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# Cloning of the Vaccinia Virus Ribonucleotide Reductase Small Subunit Gene

## CHARACTERIZATION OF THE GENE PRODUCT EXPRESSED IN *ESCHERICHIA COLI**

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**During its infectious cycle, vaccinia virus expresses a virus-encoded ribonucleotide reductase which is distinct from the host cellular enzyme (Slabaugh, M. B., and Mathews, C. K. (1984) *J. Virol.* 52, 501–506; Slabaugh, M. B., Johnson, T. L., and Mathews, C. K. (1984) *J. Virol.* 52, 507–514). We have cloned the gene for the small subunit of vaccinia virus ribonucleotide reductase (designated VVR2) into *Escherichia coli* and expressed the protein using a T7 RNA polymerase plasmid expression system. After isopropyl β-D-thiogalactopyranoside induction, accumulation of a 37-kDa peptide was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and this peptide reacted with polyclonal antiserum raised against a TrpE-VVR2 fusion protein. The 37-kDa protein was purified to homogeneity, and gel filtration of the purified protein revealed that the recombinant protein existed as a dimer in solution. Purified recombinant VVR2 protein was shown to complement the activity of purified recombinant ribonucleotide reductase large subunit, with a specific activity that was similar to native VVR2 from a virus-infected cell extract. A CD spectrum of the recombinant viral protein showed that like the mouse protein, the vaccinia virus protein has 50% α-helical structure. Like other iron-containing ribonucleotide reductase small subunits, recombinant VVR2 protein contained a stable organic free radical that was detectable by EPR spectroscopy. The EPR spectrum of purified recombinant VVR2 was identical to that of vaccinia virus-infected mammalian cells. Both the hyperfine splitting character and microwave saturation behavior of VVR2 were similar to those of mouse R2 and distinct from *E. coli* R2. By using amino acid analysis to determine the concentration of VVR2, we determined that ≈ 0.6 radicals were present per R2 dimer. Our results indicate that vaccinia virus small subunit is similar to mammalian ribonucleotide reductases.**

Ribonucleotide reductase is an essential enzyme in DNA replication, providing the only source of *de novo* synthesized deoxyribonucleotides. In both *Escherichia coli* and eukaryotic organisms, the holoenzyme is composed of two homodimers: an allosteric/catalytic subunit, designated R1,[1] whose protomers have molecular weights of 83,000–87,000, and a smaller iron-containing subunit, designated R2, with protomer molecular weights of 37,000–45,000 (Sjöberg and Gräslund, 1983). Although several types of ribonucleotide reductase have been described, accumulating evidence suggests that all forms of the enzyme utilize a free radical mechanism to initiate reduction of the four ribonucleotide substrates (reviewed in Stubbe, 1990). In the iron-containing reductases, the source of the radical is a tyrosine side chain (Larsson and Sjöberg, 1986) that has recently been shown by x-ray crystallography to reside within a hydrophobic pocket in each R2 protomer, adjacent to a diferric iron center (Nordlund *et al.*, 1990).

The mechanism by which the diferric tyrosyl radical center in R2 is formed and maintained is currently the subject of intense research (Sahlin *et al.*, 1989, 1990; Ochiai *et al.*, 1990; Fontecave *et al.*, 1990; Stubbe, 1990). Iron binding to mouse R2 apoprotein appears to be highly cooperative (Ochiai *et al.*, 1990), and evidence from the *E. coli* and mouse proteins indicates that generation of the radical is concomitant with oxidation of the diferrous protein to the oxo-bridged diferric state. However, tyrosyl radical quantitation by EPR of R2 proteins from various sources has yielded variable results, usually around 0.5 radical/polypeptide chain, rather than the theoretical maximum of 1.0/R2 monomer (Lynch *et al.*, 1989; Sahlin *et al.*, 1989). Additionally, the mechanistic role of the iron center and its associated radical remains obscure since changes in the redox state of either entity have never been detected under substrate turnover conditions (reviewed in Stubbe, 1990).

We have shown previously that ribonucleotide reductase is encoded by the eukaryotic orthopoxvirus vaccinia and is actively expressed during viral infection. The genes for the large and small subunits, which are separated by 35 kilobases on the vaccinia genome, are transcribed early in the infective cycle (Roseman and Slabaugh, 1990; Tengelsen *et al.*, 1988). Because of the difficulties involved in obtaining large amounts of enzyme from vaccinia-infected cell extracts, we chose to overexpress the genes for both subunits of ribonucleotide

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[1] The abbreviations used are: R1 and R2, large and small subunit of ribonucleotide reductase, respectively; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; mW, milliwatts.

reductase in a bacterial expression system. Comparison of the amino acid sequences of the vaccinia gene products with other ribonucleotide reductase subunits revealed that the vaccinia virus enzyme has far greater similarity to the mammalian and yeast reductases than it does to the corresponding enzyme from *E. coli*, T4 bacteriophage, or the herpes viruses (Slabaugh *et al.*, 1988). In fact, the vaccinia proteins share 70–80% amino acid identity with the respective mouse subunits. In contrast to the herpes virus ribonucleotide reductase, which is unregulated (Averett *et al.*, 1983), the vaccinia virus-induced enzyme exhibits the same pattern of allosteric regulation as the mammalian enzyme (Slabaugh *et al.*, 1984). The regulatory and structural similarity of the VVR2 protein to the mammalian R2 subunits as well as the genetic advantages offered by the readily manipulated nature of the vaccinia virus genome (Traktman, 1990) have led us to pursue study of the vaccinia virus ribonucleotide reductase.

