The rat cytomegalovirus R78 G protein-coupled receptor gene is required for production of infectious virus in the spleen


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The rat cytomegalovirus (RCMV) R33 and R78 genes are conserved within members of the subfamily Betaherpesvirinae and encode proteins (pR33 and pR78, respectively) that show sequence similarity with G protein-coupled receptors. Previously, the biological relevance of these genes was demonstrated by the finding that R33- and R78-deleted RCMV strains are severely attenuated in vivo. In addition, R78-deleted strains were found to replicate less efficiently in cell culture. To monitor of the expression of R33- and R78-encoded proteins, recombinant RCMV strains, designated RCMV33G and RCMV78G, were generated. These recombinants expressed enhanced green fluorescent protein (EGFP)-tagged versions of pR33 and pR78 instead of native pR33 and pR78, respectively. Here it is reported that, although RCMV33G replicates as efficiently as wt virus in rat embryo fibroblast cultures, strain RCMV78G produces virus titres that are 3- to 4-fold lower than wt RCMV in the culture medium. This result indicates that the pR78-EGFP protein, as expressed by RCMV78G, does not completely functionally replace its native counterpart (pR78) in vitro. Interestingly, in infected rats, infectious RCMV33G was produced in significantly lower amounts than infectious wt RCMV, as well as RCMV78G, in the salivary glands. Conversely, although RCMV33G replicated to similar levels as wt virus in the spleen, both RCMV78G and an R78 knock-out strain (RCMV ΔR78a) replicated poorly in this organ. Together, these data indicate that R78 is crucial for the production of infectious RCMV in the spleen of infected rats.

INTRODUCTION

Cytomegaloviruses (CMVs) employ a panoply of strategies that are aimed at subversion of antiviral defence mechanisms of their hosts. Among the CMV proteins that are likely to play a key role in some of these strategies are proteins that show sequence similarity with G protein-coupled receptors (GPCRs). It is generally believed that the CMV GPCR genes have been pirated by an ancestral virus during the long coevolution of pathogen and host. GPCRs form a large family of receptors that function in signal transduction through cell membranes. These proteins invariably consist of seven transmembrane helices that are connected by three intracellular and three extracellular loops. The majority of GPCRs activate G proteins and are capable of transducing a wide variety of messages. Within the genomes of all CMVs sequenced, genes have been identified that encode GPCR homologues. Human CMV (HCMV) carries four putative GPCR genes: US27, US28, UL33 and UL78 (Chee et al., 1990a, b; Gompels et al., 1995). Only two of these, UL33 and UL78, have homologues in each of the betaherpesvirus genomes sequenced currently (Bahr & Darai, 2001; Beisser et al., 1998; Chee et al., 1990a, b; Gompels et al., 1995; Liu & Biegalke, 2001; Nicholas, 1996; Rawlinson et al., 1996), which may reflect the biological relevance of these genes.

The biological significance of the UL33 family members has been demonstrated previously in studies using recombinant CMVs that carry either a disrupted UL33 (Margulies et al., 1996), M33 (Davis-Poynter et al., 1997) or R33 gene (Beisser et al., 1998) in their genomes. In cell culture, each of these mutant viruses replicated with similar efficiency as the corresponding wt viruses (Beisser et al., 1998; Davis-Poynter et al., 1997; Margulies et al., 1996). However, during in vivo infection, significant differences were observed between animals infected with the recombinants and those infected with the wt viruses. In contrast to their wt counterparts, M33- and R33-deleted viruses could not be detected within the salivary glands of infected mice and rats, respectively (Beisser et al., 1998; Davis-Poynter et al., 1997). This indicated that M33 and R33 play a role in virus dissemination to or replication in the salivary glands (Beisser et al., 1998;
Davis-Poynter et al., 1997). Furthermore, it was shown in the RCMV/rat model that R33 plays an important role in the pathogenesis of RCMV disease, since a significantly lower mortality was seen among rats infected with R33-deleted RCMV (RCMVAR33) than among those infected with wt RCMV (Beisser et al., 1998).

It has been established firmly that the members of the UL33 gene family encode GPCRs. The human herpesvirus type 6B (HHV-6B) member of the UL33 family, pUL12, was reported to be a calcium-mobilizing receptor for several CC chemokines (Isegawa et al., 1998). In addition, we found the RCMV R33-encoded protein to signal in a ligand-independent, constitutive fashion (Gruijthuijsen et al., 2002). Recently, similar activities have also been attributed to the murine CMV (MCMV) and HCMV counterparts of these proteins (Waldhoer et al., 2002). Like the UL33 family members, the UL78 gene family members were found to have important roles in the pathogenesis of infection. A significantly lower mortality was observed among rats infected with R78-deleted RCMV strains (RCMVAR78a and RCMVAR78c) than among animals infected with wt RCMV (Beisser et al., 1999). Additionally, cells infected with these recombinant viruses produced virus titers that were 10- to 100-fold lower than their wt counterpart. Similar observations have been made in the MCMV/murine model (Oliveira & Shenk, 2001). Despite the relatively low sequence similarity with known chemokine receptors, the HHV-6A pUL78 homologue (pUL51) was reported to bind several CC chemokines, such as CCL2, CCL5, CCL7, CCL11 and CCL13, as well as an HHV-8-encoded chemokine, vMIP-II (Milne et al., 2000). These binding characteristics strongly resemble those of the HHV-6B homolog of pUL33, pUL12. Nevertheless, signalling activities have hitherto not been identified for any other member of the UL78 family.

To monitor the expression of both pR33 and pR78 in vitro and in vivo, we set out to generate recombinant RCMV strains expressing either pR33-enhanced green fluorescent protein (EGFP) or pR78-EGFP instead of native pR33 and pR78, respectively. Here, we show that these recombinant viruses (RCMV33G and RCMV78G, respectively) differ from wt RCMV in various aspects of replication in vitro and in vivo. Most notably, while strain RCMV33G is defective in producing infectious virus in the salivary glands of infected rats, strain RCMV78G is incapable of producing virus progeny in the spleen. In all other organs and tissues tested, these strains replicate in a fashion indistinguishable from that of wt virus.

