

DNA sequencing and phylogenetic analysis of the protease gene of ovine adenovirus 3 suggest that adenoviruses of sheep belong to two different genera

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Abstract

Until now, the only published ovine adenovirus DNA sequence was the complete genome of ovine adenovirus isolate 287 (OAV287) which, compared to other mammalian adenoviruses, possesses strikingly unique genomic organisation and should properly be classified into a new adenovirus genus. The protease gene sequence of ovine adenovirus type 3 (OAdV-3) was determined and analysed. The results of phylogenetic analysis of the 205 residue long protein demonstrated that OAdV-3 belongs to the genus *Mastadenovirus*, and is surprisingly closely related to bovine adenovirus type 2. In spite of the common host origin, the evolutionary distance between OAdV-3 and OAV287 proved to be great suggesting that sheep, similarly to cattle and fowl, might be infected by distantly related adenoviruses belonging to different genera. © 2000 Elsevier Science B.V. All rights reserved.

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The family Adenoviridae presently contains two genera (*Aviadenovirus* and *Mastadenovirus*) which comprise adenoviruses isolated from birds and mammals, respectively. Although sheep adenoviruses are in general not considered severe pathogens, several clinical manifestations have been associated with adenovirus infection

(Lehmkuhl et al., 1993; Smyth et al., 1994). Based on the results of serum neutralisation tests, six different serotypes of ovine adenoviruses are officially accepted and classified into the *Mastadenovirus* genus (Belák, 1990). Additionally, bovine adenovirus type 2 (BAdV-2) has also been repeatedly isolated from healthy or diseased lambs (Belák and Pálfi, 1974). Although RFLP analysis has suggested that ovine adenoviruses are fairly similar to each other and to mastadenoviruses (Benkő, 1990), until recently no sequence information was available to confirm this hypothesis.

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The complete genomic sequence of an adenovirus of ovine origin designated isolate 287 (OAV287) has been determined, and its genome organisation was shown to be strikingly different from that of the other mammalian adenoviruses (Vrati et al., 1996; Venkatesh et al., 1998). OAV287 was isolated from sheep in 1983 (Peet et al., 1983), but has not been classified as a new OAdV serotype, because of its cross-reaction in neutralisation test with BAdV-7 (Adair et al., 1986; Boyle et al., 1994).

In the GenBank and other data banks, the sequences of OAV287 are still the only entries referred to as ovine adenovirus. This may lead to the inaccurate assumption, that ovine adenoviruses in general are not typical mastadenoviruses. Earlier observations, however, such as the presence of common complement fixing antigen, replication characteristics, inclusion body morphology, genome length, etc (Adair et al., 1985; Belák, 1990; Benkő, 1990) suggested that the officially accepted OAdV types are not exceptional and resemble other members of the *Mastadenovirus* genus.

Since the genome of OAdV-3 had earlier been partially cloned (unpublished), we have decided to identify and sequence its protease gene to investigate the evolutionary relationship between OAdV-3 and OAV287. The well-characterised protease gene (Webster and Kemp, 1993; Grierson et al., 1994; Rancourt et al., 1994) has been chosen for sequencing, since this gene sequence is available for many adenoviruses from different hosts, and it is a very suitable subject for phylogenetic calculations due to the size and conservative structure of the coded enzyme (Harrach and Benkő, 1998).

The prototype (PX611) strain of OAdV-3 (McFerran et al., 1969) was propagated in ovine embryonic kidney cells, and the viral DNA was extracted from the infected cells as described by Shinagawa et al. (1983). Based on the results of preliminary restriction endonuclease analysis (Benkő, 1990), DNA fragments generated by *HindIII* and *PstI* enzymes were cloned randomly into pUC19 plasmid. Both ends of selected clones were sequenced by automated laser fluorescence sequencing (cycle sequencing using labelled T3 and T7 primers) performed on an ABI 373A

DNA Sequencer (at the Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary). By computer based homology analysis of the translated sequences (BLASTX), two *HindIII* clones with the same 2000 base pair (bp) long insert (in opposite directions) predicted to encode the protease gene were selected for further sequence analysis. According to our preliminary physical map, this was the *HindIII* D fragment of OAdV-3 (Benkő, 1990). For the generation of overlapping sequences, inner restriction enzyme cleavage sites (*ApaI*, *KpnI*, and *PstI*) were used to introduce deletions (simple 'cut-backs'). The final sequence was assembled and analysed using the PC/Gene program package (IntelliGenetics).

