The *REC1* Gene of *Ustilago maydis*, Which Encodes a 3′ → 5′ Exonuclease, Couples DNA Repair and Completion of DNA Synthesis to a Mitotic Checkpoint

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ABSTRACT

Mutation in the *REC1* gene of *Ustilago maydis* results in extreme sensitivity to killing by ultraviolet light. The lethality of the *rec1-1* mutant was found to be partially suppressed if irradiated cells were held artificially in G2-phase by addition of a microtubule inhibitor. This mutant was also found to be sensitive to killing when DNA synthesis was inhibited by external means through addition of hydroxyurea or by genetic control in a temperature-sensitive mutant strain defective in DNA synthesis. Flow cytometric analysis of exponentially growing cultures indicated that wild-type cells accumulated in G2 after UV irradiation, while *rec1-1* cells appeared to exit from G2 and accumulate in G1/S. Analysis of mRNA levels in synchronized cells indicated that the *REC1* gene is periodically expressed with the cell cycle and reaches maximal levels at G1/S. The results are interpreted to mean that a G2-M checkpoint is disabled in the *rec1-1* mutant. It is proposed that the *REC1* gene product functions in a surveillance system operating during S-phase and G2 to find and repair stretches of DNA with compromised integrity and to communicate with the cell cycle apparatus.

SUCCESSFUL transmission of genetic information requires not only that the cell provide efficient means for correcting errors introduced by replication and DNA damage, but also that the cell maintain a highly ordered mechanism for cell-cycle progression. Replication of damaged DNA or segregation of defective chromosomes can lead to cellular catastrophe. To avoid such calamities, cells require feedback controls by which events within the cell cycle are coupled. These controls regulate the ordered progression of events within the cell cycle and ensure that the initiation of one event is delayed until the prior successful completion of an earlier event. Hence, genomic integrity following DNA replication must be confirmed before chromosome segregation and cell division can occur.

Part of the cellular response to DNA damage to avoid the transmission of defective chromosomes is imposition of a delay in cell division by arresting progression through the mitotic cycle. Delay in G2 appears to be required by the cell to effect repair and to prevent a potentially lethal attempt at the segregation of damaged chromosomes (for a review, see HARTWELL and WEINERT 1989). A consequence of failure to arrest the cell cycle after DNA damage is loss of viability in the progeny, as shown in studies on *Saccharomyces cerevisiae* in which it was demonstrated that the radiation-sensitive mutant *rad9* is defective in G2 arrest (WEINERT and HARTWELL 1988). These studies have led to the concept of cell-cycle checkpoints that link DNA repair activities to cell-cycle arrest as a means for ensuring completion of repair and that couple progression into mitosis with the prior successful completion of replication as a means of ensuring genomic integrity (WEINERT and HARTWELL 1988; MURRAY 1992; LI and DESHAIES 1993; NURSE 1994).

Radiation-sensitive mutants of *Ustilago maydis* were the earliest examples of DNA repair defective mutants isolated in eukaryotes (HOLLIDAY 1965a), but not until recently has progress been made in understanding the molecular basis for the genetic defects. *rec1-1*, which was the first of these mutants isolated, has an extremely complicated phenotype characterized by sensitivity to radiation and alkylating chemicals, defects in recombination, aberrant meiosis, lethal sectoring during mitotic growth, and elevated spontaneous mutation (HOLLIDAY 1967; HOLLIDAY *et al.* 1976). The mutator phenotype was one distinguishing property suggesting that the *REC1* gene plays a constitutive role to ensure the integrity of the genome. Likewise, the sensitivity to radiation and chemicals suggested a central role for *REC1* in the response to DNA damage, again indicative of a role in guarding the integrity of the genome. However, there has appeared to be no easy way to rationalize the diverse nature of the *rec1* phenotype on the basis of a single biochemical defect. In light of the global effects caused by mutation in the *REC1* gene, it has been suggested that the gene must encode some regulatory function (HOLLIDAY *et al.* 1976).

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The *REC1* gene has been found to encode a 3′ → 5′ exonuclease (THELEN *et al.* 1994). This discovery has provided a framework for beginning to rationalize the *rec1* phenotype. For example, loss of an exonuclease activity involved in removing unpaired or damaged bases might be imagined to account for the radiation sensitivity, the elevated spontaneous mutation rate, and the recombination defects, which include deficiency in induced gene conversion and increased allelic recombination. Nevertheless, certain observations do not provide support for the interpretation that the radiation sensitivity is a direct consequence of the loss of exonuclease activity. For instance, pyrimidine dimer excision is unimpaired in *rec1-1* (UNRAU 1975). Further, the 3′ → 5′ exonuclease activity was discovered to be virtually undiminished in the *rec1-1* allele, which is extremely radiation sensitive (ONEL *et al.* 1995). Finally, strong liquid holding recovery in *rec1-1* after radiation indicates that the mutant retains the ability to repair damage. This feature describes a phenomenon in which the severity of radiation-induced killing can be partially attenuated by maintaining cells in a nutrient-free medium for several hours before plating (MOUSTACCHI and ENTERIC 1970). Thus, the biochemical deficiency in *rec1* does not fully account for the radiation sensitivity.

U. maydis is extremely resistant to radiation (RESNICK 1978; LEAPER *et al.* 1980). A factor of importance in its survival after irradiation is the significant G2 phase fraction of the mitotic cell cycle, which provides a large window of opportunity for recombinational and other postreplicational pathways to enable repair before mitosis (HOLLIDAY 1965b). Given that *U. maydis* cells arrested in growth by starvation are known to re-enter the cell cycle in G2 (ESPOSITO and HOLLIDAY 1964; HOLLIDAY 1965b), it seems evident that cell-cycle dynamics at G2 contribute fundamentally to the repair of DNA damage in *U. maydis* (LEAPER *et al.* 1980). One explanation for the pronounced survival of irradiated *rec1-1* cells after liquid holding is that cell-cycle delay or arrest caused by nutrient deprivation enables DNA repair. This implies that the DNA repair deficiency in *rec1-1* is at least in part a manifestation of some cell-cycle defect.

