Journal of General Virology (1998), **79**, 509–516. Printed in Great Britain

Ovine adenovirus (OAV287) lacks a virus-associated RNA gene

Aruna Venktesh, Fujiko Watt, Zheng Zhou Xu and Gerald W. Both

Molecular Science, CSIRO, North Ryde, NSW 2113, Australia

Ovine adenovirus OAV287 (OAV) is the prototype of a virus group which is phylogenetically distinct from the mastadenoviruses and aviadenoviruses. The genome arrangement of OAV showed that virus-associated (VA) RNA genes were not located between the reading frames for p52/55K and terminal protein as these overlapped. To determine whether VA genes were located elsewhere, several approaches were used. Nuclear extracts containing RNA polymerase III activity were used to transcribe OAV genome fragments *in vitro*. A product of ~ 120 bp was produced from two widely separated coding regions of the genome. However, when these were subcloned and used as radiolabelled probes to analyse RNA from OAV-infected cells, no VA-like RNA was detected, although late mRNAs that were transcribed from the regions were identified. In addition, 5′ radiolabelling of small RNA species in control- and OAV-infected cells followed by gel analysis did not identify candidate VA RNAs. Radiolabelling of proteins in control- and OAV-infected cells showed that there was little preferential translation of viral proteins compared with host polypeptides, in contrast to the situation for adenovirus 5 (Ad5). In addition, the kinetics of OAV infection were slower than observed for human adenoviruses. Collectively, the data suggest that OAV is unique in that it does not produce VA RNA during infection. This conclusion is supported by a comparison of the genomes of the phylogenetically related OAV and egg drop syndrome viruses which shows that the VA gene identified in the latter is located in a region absent from OAV.

Introduction

Virus-associated (VA) RNAs, first characterized for adenovirus 2 (Ad2), are small, partially double-stranded RNA species that have been shown to fulfil an important role during replication. Members of the genus *Mastadenovirus*, which include all known human and many animal adenoviruses, have one or two VA RNA genes that are located between the genes for terminal protein (pTP) and 52/55K proteins (Mathews, 1975; Roberts *et al.*, 1984). The VA genes are transcribed in a rightward direction by RNA polymerase III (pol III) (Weinmann *et al.*, 1976; Wu, 1978). In chicken embryo lethal orphan (CELO) virus, the prototype aviadenovirus, a single VA RNA gene of 90 nucleotides is located near the right-hand end of the genome in a region which is unique to CELO virus. This gene is transcribed leftwards (Larsson *et al.*, 1986; Chiocca *et al.*, 1996). During Ad2 infection, VA RNAs are produced in abundance, especially late in the infectious cycle (Soderlund *et al.*, 1976). Although VA RNAs from different adenoviruses vary in length and sequence they probably share structural features which are important for function (Pe'ery *et al.*, 1993; Ma & Mathews, 1996). Ad2 VA RNA binds to a protein kinase which phosphorylates eIF-2, a key factor in the initiation of translation. The inhibition of phosphorylation that results from VA RNA binding prevents the shut-down of protein synthesis in the cell, thereby subverting a host defence mechanism (reviewed by Mathews & Shenk, 1991). At later times, when capsid proteins are required for virus assembly, VA RNAs appear to associate with ribosomes to enhance the translation of viral mRNAs (O'Malley *et al.*, 1989). As the transport of cellular versus viral RNAs is inhibited at late times (Beltz & Flint, 1979; Babiss *et al.*, 1985; Pilder *et al.*, 1986), an apparent shut-down of host protein synthesis results. Thus VA RNAs appear to be important in allowing the virus to subvert host cell functions during replication. Perhaps as a consequence, the replication cycle of Ad2 is rapid, virus production nearing completion within 24 h post-infection (p.i.) (reviewed by Horwitz, 1990).

OAV287 (OAV) is an ovine adenovirus which appears to be the prototype of a new group of adenoviruses that are phylogenetically distinct from the mastadenoviruses and aviadenoviruses. The OAV group also includes BAV-7 and avian egg drop syndrome (EDS) virus (Harrach *et al.*, 1997). The structure of the OAV genome, which was previously determined, revealed that the pTP and 52/55K genes over-

Author for correspondence: Gerald Both.
Fax +61 2 9490 5005. e-mail gerry.both@molsci.csiro.au

0001-5212

lapped, leaving no space to code for VA RNA genes (Vrati *et al.*, 1995, 1996*a*, *b*). As homology searches did not identify candidate VA RNA sequences in OAV, it was not clear whether they were absent or undefined. In this study several approaches were used to investigate whether VA RNA is produced during OAV infection in permissive cells.

Methods

■ **Cloning of genome fragments.** OAV *Bam*HI fragments a–f representing the entire genome (Fig. 1) (Vrati *et al.*, 1995, 1996*a*, *b*) were subcloned into pUC13 plasmids using standard techniques (Sambrook *et al.*, 1989; Boyle *et al.*, 1994). Similarly, clones representing the left-hand ~ 21·5 kb *Kpn*I–*Sph*I, 11 kb *Bam*HI D/B, 12·3 kb *Sma*I–*Sph*I and the 8·1 kb *Sac*I–*Sph*I fragments were cloned into convenient plasmids. *Eco*RI–*Sna*BI (542 bp) and *Eco*RI–*Xba*I (531 bp) fragments were derived from the larger clones by generating appropriate deletions. The Ad2 VA RNA_I gene was subcloned as a blunt-ended *Xba*I–*Hin*dIII fragment into pUC8.

