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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 parvoviral *rep* genes (Dominguez *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

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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 best characterized *rep* gene product is Rep68/78 of AAV-2 (reviewed by Berns, 1996). Rep68/78 is involved in many aspects of the virus replication cycle of AAV-2. It is required for viral DNA replication, modulation of viral and cellular gene expression and site-specific integration of the viral genome into human chromosome 19. Rep68/78 possesses several activities that correspond to its role in the virus replication cycle, including sequence-specific DNA-binding activity, site- and strand-specific endonuclease activity and ATP-dependent helicase activity (Berns, 1996).

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 immediate 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 H_2O. 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′-ACGTGGATCCATGAAGACTAGAACCGG-3′) and 3′-r127 (5′-ACGTAAGCTTAACATCCTAGTCAC-3′). These primers introduced unique *Bam*HI and *Hin*dIII restriction sites into the amplified fragment. Construct pRXO, which contains the RCMV *Xba*I O fragment (Meijer *et al.*, 1986), was used as target DNA. The amplified fragment of 1·0 kb was digested with *Bam*HI and *Hin*dIII 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 ALF*express* automated DNA sequencer (Amersham Biosciences). Construct p302 was then digested with *Bam*HI and *Hin*dIII and the resulting fragment containing ORF r127 was labelled with [α-^{32}P]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′-GGACTCCCGGGTTCCCAAGTACCTC-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 1×10^5 p.f.u. RCMV and sacrificed at either 1 week or 4 months p.i. 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 H_2O and quantified by determination of the absorbance at 260/280 nm.

Generation of *in vitro*-transcribed RNA. Construct p302 (see above) was digested with *Bam*HI and *Hin*dIII and the resulting 1·0 kb fragment containing ORF r127 was cloned into *Bam*HI- and *Hin*dIII-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 H_2O 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 μl^{-1} (Roche Applied Science). Samples were then chilled on ice and added to 50 μl pre-chilled reaction mixture containing 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 3 mM $MgCl_2$, 2 mM DTT, 0·2 mM each dNTP, 10 U RNAguard (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′-CGTATCGTCTGATCCGAACC-3′; position 178908 to 178889 of the RCMV genome) and P4 (5′-GGGATCTCTCCACCAGATGA-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′-CCATAACCTCAACCCTCGTG-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′-GGTGGGTATGGGTCAGAAGG-3′) and RT-ACT-F (5′-TGCCGATAGTGATGACCTGA-3′) to confirm the integrity of the sample.

Generation of MBP–pr127 and 6H–pr127 expression constructs. Construct p302 (see above) was digested with *Bam*HI and *Hin*dIII and the resulting 1·0 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)p*LysS* and the resulting strains were grown overnight at 37 °C in LB medium containing 50 μg ticarcillin ml^{-1} and 20 μg chloramphenicol ml^{-1}. The cultures were diluted 1 : 100 in 300 ml LB medium with ticarcillin and chloramphenicol and grown at 37 °C to an OD_{600} 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)p*LysS* 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 antiserum 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. During 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 antiserum 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 Axiovert 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 Gy of total-body Röntgen irradiation 1 day prior to infection, as described previously (Stals *et al.*, 1990). Rats were either mock-infected or infected by intraperitoneal injection of 3×10^5 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 antiserum 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-*α* (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 *Xho*I and the resulting 2·1 kb fragment containing a neomycin resistance gene (*neo*) was cloned into *Sal*I-digested pBluescript SK (+) vector (Stratagene), generating plasmid p474. Construct p474 was then digested with *Cla*I and *Xho*I and the 2·1 kb *neo* fragment was used to replace the 0·5 kb *Cla*I–*Xho*I fragment within ORF r127 of construct pRXO. The resulting RCMVΔr127 recombination plasmid was designated p475.

Generation of an RCMV r127 deletion mutant. Approximately 2×10^7 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 *Xba*I and *Xho*I. 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 $<0{\cdot}05$ 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 1×10^6 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 $<0{\cdot}05$ 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 parvoviruses and to the proteins that are encoded by the U94 genes of HHV-6A and -6B.

