GENOMIC VARIATION IN THE CANINE ADENOVIRUSES

by

HEATHER MARGARET ANN CAVANAGH, B.Sc.

Thesis submitted for the degree of Doctor of Philosophy,
Faculty of Veterinary Medicine, University of Glasgow.

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Department of Veterinary Pathology,
University of Glasgow.

A C K N O W L E D G E M E N T S

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A B S T R A C T

Considerable differences exist in the pathogenicity of wild-type and vaccine strains of canine adenovirus. An analysis of their genome structure was undertaken in orrder to investigate the molecular basis of their differing pathogenicities. Six vaccine strains and two wild-type strains were examined. Preliminary investigations revealed significant variation near the genome termini.

A restriction map of one vaccine strain, Vaxitas CAV-2, was determined. The origin of the additional DNA near the right-hand end of the genome was also determined for four of the vaccine strains, including Vaxitas. These novel sequences included a reiterated E2 promoter, reiterated ITR's and adjacent sequences and one of unknown, possibly cellular, origin.

The right-hand terminal DNA sequence of Vaxitas (1.7kb) and a wild-type strain (Glasgow CAV-2: 0.65kb) was determined, to reveal the exact position of the additional DNA. The Vaxitas "insertion" was found to consist of a copy of the left-hand terminal 1.352kb (4.3% of the genome) reiterated at the right-hand terminus. This constituted a copy of the E1a region at the right-hand end of the genome, upstream of the Vaxitas E4 region. 250bp of the "normal" right-hand terminus had been displaced by the reiterated E1a.

The reiterated E1a was shown to encode no products capable of transactivation. A fully functional E1a promoter was retained however. Transcription through the Vaxitas E4 promoter was found to originate at the reiterated E1a promoter. The Vaxitas E4 gene does not require transactivation by E1a products.

The effect of the enlarged genome termini upon the pathogenicity of the canine adenovirus vaccine strains and the mechanism by which the aberrant genomes arose is discussed.

A B B R E V I A T I O N S

AdV	Adenovirus
CAV-1	Canine adenovirus type 1
CAV-2	Canine adenovirus type 2
MDCK	Madin and Derby canine kidney (cell line)
NMF	Normal mink fibroblasts (cell line)
Mv-1-Lu	Mink lung (cell line)
TCID	Tissue culture infectious dose
pfu/ml	plaque forming units per millilitre
CPE	Cytopathic effect
HMP	High multiplicity passage
EDS	Egg drop syndrome
BAdV	Bovine adenovirus
IM	Intramuscular
SC	subcutaneous
IV	intravenous
A	Adenosine
C	Cytosine
G	Guanosine
T	Thymidine
m.w.	molecular weight
µg	micrograms
ng	nanograms
MegaD	megadaltons
kD	kilodaltons
kb	kilobase
nm	nanometeres
λ	Bacteriophage Lambda

RHT	Right-hand terminal
LHT	Left-hand terminal
TP	Terminal protein
pTP	Terminal protein precursor
ITR	Inverted terminal repeat
DBP	DNA binding protein
MLP	Major late promoter
m.u.	Map units
NFI	Nuclear factor I
NFII	Nuclear factor II
NFIII	Nuclear factor III
a.a.	amino acids
ORF	Open reading frames
URF	Unidentified reading frames
CAT	Chloramphenicol acetyltransferase
ts	Temperature sensitive

GENERAL INTRODUCTION

Adenoviruses (AdV) were first isolated in 1953 by Rowe et al from the adenoids of children. The group name Adenoviruses, indicating the occurrence in adenoid tissue, was created in 1956 and the group was given a family status Adenoviridae in 1976 with two genera Mastadenovirus and Aviadenovirus. They encompass AdV occurring in mammals and birds respectively (Doerfler, 1986 and references therein). The viruses are widely distributed in nature in many mammalian and avian species.

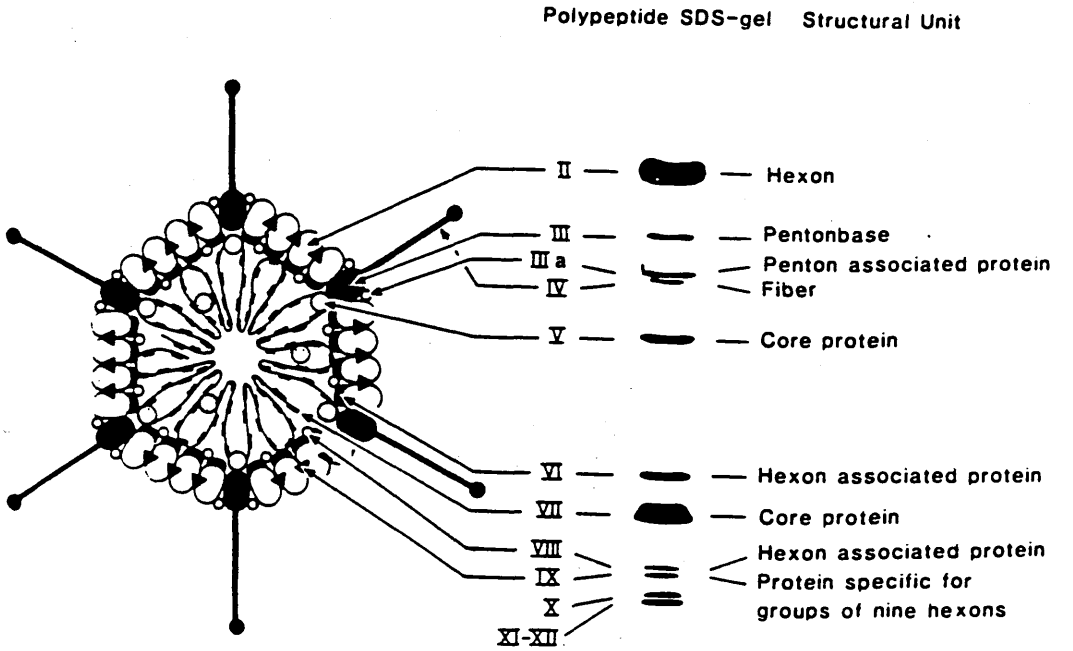
AdV are non-enveloped particles, that replicate and assemble their virions in the nucleus of infected cells, with a linear double-stranded DNA, molecular weight (m.w.) 20-30 Megadaltons (MegaD) corresponding to 30-45.5 kilobase pairs (kb) and with at least 10 structural polypeptides ranging from 5-120 kilodaltons (kD). The isometric particles, 70-90 nanometres (nm) in diameter, have an icosahedral symmetry with 252 capsomers. The 12 penton bases (vertex capsomeres) carry one or two filamentous projections (fibres) for the genus mastadenovirus or aviadenovirus respectively (figure 1).

The Canine Adenoviruses

Two distinct but related adenoviruses, canine adenovirus type 1 (CAV-1) and canine adenovirus type 2 (CAV-2), have been isolated from the dog. They differ in structure, pathogenicity and antigenicity. CAV-1, formerly known as infectious canine hepatitis virus (ICHV), was first

F I G U R E 1

Structure of the Adenovirus Virion



Schematic representation of an adenovirus. The mobilities and relative amounts of each protein after electrophoresis of the dissociated virus on a sodium dodecyl sulphate-containing polyacrylamide gel are shown on the right. The position of each polypeptide in the virion is designated; however, the configuration of the DNA does not imply the actual structure within the core. The roman numerals refer to a polypeptide designation described by Maizel et al (1968). The hexon (II), penton base (III), fibre (IV) and hexon-associated proteins (IIIa, VI, VIII and IX) are subunits of the capsid. The core contains proteins V, VII and the 55kD terminal protein (TP) covalently linked at each of the 5'ends of the DNA. The two molecules of TP per virion are too few to be demonstrated by the coomassie stain of the viral polypeptides. Adapted from Persson and Philipson (1982).

isolated by Cabasso et al (1954) from a dog with hepatitis and has since been associated with a range of clinical syndromes, the best known being a systemic disease with hepatitis (Rubarth's disease), reviewed by Koptopoulos and Cornwell (1981).

CAV-1 infection is known to be widespread in the canine population and most infections are probably subclinical (Ablet and Baker, 1960). The virus replicates in vascular endothelial tissue and can affect many organs (Appel et al, 1973). As well as the classical hepatitis described by Rubarth (1947), CAV-1 is also associated with focal intestinal nephritis (Wright, 1976), glomerulonephritis (Wright et al, 1974), chronic hepatitis (Gocke et al, 1970) and respiratory tract infections.

The route of infection plays a significant role in the type of disease produced by the virus. Intravenous or intraperitoneal inoculation leads to hepatitis with focal nephritis and anterior uveitis (the blue eye syndrome) in recovering animals. Aerosol infection however causes severe pneumonia but hepatitis is rarely seen (Koptopoulos and Cornwell, 1981).

CAV-2 (formerly called canine laryngotracheitis virus) was first isolated by Ditchfield and co-workers (1962) from kennelled dogs during an outbreak of respiratory tract disease. This virus was designated Toronto A26/61 and is one of the prototypic isolates of CAV-2. CAV-2 has been

isolated from throat swabs or from the lungs of dogs with pneumonia, bronchitis, tonsillitis and pharyngitis in North America where it is recognised as one of the aetiological agents of "kennel cough". Unlike CAV-1, when CAV-2 is inoculated by the IM, SC or IV routes, clinical signs are rarely seen except for the occasional mild fever and nasal discharge. Inoculation by the oro-respiratory route results in a mild fever with discharge in the majority of cases. By contrast, aerosol infection of dogs with CAV-2 leads to severe respiratory disease. Hepatitis has not been associated with CAV-2.

There are only two reports of the isolation of CAV-2 from dogs in Britain (Danskin, 1973; Macartney et al, 1988). Danskin (1973) isolated both CAV-1 and CAV-2 from dogs with respiratory disease although no dogs were found to be co-infected with both viruses. Hamelin et al (1986) recently isolated CAV-2 from the faeces of dogs suffering from haemorrhagic diarrhoea and it may therefore be implicated as an enteric as well as a respiratory pathogen. The molecular basis of the pathogenicity of the canine adenoviruses has not yet been established.

Cornwell et al (1970) were the first to propagate CAVs in the Madin and Darby canine kidney (MDCK) continuous cell line. The sensitivity of these cells was comparable to that of secondary dog kidney cultures but the yields of virus obtainable from them were greater by 0.5 to 0.8

$\log_{10}$ $TCID_{50}$ /ml. The cytopathic effect (CPE) is essentially the same in all types of susceptible cells, with infected cells rounding up, becoming refractile and clumping together to form characteristic "bunches of grapes" before detaching from the glass (Cabasso et al, 1954).

Moulton and Frazier (1965) reported that viral DNA synthesis occurred 8-10 hours post infection (pi) and preceded the production of protein by approximately two hours. CAV-1 release occurs by degeneration of the nuclear membrane whereas release of CAV-2 into the cytoplasm occurs by the formation of protrusions of the nuclear membrane which, when pinched off, release a membrane-bound aggregate of virions. Although the formation of intracellular spheres has been described for CAV-1 infection, the production of crystalline aggregates and their release by protrusion of the nuclear membrane seems to be peculiar to CAV-2 infection (Koptopolous and Cornwell, 1981). The relevance of this difference in the "release" mechanism of the two strains is not yet apparent nor is its role,if any, in the pathogenicity of the viruses.

Electron microscopy has shown that the virions of both CAVs are characteristic of the adenoviridae, i.e. are icosahedral structures made up of 252 capsomeres (Yamamoto and Marusyk, 1968). However, the fibres of CAV-2 (35-37nm long) are approximately 10nm longer than those of

CAV-1. This difference in fibres may account for the fact that, although both viruses agglutinate human and rat erythrocytes (RBC), they use different receptors on the RBC surface. The CAV-2 receptor complex is sensitive to trypsinisation whereas that of CAV-1 is not (Marusyk and Yamamoto, 1971). Ditchfield et al (1962) showed that antisera to CAV-1 and CAV-2 prevented haemagglutination with the homologous viruses but completely failed to inhibit the heterologous viruses whereas levels of neutralising and complement fixing antibodies (Ab) to the heterologous viruses were found in both types of antiserum. Using the haemagglutination-inhibition (HI) test, Swango et al (1969) compared seven CAV isolates and confirmed that they formed two distinct but related serological groups. In 1970, Marusyk et al reported that the hexons carried a common group specific, complement-fixing antigen (Ag); the penton oligomers acted as a complete soluble haemagglutinin while the penton and fibre monomers were able to haemagglutinate only when bound by selected antisera against human AdV soluble Ags (haemagglutination enhancement). CAV-1 and CAV-2 were also found to have a distinct polypeptide pattern when compared to human, simian and equine AdV (Marusyk and Cummings, 1978).

The first modified live CAV vaccine was described by Fieldsteel and Emery (1954) and consisted of the 51t serial passage of CAV-1 in dog kidney tissue cultures.

Although this proved safe for the inoculated animals, it was later shown to be excreted in the urine and, after four serial passages in dogs, could revert to virulence (Cabasso et al, 1958). Fortunately it was found that this problem could be overcome by growing the virus in pig kidney cells (PKC) and these are now used to produce the CAV-1 vaccines.

Provided that maternal Ab is absent, a single dose of the live vaccine produces good immunity in dogs of any age (Cabasso, 1962). However, several side-effects of CAV-1 vaccines became apparent, notably nephritis and a characteristic ocular reaction consisting of corneal oedema and inflammation of the anterior uveal tract, often known as "blue eye". The isolation of CAV-2 in 1962 and the discovery that it does not produce an acute generalised disease, is not excreted in urine and does not produce renal and ocular lesions, led several groups to show that both CAVs can protect against each other through heterotypic Ab production (Appel et al , 1970; Wright et al, 1974). Appel et al (1973) reported that dogs inoculated intranasally or intramuscularly with attenuated CAV-2 did not become ill and were completely immune to intravenous challenge inoculation with virulent CAV-1. Koptopolous (1979) compared the efficacy of two live attenuated vaccines, one CAV-1 and one CAV-2, in protecting dogs against an aerosol of virulent CAV-2. The CAV-2 vaccine was found to give better protection. It is believed that CAV-2 vaccines confer greater protection

than CAV-1 vaccines as the CAV-1 vaccines produce systemic immunity only (cell and humoral mediated) whereas CAV-2 vaccines produce both systemic and local immunity (Koptopolous and Cornwell, 1981). Killed virus vaccines were found to be much less efficient than live viral vaccines (Cornwell et al, 1982). No immunological relationship to human AdVs has been detected (Willimzik et al, 1981).

The advent of molecular biology enabled a direct comparison of CAV-1 and CAV-2 genomes using restriction enzyme analysis. The difference between the restriction enzyme cleavage patterns of CAV-1 and CAV-2 confirms the distinctiveness of the two species (Assaf et al, 1983; Shinagawa et al, 1983; Hamelin et al, 1984; Darai et al, 1985; Hamelin et al, 1986; Jouvenne and Hamelin, 1986). All strains and isolates examined thus far appear to have virtually identical restriction enzyme cleavage patterns to their respective prototypic strains: i.e. all CAV-1 isolates appear virtually identical to the prototypic strain Lederle 255; all CAV-2 isolates to the prototypic strain Toronto A26/61. However, small variations have been observed. Hamelin et al (1986) reported enteric isolates of CAV-2 which revealed variation in the HpaII cleavage patterns of different isolates but all other restriction enzyme cleavage patterns examined appear to be identical to the prototypic Toronto A26/61.

The BamHI, ClaI, SacI, SalI and SmaI restriction maps of the CAV-1 strain Behring HCC 299 have been reported (Darai et al, 1985) but none of CAV-2. Marusyk and Hammarskjold (1972) reported the CAV-1 and CAV-2 genomes to be 70% homologous and Hamelin et al (1986) showed that CAV DNA was unmethylated as defined by HpaII/MspI digestion. A terminal protein was found to be covalently linked to the 5′ ends of the CAV genome (Darai et al, 1985). This was confirmed by the use of λ 5′-exonuclease and Escherichia coli (E.coli) 3′-exonuclease III. The CAV DNA was resistant only to λ 5′-exonuclease and was degraded by E.coli 3′-exonuclease III. Terminal proteins have been described for all AdVs, irrespective of species of origin, so far examined (Ginsberg, 1984).

Adenovirus Subclassification

The first isolation of a human AdV was reported by Rowe et al (1953) from cultured, uninoculated explants of human tonsils and adenoids. Since then over 40 distinct antigenic types have been found to infect humans and numerous other types infect a wide variety of lower animal species including cattle, pigs, horses, monkeys, rodents, fowl and, of course, dogs. An AdV has even been isolated from the frog (for review of AdV isolations see Doerfler, 1986). A further group of AdV of avian origin has been described. Egg-drop syndrome (EDS), a disease which causes infected birds, mainly ducks, to produce fewer eggs and eggs with defective shells has been isolated which has been found to be immunologically distinct from

other avian AdV. Analysis to date has demonstrated the EDS virus to be similar in structure and organisation to the mammalian AdV (personal communication, Dr. N. Spibey) and possibly represents a species of avian AdV phylogenically less diverged from the mammalian AdV than other avian AdV?

The original subclassification of the human AdVs was based on the haemagglutination patterns of rat and rhesus monkey RBC. A different type of subclassification is based on the oncogenicity of the human AdV. The different serotypes have been subdivided into a highly oncogenic subgroup A (e.g. Ad12, Ad18, Ad31), a weakly oncogenic subgroup B (e.g. Ad3, Ad7) and a non-oncogenic subgroup C (e.g. Ad2, Ad5). It is interesting to note that there is a correlation between the guanine-cytosine (GC) content of the human AdVs and their oncogenicity. The GC content of the DNAs decreases with increasing oncogenicity (Doerfler, 1986).

Subsequent grouping on the basis of DNA homology has agreed with the original classifications very well. Different human AdV serotypes were divided into six different subgroups, A-F (Green et al, 1979). In general, members of the same subgroup have genomes that are homologous for more than 90%. However, members of highly oncogenic subgroup A share only 50-70% of their DNA sequences. The homology between members of different

subgroups is less than 20%. The poor homology between subgroups demonstrates that the DNA sequences of different serotypes have significantly diverged in the course of viral evolution but the complex organisation of the AdV genome has been rigidly conserved (Tibbets, 1977).

By analogy, the extensive homology between CAV-1 and CAV-2 places them in the same group. In addition, the reported homology of 70% between CAV-1 and CAV-2 (Marusyk and Hammarskjold, 1972) is comparable to that displayed by members of the highly oncogenic (group A) human AdVs. It is therefore interesting that both CAV-1 and CAV-2 induce tumours in newborn hamsters (Sarma et al, 1967; Dulac et al, 1969). Hoggan et al (1965) reported that CAV-1 and CAV-2 share a common T-Ag. The virus specific, non-virion T-Ag produced during acute infection of cell cultures with AdV have been shown to be essentially indistinguishable from tumour specific Ag in viral-induced tumours. Highly oncogenic AdV 12, 18 and 31 share a common T-Ag which does not cross-react with the common T-Ag shared by the moderately oncogenic Ad3, 7, 14, 16 and 21 (Heubner, 1967).

The pathogenesis and tissue tropism of each strain of human AdV also appears to be associated with its subclassification. Again the GC content appears significant with the highly oncogenic respiratory tract associated group A having a relatively low GC content, as previously defined by SmaI digestion (CCC/GGG), with 4-5

sites whereas the non-oncogenic enteric group F exhibits a high GC content with 9-11 SmaI sites.

The greatest number of human AdV infections has always been associated with diseases of the respiratory tract (groups A-C) followed by those of the gastrointestinal tract (groups E and F) and finally as casual agents of opthalmic disease (group D). The pathological divisions between groups A-C and D are not strictly defined as certain members of group B have been associated with conjunctivitis, e.g. Ad3, although usually in conjunction with respiratory symptoms. In addition, members of group B have also been associated with urogenital infections, mainly haemorrhagic cystitis. The number of all respiratory infections associated with the human AdV is lower than that caused by the influenza and rhinoviruses but higher than all other respiratory viruses. Most severely infected are the young and immunologically compromised hosts and, although most infections are upper respiratory, lower respiratory tract infections can lead to severe, occasionally fatal, pneumonia. Of the diseases of the gastrointestinal tract, infantile gastroenteritis is the most common. Opthalmic infection most commonly takes the form of keratoconjunctivitis and is widespread in eye clinics, usually due to transmission from patient to patient by clinic staff. This tissue tropism is not well understood at the present time but is known to be associated with more than the ability of each strain to

gain access to the relevant cell type.

Studies in animal models (e.g. cotton rats) show that type C AdV demonstrate a limited ability to replicate in rodent cells, a capacity not found in other subgroups.Conversely, subgroup F viruses, the causative agents of infantile gastroenteritis, are extremely difficult to grow *in vitro* although they are capable of entry into the majority of cell types commonly used for AdV replication, namely HeLa, KB and HEK cells. They appear to be blocked at an early step in the growth cycle when introduced into these cells (Ishino *et al*, 1988). A recent isolate of a group D AdV has been found to be capable of causing gastroenteritis. Its restriction enzyme pattern is unique compared to all other AdV but it is immunologically a member of group D. All variation within the genome of this new strain appears to be restricted to the right-hand 25% of the genome (Gomes and Neil, 1987). Thus attachment and entry of the virus into various cell types is not the only limiting factor in "tissue tropism", a fact which may have direct significance on the isolation of a canine adenovirus from the gastrointestinal tract when its genome is compared to the wild-type respiratory virus.

This tropism is found in other non-human AdV for example within the Bovine AdV (BAdV). The 10 serotypes of BAdV are subdivided into two groups, I and II, as determined by immunological tests and their tissue tropism. Both groups grow well in bovine testicle cells but only subgroup I can

replicate in bovine kidney cells. Subgroup I appears to be a bovine specific respiratory disease whereas subgroup II, the causative agent of pneumoenteritis, has been routinely isolated from sheep as well as cattle but remains immunologically distinct from all ovine AdV species (Belak et al, 1986).

The relationship of the various areas of the AdV genome will be discussed with relevance to their effect on pathogenicity as each gene is described in the following pages.

Structure of the Adenovirus Genome

The genomes of the AdVs are linear, double-stranded molecules. Both ends of the viral genome function as origins for DNA replication and contain a terminal protein (TP) which is covalently linked to the 5'-end of each DNA strand via a serine residue (Ginsberg, 1984). The ends of the viral DNA contain an inverted terminal repetition (ITR). AdV gene expression is strictly regulated at multiple levels during a productive infection. The lytic cycle is traditionally divided into two distinct phases; an early phase precedes viral DNA replication and is triggered by gene products from early region E1a and a late phase which follows DNA replication and is characterised by the expression of structural proteins. A schematic diagram of the AdV genome is shown in fig. 2. It should be noted that the descriptions of the various

components of the AdV genome are based on the extensive studies carried out on the human AdV type 2 unless otherwise stated.

Inverted Terminal Repetition

The existence of an ITR in AdV DNA was discovered when denatured DNA was reannealed at low concentrations and examined by EM. A high percentage of the single strands were present in a circular form indicating the presence of an ITR (Garon et al, 1975). ITRs have been detected in every serotype so far examined. The length of the ITR is very variable, ranging in size from 52bp in the avian AdV EDS-76, the smallest found so far, to 196bp in CAV-2. All other AdV ITRs range in size between these two.The ITR DNA sequences of CAV-1 (strain CCl-64) and CAV-2 (strain Toronto A26/61) were reported by Sira et al (1987) and Shinagawa et al (1987) respectively, the length of the CAV-1 ITR reported as being 160bp. The first nucleotide of the 5'-end of the ITR is always dC (deoxycytosine) with the exception of chick embryo lethal orphan AdV (CELO) which has a terminal dG (deoxyguanosine).

A striking property of the ITRs is the asymmetrical distribution of GC and AT (adenosine/thymidine) base pairs; a terminal AT rich region an internal GC rich region. DNA sequences within the AT rich region are highly conserved among all human AdV and partially conserved in the simian, murine, canine, equine and avian AdVs. Particularly notable are the 7bp sequence ATAATAT

located from nucleotides 9-15, thought to be involved in the initiation of DNA replication, and the 8bp sequence AATTTGGG, thought to be essential for encapsidation. The GC rich region is not highly conserved although some short blocks of conserved sequences can be found. One such sequence, GGGCGG, appears in multiple copies in the GC rich region of all human AdVs. Interestingly, this sequence is similar to the sequence GGGXGGAG which is present in multiple copies at the origin of the BK, SV40 and polyoma viruses (Ginsberg, 1984) The second sequence is TGACGT and this 6bp block is always present at or near the end of the ITR of human AdVs and the 5bp block TGACG in all other AdV ITRs. The function of the TGACG(T) block is at present unknown.

The phylogenetic relationships between AdVs as inferred from the nucleotide sequences of the ITRs, from a wide range of AdVs, has recently been examined by Shinagawa et al (1987). It was found that members belonging to the same AdV subgenus are closely related to each other whereas representatives of different human subgenera are distributed rather divergently among animal AdV. Thus the conclusion was that human AdV have not necessarily evolved from a common human AdV ancestor although it is almost certain that the two CAVs, CAV-1 and CAV-2, have arisen from a common CAV ancestor.

Terminal Protein

F I G U R E 2

Organisation of the Adenovirus Genome

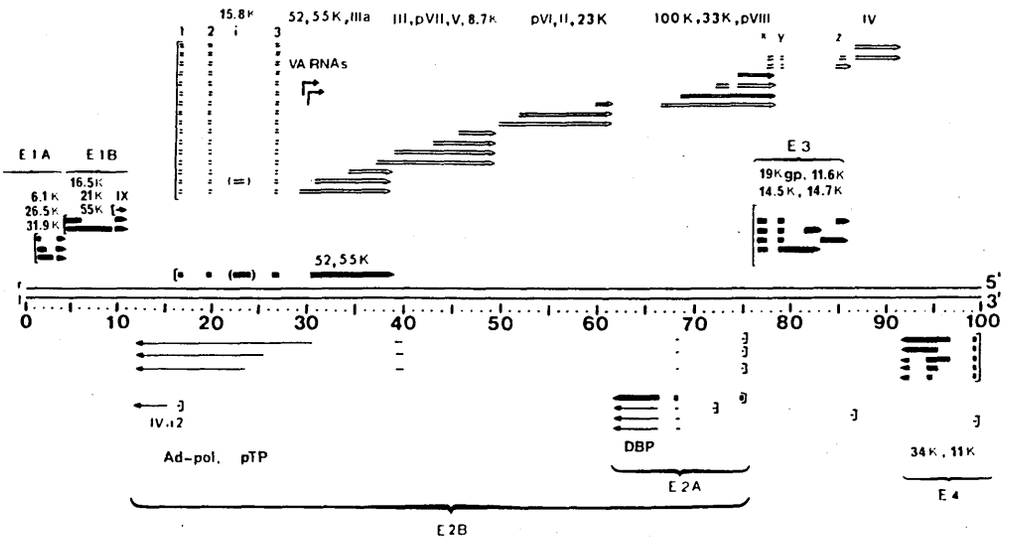


Fig. 2 Organisation of the Ad2 genome. Arrows show the location of the major RNA species. Thick lines illustrate the mRNAs expressed early after infection and thin lines mRNAs expressed at intermediate times after infection. Open arrows show sequences present in late mRNA. Polypeptides that have been assigned to the different regions are indicated. Adapted from 'The Adenoviruses', Doerfler (ed.), 1984.

The terminal protein (TP) is a highly conserved, approximately 55kD, virus-coded protein which is synthesised as an 80kD precursor (pTP). The pTP is the active form of the TP in AdV DNA replication. Stillman et al (1981) showed that cell free translation of mRNA selected from a region between co-ordinates 11 and 31.5 on the Ad2 genome l-strand (see fig.2) leads to synthesis of the three proteins, one of which is the 80kD pTP.

The TP is believed to play an essential role in the initiation of DNA replication. The protein is thought to recognise a specific sequence within the ITR, probably the conserved sequence ATAATAT (bp9-15), which might be involved in binding the pTP to the DNA, the first step in the replication of the AdV DNA. It has also been proposed that the TP protects the viral DNA against nucleolytic degradation. This function would explain the high infectivity of DNA-protein complexes. Deproteinised DNA is infectious when assayed by transfection into permissive cell lines but the infectivity of the DNA-TP complexes is 50-100 times higher (Sharp et al, 1976). Thus perhaps the two functions, aid to replication and DNA protection, are inextricably linked.

Early Region 1 (E1)

E1 is transcribed from the left-hand end of the viral r-strand (see fig.2) and contains the genes which are involved in the regulation of transcription and are responsible for cell transformation (Graham et al, 1974;

Berk and Sharp, 1977). The overall organisation of this region appears to be very similar for all the different serotypes of AdV. The region, map units (m.u.) 1.3-11.2, can be subdivided into three transcription units designated E1a (m.u. 1.4-4.5), E1b (m.u. 4.7-11.3) and region pIX (m.u. 9.9-11.3). The pIX transcription unit overlaps completely with E1b, encodes the structural virion polypeptide IX and has the only AdV mRNA which does not have a spliced structure. pIX is a minor structural component of the virion located between the hexons in the AdV icosahedron. Mutants lacking this gene are viable although less heat stable than wild-type virions, suggesting that the protein plays a role in stabilising the capsid structure. The gene is translated throughout both early and late phases of replication, although with the highest efficiency at late times, and is often referred to as an "intermediate" gene (Doerfler, 1986). In addition, a number of unidentified reading frames (URF) have been detected; in particular, a 22S mRNA which spans the border between E1a and E1b and encodes an 11kD polypeptide. However, the exact function of this transcription unit remains to be established.

E1A

The three mRNAs produced in region E1a: 13S, 12S and 9S: generated by differential splicing of a common nuclear precursor RNA, have common 5′ and 3′-ends but differ from each other by the size of the intron which is removed

during mRNA maturation. Analysis of the sequence of the E1a region, of all AdV serotypes so far examined, reveals the presence of a TATA box, e.g. TATTTATA in Ad2, and a polyadenylation site for all E1a mRNAs. The polyadenylation site is always preceded by the polyadenylation signal, AATAAA, which is specifically required for the cleavage of the precursor RNA prior to its polyadenylation (Doerfler, 1986). The E1a mRNAs accumulate with different kinetics during the infectious cycle. The 12S and 13S being the most abundant species early after infection, the 9S becoming the most abundant at late times. The polypeptides encoded by the 13S, 12S and 9S mRNAs are 289, 243 and 55 amino acid residues (a.a.) long with molecular weights of 31.9kD, 26.5kD and 6.1kD respectively.

Region E1a is the first to be expressed during the infectious cycle, transcripts can be detected 45 minutes after infection, and the 289a.a. protein encoded by the 13S mRNA is responsible for the stimulation of early viral transcription. This 289a.a. protein has been shown to be a nuclear antigen and stimulates transcription by RNA polymerases II and III from many promoters. The detailed mechanism of transcriptional activation (transactivation) by the E1a protein remains unclear but preliminary results suggest that the E1a product might act to stimulate the activity of cellular transcription factors (Kovesdi et al, 1986; Wu et al,1987). This process of transactivation by E1a may, in part, contribute to oncogenesis and

immortalisation which will be discussed later.

Studies in an animal model (cotton rats) revealed that a functional E1a was required for the onset of viral pneumonia (Ginsberg et al,1987). This was evinced by the use of AdV mutants. Mutants defective in all late functions were found to produce a pneumonia as extensive as that induced by wild-type virus. The pneumonia occurred even with mutants which were temperature sensitive (ts) at late times. These viruses do not replicate in the lungs because viral DNA cannot replicate at lung temperature and therefore viral structural proteins (i.e. late gene products) are not synthesised. E1a deletion mutants produced no pneumonia.

Recent studies have shown (Ishino et al, 1988) that the enteric AdV, Ad40, codes for only two E1a products unlike the respiratory AdV which produce those outlined above. In addition, the ability of Ad40 E1a to induce transformation is considerably reduced when compared to that of Ad5 and Ad12. The transactivation capability of the E1a gene is also much lower than that of other respiratory AdV to which it was compared by CAT (chloramphenicol acetyltransferase) assay. This variation is thought to be associated with the inability to propagate the virus in the standard cell lines in which it is blocked at an early stage in the replicative cycle. The relevance of these changes to the pathogenicity of the

virus is not discussed however but it seems likely that, as a fully functional E1a, as defined by the Ad2/Ad5 E1a, is necessary for the onset of viral pneumonia, this alteration in the coding capacity of the Ad40 E1a will prove significant in the pathogenesis of the virus. It also raises the question as to whether the newly isolated "enteric" CAVs will differ in the structure of their E1a regions as compared to the wild-type respiratory CAVs. The effect of E1a on pathogenesis other than respiratory infections is not clear. A new strain of the group D viruses, previously associated with conjunctivitis, has been found to cause gastroenteritis but the E1a region, on preliminary examination, appears identical to other "normal" group D viruses and the only alteration appears to be in the right-hand 25% of the genome (Gomes and Neil, 1987). This would indicate that other parts of the genome are responsible for/involved in gastrointestinal infections.

E1B

Region E1b is located immediately adjacent to region E1a, is also transcribed in a rightward direction and is stimulated by the E1a 289a.a. (fig.2). E1a is believed to mediate its effect on the E1b promoter through the TATA box transcription factor (Wu et al, 1987). Two major mRNAs (22S and 13S) and two minor mRNAs (14.5S and 14S) are generated by the splicing of a common precursor RNA. The accumulation of the E1b mRNAs is subjected to post-transcriptional regulation. The stability of the E1b

mRNAs is influenced by the E2A-72kD DNA binding protein (DBP) and at late times by a change in the specificity of the RNA splicing machinery (reviewed in Doerfler, 1986).

Region E1b contains three long open reading frames (ORF) which encode polypeptides with m.w. of 14.3kD, 21kD and 55kD. The 14.3kD polypeptide corresponds to the virion polypeptide IX. Gosh-Choudhury et al (1987) revealed that protein IX is essential for the packaging of full length genomes and is dispensable only for virions containing DNA of less than genomic size. Edbauer et al (1987) revealed that, in Ad12 at least, this protein is not required for oncogenic transformation. The 21kD polypeptide is associated with both the nuclear and the plasma membrane fractions of both infected and transformed cells and its localisation on the cell surface makes it a likely candidate for the tumour specific transplantation antigen (TSTA) expressed by AdV-transformed cells (Rowe et al, 1983). The 21kD polypeptide is also believed to function as a nuclease inhibitor as, in mutants defective for the 21kD protein, its absence results in the degradation of both viral and cellular DNA during infection (Pilder et al, 1984).

The 55kD protein is reported to be essential for efficient shut-off of host protein synthesis, demonstrated by the use of engineered mutants (Babiss and Ginsberg, 1984). The translation of late proteins and the synthesis of late

cytoplasmic mRNAs are drastically reduced if an intact E1b region is not present. It therefore appears that the 55kD protein is required for the transition from the early to the late mode of gene expression. The 55kD protein also appears to be one of the products responsible for the production of AdV pneumonia (Ginsberg et al, 1987) which could be related, either directly or indirectly, to its association with the shut-off of host cell protein synthesis.

The role of E1b has also been investigated by Mautner and Mackay (1987) who demonstrated the effectiveness of Ad40, an enteric AdV, as compared to Ad2 and Ad5. Studies in a range of host cells and using complementation with Ad2 and Ad5 mutants showed that Ad40 had a functional E1a as defined by its ability to complement E1a defective Ad2 and Ad5. However the Ad40 E1b could not complement E1b defective Ad2 or Ad5. Ad40 growth in normal non-permissive cell lines, e.g. HeLa cells, was made possible in the presence of a functional E1 region from Ad2 or Ad5 but not in the presence of E1a alone. It was suggested that this "dysfunction" of Ad40 E1b plays a role in the host range of the virus.

Early Region 2 (E2)

Region E2, which is transcribed from the viral l-strand (fig.2), differs from other AdV transcription units by using alternative promoter sites for the initiation of transcription (Chow et al, 1979). An early promoter (E2-

E) is activated at early times after infection, m.u. 75.4 in Ad2, following which a promoter shift occurs at late times during infection to the E2 late promoter (E2-L), located at m.u. 72.2 in Ad2. The two E2 promoters are regulated differently; the E2-E promoter is E1a inducible whereas the E2-L promoter appears to be inhibited by E1a. The transactivation of E2-E by E1a appears to involve a cellular transcription factor, the concentration or binding activity of which increases as a result of the action of the E1a gene product (Kovesdi et al, 1986). This regulation can occur even when the E2 promoter region is stably integrated into the cellular genome (Kingston et al, 1984).

Two major classes of transcripts are generated from E2. The first mRNA class, designated E2A, extends from the transcription initiation site to a polyadenylation site, at m.u. 62.4 in Ad2, and codes for the single-strand-specific DNA-binding protein (DBP). The DBP is a multifunctional protein, is phosphorylated and has an apparent m.w. of 72kD. It is involved in DNA replication, in regulation of early and late gene expression and in cell transformation. In the absence of a functional DBP the normal turn off of early mRNA expression does not occur (Carter and Blanton, 1978). The DBP also influences the host range of the virus: Brough et al (1985) reported that a very limited number of changes in the 5' portion of the DBP gene gives rise to Ad2 mutants which can replicate

efficiently in monkey cells. Wild-type Ad2 fails to multiply in the same monkey cells due to a block to late viral gene expression. Additionally, they found that minor mutations in the 3' portion of the DBP gene gave rise to ts phenotypes. Conversely, mutations in the DBP appear to have no effect upon the pathogenicity of the virus in that DBP mutants produce viral pneumonia to the same extent as wild-type virus although the mutants are ts and could not replicate at lung temperature (Ginsberg et al, 1987). A comparison of the DBP of animal AdVs demonstrated to replicate (altered host range) and cause disease (retained pathogenicity) in animals other than the host of origin, e.g. enteritis in sheep due to bovine AdV and Rubarth's disease due to CAV-1 in bears (Koptopolous and Cornwell, 1981: Kapp and Lehoczki, 1966), may therefore prove valuable in the understanding of AdV pathogenicity and determination of host range.

The second major class of transcripts, designated E2B, by-pass the polyadenylation signal used by the E2A mRNAs and extend to a second polyadenylation site, at m.u. 11.3 in Ad2. Three differentially spliced mRNAs have been identified from region E2B (Ginsberg, 1984). The longest of the E2B mRNAs encodes the pTP which serves as a primer for DNA replication and which is later cleaved to its mature 55kD form by a viral encoded protease. The shortest of the E2B mRNAs encodes a 140kD polypeptide which is the DNA polymerase required to replicate the DNA. Mutations in the DNA polymerase gene had no effect upon the

pathogenicity of the virus as viruses defective in the production of the viral polymerase were capable of producing a wild-type pneumonia in infected animals (Ginsberg et al, 1987). The function of the intermediate-sized E2B mRNA is at present unknown.

Early Region 3 (E3)

Region E3 is transcribed from the viral r-strand and is located completely within the major late transcription unit (fig.2). The E3 mRNAs are generated by differential processing of two major RNA precursors which have a common cap site but different 3'-termini. In Ad2 the major polyadenylation site is located at m.u. 85.9, and is preceded by the hexanucleotide sequence AAUAAA. A second polyadenylation site, at m.u. 82.9, is less frequently used.

At least nine E3 mRNAs are generated by differential splicing and most of the splice junctions have been characterised by sequence analysis of several human AdVs. Based on the DNA sequence, seven of the proteins encoded by this region are believed to be membrane proteins (Cladaras and Wold, 1985). Functional studies on proteins derived from E3 have been limited mainly because E3 can be deleted without apparent effect on virus growth in cultured cells (Kelly and Lewis, 1973). E3 is not required for replication of Ad5 in the lungs of acutely infected hamsters. However, the overall organisation of E3

is highly conserved between even distantly related serotypes implying that E3 serves an important function when the virus infects its natural host.

Support for this theory is provided by Belak et al (1986) who compared the genomes of two bovine AdV, one bovine specific and one capable of producing, and routinely isolated from, enteritis in sheep as well as cattle. Ninety-five precent of the two genomes proved highly homologous but major differences occurred between their E3 regions. It is also feasible that the human group D AdV (normally associated with ocular infections) isolate described by Gomes and Neil (1987) which was found to be the causative agent of an outbreak of gastroenteritis also contains variation within E3. All variation noted, by restriction enzyme analysis, between the enteric group D virus and other group D viruses was confined to the right-hand 25% of the genome which includes the E3 region. It seems apparent therefore that E3 must serve an important biological function.

Only three proteins have thus far been purified and mapped to E3; a 19kD glycoprotein, an 11.6kD polypeptide and a 14.7kD polypeptide (Tollefson and Wold, 1988). The E3/19kD has been demonstrated to bind to human histocompatability class I antigens (HLA). The formation of an HLA-E3/19kD complex prevents the HLA antigens from being correctly processed by inhibiting their terminal glycosylation. This effect is specific for HLA antigens

and does not generally involve the glycosyltransferases. In addition, the HLA-E3/19kD association dramatically reduces the cell surface expression of the HLA antigens. This reduced level of antigens may influence the cytotoxic T-cell response and might show a possible molecular mechanism whereby AdV escape the cellular immune system of the host (Burgert and Kvist, 1985; Paabo et al, 1986). All serotypes so far examined have demonstrated this capability. No functions have been ascribed to any other E3 products as yet although it has been proposed that several of them may be cell membrane proteins.

The findings of Belak et al (1986) support this hypothesis. They observed differences between the E3 regions of two bovine AdV, one of which was capable of replicating and causing disease in two distinct animal species (bovine and ovine). They propose that the virus in this specific case has been "placed under pressure to alter the genes that influence the interaction between the virus and its host, rather than the genes which determine the structure of epitopes that are exposed on the surface of the viral capsid (hexon and fibre)". Other reports of natural transmission of AdV to an animal species different from the host of origin are rare and have not, as yet, been clearly associated with a specific area of the genome. It is interesting to note however that one such occurrence includes the transmission of CAV-1 which resulted in an outbreak of Rubarth's disease (canine

hepatitis) in bears (Kapp and Lehoczki, 1966).

Deletions and/or mutations in the E3 region led to latency of the virus in lymphocytes when Ad5 mutants were inoculated into cotton rats. Latent infection was maintained indefinitely *in vitro* when excess neutralising antibodies were added to the culture. Virus became detectable and the amount of infectious virus rapidly increased after the antibodies were removed (Ginsberg *et al*, 1987). The cause and effect of these results are not clear at present.

Early Region 4 (E4)

Region E4 is transcribed from the l-strand and is located at the extreme right end of the genome (Fig.2). The E1a 289 a.a. protein induces E4 transcription early after infection whereas the E2A 72kD-BP has been shown to down regulate E4 transcription at intermediate to late times (Raychaudhuri *et al*, 1987a,b: Handa *et al*, 1983). The E1a-mediated induction of E4 , as with E2 transcription, involves the post-translational activation of previously limiting cellular factors. Two binding sites for the E1a induced cellular factors have been demonstrated upstream of the E4 TATA box both comprising multiple copies of hepatanucleotide sequences (Cortez *et al*, 1987; Leza and Hearing, 1987). These cellular factors are independent of the cellular factors involved in E1a transactivation of other AdV promoters thereby confirming that E1a control involves an activation of multiple promoter specific

binding proteins.

The primary transcript from E4 is spliced into a complicated set of cytoplasmic mRNAs (Tigges and Raskas, 1984); alternate splicing of the precursor RNA can generate as many as 25 mRNAs that encode at least 16 polypeptides. Of particular interest among the E4 mRNAs is an extensively spliced class which includes multiple species with sizes ranging from 1.1 to 0.75kb which were found to specify at least 10 polypeptides in vitro.

Identification of E4 proteins produced in vivo has been difficult because E4 is not expressed at high levels during productive infections. The only viral protein conclusively identified as an in vivo product of E4, an 11kD polypeptide, has been found to be tightly bound to the nuclear matrix. This protein is highly conserved in different AdV serotypes (Sarnow, et al, 1982). Expression of this product is not essential for virus yield in cultured cells.

In the absence of E4 AdV are defective in the synthesis of late mRNAs and proteins. Viral DNA replication appears normal but the shut off of host cell macromolecule synthesis seems to be incomplete. Since mutants in the E1b-55kD polypeptide are also defective in late mRNA synthesis and in the control of cellular gene expression it seems likely that the physical complex formed between

the E1b-55kD and the E4 product (possibly a 34kD polypeptide) is of major significance for the switch from early to late AdV gene expression. The E4 region has been demonstrated to be capable of increasing the cytoplasmic level of mRNAs from the major late promoter transcription unit up to 10-fold (Nordivist et al, 1987). This function appears to be associated with the left-hand end of the E4 transcription unit (Sandler and Ketner, 1989). An unknown product from region E4 is also required for adeno-associated virus (AAV) to replicate in human cells (Doerfler, 1986).

An unidentified diffusible E4 product has been demonstrated to be essential for efficient virus particle assembly (Falgout and Ketner, 1987). Nothing is known about the functions of other E4 proteins.

VA RNA and Late mRNA Translation

At late times after AdV infection, the majority of viral mRNAs are transcribed from the major late promoter (MLP). Five families of 3' coterminal mRNAs (L1-L5) are derived from a primary transcript, all of which contain an identical 5' noncoding tripartite leader segment 200 nucleotides in length, joined to the various coding regions by the splicing of three small exons (fig.3). Transcription from the MLP accounts for nearly 30% of the total RNA polymerase II activity at late times (Ginsberg, 1984). The MLP of AdV has been intensively studied as it is reported to be the strongest promoter in most in vivo

transcription systems (Ginsberg, 1984).

The AdV genome encodes two low m.w. RNAs designated virus associated RNA (VA RNA) I and II which are produced in large amounts late during infection. Neither VA RNAI or VA RNAII is post-transcriptionally processed. Both RNAs are approximately 160 nucleotides long and are transcribed from two closely spaced transcription units located within the L1 cotermination family. Studies of viral mutants have shown the VA RNAI is required for efficient translation of viral mRNAs at late times. Svensson and Akusjarvi (1986 and 1984b) reported that mutants defective in the production of VA RNAI have substantially reduced levels of L2, L3 and L5 mRNAs. Although normal levels of mRNA were found from L1 and L4, the L1 pre-mRNA was found to be defective in splicing all except one of its normal three mRNA products. A double mutant lacking both VA RNAs was shown to grow very poorly, producing 5- to 6-fold less virus compared to wild-type. Since a virus lacking the VA RNAII species grows like wild-type, it is likely that VA RNAII serves a similar function to VA RNAI. VA RNAII has been shown to partially substitute for defective VA RNAI species in lytic growth.

Virion component and abundant non-virion proteins are encoded by late mRNAs. Each member of a family has the tripartite leader spliced to the body of the mRNA (Chow et al, 1977). Although the abundant form of each late mRNA

has the standard tripartite leader, variant forms of almost any late mRNA can be detected. These variant forms frequently have a fourth leader mapping at 22 m.u., spliced between the second and third leader of the standard set (fig.3). The structure of the mRNA for fibre is particularly complex. Various combinations of leader sequences, i.e. X, Y or Z, mapping at 77, 79 and 85 m.u. respectively, are inserted between the standard third leader and the main body of fibre mRNA (Chow et al, 1979). It is believed that E3 splice sites in this area are interfering with the fibre splice sites and, as no function can be found for these aberrant fibre mRNAs, that they are a non-essential by-product of the splicing process. Only 10% of the virion components synthesised during the late stage are assembled into viral particles.

The major event governing the transition from the early mode to the late mode of gene expression appears to be DNA replication. Unreplicated DNA, when introduced into AdV infected cells which are in late phase, still fail to express the late viral genes (Ginsberg, 1984). A termination site for late transcription has been mapped at 99 m.u. RNA sequences from the r-strand between 91.9 m.u., the poly(A) site of fibre mRNA, and 99 m.u. are confined to the nucleus and rapidly degraded (Dressler and Fraser, 1987).

The protein products of each MLP family are shown in figure 3. Briefly, the proteins with known function are

F I G U R E 3

Structure of mRNAs Originating at the Major Late Promoter

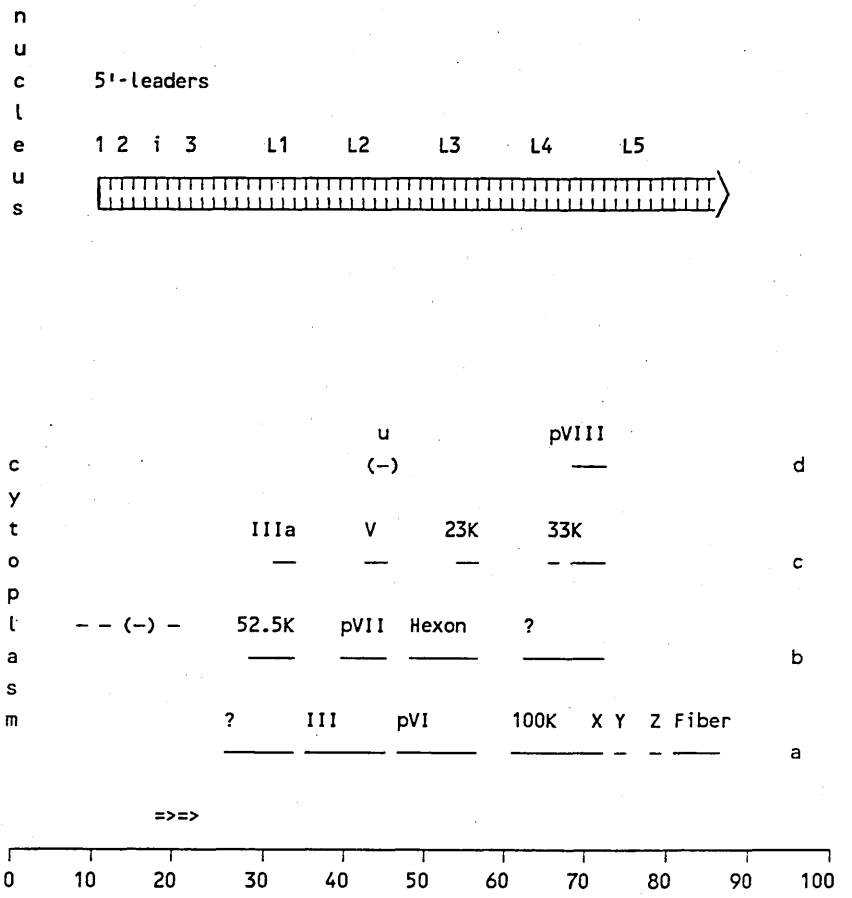


Fig.3 Structure of mRNAs originating from the MLP at late times after infection. The mRNAs are divided into five families (L1-L5), each having coterminal 3'-ends and a common set of 5' leaders spliced onto the RNA body. The i-leader, which is found on a fraction of the mRNAs, is shown in parenthesis. The position of the VA RNA genes are shown by open arrows (=>).

as follows:-

L1 - the most abundant mRNA encodes the virion polypeptide IIIa which is a minor component of the virus particle located in the vertex region of the virion.

L2 - three polypeptides are coded by this region, designated III, V and pVII. Polypeptide III forms part of the penton base. Polypeptides V and pVII constitute the major proteins found in AdV chromatin. pVII is tightly bound to the viral DNA and it has been proposed that the polypeptide V forms a protein shell around a nucleoprotein particle consisting of polypeptide VII and the viral DNA.

L3 - this region encodes three extensively studied polypeptides, pVI, hexon and 23kD. pVI is believed to be associated with the hexon capsomere on the inside of the virus particle. The hexon is the major structural component of the virion and the 240 hexon capsomeres form the facets of the icosahedron. The hexon appears to be well conserved throughout most mammalian AdV. Boursnell and Mautner (1981) revealed that the two ends of the hexon molecule were well conserved between the strains examined and that variation is more common within the central region which is thus likely to contain the type-specific antigenic determinants. p23kD has been shown to be responsible for the proteolytic cleavage of many of the AdV polypeptides.

L4 - polypeptide VIII is known to be a minor component

of the virus particle however no function is known for the other products of this region except that they are believed to be non-structural.

L5 - a single polypeptide, the fibre protein, is encoded by this region. The fibre is present at the vertices of the virion and attaches to the cellular receptors. It has been shown to be composed of a dimeric structure with a characteristic rod and knob appearance. For a review of the above see Ginsberg (1984) and Doerfler, (1986).

The number and length of the fibre varies according to the species and subgroup of AdV. Four of the six human AdV subgenera are well endowed with fibres of different length:- A - 28-31nm; B - 9-11nm; C - 23-31nm; D - 12-13nm; E - 17nm; F - 28-33nm. The relevance of these differences to the pathogenicity, if any, is not yet known although it must play a significant role in the tissue tropism as defined by attachment of the virus, not ability to replicate, to various cell types. Avian AdV, except the EDS virus, have been found to code for two fibres of different lengths extending from different sites on the penton base (Ginsberg, 1984).

mRNAs have been detected from the L1 family at early times after infection which initiate at the MLP. The level of early mRNAs at the MLP is approximately one tenth that of other early promoters. One of the mRNAs found is thought,

due to finding a polypeptide product of 14kD, to be functional (Akusjarvi and Persson, 1980).

DNA Replication

A model for AdV DNA replication is shown in figure 4. As described previously, the origins of AdV DNA replication are known to lie at the ends of the viral genome. Hay et al (1984) isolated the AdV origins of replication from both the right and left ends of the genome which were functional on linear autonomously replicating mini-chromosomes. The mini-chromosomes contained two cloned ITRs and required non-defective AdV as a helper. Replicated molecules were found to be covalently attached to protein and DNA synthesis was initiated at the correct nucleotide even when the origins were not located at molecular ends. The activity of the embedded origins led to the generation of linear mini-chromosomes from circular or linear molecules thus suggesting that the sequences within the AdV origin of replication position the protein priming event at the AdV terminus.

Following each initiation event a daughter strand is synthesised in a 5′-to-3′ direction. Initiation events that produce type I and type II molecules occur at approximately the same frequency at either end. Thus, when initiation occurs at the right molecular end the parental l-strand serves as a template and the parental r-strand is displaced and vice versa for the left end. Since the 3′- terminus of the l- and r-strands are

identical, it seems likely that all initiation events proceed by the same molecular mechanism.

One of the remarkable features of AdV DNA replication is the use of a protein to prime DNA synthesis. The initial event in DNA replication is believed to be the binding of the pTP to the terminus of the parental genome. This is thought to be mediated by the conserved terminal nucleotide sequences and may be aided by the presence of the TP attached to the parental DNA. Berkner and Sharp (1983) demonstrated that cloned viral DNA with EcoRI linkers on the termini were 100-fold less efficient in DNA replication than wild-type virus. This was thought to be due to the lack of a residual peptide, after proteolytic digestion of viral DNA, at the termini.

A total of five proteins are required for efficient DNA replication in Ad2, the most intensively studied so far. The pTP, the DBP, AdV DNA polymerase and two cellular factors, nuclear factors I and II (NFI and NFII). DNA replication does not occur in the absence of the DBP which binds strongly to the single-stranded DNA (ssDNA) during replication, which is thought to stabilise the event, and interacts with the AdV DNA polymerase. AdV DNA polymerase contains an intrinsic exonuclease activity. It is thought that an associated nuclease removes misincorporated and mispaired nucleotides thus increasing the fidelity of the DNA synthesis.

