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Sequence of Ovine Adenovirus Homologs for 100K Hexon Assembly, 33K, pVIII, and Fiber Genes: Early Region E3 Is Not in the Expected Location

SUDHANSHU VRATI,[1] DAVID BOYLE,* ROLF KOCHERHANS,* and GERALD W. BOTH†[2]

†Division of Biomolecular Engineering, CSIRO, North Ryde, Sydney 2113, Australia; and
*Australian Animal Health Laboratories, CSIRO, Geelong, Victoria, Australia

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Ovine adenovirus OAV287 was previously isolated from sheep in Western Australia. As a first step in characterizing the genome of this virus we have determined the sequence of its genome between map units 65 and 81. This region was expected to contain the nonessential E3 region which, in other adenoviruses, lies between the genes encoding the pVIII and fiber proteins, although its size and complexity varies. OAV287 genes coding for the hexon assembly, 33K, pVIII, and fiber proteins were identified by their homologies with human Ad2. These genes lie in the same relative positions in the OAV287 genome, but the intergenic region between the pVIII and the fiber genes is only 197 nucleotides and these appear to be incapable of coding for any protein. Thus, the ovine adenovirus E3 region is not present in the expected location. In addition, using cDNA synthesis, PCR amplification, and nucleotide sequencing we determined the location of splice junctions and transcription termination signals in mRNA species encoding these proteins. This showed that a family of variably spliced L4 RNAs is produced and that the region between the pVIII and the fiber genes contains several signals for RNA synthesis and processing. As the E3 region in human adenoviruses is nonessential for replication, in many instances it has been replaced with foreign DNA during the construction of recombinants. Because of this unexpected difference in the organization of the OAV287 genome further experimentation will be required to determine whether potential vaccine recombinants can be constructed for this adenovirus by making insertions into the pVIII/fiber intergenic region. © 1995 Academic Press, Inc.

INTRODUCTION

Adenoviruses (Ads) are widely distributed among the human and the animal kingdoms. Several human adenoviruses have been well studied at the molecular level and the complete sequences of the ~36-kb dsDNA genome has been determined for types 2, 5, 12, and 40 (see EMBL and Genbank databases). Genes encoding the structural and nonstructural proteins have been identified and, at the transcriptional level, regions of the genome expressed early and late have been characterized. Most of the promoters and the RNA products derived from them have been identified and mapped (reviewed in Ginsberg, 1984; Horwitz, 1990). From the study of Ad/SV40 mutants, a region of the Ad genome known as E3 has been identified as nonessential for replication *in vitro* (Lewis *et al.*, 1973). The E3 region (of ~2.5 kb) lies between the genes coding for the structural proteins pVIII and fiber. E3 genes code for a suite of proteins which appear to modulate the immune response of the host during infection (Gooding, 1992; Wold and Gooding, 1991). Because of the nonessential nature of this region, several groups have constructed E3-substituted recombinants expressing a variety of genes (e.g., Graham and Prevec, 1992; Morin *et al.*, 1987; Natuk *et al.*, 1992).

The genomes of murine, canine, and bovine adenoviruses have also been characterized across the E3 region and compared with the human viruses (Dragulev *et al.*, 1991; Mittal *et al.*, 1992; Elgadi and Hajahmad, 1992; Raviprakash *et al.*, 1989). In these genomes also the pVIII and fiber proteins (identified by their homology with sequences in Ad2) define the boundaries of E3 although the regions range in size from ~1.0–1.5 kb for canine Ads (Dragulev *et al.*, 1991; Linne, 1992) and ~1.3 kb for bovine Ad type 3 (Mittal *et al.*, 1992, 1993; Elgadi and Hajahmad, 1992) to only ~0.5 kb for mouse (Raviprakash *et al.*, 1989). The smaller sizes of E3 in these animal Ad genomes reflects a decreasing complexity in the number of open reading frames. As for human Ads, certain genes in the E3 region of canine Ad type 1 appear to be nonessential for virus growth *in vitro* (Dragulev *et al.*, 1991). However, it is not known whether all the animal Ad E3 regions contain such nonessential genes. By analogy with human Ads, these regions would be prime candidates for modification during the construction of recombinants.

We are characterizing an ovine adenovirus, OAV287, which was recovered from sheep in Western Australia (Adair *et al.*, 1986; Boyle *et al.*, 1994). The basic properties of the virus, its serotyping, and its passage in sheep have been described (Boyle *et al.*, 1994). We set out to characterize the E3 region of OAV287. Here we report the sequence of the genome between map units 65 and 81 of the genome (1 m.u. = ~295 bp). Genes encoding

[1] Present address: National Institute of Immunology, New Delhi 110067, India.

[2] To whom correspondence and reprint requests should be addressed.

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the OAV287 equivalents of the 100K hexon assembly and 33K proteins and the structural pVIII and fiber proteins are described. These were clearly identifiable by homology with their counterparts in other Ads. Surprisingly, however, in the OAV287 genome the pVIII and fiber genes were separated by only 197 nucleotides which appeared not to code for any protein. Thus, the OAV287 E3 transcription unit is missing from its expected location.

MATERIALS AND METHODS

Growth of virus and preparation of DNA

Virus was propagated and plaqued in CSL503 cells grown in Eagle's MEM supplemented with 10% fetal calf serum (Pye, 1989). OAV287 was twice plaque-purified before use. DNA was prepared from virus purified on CsCl gradients as previously described (Boyle *et al.*, 1994).

Nucleotide sequencing

The determination of the *Bam*HI restriction map for OAV287 and cloning of the *Bam*HI fragments A–F has been described (Boyle *et al.*, 1994). Sequences spanning map units 65–81 are contained within the *Bam*HI E, F, and A fragments which are located from left to right in that order. The *Bam*HI F fragment was sequenced on both strands by subcloning fragments into bacteriophage M13 and by using synthetic oligonucleotide primers made on a model 391 DNA synthesizer (Applied Biosystems, Foster City, Ca). Nucleotide sequences for one strand of the *Bam*HI A and E fragments was determined by sequencing a family of overlapping deletion mutants generated using a nested deletion kit (Pharmacia). The opposite strand was sequenced using a series of synthetic oligonucleotide primers which were complementary to the sequence determined above. Nucleotide sequences were determined manually (Sanger *et al.*, 1977) or using an Applied Biosystems DNA sequencer.

