A mutant of canine adenovirus type 2 with a duplication of the E1a region exhibits altered expression of early region 4

Heather M. A. Cavanagh,† Carina F. Gallagher and Norman Spibey*

Department of Veterinary Pathology, University of Glasgow Veterinary School, Bearsden Road, Glasgow, U.K.

The genomic DNA of a vaccine strain of canine adenovirus type 2 (Vaxitas; ICI Tasman) has been shown to contain two copies of the E1a region, the second being at the far right end of the genome. DNA sequence analysis of the right terminal 2.8 kbp of this vaccine strain showed that numerous point mutations have occurred in the second copy, which would preclude the synthesis of any functional products. However, expression vectors in which the E1a promoter from the right terminus were linked to the chloramphenicol acetyltransferase gene showed that the promoter was fully functional. Furthermore, the activity of the reiterated E1a promoter was considerably greater than that of the normal E4 promoter. This dramatic change in the regulation of E4 expression may be an important factor in determining the altered host cell specificity displayed by this vaccine strain virus.

Introduction

Canine adenovirus type 2 (CAV-2) is a typical member of the Adenoviridae (Spibey & Cavanagh, 1989; Spibey et al., 1989), and has been associated with respiratory and possibly enteric disease in the dog (Ditchfield et al., 1962; Hamelin et al., 1984, 1986; Macartney et al., 1988). Laboratory attenuated strains of the virus are routinely administered to young dogs as a live vaccine against infectious canine hepatitis and respiratory disease (Koptopoulos & Cornwell, 1981).

The CAV-2 genome consists of a linear dsDNA molecule 32.2 kb in length (Spibey & Cavanagh, 1989). Studies with human adenoviruses have shown that viral DNA replication initiates within the inverted terminal repeat (ITR) and requires cooperative interaction between nuclear factor 1 and the virus-encoded DNA-binding protein (De Vries et al., 1985; Cleat & Hay, 1989). The E2b-encoded pre-terminal protein also participates in the initiation of viral DNA replication by forming a complex with the adenovirus-encoded DNA polymerase and becoming covalently linked to dCMP, which then serves as the primer for chain elongation (Stillman et al., 1981; Lichy et al., 1982).

Genomic variants of both human and canine adenoviruses have been reported in which the alterations result in multiple reiterations within the ITRs (Schwartz et al., 1982; Larsen & Tibbetts, 1985; Sira et al., 1987; Salewski et al., 1989). The mechanisms proposed for the origin of these tandem repeats suggest that a direct repeat of part of the ITR already present at one end of the genome can go through a series of duplications as a result of (inaccurate) panhandle formation by the displaced strand during DNA replication. Mutants of adenovirus types 2 and 5 which possess copies of left end sequences inserted at the right end have also been isolated (Brusca & Chinnadurai, 1983; Hanahan & Gluzman, 1984). However, these mutants, which were either unstable or constructed in vitro, retained the right-hand ITR within the genome. Although the mechanisms responsible for the formation of these rearrangements may be unclear, it does appear that repeated high multiplicity passage of the virus leads to the formation of some of these aberrant forms. What is not clear is the effect on DNA replication and expression of the E1 and E4 regions which border the ITRs.

Here we describe genomic rearrangements in a vaccine strain of CAV-2 which resulted in a duplication of the entire E1a region at the right end of the genome, with concomitant loss of right terminal sequences. DNA sequence analysis together with E1a and E4 promoter–chloramphenicol acetyltransferase (CAT) constructions have been used to assess the effect of this duplication on expression of the E4 region in this vaccine strain.
Methods

Cell lines and viruses. Virus strains were propagated as previously described (Spibey & Cavanagh, 1989); the feline isolate, Glasgow CAV-2, was propagated in the dog MDCK cell line and the vaccine strain, Vaxitas CAV-2, (ICI Tasman) in the mink Mv-l-Lu cell line. The A72 canine tumour and the NBL-7 mink cell lines were grown in Dulbecco's modification of Eagle's medium containing 10% foetal calf serum. Viral and plasmid DNAs were prepared as described previously.

