Construction and initial characterization of an infectious plasmid clone of a newly identified hamster parvovirus

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The construction and characterization of a full-length infectious plasmid clone of the newly identified hamster parvovirus (HaPV) are described. Following transfection of hamster BHK cells with the infectious clone, pHaPV, the specific intracellular DNA replicative forms, RNA transcripts and viral proteins that were expected for this rodent parvovirus were generated. Infected cells were lysed and progeny virus was produced, demonstrating that pHaPV could generate a productive virus infection. The complete sequences of both hairpin termini, which had not been previously determined, were obtained. Preliminary host-range studies, which compared virus production and macromolecular synthesis in various cell lines following either HaPV infection or pHaPV transfection, demonstrated an early block of infection of HaPV in both monkey COS-1 and murine A9 cells. The availability of an HaPV infectious clone will facilitate its genetic analysis and allow the elucidation of the determinants important in host range, tissue tropism and pathogenicity of this newly identified rodent parvovirus.

Introduction

Parvoviruses are small single-stranded DNA viruses that infect a wide variety of animal species ranging from insects to humans. They are nonenveloped and have an icosahedral capsid which is 20 nm in diameter. Members of the Parvovirus genus, as opposed to the Dependovirus genus, are autonomously replicating viruses of vertebrates that do not require coinfection of a helper virus (Berns, 1996).

In addition to the well-characterized rodent parvoviruses such as rat virus (RV), H-1 virus and minute virus of mice (MVM), several other rodent parvoviruses have recently been discovered that are closely related but distinct from these prototypes. These include mouse parvovirus (MPV), rat parvovirus, hamster parvovirus (HaPV) and their variants (Ball-Goodrich & Johnson, 1994; Besselsen et al., 1996; Ball-Goodrich et al., 1998). HaPV represents a distinct serotype of rodent parvovirus and encapsidates approximately 93% of its negative strand DNA (Besselsen et al., 1996). In contrast, MVM encapsidates 99% of its negative strand DNA. The HaPV genome shows 94.5% nucleotide similarity to MPV-1, 88.5% to LuIII virus, 87% to MVMp (prototype for this parvovirus group), and 80% to RV and H-1 virus (Besselsen et al., 1996).

Parvoviruses have compact genomes and so have maximized their coding capacity by using an overlapping transcriptional pattern, alternative splicing and multiple reading frames of the positive (sense) strand. Comparison of the nucleotide sequence of HaPV suggests a genetic organization similar to MVMp, which has been extensively characterized (Cotmore & Tattersall, 1987; Berns, 1996) and can be used as a model for comparison.

Rodent parvoviruses are frequent contaminants of laboratory animal colonies as well as of cell cultures (Hallauer et al., 1971; Tattersall & Cotmore, 1986; Jacoby et al., 1996). These viruses seriously threaten biomedical research in a number of ways: they suppress tumours, accelerate graft rejection, induce interferon production and alter lymphocyte proliferation and activity (Tattersall & Cotmore, 1986; Jacoby et al., 1996). Although a significant amount is known about the determinants that govern the host range of MVM, canine parvovirus (CPV) and feline panleukopenia virus, it is not yet known what determines the differences in host range between any of the newly identified closely related rodent parvoviruses.

This work describes the construction of a full-length infectious (plasmid) clone of HaPV. Infectious clones of a number of other autonomous parvoviruses have previously
been made, e.g. MVM, bovine parvovirus, Aleutian (mink) disease virus, mink enteritis virus and CPV (Merchlinsky et al., 1983; Shull et al., 1988; Bloom et al., 1990; Kariatsumari et al., 1991; Parrish, 1991) as well as adeno-associated virus (Samulski et al., 1982). Such clones are extremely helpful tools for studying virus replication, transcription and translation functions in the cell as well as host-range determinants. We constructed an infectious clone of HaPV by applying PCR-based strategies, the use of portions of the viral DNA replicative forms (RFs), and inclusion of synthesized oligonucleotides. The resulting infectious clone was transfected into different cell lines where its RF DNA, RNA transcripts and viral proteins were characterized. A preliminary study of the host-cell range of HaPV is presented as well as the previously unknown sequence of the viral genomic hairpin termini.

