rhp6+
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Fig. 2. Homology between the *S.cerevisiae* RAD6 and the *S.pombe* rhp6$^+$ proteins. The 172 amino acid long RAD6 protein is aligned with the 151 amino acid long rhp6$^+$ protein. The alignment of the two sequences is continuous throughout with the exception of the absence of the acidic carboxyl terminus in the rhp6$^+$ protein. Vertical lines between amino acids indicate identical residues and asterisks between amino acids indicate similar residues based on Dayhoff (1978).

methods) confirmed that all the introns are excised at these splice sites. The *rhp6*$^+$ ORF encodes a protein of 151 amino acids with an M_r of 17 097, containing 11.9% acidic and 12.6% basic residues. The predicted mol. wt of rhp6$^+$ protein is in good agreement with the 17 kd size estimated by SDS−PAGE.

Homology between rhp6$^+$ and RAD6 encoded proteins

The alignment of the amino acid sequences of the *S.cerevisiae RAD6* and *S.pombe rhp6*$^+$ encoded proteins is shown in Figure 2. RAD6 protein is 172 amino acids long and 20 of its 23 carboxyl-terminal residues are acidic, whereas the *rhp6*$^+$ encoded protein contains 151 amino acids, and lacks the polyacidic carboxyl-terminal sequence. The first 151 residues of RAD6 share a high degree of homology with the rhp6$^+$ protein sequence. The alignment of the two sequences is continuous throughout, and 77% of the residues in these two sequences are identical. Although conservation of residues extends throughout the RAD6 and rhp6$^+$ proteins, the similarity between them is greater in the first 127 residues, which show 82% identity, than in the terminal 24 residues, with 50% identity. When conservative amino acid replacements are grouped according to Dayhoff (1978), the similarity between rhp6$^+$ and RAD6 proteins becomes 90%. Both proteins contain a single cysteine residue at position 88. Mutational studies with cysteine 88 in RAD6 indicate that this residue is essential for the E2 activity (Sung *et al.*, 1990).

rhp6$^+$ function is required in DNA repair, UV mutagenesis and sporulation in S.pombe

The high degree of homology between the *rhp6*$^+$ and *RAD6* encoded proteins strongly suggested that they are functionally similar. To determine this, we constructed a null mutation of *rhp6*$^+$ (*rph6Δ*) in which the genomic *rhp6*$^+$ sequence from the *Eco*RV site at position −93 to the *Pvu*II site at position +795 (Figure 1B) was deleted and replaced by the *ura4*$^+$ gene of *S.pombe*. We examined the effect of the *rhp6Δ* mutation on growth, sensitivity to DNA-damaging agents, UV mutagenesis and sporulation. We found that *rhp6Δ* mutants have a much slower growth rate than the *rhp6*$^+$ wild-type strains (Figure 3), and *rhp6Δ* strains accumulate longer cells. The *rhp6Δ* mutants are sensitive to UV light (Figure 4) and to γ-rays and to the alkylating agent methyl methanesulfonate (results not shown). The

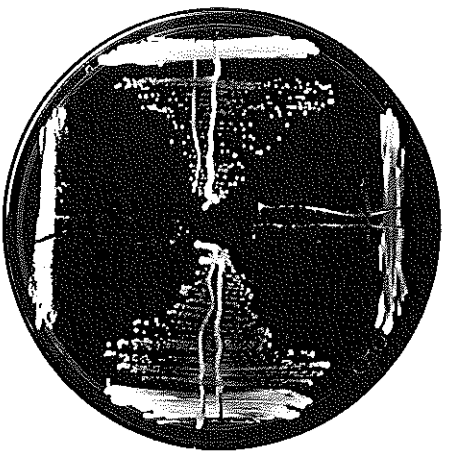

Fig. 3. Deletion mutation of the *rhp6*$^+$ gene of *S.pombe* causes slow growth of cells. Cells were streaked onto minimal medium and plates were incubated at 30°C for 3 days. **Top:** *rhp6*$^+$ haploid; **left:** *rhp6Δ* haploid; **bottom:** *rhp6*$^+$/*rhp6*$^+$ diploid; **right:** *rhp6Δ*/*rhp6Δ* diploid.

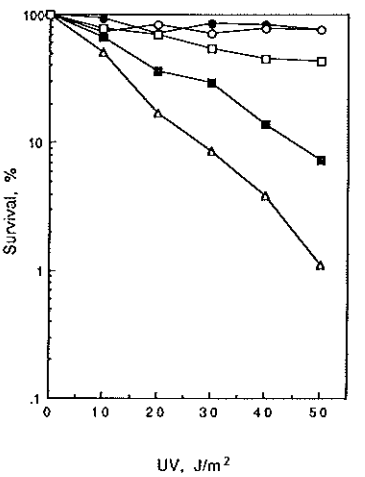

Fig. 4. Survival after UV irradiation of the *S.pombe rhp6Δ* haploid strain PRZ61 carrying various plasmids. Strains were grown on media for maintaining selection for the plasmid. Strains PRZ55 and PRZ61 are isogenic (Table III). Symbols: ●, PRZ55 (*rhp6*$^+$); △, PRZ61 (*rhp6Δ*); ○, PRZ61 + plasmid pRR413 (*rhp6*$^+$); ■, PRZ61 + plasmid pRR415 (*RAD6*); □, PRZ61 + plasmid pRR417 (*rad6-149*).

rph6Δ mutant is not as UV sensitive (Figure 4) as the *S.cerevisiae rad6Δ* mutant (Figure 6); however, the UV sensitivity of *rph6Δ* mutants is equal to that of the most UV-sensitive mutants of *S.pombe* (Schüpbach, 1971; Nasim and Smith, 1975). The lower sensitivity of the *rhp6Δ* strain to UV light than that of *rad6Δ* may reflect the fact that *S.pombe* is more radioresistant than *S.cerevisiae* (Phipps *et al.*, 1985).

To determine the effect of *rhp6Δ* mutation on UV mutagenesis, we examined the frequency of forward

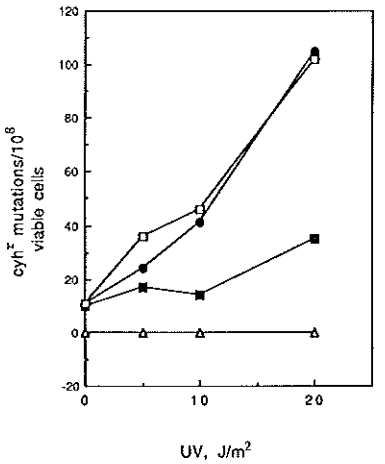

Fig. 5. UV-induced forward mutation to *cyh*^{r}. *rhp6*Δ strains carrying various plasmids were irradiated with UV light and the frequency of *cyh*^{r} mutants determined. Symbols: ●, PRZ55 (*rhp6*$^+$); △, PRZ61 (*rhp6*Δ); ■, PRZ61 + plasmid pRR415 (*RAD6*); □, PRZ61 + plasmid pRR417 (*rad6-149*).

Table I. Sporulation of the *S.pombe rhp6*Δ/*rhp6*Δ strain in the presence of the *RAD6* or *rad6-149* gene

Strain	Genotype	% sporulation[a]
ZD6	*rhp6*$^+$/*rhp6*$^+$	57
ZD14	*rhp6*$^+$/*rhp6*Δ	54
ZD16	*rhp6*Δ/*rhp6*+	70
ZD18	*rhp6*Δ/*rhp6*Δ	0
ZD18 (pRR413)	*rhp6*Δ/*rhp6*Δ + *rhp6*$^+$	47
ZD18 (pRR415)	*rhp6*Δ/*rhp6*Δ + *RAD6*	29
ZD18 (pRR417)	*rhp6*Δ/*rhp6*Δ + *rad6-149*	43

[a]Based on a count of >500 cells for each strain.

mutations to cycloheximide resistance (*cyh*^{r}) in *rhp6*$^+$ and *rhp6*Δ strains. *cyh*^{r} mutations were induced by UV light in the *rhp6*$^+$ strain: at 10 J/m^2, the frequency of *cyh*^{r} mutants was 40/10^8 viable cells; and at 20 J/m^2, this frequency increased to >100/10^8 viable cells. In contrast, no UV-induced *cyh*^{r} mutants were observed in the *rhp6*Δ mutant strain (Figure 5).

To determine the role of *rhp6*$^+$ in sporulation, we examined sporulation in isogenic diploid strains *rhp6*$^+$/*rhp6*$^+$, *rhp6*Δ/*rhp6*$^+$ and *rhp6*Δ/*rhp6*Δ (Table I). Sporulation occurred at a frequency of 50–70% in *rhp6*$^+$ homozygous and heterozygous strains, whereas we observed no sporulation in *rhp6*Δ/*rhp6*Δ diploids. Thus, like the *rhp6*Δ mutation of *S.cerevisiae*, the *rhp6*Δ mutation of *S.pombe* results in defective DNA repair, UV mutagenesis and sporulation.

rad6-149 complements the rhp6Δ mutation of S.pombe more efficiently than the complete RAD6 gene

Next, we examined whether the *RAD6* gene of *S.cerevisiae* can functionally substitute for the *rhp6*$^+$ gene in *S.pombe*. Since the *rhp6*$^+$-encoded protein is devoid of the acidic carboxyl terminus, we also examined whether the rad6-149 protein lacking the carboxyl-terminal 23 predominantly acidic residues differs from the complete RAD6 protein in its capacity to complement the *rhp6*Δ mutation. To ensure adequate expression of the *RAD6* and *rad6-149* genes in *S.pombe*, these genes were placed downstream of the *rhp6*$^+$ promoter in the *S.pombe* vector pRR399 (see Materials and methods). As a control, the *rhp6*$^+$ gene was also cloned into this *S.pombe* vector. Western blots of total cellular protein from an *S.pombe rhp6*Δ strain transformed with these three plasmids—pRR413, pRR415 and pRR417—were probed with anti-RAD6 antibody. The RAD6, rad6-149 and rhp6$^+$ proteins were all present at about equal levels, and the amount of these proteins was somewhat higher than the amount of rhp6$^+$ protein present in the wild-type *S.pombe* strain (results not shown).

We examined the response to UV irradiation of an *rhp6*Δ strain carrying the *RAD6* or *rad6-149* gene (Figure 4). As expected, the *rhp6*$^+$ gene in plasmid pRR413 fully complements the UV sensitivity of the *rhp6*Δ mutation. The *rhp6*Δ mutant carrying the complete *S.cerevisiae RAD6* gene on plasmid pRR415 shows UV sensitivity that is intermediate between *rhp6*Δ and *rhp6*$^+$ strains, whereas a much higher level of UV resistance occurred with the *rad6-149* gene on plasmid pRR417.

Complementation for the UV mutagenesis defect of *rhp6*Δ by *RAD6* and *rad6-149* was tested by measuring the forward mutation frequency to *cyh*^{r} (Figure 5). The *rad6-149* gene restored wild-type levels of UV mutagenesis to the *rhp6*Δ strain, whereas the level of UV mutagenesis with the complete *RAD6* gene was intermediate between that of the wild-type *rhp6*$^+$ and *rhp6*Δ mutant strains.

The *rad6-149* gene restored nearly wild-type levels of sporulation in the *rhp6*Δ/*rhp6*Δ diploid, whereas sporulation was somewhat less efficient with the complete *RAD6* gene (Table I). The growth and morphology defects associated with the *rhp6*Δ mutation were also complemented to near wild-type levels by the *rad6-149* gene, and to a lesser extent, by the complete *RAD6* gene (results not shown). Thus, our observations clearly show that the *rad6-149* gene can carry out all of the functions of *rhp6*$^+$ in *S.pombe*. The lower efficiency with which the complete *RAD6* gene functionally substitutes for the *rhp6*$^+$ gene suggests that the polyacidic carboxyl-terminal region present in RAD6 interferes with its functioning properly in *S.pombe*.

The rhp6$^+$ *gene complements the rad6Δ mutation of S.cerevisiae*

We also examined whether the *rhp6*$^+$ gene complements the DNA repair, UV mutagenesis and sporulation defects of the *rad6*Δ strain of *S.cerevisiae*. Since *S.pombe* introns are spliced inefficiently in *S.cerevisiae* (Beach *et al.*, 1982; Booher and Beach, 1986), we cloned the *rhp6*$^+$ cDNA into *S.cerevisiae* low copy *CEN* and multicopy 2μ plasmid vectors (see Materials and methods). We also cloned the *rhp6*$^+$ cDNA downstream of the highly expressed *S.cerevisiae* alcohol dehydrogenase I (*ADC1*) promoter. These plasmids were introduced into the *S.cerevisiae rad6*Δ strain, and the level of the rhp6$^+$ protein examined by Western analysis using the anti-RAD6 antibodies. The level of rhp6$^+$ protein in the *rad6*Δ *S.cerevisiae* strain carrying the *rhp6*$^+$ gene on the *CEN* plasmid pRR425 was about the same as the level of RAD6 protein present in the wild-type *S.cerevisiae* strain. The rhp6$^+$ protein level increased ~10-fold in *rad6*Δ cells

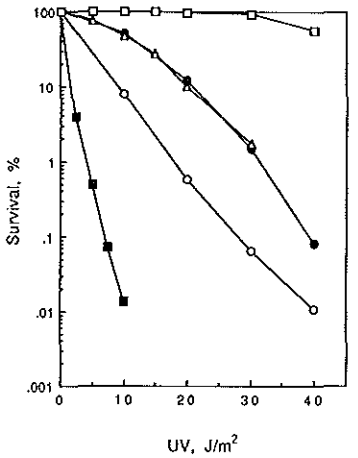

Fig. 6. Survival after UV irradiation of the *S.cerevisiae rad6*Δ haploid strain EMY7 carrying various plasmids. Strains were grown on media for maintaining selection for the plasmid. Symbols: ■, EMY7 + plasmid pTB236 (2μ vector); ○, EMY7 + plasmid pRR425 (*CEN rhp6*$^+$); ●, EMY7 + plasmid pRR428 (2μ *rhp6*$^+$); △, EMY7 + plasmid pRR429 (*ADC1::rhp6*$^+$); □, EMY7 + plasmid pR67 (*CEN RAD6*).

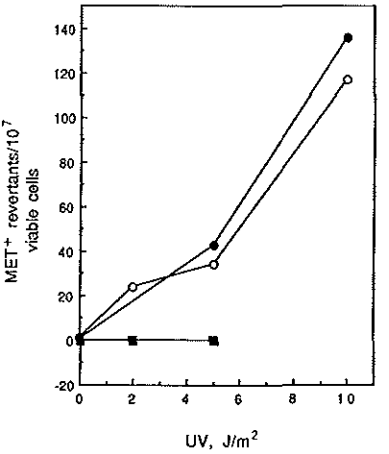

Fig. 7. UV-induced reversion of *met14* in the *rad6*Δ strain EMY7 carrying various plasmids. Cells were irradiated with UV light and the frequency of *MET*$^+$ revertants determined. Symbols: ■, EMY7 (*rhp6*Δ); ○, EMY7 + plasmid pRR425 (*CEN rhp6*$^+$); ●, EMY7 + plasmid pR67 (*CEN RAD6*).

carrying the *rhp6*$^+$ gene on a 2μ multicopy plasmid pRR428, and a further ~10-fold increase occurred with plasmid pRR429 in which the *rhp6*$^+$ gene is fused on the *ADC1* promoter (results not shown).

The *rad6*Δ mutant is highly sensitive to UV light: at 10 J/m^2, survival is reduced 10^{-4}-fold (Figure 6). Transformation of the *rad6*Δ strain with the low copy *CEN rhp6*$^+$ plasmid pRR425 greatly enhanced the UV resistance of the *rad6*Δ strain, such that at 10 J/m^2, UV survival increased 10^3-fold to 10% (Figure 6). The *rad6*Δ strain carrying the multicopy 2μ *rhp6*$^+$ plasmid pRR428 showed a further increase in UV resistance; however, the UV resistance of these cells was still below the wild-type level. Additional overproduction of *rhp6*$^+$ protein by the *ADC1::rhp6*$^+$ plasmid pRR429 did not raise the UV resistance of *rad6*Δ cells further.

To examine whether the *rhp6*$^+$ gene can perform the UV mutagenesis function of *RAD6* in *S.cerevisiae*, we examined the reversion of a *met14* mutation in a *rad6*Δ strain carrying the *CEN rhp6*$^+$ plasmid pRR425. As shown in Figure 7, the *CEN rhp6*$^+$ plasmid restored UV mutability to the *rad6*Δ strain to the same extent as does the *CEN RAD6* plasmid pR67. Similar results were obtained with the 2μ *rhp6*$^+$ plasmid pRR428 and the *ADC1::rhp6*$^+$ plasmid pRR429 (results not shown).

Although the *rhp6*$^+$ gene of *S.pombe* restored the UV resistance and UV mutability to the *rad6*Δ strain, the sporulation defect of the *rad6*Δ/*rad6*Δ strain was not complemented by the *rhp6*$^+$ gene carried on the low copy *CEN* plasmid pRR425 (Table II). Little sporulation occurred with the *rhp6*$^+$ gene on the multicopy 2μ plasmid pRR428, but the introduction of the *ADC1::rhp6*$^+$ plasmid pRR429 in the *rad6*Δ/*rad6*Δ strain increased sporulation to 7% (Table II). Even though *rad6-149* mutants of *S.cerevisiae* are defective in sporulation (Morrison *et al.*, 1988), overproduction of the rad6-149 protein from the *ADC1* promoter also conferred a low level of sporulation ability to the *rad6*Δ/*rad6*Δ strain (Table II).

Table II. Sporulation of *S.cerevisiae rad6*Δ/*rad6*Δ diploids carrying the *rhp6*$^+$ gene on different plasmids

Plasmids		Vector	Gene	% sporulation[a]
S.cerevisiae	*S.pombe*			
pR611		CEN	*rad6*Δ	0
pR67		CEN	*RAD6*	34
	pRR425	CEN	*rhp6*$^+$	0
	pRR428	2μ	*rhp6*$^+$	1
	pRR429	ADC	*rhp6*$^+$	7
pR619		ADC	*rad6-149*	12

All CEN and 2μ plasmids are in *S.cerevisiae* strain EMY26, whereas the ADC plasmids are in strain EMY28.
[a]Based on a count of >500 cells for each strain.

Discussion

We have cloned the *rhp6*$^+$ gene of *S.pombe* and show that it bears strong structural and functional homology to the *RAD6* gene from the distantly related yeast *S.cerevisiae*. The rhp6$^+$ protein differs from RAD6 in not possessing the last 21 residues, of which 18 are acidic. The two proteins are highly homologous, sharing 77% identical residues and 90% similar residues when conservative replacements are grouped together. The conservation of RAD6 suggests that the other components of the ubiquitin conjugation pathway, such as the ubiquitin-activating enzyme E1, which transfers ubiquitin to a cysteine residue in the E2 enzymes, and the other E2 enzymes, are likely to be conserved among eukaryotes.

The biological functions of the *rhp6*$^+$ gene product in *S.pombe* are identical to those of *RAD6* in *S.cerevisiae*. Strains carrying null mutations of both genes are defective in DNA repair, UV mutagenesis and in sporulation. In addition, both mutations affect growth rate and plating

Table IIIA. Strains used

Strain	Genotype	Source
S.pombe strains		
PRZ55	*h⁻ leu1-32 ura4.D18 lys1-131*	this study
PRZ61	*h⁻ leu1-32 ura4.D18 lys1-131 rhp6Δ::ura4⁺*	this study
PRZ107	*h⁺ leu1-32 ura4.D18 lys1-131 ade6-210*	this study
PRZ109	*h⁻ leu1-32 ura4.D18 lys1-131 ade6-216*	this study
PRZ119	*h⁺ leu1-32 ura4.D18 lys1-131 ade6-210 rhp6Δ::ura4⁺*	this study
PRZ121	*h⁻ leu1-32 ura4.D18 lys1-131 ade6-216 rhp6Δ::ura4⁺*	this study
ZD6	*rhp6⁺/rhp6⁺* (PRZ107 × PRZ109)	this study
ZD14	*rhp6⁺/rhp6Δ* (PRZ107 × PRZ121)	this study
ZD16	*rhp6Δ/rhp6⁺* (PRZ109 × PRZ119)	this study
ZD18	*rhp6Δ/rhp6Δ* (PRZ119 × PRZ121)	this study
S.cerevisiae strains		
EMY1	*MATα leu2-3 leu2-112 trp1Δ ura3-52 rad6Δ::LEU2⁺*	Morrison *et al.* (1988)
EMY7[a]	*MATα ade5 his7 leu2-3 lys1 met14 pet5 ura3 rad6Δ::LEU2⁺*	Morrison *et al.* (1988)
EMY8	*MATα ade5 his7 leu2-3 lys1 met14 pet5 ura3 trp1Δ::URA3⁺ rad6Δ::LEU2⁺*	this study
EMY26	*rad6Δ/rad6Δ* (EMY1 × EMY7)	
EMY28	*rad6Δ/rad6Δ* (EMY1 × EMY8)	

[a]EMY7 is isogenic with EMY8. They differ only in that EMY8 was made *trp1Δ* by replacing the *TRP1* gene with the *URA3* gene, thus making EMY8 Ura⁺.

efficiency adversely. We find that the *rhp6⁺* and *RAD6* genes can functionally substitute for one another. In the presence of the *rhp6⁺* gene on a low copy plasmid, the UV resistance of the *S.cerevisiae rad6Δ* strain is greatly enhanced and UV mutagenesis occurs at wild type rates. The *rhp6⁺* gene did not complement the sporulation defect of the *rad6Δ/rad6Δ* strain, unless the *rhp6⁺* gene product was overproduced in high amounts from the *ADC1* promoter. The *rad6-149* allele, which resembles *rhp6⁺* in the absence of the polyacidic carboxyl terminus, also does not support sporulation (Morrison *et al.*, 1988) except when rad6-149 protein is overproduced from the *ADC1* promoter (Table II). The *RAD6* and *rad6-149* genes of *S.cerevisiae* differ in their ability to function in *S.pombe*. Interestingly, the complete *RAD6* gene functions less efficiently in *S.pombe* than the *rad6-149* gene. In the *rhp6Δ S. pombe* strain carrying the *rad6-149* gene, UV resistance increases to near wild-type levels, and UV mutagensis and sporulation occur at normal frequencies, whereas the complete *RAD6* gene provides a lower level of complementation of all these defects, indicating that the polyacidic carboxyl terminus of RAD6 inhibits its proper functioning in *S.pombe*. Thus, it appears that the *S.cerevisiae* DNA repair proteins have evolved to adapt the RAD6 polyacidic sequence.

The acidic carboxyl terminus of RAD6 is required for sporulation in *S.cerevisiae* (Morrison *et al.*, 1988) and for efficient polyubiquitination of histones *in vitro* (Sung *et al.*, 1988). Our observation that the rhp6⁺ protein lacking the polyacidic carboxyl terminus is essential for sporulation in *S.pombe* raises the possibility that the rhp6⁺ and RAD6 proteins ubiquitinate non-histone protein substrates in sporulation. The absence of the polyacidic sequence from the rhp6⁺ protein may mean that either polyubiquitination of histones is not as necessary for sporulation in *S.pombe* as in *S.cerevisiae*, or there is an alternate E2 in *S.pombe* that mediates histone polyubiquitination during sporulation.

The high degree of structural and functional homology between the *RAD6* and *rhp6⁺* genes lends credence to the idea that the other proteins with which RAD6 and rhp6⁺ proteins interact in mediating their different cellular roles have also been conserved during evolution. The various proteins involved in DNA repair and mutagenesis in *S.cerevisiae* with which RAD6 may interact could include the proteins encoded by genes in the *RAD6* epistasis group, such as RAD18, REV1, REV2 and REV3. The *RAD18*-encoded protein contains three putative DNA binding zinc finger domains and a Walker type A sequence for the binding and hydrolysis of purine nucleotide(s) (Jones *et al.*, 1988). Both *rad6* and *rad18* mutants are highly defective in post-replication repair of UV-damaged DNA (Prakash, 1981). Since the RAD6 protein by itself does not bind DNA (P.Sung, unpublished observations), presumably RAD6 is brought to the site of DNA damage via its interaction with other proteins that bind the damage sites in DNA. The RAD18 protein could be the damage recognition factor and the interacton of RAD6 with RAD18 could target RAD6 to the sites of DNA lesions, where it may facilitate repair via ubiquitination of chromosomal proteins. The *REV* genes are required for UV mutagenesis (Lemontt, 1971) and *REV3* encodes a protein that shows homology to DNA polymerases

Table IIIB. Plasmids used in this study

Plasmids	Gene; vector
pRR399	*S.cerevisiae LEU2⁺; S.pombe ars1* vector
pRR413	*rhp6⁺* in *S.pombe ars1* vector
pRR415	*rhp6⁺ promoter::RAD6* in *S.pombe ars1* vector
pRR417	*rhp6⁺ promoter::rad6-149* in *S.pombe ars1* vector
pRR425	*RAD6 promoter::rhp6⁺* in *S.cerevisiae CEN* vector
pRR428	*RAD6 promoter::rhp6⁺* in *S.cerevisiae 2μ* vector
pRR429	*ADC1 promoter::rhp6⁺* in *S.cerevisiae 2μ* vector
pR67	*RAD6* gene in *S.cerevisiae* CEN vector
pR611	*rad6Δ* gene in *S.cerevisiae CEN* vector
pR619	*ADC1 promoter::rad6-149* gene in *S.cerevisiae 2μ* vector

(Morrison *et al.*, 1989). RAD6 could also be an integral part of the error-prone repair complex. Our finding of strong conservation between the *RAD6* and *rhp6*$^+$ genes suggests that other components of RAD6/rhp6$^+$-dependent DNA repair and mutagenesis machinery have also been conserved among eukaryotes.

Materials and methods

Yeast strains and media

S.pombe strains, originally obtained from A.Klar, A.Nasim and V.Simanis, were used to generate the strains listed in Table III(A). *S.cerevisiae* strains used in this study are also listed in Table III(A). Growth, minimal and sporulation media for *S.cerevisiae* were prepared as described previously (Sherman *et al.*, 1986), and media for *S.pombe* were prepared as described by Gutz *et al.* (1974) and Nurse (1975). *S.pombe* strain CBS356 (Yeast Stock Center, Delft, The Netherlands) was used for preparing the genomic DNA library.

Genetic analyses

Standard genetic techniques for *S.pombe* (Gutz *et al.*, 1974) and for *S.cerevisiae* (Sherman *et al.*, 1986) were used.

Transformation and other procedures

Yeast transformations were performed according to the method of Ito *et al.* (1983). *E.coli* transformations and DNA treatment were carried out by previously published methods (Maniatis *et al.*, 1982; Frischauf *et al.*, 1983).

Survival after UV irradiation and induction of mutations by UV light were as described previously (Morrison *et al.*, 1988).

Isolation of total RNA and poly(A) RNA from *S.pombe* and Northern hybridizations were as described by Madura and Prakash (1986). Polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970). Preparation of anti-RAD6 antibody and Western blotting were as described by Morrison *et al.* (1988).

The nucleotide sequence of the *rhp6*$^+$ gene was determined by the deoxy chain termination method of Sanger *et al.* (1977) using ([α-^{35}S]-thio)triphosphate (Biggin *et al.*, 1983). DNA fragments obtained by a variety of restriction enzymes recognizing six-base and four-base sequences were cloned into M13 derivative phages.

Construction of S.pombe plasmids and generation of a genomic rhp6$^+$ *deletion mutation in S.pombe*

To facilitate genetic manipulations with the *rhp6*$^+$ gene, the 3.2 kb *Hin*dIII DNA fragment containing the *S.pombe rhp6*$^+$ gene (Figure 1A) was cloned into pUC18 in which the *Aat*II site had been deleted and the 322 bp *Pvu*II fragment spanning the polylinker had been replaced by a *Hin*dIII site for cloning the 3.2 kb *Hin*dIII fragment, generating the plasmid pRR404.