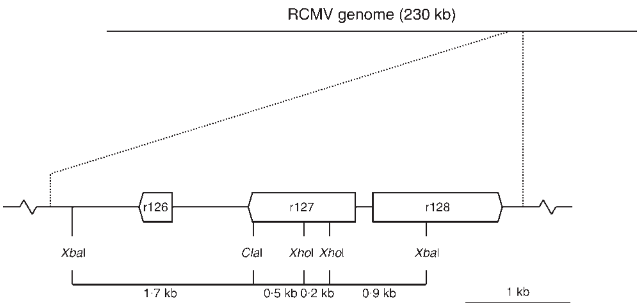

Fig. 1. Map of the 230 kb RCMV genome and position of ORFs r126, r127 and r128. A section of the map, containing the RCMV *Xba*I O fragment (Meijer *et al.*, 1986), has been enlarged below the main map. The direction of the arrow indicates the orientation of the sense strand of the corresponding ORF.

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′ and 3′ 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′

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).

Protein	Amino acids	RCMV pr127	HHV-6A RepH6	BDPV Rep1	MDPV Rep1	GPV Rep1
RCMV pr127	337					
HHV-6A RepH6	490	21				
BDPV Rep1	627	28	22			
MDPV Rep1	626	28	22	98		
GPV Rep1	627	27	22	90	89	
AAV-2 Rep78	621	24	24	47	47	46

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 1·1 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 50 (two clones), 49 (one clone) or 48 (two clones) nucleotides upstream of the predicted ATG initiation codon. Nine independent 3′-RACE clones were sequenced. In seven of these clones, the poly(A)$^+$ tail was identified immediately 3′ of 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 1·1 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*.