In this report we describe the cloning and overexpression of the vaccinia small subunit ribonucleotide reductase gene in *E. coli*. We describe purification of VVR2 and compare the specific activities and migration on an SDS-polyacrylamide gel, of the native and recombinant proteins. We show that the recombinant protein is susceptible to hydroxyurea inactivation, as evidenced by a decrease in the light absorption spectrum of the R2 free radical and iron center. The radical content of the small subunit is quantitated, and the EPR and CD spectra of recombinant protein are compared with spectra of small subunit proteins from other sources.

## MATERIALS AND METHODS

*Buffers*—Buffers used in this work were as follows. Buffer A was 50 mM HEPES (pH 7.6), 2 mM dithiothreitol, 1 mM PMSF, 5% glycerol, 2 mM $MgCl_2$. Buffer B was 100 mM potassium phosphate (pH 7.6), 2 mM dithiothreitol, 5% glycerol, 1 mM PMSF. Buffer C was 50 mM Tris-HCl (pH 7.6), 2 mM dithiothreitol, 5% glycerol, 1 mM PMSF. Buffer D was 50 mM Tris-HCl (pH 9.5), 150 mM NaCl, 2 mM EDTA, 0.1% polyoxyethylene sorbitan monolaurate. Dithiothreitol and PMSF were added immediately before use of the buffer.

*Bacterial Strains and Media*—The *E. coli* strain BL21(DE3) is a λ lysogen that carries the gene for T7 RNA polymerase under the control of the inducible $p_L$ promoter (Rosenberg *et al.*, 1987). The plasmids pET11d (Studier *et al.*, 1990) and pET11d-R2 were transformed into BL21(DE3) by the method of Chung *et al.* (1989). pET plasmids and suitable bacterial hosts were generously supplied by William Studier, Department of Biology, Brookhaven National Laboratory. Bacteria containing plasmids were routinely grown at 30 °C in Luria broth medium (per liter: 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 1 ml of 1 N NaOH) containing 100 μg/ml ampicillin.

The *E. coli* strain N6405/pSPS2, which overproduces the R2 subunit of *E. coli* ribonucleotide reductase, was a gift of JoAnne Stubbe (Salowe and Stubbe, 1986).

*Cloning and Overexpression*—The gene for the 37-kDa VVR2 protein was previously localized to the left end of the *Hin*dIII F fragment of the vaccinia virus genome (Slabaugh *et al.*, 1988). A 2.3-kilobase *Xba*I fragment containing the gene was cloned into the *Xba*I site in the polylinker of the phagemid vector pIBI30 (International Biotechnologies, Inc., New Haven, CT). An *Nco*I site was engineered at the initiating ATG start codon of the VVR2 gene by site-directed mutagenesis (Kunkel *et al.*, 1987) using the oligonucleotide 5′-GGATGGGTTCCATGGCTAAATTAACAATAA-3′. This generated a 2-base pair change $\overline{AA}$ to GG; underlined in the oligonucleotide sequence) directly upstream of the start codon. The manipulation did not change the coding sequence of the VVR2 gene. The new construct, pXR2a-*Nco*I, was digested with *Nco*I and *Hin*dIII, and the fragment containing the VVR2 tene was ligated into the expression vector pET11d (Rosenberg *et al.*, 1987). Routinely, a 1-liter culture of BL21(DE3) cells harboring the plasmid pET11d-R2 was grown overnight at 30 °C. The saturated culture was diluted 1:1 with Luria broth and incubated at 30 °C for 1 h before induction for 4 h with 0.4 mM isopropyl β-D-thiogalactopyranoside.

*Large-scale Purification of Recombinant VVR2 protein*—The purification scheme used to isolate the recombinant VVR2 protein involved streptomycin sulfate and ammonium sulfate precipitations, gel filtration, and anion exchange chromatography. Precipitation steps were performed at 0–4 °C, and column chromatography was done at ambient temperature. Beyond the ammonium sulfate step we found it advantageous to include glycerol (5%, v/v) in purification buffers to avoid precipitation of VVR2 protein. Cells were harvested by centrifugation at 3,500 × *g*, and the cell pellet was resuspended in 5 volumes of Buffer A and lysed in a French press. Debris was pelleted by centrifugation at 10,000 × *g* for 20 min. To remove nucleic acids, solid streptomycin sulfate was added to the clarified extract to 0.5% (w/v). After stirring on ice for 30 min, the solution was centrifuged at 8,500 × *g* for 20 min to remove insoluble material. To concentrate the protein extract, solid ammonium sulfate was added to 40% saturation with stirring for 30 min on ice, and the following suspension was centrifuged at 8,500 × *g* for 20 min. The supernatant was discarded, and the precipitate was resuspended in Buffer A and applied directly to a Superose 6 HR16/50 fast protein liquid chromatography gel filtration column (16 × 50 mm, Pharmacia LKB Biotechnology Inc.) that had been preequilibrated with Buffer B. After elution in Buffer B, fractions were analyzed for the presence of VVR2 by SDS-polyacrylamide gel electrophoresis. The recombinant protein eluted as a single peak from the gel filtration column. For the final step in purification, anion exchange chromatography, pooled eluate from the Superose 6 column was loaded onto a Mono Q HR5/5 fast protein liquid chromatography column and eluted with a triphasic salt gradient of 0–0.3 M NaCl in Buffer C.

*SDS-Polyacrylamide Gel Electrophoresis and Western Blots*—Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels as described by Laemmli (1970). Peptide bands were visualized by staining with Coomassie Brilliant Blue. For immunoblots, proteins were transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA) for 30 min at 200 mA by using a semidry electroblotter (Idea Scientific, Minneapolis, MN) as described by Towbin *et al.* (1979). The membrane was blocked in Buffer D with 1% gelatin, incubated in Buffer D containing polyclonal antiserum to the vaccinia R2 protein at a dilution of 1:2,000, and then incubated in Buffer D containing goat anti-rabbit antibody conjugated to alkaline phosphatase at a dilution of 1:5,000 (Boehringer Mannheim).

