DEVELOPMENTAL BIOLOGY **173,** 119–132 (1996)
Article No. 0011

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

Marcel H. M. Koken,*[,1] Jos W. Hoogerbrugge,† Iris Jaspers-Dekker,* Jan de Wit,* Rob Willemsen,* Henk P. Roest,* J. Anton Grootegoed,† and Jan H. J. Hoeijmakers*[,2]

**Department of Cell Biology and Genetics, Medical Genetics Centre, Erasmus University, P.O. Box 1738 3000DR Rotterdam, The Netherlands; and †Department of Endocrinology and Reproduction, Erasmus University, Rotterdam, The Netherlands*

***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_r 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-

[1] Present address: CNRS UPR43 Hôpital St. Louis, Bâtiment INSERM, 16 Rue de la Grange aux Belles, 75010 Paris, France.

[2] To whom correspondence should be addressed.

0012-1606/96 $12.00

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.

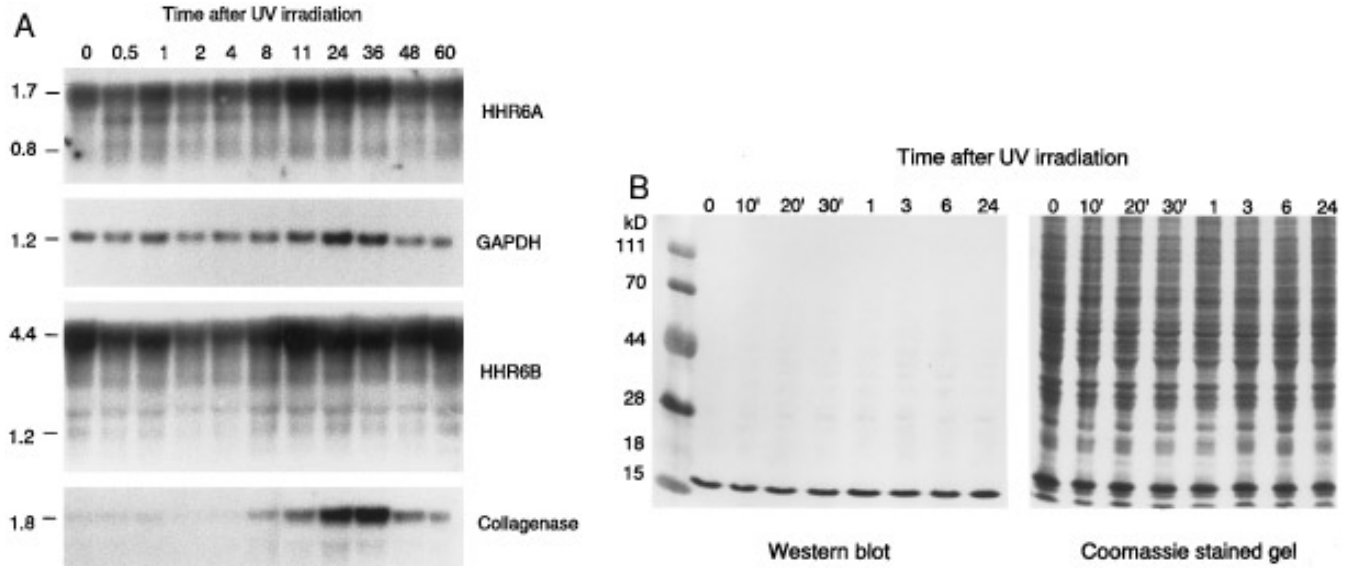


FIG. 1. (A) Northern blot analysis of *HHR6A* and *HHR6B* after UV irradiation. Primary fibroblasts were irradiated with 1 J/m^2 and total RNA was used for Northern blot analysis. The time scale is given in hours and the probes used are indicated at the right. Transcript length is given in kilobases 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

