The REC1 Gene of *Ustilago maydis* Involved in the Cellular Response to DNA Damage Encodes an Exonuclease*

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Mutation in the **REC1** gene of *Ustilago maydis* is known to lead to a complex phenotype with alterations in DNA repair, recombination, mutagenesis, meiosis, and cell division. The predicted product of the **REC1** gene is a polypeptide of 522 amino acid residues with a molecular mass of 56,886 daltons, with no overall sequence homology to any other known protein. The open reading frame of the **REC1** gene placed by itself in an *E. coli* expression vector was found to be sufficient to complement the **rec1** mutant. Overexpression of **REC1** in *Escherichia coli* gave rise to the anticipated 57-kDa product together with a 3'-5' exonuclease activity. This activity was only present in cells overexpressing **REC1** and its characteristics were distinguishable from the major bacterial nuclease, but it had certain enzymatic features in common with ε, the proofreading exonuclease subunit of *E. coli* DNA polymerase III holoenzyme. To facilitate isolation of the protein product from bacteria, the **REC1** gene was overexpressed from a vector that fused a hexa-histidine-leader sequence onto the amino terminus, enabling the isolation of the His**REC1** product on an immobilized metal ion affinity column. The His**REC1** protein co-eluted with the novel exonuclease activity. Alignment of the amino acid sequence of the **REC1** gene product with the conserved proofreading exonuclease motifs of DNA polymerases indicated significant homology.

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1 The use of the term "error-prone" indicates a phenomenon which occurs during DNA damage-induced mutagenesis, and is not to be confused with the specific error-prone repair pathway of the SOS response in *E. coli* (see Friedberg et al. (1991)).
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The relevant genotype

**Table I**

| Strains       | Plasmid* | Description                                                                 |
|---------------|----------|------------------------------------------------------------------------------|
| UCM3 REC1     | pCM130   | ARS HPH                                                                      |
| UCM21 rect-1  | pCM224   | ARS HPH                                                                      |
| Plasmid*      | pCM251   | ARS HPH                                                                      |
| pCM254        | phap70   | ARS HPH                                                                      |
| pCM306        | pHI0:RECl| ARS HPH                                                                      |
| pCM322        | pHI0:HisRECl|                                                              |

* All plasmids indicated also contain the ColEl origin of replication and the β-lactamase gene conferring ampicillin resistance in E. coli.

NdeI site of pCM317, a shuttle vector constructed by B. Rubin of this laboratory, containing the NdeI fragment containing the RECl DNA fragment. The DNA sequence of this fragment was determined (Holden et al., 1989a). The gene was then subcloned from pCM251 containing the RECl ORF was then inserted into the NdeI site of pET14b (Novagen, Madison, WI). The relevant features of pCM317 are that it was derived from pCM284 (Tsukuda et al., 1989a), but no insight into its function was obtained at that time after a search of the protein data bases (Holden et al., 1991). The aim of the present work was to overexpress and purify the REC1 gene product so as to discover its biochemical activity and to gain insight into its function in the cellular response to DNA damage.

**MATERIALS AND METHODS**

**U. maydis Strains and Plasmids**—Cell cultures were grown on minimal or YEPS medium as described before (see Table I and Fotheringham and Holohan (1989, 1990)). The REC1 gene was previously isolated in this laboratory on a 6.9-kbp U. maydis genomic fragment that complemented all aspects of the rec1 phenotype (Tsuchida et al., 1989). The gene was then subcloned from pCM130 on a 2.7-kbp Smal-HindIII DNA fragment. The DNA sequence of this fragment was determined and an open reading frame of 1569 nucleotides was identified identical to that reported by Holden et al. (1989a). Two plasmids used in cloning portions of REC1 were constructed by inserting fragments into the EcoRV site of pBluescriptII (Stratagene, La Jolla, CA): pCM224 contains the Smal-HindIII 2.7-kbp fragment, and pCM251 contains the complete REC1 open reading frame (ORF) generated by the polymerase chain reaction (PCR), described below. For bacterial overexpression, the PCR-amplified ORF was removed from pCM251 as a 1.6-kbp NdeI fragment and inserted into the NdeI site of pET3c (obtained from Novagen, Madison, WI), yielding pCM317. The relevant features of pCM317 are that it was derived from pCM254, which is pBluescriptII with the U. maydis ARS (Tsuchida et al., 1988), and the HPH gene conferring resistance to hygromycin B (Wang et al., 1988). pCM317 was constructed by insertion into pCM254 of a 0.3-kbp fragment containing part of the U. maydis hap70 promoter element (Holden et al., 1992b), generated by PCR to contain an NdeI cloning site for translational initiation during expression. The 1.6-kbp NdeI fragment from pCM251 containing the REC1 ORF was then inserted into the NdeI site of pCM317 to yield pCM306.

