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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.

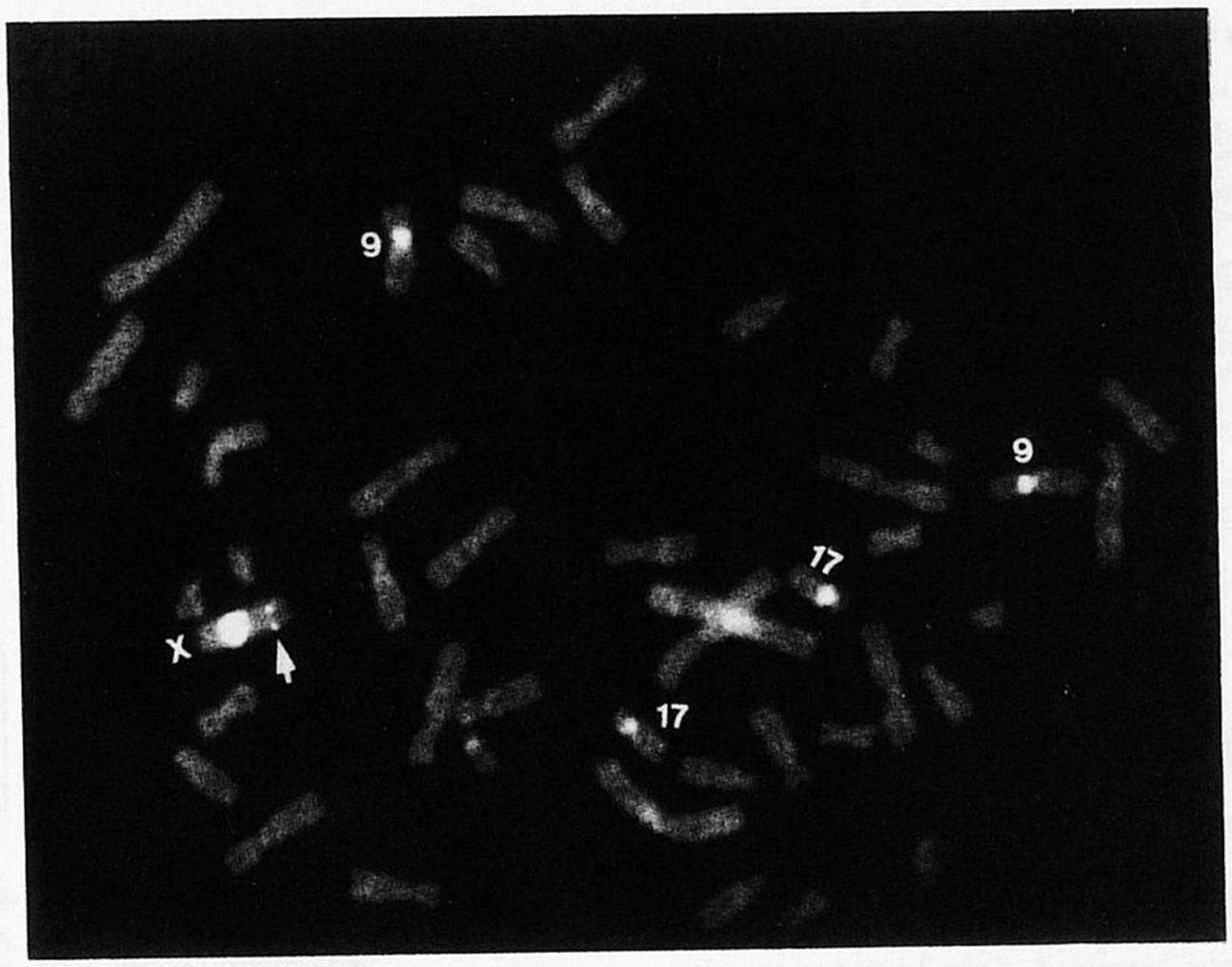
Figure 1B shows the hybridization with biotinylated *HHR6B* gene probes (arrows). Using the DAPI staining procedure, the hybridizing chromosome was identified as chromosome 5 (Fig. 1B). Therefore, the gene was unambiguously assigned to 5q23–q31.