Plasmid pRR394 contains the *rhp6*$^+$ gene on the 3.2 kb *Hin*dIII DNA fragment (Figure 1A), in which the blunt-ended 1.8 kb *Hin*dIII *ura4*$^+$ fragment (Grimm *et al.*, 1988) has replaced the *rhp6*$^+$ gene from the *Eco*RV site at −93 to the *Pvu*II site at +795 (Figure 1B). The resulting 4.1 kb *Hin*dIII fragment from pRR394 was used to transform *ura4.D18* *S.pombe* strains to Ura$^+$. The slow growing transformants were examined by Southern blotting of genomic DNA and shown to carry the *rhp6*Δ mutation (results not shown). The frequency of genomic *rhp6*Δ mutations among Ura$^+$ transformants was ~2%.

Isolation of rhp6$^+$ *cDNA and cloning into S.cerevisiae vectors*

Plasmid pRR404 was gapped at the unique *Aat*II and *Pvu*II sites in the first and last exons of *rhp6*$^+$ (Figure 1). The gap was filled by a 362 nt *Aat*II−*Pvu*II fragment containing *rhp6*$^+$ cDNA prepared by PCR (Saiki *et al.*, 1985), using the protocol described by Rotenberg *et al.* (1989). The two oligonucleotide primers employed for PCR were 89.023: 5′-TTTCACGATGCAGCTGAGCA-3′, which hybridizes to *rhp6*$^+$ mRNA and spans the *Pvu*II site in the last exon of the *rhp6*$^+$ gene; and 89.024: 5′-ACCGCAAGAAGACGTCTCAT-3′, which hybridizes to the DNA strand coding for *rhp6*$^+$ mRNA and spans the *Aat*II site. The *Pvu*II site and the *Aat*II sites are indicated in bold letters in 89.023 and 89.024 respectively. The 362 nt reaction product was purified from an agarose gel and subjected to a second round of PCR. This amplified fragment was digested with *Aat*II and *Pvu*II and cloned into gapped plasmid pRR404, generating plasmid pRR405. The cDNA sequence of *rhp6*$^+$ in plasmid pRR405 was confirmed by dideoxy sequencing using oligonucleotides 89.023 and 89.024 as primers.

The *rhp6*$^+$ cDNA was cloned downstream of the *RAD6* promoter in various *S.cerevisiae* vectors by using the following strategy. *Eco*RI linkers were inserted at the filled in *Cla*I site 61 nt upstream of the first ATG codon in the rhp6$^+$ open reading frame (ORF) and at the filled in *Xba*I site 481 nt downstream of the TGA termination codon of rhp6$^+$ (Figure 1B) The resulting 1 kb *Eco*RI fragment containing the entire *rhp6*$^+$ ORF was cloned downstream of the *RAD6* promoter in the *CEN* plasmid pR611 and the 2μ multicopy plasmid pTB236 (Morrison *et al.*, 1988), generating plasmids pRR425 and pRR428 respectively. To obtain higher levels of expression of *rhp6*$^+$, the *rhp6*$^+$ ORF was cloned downstream of the *S.cerevisiae* alcohol dehydrogenase promoter I (*ADC1*) in plasmid pSCW231 (Sung *et al.*, 1987), generating plasmid pRR429.

Cloning of RAD6 and rad6-149 into plasmids for propagation in S.pombe

A new plasmid vector, designated pRR399, was constructed for propagation in *S.pombe*. A 1.1 kb *Eco*RI fragment containing the *S.pombe arsl* sequence (Losson and Lacroute, 1983; Heyer *et al.*, 1986) was inserted into YIplac128, an *S.cerevisiae* integrating vector carrying the *LEU2* gene (Gietz and Sugino, 1988), to generate pRR399. Prior to cloning into pRR399, the *RAD6* and *rad6-149* genes were placed under the control of the *rhp6*$^+$ promoter by first cloning each of them into pRR381. pRR381 was constructed by cutting pRR404 with *Cla*I at position −61 (Figure 1), filling in the *Cla*I site, then digesting with *Pvu*II, and attaching *Eco*RI linkers; this creates a gap deleting 85% of the *rhp6*$^+$ ORF. The *RAD6* gene on the 0.61 kb *Eco*RI fragment from positions −48 to +565, which includes the entire RAD6 ORF along with 49 5′ flanking nucleotides and 66 3′ flanking nucleotides (Reynolds *et al.*, 1985), and the *rad6-149* gene on the 0.57 kb *Eco*RI fragment (Morrison *et al.*, 1988) were then each inserted into the *Eco*RI site of pRR381, generating plasmids pRR409 and pRR411 respectively. The *rhp6*$^+$ promoter::*RAD6* and *rhp6*$^+$ promoter::*rad6-149* genes from plasmids pRR409 and pRR411 were cloned into pRR399 as 3 kb *Hin*dIII fragments, generating plasmids pRR415 and pRR417 respectively.

A summary of plasmids used in this study is given in Table III(B).

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Chapter III

Dhr6, a Drosophila homologue of the yeast DNA repair gene RAD6

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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-

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).

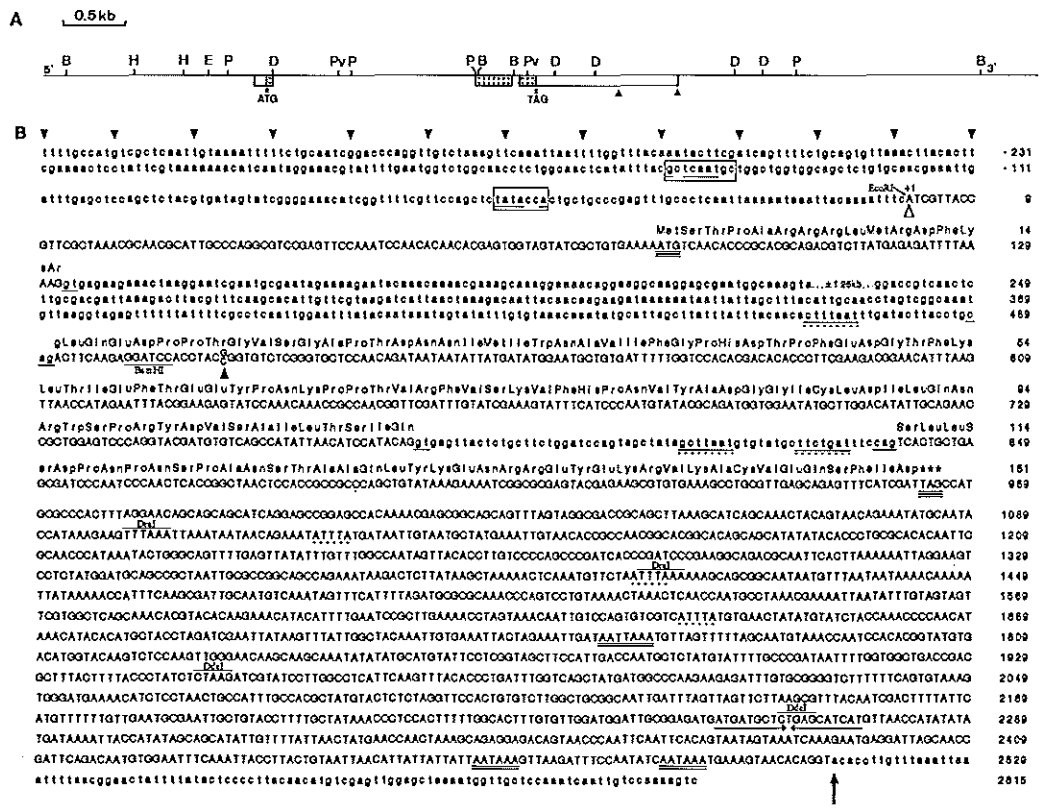

FIG. 1. Genomic organization, nucleotide sequence, and transcripts of the *Drosophila Dhr6* gene. (*A*) Partial restriction map of the three *Bam*HI fragments harboring the entire *D. melanogaster Dhr6* genomic sequence. Exons are indicated as boxes; parts with dots indicate coding region of the mRNA 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).

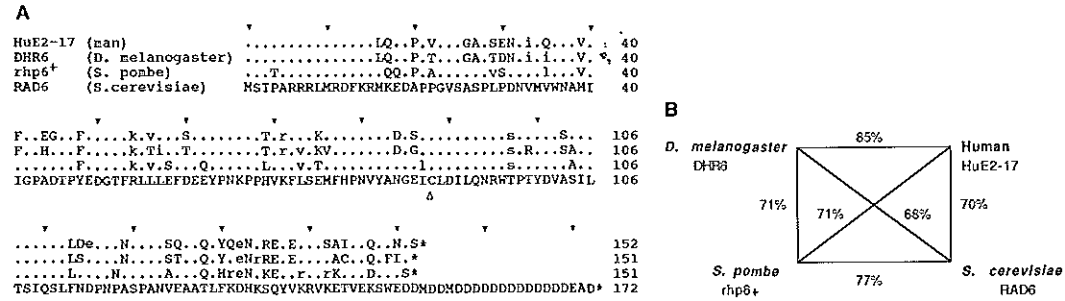

FIG. 2. (*A*) Comparison of amino acid sequences of RAD6 homologs from *Sa. cerevisiae*, *Sc. pombe*, *D. melanogaster*, and human. The 172-amino acid-long RAD6 protein is aligned with the 151 amino acids of Dhr6 and rhp6$^+$ proteins and the 152 amino acids of the human homolog. The position of Cys-88, 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.

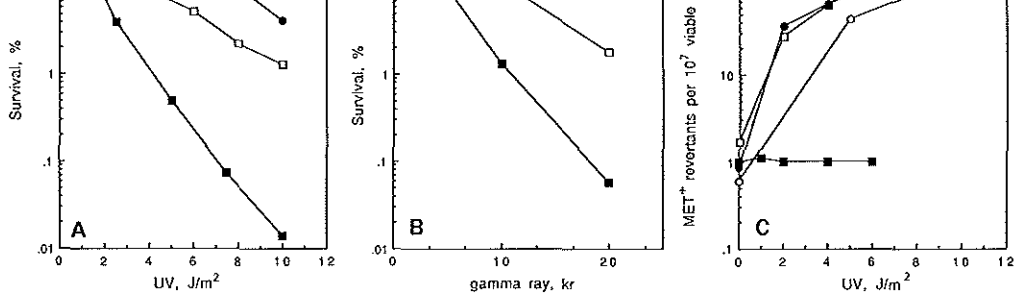

FIG. 3. Complementation of the radiation sensitivity and UV immutability of the *Sa. cerevisiae rad6*Δ mutation by the *D. melanogaster Dhr6* gene. Survival after UV (*A*) or γ-ray irradiation (*B*), and UV-induced reversion of *met14* (*C*) in *Sa. cerevisiae rad6*Δ strains carrying the *Dhr6* gene 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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Chapter IV

Structural and functional conservation of two human homologues of the yeast DNA repair gene RAD6

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Biochemistry

Structural and functional conservation of two human homologs 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 *Saccharomyces cerevisiae* encodes a ubiquitin-conjugating enzyme (E2) that is required for DNA repair, damage-induced mutagenesis, and sporulation. We have cloned the two human *RAD6* homologs, designated *HHR6A* and *HHR6B*. The two 152-amino acid human proteins share 95% sequence identity with each other and ≈70% and ≈85% overall identity with the homologs from yeasts (*S. cerevisiae* and *Schizosaccharomyces pombe*) and *Drosophila melanogaster*, respectively. Neither of the human RAD6 homologs possesses the acidic C-terminal sequence present in the *S. cerevisiae* RAD6 protein. Genetic complementation experiments reveal that *HHR6A* as well as *HHR6B* can carry out the DNA repair and mutagenesis functions of *RAD6* in *S. cerevisiae rad6Δ* mutants.

The *Saccharomyces cerevisiae RAD6* gene plays a key role in DNA repair and DNA damage-induced mutagenesis. *rad6* mutants are extremely sensitive to a plethora of DNA-damaging agents, including UV irradiation, x-rays, and alkylating agents; they are defective in postreplication repair of UV-damaged DNA, in mutagenesis induced by DNA damaging agents, and in sporulation (for a review, see ref. 1). Transposition of Ty elements is enhanced in *rad6* mutants (2). *RAD6* encodes a protein of 172 amino acids (3) with a globular domain consisting of approximately the first 149 amino acids and an extended, predominantly acidic C terminus (4). RAD6 is a ubiquitin-conjugating enzyme (5, 6). Ubiquitin, a highly conserved, 76-amino acid polypeptide is covalently attached to many cellular proteins and targets them for selective degradation, (re)folding, or stabilization (for recent reviews, see refs. 7–9). Ubiquitination is carried out by a family of proteins in a multistep reaction involving a ubiquitin-activating enzyme (E1) that binds and subsequently transfers a ubiquitin moiety to one of a set of ubiquitin-conjugating enzymes (E2). The E2 enzyme ligates ubiquitin directly to a target protein, with or without the help of a ubiquitin protein ligase (E3). RAD6 polyubiquitinates histones H2A and H2B *in vitro* without the involvement of E3, and the acidic domain of RAD6 is required for multiple ubiquitination of histones (6). It is possible that the protein is implicated in modifying chromatin structure as part of the processes that are disturbed in a *rad6* mutant, including repair and mutagenesis.

RAD6 is highly conserved among eukaryotes. Previously, we cloned the *rhp6*$^+$ gene of the fission yeast *Schizosaccharomyces pombe* and showed that it is a structural and functional homology of *RAD6* (10). We have also isolated a *RAD6* homolog, *Dhr6*, from *Drosophila melanogaster* (11). In this paper, we present the cloning, sequence analysis, and functional studies with the *RAD6* homologs from human.¶ In contrast to yeast and *Drosophila*, where *RAD6* is a single copy gene, interestingly, in human, the *RAD6* homologous gene is duplicated, and the proteins encoded by the two genes *HHR6A* (human homolog of RAD6) and *HHR6B* share 95% identical amino acid residues. We also show that the *HHR6A* and *HHR6B* genes complement the DNA repair and UV mutagenesis defects of the *S. cerevisiae rad6* mutant.

MATERIALS AND METHODS

Restriction Enzyme Digests and Southern Blot Library Hybridizations. Restriction enzyme digestions were performed according to the manufacturer's descriptions. Blots were prepared on Zeta-Probe (Bio-Rad) using the alkaline-blotting procedure as recommended by the manufacturer. DNA probes were labeled by the random-priming method as described (12). Unless stated otherwise, hybridization of *Sc. pombe* and *Drosophila* probes to human DNA occurred overnight at 55°C and hybridization of human probes to human DNA was at 65°C in a hybridization mixture containing 10× Denhardt's solution (2% Ficoll/2% bovine serum albumin/2% polyvinylpyrrolidone)/10% dextran sulfate/0.1% SDS/3× standard saline citrate (SSC)/50 mg of sonicated salmon sperm DNA per liter. Washings for hybridizations involving different species were performed for 5 min in 3× SSC twice and for 5 min in 1× SSC once at 55°C. For hybridizations within a species, washings were done twice for 20 min each in 3× SSC, twice for 20 min each in 1× SSC, and twice for 20 min each in 0.3× SSC at 65°C.

Northern Blotting and Nucleotide Sequence Analysis. Isolation of total RNA by the LiCl/urea method, preparation of poly(A)$^+$ RNA by two consecutive passages over oligo(dT) cellulose columns, and Northern blotting protocols were all according to Sambrook *et al.* (13). Sequence analysis on double-stranded DNA was done by the T7 polymerase modification (Pharmacia) of the dideoxynucleotide chain-termination method (14) using sequence-derived oligonucleotides and exonuclease III prepared deletion clones for sequencing both strands. For separation of the fragments we used Hydrolink (AT Biochem, Malvern, PA) sequencing gels.

Yeast Strains, Media, and Genetic Analyses. The *S. cerevisiae* haploid strains used in this study were the *rad6Δ* strains EMY1 (*MATa leu2-3 leu2-112 trp1 ura3-52 rad6::LEU2*$^+$) and EMY8 (*MATα ade5 his7 leu2-3 lys1 met14 pet15 ura3 trp1::URA3*$^+$ *rad6::LEU2*$^+$). The *rad6Δ/rad6Δ* diploid EMY28 was constructed by mating EMY1 and EMY8. UV irradiation conditions and media for determining survival and mutagenesis after UV exposure and sporulation

media were as described (4). Standard genetic techniques for *S. cerevisiae* (15) were used.

Plasmids. The following yeast plasmids were used in this study: pR67 contains the *RAD6* gene within a 2-kilobase (kb) *Hin*dIII/*Bam*HI DNA fragment inserted into the yeast *CEN4* plasmid YCp50 as described (4). pR611 is derived from pR67 by deleting the 0.6-kb *RAD6 Eco*RI fragment containing the entire *RAD6* open reading frame (ORF). Plasmids pR67 and pR611 are maintained in yeast as low copy plasmids. For expression of the human *RAD6* homologs *HHR6A* and *HHR6B* in *S. cerevisiae*, the human genes were cloned downstream of the highly expressed *S. cerevisiae ADC1* promoter in the yeast expression vector described previously (16), yielding plasmids pRR510 and pRR518, respectively.

RESULTS

Cloning of Human cDNAs Cross-Hybridizing to *RAD6* Derivatives. Southern and Western blot analyses indicated that the *RAD6* gene and protein are conserved in eukaryotes. For cloning the human *RAD6* homolog, a human λ cDNA library prepared from human testis RNA was screened. One set of filters was hybridized with the *Drosophila Dhr6* probe and a duplicate filter set was hybridized with the *Sc. pombe rhp6*$^+$ gene probe. Of the many clones cross-hybridizing, those reacting to some extent with both probes (≈30 in 10^6 plaques) were picked and examined by restriction enzyme analysis. Unexpectedly, restriction maps indicated the presence of two classes, corresponding to the *HHR6A* and *HHR6B* genes as described below.

Northern Blot Analysis. Representative cDNA inserts of each class were hybridized to Northern blots containing total or poly(A)$^+$ RNA from various cell lines. As shown in Fig. 1 (lane 2) the *HHR6A* probe detects transcripts of 1.7 and 0.8 kb in HeLa RNA; the *HHR6B* probe (lane 1) mainly hybridizes to a mRNA species of 4.4 kb. Similar results were

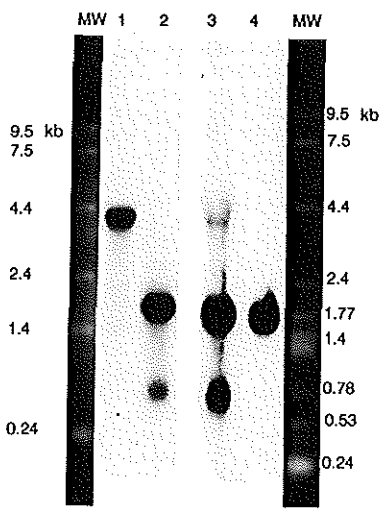

FIG. 1. Northern blot analysis of human *HHR6A* and *HHR6B* transcripts. Poly(A)$^+$ RNA was size fractionated on a 1% agarose gel containing formaldehyde. Lanes: MW, RNA molecular size markers; 1, hybridization with a *Pvu* II/*Hin*dIII fragment containing the coding region of *HHR6B*, recognizing a 4.4-kb mRNA; 2, hybridization with a probe (1.2-kb *Eco*RI fragment) of human *HHR6A* cDNA, which hybridizes to both a 0.8- and a 1.7-kb mRNA; 3 and 4, hybridization with the 293-bp *Sma* I/*Hin*dIII and the 392-bp *Sac* I *HHR6A* cDNA probe, respectively (see Fig. 2 *A* and *B* for location on the *HHR6A* cDNA maps).

obtained with RNA from the myelocytic cell line K562, a primary human fibroblast line and mouse and rat tissues (data not shown). Clones of each class with insert sizes expected for full-length cDNAs (two for the two transcripts of *HHR6A*, one for the 4.4-kb RNA of *HHR6B*) were selected for further analysis.

Nucleotide and Predicted Amino Acid Sequences of *HHR6A* and *HHR6B*. Restriction maps for the three cDNA inserts representing the *HHR6A* and *HHR6B* genes are shown in Fig. 2*A*. The nucleotide and deduced amino acid sequences of the regions of interest in *HHR6A* and *HHR6B* are shown in Fig. 2 *B* and *C*, respectively.

Sequence analysis of the *HHR6A* cDNAs indicates that the minor 0.8-kb mRNA is identical to the major 1.7-kb mRNA species for the 5′ untranslated region (UTR), ORF, and the first part [≈100 base pairs (bp)] of the 3′ UTR. The 3′ UTR of the larger transcript extends for an additional ≈1 kb. This mRNA species harbors an AATAAA polyadenylylation signal (directly followed by a suboptimal one: AATAAC) 13 bp before the presumed polyadenylylation site (18, 19). The short 0.8-kb transcript also has two potential but suboptimal polyadenylylation signals close to the 3′ terminus. Hence, the difference between the two *HHR6A* transcripts can be explained as a result of alternative polyadenylylation site selection. This is confirmed by the Northern blot hybridization shown in Fig. 1 (lanes 3 and 4) using 3′ UTR probes derived from the region common to both cDNAs (293-bp *Sma* I/*Hin*dIII probe; lane 3) and from the area unique to the 1.7-kb species (392-bp *Sac* I probe; lane 4). The *HHR6A* sequence contains a single long ORF that encodes a protein of 152 amino acids with a calculated M_r of 17,243. The *HHR6B* ORF specifies a protein of 152 amino acids with a calculated M_r of 17,312. The expected sizes of both proteins are consistent with the results of the Western blot analysis (data not shown). The *HHR6B*-encoded protein shares a high degree of identity (95%) with the HHR6A amino acid sequence with only eight amino acid substitutions, two of which are conservative changes (Fig. 3, top two lines). At the nucleotide level, the coding sequence is much less conserved (80%) and the 5′ and 3′ UTR sequences are very different. The HHR6B protein sequence is identical to the predicted gene product [termed E2 (M_r 17,000)] of a partial cDNA clone recently described by Schneider and coworkers (17). As shown by the alignment in Fig. 3 (*Upper*) and the quantitative data summarized in Fig. 4, both the HHR6A and HHR6B polypeptides share extensive amino acid sequence similarity with RAD6 homologs of other species. However, both human proteins resemble those of *Drosophila* and *Sc. pombe* in lacking the acidic C terminus characteristic of *S. cerevisiae* RAD6. In addition, there is significant similarity to the other ubiquitin-conjugating enzymes (Fig. 3 *Lower*).

Functional Complementation of the *rad6* Mutation of *S. cerevisiae* by the *HHR6A* and *HHR6B* Genes. The high degree of amino acid identity between the *HHR6*- and *RAD6*-encoded proteins suggests that the human genes function similarly to *RAD6*. To examine this possibility, we determined whether the *HHR6A* and *HHR6B* genes complement the DNA repair, UV mutagenesis, and sporulation defects of the *rad6*Δ mutation of *S. cerevisiae*. Plasmids pRR510 and pRR518, containing the human *HHR6A* and *HHR6B* genes fused to the yeast *ADC1* promoter, respectively, were introduced into the *S. cerevisiae rad6*Δ strain EMY8 by transformation and the level of HHR6 protein was examined by Western analysis using affinity-purified anti-rad6-149 antibodies. The level of HHR6A and HHR6B proteins in *rad6*Δ cells was somewhat higher than the level of RAD6 protein present in the wild-type *S. cerevisiae* cells (data not shown). Both the *HHR6A* and *HHR6B* genes substantially increase the UV resistance of the *rad6*Δ strain. At 10 J/m^2, the survival of the *rad6*Δ strain is enhanced >500-fold and

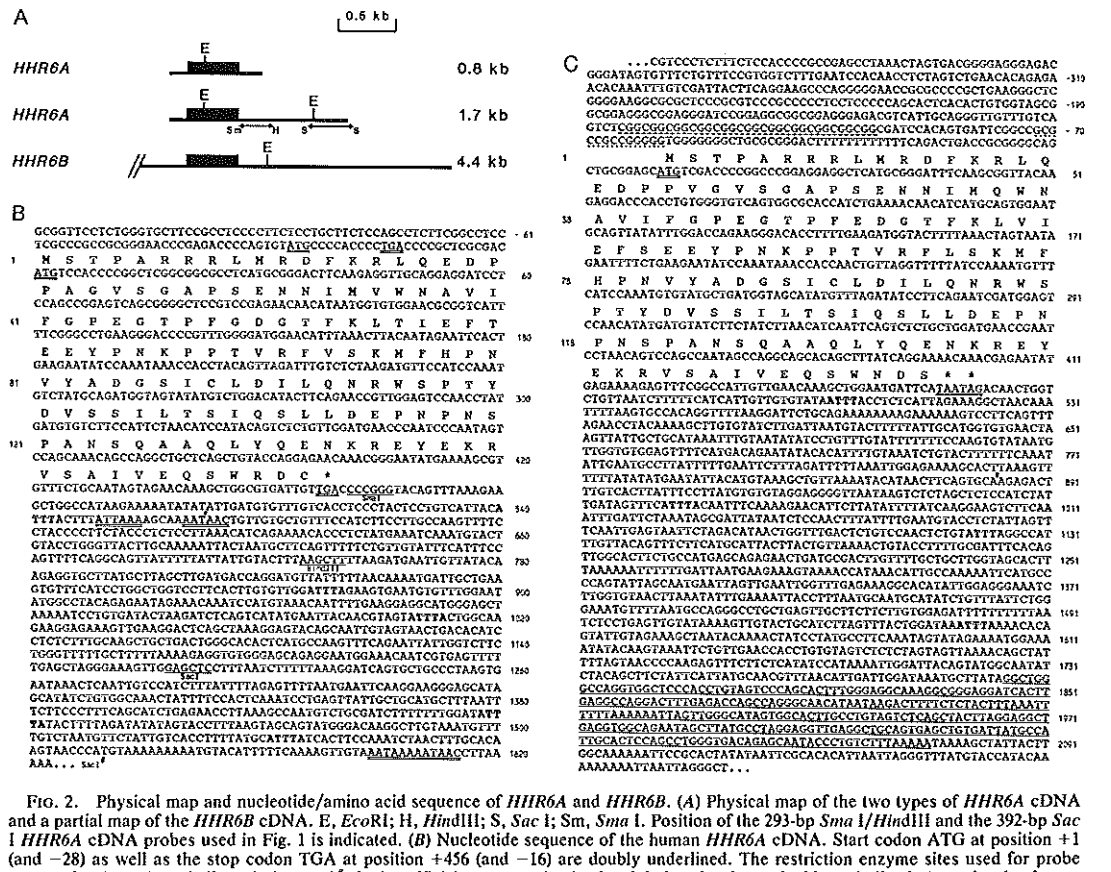

FIG. 2. Physical map and nucleotide/amino acid sequence of *HHR6A* and *HHR6B*. (*A*) Physical map of the two types of *HHR6A* cDNA and a partial map of the *HHR6B* cDNA. E, *Eco*RI; H, *Hin*dIII; S, *Sac* I; Sm, *Sma* I. Position of the 293-bp *Sma* I/*Hin*dIII and the 392-bp *Sac* I *HHR6A* cDNA probes used in Fig. 1 is indicated. (*B*) Nucleotide sequence of the human *HHR6A* cDNA. Start codon ATG at position +1 (and −28) as well as the stop codon TGA at position +456 (and −16) are doubly underlined. The restriction enzyme sites used for probe preparation (see *A*) are indicated (the *Sac* I$^{\#}$ site is artificial). Presumed polyadenylylation signals are doubly underlined. Arrowhead points to the position where the cDNA for the 0.8-kb mRNA terminated. Amino acids are given in the single-letter code. (*C*) Nucleotide sequence of the human *HHR6B* cDNA (not the entire 5′ and 3′ UTR sequence is shown). Start codon and stop codon are doubly underlined. A trinucleotide tandem repeat $(CGG)_{11}$ is indicated by interrupted underlining. Dotted underlining points to a region with very strong secondary structure. The 3′ UTR contains an *Alu* repeat (singly underlined). ATTTA boxes are in boldface. The segment from nucleotides −39 to +875 is identical with the sequence of a partial cDNA clone published by Schneider *et al.* (17) except for the presence of an extra G residue at −2 in our sequence, which changes the −3 position important for translation initiation from a G into a more optimal A. Amino acids are indicated in the single-letter code.