**DNA Methods**—DNA used as exonuclease substrate was either uniformly 32P-labeled P22 DNA prepared as described before (Holloman et al., 1981), 3'-end labeled pBluescriptII DNA made linear with Sau3AI and labeled with [α-32P]dATP or [α-35S]deoxycytidine triphosphate (dNTP) with the Klenow fragment of Escherichia coli DNA polymerase I, or 5'-end labeled Sau3AI fragments identical to those just mentioned but labeled at the 5'-termini with [γ-32P]ATP using polynucleotide kinase (cacodylate buffer) prior to filling in 3'- recessed termini with unlabeled dNTPs. DNA restriction fragments were recovered from agarose gels by centrifugation of gel slices through a small wad of glass wool. DNA sequence was determined by the enzymatic chain termination method using Sequenase 2.0 (U. S. Biochemical Corp.) and [α-35S]dATP according to Tabor and Richardson (1987). Denatured double-stranded DNA was sequenced directly (Toneguzzo et al., 1988) on 6% acrylamide gels containing 7.7 M urea using oligonucleotide primers synthesized as needed (Oligos Etc., Wilsonville, OR). The termini of sequencing gels were aligned using the computer program DNASTAR (DNASTAR, Inc., Madison, WI). Homology searches and amino acid sequence alignments were performed using the programs FASTA (Pearson and Lipman, 1988), BLAST (Altschul et al., 1990), and Genalign (Martin, 1988).

**Isolation of the REC1 Gene**—A 1569-base pair sequence including the first ATG and extending to 30 nucleotides past the TAA termination codon of the REC1 gene was amplified by PCR. The sequence amplified was contained within the cloned 2.7-kbp Smal-HindIII fragment from pCM224. The two primers used in the amplification reaction were each designed to contain a NdeI site (underlined below) distal to the sequence overlapping either the 5'- or 3'-region of the REC1 ORF. Primer 1 was 5'-GGATCATTATGCCGGAGGCAGCTT and was complementary to the first 19 nucleotide residues of the REC1 ORF noncoding strand. Primer 2 was 5'-GGATCATTATGCCGGAGGCTGACTGCGT and was complementary to nucleotides 1579-1596 of the coding strand. The reaction (100 μl) contained 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 50 mM KCl, 0.2 mM each dNTP, 1 μM each primer, 1 ng of pCM224, and 0.01% gelatin, was held at 95 °C for 5 min. Thermus aquaticus DNA polymerase (5 units, Perkin-Elmer Cetus) was added to begin the reaction and the temperature was then cycled 30 times from 72 °C (3 min) to 94 °C (1 min). Following incubation, the 1.6-kbp product was isolated after electrophoresis through a 1% agarose gel. Its termini were made flush by repair with the Klenow fragment of E. coli DNA polymerase I and then phosphorylated with T4 polynucleotide kinase and ATP. The linear product was then inserted into the EcoRV site of pBluescriptII to yield pCM251. The DNA sequence of the amplified REC1 ORF was determined and was found to be identical to that of the REC1 ORF in the genomic clone pCM224.

