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Short communication

Cloning of a Ribonucleotide Reductase Gene of the Herpes Simplex Virus Type 2 Strain G

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The ribonucleotide reductase (RR) 2 gene of the HSV-2 strain G was cloned, sequenced, and expressed in an *E. coli* cell. The RR2 gene was located on the *Pst*I 2.4 kb fragment, which was cloned and sequenced. The ORF of the gene was 1,011 bp and its termination codon was TAG; also, the CATATAA sequence was present in the promoter of the RR2 gene. A Poly A signal sequence (AATAAA) was found in the 3'-noncoding region. The RR2 proteins that were produced in the *E. coli* and Vero cells were confirmed using a Western blot analysis. SDS-PAGE revealed that the molecular weights of the fusion-RR2 that was produced in the *E. coli* cells were approximately 24 kDa and 38 kDa in the Vero cells. The RR2 proteins were soluble. The differences in the molecular weights might be due to modifications in the Vero cells.

Keywords: HSV-2, Ribonucleotide reductase gene

Introduction

Herpes simplex virus (HSV) ribonucleotide reductase (RR) is a delayed, early (DE) protein (Langelier *et al*., 1978). It is associated with the arrest and incompatibility in regard to the induction of gene amplification and long-term cell survival (Becker and Sarov, 1968; Langelier *et al*., 1978). These enzymes consist of RR1 and RR2 subunits (Duita, 1983); RR1 with a molecular weight of 140 kDa and RR2 with a molecular weight of 38 kDa, as reported by several investigators (Galloway and McDougall, 1981; Bachetti *et al*., 1986; Wymer *et al*., 1989). The nucleotide sequences of the RR gene of the HSV-2 strain 333 (Galloway and Swain, 1984) and HSV-1 (Draper *et al*., 1982) were determined and analyzed. However, they did not perform the genes expression in the *E. coli* cells as well as they did the sequencing of other strains. Therefore, we cloned and expressed the gene of the HSV-2 strain G, which is a mild pathogen for humans. The aims of this study were to clone, sequence, and express the RR2 gene of the HSV-2 strain G in a prokaryotic cell.

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Materials and Methods

Viruses, bacterial strains, cells, and plasmids The HSV-2 strain G (ATCC VR-734) was obtained from the Korean AIDS Center, Seoul, Korea. Vero cell lines (ATCC-CCL 81) were obtained from the Korean Type Culture Collection (KTCC), Seoul, Korea. The Vero cells were grown in an Eagles minimum essential medium (MEM) (Gibco, Detroit, USA) that contained 10% fetal bovine serum (Gibco), 0.22% sodium bicarbonate (Sigma, St. Louis, USA), and 50 μg/ml gentamycin (Gibco). The HSV strains were grown in the Vero cells at 37°C. The pBacPak 9 plasmid that was harbored in *E. coli* XL-1 blue (Clontech Co., Palo Alto, USA), pBluescript SK(+), and pMal-cRI in *E. coli* DH5α (Stratagene, La Jolla, USA) were used for recombinant efforts.

Preparation of viral DNAs Vero cell monolayers in a 75 cm^2 tissue culture flask were inoculated with HSV-2, approximately 0.1 plaque forming units (pfu) per cell. The culture was incubated at 37°C until an advanced cytopathic effect was observed. When a complete cytopathic effect was observed in the infected cells (within 5 d postinfection), they were scraped into the media with a rubber policeman. The cells and virus fluids were centrifuged at 15,000 × *g* for 30 min at 4°C, then the cell debris was removed. Viral supernatant was ultracentrifuged at 100,000 × *g* for 2 h at 4°C and the supernatants were collected separately. The pellets were resuspended in a small volume of H_2O and then treated with proteinase K (0.5 mg/ml) (Sigma) and 0.5% SDS in a 50°C water bath for 24 h. Next, the viral DNA mixture was extracted three times with saturated phenol and chloroform-isoamylalcohol (24 : 1, v/v). After the extraction, the aqueous phase was adjusted at a final concentration of 0.2 M ammonium acetate and centrifuged at

12,000 × *g*. DNAs in the supernatant were precipitated overnight using 2.5 volumes of 95% ethanol at −20°C. The DNA precipitates were pelleted by micro-centrifugation at 14,000 × *g* for 15 min at 4°C. The pellets were washed with 70% ethanol, dried, resuspended in 5 mM Tris-HCl (pH 7.8) and a 0.1 mM EDTA buffer, and stored at 4°C.

