High level expression in 293 cells of the herpes simplex virus type 2 ribonucleotide reductase subunit 2 using an adenovirus vector

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The herpes simplex viruses (HSV-1 and HSV-2) encode a ribonucleotide reductase consisting of two non-identical subunits (RR1 and RR2) which associate to form the active holoenzyme. To facilitate the purification and subsequent biochemical characterization of this enzyme, we have cloned the small subunit 2 of the HSV-2 ribonucleotide reductase (RR HSV-2) in a helper-independent adenovirus type 5 vector under the control of the adenovirus type 2 major late promoter. After infection of 293 cells with the recombinant virus, the amount of RR HSV-2 protein produced was eightfold higher than in HSV-2-infected cells. The specific activities of the RR HSV-2 recombinant subunit and the RR HSV-2 protein in HSV-2-infected cells were determined by their mixing with saturating amounts of isolated RR HSV-1 subunit. By comparison of the relative amount of each RR HSV-2 subunit with its specific activity, we calculated that the recombinant protein intrinsic activity was similar to that of the protein produced in HSV-2-infected cells. These results demonstrated that the adenovirus expression vector is a good system to produce an active RR HSV-2 subunit in fairly high amounts.

Introduction

Ribonucleotide reductase is an essential enzyme for the synthesis of deoxyribonucleotides in prokaryotic and eukaryotic cells. In nearly all systems, two different subunits (RR1 and RR2) associate to form the active enzyme (Thelander & Reichard, 1979). Herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2) encode their own RR HSV (136K) and RR HSV (38K) subunits which associate tightly to form the active holoenzyme (Cohen et al., 1985; Dutia, 1983; Huszar et al., 1983). Attempts to purify HSV ribonucleotide reductases to homogeneity have so far been largely unsuccessful. However partially purified preparations have been used to demonstrate that the enzyme has altered properties compared to the bacterial and mammalian isoenzymes. The tight association of the two subunits is independent of Mg2+, and its enzymic activity which does not require ATP is insensitive to dTTP and dATP inhibition (Langelier & Buttin, 1981; Huszar & Bacchetti, 1981; Averett et al., 1983, 1984; Cohen et al., 1985). Recent in vivo experiments with a mouse model have suggested that, as this enzyme is essential to the virus pathogenicity, it could be a good target for specific inhibitors of HSV replication (Cameron et al., 1988; Jacobson et al., 1989).

Our laboratory, and others (Cohen et al., 1986a; Dutia et al., 1986), have demonstrated that HSV ribonucleotide reductases are specifically inhibited by a nonapeptide which corresponds to the carboxy-terminal sequence of the RR HSV subunit. The finding that the specific binding of the nonapeptide to the RR HSV subunit prevents association of the two subunits, demonstrates that the carboxy-terminal sequence of RR HSV is involved in the interaction with RR HSV (McClements et al., 1988; Paradis et al., 1988). These observations have led to the attractive suggestion that the association of the two viral subunits could be a specific target for antiviral chemotherapy. A knowledge of the tertiary structure of the HSV ribonucleotide reductase holoenzyme would be very useful for the development of inhibitory peptides with greater potency. However these structural studies have been hampered due to the lack of large amounts of purified proteins. Attempts to express enzymically active HSV RR1 or RR2 subunits separately in, respectively, cultured human cells using a recombinant adenovirus vector or in Escherichia coli have been reported (Huang et al., 1988; Ingemarson et al., 1989), but the yield and activity of these recombinant proteins were not greater than in HSV-infected cells. Here we report a recombinant adenovirus expression vector that produced eightfold more RR HSV-2 protein than HSV-2-infected cells. Moreover by means of an efficient method for obtaining
RRHSV preparations devoid of RRHSV, we could determine that the recombinant protein has an intrinsic activity identical to that of the protein synthesized in HSV-2-infected cells.