Methods

**Virus, infectious clones, cell lines and growth conditions.** HaPV was initially isolated from a clinically ill hamster (Gibson et al., 1983) and later characterized by Besselsen et al. (1996). In this study, HaPV was initially propagated in baby hamster kidney (BHK-21; ATCC CCL-10) cells. Other cell lines used in this study were: RN (rat nephroma cell line; a gift from Solon Rhode III, University of Nebraska, NE, USA); A9G (mouse connective tissue cell line; ATCC CCL-14); NIH 3T3 (mouse fibroblast cell line); PY 3T3 (polyomavirus-transformed NIH 3T3 cell line; a gift from W. R. Folk, University of Missouri, MO, USA); NB324K (SV40-transformed human newborn kidney cell line; Tattersall & Bratton, 1983) and COS-1 (SV40-transformed monkey kidney cell line). All cells were grown, unless otherwise stated, as monolayers in Dulbecco’s Modified Eagle Medium supplemented with 5% foetal calf serum (Sigma) in a humid incubator with 5% CO₂. DNA was isolated essentially as previously described (Gersappe & Pintel, 1999) or as recombinant MVM, pMVMp (Stratagene). The 5’ HinPI–HinPI fragment was first inserted into pBS by the DraI–DraI fragment at a EcoRI site. The EcoRI–Xbal fragment was inserted into the NorI site and the full-length HaPV clone, pHaPV.

**Isolation of virus and its DNA.** HaPV was propagated in BHK cells following infection at an m.o.i. of 0.5 and harvested on day 5 post-infection (p.i.). Both cells and medium were collected separately. Virus was concentrated from the medium by polyethylene glycol (PEG 8000) precipitation followed by an overnight dialysis. Cell pellets were resuspended in 1/10 volume of 50 mM Tris and 1 mM EDTA (pH 8.7) and subjected to four freeze–thaw cycles. Cell debris was removed by centrifuging for 5 min at 1000 g. DNA was released from the virus capsids by treatment of the cell extracts or concentrated medium with 2% SDS at 55 °C for 2–16 h at 37 °C. The viral single-stranded DNA and intracellular double-stranded RFs were separated by preparative agarose gel electrophoresis and extracted from the gel with gel-extraction kits (Qiagen).

**Cloning strategy**

**Left (3’) end.** The 3’ hairpin region of the genome was obtained by PCR using cultured HaPV as template (Fig. 1). A Sall restriction site was introduced into the 5’ end of the forward primer (pF) to facilitate insertion of the product into a cloning vector. The reverse primer (pR) was chosen downstream of an EcoRI site at nt 1089 in HaPV. PCR was performed using the Expand High Fidelity enzyme mix (Boehringer Mannheim) in a Perkin Elmer 2400 thermocycler with 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 5 min at 72 °C. The elongation steps in the last 20 cycles were prolonged by 20 s each. The 1500 bp PCR product was digested with Sall and EcoRI and the desired 1086 bp fragment was ligated into a Sall/EcoRI-digested pUC18 vector (New England Biolabs). Competent Escherichia coli strain JC8111 (Boissier & Astell, 1985) was used for transformations.

**Middle region.** Gel-purified HaPV RF DNA was digested with specific restriction enzymes and the desired restriction fragments were recovered from agarose gels and ligated separately into pUC18 plasmids. Digestion with EcoRI and XbaI resulted in a 3259 bp fragment comprising a large portion of HaPV. Digestion with DraI gave a partly overlapping 1875 bp fragment from the right-hand side of the genome. Digestion with HinPI1 yielded an 889 bp fragment, which extends into the 5’ hairpin (Fig. 2). The EcoRI–Xbal fragment was directly ligated to an identically cut pUC18 vector. The DraI–DraI fragment was blunt-end ligated into the pUC18 Smal site and the HinPI1–HinPI1 fragment was cloned into the AclI site, which resulted in the deletion of those restriction sites. The correct clones were selected by restriction enzyme analysis and sequencing.

