THE DIAGNOSIS OF ADENOVIRUS IN GASTROENTERITIS BY DNA HYBRIDIZATION

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by

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This work is dedicated to my parents who taught me that perseverance is a large measure of all achievement.

ABSTRACT

The purpose of the project was to develop a DNA hybridization test for the detection of enteric adenovirus species Ad40 and Ad41, in particular, and to examine the involvement of these and other species in the local aetiology of gastroenteritis. The initial hypothesis was that the gene coding for internal hexon antigens shared by all genus Mastadenovirus species and the serotypic epitopes on the external surface of the hexon would supply probe sequences for both the detection of all adenoviruses and the specific detection of individual species from the single hexon gene. This hypothesis was tested by the examination of the adenovirus genome for conserved sequences for use as a hybridization probe capable of detection of all human species. Adenovirus DNA from prototype strains representing each subgenus of human species were prepared. Several improvements on fastidious adenovirus culture and DNA extraction were made during this process. A cocultivation technique, forcing infected and fresh cells into close contact for viral transmission, gave an improvement in yield of several log dilutions of enteric adenoviruses. Hybridization with genomic Ad41 DNA probes under stringent conditions showed a consistent pattern of gene conservation in the genome of each species from all the subgenera. Three principle areas, most closely defined on the Ad2 genome as sequences transcribing the pIVa2 scaffold protein gene and part of the DNA polymerase between 11.4 and 16.1 map units, the C terminal half of the terminal protein gene between 27.4 and 29.7 map units, and the protein V, precursor protein VI, the hexon gene and the 23K protease, contiguous genes in the center of the genome from 46.5 to 65.2 map units were found most conserved. These genes are involved in replication or

in early capsid formation in intimate association with the hexon. The hexon gene, which hybridized most strongly of the Ad2 sequences with Ad41 DNA and detected the DNA of other subgenera evenly, constituted the best common probe for detection of all human adenovirus species. Various cloning strategies failed to isolate sequences of the Ad41 genome which detected solely Ad41 DNA in hybridization. Characterization of the cloned plasmids demonstrated that the present version of some Ad41 restriction maps were misaligned, and *Bam*HI, *Hin*dIII, *Pst*I and *Sma*I maps of the prototype strain Tak of Ad41 were reformulated and the *Bgl*II map newly presented. In this process a novel method using exonuclease III to sequentially digest fragments was established to determine problematic terminal fragments. Cloned plasmids containing the *Eco*RI B or C or the *Bgl*II D fragments detected both subgenus F species Ad40 and Ad41 very specifically. Synthetic oligomers from species specific sequences of the hexon gene were manufactured from hexon gene sequences for specific detection of Ad41 and evaluated for sensitivity and specificity in comparison to other hybridization probes and other diagnostic tests against 200 clinical stool samples. Hybridization with the Ad2 hexon gene probe, allowed to develop for up to one week, was much more sensitive than electron microscopy or enzyme immunoassay or tissue culture in the detection of all species of adenovirus, with a 92.7% sensitivity compared to 54.6%, 61.8% or 45.5% respectively. The most sensitive of the Ad41 DNA probes were the *Bgl*II D and Hex5B synthetic probes which detected 92.5% and 85.7% of known subgenus F and Ad41 specimens respectively, better than the subgenus F specific enzyme immunoassay with 84.6% sensitivity. The hybridization test had advantages beside improved sensitivity over

other tests in that it enabled detection of more than one adenovirus agent in faecal specimens and allowed an estimation of the quantity of virus present in each specimen.

Restriction analysis of adenovirus isolates from patients in Manitoba over the last five years demonstrated several trends that indicate that the aetiology of adenoviral gastroenteritis is constantly evolving. The prevalence of Ad40 in Manitoba has fallen dramatically since 1980 to 1983 when a similar local survey was carried out. The proportion of genomic variant strains of non-enteric species that were not neutralized by specific National Institute of Health antisera to these species has steadily increased. The most prevalent strain of adenovirus in paediatric gastroenteritis in Manitoba was determined to be a genomic variant of species Ad41 with restriction site differences from the prototype strain with enzymes *Bam*HI, *Cla*I, *Hin*dIII and *Sma* I. This strain has been increasing in prevalence and in recent years has alone accounted for over a third of the total adenovirus isolates. The restriction site differences from the prototype strain were mapped to either the hexon or fiber genes which are both neutralizable and therefore under immunological selection pressure to vary. This prevalent variant strain was not detected by the first commercial monoclonal antibody enzyme immunoassay to be marketed. These observations suggest that immunological selection of variant strains will tend to nullify the long-term efficacy of highly specific serological detection methods. DNA hybridization tests, less affected by single point mutations in target sequences, may be preferable for continued diagnosis of rapidly evolving etiological agents of gastroenteritis.

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ABBREVIATIONS

dH_20	= distilled water
Na	= sodium
K	= potassium
HCl	= hydrochloric acid
EDTA	= ethylenediamine tetraacetic acid
μl	= microlitre, litre $x10^{-6}$
ng	= nanogram, gram $x10^{-9}$
pg	= picogram, gram $x10^{-12}$
pM	= picomole, mole $x10^{-12}$
mM	= millimole, mole $x10^{-3}$
d	= dalton
μg	= microgram, gram $x10^{-6}$
ml	= millilitre
bp	= base pair
mol. wt.	= molecular weight
mCi	= millicurie
mBq	= millibequerel
CPE	= cytopathic effect

INTRODUCTION

1. Adenoviruses, Discovery and Significance.

Adenoviruses were first documented 39 years ago as a cause of degeneration of experimental adenoid cell culture (Rowe *et al.*, 1953). At the same time similar viruses were established as the primary agent in acute respiratory disease (ARD) (Hillman and Werner, 1954), a major cause of morbidity in military recruits (Commission on Acute Respiratory Disease, 1947). The relatedness of these agents with agents isolated from conjunctivitis and pharyngitis (Parrott *et al.*, 1954) and skin inflammation (Neva and Enders, 1954) was quickly demonstrated (Huebner *et al.*, 1954). In retrospect, descriptions of ARD can be found dating back more than a hundred years (Dudding *et al.*, 1973). Adenoviruses have also been linked to epidemic keratoconjunctivitis which caused over 10,000 cases of eye infection in Pearl Harbour in the summer of 1941 (Jawetz, 1959) and may have been first described in the 1880s by Fuchs (1889). These viruses were variously called adenoid degeneration, acute respiratory disease and adenoid-pharyngeal conjunctival agents, the current nomenclature was adopted in 1956 (Enders, 1956). Adenovirus taxonomy has since progressed further than any other group of viruses. At the fourth meeting of the International Committee on Viral Taxonomy (ICVT) in 1976 the 34 adenovirus serotypes then known were designated species in the genus Mastadenovirus (Norrby, 1976). The term species has not gained wide acceptance in virology, type being preferred in referring to varieties of adenovirus. However, to adhere to scientific accuracy, species will be used in this volume. The family Adenoviridae contains one other genus, that of Aviadenovirus which consists of fourteen

species described from chickens, ducks, turkeys, geese and pheasants distinguished by a pair of fibers projecting from each apex (Wigand *et al.*, 1982). Mastadenoviruses include a number of species in a range of animals including dogs, mice, cattle, pigs, sheep, monkeys and the range is growing as more exotic animals are examined (Gibson *et al.*, 1990). The length of the fiber in different species is highly variable (Wadell, 1979) but this feature is rarely preserved in fixed preparations and all the human adenoviruses have an identical appearance under the electron microscope. Mastadenoviruses also share a common antigen on the internal aspect of the major capsid component, the hexon. Antisera to disrupted virus or purified hexon capsomeres cross-reacts with all Mastadenovirus species and forms an important characteristic to distinguish the two genera.

With the increase in adenovirus serotypes to the current 47 human species, additional clinical syndromes have been associated with adenovirus infection. Most notably, adenoviruses cause outbreaks of pharyngoconjunctival fever, usually at lakeside camp-sites in summer (Bell *et al.*, 1955) and acute haemorrhagic cystitis, a self limiting infection of the urinary tract (Numazaki *et al.*, 1973). Additionally, adenoviruses have been implicated in a number of important syndromes such as intussusception (Clarke, Philips and Alexander, 1969; Gardner *et al.*, 1962; Ross, Potter and Zachary, 1962), pertussis (Connor, 1970; Klenk, Gwaltney and Bass, 1972), and infection of immunocompromised transplant recipients (Hierholzer, Atuk and Gwaltney, 1975; Stolder, Hierholzer and Oxman, 1977; Zahradnik, Spencer and Porter, 1980). Adenoviruses are very widespread, usually as causes of paediatric enteritis and colds.

Extensive surveys of North American and European children suggest that adenoviruses are responsible for about 10% of juvenile pneumonias and lower respiratory illnesses (Brandt *et al.*, 1969; Foy *et al.*, 1973). Channock (1974) estimated from serological prevalence studies, allowing for the fact that only 45% (Fox *et al.*, 1969) or 50% (Brandt *et al.*, 1972) of adenoviral infections develop into illness, that the average individual undergoes a minimum of two or three clinical episodes of adenovirus infection during childhood.

In trying to establish laboratory hosts for the new adenovirus species it was discovered early on that Ad12 and Ad18 (Huebner, Rowe and Lane, 1962; Trentin *et al.*, 1962) could cause tumours in rodents. Subsequent studies have shown that several other species, including Ad31, Ad3, Ad7, Ad11, Ad14, Ad16, Ad21 can induce similar sarcomas and that newborn rats and mice as well as hamsters are susceptible. As is the case with polyoma virus and SV40, infectious virus is not detectable and tumours have not been reliably associated with permissive hosts. While adenoviruses almost certainly do not cause malignancies in humans, these were the first descriptions of the oncogenic capacity of human viruses and the discovery led to an intensification of research and to many advances in molecular biology. Calcium phosphate coprecipitation, an important technique that allows introduction of DNA fragments into cells, was developed to demonstrate cellular transformation by adenoviruses (Graham and van der Eb, 1973). Splicing was first observed when Ad2 mRNA was examined in the electron microscope (Berget, Moore and Sharp, 1977; Chow *et al.*, 1977). Hybrid arrested translation and S1 nuclease digestion are among a number of methods first evolved to localize mRNAs on

the adenovirus genome (Berk and Sharp, 1979; Ricciardi, Miller and Roberts, 1979). The establishment of eukaryotic DNA replication *in vitro* with extracts of Ad2 infected cells resulted in the definition of both viral and cellular factors for transcription (Challberg and Kelly, 1979). Adenovirus emerged as an early model of eukaryotic molecular biology and extensive knowledge of the viral structure and function has been accumulated.

2. Structure of Adenoviruses

Mastadenoviruses are non-enveloped particles 60-80 nanometres in diameter in the shape of an icosahedron, having 20 triangular surfaces and 12 vertices. The majority of the outer shell or capsid is composed of 240 "hexon" subunits, each surrounded by six identical subunits. At each vertex is a five sided "penton" from which projects a single "fiber" thought to make initial cell contact. The capsid consist of at least 10, perhaps 12 polypeptide components, numbered according to their order of migration in sodium dodecyl sulphate containing polyacrylamide (SDS-PAGE) gels (Maizel, White and Scharff, 1968B). The hexon integrates with four polypeptides in the formation of the early intermediate capsid, polypeptide IX bridges between hexon monomers, polypeptide IV is found on the inner aspect of the outer capsid and polypeptide IIIa is positioned between the hexon and the penton. The integration of the hexon with its bridging proteins has been the subject of some highly sophisticated x-ray analysis (Burnett, 1987) and has similarities with some of the simpler and most studied viruses, including influenza and polioviruses (Hogle, Chow and Filman, 1985; Rossman *et al.*, 1985) which may indicate the sharing of an ancestral gene. The hexon capsomere is a trimer of three identical proteins linked by non-covalent bonds (Horowitz, Maizel and Scharff, 1970). Formation

of the trimer requires the intervention of a 100K scaffold protein (Cepko and Sharp, 1983; Oosterom-Dragon and Ginsberg, 1981). The adenovirus hexon was the first viral protein to be crystallized (Periera, Valentine and Russel, 1968) and a three dimensional map charting the length of the entire protein strand (Roberts *et al.*, 1986) has been formulated by x-ray analysis. Simplistically, the monomer consists of two semicircular shells (P1 and P2), composed of anti-parallel, beta-pleated sheets from which extend three epitopic loops (L1, L2 and L4) with the L3 loop forming an internal, stabilizing bridge between the two shells, and a free amino-terminal tail which links with adjacent capsomeres.

The outer portion of the capsid can be stripped from the virion by mild disruption with acetone (Laver, Suriana and Green, 1967) or urea (Maizel, White and Scharff, 1968A) to release four more viral proteins associated with the genomic core. The function and position of protein u inside the viral core is unknown. This protein is absent from protease deficient mutants (Farber and Baum, 1978) and is surmised to be a cleavage product, equating with protein X of SDS PAGE gels (Mizra and Weber, 1982). The second protein released from the core is the 55K terminal protein covalently linked to the 5' cytidine residue at both ends of the DNA genome (Rekosh *et al.*, 1977). The terminal proteins can interact non-covalently to form circles of the DNA genome (Robinson, Younghusband and Bellet, 1973) and may act as the orientation site for the 140K viral DNA polymerase in replication (Lichy *et al.*, 1983). The terminal protein is not completely removed by extensive proteolysis (Carusi, 1977; Roninson and Padmanabhan, 1980) and forms an obstruction to cloning of the terminal fragments that can only be

circumvented by sequential treatment with exonuclease III, S1 nuclease and Klenow polymerase (Bos *et al.*, 1981; Graham *et al.*, 1974; McKinnon *et al.*, 1982) or by piperidine treatment (Tokunga *et al.*, 1986) followed by poly A tailing (Alestrom *et al.*, 1982; Stenlund *et al.*, 1980) or ligation of linkers. The capacity of the terminal protein to prevent attached DNA fragments from entering agarose gels has been used to identify terminal fragments in restriction enzyme mapping (Kitchingman, 1982).

The two remaining core proteins are responsible for the organization of the DNA within the virus particle. Protein VII is the most numerous and is clustered as three dimers in a nucleosome-like structure associated with approximately 150 base pairs of coiled DNA (Mizra and Weber, 1982). The six molecule protein VII complex does not seem to be positioned regularly on the DNA duplex (Corden, Engelking and Pearson, 1976) but loops out a range of DNA lengths, averaging about 200 base pairs. Each complex of protein VII is associated with one molecule of protein V which occupies a position equivalent to histone H1 on chromatin nucleosomes. Protein V also binds to the penton and this linkage between the DNA winding protein VII complex and the outer capsid could have important implications in the packaging of the virion DNA (Everitt, Lutter and Philipson, 1975). A very delicate technique, using a bombardment of argon ions to etch away the capsid, produced a view of the inner core of Ad2 that could extend this observation. Newcomb, Boring and Brown (1984) found the interior of an adenovirus particle to consist of twelve spheres, filling the core, one directly beneath each vertex, a level of organisation probably brought about by the dual binding capacities of the two core proteins.

3. The Adenovirus Genome, Its Products and Replication.

The genome of Mastadenoviruses is a linear, duplex DNA molecule. The nucleotide code of the entire Ad2 genome of 35,937 +/- 9 base pairs has been compiled from the overlapping sequences of various authors (Akusjarvi and Pettersson, 1979; Akusjarvi *et al.*, 1984; Alestrom *et al.*, 1984; Gingeras *et al.*, 1982; Herisse, Courtois, and Galibert, 1980; Herisse *et al.*, 1981; Kruijer, Shaik and Sussenbach, 1982; Roberts, O'Neill and Yen, 1984; Shinagawa, Padmanabhan and Padmanabhan, 1980; van Ormondt and Galibert, 1984) and is available from GenBank DataBase Systems. There is an ambiguity of 18 base pairs due to two regions of heterogeneity (at map units 39.14 and 95.60) between stocks of prototype virus held in different laboratories. The nucleotide code can be manipulated by current software to generate restriction enzyme maps accurate to the exact base pair from the enzyme recognition sequences. A useful array of Ad2 maps, for virtually all common restriction enzymes, has been presented in the second edition of the semi-annual series Genetic Maps (1984).

The position of genes on the adenovirus genome was elucidated in studies that pioneered the use of transcript mapping. Heteroduplexes of DNA and mRNA from the nucleus or from cellular cytoplasm were viewed in the electron microscope to identify the position of genes or splice junctions of introns (Chow and Broker, 1981). The lengths of Ad2 exons and introns were evaluated from alkaline electrophoresis gels after digestion with exonuclease VII or S1 nuclease (Berk and Sharp, 1977). Four families of "early" genes, E1 to E4, producing a range of spliced transcripts from individual promoters early in infection were mapped (reviewed by Persson and Philipson, 1982) at diverse positions

on both the L and R strands. The production of adenovirus mRNA appears to proceed as a cascade of transcripts from "delayed early" genes EIB to E4 initiated by an "pre-early" EIA gene product (Lewis and Mathews, 1980). The increase in viral transcription rate with the addition of translation inhibitors (Craig and Raskas, 1974) suggests that a cellular protein repressor normally inhibits viral gene expression. Complementation of E1A defective adenovirus by the completely unrelated pseudorabies virus (Feldman, Imperiale and Nevins, 1982) indicates that a common cellular control factor is inactivated in infection with both viruses. There are, however, two mRNA transcript species from the first of the five "late" families of genes that are independent of the E1A effect and are still detected in the presence of inhibitors of protein synthesis (Lewis and Mathews, 1980). These transcripts, relabelled "immediate early", debase the absolute need for viral products to enable viral transcription. The progression of transcription from pre-early to delayed early to late genes forms a pattern similar to many other virus groups, the independence of the immediate early genes from the E1A initiation of transcription is unusual.

The E1A gene produces at least six post-translationally modified nuclear phosphoproteins from two primary products of 243 and 289 amino acids of two overlapping mRNAs that sediment at 12 and 13S. These products are referred to by their length rather than molecular weight as the degree of phosphorylation (Yee and Branton, 1985) and possibly the high content of glutamic acid, which prevents SDS binding and results in a slower migration rate in electrophoresis (Spindler and Berk, 1984), cause heterogenous banding pattern in SDS polyacrylamide gels. Two further mRNAs of 10 and 11S, to which no

functions have yet been assigned, have also been detected (Stephens and Harlow, 1987). A 9S mRNA is transcribed in a different reading frame from an independent promoter at late times and encodes polypeptide IX, a structural component of the adenovirus capsid (Boulanger *et al.*, 1989).

Sequence comparison of various adenovirus types has identified three conserved domains and a nuclear targeting sequence in the E1A gene (Kimelman *et al.*, 1985). The first two conserved domains occur in both the 289 and 243 products, while the third domain of 46 amino acids is confined to the 13S transcript by differential splicing. Topological definition of the functional regions of E1A proteins has been discerned by point mutation, deletion analysis, synthetic peptides, and anti-peptide sera (Boulanger and Blair, 1991) and shows the activity to map to the conserved domains. Changes to the first domain affect the ability of the E1A proteins to stimulate DNA synthesis and activate certain cellular genes and to immortalize cells (Smith and Ziff, 1988). A repressor function for other viruses, some cellular genes and E1A self regulation, mediated via enhancer elements, involves both the first and second domains (Schneider et al., 1987).

The second conserved domain consists of a series of acidic amino acids, residues 121 to 136, following a serine. The same motif is present in myc oncoprotein, a variety of oncogenic papovaviruses and SV40 T antigen where it is essential for transformation. This suggests that the motif is utilized by a number of oncoproteins of disparate origin in a common biological function, a hypothesis supported when a chimera, created by the substitution of domain 2 in the 243 amino acid protein with the SV40 motif, retained all

the transforming properties of the wild type protein (Moran, 1988). The motif constitutes a recognition site for cellular casein kinase II (Kuenzel *et al.*, 1987). Casein kinase II is active in S phase with the cell cycle and is thought to phosphorylate oncoproteins to their active dimeric forms (Robertson, 1988).

The transactivating properties of the E1A gene localize to the third conserved domain unique to the 289 amino acid protein (Moran *et al.*, 1986). The E1A products have not been found to bind directly to any consensus DNA sequence (Ferguson *et al.*, 1985) implying that the E1A proteins act by inducing cellular factors which then activate the other early viral promoters. A variety of cellular proteins, ATF, E2F, E2A-EF, E4F1, E3F1, E3F2, E3F4 (Hurst and Jones, 1987; Lee and Green, 1987; Reichel, Kovesdi and Nevins 1988; SivaRaman, Subramanian and Thimmappaya, 1986) have been described which attach to various sequences in some but not all of the viral early promoters, indicating that a number of factors with diverse targets may be mobilized in the infected cell. The induction of these factors is independent of host protein synthesis and transactivation probably results from post-translational modification of host promoter-binding proteins. This supposition was supported by the finding of a second form of the polymerase III transcription factor TFIIIC that bound well to the VA promoter in HeLa cells transcribing E1A (Reichel, Kovesdi and Nevins, 1988). The TFIIIC binding activity was abrogated in acid phosphatase, implicating phosphorylation as the activating principle.

The 289 amino acid polypeptide has greatly enhanced activity in the vicinity of the

promoter (Lillie and Green, 1989), indicating that it may bind to cellular proteins attached to the promoter. This indirect mechanism occurs in the association of proto-oncogenes c-fos with c-jun (Sassone-Corsi *et al.*, 1988) and the herpes simplex virus regulatory protein Vmw65 association with the cellular protein designated TRF (Preston, Frame and Campbell, 1988). Construction of fused GAL4 and E1A gene products has been used to formulate a model of the 289 amino acid protein with a binding and an activating domain separated by a zinc-finger (Lillie and Green, 1989) which is very typical of the structure of other cellular activators (Ptashne, 1988).

The E1A gene is alone able to immortalize non-permissive cells (Houweling *et al.*, 1980). The E1A transforming activity maps to the first and second conserved domains (Moran *et al.*, 1986) but both the 289 and the 243 amino acid proteins are required for complete and stable transformation (Jones and Shenk, 1989). This suggests there may be a difference in the folding of the E1A proteins and may explain the need for two overlapping transcripts. E1A proteins have been shown to associate with host cell peptides of a variety of molecular weights (Harlow *et al.*, 1986; Yee and Branton, 1985). The sites interacting with the three major cellular proteins, p300, p105 and p107 have been mapped and coincide with the transforming regions (Whyte, Williamson and Harlow, 1989). Mutations in E1A that destroy binding to these three peptides also abolish the ability of E1A to cooperate with ras oncogene in transforming primary neonatal rat cells (Whyte, Ruley and Harlow, 1988). The 105K peptide has been identified as the retinoblastoma susceptibility gene product, a protein that seems to act as an "anti-oncogene" (Klein, 1988). Families with an inherited tendency to develop

retinoblastomas and osteosarcomas grow tumours composed of cells that invariably have lost at least one normal copy of this gene (Cavenee *et al.*, 1983; Murphee and Benedict, 1988) and it has been inferred that the 105K product normally suppresses cellular proliferation. It can be postulated that one method of malignant induction by adenoviruses might be the physical interaction of E1A proteins with anti-oncogenes.

Full transformation of adenovirus infected cells to those able to form tumour nodules in syngeneic hosts requires at least two steps involving the participation of the E1B gene (Byrd *et al.*, 1988). The E1A function in transformation is analogous to a malignant myc oncogene, with which it shares DNA homology, while the E1B function can be substituted with the ras oncogene (Ruley, 1983). Fully transformed cells can lack the 20K and 55K polypeptide products of EIB but always contain the 19K product (Matsuo *et al.*, 1982). The 19K protein is fatty acylated, covalently linked to myristic or palmitic acid, and localises to cellular membranes, accumulating in the nuclear envelope late in infection (White *et al.*, 1984). It has been found to associate with the cytoskeletal protein virmentin and may contribute to the disruption of adhesion plaques in transformed cells (White and Cipriani, 1990). The 55K protein of E1B forms a complex with the 34K E4 gene protein (Cutt, Shenk and Hearing, 1987), a complex that is located in the nucleus and appears to be involved in host cell shut-off and in late gene expression (Halbert, Cutt and Shenk, 1985). The 55K protein is also found in conjunction with the cellular p53 protein, another anti-oncogene which is found elevated and mutated in transformed cells (Finlay *et al.*, 1988). The wild type p53 has the ability to suppress transformation by the mutated version, suggesting that the 55K-p53 complex could release cells from controlled

growth in a similar manner to the E1A-p105 interaction. The p53 binds to the SV40 T antigen and to the transforming E6 proteins of the malignant papillomavirus types 16 and 18 (Werness, Levine and Howley, 1990), forming yet another common pathway by which DNA tumour viruses may generate transformation.

The E3 region may also contribute to oncogenicity. Consisting of a non-essential sequence substituted in adenovirus vector constructs, the E3 gene codes for a series of transcripts which alter the host reaction to infection. A 19K protein binds and interferes with the immune recognition of infected cells by blocking transport of the major histocompatibility complex heavy chain to the cell surface (Burgert, Maryanska and Kvist 1987). Another 14.7K E3 product prevents lysis of infected cells by tumour necrosis factor (Gooding *et al.*, 1988) whilst a third E3 protein has been found to bind the epidermal growth factor (EGF) receptor (Carlin *et al.*, 1989). Similarly, vaccinia virus also affects EGF secretion and HIV blocks transport of the CD4 molecule to the surface (Koga *et al.*, 1990). Thus it appears that the early region products of adenoviruses are used to transform infected cells and modulate the host immune reaction in a variety of complex mechanisms, most of which have been exactly duplicated by other viruses.

The E2 region products, the DNA binding protein, the DNA polymerase and the terminal protein, are all required for DNA replication. It has been shown conclusively in an *in vitro* replication system that the 80K precursor terminal protein is a protein primer, binding the first nucleotide of the genome in a complex formed with the DNA polymerase and the bound terminal protein at the 5' ends of the DNA duplex (Friefeld,

Krevolin and Horwitz, 1983). Extension of the primer is then accomplished in the presence of the DNA binding protein and a number of cellular factors (Chalberg, Osterove and Kelly, 1982). Nuclear Factor I, which corresponds to CTF, Nuclear Factor III and ORP A are polypeptides ordinarily involved in regulation of transcription in the uninfected cell whose functions have been subjugated by the virus. These factors recognize consensus sequences within the first fifty five nucleotides of the origin of the adenovirus genome (Rosenfeld *et al.*, 1987) and initiate replication. Elongation requires Nuclear Factor II, a complex of two proteins with topoisomerase I activity, which unwinds the duplex genome. Each new strand of DNA advances in a continuous 5' to 3' direction against a parental template in a semi-conservative fashion, without the need for Okazaki fragments (Lechner and Kelly, 1977).

DNA replication coincides with the start of late mRNA transcription some 6-8 hours after Ad2 infection (Lucas and Ginsberg, 1971). The switch to late gene transcription is thought to be controlled by a cis acting product as infected cells transcribing adenovirus late genes do not induce late transcription from superinfecting DNA (Thomas and Mathews, 1980; Falke-Pederson and Logan, 1989). Conformational changes (Brison *et al.*, 1979) and methylation (Vardimon *et al.*, 1980) have been discounted as the late transition signal. Compartmentalization of progeny genomes (Thomas and Mathews, 1980), nucleosome aggregation (Tate and Philipson, 1979) and the cleavage of the precursor terminal protein are proposals for the signal that have not been tested.

Unlike the early gene products, adenovirus late proteins are almost all derived from a

single RNA transcript comprising 80% of the coding capacity of the R strand from map units 16.4 to 99 (Evans *et al.*, 1979; Ziff and Evans, 1978). The exceptions are the proteins IX and IVa2, located within the first 20 map units, which are transcribed from their own promoters even earlier in infection (Persson and Philipson, 1982). The sequence of late mRNA begins with a eleven-nucleotide cap structure at position 16.4 (Gelinas and Roberts, 1977) and proceeds through five families of late genes, L1 to L5, arranged in contiguous fashion on the R strand. Internal splicing of this transcript to a single gene product with a tripartite leader, assembled from sequences mapped at 16.6, 19.6 and 26.6 map units (Berget, Moore and Sharp, 1977; Chow *et al.*, 1977), occurs after selection of one of the five cleavage sites and polyadenylation (Nevins and Darnell, 1978).

Control of splicing may be performed by two virus associated RNAs, VA RNA I and II, transcribed within the L1 region (Ohe and Weissman, 1970). These RNAs have many features in common with their mammalian counterparts, small nuclear ribonucleoproteins or SNRNPs (Lerner and Steitz, 1981), including size, transcription by RNA polymerase III instead of II, lack of translation, and binding to nucleoprotein complexes (Celma, Pan and Weissman, 1977; Thimmappaya *et al.*, 1982). The VA RNAs do not have any homology to splice junction sequences, however, and they have been shown to prevent the shut down of translation in adenovirus infected cells and to antagonize the antiviral effects of interferon (Mathews and Shenk, 1991). VA RNAs combine with and inactivate the cellular P1 kinase, an enzyme which is induced 5 to 10 fold by interferon and acts to stop translation by phosphorylating eIF-2. The phosphorylated form of eIF-2 forms

a tight complex with GEF, the guanosine exchange factor, and is thereby prevented from ferrying methionyl tRNA for polypeptide initiation. Besides maintenance of the cellular translation machinery, VA RNAs may also contribute to the selective translation of viral transcripts. VA RNAs have an affinity for newly synthesized viral mRNA (Mathews, 1980) and their immediate vicinity blocks activation of the P1 kinase, allowing selective translation of viral mRNA (O'Malley *et al.*, 1986). The selective attachment of VA molecules to enable translation is not the only means by which adenoviruses can subjugate the cellular translation process. Despite apparently normal polyadenylation, the cellular mRNA transcripts produced in infection fail to migrate from the nucleus (Babich *et al.*, 1983) and virtually all the ribosome-associated mRNA is viral (Beltz and Flint, 1979). By disruption of cellular processing at these pivotal points adenoviruses ensure exclusive production of viral proteins.

Virion assembly begins in the cytoplasm with the formation of complete capsomeres. The hexon monomer is translated from the gene between 50.4 and 60.9 map units and assembled into trimeric capsomeres by the 100K scaffold protein within 4 minutes after synthesis (Cepko and Sharp, 1983). The penton self-assembles more slowly from five penton base molecules and three fiber polypeptides. Capsid formation occurs in the nucleus initiated by the association of pVI, pVIII and IIIa with the hexon (D'Halluin *et al.*, 1978). This "light-intermediate capsid" is probably constructed by the 50K IVa2 and the 39K scaffold proteins as these are subsequently removed from the capsid (D'Halluin *et al.*, 1978). The naked DNA genome, directed by a packaging signal between 290 and 390 base pairs from the left terminus (Hammarskjold and Winberg, 1980) is thought to

enter the capsid next through one of the vertices. Pentons are added to the capsid after the addition of the core proteins at a final "young virion" stage (Ishibashi and Maizel, 1974) when the 23K protease cleaves the precursor proteins as the virion tightens its configuration and becomes impermeable to nucleases. Free penton capsomeres, produced in greater quantity than required for viral assembly, are largely responsible for the rounding and cell detachment associated with adenoviral cytotoxicity (Valentine and Periera, 1965; Philipson *et al.*, 1975). The process of virus multiplication is completed with the accumulation of mature virions as crystalline arrays in intranuclear inclusions. The most efficiently multiplying adenoviruses can produce 10,000 viral particles within 24 hours after infection of human embryonic kidney cells (Wordworth *et al.*, 1986).

4. Classification of the Human Adenovirus.

Adenoviruses comprises over 100 serotypes found in humans, cattle, rodents, monkeys, sheep, pigs, dogs, horses, opossums and chickens. The International Congress on Taxonomy of Viruses (ICTV), in accordance with their mandate to attribute binomial nomenclature to all viruses, chose adenoviruses as the test case and designated the known serotypes as species in the family Adenoviridae (Norrby, 1976). Two genera were defined. The genus Mastadenovirus have an identical appearance in the electron microscope and share a common antigen on the internal aspect of the hexon. Antisera to disrupted virus or purified hexons cross-reacts with Mastadenoviruses from different animals. The viruses found in birds have a pair of fibers projecting from the penton base and lack the common Mastadenovirus antigen and are classified in the separate genus Aviadenovirus. Although it is still a matter of intense debate whether an inert object

warrants the same binomial designation as more sentient animals (Regenmortel, 1990; Ward, 1986), the ICTV has extended the binomial classification to other groups of viruses and has embarked on plan to name all groups of viruses in the Linnaean fashion.

The human adenoviruses were first classified on the basis of the property of the fiber tip to bind to unknown proteins in the red blood cell and cause cross-linkage. The human adenovirus species were divided into four groups according to their ability to agglutinate rhesus monkey or rat erythrocytes (Rosen, 1960; updated by Hierholzer, 1973), as shown in Table 1. The agglutination patterns of the most recently described adenoviruses, serotype 42, isolated from an apparently healthy child after bowel surgery (Wigand, Adrian and Bricout, 1987) and serotypes 43 to 47, all isolated from AIDS patients (Hierholzer *et al.*, 1987) conform with subgroup II (Table 1) and will probably be placed in subgenus D. The length of the fiber and the molecular weight of some of the virion structural proteins, variable factors that are consistent in related adenoviruses, also correspond to the haemagglutination grouping and have been used as a separate classification system (Wadell, 1979). When Ad12 was shown to induce tumours in hamsters the oncogenic potential of other species was investigated and a further classification system devised (Huebner *et al.*, 1965). The genomes of tumorigenic adenoviruses (see Table 1) were found to have a low guanine (G) and cytosine (C) content (Pina and Green, 1965) but the idea that oncogenicity correlates with low GC content was disrupted when malignant simian virus SA7 DNA was shown to have a greater proportion of guanine and cytosine than the DNA of other simian adenoviruses (Goodhearst, 1971; Pina and Green, 1968). The nucleotide code underlies all of these

Table 1: Classification of Human Adenoviruses.

Subgenus	Species	Haemagglutination Group	Oncogenic Potential	% G+C Genome	DNA Homology Inter Subg.	DNA Homology Intra Subg.
A	12,18,31	IV (3B) Little or no agglutination	High	47-49%	48-69%	8-20%
B	3, 7,11, 14,16,21, 34,35	I (1A, 1B) Complete aggl. of monkey RBC	Moderate	49-52%	89-94%	9-20%
C	1, 2, 5, 6	III (3A) Partial aggl. of rat RBC	Low	57-58%	99-100%	10-16%
D	8, 9,10, 13,15,17, 19,20,22--30,32,33, 36-39,42-47	II (2A-F) Complete aggl. of rat RBC	Low	57-59%	94-99%	4-17%
E	4	III (3A) Partial aggl. of rat RBC	Low	57%	-	4-23%
F	40,41	III (3A) Partial aggl. of rat RBC	Low	52%	62-69%	15-22%

properties and subsequent attention in classifying the human species has focused on the DNA as the most definitive way of determining interspecies relationship (Green, 1970). The amount of sequence homology between the genomes of two viruses as measured by the melting temperature of the hybrid in liquid hybridization (Garon *et al.*, 1973) provides an accurate assessment of the divergence between species and shows a great distinction between members of one group and another. DNA homology within a single group is generally greater than 85% except for the variable subgenus A members where homology among its three members is 48-69% (Green, 1970) and the two subgenus F viruses which share approximately 66% of their genomic sequence. The genomic homology between members of different subgenera, on the other hand, is only 4 to 25% of the sequence. The grouping of human adenovirus species by haemagglutination, protein analysis, GC content and DNA homology agree very well and are compared in Table 1. The general concurrence of all the systems of classification, indicating close correlation of different characteristics in related viruses, probably shows that the different factors are diversifying at a concurrent rate in related viruses.

5. Association of Adenovirus with Gastroenteritis.

Numerous surveys of diarrhoea in children show adenovirus in 5-17% of stools from sick infants usually of 1-5 years of age (see Table 2). However, adenovirus has been consistently found during surveillance programs in the stools of apparently healthy children (Brandt *et al.*, 1969; Fox *et al.*, 1969; Galbraith, 1965). This ubiquity of adenoviruses makes it difficult to establish criteria to define adenoviral agents of gastroenteritis (discussed by Flewett, 1976) and even prevents unequivocal substantiation

Table 2. Incidence of Adenovirus in Gastroenteritis Studies.