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

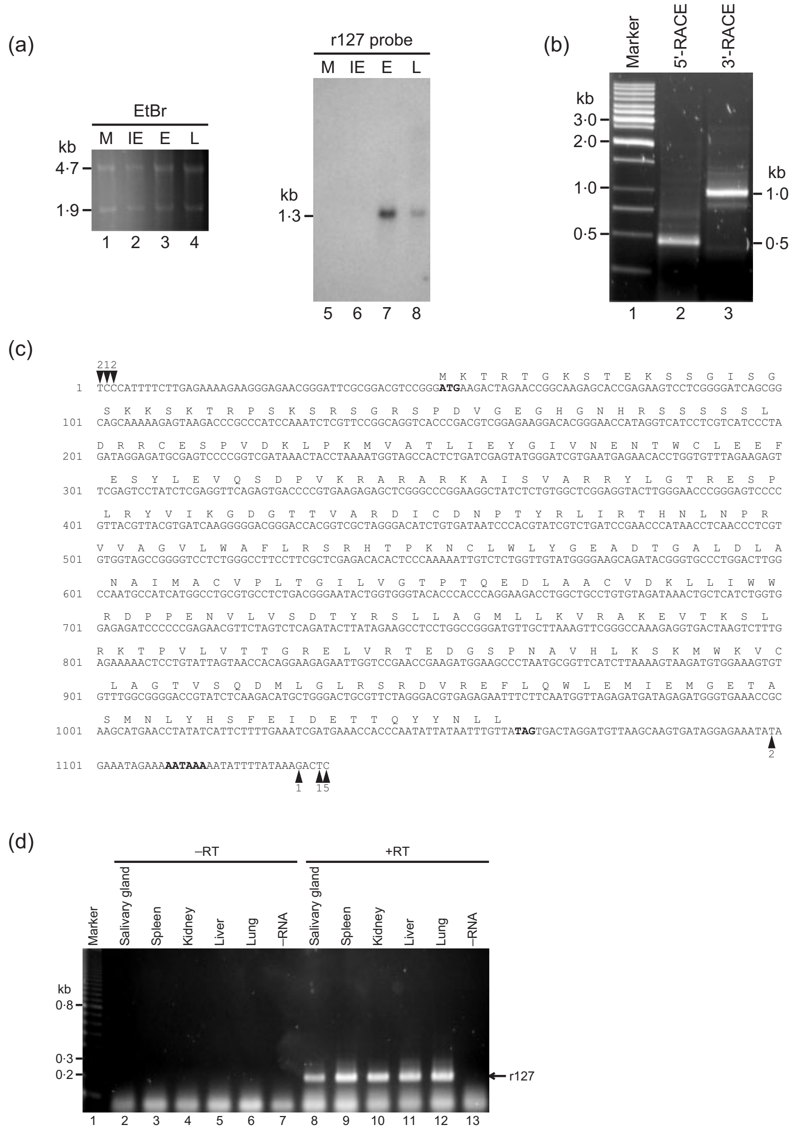

Fig. 2. The r127 gene is transcribed during RCMV infection *in vitro* and *in vivo*. (a) Northern blot analysis. Samples of mock (M; lanes 1 and 5), IE (lanes 2 and 6), E (lanes 3 and 7) and L (lanes 4 and 8) mRNA were separated by agarose/formaldehyde gel electrophoresis, analysed by ethidium bromide staining (EtBr; lanes 1–4), blotted onto a Hybond-N nylon membrane and hybridized to an r127-specific probe (lanes 5–8). The lengths of the rRNA bands and the estimated length of the r127 transcript are shown on the left-hand side of the gel and blot, respectively. (b) 5′- and 3′-RACE. Samples of L mRNA were subjected to 5′- and 3′-RACE experiments (lanes 2 and 3). 5′- and 3′-RACE products were analysed by agarose gel electrophoresis and ethidium bromide staining. The sizes of the DNA markers (lane 1) and the estimated lengths of the 5′- and 3′-RACE products are shown on the left- and right-hand side, respectively, of the gel. (c) Primary structure of the r127 cDNA. The sequence of the r127 cDNA was deduced from the sequences of the 5′- and 3′-RACE products. The translation start (ATG) and stop (TAG) codons and the consensus polyadenylation sequence (AATAAA) are marked. Arrowheads pointing down indicate the number of 5′-RACE clones in which the transcription start site was mapped to a particular residue and arrowheads pointing up indicate the number of 3′-RACE clones in which the poly(A)$^+$ tail was identified immediately 3′ of a particular residue. The amino acid sequence that is predicted to be encoded by the r127 transcript is indicated above the cDNA sequence. (d) RT-PCR. Samples of total RNA from salivary gland (lanes 2 and 8), spleen (lanes 3 and 9), kidney (lanes 4 and 10), liver (lanes 5 and 11) and lung (lanes 6 and 12) of RCMV-infected rats at 1 week p.i. were subjected to a nested RT-PCR assay either with (+RT; lanes 8–13) or without (–RT; lanes 2–7) the addition of reverse transcriptase. The assay was also performed in the absence of RNA (–RNA; lanes 7 and 13). RT-PCR products were analysed by agarose gel electrophoresis and ethidium bromide staining. The sizes of the DNA markers (lane 1) are shown on the left-hand side of the gel. The position of the 200 bp RT-PCR product is indicated on the right (r127).