Studies performed largely in *S. cerevisiae* and *Schizosaccharomyces pombe,* but in a few other systems as well, have established that the mitotic checkpoint is enforced by multiple genetic pathways. A hallmark defining participation of more than one pathway to the G2-M checkpoint is sensitivity of a mutant to radiation and hydroxyurea. This indicates that there is dysfunction in checkpoint pathways that are dedicated to recognition of DNA damage as well as to incomplete DNA synthesis. Genes in *S. cerevisiae* and *S. pombe* have been identified that function in one or contribute to multiple overlapping checkpoint pathways. In *S. cerevisiae,* mutants in *ESR1/MEC1* and *RAD53* are defective in S phase and G2 phase checkpoint pathways (ALLEN *et al.* 1994; WEINERT *et al.* 1994; PAULOVICH and HARTWELL 1995), while mutants in *RAD9, RAD17, RAD24,* and *MEC3* are defective in the G2 checkpoint pathway (WEINERT *et al.* 1994), mutants in *RAD9, RAD24, RAD53, DBF4,* and *CDC7* are defective in a G1 checkpoint pathway (SIEDE *et al.* 1994; TOYN *et al.* 1995), and mutants in *POL2* and *CDC6* appear defective in the S phase checkpoint pathway only (BUENO and RUSSELL 1992; NAVAS *et al.* 1995). Similarly in *S. pombe* mutants defining the multifold nature of mitotic checkpoint control have also been identified (ENOCH and NURSE 1990; AL-KHODAIRY and CARR 1992; ENOCH *et al.* 1992; ROWLEY *et al.* 1992; KELLY *et al.* 1993; SAKA and YANAGIDA 1993; AL-KHODAIRY *et al.* 1994; CARR *et al.* 1995).

The functions of only a few of the checkpoint genes are known. In *S. cerevisiae,* the functions of some genes known to overlap both the S phase and G2 phase checkpoint pathways have been partially uncovered. *ESR1/MEC1* has been found to share homology with phosphoinositol kinases and is functionally related to another yeast gene, *TEL1,* which controls telomere length, and to *ATM,* a human gene responsible for the genetic disorder ataxia telangiectasia (KATO and OGAWA 1994; WEINERT *et al.* 1994; GREENWELL *et al.* 1995; MORROW *et al.* 1995; PAULOVICH and HARTWELL 1995). The *S. pombe* checkpoint gene $rad3^+$ shares homology with *ESR1/MEC1* (JIMENEZ *et al.* 1992; GREENWELL *et al.* 1995; MORROW *et al.* 1995). *RAD53* is a serine/threonine protein kinase (STERN *et al.* 1991; ALLEN *et al.* 1994; WEINERT *et al.* 1994). For the S phase checkpoint pathway, the *POL2* gene encodes DNA polymerase ϵ (NAVAS *et al.* 1995), while *CDC6* encodes a protein that interacts with the origin recognition complex (BUENO and RUSSELL 1992; HOGAN and KOSHLAND 1992; LIANG *et al.* 1995) and is homologous to the *S. pombe* gene $cdc18^+$ (KELLY *et al.* 1993). None of the *S. cerevisiae* genes in the G2 phase checkpoint pathway has yet been functionally defined, but the *S. pombe* $chk1^+$ gene, which appears to function only in the G2 pathway, encodes a protein kinase (WALWORTH *et al.* 1993).

In this study, the aim was to investigate if there was a role for the *REC1* gene in coupling DNA repair and cell-cycle progression. To approach this issue, we examined survival of cells defective in *REC1* function for response to both DNA damage and incompletely replicated DNA and studied the effect of artificially delaying progression through the cell cycle at the G2/M boundary. As an additional but more oblique means for gaining understanding of the role of *REC1,* we investigated the mode of gene expression in relation to the cell cycle. The results of the study led to the conclusion that *REC1* serves in checkpoint control during the mitotic cell cycle of *U. maydis* and raised the general question of how the cell cycle machinery harnesses an enzyme with catabolic activity on DNA to a signaling mechanism.

MATERIALS AND METHODS

U. maydis procedures: *U. maydis* strains used in this study from the Cornell Medical College collection were UCM3 (wild type), UCM21 (*rec1-1*), UCM54 (*rec2-1*), UCM334

(*uvs3-1*), UCM266 (*pol1-1*) and UCM268 (*rec1-1 pol1-1*). Cultures were maintained in YEPS medium as described (FOTHERINGHAM and HOLLOMAN 1990). Strains with the *pol1-1* marker were grown at 22°. *U. maydis* cells were synchronized by a feed-starve regimen as described by HOLLIDAY (1965b). Strains to be irradiated with 254 nm ultraviolet light were suspended in water at 10^7 cells/ml and irradiated with vigorous agitation at a fluence of 6.7 erg/mm^2/sec. After appropriate dilutions, cells were plated and survivors were counted as colonies visible after incubation for 3 days at 32°. Cells were synchronized in G2/M by incubation at 32° with shaking in 200 μg/ml methyl-1-(butylcarbamoyl)-2-benzimidazole-carbamate (MBC) (Southern Agricultural Insecticides, Inc., Palmetto, FL) for 2 hr before irradiation.

