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Identification of the Gene for the Yeast Ribonucleotide Reductase Small Subunit and Its Inducibility by Methyl Methanesulfonate

HOLLY K. HURD, CHRISTINE W. ROBERTS, AND JEFFREY W. ROBERTS*

Section of Biochemistry, Molecular & Cell Biology, Cornell University, Ithaca, New York 14853

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We have identified, cloned, and sequenced the gene for the small subunit of ribonucleoside diphosphate reductase of *Saccharomyces cerevisiae*. The protein and its transcript are induced about 10-fold by the alkylating agent methyl methanesulfonate, a result which suggests that the gene is induced by DNA damage.

The highly conserved and universal enzyme ribonucleotide reductase catalyzes the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, precursors for DNA synthesis. Along with S. Elledge and R. W. Davis (2a), we have cloned and sequenced the gene *RNR2* for the small subunit of ribonucleotide reductase from *Saccharomyces cerevisiae*. As recounted by Elledge and Davis (2a), the identification of *RNR2* was an accident resulting from cross-reaction between the small subunit of reductase and antibody to *Escherichia coli* RecA protein. Because the regulation of this gene may be related in an interesting way to DNA damage or DNA replication, and because availability of the clone should promote further study of both the protein and the gene, we report here (i) the characteristics of the clone, (ii) a procedure for purification of the small subunit, and (iii) features of the regulation of the gene. In particular, we show that *RNR2* is induced at the transcriptional level by an agent that damages DNA, a property that *RNR2* shares with genes for the ribonucleotide reductase subunits of *E. coli* (3). Furthermore, we show that the amino-terminal sequence of the purified protein is that predicted by the DNA sequence of *RNR2*.

MATERIALS AND METHODS

Strains, growth, and transformation. *S. cerevisiae* TD4 (*MATa his4-519 ura3-Δ52 leu2-3,112 trp1-289*) was obtained from B.-K. Tye. The YEp13 plasmid library consists of *Sau*3A partial digests (average size, 10 kilobases [kb]) of genomic DNA from *S. cerevisiae* AB320 (*HO ade2-1 lys2-1 trp5-2 leu2-1 can1-100 ura3-1* and/or *ura1-1 met4-1*) cloned into the *Bam*HI site of the 2-μm plasmid YEp13; it was obtained from K. Nasmyth (7) via B.-K. Tye. The λgt11 yeast expression library (19) was obtained from Clontech Laboratories, Inc., (Palo Alto, Calif.); it contains randomly sheared genomic DNA (average size, 3 kb) from *S. cerevisiae* X2180, ended with *Eco*RI linkers, and fused near the carboxy-terminal end of the *E. coli lacZ* gene.

E. coli HH49 [*ΔlacU169 proA*$^+$ *lon araD139 rpsL supF* Δ(*srlR-recA*)*306*::Tn*10* (pMC9)] was constructed by P1 transduction (6) of the Δ(*srlR-recA*)*306*::Tn*10* locus of strain JC10289 [F$^-$ Δ(*srlR-recA*)*306*::Tn*10 thr-1 leuB6 thi-1 lacY-1 galK2 ara14 xgl-5 mtl-4 proA2 his4 argE2 rpsL31 tsk-33 supE44*] obtained from A. J. Clark, into the standard λgt11 strain Y1090 (18) obtained from R. A. Young and R. W. Davis via K. Holzer. Y1090 was first cured of plasmid pMC9 and transduced to *trpC*$^+$ to remove its resident Tn*10*. The *lacI* plasmid pMC9 was then replaced by transformation.

Yeast strains were cultured in yeast extract-peptone-glucose (YEPD)-rich medium or Bacto-yeast nitrogen base-glucose (SD) minimal medium supplemented with appropriate amino acids and were transformed by using the glusulase method (12). *E. coli* transformation was done as described previously (5).

