Divergent replication kinetics of two phenotypically different parvoviruses of rats

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Rat virus (RV) is an important infectious agent of laboratory rats because of its high prevalence and capacity to disrupt research. Additionally, RV infection serves as a model for characterizing virus–host interactions during acute, persistent and prenatal infection. Our research has examined the pathogenesis of two RV strains, RV-UMass and RV-Y. RV-UMass is more pathogenic, causes a higher level of persistent infection and transmits to the foetus after oronasal inoculation of the pregnant dam. To determine in vitro distinctions between the strains that may account for these differences and to provide a benchmark for characterizing virus replication in vivo, synchronized in vitro replication of both RV strains was defined and compared. The results demonstrated that RV replication has replicative intermediates, virus transcripts and proteins similar to those reported for the prototype parvovirus, minute virus of mice. However, the replicative cycle of RV-UMass was 12 h compared with 24 h for RV-Y, and RV-UMass and RV-Y differed in kinetics of virus DNA replication, transcription and protein accumulation. Additionally, in situ analysis correlated well with kinetics data as determined by Southern and Northern blot analysis. Sequence comparisons between the strains also determined coding differences that may contribute to phenotypic differences.

Introduction

Autonomous rodent parvoviruses are small, non-enveloped viruses that replicate without a helper virus. Virus replication in vitro is best defined for minute virus of mice (MVM). After infection of permissive cells, the 5 kb single-stranded (ss)DNA genome is converted to a 5 kb double-stranded replicative form (mRF). The double-genome length 10 kb replicative form (dRF), mRF and newly synthesized ssDNA accumulate by a rolling hairpin mechanism (Astell et al., 1985; Cotmore & Tattersall, 1996). The viral genome has two promoters that transcribe three alternatively spliced mRNAs. R1 encodes nonstructural protein 1 (NS1), R2 encodes NS2 and R3 encodes the two capsid proteins, VP1 and VP2 (Cotmore & Tattersall, 1987).

Rodent parvoviruses are prevalent infectious agents of laboratory mice and rats with the capacity to disrupt animal-based research. In the case of rat virus (RV), infection can result in clinical morbidity and mortality, disruption of immune function or alteration of tumour cell growth both in vitro and in vivo (Jacoby & Ball-Goodrich, 1995). Transplacental infection can cause teratogenic effects or foetal deaths (Jacoby & Ball-Goodrich, 1995). Additionally, RV can persist for extended periods in rats infected as infants and can be transmitted for more than 2 months (Jacoby et al., 1988).

In addition to its importance as an infectious agent, RV is a model for characterizing virus–host interactions during acute, persistent and prenatal infection. Infection of 6-day-old or older rats is usually clinically silent, whereas severe or lethal infection may occur after infection of foetal or neonatal rats. Non-fatal infection of neonatal rats with the RV Yale strain (RV-Y) leads to virus persistence for at least 6 months, despite development of host immunity (Jacoby et al., 1991). However, the prevalence of persistence is not high enough to study corresponding virus–host interactions, and transplacental transmission does not occur after oronasal inoculation of pregnant dams (Jacoby et al., 1988).

To increase the prevalence of RV persistence and transplacental transmission, we characterized infection with a more...
infection will allow for a more detailed evaluation of virus replication kinetics and reagent sensitivity during a controlled infection. Median infectious dose (TCID₅₀) was prepared as described previously (Ball-Goodrich & Johnson, 1994). Propagated in the normal rat kidney (NRK) cell line and stocks were used to examine virus–host interactions. Analysis of in vivo infection has relied significantly on the assumption that RV replication is similar or identical to that of MVM or H-1 virus, another parovirus of rats. To improve our interpretation and understanding of RV replication in vivo, we have defined the replicative cycle of RV-UMass during single-round infection of synchronized cells. Definition of virus replication kinetics and reagent sensitivity during a controlled infection will allow for a more detailed evaluation of virus status during persistent in vivo infection. Because of differences in vivo between infection with RV-UMass compared with RV-Y, we compared in vitro replication kinetics of RV-UMass and RV-Y and DNA sequences of both strains to determine if strain differences in pathogenicity correlate with differences in replication kinetics and nucleotide sequence.