DNA methods: A DNA fragment of the *U. maydis* histone H4 gene (*HHF*) encompassing almost the entire coding sequence (300 of 312 residues) was cloned for use as a cell-cycle marker. Degenerate oligonucleotide primers flanking nearly the entire histone H4 consensus sequence (WELLS and MCBRIDE 1989; WEELS and BROWN 1991) were designed and used to prime amplification of the *U. maydis* gene by PCR. Primer 1, 5′-GG(T/C/G/A) AA(A/G) GG(T/C/G/A) GG(T/C/G/A) AA(A/G) corresponds to nucleotides 13–27 of the consensus histone H4 ORF, while primer 2, 5′-(T/C/G/A)CC (T/C/G/A)CC (G/A)AA (T/C/G/A)CC (G/A)TA, is complementary to nucleotide 295–309. After polymerase chain reaction (PCR) using *Taq* DNA polymerase, the resulting major amplification product of 300 bp was then purified following agarose gel electrophoresis, its ends were made flush by Klenow DNA polymerase and dNTPs, phosphorylated by T4 polynucleotide kinase and ATP, and inserted into the *Eco*RV site of pBluescript II SK$^+$. DNA sequence analysis confirmed the identity of the histone H4 gene. pCM534 is pBluescript II SK$^+$ containing the 300-bp fragment of the *U. maydis* histone H4 gene (*HHF*)isolated by PCR inserted into the *Eco*RV site. Two plasmids used in cloning portions of *REC1* were constructed by inserting fragments into the *Eco*RV site of pBluescriptII (Stratagene, La Jolla, CA): pCM224 contains the *Sma*I-*Hin*dIII 2.7-kb fragment, and pCM251 contains the complete *REC1* open reading frame (ORF) generated by the PCR. DNA probes were labeled with ^{32}P by random priming with the Klenow fragment of *E. coli* DNA polymerase I and [α-^{32}P]dATP (FEINBERG and VOGELSTEIN 1983).

RNA methods: Cell cultures (5 ml) were collected by centrifugation, suspended in 300 μl 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1 M LiCl, 0.2% SDS, (0.1 M LETS) and disrupted by violent agitation on a vortex mixer with 0.9 g nitric acid-washed glass beads (0.5 mm) in 0.25 ml phenol saturated with 0.1 M LETS for 5 min, or with a phenol/guanidinium thiocyanate reagent (RNA STAT-60, Tel-Test "B," Inc.) alternating 30 sec of vortexing with 30 sec of cooling on ice. After two phenol:chloroform extractions and one chloroform extraction, total RNA was precipitated with 0.5 M LiCl and one volume of isopropanol. After 3–6 hr at −20°, the precipitate was then washed three times in 2 M LiCl, once in 70% ethanol, and was finally dissolved in 10 μl 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (TE) treated with diethylpyrocarbonate (DEPC). Contaminating DNA was removed by incubation with (26 mg) RNase-free DNase (GIBCO BRL) in the presence of 10 units human placental ribonuclease inhibitor (GIBCO BRL) for 30 min at 37°. After one phenol:chloroform extraction and one chloroform extraction, the RNA was precipitated in 0.3 M sodium acetate and 2.5 volumes ethanol, washed in 70% ethanol, and dissolved in DEPC-treated TE. Electrophoretic separations of RNA (5–50 μg) were performed in 1.1% agarose gels containing 2.2 M formaldehyde, and blot hybridization was carried out as described (TSUKUDA *et al.* 1989). In preparation for reprobing, RNA blots were stripped by scalding with two washes of a solution containing 1.5 mM sodium citrate, 15 mM NaCl, and 0.5% SDS.

Flow cytometry: Aliquots of cell cultures (1×10^7 cells) were aliquoted into 15-ml screw cap tubes, pelleted, and rinsed in TBS (50 mM Tris-Cl, pH 7.5, 150 mM NaCl). Cells were fixed in 1 ml 70% cold ethanol by incubation with shaking overnight at 4°. Cells were collected and rinsed and resuspended in 1 ml TBS, and digested with RNaseA (100 μg/ml) for 2 hr at 37°. After a rinse in TBS, cells were stained in propidium iodide (10 μg/ml in TBS) overnight at room temperature and analyzed by flow cytometry using a Becton Dickinson FACSCAN (SAZER and SHERWOOD 1990). DNA content was determined by multivariate flow cytometry. To determine the appropriate gates, 10^4 data points were collected in both DNA content (FL2) and cell size (FSC).

RESULTS

The *rec1-1* mutant is defective in DNA damage-induced G2 arrest: To determine whether artificially induced cell-cycle arrest could enhance the survival of *rec1-1* cells after DNA damage, exponentially growing cells were irradiated with UV light and then held in medium containing methyl-1-(butylcarbamoyl)-2-benzimidazole-carbamate (MBC), a microtubule inhibitor that prevents tubulin polymerization (QUINLAN *et al.* 1980). This treatment blocks chromosome segregation by inhibiting spindle assembly and holds cells in G2-phase. Killing of the wild-type and the radiation-sensitive mutant *rec2* (HOLLIDAY 1967; RUBIN *et al.* 1994a) was unaffected by MBC treatment (Figure 1, A and C), but killing of the *rec1-1* mutant was partially suppressed when cells were held for 8 hr in MBC (Figure 1B).

To gain more understanding of the relationship between radiation sensitivity and damage-induced G2 arrest, *rec1-1* cells synchronized in G2 were tested for UV sensitivity. Synchronization was accomplished by addition of MBC to cell cultures for 2 hr. Cells thus synchronized were irradiated, then washed free of MBC and plated for determination of survival, or else were prevented from progressing through the cell cycle by arresting for 8 more hr in MBC before plating. In controls, the results indicated that survival of wild type was unaffected either by synchronization or by synchronization and subsequent G2 arrest (Figure 1A). Similarly, survival of *rec2-1*, which is defective in a RecA-like protein (RUBIN *et al.* 1994a), and *uvs3-1*, which is sensitive to UV-irradiation because of a defect in excising pyrimidine dimers (UNRAU 1975), was not enhanced by G2 arrest (Figure 1, C and D). Indeed, MBC potentiated the sensitivity of *uvs3* to killing. In contrast, killing of synchronized *rec1-1* cells was strongly, although not completely suppressed by G2 arrest (Figure 1B). Two other features of note were evident. First, killing of the *rec1-1* strain was significantly suppressed by the synchronization procedure itself. This could result from elimination of the very UV-sensitive G1/S population from the culture. Second, the survival curve of the *rec1-1* mutant after synchronization and G2 arrest was biphasic, with a shoulder evident at low doses of UV-irradiation. These results suggest that the *rec1-1* mutant retains a capacity for repairing DNA damage and that repair abil-

