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Molecular Cloning and Restriction Endonuclease Mapping of Two Strains of Canine Adenovirus Type 2

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SUMMARY

The DNA of a field isolate and of a vaccine strain of canine adenovirus type 2 (CAV-2) were analysed by digestion with several restriction endonucleases. The *Pst*I restriction fragments of the field isolate (CAV-2 Glasgow) and the vaccine strain were cloned into the plasmid pBR322. Physical maps of the two viral genomes were constructed by molecular hybridization of *Pst*I, *Eco*RI, *Sma*I, *Bam*HI and *Kpn*I digests of the viral DNA with the cloned *Pst*I fragments. The restriction profile of CAV-2 Glasgow was shown to be virtually identical to those of the two prototype CAV-2 strains, Toronto A26/61 and Manhattan. However, the restriction fragment pattern of the vaccine strain of CAV-2 showed characteristic alterations, in particular additional sequences at or near the genome termini.

INTRODUCTION

The canine adenoviruses are typical members of the Adenoviridae. They are divided into two types, based on haemagglutination inhibition and neutralization tests, canine adenovirus type 1 (CAV-1) and canine adenovirus type 2 (CAV-2). CAV-1, formerly called infectious canine hepatitis virus, replicates in vascular endothelial tissue and affects most of the major organs of the dog (Rubarth, 1947; Appel *et al.*, 1973). CAV-2 also referred to as canine laryngotracheitis virus was previously thought to replicate solely in the respiratory epithelium (Appel *et al.*, 1973; Wright *et al.*, 1974).

Recently, however, CAV-2 has been isolated from the faeces of dogs suffering from diarrhoeal disease (Hamelin *et al.*, 1986; Macartney *et al.*, 1988) indicating that replication of the virus was taking place in the intestinal tract, which may implicate CAV-2 as an enteric as well as respiratory pathogen.

Despite differences in their pathogenicity and sites of replication CAV-1 and CAV-2 share common antigenic properties. This allows the use of a laboratory-attenuated strain of CAV-2 to be given as a live vaccine to protect against infectious canine hepatitis and laryngotracheitis. An attenuated strain of CAV-2 is generally preferred over CAV-1 because vaccination using the type 1 virus has frequently given rise to clinical problems, in particular corneal oedema.

In this study we have constructed recombinant plasmids which contain restriction fragments covering the entire genomes of a CAV-2 (Glasgow) field isolate and a vaccine strain. These data were used to construct cleavage maps for the two genomes for a variety of restriction enzymes. The restriction profiles of the prototype CAV-2 strains Toronto A26/61 and Manhattan are compared with those of the Glasgow strain.

METHODS

Preparation of virus and virus DNA. The Glasgow, Manhattan and Toronto strains of CAV-2 were propagated in the MDCK cell line and the Vaxitas vaccine strain in the Mv-1-Lu (mink) cell line. Conditions for growth of the cells and preparation of virus and viral DNA have been described previously (Macartney *et al.*, 1988).

Construction and analysis of recombinant plasmids. Restriction endonucleases were purchased from Pharmacia and used according to the manufacturer's instructions. Restriction enzyme digests were analysed by both agarose and polyacrylamide gel electrophoresis. CAV-2 DNA was digested with *Pst*I and the fragments cloned into the *Pst*I site of pBR322. The left and right termini of the CAV genomes were cloned as blunt-ended *Eco*RI and blunt-ended *Sal*I fragments respectively, into appropriately cut Bluescript plasmids (Stratagene). The terminal cloning was carried out using DNA which had been prepared using proteinase K, but without any sodium hydroxide or S1 nuclease treatment. The pBR322 clones were selected on the basis of their tetracycline resistance and ampicillin sensitivity. The terminal clones in the Bluescript plasmid were initially selected as white colonies on X-gal-containing media. Recombinant plasmids were prepared from each transformant by the method of Holmes & Quigley (1981) and characterized by digestion with *Pst*I and *Sal*I.