Children with Gastroenteritis			Control Children		
Number Tested	Adenovirus Positive(%)	non-cultivable Adenovirus(%)*	Number Tested	Adenovirus Positive(%)	Source
59	11.8	5.1	141	0.7	Appleton et al., 1978
690	5.1	2.2	NT		Birch et al., 1977
604	5.1	4.3	522+	1.9	Brandt et al., 1979
1274	5.2	NT	NT		Brandt et al., 1983
654	8.0	7.2	709+	8.2	Brandt et al., 1985
192	7.3	6.8	NT		Gary, Hierholzer & Black, 1979
74	10.8	NT	62	4.8	Joncas & Pavilanis, 1960
143	11.2	7.0	NT		Kapikian et al., 1976
538	10.6	5.2	372	1.8	Kotloff et al., 1989
506	5.7	NT	NT		Konno et al., 1978
834	15.2	8.3	NT		Krajden et al., 1990
183	11.5	5.5	NT		Madeley et al., 1977
669	12.9	4.0	NT		Middleton, et al., 1977
167	16.8	NT	95	5.3	Moffet et al., 1968
322	8.1	NT	419	21.2(4+)	Parks et al., 1966
246	8.5	NT	158	0.6	Ramos-Alvarez and Olarte, 1964
2606	15.0	6.8	NT		Retter et al., 1979
416	13.5	7.9	200	1.5	Uhnoo et al., 1984
283	10.2	NT	NT		Vesikari et al., 1981
96	27.3	14.1	72	8.3	Yolken et al., 1982
167	1.8	NT	172	2.3	Yow et al., 1966

*Where non-cultivable adenovirus were detected by E.M. or EIA but not grown.

+ = Controls from children hospitalized for respiratory tract infection. NT = not tested

of adenoviral causation of diarrhoea (Madeley, 1983). In a number of studies in Table 2, where the source of controls have been specified, the use of stools from children with respiratory illness (e.g. Brandt *et al.*, 1985) may have masked the differentiation of control and gastroenteritis isolation rates, as was experienced by Parks *et al.*, (1966). Many of the studies argued that a statistically higher presence of adenoviruses in diarrhoea than in faeces of controls strongly indicated a causative association of adenovirus with gastroenteritis (Appleton *et al.*, 1978; Joncas and Pavilanis, 1960; Moffet, Shulenburger, and Burkholder, 1968; Parks *et al.*, 1966; Ramos-Alvarez and Olarte, 1964; Uhnoo *et al.*, 1984).

The earlier studies in the 1960s and early 1970s used routine cell culture to detect the viruses and found the lower-numbered species to predominate. A high proportion of the adenovirus isolates observed in the 1970s under the electron microscope could not be cultured in conventional cell lines (see column 3, Table 2). A number of workers commented on a "paradox" wherein the more virus visualized by the electron microscope the less likely it seemed culture could be achieved (Appleton *et al.*, 1978; Brandt *et al.*, 1979; Madeley *et al.*, 1977). In a four year study in Washington, D.C., for example, Brandt *et al.* (1979) observed significantly more adenovirus in paediatric inpatients with gastroenteritis than in inpatient controls, both in terms of frequency and the amount of viral particles, and only 5 of the 31 gastroenteric isolates grew in conventional culture. The high proportion of the non-cultivable viruses among adenovirus isolates and their low level in controls indicated that they played the major role in acute enteric disease.

Adenoviruses refractory to conventional culture form the majority of isolates in most studies and are responsible for 2-14% of the total incidence of gastroenteritis in children. Characterization of these viruses was finally achieved by Jacobson, Johansson and Wadell (1979) using Hovi-X, a non-cultivable adenovirus purified by density gradient centrifugation from a large amount of the stool of a five-year old boy (provided by Dr. Tapani Hovi of Helsinki University in 1976). Antisera to Hovi-X was still reactive after absorbtion with antigens of the known subgenera, indicating a novel species and Wadell *et al.*, (1980) showed the isolate to have distinct proteins and DNA banding patterns in electrophoresis and classified it in a separate subgenus.

Renewed efforts to cultivate these refractory species led to identification of a number of semi-permissive cell lines. Kidd and Madeley (1981) demonstrated the usefulness of Chang conjunctival cells and Takiff *et al.*, (1981) succeeded with 293 cells in growing some refractive isolates. The innovative use of cynomologous monkey kidney cells led to the original isolation of another strain, Dugan (Johansson *et al.*, 1980). These three cell lines were used in a painstaking study (de Jong *et al.*, 1983) to evaluate the haemagglutination and neutralization activities of antisera to refractory isolates according to criteria established for speciation of adenoviruses by Matthew (1982). No cross-reaction was observed between adenovirus antisera to the first 39 species and the refractory strains which could be separated into two groups by serum neutralization tests. Strains Hovi-X and Dugan gave the same reactions and were named species 40 whilst a second new species 41 was designated for a strain Tak, isolated on HeLa cells as far back as 1973 (de Jong *et al.*, 1983). Initially considered as forming a single new

subgenus from their antigenic relatedness (de Jong *et al.*, 1983), these species were separated into two subgenera due largely to the variation in molecular weight of internal polypeptides (Wadell *et al.*, 1980) and their lack of comigrating DNA restriction endonuclease fragments (Uhnoo *et al.*, 1983). It was evident from the high level of nucleotide homology shared by the two genomes (van Loon *et al.*, 1985) that Ad40 and Ad41 should be reclassified as a single subgenus F (Wadell *et al.*, 1987). The definitions "fastidious" and "enteric", suggested to denote the viruses' refractory culture characteristics and their exclusive association with gastroenteritis (Madeley, 1986; Petric *et al.*, 1983) are both currently used to refer to these species. Some authors have used "enteric" to describe viruses generally associated with the alimentary tract, in this volume the usage will be restricted to distinguish Ad40 and Ad41 from conventional species.

Despite great interest no further species of fastidious adenovirus have been found although the number of variants described is extensive (Allard *et al.*,1985; Buitenwerf, Louwerens and de Jong, 1985; Hammond *et al.*, 1985; Kidd, 1984; Kidd *et al.*, 1984; Shinozoki *et al.*, 1988; van der Avoort *et al.*,1989). There have been no recent improvements in cell culture although media supplemented with less than 1% serum allowed efficient multiplication of Ad41 strain Tak in several primary cell lines (Pieniazek *et al.*, 1990A). A549 cells (Lieber *et al.*, 1976) have been applied to adenoviruses (Smith *et al.*, 1986) and seem to have advantages over traditional HeLa and HEp 2 lines which tend to produce an abundance of unassembled capsomeres (Hierholzer, J., personal communication). A549 cells did produce the highest yields of Ad40 (Witt and Bousquet, 1988) when applied to enteric adenoviruses, although the

improvement was marginal. It is evident that conventional HEp 2 culture can propagate some isolates of fastidious species (Brown, Petric and Middleton, 1985) and may produce a high yield of the prototype strain Tak of Ad41 after serial passage (Pieniazek *et al.*, 1990B). Nevertheless, efficient isolation and propagation of subgenus F adenoviruses in quantity, particularly of Ad40 (Chiba *et al.*, 1983; Uhnoo *et al.*, 1983), remain difficult propositions.

The evidence to associate species 40 and 41 with paediatric gastroenteritis is now very extensive. These species have so far been isolated only from the gastrointestinal tract (Petric *et al.*, 1982) and, apart from two exceptions discussed by Brandt *et al.*, (1985), of a patient with concurrent influenza infection and a boy with a neurological disease characterized by chronic constipation (Yolken *et al.*, 1982), always from diarrhoea. At the acute stage of disease fastidious adenoviruses are excreted in excess of 10^{11} particles per gram of stool (Gary, Hierholzer and Black, 1979; Retter *et al.*, 1979). The high level of excretion must result from active multiplication in the intestinal tract and would indicate considerable cell destruction. In support of this, a large number of fastidious adenovirus particles were found in the duodenal fluids of three children (Mavromichelis *et al.*, 1977) and crystalline arrays of virus in the nuclei of small intestinal mucosal cells were observed in biopsy tissue from a fatal case of Ad41 gastroenteritis (Whitelaw, Davies and Parry, 1977). Lactose intolerance or xylose malabsorption are consistently reported after infection with Ad40 or 41 (Albert, 1986; Uhnoo *et al.*, 1984; Uhnoo *et al.*, 1986) and serconversion is a common feature (Chiba *et al.*, 1983; Uhnoo *et al.*, 1984) not usually found with intestinal infection with other species. The presentation of

symptoms of adenoviral gastroenteritis ranges from mild afebrile diarrhoea with a duration of two to four days (Flewett *et al.*, 1975) to pronounced diarrhoea for up to two weeks with vomiting, low fever and respiratory symptoms (Kidd *et al.*, 1982; Uhnoo *et al.*, 1984). Clinical signs of respiratory tract infection concurrent with diarrhoea are not uncommon. In one study 13 of the 14 patients with adenovirus detected in faeces also had a cough, wheeze, rhinorrhoea or x-ray evidence of pneumonia (Yolken *et al.*, 1982). Respiratory symptoms are more frequent with the non-fastidious species, especially with the lowest numbered species associated with latent adenoid infection (Krajden *et al.*, 1990). Antecedent respiratory symptoms of these species were observed, raising the possibility that some virus particles may survive alimentary passage to be observed in stool or that enteric infection may be secondary to an upper respiratory tract infection. Respiratory symptoms occur rarely in patients infected with Ad40 or Ad41 and these species are probably restricted to the intestinal tract (Uhnoo, Olding-Stenkvist and Kreuger, 1986). The severity of diarrhoea due to fastidious adenovirus infection exceeds that of the conventional adenovirus species (Kotloff *et al.*, 1989) and can have a more prolonged duration than diarrhoea due to rotavirus (Uhnoo *et al.*, 1984; Yolken *et al.*, 1982B). Two fatalities in children with enteric adenovirus gastroenteritis have been reported (Retter *et al.*, 1979; Whitelaw, Davies and Parry, 1977) and Flewett mentions a third case from Australia (Wadell *et al.*, 1987, discussion). Krajden *et al.*, (1990) document five deaths from adenoviral gastroenteritis, all of immunocompromised children, attributed to Ad12, Ad31, Ad40 and twice to Ad41. The preponderance of the enteric species in the fatal reports, the severity of the diarrhoea and the indications of destructive intestinal multiplication, mark Ad40 and Ad41 as a cause of considerable

disease in young children.

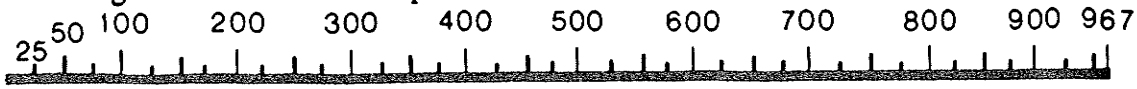
6. Genetic Homology Among Adenovirus Species.

The degree of nucleotide homology of the entire genomic sequences of human adenovirus species have been well evaluated by liquid hybridization (Garon *et al.*, 1973; Green *et al.*, 1978) for the purposes of classification. Melting temperatures of interspecies hybrids show the amount of nucleotides shared by species of different adenovirus subgenera to be between 4 and 25%. Mapping of the homologous areas detected in these experiments, however, has not been detailed. The best studies have concerned the relationship between animal species and Ad2. When mouse Mastadenovirus FL and Ad2 DNA genomes were digested with restriction enzymes and the fragments separated by agarose electrophoresis, Southern blotted and hybridized with the reciprocal DNA, two small areas of homology were detected (Larsen, Margolskee and Nathans, 1979). In experiments with the probe and target DNA reversed, the homologous areas were mapped to similar positions on the two genomes. Hybridization was demarcated to Ad2 DNA fragments between 12-18 map units (mu) and FL DNA fragments of 6-16 m.u. and a central portion of both genomes of 51-62 m.u. with Ad2 and 54-66 m.u. with mouse FL DNA, respectively. The central portion is close to the start and termination sites of the Ad2 hexon gene. The area of homology towards the left end of the genome has multiple purposes. The R strand in this region codes for part of the protein IVa2 and the overlapping terminal protein while the L strand transcribes the first sequence of the tripartite leader of late gene transcripts (Akusjarvi and Wadell, 1986).

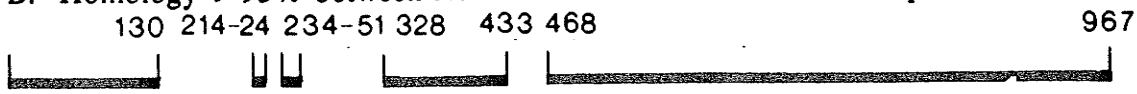
Other studies in which restriction fragments of bovine adenovirus 3 (BAd3) (Hu, Hays and Potts, 1984) and chicken embryo lethal orphan (CELO) virus (Alestrom *et al.*, 1982) have been hybridized with human Ad2 DNA also demarcated two areas of high nucleotide homology in the genomic sequence. Hu *et al.*, (1984) used plasmids containing cloned *Hin*dIII fragments of bovine DNA to react with a single enzyme digest of Ad2 and found that a large area between 8-80 m.u. of the genomes would react in non-stringent conditions. The hexon gene was found to be the most reactive sequence and nucleotide sequencing showed the bovine hexon gene to code for a protein with greater than 80% overall amino acid identity with the Ad2 hexon. This sequence homology fell distinctly into three separate regions in the hexon gene as shown in Figure 1. CELO virus DNA was hybridized with Ad2 DNA at increasing degrees of stringency (Alestrom *et al.*, 1982). At the highest concentration of formamide the cross-hybridization was reduced to two discrete areas very similar in position to those found with mouse FL virus. These areas were cloned and subdivided. Sequences from 18.1 to 19.3 m.u. and 56.5 to 58 m.u. on the Ad2 genome were defined as having the closest identity to CELO virus sequences. The sequences towards the left terminus of the Ad2 genome with demonstrated homology to mouse and chicken virus DNAs do not overlap. It is very likely, in view of their proximity, that the detected sequences code for the same conserved protein(s) and the difference in position may result from experimental variability. The position of the central sequence of the Ad2 genome homologous with CELO virus does correspond to the sequence shared in the middle of FL and Ad2 genomes and both map to the hexon gene (Figure 1). Thus, the single sequence found in common between three adenoviruses from three different animals and two different

Figure 1: Functional Regions of the Adenovirus Hexon Monomer.

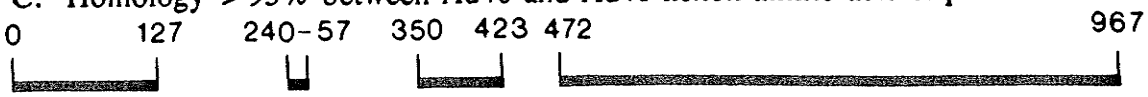
A: Length of the Ad2 hexon protein in amino acid residues.



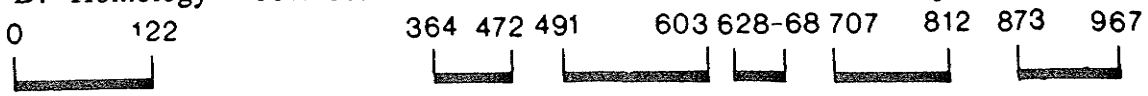
B: Homology >95% between Ad2 and Ad5 hexon amino acid sequences.



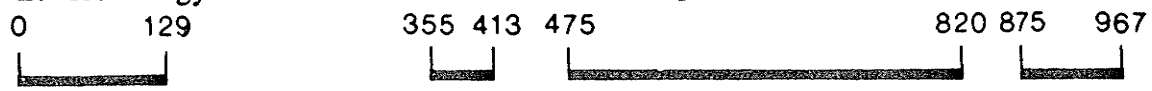
C: Homology >95% between Ad40 and Ad41 hexon amino acid sequences.



D: Homology >80% between Ad2 and Ad41 hexon amino acid sequences.



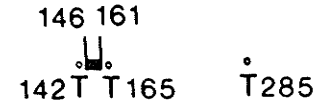
E: Homology >80% between Ad2 and bovine species hexon 2 amino acid sequences.



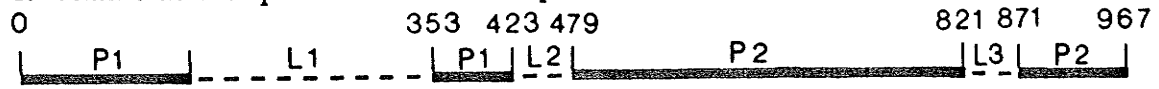
F: Homology >80% between Ad2 and CELO virus hexon amino acid sequences.*

G: Distribution of frequent proline residues in Ad2 hexon sequence.

H: Acid labile region and (T) trypsin incision sites of Ad2 hexon.+



I: Amino acid sequence in relation to position in 3 dimensional monomer.x



* Positioned exactly by restriction sites in the Ad2 sequence.

+ Adapted from Jornval et al. (1981). x Taken from Roberts et al. (1986).

genera codes for part of the hexon, the capsomere that forms the major part of the external surface of the virion. Further evidence of the conservation of the hexon between adenovirus species is provided by the fact that antibody to disrupted virions agglutinates virus of any Mastadenovirus species (Periera *et al.*, 1963; Petterson, Philipson and Hoglund, 1967; Wilcox and Ginsberg, 1961) and that monoclonal antibody to an internal hexon epitope detect all Mastadenoviruses in infected cells (Cepko *et al.*, 1983). CELO virus belongs to the second genus, Aviadenovirus, which lack the common internal hexon antigen (Clemmer, 1964) and the hybridization between hexon sequences of Ad2 and the chicken virus is interpreted as due to the architectural constraints on the hexon as the major capsid component (Alestrom *et al.*, 1982).

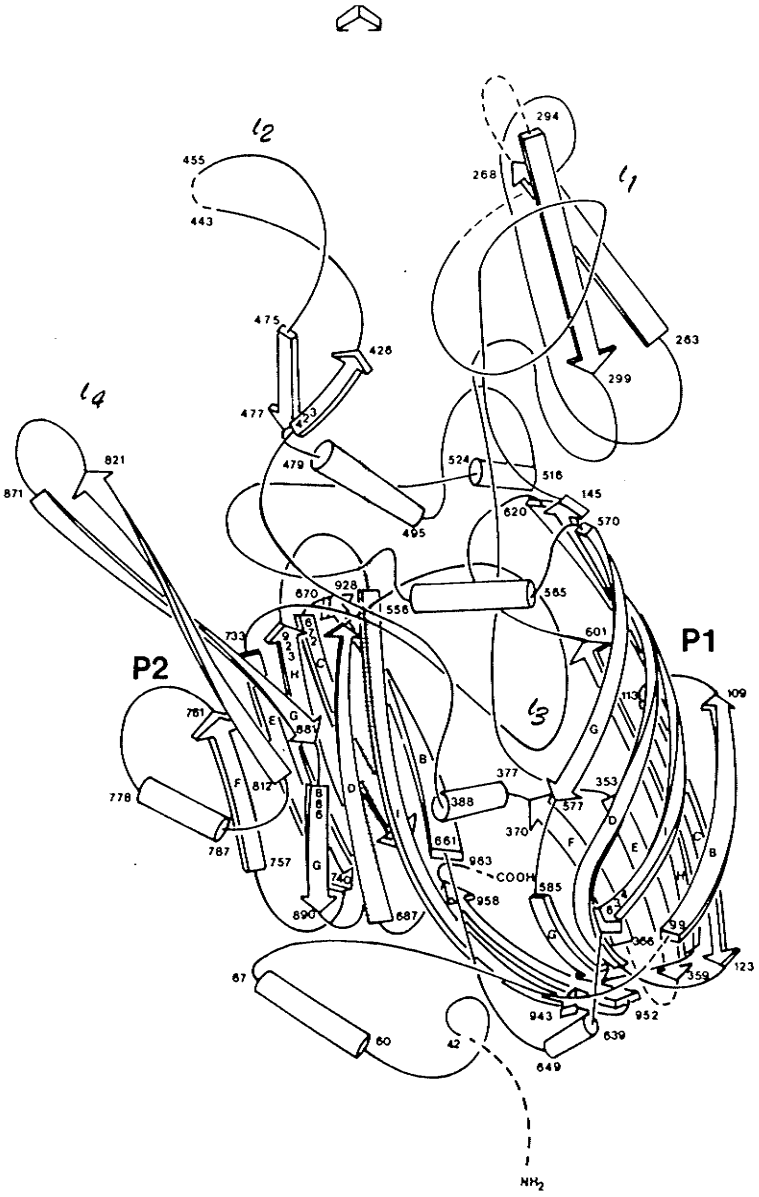
Much less is known of the interrelation of human adenovirus species. There are a large number of studies demonstrating a serological relationship between pairs of viruses, some of which may have phylogenic relevance. However, there are few adenovirus species without serological cross-reactions of serotypic and neutralizing antibodies with various other species. The surface epitopes account for a small proportion of the genomic code and can poorly reflect the genetic relationship between viruses. There is little data on the sequence homology of human adenoviruses. In a simple experiment van Loon *et al.*, (1985) hybridized one lane each of a single restriction digest of Ad5, Ad40 and Ad41 genomes with each DNA in turn as a probe. In the conditions of low stringency used, the only fragments that did not hybridize belonged to both the left and right termini of the genomes. The left terminus is the area of the genome of human adenoviruses that has been most frequently sequenced. The E1 region has often been presumed to be conserved

because of its importance in controlling viral and cellular transcription. However, comparison of sequences from three species of different subgenera by Sambrook *et al.*, (1980) evaluates the sequence homology at the left terminus at about 30%. Other sequences that can be compared show other genes to have a much higher sequence homology (Sambrook *et al.*, 1980; Tooze, 1982; Quin and Kitchingman, 1984). The nucleotide sequence of the IVa2 gene shows the highest level of conservation of those areas of the genome for which sequences from a variety of virus species in multiple subgenera have been collated and compared (Sambrook *et al.*, 1980).

The hexon genes of four species of human adenovirus, Ad2 and Ad5 of subgenus C and Ad40 and Ad41 of subgenus F have been sequenced and it is possible to make comparisons of the patterns of sequence homology both within and among subgenera for this gene and assess whether the pattern is consistent. The subgenera C and F have no special links and the pattern of hexon gene comparisons is probably typical of the relationship of hexon sequences of other subgenera. The hexon sequences of Ad5 and Ad2 (Kinloch, MacKay and Mautner, 1984), have long sequences with 90% or more of the nucleotides and 100% of the amino acid code in common (Kinloch, MacKay and Mautner, 1984) as shown in Figure 1, row B. Virtually every base pair difference between the two hexon genes occurs in the third position of the codons and does not affect the amino acid sequence. Similarly, the Ad40 and Ad41 hexon sequences (Toogood and Hays 1988: Toogood *et al.*, 1989) code for proteins with long stretches of identical amino acids (Figure 1, row C). These stretches are interrupted by sequences with very little or no sequence homology even among species within one subgenus. The pattern of

sequence homology in the hexon genes is rigidly demarcated so that the transition points between zones of sequence homology and sequence disparity occur within a few nucleotides, even in the comparisons of species from different subgenera (Figure 1, row D) and between viruses from different animals (Hu, Hay and Potts, 1984) (Figure 1, row E). The pattern of variable and conserved hexon sequences are paralleled by some of the structural information available from previous chemical analyses. Proline residues which allow short stabilizing bridges between strands (Richardson, 1981) are mostly coded by conserved sections (Figure 1, row G) while trypsin sensitive sites of the Ad2 hexon fall within a variable region (Figure 1, row H) (Jornvall *et al.*, 1981). The reason for the sudden dichotomy between strictly conserved and divergent sequences is apparent from the position of the transition sites in the three dimensional structure of the hexon monomer, portrayed in Figure 1, row I. Comparison of the pattern of homology with the position of amino acid residues in the three dimensional map of the Ad2 hexon (Roberts *et al.*, 1986) shown in Figure 2 demonstrates that the conserved sequences code for the two semicircular structural shells of antiparallel, beta-pleated sheets, P1 and P2. Prolines are most often found at the first and last residues of the antiparallel strands in the shell and evidently play an important role in sustaining the folding of the chain. The fact that the third nucleotide of the codons of conserved sequences is by far the most variable is evidence that the P1 and P2 shells of the hexon are under strong constraint as the major component of the virion to retain an unchanging structure. The variable regions correspond precisely with the three epitopic loops that extend from the P1 and P2 shells (Figure 2). These loops project to the external surface of the hexon, creating serotypic and neutralizable epitopes (Norrby, 1969) that form the major part of the surface of the

The Figure 2. Diagram of the Three Dimensional Conformation of the Ad2 Hexon Protein.



This diagram, showing the sequence of amino acid residues that form the semicircular P1 or P2 shells comprising the body of the hexon capsomere and the L1 to L4 loops which extend from the shells to form the epitopes of the hexon surface, was taken from Roberts *et al.*, (1986).

virion. The L1, L2 and L4 are responsible for the antigenic presentation of the virion and must be under selective pressure from the immune system to vary. L3 loop, which extends between the P1 and P2 shells, is an internal, stabilizing structure and is more conserved. The trypsin sensitive sites of the surface L1 loop of the Ad2 hexon are absent from the Ad40 and Ad41 proteins and may help explain the difference in tropism of the viruses, contributing to the survival of the enteric viruses in the alimentary tract. Thus, the hexon gene contains both the most conserved nucleotide sequences of any adenovirus gene sequenced to date and the most divergent. The dichotomy between these conserved and divergent sequences is precise and pertains to the exact function of the amino acid structure.

7. Problem and Proposal.

The causes of nonbacterial gastroenteritis were entirely unknown until relatively recently. The examination of clinical specimens with the electron microscope first showed the presence of a number of viruses in diarrhoea and correlation of these viruses with disease symptoms has established belief that they have a pathogenic role. Determination of the involvement of viruses in the aetiology of enteritis has been beset with complications. The common and readily grown enteroviruses cause few cases of gastroenteritis whilst those viruses that do cause disease, including rotaviruses, caliciviruses, the coronaviruses, astroviruses, some adenoviruses, Norwalk and similar small round viruses, tend to be noncultivable. The difficulty in cultivation has hampered progress in studying these viruses and there is still a dearth of information on the clinical features, immunology, molecular biology and epidemiology of viral gastroenteritis. The

identification and understanding of these agents is of great importance as viral gastroenteritis is one of the most common causes of disease in the world. Exceeded in frequency only by viral respiratory tract infection in North America (Kapikian et al., 1980), viral gastroenteritis is the most common clinical syndrome in developing countries, affecting over 500 million children annually (Tolia and Dubois, 1985). Contributed to by malnutrition, poor sanitation and crowded conditions in developing countries, acute gastroenteritis is estimated to claim the lives of 5 million children each year (de Zoysa and Feachem, 1985).

Adenoviruses have been associated with 5.0 to 17% of sick children in gastroenteritis surveys. This association rate is high for one group of agents in the diverse and multiple aetiology of gastroenteritis. Adenoviruses have been found to be the second most common virus detected by the electron microscope in a number of gastroenteritis surveys from the United States (Brandt *et al.*, 1979; 1983), the United Kingdom (Flewett *et al.*, 1975), Canada (Krajden *et al.*, 1989), Scandinavia (Uhnoo, Olding-Stenkvist and Kreuger, 1986) and South Africa (Kidd *et al.*, 1986; Schoub *et al.*, 1975). Several studies have enumerated adenoviruses as second in frequency as a cause of paediatric gastroenteritis only to rotavirus of any infantile enteric pathogen, viral, bacterial or protozoan (Brandt *et al.*, 1985; Uhnoo *et al.*, 1984; Vesikari *et al.*, 1981). Adenovirus infection is evidently widespread and endemic. Epidemic outbreaks do occur in nurseries and paediatric wards (Yolken *et al.*, 1982B; Chiba *et al.*, 1986) where nosocomial transmission of adenovirus can be extensive once introduced (Kotloff *et al.*, 1989; Krajden *et al.*, 1990), causing considerable morbidity in surgical patients (Yolken and

Franklin, 1985). Thus, adenoviruses are known to form a large contribution to diarrhoeal disease, one of the most significant health problems of young children. However, the incidence and epidemiology of adenoviruses are ill-defined because of the lack of a convenient and definitive means of diagnosis. In particular, the medical importance of fastidious adenoviruses in gastroenteritis in various populations and age groups has not been adequately assessed due to the problems presented as a consequence of their fastidious growth characteristics.

The methods presently in use for isolation and identification of adenoviruses have several drawbacks. Electron microscopy is expensive, labour intensive, has a low sensitivity, able to identify viruses only when present at greater quantity than 10^6 particles per gram of stool (Flewett, 1976) and is not able to distinguish between the morphologically identical adenovirus species. Identification methods that are based on tissue culture, such as immunofluorescence and neutralization are again labour intensive, are slow, subjective and use antibodies that can cross-react between adenovirus species, giving erroneous results. The enteric adenoviruses, which constitute over half of the adenovirus isolates of most studies, necessitate the use of several cell lines for culture and present particular problems in cultivation and identification. Although cell lines have been found for better growth of fastidious adenoviruses, efficient cultivation of these species is not yet possible. Growth of enteric adenoviruses, where successful, is slow, producing a cytopathic effect (CPE) in isolated cells after several days so that cell infection can be difficult to differentiate from cellular degeneration. Moreover, dual infections of intestinal viruses are not uncommon (Brandt *et al.*, 1986; Brown, 1990; Kidd *et*

al.,1982; Wigand *et al.*, 1983) and when a specimen containing Ad40 or Ad41 and another virus is cultured the fastidious adenovirus is usually overgrown. In one study, 4 of 15 Ad40 positive specimens contained a second adenovirus species, and in each case the fastidious species was overgrown (Brown, 1985). Longterm excretion of the nonfastidious species of adenovirus following intestinal or respiratory infection is well documented (Fox *et al.*, 1969; Fox, Hall and Cooney, 1977; Kidd *et al.*, 1982). Even when present in low proportion relative to an enteric adenovirus species in concurrent infection, the nonfastidious species are favoured in neutralization tests because of their higher infectious titre (Brown, 1990). To improve adenovirus diagnosis, tests that circumvent these problems need to be designed. Ideally, the test should have higher sensitivity than electron microscopy, retain the sensitivity of culture in less labour and time and must be performed directly on the specimen to overcome the difficulties with overgrowth and fastidious viruses.

It is important for the test to distinguish the fastidious adenovirus species because these viruses are particularly poorly known and they have a specific association with gastroenteritis. A number of authors have addressed this problem with techniques to improve fastidious virus detection directly in the specimen prior to tissue culture by presumptive, quantitative electron microscopy (Brandt *et al.*, 1984), by restriction enzyme analysis (Brown, Petric and Middleton, 1984; Buitenwerf, Louwerens and de Jong, 1985), by immune electron microscopy (Wood and Bailey, 1986; Wood *et al.*, 1989), by enzyme immunoassay (Johansson *et al.*, 1980; 1985) or by radioimmunoassay (Holonen *et al.*, 1980). The ability of polyclonal antibodies to detect faecal adenoviruses

equally has been shown (Johansson *et al.*, 1980; Cepko *et al.*, 1983), but antisera able to distinguish Ad40 and Ad41 from other species can be made only by lengthy absorbtion with other subgenera (Jacobson, Johansson and Wadell, 1979; de Jong *et al.*, 1983; Wood and Bailey, 1986). Monoclonal antibodies able to distinguish Ad40 (Singh-Naz and Naz, 1986) or both Ad40 and 41 (Hermann, Perron-Henry and Blacklow, 1987; van der Avoort *et al.*, 1989) have great potential in the diagnosis of enteric gastroenteritis and have rapidly been marketed as viable immunoassay kits (Sing-Naz *et al.*, 1988; Wood *et al.*, 1989B). The suitability of the enzyme immunoassay for epidemiological study of enteric adenoviruses is borne out by the ease with which it was possible to screen large numbers of samples in recent studies (Kotloff *et al.*, 1989). Ironically, the specificity of the monoclonal antibody may be the main detracting factor to these immunoassays. The monoclonal antibodies so far developed react with hexon antigens, surface epitopes exposed to the immune system and are liable to change with antigenic variation due to antibody selection. Nonreactive fastidious adenovirus strains are already known (Scott-Taylor *et al.*, 1990; Wood *et al.*, 1989B).

The other main avenue of current research in diagnostic tests is based on the specific binding capacity of nucleotide pairs in the DNA duplex, culminating in the description of polymerase chain detection of amplified adenovirus DNA (Allard *et al.*, 1990). Unfortunately, faecal matter contains as yet unknown inhibitors of the polymerase enzyme, necessitating labour intensive extraction of sample prior to amplification. By far the most attention has been devoted to the practicality of devising a DNA hybridization test which would have a number of conspicuous advantages over serological tests (Ranki

et al., 1983A; Engleberg and Eisenstein, 1984). The chemical stability of DNA enables its survival of drastic pretreatment of samples of mucous, whole cells or stool, while cloning allows an inexhaustible supply of pure reagents. The specificity of the test is open to absolute manipulation by selection of unique or common sequences of DNA as probes. At present the dangers associated with the use of radioactivity as a label is a major impediment to the use of hybridization as a routine diagnostic technique. However, some progress towards a biotin label has been made (Brigati *et al.*, 1983; Langer, Waldrop and Ward, 1981) and other possibilities such as immunological detection of chemically modified DNA (Tchen *et al.*, 1984) and polyethyleneimine bound enzyme detection (Renz and Kurz, 1984) are being investigated. It is reasonable to assume, with the rapid progress of nucleic acid chemistry, that a harmless alternative label will be developed in the near future.

DNA hybridization was devised as a means of detecting some cryptic viral infections (Brandsma and Miller, 1980). The first assays of nucleic acids in clinical specimens were described for adenoviruses (Hyypia and Pettersson, 1985; Virtanen *et al.*, 1983). A genomic adenovirus probe has twice been compared to radioimmunoassay using hexon antisera, concluding that the two tests had equivalent sensitivity (Stalhandske *et al.*, 1983; Virtanen *et al.*, 1983). In another comparative study (Hammond *et al.*, 1987), hybridization using the Ad2 genome as a probe detected fewer adenoviruses in faecal samples than electron microscopy by an improved ultracentrifugation technique (Hammond *et al.*, 1981) but was able to correct some false negative EM or tissue culture results.

Most attention has centred on the reactivity of various cloned sections of the genome as probes in attempts to improve sensitivity and specificity of hybridization with a renewable reagent. A *Bam*HI J fragment from Ad2 has been shown to have broad cross-reactivity with the different subgenera except group A (Allard *et al.*, 1985). *Bam*HI C and D fragments of Ad2 were reported to react strongly with adenoviruses of subgenera B, C and E (Gomes *et al.*, 1985) while a *Hin*dIII D containing plasmid detected viruses of groups A, B and C (Schuster *et al.*, 1986). The Ad2 *HindIII* A fragment, enclosing the hexon gene sequence, has been tested against DNA of subgenera A to E and found to have a range of cross-reactivity equivalent to the whole genome (Huang and Deibel, 1988) but has yet to be evaluated with Ad40 and Ad41. In this study, the Ad2 *Hin*dIII A fragment was able to detect the DNA of other species with no less sensitivity than the genomic Ad2 probe. In previous tests, however, the *Hin*dIII A fragment probe had lower reactivity with heterologous DNA than the genomic probe by at least one log dilution of DNA (Hyypia, 1985).