*Polyclonal Antisera*—An *Eco*RV-*Xba*I DNA fragment encoding sequences for 30 kDa of the vaccinia virus R2 gene product was cloned into a pATH bacterial expression vector (Dieckmann and Tragloff, 1985). TrpE-VVR2 fusion protein was purified by SDS-polyacrylamide gel electrophoresis and injected into rabbits by using established procedures. Antiserum to VVR2 was demonstrated to be specific for vaccinia virus R2 by Western blot and immunoprecipitation.[2]

*Enzyme Activity Measurements and Preparation of Native VVR2 Extracts*—The assay for ribonucleotide reductase measured conversion of [$^3$H]CDP to [$^3$H]dCDP and was performed as described previously (Slabaugh *et al.*, 1984). Bovine serum albumin was added to reaction mixtures to normalize the protein concentration. Assay reactions were run in triplicate, and the results were averaged.

A unit of enzyme activity is defined as the amount of VVR2 protein which catalyzes the formation of 1 nmol of dCDP in 1 min in the presence of an excess of complementary vaccinia virus ribonucleotide reductase large subunit (VVR1) protein at 30 °C. Specific activity is defined as units of enzyme activity/mg of protein. The most reliable measurements of enzyme activity were made when VVR2 protein was present at 0.01–0.5 units/20-μl reaction, and the complementary VVR1 protein was present in a 5-fold molar excess. In preliminary experiments we incubated preparations of recombinant VVR1 and VVR2 together before initiation of the reaction to determine whether preassociation of the two subunits affected enzyme activity. Preincubation periods of 1, 10, or 40 min at 30 or 37 °C did not affect the activity detected in a 10-min period immediately after the preincubation.

Virus-infected and uninfected cell extracts were prepared as described previously (Slabaugh and Mathews, 1984). VVR1 and host cell R1 protein were specifically removed from virus-infected and uninfected extracts by using an affinity matrix, dATP-Sepharose. Crude extracts were incubated at 4 °C for 30 min with dATP-Sepharose resin and separated from the resin by brief centrifugation. This resulted in extracts with no endogenous ribonucleotide reductase activity and no immunoreactive VVR1. For specific activity measurements, VVR2 protein was complemented with purified recombinant

[2] N. A. Roseman and M. B. Slabaugh, manuscript in preparation.

vaccinia virus large subunit.[3] We choose to use recombinant VVR1 in our complementary enzyme assay because although native VVR1 can be recovered from the dATP-Sepharose affinity resin, it is contaminated with host R1. For the activity assay measurements in Fig. 3, 0.27 μg of VVR1 was included in each reaction.

To add equivalent amounts of recombinant and native VVR2 to the reactions we estimated the concentration of native VVR2 in the cellular extract by quantitative Western blot analysis. Incremental amounts of purified recombinant VVR2 (0.07–0.70 μg) were electrophoresed on an SDS-polyacrylamide gel alongside aliquots of extract from virus-infected cells. This procedure allowed us to estimate the VVR2 concentration in the extract to within 10%.

*Protein Concentration Determination*—Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. The relative amount of VVR2 protein in extracts was determined by scanning laser densitometry of Coomassie-stained extracts in SDS-polyacrylamide gels using a Zeineh model SL-504-XL instrument (Biomed Instrument Inc., Fullerton, CA). The extinction coefficient of purified VVR2 was determined by amino acid analysis. This procedure was performed as described by Malencik *et al.* (1990). Briefly, vacuum-dried VVR2 protein (approximately 5 μg) was subjected to acid hydrolysis with 6 M HCl in the vapor phase for 24 h at 160 °C. After dabsyl chloride derivatization, approximately 100 total nmol of amino acid was chromatographed on a reverse-phase Ultracarb 20 HPLC column. Absorbance was monitored at 460 nm, and peak areas were determined by using Dynamic Solutions' base-line data acquisition system (version 3.0). Based on parallel analysis of a known amount of chicken lysozyme, recovery of VVR2 was quantitative.

Amino acid analysis yielded a molar extinction coefficient for active VVR2 of 119,000 $M^{-1}$ $cm^{-1}$ at 280 nm.

*Circular Dichroism*—The circular dichroism spectrum for VVR2 was recorded on a Jasco J-41A spectropolarimeter that was calibrated by using (+)-10-camphorsulfonic acid, $\Delta\Sigma \approx 2.42$ at 290.5 nm. The measurement was made using a 200-μm pathlength cell, and the protein solution was 5.7 μM in 50 mM sodium phosphate buffer (pH 7.8). The secondary structure prediction was made using the variable selection method computer program outlined by Compton and Johnson (1986), with a basis set of 26 proteins.

*Electron Paramagnetic Resonance Measurements*—EPR spectra were obtained on a Varian E-109 spectrometer equipped with an E-102 microwave bridge and an Air Products liquid helium cryostat and appropriate cavity for measurements at 30 K. For quantitation of the tyrosyl radical content, measurements were performed at 108 K (by use of a Varian variable temperature accessory that flows chilled nitrogen gas over the sample) at a power of 1 mW and a modulation amplitude of 5 gauss. The area of the double integral was compared with that obtained from a 1.0 mM $Cu(ClO_4)_2$ standard in 2 M $NaClO_4$, 10 mM HCl (pH 2) under similar instrumental conditions.

