Identification of a Human Homologue of the Schizosaccharomyces pombe rad17* Checkpoint Gene*

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In the fission yeast Schizosaccharomyces pombe the rad17* gene is required for both the DNA damage-dependent and the DNA replication-dependent cell cycle checkpoints. We have identified a human cDNA homologue of the S. pombe rad17* checkpoint gene, designated Hrad17. Hrad17 has 49% identity to the S. pombe rad17* sequence at the DNA level and 49% identity and 72% similarity at the amino acid level. Northern blot analysis indicates elevated levels of expression in testis and in cancer cell lines. Chromosomal localization by fluorescence in situ hybridization indicates that Hrad17 is located on chromosome 4q13.3–21.2. This region is subject to loss of heterozygosity in several human cancers. To begin to understand the protein-protein interactions of the human checkpoint machinery, we have used the yeast two-hybrid system to examine potential interactions between Hrad1, Hrad9, and Hrad17. We demonstrate a physical interaction between Hrad17 and Hrad1 but no interaction with Hrad9.

Cell cycle checkpoints are regulatory pathways ensuring that the events of the cell cycle are completed with high fidelity and in an orderly fashion (reviewed in Refs. 1–3). When cells are subjected to conditions that interfere with DNA replication or cause damage to DNA, a signal is sent to halt cell cycle progression, thus permitting cell cycle phase completion or DNA repair (1–3). The loss of checkpoint control in mammalian cells results in genomic instability, leading to the amplification, rearrangement, or loss of chromosomes, events associated with tumor progression (2, 4). In Schizosaccharomyces pombe the products of six genes: rad1, rad3, rad9, rad17, rad26, and hus1 have been identified as essential components of the checkpoint pathways (5). Several of the S. pombe checkpoint genes have structural homologues in the budding yeast, and further conservation across eukaryotes has recently been demonstrated with the cloning of two human homologues of S. pombe rad3*, ATM (gataxia telangiectasia mutated) (6) and ATR (gataxia telangiectasia and rad3* related) (7, 8); a human homologue of S. pombe rad9*, Hrad9 (9); and a human homologue of S. pombe rad1*, Hrad1 (49). Currently, little is known about the biochemistry of checkpoint control; however, the identification and characterization of human homologues of yeast checkpoint genes provides clear evidence that checkpoint pathways are conserved between mammals and yeast. The genetic data in yeast suggest that a complex of proteins mediates the monitoring of replication-specific structures and damaged DNA (10), and recent biochemical studies in yeast and humans suggest that the cell DNA replication response is mediated by a protein kinase. The activation of a signal transduction pathway involving the protein kinases ATM/ATR and Hchk1 resulting in inhibitory phosphorylation of Cdc2 and subsequent stabilization of the inhibitory Tyr15 phosphorylation of Cdc2 (7, 11–13).

The S. pombe rad17* mutant is defective in both the DNA damage-dependent and the DNA replication-dependent checkpoints (14). The rad17* gene has been cloned (15) and shows significant sequence similarity with Saccharomyces cerevisiae RAD24 and components of mammalian replication factor C, which are required to load the replicative DNA polymerases δ and ε onto primed DNA templates (16–19). Rad17 is not a functional homologue of a replication factor C subunit, although the sequence similarity may reflect some shared biological activity such as association with elements of the replication machinery or binding of specific DNA structures (15).