A variety of cloned Ad41 fragments have been evaluated as probes for specificity to fastidious adenoviruses. The *Bam*HI H fragment, adjacent to the cross-reactive J fragment in the EIB region had no apparent reaction with titrated DNA of subgenera A to E and almost the same sensitivity with Ad40 as with homologous DNA (Allard *et al.*, 1985). Another Ad41 EIB cloned fragment, designated 41-27 (Niel *et al.*, 1986), also apparently specifically detected fastidious adenovirus isolates. Takiff *et al.*, (1985) found a fragment from the right terminus of the genome, the Ad41 *Eco*RI B fragment, to be the least cross-reactive of their cloned fragments with Ad2. The specificity of the *Eco*RI

fragment was localized by subcloning, resulting in a *Bgl*II D plasmid that retained the specificity of the parent plasmid. This *Bgl*II D cloned plasmid was used by Hammond *et al.*, (1987) to specifically detect fastidious adenoviruses in clinical faecal specimens. Kidd, Harley and Erasmus (1985) have also developed two probe N26 and M9 from unspecified areas of the Ad40 and Ad41 genomes respectively. The sensitivity or specificity of these probes has not been compared to other detection methods. Fastidious isolates cross-react with these probes and specific diagnosis of Ad40 and Ad41 is presumed from intensity of reaction with one or other probe (Tiemessen *et al.*, 1989).

Most of the hybridization studies have relied on shotgun cloning and random evaluation of their cloned fragments to find probes of narrow or broad cross-reactivity. The Ad41 sequences and the information available on conserved and species specific areas of the adenovirus genome have not been taken into account in the designing of diagnostic probes. It is proposed in this study to first define the areas of cross-homology in the adenovirus genome, conserved between subgenera, that would logically make the best hybridization probe for detection of all adenovirus species. One species from each subgenus of adenovirus will be tested to ensure the pattern of gene conservation is common to all the human species. The genomic DNA of each species, digested with restriction enzymes and separated by agarose electrophoresis, will be Southern blotted to nylon membrane and hybridized in stringent conditions with genomic DNA of another species. This format, previously used to map the homology between mouse FL virus and Ad2 (Larsen, Margolskee and Nathans, 1978), gives the clearest definition of the position of sequences of homology. It is important to note that it is indicated, by the comparison

of sequences of various parts of the genomes of different species, that the order of genes appears to be preserved throughout human adenovirus subgenera (Sambrook *et al.*, 1980; Tooze, 1981; Wigand and Adrian, 1986). Specific probes for the diagnosis of fastidious adenoviruses will be defined by the testing of different fragments of the Ad41 genome for specificity when hybridized against titrated DNA of each subgenus. The species specific sequences could be identified by their failure to hybridize in conditions of low stringency, a reversal of the method proposed for location of conserved sequences. This method is less likely to work in reverse, however, as species specific sequences will still hybridize unless completely separated from sequences with homology to other viruses, an accuracy of cleavage unlikely with restriction enzymes. Thus a prior concept of the location of species specific sequences would be invaluable. It was hypothesized on the basis of the existence of antigenically distinct epitopes on the surface of the hexon, responsible for the unique serotypic identity of each species (Norrby, 1969), that species specific probe sequences could be found in the hexon gene. Due to the reaction of antisera to the internal aspect of the hexon with all species of Mastadenovirus (Periera *et al.*, 1963; Cepko *et al.*, 1983) and the hybridization of Ad2 DNA with central areas of the genomes of bovine, murine and avian adenovirus species (Alestrom et al., 1982; Hu, Hays and Potts, 1984; Larsen, Margolskee and Nathans, 1979) it is probable that highly conserved sequences are also liable to be found in the code for the hexon. Therefore, it was hypothesized that DNA probes for the diagnosis of both specific as well as all adenovirus species were to be found in the hexon gene. These various probes will be compared to other methods used for the detection of adenoviruses and the sensitivity and specificity for diagnosis of adenovirus in clinical specimens assessed. An examination

of the incidence of different adenovirus species in local children will be undertaken and restriction analysis of prevalent variants performed to form an assessment of the various species involved in the etiology of adenoviral gastroenteritis in Manitoba.

MATERIALS AND METHODS

1. Cells and Viruses

293 cells (Graham *et al.* 1977) A549 cells (Lieber *et al.*, 1976) and HEp 2 cells (Moore, Sabachewsky and Toolan, 1952) were obtained from American Type Culture Collection (ATCC, 12301 Parklawn Drive, Rockville, Maryland 20852, USA). These three continuous human cell lines were received at passage 35, 54 and 362 and maintained within passage 60, 100 and 460 respectively. Aliquots of the first passage of each cell line were stored at -198°C in liquid nitrogen and reconstituted when the passage number reached the upper limit. Cells were grown in Eagles Minimal Essential Medium (MEM, Gibco Cat. no. 410-1100, Burlington, Ontario) or later L13 (Liebovitz, 1963) (Gibco no. 430-1300) and kept in an atmosphere of 3.5% CO_2 in a 37°C incubator (model 3158, Forma Scientific, P.O. Box 649, Marietta, Ohio). The media, supplemented with 5% fetal calf serum (FCS, Gibco no. 200-624) for A549 and HEp 2 cells, 10% FCS for 293 cells, was changed every three days.

Cell cultures were tested for the presence of mycoplasma at three monthly intervals. Trypsinized cells were allowed to settle in wells created by a well clamp on a glass slide, washed and incubated with fresh medium for several days until 20 - 50% confluency was reached. Cells were then fixed by addition of a mixture of glacial acetic acid and methanol in a 1:3 ratio to the wells for 10 minutes, washed with phosphate buffered saline (PBS) and blocked with PBS containing 2% bovine serum albumin for 10 minutes at room temperature. The slide was flooded with Hoechst 33258 stain (Flow Labs Inc.,

McLean, Virginia), a fluorescent dye which binds specifically to DNA (Chen, 1977), diluted in Hank's balanced salt solution (Gibco no. 450-1200EL) and left for ten minutes. Cells were washed thoroughly with PBS and mounted with a coverslip. The cells were then examined for fluorescence other than over the nucleus under a (Lietz, HM-Lux model) fluorescent microscope equipped with a Ziess 53/44 filter. If mycoplasms were detected by particulate or filamentous fluorescent patterns on the cell surface fresh cells were thawed from frozen stock and the contaminated cells discarded.

The prototype strain of adenovirus species from each subgenus; adenovirus species 31 (Ad31), strain 1315 and Ad12, strain Huie of subgenus A; Ad7, strain Gomen, of subgenus B; Ad2 strain Adenoid 6 of subgenus C; Ad8, strain Trim of subgenus D; Ad4, strain RI-67, of subgenus E; and both prototype strains Dugan of Ad40 and Tak of Ad41 of subgenus F, were also obtained from ATCC. The first passage of virus after receipt was aliquoted and stored at -198°C in liquid nitrogen. The passage number of the nonfastidious species of viruses was limited to 10, species Ad40 and Ad41 were propagated to the sixth passage before a fresh aliquot of virus stock was thawed from storage.

2. Viral Growth and Purification

Large amounts of each virus were propagated according to a procedure adapted from Wold, Green and McKay (1978). Cells were washed and infected with the relevant virus suspended in MEM without FCS supplementation at a multiplicity of approximately 1 to 10 $TCID_{50}$/cell (50% tissue culture infection dose per cell). Batches of 10 semiconfluent

150 mm^2 flasks (Corning no. 25120-150, Richmond Hill, Ontario) were infected at one time. The viral inoculum was replaced after 1 to 2 hours with MEM supplemented with 2% FCS and the media changed every third day.

Species Ad40 and Ad41 were propagated in 293 cells, all other species were grown in HEp 2 cells. The infected cells were checked every day with an inverted microscope for the appearance of cellular cytopathic effect. In adenovirus infection this takes the form of swollen, refractile cells and cellular clustering, typically in the shape of a bunch of grapes. On the fifth day post infection, or once the cytopathic effects reached 3-4+ (involving 75 to 100% of the cells), the cells were harvested using a rubber policeman and collected in 50 ml tubes. After 10 minutes centrifugation at 800 g (1500 rpm, Sorvall RT600 centrifuge) at 4°C the cells were pooled in a volume of supernatant corresponding to 1 ml per flask. The remaining supernatant was titred to assess the concentration of infectious virus and used as stock for infection. Each batch of cells was subjected to 5 cycles of freeze-thawing, then clarified by two rounds of centrifugation at 800 g and 12,000 g (10,000 rpm, Sorvall RC-S8 superspeed centrifuge). One ml of clarified cell lysate was put aside for confirmation of identity by neutralization and restriction analysis prior to use. The remaining cell lysate was diluted in fresh MEM and homogenized in an ice waterbath in the 90 ml tube of a Sorvall omnimixer (Cat. no. 17105, Norwalk, Connecticut) with an equal volume of freon (trichloro, trifluoro ethane; Fisher Scientific Inc., no. T180) for two 30 second bursts to remove lipids. Freon and aqueous phases were separated in 50 ml (Falcon 2070, Becton Dickinson, Lincoln Park, New Jersey 07035) tubes at 800g for 3 minutes. Cesium chloride (CsCl) (optical grade, Sigma Chem.

Co., no. 3139, P. O. Box 14508, St. Louis 63178) was added to the supernatant until a density of 1.344, at which adenovirus particles band, as measured by (Bausch and Lomb 33-45-58) refractometer, was reached. Five ml of the treated cell lysate was added to 50 Ti rotor tubes (Beckman, Cat. No. 326 221) which were balanced and filled with mineral oil through the cap. The caps were tightened and the tubes centrifuged at 45,000 rpm (25,000 g) for 48 hours at 4°C in a (Beckman model 637 565) ultracentrifuge. The virus, visible as an iridescent blue band in the centre of the tube, was taken off using a fractionator (Beckman no. 343890) driven by a peristaltic pump (LKB 2120 Varperpex II pump, Fischer Scientific P4713) and carefully applied to the top of a continuous density gradient in a clear 14 ml cellulose nitrate tube (Beckman, cat. no. 344089). The continuous density gradients were formed by mixing equal quantities of solutions of 1.2 g/ml and 1.5 g/ml CsCl in the wells of a gradient maker (Hoefer Scientific Instruments, 654 Minnesota Street, San Francisco, California 94107). The preformed gradients were ultracentifuged at 35,000 rpm (17,500 g) in a SW40 rotor (Beckman no. 331301) for 90 minutes. The single virus band was taken off and dialysed several times against 40 mM Tris-HCl pH 8.0/20 mM $MgCl_2$. Dialysis tubing was prepared as in appendix A. An assessment of the yield of virions was made of some preparations according to the estimation that an absorbance value of 1.0 at 260 nm is given by 1.1×10^{12} particles per ml (Maizel, White and Sharff, 1968). Dialysed virus was stored at 4°C until processed for DNA.

Species Ad8 and Ad12, as well as the fastidious Ad40 and Ad41, were found difficult to passage successively and poor yield commonly resulted from pools of up to three

batches, totalling 45,00 cm^2 of HEp 2 or 293 cells, pooled for extraction. Attempts to implicate the presence of proteases in the cell extract or harsh solvents in the degradation of virus were unsuccessful. The possibility that repeated round of freeze-thawing were damaging virions was also disproved. No evidence that viral decreased during purification was found. It was concluded that the poor yield was an intrinsic characteristic of these virus species and a variety of different protocols were assayed in an effort to improve the yield over that of standard methods (Wold, Green and McKay, 1978).

Hela, rhesus monkey kidney cells and A549 cells were cultured as an alternative to HEp 2 or 293 cells. A549 cells were found to have several advantages; the flat, even monolayer facilitated the visualization of CPE while the growth rate, lower than HEp 2 cells, allowed development of greater titres of slow growing viruses such that the yields of Ad31 and Ad40 virions from A549 cells were consistently greater than those of other cell lines. A549 cells were also found amenable to cocultivation, where virus infected cells were trypsinized and mixed with fresh cells. This technique, routinely used for growth of CMV (Benyesh-Melnick *et al.*, 1964) allowed a major improvement in yield of fastidious adenoviruses Ad40 and Ad41.

The cocultivation technique was performed by mixing infected and target cells by stir bar in a small volume of L15 medium augmented with 20% fetal calf serum for prolonged periods at 37°C before being allowed to settle in tissue culture flasks. A549 cells could be mixed at a 2:1 ratio with infected cells at a concentration of approximately 10^8 cells

per ml and commonly grew into a completely infected monolayer within several days. This was a major advantage over 293 cell culture since trypsinized infected 293 cells did not settle and the process of mixing proved too traumatic for most 293 cells even after inhibition of trypsin with FCS. The end point of each dilution series were confirmed by electron microscope and/or enzyme immunoassay analysis of the culture well supernatant. Cocultivation had a minimal effect on the yield of Ad2 but generated a relatively large increase in the titre of the fastidious adenoviruses. The cocultivation technique, used in combination with direct extraction of viral DNA with phenol (Shinagawa *et al.*, 1983), enabled purification of the viral DNAs that could not previously be achieved by extraction of several times the surface area of cells cultured and prepared by conventional protocols.

3. Determination of Infectious Titre of Virus Stocks

To assess the concentration of infectious virus in cell culture supernatants four monolayers of HEp 2 or 293 cells in 24 well (Nunc, Nuclon Multidish, no. 143982) plates were exposed to a series of log 10 dilutions of clarified cell supernatant. The infected monolayers were incubated at 37°C and monitored daily for cytopathic effect. The method of Reed and Muench (1938) was used to determine the viral titre in $TCID_{50}$ (50% tissue culture infectious dose) per ml from the proportion of the four cell sheets showing cytopathic effect at the greatest infectious dilution of culture supernatant. Culture supernatants of unlysed 293 cells infected with Ad40 or Ad41 rarely contained sufficient infectious virus for useful stock and it was necessary to first release the cell associated virus by several rounds of freeze-thawing prior to the assessment of fastidious

adenovirus cultures intended for use as stock virus. The end point of Ad40 and Ad41 dilution series were confirmed by examination of lysed cell supernatants from the least dilution in which CPE was evident and from several additional dilutions by electron microscopy and/or enzyme immunoassay. The concentration of culture supernatants and passage history of each virus were recorded. Titred supernatants were stored at -70°C until required for infection of flasks of HEp 2 or 293 cells.

4. Preparation of DNA

Dialysed virus was first treated with 50 μg/ml of DNAse (RNAse free, Boehringer Mannheim 776785) and 50 μg/ml RNAse (Boehringer Mannheim 109142) at 37°C for 1 hour. To prevent further nuclease activity EDTA was added to 20mM along with 50 μg/ml of proteinase K (Boehringer Mannheim 745723) and the temperature raised to 65°C for 1 hour. 350 μl of the preparation per Eppendorf® tube (1.5 ml microfuge tube, Can-Lab C3515-100, Mississauga, Ontario) was then extracted by repeated inversion with an equal volume of distilled phenol aqueous (Fischer Scientific A931-1). Phenol was prepared according to the protocol in appendix B. A further 350 μl of chloroform, mixed 20:1 with isoamyl alcohol to prevent foaming, was added to increase surface tension and the two phases were separated by microcentrifugation (Beckman Eppendorf Centrifuge 5414) for 3 minutes. This extraction was repeated with both phenol and chloroform and then with chloroform alone to extract remaining phenol. Finally the DNA was precipitated by addition of 1/10th volume of 3M sodium acetate pH 5.6 and 2 volumes of 100% ethanol and holding at -20°C overnight or -70°C until viscous (30-60 minutes). The precipitate was pelleted by 30 minutes microcentrifugation at 15,000 g and washed

in 70% ethanol. The ethanol was decanted and the pellet air dried. The DNA was dissolved in 1 ml 10 mM Tris pH 7.6/1 mM EDTA (TE) and the quantity and purity checked by the absorption values (Beckman spectrophotometer model DU-40) at 250, 260, and 280 nm. A ratio of 280 to 260 nm readings smaller than 0.55 indicates RNA or protein contamination, and a ratio of 250 to 260 nm readings larger than 0.9 indicates the presence of phenol. The optical density of the solution at 260 nm, multiplied by a factor of 48, gives an approximate value of DNA concentration in μg/ml. The concentration was determined more accurately by comparison of the intensity of an aliquot of the DNA, stained with ethidium bromide, with a range of known quantities of undigested λ phage or plasmid DNA in agarose electrophoresis (see Figures 21 and 22). Identity of the DNA was confirmed by comparison of the *Bam*HI and *Hin*dIII restriction enzyme patterns on electrophoretic gels with those published by Adrian *et al.*, (1986). DNA that failed to digest with restriction endonucleases was dialysed against distilled water and retested. Finally, DNA preparations were precipitated and redissolved in TE to a concentration of 100 to 200 ng/μl.

Cellular DNA preparations were purified from 25 cm^2 flasks of uninfected HEp 2 cells lysed in PBS containing 1% SDS and 250 μg/ml proteinase K. The DNA was treated with DNAse-free RNAse and extracted several time in succession with phenol, followed by chloroform, and precipitated. The DNA pellet was washed with 70% ethanol, resuspended in 1.5 ml TE and sonicated at 30% output (Fischer, Sonimax model) for two 30 second burst in ice water. The DNA was then dialysed against several changes of TE and the concentration evaluated by optical density.

5. Electrophoresis and Electroelution

DNA aliquots of 200 ng to 2.5 μg were incubated with 5-10 units of the appropriate restriction enzyme and 2 μl of 10x concentrated buffer at 37°C for 3 hours in a total reaction volume of 20 μl. Enzymes *Ava*I, *Kpn*I and *Sac*I were incubated with low salt buffer (Maniatis, Fritsch and Sambrook, 1982) the ingredients of which are specified in see appendix C. Enzymes *Bam*HI, *Cla*I, *Hinc*II, *Hind*III, *Pst*I, *Sau*3A were incubated in medium salt buffer and enzymes *Bgl*II, *Eco*RI, *Hinf*I, *Nco*I, *Pvu*II, *Sal*I, and *Xho*I in high salt buffer while *Sma*I was incubated in its own buffer without NaCl. The reaction mixture was mixed with 10 μl of tracking dye (see appendix D) and pipetted into wells in a 150 ml agarose slab gel. Reaction mixtures of digested viral genomic DNA, containing fragments of 0.5 to 10 kb were electrophoresed in 0.8% agarose gels. Plasmid DNA or DNA fragments of smaller size were run on gels containing higher concentrations of agarose specified in the legend to the figures. 0.5 to 2 μg quantities of λ DNA (Boehringer Mannheim 208396) digested with *Hind*III or both *Hind*III and *Eco*RI were added to wells on either side of the test DNA to act as molecular size markers. The sizes of λ DNA fragments in molecular weight standards are given in appendix E. Gels were run in a BioRad DNA Sub Cell apparatus (BioRad cat. no. 170-4300, Mississauga, Ontario) in 1500 ml of 0.089 M Tris borate pH 8.0/10 mM EDTA pH 8.0 (TBE) with 0.5 μg/ml ethidium bromide. 0.8% agarose gels were run at 25 volts/5 milliamps overnight, gels containing higher concentrations of agarose required higher voltages, as specified in the legend. When the dye front neared the end of the gel the gel was transferred to an ultraviolet transilluminator (Ultraviolet Products Inc., San Gabriel, California) and photographs were taken with approximately 8 second exposures on Type

57 Polaroid Film using a Polaroid MP-4 land camera (Johns Scientific, Winnipeg).

Restriction fragments destined for use as probes were cut out of the original gel and run at least once through gels composed of low concentrations, 0.3 or 0.5 g per 100 ml, of SeaKem agarose (FMC Bioproducts, no. 50072, Rockland, ME). Small blocks of agarose containing the fragment band were laid on the gel tray and a new agarose gel poured around the fragment. This process was repeated up to three times in an attempt to remove all traces of spurious DNA. Fragment bands were usually excised from the final gel prior to photography to protect the DNA from UV irradiation. Electroelution was conducted on excised agarose blocks sealed in a dialysis tube oriented perpendicular to the flow of current in 0.5x TBE for 1 to 2 hours at 75 volts/20 milliamps. The electroeluted DNA was washed out of the tubing with TE and extracted several times with butanol to remove ethidium bromide and concentrate the sample in a smaller aqueous volume. The butanol was removed by extraction with chloroform and the DNA precipitated, washed in 70% ethanol and resuspended. Evaluation of the DNA concentration was performed by electrophoresis of an undigested aliquot and comparison of the intensity of the band to λ or plasmid DNA standards.

6. Hirt and Shinagawa Methods of DNA Extraction

The following protocol was adapted for the rapid identification of virus from the original description (Hirt 1967). Round-bottom, semi-confluent tubes (Corning no. 25200), seeded with 40,000 HEp 2 or 250,000 293 cells several days previously, were infected with 100 or 200 μl of viral supernatant, depending on the concentration of virus. Seven

days post infection or once 3-4+ CPE was reached the media was discarded and the cells carefully resuspended by agitation in 350 μl of 100 mM Tris/10 mM EDTA pH 8.0 and transferred to a microcentrifuge tube. The suspended cells were lysed by the addition of 30 μl of 10% sodium dodecyl sulphate and 275 μg of proteinase K. The cells were incubated at 37°C with occasional mixing until visible traces of cell structure had dissolved. The salt concentration was adjusted to 1 M by addition of 100 μl of 5 M NaCl and the tubes incubated at +4°C overnight to precipitate the large molecular weight cellular DNA. The cellular DNA was pelleted by microcentrifugation for 30 minutes at 15,000 g and the supernatant carefully transferred to a fresh tube. 10 μg RNAse was added and the supernatant incubated for 1 hour at 37°C. The preparation was then extracted with phenol to remove the protein and the DNA precipitated as previously described. The viral DNA was washed twice in 70% ethanol. The salt free pellet was resuspended in 50 μl TE and 5-10 μl samples of the DNA digested with *Bam*HI and *Hind*III enzymes before overnight electrophoresis. The resulting banding pattern was compared to the 41 prototype patterns published by Adrian *et al.* (1986) for identification.

The Shinagawa method (Shinagawa *et al.*, 1983) also disrupted both virus and cells simultaneously with SDS and separated the viral DNA from the cellular components. Separating cellular DNA from viral DNA by precipitation with high molarity salt (Hirt, 1967) gave viral preparations with quite clear restriction enzyme digestion patterns (e.g. Figure 13). A smear of cellular DNA accompanied most Hirt extracts and could occlude higher molecular weight fragments (e.g. Figures 61, 62). The technique developed by

Shinagawa *et al.* (1983) employs phenol to separate viral DNA from cellular lysates by means of the adenovirus terminal protein. This 55 kilodalton protein attached to the last residue of both 5' ends of the linear DNA (Rekosh *et al.*, 1977) is used to sequester the viral DNA in the phenol phase. DNA prepared by this means was visibly clearer in restriction analysis than Hirt extracts and was more suitable for restriction analysis.

Twenty five cm^2 flasks of fastidious adenovirus infected cells were scraped with a rubber policeman and the cells transferred to an Eppendorf® tube. SDS and EDTA were added to concentrations of 1.5% and 0.1 M, respectively, and the mixture incubated for 30 minutes at 37°C. The mixture was divided into 0.75 ml aliquots and extracted with an equal volume of phenol by careful inversion. After centrifugation for 5 minutes, the aqueous was discarded and the phenol phase extracted twice with an equal volume of TE buffer to reduce the RNA contamination. 0.6 volumes of cold absolute alcohol were added and the DNA precipitate pelleted within five minutes to avoid the protein which precipitated more slowly. The pellet was washed with 70% alcohol and treated with RNAse and then proteinase K as previously described. In the original description (Shinagawa *et al.*, 1983), 2 to 5 μg of viral DNA resulted from extraction of 38 cm^2 surface area of Ad2 infected cells. Yields of Ad40 or Ad41 from equivalent amounts of cells were found to be very much poorer than this but the technique was amenable to expansion to large scale preparations with batches of 293 cells or cocultivated A549 cells and large amounts of the DNA of fastidious viruses were produced. Frequently, cells showing an advanced degree of CPE failed to yield any DNA precipitate from the phenol phase in the sequestration technique and the virus extract was lost. The technique did

occasionally yield large amounts of viral DNA ostensibly free of cellular DNA from fastidious adenovirus preparations and was found of great benefit.

7. Neutralization

Antisera to adenovirus species Ad1, Ad2, Ad3, Ad4, Ad7, Ad8, were obtained from ATCC (VR 1078, VR 846, VR 847, VR 1081, VR 1032, VR 1083, VR 1084, and VR 1085 respectively) and used to confirm the identity of virus species in stock cultures. Antisera to Ad40 and Ad41 were not available. All antisera were standardized to an arbitrary concentration of 100 units/ml, where 1 unit is the quantity required to neutralize 1 $TCID_{50}$ of virus stocks of known purity and concentration. In preliminary tests, 1 ml aliquots of a series of double dilutions of the antisera were incubated with 100 $TCID_{50}$ of the relevant stock virus prior to addition to semiconfluent tubes of HEp 2 cells. Based on the results of these tests the stock antisera was then diluted with phosphate buffered saline to the lowest dilution that completely neutralized the 100 $TCID_{50}$ of stock virus. In subsequent neutralization tests, 200 μl of quantified antisera containing 20 U, was incubated for 1 hour at 37°C with 100 μl of culture supernatant prior to addition to round-bottom, semi-confluent HEp 2 tube cultures seeded with 40,000 cells several days previously. The CPE was monitored twice daily and a retardation in growth relative to the control greater than 2+ CPE, equivalent to a delay in infection of more than 50% of the cells, was regarded as indicative of identity. Equivocal results were repeated with twice the concentration of antiserum and doubling dilutions of viral inoculum.

8. Southern Blot

An adaption (Reed and Mann, 1985) of the Southern (1975) blot was used to transfer DNA from agarose gels to a nylon matrix prior to hybridization. Gels were gently agitated at room temperature for 30 minutes in 500 ml of a denaturation solution consisting of 15 M NaCl/0.5 M NaOH. The denaturation solution was decanted and 500 ml of fresh solution applied and the gels rocked again for 30 minutes. Gels were then washed twice for 30 minutes in 250 ml of a transfer solution of 1M ammonium acetate/0.02 M NaOH. The DNA was transferred to a nylon membrane (Micron Separations Inc., 0.45 μm Mesh, Fischer Scientific, Cat. No. NJ4HY00010) in 1500 ml of the same transfer solution through an arrangement of wicks (Whatman 3 mm chromatography paper, Cat. no. 3030917) and paper towels as described by Maniatis, Fritsch and Sambrook (1982). The gel was restained and checked for residual DNA before the membrane was briefly rinsed and baked at 68°C for 6-16 hours. Blotted membranes were sealed in a plastic envelope using a Sears Seal a Meal® apparatus and stored at 4°C until hybridized.

9. Hybridization

Membranes in a plastic envelope were prehybridized at 68°C in a rocking waterbath in 15 mls of filtered 0.5% sodium dodecyl sulphate (SDS)/6 x SSC (1x SSC = 0.15M NaCl + 0.015 M Na citrate)/5x Denhardt's solution (1x Denhardt's = 0.3% Ficoll 400/0.3% polyvinyl pyrolidone/0.3% bovine serum albumen) containing 100 μg/ml of denatured, sheared herring sperm DNA (Boehringer Mannheim, Cat. No. 223646). The prehybridization buffer was discarded through a clipped corner of the envelope and

replaced with the same solution containing 1 mg of freshly denatured herring sperm DNA and at least 10^7 cpm of radiolabelled probe DNA at a quantity of 1 ml for each 1.5 cm^2 of membrane. The envelope was resealed, enclosed in a second envelope, and rocked at 68°C or 42°C in conditions of high or low stringency, respectively (Maniatis, Fritsch and Sambrook, 1982) for a minimum of 12 hours. Subsequently the membranes were given four 30 minute washes, twice in 2x SSC/0.1% SDS and twice in 0.2% SSC/0.1% SDS at the same temperature as used for hybridization. After blotting excess moisture, membranes were resealed in plastic envelopes and enclosed with X-ray film (Kodak X-OMAT XAR-5, Cat. no. 251 895, Picker International, Winnipeg) between intensifying screens (Dupont Quanta III, no. 224032, Picker International, Winnipeg) at -70°C until the required detail had developed. The comparative strength of hybridization was determined by reading the bands with a densitometer (Model GS-300, Hoefer Scientific Instruments) under an IBM GS360 Data System program (Mind Computers, Winnipeg). Probes were removed from membranes after autoradiography by two 15 minute washes, first in 250 ml 0.4 M NaOH followed by 250 ml of 0.1x SSC/0.1% SDS/0.2 M Tris pH 7.5 in a rocking water bath at 45°C. The membranes were checked for residual radioactivity prior to resealing and storage. Nylon membranes could be hybridized two or three times , with different probes, by the same procedure with little loss of intensity.

10. Radiolabelling of Probe DNA

Initially probes were radiolabelled using a nick translation kit (Cat. No. n5000, Amersham Co., 505 Iroquois Shore Rd., Oakville, Ontario). In this procedure 200 ng

of purified DNA, 2 μl of solution A, 4 μl of solution B and 7 μl of ^{32}P-dCTP (deoxycytosine triphosphate nucleotide phosphorylated with a radioactive isotope in the α position) (supplied as 50 μl/50 mCi = 18.5 MBq, by ICN Biomed Corp., Cat. No. R33004X, P. O. Box 540, Succ. St. Laurent, Monterey, Quebec) were mixed in a total volume of 20 μl and incubated at precisely 16°C for 90 minutes. Solution B contains ATP, a buffer and DNA polymerase 1 which creates nicks in the probe DNA with exonuclease activity, extends the nick in the 5' direction by 3' to 5' endonuclease activity and then incorporates the ^{32}P-dCTP and the nucleotides in solution A to close the nick. Probes radiolabelled by nick translation had a specific activity ranging between 10^7 and 10^8 cpm per μg of DNA.

A higher rate of radionucleotide incorporation was achieved later with probes radiolabelled by random prime labelling (Boehringer Mannheim kit, Cat. no. e3456). In this procedure 25 ng of denatured DNA, boiled for ten minutes and immediately placed in ice water, was mixed with one μl each of solutions of the deoxynucleotides, dATP, dTTP and dGTP, 4 μl of a solution containing the 4096 possible permutations of 6 base primers, 5 μl of ^{32}P-dCTP and 1 μl of the Klenow fragment of DNA polymerase 1 in Tris buffer. This truncated version of DNA polymerase 1 carries the 3' to 5' endonuclease and 5' to 3' polymerase activities but lacks the last 22 amino acids of the C terminus of the full polymerase which carry the 3' to 5' exonuclease activity. The reaction mixture was made up to a total volume of 20 μl and kept at room temperature for 30 minutes. The hexanucleotide primers can attach and initiate strand polymerisation against any single stranded sequence and tend to generate longer stretches of

radionucleotide-containing DNA than nick translation (Scott, 1978).

5' prime end labelling (Boehringer Mannheim kit, Cat. no. D56342) was found to generate the highest cpm in synthetic oligonucleotides manufactured for specific Ad41 identification. This reaction utilizes the enzyme polynucleotide kinase from T4 phage to attach a γ ^{32}P-dCTP (deoxycytosine triphosphate phosphorylated with a radioactive isotope in the γ position) molecule to the 5' terminal nucleotide. The radiolabelling reactions were terminated by the addition of 5 μl of 0.5 M EDTA to reaction mixtures involving random priming and end labelling or 30 μl of 0.2% SDS/2 mg/ml herring sperm DNA/20 mM EDTA to nick translation reaction mixtures. The unincorporated nucleotides were separated from the DNA probe by passage of the mixture through a 5 ml G100 Sephadex (Pharmacia, 17-006-01, 2044, Boul. St. Regis, Quebec) pipette column and the progress of the two peaks of radioactivity was monitored with a Geiger counter behind a perspex shield. The principle behind the separation is that individual nucleotides enter the beads while sequences of nucleotides are excluded by the Sephadex and descend more quickly. 2 μl from each 0.5 ml aliquot collected off the column were analyzed for total radioactivity and trichloro acetic acid (TCA) precipitable activity (DNA without free nucleotides) by liquid scintillation in Aquasol (Dupont, Cat. No. NEF939, P. O. Box 2200, Streetsville, Mississauga, Ontario). The TCA precipitable activity was measured as follows: 2 μl of each aliquot was added to 5 ml ice cold 10% TCA and held for 5 minutes on ice. The precipitate formed was captured on a GFC filter (Whatman cat. no. 35583) and rinsed with a large volume of TCA and a further 10 ml of ice cold absolute ethanol, dried, immersed in scintillation cocktail and counted. Nucleotide free

aliquots were pooled and the probe DNA denatured by boiling for 5 minutes then immediately cooled in ice water. The specific activity of nick translation and 5' end labelling reactions was calculated simply; the cpm sum of 2 μl samples of pooled aliquots, multiplied by 250 and 5 to correct for volume and DNA quantity, gives the cpm/μg of probe DNA. The evaluation of specific activity of random prime labelled DNA has to take into account the DNA synthesized in the reaction and involved a three step calculation beginning with the rate of incorporation. The amount of radioactivity in the total probe preparation was estimated from assessment of an aliquot in the scintillation counter and divided by the quantity of radioactivity initially added to the reaction gave the % incorporation. The amount of newly synthesized DNA was evaluated from the % incorporation multiplied by the amount of radionucleotide added and corrected for the specific activity of the nucleotide. Finally, the incorporated radioactivity, divided by the input and new DNA, gave the specific activity of the probe in dpm/μg of DNA. Radiolabelled DNA was added to the final hybridization reaction if the specific activity exceeded 10^7 cpm/μg so that the concentration of label in the final hybridization was at least 10^6 cpm/ml. This level was always achieved if the DNA used was of sufficient quality to band properly in electrophoresis after restriction endonuclease digestion.

11. Cloning

Plasmids pBR322 and pAT153 were obtained from Boehringer Mannheim (Cat. No. 431238). Plasmid pGEM 3Z was ordered from Promega Biotec (Cat. No.P2131, Bio/Can Sci. Inc., 2368 Dunwin Drive, Mississauga, Ontario). Approximately 2 μg of plasmid

DNA were digested with the same enzyme(s) used to restrict the viral DNA destined for insertion. The plasmid DNA was then extracted with an equal volume of phenol and then chloroform before precipitation with ethanol and 1/10th volume of 3 M Na acetate (pH 5.6) as previously described. The DNA was resuspended at a concentration of 50 ng/μl and two aliquots of 2 μl put aside to test the efficacy of enzymatic digestion and ligation of the DNA. The main part of the DNA was treated with 1 unit of calf alkaline phosphatase (Boehringer Mannheim, Cat. No. 108 138) in high salt buffer (Appendix C) at 22°C for 1 hour. Another 1 unit (where 1 unit is the amount required to dephosphorylate 1 μg of linearized pBR322 DNA) was added and the DNA incubated for a further hour. This process removes the 5' phosphate groups from the two plasmid 5' termini and prevents the plasmid ends from religating to form an intact circle without insert DNA. The DNA was extracted with phenol, precipitated a second time, then resuspended in distilled water at a concentration of 0.05 pM/μl (where 1 pmol is the length of plasmid sequence in base pairs x 660 d x 10^{-12}). 100 ng of phosphatase treated plasmid were put aside to transform 200 μl of competent E. coli as the phosphatase control.

Approximately 5 to 10 μg of viral DNA was digested simultaneously with the same restriction enzyme(s) as used to digest the plasmid. The viral DNA was extracted with phenol, precipitated to remove the enzyme(s), then resuspended at a concentration of 0.1 pmol/μl in distilled water. 2 μl of plasmid DNA and 4 μl of viral DNA were mixed, in a ratio of 4 pmol:1 pmol to increase insert to vector ligation efficiency, in a total volume of 20 μl of ligation buffer (see appendix G) containing 2 units of T4 DNA ligase

(Boehringer Mannheim, Cat. No. 481 220). The DNA aliquots put aside to monitor ligation and phosphatase reactions were also treated with 2 units of T4 DNA ligase in 20 μl of ligation buffer. Ligation was conducted at 16°C for 36 to 48 hours before the DNA was extracted with phenol, precipitated and resuspended at 10 ng/μl of combined plasmid and viral DNA.