**Complementation of DNA Repair Deficiency**—Transformed U. maydis recl strains were tested after treatment with 4-nitroquinoline-1-oxide (4-NQO) or 4-nitroquinoline-2-oxide (4-NNO) at 1 μg/ml, and further incubated with shaking at room temperature. Aliquots (0.1 ml) were removed at timed intervals, washed twice in water, appropriately diluted and plated on selective medium. Survivors were counted as colonies visible after incubation for 3 days at 32 °C.

**Overexpression of the REC1 Gene**—In E. coli—E. coli BL21(DE3) [pBADgal/ Int / lacUV5/ Tet/ genel imm15 nin5 Sam7] was transformed with plasmid pCM317 containing plasmid was induced for expression with isopropyl-β-D-thiogalactoside (IPTG) as described by Studier et al. (1990). A cell culture (from 5 to 1,000 ml) of BL21(DE3)/pCM322 was grown at 37 °C to A600 of 0.6 and induced by addition of 1 mM IPTG. After 3 h, cells were harvested by centrifugation in a 2-liter graduated centrifuge beaker, were washed with 0.2 N HCl (0.5 M NaCl, 1 mM EDTA) and stored at −70 °C. After thawing, cells were resuspended in 0.05 volume of TNE and lysozyme was added to 1.0 mg/ml. The suspension was incubated on ice for 1 h. EDTA was then added to 10 mM and Triton X-100 to 0.1% and the reaction was held at 37 °C until a clear viscous reaction suspension was sonicated with 3×30-s bursts using an immersion probe (Branson Sonifier), then centrifuged for 15 min at 20,000 × g. Afterwards, all of the REC1 product was contained in the precipitate, as demonstrated by protein staining on SDS-polyacrylamide gels (see for example, Fig. 2, lanes 2 and 3). The supernatant was therefore discarded, and the precipitate was re-suspended and centrifuged again (see above), containing 0.2 pmol of either 32P-labeled as terminal label at a specific activity of 2×106 cpm/μmol or 1 pmol of [35S]labeled as terminal label at a specific activity of 2×108 cpm/μmol. Reaction mixtures were incubated at 37 °C for 10 min in 4×104 cpm/μmol. Reactions were started by the addition of 1–2 μl of enzyme, incubated at 37 °C for 10 min.
min (or the time indicated), and terminated by the addition of ice-cold solutions of carrier (300 μl, 0.5 mg/ml DNA (sonicated salmon sperm DNA), 25 mM EDTA) and 10% trichloroacetic acid (500 μl). The mixture was held on ice for 10 min, centrifuged at 10,000 × g for 10 min, and the supernatant was removed and mixed with 5 volumes of scintillant (Ecolume, ICN Biomedicals Inc.) for determination of radioactivity. Each experiment included at least three protein concentrations to determine the linear relationship between activity and protein. One unit of exonuclease activity is that amount of enzyme that converts 1 pmol of radiolabeled DNA to an acid-soluble form in 10 min under these conditions. Activity in protein fractions containing guanidine HCl were assayed by adding a 1-μl sample directly to the standard assay; under these conditions the RECl protein is soluble to at least 10 μg/ml, determined by measuring the radioactivity in protein labeled in vitro during overexpression (data not shown). Comparisons of purified E. coli exonucleases were performed using DNA polymerase I Klenow fragment (New England Biolabs, Inc.), exonuclease III (New England Biolabs, Inc.), exonuclease I (U. S. Biochemical Corp.), and 3′-exonuclease (Life Technologies Inc.). The e-subunit of DNA polymerase III holoenzyme (DNAQ gene product) was isolated from the overproducing E. coli strain MC1000 transformed with pRK248-tls and pNS380 (provided by M. O'Donnell of this Department) as described by Scheuermann and Echols (1984).