In this report we describe the cloning and characterization of a novel human cDNA, designated Hrad17, which is highly similar to the S. pombe rad17* checkpoint gene. We also demonstrate that Hrad17 interacts with the recently identified Hrad1 but not Hrad9.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of Human rad17—A search for sequences similar to S. pombe rad17* was carried out using the TBLASTN program (20) against the proprietary LifeSeq® data base (Incyte Pharmaceuticals Inc., Palo Alto, CA) as well as public domain data bases such as GenBank®. Deduced amino acid sequences were aligned using the CLUSTALW program, and similarity was determined with a blust32 amino acid substitution matrix. Clones with significant sequence similarity were ordered from Incyte or the Merck/Washington University EST® collection, and the complete sequence of their inserts was determined. DNA sequencing was carried out on double-stranded plasmid DNA with dye-terminator chemistry as prescribed by the manufacturer (Perkin-Elmer/Applied Biosystems), and the products were resolved on an ABI Prism™ 377 Automated Sequencer. The complete DNA sequences of relevant inserts were aligned as described. The 5′ end of the longest cDNA was extended by 5′ RACE-PCR according to the manufacturer’s instructions (CLONTECH). Two gene-specific primers were designed to the 5′ end of the putative Hrad17 ORF: GSP1 (5′-TTCTATGAGTTGGTATATGCACCC-3′) and GSP2 (5′-GGCAAGTATTGATAGAGACGGCCAC-3′). These were used in a nested PCR reaction with Marathon-Ready human placental cDNA (CLONTECH) as the template. The first PCR reaction made use of the GSP1 PCR primer for the 3′ end, combined with the AP1 primer from CLONTECH that is com-

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The abbreviations used are: EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; ORF, open reading frame; FISH, fluorescence in situ hybridization; DAPI, 4′,6-diamidino-2-phenylindole; kb, kilobase pair(s).
Identification of Human Rad17 cDNA

Identification of a Human Homologue of S. pombe rad17—
A human expressed sequence tag cDNA clone (number 515944) was identified in the proprietary LifeSeq® data base (Incyte Pharmaceuticals Inc., Palo Alto, CA) using the tblASTN homology searching program (20) with the S. pombe rad17+ amino acid sequence as the query. This clone was purchased from Incyte Pharmaceuticals and DNA sequence analysis of the 1.9-kb insert revealed an incomplete ORF that was highly similar to the S. pombe Rad17 amino acid sequence. Further searches of the public data bases using the clone 515944-derived DNA sequence as the query sequence identified several expressed sequence tags with significant sequence similarity. One of these (accession number AA287094) extended the putative Hrad17 sequence in a 5′ direction and maintained the similarity to S. pombe rad17. This sequence did not extend to the 5′ end of the ORF, and comparison of the derived amino acid sequence with the S. pombe rad17+ sequence suggested that approximately 60 nucleotides were missing. To complete the putative Hrad17 ORF, a 5′ RACE-PCR was carried out using a Marathon-Ready human placental cDNA library (CLONTECH) and two gene-specific primers. Approximately 350 nucleotides of novel 5′ DNA sequence were obtained, including 57 nucleotides required to complete the Hrad17 ORF and 300 nucleotides of 5′-untranslated region. Termination codons were present in all three reading frames in the 80 nucleotides immediately 5′ to the putative Hrad17 initiation codon, indicating that we had isolated the complete coding region (Fig. 1). The full-length Hrad17 ORF was then amplified from cDNA prepared from human SK-N-MC neuroblastoma cells, and the insert DNA sequence from three independent clones was determined. The complete coding region of Hrad17 is 2010 nucleotides in length and has 49% identity to the S. pombe rad17+ sequence at the DNA level and 49% identity and 72% similarity at the amino acid level (Fig. 2). The Hrad17 sequence has been deposited into GenBank™ under accession number AJ004977. Hrad17 also has significant sequence similarity to S. cerevisiae RAD24 (Fig. 2). The Hrad17 cDNA encodes a protein with a predicted molecular mass of approximately 71 kDa.