■ **Preparation of nuclear extracts.** Nuclear extracts were prepared from a pre-promyelocytic K562 leukaemia cell line (Borellini *et al.*, 1991) as described previously (Prywes & Roeder, 1986) except that protease inhibitors were added to the hypotonic buffer as well as the nuclear suspension buffer (Borellini *et al.*, 1991) and an additional step of concentrating nuclear extract by ammonium sulphate precipitation (0·33 g/ml) was included (Shapiro *et al.*, 1988). The protein concentration (~ 8 mg/ml) was measured using a kit from Bio-Rad (Hercules).

■ ***In vitro* transcription.** Transcription reactions were performed for 1 h at 30 °C in a 10 µl reaction volume with 4·5–6 µl K562 nuclear extract and 50–500 ng (supercoiled) DNA templates. The standard reaction mixtures contained 12 mM HEPES–KOH pH 7·9, 60 mM KCl, 5 mM $MgCl_2$, 1 mM dithiotheitol, 500 µM each ATP, CTP and GTP, 25 µM UTP, 10 µCi [^{32}P]UTP, 5 mM creatine phosphate and 0·25 U RNasin (Promega). Some reactions contained α-amanitin (1 mg/ml). Reactions were stopped by the addition of 100 µl stop solution (0·1% SDS, 10 mM EDTA and 2 µg tRNA), followed by phenol–chloroform (1:1) extraction and ethanol precipitation. The pellet was resuspended in a formamide–dye mixture, heat-denatured and analysed by gel electrophoresis as described below.

■ **Preparation and analysis of RNAs.** Foetal ovine lung CSL503 cells are permissive for OAV replication (Boyle *et al.*, 1994). Cells cultured in EMEM plus 10% foetal calf serum (FCS), were left uninfected or were infected at an m.o.i. of 20 p.f.u. per cell with wild-type OAV. Total RNA was prepared at various times between 8 and 36 h p.i. as described previously (Chirgwin *et al.*, 1979; Chomczynski & Sacchi, 1987) using reagents from Advanced Biotechnologies. RNAs were denatured in formamide and separated for Northern analysis by electrophoresis on 1·5% agarose gels under denaturing conditions (Sambrook *et al.*, 1989). RNAs were probed using plasmids which were radiolabelled using a random priming kit (Gibco/BRL). RNase protection assays were carried out using a kit (Ambion) and radiolabelled RNA probes. To generate RNA probes corresponding to either potential orientation of pol III transcription products, clones of *Eco*RI–*Sna*BI (542 bp) and *Eco*RI–*Xba*I (531 bp) fragments in pAlter-1 (Promega) (1 µg DNA), corresponding to the left- and right-hand pol III transcribed regions, respectively, were digested with *Pvu*II and transcribed for 60–90 min at 37 °C in a 20 µl reaction volume by T7 or SP6 RNA polymerase (5 U) in the presence of 7·5 µM unlabelled CTP and 25 µCi [^{32}P]CTP (800 Ci/mmol; 10 mCi/ml; NEN Life Science Products) as described in a protocol by Epicentre Technologies. DNA templates were destroyed by incubation with RNase-free DNase I. Total RNA (5 µg) prepared at 36 h p.i. from mock-infected or OAV-infected CSL503 cells was annealed with radiolabelled RNA probes at 42 °C overnight. Annealed samples were divided into four parts. Three were digested with a mixture of pancreatic and T1 ribonucleases at dilutions of 1/50, 1/100 and 1/250, in buffer supplied in the kit. Digested samples were denatured and analysed on polyacrylamide gels as described below. For direct radiolabelling, RNA (20 µg) was digested with calf intestinal alkaline phosphatase which was then inactivated by heat (65 °C, 20 min) and extraction with phenol. After precipitation with ethanol, the RNA was radiolabelled by incubation with T4 polynucleotide kinase and [γ-^{32}P]ATP (10 µCi, 6000 Ci/mmol). RNA was denatured by heating in formamide and analysed by gel electrophoresis on 6% polyacrylamide sequencing gels (Sambrook *et al.*, 1989). Denatured, radiolabelled DNA fragments of known size were used as markers.

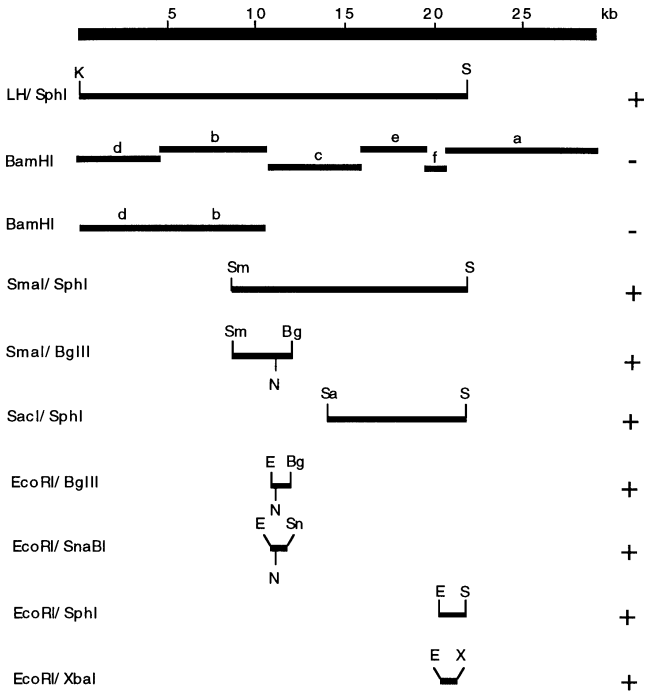

Fig. 1. Summary of OAV genome fragments subcloned and transcribed *in vitro*. The complete sequence of the OAV genome (represented by the top bar) is known (Vrati *et al.*, 1995, 1996*a*, *b*). Subclones of restriction fragments derived from various parts of the genome are depicted. (+) and (−) indicate the presence or absence of a 120 bp product transcribed from the genomic fragments by nuclear extracts of K562 cells containing pol III activity. K and N signify *Kpn*I and *Nco*I sites, respectively.