Two hundred μl aliquots of frozen competent E. coli JM109 were thawed rapidly in a 37°C water bath and transformed with 10 to 20 ng aliquots of DNA. The ligated test DNA was added to five to ten tubes while the DNA put aside for testing the efficiency of restriction enzyme digestion, ligation and phosphatase digestion, as well as 10 to 20 ng of intact plasmid DNA were added to a pair of competent E. coli aliquots. Each tube was then vortexed briefly, incubated on ice for 30 minutes and heat shocked by immersion in a 42°C waterbath for 2 minutes. 400 μl of warm LB broth, composed of 1.5% low endo osmotic agar type I (Sigma Chem., No. A-6013) in 1% trypticase/0.5% yeast extract (Difco, Nos. 0123-01 and 0127-01)/1% NaCl, was added and each tube incubated for 1 hour at 37°C to allow expression of antibiotic resistance.

Two hundred μl transformed cells were plated as a lawn on LB plates supplemented with the appropriate antibiotic (50 μg/ml ampicillin or 12.5 μg/ml tetracycline). Colonies were visible 16 to 24 hours after incubation at 37°C in an inverted position. In this system, intact plasmid resulted in approximately 1 x 10^8 transformants per μg DNA. If the various reactions were performed efficiently, the plasmid only digested with restriction enzyme(s) resulted in about 10 colonies per μg of DNA while the religated plasmid DNA

gave approximately 5 x 10^6 transformants per μg. The plasmid ligated after phosphatase treatment, sheared of phosphate groups on the 5' termini, gave a negligible level of transformants. The different amounts of transformation of the control samples results from the relatively poor ability of linear DNA to cross the bacterial membrane.

Colonies transformed with pGEM 3Z plasmids were transferred to media additionally supplemented with 40 μg/ml X-gal (BRL, Cat. No. 5520-UB, Gaithersberg, MD, 20877) and 0.5 mM IPTG (isopropylthio-D-galactoside) (BRL, Cat. No. 5529-UA). The pGEM 3Z plasmid has a B galactosidase gene whose product will convert X-gal into a blue compound, when induced by IPTG, if the gene has not been disrupted by insertion of viral DNA. Bacteria transformed with plasmids containing viral DNA were differentiated as white colonies from the blue colonies transformed with intact plasmid DNA. Bacterial transformants containing viral DNA inserted into pBR322 and pAT153 plasmids were identified by replica plating. The underside of pairs of LB plates, one supplemented with 100 μg/ml ampicillin, the other with 25 μg/ml tetracycline, were scored with a grid of squares. Transformant colonies were replica plated to equivalent squares on pairs of plates. Organisms containing viral DNA inserted in one of the antibiotic resistance genes of the plasmid were identified by the inability to grow on one of the LB plates.

The identity of the inserted viral fragment was then found by matching bands produced by electrophoresis of Quick Plasmid Preparations of each colony to bands of viral DNA digested with the same restriction enzymes. Large quantities of plasmid DNA for analytical purposes were prepared by Large Plasmid Preparations. Bacterial clones were

grown overnight in triplicate in 1.275 ml of LB broth at 37°C, vortexed with 225 μl glycerol (15%) and frozen at -70°C. Subcultures were chipped from a stored aliquot with a cold, sterile loop. Stored clones were plated out and regrown from a single colony every six months.

12. Competent E. coli

Eschericia coli JM109, a Rec A variant of strain JM103 (Messing *et al.*, 1981) was obtained from Promega Biotec. 25 ml of LB broth was inoculated with a single colony and incubated overnight at 37°C. The cells were diluted in 1 litre of LB broth and shaken at 37°C till an absorbance between 0.4 and 0.5 ODU at 550 nm was reached, in approximately 5 hours. The culture was chilled on ice and then centrifuged in 250 ml capacity tubes (Nalgene, Cat. No. 3123-0250, Fischer, Scientific Co., Winnipeg) in a GSA rotor (DuPont, Mississauga, Ontario) for 10 minutes at 4°C in a Sorvall RC-5B centrifuge (DuPont, Mississauga, Ontario) at 5000 rpm (equivalent to 4,000 g). The cell pellet was vortexed to break up clumps and uniformly suspended in 500 ml ice cold 1M $CaCl_2$ by repeated pipetting with a wide bore pipette. The suspended cells were kept on ice for 30 minutes and repelleted at 5000 rpm for 10 minutes at 4°C. The cell pellet was again vortexed and resuspended in 40 ml, ice cold 100mM $CaCl_2$/15% glycerol. Aliquots of 200 μl of cells were distributed in sterile Eppendorf tubes and kept on ice for 12-24 hours. This modification to the original protocol (Mandel and Higa 1970) was found to greatly increase bacterial competency (Dagert and Ehrlich, 1979).

Each batch of competent cells was tested with a range of quantities of intact plasmid to

determine transformation efficiency. Aliquots were frozen in liquid nitrogen and stored at -198°C. The cells were found to retain their original transformation efficiency for at least 6 months.

13. Quick Plasmid Preparation

The alkali method of Birnboim and Doly (1979) was found the most convenient method for preparing 2-4 μg of plasmid DNA for restriction analysis. 1.5 ml of LB broth, supplemented with 100 μg/μl ampicillin or 12.5 μg/ml tetracycline, was inoculated with bacterial transformant clones from stored cultures and incubated overnight at 37°C with shaking. The cells were pelleted by 15,000 g microcentrifugation for 3 minutes, and resuspended in 80 μl of 15% glucose/25 mM Tris-HCl pH 8.0/10 mM EDTA/2 mg/ml lysozyme by repeated pipetting. The mixture was held for 10 minutes on ice, 200 μl of 200 mM NaOH/1% SDS was added and the tubes kept on ice for a further 10 minutes. 150 μl of 3 M sodium acetate pH 5.6 was added and the tube held at -20°C for 10 minutes to precipitate the bacterial cell DNA. The tubes were spun at 15,000g in the microcentrifuge for 15 minutes and the supernatant carefully transferred to a fresh tube. The supernatant was then extracted with phenol and precipitated in ethanol as previously described.

The plasmid DNA pellet was pelleted in the microcentrifuge, air dried and resuspended in 50 μl of TE. 10 μl of the DNA solution was digested for 3 hours with 2 μg RNAse and the same restriction enzyme used for cloning. The digested sample was run on an

agarose electrophoresis gel. The DNA insert, released from the plasmid by the restriction enzyme, was identified by comparison to the bands formed from genomic viral DNA digested with the same enzyme.

14. Large Plasmid Preparation

One litre of LB broth was infected with a chip of a frozen culture of the appropriate bacterial transformant. The appropriate antibiotic (12.5 μg/ml tetracycline or 100 μg/ml ampicillin) was added and the culture was shaken at 37°C overnight in a 2 litre Erlenmeyer flask. Cells were pelleted in 250 ml volume perspex tubes at 5000 rpm (4,000 g) for 10 minutes and resuspended in 20 mls of 15% glucose/25 mM Tris HCl pH 8.0/10 mM EDTA/2 mg/ml lysozyme solution and divided into four 30 ml perspex (Nalgene, Cat. No. 3118-0050) centrifuge tubes. The mixture was held on ice for 10 minutes before 10 ml of 200 mM NaOH/1% SDS were added and the tubes returned to ice for 10 minutes more. 7.5 ml of 3M sodium acetate pH 5.6 was added and the tubes chilled at 4°C overnight. Cellular DNA was then pelleted by centrifugation at 15,000 rpm (14,000 g) for 15 minutes. Supernatant was carefully transferred into four clean tubes and centrifuged for another 10 minutes at 15,000 rpm.

The supernatant was pooled in a clean plastic tube and extracted with an equal volume of phenol as previously described. Phases were separated by centrifugation at 5,000 rpm (4,000 g) for 5 minutes. The aqueous phase was then extracted with chloroform and treated with 0.1 volume 3M sodium acetate and 2 volumes of ethanol before the tube was

chilled on ice for 20 minutes. The plasmid DNA was then pelleted at 15,000 rpm (14,000 g) for 30 minutes. A 5 or 10 ml solution of 1 g/ml CsCl/0.3 mg/ml ethidium bromide, at a density of 1.59 g/ml as measured by the refractive index of 1.3893 at room temperature, was used to resuspend the DNA. The DNA solution was placed in 50 Ti rotor tube(s) (Beckman, Cat. No. 326 221) which were balanced and filled with mineral oil through the cap. The cap was then tightened and the tubes centrifuged at 45,000 rpm (25,000 g) for 36 to 48 hours at 4°C.

The isopycnically centrifuged DNA separated into bands visible under UV light. Nicked circular or linear plasmid bands were concentrated at lower density above the band of supercoiled plasmid DNA. The bacterial RNA formed a pellet as the 1.8 g/ml buoyant density of RNA exceeded the density of CsCl formed in this gradient. The lower band of intact plasmid DNA was taken off by fractionator and shaken with an equal volume of CsCl saturated butanol to remove the ethidium bromide. The ethidium bromide partitions into the upper butanol phase and DNA remains in the lower aqueous phase. The extraction was repeated 3 to 4 times until all traces of stain under ultra violet light had been removed. The plasmid fraction was diluted in 2 volumes of distilled water to avoid precipitating salt and chilled in 6 volumes of ice cold ethanol for 30 minutes. This mixture was centrifuged for 20 minutes at 15,000 rpm and the plasmid DNA pellet was washed several times in 70% ethanol and resuspended in 1 ml TE. Aliquots of purified plasmid DNA, diluted 100 or 1,000 fold, were used for quantitation of the DNA by spectrophotometry. 5 mg of pure plasmid DNA was routinely prepared by this method.

RESULTS

1. The Infectious Titre of Viral Stocks

The titres of three comparable batches of ten 150 cm^2 flasks of cells infected with each of the different species of adenovirus grown are given in Table 3. Ad2 titres typically ranged from 1 x 10^7 to 1 x 10^{12} per ml of culture supernatant. Since the average cell density of HEp 2 cells reaching confluency was counted at 2 x 10^5 cells per cm^2 and 1 ml of media was used for 3 cm^2, this is equivalent to approximately 23 to 3 x 10^4 infectious virions per cell when an estimated 75% and 100% of the cells, respectively, were infected, as observed from the cytopathic effect. The other nonfastidious virus species produced titres ranging from 4 x 10^4 to 1 x 10^{10} $TCID_{50}$ per ml. Grape-like clustering of infected cells, characteristic of the cytopathic effect of adenovirus replication, was observed frequently with Ad41 infected cultures but was seldom seen with Ad40 infected cells. In some experiments with Ad41 and almost always with Ad40, the proportion of cells infected by the undiluted viral inoculum was very low yet several log dilutions of the initial viral stock were infective. The correlation between infection and evident cytopathic effect could not be made in these situations and it was necessary to confirm the endpoints of Ad41 and Ad40 dilution series with electron microscope examination of the lysed cell supernatant and by EIA. Titres of Ad41 could reach 1 x 10^5 but the titres of Ad40 in the supernatant of unlysed cells were most frequently undetectable and it was necessary to prepare stocks of Ad40 with virus released from the cells by freeze-thawing. Ad40 concentrations in the clarified supernatant of lysed cells was documented to be between 10 and 10^4 per ml. The cell density of 293 cells averaged

Table 3. Yield of Infectious Virus

Virus Species	Host Cell	Cell Culture	Hours p.i.	Cytopathic Effect	Viral Titre	Mean Titre
Ad2	HEp2	conventional	72	3+	0.5×10^{7}	
			72	4+	0.66×10^{9}	$10^{8.89}$ $TCID_{50}$
			72	4+	0.5×10^{12}	
	A549	cocultivation	48	4+	0.5×10^{9}	
			120	4+	0.5×10^{10}	$10^{9.6}$ $TCID_{50}$
Ad4	HEp2	conventional	72	3 to 4+	0.5×10^{7}	
			72	2 to 3+	0.5×10^{8}	$10^{7.22}$ $TCID_{50}$
			96	3 to 4+	0.66×10^{8}	
Ad7	HEp2	conventional	72	3 to 4+	0.25×10^{9}	
			72	4+	0.66×10^{9}	$10^{8.98}$ $TCID_{50}$
			72	4+	0.5×10^{10}	
Ad8	HEp2	conventional	96	4+	0.25×10^{4}	
			120	3 to 4+	0.66×10^{4}	$10^{5.13}$ $TCID_{50}$
			120	3 to 4+	0.5×10^{7}	
Ad31	HEp2	conventional	96	2+	0.5×10^{3}	
			168	3+	0.5×10^{4}	$10^{3.22}$ $TCID_{50}$
			120	1 to 2+	0.66×10^{4}	
Ad40	293	conventional	120	1+	0.5×10^{1}	
		*	168	<1+	0.5×10^{2}	$10^{1.15}$ $TCID_{50}$
		*	168	2+	0.66×10^{2}	
	A549	cocultivation*	120	3+	0.5×10^{4}	
		*	120	3+	0.5×10^{4}	$10^{3.58}$ $TCID_{50}$
Ad41	293	conventional	120	3+	0.66×10^{4}	
		*	168	2 to 3+	0.5×10^{5}	$10^{4.22}$ $TCID_{50}$
		*	168	3+	0.5×10^{5}	
	A549	cocultivation*	120	3 to 4+	0.5×10^{6}	
		*	120	3+	0.5×10^{7}	$10^{6.98}$ $TCID_{50}$

The supernatant from harvested infected cells was clarified by centrifugation and applied in log dilutions to confluent monolayers in multiwell plates. The titre in $TCID_{50}$ was calculated by the method of Reed and Muench (1938). * Some cultures of fastidious adenoviruses were lysed by freeze-thaw to release the virus before evaluation of the $TCID_{50}$.

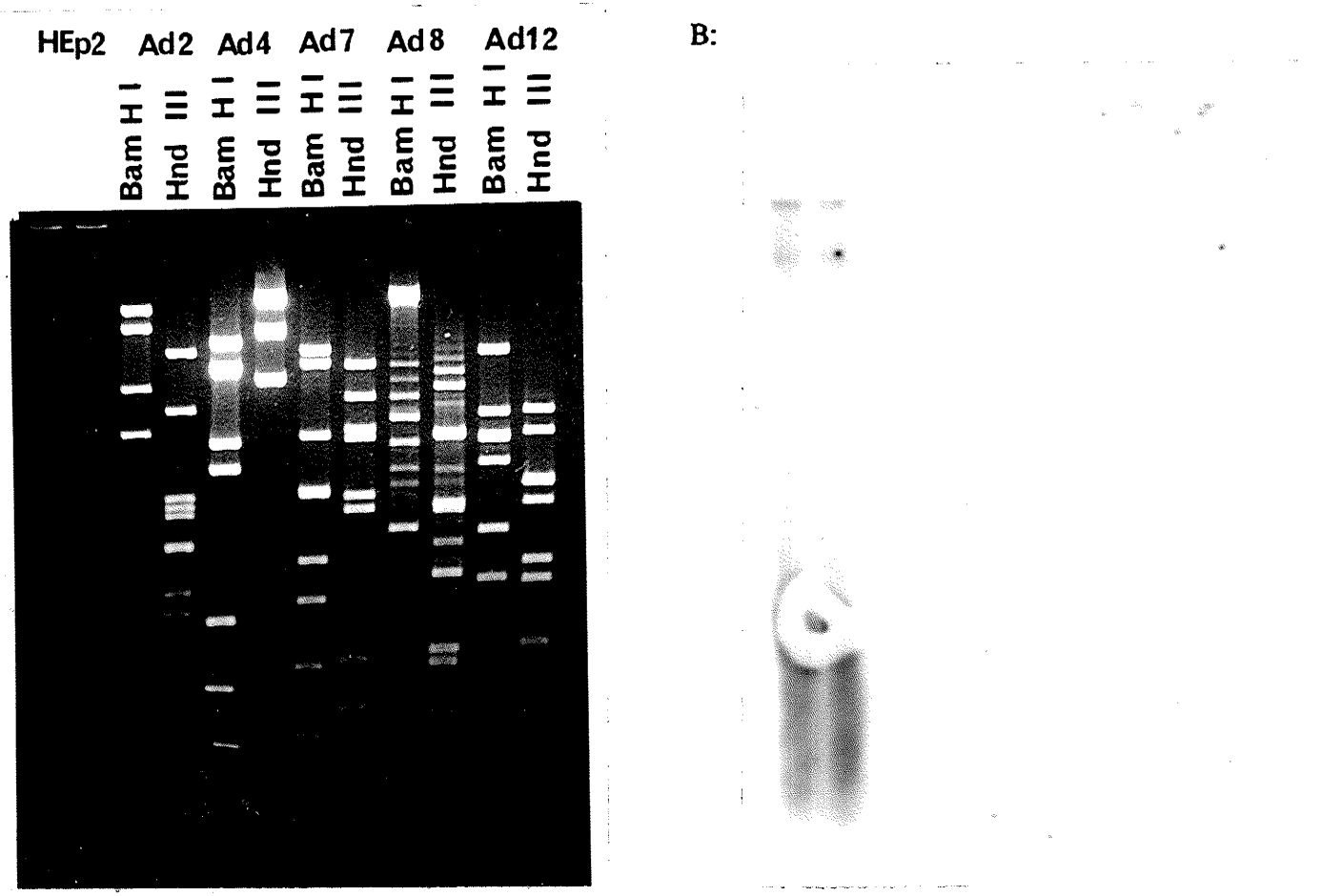
about half of the HEp 2 cell density. Despite this, the yield of Ad40 virions per cell was very low compared to Ad2, ranging from approximately 1.7×10^{-3} to 6.8×10^{-2} virions per cell when about 1% to 50% of the cells were infected, respectively. The highest yields of fastidious adenovirus species were achieved with the cocultivation technique. Infected cells were mixed in close contact with fresh A549 cells before seeding in flasks. This treatment augmented the yield of batches of Ad40 or Ad41 by at least one log relative to conventional culture (Table 3). The increase in yield was relative to the rate of growthof the virus species. Ad2 titre was not increased relative to conventional culture while Ad40 yield with cocultivation commonly exceed the concentration achieved with conventional culture by over 1,000 fold.

2. Hybridization Between Viruses of Different Subgenera.

i) Evaluation of Purity and Quality of Viral DNA

Adenoviruses of one representative of each human subgenus A to F, were purified by centrifugation through two density gradients. DNA extracted from these preparations was used in experiments to find the conserved sequences of the adenovirus genome. Restriction digests of each genomic DNA were analyzed by agarose gel electrophoresis shown in Figure 3 to ensure that the restriction patterns corresponded with those of prototype strains of each species (Adrian *et al.*, 1986). The Ad41 DNA used as a probe against other subgenera in this study is shown in an electrophoresis gel in Figure 44 in three restriction patterns that match those of the prototype strain Tak (Adrian *et al.*, 1983). Ad8 and Ad12 DNA preparations were of poor quality, producing smears and bands of varying intensity. Ad8 DNA appears to have partial or star digest restriction

Figure 3 A and B. Adenovirus Subgenera A to E DNA Preparations Hybridized with a Cellular Probe.

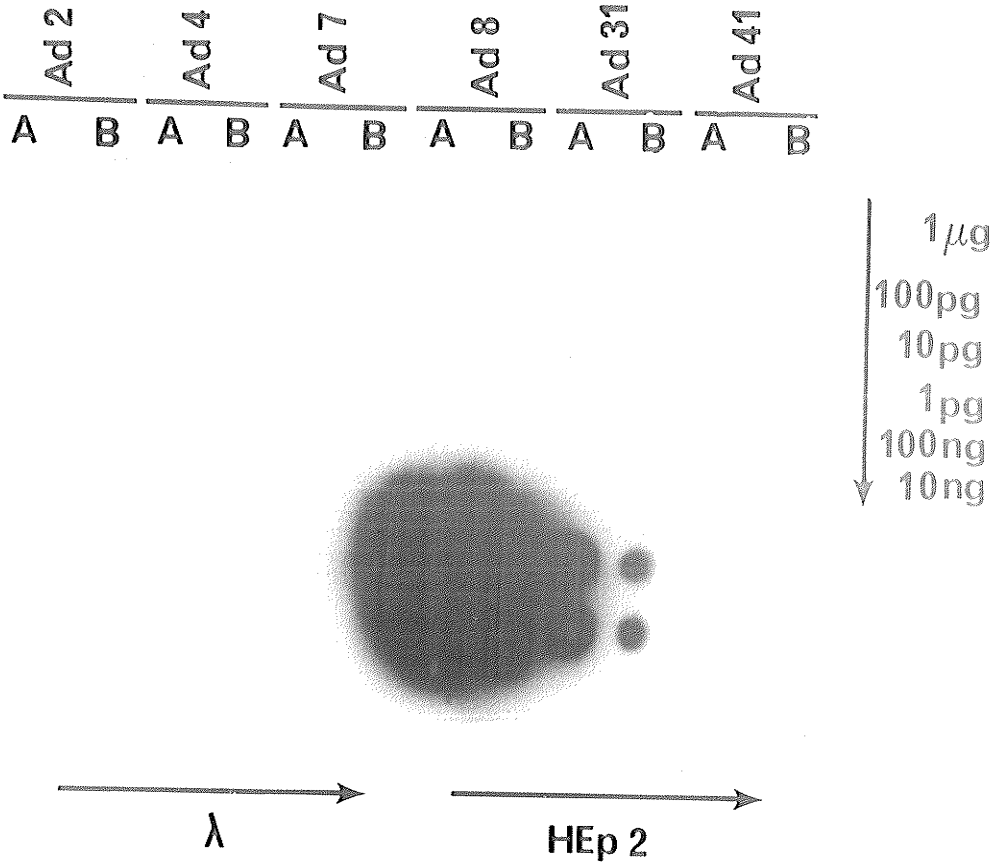


A: 2 μg aliquots of Ad2, Ad4, Ad7, Ad8, Ad12 DNA of subgenus C, E, B, D and A respectively, were digested with *Bam*HI and *Hin*dIII and run on 0.8% agarose overnight at 25 volts. 0.25 μg of *Bam*HI digested HEp 2 cell DNA was run in lanes 1 and 2.
B: The gel was blotted and hybridized with a HEp 2 probe at 68°C. A 76 hour autoradiograph is shown.

problems which were not alleviated by dialysis to remove salt or by purifying Ad8 DNA by Hirt (1967) or Shinagawa *et al.*, (1983) techniques after first passage, and seems to be an intrinsic character of the DNA. The Ad12 DNA preparation failed to digest completely with a number of restriction enzymes. The poor quality of this DNA was related to low titre of initial stock virus and excessive handling of the small DNA yield. It was found expedient to substitute Ad12 with another species of subgenus A, Ad31, of which better stocks were available.

The genomic DNA of each subgenus were also tested for the presence of cellular DNA contamination. HEp 2 cell DNA was electrophoresed in wells alongside the adenoviral DNA in the agarose gel shown in Figure 3. Only the duplicate cellular DNA lanes react when a Southern blot of this gel was probed with uninfected HEp 2 DNA as shown in Figure 3 B. DNA from each subgenus A to F was also applied directly to nylon by dot blot apparatus (Figure 4) and shows no detectable hybridization with a cellular probe while the homologous control reaction is visible at 100 pg of HEp 2 DNA. These preliminary tests showed that the DNA preparations of each virus was (a), from the prototype strain of each species, (b), of sufficient quality to be recognizable to restriction enzymes and (c), free of cellular DNA contamination at levels that would interfere with the hybridization. Cellular DNA in both the probe and target DNA would provide an erroneous source of hybridization signal and it was important to prove that the adenovirus DNA preparations were pure. It was found that DNA that could be cleaved cleanly by restriction enzymes was always amenable to manipulation with DNA polymerase I and other enzymes and presumably was of sufficient quality for unrestrained hybridization.

Figure 4. Adenovirus Subgenera DNA Dot Blot Preparations Hybridized with a Cellular Probe.

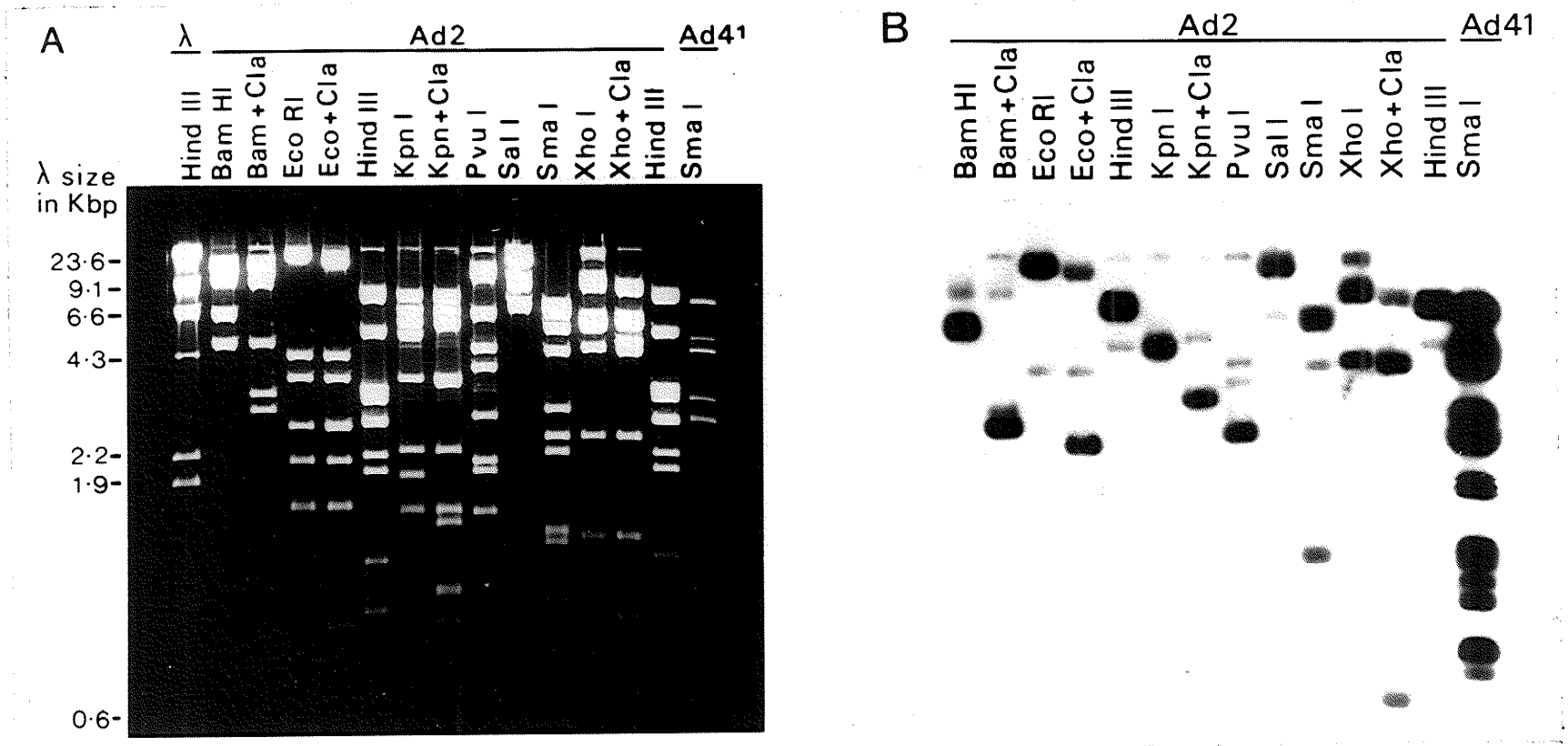


Adenovirus DNA of each subgenus were prepared from virus purified by density gradient centrifugation in CsCl. Adenovirus, λ and HEp 2 DNA were denatured by boiling and applied in duplicate to a nylon membrane by dot blot apparatus. A dilution series of each adenovirus DNA from 1 μg to 10 pg were spotted in vertical columns. λ and HEp 2 controls from 1 μg to 10 pg (6 log dilutions) were spotted in the lower 2 horizontal rows.

ii) The Cross-Reaction of Ad2 DNA with Ad41.

Ad2 virus, purified twice through CsCl, were treated with proteinase K and extracted with phenol until the optical density at 260 and 280 nm formed a ratio of 1.8, consistent with that of pure DNA. 2.5 μg aliquots were digested for 3 hours with a range of commonly used restriction enzymes, namely *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pvu*I, *Sal*I, *Sma*I and *Xho*I. Enzymes that produced a wide range of fragment sizes, without comigrating bands, were used. *Cla*I was used in conjunction with a number of enzymes since the Ad2 sequence has a *Cla*I restriction site close to the N terminus of the hexon gene. Double digestion with *Cla*I, therefore, produced a 3,000 to 4,000 base pair fragment containing the isolated hexon gene, enabling examination of the intensity of hybridization of this specific sequence. A 2.0 μg aliquot of λ DNA, digested with *Hin*dIII to act as molecular weight markers were placed before the Ad2 DNA lanes. 400 ng of Ad41 DNA, digested with *Sma*I to produce short fragments was included in the fifteenth lane as a control. The gel was run overnight at 25 volts (5 ma) and photographed the following morning (Figure 5). The gel was washed twice in NaOH to denature the DNA, and transferred to a nylon membrane in ammonium acetate by Southern blotting. The nylon was then baked at 68°C for 6 hours and hybridized with a genomic Ad41 radiolabelled probe. The membrane was washed under stringent conditions at 65°C and exposed for autoradiography at -70°C. Figure 5 B shows the Ad2 bands that hybridize with Ad41 DNA on a film developed after 48 hours. The visible bands are discrete and can be attributed to fragments in the various restriction enzyme maps presented in Figure 5. Some bands comigrate and the relative intensity of reaction for each band cannot be distinguished. An Ad2 fragment of 670 bp can be seen

Figure 5 A and B. Hybridization of Ad2 DNA Fragments with an Ad41 DNA Probe.



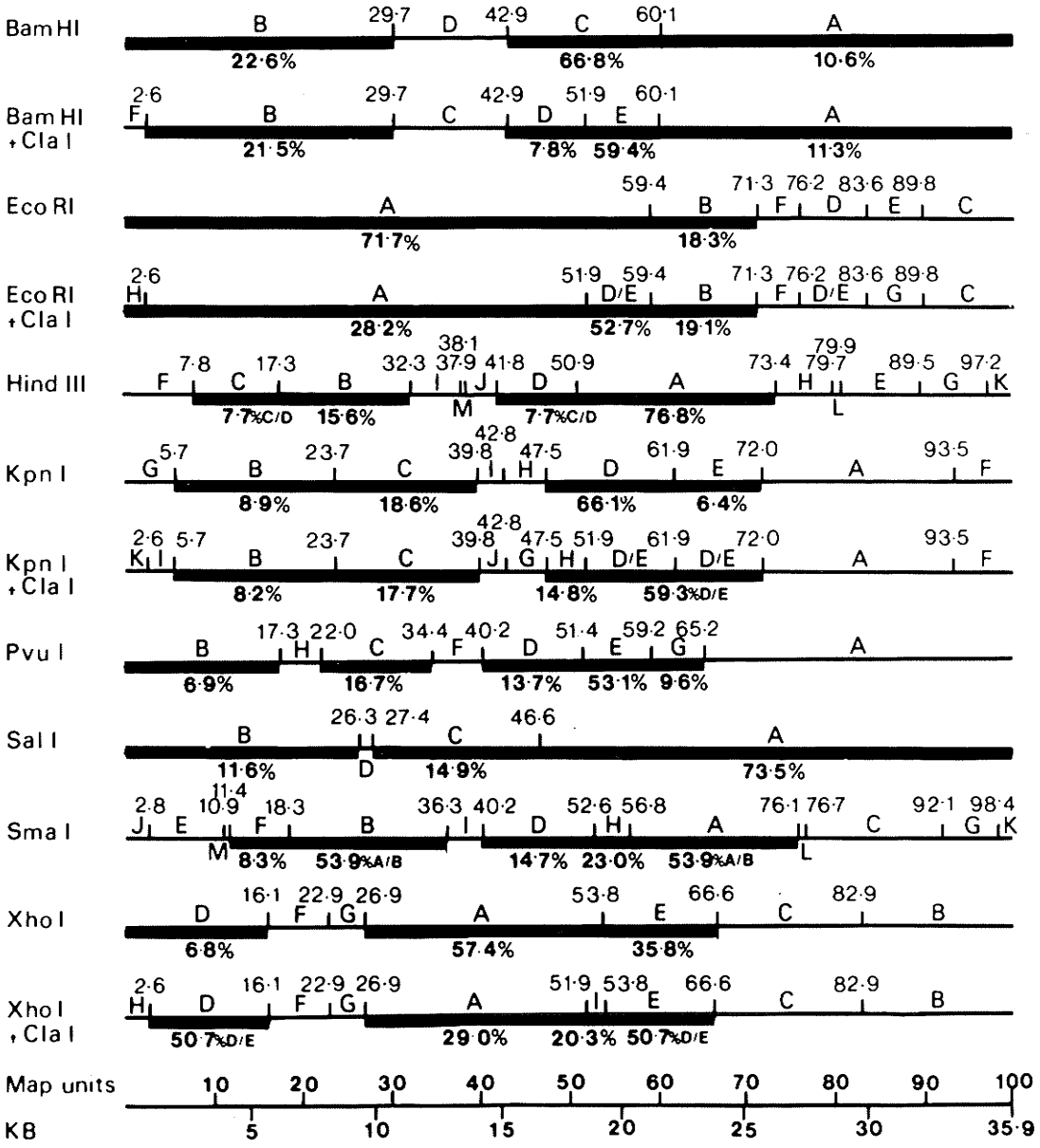
A: Ad2 DNA in 2.5 aliquots was digested with a variety of restriction enzymes. 2 μg of λ DNA digested with *Hin*dIII in lanes 1 and 0.4 μg Ad41 DNA digested with *Sma*I in lane 15 were applied to 0.8% agarose and the gel run at 25 v for 16 hours.

B: This gel was blotted and the membrane enclosed with film for 48 hours after hybridization with an Ad41 genomic probe at 68°C.

hybridizing in the *Xho*I + *Cla*I digest lane, indicating that there was sufficient transfer of small molecular weight DNA. The film was read by a densitometer, set for 100% gain on the maximum control reaction, and a value derived for each positive fragment as a proportion for the total absorbance value for each digest. These % values of the total absorbance were printed below the position of the fragment in the Ad2 restriction maps in Figure 6.

The sequences of the Ad2 genome homologous with Ad41 can be plotted from the fragments that consistently hybridize in the different restriction digests. Fragments that do not hybridize can be discounted as containing any sequence of homology with Ad41 DNA. Subtraction of non-hybridizing fragments leaves sequences that overlap in the different digests, containing the areas of sequence homology between Ad2 and Ad41. Three sequences can be identified in this way, two toward the left terminus from 11.4 to 16.1 and 27.4 to 29.7 map units and a central one from 47.5 to 65.2 map units (Figure 6). A fourth area of hybridization occurs marginally in some lanes from 65.2 map units to 72 map units in Figure 6. The fragments of different areas of the Ad2 genome do not react equally, the fragments enclosing either of the homologous sequences toward the left terminus consistently react less than the central area of the Ad2 genome. The different levels of hybridization intensity are most clearly illustrated with the *Pvu*I digest of Ad2 where a small fragment of the central area of the genome is responsible for more than half of the total absorbance for the lane.

Figure 6. Restriction Endonuclease Cleavage Maps of DNA from Human Adenovirus 2 (Subgenus C) Strain Adenoid 6.



A thick line indicates fragments positive by hybridization, values below line represent the densitometry reading in % absorbance/lane. Ad2 restriction maps were constructed on the genomic sequence from the Genbank library with the Nucalgn computer program.