Molecular hybridization. CAV-2 DNA digested with *Bam*HI, *Eco*RI, *Pst*I, *Kpn*I and *Sma*I was separated in a 0·8% agarose gel and transferred to nitrocellulose by the method of Southern (1975). The nitrocellulose filters were then hybridized in 50% (v/v) formamide in 2 × SSC (0·3 M-NaCl, 30 mM-sodium citrate) plus 200 μg/ml salmon sperm DNA, at 42 °C for 24 to 48 h with cloned adenovirus DNA, which had been labelled with ^{32}P by nick translation (Rigby *et al.*, 1977). Less stringent conditions were used when probing with cloned human adenovirus DNA, usually 50% formamide and 37 °C. The filters were washed three times in 2 × SSC plus 0·1% SDS, then three times in 0·2 × SSC plus 0·1% SDS, dried and subjected to autoradiography.

RESULTS

Restriction enzyme analysis of CAV-2 DNA

The restriction endonuclease cleavage patterns obtained by digestion of CAV-2 DNA with *Bam*HI, *Eco*RI, *Kpn*I, *Pst*I, *Sal*I and *Sma*I are shown in Fig. 1. The fragment lengths calculated from the electrophoretic mobilities are given in Table 1. The fragmentation patterns of the field isolate (Glasgow strain) and the vaccine strain (Vaxitas) are essentially the same; however, some differences do occur and these will be described and discussed later. Digestion of CAV-2 Glasgow DNA with *Pst*I yields 11 fragments, two of these fragments (C and D) comigrate at 4·4

Table 1. *Sizes of fragments generated after cleavage of CAV-2 DNA with* Bam*HI,* Eco*RI,* Kpn*I,* Pst*I,* Sal*I and* Sma*I**

Enzyme	Fragment	CAV-2 Glasgow (kbp)	CAV-2 Vaxitas (kbp)	Enzyme	Fragment	CAV-2 Glasgow (kbp)	CAV-2 Vaxitas (kbp)
*Bam*HI	A	13·86	14·06	*Kpn*I	A	10·2	10·5
	B	8·1	8·1		B	5·2	5·2
	C	6·2	8·1		C	3·6	5·2
	D	2·0	2·0		D	3·05	3·6
	E	0·7	0·7		E	3·05	3·05
	F	0·6	0·6		F	2·85	3·05
					G	2·3	2·85
					H	0·7	0·7
*Eco*RI	A	19·0	20·4		I	0·6	–
	B	8·8	8·8				
	C	3·5	3·8	*Sal*I	A	28·0	28·3
					B	3·2	5·2
*Pst*I	A	6·0	6·0	*Sma*I	A	6·75	6·75
	B	4·6	4·6		B	5·3	6·1
	C	4·35	4·6		C	5·3	5·5
	D	4·35	4·1		D	4·55	5·3
	E	3·9	3·9		E	4·4	4·4
	F	3·1	3·1		F	3·85	3·85
	G	3·0	3·1		G	0·72	0·72
	H	1·3	3·0		H	0·233	0·233
	I	0·32	0·32		I	0·233	0·233
	J	0·26	0·26				
	K	0·2	0·2				

* Fragment sizes were calculated from electrophoretic mobilities in 0·8% agarose and 6% polyacrylamide as shown in Fig. 1 and 2.

