A Human Homologue of the Schizosaccharomyces pombe rad1* Checkpoint Gene Encodes an Exonuclease*

(Received for publication, December 29, 1997, and in revised form, April 22, 1998)

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In the fission yeast Schizosaccharomyces pombe the rad11 gene is required for both the DNA damage-dependent and the DNA replication-dependent cell cycle checkpoints. We have identified a human homologue of the S. pombe rad11 gene, designated Hrad1, as well as a mouse homologue: Mrad1. Two Hrad1 alternative splice variants with different open reading frames have been identified; one codes for a long form, Hrad1A, and the other encodes a short form because of N-terminal truncation, Hrad1B. Hrad1A has 60% identity to the S. pombe rad11 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 cancer cell lines. Chromosomal localization by fluorescence in situ hybridization indicates that Hrad1 is located on chromosome 5p13.2–13.3. This region is subject to loss of heterozygosity in several human cancers. Hrad1 also shares homology with the Saccharomyces cerevisiae RAD17 and Ustilago maydis REC1 proteins. REC1 has previously been characterized as a 3' → 5' exonuclease with a C-terminal domain essential for cell cycle checkpoint function. We have expressed and purified polyhistidine-tagged fusions of Hrad1A and Hrad1B and show that HisHrad1A has 3' → 5' exonuclease activity, whereas HisHrad1B lacks such activity. The biological functions of the two proteins remain to be determined.

For any dividing cell it is essential to maintain the integrity of the genome ensuring that DNA replication occurs once per cell cycle and that identical chromosomal copies are distributed equally to two daughter cells at mitosis. When cells are subjected to conditions that interfere with DNA replication or spindle assembly or that cause damage to DNA, cell-cycle progression halts, permitting cell cycle phase completion or DNA repair. These control mechanisms are referred to as "checkpoints" (reviewed in Refs. 1–3). The loss of checkpoint control in mammalian cells, most notably through the inactivation of p53, results in genomic instability leading to the amplification, rearrangement, or loss of chromosomes, events that commonly occur in cancer cells (2, 4).

Much of our current knowledge about checkpoint control has been obtained from studies using budding (Saccharomyces cerevisiae) and fission (Schizosaccharomyces pombe) yeast (reviewed in Ref. 5). In the fission yeast at least two distinct checkpoint pathways have been identified: the DNA replication-dependent checkpoint pathway that ensures that S phase is completed before M phase is initiated and the DNA damage-dependent checkpoint pathway that acts to halt the cell cycle when genomic integrity is compromised (5). The products of six genes, rad11, rad31, rad91, rad171, rad261, and hus11, have been identified in S. pombe as essential components of both cell cycle pathways (5). In S. cerevisiae the two checkpoint pathways are genetically separable; four gene products, RAD9, RAD17, RAD24, and MEC3, have been identified as essential for the DNA damage-dependent checkpoint pathway, and three gene products, POL2, RFC5, and DPB1, are essential for the DNA replication-dependent checkpoint pathway (reviewed in Refs. 5–6). 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 rad31, ATM (ataxia telangiectasia mutated) (6) and ATR (ataxia telangiectasia and rad31 related) (7, 8), and a human homologue of S. pombe rad91, Hrad9 (9).

The identification and characterization of human homologues of yeast checkpoint genes provides clear evidence that at least some checkpoint pathways are conserved between mammals and yeast. Currently little is known about the biochemistry of checkpoint control. The genetic data in yeast suggest that a complex of proteins mediates the monitoring of replication-specific structures and damaged DNA (10). Furthermore, recent biochemical studies in S. pombe and humans suggest that the cell cycle arrest in response to DNA damage is brought about by the activation of a signal transduction pathway involving the putative protein kinases ATM/ATR and Hchk1, resulting in inhibitory phosphorylation of Cdc25 and subsequent stabilization of the inhibitory Tyr15 phosphorylation of Cdc2 (7, 11–14).