>1000-fold by *HHR6A* and *HHR6B*, respectively (Fig. 5*A*). The *HHR6A* and *HHR6B* genes also restore UV mutagenesis in the *rad6*Δ strain to wild-type levels (Fig. 5*B*). In contrast, the two human homologs confer only a low level of sporulation ability (≈5%) to the *rad6*Δ/*rad6*Δ strain.

DISCUSSION

In this paper, we have identified two closely related homologs of the *S. cerevisiae RAD6* gene in human, one of them being identical to the E2 (M_r 17,000) protein recently described by Schneider *et al.* (17), who isolated an incomplete cDNA on the basis of a partial amino acid sequence. Our extensive analysis of a large number of independent genomic DNA clones points to the existence of only a single *RAD6* gene in *S. cerevisiae*, *Sc. pombe*, and *D. melanogaster*. The very high degree of amino acid sequence conservation throughout eukaryotic evolution points to extremely strong sequence constraints imposed on the RAD6 protein. As shown in Fig. 4, the human and yeast *RAD6* homologs share ≈70% sequence identity and the *Drosophila* homolog is the one most closely related to the human HHR6 proteins (85–87% identity). The Dhr6 and HHR6 proteins share almost the same degree of sequence homology (68–69% identity) to RAD6, whereas the rhp6^{+} gene product is only somewhat more homologous to the *S. cerevisiae* protein (77% identity). Based on the degree of divergence between the various RAD6 homologs, we calculate that the duplication found in humans (and also in mouse and kangaroo; unpublished results) must have occurred ≈200 × 10^6 years ago, in the Jurassic era.

Fig. 3 (top five lines) shows that among the RAD6 homologs, the N-terminal part and the central region, in particular, have been highly conserved. The middle portion contains the invariant Cys-88 residue that is involved in thiol ester linkage with ubiquitin and that is crucial for all *RAD6* functions, as its substitution by valine or alanine produces a *rad6* null phenotype (24). The C terminus, on the other hand, has diverged much more. The *S. cerevisiae* RAD6 protein is unique in harboring an acidic tail sequence. Mutational analysis has shown the acidic domain to be essential for sporulation in *S. cerevisiae* (4). A possible explanation for the absence of an acidic C-terminal extension in other RAD6

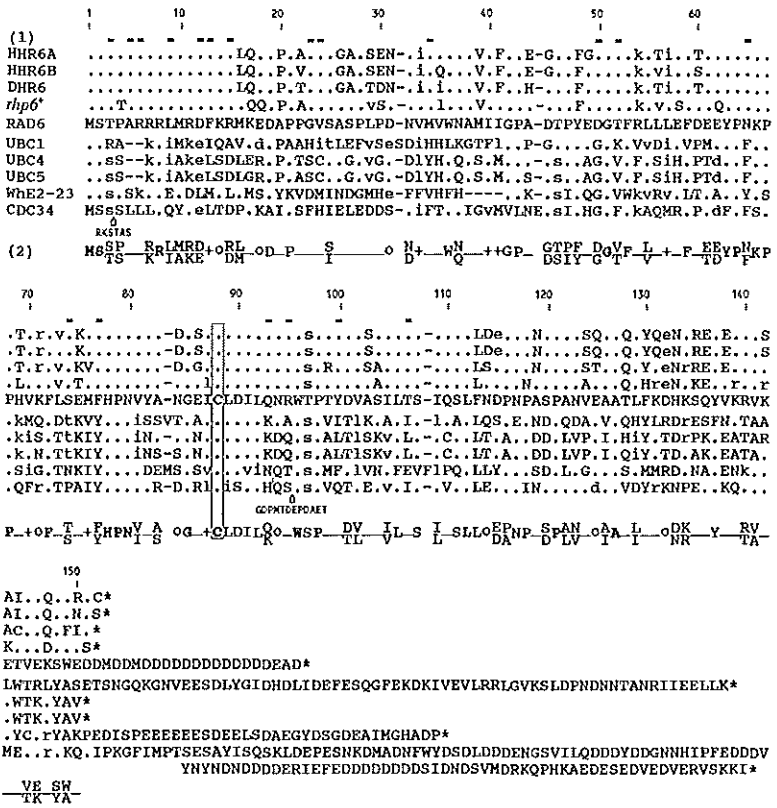

FIG. 3. Comparison of amino acid sequences of HHR6A and HHR6B proteins with various RAD6 homologs and with other ubiquitin-conjugating enzymes. (*Upper*) Comparison of various RAD6 homologs: *S. cerevisiae* RAD6 (3), *Sc. pombe* rhp6$^+$ (10), *D. melanogaster* Dhr6 (11), and human HHR6A and HHR6B (human E2; M_r 17,000) (this paper; ref. 17). (*Lower*) Comparison of the other published ubiquitin-conjugating (E2) proteins; *S. cerevisiae* UBC1 (20); UBC4 and UBC5 (21), involved in protein degradation; *S. cerevisiae* CDC34, involved in cell cycle regulation (22); and wheat E2 (M_r 23,000) (23). Dots indicate identity, whereas lowercase letters indicate strongly conserved residues compared with the yeast RAD6 protein. Conserved amino acids: R and K; E and D; I, V, and L; T and S. (1), Horizontal bars, amino acid residues exclusively conserved in all members of the *RAD6* family; (2), consensus sequence present in all 10 E2 enzymes. Boldface letters, amino acid residues occurring at this position in all 10 ubiquitin-conjugating enzymes; lightface letters, the most likely possibility at this position (occurring in 80% or more of the cases); circles, hydrophilic residue at this position in all the proteins; crosses, hydrophobic residues in all 10 positions. Cys-88 residue, used for ubiquitin attachment, is boxed in all E2 family members.

homologs is that in the other species this domain may have evolved into a protein of its own or it may have become incorporated into a different protein.

The comparison of RAD6 with the other ubiquitin-conjugating enzymes presented in Fig. 3 (bottom six lines)

	RAD6	rhp6$^+$	DHR6	HHR6A	HHR6B	
S. cerevisiae RAD6	-	77	68	68	69	
S. pombe rhp6$^+$	84	-	70	71	71	
D. melanogaster DHR6	74	77	-	87	85	% Identity
Man HHR6A	74	76	90	-	95	
Man HHR6B	74	76	89	96	-	

% Similarity

FIG. 4. Identical and similar amino acid residues shared among *RAD6* homologs. Percentage identity is given above the diagonal, and percentage similarity is given below the diagonal. See Fig. 3 legend for classification of conserved residues.

reveals marked similarity, especially in the central part around the Cys-88 residue (see overall consensus sequence 2 in Fig. 3). This segment is likely involved in binding of ubiquitin and/or interaction with the ubiquitin-activating enzyme E1 that donates a ubiquitin moiety from an internal cysteine residue to the cysteine in E2 enzymes. The amino acid sequence around Cys-88 in E2 enzymes bears resemblance to the sequence context of Cys-908 and -866 of the recently cloned ubiquitin-activating enzymes (E1) of wheat and human, respectively (25, 26), and may define a ubiquitin binding domain in E1 enzymes as well.

The strict conservation of the N terminus among RAD6 homologs does not extend to the other E2 enzymes. This part may therefore be implicated in important RAD6-specific functions such as interaction with protein components of the DNA repair and mutagenesis machinery. Finally, it is remarkable that all E2 proteins begin with the sequence MS(S/T). Proteins starting with serine are frequently subject to N-terminal acetylation (27). It is not known whether RAD6 or any other E2 enzyme is acetylated at the N terminus.

The high degree of amino acid sequence conservation of RAD6 is also reflected at the functional level. Both human

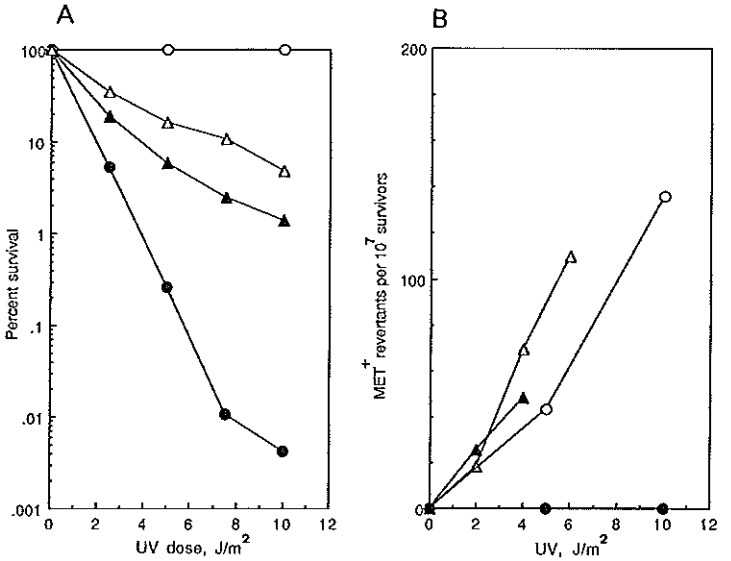

FIG. 5. Complementation of UV sensitivity and UV immutability of the *S. cerevisiae rad6*Δ mutation by human *HHR6A* and *HHR6B* genes. Survival after UV irradiation (*A*) and UV-induced reversion of *met14* (*B*) in the *S. cerevisiae rad6*Δ strain EMY8 carrying the *HHR6A* or *HHR6B* gene on the *ADC1* plasmid. Cells were grown in synthetic complete medium lacking tryptophan for selection of the plasmid and were harvested in midexponential phase. After plating on appropriate medium, cells were irradiated with UV light at a dose rate of 0.1 $J \cdot m^{-2} \cdot sec^{-1}$ and incubated in the dark to avoid photoreactivation. ○, EMY8 + pR67 (*CEN RAD6*); ●, EMY8 + pR611 (*rad6*Δ); ▲, EMY8 + pRR510 (*ADC HHR6A*); △, EMY8 + pRR518 (*ADC HHR6B*).

homologs restore normal levels of UV mutagenesis and effect a substantial increase in UV resistance in *S. cerevisiae rad6* mutants. On the other hand, human homologs confer only a very low level of sporulation ability to *rad6/rad6* mutants. This result is expected in view of the absence of the acidic tail sequence in the human proteins and previous observations that this domain is essential for sporulation but not for DNA repair or UV mutagenesis (4).

The availability of *HHR6* genes should make it possible to examine their role in various cellular processes in mammals such as mutagenesis, postreplication repair, and recombination. Because of the involvement of *RAD6* in sporulation, it will be of special interest to examine whether the *HHR6* genes are implicated in meiosis and gametogenesis. At the final stages of spermatogenesis, histones are replaced by protamines. One can envisage that the capability of RAD6 to polyubiquitinate histones is utilized at this stage to mark histones for degradation by the ATP-dependent ubiquitin-specific protease complex. For these studies, it will be necessary to obtain *HHR6* mutants. One way toward identifying such mutants will be to screen mutant cell lines from human DNA repair disorders or from the existing collection of *in vitro* generated repair-deficient rodent cell lines. Alternatively, *HHR6* mutants could be generated by gene disruption utilizing recently developed methods of gene replacement (28). It is possible to perform this in totipotent mouse embryonic stem cells and in that way to create an *HHR6* defective mouse model. An obvious complication, however, is the presence of two genes, whose function is likely to overlap considerably, necessitating the simultaneous inactivation of both genes.

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Chapter V

Localization of two human homologues, HHR6A and HHR6B, of the yeast DNA repair gene RAD6 to chromosomes Xq24-25 and 5q23-31

GENOMICS 12, 447–453 (1992)

Localization of Two Human Homologs, *HHR6A* and *HHR6B*, of the Yeast DNA Repair Gene *RAD6* to Chromosomes Xq24–q25 and 5q23–q31

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The chromosomal localizations of two closely related human DNA repair genes, *HHR6A* and *HHR6B*, were determined by *in situ* hybridization with biotinylated probes. *HHR6A* and *HHR6B* (human homolog of yeast *RAD6*) encode ubiquitin-conjugating enzymes (E2 enzymes), likely to be involved in postreplication repair and induced mutagenesis. The *HHR6B* gene was assigned to human chromosome 5q23–q31, whereas the *HHR6A* gene was localized on the human X chromosome (Xq24–q25). This latter assignment was confirmed with an X-specific human–mouse/hamster somatic cell hybrid panel. Southern blot analysis points to an X and an autosomal localization of *HHR6A* and *HHR6B*, respectively, in the mouse. The potential involvement of these genes in human genetic disorders is discussed.

INTRODUCTION

Recently, we reported the cloning of two human genes, designated *HHR6A* and *HHR6B*, homologous to the *Saccharomyces cerevisiae RAD6* gene (Koken *et al.*, 1991b). As deduced from the very pleiotropic phenotype of yeast *rad6*Δ mutants, the RAD6 protein plays an important role in various cellular processes, including postreplication repair (a poorly defined, error-prone repair pathway), damage-induced mutagenesis, sporulation, and recombination (for a review, see Prakash *et al.*, 1990). The RAD6 functions are accomplished by a 172-amino-acid protein with an N-terminal globular structure and an extended C-terminal acidic tail (Reynolds *et al.*, 1985). The acidic domain is specifically required for sporulation but is not essential for the other RAD6 functions (Morrison *et al.*, 1988). An important finding concerning the biochemical activity of the RAD6 protein was the discovery that the gene encodes a ubiquitin-conjugating enzyme (Jentsch *et al.*, 1987). Ubiquitin, a widespread, highly conserved 76-amino-acid polypeptide, is covalently attached to specific cellular proteins that in this way are targeted for selective degradation, (re)folding, or stabilization (for recent reviews, see Hershko, 1988; Rechsteiner, 1988; Jentsch *et al.*, 1990). Ubiquitination of proteins occurs in a multistep reaction. First, a ubiquitin-activating enzyme (or E1 enzyme) binds and activates a ubiquitin molecule. This is subsequently transferred to one of a set of ubiquitin-conjugating enzymes (or E2 enzymes). The E2 enzyme ligates the ubiquitin moiety to a target protein with or without the help of an E3 ubiquitin protein ligase molecule. The RAD6 protein was found to attach one (Jentsch *et al.*, 1987) or multiple (Sung *et al.*, 1988) ubiquitin moieties to histones H2A and H2B *in vitro*. If histones are also the main targets of RAD6 *in vivo*, it is likely that RAD6 mediates chromatin remodeling required for the processes impaired in a *rad6*Δ mutant.

RAD6 is very strongly conserved in eukaryotic evolution, and this property permitted us to clone by evolutionary walking two human homologs (Koken *et al.*, 1991b) using the *Schizosaccharomyces pombe* (Reynolds *et al.*, 1990) and *Drosophila melanogaster* (Koken *et al.*, 1991a) homologs as "intermediates." The human HHR6A and HHR6B proteins (HHR for human homolog of RAD6) share ≈95% amino acid sequence identity with each other and ≈70% amino acid identity with their yeast counterparts, but notably lack the acidic C-terminal domain, the occurrence of which seems to be limited to *S. cerevisiae* RAD6. Moreover, the human polypeptides were found to substitute functionally for the repair and mutagenesis functions of RAD6 in a *S. cerevisiae rad6*Δ mutant but not for its role in sporulation. This indicates that the proteins of the repair and mutagenesis machinery with which RAD6 interacts are also conserved to a significant extent between man and yeast. Furthermore, it is likely that the HHR6 proteins in man have a function similar to that of RAD6 in yeast, i.e., catalyzing ubiquitin conjugation as an essential step in the repair and mutagenesis pathways. This conclusion makes the gene a candidate for human inherited

HHR6A and *HHR6B* are not HGMW approved gene symbols.

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repair disorders, in particular the variant complementation group of the cancer-prone repair syndrome xeroderma pigmentosum (XP) in which the postreplication repair pathway is considered to be impaired (Lehmann *et al.*, 1975). Here we present the chromosomal localization of these two human genes by *in situ* hybridization using biotinylated probes and by Southern blot hybridizations to DNA of rodent/human cell hybrids.

MATERIALS AND METHODS

Cell lines/DNAs. The somatic cell hybrids containing various parts of the human X chromosome used in this study have been described elsewhere. The hamster/human hybrids were X3000, Xq24–qter (Nussbaum *et al.*, 1986); 908K1B18, Xq24–q26 (Schonk *et al.*, 1989); 8121, Xpter–q27.1; and 2384, Xpter–q27.2 (Patterson *et al.*, 1987). The mouse/human hybrids were RJK734, Xq26–qter (Scott *et al.*, 1979); and CY34A, Xq24–q27 (Suthers *et al.*, 1989) See Fig. 2B for schematic diagram of the human X-chromosome segments in these hybrids.

Restriction enzyme digests and Southern blot hybridizations. Enzyme digestions and Southern blotting procedures were essentially the same as described previously (Koken *et al.*, 1991b; Sambrook *et al.*, 1989). In brief, 20 μg of restriction enzyme-digested genomic DNA was size-fractionated on 0.8% agarose gels and transferred onto nylon (Zetaprobe) membranes. Hybridization occurred overnight at 65°C in a hybridization buffer containing 10× Denhardt's solution, 10% dextran sulfate, 0.1% SDS, 3× SSC, and 50 μg/ml sonicated salmon sperm DNA. Washings were performed extensively up to 0.3× SSC containing 0.1% SDS at 65°C. The 1.7-kb *HHR6A* cDNA probe, H28, contains a full-length *HHR6A* cDNA on a *Sal*I fragment (Koken *et al.*, 1991b). The *HHR6B* cDNA probe, $H13_{0.8}$, harbors the complete *HHR6B* open reading frame on an 0.8-kb fragment starting with an artificial *Eco*RI site at the position of the ATG and ending at a natural *Eco*RI site in the cDNA (Koken *et al.*, 1991b).

In situ hybridization. *In situ* hybridization was performed essentially as described (Landegent *et al.*, 1985; Pinkel *et al.*, 1986). Human lymphocyte metaphase spreads were treated with 100 μg RNase A/ml in 2× SSC for 1 h at 37°C, rinsed in 2× SSC, and dehydrated in alcohol. After a pepsin (0.1 μg/ml 0.01 *N* HCl) treatment at 37°C for 10 min, the slides were washed in PBS, postfixed with 1% formaldehyde in PBS containing 50 m*M* $MgCl_2$, washed for 5 min in PBS, dehydrated in ethanol, and air-dried. The hybridization mixture (10 μl per slide) consisted of 50% formamide, 2× SSC, 40 m*M* sodium phosphate (pH 7.0), 10% dextran sulfate 50 ng labeled probe, 1 μg sonicated salmon sperm DNA, and 1 μg *Escherichia coli* tRNA. The genomic probes, B3.0, B2.3, H2.7, H0.75, and HS2.7 (*HHR6A*) and E2.3, E6.0, E4.5, and E1.3 (*HHR6B*), representing most of the genomic region of both genes (Koken *et al.*, manuscript in preparation), were biotin-labeled. A cocktail of the genomic probes for each gene was used for *in situ* hybridization. Probes were denatured at 70°C for 5 min in hybridization mixture (specified above). Competition for repeat sequences present in the genomic subclones was achieved by incubation for 6 h (*HHR6A* probes) or 2 days (*HHR6B*) with a 100 times excess of thymus DNA (*HHR6A*) or a 1000 times excess of human C_0t1 DNA (*HHR6B*) at 37°C in hybridization buffer. This was necessary because of the extremely high content of repeats in the genomic clones used as probes. The chromosome spreads were denatured in 70% formamide for 2.5 min at 70°C. After competition, the probes were incubated overnight with the slides and then washed once with 50% formamide in 2× SSC at 39°C followed by three times for 5 min in 2× SSC, three times for 5 min in 0.1× SSC at 60°C, and once for 5 min in 4× SSC, 0.05% Tween20 at room temperature. Finally, the slides were blocked in 4× SSC, 5% nonfat dry milk for 20 min at 37°C. Slides were incubated with 5 μg avidin D–FITC (Vector, U.S.A.), and the fluorescent signal was amplified with biotinylated goat anti-avidin D, washed, dehydrated with ethanol, and air-dried. The slides were embedded and stained in 9 parts glycerol containing 2.3% (w/v) 1,4-diazobicyclo-(2,2,2)-octane (DABCO) and 1 part 0.2 *M* Tris–HCl, 0.02% NaN_3, pH 8.0, containing 4',6'-diamino-2-phenylindole (DAPI) to a final concentration of 0.5 μg/μl.

RESULTS

In Situ Hybridization to Metaphase Chromosomes

For mapping the *HHR6A* and *HHR6B* loci, *in situ* hybridization experiments on metaphase spreads were performed using biotinylated genomic probes. A representative *in situ* hybridization for each of the two genes of the more than 50 metaphases analyzed is depicted in Fig. 1. As shown in Fig. 1A (*HHR6A*), a specific signal (arrow) is found on the long arm of only one chromosome in every metaphase analyzed. Because cells in this experiment were derived from a male donor, this finding strongly suggests that the gene is located on the X chromosome. This interpretation was confirmed by simultaneous hybridization with an X-specific centromere probe, pBamX5 (Willard *et al.*, 1983), clearly identifying the hybridizing chromosome as the X chromosome. [The weak hybridization with the centromeric regions of four other chromosomes (9 and 17) is due to cross-hybridization of the X-centromere probe to the centromeres of chromosomes 9 and 17 (Willard and Waye, 1987).] From these results we deduce that the *HHR6A* gene resides on the lower part of the q arm of the X chromosome.

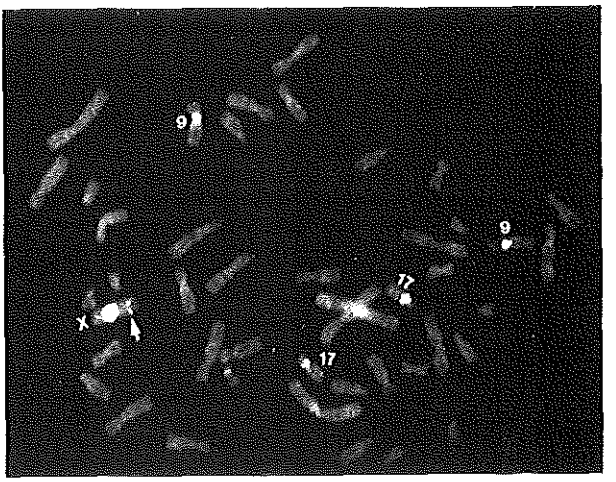
Figure 1B shows the hybridization with biotinylated *HHR6B* gene probes (arrows). Using the DAPI staining procedure, the hybridizing chromosome was identified as chromosome 5 (Fig. 1B). Therefore, the gene was unambiguously assigned to 5q23–q31.

Southern Hybridization of HHR6A Probes to DNA of a Panel of Human/Rodent Somatic Cell Hybrids

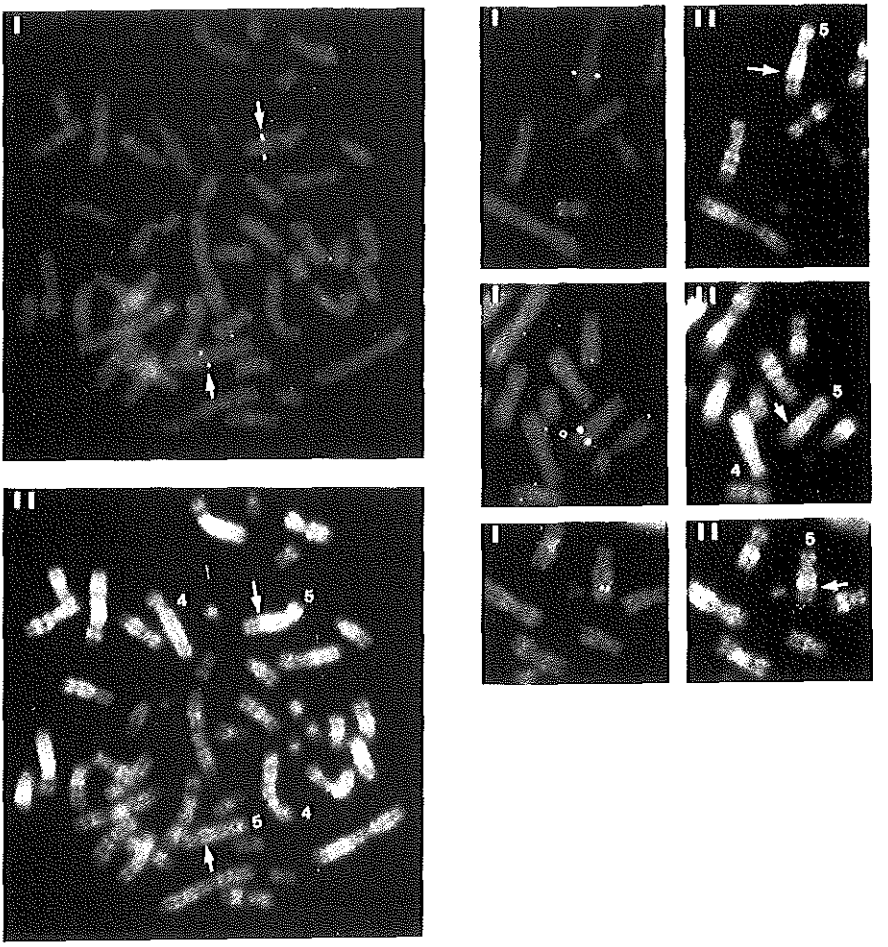
To confirm the assignment of *HHR6A* and to obtain a more precise subchromosomal localization, Southern blot analysis was carried out using genomic DNA from a panel of human–mouse/hamster hybrids containing specific parts of the human X chromosome (Fig. 2B). As shown in Fig. 2A the *HHR6A* cDNA probe recognizes the human fragments (3.0, 2.6, and 0.75 kb, indicated by arrowheads) in hybrid cell lines X3000, 8121, and 2384. This indicates that the *HHR6A* gene maps on Xq24–q25 centromeric of the breakpoint in the X chromosome found in the RJK734 hybrid and distal of the breakpoint

FIG. 1. *In situ* hybridization of metaphase chromosomes to biotinylated genomic *HHR6* probes. (A) Hybridization with a cocktail of all genomic *HHR6A* probes specified under Material and Methods. The arrow indicates the hybridization signal on chromosome Xq. This chromosome shows also the X-specific hybridization of the pBamX5 probe. The probe weakly cross-hybridizes to chromosomes 9 and 17 as indicated. (B) Hybridization with a cocktail of all genomic *HHR6B* probes (indicated under Materials and Methods). The arrows point to the regions with a specific signal on chromosome 5q23–q31. In panels I the *in situ* hybridization is shown. In panels II the DAPI banding of the same metaphases is shown.