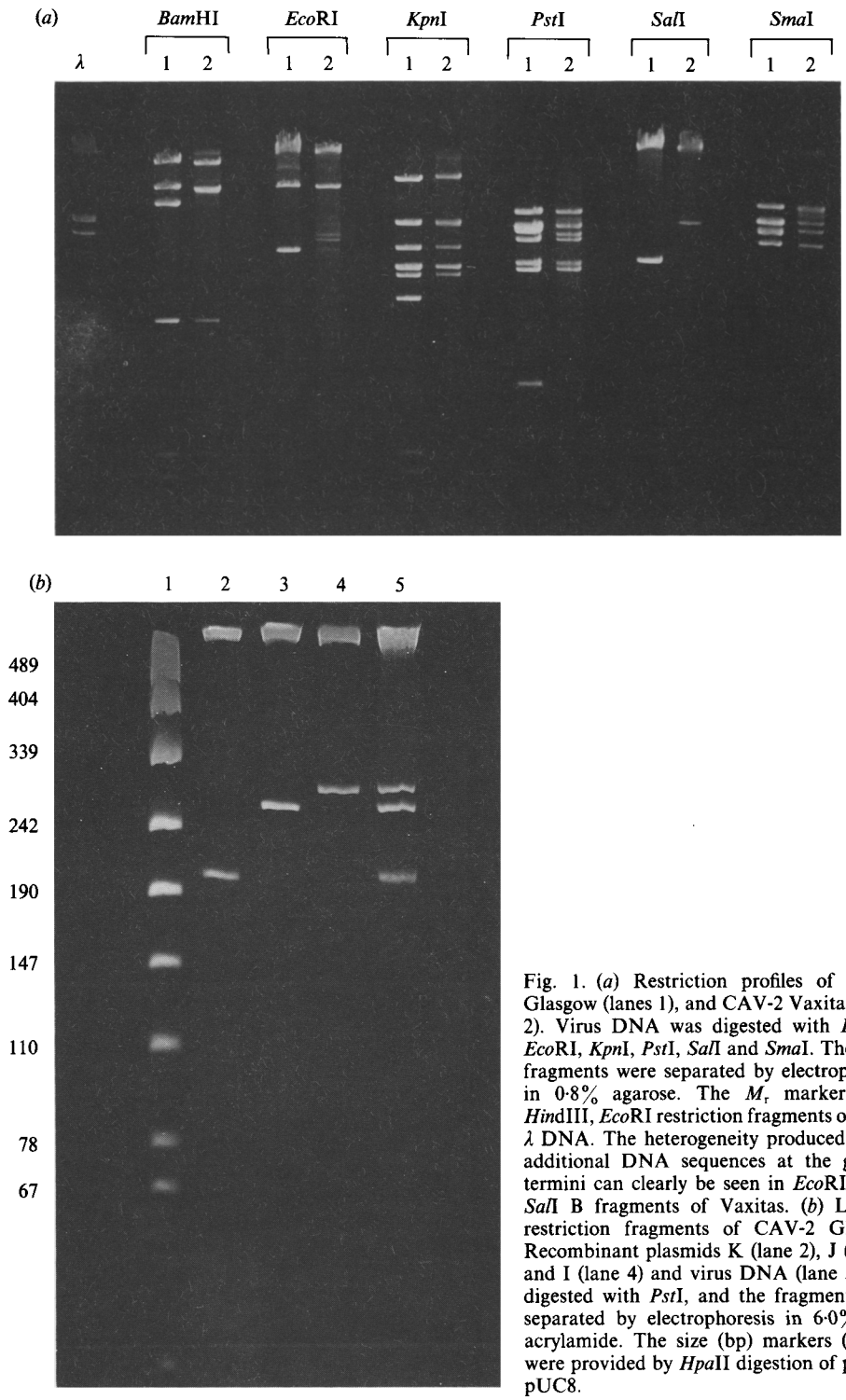

Fig. 1. (*a*) Restriction profiles of CAV-2 Glasgow (lanes 1), and CAV-2 Vaxitas (lanes 2). Virus DNA was digested with *Bam*HI, *Eco*RI, *Kpn*I, *Pst*I, *Sal*I and *Sma*I. The DNA fragments were separated by electrophoresis in 0·8% agarose. The M_r markers were *Hin*dIII, *Eco*RI restriction fragments of phage λ DNA. The heterogeneity produced by the additional DNA sequences at the genome termini can clearly be seen in *Eco*RI C and *Sal*I B fragments of Vaxitas. (*b*) Low M_r restriction fragments of CAV-2 Glasgow. Recombinant plasmids K (lane 2), J (lane 3) and I (lane 4) and virus DNA (lane 5) were digested with *Pst*I, and the fragments were separated by electrophoresis in 6·0% polyacrylamide. The size (bp) markers (lane 1) were provided by *Hpa*II digestion of plasmid pUC8.