Northern Blot Analysis for Hrad17—The transcript profile of Hrad17 was examined by probing two multiple tissue Northern blots (CLONTECH) and a cancer cell line Northern blot (CLONTECH) with a probe corresponding to the complete Hrad17 ORF. A single transcript of approximately 3.0 kb was identified for Hrad17. The blots were also hybridized with a β-actin probe to demonstrate equal loading of RNA across all lanes. The Hrad17 transcript was present in all tissues examined and was highly elevated in testis. Although the β-actin signal is slightly elevated in the cancer cell lines when compared with the normal tissue blots, it is clear that the level of Hrad17 transcription is dramatically elevated in the cancer cell lines examined (Fig. 3). Because the Hrad17 sequence that we have identified is over 2800 bp long, it is likely to include most of the 5′- and 3′-untranslated region, considering that a typical mammalian poly(A) tail can be several hundred nucleotides long. The presence of an oligo(dT) stretch at the 3′ end of the clone supports the conclusion that we have the full 3′-untranslated region.

Hrad17 Does Not Complement the S. pombe rad17 Checkpoint Phenotypes—Complementation has often been used to demonstrate biological activity for mammalian homologues of yeast proteins (9, 28). We examined whether Hrad17 could complement the UV irradiation (DNA damage-dependent checkpoint) and hydroxyurea (DNA replication-dependent checkpoint) sensitivity phenotypes of an S. pombe rad17+ strain.
Identification of Human Rad17 cDNA