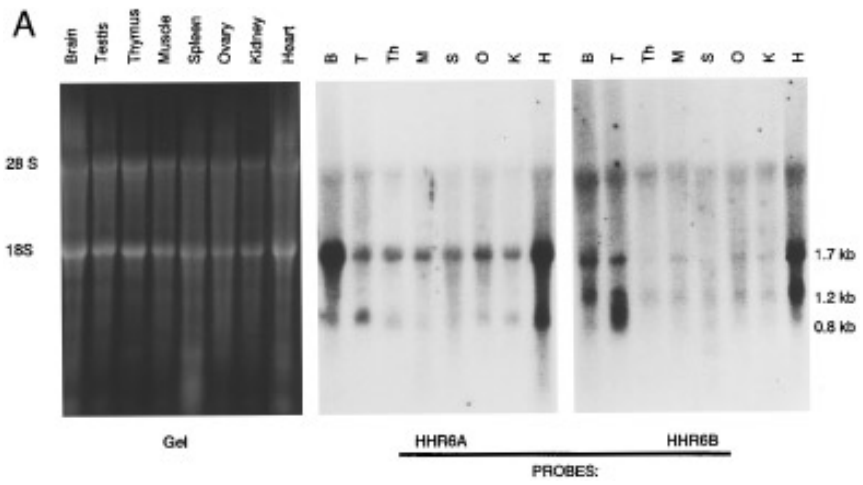
The ubiquitin system has an important function in selective degradation or refolding of misfolded proteins due to, for instance, heat shock. Several members of the UBC family are implicated in general stress responses and two of the three human ubiquitin genes are induced after heat shock (Fornace *et 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).

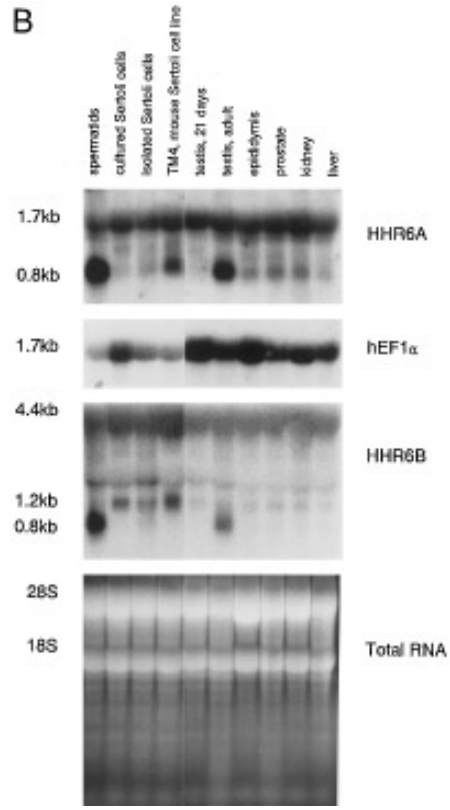
Expression during Mitotic Cell Cycle

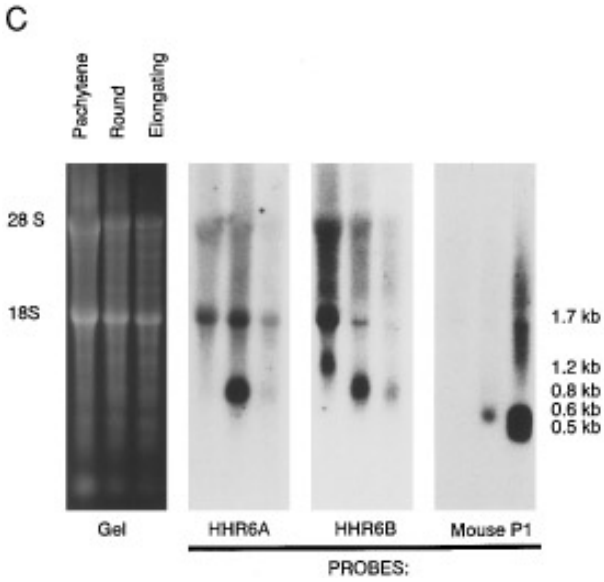
Several lines of evidence associate RAD6 with chromatin remodeling. To investigate whether the human homologues are involved in any of the chromatin transitions which occur during the mitotic cell cycle, HeLa cells were synchronised by a double thymidine block, and mRNA and protein expression were analysed 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).