The sequenced *HindIII* D fragment proved to be 2099 bp long (GenBank/EMBL/DDJB accession number: AF153447) and contained the 3' end of the hexon gene, the entire putative protease gene, and (on the complementary strand) the 3' end of the DNA-binding protein gene (Fig. 1). The OAdV-3 protease gene was predicted to be 618 nucleotides (nt) long coding for a 205 amino acid long protein. The distance between the genes of the hexon and the protease was 9 nt long in OAdV-3, very different to that observed for OAV287 where the hexon and protease genes overlap each other by 3 nucleotides. Similarly, the distance between the ends of the protease and the DNA-binding protein genes is 40 bp in OAdV-3, while it is only 28 bp in OAV287. These findings corresponded to the characteristic difference observed earlier between the genomes of mastadenoviruses and the more compact egg drop syndrome (EDS) virus (Harrach et al., 1997) the official taxonomical name of which is duck adenovirus 1.

The protease gene sequence of OAdV-3 had an AT content of 54.4% which is significantly lower than the 69.7% found in OAV287, and is closer to the corresponding values of BAdV-2, human adenovirus 12 (HAdV-12), and canine adenovirus 1, that have 55.5, 53.8 and 53% AT content in their protease gene, respectively. As noted before (Harrach et al., 1997), the AT content of the protease gene of OAV287 is more similar to that of BAdV-4 (69.9%), BAdV-7 (69.7%), and the EDS virus (61%).