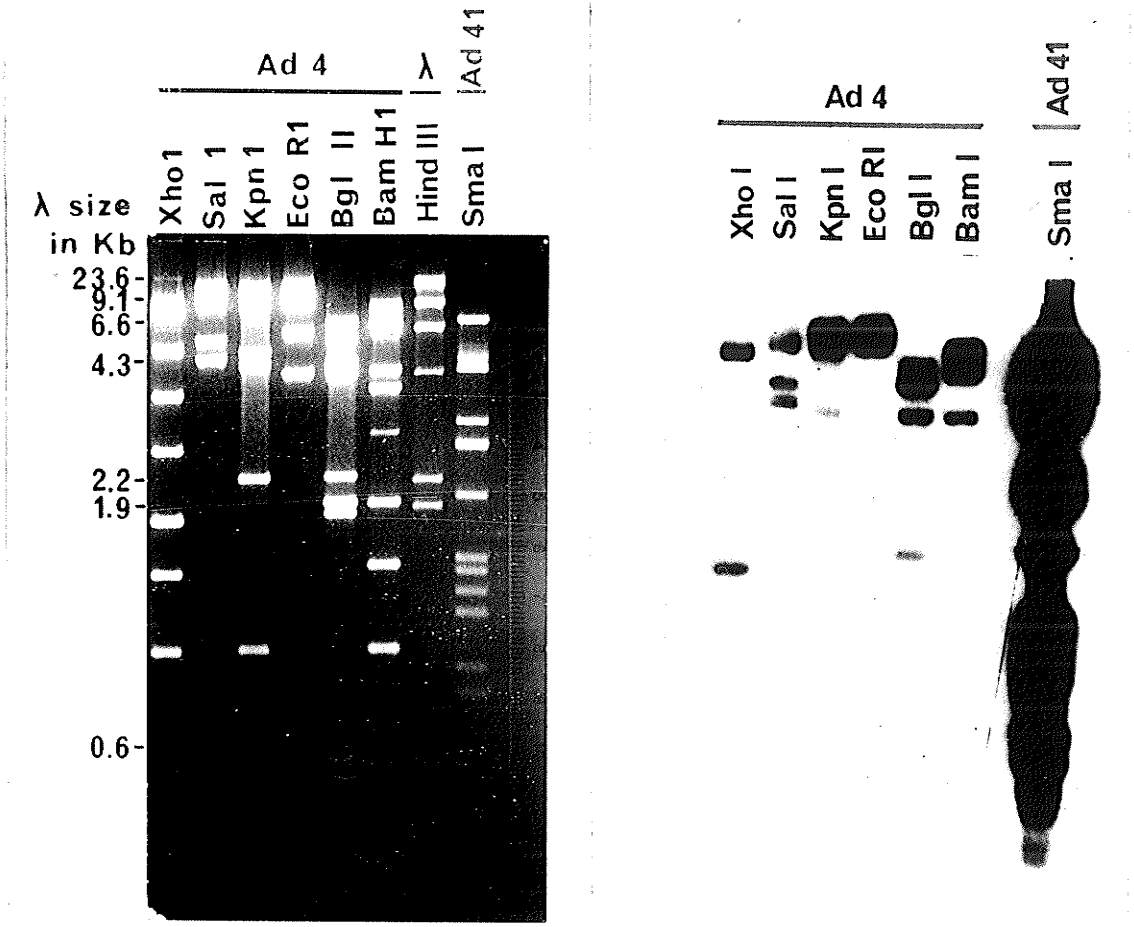
iii) Homology of Ad41 DNA with the Genomes of Ad4, Ad7, Ad8 and Ad31.

DNA of viruses of the remaining subgenera were hybridized after Southern blotting with an Ad41 genomic DNA probe. The amounts of DNA digested per lane of the adenovirus species and the controls vary and are printed in the legend to each figure. Ad4 DNA was digested with the small number of restriction endonucleases for which maps of the genomic restriction site positions have been formulated by Tokunaga, Shinagawa and Padamabhan (1982) and Kitchingman (1982). The DNA digests were electrophoresed as shown in Figure 7. The banded DNA was Southern blotted, hybridized with genomic Ad41 DNA and the homologous fragments detected by autoradiograph. Bands in the film were read by densitometer and the relative intensity of hybridization of Ad4 fragments, measured as the proportion of the total absorbance of their lane, are shown below the position of the fragment in the restriction maps in Figure 8.

Ad7 DNA, digested with a small range of enzymes (Tibbetts, 1977) was electrophoresed in the agarose gel pictured in Figure 9. The limited number of restriction fragments generated limits the size of the area that can be defined as homologous and these remain mostly in the larger fragments of the genome in the autoradiograph of the hybridized Southern blotted gel shown in Figure 9 B. The poor selection of restriction maps available is largely responsible for the limitation in the precision of restriction site cleavage of the genomes. This limitation prevents finer definition of the sequences of homology in the genomes of species other than Ad2 with Ad41. Consequently, the area of homologous reaction defined for the Ad7 genome by the overlap of hybridizing fragments at the base of the restriction maps in Figure 10, is much broader than the

Figure 7 A and B. Hybridization of Ad4 DNA Fragments with an Ad41 Probe.

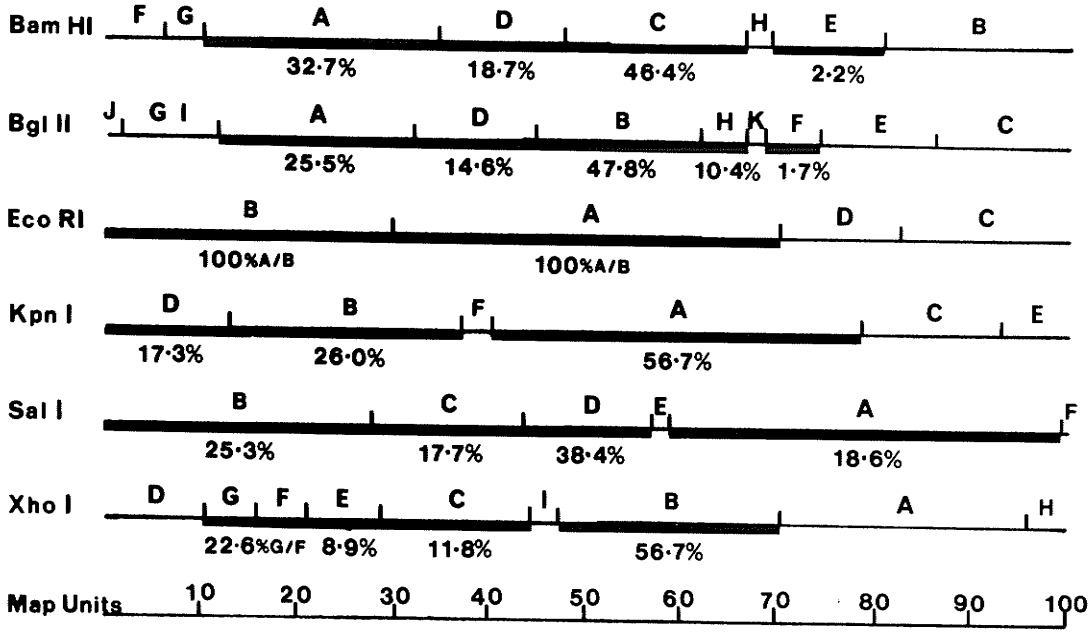
A: B:



A: Ad4 DNA, extracted from virus purified by CsCl density gradient centrifugation, was digested in 2 μg aliquots with enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Kpn*I, *Sal*I and *Xho*I and run in duplicate on a 0.8% agarose gel. 1 μg of λ DNA digested with *Hin*dIII and 1 μg of Ad41 DNA were applied to outside lanes.

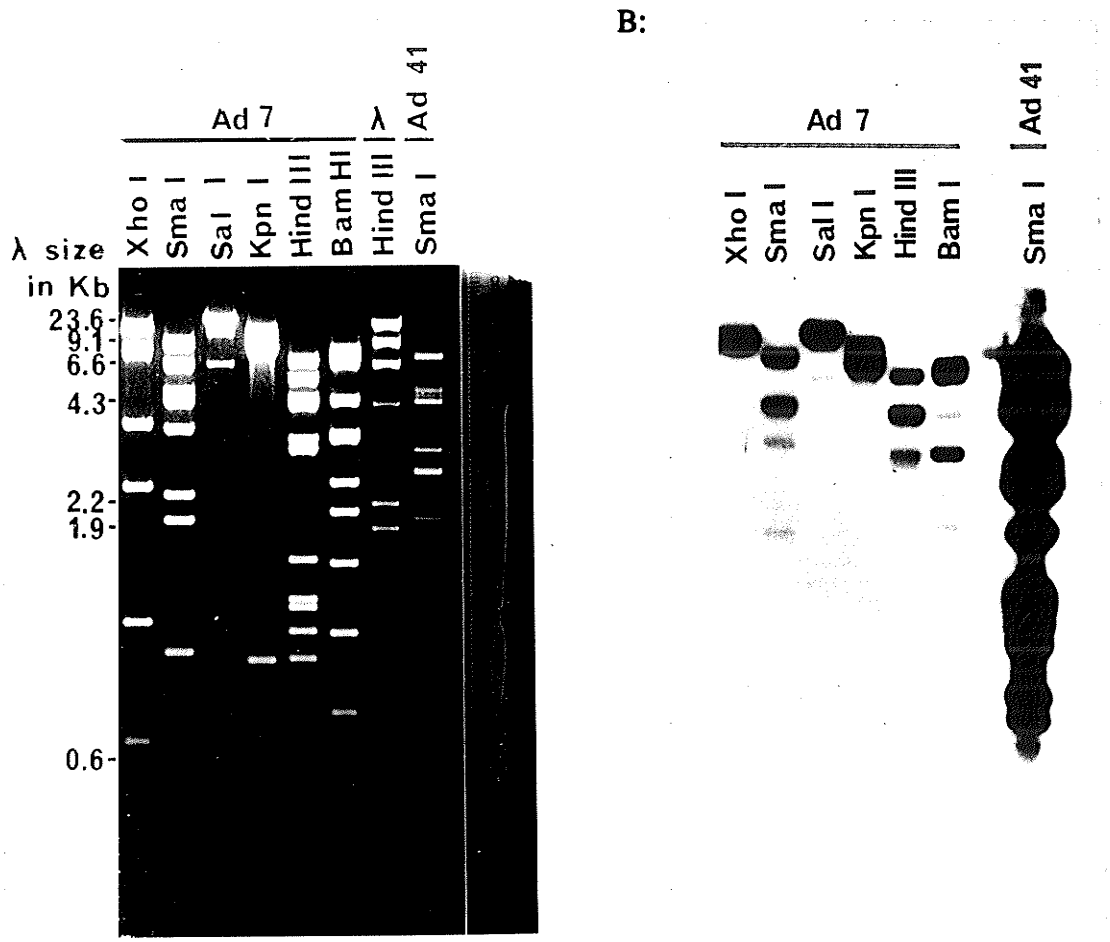
B: The DNA was transferred to a nylon membrane and hybridized at 68°C with an Ad41 genomic probe. The membrane was exposed to film for 24 hours.

Figure 8. Restriction Endonuclease Cleavage Maps from Adenovirus 4, Strain R1-67 (Subgenus E).*



Thick lines denote hybridizing fragments. Densitometry reading in % absorbance/lane is printed below positive fragments. *Maps were taken from Tokunaga, Shinagawa and Padmanabhan (1982) and Kitchingman (1982).

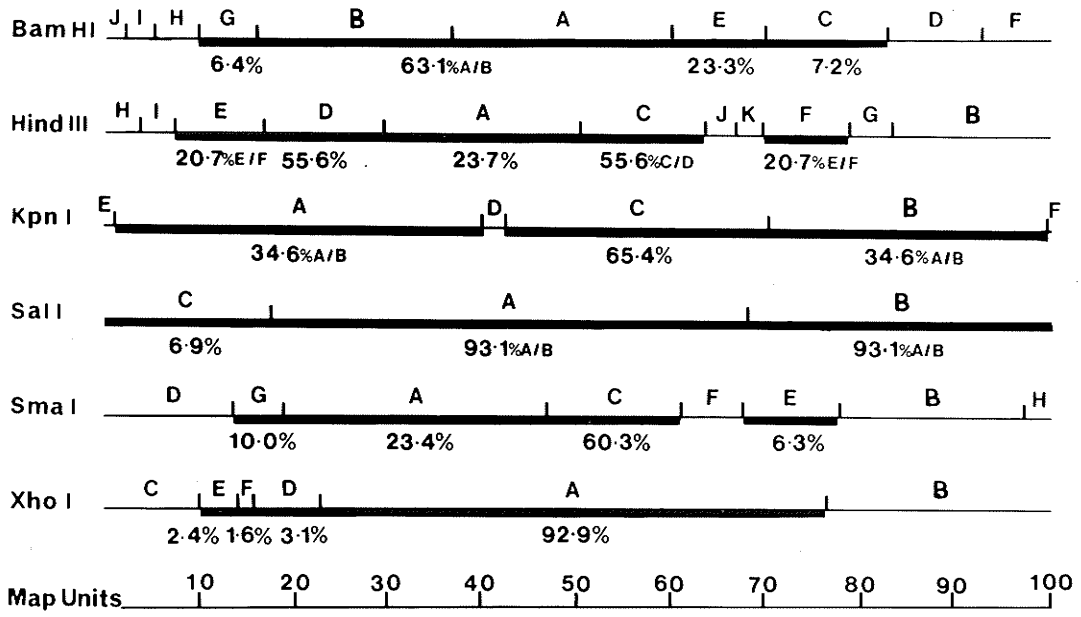
Figure 9 A and B. Hybridization of Ad7 DNA Fragments with Ad41 Probe.



A: Ad7 DNA, extracted from virus purified by density gradient centrifugation, was digested with various enzymes and electrophoresed on an 0.8% agar gel. Lanes 1 to 6, 2 μg Ad7 DNA + *Xho*I, *Sma*I, *Sal*I, *Kpn*I, *Hin*dIII, *Bam*HI; lane 7, 1 μg λ DNA + *Hin*dIII; lane 8, 0.4 μg Ad41 DNA + *Sma*I.

B: Gel A was blotted to a nylon membrane and hybridized with an Ad 41 genomic probe. Film was developed after 72 hours exposure to membrane and photographed at 0.6 magnification.

Figure 10. Restriction Endonuclease Cleavage Maps of DNA from Human Adenovirus 7, Strain Gomen (Subgenus B).*

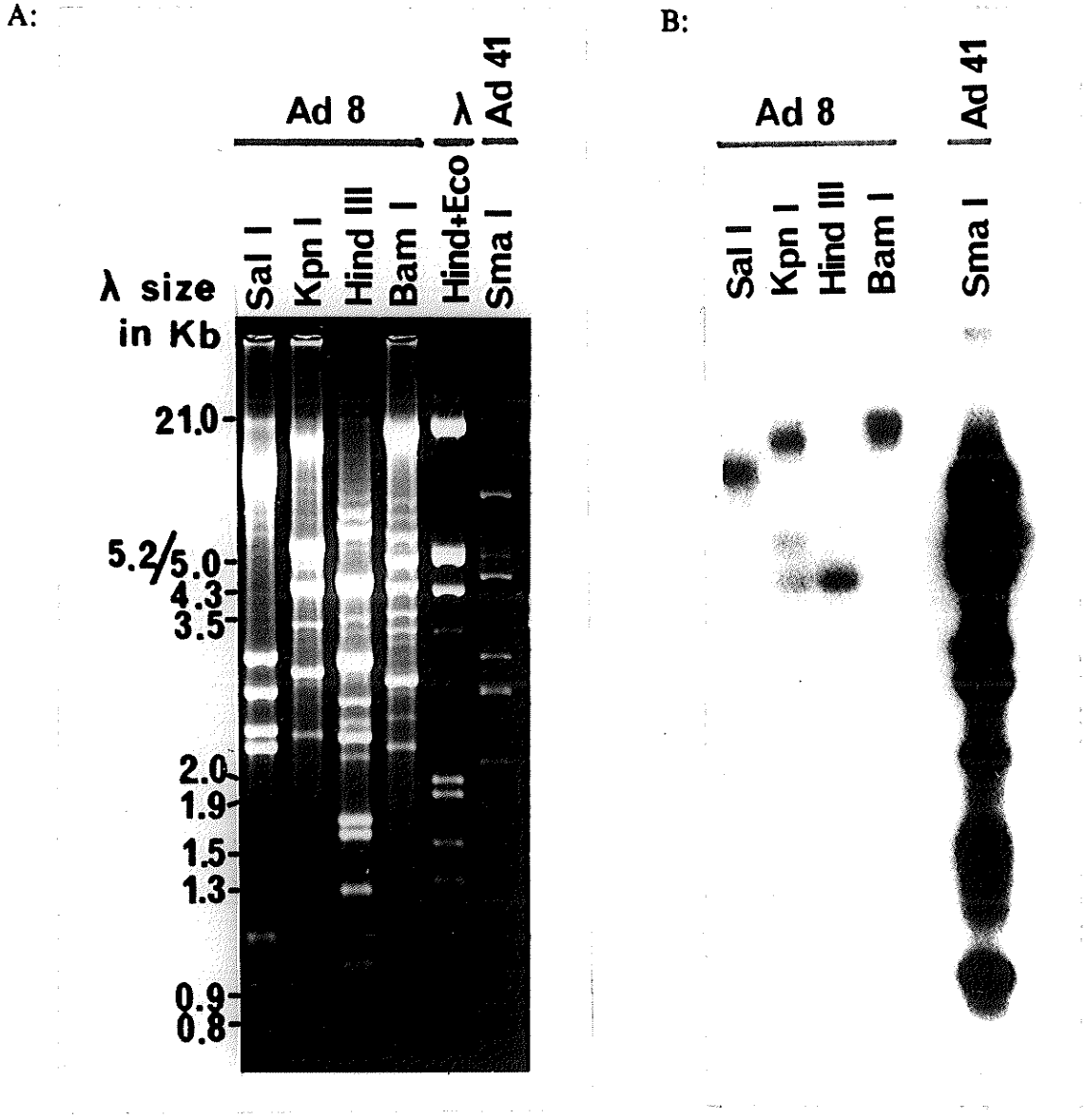


Underlining denotes hybridizing fragments. Densitometer values for fragments as the % absorbance of the total per lane are printed below each fragment. * Ad7 restriction maps taken from Tibbetts, 1977.

corresponding areas in the Ad2 genome. Both Ad4 and Ad7 have a small area of their genomes beyond 65 map units, seen at a marginal level in only some Ad2 lanes, that consistently hybridizes with Ad41 DNA. *Sma*I fragments of Ad7, in particular, show that the areas of the Ad7 genome react in a disproportionate pattern with Ad41 DNA probes in a similar pattern given by the Ad2 genome.

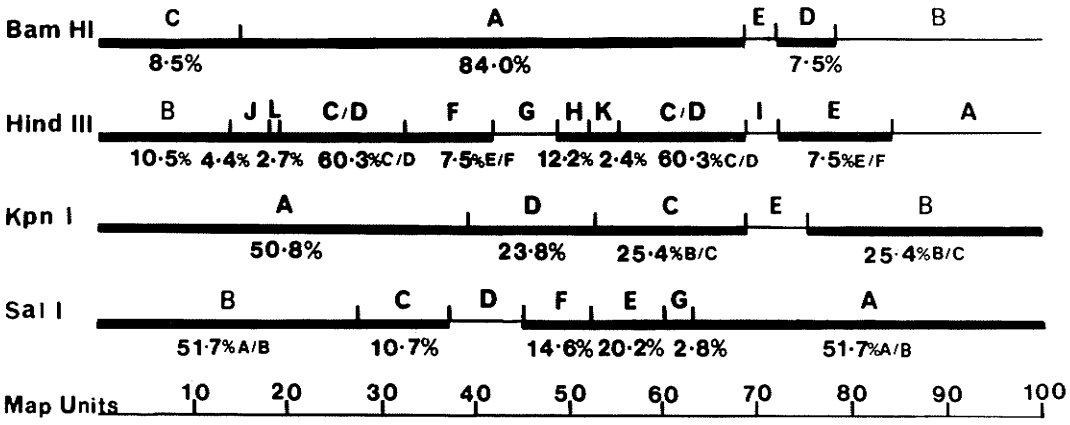
The restriction enzyme maps available for Ad8 (Takacs *et al.*, 1983) are more limited than any of the other viruses analyzed. Despite attempts to improve the quality of Ad8 DNA preparations partial bands were not eliminated. The DNA shown in Figure 11 had a consistent pattern of poor digestion with *Bam*HI, in particular, while the restriction pattern with *Sal*I was always clear. The *Bam*HI digest fits a 4 band pattern found by Adrian *et al.* (1986) for the prototype strain Trim of Ad8 but not the 5 fragment pattern of the published map (Takacs *et al.*, 1983) of an undefined Hungarian strain. The *Hind*III digest has the required number of fragments but does not migrate in the same pattern as the prototype strain (Adrian *et al.*, 1986). Additionally, the relative intensity of hybridization of Ad8 fragments, when attributed to positions correct for the Hungarian strain in Figure 12, are totally at variance with the positions of sequences of homology with Ad41 DNA in other genomes. Therefore, all the indications are that the prototype and Hungarian strains of Ad8 have different restriction maps and not that the Ad8 sequences of homology with Ad41 differ in position from the other species. Only the relatively simple *Bam*HI restriction map, with a single extra restriction site relative to the prototype map, has an order of fragments that conforms with the positions of sequence homology in other genomes. The simple *Bam*HI map is probably the least dissimilar of

Figure 11 A and B. Hybridization of Ad8 DNA fragments with Ad41.



A: 2 μg aliquots of Ad8 DNA, extracted from purified virions and digested with *Bam*HI, *Hin*dIII, *Kpn*I, and *Sal*I were run on an 0.8% agarose gel in duplicate. Lanes 5 and 6 were spotted with 1 μg λ DNA + *Hin*dIII and *Eco*RI or 0.4 μg Ad41 DNA + *Sma*I. B: This gel was blotted to a nylon membrane and hybridized with Ad41 DNA. Film was photographed after 12 hours in an intensifying cassette with the hybridized membrane.

Figure 12. Restriction Endonuclease Maps of Adenovirus 8, Strain TRIM (Subgenus D)*.

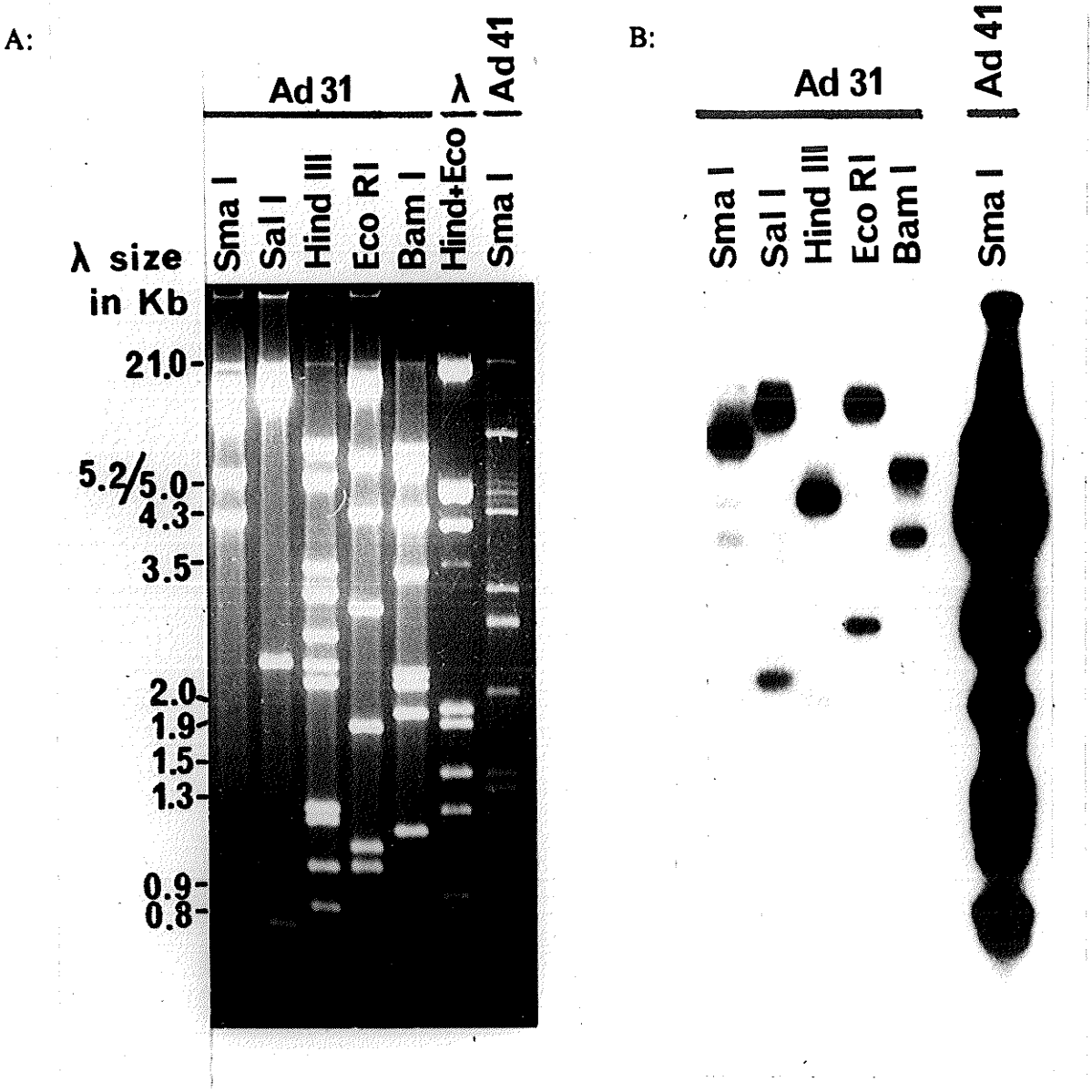


Underline denotes hybridizing fragments. Densitometer value of the % absorbance/lane represented by each fragment are placed below line. * maps taken from Takacs *et al.*, (1983).

the Hungarian strain maps to the prototype pattern.

Ad31 of subgenus A, was found to be much more amenable to culture than Ad12, although the availability of restriction enzyme maps for Ad31 is limited (Broker, 1982). The Ad31 DNA electrophoresed in Figure 13 was purified by phenol sequestration (Shinagawa *et al.*, 1983). Very little background reaction is visible in the autoradiograph taken of the membrane in Figure 13 B after DNA transfer and hybridization with an Ad41 probe from purified virions. Subtracting those fragments that do not hybridize with Ad41 DNA, as shown in prototype restriction maps in Figure 14, the cross-homologous sequences of the Ad31 genome are defined as sequences between the left terminus and 6.7 map units, 10.8 and 17.4 map units, 23.5 and 30.3 map units and a central area between 42.6 and 66.8 map units. The intensity of hybridization reactions of the two homologous sequences near the left terminus and the central area of Ad31 closely describe the relative levels of intensity observed with genomic fragments of the other species. The cross-reaction of the left terminus of Ad31 when isolated as a *Hind*III G fragment may only occur with Ad8 of the species analyzed. The distribution of the other 3 hybridizing areas of the Ad31 genome in Figure 14 very closely match the positions of sequence homology found with other species. The similarity between the positions of sequences of homology in the different genomes is evident in the comparison of the areas of fragment overlap of each species in Figure 15. Although this figure does not take into account the variation in the size of the different viral genomes, estimated at up to 2,000 base pairs or 5.5% variation with some species, the hybridizing fragments of each species overlap in similar positions of the genome. The limitation to the precision of

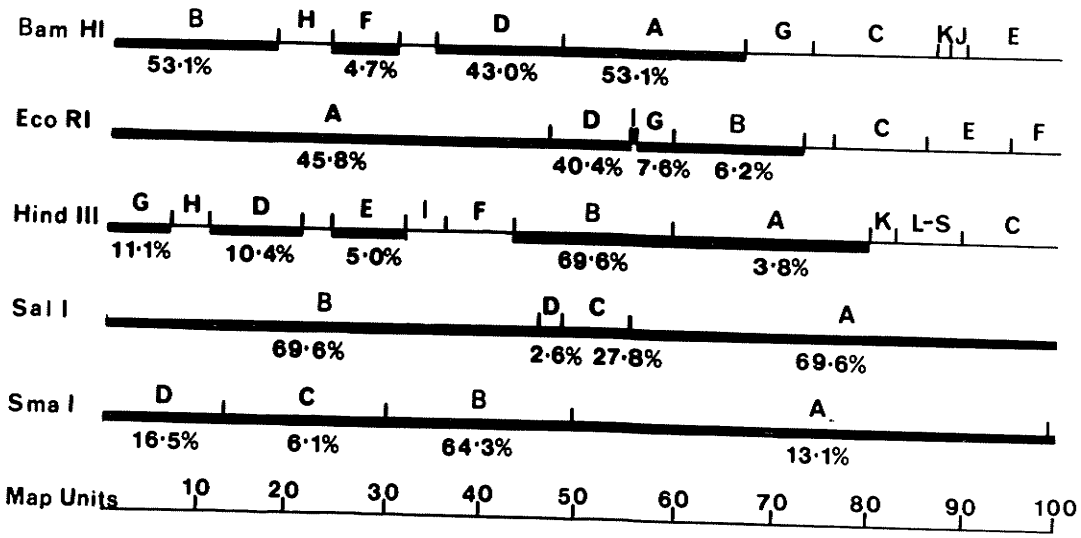
Figure 13 A and B. Ad31 Cross-Reaction with an Ad41 DNA Probe.



A: Ad31 DNA purified as by Shinagawa *et al.*, (1983), was digested with five restriction enzymes and run on a 0.8% agar electrophoresis gel. Lane 1 to 5, 2.5 μg Ad31 DNA + *Bam*HI, + *Eco*RI, + *Hin*dIII, + *Sal*I, + *Sma*I; lane 6; 2 μg λ DNA + *Hin*dIII and *Eco*RI; Lane 7, 400 ng Ad41 DNA + *Sma*I.

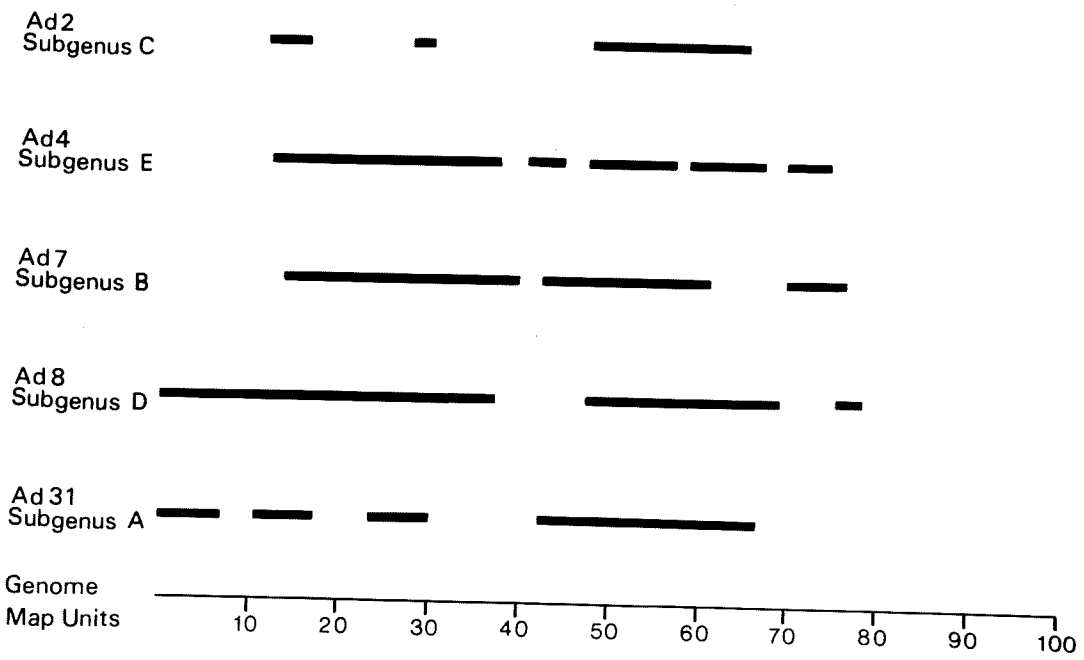
B: The gel was blotted and the membrane hybridized with an Ad41 DNA probe. The film was developed after 20 hour exposure to membrane between intensifying screens.

Figure 14. Restriction Endonuclease Cleavage Maps of DNA from Adenovirus 31, Strain 1315, Subgenus A.



Hybridizing fragments are underlined. The % absorbance /lane of each fragment are ascribed below the line. Ad31 Restriction maps were taken from Broker (1982).

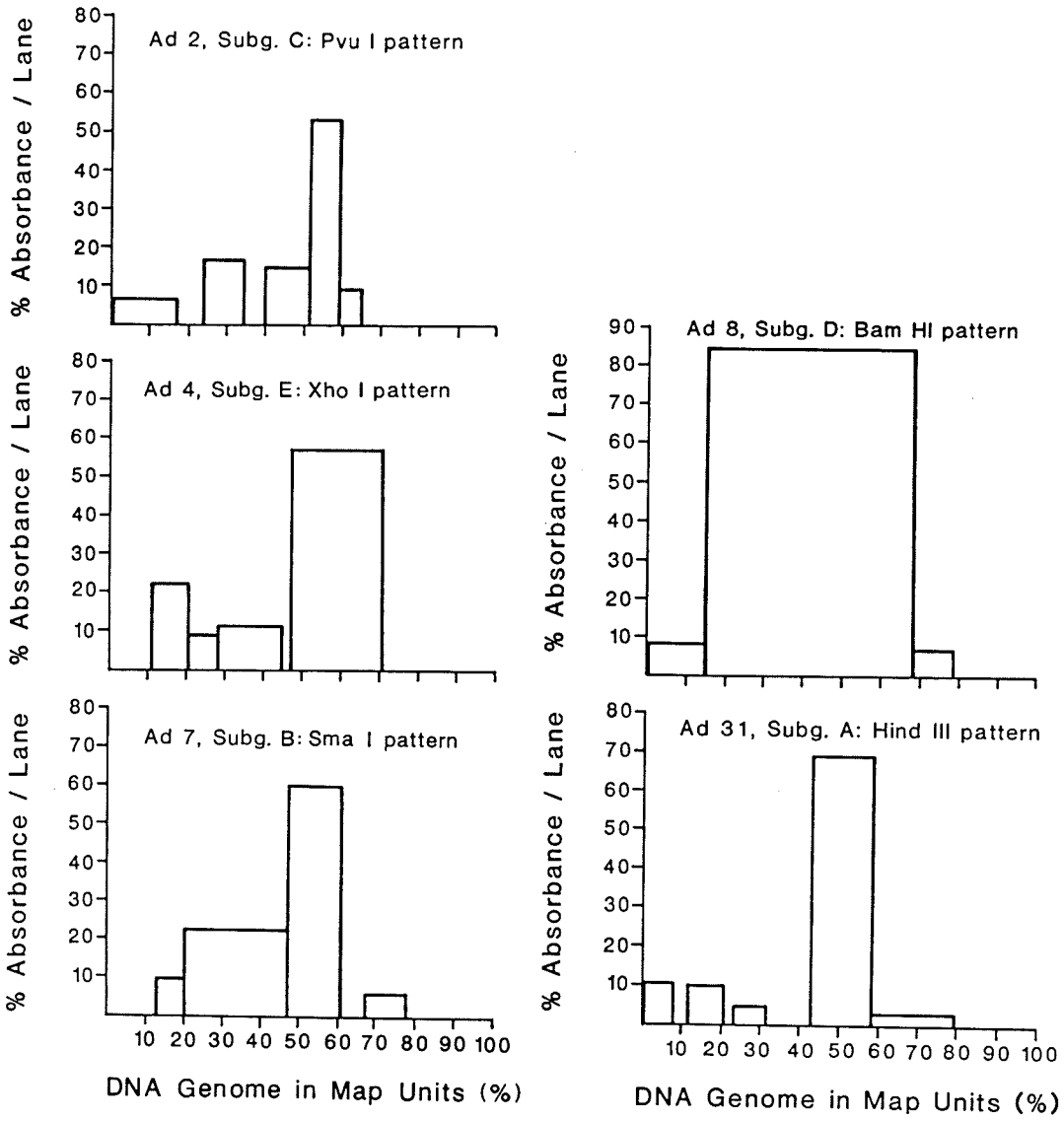
Figure 15. Areas of Closest Homology of the Genomes of Viruses from Subgenera A to E with Ad41 Genomic DNA.