Although a great deal of progress has been made in identifying the kinase components of the signal transduction pathway mediating cell cycle arrest (6–8, 12), there has been little progress in identifying the sensing mechanisms that activate the checkpoint pathways. The S. pombe rad11 gene acts early in the checkpoint pathway (15) and presumably plays a role in the transmission of information regarding the state of the genome to the checkpoint control mechanism. The rad11 mutant is extremely sensitive to all DNA damaging agents (15, 16) and fails to invoke a G2 arrest in response to ionizing radiation yet is DNA repair-competent, indicating a loss in the ability to recognize the presence of damaged DNA (15). The rad11 mutant is also sensitive to hydroxyurea, indicating an uncoupling between the completion of S phase and entry into mitosis (15), and is synthetically lethal when combined with mitosis-promoting mutations such as wee1-50 (15). The S. pombe rad11 mutant...
gene has been cloned (17), and the predicted amino acid sequence shows significant sequence similarity to the Ustilago maydis protein Rec1 (18) and S. cerevisiae RAD17 (20). Mutations in these genes lead to cell cycle checkpoint defects and other phenotypes associated with alterations in DNA repair and recombination (19, 20).

Purified Rec1 protein has been shown to have a 3′→5′ exonuclease activity (21), suggesting that Rec1/Rad1/RAD17 may be involved in modifying DNA damage (22, 23). Studies of Rec1 truncation mutants have demonstrated that the nucleolytic activity is located in the N-terminal half of the protein. However, the Rec1-dependent checkpoint function requires the C-terminal region of the protein and does not require the exonuclease activity (24, 25). In addition, Rec1 mutants that lack exonuclease activity have a 100-fold higher rate of spontaneous mutation, indicating that the exonuclease function may be required for mismatch repair (24). Thus it would appear that the Rec1 protein is bifunctional, having an N-terminal exonuclease domain and a C-terminal checkpoint domain.

In this report we describe the cloning and characterization of a novel human cDNA, designated Mrad1, which is highly similar to the S. pombe rad1+ checkpoint gene. We also report the cloning of a mouse homologue designated Mrad1. We show that Mrad1 is subject to alternative splicing giving rise to two potential open reading frames (ORFs) coding for a full-length and a truncated form of Mrad1, designated Mrad1A and Mrad1B, respectively. We have expressed in Escherichia. coli and affinity purified N-terminal polyhistidine-tagged fusions of Mrad1A and Mrad1B and show that Mrad1A but not Mrad1B has 3′→5′ exonuclease activity. Mrad1B corresponds to the C-terminal domain of Rec1 that has been implicated solely in checkpoint function. Thus we can speculate that Mrad1A plays a role in the recognition and processing of DNA ends and also in checkpoint function, whereas the role of Mrad1B may be restricted to checkpoint control.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of Human and Mouse rad1—A search for sequences similar to S. pombe rad1+ was carried out using the TBLASTN program (26) against the GenBank™ data base. Deduced amino acid sequences were aligned using the CLUSTALW program, and similarity was determined with a blustatn matrix. An expressed sequence tag (EST) clone with significant sequence similarity to Mrad1 was identified and obtained from the Integrated Molecular Analysis of Genomes and their Expression (I.M.A.G.E.) Consortium.

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 insert sequence of the EST clone was determined.

To extend this sequence and to examine the possibility of alternative splicing, 5′ and 3′ RACE-PCR was carried out according to the manufacturer’s instructions (CLONTECH). For the 5′ RACE, two gene-specific primers were designed to the 5′ end of the putative Hrad1 ORF. GSP1 (5′-ATTTGAGCCATTCTGCTATATG-3′) and GSP2 (5′-TGGATTGAGTTGCACTGACACTGCTATA-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 complementary to an adaptor ligated to the 3′ end of all Marathon-Ready cDNAs. The second PCR reaction started from 1–5 μl of the first and used the GSP4 primer in combination with a nested AP2 primer (CLONTECH). After a 1-min denaturation at 94 °C, the reaction conditions for the first RACE-PCR were 30 cycles of 30 s at 95 °C and 4 min at 68 °C, concluding with a 7-min extension at 72 °C. The subsequent nested PCR also used two-step cycles, each consisting of a 30 s denaturation step at 94 °C and a combined annealing and elongation step for 4 min, which was carried out at 72 °C for the first five cycles, at 70 °C for the five subsequent cycles and at 68 °C for the remaining 25 cycles. A final 7-min extension step at 72 °C concluded the PCR.

The PCR reaction products were resolved by agarose gel electrophoresis. Two reaction products were excised, and DNA was purified using the QIAquick gel extraction kit (Qiagen), and ligated into the pcR2.1-TOPO vector (Invitrogen). The insert sequence of 10 independent clones was determined and compared with the putative Hrad1 cDNA sequence. The sequences obtained separated into two classes generating two ORFs that we designated Hrad1A and Hrad1B.