Enzymes and chemicals. Enzymes were purchased from New England BioLabs, Inc. (Beverly, Mass.), Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Methyl methanesulfonate (MMS) was obtained from Eastman Kodak Co. (Rochester, N.Y.).

DNA sequencing. Restriction fragments were cloned into M13 and sequenced by the dideoxy chain termination method of Sanger et al. (10).

Nucleic acid preparation. Plasmid DNA was prepared by the alkaline extraction method of Birnboim and Doly (2). Total yeast DNA was prepared as described by Sherman et al. (12). Total yeast RNA was isolated as follows. Strain TD4 was grown in YEPD medium, harvested by centrifugation, and washed with ice-cold glass-distilled water containing 0.2% diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Mo.). The pelleted cells were then suspended in 1/20 volume RNA extraction buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris hydrochloride [pH 7.6], 5% [wt/vol] sodium dodecyl sulfate, 0.2% diethyl pyrocarbonate). An equal volume of phenol-chloroform (1:1 [vol/vol]) and a half volume of acid-washed 0.45-mm-diameter glass beads (VWR Scientific, Piscataway, N.J.) were added. The cells were lysed by repeated high-speed vortexing for 30 s, followed by 30-s intervals on ice. The lysate was extracted with phenol-chloroform (1:1 [vol/vol]), and nucleic acid was precipitated from the aqueous phase by the addition of 0.3 M sodium acetate, pH 7.0, and 2 volumes of 95% ethanol, followed by storage at −70°C for several hours. The pellet was suspended in water containing 0.2% diethyl pyrocarbonate to a concentration of 1 to 5 mg of RNA per ml.

Nucleic acid hybridization. Southern blot hybridization was done as previously described (5). For RNA blotting, RNA was electrophoresed through 1.2% formaldehyde gels in morpholinepropanesulfonic acid buffer, pH 7.0, and transferred to Gene Screen Plus (New England Nuclear Corp., Boston, Mass.) as recommended by the supplier. Hybridization probes were prepared by nick translation (5) with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.).

* Corresponding author.

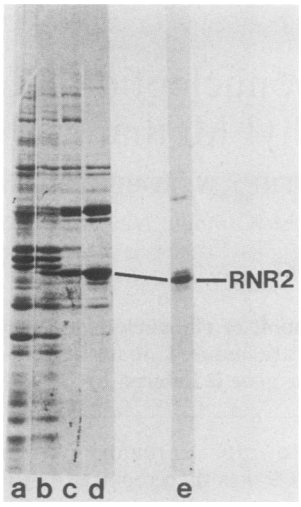

FIG. 1. Purification of yeast ribonucleotide reductase small subunit. Samples were resolved on 10% polyacrylamide gels in sodium dodecyl sulfate and visualized by staining with Coomassie blue. Lanes: a, crude extract from 0.2 mg of cells containing YEp13; b, crude extract from 0.2 mg of cells containing plasmid YEp13*rnr*, grown for 8 h in 0.01% MMS; c, 5-μg ammonium sulfate fraction; d, 2.2-μg heptyl-agarose peak; e, 1.8-μg DEAE fraction.

Immunoblotting. Yeast extracts were prepared by lysis of cells in 1.8 M NaOH–7.4% β-mercaptoethanol, followed by trichloroacetic acid precipitation and washing in cold acetone. Extracts were electrophoresed through 8 to 10% polyacrylamide gels (acrylamide-bisacrylamide, 30:1) and transferred electrophoretically to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) (15). The nitrocellulose was blocked for 30 min with 3% gelatin in Tris-buffered saline (50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl) and incubated for 3 h with rabbit anti-*E. coli* RecA protein antiserum. Cross-reactive proteins were detected by incubation for 1 h with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad Laboratories, Richmond, Calif.), followed by two 15-min washes in Tris-buffered saline and color development with 4-chloro-1-naphthol as recommended by the supplier (Bio-Rad).