Preparation of RNA from OAV287-infected cells

Total RNA was prepared from uninfected and OAV287-infected CSL503 cells using an RNAgents kit (Promega, Madison, WI). Cells were infected at 10 PFU/cell in the presence or absence of AraC and harvested at 45 hr p.i. as per the instructions provided in the kit. Cells incubated with AraC (40 μg/ml) were pretreated for 1 hr prior to infection and maintained in that concentration of drug.

Determination of RNA transcript splice and termination points

RNA prepared as above from uninfected and infected cells was copied into cDNA using AMV reverse transcriptase (Promega) as previously described (Sleigh *et al.*, 1979) using one of the cDNA primers listed in Table 1. Portions of the cDNAs were amplified by PCR using *Taq* DNA polymerase from one of several suppliers, cloned directly using the pGEM-T vector (Promega) and sequenced. The PCR primers are listed in Table 1 and indicated in Fig. 1.

TABLE 1

Primers Used for cDNA Synthesis and PCR Amplification of OAV287 RNA Species

Purpose	cDNA synthesis[a]	PCR amplification[a]
TLS/fiber splice junction	3177–3199 (−)	TLS exon 1 oligo (+)[b]
TLS/pVIII splice junction	2381–2401 (−)	TLS exon 1 oligo (+)[b]
TLS/33K splice junction	1843–1862 (−)	TLS exon 1 oligo (+)[b]
33K intron/L4 termination	T_{20}	1761–1781 (+)
pVIII/L4 termination	T_{20}	2668–2685 (+)
Fiber/L5 termination	T_{20}	4620–4639 (+)
pVIII–fiber gap RNA (putative right to left transcript)	T_{20}	3177–3199 (−)

[a] Nucleotides according to Fig. 1.
[b] Manuscript in preparation.

RESULTS

Nucleotide sequence and landmark genes

The nucleotide sequence of part of the *Bam*HI E fragment, the complete F fragment, and the A fragment from its left-hand end to the termination codon of the fiber gene (corresponding to map units 65–81) has been determined (GenBank Accession No. U18755). The nucleotide and predicted amino acid sequences for this region are presented in Fig. 1. Reading frames corresponding to the 100K hexon assembly, pVIII, and fiber proteins were clearly identifiable by their homologies with the equivalent proteins of human Ad2 using the GCG Bestfit program (Fig. 2). Two open reading frames between nucleotides 1751–1927 and 2003–2935 (Fig. 1) showed limited homology with the Ad2 33K protein which is generated from two exons. The proposed start and finish points for all reading frames are indicated in Fig. 1 and their arrangements are indicated in Fig. 3. The relative order of these genes corresponds to that observed for other adenovirus genomes (Dragulev *et al.*, 1991; Horwitz, 1990; Mittal *et al.*, 1992, 1993; Elgadi and Hajahmad, 1992; Raviprakash *et al.*, 1989).

Location of splice junctions for 33K, pVIII, and fiber transcripts

In the Ad2 genome, late mRNAs are spliced to a tripartite leader sequence (TLS) which is transcribed from the major late promoter (MLP) located upstream of the late genes at map unit 15 (reviewed in Horwitz, 1990). We