**Methods**

**Cells and viruses.** 293 cells and BHK-21/C13 cells were grown at 37 °C in, respectively, Dulbecco’s modified Eagle’s medium (DMEM) or α-modified Eagle’s medium supplemented with 10% foetal bovine serum, glucose and antibiotics. Ad5ΔE1ΔE3 is an adenovirus type 5 strain from which the E1 and E3 transcription units had been deleted (Gluzman et al., 1982). These adenoviruses and Ad5-derived recombinant viruses were propagated by infecting 293 cells, a human embryo kidney cell line expressing Ad5 E1A and E1B functions (Graham et al., 1977). Virions were released from the infected cells by three cycles of freezing and thawing. HSV-1 (strain F) and HSV-2 (strain HG52) stocks were produced in BHK-21/C13 cells using a low m.o.i. as described previously (Langelier et al., 1978).

**Construction of plasmids and recombinant viruses.** Plasmid pAdBM1 was derived from pPyMT-2 (Davidson & Hassell, 1987; Logan & Shenk, 1984) by sequentially subcloning a 590 bp Sau3AI fragment containing the Ad5 hexon polyadenylation signal at the BglII site located at the 3’ end of the polyomavirus (PyV) middle T antigen coding sequences, and then a 750 bp Xhol-XbaI fragment from pD1 (Berkner et al., 1987) in replacement of the Xhol-XbaI fragment containing most of the PyV middle T antigen. Thus pAdBM1 contains DNA segments which, going counter-clockwise, include (Fig. 1): the EcoRI-SalI large fragment of the E. coli replicon pML2 (Lusky & Botchan, 1981); Ad5 sequences from 15.5 to 9.4 map units (m.u.); the Ad2 major late promoter; a cDNA copy of the entire Ad2 tripartite leader sequence including the 5’ splicing donor site of the third leader and a 3’ acceptor site from an immunoglobulin gene; a unique BamHI cloning site; the simian virus 40 (SV40) late polyadenylation signal and a 3’ acceptor site from an immunoglobulin gene; a unique HindIII site (nucleotide 2522) to the XbaI site (nucleotide 2533) of the MLP to the BclI site (nucleotide 2770); pBR322 sequences (nucleotides 375 to 650); the PyV early polyadenylation signal from the XbaI site (nucleotide 2522) to the HindIII site (nucleotide 2962); the Ad5 hexon polyadenylation signal from 64.0 to 62.3 m.u. and the 5’ 356 bp or 1.0 to 0 m.u. of the Ad5 genome.

The cloning of the RRHSV gene into pAdBM1 proceeded as follows (Fig. 1). The sequence encoding RRHSV was isolated as a 2.2 kb Xhol fragment from the HSV-2 genome and cloned into the unique Xhol site of the plasmid pSV103 (Paterson et al., 1985) to generate the plasmid pSVRRHSV. We then cloned a synthetic double-stranded 16mer oligonucleotide (5’-GATC[AGATCT]GCCATG-3’) containing the ATG in-frame and created a unique BglII site in adjacent upstream sequences. The fragment containing the RRHSV coding sequence and including the SV40 polyadenylation signal found in pSV103, was then released from pSVRRHSV by BglII-BstI double digestion and then inserted into the unique BamHI site of pAdBM1.

As shown in Fig. 2, the resulting plasmid, pAdBM1-RRHSV, was used to generate recombinant virus by *in vitro* homologous recombination between the overlapping sequences on the linearized plasmid pAdBM1-RRHSV and the large 3’ fragment of the Ad5 genome (Berkner & Sharp, 1983). In a typical experiment, 2 μg of pAdBM1-RRHSV linearized with Clai was mixed with 2 μg of Clai-cut Ad5/ΔE1ΔE3 viral DNA and 6 μg of calf thymus DNA. This DNA mixture was transfected onto 60 mm diameter dishes of subconfluent 293 cells using the calcium phosphate technique (Graham & Van Der Eb, 1973). After the appearance of c.p.e., the culture was harvested and the viral lysates were subjected to plaque purification. Viral plaques were picked and amplified by infecting 293 cells in 24-well plates (at a cell density of 10⁶ cells per well). Recombinants were identified by restriction endonuclease analysis of viral DNA extracted from infected cells by the Hirt procedure (Hirt, 1967). After positive identification, the Ad5-RRHSV recombinant virus was further plaque-
purified and virus stocks were prepared by infecting confluent 293 cells.