**Right (5’) end and final full-length HaPV clone.** To construct the final infectious clone, the fragments described above were cut out of pUC18 and joined together in a single pBluescript (pBS) vector. The 5’ HinPI1–HinPI1 fragment was first inserted into pBS by the DraI–DraI fragment at an EcoRI site. The EcoRI–Xbal fragment was inserted into the NorI site and the Sall–EcoRI fragment at the EcoRI site, which resulted in an almost full-length Sall–HinPI1 HaPV clone (pBS-SH). The missing 80 bp of the 5’ hairpin were generated by synthesizing two complementary oligonucleotides (108 and 110 nt) with Banking MspI and BamHI open restriction sites (Fig. 2). These oligonucleotides were annealed to each other and directly ligated in a two-step ligation reaction. The MspI site of an excised 2910 bp PstI–MspI pBS-SH fragment and the whole PstI–BamHI fragment were ligated into a pBS vector and transformed into E. coli JC8111. This PstI–BamHI fragment was finally inserted into pBS-SH to create the full-length HaPV clone, pHaPV.

**Sequencing of the 3’ and 5’ ends.** The previously unknown sequences of the 3’ and 5’ ends of HaPV were determined either by ABI automated (Perkin Elmer) or by manual sequencing using primers specific for either negative- or positive-strand DNA and pHaPV as template.

**Characterization of pHaPV**

**Cell transfections.** Subconfluent cells were transfected using CaPO₄, FuGene6 (Boehringer Mannheim) or Lipofectamine (Life Technologies) either as previously described (Gersappe & Pintel, 1999) or as recommended by the supplier.

**Infectivity study.** In order to evaluate the infectivity of pHaPV and to study its replication, transcription and translation, CsCl-purified pHaPV was transfected initially into BHK cells by the CaPO₄, FuGene or Lipofectamine method. Mock and pMVMp transfections, as well as HaPV infections (m.o.i. of 0.1), were performed as controls. DNA, RNA and protein were collected on days 1 to 4 as described.

**DNA isolation.** Total DNA was isolated essentially as previously described (Tullis et al., 1994). Briefly, the transfected or infected cell monolayers were washed twice with PBS and scraped off the plates in 1.5 ml PBS per plate and centrifuged for 2 min in a microcentrifuge. Cell pellets were lysed in 200 μl lysis buffer (10 mM Tris, pH 8.0, 10 mM EDTA, 150 mM NaCl, 2% SDS) per tube for 30 min at 55 °C followed by proteinase K treatment (0.5 mg/ml) for 2–16 h at 37 °C. Duplicate tubes were combined and DNA was sheared by ten passages through...
**Construction of an infectious parvovirus clone**

Fig. 1. PCR and cloning strategy of the 3′ hairpin of HaPV. The forward primer, pF, was designed on the basis of the consensus sequence of rodent parvoviruses (Astell *et al.*, 1979) but with a SalI site added to its tail. The reverse primer, pR, downstream of an EcoRI site, leaves a 1089 bp piece after digestion with SalI/EcoRI, which was cloned into a plasmid. (Above sequence given as the negative strand based on its preferred encapsidation).

Fig. 2. Cloning strategy of the 5′ hairpin of HaPV. The sequence of the HaPV negative strand (top) is shown. Restriction sites that were used for cloning are underlined. The continuous line (centre) is the part of HaPV already obtained in pBS-SH and the dotted extended line is the missing 80 bp of the 5′ end of HaPV. TTT/AAA is the loop region. The dashed line underneath indicates the annealed synthesized oligonucleotides producing open ‘sticky’ ends. The ligated PstI–MspI fragment + oligonucleotide (bottom) to produce the PstI–BamHI fragment described in the text is also shown.