Methods

Preparation and titration of virus stocks. RV-UMass was propagated in the normal rat kidney (NRK) cell line and stocks were prepared as described previously (Ball-Goodrich & Johnson, 1994). Median infectious dose (TCID₅₀) was determined in NRK cells. RV-Y was grown in 324K cells (SV40-transformed human kidney cells), and its TCID₅₀ was found to be the same in both 324K and NRK cells. A stock of the lymphotropic strain of MVM (MVMi) was prepared as described previously (Ball-Goodrich & Tattersall, 1992).

Synchronized virus infection. NRK cells were synchronized using a modification of the double-block method of isoleucine-deprivation followed by incubation in the DNA polymerase inhibitor aphidicolin (Cotmore & Tattersall, 1987). We used charcoal/dextran-treated foetal bovine sera (FBS) rather than dialysed FBS during the 44 h incubation in isoleucine-deficient media. Monolayers were washed and then complete media containing 10 µg/ml aphidicolin and either RV-UMass or RV-Y, at an m.o.i. of 1, was added. Cells were washed 20 h later and fed with complete media (T = 0). Neuraminidase was added to the cultures at T = 3 (RV-UMass) or T = 5 (RV-Y) to prevent second-round infection. RV-UMass-infected and RV-Y-infected cultures were harvested at the times indicated in Results. At each time-point, cells were washed and scraped into PBS, centrifuged and flash-frozen for later preparation of RNA, DNA and protein. Cytospin preparations of RV-UMass-infected or mock-infected cells were prepared and fixed in acetone. Additionally, uninfected cells and RV-UMass-infected cells were suspended in paraformaldehyde–lysine–periodate fixative (PLP) and held overnight at 4 °C. After two PBS washes, cells were pelleted, resuspended in PBS and mixed with warm 2% agarose. After the agarose had hardened, the cell suspensions were embedded in paraffin and sectioned at 5 µm for in situ hybridization (ISH).

Asynchronous virus infection. 324K cells were infected with MVMi at an m.o.i. of 1. At 24 h post-infection (p.i.), monolayers were washed and scraped into PBS. Cells were then centrifuged and flash-frozen.

DNA purification and Southern blot analysis. Total cellular DNA was prepared from 4 × 10⁶ virus-infected or mock-infected cells (Ball-Goodrich & Tattersall, 1992). Duplicate gels were transferred to Hybond-N+ membranes (Amersham/Pharmacia) as described (Ball-Goodrich & et al., 1998) and hybridized with 3²P-labelled positive- and negative-sense riboprobes made using a HindIII (nt 2655) to HindIII (nt 4274) RV template. Hybridization and wash conditions were as described previously (Ball-Goodrich & Tattersall, 1992). Membranes were exposed to Kodak X-OMAT films and bands were quantified using a Phosphorimager SI and ImageQuaNT software (Molecular Dynamics).

RNA purification and Northern blot analysis. Total cellular RNA was prepared from 6 × 10⁶ virus-infected or mock-infected cells using the Qiagen QIAshredder and RNeasy mini-kit as described by Qiagen. Because purified RV-UMass RNA was contaminated with small amounts of low molecular mass viral DNA, it was subsequently digested with 1 U of RNase-free DNase, extracted with phenol–chloroform and ethanol precipitated. To eliminate viral DNA from RV-Y-infected cell RNA, poly(A) RNA was purified on a QiaGene oligo(dT) column. RNA was electrophoresed on 1% formaldehyde–agarose gels containing 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA. 22 mM formaldehyde at 6 V/cm in buffer containing 246 mM formaldehyde. Gels were washed twice in DEPC-treated water and RNA was transferred to Hybond-N+ membranes with 20 × SSC. Baked blots were prehybridized and hybridized as described above except 3²P-labelled random-prime probes were made from EcoRI (nt 1086) to HindIII (nt 4274) template DNA or from a PCR fragment encompassing the common 5′ region of R1 and R2. To control for RNA quantity, membranes were hybridized using a random-prime probe that detects cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion).