kbp. A double digest of CAV-2 (Glasgow) DNA with *Pst*I and *Sal*I shows the single *Sal*I site of CAV-2 to be in one of this pair (fragment D). In the *Pst*I digest of CAV-2 (Vaxitas) the fragments B and C comigrate at 4·8 kbp, and the fragments F and G comigrate at 3·1 kbp. The mean genomic sizes of CAV-2 (Glasgow) and CAV-2 (Vaxitas) calculated from the restriction fragment lengths for each restriction enzyme digest are 31·26 ± 0·18 and 33·18 ± 0·18 kbp respectively.

Molecular cloning of CAV-2 Pst*I fragments*

Digestion of CAV-2 with *Pst*I gives 11 fragments (Fig. 1) ranging in size from 6·0 kbp to 0·2 kbp. This number of fragments and range of sizes make them amenable to cloning in a plasmid vector. Nine of the 11 *Pst*I fragments were cloned into the *Pst*I site of pBR322. The two remaining fragments C and H (in Glasgow strain) and C and G (Vaxitas strain) were not initially cloned as *Pst*I fragments but were instead cloned as *Sal*I blunt-ended (*Sal*I B) and *Eco*RI blunt-ended (*Eco*RI C) fragments. The terminal *Pst*I fragments were subcloned from these.

Recombinant plasmids were digested with *Pst*I and the electrophoretic mobility of the inserted CAV-2 DNA was compared with *Pst*I-cleaved CAV-2 DNA. The recombinant plasmids were characterized further by hybridization against Southern transfers of *Pst*I-digested CAV-2 DNA (Fig. 2); all the *Pst*I-cloned fragments hybridized with a single *Pst*I fragment in these experiments, but the cloned termini showed the characteristic homology due to the terminal repeats. In the case of the *Pst*I I, J and K cloned fragments, they were used to probe *Pst*I-digested DNA which had been separated on 2% agarose in order to show hybridization to themselves only.

Physical maps of CAV-2 DNA

The maps shown in Fig. 3 were constructed using the data from molecular hybridization experiments (Fig. 2) and restriction fragment sizes (Table 1). The *Sma*I sites at map units 0·75 and 99·25, giving rise to *Sma*I fragments H and I, were also located by sequence analysis of the CAV terminal fragments (unpublished data). Hybridization against small DNA fragments (*Pst*I I, J, K and *Sma*I H and I) was performed on Southern transfers from 2% agarose.

The terminal *Pst*I fragments were identified as fragments C and H of the Glasgow strain, and C and G of Vaxitas. The terminal fragments of CAV-2 Vaxitas show considerable heterogeneity (Fig. 1), which is most clearly visible with *Eco*RI C and *Sal*I B. In constructing the restriction map (Fig. 3) the size of the most predominant species was taken. Cross-hybridization was observed between the terminal fragments (Fig. 2) which is probably due to the homologous inverted terminal repeats found in all adenoviruses (Tooze, 1981; Sussenbach, 1984).

The order of *Bam*HI fragments within *Pst*I B (Glasgow) was determined by *Kpn*I and *Bam*HI double digests of the cloned *Pst*I B fragment (data not shown).

Data from the molecular hybridization experiments was confirmed by restriction endonuclease digestion of the CAV-2 recombinant plasmids; for example the clone of *Pst*I G was shown to be cut by *Eco*RI, whereas no other cloned fragment has a site for *Eco*RI.

Comparison of physical maps of CAV-2 Glasgow and Vaxitas DNA

From Fig. 3 it can clearly be seen that the restriction maps of the field isolate and vaccine strain are very similar, but some differences do occur in the terminal fragments and in the *Pst*I D fragment of Vaxitas. The right terminal *Pst*I fragment (fragment H) of Glasgow strain is 1·3 kbp, whereas its counterpart in Vaxitas is 3·3 kbp. The nature and exact point(s) of insertion of the additional DNA sequences in the vaccine strain is under investigation and initial studies indicate that the point of insertion is within 500 bp of the terminus. Additional DNA sequences are also present at the left terminus of Vaxitas, the terminal *Pst*I fragment measuring 4·7 kbp compared with 4·4 kbp in the Glasgow strain.