A



B



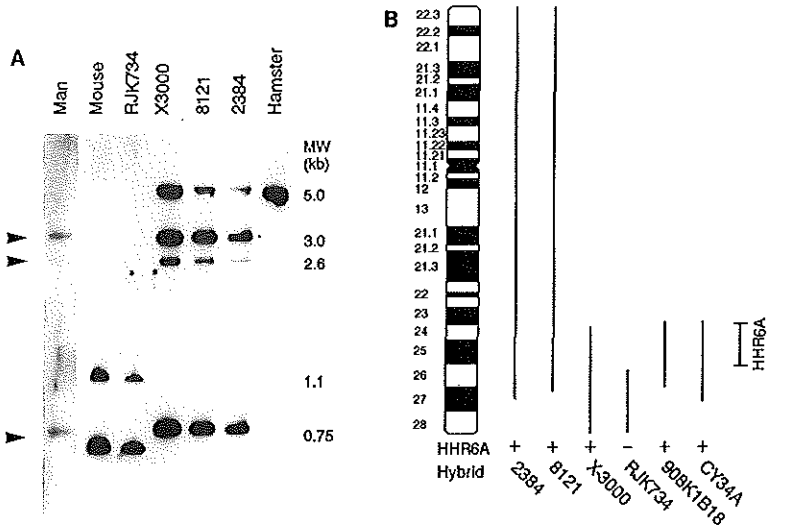


FIG. 2. (A) Southern blot analysis of X-specific hybrid panel with the *HHR6A* cDNA probe. The source of the genomic DNA is indicated. DNA is digested with *Hin*dIII and size-fractionated on an 0.8% agarose gel. The molecular weight (MW) indicated on the right refers to the hybridizing fragments at the corresponding positions in the autoradiogram. The fragments of 3.0, 2.6, and 0.75 kb are of the human *HHR6A* gene. (B) Representation of the human X-chromosome fragments (indicated by lines) retained in the rodent/human hybrids used in this study. The + or − sign above the hybrid-names indicates whether or not DNA from this specific cell line hybridizes with the human probe.

in the X3000 hybrid cell line, confirming the data found via *in situ* hybridization. Hybridizations to the somatic cell hybrids 908K1B18 and CY34A were also positive with *HHR6A* probes (data not shown).

Chromosomal Localization of the Mouse Homologs of HHR6A and HHR6B

To assess whether also in the mouse one gene is located on the X chromosome and the other on an autosome, a Southern blot with equal amounts of genomic DNA from a male and female mouse was hybridized consecutively with both human cDNA probes. As shown in Fig. 3, the hybridization with the *HHR6A* gene clearly shows an approximately twofold difference in hybridization intensity between the DNA of the male and the female mouse, whereas with the *HHR6B* probe and the same blot, no difference between male- and female-derived DNA is detectable. This strongly suggests that also in the mouse the *HHR6A* gene is X-linked, whereas the *HHR6B* gene is on an autosome.

DISCUSSION

Localization of Genes Involved in DNA Repair or in Ubiquitin Systems

This paper describes the localization of two human homologs, *HHR6A* and *HHR6B*, of the yeast DNA repair gene *RAD6* to human chromosomes Xq24–q25 and 5q23–q31, respectively. The *HHR6A* gene is the first human DNA repair gene located on X. Among the DNA repair genes isolated thus far, no clustering is apparent, with the possible exception of the q13.2 area of chromosome 19 onto which at least three repair genes have been localized (Mohrenweiser *et al.*, 1989; Weeda *et al.*, 1991; Smeets *et al.*, 1990; Thompson, 1989). This, however, could be due at least in part to the presence of large regions of hemizygosity in the Chinese hamster cells used to generate the repair mutant cell lines with which these three genes were cloned. The hemizygosity favors the isolation of mutants in genes located in those areas (Siciliano *et al.*, 1983).

In contrast to a dispersed localization of DNA repair genes over the genome, it is of interest to note that a clustering of genes for different components of the ubiquitin system may exist on the X chromosome. With the exception of ubiquitin itself, encoded by several polyubiquitin and ubiquitin fusion genes on a number of different autosomes (Webb *et al.*, 1990), the other two ubiquitin-system genes cloned thus far are both located on X. The GdX gene (HGMW symbol DXS254), with extensive homology to ubiquitin, has been localized onto Xq28 (Toniolo *et al.*, 1988). Moreover, the gene for one of the human ubiquitin-activating enzymes (E1, HGMW gene symbol UBE1) has been assigned to the X chromosome (Ohtsubo and Nishimoto, 1988; Kudo *et al.*, 1991), more precisely to Xp11.2–p11.4 (Zackenhaus and Sheinin, 1990; Handley *et al.*, 1991; McGrath *et al.*, 1991).

Duplication of HHR6

In the lower eukaryotes (*S. cerevisiae, S. pombe,* and *D. melanogaster*), we could identify only a single *RAD6* locus situated on an autosome (Reynolds *et al.*, 1990; Koken *et al.*, 1991a). As calculated from divergence data,

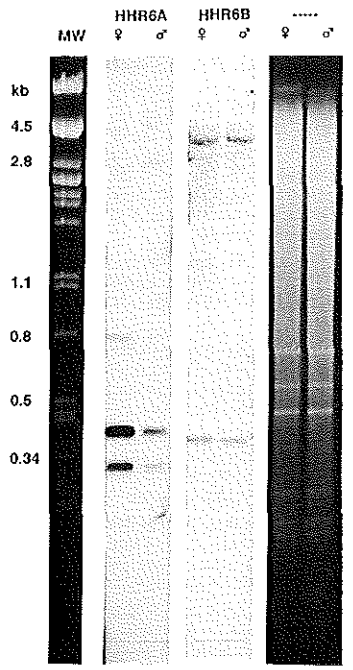


FIG. 3. Southern blot analysis of genomic liver DNA from a male and a female mouse. MW: Molecular weight marker, i.e., phage λ DNA digested with *Pst*I. Left: The autoradiogram of a blot with *Hin*dIII + *Eco*RI + *Bam*HI triply digested mouse DNA hybridized with a human *HHR6A* cDNA probe. Middle: An autoradiogram of the same blot hybridized with a human *HHR6B* cDNA probe. Right: A photograph of the ethidium-stained genomic gel. The probes used and the ♂ (male) or ♀ (female) sex of mouse from which the DNA was isolated are indicated above the autoradiogram.

the two *HHR6* genes in human and mouse (unpublished data) may have arisen from a gene duplication event in the Jurassic era about 200 million years ago, early in the history of mammals, i.e., well before the separation of evolutionary lines leading to rodents and primates. Duplication has several advantages and is more often found for essential genes. One advantage could be that it permits differential gene regulation and/or functional divergence of the proteins.

Finally, the synteny conservation for the X chromosome between mouse and man as found for *HHR6A* supports Ohno's law that there is a strong selection against chromosomal rearrangements involving the sex chromosomes and autosomes (Ohno, 1969; Nadeau, 1989).

The Chromosomal Context of HHR6A and HHR6B; Possible Involvement of HHR6 in Human Disorders

Yeast *rad6*Δ mutant cells show a very pleiotropic phenotype, with sensitivity to many DNA damaging agents, a defect in postreplication repair, no induced mutagenesis, and a complete lack of sporulation. In human, only cells of a single syndrome are known to be affected in postreplication repair: the variant complementation group of the rare DNA repair disorder, xeroderma pigmentosum (XP) (Lehmann *et al.*, 1975). In this complementation group, constituting about 30% of all XP patients, no indications favoring an X-linkage have been found. This renders it unlikely that *HHR6A* is the gene responsible for this disorder. However, the *HHR6B* gene remains a possible candidate, although cells from XP variant patients have an elevated frequency of uv-induced mutations, and in that respect differ from the yeast phenotype (Maher *et al.*, 1976; Myhr *et al.*, 1979). A systematic search for abnormalities in DNA, mRNA, or protein structure or expression in (families of) XP variant patients should resolve this issue. In addition, two mammalian postreplication repair-deficient cell mutants that are potential *HHR6A* mutants have been characterized, UV1 of Chinese hamster origin (Hentosh *et al.*, 1990) and SVM (derived from Indian Muntjac) (Pillidge *et al.*, 1986).

Two human disorders have been assigned to the q24–q25 region of the X chromosome where *HHR6A* is located (Human Gene Mapping 10 and 10.5): first, the X-linked lymphoproliferative syndrome, which results in fatal infectious mononucleosis, hypogammaglobulinemia, and malignant lymphoma—cells from these patients seem to be disturbed in the appropriate immune response to Epstein–Barr virus (Skare *et al.*, 1989); and second, the oculocerebrorenal syndrome of Lowe, characterized by congenital cataract, mental retardation, and a defective renal tubular function (Reilly *et al.*, 1988). Although these diseases apparently map to the same region of the X chromosome as *HHR6A*, to our knowledge there is no evidence for a DNA repair defect associated with any of them. A final X-linked disorder not assigned to a certain subchromosomal region with a possible defect in DNA repair is the N syndrome. Patients suffering from this disease display mental retardation, malformations, development of T-cell leukemia, and chromosome breakage (Floy *et al.*, 1990). The last two phenotypic traits resemble those of the DNA repair disorder Fanconi anemia. Although it has been proposed that malfunction of DNA polymerase α (X-linked) could be the cause for N syndrome, the evidence is based on aphidicolin inhibition studies which provide only indirect indications.

HHR6B resides in a region of chromosome 5 containing a large cluster of growth factor genes, i.e., the genes for IL3, IL4, IL5, and CSF2 (Human Gene Mapping 10 and 10.5). These genes have recently been assigned to chromosome 11 in mouse (ATCC/NIH, 1990). The possibility exists that—due to synteny conservation—the murine *HHR6B* gene is also located on this chromosome. *In situ* hybridization should be performed to verify this proposition. Thus far, the human 5q23–q31 region has not been associated with any hereditary disease (Human Gene Mapping 10 and 10.5). To our knowledge, the only syndrome to be linked to chromosome 5 with a possible defect in DNA repair is Gardner syndrome (HGMW gene symbol APC), a dominant disorder with a predisposition to cancer, especially of the large intestine. It has been found that cells from some of these patients are

hypersensitive to uv light, X rays, and mitomycin C (Little *et al.*, 1980); however, thus far no specific repair defect has been reported in cells of these patients (Henson *et al.*, 1983). Because postreplication repair was not investigated, a possible involvement of *HHR6B* in this disorder is not ruled out on the basis of these findings. However, the recent cloning of the APC gene, responsible for familial adenomatous polyposis (FAP) and Gardner syndrome (Kinzler *et al.*, 1991), excludes any link with *HHR6B*.

It is reasonable to assume that *HHR6A* and *HHR6B* have largely overlapping functions in view of their high sequence homology and their ability to complement yeast *rad6* repair functions. This functional redundancy would require the unlikely event of simultaneous inactivation of both *HHR6* genes for clinical symptoms to become manifest. Alternatively, considering the pleiotropic and severe yeast *rad6* phenotype, it is possible that inactivation of one or both *HHR6* genes is lethal in mammals. These propositions could provide an explanation for the possible absence of known disorders associated with *HHR6*. The recently developed methodology of targeted gene replacement in mouse embryonal stem cells (Capecchi, 1989) opens the possibility of generating *HHR6*-defective cell lines or mice in the laboratory. In that way the role of these genes at the level of the cell and organism can be established.

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Chapter VI

Expression of the human ubiquitin-conjugating DNA repair enzymes HHR6A and 6B suggests a role in spermatogenesis and chromatin modification

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Expression of the Ubiquitin-Conjugating DNA Repair Enzymes HHR6A and B Suggests a Role in Spermatogenesis and Chromatin Modification

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RAD6, a member of the expanding family of ubiquitin-conjugating (E2) enzymes, functions in the so-called "N-rule" protein breakdown pathway of *Saccharomyces cerevisiae*. *In vitro*, the protein can attach one or multiple ubiquitin (Ub) moieties to histones H2A and B and trigger their E3-dependent degradation. Rad6 mutants display a remarkably pleiotropic phenotype, implicating the protein in DNA damage-induced mutagenesis, postreplication repair, repression of retrotransposition, and sporulation. RAD6 transcription is strongly induced upon UV exposure and in meiosis, suggesting that it is part of a damage-induced response pathway and that it is involved in meiotic recombination. It is postulated that the protein exerts its functions by modulating chromatin structure. Previously, we have cloned two human homologs of this gene (designated HHR6A and HHR6B) and demonstrated that they partially complement the yeast defect. Here we present a detailed characterisation of their expression at the transcript and protein levels. Both HHR6 proteins, resolved by 2-dimensional immunoblot analysis, are expressed in all mammalian tissues and cell types examined, indicating that both genes are functional and constitutively expressed. Although the proteins are highly conserved, the UV induction present in yeast is not preserved, pointing to important differences in damage response between yeast and mammals. Absence of alterations in HHR6 transcripts or protein upon heat shock and during the cell cycle suggests that the proteins are not involved in stress response or cell cycle regulation. Elevated levels of HHR6 transcripts and proteins were found in testis. Enhanced HHR6 expression did not coincide with meiotic recombination but with the replacement of histones by transition proteins. Immunohistochemistry demonstrated that the HHR6 proteins are located in the nucleus, consistent with a functional link with chromatin. Electron microscopy combined with immunogold labeling revealed a preferential localisation of HHR6 in euchromatin areas, suggesting that the protein is associated with transcriptionally active regions. Our findings support the idea that both HHR6 genes have overlapping, constitutive functions related to chromatin conformation and that they have a specific role in spermatogenesis, involving Ub-mediated histone degradation.

INTRODUCTION

Saccharomyces cerevisiae rad6 mutants display an extremely pleiotropic phenotype: hypersensitivity to a remarkably wide spectrum of genotoxic agents, defects in damage-induced mutagenesis, postreplication repair, repression of retrotransposition and sporulation (for a recent review see Lawrence, 1994). The *RAD6(UBC2)* gene encodes a 172-amino-acid protein (*M*, 19.7 kDa) (Reynolds *et al.*, 1985) which was shown to be a ubiquitin-conjugating (E_2) enzyme (Jentsch *et al.*, 1987). Ubiquitin (Ub), a highly conserved 76-amino-acid polypeptide, can be attached to many cellular proteins, after which it functions as a signal for selective degradation, (re)folding, or stabilisation. This implicates the protein in a variety of processes, ranging from cellular stress response, mitochondrial protein import, and peroxisomal biogenesis to apoptosis (for reviews see: Cie-

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chanover, 1994; Finley and Chau, 1991; Hershko and Ciechanover, 1992; Jentsch, 1992; Jentsch *et al.*, 1990; Rechsteiner, 1988). Ubiquitination occurs by a cascade of reactions: ATP-dependent activation of Ub by binding to a Ub-activating enzyme (E1), transfer of the Ub moiety to a member of a family of Ub-conjugating enzymes (UBC, or E2 enzymes), which in turn covalently attaches its activated Ub to the target protein, with or without the assistance of a ubiquitin–protein ligase (E3). Experiments have shown that yeast RAD6 is able to link *in vitro* one (Jentsch *et al.*, 1987) or multiple (Sung *et al.*, 1988) ubiquitin molecules to a specific lysine residue in the carboxyl terminus of histones H2A and H2B (Thorne *et al.*, 1987). *In vitro*, the presence of an E3 molecule causes tagged artificial substrates to be quickly broken down (Dohmen *et al.*, 1991; Sung *et al.*, 1991). RAD6 is one of the E2s involved in breakdown of proteins via the so-called "N-rule" (Bachmair *et al.*, 1986; Dohmen *et al.*, 1991; Varshavsky, 1992; Watkins *et al.*, 1993). The N-rule, which is conserved throughout evolution from bacteria to mammals, is derived from the observation that the N-terminal amino acid of a protein is one of the determinants for its *in vivo* half-life (Bachmair *et al.*, 1986). When histones are also one of the targets of RAD6 *in vivo*, it is likely that this E2 enzyme mediates the chromatin changes required for postreplication repair and sporulation.

Via evolutionary walking using nucleotide homology we have isolated homologous *RAD6* genes from *Schizosaccharomyces pombe* (named *rhp6*$^+$) (Reynolds *et al.*, 1990), *Drosophila melanogaster* (*Dhr6*) (Koken *et al.*, 1991a), and a duplicated locus in man, *HHR6A* and *HHR6B* (Koken *et al.*, 1991b). *HHR6A* is located on the X chromosome at position q24–25, and *HHR6B* on 5q23–31 (Koken *et al.*, 1992). The RAD6 homologs of these species share >68% sequence identity. A unique feature of yeast RAD6, not found in the other homologs, is the presence of a 20-amino-acid acidic C-terminus, which is essential for sporulation and the polyubiquitination of histones *in vitro*, but not for monoubiquitination (Sung *et al.*, 1988). Due to their high structural conservation, all *RAD6* homologues are able to functionally correct yeast *rad6*Δ mutants with respect to UV survival and UV-induced mutagenesis, but not with respect to the defect in sporulation, which requires the acidic tail (Koken *et al.*, 1991a,b; Reynolds *et al.*, 1990). *RAD6* in yeast is UV-inducible consistent with the idea that RAD6 plays an important role in an inducible cellular response to genotoxic insult. In addition, the gene is induced in meiosis coinciding with meiotic recombination. In mammals damage-induced cellular responses have received considerable attention in recent years. Links observed with cell cycle progression, DNA repair processes and genome stability, replication and transcription, as well as apoptosis have been the topic of intensive research. Therefore, it was of considerable interest to investigate whether the strong structural and functional conservation of RAD6 also includes its gene-regulatory properties. In view of the presumed role of RAD6 in chromatin transactions and sporulation we examined in detail the involvement of HHR6 in spermatogenesis, in which meiotic recombination and extensive chromatin remodeling take place. Finally, this study intended to provide clues as to whether the two highly homologous human genes have acquired distinct functions detectable in the way they are expressed, thus giving an evolutionary rationale for the gene duplication that has occurred at the beginning of the origin of mammals.

MATERIALS AND METHODS

RNA and DNA Manipulations

Standard DNA manipulations were done as described (Sambrook *et al.*, 1989). Total RNA was isolated by means of the LiCl/urea precipitation procedure (Sambrook *et al.*, 1989). RNA samples were fractionated on 1% agarose formaldehyde gels (Fourney *et al.*, 1988) and transferred onto nylon membranes (Zetaprobe, Hybond). RNA hybridisation's took place overnight at 56°C in 3× SSC and washings until 0.3× SSC were performed as detailed elsewhere (Sambrook *et al.*, 1989). Although HHR6A and B are very homologous at the protein level the genes do not show any cross-hybridisation at the nucleotide level under the conditions specified above (as shown by Figs. 1A and 2A).

UV-Light and Heat Shock Treatment

HeLa cells, primary human fibroblasts, or primary human keratinocytes (Gibbs *et al.*, 1990) were grown in a 1:1 mixture of Dulbecco–Vogt medium (DMEM) and Ham's F10 medium supplemented with 10% foetal calf serum, 0.75 mg/ml penicillin, 1.25 mg/ml streptomycin, and 2.92 mg/ml glutamine. When reaching near confluency cells were washed twice in phosphate-buffered saline (PBS) and irradiated with 1, 5, or 10 J/m^2. Keratinocytes were cultured as described (Gibbs *et al.*, 1990) (40 J/m^2). After UV exposure and two additional washings with PBS, the conditioned medium was added to the cells. For the heat-shock treatment, cells were placed at 41°C for 2 hr, after which they were returned to 37°C. The time point of irradiation or transfer to the 41°C incubator was considered $t = 0$. At different time points cells were isolated by scraping, washed in PBS, and quickly frozen in liquid nitrogen.

Cell Cycle Synchronisation by Double Thymidine Block

The synchronisation of HeLa cells was done with a double thymidine (TdR) block (Galavazi *et al.*, 1966). The synchronisation was checked on a fluorescence activated cell sorter; a cell sample was stained with propidium iodide and the DNA content was determined (Vindelov *et al.*, 1983). The S phase peak appeared 1 hr after the second TdR block had been released. After 8 hr the peak of G2 cells appeared (74% pure), after 9 hr that of mitotic cells (58% pure) ap-

peared, and finally after 14.5 hr the G1 cells (84% pure) could be collected.

Isolation of Different Cell Types from Rat Testis

Spermatocytes, round and elongating spermatids. Spermatogenic cells were isolated from 40- to 50-day-old rats using collagenase and trypsin treatment, and purified using sedimentation at unit gravity (StaPut procedure) followed by density gradient centrifugation (Percoll gradients) (Grootegoed *et al.*, 1986). The purity of the cell preparations was analysed using DNA-flow cytometry as described (Toebosch *et al.*, 1989); the preparations enriched in spermatocytes and spermatids contained more than 90% of cells with a 4 or 1 C amount of DNA per cell, respectively.

Sertoli cells from young (21-day-old) and adult rats. Highly purified Sertoli cells were isolated from immature rats as described (Themmen *et al.*, 1991). Essentially the same method was used to obtain a preparation of adult Sertoli cells which was contaminated with 50% germinal cells.

The mouse Sertoli cell line TM4, which was used in some of the experiments, is described elsewhere (Mather, 1980).

Peptide Synthesis and Coupling to Affi-gel 10

Because the original antiserum against yeast RAD6 (Morrison *et al.*, 1988) shows some cross-reactivity with higher molecular weight proteins next to the 17-kDa HHR6 proteins, this serum (designated total RAD6 antiserum) was affinity purified with an N-terminal HHR6 oligopeptide coupled to Affi-gel. This serum (designated AP-RAD6 antiserum) reacts almost exclusively with a major band of 17 kDa, and a faint band of 25 kDa, which may represent a ubiquitinated form of HHR6 (Fig. 3D). The N-terminal HHR6 peptide MSTPARRRLMRDFKC, conserved in all the RAD6 homologous proteins, was prepared by the 9-fluorenylmethoxycarbonyl (Fmoc) method using solid-phase synthesis according to Merrifield (1963) on an automated peptide synthesiser (NovaSyn-Crystal, Novabiochem). After completion of the synthesis, the peptide was cleaved from its support and amino acid side-chain protecting groups were removed. Peptides were purified by high-performance liquid chromatography on a reverse-phase C_{18} column (Merck, LiChroCart) using a gradient of 0–60% acetonitril/H_2O in 0.1% trifluoracetic acid. Amino acid analysis was performed (473A protein sequencer, Applied Biosystems) to ensure correct residue composition. Coupling to Affi-gel 10 (Bio-Rad) was according to the manufacturer's description in a 0.1 *M* Mops buffer, pH 7.5, for 2 hr at 4°C. To block any remaining active esters the slurry was incubated with 0.1 vol of 1 *M* ethanolamine·HCl for 1 hr at 4°C. After extensive washing with water the column was equilibrated with PBS. The sample was loaded onto the column in PBS. After extensive washing with PBS, column elution was done with 0.1 *M* glycine, 0.5 *M* NaCl, pH 2.5, and the fractions were immediately neutralised in 0.5 *M* phosphate buffer, pH 7.2.

Immunohistochemistry

Light microscopy. Small tissue samples (ϕ0.5cm) were extracted from mouse or rat and fixed in 4% paraformaldehyde–PBS for 4 hr at 4°C, dehydrated, and embedded in paraffin (Fluka, Switzerland) according to standard procedures (Zeller *et al.*, 1987, 1991). (For optimal detection of RAD6 protein in tissue sections we determined that the fixation time should not exceed 4 hr). Seven-micrometer tissue sections were made, collected on gelatin\CrK-$(SO_4)_2 \cdot 12H_2O$-coated microscopic slides, and dried overnight. After deparaffination in a xylol–ethanol–PBS sequence, endogenous peroxidase was blocked by incubation in 100% methanol/1% H_2O_2 for 30 min. Slides were rinsed in PBS/0.5% Tween 20, 3× 5 min, and incubated with primary antibody (total anti-RAD6 serum 1:600, affinity-purified serum 1:40) in a moist incubation chamber at room temperature (RT) for 1 hr. After extensive washing (3× 5 min) with PBS/Tween, the slides were incubated with horseradish peroxidase-labeled swine anti-rabbit conjugate (DAKO, Denmark) (1:100 diluted) as second step antibody for 45 min at RT. After washing (3× 5 min PBS/Tween) peroxidase was visualised with 0.1% 3,3′-diaminobenzidine·HCl (Serva, FRG), 0.01% H_2O_2. Counterstaining was done with haematoxilin for half a minute, after which the slides were passed through a PBS–ethanol–xylol sequence and embedded in Entellan (Merck, FRG).

Immunoelectron microscopy. Mouse testis were fixed in 0.1 *M* phosphate buffer, pH 7.3, containing 2% paraformaldehyde. After fixation for 1 hr at 4°C, samples were embedded in Lowicryl K4M (Roth *et al.*, 1981). For antigen localisation on thin sections the immunogold technique was used (Willemsen *et al.*, 1988). Subcellular quantitation of the gold particles was done in Leydig cells, according to Willemsen *et al.* (1991).

For light as well as electron microscopy two immunocytochemical controls were always included: (a) omission of the primary antibody incubation step and (b) incubation with normal rabbit serum as substitution for the primary antibody. Background was negligible.

SDS–PAGE Gel Electrophoresis and 2-Dimensional Gel Electrophoresis

Mouse or rat tissues were fragmented under liquid nitrogen in a mortar. The fragments or cultured cells were collected in PBS at 4°C and subjected to 10 cycles of 10-sec sonification at full amplitude at 4°C. These crude extracts were used in all experiments. The preparation of nuclear and cytoplasmic extracts was performed following three different methods: the methods of Dignam (Dignam *et al.*, 1983) and Lee (Lee *et al.*, 1988) and a combination of methods described by Lue (Lue and Kornberg, 1987) and Radke (Radke *et al.*, 1983). Protein concentration was determined with the BCA assay (Pierce) as described by the manufacturer. Two-dimensional gel electrophoresis was principally done according to the Bio-Rad mini-protean 2-D cell in-

struction manual and as described by Luider *et al.* (1992). Gels with a length of 6.5 cm were prepared in glass tubes 2-mm diameter. Next, 0.25 ml Bio-lyte 3/5 and 0.25 ml Bio-lyte 4/6 ampholytes (Bio-Rad), 2 ml 10% Triton X-100, 5.5 g urea (Merck), 1.33 ml acrylamide solution (28.3% acrylamide and 1.62% piperazinediacrylamide (Bio-Rad)), 1.97 ml distilled water, 10 μl 10% ammonium persulfate (Bio-Rad), and 10 μl *N,N,N',N'*-tetramethylethylenediamine (Bio-Rad) were mixed and allowed to polymerise in the glass tubes at 37°C for about an hour. The gels were covered with overlay buffer (9 *M* urea, 0.8% Bio-lyte 3/5, 0.2% Bio-lyte 5/7, bromophenol blue) and preelectrophoresis was performed at 200, 300, 400 V for 10, 20, and 20 min, respectively. As upper chamber buffer a 20 m*M* NaOH solution was used, whereas 100 m*M* H_3PO_4 served as lower chamber buffer.

Subsequently, samples were diluted with an equal volume of sample buffer (2% SDS, 10% glycerol, 62.5 m*M* Tris/HCl, pH 6.8, bromophenol blue, 0.1% dithiotreitol (DTT)), boiled for 3 min, and chilled on ice. Samples were diluted with an equal volume of lysis buffer (0.5 *M* urea, 2% Triton X-100, 0.1% DTT, 1.6% Bio-lyte 3/5, 0.4% Bio-lyte 5/7 in distilled water). One hundred microliters containing 50 μg protein was loaded under 40 μl of 1:1 diluted overlay buffer and electrophoresis was performed for 3.5 hr at 600 V.

The gels were gently removed from the glass tubes and equilibrated against sample buffer for about 90 min until the pH indicator in the acid part of the tube gel became blue. The tubes were directly loaded on a 2.25-mm-thick 15% SDS–polyacrylamide minigel (Bio-Rad) and electrophoresis was performed with 0.1 *M* sodium acetate in the anode buffer (Christy *et al.*, 1989) at 100 V (stacking) and subsequently 150 V until the bromophenol blue reached the bottom of the gel. Blotting was done onto 0.45-μm polyvinylidenedifluoride (PVDF) membranes (Millipore), according to the manufacturer's description, using electrotransfer. Blots were blocked in nonfat milk for 1 hr and incubated with the primary antibody diluted in nonfat milk (anti-yeast RAD6, 1:2000 or AP-RAD6 antiserum, 1:100) at 4°C, overnight. After extensive washing with PBS/0.5% Tween 20, the second antibody (goat anti-rabbit antibodies, alkaline phosphatase labeled (TAGO, Inc.)) was incubated for 1 hr at 4°C, in a 1:1000 dilution in PBS/Tween. After several additional washings with PBS/Tween the antigen–antibody complexes were visualised with the staining method described by Blake (Blake *et al.*, 1984). Carbamylate carbonic anhydrase (CA) (Pharmacia lab.) and 2-D SDS–PAGE standards (Bio-Rad) were used as standards for isoelectrofocusing. Prestained protein molecular weight markers (Gibco\BRL) were used in the second dimension.