Generation of antibodies specific for RV VP and NS proteins. RV VP2 protein-coding sequence was amplified by PCR using primers incorporating BamHI and HindIII sites in-frame with both VP2 and pET20b+ vector (Novagen), an IPTG-inducible vector that adds a His-tag onto the C terminus of the expressed protein. The PCR product was cleaved with BamHI and HindIII, ligated into the predigested vector and electroporated into Escherichia coli strain JM109. Plasmids containing the VP2 gene were purified and electroporated into E. coli expressor strain BL21(DE3). Expression of RV VP2 was induced and the protein was purified using denaturing metal-chelation column chromatography. Polyonal VP2-specific sera were produced in rabbits by immunization with purified denatured protein following standard protocols.

The common N-terminal coding region of RV NS1 and NS2 (nt 266–511 in the RV genome; NS1 aa 2–83) was amplified by PCR, cloned into pET20b+ and protein was expressed and purified as above. Purified protein was used to immunize guinea pigs. Specificity of VP2 and NS antisera was tested by indirect immunofluorescence assay (IFA) and Western blot analysis. Antisera reacted with RV-infected but not uninfected cells by IFA and appropriate size bands were detected in infected but not uninfected cell lysates. Because the NS and VP antigens were denatured, antisera cross-react with conserved amino acids in MVM NS and VP proteins on Western blots.

Western blot analysis of viral proteins. Western immunoblots were performed as described previously (Ball-Goodrich & Tattersall, 1992). Viral proteins were detected using either a 1:1300 dilution of...
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Fig. 1. Southern blot of RV DNA replication. Total cellular DNA (1–44 × 10⁵ cell equivalents) from uninfected, RV-UMass-infected (A, B) or RV-Y-infected (C, D) synchronized NRK cells as fractionated by electrophoresis on a 1% agarose gel and transferred to nylon membranes. Membranes were hybridized with positive-sense (A, C) or negative-sense riboprobes (B, D). Labelling on top of lanes corresponds with uninfected cells (M) or to the time-point of the sample. Replicative forms are labelled and 10 ng and 1 ng of RV plasmid DNA (labelled 10 or 1) served as quantified controls.

guinea pig polyclonal sera specific for the N terminus of NS1 and NS2 and a 1:1500 dilution of horseradish peroxidase (HRP)-conjugated anti-guinea pig antibody (ICN) or a 1:5000 dilution of a rabbit polyclonal sera specific for VP1 and VP2 and a 1:10 000 dilution of HRP-linked goat anti-rabbit antibody (ICN). Detection was performed using the Renaissance chemiluminescence reagent plus system (NEN).

In situ hybridization (ISH). Sections of PLP-fixed paraffin-embedded cells mounted on positively charged slides were processed for detection of viral DNA using a positive-sense probe as described previously (Ball-Goodrich et al., 1998). Sections hybridized with a negative-sense probe for detecting viral RNA were dehydrated, hydrated and treated with 0.2 M HCl for 20 min at room temperature. Slides were rinsed in DEPC-treated water, covered with 2 x SSC and placed on a 70 °C hot-plate. Tissues were re-fixed in cold 4% paraformaldehyde in PBS, washed in PBS and treated with 0.25% acetic anhydride in 0.1 M triethanolamine. After rinsing in 2 x SSC and DEPC-treated water, cells were dehydrated. Strand-specific 32P-labelled riboprobes (sp. act. 2 × 10⁷ c.p.m./µg of RNA) were used to detect either viral DNA or mRNA. Hybridization conditions, washing and detection were as previously described (Ball-Goodrich et al., 1998). Level of infection and sensitivity of ISH were determined by counting more than 200 cells at multiple time-points after ISH with a positive-sense riboprobe or indirect IFA using anti-VP antibodies. Both methods determined that approximately 30% of cells were infected.

Immunocytochemistry (ICC) for viral proteins. Cytospin preparations of cells from all time-points were blocked with 5% goat serum. Viral proteins were detected using a 1:500 dilution of anti-VP polyclonal rabbit serum or a 1:50 dilution of anti-NS polyclonal guinea pig serum, both diluted in 1% goat serum in PBS. Slides were washed in PBS and stained with a 1:100 dilution of appropriate secondary antibody (FITC-conjugated goat anti-rabbit IgG or rhodamine-conjugated goat anti-guinea pig IgG; ICN). Slides were washed in PBS and cover-slipped with 80% glycerol in PBS.

