A parvovirus isolated from royal python (Python regius) is a member of the genus Dependovirus

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Paroviruses were isolated from Python regius and Boa constrictor snakes and propagated in viper heart (VH-2) and iguana heart (IgH-2) cells. The full-length genome of a snake parovirus was cloned and both strands were sequenced. The organization of the 4432-nt-long genome was found to be typical of paroviruses. This genome was flanked by inverted terminal repeats (ITRs) of 154 nt, containing 122 nt terminal hairpins and contained two large open reading frames, encoding the non-structural and structural proteins. Genes of this new parovirus were most similar to those from waterfowl paroviruses and from adeno-associated viruses (AAVs), albeit to a relatively low degree and with some organizational differences. The structure of its ITRs also closely resembled those of AAVs. Based on these data, we propose to classify this virus, the first serpentine parovirus to be identified, as serpentine adeno-associated virus (SAAV) in the genus Dependovirus.

Paroviruses are small, isometric viruses, with a diameter of approximately 20–25 nm, containing a linear, single-stranded DNA genome of about 4–6 kb, and have been classified in the family Paroviridae (Berns et al., 2000). Their capsids consist of 60 structural proteins, some of which have N-terminal extensions that play a key role in the viral cycle. An example is the phospholipase A₂ domain in the structural protein with the largest N-terminal extension (VP1, a minor constituent of the capsid) that is essential for most paroviruses during cell entry (Zádori et al., 2001). These N-terminal extensions of the structural proteins are obtained by alternative splicing or leaky scanning of the transcripts carrying the gene for the structural protein (Pintel et al., 1996; Tijssen et al., 2003). The structure of several paroviruses has been determined by cryoelectron microscopy and X-ray crystallography to near-atomic resolutions of about 2.5–3.5 Å (Tsao et al., 1991; Agbandje-McKenna et al., 1998; Simpson et al., 1998, 2002; Xie et al., 2002).

These viruses are known to infect invertebrates, such as insects and shrimp (Bergoin & Tijssen, 2000), and higher vertebrates, such as birds (Zádori et al., 1995) and mammals (Brown & Young, 2000; Truyen & Parrish, 2000). Historically, the adeno-associated paroviruses (AAVs) that depend on helper viruses were classified in a separate genus, the genus Dependovirus (Berns et al., 2000). However, some autonomous avian paroviruses that are closely related to AAVs (Zádori et al., 1995) have now also been classified in this genus.

Diseases of reptiles and their pathogens have become a more common subject of veterinary research due to the increased popularity of reptiles as pets. The isolation and successful propagation of a reptilian parovirus-like virus in tissue culture has been reported (Ahne & Scheinert, 1989), but no studies on the further identification and molecular characterization of this virus have as yet been provided. Several case reports state that parovirus-like particles have been detected by histopathological examination and electron microscopy in samples from lizards, including bearded dragons (Pogona vitticeps; Kim et al., 2002), and from snakes such as Aesculapian snake (Elaphe longissima) and four-lined snake (Elaphe quatuorlineata; Heldstab & Bestetti, 1984), corn snakes (Elaphe guttata; Ahne & Scheinert, 1989) and California mountain king-snakes (Lampropeltis zonata multiscintia; Wozniak et al., 2000). These parovirus-like particles were often detected in the presence of adenovirus and herpesvirus or other pathogens, such as picornaviruses (Ahne & Scheinert, 1989) and Isospora (protozoa) species (Kim et al., 2002). Reptiles infected by parovirus-like viruses have shown different clinical signs such as gastroenteritis (Ogawa et al., 1992;
Wozniak et al., 2000), necrotic duodenum and liver (Heldstab & Bestetti, 1984; Jacobson et al., 1996), pneumonia (Ahne & Scheinert, 1989) and neurological signs (Kim et al., 2002), but the link between these virus infections and the pathologic features have as yet not been established. The purpose of this study was the molecular characterization of these parvovirus-like viruses from snakes.