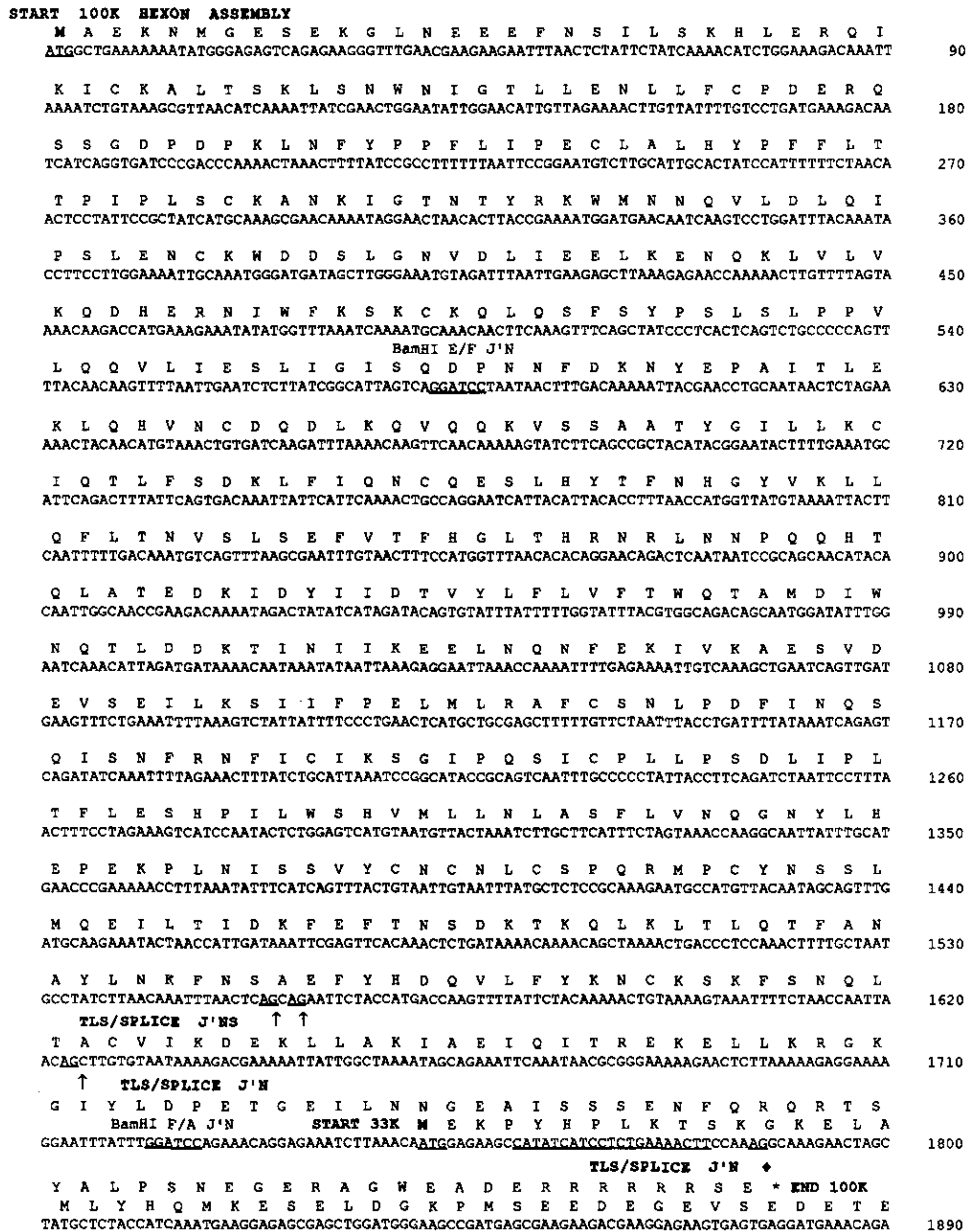

FIG. 1. Nucleotide and predicted amino acid sequence for the genome of OAV287 spanning the 100K, 33K, pVIII, and fiber genes. Long underlined sequences correspond or are complementary to the primers described in Table 1. Upward thin arrows (↑) indicate the location of splice junctions with the tripartite leader sequence (TLS). Open arrows (⇑) show the splice junctions for the 33K intron. Diamonds (♦) indicate the location of alternative splice sites with base 1922 in the pVIII transcript. Downward arrows (⇓) indicate predicted sites for cleavage by the viral 23-kDa protease.

have identified the probable MLP of OAV287 at map unit 17 in the *Bam*HI B fragment of the genome (manuscript in preparation). Exon 1 of the TLS was assumed to be nearby. An oligonucleotide complementary to mRNA from the fiber gene (Fig. 1 and Table 1) was used to prime cDNA synthesis by reverse transcriptase using RNA prepared from uninfected and infected cells (without AraC) as template. A part of the cDNA was then amplified by PCR using the primer from putative TLS exon 1 and the primer complementary to the fiber mRNA. No significant PCR products were obtained from RNA derived from uninfected cells (data not shown). However, major and minor products of ~235 and ~275 bp, respectively, were detected when infected cell RNA was used as template. The major product was cloned and several clones were sequenced. This showed that the leader sequence of 157 nucleotides was composed of three exons (manuscript in preparation) with the third exon being spliced to the A of the first ATG codon in the fiber open reading frame at base 3135 (Fig. 1). The minor PCR product had an exon 2 sequence which was 50 nucleotides larger than observed in most clones. Its origin was not examined further.