Sequencing and analysis of viral DNA. Sequencing of RV-Y clones containing EcoRI (nt 1086) to HindIII (nt 2655) and HindIII (nt 2655) to HindII (nt 4274) fragments was performed by the DNA Sequencing Facility at the W. M. Keck Foundation Biotechnology Laboratory at the Yale School of Medicine, USA. Sequence analysis was performed using Genetics Computer Group (GCG) analysis programs on the VAX at Yale School of Medicine, USA. The programs used were Pileup, Pretty, Gap and Best Fit. The parameters used were default settings. RV-Y sequence was compared with RV-UMass (Ball-Goodrich et al., 1998) and both RV strains were aligned with the MVMi sequence.
Results

Comparison of RV-UMass and RV-Y replication

Synchronized monolayer cultures of NRK cells were infected with RV-UMass. Cytopathic effects (CPE), including increased granularity and refractivity, had occurred by 6 h after release from the aphidicolin block and cytolysis was extensive at 12 h. Preliminary experiments showed that, by 18 h, monolayers were sparse and little viral DNA could be purified from cells. When RV-Y was used to infect synchronized monolayer cultures of NRK cells, CPE was first evident by 18 h and had increased by 24 h. However, cytolysis at this time was minimal compared with that caused by RV-UMass-infected cultures.

DNA replication during synchronized infection was examined by Southern blot analysis. Total cellular DNA from RV-UMass- (Fig. 1A, B) and RV-Y-infected cultures (Fig. 1C, D) was prepared for each time-point and identical membranes were hybridized using positive-sense (Fig. 1A, C) or negative-sense (Fig. 1B, D) riboprobes. After a 1 week exposure, the positive-sense riboprobe detected input RV-UMass negative-sense ssDNA at the time of release from aphidicolin (T = 0), which disappeared by 3 h with conversion to mRF. After 1 h exposure of the blot, mRF, dRF and newly synthesized ssDNA were evident at the 4–5 h time-point (Fig. 1A). Replication intermediates peaked and plateaued by 9 h, at which time levels of intracellular ssDNA surpassed those of mRF and dRF. Levels of ssDNA continued to increase through 12 h. Hybridization with a negative-sense riboprobe (Fig. 1B) confirmed that virtually all ssDNA was negative-sense throughout the infection, although trace amounts of positive-sense ssDNA were observed at 4–5 and 6 h. Based on comparison with RV DNA standards, the 4–5 h time-point sample contained approximately 1 ng of negative-sense ssDNA (1.46 × 10⁸ total copies; 2.6 × 10⁸ copies per cell) and 0.01 ng of positive-sense ssDNA (1.46 × 10⁸ total copies; 2.6 × 10⁸ copies per cell).

Southern blot analysis of RV-Y DNA showed that the kinetics of replication were different from those of RV-UMass. After a 3 day exposure of the blot, trace amounts of input viral DNA were detected at T = 0, mRF was detected by 3 h and dRF was detected by 4–5 h. After 1 h exposure of the blot hybridized with a positive-sense riboprobe (Fig. 1C), mRF and dRF accumulated by 6 h and trace amounts of newly synthesized ssDNA were evident. The peak of replication was reached by 12 h. Levels of replicative forms were sustained through 24 h, but levels of ssDNA decreased. Hybridization with a negative-sense riboprobe (Fig. 1D) verified that the majority of ssDNA was negative-sense. However, trace amounts of positive-sense ssDNA were present from 6 through 12 h, with a peak at 9 h.

RV-UMass and RV-Y transcription were examined by Northern blot analysis (Fig. 2A–C). At T = 0, RV-UMass viral transcripts were barely detected after a long exposure of the membrane. By 3 h, moderate levels of all three viral transcripts, R1 (4–8 kb), R2 (3–3 kb) and R3 (3–0 kb), were present, but R2 and R3 were not well-resolved because of their similar size (Fig. 2A). R1 mRNA levels peaked between 4–5 and 6 h and decreased through subsequent time-points, and the R2/R3 doublet was more abundant than R1 at all time-points. To determine the levels of the R2 transcript, a second Northern blot was probed using a random-primed PCR fragment specific for the 5’ common region of R1 and R2 (Fig. 2B). Ratios of R1 and R2 can be directly compared within each lane, but rRNA levels were not equivalent in all samples based on ethidium bromide staining. Levels of R1 and R2 were roughly equivalent