F I G U R E 4

A Model For Adenovirus DNA Replication

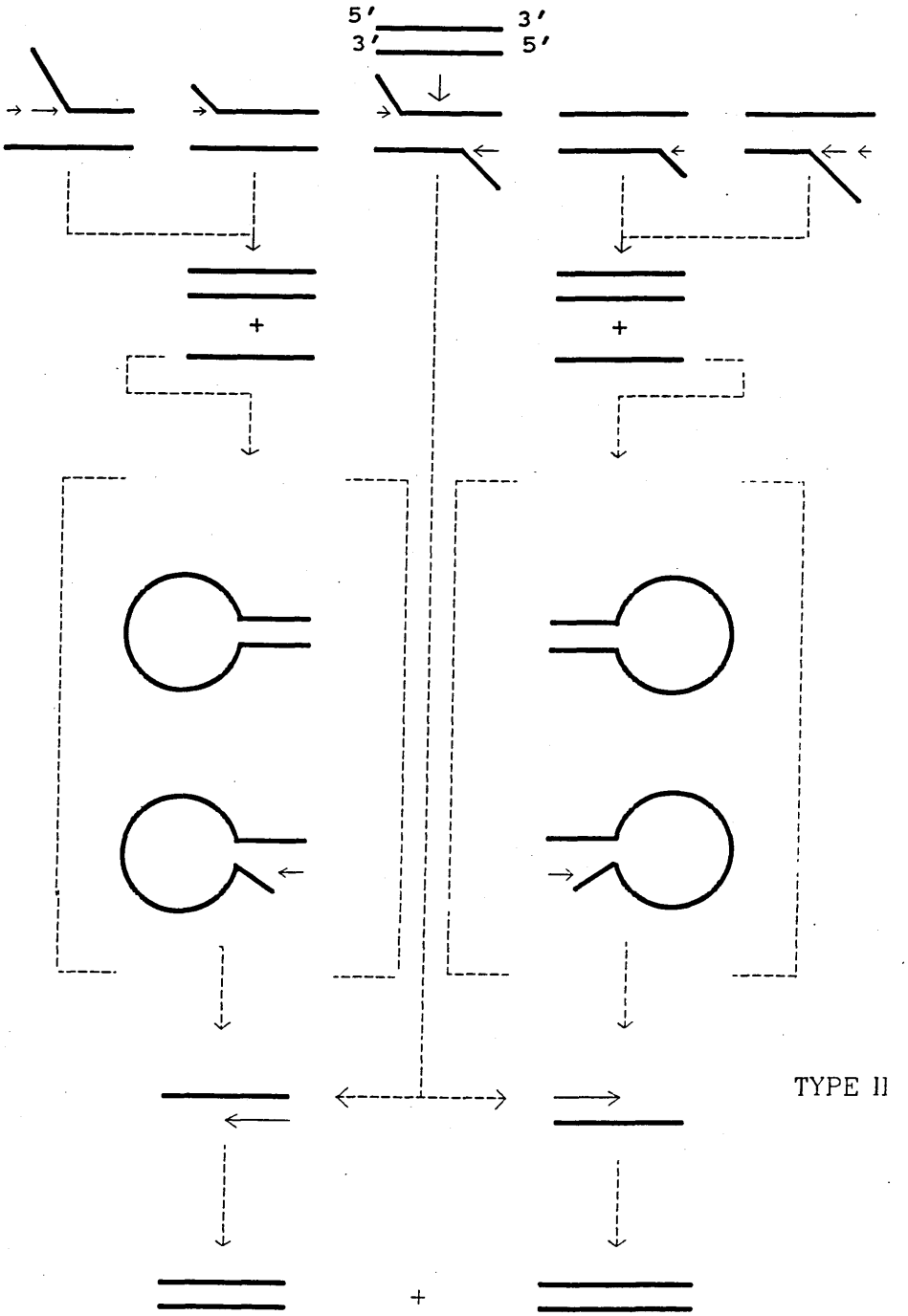


Fig. 4 Model for AdV DNA replication. Newly synthesised DNA strands are depicted by bold lines, parental DNA strands by light lines. Arrows indicate the direction of chain elongation. The bracketed circular "panhandle" forms are theoretical intermediates. Adapted from Ginsberg (1984).

NFI is known to be a sequence-specific DNA binding protein. The first 18bp of the viral genome have been demonstrated to be adequate to support a limited degree of initiation and replication (Hay and McDougall, 1986). However, the presence of sequence elements in the region between nucleotides 19-48, the NFI binding site, greatly enhances the efficiency of the initiation reaction. NFII has been shown to possess topoisomerase I activity but the enhancement of DNA replication by NFII is completely dependant on the presence of NFI; in the absence of NFI, NFII does not stimulate DNA replication.

Studies on nuclear factors have also been used to demonstrate the relationship of different AdV species to each other. Interestingly, the question of the origin of EDS virus (mammalian origin or distinct from both avian and animal AdV?) is further confused by these studies to date. Although most information indicates that EDS virus is an AdV of mammalian origin (personal communication, Dr. N. Spibey), Endo et al (1986) demonstrated that while a nuclear factor from MDBK cells would bind, with varying efficiency, to all mammalian AdVs examined, it did not demonstrate any binding capacity to the EDS virus genome.

It has recently been reported that a sixth protein, nuclear factor III (NFIII), may enhance Ad2 DNA replication. NFIII is also a sequence-specific DNA binding protein and has been found to bind to a region in the AdV origin, adjacent to the NFI binding site (Pruijn

et al, 1987).

AdV DNA replication is therefore controlled in a complex manner. In addition, the method of DNA replication for each type of AdV, although requiring the conserved sequences contained within the terminal 18bp of the ITR, does not necessarily utilise, or be enhanced by, NFI, II or III. Harris and Hay (1988) demonstrated that initiation of Ad4 was not enhanced by either NFI or NFIII, or by a combination of the two as is Ad2, but was shown to be stimulated by an unknown cellular factor which has not been demonstrated to be a stimulant for Ad2 initiation. Thus different AdVs have different DNA sequence requirements for the initiation of DNA replication involving a variety of cellular enhancers many of which are, as yet, unidentified.

The model in fig.4 indicates two possible pathways for AdV DNA replication. Both pathways have been deduced from EM studies of AdV DNA replication. Support of the "panhandle" structure during DNA replication is provided by Hay et al (1984). Experiments investigating the regeneration of deleted viral ITR sequences showed a sequence-independent requirement for inverted sequences in this process, suggesting that repair results from the formation of a "panhandle" structure by a displaced single strand. However, whether "panhandle" formation is an obligatory, or major, pathway in AdV replication remains

to be seen.

Oncogenicity and Transformation

Based on their ability to induce tumours in newborn hamsters, the human AdV were classified into different subgroups as described earlier. The highly oncogenic group A viruses induce tumours with high frequency and the latent period is very short whereas the moderately oncogenic group B cause tumours in only a small fraction of the animals and tumours become apparent only after a relatively long latent period. Cells transformed by AdVs can have a wide range of properties, ranging from the ability to divide indefinitely in tissue culture (immortalisation) to the ability to cause tumour formation in immunocompetent animals. Thus AdV transformed cells are usually defined as mammalian cells that have an altered phenotype resulting from infection with virus or from transfection with viral DNA. The CAVs are reported to be highly oncogenic as defined by the phenotypic changes occurring in CAV transformed cells and their ability to induce tumours rapidly in newborn hamsters (Sarma et al, 1967; Kinjo et al, 1968; Dulac et al, 1969).

Transformation by AdVs requires the integration of the left 14%, E1 region, of the virus genome into the cellular chromosome although the entire viral genome can be inserted. Analysis of the integration pattern of the viral DNA sequences appears to indicate that there is no specific integration site into the cellular DNA. In

addition, the site of integration, as well as all viral genes except E1a, plays no important role in the maintenance of the transformed phenotype (Graham et al, 1977; Spector, 1983; Ginsberg, 1984). The relative role of each part of the E1 region in the transformation of cells is shown in figure 5. The areas described have been defined by a variety of workers using both constructed and natural mutants of various AdVs (for a review see Doerfler, 1986).

E1a proteins are required for the efficient expression of early viral genes whose products are essential for DNA replication. E1a proteins can activate transcription of viral and non-viral genes both transiently and stably introduced into cells. E1a proteins can also stimulate the expression of some endogenous cellular genes. The observation that E1a proteins transcriptionally stimulate cellular genes has led to the suggestion that the basis of E1a mediated transformation may be the activation of cellular genes involved in normal growth control. Consistent with this possibility, E1a mutants that fail to activate transcription are also transformation-defective (Lillie et al, 1986). Two functional regions have been localised on the closely related E1a products: the 243 and the 289 a.a. phosphoproteins, one of which is required for efficient transactivation, the other for transcriptional repression. The 289 a.a. protein contains both regions and appears to function mainly as a transactivator whereas

the 243 a.a. protein lacks the transcriptional activation domain and appears to function as a transcriptional repressor. It is possible that E1a proteins deregulate cell growth by repressing the transcription of specific cellular regulatory genes.

This is in agreement with the findings of Jochemsen et al (1986) who reported that the difference in oncogenic potential of Ad5 and Ad12 was due to the different capacities of the Ad5 and Ad12 E1a-encoded proteins to modulate cellular gene expression. Support is also given to this theory by the fact that the E1a regions of all highly oncogenic AdV so far examined, human and non-human, are closely related (Kimelman et al, 1985).

One of the cellular targets implicated in the process of transformation by the AdV E1a proteins is a 105kD cellular protein. This protein has been shown to form stable protein/protein complexes with the E1a polypeptides. It has recently been identified as the product of the retinoblastoma gene and subsequently is the first demonstration of a physical link between an oncogene and an anti-oncogene (Whyte et al, 1988).

Adenovirus Vectors

The use of AdV as vaccine vectors is a rapidly growing field of study. The observation that certain regions of the AdV genome may be deleted without detectable loss of replication ability and stability of the virus in vitro,

F I G U R E 5

Genomic Regions Required For Adenovirus Transformation

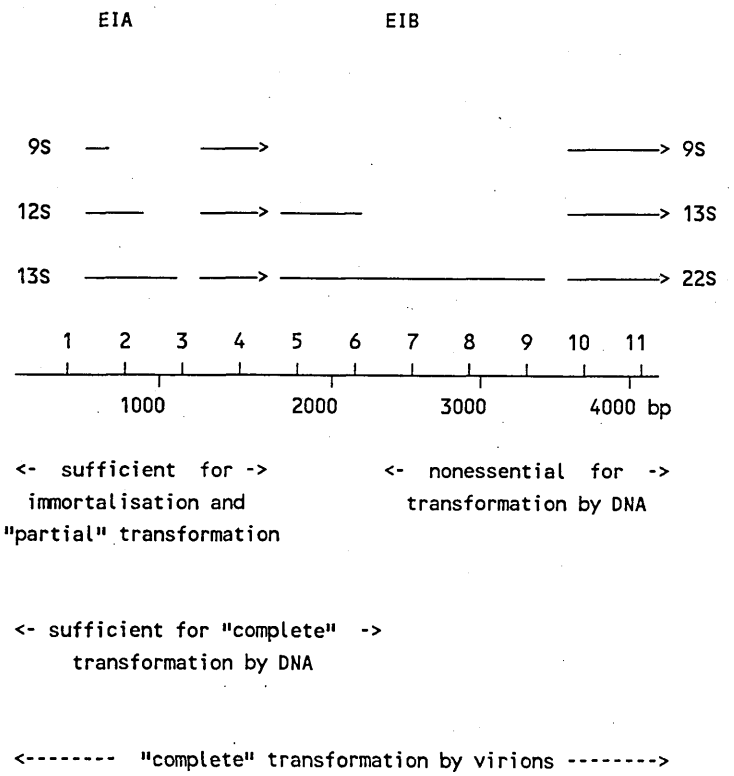


Fig.5 Sequences required for cell transformation by AdV DNA or virions. Adapted from Ginsberg (1984).

for example E3, E1 if the virus is grown in 293 cells and certain areas of E4 (for a review see Berkner, 1988), has lead to many groups investigating the virus as both a vaccine vector and as a complex expression vector. Without deletion of viral sequences the human AdV virion capsid can accommodate up to 2kb of additional DNA and, if compensating deletions of viral DNA are made, as much as 7kb of foreign DNA can be inserted. It has been reported that AdV genomes greater than 105% of the wild type genome in size will not be packaged (Munz and Young, 1987).

AdV vectors used as expression vectors have been demonstrated to be efficient in post-translational processing, including glycosylation, phosphorylation and carboxylation. This ability, combined with the transforming capabilities of AdV generating stable integrants and the wide host range of AdVs in general, makes it an ideal expression vector. Inserted genes have already been demonstrated to be expressed at high levels by the AdV vectors e.g. the HSV1 thymidine kinase (TK) gene; human B-globin genes; polyomavirus middle T antigen; and SV40 large T antigen (Yamada et al, 1985: Karlsson et al, 1986: Davidson and Hassell, 1987; Berkner et al, 1987: Thummel and Tjian, 1981 respectively). In addition to the expression of these foreign genes, the AdV expression vectors facilitate the study of gene expression of otherwise "difficult" genes. This aspect was clearly demonstrated by Saito et al (1986) who utilised an Ad5-hepatitis B virus (HBV) construct, carrying 87% of the

total HBV genome, to analyse the genomic organisation of the virus, a feat which had hitherto proved limited due to the lack of an *in vitro* system to propagate HBV in human cells. Control of these foreign genes has been tested under a number of promoters e.g. the AdV major late promoter (MLP) and the SV40 early promoter.

Adenovirus Vectors and Vaccines

AdV vaccines have already been demonstrated to be safe and effective with, for example, Ad4 and Ad7 being used as human vaccines and the CAV vaccines being one of the most widely used and effective veterinary viral vaccines. In addition, the ability of AdV to be administered orally makes the potential use of of AdV vectors for vaccine production particularly attractive.

Again, as with the expression vectors, a variety of models have been tested to determine the efficacy of AdV vaccine vectors. A herpes simplex virus glycoprotein B (HSVgpB)/AdV vector has been shown not only to induce production of circulating antibody in infected mice but to be able to protect mice against a subsequent challenge with HSV (McDermott *et al*, 1989). A variety of AdV vectors encoding HIV products, e.g. gp160, gp140, *env* protein, are presently being studied by a large number of groups but more importantly their efficacy as vaccines is being evaluated.

However, the inability to test any human vaccine vectors in their natural host (testing in simian species has been used to date) has proved a stumbling block in the complete testing and use of these engineered viruses. The finding that cotton rats are permissive for human AdV, producing very similar clinical symptoms, may aid to some degree but it does not overcome the problem altogether (Pacini et al, 1984). The study of both wild-type and mutant AdV in cotton rats has raised some interesting questions. Ginsberg et al (1987) revealed that E1a deletion mutants : and therefore by analogy E1a/insertion vectors: produced no viral pneumonia, an extremely beneficial factor for vectors deleted in this region. However, it was also found that E1a and E3 deletions in the viral genome lead to the viruses becoming latent within lymphocytes. The significance of this finding will obviously have great relevance to the use of AdV vectors. Questions remain, however, as to whether this latency is of significance to the host. The continued expression of the inserted "foreign" gene and AdV proteins might be either beneficial or extremely detrimental. Additionally, latency might be due to the fact that the virus and viral mutants are infecting/replicating in a non-host animal which poses the questions whether an E1a or E3 deletion would mutant lead to latency in its natural host.

The CAVs therefore provide an excellent solution to this problem. If the genome organisation could be determined and the position of E3 mapped accurately the CAVs would

provide an excellent animal model for the AdV vectors. In addition, when this is combined with the fact that excellent CAV vaccines are already in widespread use, the use of a vaccine strain as an AdV vector may overcome many of the problems that it is feared may occur when trying to initiate the use of AdV hosts as vaccine vectors as the "safety" of the host itself, unless altered dramatically by the insertion of foreign genes which could be quickly checked by passage and testing of the vector in controlled dogs, is already beyond question. Latency of vectors within lymphocytes could be fully examined if present and its long term effect upon the inoculated animals investigated. The vectors, expressing for example the rabies virus glycoprotein, could be fully tested *in vivo* in their natural hosts providing not only a good animal model for the human, and other, AdV but, if successful, perhaps one of the first commercially viable mammalian engineered viral vaccines.

Objectives

The purposes of the present study were two-fold. Firstly an investigation into the genomic organisation of the CAVs was required as a prerequisite to their development as viral vectors along the lines of those developed by Haj-ahmed and Graham (1986b). Secondly, an investigation into the molecular basis of pathogenicity in the CAVs was undertaken. To this end commercially available vaccine strains were utilised in order to compare their genomic

organisation to both the human adenoviruses and wild-type CAVs.

C H A P T E R 1

General Materials and Methods

The material and methods detailed below are used repeatedly throughout the experiments in the following chapters. Any method specifically relating to work in an individual chapter is detailed in the materials and methods section of that chapter.

Viral Strains

Canine Adenovirus Type 2 Strains:-

Toronto - kindly given by Dr.C. Cornwell (University of Glasgow).

Manhattan - kindly given by Dr.W. Wunner (Wistar Inst., Philadelphia, U.S.A.).

Glasgow - a faecal isolate from dogs with enteric disease (Macartney et al., 1988).

Epivac (2) - Epivac DH_2 vaccine strain (Wellcome Foundation) lot number B16013A.

Kavak - Kavak DA_2 vaccine strain (Duphar) lot number 16014-B.

Nobivac - Nobivac DH_2 vaccine strain (Intervet) batch number 107.

Vaxitas - Vaxitas DA_2 vaccine strain (ICI Tasman) lot number 438.

Canine Adenovirus Type 1 Strains:-

RI 127 - kindly given by Dr.C. Cornwell, University of Glasgow.

Boostervac - Boostervac DH vaccine strain (C-Vet) lot number 2012

Epivac (1) - Epivac DH vaccine strain (Wellcome) lot number B TCD 280A

Culture of Monolayer Cells

Cell Types

CAV is routinely grown in Madin and Darby canine kidney cells (MDCK). However, the Vaxitas strain of CAV-2 is grown in normal mink fibroblasts (NMF) or Mv-1-Lu a mink cell line established by Kniazoff, Nelson, Rees and Darby in 1964 (personal communication, ICI Tasman) from near term Aleutian mink foetuses (American type culture collection number CCL 64). MDCK and NMF cell lines kindly provided by Prof. O. Jarrett, University of Glasgow; Mv-1-Lu kindly provided by ICI Tasman.

Media

MDCK and Mv-1-Lu cell lines were maintained in Eagle's Minimum Essential Medium supplemented with 2mM L-glutamine, 400 units/ml penicillin/streptomycin, 2.5µg/ml Amphotericin B, 1x Eagle's non-essential amino acids and 10% foetal bovine serum. The complete medium is referred to as 10% MEM.

NMF cells were maintained in Dulbecco's Minimum Essential Medium supplemented with 2mM L-glutamine, 400 units/ml penicillin/streptomycin, 2.5µg/ml Amphotericin B and 10% foetal bovine serum. The complete medium is referred to as 10% DMEM.

All media and supplements were purchased from Imperial Laboratories Ltd., Wiltshire.

Cell Culture

Stock monolayer cells were passaged in 180cm^2 plastic flasks with 40ml of media and routinely subcultured 1:3 twice weekly. Cells were removed from the plastic by rinsing with versene (0.02% Ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS)) followed by 0.01% trypsin in 0.02% EDTA. Resuspended cells were transferred to new flasks and gassed with 5% carbon dioxide in air. The flasks were then incubated on a level surface at 37^{o}C.

Preparation of Viral Stocks

Vaccinal strains were prepared by resuspending the vaccines in 1ml sterile distilled water (dH_2O) and then mixing with an equal volume of chloroform to remove distemper virus. The aqueous phase was removed and passed through a 0.22μm filter (Millipore). The resultant suspensions of Epivac, Kavak, Nobivac, Vaxitas and Boostervac were then ready for inoculation into the relevant cell cultures. Cell monolayers (70% confluent) were inoculated with virus and incubated for 3 - 4 days until a strong cytopathic effect (cpe) became apparent. The flasks containing infected cells were then freeze thawed 3x at -20^{o}C. The supernatant was then centrifuged at 2000r.p.m. for 10 minutes and the clarified supernatant stored at -20^{o}C until required.

Isolation of Viral DNA

Viral DNA was extracted from infected monolayers when 70%

of the cells showed cpe. All media was removed and 5ml of cell lysis buffer (10mM Tris(hydroxymethyl)aminomethane (Tris), 10mM EDTA, 1% sodium dodecylsulphate (SDS) added to each 180cm^2 roux. The cultures were incubated for 10 minutes at room temperature. The viscous lysate was decanted and 5M sodium chloride (NaCl) was added to a final concentration of 1.25M. This solution was incubated on ice for 3 hours and precipitated chromosomal DNA was removed by centrifugation at 28,000g for 20 minutes in a Beckman JA20 rotor. Protease (type XI, Sigma) was added to the supernatant to a final concentration of 300µg/ml and the solution was incubated at 37^{o}C for 3 hours (Hirt,1967).

The solution was then extracted twice with phenol and twice with chloroform, a 1/10th volume of 5M NaCl was added and the DNA precipitated with ethanol (EtOH) at -20^{o}C overnight. The precipitated viral DNA was washed twice with 70% EtOH and dried in a vacuum dessicator. Finally the viral DNA was resuspended in TE buffer (10mM Tris pH7, 1mM EDTA) and stored at -20^{o}C until required.

Restriction Endonuclease Cleavage of DNA

The required volume of DNA in TE buffer was transferred to a microfuge tube. To this was added 0.2 volumes of the relevant restriction buffer; either low (10mM Tris pH7.5, 10mM $MgCl_2$, 1mM dithiothreitol (DTT)), medium (50mM NaCl, 10mM Tris pH7.5, 10mM $MgCl_2$, 1mM DTT) or high salt (100mM

NaCl, 50mM Tris pH7.5, 10mM $MgCl_2$, 1mM DTT) according to the manufacturers specifications. The restriction enzyme was added (one enzyme unit per µg of DNA to be digested), followed by sufficient distilled water to bring the buffer to the correct concentration. The mixture was incubated at 37°C for 60 minutes.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was conducted on a horizontal perspex bed submerged in TBE buffer (table 1.1). Agarose (2.4g) was suspended in 300ml of TBE and was dissolved by boiling in a microwave oven. This solution of 0.8% agarose in TBE was used consistently throughout this study unless otherwise stated in each individual chapter. When the molten agarose had cooled to approximately 50°C it was poured onto the perspex bed and left to solidify with the well-forming comb in position. The comb was then removed and the gel submerged in the electrophoresis tank.

Samples were prepared for electrophoresis by the addition of a 1/4 volume of loading solution (10% Ficoll (mw 400,000), 0.05% Bromophenol Blue, 0.05% Orange G, 0.05% Xylene Cyanol FF). The samples were then loaded into the wells and a constant potential difference of 50V was applied across the gel for approximately 16 hours. The gel was then removed from the electrophoresis tank, immersed in distilled water containing 0.5µg/ml ethidium bromide (EtBr) for 20 minutes and then left to destain in distilled water for a further 20 minutes. The DNA was

visualised on a 302nm transilluminator (UV Products Ltd.) and photographed on polaroid film.

Polyacrylamide Gel Electrophoresis

Restriction enzyme digests were also subjected to electrophoresis in 6% polyacrylamide, in order to visualise fragments between 50 and 500 base pairs (bp) in size. Polyacrylamide gels were conducted on a vertical gel set (Bio-Rad). The glass plates were thoroughly cleaned and placed into the casting stand as per manufacturers specifications.

The constituents of the gel were as follows:- 25.3ml dH_2O, 4ml TBE buffer, 10ml stock acrylamide (20% acrylamide, 1% methylenebisacrylamide in water), 24µl tetramethylethylenediamine (TEMED) and finally 480µl 10% of ammonium persulphate (APS) freshly prepared in water). This mixture was then poured between the glass plates, the well forming comb inserted and the gel left to polymerise for 45 minutes.

All polyacrylamide gels were run under a constant current of 36mA for 3 hours or 3mA overnight. Samples were prepared for electrophoresis by the addition of a 1/4 volume of loading dye (as for agarose gel electrophoresis). Gels were stained in EtBr, visualised and photographed as stated above.

Isolation of Plasmid DNA

Small-scale

Small-scale plasmid isolations were carried out after the method of Holmes and Quigley (1981). Bacteria were patched out on L-agar plates (table 1.1) and grown overnight at 37°C. A large loopfull was then placed directly into microfuge tubes containing 100µl of STET buffer (table 1.1) and resuspended thoroughly. Then 8µl of a freshly prepared solution of lysozyme (10mg/ml in water) was added and the tubes left at room temperature for 5 minutes. The tubes were placed into a boiling waterbath for 40 seconds and then spun in a bench-top microfuge at 13,000g for 10 minutes. The supernatants were removed to fresh tubes containing 100µl TE where the solutions were extracted 2x with phenol, 2x with chloroform and finally EtOH precipitated.

The DNA was pelleted in a bench-top microfuge at 13,000g for 10 minutes, washed 2x with 70% ethanol and dried in a vacuum dessicator. The pellets were resuspended in TE and stored at -20°C until required.

Large-scale

Bacteria carrying the required plasmid were grown overnight in 200ml L-broth (table 1.1) containing the appropriate antibiotics. The cells were harvested by centrifugation (9000r.p.m./10 minutes/5°C), resuspended in 2ml of 25% sucrose in 0.05M Tris pH8.0, and left on ice for 5 minutes. Lysozyme, freshly prepared at 20mg/ml in

0.25M EDTA pH8.0, was added (0.3ml) and the mixture left on ice for a further 15 minutes. 2ml of 0.25M EDTA pH8.0 was added and the mixture left on ice for a further 5 minutes. Next, 4ml of Brij/Doc solution (table 1.1) was added, mixed gently and left on ice for 30 minutes.

The mixture was then centrifuged (18,000r.p.m./45 minutes/4 ^{o}C) in a Beckman JA20 rotor. The supernatant was gently removed to a sterile universal container and CsCl added (0.95g/ml supernatant). EtBr was added to a final concentration of 200µg/ml and the mixture transferred to a 12ml heat sealable tube (Beckman). The tubes were sealed using a Beckman heat sealing apparatus and then centrifuged in a Beckman Ti50 fixed angle rotor at 38,000r.p.m. for 60 hours at 18 ^{o}C. The plasmid DNA band was easily visualised in the centrifuge tube and was extracted by side puncture of the tube using a 1ml syringe and a 23 gauge needle.

EtBr was removed from the DNA by repeated extraction with CsCl saturated iso-propanol. Extractions were continued until no visible colour remained in the aqueous phase. The CsCl was removed by dilution of the sample to 5ml with TE followed by ethanol precipitation at -20 ^{o}C for one hour. Following this, the DNA was pelleted in a Beckman JA20 rotor (15,000r.p.m./45 minutes/4 ^{o}C) and washed 3x with 70% ethanol in water (v/v). The pellets were then dried in a vacuum dessicator, resuspended in TE pH7.5 and stored at -20 ^{o}C until required.

T A B L E 1.1

Preparation of Stock Solutions

20X SSC		
	NaCl	175.3g
	Sodium citrate	88.2g
	dH_2O	to 1l
	adjusted to pH7.0 with NaOH	
20x SSPE		
	NaCl	174g
	$NaH_2PO_4.H_2O$	27.6g
	EDTA	7.4g
	dH_2O	to 1l
	adjusted to pH7.4 with NaOH	
Ethidium Bromide(10mg/ml)		
	EtBr	0.1g
	dH_2O	to 10ml
	store at 4 °C in the dark	
5M NaCl		
	NaCl	292.2g
	dH_2O	to 1l
0.5M EDTA		
	EDTA	186.1g
	dH_2O	to 1l
	adjust to pH8.0 with NaOH	
1M Tris		
	Tris base	121.1g
	dH_2O	to 1l
	adjust to required pH with HCl	
10x TBE		
	Tris base	108g
	Boric acid	55g
	0.5M EDTA	20ml
	dH_2O	to 1l

Denhardt's Solution

Ficoll	5g
Polyvinylpyrrolidone (PVP)	5g
BSA (pentax fraction V)	5g
dH_2O	to 500ml

Store at -20°C

20% SDS

Sodium dodecylsulphate	20g
dH_2O	to 100ml

L-Broth

Tryptone	15g
Yeast extract	5g
NaCl	5g
dH_2O	to 1l

adjust to pH7.2 with NaOH

L-Agar

L-broth	100ml
Difco agar	1.5g

Ampicillin

Ampicillin (sodium salt) 50mg/ml in dH_2O

Store at -20°C
Use at final concentration of 50µg/ml

Tetracycline

Tetracycline hydrochloride 15mg/ml in dH_2O/ethanol (50%v/v)

Store at -20°C
Use at final concentration of 15µg/ml

Streptomycin

Streptomycin sulphate 50mg/ml in dH_2O

Store at -20°O
Use at final concentration of 50µg/ml

Prehybidisation Solution for Nitro-cellulose

5xSSPE
50% Formamide
1x Denhardts
200µg/ml Denatured Calf Thymus DNA

Denatured Calf Thymus DNA (10mg/ml)

Calf Thymus DNA 10mg/ml in dH_2O
Solution boiled in a microwave oven and then sonicated for 3 minutes.

STET Buffer

Sucrose	8g
Triton X-100	5ml
0.5M EDTA pH8.0	10ml
1M Tris pH8.0	5ml
dH_2O	to 100ml

Brij/Doc

Brij 58	10g
Sodium deoxycholate (DOC)	4g
TE pH8.0	to 1l

X-gal(40mg/ml)

5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal)	40mg
dimethyl formamide	1ml

Store at -20°C

IPTG(50mg/ml)

Isopropyl-thiogalactoside	50mg
dH_2O	1ml

Store at -20°C

AIX Plates

IPTG (50mg/ml)	1ul	per
X-gal (40mg/ml)	1ul	100ml
Ampicillin (50mg/ml)	1ul	L-Agar

C H A P T E R 2

Comparison of Wild-type, Vaccine and Reference Strains of Canine Adenovirus and Construction of a Physical Map of the Vaccine Strain Vaxitas CAV-2

I N T R O D U C T I O N

Respiratory disease is one of the most important problems encountered in canine medicine. Two serologically related, but not identical, canine adenoviruses (CAV-1 and CAV-2) now appear as the primary cause of respiratory infections in dogs. Different immunological techniques have been used to distinguish between the CAVs but many of these typing procedures are either tedious or require further experimentation to establish their specificity and/or sensitivity. Preparation and evaluation of the adequate reagents for serological analysis of such closely related viruses is also a time consuming task especially when the number of "atypical" or "intermediate" results must be kept to a minimum.

Restriction endonuclease analysis has recently been found to be the most useful tool for the identification of the CAVs. However, the published data on the restriction enzyme analysis of the CAVs has been limited. Many groups have reported the restriction enzyme cleavage patterns of CAV-1 and CAV-2, confirming their distinctiveness, but in the majority of cases this has involved only the reference strains and as yet only one physical map of a CAV, that of a reference strain of CAV-1, has been published (reviewed in the general introduction).

The interest in CAV as a virus vector indicated that genomic analysis of the existing vaccine strains may prove

valuable and, in addition, may help to elucidate the mechanism of attenuation at the DNA level. A physical map of CAV-2 would also enable a more direct comparison with the CAV-1 genome and the human AdV.

It was hoped that the use of a range of restriction enzymes would reveal one or two which would yield a range of fragments of suitable size to enable the cloning of the entire CAV-2 genome. This would enable a physical map to be constructed and also the orientation of the CAV genome to be established with respect to the human AdVs. Any alteration in the genome structure or function of the CAVs compared to the human AdVs could then be investigated.

The wild-type strains used in this study were designated Glasgow CAV-2 and RI127 CAV-1. Glasgow CAV-2 was obtained from the faeces of pups with enteric disease, an unusual site of isolation for CAV-2, thus indicating that it may prove to be a variant strain when compared to the reference strains Manhattan and Toronto (Macartney et al, 1988). The CAV-1 wild-type strain, RI127, was obtained as a routine isolate by the Canine Infectious Disease Research Unit at the University of Glasgow.

MATERIALS AND METHODS

Restriction Enzyme Analysis

Restriction endonucleases were purchased from Pharmacia and used according to the manufacturers specifications. Restriction enzyme digests were subjected to electrophoresis in 0.8% agarose, prepared and run in TBE. Lambda phage (λ) DNA, cleaved by HindIII (Pharmacia), was used as a size marker.

Restriction digests were also subjected to electrophoresis in 6% polyacrylamide prepared and run in TBE. Puc8 plasmid DNA, cleaved by either HpaII or HaeIII, was used as a size marker.

Construction and Analysis of Recombinant Plasmids

Preparation and Ligation of DNA

The plasmid pBR322 was digested with PstI as was Vaxitas DNA. The restriction enzyme reactions were terminated by phenol extraction, followed by chloroform extraction and ethanol precipitation. The plasmid vector was then dephosphorylated using bacterial alkaline phosphatase (Maniatis et al., 1982).

Eight units of phosphatase/buffer mix (as supplied by Pharmacia) was added to the vector DNA which had been resuspended in 20µl TE and the mixture incubated at 37°C for 30 minutes after which the mixture was phenol/chloroform extracted and reprecipitated with

ethanol.

The DNA samples were redissolved in water and mixed in the ratio 3:1, Vaxitas:pBR322. The mixture was then incubated with T4 DNA ligase (Pharmacia) in 66mM Tris pH7.2, 10mM $MgCl_2$, 10mM DTT, 1mM ATP at 14°C for 12 hours (Maniatis et al., 1982).

Transformation with Plasmid DNA

Transformation of the ligated DNA into E.coli JM101 was carried out as described by Maniatis et al (1982).

An overnight culture of JM101 was diluted 1:25 into fresh L-Broth (table 1.1) at 37°C. When the culture reached an OD_{650} of 0.2 the cells were harvested by centrifugation (8K/10 minutes/4°C) in a Beckman JA20 rotor. The supernatant was discarded and the pellet resuspended in 3.8ml 0.1M $MgCl_2$. The cells were again sedimented by centrifugation as above and the pellet resuspended in 1.9ml 0.1M $CaCl_2$. This step was then repeated once more and the final suspension of cells in 1.9ml 0.1M $CaCl_2$ was left on ice for a minimum of 1 hour.*

200µl of the cell suspension was then added to 100µl of the ligated DNA solution and incubated at 0°C for 30 minutes. The cell/DNA mixture was then heat-shocked by incubation at 42°C for 2 minutes and returned to ice for a further 30 minutes. The mixture was then added to 1ml of

L-broth and incubated at 37°C for 1 hour to allow expression of the antibiotic resistance genes. Finally, 0.1ml samples of the transformation mix were spread directly onto agar plates containing antibiotics.

Transformants were initially selected on L-agar (table 1.1) containing 15µg/ml tetracycline. Individual colonies were then patched out onto L-agar containing either tetracycline (15µg/ml) or ampicillin (50µg/ml).

Transformants containing recombinant plasmids were selected as ampicillin sensitive. Recombinant plasmids were initially analysed using small-scale plasmid isolation techniques and those containing the required insertions were isolated by the large-scale plasmid isolation method (chapter 1).

* Excess cells were mixed with a sterile solution of 50% glycerol in water (v/v) in a ratio of 2:1 to give a final concentration of approximately 16%. This mixture was then divided into 300µl aliquots which were stored at -70°C until required.

Cloning of the Terminal Fragments of Glasgow and Vaxitas Vector

The plasmid vector Bluescript M13+ (Stratagene) was double digested with either SalI/SmaI or EcoRI/SmaI. The entire reaction from each digestion was run on a 0.8% agarose gel and the linear SalI/SmaI and EcoRI/SmaI fragments were cut

out and purified. This method facilitated the removal of any uncut vector and also the small polylinker fragments (SalI to SmaI 33bp: EcoRI to SmaI 8bp) thus preventing recirculisation of the vector.

Viral DNA

100µg of Vaxitas DNA and 200µg of Glasgow DNA were pretreated with proteinase K (Sigma) before digestion to remove any remaining terminal protein. In addition, 50% of each sample was pretreated with S_1 nuclease (Pharmacia). Briefly, 8 units of S1 nuclease were added to the DNA along with a 1/10 volume of 10xS1 buffer (2M NaCl, 0.5M sodium acetate (pH4.5), 10mM $ZnSO_4$, 5% glycerol) and water to make up the correct volume. The mixture was incubated at 37°C for 15 minutes. A 1:100 volume of 0.5M EDTA was then added to stop the reaction.

Next, protease type X1, at 10mg/ml in water, was added to S_1 treated and untreated samples to a final concentration of 500µg/ml and the mixture returned to 37°C for a further 3 hours. The mixture was then extracted with an equal volume of phenol (2x) and then chloroform (2x) before ethanol precipitation. The washed (70% EtOH) and dried pellets were then resuspended in TE ready for restriction endonuclease digestion.

The viral DNA was then divided into two; one half was digested with EcoRI, the other half with SalI. Upon

completion of the digestions, the DNA was again extracted 2x with phenol, 2x with chloroform and finally ethanol precipitated. The DNA was resuspended in TE and was then ready for ligation to Bluescript.

Ligation and Transformation

The ligations were carried out as described previously with the difference that excessive vector was present giving an approximate ratio of 1:1, vector:viral DNA. The ligated mixture was then transformed into E.coli DS941 (provided by Prof. D. Sherrat, Dept. of Genetics, University of Glasgow) as described above.

The DNA/cell mixture was plated directly onto AIX plates (table 1.1) in 0.1ml aliquots. The agar plates were incubated at 37°C for 24 hours and any white colonies then screened, following small scale plasmid isolation, by restriction enzyme analysis. Those recombinants containing the required insert were then re-isolated using the large scale plasmid isolation technique (chapter 1).

Transfer of DNA from Agarose Gels to Nitro-cellulose

CAV-2 DNA digested with BamHI, EcoRI, PstI and KpnI was separated on a 0.8% agarose gel and transferred to nitro-cellulose by the method of Southern (1975).

Briefly, the gel was denatured in 1.5M NaCl/0.5M NaOH for one hour at room temperature and the neutralised in a solution of 1M Tris (pH8.0)/1.5M NaCl for a further hour

at room temperature. The gel was then ready for blotting. 20xSSC (table 1.1) was used consistently as the transfer medium and the gel was allowed to blot overnight to allow complete transfer to the nitro-cellulose. The nitro-cellulose was then removed form the gel, cut with a scalpel as required, briefly washed in 2xSSC, air dried and finally baked at 80 °C for two hours.

Nick Translation of DNA

DNA was nick translated to prepare ^{32}P-labelled DNA (Rigby et al., 1977) using a commercial nick translation kit (Amersham). The radioactive label, α-^{32}P dATP, was purchased from Amersham at a specific activity of 3000μCi/mM.

The reactions were carried out according to the manufacturer's instructions and the mixtures were incubated at 14 °C for 2 hours. Unincorporated label was removed from the incorporated label by passing the reaction through a Sephadex G-50 column equilibrated with STE (table 1.1). Orange G (10mg/ml) was used as a marker for the unincorporated label. All nick translations yielded labelled DNA with >10^8 cpm. The labelled DNA was stored at -20 °C in lead containers and used within four days of nick translation.

Molecular Hybridisation

The baked nitro-cellulose filters were placed in a plastic

bag and prehybridised (table 1.1) overnight at 42°C.

The nick translated DNA (200µl) was denatured by adding 10µl 0.5M NaOH, boiling for 3 minutes, cooling on ice and finally neutralised with 15µl 1M HCl and 50µl 1M Tris pH7.0. The probe was then injected into the plastic bag containing the filter and the bag was resealed. The bag was further incubated at 42°C overnight. The filters were then removed from the bags, washed 3x in 2xSSC/0.1%SDS, then 3x in 0.2xSSC/0.1%SDS at 42°C, dried and subjected to autoradiography.

R E S U L T S

Restriction Enzyme Analysis of CAV-2 DNA

Three restriction enzymes, BamHI, PstI and HpaII, were used to cleave the purified DNA of the Glasgow isolate, the CAV-2 reference strains Manhattan and Toronto A26/61 and a reference strain of CAV-1, RI127. The separated DNA fragments are shown in Fig. 2.1. CAV-1 is easily distinguishable from the CAV-2 reference strains and the Glasgow isolate. The Glasgow strain appears identical to both prototypic strains of CAV-2 using BamHI and PstI. However, the HpaII digestion shows slight variation between Glasgow and the reference strains. At least one additional HpaII site is apparent resulting in the loss of a 1.8 kilobase (kb) fragment and the appearance of a 1.0kb fragment.

Two enzymes, BamHI and PstI, were then used to digest the purified DNA of the vaccine strains Vaxitas CAV-2, Kavak CAV-2, Nobivac CAV-2 and Epivac CAV-2 together with that of the Glasgow isolate (Glasgow CAV-2). The pattern of fragments produced is shown in figure 2.2.

PstI fragments A,B,E,F and G are common to all strains. Fragment C is common to all strains except Vaxitas in which it is not apparent. Further, fragment C of the other vaccine strains is slightly increased in size compared to Glasgow CAV-2. Fragment D is again common to all isolates but is decreased in size in all vaccine

strains with the maximum deletion, approximately 0.3kb, in Vaxitas. Fragment H is represented as a single discreet fragment in Glasgow but in the vaccine isolates varies from 1.3kb to 3.0kb in size. In addition, three fragments were visible on acrylamide which were of a constant size in all strains.

BamHI fragments A,B,D,E and F are common to all isolates. However, fragment A varies in size, with Vaxitas having the largest and Glasgow the smallest fragment. Fragment C, while not apparent in Vaxitas, is common to all other isolates but is slightly larger in the vaccinal strains than in Glasgow.

A direct comparison of Glasgow CAV-2, as a wild-type strain, and Vaxitas CAV-2, as the vaccine strain differing most widely from wild-type, was now undertaken. Restriction profiles of Glasgow (A) and Vaxitas (B) can be seen in figure 2.3. Virus DNA was digested with BamHI, EcoRI, KpnI, PstI, SalI and SmaI. The size of fragments generated by digestion with each of these enzymes can be seen in table 2.1. PstI fragments B and F in Vaxitas were demonstrated to consist of two co-migrating fragments by double digestion of the PstI clones with a range of restriction enzymes.

Molecular Cloning of Vaxitas CAV-2 Fragments

The number (11) and range of sizes (0.2kb - 6.0kb) of

fragments generated by digestion with PstI indicated these as suitable fragments for cloning into a plasmid vector. Nine of the eleven PstI fragments were cloned into the PstI site of pBR322. Two terminal fragments, SalI B and EcoRI C, were cloned as SalI-blunt and EcoRI-blunt fragments in the plasmid vector Bluescript M13+ (Stratagene: Fig. 2.4). The right-hand terminal PstI fragment, PstI G (Glasgow)/PstI H (Vaxitas), was subcloned from this. The single Vaxitas SalI B clone isolated contained an insert of approximately 1kb, as compared to the Glasgow SalI B fragment, and this clone was used for subcloning the terminal PstI fragment. The left-hand terminal clone, EcoRI C, was used in the hybrisiation mapping experiments instead of PstI C.

Recombinant plasmids were digested with PstI and electrophoretic mobility of the inserted Vaxitas DNA fragments compared with that of PstI cleaved whole virus DNA. Final confirmation of each cloned fragment was obtained by hybridisation of each clone against Southern transfers (Southern, 1975) of PstI digested Vaxitas DNA. All the PstI clones and the EcoRI C clone hybridised with a single PstI fragment of Vaxitas whole virus DNA except the cloned termini which exhibited the characteristic homology due to the terminal repeats (Fig.2.5). Homologous inverted terminal repeats (ITR) are reported to have been found in all adenoviruses so far examined (Ginsberg, 1984).

Physical Map of Vaxitas CAV-2

The maps shown in Fig. 2.6 were constructed using the data from molecular hybridisation experiments and restriction fragment sizes. The hybridisation results for one restriction enzyme, BamHI, digestion of Vaxitas DNA are given as an example in figure 2.7. The hybridisation results are summarised in table 2.2. Due to the considerable heterogeneity at the termini of Vaxitas, most clearly seen in EcoRI C and SalI B fragments (fig. 2.3), the size of the most predominant species was taken. The SmaI sites at map units (m.u.) 0.75 and 99.25 were also located by sequence analysis which is discussed later. As an area existed within the genome which was not covered by any of the above clones, the region between the end of EcoRI C and the beginning of PstI A (1.26kb) the cloned EcoRI B fragment was used to confirm the position of the PstI fragments J and K within this region.

The physical maps of Glasgow CAV-2 DNA used for comparison were kindly provided by Dr. N. Spibey. The order of BamHI fragments within PstI B was determined by KpnI/BamHI double digestion of the cloned PstI B fragment. The physical maps of the CAV genomes were orientated with respect to the human adenovirus genome (HuAdV) by probing southern transfers of Glasgow DNA with cloned fragments of HuAdV types 5 and 12 hexon coding region (Spibey and Cavanagh, 1989). All data from the molecular hybridisation experiments were confirmed by restriction

endonuclease digestion of the Vaxitas recombinant plasmids.

Comparison of the Physical Maps of Vaxitas and Glasgow CAV-2

From figure 2.6 it is apparent that the restriction maps of Glasgow and Vaxitas are very similar. However, three sites of variation between the two strains can clearly be seen. The first two involve insertions at either end of the Vaxitas genome: right terminal PstI fragment Glasgow 1.3kb, Vaxitas 3.0kb; left terminal PstI fragment Glasgow 4.4kb, Vaxitas 4.7kb. The third change occurs in the PstI D fragment. This fragment is present as 4.4kb in Glasgow but 4.1kb in Vaxitas. The deletion which has occurred in the vaccine strain removes a KpnI site resulting in the loss of the 0.6kb KpnI fragment.

Table 2.1 lists the size of each restriction fragment for each virus to enable a clearer comparison.

Restriction Enzyme Analysis of CAV-1 DNA

Two restriction enzymes, PstI and SalI were used to cleave the purified DNA of the CAV-1 reference strain RI 127 and the CAV-1 vaccine strains Boostervac CAV-1 and Epivac CAV-1. The separated DNA fragments are shown in figure 2.8. The physical map of CAV-1 DNA (RI 127) is shown in figure 2.9 courtesy of Dr. A. M Clory (University of Glasgow). PstI digestion of CAV-1 DNA produces 11 fragments, 8 of which are visible on 0.8% agarose. SalI digestion

produces three fragments. The terminal SalI C fragment exhibits considerable heterogeneity within the vaccine strains with Boostervac appearing to consist of two distinct species. Variation is also apparent in the larger, SalI A and B, fragments but the size of the large fragments produced by this enzyme , 17.7kb and 11.9kb, does not facilitate easy interpretation of this. A similar observation can be made in the PstI digest. Heterogeneity can clearly be seen in the PstI G fragment. Unfortunately, it is impossible to distinguish whether the variation in the larger fragments occurs in PstI B, C or D or a combination thereof. Further digests with other enzymes were therefore undertaken.

Figure 2.10 displays the separated fragments obtained from digestion of the three CAV-1 strains with KpnI, BamHI, SmaI and EcoRI. The EcoRI 2.3kb and 0.8kb fragments show the characteristic heterogeneity very clearly and confirms the variation as terminally associated insertions within the CAV-1 vaccine strains. The EcoRI 3.6kb fragment similarly shows the presence of a deletion in this fragment of the vaccine strains. The remaining enzyme digestions; KpnI, BamHI and SmaI; exhibit the same features although less clearly. The insertions can be noticed in the KpnI E and BamHI C fragments but the size and separation of the larger terminal fragments does not allow for a clear demonstration of relatively small changes in size. The deletion can similarly be noticed in

KpnI C and SmaI A although still to a lesser degree than in EcoRI D due to their larger size and lesser separation. The restriction pattern obtained by digestion of the CAV-1 strains with BamHI was also analysed on acrylamide, figure 2.11. This demonstrated that the 0.4kb fragment D present in wild-type CAV-1 was no longer present in the vaccine strains thus indicating that the deletion in the genomes of the vaccine strains was contained within this region, around 80 m.u.

F I G U R E 2.1

Restriction Enzyme Analysis of Wild-type and Reference Strains of Canine Adenovirus

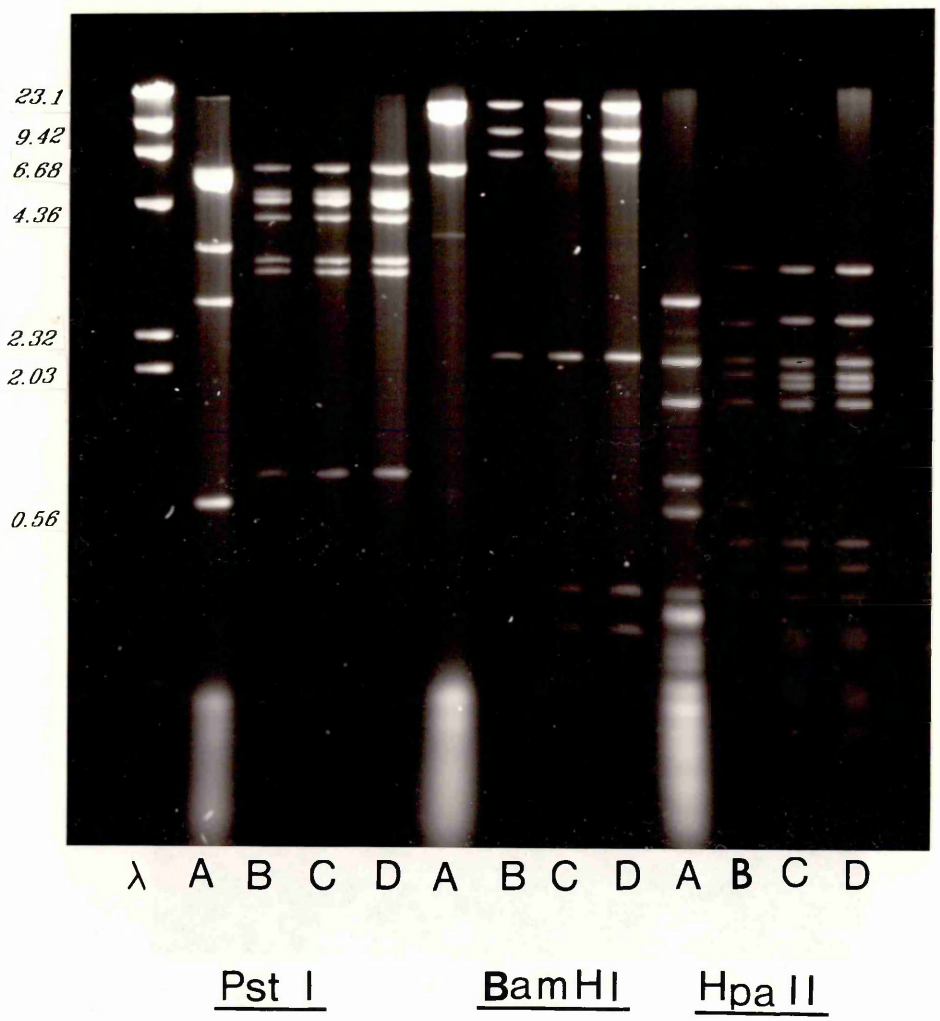


Fig.2.1 Restriction enzyme analysis of the CAV genome. The wild-type isolates RI 127 CAV-1 (lane A) and Glasgow CAV-2 (lane B) are compared to the two prototypic CAV-2 strains, Manhattan (lane C) and Toronto (lane D), using the restriction enzymes PstI, BamHI and HpaII. The CAV-1 isolate is clearly distinguishable from the CAV-2 strains. Variation in the HpaII digestion of the CAV-2 strains is marked. HindIII digested λ DNA fragments are used as m.w. markers.

F I G U R E 2.3

Comparison of the Restriction Enzyme Profiles of Glasgow and Vaxitas CAV-2

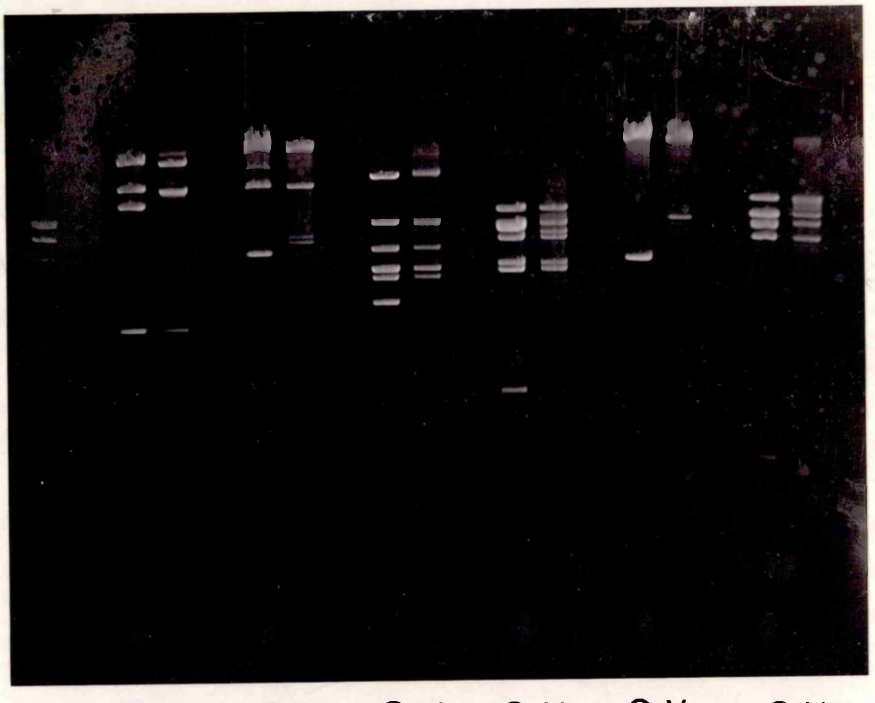
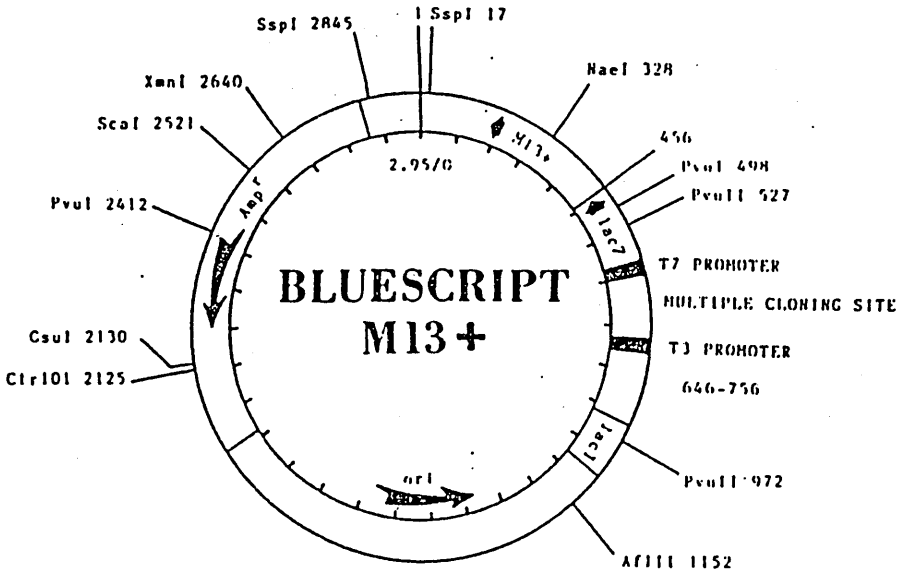
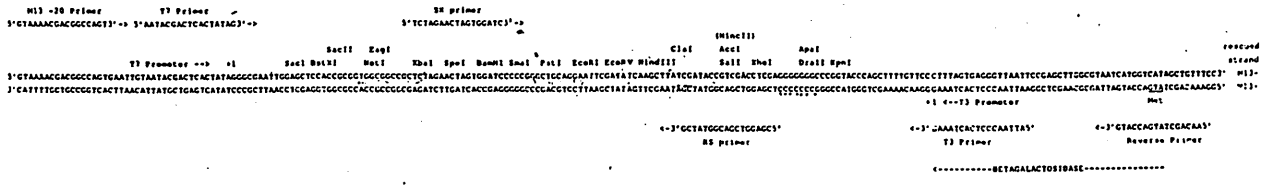


Fig.2.3 A direct comparison of the restriction profiles of Glasgow (G) and Vaxitas (V) CAV-2. Viral DNA was digested with BamHI (1), EcoRI (2), KpnI (3), PstI (4), SalI (5) and SmaI (6). Variation in size between the terminal fragments of Glasgow and Vaxitas can easily be seen and are described within the text. HindIII digested λ DNA fragments represent m.w. markers.