For normal SDS–polyacrylamide gel electrophoresis 1.5-mm-thick 11 or 15% gels were used with a 4% stacking gel. About 20 μg protein extract was loaded, and electrophoresis was performed with 0.1 *M* sodium acetate in the anode buffer (Christy *et al.*, 1989) at 50 V (stacking) and 150 V (running). Blotting, antibody incubations, and staining were done as described above.

In Vitro Transcription and in Vitro Translation

The construct H28^{ccc}, containing a 1.7-kb cDNA of human *HHR6A* (Koken *et al.*, 1991b), was linearised with *Xba*I. The H28^{ccc} construct differs from the previously reported *HHR6A* sequence in that at nucleotide position 146 (Koken *et al.*, 1991b), a mutation derived from cDNA amplification in *Escherichia coli* or as PCR artefact (resulting in the reported guanine) has been changed into the genomically encoded adenine, resulting in a change of the previously reported glycine into aspartic acid (amino acid position 49). pPHB2 containing an 0.8-kb *Eco*RI fragment, harbouring the complete open reading frame of *HHR6B* (Koken *et al.*, 1991b), was linearised with *Bam*HI. *In vitro* transcription as well as *in vitro* translation were done exactly as described (Promega, 1991).

RESULTS

UV-Induced Expression

In yeast, *RAD6* transcription is induced upon UV irradiation (≥sixfold) within 30 min after exposure (Madura *et al.*, 1990). To investigate whether this feature of *RAD6* is evolutionarily conserved, Northern blot analysis for *HHR6A* and *HHR6B* was performed on RNA from exponentially growing cells, UV irradiated with 1, 5, or 10 J/m^2, and harvested after different incubation periods. The blot was subsequently probed with the cDNA probes for *HHR6A* (1.7-kb *Sal*I fragment) and *HHR6B* (0.8-kb *Eco*RI fragment) (Koken *et al.*, 1991b), the UV-inducible collagenase or methallotheionin IIa genes (Angel *et al.*, 1986) (positive control for UV induction), and GAPDH (Benham *et al.*, 1984) (included as an internal standard for quantitation). HeLa cells, primary human fibroblasts, and primary foreskin-derived keratinocytes were investigated. The latter cell types are also subjected to UV irradiation in the body and are therefore the most relevant targets to study UV induction. Figure 1A shows the result of a "physiological" dose of 1 J/m^2 UV irradiation on primary fibroblasts. When corrected for the slight variation in RNA amounts per lane (see GAPDH hybridisation) it appears that irradiation did not result in a significant increase of human *HHR6A* (0.8 and 1.7 kb) and *HHR6B* (1.2 and 4.4 kb) mRNAs, under conditions where collagenase (Fig. 1A) and metallotheionin (not shown) displayed clear induction. Also experiments using other UV doses, time courses, and cells did not result in detectable induction (data not shown). To examine the possibility that HHR6A and 6B may be induced at the protein (translational) level instead of the mRNA level like in yeast, protein expression was studied using affinity-purified anti-yeast RAD6 antiserum. Western analysis after 10 J/m^2 UV irradiation of HeLa cells (Fig. 1B) failed to reveal a significant increase in the amount of the 17-kDa HHR6 proteins (for details on the antiserum see below, and under Materials and Methods). We conclude that in contrast to *S. cerevisiae* neither of the human genes is inducible by UV.

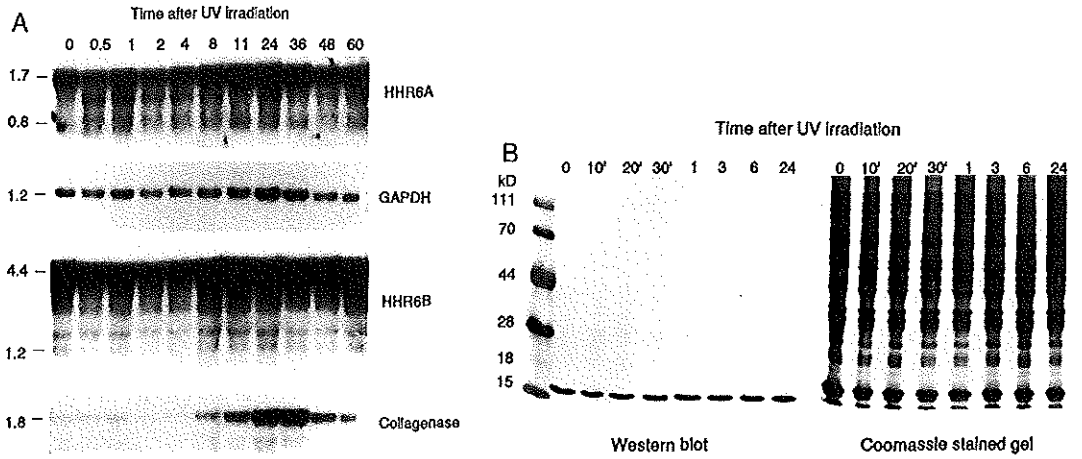


FIG. 1. (A) Northern blot analysis of *HHR6A* and *HHR6B* after UV irradiation. Primary fibroblasts were irradiated with 1 J/m^2 and total RNA was used for Northern blot analysis. The time scale is given in hours and the probes used are indicated at the right. Transcript length is given in kilobases to the left. A probe for GAPDH was used as control for the amount of RNA loaded (Benham *et al.*, 1984), whereas a collagenase probe was used to check the UV induction (Angel *et al.*, 1986). The probes for *HHR6A* and *HHR6B* do not show any crosshybridization as apparent from the different patterns of hybridisation. (B) Western blot analysis of HHR6 proteins in UV-irradiated HeLa cells. Subconfluent HeLa cells were UV irradiated with 10 J/m^2 and incubated at 37°C during the indicated chase time (given in minutes (') and hours). The blot (left) was incubated with affinity-purified anti-RAD6 (AP-RAD6) antiserum, whereas the Coomassie brilliant blue (CBB)-stained gel (right) serves as quantity control. Molecular weight markers are indicated to the left.

Heat Shock Treatment

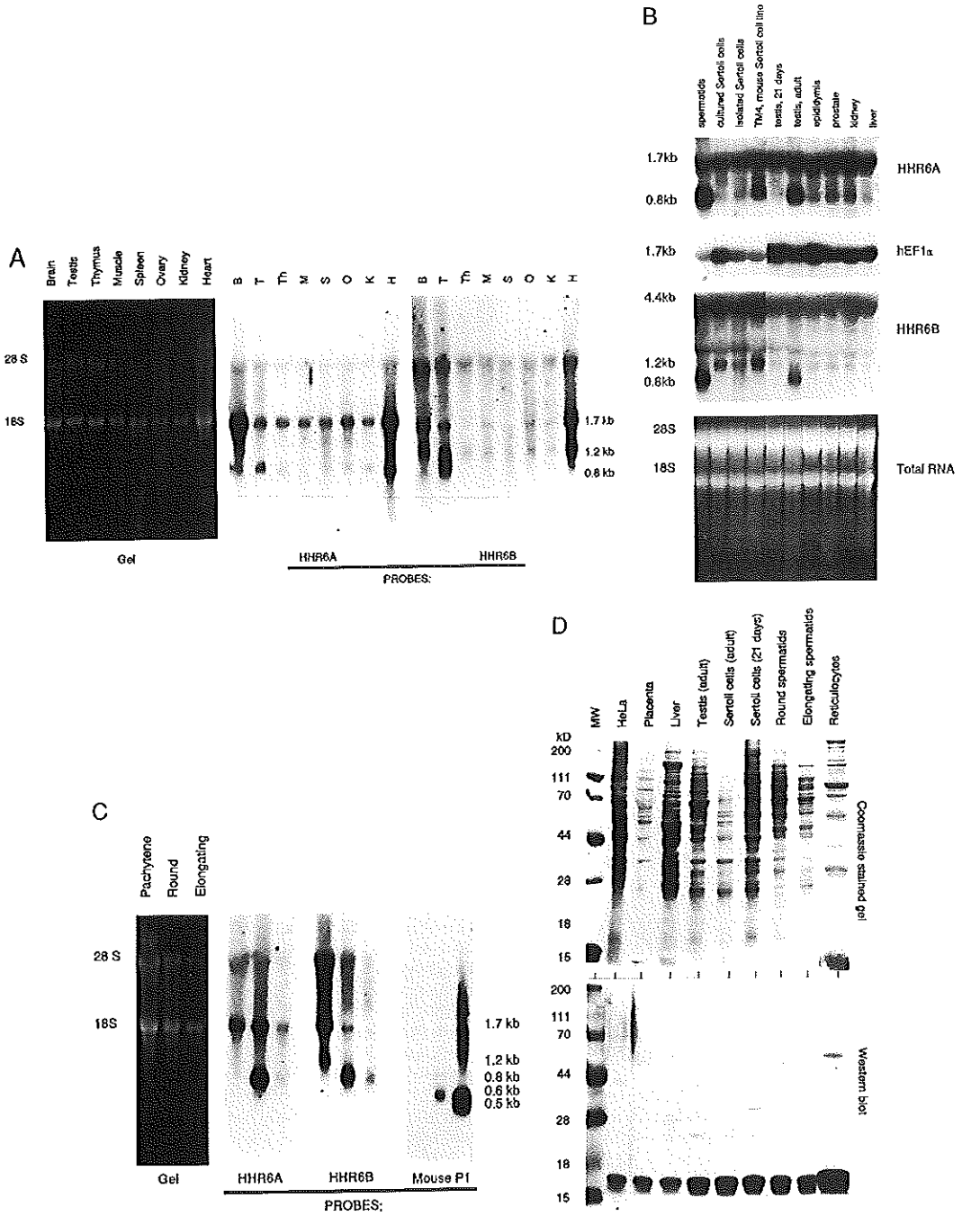
The ubiquitin system has an important function in selective degradation or refolding of misfolded proteins due to, for instance, heat shock. Several members of the UBC family are implicated in general stress responses and two of the three human ubiquitin genes are induced after heat shock (Fornace *et al.*, 1989). Therefore it may be that also *HHR6A* or *B*, as they are probably part of the protein degradation machinery, are inducible by such a treatment. mRNA and protein expression were analysed in HeLa cells cultured for 2 hr at 41°C. No significant increase in *HHR6* transcripts could be detected by Northern blot analysis under conditions that clearly induced ubiquitin mRNA. Similarly, no obvious enhancement of HHR6 proteins was observed by Western blot analysis (data not shown).

Expression during Mitotic Cell Cycle

Several lines of evidence associate RAD6 with chromatin remodeling. To investigate whether the human homologues are involved in any of the chromatin transitions which occur during the mitotic cell cycle, HeLa cells were synchronised by a double thymidine block, and mRNA and protein expression were analysed following release of the cells. The various stages of the cell cycle were monitored by FACS analysis. No substantial differences during the mitotic cell cycle could be registered in the quantities of both *HHR6* mRNAs nor proteins (data not shown).

Tissue-Specific Expression of HHR6A and HHR6B

To study tissue-specific differences in *HHR6A* or *HHR6B* expression, Northern blots containing total RNA from various organs of mouse and rat were hybridised with mouse HHR6 probes designated: *MHR6A* (for mouse homolog of RAD6) (1.7-kb insert of clone B4) and *MHR6B* probe (830-bp *Eco*RI fragment of clone 44) (the isolation and characterisation of the mouse *MHR6A* and *MHR6B* will be described elsewhere; manuscript in preparation) (Figs. 2A and 2B). The *MHR6A* probe recognises mRNA species with the same length as in human (1.7 and 0.8 kb), whereas the *MHR6B* probe in mouse and rat visualises 1.7-, 1.2-, and 0.8-kb transcripts. In analogy with the human genes we have indications that the different mouse transcripts result from differential polyadenylation (data not shown). Overall, mRNA amounts of both genes do not vary much between the organs tested. Three tissues, however, have significantly higher levels of transcript: brain, heart, and testis. The 0.8-kb *MHR6A* and 0.8- and 1.2-kb *6B* transcripts seem significantly elevated in testis and heart (and brain for *MHR6B*). In view of the involvement of yeast RAD6 in sporulation and the induction of RAD6 transcription in meiosis it was


A
Brain
Testis
Thymus
Muscle
Spleen
Ovary
Kidney
Heart
28 S
18S
Gel
B T Th M S O K H
1.7 kb
1.2 kb
0.8 kb
HHR6A
HHR6B
PROBES:
B
spermatids
cultured Sertoli cells
isolated Sertoli cells
TM4, mouse Sertoli cell line
testis, 21 days
testis, adult
epididymis
prostate
kidney
liver
1.7kb
0.8kb
HHR6A
hEF1α
4.4kb
1.2kb
0.6kb
HHR6B
28S
18S
Total RNA
C
Pachytene
Round
Elongating
28 S
18S
1.7 kb
1.2 kb
0.8 kb
0.6 kb
0.5 kb
Gel
HHR6A
HHR6B
Mouse P1
PROBES:
D
kD
MW
HeLa
Placenta
Liver
Testis (adult)
Sertoli cells (adult)
Sertoli cells (21 days)
Round spermatids
Elongating spermatids
Reticulocytes
200
111
70
44
28
18
15
Coomassie stained gel
Western blot

of interest to investigate the *HHR6* expression in testis in more detail.

Testis-Specific Expression of HHR6 mRNAs

The mammalian testis is organised in tubular structures surrounded by peritubular myoid cells, hormone producing Leydig cells, and blood vessels (for a general review see Johnson and Everitt, 1984). In the tubules Sertoli cells support the developing germ cells. Complicated processes of differentiation, growth, and mitotic and meiotic divisions take place when spermatogonia develop into spermatozoa. After several mitotic divisions the spermatogonia differentiate and give rise to primary spermatocytes. These cells undergo meiotic divisions which convert them first into secondary spermatocytes and then into round spermatids. Subsequent differentiation results in elongated spermatids and finally spermatozoa, which have lost most of their cytoplasm and possess highly compacted DNA. Dramatic changes in chromatin composition occur throughout spermatogenesis, starting with synthesis of testis-specific histones in spermatocytes and culminating in the total replacement of histones by transition proteins and ultimately by protamines (Bucci *et al.*, 1982; Smith *et al.*, 1992).

To examine *HHR6* expression during this process, germ cells and Sertoli cells of rat testis were isolated and tested for *HHR6* mRNA and protein expression (Figs. 2B–2D). RNA from testes of 21-day-old rats, in which spermatogenesis is not yet complete and only a small number of round spermatids is present, did not contain detectable amounts of the abundant testis-specific 0.8-kb transcripts (Fig. 2B). To determine when these transcripts appear during spermatogenesis, *HHR6* expression was followed in testis of young rats during the first 58 days of postnatal life. Increase of 0.8-kb transcripts takes place around Days 28–34 after birth of the rat, at the time when round and elongating spermatids become abundant (data not shown). Figure 2C shows that the highly expressed 0.8-kb mRNAs of both *HHR6A* and *HHR6B* are found mainly in round spermatids with a low level of expression in elongating spermatids. As a control the elongating spermatids are shown to contain protamine 1 mRNA. The 1.2-kb *HHR6B* transcript is elevated in Sertoli cells (Fig. 2B), and both the 1.2- and the 1.7-kb mRNA species in pachytene spermatocytes (Fig. 2C), whereas they are absent in the haploid cell types.

For several other genes, the observation has been made that although significant amounts of mRNA are present in the testis no corresponding protein can be detected. It is speculated that these transcripts result from dysregulated gene expression due to the extensive chromatin remodeling taking place in spermatogenesis (reviewed by Ivell, 1992). To examine whether both *HHR6* mRNAs in testis are translated into protein, Western blot analysis was performed. Total cell extracts of different tissues and of various cell types were incubated with affinity-purified anti-yeast RAD6 antibody (AP-RAD6 antibody). This antiserum detects exclusively a major protein band of 17 kDa, the calculated molecular weight of HHR6A/B (Koken *et al.*, 1991b), in addition to a faint band of 25 kDa, which may represent a ubiquitinated form of HHR6 (Figs. 2D and 3). HHR6 quantities vary from tissue to tissue; reticulocytes and adult Sertoli cells contain high amounts, whereas immature Sertoli cells and liver harbour relatively small quantities. (Compare also the amounts of protein loaded in each lane, Fig. 5D, upper panel.) HHR6 proteins are also clearly detected in round and elongating spermatids.

Identification of HHR6A and HHR6B in Total Protein Extracts and Determination of Relative Amounts

The two highly homologous and similar-sized HHR6 proteins are not separated in one dimension (Fig. 2D). To verify that both 17-kDa proteins are synthesised and to determine their relative abundance in total HeLa cell extracts, 2-D gel electrophoresis followed by immunodetection with the polyclonal anti-yeast RAD6 serum was applied. Figure 3 (top panel, arrowheads) shows the presence of two 17-kDa polypeptides visualised by the total anti-yeast RAD6 antiserum. (The other dots present on the immunoblot probably represent other E2 enzymes recognised by the polyclonal anti-yeast RAD6 antiserum.) To establish which of the 17-kDa spots represents human HHR6A (calculated p*I* 4.91 (Skoog and Wichman, 1986)) or HHR6B (calculated p*I* 4.76),

FIG. 2. (A) Expression of *HHR6A* and *HHR6B* mRNAs in mouse tissues. The tissues used are indicated (abbreviated) above the panels. Left panel: Total RNA gel to serve as control for the amount of RNA loaded. The two right panels: autoradiograms obtained after hybridisation with the probes indicated. (B) Expression of *HHR6A* and *HHR6B* mRNAs in testicular cells and tissues of the rat. The probes used are indicated to the right. The stained total RNA gel and hybridisation with a human elongation factor 1 α (hEFα) probe (Brands *et al.*, 1986) serve as quantity controls. The length of the different transcripts is indicated to the left. (Isolated and cultured) Sertoli cells and testis were isolated from 21-day-old rats and spermatids from 40- to 50-day-old rats; all other tissues from adult animals. (C) Northern blot analysis of *HHR6A* and *HHR6B* during the later stages of rat spermatogenesis. A blot containing total RNA isolated from different germ-line cells was hybridised with the indicated probes. The ethidium bromide-stained gel serves as quantity control, whereas hybridisation with a mouse protamine 1 gene probe (mouse P1) was used to check the purity of the cell fractions used (Hecht, 1986). The transcript length (kb) is indicated to the right. (D) Expression of HHR6 proteins in different human, rabbit, and rat tissues. A Western blot containing total protein extracts of rat, human (placenta/HeLa), and rabbit (reticulocyte lysate) cells or tissues was incubated with AP-RAD6 antiserum. Top: the CBB-stained gel; bottom: Western blot. Tissues are indicated above the CBB-stained gel. The molecular weight marker (MW) is indicated to the left.

the HeLa cell extract was mixed with [^{35}S]methionine-labeled HHR6A or HHR6B obtained through *in vitro* translation (Fig. 3, bottom four panels). The left spot comigrates with the *in vitro*-translated ^{35}S-labeled HHR6A, whereas the protein at the right coincides with the position of ^{35}S-labeled HHR6B. In support of this identification is our observation that the right dot (HHR6B) migrates at a slightly higher molecular weight than the left dot (HHR6A). This is consistent with the calculated molecular weight for both proteins (HHR6A 17.243, HHR6B 17.312). The two proteins

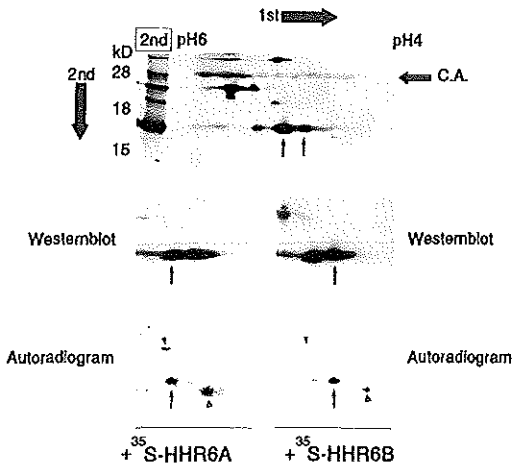


FIG. 3. Identification of HHR6A and HHR6B in total cell extracts by 2-dimensional gel electrophoresis. Top: Total HeLa extract was fractionated by 2-dimensional gel electrophoresis. From left to right the first dimension: isoelectrofocusing over a gradient from pH 6 to 4, and from top to bottom: size-fractionation by SDS–PAGE. The boxed "2nd" indicates the molecular weight marker for the second dimension given in kDa. C.A. marks the position of the carbamylated marker (Carbonic anhydrase, Bio-Rad), used as standard in IEF. The small 17-kDa band to the left of HHR6A and HHR6B (arrows) is a gel artefact which is sometimes encountered. Bottom 4 panels: Mixing experiment to prove the HHR6A and HHR6B identity. Total HeLa extract was mixed with [^{35}S]-methionine-labeled *in vitro* translated *HHR6A* (left) or *HHR6B* RNA (right) and separated in 2-dimensions. The gels were blotted onto PVDF membranes, and HHR6A and HHR6B were visualised with anti-yeast RAD6 antibodies (not affinity purified) (Western blot). The blots were subsequently exposed to Kodak XAR5 film, and the resulting autoradiogram is shown in the lower panels. The arrows indicate HHR6A (left two panels) and HHR6B (right two panels). The arrowhead indicates a form of HHR6A or HHR6B protein which was formed in the reticulocyte lysate, and which may represent a ubiquitinated form of HHR6: calculated *pI* 5.26 (HHR6A+ubiquitin) and *pI* 5.10 (HHR6B+ubiquitin) (Arnold and Gevers, 1990). The lower molecular weight spots on the autoradiogram probably represent breakdown products or are derived from incomplete synthesis of the HHR6 proteins.

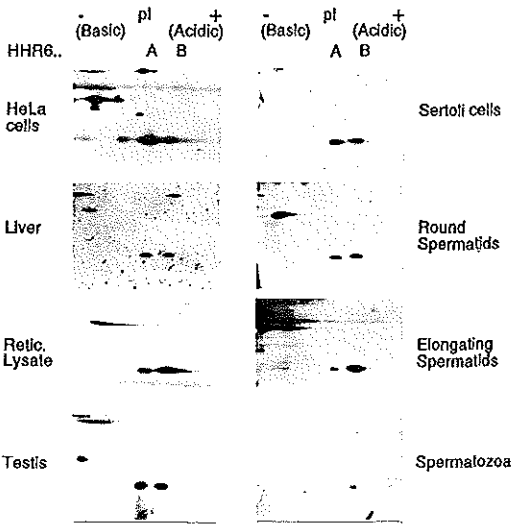


FIG. 4. HHR6A and HHR6B protein expression in different tissues. 2-Dimensional gel electrophoresis was performed to separate the two human HHR6 proteins in different human (HeLa/placenta), rabbit (reticulocyte lysate), and rat cells or tissues (young Sertoli cells, adult liver, testis, round spermatids, elongating spermatids, and spermatozoa). (A minor contamination with some elongating spermatids (or cytoplasmic fragments thereof) cannot be completely excluded in the spermatozoa fraction.) The cell or tissue source is indicated to the right and left. The direction of the pH gradient is given above the figure.

seem to behave on gel according to their calculated isoelectric points as judged by comparison with 2-D SDS–PAGE standards (Bio-Rad) and carbamylated carbonic anhydrase (Pharmacia). Minor modifications, such as phosphorylation or acetylation, which shift proteins in a pH gradient, are not entirely excluded. Preliminary *in vivo* phosphorylation experiments, however, do not provide indications that a significant fraction of one of the HHR6 proteins is phosphorylated.

Two-dimensional gels were also used to examine the tissue-specific expression of both HHR6 proteins. Figure 4 shows that the ratios between HHR6A and HHR6B proteins may vary significantly between different cells and tissues. HeLa cells have more HHR6A than HHR6B, total testis harbours equal amounts, whereas placenta contains more of the B protein. Both HHR6 proteins are present in round and elongating spermatids, and trace amounts of protein may be detected even in rat epididymal spermatozoa.

(Subcellular) Localisation of HHR6A and HHR6B

The affinity-purified antibodies were also used for tissue sections of different mouse organs. Cells with a clear,

mainly nuclear, staining reaction could be detected with this antiserum in all tissues examined (data not shown). In view of the RNA expression (Fig. 2) and the induction of yeast RAD6 during meiotic recombination, mouse testis was studied in more detail. In testis sections from 21-day-old mice only a weak reaction with the antibody was visible (data not shown). However, specifically in adult testis (Fig. 5D) strong positive cells were observed. Round spermatids which contain high amounts of *HHR6A* and *HHR6B* transcripts (Fig. 2) display a positive antibody staining (arrowheads), whereas elongating spermatids do not show a visible reaction.Some staining may also be present in the primary spermatocytes. Somewhat to our surprise, the strongest signal was detected in adult Sertoli cells (Fig. 5D). In addition, the Leydig cells in the interstitial tissue showed staining (Fig. 5D).

The immunohistochemical analysis of tissue sections examined by light microscopy strongly suggests that the HHR6 proteins are localised in the nucleus. To independently confirm and further extend this intracellular localisation, immunoelectron microscopy was used, applying different fixation and embedding conditions. Thin sections of mouse testis tissue were incubated with antiserum, and the reaction was visualised by immunogold labeling. As shown by Fig. 5A most HHR6 can be found in the Sertoli cell nucleus, whereas cytoplasm and the characteristic nucleolus (Fig. 5B) are almost devoid of gold particles. Leydig cells and to a somewhat lesser extent round spermatids (Fig. 5C) present a similar picture, although, like in light microscopy, the amount of protein detected is lower. Table 1 shows that the proteins are strongly enriched in the euchromatin regions of the nucleus. Heterochromatin and nucleolus are markedly devoid of gold particles. Similar observations were made in other cell types. The Sertoli cell in Fig. 5A may even provide evidence for clustering of gold particles in certain restricted areas of the nucleus. The Lowicryl-embedding procedure allows only the direct surface to react and therefore avoids penetration and accessibility problems. Therefore a quantitative analysis of gold particles is permitted. The subnuclear localisation of the protein in Leydig cells was determined by counting the grains in the different compartments. In these cells the distinction between eu- and heterochromatin can be made easily in these cells which display relatively high levels of RAD6 proteins (Fig. 5D).

DISCUSSION

The present report concerns a systematic analysis of the expression of two closely related HHR6 genes at the RNA and protein level. Our findings are relevant in two directions: the involvement of repair genes in the UV response in higher organisms and the relationship of HHR6 with modification of chromatin structure in the process of spermatogenesis.

In recent years, cellular responses to environmental genotoxic stresses have gained increasing interest. Exposure of cells to DNA injury triggers a cascade of reactions, including intricate signal transduction pathways resulting in altered expression of numerous genes. A universal response to inflicted gene damage in normal cells is arrest of cell cycle progression. This gives DNA repair mechanisms time to remove the DNA lesions before they can give rise to permanent mutations. In lower organisms several repair systems have been shown to be inducible by genotoxic agents. The SOS response, controlled by the LexA/RecA regulon, mediates a rapid activation of the main components of the nucleotide excision repair in *E. coli*. In yeast a specific subset of repair genes including RAD6 is damage-induced (for a review see Hoeijmakers, 1993a,b). Among the numerous mammalian damage-inducible genes analysed to date, however, no known repair genes have been recognised except for the O6G-methyl transferase. On the other hand, several members of the ubiquitin pathway including ubiquitin itself appeared to be induced upon various stress treatments (for a review see Fornace, 1992). The availability of a human repair gene, whose UV inducibility in yeast is well-documented, permitted for the first time investigations regarding to what extent the involvement of repair in this response is conserved in eukaryotic evolution. Careful analysis in a variety of human cells and utilizing different UV doses failed to provide indications for any significant UV inducibility of HHR6A nor HHR6B. This suggests that the DNA repair component of the UV responses in yeast and man are different. Our recent findings with another repair gene, RAD23, whose transcription is enhanced after UV irradiation in yeast, but not in man, confirm this idea (Madura and Prakash, 1990, and manuscript in preparation).