Astell et al., 1986; Sahli et al., 1985). The RV-Y sequence was submitted to GenBank and assigned accession number AF317513.
between 3 and 4.5 h, but band intensity between 7.5 and 9 h indicated that R1 levels decreased faster. Because the intensity of the 7.5 and 9 h R1 bands was equivalent in Fig. 2(A, B), we determined that the predominant RNA late in infection was R3. By matching exposures of the R1 band at the 4.5 h time-point, it can be deduced that this sample contained roughly equivalent amounts of R1, R2 and R3.

Kinetics of RV-Y transcription correlated with those seen for RV-Y DNA replication. Trace amounts of R1 were evident at T = 0 after a long exposure. At 3 h (Fig. 2C), R1, R2 and R3 were present and levels of all three transcripts increased during the next 9 h. The level of R1 decreased by 18 h and stabilized through 24 h. R2/R3 remained high throughout the time-course. Northern blot analysis confirmed that R1, R2 and R3 of RV-Y, RV-UMass and MVM correlated in size (data not shown).

RV-UMass protein accumulation was examined by Western blot analysis using antisera specific for the N terminus of NS1 and NS2 (Fig. 3 A) or VP1 and VP2 (Fig. 3 B). At T = 0, there were trace amounts of NS1 and no NS2. NS1 and NS2 were detected by 3 h, quantities increased and peaked by 9 h and levels decreased by 12 h. The NS1 doublet contained roughly equivalent bands, whereas bands of the NS2 doublet differed in intensity during the time-course. Input capsid VP1 and VP2 were detectable at T = 0 (Fig. 3 B). Levels of both proteins increased maximally through 7.5 h, with only a small increase thereafter.

RV-Y protein accumulated later than that for RV-UMass (Fig. 3 C, D). A trace NS1 band was detected at T = 0 (Fig. 3 C), levels increased by 3 h, a doublet appeared by 4.5 h and levels plateaued by 12 h. A faint NS2 doublet was evident at 6 h and levels increased through 12 h. Levels of both NS1 and NS2 dropped by 24 h post-release. VP1 and VP2 (Fig. 3 D) were first detected at 4.5 h. Levels increased through 9 h, when accumulation peaked and plateaued. RV-Y and RV-UMass proteins were compared with those of MVM by Western blot analysis (data not shown) and the RV and MVM proteins correlated in size.

Characterization of RV-UMass-infected cell cultures in situ

A major focus of our current research is examining the pathogenesis of RV-UMass infection in rats. In order to determine the sensitivity of riboprobes used for ISH of fixed and embedded tissue, we used synchronized infections to correlate copy number of viral genomes detected by quantification of the Southern blot with ISH of the identical, infected...
Fig. 4. ISH of RV-infected synchronized cells. Cells were fixed in PLP and spun into agarose. The agarose pellets were embedded in paraffin, sectioned at 5 µm and adhered to glass slides. Sections were hybridized with either a positive-sense riboprobe for detection of viral DNA (A, C, E, G, I) or a negative-sense riboprobe for detection of RNA (B, D, F, H, J). Photomicrographs correspond with the following times after release from aphidicolin block: A, B = 0; C, D = 4–5 h; E, F = 6 h; G, H = 7–5 h; I, J = 12 h.

cell population. Histological sections of pelleted, synchronized RV-UMass-infected cells were in situ-hybridized with positive-sense riboprobe to detect viral DNA. At T = 4.5 (Fig. 4C), localized nuclear signal was first evident after a 3 h exposure. Using phosphorimager analysis, we quantified the T = 4.5 bands compared with standards on the Southern blot, where
Table 1. Comparison of protein-coding sequences from RV-UMass and RV-Y

For sequence comparison, the carboxy 60% of the NS1 protein-coding sequence was used. The sequence located after the major splice-site, including the 3' carboxy terminus, was used for the comparison of the NS2 protein-coding sequence.

