The rat cytomegalovirus homologue of parvoviral rep genes, r127, encodes a nuclear protein with single- and double-stranded DNA-binding activity that is dispensable for virus replication


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An intriguing feature of the rat cytomegalovirus (RCMV) genome is open reading frame (ORF) r127, which shows similarity to the rep genes of parvoviruses as well as the U94 genes of human herpesvirus type 6A (HHV-6A) and 6B (HHV-6B). Counterparts of these genes have not been found in other herpesviruses. Here, it is shown that the r127 gene is transcribed during the early and late phases of virus replication in vitro as an unspliced 1.1 kb transcript containing the complete r127 ORF. Transcripts of r127 were also detected in various organs of RCMV-infected rats at 1 week post-infection (p.i.), but only in the salivary gland at 4 months p.i. Using rabbit polyclonal antibodies raised against the r127-encoded protein (pr127), pr127 was found to be expressed as early as 12 h p.i. within the nuclei of RCMV-infected cells in vitro. Expression of pr127 was also observed within the nuclei of cells in various organs of RCMV-infected rats at 3 weeks p.i. Moreover, pr127 was demonstrated to bind single- as well as double-stranded DNA. Finally, an RCMV r127 deletion mutant (RCMVΔr127) was generated, in which the r127 ORF was disrupted. This deletion mutant, however, was shown to replicate with a similar efficiency as wild-type RCMV (wt RCMV), both in vitro and in vivo. Taken together, it is concluded that the RCMV r127 gene encodes a nuclear protein with single- and double-stranded DNA-binding activity that is dispensable for virus replication, not only in vitro, but also during the acute phase of infection in vivo.

INTRODUCTION

Cytomegaloviruses (CMVs) are widespread species-specific betaherpesviruses that cause acute, persisting and latent infections in both humans and animals. Infections with human CMV (HCMV) are mainly asymptomatic in immunocompetent individuals, but can be life-threatening in immunocompromised individuals, such as AIDS patients and organ transplant recipients.

In our laboratory, we are studying the interaction between rat CMV (RCMV) and its host as a model for HCMV infection and disease (Bruggeman et al., 1982). RCMV contains a linear, double-stranded DNA genome of 230-1 kb. The complete DNA sequence of the RCMV genome has been determined recently and was found to contain at least 167 open reading frames (ORFs) (Vink et al., 2000). Most of these ORFs have counterparts in the genomes of both HCMV and murine CMV (MCMV) (Chee et al., 1990; Rawlinson et al., 1996; Vink et al., 2000). However, an exception is RCMV ORF r127, which is unique among the CMVs. This ORF has the capacity to encode a 337 amino acid protein (pr127) which shows similarity to the non-structural proteins (NS or Rep proteins) that are encoded by the rep genes of parvoviruses (Vink et al., 2000). The predicted amino acid sequence of the r127-encoded protein is most closely related to the sequences of the Rep1/2 proteins of three avian parvoviruses, namely barbary duck parvovirus (BDPV), muscovy duck parvovirus (MDPV) and goose parvovirus (GPV) (Vink et al., 2000; Zadori et al., 1995). Although the RCMV rep gene homologue is unique among the CMVs, it is not unique among the betaherpesviruses: the U94 genes of human herpesvirus type 6A (HHV-6A) and 6B (HHV-6B) also show similarity to the paroviral rep genes (Domínguez et al., 1999; Gompels et al., 1995; Isegawa et al., 1999; Thomson et al., 1991). The U94 ORF was first discovered in HHV-6A and was found to encode a
490 amino acid protein (RepH6) that is most closely related to the Rep proteins of the adeno-associated viruses (AAVs), including Rep68/78 of AAV-2 (Srivastava et al., 1983; Thomson et al., 1991). Interestingly, a counterpart of U94 has not been identified in the genome of HHV-7, which is closely related to HHV-6A and -6B (Nicholas, 1996). Remarkably, although RCMV r127 and HHV-6A and -6B U94 have a conserved genomic position as well as orientation (Dominguez et al., 1999; Gompels et al., 1995; Isegawa et al., 1999; Vink et al., 2000), rep gene homologues have so far not been found in the genomes of other herpesviruses.


The role of RepH6 in the virus replication cycles of HHV-6A and -6B is less well documented. However, RepH6 seems to have a conserved function with its AAV-2 counterpart, since HHV-6A U94 is able to complement the replication of a rep-deficient AAV-2 genome (Thomson et al., 1994). In addition, U94 is transcribed in latently infected peripheral blood mononuclear cells (PBMCs) from HHV-6A-infected individuals (Rotola et al., 1998), suggesting a role for RepH6 in the regulation of latency. This possibility is supported by the fact that virus replication and expression of viral genes are restricted in HHV-6A-infected lymphocytes expressing HHV-6B U94 in vitro (Rotola et al., 1998). Nevertheless, not much is known about the role of RepH6 in the pathogenesis of viral infection. Moreover, it is not possible to study the role of RepH6 in animal models. We therefore set out to study the biological function of pr127, the RepH6 homologue of RCMV. Here, we show that the RCMV r127 gene encodes a nuclear protein with single- and double-stranded DNA-binding activity. Furthermore, we demonstrate that the r127-encoded protein is dispensable for virus replication in vitro and in vivo.

**METHODS**

**Cells and virus.** Primary rat embryo fibroblasts (REFs) and the rat fibroblast cell line Rat2 (ATCC CRL 1764) were cultured as described previously (Bruggeman et al., 1982, 1985). RCMV (Maastricht strain) was propagated in REFs and virus titres were determined by plaque assays according to standard procedures (Bruggeman et al., 1982, 1985).