Restriction enzyme analysis. Restriction endonucleases were purchased from Pharmacia and used according to the manufacturer's specifications. Restriction enzyme digests were subjected to electrophoresis on a 0.8% agarose gel, prepared and run in Tris-borate-EDTA buffer.

Double-stranded deoxyribonucleotide chain termination sequencing. Deoxyribonucleotide chain termination sequencing was carried out on dsDNA using the method of Sanger et al. (1977) with deoxyadenosine 5'-triphosphate triethoxythionin salt (Sp isomer; Amersham) being used as the radioactive label throughout. Primers SK, KS, T3 and T7 were purchased from Northumbria Biologicals and used at a final concentration of 2.5 ng/μl. Additional primers were synthesized within the laboratory on an Applied Biosystems 381A DNA synthesizer and used at a concentration of 5 ng/μl. Polyacrylamide sequencing gels were run on Bio-Rad gel systems at 56 °C. Gels were fixed in 10% methanol/10% acetic acid for 15 min before being dried onto Whatman 3MM paper and subjected to autoradiography overnight.

Construction of recombinant plasmids. Plasmid pCAT-12 (Spandidos & Riggio, 1986) consists of the CAT gene inserted between the BamHI and XbaI sites of pUC12 and was the vector used for subsequent cloning. The vector was cut with Smal, phosphatase-treated, ethanol-precipitated, resuspended in Tris-EDTA and stored at −20 °C until use. The terminal SalI clones of Vaxitas (V27) and Glasgow CAV-2 (G11) (Spibey & Cavanagh, 1989) were then digested with SsrI, producing two blunt-ended fragments of 1.6 kb and 0.5 kb, respectively, to be cloned into the pCAT-12 vector. The viral DNA fragments were purified from an agarose gel by electroelution and ligated into the Smal-cut vector. The orientation of the viral fragments with respect to the CAT gene was confirmed by restriction enzyme analysis. The plasmid carrying the Vaxitas E4 and reiterated E1 promoters fused to the CAT gene is pVE1a/CAT; similarly carrying the Glasgow CAV-2 E-4 promoter linked to the CAT gene is pGE4/CAT. Blunt-ended fragments containing the Glasgow E2 and E3 promoters were cloned into the Smal site of pCAT-12 (pGE2/CAT and pGE3/CAT respectively) to be used both for comparison and as controls (N. Spibey et al., unpublished results). The EcoRI C fragment of Glasgow CAV-2, which contains the E1 region (pGRIC) was cloned into the plasmid vector Bluescript (Stratagene) and utilized in cotransfection experiments, demonstrating that the early promoters were transactivated (Spibey & Cavanagh, 1989).

Transfections for CAT assay. Transfections were carried out by the calcium phosphate precipitation method (Graham & van der Eb, 1973). Mv-l-Lu cells were split 1:10 and allowed to settle at 37 °C overnight. Donor DNA (0.25 ml; pCAT12 clones) at a concentration of 80 μg/ml in one-tenth TE buffer (0.1 M-EDTA, 10 mM-Tris-HCl pH 8.0) was placed into a sterile bijou bottle, 0.2 ml one-tenth TE and 0.05 ml of 2.5 M-CaCl2 were added, and the solution was mixed well. The DNA solution was then slowly added, with continuous mixing, to 0.5 ml 2 × HBS (50 mM-HEPES, 280 mM-NaCl, 1.5 mM-Na2HPO4, adjusted to pH 7.1) and mixed thoroughly; this mixture was left at room temperature for 30 min.

The DNA/calcium phosphate suspension was added to a 75 cm2 flask which was then incubated overnight at 37 °C to allow adsorption. The medium containing the DNA precipitate was then removed and replaced with fresh medium.