**Protein analysis.** Confluent 293 cells (2 x 10^6) in 60 mm diameter dishes were infected with HSV-2, Ad5/AE1/AE3, or Ad5 derived recombinant virus at a multiplicity of 20 p.f.u./cell. At different times post-infection (p.i.), cells were harvested and lysed on ice in 100 mM-NaCl, 100 mM-Tris-HCl pH 8.0, 0.5% NP40 and the protease inhibitors 1 mM-PMSF, 10 μg/ml leupeptin, 0.3 trypsin inhibitor units/ml aprotonin. Proteins were separated by SDS-PAGE (Laemmli, 1970) and then stained with Coomassie blue or, for immunoblotting, were electrophoretically transferred to nitrocellulose paper in 25 mM-Tris-HCl pH 8.3 and 192 mM-glycine (Towbin et al., 1979). The nitrocellulose was then incubated with P9 serum (diluted 1:500 with 5% milk in phosphate-buffered saline (PBS)), a polyclonal rabbit serum raised against a synthetic nonapeptide corresponding to the carboxy terminus of the RR_HSV subunit (Cohen et al., 1986b), washed in PBS including 0.1% Triton X-100 and further incubated with 0.1 μg/ml of 125I-labelled protein A (Amersham).

To measure the rate of synthesis of the RR_HSV protein, confluent 293 cells were infected with Ad5-RR_HSV, Ad5/AE1/AE3, or HSV-2. At selected times after infection, cells were labelled with 30 μCi/ml of [35S]methionine (Amersham) in methionine-free medium for 2 h. The labelling of some dishes of infected cells was chased for 6 h by the replacement of the labelling medium for fresh normal medium. Protein extracts were prepared as described above, separated by SDS-PAGE and analysed after autoradiography.

**Assays for ribonucleotide reductase activity.** HSV-1 or HSV-2 ribonucleotide reductases were partially purified from quiescent BHK-21/C13 cells infected, respectively, with strains F or HG52 whereas the RR_HSV recombinant protein was obtained from confluent 293 cells infected with Ad5-RR_HSV recombinant virus, as described previously (Cohen et al., 1985; Huszar & Bacchetti, 1981). Protein concentration was measured by the Coomassie blue staining method of Bradford (1976) using serum albumin as a standard.

Ribonucleotide reductase activity was assayed by monitoring the reduction of [3H]CDP for 30 min in a final volume of 60 μl, as detailed previously (Cohen et al., 1985). The standard reaction mixture contained 50 mM-HEPES pH 7.8, 4 mM-sodium fluoride, 20 mM-DTT, 50 μM-CDP, 0.25 μCi of [3H]CDP (and 1 mM-bacitracin when inhibitory peptide was added to the assay). One unit of ribonucleotide reductase is defined as the amount of enzyme generating 1 nmol of dCDP/h under the standard assay conditions.

**Slot blot densitometric quantification of RR_HSV protein.** Partially purified HSV-2 ribonucleotide reductase and RR_HSV recombinant protein extracts were serially diluted in 20 mM-Tris·HCl pH 7.5 and 500 mM-NaCl. The diluted amounts of protein were fixed on nitrocellulose paper using a slot blot apparatus. The nitrocellulose was then incubated with P9 serum (1:500), washed and reacted with 125I-labelled protein A as described above. The relative amount of RR_HSV protein was determined by densitometry with a LKB Ultrascan XL instrument.