**Poly(A)+ RNA isolation from RCMV- and mock-infected REFs.** Poly(A)+ RNA was isolated from RCMV-infected REFs at intermediate early (IE), early (E) and late (L) times of infection. During the 1 h infection period, the REFs were exposed to RCMV at an m.o.i. of 1.0. IE mRNA was extracted from REFs that had been treated with 100 µg cycloheximide ml−1 from 1 h prior to infection until they were harvested at 16 h post-infection (p.i.). E mRNA was obtained from REFs that had been treated with 200 µg phosphonoacetic acid ml−1 from 3 h p.i. until harvesting at 16 h p.i., and L mRNA was isolated from REFs that were harvested at 72 h p.i. Mock mRNA was extracted from REFs that had not been infected with RCMV. Poly(A)+ RNA was purified with the QuickPrep Micro mRNA purification kit (Amersham Biosciences) and dissolved in RNase-free H2O. mRNA was quantified by determination of the absorbance at 260/280 nm.

**Northern blot analysis.** Northern blot analysis was performed on 5 µg portions of mRNA from RCMV- and mock-infected REFs (see above). Samples were separated by electrophoresis through an agarose/formaldehyde gel, blotted onto a Hybond-N nylon membrane (Amersham Biosciences) and hybridized to a DNA probe representing the full-length r127 ORF. This probe was generated after amplification of ORF r127 by PCR using primers 5′-r127 (5′-ACGTTGATCCATGAACTAGAACCGG-3′) and 3′-r127 (5′-AAGTAAGCTTTAACATCCTAGTCAC-3′). These primers introduced unique BamHI and HindIII restriction sites into the amplified fragment. Construct pRXO, which contains the RCMV Xbal O fragment (Meijer et al., 1986), was used as target DNA. The amplified fragment of 1.0 kb was digested with BamHI and HindIII and cloned into the corresponding restriction sites of cloning vector pUC119, generating plasmid p302. The sequence of ORF r127 was confirmed by DNA sequencing using the Thermo Sequenase cycle sequencing kit (Amersham Biosciences) with Cy5-labelled M13 universal primers and an ALFexpress automated DNA sequencer (Amersham Biosciences). Construct p302 was then digested with BamHI and HindIII and the resulting fragment containing ORF r127 was labelled with [α-32P]dATP (ICN) using the Random Primed DNA labelling kit (Roche Applied Science). The radioactive signal was visualized by autoradiography.

**5′- and 3′-RACE.** Rapid amplification of 5′ and 3′ cDNA ends (5′- and 3′-RACE) was performed on L mRNA (see above) using the SMART RACE cDNA amplification kit (BD Biosciences) and the Marathon cDNA amplification kit (BD Biosciences). Gene-specific primers r127P4 (5′-GGACTCCCGGGGTTCCTCCAAAAGCTC-3′; position 178973 to 178997 of the RCMV genome; Vink et al., 2000) and r127P3 (5′-AGATGCGAGTCCCCGGTCGATAAAC-3′; position 179164 to 179140) were used for the 5′- and 3′-RACE, respectively. Amplified fragments were cloned into vector pGEM-T Easy (Promega) and their sequence was determined by DNA sequencing, as described above.

**Total RNA isolation from tissues of RCMV-infected rats.** Three-week-old, male Wistar rats (Central Animal Facilities, University of Maastricht) were infected by intraperitoneal injection of 1 × 103 p.f.u. RCMV and sacrificed at either 1 week or 4 months post-infection. Salivary gland, spleen, kidney, liver and lung tissues were excised and total RNA was isolated as described previously (Gauthier et al., 1997). RNA was dissolved in RNase-free H2O and quantified by determination of the absorbance at 260/280 nm.

**Generation of in vitro-transcribed RNA.** Construct p302 (see above) was digested with BamHI and HindIII and the resulting 1.0 kb fragment containing ORF r127 was cloned into BamHI- and HindIII-digested vector pGEM-3Z (Promega), generating plasmid p271. In this construct, ORF r127 is located downstream of the T7 RNA polymerase promoter. A 1.2 kb fragment of construct p271 containing the T7 promoter and ORF r127 was amplified by PCR using M13 universal primers. A sample (0.5 µg) of the amplified fragment was transcribed in the presence of T7 RNA polymerase (Amersham Biosciences) and subsequently treated with DNase I (Amersham Biosciences). RNA was purified by extraction with
phenol/chloroform, recovered by ethanol precipitation, dissolved in RNase-free H₂O and quantified by determination of the absorbance at 260/280 nm.