**Isolation of the HsRECl Protein**—E. coli BL21(DE3)pCM391 cells (from 1-liter cultures) were induced and lysed as described above, and the insoluble precipitate containing the HsRECl protein was prepared. The washed precipitate was dissolved in 20 ml of Buffer A (6 M guanidine HCl, 10 mM Tris-HCl, 0.1 mM sodium phosphate) at pH 8.0. The clear protein solution (Fraction 1) was centrifuged to remove any minor insoluble material, and loaded onto a column (1 × 5.5 cm) containing nitrocellulose acid-agarose (NTA) which had been charged with Ni²⁺ and equilibrated with Buffer A according to the vendor's protocol (Qiagen, Inc.). The Ni²⁺-NTA column was developed by gravity (10 ml/h) with a discontinuous pH gradient in Buffer A: first at pH 8.0 (50 ml), followed by pH 6.3 (25 ml), pH 5.9 (20 ml), and finally pH 4.5 (20 ml). Fractions of 1.5 ml were collected and assayed for exonuclease activity (see above) and fractions containing activity were combined (Fraction II). Guanidine HCl was removed by dialysis of Fraction II for 12-18 h against one change of 0.5 M NaCl in Buffer Z (50 mM Tris-HCl, pH 8.5, 20% glycerol, 5 mM dithiothreitol, 0.01% Triton X-100), and then against two changes of 6 M each against Buffer Z containing 10 mM NaCl. About 90% of the protein was precipitated below a concentration of 0.5 M NaCl, and this was removed from the dialysate by centrifugation. The supernatant was applied to a Mono Z column (Pharmacia LKB Biotechnology Inc.) and fractions containing activity were collected and assayed for exonuclease activity. A single peak of exonuclease activity emerged at a salt concentration of 0.2 M. In order to estimate the size of the HsRECl protein, a 100-μl sample from the peak Mono-Z fraction was loaded on a 30-ml Superose 12 HR10/30 (Pharmacia) and the column developed with 30 ml of Buffer Z containing 50 mM NaCl. Fractions (0.5 ml) were collected and assayed for exonuclease activity, and a single sharp peak of exonuclease activity was detected. Column fractions were analyzed for protein content with Coomassie Blue dye reagent (Bio-Rad), using the column buffer as blank. Samples containing guanidine HCl to be analyzed by SDS-gel electrophoresis were precipitated in 3 volumes of ethanol at -20 °C, a step necessary to remove guanidine HCl which precipitates SDS. The precipitates were washed with 75% ethanol before dissolving in SDS-sample buffer (1% SDS, 100 mM dithiothreitol, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.1% bromphenol blue).

**RESULTS**

The Functional Unit of the RECl Gene—In previous work from this laboratory a genomic DNA fragment that complemented the DNA repair and recombination phenotype of the recl mutant was isolated and established by one-step gene disruption to contain the RECl gene (Tsukuda et al., 1989). After subcloning and sequencing, we found that the product of the RECl gene should be a polypeptide of 522 amino acid residues with a molecular mass of 56,866 daltons. The ORF of 1569 nucleotides was amplified by polymerase chain reaction, its complete DNA sequence rechecked, and then inserted in a U. maydis expression vector (Fig. 1A) to test for biological activity. Following 4-nitroquinoline-1-oxide treatment of a recl strain transformed with this plasmid (Fig. 1B), the DNA repair deficieny was restored to the wild-type level providing independent proof that the isolated ORF contains the structural portion of the RECl gene.

**Identification of an Exonucleolytic Activity in the RECl Gene Product**—The RECl ORF was inserted behind the phoA beta-galactosidase T7 promoter in the pET vector system designed for overexpression of cloned genes in E. coli (Studier et al., 1990). After induction with IPTG, a protein with the predicted mass of the RECl gene product accumulated in the cells, as determined by SDS-gel electrophoresis (Fig. 2, lanes 1 and 2). The 57-kDa protein was specifically labeled when [35S]methionine and rifampicin were added to the induced culture, as would be expected for a gene expressed under the control of a T7 promoter. The overexpressed RECl protein was insoluble even when cell extracts were prepared in the presence of 6 M urea. Solubilization was achieved only by addition of 4 M guanidine HCl, 1% SDS, or 1% Sarkosyl. Because of the insoluble nature of the overproduced protein, most bacterial proteins in extracts were easily removed when the precipitate was washed in buffer containing urea (Fig. 2, lanes 3 and 4). The insoluble RECl gene product remaining after extensive washing was dissolved in concentrated guanidine HCl and then