F I G U R E 2.4

Physical Map and Polylinker of the Plasmid Bluescript





KS Polylinker

Fig. 2.4 Physical map and the polylinker of the plasmid vector Bluescript M13+ (Stratagene). Primer recognition sequences are indicated.

F I G U R E 2.5

Confirmation, by Hybridisation, of the Isolation of the PstI fragments of Vaxitas CAV-2

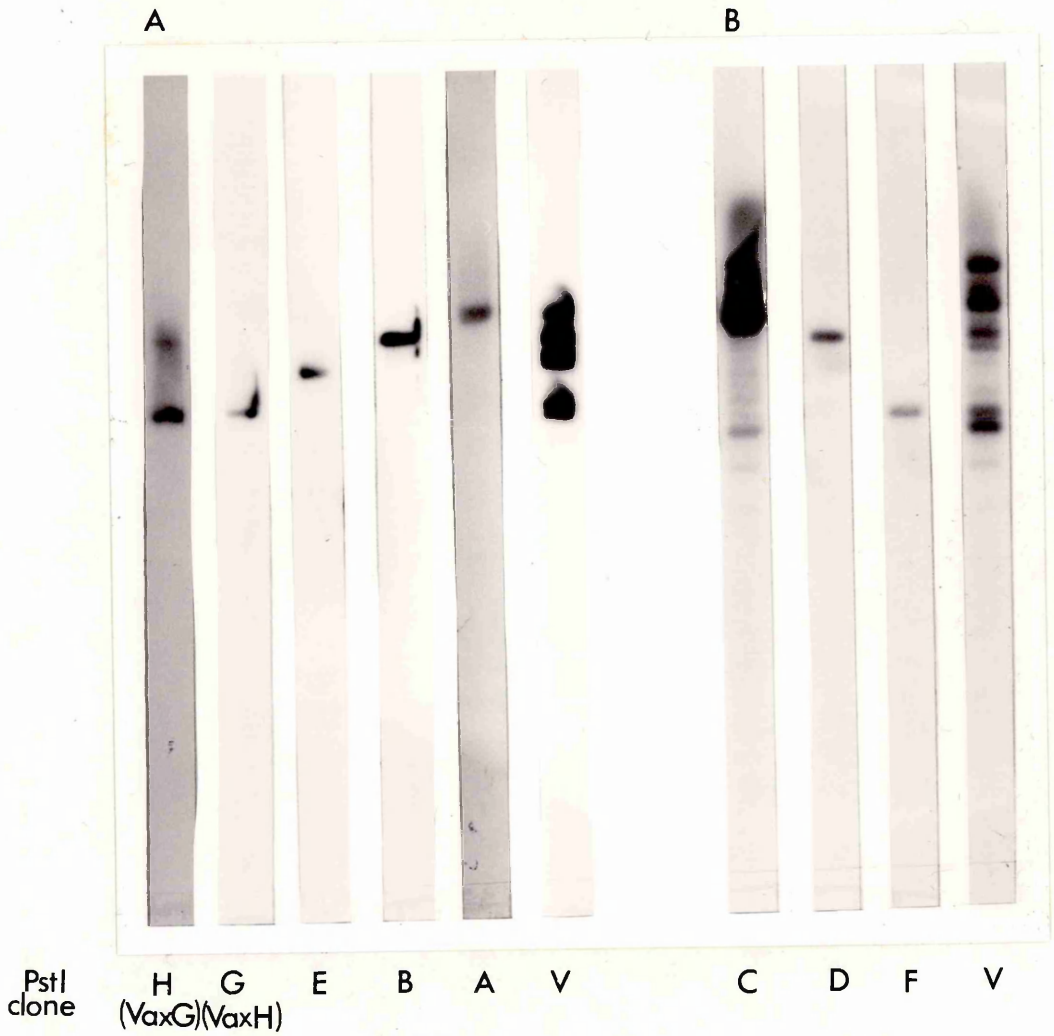


Fig. 2.5 Confirmation of the isolation and cloning of the PstI fragments of Vaxitas CAV-2. Each fragment was nick translated and hybridised to Southern transfers of viral DNA cleaved with PstI. Each fragment bound only to a single viral PstI fragment of identical size except the terminal fragments which bound to themselves and to each other due to the presence of an inverted terminal repeat within the viral genome. Group A and group B represent Southern transfers of PstI digested Vaxitas DNA from two different 0.8% agarose gels. PstI digested Vaxitas DNA probed with ^{32}P-labelled whole virus DNA is demonstrated for each gel (V). Confirmation of the clones of Pst K, J and I was carried out by probing transfers of PstI digested viral DNA from 2% agarose gels (data not shown).

T A B L E 2.1

Sizes of fragments generated after cleavage of CAV-2 DNA with BamHI, EcoRI, KpnI, PstI, SalI and SmaI

Restriction enzyme	Fragment	CAV-2 Glasgow length (Kbp)	CAV-2 Vaxitas length (Kbp)
BamHI	A	13.86	14.06
	B	8.1	8.1
	C	6.2	8.1
	D	2.0	2.0
	E	0.7	0.7
	F	0.6	0.6
EcoRI	A	19.0	20.4
	B	8.8	8.8
	C	3.5	3.8
PstI	A	6.0	6.0
	B	4.6	4.6
	C	4.35	4.6
	D	4.35	4.1
	E	3.9	3.9
	F	3.1	3.1
	G	3.0	3.1
	H	1.3	3.0
	I	0.32	0.32
	J	0.26	0.26
	K	0.2	0.2

KpnI	A	10.2	10.5
	B	5.2	5.2
	C	3.6	5.2
	D	3.05	3.6
	E	3.05	3.05
	F	2.85	3.05
	G	2.3	2.85
	H	0.7	0.7
	I	0.6	-
SalI	A	28.0	28.3
	B	3.2	5.2
SmaI	A	6.75	6.75
	B	5.3	6.1
	C	5.3	5.5
	D	4.55	5.3
	E	4.4	4.4
	F	3.85	3.85
	G	0.72	0.72
	H	0.233	0.233
	I	0.233	0.233

Table 2.1. Fragment sizes were calculated from electrophoretic mobilities in 0.8% agarose (>500bp) and 6% polyacrylamide (50 - 500bp).

T A B L E 2.2

Hybridisation between PstI fragments and restriction enzyme digests of CAV-2 DNA

Cloned PstI fragment	Bam HI	EcoRI	KpnI	PstI	SmaI
A	A	B	A D	A	C E G F
B	B C D E F	A	B F	B	B
C	A C	A C	A G	C G	D H I * *
D	C	A	B I G	D	B D
E	B	A	C E H	E	A F
F	B	A	E F	F	A
G	A	A B	D H	G	F G
H	A C	A C	A G	C H	D H I * *
I	B	A	E	I *	A
J	A	B	A	J *	C
K	A	B	A	K *	C

Hybridisation relatively weak, and due to inverted terminal repeated sequences.

* Hybridisation detected on DNA transferred to nitro-cellulose from 2% agarose

Table 2.2 Hybridisation results from nick translated PstI clones probed against Southern transfers of Vaxitas DNA digested with a range of restriction enzymes.

F I G U R E 2.6

Physical Maps of the Vaxitas and Glasgow CAV-2 Genomes

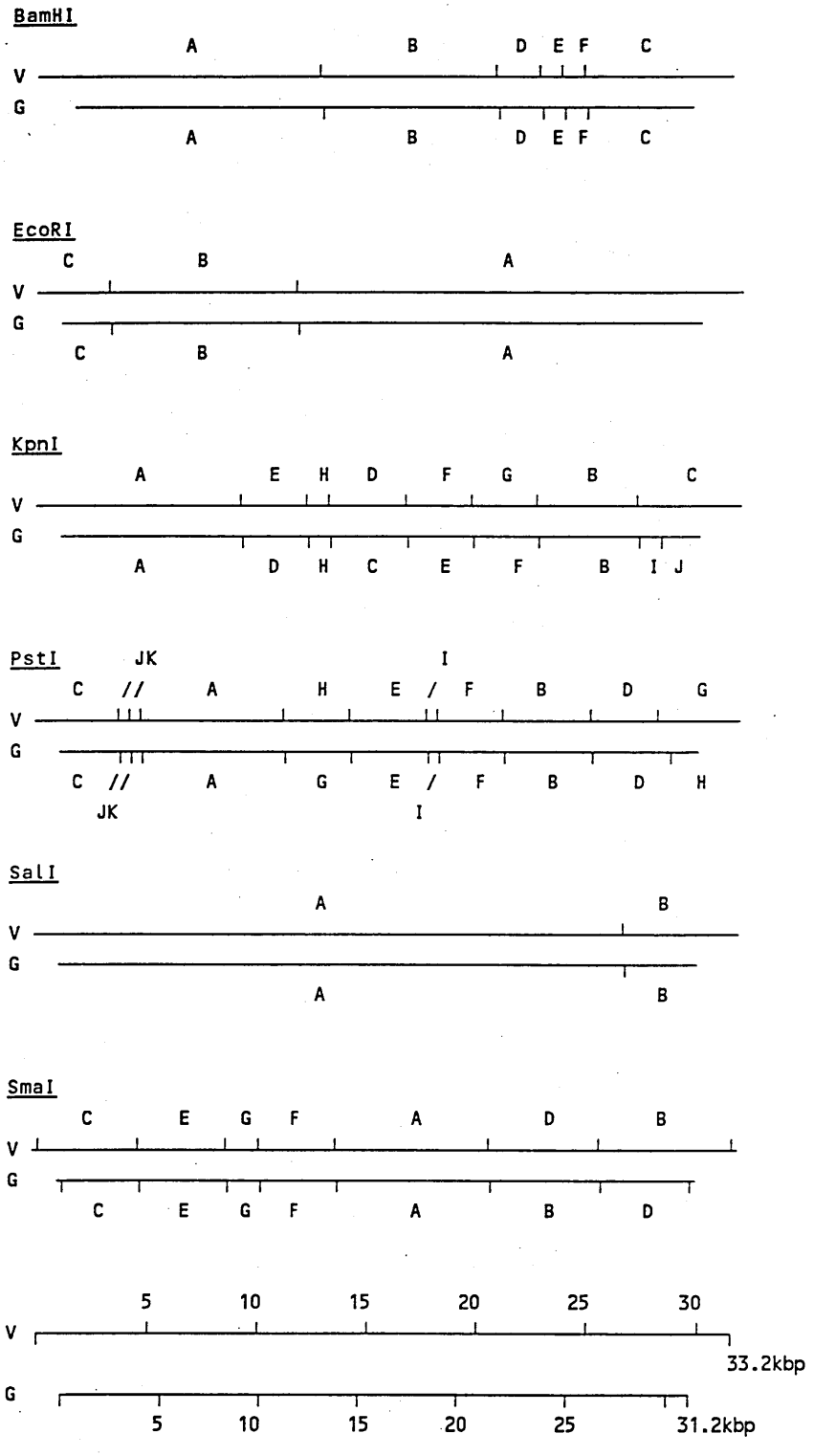


Fig. 2.6 Schematic restriction maps of Vaxitas and Glasgow CAV-2 genomes. Accurate sizes of each fragment given in table 1. Glasgow map and fragment sizes courtesy of Dr. N. Spibey, Glasgow University (Spibey and Cavanagh, 1988).

F I G U R E 2.7

Hybridisation Results From Southern Transfers of BamHI Digested Vaxitas DNA

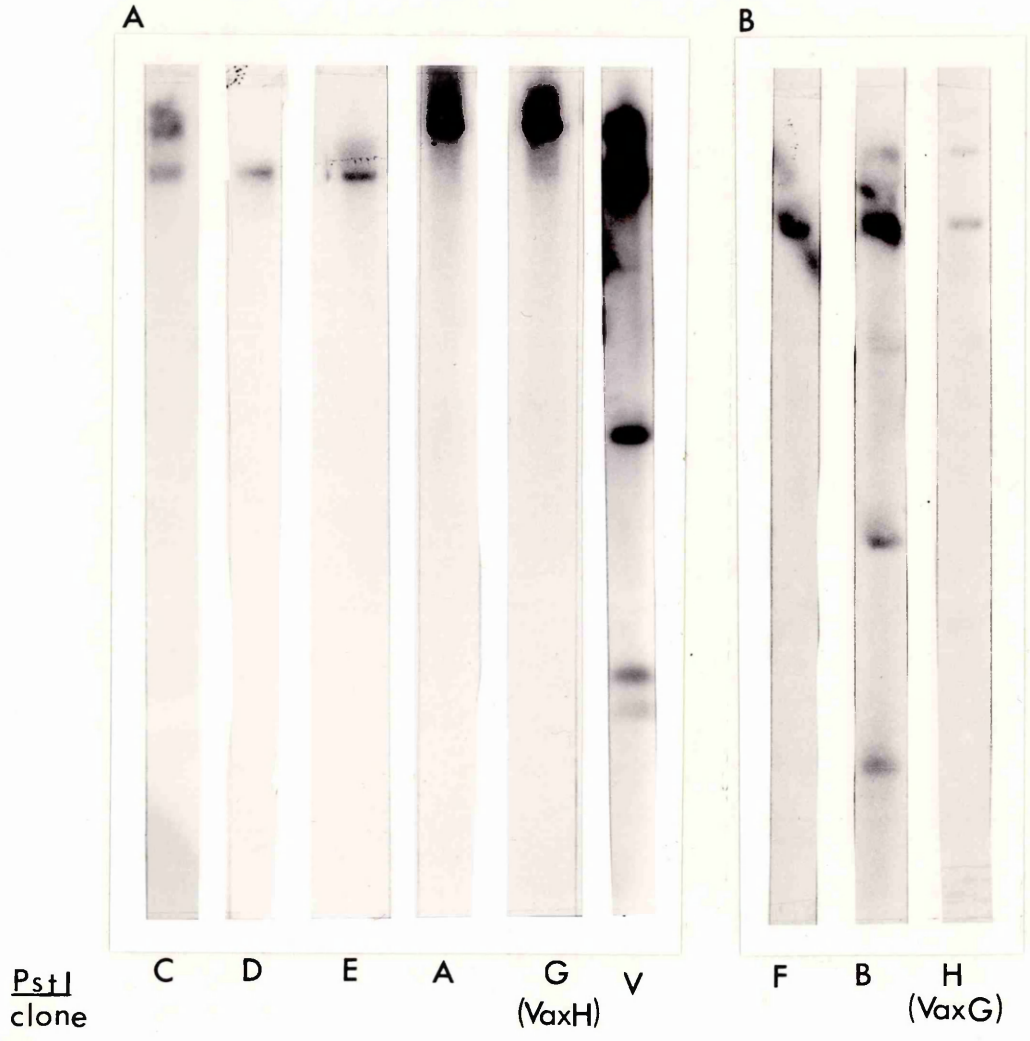


Fig. 2.7 Hybridisation results from BamHI digested Vaxitas DNA probed with nick translated PstI clones. Groups A and B represent Southern transfers of BamHI cut ^{32}P-labelled Vaxitas DNA from two distinct 0.8% agarose gels. Whole virus DNA was used to probe a single track (V). The PstI clone used for probing each track is indicated. Results for PstI clones I, J and K are not shown but are consistent with their position on the viral genome.

F I G U R E 2.8

Preliminary Restriction Enzyme Analysis of CAV-1 Vaccine Strains

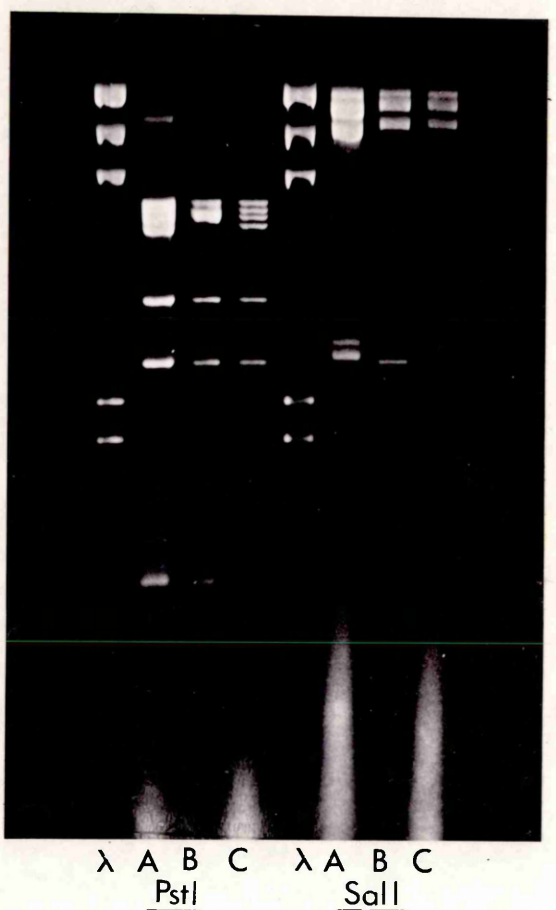


Fig. 2.8 Restriction enzyme analysis of CAV-1 vaccine strains Boostervac (lane A) and Epivac (lane C) compared to the wild-type isolate RI 127 (lane B). RI 127 and the two vaccine strains were digested with PstI and SalI. Variation in the terminal fragments is apparent. HindIII digested λ DNA fragments were used as m.w. markers.

F I G U R E 2.9

Physical Map of the Wild-type CAV-1 Strain RI 127

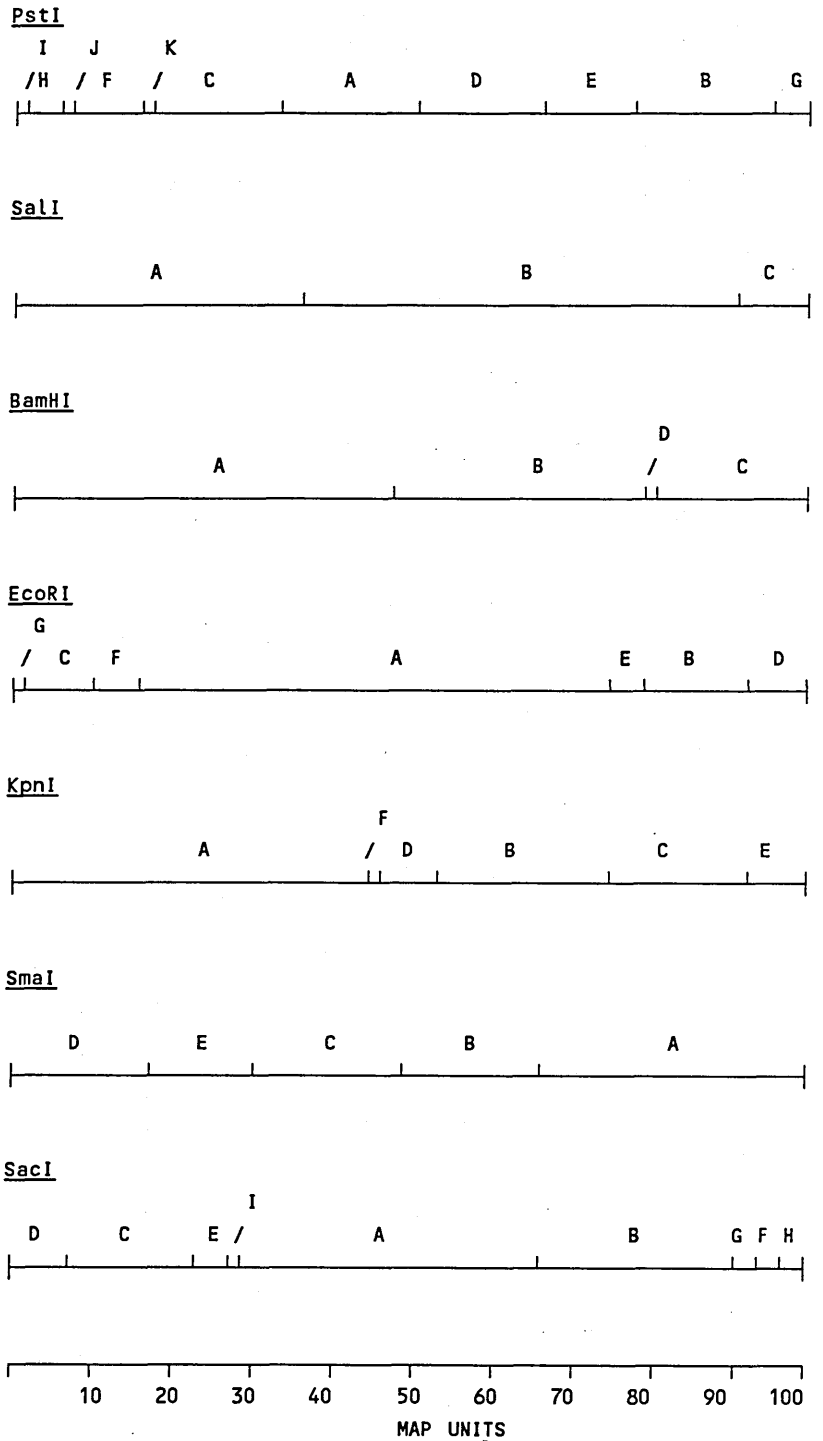


Fig. 2.9 Physical Map of the genome of the wild-type isolate of CAV-1, RI 127. Map kindly provided by Dr. A. S. M^{C}Clory, University of Glasgow. Fragments are labelled alphabetically according to size i.e. largest labelled A etc.

F I G U R E 2.10

Restriction Enzyme Analysis of CAV-1 Vaccine Strains

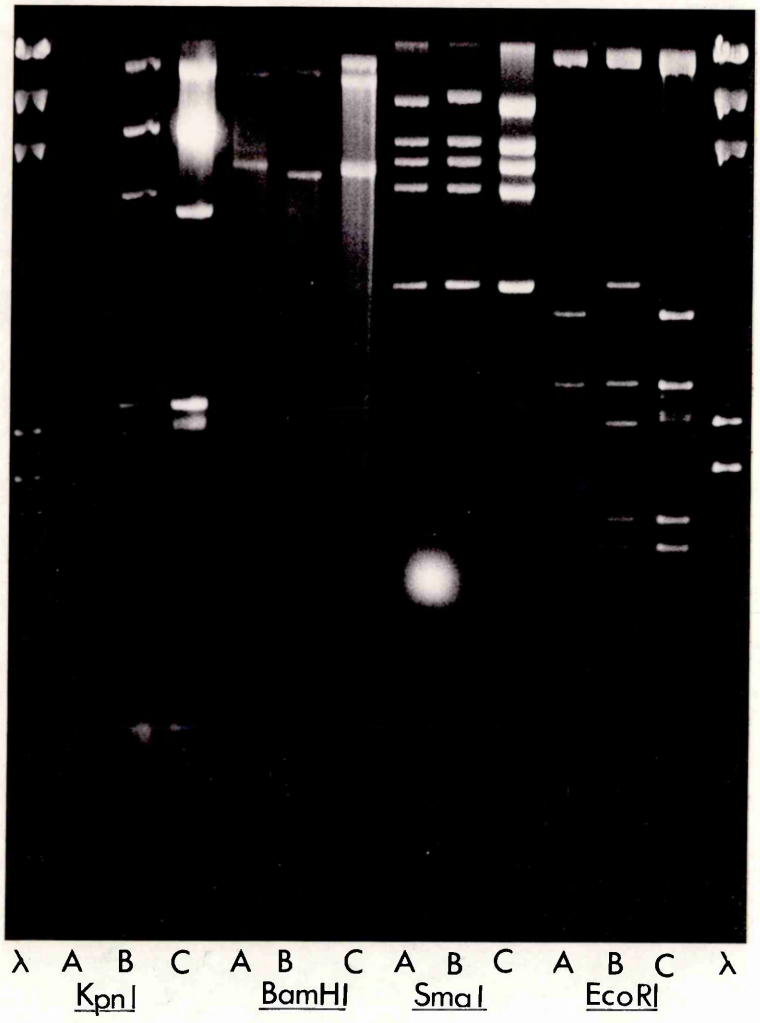


Fig. 2.10 Restriction profiles of the CAV-1 vaccine strains: Boostervac (lane A) and Epivac (lane C): and wild-type strain RI 127 (lane B) obtained using the restriction enzymes KpnI, BamHI, SmaI and EcoRI. Variation within the terminal fragments and an internal deletion is most apparent in the EcoRI digest. Other digests exhibit the variation less clearly due to the size of the fragments (see text). HindIII digested λ DNA fragments were used as m.w. markers.

F I G U R E 2.11

Analysis of BamHI Digestion of the CAV-1 Strains on Acrylamide

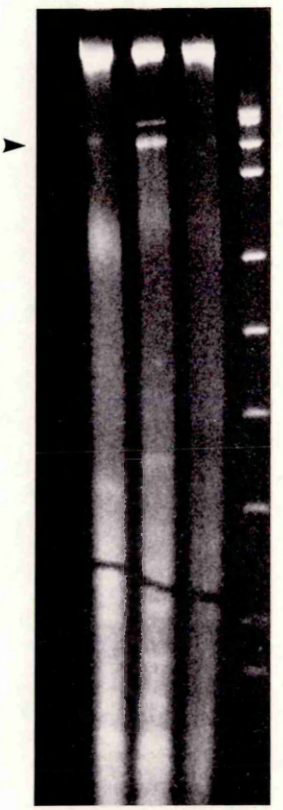


Fig. 2.11 BamHI digested Boostervac (lane A), RI127 (lane B) and Epivac (lane C) CAV-1. The 0.4kb Bam D fragment demonstrated by wild-type is no longer present in the vaccine strains. HpaII digested pUC8 was used as a size marker.

D I S C U S S I O N

The distinct DNA cleavage patterns of CAV-1 and CAV-2 reported here are consistent with the published findings of several authors (Hamelin *et al*, 1984; Assaf *et al*, 1983; Darai *et al*, 1985; Hamelin *et al*, 1986; Macartney *et al*, 1988).

Both the present isolate, Glasgow CAV-2, and that of Hamelin *et al* (1986) show a similar *HpaII* polymorphism. However, the two isolates are not identical, evinced by a difference in digestion with *BamHI*. The difference between the restriction patterns of the enteric isolate of Hamelin *et al* and the prototypic Toronto A26/61 strain led that group to postulate that "relatively small changes in the original CAV-2 DNA sequence allowed the virus to replicate at an unusual site in the dog". While it is unlikely that the variation in the *HpaII* digestion patterns between the various strains of CAV-2 is responsible for the ability of the virus to replicate enterically, it is likely that the *HpaII* variation is indicative of other, more significant, genomic alterations.

The cleavage patterns of all the CAV-2 strains are essentially the same. From the published data on the restriction enzyme analysis of the prototypic Manhattan and Toronto strains, there is no evidence to suggest that they are different strains of CAV-2. No method has been

found with which to differentiate between Manhattan and Toronto, either by immunological or molecular techniques. All the available evidence points to the fact that they are simply different isolates of the same strain of CAV-2.

Minor differences are apparent, however, when the wild-type Glasgow isolate and the vaccine strains are compared to each other and to the prototypic strains. The termini of each vaccine strain are increased in size with regard to the wild-type Glasgow isolate and the two prototypic strains. This increase cannot simply be due to strain variation as all the vaccine strains are derived from the Manhattan or the Toronto A26/61 prototype isolates. As stated, minor differences were observed in the HpaII restriction profiles of Glasgow and the two prototype isolates but no large increase or variation in the size of the terminal fragments can be seen. Thus it must be concluded that the terminal variation is not strain dependent but has occurred via some other route.

Similarly, the CAV-1 vaccines exhibit terminal variation when compared to the wild-type reference strain although none showed variation as marked as that of Vaxitas CAV-2 compared to wild-type CAV-2. Liu et al (1988) described viable isolates with aberrant genomes from a CAV-1 vaccine strain (CCL, Conaught Labs.). The genome length of the viable isolates was found to be 480bp shorter than wild-type CAV-1. The 480bp deletion, from the information and

restriction enzyme map given in the paper, would appear to be in the same region as the deletion in the CAV-1 vaccine strains described here, i.e. BamHI D is no longer apparent within the vaccine strain genome. Liu et al speculate that this deletion, 83-91m.u., is situated in E3 and is directly involved in the attenuation of the virus, thus implying that E3 is responsible for virulence. The vaccine strain was found to still display the 480bp deletion following several passage in dogs.

However, their assumption that the deletion is based in E3 was solely due to the fact that Tsukiyama et al (1988) reported that the left-hand terminal region of the canine adenoviruses demonstrated transforming ability; presumed due to the presence of E1a at this end of the genome; and that by alignment of the CAV-1 genome with the human adenoviruses, the deletion occurs in E3. No analysis of the CAV-1 vaccine strain genome was carried out to confirm this speculative position of the deletion.

The most significant alteration in restriction enzyme profile among the canine adenoviruses examined was however displayed by Vaxitas CAV-2. Therefore, it was selected for further analysis and the construction of a physical map of the virus genome.

Considerable difficulty was experienced in cloning the terminal fragments of both Vaxitas and Glasgow. Several methods were attempted repeatedly without success.

Presumably, this was due to an inability to remove all of the terminal protein from the genome even with extensive Proteinase K, NaOH or S_1 nuclease treatment. Finally, only by using vast amounts of viral DNA were the terminal clones obtained. However, it is not known whether the S_1 treatment was effective or if a few genomes within the vast amount used were simply derelict of the TP. Similar numbers of terminal clones were obtained with and without S_1 treatment, except for the SalI B clone from Vaxitas where only one clone was ever obtained.

Alternatively, residual protein may have been present at the time of ligation of the terminal fragments to the vector. It is feasible that the remaining protein was then removed following ligation and transformation into E. coli by DNA repair. Finally however, all four terminal clones were obtained and confirmed by restriction enzyme analysis and hybridisation.

Unfortunately, the only SalI B clone of Vaxitas, which exhibits considerable heterogeneity in the size of this fragment, obtained did not contain the 2.0kb insertion of the dominant species but instead contained approximately 1.0kb of additional DNA as compared to wild-type. Repeated attempts to secure a clone of the larger SalI B fragment also failed and it was therefore necessary to confine further studies of this area to the clone already obtained. It should be noted however that the clone

hybridised strongly to all sizes of SalI B exhibited by Vaxitas and contained all known restriction enzyme sites belonging to the larger species. Thus the only difference between the heterogeneously sized SalI B fragments appears to be restricted to the size of the additional DNA present. Plaque purification of Vaxitas was also impossible at this time as no cell line could be located which would allow plaque formation by this virus. Attempted purification by high-dilution methods resulted in the normal heterogeneic population by the DNA analysis stage.

The cleavage maps of Vaxitas, which was originally derived from the Manhattan strain, and Glasgow are essentially the same. The differences which do exist between Vaxitas and the field isolate, i.e. the additional DNA sequences near the genome termini and a small deletion at around 90 m.u., have probably come about as a result of continuous passage in tissue culture. Variation in the genomic termini of human AdV, consisting of either multiple repeats of viral DNA sequences or the insertion of mammalian DNA into the viral genome, have been described.

Larsen and Tibbetts (1985) and Larsen et al (1986) described variant species within Ad3 containing both insertions and deletions within the ITR. Their initial studies examined only high multiplicity passage (HMP)

virus but upon further examination it was found that even parental low level passage Ad3 contained some variants at low levels. Therefore it was maintained that the variation was not due to HMP but may be due to serial passage in tissue culture. All the variant species examined contained multiple tandem repeats of HeLa cell DNA with no relevant homology to the viral DNA. However, they found that insertions in the left-hand terminal (LHT) rendered E1a defective and the resultant virus was only competent for growth in 293 cells which constitutively express E1a. Brusca and Chinnadurai (1983) described Ad2 mutants, which were stable and viable, containing inserts of up to 1110bp at the right-hand terminal (RHT) but, as with Ad3, those containing insertions or deletions at the LHT were defective for E1a expression and were viable only in 293 cells. This is a significant difference between the terminal insertions of Ad3/Ad2 and CAV-2 as, although Vaxitas is non-permissive in MDCK cells but only in mink fibroblasts and vice versa for Glasgow, there is no evidence to suggest that the Vaxitas E1a is not functional. Both Glasgow and Vaxitas replicate extremely well within their respective cell lines yielding titres of 10^{7} -10^{8} pfu/ml consistently. In addition, although the size of the insertions or duplications at the LHT of the other vaccine strains, both CAV-1 and CAV-2, are much reduced compared to Vaxitas, they are fully competent for growth in MDCK cells although they do not yield such high titres as wild-type virus. This is however probably associated with the attenuation process. Little is known

of the conditions under which the vaccine strains were attenuated except that Vaxitas was attenuated in mink fibroblasts and the CAV-1 vaccines in pig kidney cells i.e. "foreign" cell lines, a process which would certainly require initial passage of the virus at high multiplicity. It is therefore conceivable that the additional DNA sequences in CAV have arisen by a similar mechanism to those in human AdVs.

Hammarskjold and Winberg (1980) reported that a DNA sequence located between 290 and 390bp from the LHT of Ad16 contained recognition signals for encapsidation of the viral DNA. The location was determined by analysing a series of spontaneous mutants of Ad16 which arose during serial passage in HeLa cells and carried duplications of 200-500bp of left terminal sequences at the right end of the genome. A duplication of about 390bp sequences from the left end at the right end enabled the subgenomic viral DNAs containing both molecular ends to be encapsidated. However, the incomplete virus particles containing the left end sequences at the right end did not promote encapsidation of the RHT sequences.

Serial passage of Ad12 in Vero cells (Werner and zur Hausen, 1978) resulted in insertions of 190-280bp at the RHT and deletions of 70bp at the left end of the genome. Similar alterations were observed when the virus was serially passaged in other, normally non-permissive, cell

lines. Serial passage resulted in the generation of host range mutants with duplications of the ITR and its adjacent sequences from the LHT at the RHT. Similarly, Schwartz et al (1982) reported that after serial passage of Ad12, in cells of the human melanoma cell line, variants with enhanced growth properties were isolated which carried additional sequences of regularly increasing size at the right end of the genome. Sequence analysis of these variants indicated that these extra sequences were of viral origin. They originated from either the extreme right or left ends of the genome, consisting of the ITR and different segments of the adjacent unique sequences.

Equine AdV (EAdV) DNA prepared from infected bovine kidney cells (MDBK) was found to contain additional sequences of 100-700bp at the LHT (Ishiyama et al, 1986). These aberrant viral genomes were produced even after the first passage of the wild-type EAdV in MDBK cells and their relative amounts did not significantly change during serial passage. One viral DNA contained a duplication of the ITR and another contained 270bp of additional sequences derived from the right terminal region added to the left terminal ITR. While the former DNA was packaged into virions the latter was not. This was presumed to be due to the alteration of the distance from the LHT to the DNA packaging signal. It is interesting to note however, that these aberrant viral genomes were only produced in MDBK cells and not in equine cells in which the virus grows extremely well and produces wild-type genomes.

Reiterated DNA sequences have been found in a number of different viruses. Moss et al (1981) described the presence of a set of 13-17 tandem 70bp repeats near both ends of the genome of Vaccinia virus, the evolution of which appeared to be mediated by recombinational events. Analysis of virulent and vaccine strains of bovine herpes virus type 1 (BHV-1) revealed a 14bp sequence at the left end of the genome repeated between 8 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 that the repeated sequences in BHV-1, the human and equine AdVs and possibly CAV are in the regions of the genome associated with viral DNA replication. Furthermore, the growth characteristics of the CAV vaccine strains, except Vaxitas, suggests that DNA replication is impaired to a limited degree.

Further work was then undertaken to define the nature and location of the additional DNA sequences within the vaccine strains and their effect, if any, on viral DNA replication.

C H A P T E R 3

Sequence Analysis of the Glasgow CAV-2 ITR and the Additional DNA Sequences Present in the CAV Vaccine Strains

The physical maps of CAV-1 and CAV-2 described in the previous chapter indicate that the heterogeneity in all the vaccine strains is confined to the terminal regions. The exact point of insertion/duplication, however, remains unclear. The possibilities are that the variation is confined to the ITR or that they occur directly before or within the E1a (LHT) and E4 (RHT) genes. AdV variants have never been described with additional DNA sequences within a functional gene that are still competent for normal replication without helper viruses. It is therefore unlikely that the additional sequences lie directly within E1 or E4 although their function may be altered in some way, either suppressed or enhanced.

Sira et al (1987) reported that the ITR of a vaccine strain of CAV-1 [Connaught Laboratories Ltd., CAV-1 (CCL)] contained multiple reiterations of a 40bp sequence. A range of terminal clones were isolated that contained varying numbers of this repeat, with a minimum of 1 to a maximum of 7. The repeat contained the sequence AGG(N)$_4$GCCTA (nucleotides 27-39) which closely resembles the consensus sequence of the human AdV NFI binding site (TG(N)$_{6-7}$GCCAA: nucleotides 25-38). The virus therefore appears to have reiterated copies of an essential part of the AdV origin of DNA replication. It is possible that the CAV vaccine strains presently under examination may reveal a similar structure. The human AdVs, Ad16 and Ad12,

also exhibited repeats of the ITR, and adjacent sequences (reviewed in chapter 2), although the repeats contained the whole ITR and not small fractions of it as in the CAV-1 strain reported by Sira et al (1987).

The final alternative, from all variation found so far in AdV, is that the additional sequences are cellular in origin as with the Ad3 variants (reviewed in chapter 2).

This chapter therefore deals with the attempted localisation of the additional sequences within the vaccine strains by means of restriction enzyme analysis and nucleotide sequencing. Sequence analysis was carried out to show any homology to known sequences, either viral or cellular. In addition, due to repeats of the ITR, either whole or partial, being the most common reported phenomenon to explain additional terminal sequences within AdV genomes, it was necessary to define the DNA sequence of the Glasgow CAV-2 ITR. This would allow direct comparison of a wild-type ITR and the additional sequences within the vaccine strains.

Kavak CAV-2 was discarded from future analysis upon discovery that it was produced by Wellcome for Duphar and was identical to Epivac CAV-2. All DNA sequence analysis was carried out on an IBM PC using the Beckman Microgenie DNA Sequence Analysis Package.

Extraction of DNA from Agarose Gels

The required DNA bands, from restriction enzyme digested DNA run on agarose gels, were cut out from the gel using a sterile scalpel. The fragment of agarose was then placed in a sterile microfuge tube and left at -20°C until frozen (approximately 30 minutes). The fragment was then thawed and placed in the column of a Spin-x tube (NBL), spun in a bench-top microfuge for 15 minutes after which the DNA in TBE had spun through the filter and the agarose was retained by it.

The DNA solution was then extracted with an equal volume of phenol (2x) and chloroform (2x) and finally ethanol precipitated. After pelleting, washing and drying, the DNA was resuspended in TE and stored at -20°C until required.

Electroelution of DNA From Polyacrylamide Gels

Polyacrylamide gels were prepared and run as described previously (chapter 2, materials and methods).

The required DNA bands were cut out from the gels using a sterile scalpel. The slice of acrylamide was then placed in a 5cm length of dialysis tubing which had previously been boiled in 0.5xTBE. The dialysis tubing was then clamped at one end, 0.5ml of 0.5xTBE placed into the tubing and the other end was also clamped after ensuring

that all air was removed.

The tubing was then placed in a horizontal gel tank, filled with 0.5xTBE, parallel to the electrodes. A constant potential difference of 120V was applied across the gel tank for 2 hours. The current was then reversed 3x for 5 minutes each time to ensure that no DNA was attached to the tubing which was then removed from the tank and the buffer removed from within it to a sterile tube. The slice of acrylamide was discarded after a visual check on a transilluminator that all the DNA had been extracted.

The buffer containing the DNA was extracted with an equal volume of phenol (2x) followed by chloroform (2x) and the DNA was recovered by ethanol precipitation. The DNA pellet was washed 2x in 70% EtOH, dried, resuspended in TE and stored at -20 °C until required.

Cloning Of HpaII Fragments In Bluescript

Bluescript vector was digested with ClaI, extracted once with phenol, twice with chloroform and the DNA was recovered by ethanol precipitation. After washing twice with 70% EtOH and drying, the DNA pellet was resuspended in TE and stored at -20 °C.

Viral DNA was initially digested with SalI and the entire reaction was loaded onto an agarose gel. The right-hand

terminal (RHT) SalI fragments were then extracted from the agarose using Spin-x columns as previously described. Each of the SalI fragments were then digested with HpaII and loaded onto either a polyacrylamide gel (Epivac, Nobivac, Boostervac) or an agarose gel (Vaxitas). The required HpaII fragment was removed from the relevant gel type, after electrophoresis and staining, and the DNA extracted as previously described.

The DNA samples were redissolved in TE pH7.5 and mixed in the ratio 3:1, CAV HpaII fragment:ClaI cut Bluescript. The mixture was then incubated with T_4 ligase as described previously. The ligated DNA was transformed into E.coli DS941. Recombinants were selected and screened as described previously.

Transfer Of DNA In Solution To Nitro-cellulose Paper (Dot-blots)

A small sheet of nitro-cellulose was marked into 1cm squares and 2µl of recombinant plasmid DNA (0.5mg/ml) was placed onto the centre of each square. The nitro-cellulose was then allowed to air dry. Next, the nitro-cellulose was floated, DNA side up, on a solution of 1.5M NaCl/ 0.5M NaOH for two minutes to denature the DNA. This was followed by floating the squares on a neutralising solution (1.5M NaCl/ 1M Tris pH8.0) for a further two minutes. The nitro-cellulose was then air dried and baked at 80 °C for two hours. The filters were then prehybridised and hybridised as described previously.

Empty Bluescript vector was used as a control.

Double Stranded Dideoxy Chain Termination Sequencing

Dideoxy chain termination sequencing was carried out on double stranded DNA after the method of Sanger et al (1977). All stock solutions and buffers were prepared as described in table 3.1.

Radioactive Label

Deoxyadenosine 5-[alpha-^{35}S]thiotriphosphate triethyl-ammonium salt (dATP, Sp isomer, Amersham) was used as the radioactive label throughout. The ^{35}S-dATP was purchased at a specific activity of 1200 curies/mmol and a concentration of 10mCi/ml. The label was stored in 12µl aliquots in vapour phase liquid nitrogen.

Primers

The primers SK, KS, T3 and T7 were purchased from NBL and diluted to 2.5ng/µl. Additional primers were synthesised within the laboratory on an Applied Biosystems 381A DNA synthesiser. Laboratory synthesised primers were initially used at a concentration of 5ng/µl but an increase of this amount was often necessary and the final concentration used in each reaction is stated with each individual sequence if other than 5ng/µl.

All primers were aliquoted and stored at -20^{o}C until required.

Denaturation

To 2µg of DNA was added 4µl 1M NaOH/ 1M EDTA. Sterile dH_2O was added to 20µl and the solution was incubated at room temperature for 5 minutes. 2µl of 2M NH_4Ac (pH5.4) was then added and the mixture placed on ice immediately. 2.5 volumes (55µl) of ethanol was added and the solution placed at -70°C for 15 minutes. The ethanol precipitation was centrifuged in a bench top microfuge for 10 minutes and the pellet washed (70% EtOH) and dried. The DNA was resuspended in 8.5µl TE pH8.0 and kept on ice.

Annealing

To the 8.5µl of DNA in TE was added 2µl of primer, 1.5µl 10x annealing buffer and 3µl ^{35}S-dATP. The mixture was incubated at 37°C for 15 minutes when 1µl of Klenow (1u/µl, Pharmacia) was added and the solution carefully mixed. This now constituted the template/primer/label/ enzyme mix (T/P/L/E mix).

Sequencing Reaction

To 3µl of the T/P/L/E mix was added 2µl of the A, C, G or T mix. The four reactions were then incubated at 42°C for 20 minutes after which 2µl of chase solution was added. The reactions were then incubated at 42°C for a further 15 minutes. Finally 4µl of formamide/dye mix was added and the samples heated to 95°C for 3 minutes prior to loading onto the sequencing gel. 5µl of each reaction was loaded onto the gel for the first long run and the remainder of

the reaction loaded, after reheating, for the short run.

Polyacrylamide Sequencing Gel

All solutions are as stated in table 3.2. Gel sets were purchased commercially and used as per manufacturer's instructions (Bio-Rad).

After the gels had been plugged and poured, with the well forming comb inserted, they were left to set at room temperature. All gels were then pre-run, in 1xTBE at a constant current of 30mA, for a minimum of 1 hour ensuring that the temperature of the gel and buffer had reached 50 ^{o}C. The gel was then loaded and the current altered to approximately 25mA, to retain a constant temperature of 50 ^{o}C, and the gel run until the bromophenol blue band reached the foot of the gel plates. If a long and short run were required, the remaining half of the sequencing reaction was then loaded and the gel run until the bromophenol blue band from the second loading reached the bottom.

All gels were fixed in 10% methanol/10% acetic acid for 15 minutes before being dried onto Whatman 3MM paper and subjected to autoradiography overnight.

T A B L E 3.1

Double Stranded DNA Sequencing Stock Solutions and Buffers

1) DeoxyNTP Stock Solutions

Stock solutions of 10mM dATP, 10mM dCTP, 10mM dGTP and 10mM dTTP were prepared in dH_2O and stored at -20°C.

2) dNTP Working Solutions

Stock solutions of dATP, dCTP, dGTP and dTTP diluted 1:20 in dH_2O to give 0.5mM working solutions.

3) dNTP Mixes

(for use with α-^{35}S dATP)

	A°	C°	G°	T°
0.5mM dCTP	20µl	1µl	20µl	20µl
0.5mM dGTP	20µl	20µl	1µl	20µl
0.5mM dTTP	20µl	20µl	20µl	1µl
TE pH8.0	20µl	20µl	20µl	20µl

4) DideoxyNTP Stock Solutions

Stock solutions of 10mM ddATP, 10mM ddCTP, 10mM ddGTP and 10mM ddTTP were prepared in dH_2O and stored at -20°C until required.

T A B L E 3.1 (continued)

5) ddNTP Working Solutions

10mM ddNTP	Dilution in dH_2O	Final Concentration
ddATP	3:200	0.15mM
ddCTP	1:500	0.02mM
ddGTP	1:200	0.05mM
ddTTP	1:20	0.50mM

6) dNTP/ddNTP Mixes

A mix	25µl A^o	+	25µl 0.15mM ddATP
C mix	25µl C^o	+	25µl 0.02mM ddCTP
G mix	25µl G^o	+	25µl 0.05mM ddGTP
T mix	25µl T^o	+	25µl 0.50mM ddTTP

7) Chase Solution

10mM dATP	5µl	0.5mM dNTP mix
10mM dCTP	5µl	
10mM dGTP	5µl	
10mM dTTP	5µl	
dH_2O	80µl	

8) 10x Annealing Buffer

100mM Tris.Cl pH8.0

50mM $MgCl_2$

T A B L E 3.1 (continued)

9) Formamide/Dye Mix

100ml formamide — stir gently for
5g Amberlite™ Mixed Bed Resin — 30 min then filter

Add:-

0.1g Xylene cyanol FF

0.1g Bromophenol Blue

4ml 500mM EDTA

Store at room temperature

10) Gel Fixing Solution

Methanol	200ml
Acetic Acid	200ml
dH_2O	to 2L

T A B L E 3.2

Sequencing Gel Stocks and Solutions

1) Stock Acrylamide

Acrylamide	76g
methylene-bis-acrylamide	4g
dH_2O	to 200ml

2) Urea Mix

Stock acrylamide	30ml
10x TBE	20ml
Urea	84g
dH_2O	to 200ml

3) Gel Plug

Urea mix	10ml
25% APS	70µl
TEMED	50µl

4) Sequencing Gel

Urea mix	50ml
25% APS	50µl
TEMED	50µl

5) 25% APS

Ammonium persulphate	0.25g
dH_2O	to 1ml

Divided into 120µl alliquots and stored at $-20^{o}C$

RESULTS

Restriction Enzyme Analysis of the SalI B Fragments from Vaxitas and Glasgow

The two terminal SalI B clones G11 (Glasgow) and V27 (Vaxitas) were further analysed by restriction endonuclease digestion in order to localise the site of insertion of the 1.0kb fragment in Vaxitas. A total of 16 enzymes were initially investigated as shown in figure 3.1. By utilising the known sites of these enzymes in the Bluescript vector (see Fig. 2.4) it was possible to construct a putative restriction endonuclease map of the SalI B fragments (Fig. 3.2). The site of insertion of the 1.0kb fragment in Vaxitas was now localised between the SmaI site, constant in both Vaxitas and Glasgow, 0.2kb from the right-hand terminal (RHT) and the PvuII site 1.1kb from the RHT in Glasgow and 2.2kb in Vaxitas.

HpaII Analysis of the Right-hand Termini of CAV-2 and CAV-1

Right-hand terminal SalI fragments (SalI B in CAV-2; SalI C in CAV-1) were electroeluted from agarose for all vaccine strains and the wild-type strains,Glasgow CAV-2 and RI 127 CAV-1. The most dominant species within each vaccine of the SalI B (CAV-2) and SalI C (CAV-1) were selected. Boostervac CAV-1 exhibits two equally dominant species therefore both Boostervac SalI C fragments were taken and examined individually. HpaII digestion of all strains is shown in figure 3.3 on 5% acrylamide. Vaxitas

and Glasgow SalI B fragments digested with HpaII are also shown on 0.8% agarose to further demonstrate the deletion in PstI D (fig. 3.3).

All inserts could now be clearly differentiated from wild-type fragments. Every vaccine strain examined appeared to exhibit an insert contained within a single HpaII fragment either by the appearance of a unique fragment (CAV-2 vaccines) or enlargement of an existing fragment (CAV-1 vaccines).

CAV-2

HpaII digestion of the wild-type SalI B fragment from Glasgow CAV-2 (Fig. 3.3) appears to comprise of four distinct fragments on acrylamide: 500, 180, 170 and 80 base pairs (bp): and one on agarose: 2.4kb. Epivac has an additional fragment at approximately 160bp, Nobivac at approximately 230bp and both appear to retain all other fragments associated with wild-type. Conversely Vaxitas no longer contains the 80bp fragment and three additional fragments can now be seen at 60bp, 190bp and 600bp. In addition, the deletion associated with Vaxitas in PstI D, resulting in the loss of the 0.6kb KpnI fragment, is now clearly exhibited on the agarose gel with the wild-type 2.4kb fragment being reduced to 2.0kb in size.

CAV-1

HpaII digestion of the wild-type RI 127 isolate appears to

result in three distinct fragments on acrylamide: 440, 180 and 170bp: and 1 on agarose: 2.0kb. Boostervac SalI C exhibits four fragments on acrylamide when digested with HpaII, 440bp,350bp, 240bp and 180bp. The 170bp fragment of wild-type is no longer apparent. Further analysis showed that the 350bp fragment was associated with the larger of the two SalI C fragments and the 240bp with the smaller of the two. Again the 170bp fragment is not present in the HpaII digest of the Epivac CAV-1 SalI C fragment but an additional fragment is present at 330bp with the 440bp and 180bp fragments still constant. The 2.0kb wild-type fragment remains constant for all vaccine strains showing that the 0.6kb deletion does not extend into SalI C.

Molecular Cloning of the Variant HpaII Fragments

Further investigation into the nature of the additional DNA found within the terminal regions of the vaccine strains necessitated that all the varying HpaII fragments of each vaccine strain be cloned. The enlarged (CAV-1) or unique (CAV-2) HpaII fragments from Boostervac CAV-1, Nobivac CAV-2 and Epivac CAV-2 were cloned into the ClaI site of the plasmid vector Bluescript. Boostervac was chosen as the sole CAV-1 vaccine strain examined due to its uniqueness in having two distinct species.

The size of the cloned HpaII fragments were confirmed by restriction endonuclease digestion, visualised by acrylamide gel electrophoresis alongside a HpaII digest of

the relevant RHT SalI fragment. As an additional confirmation all clones were checked by molecular hybridisation against each vaccine strain i.e. Boostervac clones BT1 (350bp) and BL2 (240bp) were hybridised to ^{32}P labelled Boostervac DNA; the Nobivac clone (N3) to Nobivac DNA; the Epivac clone (E4) to Epivac DNA etc. All hybridisations were carried out using the dot-blot method.

Both Vaxitas SalI B HpaII fragments at 600bp and 190bp were electroeluted and cloned into the ClaI site of Bluescript also. Unfortunately a sufficiently large or clean enough yield of the 60bp fragment was not obtained to subclone. Analysis of this small fragment will be discussed later. The Vaxitas clones were confirmed as above. An example of the dot-blots for various clones is given in figure 3.4.

DNA Sequence and Analysis of the Glasgow ITR

As all extra DNA associated with the vaccine strains appeared to be terminally associated it was necessary to obtain the DNA sequence of the Glasgow CAV-2 inverted terminal repeat from the SalI B and EcoRI terminal clones. This could then be used to compare to the vaccine inserts to confirm or eliminate direct or indirect repeats of this region. Both clones were sequenced from the termini using SK and T7 primers. The consensus sequence of the inverted terminal repeat is presented in figure 3.5. Sequence analysis (figure 3.6) demonstrated all known

conserved AdV sequences exhibited by the ITR. The CAV-2 ITR was demonstrated to be 82.5% homologous to the CAV-1 ITR over 63bp. Three base changes were found to occur however between the 3' and 5' ITRs. Repeated sequence analysis confirmed that these base changes were real and not reading errors. However other, aberrant, clones of the EcoRI LHT fragment were also obtained. These consisted of minor variations, usually single base changes, in the ITR. One clone consisted of an additional 60bp outwith the ITR but also replacing the initial 10bp, CATCATCAAT, demonstrated by the majority of the LHT clones and all RHT clones obtained (data not shown). These additional sequences were not included in the sequence analysis and comparisons with CAV-1 as they are believed to be the result of cloning aberrant genome termini.

DNA Sequence and Analysis of Vaccine Inserts

The DNA sequence of the multiple cloning site and primers of the plasmid vector Bluescript are shown in figure 2.4. The figures associated with each vaccine insert are described below and a representative sequencing gel of each clone is shown as an example.