The *Pst*I D fragment of CAV-2 Glasgow (4·4 kbp) is present in Vaxitas as a 4·1 kbp fragment. The deletion which has occurred in the vaccine strain removes a *Kpn*I site, resulting in the loss of the 0·6 kbp *Kpn*I fragment.

The restriction profiles of CAV-2 Toronto (A26/61) and Manhattan appear identical to the Glasgow isolate in all the restriction enzyme digests apart from the *Hpa*II digest.

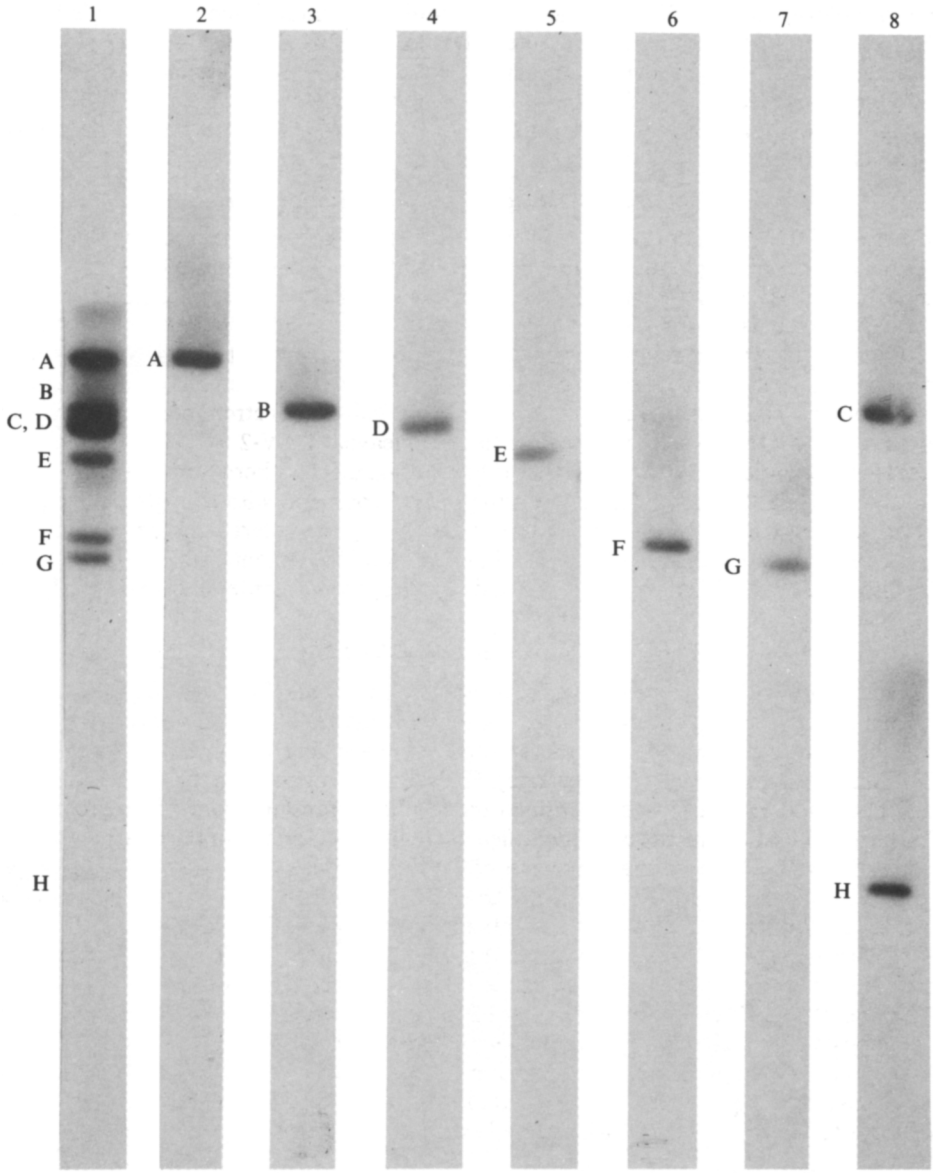

Fig. 2. Hybridization of ^{32}P-labelled recombinant plasmids, containing *Pst*I fragments of CAV-2 Glasgow, with Southern transfers of *Pst*I-digested CAV-2 Glasgow. The Glasgow *Pst*I fragments used as probes are as follows: lane 2, A; lane 3, B; lane 4, D; lane 5, E; lane 6, F; lane 7, G; lane 8, H. To provide a reference one filter was hybridized with ^{32}P-labelled whole virus DNA (lane 1). Hybridization of cloned Vaxitas *Pst*I fragments and Southern transfers of Vaxitas DNA were also carried out (data not shown).