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**Fig. 1. Complementation of recl-1 with a chimeric RECl transcription unit.** A, the expression vector pCM317 is a 5.1-kbp plasmid with a fragment of the hsp70 promoter (phsp70) for expression of genes in U. maydis. The unique NdeI site serves as the cloning site for translational initiation. The plasmid contains a hygromycin resistance cassette (HPH), an ARS for selection and replication in U. maydis, and bla and ori for E. coli. B, U. maydis strains UCM21 (recl-1) and UCM3 (RECl) were transformed to hygromycin resistance with pCM306 which contains the PCR-amplified RECl ORF cloned into the NdeI site of the expression vector pCM317. After growth in YEPS containing 100 µg/ml hygromycin, cultures were treated with 4-nitroquinoline-1-oxide and cell survival was measured. UCM21/pCM317 (circles); UCM21/pCM306 (diamonds); UCM3/pCM306 (triangles).
processed through a renaturation regime involving the slow removal of denaturant by dialysis. A potent nucleolytic activity which released radiolabeled nucleotide preferentially from the 3'-end of DNA was detected in the renatured preparation of RECl protein (Fig. 3). Over an extended time uniformly labeled DNA could be converted almost completely to an acid-soluble form. The conditions for optimal activity were a low ionic strength buffer at pH 8.5-9.0 containing Mg\(^{2+}\) (Table II). Activity was unaffected by ATP and dNTPs but was inhibited by dAMP. Salt at 100 mM reduced the activity nearly 80%. Little to no activity was observed when Mg\(^{2+}\) was replaced with Ca\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\), or Co\(^{2+}\). A reducing agent was necessary for maintaining enzymatic stability over time during storage at 4 or -20 °C.

Distinct Characteristics of the RECl-associated Exonuclease Activity—The activity observed in renatured preparations of RECl gene product was examined for properties that could distinguish it from the major nucleases of E. coli and λ-lysogens which might potentially contaminate the bacterial preparation. Based on the published properties of well characterized E. coli nucleases, exonuclease V and exonuclease VII were ruled out as possible sources of contamination, since the former is dependent upon ATP (Wright et al., 1971) as a cofactor and the latter has no divalent cation requirement for activity (Chase and Richardson, 1974). We considered in more detail Mg\(^{2+}\)-requiring nucleases that are known to render DNA acid soluble. These include exonuclease I (Lehman and Nussbaum, 1964), the 3'→5' proofreading activity of DNA polymerase I (Brutlag and Kornberg, 1972), exonuclease III (Richardson et al., 1964), and endonuclease I (Lehman et al., 1962). We also considered λ-exonuclease (Carter and Radding, 1971), because the E. coli strain that we used for overexpression was a λ-lysogen, but this possibility seemed unlikely since the pL-promoted transcription of the red genes, which occurs under conditions of DNA damage induction, would not be expected with IPTG induction.

When assayed under the same conditions it was apparent that the exonuclease activity present after overexpression of RECl was different from several exonucleases. The RECl-associated exonuclease showed only a 5-fold preference for single-stranded over duplex DNA as substrate (Table III), unlike exo-

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**Fig. 2.** Analysis of protein fractions from bacterial overexpression preparations. Portions of each sample were analyzed by Coomassie Blue staining after SDS-polyacrylamide gel electrophoresis. Size standards are indicated in kilodaltons on the left of the photograph; the positions of the protein bands are also indicated. Lane 1, lysate (20 μl) of uninduced cells harboring expression plasmid containing RECl (pCM322); lane 2, lysate (20 μl) of induced cells containing pCM322; lane 3, soluble fraction from sample in lane 2 (20 μl); lane 4, washed precipitate fraction from lysate of lane 2, resuspended to original volume (20 μl); lane 5, lysate (4 μl) of induced cells harboring expression plasmid containing histidine-leader fused to RECl (pCM391); lane 6, washed precipitate fraction from lysate of lane 5, resuspended to original volume (1 μl); lane 7, Fraction II (20 μl) from Ni\(^{2+}\)-NTA affinity column.