It should be noted that the orientation of these fragments within the Bluescript vector is not necessarily their orientation with respect to the virus genome. This aspect of the insertions will be resolved later.

Boostervac CAV-1

BT1 (349bp): The larger of the Boostervac variant HpaII fragments was sequenced using T3 and T7 primers (Fig.3.7). The consensus sequence is shown in Fig. 3.8. The sequence analysis (Fig. 3.9) revealed a 70% similarity over 184bp in the region of the HuAd2 (human adenovirus type 2) E2 promoter and pVIII regions. When compared to the DNA sequence of Glasgow CAV-2 E2/E3 region (kindly provided by Mr. M. Macdonald, University of Glasgow) homology of >90% was revealed from bp1 to bp247 of BT1.

BL2 (249bp): the smaller of the two variant HpaII Boostervac fragments was sequenced using the KS primer (Fig. 3.7). The DNA sequence is shown in figure 3.10. Sequence analysis (Fig. 3.11) demonstrated >90% homology over 176bp to the CAV-1 E1 region (E1 sequence courtesy of Dr. A. M^{C}Clory, University of Glasgow).

Nobivac CAV-2

N3 (231bp): The unique 231bp HpaII fragment was sequenced using T3 and T7 primers (Fig. 3.12). The DNA sequence is shown in figure 3.13. The sequence analysis (Fig. 3.14) revealed N3 was 97% homologous to the right-hand terminus of Glasgow CAV-2 over 167 bases. This homology extended from bp10 of the ITR sequence to the end of the known Glasgow terminal sequences. It is therefore feasible that the homology will be continued further than the end of the ITR. This will be investigated in chapter 4.

Epivac CAV-2

E4 (164bp): the unique 164bp HpaII fragment of Epivac was sequenced using T3 primer (Fig. 3.12). The DNA sequence is shown in figure 3.15. Sequence analysis only revealed short regions of homology to other known AdV sequences (Fig. 3.16): 83.3% over 24bp to the Ad5 E3 region and 89.5% over 19bp to the Tupaia AdV E1b region. The two regions of homology however, are probably too short to be of any significance. However, homology of 67.1% over 79bp was also displayed against X. laevis mitochondrial DNA.

Vaxitas CAV-2

Vaxitas (193bp): the unique 193bp HpaII fragment of Vaxitas was sequenced using the T7 and T3 primers (Fig. 3.17). The DNA sequence is shown in Fig. 3.18. Sequence analysis revealed 98.9% homology over 190bp to the E1a region of CAV-2 and 70.9% over 172bp to the E1a region of CAV-1 (Fig. 3.20). In addition, several small regions of human AdV E1a's were found to display short regions of homology.

Vaxitas (631bp): the unique 631bp HpaII fragment of Vaxitas was sequenced using the primers T7 and T3. The DNA sequence is shown in Fig. 3.19. Sequence analysis revealed 98.1% homology to the E1a region of CAV-2 and an average of 74.2% to the E1a region of CAV-1 (figure 3.20). This is consistent with the results found from the sequence of the 193bp insert.

Fig. 3.1 Restriction enzyme analysis of the Glasgow CAV-2 (G) and Vaxitas CAV-2 (V) SalI B fragments in SalI/SmaI Bluescript. The terminal clones were digested with
Gel A:- SnoI (1), XbaI (2), SspI (3), AluI (4), NheI (5), AccI (6), AvaI (7), NdeI (8), SstII (9).
Gel B:- XhoI (10), XcyI (11), Sau3A (12), HindIII (13), PvuII (14), SphI (15), HaeII (16). HindIII digested λ DNA fragments were used as m.w. markers.

F I G U R E 3.1

Restriction Enzyme Analysis of the Glasgow and Vaxitas CAV-2 SalI B Fragments

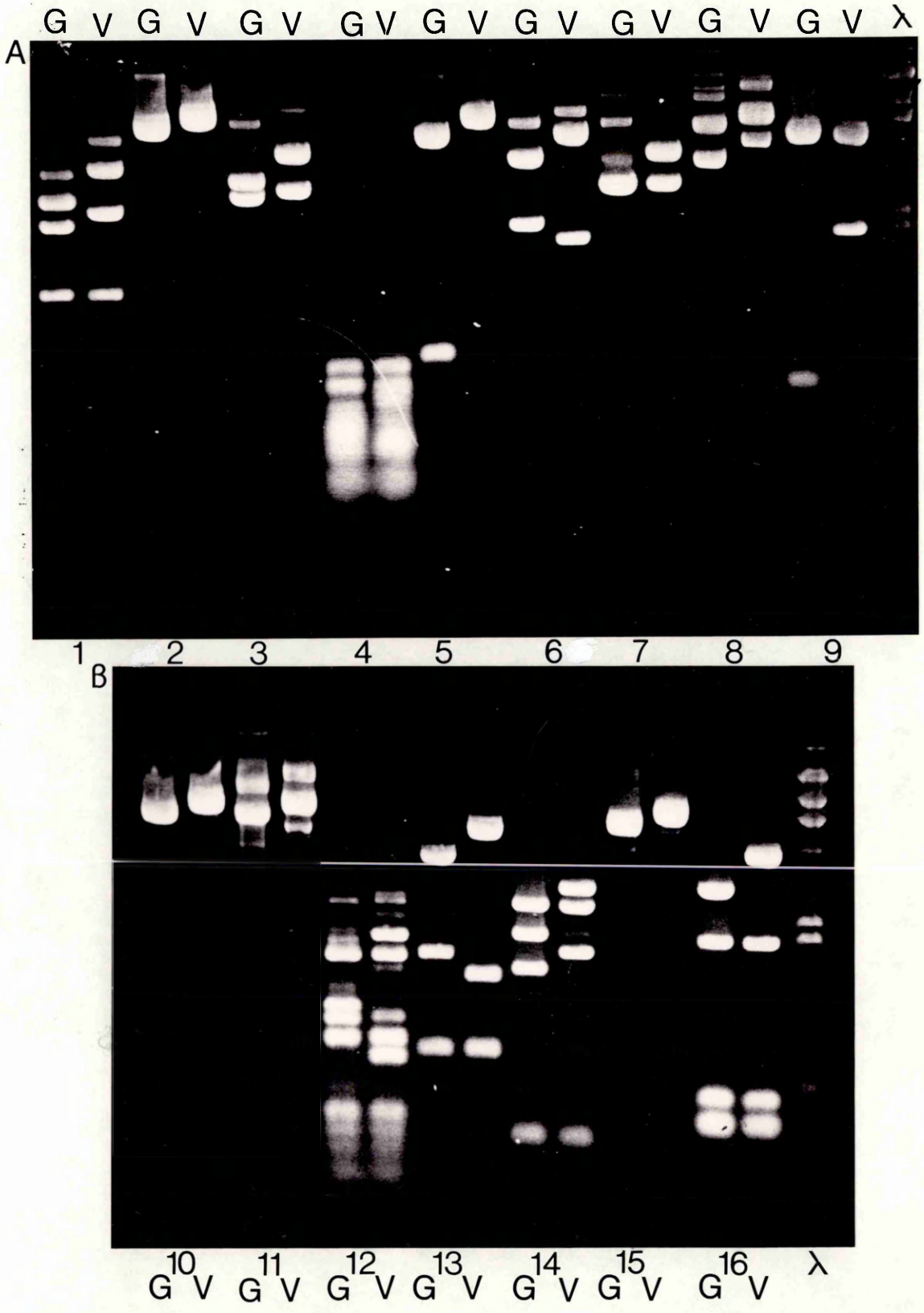


Fig. 3.2 Schematic physical map of the terminal SalI B clones of Glasgow and Vaxitas CAV-2. The SmaI sites represent the terminals of the clones (inserted into SalI/SmaI cut Bluescript as SalI/blunt fragments). Position of sites based on fragment size generated as indicated on agarose gels. Restriction enzyme sites marked in close proximity to each other e.g. AvaI/HpaII/SstII near terminal SmaI, are not necessarily in the order indicated as such precise mapping is not possible by restriction enzyme analysis alone. The unique NheI site of Vaxitas is thought to be positioned near the KpnI site and that the second NheI site present in Glasgow has been lost in Vaxitas along with the second KpnI site.

RE With No Sites in SalI B Clones:-

XbaI
NdeI
XhoI
XcyI

RE With A Single Site in SalI B Clones:-

AccI
AvaI
SphI (position unknown)
NheI (Vaxitas only)

F I G U R E 3.2

Schematic Restriction Enzyme Map of the Glasgow and Vaxitas SalI B Fragments

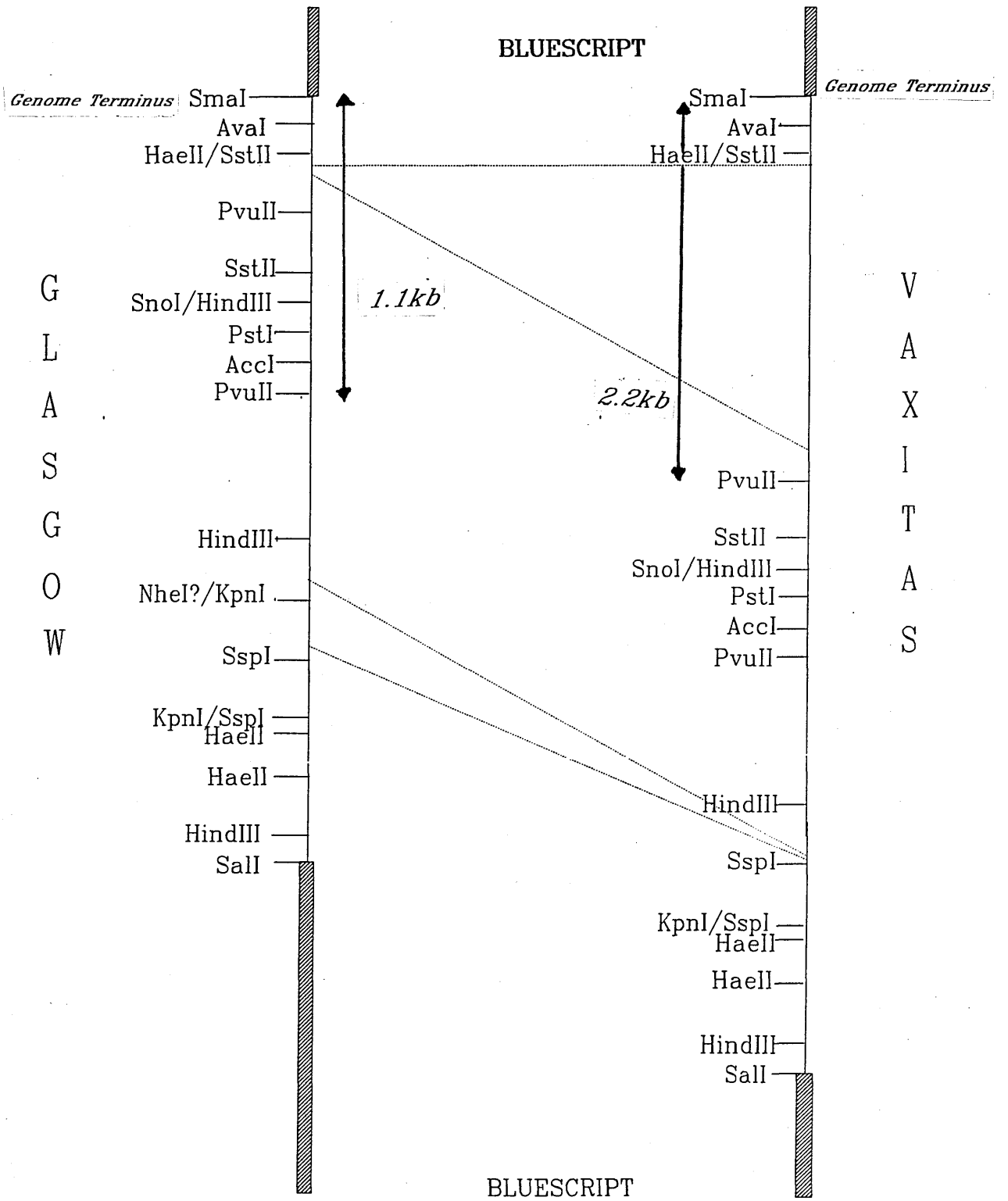
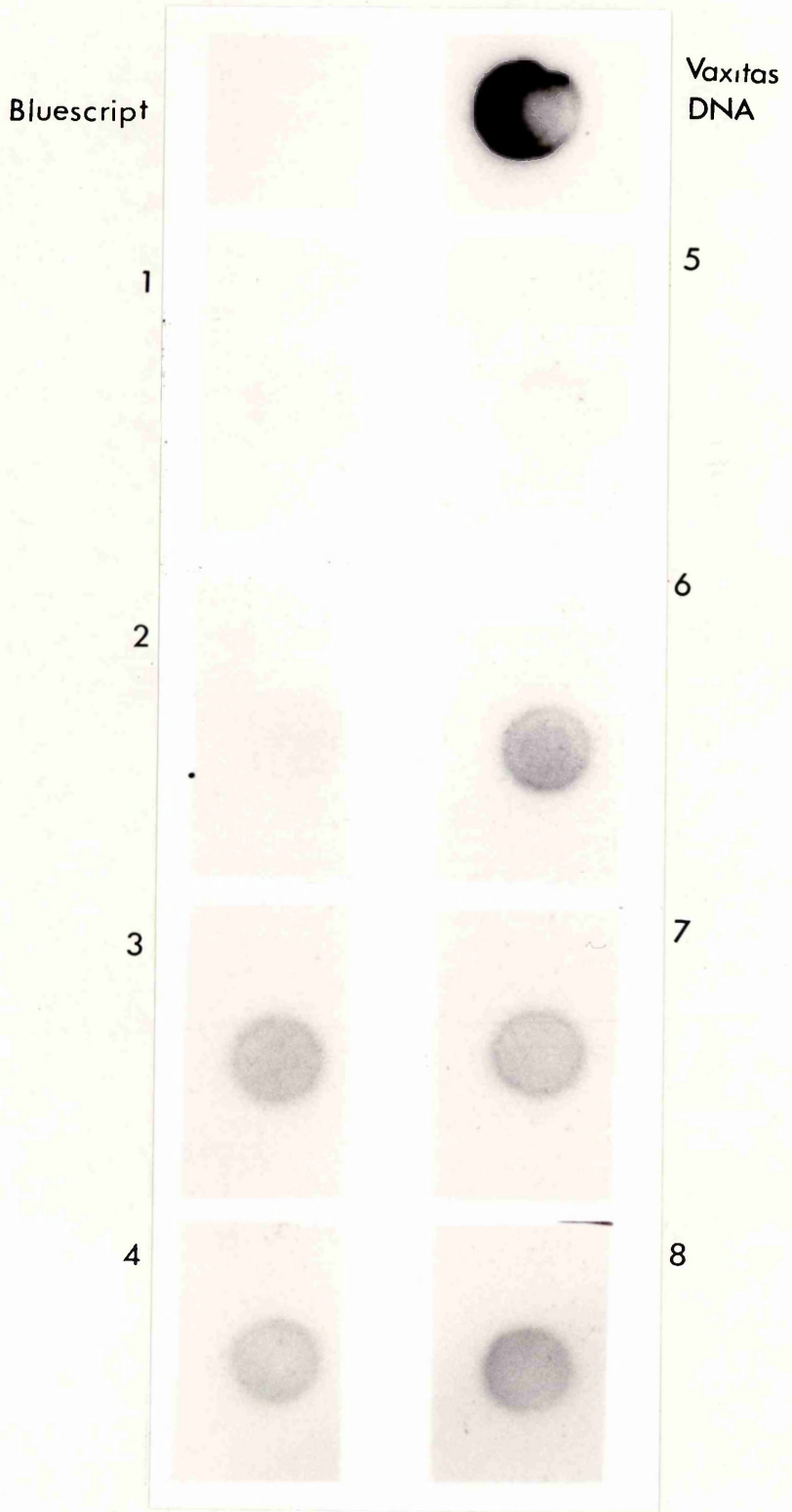


Fig. 3.4 HpaII clones in Bluescript were spotted onto nitro-cellulose and probed with ^{32}P-labelled whole virus. A representative sample of the results obtained during the cloning of the 190bp and the 660bp fragments of Vaxitas SalI B are shown.

Spots 1, 2 and 5 show aberrant clones obtained during the cloning procedure, probably chromosomal or fragmented Bluescript. Spots 3 and 4 represent two clones of the 190bp fragment and spots 6, 7 and 8, three isolates of the cloned 600bp fragment. All were probed with Vaxitas DNA.

F I G U R E 3.4

Molecular Hybridisation of CAV DNA to HpaII Clones



F I G U R E 3.5

Glasgow CAV-2 Inverted Terminal Repeat

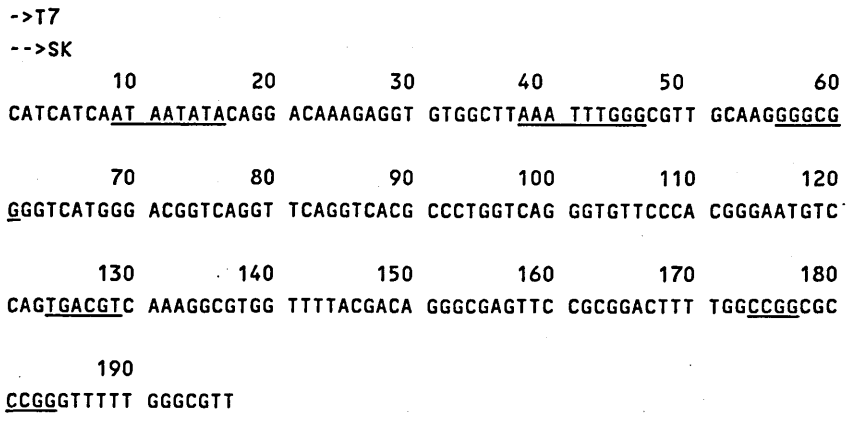


Fig. 3.5 DNA sequence of the inverted terminal repeat (ITR) of Glasgow CAV-2. SalI B and EcoRI C terminal clones sequenced using the T7 and SK primers of Bluescript as indicated above (->). The SalI B fragment cloned as a SalI/blunt fragment into the SalI/SmaI sites of Bluescript and the EcoRI C as an EcoRI/blunt into the EcoRI/SmaI sites of Bluescript thus allowing a known orientation (see Fig. 3.8). All HpaII sites within this region are underlined. Sequence conserved between species are also underlined.

F I G U R E 3.6

Sequence Analysis of the Glasgow CAV-2 Inverted Terminal Repeat

```
GLASGOW ITR
CAV1 (RI 127) ITR

83  AGGTCACGCCCTGGTCAGGGTGTT CCCACGGGAATGTCCAGTGACGTCAAAGGCGTGGT
    ||||||| ||||| |||||| ||| |||||||||| | ||| |||||||||  | ||| |
55  AGGTCACACCCTGTTCAGGGCGTTTCCCACGGGAAAGACCA TGACGTCAATTGGGTGTT

    TTT     144
    |||
    TTT     116

 Matches = 52        Length = 63        Matches/length =  82.5 %


GLASGOW 3'-ITR
GLASGOW 5'-ITR

1   CATCATCAATAATATACAGGACAAAGAGGTGTGGCTTAAATTTGGGCGTTGCAAGGGGCGG
    ||||||||| || ||||||||||||||||||||||||||||||||||||||||||||||||
1   CATCATCAATGATGTACAGGACAAAGAGGTGTGGCTTAAATTTGGGCGTTGCAAGGGGCGG

61  GGTCATGGGACGGTCAGGTTCAGGTCACGCCCTGGTCAGGGTGTTCCCACGGGAATGTCCA
    |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
61  GGTCATGGGACGGTCAGGTTC GGTCACGCCCTGGTCAGGGTGTTCCCACGGGAATGTCCA

121 GTGACGTCAAAGGCGTGGTTTTACGACAGGGCGAGTTCCGCGGACTTTTGGCCGGCGCCCG
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
120 GTGACGTCAAAGGCGTGGTTTTACGACAGGGCGAGTTCCGCGGACTTTTGGCCGGCGCCCG

181 GGTTTTTGGGCGTT   197
    |||||||||||||
180 GGTTTTTGGGCGTT   196

 Matches = 194       Length = 197       Matches/length = 98.4%
```

GLASGOW ITR
Ad10 ITR

```
1   CATCATCAATAATATACAGGACAAAG      26
    | ||||||||||||||||   ||||||
1   CTTCATCAATAATATACCCCACAAAG      26
```

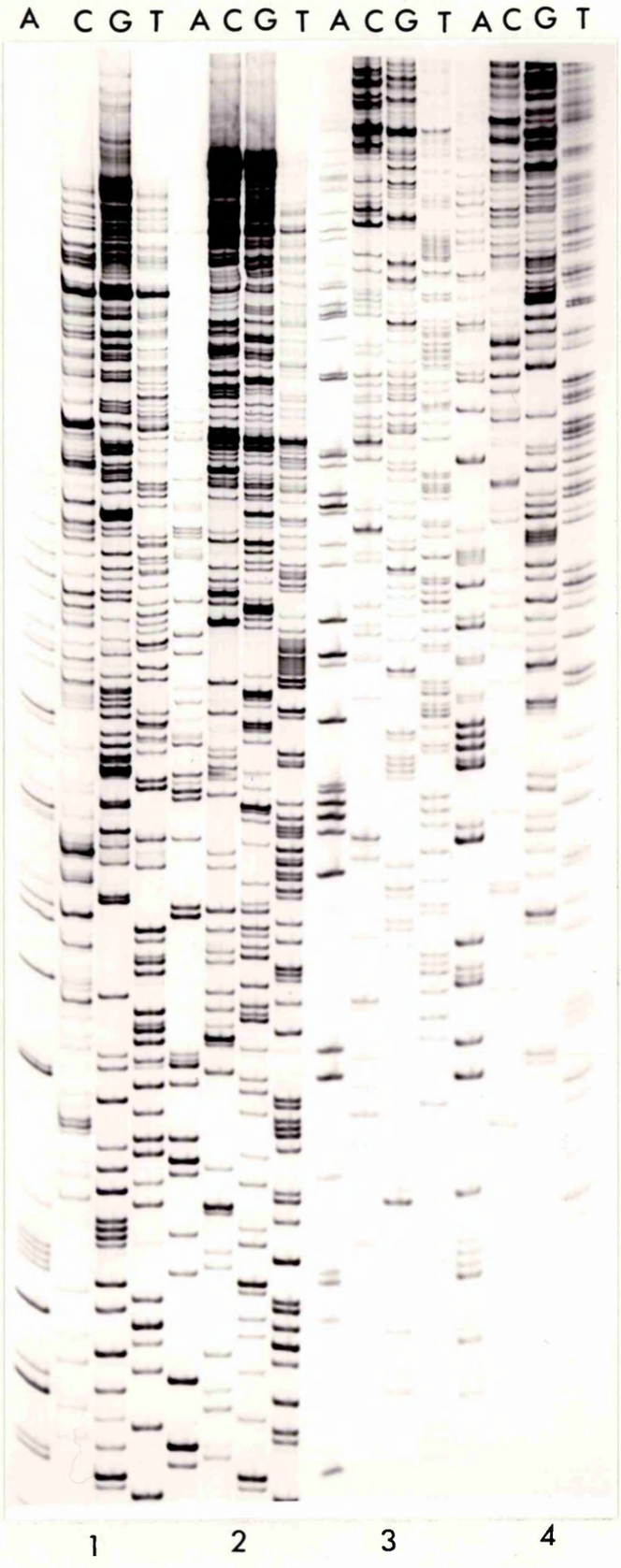
Matches = 22 Length = 26 Matches/length = 84.6%

				Matches/length cf. Glasgow ITR
GLASGOW ITR	1	CATCATCAATAATATAC	17	
		||||||||||||||||		
Ad12 ITR	1	CATCATCAATAATATAC	17	100%
		||||||||||||||||		
Ad18 ITR	1	CATCATCAATAATATAC	17	100%
		||||||||||||||||		
Ad31 ITR	1	CATCATCAATAATATAC	17	100%
		||||||||||||||||		
Ad5 ITR	1	CATCATCAATAATATAC	17	100%
		| |||||||||||||||		
Ad10 ITR	1	CTTCATCAATAATATAC	17	94.1%

Fig. 3.7 Long and short runs of sanger dideoxy sequencing reactions on the HpaII clones BT1 and BL2 using the primer KS. Lanes 1 and 4 display long and short runs of BL2 respectively. Lanes 2 and 3, long and short runs of BT1 respectively.

F I G U R E 3.7

Boostervac CAV-1 Insertions BT1 (600BP) and BL2 (190BP)
- Using the Bluescript Primer KS



F I G U R E 3.8

Boostervac CAV-1 - BT1

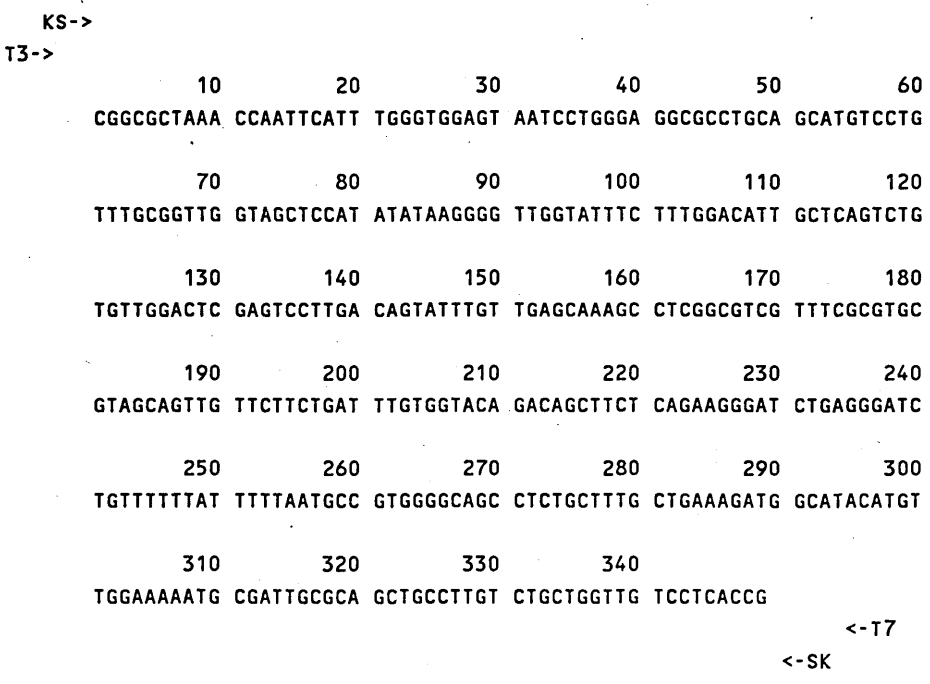
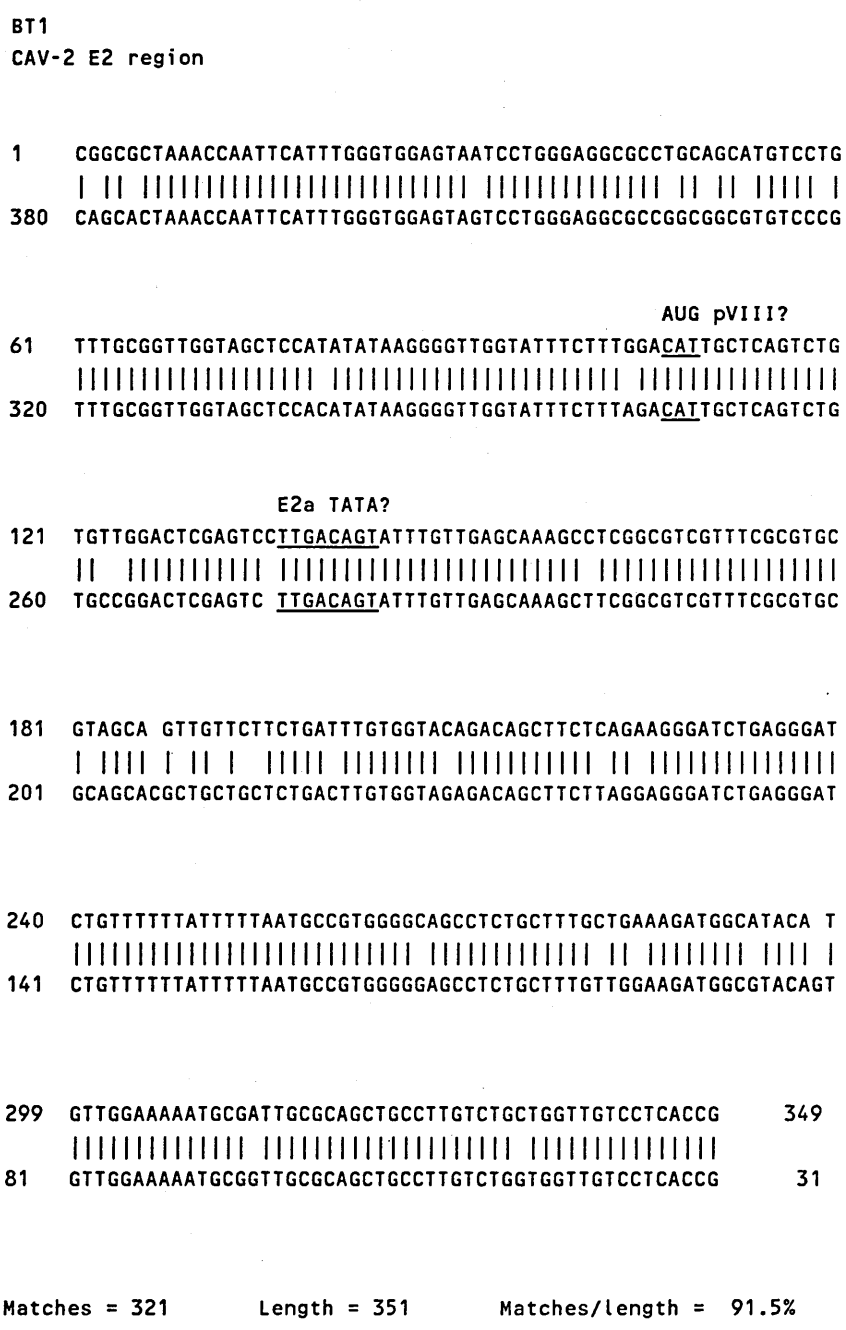


Fig. 3.8 DNA sequence of Boostervac CAV-1 clone BT1, the larger of the two variant HpaII fragments contained within the vaccine strain. DNA sequence obtained using T3 and T7 primers of Bluescript (-> and <-) as shown above.

F I G U R E 3.9

Sequence Analysis of the Boostervac Insertion BT1



Matches = 321 Length = 351 Matches/length = 91.5%

```
BT1
Adenovirus type 2, complete genome                    STRAND L


 141    CAGTATTTGTTGAGCAAAGCCTCGGCGTCGTTTCGCGTGCGTAGCAGTTGTTCTTCTGAT
        ||||||||| |||  | ||||||  |||||  |   |||||||  | ||  ||  |||||   |
27105   CAGTATTTGCTGAAGAGAGCCTCCGCGTCTTCCAGCGTGCGCCGAAGCTGATCTTCGCTT
                 / **
                 Ad2 cap sites for 72kD mRNA early (E2a)
 201    TTGTGGTACAGACAGCTTCTCAGAAGGGATCTGAGGGATCTGTTTTTTATTTTTAATGCC
        |||||  |||||  |||||  |     |||||  |  ||  ||  ||||||||||||||  |   |
27045   TTGTGATACAGGCAGCTGCGGGTGAGGGAGCGCAGAGACCTGTTTTTTATTTTCAGCTCT
                        / *
                        donor splice 72kD mRNA (E2a)

 261    GTGGGGCAGCCTCTGCTTTGCTGAAAGATGGCATACATGTTGGAAAAA TGCGATTGCGC
                ||     ||||  |||||  ||  |||||||   |||  ||||  |  |  |||||
26985   TGTTCTGGCCCCTGC TTTGTTGAAATATAGCATACAGAGTGGGAAAAATCCTATTTCTA


320     AGCT     323
        ||||
26918   AGCT   26921


 Matches = 129       Length = 184       Matches/length =  70.1%
```

Fig. 3.9 Sequence analysis of the larger of the two Boostervac CAV-1 insertion sequences. 70.1% similarity is demonstrated to the E2/E3 promoter regions of Ad2 and the presumptive E2/E3 promoter region of Glasgow CAV-2. The Ad2 E2a TATA is situated at bp27118, ACTCTTAA: 13bp before the start of the demonstrated homology. No such sequence is demonstrated in either the Boostervac insertion sequence or the Glasgow sequence. However the Boostervac insertion sequence and the Glasgow sequence demonstrate possible E2a TATA's: ACTgTcAA: in the same region as the Ad2 E2a TATA.

F I G U R E 3.10

Boostervac CAV-1 - BL2

T3
->

```
       10         20         30         40         50         60
CGGCCCTCCT CTCCTCAGCC TCAATCAAGC TAAAATCTAC CTGCCCTCTA GGCATTTCCA

       70         80         90        100        110        120
AAGCATGGTC CCGTTGCAGA GCTCGCGTAA CATACAGGGA ATCTGGAAGC TGCAGATCGT

      130        140        150        160        170        180
CTAGTCGAAA AGGGGATCGT CGCTGTCCCT CGCTAAGTGC TCTTCTCCGC ACTCTTCCTC

      190        200        210        220        230        240
TACAGGACCC TGCCCGTCGA ACAGGACTCT GGAGGTCGTT GATTAGTCGC GCGGCCTGAC


TCAGATCTG
```

Fig.3.10 DNA sequence of Boostervac CAV-1 BL2 clone, the smaller of the two variant HpaII fragments within the SalI C fragment of the vaccine strain. DNA sequence obtained using the T3 of Bluescript (->) as shown above.

F I G U R E 3.11

Sequence Analysis of the Boostervac Insertion BL2

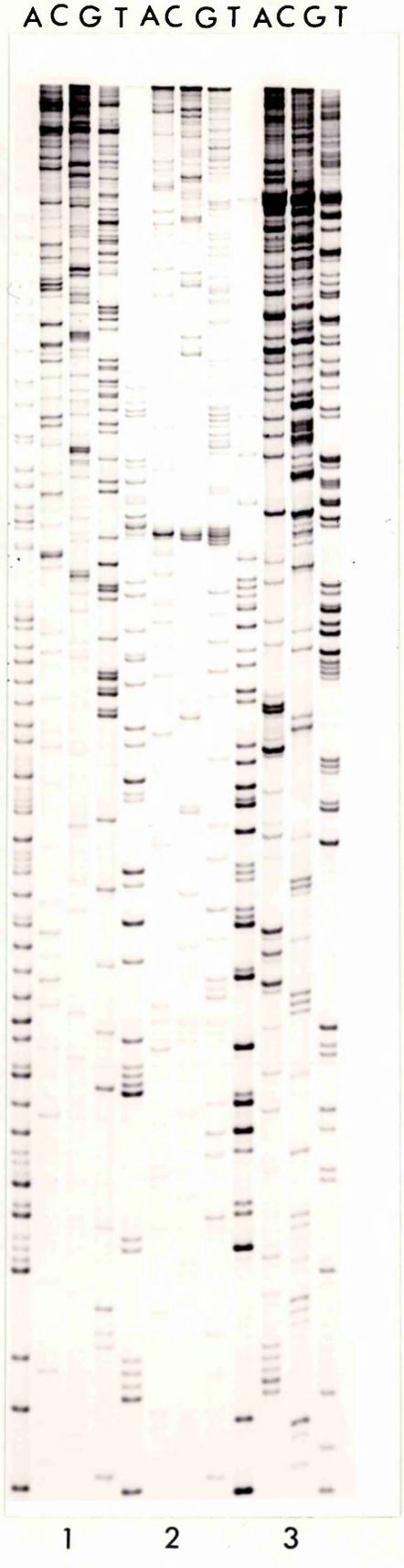
```
BL2
CAV1E1                                          STRAND L

110   CTGCAGATCGTCTAGTCGAAAAGGGGATCGTCGCTGTCCCTCGCTAAGTGCTCTTCTCCG
      ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
1853  CTGCAGATCGTCTAGTCGAAAAAGGGATCGTCGCTGTCCCTCGCTAAGTGCTCTTCTCCG


170   CACTCTTCCTCTAC A GGACCC TGCCCGTCGAACA GGACTCTGG AGGTCGTTGATT
      |||||||||||||| | |||||| |||||||||||| ||||||||| ||||||||||||
1793  CACTCTTCCTCTACCACGGACCCCTGCCCCTCGAACACGGACTCTGGGAGGTCGTTGATT


225   AGTCGCGCGGCCTGACTC AGATCTG     249
      |||||||||||||||||| |||||||
1733  AGTCGCGCGGCCTGACTCCAGATCTG    1708

Matches = 138       Length = 146       Matches/length =  94.5%
```

```
BL2
CAV2E1                                          STRAND L

80    AGCTCGCGTAACATACAGGGAATCTGGAAGCTGCAGATCGTCTAGTCGAAAAGGGGATCG
      |||| || ||||||||||||   | || |||||||  || ||||  |||| |  || |||
2097  AGCTTGCATAACATACAGGGGG CGGGCAGCTGCAACTCATCTACCCGAATAATGGGTCG


140   TCGCTGTCCCTCGCTAAGTGCTCTTCTCCGCACTCTTCCTCTACAG G ACCC TGCCCG
      ||||||||||  |||||  || ||||| |||||||||||||| | | | |||| ||| |
2038  TCGCTGTCCCCTGCTAAAAGCGCTTCTTCGCACTCTTCCTCTTCCGCGGACCCCTGCTCC


197   TCGAACA GGACTCT GGAGGTCGTTGATTAGTCGCGCGGCCTGACTC AGATCTG  249
      |||| || ||| ||| ||||||||||||||   | |||| |||  || | |||||
1978  TCGAGCACGGAATCTTGGAGGTCGTTGATTAACTG GCGGTCTGGGTCCATATCTG 1924

Matches = 134       Length = 176       Matches/length =  76.1%
```

Fig. 3.12 Lanes 1 and 2 show the result of a Sanger dideoxy sequencing reaction using the primer T3 on the HpaII clones E4 (Epivac CAV-2 additional SalI B HpaII fragment) and N3 (Nobivac CAV-2 additional SalI B HpaII fragment) respectively. Lane 3, sequence obtained of the Nobivac insertion (N3) using the primer T7.

F I G U R E 3.12

Nobivac 230BP Insertion (N3) and Epivac 165BP Insertion (E4) - Using the Bluescript Primers T3 and T7



F I G U R E 3.13

Nobivac CAV-2 - N3

```
T3
->
         10         20         30         40         50         60
   CGGCCAAAAG TCCGCGGAAT CGCCCTGTCG TAAAACCACG CCTTTGACGT CACTGGACAT

         70         80         90        100        110        120
   TCCCGTGGGA ACACCTGACC AGGGCGTGAC CTGAACCTGA CCGTCCCATG ACCCCGCCCC

        130        140        150        160        170        180
   TTGCAAGACC CAAATTTAAG CCACACCTGT TTGTCCTGTA TATTAAGTAC AGTCATAAAA

        190        200        210        220        230
   TGTGGCGCGT GGTAAATGTT AAGTGCAAGT TTCCCTTTGG CGGTTGGCCC G
```

Fig. 3.13 DNA sequence of Nobivac CAV-2 unique HpaII fragment sequenced using the Bluescript primer T3 (->) as indicated above.

F I G U R E 3.14

Sequence Analysis of the Nobivac Insertion N3

N3
GLASGOW 5'-ITR STRAND L

```
1    CGGCCAAAAGTCCGCGGAA TCGCCCTGTCGTAAAACCACGCCTTTGACGTCACTGGACA
     |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
176  CGGCCAAAAGTCCGCGGAACTCGCCCTGTCGTAAAACCACGCCTTTGACGTCACTGGACA

60   TTCCCGTGGGAACACC TGACCAGGGCGTGACCTGAACCTGACCGTCCCATGACCCCGCC
     |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
116  TTCCCGTGGGAACACCCTGACCAGGGCGTGACCTGAACCTGACCGTCCCATGACCCCGCC

119  CCTTGCAAGACCCAAATTTAAGCCACACCTGTTTGTCCTGTATATTA     165
     ||||||||  |||||||||||||||||||| ||||||||||||||||
56   CCTTGCAACGCCCAAATTTAAGCCACACCTCTTTGTCCTGTATATTA      10
```

Matches = 162 Length = 167 Matches/length = 97.0%

F I G U R E 3.15

Epivac CAV-2 - E4

```
T3
->
          10         20         30         40         50         60
   CGGCAGCCAT TCTGCAAAAT AACAAAACAC CACTAAGCAT AGCACCATCA CCAAGCATGA

          70         80         90        100        110        120
   AAACAGGTAA AAACAAAAGC AACACTTACT TATTCAGCAG TCACAAGAAT GTTGGGCTCC

         130        140        150        160
   CAAGTGACAG ACCAAGCCTA ATGCAAGGTG GGCACAGTCC TCCG
```

Fig. 3.15 DNA sequence of the Epivac CAV-2 unique HpaII fragment. Sequenced using the Bluescript primer T3 (->) as indicated above.

F I G U R E 3.16

Sequence Analysis of the Epivac Insertion E4

E4
Ad5 E3 transcription region

```
69   AAAAACAAAAGCAACACTTACTTA     92
     ||| |  |||||| ||||||||||
3517 AAATAATAAAGCATCACTTACTTA   3540
```

Matches = 20 Length = 24 Matches/length = 83.3%

E4
Tree shrew (Tupaia) adenovirus early region E1b STRAND L

```
6    GCCATTCTGCAAAATAACA      24
     | ||||||| |||||||||
1613 GACATTCTGAAAAATAACA    1595
```

Matches = 17 Length = 19 Matches/length = 89.5%

E4
X.laevis mitochondrial DNA containing the D-loop and the 12S rRNA

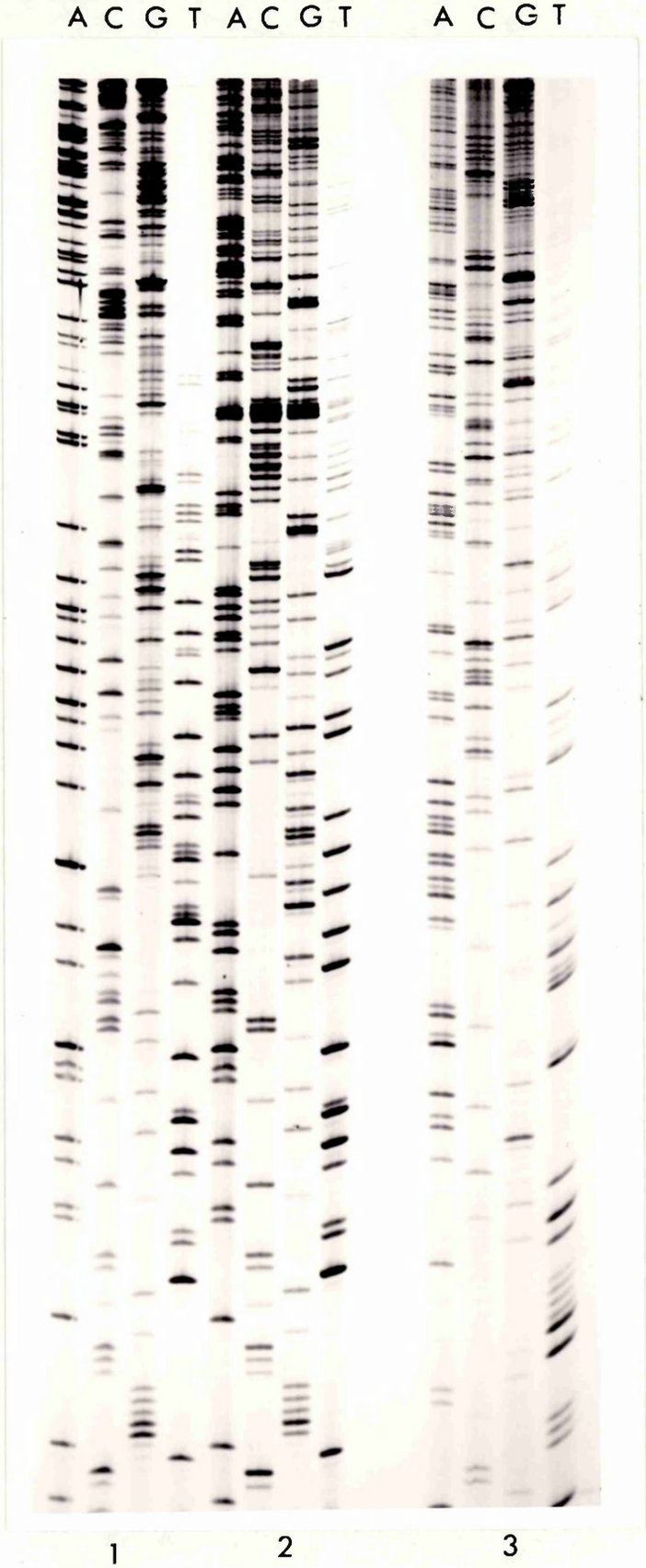
```
1    CGGCAGCCATT CTGCAAAATAACAAAACACCACTAAGCATAGCACCATCACCAAGCATG
     | |||||||   ||||| ||||   |||||| ||||| |||  ||||   |  || ||
1    CTGCAGCCAACGCTGCAGAATAGGCAAACACTACTAA CATCCCACCTAAATAAATCAAA


60   AAAACAGGTAAAAACAAAA      78
     ||||  |   | | ||||||
60   AAAAGAACAATAGACAAAA      78
```

Matches = 53 Length = 79 Matches/length = 67.1%

Fig. 3.17 Sanger dideoxy sequencing reaction on the Vaxitas 631bp and 193bp SalI B HpaII fragments using the primers T3 and T7. Lane 1 represents the 193bp HpaII fragment sequence, on a short run, with the primer T7. Lanes 2 and 3 show short runs of the sequence obtained, using the primers T7 and T3 respectively, from the 631bp HpaII fragment.

F I G U R E 3.17

Vaxitas CAV-2 631bp and 193bp HpaII Fragments
- Using the Bluescript Primers T3 and T7



F I G U R E 3.18

DNA Sequence
of the 193bp HpaII Fragment of Vaxitas CAV-2 SalIB

```
T7
-->
          10         20         30         40         50         60
    CCGGACTGCC TTGACTGTGA GTATTCTCAT GGCAGCCCCT CGCCGCCTAC TCTGCACGAT

          70         80         90        100        110        120
    CTTTTTGATG TTGAGCTGGA GACTTCTCAC AGCCCTTTTG TGGGCCTGTG TGATTCCTGT

         130        140        150        160        170        180
    GCGGAGGCTG ACACTGATTC GAGTGCGAGC ACTGAGGGTG ATTCTGGGTT TAGTCCTTTA

         190
    TCCACTCCGC CGG
```

Fig. 3.18 DNA sequence of the 193bp HpaII fragment unique to Vaxitas CAV-2. Sequence data obtained using the primer T7 of Bluescript as indicated above (-->).

F I G U R E 3.19

DNA Sequence of the 631bp HpaII Fragment of Vaxitas CAV-2 SalIB

T7
-->

```
        10         20         30         40         50         60
CCGGTTTCAC TATTCCACCG CATCCCACCT CTCCTGCTAG CATTTCTGAC GACATGTTGC

        70         80         90        100        110        120
TGTGCTTAGA GGAAATGCCC ACCTTTGATG ACGAGGACGA GGTTCGAAGC GCGGCGACCA

       130        140        150        160        170        180
 CTTTGAGCG GTGGGAAAAC ATTTTGACCC CCATGTGGGT CCTATTTTTG GCTGTTTGCG

       190        200        210        220        230        240
CTGTGCTTTT TATCAAGAGC AGGATGATAA TGCACTTTGT GGGCTTTGCT ATCTAAAGGC

       250        260        270        280        290        300
CCTTGCCGAA GTAAGTTTTA ATTTAAATGT TTGGGCAGGT TAAATGTTTG GGCAGGTTAA

       310        320        330        340        350        360
ATGTTTTAGG TGTGTATTGA TTTTTAATTT TGCTTTTTAG TGCCTTTTGC TATGCCTGTA

       370        380        390        400        410        420
CGTTCAGAAC CCGCTTCGGC TGGAGCTGAG GAGGAAGATG ATGAAGTTAT TTTTGTGTCT

       430        440        450        460        470        480
GCCAAACCTG GGGGCAGAAA GAGGTCAGCA GCTACTCCCT GTGAGCCAGA TGGGGTCAGC

       490        500        510        520        530        540
AAACGCCTTG CGTGCCAGAG CCTGAGCAAA CAGAACCTTT GGATTTGTCT TTGAAGCCAC

       550        560        570        580        590        600
GCCCGAACTA ATCTCCTTGA GCACAAAGCA ATAAAGTAAT CTTGTTTAAC AAGTTGACAA

       610        620        630
GGACGTGCTG TACTTTTTGT GAACTTTCCG G
                                  <--T3
```

Fig. 3.19 DNA sequence of the unique 631bp HpaII fragment of Vaxitas CAV-2 obtained using the primers T3 and T7 of Bluescript as indicated (->).

FIGURE 3.20

Sequence Analysis of the 631bp and the 193bp HpaII Fragments of Vaxitas CAV-2 SalIB

193bp Fragment
CAV1E1

```
1    CCGGACTGCCTTGACTGTGAGTATTCTCATGGCAGCCCCTCGCCGCCTACTCTGCACGAT
     ||||| || ||  ||||||||| || |||||  || | || |  || | ||| || |||
492  CCGGAATGTCTCTCCTGTGAGTACTCCCATGGTGGCTCGTCACA CCGAGTCTTCATGAT

61   CTTTTTGATGTTGAGCTGGAGACTTCTCACAGCCCTTTTGTGGGCCTGTGTGATTCCTGT
     ||||||||| |||||||||| | |||||    |||| |  |  |||||||||| |||
551  CTTTTTGATCTTGAGCTGGAAAATTCTCGTTCTCCTTCTCCGCTGCTGTGTGATTGGTGT

121  GCGGAGGCTGACACTGATTCGAGTGCGAGCACTGAGGGTGATTCTGGGTTTA
     |||||||||||||| ||| || |      ||| |||  ||||  ||| ||||
611  GCGGAGGCTGACAGTGAATCTACAATTTCCACGGAGACTGATGTTGGCTTTA
```

Matches = 122 Length = 172 Matches/length = 70.9%

193bp Fragment
Ad41E1a

```
47   TACTCTGCACGATCTTTTTGATGTTGAGCTGGA   80
     | | || ||| |||||||||||||| ||||||||
600  TTCGCTTCACAATCTTTTTGATGTGGAGCTGGA   633
```

Matches = 28 Length = 33 Matches/length = 84.8%

193bp Fragment
CAV2E1

```
1    CCGGACTGCCTTGACTGTGAGTATTCTCATGGCAGCCCCTCGCCGCCTACTCTGCACGAT
     ||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||
624  CCGGACTGCCTTGACTGTGAGTATCCTCATGGCAGCCCCTCGCCGCCTACTCTGCACGAT

61   CTTTTTGATGTTGAGCTGGAGACTTCTCACAGCCCTTTTGTGGGCCTGTGTGATTCCTGT
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
684  CTTTTTGATGTTGAGCTGGAGACTTCTCACAGCCCTTTTGTGGGCCTGTGTGATTCCTGT
```

```
121  GCGGAGGCTGACACTGATTCGAGTGCGAGCACTGAGGGTGATTCTGGGTTTAGTCCTTTA
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
744  GCGGAGGCTGACACTGATTCGAGTGCGAGCACTGAGGGTGATTCTGGGTTTAGTCCTTTG


181  TCCACTCCGC
     ||||||||||
804  TCCACTCCGC
```

Matches = 188 Length = 190 Matches/length = 98.9%

193bp Fragment
Adenovirus 3 left end, with inverted terminal rpt

```
27   CATGGCAGCCCCTCG  CCGCCTACTCTGCACGATCTTTTTGATGTTGAGCTGGA   81
     ||  ||||||  |||  || ||||| ||||||||||| | |||| | ||| | ||
690  CAGTGCAGCCTTTCGATCCACCTACGCTGCACGATCTGTATGATTTAGAGATAGA   744
```

Matches = 41 Length = 55 Matches/length = 74.5%

193bp Fragment
Adenovirus type 7 left end of the genome

```
42   CCGCCTACTCTGCACGATCTTTTTGATGTTGAGCTGGA   79
     || ||||| |||||||||||| | |||| | ||| | ||
699  CCACCTACGCTGCACGATCTGTATGATTTAGAGGTAGA   736
```

Matches = 30 Length = 38 Matches/length = 78.9%

631bp HpaII fragment
CAV2E1

```
1    CCGGTTTCA CTATTCCACCGCATCCCACCTCTCCTGCTA GCATTTCTGACGACATGTT
     ||||||||| |||||||||||||||||||||||||||||| |||||||||||||||| |
813  CCGGTTTCACCTATTCCACCGCATCCCACCTCTCCTGCTACGCATTTCTGACGACATGGT


59   GCTGTGCTTAGAGGAAATGCCCACCTTTGATGACGAGGACGAGGTTCGAAGCGCGGCGAC
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
873  GCTGTGCTTAGAGGAAATGCCCACCTTTGATGACGAGGACGAGGTTCGAAGCGCGGCGAC


119  CACCTTTGAGCGGTGGGAAAACA TTTTGACCCCCATGTGGGTCCTATTTTTGGCTGTTT
     |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
933  CACCTTTGAGCGGTGGGAAAACACTTTTGACCCCCATGTGGGTCCTATTTTTGGCTGTTT


178  GCGCTGTGCTTTTTATCAAGAGCAGGATGATAATGCACTTTGTGGGCTTTGCTAT CTAA
     |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
993  GCGCTGTGCTTTTTATCAAGAGCAGGATGATAATGCACTTTGTGGGCTTTGCTATCCTAA
```

```
237  AGGCCCTTGCCGAA GTAAGTTTT AATTTAAATGTTTGGGCAGGTTAAATGTTTGGGCA
     ||||||||||||||| ||||||||| ||||||||||||||||||||||||||||||||||
1053 AGGCCCTTGCCGAAGGTAAGTTTTGAATTTAAATGTTTGGGCAGGTTAAATGTTTGGGCA


295  GGTTAAATGTTTTAGGTGTGTATTGATTTTTAATTTT GCTTTTTAGTGCCTTTTGCTAT
     |||||||||||||||||||||||||||||||||||| |||||||||||||||||||| |
1113 GGTTAAATGTTTTAGGTGTGTATTGATTTTTAATTTTGGCTTTTTAGTGCCTTTTGCTTT


354  GCCTGTACGTTCAGAACCCGCTTCGGCTGGAGCTGAGGAGGAAGATGATGAAGTTATTTT
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1173 GCCTGTACGTTCAGAACCCGCTTCGGCTGGAGCTGAGGAGGAAGATGATGAAGTTATTTT


414  TGTGTCTGCCAAACCTGGGGGCAGAAAGAGGTCAGCAGCTACTCCCTGTGAGCCAGATGG
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1233 TGTGTCTGCCAAACCTGGGGGCAGAAAGAGGTCAGCAGCTACTCCCTGTGAGCCAGATGG


474  GGTCAGCAAACGCCCTTGCGTGCCAGAGCCTGAGCAAACAGAACCTTTGGATTTGTCTTT
     || |||||||| |||||||||| ||||||||||||||||||||||||||||||||||||
1293 GGCCAGCAAACCCCCTTGCGTGGCAGAGCCTGAGCAAACAGAACCTTTGGATTTGTCTTT


534  GAAGCCACGCCCGAACTAATCTCCTTGAGCACAAAGCAATAAAGTAATCTTGTTTAACAA
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1353 GAAGCCACGCCCGAACTAATCTCCTTGAGCACAAAGCAATAAAGTAATCTTGTTTAACAA


594  GTT
     |||
1413 GTT
```

Matches =591 Mismatches =5 Unmatched =7 Length =603 Matches/length =98.1%

631bp Fragment
CAV1E1

```
279  TTAAATGTTTGGGCAGGTTAAATGTTTTAGGTGTGTATTGATTTTTAATTTTGCTTTTTA
     |||||| | ||||  ||| ||  |||| |     ||  | || | |   | |   |||||
943  TTAAAT TATGGGTGGGTCAATAGTTTCACAAAGGTTCTTATGTGTTCCTCTCTGTTTTA


339  GTGCCTTTTGCTATGCCTGTACGTTCAGAACCCGCTTCGGCTGGAGCTGAGGAGGAAGAT
     ||||| |  || |||||||| ||  ||||||| | |   ||||  || | | || | |||
1002 GTGCCCTGCGCCATGCCTGTTCGCGCAGAACCTGTTGATGCTGATGCCG G AG AGGAT


399  GATGAAGTTATTTTTGTGTCTGCCAAACCTGGGGGCAGAAAGAGGTCAGCAGCTACTCCC
     ||||| || |||||||||||||| ||||||||   |||||||||||||| |  ||| ||
1059 GATGAGGTCATTTTTGTGTCTGCTAAACCTGGTTCCAGAAAGAGGTCAGCGGTGACTTCC
```

=

```
459  TGTGAGCCAGATGGGGTCAGCAAACGCC TTGCGTGCCAGAGCCTGAGCAAACAGAACCT
      | ||  ||| ||    ||| ||||||| |||| |||||||||||| ||| | ||||||
1119 CGCGACTCAGTTGAAAGCAGTAAACGCCCTTGCTTGCCAGAGCCTGAACAA CTGAACCT


518  TTGGATTTGTCTTTGAAGCCACGCCCGAACTAATCTC CTTGAG    560
     |||||| | ||  | ||||||||||  ||  |||  | ||||||
1178 TTGGATCTTTCCCTAAAGCCACGCCGCAATAAATTACTCTTGAG    1221

Matches = 203     Length = 284     Matches/length = 71.5%


1    CCGGTTTCAC TATTCCACCGCATCCCACCTCTCCTGCTAGCATTTCTGACGACATGTTG
     |||||||| | | ||||  || || |||| |||||||| || ||| |||| |||||| ||
677  CCGGTTTCTCCTCTTCCTTCGTATTCCACTTCTCCTGCAAGTATTCCTGAGGACATGCTG


59   CTGTGCTTAGAGGAAATGCCCACCTTTGATGACGAGGACGAGGTTCGAAGCGCGGCGACC
      ||||  ||||||| ||||| |||||||||||||| ||| |||||||| || ||| | |||
737  TTGTGTCTAGAGGAGATGCCTACCTTTGATGACGGGGATGAGGTTCGGAGTGCGACCACC


119  ACCTTTGAGCG  GT GGGAAAACAT TTTGACCCCCATGTGGGTCCTATTTTTGGCTGT
      ||||||||  |  |  ||||||| |  |||||||  |||| ||  || ||||||| |||
797  TCCTTTGAGAGCAGACGGGAAAAAAACTTTGACCCTAATGTTGGGTCTTTTTTTGGTTGT


175  TTGCGCTGTGCTTTTTATCAAGAGCAGGATGATAATGCACTTTGTGGGCTTTGCTATCTA
      |||| ||||| | ||| || |||||||  || ||| |  |||||||||||| ||  ||
857  CTGCGTTGTGCCTATTACCAGGAGCAGGGGGAGAATTCCATTTGTGGGCTTTGTTACTTA


235  AAGGCCCTTGCCGAAG TAAGTTTTAATTTAAATGTTTGGGCAGGTTAAATGTTT  288
     |||||||||| |||| |||| | |  ||||||| | ||||  ||| ||  ||||
917  AAGGCCCTTGCTGAAGGTAAGATAT  TTTAAAT TATGGGTGGGTCAATAGTTT  968

Matches = 227     Length = 295     Matches/length = 76.9%


631bp fragment
Adenovirus type 7 (simian) left end of genome


497  GAGCCTGAGCAAACAGAACCTTTGGATTTGTCTTTGAAGC    536
     |||  ||| |||||||| |||||||||  ||||  | ||||
1351 GAGGTTGAACAAACAGTACCTTTGGACCTGTCCCTAAAGC    1390

Matches = 31      Length = 40      Matches/length = 77.5%
```

D I S C U S S I O N

Restriction enzyme analysis of the SalI B Glasgow and Vaxitas clones failed to reveal any restriction enzyme which cut within the additional Vaxitas DNA. The additional DNA always appeared to be excised within an existing Glasgow fragment, simply increasing it in size. Only by using a frequent 4bp cutter (HpaII) was it possible to isolate the additional sequences.