## RESULTS

*Overexpression of the VVR2 Subunit in E. coli*—As shown in Fig. 1, induction of BL21(DE3) cells containing pET11d-R2 with isopropyl β-D-thiogalactopyranoside resulted in high level expression of a 37-kDa peptide (*lanes 2–5*). This peptide was not detected in BL21(DE3) cells containing the control plasmid, pET11d (*lane 1*). After 4 h of induction, VVR2 accumulated to approximately 15% of the total soluble protein as determined by densitometric scanning of Coomassie-stained electrophoretic bands. Approximately 40 mg of recombinant VVR2 was produced per liter of bacterial culture.

pET11d-R2 was induced at several different temperatures in an attempt to increase the ratio of soluble to insoluble VVR2 protein in the cell extracts. When cultures were induced at 37 °C, only 50% of the total amount of expressed VVR2 protein was soluble. However, when the cultures were induced at 30 °C, more than 70% of the recombinant protein was expressed in soluble form. Induction at temperatures below 30 °C under the same growth conditions decreased the level of total protein expression.

*Summary of VVR2 Purification*—A typical purification of recombinant VVR2 is summarized in Table I. Two liters of bacterial culture yielded 18.8 mg of purified VVR2 protein with a specific activity of 48 units/mg. The final step in purification, chromatography on a strong anion exchange matrix, separated full-length VVR2 monomers from partially degraded VVR2 polypeptides. As shown in Fig. 2*A*, VVR2 eluted in two peaks. The first peak, eluting at 0.20 M NaCl, contained full-length 37-kDa VVR2 as well as several peptides of discrete sizes (35.5, 34.5, and 33 kDa) whose electrophoretic mobilities were slightly greater than full-length VVR2 (Fig. 2*B*, Fractions 23–25). All peptides in these fractions reacted positively to anti-VVR2 antiserum in Western blot analysis, suggesting that the shorter fragments were derived from full-length VVR2. The second peak, which eluted at 0.25 M NaCl, contained predominantly 37-kDa monomer.

Sjöberg *et al.* (1987), described fractionation on a Mono Q HPLC anion exchange column as a method for separating *E. coli* R2 homodimers from heterodimers consisting of one full-length and one R2 polypeptide of smaller molecular mass. The truncated *E. coli* polypeptide was shown to be a carboxyl-terminal proteolytic digestion product that was present when PMSF was omitted from purification buffers. Mann *et al.* (1991) reported that recombinant mouse R2 is also susceptible to partial degradation during purification even in the presence of protease inhibitors, but that in this case, degradation was from the amino-terminal end of the protein. Similarily, addition of the protease inhibitors PMSF, pepstatin, leupeptin, and aprotinin to purification buffers did not prevent formation of the degradation products that we observed. Analogous to the formation of *E. coli* heterodimers, we hypothesize that the first fraction eluted from the Mono Q column represents various VVR2 heterodimers of different molecular masses.

The mobility of purified recombinant VVR2 was compared

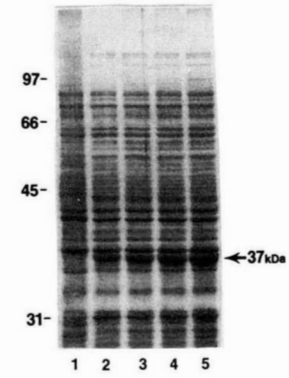


FIG. 1. **Expression of recombinant VVR2 protein in *E. coli* at 30 °C.** Isopropyl β-D-thiogalactopyranoside was added to cultures to 0.4 mM, and 1-ml aliquots were removed from cultures of pET11d-R2 at 0, 1, 2, and 4 h postinduction (*lanes 2–5*) and from pET11d at 4 h (*lane 1*). Aliquots were centrifuged, and each cell pellet was resuspended in 200 μl of SDS-containing sample buffer. Extracts were sonicated, heat denatured at 100 °C for 2 min, and 20 μl was loaded onto an SDS-polyacrylamide gel.

TABLE I
*Purification of recombinant R2*

| Purification step | Total volume | Total protein | Enzyme activity | Specific activity | Yield | Purification |
|---|---|---|---|---|---|---|
| | *ml* | *mg* | *units* | *units/mg* | *%* | *-fold* |
| Crude extract[a] | 30 | 630 | 5,090 | 8.1 | 100 | 1.0 |
| Ammonium sulfate | 5 | 151 | 1,330 | 8.8 | 26 | 1.1 |
| Gel filtration | 12 | 73 | 1,190 | 16.3 | 23 | 2.0 |
| Anion exchange | 6 | 18.8 | 900 | 48 | 17.6 | 6.0 |

[a] Crude extract denotes the supernatant fraction obtained after the removal of cell debris.

[3] M. B. Slabaugh, N. A. Roseman, and M. L. Howell, manuscript in preparation.