AAGCTTATCAGGAAGTTACTATTTCAAACCAGCACAAATAATTCAGGATTTACAGCTTTTGCTAATGCGTCTTTA 74
hexon> A Y Q E V T I S N Q H N N S G F T A F A N A S L
 CCTAGAGAAGGCCACCTTACCCAGCTAATTGGCCTTATCCGCTCATTTGGAGAAAACGTGGCCCCGTGTACCACCCAG 152
 P R E G H P Y P A N N W P Y P L I G E N V A P C T T Q
 AAAAAGTTTTGTGTGACAAGACTCTGTGGCGCATCCCATTCTAGCAATTTTATGTCAATGGGCTCGCTCACTGAC 230
 K K F L C D K T L W R I P F S S N F M S M G S L T D
 TTGGGGCAAACCTTGTGTACGCTAACGCCGCTCATGCGCTAGACATGACTTTTGAAGTGGATGCTATGGATGAACCT 308
 L G Q N L L Y A N A H A H A L D M T F E V D A M D E P
 ACATTGCTGTATGTGTTTGAAGTATTTGATGTTGTGCGGTGCATCAACCACAGGGCGCTCATGAAACCGTG 386
 T L L Y V L F E V F D V V R V H Q P H R G V I E T V
 TACCTGAGAACTCCGTTCTCTGCTGGCAACGCCACTACATAATCGCCGCGATGGGCTCCATGGAAGAAGATTGCGAG 464
 Y L R T P F S A G N A T T - **protease**>M G S M E E E L R
 CTATTGTTTCGCGACTAGGCATAAGTCTTACTTTTTAGGAACCTTTTGACAAGCGCTTCCCGAGTTTCTTGCATAGAG 542
 A I V R D L G I S P Y F L G T F D K R F P G F T C L H R
 ATAAACTGAGTTGGCGGATTTGGAATACAGCGCCGCGAGACCGGTGGGGCCACTGGCTGGCGCTAGCTTGGTTTC 620
 D K L S C A I V N T A A R E T G G A H W A L A L A W F
 CAATAGCTAAAAACTTTTACTTTTGGACCCATTTGGGTTTTCTGACATAAACTTAAACAGATTTTACAGTTTGAAT 698
 P N A K N F Y F F D P F G F S D H K L K Q I Y Q F E
 ATGAAGGCTTGTTCGCCGAAGCGCGCTAGCGGGCGATGGCTGTGTAATTTAGTGAAGAAGCACTGAAACAGTTCAAG 776
 Y E G L L R R S A L A G D G C V N L V K S T E T V Q
 GGCAAACAGCGCCCTGCTGAGTTGTTTGTGTCATGTTTTCACAGCTTTTGTAAATTGGCCAGCCGCCCAATGA 854
 G P N S A A C G L F C C M F L H A F V N W P D R P M
 GCCGCAACCCACATGGATTGCTAACTGGGGTACCTAATGCTGATATGATGAAACCGTCTGCTGCGTTGCGATTTTAA 932
 S R N P T M D L L T G V P N A D D M M K P S S L A I L
 GAGAAAACAAAATCAGCTGTATAAAATTTTGTATCCCACTCCCACTTTTCGCGCTGACCCGCCAGATTTGAAC 1010
 R E N Q N Q L Y K F L S S H S P Y F R A H R P Q I E
 GCGATACCTCTTTT**AATAA**CTGCTAGAACTCAAAAATCA**AATAA**CTGAAC**TTTATT**GAACTTTTTCGCTGTCAGTATT 1088
 R D T T S F N K L L E L K N ↑ Q ↑ - **[protease]** ↑
 RTGTTTAAAAAGACATCTCGTCCGCATCATCTTGGCCGGTGGGGAGGAGATTTTGGACTCTGTACTGAGGTTGCC 1166
[DBP]- F L C E D A D Q G T P L L T N Q V R Y Q P Q W
 ACTTGAACCTTGAACCACAATGGAAGCCTTAGTGCCAGTGAAGACACATTTGCTTAGCCAGCTGCAGAGCCA 1244
 K F E Q V V I S A K T G T L S S W M Q K A L Q L A M
 TCACAACATCAGTTGAGCTTATTTTAAATCACAATTTTCTGAGGATTCGCTTTAGTGTTCGGGAACACTGGGTTGC 1322
 V V D T S S I K F D C N K Q P N A K T N R F V S N C
 AGCACTGGAACACGAGAACCACAGGGTTGTTTAAAGTGGCCAGCACCTTAGCATCTCCACTAAAGAACGGTCGATGT 1400
 C Q F V L V V P N N L T A L V K A D E V L S R D I N
 TGCTGACGGCATTCAAAGCAAAGGGGTAACCTTGCAGTTTGTCTGCCAAGAAGAGGAATAACGTTGGTGGCCGTTAG 1478
 S V A N L A G F P T V K C T Q K G L L P I V H H G Y N
 TGCATTCACACCAACGGCATCAACAGCATCTCGCCAGCCTTGGGCATCTGCGGATACATGGCTTTTACAAAATCTC 1556
 C E C V L P M L L M E G A K P M Q P Y M A K V F D R
 TAATTTGGTAAAAGCCTTGACGGGCTTTGTTCCCGTCAGAAATAAAAGTATCCGCAAGATTTCAGAGCTAAAAGAATTTA 1634
 I Q Y F G Q R A K N G D S Y F Y G C S E S F S N L
 AAGAAGATTTTAGATCGTAGAAGCAGCACATGGCATCAGCATTCTTCATCTGCACCACACTGCGCCCACTTGTGGTG 1712
 S S K L D Y F C C M A D A N K M Q V V S R G S T P S
 ACAATTTAGCCCTCTCGACTCTTTTAGAGCGCCTTGACCGTTCTCGCTGTTTATATCCATCCACAATTTGCT 1790
 L K L G R Q S E K L A R Q G G N E S N I D M E V I Q E
 CCTTGTTAATCATAGGCAGCCCGTGCAGACAGTAGAGCTTTTCTTCATTTGTTTATGTTCCACACGACGCACCCGT 1868
 K N I M P L G H L C Y L K E E N T K H E W V V C G N
 TAGGGTTCCAATCAGAAGGGCAAATTTAGCCGCTCTTAGCACAAAATCAAAAACAAATCTAGCCACCACAGTCTGTA 1946
 P N W D S P C I E A A R L V F D F V F R A V V T Q L
 AAGACTTTTGTGTTGAAAAAGTAAGCGGCACAAATCTCTCTGCTCGTTGAGCCAGCTTTGGACGCCTTTTCTGAAGC 2024
 S K Q T S F T L P V F R K Q E N L W S Q V G K R F C
 ACTCCATAGTGCCTGCTGGAAGCAGCGTAAGATCTTTTATCCACTTTTAAATGGCACTAAAAGCTT 2095
 E M T G A D P L L T L D K I D V K L P V L F S < **DBP**