The lines represent the areas of overlap of fragments that hybridize with an Ad41 genomic probe. With the subtraction of nonhybridizing fragments these areas contain the sequences of nucleotide identity with the Ad41 genome as closely as can be defined by the available restriction enzymes with known maps.

genomic cleavage with the available restriction maps prevents such fine definition of the sequences of Ad41 homology as performed with Ad2 and the area of the Ad4, Ad7, Ad8 and Ad13 genomes hybridizing with Ad41 are much broader than that of found with Ad2 DNA, but they cover the same areas of the genome. Each of the Ad2 sequences of homology with Ad41 DNA fit into the areas of homology of the other species, indicating a pattern of sequence homology and conservation of specific genes consistent throughout all subgenera of human adenovirus species. The consistent pattern of sequence conservation is emphasized in Figure 16 where the relative intensities of hybridization of fragments of one digest of each species are compared. An enzymatic digest that divides the genome between the areas of sequence homology, separating homologous fragments for individual assessment, was used with the exception of *Bam*HI digestion of Ad8. The separated fragments show levels of hybridization intensity corresponding to fragments of similar position in the other genomes. Either of the sequences toward the left terminus emit 5 to 20% of the total hybridization signal, while the central area of the genome, particularly a sequence between approximately 50 to 60 map units of each genome, is responsible for over half of the total signal of the genome. The similarity in intensity of hybridization of the same areas of the genomes indicates that not only is the position of conserved sequences preserved in all the human species of adenovirus but that the degree of sequence homology in those conserved genes is also similar in each species.

Figure 16. Proportional Cross-reaction of Restriction Fragments of Different Subgenera with Ad41 DNA Probes.



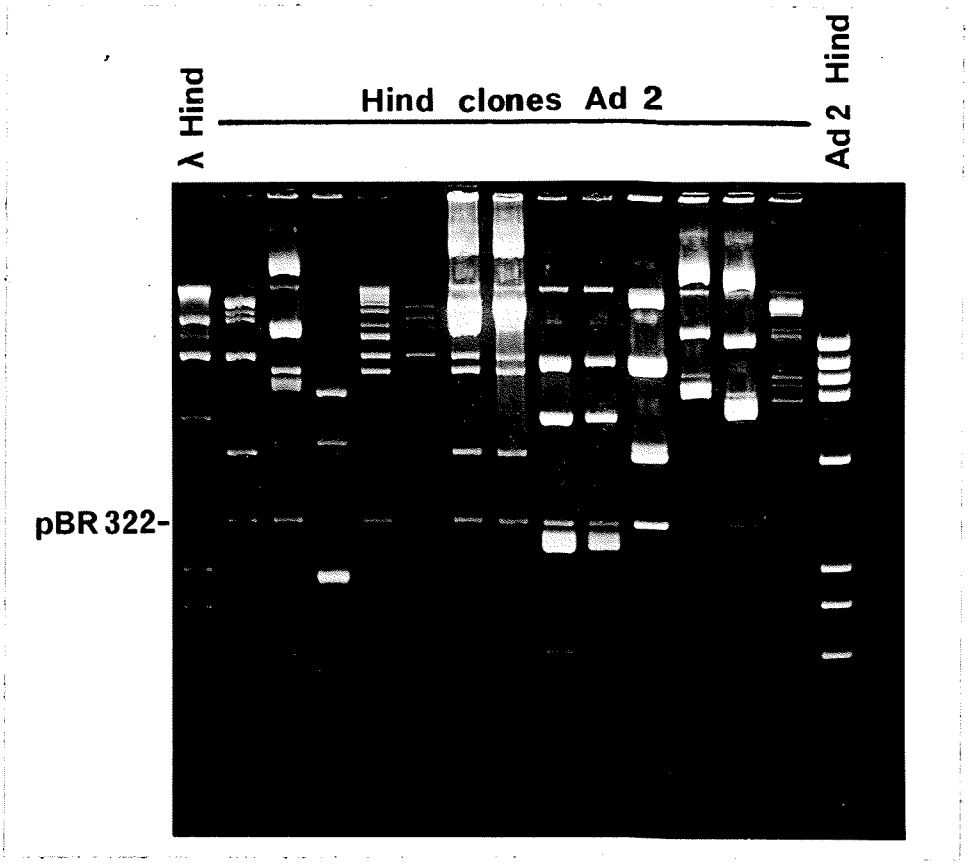
The relative intensity of hybridization of the different fragments of adenovirus DNA with Ad41 DNA were measured by densitometer. The intensity value of each fragment was expressed as the % of the total absorbance of the lane. One lane of each species in which the areas of homology were separated by cleavage were depicted.

3. Localization of Sequences of Homology of the Ad2 Genome with Ad41

i) Cloning of the *Hin*dIII-*Bgl*II Fragment of the Ad2 Genome.

In the preceding experiments, a central area of the genome of each species, most closely defined with the Ad2 genome between 47.5 and 65 map units, was found to harbour the most conserved sequences of the adenovirus genome. A small section of the central homologous area, between approximately 50 and 60 map units, hybridized with by far the most intensity when isolated as a separate fragment, emitting over half of the total signal and evidently contained sequences with much greater nucleotide identity with Ad41 than the rest of the genome. It was suspected from the initial hypothesis and the central position of the conserved sequences that the intense hybridization was due to the hexon gene. However, in order to isolate the best cross-reactive probe sequence it was decided to localize the conserved sequences empirically. The strategy used was to first clone the entire central area of homology and subsequently subclone fragments of the parent plasmids and test their ability to cross-react with other adenovirus DNA. The Ad2 genome was cleaved with *Hin*dIII and ligated with the vector pAT153. Insert containing plasmids were identified by resistance of host bacteria to ampicillin and their sensitivity to tetracycline. Quick plasmid DNA preparations were quantified, boiled and spotted on a nylon membrane and hybridized at 65°C with an Ad41 genomic probe. The viral DNA insert enclosed in the most cross-reactive of the plasmids was identified as the *Hin*dIII A fragment by digestion of the plasmids with *Hin*dIII and electrophoresis of the plasmid digests adjacent to a lane of similarly digested genomic DNA in the agarose gel pictured in Figure 17. The *Hin*dIII A fragment of Ad2 spans from 50.9 to 73.4 map units on the

Figure 17. Restriction Endonuclease Patterns of Ad2 *Hin*dIII Fragment Containing Plasmids

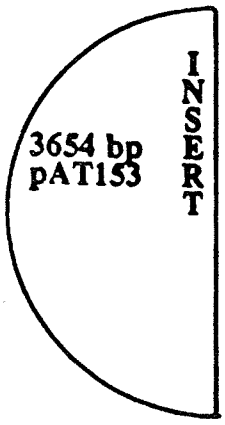


Ad2 DNA digested with *Hin*dIII was ligated with pAT153 DNA in a 4:1 ratio. This DNA was used to transform competent JM109 E. coli and quick plasmid preparation of plasmid DNA made from the tetracycline sensitive clones. 2 μg of λ DNA in lane 1, 13 quick plasmid preparation samples in lanes 2 to 14, and 2 μg of genomic Ad2 DNA, all digested with *Hin*dIII, were run in 1.2% agarose in a minigel apparatus at 100v for 2 hours. Multiple bands of supercoiled and relaxed plasmid and plasmid fragments are evident. The *Hin*dIII fragment plasmid reacting with Ad41 with the greatest intensity was run in lane 4.

Ad2 genome and contains almost all of the central area of homology with Ad41. The plasmid containing the Ad2 *Hin*dIII A fragment, called p2HA, was subcloned with *Hin*dIII and *Bgl*II or *Pvu*I enzymes, which have several cut sites in this fragment. Plasmid fragments digested with *Pvu*I and *Hin*dIII were ligated with vector pBR322 cleaved with the same enzymes and bacterial transformants were selected on ampicillin plates. *Bgl*II was used because of the restriction site close to the C terminus of the hexon gene. Plasmid fragments cleaved with *Bgl*II were ligated with the compatible sticky ends of pBR322 cut with isoschizomer *Bam*HI. In spot blot hybridization tests a number of the *Hin*dIII and *Bam*HI subcloned plasmids showed apparently stronger reactivity to the Ad41 probe than an equal quantity of the parent p2HA plasmid. *Bam*HI enzyme could not be used to cut out the insert and identify these subclones as the sequence created by ligation of *Bam*HI and *Bgl*II compatible ends is altered and not recognized by either enzyme. Therefore, the subclones were digested with *Hin*dIII and *Pvu*II, which has a number of restriction sites in the *Hin*dIII A fragment, and identified by comparison to the p2HA plasmid digested with the same enzymes. The *Hin*dIII and *Pvu*II restriction map of the parent p2HA plasmid and the sizes and migration order of fragments resulting from digestion with these enzymes are detailed in Figure 18 A. The *Hin*dIII and *Bgl*II subclones that reacted strongly with Ad41 DNA were electrophoresed in lanes 3, 7, 12 and 13 of the agarose gel shown in Figure 18 C after digestion with enzymes *Hin*dIII and *Pvu*II. All the highly cross-reactive subclones were identical and comparison with the parent plasmid in lane 15 of Figure 18 C shows that they retained the 3,453 base pair band of the parent plasmid. This fragment comes from the end of the *Hin*dIII A parent plasmid insert nearer the left terminus of the adenovirus genome (Figure 18 A) and the

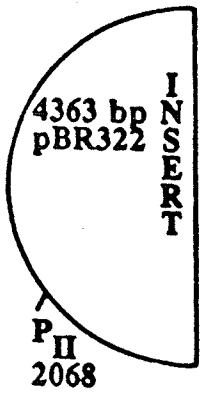
Figure 18 A and B. Restriction Patterns of Plasmids p2HA and p2HB with *Hind*III (H) and *Pvu*II (P_{II}).

A: 2HA



Restriction site (bp)	Fragment size (bp)	Migration order (bp)
- H29 / 18316	3453	3658
- P_{II} 21769	1617	3453
- Bgl 21816	-	1617
- P_{II} 23386	1300	130
- P_{II} 24696	360	1216
- P_{II} 25046	1216	360
- P_{II} 26262	107	107
- H29 / 26369	3654	

B: p2HB



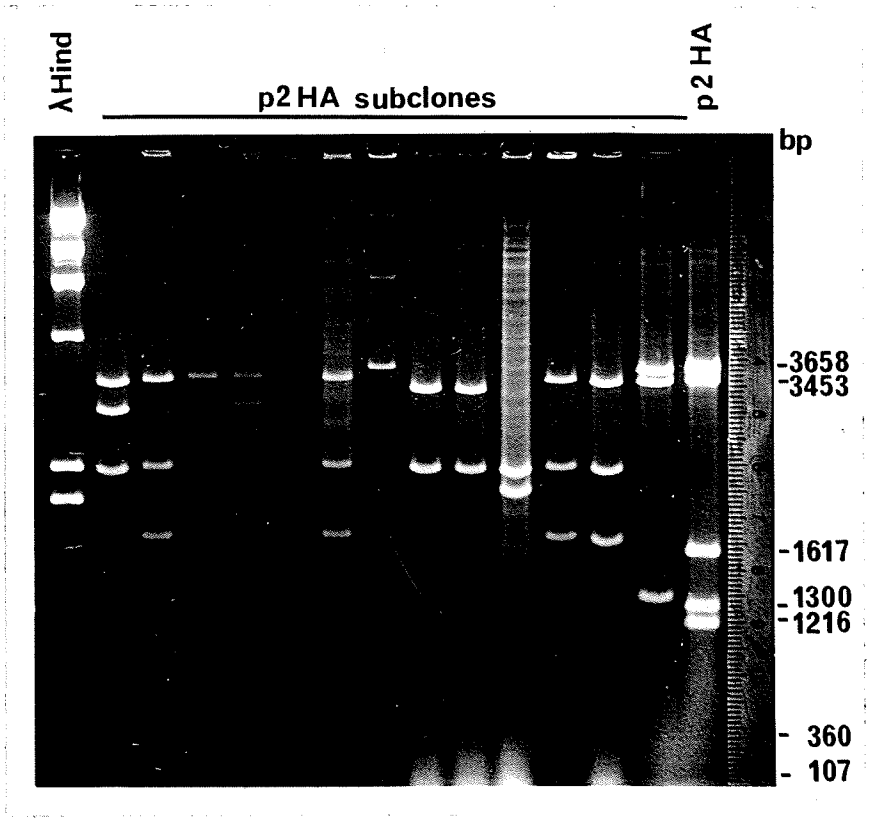
- H29 / 18316	3453	3453
- H 18801 EXON		
- N 21697		
- P_{II} 21769	1765	2324
- Bam 350 / Bgl 21816	2324	1765

* Where restriction site in the insert is given in terms of the position in the Ad2 genome of 35,937 bp.

reactive subclone, called p2HB, is drawn in Figure 18 B. The subclone p2HB contains the Ad2 sequence between base pair 18,316 at the *Hin*dIII restriction site at 50.9 map units and base pair 21,816 at the 60.7 map unit *Bgl*II restriction site. This insert neatly encloses the hexon gene sequence of Ad2, the start codon of which is at base pair 18,801 and which terminates at base pair 21,697 in the Genbank library sequence, 485 base pairs downstream and 72 base pairs upstream of the beginning and end of the insert respectively. This process of cloning and subcloning the *Hin*dIII A fragment of Ad2 confirmed that the hexon gene is the most cross-reactive sequence of the Ad2 genome with Ad41 and liable to make the best probe for detection of all adenoviruses.

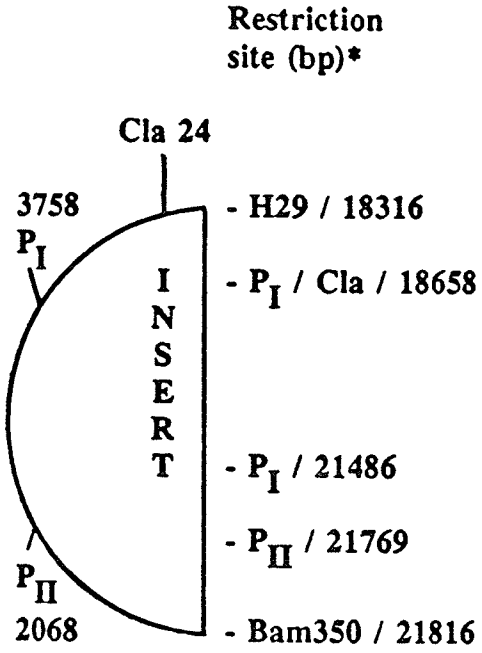
Attempts to cut the *Hin*dIII A subclone p2HB closer to the hexon initiation codon with *Cla*I at the restriction site 450 base pairs downstream of the *Hin*dIII site were unsuccessful. *Cla*I will not recognize methylated DNA (McClelland, 1981). Transformed bacterial hosts methylate DNA differently from eukaryotes so that DNA amplified in most strains of E. coli has altered restriction patterns for methylation sensitive enzymes (Backman, 1980). One in every four *Cla*I recognition sites, which cleaves the sequence ATCGAT, is prone to methylation by the dam methylase which introduces methyl groups at adenine in the sequence GATC (Hattman, *et al.*, 1978). To circumvent this problem, the p2HB plasmid was transferred into methylase negative mutants (Arraj and Marinus, 1983) 2929 and 271 of E. coli supplied by Mike Marinus. DNA purified after amplification in these strains still did not give the complete digestion pattern with *Cla*I. The restriction map of plasmid p2HB with enzymes *Cla*I, *Hin*dIII, *Pvu*I and *Pvu*II is shown in Figure 19 A and the patterns of fragments expected from p2HB with these

Figure 18 C. Quick Plasmid Preparations of p2HA Plasmid Subclones.



Plasmid p2HA was cut with *Hin*dIII and *Bgl*II and ligated with *Hin*dIII and *Bam*HI digested pBR322 vector. Tetracycline sensitive transformants of JM109 strain E. coli were grown to late log phase and plasmid DNA extracted by the alkali method of Birnboim and Doly (1972). Subclone DNAs were resuspended in 50 μl and 10 μl of each was digested with both *Hin*dIII and *Pvu*II, then electrophoresed in lanes 2 to 14 of an 0.8% agarose gel. 2 μg λ DNA + *Hin*dIII and 2 μg p2HA plasmid + *Hin*dIII and *Pvu*II were applied to lanes 1 and 15, respectively.

Figure 19 A. Restriction Map of Plasmid p2HB with Various Enzymes.



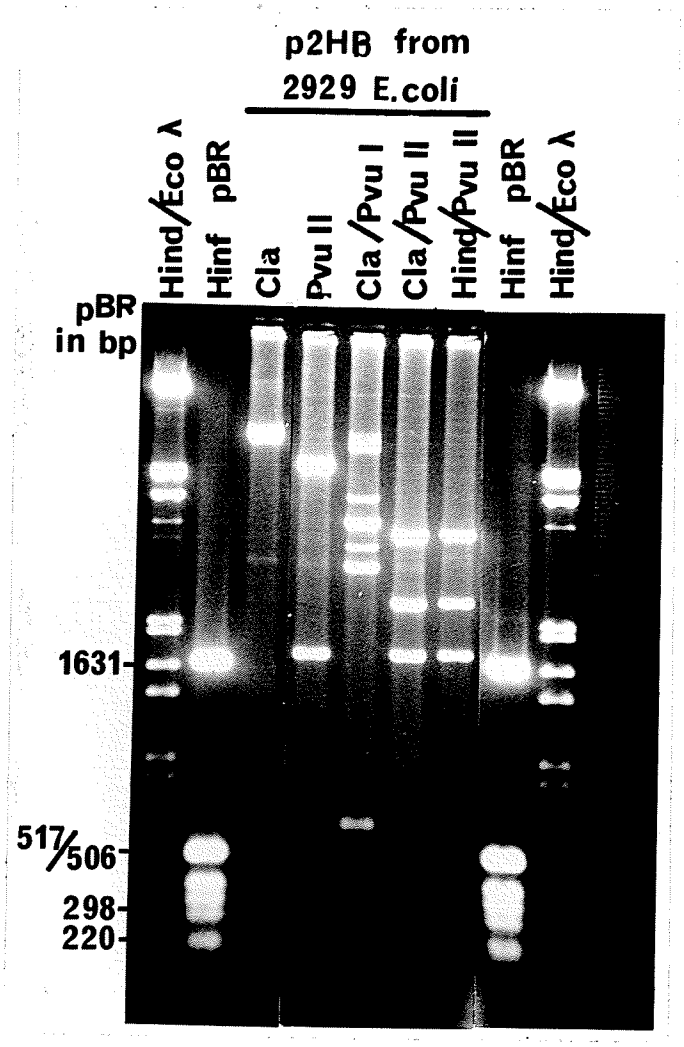
* Where restriction site in the insert = position on Ad2 genome of 35,937 bp.
Where H = *Hin*dIII, PI = *Pvu*I, PII = *Pvu*II, Cla = *Cla*I, Bam = *Bam*HI

Figure 19 B. Expected fragment pattern from p2HB with various enzymes in base pairs.

*Cla*I	*Pvu*II	*Cla*I + *Pvu*I	*Cla*I + *Pvu*II	*Pvu*II + *Hin*dIII
7,195	5,777	3,738	3,111	3,453
347	1,765	2,828	2,319	2,324
		629	1,765	1,765
		347	347	

enzymes are defined in Figure 19 B. The bands that were actually produced by these enzymes after amplification in the methylase mutant bacterial strains are shown in the agarose gel in Figure 20 and it can be seen that, whilst the *Pvu*II and *Pvu*II with *Hin*dIII digests give the expected pattern of bands, the *Cla*I digest lacks the 347 base pair fragment. The DNA was not deleted because *Pvu*I, which cuts within the *Cla*I recognition sequence in the insert, does produce the 347 bp band in combination with *Cla*I. Therefore, the DNA has been altered by methylation so that only the *Cla*I site on the vector DNA was recognized by the enzyme. No further efforts to eliminate nonhexon sequences from the p2HB plasmid were made and the intact plasmid or the 3,453 base pair *Hin*dIII and *Pvu*II fragment, electroeluted from large preparations of the plasmid and referred to as HPII, were used as a probe.

Figure 20. Restriction Patterns of Plasmid p2HB after Amplification in Methylase Deficient Mutant E. coli.



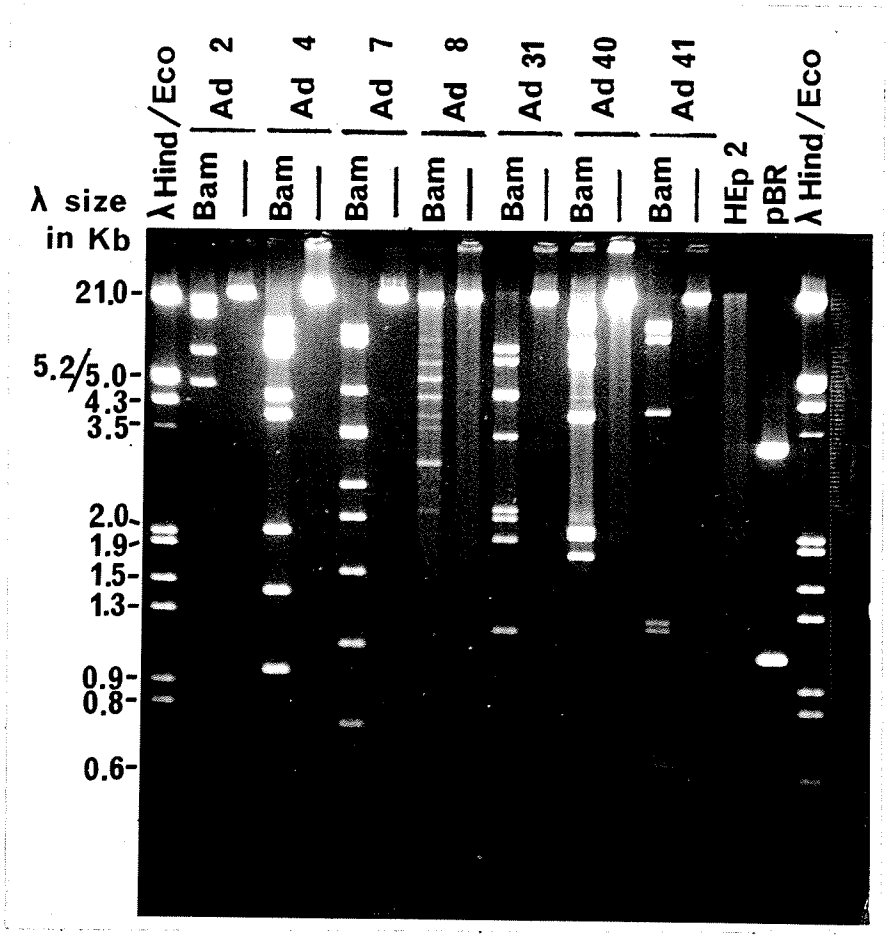
Plasmid p2HP was transformed into competent strain 2929 E. coli. The bacteria were harvested during log phase growth and the plasmid extracted. After digestion with restriction enzymes the DNA was electrophoresed in 0.8% agarose. Lanes 1 and 9, λ DNA 1.5 μg/lane; lanes 2 and 8, pBR322 DNA 1.5 μg/lane; lanes 3-7, p2HP DNA. Cla = *Cla*I, Eco = *Eco*RI, Hind = *Hin*dIII, Hinf = *Hin*fI.

4. Evaluation of Sequences of the Adenovirus Species 41 Genome for Specific Detection of Enteric Adenovirus.

A) Preliminary Quality and Quantity Controls.

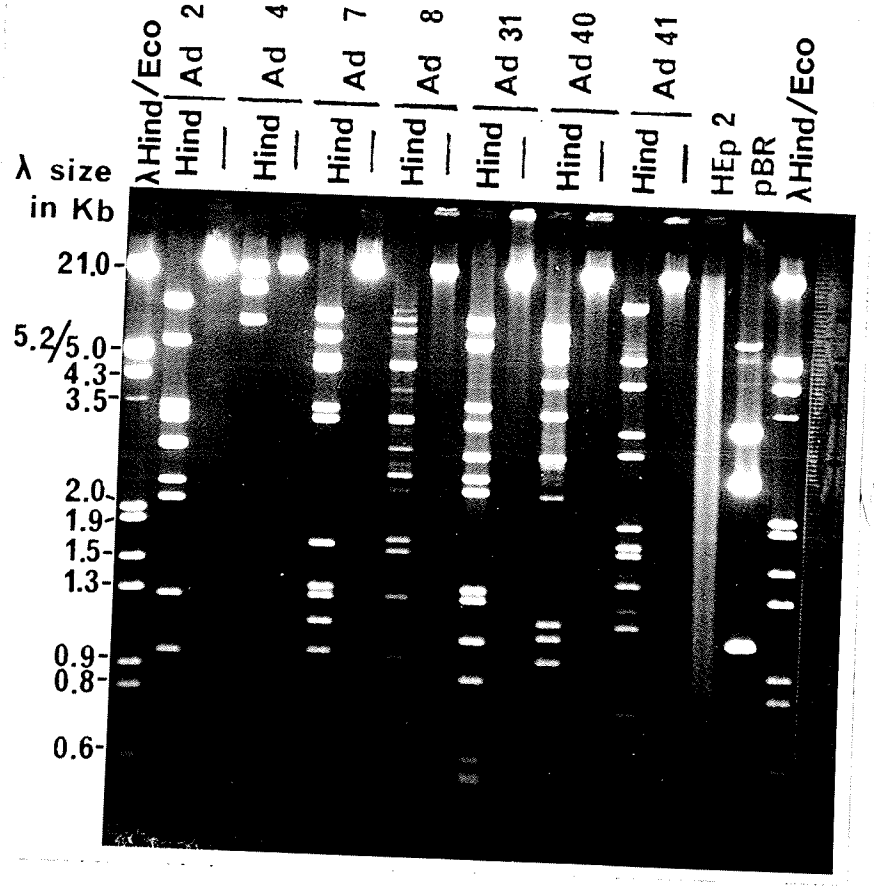
These experiments were performed with a new series of subgenera DNA preparations. The DNA was subjected to the same process of quantification by optical density and comparison to standards in electrophoresis, examination of the restriction patterns for identification and quality assurance, and hybridization with HEp 2 DNA to check for cellular DNA contamination. DNA of the prototype strains of Ad2, Ad4, Ad7, Ad8, Ad31, Ad40 and Ad41 were purified from virus banded twice in CsCl density gradients. Ad31 and Ad41 DNA preparations were directly extracted from cocultivated A549 cells. Ad40 DNA, also from subgenus F, was extracted in small quantity from lysed cocultivated A549 cells by sequestration in the phenol phase by the undigested terminal protein of the genome (Shinagawa *et al.*, 1983). After preliminary estimation of the DNA concentration by measurement of the absorbance by spectrophotometer, each subgenus DNA was visualized by gel electrophoresis after digestion with *Bam*HI in Figure 21 and *Hin*dIII in Figure 22. The restriction patterns of the DNA of each species, apart from the Ad8 preparation, were consistent with the results of Adrian *et al.*, (1986) for the respective prototype strains. Undigested DNA of each species was included for comparison to pBR322 DNA standards. These standards were made with *Hin*dII which cuts the plasmid twice, releasing 2 fragments one of which is three times the size, and has thrice the quantity of DNA, of the other. In the agarose gel in Figure 22 1 μg of pGEM plasmid, digested with an enzyme with a single restriction site in the plasmid, was

Figure 21. *Bam*HI Patterns of Adenovirus Subgenera DNA Preparations



1 μg aliquots of Ad2 (Subgenus C), Ad4 (Subgenus E), Ad7 (Subgenus B), Ad8 (Subgenus D), Ad31 (Subgenus A), Ad40 and 41 (Subgenus F) DNA preparations undigested and digested with *Bam*HI in alternate lanes, were run on an 0.8% agarose overnight at 30 v/15ma. Lanes 1 and 18, 2 μg λ DNA + *Hin*dIII and *Eco*RI; lanes 2 to 15, adenovirus subgenera DNA preparations; lane 16, 0.5 μg HEp 2 cellular DNA; lane 17, 1.2 μg pBR322 + *Hin*dII.

Figure 22. *Hind*III Patterns of Adenovirus Subgenera DNA Preparations



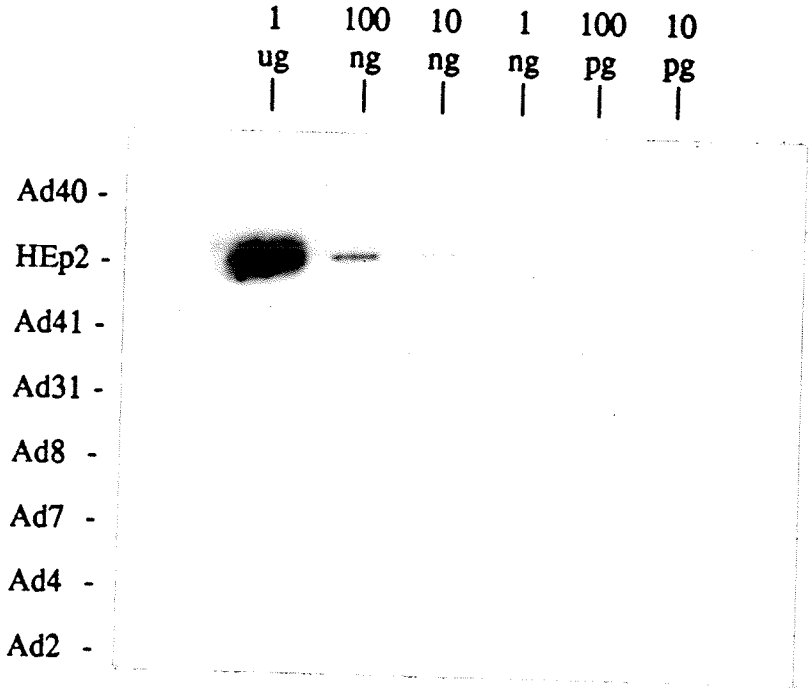
1 μg aliquots of Ad2 (Subgenus C), Ad4 (Subgenus E), Ad7 (Subgenus B), Ad8 (Subgenus D), Ad31 (Subgenus A), Ad40 and 41 (Subgenus F) undigested and digested with *Hind*III in alternate lanes, were run overnight at 30 v/15ma in 0.8% agarose. Lanes 1 and 18, 1 μg λ DNA + *Hind*III and *Eco*RI; lanes 2 to 15, subgenera DNA preparations; lane 16, 2 μg HEp 2 DNA; lane 17, 1.0 μg pBR322 DNA + *Hind*II and 1 μg pGEM DNA + *Eco*RI.

included with the DNA standards in lane 17, to give three bands of 1 μg, 750 ng and 250 ng. Unfortunately, only a small proportion of pGEM was linearized and an unknown amount migrates as a supercoiled band. Estimates of DNA concentrations from the band intensities in electrophoresis were used to calculate the quantities required to make accurate dilutions of species' DNA in subsequent slot blot experiments. DNA preparation of each species, Ad2, Ad4, Ad7, Ad31, Ad40, Ad41, representing subgenera A to F, and HEp 2 DNA were spotted on a membrane in quantities from 1 μg to 10 pg. The membrane was hybridized with a HEp 2 DNA probe and spots developed on the autoradiograph only in the homologous DNA lane (Figure 23). The subgenus DNA preparations were thereby shown to contain quantities of cellular DNA less than 1 ng per 1 ug, quantities unlikely to influence the hybridization of related adenovirus DNA preparations.

B) Cloning of the *Eco*RI A, B and C Fragments of the Ad41 Genome

To alleviate the problems in culturing fastidious adenoviruses to produce sufficient quantities of genomic DNA for use in cross-hybridization studies the Ad41 genome was cloned. The restriction endonuclease *Eco*RI was used to fragment the genome as it has few sites in the Ad41 genome, resulting in few, large fragments, and the fragments at either termini are small relative to most other enzyme digests. This is a consideration as the termini of the adenovirus genome are difficult to clone since the 55K terminal protein attached to the 5' end of each strand is not completely removed by proteolytic digestion and a portion remains to interfere with ligation. The *Eco*RI fragments were ligated with pGEM plasmid vector and used to transform JM109 strain E. coli. The bacterial clones

Figure 23. Adenovirus Subgenera DNA Preparations Hybridized with a Cellular Probe.



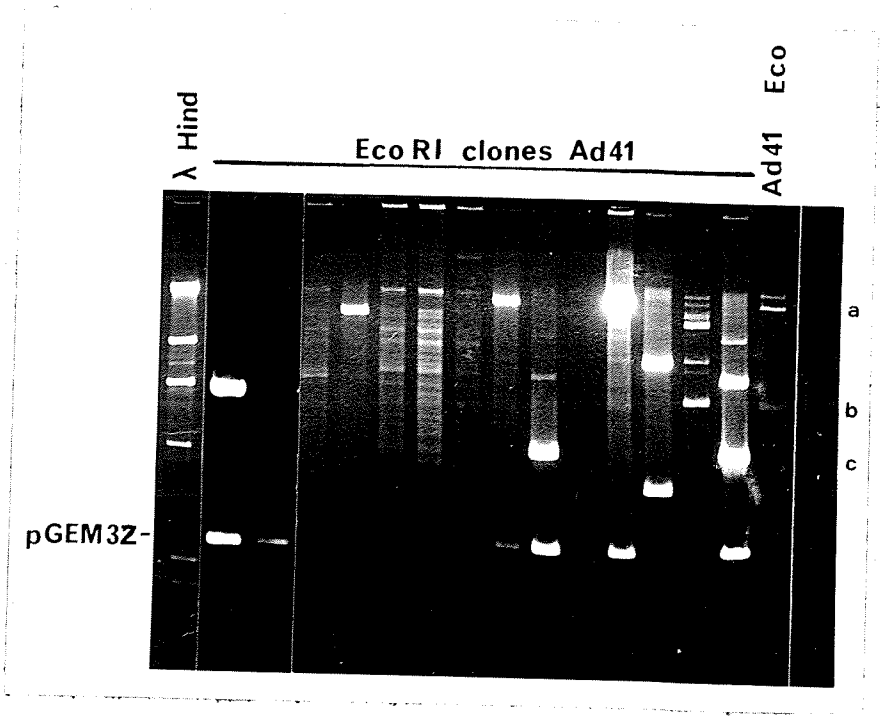
Adenovirus subgenera A to F DNA preparations, used for cross-hybridization experiments, were first probed with HEp 2 to detect cellular contamination. 100 μl of boiled adenovirus subgenera, HEp 2 and λ DNA were spotted in horizontal rows using 48 slot blot apparatus in log dilutions from 10 μg/ml. Membranes were then hybridized at 68°C and enclosed with film for 48 hours.

containing plasmids with viral DNA inserts were identified by their white colour on X-gal supplemented plates, denoting insertional inactivation of the β lactamase gene in the plasmid. The DNA of these transformant colonies were digested with *Eco*RI and analyzed by agarose gel electrophoresis (Figure 24). The enzyme releases the insert which can be identified by comparison to the bands in the genomic digest in the outermost lane. Bands corresponding to fragments A, B and C are evident in different lanes. These three are the non-terminal fragments of the Ad41 *Eco*RI restriction map, together representing 84% or approximately 29,000 base pairs of the Ad41 genome. The four insert containing plasmids were called p41EAC, p41EA, p41EB and p41EC, according to the size of the fragment(s) they contained and their reaction with Ad41 DNA fragments in Southern blots. Attempts to procure clones of the terminal fragments D and E by blunt end ligation were unsuccessful. Further attempts to improve the ligation efficiency of terminal fragments with the vector by means of polylinkers, short sequences of double stranded DNA containing a restriction site which can be tagged at a high ratio to blunt ended DNA, digested and ligated to the sticky ends of cleaved plasmid DNA were also unsuccessful and clones containing the terminal fragments were not isolated.

C) Hybridization of DNA of Each Adenovirus Subgenus with Ad41 *Eco*RI Fragment Containing Plasmids.

Ad41 *Eco*RI fragment plasmids p41EA, p41EB and p41EC were radiolabelled and used as probes to hybridize with a series of log dilutions of adenovirus DNA preparations of subgenera A to F from 1 μg to 1 pg. The reaction of Ad2, Ad4, Ad7, Ad8, Ad31 and Ad41 DNA with a whole Ad41 genomic probe is shown as a baseline comparison in the

Figure 24. Quick Plasmid Preparations from Ad41 DNA Transformants.