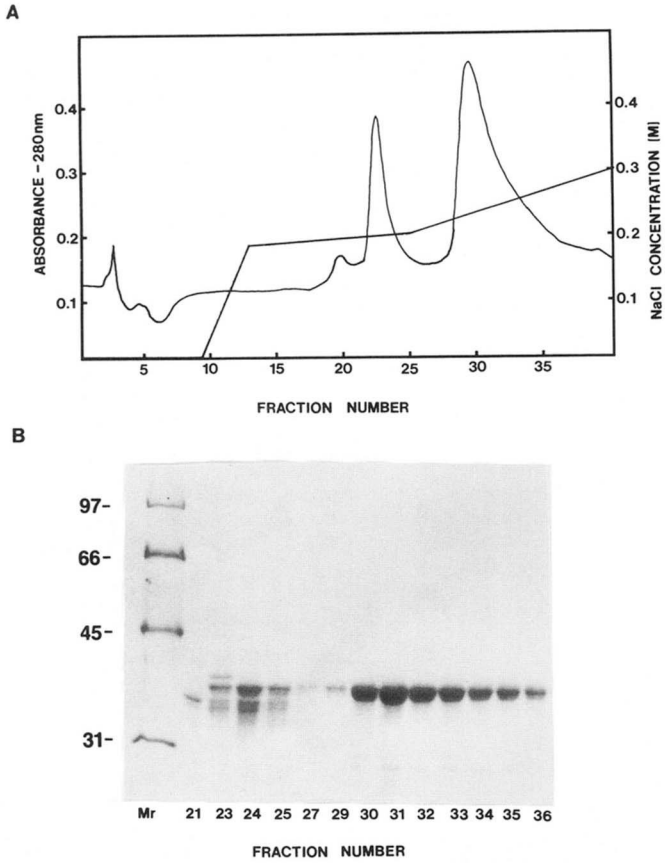


FIG. 2. **Anion exchange chromatography of recombinant VVR2 small subunit.** Partially purified VVR2 (~100 $\mu$g) was applied to a Mono Q HR5/5 column and eluted using a triphasic salt gradient of 0–0.3 M NaCl in Buffer B. *Panel A*, NaCl gradient and absorbance at 280 nm of the Mono Q eluate. *Panel B*, 50 $\mu$l of each 0.5-ml fraction analyzed on an SDS-polyacrylamide gel. The fraction numbers for the sample are indicated below the elution profile and below the corresponding *lanes* of the gel.

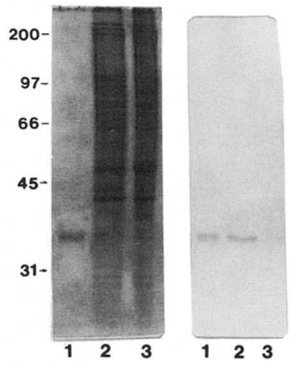


FIG. 3. **Protein gel and Western blot analysis of recombinant and native VVR2.** *Lane 1*, Purified recombinant VVR2 (0.35 $\mu$g). *Lane 2*, 8 $\mu$l of a vaccinia virus-infected cell extract (1.2 mg/ml total protein). *Lane 3*, 8 $\mu$l of an uninfected cell extract (1.2 mg/ml total protein).

with that of native VVR2 from a vaccinia-infected cell extract by migration of the two proteins in SDS-polyacrylamide gel electrophoresis (Fig. 3). Staining of a blot of the gel with antiserum against a TrpE-VVR2 fusion protein revealed a single 37-kDa band in the lanes containing recombinant and native VVR2 but no band in the lane containing extract from uninfected cells. Native VVR2 degradation fragments were not detected in the lane containing VVR2. Additional Western blot analysis of recombinant VVR2 during purification revealed that the protein is not proteolytically degraded until after lysis of the bacterial cells.

To determine whether the recombinant vaccinia ribonucleotide reductase small subunit was biologically active, VVR2 was combined with purified recombinant VVR1. Neither VVR2 nor VVR1 preparations exhibited enzyme activity when assayed alone. However, as shown in Fig. 4, recombinant VVR2 did complement the recombinant VVR1 preparation. To compare directly the specific activities of recombinant and native VVR2 proteins, equivalent amounts of VVR2 were assayed for enzyme activity in the same experiment. (Native VVR2 concentration was estimated in crude extracts from infected cells as described under "Materials and Methods.") The saturation curve obtained by assaying increasing amounts of R2 with a constant amount of R1 showed that the specific activity of the purified recombinant VVR2 was equivalent to that of VVR2 produced in an infected eukaryotic cell.

*Determination of the Apparent Molecular Mass for Native VVR2 Protein*—A Superose 12 HR10/30 fast protein liquid chromatography gel filtration column (10 × 30 mm) was used to estimate the molecular mass of native R2 protein in solution. The molecular mass of VVR2 monomer calculated from the deduced amino acid sequence is 36,975 Da, and the recombinant protein has an apparent size of 37 kDa as compared with standard proteins on an SDS-polyacrylamide gel. Purified VVR2 eluted as a single peak on a Superose 12 gel filtration column at a retention time of 67.4 min (Fig. 5), corresponding to an estimated molecular weight of 64,000. We conclude that the VVR2 protein exists in solution as a dimer. These results agree with experiments on corresponding proteins from *E. coli* (Thelander, 1973) and mouse (Thelander *et al.*, 1985), which also describe the native R2 unit as a dimer.

*Circular Dichroism*—The circular dichroism spectrum recorded for VVR2 is shown in Fig. 6. Analysis of the spectrum revealed the secondary structure composition of VVR2 to be 47% $\alpha$-helix, 6% $\beta$-sheet, 21% $\beta$-turn, and 29% other structures. Recent CD spectra of mouse and *E. coli* R2 and three-dimensional structure analysis of *E. coli* R2 have distinguished the two proteins on the basis of secondary structure (Mann *et al.*, 1991; Nordlund *et al.*, 1990). It was demonstrated that *E. coli* R2 has a high $\alpha$-helical content (70%) corresponding to the helix-turn-helix structural motif. Mouse R2 exhibited a lower $\alpha$-helical content (50%) and had a significant secondary structure contribution from $\beta$-sheet (17%).

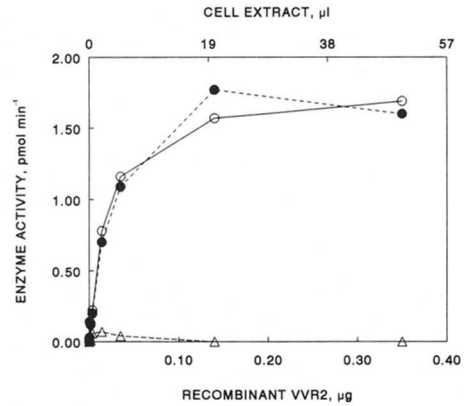


FIG. 4. **Complementation of recombinant and native VVR2 with purified recombinant VVR1.** Vaccinia and host R1 were removed from the cell extracts by adsorption with dATP-Sepharose. Assay parameters and the method for determining equivalent amounts of recombinant and native VVR2 are described under "Materials and Methods." *Solid line/open circles*, purified recombinant VVR2; *dashed line/solid circles*, vaccinia virus-infected cell extract; *dashed line/open triangles*, uninfected cell extract.