Fig. 1. Nucleotide and translated amino acid sequence of the OAdV-3 protease gene flanked by the C-terminals of the hexon and the DNA-binding protein genes. The recognition sites of the restriction enzymes applied in generating the overlapping sequences are underlined. Potential polyadenylation signals are shown by bold letters, possible poly(A) sites are indicated by simple arrows for the presumed common hexon–protease pre-RNA transcripts, and by a double arrow for the presumed mRNA of the DNA-binding protein. Potential $(T)_n(A)_p(T)_q$ motifs are in italics.

As expected, the hexon and protease genes of OAdV-3 share a common polyadenylation signal. There are two putative polyadenylation signals (starting at nt 1025 and 1050), that might fulfil the criteria suggested by Le Moullec et al. (1983): AATAAA-N₁₂₋₃₀-A₁₋₃-N₄₋₂₄-(T)_n(A)_p(T)_q, where n, p, q ≥ 1. Either the adenosine at nt 1053 is the exact poly(A) addition site, since this is the first possible site that fulfils the Le Moullec criteria and contains the entire protease gene including the termination signal. Alternatively, the second polyadenylation signal at nt 1050 is the actual signal, in this case the poly(A) addition site could be at nt 1069 (Fig. 1). Experimental proof is needed to determine which of the two possible sites is active. The presumed polyadenylation signal for the supposed transcript of the DNA-binding protein gene is at nt 1085–1090, and the poly(A) site is at position 1048 (Fig. 1).

Adenovirus proteases are 23 kDa cysteine endoproteases involved in proteolysis during virus replication and maturation (Tihanyi et al., 1993; Webster and Kemp, 1993). The enzymatic activity is controlled by another adenoviral peptide (pVlc) and by the viral DNA itself (Webster et al., 1993; Mangel et al., 1997). The predicted 205 residue long OAdV-3 protease showed 97% identity with the BAdV-2 protease, whereas only 35% identity with the OAV287 protease (Fig. 2). The protease of OAdV-3 as all other known adenovirus proteases including that of OAV287 seemed to share similar active site residues. The catalytic triad responsible for the proteolytic activity had been determined to be His₅₄-Glu₇₁-Cys₁₂₂ in HAdV-2 (Grierson et al., 1994; Rancourt et al., 1994; Ding et al., 1996) (the glutamine [E] is 'replaced' by aspartic acid [D] in all other adenovirus proteases except HAdV-5), while Cys₁₀₄ to