**RT-PCR.** RT-PCR was performed on 0.5 μg samples of total RNA from tissues of RCMV-infected rats (see above). Each sample was denatured for 5 min at 65 °C in the presence of 0-4 μg tRNA ρ-1 (Roche Applied Science). Samples were then chilled on ice and added to 30 μl pre-chilled reaction mixture containing 10 mM Tris/HCl (pH 8.5), 50 mM KCl, 3 mM MgCl₂, 2 mM DTT, 0.2 mM dNTP, 10 μg RNaseguard (Amersham Biosciences), 0.25 U HotStarTaq DNA polymerase (Qiagen), 5 U SuperScript II reverse transcriptase (Invitrogen) and 0.2 μM each of primer P3 (5′-CGTATGCTGCTGTCCGACC-3′; position 178908 to 178889 of the RCMV genome) and P4 (5′-GGGATCTCTCCACCGATAG-3′; position 178661 to 178680). Each sample was reverse transcribed for 20 min at 50 °C. Subsequently, the HotStarTaq DNA polymerase was activated for 15 min at 95 °C. The reverse-transcribed target was then amplified by 40 cycles of denaturation for 30 s at 94 °C, primer annealing for 30 s at 59 °C and primer extension for 30 s at 72 °C. A 1 μl aliquot of the RT-PCR product was subjected to another 30 cycles of PCR with nested primers P1 (5′-CCATAACCTCAACCTGTGTTG-3′; position 178889 to 178870 of the RCMV genome) and P2 (5′-CTACACAGGCAGCCAGGTCT-3′; position 178690 to 178709). Each sample was also processed in the absence of reverse transcriptase to monitor any residual DNA contamination. Furthermore, two additional reactions were run in parallel for each sample. In one of these reactions, the sample was spiked with 10–20 copies of in vitro-transcribed RNA (see above) to address whether possible contaminants interfered with the amplification. In the other reaction, the sample was subjected to RT-PCR with rat β-actin gene-specific primers RT-ACT-B (5′-GGTGGG-TATGGGTCAGAAGG-3′) and RT-ACT-F (5′-TGCGCATAGTGATGCCCTGA-3′) to confirm the integrity of the sample.

**Generation of MBP-pr127 and 6H-pr127 expression constructs.** Construct p302 (see above) was digested with BamHI and HindIII and the resulting 1 kb fragment containing ORF r127 was cloned into the corresponding restriction sites of expression vectors pMAL-c (New England Biolabs) and pRSET A (Invitrogen), generating plasmids p248 and p232. These constructs encode proteins containing the complete r127-derived amino acid sequence fused to the C terminus of either maltose-binding protein (MBP-pr127; construct p248) or a tag consisting of six consecutive histidine residues (6H-pr127; construct p232).

**Expression and purification of MBP-pr127 and MBP-β-gal-α.** Construct p248 (see above) and expression vector pMAL-c were introduced into Escherichia coli BL21(DE3) pLysS and the resulting strains were grown overnight at 37 °C in LB medium containing 50 μg tetracycline ml⁻¹ and 20 μg chloramphenicol ml⁻¹. The cultures were diluted 1:100 in 300 ml LB medium with tetracycline and chloramphenicol and grown at 37 °C to an OD₆₅₀ of 0-6. Protein expression was then induced by the addition of IPTG to a final concentration of 0-3 mM. After 3 h of protein expression at 37 °C, the bacteria were harvested by centrifugation and resuspended in 10 ml of buffer A (10 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA, 1 mM β-mercaptoethanol) supplemented with 1 M NaCl. The suspensions were sonicated and cleared by centrifugation. To the supernatants, 10 ml of buffer A was added. The materials were then loaded onto 2 ml amylose columns (New England Biolabs). The columns were washed with 7.5 ml buffer B (buffer A supplemented with 0.5 M NaCl) containing 0.25% Tween 20 and subsequently with 7.5 ml buffer B. Proteins were eluted with 7.5 ml buffer B containing 10 mM maltose. Fractions of 0.5 ml eluted protein were collected and analysed by SDS-PAGE. Peak fractions were pooled, dialysed into buffer B containing 10% glycerol and stored at –80 °C.

**Generation of rabbit polyclonal antibodies directed against MBP-pr127.** A rabbit was immunized by intramuscular injection of 1 mg purified MBP-pr127 (see above) in Specol adjuvant (Animal Sciences Group, Wageningen University and Research Centre). An intramuscular booster injection, containing 1 mg purified MBP-pr127, was given at week 9. Blood was obtained before and 13 weeks after immunization, and sera were prepared as described previously (Harlow & Lane, 1988). The reactivity of these sera against the RCMV pr127 protein was determined by Western blot analysis.

**Western blot analysis.** Constructs p248 and p232 (see above) and expression vector pMAL-c were introduced into E. coli BL21(DE3)pLysS and protein expression was induced essentially as described above. Proteins from crude bacterial extracts were separated by SDS-PAGE and transferred to a PROTRAN BA 83 nitrocellulose membrane (Schleicher & Schuell). The blots were then incubated with a 1:1000 dilution of either rabbit anti-MBP-pr127 antisera or rabbit pre-immune serum (see above) and subsequently with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulins (DakoCytomation). The blots were stained with diaminobenzidine.

**Immunocytochemical analysis of RCMV- and mock-infected Rat2 cells.** To perform a 1 h infection period, Rat2 cells were either mock-infected or infected with RCMV at an m.o.i. of 0-1. The cells were fixed and permeabilized at 8, 12, 24 and 72 h.p.i. The cells were then incubated with a 1:100 dilution of either rabbit anti-MBP-pr127 antisera or rabbit pre-immune serum (see above) and subsequently with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulins (DakoCytomation). The fluorescent label was visualized with an Axiosvert 100 fluorescence microscope (Zeiss). Staining of cells with monoclonal antibody RCMV8 was carried out as described previously (Bruning et al., 1987; Kaptein et al., 2001).