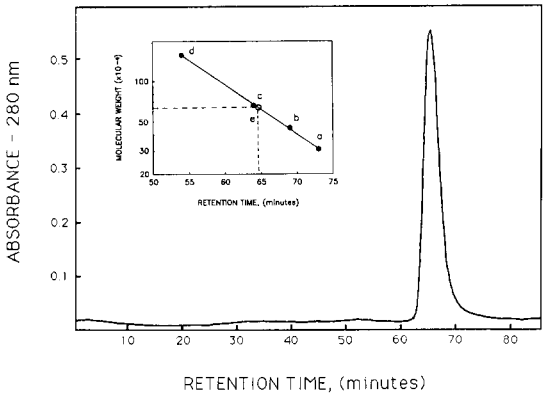


FIG. 5. **Gel filtration column chromatography of VVR2.** Purified VVR2 protein was chromatographed on a Superose 6 molecular sieve column in 50 mM Tris-HCl (pH 7.6), 5% glycerol, and 2 mM dithiothreitol. The flow rate was 1.0 ml/min, and 1-ml fractions were collected. The *inset panel* depicts the correlation between the migration of molecular weight standards and the retention time from the column. *Letters* indicate elution of standards: *d*, aldolase at 148,000 Da; *c*, bovine serum albumin at 68,000 Da; *b*, ovalbumin at 45,000 Da; *a*, cytochrome *c* at 12,500 Da. *e*, migration of recombinant VVR2 relative to standards.

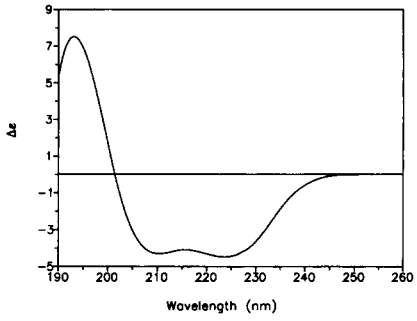


FIG. 6. **Circular dichroism spectrum of recombinant VVR2.** The spectrum of recombinant protein (0.42 mg/ml) in 50 mM sodium phosphate buffer (pH 7.6) was recorded with a 200-μm path length at 25 °C.

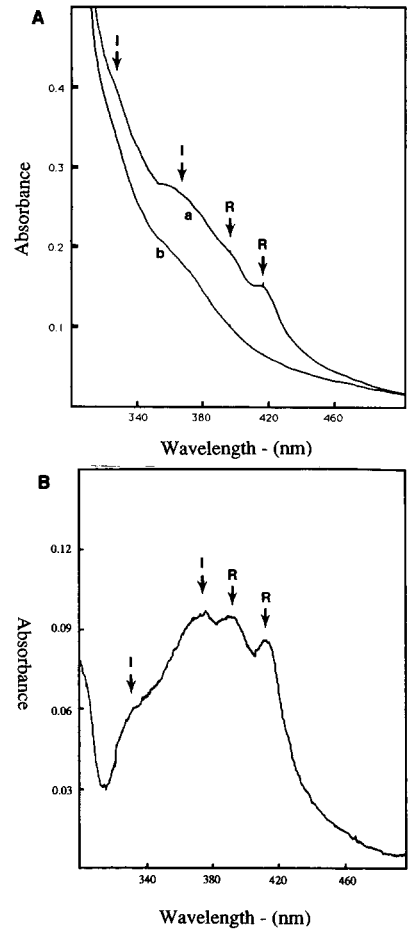


FIG. 7. **Light absorption spectra above 300 nm for the VVR2 subunit of ribonucleotide reductase.** *Panel A*, the electronic spectra of *a*, protein VVR2 (19 μM) in Buffer C and *b*, an identical sample after incubation with 10 mM hydroxyurea for 20 min at 25 °C. The absorbance peaks exhibited by mouse R2 protein are indicated by *arrows*. Spectral features are assigned to iron center (*I*) or radical (*R*). *Panel B*, difference spectrum derived from data in *A*.

*Effect of Hydroxyurea on the Absorption Spectrum of VVR2 Protein*—The near UV absorption spectra of the iron-containing ribonucleotide reductases exhibit characteristic bands that have been ascribed to the dinuclear iron center (325 and 370 nm) and the tyrosyl radical (390 and 410/416 nm) (Atkin *et al.*, 1973; Thelander *et al.*, 1985). The mammalian radical peak at 416 nm (Mann *et al.*, 1991) is slightly shifted from the sharp *E. coli* radical peak at 410 nm (Petersson *et al.*, 1980). The absorption spectrum of purified VVR2 protein from 300 to 500 nm is shown in Fig. 7*A*, *curve a*. In addition to absorption bands at 325, 370, and 390 nm and similar to the published spectrum for mouse R2, a VVR2 peak is also detected at 416 nm.

Vaccinia virus ribonucleotide reductase activity is inhibited by the radical scavenger, hydroxyurea (Slabaugh and Mathews, 1986). Inactivation of mouse ribonucleotide reductase by hydroxyurea results in a decrease in intensity of all the near-UV absorbance bands (Thelander *et al.*, 1985). In contrast, hydroxyurea inhibition of *E. coli* R2 is characterized by the marked decrease in intensity of the maxima at 390 and 410 nm but only slight changes in the ferric iron center absorbances. (The 360–390 nm region contains overlapping contributions from both the free radical and the iron center, and this has made quantitative analysis of the absorption signals somewhat ambiguous.)