CAT assay. Cells were harvested 48 h post-transfection and the cell extract was assayed for CAT activity as described by Gorman et al. (1982) with slight modifications. Briefly, the enzyme assay was performed in a final volume of 155 μl containing 75 μl of cell extract, 2.5 μl of 40 mM-acetyl coenzyme A (sodium salt; Sigma), 2.5 μl (0.1 μCi) of [14C]chloramphenicol and 75 μl 250 mM-Tris-HCl pH 7.8. The mixture was incubated at 37 °C for 60 min and stopped by the addition of 100 μl ethyl acetate. Chloramphenicol was extracted from the mixture by two treatments with 400 μl of ethyl acetate, which was then evaporated by centrifugation under vacuum.

The chloramphenicol sample was re-dissolved in 30 μl of ethyl acetate and spotted onto a plastic-backed silica thin-layer chromatography plate in 5 μl aliquots. The unreacted chloramphenicol was separated from the 1C-acetylated, 3C-acetylated and the 1C,3C-diacylated forms by ascending chromatography in chloroform:methanol (95:5). The plate was air-dried and subjected to autoradiography overnight.

Results

Restriction enzyme analysis of cloned viral DNA fragments

The two terminal SalI clones, G11 (Glasgow) and V27 (Vaxitas) (Spibey & Cavanagh, 1989), were analysed by restriction endonuclease digestion to locate more precisely the additional 1-0 kb of DNA in Vaxitas. A total of 16 enzymes were used to obtain a detailed restriction map of the right terminal SalI fragments (Fig. 1). These data showed that the position of the SalI site (0-2 kb from the right-hand terminus) is retained in Vaxitas, whereas other sites, such as the PvuII site 1-1 kb from the right terminus in the Glasgow strain, have been displaced to the left. These restriction enzyme data were used, together with DNA sequence information, to decide which would be the most appropriate fragments to clone into the CAT expression plasmids.

DNA sequence analysis of the right-hand terminus of Glasgow and Vaxitas CAV-2

Sequence data were most easily obtained by using both commercially available and custom synthesized primers. The DNA sequences were aligned to reveal the position at which the Glasgow and Vaxitas sequences diverged. The DNA sequences of the ITRs from the Glasgow and Toronto CAV-2 isolates have been reported to be 197 and 196 bp in length (Spibey et al., 1989; Shinigawa et al., 1987). It was clear from this alignment that the Vaxitas and Glasgow sequences diverged immediately after the end of the ITR, the only difference in the ITRs being a single base change at position 27.
CAV-2 duplication mutant

Fig. 1. Detailed restriction endonuclease cleavage maps of the right terminal SalI fragments from the Glasgow (a) and Vaxitas (b) strains of CAV-2. The dotted lines show the size and position of the modifications in Vaxitas.

The DNA sequence of the Vaxitas insertion was compared with sequences in the EMBL and GenBank databases and found to be an almost exact duplication of the CAV-2 Ela region (Spibey et al., 1989; Shibata et al., 1989); an example alignment with the Glasgow Ela region is shown in Fig. 2. A number of single base pair differences were apparent in the Vaxitas Ela region compared to that from the Glasgow isolate. Translation of the reiterated Ela sequence data showed that these four mismatched bases give rise to significant changes in the open reading frames (ORFs).

Further analysis of the Vaxitas terminal sequence also revealed that there is a 28 bp stretch of DNA present in the Glasgow isolate E4 region which is missing from the Vaxitas strain. The loss of this 28 bp sequence appears to have occurred at the site of insertion of the reiterated Ela copy (Fig. 3).