HpaII digestion of the SalI C fragment of CAV-1 vaccines demonstrated an increase in size in a single wild-type fragment thus creating a fragment of a size unique to each vaccine strain : 349bp (BT1), 249bp (BL2): and the "loss" of one wild-type fragment : the 170bp fragment was consistently lost, the 440bp and 180bp fragments always retained.

The additional sequences within the CAV-2 vaccines however appear to have inserted either directly into a HpaII site (unlikely) or very near to an existing HpaII site. If the point of insertion is adjacent to an existing, i.e. wild-type, HpaII site and the insertion carried a HpaII site near its own terminus, it is possible that any new fragment created in addition to those noted could be too small to be detected on acrylamide. This would also require that the insertion carried no other HpaII sites unless the fragments generated by digestion with HpaII were of a similar size to those generated upon digestion

of wild-type SalI B, i.e. would co-migrate with wild-type fragments on acrylamide. Alternatively, but also unlikely, the HpaII fragments demonstrated by HpaII digestion of the vaccine strain SalI B fragments, may not be "wild-type" but may be HpaII fragments encoded by the insertion which are of a comparable size to one, or more, of the wild-type fragments. This would require large deletions in the RHT of each genome however and the generation of HpaII fragments almost exactly the same size as those generated by digestion of the Glasgow RHT with HpaII which is most improbable.

Upon HpaII digestion of the SalI B fragments from the CAV-2 vaccines, all wild-type bands appear to be retained: 80, 160, 180, 500 and 2400bp (the 2400bp fragment varies in size due to the deletion in Pst D), and an additional fragment, varying in size for each vaccine, becomes apparent. The CAV-2 vaccines Nobivac and Epivac exhibit one unique fragment each in addition to the wild-type fragments : 231bp and 164bp respectively. Vaxitas however exhibits three additional fragments (60bp, 193bp and 631bp) but all wild-type fragments except the 80bp are, apparently, again retained. The HpaII digest of the vaccine strains also demonstrates more clearly the deletion within Pst D.

The DNA sequence of the Glasgow CAV-2 ITR exhibits all known conserved AdV ITR sequences. The three base changes

between the RHT and LHT ITRs cannot be explained but may be related to the aberrant SalI B clones obtained. As stated in chapter 2, it is not known whether the S_1 treatment of the viral genomes was successful or whether the terminal clones obtained arose from aberrant genomes that were derelict of the terminal protein. Alternatively, any residual protein may have been removed, following ligation and transformation, by DNA repair. It seems likely however that aberrant genomes are present within the Glasgow CAV-2 stock due to the isolation of RHT clones with either base changes in the ITR or additional DNA "external" to the normal ITR. As the sequence of the RHT found in the majority of SalI B clones obtained contains all conserved sequences and a highly conserved initial 20bp (100% homologous to many other AdV both human and non-human) it was assumed to be a correct assessment of the DNA sequence of the Glasgow CAV-2 ITR.

The DNA sequence of the Toronto strain of CAV-2 has been published (Shinagawa et al, 1987). Three base changes occur between the published Toronto CAV-2 ITR sequence and the Glasgow CAV-2 ITR sequence: at bp47, 130 and 157 the Glasgow ITR has C, C and G respectively; at bp 47, 130 and 157 the Toronto ITR has T, G, and C respectively. The Glasgow ITR also contains one additional base, at bp185 (G), not present in the Toronto ITR. The additional base in the Glasgow ITR gives rise to a SmaI site which has been confirmed by restriction enzyme analysis.

HpaII analysis of the SalI C fragments of the CAV-1 strains revealed one fragment which was increased in size in the vaccine strains and three others which remain constant. The small increases in the 160bp fragment of wild-type, becoming 300bp in Epivac, 240bp and 350bp in Boostervac, do not account for the difference in size of the SalI C fragments. It must therefore be assumed that either the enlarged fragments are present in multiple copies or other fragments, which co-migrate with normal wild-type fragments, are present.

Sequence analysis demonstrated that no one region of the viral genome is exclusively repeated. The wide range of "type" of insertion appears to be unique when compared to other known additional sequences at the genome termini of AdV. Consistent with the Ad12, Ad16 and equine AdV additional sequences however (Werner and zur Hausen, 1978; Schwartz et al, 1982: Hammarskjold and Winberg, 1980: Ishiyama et al, 1986), Nobivac CAV-2 appears to consist of a repeat of the ITR and perhaps the adjacent sequences. The smaller species of Boostervac CAV-1 also appears to be a repeat of the sequences adjacent to the LHT ITR, showing homology of >90% to the RI127 wild-type CAV-1 E1 region, which appears to have been duplicated at the RHT.

The Epivac CAV-2 insertion may be similar to those found within Ad3 genomes (Larsen and Tibbetts, 1985; Larsen et al, 1986). The variant Ad3 genomes were found to contain

inserts of HeLa cell DNA. The Epivac clone does show homology to short regions of other AdV genomes (83.3% over 24bp against the Ad5 E3 region, 89.5% over 19bp against the Tupaia AdV E1b region) but the length of the region of homology against X. laevis mitochondrial DNA (67.1% over 194bp) gives the latter more credibility than the short regions of homology to other AdVs. This perhaps indicates that, as with Ad3, the Epivac CAV-1 insertion is derived from cellular DNA. However, there is not enough canine cellular DNA information available upon the data bank used to allow for more detailed comparison. Nor is there any further detailed information available on the CAV-2 genome, other than the CAV-2 ITR (Shinagawa et al, 1987), the Glasgow CAV-2 ITR, E1 and E3 regions, so it may be that the Epivac insertion has arisen from a part of the viral genome, with little or no homology to the human AdVs, for which there is no sequence data available at this time.

The larger of the two Boostervac CAV-1 species, however, contains a copy of the overlapping region between E2 and E3, a region with strong similarity to the Ad2 E2a cap site and a possible E2a TATA box. Homology of >90% is demonstrated over 247bp of the HpaII clone containing the insert when the sequence is compared to the CAV-2 E2/E3 region. Duplications of this area of the genome have not been reported for any other AdV.

Finally, the Vaxitas insertions (193 and 631bp) appear to

consist exclusively of repeats of the CAV-2 E1 region. In addition, the two clones are highly homologous to adjacent areas of the E1 gene perhaps indicating that the two fragments exist adjacent to each other within the LHT of Vaxitas. Although this appears to be an almost exact copy of the Glasgow CAV-2 E1 region, the "mismatches" may be accounted for by strain differences. The orientation of the RHT E1 sequences within the LHT and their exact position with reference to the E4 gene cannot be revealed by restriction enzyme analysis alone. It was therefore necessary to analyse the site of insertion using DNA sequencing techniques.

It has been demonstrated, from the restriction enzyme map, that the vaccine strain insertions are not terminal, the 0.2kb terminal SmaI site is retained, but their exact location can only be found, as described above, by detailed sequence analysis. However, this raises the possibility that the insertions interfere with viral gene products. The site of insertion may either be between the end of the ITR and the E4 promoter or may be within the E4 coding region. This may result in an alteration of the level of expression of E4 or in defective E4 products being produced.

It is known that mutants with deletions in E4 are defective for growth in normal AdV hosts. E4 deletion mutants are also defective for viral DNA accumulation,

late mRNA and protein synthesis, fail to inhibit host cell protein synthesis and leads to the over-production of the 72kD DNA binding protein, a product of E2. A diffusible E4 product is also believed to be involved in virus particle assembly (Falgout and Ketner, 1987). Sandler and Ketner (1989) also revealed that E4 deletion mutants were defective in the accumulation of both nuclear and cytoplasmic late mRNAs. However, they suggest that an E4 product(s) acts directly or indirectly to stabilise viral late RNAs but does not directly affect the levels of transcription/translation. It is also hypothesised that E4 products may serve to circumvent the host cell defence response, designed to destabilise foreign RNAs. Alterations in the structure of this gene and its products may therefore have profound effects upon the vaccine strains which may not be easily detected while the vaccines are grown <u>in vitro</u> but may have a significant effect upon the functioning of the viruses within the natural host. However, to examine the site and effect of the insertions it was impractical to obtain the information necessary on all the vaccine strains. One vaccine strain was therefore chosen, Vaxitas CAV-2, for further detailed analysis for three reasons.

First, Vaxitas contains the largest of all the inserts examined so far and therefore demonstrates the widest variation from wild-type. Secondly, sequence data would provide the exact site of insertion with reference to E4 and the origin of replication of the virus. In addition,

chloramphenicol acetyltransferase (CAT) assays should provide sufficient data to demonstrate whether the insertion interferes with the functioning of E4. This would, in turn, perhaps enable the mechanism by which these aberrant genome arose to be elucidated upon. Additionally, it would reveal the sequence of the Vaxitas ITR and confirm the DNA sequence obtained, allowing for a few strain associated differences, from Glasgow for the CAV-2 ITR.

Finally, Vaxitas, alone amongst all the vaccine strains thus far examined, fails to replicate in dog cells even after continuous high multiplicity passage. This, combined with the size of the insertion (and perhaps the 0.4kb deletion in Pst D) raises questions as to the viability of the vaccine. It is possible that the vaccine is acting more like a killed vaccine when inoculated into the dog rather than a live, attenuated vaccine. This may still allow the vaccine to raise a protective immunity. Previous research, however, has shown that killed CAV-2 vaccines are significantly less effective than CAV-2 live vaccines (Koptopolous and Cornwell, 1981). It may be that analysis of the Vaxitas insertion may help to elucidate upon this although it may be that the presence of the insertion is only a "symptom" of another, more significant, alteration in a major function of the viral genome.

C H A P T E R 4

Exact Position of the Additional DNA in the SalI B Fragment of Vaxitas CAV-2

The use of "designer" primers, i.e. primers synthesised to the specifications of the individual researcher, has considerably eased the process of sequencing large sections of DNA without the need to subclone small, usually HpaII or HaeIII, fragments. They eliminate the requirement for sequencing overlapping fragments which duplicates much of the required sequence and vastly increases the bench time required, in order to correctly orientate the fragments in relation to each other. Commercially available and "in-house" synthesised primers were therefore exclusively used in the sequencing of the Vaxitas and Glasgow RHT from the SalI B fragments already cloned into the Bluescript vector.

Although the function of the E4 region in vivo has not been well characterised, the organisation of the E4 promoter region and the DNA sequence of several AdV has been described (Ginsberg, 1984: Babiss, 1989). A summary of the known E4 control sequences and a schematic organisation of the human AdV type 2 E4 promoter region are shown in figures 4.1, 4.2 and 4.3. E4 is positioned at map units 91.3 - 99.1 and is transcribed in a leftward direction from a promoter at m.u. 99.1. With the exception of three minor species, all E4 mRNAs have a 61-nucleotide leader sequence devoid of ATG codons, spliced to mRNA species, with or without additional introns, which extend to a polyadenylation signal at m.u. 91.3. As many as 20

genes may be encoded within approximately 3kb of DNA. E1a and E2a gene products regulate E4 transcription in a positive- and negative-acting fashion respectively. The 72kD DNA binding protein specifically suppresses E4 transcription (Handa and Sharp, 1984). An E1a product is also reported to act directly upon an E4 enhancer, E4F, increasing levels of transcription 10-fold (Raychaudhuri et al, 1987a).

This details the type of organisation that the CAV-2 RHT would be expected to demonstrate. CAV E1a sequence has been shown to contain regions highly homologous to the binding sites of nuclear factors utilised by Ad2 E1a (Spibey et al, 1989). It is feasible therefore, that some homology to the factors involved in the control of Ad2 E4 would exist in the CAV-2 E4 promoter region. Alignment of any such sequences together with the identification of a possible TATA box (again initially by comparison with other E4 TATA regions) should help to elucidate on the effect of the Vaxitas insertion upon E4 and define its position within the virus RHT.

The NMF cell line used to date for the replication of Vaxitas was a cell line that had been isolated within the Department of Veterinary Pathology at the University of Glasgow. It is not the cell line used by ICI Tasman, the manufacturers of the Vaxitas CAV-2 vaccine. Due to a combination of factors the cell line was lost. All

commercially available mink cell lines were purchased but none would facilitate the replication of the Vaxitas strain even after repeated attempts with high multiplicity passage virus.

ICI Tasman therefore kindly provided the Mv-1-Lu cell line in which the vaccine is grown commercially. This cell line of mink fibroblasts was derived from the CCL 64 line which is commercially available from Flow Laboratories but the commercially available CCL 64 proved non-permissive for growth of Vaxitas. The growth of Vaxitas in the Mv-1-Lu cells was therefore investigated with the hope that, as it was the virus "natural" <u>in vitro</u> cell line, virus yield would be comparable to, if not an improvement on, that from the NMF cell line.

F I G U R E 4.1

Schematic Organisation of the Human Adenovirus Type 2 E4 Promoter

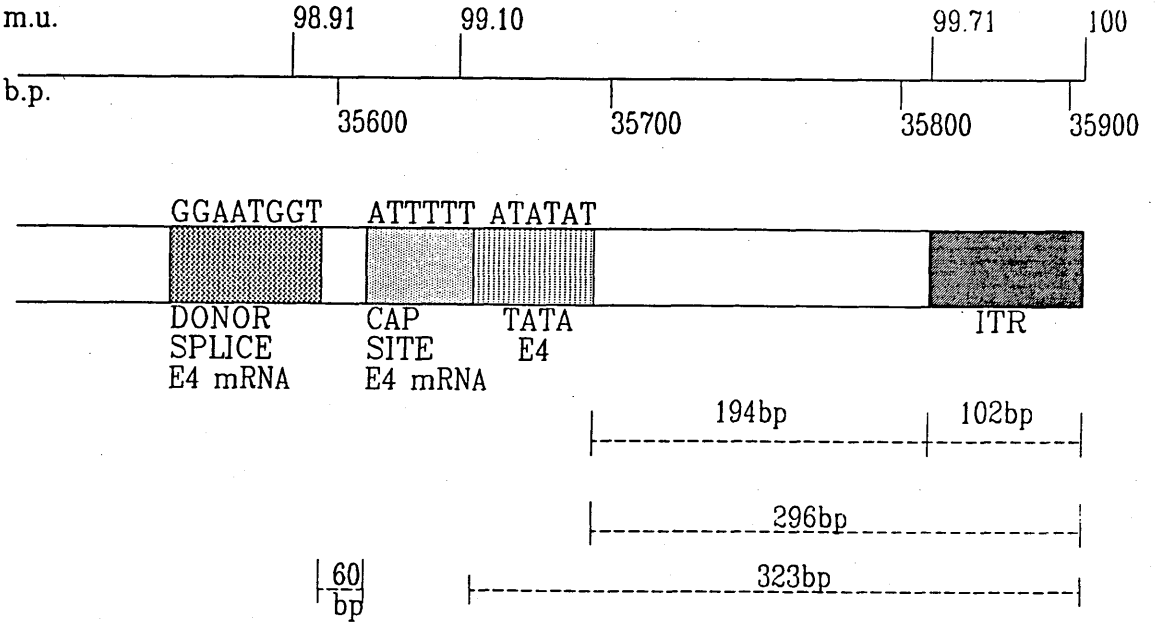


Fig. 4.1 Schematic organisation of the E4 promoter region of Ad2 as derived from the published DNA sequence of the Ad2 genome (Ginsberg, 1984).

F I G U R E 4.2

E4 RNA Splicing

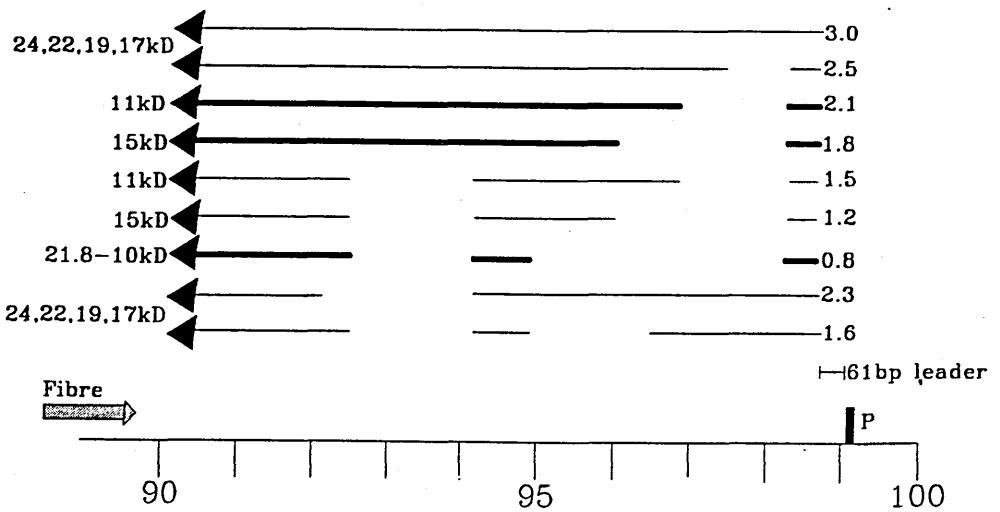
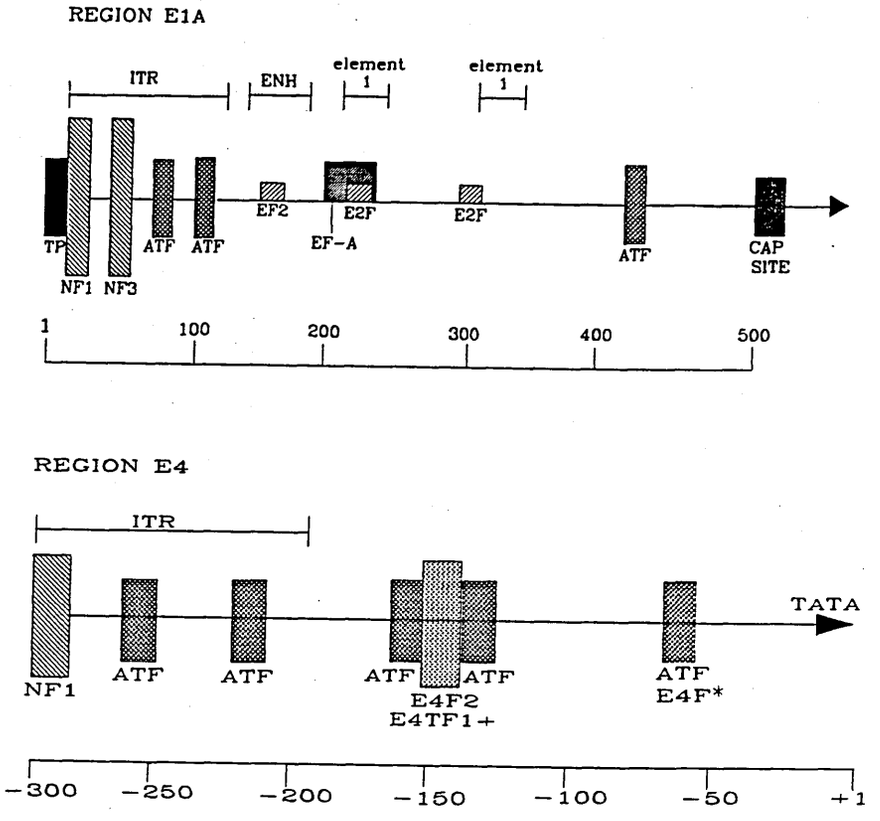


Fig. 4.2 Organisation of E4. E4 RNAs are represented as arrows with the arrowhead at the 3′ end. Breaks in the lines represent the genomic locations and sizes of introns. The most abundant E4 mRNAs that accumulate in the presence of cyclohexamide are indicated by the heavy arrows. the sizes of the mRNAs are shown to the right and the peptides they encode when translated in vitro are shown to the left. Position "P" at m.u. 99.1 denotes the E4 promoter.

F I G U R E 4.3

Control of E1a and E4 Transcription



Factor	Binding Site#
NF1	TGGCT(N3)AGCCAA
NF3	TATGATAATGAG
ATF/E4F*	(T/G)(T/A)CGTCA
E2F	TTTCGCGC
E4F2	TGGGAATT
E4TF1+	GGAAGTG

Fig. 4.3 DNA-protein interactions within the upstream promoter region of the human AdV E1A and E4 genes, derived from Jones et al (1988). The position of the binding sites and the names of the factors are indicated.
binding sites shown represent consensus sequences.
*E4F binds to the same sequences as ATF but its activity is induced upon infection and it is only seen in whole cell extracts.
+E4TF1 binds in the same region as E4F2 but the binding site is different.

MATERIALS AND METHODS

Plaque Purification of Vaxitas

Cell Line

The Mv-1-Lu cell line was passaged in 10% MEM with the addition of 5% lactalbumin hydrolysate (LH) to the media every third passage (25ml of a 25% solution of lactalbumin hydrolysate was added to each 500ml of 10% MEM). The regular enrichment of the media was recommended by ICI Tasman who kindly supplied the cell line. LH was not present in the media during any transfections. The LH was purchased from Gibco (UK) Ltd.

Plaque Purification Media

A stock media consisting of 2xMEM with 20% HFCS was prepared. Sea plaque agarose was dissolved by boiling in water to a concentration of 1.2%. The stock agarose was allowed to cool and was stored at 4^{o}C until required. The final media was prepared by melting the 1.2% agarose in a microwave oven, allowing it to cool to 40^{o}C and mixing it with an equal volume of 2x MEM which had been prewarmed to 37^{o}C.

Preparation and Infection

Confluent flasks of cells were split 1:20 into six well plates (Sterilin) and grown to 80% confluency. A volume of 0.1ml of a range of stock virus concentration was then added, ranging from 10^{6} p.f.u./ml to 10^{1} p.f.u./ml. The virus stock was incubated on the cells at 37^{o}C for 1 hour

when it was removed. Two ml of media containing 0.6% agarose was added and the cells incubated at 4°C for 30 minutes or until the agarose had set. The plates were then transferred to a 37°C incubator and left for 3-5 days.

Plaque Isolation

The tip of a sterile plastic pasteur pipette was removed by a sterile scalpel within the confines of a class II tissue culture hood. The end of the pastette was then inserted through the agarose and the piece of agarose directly over the plaque removed. It was quickly dropped into a plastic microfuge tube containing 0.5ml of 10% MEM. The agarose was then dispersed in the media and the mixture added to a confluent test-tube monolayer of Mv-1-Lu cells.

Amplification

The infected test-tube monolayers were incubated at 37°C for 5-7 days, or until a CPE became apparent, when they were freeze/thawed 3x and the supernatant was clarified. Half of the supernatant was then inoculated again onto test-tube monolayers until a CPE appeared when they were freeze/thawed 3x, the supernatant was clarified and the entire supernatant placed onto a 25cm^2 flask. The other half was kept as stock virus. This process was continued through a 75cm^2 flask to a 150cm^2 flask and finally to two 180cm^2 flasks, one of which was kept for stock virus, the other was used for the standard viral DNA preparation.

R E S U L T S

SstII Analysis of the SalI B Fragments of Glasgow and Vaxitas CAV-2

The SalI B clones of Glasgow and Vaxitas yield three fragments upon digestion with the restriction enzyme SstII (Fig. 3.1). The Glasgow SalI B clone gave fragments of 0.2kb, 0.5kb and approximately 6.0kb while the Vaxitas SalI B clone gave fragments at 0.2kb, 1.6kb and approximately 5.6kb. The Vaxitas insert was therefore contained totally within the 1.6kb SstII fragment. As further proof, the SstII fragments 0.2kb and 0.5kb (Glasgow) and the 0.2kb and 1.6kb (Vaxitas) were isolated from an agarose gel using Spin-x columns. These fragments were then digested with HpaII and examined on an acrylamide gel alongside the purified SalI B fragments from each virus (Fig. 4.4).

Fragment sizes and a schematic diagram of the SstII and HpaII sites are shown in Fig. 4.5. Obviously the orientation of the HpaII fragments within the 1.6kb SstII fragment of Vaxitas cannot be determined except for the 174bp fragment (terminal, with an overlap into the 1.6kb SstII confirmed by sequence data from the ITR (Fig. 3.6)) and the 560bp fragment which is reduced to the 400bp and 160bp fragments upon digestion with SstII. The orientation of these two HpaII fragments is confirmed by the digestion of the Glasgow SalI B and the Glasgow SstII 0.5kb fragments. As no other suitable restriction enzyme

sites had been found with which to isolate and clearly map the multiple Vaxitas insertions as defined by the additional HpaII fragments it became necessary to determine the sequence of the Glasgow and Vaxitas SalI B clones from the termini of the virus to the internal SstII site i.e. approximately 0.7kb from the terminus in Glasgow, approximately 1.8kb in Vaxitas.

DNA Sequence of the Right-hand Terminal of Glasgow CAV-2

As the SalI B fragment of Glasgow was already cloned into the vector Bluescript, continuous sequence was most easily obtained by using both commercially available primers and unique primers synthesised to known sequence as it became available. All non-commercial primers were synthesised using an Applied Biosystems DNA Synthesiser. A schematic representation of the Glasgow SalI B clone within Bluescript and the primers used, both commercial and non-commercial, is shown in Fig.4.6. The DNA sequence obtained using this method is shown in Fig. 4.7.

Confirmation of the exact size and positioning of the HpaII fragments within this region of Glasgow CAV-2 is now possible. Three HpaII sites are contained within this region as expected; at 174bp, 181bp and 262bp from the terminus, yielding fragments of 174bp, 7bp, 81bp and 390bp, the latter being the foremost part of the 560bp HpaII fragment which extends past the SstII site. The size of the SstII fragments can also be confirmed as 160bp

and 474bp.

DNA Sequence of the Right-hand Terminal of Vaxitas CAV-2

As the Vaxitas SalI B fragment was already cloned into Bluescript, as with Glasgow SalI B, it too was directly sequenced using commercial and non-commercial synthesised primers as before. The sequencing strategy using these primers is shown in Fig.4.8. The DNA sequence obtained by using this method is shown in Fig. 4.9.

All HpaII sites can now be confirmed and, in addition, it is apparent that several of the fragments appearing on acrylamide consist of two co-migrating fragments. HpaII sites can be found 174bp, 181bp, 354bp, 516bp, 570bp, 759bp and 1386bp from the terminus of the genome thus yielding eight fragments in this area of the RHT: 627bp, 390bp, 189bp, 174bp, 173bp, 162bp, 54bp and 7bp in size. The 174bp and 173bp would co-migrate; the 162bp and the wild-type 160bp SalI/HpaII fragment, positioned past the SstII site to which the sequence data runs, would co-migrate. The 390bp fragment is part of the 560bp wild-type HpaII fragment which contains the SstII site. The 627bp, 189bp and 54bp fragments were apparent as "additional" fragments on acrylamide.

Therefore, it is apparent that the RHT of Vaxitas contains six unique HpaII fragments. As the 627bp and the 189bp fragments had already been sequenced this allowed for confirmation of the sequence data and synthesised primer

recognition sites.

The exact size of the Vaxitas RHT SstII fragments are shown to be 160bp and 1.598kb, whereas the Glasgow RHT SstII fragments are 160bp and 474bp. Thus the additional DNA in the RHT of this particular isolate of Vaxitas CAV-2 amounts to 1.124kb.

The Vaxitas ITR can also be seen to contain all recognised AdV conserved sequences in this region.

DNA Sequence Analysis of the Glasgow RHT

The analysis of the ITR has already been discussed in chapter 3. No similarity was demonstrated to any other AdV sequence available on the data bank.

Sequence Analysis of the Vaxitas CAV-2 RHT

The DNA sequence analysis data is shown in Fig. 4.10. From the data base, all similarity demonstrated to the Vaxitas ITR was identical to that demonstrated to the Glasgow ITR and was therefore, apart from a few confirmatory examples, omitted from Fig. 4.10. However, the inserted DNA found in Vaxitas revealed strong homology to the E1 region of several adenovirus strains: HuAdV 3, HuAdV 12, simian AdV 7, CAV-1 and Glasgow CAV-2. The strongest similarity was demonstrated against the Glasgow CAV-2 E1 region which exhibited 98.4% homology over 1380bp. The "copy" of the E1 region is found in the opposite orientation from the E1

region at the RHT, i.e. with the duplicated E1 promoter adjacent to the ITR. A direct comparison with the entire RHT of Glasgow CAV-2 was now undertaken to reveal the exact point of insertion with reference to Glasgow.

Comparison of Glasgow CAV-2 RHT and Vaxitas CAV-2 RHT

A direct comparison of the DNA sequences of the Glasgow RHT and the Vaxitas RHT can be found in Fig.4.11. The ITR of both strains (bp 1 - 197) show 99.5% homology, the only difference being a single base change at bp27 from a C in Glasgow to a T in Vaxitas. Homology between the two then ceases until past the Vaxitas insertion where it resumes at bp229 in Glasgow and bp1353 in Vaxitas. The homology then continues until the end of the known sequence with only four mismatched bases giving a homology of 99.1% over 424 bases. This is a perfect join in the Vaxitas sequence between the E1a insertion and reversion to wild-type sequence. The loss of 30bp of the Glasgow sequence has occurred directly at the site of the E1a insertion into Vaxitas thus explaining the loss of the 81bp HpaII fragment from Vaxitas SalI B. The "insertion" has therefore occurred within a HpaII fragment as follows :-

a) the initial 16bp of the Glasgow 81bp HpaII fragment have been retained by both species - point *.

b) the Glasgow 81bp HpaII fragment then contains 30bp not found in the Vaxitas sequence.

c) at the same point *, Vaxitas contains an almost perfect 1.115kb copy of the Glasgow CAV-2 E1a.

d) finally, the last 35bp of the 81bp Glasgow HpaII

fragment are retained by both species. The sequence of both then reverts back to being highly homologous until the end of the known sequence of this area of the genome. This is demonstrated schematically in figure 4.12.

The DNA sequences of both Glasgow and Vaxitas were also translated, in all three reading frames, to demonstrate the predicted amino acid sequences and to demonstrate any open reading frames. These are demonstrated in figures 4.13 (Vaxitas RHT) and 4.14 (Glasgow RHT). The Glasgow RHT can again be seen to demonstrate all presumptive control signals for the E4 promoter region. The Vaxitas RHT confirms that all presumptive control sequences are present for both the reiterated E1a and E4. However, a number of translational stops are apparent which are not present in the open reading frames of the Glasgow CAV-2 E1a. The frame shifts demonstrated when the two DNA sequences were compared (Vaxitas reiterated E1a vs Glasgow E1a in figure 4.10) have thus introduced a number of translational stops in the Vaxitas predicted amino acid sequences of the reiterated E1a. A comparison of the Glasgow 1a orf1 and the Vaxitas reiterated E1a equivalent "orf"1 is shown in figure 4.15.

A schematic representation of the organisation of the right-hand terminal region of Vaxitas CAV-2 is shown in figure 4.16.

Nobivac CAV-2 Analysis

Further analysis of the Nobivac insertion was carried out to elucidate whether the remainder of the Nobivac insertion would show further homology to newly sequenced areas of the Glasgow genome. The 231bp insertion had already demonstrated 97% homology to the Glasgow ITR over 167 bases. The homology demonstrated against the newly available sequence of the Glasgow and Vaxitas RHT to the Nobivac insert is shown in figure 4.17. As before, the insert shows 97% homology over 167 bases to the Glasgow ITR and now also 97.6% homology over the same 167 bases to the Vaxitas ITR (comprising bases 1-165 of the Nobivac insertion, two bases of the Glasgow and Vaxitas ITRs being unmatched). In addition, the remainder of the entire Nobivac insertion, bases 165-231, exhibit 95.6% homology to 68 bases of the Glasgow RHT past the end of the ITR and 97.1% homology to the same region of the Vaxitas RHT i.e. past the ITR and the site of the Vaxitas insertion. The two areas are not directly continuous, however, the first 165bp of the insertion being in the opposite orientation to the remaining 66 bases as judged by their relative homologies to the Vaxitas and Glasgow genomes. This remains true irrespective of the orientation of the inserted fragment within the Nobivac genome which is at present unknown. Figure 4.18 demonstrates, schematically, the homology between the Nobivac insertion and the Vaxitas and Glasgow RHT.

Effect on the Genome of Vaxitas when Passaged in a New Permissive Cell Line (Mv-1-Lu)

Stock virus from the cell line NMF was inoculated into the cell line Mv-1-Lu. The supernatant from the first pass infected cells was clarified as before and kept as first passage virus stock. The infected cells were lysed and the viral DNA purified as before. The CPE and rate of infection appeared identical to that exhibited by infection of NMF but remarkably, the viral DNA showed an increase of approximately 2kb at the LHT after only one pass in the new cell line (Fig. 4.19). The size of the RHT appears not to have altered by any degree that can be detected by restriction enzyme analysis on an agarose gel. The variation in genome size remained constant for each first pass of "old" stock virus i.e. stock from the NMF cell line.

Plaque Purification of Vaxitas from the Mv-1-Lu Cell Line

Mv-1-Lu cells infected with a low dose of the Vaxitas strain (<1p.f.u./cell) demonstrated that Mv-1-Lu cells were permissive for the formation of plaques. Viral plaques were therefore isolated and the virus from each individual plaque amplified to yield sufficient viral DNA to be isolated for restriction enzyme analysis. All plaque purifications demonstrated a heterogeneous mixture of the virus as before and revealed that the additional excess 2.0kb at the LHT was now a stably integrated part of the viral genome (Fig. 4.20).

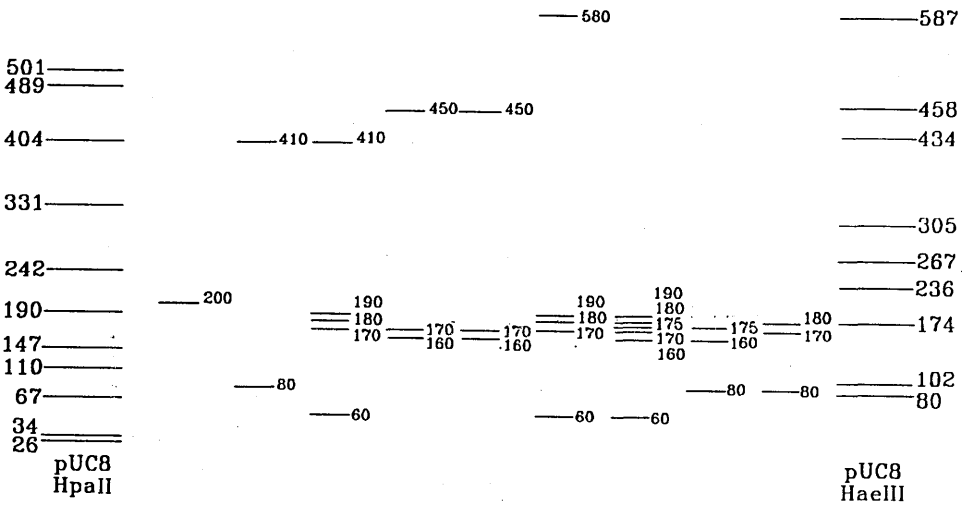


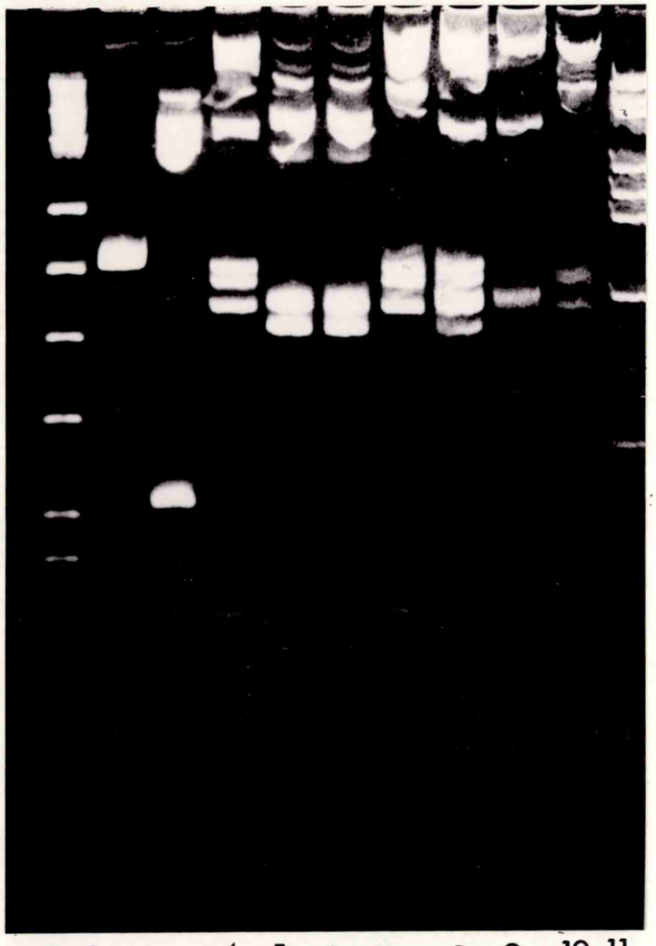
Fig. 4.4 The SstII fragments 0.2kb, 0.5kb (Glasgow) and 1.6kb (Vaxitas) were purified from the cloned SalI B fragments and digested with HpaII to demonstrate the additional HpaII fragments within the RHT of the vaccine strain. The 0.7kb PstI/SstII fragment of both the Glasgow and Vaxitas SalI B clones were also purified and digested with HpaII.

Lane (1) pUC8 DNA digested with HpaII.
Lane (2) Glasgow 0.2kb SstII fragment digested with HpaII.
Lane (3) Glasgow 0.5kb SstII fragment digested with HpaII.
Lane (4) Vaxitas 1.6kb SstII fragment digested with HpaII.
Lane (5) Glasgow PstI/SstII 0.7kb fragment digested with HpaII.
Lane (6) Vaxitas PstI/SstII 0.7kb fragment digested with HpaII.
Lane (7) Vaxitas 5.2kb SalI B fragment digested with HpaII.
Lane (8) Vaxitas 5.2kb SalI B fragment digested with HpaII/SstII.
Lane (9) Glasgow 3.2kb SalI B fragment digested with HpaII/SstII.
Lane (10) Glasgow 3.2kb SalI B fragment digested with HpaII.
Lane (11) pUC8 DNA digested with HaeIII.

A schematic diagram of the fragments obtained is provided above. All fragment sizes are as judged from the digested pUC8 DNA m.w. markers.

F I G U R E 4.4

HpaII/SstII Analysis of the SalIB Fragments of Glasgow and Vaxitas CAV-2



F I G U R E 4.5

Schematic Diagram of the HpaII and SstII Sites in the Right-hand Terminal Region of Glasgow and Vaxitas CAV-2

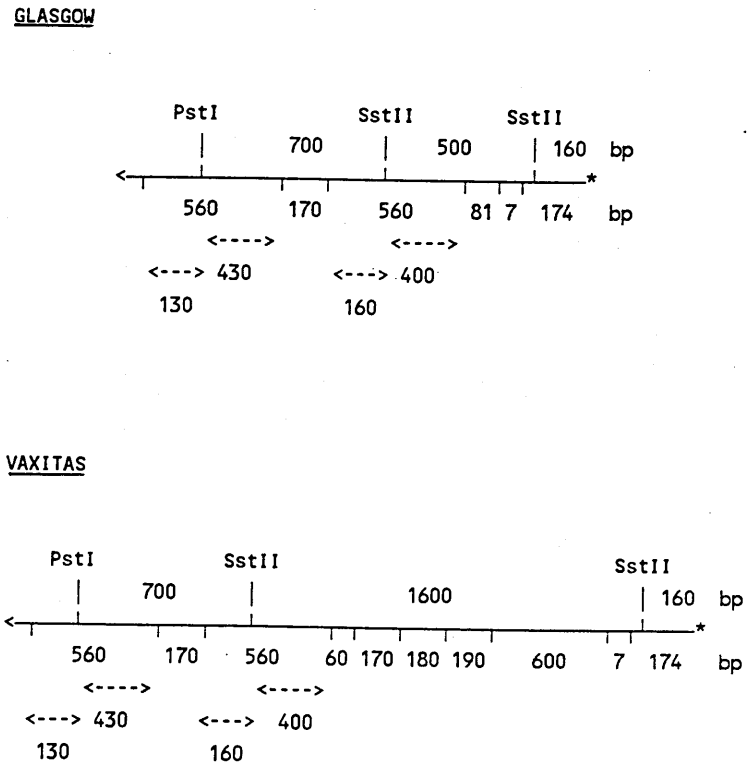


Fig.4.5 Schematic diagram shows estimated sizes and positions of all HpaII fragments shown on an acrylamide gel with SstII and PstI sites shown for reference. The orientation of the five HpaII fragments within the 1.6kb SstII fragment is hypothetical. All fragment sizes are shown in base pairs (bp).

F I G U R E 4.6

DNA Sequencing Strategy For Use On The Glasgow Right-hand Terminus Of Glasgow CAV-2

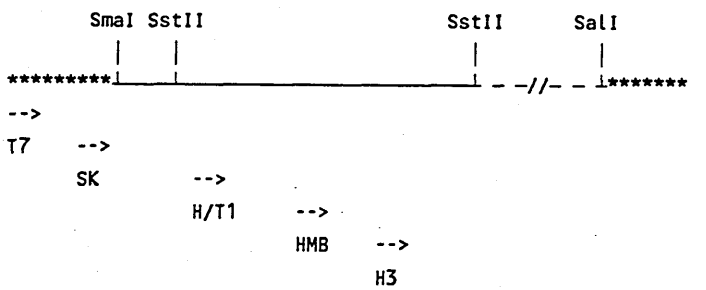


Fig. 4.6 Sequencing strategy to obtain the DNA sequence of the Glasgow right-hand terminal up to and including the internal SstII site nearest the SalI site.

F I G U R E 4.7

DNA Sequence of the Right-hand Terminal 652bp of Glasgow CAV-2

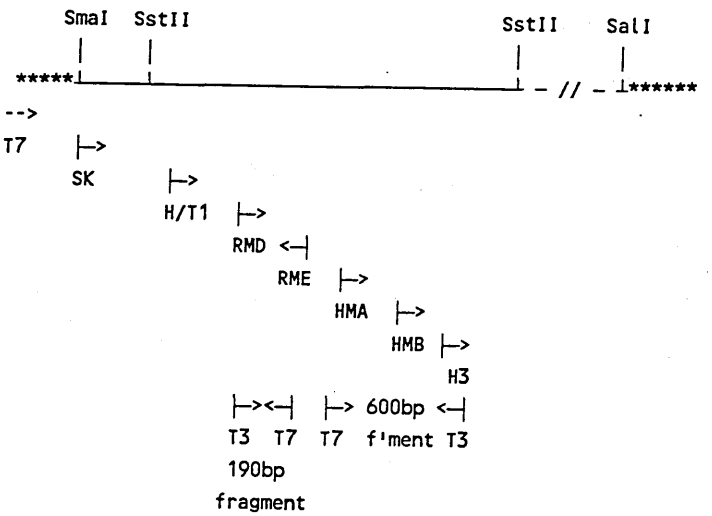
```
T7/SK
 -->        10         20         30         40         50         60
    CATCATCAAT AATATACAGG ACAAAGAGGT GTGGCTTAAA TTTGGGCGTT GCAAGGGGCG

           70         80         90        100        110        120
    GGGTCATGGG ACGGTCAGGT TCAGGTCACG CCCTGGTCAG GGTGTTCCCA CGGGAATGTC

          130        140        150        160        170        180
    CAGTGACGTC AAAGGCGTGG TTTTACGACA GGGCGAGTTC CGCGGACTTT TGGCCGGCGC
    ------------------>H/T1                   SstII           HpaII
          190        200        210        220        230        240
    CCGGGTTTTT GGGCGTTGTG CCTATTTGTG GTTTTGTGGT TGACAGGGTG ACAAGGACGT
    HpaII                                                        --
          250        260        270        280        290        300
    GCTGTACTTT TTGTGAACTT TCCGGGCCAA CCGCCAAAGG GAAACTGCAC TTAACATTTA
    ---------------->HMB   HpaII
          310        320        330        340        350        360
    CCACGCGCCC ACAATTTATG ACTGTACTTG GCACCACTTC CTCAAACGCC CCGTTATATT

          370        380        390        400        410        420
    CCTTTTCGTT TTCCACACGC CCTACTTTGA GGACTATATA AACGCTGTGC TTGGCATTTC
                                          TATA E4
          430        440        450        460        470        480
    ATCCTCATAG CTCTCCTCTG ACAGCCAGCC GTCCGTGAGT ACTATGGCAG CTTTAGGAGT
    ------------------>H3         donor splice?    met?
          490        500        510        520        530        540
    GTCTATGGGA GCATGTTTTT GTCTGAGGCT TCACAAGAGT CTAGTGGAGA GTGTGTGTGC

          550        560        570        580        590        600
    TCAACTGAGA CTTACGAACT TTTTGCCTTC TGAACTCGCC GTGTGGTGTT TAGCCTTATT
                                                 donor splice?
          610        620        630        640        650
    AGGGCCCAGG AAGTGCGTCC GTGTCCTACC TTGCCGCGGC TGTTTCGGTT TA
                                      SstII
```

Fig 4.7 HpaII sites are underlined for ease of recognition and comparison to the restriction enzyme data. SstII sites and sequences conserved between all species of adenovirus so far examined are also underlined. Recognition sequences for synthesised primers are indicated by arrows (------>).

Presumptive TATA, initiating methionine and two possible donor splice sites (as judged by sequence homology to human AdV E4 regions) are indicated. Ad2 initial donor splice is situated 61bp downstream of the E4 promoter and contains no ATG prior to the first intron. The consensus sequence for the Ad2 E4 donor splice at 99m.u. is AAC/GTGAGT and at 94.4m.u. is CTG/GTAAGG. Two possible donor splice sites are indicated on the sequence above one at 451bp, tcC/GTGAGT and one at 584bp, gTGGTGttT. It is likely that the presumptive splice site at 451bp is correct, thus placing the folowing ATG in a non-coding region.

F I G U R E 4.8

DNA Sequencing Strategy For The Right-hand Terminus Of Vaxitas CAV-2



Commercial Primers

T7 : 5' AATACGACTCACTATAG 3'
SK : 5' TCTAGAACTAGTGGATC 3'
T3 : 5' ATTAACCCTCACTAAAG 3'

Synthesised Primers

H/T1 : 5' CAGTGACGTCAAAGGCG 3'
RMD : 5' GGTGCCAGTTCCTCCAC 3'
RME : 5' GAGGTGGGATGCGGTGG 3'
HMA : 5' TATTTTTGGCTGTTTGC 3'
HMB : 5' GTGCTGTACTTTTTGTG 3'
H3 : 5' ATCCTCATAGCTCTCCT 3'

Fig. 4.8 Sequencing strategy used to obtain the DNA sequence of the right-hand terminal of Vaxitas CAV-2 up to and including the SstII site nearest to the SalI site. The subcloned fragments, 190bp and 600bp, which had already been sequenced are indicated within the overall picture and were used as confirmation of sequence data obtained from the synthesised primers.

FIGURE 4.9

DNA Sequence of the Right-hand Terminal 1.776KB of Vaxitas CAV-2

```
        10         20         30         40         50         60
CATCATCAAT AATATACAGG ACAAAGAGGT GTGGCTTAAA TTTGGGTGTT GCAAGGGGCG

        70         80         90        100        110        120
GGGTCATGGG ACGGTCAGGT TCAGGTCACG CCCTGGTCAG GGTGTTCCCA CGGGAATGTC

       130        140        150        160        170        180
CAGTGACGTC AAAGGCGTGG TTTTACGACA GGGCGAGTTC CGCGGACTTT TGGCCGGCGC
------------------>H/T1                  SstII           HpaII
       190        200        210        220        230        240
CCGGGTTTTT GGGCGTTTAT TGATTTTGCG GTTTAGCGGG TGGTGCTTTT ACCATGTTTG
HpaII
       250        260        270        280        290        300
CGGAAGATTT AGTTGTTTAT GGAGCTGGTT TTGGTGCCAG TTCCTCCACG GCTAATGTCA
                                  ------------------>RMD
       310        320        330        340        350        360
AAGTTTATGT CAATATAACA GAAACACTCT GTTCTCTGTT TACAGCACCC CACCCGGTGG
                                                          HpaII
       370        380        390        400        410        420
TTTTTCGCCA CGCCTTTGGG TTAATTTTAT TTCCCTATAC GCGGCCTTAA ATTCTCAGTG

       430        440        450        460        470        480
CAGACGAAAG AGGACTACTC TTGAGTGCGC ACGGAGAAGA GTTTTCTCTT CGCTGTGTCT

       490        500        510        520        530        540
CATATATTTT CTGAAAAATG AAATATACTA TTGTGCCGGC GCCGCGCAAT CTCCATGATT
                                    HpaII
       550        560        570        580        590        600
ATGTTTTAGA GCTACTGGAA GAGTGGCAGC CGGACTGCCT TGACTGTGAG TATTCTCATG
                      T3*->HpaII
       610        620        630        640        650        660
GCAGCCCCTC GCCGCCTACT CTGCACGATC TTTTTGATGT TGAGCTGGAG ACTTCTCACA

       670        680        690        700        710        720
GCCCTTTTGT GGGCCTGTGT GATTCCTGTG CGGAGGCTGA CACTGATTCG AGTGCGAGCA
                                       HpaII
       730        740        750        760/       770        780
CTGAGGGTGA TTCTGGGTTT AGTCCTTTAT CCACTCCGCC GGTTTCACTA TTCCACCGCA
                                  T7*-> <-T7*     RME<--------
       790        800        810        820        830        840
TCCCACCTCT CCTGCTAGCA TTTCTGACGA CATGTTGCTG TGCTTAGAGG AAATGCCCAC
---------
       850        860        870        880        890        900
CTTTGATGAC GAGGACGAGG TTCGAAGCGC GGCGACCACC TTTGAGCGGT GGGAAAACAT

       910        920        930        940        950        960
TTTGACCCCC ATGTGGGTCC TATTTTTGGC TGTTTGCGCT GTGCTTTTTA TCAAGAGCAG
                     ---------------->HMA
       970        980        990       1000       1010       1020
GATGATAATG CACTTTGTGG GCTTTGCTAT CTAAAGGCCC TTGCCGAAGT AAGTTTTAAT
```

```
      1030        1040        1050        1060        1070        1080
TTAAATGTTT GGGCAGGTTA AATGTTTGGG CAGGTTAAAT GTTTTAGGTG TGTATTGATT

      1090        1100        1110        1120        1130        1140
TTTAATTTTG CTTTTTAGTG CCTTTTGCTA TGCCTGTACG TTCAGAACCC GCTTCGGCTG

      1150        1160        1170        1180        1190        1200
GAGCTGAGGA GGAAGATGAT GAAGTTATTT TTGTGTCTGC CAAACCTGGG GGCAGAAAGA

      1210        1220        1230        1240        1250        1260
GGTCAGCAGC TACTCCCTGT GAGCCAGATG GGGTCAGCAA ACGCCCTTGC GTGCCAGAGC

      1270        1280        1290        1300        1310        1320
CTGAGCAAAC AGAACCTTTG GATTTGTCTT TGAAGCCACG CCCGAACTAA TCTCCTTGAG

      1330        1340        1350        1360        1370        1380
CACAAAGCAA TAAAGTAATC TTGTTTAACA AGTTGACAAG GACGTGCTGT ACTTTTTGTG
                                                HMB---------------->
      1390        1400        1410        1420        1430        1440
AACTTTCCGG GCCAACCGCC AAAGGGAAAC TGCACTTAAC ATTTACCACG CGCCCACATT
     HpaII<--T3*
      1450        1460        1470        1480        1490        1500
TTATGACTGT ACTTGGCACC ACTTCCTCAA ACGCCCCGTT ATATTCCTTT TCGTTTTCCA

      1510        1520        1530        1540        1550        1560
CACGCCCTAC TTTGAGGACT ATATAAACGC TGTGCTTGGC ATTTCATCCT CATAGCTCTC
                                                    ---------------
      1570        1580        1590        1600        1610        1620
CTCTGACAGC CAGCCGTCCG TGAGTACAAT GGCAGCTTTA GGAGTGTCTA TGGGAGCATG
-->H3
      1630        1640        1650        1660        1670        1680
TTTTTGTCTG AGGCTTCACA AGAGTCTAGT GGAGAGTGTG TGTGCTCAGC TGAGACTTAC

      1690        1700        1710        1720        1730        1740
GAACTTTTTG CCTTCTGAAC TCGCCGTGTG GTGTTTAGCC TTATTAGGGC TCAGGAAGTG

      1750        1760        1770
CGTCCGTGTC CTACCTTGCC GCGGCTGTTT CGGTTTA
                  SstII
```

Fig. 4.9 Sequence of the RHT of Vaxitas CAV-2 including the ITR. All HpaII sites are underlined. Sequences conserved between all species of adenovirus so far examined and SstII sites are also underlined. Recognition sequences of synthesised primers are depicted as arrows (--->). Sequence data from the subcloned 160bp and 600bp fragments used as confirmation are indicated as T3* and T7*.