Carboxypeptidase digestion. Purified reductase small subunit (400 to 500 ng) was digested with 5.1 μg (0.12 U/ml) of carboxypeptidase A (Sigma) per ml in 50 mM NaCl–10 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$–1 mM dithiothreitol (DTT) for 10 min at 37°C (16), after which the reaction was stopped by precipitation of the proteins in 15% trichloroacetic acid.

Purification of RNR2 small subunit. Since the polypeptide is visible in Coomassie blue-stained gels of crude extracts of cells carrying the YEp13*rnr* plasmid, purification was followed by gel electrophoresis. *S. cerevisiae* TD4 transformed with YEp13*rnr* was grown in SD minimal medium to an optical density at 550 nm of 1, about 10^7 cells per ml. MMS was added to 0.01%, and the culture was enriched with 1/10 volume 10× YEPD medium. After 8 h of additional growth, cells were harvested and washed with water; sometimes they were frozen at −20°C before use. Two to four volumes of cracking buffer (0.2 M NaCl, 100 mM Tris chloride [pH 7.5], 1 mM DTT, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol) were added, and the cells were lysed by being ground for 3 min in a Bead Beater (Biospec Products, Bartlesville, Okla.), keeping the mixture ice-cold, as recommended by the manufacturer. All further procedures were done at 0 to 4°C. The mixture of cells, beads, and buffer was allowed to settle, and the supernatant was decanted; the beads were extracted with half the original volume of buffer. The combined supernatants were clarified by low-speed centrifugation, made 0.8% in Polymin P (Miles Laboratories, Inc., Elkhart, Ind.), mixed 15 min in the cold, and centrifuged for 20 min at 9,000 × *g*, after which the RNR2 protein was in the supernatant. The supernatant was mixed with 38.5 g of enzyme grade ammonium sulfate (Schwarz/Mann, Orangeburg, N.Y.) per 100 ml, and after 2 h, the precipitate was collected by centrifugation for 30 min at 9,000 rpm. The pellet was washed two to four times, until the supernatant was clear, with an ammonium sulfate solution made up to the original concentration in cracking buffer (ammonium sulfate fraction). The pellet was suspended in 20 mM sodium phosphate (pH 7.5)–10% glycerol–1 mM DTT–0.2 M NaCl–1 mM EDTA (phosphate buffer), and ammonium sulfate was added until the conductivity of the solution was the same as that of 1.5 M ammonium sulfate. Protein was adsorbed to a column of heptyl-agarose (15 mg of protein per ml of resin) and eluted by a 16-volume linear

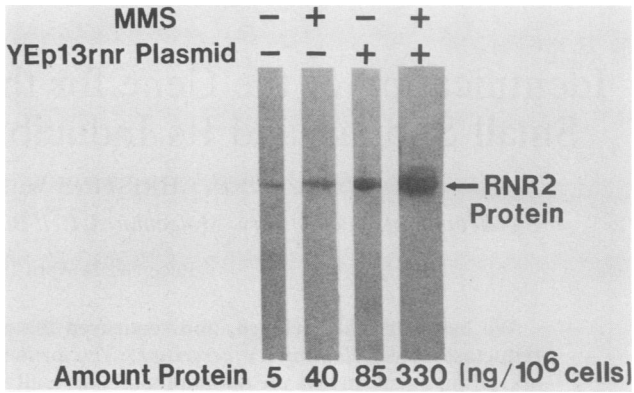

FIG. 2. Overproduction of the RNR2 protein and its induction by MMS. Crude extracts of cells carrying or lacking the YEp13*rnr* plasmid and cells grown in the presence or absence of MMS were immunoblotted with RecA antibody to identify the RNR2 protein.