Transfection with CAT plasmids

To assess the effect of the Vaxitas insertion on expression of the E4 region, virus DNA–CAT clones were used both singly and in conjunction with others in transient transfection experiments. Both viral SalI B clones yielded three fragments upon digestion with SstII (Fig. 1); the Glasgow SalI B clone gave fragments of 0.2 kb, 0.5 kb and approximately 6.0 kb, whereas the Vaxitas SalI B clone gave fragments of 0.2 kb, 1.6 kb and approximately 6.0 kb. Therefore the Vaxitas Ela insert was contained totally within the 1.6 kb SstII fragment and it was predicted that the Glasgow CAV-2 E4 regulatory region would be carried on the 0.5 kbp SstII fragment. These fragments were therefore cloned into the pCAT-12 vector to bring the CAT gene under the control of the Ela and E4 promoters. Transfections were performed using the mink cell line Mv-1-Lu, to which Vaxitas has become adapted, and the canine cell lines MDCK and A72. Transfections also were performed using a foetal mink lung cell line, NBL-7, that does not support CAV-2 replication (data not shown).

The data presented in Fig. 4 demonstrate that CAT expression driven by the CAV-2 (Glasgow) E4 promoter is minimal in Mv-1-Lu and A72 cells transfected with this plasmid alone. However, when cotransfected into cells with a cloned viral fragment expressing the CAV-2 E1 proteins (Glasgow EcoRI C fragment; Spibey & Cavanagh, 1989), the E4 promoter was activated. It was also clear that the 1.6 kbp SstII fragment containing the Vaxitas insertion carries a strong promoter able to direct expression of the CAT gene, which is further stimulated by cotransfection with the Glasgow E1 clone, or by transfection into CAV-infected cells. Similar results were obtained when MDCK cells were transfected, but transfected NBL-7 cells did not produce such high levels of CAT activity (data not shown). These data showed that in a transient transfection assay, even when fully trans-activated, the maximal level of activity of the wild-type Glasgow E4 promoter is far less than that of the Vaxitas Ela/E4 promoter. Plasmids pGE2/CAT and pGE3/CAT, containing the promoters of early regions 2 and 3 coupled to the CAT gene (unpublished results), are included to show the relative activity of the early promoters.

To investigate the possibility that functional products were expressed from the duplicated Ela gene, the Vaxitas SalI B clone was cotransfected with the CAV-2 (Glasgow) E4-CAT plasmid. Whereas trans-activation of the E4 promoter occurs with the Glasgow E1 clone, no trans-activation of the E4 promoter was discernible with the Vaxitas SalI B clone containing the reiterated Ela (data not shown).

Discussion

Adenovirus DNA replication originates in the ITR and involves cooperative interactions between viral and
Fig. 2. Nucleotide sequence of the right-hand terminus of the Vaxitas isolate (top line) aligned with that of the Glasgow left-hand ITR and Ela region (bottom line). The sequences are aligned with the terminal bases of the two genomes numbered 1. Base pair differences are indicated. The SstII site within the ITR is underlined and other features are described below the sequence.

cellular proteins (van der Vliet et al., 1977; De Vries et al., 1985; Cleat & Hay, 1989). It has been postulated that during the replication process the displaced DNA strand is able, by virtue of the ITRs, to form a panhandle structure (Daniell, 1976; Rekosh et al., 1977). This panhandle structure would in turn become a viable template for DNA replication. There have been numerous reports (Schwartz et al., 1982; Larsen & Tibbetts, 1985; Larsen et al., 1986; Sira et al., 1987) of adenovirus mutants in which the genomes have tandem reiterations within the ITR. Some of these describe adenoviruses which have enhanced growth properties, presumably as a result of having additional copies of the replication origin. The mechanisms put forward to account for these
short reiterations (Haj-Ahmad & Graham, 1986; Sira et al., 1987) suggest that inaccurate panhandle formation could lead to a direct repeat at either end being copied to the other end, and this process could occur many times, resulting in an amplification of the original single repeat.