METHODS

**Cells and virus.** Primary rat embryo fibroblasts (REFs), the rat fibroblast cell line Rat2 (Rat2 TK−, ATCC CRL-1764) and the monocyte/macrophage cell line R2 were cultured as described previously (Bruggeman et al., 1982; Damoiseaux et al., 1994). REFs were utilized for the propagation of both wt RCMV (Maastricht strain; Bruggeman et al., 1982) and recombinant RCMV strains, as well as for virus titration by plaque assay (Bruggeman et al., 1985). Rat2 cells were utilized for transfection (Beisser et al., 1998) and confocal laserscan microscopy studies. RCMV DNA was isolated from culture medium as described by Vink et al. (1996).

**RCMV33G recombinant plasmid construction.** To generate an RCMV strain in which the R33 ORF is fused in-frame to the 5’ end of the EGFP ORF, a recombinant plasmid was generated. This plasmid, designated p388 (Fig. 1A), was constructed by cloning a 2.4 kb Nhel–XbaI fragment, containing both the EGFP ORF and a neo-mycin resistance gene (neo), into the XbaI site of plasmid p384. This fragment was designated the EGFP-neo cassette. A detailed description of the construction of p384 has been described previously (Gruijthuijsen et al., 2002). Plasmid p384 contains the complete R33 gene with its translation termination codon changed into a leucine codon and an XbaI site. The 2.4 kb Nhel–XbaI insert of p388 was derived from plasmid p374, which was generated as follows. First, a 1.4-kb fragment containing the simian virus 40 (SV40) early promoter and neo was amplified by PCR, using primers NEO.C-F and NEO.C-R (Table 1) and, as template, plasmid RcCMV (Invitrogen). The amplified fragment was digested with EcoRI/XbaI and cloned into EcoRI- and XbaI-digested pUC119, generating plasmid p370. Subsequently, the EGFP ORF was cloned upstream of the neo ORF. To this purpose, the EGFP ORF was amplified by PCR using primers GFP.CN-F and GFP.C-R (Table 1) and, as template, plasmid p368. Plasmid p368 was derived from vector pEGFP-N1 (Clontech) by deletion of the 51 bp BamHI–BglII fragment. The 1.1-kb PCR fragment containing the EGFP ORF was digested with EcoRI and cloned into EcoRI-digested p370, resulting in plasmid p374. The integrity of all DNA constructs was verified by sequence analysis.

**RCMV78G recombinant plasmid construction.** To generate an RCMV strain in which the R78 ORF is fused in-frame to the 5’ end of the EGFP ORF, a recombinant plasmid, designated p390 (Fig. 1A), was generated as follows. First, the XbaI site of pUC119 was destroyed by digestion of this vector with XbaI, treatment of the linearized vector with the Klenow fragment of DNA polymerase I (Klenow) in the presence of dNTPs, followed by ligation using T4 DNA ligase. The resulting plasmid was called pre-p377. Next, the RCMV Xbal B fragment, which was cloned in vector pSP62-PL by Meijer et al. (1986), was digested with Ncol, and a 4.6-kb fragment, containing the complete R77, R78 and R79 ORFs (positions 96,996–101,556 of the RCMV genome; Vink et al., 2000), was treated with Klenow and cloned into the HinflI site of plasmid p377, generating plasmid p377. To enable the in-frame fusion of the EGFP ORF to the 3’ end of the R78 ORF, the R78 stop codon was altered into an XbaI restriction site using the following PCR-based procedure. First, the sequence downstream of the R78 stop codon was amplified by PCR with primers R79.C-F and R79.C-R (Table 1), using p377 as a template. The resulting 358 bp PCR product was treated with T4 DNA polymerase and then digested with XbaI. Then, the XbaI-blunt PCR product was cloned into pUC119, which had been successively treated previously with HinflI, Klenow and XbaI. The resulting plasmid was termed pre-p382. Next, the 3’ end of the R78 ORF was amplified by PCR with primers R78.C-F and R78.C-R (Table 1), using p377 as a template. The amplified 667 bp fragment was treated with T4 DNA polymerase and then digested with XbaI. The resulting blunt-XbaI fragment was cloned into pre-p382 using the filled-in EcoRI site of the polylinker and the newly introduced XbaI site at the 3’ end of the R78 ORF, generating plasmid p382. Finally, the 682 bp Ascl–Clal fragment from p377 was exchanged for the Ascl–Clal fragment from p382, resulting in plasmid p386. Next, the 2.4-kb Nhel–XbaI fragment from plasmid p374 (see above) was cloned into the newly introduced XbaI site at the 3’ end of ORF R78 in p386, resulting in plasmid p390. In this plasmid, the EGFP ORF is fused in-frame to the 3’ terminus of the R78 ORF. The integrity of all DNA constructs was verified by sequence analysis.
Fig. 1. Construction of RCMV strains RCMV33G and RCMV78G. (A) Recombinant strains were generated by homologous recombination between RCMV genomic DNA and plasmids containing an EGFP-neo cassette. The RCMV genome is represented by a black line at the top of the diagram. The R33 and R78 loci are highlighted below the genome. The ORF positions, sizes and orientations are indicated by white arrows. Consensus polyadenylation sequences closest to the 3’ end of either R33 or R78 are indicated by black triangles. Recombinant plasmid diagrams are indicated below the R33 and R78 loci. ORFs within the EGFP-neo cassette are indicated by black arrows. (B) Location of NcoI restriction sites at the R33 locus within the genomes of wt RCMV (top) and RCMV33G (bottom). Black boxes indicate the locations that correspond with each of the probes used for hybridization. NcoI restriction sites and predicted lengths of restriction fragments are indicated below each of the loci. (C) Chemiluminescence exposure from a Southern blot of NcoI-treated wt RCMV DNA (lanes 1 and 3) and RCMV33G DNA (lanes 2 and 4) hybridized with either the PvuI probe (lanes 1 and 2) or the EGFP-neo probe (lanes 3 and 4). The lengths of the fragments detected are indicated on the left in kb. (D) Location of NcoI restriction sites at the R78 locus within the genomes of wt RCMV (top) and RCMV78G (bottom). (E) Chemiluminescence exposure of a Southern blot containing NcoI-treated wt RCMV DNA (lanes 1 and 3) and RCMV78G DNA (lanes 2 and 4) hybridized with either the NcoI probe (lanes 1 and 2) or the EGFP-neo probe (lanes 3 and 4).
Table 1. PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Restriction site</th>
<th>Source</th>
<th>Position</th>
<th>Orientation</th>
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<tbody>
<tr>
<td>NEO.C-F</td>
<td>CTTTCTGATTATCAAACGGGTTGGTACC</td>
<td>XbaI</td>
<td>R/CMV</td>
<td>1804–1833</td>
<td>+</td>
</tr>
<tr>
<td>NEO.C-R</td>
<td>TCATCTAGATGGGGTGAGAAGACTC</td>
<td>XbaI</td>
<td>R/CMV</td>
<td>3201–3228</td>
<td>–</td>
</tr>
<tr>
<td>GFP-CN-F</td>
<td>ATAAAGATTCGATGCGGCTAGGCTACG</td>
<td>EcoRI</td>
<td>pEGFP-N1</td>
<td>574–603</td>
<td>+</td>
</tr>
<tr>
<td>GFP.C-R</td>
<td>AGCGAATTCGATGCGGCTAGGCTACG</td>
<td>EcoRI</td>
<td>pEGFP-N1</td>
<td>1622–1659</td>
<td>–</td>
</tr>
<tr>
<td>R78.C-F</td>
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<td>XbaI</td>
<td>R/CMV genome</td>
<td>99860–99884</td>
<td>+</td>
</tr>
<tr>
<td>R78.C-R</td>
<td>AATAATCAGGAACGCGCTTCGGCG</td>
<td>XbaI</td>
<td>R/CMV genome</td>
<td>10053–100526</td>
<td>–</td>
</tr>
<tr>
<td>R79.C-F</td>
<td>CTTTCTGATTATCAAACGGGTTGGTACC</td>
<td>XbaI</td>
<td>R/CMV genome</td>
<td>10051–100540</td>
<td>+</td>
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<tr>
<td>R79.C-R</td>
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<td>XbaI</td>
<td>R/CMV genome</td>
<td>101048–101068</td>
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<td>RCMVfor</td>
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<td>XbaI</td>
<td>RCMV genome</td>
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<td>+</td>
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<td>RCMVpro</td>
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<td></td>
<td>RCMV genome</td>
<td>172826–172850</td>
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Generation of strains RCMV33G and RCMV78G. Approximately 1×10^6 Rat2 cells were trypsinized and centrifuged for 5 min at 500 g. The cells were resuspended in 0.5 ml culture medium, after which 10 μg of either plasmid p388 or p390 was added. The suspension was transferred to a 0.4 cm electroporation cuvette (Bio-Rad) and pulsed at 0.25 kV and 500 μF in a Bio-Rad Gene Pulser electroporator. Cells were then seeded in T75 culture flasks. At 14 h after transfection, the cells were infected with low-passage RCMV at an m.o.i. of 1. The culture medium was supplemented with 50 μg G418 ml⁻¹ at 16 h post-infection (p.i.). Recombinant viruses were cultured on REF monolayers and plaque-purified as described earlier (Beisser et al., 1998, 1999, 2000).