Determination of genome orientation

The physical maps of the CAV genomes were orientated with respect to human adenovirus genomes by probing Southern transfers of CAV (Glasgow) DNA with cloned fragments of human adenovirus (Ad) types 5 and 12. The *Bgl*II C fragment of Ad5 (map units 45·3 to 60·2) carries hexon-coding sequences, and this fragment was used to probe the CAV genome (Fig. 4*b*).

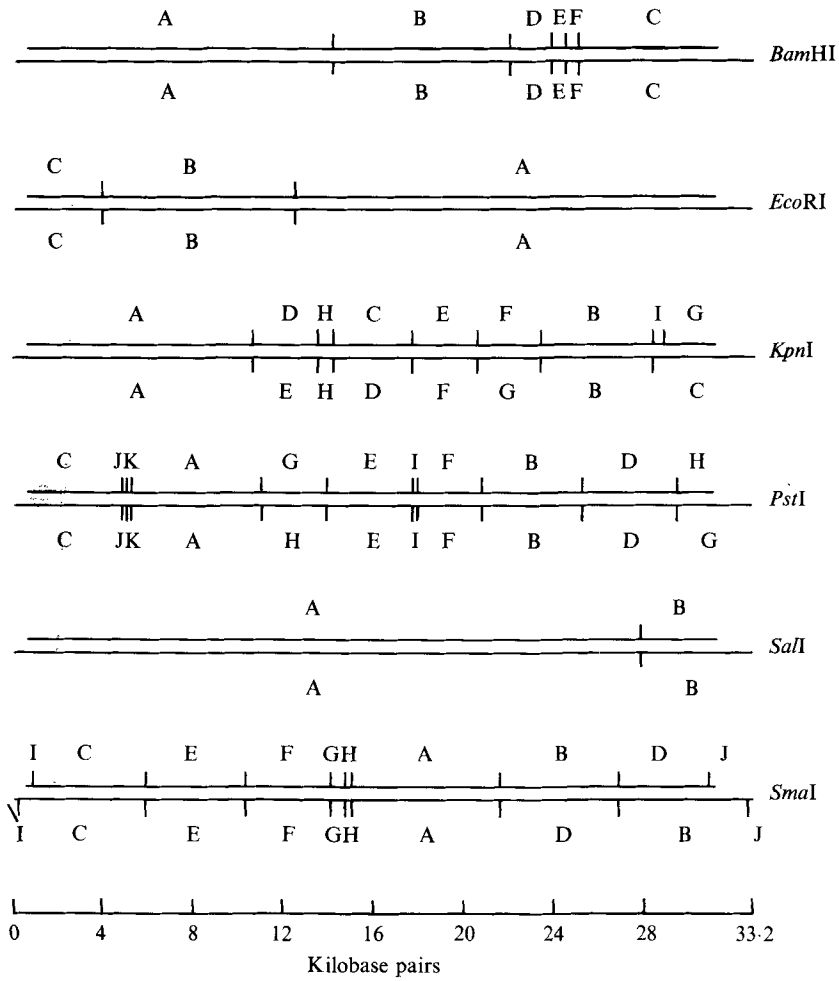

Fig. 3. Restriction maps of CAV-2 Glasgow (upper lines) and CAV-2 Vaxitas (lower lines) for the enzymes *Bam*HI, *Eco*RI, *Kpn*I, *Pst*I, *Sal*I and *Sma*I. The relative positions of the *Pst*I fragments J and K has not been determined. The maps are shown in the conventional left to right orientation.