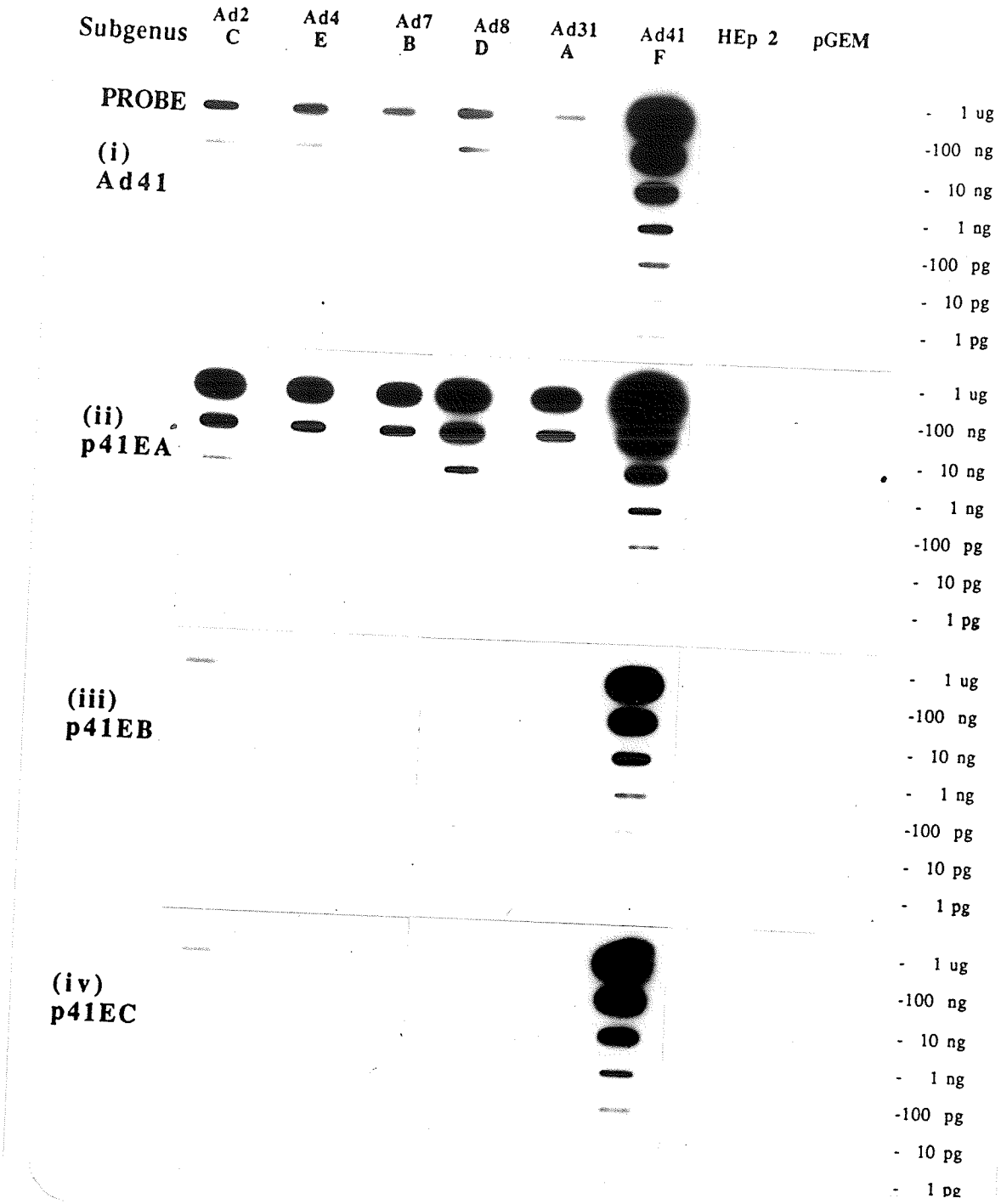


White ampicillin resistant colonies transformed with Ad41 *Eco*RI digested DNA ligated with plasmid pGEM 3Z were grown and plasmid preparations performed by the method of Birnboim and Doly (1972). Plasmid DNA precipitates were resuspended in 50 μl and 10 μl aliquots, digested with *Eco*RI, were run on a number of 0.8% agarose gels. A composite of lanes from two such gels is shown with 1 μg λ DNA + *Hind*III in Lane 1, and 0.5 μg Ad41 DNA + *Eco*RI in lane 16. The largest EcoRI fragments A, B and C are indicated on the figure.

first panel of Figure 25. The reaction of p41EA, containing over 50% of the Ad41 genome from 8 to 61 map units, with the subgenera DNA as shown in panel (ii) is less specific than the whole Ad41 genome, detecting lower amounts of heterologous subgenus DNA relative to the quantity of Ad41 DNA detected in the same interval. The parameters of the p41EA plasmid, if the position of genes are similar in Ad2 and Ad41, should encompass the two sequences of homology towards the left terminus and the hexon gene. Concentration of the sequences with nucleotide identity in a smaller quantity of DNA should enable the p41EA plasmid to react with higher intensity with other adenovirus species DNA preparations than the genomic DNA probe, as observed. Accordingly, the p41EB and p41EC plasmids, containing Ad41 DNA towards the right terminus, react with the subgenera DNA in panels (iii) and (iv), respectively, with greater specificity than the genomic probe. The difference between sensitivity for Ad41 DNA and non homologous subgenera DNA is at least 4 to 5 logs with either p41EB or p41EC probe, greater than the homologous to heterologous difference found with Ad2 probes. This suggests that the Ad41 DNA sequence has less overall identity with the other species than the sequence of Ad2 DNA and, perhaps, that subgenus F has diversified more than other subgenera. Again Ad31 DNA reacts less strongly than other species, indicating that subgenus A may be more distantly related to both Ad41 and Ad2 than the other subgenera. Both probes p41EB and p41EC have great specificity for Ad41 DNA and either could be used to differentiate Ad41 DNA from DNA of species of other subgenera.

Figure 25. Adenovirus Subgenera DNA Hybridized with Ad41 Genomic DNA and Cloned *Eco*RI Fragments.

100 μl aliquots of plasmid, cellular and adenovirus DNA of one species from each human subgenus were spotted in ten fold dilutions from 10 μg/ml to 1 pg/ml on nylon membranes. The membranes were hybridized at 68°C with genomic and cloned *Eco*RI fragments of Ad41 DNA. The autoradiograph of the hybridization reaction of a genomic Ad41 probe is shown in panel (i); plasmid p41EA in panel (ii); plasmid p41EB in panel (iii); plasmid p41EC in panel (iv).

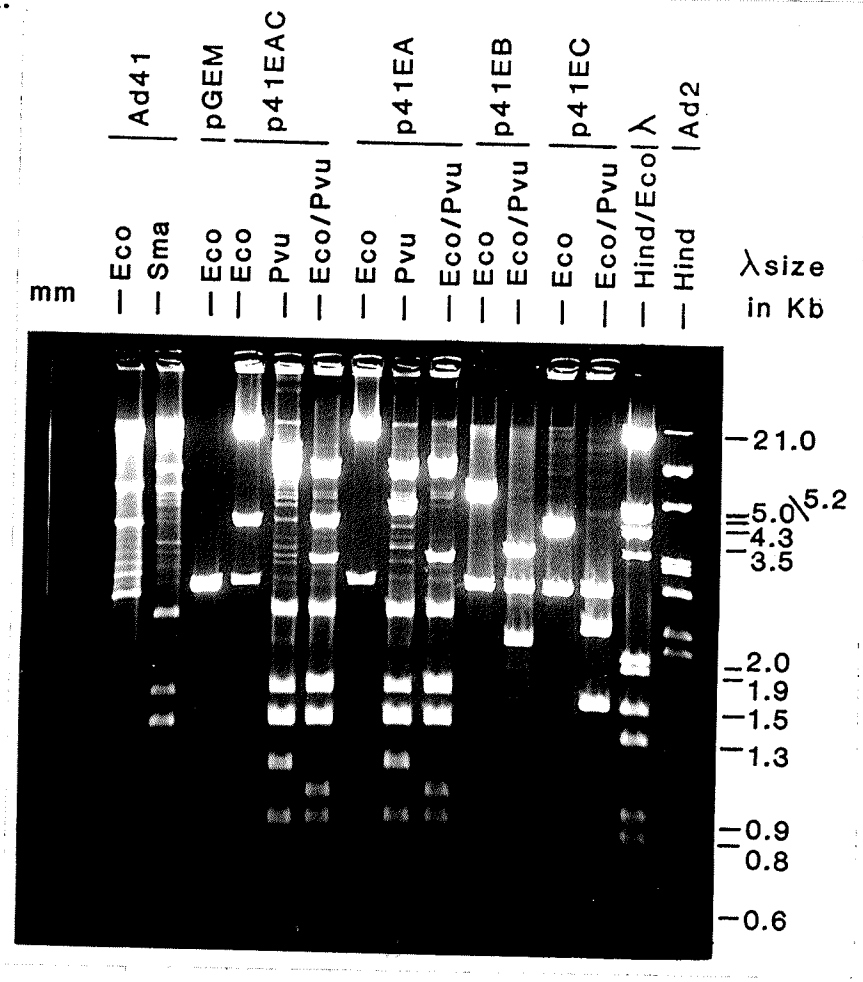

Subgenus
Ad2 C
Ad4 E
Ad7 B
Ad8 D
Ad31 A
Ad41 F
HEp 2
pGEM
PROBE
(i) Ad41
(ii) p41EA
(iii) p41EB
(iv) p41EC
- 1 ug
-100 ng
- 10 ng
- 1 ng
-100 pg
- 10 pg
- 1 pg

D) Hybridization of Subdivided *Eco*RI Fragments of Ad41 with Ad2 DNA at Different Levels of Stringency.

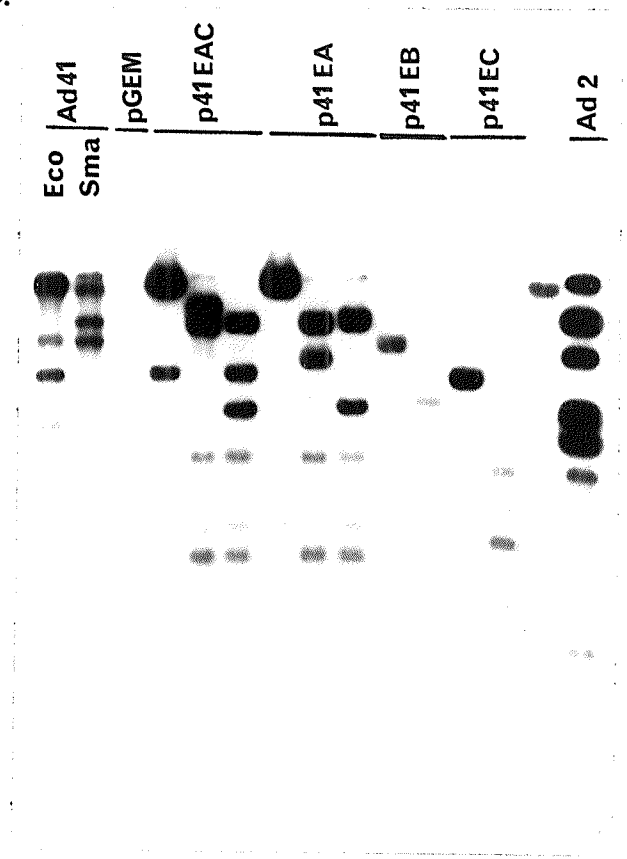
Due to the large size of the cloned *Eco*RI fragments, the EcoRI fragment containing plasmids were subdivided with *Pvu*I in an attempt to localize a sequence with species specificity between map units 8 to 61 of the Ad41 genome in plasmid p41EA. A preliminary indication of the cross homology of these fragments was gained by reacting the Ad41 plasmids with a genomic Ad2 DNA probe at different conditions of stringency in Figure 26. The agarose gel with the *Pvu*I cleaved fragments of all three *Eco*RI fragment containing plasmids is shown in panel A of Figure 26. The Southern blot of this gel was hybridized with a genomic Ad2 DNA probe and washed at low stringency and the temperature and the dilution of salt increased in subsequent washes of the membrane. The autoradiographs of these hybridizations, each exposed to film for the same length of time, are displayed in their original order. Ad41 *Eco*RI A, *Pvu*I D and B fragments evidently contain the most cross homologous sequences and are the only bands seen after reaction with Ad2 DNA in the conditions of high stringency (Figure 26 D; 0.1 x SSC washes at 68°C). These fragments originate from central areas of the Ad41 genome and confirm the position of sequences of the closest homology between Ad2 and Ad41 observed when the probe and target DNA were reversed. After washing at intermediate stringency (washes in 2 x SSC at 55°C) only fragments *Eco*RI A and *Pvu*I B and D, with faint hybridization from *Pvu*I fragment F, plasmid pGEM 3Z and the Ad2 *Hind* A control are retained (Figure 26 C). Fragments positioned towards either end of the cloned plasmids were the first to disappear with increasing stringency. At low stringency (42°C washes in 2 x SSC) in Figure 26 B the least intense bands, from which the probe was

Figure 26 A and B. Hybridization of Ad41 *Eco*RI Fragments with Ad2 DNA at Varying Stringency.

A:

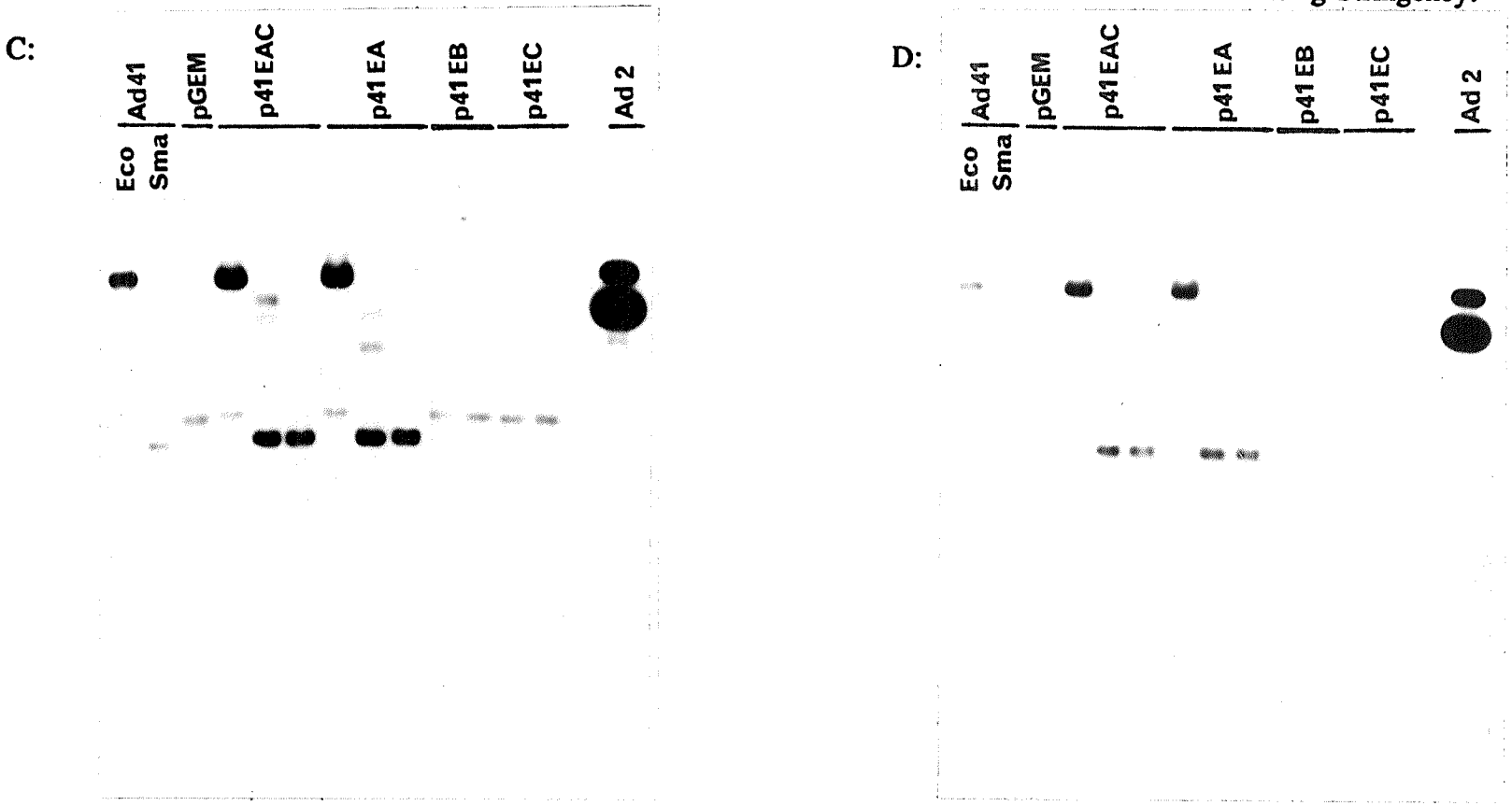


B:



A: 2 μg aliquots of *Eco*RI fragment containing plasmids were digested and run in 0.8% agarose. B: The DNA was blotted and hybridized with Ad2 DNA at 42°C. The membrane was washed 4 times at 42°C in 2x SSC/0.1% SDS and a 48 hour film shown.

Figure 26 C and D. Hybridization of Ad41 *Eco*RI Fragments with Ad2 DNA in Conditions of Increasing Stringency.

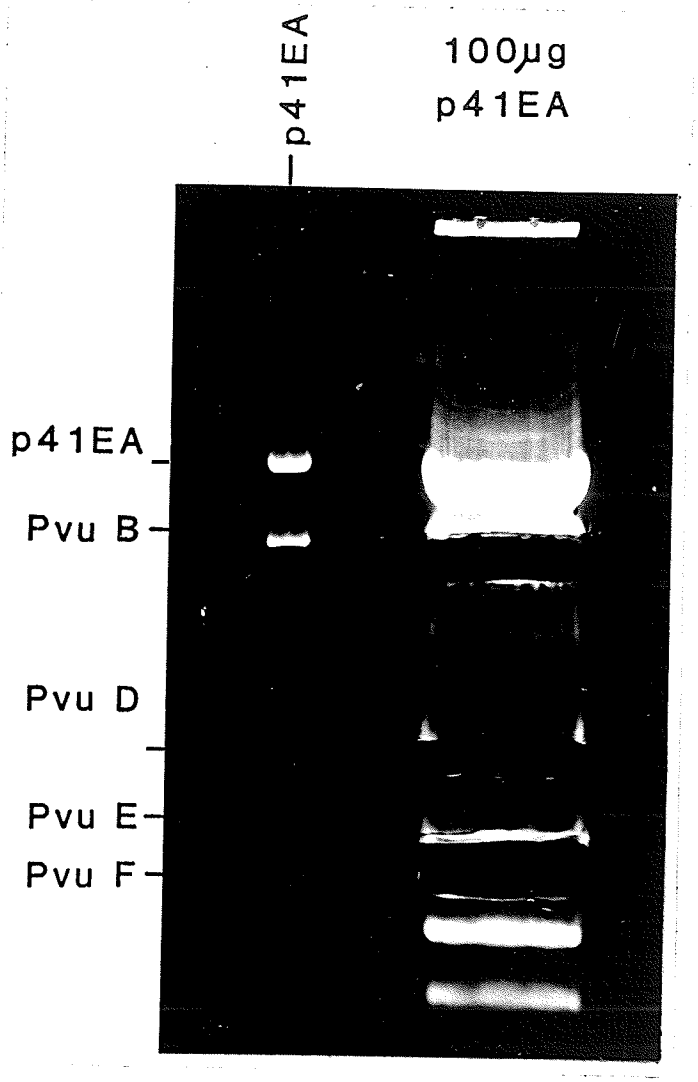


C: The membrane was rewashed at 55°C in 4 successive changes of 2x SSC/0.1% SDS and enclosed with film for a further 48 hours in an intensifying cassette. D: The membrane was rewashed 4 times in 0.1x SSC/0.1% SDS at 68°C and placed between film in an intensifying cassette for another 48 hours.

removed by subsequent washing, are of fragments *Pvu*I E towards the left terminus and the portion of the *Eco*RI B fragment clone nearer the right terminus. The indication that the sequences nearer the Ad41 genomic termini are the least homologous with Ad2 is again in keeping with the results of the previous experiments where Ad41 DNA was the probe.

The reaction of the various *Pvu*I fragments of the large cloned p41EA plasmid were also assessed against other adenovirus subgenus DNA preparations. Ad40 DNA was included to evaluate whether these fragments could be employed to differentiate between species of subgenus F. *Pvu*I fragments B, D, E and F of Ad41 DNA were cut out from plasmid p41EA and separated by electrophoresis in 0.5% Seakem agarose (Figure 27). The fragment DNA bands were cut out and reimbedded in 0.3% agarose and electrophoresed again to remove plasmid DNA. The purified fragments, electroeluted from the agar, were tested by digestion with restriction enzymes and subjected to electrophoresis prior to radiolabelling. Their reaction as probes against adenovirus subgenera DNA preparations is shown in Figure 28 compared to the reaction of p41EA plasmid DNA. The hybridization of the probes is compared when 1 to 10 pg of Ad41 DNA had been detected. This length of time allowed visualization of some non specific reaction particularly with a poor *Pvu*I F fragment probe. The difference in reactivity of the whole p41EA plasmid probe with the DNA of subgenus F as opposed to the DNA of other subgenera is 1,000 to 10,000 fold, the plasmid detected the DNA of the heterologous subgenera 3 to 4 log dilutions more concentrated than the reaction with Ad41 DNA (panel (i), Figure 28). Ad40 DNA can only be detected at a 1 to 2 log higher

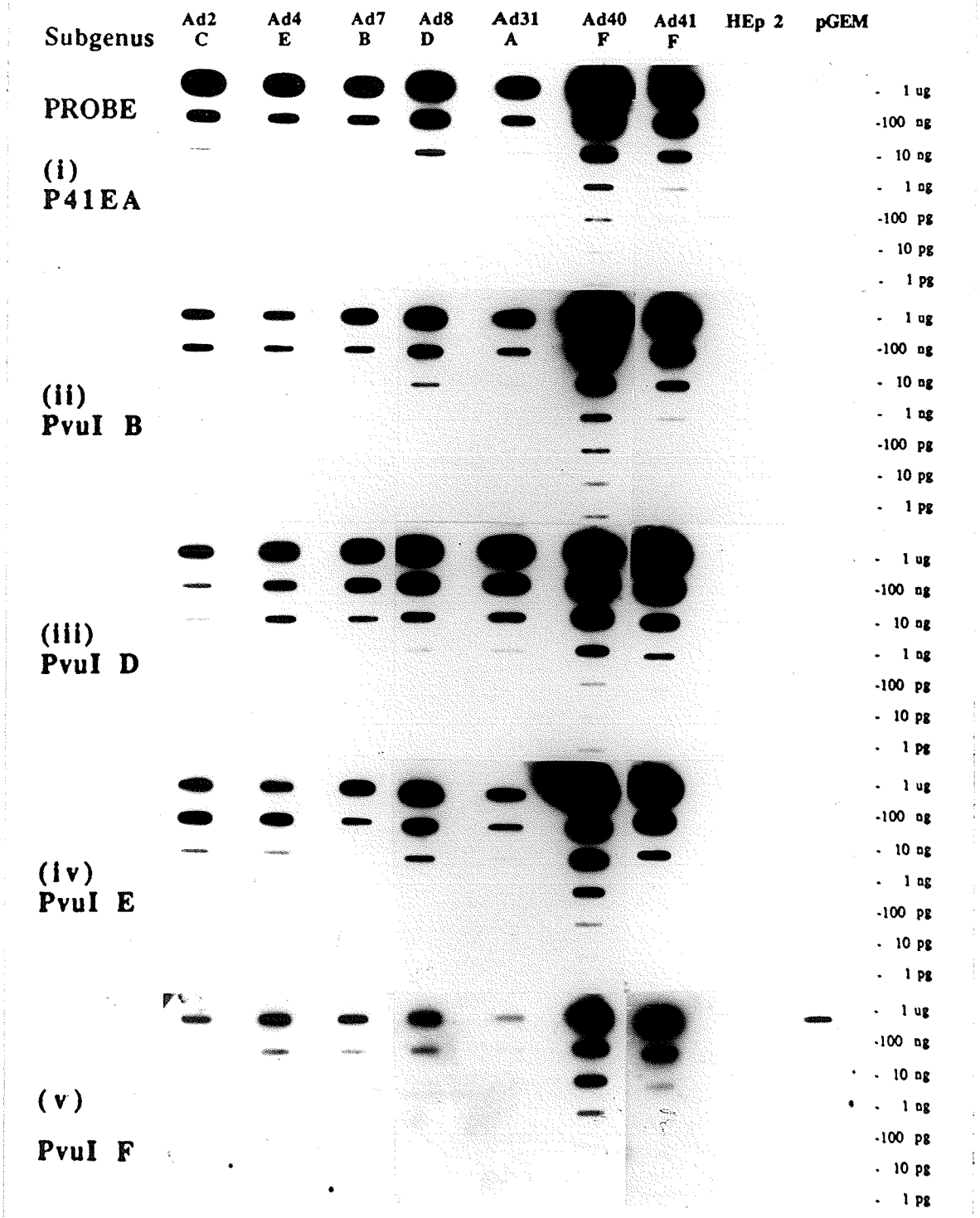
Figure 27. Electroelution of Ad41 *Pvu*I Fragments B, D, E and F.



100 µg of plasmid p41EA, purified by isopycnic centrifugation and digested with *Pvu*I, was electrophoresed in 0.5% Seakem agarose. *Pvu*I fragments B, D, E and F were cut out and run again in 0.3% agarose to separate traces of plasmid DNA. The gel has been photographed after excision of the fragment bands to protect the DNA from excessive ultra violet light exposure.

Figure 28. Hybridization of Adenovirus Subgenera and Ad40 DNA with *Pvu*I Fragment Probes.

Ad40 DNA, extracted from lysed cells and shown to contain less than 10 ng per μg of cellular DNA, was spotted with purified λ phage, HEp 2 and adenovirus DNA preparations of each subgenus in amounts from 1 μg to 1 pg. Membranes were hybridized at 68°C with p41EA plasmid DNA probe in panel (i); the electroeluted *Pvu*I fragment B in panel (ii); *Pvu*I fragment D in panel (iii); *Pvu*I fragment E in panel (iv); *Pvu*I fragment F in panel (v).


Subgenus
Ad2 C
Ad4 E
Ad7 B
Ad8 D
Ad31 A
Ad40 F
Ad41 F
HEp 2
pGEM
PROBE
(i) P41EA
(ii) PvuI B
(iii) PvuI D
(iv) PvuI E
(v) PvuI F
1 ug
100 ng
10 ng
1 ng
100 pg
10 pg
1 pg

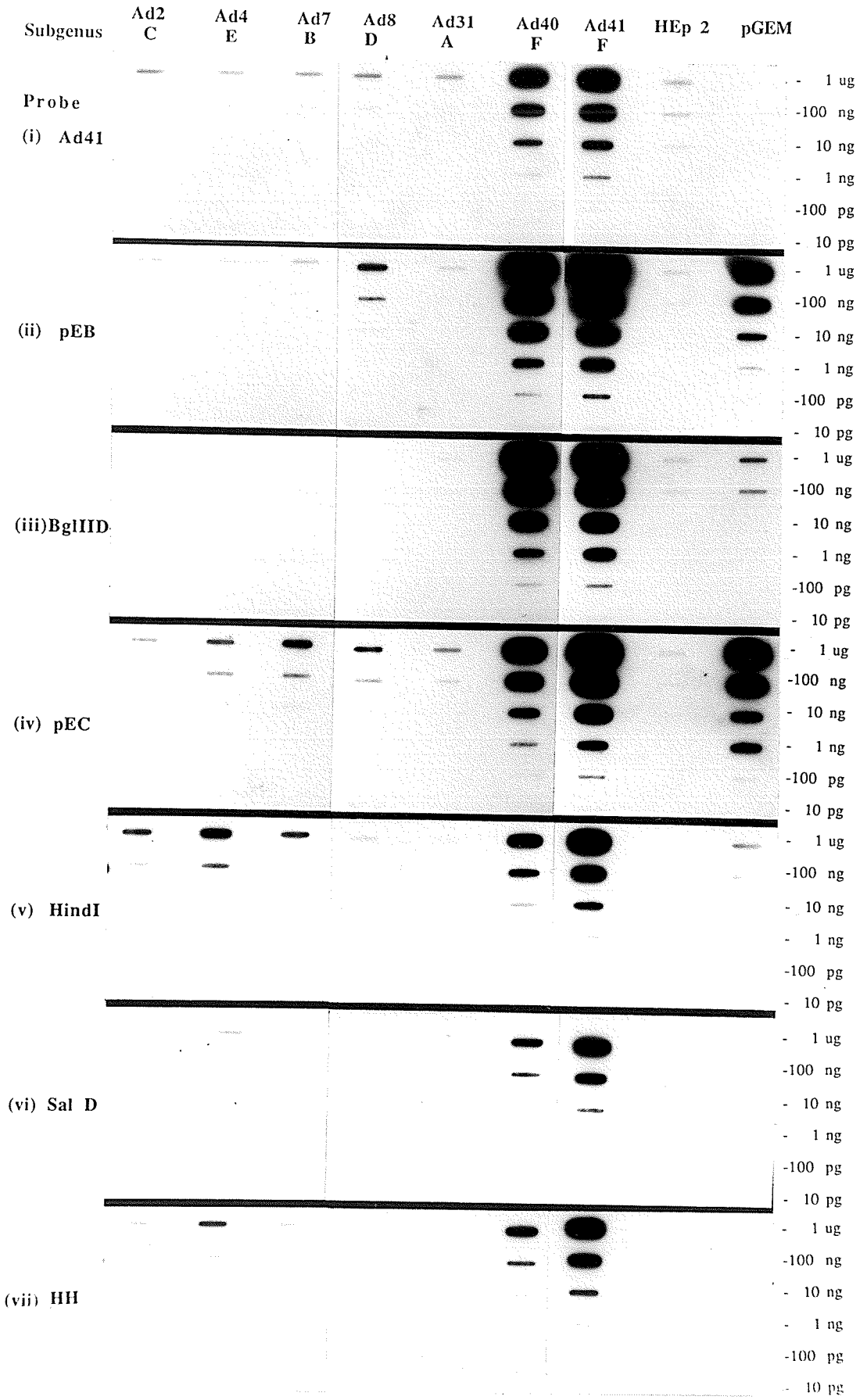
concentration than homologous Ad41 DNA by the p41EA plasmid, demonstrating a 10 to 100 fold ability to distinguish between the DNA of the species of subgenus F. The *Pvu*I fragment probes all show approximately equal reactivity with the DNA of other subgenera relative to the parent plasmid, the B and F fragments may show a slight increase between the relative concentrations of homologous and heterologous DNA required for detection. However, none of the *Pvu*I fragment probes hybridize with the degree of difference with Ad41 DNA and the DNA of other species shown by the *Eco*RI B and C fragments and would not make as effective specific probes. Isolation of segments of the *Eco*RI A fragment by cleavage with *Pvu*I has not demarcated any fragment able to better distinguish between Ad41 and Ad40. All of the *Pvu*I fragments have a reactivity with Ad40 DNA equivalent or greater than that of the parent plasmid. It would be useful, however, to develop a probe capable of detecting both Ad41 and Ad40 equally. The *Pvu*I F fragment in panel (v) appears to detect Ad40 DNA less than one log more concentrated than Ad41 DNA and distinguish between other subgenera by at least 3 log dilutions. *Pvu*I F fragment probe can thus detect quantities of Ad40 DNA within 1/10th of the amount of Ad41 DNA detected in the same time interval and would not react with other adenovirus DNA unless present at 1,000 times the Ad41 DNA quantity. The *Pvu*I F fragment would be useful as a subgenus F specific probe.

E) Further Strategies to Isolate an Ad41 Species Specific Probe.

A number of fragments from different sequences of the Ad41 genome were examined for their ability to differentiate between species Ad40 and Ad41. The reaction of these various probes are shown in a succession of panels in Figure 29 as tested against both

Figure 29. Hybridization of Ad41 *Eco*RI and Hexon Gene Fragments with the DNA of Each Subgenera.

The DNA of Ad40 and one species from each subgenus, spotted in log dilutions from 10 μg to 10 pg per ml in 100 μl aliquots on membrane, were hybridized under stringent conditions with genomic Ad41 DNA in panel (i); pGEM 3Z plasmid containing *Eco*RI fragment B in panel (ii); pAT153 plasmid with a *Bgl*II D fragment insert in panel (iii); pGEM 3Z plasmid containing the *Eco*RI C fragment in panel (iv); fragment *Sal*I D in panel (v); fragment *Hin*dIII I in panel (vi); pGEM 3Z plasmid containing a hexon *Hin*dII fragment in panel (vii).

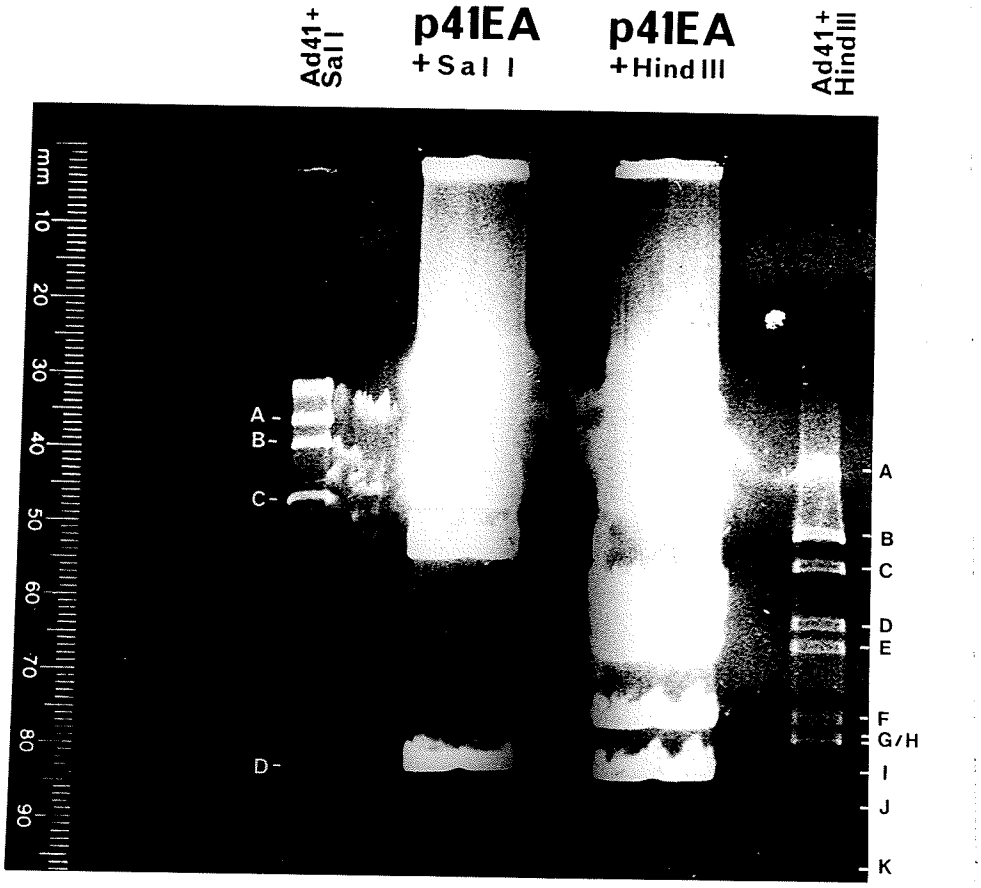

Subgenus
Ad2 C
Ad4 E
Ad7 B
Ad8 D
Ad31 A
Ad40 F
Ad41 F
HEp 2
pGEM
Probe
(i) Ad41
(ii) pEB
(iii)BglIID
(iv) pEC
(v) HindI
(vi) Sal D
(vii) HH
- 1 ug
-100 ng
- 10 ng
- 1 ng
-100 pg
- 10 pg

species Ad40 and Ad41 DNA preparations on membrane spotted with DNA of representative species of each subgenus. In comparison to the whole Ad41 DNA probe in the uppermost panel, the *Eco*RI B fragment in the pGEM 3Z vector (panel (ii), Figure 29), has a relatively insensitive reaction with the DNA of other subgenera, corresponding with its position at the nonconserved right hand end of the genome. The 100 fold higher sensitivity of the *Eco*RI B fragment probe to Ad41 than Ad40 DNA would neither enable equally sensitive hybridization with both subgenus F species nor specific detection of only Ad41 DNA. The plasmid containing the *Eco*RI C fragment in panel (iv) of Figure 29 demonstrated a similar reaction, a much greater but uneven sensitivity to the subgenus F adenoviruses, which detracts from its use either as a species specific or subgenus F specific probe. The *Bgl*II D fragment was cloned (Takiff *et al.*, 1985) from the portion of the *Eco*RI B fragment nearer the right terminus of the genome (see section 7 of Results for the Ad41 map) and was kindly donated in the form of an insert in pAT153 vector. As noted by Takiff *et al.*, (1985), the *Bgl*II D fragment has much greater reactivity with homologous Ad41 DNA than the DNA of species of other subgenera. It is shown in panel (iii) of Figure 29 that the reaction of *Bgl*II D fragment with Ad40 DNA is highly equivalent in reactivity with the homologous Ad41 DNA reaction. The difference in sensitivity for Ad41 and Ad40 DNA, apparently only 2 to 4 fold, is the least of any of the Ad41 fragments tested and the *Bgl*II D fragment is the best prospect for use as a subgenus F specific probe discovered.