■ **Radiolabelling and analysis of proteins.** CSL503 cells were left uninfected or were infected with OAV at an m.o.i. of 20–50 p.f.u. per cell. Similarly, 293 cells cultured in MEM F-11 medium plus 10% FCS, were infected with Ad2 at an m.o.i. of 20. At 12–66 h p.i., cells were incubated in methionine/cysteine-free medium for 1 h and then radiolabelled for 1 h with [^{35}S]Translabel (ICN Pharmaceuticals). Cells were lysed in RIPA buffer (100 µl) (Ericson *et al.*, 1982) and a portion of the lysate was analysed by SDS–PAGE in 12% gels.

Results

To search for a VA gene and its product, complementary approaches were used: (i) OAV genome fragments were transcribed *in vitro* by nuclear extracts containing pol III

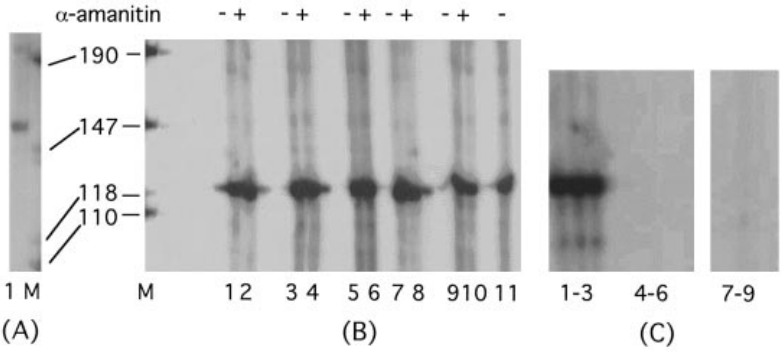

Fig. 2. Transcription of pol III products from Ad2 and OAV genome fragments. Ad2 VA_I RNA (A, lane 1) and OAV fragments (B, lanes 1–11) were transcribed *in vitro* in the presence of [^{32}P]ATP using K562 cell nuclear extracts. Products were denatured and analysed against markers of known size (lanes M) by electrophoresis on a 6% sequencing gel. Ad2 VA RNA_I and OAV products were produced in separate experiments. In (B) DNA template fragments corresponded to: lanes 1 and 2, *Sma*I–*Bgl*II; lanes 3 and 4, *Eco*RI–*Bgl*II; lanes 5 and 6, *Eco*RI–*Sna*BI; lanes 7 and 8, *Eco*RI–*Sph*I; lanes 9 and 10, *Eco*RI–*Xba*I; lane 11, *Sma*I–*Sac*I. In (C) the *Sma*I–*Bgl*II fragment was transcribed before (lanes 1–3) and after (lanes 4–6) digestion with *Nco*I. pAlter-1 plasmid vector (Promega) was transcribed in lanes 7–9.

activity; (ii) pol III-transcribed regions were subcloned and used as probes to analyse total infected cell RNA by Northern analysis and RNase protection assays; and (iii) RNA from infected cells was radiolabelled and analysed directly.

Transcription of OAV genome fragments *in vitro*

To characterize the *in vitro* transcription system, the Ad2 VA RNA_I gene was transcribed by K562 nuclear extracts. As expected, products of ~ 160 and ~ 195 nucleotides were obtained (Fowlkes & Shenk, 1980) (Fig. 2A). Next, OAV genome fragments were transcribed *in vitro* to determine whether any RNA was produced. When the left-hand ~ 21·5 kb *Kpn*I–*Sph*I and the central 12·3 kb *Sma*I–*Sph*I fragments (Fig. 1) were used as templates, a product of ~ 120 bp was detected (data not shown). The transcription product was never seen in control reactions containing plasmid vector only (Fig. 2C, lanes 7–9), nor when any of the *Bam*HI A fragments were used (data not shown). Further subcloning revealed that the transcription product was produced by both the 2·5 kb *Sma*I–*Bgl*II and 8·1 kb *Sac*I–*Sph*I clones (Fig. 2B, lanes 1 and 2, and data not shown), indicating that more than one region of the OAV genome was transcribed by pol III. Additional subcloning showed that the transcription products were derived from a 542 bp *Eco*RI–*Sna*BI fragment and from a 531 bp *Eco*RI–*Xba*I fragment (Fig. 2B, lanes 5 and 6, lanes 9 and 10, respectively). These products were also produced in the presence of α-amanitin (Fig. 2B), indicating transcription by pol III. The left-hand transcribed region fell within the ORF for pIIIa and the right-hand region occurred at the 3′ end of the ORF for 100K hexon assembly where it overlaps the 5′ end of the ORF for 33K protein (Vrati *et al*., 1995, 1996*b*). In addition, as none of the *Bam*HI fragments produced a signal, both transcribed regions were apparently inactivated by *Bam*HI. The signal from the left-hand region was also lost following digestion with *Nco*I (Fig. 2C, lanes 1–3, 4–6). Thus the left-hand transcribed region was located in the vicinity of the *Bam*HI and *Nco*I sites between positions 10964 and 11123 of the genome, respectively. Similarly, the right-hand transcribed region apparently overlapped the *Bam*HI site at base 20860.

Are these regions transcribed during OAV infection?