Oligonucleotides and restriction mapping One oligonucleotide probe (5'-CGCGATCGCCTGCTGGGCC-3') was deduced from the terminal sequences of the published sequences of the RR1 gene of HSV-1 (Draper *et al.*, 1982) and synthesized by Korea Biotech Inc. (Daejeon, Korea). All of the restriction endonuclease digestions were performed according to the manufacturers instructions.

The physical restriction map of the *Pst*I fragment was constructed with seven enzymes (*Dra*II, *Bam*HI, *Sac*I, *Pvu*II, *Bst*EII, *Eco*RV, and *Hinc*II). The digested DNA fragments were electrophoresed on a 0.6% agarose gel (Sambrook *et al*., 1989; Lee *et al*., 1998; Ju *et al*., 2001; Uh *et al*., 2001).

Preparation of plasmid DNAs and agarose gel electrophoresis *E. coli* that contained the recombinant plasmids was cultured in a LB broth that contained the proper antibiotics at 37°C. The plasmid DNA was purified according to the procedure that was described by Birnboim and Doly (1979) and Hwang *et al*., (1998). A rapid, small-scale plasmid analysis was performed using micro-centrifugation at 15,000 rpm for 1 min. The purified DNAs were then examined by 0.8% agarose gel electrophoresis (Sambrook *et al*., 1989).

Molecular cloning The purified viral DNAs from HSV-2 were digested with *Bgl*II, cloned into the pBackPak9 vector (Sambrook *et al*., 1989; Hwang *et al*., 1998, Kang *et al.*, 2000; Uh *et al.,* 2001), and run on 0.7% agarose gel (Bollag *et al*., 1996). Then the *Bgl*II fragment that contained the RR2 gene was detected with a probe by a Southern blot analysis (Southern, 1975). The hybridized 7.1 kb *Bgl*II fragment, which was cloned and named the pH2Bg-8 plasmid, was redigested with 5 different restriction enzymes (*Bst*EII, *Eco*RV, *PstI, Sac*I, *and Xho*I) and rehybridized with the probe. The hybridized 2.4 kb *Pst*I fragment was cloned into the *Pst*I site of pBluescript SK (+). The resulting recombinant was named pHS-RR (5.36 kb). The RR2 gene in the plasmid was digested with *Bam*HI and treated with a Klenow enzyme with dATP and dGTP. It was then digested with *Hind*III. The resulting fragment (2.0 kb) was cloned into the *Sal*I (treated with Klenow enzyme with dCTP and dTTP) and *Hind*III sites of the pMal-cRI vector. The resulting clone was named the pMal-RR recombinant plasmid (8.15 kb) and transformed in *E. coil* DH5α. The molecular weight of each DNA fragment was determined by comparing its mobility with the *Hind*III-digested λ phage DNA fragments. The recombinant plasmids were analyzed by *KpnI* and *Hind*III digestion. The 20 kb fragment was then partially sequenced using a Sequence Version 2.0 system kit (United States Biochemical Co, USA), if the RR2 gene orientation was either correct or reversed in relation to the gene promoter. The transformed *E. coli* was isolated on a nutrient agar plate that contained 50 μg/ml of ampicillin, 2% X-gal (5-bromo-4-chloro-3-indol-β-galactoside), and 100 mM IPTG (isopropyl-β-D-thio-galactopyranoside) (Sigma).

Determination of the nucleotide sequence The DNA fragment that contained the RR2 gene was digested with restriction enzymes (*Bst*EII, *Eco*RV, *Sac*I, *Pst*I, and *Xho*I) using the procedure that was described by Lee *et al.*, (1998). The DNA fragment was inserted in pBluescript SK(+) and sequenced by the procedure that was described by Sanger *et al*., (1977).

Purification of MBP-RR2 fusion proteins The *E. coli* / pMal-RR was cultured overnight in 10 ml of a LB broth at 37°C and then inoculated in 1 liter of the LB broth and grown to 2×10^8 cells per ml. Next, 1.0 mM of IPTG was added and the cells were grown for 4 h. The cells were harvested at 4,000 × *g*, washed, and lysed. The lysates were centrifuged at 9,000 × *g* for 10 min and the resultant pellets were resuspended in 5 ml of the lysis buffer. The supernatants were applied to a 15 ml column of amylose resin (New England Biolabs, Beverly, USA) using the procedure that was described by Guan *et al.,* (1987) and Malna *et al*., (1988). The column was then washed with three column volumes of the column buffer with 0.25% Tween. The fusion protein was eluted with 50 ml of the column buffer that contained 10 mM maltose. The fractions that contained the proteins were pooled and put into a concentrator (Millipore, Bedford, USA), then used for further efforts. The purified proteins were analyzed using SDS-PAGE (Bollag *et al.*, 1996).