A similar approach was used to map the TLS splice

```
T  T  I  P  K  K  M  K  F  T  S  K  *
GACAACAATTCCAAAGAAAATGAAGTTTACAAGTAAGTAAGCTCTAAATTTTTTATATTAAAAACTGAATTTTTTTAGACAAAATTATTT   1980
        INTRON SPLICE J'N  ⇑                    33K  INTRON
                          Q  L  I  F  V  R  F  S  E  N  S  S  V  Q  S  Y  Q  V  H  L  P  L  K
TAAATTAAATCTTTATAGCTAGCAGTTGATCTTTGTTCGTTTTTCAGAAAACTCAGTGTTCAGTCATATCAAGTTCACTTGCCTCTGAA   2070
              •           • INTRON SPLICE J'NS  ⇑              •
 H  E  I  A  E  I  L  E  K  I  R  L  E  S  K  K  Y  P  G  K  V  Y  Q  I  R  N  R  T  P  A
ACACGAAATTGCGGAAATTCTAGAAAAAATTAGACTAGAATCTAAAAAATATCCAGGAAAAGTTTATCAAATAAGAAATAGAACTCCAGC   2160

 S  I  T  K  R  Y  L  Y  E  R  D  L  K  K  L  F  Q  Y  L  E  D  A  K  K  L  Y  A  K  Y  Q
AAGTATTACAAAACGATACCTGTATGAAAGAGATCTGAAGAAACTGTTCCAGTATCTAGAAGACGCAAAGAAGCTTTACGCTAAGTACCA   2250

 S  * END 33K   START pVIII  M  A  Q  P  V  T  P  Y  V  W  K  Y  Q  P  E  T  G  Y  T
AAGCTGAGGCTTTATAGTTTTAAATTTTCCCGCCATGGCTCAACCAGTGACGCCTTACGTCTGGAAATACCAACCAGAAACAGGATATAC   2340

 A  G  A  H  Q  N  Y  N  T  V  I  N  W  L  H  A  N  P  Q  M  F  A  R  I  Q  H  I  N  T  A
TGCTGGAGCCCATCAAAATTATAACACTGTTATCAACTGGTTGCATGCCAATCCACAAATGTTTGCCAGAATTCAACATATAAACACCGC   2430

 R  N  V  M  D  K  F  R  S  D  L  T  R  D  D  I  A  V  N  I  N  N  W  P  A  E  D  L  M  Q
ACGCAATGTTATGGACAAATTCCGCTCTGATTTGACCCGAGATGACATCGCGGTTAACATCAACAACTGGCCTGCAGAGGATTTAATGCA   2520

 P  P  N  F  P  Y  I  P  A  T  S  K  S  A  S  T  I  N  D  W  L  A  T  T  Q  G  I  Q  L  S
ACCTCCTAATTTTCCTTACATTCCTGCGACCTCTAAATCCGCTTCAACCATAAATGACTGGTTGGCTACCACTCAAGGAATTCAACTCAG   2610
PROTEASE  CLEAVAGE  SITE? ⇓
 G  T  S  E  L  N  G  W  G  S  N  R  L  T  S  Y  P  D  I  P  P  I  L  K  Y  E  R  P  G  Q
TGGAACTAGTGAACTAAACGGGTGGGGATCTAACCGCCTGACTTCCTATCCGGATATTCCACCCATTTTAAAGTATGAAAGGCCTGGTCA   2700
                  ⇓  PROTEASE  CLEAVAGE  SITE?
 Q  L  Q  G  Q  G  L  F  K  Q  E  N  I  H  L  F  Y  E  S  P  R  L  P  R  S  G  G  L  T  P
ACAACTTCAAGGCCAAGGACTTTTTAAGCAAGAAAATATTCATTTATTTTACGAATCTCCGCGCCTCCCTCGCTCTGGAGGATTAACTCC   2790

 Q  Q  F  V  K  E  F  P  P  V  V  Y  N  N  P  F  S  E  S  M  S  V  F  P  K  E  F  S  P  L
CCAACAATTTGTAAAAGAATTTCCGCCTGTTGTTTATAATAACCCCTTCTCAGAATCTATGAGTGTATTTCCGAAAGAATTTAGTCCTTT   2880

 F  N  P  S  E  S  L  K  K  T  S  S  Q  T  L  Q  Y  K  *  END pVIII
GTTTAACCCTTCAGAATCTTTGAAAAAAACATCCAGTCAAACTTTACAATATAAATAAAAAACTTCTATTGATCTTTATACTTACACTAA   2970
                                                                     END  L4  RNAs ↑  ↑
AGCATCGCGTTTATTTTCGTCGCCATAAAAATATATCAAAGACCCGTAATTCTCTAACTTTAAATCATTTTTTGAACTAATCTTAATCCA   3060

                                                               START FIBER   M  K  R  A  R
TTTAAATGTAGGAATTAATATATCAGAAACCAGTAACAAGCCAGAATTAAAATATACTTGTGTCATTTTTACAGATGAAGCGAGCACGCT   3150
                                                   TLS/FIBER  SPLICE  J'N ↑
 W  D  P  V  Y  P  F  S  E  E  R  L  V  P  L  P  P  F  I  E  A  G  K  G  L  K  S  E  G  L
GGGACCCGGTTTATCCCTTTTCTGAAGAGAGACTGGTTCCTCTGCCTCCTTTTATTGAAGCCGGAAAAGGGCTAAAAAGCGAAGGGTTGA   3240

 I  L  S  L  N  F  T  D  P  I  T  I  N  Q  T  G  F  L  T  V  K  L  G  D  G  I  F  I  N  G
TCTTATCTTTAAACTTTACTGATCCTATCACTATAAATCAAACCGGTTTCTTAACTGTAAAATTGGGAGATGGAATATTCATAAACGGAG   3330

 E  G  G  L  S  S  T  A  P  K  V  K  V  P  L  T  V  S  D  E  T  L  Q  L  L  L  S  N  S  L
AGGGTGGCCTATCAAGCACTGCTCCAAAAGTCAAAGTTCCCCTGACTGTCTCAGATGAAACATTGCAACTGCTATTAAGTAATTCTCTAA   3420

 T  T  E  S  D  S  L  A  L  K  Q  P  Q  L  P  L  K  I  N  D  E  G  S  L  V  L  N  L  N  T
CAACTGAGTCAGACTCTTTAGCTTTAAAACAACCGCAACTTCCCCTAAAAATAAATGATGAGGGGAGTTTAGTATTGAACTTAAATACTC   3510

 P  L  N  L  Q  N  E  R  L  S  L  N  V  S  N  P  L  K  I  A  A  D  S  L  T  I  N  L  K  E
CTTTAAATCTACAAAATGAGAGATTGAGTTTAAATGTTTCAAATCCACTAAAGATAGCGGCAGATTCTTTAACTATAAACTTAAAGGAAC   3600

 P  L  G  L  Q  N  E  S  L  G  L  N  L  S  D  P  M  N  I  T  P  E  G  N  L  G  I  K  L  K
CCCTAGGATTGCAAAATGAAAGTTTGGGCTTAAATCTAAGTGATCCTATGAATATAACTCCAGAAGGAAATTTAGGTATTAAATTGAAAA   3690

 N  P  M  K  V  E  E  S  S  L  A  L  N  Y  K  N  P  L  A  I  S  N  D  A  L  S  I  N  I  A
ATCCTATGAAAGTTGAAGAAAGTTCTTTAGCCTTAAACTATAAGAATCCTCTCGCCATTAGTAATGATGCGTTAAGTATAAACATTGCGA   3780

 N  P  L  T  V  N  T  S  G  S  L  G  I  S  Y  S  T  P  L  R  I  S  N  N  A  L  S  L  F  I
ATCCATTAACTGTTAATACAAGCGGATCTCTAGGAATATCTTATTCTACTCCCTTACGAATTTCAAATAATGCTTTATCATTATTTATAG   3870


 G  K  P  L  G  L  G  T  D  G  S  L  T  V  N  L  T  R  P  L  V  C  R  Q  N  T  L  A  I  N
GAAAACCTTTAGGATTAGGAACTGACGGCTCTTTAACTGTAAATTTAACTAGGCCTCTGGTATGTCGTCAGAACACTTTGGCCATAAACT   3960

 Y  S  A  P  L  V  S  L  Q  D  N  L  T  L  S  Y  A  Q  P  L  T  V  S  D  N  S  L  R  L  S
ACTCAGCCCCACTAGTGTCATTGCAAGACAATCTTACTTTAAGTTATGCTCAACCATTAACTGTAAGCGATAATTCTTTAAGATTGTCTC   4050

 L  N  S  P  L  N  T  N  S  D  G  K  L  S  V  N  Y  S  N  P  L  V  V  T  D  S  N  L  T  L
TAAATTCTCCACTAAACACAAATAGTGATGGAAAACTTAGTGTAAACTATTCTAATCCTTTAGTTGTGACTGACTCTAATCTTACCCTCA   4140

 S  V  K  K  P  V  M  I  N  N  T  G  N  V  D  L  S  F  T  A  P  I  K  L  N  D  A  E  Q  L
GTGTTAAAAAACCTGTAATGATTAACAACACAGGTAATGTTGACTTAAGCTTTACAGCTCCCATAAAATTAAATGATGCAGAACAGTTGA   4230

 T  L  E  T  T  E  P  L  E  V  A  D  N  A  L  K  L  K  L  G  K  G  L  T  V  S  N  N  A  L
CTTTAGAAACCACTGAGCCCTTGGAAGTGGCCGATAACGCTCTAAAACTGAAACTTGGAAAAGGCTTAACTGTTAGTAATAATGCTTTAA   4320

 T  L  N  L  G  N  G  L  T  F  Q  Q  G  L  L  Q  I  K  T  N  S  S  L  G  F  N  A  S  G  E
CCTTAAACCTTGGAAACGGTTTGACTTTCCAACAAGGTCTTTTACAAATTAAAACTAATAGCTCTCTAGGGTTTAATGCTTCTGGGGAAT   4410

 L  S  T  A  T  K  Q  G  T  I  T  V  N  F  L  S  T  T  P  I  A  F  G  W  Q  I  I  P  T  T
TATCAACAGCTACAAAGCAGGGAACCATAACCGTTAACTTTCTAAGCACAACTCCTATAGCTTTTGGGTGGCAAATAATACCTACTACTG   4500

 V  A  F  I  Y  I  L  S  G  T  Q  F  T  P  Q  S  P  V  T  S  L  G  F  Q  P  P  Q  D  F  L
TAGCTTTCATTTATATTTTATCAGGAACACAATTTACTCCTCAATCCCCAGTAACTTCTTTAGGTTTTCAACCCCCACAAGACTTTTTGG   4590

 D  F  F  V  L  S  P  F  V  T  S  V  T  Q  I  V  G  N  D  V  K  V  I  G  L  T  I  S  K  N
ATTTCTTCGTTTTAAGTCCGTTTGTTACATCTGTAACTCAAATTGTGGGAAATGATGTTAAGGTTATTGGCCTAACTATTTCTAAAAACC   4680

 Q  S  T  I  T  M  K  F  T  S  P  L  A  E  N  V  P  V  S  M  F  T  A  H  Q  F  R  Q  *  END FIBER
AATCTACCATAACTATGAAATTTACTTCTCCCTTAGCTGAAAATGTACCAGTTAGTATGTTTACAGCACATCAATTCAGACAATGAA   4767
```