Viruses were obtained from a diseased royal python (Python regius) and a boa constrictor (Boa constrictor) and submitted for pathological and microbiological examinations to the University of Hohenheim (described in detail in Ogawa et al., 1992). The viruses were separately isolated from the spleen and liver of the Boa constrictor and the heart, liver and kidney of the Python regius. Tissue specimens were removed aseptically from the carcasses and homogenized. Bacteria-free organ suspensions were inoculated into monolayers of viper heart (VH-2; ATCC CCL 140) and iguana heart (IgH-2; ATCC CCL 108) cells and incubated at 28°C in minimal essential medium supplemented with 10% foetal calf serum. The cultures were checked daily for cytopathic effects (CPE). IgH-2 and VH-2 cells inoculated with the tissue homogenates from either boa or python exhibited CPE that were undistinguishable and characterized by rounding of cells by 3–5 days and cell lysis within 7–10 days. Both virus cultures investigated by electron microscopy revealed two different particles. Ultrathin sections of infected IgH-2 cells revealed the assembly of icosahedral particles with mean sizes of ~75 nm in the nucleus (Fig. 1A).

To concentrate the virus, infected cell cultures showing CPE were frozen at -20°C. The cells were frozen and thawed three times and were subsequently pelleted at 5000 g for 30 min at 4°C. Supernatants were centrifuged at 35 000 g for 2 h at 4°C. The virus pellets were resuspended in TNE buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA). Negatively stained preparations of purified viruses similarly exhibited particles measuring ~75 nm and, additionally, particles measuring ~25 nm (Fig. 1B). The larger particles resembled adenoviruses, while the small virions resembled parvoviruses.

DNA was isolated from virus particles using the SDS/proteinase K method. Electrophoretic analysis of the extracted DNA showed two bands with sizes of approximately 4.4 and 30 kb, which correspond to the sizes of the snake parvovirus and the snake adenovirus genomes, respectively. The ratio of the two virus genomes was approximately equal in the royal python sample, whereas the Boa constrictor isolate contained approximately 10 times more adenovirus than parvovirus DNA.

Digestion of the DNA extracted from the royal python sample with PstI revealed three fragments, of which the 2.7 kb fragment was cloned into PstI-digested pBluescript II KS. The two terminal fragments were cloned into PstI/EcoRV-digested pBluescript II KS and the complete genome was generated as described above. PCR amplification using primers that flank the two PstI sites and sequencing showed that no small fragments were missing (primer pairs 5’-GGACTACAAGGAAGACAAGC-3’ plus 5’-ACCATACC-TGGCATTGTCTC-3’, and 5’-TCGTCGGCTGGAACGGC-ATT-3’ plus 5’-CTTCTGTGTCAGGTAATC-3’). The use of the Sure-2 bacterial host and incubation at 30°C decreased recombination and deletion in the inverted terminal repeats (ITRs) (Tijssen et al., 2003).

Direct cloning of the complete viral DNA was unsuccessful. Therefore, the clone containing the 3’ terminus was cut with HindIII, blunt-ended with T4 polymerase for 15 min at 12°C, then cut with PstI and cloned into the clone containing the 5’ terminus, which had been digested by PstI and SmaI. The PstI clone containing the core of the genome was inserted between the two ends. The complete...
A royal python parvovirus is a Dependovirus

The genome of the parvovirus isolated from royal python was sequenced on an ABI310 automated DNA sequencer and found to be relatively short, 4432 nt long (Fig. 2A), with an organization that is typical of the AAVs (Fig. 2B). Phylogenetic analysis also demonstrated that this new virus belonged to the genus Dependovirus (Fig. 2C). The ends of the genome were flanked by identical, short ITRs of 154 nt, of which 122 nt could fold up forming a Y-shaped double-stranded hairpin structure with a putative Rep protein binding motif located 17 nt from the terminal resolution site (trs) (Fig. 2D).