F I G U R E 4.10

Sequence Analysis of the Vaxitas CAV-2 Right-hand Terminus

VAXITAS RHT
Adenovirus type 3 E1a

```
597  CATGGCAGCCCCTCG  CCGCCTACTCTGCACGATCTTTTTGATGTTGAGCTGGA   649
690  CAGTGCAGCCTTTCGATCCACCTACGCTGCACGATCTGTATGATTTAGAGATAGA   744
       **      **   **  *     *          * *    * *   * *
```

Matches = 41 Length = 55 Matches/length = 74.5%

```
428     AAGAGGACTACTCTTGAGTGCGCACGGAGAAGAGTTTTCTCTTC     471
512     AAGAGGCC ACTCTTGAGTGC CAGCGAGAAGAGTTTTCTCCTC     553
            * *           *  **              *
```

Matches = 38 Length = 44 Matches/length = 86.4%

VAXITAS RHT
Adenovirus type 12 E1a

```
428     AAGAGGACTACTCTTGAGTGCGCACGGAGAAGAGTTTTCTCT     469
437     AAGAGGCC ACTCTTGAGTGC CAGCGAGAAGAGTTTTCTCT     476
            * *           *  **
```

Matches = 37 Length = 42 Matches/length = 88.1%

VAXITAS RHT
Adenovirus type 7 (simian) E1a

```
1256    GAGCCTGAGCAAACAGAACCTTTGGATTTGTCTTTGAAGC    1295
1351    GAGGTTGAACAAACAGTACCTTTGGACCTGTCCCTAAAGC    1390
          **   *       *        **    ** *
```

Matches = 31 Length = 40 Matches/length = 77.5%

VAXITAS RHT
Adenovirus type 7 E1a

```
612     CCGCCTACTCTGCACGATCTTTTTGATGTTGAGCTGGA     649
699     CCACCTACGCTGCACGATCTGTATGATTTAGAGGTAGA     736
          *     *          * *    * *   * *
```

Matches = 30 Length = 38 Matches/length = 78.9%

```
428     AAGAGGACTACTCTTGAGTGCGCACGGAGAAGAGTTTTCTCTTC     471
504     AAGAGGCC ACTCTTGAGTGC CAGCGAGAAGAGTTTTCTCCTC     545
            * *           *  **              *
```

Matches = 38 Length = 44 Matches/length = 86.4%

VAXITAS RHT
equine adenovirus (ead) 5′ inverted terminal repeat

```
1       CATCATCAATAATATACAGGACAAAGAGGTGTGG      34
1       CATCATCAATAATATACAGGACACACGGGCATGG      34
                           * **  **
```

Matches = 29 Length = 34 Matches/length = 85.3%

VAXITAS RHT
CAV-1 ITR

```
1     CATCATCAATAATATACAGGACAAAGAGGTGTGGCTTAAAT TTGGGTGTTGCAAGGGGC
      ||||||||||||||||||||||||||||||||||| ||||| ||| | ||   |
1     CATCATCAATAATATACAGGACAAAGAGGTGTGGCCTAAATGTTGTTTTTTTTTAAAAAA

60    GGGGTCATGGGACGGTCAGGTTCAGGTCACGCCCTGGTCAGGGTG TTCCCACGGGAATG
      |   | | || ||   |  ||||||| ||||| |||||| | |||||||||||| |
61    GTTTTTGTCTGATTGTTTTGACAAGGTCACACCCTGTTCAGGGCGTTTCCCACGGGAAAG

119   TCCAGTGACGTCAAAGGCGTGGTTTTACGACAGGGCGAGTTCCGCGGACTTTTGGCCGGC
       ||| |||||||||  | ||| ||||           ||     |||| ||||||| |
121   ACCA TGACGTCAATTGGGTGTTTTT           GT     GGAC TTTGGCCCGT

179   GCCCGGGTTTTTGGG
      |    ||||||||||
162   G   TGGTTTTTGGG
```

Matches = 129 Mismatches = 38 Unmatched = 24
Length = 191 Matches/length = 67.5%

VAXITAS RHT
CAV1 (RI 127) E1 REGION

```
404   GCCTTAAATTCTCAGTGCAGACGAAAGAGGACTACTCTTGAGTGCGCACGGAGAAGAGTT
      || |||||||| | |   |||||||||||||| |||||||||||||||||||||||||||
340   GCTTTAAATTCACTGCAGAGACGAAAGAGGAGTACTCTTGAGTGCGCACGGAGAAGAGTT
          TATA E1a
464   TTCTCTTCGCTGTGTCTCATATATTTTCTGAAAAATGAAATATACTATTGTGCCGGCGCC
      |||           |||||   | | |   |||||||| |  ||| ||| |||||| ||
400   TTC           TCTCAGCGAATCTGCCAAAAATGAAGTTAACTCTTGAGCCGGCACC
                                      met E1a orf1
```

```
 524  GCGCAATCTCCATGATTATGTTTTAGAGCTACTGGAAGAGTGGCAGCCGGACTGCCTTGA
      ||||  ||| |||||  |||||||   |||||||||||| |||||||||| || ||
 449  GCGCTGTCTTCATGAATATGTTTCTCAGCTACTGGAAGATTGGCAGCCGGAATGTCTCTC

 584  CTGTGAGTATTCTCATGGCAGCCCCTCGCCGCCTACTCTGCACGATCTTTTTGATGTTGA
      |||||||||  || |||||  || | ||  || ||  | ||| || ||||||||||||  ||||
 509  CTGTGAGTACTCCCATGGTGGCTCGTCACCACCGAGTCTTCATGATCTTTTTGATCTTGA

 644  GCTGGAGACTTCTCACAGCCCTTTTGTGGGCCTGTGTGATTCCTGTGCGGAGGCTGACAC
      ||||||  |  |||||     ||||  |  |  ||||||||||  ||||||||||||||||
 569  GCTGGAAAATTCTCGTTCTCCTTCTCCGCTGCTGTGTGATTGGTGTGCGGAGGCTGACAG

 704  TGATTCGAGTGCGAGCACTGAGGGTGATTCTGGGTTTAGTCCTTTATCCACTCCGCCGGT
      |||  ||  |       |||  |||  ||||  |||  ||||   ||||   ||||||||||||
 629  TGAATCTACAATTTCCACGGAGACTGATGTTGGCTTTA   CTTTGAACACTCCGCCGGT

 764  TTC ACTATTCCACCGCATCCCACCTCTCCTGCTAGCATTTCTGACGACATGTTGCTGTG
      |||  ||  ||||  ||  ||  ||||  ||||||||  ||  |||  ||||  ||||||  ||  ||||
 686  TTCTCCTCTTCCTTCGTATTCCACTTCTCCTGCAAGTATTCCTGAGGACATGCTGTTGTG

 823  CTTAGAGGAAATGCCCACCTTTGATGACGAGGACGAGGTTCGAAGCGCGGCGACCACCTT
        |||||||  |||||  ||||||||||||||  |||  ||||||||  ||  |||  |  |||  ||||
 746  TCTAGAGGAGATGCCTACCTTTGATGACGGGGATGAGGTTCGGAGTGCGACCACCTCCTT

 883  T  GAGC GGTGGG AAAACATTTTGACCCCCATGTGGGTCCTATTTTTGGCTGTTTGCG
      |  ||||  |  |||  ||||  |  ||||||||  ||||  ||  ||  |||||||  |||  ||||
 806  TGAGAGCAGACGGGAAAAAAACTTTGACCCTAATGTTGGGTCTTTTTTTGGTTGTCTGCG

 939  CTGTGCTTTTTATCAAGAGCAGGATGATAATGCACTTTGTGGGCTTTGCTATCTAAAGGC
       |||||  |  |||  ||  |||||||  ||  |||  |  ||||||||||||  ||  |||||||
 866  TTGTGCCTATTACCAGGAGCAGGGGGAGAATTCCATTTGTGGGCTTTGTTACTTAAAGGC

 999  CCTTGCCGAA GTAAGTTTTAATTTAAATGTTTGGGCAGGTTAAATGTTTGGGCAGGTTA
      ||||||  |||  |||||   ||  |||||||  |  ||||  |||  ||  ||||   ||    |
 926  CCTTGCTGAAGGTAAG  ATATTTTAAAT TATGGGTGGGTCAATAGTTT   CA   CA
                    end orf1
1058  AATGTTTTAGGTGTGTATTGATTTTTAATTTTGCTTTTTAGTGCCTTTTGCTATGCCTGT
      ||  |||  |   |  |||  ||  |       |  ||  ||||||||||  |  ||  ||||||||
 977  AAGGTTCT   TATGTGTTCCT      CTCTG  TTTTAGTGCCCTGCGCCATGCCTGT
         met orf2
1118  ACGTTCAGAACCCGCTTCGGCTGGAGCTGAGGAGGAAGATGATGAAGTTATTTTTGTGTC
       ||  |||||||  |  |   ||||  ||   |||  ||  ||||||||  ||  ||||||||||||
1026  TCGCGCAGAACCTGTTGATGCTGATGC  CGGA GAGGATGATGAGGTCATTTTTGTGTC

1178  TGCCAAAACCTGGGGGCAGAAAGAGGTCAGCAGCTACTCCCTGTGAGCCAGATGGGGTCAG
      |||  ||||||||   ||||||||||||||||  |  |||  ||  |  ||  |||  ||    |||
1083  TGCTAAACCTGGTTCCAGAAAGAGGTCAGCGGTGACTTCCCGCGACTCAGTTGAAAGCAG

1238  CAAACGCCCTTGCGTGCCAGAGCCTGAGCAAACAGAACCTTTGGATTTGTCTTTGAAGCC
       ||||||||||||  ||||||||||||||  |  |||  ||||||||||||  |  ||  |  |||||
1143  TAAACGCCCTTGCTTGCCAGAGCCTGAACCAACTGAACCTTTGGATCTTTCCCTAAAGCC

1297  ACGCCCG AACTAATCTC CTTGAG
      |||||||  ||  |||  |  ||||||
1203  ACGCCCGCAATAAATTACTCTTGAG
            end orf2
Matches=682  Mismatches=198  Unmatched=45  Length=925  Matches/length=73.7%
```

Vaxitas RHT

Glasgow LHT and E1a Region (E1a sequence courtesy of Dr A. M^{c}Clory)

```
1     CATCATCAATAATATACAGGACAAAGAGGTGTGGCTTAAATTTGGGTGTTGCAAGGGGCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
1     CATCATCAATAATATACAGGACAAAGAGGTGTGGCTTAAATTTGGGCGTTGCAAGGGGCG

61    GGGTCATGGGACGGTCAGGTTCAGGTCACGCCCTGGTCAGGGTGTTCCCACGGGAATGTC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61    GGGTCATGGGACGGTCAGGTTCAGGTCACGCCCTGGTCAGGGTGTTCCCACGGGAATGTC

121   CAGTGACGTCAAAGGCGTGGTTTTACGACAGGGCGAGTTCCGCGGACTTTTGGCCGGCGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
121   CAGTGACGTCAAAGGCGTGGTTTTACGACAGGGCGAGTTCCGCGGACTTTTGGCCGGCGC
               end ITR
181   CCGGGTTTTTGGGCGTT TATTGATTTTGCGGTTTAGCGGGTGGTGCTTTTACCA TGTTT
      |||||||||||||||| |||||||||||||||| |||||||||||||||||| |||||
181   CCGGGTTTTTGGGCGTT TATTGATTTTGCGGTTTATCGGGTGGTGCTTTTACCACTGTTT

240   GCGGAAGATTTAGTTGTTTATGGAGCTGGTTTTGGTGCCAGTTCCTCCACGGCTAATGTC
      |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
241   GCGGAAGATTTAGTTGTTTATGGAGCTGGTTTTGGTGCCAGTTCCTCCACGCCTAATGCT

300   AAAGTTTATGTCAATATAACAGAAACACTCTGTTCTCTGTTTACAGCACCCCACCCGGTG
      |||||||||||||||||||||||||||||||||| |||||||||| |||||||||| |
301   AAAGTTTATGTCAATATAACAGAAACACTCTGTTCTTTGTTTACAGCGCCCCACCCGGCG

360   GTTTTTCGCCACGCCTTTGGGTTAATTTTATTTCCCTATACGCGGCCTTAAATTCTCAGT
      |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||| |
361   GTTTTTCGCCACGCCTTTGGGTTAATTTTATTTCCCTATACGCGGCCCTAAATTCTCA T
                                                        TATA E1a
420   GCAGACGAAAGAGGACTACTCTTGAGTGCGCACGGAGAAGAGTTTTCTCTTCGCTGTGTC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
420   GCAGACGAAAGAGGACTACTCTTGAGTGCGCACGGAGAAGAGTTTTCTCTTCGCTGTGTC

480   TCATATATTTTCTGAAAAATGAAATATACTATTGTGCCGGCGCCGCGCAATCTCCATGAT
      ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
480   TCATATATTCTCTGAAAAATGAAATATACTATTGTGCCGGCGCCGCGCAATCTCCATGAT
                  met E1a (orf1)
540   TATGTTTTAGAGCTACTGGAAGAGTGGCAGCCGGACTGCCTTGACTGTGAGTATTCTCAT
      |||||||||||||||||||||||||||||| |||||||||||||||||||||| |||||
540   TATGTTTTAGAGCTACTGGAAGAGTGGCACCCGGACTGCCTTGACTGTGAGTATCCTCAT

600   GGCAGCCCCTCGCCGCCTACTCTGCACGATCTTTTTGATGTTGAGCTGGAGACTTCTCAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
600   GGCAGCCCCTCGCCGCCTACTCTGCACGATCTTTTTGATGTTGAGCTGGAGACTTCTCAC

660   AGCCCTTTTGTGGGCCTGTGTGATTCCTGTGCGGAGGCTGACACTGATTCGAGTGCGAGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
660   AGCCCTTTTGTGGGCCTGTGTGATTCCTGTGCGGAGGCTGACACTGATTCGAGTGCGAGC

720   ACTGAGGGTGATTCTGGGTTTAGTCCTTTATCCACTCCGCCGGTTTCA CTATTCCACCG
      |||||||||||||||||||||||||||||| |||||||||||||||||| ||||||||||||
720   ACTGAGGGTGATTCTGGGTTTAGTCCTTTGTCCACTCCGCCGGTTTCACCTATTCCACCG

779   CATCCCACCTCTCCTGCTAGCATTTCTGACGACATGTTGCTGTGCTTAGAGGAAATGCCC
      |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
780   CATCCCACCTCTCCTGCTAGCATTTCTGACGACATGGTGCTGTGCTTAGAGGAAATGCCC
```

```
839   ACCTTTGATGACGAGGACGAGGTTCGAAGCGCGGCGACCACCTTTGAGCGGTGGGAAAAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
840   ACCTTTGATGACGAGGACGAGGTTCGAAGCGCGGCGACCACCTTTGAGCGGTGGGAAAAC

899   A TTTTGACCCCCATGTGGGTCCTATTTTTGGCTGTTTGCGCTGTGCTTTTTATCAAGAG
      | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
900   ACTTTTGACCCCCATGTGGGTCCTATTTTTGGCTGTTTGCGCTGTGCTTTTTATCAAGAG

958   CAGGATGATAATGCACTTTGTGGGCTTTGCTAT CTAAAGGCCCTTGCCGAA GTAAGTT
      |||||||||||||||||||||||||||||||| |||||||||||||||||| |||||||
960   CAGGATGATAATGCACTTTGTGGGCTTTGCTATCCTAAAGGCCCTTGCCGAAGGTAAGTT
                                                          end

1016  TT AATTTAAATGTTTGGGCAGGTTAAATGTTTGGGCAGGTTAAATGTTTTAGGTGTGTA
      || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1020  TTGAATTTAAATGTTTGGGCAGGTTAAATGTTTGGGCAGGTTAAATGTTTTAGGTGTGTA
      orf1                                      met E1a (orf2)
1075  TTGATTTTTAATTTT GCTTTTTAGTGCCTTTTGCTATGCCTGTACGTTCAGAACCCGCT
      ||||||||||||||| |||||||||||||||||||| ||||||||||||||||||||||
1080  TTGATTTTTAATTTTGGCTTTTTAGTGCCTTTTGCTTTGCCTGTACGTTCAGAACCCGCT

1134  TCGGCTGGAGCTGAGGAGGAAGATGATGAAGTTATTTTTGTGTCTGCCAAACCTGGGGGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1140  TCGGCTGGAGCTGAGGAGGAAGATGATGAAGTTATTTTTGTGTCTGCCAAACCTGGGGGC

1194  AGAAAGAGGTCAGCAGCTACTCCCTGTGAGCCAGATGGGGTCAGCAAACGCCCTTGCGTG
      |||||||||||||||||||||||||||||||||||||||| |||||||| ||||||||||
1200  AGAAAGAGGTCAGCAGCTACTCCCTGTGAGCCAGATGGGGCCAGCAAACCCCCTTGCGTG

1254  CCAGAGCCTGAGCAAACAGAACCTTTGGATTTGTCTTTGAAGCCACGCCCGAACTAATCT
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1260  GCAGAGCCTGAGCAAACAGAACCTTTGGATTTGTCTTTGAAGCCACGCCCGAACTAATCT
                                                        end orf2

1314  CCTTGAGCACAAAGCAATAAAGTAATCTTGTTTAACAAGTT
      |||||||||||||||||||||||||||||||||||||||
1320  CCTTGAGCACAAAGCAATAAAGTAATCTTGTTTAACAAGTT
                  polyA E1a

Matches = 1335   Mismatches = 18     Unmatched = 8
Length = 1361    Matches/length =  98.1 percent
```

Fig. 4.10 Presumptive TATA, initiating methionines and polyA signal are indicated on both the CAV-1 E1a/Vaxitas insertion and the Glasgow E1a/Vaxitas insertion sequence; demonstrating that the Vaxitas insertion consists of a copy of the LHT E1a region at the RHT.

FIGURE 4.11

Comparison of the Glasgow and Vaxitas Right-hand Ends

```
VAXITAS RHT
GLASGOW RHT

1     CATCATCAATAATATACAGGACAAAGAGGTGTGGCTTAAATTTGGGTGTTGCAAGGGGCG
1     CATCATCAATAATATACAGGACAAAGAGGTGTGGCTTAAATTTGGGCGTTGCAAGGGGCG
                                                      *
61    GGGTCATGGGACGGTCAGGTTCAGGTCACGCCCTGGTCAGGGTGTTCCCACGGGAATGTC
61    GGGTCATGGGACGGTCAGGTTCAGGTCACGCCCTGGTCAGGGTGTTCCCACGGGAATGTC

121   CAGTGACGTCAAAGGCGTGGTTTTACGACAGGGCGAGTTCCGCGGACTTTTGGCCGGCGC
121   CAGTGACGTCAAAGGCGTGGTTTTACGACAGGGCGAGTTCCGCGGACTTTTGGCCGGCGC

181   CCGGGTTTTTGGGCGTT   197
181   CCGGGTTTTTGGGCGTT   197

Matches = 196          Length = 197        Matches/Length = 99.5%
Mismatches = 1         Unmatched = 0


1353      TGACAAGGACGTGCTGTACTTTTTGTGAACTTTCCGGGCCAACCGCCAAAGGGAAACTGC
229       TGACAAGGACGTGCTGTACTTTTTGTGAACTTTCCGGGCCAACCGCCAAAGGGAAACTGC

1413      ACTTAACATTTACCACGCGCCCACATTTTATGACTGTACTTGGCACCACTTCCTCAAACG
289       ACTTAACATTTACCACGCGCCCACAATTTATGACTGTACTTGGCACCACTTCCTCAAACG
                              *
1473      CCCCGTTATATTCCTTTTCGTTTTCCACACGCCCTACTTTGAGGACTATATAAACGCTGT
349       CCCCGTTATATTCCTTTTCGTTTTCCACACGCCCTACTTTGAGGACTATATAAACGCTGT
                                                      TATA E4
1533      GCTTGGCATTTCATCCTCATAGCTCTCCTCTGACAGCCAGCCGTCCGTGAGTACAATGGC
409       GCTTGGCATTTCATCCTCATAGCTCTCCTCTGACAGCCAGCCGTCCGTGAGTACTATGGC
                                                  donor splice? *met E4?
1593      AGCTTTAGGAGTGTCTATGGGAGCATGTTTTTGTCTGAGGCTTCACAAGAGTCTAGTGGA
469       AGCTTTAGGAGTGTCTATGGGAGCATGTTTTTGTCTGAGGCTTCACAAGAGTCTAGTGGA

1653      GAGTGTGTGTGCTCAGCTGAGACTTACGAACTTTTTGCCTTCTGAACTCGCCGTGTGGTG
529       GAGTGTGTGTGCTCAACTGAGACTTACGAACTTTTTGCCTTCTGAACTCGCCGTGTGGTG
                       *                                  donor splice
1713      TTTAGCCTTATTAGGGCTCAGGAAGTGCGTCCGTGTCCTACCTTGCCGCGGCTGTTTCGG
589       TTTAGCCTTATTAGGGCCCAGGAAGTGCGTCCGTGTCCTACCTTGCCGCGGCTGTTTCGG
          E4?              *
1773      TTTA
649       TTTA

Matches = 420    Mismatches = 4      Unmatched = 0
Length = 424     Matches/length =  99.1 percent
```

Fig. 4.11 Alignment of the Glasgow and Vaxitas RHT sequences excluding the Vaxitas insertion. Presumptive control signals, i.e. TATA, ATG and possible donor splice sites, as inferred by homology to human AdV E4 regions, are indicated.

F I G U R E 4.12

Schematic Diagram of the Vaxitas Additional DNA In Relation to the Glasgow Right-hand End and E1a Region

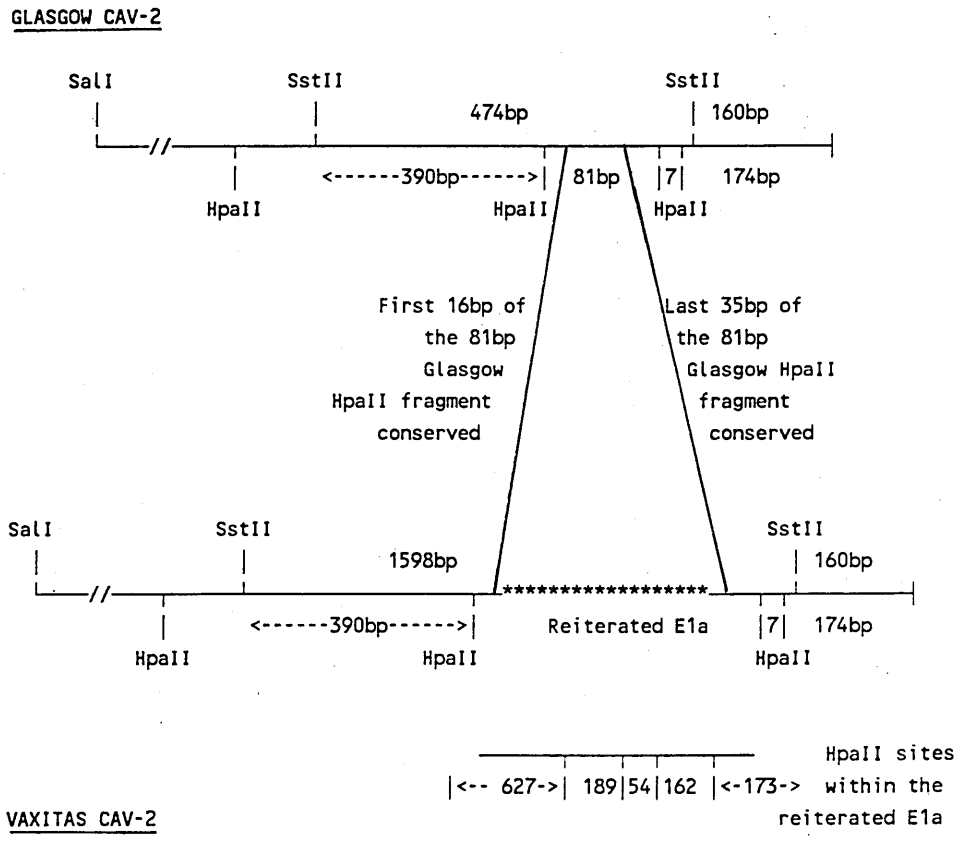


Fig. 4.12 Schematic representation of the right-hand terminal regions of Glasgow and Vaxitas CAV-2 demonstrating SstII and HpaII sites. All fragment sizes are indicated in bp.

F I G U R E 4.13

Translation in all Three Reading Frames of the Vaxitas Right-Hand End

```
        10         20         30         40         50         60
CATCATCAATAATATACAGGACAAAGAGGTGTGGCTTAAATTTGGGTGTTGCAAGGGGCG
HisHisGlnEndTyrThrGlyGlnArgGlyValAlaEndIleTrpValLeuGlnGlyAla
 IleIleAsnAsnIleGlnAspLysGluValTrpLeuLysPheGlyCysCysLysGlyAr
  SerSerIleIleTyrArgThrLysArgCysGlyLeuAsnLeuGlyValAlaArgGlyG

        70         80         90        100        110        120
GGGTCATGGGACGGTCAGGTTCAGGTCACGCCCTGGTCAGGGTGTTCCCACGGGAATGTC
GlySerTrpAspGlyGlnValGlnValThrProTrpSerGlyCysSerHisGlyAsnVal
gGlyHisGlyThrValArgPheArgSerArgProGlyGlnGlyValProThrGlyMetSe
lyValMetGlyArgSerGlySerGlyHisAlaLeuValArgValPheProArgGluCysP

       130        140        150        160        170        180
CAGTGACGTCAAAGGCGTGGTTTTACGACAGGGCGAGTTCCGCGGACTTTTGGCCGGCGC
GlnEndArgGlnArgArgGlyPheThrThrGlyArgValProArgThrPheGlyArgArg
rSerAspValLysGlyValValLeuArgGlnGlyGluPheArgGlyLeuLeuAlaGlyAl
roValThrSerLysAlaTrpPheTyrAspArgAlaSerSerAlaAspPheTrpProAlaP
      end ITR     start reiterated E1a
       190    \ |/200        210        220        230        240
CCGGGTTTTTGGGCGT|TTATTGATTTTGCGGTTTAGCGGGTGGTGCTTTTACCATGTTTG
ProGlyPheTrpAlaP|heIleAspPheAlaValEndArgValValLeuLeuProCysLeu
aArgValPheGlyArg|LeuLeuIleLeuArgPheSerGlyTrpCysPheTyrHisValCy
roGlyPheLeuGlyVa|lTyrEndPheCysGlyLeuAlaGlyGlyAlaPheThrMetPheA

       250        260        270        280        290        300
CGGAAGATTTAGTTGTTTATGGAGCTGGTTTTGGTGCCAGTTCCTCCACGGCTAATGTCA
ArgLysIleEndLeuPheMetGluLeuValLeuValProValProProArgLeuMetSer
sGlyArgPheSerCysLeuTrpSerTrpPheTrpCysGlnPheLeuHisGlyEndCysGl
laGluAspLeuValValTyrGlyAlaGlyPheGlyAlaSerSerSerThrAlaAsnValL

       310        320        330        340        350        360
AAGTTTATGTCAATATAACAGAAACACTCTGTTCTCTGTTTACAGCACCCCACCCGGTGG
LysPheMetSerIleEndGlnLysHisSerValLeuCysLeuGlnHisProThrArgTrp
nSerLeuCysGlnTyrAsnArgAsnThrLeuPheSerValTyrSerThrProProGlyGl
ysValTyrValAsnIleThrGluThrLeuCysSerLeuPheThrAlaProHisProValV
                                                  TATA E1a
       370        380        390        400        410 /      420
TTTTTCGCCACGCCTTTGGGTTAATTTTATTTCCCTATACGCGGCCTTAAATTCTCAGTG
PhePheAlaThrProLeuGlyEndPheTyrPheProIleArgGlyLeuLysPheSerVal
yPheSerProArgLeuTrpValAsnPheIleSerLeuTyrAlaAlaLeuAsnSerGlnCy
alPheArgHisAlaPheGlyLeuIleLeuPheProTyrThrArgProEndIleLeuSerA

       430        440        450        460        470        480
CAGACGAAAGAGGACTACTCTTGAGTGCGCACGGAGAAGAGTTTTCTCTTCGCTGTGTCT
GlnThrLysGluAspTyrSerEndValArgThrGluLysSerPheLeuPheAlaValSer
sArgArgLysArgThrThrLeuGluCysAlaArgArgArgValPheSerSerLeuCysLe
laAspGluArgGlyLeuLeuLeuSerAlaHisGlyGluGluPheSerLeuArgCysValS
```

```
            met E1a orf1
      490      \500       510       520       530       540
CATATATTTTCTGAAAAATGAAATATACTATTGTGCCGGCGCCGCGCAATCTCCATGATT
HisIlePheSerGluLysEndAsnIleLeuLeuCysArgArgArgAlaIleSerMetIle
uIleTyrPheLeuLysAsnGluIleTyrTyrCysAlaGlyAlaAlaGlnSerProEndLe
erTyrIlePheEndLysMetLysTyrThrIleValProAlaProArgAsnLeuHisAspT

      550       560       570       580       590       600
ATGTTTTAGAGCTACTGGAAGAGTGGCAGCCGGACTGCCTTGACTGTGAGTATTCTCATG
MetPheEndSerTyrTrpLysSerGlySerArgThrAlaLeuThrValSerIleLeuMet
uCysPheArgAlaThrGlyArgValAlaAlaGlyLeuProEndLeuEndValPheSerTr
yrValLeuGluLeuLeuGluGluTrpGlnProAspCysLeuAspCysGluTyrSerHisG

      610       620       630       640       650       660
GCAGCCCCTCGCCGCCTACTCTGCACGATCTTTTTGATGTTGAGCTGGAGACTTCTCACA
AlaAlaProArgArgLeuLeuCysThrIlePheLeuMetLeuSerTrpArgLeuLeuThr
pGlnProLeuAlaAlaTyrSerAlaArgSerPheEndCysEndAlaGlyAspPheSerGl
lySerProSerProProThrLeuHisAspLeuPheAspValGluLeuGluThrSerHisS

      670       680       690       700       710       720
GCCCTTTTGTGGGCCTGTGTGATTCCTGTGCGGAGGCTGACACTGATTCGAGTGCGAGCA
AlaLeuLeuTrpAlaCysValIleProValArgArgLeuThrLeuIleArgValArgAla
nProPheCysGlyProValEndPheLeuCysGlyGlyEndHisEndPheGluCysGluHi
erProPheValGlyLeuCysAspSerCysAlaGluAlaAspThrAspSerSerAlaSerT

      730       740       750       760       770       780
CTGAGGGTGATTCTGGGTTTAGTCCTTTATCCACTCCGCCGGTTTCACTATTCCACCGCA
LeuArgValIleLeuGlyLeuValLeuTyrProLeuArgArgPheHisTyrSerThrAla
sEndGlyEndPheTrpValEndSerPheIleHisSerAlaGlyPheThrIleProProHi
hrGluGlyAspSerGlyPheSerProLeuSerThrProProValSerLeuPheHisArgI

      790       800       810       820       830       840
TCCCACCTCTCCTGCTAGCATTTCTGACGACATGTTGCTGTGCTTAGAGGAAATGCCCAC
SerHisLeuSerCysEndHisPheEndArgHisValAlaValLeuArgGlyAsnAlaHis
sProThrSerProAlaSerIleSerAspAspMetLeuLeuCysLeuGluGluMetProTh
lePro ProLeuLeuLeuAlaPheLeuThrThrCysCysCysAlaEndArgLysCysProP

      850       860       870       880       890       900
CTTTGATGACGAGGACGAGGTTCGAAGCGCGGCGACCACCTTTGAGCGGTGGGAAAACAT
LeuEndEndArgGlyArgGlySerLysArgGlyAspHisLeuEndAlaValGlyLysHis
rPheAspAspGluAspGluValArgSerAlaAlaThrThrPheGluArgTrpGluAsnIl
roLeuMetThrArgThrArgPheGluAlaArgArgProProLeuSerGlyGlyLysThrP

      910       920       930       940       950       960
TTTGACCCCCATGTGGGTCCTATTTTTGGCTGTTTGCGCTGTGCTTTTTATCAAGAGCAG
PheAspProHisValGlyProIlePheGlyCysLeuArgCysAlaPheTyrGlnGluGln
eLeuThrProMetTrpValLeuPheLeuAlaValCysAlaValLeuPheIleLysSerAr
heEndProProCysGlySerTyrPheTrpLeuPheAlaLeuCysPheLeuSerArgAlaG
                                                    end orf1
      970       980       990      1000      1010      \1020
GATGATAATGCACTTTGTGGGCTTTGCTATCTAAAGGCCCTTGCCGAAGTAAGTTTTAAT
AspAspAsnAlaLeuCysGlyLeuCysTyrLeuLysAlaLeuAlaGluValSerPheAsn
gMetIleMetHisPheValGlyPheAlaIleEndArgProLeuProLysEndValLeuIl
lyEndEndCysThrLeuTrpAlaLeuLeuSerLysGlyProCysArgSerLysPheEndP
```

```
                                        met orf2
      1030       1040       1050       1060/      1070       1080
TTAAATGTTTGGGCAGGTTAAATGTTTGGGCAGGTTAAATGTTTTAGGTGTGTATTGATT
LeuAsnValTrpAlaGlyEndMetPheGlyGlnValLysCysPheArgCysValLeuIle
eEndMetPheGlyGlnValLysCysLeuGlyArgLeuAsnValLeuGlyValTyrEndPh
heLysCysLeuGlyArgLeuAsnValTrpAlaGlyEndMetPheEndValCysIleAspP


      1090       1100       1110       1120       1130       1140
TTTAATTTTGCTTTTTAGTGCCTTTTGCTATGCCTGTACGTTCAGAACCCGCTTCGGCTG
PheAsnPheAlaPheEndCysLeuLeuLeuCysLeuTyrValGlnAsnProLeuArgLeu
eLeuIleLeuLeuPheSerAlaPheCysTyrAlaCysThrPheArgThrArgPheGlyTr
heEndPheCysPheLeuValProPheAlaMetProValArgSerGluProAlaSerAlaG

      1150       1160       1170       1180       1190       1200
GAGCTGAGGAGGAAGATGATGAAGTTATTTTTGTGTCTGCCAAACCTGGGGGCAGAAAGA
GluLeuArgArgLysMetMetLysLeuPheLeuCysLeuProAsnLeuGlyAlaGluArg
pSerEndGlyGlyArgEndEndSerTyrPheCysValCysGlnThrTrpGlyGlnLysGl
lyAlaGluGluGluAspAspGluValIlePheValSerAlaLysProGlyGlyArgLysA

      1210       1220       1230       1240       1250       1260
GGTCAGCAGCTACTCCCTGTGAGCCAGATGGGGTCAGCAAACGCCCTTGCGTGCCAGAGC
GlyGlnGlnLeuLeuProValSerGlnMetGlySerAlaAsnAlaLeuAlaCysGlnSer
uValSerSerTyrSerLeuEndAlaArgTrpGlyGlnGlnThrProLeuArgAlaArgAl
rgSerAlaAlaThrProCysGluProAspGlyValSerLysArgProCysValProGluP
                                                  end orf2
      1270       1280       1290       1300       1310/      1320
CTGAGCAAACAGAACCTTTGGATTTGTCTTTGAAGCCACGCCCGAACTAATCTCCTTGAG
LeuSerLysGlnAsnLeuTrpIleCysLeuEndSerHisAlaArgThrAsnLeuLeuGlu
aEndAlaAsnArgThrPheGlyPheValPheGluAlaThrProGluLeuIleSerLeuSe
roGluGlnThrGluProLeuAspLeuSerLeuLysProArgProAsnEndSerProEndA
     polyA signal E1a
      1330 /     1340       1350       1360       1370       1380
CACAAAGCAATAAAGTAATCTTGTTTAACAAGT|TGACAAGGACGTGCTGTACTTTTTGTG
HisLysAlaIleLysEndSerCysLeuThrSer|EndGlnGlyArgAlaValLeuPheVal
rThrLysGlnEndSerAsnLeuValEndGlnVa|lAspLysAspValLeuTyrPheLeuEn
laGlnSerAsnLysValIleLeuPheAsnLysL|euThrArgThrCysCysThrPheCysG
              end reiterated E1a   revertion to wild-type RHT
      1390       1400       1410       1420       1430       1440
AACTTTCCGGGCCAACCGCCAAAGGGAAACTGCACTTAACATTTACCACGCGCCCACATT
AsnPheProGlyGlnProProLysGlyAsnCysThrEndHisLeuProArgAlaHisIle
dThrPheArgAlaAsnArgGlnArgGluThrAlaLeuAsnIleTyrHisAlaProThrPh
luLeuSerGlyProThrAlaLysGlyLysLeuHisLeuThrPheThrThrArgProHisP

      1450       1460       1470       1480       1490       1500
TTATGACTGTACTTGGCACCACTTCCTCAAACGCCCCGTTATATTCCTTTTCGTTTTCCA
LeuEndLeuTyrLeuAlaProLeuProGlnThrProArgTyrIleProPheArgPhePro
eTyrAspCysThrTrpHisHisPheLeuLysArgProValIlePheLeuPheValPheHi
heMetThrValLeuGlyThrThrSerSerAsnAlaProLeuTyrSerPheSerPheSerT
                    TATA E4
      1510       1520  /    1530       1540       1550       1560
CACGCCCTACTTTGAGGACTATATAAACGCTGTGCTTGGCATTTCATCCTCATAGCTCTC
HisAlaLeuLeuEndGlyLeuTyrLysArgCysAlaTrpHisPheIleLeuIleAlaLeu
sThrProTyrPheGluAspTyrIleAsnAlaValLeuGlyIleSerSerSerEndLeuSe
hrArgProThrLeuArgThrIleEndThrLeuCysLeuAlaPheHisProHisSerSerP
```

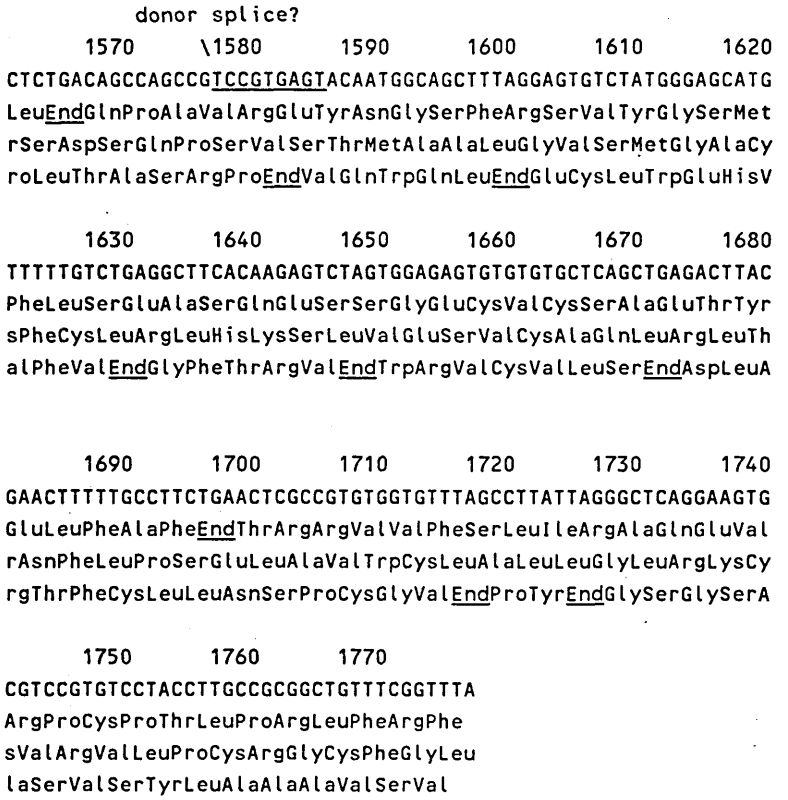


Fig. 4.13 Translation in all three reading frames of the Vaxitas RHT. The end of the ITR/start of reiterated E1a: end of reiterated E1a/revertion to wild-type RHT sequences are marked. Presumptive TATA boxes, donor splice sites and termination codons are also indicated. Explanation of splice site consensus sequences given in figure 4.14.

F I G U R E 4.14

Translation in all Three Reading Frames of the Glasgow Right-hand end

```
      490        500        510        520        530        540
GTCTATGGGAGCATGTTTTTGTCTGAGGCTTCACAAGAGTCTAGTGGAGAGTGTGTGTGC
ValTyrGlySerMetPheLeuSerGluAlaSerGlnGluSerSerGlyGluCysValCys
lSerMetGlyAlaCysPheCysLeuArgLeuHisLysSerLeuValGluSerValCysAl
ysLeuTrpGluHisValPheValEndGlyPheThrArgValEndTrpArgValCysValL


                                                 donor splice E4?
      550        560        570        580     / 590        600
TCAACTGAGACTTACGAACTTTTTGCCTTCTGAACTCGCCGTGTGGTGTTTAGCCTTATT
SerThrGluThrTyrGluLeuPheAlaPheEndThrArgArgValValPheSerLeuIle
aGlnLeuArgLeuThrAsnPheLeuProSerGluLeuAlaValTrpCysLeuAlaLeuLe
euAsnEndAspLeuArgThrPheCysLeuLeuAsnSerProCysGlyValEndProTyrE

      610        620        630        640        650
AGGGCCCAGGAAGTGCGTCCGTGTCCTACCTTGCCGCGGCTGTTTCGGTTTA
ArgAlaGlnGluValArgProCysProThrLeuProArgLeuPheArgPhe
uGlyProArgLysCysValArgValLeuProCysArgGlyCysPheGlyLeu
ndGlyProGlySerAlaSerValSerTyrLeuAlaAlaAlaValSerVal
```

Fig. 4.14 Translation in all three reading frames of the Glasgow CAV-2 right-hand terminal region, encoding the presumptive E4 promoter. Presumptive **TATA** marked. All translational stops are underlined. In addition, possible donor splice sites are indicated. The Ad2 E4 donor splice site is situated 60bp from the E4 TATA and demonstrates the consensus sequence (C/A)(T/A)(G/C)GT(A/G)AG(G/T) (Rigolet and Galibert, 1984):- the possible donor splice of CAV-2 E4 at 452bp shows a 78% similarity to the consensus sequence tcCGTGAGT and is situated 57bp from the presumptive TATA: the possible donor splice site at 584bp shows a 50% similarity to this sequence TGGTgttt but is situated 120bp from the presumptive TATA. Additionaly, the second possible donor splice, 120bp from the presumptive TATA would involve several ATG codons prior to the first splice site, contrary to the human adenovirus E4 region.

Fig. 4.15 DNA sequence of presumptive Glasgow E1a orf1 and Vaxitas reiterated E1a orf1 transformed to protein. Alignment of the two sequences demonstrates the effect of the frame shifts and base changes on the presumed orf1 product of the Vaxitas RHT E1a.

F I G U R E 4.15

Comparison of Open Reading Frame 1 (ORF1) of Glasgow E1a (LHT) and the Vaxitas Reiterated E1a (RHT)

Fig. 4.16 Organisation of the right-hand terminal of Vaxitas CAV-2 as defined by sequence analysis. Presumptive TATA boxes and initiating methionine codons are indicated.

F I G U R E 4.16

Schematic Organisation of the Right-hand Terminal of Vaxitas CAV-2

F I G U R E 4.17

Sequence Analysis of the 231bp Nobivac CAV-2 Insertion

```
NOBIVAC
GLASGOW CAV-2 RHT                                    STRAND L


1   CGGCCAAAAGTCCGCGGAA TCGCCCTGTCGTAAAACCACGCCTTTGACGTCACTGGACA
176 CGGCCAAAAGTCCGCGGAACTCGCCCTGTCGTAAAACCACGCCTTTGACGTCACTGGACA
                      *

60  TTCCCGTGGGAACACC TGACCAGGGCGTGACCTGAACCTGACCGTCCCATGACCCCGCC
116 TTCCCGTGGGAACACCCTGACCAGGGCGTGACCTGAACCTGACCGTCCCATGACCCCGCC
                  *

119 CCTTGCAAGACCCAAATTTAAGCCACACCTGTTTGTCCTGTATATTA     165
56  CCTTGCAACGCCCAAATTTAAGCCACACCTCTTTGTCCTGTATATTA      10
          **                  *

Matches = 162    Length = 167    Matches/length = 97.0%


165 AAGTACAGTCATAAAATGTGG CGCGTGGTAAATGTTAAGTGCAAGTTTCCCTTTGGCGG
329 AAGTACAGTCATAAATTGTGGGCGCGTGGTAAATGTTAAGTGCA GTTTCCCTTTGGCGG
                  *    *                    *

224 TTGGCCCG     231
270 TTGGCCCG     263


Matches = 65      Length = 68      Matches/length = 95.6%


NOBIVAC
VAXITAS RHT                                          STRAND L

1   CGGCCAAAAGTCCGCGGAA TCGCCCTGTCGTAAAACCACGCCTTTGACGTCACTGGACA
176 CGGCCAAAAGTCCGCGGAACTCGCCCTGTCGTAAAACCACGCCTTTGACGTCACTGGACA
                      *

60  TTCCCGTGGGAACACC TGACCAGGGCGTGACCTGAACCTGACCGTCCCATGACCCCGCC
116 TTCCCGTGGGAACACCCTGACCAGGGCGTGACCTGAACCTGACCGTCCCATGACCCCGCC
                  *

119 CCTTGCAAGACCCAAATTTAAGCCACACCTGTTTGTCCTGTATATTA     165
56  CCTTGCAACACCCAAATTTAAGCCACACCTCTTTGTCCTGTATATTA      10
          *                   *

Matches = 163     Length = 167     Matches/length = 97.6%
```

```
165  AAGTACAGTCATAAAATGTGG CGCGTGGTAAATGTTAAGTGCAAGTTTCCCTTTGGCGG
1453 AAGTACAGTCATAAAATGTGGGCGCGTGGTAAATGTTAAGTGCA GTTTCCCTTTGGCGG
                          *                     *

224  TTGGCCCG     231
1394 TTGGCCCG    1387

Matches = 66      Length = 68      Matches/length = 97.1%
```

```
NOBIVAC
CAV-1 ITR

32  AAAACCACGCCTTTGACGTCACTGGACATTCCCGTGGGAA CACC TGACCAGGGCGTGA
22  AAAAACACCCAATTGACGTCA TGGTCTTTCCCGTGGGAAACGCCCTGAACAGGGTGTGA
       *   * **         *   * *           * *  *   *     *

90  CCTGAACCTGACCGTCCCATGACCCCGCCCCTTGCAAGACCCAA ATTTAAGCCACACCT
81  CCTTGTCAAAACAATCAGACAAAAACTTTTTTAAAAAAAAACAACATTTAGGCCACACCT
      *** ***  **  ** ** *** ***** ***  * **   *     *

149 GTTTGTCCTGTATATTA     165
141 CTTTGTCCTGTATATTA     157
    *

Matches = 96      Length = 137     Matches/length = 70.1%
```

Fig. 4.17 Additional sequence analysis to the Nobivac CAV-2 insertion. Previous similarity to the Vaxitas and Glasgow ITRs now extended to include the area of the Vaxitas and Glasgow right-hand terminals immediatly prior to the E4 promoter.

F I G U R E 4.18

Schematic Representation of the Nobivac 231bp Insertion as Compared to the Glasgow and Vaxitas Right-hand Termini

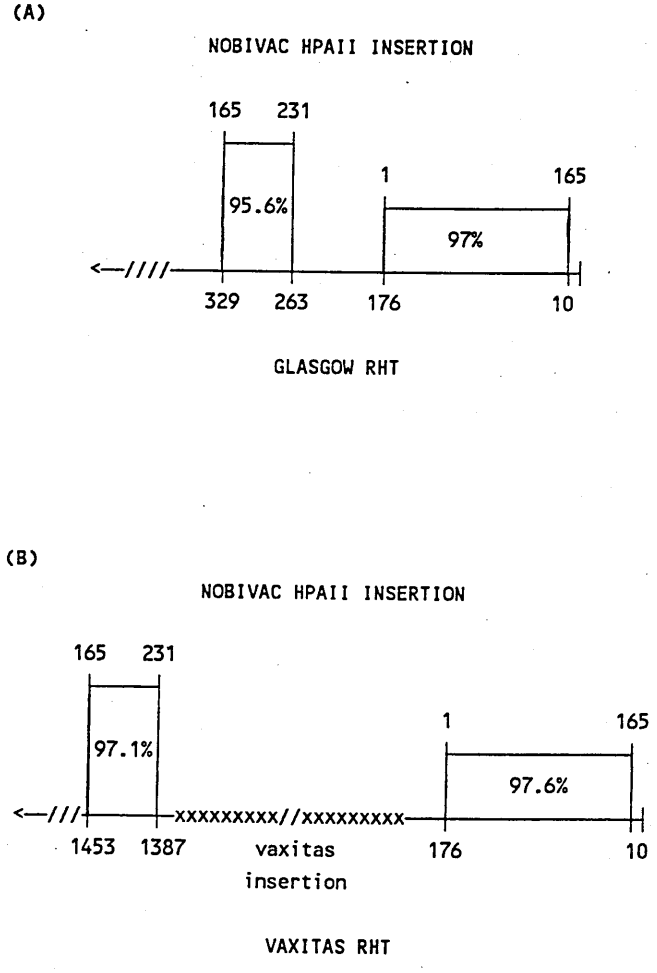


Fig. 4.18 HpaII fragment containing the Nobivac additional DNA was sequenced as a single fragment, in both orientations, from bp1 to bp 231. The numbers above the areas of the Nobivac clone which show homology indicate the part of the fragment with reference to the whole 231bp in the orientation indicated by the sequence given in chapter 3. The percentage homology of each region of Glasgow (A) and Vaxitas (B) is noted within each block.

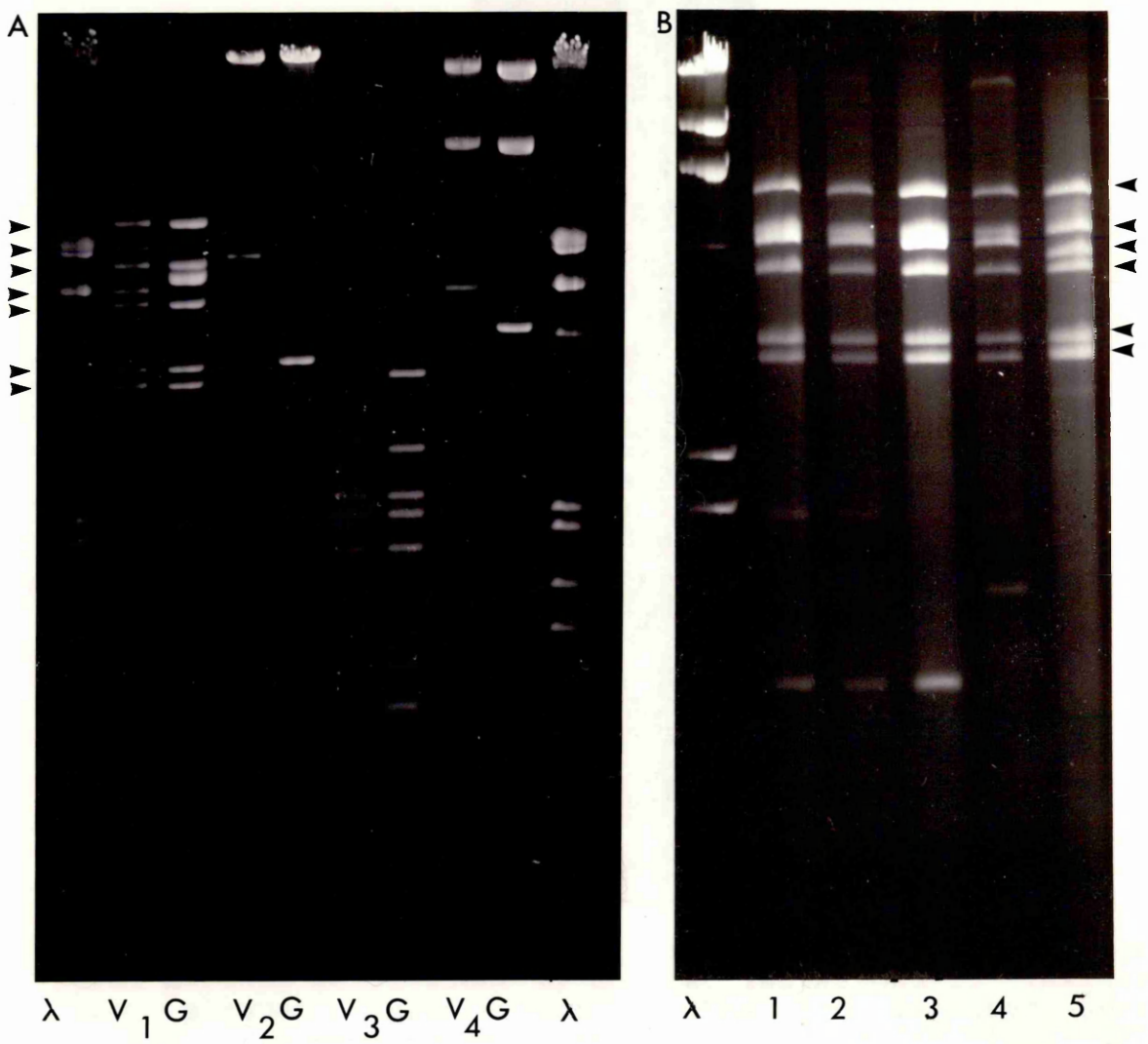
Fig. 4.19 Restriction enzyme profile of the Vaxitas (V) genome following a single pass in the Mv-1-Lu cell line as compared to the genome prior to passage in the new cell line. The Glasgow (G) CAV-2 genome is shown for comparison.