**Fig. 3.** Exonucleolytic activity in the overexpressed and renatured RECl preparation. A, 100-ml culture of BL21(DE3)/pCM322 containing RECl was induced for expression and further processed as described to obtain the washed precipitate fraction. Protein (approximately 3.5 mg) was solubilized in 2 ml of 4 M guanidine HCl in Buffer Z, diluted to 1 M guanidine HCl with buffer, then slowly renatured by dialysis against Buffer Z. The final volume of the dialysate was 7.5 ml containing about 0.34 mg of protein, representing a final yield of 10%. Precipitated material was removed by centrifugation. Portions containing 90 ng each were incubated for activity in the standard reaction mixture with end-labeled linear duplex plasmid DNAs for the times indicated. Filled circles, 3'-32P-DNA substrate; open circles, 5'-32P-DNA substrate.

**Table II**

Characterization of nucleolytic activity in an overexpressed RECl preparation

The description of RECl sample preparation is given in the legend to Fig. 3.

| Condition* | Activity* | %max |
|------------|-----------|------|
| pH 9.0     | 100       |
| pH 7.0     | 53        |
| pH 5.0     | 2         |
| +EDTA      | <1        |
| -Mg\(^{2+}\) | >1       |
| Mg\(^{2+}\) | 0.1 mM    |
| Mg\(^{2+}\) | 1.0 mM    |
| Ca\(^{2+}\) | 1.0 mM    |
| Mn\(^{2+}\) | 5         |
| Zn\(^{2+}\) | 1.0 mM    |
| Co\(^{2+}\) | <1        |
| +NaCl      | >1        |
| 10 mM      | >1        |
| 100 mM     | >1        |
| +ATP       | 90        |
| +dATP      | 99        |
| +dAMP      | 49        |

*Standard incubation conditions were at 37 °C for 30 min in 25 mM Tris acetate, pH 9.0, and 10 mM Mg\(^{2+}\). Listed in the table are individual modifications to these conditions (e.g., replacement of Mg\(^{2+}\) with the other divalent cations) or test conditions which were added to the standard mixture (noted as *+*).

*Activity was measured in each reaction mixture using 0.9 μg of total protein from the RECl sample and 1 nmol (as total DNA nucleotide) heat denatured, uniformly labeled [PH]P22 DNA. For this experiment, the maximal activity was 100 pmol of nucleotide released in 30 min at 37 °C.
nuclease I of *E. coli* (Lehman and Nussbaum, 1964). The activity also differed from the 3'-5' exonuclease activities of exonuclease III and DNA polymerase I in its relaxed specificity for the type of diester-linkage. These two bacterial enzymes are reduced more than 100-fold in activity when the linkage of the substrate DNA to the 3'-terminal nucleotide was through a 3'-phosphorothioate ester linkage instead of the standard 3'-phosphodiester linkage (Putney et al., 1981; Gupta et al., 1984).

Under the conditions tested, the exonuclease in the *E. coli* preparation exhibited a 60-fold preference for 3'-labeled substrate, unlike α-exonuclease (Carter and Radding, 1971), which preferentially degrades DNA in the 5'→3' direction (see also Fig. 3). The level of exonuclease activity was not reduced when the *REC1* gene product was overexpressed and prepared similarly from an *endA* strain of *E. coli* (see Table IV), indicating the activity is distinct from *E. coli* endonuclease I.