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<th>Protein</th>
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<tr>
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<td>VP1 unique region</td>
<td>1</td>
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<td>VP1/VP2 common region</td>
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1·44 × 10^6 cell equivalents were loaded per lane. The level of virus infection, 39%, was determined in the ISH-positive and virus antigen-positive cells. Therefore, the amount of replicative and viral ssDNA easily detected was 2·6 × 10^6 copies per cell. When time of exposure of the Southern blot and the in situ-hybridized slides was increased, a signal was detected at both 3 h post-release, at which time there were approximately 4·08 × 10^2 ssDNA copies per cell. These calculations will allow estimation of copy number of genomes detected by ISH on infected tissues.

In addition to defining the sensitivity of riboprobes, this experiment also allowed us to examine the kinetics of viral RNA and DNA in situ at multiple time-points in the replicative cycle. Histological sections of fixed and embedded synchronized RV-UMass-infected cells (Fig. 4 A–J) were prepared for detection of viral DNA and hybridized with a positive-sense riboprobe (Fig. 4 A, C, E, G, I). After exposure to emulsion for 18 days, a low level of signal was detected at T = 0 (Fig. 4 A) and 3 h, which was attributed to input virus. After a short exposure (3·5 h), T = 4·5 was the first time-point where signal was detected over infected cell nuclei (Fig. 4 C). DNA signal intensity increased by 6 h, peaked at 7·5 h and plateaued at 12 h (Fig. 4 E, G, I). At T = 0, cells prepared for RNA detection and hybridized with a negative-sense riboprobe were negative after an 18 day exposure to emulsion (Fig. 4 B). By T = 3, a low level of RNA was detected over individual cells after a short (3·5 h) exposure. Signal strength increased from 4·5 to 7·5 h (Fig. 4 D, F, H) and decreased through 12 h (Fig. 4 J).

Sequence analysis of RV-Y

To examine the genetic basis of phenotypic differences between RV-Y and RV-UMass, the RV-Y genome was sequenced from the EcoRI site (nt 1087) to the HincII site (nt 4274) and compared with the previously reported sequence of RV-UMass. The results are summarized in Table 1. The sequence of RV-Y was 99·3% identical to RV-UMass and the two genomes had a total of seven silent and 11 protein-coding differences within the sequenced region, the majority of which were in the VP2 gene. RV-Y and RV-UMass VP2 sequences were aligned with the previously reported MVMi sequence using the GCG sequence analysis software. Using the MVMi ‘roadmap’ published by Agbandje-McKenna et al. (1998) where surface amino acids on the virion were identified, we used an alignment of RV and MVMi VP2 to project which RV-UMass/RV-Y VP2-coding differences were on the surface of the virion. The results are summarized in Fig. 5. In addition, when the locations of RV-coding differences were compared with those of the allotropic MVM changes, many of the RV-coding differences were in the proximity of MVM allotropic changes.

Discussion

Previous research has examined individual elements of RV replication (Mitra et al., 1983; Salzman & Fabisch, 1978). This report presents the first comprehensive assessment of RV replication in synchronized cells, including the time-course of RV replication, transcription and translation during a single-round infection. Additionally, prior results from our laboratory indicate that RV-Y differs from RV-UMass in both tropism and virulence. In vitro comparison determined that RV-Y infects both 324K and NRK cells, whereas RV-UMass infects only NRK cells (data not shown). A lower dose of RV-UMass is required to achieve seroconversion in adult rats or lethal infection in infant rats; RV-UMass causes a higher incidence of persistent infection and transmits more efficiently to the foetus after oronasal inoculation of dams than does RV-Y (Gaertner et al. 1996). The current study showed that in vitro replication strategies of RV-Y and RV-UMass were similar, but specific differences in replication kinetics and/or coding sequence could account for the phenotypic differences.

Analysis of RV-UMass and RV-Y replication showed an accumulation of replicative intermediates identical to those
found during synchronized MVM infection. Early in infection, input negative-sense ssDNA was converted to mRF and levels of mRF and dRF accumulated prior to the synthesis of ssDNA. Minor species present during RV-UMass and RV-Y replication may correspond to replicative intermediates described during MVM replication (Faust & Gloor, 1984; Tullis et al., 1994). One MVM intermediate, designated band X (Tullis et al., 1994), is an RNA–DNA duplex migrating between mRF and ssDNA that may correlate with a similar size RV band detected using the positive-sense riboprobe. A second MVM intermediate (Faust & Gloor, 1984), an 8 kb partially replicated dimer, may correspond with an RV band detected using both riboprobes that migrated between mRF and dRF.