(A) digestion of the Vaxitas and Glasgow genomes with; lane 1, PstI; lane 2, SalI; lane 3, HpaII; lane 4, EcoRI. HindIII/EcoRI digested λ DNA fragments were used as m.w. markers.

(B) Pattern of fragments produced by Vaxitas (lane 5) following digestion with PstI prior to being passed in the Mv-1-Lu cell line. PstI digestion of Kavak CAV-2 (lane 1), Epivac CAV-2 (lane 2), Glasgow CAV-2 (lane 3) and Nobivac CAV-2 (lane 4) are shown for comparison. HindIII digested λ DNA fragments were used as m.w. markers.

F I G U R E 4.19

Alterations in the Vaxitas Genome Following One Pass In the Mv-1-Lu Cell Line



F I G U R E 4.20

Plaque Purification of Vaxitas CAV-2

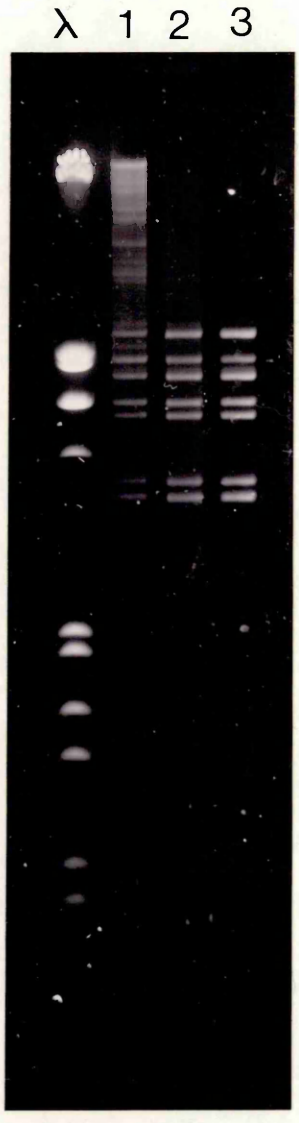


Fig. 4.20 Comparison of PstI digestion of Vaxitas stock from Mv-1-Lu cells (lane 1) and two isolations of Vaxitas from plaques (lanes 2 and 3). The two isolates were picked from plaques and amplified to allow for viral DNA isolation. The appearance of a heterogeneous stock following plaque isolation, displayed by the two isolates shown above, is consistent with all plaque isolates examined. HindIII/EcoRI digested λ DNA fragments were used as m.w. markers.

D I S C U S S I O N

The DNA sequence of the Glasgow RHT demonstrates several of the conserved regions expected from other AdV sequences in the E4 region. A presumptive TATA is situated at bp 395 with an initiating methionine 70bp downstream which initiates an open reading frame until the end of the known sequence. The human AdVs, however, contain no ATG before the initial donor splice site situated 60bp from the E4 cap site. The only Glasgow RHT/E4 sequences with a strong similarity to this consensus splice site are situated at bp 452, 57bp downstream of the presumptive TATA: tcCGTGAGT (78% similarity):, and at bp584, 130bp downstream of the presumptive E4 TATA: TGGTgttt (50% similarity). S_1 analysis and mRNA mapping will be required to determine whether the organisation of the CAV-2 E4 region varies from that of Ad2. It may initiate at an ATG prior to the initial donor splice site (if splice at 584bp), the ATG may be in a non-coding region (if splice at 452bp) or the initial CAV-2 donor splice site may have no similarity to the Ad2 E4 donor splice consensus sequence and is situated upstream of the ATG thus again placing the ATG in a non-coding region. A summary of the presumptive organisation of Glasgow CAV-2 E4 promoter region, as defined by sequence analysis, is provided in figure 4.21.

The HpaII analysis of the SalI B and SstII fragments, combined with the sequence data from the RHT of both

F I G U R E 4.21

Schematic Organisation of the Glasgow CAV-2 Right-hand Terminus

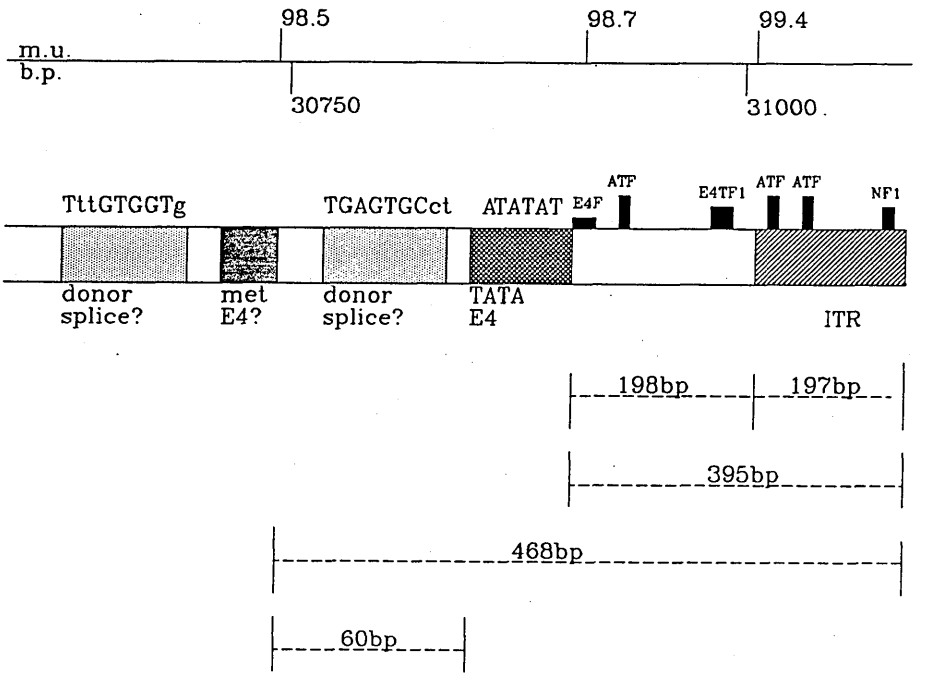


Fig. 4.21 Schematic organisation of the right-hand terminal region of Glasgow CAV-2 as inferred by DNA sequence analysis. It should be noted that the distance from the end of the ITR to the presumptive TATA is 198bp. This is consistent with the spacing of the end of the ITR and E4 TATA in Ad2 and Ad5, 194bp. The ITR itself however is almost twice the length of that in Ad2, 197bp vs 102bp.

Enhancer binding sites are also indicated based upon sequence similarity to known consensus sequences. ATF, consensus sequence (T/G)(T/A)CGTCA, is thought to have binding sites at 124bp GACGTCA, 145bp TACaCA and 387bp TGA gGAC (upper strand). E4TF1, consensus sequence GGAAGTG, is believed to have a binding site at 335bp CACTTCC (upper strand) and E4F, consensus sequence identical to ATF but only recorded as binding to one site adjacent to E4 TATA's (Jones et al, 1988), at bp387 TGAgGAC (upper strand). No strong similarity is demonstrated to the consensus sequence of NF1, TGGCT(N)AGCCAA except at bp31 TGGCT(TAA)Atttgg (64.2%).

Glasgow and Vaxitas, demonstrated that the mode of insertion of excess DNA into the Vaxitas genome at the RHT was similar to the other vaccine strains. All other vaccine strains exhibited insertion of DNA into one, or possibly more, HpaII fragments. Vaxitas exhibits the same properties the only difference being that the insertion itself contained additional HpaII fragments, a possibility that cannot be excluded from the other vaccine strains until their full sequence, i.e. past the site of insertion, can be ascertained.

The striking homology between the Vaxitas/Glasgow RHT and the Vaxitas RHT/Glasgow E1a implies that replication error of some type has occurred in order to facilitate the construction of this aberrant genome. The fact that the genomes are not identical is evinced by the single base change in the ITR and the 4 base changes in the E4 region following the end of the Vaxitas insertion. The base changes within these regions do not however affect the presumptive control sequences or any presumptive open reading frames.

The reiterated E1a within the RHT of Vaxitas also appears to retain all the presumptive control sequences demonstrated by the Glasgow LHT E1a region (Spibey et al, 1989). However, numerous base changes and frame shifts can be seen to have occurred when the overall sequence is compared to Glasgow E1a. The frame shifts in particular introduce several additional translational stops within

both orf1 and orf2 (figs.4.13, 4.14 and 4.15). When the sequence is transformed to demonstrate the protein sequence, the predicted amino acid sequence implies that any products of the Vaxitas reiterated E1a should be non-functional. This is of course based on the assumption that the presumptive control sequences are correct. A highly conserved region exists in the majority of AdV, downstream of the E1a TATA, which is demonstrated downstream of the presumptive CAV-2 E1a TATA thus supporting this area as the candidate TATA box (fig. 4.10).

When the presumptive orfs of Glasgow E1a and the Vaxitas reiterated E1a are compared it can be seen that the frame shifts/base changes cause major alterations in the continuity of the orfs. An alignment of orf1 from Glasgow E1a and orf1 from the Vaxitas RHT E1a is demonstrated in figure 4.15. An assay was therefore required to assess whether the reiterated E1a at the RHT of Vaxitas was indeed non-functional or what effect it had on the function of E4. Chloramphenicol acetyl transferase (CAT) assays should answer both the above questions.

It should be noted that, originally, the dominant SalI B fragment within the genome of Vaxitas DNA contained an insertion of 2.0kb as opposed to the 1.1kb insertion examined. Preliminary sequence examination of this 2.0kb fragment has indicated that it is of a comparable

structure to the 1.1kb fragment i.e. it also appears to consist of a reiterated E1a at the RHT. However, it is not yet clear whether the difference in the size of the insertion is due to two copies of a RHT E1a being present or whether the 2.0kb reiterated "E1a" in this case also includes E1b. Further work will resolve this and the results may elucidate upon the mechanism by which these aberrant genomes arise.

Growth of the Vaxitas strain in the new cell line, Mv-1-Lu, was directly comparable to the growth of the wild-type Glasgow in MDCK cells and growth of Vaxitas in the former NMF cell line. The ability of the new cell line to support plaque formation was an additional bonus and facilitated the plaque purification of the virus which, it was hoped, would allow the individual isolation of different strains of Vaxitas contained within the heterogeneous stock virus.

However, plaque purification techniques require amplification of the plaque purified virus before there is an adequate supply for a viral DNA preparation. During this amplification, facilitated by passage of the plaque purified virus through increasing numbers of permissive cells, all plaque purified virus reverted to the standard heterogeneous mixture and repeated attempts could not produce virus on a large enough scale to facilitate DNA analysis that yielded one single genomic type without a heterogeneous background species.

The remarkable effect upon the LHT of the Vaxitas genome of a single pass in Mv-1-Lu cells is not a unique occurrence. Ishiyama et al (1986) described equine AdV which was found to contain additional sequences of 100-700bp after a single pass in a new cell line. As with Vaxitas, the relative amounts of each genome "type" present within the heterologous mixture did not appear to vary during serial passage. However, the EAdV insertions occurred at the LHT not the RHT and insertions of greater than 200bp rendered the virus defective for replication and/or packaging.

The additional homology now available to the Nobivac insertion is in agreement to several other human AdV insertions mentioned previously (CH 2, Discussion). The Nobivac insertion comprises of a copy of the ITR plus adjacent sequences. It is presumed, until the exact sequence of the Nobivac ITR can be ascertained, that the few base changes that occur between the Nobivac ITR copy and the Glasgow/Vaxitas ITRs are due to strain differences. However, it is not known whether the Nobivac insertion is a direct copy: i.e. does it run from specific point within the ITR continuously until the end of the area of repeat?

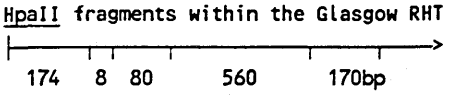
The insertion is divided into two parts; the first 165bp, i.e. bases 1 -> 165, are a repeat of the ITR matching

bases 176 -> 10 of the Glasgow/Vaxitas ITRs; the second 66 bases, i.e. bases 165 -> 231 (the complete HpaII fragment of the Nobivac insertion), are a repeat of the area covered by bases 329 -> 263 of the Glasgow RHT and the corresponding area, bases 1453 -> 1387, of the Vaxitas RHT. This is clarified schematically in figure 4.18. Two possible structures of the Nobivac RHT are described in figure 4.22. The exact site and nature of the Nobivac insertion can however only be ascertained by obtaining a complete sequence of the Nobivac RHT from the terminus until past the site of insertion as has already been carried out upon the Vaxitas RHT.

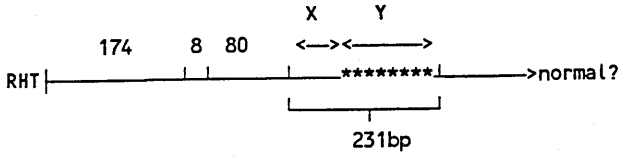
F I G U R E 4.22

Proposed Structure of the Nobivac Right-hand Terminus

For reference:-

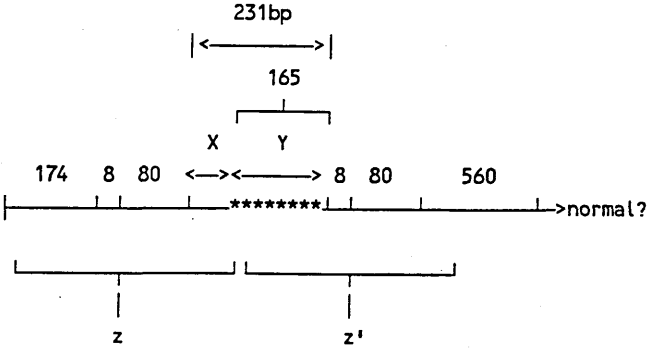


If assume Nobivac is normal until bp329 from the RHT:-



Where X+Y=the 231bp HpaII fragment unique to Nobivac. Section X of this fragment represents the 66bp homologous to bp1387-1453 of the Glasgow RHT (see fig. 19) and section Y represents the 165bp homologous to bp10-175 of the Glasgow ITR.

(i) The whole area of the genome, until past the insertion (X and Y), may be repeated as this would result in co-migrating fragments which may not be apparent on an acrylamide gel.



The two 8 and 80bp fragments would co-migrate, the 231bp fragment would appear as a distinct band and all other fragments would appear as normal unless the "insertion" continues past the HpaII site encoded at the terminus of the ITR (Y) in which case other fragments may also be doubles until the genome presumably reverts to normal.

(ii) If the excess DNA is a straightforward insertion the genome would simply contain all normal fragment plus the additional HpaII fragment. The genome may then continue as normal:-

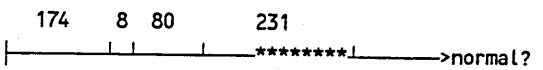


Fig. 4.22 Two possible descriptions of the additional sequences present at the RHT of the Nobivac genome are presented, (i) and (ii). It should be noted that (i) and (ii) rely on the site of "insertion" being within the 560bp fragment of wild-type i.e. the first 66bp of this fragment have been retained, followed by the copy of the ITR as far as the HpaII site of the 231bp fragment. When comparing this to the Vaxitas insertion, it seems unlikely that the copy of the ITR ends exactly on a HpaII site and, as no part of the opposite end of the 560bp fragment is present in the sequence, that more than one excess HpaII fragment is present within the Nobivac RHT. The evidence obtained from analysis of Vaxitas would therefore favour version (i) as a copy of the 560bp fragment is apparent after HpaII digestion of the Nobivac genome analysed on an acrylamide gel.

C H A P T E R 5

Effect of the Additional DNA on the E4 Region of the Vaxitas CAV-2 Genome

INTRODUCTION

Expression of the E1a gene facilitates transcription from early viral promoters E1b, E2, E3 and E4. In addition, E1a gene products are able to activate transcription of the mammalian heat shock gene (Nevins, 1982) as well as certain cellular genes introduced into cells as recombinant DNA molecules by transfection or infection (Svensson and Akusjarvi, 1984a). The E1a gene also plays a critical role in the ability of an AdV to morphologically transform cells in vitro. Ginsberg et al (1987) reported that E1 plays an essential role in the pathogenesis of adenovirus pneumonia. The significant sequence differences between the Glasgow and reiterated Vaxitas E1a's, if the Vaxitas reiterated E1a is functional, may be an important factor in the ability of Glasgow to replicate in the intestine, an unusual site of isolation for a CAV-2. The Vaxitas 'normal' left-hand terminal E1a may however bear little resemblance to the reiterated E1a at the right-hand end of the genome due to mutations in the reiterated sequence. If however, the reiterated E1a encodes functional protein products then it may be considered as closely resembling, if not identical to, the E1a present at the left-hand terminus. The differences between the Glasgow E1a sequence and Vaxitas reiterated E1a sequence may then be considered for their relevance in the pathogenicity of the two strains.

The presence of a functional reiterated E1a at the right-

hand terminus of Vaxitas could have profound effects upon the virus both in vivo and in vitro. Presumably excess amounts of E1a products would be present in cells soon after infection. The effect this would have upon the expression of viral genes however is not clear. Initial expression of the other early genes may initially be enhanced with transcription occurring more quickly after infection and perhaps to higher levels due to the excess of E1a products. Conversely, it is believed that E1a is self-regulatory. It is therefore feasible that the regulation of E1a would lead to a premature reduction of transcription from the other early genes which may in turn lead, via a cascade effect, to reduced production from all viral genes.

The effect of the reiterated E1a is likely to be most pronounced upon E4. The initiation of expression from the reiterated E1a may affect the E4 promoter region. Perhaps the presence of the reiterated E1a interferes with the E4 promoter to such an extent that it is either no longer functional or poorly functional due to the increased distance from several known enhancer sequences which, by sequence similarity between the CAV-2 RHT and the human AdV RHT, lie terminal to the insertion sequence and hence greater than 1.2kb from their initial site with reference to the E4 promoter.

Alternatively, the initiation of expression from the

reiterated E1a may lead to the initiation of transcription from the E4 promoter by transcripts initiating at the reiterated E1a and continuing through E4. Thus E4 would be transcribed much earlier in infection than is normal and at much higher levels. What effect this would have upon viral infection *in vitro* or *in vivo* is not clear.

An efficient procedure has been developed to measure the function and activity of putative promoter sequences. Gorman *et al* (1982) constructed recombinant genomes that direct the expression of an enzyme, chloramphenicol acetyltransferase (CAT), in mammalian cells. When cells were transfected with a recombinant plasmid, e.g. pSV2/CAT, consisting of the origin of replication from pBR322 coupled to simian virus (SV40) early transcription unit and the CAT coding sequences, CAT activity was easily detected. Similar recombinants with the SV40 promoter removed serve as useful vectors with which to test promoter activity. This test is carried out by insertion of a putative promoter sequence 3' to the CAT gene; any promoter activity from the inserted DNA will result in expression of the CAT gene upon transfection into mammalian cells. The assay can therefore be used to demonstrate both the fact that the promoter is functional and the level at which it functions, i.e. a strong or weak promoter. There is no endogenous CAT activity present in eucaryotic cells.

CAT assays have been used to examine a range of human AdV early promoters e.g. E3 and E4 (Glenn and Ricciardi, 1985 and 1987: Lillie and Green, 1989). The assay has been used to demonstrate the effect of E1a gene products upon the function of other AdV early promoters. This was achieved by transfecting, e.g., an E3/CAT construct with and without E1a. Minimal levels of CAT expression were detected from the E3/CAT construct in the absence of E1a. However, when the construct was transfected in the presence of E1a, high levels of CAT activity were observed (Weeks and Jones, 1983). This was again demonstrated for the effect of E1a upon the E4 promoter (Lillie and Green, 1989). CAT assays therefore form a convenient assay which can discriminate between a functional and a non-functional E1a gene.

Sequence analysis has revealed that the reiterated RHT E1a of Vaxitas is situated between the ITR and the presumptive E4 promoter. It also suggests that the reiterated E1a is non-functional. The Glasgow and Vaxitas RHT SmaI/PvuII fragments (fig. 3.2, the RHT PvuII site being between the two SstII sites and situated after the end of the reiterated E1a in Vaxitas) contain the presumptive E4 promoter and, in Vaxitas, the reiterated E1a. CAT constructs containing these fragments should therefore demonstrate whether the CAV-2 E4 will function independently of E1a, whether the reiterated E1a promoter is functional and, by utilising the SalI B clone of

Vaxitas containing the reiterated E1a, whether the reiterated E1a produces functional products capable of transactivation of the E4 promoter. The Glasgow E1a clone, EcoRI C, will provide a positive control to demonstrate the effect of a functional E1a upon the E4 promoter.

Chloramphenicol Acetyltransferase (CAT) Assay

Cloning in pCAT12

A pUC12/CAT construct was obtained from Mr M. Riggio (Department of Veterinary Pathology, University of Glasgow). The CAT reporter plasmid consisted of an insertion of the CAT gene into the BamHI/XbaI sites of pUC12. The vector was cut with SmaI, phosphatased, ethanol precipitated and finally resuspended in TE and stored at -20°C until required. A second CAT reporter plasmid, pSV2, comprising of a pBR322/CAT construct with the CAT gene under the control of an SV40 promoter was utilised as a positive control for transfections and CAT assays (courtesy of Mr M. Riggio).

The terminal SalI B clones of Vaxitas and Glasgow were then digested with SmaI/PvuII, thus providing two blunt fragments of 1.5kb and 0.4kb respectively for cloning into the pUC12/CAT vector. The viral fragments were purified from an agarose gel as previously described and ligated to the SmaI cut vector. Orientation of the viral fragments within the new CAT reporter plasmids were checked by restriction enzyme analysis. DNA was then prepared by large-scale plasmid preparations through CsCl as described previously.

Transfections for CAT assay

Transfections were carried out after the method of Graham

and Van der Eb (1973). Briefly, Mv-1-Lu cells were split 1:15 and allowed to settle at 37°C overnight. Meanwhile, 0.25ml of donor DNA (pCAT12 clones) at a concentration of 80µg/ml in 1/10 TE (0.1mM EDTA, 1.0mM Tris pH8.0) was placed into a sterile bijou and 0.2ml 1/10 TE added. Next, 0.05ml of 2.5M $CaCl_2$ was added and the solution mixed well. The DNA solution was then added slowly, with continuous mixing, to 0.5ml 2xHBS (table 5.1) and thoroughly mixed on a bench-top whirlimix. This mixture was then left at room temperature for 30 minutes.

The DNA/calcium phosphate suspension was added to a 75cm^2 flask which was incubated at 37°C overnight to allow adsorption. The media containing the DNA precipitate was then removed and replaced with fresh media.

Cell Harvest for CAT Assay

Cells were harvested 48 hours after transfection. Cells were first washed 3x with Dulbecco's PBS (calcium- and magnesium-free) followed by addition of 1.5ml of harvest TEN (table 5.1) to the flasks. Cells became rounded and loose after 5 minutes and were removed by scraping. After being transferred to a microfuge tube, cells were collected by centrifugation in a bench-top microfuge for 10 minutes at 4°C. The cell pellet was resuspended in 150µl of 250mM Tris pH7.8 buffer and the suspension freeze/thawed 3x at -20°C. Cell debris was pelleted by centrifugation for 10 minutes in a bench-top microfuge.

The cell extract containing the CAT enzyme was stored at -20 °C until required.

CAT Assay

The extract from transfected cells was assayed for CAT enzyme activity as described by Gorman et al (1982) with slight modifications. Briefly, the enzyme assay was performed in a final volume of 155µl containing 75µl of cell extract, 2.5µl of 40mM acetyl coenzyme A (sodium salt, Sigma), 2.5µl (0.1 µCi) of ^{14}C-chloramphenicol (Amersham) and 75µl tris-HCl (250mM, pH7.8). The mixture was incubated at 37 °C for 60 minutes and stopped by the addition of 200µl ethyl acetate. CAT enzyme (Sigma), 8 units, was used as a positive control for the CAT assays. Chloramphenicol was extracted from the mixture by three treatments with 150µl of ethyl acetate and the solvent was evaporated by centrifugation under vacuum.

The chloramphenicol sample was dissolved in 30µl of ethyl acetate and spotted onto a plastic-backed silica thin layer chromatography plate in 5µl aliquots. The unreacted chloramphenicol was separated from the 1C-acetylated, 3C-acetylated and the 1C, 3C diacetylated forms by ascending chromatography in chloroform/methanol (95:5) solvent. The plate was air dried and subjected to autoradiography at -70 °C overnight.

T A B L E 5.1

CAT Assay and Transfection Solutions

2x Hepes Buffered Saline (HBS)

NaCl	1.63g		280mM
HEPES	1.19g	to give	50mM
$Na_2HPO_4.2H_2O$	0.023g		1.5mM
dH_2O	to 100ml		

adjust pH to 7.1 - 7.25.

TEN

Tris (pH 7.5)		40mM
0.5M EDTA	to give	1mM
5M NaCl		150mM
dH_2O		

R E S U L T S

CAT Assay

To assess the effect of the Vaxitas insertion upon the function of E4 a combination of virus DNA clones were used both singly and in conjunction with others in the transfections. The SmaI/PvuII/CAT clones of Glasgow and Vaxitas were used to monitor the transcription through the CAT gene. The SmaI/PvuII/CAT clones were transfected as follows:-

a) Glasgow SmaI/PvuII/CAT clone (pGE4/CAT) alone

b) Glasgow SmaI/PvuII/CAT clone (pGE4/CAT) co-transfected with the Glasgow E1 clone (pGE1a) which consisted of Bluescript containing the EcoRI C fragment

c) Glasgow SmaI/PvuII/CAT clone (pGE4/CAT) co-transfected with the Vaxitas SalI B clone (pVE1a(r))

d) Vaxitas SmaI/PvuII/CAT clone (pVE4/CAT) alone

e) Vaxitas SmaI/PvuII/CAT clone (pVE4/CAT) co-transfected with the Glasgow EI clone (pGE1a)

The positive control was provided by Mr. M. Riggio, University of Glasgow in the form of a pUC12/CAT reporter plasmid under the control of the SV40 promoter, pSV2. All transfections were performed in the Mv-1-Lu (mink) cell line to ensure that the performance of the E1a promoter within the Vaxitas insert was not inhibited by cellular factors in a cell line non-permissive to Vaxitas.

Figure 5.1 shows the results of the CAT assays, summarised in table 5.2. The Glasgow SmaI/PvuII/CAT clone, as

expected, is virtually non-functional when transfected without the presence of a functional E1. When the Vaxitas SmaI/PvuII/CAT clone is transfected alone, however, a high level of CAT expression can be detected.

The Glasgow E4/CAT reporter plasmid is fully functional and the CAT gene is strongly expressed when the SmaI/PvuII/CAT clone is co-transfected with the EcoRI C (E1 region) clone. Again, CAT expression is demonstrated by the Vaxitas SmaI/PvuII/CAT construction.

When the Glasgow SmaI/PvuII/CAT construction is co-transfected with the Vaxitas SalI B clone containing the reiterated E1a, no CAT expression can be detected.

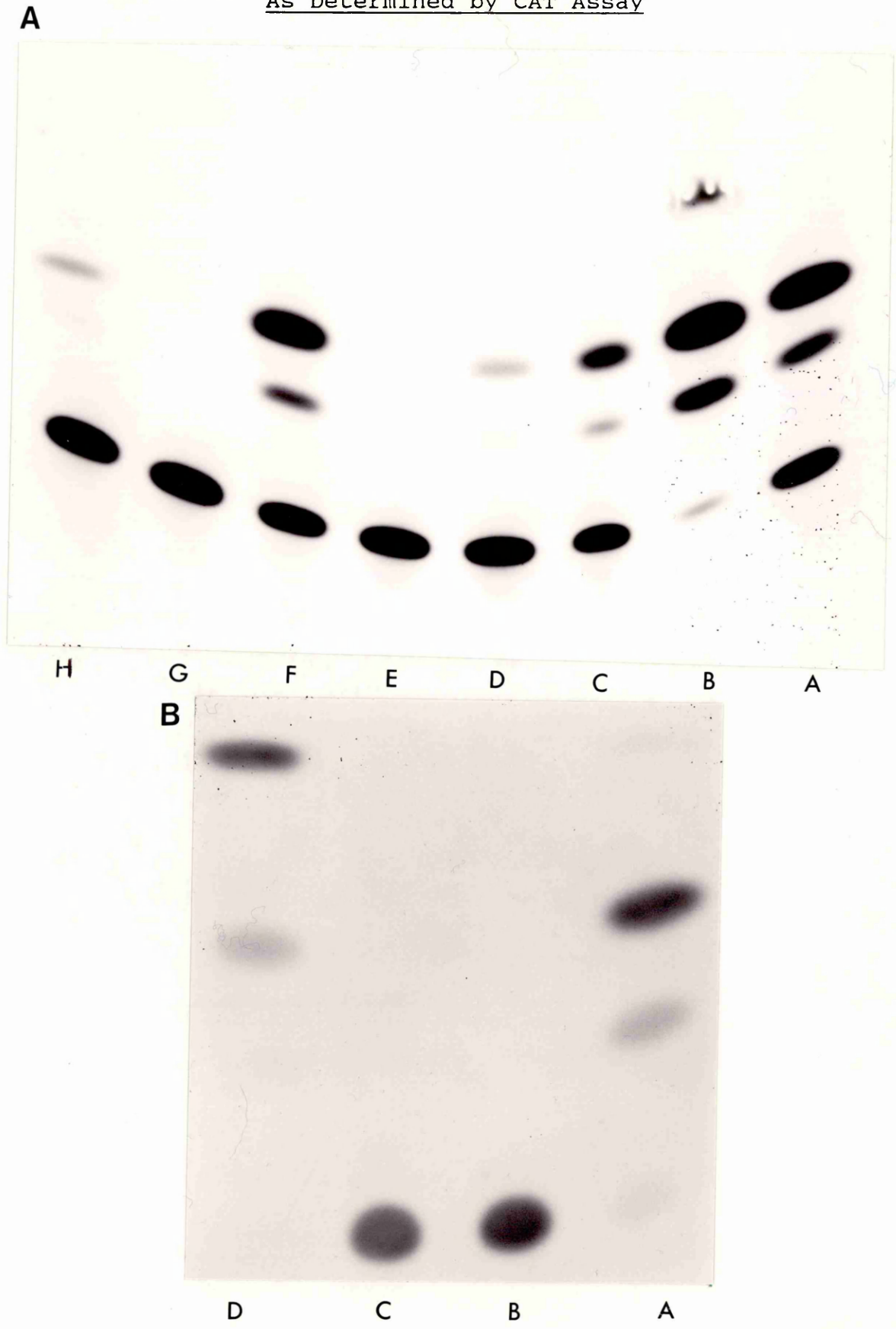
Fig. 5.1 (A) CAT assay to demonstrate the effect of co-transfection with E1a upon the expression of the CAV-2 E4 region. Lanes A and F are positive controls, using the CAT reporter plasmid pSV2/CAT which consists of the CAT gene under the control of the SV40 promoter. Lanes B (Vaxitas) and E (Glasgow) show the result of transfection with the E4 region/CAT constructs alone. Lanes C (Vaxitas) and D (Glasgow) demonstrate the effect of the same clones co-transfected with the Glasgow E1a region.

Transfections of the Glasgow E3 promoter with and without E1a, lanes H and G respectively, are also demonstrated.

(B) CAT assay to demonstrate the effect of the Vaxitas RHT reiterated E1a upon the function of the E4 promoter of Glasgow CAV-2. Lanes B and C show the effect of the Glasgow E4 promoter region transfected with (lane C) and without (lane B) the Vaxitas SalI B clone containing the reiterated E1a. Lanes A and D represent positive controls consisting of A - pSV2/CAT and B - CAT enzyme.

F I G U R E 5.1

Activity Of The Vaxitas and Glasgow E4 Promoters
As Determined by CAT Assay



T A B L E 5.2

CAT Assay Results

	pGE4/CAT	pVE4/CAT	pSV2
pGE4/CAT	-	nd	nd
pVE4/CAT	nd	+	nd
pGE1A	+	+	nd
pVE1A(r)	-	nd	nd
pSV2	nd	nd	+

where nd = not tested

\+ = positive expression of CAT

\- = negative expression of CAT

Table 5.2 Summary of results of CAT assays to examine the effect of the Vaxitas reiterated E1A upon the function of E4. The Glasgow and Vaxitas clones containing the presumptive E4 promoters in conjunction with the CAT gene, pGE4/CAT (Glasgow) and pVE4/CAT (Vaxitas) were transfected into the Mv-1-Lu cell line alone and co-transfected with a plasmid containing a functional E1A gene derived from the Glasgow CAV-2 genome, pGE1A. The Glasgow E4/CAT construct was also co-transfected with a plasmid containing the Vaxitas RHT reiterated E1A, pVE1A(r). The Glasgow E4 promoter is virtually non-functional in the absence of a functional E1A; high levels of CAT expression were however obtained in the presence of the Glasgow E1A. The Glasgow pGE4/CAT shows no CAT expression in the presence of the reiterated E1A of the Vaxitas RHT thus indicating that the reiterated E1A encodes no functional products. High levels of CAT expression can be detected from the Vaxitas E4 clone, pVE4/CAT, both in the presence and in the absence of the Glasgow E1a. The Vaxitas E4 promoter therefore does not require transactivation by E1a.

D I S C U S S I O N

The Glasgow E4 promoter is fully functional only in the presence of the Glasgow E1a gene. No expression was detected either in the absence of E1a or in the presence of the Vaxitas RHT reiterated E1a. Therefore, concurring with the sequence data, the Vaxitas RHT E1a appears to encode no functional products. Expression of the CAT gene in the Vaxitas SmaI/PvuII/CAT reporter plasmid, in the absence of a functional E1a, indicates that, as expected from the DNA sequence, the reiterated E1a at the RHT contains a functional E1a promoter. Assurance that the positive CAT assay result obtained by transfection of the Vaxitas E4/CAT reporter plasmid alone is not the result of an Mv-1-Lu cellular product with E1a-like activity, comes from the negative Glasgow E4/CAT assay results under the same circumstances i.e. the Glasgow E4 promoter would also be transactivated by the same cellular factor.

The first CAT assay experiment, fig. 5.1(a), described the effect of E1a on expression from the the E4 promoter. It should be noted that the amount of DNA used in single transfections and co-transfections varies. When the Glasgow and Vaxitas E4/CAT constructs were transfected alone, 20ug of each plasmid was used. However, when the experiment involved a co-transfection, i.e. pGE4/CAT + pGE1a, pVE4/CAT + pGE1a, 10ug of each plasmid was used. Thus the very strong expression of the Vaxitas E4/CAT clone compared to the less strong Vaxitas E4/CAT+pGE1a co-

transfection CAT assay results, is due partly to a quantitative DNA effect, i.e. half the amount of the CAT gene is present in the co-transfection, and partly to the strength of the E1a promoter.

The Vaxitas E4 promoter appears to be fully functional both in the presence and in the absence of a functional E1a. However, further work will be needed to clarify whether any of the CAT activity detected from the Vaxitas E4 promoter during co-transfection with the Glasgow functional E1a was due to E1a transactivation or whether all CAT activity displayed by this region is due to the reiterated E1a promoter. It is likely that, in the co-transfection, E1a products from the pGE1a clone would compete, by transactivating the E4 promoter, with transcripts running directly from the reiterated E1a promoter to express the CAT gene.

The Vaxitas E4 region therefore does not require transactivation by E1a. The effect of this <u>in vivo</u> however is not clear although it does raise many interesting questions. Handa and Sharp (1984) reported that distal upstream sequences were required for the maximum transcription of E4: deletion of sequences between -325 and -140 resulted in a 10-fold loss of transcription. It is interesting that although the majority of this sequence is still present at the right-hand terminus of Vaxitas, it is now placed more than 1000bp further

upstream of the E4 promoter. Presumably therefore, "normal" transcription of the Vaxitas E4 promoter is reduced compared to transcription of the Glasgow E4 promoter.

The effect of the viral DNA binding protein, encoded by E2a, upon transcription of Vaxitas E4 also remains unclear as yet. Normally E4 transcription is negatively regulated in the presence of the viral DNA binding protein (Raychaudhuri et al, 1987a). Would this negative control of E4 expression still function in the presence of an E4 gene being driven by an E1a promoter? Would the DNA binding protein prevent transcription of E4 leading to abortive transcripts being formed from the reiterated E1a promoter or would E4 continue to be expressed as long as the reiterated E1a promoter remained active? Is transcription through the E4 region of Vaxitas totally dependent upon the reiterated E1a promoter? Does the regulation of the left-hand end E1a, in the presence of excess E1a products, negatively regulate the reiterated E1a promoter at the right-hand terminus thus leading to an E1a dependent regulation of transcription from E4?

If the Vaxitas RHT can contain an E4 region which does not require transactivation by E1a and yet is presumably strongly expressed by way of the E1a promoter, why has the AdV genome evolved to contain such a complicated means of ensuring the expression of each of its early genes? E4 has been demonstrated to be essential for the stability of

late viral nuclear RNA's (Sandler and Ketner, 1989), late viral protein synthesis, viral DNA accumulation (Bridge and Ketner, 1989) and efficient virus particle assembly (Falgout and Ketner, 1987). Functions which are vital to the virus and therefore it would seem, should only enhance the replicative efficiency of Vaxitas. The effect of excess E4 products upon the replication of AdVs has however not been reported. It is possible that an increased replicative efficiency, in the absence of any controlling element, would prove detrimental to the virus. It may lead to a highly cytopathic virus which in turn leads to cell death before completion of viral replication and packaging. The Vaxitas RHT will prove a valuable asset in studying the effect of any excess E4 products in vitro.

GENERAL DISCUSSION

This study was concerned with the genomic analysis of the CAVs in order that the genome organisation could be compared to that of the HuAdVs and that the feasibility of using the CAVs as viral vectors could be examined.

The general organisation of the genome is now known to be similar to that of the human adenoviruses with E1a, hexon, E3, fibre and E4 having been positioned at comparable sites on the genome of the CAVs (Macartney et al, 1988: Spibey and Cavanagh, 1989: Spibey et al, 1989).

Restriction enzyme analysis of both wild-type and vaccine strains and the construction of physical maps of a wild-type and vaccine strain of CAV-2 demonstrated the distinctiveness of CAV-1 and CAV-2. Both CAV-1 and CAV-2 demonstrate a low GC content as defined by the number of SmaI sites (6 SmaI sites in CAV-2, 4 in CAV-1) thus suggesting that they should be placed in an equivalent of group A, highly oncogenic, human adenoviruses. This is in agreement with the published data on the oncogenicity of the canine adenoviruses (Tsukiyama et al, 1988).

In addition, it was revealed that all vaccine strains examined contained additional DNA near the genome termini. This raised the question as to whether the inserted DNA could have any relevance to the attenuation of the CAVs. It also affected the use of the CAVs as viral vectors. The fact that the vaccine strains were competent for growth with up to 4kb of stably integrated additional

DNA, implied that a similar amount of "foreign" DNA could be inserted into a "normal" CAV genome with no adverse affects upon the replication efficiency of the virus. This indicated that the CAVs would be suitable for use as viral vectors.

Conversely, however, the same insertions, although appearing to be stable, have been demonstrated to undergo rapid alterations under certain conditions. When the vaccine strain Vaxitas was introduced into an alternative cell line the previously dominant species of 33.2kb was replaced, in one passage, by a dominant species of 35kb. It is not yet known whether this major alteration in the size of the genome is due to a further insertion of DNA into the genome or whether the new cell line has selected for a species which was previously present only as a limited background species amongst the very heterogeneous species that represents Vaxitas.

The availability of the DNA sequence of the right-hand termini of Glasgow and Vaxitas clearly defined the site of insertion. The Vaxitas reiterated E1a would seem to have "inserted" into the wild-type 80bp HpaII fragment, with the initial 16bp and terminal 35bp of the wild-type HpaII fragment being retained either side of the reiterated E1a. As the initial 16bp conserved sequence forms the end of the ITR, it is more likely that the reiteration has occurred not by insertion into the right-hand end but by

replacement, up to bp229, with the initial 1.353bp of the left-hand end.

The phenomenon of an adenovirus left-hand terminus present at the right-hand end, as in the Vaxitas genome, has been reported previously. Brusca and Chinnadurai (1983) described Ad2 mutants which contained the left 3% (1110bp) of the genome duplicated at the right-hand terminus. However, these mutants were observed only after treatment with an intercalating agent, Proflavin, which typically causes small deletions or insertions (Orgel and Brenner, 1961). The Ad2 mutants were demonstrated to grow 2- to 4-fold slower than their wild-type counterparts. This was hypothesised to be due to a retarded replication of the mutant viral DNA and/or the increased size of the genome causing the virion to be unstable after packaging. In addition, the mutants were found to revert to wild-type after a relatively low number, 5, of passages in vitro. Thus although the type of genome variation is similar, if not identical to that of Vaxitas, the effects and stability of the reiterated E1a are not. The rate of growth and yield of Vaxitas are directly comparable to that of wild-type CAV-2 and after multiple passage, >50, no reversion to wild-type genome was evident.

The vaccine strains Boostervac (CAV-1), Nobivac (CAV-2) and Epivac (CAV-2) appear to differ from Vaxitas in more than one respect. Not only are the inserted DNA sequences of a different origin (BT1, E2/E3 promoter region: BL2,

E1: N3, ITR/E1: E4, unknown) but neither end of the HpaII fragments that appear to encase the inserted sequences demonstrate any homology to any known right-hand terminal sequences. However, this is probably an indication of the similarity in the structure of the right-hand termini and not, as previously thought, indicative of differences. When the final structure of the Vaxitas right-hand end was revealed, it was discovered that the "insertion" contained additional HpaII fragments which were not detected on acrylamide gels. The initial isolation and sequencing of the variant HpaII fragments, 190bp and 600bp, thought to constitute the inserted DNA sequences, revealed only that they were homologous to the Glasgow E1a region. Only by sequencing the entire Vaxitas right-hand terminus was it shown that the inserted sequence contained additional HpaII sites and that reversion to wild-type right-hand terminal sequences occurred past the site of insertion. Thus, until the DNA sequence of the right-hand end of the other vaccine strains can be ascertained, it would be impossible to determine the exact position or mode of insertion of the additional DNA. It will also be necessary to obtain the DNA sequence of the right-hand terminus of a wild-type strain of CAV-1 to enable a comparison with the right-hand terminus of Boostervac CAV-1.

The preliminary investigations into the exact site and effect of the 0.4kb deletion in Vaxitas PstI D, and

smaller deletions in the same area in the other CAV-2 vaccine strains, have shown that the deletion is placed within E4, most probably, using an assumed co-linearity with the human adenovirus E4 region, within the final open reading frame, orf6 by analogy to the HuAdVs. Huang and Hearing (1989) reported that deletion mutants of Ad5 E4 varied in their replicative ability dependent upon where in E4 the deletion occurred. Initially HeLa cells were infected with 200 particles per cell and assayed for plaque forming units per ml (pfu/ml). Those genomes with an intact orf6 and deletions elsewhere, or even the remainder of the E4 orf's deleted, retained a wild-type level of replication. Genomes which were orf6^{-}/orf3^{+} demonstrated a 100-fold drop in yield compared to wild-type, and those which were orf6^{-}/orf3^{-} demonstrated a 10^{4} to 10^{5}-fold drop. When viral DNA accumulation was examined it was discovered that viruses with deletions in orf's 1-4 demonstrated normal viral DNA accumulation. Viruses which also contained deletions or insertions in orf6 exhibited a lag phase of almost 48hrs before the onset of viral DNA replication after which the levels of accumulated viral DNA within the cell were comparable to wild-type. Genomes which were orf6^{-}/orf3^{+} accumulated viral DNA at almost wild-type levels. Orf6^{-}/orf3^{-} mutants display dramatically reduced levels of viral late protein synthesis and virtually fail to switch off host cell protein synthesis, resulting in a 10^{3} to 10^{5}-fold decrease in final virus yield.

These results may be of significance when observing the replicative abilities of the CAV-2 vaccine strains. All the CAV-2 vaccine strains demonstrate a deletion in PstI D, now believed to be in the equivalent of the HuAdV E4 orf6. All of the CAV-2 vaccine strains show a much reduced viral yield compared to wild-type except Vaxitas. Whereas Nobivac and Epivac exhibit a viral concentration of 10^6 pfu/ml, Vaxitas and the wild-type Glasgow repeatedly yield 10^8 to 10^9 pfu/ml. The loss of replicative ability in the Nobivac and Epivac strains can perhaps be explained by the presence of a deletion in E4 orf6. The same deletion however (or a very similar one at the same site on the viral genome) does not appear to affect the replicative ability of Vaxitas. It is feasible that the presence of the reiterated E1a compensates in some way for the proposed deletion in orf6, increasing the yield of orf3 and the other E4 orf's leading to wild-type levels of replication. This would also provide an insight into why a genome so much larger than wild-type has been selected for, is retained as the dominant species within the population and any reversion to a wild-type genome (which without the presence of a wild-type virus would not be able to undergo recombination/repair of E4) within the population is not selected for.

Again, correlation begins to emerge between the function of the additional DNA at the genomic termini of the other CAV vaccine strains. Perhaps enhancement of E4 in order

to compensate for deletions in orf6 may again be applied to the origin and stability of the additional terminal DNA in all the vaccine strains. The five vaccine strains examined display additional DNA comprised of the E1a promoter (Vaxitas CAV-2), the E2 promoter (Boostervac CAV-1, larger species), the ITR and adjacent sequences from the right-hand terminal (Nobivac CAV-2), part of E1b (Boostervac CAV-1, smaller species) and a sequence of unknown origin (Epivac CAV-2). The duplicated E2 promoter present at the right-hand terminal of the larger Boostervac species may again, as with Vaxitas, enhance transcription through E4. It may even remove the requirement of the E4 promoter to be transactivated by E1a products as preliminary results indicate that the CAV E2 promoter does not require transactivation by E1a (N. Spibey, personal communication). Nobivac displays additional DNA upstream of the E4 promoter which would appear to be a direct repeat of part of the ITR (all except the initial 10bp) and adjacent sequences.

Handa and Sharp (1984) reported that Ad5 mutants with deletions in the region -140 to -325 upstream of the E4 promoter displayed a ten-fold decrease in transcription through E4. This same region forms part of the duplicated ITR and adjacent sequences present at the right-hand terminus of Nobivac. Pasananti et al (1987) reported that duplication of enhancer sequences is a common method of gene amplification in mammalian cells brought about by illegitimate recombination events. Perhaps the

duplication of this known E4 enhancer sequence again confers selective advantages to viral genomes with deletions in orf6 by amplifying the remaining E4 gene products. The smaller of the Boostervac CAV-1 species displays an internal part of E1b at its right-hand terminus. No promoter function can be associated with this sequence. It may be however that the sequence contains an enhancer activity or that other additional sequences connected to this region exist at the right-hand terminus of this viral species, again perhaps acting as an E4 enhancer. Epivac CAV-2 contains additional DNA of unknown origin at the right-hand terminus. Sequence analysis to date implies that the additional DNA may be cellular in origin, although as more sequence data becomes available for the CAV-2 genome it may reveal a viral origin. Further analysis of the Epivac CAV-2 and Boostervac CAV-1 (smaller species) right-hand termini will be required to assess the extent of the additional DNA and whether they do infact contain promoter or enhancer sequences.

The different replicative abilities of each strain may again be related to the deletion in E4 and to the presence of the additional terminal DNA. The relative strength of the promoter or enhancer sequences upstream of the E4 promoter would determine the extent to which these additional sequences enhance transcription of the remaining functional E4 orf's. Further examination may

reveal that the replicative abilities of the vaccine strains varies according to the strength of any enhancer present at the right-hand terminal.

The evidence obtained to support the theory that the reiterated E1a of Vaxitas is non-functional again would correlate with the above. E1a is believed to be self-regulatory and therefore it can be assumed that an excess of E1a products very early in infection would be detrimental to the expression of all other early genes in which E1a plays an essential role. A viral genome expressing E1a products at either end simultaneously would lead to vastly increased levels of all E1a products very early in infection. Premature regulation of E1a expression relatively early in expression may lead to decreased levels of expression from all other early genes. Such a genome would have no selective advantage over a wild-type genome and might in fact be almost self-destructive, leading perhaps to less transcription through the other early E1a dependent genes. However, once the replicative error/mutation/recombination which allowed a copy of E1a to form at the right-hand terminus had occurred (especially in an E4 orf6$^-$ mutant), great replicative and therefore selective advantages would be conferred upon any genome in which mutations had occurred within the reiterated E1a, such that no functional proteins were encoded by it, but the promoter remained fully functional leading to increased expression of the remaining E4 orf's. This perhaps explains to some degree

the conservation of the aberrant Vaxitas genome but leads to the question of which came first, the E4 deletion or the reiterated E1a? Theoretical evidence would imply that there is no selective pressure for increased levels of E4 expression in the absence of an E4 orf6 deletion in which case it would be expected that the original mutation/replicative error would have been corrected by recombination with a wild-type genome. The relevance of the reiterated E1a and deletion in E4 orf6 to the attenuated form of the virus, if any, is not clear. However, as the deletion appears to be a consistent part of CAV-2 attenuated vaccine strain genome it is likely that E4 plays some role in the attenuated state which is as yet unknown. The origin of the original CAV strains used for the attenuation of each vaccine is not known. It is feasible that only one attenuated strain, containing the deletion, was obtained from a common source by each vaccine company for utilisation as a vaccine. Subsequent differences in cell lines and production methods may have then given rise to the individual terminal DNA sequences. Ginsberg et al (1987) reported that deletions in E3 led to viral latency in vivo which may confer additional advantages to an attenuated vaccine strain but this function has not been reported for E4 deletion mutants. Paradoxically, the same group also reported that sequence alterations in the Ad5 E1 region led to increased pneumonia in cotton rats. Any correlation between these findings and the Vaxitas aberrant right-hand terminal E1a

will be investigated as the DNA sequence of the left-hand terminal becomes available.

Liu et al (1988) described a vaccine strain of CAV-1 (CCL - Connaught Laboratories Ltd.) which, following eight passes in DK-N (dog kidney) cells, led to the production of 3-4kb deletions at the right-hand terminus. These aberrant genomes were not viable and were isolated only from plaques containing full length genomes in addition to the aberrant genomes. This does indicate however that replicative errors do occur in the CAVs genome under certain conditions and, it would seem, with higher frequency in certain cell lines. It is also interesting to note that the aberrant genome described by Liu et al involved deletions at the right-hand terminus. No aberrant genomes were described with large deletions at the left-hand terminus. The right-hand end would therefore, for some unknown reason, seem to be more prone to replication errors than the left-hand end. This is in agreement with the vaccine strains described in this study where, although both termini exhibit additional DNA sequences, the largest "insertion" in the terminal sequences was always demonstrated at the right-hand end.

In addition, Liu et al (1988) described viable isolates with aberrant genomes from the CCL CAV-1 vaccine strain. The genome length of the viable isolates was found to be 480bp shorter than wild-type CAV-1. Liu et al speculate that this deletion, 83-91m.u., is situated in E3 and is

directly involved in the attenuation of the virus, by which they concluded that E3 is responsible for virulence. The vaccine strain was found to still display the 480bp deletion following passage in dogs. This assumption is not supported by the data produced by Ginsberg et al (1987) who found that deletions in E3 led to latency and may be related to increased viral pathogenicity. It may be, however, that the CAVs are more widely diverged from the human adenoviruses than is presently believed.

However, their assumption that the deletion is based in E3 was solely due to the fact that Tsukiyama et al (1988) reported that the left-hand terminal region of the canine adenoviruses demonstrated transforming ability; presumed due to the presence of E1a at the LHT; and that by alignment of the CAV-1 genome with the human adenoviruses, the deletion occurs in E3. No analysis of the CAV-1 vaccine strain genome was carried out to confirm this speculative position of the deletion.

Analysis of the E3 region of CAV-2 shows that the E3 region of CAV-2 is much smaller than that described for the HuAdVs thus placing the organisation of the right end of the genome out of alignment with the HuAdVs although all regions are conserved i.e. E3->, fibre->, <-E4 (M. Macdonald, University of Glasgow, personal communication). Perhaps the function of E3 in the CAVs is altered somewhat from that of the HuAdVs, hence the requirement for a

smaller E3 region. If the function has indeed altered in some way, the findings of Ginsberg et al (1987), that E3 deletions led to latency in Ad5 infections, may not be applicable to the canine adenoviruses.

The deletion displayed near the right-hand terminal of the Boostervac CAV-1 vaccine strain genome appears to be situated at the same position on the CAV-1 genome as that described by Liu et al and by alignment to the Glasgow and Vaxitas genomes would appear to be situated in E3.

The fact that the deletion described by Liu et al was retained by the CAV-1 vaccine strain following several passages through animals is presumably due to the fact that no wild-type CAV-1 was available in vivo for recombination/repair. Perhaps selective advantages may be conferred upon attenuated strains with the ability for latency. Preliminary results indicate that in the CAV-2 vaccine strains described in this work, the additional DNA sequences/deletions are also stable following passage through dogs (Dr. N. Spibey, personal communication).

No explanation has been found to explain the varying cell specificity of the CAV-2 strains. The wild-type Glasgow used throughout this study was isolated from the faeces of a pup, an unusual site of isolation for a CAV-2 and indicating enteric replication. Vaxitas appears to be unable to replicate in all the canine cell lines examined and most probably is blocked at the stage of entry into

the cell as no intracellular viral DNA has been detected following infection of canine cells with Vaxitas. This would imply that the Vaxitas fibre has a role to play in this cell specificity.