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

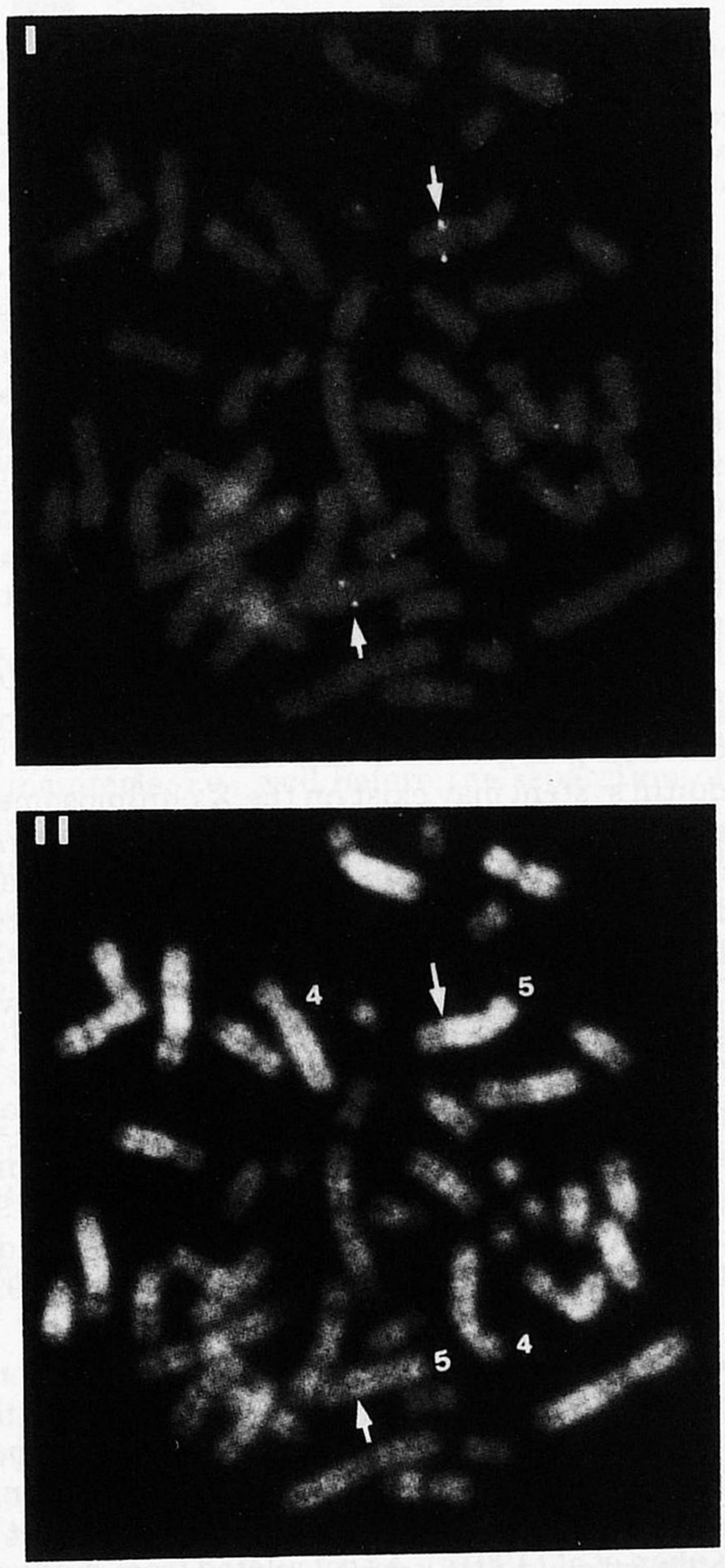
To confirm the assignment of *HHR6A* and to obtain a more precise subchromosomal localization, Southern blot analysis was carried out using genomic DNA from a panel of human–mouse/hamster hybrids containing specific parts of the human X chromosome (Fig. 2B). As shown in Fig. 2A the *HHR6A* cDNA probe 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