**Comparison of Exonuclease Activity in Overexpressed Proteins**—Further evidence that overexpression of the *REC1* gene was responsible for the 3'-5' exonuclease activity was obtained by a controlled comparison of several different protein extracts. In addition to the renatured preparation of *REC1*, samples containing the overexpressed products of *HisREC1* (with a hexa-histidine-leader, described below), and the α-subunit of *E. coli* DNA polymerase III were prepared in parallel and tested for activity using the 3'-thiophosphoryl substrate. Exonuclease activity was observed in *REC1*, *HisREC1*, and α preparations but was at the limit of detection in the control samples which were not expected to overproduce a protein (Table IV). The activity measured in the *HisREC1* preparation was lower than that of *REC1*, but the characteristics of the activity were nearly identical. The α-subunit of DNA polymerase III holoenzyme remains the notable *E. coli* exonuclease with properties in line with the observations here. It has the same polarity and preference for single-stranded substrate (Scheuermann and Echols, 1984; Brenowitz et al., 1991). Furthermore, α is also active on the thiophosphoryl end-labeled DNA (Griep et al., 1990; Table IV). In direct comparison with the *REC1* sample, the highest yield of activity after overexpression was obtained from the preparation of α. This protein was also insoluble after overexpression (Scheuermann and Echols, 1984), but following the denaturation/renaturation regime much more of the product was soluble than the products of *REC1* and its derivatives. However, the specific activity of the *REC1* sample was 2-fold higher than that of α in these preparations.

Another difference was found with respect to cofactor requirements: Mn²⁺ stimulates the activity of overexpressed α by as much or slightly more than Mg²⁺ (Thompson, 1992) in contrast to that observed with the *REC1*-associated exonuclease (see Table II). Furthermore, although all four dNMPs inhibit the activity of α, dAMP in particular appears to have much less of an effect on α than it does on the activity in the *REC1* preparation (Scheuermann and Echols, 1984; Thompson, 1992; Table II).

The comparison of the overexpressed proteins as well as the purified nucleases with the *REC1* sample thus provide several independent lines of evidence which strongly point to the conclusion that the exonuclease activity we observe is inherent in the *REC1* gene product.

**Chromatographic Isolation and Size Estimation of the 3'-5' Exonuclease**—By visual inspection of stained SDS gels it was estimated that greater than 10 times if not more of the overexpressed protein was observed when the *REC1* ORF was preceded by a 60-base pair leader sequence containing 6 consecutive histidine codons provided by the pET14b vector (Fig. 2, lanes 2 and 5). The reason for the increase in expression of the fusion protein is not understood. It is possible that there was stimulation by the artificial leader sequence at the level of transcription or translation, or else that this construct was more stably maintained in the cells; nevertheless, the insolvency of the protein was apparently unaffected. Due to the utility of the histidine-leader sequence in affinity chromatography, the resulting 50-kDa *HisREC1* product was considered more amenable for biochemical studies than the *REC1* product.

The insoluble fusion protein was isolated from bacteria and processed exactly as in the *REC1* preparation (Fig. 2, lanes 5 and 6). After solubilization with guanidine HCl, the *HisREC1* protein was isolated using immobilized metal ion affinity chromatography (reviewed by Forath (1992)). The denatured protein (Fraction I) bound stably to a Ni²⁺-NTA column and contaminating proteins which lacked the histidine-leader sequence were removed by washing sequentially with buffers at pH 8.0 and 6.3 (data not shown). A broad peak of exonuclease activity coinciding with the peak of *HisREC1* protein eluted with buffer at pH 5.9, giving rise to Fraction II (Fig. 4A and Fig. 2, lane 7). At pH 5.9, the complex made with the immobilized Ni²⁺ through two or more consecutive histidines on the protein is disrupted. The total exonuclease activity measured in Fraction II was significantly higher (usually 10-fold) than that in the sample loaded, and consisted of about 10% of the total
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Fig. 4. Chromatographic isolation of exonuclease activity from the HisREC1 preparation. A, purification on a Ni²⁺-NTA affinity column. HisREC1 protein was bound to the column in 6 M guanidine HCl at pH 8, washed further at pH 6.3, and then eluted at pH 5.9 as described (loading and wash steps are not shown). Exonuclease was assayed from 1 μl of each 1.5-ml fraction. The activity recovered in Fraction II was 50-fold over that in the sample loaded. Circles, activity; dots, protein. B, recovery of renatured exonuclease. Protein in Fraction II was renatured by dialysis and loaded onto a Mono-S cation exchange column. The exonuclease was isolated by fast protein liquid chromatography on a Superose-12 column (Fig. 4B). This value was estimated at 60 kDa which is in agreement with the molecular mass calculated from SDS gel analysis of the HisREC1 protein, and was clearly resolved from the 28-kDa exonuclease III of E. coli. The size estimation indicates that the active species of HisREC1 is monomeric under the chromatographic conditions.