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

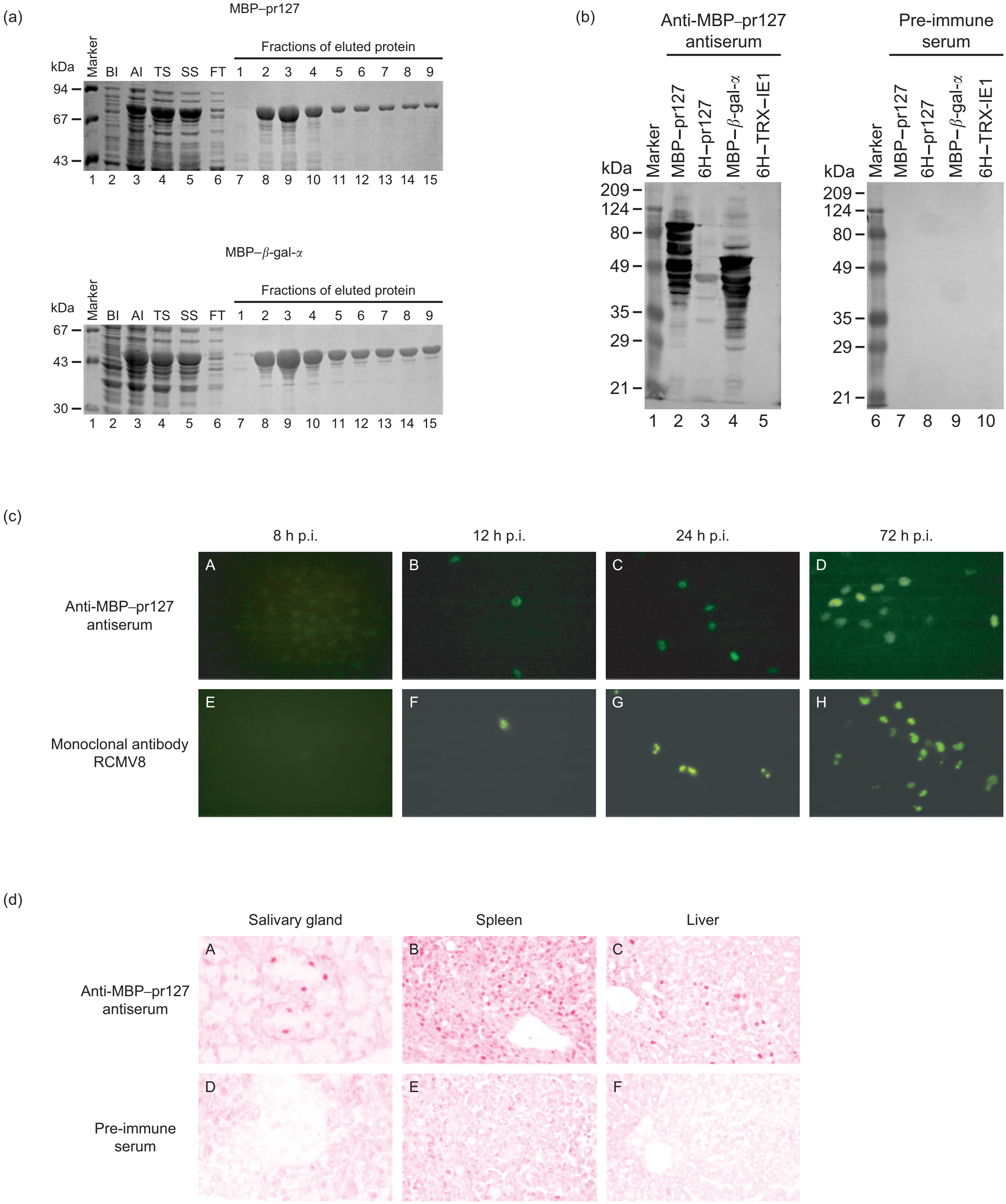

Fig. 3. For legend see page 2008.