RV-UMass and RV-Y ssDNA were predominantly negative-sense, but small amounts of positive-sense ssDNA were detected early in infection. Such an intermediate is not included in the current modified rolling hairpin model proposed for MVM replication (Astell et al., 1985; Cotmore & Tattersall, 1996), but small amounts of positive-sense ssDNA have also been detected in purified virion preparations of MVM (Gardiner & Tattersall, 1988a). Its appearance early in the replicative cycle may provide additional templates for transcription and replication.

Late in infection, RV-UMass and RV-Y negative-sense ssDNA migrated at a slightly higher molecular mass and was more heterogeneous than early in infection. The altered migration may be due to the accumulation of partially double-stranded forms, defective interfering genomes or linkage of NS1 to the terminus of virion DNA, as was observed with MVM (Cotmore & Tattersall, 1989). In addition, RV-Y had reduced levels of intracellular ssDNA whereas RV-UMass accumulated high levels of intracellular ssDNA. Because RV-UMass is the more cytotoxic virus, it is possible that accumulation of virus correlates with cytotoxicity. Reduced intracellular RV-Y ssDNA cannot be explained by abortive infection of NRK cells. The TCID<sub>50</sub> of RV-Y is the same in both NRK and 324K cells, indicating equivalent infectivity in both cell types. A similar decrease in intracellular virus was reported for synchronized MVM infections (Cotmore et al., 1989; Tullis et al., 1992). Titration of extracellular and intracellular virus determined that low levels of intracellular ssDNA were due to preferential export of progeny virions from the cell. Although extracellular virus was not examined as a part of this study, the differences in levels of intracellular ssDNA could involve the use of a similar mechanism of virus export by RV-Y.

Another difference between the two strains involved the time-course of replication. The replicative cycle of RV-UMass was approximately 12 h, whereas RV-Y monomer and dimer intermediates did not accumulate until 9 h and maximal DNA replication continued through 24 h. Therefore, the replicative cycle of RV-Y was similar to that of MVM (Cotmore & Tattersall, 1987; Cotmore et al., 1989). RV-UMass replication kinetics were similar to those described previously for the Kilham strain of RV (Salzman & Fabisch, 1978), although direct comparison is difficult because of differences in methods of cell synchronization and analysis. RV-Y transcription closely overlapped DNA replication. In contrast, RV-UMass transcription peaked early when DNA replicative intermediates were first evident and levels of nonstructural transcripts decreased with increased DNA replication. This sequence was also observed during asynchronous infection with the Kilham strain of RV (Salzman & Redler, 1974). The sizes of RV-UMass and RV-Y transcripts were similar to those reported for MVM (Pintel et al., 1983) and RV (Salzman et al., 1982).

RV-UMass proteins accumulated after transcription but prior to maximal DNA replication. In contrast, the accumulation of RV-Y proteins more closely overlapped DNA replication. NS and VP proteins of both virus strains were similar in size to those encoded by MVM (Cotmore et al., 1983) and H-1 virus (Cotmore et al., 1983; Paradiso, 1984) and the NS1:NS2 ratios were similar to those of MMO1 (Ball-Goodrich & Tattersall, 1992). Both RV NS1 and NS2 migrated as doublets on SDS–polyacrylamide gels, whereas an MVM preparation on the same gel only had an NS2 doublet (data not shown). While some preparations of MVM NS1 migrate as two species of 83 and 65 kDa, the two forms migrate with a larger band separation on SDS–PAGE (Cotmore & Tattersall, 1987) than was seen for the RV doublet in our study. NS1 of H-1 virus has been shown to migrate as a doublet and the higher molecular mass form was characterized as a highly phosphorylated, post-translationally modified form (Paradiso, 1984). There are two possible explanations for the RV NS2 doublet. As with MVM (Cotmore & Tattersall, 1990), the RV genome contains sequences for alternative splicing of the R2 transcript to encode a mixture of different carboxy-terminal forms of NS2. Secondly, Clemens et al. (1990) found that transfected cells expressing individual cDNA versions of MVM NS2 had NS2 doublet bands and that the two species differed in their level of phosphorylation.