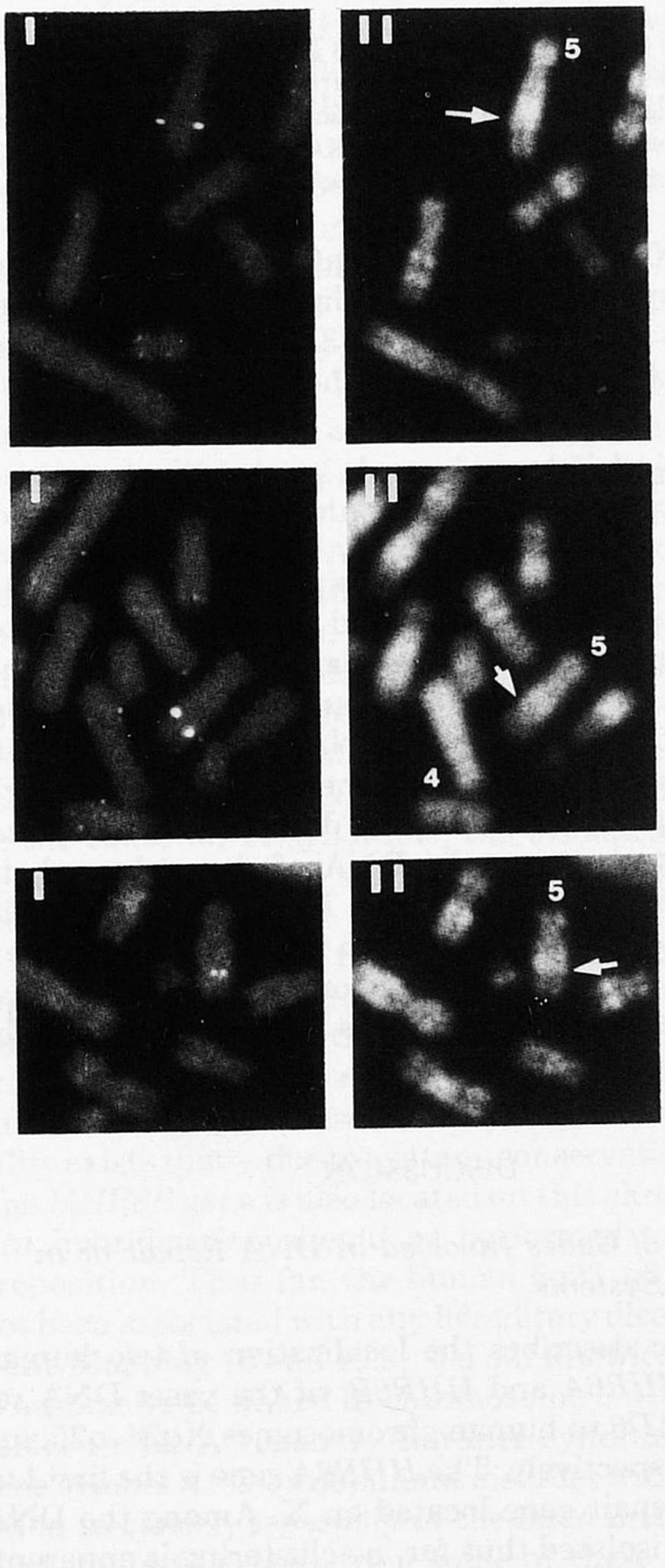
FIG. 1. *In situ* hybridization of metaphase chromosomes to biotinylated genomic *HHR6* probes. (**A**) Hybridization with a cocktail of all genomic *HHR6A* probes specified under Material and Methods. The arrow indicates the hybridization signal on chromosome Xq. This chromosome shows also the X-specific 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