Southern blot hybridization. DNA isolated from wt RCMV, RCMV33G and RCMV78G was digested with NcoI, electrophoresed through a 1% agarose gel and blotted onto a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech), as described previously (Brown, 1993). The integrity of genomic DNA from strain RCMV33G was checked using plasmids p375 (containing R32, R33 and R34 sequences) and p374 (containing EGFP and neo sequences) as probes. The integrity of the RCMV78G DNA was verified using p377 (which contains R77, R78 and R79 sequences) and p374 as probes. Hybridization and detection experiments were performed with digoxigenin DNA-labeling and chemiluminescence detection kits (Roche).

Isolation of poly(A)^+ RNA and Northern blot hybridization. Poly(A)^+ RNA was isolated from wt RCMV-, RCMV33G- or RCMV78G-infected REFs (m.o.i. of 0.01) at day 6 p.i. using a QuickPrep Micro mRNA Purification kit (Amersham Pharmacia Biotech), according to the manufacturer’s protocol. Electrophoresis of RNA under denaturing conditions and transfer to Hybond-N membranes have been described previously (Brown & Mackey, 1997). The blots were hybridized with probes specific for R32, R33, R34, R77, R78, R79–80, EGFP, neo or the SV40 early antigen promoter. The 426 bp BamHI, 976 bp ScaI, 501 bp Asp718–HindIII, 1432 bp BamHI–BglII, 734 bp Ncol–EcoRI and 1380 bp EcoRI–XbaI fragments from plasmid p388 were used as R32-, R33-, R34-, R79–80-, EGFP- and neo-specific probes, respectively. R77- and R78-specific probes were generated as described previously by Beisser et al. (1999). Hybridization and detection experiments were performed with digoxigenin DNA labelling and chemiluminescence detection kits (Roche).

Western blot analysis. REF cells were infected with wt RCMV, RCMV33G or RCMV78G at an m.o.i. of 0.01. At day 6 p.i., cells were harvested and resuspended in lysis buffer [150 mM NaCl, 50 mM NaF, 25 mM Tris/HC1 pH 7.5, 2 mM EDTA, 1% (w/v) NP-40]. Subsequently, lysates from 3×10^6 infected cells were separated by 12% SDS-PAGE, essentially according to the Laemmli method. The gel was transferred to a nylon filter (NYTRAN NY 12 N; Schleicher & Schuell) and incubated successively with rabbit anti-EGFP polyclonal antiserum (Living Colours A.v. Peptide Antibody; Clontech) and peroxidase-conjugated, goat anti-rabbit immunoglobulins (Dako). The blot was developed using a luminescent detection system (ECL; Amersham Pharmacia Biotech).

Confocal laserscan microscopy. Rat2 cells were grown on glass coverslips and infected with wt RCMV, RCMV33G or RCMV78G. At several time-points p.i., the cells were fixed for 20 min with 3:7% paraformaldehyde in PBS. Confocal laserscan microscopy images were collected at wavelengths of 488 nm using a MRC 600 confocal microscope equipped with an oil immersion objective (40× magnification, numerical aperture=1.3; Bio-Rad), as described previously (Broers et al., 1999). Digital images were processed using Confocal Assistance software from Bio-Rad.