| | | |
|--|---|-----|
| HAdV-2 | M-GSSEQELKAIIVKDLGCGPYFLGTYDKRFPGFVSPHKLACAIVNTAGRETGGVHWMAFAWNPRSKTCYL | 69 |
| BAdV-1 | M-GSREEELRAIVRDLGVGPYFLGTDFKRFPGFLNNSKPSCAIVNTAGRETGGAHWLAALAWFPKSKAFYF | 69 |
| BAdV-2 | M-GSREEELRAIVRDLGISPYFLGTDFKRFPGFLHDKLSCAIVNTAARETGGAHWLAALAWFPNAKNFYF | 69 |
| OAdV-3 | M-GSMEEELRAIVRDLGISPYFLGTDFKRFPGFLHRDKLSCAIVNTAARETGGAHWLAALAWFPNAKNFYF | 69 |
| OAV287 | MSGTSESELKNLISLHLNNGFLGIFDCRFPGFLOKSKIQTAIINTGPREQGGIHWITLALAPI SYKLF I | 70 |
| BAdV-4 | MSGTSESELKHLSSLHLTYGFLGTDFCRFPGFLOKKNVQTAIVNTGPREKGGVHWVAMAWDPIYKMYI | 70 |
| BAdV-7 | MSGLSEKEVFLLSLQCTHGFLGTDFCRFPGFINKVKVQTAIINTGPREQGGIHWIALAWDPKSYQMF I | 70 |
| EDS | MSGTSESELKALMKSGLGIAGNFLGTDFCRFPGFINKHKRQTAIINTGSRASGGLHWLAFADWPLRYTIY | 70 |
| FAdV-1 | MSGTTEQLRDLLSSMHLRHRFLGVDFKSFPGFLDPHVPASAI VNTGSRASGGMHWIGFAFDPAAGRCYM | 70 |
| ↑H54/triad | | |
| HAdV-2 | FEPPFGFSQRLKQVYQFEYESLLRRSAIASSPDRCTITLEKSTQSVQGPNSAACGLFCCMFLHAFANWPQT | 139 |
| BAdV-1 | FDPPFGFSDSKLKQIYEFYEGLLRRS-LAATGDGCINLVKSSSESVQGPNSAACGLFCCMFLHAFAHWPHS | 138 |
| BAdV-2 | FDPPFGFSDHKLKQIYQFEYEGLLRRSAL--AGDGCVNLVKSTETVQGPNSAACGLFCCMFLHAFVNWPD | 137 |
| OAdV-3 | FDPPFGFSDHKLKQIYQFEYEGLLRRSAL--AGDGCVNLVKSTETVQGPNSAACGLFCCMFLHAFVNWPD | 137 |
| OAV287 | FDPLGWKDTQLIKFYNFSLNLIKRSAL-NNSDRCTITVERNTQSVQCTCAGSCGLFCIFFLYCFHFYKQN | 139 |
| BAdV-4 | FDPLGWKESQLQSLYNYSYQSMKRSAL-TESERCITVEKNTQSVQCTCSGACGLFCVFFLYCFYKYRKG | 139 |
| BAdV-7 | FDPLGWKNDQLMKYKFSYSNLIKRSAL-SSPDKCVKVIKNSQSVQCTCAGSCGLFCVFFLYCFYKYKSN | 139 |
| EDS | FDPLGWKEKDLFLKLYGFSYKTMIKRSAL-QSDNRCVKLKVNTEAVQCTCAGSCGLFCVFFLYCFNLCHIN | 139 |
| FAdV-1 | FDPPFGWSDQKLWELRYVKYNAFMRRTGL-RQPDRCTTLVRSTEAVQCPCSAACGLFSAFIVSFDTRYRSK | 139 |
| ↑"E"71/triad C104/pVlc↑ C122/triad↑ P137/traffic↑ | | |
| HAdV-2 | PMDHNPMTNLIITGVPNSMLNSPQVQPTLRRNQELYSFLERHSPYFRSHSAQIRSATSFCHLKNM | 204 |
| BAdV-1 | PMTHNPMTDLLTGVPNHNIMSPSAQPTLRENQVKLYKFLAAHSQYFRTHRPQIERDTSFNKLLLESKLQ | 206 |
| BAdV-2 | PMTRNPMTDLLTGVPNADMNKPSLAILRENQQLYKFLSTHSQYFRTHRPQIERDTSFNKLLLEKLNQ | 205 |
| OAdV-3 | PMSRNPTMDLLTGVPNADMMKPSLAILRENQQLYKFLSSHSPYFRAHRPQIERDTSFNKLLLEKLNQ | 205 |
| OAV287 | VFKSWLFQKLNSTPS---LIPCEPHLLHENQTFLYDFLNAKSVYFRKNYRTFIENTKTGLIKTH | 201 |
| BAdV-4 | AFNNELFQSLNGASPS---LTPSDPSSLHKNQDILYDFFCIKSSYFRHNKMLISNTKLGLIKSH | 201 |
| BAdV-7 | AFKNCLFQSLYGSIPS---LTPPNPTNLHKNQDFLYKFFKEKSLYFRQNEEYIVSNTKIGLIKSHI | 202 |
| EDS | PFEASIFQAMHGTSIPA---LYPSKPHLLHANQQLYDFLRSHSSYFVNNERTLVCNTKLNLIHQ | 202 |
| FAdV-1 | PMDGNPVIDTVGVKHEMNNSPPYRDILHRNQERTYYWTKNSAYFRAHQEELRRETALNALPENHV | 206 |