303  atg  aat  cag  gta  aca  gac  tgc  gtt  gac  cca  tca  ttt  gat  gat  ttt  cta  gag  tgg  aat  ggc  gcc
308  M  N  Q  U  T  V  W  D  P  S  F  D  D  F  L  E  C  S  G  V
313  tac  act  act  act  gcc  aca  tca  tta  ggt  ggc  aat  aac  tca  act  agt  cat  aca  aca  aac  aat  ggg  gct
318  S  T  S  H  L  G  V  W  E  N  N  S  S  H  G  S  E  N  H  A  S  N  P  R
323  tct  aca  tta  gaa  aag  aca  gag  aga  ttt  cca  ggg  gag  aca  aag  gaa  aca  cta  tct  ttc  taa  gaa  cag
328  S  T  L  E  S  S  R  P  F  P  A  R  K  R  G  N  L  S  S  L  E  Q
333  att  tat  ggt  tta  gaa  aat  aca  aac  cag  ttt  tct  tca  gat  gaa  aca  cca  tga  ggg  gaa  aat  tat  cag  tac
338  I  Y  G  L  E  N  S  K  R  Y  L  S  E  N  E  P  W  D  K  Y
343  aac  cca  gaa  act  cag  cat  gaa  cct  ggt  gac  cat  aca  aag  aat  gaa  gaa  gta  gag  acc  tgg
348  K  F  E  Q  S  H  E  L  A  H  V  K  K  B  V  T  V  E  V  W
353  tta  aac  gaa  gat  gtt  tta  gaa  aag  cca  aac  aag  cag  gtt  gaa  aag  tct  cta  tct  tta  aca  atg  aca  gtt
358  L  K  A  Q  V  L  E  R  O  P  K  Q  G  S  I  L  L  I  T  G
363  cct  cct  gga  tgt  gga  aag  aca  aca  acc  tca  aca  aca  ata  cta  tca  aca  aag  cag  cct  gaa  gat  ctt  gtt  cta
368  P  P  G  C  G  K  G  T  T  T  L  K  I  L  S  K  R  H  G  I  Q  V
373  caa  gag  tgg  att  aat  cca  ggt  ttt  cca  gac  ttc  cca  aca  gat  gtt  cgg  cag  gga  act  ctc  gca  aat  ttt  gat
378  Q  E  W  I  N  P  V  L  P  F  D  Q  K  D  F  K  G  M  F  N
383  act  gaa  tca  ggc  tct  atg  tat  cag  ttt  tct  cag  cag  aac  ctc  gag  cct  cta  aca  aac  gtt  caa  gat  ggt
388  T  E  P  E  P  P  Y  K  P  D  V  Q  S  Q  H  I  E  K  L  T
393  cta  aca  ggc  aca  aag  ttt  acc  tga  agt  atc  cct  gaa  aac  tag  gta  cgg  aga  act  cgg  act  aag  aag
398  L  R  A  T  K  Y  N  L  K  Q  M  L  D  D  L  R  T  D  K
403  ata  att  ctc  gtt  gaa  gat  tta  cct  aac  cag  ttt  tat  cgg  gat  ttc  act  ata  cat  gaa  gat  ggt
408  I  L  V  E  D  L  P  N  O  Y  R  D  S  H  T  L  H  E  V
413  cta  aag  aag  tag  ggt  gaa  aat  acc  taa  tct  att  ttt  cta  aca  gaa  gat  ggt  gtt  aag  gaa  gaa  ggt
418  L  R  X  Y  V  R  I  G  C  P  L  I  I  S  D  S  L
423  gga  gat  aat  aat  caa  gag  ttt  ttc  gcc  aat  caa  gag  gaa  gat  ggt  gtt  aag  gaa  gaa  ggt
428  K  D  V  S  L  F  L  F  R  A  L  S  G  I  L  Y  L  C  K  R  A  S
433  tta  aca  gaa  gaa  tca  cct  cag  ttg  ctc  tct  tct  tca  gat  gaa  gat  aca  tta  lts  rly  lvp  swl  s  h  s  e  l  s  s  t
438  D  S  R  Y  L  L  F  S  H  L  S  Y  F  L  F  P  S  S  L
443  ctt  gtt  gaa  ctt  ggt  gag  ggt  gaa  arg  tca  cac  aag  cct  gca  gga  tta  aat  aag  gaa  cca  tca  ggt  tgg
448  L  V  E  P  E  V  B  K  X  S  H  P  G  O  D  L  F  N  L  Y
453  ctt  cac  caa  aac  tac  ata  gat  ttc  ttt  att  gat  aag  aat  cat  gaa  aac  cag  atg  ggc  aat  gaa  ggt
458  L  Q  H  Q  N  Y  I  M  C  I  D  D  I  V  R  A  S  E  H
463  ctt  gtt  gaa  gat  ttc  aac  cag  ttt  ctt  aag  tga  aac  cgg  acc  tgc  ttc  aat  cgg  aat  gga  gat  gtt
468  S  F  A  D  I  L  S  G  D  W  N  R  S  L  D  E  Y  S
473  aca  tct  ata  gct  aag  aag  ggt  gtc  act  tca  aca  gtc  gca  gga  gaa  gat  gga  cag  tgg  aat  gca  gag  gaa
478  T  R  O  Y  V  A  H  C  Q  G  S  F  P  L  E  S  G  A  P  F  S
483  cgg  gaa  gga  tca  agt  ttt  cga  ccc  tca  gac  cag  cct  gaa  ata  aat  aca  aag  gat
488  C  G  S  F  S  F  R  P  L  H  E  P  Q  W  F  L  N  K  K  E  Y
493  cgg  gaa  aat  tgg  tgc  gca  aca  tca  att  tca  cta  gcc  ttc  gtc  tca  cca  cca  gag  att  taa  gtt
498  R  E  N  C  L  A  A  K  A  L  F  P  D  F  C  L  P  A  L  C
503  caa  act  cag  tta  tgc  cca  cac  ctt  gct  gct  tca  tca  gat  cca  gag  act  tta  gga  gat  ggt
508  Q  T  Q  L  L  L  P  L  L  L  L  T  I  P  Q  N  H  Q  T
513  tct  ttc  ata  caa  gat  ggc  tgg  cct  cgc  ctc  aag  cca  tgg  gga  act  ctt  cag  cag  atg  ggc
518  S  P  E  I  D  O  L  P  E  L  P  K  E  R  F  L  K  E  M
523  ggg  cgg  cgt  act  aag  gaa  gtt  act  gtt  gca  gat  ggt  gac  ggc  aca  cag  ggc  ggt  ctt  gtt  gtt
528  A  L  T  D  R  H  G  M  I  D  P  S  D  G  D  E  A  Q  L  N
533  ggg  gga  cat  tct  gca  gag  tgg  cgg  cct  gcc  aca  ggc  aat  cgg  aac  acc  aat  cgg
538  G  H  G  S  A  E  S  L  G  C  E  P  T  Q  A  T  V  P  E  T  W
543  tct  ctt  ctc  ttg  aag  gat  ggt  gac  ctt  gct  gcc  cgg  ccc  tca  aat  ggg  ggc  aag  ctg  gg
548  G  C  G  A  A  T  C  A  G  G  G  G  C  G  G  C  G  G  G  C  G
553  gcc  caa  gga  aag  gat  aag  aca  aag  aca  ata  aca  aag  gag  cag  gag  aat  ggg  gca  aat  aaag  tgg
558  A  Q  D  G  M  E  N  I  I  E  D  Y  E  S  D  G  T