**Immunohistochemical analysis of tissues of RCMV- and mock-infected rats.** Ten-week-old, male Brown Norway rats (Central Animal Facilities, University of Maastricht) were immunocompromised by 5 g of total-body Rontgen irradiation 1 day prior to infection, as described previously (Stals et al., 1990). Rats were either mock-infected or infected by intraperitoneal injection of 3×10⁶ p.f.u. RCMV. The animals were sacrificed at 3 weeks p.i. and 4 μm paraffin tissue sections were prepared from salivary gland, spleen and liver. Serial tissue sections were mounted on glass slides and deparaffinized. Sections were then incubated with a 1:100 dilution of either rabbit anti-MBP-pr127 antisera or rabbit pre-immune serum (see above) and subsequently with biotin-conjugated swine anti-rabbit immunoglobulins (DakoCytomation) and streptavidin–biotinylated alkaline phosphatase (AP) complex (DakoCytomation). Sections were stained with Fast Red (Speel et al., 1992). Staining of sections with monoclonal antibody RCMV8 was performed as described previously (Bruning et al., 1987; Kaptein et al., 2001).

**DNA-binding assay.** Aliquots of 150 μg of either purified MBP-pr127 or MBP-β-gal-s (see above) in 1 ml binding buffer (10 mM Tris/HCl, pH 7-4, 25 mM KCl, 0.5 mM EDTA, 0.05% Tween 20, 100 mM NaCl) were added to 1 ml single- and double-stranded DNA-cellulose columns (Amersham Biosciences). The columns were washed with 5 ml binding buffer and proteins were eluted with 0-5 ml fractions of binding buffer supplemented with 0, 100, 200, 400, 600, 800, 1000 and 1500 mM NaCl. Fractions of eluted protein were collected and analysed by SDS-PAGE.

**Generation of an RCMVΔr127 recombination plasmid.** Vector pRc/CMV (Invitrogen) was digested with XhoI and the resulting 2-1 kb fragment containing a neomycin resistance gene (neo) was cloned into SalI-digested pBluescript SK (+) vector (Stratagene),
generating plasmid p474. Construct p474 was then digested with Clal and Xhol and the 2-1 kb neo fragment was used to replace the 0-5 kb Clal–Xhol fragment within ORF r127 of construct pRXO. The resulting RCMVr127 recombination plasmid was designated p475.

**Generation of an RCMV r127 deletion mutant.** Approximately 2 x 10⁶ Rat2 cells were trypsinized and harvested by centrifugation. The cells were washed and resuspended in 500 μl of serum-free culture medium. To the cell suspension, 20 μg of construct p475 (see above) 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 Gene Pulser electroporator (Bio-Rad). The transfected cells were subsequently seeded in culture flasks. At 16 h after transfection, the cells were infected with low-passage RCMV at an m.o.i. of 1-0. The culture medium was supplemented with 50 μg G418 ml⁻¹ at 24 h p.i. Recombinant virus was plaque-purified and cultured on REFs as described previously (Beisser et al., 1998, 1999, 2000; Kaptein et al., 2003). The integrity and plaque purity of the RCMV r127 deletion mutant (RCMVΔr127) were determined by Southern blot analysis.

**Southern blot analysis.** DNA was isolated from wild-type (wt) RCMV- and RCMVΔr127-infected REFs and digested with Xbal and Xhol. The digested samples were separated by electrophoresis through an agarose gel and blotted onto a Hybond-N+ nylon membrane (Amersham Biosciences). Constructs pRXO and p474 (see above) were used as RXO- and neo-specific probes, respectively. These constructs were labelled with the DIG DNA labelling kit. The DIG Easy Hyb solution was used for hybridization, and the DIG wash and block buffer set and the DIG luminescent detection kit (all from Roche Applied Science) were used for detection.

**Replication of wt RCMV and RCMVΔr127 in vitro.** During a 1 h infection period, REFs were infected with either wt RCMV or RCMVΔr127 at an m.o.i. of 0-01. At days 1, 3, 5 and 7 p.i., culture medium samples were taken and subjected to plaque assays. The data were statistically analysed by applying Student’s t-test. P values of <0-05 were considered to indicate statistical significance.

**Replication of wt RCMV and RCMVΔr127 in vivo.** Two groups of ten 7-week-old, male, specific-pathogen-free (SPF) Lewis/M rats (Central Animal Facilities, University of Maastricht) were immunocompromised by 5 Gy of total-body Röntgen irradiation at 1 day prior to infection, as described previously (Stals et al., 1990). Rats were infected by intraperitoneal injection of 1 x 10⁶ p.f.u. of either wt RCMV or RCMVΔr127. At days 4 and 28 p.i., 5 rats from each group were sacrificed. Salivary gland, spleen, kidney, liver, pancreas and thymus tissues were collected and subjected to plaque assays. The data were statistically analysed by applying Student’s t-test. P values of <0-05 were considered to indicate statistical significance.

**RESULTS**

The RCMV r127 gene

The 1011 bp ORF of the r127 gene was found to be localized between nucleotides 178310 and 179320 of the 230138 bp RCMV genome. ORF r127 runs from right to left and is located immediately 5′ of ORF r128, which runs in the opposite direction (Fig. 1). A potential TATA box (5′-TTAAAA-3′; position 179398 to 179393 of the RCMV genome) and a consensus polyadenylation sequence (5′-AATAAA-3′; position 178260 to 178255) are located up- and downstream, respectively, of ORF r127 (Vink et al., 2000). This ORF has the potential to encode a 337 amino acid protein with a calculated molecular mass of 37-8 kDa, which was found to be 28% identical to the Rep1/2 proteins of BDPV and MDPV and 27% identical to the Rep1/2 protein of GPV (Table 1) (Vink et al., 2000; Zadori et al., 1995). Lower similarities were found to the rep gene products of other paroviruses and to the proteins that are encoded by the U94 genes of HHV-6A and -6B.