To determine whether the transcription products produced *in vitro* were representative of pol III RNAs produced during productive infection, clones containing these regions were radiolabelled and hybridized with total RNA prepared at 8 and 24 h p.i. from OAV-infected CSL503 cells. RNA from mock-infected cells was prepared at 24 h. RNAs were probed by Northern hybridization using radiolabelled *Eco*RI–*Sna*BI (left-hand) or *Eco*RI–*Xba*I (right-hand) subclones (Fig. 1). Neither probe hybridized to RNA from uninfected cells (Fig. 3A, lane 3, which was divided vertically) or to RNAs that were present at 8 h p.i. (Fig. 3A, lanes 1 and 4). The left-hand *Eco*RI–*Sna*bI probe hybridized to discrete MLP transcripts of ~ 1·9 and ~ 2·9 kb present at 24 h p.i. and to some more diffuse material near the origin (Fig. 3A, lane 2). The transcripts were of a size predicted from the known sequence and the transcription map of OAV (Vrati *et al*., 1996*a*, *b*; A. Khatri & G. W. Both, unpublished data) and corresponded to the L1 52/55K and pIIIa late mRNAs. Similarly, the right-hand *Eco*RI–*Xba*I probe hybridized to transcripts of ~ 3·1, 1·5 and 1·31 kb (Fig. 3A, lane 5) predicted to correspond to the L4 RNAs for homologues of the 100K hexon assembly protein, 33K protein and pVIII, respectively (Vrati *et al*., 1995; A. Khatri & G. W. Both, unpublished data). However, with either probe the signal obtained from the low molecular mass region of the gel (~ 100–200 bp) was weaker in both cases than that observed for the late mRNAs described above (Fig. 3A, lanes 2 and 5), suggesting that little if any pol III RNA was transcribed from these regions *in vivo* at a time when late transcription was active.

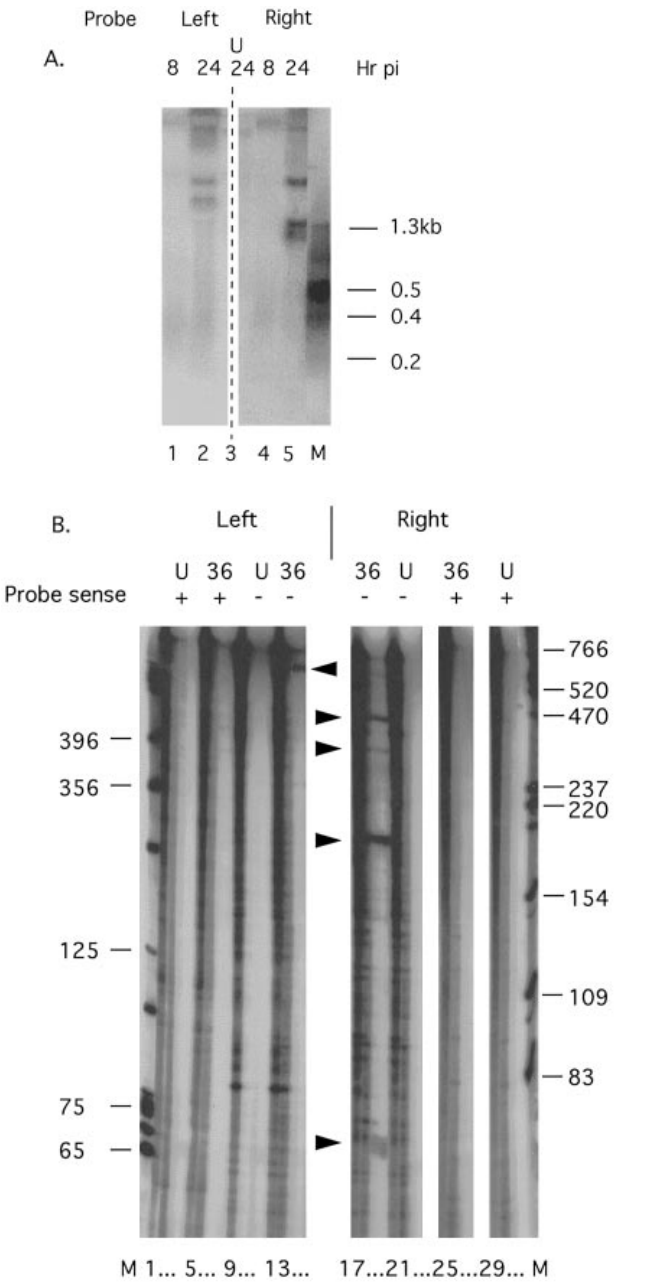

Fig. 3. Analysis of RNAs in mock and OAV-infected CSL503 cells. (A) Total RNA from uninfected and OAV-infected cells was prepared at 8 and 24 h p.i., separated under denaturing conditions by electrophoresis on a 1·5% agarose gel and blotted to a membrane. The lane containing 24 h uninfected cell RNA was cut vertically such that half was available for each probe. The left-hand *Eco*RI–*Sna*BI and right-hand *Eco*RI–*Xba*I subclones containing pol III-transcribed regions were radiolabelled by random priming and hybridized with RNA samples on one half of the membrane. In (B) T7 or SP6 RNA polymerase was used to generate internally radiolabelled (+) or (−) sense probes, respectively, from the left- and right-hand subcloned regions. RNAs were hybridized with total RNA harvested from mock- (U) and OAV-infected cells at 36 h p.i. Annealed RNA was left undigested or was digested with ribonuclease at dilutions of 1/250, 1/100 and 1/50. Protected fragments were analysed on the same DNA sequencing gel. Each set of four samples was loaded from left to right in the following order: undigested; + RNase 1/250; 1/100; 1/50. The position of end-labelled, denatured DNA markers of known size is indicated. Arrowheads indicate the protected fragments.