FIG. 1 — *Continued*

A

```
Ad2/100K x OAV/100K
 64 EDKKQDQDDAEANEEQVGRGDQRHGDYLDVGDDVLLKHLQRQCAIICDAL 113
    .:|. ::.: : |||:.                :.:| |||:||.  ||.||
  2 AEKNMGESEKGLNEEEF..............NSILSKHLERQIK.ICKAL 36

114 QERSDVPLAIADVSLAYERHLFSPRVPPKRQENGTCEPNPRLNFYPVFAV 163
     .:     |.  :::  .|. ||:    |..||..|  :|:|:|||||.| :
 37 TSK....LSNWNIGTLLENLLFC...PDERQSSG..DPDPKLNFYPPFLI 77

164 PEVLATYHIFFQNCKIPLSCRANRSRADKQLALRQGAVIP.DIASLDEVP 212
    ||.|| .. || ...||||:||:   .:.    :....|:. :|:||:::..
 78 PECLALHYPFFLTTPIPLSCKANKIGTNTYRKWMNNQVLDLQIPSLENCK 127

213 KIFEGLGRDEKRAANALQQENSENESHCGVLVELEGDNARLAVLK.RSIE 261
           :|:. :. .| :| .||:.     || :..|:.|    :| :: :
128 .......WDDSLGNVDLIEELKENQK....LVLVKQDHERNIWFKSKCKQ 166

262 VTHFAYPALNLPPKVMSTVMSELIVRRARPLERDANLQEQ.TEEGLPAVG 310
    :   |.||.|.||| :  .::..||. .. | : | |.:.. | | |. |.
167 LQSFSYPSLSLPPVLQQVLIESLIGISQDPNNFDKNYEPAITLEKLQHVN 216

311 DEQLARWLETREPADLEERRKLMMAAVLVTVELECMQRFFADPEMQRKLE 360
     :|             ||.: .. : .|.  .: |.|:| :|.|. : .. :
217 CDQ...........DLKQVQQKVSSAATYGILLKCIQTLFSDKLFIQNCQ 255

361 ETLHYTFRQGYVRQACKISNVELCNLVSYLGILHENRLGQNVLHSTLKGE 410
    |.|||||.:|||:      :.||.|:::|.: |: | |||..    |. | .|
256 ESLHYTFNHGYVKLLQFLTNVSLSEFVTFHGLTHRNRLNNPQQHTQLATE 305

411 ARRDYVRDCVYLFLCYTWQTAMGVWQQCLEERNLKELQKLLKQNLKDLWT 460
    .: ||: |.|||||.:||||||||::|.|.|:::.:. :.. |.||:..: .
306 DKIDYIIDTVYLFLVFTWQTAMDIWNQTLDDKTINIIKEELNQNFEKIVK 355

461 AFNERSVAAHLADIIFPERLLKTLQQGLPDFTSQSMLQNFRNFILERSGI 510
    | .   .|.. | .||||| :|:.:  .||||..|| : ||||||  :|||
356 AESVDEVSEILKSIIFPELMLRAFCSNLPDFINQSQISNFRNFICIKSGI 405

511 LPATCCALPSDFVPIKYRECPPPLWGHCYLLQLANYLAYHSDIMEDVSGD 560
     ...|. ||||::|:.: |:.| ||:|. ||.||.:|. :::.:.:.. .
406 PQSICPLLPSDLIPLTFLESHPILWSHVMLLNLASFLVNQGNYLHEPEKP 455

561 .GLLECHCRCNLCTPHRSLVCNSQLLSESQIIGTFELQGPSPDEKSAAPG 609
     .: ...|.||||.|:|   .:|| |: |   .|:.||: .......
456 LNISSVYCNCNLCSPQRMPCYNSSLMQEILTIDKFEFTNSDKTKQ..... 500

610 LKLTPGLWTSAYLRKFVPEDYHAHEIRFYEDQSRPPNAELTACVITQGHI 659
    |||| . :..|||.|| ..::. .:: ||.: ... ..:|||||||.::.:
501 LKLTLQTFANAYLNKFNSAEFYHDQVLFYKNCKSKFSNQLTACVIKDEKL 550

660 LGQLQAINKARQEFLLRKGRGVYLDPQSGEELNPIPPPPQPYQQPRALAS 709
    |:.:..|. .|:. ||::|:|:||||:.|| ||          .. |:.|
551 LAKIAEIQITREKELLKRGKGIYLDPETGEILN..........NGEAISS 590

710 QDGTQKEAAAAAAATHGRGGILGQSGRGGFGRGGGDDGRLGQPRRSFRGR 759
     :. |::                    |.::: .:.:::| |    :.  :|
591 SENFQRQ...................RTSYALPSNEGERAG...WEADER 618

760 RGVRRNT 766
    |  ||..
619 RRRRRSE 625
```

B

```
Ad2 33K x OAV287 33K

102 TRAGAPTAPAAAAAAATAAVTQKQRRPDSKTLTKPKKSTAAAAAGGGA.. 149
     .::  |  .. : . |       |:.  |:|.:........:......
  2 EKPYHPLKTSKGKELAMLYHQMKESELDGKPMSEEDEGEVSEDETETTIP 51

150 ..LRLAPNEPVSTRELRNRIFPTLYAIFQQSRGQEQE.....LKIKNRSL 192
      :::..|..| . ::: .: ..: .|::. | :...      ..|:||.
 52 KKMKFTKNSSVQSYQVHLPLKHEIAEILEKIRLESKKYPGKVYQIRNRTP 101

193 RSLTRSCLYHKSEDQLRRTLEDAEALFSKY 222
     |:|:.:||.:. ..| . ||||. |:.||
102 ASITKRYLYERDLKKLFQYLEDAKKLYAKY 131
```