Luciferase assay. Luciferase assays were performed as previously described (Lorson *et al.*, 1998). Briefly, cells from 200 µl of the pre-centrifugation cell–PBS suspensions described above were pelleted and lysed in 100 µl lysis buffer (1% Triton X-100, 25 mM glycyglycine, pH 7.8, 4 mM EGTA, 15 mM MgSO₄, 1 mM DTT) for 15 min at room temperature and centrifuged for 5 min at 12,000 g. The supernatant was combined with 360 µl assay buffer (25 mM glycyglycine, pH 7.8, 15 mM KPO₄, 15 mM MgSO₄, 4 mM EGTA, 2 mM ATP, 1 mM DTT) and the expression of luciferase was measured by a luminometer after injection of 200 µl substrate solution [0.5 M glycyglycine, 1 mM DTT, 200 µM luciferin (Sigma)] per assay tube.

RNase protection of RNA. Total RNA was isolated following guanidine isothiocyanate lysis and centrifugation through a CsCl cushion exactly as previously described (Schoborg & Pintel, 1991). RNase protections were performed as previously described (Schoborg & Pintel, 1991). To make an HaPV-specific RNA probe, a 454 bp NcoI–AseI fragment from pHaPV was blunt-ended by Klenow polymerase (New England Biolabs) and then ligated into the Smal site of pGEM (Promega). The 524 bp
MVMp-specific probe has been previously described (Clemens & Pintel, 1988). RNA probes were made by in vitro transcription of 1 μg of linearized template plasmid in the presence of [32P]UTP using SP6 polymerase (Promega) as described (Schoborg & Pintel, 1991).

Protein collection and Western blots. Duplicate plates of transfected or infected cells were washed twice with PBS and scraped into 1.5 ml PBS per plate. Cell pellets were resuspended in 200 μl reduced sample buffer per tube containing 20% glycerol, 4% SDS and 10 mM DTT in 0.125 M Tris (pH 8.0). Proteins were separated by SDS–PAGE (Bio-Rad mini PAGE apparatus) in a 10% gel and then electrophoretically transferred to nitrocellulose NitroBind filters (MSI). Immunoblotting was performed by the chemiluminescent ECL system (Amersham) according to the manufacturer’s instructions. Rabbit antibodies against MVMp proteins NS1 and NS2 (Cutmore & Tattersall, 1986), VP1 N-terminal unique region and VP1/2 capsids (Schoborg & Pintel, 1991) were used at a 1:10000 dilution and horseradish peroxidase-conjugated anti-rabbit antibodies (ICN) were used at a 1:1000 dilution. Substrate reaction was detected by exposing the filters to Kodak RA autoradiograph film for 5 s to 5 min.

Host-range study. To study the host range of HaPV, the stock of virus originally isolated from a hamster (Gibson et al., 1983) was used to infect, and pHaPV was used to transfect, a variety of cell lines that are originally isolated from a hamster (Gibson et al., 1983). RNA probes were made by the consensus 3′ sequence determined for the other rodent parvoviruses (Astell et al., 1979) for the forward primer, pF, as the DNA sequence of the HaPV hairpin was not previously known. The pF sequence was later confirmed by comparison with the sequence that was determined for the complementary strand of the hairpin (Fig. 1). For the design of primer pF, advantage was taken of the mismatch or ‘bubble’ region in the double-stranded portion of the hairpin (Fig. 1). By placing the 3′-OH end of the primer within this bubble, only one site of the hairpin was fully complementary to the primer. PCR produced a single band of 1500 bp that was cloned, following Sall/EcoRI digestion, into pUC18.

The middle region. To minimize PCR-mediated errors in the cloned DNA sequences, as much as possible of the HaPV genome was directly excised from viral DNA. As described in Methods, an internal region of 3954 nt (nt 1086–5040) was cloned in this way, including the internal half of the 5′ hairpin (to the HinP11 site). Digestion of the HinP11–HinP11 transformants with XbaI/PstI revealed two distinct restriction patterns consistent with the two forms, ‘flip’ and ‘flop’, of the 5′ ends of the virus (Astell et al., 1985). The flip transformant harbouring the longer insert of 889 bp and containing the actual loop of the hairpin was chosen to be incorporated into the HaPV clone.