Brusca & Chinnadurai (1983) describe the isolation of a proflavin-induced mutant with a 1.1 kbp sequence from the left end of the genome duplicated at the right end. This mutant has a low but significant reversion rate, wild-type virus being detectable after five passages, and retains all but 10 bp of the original right-hand ITR, which is embedded between the E4 region and the duplicated sequences. Hanahan & Gluzman (1984) have shown that it is possible to construct viral genomes in vitro which carry left end sequences duplicated at the right end; transfection with this DNA produces stable infectious virus. These viruses again contain the original right end ITR embedded in the genome.

We describe a duplication of the Ela region of a vaccine strain of CAV-2. Unlike the left end reiterations hitherto described in adenovirus types 2 and 5, the event which occurs in the Vaxitas genome results in the deletion of the original right end ITR. That the ITR at the right end originates from the left end is suggested by the single base difference at base pair 27 between right end ITRs of the Vaxitas and Glasgow isolates. The left end ITR of both Glasgow and Vaxitas has a C at this position (Spibey et al., 1989; unpublished results). The junction between the ITR and the reiterated Ela region corresponds perfectly with that at the left end; if the ITR at the right end was the original then the insertion of the left end sequence would be expected to result in some small deletion, as has occurred between the reiterated Ela and E4 regions.

Proteins predicted to be encoded by the reiterated Ela at the right end of the Vaxitas genome would comprise one severely truncated form (65% of the wild-type Ela ORF1 plus three short ORFs. It is thought that the trans-activating activity of Ela is associated with the metal-binding domain within the putative zinc finger near the C terminus of Ela ORF1 (Culp et al., 1988). This region is no longer part of the major protein encoded by the

Fig. 3. The 28 bp deletion at the junction of the reiterated Ela gene and the E4 region in Vaxitas CAV-2. The ITR sequences are underlined. Sequences common to the Glasgow and Vaxitas strains are shown in bold and the 28 bp of the E4 sequence absent from Vaxitas is shown in italics.

Fig. 4. Results of CAT assays comparing the activity of the reiterated Ela promoter in Vaxitas with the E4 promoter of CAV-2 (Glasgow). (a) transfection of Mv-1-Lu cells with pVEla(r)/CAT (lane 1), pVEla(r)/CAT and pGRIC (lane 2), pGE4/CAT (lane 3), and pGE4/CAT and pGRIC (lane 4). (b) Transfection of A72 cells with pVEla(r)/CAT (lane 1), pVEla(r)/CAT and pGRIC (lane 2), pGE4/CAT (lane 3), pGE4/CAT and pGRIC (lane 4), pGE3/CAT (lane 5), pGE3/CAT and pGRIC (lane 6), pGE2/CAT (lane 7), and pGE2/CAT and pGRIC (lane 8). Plasmids pGE2/CAT and pGE3/CAT contain the promoters of early regions 2 and 3, respectively, from Glasgow CAV-2 coupled to the CAT gene. These results are included to show the relative activity of the early promoters.
Vaxitas reiterated Ela. Thus, it would appear that the reiterated Ela has no, or severely reduced, trans-activating ability. This prediction is confirmed by the CAT assay results; Glasgow CAV-2 Ela appears to be capable of trans-activating the Vaxitas E4 promoter, but the Vaxitas reiterated Ela does not appear to be capable of trans-activating the Glasgow E4 promoter.

A model to explain the juxtaposition of left and right end sequences in defective adenovirus virions has been put forward by Daniell (1976). This proposes that displaced parental strands break before type II replication begins, and that without the second ITR they can not form the correct panhandle. However, the broken strands may be able to fold back at regions of partial complementarity and continue synthesis using the 5' half of the same molecule as a template. This model would explain the rearrangement observed in Vaxitas, but it is not obvious from the DNA sequences involved whether there is sufficient similarity for CAV strands to fold back.