Preparation of polyclonal antiserum The fusion proteins (0.5 g) were mixed with an equal volume of complete Freunds adjuvant (Gibco) and injected into the peritoneal cavity of a mouse. After 3 weeks, 0.5 g of the antigen and an equal volume of incomplete Freund's adjuvant (Gibco) were also mixed and injected into the peritoneal cavity. To obtain an antiserum, the fluid was collected from the heart after 10-14 d, incubated at 4°C for 18 h, centrifuged at 2,700 × *g* for 10 min, and then the resultant supernatant was stocked at −20°C.

SDS-PAGE and Western blot assay The RR2 proteins that were produced in the *E. coli* and/or Vero cells were confirmed by 7.5% polyacrylamide gel electrophoresis and Western blot, as described by Bollag *et al*., (1996). The Vero cell monolayers in the 75 cm^2 tissue culture flask were inoculated with HSV-2 of approximately 0.1 pfu per cell. The cultures were incubated for 5 d at 37°C. The media was then ultracentrifuged at 100,000 × *g* for 2 h at 4°C. The resultant supernatants were separately collected and the pellets were discarded. The supernatants were used as a source of RR2 that is produced by virus in the Vero cells. The RR2 enzymes were confirmed by the antiserum that is produced by mice, post injection of the fusion that are proteins produced in *E. coli* cells.

Results and Discussion

Cloning and nucleotide sequence analysis of the RR2 gene To clone and obtain the complete nucleotide sequence of the DNA fragment that encodes the RR2 gene of the HSV-2 strain G, we digested the HSV-2 genomic DNAs with the *Bgl*II enzyme. It was then cleaved into 15 fragments that ranged from 1.2 −16.92 kb (1.2, 1.9, 3.34, 3.52, 4.3, 4.43, 6.4, 7.1,

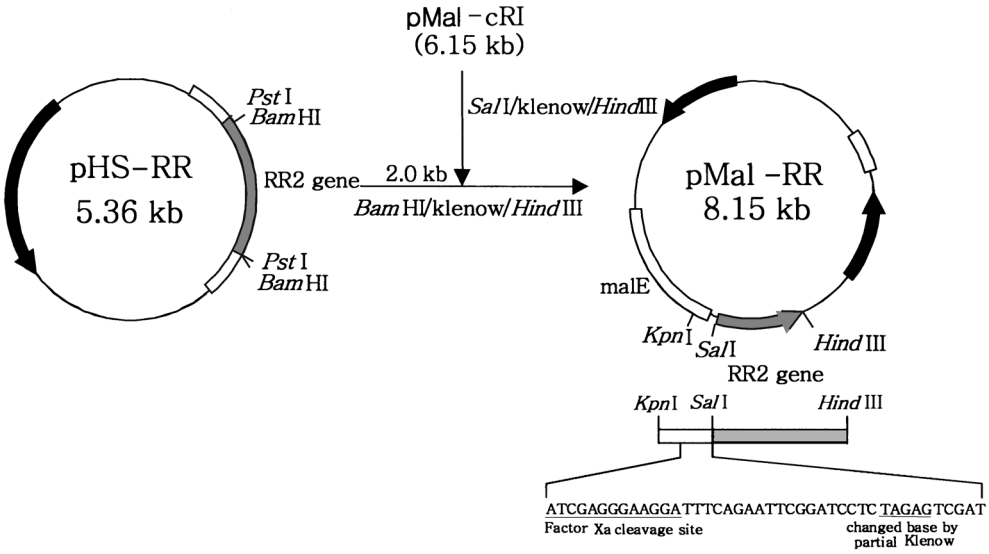

Fig. 1. Construction scheme for pHS-RR and pMal-RR plasmids containing the RR2 gene sequence. The 7.1 kb DNA fragment that contained the RR2 gene was cloned into the pBacPAK9 vector. The clone was then redigested with the *Pst*I enzyme to cut out the RR2 gene sequence, which was cloned into the *Pst*I site of the pBluescript SK(+) and named pHS-RR plasmid. The RR2 gene was transferred into the *Sal*I and *Hind*III sites of the pMal-cRI vector to construct the pMal-RR recombinant plasmid (8.15 kb). The cloning site was partially sequenced.