Final full-length pHaPV clone. To construct the final infectious clone, the four individual cloned pieces were excised from pUC18 and joined together in a single pBS vector, resulting in an almost full-length clone, pBS-SH. The missing 80 nucleotides of the very end of the 5′ hairpin of HaPV were synthesized as two long oligonucleotides and inserted into pBS-SH to create the full-length pHaPV plasmid. The sequence of the terminal 5′ region was obtained from the already cloned complementary strand of the 5′ hairpin in pBS-SH. The recombination-deficient E. coli strain JC8111 (recBC sbCB recF; Boissy & Astell, 1985) was used throughout as the bacterial host in order to maintain the deletion-generating palindromic hairpin structures intact in the plasmid.

Results and Discussion

Construction of pHaPV

We first describe the construction of a full-length infectious clone of HaPV. From previous work generating the first parvovirus plasmid clones, we expected that cloning HaPV would be difficult because the terminal genomic palindromes form tight hairpin structures, and because NS1 would be expected to be covalently attached to the 5′ end of the intracellular RF DNA. These obstacles were circumvented by first sub-cloning individual segments of the genome and then combining these cloned segments together to generate the final full-length HaPV clone. Three different cloning strategies were applied in order to clone the left, middle and right regions of the HaPV genome, respectively.

The left (3′) end. Because of difficulties in the direct cloning of the 3′ hairpin of the viral genome into a plasmid vector, the 3′ end region was first amplified by PCR. We chose the consensus 3′ sequence determined for the other rodent parvoviruses (Astell et al., 1979) for the forward primer, pF, as the DNA sequence of the HaPV hairpin was not previously known. The pF sequence was later confirmed by comparison with the sequence that was determined for the complementary strand of the hairpin (Fig. 1). For the design of primer pF, advantage was taken of the mismatch or ‘bubble’ region in the double-stranded portion of the hairpin (Fig. 1). By placing the 3′-OH end of the primer within this bubble, only one site of the hairpin was fully complementary to the primer. PCR produced a single band of 1500 bp that was cloned, following Sall/EcoRI digestion, into pUC18.

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Sequence confirmation and determination of the ends

The regions joining all the individual inserts utilized to construct the infectious clone were sequenced to confirm that correct ligation had occurred. The DNA sequence from nt 252 to 4912 of HaPV had been reported previously (Besselsen et al., 1996) but the critical 3′ hairpin and 5′ termini had not. Cloning of the HaPV genome in a plasmid clone permitted determination of the sequence of these regions (Fig. 3). Comparison of these sequences (shown in Fig. 3 as the positive-strand sequence), with analogous sequences from two immunosuppressive MVM (MVMi) strains, #X02481 (Sahli et al., 1985) and #M12032 (Astell et al., 1986) diverging from each other by 11 nucleotides, and one MVMp strain, #J02275 (Astell et al., 1986), revealed several differences as follows: the 3′ hairpin region (nt 1–116) of HaPV contained four nucleotide
Construction of an infectious parvovirus clone

Fig. 3. The positive sense nucleotide sequences obtained for (A) the 3′ (nt 1–140) and (B) the 5′ (nt 4913–5147) end regions. Nucleotide differences to MVMp (gJ02275) or MVMi (gX02481 or gM12032) are capitalized. The 3′ end of HaPV contained no unique changes whereas the 5′ end contained ten changes, shown in italics. The hairpin mismatch bubble (A) and the hairpin loop and HinP1 site (B) regions are underlined. GenBank accession numbers of the sequences shown above are (A) AF288060 and (B) AF288061.

differences as compared with MVMp, all of which were identical to MVMi #X02481; two nucleotide differences as compared with MVMi #X02481, which were identical to MVMp; and four nucleotide changes different from MVMi #M12032 but identical to both MVMi #X02481 and MVMp (Fig. 3). The bubble region (nt 89–92; TCTC) of HaPV was identical to that of MVMi #X02481 but differed from that of MVMi #M12032 and MVMp (TTTC). HaPV, like both MVMi strains, had a T residue at nt 61, which is absent from MVMp. A nucleotide switch at nt 70–71 (CA in HaPV and MVMi #X02481; AC in MVMp and MVMi #M12032) allows the two strands to anneal completely (Fig. 1).