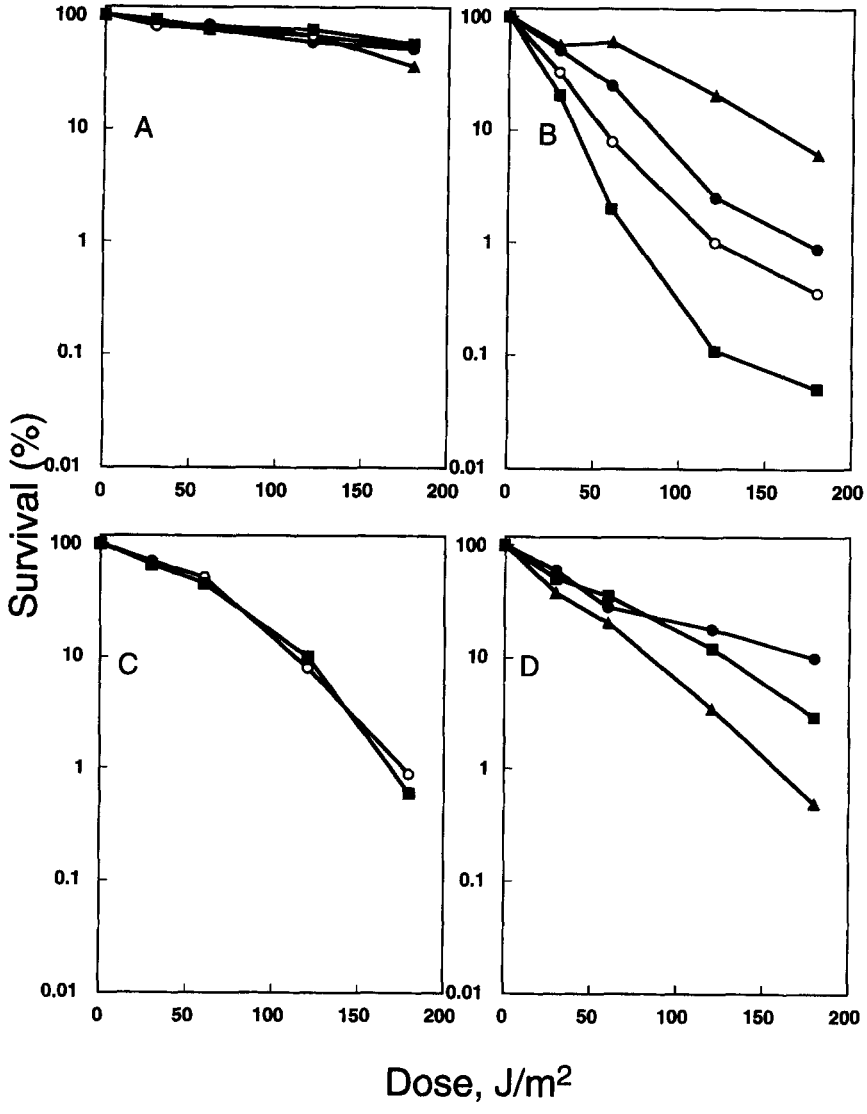

FIGURE 1.—Cell cycle arrest of *rec1* in G2 suppresses the killing caused by UV irradiation. Cell cultures were grown to mid-log phase, washed and irradiated at a cell density of 10^7 per milliliter with the indicated doses of UV. For synchronization in G2-phase, cells were incubated in MBC (100 μg/ml) for 2 hr before irradiation. After irradiation, cells were washed free of MBC and plated immediately or else arrested in growth by holding in MBC for an additional 8 hr before plating. Aliquots were diluted appropriately then plated. Survivors were determined as colonies visible after incubation for 3 days at 32°. ■, irradiated cells plated immediately; ○, irradiated cells held in MBC for 8 hr then plated; ●, cells synchronized in G2, irradiated, then plated; ▲, cells synchronized in G2, irradiated, arrested by holding in MBC for 8 hr, then plated. (A) Wild type; (B) *rec1-1*; (C) *rec2-1*; (D) *uvs3-1*.

ity is most active when G2 phase is lengthened. Alternative models to account for these findings are that *rec1-1* is defective (or at least leaky) in a G2 specific repair system and that *rec1-1* is defective cell-cycle delay after DNA damage that normally enables repair before mitosis is permitted.

The *rec1-1* mutant is unable to recognize incomplete DNA synthesis: Coupling mitosis to the prior completion of DNA synthesis provides a servomechanism ensuring that entry into mitosis is dependent upon the prior repair of DNA damage (AL-KHODAIRY and CARR 1992; ENOCH *et al.* 1992; ROWLEY *et al.* 1992; WEINERT *et al.* 1994). To determine whether the *rec1-1* mutant is defective for the recognition of unreplicated DNA, sensitivity to hydroxyurea (HU) was assessed. HU blocks DNA synthesis by inhibiting the first and rate-limiting step in the formation of deoxyribonucleotides from ribonucleotides. It does not directly damage DNA but prevents the completion of S phase. When wild-type cells were held in HU for ≤8 hr, viability was largely unaffected (Figure 2A) although cell division was blocked (not shown). In contrast, *rec1-1* was quite sensitive and rapidly lost viability when held in HU (Figure 2C).