**DISCUSSION**

The principal conclusion from biochemical experiments with the REC1 gene product isolated from E. coli after overexpression is that an exonuclease activity is inherent in the REC1 protein. Evidence supporting this conclusion comes from sev-
eral independent lines of investigation. 1) A potent 3'→ 5' exo-
nuclease activity with novel properties appears in preparations of the REC1 gene product, but is not found in extracts from bacterial cells where the REC1 gene has not been expressed. 2) The exonuclease activity was isolated after three column chromato-
graphy steps: in the first of these steps the activity was bound to an immobilized Ni²⁺ column under denaturing condi-
tions, and released at the pH predicted to disrupt this specific and high affinity complex with the histidine-leader on the fusion protein HisREC1. After a second column step the size of the exonuclease was estimated to be 60,000 daltons, corre-
sponding to the molecular mass calculated from the deduced amino acid sequence, and to the molecular mass of the over-
expressed fusion gene product as determined by SDS-gel electro-
phoresis. 3) The activity of the REC1 preparation on duplex DNA, the ability to hydrolyze a phosphorothioate 5' end, the lack of ATP requirement, the requirement for Mg²⁺, the polarity of DNA degradation, and the presence of the nuclease activity in an endA strain are all observations that place this activity in a class apart from E. coli exonuclease I, exonuclease III, exo-
nuclease V, exonuclease VII, λ-exonuclease, DNA polymerase I, and endonuclease I which are abundant enzymes and the most likely sources of contamination by cellular nucleases. We cannot completely rule out contamination by the €-subunit of DNA polymerase III holoenzyme. However, we think this is unlikely since it is present at only a few copies per cell. 4) The amino acid sequence alignments of the REC1 gene product with those of E. coli DNA polymerase I and ε, both in the conserved "Exo-
domain" landmark amino acids and in the order and spacing of the three segments constituting these motifs (Fig. 5), are consistent with the alignments of many DNA polymerases with real or predicted exonuclease proofreading activity. The simplest and most straightforward interpretation of these various observations is that the novel 3'→ 5' exonuclease activity appearing in cells overexpressing the REC1 gene is an intrinsic feature of the REC1 protein.

The exonuclease activity identified in the REC1 gene product provides a mechanistic framework for beginning to rationalize the complex phenotype of the rec1 mutant, which is characterized by radiation sensitivity, lethal sectoring, elevation in spontaneous mutation frequency, aberrant meiosis, elevated spontaneous allelic recombination, and an altered form of crossing over associated with a high rate of chromatid breakage and loss (Holliday et al., 1976). For instance, sensitivity to radiation could result from failure of a defective exonuclease to function in repair of DNA lesions. The mutator phenotype of the rec1 mutant could arise as a consequence of a dysfunctional exo-
nuclease, as postulated for some mutants in dnaQ (Echols et al., 1983; Takano et al., 1986; Schaaper, 1989). Other aspects of the phenotype such as lethal sectoring, immutability by ultraviolet irradiation, and crossing over associated chromatid breakage are less easy to rationalize in terms of loss of an exonuclease function and could be indicative of some intricate role of REC1 in controlling or interacting with other genes as postulated by Holliday (e.g., Holliday et al., 1976; Holliday, 1977). More biochemical and molecular genetic studies will be necessary to gain a better understanding of the in vivo function of the REC1 gene product. It would come as no surprise to find interplay between the REC1 gene product and other components important in error-prone DNA repair.

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Fig. 5. Alignment of REC1 with the Exo motifs of E. coli DNA polymerase I (polA) and the €-subunit of DNA polymerase III (dnaQ).
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