These results were confirmed using a more sensitive RNase protection assay. RNA probes corresponding to both orientations of the *Eco*RI–*Sna*BI (left-hand; +, 784, −, 681 nucleotides in length, respectively) and *Eco*RI–*Xba*I (right-hand; +, 755, −, 652 nucleotides) subclones were produced. These were annealed with total RNA harvested at 36 h p.i. from mock- and OAV-infected CSL503 cells. Plus- and minus-sense RNA probes derived from either the left- or right-hand regions failed to protect any products from RNase digestion following hybridization with RNA from mock-infected cells (Fig. 3B, lanes 1–4, 9–12, 21–24, 29–32). In contrast, each minus-sense probe protected fragments which were expected from the transcription map of the OAV genome. The left-hand (−) probe protected a ∼ 540 nucleotide fragment expected as part of the transcripts for p52K and pIIIa (Fig. 3B, lanes 13–16). The right-hand (−) probe protected several fragments between ∼ 450 and ∼ 60 nucleotides in length (Fig. 3B, lanes 17–20) derived from the transcript encoding 100K hexon assembly protein and previously characterized spliced RNAs encoding the 33K and pVIII proteins (Vrati *et al.*, 1995). However, no probe of (+) or (−) sense protected any RNA fragment of ∼ 120 nucleotides in size (Fig. 3B, lanes 5–8, 13–16, 17–20, 25–28), confirming that pol III RNA products synthesized *in vitro* were not detectably transcribed from these regions of the genome during permissive OAV infection.

Direct analysis of RNAs

To determine whether VA-like RNAs might be derived from other regions of the genome during permissive infection, total RNA was prepared from OAV-infected and uninfected CSL503 cells at 12–36 h p.i., treated with alkaline phosphatase, radiolabelled at the 5′ end by polynucleotide kinase and analysed by gel electrophoresis. For mock-infected cells, prominent RNAs of ∼ 150, ∼ 120 and 75–83 nucleotides were observed (Fig. 4, lanes 1, 3 and 5). These probably corresponded to ovine 5.8S rRNA, 5S RNA and various tRNA species, respectively. Very similar profiles of low molecular mass RNAs were observed in OAV-infected cells at the same time-points (Fig. 4, lanes 2 and 4), although the 154 nucleotide species increased marginally at 36 h p.i. (lane 6). However, no RNA species increased greatly in amount during infection in a manner similar to that described for Ad2 (Soderlund *et al.*, 1976).

Kinetics of protein synthesis

Ad5 replication is aided by the production of VA RNAs which enhance the synthesis of virus proteins relative to host proteins (Mathews & Shenk, 1991). Thus when 293 cells were infected with Ad5 at an m.o.i. of 20 p.f.u. per cell and radiolabelled at 24 h p.i., viral proteins dominated the nascent protein profile (Fig. 5A, lane 1) as expected (Reichel *et al.*, 1985). In contrast, CSL503 cells infected with OAV at an m.o.i. of 20 p.f.u. per cell showed complete cytopathic effect only

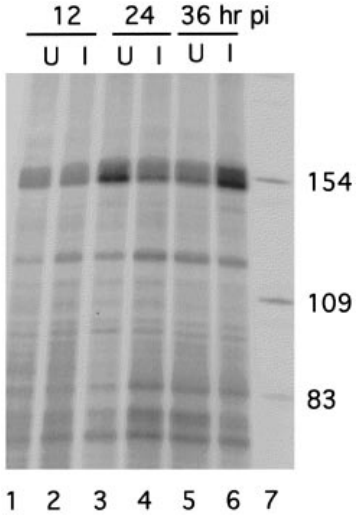

Fig. 4

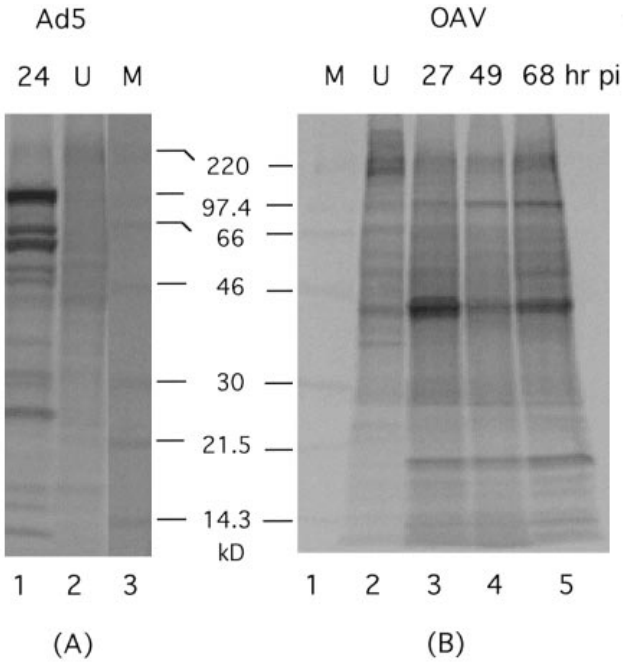

Fig. 5

Fig. 4. Analysis of low molecular mass RNAs. Total RNA was prepared at various times p.i. from uninfected (U) and OAV-infected (I) CSL503 cells, treated with calf intestinal phosphatase, 5′-radiolabelled with [γ-^{32}P]ATP, denatured in formamide and separated by electrophoresis on a 6% DNA sequencing gel.