These findings support the notion that a fundamental difference exist in this regard between the unicellular organisms *E. coli* and yeast, and mammals. The relatively constant environment in which mammalian cells live may have obviated the need for a UV repair response.

Like with UV, we failed to find an induction of HHR6 RNA or protein upon heat shock and during the mitotic cell cycle. Instead Northern and immunoblot analysis demonstrate that both genes are constitutively expressed. These results are consistent with a function of HHR6 that does not involve the general stress response (which seems to require the ubiquitin-conjugating enzymes, UBC1, 4, and 5) nor cell cycle-related processes (in which UBC3 and 9 are thought to be implicated) (Ciechanover, 1994).

The two very similar 17-kDa HHR6 proteins were separated by 2-dimensional gel electrophoresis and visualised by Western blot analysis. Mixing with *in vitro*-labeled HHR6A or B protein permitted identification of each of the gene products. The results suggest that—except for a possible ubiquitination—no extensive posttranslational modifications detectable by 2-D gel electrophoresis occur in a significant fraction of the HHR6 molecules. Overall, the expression patterns of HHR6A and B ressemble each other, and on this basis we cannot assign a specific distinct function to each of the genes.

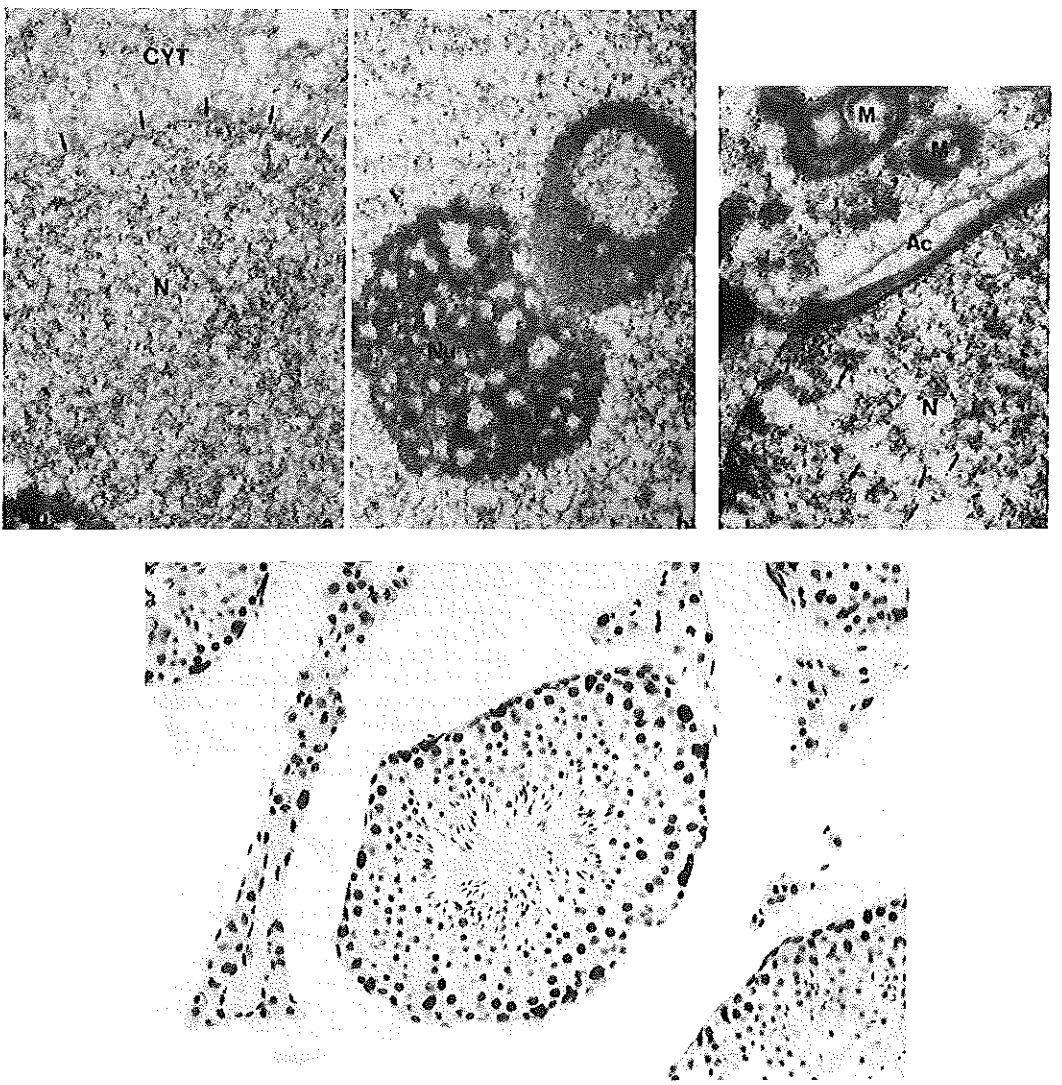


FIG. 5. Immunohistochemical detection of the HHR6 proteins by electron and light microscopy. Abbreviations used: N, nucleus; CYT, cytoplasma; Nu, nucleolus; M, mitochondrion; and Ac, acrosome. (A) Sertoli cell incubated with anti-RAD6 serum. The arrows indicate the nuclear membrane. (B) Higher magnification of A: Sertoli cell nucleolus. (C) Round spermatid incubated with anti-RAD6 antiserum. The arrows point to the gold particles found in the nucleus. (D) Light microscopic picture of a mouse testis cross section, incubated with anti-yeast RAD6 serum. Arrows point to the strongly positive Sertoli cell nuclei at the basement membrane of the tubule. Arrowheads indicate the round spermatids exhibiting a more weak, but clearly detectable, staining.

TABLE 1
Quantitation of HHR6 Protein in Leydig Cells

	Total no. of gold particles counted	Surface counted μm^2	No. of gold particles per μm^2	Factor of enrichment
Euchromatin	1820	87.1	20.9	21
Heterochromatin	43	14.0	3.1	3
Nucleolus	8	7.86	1.0	1

However, clear quantitative differences exist for the mammalian RAD6 proteins and mRNAs between the cells and tissues examined. Brain, heart, and testis show elevated levels of specific mRNAs when compared to the other tissues tested. Yeast *RAD6* has been shown to be meiotically induced, coinciding with the time when meiotic recombination takes place (Madura *et al.*, 1990). However, we find high amounts of shorter mammalian *RAD6* transcripts specifically in postmeiotic cells, i.e., round and elongating spermatids, whereas pachytene spermatocytes have levels below our detection. This renders a specific involvement in meiotic recombination less likely. The analysis presented here shows that the increase in mRNA and protein quantities of both RAD6 homologues during spermatogenesis coincides with the developmental stage when somatic and testis-specific histones are removed from the chromatin and replaced by transition proteins and subsequently protamines (Kistler *et al.*, 1987). The induction of the two *HHR6* genes as representatives of the ubiquitin pathway at this time of spermatogenesis is not without precedent. The chicken ubiquitin II gene is induced at approximately the same stage of spermatogenesis (Mezquita and Mezquita, 1991; Rocamora and Agell, 1990) and increased ubiquitination of histone H2A is observed (Oliva and Dixon, 1991). A ubiquitin-activating enzyme E1 encoded by the Y-chromosomal *Sby* gene exhibits testis-specific expression (Mitchell *et al.*, 1991). This gene and the X-chromosomal homolog *Sbx* show postmeiotic transcription in the mouse (Hendriksen *et al.*, 1995). Interestingly, recently another E2 enzyme ($E2_{17kD}$) was also found to be highly expressed in rat testis (Wing and Jain, 1995). These observations together with the *in vitro* demonstration that RAD6 is able to ubiquitinate histones (Jentsch *et al.*, 1987; Sung *et al.*, 1988), provide indirect evidence for involvement of ubiquitin and HHR6 in the chromatin remodeling processes during spermatogenesis. The idea that HHR6 plays a role in restructuring chromatin may also further pertain to the present finding of very high amounts of HHR6 in reticulocyte lysates (see Fig. 4), since these cells have undergone extensive chromatin modification prior to nuclear elimination. Finally, a conserved role of RAD6 in gross structural alterations of chromatin is consistent with the observation that RAD6 as well as rhp6+ are essential for sporulation (Morrison *et al.*, 1988; Reynolds *et al.*, 1990) again a process known to involve drastic chromosomal changes. In this respect spermatogenesis may represent an advanced phenocopy of the process of sporulation.

The intracellular location of the HHR6 proteins in the euchromatic part of the nucleus as indicated in this study by immunoelectron microscopy is consistent with a function in chromatin transactions. In the immunogold labeling experiments we have used the Lowicryl method which allows only the direct surface to react. This eliminates possible artefacts inherent to some immunohistochemical procedures that are caused by unequal accessibility of antigenic determinants in different locations (Posthuma *et al.*, 1987). Biochemical cell fractionation studies (see Materials and Methods for details on the procedure) suggest that a substantial fraction of the HHR6 proteins is only weakly associated with the euchromatin.

High transcript levels of both HHR6 genes are found in round spermatids at a stage more than 2 weeks after the formation of the heterochromatic sex vesicle which becomes visible in early pachytene spermatocytes (Stefanini *et al.*, 1974). Since the human and the mouse *HHR6A* gene are located on the X chromosome (Koken *et al.*, 1992), it is remarkable that the total amount of *HHR6A* transcripts seems to even increase during the transition of pachytene spermatocytes into round spermatids. This finding has been described elsewhere in more detail (Hendriksen *et al.*, 1995) and is explained by postmeiotic transcription. Hence, *HHR6A* is likely to represent one of the few recently discovered examples of X/Y-chromosomal genes whose gene products may play an important role in postmeiotic stages of spermatogenesis (reviewed in Hendriksen *et al.*, 1995). Western blot analysis confirmed that both HHR6 proteins are indeed present in the germ cells. This was important to verify, since it has been found for other genes that significant quantities of testis-specific, shorter transcripts accumulate in spermatids, but that no corresponding proteins can be detected (Capel *et al.*, 1993; Ivell, 1992). It has been speculated that these transcripts could result from dysregulated gene expression due to the extensive chromatin remodeling events that take place in this stage of spermatogenesis (Ivell, 1992). It cannot entirely be ruled out that the presence of HHR6A protein in spermatids results from an extremely long protein half-life following synthesis in earlier stages of spermatogenesis. The *HHR6B* mRNA is translated more efficiently or the protein is more stable than *HHR6A* as may be concluded from the larger amount of HHR6B in elongating spermatids (Fig. 4). The transcription of the *HHR6A* gene in spermatids, together with the notion that different ratios of the A and B proteins are present in different cells and tissues, suggests that both proteins have a specific task which cannot be completely taken over by the very homologous counterpart. Selective inactivation of either one or both of these genes, e.g., by gene targeting in totipotent mouse ES cells from which mutant mice strains can be obtained, should reveal what is the specific role of each of the proteins and to which extent their functions overlap. Very recently, we have succeeded in generating

HHR6B knockout mouse mutants (H.P.R., unpublished results). The phenotype of these mice comprises specific defects in spermatogenesis that are completely consistent with the above ideas. This finding underlines the significance of the observations described here. Thus the role of this ubiquitin-conjugating enzyme in spermatogenesis in mammals may be an advanced phenocopy of the involvement of yeast RAD6 in sporulation.

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Chapter VII

Inactivation of the HR6B ubiquitin-conjugating DNA repair enzyme in mice causes male sterility associated with chromatin modification

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Inactivation of the HR6B ubiquitin-conjugating DNA repair enzyme in mice causes male sterility associated with chromatin modification

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Summary

The ubiquitin-conjugating yeast enzyme RAD6 and its strongly conserved human homologs hHR6A and hHR6B, are implicated in post replication repair and damage-induced mutagenesis. The yeast protein is also required for sporulation, and may modulate chromatin structure via histone ubiquitination. We report the phenotype of the first animal mutant in the ubiquitin pathway: inactivation of the *hHR6B*-homologous gene in mice causes male infertility. Derailment of spermatogenesis becomes overt during the post-meiotic condensation of chromatin in spermatids. These findings provide a parallel between yeast sporulation and mammalian spermatogenesis, and strongly implicate hHR6-dependent ubiquitination in chromatin remodelling. Since heterozygous male mice and even knockout female mice are completely normal and able to transmit the defect, similar *hHR6B* mutations may cause male infertility in man.

Introduction

The ubiquitin system plays a key role in numerous cellular processes, including metabolic homeostasis, stress response, organelle biosynthesis, cell cycle regulation, DNA repair, apoptosis, antigen processing and gene expression (for recent reviews see (Ciechanover, 1994; Hochstrasser, 1995)). This selective, non-lysosomal proteolytic pathway mediated by the 26S protease complex determines the half-life of crucial proteins such as p53, cyclins, transcription factors, and cytosolic polypeptides (Chau et al., 1989; Ciechanover et al., 1991; Glotzer et al., 1991; Kornitzer et al., 1994; Seufert et al., 1995). Furthermore, this pathway is implicated in stabilization, refolding, and translocation of a diverse range of proteins. The ubiquitin pathway involves a three or four step ubiquitin thioester cascade. The highly conserved 76 amino acid ubiquitin molecule is first activated by the ubiquitin-activating enzyme (E1) through formation of a high-energy thioester linkage of its C-terminal glycine with a cysteine residue in the E1 protein itself. Subsequently, the ubiquitin moiety is transferred to a cysteine of one of the ubiquitin-conjugating (UBC or E2) enzymes, which in turn attach the ubiquitin part onto a target protein with or without the assistance of a ubiquitin-protein ligase (E3). The latter step may involve a third thioester linkage with the E3 enzyme (Scheffner et al., 1995) and results in the formation of an isopeptide bond of the activated C-terminal glycine of ubiquitin with the N-terminus or the ε-NH2 group of an internal lysine residue of the substrate polypeptide. Poly-ubiquitination is thought to mark proteins for degradation, whereas mono-ubiquitination may serve other functions (Chau et al., 1989).

A key role in this pathway is exerted by a growing family of UBC enzymes, which in yeast already comprises 12 members (Hochstrasser, 1995). An extreme example of the pleiotropic involvement of this class of proteins in cellular processes is presented by the yeast *S.cerevisiae RAD6* gene product. Strains mutated in *RAD6* display defects in post-replication daughter strand gap repair, causing pronounced sensitivity to all kinds of DNA damaging agents, increased spontaneous mutation frequency and concomitant loss of damage-induced mutagenesis. In addition, rad6 null alleles exhibit cell cycle perturbance, temperaturesensitive growth, inability to sporulate, and increased retrotransposition (see Lawrence, 1994). The crucial finding concerning the function of the protein was made by Jentsch et al. (1987), who identified RAD6 as the first ubiquitin-conjugating enzyme, able to mono- and poly-ubiquitinate histones 2A and 2B *in vitro*. The protein has been demonstrated to mediate N-end rule protein degradation (Dohmen et al., 1991) and its highly conserved N-terminus is implicated in interaction with the UBR1 protein (Madura et al., 1993). Its *in vitro* ability to ubiquitinate histones has led to the suggestion that RAD6 mediates its functions by modulating chromatin structure as an essential part of the DNA transactions (repair, replication) in which it is implicated. However, direct demonstration of the role of the protein in chromatin dynamics is still lacking. In normal mammalian cells, a substantial fraction of histone H2A, and, to a lesser extent, H2B, is present in a ubiquitinated form (Goldknopf and Busch, 1980; Thorne et al., 1987). Although histones are frequently utilized as substrates for ubiquitin-conjugation, the function of ubiquitination of histones *in vivo* is largely unexplored, and it is unknown whether and to what extent RAD6 is involved in this post-translational modification.

Previously, we cloned *RAD6* homologs of several higher eukaryotes including two closely related human homologs, de signated *hHR6A* and *hHR6B* (for human Homologs of RAD6) (Koken et al., 1991b). The

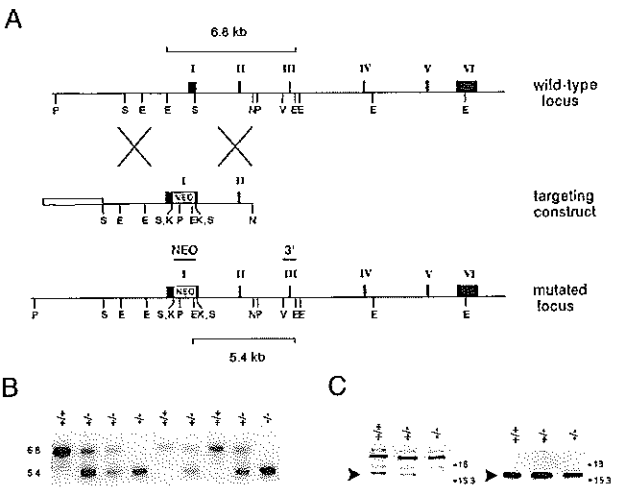

Figure 1. Targeted disruption of the *mHR6B* gene by homologous recombination. (A) Genomic organisation and disruption strategy for *mHR6B* showing the gene, the targeting construct and the targeted mHR6B allele. The *neo* cassette is inserted in the *SalI* site of exon I, introducing a diagnostic *Eco*RI site. Note that insertion of the dominant marker disrupts the gene immediately behind the ATG translation initiation codon. Shown are the relevant restriction sites (E, *Eco*RI; S, *SalI*; N, *NsiI*; K, *KpnI*; V, *Eco*RV; P, *SphI*). The position of the 3' probe and the *neo* probe for Southern blot analysis are indicated above the mutated locus. Lines on top and bottom indicate the estimated length of the fragments detected in Southern blot analysis of *Eco*RI digested DNA. Roman numerals mark the exons. (B) Southern analysis of *Eco*RI digested DNA from nine littermates after hybridization with the 3' probe. The position of the wild-type allele (6.8 kb) and the targeted allele (5.4 kb) are indicated. (C) Western blot analysis of testes extract of wild-type (+/+), heterozygous (+/-) and homozygous mutant (-/-) animals. In the left panel mHR6B protein was detected using the antiserum raised against the C-terminus of mHR6B/hHR6B (α-AB1). In the right panel the reaction with the α-RAD6 antiserum is presented. On the left of both panels the position of mHR6B is indicated (arrow). On the right side the positions of the relevant molecular weight markers are shown.

encoded human proteins are both structurally and functionally highly conserved: they share approximately 70% sequence identity with *S.cerevisiae* RAD6, are able to ubiquitinate histones *in vitro*, and both human gene products can substitute for the mutagenesis and UV resistance function of the yeast protein, but not for its role in sporulation. This latter function requires in *S.cerevisiae* an acidic C-terminal extension. In *S.pombe* however, like in the Drosophila and mammalian homologs, the acidic tail is absent and not needed for sporulation (Koken et al., 1991a and 1991b; Reynolds et al., 1990; Schneider et al., 1990). The subcellular localization of HR6 in the euchromatic regions of the nucleus (Koken et al., 1996) suggests that its function is related to active chromatin conformation. Both mammalian genes are expressed in all organs and tissues and are not subject to mitotic cell cycle regulation. Furthermore, expression of both genes is elevated in mouse spermatids (post-meiotic spermatogenic cells), coinciding with the developmental steps at which a complex series of chromatin modification events takes place (Koken et al., 1996). These events involve replacement of somatic and testis-specific histones by transition proteins TP1 and TP2, and subsequently by protamines P1 and P2 (Balhorn, 1989; Kistler, 1989; Meistrich, 1989). In rat spermatids, occurrence of highly acetylated histone H4 is found to be associated with histone displacement (Meistrich et al., 1992). Ubiquitination of histones and other nuclear proteins might also be involved in this process, because ubiquitination of histones has been observed during chicken and trout spermatogenesis (Agell et al., 1983; Agell and Mezquita, 1988; Nickel et al., 1987; Oliva and Dixon, 1991).

Studies on the biological and molecular function of HR6 and other enzymes implicated in the ubiquitin pathway in higher organisms is hampered by lack of mutants. The central role of RAD6 in multiple processes makes it an interesting target for generating a knockout mouse mutant. Here we demonstrate that mice deficient for the murine version of *HR6B* (*mHR6B*) are viable and phenotypically normal, presumably due to functional redundancy with *mHR6A*. The *mHR6B*-deficient male mice, however, are infertile, whereas *mHR6B*-deficient females show normal fertility. The defect in spermatogenesis is consistent with impairment of the complex post-meiotic chromatin remodelling process, and provides evidence for involvement of the ubiquitin pathway in chromatin dynamics. Moreover, our findings may have clinical implications for understanding male infertility in man.

Results

Main features of the mouse *HR6B* gene and cDNA

To permit the design of targeting constructs, mouse cDNAs and the corresponding gene were isolated using cross-hybridization to a human *HR6B* (*hHR6B*) probe. To facilitate homologous recombination with high efficiency, the mouse homolog of *RAD6* (*mHR6B*) was cloned from a λ phage library of genomic mouse strain 129/Sv DNA, isogenic to the embryonal stem cell line used for gene targeting. The high conservation of the gene is apparent from the finding that its predicted amino acid sequence is completely conserved between mouse and man. A notable feature is the 100% conservation of a sequence of at least 309 base pairs in the 3'UTR of the *mHR6B* mRNA between all mammals investigated (man, mouse, rat and rabbit). This stretch corresponds with nucleotides 575 to 884 of the published human cDNA sequence (Koken et al., 1991b). To our knowledge this represents the longest nucleotide stretch strictly preserved over such an evolutionary distance. The function of this exceptionally stable, non-coding

nucleotide sequence element is unknown.

Figure 1A presents the architecture of the murine *mHR6B* gene. The gene spans a region of approximately 15 kb and is comprised of six exons. Interestingly, the location of two introns is exactly preserved in Drosophila and even *S. pombe* (Koken et al., 1991a; Reynolds et al., 1990), presumably reflecting a high importance for the gene. The gene was mapped on mouse chromosome 13 in a region syntenic with human chromosome 5, and evidence was obtained for a pseudogene on mouse chromosome 11 (Roller et al., 1995).

Inactivation of the mHR6B gene and generation of mouse mutants

In designing a knockout targeting construct, we envisioned the possibility that any truncated mHR6B protein may exert unpredictable effects. Particularly the highly conserved N-terminus, encoding a site for protein-protein interaction, could interfere with other processes resulting in semi-dominant consequences. Therefore, we chose to inactivate the *mHR6B* gene immediately after the translational start codon by insertion of the dominantselectable neomycin or hygromycin marker, ruling out the synthesis of any part of the protein. The targeting construct depicted in Figure 1A contains 3.5 kb and 2.8 kb of homology at the 5' and 3' side flanking the dominant-selectable marker, respectively. Two versions, each with a different selectable marker, were constructed to permit inactivation of both autosomally located alleles in ES cells.

Transfection of the *neo* cassette-containing targeting construct (Figure 1A) by electroporation and selection for stable uptake of the dominant selectable marker gene yielded a frequency of 16% targeted transformants (27 homologous recombinants/166 total transformants; no selection was applied against random integration). Homologous recombinants were checked for accurate integration of the construct by Southern blot analysis using external and internal probes and were found to be correct (data not shown). The multiple engagements of RAD6 on the one hand and the presence of a 95% identical mHR6A protein on the other, make it difficult to predict a phenotype for a *mHR6B*deficient mouse. To find out whether a homozygous *mHR6B* inactivation is viable, at least at the cellular level, the second allele was targeted using the *hygro* cassette-containing construct. The frequency of targeting directed to the wild-type allele was 8% (11/143), indicating that there was no selection against *mHR6B* inactivation and that inactivation of both *mHR6B* alleles is not lethal. Therefore, we performed injection of ES cells of two independent, neomycin-resistant clones (80 and 134) into blastocysts of C57BL/6 mice, resulting in the generation of chimaeras. Male chimaeras from both independent clones were bred and both gave germline transmission. Southern blot analysis on DNA isolated from tail biopsies was used to determine the genotype of the offspring. Hybridization with the 3' external probe visualized a 6.8 kb *Eco*RI fragment in the case of a normal allele and a 5.4 kb fragment for a targeted allele (Figure 1B). Heterozygotes were interbred and yielded homozygous *mHR6B* mutants with the expected Mendelian frequency. The results of a representative litter are shown in Figure 1B.

We verified that the targeting of *mHR6B* indeed resulted in a null-mutation at the RNA and protein levels. Northern blot analysis confirmed absence of significant amounts of *mHR6B* transcripts (data not shown), indicating that the presence of the dominant marker interfered with proper transcription and/or processing of the altered mRNA. The mHR6A and mHR6B proteins, like hHR6A and hHR6B, are 95% identical and migrate at the same molecular weight in SDS-PAGE. To distinguish between these highly homologous polypeptides, we took advantage of the fact that within the 14 C-terminal amino acids, the A and B products differ at 2 positions. A peptide identical to the 14 C-terminal amino acids of HR6B was synthesized and utilized to raise a polyclonal antiserum that specifically recognizes this protein. Since in testis both proteins are expressed in high quantities, total testis extracts were analysed. Figure 1C (left panel) shows that no mHR6B protein is detected in *mHR6B*$^{-/-}$ mice, whereas the protein is present in *mHR6B*$^{+/-}$ and *mHR6B*$^{+/+}$ littermates. The decrease in intensity in the testis extract of the heterozygous animal suggests that these animals contain roughly approximately half the amount of mHR6B protein as compared to the normal animals. This argues against upregulation of the untargeted allele to compensate for the loss of expression of the targeted copy. An antiserum against yeast RAD6, recognizing both HR6A and HR6B (Koken et al., 1996), shows a positive reaction in the mHR6B$^{-/-}$ sample, indicating that the *mHR6A* gene is expressed (Figure 1C, right panel). These results verify the null status of the *mHR6B* mutation and also show that mHR6A protein is still present.

Phenotypic characteristics of *mHR6B*$^{-/-}$ mice and cells

The *mHR6B*$^{-/-}$ mice proved normally viable with a lifespan exceeding 14 months. Except for the feature discussed below, no apparent phenotypical or pathological abnormalities were found. Furthermore, no differences were noted between the main phenotypic characteristics of the *mHR6B*$^{-/-}$ mice derived from the independently targeted ES recombinants and between mice from crossings between different strains (129xFVB/J, 129x C57BL/6). This rules out the possibility that by accident other genetic alterations had occurred that might influence the phenotype or that the genetic background is of major importance. Since RAD6 in yeast accounts for much of the cellular resistance against a wide spectrum of genotoxic agents, we investigated UV and γ-ray sensitivity in mouse cells. To test UV sensitivity, mouse embryonic fibroblast cell lines were established from *mHR6B*$^{+/+}$, $^{+/-}$, and $^{-/-}$ mice and tested for their cellular survival, as measured by [3H]-thymidine incorporation, after irradiation with different doses of UV. For γ-ray sensitivity the doubletargeted ES cell line was irradiated and cloning efficiency was compared with irradiated, nontargeted ES cells. No differences between *mHR6B*-deficient and -proficient cells were observed for these DNA damaging agents (data not shown). Thus no overt defect in DNA repair was detected. This is possibly caused by a redundant effect of a functional *mHR6A* gene.