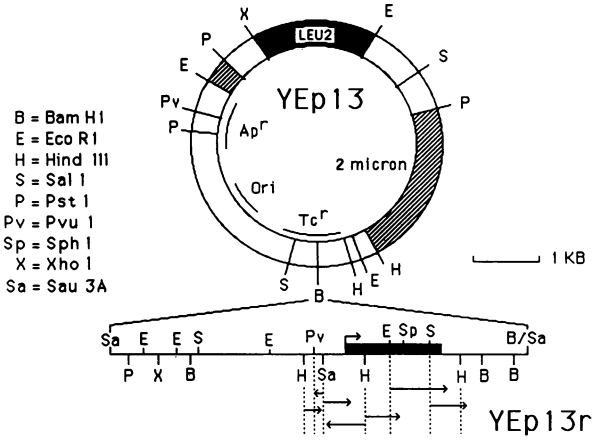

FIG. 3. Restriction map of the plasmid containing the *RNR2* gene, and the sequencing strategy. Arrows show the direction and length of sequence determined by the M13 dideoxy chain termination method. Arrows pointing leftward represent sequence of the sense strand, and rightward-pointing arrows represent sequence of the antisense strand. The black bar shows the single open reading frame; the direction of transcription is indicated by an arrow.

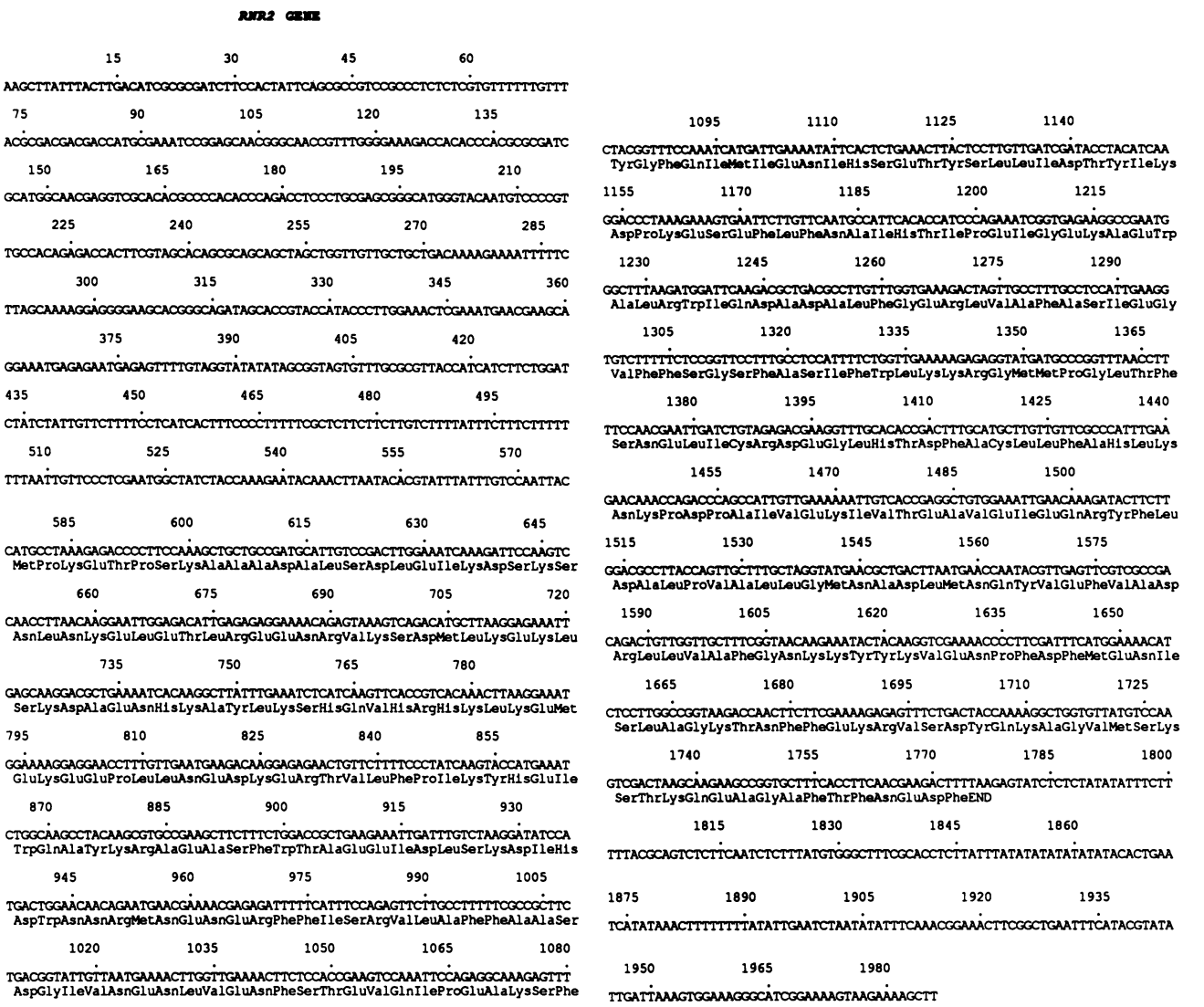

FIG. 4. DNA sequence of the *RNR2* gene. There is a single difference (C for T at position 751) between the sequence of *RNR2* of strain AB320 determined here and its sequence in the unidentified strain used by Elledge and Davis (2a). Both codons specify tyrosine, suggesting a strain-specific variation.

gradient from 1.5 M ammonium sulfate to 0.1 M ammonium sulfate in phosphate buffer. Fractions containing RNR2 protein were pooled and dialyzed into a buffer containing 0.05 M NaCl, 50 mM Tris chloride (pH 7.5), 1 mM EDTA, 1 mM DTT, and 10% glycerol (D buffer). The dialysate was clarified by low-speed centrifugation and loaded onto a DEAE-A50 column; protein was eluted with a 10-volume linear gradient from 0.1 to 0.45 M NaCl in D buffer. Fractions containing RNR2 protein (DEAE fraction) were concentrated by dialysis against 