Dissemination of wt RCMV, RCMV33G and RCMV78G in vivo. Male specific-pathogen-free Lewis/M rats (Central Animal Facility, Maastricht University, Maastricht, The Netherlands) were kept under standard conditions (Stals et al., 1990). All experimental protocols mentioned in this paper were approved by the Maastricht University Animal Experiments Committee and were consistent with the Dutch Laboratory Animal Care Act. Five groups of 12 male specific-pathogen-free Lewis/M rats (7 weeks old, 250–300 g body weight, immunosuppressed 1 day before infection) were infected with 9×10^5 p.f.u. wt RCMV, RCMV33G, RCMV33AR33, RCMV78G or RCMV78AR78. On days 5 and 28 p.i., six rats from each group were sacrificed and their internal organs were collected. These organs were subjected to immunohistochemical analysis, plaque assay (as described previously by Bruggeman et al., 1982) and quantitative PCR as described below. The plaque test and PCR data were analysed statistically by applying the Mann–Whitney U-test using SPSS (SPSS International).

Real-time quantitative PCR. Total cellular DNA was extracted from the salivary glands, spleen, kidneys, liver, lungs, heart, pancreas and thymus as follows. Frozen tissue (approximately 4 mm^3 in size)
was lysed in lysis buffer (100 mM NaCl, 10 mM Tris/HCl pH 8·0, 25 mM EDTA, 0·5 % SDS) supplemented with 50 ng proteinase K ml⁻¹ (Roche) and 5 μg RNAse A ml⁻¹ (Amershams Pharmacal Biotech), followed by homogenization and incubation for 30 min at 56 °C. Next, DNA was extracted with phenol/chloroform (1:1) and ethanol-precipitated. Before the samples were subjected to real-time PCR, they were analysed by both agarose gel electrophoresis, to establish their integrity, and spectrophotometry, to determine their DNA concentrations. The sequences of the TaqMan primers (RCMVfor and RCMVrev; Table 1) and that of the TaqMan probe (RCMVpro; Table 1) used to quantify CMV were selected from the immediate-early 1 (IE1) gene with Primer Express software, version 2·0 (Perkin Elmer). The TaqMan probe selected between the primers was fluorescently labelled at the 5’ end with FAM, as the reporter dye, and at the 3’ end with TAMRA, as the quencher (Table 1). PCR was performed with 12·5 μl TaqMan Universal PCR Master mix (Perkin Elmer), 900 nM forward primer, 300 nM reverse primer, 125 nM TaqMan probe and 100 ng sample DNA in a total volume of 25 μl. PCR was performed in 96-well microtitre plates under the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by 42 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were analysed using the ABI PRISM 7000 Sequence Detection System software (Perkin Elmer). For quantification, standard curves were generated using dilutions of RCMV DNA preparations of known concentration.

RESULTS

Generation of RCMV strains with EGFP genes fused at the 3’ end of either R33 or R78

We demonstrated previously that RCMV pR33 is a GPCR that signals in a constitutive fashion in cells transfected with an expression construct containing R33 (Grujthuijsen et al., 2002). In addition, we found that a modified version of this protein, containing EGFP at its cytoplasmic C-terminal tail, possesses similar signalling activities as native, nonfused pR33 (Grujthuijsen et al., 2002). This indicated that the EGFP tag at the C terminus of pR33 does not interfere with its signalling properties and, hence, that EGFP may be a suitable tag to study pR33 expression in vivo. To investigate the expression of both pR33 and pR78 during RCMV infection, we set out to generate two recombinant RCMV strains, which express either pR33-EGFP or pR78-EGFP instead of native pR33 and pR78, respectively. To create these strains, we first designed two plasmid constructs containing either the R33 or the R78 gene. Subsequently, the EGFP ORF was inserted into these plasmids at the 3’ end of either R33 or R78, such that their ORFs were fused in-frame to the EGFP ORF. The resulting R33-EGFP and R78-EGFP genes were then shuttled into the RCMV genome by homologous recombination between the plasmids containing these genes and the RCMV genome during infection in cultured fibroblasts (Fig. 1A). Recombinant virus was subsequently enriched for and plaque purified, as outlined in Methods. To verify the genomic integrity of the recombinant strains, Southern blot analysis was performed. First, we analysed the R33-EGFP-expressing recombinant strain, which was designated RCMV33G. DNA from both RCMV33G and wt RCMV was digested with NcoI, electrophoresed, blotted and hybridized with either an RCMV DNA-specific probe or an EGFP-specific probe. As shown in Fig. 1(B, C), the hybridization patterns observed with each of these probes were as predicted, both for wt RCMV and for RCMV33G. This indicated that (i) the R33 gene was replaced correctly by the R33-EGFP gene in the RCMV33G genome and (ii) the RCMV33G pool is plaque pure. The genomic integrity of the R78-EGFP-expressing strain, termed RCMV78G, was checked in a similar fashion as described above for RCMV33G. Fig. 1(D, E) shows that hybridization of NcoI-digested RCMV and RCMV78G DNA with either an RCMV DNA-specific or an EGFP DNA-specific probe yielded results indicating that (i) R78 was replaced properly by R78-EGFP in the RCMV78G genome and (ii) the RCMV78G pool is plaque pure. The correct genomic integrity of each of the recombinant strains was also confirmed by Southern blot hybridization of PstI-digested RCMV33G and RCMV78G DNA (data not shown).

Transcription of R33-EGFP, R78-EGFP and neighbouring genes

To evaluate transcription of R33-EGFP, R78-EGFP and their neighbouring genes, Northern blot analysis was performed with poly(A)⁺ RNA extracted from RCMV-, RCMV33G- and RCMV78G-infected cells. Two different sets of Northern blots were prepared. The first set contained RNA from wt RCMV- and RCMV33G-infected cells. These blots were treated with probes specific for R32, R33, R34, EGFP or neo (Fig. 2A, B). We found previously the RCMV genomic region containing R33 to be transcribed in a highly complex fashion (Beisser et al., 1998). Whereas R32 was reported to be transcribed as a single, major 2·5 kb mRNA (Beuken et al., 1999), numerous cotranscripts were identified that contained both R33 and R34 sequences (Beisser et al., 1998). In light of the expression of these large, 4–6 kb cotranscripts, it was to be expected that insertion of both the EGFP ORF and the neo expression cassette into the RCMV genome would have a significant impact on the transcription patterns of the R33 and R34 genes. Indeed, Fig. 2(C) shows clear differences between strain RCMV33G and wt RCMV in the expression of these genes. Most notably, strain RCMV33G expresses two unique transcripts, with lengths of 3·1 and 3·5 kb, respectively, which hybridize with both the R33- and EGFP-specific probe (Fig. 2C, lanes 1 and 3). These mRNAs are likely to represent transcripts for the pR33-EGFP fusion protein. In contrast to the R33 and R34 genes, the R32 gene was transcribed by both RCMV33G and wt virus in a similar fashion (Fig. 2C, lanes 1 and 2). Given the complexity of transcription of the RCMV R33–R34 genomic region, and in the absence of antibodies directed against either pR33 or pR34, it is difficult to predict the physiological consequences of the transcriptional differences between RCMV33G and wt RCMV. Therefore, we cannot exclude the possibility that potential differences in replicative potential between RCMV33G and wt virus, either in vitro or in vivo, can be attributed to differences in
transcription of genes other than, and downstream of, the R33 gene.