Spermatogenesis in *mHR6B*-deficient mice

In breeding experiments, it soon became apparent that the *mHR6B*$^{-/-}$ male mice were consistently infertile. Copulatory behaviour was judged to be normal,

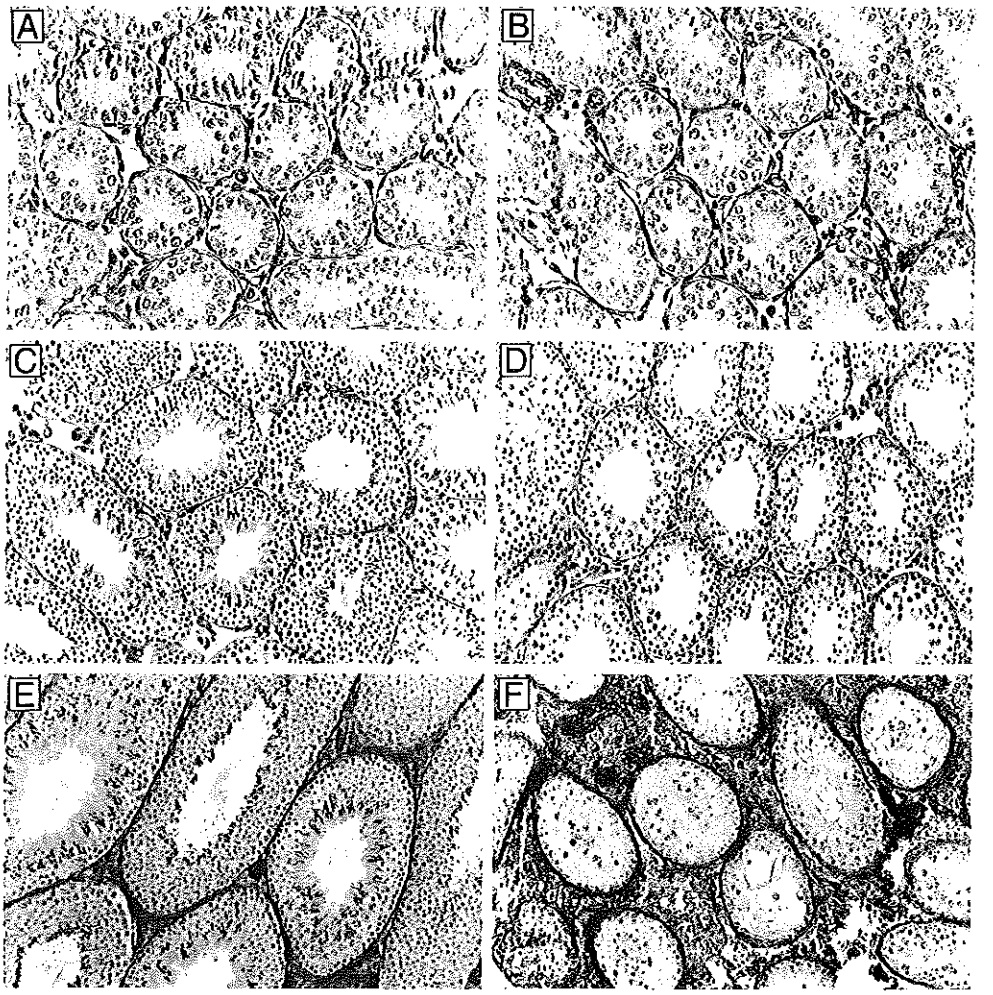

Figure 2. Testicular histology of normal and *mHR6B* knockout mice.
The histological sections were prepared as described in Experimental Procedures, and stained with periodic acid Schiff (PAS). The panels to the left (A,C,E) show the testicular histology of normal mice, the panels to the right (B,D,F) that of knockout animals. A,B: 8-day-old mice (x400); C,D: 40-day-old mice (x200); E,F: 9-month-old mice (x200).

and copulation plugs were found, but none of the tested males induced pregnancy in fertile females (out of at least 27 matings with 11 knockout males no pregnancies were recorded). Histological evaluation of the testes and epididymides of adult *mHR6B*$^{-/-}$ males showed a strong derailment of spermatogenesis (>10 males investigated). However, considerable variation in the severity of the deficiencies in different adult mice was observed, involving early as well as later steps of spermatogenesis, precluding identification of the exact step at which spermatogenesis is affected. Therefore, the onset of spermatogenesis was closely followed in these mice.

In immature *mHR6B*$^{-/-}$ mice, an intact tubularstructure with normal development of Sertoli cells was observed (Figure 2A and B). Subsequently, initiation of spermatogenesis showed no overt abnormalities, with proper development of spermatogonia, and timely onset and progression of the meiotic prophase and divisions. It is unlikely that mHR6B is indispensable for meiosis, also because the *mHR6B*$^{-/-}$ females showed normal fertility (not shown). Clear signs of spermatogenic failure were observed when the first waves of spermatogenic cells reached the more advanced steps of spermiogenesis, in 4 to 5-week-old *mHR6B*$^{-/-}$ mice (mice analysed at 8 days, 2.5, 3.5, 4.5, and 5.5 weeks). In general, the spermatogenic epithelium started to show a number of irregularities,

Table 1. Body and organ weights, and epididymal sperm count in normal and *mHR6B* knockout mice.

	Intact* (Mean±SD)	Knockout** (mice no.1;2;3)
Body weight(g)	44 ±6	40; 49; 63
Testis (mg)	99 ±17	55; 26; 48
Epididymis (mg)	42 ±5	38; 29; 39
Seminal vesicles(mg)	109 ±16	86; 79; 109
Sperm count ($x10^6$)	15.5±2.7	0.9; <0.1; 0.9

*Control group consisted of five 8-month-old mice (two $^{+/+}$ and three $^{+/-}$)

**Individual data of three 8-month-old $^{-/-}$ mice (no.1;2;3)

Including the formation of vacuoles within the epithelium and shedding of immature germ cells, in particular round and more advanced spermatids. Figures 2C and 2D show histological sections of testes from control and knockout mice isolated at the age of 40 days. From this point on, heterogeneity in testicular histology and variation in regression of spermatogenesis was observed between individual mice. Occasionally (in 10 - 20% of *mHR6B*$^{-/-}$ males) nearly total absence of all germ cell types was found (Figure 2F), but in most knockout males we registered ongoing spermatogenesis with only low numbers of predominantly abnormal spermatozoa (see below).

A marked, but variable reduction in testis weight (Table 1) illustrated the pronounced overall regression of spermatogenesis, although inter-individual heterogeneity was apparent. In *mHR6B*$^{-/-}$ mice #1 and #3 (Table 1), the epididymis weights were not significantly decreased, despite the fact that the epididymal sperm counts were less than 10% of the numbers found in *mHR6B*$^{+/+}$ and $^{+/-}$ mice. This is probably explained by the abundant presence of immature germ cells in the epididymal lumen (Figure 5; compare C and D). Epididymis weight of mutant mouse #2 was lower, due to the complete absence of germ cells. Mutant mice #1 and #3 still contained many immature germ cells in the epididymal lumen.

Seminal vesicle weight is an excellent marker of long-term testosterone action, and the data in Table 1 therefore indicate that the plasma testosterone concentration in the *mHR6B* knockout mice was maintained within the normal range. Furthermore, the plasma follicle-stimulating hormone (FSH) concentration was not different between *mHR6B*-deficient mice (37, 38, and 51 ng/ml in three mice) and intact mice (40 ± 6 ng/ml in five mice).

To study the remaining spermatozoa of *mHR6B*$^{-/-}$ mice in more detail, morphology and motility were examined using Nomarski optics of unfixed material and phase contrast microscopy, respectively. In knockout mice more than 90% of the spermatozoa were clearly morphologically abnormal. At least 70% of these spermatozoa had an aberrant head morphology, in most cases combined with middle piece deformation (see Figure 3). Moreover, the residual spermatozoa appeared almost immotile: a few spermatozoa (about 5%) displayed a sluggish progressive or non-progressive motility. These findings confirmed that the *mHR6B* gene knockout does not cause a complete and uniform block of spermatogenesis at a givenpoint in adult animals.

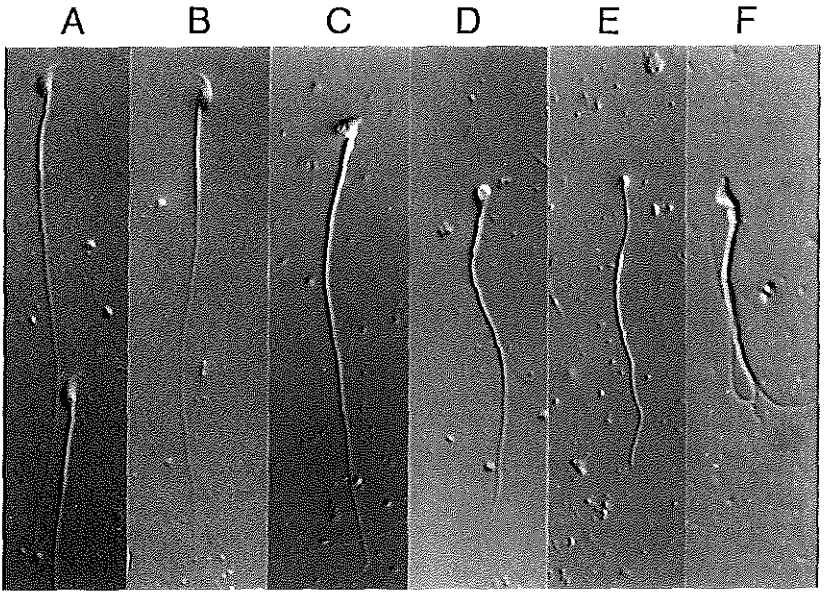

Figure3. Normal (A) and abnormal (B-F) morphology of spermatozoa from mHR6B knockout mice. The spermatozoa were collected from the cauda epididymis, and photographed without fixation using Normarski optics (x400).

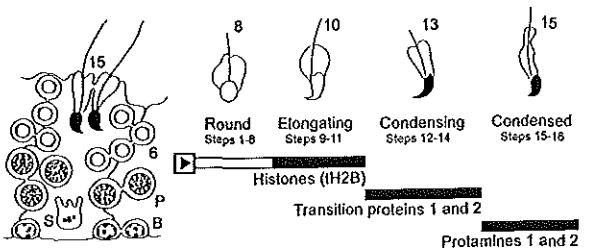

Figure 4. Schematic presentation of the his-tone-to-protamine replacement in mouse spermatids. The figure to the left is a schematic representation of a part of a cross-section of a tubule at Stage VI of the spermatogenic cycle (Russell et al., 1990), showing the interrelationship between a Sertoli cell (S), spermatogonia type B (B), pachytene spermatocytes (P), round spermatids Step 6 (6), and condensing spermatids Step 15 (15). The right part of the figure shows selected steps of spermatid development (Steps 8,10,13, and 15 of spermiogenesis). The bars represent (from left to right) the following: testisspecific histone 2B (tH2B) is present in round spermatids (and in spermatocytes), but the immunoexpression of this protein is increased in elongating spermatids Steps 9-11 (Unni et al., 1995); nuclear deposition of transition proteins 1 and 2 (TP1 and TP2) occurs in condensing spermatids Steps 12-14 (Alfonso and Kistler, 1993), followed by replacement of the TPs by the protamines (P1 and P2).

The low number of cells at the critical step where the first abnormalities were seen precluded biochemical analysis of these cells in the *mHR6B*$^{-/-}$ mice. However, the impairment of spermatogenesis in these mice was defined more precisely, using immunohistochemistry.

As an introduction to these studies, a brief description of chromatin rearrangement during spermatogenesis, in particular during the post-meiotic development of spermatids (spermiogenesis), is presented (see also Figure 4).

Spermatogonia, proliferating through mitotic divisions, contain somatic histones. With the progression of spermatogenesis, a number of testis-specific histones (tH2B) are synthesized, mainly in primary spermatocytes, during the prophase of the meiotic divisions. The round and elongating spermatids contain a mixture of somatic and testis-specific histones (Brock et al., 1980; Meistrich et al., 1985). Following the elongation phase (Steps 9-11 of spermiogenesis in the mouse), the elongated spermatids start with the process of nuclear condensation (Steps 12-14), involving the synthesis of transition proteins 1 and 2 (TP1 and TP2) and protamines 1 and 2 (P1 and P2). The transition proteins appear in the nucleus at Step 12 and are lost at Step 14 when further condensation of the nucleus takes place, concurrent with the nuclear deposition of the protamines (Alfonso and Kistler, 1993; Kistler, 1989; Meistrich, 1989).

Testis-specific histone H2B (tH2B) is synthesized and deposited onto the chromatin, beginning in early primary spermatocytes (Brock et al., 1980; Meistrich et al., 1985). It represents a good marker for the elongation phase, showing intense immunostaining, due to increased accessibility of the epitope in spermatids (Unni et al., 1995). Figures 5A and 5B show that the tH2B-immunopositive spermatids that remain present in the testis of *mHR6B-/-* mice, display an irregular orientation and distribution, in contrast to the well-organized structure of the spermatogenic epithelium in control mice (Figure 5A and B). Interestingly, tH2B-immunopositive cells were also detected in the lumen of the epididymis of *mHR6B*$^{-/-}$ mice (Figure 5D). These cells were virtually absent in the epididymis from intact adult mice, which was filled with mature spermatozoa (Figure 5C). Many of the epididymal tH2Bimmunopositive cells are round and elongating spermatids that have been prematurely released from the spermatogenic epithelium and have not undergone further elongation and nuclear condensation.

Immunostaining with an antibody against TP2 showed pronounced staining of elongated/condensing spermatids, at Steps 12-14 of spermiogenesis (Alfonso and Kistler, 1993). In control mice, these spermatids are arranged in groups of cells and in a regular pattern, at Stages XII and I-III of the spermatogenic cycle (Figure 5E). In *mHR6B*$^{-/-}$ mice, a relatively small number of elongated spermatids showed TP2 immunostaining, and a proportion of these cells showed abnormal morphology and were not well positioned within the spermatogenic epithelium (Figure 5F). Our findings indicate that *mHR6B*$^{-/-}$ mice synthesize TPs, but that these proteins are not uniformly located in the nucleus as observed during normal spermatogenesis.

Since the general picture is an overall impairment of spermatogenesis as a consequence of a primary defect in the elongation stage of spermiogenesis, we investigated whether apoptosis is elevated in *mHR6B*$^{-/-}$ mice. Figure 6A shows sections through seminiferous tubules of testis of 6-week-old *mHR6B*$^{+/+}$ and $^{-/-}$ mice stained using the TUNEL assay. A 4-fold increase in the number of apoptotic cells was calculated and represented as the number of positively-stained cells per 100 tubuli (Figure 6B). Moreover, the apoptotic cells were clustered and predominantly localized in the germ cell layers that contain primary spermatocytes. These data indicate an elevated level of apoptosis as a consequence of *mHR6B*-deficiency.

Discussion

In spite of the pleiotropic functions and fundamental importance of the ubiquitin system, no mammalian mutants affected in this pathway are available that reveal the biological ramifications and impact of this process at the level of the organism. In the present report, we describe the phenotype of mice deficient in the ubiquitin-conjugating enzyme mHR6B. In both ES cells and in mice, the loss of function of mHR6B is compatible with viability. Although yeast rad6 deletion mutants are viable, they display a severe phenotype. The finding that this is not the case in the *mHR6B* knockout mouse can be explained by functional redundancy of the *HR6A* and *HR6B* gene products. The hHR6A and hHR6B proteins are expressed to approximately the same extent in most somatic cells and tissues (Koken et al., 1996). The two gene products show 95% amino acid sequence identity, and thus probably catalyze very similar reactions. Furthermore, both proteins are functional and complement the same defects of a rad6 null allele (Koken et al., 1991b). Apparently, the approximal 50% of remaining activity derived from the mHR6A gene is sufficient to permit relatively normal development.

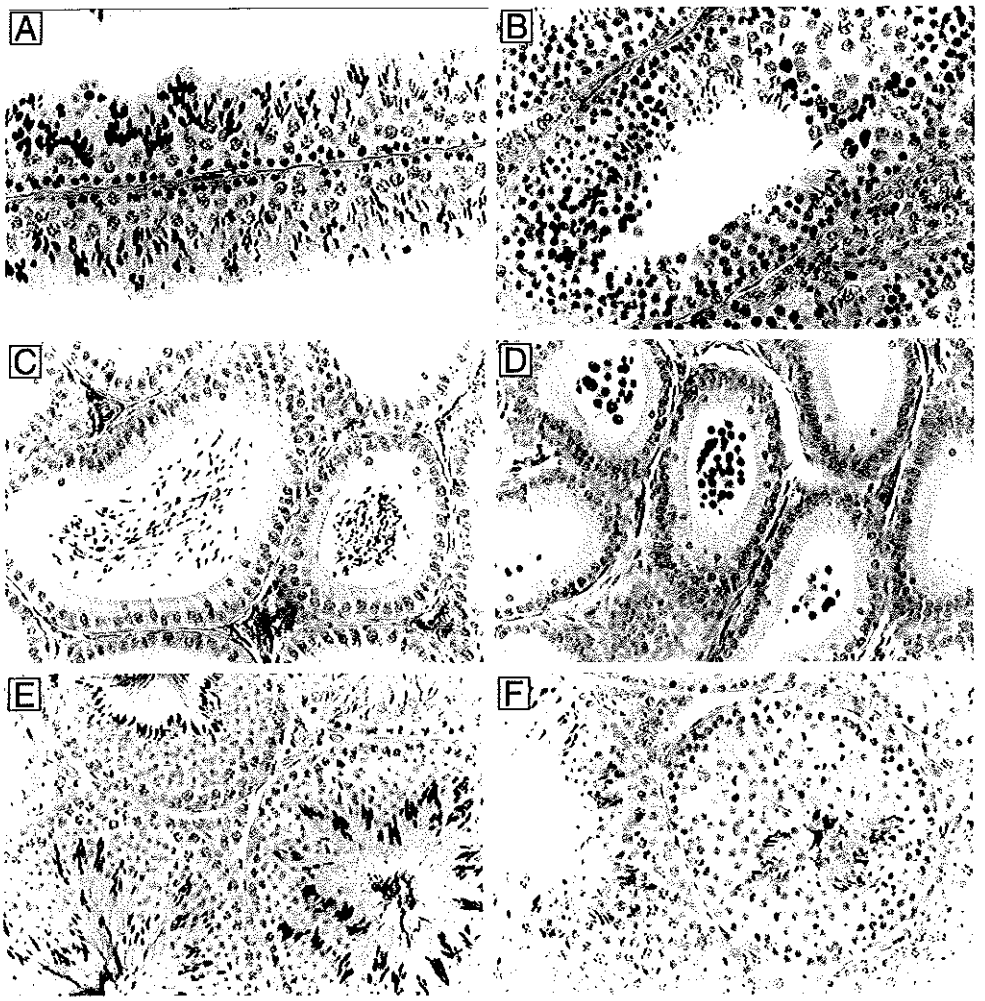

Figure 5. Immunohistochemical localization of testis-specific histone H2B (tH2B) and transition protein 2 (TP2) in testis and epididymis of intact and *mHR6B* knockout mice. The immunohistochemistry was performed as described in Experimental Procedures. The panels to the left (A,C,E) show tissues from a 9-month-old intact mouse, and the panels to the right (B,D,F) represent a 9-month-old *mHR6B* knockout mouse. A,B: aH2B immunostaining of testis; C,D: tH2B immunostaining of epididymis; E,F: TP2 immunostaining of testis (x400).

We failed to observe any defect in DNA repair. However, this does not exclude a subtle effect of partial loss of mHR6 activity on mutagenesis and carcinogenesis, which remains to be studied. Experiments aimed at generating *mHR6A*deficient mice, in order to assess the phenotype of these and full *mHR6A/mHR6B* double knockout mice, are in progress.

The most prominent phenotypic expression of the *mHR6B* gene knockout detected to date is impairment of spermatogenesis, resulting in greatly reduced numbers of mainly abnormal spermatids and spermatozoa. However, in the adult testis, the causative step is difficult to pinpoint, because of the considerable interindividual variation in the manifestations, and the fact that early as well as late steps of spermatogenesis seem to be impaired. Detailed analysis of the first wave of spermatogenesis, however, allowed identification of the primary defective stage: progression through the elongating and condensing steps of spermatid development is impaired. Probably as a secondary consequence, earlier steps of spermatogenesis also become deregulated (see below).

Previously, we found elevated levels of *mHR6A* and *mHR6B* mRNAs in spermatids during normal rat

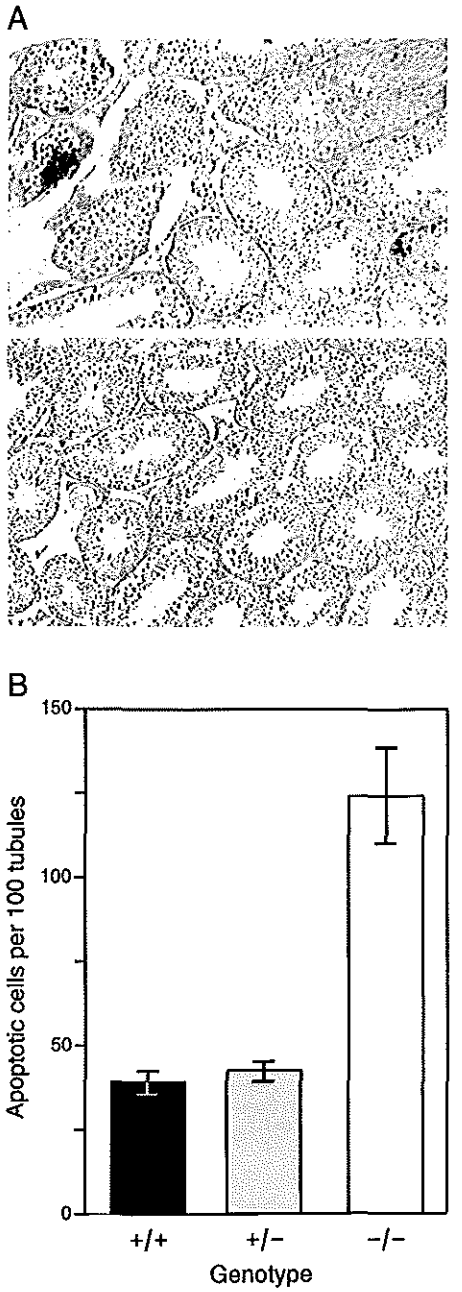

Figure 6. Analysis of apoptosis in seminiferous tubule cross sections of six-week-old mice.
(A) Nuclear DNA fragmentation visualized using the TUNEL assay. The upper panel shows a section through a testis of a homozygous mutant, and the lower panel is a testis section of a wild-type animal. A cluster of apoptotic cells is present in the upper left corner of the upper panel.
(B) Quantification of apoptosis in wild-type (+/+), heterozygous (+/-) and homozygous mutant (-/-). Slides were randomly manoeuvred under a light microscope and all apoptotic cells present in at least 100 tubule cross sections were counted and divided by the number of tubules. These data were recalculated to give the number of apoptotic cells per 100 tubules cross sections.

spermatogenesis (Koken et al., 1996). In fact, *HR6A* is the first X-linked gene for which post-meiotic expression, in mouse spermatids, has been documented (Hendriksen et al., 1995). In addition, immunohistochemical experiments show the presence of HR6 proteins in the nuclei of round and elongating rat spermatids. However, it is important to note that in two-dimensional immunoblot analysis of all cells tested, elongating spermatids and spermatozoa were the only cell types in which the mHR6A level appeared significantly lower relative to that of mHR6B (Koken et al., 1996). Thus, it is conceivable that in the absence of mHR6B, the relatively low levels of mHR6A are insufficient for performing the HR6 function required in these cells. Unfortunately, the low number of elongating spermatids in immature *mHR6B*$^{-/-}$ mice precludes biochemical analysis of HR6 activity in this way.

Although different hypotheses can be put forward to explain our findings, such as defects in Sertoli cells, which like germ cells, express high levels of both mHR6A and mHR6B (Koken et al., 1996), we consider the following scenario most consistent with all observations. The nuclei of early round spermatids contain a mixture of somatic histones and testis-specific histones. Following elongation of spermatids, chromatin is reorganized, and the histones are replaced by transition proteins (TPs) and then by protamines (Balhorn, 1989; Kistler, 1989; Meistrich, 1989). Two types of histone modification have been documented during spermatogenesis. In rat spermatids, occurrence of highly acetylated H4 is associated with histone displacement (Meistrich et al., 1992), and during chicken and trout spermatogenesis poly-ubiquitination of histone H2A has been observed (Agell et al., 1983; Agell and Mezquita, 1988; Nickel et al., 1987; Oliva and Dixon, 1991). In preliminary experiments we have detected mono- and poly-ubiquitinated forms of histones in nuclear extracts of mouse spermatocytes and spermatids (our unpublished observations). Considering the ability of RAD6 to polyubiquitinate histones *in vitro*, and its *in vivo* role in yeast sporulation, the most plausible hypothesis is that, in mammalian spermatids, the functional homologs of RAD6 poly-ubiquitinate histones. This allows for their degradation and replacement by transition proteins and, subsequently, by protamines. A shortage of the enzyme at this critical stage could interfere with this process. However, it remains to be shown that the ubiquitin-conjugating enzyme activity targeting specific histones in spermatids is below a critical threshold level in spermatids from *mHR6B* knockout mice.

Spermatogenesis involves elimination and modification of many proteins. A novel ubiquitin-conjugating enzyme E2 (E2 17kB) was recently also found to be highly expressed in testis (Wing and Jain, 1995). In addition, the Y-chromosomal gene *Sby* or *Ube1y*, encoding ubiquitin-activating enzyme E1, shows testis-specific expression in the mouse, and is considered a candidate spermatogenesis gene (Kay et

al., 1991; Mitchell et al., 1991). Testicular expression is dependent on the presence of germ cells (Mitchell et al., 1991) and *Ube1y* mRNA has been detected in round spermatids (Hendriksen et al., 1995). The homologous gene on the X chromosome (*Sbx* or *Ube1x*) is expressed in all male and female tissues (Mitchell et al., 1991), and also in spermatogenic cells (Hendriksen et al., 1995). Assuming that disturbance of chromatin remodeling in spermatids of *mHR6B*-deficient mice is the primary cause of the infertility, how can this defect lead to formation of vacuoles in Sertoli cells and the release of immature germ cells? A clue is provided by a recent finding on the effect of ectopic expression of avian protamine (galline) in spermatids of transgenic mice. This expression induces disruption of the normal dense chromatin structure of spermatozoa, and results in infertility (Rhim et al., 1995). As for *mHR6B*$^{-/-}$ mice, the spermatogenic epithelium of these transgenic males showed many vacuoles and loss of immature germ cells. Thus, disruption of chromatin conformation by ectopic protamine expression leads to very similar types of spermatogenic abnormalities as observed in *mHR6B*$^{-/-}$ mice, in agreement with the idea that *HR6B* deficiency affects chromatin conformation. Possibly, Sertoli cells are adversely affected by degenerating late spermatids. These spermatids might release protamines, which are known to exert toxic effects on epithelial cells (Peterson and Gruenhaupt, 1992). In concordance with this, the clustered apoptosis of primary spermatocytes in testis of *mHR6B*$^{-/-}$ mice (Figure 6) points to local Sertoli cell damage.

It is not clear why most of the male *mHR6B* knockout mice show production of spermatozoa with a wide range of morphological abnormalities. Other defects in spermatogenesis affecting spermatocytes rather than spermatids, can also give rise to abnormal spermatozoa. Such a defect in spermatogenesis was observed in mice that were mutated in the DNA mismatch repair gene *PMS2* (Baker et al., 1995). This defect results in abnormal chromosomal synapsis in meiosis and male infertility, with production of a small number of spermatozoa with abnormal morphology.