PCR primers were designed to amplify Hrad1A, OML003 (5′-AAGGATCCGCGATGCCTCGCGCCACGAGAGAGA-3′) and OML001 (5′-TAACTGCTGTCGAGACTGCAGATCTCAGGATCTCAGTCCAGATCCAGGATCTCAGTCCAG-3′), and Hrad1B, OAP034 (5′-CCGCTCGAGAGTTAGTTCAAAGGTAT-3′) and OAP033 (5′-GGCCGAATTCGCAAGTCTGACAGATCTCAGGAGAAA-3′), to subclone into various expression vectors. The complete ORFs of Hrad1A and Hrad1B were PCR-amplified starting from cDNA prepared from human SK-N-MC neuroblastoma cells and the Marathon-Ready human placenta cDNA (CLONTECH), respectively. The amplification products were directly cloned into the pcR2.1-TOPO vector (Invitrogen), and the insert sequence from three independent clones of Hrad1A and Hrad1B was determined.

To determine the DNA sequence of mouse rad1+ (Hrad1), mouse EST clones were identified by screening the GenBank™ with Hrad1, and then the 5′-end EST clones were obtained from the I.M.A.G.E. consortium. The insert sequence for each clone was determined and aligned as described for Hrad1.

Northern Blot Analysis—Two multiple human tissue Northern blots (CLONTECH) and a human cancer cell line Northern blot (CLONTECH) were hybridized with a full-length Hrad1A cDNA probe, labeled with [α-32P]dCTP by random hexamer priming with the Prime-a-Gene (I.M.A.G.E. C.) and the PRIME-A-GENE (I.M.A.G.E. C.) kits. The blots were processed and washed according to the manufacturer’s instructions (CLONTECH) and washed at high stringency (0.1× SSC, 0.1% SDS, 50 °C, 2× 20 min) and exposed to Kodak X-Omat autoradiography film with intensifying screens at −70 °C. The blots were then rehybridized with a human β-actin probe (CLONTECH) labeled with [α-32P]dCTP as described above to verify the intactness of the RNA samples and confirm the presence of comparable amounts of RNA across lanes.

After stripping (0.1× SSC, 0.1% SDS, 90 °C, 2× 20 min), one of the blots was rehybridized with an oligonucleotide specific to the alternatively spliced region of Hrad1A, OAP103 (5′-CTGGAATATTTCTGCCAGTTAAA3′), to discriminate between the transcripts. The oligonucleotide was end-labeled in a standard reaction using T4 polynucleotide kinase and [α-32P]dATP and hybridized to the blot at 37 °C, and the blot was washed as described for the previous hybridizations.

In addition, a multiple mouse tissue Northern blot (CLONTECH) and a mouse developmental blot (CLONTECH) were hybridized with a mouse rad1+ cDNA probe (Avet/Plat fragment) labeled by random hexamer priming as described above. The blots were washed and exposed to film and then rehybridized with the control β-actin probe; all of these steps were carried out as described for the previous hybridizations. S. pombe Strains, Culture, and Plasmids—S. pombe was cultured by standard techniques (27). The genotypes of the strains used are as follows: APY002, h+ leu1-32 ura4-D18 ade6-M216, and GBY190, h+ rad1::ura4-1;ura4-1, leu1-32, ura4-D18, ade6-M216. The complete ORF coding for Hrad1 was cloned into the Smal site of the S. pombe expression
vector pREP3X (28) using standard techniques. Transformation of S. pombe was carried out by electroporation (29).

**Bacterial Expression and Purification of HisHrad1A and HisHrad1B**—The following primers were used to amplify the complete coding region of Hrad1A (accession number Y18334) and HisHrad1B (Y18334): CCGGGCCTACTGACCGCAGACTCTC-3′ and 5′-GCCAGATTCTGTTACAGGTATGTTGACT-3′ for the 5′ end of Hrad1A and His1B, respectively, each in combination with the 3′ PCR primer OAP033 (5′-GCCGAATTCAGCTACGATTCAGTACGAA-3′). The PCR product was digested with BglII and EcoRI and cloned into the corresponding sites of pSETB (Invitrogen). This creates a fusion protein with the following amino acid sequence N-terminal to the BglII sites of Hrad1A or His1B: MBGGHHHHGMSMTGQGMRDLYDDDKPSS. The insert sequence of both constructs was verified.