The second set of Northern blots contained RNA from wt RCMV- and RCMV78G-infected cells. These blots were treated with probes specific for either R77, R78, R79–R80, EGFP or neo (Fig. 2D, E). In accordance with our previous findings (Beisser et al., 1999), wt RCMV was found to express three different R78 transcripts, with lengths of 1·8, 3·7 and 5·7 kb (Fig. 2F, lane 13). Interestingly, strain RCMV78G also expresses three different R78 mRNAs, the sizes of which correspond roughly to the sum of the length of the EGFP expression cassette and the lengths of the respective wt RCMV R78 mRNAs (Fig. 2F, lane 14). Accordingly, each of the three R78 transcripts expressed by RCMV78G also hybridize with the EGFP-specific probe (Fig. 2D, lane 18). Contrary to strain RCMV33G, which expresses the neo gene predominantly as a cotranscript (Fig. 2C, lane 10), RCMV78G transcribes the neo gene into a major, monocstronic message of 1·2 kb (Fig. 2F, lane 20).

We conclude that the transcription of genes neighbouring R78 in the RCMV genome is not altered significantly by...
the introduction of the EGFP expression cassette at the 3’ terminus of the R78 ORF.

**Strain RCMV78G replicates less efficiently than wt RCMV in vitro**

In previous experiments, it was shown that deletion of the R33 gene from the RCMV genome does not affect virus replication in vitro (Beisser *et al.*, 1998). In contrast, the R78 gene was found to play a crucial role during in vitro replication, as 10- to 100-fold lower virus titres were recovered from cultures of cells infected with R78 null mutant RCMV strains (RCMVΔR78a and RCMVΔR78c) (Beisser *et al.*, 1999). To determine the impact of replacing the R33 and R78 genes in the RCMV genome with R33-EGFP and R78-EGFP, respectively, multi-step growth curves were generated for wt RCMV, RCMV33G and RCMV78G. As a control for attenuation due to R78 dysfunction, RCMVΔR78a was also included in these experiments. REFs were infected with either of these virus strains at an m.o.i. of 0.01. Subsequently, culture medium samples were taken at days 1, 3, 5 and 7 p.i. and subjected to plaque titration assays. Since R33-deleted virus was shown previously to replicate with a similar efficiency as wt RCMV in vitro, it was not surprising to find that strain RCMV33G also replicates in a similar fashion as wt virus (Fig. 3A). In contrast, strain RCMV78G was found to produce virus titres 3- to 4-fold lower than wt virus titres at days 5 and 7 p.i., respectively (P<0.05; Fig. 3B). Although the level of virus production of strain RCMV78G is significantly higher than that of RCMVΔR78a, which produces virus titres approximately 50-fold lower than wt virus at days 5 and 7 p.i. (P<0.05; Fig. 3B), these data indicate that strain RCMV78G may be attenuated, albeit moderately, in comparison with wt RCMV. Therefore, it is likely that the R78-EGFP gene can only partly functionally replace the R78 gene.

**pR33-EGFP and pR78-EGFP expression in vitro**

To assess the expression of the R33-EGFP- and R78-EGFP-encoded proteins (pR33-EGFP and pR78-EGFP, respectively) during infection in vitro, Rat2 cells were infected with wt RCMV, RCMV33G or RCMV78G and subjected to confocal laserscanning microscopy at various times p.i. Upon infection of the cells with RCMV33G, a focal green fluorescent signal was observed in the cytoplasm as early as 4 h.p.i. (Fig. 4). At early times after infection, between 4 and 8 h, a granular, perinuclear signal was seen in the RCMV33G-infected cells. This pattern of expression is very similar to that observed in COS-7 or Rat2 cells after transfection with R33-EGFP expression constructs (Gruijthuijsen *et al.*, 2002). However, a clear colocalization of the fluorescent signal with cell membranes, as seen previously in transfected cells, could not be observed in the RCMV33G-infected cells. At late times after infection (24 and 48 h), fluorescence was distributed evenly throughout the cells (Fig. 4).

After infection of Rat2 cells with RCMV78G, green fluorescence was seen first at 2 h.p.i. as a granular, perinuclear signal (Fig. 4). While the intensity of the signal increased between 4 and 48 h.p.i., granular distribution of the signal remained visible until 24 h.p.i. At 48 h.p.i., fluorescence was distributed more evenly throughout the RCMV78G-infected cells. As in RCMV33G-infected cells, a clear colocalization of green fluorescence with the cell membrane is not seen clearly in RCMV78G-infected cells. Nevertheless, such a colocalization is also absent in Rat2 cells transfected with R78-EGFP expression constructs, which give rise to an expression pattern highly similar to that seen in RCMV78G-infected cells at 8 h.p.i. (data not shown).

To characterize further the expression of pR33-EGFP and pR78-EGFP by the recombinant viruses, Western blot analysis was performed. First, protein extracts were made from REFs infected with either RCMV33G or RCMV78G. Subsequently, extracts were electrophoresed and blotted onto nitrocellulose filters, which were developed using commercial anti-EGFP antibodies. Contrary to the green fluorescent signal evident in RCMV33G-infected cells, as seen by confocal microscopy (Fig. 4), we observed a weak EGFP-specific signal in extracts of these cells using Western blot analysis (Fig. 5, lane 2). Accordingly, we were previously unable to detect any EGFP signal in extracts of transfected cells expressing pR33-EGFP by Western blot analysis, whereas the expression of this protein could be monitored easily under the microscope (Gruijthuijsen *et al.*, 2002). We hypothesize that pR33-EGFP forms large, insoluble aggregates which do not enter polycrylamide gels efficiently. Nevertheless, the weak EGFP band in Fig. 5 corresponds to a protein with an estimated molecular mass of approximately 73 kDa, which is similar to the predicted molecular mass of pR33-EGFP (71 kDa). This suggests that the R33-EGFP fusion gene within the RCMV33G genome expresses a full-length pR33-EGFP fusion protein.