20% polyethylene glycol (J. T. Baker Chemicals Co., Phillipsburg, N.J.) in 0.1 M NaCl–50 mM Tris chloride (pH 7.5)–1 mM EDTA–1 mM DTT–10% glycerol. From gel electrophoresis, we estimate the final purity to be about 80% (Fig. 1). We obtained about 5 mg of RNR2 protein from 50 g of yeast. A sample of this material was given to the Cornell Biotechnology sequencing facility for determination of 13 amino-terminal amino acids. Because the multimeric reductase enzyme contains polypeptides encoded by two separate genes, we have not attempted to determine whether the isolated small polypeptide is active.

RESULTS AND DISCUSSION

Cloning the *RNR2* gene. The *RNR2* gene was isolated by screening a genomic λgt11 expression library for antigens cross-reactive with anti-*E. coli* RecA antibody (18). To enhance specific reaction with a phage-encoded fusion protein, the *E. coli* strain used for plating the bacteriophage contained a deletion in the *recA* gene, and the polyclonal antiserum was preadsorbed with proteins from an *E. coli recA* deletion strain coupled to Sepharose. Figure 2 displays the cross-reactive proteins in a yeast crude extract immunoblotted with purified anti-RecA antibody. There is one major protein of about 43 kilodaltons that cross-reacts with two independently generated anti-RecA antisera, as well as to antisera affinity purified on a RecA column.

