Analysis of the hexon gene sequence of bovine adenovirus type 4 provides further support for a new adenovirus genus (Atadenovirus)

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The putative hexon gene of bovine adenovirus type 4 (BAV-4), encoding 910 amino acid residues, has been identified and sequenced. A characteristic codon usage biased towards the use of AT-rich triplets was observed. Comparative analysis with other hexon sequences detected a high level of amino acid identity in the regions corresponding to the pedestals of the hexon. Substitutions, insertions and deletions were identified mainly in the variable regions forming the loops which are exposed on the outer surface of the virion. In these variable regions, BAV-4 shared similarity only with egg drop syndrome (EDS) virus and ovine adenovirus isolate 287 (OAV287). The close relationship of these viruses was also demonstrated by phylogenetic analysis of the hexon gene. In addition to the two groups of the Mastadenovirus and Aviadenovirus genera, a third cluster appeared comprising BAV-4, OAV287 and EDS virus.

The family Adenoviridae is divided into the genera Mastadenovirus and Aviadenovirus comprising adenoviruses isolated from mammals or birds, respectively. The separation of the two genera was partially based on the lack of immunologically cross-reactive proteins between members of the two groups (Norrby et al., 1976).

The genus Mastadenovirus presently contains 10 bovine adenovirus (BAV) serotypes. Because of the lack of the genus-specific complement-fixing antigen and some obvious biological differences observed in certain serotypes, subdivision of BAVs was introduced (Bartha, 1969). BAVs which were similar to human and other mammalian adenoviruses were classified into subgroup 1, while the slow-growing, non-cross-reacting BAV serotypes (4–8) were separated into subgroup 2.

After DNA analysis of the genome, remarkable genetic differences between the members of the two subgroups were reported (Hu et al., 1984a; Benkő et al., 1988, 1990). More recently, based primarily on phylogenetic analysis performed on protease gene sequences, a proposal was made for the establishment of a new adenovirus genus for these BAV serotypes (i.e. members of subgroup 2) along with egg drop syndrome (EDS) virus and ovine adenovirus isolate 287 (OAV287) (Harrach et al., 1997; Harrach & Benkő, 1998).

In addition to the complete genome sequence of OAV287 (Vrati et al., 1996) and EDS virus (Hess et al., 1997), DNA sequences are available from different subgroup 1 BAVs (Hu et al., 1984b; Cai et al., 1990a; Mittal et al., 1992; Elgadi et al., 1993; Esford & Haj-Ahmad, 1994; Salmon & Haj-Ahmad, 1994; Fitzgerald et al., 1997). From subgroup 2 BAVs, however, only the protease gene (Cai et al., 1990b) and the major late promoter sequence (Song et al., 1996) of BAV-7 have been published so far. The information concerning the phylogenetic relatedness between subgroup 1 and subgroup 2 BAVs, and between human and animal adenoviruses, is therefore deficient (Bailey & Mautner; 1994; Harrach et al., 1997).

To enhance the reliability of comparisons aiming to clarify the taxonomic place and relationships of BAVs, we decided to sequence the complete genome of BAV-4, a typical representative of subgroup 2. In the present paper, the hexon gene sequence of BAV-4 is described. Since the hexon is the major structural component of the adenovirus capsid, containing type, subgenus- and genus-specific antigenic determinants (Adám et al., 1996; Norrby & Wadell, 1969; Wilcox & Mautner, 1976), it has been widely investigated. Hexon gene sequences from numerous human and animal adenovirus types have been published; thus a very comprehensive amino acid sequence alignment could be assembled for the examination of genus-specific characteristics. The results of the phylogenetic analysis and the putative three-dimensional structure of the BAV-4 hexon protein are presented.

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The GenBank accession number of the sequence reported in this paper is AF036092.
Fig. 1. Nucleotide and predicted amino acid sequence of the BAV-4 genome between map units 45 ± 7 and 57 ± 0 containing the C-terminal (284 nt) part of the pVI protein, the complete hexon gene and the N-terminal (188 nt) part of the protease gene. The putative recognition site of the second protease cleavage on the pVI protein is underlined, and the hypothetical cleavage site is marked by an arrow. The putative cleavage yields an 11-residue-long protease cofactor (pVIc).
The genome of the reference strain (THT/62) of BAV-4 had previously been cloned and mapped (Benkö et al., 1990). The viral insert of clone pBAV402, a HindIII–BamHI fragment was subcloned into plasmid pMOB, a vector designed specifically for use in a transposon insertion system. Using a TN1000 kit (according to the instructions of the manufacturer, Gold Biotechnology) the transposon was randomly introduced into the viral fragment. By restriction enzyme digestion of the positive clones, the insertion site of the transposon was determined, and a nested set of clones was selected for the generation of overlapping sequence data. Sequencing was performed at the two ends of the viral insert (using T3 and T7 primers), and from the two ends of the transposon (using the G186 and G187 primers complementary to the transposon sequence, and supplied with the kit). Compared to the published physical maps of BAV-4 (Benkö et al., 1990), an additional HindIII site at map position 46±4 yielding an additional small fragment (N) was found. Similarly, an additional XbaI site (at map position 53±9) and fragment (F) could be identified. These internal fragments were subcloned into plasmid pBluescript (pBS) SK (Stratagene), and sequenced with T7 and T3 primers. The PRISM Ready Reaction Dye Deoxy Cycle sequencing protocol (Perkin-Elmer) and an ABI 373A automated DNA sequencer (Applied Biosystems) were used. The nucleotide sequences were read using the Applied Biosystems 373A DNA Sequencer Data Analysis Program and assembled by the program package Largenex (DNASTAR). The coded proteins were identified using the BLAST search program (Altschul et al., 1990).