Fig. 3. *(on page 2007)* The pr127 protein is expressed within the nuclei of RCMV-infected cells *in vitro* and *in vivo*. (a) Expression and purification of MBP–pr127 and MBP–β-gal-α. MBP–pr127 (upper gel) and MBP–β-gal-α (lower gel) were purified by affinity chromatography on an amylose column and samples were analysed by SDS-PAGE and Coomassie blue staining. Samples include crude extracts of bacteria expressing MBP–pr127 and MBP–β-gal-α before (BI; lane 2) and after (AI; lane 3) induction with IPTG, the total sonication suspension (TS; lane 4), the supernatant of the suspension after centrifugation (SS; lane 5), the flow-through from the column (FT; lane 6) and the collected fractions of eluted protein (lanes 7–15). The sizes of the protein markers (lane 1) are shown on the left-hand side of each gel. (b) Western blot analysis. Proteins from crude extracts of bacteria expressing MBP–pr127 (lanes 2 and 7), 6H–pr127 (lanes 3 and 8), MBP–β-gal-α (lanes 4 and 9) and 6H–TRX–IE1 (lanes 5 and 10) were separated by SDS-PAGE and transferred to a PROTRAN BA 83 nitrocellulose membrane. Blots were immunologically stained using either rabbit anti-MBP–pr127 antiserum (lanes 2–5) or rabbit pre-immune serum (lanes 7–10). The sizes of the pre-stained protein markers (lanes 1 and 6) are shown on the left-hand side of each blot. (c) Immunocytochemical analysis of RCMV-infected cells. RCMV-infected cells were fixed, permeabilized and immunologically stained using either rabbit anti-MBP–pr127 antiserum (panels A–D) or, as a control, anti-pR44 monoclonal antibody RCMV8 (E–H) at 8 (panels A and E), 12 (B and F), 24 (C and G) and 72 h p.i. (D and H). (d) Immunohistochemical analysis of tissues of RCMV-infected rats. Paraffin tissue sections of salivary gland (A and D), spleen (B and E) and liver (C and F) of RCMV-infected rats at 3 weeks p.i. were deparaffinized and immunologically stained using either rabbit anti-MBP–pr127 antiserum (A–C) or, as a control, rabbit pre-immune serum (D–F).

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-α (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

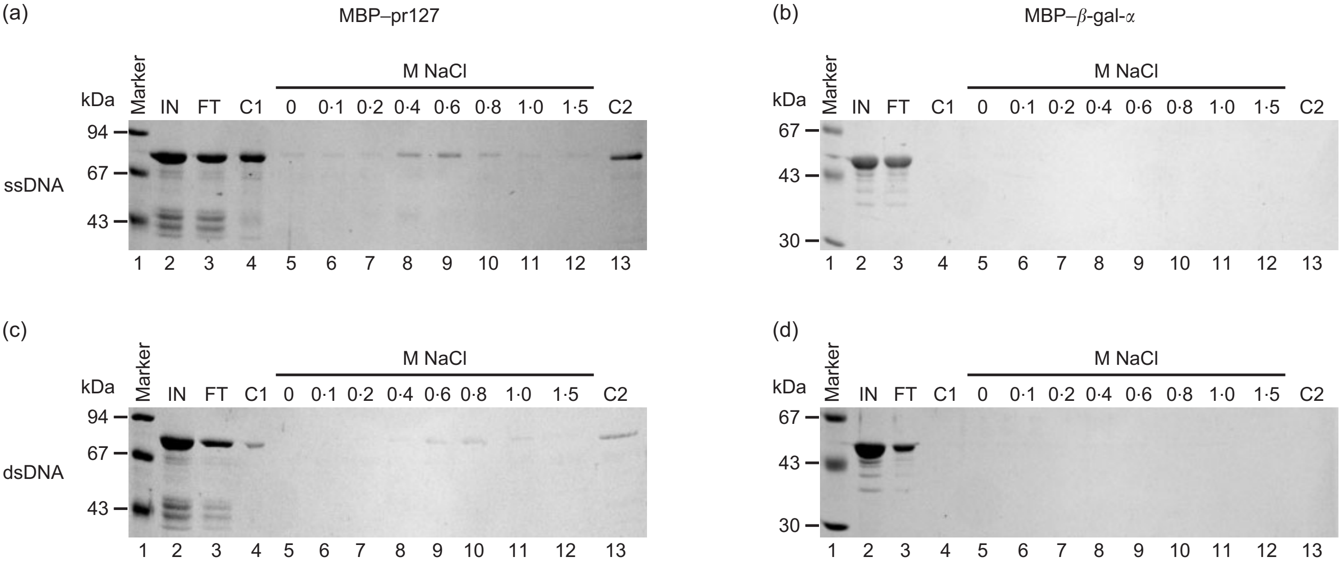

Fig. 4. The RCMV pr127 protein has single- and double-stranded DNA-binding activity. Purified MBP–pr127 (a and c) and MBP–β-gal-α (b and d) were added to single- (ssDNA; a and b) and double-stranded (dsDNA; c and d) DNA-cellulose columns. The columns were washed and proteins were eluted with increasing concentrations of NaCl. Samples were analysed by SDS-PAGE and Coomassie blue staining. Samples include the input (IN; lane 2), the flow-through from the column (FT; lane 3), the material on the column before elution (C1; lane 4), the collected fractions of eluted protein (lanes 5–12) and the material on the column after elution (C2; lane 13). The sizes of the protein markers (lane 1) are shown on the left of each gel.