**The r127 gene is transcribed during RCMV infection in vitro**

Northern blot analysis was performed to investigate transcription of the r127 gene during RCMV infection in vitro. Transcription of r127 was examined in RCMV-infected cells at IE, E and L times of infection. As shown in Fig. 2(a), r127-specific transcripts were exclusively detected during the E and L phases of infection (lanes 7 and 8) and not during the IE phase of infection (lane 6). Transcription of r127 was dependent on RCMV infection, since it was not observed in mock-infected cells (Fig. 2a, lane 5). One major r127-specific mRNA was identified with an estimated length of approximately 1·3 kb (Fig. 2a, lanes 7 and 8). This mRNA is most abundantly transcribed during the E phase of infection. Ethidium bromide staining of the agarose/formaldehyde gel confirmed the integrity of all mRNA samples and clearly demonstrated that similar amounts of mRNA were used for each experiment (Fig. 2a, lanes 1–4). Since we used a double-stranded DNA probe to detect the r127-specific transcripts, we could not designate these transcripts as either sense or antisense. Nevertheless, we predicted the 1·3 kb mRNA to represent a sense transcript containing the complete 1·0 kb r127 ORF. This prediction was based on the position of a potential TATA box and a consensus polyadenylation sequence near the 5′ end, respectively, of ORF r127 (see above).

In order to determine the structure of the r127 transcript, 5′- and 3′-RACE experiments were performed. Gene-specific primers r127P4 and r127P3 (see Methods) were designed in such a way that they would generate overlapping 5′ and 3′
cDNA ends, which allowed us to determine the complete structure of the r127 transcript. As shown in Fig. 2(b), the 5' and 3'-RACE experiments resulted in major amplified fragments with lengths of approximately 0.5 and 1.0 kb, respectively (lanes 2 and 3). These fragments were cloned and sequenced, and it was found that they represented the overlapping 5' and 3' ends of the r127 cDNA. The sequence of this cDNA is shown in Fig. 2(c). The r127 transcript is unspliced, has a length of approximately 1 kb and contains the complete r127 ORF. Five independent 5'-RACE clones were sequenced. In these clones, the r127 transcription start site was mapped to a residue located either 18 (five clones), 17 (one clone) or 14 (one clone) nucleotides downstream of the consensus polyadenylation sequence. The 3' end of the other two 3'-RACE clones was located 12 nucleotides upstream of the polyadenylation signal. These clones may have been generated after annealing of the oligo(dT) primer, which was used for first-strand cDNA synthesis, on a stretch of A residues near the 3' end of the transcript rather than on the poly(A)+ tail.

The 1-1 kb r127 transcript that was identified by the RACE experiments (Fig. 2c) is likely to correspond to the 1.3 kb mRNA that was detected by Northern blot analysis (Fig. 2a). We therefore conclude that the RCMV r127 gene is transcribed during the E and L phases of virus replication in vitro as an unspliced transcript of approximately 1 kb, comprising the full-length r127 ORF.

The r127 gene is transcribed during RCMV infection in vivo

To examine whether the r127 gene is transcribed during the acute phase of RCMV infection in vivo, an r127-specific, nested RT-PCR assay was performed on total RNA purified from salivary gland, spleen, kidney, liver and lung of RCMV-infected rats at either 1 week or 4 months p.i. The assay, which has a lower detection limit of approximately 10 copies of RNA (data not shown), was designed to amplify a 200 bp fragment. At 1 week p.i., r127-specific transcripts were detected in all five organs tested (Fig. 2d, lanes 8–12). As expected, transcripts of r127 were not detected when RNA was omitted from the reaction mixture (Fig. 2d, lane 13). Furthermore, amplified fragments were derived from RNA rather than from contaminating DNA, since they were not generated when samples were processed in the absence of reverse transcriptase (Fig. 2d, lanes 2–7). Spiking each sample with in vitro-synthesized RNA and subjecting each sample to an RT-PCR assay with a primer set specific for the rat β-actin gene confirmed the efficiency of all enzymic reactions and the integrity of all RNA samples (data not shown). At 4 months p.i., r127-specific transcripts were only detected in the salivary gland and not in spleen, kidney, liver or lung (data not shown). This pattern of r127 transcription parallels the temporal production of infectious virus in organs of RCMV-infected rats (Bruggeman et al., 1985). Taken together, the RCMV r127 gene is widely transcribed during productive infection, both in vitro and in vivo.

**Table 1. Identities among parvoviral Rep(-like) proteins**

Percentages of identity were determined using local alignment tool BLAST 2 Sequences (Tatusova & Madden, 1999). The amino acid sequences of the proteins were derived from Vink et al. (2000) (RCMV pr127), Thomson et al. (1991) (HHV-6A RepH6), Zadori et al. (1995) (BDPV, MDPV and GPV Rep1) and Srivastava et al. (1983) (AAV-2 Rep78).