C

```
Ad2 pVIII x OAV287 pVIII.

  4 EIPTPYMWSYQPQMGLAAGAAQDYSTRINYMSAGPHMISRVNGIRAHRNR 53
    : .|||:||.|||: |..||| |:|.| ||:: |.|:||:.|:. |.. ||
  3 QPVTPYVWKYQPETGYTAGAHQNYNTVINWLHANPQMFARIQHINTARN. 51

 54 ILLEQAAITTTPRNNLNPRSWPAALVYQESPAPTTVVLPRDAQAEVQ..M 101
    :: .   .   |  .   .:| ..|||. : |..   | ... .:.| .  :
 52 VMDKFRSDLTRDDIAVNINNWPAEDLMQPPNFPYIPATSKSASTINDWLA 101

                                     ⇓
102 TNSGAQLAGGFRHRVRSPGQGITHLKIRGRGIQLNDESVSSSLGL.RPDG 150
    |. | ||.|.                 ..|. :|..   . ..:.. | . ||:.
102 TTQGIQLSGT............SELNGWGSNRLTSYPDIPPILKYERPGQ 139
                                   ⇑
        ⇓
151 TEQIGGAGRSSFTPRQAILTLQTSSSEPRSGGIGTLQFIEEFVPSVYFNP 200
     :| .|      : ..:.|   : .|.. |||||:.. ||:.|||.| || ||
140 QLQGQG.....LFKQENIHLFYESPRLPRSGGLTPQQFVKEFPPVVYNNP 184
       ⇑

201 FSGPPGHYPDQFIPNFDAV....KDSADGYD 227
    ||:. : :|.:|| | |::     |.|.:..:
185 FSESMSVFPKEFSPLFNPSESLKKTSSQTLQ 215
```

FIG. 2. Homology between human Ad2 and OAV287 proteins. Sequences were compared using the Bestfit program in the GCG package using a gap weight of 3.0, length weight of 0.1, average match of 0.54, and average mismatch of −0.396 as parameter settings. ⇓ indicates the 23-kDa protease cleavage sites determined for Ad2 and predicted for OAV287 pVIII.

sites for mRNAs encoding 33K and pVIII. It was reasoned, given the relative order of the genes (Fig. 3), that the PCR products of the TLS/33K primer combination would be a subset of the products derived from the TLS/pVIII primer pair. Oligonucleotides complementary to 33K and pVIII mRNAs (Fig. 1 and Table 1) were used for cDNA synthesis and these primers, in conjunction with the TLS primer, were used for PCR amplification of the cDNA products (Table 1). For both combinations of primers, infected cell-specific PCR products were obtained, the TLS/33K primer pair generating DNAs of ~400 and ~470 bp (Fig. 4A, lane 2), the TLS/pVIII primer combination producing DNAs of ~770, ~600, and ~530 bp (Fig. 4A, lane 5). Each mixture of products was cloned and several were sequenced using a primer from within exon 2 of the TLS to ascertain the location of the splice junctions. Three clones from the TLS/33K and five from the TLS/pVIII group were examined. These sequences showed that the TLS was spliced to transcripts potentially coding for 33K and pVIII at four different locations between bases 1555 and 1787 (Fig. 1). These splices all occurred 3′ to an AG dinucleotide, as did the splice for the fiber transcript (Fig. 1). The last splice site (position 1787) is downstream of the probable initiation codon for 33K protein and transcripts with this TLS junction presumably code for pVIII. Thus, unlike the fiber gene (Fig. 1), the TLS splice sites for these genes map to locations well upstream of the coding sequences.

The Ad2 33K gene is interrupted by an intron. All PCR-amplified clones examined here had a splice junction 5′ to the GT dinucleotide at base 1923 but were joined to one of four different downstream locations. In one clone splicing between bases 1922 and 1999 occurred, i.e., using an AA′GT donor and a AG′CT acceptor site (Fig. 1). However, in this reading frame a stop codon was soon encountered. Splicing in other clones was identified between bases 1922 and 2006 and 1922 and 2045. These also produced reading frames in which stop codons were soon encountered (Fig. 1). In four of seven clones examined, base 1922 was spliced to base 2028 (AA′GT to AG′AAAA) which linked a second extended reading frame with the first, predicting that the 33K gene product is a protein of 133 amino acids. The 33K gene therefore appears to have an intron of 104 bp. Presumably the other splicing events generate mRNAs which code for pVIII.

Termination of the L4 and L5 transcripts

Late transcription of the Ad2 genome generates the L4 family of RNAs which encode the 100K hexon assem-

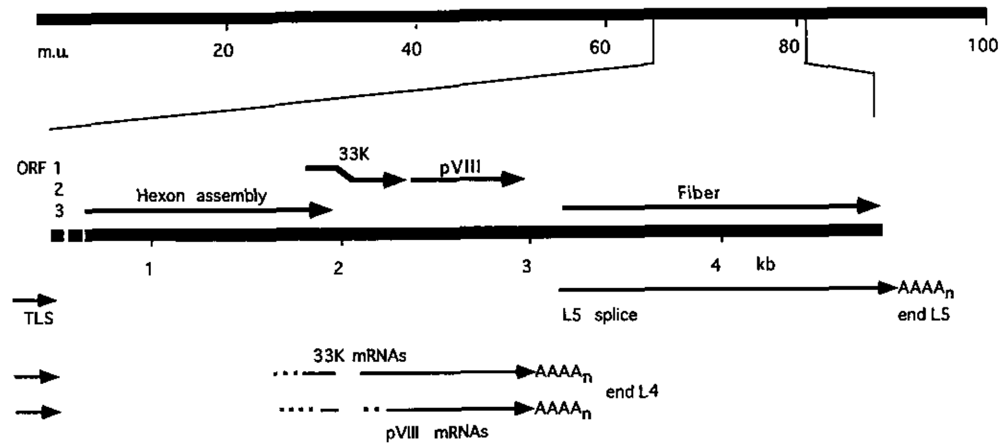

FIG. 3. Summary of the open reading frames and transcripts in the 100K, 33K, pVIII, and fiber genes. Broken lines indicate multiple splice sites in L4 (see Fig. 1).