The plasmids were transformed into E. coli BL21(DE3) cells, and a single colony of each was inoculated into 10 ml of LB medium (containing 10 g/liter Bacto-Tryptone (Difco), 5 g/liter Bacto Yeast extract (Difco), and 10 g/liter NaCl (Merck)), brought to pH 7 with 10 x NaOH and supplemented with 100 μg/ml ampicillin) and incubated at 250 rpm at 30 °C overnight. 4 ml of the overnight culture was added to 400 ml of TB medium containing 2 mg/ml ampicillin and 0.5 mM PMSF, Buffer RB (pH 6.3) and Buffer RB (pH 5.5). The bound protein was then eluted in 10 ml of Buffer RB, pH 6.3 and Buffer RB, pH 5.5. The bound protein was eluted in Buffer RB, pH 6.3, containing 250 mM imidazole. Nine 1-ml fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis. Those fractions containing HisHrad1A or His1B were pooled and dialyzed overnight (Spectra/Por 6,000 molecular weight cut-off membrane) at 4 °C against 1 liter of Buffer RB. The dialyzed material was centrifuged for 1 h at 4 °C and 100,000 × g. No pellet was visible by naked eye inspection. For His1B, the expressed protein was found to be almost exclusively present in the insoluble fraction. Affinity purification was carried out using Ni-NTA-agarose essentially as described by the manufacturer (Qiagen). The soluble material was incubated with the Ni-NTA-agarose in buffer in a rotating wheel. The column was then poured and washed with 5 volumes of sonication buffer (50 mM Tris acetate, pH 9, 10 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM EDTA and substrate DNA. Exonuclease assays were carried out with the T4 polynucleotide kinase, prior to filling in 3′ recessed termini with unlabeled dNTPs.

The exonuclease assay was carried out in a 20-μl volume containing 50 mM Tris acetate, pH 9, 10 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM EDTA and substrate DNA. Exonuclease assays were carried out with 2 nmol (as total DNA nucleotide) of end-labeled DNA containing 1.6 pmol of 32P-nucleotide as the terminal label at a specific activity of 2 x 107 cpm/pmol. Reactions were started by the addition of enzyme, incubated at 37 °C for 30 min unless otherwise stated and terminated by the addition of 30 μl of an ice-cold solution of salmon sperm DNA (0.5 mg/ml) in 25 mM EDTA and 50 μl of 10% trichloroacetic acid. The mixture was held on ice for 10 min and centrifuged at 4 °C and 10,000 × g for 10 min. A 75-μl aliquot of the supernatant was removed and mixed with 400 μl of scintillant (UltimaGold™, Packard) for determination of radioactivity by liquid scintillation counting (Tri-Carb 2100TR, Packard).

**Fluorescence in Situ Hybridization Studies (FISH)**—Chromosomal localization was carried out by SeeDNA Inc. (Toronto, Ontario, Canada). Lymphocytes isolated from human blood were cultured in α-minimal essential medium supplemented with 10% fetal calf serum and phorbol-12-myristate-13-acetate at 37 °C for 68–72 h. The lymphocyte cultures were treated with bromodeoxyuridine (0.18 mg/ml) to synchronize the cell population. The synchronized cells were washed three times with serum-free medium to release the block and recultured at 37 °C for 6 h in α-minimal essential medium with thymidine (2.5 μg/ml). Cells were harvested, and slides were prepared by using standard procedures including proteinase K treatment, fixation, and air drying.

The Hrad1B clone was purified and biotinylated with dATP using the Life Technologies, Inc. BioNick labeling kit (15 °C, 1 h) (31). The procedure for FISH detection was performed as described previously (31, 32). Briefly, slides were baked at 55 °C for 1 h. After RNase treatment, the slides were denatured in 70% formamide in 2 x SSC for 2 min at 70 °C followed by dehydration with ethanol. Probes were denatured at 75 °C for 5 min in a hybridization mix consisting of 50% formamide and 10% dextran sulfate. Probes were loaded on the denatured chromosomal slides. After overnight hybridization, slides were washed, and the signal was detected. FISH signals and the 4,6-diamidino-2-phenylindole (DAPI)-banding pattern were recorded separately by taking photographs, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with DAPI-banded chromosomes (33).