The 5′ hairpin sequence of HaPV confers a full match between the two strands, with the exception of the HinP1 site and the three-nucleotide loop itself (underlined in Fig. 3; see also Fig. 2). The 5′ hairpin of HaPV (nt 4942–5147) comprises 17 differences from that of MVMp (the three-nucleotide loop TTT instead of AAA plus seven complementary nucleotides on each strand of the hairpin) and ten differences as compared to both MVMi strains (five complementary nucleotides on each side of the loop). All in all, ten nucleotide differences were unique to HaPV (shown in italics in Fig. 3).

Infectivity of pHaPV

BHK cells are productive hosts for HaPV (Besselsen et al., 1996) and so we first determined whether HaPV would replicate following the transfection of BHK cells with pHaPV. BHK cells typically started to exhibit CPE, following HaPV infection at an m.o.i. of 0.1, by day 3 p.i. CPE was seen on day 4 and total cell lysis was observed 1–2 days later following transfection of BHK with pHaPV. Transfection of BHK cells with pMVMp generated similar titres of virus but virus growth was 1–2 days slower than that following pHaPV transfection. Supernatants of pHaPV-transfected BHK cells, taken at day 6 p.i., generated dramatic CPE on fresh BHK cells within 3 days.

Southern hybridization of DNA extracted from both transfected and infected BHK cells showed the expected viral RFs (10 kb, 5 kb and ~3 kb, representing dRF, mRF and ssDNA, respectively; Fig. 4). The DNA RFs originating from pHaPV-transfected BHK cells were indistinguishable in size from those obtained from HaPV-infected or pMVMp-transfected BHK cells.

Quantitative RNase protection assays showed that both HaPV and pHaPV generated similar RNA species in matching abundancies to that of the well-characterized prototypic
MVMp (Fig. 5). This was expected since the primary sequence of HaPV from nt 1975 to 2400, including the p38 TATA box, the R2 large intron 3' splice sites of the large (D2) intron and the minor small (A2) intron. The change at nt 2396 does not seem to diminish the relative frequency at which A2 is used. However, HaPV, which shares with MVMi a C at nt 1985, apparently, like MVMi, generates less R2 relative to R1 than has been reported for MVMp (Schoborg & Pintel, 1991).

The primary translation products of pHaPV in BHK cells were also indistinguishable from those of the HaPV virus and similar to those of MVMp in terms of size and antibody reactivity. Rabbit anti-MVM antibodies cross-reacted well with all corresponding HaPV proteins. Fig. 6 shows the comparative accumulated protein profile of NS1 (83 kDa), NS2 (20–25 kDa), VP1 (83 kDa) and VP2 (64 kDa) proteins following pHaPV-, pMVMp-, mock-transfected and HaPV-infected BHK cells. Interestingly, antibody raised to the allotropic capsid determinant of MVMp (Colmore et al., 1997) did not react with HaPV capsid proteins (data not shown). This is most likely to be due to the numerous amino acid differences in this region: 310'RGRGSGTVQFGSNVTE237 for HaPV and 310'QGSRHGTQMGVNWVSK237 for MVMp, where residues indicated in bold, important for the fibrotropism of MVMp, are A and E in MVMi (Ball-Goodrich & Tattersall, 1992).

The HaPV clone pBS-SH that lacked the last 80 nt of the 5' end was also tested for replication following transfection into BHK cells. CPE was observed and virus could be isolated, albeit much later (day 6) than that observed following pHaPV transfection. RNA transcripts were visible by RNase protection assays on day 2 and DNA RFs could be seen by day 3 post-transfection (data not shown). These results indicated that this abbreviated viral genome could indeed replicate (although less efficiently), even without the last 80 nt of the 5' hairpin. Whether this truncated HaPV genome actually repaired itself in the cell is not known; however, the capability of self-repair of parvovirus genomes has been previously reported (Gardiner & Tattersall, 1988; Parrish, 1991; Costello et al., 1995).