bly, 33K, and pVIII polypeptides. The fiber protein is generated from a separate transcript, L5. The splice junction between the TLS and L5 was mapped as described above. To ascertain whether L4 transcripts terminated after the pVIII coding sequences, polyadenylated mRNAs from non-AraC-treated OAV287-infected cells were copied into cDNA using a T_{20} primer. These were then amplified by PCR using T_{20} and primers from within the 33K and pVIII genes (Fig. 1 and Table 1). Single, infected-cell-specific products of ~1100 (Fig. 4B, lane 3) and ~300 bp were obtained, cloned and sequenced. This revealed that the transcripts terminated and were polyadenylated at two nearby sites (bases 2962 and 2965), i.e., close to the TAA stop codon for pVIII (Fig. 1). This codon occurs as part of an AATAAA sequence which functions as a signal for termination/polyadenylation of transcripts (Fitzgerald and Shenk, 1981; Proudfoot and Brownlee, 1976). Thus, L4 terminates 169–172 bases upstream of the splice site for fiber mRNA. These data predict that L4 transcripts would be a family of mRNAs of ~1.3–1.4 kb in size, although the resolution of a Northern blot would probably not show this heterogeneity.

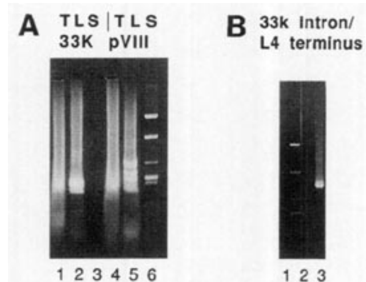

FIG. 4. PCR amplification of cDNAs transcribed from the OAV287 L4 family of mRNAs. Total RNA prepared from uninfected (A, lanes 1 and 4; B, lane 2) and OAV287-infected (A, lanes 2 and 5; B, lane 3) CSL503 cells was reverse transcribed as described under Materials and Methods and amplified by PCR using primers indicated in Table 1 and Fig. 1. Products were analyzed by agarose gel electrophoresis against marker fragments (A, lane 6; B, lane 1) of 3019, 1429, 766, 543/525/517, 470, and 237/220 bp.

The termination point of the L5 transcript was similarly mapped using oligonucleotide T_{20} and the primer shown in Table 1 and Fig. 1. A PCR product of ~180 bp was obtained and cloned. Two clones were sequenced. This showed that L5 transcription termination and polyadenylation occurred at a point 41 nucleotides past the TGA stop codon of the fiber gene. This codon forms part of a AATGAA sequence (Fig. 1) which appears to function as the polyadenylation signal in this case as no other similar sequence motif exists in the region.

Features of individual genes and proteins

100K hexon assembly. The gene encoding the OAV287 100K hexon assembly protein overlaps the gene coding for the 33K protein at its 3′ end (Figs. 1 and 3). The hexon assembly protein is predicted to be a polypeptide of 625 amino acids with an apparent molecular weight of 72.2 kd. The predicted protein is 141 amino acids shorter than the equivalent Ad2 protein.

33K protein. The OAV287 equivalent of the Ad2 33K coding sequence probably begins at bases 1751–1753 since this is the first favorable initiation codon in a significant reading frame following the TLS splice sites (Fig. 1). The OAV 33K protein is predicted to be a polypeptide of 134 amino acids with a size of 15.7 kDa. This is very much smaller than its Ad2 counterpart which is 230 residues long. The two proteins appear only distantly related (Fig. 2B). Nevertheless, both genes retain an intron which interrupts the coding sequence (Fig. 1 and Horwitz, 1990).

The RNA cap site (and presumably the promoter) for the E2A region of Ad2 lies on the complementary strand within the second exon of the 33K gene (Horwitz, 1990). We have not detected any homology between the two genomes in this area and the OAV287 transcription map on the complementary strand has not been determined.

pVIII precursor. The 33K gene is closely followed in the same reading frame by the gene for structural protein VIII, beginning at nucleotides 2285–2287 (Fig. 1). This is synthesized as a precursor (pVIII) in other Ads. OAV287

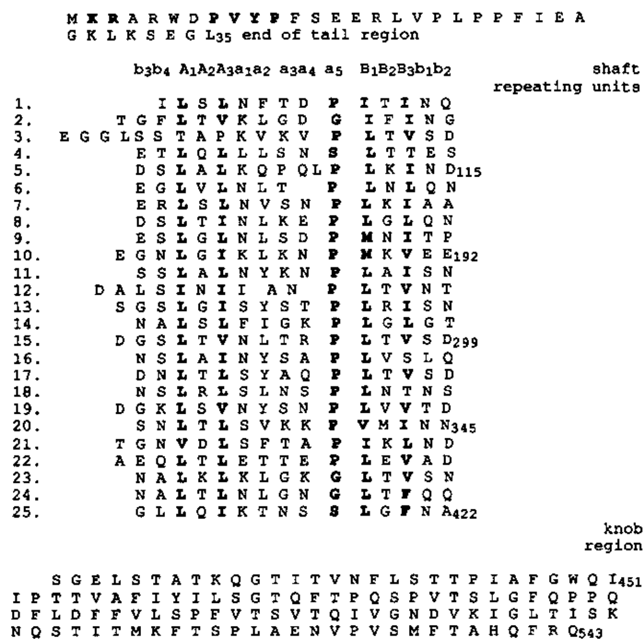

FIG. 5. Predicted amino acid sequence for the fiber protein of OAV287 showing the various domains described by others (see text). The letters in bold in the N-terminal region are widely conserved among adenoviruses or form part of the repeat structure which makes up the shaft.

pVIII (24.7 kDa) is similar in size to the Ad2 protein (218 vs 227 aas) but has only 32.5% identity with it (Fig. 2C). In spite of this, the cleavage sites HyGX'G and HyXGG' (where Hy is usually Ile, Leu, or Met), known to be used by the 23-kDa virus-encoded protease (Freimuth and Anderson, 1993; Webster *et al.*, 1989), appear to be conserved in the OAV287 protein (Fig. 2C). Other possible cleavage sites in Ad2 pVIII are not conserved in the ovine Ad protein nor in the type 3 bovine Ad pVIII (Mittal *et al.*, 1992, 1993).