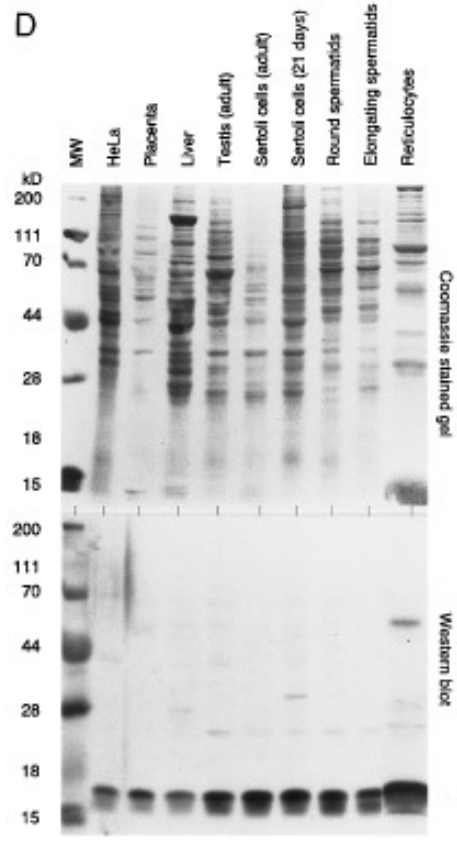
Tissue-Specific Expression of HHR6A and HHR6B

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
B T Th M S O K H
B T Th M S O K H
28 S
18S
1.7 kb
1.2 kb
0.8 kb
Gel
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
1.7kb
hEF1α
4.4kb
HHR6B
1.2kb
0.8kb
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
MW
HeLa
Placenta
Liver
Testis (adult)
Sertoli cells (adult)
Sertoli cells (21 days)
Round spermatids
Elongating spermatids
Reticulocytes
kD
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

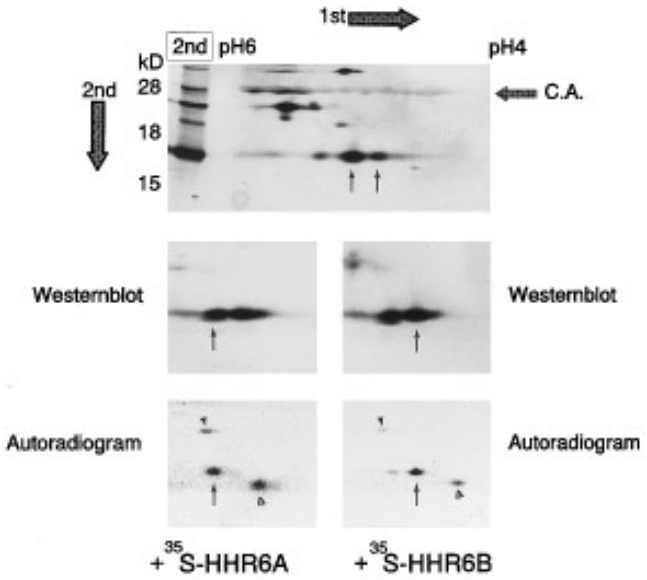


FIG. 3. Identification of HHR6A and HHR6B in total cell extracts by 2-dimensional gel electrophoresis. Top: Total HeLa extract was fractionated by 2-dimensional gel electrophoresis. From left to right the first dimension: isoelectrofocusing over a gradient from pH 6 to 4, and from top to bottom: size-fractionation 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 p*I* 5.26 (HHR6A+ubiquitin) and p*I* 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.

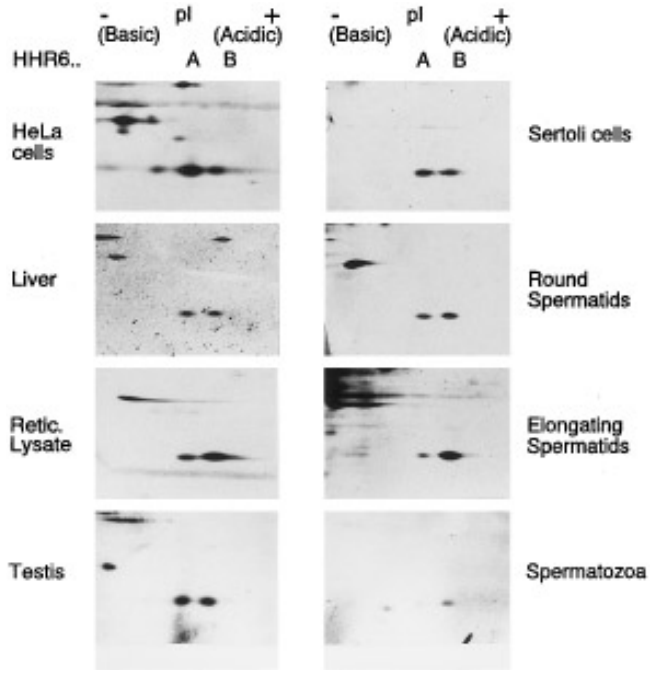


FIG. 4. HHR6A and HHR6B protein expression in different tissues. 2-Dimensional gel electrophoresis was performed to separate the two human HHR6 proteins in different human (HeLa/placenta), rabbit (reticulocyte lysate), and rat cells or tissues (young Sertoli cells, adult liver, testis, round spermatids, 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.