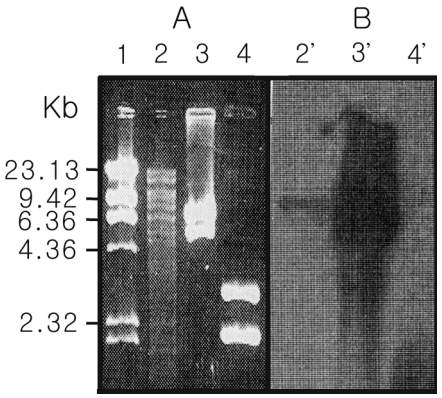

Fig. 2. Agarose gel analysis of RR2 gene fragments (A plate) and identification of the RR2 gene fragments in the recombinant clones by Southern blot analysis (B plate). The digested DNAs were electrophoresed for 17 h on a 0.6% agarose gel. The Southern blot of plate A was as follows: Lanes in A plate 1, λ phage DNAs digested with *Hind*III; 2, HSV-2 strain G genomic DNAs digested with *Bgl*II; 3, pH2Bg-8 plasmid DNAs digested with *Bgl*II; 4, pHS-RR plasmid DNAs digested with *Pst*I. Lanes in B plate 2, 3 and 4.

8.05, 8.32, 9.34, 11.2, 14, 15.9, 16.92 in kb) (Fig. 2, lane 2) on 0.6% agarose gel. These fragments were cloned into the *Bgl*II site of the pBacPAK9 vector (Fig. 1). Each clone was named pH2Bg-1, 2, –15 according to the lowest order of electrophoretic mobilities in the agarose gel. They were confirmed by redigestion with the enzyme and Southern blot analysis with the *Bgl*II HSV-2 DNA fragments (data not shown). Using the probe, the fragment that contained the RR2 gene of HSV-2 was identified on the pH2Bg-8 that contained the 7.1 kb DNA fragment (Fig. 2, lanes 3). Then the RR2 gene was digested with *Pst*I. The resulting 2.4 kb DNA fragment was subcloned into the *Pst*I site of the pBluescript SK(+) vector. The generated clone was named pHS-RR (5.36 kb) (Fig. 1). The 2.4 kb DNA fragment was analyzed with seven restriction enzymes, and a physical restriction map of the fragment was constructed (data not shown).

The 2.4 kb RR2 gene fragment was digested with *Bam*HI, *Pvu*II, *Eco*RV, and *Dra*II. The resulting fragments were cloned into the pBluescript SK (+) vector and sequenced. The nucleotide sequences of the noncoding strand and deduced amino acid sequences of the RR2 gene of the HSV-2 strain G are illustrated in Fig. 3. A total of 1,711 nucleotides were sequenced; 1014 bases of the open-reading frame, 369 bases 5'-upstream of the ORF, and 328 bases downstream from the termination codon (Fig. 3). The open-reading frame (ORF) was 1,014 kb from the ATG translation initiation codon to the TGA termination codon (boldfaced), which could encode a 337-amino acid polypeptide (GenBank accession No. AF305570). The G strain had a termination codon, TAG, which was different than the strain 333, where it was TGA (Galloway and Swain, 1984). Nucleotides at 454 and 904 (shaded) in the ORF of the G strain were different than those with the strain 333. The codons A**C**C and **T**CC in the G strain were changed into codons A**A**C and **C**CC in the strain 333, respectively (Galloway and Swain, 1984). These results indicated that the threonine and serine residues in the G strain were changed into asparagine and proline in the strain 333 at the nucleotide 454 and 904 codons (Table 1). The sequence homology between the G and 333 strains appeared to be