A sequence is provided that encodes a protein with the given identification for human Rad17 cDNA.
Hrad17 was cloned into the S. pombe expression vector pREP3x (22) and transformed into wild type and rad17::ura4 cells. Transformants were exposed to varying doses of UV or transiently exposed to 10 mM hydroxyurea as described previously (15). We observed no complementation of the UV or hydroxyurea sensitivity phenotypes (data not shown).

Hrad17 Is Located on Chromosome 4q13.3–21.2—The chromosomal position of Hrad17 was determined to establish whether loss of heterozygosity associated with Hrad17 might be linked with any known disease. The 2.6-kb cDNA corresponding to expressed sequence tag clone number AA287094 was used as a probe for FISH mapping. Under the conditions used, the hybridization efficiency was approximately 71% for the probe (among 100 checked mitotic figures, 71 showed signals on one pair of chromosomes). No additional locus was picked up by FISH detection under the conditions used. The 4',6-diamidino-2-phenylindole (DAPI) banding pattern was used to establish that Hrad17 localizes to the long arm of chromosome 4 (25). The detailed position was further determined based upon the analysis of 10 photographs leading to the conclusion that Hrad17 is located on human chromosome 4q13.3-q21.2 (Fig. 4). Loss of heterozygosity of this region of chromosome 4 has been linked to a variety of human neoplasias including breast cancer, hepatocellular carcinoma, and small cell lung cancer (29–34).

Hrad17 Interacts with Hrad1—The yeast two-hybrid system has been used extensively to examine protein-protein interactions (35–37). We cloned Hrad17, Hrad9, and two forms of Hrad1 generated by alternative splicing (49), into the yeast two-hybrid expression vectors pAS2-1 and pACT (CLONTECH) and then transformed them individually or as pair-wise combinations into S. cerevisiae strain Y187 (CLONTECH). Transformants were assayed for β-galactosidase activity either on filter lifts or using O-nitrophenyl β-D-galactopyranoside as substrate as described by the manufacturer (CLONTECH). The β-galactosidase activity was high in cells transformed with Hrad17 combined with either form of Hrad1, indicating a positive interaction (Fig. 5). In addition, Hrad17 was able to interact with itself to a lesser extent. Hrad9 did not show any interaction with itself nor with Hrad1 or Hrad17 (Fig. 5). The interaction between Hrad17 and Hrad1 was only observed when Hrad1 was fused with the GAL4 activation domain. We interpret this to mean that the 163-amino acid GAL4 DNA-binding domain is underlined.

**Fig. 1.** The nucleotide sequence (GenBank™ accession code AJ004977) and predicted amino acid sequence of human Rad17. The consensus polyadenylation site is underlined; start and stop codons are in bold type.