Two *Pst*I fragments (E and F) and two *Kpn*I fragments (C and E) hybridize to this Ad5 clone. A *Sac*I/*Bgl*II subclone of this fragment (map units 56·6 to 60·2) only hybridizes to the *Pst*I F and *Kpn*I E fragments (data not shown). The cloned *Eco*RI C fragment of Ad12 (map units 0 to 16·2) was shown to hybridize to *Bam*HI A and *Eco*RI C. These data indicate that the orientation of the CAV genome is as shown in Fig. 3.

DISCUSSION

In this paper we have described the construction of recombinant plasmids containing *Pst*I restriction fragments of the DNA from two strains of CAV-2. These recombinant plasmids were used to construct *Bam*HI, *Eco*RI, *Kpn*I, *Pst*I, *Sal*I and *Sma*I cleavage maps of CAV-2 DNA. These data show that the first U.K. isolate of CAV-2 (Glasgow strain) has the same restriction enzyme profile as two prototype CAV-2 strains, Toronto A26/61 and Manhattan.

The cleavage maps of the CAV-2 Glasgow strain and a vaccine strain of CAV-2 (Vaxitas), which was originally derived from the Manhattan strain, are essentially the same. The differences which do exist between Vaxitas and the field isolates, i.e. the additional DNA

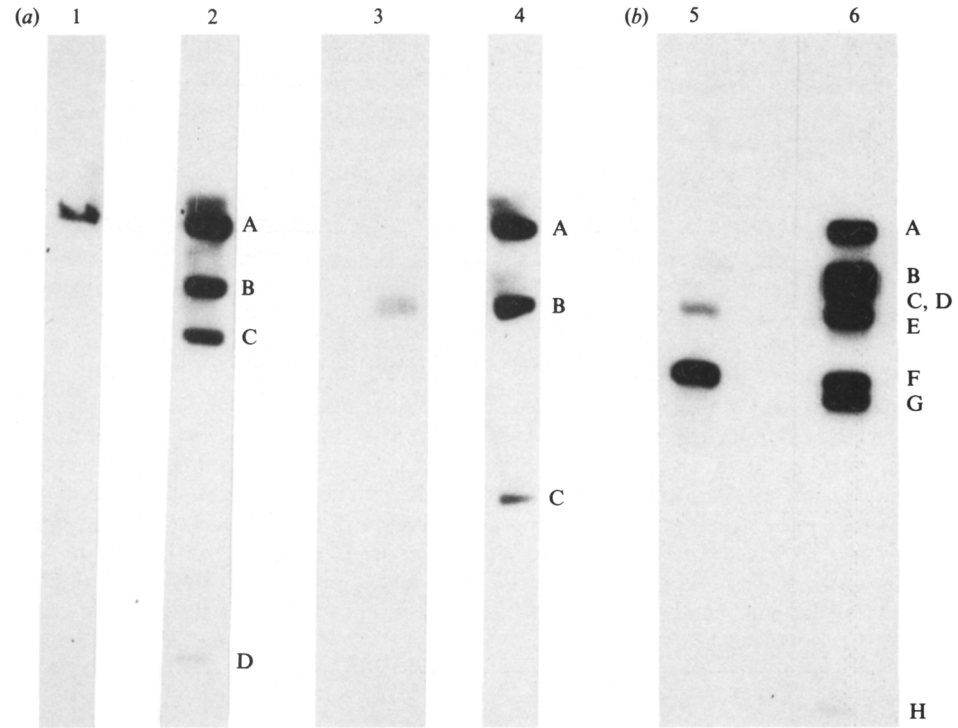

Fig. 4. Hybridization of ^{32}P-labelled recombinant plasmids, containing (*a*) *Eco*RI C fragment of human Ad12, (*b*) *Bgl*II C fragment of human Ad5, with Southern transfers of CAV-2 Glasgow DNA digests. Lanes 1 and 2, *Bam*HI; lanes 3 and 4, *Eco*RI; lanes 5 and 6, *Pst*I. For reference one filter of each was probed with ^{32}P-labelled whole virus (lanes 2, 4 and 6).