Fig. 5. Nascent protein synthesis in adenovirus-infected cells. (A) Human 293 or (B) ovine CSL503 uninfected and infected cells were radiolabelled with [^{35}S]methionine at various times p.i., as indicated. For OAV, uninfected cells were labelled at the same time as the 68 h infected cells. Proteins were analysed under denaturing conditions by SDS–PAGE on a 12% Tris–glycine gel. The position of standard marker proteins (M) is indicated.

after 3–4 days (data not shown). Although OAV proteins of ~ 97, 43 and 20 kDa were detected by radiolabelling at 27–68 h p.i. (Fig. 5B, lanes 3–5), virus polypeptide synthesis did not dominate nascent protein synthesis, i.e. the preferential translation of OAV proteins was inefficient compared with Ad5 infection in 293 cells. This observation is also consistent with the apparent lack of a VA RNA gene in the OAV genome.

Discussion

The importance of VA RNA function in adenovirus replication is reflected in the fact that all known adenovirus genomes have at least one, and usually two VA RNA genes (Larsson *et al.*, 1986; Kidd & Tiemessen, 1993; Ma & Mathews, 1993). Thus it was anticipated that OAV would also have a VA RNA gene. For the mastadenoviruses, VA RNAs are located between the 52/55K and pTP ORFs and transcribed rightwards. In CELO, an aviadenovirus, a single shorter gene is transcribed leftwards. It is now known to be located towards the right-hand end of the genome (Chiocca *et al.*, 1996). As the OAV VA RNA gene(s) were unlikely to be located in the region of the 52/55K and pTP ORFs, as these overlap (Vrati *et al.*, 1996*a*), the situation in CELO virus suggested that an alternative location was possible.

For CELO virus the approximate location of the VA gene was determined using pol III-containing nuclear extracts to transcribe genomic clones *in vitro* (Larsson *et al.*, 1986). Using this approach for OAV, an α-amanitin-resistant transcription product of ~ 120 bp was mapped to two ~ 500 bp regions which were widely separated in the genome. The left-hand region fell within the ORF encoding pIIIa while the right-hand region included portions of both the 100K and 33K ORFs where these overlapped (Vrati *et al.*, 1995). As VA RNA genes in other adenoviruses occupy exclusive coding regions in the genome, the location of these pol III-transcribed regions in the OAV genome raised doubts as to their authenticity.

Recognizing that the pol III-transcribed regions must contain recognizable promoter elements, an attempt was made to identify these within the ~ 500 bp subclones of OAV. The consensus promoter sequences (A and B boxes) for pol III recognition have previously been characterized for various genes (Fowlkes & Shenk, 1980) and for human and simian adenoviruses (Ma & Mathews, 1993; Kidd *et al.*, 1995). For CELO virus VA RNA, only minimal similarity is shared in the region of the B box (Larsson *et al.*, 1986; Ma & Mathews, 1993). The element GGTTC is highly conserved in the B box of many pol III-transcribed genes, but GGaTC is allowed in some cases (Fowlkes & Shenk, 1980; Bandea *et al.*, 1992; Ma & Mathews, 1993; Kidd *et al.*, 1995). Within the left-hand OAV

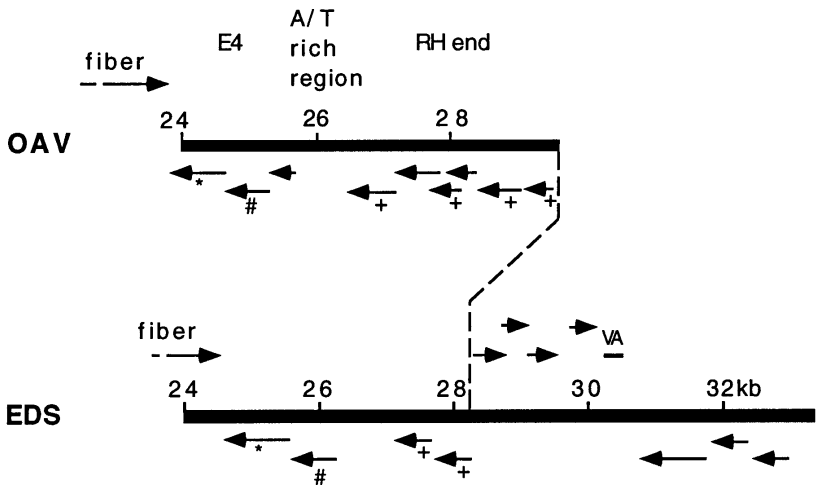

Fig. 6. Comparison of OAV and EDS virus genomes near their the right-hand ends. The EDS data were taken from GenBank (accession no. Y09598; P. Brandt, H. Bloecker & M. Hess, unpublished data). The E4, A/T-rich and right-hand end regions in OAV have counterparts in EDS. ORFs which show homology within (Xu *et al.*, 1997) and across genomes are indicated by the same symbol. Note that the EDS VA RNA gene occurs beyond the boundary of the OAV genome.

region the sequence GGTTC occurs only once for both orientations, 50 nucleotides upstream of the *Nco*I site at position 11073. Potential T-rich transcription termination sequences occur 33 and 45 nucleotides downstream of the *Nco*I site. This would place the 5′ end of the 120 bp transcript ~ 70 nucleotides downstream of the *Bam*HI site. Alternatively, if the *Bam*HI site itself constituted the B box, i.e. GGaTC, transcription could occur in either orientation. Leftward transcription would leave the *Nco*I site in a distant position relative to the transcript. Rightward transcription would place the *Nco*I site ~ 55 bp 3′ to a possible termination signal. On balance, it would seem more likely that transcription might occur in a rightward direction, from position 11037 to ~ 11160 or from ~ 10907 to 11030.