The observed killing of *rec1-1* seemed unlikely a result of activity of HU as a radiomimetic agent since the radiation-sensitive mutants *rec2* and *uvs3* were no different from wild type in sensitivity to the drug (Figure 2B). A more likely explanation of the sensitivity of *rec1-1* mutant to HU appeared to be that there was a defect in the recognition of unreplicated DNA and concomitant failure to arrest progression of the cell cycle. To assess this possibility, cultures were synchronized in G2 with MBC and then treated with HU while MBC-induced G2 arrest was maintained; viability of wild-type cells was unaffected (Figure 2A). On the other hand, HU-induced killing of *rec1-1* cells was strongly suppressed by G2 arrest (Figure 2C). This result demonstrates that the HU-sensitivity of the *rec1-1* mutant is probably not due to an inherent repair defect, but is instead more likely the result of a defect in blocking progression through the cell cycle in response to unreplicated DNA.

As an independent means for assessing the inability of *rec1-1* to recognize and respond to incomplete DNA replication, survival was determined under conditions in which DNA synthesis was blocked *in vivo*. The experi-

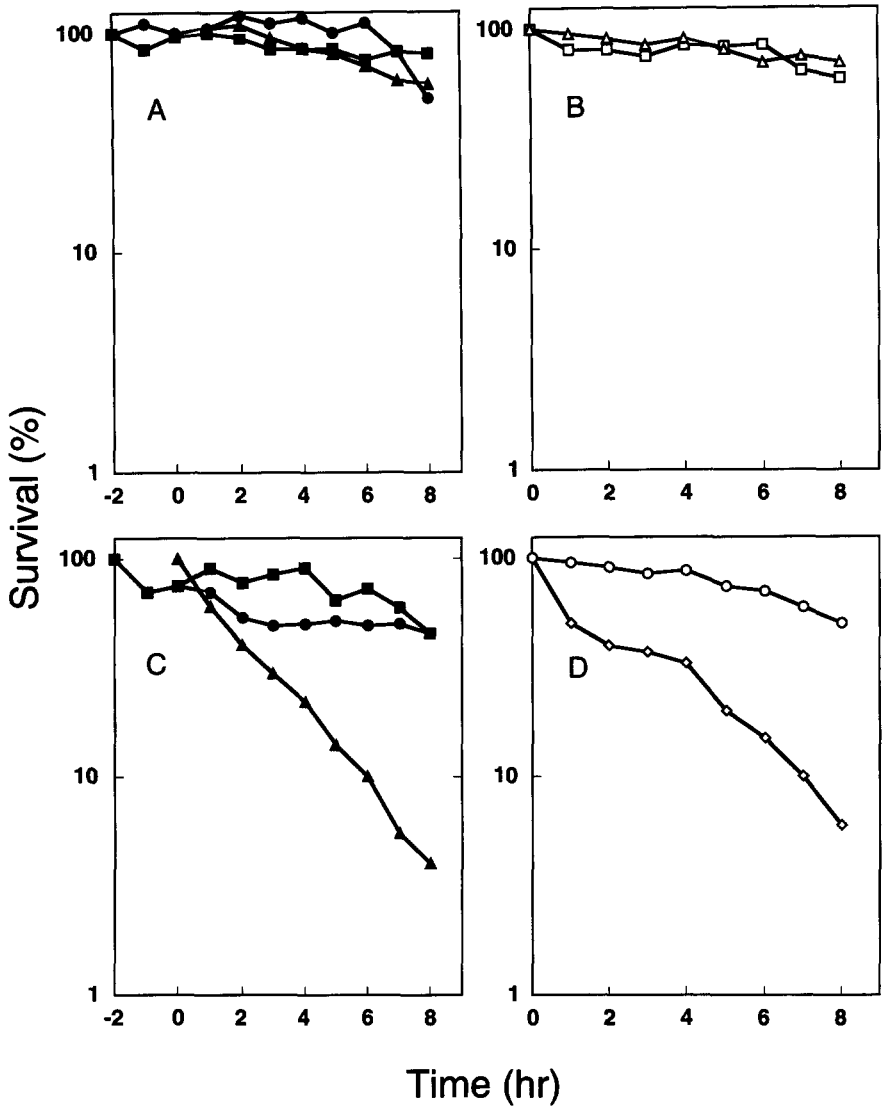

FIGURE 2.—The *rec1* mutant is sensitive to incomplete DNA synthesis. For A (wild type) and C (*rec1*), cell cultures were grown to mid-log phase then split into three cultures containing either 50 mM hydroxyurea (▲), or 100 μg/ml MBC (■), or else were held in 100 μg/ml MBC for 2 hr before addition of 50 mM hydroxyurea (●). (B) *rec2* (□) or *uvs3* (△) cell cultures were grown to mid-log phase, hydroxyurea was added to 50 mM, then samples were removed at the indicated times and plated. (D) *pol1* (○) and *rec1 pol1* (◇) cultures were grown at 22° to mid-log phase then shifted to the restrictive temperature of 32°. At the indicated times aliquots were removed and plated and survival was determined at 22°.

mental design adopted was based on a strategy developed in *S. cerevisiae* for isolating checkpoint mutants. Hyperlethality in a *cdc13* mutant strain, which is defective in a gene essential for DNA synthesis, was exploited as the basis of a screen that led to the identification of several checkpoint genes (WEINERT *et al.* 1994). For analysis of the role of *rec1-1* in recognition of incomplete synthesis, survival was examined in a *pol1* background. *pol1-1* is a conditional lethal mutant that is temperature sensitive for growth and is unable to synthesize DNA at the restrictive temperature (JEGGO *et al.* 1973). When the double mutant was shifted to the restrictive temperature, there was a pronounced time-dependent loss of viability in contrast to the *pol1-1* single mutant (Figure 2D). This observation supports the interpretation that *rec1-1* fails to recognize incomplete DNA synthesis.