While we have no direct proof that the replication pace of RV correlates with cytotoxicity, this concept has been demonstrated for other viruses. For example, reovirus strain Dearing 3 (serotype 3) forms larger plaques and exhibits greater cytopathogenicity than strain D/5 Jones (serotype 2) (Gaillard & Joklik, 1985). At 12 h p.i., serotype 2-infected cells formed only 20–30% as much viral ssRNA, dsRNA and protein as cells infected with serotype 3 (Moody & Joklik, 1989). Faster replication resulting in increased cytopathogenicity of RV-UMass also may correlate with accumulation of NS1 and NS2 during infection. RV-UMass NS1 and NS2 were expressed early, achieved peak levels at comparable time-points and CPE was apparent early in infection. In contrast, RV-Y NS1 reached peak levels later, NS2 accumulation peaked later still and infection resulted in less severe CPE, which appeared by 18 h p.i. NS1 is the major cytotoxic protein of MVM and co-expression of NS2 enhances CPE of NS1 (Legrand et al., 1993). By extrapolation, early accumulation of both proteins may increase the virulence of RV-UMass in vitro.
and also may influence its pathogenicity in vivo. A precedent for correlation between virulence in vitro and in vivo is provided by lymphocytic choriomeningitis virus. One virus type causes a clear plaque and CPE in indicator cells and lethal choriomeningitis in adult mice, whereas a second type causes a turbid plaque with minimal CPE in indicator cells and is not lethal for adult mice (Hothcin et al., 1971, 1975).

Sequence analysis of RV-UMass and RV-Y from nt 1086 to 4262 revealed one coding difference in the carboxy-half of NS1, one coding difference in the VP1-specific region and nine coding differences in the VP1/VP2 common region. Mitra et al. (1982) established a correlation between RV capsid region nucleotide changes and differences in pathogenicity. They found different restriction enzyme patterns in the capsid-coding region of two RV strains with different tropisms in vitro and pathogenicity in vivo. Evidence correlating coding differences in the capsid genes with phenotypic changes has also been found for other parvoviruses. Studies with MVM (Antonietti et al., 1988; Ball-Goodrich & Tattersall, 1992; Gardiner & Tattersall, 1988b) and canine parvovirus (Chang et al., 1992) have defined the capsid as the determinant of host cell tropism in vitro and pathogenicity of MVM in vivo (Brownstein et al., 1992). Based on the VP2 alignment of RV-Y, RV-UMass and MVM, most RV VP2-coding differences are located on the surface of the virion near amino acids involved in host range and pathogenicity for MVM. Because we did not sequence the P4 promoter region or the N terminus of NS1, we cannot conclude that capsid region changes result in the increased pathogenicity of RV-UMass. However, intertypic recombinants between RV-UMass and RV-Y would define the genetic basis of accelerated replication kinetics and greater pathogenicity of RV-UMass.

Reagents developed during this study are being used to analyse RV-UMass infection in rats (Jacoby et al., 2000). Therefore, it was important to assess their ability to detect viral DNA, RNA and proteins under conditions for ISH and ICC. With synchronized-infected cells substituting for rat tissues, the prevalence of ISH signal correlated well with results obtained by ICC. The negative-sense riboprobe detected transcription in infected cells prior to DNA replication. Signal was strongest at a time-point corresponding to peak mRNA accumulation by Northern blot analysis and declined late in infection when viral DNA replication was high. The positive-sense riboprobe produced an early low signal, which increased throughout the course of infection. Thus, results from Southern and Northern blot analysis correlated well with ISH results and facilitated estimation of virus copy numbers in infected cells. In addition, immunohistochemistry confirmed the specificity and reactivity of anti-NS and anti-VP antibodies with virus antigens in situ. Information about RV replication kinetics gained during controlled in vitro infection should facilitate analysis of infection in vivo.

We thank Frank Paturzo for expert technical assistance. This work was supported by a National Institutes of Health grant to R.O.J. (RO1-RR11740).

References


Received 24 July 2000; Accepted 14 November 2000