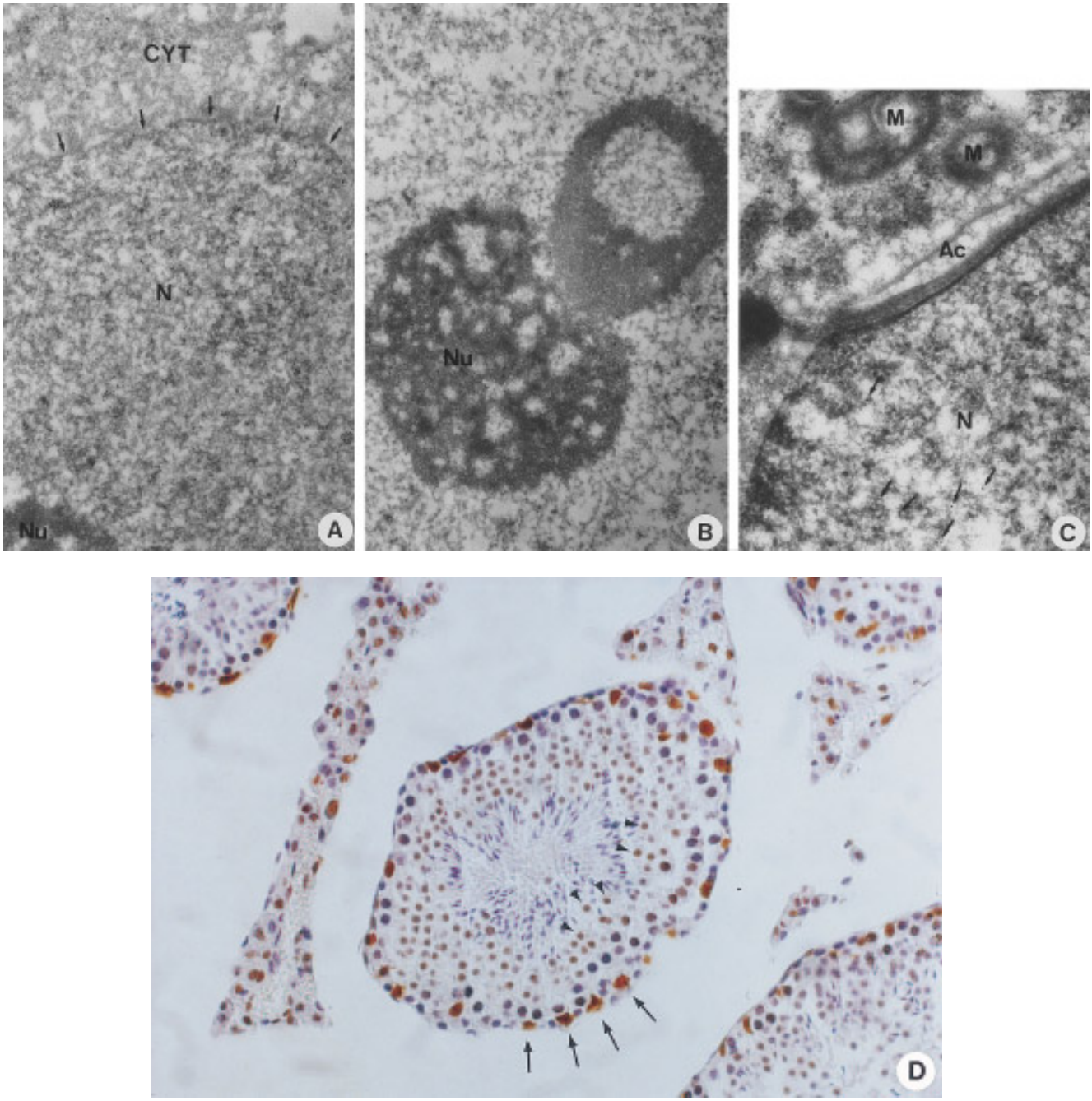


FIG. 5. Immunohistochemical detection of the HHR6 proteins by electron and light microscopy. Abbreviations used: N, nucleus; CYT, cytoplasma; Nu, nucleolus; M, mitochondrion; and Ac, acrosome. (A) Sertoli cell incubated with anti-RAD6 serum. The arrows indicate the nuclear membrane. (B) Higher magnification 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.