Progression of the cell cycle following DNA damage is aberrant in *rec1-1*: The *rec1-1* mutant was examined by flow cytometry for the purpose of gaining insight into the dynamics of DNA replication and cell division after UV irradiation. Cells were removed from cultures after UV irradiation, stained with propidium iodide, and analyzed for DNA content as a function of cell number and cell size. Examination of populations from unirradiated cultures of wild type and *rec1-1* revealed in both instances a similar bimodal distribution (Figure 3). We interpret this as representing approximately equal proportions of cells in G1 and G2-phase. After UV irradiation there were marked changes in the distributions with significant differences in how the wild-type and *rec1-1* strains responded. In the case of wild type, it was evident that over the course of 6 hr after irradiation there was a shift in the population such that cells accumulated in G2. However, in the case of *rec1-1*, there was an overall decrease in the steady-state level of cells in G2 and concomitant accumulation in G1/S. The results indicate that the dynamics of cell-cycle progression after DNA damage are deranged in *rec1-1* with an apparent failure in preventing cells from proceeding through the G2 → M checkpoint. The accumulation of cells in G1/S indicates that another checkpoint at this stage remains operational in *rec1-1*.

Expression of the *REC1* gene is cell cycle regulated:

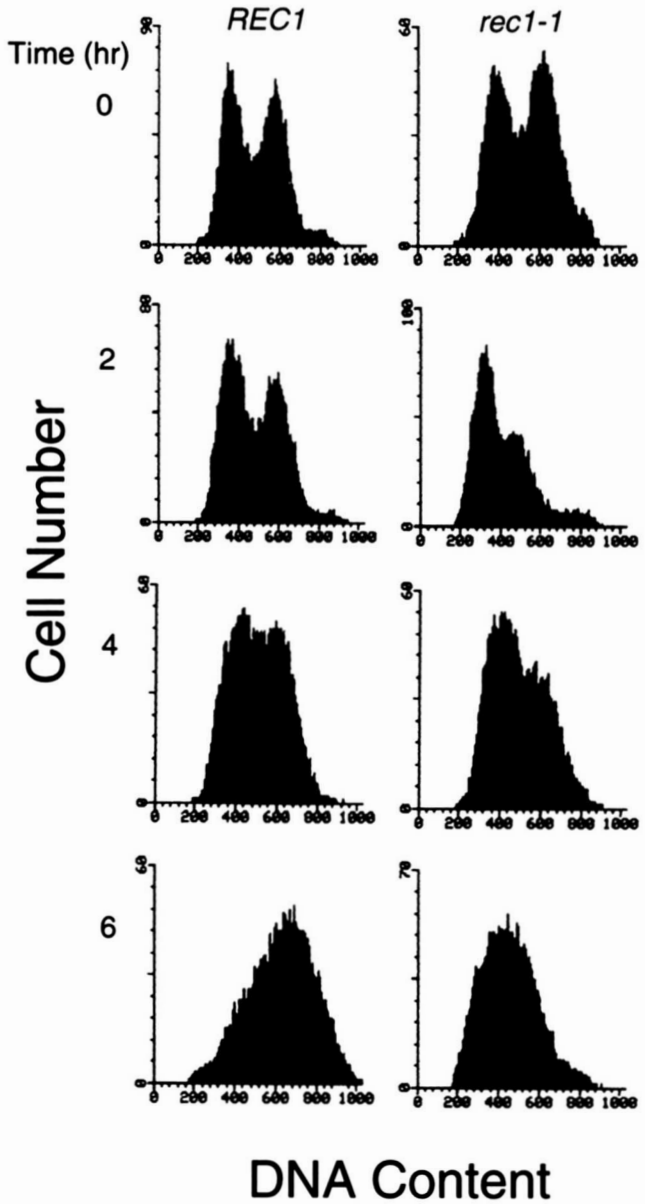

FIGURE 3.—Cell-cycle progression after DNA damage is aberrant in *rec1*. Logarithmically growing cultures of wild-type and *rec1* cells were irradiated with UV at 40 J/m^2 then diluted into fresh medium at a density of 1×10^6 per milliliter. Samples were removed at the indicated times, fixed, stained with propidium iodide, and analyzed by flow cytometry for DNA content *vs.* cell number.

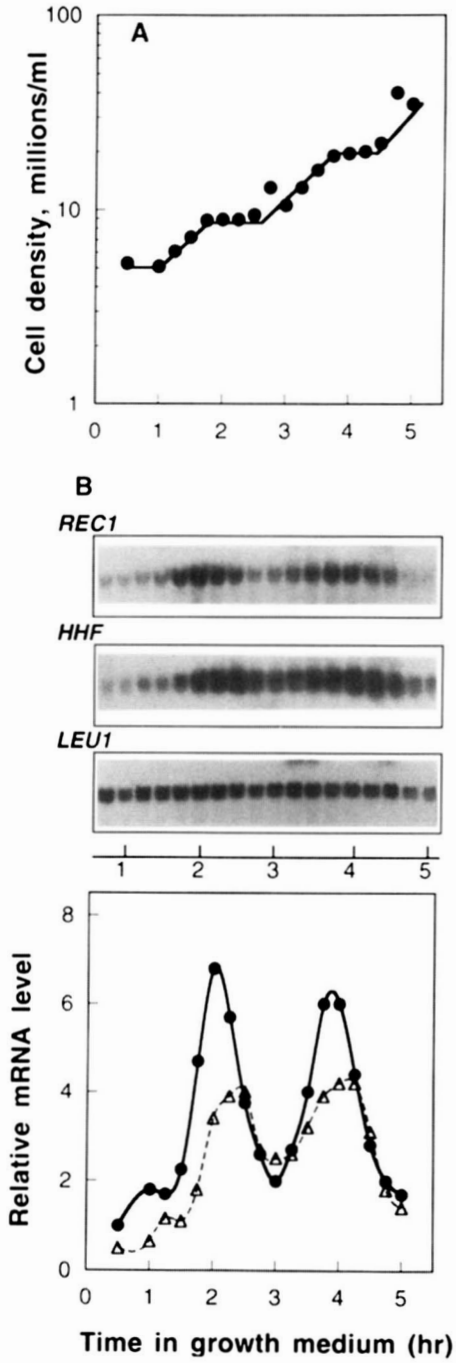

FIGURE 4.—Periodic expression of the *REC1* gene. RNA was prepared from samples removed at the indicated times from a synchronized cell culture (A) and was analyzed by northern blot hybridization (B) as described in MATERIALS AND METHODS. *REC1* mRNA was detected using the 1-kb *Eco*RV fragment internal to the *REC1* gene as probe. After hybridization and autoradiography, the blot was reprobed with the 3-kb *Eco*RI-*Hin*dIII fragment containing the biosynthetic gene *LEU1* (46), or the 0.3-kb frament comprising the *HHF* gene. The relative mRNA levels were obtained by scanning autoradiographs in a densitometer and normalizing to the *LEU1* mRNA. *REC1* mRNA (●); *HHF* mRNA (▲). For clarity the *HHF* level has been reduced by half.