The PCR primers used for the parvovirus isolated from Python regius were also used to amplify two ~300 bp fragments from the Boa constrictor parvovirus. An additional ~1000 bp fragment was amplified from the Boa constrictor virus with the 5'-TCTTCTACGGCTGGACCT-GC-3' plus 5'-AACTGTTCGCCTGATT-3' primers. The sequence between the primers (total of 1569 nt) of the three PCR fragments from the parvovirus isolated from the Boa constrictor was found to be identical to that isolated from the royal python.

Two large open reading frames (ORFs) were found in one strand ('positive strand'), encoding the putative non-structural (Rep1, Rep2) and capsid (VP1, VP2, VP3) proteins, respectively. These ORFs proved to be most similar to those of members of the genus Dependovirus using BLAST homology searches. We named this virus serpentine adenovirus (SAAV) by analogy with ovine and avian (Bossis & Chiorini, 2003) AAV. In the genome, three putative transcriptionally active promoters could be identified from which, by analogy with the mammalian and bird AAVs, the Rep and VP proteins are most likely generated (Fig. 2B). The left ORF encoded a putative unspliced non-structural protein (NS1) of 562 aa (Fig. 2B), which showed 46±1% similarity to the goose parvovirus and 49±9% to the AAV-5 NS1 proteins, respectively. The putative VP1 gene consisted of 2181 nt encoding 726 aa residues. The virion contained three putative capsid proteins. The VP1 gene was found to be more conserved than the NS1 gene, showing 64±9% similarity to the goose parvovirus and 63±8% to AAV-5, respectively. Translation of the VP1 and VP3 genes most probably initiates from AUG codons at nt 2030 and 2600, respectively. The translation of the capsid protein VP2 was hypothesized to start from an atypical ACG start codon at nt 2441, as in other dependoviruses.

The most conserved region in the genome was the domain encoding the viral phospholipase A2 within VP1. This capsid enzyme is critical for infection (Zádori et al., 2001; Girod et al., 2002). The catalytic dyad (HD) and calcium-binding loop (GPG) in these distinct AAVs are equidistant from the initiation codon of VP1, which in turn is 2 nt downstream from the first acceptor site (Fig. 2B) and about 27 nt upstream from a second, alternative, splice acceptor site. The facultative splice donor, which would also cause a swapping of the C terminus of the Rep products, could use either acceptor site so that translation would start at the initiation codon of either VP1 or VP2/VP3. The phospholipase A2 domain is thus only present in a minority of the structural proteins. The Rep C terminus after splice acceptor site 1 is only 5 aa long and absent after acceptor site 2. For AAV-2, both alternative Rep products have short tails (16 and 7 aa, respectively), whereas for AAV-5 there are no tails because of the polyadenylation of the Rep transcripts within the intron region (Qiu et al., 2002).

A surprising difference was the small size of the SAAV genome. This was reflected in smaller Rep products, particular at their C termini (Fig. 3). Sequences of two independent clones confirmed this organization. Moreover, the sequence of the boa isolate was identical from nt 1269 to 2305, which overlaps these different regions.

The alignment of the NS1 gene with that of other dependoviruses identified the most conserved region in the core of the gene. This region has been shown to contain the replication initiator motif (I and II) (Ilyina & Koonin, 1992) and the tripartite helicase superfamily III motifs (A, B and C), which are conserved among mammalian parvoviruses and densoviruses of invertebrates (Tijssen & Bergoin, 1995; Li et al., 2001; Fédire et al., 2002) and may be involved in the initiation of DNA replication (Koonin, 1993). The 'P loop' ATP/GTP binding motif (GXXXXGKT; Saraste et al., 1990), which is highly conserved among parvoviruses, was also identified in the snake parvovirus as GPATTGKT. In spite of the low degree of similarity, one putative zinc-binding motif was also recognized within the C-terminal end of the non-structural proteins.