For the right-hand region, the closest match to the B box element occurs in the *Bam*HI site at 20860. However, considering the location of possible transcription terminators, transcription is unlikely to occur leftwards as there are several T runs nearby. For rightward transcription incorporating the *Bam*HI element there are no T-rich termination sequences for ~ 200 nucleotides unless a TCCTCT sequence at position 20906 can suffice. This would place the start site for transcription at ~ 20786. The transcribed regions have not been defined more accurately because it is thought that they do not reflect events which occur during OAV infection, as discussed below.

We initially sought to determine whether the *in vitro*-transcribed pol III products were produced from the viral genome during infection by using clones of these regions as DNA probes in a Northern analysis of total infected cell RNA. While expected transcripts of ~ 1·3–3·1 kb were detected, corresponding to L1 and L4 late mRNAs (based on the OAV transcription map: A. Khatri & G. W. Both, unpublished), in the low molecular mass region of the gel only a weak signal was obtained. This was marginally stronger than that seen in uninfected cells and significantly weaker than the signal for the L1/L4 transcripts (Fig. 3A). In a separate analysis, internally radiolabelled RNA probes derived from the plus and minus strands of the subcloned pol III transcribed regions were used to search for complementary RNA species by an RNase protection assay. This approach was related to the S1 nuclease mapping strategy used successfully for the identification of CELO virus VA RNA, when end-labelled DNA probes were employed (Larsson *et al.*, 1986). The expected fragments derived from late mRNAs were identified by the minus sense probes in RNA derived from OAV-infected cells at 36 h p.i. However, no RNA species of ~ 120 nucleotides was detected with a probe of either sense (Fig. 3B), confirming that the regions transcribed *in vitro* were not detectably transcribed during infection, even at a time when late protein synthesis was well advanced.

Finally, total RNA from OAV-infected cells was analysed directly by 5′ radiolabelling. This approach also failed to identify any prominent low molecular mass RNA species which were not also seen in uninfected cells. These observations contrast with Ad2 infection of HeLa cells where VA RNA species increased dramatically in amount during infection (Soderlund *et al.*, 1976). Collectively, therefore, the data suggest that VA RNAs are not detectably transcribed from the OAV genome. However, the possibility cannot be excluded that pol III RNAs lacking homology with other known products are transcribed in minor amounts during infection, and obscured during gel analysis by RNA species which are also present in uninfected CSL503 cells (Fig. 4). In any event, if any VA-like RNA was synthesized during OAV infection, it did not enhance the synthesis of viral polypeptides relative to host proteins as shown by radiolabelling of nascent proteins (Fig. 5B).

The recent determination of the complete sequence of the EDS virus (Hess *et al.*, 1997) has revealed features which are consistent with the above data. Phylogenetic analysis of adenovirus protease genes shows that OAV and EDS, together with BAV-7, represent a new and distinct adenovirus group (Harrach *et al.*, 1997). This grouping is

supported by a more extensive comparison of the EDS-76 and OAV sequences and by their genome arrangements. It is clear that over the first ~ 25 kb of its genome, almost every ORF of OAV has a homologue in EDS (not shown). Differences emerge towards the right-hand end where OAV is truncated relative to the EDS genome (Fig. 6). Importantly, the VA RNA gene, which was identified in EDS virus on the basis of its homology with CELO virus VA RNA (Larsson *et al.*, 1986; Chiocca *et al.*, 1996), lies between two ORFs in a region of EDS which is outside the region of similarity between OAV and EDS (Fig. 6). Collectively, therefore, the data indicate that OAV is a unique virus in that it does not contain a VA RNA gene.

The absence of a VA RNA gene has implications for the future use of OAV as a gene therapy vector. Known OAV promoters are not detectably active in some human cell types (Khatri *et al.*, 1997). Thus in the absence of a pol III-transcribed VA gene, there may be minimal production of double-stranded RNA following infection of some cell types. It remains to be determined whether this might be beneficial for achieving persistent gene expression in these cell types.

The authors wish to thank Dr P. Molloy for the pUC/VARNA$_I$ plasmid and for reviewing the manuscript.

References

Babiss, L., Ginsberg, H. & Darnell, J. E., Jr (1985). Adenovirus E1B proteins are required for accumulation of late viral mRNA and for effects on cellular mRNA translation and transport. *Molecular and Cellular Biology* **5**, 2552–2558.

Bandea, C., Wu, M. & Wu, G. (1992). Adenovirus VARNA1 gene B block promoter element sequences required for transcription and for interaction with transcription factors. *Journal of Molecular Biology* **227**, 1068–1085.

Beltz, G. & Flint, S. (1979). Inhibition of HeLa cell protein synthesis during adenovirus infection. Restriction of cellular messenger RNA sequences to the nucleus. *Journal of Molecular Biology* **131**, 353–373.

Borellini, F., He, Y., Aquino, A., Yu, G., Josephs, S. & Glazer, R. (1991). Increased DNA binding and transcriptional activity associated with transcription factor Sp1 in K562 cells transfected with the myeloid-specific c-*fes* tyrosine kinase gene. *Journal of Biological Chemistry* **266**, 15850–15854.

Boyle, D. B., Pye, A. D., Kockerhans, R., Adair, B. M., Vrati, S. & Both, G. W. (1994). Characterisation of Australian ovine adenovirus isolates. *Veterinary Microbiology* **41**, 281–291.

Chiocca, S., Kurzbauer, R., Schaffner, G., Baker, A., Mautner, V. & Cotten, M. (1996). The complete DNA sequence and genomic organization of the avian adenovirus CELO. *Journal of Virology* **70**, 2939–2949.