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**Generation of rabbit polyclonal antibodies directed against the RCMV pr127 protein**

In order to study expression of the RCMV r127-encoded protein, we set out to generate rabbit anti-pr127 polyclonal antibodies. To this end, an MBP–pr127 fusion protein was expressed in E. coli and purified by affinity chromatography (Fig. 3a, upper gel). The purified protein (Fig. 3a, upper gel, lanes 7–15) had a calculated molecular mass of 80 kDa and was approximately 90 % pure. Minor proteins in the MBP–pr127 preparation with molecular masses lower than 80 kDa probably represented degradation products of the full-length fusion protein. Peak fractions of purified MBP–pr127 (Fig. 3a, upper gel, lanes 8–15) were pooled, dialysed and used to immunize a rabbit. The reactivity of the resulting
rabbit anti-MBP–pr127 antiserum against pr127 was tested by Western blot analysis (Fig. 3b). This antiserum reacted with MBP–pr127 (Fig. 3b, lane 2) as well as with 6H–pr127 (lane 3). As might be expected, reactivity was also seen with a protein containing the bacterial β-galactosidase-α protein fused to MBP (MBP–β-gal-α; Fig. 3b, lane 4), but not with a
Characterization of the RCMV r127 gene

Fig. 3. For legend see page 2008.
protein containing part of the RCMV IE1 protein fused to a combined 6H-thioredoxin tag (6H–TRX–IE1; lane 5) (Beuken et al., 1999). Rabbit pre-immune serum did not react with any of these proteins (Fig. 3b, lanes 7–10). These data clearly indicate that the rabbit anti-MBP–pr127 antiserum contains antibodies directed against both MBP and pr127. Since eukaryotic cells do not express MBP, this antiserum is a useful tool to study expression of the pr127 protein in RCMV-infected cells.

**The pr127 protein is expressed within the nuclei of RCMV-infected cells in vitro**

To investigate expression of the pr127 protein during RCMV infection *in vitro*, and to determine the cellular localization of this protein, we applied the rabbit anti-MBP–pr127 antiserum (see above) in immunocytochemical analysis of RCMV-infected cells at 8, 12, 24 and 72 h.p.i.

Fig. 3(c) shows that pr127 is expressed within the nuclei of RCMV-infected cells at 12, 24 and 72 h.p.i. (panels B–D), but not at 8 h.p.i. (panel A). Expression of pr127 was dependent on RCMV infection, since it was not observed in mock-infected cells (data not shown). In addition, specific staining was not seen after incubation with rabbit pre-immune serum (data not shown). Staining with monoclonal antibody RCMV8, which is directed against a nuclear RCMV protein (pR44), also resulted in nuclear staining of RCMV-infected cells (Fig. 3c, panels E–H), as shown previously (Bruning et al., 1987; Kaptein et al., 2001).

In conclusion, the pr127 protein is a nuclear protein that is expressed as early as 12 h.p.i. in RCMV-infected cells *in vitro*. The kinetics of pr127 expression are in accordance with the early-late kinetics of r127 transcription (see above).

**The pr127 protein is expressed within the nuclei of RCMV-infected cells in vivo**

To examine *in vivo* expression of the pr127 protein during the acute phase of RCMV infection, immunohistochemical analysis using the rabbit anti-MBP–pr127 antiserum (see above) was performed on salivary gland, spleen and liver of RCMV-infected rats at 3 weeks p.i. As shown in Fig. 3(d), pr127 is expressed within the nuclei of RCMV-infected cells in all three organs tested (panels A–C). As expected, specific staining was not observed either in corresponding tissue sections from mock-infected rats (data not shown) or after incubation with rabbit pre-immune serum (Fig. 3d, panels D–F). As shown previously (Bruning et al., 1987; Kaptein et al., 2001), staining with anti-pR44 monoclonal antibody RCMV8 also resulted in nuclear staining of RCMV-infected cells in these organs (data not shown). Taken together, the pr127 protein is widely expressed within the nuclei of RCMV-infected cells during productive infection, both *in vitro* and *in vivo*.

**The RCMV pr127 protein has single- and double-stranded DNA-binding activity**

There are several indications that the RepH6 proteins of HHV-6A and -6B might be involved in the regulation of viral and/or cellular gene expression. One of these is the ability of HHV-6B RepH6 to bind single-stranded DNA (Dhepakson et al., 2002). To determine whether the RCMV pr127 protein also possesses DNA-binding activity, we tested its capacity to bind to DNA-cellulose. First, fusion proteins MBP–pr127 (80 kDa) and MBP–β-gal-z (51 kDa) were purified from *E. coli*, as described above (Fig. 3a). The purified proteins were then tested for their affinity for single- and double-stranded DNA-cellulose columns. As shown in Fig. 4, MBP–pr127 was found to bind single- (a, lane 4) as well as double-stranded DNA-cellulose (c, lane 4), although its affinity for single-stranded DNA-cellulose seemed somewhat more pronounced. Since MBP–pr127 does not bind to cellulose alone (data not shown), we conclude that the affinity of this protein for DNA-cellulose is the result of DNA binding by MBP–pr127. The DNA-binding activity of this protein appears to be strong, given that the protein was only partially eluted from the columns.
with increasing concentrations of NaCl (Fig. 4a and c, lanes 5–13). Furthermore, the DNA-binding activity of MBP–pr127 was pr127-specific, since the columns did not retain MBP–β-gal-a (Fig. 4b and d, lane 4). These results indicate that the RCMV pr127 protein has single- as well as double-stranded DNA-binding activity.