The rearrangement of terminal sequences in the adenovirus genome could have potentially dramatic effects on the virus infectious cycle. We have shown that in transient transfection assays the Vaxitas Ela/E4 promoter is far more active than the E4 promoter alone. However, there still appears to be a minor component of the hybrid promoter which is trans-activated by the Ela gene product. These data strongly suggest that during growth on Mv-1-Lu cells the Vaxitas E4 region is no longer solely under the control of its own promoter, but is also being expressed from the reiterated Ela promoter. The presence of the reiterated Ela polyadenylation signal prior to the E4 promoter may not determine transcription termination at this point; Dressler & Fraser (1989) have demonstrated that transcription termination signals and polyadenylation signals are not necessarily coincident. More detailed transcriptional studies will be needed to clarify the pattern of transcription through the Vaxitas reiterated Ela/E4 region.

The cell line which Vaxitas has been adapted to grow in is derived from mink lung and is not normally permissive for growth of canine adenoviruses; therefore it is possible that this modification has conferred a growth advantage on the Vaxitas strain. Adenovirus type 12 mutants have adapted to growth in a number of different cell lines (Werner & zur Hausen, 1978; Schwartz et al., 1982; Salewski et al., 1989). Most of these viruses have been shown to have terminal modifications resulting in repetitions within the ITR; others had small deletions within the Ela region. In these adenovirus type 12 mutants, as with other adenoviruses which have terminal repetitions, the suggestion has been that this type of mutation in some way increases the replication efficiency, thereby conferring a selective advantage. In the case of the Vaxitas strain the right-hand ITR is normal, and therefore, as stated previously, we might expect the left ITR to be normal also. This suggests that the selective advantage which this strain has acquired is not related directly to ITR function.

The precise functions of the adenovirus E4 proteins have yet to be determined, however their functions do appear to be largely regulatory. For example, Babiss (1989) has shown that E4 protein(s) are probably involved in the activation of a cellular transcription factor, E2f, which is required in turn for efficient expression of the viral E2 region. E4 products have also been implicated with late protein synthesis, host cell shutoff and virus particle assembly (Weinberg & Ketner, 1986; Bridge & Ketner, 1989; Faiquot & Ketner, 1987; Huang & Hearing, 1989). Given the proposed regulatory role of the E4 proteins, it is entirely possible that the altered regulation of Vaxitas CAV-2 E4 is advantageous for growth in the normally non-permissive mink cell line.

The adaptation of CAV-2 to grow in the Mv-1-Lu cell line, which has resulted in the Vaxitas strain, also appears to have reduced its ability to grow in MDCK cells, which are often used for propagation of CAV-2. In fact, some MDCK stocks fail to allow any Vaxitas replication. The growth of Vaxitas in a different dog kidney cell line (DK-N, Cornell University) obtained recently is also reduced compared to that of wild-type CAV-2. Of course many other mutations may have occurred in the Vaxitas genome which could contribute to the altered cell specificity of the virus; for example, there is a deletion of approximately 400 bp at around 90 map units (Spibey & Cavanagh, 1989). The map location, together with DNA sequence data, show that this deletion is at the end of the E4 region. However, several other CAV vaccine strains appear to have deletions in this and other regions (Liu et al., 1988, N. Spibey & H. M. A. Cavanagh, unpublished results), but retain the ability to replicate in MDCK cells. An examination of the left end of the Vaxitas genome shows some variation; however, the exact nature of the events which have taken place has still to be determined.

In conclusion, we have shown that a CAV-2 vaccine strain has undergone a genomic rearrangement resulting in a duplication of the Ela region and left ITR at the right-hand terminus. This has resulted in the E4 region being controlled by the reiterated Ela promoter. It is tempting to speculate that it is this change in E4 expression which has allowed Vaxitas to replicate in the Mv-1-Lu mink cell line. Experiments to verify this, involving transfer of this novel right terminal arrangement to otherwise normal genomes, are under way.

This work was supported by the Wellcome Trust.
References


(Received 31 October 1990; Accepted 21 May 1991)