**Results**

**Construction of the Ad5-RR_HSV recombinant virus**

To produce the RR_HSV subunit at high levels in mammalian cells, we have constructed a helper-free recombinant adenovirus, Ad5-RR_HSV, expressing the RR_HSV coding sequence under the control of the Ad2 major late promoter in the opposite transcriptional orientation from the overall direction of late gene transcription. The construction of the Ad5-RR_HSV hybrid virus proceeded in two major steps. The first step involved the subcloning of the RR_HSV gene into the adenovirus shuttle plasmid pAdBM1 (Fig. 1). To achieve optimal expression, the junction between the coding region of the RR_HSV subunit and the expression vector was engineered such that the ATG was positioned next to the splice donor and acceptor site of the vector. The resulting plasmid, pAdBM1-RR_HSV, contained a hybrid transcription unit which consists of the Ad2 major late promoter, a cDNA copy of the entire Ad2 tripartite leader, a splice donor and acceptor site, and the complete genomic segment encoding the RR_HSV protein followed by several polyadenylation signals. Multiple polyadenylation signals were included at the 3′ end of the transcription unit to ensure efficient polyadenylation of the majority of the mRNAs, since transcription from the strong Ad2 major late promoter often leads to read-through transcripts as seen in a number of other recombinant adenoviruses (Alkhatib & Briedis, 1988; Alkhutib et al., 1988; Berkner & Sharp, 1984; Davidson & Hassell, 1987).

The second step consisted of the rescue of the hybrid transcription unit into infectious Ad5 by cotransfecting, in 293 cells, the plasmid pAdBM1-RR_HSV linearized at the unique ClaI site, with ClaI-cut Ad5/AE1/AE3 viral DNA (Gluzman et al., 1982) (Fig. 2). DNA extracted from 13 out of 24 plaque isolates had the structure corresponding to the genome of Ad5-RR_HSV depicted at the bottom of Fig. 2, whereas the remaining viruses had the structure of Ad5/AE1/AE3 as determined by restriction endonuclease analysis followed by Southern blot hybridization with the appropriate DNA probes (data not shown). The genome of Ad5-RR_HSV, which was approximately 1% smaller than wild-type Ad5, remained stable upon further rounds of plaque purification and amplification to produce large stocks of virus. Similarly to its parent, Ad5/AE1/AE3, the recombinant virus grew routinely to high titres in 293 cells (> 10^9 p.f.u./ml).

**Expression of the RR_HSV protein in recombinant adenovirus-RR_HSV-infected cells**

To study the synthesis of RR_HSV protein, protein extracts were prepared from 293 cells infected with...
Ad5/ΔE1ΔE3, Ad5–RR<sub>HSV</sub> or HSV-2 at various times after infection and then subjected to SDS-PAGE. As shown in Fig. 3(a), where the resolved proteins were stained with Coomassie blue, the recombinant adenovirus synthesized a new polypeptide in large amounts (lanes 3 to 7) that was detected in neither the Ad5/ΔE1ΔE3-infected (lane 2) nor in the mock-infected cells (lane 1). This protein of 38K has the same electrophoretic mobility as the RR<sub>HSV-2</sub> protein produced in HSV-2-infected cells (lanes 8 and 9). The amount of recombinant protein increased until 30 h p.i. and reached a plateau thereafter, as expected for a protein expressed under the control of the Ad2 major late promoter. By densitometric scanning of the stained bands, this maximal amount was established to be 4% of the total intracellular proteins and contrasted with the low level of the RR<sub>HSV-2</sub> protein synthesized in HSV-2-infected cells which was barely detectable by Coomassie blue staining.

To confirm the identity of the RR<sub>HSV-2</sub> recombinant polypeptide, protein extracts were immunoblotted with P9 serum. As shown in Fig. 3(b), this serum reacted with an identical polypeptide in either HSV-2- or Ad5–RR<sub>HSV</sub>-infected cells (lanes 8 and 9, and 3 to 7, respectively). As expected, none of the proteins reacted with the P9 serum in the mock-infected (lane 1) or the Ad5/ΔE1ΔE3-infected cells (lane 2). These results clearly demonstrated that the RR<sub>HSV-2</sub> recombinant protein produced at high levels in Ad5–RR<sub>HSV</sub>-infected 293 cells is similar to the ribonucleotide reductase RR<sub>HSV-2</sub> subunit synthesized in the HSV-2-infected cells.
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Fig. 4. Rate of synthesis of the RRHSV-2 protein in Ad5-RRHSV-infected cells. 293 cells were infected with Ad5/AEtAE (lanes 2 and 3), Ad5-RRHSV (lanes 4 to 7, 11 and 12) and HSV-2 (lanes 8 to 10). At various times after infection, they were labelled for 2 h with [35S]methionine, harvested or chased for 6 h in non-radioactive medium prior to harvesting. Protein extracts were resolved by SDS-PAGE and revealed by autoradiography. Lane 1, mock-infected cells; lanes 2 and 3, Ad5/AEtAE-infected cells, 20 h and 24 h p.i.; lanes 4 to 7, Ad5-RRHSV-infected cells, 12 h, 16 h, 20 h and 24 h p.i.; lanes 8 and 9, HSV-2-infected cells, 8 h and 20 h p.i.; lane 10, HSV-2-infected cells, 8 h p.i. plus chase; lanes 11 and 12, Ad5-RRHSV-infected cells, 16 h and 20 h p.i. plus chase. Mr markers are shown on the left.