ACKNOWLEDGMENTS

We thank Drs. L. and S. Prakash for the generous gift of the RAD6 polyclonal antiserum, Drs. F. J. Benham for the GAPDH probe, P. Herrlich for the probes for collagenase and metallotheionin, T. Butt for ubiquitin probes, I. Laird for the human elongation factor 1α gene, and D. Meijer for the mouse protamine 1 probe. We are very thankful for the help of the following colleagues: Dr. Theo Luider for help with the 2-dimensional gel electrophoresis, Ton Verkerk for FACS analysis, Dr. Claude Backendorf and Li Bin Ma for some of RNA samples of UV-irradiated cells, Dr. André Hoogeveen for preparation of synthetic oligopeptides, Wim Vermeulen for microinjection experiments, Maarten Fornerod for the protein samples of cell cycle synchronisation experiments, Drs. Carel Meijers, Axel Themmen, and Leen Blok for helpful discussions, and Jan van Klaveren for helpful discussions and excellent technical assistance. Dr. D. Bootsma is acknowledged for critical reading of the manuscript and continuous support. We are grateful to Mirko Kuit and Tom de Vries Lentsch for photography, and Sjozef van Baal for computer assistance. This work was supported by the Dutch Cancer Society (Project IKR 88-2, 90-20 and 92-118) and the European Community (Contract B16-141-NL).

REFERENCES

Angel, P., Poting, A., Mallick, U., Ramsdorf, H. J., Schorp, M., and Herrlich, P. (1986). Induction of metallotheionein and other mRNA species by carcinogens and tumor promoters in primary human skin fibroblasts. *Mol. Cell. Biol.* **6,** 1760–1766.

Arnold, J. E., and Gevers, W. (1990). Auto-ubiquitination of ubiquitin-activating enzymes from chicken breast muscle. *Biochem. J.* **267,** 751–757.

Bachmair, A., Finley, D., and Varshavsky, A. (1986). In vivo half-life of a protein is a function of its amino-terminal residue. *Science* **234,** 179–186.

Benham, F. J., Hodgkinson, S., and Davies, K. E. (1984). A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X chromosome defines a multigene family. *EMBO J.* **3,** 2635–2640.

Blake, M. S., Johnston, K. H., Russell-Jones, G. J., and Gotschlich, E. C. (1984). A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. *Anal. Biochem.* **136,** 175–179.

Brands, J. H. G. M., Maassen, J. A., Van Hemert, F. J., Amons, R., and Moller, W. (1986). The primary structure of the α-subunit of human elongation factor 1. *Eur. J. Biochem.* **155,** 167–171.

Bucci, L. R., Brock, W. A., and Meistrich, M. L. (1982). Distribution and synthesis of histone 1 subfractions during spermatogenesis in the rat. *Exp. Cell. Res.* **140,** 111–118.

Capel, B., Swain, A., Nicolis, S., Hacker, A., Walter, M., Koopman, P., Goodfellow, P., and Lovell-Badge, R. (1993). Circular transcripts of the testis-determining gene sry in adult mouse testis. *Cell* **73,** 1019–1030.

Christy, K. G., LaTart, D. B., and Osterhoudt, H. W. (1989). Modifications for SDS–PAGE of proteins. *Biotechniques* **7,** 692–693.

Ciechanover, A. (1994). The ubiquitin-proteasome proteolytic pathway. *Cell* **79,** 13–21.

Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11,** 1475–1489.

Dohmen, R. J., Madura, K., Bartel, B., and Varshavsky, A. (1991). The N-end rule is mediated by the UBC2(RAD6) ubiquitin-conjugating enzyme. *Proc. Natl. Acad. Sci. USA* **88,** 7351–7355.

Finley, D., and Chau, V. (1991). Ubiquitination. *Annu. Rev. Cell. Biol.* **7,** 25–69.