Preliminary host-range analysis of HaPV

Replication following either infection of HaPV or transfection of pHaPV was examined in a number of different cell lines. The nucleotide sequence of HaPV is quite similar to that of MVM and therefore similarities and differences in host-specific replication might be expected to help identify important determinants for host range. The ability to replicate fell into three broad categories (Table 1).

(i) Hamster/rat cells. Highly permissive BHK cells were used as the standard against which other cell types were compared. HaPV was found to infect rat RN cells to approximately the same degree as that seen for BHK cells (Table 1). Viral RFs, visualized by Southern blot, and RNA, following RNase protection, were indistinguishable following infection of these
Table 1. Growth of HaPV in various cell types

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two cell lines (data not shown). In parallel pHaPV transfections using either CaPO₄ or FuGene, virus replication was clearly detectable (by Southern blot) in RN cells. In contrast to BHK cells, however, replication in RN cells following transfection was slightly delayed as compared to virus replication following HaPV infection (Table 1). Likewise, expression of viral RNA was also delayed in pHaPV-transfected RN cells as compared to HaPV-infected RN cells and pHaPV-transfected BHK cells. Supernatants from pHaPV-transfected RN cultures taken at late times (when full CPE was seen) were as capable as purified parent HaPV in initiating new infections of RN cells. The cause of the delay of replication following pHaPV transfection may be due to less efficient excision of the virus from the plasmid in RN cells. However, analysis of the activity of cotransfected reporter plasmid standards revealed similar levels of transfection in the two cell types.

(ii) Primate cells. In human NB324K cells, virus replication was observed after both HaPV infection and pHaPV transfection (Table 1). However, the kinetics were significantly slower than those seen in either BHK or RN cells at similar m.o.i. or concentration of DNA transfected. HaPV RF DNA was readily detected 6 days after infection at an m.o.i. of 0.1 or following transfection of 5 µg DNA. CPE was observed 1–2 days later. Monkey COS-1 cells did not support replication following infection by HaPV (Table 1). Transfection of pHaPV into COS-1 cells, however, resulted in the production of RFs by 24 h, but the infection did not progress, and by 7 days post-transfection, viral RFs had significantly diminished. These results are similar to what has been seen for interactions of MVMP with COS-1 cells (Naeger et al., 1990) and suggests that, while the intracellular environment for replication of both viruses is supportive, infections cannot progress to new cells; this is most likely to be due to a block at an early intracellular stage of infection or perhaps due to the lack of functional virus receptors.

(iii) Murine cells. Although the HaPV genome is quite similar to MVMP, none of the murine cell lines that we tested (A9, NIH 3T3, PY 3T3) supported a productive infection, as assessed by the appearance of increasing amounts of viral DNA RFs (by Southern blot) and apparent CPE, following either infection with HaPV or transfection with pHaPV (Table 1). A9 cells, a traditionally permissive cell line for both MVMP infection and
transfection by the pMVMp infectious clone, were chosen for further characterization. Infection of A9 cells by HaPV showed (by Southern blot) no increase in intracellular RFs over the input RFs (data not shown). Transfection of A9 cells with pHaPV led to transient appearance of viral RFs; the amounts of RFs at day 2 post-transfection were similar between pHaPV and pMVMp, but a drastic increase in those of MVMp could be seen 1 day later, whereas those of HaPV remained low and became undetectable by Southern blot 6 days post-transfection (Table 1). In addition, considerably less pHaPV RNA was detected in these experiments by RNase protection at 2–3 days post-transfection than pMVMp RNA. Interestingly, in a separate experiment in which the Lipofectamine reagent was used for transfection, similar amounts of RNA were generated by pHaPV, pBS-SH and a replication-deficient MVMp mutant clone at 48 h post-transcription (data not shown). These results suggest that A9 cells, as such, are not restrictive for HaPV transcription. Both the medium and a cell extract taken from A9 cells 3 days post-transfection with pHaPV were shown to contain low levels of HaPV that could grow on BHK cells. Taken together, these results suggested a model (similar to the COS-1 cells described above) in which A9 cells allowed replication following transfection of pHaPV; however, particle-mediated virus spread was reduced by a block that did not allow the newly formed virions to infect new cells.