In contrast to pR33-EGFP, pR78-EGFP could be detected

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**Fig. 3.** Strain RCMV78G replicates with a lower efficiency than wt RCMV. (A) Multi-step growth curves of wt RCMV (○) and RCMV33G (●). (B) Multi-step growth curves of wt RCMV (○), RCMV78G (■) and RCMVΔR78a (▲). Infection of REFs with either of the RCMV strains was performed at an m.o.i. of 0.01. Graphs indicate virus titres determined in samples taken from culture media at the times indicated p.i. Error bars show the SD for each of the virus titres.

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readily by Western blot analysis. By using anti-EGFP antibodies, a major polypeptide with an estimated molecular mass of approximately 75 kDa was observed in extracts from RCMV78G-infected cells (Fig. 5, lane 3). The estimated molecular mass of this major protein species corresponds with the calculated molecular mass of the pR78-EGFP fusion protein (78 kDa). Besides the ~75 kDa protein, several minor species of high molecular mass are also observed in extracts from RCMV78G-infected cells. It is likely that these polypeptides represent multimeric forms of pR78-EGFP, since multimerization is a phenomenon common to both GPCRs and green fluorescent proteins (Rios et al., 2001; Tsien, 1998).

Dissemination and replication of RCMV33G and RCMV78G in immunocompromised rats at 5 days after infection

To monitor the dissemination and replication of strains RCMV33G and RCMV78G, the following animal experiment was performed. Five groups of 7-week-old immunocompromised rats were infected with wt RCMV, RCMV33G, RCMVΔR33, RCMV78G or RCMVΔR78a. At days 5 and 28 p.i., six rats from each group were sacrificed and dissected. The salivary glands, spleen, kidneys, liver, lungs, heart,
pancreas and thymus were extracted and analysed by immunohistochemistry, plaque assay and quantitative PCR.

First, we set out to examine expression of either pR33-EGFP or pR78-EGFP in RCMV33G- and RCMV78G-infected rats, respectively. This was done by direct fluorescence microscopy and immunohistochemical analysis using both anti-pR44 antibodies (directed against an RCMV early phase protein; Kaptein et al., 2001) and anti-EGFP antibodies. We were unable to detect pR33-EGFP expression in solid organs, such as spleen and liver, at day 5 p.i. However, pR33-EGFP fluorescence was evident in cells from femoral bone marrow suspensions (Fig. 6A). These suspensions yielded 69 ± 50 (mean ± SD) pR33-EGFP-positive cells per 10⁶ bone marrow cells, similar to bone marrow suspensions from RCMV78G-infected rats, which yielded 121 ± 72 pR78-EGFP-positive cells per 10⁶ bone marrow cells. In contrast to the absence of pR33-EGFP-specific signals in spleen and liver tissue, overt expression of pR78-EGFP could be observed in these tissues in RCMV78G-infected rats (Fig. 6C, D). In additional, in double-stained sections of the spleen, pR78-EGFP could be detected not only in each pR44-positive cell (Fig. 6E) but also in pR44-negative cells in both spleen (Fig. 6F) and liver (Fig. 6G).

Similar to what was observed in RCMV78G-infected REFs in vitro, the subcellular distribution of pR78-EGFP in either bone marrow cells or spleen or liver sections appeared to be granular and predominantly perinuclear.

Next, the titres of infectious virus was determined by plaque assay. As shown in Table 2, at day 5 p.i., significant amounts of infectious virus were detected predominantly in the spleen. Interestingly, while virus was readily detectable in spleen samples from wt RCMV-, RCMV33G- or RCMVAR33-infected rats, virus could not be detected in spleen samples from RCMV78G- and RCMVAR78a-infected rats (Fig. 7A and Table 2). This indicated that strains RCMV78G and RCMVAR78a are unable to either enter or replicate in the spleen. To investigate this phenomenon further, viral DNA load in the spleen was determined by quantitative PCR. Surprisingly, viral DNA concentrations in spleen did not differ significantly between any of the five experimental groups (Fig. 7B). Taken together, these observations indicate that although RCMV78G and RCMVAR78a disseminate to the spleen with an efficiency similar to that of wt RCMV, these recombinant strains are not as efficient as wt virus in producing infectious virus. We conclude, therefore, that the R78 gene is important for efficient replication of RCMV in the spleen and that, in accordance with our previous findings, R78-EGFP is not able to functionally replace R78.

**Dissemination and replication of RCMV33G and RCMV78G in immunocompromised rats at 28 days after infection**

Tissue sections from rat organs sampled at day 28 p.i. were stained with anti-pR44 and anti-EGFP antibodies. At this time-point, both spleen and kidney sections from rats infected with wt RCMV and either of the mutant strains were found to be negative for pR44 and either pR33-EGFP or pR78-EGFP. In contrast, salivary glands were found to harbour pR44-positive cells in rats infected with wt RCMV, RCMV78G and RCMVAR78a but not in rats infected with RCMV33G or RCMVAR33 (data not shown). In additional, salivary gland sections from RCMV78G-infected rats were pR78-EGFP-positive by fluorescence microscopy (Fig. 6H).

Similar to what was observed in RCMV78G-infected REFs in vivo, the subcellular distribution of pR78-EGFP in salivary gland epithelial cells appeared to be granular and predominantly perinuclear.