Fiber protein. The OAV sequences encoding the fiber protein begin at nucleotides 3135–3137 and terminate at nucleotide 4763 (Fig. 1). The protein of 543 residues (58.2 kDa) shows only a low level (~25%) of direct homology with other adenovirus fiber proteins but shares the repeated motifs characteristic of the shaft region previously described by others (Chroboczek and Jacrot, 1987; Dragulev *et al.*, 1991; Green *et al.*, 1983; Stouten *et al.*, 1992; Hong *et al.*, 1988; Kidd *et al.*, 1993; Mittal *et al.*, 1992, 1993; Elgadi and Hajahmad, 1992; Raviprakash *et al.*, 1989). There is an N-terminal region of about 35 residues which precedes the domain comprising the shaft of the fiber. The latter is composed of 25 pseudorepeat motifs which can be adapted to either of the models proposed for the structure of the Ad fiber (Green *et al.*, 1983; Stouten *et al.*, 1992). As in other Ads, the third motif is extended (Fig. 5) and may be associated with a kink in the shaft (Ruigrok *et al.*, 1994). In the regularity and the number of the pseudorepeats, the OAV287 shaft region more closely resembles the human Ads (Kidd *et al.*, 1993) rather than bovine Ad3 which is much longer (Mittal *et al.*, 1992, 1993). The remaining 121 residues of OAV287 fiber sequence probably constitute the knob, or C-terminal region, although the start of this region is less well defined as the sequence TLWT, which is conserved in many Ads, but is missing in OAV287 (Fig. 5). Thus, the C-terminal head region appears to be considerably shorter than those described for other Ad fiber proteins (Kidd *et al.*, 1993) and the level of homology is also very low.

DISCUSSION

This paper describes the partial sequence and genome arrangement of an ovine adenovirus OAV287, isolated from sheep in Western Australia. By restriction enzyme mapping this isolate is very closely related to another isolate, OAV1537, recovered earlier from the same region and may therefore be representative of a group of viruses circulating in the area. These viruses may represent a new serotype as they are only distantly related (one-way cross-reactivity) to bovine adenovirus type 7 and are serotypically and genetically distinct from adenoviruses recovered from sheep in the state of Victoria in southeastern Australia (Boyle *et al.*, 1994).

The TLS of Ad2 is spliced to all late RNA transcripts promoting the efficient translation of late viral proteins. As for Ad2, there are no initiation codons within the TLS of OAV287 (manuscript in preparation) and for the fiber gene, RNA splicing generates an AUG immediately after the TLS. This must be used since there are no other initiation codons in the region. This fits the pattern observed for most eukaryotic mRNAs where the first AUG codon from the 5′ end is used for initiation (Kozak, 1984). Short reading frames which are close to preferred AUG codons can also attenuate initiation of translation (Kozak, 1984). The complex splicing pattern observed for 33K and pVIII RNAs generates a short open reading frame of 16 residues upstream of the AUG proposed for 33K protein. Numerous AUGs and short reading frames exist upstream of the proposed AUG for pVIII. However, all but one of these lie upstream of the 33K intron sequence and most are in a very weak context for initiation according to Kozak's rules (Kozak, 1987). Perhaps, therefore, they have little influence on the efficiency of initiation from the pVIII AUG.

PCR amplification of cDNAs reverse transcribed from total infected cell RNA yielded numerous products, indicating the existence of multiple RNA transcripts from the region encompassing the 33K and pVIII genes. This was surprising because following similar amplification of transcripts from other regions of the genome, e.g., using TLS/fiber oligonucleotides, only a single product was detected. The reason for the existence of multiple splice junctions is not clear but it is possible that they create potential for generating new proteins, e.g., an RNA species in which the TLS is spliced to base 1787 (Fig. 1) and from which the 33K intron sequences are removed would generate a protein lacking only the N-terminal 12 amino acids of 33K protein.

In Ad2 and Ad5 the E3 transcription unit of about 2.5 kb is located between the end of pVIII and beginning of fiber protein gene. Therefore, the most surprising finding from this study was that in OAV287, the intergenic region between the pVIII and fiber consists of only 197 nucleotides (Figs. 1 and 3). This region incorporates signals for termination and polyadenylation of the L4 transcript derived from the MLP and for TLS/fiber RNA splicing (Fig. 1). There is no significant open reading frame encoded by the top strand but on the bottom strand one reading frame of 79 codons runs from nucleotide 3201 in the fiber gene toward nucleotide 2962 (Fig. 1) and there is a potential polyadenylation signal at nucleotides 2979–2984. Therefore a spliced transcript could possibly traverse this region. However, when the T_{20} primer and the TLS/fiber oligonucleotide (Table 1), i.e., a primer from within the 79 residue ORF (Fig. 1) were used for cDNA synthesis and PCR amplification, no amplified product was obtained under conditions where the pVIII/L4 termination product was amplified. This suggests that the region is not transcribed from right to left but it must be transcribed from left to right to produce the L5 RNA encoding the fiber protein.

In human adenoviruses, the E3 transcription unit codes for numerous polypeptides, some of which interact with components of the immune system (Gooding, 1992; Wold and Gooding, 1991). While the region varies in size and complexity in other animal adenoviruses (Dragulev *et al.,* 1991; Linne, 1992; Mittal *et al.,* 1992; Raviprakash *et al.,* 1989), in most cases it has the potential to code for several protein products. Some of these show direct sequence homology with human Ads (Mittal *et al.,* 1992; Elgadi and Hajahmad, 1992) or functional homology with human Ad E3 proteins, e.g., proteins which may be equivalent to E3 19 kDa (Dragulev *et al.,* 1991; Linne, 1992; Raviprakash *et al.,* 1989). This poses the question of whether OAV287 lacks an E3 region completely, or whether sequences elsewhere in the genome fulfill this function. Compared with the Ad2 genome, there are additional open reading frames in OAV287 opposite to the expected orientation and 3′ to the fiber gene (our unpublished data). However, no obvious amino acid homology with other E3 genes or functional homology, e.g., the presence of hydrophobic sequences characteristic of the Ad2 E3 19- and 6.7-kDa polypeptides (Wilson-Rawls and Wold, 1993; Wold and Gooding, 1991), has so far been found.

Many recombinant human adenoviruses have now been constructed by making substitutions into the nonessential E3 region between the pVIII and the fiber genes (reviewed in Berkner, 1988; Graham and Prevec, 1992). As the OAV287 genome has no E3 region in this location and the short intergenic region of 197 nucleotides contains multiple signals for the termination, polyadenylation, and splicing of the L4 and L5 transcripts, it will have to be determined experimentally whether foreign DNA can be inserted into this region of the genome.

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