The three-dimensional structure of BAV-4 was predicted using Swiss-Model (Peitsch, 1996). Adenovirus hexon sequences for multiple alignments were retrieved from the GenBank and EMBL databases. Multiple alignment of the amino acid sequences was performed with the MultAlin program (Altschul et al., 1990). In the BAV-4 hexon, the candidate members of the proposed third adenovirus genus were placed in the middle. In addition to the most conservative regions of the hexon retained in all the three groups (underlined residues), a large number of identical amino acids shared only by the members of one or two of the groups.
could be identified (amino acid residues printed in bold). However, no sequence motifs were identified which were conserved throughout the different adenovirus types of any individual host species and could have been considered as host-specific determinants. Thus, for example, no common patterns were recognized between BAV-3 and BAV-4, or between EDS virus and fowl adenovirus (FAV) type 1 or 10, although these viruses share distant evolutionary but common host origins. No further evidence was found for the hypothesis that \( l_4 \) is a host species-specific region (Crawford-Miksza & Schnurr, 1996; Vrati et al., 1996; Reubel & Studdert, 1997a, b).

Interestingly, there were some instances where all the sequences except those from one virus were identical. For example at the end of \( l_4 \), before the beginning of the P2 region,
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Fig. 3. For legend see page 1459.
there is a very conserved stretch of amino acid sequence which was present in every type but BAV-3. The published nucleic acid sequence of the BAV-3 hexon at this critical point indicates a suspected frame shift possibly caused by the compression of four consecutive C bases. The questionable conserved amino acid sequence is in fact present in BAV-3, but in another reading frame. Several similar situations could be observed in the hexon sequences of EAV-2 and FAV-10. It would be interesting to repeat the sequencing or read the original gels again in order to confirm the amino acid sequence.

The result of the phylogenetic (distance matrix) analysis performed with the same set of data (22 hexon sequences) is shown in Fig. 3(b). In addition to the clusters of the genera Mastadenovirus and Aviadenovirus, a third, well separated cluster appeared, which contained BAV-4, OAV287 and EDS virus. Phylogenetic analysis of these sequences thus provides persuasive data about the phylogenetic relations, but unfortunately a limitation of the approach is the poor availability of DNA sequences representing different genome regions from many different adenovirus types. The unrooted tree presented here is, however, almost identical with those obtained earlier by analysis of the protease (Harrach et al., 1997) and DNA polymerase (Harrach & Benko, 1998) sequences. Similar tree topology (and maximal bootstrap values for the proposed Atadenovirus cluster) were generated using maximum parsimony analysis, or when the DNA sequence alignment was analysed with either of the two programs (data not shown but available on request).

The present results confirm our earlier findings concerning the distinctiveness of subgroup 2 BAVs, and support the proposal for the establishment of a third genus within the family Adenoviridae. In addition to BAV-4 and its close relatives...
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Fig. 3. (a) Multiple alignment of the amino acid sequences of representative adenovirus hexons including BAV-4. Amino acids conserved in every type examined are underlined, while the amino acids shared by all members of one or two groups only are in bold. The positions where each group, corresponding to the three proposed genera, contains a different (but identical within-group) amino acid, are emphasized by italics in addition to bold. The locations of the loops and pedestals are marked according to the Ad2 sequence. The aligned sequences (and their abbreviations) are: human adenovirus types 2 (H2) and 40 (H40), equine adenovirus type 1 (E1), canine adenovirus type 1 (C1), porcine adenovirus type 3 (P3), BAV-3 (B3), murine adenovirus type 1 (M1), BAV-4 (B4), OAV287 (O), EDS virus (EDS), fowl adenovirus types 1 (F1) and 10 (F10). (b) Phylogenetic tree of 22 adenovirus hexon sequences showing the homology of BAV-4, OAV287 (O287) and EDS virus, and their distinctiveness from all other sequenced mastadenoviruses and aviadenoviruses. The length of the branches indicates the phylogenetic distance between the different viruses. The tree was generated by distance matrix analysis (PROTDIST, using the Dayhoff PAM 001 scoring matrix, followed by FITCH, applying Global search option). The high statistical significance of the tree topology is shown by the bootstrap values obtained by analysis of 100 randomly re-sampled data sets from the aligned sequences. The bootstrap values of human adenoviruses are not shown. The aligned sequences and their accession numbers are: human adenovirus (marked by serotype number only) type 2 (2), J01917; 3, X76549; 4, X84646; 5, M73260; 7, X76551; 12, X73487; 16, X74662; 40, X51782; 41, X51783; 48, U20821; B3, K01264; B4, AF036092; C1, U55001; C2, U77082; E1, L79955; E2, L80007; EDS, Y09598; F1, U46993; F10, U6221; M1, U57336; OAV287, U40837; P3, U34592. The edited alignment (in PHYLIP format) of the 22 hexon sequences used as infile for the phylogenetic analysis is available at http://www.vmri.hu/~harrach.

(BAV serotypes 5–8), OAV isolate 287 and EDS virus should be classified into this new taxon. The descriptive genus name Atadenovirus, referring to the high genomic AT content of the candidate members, has been proposed (Harrach & Benkö, 1998).

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