Time course of RRHSV-2 polypeptide synthesis in Ad5–RRHSV-infected 293 cells

The rate of synthesis of the RRHSV-2 protein was analysed by a 2 h labelling of the infected cells with [35S]methionine at different times after infection, followed by SDS-PAGE. De novo synthesis of the RRHSV-2 recombinant protein was first observed at 12 h p.i. and rapidly increased up to 16 h. Thereafter, the rate of synthesis was maintained until 30 h (Fig. 4, lanes 4 to 7 and data not shown). In 293 cells infected with HSV-2, the synthesis of RRHSV-2 protein was maximal at 8 h p.i. and decreased until 20 h where its synthesis ceased (lanes 8 and 9) as previously reported (Huszar & Bacchetti, 1981). The maximum rate of synthesis of the RRHSV-2 recombinant protein was threefold higher than the rate observed in HSV-2-infected cells and was maintained over a longer period of time.

The in vivo stability of the recombinant protein was studied, at 16 h and 20 h p.i. by labelling the infected cells for 2 h followed by a chase in non-radioactive medium for 6 h. The results presented in Fig. 4 (lanes 11 and 12) indicated that the labelling of the recombinant polypeptide remained as stable as the protein synthesized in HSV-2-infected cells during the 6 h chase period for both time points. This showed that the recombinant protein was fairly stable. Therefore, we concluded that the sustained and high rate of synthesis of the stable RRHSV-2 recombinant subunit is responsible for its higher accumulation in Ad5–RRHSV-infected 293 cells than in the parallel infection by HSV-2.

Measurement of the RRHSV-2 recombinant protein activity

As extracts of 293 cells infected with Ad5–RRHSV contain only the small subunit of HSV-2 ribonucleotide reductase, they were completely inactive when the ribonucleotide reductase assays were performed in the absence of ATP and Mg2+ even with high amounts of extract (200 μg protein/tube). Hence, to measure the activity of the RRHSV subunit, a method was devised to obtain preparations of active RRHSV free of RRHSV. As P9 serum immunoprecipitates the RRHSV protein without co-immunoprecipitation of the RRHSV protein (Cohen et al., 1986b), its use easily provided supernatants depleted of RRHSV as demonstrated by immunoblotting (data not shown) and loss of reductase activity (Fig. 5). The addition of increasing amounts of RRHSV recombinant extracts to a small amount of such supernatants produced a rapid increase in reductase activity followed by a plateau which corresponds to the RRHSV specific activity (approx. 40 units/mg) (data not shown). This result indicates also that the recombinant small subunit was highly active. When comparable amounts of extracts of Ad5/AEtAE-infected cells (control) were added no enzymic activity was observed.

Fig. 5 illustrates an experiment done to measure the recombinant RRHSV specific activity using two amounts of recombinant extract (2 and 5 μg). The plateau value at 2 μg gave a specific activity of 330 units/mg, whereas at 5 μg it was more difficult to attain saturating amounts of the large subunit. One additional control performed in this experiment demonstrated that the measured reductase activity was virus-specified. Indeed, addition of 100 μM-acetyl RRHSV-(329 to 336) synthetic peptide to the mixture of the two subunits giving the highest specific activity completely abolished it. This peptide which by its specific binding to RRHSV impairs the formation of active HSV holoenzyme has no effect on
Table 1. Correlation between ribonucleotide reductase specific activity and amount of the RR\textsubscript{HSV-2}\textsuperscript{2} subunit