Four phage that encoded cross-reactive protein and contained yeast DNA inserts of different sizes were isolated

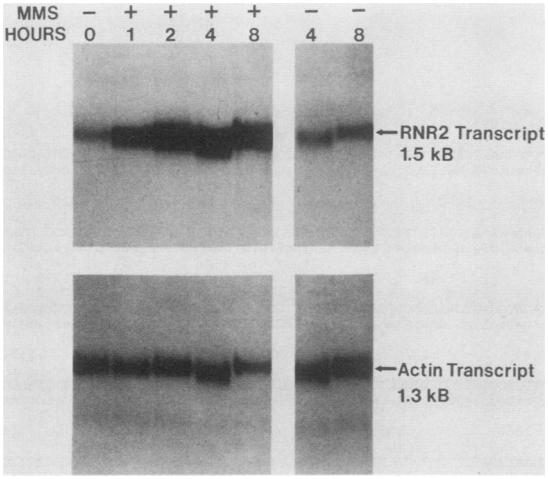

FIG. 5. Induction of the *RNR2* transcript by MMS. RNA was prepared from log-phase cells grown in the presence or absence of MMS for the indicated times and blotted to nitrocellulose. The upper panels show the 1.5-kb transcript that hybridizes to a RNR2-specific probe, and the lower panels show the same filter probed with pYActI, a plasmid containing the single yeast actin gene (8). Comparison of the intensity of the *RNR2* transcript to the actin transcript indicates the relative induction of the *RNR2* mRNA. The size of the *RNR2* transcript was determined by measuring its mobility relative to 26S rRNA (3.4 kb), 18S rRNA (1.5 kb), and actin mRNA (1.3 kb).

from 10^6 plaques screened. Two DNA inserts overlapped and had portions of the gene we infer to be *RNR2*; the other two contained unrelated DNA. One phage containing *RNR2* DNA encoded a β-galactosidase-RNR2 fusion protein, whereas the other did not; presumably the second phage expressed RNR2 protein, or some portion of it, independently of *lacZ* (13). The yeast DNA insert from the clone that made the fusion protein was nick translated and used as a probe to screen the YEp13 yeast genomic plasmid library in order to isolate the entire gene by colony hybridization. When a YEp13 clone homologous to the insert (Fig. 3) was transformed into yeast strain TD4, the cross-reactive yeast protein was overproduced 15- to 20-fold (Fig. 2), typical for yeast genes carried on 2-μm plasmids (9). Southern blot analysis suggested that the *RNR2* gene is present in single copy (data not shown).

Sequence and identification of the *RNR2* gene. Yeast DNA sequences encoding portions of the cross-reactive protein were identified in the phage encoding the largest fusion protein, and these, in turn, were localized in the 6.8-kilobase-pair *Bam*HI fragment of YEP13*rnr* by restriction mapping and Southern blot analysis. DNA sequence analysis of this region of YEP13*rnr* by the strategy shown in Fig. 3 revealed a single open reading frame of 1,197 nucleotides, sufficient to encode a polypeptide of 399 amino acids with a predicted molecular weight of 45,968 (Fig. 4). Elledge and Davis (2a) also obtained this sequence with one difference (mentioned in the legend to Fig. 4), and they identified it as the small subunit of ribonucleotide reductase on the basis of protein sequence homology to ribonucleotide reductase from other organisms. We note that the nucleotide sequences surrounding the ATG translational start are characteristic of yeast genes; an interval of 15 nucleotides immediately upstream of the ATG start has few G residues and contains the sequence CAA, as well as an invariant A at −3 relative to ATG and a prevalent T at +6 (11).

Induction of the RNR2 protein and transcript by MMS. We found that the DNA-damaging agent and mutagen MMS strongly induces expression of both the chromosomal and the plasmid-borne *RNR2* gene. *RNR2* thus shares with ribonucleotide reductase of *E. coli* (3) the ability to be induced by agents that damage cellular DNA, such as UV light and nitroquinoline oxide (1, 2a). For the experiment shown in Fig. 2, strains TD4 and TD4(YEp13*rnr*) were grown in the presence or absence of 0.01% MMS, and cellular proteins were analyzed by immunoblotting with anti-RecA antibody as described in Materials and Methods. RNR2 protein was measured by densitometric scanning of the color produced by the enzyme-linked immunoassay, by using purified protein (see below) as a standard. After 8 h, when maximum induction occurred (data not shown), MMS provided an eightfold increase in RNR2 protein in cells lacking the plasmid. The plasmid alone gave a 17-fold increase, and inducing the transformed cell gave more than 60 times the basal level of RNR2 protein. By comparison with the standard, we calculate that the basal level is about 60,000 molecules per cell.