Fornace, A. J. (1992). Mammalian genes induced by radiation: Activation of genes associated with growth control. *Annu. Rev. Genet.* **26,** 507–526.

Fornace, A. J., Alamo, I., Hollander, M. C., and Lamoreaux, E. (1989). Ubiquitin mRNA is a major stress-induced transcript in mammalian cells. *Nucleic Acids Res.* **17,** 1215–1230.

Fourney, R. M., Miyakoshi, J., Day, R. S., and Paterson, M. C. (1988). Northern Blotting: Efficient RNA staining and Transfer. *FOCUS* **10,** 5–7.

Galavazi, G., Schenk, H., and Bootsma, D. (1966). Synchronization of mammalian cells *in vitro* by inhibition of the DNA synthesis I: Optimal conditions. *Exp. Cell Res.* **41,** 428–437.

Gibbs, S., Lohmann, F., Teubel, W. J., Van der Putte, P., and Backendorf, C. (1990). Characterization of the human *spr2* promoter: Induction after UV irradiation of TPA treatment and regulation during differentiation of cultured primary keratinocytes. *Nucleic Acids Res.* **18,** 4401–4407.

Grootegoed, J. A., Jansen, R., and Van der Molen, H. J. (1986). Effect of glucose on ATP dephosphorylation in rat spermatids. *J. Reprod. Fertil.* **77,** 99–107.

Hecht, N. B. (1986). Molecular and cellular endocrinology of the testis. *In* ''Molecular and Cellular Endocrinology of the Testis'' (M. Stefanini, M. Conti, R. Geremia, and E. Ziparo, Eds.), pp. 199–213. Elsevier, Amsterdam.

Hendriksen, P. J. M., Hoogerbrugge, J. W., Themmen, A. P. N., Koken, M. H. M., Hoeijmakers, J. H. J., Oostra, B. A., Van der Lende, T., and Grootegoed, J. A. (1995). Postmeiotic transcription of X and Y chromosome genes during spermatogenesis in the mouse. *Dev. Biol.* **170,** 730–733.

Hershko, A., and Ciechanover, A. (1992). The ubiquitin system for protein degradation. *Annu. Rev. Biochem.* 761–807.

Hoeijmakers, J. H. J. (1993a). Nucleotide excision repair I; from *E. coli* to yeast. *Trends Genet.* **9,** 173–177.

Hoeijmakers, J. H. J. (1993b). Nucleotide excision repair II; from yeast to mammals. *Trends Genet.* **9,** 211–217.

Ivell, R. (1992). ''All that glisters is not gold'' Common testis gene transcripts are not always what they seem. *Int. J. Androl.* **15,** 85–92.

Jentsch, S. (1992). The ubiquitin-conjugation system. *Annu. Rev. Genet.* **26,** 179–207.

Jentsch, S., McGrath, J. P., and Varshavsky, A. (1987). The yeast DNA repair gene RAD6 encodes a ubiquitin-conjugating enzyme. *Nature* **329,** 131–134.

Jentsch, S., Seufert, W., Sommer, T., and Reins, H. A. (1990). Ubiquitin-conjugating enzymes: Novel regulators of eukaryotic cells. *Trends Biochem. Sci.* **15,** 195–198.

Johnson, M. H., and Everitt, B. J. (1984). ''Essential Reproduction.'' Blackwell Sci., Oxford, London, Edinborough, Boston.

Kistler, W. P., Heidaran, M. A., Cole, K. D., Kandala, J. C., and Showman, R. M. (1987). *In* ''Cell Biology of the Testis and Epididymis'' (B. J. D. M. C. OrgebinCrist, Ed.), pp. 102–111. The New York Academy of Sciences, New York.

Koken, M. H. M., Reynolds, P., Bootsma, D., Hoeijmakers, J. H. J., Prakash, S., and Prakash, L. (1991a). *Dhr6*, a *Drosophila* homolog of the yeast DNA-repair gene *RAD6*. *Proc. Natl. Acad. Sci. USA* **88,** 3832–3836.

Koken, M. H. M., Reynolds, P., Jaspers-Dekker, I., Prakash, L., Prakash, S., Bootsma, D., and Hoeijmakers, J. H. J. (1991b). Structural and functional conservation of two human homologs of the yeast DNA repair gene *RAD6*. *Proc. Natl. Acad. Sci. USA* **88,** 8865–8869.