To monitor the effects of hydroxyurea on the VVR2 protein, the electronic signals of the free radical and the iron species were measured after treatment of purified VVR2 with the inhibitor (Fig. 7*A*, *curve b*). Hydroxyurea was added to the purified VVR2 sample to 10 mM in the presence of 2 mM dithiothreitol, and the preparation was incubated at 25 °C for 30 min. A difference spectrum (Fig. 7*B*), derived by subtracting *curve b* from *curve a*, showed that absorbance maxima from the tyrosyl radical (390 and 416 nm) were decreased by 45 and 40%, respectively, and that the iron center absorbance maxima (325 and 370 nm were also significantly decreased (20 and 35%, respectively).

*Characterization of Tyrosyl Radical by EPR Spectroscopy*—Purified vaccinia virus R2 protein exhibited a $g = 2.0$ EPR signal (Fig. 8*B*) that is characteristic of a tyrosyl radical (Sjöberg and Gräslund, 1983). The EPR spectrum showed a hyperfine splitting pattern that was identical to that of native vaccinia protein expressed in virus-infected cells (Fig. 8*C*). No such signal was present in uninfected cells (Fig. 8*D*). Treatment of recombinant VVR2 with hydroxyurea, which destroys the enzymatic activity and the 416-nm absorption band associated with the tyrosyl radical (see above), also caused the disappearance of the tyrosyl radical EPR signal. (The spectrum of VVR2 treated with 10 mM hydroxyurea for 30 min looked the same as the spectrum in Fig. 8*D*.)

The hyperfine splitting of the tyrosyl radical signal appears to be extremely sensitive to the orientation of the β-$CH_2$

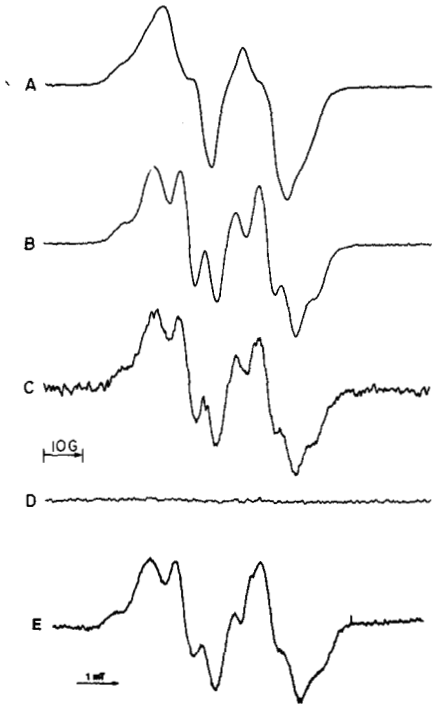


FIG. 8. **EPR spectra at 30 K of tyrosyl radicals in R2 proteins.** *A*, *E. coli* R2 protein from strain N6405/pSPS2 (0.1-mW power, 1.6 gauss modulation amplitude). *B*, purified recombinant VVR2 protein, 10 $\mu$M in R2 dimer (3 mW, 1.6 gauss). *C*, VVR2 protein from virus-infected $BSC_{40}$ cells (3 mW, 4 gauss). *D*, protein from uninfected $BSC_{40}$ cells (3 mW, 4 gauss). *E*, hydroxyurea-resistant, R2-overproducing mouse fibroblast 3T6 cells (data from Lankinen *et al.*, 1982). Samples in *A*, *C*, and *D* were partially purified by ammonium sulfate fractionation. *mT*, millitesla.

group with respect to the tyrosine ring (Gräslund *et al.*, 1982). The close similarity of the EPR spectra in Fig. 8, *B* and *C*, indicates that expression of the vaccinia virus R2 gene in *E. coli* yields a protein whose metal center is indistinguishable from that of the native virus. The hyperfine splitting pattern of vaccinia virus R2 is readily distinguished from that of *E. coli* (Fig. 8*A*). In fact, the EPR spectrum of vaccinia virus R2 closely matches the spectra of ribonucleotide reductases from eukaryotic sources such as mouse (Fig. 8*E*; Gräslund *et al.*, 1982) and yeast (Harder and Follmann, 1990). The spectra of the R2 radicals from the pseudorabies virus (Lankinen *et al.*, 1982), herpes simplex virus (Mann *et al.*, 1991), and bacteriophage T4 (Sahlin *et al.*, 1982) are all distinctively different. These findings suggest that the vaccinia R2 gene is closely related to and presumably derived from a mammalian gene, in agreement with amino acid sequence comparisons (Slabaugh *et al.*, 1988).

Another point of similarity between vaccinia and mouse R2 proteins is in the power dependence for microwave saturation. As in the case of mouse R2 (Mann *et al.*, 1991), recombinant VVR2 begins to show saturation of its EPR signal (*i.e.* > 10% reduction in $S/P^{1/2}$) when the microwave power exceeds 1 mW at 30 K. In contrast, the EPR signal of *E. coli* R2 begins to saturate already at 0.1 mW at 30 K, whereas that of herpes simplex R2 saturates only above 10 mW at 30 K (Mann *et al.*, 1991). The differences in saturation behavior are believed to be related to the degree of magnetic interaction between the tyrosyl radical and the dinuclear iron center (Sahlin *et al.*, 1987). Such an interaction enhances the spin-lattice relaxation rate, thereby making the signal less susceptible to saturation. Thus, it would appear that the tyrosyl radical is more closely associated with the dinuclear iron center in vaccinia and mouse R2 than it is in *E. coli* R2.

The spin concentrations in two different preparations of purified recombinant VVR2 were calculated by comparison with a copper standard. The protein concentration was determined by amino acid analysis. These measurements indicated that at the completion of the purification procedure, 0.3 tyrosyl radicals were present per VVR2 polypeptide chain.