sequences at the genome termini and a small deletion at around 90 map units, have probably come about as a result of continued passage in tissue culture. Indeed, preliminary experiments indicate that other laboratory-attenuated vaccine strains of both CAV-1 and CAV-2 have inserted or duplicated sequences near the genome termini (data not shown).

Multiple repeats of viral DNA sequences have been observed at both the left and right termini of human Ad3 (Larsen & Tibbetts, 1985; Larsen *et al.*, 1986). These repeated sequences appear to have arisen because of continued passage of the virus at high multiplicity. The reiterations at the left end of the Ad3 genome were found to be 20 to 300 bp stretches of viral DNA repeated two to four times, creating viral genomes whose left termini were increased by as much as 700 bp compared with the parental virus. The same authors observed even larger repeats at the right terminus of the Ad3 genome, creating increases of 1 to 2 kbp. The variable increases in terminal restriction fragment lengths we have observed in CAV-1 and CAV-2 appear very similar to those described in human Ad3 (Larsen & Tibbetts, 1985). However, we do not know the conditions under which all the vaccine viruses were produced, except that the Vaxitas vaccine strain has been attenuated in mink fibroblasts, a procedure which would certainly require initial passage of the virus at high multiplicity. Whilst it is quite possible that the extra sequences in CAV have arisen by a mechanism similar to those in Ad3, a sequence analysis of the right terminus of the Vaxitas genome has revealed that the extra DNA is a single duplicated sequence and not a series of tandem reiterations (unpublished results). However, the nature of any modifications at the left terminus is still unclear.

Reiterated DNA sequences have been found in a number of different viruses. Brockman *et al.* (1974) described variants of simian virus 40 in which major portions of the viral genome were deleted and the remaining sequences reiterated to maintain an approximately full-length genome. These variants had multiple copies of the origin of replication and appeared to replicate

at an advantage over the wild-type helper virus in mixed infections. Analysis of virulent and vaccine strains of bovine herpesvirus type 1 (BHV-1) revealed at 14 bp sequence at the left end of the genome repeated between eight and 38 times (Hammerschmidt *et al.*, 1986).

Illegitimate recombination and aberrant replication have been proposed as mechanisms by which reiterated DNA sequences arise (Smith, 1976). Subsequent homologous recombination could increase the number of copies of the repeated sequence. Selection of variant genomes would be based on replication and encapsidation efficiency. It is interesting to note that the repeated sequences in BHV-1, Ad3 and possibly CAV are in regions of the genomes associated with viral DNA replication. Furthermore the growth characteristics of CAV-2 Vaxitas and other vaccine strains of CAV suggest that in these strains DNA replication is impaired.

Work is now in progress to define more precisely the nature and location of the additional DNA sequences in the CAV vaccine strains, and to investigate the effect they have on DNA replication and gene expression.

The Glasgow strain of CAV-2 was isolated from the faeces of a young dog with diarrhoeal disease (Macartney *et al.*, 1988), which suggests that viral replication may have been taking place in the intestinal tract, an unusual site of replication for a virus associated with respiratory disease (Koptopoulos & Cornwell, 1981). The apparent ability of the virus to replicate in the gut does not appear to be accompanied by any major changes in the restriction enzyme cleavage patterns. However, some minor differences were observed in the *Hpa*II restriction profiles of CAV-2 Glasgow and the two prototype strains. It is hoped that further work currently being undertaken will explain the relevance, if any, of these minor differences between the Glasgow and prototypic strains of CAV-2.

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