Koken, M. H. M., Smit, E. M., Jaspers-Dekker, I., Oostra, B. A., Hagemeijer, A., Bootsma, D., and Hoeijmakers, J. H. J. (1992). Localization of two human homologs, HHR6A and HHR6B, of the yeast DNA repair gene RAD6 to chromosomes Xq24-q25 and 5q23-q31. *Genomics* **12,** 447–453.

Lawrence, C. (1994). The RAD6 DNA repair pathway in *Saccharomyces cerevisiae:* What does it do, and how does it do it? *BioEssays* **16,** 253–258.

Lee, K. A. W., Bindereif, A., and Green, M. R. (1988). A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. *Gene Anal. Tech.* **5,** 22–31.

Lue, N. F., and Kornberg, R. D. (1987). Accurate initiation at RNA polymerase II promoters in extracts from *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **84,** 8839–8843.

Luider, T. M., Peters-van der Sanden, M. J. H., Molenaar, J. C., Tibboel, D., van der Kamp, A. W. M., and Meijers, J. H. C. (1992). Characterization of HNK-1 antigens during formation of the avian enteric nervous system. *Development* **115,** 561–572.

Madura, K., and Prakash, S. (1990). Transcript levels of *S. cerevisiae* DNA repair gene *RAD23* increase in response to UV light and in meiosis but remain constant during the mitotic cell cycle. *Nucleic Acids Res.* **18,** 4737–4742.

Madura, K., Prakash, S., and Prakash, L. (1990). Expression of the *Saccharomyces cerevisiae* DNA repair gene RAD6 that encodes a ubiquitin conjugating enzyme, increases in response to DNA damage and in meiosis but remains constant during the mitotic cell cycle. *Nucleic Acids Res.* **18,** 771–778.

Mather, J. P. (1980). Establishment and characterization of two distinct mouse testicular epithelial cell lines. *Biol. Reprod.* **23,** 243–252.

Merrifield, R. B. (1963). Solid phase peptide synthesis. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* **85,** 2149–2154.

Mezquita, J., and Mezquita, C. (1991). Characterization of a chicken polyubiquitin gene preferentially expressed during spermatogenesis. *FEBS Lett.* **279,** 69–72.

Mitchell, M. J., Woods, D. R., Tucker, P. K., Opp, J. S., and Bishop, C. E. (1991). Homology of a candidate spermatogenic gene from the mouse Y chromosome to the ubiquitin-activating enzyme E1. *Nature* **354,** 483–486.

Morrison, A., Miller, E. J., and Prakash, L. (1988). Domain structure and functional analysis of the carboxyl-terminal polyacidic sequence of the RAD6 protein of Saccharomyces cerevisiae. *Mol. Cell. Biol.* **8,** 1179–1185.

Oliva, R., and Dixon, G. H. (1991). Vertebrate protamine genes and the histone-to-protamine replacement reaction. *Progr. Nucleic Acid Res. Mol. Biol.* **40,** 25–94.

Posthuma, G., Slot, J. W., and Geuze, H. J. (1987). Usefulness of the immunogold technique in quantitation of a soluble protein in ultra-thin sections. *J. Histochem. Cytochem.* **35,** 405.

Promega (1991). Promega protocols and applications guide. Madison, WI.

Radke, K., Carter, V. C., Moss, P., Dehazya, P., Schliwa, M., and Martin, G. S. (1983). Membrane association of a 36,000-dalton substrate for tyrosine phosphorylation in chicken embryo fibroblasts transformed by avian sarcoma viruses. *J. Cell Biol.* **97,** 1601–1611.

Rechsteiner, M. (1988). ''Ubiquitin.'' Plenum, New York.

Reynolds, P., Koken, M. H. M., Hoeijmakers, J. H. J., Prakash, S., and Prakash, L. (1990). The rhp6+ gene of *Schizosaccharomyces pombe:* A structural and functional homolog of the RAD6 gene from the distantly related yeast *Saccharomyces cerevisiae*. *EMBO J.* **9,** 1423–1430.

Reynolds, P., Weber, S., and Prakash, L. (1985). *RAD6* gene of *Saccharomyces cerevisiae* encodes a protein containing a tract of 13 consecutive aspartates. *Proc. Natl. Acad. Sci. USA* **82,** 168–172.