<table>
<thead>
<tr>
<th>Cell</th>
<th>Virus</th>
<th>Ad5-RR\textsuperscript{HSV-2} infected cell extract</th>
<th>Relative amount of RR\textsuperscript{HSV-2} protein*</th>
<th>CDP reductase specific activity of RR\textsuperscript{HSV-2} protein (units/mg)\dagger</th>
<th>Relative RR\textsuperscript{HSV-2} activity\dagger *</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-21/C13</td>
<td>HSV-2</td>
<td>-</td>
<td>1.0</td>
<td>36.0 (1.0)</td>
<td>1.0</td>
</tr>
<tr>
<td>293</td>
<td>Ad5-RR\textsuperscript{HSV-2}</td>
<td>No. 1</td>
<td>9.0</td>
<td>330.0 (9.2)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. 2</td>
<td>8.0</td>
<td>251.0 (7.0)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. 3</td>
<td>8.0</td>
<td>302.0 (8.4)</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Slot blot densitometric quantification of the RR\textsuperscript{HSV-2} protein was performed as described in Methods and the values are expressed in comparison with HSV-2-infected cell extract.
† RR\textsuperscript{HSV-2} specific activities are plateau values obtained with saturating amounts of isolated RR\textsuperscript{HSV-2} subunit prepared from HSV-1-infected BHK-21/C13 cells as described in Methods. Values in parentheses are expressed in relation to reductase specific activity of the RR\textsuperscript{HSV-2} protein from HSV-2-infected cell extract.
§ Relative activity is calculated as the ratio of relative specific activity to the relative amount of the RR\textsuperscript{HSV-2} protein.

Fig. 5. Measurement of the RR\textsuperscript{HSV-2} recombinant protein specific activity. Saturating amounts of the isolated RR\textsuperscript{HSV-1} subunit from HSV-1-infected BHK-21/C13 cell extract was added to 2 μg (●) or 5 μg (□) of the RR\textsuperscript{HSV-2} recombinant protein partially purified from Ad5-RR\textsuperscript{HSV-2} infected 293 cell extract, and CDP reductase was measured as described in Methods. (●) Reductase activity of isolated RR\textsuperscript{HSV-1} subunits without Ad5-RR\textsuperscript{HSV-2} infected cell extract. (△) Reductase activity obtained when 100 μM of synthetic peptide acetyl RR\textsuperscript{HSV-2}-(329 to 336) was added to the mixture containing 5 μg of Ad5-RR\textsuperscript{HSV-2} infected cell extract and 50 μg of isolated RR\textsuperscript{HSV-1} subunit.

Here we report the construction and characterization of a helper-free recombinant adenovirus that expresses the RR\textsuperscript{HSV-2} subunit. The RR\textsuperscript{HSV-2} recombinant protein accumulated at a level of approximately 4% of the total intracellular proteins. This high accumulation, which corresponds to an 8.5-fold overproduction compared to HSV-infected cells, results from a high rate of synthesis sustained over a period of at least 18 h in Ad5-RR\textsuperscript{HSV-2} infected cells compared with approximately 6 h in HSV-2-infected cells. Owing to a rapid and efficient procedure to obtain an RR\textsuperscript{HSV} preparation free of RR\textsuperscript{HSV} from HSV-infected cells, we could evaluate that the intrinsic activity of the recombinant protein is as high as for the protein produced in HSV-2-infected cells. Other strategies that we and others used previously to obtain preparations of large subunit free of small subunit have yielded preparations with very low specific activities (Cohen et al., 1985; Bacchetti et al., 1986; see also below).