Insight into the biological function of DNA metabolic genes in *S. cerevisiae* and *S. pombe* has come from analysis of the regulation of gene expression. In general, DNA metabolic genes that function in DNA replication have been found to be periodically expressed with the cell cycle in G1/S-phase while those dedicated to DNA repair tend to be expressed constitutively throughout the cell cycle (*e.g.*, JOHNSTON 1992). The level of the *REC1* mRNA was measured in synchronously growing cells (Figure 4) to determine the mode of *REC1* gene expression. Cells synchronized after a regimen of feeding and starving were observed to proceed through two successive rounds of division. Analysis of RNA extracted from these synchronized cells indicated that the level of *REC1* mRNA periodically fluctuated with accumulation and depletion in concert with cell division while the level

of mRNA from the amino acid biosynthetic gene *LEU1* (RUBIN *et al.* 1994b) remained constant.

To define the cell cycle in more detail, the periodicity of histone H4 (*HHF*) gene expression was measured. Histone H4 was chosen as a cell-cycle marker since histone gene expression is tightly controlled and is restricted to G1 and S phase and since the level of *HHF* mRNA is known to peak during S phase (HEREFORD *et al.* 1982; SCHUMPERLI 1986; CROSS and SMITH 1988). Therefore the time of the maximum state of *HHF* mRNA accumulation was taken as an S-phase landmark of the *U. maydis* cell cycle. *REC1* gene expression was found to coincide almost precisely with *HHF* gene expression with the peak in accumulation of *REC1* mRNA slightly preceding *HHF* mRNA. These results indicate that *REC1* is expressed periodically most likely during G1/S-phase of the cell cycle. This mode of regulated expression is like that seen with genes that function during DNA replication.

DISCUSSION

A 3′ → 5′ exonuclease is encoded by the *REC1* gene. Structure/function analysis of a series of deletion mutants has revealed that the exonuclease activity appears to be directly related to proficiency in mutation avoidance. A working model that has emerged based on this observation is that the exonuclease acts in removing mismatched bases from DNA (ONEL *et al.* 1995). Such a function would be consistent not only with the observed elevated spontaneous mutation rate, but also with numerous additional features of the *rec1-1* phenotype. The hyperrecombination between heteroalleles noted in mitotic cells, the absence of radiation-induced gene conversion, and the poor viability of meiotic products are characteristics expected of a mutant defective in removing mismatched bases. Similar properties are seen in *pms1,* (WILLIAMSON *et al.* 1985; KRAMER *et al.* 1989) *msh2* (REENAN and KOLODNER 1992; ALANI *et al.* 1994) and *mlh1* (PROLLA *et al.* 1994) mutants of *S. cerevisiae* that are known to be defective in mismatch repair. Given a mismatch repair deficiency, it might have been supposed that the radiation sensitivity could have been accounted for by an exonuclease defect. For instance, the *exo1* gene of *S. pombe* encoding a 5′ → 3′ exonuclease that appears to function in mismatch repair is a member of a family of related DNA repair genes that functions in UV excision repair (SZANKASI and SMITH 1995). Nevertheless, the structure/function analysis of *REC1* indicated that the exonuclease activity did not appear to be directly coupled to resistance to radiation damage. Furthermore, the analogy with the mismatch repair mutants of *S. cerevisiae* and *S. pombe* is somewhat limited since those mutants exhibit no radiation sensitivity. Thus the question is raised of how to rationalize the DNA repair defect. The solution to the puzzle would appear to be that the *REC1* gene is at least bifunctional. One function is in service in repair of mismatched bases, while another, as demonstrated in this study, is in regulation of the cell cycle.

The partial suppression of radiation- and hydroxyurea-induced killing of *rec1-1* by arresting cells in G2 with MBC and the decrease in the G2 phase cell population after UV irradiation indicate that the mutant is defective in some aspect of cell-cycle arrest brought on by DNA damage or incomplete DNA replication. The results imply that *REC1* couples the onset of mitosis to the prior repair of DNA damage and successful completion of S phase. Mutants of *S. cerevisiae* and *S. pombe* that trespass illicitly through M can be grouped into categories that define elements of the cell cycle. By studying the response of these mutants to radiation and hydroxyurea as well as by examining their interaction with genes that encode components of the DNA replication apparatus, investigators have defined S-phase, G1, and G2 phase pathways for the mitotic checkpoint. These pathways include components that are responsive to DNA damage or DNA replication or both. Mutants of *S. cerevisiae* that are simultaneously defective in the S-phase and DNA damage checkpoint component include *ESR1/MRC1* (KATO and OGAWA 1994; WEINERT *et al.* 1994; PAULOVICH and HARTWELL 1995), *RAD53* (ALLEN *et al.* 1994; WEINERT *et al.* 1994) and *POL2* (NAVAS *et al.* 1995). In *S. pombe,* a number of mutants have been identified as defective in overlapping S phases and DNA damage phases including *rad1, rad3, rad9, rad17, hus1,* and *rad26* (AL-KHODAIRY and CARR 1992; ENOCH *et al.* 1992; ROWLEY *et al.* 1992; AL-KHODAIRY *et al.* 1994).