Chirgwin, J., Przybyla, A., MacDonald, R. & Rutter, W. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162**, 156–159.

Ericson, B. L., Graham, D. Y., Mason, B. B. & Estes, M. K. (1982). Identification, synthesis, and modifications of simian rotavirus SA11 polypeptides in infected cells. *Journal of Virology* **42**, 825–839.

Fowlkes, D. & Shenk, T. (1980). Transcriptional control regions of the adenovirus VAI RNA gene. *Cell* **22**, 405–413.

Harrach, B., Meehan, B. M., Benko, M., Adair, B. M. & Todd, D. (1997). Close phylogenetic relationship between egg drop syndrome virus, bovine adenovirus serotype 7, and ovine adenovirus strain 287. *Virology* **229**, 302–306.

Hess, M., Blöcker, H. & Brandt, P. (1997). The complete nucleotide sequence of the egg drop syndrome virus: an intermediate between mastadenoviruses and aviadenoviruses. *Virology* **238**, 145–156.

Horwitz, M. S. (1990). Adenoviridae and their replication. In *Fields Virology*, pp. 1679–1721. Edited by B. N. Fields & D. M. Knipe. New York: Raven Press.

Khatri, A., Xu, Z. Z. & Both, G. W. (1997). Gene expression by atypical recombinant ovine adenovirus vectors during abortive infection of human and animal cells in vitro. *Virology* (in press).

Kidd, A. H. & Tiemessen, C. T. (1993). Characterization of a single SA7-like VA-RNA gene in subgroup-F adenoviruses. *Journal of General Virology* **74**, 1621–1626.

Kidd, A. H., Garwicz, D. & Oberg, M. (1995). Human and simian adenoviruses: phylogenetic inferences from analysis of VA RNA genes. *Virology* **207**, 32–45.

Larsson, S., Bellett, A. & Akusjarvi, G. (1986). VA RNAs from avian and human adenoviruses: dramatic differences in length, sequence and gene location. *Journal of Virology* **58**, 600–609.

Ma, Y. L. & Mathews, M. B. (1993). Comparative analysis of the structure and function of adenovirus virus-associated RNAs. *Journal of Virology* **67**, 6605–6617.

Ma, Y. L. & Mathews, M. B. (1996). Secondary and tertiary structure in the central domain of adenovirus type 2 VA RNA(I). *RNA* **2**, 937–951.

Mathews, M. (1975). Genes for VA-RNA in adenovirus 2. *Cell* **6**, 223–229.

Mathews, M. & Shenk, T. (1991). Adenovirus virus-associated RNA and translation control. *Journal of Virology* **65**, 5657–5662.

O'Malley, R., Duncan, R., Hershey, J. & Mathews, M. (1989). Modification of protein synthesis initiation factors and the shut-off of host protein synthesis in adenovirus-infected cells. *Virology* **168**, 112–118.

Pe'ery, T., Mellits, K. H. & Mathews, M. B. (1993). Mutational analysis of the central domain of adenovirus virus-associated RNA mandates a revision of the proposed secondary structure. *Journal of Virology* **67**, 3534–3543.

Pilder, S., Moore, M., Logan, J. & Shenk, T. (1986). The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Molecular and Cellular Biology* **6**, 470–476.

Prywes, R. & Roeder, R. (1986). Inducible binding of a factor to the c-*fos* enhancer. *Cell* **47**, 777–784.

Reichel, P. A., Merrick, W. C., Siekierka, J. & Mathews, M. (1985). Regulation of a protein synthesis initiation factor by adenovirus-associated RNA$_I$. *Nature* **313**, 196–200.

Roberts, R. J., O'Neill, K. E. O. & Yen, C. T. (1984). DNA sequences from the adenovirus 2 genome. *Journal of Biological Chemistry* **259**, 13968–13975.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Shapiro, D., Sharp, P., Wahli, W. & Keller, M. (1988). A high-efficiency HeLa cell nuclear transcription extract. *DNA* **7**, 47–55.

Soderlund, H., Pettersson, U., Vennstrom, B., Philipson, L. & Mathews, M. B. (1976). A new species of virus-coded low molecular weight RNA from cells infected with adenovirus type 2. *Cell* **7**, 585–593.

Vrati, S., Boyle, D., Kocherhans, R. & Both, G. W. (1995). Sequence of ovine adenovirus homologs for 100K hexon assembly, 33K, pVIII, and fiber genes: early region E3 is not in the expected location. *Virology* **209**, 400–408.

Vrati, S., Brookes, D. E., Boyle, D. B. & Both, G. W. (1996*a*). Nucleotide sequence of ovine adenovirus tripartite leader sequence and homologues of the IVa2, DNA polymerase and terminal proteins. *Gene* **177**, 35–41.

Vrati, S., Brookes, D. E., Strike, P., Khatri, A., Boyle, D. B. & Both, G. W. (1996*b*). Unique genome arrangement of an ovine adenovirus: identification of new proteins and proteinase cleavage sites. *Virology* **220**, 186–199.

Weinmann, R., Brendler, T., Raskas, H. & Roeder, R. (1976). Low molecular weight viral RNAs transcribed by RNA polymerase III during adenovirus 2 infection. *Cell* **7**, 557–566.

Wu, G. J. (1978). Adenovirus DNA-directed transcription of 5.5S RNA *in vitro*. *Proceedings of the National Academy of Sciences, USA* **75**, 2175–2179.

Xu, Z. Z., Hyatt, A., Boyle, D. B. & Both, G. W. (1997). Construction of ovine adenovirus recombinants by gene insertion or deletion of related terminal region sequences. *Virology* **230**, 62–71.

Received 16 September 1997; Accepted 27 October 1997