**Fig. 2.** Amino acid sequence alignment between human Rad17, S. pombe Rad17, and S. cerevisiae RAD24 obtained with the CLUSTALW alignment program. Identical residues are highlighted in black and conserved residues are highlighted in gray. The putative nucleotide-binding domain is underlined.
DISCUSSION

We have identified a human cDNA encoding a protein that shares significant sequence similarity with the *S. pombe* Rad17 protein and the *S. cerevisiae* RAD24 protein. Alignment of these sequences identifies several blocks of sequence similarity that are conserved between the human and yeast proteins and the human replication factor C subunits (15). One of these contains the consensus sequence for a nucleotide-binding domain. This domain is required for Rad17 function in *S. pombe* (15). The other regions of sequence similarity are not reminiscent of any defined functional domain but may reflect shared biological activity such as association with elements of the replication machinery or binding of specific DNA structures (15).

The *Hrad17* transcript is present in all tissues that we examined and increased in all the cancer cell lines examined, suggesting either that transcription of *Hrad17* is proliferation-dependent or that it is increased in response to the genomic instability of cancer cell lines. The level of *Hrad17* transcript in testis is approximately 10-fold that seen in other tissues. Several yeast cell cycle checkpoint genes play important roles in meiosis (38), and recently ATM and ATR (the human homologue of *S. pombe* Rad3) were shown to be highly expressed in testis where they interact with meiotic chromosomes. This

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**Fig. 3. Northern blot analysis of Hrad17.**

A, the multiple tissue and cancer cell line Northern blots (CLONTECH) were hybridized in parallel with a cDNA probe corresponding to the *Hrad17* ORF. RNA size markers are indicated. The blots were then rehybridized with a human β-actin cDNA probe (CLONTECH) to verify that comparable amounts of RNA are present across lanes and that the RNA samples show no sign of degradation. Exposure times were overnight for the *Hrad17* probe and 30 min for the β-actin probe. For either probe, all steps of the procedure (hybridization, washing, and exposure) were carried out in parallel for the three blots. B, quantitation (arbitrary units) using scanning densitometry of autoradiograph of blot shown in panel A.
suggests a direct role for these proteins in recognizing and responding to DNA strand interruptions that occur during meiotic recombination (39). Hrad1 is also highly expressed in testis (49), suggesting that Hrad1 and Hrad17 may form part of a recognition complex in association with ATM or ATR.

Loss of checkpoint function has been shown to lead to genomic instability even in the absence of exogenous DNA damage (40). In man the p53 gene and the ATM gene are required for the G1-S phase checkpoint (41, 42). These genes also act as tumor suppressors (43–46), suggesting that it is likely that other checkpoint genes will act as tumor suppressors. We have shown that Hrad17 resides on chromosome 4 position q13.3–21.2. Loss of heterozygosity of this region of chromosome 4 has been linked to a variety of human neoplasias including breast cancer (29), hepatocellular carcinoma (30), and small cell lung cancer (13, 32). Consequently, Hrad17 should be considered as a candidate tumor suppressor gene on chromosome 4q. There is also evidence for the presence of an as yet unidentified gene on chromosome 4q that regulates DNA replication in response to DNA damage (33) and that is involved in the G1 arrest in cellular senescence (34). Hrad17 should also be considered as a potential candidate for these gene functions.

Demonstration of an interaction between Hrad17 and Hrad1 provides further evidence that we have identified the human homologue of S. pombe rad17. In fission yeast these two gene products act early in both the DNA damage-dependent and DNA replication-dependent checkpoint pathways, and mutants in either gene have similar phenotypes. In the budding yeast, RAD24 and RAD17 (the homologue of S. pombe rad1) are only required for the DNA damage-dependent checkpoint and have been shown to function in conjunction with MEC1 to activate DNA degradation (47), leading to the suggestion that there is a requirement to process single- or double-stranded breaks such that single-stranded DNA is exposed to activate the checkpoint (47, 48). Two forms of Hrad1 have been identified; Hrad1A which has an exonuclease activity and Hrad1B, an inactive N-terminal truncation of Hrad1A (49). We show in our two-hybrid assay that Hrad17 interacts with both forms of Hrad1. We also find that Hrad17 is able to interact with itself suggest-
ing the presence of a multi-subunit complex consisting of Hrad1 and one or more Hrad17 molecules. We were unable to

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