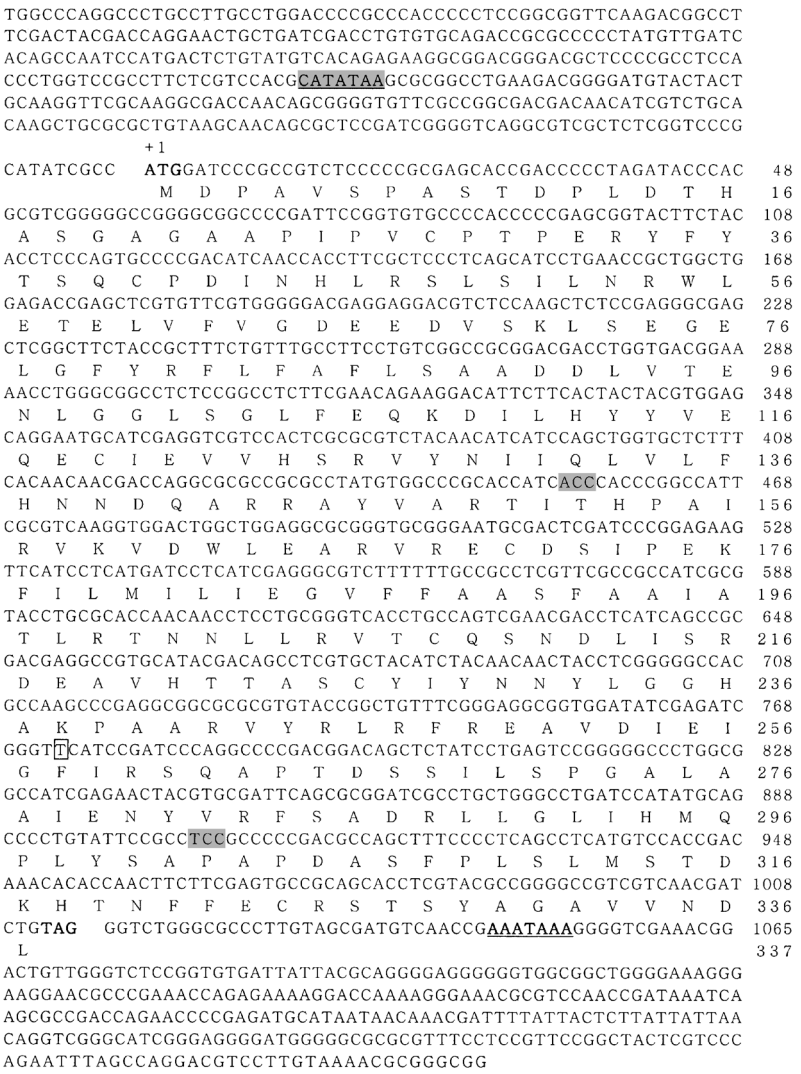

Fig. 3. Nucleotide and deduced amino acid sequence of the RR2 gene from HSV-2 strain G (GenBank accession No. AF305570). Putative promoter sequence and 3'-noncoding region of the RR2 gene of HSV-2 appeared. Nucleotide residues are numbered from the 5' to 3' end on the right. The predicted amino acid sequence is displayed under the nucleotide sequence. Amino acid residues are numbered, beginning with the first methionine on the right. The sequence of the noncoding strand is given. The initiation and termination codons are boldfaced. The TATA box in the promoter region is shaded. The polyadenylation signal sequence is underlined.

Table 1. Nucleotide and amino acid changes in RR2 gene between HSV-2 strains G and 333

NT site	NT and codon difference in the strains	
	G	333
454	A**C**C (thr)	A**A**C (asn)
904	**T**CC (ser)	**C**CC (pro)
1013-1014	T**AG** (stop)	T**GA** (stop)

The NT (nucleotide) sequences of the RR2 gene and protein of HSV-2 strain 333 were derived from the report of Galloway and Swain (1984). Comparisons were made to the strain G nt and deduced amino acid sequences. Base differences at the codons are boldfaced.

99.9%. In the promoter region upstream of the ORF, the CATATAA sequence was located from nucleotides −159 to −165 sites, and was identical in both HSV-1 and HSV-2 (McLaughlan and Clement, 1983; Galloway and Swain, 1984). The nucleotide sequence of the promoter region of the G strain was almost identical to the HSV-2 strain 333 (Galloway and Swain, 1984). The sequences that followed the coding region for the RR2 gene of the HSV-2 strain G were almost identical to that of the strain 333 (Galloway and Swain, 1984). The poly A signal sequence (AATAAA) was found in the 3'-noncoding region, which is located from nucleotides 32 to 38 downstream of the termination codon (Fig. 3). However, in the strain 333 the sequences were located from nucleotides 35 to 40 (Galloway and Swain, 1984). The hydrophobility of

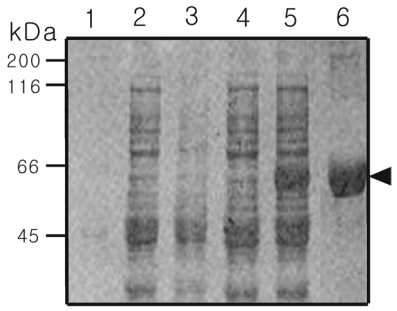

Fig. 4. SDS-PAGE patterns of MBP-RR-2 fusion protein produced in *E. coli.* Lanes 1, standard molecular markers; 2, normal *E. coli* lysates; 3, lysates of *E. coli*/pMal-cRI; 4, lysates of *E. coli* /pMal-RR plasmid; 5, lysates of *E. coli*/pMal-RR plasmid induced with 1.0 mM IPTG for 4 h; 6, MBP-RR2 protein purified with the affinity chromatography. Numbers at the left correspond to the positions of the molecular weight markers (myosin 205, β-galactosidase 116, phosphorylase 97.4, bovine serum albumin 66, ovalbumin 45, carbonic anhydrase 31 in kDa (Sigma).