**RESULTS**

**Identification of Human and Mouse Homologues of S. pombe rad1**—A human EST cDNA clone (accession number AA029300) was identified in the EMBL DNA data base using the TBLASTN homology searching program (26) with the S. pombe rad1-derived amino acid sequence as the query. This clone was obtained from the I.M.A.G.E. Consortium (EST 470124), and DNA sequence analysis of the 1.2-kb insert (Figs. 1 and 2A) revealed an ORF that was highly similar to S. pombe Rad1. The predicted amino acid sequence of this ORF, however, was significantly shorter than the S. pombe Rad1 protein. To ensure that we had identified the complete sequence of the putative Hrad1 cDNA, we carried out 3′ and 5′ RACE-PCR. The 3′ RACE clones that we obtained were all identical to the original EST cDNA sequence. The 5′ RACE clones that we obtained separated into two classes. The first was identical to our original cDNA clone, and the second contained a 119-nucleotide insertion presumably originating from an alternatively spliced mRNA. This additional sequence changed the reading frame immediately upstream of the initiation codon in our original cDNA sequence and resulted in a second and longer ORF (Figs. 1 and 2A). We have named the long and short ORFs Hrad1A and Hrad1B, respectively, and their sequences have been deposited into GenBank™ under accession codes AJ004974 and AJ004975, respectively). Hrad1A has 60% identity to the S. pombe rad1* sequence at the DNA level and 49% identity and 72% similarity at the amino acid level (Fig. 2B). The Hrad1A cDNA encodes a protein with a predicted molecular mass of approximately 31.5 kDa, and the Hrad1B protein has a predicted molecular mass of approximately 19.5 kDa.

A subsequent search of the data bases resulted in the identification of several mouse ESTs homologous to the Hrad1 cDNA. Three clones were obtained from the I.M.A.G.E. Consortium (accession codes AA357891, AA357843, and AA356881) and sequenced, resulting in the identification of a single ORF that encodes a protein with a predicted molecular mass of approximately 31.5 kDa (data not shown). The nucleotide sequence was deposited into GenBank™ under accession code AJ004976). Mouse and human Rad1A are 91% identical at the amino acid level. None of the mouse clones sequenced corresponded to Hrad1B.

**Northern Blot Analysis of Human and Mouse rad1**—The transcript profiles of human and mouse rad1 were examined by probing several multiple tissue Northern blots (CLONTECH),
a cancer cell line Northern blot (CLONTECH), and a mouse developmental Northern blot (CLONTECH). Three transcripts of approximately 5, 3, and 1.3 kb were identified for \textit{Hrad1}, and all were elevated in the cancer cell lines (Fig. 3A). All three transcripts were present in differentiated tissues with the highest levels in testis, skeletal muscle, placenta, and heart. There was a specific increase in the shortest transcript in testis. The alternative splice we identified represents a difference of 119 nucleotides, which is too small to account by itself for the differences between the three transcripts.

To discriminate between the transcripts we probed the blot containing the testis sample with an oligonucleotide (OAP103) derived from the alternatively spliced region. This hybridized specifically to the 3-kb transcript indicating that the 1.3-kb transcript, which is highly elevated in testis does not contain the alternatively spliced region and therefore does not correspond to \textit{Hrad1A} (Fig. 3B). Further searches of the EST databases have revealed a possible alternative 3'-untranslated region (accession number AA464502), which may explain at least one of the other transcripts (data not shown).

A single transcript of approximately 2.2 kb was identified for mouse \textit{rad1} and was expressed at comparable levels in all tissues and at all stages of development tested (Fig. 3C). We have not established whether mouse \textit{rad1} is subject to alternative splicing as demonstrated for \textit{Hrad1}. Our Northern blot experiment does not provide the resolution to display two transcripts differing by a 119-nucleotide alternative splice.

\textbf{Complementation of S. pombe rad1—}Complementation has often been used to demonstrate biological activity for mammalian homologues of yeast proteins (34, 35). We examined whether \textit{Hrad1} could complement the UV irradiation (DNA damage-dependent checkpoint) or hydroxyurea (DNA replication-dependent checkpoint) sensitivity phenotypes of a \textit{S. pombe} \textit{rad1::ura4} strain. \textit{Hrad1A} and \textit{Hrad1B} were cloned into the \textit{S. pombe} expression vector pREP3x (28) and transformed into wild-type and \textit{rad1::ura4} cells. Transformants were exposed to varying doses of UV or transiently exposed to 10 mM hydroxyurea as described previously (16). We observed no complementation of the UV or hydroxyurea sensitivity phenotypes (data not shown).