**Generation of an RCMV r127 deletion mutant**

The role of the herpesviral homologues of the parvoviral Rep proteins in the pathogenesis of viral infection is still enigmatic. We therefore decided to characterize the biological significance of the RCMV pr127 protein by generating and studying a recombinant RCMV strain (RCMVΔr127), in which the r127 gene is disrupted by replacing part of its ORF with a neo expression cassette (Fig. 5a). The mutation was first introduced into a plasmid that contains the RCMV XbaI O fragment (Meijer et al., 1986). The r127 gene within the RCMV genome was subsequently replaced with the mutated r127 gene by homologous recombination, after transfection of cells with the recombination plasmid followed by infection with RCMV. Recombinant virus was purified by several rounds of G418 selection and subsequent plaque purifications. The integrity and plaque purity of the RCMV r127 deletion mutant were verified by Southern blot analysis (Fig. 5b). Hybridization of the neo-specific probe with Xbal- and XhoI-digested DNA from wt RCMV and RCMVΔr127 should reveal 0.9 and 2.8 kb fragments for both viruses and either a 2.2 or 3.8 kb fragment for wt RCMV and RCMVΔr127, respectively. Hybridization of the neo-specific probe with Xbal- and XhoI-digested DNA from wt RCMV and RCMVΔr127 should only reveal a 3.8 kb fragment for RCMVΔr127. As shown in Fig. 5(b), the hybridization patterns were as predicted and therefore confirm the integrity and the plaque purity of the RCMV r127 deletion mutant.

**The r127 gene is dispensable for RCMV replication in vitro**

To compare the in vitro replication characteristics of wt RCMV and RCMVΔr127, multi-step growth curves were generated. The amount of infectious virus produced by wt RCMV- and RCMVΔr127-infected cells was determined at 1, 3, 5 and 7 days p.i. Fig. 5(c) shows that the virus titres produced by wt RCMV- and RCMVΔr127-infected cells did not differ significantly at any of the time-points tested. These data indicate that wt RCMV and RCMVΔr127 replicate with similar efficiencies. We therefore conclude that the RCMV r127 gene is dispensable for virus replication in vitro.

**The r127 gene is dispensable for RCMV replication in vivo**

To study the role of the pr127 protein in the pathogenesis of RCMV infection, we compared the in vivo replication characteristics of wt RCMV and RCMVΔr127 during productive infection by monitoring their dissemination in infected rats. The amount of infectious virus produced in salivary gland, spleen, kidney, liver, pancreas and thymus of...
The r127 gene is dispensable for RCMV replication in vitro and in vivo. (a) Generation of an RCMV r127 deletion mutant. RCMVΔr127 was produced by replacing the 0.5 kb Xba–XhoI fragment within RCMV ORF r127 with a 2-1 kb neo expression cassette. The positions of the RXO- and neo-specific probes that were used for Southern blot analysis are indicated. (b) Southern blot analysis. XbaI- and XhoI-digested DNA from wt RCMV (wt; lanes 1 and 3) and RCMVΔr127 (Δr127; lanes 2 and 4) was separated by agarose gel electrophoresis, blotted onto a Hybond-N+ nylon membrane and hybridized to either an RXO- (lanes 1 and 2) or neo-specific (lanes 3 and 4) probe. The lengths of the restriction fragments are shown on the left of each blot. (c) Replication of wt RCMV and RCMVΔr127 in vitro. The graph shows the virus titres in the culture media of wt RCMV- and RCMVΔr127-infected cells at 1, 3, 5 and 7 days p.i. Standard deviations are indicated by vertical bars. <D<, Below detection level. (d) Replication of wt RCMV and RCMVΔr127 in vivo. The diagram shows virus titres in the salivary glands of wt RCMV- and RCMVΔr127-infected rats at 28 days p.i. Each bar represents the result from a single rat. <D<, Below detection level.

wt RCMV- and RCMVΔr127-infected rats was determined at 4 and 28 days p.i. Table 2 shows that there was no significant difference between wt RCMV and RCMVΔr127 in tissue distribution at both 4 and 28 days p.i. High virus titres were predominantly detected in the salivary glands of infected rats at 28 days p.i. As shown in Fig. 5(d), these titres did not differ significantly between wt RCMV and RCMVΔr127. The unlikely possibility that, within the RCMVΔr127-infected rats, the recombinant virus was overgrown by contaminating wt RCMV, e.g. because of insufficient plaque purifications, was excluded by Southern blot analysis on DNA purified from virus that was derived from salivary gland homogenates of RCMVΔr127-infected rats at 28 days p.i. (data not shown).

In general, the inclusion of either more than one type of mutant or a rescued virus is obligatory when assigning functions to mutated genes. However, since wt RCMV and RCMVΔr127 were indistinguishable in their growth characteristics in vitro and in vivo, we decided not to test another independent RCMV r127 deletion mutant in our experimental systems.

Taken together, the RCMV r127 gene is dispensable for virus replication, not only in vitro, but also during the acute phase of infection in vivo.