In a considerable number of male infertility patients, the cause of the infertility might be related to disturbance of the histone-to-protamine replacement during spermatogenesis. Several reports describe that sperm from infertile men can show abnormal protein complements, with persistent elevated levels of histones and/or an altered protamine P1/P2 ratio (Chevaillier et al., 1987; De Yebra et al., 1993; Foresta et al., 1992). Notwithstanding the relative genetic uniformity of *HR6B*$^{-/-}$ mice, a marked variation of testis histology and sperm morphology was observed. The pronounced variability in features is reminiscent of the testicular manifestations associated with infertility in man. The fact, that an *HR6B* defect in mice can be transmitted not only by heterozygous carriers but even by homozygous knockout females enhances the possibility, that the identical human enzyme may be implicated in male infertility conditions. Probably as many as one in three of all cases of human male infertility are of unknown testicular origin. These cases cannot be explained by chromosome abnormalities, endocrine dysfunction, etc. (Wong et al., 1973). In unexplained male infertility, there is often the production of a low number of spermatozoa (oligozoospermia) and/or abnormal sperm morphology (teratozoospermia) (Aitken et al., 1995). Several hallmarks of this variable condition are shared with *mHR6B*$^{-/-}$ mice. A potential involvement of a defect in the ubiquitin-pathway in cases of human male infertility is presently under investigation.

A final implication from the findings reported here is the parallel emerging between spermatogenesis in mammals and sporulation in yeast (Game et al., 1974, Montelone et al., 1981). The latter process also appeared to be accompanied by gross changes in chromatin conformation in which RAD6 may play a similar role as HR6B in higher organisms. Interestingly, the yeast UBC1 enzyme is found to be required for recovery of growth after germination of ascospores (Jentsch, 1992). This enzyme may thus accomplish the reverse of the reaction catalysed by RAD6, namely the decondensation of chromatin.

Experimental Procedures

Isolation and sequence of murine *mHR6B* cDNA clones

A 784 bp *Hin*dIII-*Bam*HI cDNA fragment containing the complete open reading frame (ORF) of the human *hHR6B* gene, including 5' and 3' flanking sequences (176 and 149 nucleotides, respectively) (Koken et al., 1996), was used to screen an 129/Ola mouse testis library (λZAP) for *hHR6B* homologous mouse cDNAs. Seven positive plaques were isolated of which 2 contained the complete ORF. The nucleotide sequence of the ORF of *mHR6B* and (part of) the 3' untranslated region were determined using T7-polymerase (Pharmacia Biotech, Uppsala, Sweden) and deposited in the Genbank/EMBL nucleotide sequence database under accession number X96859.

Construction of the mouse *mHR6B*-targeting vector and transfection

An EMBL-3 λ phage genomic library constructed from the CCE ES cell line derived from mouse strain 129/Sv (a kind gift of dr. G. Grosveld) was screened with the 784 bp human *HR6B* cDNA fragment. Positive genomic clones were rescreened with a γ-,32P-ATP labeled primer, complementary to nucleotides 28-69 of the mouse *HR6B* coding region. A genomic clone was isolated, designated G28, encompassing the exons encoding the 5' end of the mHR6B coding region. This genomic clone was digested with *Sal*I and subcloned in pTZ19R (Pharmacia Biotech). The two *Sal*I subclones flanking the *Sal*I-site at the 5' end of the ORF, were cloned into the vector pGEM-7Zf(+) (Promega Corp., Madison WI). In this way, a unique *Kpn*I-site was created at this position. A cassette with the neomycin resistance gene driven by the TK-promoter (Thomas and Capecchi, 1987) was inserted at this *Kpn*I site, resulting in a targeting vector with 3.2 and 3.5 kb of homologous sequences flanking the mutation at the 3' and 5' position, respectively. This *neo* cassette was inserted in the antisense orientation with respect to the transcriptional orientation of the *mHR6B* gene. The resulting plasmid was linearized with *Nsi*I, reducing the homologous region 3' of the neo cassette to 2.6 kb, and electroporated into 129/Ola-derived E14 ES cells (a kind gift of dr. A. Berns, NKI, Amsterdam, The Netherlands) as described earlier (Zhou et al., 1995). G418 (Geneticin®; Gibco BRL, Gaithersburg MD) was added 24 hrs after electroporation (final concentration: 200μg/ml), and the cells were maintained under selection for 6-8 days. Genomic DNA from individual, neomycin-resistant clones was digested with *Eco*RI or *Sph*I and analyzed by Southern blotting using a 0.7 kb *Eco*RI-*Eco*RV probe positioned immediately 3' of the targeting construct. Targeted clones, with the correct hybridizing *Eco*RI fragments, were subsequently screened with a fragment of the neo-resistance gene as a probe to confirm proper homologous recombination. To obtain double targeted ES cells, a second targeting construct was made in a similar way with a cassette containing a hygromycin resistance gene under the control of the PGKpromoter (Riele te et al., 1990) instead of the neo cassette.

Generation of *mHR6B*-deficient mice

Cells of G418-resistant, homologous recombinant clones were karyotyped and ES cells from two independent clones with 40 normal chromosomes were used for injection into 3.5 day-old blastocysts isolated from pregnant C57BL/6 females as described previously (Zhou et al., 1995). Male chimaeric mice were mated with FVB/J females to obtain heterozygote animals. Germline transmission was observed in the coat color of the F1 offspring. Genomic DNA was isolated from tail biopsies, digested with *Eco*RI, resolved in 1% agarose, blotted and probed with the 0.7 kb *Eco*RI-*Eco*RV diagnostic probe to assess the genotype (Figure 1A). Heterozygous siblings were mated to generate *mHR6B*$^{-/-}$ animals.

Antibody production and immunoblotting

A peptide of 15 amino acids, resembling the C-terminal end of the mHR6B/hHR6B protein including an additional cysteine, KRVSAIVEQSWNDSC, was synthesized on an automated peptide synthesizer (Novabiochem AG, Laufelfingen, Switserland) as described earlier (Koken et al., 1996). One mg peptide was dissolved in PBS and coupled to activated carrier protein (keyhole lampet hemocyanin) according to the manufacturer's guidelines (Pierce, Rockford, IL). A rabbit was primed by intracutaneous application of the antigen mixed with Freund's complete adjuvans. The first boost of the antigen was applied after five weeks and the second boost after 10 weeks. For boosting the antigen was mixed with Freund's incomplete adjuvans. Blood was collected 14 days after the second boost. Preparation of crude tissue extracts, separation of sample contents, electroblotting and antigen detection were carried out as described by Koken et al. (1996). The primary antibody, raised against the C-terminal petide, was used in a 1:250 dilution. The blots were developed using horseradish peroxidase (Biosource International, Camarillo, CA) as the secondary antibody and visualized using ECL (Amersham International plc, Little Chalfont, England).

Cell survival after irradiation

UV sensitivity was determined assaying the incorporation of [3H]-thymidine by proliferating fibroblasts at various doses of UV. In short, cells were pulse-labelled for one hour, incubated in unlabelled medium for one hour, lysed and incorporation was quantified using a scintillation counter. Cell survival is expressed as the ratio of [3H] incorporation in irradiated and non-irradiated primary mouse embryonic fibroblasts (A.M. Sijbers et al., submitted). Ionizing radiation sensitivity was determined by comparing the colony forming ability of targeted ES cells after ^{60}Co-irradiation essentially as described by (Taalman et al., 1983). After irradiation cells were seeded on BRL-conditioned ES medium in 60 mm petridishes. Cells were grown for 6-10 days, fixed and stained. The number of colonies were counted and compared with non-targeted ES cells treated in the same way.

Hormone and tissue weight determination

Blood was collected by orbital sinus puncture under ether anaesthesia. Then, the animals were killed by cervical dislocation and the testes, epididymides and seminal vesicles were dissected out. Of each animal, one testis and its attached epididymis were fixed using Bouin's solution, and the other testis and epididymis were weighed. The latter epididymis was homogenized in phosphate-buffered saline, to count the number of spermatozoa using a Neubauer haemocytometer. Cells with a head and a tail were regarded as sperm cells, irrespective of morphological abnormalities. The concentration of follicle-stimulating hormone (FSH) in the circulation was estimated by radioimmunoassay in 2 volumes of serum (Dullaart et al., 1975). The concentration of FSH is expressed in terms of the standard NIDDK RP-2. The inter-assay coefficient of variation was 10%.

Histology

Testes were isolated and punctured for good penetration of the fixative. Testes and epididymides were fixed for 48 hours in Bouin's fixative at room temperature, the fixative was extracted with 70% ethanol for 2-3 days and the tissues were embedded in paraffin. Mounted sections (4-6 µm) were deparaffinized, rehydrated and stained with the periodic acid/Schiff sulfite leucofuchsin (PAS) reaction or used in immunohistochemistry.

Immunohistochemistry

Immunohistochemical localization of testis-specific transition protein 2 (TP2) was performed using rabbit anti-rat polyclonal antibody (Alfonso and Kistler, 1993) (kindly provided by dr. Kistler). Immunolocalization of testis-specific histone 2B (tH2B) was performed using a mouse monoclonal IgG raised against tyrosine hydroxylase (TH; Boehringer Mannheim GmbH, Mannheim, Germany), which is known to also immunoreact with rat tH2B in tissue sections because of sequence homology at the N-termini of TH and tH2B (Unni et al., 1995).

Testis and epididymis sections were mounted on slides coated with 3-aminopropyltrietoxysilane (Sigma Co., St. Louis MO), and kept at 60°C overnight. The tissues were dewaxed in xylene, and endogenous peroxidase was blocked with a 20 min incubation in 3% H2O2 in methanol. An antigen retrieval step was performed, for tissues prepared for anti-TP2 staining, by heating the sections in 0.01M sodium citrate buffer (pH 6.0) in a microwave oven at 700W (4x5 min). This was not necessary for sections prepared for anti-TH immunostaining. Non-specific antibody binding was blocked with normal goat serum (Dako, Glostrup, Denmark), diluted 1:10 in 5% (w/v) BSA in PBS (pH 7.4). The tissues were then placed in a Sequenza Immunostainer (Shandon Scientific Ltd, Runcorn, England) and incubated at 4°C overnight with the primary antibody, diluted 1:10,000 for anti-TP2 and 1:100 for anti-TH in 5% BSA (w/v) in PBS. Immunostaining was performed using biotinylated goat anti-rabbit or goat anti-mouse immunoglobulin where appropriate (Dako) for 30 min, streptavidin-peroxidase (Dako) for 30 min, and metal-enhanced diaminobenzidine (Pierce) for 7 min. The sections were counterstained for 15 sec with Mayer's hematoxylin, and viewed with a Zeiss Axioskop 20 light microscope at magnifications 100x and 400x. Control sections were incubated with 5% BSA (w/v) in PBS without the primary antibody and subsequently processed as described above.

Nuclear DNA fragmentation labeling (TUNEL)

Tissues were fixed for 16 hours at 4°C in PBS containing 3.6% formaldehyde and embedded in paraffin. Sections (4-6 µm) were mounted on AAS-coated glass slides, dewaxed and pretreated with proteinase-K (Sigma) and peroxidase as described elsewhere (Gavrieli et al., 1992). Slides were subsequently washed in TdT-buffer for 5 minutes (Gorczyca et al., 1993), and incubated for at least 30 minutes in TdT-buffer containing 0.01 mM Biotin-16-dUTP (Boehringer Mannheim) and 0.4U/µl TdT-enzyme (Promega). The enzymatic reaction was stopped by incubation in TB-buffer and the sections were washed (Gavrieli et al., 1992). Slides were then incubated with streptABComplex/horseradish peroxidase conjugate (Dako) for 30 minutes and washed in PBS. dUTP-biotin labeled cells were visualized with Diaminobenzidine.4HCl (Sigma). Cells were counterstained with 0.2% (w/v) nuclear fast red/5% (w/v) $Al_2(SO4)3$ for 10 seconds and rinsed in tap water for 10 min.

Acknowledgements

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Summary
Samenvatting

Summary

For each organism it is very important to ensure that the genetic information encoded by its DNA is kept intact and that it is transmitted faithfully to daughter cells or to the next generation. However, many chemical and physical agents (f.i. the UV component of the sunlight) cause adducts in DNA which hamper directly the transcription and replication processes. When in addition such DNA damage is fixed into mutations, the coding information for proteins or the regulatory regions of genes can be affected which may result in inborn defects, cell death or malfunctioning including onset of cancer.

To avoid these deleterious consequences all organisms have developed a complex network of DNA repair mechanisms, two of which "nucleotide excision" and "postreplication" repair are of special interest for the studies performed in this thesis. Since recently, it is known that both processes are strongly conserved in evolution. The main objective of the thesis project was the exploitation of this (at the beginning of the thesis-period putative) evolutionary conservation for the isolation of homologous genes from man starting from known yeast genes, and vice versa.

To this aim a general method was developed and applied for the cloning of *Drosophila melanogaster* (fruitfly), *Saccharomyces cerevisiae* (baker's yeast) and *Schizosaccharomyces pombe* homologues of the human nucleotide excision repair gene *XPB/ERCC3*. In addition this strategy was utilized starting with the *RAD6* DNA postreplication repair gene from *S. cerevisiae* for the isolation of *Ss.pombe*, *D.melanogaster* and as later appeared two human homologues of this yeast gene. To detect primarily meaningful nucleotide sequence homology and to avoid small regions with fortuitous homology, the "junction probe strategy" was developed. This approach assumes that a truely homologous gene will display cross-hybridisation over an extended DNA area. The basic idea is the use of two non-overlapping flanking DNA probes, and to search for clones which hybridize simultaneously with both probes. This introduces a selectivity for extended regions of nucleotide sequence identity and greatly diminishes fortuitous cross hybridization which is one of the major problems when low-stringency hybridizations are performed.

The human XPB/ERCC3 protein is part of a multi-protein complex called TFIIH with a dual function: initiation of basal transcription and nucleotide excision repair. The isolation of the homologous genes from *D.melanogaster*, *Ss. pombe* and *S.cerevisiae* using the above approach is described in references 12, 14 and 15 of the publications list (pg. 117).

The PhD thesis concerns the different homologues of the yeast gene *RAD6*. Yeast *RAD6* is the "master gene of post replication repair", an ill-defined process of DNA damage-tolerance and damage-induced mutagenesis. The *RAD6* gene encodes a small 172 amino acids protein with a highly acidic C-terminal tail sequence. The protein was shown to be a ubiquitin-conjugating enzyme i.e. an enzyme capable of coupling ubiquitin, the most conserved polypeptide known, to a variety of protein targets. Ubiquitin is implicated in an extremely large number of functions ranging from protein breakdown, DNA repair, or cell cycle control to protein folding and protection against proteolysis. As the RAD6 protein

corrects a pleiotropic mutant phenotype in yeast (Table IV, pg.29) this suggests that this polypeptide too is important for processes as different as DNA damage-induced mutagenesis, postreplication repair, protein degradation via the N-rule pathway, repression of retrotransposition and sporulation.

The cloning and characterisation of the different homologues led to several important conclusions concerning the mode of action of the RAD6 protein. Analysis of the *Ss.pombe rhp6+* (Chapter II), Drosophila *Dhr6* (Chapter III), and the duplicated human genes, *HHR6A* and *6B* (Chapters IV, VI and VII), indicated that they all lack the acidic tail sequences. Thus this C-terminal domain of yeast rad6 protein was probably lost in the course of evolution in the other species or, alternatively, it was gained by the yeast gene. The presence of this protein region which was shown to be absolutely necessary for sporulation and N-rule protein degradation in the baker's yeast, was found to hamper the complete correction of the sporulation defect of an *Ss.pombe* $rhp6^{+}$ deletion mutant. However, the homologous *Ss.pombe* gene, which doesnot encode itself a tail sequence can correct almost completely the full *rad6* mutant phenotype (including the sporulation deficiency). The Drosophila gene *Dhr6*, and the two human homologues *HHR6A* and *6B*, correct the RAD6 defects in UV-induced mutagenesis and sensitivity to DNA damaging agents, but are unable to complement the sporulation deficiency of a yeast *rad6Δ* mutant.

Based on sequence divergence we estimated that about 200 million years ago a gene-duplication took place leading to two *RAD6* homologous genes in probably all mammals. In man the two genes, *HHR6A* and *HHR6B*, are localised on chromosomes X and 5, respectively (Chapter V). The study of the expression of both these genes first showed that in contrast to the situation encountered in yeast the two human or mouse genes are not induced upon UV irradiation (Chapter VI). This points probably to a fundamental difference in UV response between unicellular organisms which have to continuously adapt to a changing environment, and the situation encountered in higher multicellular eukaryotes which have created a relatively stable microenvironment for their cells.

An extensive study of mRNA and protein expression in different tissues showed furthermore that both genes and proteins are ubiquitously expressed. However, quantitative differences between various tissues exist and the relatively high mRNA content in testis led us to examine this tissue in more detail. Both the *HHR6A* and *HHR6B* genes are expressed until very late during spermatogenesis on the RNA and protein level. This is rather remarkable for two reasons. First, for many genes, RNA is found in the different stages of spermatogenesis, however only for a very limited set also the corresponding proteins are present, probably implicating that only these few are indeed functioning in the complicated processes of differentiation, growth and mitotic and meiotic divisions taking place during spermatogenesis. For HHR6 its functional presence is indeed corroborated by the results of the *HHR6B* knockout mice (Chapter VII), which present a severe spermatogenesis defect.

Second, it was generally assumed that during late spermatogenesis X- and Y-linked genes are transcriptionally silenced. However, the mRNA and protein of the X-linked *HHR6A* gene are present in large amounts several days after meiosis took place. This renders the inactivation dogma questionable at least for this gene.

The specific expression during late spermatogenesis is in favour of a function for both genes in the processes taking place after meiosis, in particular the replacement of normal histones by transition proteins or protamines, necessary for the chromatin condensation which occurs during the formation of spermatozoa. Moreover, the phenotype of the *HHR6B*-knockout mouse model, recently constructed in the laboratory, shows a deregulation of the spermatogenesis process which becomes obvious post-meiotically and results in a male-sterile phenotype because only abnormal sperm cells are formed. Female knockout mice are completely normal as probably the *HHR6A* gene complements for the *HHR6B* deletion. In conclusion, it is reasonable to assume that both genes have largely complementary functions, and that the implication of the human *HHR6* genes in late spermatogenesis may represent a phenocopy of the implication of yeast *RAD6* in sporulation.

Samenvatting

Voor ieder levend organisme is het van het grootste belang om de vitale erfelijke informatie, die is opgeslagen in de volgorde van de basen in het DNA, intact te houden zodat die kopiegetrouw aan dochtercellen, of aan de volgende generatie kan worden doorgegeven. Echter veel chemische en fysische agentia (bijvoorbeeld het UV licht afkomstig van de zon) brengen schade aan in het DNA, welke het normale verloop van transcriptie en replicatie processen kunnen verstoren. Als deze DNA-beschadigingen bovendien in blijvende mutaties worden omgezet, kan de informatie, welke codeert voor eiwitten, veranderingen ondergaan, evenals de DNA-gebieden die de expressie van genen reguleren. Dit kan resulteren in erfelijke defecten, celdood of erger nog het fout functioneren van de cel, wat bijvoorbeeld kan leiden tot kanker.

Om al deze problemen te voorkomen, hebben alle organismen een complex netwerk van DNA-herstel mechanismen ontwikkeld. Het werk beschreven in dit proefschrift heeft betrekking op twee van zulke systemen, namelijk het "nucleotide excisie" en "postreplicatie" herstel. Sinds kort is het duidelijk geworden dat beide processen erg geconserveerd zijn gedurende de evolutie, en het onderzoeksproject had als hoofddoelstelling het uitbuiten van deze (bij de aanvang van het onderzoek "mogelijke") evolutionaire conservering voor het isoleren van homologe menselijke genen, gebruikmakend van reeds gecloneerde gist genen, en vice versa.

Hiertoe werd een algemeen toepasbare methode ontwikkeld. Deze is toegepast voor het isoleren van een homoloog *Drosophila melanogaster* (fruitvlieg), *Saccharomyces cerevisiae* (bakkers gist) en *Schizosaccharomyces pombe* gen van het humane nucleotide excisie herstel gen *XPB/ERCC3*. Anderzijds, werd dezelfde strategie toegepast, beginnend met het postreplicatie herstel gen RAD6 van de bakkers gist, voor de isolatie van een *Ss. pombe*, *D.melanogaster*, en zoals later bleek twee humane homologen. Om betekenisvolle nucleotide sequentie homologie te detecteren, en om te vermijden dat kleine stukjes toevallige homologie worden opgepikt, is een nieuwe methode ontwikkeld. Deze "flankerende probe benadering" heeft als grondslag de gedachte dat een "echt" homoloog gen meestal kruishybridisatie zal vertonen over een uitgebreide DNA regio. Het principe berust op het gebruik van twee niet-overlappende DNA probes, en het alleen isoleren van clones, die met beide probes hybridiseren. Dit zorgt voor het selecteren van verspreide nucleotide sequentie identiteit, en vermindert drastisch het isoleren van clones die alleen hybridiseren op grond van unieke kleine gebieden van sequentie gelijkheid. Zo wordt een van de grote beperkingen van lage stringentie hybridisatie technieken opgeheven.

Het humane XPB/ERCC3 eiwit maakt deel uit van een groot eiwit complex, TFIIH genaamd, met een dubbele functie: het initiëren van basale transcriptie en nucleotide excisie herstel. De isolatie van de homologe genen uit *D.melanogaster*, *Ss. pombe* en *S.cerevisiae*, gebruikmakend van bovenbeschreven principe, is beschreven in referenties 12, 14 en 15 van de publicatie lijst (pag. 117).

Het proefschrift behandelt daarentegen alleen de isolatie en karakterisering van verschillende homologen van het gist postreplicatie herstel gen *RAD6*. Gist *RAD6* is het dominerende gen van het postreplicatie herstel mechanisme, een belangrijk, maar grotendeels onbegrepen proces van DNA-schade tolerantie en schade-geïnduceerde mutagenese. Dit gen codeert voor een eiwit van 172 residuen met een reeks zure aminozuren aan de carboxy-terminus. Het is een ubiquitine-conjugerend enzym, dat ubiquitine, het meest geconserveerde polypeptide dat bekend is, koppelt aan een grote aantal andere eiwitten. Ubiquitine is betrokken bij talloze processen in de cel variërend van eiwit afbraak, DNA herstel, cel cyclus regulatie, tot eiwit vouwing en zelfs bescherming tegen eiwit afbraak. RAD6 corrigeert een pleiotroop gist fenotype (zie Tabel IV, pag.29), hetgeen suggereert dat ook dit gen erg belangrijk is voor diverse processen zoals DNA-schade-geïnduceerde mutagenese, postreplicatie herstel, eiwitafbraak via de "N-rule pathway", repressie van retrotranspositie en sporulatie.

Het isoleren en karakteriseren van de verschillende *RAD6*-homologe genen heeft tot verschillende belangrijke conclusies geleid met betrekking tot het werkingsmechanisme van dit eiwit. Allereerst toonde de analyse van het *Ss. pombe rhp6*$^+$ (Hoofdstuk II), het *Drosophila Dhr6* (Hoofdstuk III), en de gedupliceerde humane genen, *HHR6A* en *6 B* (Hoofdstukken IV, VI en VII), aan, dat de eiwitten, die gecodeerd worden door deze genen, allen de "zure staart" sequentie missen. Blijkbaar ging dit C-terminale domein, essentieel voor gist RAD6, gedurende de evolutie verloren, of, een andere mogelijkheid is dat het bakkersgist eiwit, in tegenstelling tot de andere organismen, de zure staart tijdens de evolutie heeft verkregen. De "zure staart"-sequentie van het bakkersgist RAD6 is absoluut noodzakelijk voor het correct verlopen van de sporulatie en "N-rule"-eiwit afbraak. Daarentegen verstoort de aanwezigheid van zulk een zure C-terminale sequentie het correcte verloop van het sporulatie proces in een *Ss. pombe rhp6*$^+$ deletie mutant. Het homologe *Ss. pombe* gen, echter, dat zoals gezegd geen "zure staart" bevat, corrigeert volledig het *rad6* mutante fenotype, inclusief de sporulatie deficiëntie. Het *Drosophila* gen, *Dhr6*, als ook de twee humane homologen, *HHR6A* en *6 B*, corrigeren alleen de defecten in de UV-geinduceerde mutagenese en de gevoeligheid voor de DNA beschadigende agentia, maar niet de problemen met de sporulatie.

Op grond van sequentie divergentie hebben we kunnen berekenen dat er ongeveer 200 miljoen jaar geleden een gen-duplicatie plaatsvond, leidend tot twee homologe *RAD6* genen in waarschijnlijk alle zoogdieren. In de mens zijn de twee genen, *HHR6A* en *HHR6B*, gelokaliseerd op, respectievelijk, de chromosomen X en 5 (Hoofdstuk V). De analyse van het transcriptie patroon van beide genen toonde aan, dat in tegenstelling tot de situatie in gist, de twee menselijke of muize genen niet door UV licht geïnduceerd kunnen worden (Hoofdstuk VI). Dit wijst waarschijnlijk op een fundamenteel verschil tussen de reactie op bestraling met UV licht tussen de eencellige organismen, die zich voortdurend moeten aanpassen aan veranderende omgevings-omstandigheden, en de multicellulaire organismen, die een redelijk stabiel milieu gecreëerd hebben voor hun cellen.

De analyse van de mRNA en eiwit expressie in verschillende weefsels toonde ook aan, dat beide genen en eiwitten waarschijnlijk overal in het lichaam tot expressie komen. Er zijn, echter, kwantitatieve verschillen tussen de verschillende weefsels, en de grote hoeveelheid *HHR6* RNA en eiwit in testis leidde ons er toe dit weefsel aan een meer gedetailleerde analyse

te onderwerpen. Beide genen worden tot laat in de spermatogenese geëxpresseerd zowel op het RNA- als het eiwit-niveau. Dit is opmerkelijk om twee redenen. Allereerst, wordt er voor veel genen RNA gevonden in de verschillende stadia van de spermatogenese, terwijl er echter voor slechts een gering aantal ook het corresponderende eiwit aanwezig is. Dit wijst er waarschijnlijk op, dat alleen deze laatste kleine groep genen daadwerkelijk werkzaam is in de gecompliceerde processen van differentiatie en groei na de mitotische en meiotische delingen die plaatsvinden tijdens de spermatogenese. Voor HHR6 wordt deze hypothese ondersteund door het fenotype van muize-mutanten, waarin wij het *HHR6B* gen hebben uitgeschakeld, hetgeen een ernstig spermatogenese defect veroorzaakt (Hoofdstuk VII). Ten tweede wordt algemeen aangenomen dat gedurende de late spermatogenese, de transcriptie van genen die op het X en Y chromosoom gelegen zijn, wordt onderdrukt. Er is echter een grote hoeveelheid mRNA en eiwit aanwezig afkomstig van het X-gebonden *HHR6A* gen, enkele dagen nadat de meiose reeds heeft plaatsgevonden. Dit gegeven maakt inactivatie, althans van dit gen, onwaarschijnlijk, hetgeen van belang is voor het concept van transcriptie-inactivatie.

De specifieke expressie van de beide genen tijdens de laatste stadia van de spermatogenese is waarschijnlijk indicatief voor een functie van beide eiwitten in de processen, die plaatsvinden na de meiose. Dit betreft ondermeer het vervangen van de normale histonen door transitie-eiwitten, en later door protamines, hetgeen nodig is voor de chromatine-condensatie gedurende de laatste etappes van de spermatogenese. De *HHR6B*-"knockout" muizen vertonen een ontsporing van de spermatogenese, die zichtbaar wordt na de meiose en resulteert in een mannelijk steriliteits syndroom. Vrouwelijke mutant muizen zijn normaal, evenals de overige weefsels in de mannelijke mutant muizen. Waarschijnlijk kan het homologe *HHR6A* gen de *HHR6B*-deletie in de "knockout" muizen complementeren. Daarom is het aannemelijk, dat beide genen een grotendeels overlappende functie vervullen. De betrokkenheid van de humane *HHR6* genen in de spermatogenese zou een feno-copie kunnen zijn van de betrokkenheid van gist *RAD6* gen in het sporulatie proces.

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Curriculum vitae

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List of Publications

List of Publications

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Nawoord

Nawoord

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