\textbf{Hrad1 Transcription Is Not DNA Damage-inducible—}Many genes involved in the response to DNA damage are induced by exposure to DNA damaging agents. To examine whether \textit{Hrad1} could complement the UV irradiation (DNA damage-dependent checkpoint) or hydroxyurea (DNA replication-dependent checkpoint) sensitivity phenotypes of a \textit{S. pombe rad1::ura4} strain. \textit{Hrad1A} and \textit{Hrad1B} were cloned into the \textit{S. pombe} expression vector pREP3x (28) and transformed into wild-type and \textit{rad1::ura4} cells. Transformants were exposed to varying doses of UV or transiently exposed to 10 mM hydroxyurea as described previously (16). We observed no complementation of the UV or hydroxyurea sensitivity phenotypes (data not shown).

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\begin{figure}
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\caption{Nucleotide sequence and predicted amino acid sequence of human rad1. The sequence presumed to be alternatively spliced is underlined, and the two presumptive initiation codons are shown in bold type. The sequences of \textit{Hrad1A} and \textit{Hrad1B} have been deposited into GenBank\textsuperscript{TM} under accession codes AJ004974 and AJ004975, respectively.}
\end{figure}
conclude that Hrad1 transcription is not increased in response to UV-induced DNA damage.

**Affinity Purified HisHrad1A Exhibits Exonuclease Activity**—
The sequence of Hrad1 is highly similar to that of REC1 from *U. maydis*, and purified hexahistidine-tagged Rec1 has been shown to have exonuclease activity (21). To test whether Hrad1 shared this biochemical activity, both Hrad1A and Hrad1B were expressed in *E. coli* as N-terminal hexahistidine-tagged fusion proteins using the pRSETB inducible expression vector (Invitrogen). The proteins designated HisHrad1A and HisHrad1B were purified by immobilized metal affinity chromatography (Qiagen). Bacterially expressed HisHrad1A was found predominantly in the insoluble fraction; however, approximately 20% of the protein as judged by Coomassie staining of SDS-polyacrylamide gels was present in the soluble fraction, allowing purification under non-denaturing conditions. HisHrad1B was found exclusively in the insoluble fraction and was isolated from inclusion bodies under denaturing conditions and refolded by rapid dilution. HisHrad1A and HisHrad1B have predicted molecular masses of approximately 35.5 and 23.5 kDa, respectively. The electrophoretic mobility of the purified proteins on SDS-polyacrylamide gels was in agreement with these predictions (Fig. 4).

First we assayed purified HisHrad1A and HisHrad1B for the ability to release labeled 5' or 3' terminal nucleotides from linear double-stranded DNA. HisHrad1A exhibited clear nucleolytic activity (Fig. 5, A and B), releasing radionucleotide from both the 5' and the 3' termini with a 6-fold preference for the 3' end. HisHrad1B did not show any nucleolytic activity. This may be a consequence of the different purification regimes.
or may reflect a true difference in biological activity.

The conditions for optimal activity were examined and found to be similar to those observed for REC1 (21). Maximum activity was obtained in a low ionic strength buffer at pH 7.4–9.0 (Table I). Activity was unaffected by ATP or dATP but was significantly reduced by 100 mM NaCl. The replacement of Mg\(^{2+}\) with Zn\(^{2+}\) or the addition of 10 mM EDTA resulted in complete loss of activity (Table I).

The substrate specificities were also examined in greater detail. HisHrad1A has an approximately 6-fold preference for 3’ termini under these assay conditions and is approximately 2-fold more active when single-stranded DNA is provided as substrate (Fig. 5B). The activity associated with HisHrad1A differs from known Mg\(^{2+}\)-dependent exonucleases (Table II), strongly suggesting that the activity we observed is inherent in the Rad1A gene product.

Chromosomal Localization of Hrad1—The chromosomal position of Hrad1As determined to establish whether a loss of heterozygosity associated with Hrad1 might be linked with any known disease. The 1.2-kb cDNA corresponding to Hrad1B (EST 470124) was used as a probe for FISH analysis. Under the conditions used, the hybridization efficiency was approximately 69% for the probe (among 100 checked mitotic figures, 69 showed signals on one pair of chromosomes). The DAPI banding pattern was used to establish that Hrad1 localizes to the short arm of chromosome 5. No additional locus was picked up by FISH detection under the conditions used. The detailed
position was further determined based upon the analysis of 10 photographs leading to the conclusion that Hrad1 is located on human chromosome 5p13.3–13.2 (Fig. 6). Loss of heterozygosity of this region of chromosome 5 has been linked to a variety of human neoplasias including lung cancer (37, 38).