The yield of recombinant protein obtained with our adenovirus vector is comparable to the best yields reported in the past few years with similar vectors expressing proteins such as PyV middle T antigen, dihydrofolate reductase (Berkner et al., 1987) or influenza virus nucleocapsid protein (Alonso-Caplen et al., 1988). However this expression system has not always been so efficient (reviewed in Berkner, 1988). For example, using a similar construction designated AdRed-1, Huang et al. (1988) reported a level of
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production of the RR\textsuperscript{HSV-2} subunit in 293 cells which can be estimated roughly as 0.1\% of total cellular proteins (i.e. approximately 40-fold lower than the level we obtained with Ad5–RR\textsubscript{2}⁡HSV\textsuperscript{2}). These authors presented results suggesting that this low yield could be attributed to an increased proteolytic degradation of the RR\textsuperscript{HSV-2} recombinant subunit compared to HSV-2-infected cells. Apart from the fact that the RR\textsuperscript{HSV-2} subunit seems to be more stable than the RR\textsuperscript{1}⁡HSV\textsuperscript{2} subunit in their respective adenovirus-infected cells, the rate of synthesis of the RR\textsubscript{2}⁡HSV\textsuperscript{2} subunit is clearly higher than that of the RR\textsuperscript{1}⁡HSV\textsuperscript{2} subunit. Therefore the higher level of accumulation of the RR\textsuperscript{HSV-2} subunit relative to the RR\textsuperscript{HSV-2} subunit might be due, at least in part, to better genetic expression of the Ad5–RR\textsuperscript{HSV} recombinant virus possibly due to differences in the construction of the two vectors. The vector carrying RR\textsuperscript{HSV-2} lacks polyadenylation signals downstream of the RR\textsubscript{1}⁡HSV\textsuperscript{2} coding sequences and Kozak’s consensus sequence, GCC (Kozak, 1986), in position –3 of the initiation translation site; these are both present in our RR\textsuperscript{HSV-2} vector. The absence of these two elements could have reduced the translation efficiency or the stability of the RR\textsuperscript{HSV-2} mRNA.

This adenovirus expression vector represents a major improvement on 293 or RAT-2 cell lines constitutively expressing the RR\textsuperscript{HSV-2} subunit under the control of its own promoter (Huang et al., 1988; A. Seguin & Y. Langelier, unpublished data). Indeed the level of RR\textsuperscript{HSV-2} protein produced in the best 293 and RAT-2 stable cell lines was respectively 16- and 40-fold lower than in Ad5–RR\textsubscript{2}⁡HSV\textsuperscript{2}-infected 293 cells and its activity was barely detectable. Furthermore the RR\textsuperscript{HSV-1} subunit was recently purified by immunoaffinity from an E. coli recombinant expressing the protein under the control of the tac promoter at a level of 30 μg/l of culture (Ingemanson et al., 1989). This low yield (approx. 0.1\% of total bacterial protein) will require 100 l of bacterial culture to obtain 1 mg of pure protein, the lower limit to start crystallographic studies. With the adenovirus system described here, 2.5 x 10⁷ infected 293 cells are sufficient to obtain the same amount of protein. In addition, as the protein is more abundant in the starting material (4\% compared to 0.1\%), it will be easier to purify in large amounts by classical chromatographic methods. Moreover, this eukaryotic system will allow the study of the importance of post-translational modifications such as phosphorylation on the biological activity of RR\textsuperscript{HSV} and also its interaction(s) with other cellular or viral proteins. Therefore, it is more advantageous in many respects than the prokaryotic system.

High level expression of the RR\textsuperscript{HSV-2} subunit, obtained in this present study, will greatly facilitate its purification to homogeneity. These preparations of the RR\textsuperscript{HSV-2} recombinant subunit have been useful already for measurement of the specific activity of the RR\textsuperscript{HSV} subunit in different extracts of infected cells or in the course of its purification. Furthermore, when both purified HSV-2 ribonucleotide reductase subunits are available, this system will be an invaluable tool for the design of better inhibitors of this essential viral enzyme.

We acknowledge the technical work of Claire Guibault and Johanne Fleurent, the secretarial assistance of Chantal Nault, the photographic work of Roger Duclos and Benoît Charon for drawings. We also thank Anne-Marie Mes-Masson for helpful discussions. This work was supported by a grant from la Société de Recherche sur le Cancer de Montréal. Nathalie Lamarche is a recipient of a studentship from la Société de Recherche sur le Cancer de Montréal.

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(Received 16 November 1989; Accepted 23 March 1990)