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-α (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 *Xba*I 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 RXO-specific probe with *Xba*I- and *Xho*I-digested DNA from wt RCMV and RCMVΔr127 should reveal 0·9 and 0·2 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 *Xba*I- and *Xho*I-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

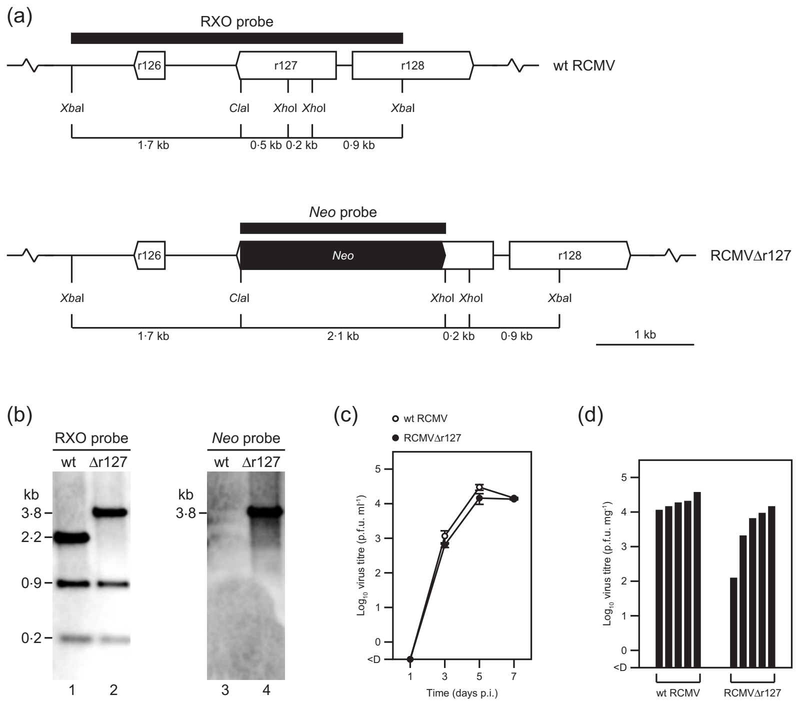

Fig. 5. The r127 gene is dispensable for RCMV replication *in vitro* and *in vivo*. (a) Generation of an RCMV r127 deletion mutant. RCMVΔr127 was produced by replacing the 0·5 kb *Cla*I–*Xho*I fragment within RCMV ORF r127 with a 2·1 kb *neo* expression cassette. The positions of the RXO- and *neo*-specific probes that were used for Southern blot analysis are indicated. (b) Southern blot analysis. *Xba*I- and *Xho*I-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).

Table 2. The r127 gene is dispensable for RCMV replication *in vivo*

Ratios represent the number of rats that were found to be wt RCMV- or RCMVΔr127-positive for a particular organ relative to the total number of rats tested. wt, wt RCMV; Δr127, RCMVΔr127.

Organ	No. of positive rats/total no. of rats tested			
	Day 4 p.i.		Day 28 p.i.	
	wt	Δr127	wt	Δr127
Salivary gland	0/5	0/5	5/5	5/5
Spleen	2/5	3/5	0/5	0/5
Kidney	1/5	0/5	0/5	0/5
Liver	0/5	0/5	0/5	0/5
Pancreas	1/5	0/5	0/5	0/5
Thymus	2/5	3/5	0/5	0/5

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 coinfecting adeno- 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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