**DISCUSSION**

One of the most interesting features of the RCMV genome is the r127 gene, which is unique among the CMVs. This gene encodes a protein with similarity to the proteins that are
encoded by the rep genes of parvoviruses as well as the U94 genes of HHV-6A and -6B (Vink et al., 2000). The proteins that are encoded by RCMV r127 and HHV-6A and -6B U94 are most closely related to the Rep proteins of the avian parvoviruses and the AAVs, respectively (Dominguez et al., 1999; Gompels et al., 1995; Isegawa et al., 1999; Srivastava et al., 1983; Thomson et al., 1991; Vink et al., 2000; Zadori et al., 1995). Interestingly, the avian parvoviruses and the AAVs are classified within different genera of the subfamily Parvovirinae within the family Parvoviridae (Lukashov & Goudsmit, 2001). Members of the family Parvoviridae possess linear, single-stranded DNA genomes of approximately 5 kb (reviewed by Berns, 1996). Their genomes contain only two genes: the cap gene, which encodes the structural proteins, and the rep gene, which encodes the non-structural proteins. The subfamily Parvovirinae consists of the genera Parvovirus, Dependovirus and Erythrovirus. Members of the genus Parvovirus can be pathogenic and are capable of autonomous replication, whereas members of the genus Dependovirus are non-pathogenic and require helper functions in order to replicate. These helper functions, which can be supplied by either genotoxic stimuli or coinfesting adenov- or herpesviruses, are needed for productive infection and rescue of integrated virus from the host genome (Berns, 1996). The avian parvoviruses and the AAVs belong to the genera Parvovirus and Dependovirus, respectively, and have different requirements for helper functions (Berns, 1996; Lukashov & Goudsmit, 2001). However, these viruses do have a close genetic relationship. Moreover, it has been suggested that the avian parvoviruses and the AAVs should be classified within the same evolutionary group of parvoviruses (Lukashov & Goudsmit, 2001). It is, therefore, likely that these viruses are derived from a common ancestor. This ancestral parvovirus may have been capable of (site-specific) integration into the host genome, a mechanism by which AAV-2 enters a latent state in the absence of helper functions (Berns, 1996). This mechanism of DNA integration might explain how the rep gene was acquired by RCMV and HHV-6A and -6B, since an ancestral parvovirus may have integrated into the genome of an ancestral betaherpesvirus during coinfection. The rep gene may subsequently have developed a novel function in the replication cycle of its new herpesviral ‘host’, while other parvoviral sequences may have been lost from the herpesviral genome. But, if the rep gene was indeed introduced into the genome of an ancestral betaherpesvirus, then the question arises why rep gene homologues have not been found in the genomes of betaherpesviruses which are genetically closely related to RCMV and HHV-6A and -6B. RCMV is more closely related to HCMV and MCMV than to the other betaherpesviruses, whereas HHV-6A and -6B are more closely related to HHV-7 than to the CMVs (Chee et al., 1990; Dominguez et al., 1999; Gompels et al., 1995; Isegawa et al., 1999; Nicholas, 1996; Rawlinson et al., 1996; Vink et al., 2000). The absence of a rep gene homologue in the genomes of the closest relatives of RCMV and HHV-6A and -6B may indicate that RCMV has acquired the rep gene independently from HHV-6A and -6B. However, this possibility is unlikely, since RCMV r127 and HHV-6A and -6B U94 are conserved not only in sequence, but also in genomic position and orientation (Dominguez et al., 1999; Gompels et al., 1995; Isegawa et al., 1999; Vink et al., 2000). These observations strongly suggest that these genes have diverged from a common ancestral betaherpesvirus origin. The absence of a rep gene homologue in the genomes of HCMV, MCMV and HHV-7 may be explained by loss of the rep gene during evolution due to lack of positive selective pressure.

To date, not much is known about the role of RepH6 in the pathogenesis of HHV-6A and -6B infection. Nevertheless, the U94 genes of HHV-6A and -6B are highly conserved (Rapp et al., 2000), which indicates that RepH6 may play an important role in the replication cycles of these viruses. Interestingly, RepH6 seems to share at least some function with its parvoviral counterparts, since HHV-6A U94 can complement the replication of a rep-deficient AAV-2 genome (Thomson et al., 1994). In addition, U94 is transcribed in latently infected PBMCs from HHV-6A-infected individuals, whereas both virus replication and expression of viral genes are restricted in HHV-6A-infected lymphocytes expressing HHV-6B U94 in vitro (Rotola et al., 1998). These observations point to a potential role for RepH6 in the regulation of latency. RepH6 may be involved in either establishment or maintenance of latency through a mechanism involving the regulation of gene expression. This notion is based on the observations that this protein can bind to the human TATA-binding protein, that it binds single-stranded DNA and that it is able to regulate expression from several promoters (Araujo et al., 1995, 1997; Dhepakson et al., 2002; Mori et al., 2000; Thomson et al., 1994).

At this point, we do not know whether pr127 has a similar role in the pathogenesis of RCMV infection. Although we found pr127 to share characteristics with RepH6, such as DNA-binding activity, we were unable to pinpoint a specific function for pr127 in RCMV replication. Furthermore, it is highly unlikely that pr127 has the same ability as RepH6 to complement parvoviral Rep proteins, since pr127 is considerably shorter than RepH6. More specifically, in comparison with the parvoviral Rep proteins, both pr127 and RepH6 are truncated at their C termini, while pr127 is also truncated at its N terminus (Vink et al., 2000). Nevertheless, although we found RCMVΔr127 to have replication characteristics indistinguishable from those of wt RCMV during the acute phase of infection in vivo, we cannot rule out the possibility that these viruses present with different features in the initiation and maintenance of latency. Due to the unavailability of an appropriate, reproducible experimental model in which to study RCMV latency and reactivation, we have not yet been able to investigate the role of pr127 in these biological processes. Although we detected transcripts of r127 at 4 months p.i. in the salivary glands of RCMV-infected rats, this does not
represent latent gene expression, as infectious virus is still produced in the salivary glands at 4 months p.i. (Bruggeman et al., 1985). It is clear, however, that the development of a model for RCMV latency and reactivation will have a high priority in future studies.

Regardless of its potential role in RCMV latency, it is to be expected that pr127 does have a function during the acute phase of infection. This notion is inferred from the finding that this protein is expressed during productive infection, both in vitro and in vivo. The function of pr127 may have been overseen, because either (i) the function is subtle, (ii) it is only apparent in specific host strains or (iii) we did not use a correct model system. Our future studies will be aimed at the elucidation of the physiological role of the nuclear localization and DNA-binding activity of pr127 and, more specifically, at the identification of viral and/or cellular proteins that bind to pr127. These studies will be likely to shed more light on the role of pr127 in RCMV replication.

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