**DISCUSSION**

In *S. pombe*, cell cycle checkpoint arrest in response to DNA damage or inhibition of replication is dependent on multiple proteins. Six gene products have been identified that act early in the process of checkpoint control, and to date human homologues of two of these proteins have been identified, Hrad9 (9) and ATR (7, 8). Based upon sequence homology and biochemical activity, we have identified a third component, Hrad1, a human homologue of the *S. pombe* rad1 cell cycle checkpoint gene. Hrad1 is highly similar to *S. pombe* rad1, *S. cerevisiae* RAD17, and the REC1 gene of *U. maydis*. Mutations in these genes lead to similar cell cycle checkpoint defects and other phenotypes associated with alterations in DNA repair and recombination (16, 19, 20).

We have shown that Hrad1 is subject to alternative splicing giving rise to two ORFs, a long form, designated Hrad1A, and an N-terminal truncation, designated Hrad1B. The Hrad1 gene has at least three transcripts of 5, 3, and 1.3 kb present in all tissues that we examined and that are increased in all the cancer cell lines examined, suggesting either that transcription of Hrad1 is proliferation-dependent or that it is increased in response to the genomic instability of cancer cell lines. In testis we observed an increase specifically in the 1.3-kb transcript. We have demonstrated that this transcript does not correspond to Hrad1A, suggesting that it corresponds to Hrad1B or an as yet unidentified third form of Hrad1. Several yeast cell cycle checkpoint genes play important roles in meiosis (39) and recently ATM and ATR (the human homologues of *S. pombe* rad3) were shown to be highly expressed in testis where they interact with meiotic chromosomes. This suggests a direct role for these proteins in recognizing and responding to DNA strand interruptions that occur during meiotic recombination (40).

Hrad1 could form part of this recognition complex in association with ATM or ATR.

When expressed in an *S. pombe rad1::ura4* strain, both Hrad1A and Hrad1B failed to complement the phenotypes associated with loss of checkpoint function. However, this should not be taken as evidence against functional homology because failure to complement has been shown for other checkpoint genes such as ATR and Hck1 (7, 12).

The Hrad1 amino acid sequence is significantly similar to the *U. maydis* REC1 protein, indicating that Hrad1 may have a 3'→5' exonuclease activity. We have confirmed the predicted 3'→5' exonuclease activity for Hrad1A by demonstrating that Hrad1A expressed as a hexahistidine fusion protein and purified from *E. coli* is an exonuclease with a 6-fold preference for 3' termini. The biochemical properties of HisHrad1A were very similar to REC1 (21). The truncated Hrad1B, expressed as a hexahistidine fusion protein, lacks detectable exonuclease activity. This may reflect the *in vivo* biological activity or may be a consequence of the purification regimes. To fully address this question, purification of endogenous Hrad1B from mammalian

| Enzyme     | single-stranded/double-stranded | 3/5 | 35S/32P |
|------------|---------------------------------|-----|---------|
| Hrad1A     | 2.3                             | 6.6 |         |
| REC1       | 4.9                             | 61  | 0.5     |
| Exonuclease I | >500                           | 0.6 |         |
| λ Exonuclease |                               | 0.002 |    |
| Exonuclease III |                             |     |         |

**TABLE II**

Comparison of nuclease activities with respect to substrate

The data for REC1, exonuclease I, λ exonuclease, and exonuclease III are taken from Thelen et al. (21). All Hrad1A reactions were carried out as described under “Experimental Procedures” for 30 min at 37 °C. Reaction conditions for the other (cited) enzymes were similar but not identical, so that exact numerical comparisons are not warranted.

2 A. Parker, unpublished results.
cells or from a heterologous expression system that yields a protein, then what role might it play in the surveillance mechanism monitoring the genome via the C-terminal checkpoint domain and the DNA damage-dependent checkpoint? It has been demonstrated that RAD17 functions in conjunction with RAD24 and MEC1 to activate DNA degradation (23), 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 (22, 23). If we consider Hrad1 as part of a complex that acts as a surveillance mechanism monitoring the genome and communicating the presence of DNA damage or unreplicated DNA to the cell cycle machinery, then it is conceivable that in higher eukaryotes two types of surveillance complexes exist separating the DNA damage- and DNA replication-dependent checkpoint pathways. Hrad1A could be specifically required for the DNA damage-dependent checkpoint, able to monitor the genome via the C-terminal checkpoint domain and process single- and double-stranded breaks via the N-terminal exonuclease domain. A link between the mismatch repair system and the DNA damage-dependent checkpoint has been demonstrated (42). Hrad1B may have a function in the DNA replication-dependent checkpoint that might require only the monitoring activity and not the nuclease function to identify replication forks. Hrad1B also contains the sequence shown to be essential for checkpoint function in a mutational analysis of S. pombe Rad1 (43). The increase in transcription that we observed in testis could also signify a role for Hrad1B in meiotic recombination in conjunction with ATR and ATM.