The cell requires three elements for the effective regulation of ordered progression through the mitotic cycle (LI and DESHAIES 1993). First, there must be a sensing mechanism by which genomic integrity can be determined and by which DNA damage, replication errors, or incompletely replicated DNA can be detected. Second, there must be a transducer by which this information is conveyed to the cell-cycle machinery. Third, there must be a responder or series of responders by which cell-cycle progression is regulated and by which the appropriate mechanism for the resolution of the genomic insult can be induced. An attractive hypothesis is that the sensor is part of the DNA synthesis machinery. Recent studies on checkpoint control mutants defective for cell-cycle arrest in the presence of unreplicated DNA in *S. cerevisiae* have identified the *POL2* gene functions as a checkpoint control (NAVAS *et al.* 1995). This gene encodes DNA polymerase ϵ, which is essential for DNA replication, but which is also necessary for transcriptional activation of damage inducible genes and for entry into mitosis. These observations suggest that DNA polymerase ϵ in some way senses unreplicated DNA as a result of direct physical interaction with DNA, perhaps by sensing DNA activity or by sensing gaps and regions of single strandedness. The replication apparatus has also been implicated as a locus from which the cell senses incomplete replication by the observation that the *S. pombe cdc18*$^{+}$ gene product serves both to

couple the onset of S phase to START, and to induce cell-cycle delay until the completion of S phase (KELLY *et al.* 1993; NISHITANI and NURSE 1995). *cdc18*$^{+}$ is a homologue of the *S. cerevisiae CDC6* gene, an essential gene that encodes a protein that plays a role in the initiation of replication by interacting with the origin recognition complex (LIANG *et al.* 1995) and that itself functions in delaying entry into mitosis (BUENO and RUSSELL 1992).

The observation that *rec1-1* cells can exit from G2 after DNA damage suggests that the machinery for cell-cycle progression is intact, but that transmission of the signal to arrest cell-cycle progression is defective. By analogy with the *S. cerevisiae* DNA polymerase ϵ, which functions directly as a DNA metabolic enzyme, and framed within the context of the function of the *REC1* gene product, which is also a DNA metabolic enzyme, it seems likely that the *rec1-1* mutant is defective for sensing DNA damage and unreplicated DNA. Further support for this notion comes from the oscillating mode of expression of the *REC1* gene. This mode of regulation is similar to that of genes in *S. cerevisiae* and *S. pombe* whose function is dedicated to DNA synthesis. A model taking all of the findings into account would hold that the 3′ → 5′ exonuclease activity encoded by the *REC1* gene functions as part of a surveillance mechanism operating during DNA replication and also as needed in G2 phase to find and repair stretches of DNA with compromised integrity and to communicate with the cell-cycle machinery.

Interallelic complementation and mutational analyses of the *S. cerevisiae POL2* gene have provided evidence that DNA polymerase ϵ is composed of two separable domains, an N-terminal DNA polymerase domain and a C-terminal checkpoint domain, each of which is essential (NAVAS *et al.* 1995). Several observations suggest a similar modular organization for the *REC1* gene product. Certain mutant alleles of *REC1* have been examined in detail (ONEL *et al.* 1995). DNA sequence analysis has revealed that *rec1-1* contains a nonsense mutation resulting in production of a truncated polypeptide of 400 amino acid residues. Similarly, the *rec1-5* allele contains a nonsense mutation that results in truncated polypeptide of 159 residues. Despite this difference in the size of the gene products, the two alleles share the same radiation-sensitive phenotype. However, the 3′ → 5′ exonuclease activity associated with the purified mutant polypeptides was quite different. The *rec1-1* encoded mutant protein retained almost full exonuclease activity while the *rec1-5* allele was completely deficient. These findings suggest that *REC1* encodes a protein consisting of two domains, an N-terminal exonuclease domain, and a C-terminal checkpoint domain.

It has recently been found that the *U. maydis REC1* gene shares homology with *rad1*$^{+}$ of *S. pombe* (LONG *et al.* 1994) and *RAD17* of *S. cerevisiae* (LYDALL and WEINERT 1995). These genes have been found to function in checkpoint control (AL-KHODAIRY and CARR 1992; ROWLEY *et al.* 1992; WEINERT and HARTWELL 1993; WEINERT *et al.* 1994), although not in an entirely equivalent manner. *rad1*$^{+}$ functions in both the S and G2 checkpoint pathways while *RAD17* appears to operate only in the G2 phase checkpoint pathway. Preliminary evidence indicates that *RAD17* appears to function together with *RAD24* and *MEC3* to activate DNA degradation. This has prompted the suggestion that some DNA processing might be required for checkpoint activation (LYDALL and WEINERT 1995). The *rec1-1* mutation interrupts a leucine-rich region homologous to similar sequences from both the *rad1*$^{+}$ and *RAD17* genes. It is possible that this region is a required domain for checkpoint function. The conservation of sequence and function of these genes in regulating cell-cycle progression in three very divergent fungi attests to central importance of this class of genes in maintaining integrity of the genome although it is not yet known if the global consequence of inactivation of the genes is the same in all three organisms. UV-induced mitotic intragenic recombination and mutagenesis were found to be reduced in frequency in the *S. pombe rad1* mutant, similar to what was observed in *rec1-1,* but spontaneous allelic recombination was unaffected, unlike *rec1-1,* and no reduction in spore viability after meiosis was reported (NASIM 1968; FABRE 1972; GROSSENBACHER-GRUNDER and THURIAUX 1981). Thus, there are several intriguing parallels between the *S. pombe rad1* mutant and *rec1-1,* although a complete analysis has not been conducted for the *rad1* or *S. cerevisiae rad17* mutant. A full comparison of genetic properties among all three mutants might give some indication if the function of this *REC1* class of genes has been conserved during evolution. Evidence suggesting a connection between the mismatch repair system and the G2 cell-cycle checkpoint in human cells (HAWN *et al.* 1995) supports the notion that the bifunctional service of *REC1* in repair of mismatch and in regulation of the cell cycle has been evolutionarily maintained.

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