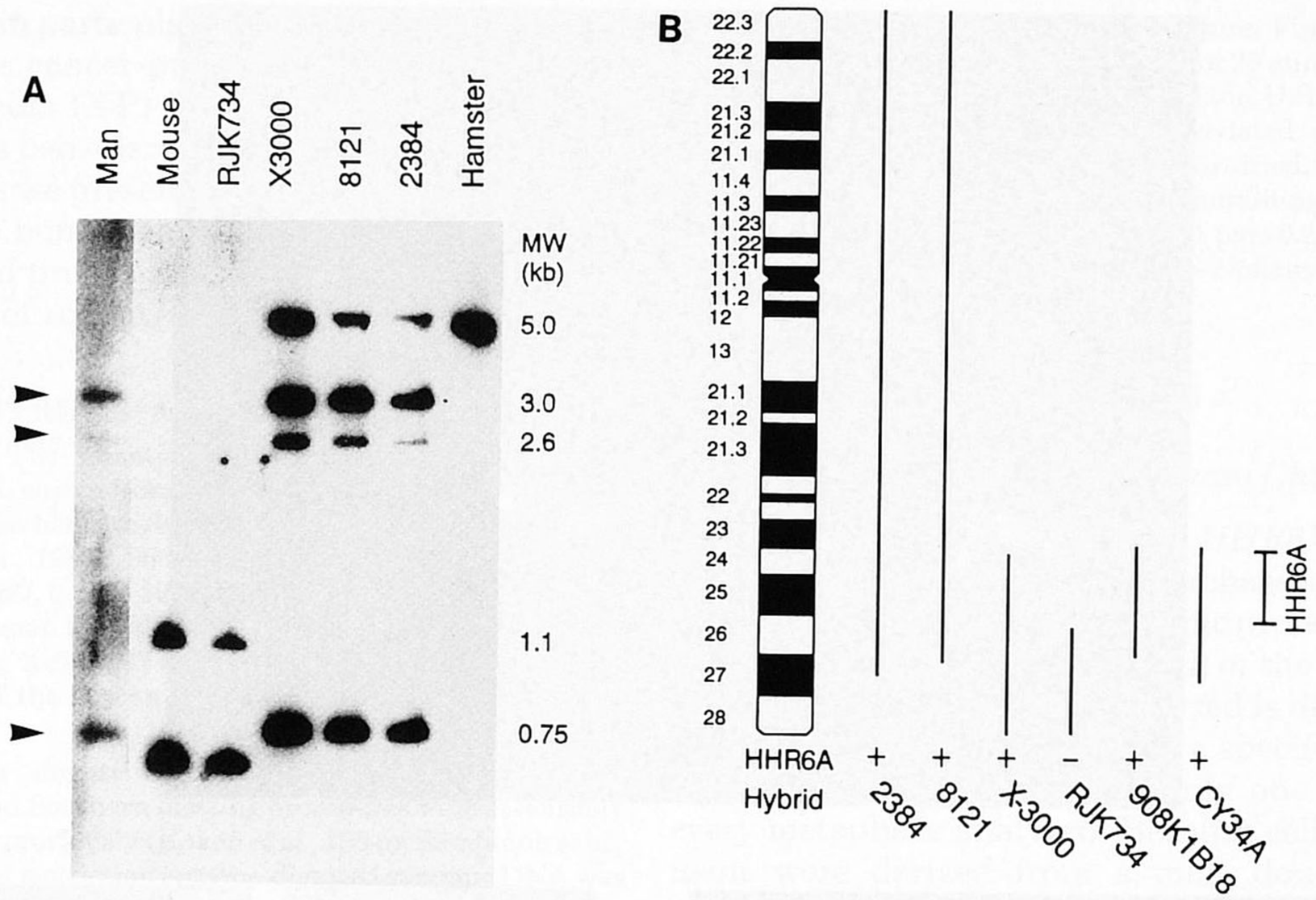


FIG. 2. (**A**) Southern blot analysis of X-specific hybrid panel with the *HHR6A* cDNA probe. The source of the genomic DNA is indicated. DNA is digested with *Hin*dIII and size-fractionated on an 0.8% agarose gel. The molecular weight (MW) indicated on the right refers to the hybridizing fragments 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,

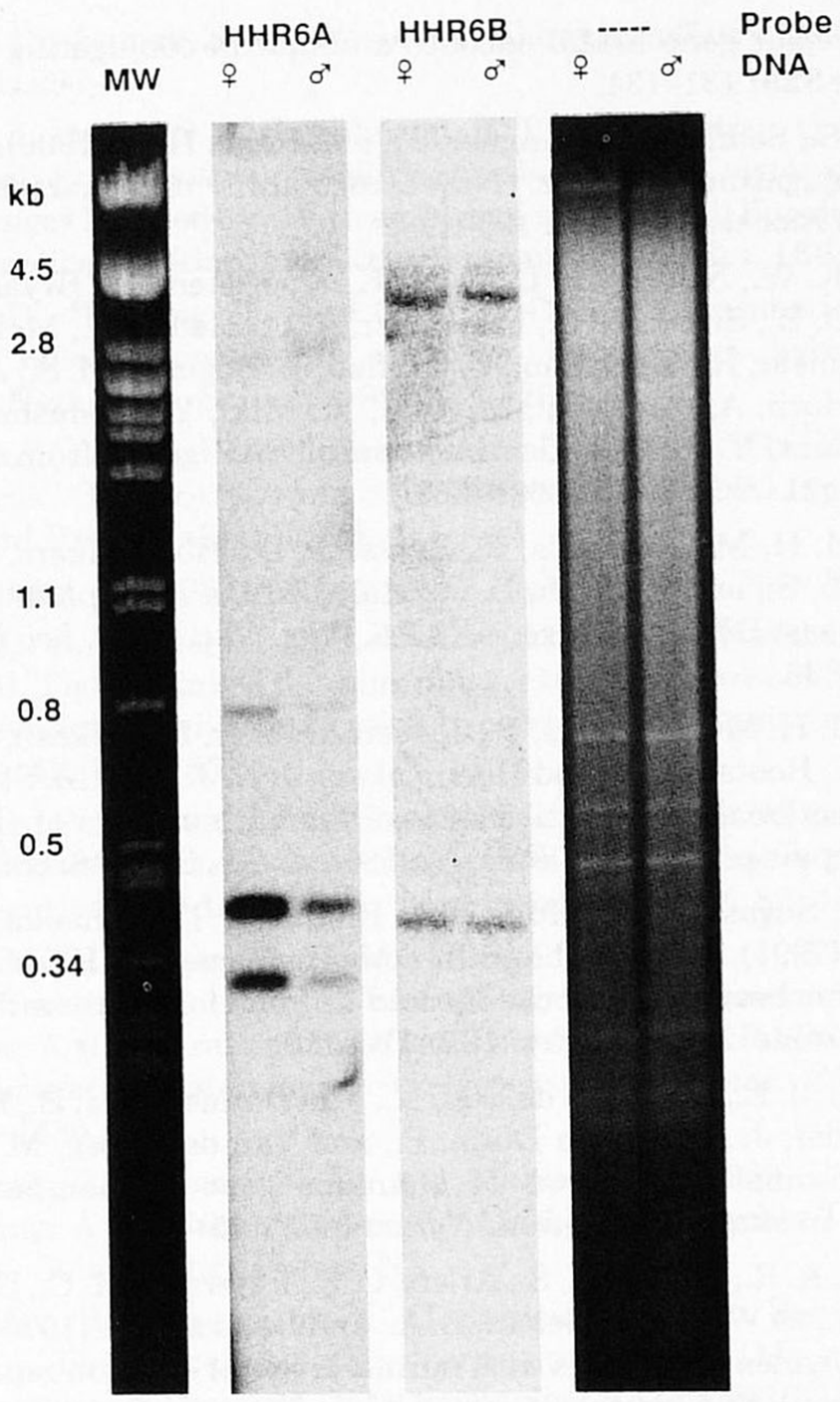


FIG. 3. Southern blot analysis of genomic liver DNA from a male and a female mouse. **MW:** Molecular weight marker, i.e., phage λ DNA digested with *Pst*I. **Left:** The autoradiogram of a blot with *Hin*dIII + *Eco*RI + *Bam*HI triply digested mouse DNA hybridized with a human *HHR6A* cDNA probe. **Middle:** 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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