The block to HaPV infection in A9 cells could be due to the absence of a HaPV-specific receptor making attachment or entry of HaPV ineffective. Alternatively, restriction could be mediated by the low of intracellular factors needed prior to DNA replication (e.g. uncoating) or during encapsidation or release of progeny virions. An intracellular particle-mediated block has been previously described for MVMi in A9 cells (Astell, C. R., Smith, M., Chow, M. B. & Ward, D. C. (1979). The different tissue tropisms of MVMi and MVMp do not depend on cellular receptors (Spalholz & Tattersall, 1983; Tattersall & Bratton, 1983) but rather on some virus- and cell-specific intracellular factors interacting either directly or indirectly with a region of the viral capsid protein VP2, termed the allotropic determinant (Gardiner & Tattersall, 1988a; Ball-Goodrich & Tattersall, 1988; Ball-Goodrich & Tattersall, 1992; Maxwell et al., 1995). The basis for the described restriction of HaPV in murine cells is similar and is probably due to some combination of the subtle differences between the HaPV and MVMp capsids.

The HaPV capsid shows significant amino acid composition differences as compared to MVM; HaPV VP1 has 76-7% amino acid identity to MVMp and 76-2% identity to MVMi (for full amino acid sequence alignment see Besselsen et al., 1996). The three-dimensional capsid structure of MVMi has been solved at 3.5 Å resolution and the genetically defined host-range determinants have been mapped to this structure (Agbandje-McKenna et al., 1998). The majority of the amino acid changes of HaPV compared to MVM are, not surprisingly, within the long surface loops inserted between the β strands of the β-barrel core of the capsid. Particularly in loop 2, which forms part of the top of the threefold protrusion of the MVM capsid, only 4 out of 19 amino acids from residues 226 to 244 of HaPV VP2 are identical to those of MVMp. Likewise, the long insertion (loops 3 and 4) between β strands G and H, including the MVM allotropic region as discussed above, contains many differences. MVM produces a small protrusion in a dimple-like depression at the twofold axis that has been shown to be involved in host tropism (Agbandje-McKenna et al., 1998). This protrusion at residues 553–559 is DNGNSYM in MVMp and YQSDQDYM (with one further insertion) in HaPV. Many of the differences between HaPV and MVM thus lie in areas of the capsid surface shown to be important in virus host range. However, in the absence of mutation studies of the pHaPV clone, it is not yet possible to predict if any HaPV-specific determinants are involved in the host-range differences seen relative to MVMp. The availability of an infectious clone will now allow such a characterization.

In this study, we have shown that a full-length HaPV clone was constructed and that it was infectious when transfected into hamster BHK cells. In the transfected cells, pHaPV produced increasing amounts of RF DNA and ssDNA, demonstrating a productive infection. RNase protection assays showed the typical transcription pattern of rodent paroviruses and immunoblot analysis revealed production of all expected viral proteins. HaPV, produced from pHaPV-transfected BHK cells, was thereby shown to be similar in replication, transcription and translation to the original parent HaPV virus as well as to MVMp. In our preliminary host-range studies, HaPV was shown to replicate well in BHK and RN cells and less well in NB324K cells. It was unable to cause a productive lytic infection in primate COS-1 cells or three different murine cell lines including A9, although a low level of viable virus was generated in pHaPV-transfected A9 cells.

Availability of this infectious HaPV clone will facilitate genetic analyses and allow us to elucidate further the virus determinants important in host range, tissue tropism and pathogenicity. Comparisons with the host range of MVM, which shares extensive sequence homology with HaPV, will be informative.

We would like to thank Lisa Burger and Greg Purdy for excellent technical assistance, Colin Parrish for advice and discussion, and Howard Wilson for photographic and computer services. This work was supported by PHHS grant RR11772 to L.K.R. and D.J.P., and by a grant from the Maud Kuistila Foundation and a UMC Molecular Biology Fellowship to M.S.-V.

References


Construction of an infectious parvovirus clone


Received 4 August 2000; Accepted 1 December 2000