Two differences have been described between the short 3' region of the fibre genes of Glasgow and Vaxitas examined to date which may or may not be involved in this altered cell specificity. However, analysis of the complete fibre genes of Glasgow, Vaxitas and a wild-type respiratory CAV-2 will be required to determine the extent of any alterations. It is not yet known whether the Glasgow isolates aptitude for enteric infection/replication is associated with cell attachment or replicative ability once within the enteric cells. The essential role of E1 in the pathogenesis of adenovirus pneumonia may also have a part to play. Ginsberg et al (1987) reported that no viral pneumonia could be detected in the absence of the E1b 55kD protein. Perhaps a comparison of this gene in Glasgow (as an enteric isolate), Vaxitas (as an attenuated vaccine strain) and a wild-type respiratory CAV-2 would reveal significant differences relating this gene product to pneumonia, perhaps altered in some way to lead to enteric replication and even to attenuation. Preliminary investigations reveal that the E1b 21kD region of Glasgow contains several point mutations when compared to CAV-1. No data is available as yet on the E1b 55kD regions of Glasgow CAV-2 and the wild-type CAV-1.

The relevance of these findings to the vaccination efficiency of the CAV vaccines can only be determined by a detailed analysis of the antibody titres of dogs examined before and after vaccination with each vaccine strain. This would require a vast number of samples for each vaccine in order that valid statistical results could be obtained. Preliminary investigations, however, into the efficiency of Vaxitas have implied that the vaccine is acting as a killed vaccine, as was expected by the inability of the virus to gain entry into canine cells. Details of the preliminary investigation are presented in table 1.

Of a random sample of 30 dogs three exhibited high Ab levels at time of vaccination and were therefore discounted on the basis that high levels of maternal antibody were still present or that the pups had been exposed to wild-type CAV-2 infection. Fourteen either failed to respond to the first inoculation or raised Ab levels of <200 (12 raised Ab levels of <100). When the criteria is taken that an antibody titre of <200 constitutes minimal protection to the animal then 46.7% of the dogs inoculated failed to give an adequate response to the vaccination. Nobivac CAV-2 however, which is fully competent for growth in dog cells, elicits an antibody response of >2000 in >95% of pups (Intervet, unpublished results)

Table 1:- Explanatory Notes

* 1st = i.e. after 1st vaccination, taken at time of second vaccination (usually 4 weeks after initial vaccination)

* 2nd = two weeks after 2nd vaccination

* 3rd = taken at time of 3rd vaccination, usually 1 year after initial vaccination

* 4th = taken two weeks after 3rd vaccination

where:-

+	= <200
++	= <1024
+++	= >1024 but <4000
++++	= >4000
-	= no response or, if titre prior to initial vaccination not known, titre of 16 or less
0	= not known
#	= initial levels probably due to maternal antibody but could be due to infection
?	= most likely due to infection following "vaccination"

! - sample 33 is noted due to the remarkable change in Ab levels. On day of initial vaccination, titre was <32. Six weeks later titre had risen to 5813, three weeks after which it again became <32.

Notes:-
Dog 26 demonstrated good maternal Ab protection which is not demonstrated in the table above. At age 6 weeks the pup exhibited an Ab level of 2048; at 7 weeks this had dropped to 734; on the day of vaccination, the Ab titre was 16 which had risen to 734 when the pup was tested four weeks later. Thus although at a young age good Ab protection against CAV existed, which lowered as the pup became older, the pup did not respond well to vaccination with Vaxitas CAV-2. Unfortunately, further blood tests on this pup were not available.

It is notable that dogs 24 and 25, showing classic Ab levels indicative of wild-type infection, are from the same litter.

TABLE 1

Anitbody Titre Against CAV-2 in Pups Before and After Vaccination with Vaxitas CAV-2

Dog/ Sample	Titre Prior to 1st Vaccination and age in weeks	Immunological Response After Each Vaccination			
		1st*	2nd*	3rd*	4th*
1	0, 10w	16	+	++++	
2	16, 12w	-	-	+	
3	16, 10w	++	++	+++	+++
4	0, 11w	90	++	++	
5	0, 8w	64	+		
6	16, 12w	+++	++		
7	16, 13w	+++	++	++	
? 8	0, 8w	16	++	++++	
9	0, 8w	64	++	+	+
10	0, 10w	24	-	-	
11	16, 12w	+++	++		
# 12	734, 9w	-	++++	+++	
13	0, 12w	4096	++		
14	16, 10w	-	+		
15	64, 8w	-	+++	+	
# 16	5813, 12w	0	11620		
17	16, 11w	+++	+		
18	16, 11w	++	++	++	
19	<16, 12w	++++	+++		
20	48, 9w	-	-	++	
21	<16, 10w	+++	++++		
22	<16, 16w	+++	++		
23	<16, 9w	++++	++		
# 24	16384, 12w	++++	+++		
# 25	11620, 12w	+++	++++		
26	16, 12w	++			
27	<16, 9w	+	+	+	++
28	367, 13w	-	++	+++	
29	32, 11w	++	++		
30	512, 17w	-	-	-	
31	<32, 10w	+			
32	16, 12w	++	++		
! 33	<32, 9w	++++	16		

Several models can be used to explain the presence of a copy of the left end of an adenovirus genome at the right end. Two types of replication are believed to be involved in the adenovirus replicative cycle. Type I replication is characterised by, after initiation, replication proceeding in a continuous fashion along a template strand to the opposite end of the genome and, in the process, displacing the parental strand whose 5′ terminus was at the origin. Type II involves the replication of the displaced parental strand, from type I replication, and can take three forms. Either type II replication might initiate at the 3′ end of the displaced single-stranded molecule or it could begin with the formation of a panhandle structure formed by hybridisation of the ITRs, generating a double-stranded origin of replication. Alternatively, recent results suggest that the single stranded parental DNA may form a circular structure, using the homology between the ITR's, and undergo replication to the double stranded form as a circular molecule (Graham et al, 1989). Circular replication may also be involved during type I replication by utilising strand displacement mechanisms.

If the displaced parental strand of type I replication breaks before type II replication can begin, then the ssDNA would lack an ITR and could not form a panhandle structure. The DNA could however, fold back onto itself at a region of partial homology and continue synthesis using the 5′ half of the same molecule as a template.

Normal type II replication would then generate a double-stranded genome with right-hand terminal sequences missing and duplicated left-hand terminal sequences.

This model, when applied to the Vaxitas genome, would require that the displaced parental l-strand from a "wild-type" genome lacked the final 238bp of the right-hand terminus. The end of this ssDNA would then hybridise to sequences to the right of position 1352 at the left end of the same strand. Replication beginning at the 3′ end would proceed to the 5′end of the left end region which would serve as a template for the new right-hand terminal. This possible mechanism for the occurrence of a reiterated E1a at the right-hand terminus is represented schematically in figure I. However, the sequences in the areas described above demonstrate only 30% homology among the first 40bp.

It is also feasible that strand displacement may occur, figure II, where a partial ssDNA copy with the polymerase still attached becomes displaced from the original parental strand. ITR homology may enable the displaced partially formed ssDNA/polymerase to attach to another parental strand, although not necessarily the same one, and by formation of stem loops, which by bulk alone may result in the non-replication of certain small regions, re-initiate replication. This method may be feasible for both the Vaxitas genome type and the Nobivac genome.

A final mechanism by which aberrant genome termini may arise could be due to physical interference of the type two replication of a ssDNA strand either by a second ssDNA strand or by template switching to the 3′ end of the growing chain during circular replication. This may cause the polymerase to change strands thus completing the type II genome with a second E1a region. This mechanism, represented in figure III, is dependent upon the fact that during type I replication of the adenovirus genome, no strand is preferentially replicated and thus either strand can be present as a displaced parental ssDNA.

The origin of the additional DNA sequences at the left-hand terminus of the vaccine strains, with Vaxitas again displaying the largest variation in size from wild-type left-hand terminus, has yet to be determined. It is possible that the additional left-hand terminal sequences have no relationship to the additional right-hand terminal sequences although it is more likely that they are related. Haj-ahmad and Graham (1986a) described the isolation of Ad5 mutants during the construction of E1/E3 deletion mutants. The mutants, thought to arise during multiple passage of Ad5 in vitro, contained two copies of the ITR at the left-end of the genome. During replication the extra terminal segment, i.e. additional copy of the ITR, was found to be copied again at the right-hand terminus. Thus prior to replication the genome consisted of two ITRs at the left end and one at the right end;

F I G U R E I

Possible Model For Rearrangement of Genome Termini

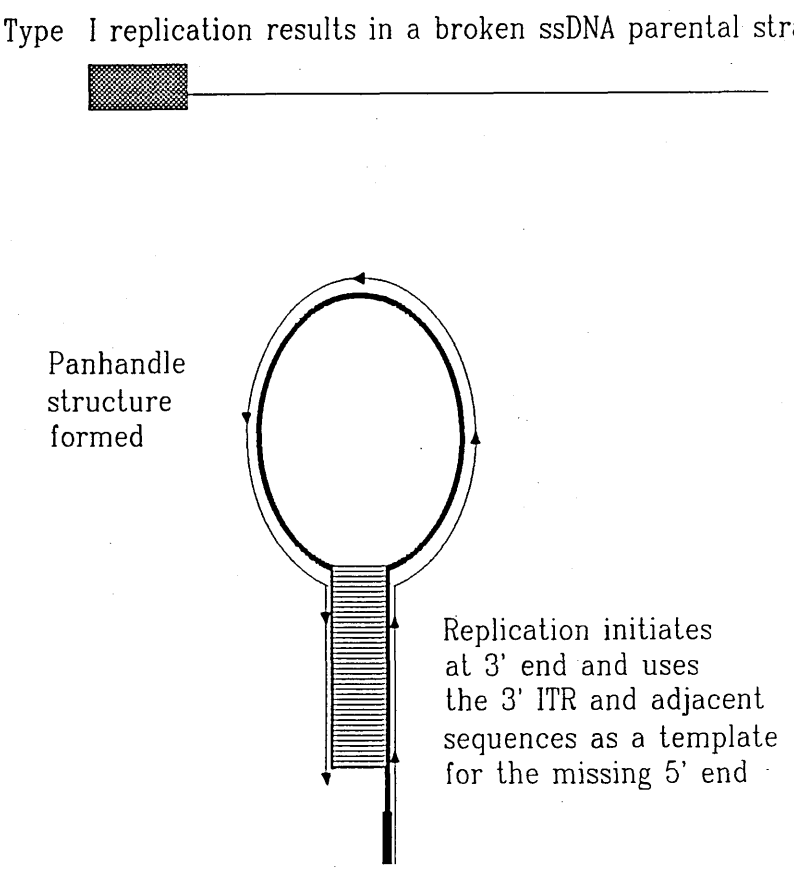


Fig. I Possible mechanism by which ssDNA strand breakage may be responsible for the reiteration of the left-hand terminus ITR/E1a region at the right-hand terminus. Full explanation given in text.

F I G U R E II

Possible Model By Which The Aberrant Genome Termini Of The Vaccine Strains May Arise

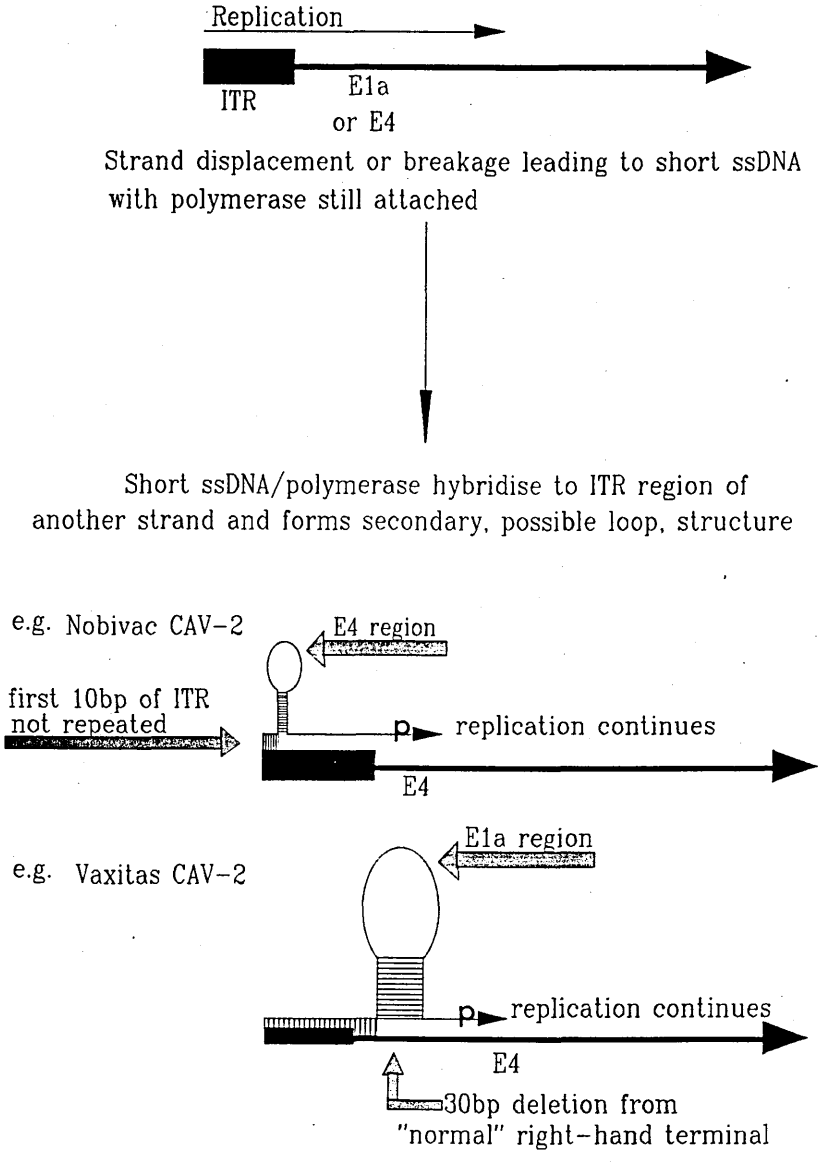


Fig. II Possible mechanism by which a displaced daughter strand, of type I or II replication, with attached polymerase (indicated as P) may be involved in the formation of a reiterated E1a at the right-hand terminus.

F I G U R E III

Alternative Mechanism for the Production of Aberrant Genome Termini During Adenovirus Replication

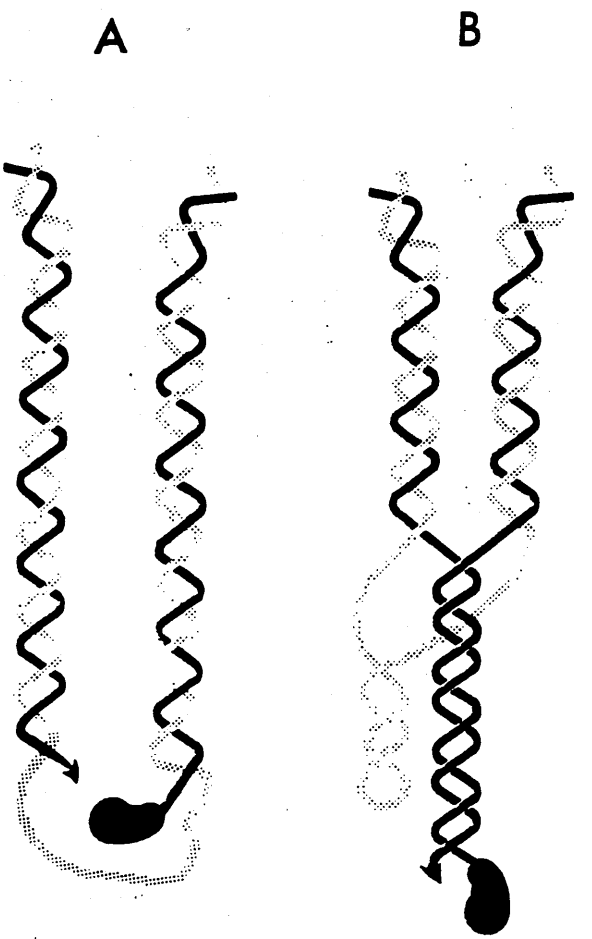


Fig. III A model for replication/aberrant replication from circular molecules. (A) Synthesis has initiated and proceeded all the way round the circular genome, by either strand displacement of by using a ssDNA template, reaching the junction region and termination near the 5′ pTP-blocked end of the newly made strand. (B) Generation of a panhandle. Annealing of newly made ITR sequences, or template switching as the 3′ end of the growing chain progresses down the 'right' ITR, generates a duplex terminus similar in structure to the ends of viral DNA. If the sequences at the 3′ end of the newly synthesised strand are incomplete they can be repaired using the 5′ sequences as a template. Model adapted from Graham et al (1989).

after replication two ITRs were present at both ends.

This may be related to the insertions at both ends of the CAV vaccine strain genome. It may be that, when the left-hand additional DNA sequences are analysed, they reveal strong homology to the additional DNA sequences at the right-hand end. The left end of the Vaxitas genome may exhibit two copies of E1a: Nobivac left end may exhibit two ITRs, one with adjacent E4 sequences and one (internal) with adjacent E1a sequences etc. However, unlike the Ad5 embedded ITRs, the CAVs do not display additional sequences of identical sizes at each end. The additional right-hand terminal DNA has always been larger than the size of any additional left-hand terminal DNA sequences. The appearance of the larger Vaxitas genome, following passage in the new mink cell line Mv-1-Lu, may indicate that the enlarged right-hand terminus has been duplicated at the left end. Restriction enzyme and sequence analysis will be required to solve this puzzle and may in turn clarify the original mechanism by which the aberrant genomes arose.

Salewski et al (1989) described mutants of Ad12 with enhanced replicative abilities which correlated with insertions of various lengths, consisting of adenoviral sequences, at the right end of the viral genome. The mutants demonstrated additional sequences of 180-560bp at the right termini following either prolonged passage in Vero cells or transfection with Ad12 DNA into Vero cells.

The replicative ability of these mutants in Vero cells exceeded their replication levels in HeLa cells, the normal Ad12 replicative host *in vitro*, whereas the activity of wild-type virus in HeLa cells exceeded that in Vero cells by 10^{1}-10^{2}. In addition, the mutants grew in primary African green monkey cells which are totally non-permissive for wild-type Ad12. The additional DNA sequences at the right-hand termini of the mutants were found to consist of either the left-hand terminal and adjacent sequences or a repeat of the right-hand terminal and adjacent sequences. Part of the right-hand terminal ITR was always retained outwith the additional sequences, i.e. 4-11bp of the "original" right-hand terminus still formed the extreme end of the genome. Thus the additional DNA sequences had inserted into the ITR. Continued passage in Vero cells favoured the accumulation of insertions in this part of the viral genome. However, all the replication mutants examined also demonstrated an identical 69bp deletion in the first exon of E1a. Recombinants containing the additional DNA sequences but not the E1a deletion behaved like wild-type virus in Vero cells; those with the deletion but no additional DNA at the genome terminus replicated as well as the mutants containing both the deletion and the insertion in Vero cells. This evidence implied that the enhanced growth potential was due to the deletion in E1a. The deletion in E1a was found to be placed at such a site as to render the mutants transformation defective but fully active in

induction of both viral and cellular genes. No mention was made of the effect of the E1a deletion upon those mutants capable of replicating in African green monkey cells. Perhaps then, further examination may reveal that the CAV vaccine strains deviate from the wild-type CAV E1a sequence, a deviation which has not as yet been detected. The presence of the additional DNA at the left-hand end of the vaccine genomes may be masking any deletion in E1 when the genomic termini are examined by restriction enzyme analysis i.e. the increase in size of fragments near the left-hand terminus hides the presence of a nearby deletion. Further restriction enzyme analysis and sequence data will resolve whether or not there is in fact a deletion in the E1 region of the CAV vaccine strains.

The isolation of the Boostervac CAV-1 vaccine strain has provided a naturally occurring E3 deletion mutant which appears to undergo normal viral replication, albeit at reduced levels. The virus remains competent for growth in canine cells and exhibits an attenuated phenotype. Thus Boostervac has provided not only a deletion mutant for the construction of viral vectors but a virus which can be used to study further the effect of E3 deletions when the virus is introduced into its natural host, a facility not amenable to investigation for ethical reasons in the human adenoviruses. The question of latency induced by E3 deletions can be investigated and any correlation between E3 and pathogenicity examined.

The construction of a right-hand terminal clone of Vaxitas has effectively created a plasmid which appears to express all E4 products, except those of the distal open reading frame, the equivalent of orf6 in the HuAdVs, controlled by the E1a promoter and which does not require transactivation by, or the presence of, a functional copy of E1a. Use of this plasmid will allow a detailed investigation into the function of E4 proteins. In addition, were this plasmid to be introduced into a cell line in which it could be stably maintained it would provide an invaluable asset in the creation and maintenance of E4 deletion mutants for use as viral vectors. The attenuation of the CAV-2 vaccine strains may or may not be related to the deletion present in E4 and the additional DNA at the genomic termini. Evidence suggests, however, that were these attenuated strains altered to be E4$^-$ and a desired protein coding region, e.g. the herpes simplex virus thymidine kinase gene or the rabies virus glycoprotein gene, inserted into the former E4 region, in the presence of the Vaxitas right-hand terminal clone, replication <u>in vitro</u> would be at wild-type levels. Evidence also suggests that when the isolated E4$^-$ recombinant CAV were grown in the absence of E4 products, replication would be much reduced compared to that of wild-type virus, a facility which may be advantageous when the virus is used for vaccination. The recombinant viruses, however, would retain the ability to replicate and, one would hope, their attenuated phenotype.

Inoculation into the host species of the recombinant viruses can now be used to correlate the information gained *in vitro* to that operating *in vivo*.

A P P E N D I X I

Preliminary Investigation of the Area of the Vaxitas Genome Containing the Deletion in PstD

INTRODUCTION

Construction of a physical map of the Vaxitas genome revealed, when compared to wild-type CAV-2, that a deletion of approximately 0.4kb had occurred in the fragment PstD resulting in the loss of a KpnI site. By comparison to the organisation of the human AdV genomes, this deletion should occur in either the fibre gene or in E4.

The fibre of adenoviruses is responsible for the attachment of the virion to cellular receptors and is therefore of major importance in the initiation of infection. Similarly, the fibre structure/composition must help to define the cell specificity of any given strain of AdV and hence, perhaps, plays a role in the pathogenesis of the virus. If the deletion has occurred in the fibre gene, it may help to explain why Vaxitas is non-permissive for growth in the MDCK cell line and yet is permissive in the mink cell lines Mv-1-Lu and NMF which do not support the replication of wild-type CAV-2. It would also reflect upon the function of Vaxitas as a viable live vaccine. If the virus strain cannot gain entry to dog cells which normally facilitate the replication of CAV-2, then how can it do so *in vivo*? The dog however consists of many cell types which are not available *in vitro*. It may be that Vaxitas is capable of replication in dog cells other than the MDCK cell line.

Alternatively, the deletion could be situated within E4. It has been reported that E4 deletion mutants are defective for viral DNA accumulation, late protein synthesis and fail to inhibit host cell protein synthesis (Falgout and Ketner, 1987). The deletion mutants are also defective in the accumulation of late viral mRNA due to the absence of an E4 product which normally stabilises late viral mRNA's (Sandler and Ketner, 1989).

DNA sequencing and the analysis of E4 RNAs have identified several open reading frames (orfs) in this region (Freyer et al, 1984; Sandler and Ketner, 1989; figure A). Three protein products encoded by E4 have been identified immunologically and include a 34kD protein from orf6, an 11kD protein from orf3 and a 19.5kD protein from orf6/7 (Sandler and Ketner, 1989 and references therein). The 34kD protein of orf6 has been shown to be physically associated with the 55kD protein encoded by E1b and the complex is thought to be involved in the transport of late viral messages from the nucleus to the cytoplasm of infected cells. The functions of the 11kD and the 19.5kD proteins are unknown as yet although the 11kD protein is known to be associated with the nuclear matrix. It has also been demonstrated that an unidentified E4 product acts, in conjunction with E1a gene products, in the transactivation of the E2 promoter. The E4 gene product is believed to stabilise the binding of cellular transcription factors, induced by E1a products, to the E2

promoter (J. Nevins, Guest Speaker, MRC Institute of Virology, University of Glasgow, September, 1989). A number of other polypeptides have been associated with E4 but they have not as yet been assigned to specific orf's (Huang and Hearing, 1989).

Bridge and Ketner (1989) have revealed that of the six E4 open reading frames of Ad2 two were interchangeable, orf3 and orf6, i.e. the presence of either would complement for the loss of the other. Deletion mutants of Ad5 E4 demonstrated that the orf6 product provides full E4 function with the products of orf's 1, 2 and 4 contributing very minor functions. Orf6/7 does not appear to contribute to E4 function (Huang and Hearing, 1989). Bridge and Ketner (1989) also revealed that only viral genomes with deletions in both orf6 and orf3 were defective for late protein synthesis and also for plaque formation. It should be noted that while the Vaxitas strain of CAV-2 was grown in the original NMF (normal mink fibroblast) cell line it was not possible to obtain plaques for the isolation of individual genome types: a facility which may be associated with the deletion in PstD.

F I G U R E A

Open Reading Frames Within Ad5 E4

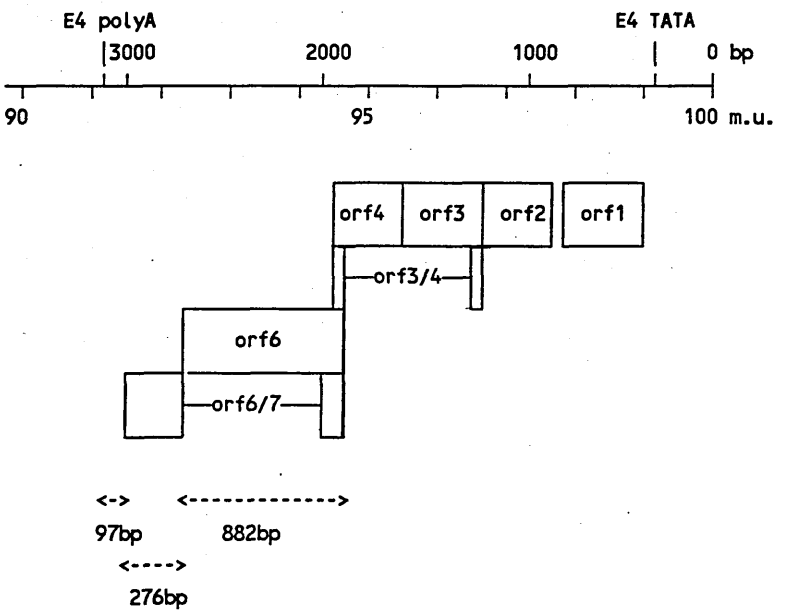


Fig. A Schematic organisation of the open reading frames of region E4 (Adapted from Bridge and Ketner, 1989).

R E S U L T S

DNA Sequence

Figure B represents the sequencing strategy used thus far to ascertain the DNA sequence, of both Glasgow and Vaxitas CAV-2, from the SalI site towards the right-hand terminus i.e. towards the region containing the 0.4kb deletion in Vaxitas SalI B. The sequences were obtained by the use of commercially available and synthesised primers to the bluescript clones of Glasgow and Vaxitas SalI B as indicated. The DNA sequences obtained are shown, in direct alignment, in figure C.

Three base changes and a single base deletion can be seen to occur between the Vaxitas and Glasgow genomes in the 448bp sequence. The sequence data has not yet reached the site of the 0.4kb deletion. The area sequenced thus far however appears, from sequence similarity to the human adenovirus genome, to encode the end of fibre/ end of E4, thus placing the deletion within an E4 coding region. This is demonstrated more clearly in figures D and E.

Figures D and E demonstrate the translation, in all three reading frames, of the above sequences. Both orientations are shown. Presumptive control signals are shown in the appropriate figures.

The effect of the base changes between the two sequences can now be seen. Two of the base changes affect fibre and

one affects E4. The initial base change (27bp from the SalI site); C in Vaxitas, T in Glasgow; produces a termination codon in one reading frame of the Glasgow fibre gene but not in Vaxitas. The second, at 62bp from the SalI site, a T in Vaxitas and a C in Glasgow leads to simple amino acid changes between the two. The effect of the translational stop in the Glasgow fibre gene, not present in the Vaxitas fibre gene, is unknown until splice patterns and coding regions can be established.

The single base deletion 178bp from the SalI site appears to have no effect upon either genome. The base deletion: AAA-AAAA in Vaxitas, AAAgAAAA in Glasgow: is situated between the presumptive fibre polyA addition signal and site thus not affecting transcription of either fibre or E4.

Analysis of DNA Sequence Data.

No similarity could be detected between the DNA sequences obtained from this region of Vaxitas or Glasgow and any other adenovirus DNA sequences of the fibre/E4 region.

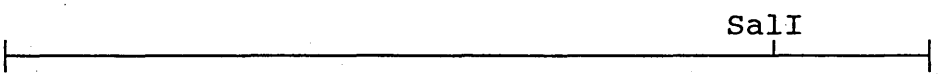
Figure F demonstrates the only similarity found between any of the predicted protein products from the 3'end of the Glasgow and Vaxitas E4 and fibre genes. Both viral predicted E4 regions show a 40.3% similarity to the 3' end of the human Ad2 E4 protein coding region. No significant similarity could be demonstrated against any of the

reading frames from the proposed fibre gene.

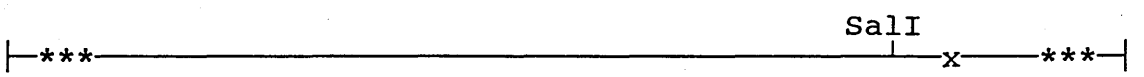
F I G U R E B

Sequencing Strategy for the Region Containing the Vaxitas 0.4Kb Deletion Within the SalI B Fragment

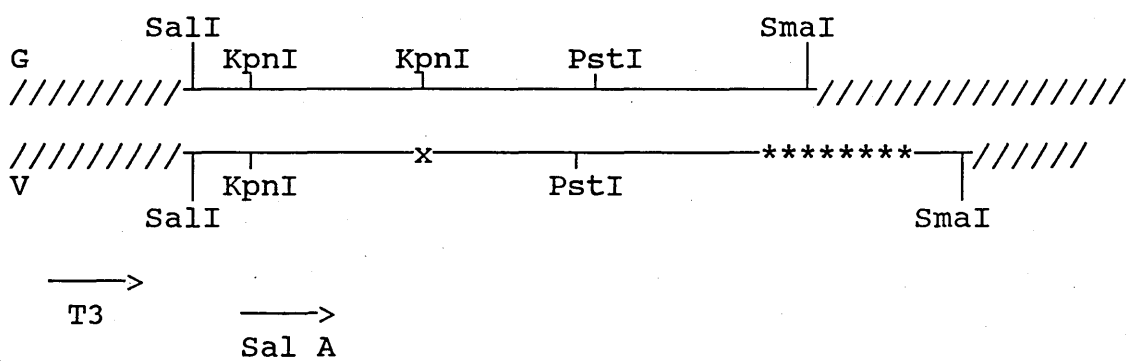
Glasgow genome



Vaxitas genome



SalI B clones in bluescript



Commercial Primers

T3 - 5' ATTAACCCTCACTAAAG 3'

Synthesised Primers

Sal A - 5' AGCTTTATTTTTCATAC 3'

Fig. B Sequencing strategy for the region containing the 0.4kb deletion of Vaxitas CAV-2. Site of deletion, as yet not precisely defined, marked as —x—. Site of Vaxitas insertions marked as —****—. The SalI and SmaI sites mark the insertion points of the terminal SalI B fragments of both viruses into Bluescript.

F I G U R E C

DNA Sequence of the Vaxitas and Glasgow Regions from the Unique SalI Site Towards the Right-hand Terminus

```
VAXITAS
GLASGOW

T3-->
1    GTCGACGGTGCCCCCAGCAGAAGTATCGACTGCATGCTAATTATTAACAAACCAAAAGGC
     ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
1    GTCGACGGTGCCCCCAGCAGAAGTATTGACTGCATGCTAATTATTAACAAACCAAAAGGC
     SalI site                *

61   GTTGCACTTACACCTTACCTTTAGGTTTTTAAACTTTAACAGACTAAGCGGAGGTACCCT
     | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61   GCTGCACTTACACCTTACCTTTAGGTTTTTAAACTTTAACAGACTAAGCGGAGGTACCCT
      *

121  GTTTAAAACTGATGTCTTAACCTTTACCTATGTAGGCGAAAATCAATAAAACAAAAA AA
     |||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
121  GTTTAAAACTGATGTCTTAACCTTTACCTATGTAGGCGAAAATCAATAAAACAAAAAGAA
                                                              *

               SalA Primer ---->
181  ATAAGTTTAAAGCTTTATTTTTCATACACGCGAGCGGTAAGGCTGCCGCCTTCAGGAAAA
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
181  ATAAGTTTAAAGCTTTATTTTTCATACACGCGAGCGGTAAGGCTGCCGCCTTCAGGAAAA


241  GTTACTCTGTAAACAGTTCTTTCACAACAGCACAAAACATAGGTATTAGTTAACAGTTCA
     |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
241  GTTACTCTGTAAACAGTTCTTTCACAACAGCACAAAACATAGGTATTAGTTAACAGATCA
                                                             *

301  TTTGGGCTATAATAATATACATTTTCTTGGGTGGCAAAGCAAGGGTCGGTAATCTCAACA
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
301  TTTGGGCTATAATAATATACATTTTCTTGGGTGGCAAAGCAAGGGTCGGTAATCTCAACA


361  AAACATCACTGGAATGCAAGAATAGTCCAGCAGGTGGGTTCAATCTAAAAATGAAGAACG
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
361  AAACATCACTGGAATGCAAGAATAGTCCAGCAGGTGGGTTCAATCTAAAAATGAAGAACG


421  CGTTGAGGTTCATAAGCAACAGGTTTTG
     |||||||||||||||||||||||||||
421  CGTTGAGGTTCATAAGCAACAGGTTTTG


Matches = 444    Mismatches = 3      Unmatched = 1
Length = 448     Matches/length =  99.1 percent
```

FIGURE D

Translation of Vaxitas DNA from the Region Shown in Figure C

a) 5′-3′ Upper Strand:- Region encoding the end of fibre

```
        10        20        30        40        50        60
GTCGACGGTGCCCCCAGCAGAAGTATCGACTGCATGCTAATTATTAACAAACCAAAAGGC
ValAspGlyAlaProSerArgSerIleAspCysMetLeuIleIleAsnLysProLysGly
 SerThrValProProAlaGluValSerThrAlaCysEndLeuLeuThrAsnGlnLysAl
  ArgArgCysProGlnGlnLysTyrArgLeuHisAlaAsnTyrEndGlnThrLysArgA

        70        80        90       100       110       120
GITGCACTTACACCTTACCTTTAGGTTTTTAAACTTTAACAGACTAAGCGGAGGTACCCT
ValAlaLeuThrProTyrLeuEndValPheLysLeuEndGlnThrLysArgArgTyrPro
aLeuHisLeuHisLeuThrPheArgPheLeuAsnPheAsnArgLeuSerGlyGlyThrLe
rgCysThrTyrThrLeuProLeuGlyPheEndThrLeuThrAspEndAlaGluValProC
                                                 polyA signal fibre
       130       140       150       160     /  170       180
GTTTAAAACTGATGTCTTAACCTTTACCTATGTAGGCGAAAATCAATAAAACCAGAAAAA
ValEndAsnEndCysLeuAsnLeuTyrLeuCysArgArgLysSerIleLysProGluLys
uPheLysThrAspValLeuThrPheThrTyrValGlyGluAsnGlnEndAsnGlnLysLy
ysLeuLysLeuMetSerEndProLeuProMetEndAlaLysIleAsnLysThrArgLysL
polyA  site fibre
/       190       200       210       220       230       240
AATAAGTTTAAAAGCTTTATTTTTCATACACGCGAGCGGTAAGGCTGCCGCCTTCAGGAA
AsnLysPheLysSerPheIlePheHisThrArgGluArgEndGlyCysArgLeuGlnGlu
sIleSerLeuLysAlaLeuPhePheIleHisAlaSerGlyLysAlaAlaAlaPheArgLy
ysEndValEndLysLeuTyrPheSerTyrThrArgAlaValArgLeuProProSerGlyL

       250       260       270       280       290       300
AAGTTACTCTGTAAACAGTTCTTTCACAACAGCACAAAACATAGGTATTAGITAACAGTT
LysLeuLeuCysLysGlnPhePheHisAsnSerThrLysHisArgTyrEndLeuThrVal
sSerTyrSerValAsnSerSerPheThrThrAlaGlnAsnIleGlyIleSerEndGlnPh
ysValThrLeuEndThrValLeuSerGlnGlnHisLysThrEndValLeuValAsnSerS

       310       320       330       340       350       360
CATTTGGGCTATAATAATATACATTTTCTTGGGTGGCAAAGCAAGGGTCGGTAATCTCAA
HisLeuGlyTyrAsnAsnIleHisPheLeuGlyTrpGlnSerLysGlyArgEndSerGln
eIleTrpAlaIleIleIleTyrIlePheLeuGlyGlyLysAlaArgValGlyAsnLeuAs
erPheGlyLeuEndEndTyrThrPheSerTrpValAlaLysGlnGlySerValIleSerT

       370       380       390       400       410       420
CAAAACCATCAACTGGAATGCAAGAATAGTCCAGCACGGTGGGTTCAATCTAAAAATGAA
GlnAsnHisGlnLeuGluCysLysAsnSerProAlaArgTrpValGlnSerLysAsnGlu
nLysThrIleAsnTrpAsnAlaArgIleValGlnHisGlyGlyPheAsnLeuLysMetLy
hrLysProSerThrGlyMetGlnGluEndSerSerThrValGlySerIleEndLysEndA

       430       440       450
GAAACGCGTTGAGGTTCATAAGCAACAGGTTTTG
GluThrArgEndGlySerEndAlaThrGlyPhe
sLysArgValGluValHisLysGlnGlnValLeu
rgAsnAlaLeuArgPheIleSerAsnArgPhe
```

b) 5′-3′ Lower Strand:- Region encoding the end of E4

```
        10         20         30         40         50         60
CAAAACCTGTTGCTTATGAACCTCAACGCGTTTCTTCATTTTTAGATTGAACCCACCGTG
GlnAsnLeuLeuLeuMetAsnLeuAsnAlaPheLeuHisPheEndIleGluProThrVal
 LysThrCysCysLeuEndThrSerThrArgPhePheIlePheArgLeuAsnProProCy
  LysProValAlaTyrGluProGlnArgValSerSerPheLeuAspEndThrHisArgA

        70         80         90        100        110        120
CTGGACTATTCTTGCATTCCAGTTGATGGTTTTGTTGAGATTACCGACCCTTGCTTTGCC
LeuAspTyrSerCysIleProValAspGlyPheValGluIleThrAspProCysPheAla
sTrpThrIleLeuAlaPheGlnLeuMetValLeuLeuArgLeuProThrLeuAlaLeuPr
laGlyLeuPheLeuHisSerSerEndTrpPheCysEndAspTyrArgProLeuLeuCysH

       130        140        150        160        170        180
ACCCAAGAAAATGTATATTATTATAGCCCAAATGAACTGTTAACTAATACCTATGTTTTG
ThrGlnGluAsnValTyrTyrTyrSerProAsnGluLeuLeuThrAsnThrTyrValLeu
oProLysLysMetTyrIleIleIleAlaGlnMetAsnCysEndLeuIleProMetPheCy
isProArgLysCysIleLeuLeuEndProLysEndThrValAsnEndTyrLeuCysPheV

       190        200        210        220        230        240
TGCTGTTGTGAAAGAACTGTTTACAGAGTAACTTTTCCTGAAGGCGGCAGCCTTACCGCT
CysCysCysGluArgThrValTyrArgValThrPheProGluGlyGlySerLeuThrAla
sAlaValValLysGluLeuPheThrGluEndLeuPheLeuLysAlaAlaAlaLeuProLe
alLeuLeuEndLysAsnCysLeuGlnSerAsnPheSerEndArgArgGlnProTyrArgS
            polyA signal E4     polyA site E4
       250   \   260        270  /   280        290        300
CGCGTGTATGAAAAATAAAGCTTTTAAACTTATTTTTTTCTGGTTTTATTGATTTTCGCC
ArgValTyrGluLysEndSerPheEndThrTyrPhePheLeuValLeuLeuIlePheAla
uAlaCysMetLysAsnLysAlaPheLysLeuIlePhePheTrpPheTyrEndPheSerPr
erArgValEndLysIleLysLeuLeuAsnLeuPhePheSerGlyPheIleAspPheArgL

       310        320        330        340        350        360
TACATAGGTAAAGGTTAAGACATCAGTTTTAAACAGGGTACCTCCGCTTAGTCTGTTAAA
TyrIleGlyLysGlyEndAspIleSerPheLysGlnGlyThrSerAlaEndSerValLys
oThrEndValLysValLysThrSerValLeuAsnArgValProProLeuSerLeuLeuLy
euHisArgEndArgLeuArgHisGlnPheEndThrGlyTyrLeuArgLeuValCysEndS

       370        380        390        400        410        420
GTTTAAAAACCTAAAGGTAAGGTGTAAGTGCAACGCCTTTTGGTTTGTTAATAATTAGCA
ValEndLysProLysGlyLysValEndValGlnArgLeuLeuValCysEndEndLeuAla
sPheLysAsnLeuLysValArgCysLysCysAsnAlaPheTrpPheValAsnAsnEndHi
erLeuLysThrEndArgEndGlyValSerAlaThrProPheGlyLeuLeuIleIleSerM

       430        440        450
TGCAGTCGATACTTCTGCTGGGGGCACCGTCGAC
CysSerArgTyrPheCysTrpGlyHisArgArg
sAlaValAspThrSerAlaGlyGlyThrValAsp
etGlnSerIleLeuLeuLeuGlyAlaProSer
```

Fig. D Translation in all three reading frames of the Vaxitas DNA sequence on both strands. Presumptive polyA signals and sites are indicated. All translational stops prior to these are underlined. The three base changes between the above sequence and the Glasgow A sequence of the same region are underlined. Underlined region of E4 reading frame 1 is discussed in fig. F.

FIGURE E

Translation of Glasgow DNA from the Region Shown in Figure C

a) 5′-3′ Upper Strand:- Region encoding the end of fibre

```
          10        20        30        40        50        60
GTCGACGGTGCCCCCAGCAGAAGTATIGACTGCATGCTAATTATTAACAAACCAAAAGGC
ValAspGlyAlaProSerArgSerIleAspCysMetLeuIleIleAsnLysProLysGly
 SerThrValProProAlaGluValLeuThrAlaCysEndLeuLeuThrAsnGlnLysAl
  ArgArgCysProGlnGlnLysTyrEndLeuHisAlaAsnTyrEndGlnThrLysArgA

          70        80        90       100       110       120
GCTGCACTTACACCTTACCTTTAGGTTTTTAAACTTTAACAGACTAAGCGGAGGTACCCT
AlaAlaLeuThrProTyrLeuEndValPheLysLeuEndGlnThrLysArgArgTyrPro
aLeuHisLeuHisLeuThrPheArgPheLeuAsnPheAsnArgLeuSerGlyGlyThrLe
rgCysThrTyrThrLeuProLeuGlyPheEndThrLeuThrAspEndAlaGluValProC
                                                polyA signal fibre
         130       140       150       160     / 170       180
GTTTAAAACTGATGTCTTAACCTTTACCTATGTAGGCGAAAATCAATAAAACCAGAAAAG
ValEndAsnEndCysLeuAsnLeuTyrLeuCysArgArgLysSerIleLysProGluLys
uPheLysThrAspValLeuThrPheThrTyrValGlyGluAsnGlnEndAsnGlnLysAr
ysLeuLysLeuMetSerEndProLeuProMetEndAlaLysIleAsnLysThrArgLysG
polyA site fibre
/        190       200       210       220       230       240
AAATAAGTTTAAAAGCTTTATTTTTCATACACGCGAGCGGTAAGGCTGCCGCCTTCAGGA
LysEndValEndLysLeuTyrPheSerTyrThrArgAlaValArgLeuProProSerGly
gAsnLysPheLysSerPheIlePheHisThrArgGluArgEndGlyCysArgLeuGlnGl
luIleSerLeuLysAlaLeuPhePheIleHisAlaSerGlyLysAlaAlaAlaPheArgL

         250       260       270       280       290       300
AAAGTTACTCTGTAAACAGTTCTTTCACAACAGCACAAAACATAGGTATTAGTTAACAGA
LysValThrLeuEndThrValLeuSerGlnGlnHisLysThrEndValLeuValAsnArg
uLysLeuLeuCysLysGlnPhePheHisAsnSerThrLysHisArgTyrEndLeuThrAs
ysSerTyrSerValAsnSerSerPheThrThrAlaGlnAsnIleGlyIleSerEndGlnI

         310       320       330       340       350       360
TCATTTGGGCTATAATAATATACATTTTCTTGGGTGGCAAAGCAAGGGTCGGTAATCTCA
SerPheGlyLeuEndEndTyrThrPheSerTrpValAlaLysGlnGlySerValIleSer
pHisLeuGlyTyrAsnAsnIleHisPheLeuGlyTrpGlnSerLysGlyArgEndSerGl
leIleTrpAlaIleIleIleTyrIlePheLeuGlyGlyLysAlaArgValGlyAsnLeuA

         370       380       390       400       410       420
ACAAAACCATCAACTGGAATGCAAGAATAGTCCAGCACGGTGGGTTCAATCTAAAAATGA
ThrLysProSerThrGlyMetGlnGluEndSerSerThrValGlySerIleEndLysEnd
nGlnAsnHisGlnLeuGluCysLysAsnSerProAlaArgTrpValGlnSerLysAsnGl
snLysThrIleAsnTrpAsnAlaArgIleValGlnHisGlyGlyPheAsnLeuLysMetL

         430       440       450
AGAAACGCGTTGAGGTTCATAAGCAACAGGTTTTG
ArgAsnAlaLeuArgPheIleSerAsnArgPhe
uGluThrArgEndGlySerEndAlaThrGlyPhe
ysLysArgValGluValHisLysGlnGlnValLeu
```

b) 5′-3′ Lower Strand:- Region encoding the end of E4

```
       10        20        30        40        50        60
CAAAACCTGTTGCTTATGAACCTCAACGCGTTTCTTCATTTTTAGATTGAACCCACCGTG
GlnAsnLeuLeuLeuMetAsnLeuAsnAlaPheLeuHisPheEndIleGluProThrVal
 LysThrCysCysLeuEndThrSerThrArgPhePheIlePheArgLeuAsnProProCy
  LysProValAlaTyrGluProGlnArgValSerSerPheLeuAspEndThrHisArgA

       70        80        90       100       110       120
CTGGACTATTCTTGCATTCCAGTTGATGGTTTTGTTGAGATTACCGACCCTTGCTTTGCC
LeuAspTyrSerCysIleProValAspGlyPheValGluIleThrAspProCysPheAla
sTrpThrIleLeuAlaPheGlnLeuMetValLeuLeuArgLeuProThrLeuAlaLeuPr
laGlyLeuPheLeuHisSerSerEndTrpPheCysEndAspTyrArgProLeuLeuCysH

      130       140       150       160       170       180
ACCCAAGAAAATGTATATTATTATAGCCCAAATGATCTGTTAACTAATACCTATGTTTTG
ThrGlnGluAsnValTyrTyrTyrSerProAsnAspLeuLeuThrAsnThrTyrValLeu
oProLysLysMetTyrIleIleIleAlaGlnMetIleCysEndLeuIleProMetPheCy
isProArgLysCysIleLeuLeuEndProLysEndSerValAsnEndTyrLeuCysPheV

      190       200       210       220       230       240
TGCTGTTGTGAAAGAACTGTTTACAGAGTAACTTTTCCTGAAGGCGGCAGCCTTACCGCT
CysCysCysGluArgThrValTyrArgValThrPheProGluGlyGlySerLeuThrAla
sAlaValValLysGluLeuPheThrGluEndLeuPheLeuLysAlaAlaAlaLeuProLe
alLeuLeuEndLysAsnCysLeuGlnSerAsnPheSerEndArgArgGlnProTyrArgS
          polyA signal E4    polyA site E4
      250   \   260       270/      280       290       300
CGCGTGTATGAAAAATAAAGCTTTTAAACTTATTTCTTTTCTGGTTTTATTGATTTTCGC
ArgValTyrGluLysEndSerPheEndThrTyrPhePheSerGlyPheIleAspPheArg
uAlaCysMetLysAsnLysAlaPheLysLeuIleSerPheLeuValLeuLeuIlePheAl
erArgValEndLysIleLysLeuLeuAsnLeuPheLeuPheTrpPheTyrEndPheSerP

      310       320       330       340       350       360
CTACATAGGTAAAGGTTAAGACATCAGTTTTAAACAGGGTACCTCCGCTTAGTCTGTTAA
LeuHisArgEndArgLeuArgHisGlnPheEndThrGlyTyrLeuArgLeuValCysEnd
aTyrIleGlyLysGlyEndAspIleSerPheLysGlnGlyThrSerAlaEndSerValLy
roThrEndValLysValLysThrSerValLeuAsnArgValProProLeuSerLeuLeuL

      370       380       390       400       410       420
AGTTTAAAAACCTAAAGGTAAGGTGTAAGTGCAGCGCCTTTTGGTTTGTTAATAATTAGC
SerLeuLysThrEndArgEndGlyValSerAlaAlaProPheGlyLeuLeuIleIleSer
sValEndLysProLysGlyLysValEndValGlnArgLeuLeuValCysEndEndLeuAl
ysPheLysAsnLeuLysValArgCysLysCysSerAlaPheTrpPheValAsnAsnEndH

      430       440       450
ATGCAGTCAATACTTCTGCTGGGGGCACCGTCGAC
MetGlnSerIleLeuLeuLeuGlyAlaProSer
aCysSerGlnTyrPheCysTrpGlyHisArgArg
isAlaValAsnThrSerAlaGlyGlyThrValAsp
```

Fig. E Translation in all three reading frames of both strands of the Glasgow DNA sequence encoding the presumed end of E4 and fibre. Presumptive polyA signals and sites are indicated and all transcription termination signals prior to these are underlined. The base changes and single additional base in this sequence compared to the Vaxitas sequence of the same area are underlined. Underlined region of E4 RF1 is discussed in fig. F.

F I G U R E F

Sequence Analysis of Open Reading Frames from the Presumed End of The E4 Region.

```
Ad2 E4
Glasgow E4


949     GlyGluPheIleAsnIleThrAspGluArgLeuAlaArgGlnGluThrValTrpAsnIle
30      Gly   PheValGluIleThrAspProCysPheAlaThrGlnGluAsnValTyrTyrTyr
          *    * *          * * *    *       *    * * *

969     ThrProLysAsnMetSerValThrHisAspMetMetLeuPheLysAlaSerArgGlyGlu
49      SerPro   AsnAspLeuLeuThrAsnThrTyrValLeuCysCysCys         Glu
         *    *    * * *    * * * *    * * * * * *

989     ArgThrValTyrSerValCysTrpGluGlyGlyGlyArgLeuAsnThrArgValLeuEnd
65      ArgThrValTyrArgValThrPheProGluGlyGlySerLeuThrAlaArgValTyrGlu
                  *    * * * *       *    * *       * *

1009    ValEnd    1010
85      LysEnd      86
         *

Matches = 25          Length = 62          Matches/length =  40.3 percent


Ad2 E4
Vaxitas E4


949     GlyGluPheIleAsnIleThrAspGluArgLeuAlaArgGlnGluThrValTrpAsnIle
30      Gly   PheValGluIleThrAspProCysPheAlaThrGlnGluAsnValTyrTyrTyr
          *    * *          * * *    *       *    * * *

969     ThrProLysAsnMetSerValThrHisAspMetMetLeuPheLysAlaSerArgGlyGlu
49      SerPro   AsnGluLeuLeuThrAsnThrTyrValLeuCysCysCys         Glu
         *    *    * * *    * * * *    * * * * * *

989     ArgThrValTyrSerValCysTrpGluGlyGlyGlyArgLeuAsnThrArgValLeuEnd
65      ArgThrValTyrArgValThrPheProGluGlyGlySerLeuThrAlaArgValTyrGlu
                  *    * * * *       *    * *       * *

1009    ValEnd    1010
85      LysEnd      86
         *

Matches = 25          Length = 62          Matches/length =  40.3 percent
```

Fig. F Computer analysis of the similarity between the predicted 3′ end of the Vaxitas and Glasgow E4 regions and the comparable region of Ad2. Each region of the CAV genes demonstrating similarity is underlined in figs. D and E.

D I S C U S S I O N

The complete sequence of the human adenovirus type 2 genome is available (Ginsberg, 1984). The end of the Ad2 E4 region contains a termination codon 97bp upstream of the polyadenylation signal. The polyadenylation site for E4 is shared, on the opposite strand, as the polyadenylation site for fibre.

Consistent with Ad2, both strains of CAV-2 appear to demonstrate polyA sites which serve for both E4 and fibre. A termination codon is apparent 95bp upstream of the proposed E4 polyA signal in reading frame three, although this is preceded by termination codons in all three reading frames. Further analysis will be required to confirm that the proposed polyA signals and sites are indeed those for fibre and E4 and, using mRNA analysis and S_1 mapping, the extent of any similarity between the organisation of the canine adenovirus fibre/E4 regions and those of the human adenovirus genomes.

Two base changes can be detected when the 3' end of the Glasgow fibre gene is compared to the same region of the Vaxitas genome. One base change introduces a translational stop in one reading frame of Glasgow: Vaxitas - Arg, Glasgow - End: and an amino acid change in another: Vaxitas - Ser, Glasgow - Leu. The second base change introduces an amino acid change in one reading frame only: Vaxitas - Val, Glasgow - Ala. The

significance of these differences cannot be ascertained until the coding regions and splice sites, if any, of the fibre gene can be determined. The human adenovirus type 2 fibre gene contains no splice sites between the initiation codon and the termination codon. Slight differences between the fibre genes of Glasgow and Vaxitas could result in the different binding capacities to various cell types and hence may determine to some degree the pathogenicity of the virus to certain cells.

By alignment with the Ad2 genome, it would appear that the 0.4kb deletion, present in the Vaxitas E4 region, is situated in the equivalent of Ad2 E4 orf6 (fig. A). This presumption is supported by the evidence of Bridge and Ketner (1989) who reported that the presence of a functional orf3 or an orf6 could compensate for the loss of the other. The absence of both orf3 and orf6 results in a 160-fold reduction in late protein synthesis, whereas a functional orf3 and/or a functional orf6 results in normal late protein synthesis and accumulation. Their results indicate that the viral phenotype would remain wild-type as long as one of these two orf's was fully functional and would display no loss of replicating ability.

Huang and Hearing (1989) revealed however, that the product of orf6 is more efficient in the regulation of viral late protein synthesis, shutoff of host cell protein synthesis, the production of infectious virus and the

augmentation of viral DNA replication than is the product of orf3. Therefore the assumption is, that although normal phenotype may be retained by mutants defective in orf6, due to compensation by orf3, they are not as efficient in the above functions as genomes encoding both orf's.

When the above data is applied to the Vaxitas genome it may provide an insight as to the selection and retention of mutant genomes encoding a reiterated E1a at the right-hand terminus. The reiterated E1a effectively increases the levels of expression of all E4 orf's including orf3. It may be possible that increased amounts of the orf3 product are advantageous in the absence of orf6, complementing for the loss of orf6 to an even greater degree. The genome containing the reiterated E1a may therefor display greater selective/replicative advantages over an E4 orf6$^-$ mutant which does not contain the reiterated functional E1a promoter at the right-hand terminus to drive the E4 promoter at greatly increased levels.

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