Fig. 2. Alignment of the amino acid sequences of selected adenovirus proteases. Arrows show the critical residues: the triad responsible for the catalytic activity ('triad'), the cysteine taking part in the disulphide bond between the protease and the activating pVlc peptide ('pVlc'), and the proline described as critical for HAdV-2 protease trafficking but missing in avi- and atadenoviruses ('traffic'). The genera are separated by continuous lines.

sequenced adenovirus of sheep, is in fact not a typical ovine adenovirus. The DNA sequence comparison and the phylogenetic calculations suggest that in agreement with serological results, OAV287 should belong to a group of viruses possibly representing a new genus. Based on the similarity observed in the DNA sequences and genomic organisation of OAV287 (Vrati et al., 1996), the EDS virus (Harrach et al., 1997), and the examined members of the subgroup 2 BAdVs, namely BAdV-4 (Dán et al., 1998), BAdV-6 (Szathmáry et al., 1997), BAdV-7 (Hu et al., 1984; Song et al., 1996), BAdV-8 (unpublished), a formal proposal has been made recently for establishing a new (third) genus in the family Adenoviridae to include these viruses (Benkő and Harrach, 1998). The proposed name of the new genus is *Atadenovirus* to reflect the characteristic high AT content of the genome of the candidate members. Nevertheless, OAdV-3 seems to be a typical member of the genus *Mastadenovirus*.

A very interesting finding of our present work is the striking similarity between OAdV-3 and BAdV-2. The distance matrix analysis showed almost no difference between the two protease sequences (length of the horizontal branches on Fig. 3A). The parsimony analysis did also group these two types together with a bootstrap (i.e. probability) value of the possible highest score (100) either for DNA (not shown) or for amino acid sequences (Fig. 3B). This finding suggests a very close genetic relationship between OAdV-3 and BAdV-2, namely a more recent evolutionary separation than that among other BAdVs.

In some known properties, BAdV-2 is indeed closer related to the six officially accepted OAdV serotypes than to other BAdVs. For example, the genome size of BAdV-2 is about 32.5 kb (Salmon et al., 1993) that is comparable to the genome size of OAdVs (30–32 kb in OAdV-1 to 5; Benkő, 1990), while the genome size of BAdV-1 and BAdV-3 is almost 36 kb (Benkő et al., 1988). Moreover, BAdV-2 has been repeatedly isolated from sheep and wide-spread seropositivity against it in sheep flocks has been described (Belák, 1990). The explanation of the very close relationship between OAdV-3 and BAdV-2 has yet to be

found. Apparently BAdV-2 is equally capable of infecting sheep and cattle, but phylogenetically is closer to OAdVs than to other BAdVs.

The International Committee on Taxonomy of Viruses (ICTV) encourages the active use of the category of virus species. The earlier concept of regarding every adenovirus serotype as a separate species would result in a classification which is unable to reflect the actual genetic relatedness of adenoviruses within a genus. It seems therefore appropriate to demarcate the species as a taxon between the level of genus and serotype. BAdV-2 and OAdV-3 (being obviously more closely related than for example BAdV-1 and 3) would certainly fit into a common 'ovine adenovirus' species. On the other hand, OAV287 is an exception, and should be classified together with BAdV-7 into the proposed genus *Atadenovirus*. Similarly, the bovine adenovirus types should be properly classified not only into several species, but even into two different genera. It remains unknown, if host animals other than cattle, sheep, and fowl might also harbour such distantly related adenoviruses.

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