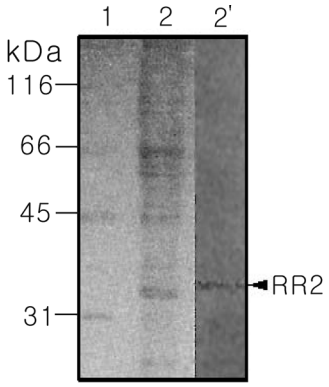

Fig. 5. SDS-PAGE and Western blot patterns of RR2 produced in Vero cells infected with HSV-2. The Vero cells were infected with the HSV-2 strain G for 5 days postinfection. The cells were then collected, lysated, and run on SDS-PAGE. The gel was analyzed using Western blot with antiserum against the fusion protein to confirm the antigen of RR2 in the infected cells. There was a hybrid band at the same site with a molecular weight of approximately 38 kDa of the RR2 that was produced by the HSV-2 infection. Lane 1, standard molecular weight markers; Lane 2, cell lysates infected with HSV-2; 2', Western blot of the lane 2 with poly-antiserum against the MBP-RR proteins. Numbers at the left correspond to the positions of the molecular weight markers (in kDa), which are described in Fig. 4.

the RR2 protein of the HSV-2 strain G was determined by a computer analysis (data not shown). Most amino acid residues were hydrophobic, and a few amino acid residues were hydrophilic. The RR2 protein was composed of 155 non-polar amino acids and 182 polar amino acids.

RR2 protein expression The expression clone, pMal-RR, was constructed for the RR2 protein expression in *E. coli* (Fig. 1). The RR2 gene in the pHS-RR plasmid was transferred into the *Sal*I and *Hind*III sites of the pMal-cRI vector. The generated clone was named the pMal-RR recombinant plasmid (8.15 kb) (Fig. 1). The *malE* gene in the pMal-cRI plasmid is located downstream of the powerful tac promoter, and the fusion of the male protein (MBP) to the RR2 protein may facilitate the purification of the RR2 protein. The ligated site of the *Sal*I site was partially sequenced to confirm the correct cloning of the RR2 gene, which was downstream of the promoter direction (Fig. 1). The partial sequence indicated that the RR2 gene sequence was correctly cloned downstream of the promoter.

The IPTG-induced *E. coli* with the pMal-RR plasmid produced MBP-RR2 fusion proteins, which were fractionated using an amylose affinity chromatography and measured at 280 nm (data not shown). The amount of the protein at the highest fraction was 0.7 μg per ml. The fusion proteins in the *E. coli* cells were examined to determine if they were soluble or insoluble. The MBP-RR2 proteins were present in the soluble cytoplasmic protein. The molecular weight on the 10% SDS-PAGE appeared to be approximately 65 kDa (Fig. 4, lanes 5 and 6). The molecular weight of the MBP is 41 kDa, and that of the RR2 protein may be estimated as approximately 24 kDa. This result indicates that the RR2 proteins might be modified in the short form in the *E. coli* system.

The production of the RR2 proteins in the Vero cells that were infected with the HSV-2 was detected by 10% SDS-PAGE (Fig. 5). The RR2 protein appeared to be approximately 38 kDa on the gel (Fig. 5, lane 2), a finding that was later confirmed by a Western blot analysis (Fig. 5, lane 2'). This indicated that the antiserum, which was provided with the fusion proteins (MBP-RR2), indirectly confirmed the production of RR2 proteins by *E. coli*. The molecular weight differences of the RR2 proteins that were produced in the *E. coli* system and Vero cells may be caused by the modification process in the cells. This result indicates that the eukaryotic proteins are stably modified in eukaryotic cells.

The cloning, sequencing, and expression in *E. coli* of RR-2 of the HSV-2 strain G were investigated. This information will provide a basis for a greater understanding of the molecular biology of the RR2 gene and enzyme.

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