The loss of checkpoint function has been shown to lead to genomic instability even in the absence of exogenous DNA damage (44). In humans, the p53 gene and the ATM gene are not only required for the G1-S phase checkpoint (45, 46) but also act as tumor suppressors (47–50). It is likely that other checkpoint genes will act as tumor suppressors. The Hrad1 gene is located on chromosome 5p13.2–13.3. Loss of heterozygosity of this region of chromosome 5 has been associated with a tumor suppressor function most notably in human lung cancer (37, 38). The CDK2/cyclin A-associated protein p45 (SKP2) has been mapped to 5p13 (51), but Hrad1 should also be considered as a candidate tumor suppressor gene in this region.

The precise roles that Hrad1A and Hrad1B play in cell cycle control, DNA repair, and tumor suppression remain to be determined. This study represents a starting point to begin to unravel the potential multiple roles for the Hrad1 gene product.

Acknowledgments—We thank Jörg Sengel for bioinformatics assistance, Grant Brown for providing S. pombe strains, George Brush, Grant Brown, Alan Richardson, and Paul Russell for their critical review of the manuscript, and the reviewer for helpful comments.

REFERENCES

Identification of Human Rad1 cDNA

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An analysis of C-terminal truncations of the Rec1 protein has established that the C-terminal portion of the Rec1 protein is not essential for exonuclease activity but is crucial for G2–M checkpoint function (24, 25). The checkpoint-compromised U. maydis rec1-1 mutant results from a C-terminal truncation lacking 122 amino acids (24), including a block of sequence containing four periodically spaced leucines that is conserved in Hrad1 and S. pombe Rad1 but not in S. cerevisiae RAD17 (24). This may represent a domain important in interactions with other checkpoint components. In addition, the rec1-5 mutant that lacks exonuclease activity has a 100-fold higher rate of spontaneous mutation, indicating that the exonuclease function may be required for mismatch repair (24). Thus, the Rec1 protein appears bifunctional, having an N-terminal exonuclease domain and a C-terminal checkpoint domain. This type of separation of enzymatic and checkpoint domains has also been described for S. cerevisiae DNA polymerase ε (41). The bifunctional nature is likely to be conserved in Hrad1A based upon our biochemical studies and the protein sequence similarity between Hrad1A and the S. cerevisiae Rec1 protein. However, in humans we have identified an additional component, Hrad1B, which corresponds to the C-terminal region of Rec1 implicated solely in checkpoint control. We cannot discount the possibility that the alternatively spliced mRNA coding for Hrad1B is not translated. However, if the Hrad1B mRNA leads to the production of a protein, then what role might it play in DNA metabolism or checkpoint control? In fission yeast, rad1 is required for both the DNA damage- and DNA replication-dependent checkpoints, whereas in S. cerevisiae RAD17 is only required for the DNA damage-dependent checkpoint. It has been demonstrated that RAD17 functions in conjunction with RAD24 and MEC1 to activate DNA degradation (23), 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 (22, 23). If we consider Hrad1 as part of a complex that acts as a surveillance mechanism monitoring the genome and communicating the presence of DNA damage or unreplicated DNA to the cell cycle machinery, then it is conceivable that in higher eukaryotes two types of surveillance complexes exist separating the DNA damage- and DNA replication-dependent checkpoint pathways. Hrad1A could be specifically required for the DNA damage-dependent checkpoint, able to monitor the genome via the C-terminal checkpoint domain and process single- and double-stranded breaks via the N-terminal exonuclease domain. A link between the mismatch repair system and the DNA damage-dependent checkpoint has been demonstrated (42). Hrad1B may have a function in the DNA replication-dependent checkpoint that might require only the monitoring activity and not the nuclease function to identify replication forks. Hrad1B also contains the sequence shown to be essential for checkpoint function in a mutational analysis of S. pombe Rad1 (43). The increase in transcription that we observed in testis could also signify a role for Hrad1B in meiotic recombination in conjunction with ATR and ATM.

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