Members of the genus Dependovirus, except the Muscovy duck and goose parvoviruses, need helper viruses (adeno-, herpes- or papillomaviruses) for efficient replication (Hoggan et al., 1968; Buller et al., 1981; McPherson et al., 1982; Walz et al., 1997). It has been reported, however, that under certain conditions AAV-2 can propagate autonomously in differentiating squamous epithelium (Meyers et al., 2000). There is no evidence as to whether SAAV can propagate autonomously or not, but so far this parvovirus has only been found with a snake adenovirus, which may be required for, or facilitate, SAAV replication. In this respect, conditions in the host may differ from those in tissue culture. It is tempting to speculate that AAVs isolated from primates and other mammals have a Sauria (Diapsida) origin. The study of additional reptilian parvoviruses may reveal whether the dependoviruses are originally parvoviruses of diapsids (birds, crocodilians, beaked reptiles and squamates), i.e. whether they co-evolved and a host switch to primates occurred only relatively recently. By analogy, a similar host switch from reptiles to ruminants, birds and marsupials is proposed to have occurred for adenoviruses (Farkas et al., 2002; Benkő & Harrach, 2003).
Fig. 2. The strategy of SAAV gene expression can be deduced from conserved motifs compared with distinct AAVs (distances in nt). (A) All these viruses have two large ORFs separated by about 20 nt. The Rep gene of SAAV is, however, somewhat smaller than those of the other AAVs. (B) The HD amino acid pair in the catalytic site of the viral phospholipase is located 73/74 aa from the start of VP1. The two alternative splice acceptor sites (2 nt upstream, 22/25 nt downstream of the initiation codon) are maintained, as well as the putative splice donor site. An internal p19 (Rep) promoter site is also conserved (distances in amino acids). (C) Unrooted phylogenetic tree of the non-structural protein sequences showing the homology between the SAAV, waterfowl parvoviruses and the AAVs. The length of the branches indicates the phylogenetic distance between the different viruses and the scale bar represents 10 mutations per 100 sequence positions. Bootstrap values are given for 100 datasets. The tree was generated by distance matrix analysis (PROTDIST, using the Dayhoff PAM 001 scoring matrix, followed by FITCH, applying the global search option). The bootstrap values of the main branches are shown. Abbreviations: AAV, adeno-associated virus; AAVV, avian AAV; B19 (V9, A6), a human parvovirus; CnMV, canine minute virus; LuII and H-1, parvoviruses originating from tissue cultures; MinkAba, mink enteritis virus strain Abashiri; MVM, minute virus of mice; Mduck, Muscovy duck virus. GenBank accession numbers: AAVV, AY186198; AAV2, J01901; AAV3b, AF028705; AAV4, U89790; AAV5, AF085716; AAV6, AF028704; AAV7, AF513851; AAV8, AF513852; A6, AY064475; Aleutian mink disease virus, M20036; B19, P07298; BPV, M14363; Bovine3 PV, AF406967; canine PV, M19296; CnMV, AF495467; chipmunk PV, U86868; feline PV, M38246; goose PV, U25749; H-1, X01457; Kilham rat PV, AF321230; LuII PV, M81888; MinkAba, D00765; MVM, J02275; Mouse1 PV, U12468; Mduck PV, U22967; pig-tailed macaque PV, AF221123; porcine PV, U44978; Rat1a PV, AF036710; Rhesus macaque PV, AF221122; simian PV, U26342; V9, A249437. (D) The predicted secondary structure of the SAAV ITR. The 154 nt ITRs contain palindromic sequences that can fold into a Y-shape double-stranded hairpin. The putative terminal resolution site (trs) cleavage site is indicated by a vertical arrow.
Fig. 3. Amino acid sequence alignment of the Rep proteins of SAAV, goose parvovirus (GPV) (Zádori et al., 1995) and AAV-2 (Srivastava et al., 1983). Replication initiator motifs (I and II) of the non-structural protein are boxed. The conserved tyrosine residues in motif II are indicated by vertical arrows. The consensus residues indicated with ‘&’ are bulky hydrophobic residues. The tripartite helicase superfamily III motifs (A, B and C) are underlined with dots. The amino acids of the NTP binding motif are in bold. The putative nuclear localization sites (NLS) are underlined. The cysteines and histidines of the putative zinc fingers are indicated by asterisks.
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