*Attempted Reconstitution of Vaccinia Virus R2*—In an attempt to increase the iron and tyrosyl radical content of recombinant VVR2 protein, purified protein was subjected to aerobic (Thelander *et al.*, 1985) and anaerobic (Mann *et al.*, 1991) reconstitution procedures. Briefly, this involved exposing the protein to excess ferrous iron in the presence of dithiothreitol (aerobic reactivation) or sodium ascorbate (anaerobic reactivation). To determine what effect reactivation had on the VVR2 protein, we compared the enzymatic activities of reconstituted and unreconstituted protein samples and measured the radical content of the protein before and after reactivation procedures. Enzyme assay results reflected no increase in activity resulting from either aerobic or anaerobic treatment of VVR2 compared with untreated protein. Additionally, inclusion of $FeCl_3$ or $Fe(NH_4)_2SO_4$ in the enzyme assay had no effect on the activity detected in treated or untreated VVR2 protein. As measured by EPR, neither reactivation procedure significantly increased the radical content of the vaccinia virus protein.

We also asked whether the activity (tyrosyl radical content) of recombinant VVR2 could be increased by supplementing the bacterial growth medium with exogenous iron or adding ferrous iron to lysis buffers. Neither of these approaches increased activity above the value of approximately 50 units/mg reported in Table I.

## DISCUSSION

In these experiments we have characterized the 37-kDa vaccinia virus ribonucleotide reductase small subunit protein that has been overexpressed in *E. coli*. By several criteria we have shown that the recombinant VVR2 subunit is functionally and physically equivalent to the R2 subunit expressed in vaccinia virus-infected mammalian cells. Polyclonal antiserum made to a fusion protein containing a major portion of the vaccinia virus R2 protein reacted with the recombinant protein expressed in *E. coli*. When native and recombinant VVR2 were complemented with purified recombinant VVR1, ribonucleotide reductase activity was readily detected, and the specific activity of both R2 proteins was the same. In addition, the EPR spectrum of recombinant protein matched that of a partially purified preparation of VVR2 protein produced by viral infection of $BSC_{40}$ cells..

The circular dichroism spectrum of VVR2 revealed the viral protein to be similar to mouse R2 in $\alpha$-helical content (~50%). Thus, the eukaryotic R2 molecule appears to differ significantly in secondary structure from *E. coli* R2 which has been shown by CD and crystallography (Mann *et al.*, 1991; Nordlund *et al.*, 1990) to be exceptionally rich in $\alpha$-helical content (70%). $\beta$-Sheet contributions to the CD spectrum in the case of VVR2 (6%) were intermediate between the reported values for *E. coli* (3%) and mouse R2 (17%).

From our spectroscopic studies we conclude that the protein environment of the tyrosyl radical in VVR2 is similar to that of the previously characterized mouse and calf thymus proteins and exhibits certain distinctions from the *E. coli* R2. The location of the radical absorbance peak at 416 nm in the electronic spectrum of the recombinant VVR2 protein differs from the 410 nm maximum for the *E. coli* protein but closely matches the 416 nm absorption band of the mouse protein

(Mann *et al.*, 1991). Hydroxyurea inhibition of VVR2 protein revealed that like analogously treated mouse R2, vaccinia virus R2 exhibits an absorption spectrum in which all four near UV electronic signals are attenuated in the presence of inhibitor. This contrasts with the effect of hydroxyurea on *E. coli* R2, in which the ferric iron center electronic signals are only slightly decreased. One interpretation of these results is that the mammalian ferric iron center is more susceptible to reduction by hydroxyurea than is the *E. coli* iron center. Either reduction of ferric iron to the ferrous form or dissociation of iron from the protein could cause the decreased intensity of the iron center electronic signals. We are continuing inhibitor studies on the VVR2 protein to examine more closely the mechanism of hydroxyurea inhibition.

The EPR spectrum of recombinant VVR2 protein more closely resembles the EPR spectrum of mouse R2 than *E. coli* R2, both in the hyperfine splitting pattern and in the susceptibility to microwave power saturation at low temperature. The former is indicative of a conserved conformation for the tyrosyl radical side chain in the mouse and vaccinia proteins. The latter suggests increased interaction between the tyrosyl radical and the dinuclear iron center in mouse and vaccinia virus R2 relative to *E. coli* protein.

Various preparations of recombinant VVR2 yielded protein with 25–30% of the theoretical maximum of one tyrosyl radical/R2 protomer. For comparison, calf thymus, mouse, and herpes virus R2 proteins purified from natural or recombinant sources exhibit even lower levels of radical content (0–14%) and corresponding low iron occupancies (6–10%) (Thelander *et al.*, 1983; Mann *et al.*, 1991). However, whereas mouse and *E. coli* R2 can be reactivated to 70–100% iron occupancy (3.2–4.0 Fe/dimer) and up to 80% radical content (1.6 radical/dimer) (Bollinger *et al.*, 1991; Mann *et al.*, 1991), identical reactivation procedures applied to the vaccinia virus enzyme did not increase the radical/R2 monomer ratio above 30%. Iron/radical centers in herpes virus R2 were also reported to be resistant to full reactivation (Mann *et al.*, 1991). Additionally, inclusion of ferrous or ferric iron in the enzyme assay had no effect on the activity measured in "unreactivated" or "reactivated" VVR2 preparations. This contrasts with the apparent lability of the iron center in calf thymus R2, which may require continual regeneration during enzyme turnover (Thelander *et al.*, 1983).

The lower specific activities of vaccinia and herpes virus R2 proteins (approximately one-sixth that of mouse R2) may be caused by the lower steady-state radical contents exhibited by these viral enzymes *in vitro*. Our results, taken together with studies of R2 from other species, emphasize the variable nature of the stability of the iron/radical center in this metalloprotein.

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