At day 28 p.i., the salivary glands were the only sites to harbour infectious virus (Table 2). High virus titres were found in the salivary glands of wt RCMV-, RCMV78G- and RCMVAR78a-infected rats (Fig. 7C). These titres did not differ significantly between the three experimental groups. In accordance with previous findings (Beisser et al., 1998), infectious virus could not be detected in salivary gland samples from RCMVAR33-infected rats (Table 2 and Fig. 7C). Surprisingly, we were also unable to detect infectious virus in salivary gland samples of RCMV33G-infected animals (Table 2 and Fig. 7C). Thus, like RCMVAR33, RCMV33G is unable to either enter or replicate efficiently in the salivary glands. To study further the presence of recombinant viruses in the salivary glands, viral genomic DNA levels were assessed by quantitative PCR (Table 2 and Fig. 7D). Fig. 7(D) shows that approximately 4 × 10⁵ copies of viral DNA μg⁻¹ tissue were found in the salivary glands from wt RCMV-, RCMV78G- and RCMVAR78a-infected rats. Viral DNA loads between the three experimental groups did not differ significantly. Interestingly, viral DNA could only be detected in one of the six RCMV33G-infected rats and in none of the RCMVAR33-infected animals (Fig. 7D). Moreover, the virus titre within the single, RCMV33G-positive salivary gland sample was very low, i.e. 27 copies of viral DNA μg⁻¹ tissue (Fig. 7D). Taken together, these data show that strain RCMV33G is defective in replication in the salivary glands.

We conclude that both strain RCMV33G and strain RCMV78G have similar in vivo replication characteristics as their corresponding null mutant viruses (RCMVR33 and RCMVR78, respectively). Although this conclusion largely disqualifies RCMV33G and RCMV78G as useful tools for studying pR33 and pR78 expression in vivo, the characterization of these recombinant strains has revealed the crucial role of the R78 gene, which is conserved among all betaherpesviruses, in the production of infectious RCMV in the spleen. In addition, these studies have underlined the essential function of the second conserved GPCR gene of RCMV, R33, in salivary gland tropism.

**DISCUSSION**

EGFP has proven to be a versatile fusion partner for studying expression and trafficking of a variety of proteins, both
Among the proteins that have been characterized previously as EGFP fusions are three alpha-herpesvirus proteins, i.e. herpes simplex virus type 1 (HSV-1) major tegument protein VP22 (Elliot & O'Hare, 1999), HSV-1 viral envelope glycoprotein gH (Lorentzen et al., 2001) and the equine herpesvirus type 1 (EHV-1) UL34 gene product (Neubauer et al., 2002). The genes encoding the EGFP fusion proteins were shuttled into the viral genomes, after which the expression of the fusions was investigated in vitro. In light of our interest in the GPCR homologues of RCMV, pR33 and pR78, and because antibodies against these proteins are currently lacking, we decided to pursue an

Fig. 6. Expression of pR44, pR33-EGFP and pR78-EGFP in several organs from rats infected with either RCMV33G or RCMV78G. (A) Expression of pR33-EGFP in bone marrow cells from RCMV33G-infected rats. The figure shows photographs taken by fluorescence (490 nm) (left-hand panel) and phase contrast (right-hand panel) microscopy. (B) Expression of pR78-EGFP in bone marrow cells from RCMV78G-infected rats. Cells were photographed as described in (A). (C) Expression of pR78-EGFP in the spleen. Sections (6 μm) were photographed as described in (A). (D) Expression of pR78-EGFP in the liver. Sections were photographed as described in (A). (E) Expression of pR44 and pR78-EGFP in the spleen. Sections were treated with both anti-pR44 and anti-EGFP polyclonal antibodies. Anti-pR44 antibodies were detected using anti-mouse IgG antibodies conjugated with alkaline phosphatase (AP). Anti-EGFP antibodies were detected using anti-rabbit IgG antibodies conjugated with horseradish peroxidase (HRP). AP staining was performed with Fast Red (red) and HRP staining with diaminobenzidine (brown). (F) Expression of pR78-EGFP in pR44-negative cells in the spleen of RCMV78G-infected rats. Sections were stained as in (E). (G) Expression of pR78-EGFP in pR44-negative cells in the liver of RCMV78G-infected rats. Sections were stained as described in (E). (H) Expression of pR78-EGFP in salivary gland ductal epithelial cells of RCMV78G-infected rats. Sections were photographed as described in (A). The micrographs shown in (A)–(G) were taken from samples extracted at day 5 p.i., whereas the micrograph shown in (H) was taken from material extracted at day 28 p.i. Bars, 20 μm. The scale bar in (E) also applies to (F) and (G). The scale bar in (H) also applies to (A)-(D).
Table 2. The presence of infectious RCMV and viral DNA in organs from infected rats

Ratios indicate the number of organs found positive for virus, relative to the total amount of organs tested (one organ per rat). PA, Plaque assay; Sg, salivary gland; ND, not determined.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Liver</th>
<th>Pancreas</th>
<th>Thymus</th>
<th>Sg</th>
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<td>PA</td>
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<td>2/3</td>
<td>2/6</td>
<td>3/3</td>
<td>0/1</td>
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<td>4/4</td>
<td>0/3</td>
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<td>3/3</td>
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<tr>
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<td>6/6</td>
<td>0/3</td>
<td>3/3</td>
<td>0/6</td>
<td>3/3</td>
<td>ND</td>
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<tr>
<td>RCMV78G</td>
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<td>6/6</td>
<td>0/6</td>
<td>3/3</td>
<td>0/6</td>
<td>3/3</td>
<td>2/5</td>
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<tr>
<td>RCMVΔR78a</td>
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<td>5/5</td>
<td>0/3</td>
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approach to study both RCMV GPCR homologues as fusions with EGFP, in a manner similar to that described for HSV-1 VP22 and gH as well as for the EHV-1 UL34 gene product. Thus, two recombinant RCMV strains were constructed, RCMV33G and RCMV78G, which express pR33-EGFP and pR78-EGFP, respectively, instead of their native counterparts. To our knowledge, RCMV33G and RCMV78G represent the first betaherpesvirus strains that express EGFP-fused versions of viral proteins.