We identified a putative mRNA for the *RNR2* gene by probing total blotted yeast RNA with the 1.7-kb *Eco*RI fragment containing about half of the *RNR2* gene. The probe annealed to a single RNA transcript of approximately 1.5 kb, sufficiently large to encode the protein. Like the RNR2 protein, this RNA was induced by MMS treatment (Fig. 5). By densitometric scanning of the autoradiogram, we determined that the RNA was induced 17-fold after 8 h of growth with MMS. Given the uncertainties of both measurements, this is probably not significantly different from the eightfold induction of the protein.

Purification and NH_2-terminal amino acid analysis of the RNR2 protein. RNR2 protein overproduced by MMS induction of the transformed cell is visible by Coomassie blue staining of total cellular proteins resolved on sodium dodecyl sulfate gel (Fig. 1). We purified it as described in Materials and Methods and obtained sufficient purity (about 80%) to match its amino-terminal amino acid sequence with the DNA sequence. The following amino acids were obtained in 13 cycles of the sequenator: 1, (Ser, Pro, Gly); 2, Lys, (Gly, Ser); 3, (Thr, Glu); 4, (Glu, Lys); 5, Pro; 6, Ser; 7, Lys, (Ser,

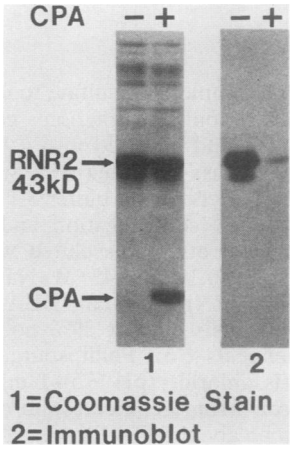

FIG. 6. Identification of the carboxy-terminal epitope of RNR2 protein. Panels: 1, a Coomassie blue-stained polyacrylamide gel of purified RNR2 protein treated identically; 2, an immunoblot of purified RNR2 protein treated (+) or not treated (−) with carboxypeptidase A (CPA).

Asn); 8, Ala, (Lys); 9, Ala; 10, Ala, (Glu); 11, Asp, (Ala); 12, Ala, (Asp); 13, Leu. Despite several uncertainties, presumably caused by the contaminants in the preparation, it is clear that the 43-kilodalton protein is encoded by the cloned sequence and is therefore the small subunit of yeast ribonucleotide reductase. Furthermore, this determination shows that the indicated translation initiation site is correct.

Basis of the immunological cross-reaction. Elledge and Davis (2a) noted that the amino acid sequences of RNR2 and *E. coli* RecA protein have significant homology only at the carboxy terminus of each protein, implying that the presence of these four amino acids at the carboxy terminus suffices to form a strong epitope for RecA antibody. This conclusion is consistent with an earlier finding that these amino acids also are present at the carboxy-terminal end of α-tubulin of yeast and that a monoclonal antibody raised to α-tubulin binds RecA protein as well as the RNR2 protein from sea urchins and clams (4, 14, 17). To provide further evidence that the carboxy terminus is the reactive part of RNR2 protein, we performed limited proteolysis of the purified ribonucleotide reductase protein with carboxypeptidase A to remove amino acids from the carboxy terminus. Figure 6 shows that this treatment eliminated the cross-reactivity of RNR2 with anti-RecA antiserum; polyacrylamide gel analysis showed that the bulk of the ribonucleotide reductase small subunit remained intact, indicating that proteolysis, and thus antibody cross-reactivity, was essentially confined to the carboxy-terminal end.

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