Rocamora, N., and Agell, N. (1990). Methylation of chick UbI and UbII polyubiquitin genes and their differential expression during spermatogenesis. *Biochem. J.* **267,** 821–829.

Roth, J., Bendayan, M., Carlemalm, E., Villiger, W., and Garavito, M. (1981). Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. *J. Histochem. Cytochem.* **29,** 663–671.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). ''Molecular Cloning: A Laboratory Manual.'' Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Skoog, B., and Wichman, A. (1986). Calculation of the isoelectric points of polypeptides from the amino acid composition. *Trends Anal. Chem.* **5,** 82–83.

Smith, F. F., Tres, L. L., and Kierszenbaum, A. L. (1992). Expression of testis-specific histone genes during the development of rat spermatogenic cells in vitro. *Dev. Dynam.* **193,** 49–57.

Stefanini, M., De Martino, C., D'Agostino, A., Agrestini, A., and Monesi, V. (1974). Nucleolar activity of rat primary spermatocytes. *Exp. Cell Res.* **86,** 166–170.

Sung, P., Berleth, E., Pickart, C., Prakash, S., and Prakash, L. (1991). Yeast RAD6 encoded ubiquitin conjugating enzyme mediates protein degradation dependent on the N-end-recognizing E3 enzyme. *EMBO J.* **10,** 2187–2193.

Sung, P., Prakash, S., and Prakash, L. (1988). The RAD6 protein of Saccharomyces cerevisiae polyubiquitinates histones, and its acidic domain mediates this activity. *Genes Dev.* **2,** 1476–1485.

Themmen, A. P. N., Blok, L. J., Post, M., Baarends, W. M., J.W., H., Parmentier, M., Vassart, G., and Grootegoed, J. A. (1991). Follitropin receptor down-regulation involves a cAMP-dependent post-transcriptional decrease of receptor mRNA expression. *Mol. Cell. Endocrinol.* **78,** R7–R13.

Thorne, A. W., Sautiere, P., Briand, G., and Crane-Robinson, C. (1987). The structure of ubiquitinated histone H2B. *EMBO J.* **6,** 1005–1010.

Toebosch, A. M. W., Brusse, R., Verkerk, A., and Grootegoed, J. A. (1989). Quantitative evaluation of the maintenance and development of spermatocytes and round spermatids in cultured tubule fragments from immature rat testis. *Intern. J. Androl.* **12,** 360–374.

Varshavsky, A. (1992). The N-end rule. *Cell* 725–735.

Vindelov, L. L., Christensen, I. J., and Nissen, N. J. (1983). A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* **3,** 323–327.

Watkins, J. F., Sung, P., Prakash, S., and Prakash, L. (1993). The extremely conserved amino terminus of RAD6 ubiquitin-conjugating enzyme is essential for amino-end rule-dependent protein degradation. *Genes Dev.* **7,** 250–261.

Willemsen, R., Brucken, R., Sorber, C. W. J., Hoogeveen, A. T., Wisselaar, H. A., Van Dongen, J. M., and Reuser, A. J. J. (1991). A quantitative immunoelectronmicroscopic study on soluble, membrane-associated and membrane-bound lysosomal enzymes in human intestinal epithelial cells. *Histochem. J.* **23,** 467–473.

Willemsen, R., Van Dongen, J. M., Aerts, J. M. F. G., Schram, A. W., Tager, J. M., Goudsmit, R., and Reuser, A. J. J. (1988). An immunoelectron microscopic study of glucocerebrosidase in type I Gauscher's disease spleen. *Ultrastruct. Pathol.* **12,** 471–478.

Wing, S. S., and Jain, P. (1995). Molecular cloning, expression and characterization of a ubiquitin-conjugating enzyme ($E2_{17kD}$) highly expressed in rat testis. *Biochem. J.* **305,** 125–132.

Zeller, R., Bloch, K. D., Williams, B. S., Arceci, R. J., and Seidman, C. E. (1987). Localized expression of the atrial natriuretic factor gene during cardiac embryogenesis. *Genes Dev.* **1,** 693–698.

Zeller, R., Rogers, M., and Watkins, S. (1991). Current protocols in molecular biology. *In* ''Current Protocols in Molecular Biology'' (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, Eds.), chapter 14. Greene Publishing Associates & Wiley-Interscience, New York.

Received for publication March 21, 1995
Accepted September 16, 1995