Previously, we reported that pR33 and pR33-EGFP possess similar constitutive signalling properties in vitro (Gruijthuijsen et al., 2002). Based on these results, we anticipated that the fusion of both pR33 and pR78 to EGFP would not affect the function of these proteins. However, we found that strain RCMV33G differed from wt virus in one major, but significant, characteristic: this recombinant strain is unable to either enter or replicate in the salivary glands of infected rats. A similar phenotype has been attributed previously to either enter or replicate in the salivary glands of infected rats. A similar phenotype has been attributed previously to strain RCMV33G, which lacks a functional R33 gene (Beisser et al., 1998). Our current data, therefore, corroborate the notion that RCMV R33, like its homologue from MCMV (Davis-Poynter et al., 1997), is essential for salivary gland tropism of the virus. Additionally, we conclude that the R33-EGFP gene is not able to functionally replace its native counterpart in vivo. Putative causes for the deficit of strain RCMV33G are the following: (i) the transcription of R33-EGFP and surrounding genes may be different from that of their native counterparts. Indeed, transcriptional analysis of the genomic region of R33-EGFP and R33 indicated that there are clear differences between RCMV33G and wt virus, not only in the expression of R33(-EGFP) but also in the transcription of the R34 gene, which is located downstream of R33. However, given the similarity between strains RCMV33G and RCMVΔR33 in their in vitro phenotypes, it is unlikely that these phenotypes are caused by aberrant expression of R34; (ii) another difference between wt RCMV and RCMV33G in salivary gland tropism may be an altered subcellular distribution of pR33-EGFP in comparison with pR33. Due to the unavailability of anti-pR33 antibodies, this hypothesis cannot be tested yet; (iii) in comparison with pR33, pR33-EGFP may be hindered in its interaction with cellular proteins due to the presence of the EGFP moiety at its C terminus. As noted previously, we did not observe any differences in the signalling properties of pR33 and pR33-EGFP in vitro (Gruijthuijsen et al., 2002). However, it is possible that these proteins carry out other, or additional, signalling activities and bind to different protein partners in vivo. An example of a process in which pR33 and pR33-EGFP may differ potentially is desensitization, which serves to modulate GPCR activity by modification of amino acid residues at the (intracellular) C terminus (Sibley & Lefkowitz, 1985). The C-terminal EGFP tag of pR33-EGFP could mask critical residues within the pR33 sequence from regulatory proteins responsible for desensitization of the receptor; (iv) finally, the expression of the pR33-EGFP fusion proteins may induce immune mechanisms that do not occur during wt RCMV infection. Similar mechanisms may also be induced by the expression of the neo gene by RCMV33G. Nevertheless, while each of the recombinant RCMV strains in our laboratory has been generated using the same neo expression cassette, each of these strains exhibits clear phenotypic differences. In addition, one of these mutants, RCMVΔr144 (Beisser et al., 2000), could not be distinguished phenotypically from wt RCMV, both in rat survival and virus dissemination experiments. Therefore, we deem it unlikely that the expression of the neo gene would induce additional immune mechanisms that are not seen during wt RCMV infection.

Although RCMV33G replicates with similar efficiency as wt RCMV in spleen and liver at day 5 p.i., pR33-EGFP could not be detected by immunohistochemistry using anti-EGFP antibodies in spleen or liver samples from RCMV33G-infected rats. In contrast, pR33-EGFP is expressed markedly in bone marrow cells from these rats. Based on these observations, it could be hypothesized that R33 is expressed specifically in bone marrow cells at early times of infection in vivo. Whether or not this expression reflects the expression of native R33 by the wt virus is currently unknown. Our future studies will, therefore, be directed at (i) the generation of anti-pR33 antibodies, which has hitherto been unsuccessful; and (ii) the construction of RCMV strains expressing GPCR homologues with alternative, smaller
protein tags, such as FLAG. In these studies, an important aim will be to address how pR33 determines the salivary gland tropism of RCMV and how this relates to RCMV virulence and putative expression of the protein in bone marrow.

The recombinant RCMV strain expressing pR78-EGFP was found to be attenuated, albeit moderately, both in vitro and in vivo. In cell culture, RCMV78G produced virus titres 3- to 4-fold lower than wt RCMV in the culture medium. In comparison, an R78-deleted virus strain, RCMVΔR78a, produced virus titres approximately 50 times lower than wt RCMV (this study; Beisser et al., 1999). Similarly, an MCMV strain with a deletion of the MCMV homologue of R78, M78, was reported to be at least 50 times less effective in producing infectious virus (Oliveira & Shenk, 2001). Taken together, these results indicate that the expression of pR78-EGFP by strain RCMV78G can complement only partly the absence of the native R78 gene. The strong similarity between wt RCMV and RCMV78G in transcription of R78 (-EGFP) and neighbouring genes in cell culture indicates that the differences between these strains in virus replication are not caused by differences in transcription but rather by dissimilarities in other processes, such as translation, subcellular distribution, protein–protein interactions and/or post-translational modification of pR78(-EGFP).

The ability of pR78-EGFP to only partially functionally replace the native pR78 protein was also observed in vivo. The most dramatic difference between wt RCMV and RCMV78G was seen at day 5 p.i. in the spleen of infected rats. While infectious virus was readily detectable in the spleen of all wt RCMV-infected rats, virus was not detected in the spleen of any of the RCMV78G-infected rats. Additionally, infectious virus was not found in the spleens of RCMVΔR78a-infected animals. Significant differences between RCMV and RCMV78G in the production of infectious virus were not seen in any of the other investigated organs. Although we studied previously the differences between wt RCMV and RCMVΔR78a in virus dissemination (Beisser et al., 1999), the replication defect of RCMVΔR78a in the spleen has hitherto remained unnoticed. Since similar viral DNA levels were found in the spleen of wt RCMV-, RCMVΔR78a- and RCMV78G-infected animals, we concluded that (i) R78 plays a crucial role in the production of infectious RCMV in the spleen; and (ii) R78-EGFP does not function (as efficiently) as R78 during infection in the spleen. We hypothesize that R78 does not play a role in virus dissemination or viral DNA replication but rather functions in intracellular processes following DNA synthesis, such as virion assembly or egress. Previously, we reported that a different cell morphology was seen in cultures infected with RCMVΔR78a as compared to cultures infected with wt virus (Beisser et al., 1999). Taken together, our findings indicate that pR78 may play a role in stabilizing intra- and/or intercellular structures required for optimal release of virions from infected cells. It is interesting to note that our data on virus dissemination differ from those reported by Oliveira & Shenk (2001) in a study of an M78-deleted MCMV strain. This strain was shown to replicate less efficiently than wt MCMV not only in spleen but also in salivary glands and liver. We do not currently have an explanation for this discrepancy, although it is clear that pR78 and the M78 gene product may show differences in function as a consequence
of differences between their predicted amino acid sequences (Vink et al., 2000). Challenges for future studies will be (i) to elucidate the putative intracellular pathways by which pM78, and maybe also pR78, mediate the accumulation of IE transcripts; (ii) to characterize how the activities of these proteins stimulate the production of infectious virus in vitro; and (iii) to identify the processes that determine the R78-dependent spleen tropism of RCMV.

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