It is known that a portion of the sequence in the interior of the hexon gene codes for species specific determinants. When simple cross-hybridization experiments involving

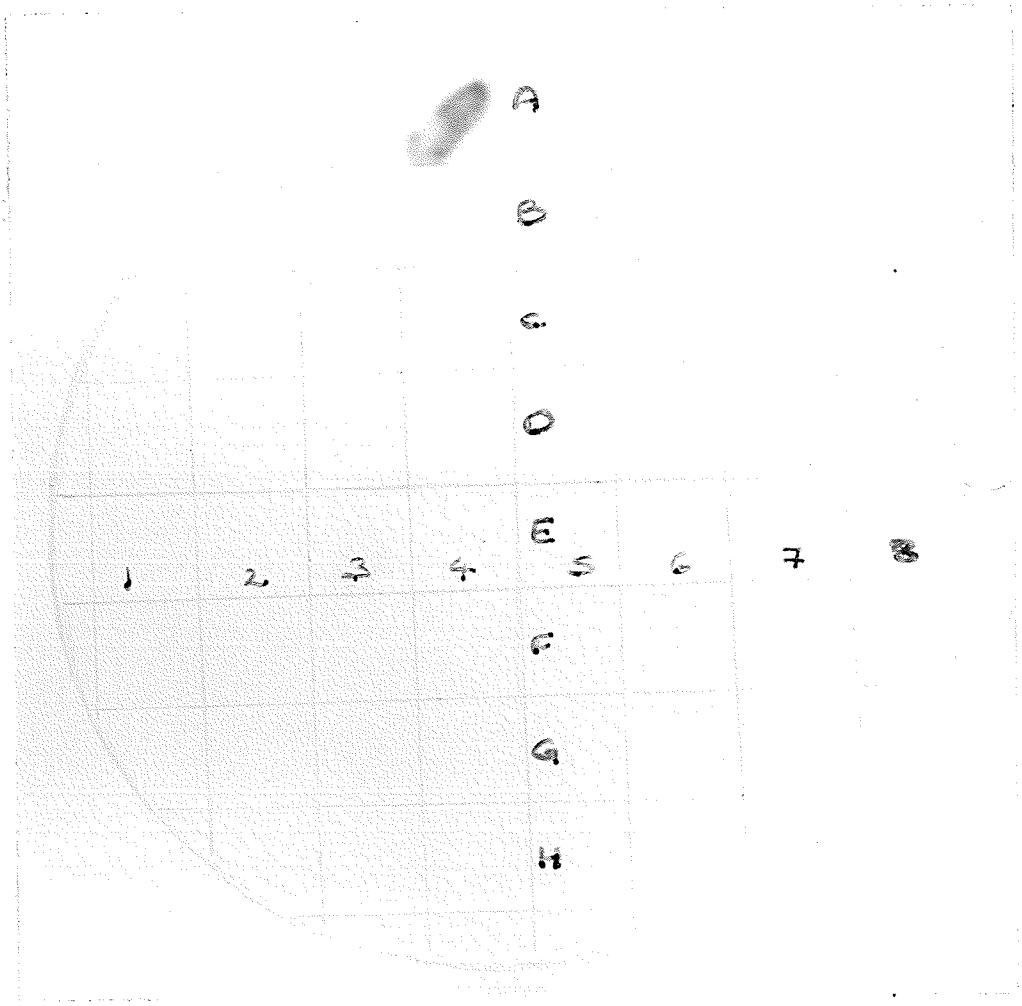
single restriction digests of Ad2 and Ad41 DNA were performed (van Loon *et al.*, 1985), the *Sal*I D fragment, positioned within the Ad41 hexon gene (see Results section 6), did not hybridize indicating that it may code for the hexon serotypic epitopes. It was therefore attempted to isolate sequences within the termini of the hexon gene for use as species specific probes. The *Sal*I D and the *Hin*dIII I fragments, which have similar positions in the Ad41 genome (see section 6 of Results), were isolated from large quantities of the p41EA plasmid by electroelution as shown in Figure 30. The reaction of the *Sal*I D and *Hin*dIII I fragment probes (Figure 29, panels (v) and (vi)) do not adequately distinguish Ad41 DNA from other species for use as specific probes. An attempt was also made to more closely isolate the species specific sequences by cloning *Hin*dII fragments of the p41EA plasmid in the blunt ended *Sma*I site in the pGEM 3Z vector. *Hin*dII has a relaxed recognition sequence in which 2 of the 6 nucleotides can be purines or pyrimidines. Consequently, *Hin*dII recognition sequences occur frequently and a large number of possible clones could arise. The useful clones were identified by hybridization with a *Sal*I D probe to a membrane blotted with the transformant colonies, arranged on a replica plate. The autoradiograph of the hybridized membrane is shown in Figure 31 and identifies two transformants (A4 and E3 of grid) as containing plasmids with inserts of internal sequences of the hexon gene. These plasmids were identical and the reaction of the cloned internal hexon probe, called plasmid p41HH, are shown in panel (vii) of Figure 29. Analysis of the Ad41 hexon gene sequence (Toogood and Hay, 1988) for *Hin*dII recognition sequences places the start of the cloned fragment within the sequence coding for the L1 epitopic loop and extending into P1 shell sequence of the three dimensional hexon capsomere (Toogood *et al.*, 1989). The conservation of the P1

Figure 30. Electroelution of Fragments *Sal*I D and *Hin*dIII I.



Two aliquots of 100 μg of plasmid p41EA, containing the Ad41 *Eco*RI A fragment, were digested with excess *Sal*I and *Hin*dIII restriction enzymes overnight before electrophoresis in 0.5% Seakem agarose between lanes of 2 μg of genomic DNA digested with the same enzymes. There has been leakage of the DNA from the wells at the top of the gel. The bands were identified by comparison to the genomic pattern, excised, run again in 0.3% agarose and then purified.

Figure 31. Ad41 *Hind*II DNA Transformants Hybridized with a *Sal*I D DNA Probe



Blunt ended *Hind*II fragments of the p41EA plasmid were ligated in a 4:1 ratio with plasmid pGEM 3Z cut with *Sma*I. Transformant colonies were replica plated and the ampicillin sensitive transformants blotted to a nylon membrane by means of a felt covered plunger. The colonies were lysed on the membrane, the DNA denatured, baked and hybridised with *Sal*I fragment D DNA at 68°C overnight. The film was enclosed with the membrane for 24 hours prior to photography.

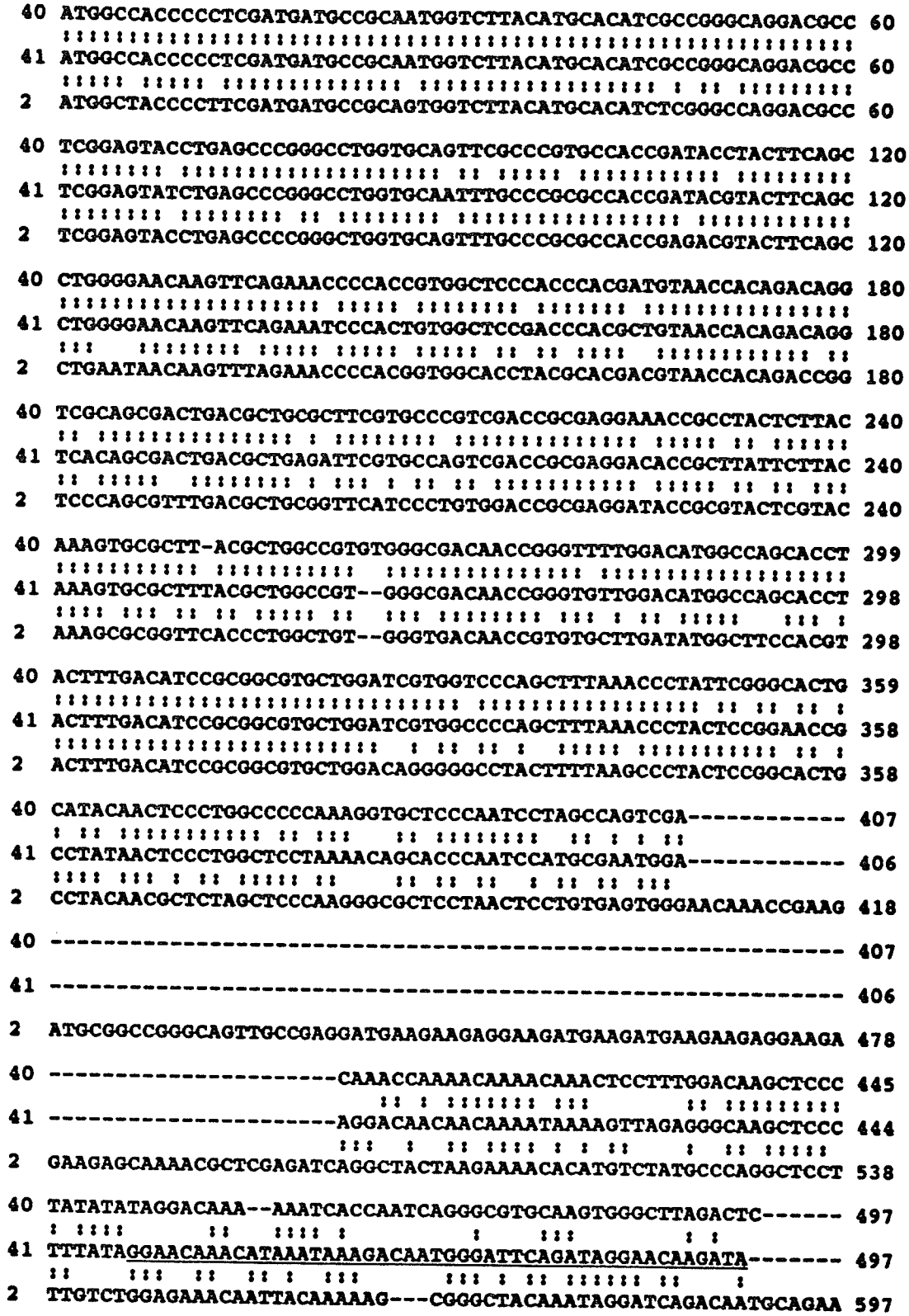
shell coding sequence undoubtedly detracts from the ability of the *Hin*dII fragment to differentiate Ad41 DNA from Ad40 DNA or DNA of other species (panel vii). In summary, no Ad41 sequence with species specific sensitivity was isolated by electroelution or cloning. It was apparent that the two subgenus F adenoviruses shared similar nucleotide sequences throughout much of the genome and that hexanucleotide restriction endonucleases would not allow the accuracy necessary to isolate the short sequences unique to Ad41 that might serve as a specific Ad41 probe.

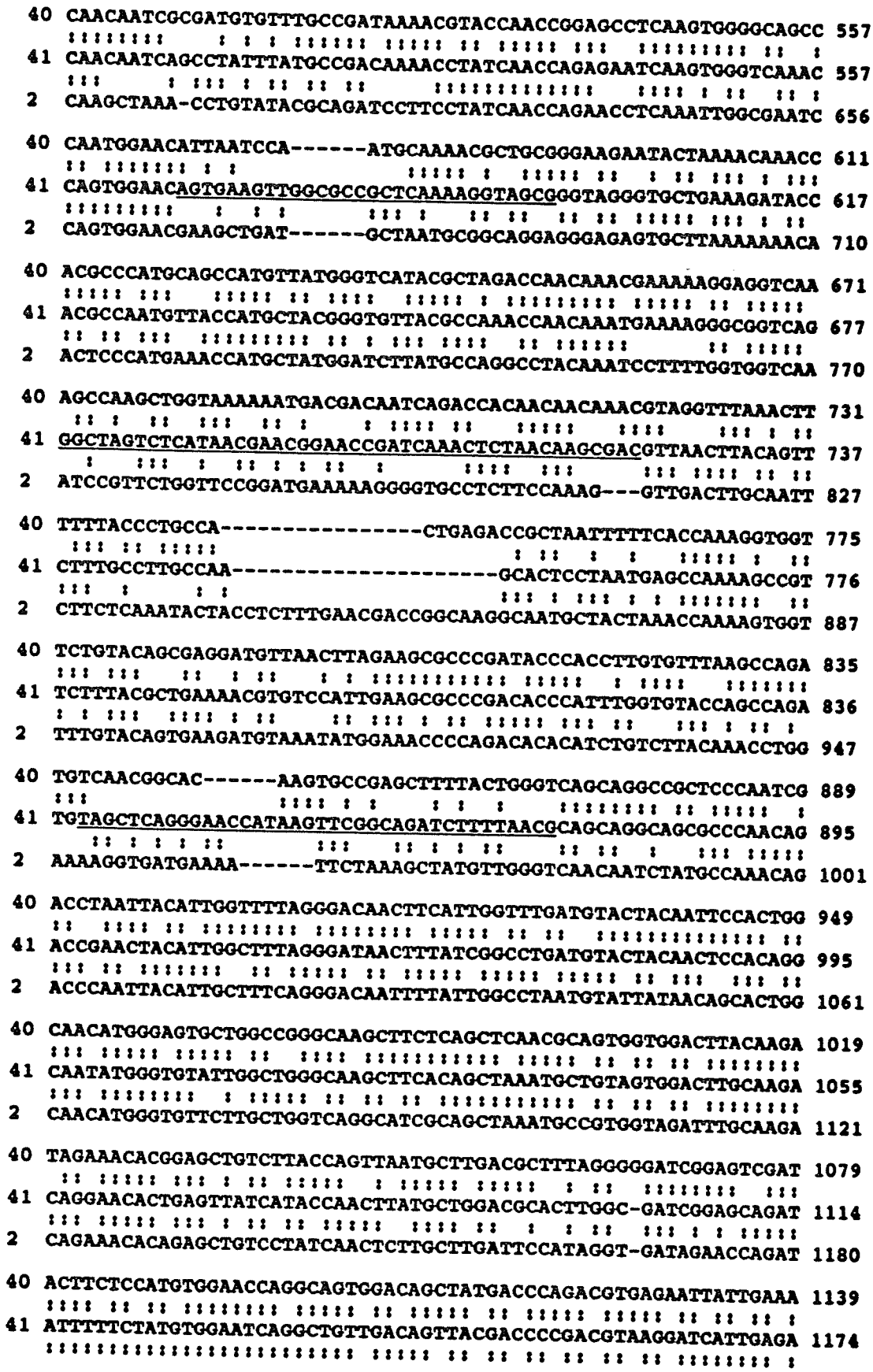
F) Ad41 Synthetic Probes.

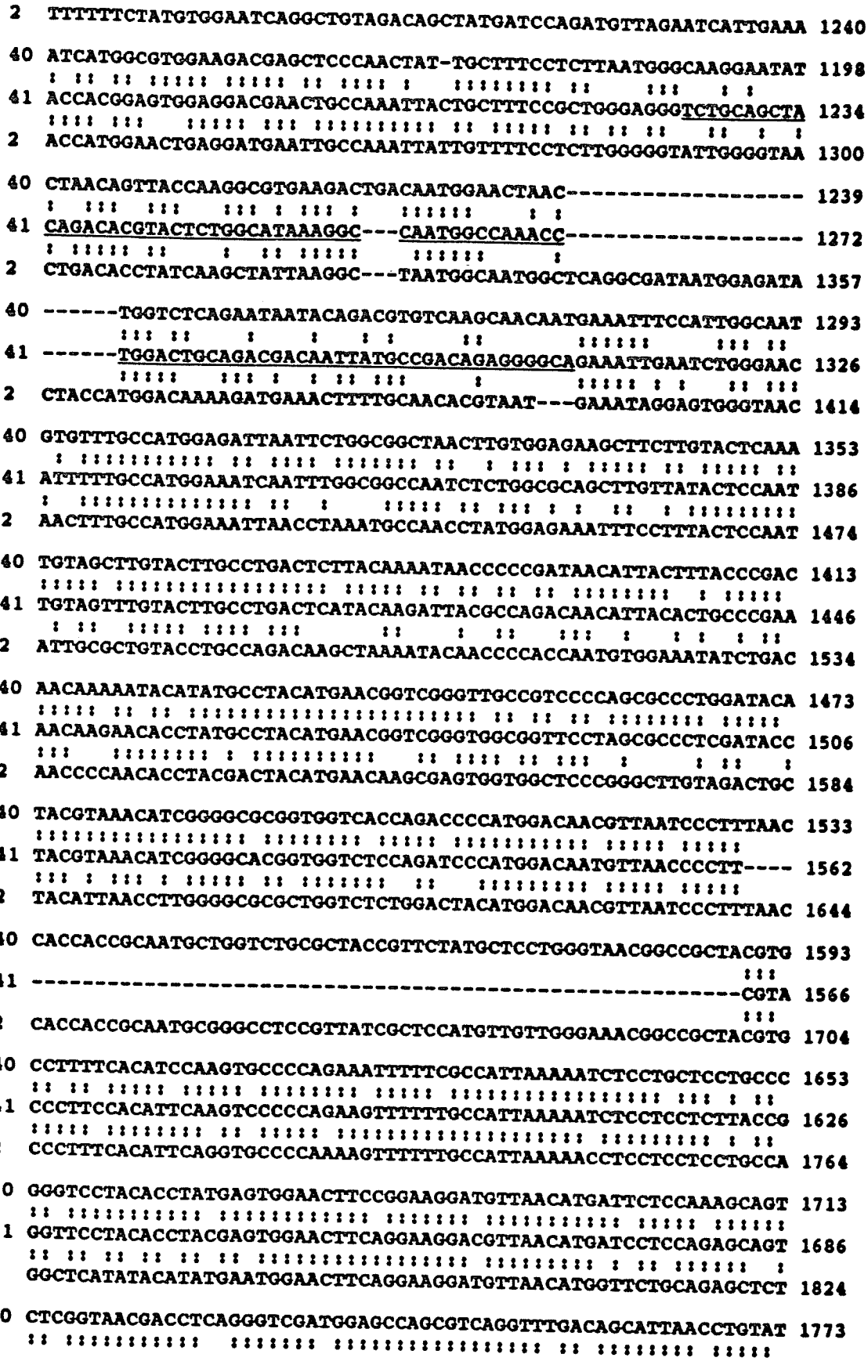
A number of Ad41 genes have been sequenced including the E1 genes (van Loon *et al.*, 1987), the DNA binding protein (Vos *et al.*, 1988), the hexon gene (Toogood *et al.*, 1989) and the fiber (Kidd, Erasmus and Tiemessen, 1990). With the failure of cloning and electroelution strategies to isolate an sequence sensitive to only Ad41, these reported sequences compared by computer and compared to the equivalent Ad2 and, where available, Ad40 sequences to determine the specific sequences unique to Ad41. Whilst the E1 genes and the fiber gene have the least nucleotide identity shared by Ad2 and Ad41, the sequence disparity is evenly distributed and it is difficult to find a lengthy sequence quite unique to Ad41 in these genes. Alignment of the sequences of fiber genes of subgenus C and subgenus F species served to demonstrate that the fiber gene of Ad40 has a deletion of 40 base pairs from a region coding for the C-terminal tip of the protein which is not well conserved. This sequence could have specificity to Ad41 but has a high content of adenine and thymine pairs and is unsuitable for use as a probe under highly stringent conditions. The hexon is known to have species specific epitopes and an

examination of the published sequences of the hexon gene identified several domains with promise as species specific probes. This gene, as shown before in Figure 1 had both conserved sequences with very high homology to Ad2 and Ad40 DNA and also abruptly demarcated sequences that have little or no homology to sequences of other viruses. Alignment of the hexon gene nucleotide sequence of Ad41 with that of Ad40 in Figure 32 elucidated a number of areas of sequence variation of thirty base pairs or more that could serve as probe sequences. The L1 loop was divided in 4 such areas by intervening sequences of homology of 15 or more base pairs, the L2 loop sequence formed a single stretch of variation while the L4 loop did not provide a prolonged stretch of nucleotide variation. Each of the four L1 and two L2 areas of nucleotide variation were assessed for their suitability as specific probes on several criteria; the length of the unique sequence which determines the likelihood of a duplicate sequence being found in cellular DNA; the melting temperature, determined by the proportion of guanine and cytosine residues in the sequence; and the ability of the sequence to pair either by folding or with complimentary nucleotides in another strand. The most suitable area was the L2 sequence of variation, nucleotides 1225 to 1308 of the Ad41 hexon sequence, a series of 84 nucleotides which had a greater than average content of guanine and cytosine residues and consequently a high melting temperature. The guanine and cytosine residues in the first half of the sequence are arranged more evenly, resulting in a higher melting temperature as calculated by nearest neighbour parameters (Figure 33) of thermodynamic values of base pairs (Breslauer *et al.*, 1986). The latter half of the sequence has the least homology to the corresponding Ad40 sequence. The two halves of the sequence, therefore, have different advantages, the latter half is naturally less likely to bind to DNA

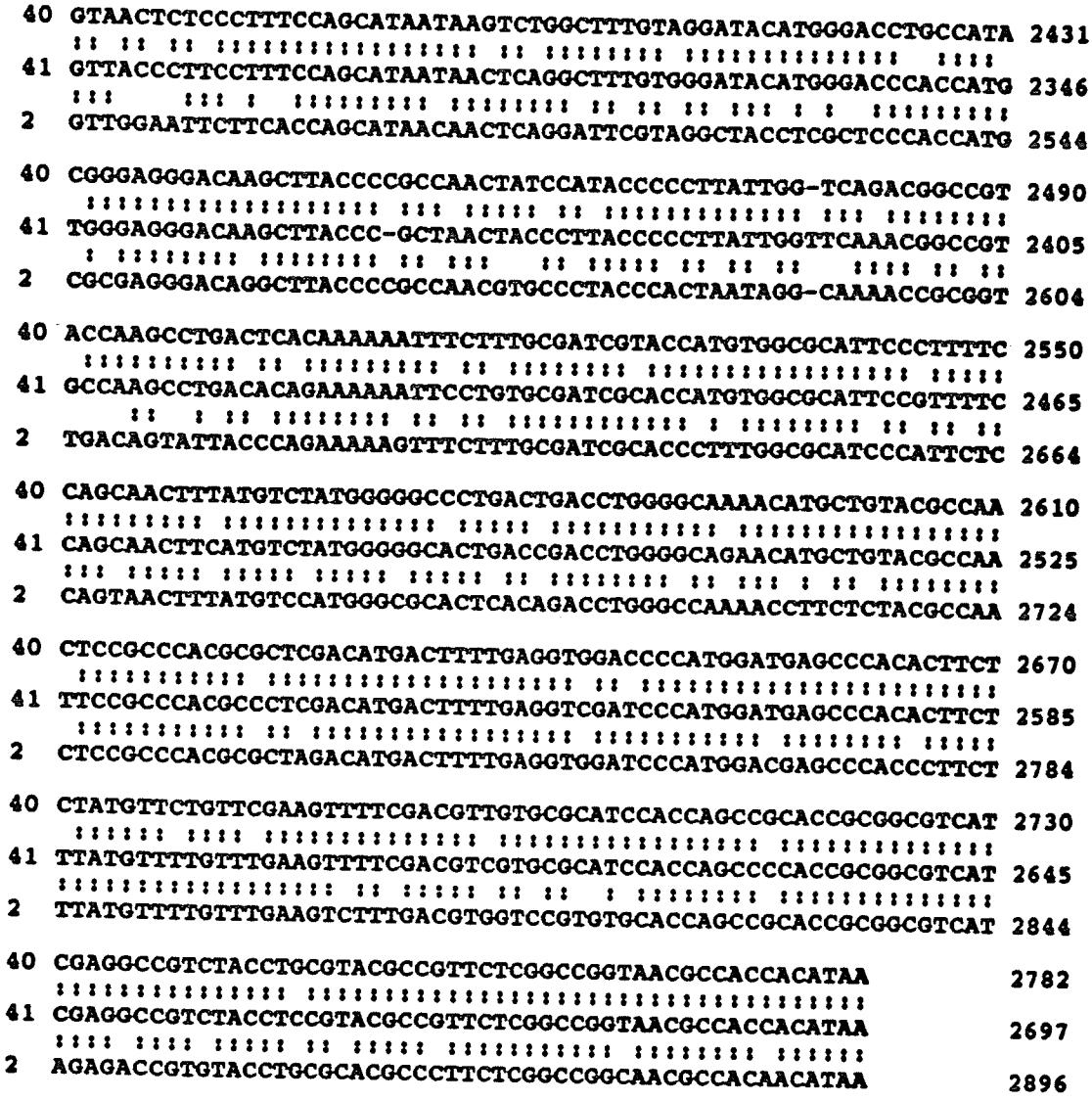
Figure 32. Alignment of the Ad40, Ad41 and Ad2 Hexon Gene Nucleotide Sequences.





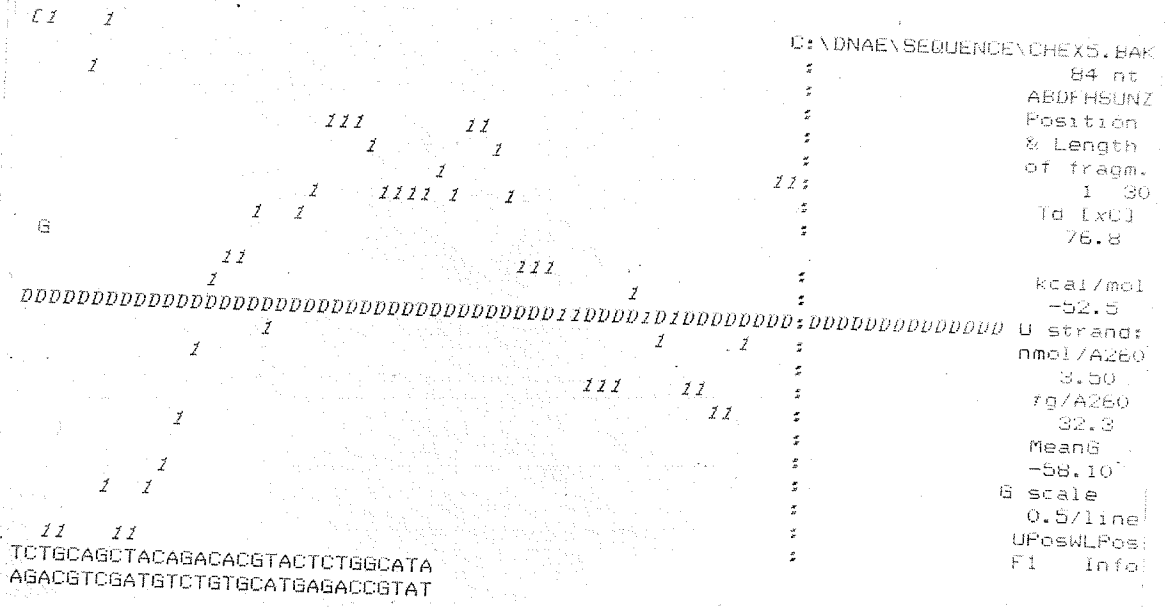


```
41 CTGGGTAACGACCTGCGGGTCGACGGAGCCAGCGTCAGGTTCGATAGCATTAATCTGTAC 1746
   ::::: ::::: ::  : :: ::::: :: ::: : : ::: :: ::::::  ::: :::
2  CTGGGAAACGATCTTAGAGTTGACGGGGCTAGCATTAAGTTTGACAGCATTTGTCTTTAC 1884

40 GCCAACTTTTTCCCCATGGCTCACAACACCGCTTCCACCTTGGAAGCAATGCTTCGTAAT 1833
   :::::::::::: :::::::::::::::::::: :::::::::::::: ::::: :: :::
41 GCCAACTTTTTTCCCATGGCTCACAACACCGCCTCCACCTTGGAAGCTATGCTGCGCAAT 1806
   :::: ::: :: :::::::: :::::::: ::::::::  ::::::: ::::: : :::
2  GCCACCTTCTTCCCCATGGCCCACAACACGGCCTCCACGCTGGAAGCCATGCTCAGAAAT 1944

40 GATACCAACGATCAGTCTTTCAACGACTACCTCTGCGCCGCAAACATGCTTTACCCCATA 1893
   :: :::::::: :: :: :: :::::::::::::::::: :::::::::::::: :::::
41 GACACCAACGACCAATCGTTTAACGACTACCTCTGCGCTGCAAACATGCTTTATCCCATT 1866
   :::::::::::::: :: ::::: :::::::: : ::: :: :::::::: :: :::::
2  GACACCAACGACCAGTCCTTTAATGACTACCTTTCCGCCGCCAACATGCTATACCCCATA 2004

40 CCCGCCAACGCTACTAGCGTGCCCATTTCTATTCCTTCGCGAAATTGGGCTGCTTTTCGG 1953
   ::  : :: :::::::::::: :: :::::::::::::::: :: ::::: :::::::::
41 CCTTCTAATGCTACTAGCGTTCCAATTTCTATTCCTTCGCGGAACTGGGCCGCTTTTCGG 1926
   ::  : :: :: :: : ::: :: :: :: :: :: ::::: :::::::: :: :::::
2  CCCGCCAACGCCACCAACGTGCCCATCTCCATCCCATCGCGCAACTGGGCAGCATTTCGC 2064

40 GGGTGGAGTTTTACTAGACTAAAAACTAAAGAAACCCCTCTTTGGGGTCCGGGTTTGATC 2013
   :: ::::::::::::: :::: ::::: :::::::::::   :: :::: :::: ::
41 GGCTGGAGTTTTACTCGACTGAAAACCAAAGAAACCCCCTCTTTGGGTTCGGGATT----- 1982
   :: :::   :: :: ::  :::: :: :: :::::::: ::  :::: :: :: :
2  GGTTGGGCCTTCACACGCTTGAAGACAAAGGAAACCCCTTCCCTGGGATCAGGCTACGAC 2124

40 CATATTTCACCTACTCTGGCTCCGTCCATACTTGGATGGCACCTTTTACCTGAA--CCAT 2071
                                                          :::
41 ----------------------------------------------------------CCAC 1986
                                                          :::
2  CCTTACTACACCTACTCTGGCTCCATACCATACCTTGACGGAACCTTCTATCTTAATCAC 2184

40 ACTTTTAAAAAGGTGTCCGTTATGTTCGACTCCTCTGTGAGCTGGCCTGGTAACGACCGA 2131
   ::::::::::::::: : : ::::: :::::::::::::: :::::::: :: ::::::
41 ACTTTTAAAAAGGTGTCCATCATGTTTGACTCCTCTGTGAGTTGGCCTGGCAATGACCGA 2046
   :: ::::: :::::: :::: :  :::::::: ::::: :: ::::: ::::: :::::
2  ACCTTTAAGAAGGTGGCCATTACCTTTGACTCTTCTGTTAGCTGGCCGGGCAACGACCGC 2244

40 CTACTTACTCCCAACGAGTTTGAAATCAAACGCACCGTGGATGGGGAAGGATACAACGTG 2191
   :: ::::: ::::: :::::::::::::::::::::::::::::::::::::: :::::::::
41 CTGCTTACCCCCAATGAGTTTGAAATCAAACGCACCGTGGATGGGGAAGGGTACAACGTG 2106
   :::::::: :::::::::::::: :: :::::: : :: :: ::::: :: ::::::::
2  CTGCTTACTCCCAATGAGTTTGAGATTAAACGCTCAGTTGACGGGGAGGGCTACAACGTA 2304

40 GCTCAATGTAACATGACCAAGGACTGGTTCCTCATACAAATGCTCAGTCACTACAATATT 2251
   :::::::::::::::::::: :::::::: :: :: :: ::::: ::::: ::::: :::
41 GCTCAATGTAACATGACCAAAGACTGGTTTCTGATTCAGATGCTTAGTCATTACAACATT 2166
   ::::: :: :::::::::::: :::::::: ::: : :::::: :      : ::::: :::
2  GCTCAGTGCAACATGACCAAGGACTGGTTCCTGGTGCAGATGTTGGCCAACTACAATATT 2364

40 GGCTACCAGGGTTTCCACGTACCAGAAAGCTACAAGGACAGGATGTACTCCTTTTTCCGA 2311
   :: :: :::::::::  : ::::: :::::::::::: :: :: ::::: ::::::::::::
41 GGATATCAGGGTTTTTATGTACCCGAAAGCTACAAAGATAGAATGTATTCCTTTTTCCGA 2226
   :: :: ::::: :: ::  : :: :::::::::::: ::  : ::::: :: :: ::: ::
2  GGCTACCAGGGCTTCTACATTCCAGAAAGCTACAAGGACCGCATGTACTCGTTCTTCAGA 2424

40 AACTTCCAACCCATGAGCCGCCAGGTGGTAGACACTACCACCTACACGGAGTATCAGAAT 2371
   :::::::: :: ::::: :: ::::::::: ::::::::::::::: ::: :::::::::
41 AACTTCCAGCCAATGAGTCGTCAGGTGGTAAACACTACCACCTACAAGGAATATCAGAAT 2286
   :::::::::: ::::: :: :: :::::  ::  ::: :  :::::::: :::::: :
2  AACTTCCAGCCCATGAGCCGGCAAGTGGTTGACGATACTAAATACAAGGAGTATCAGCAG 2484
```



The Ad41 and Ad40 hexon sequences and Ad41 and Ad2 hexon sequences were compared using the NucAlign software program. The sequence comparisons have been amalgamated and species identification and nucleotide number added at the left and right margins. Ad41 nucleotide sequences of 30 bases or greater in length with less than 50% homology to the Ad2 or Ad40 nucleotide sequences have been underlined. These sequences were analyzed for suitability for use as diagnostic probes. The first four underlined sequences occur in the L1 loop, the last in the L2 loop of the hexon genes.

Figure 33. Melting Temperature of Ad41 Sequences of the L2 Loop of the Hexon Gene.



The Oligo software program calculates the melting temperature of each possible oligomer by best neighbour parameters in which a value is assigned to each nucleotide depending on the adjacent nucleotides. This system best approximates the real melting temperatures of sequences *in vitro*. The line of Ds signifies the mean melting temperature of the 30 base pair oligomers. The data at the right margin is for the oligomer C from the beginning of the graph, the sequence of which is printed at the base of the figure. The points on the graph show that the highest melting temperatures occur in oligomers at either end of the L2 sequence.

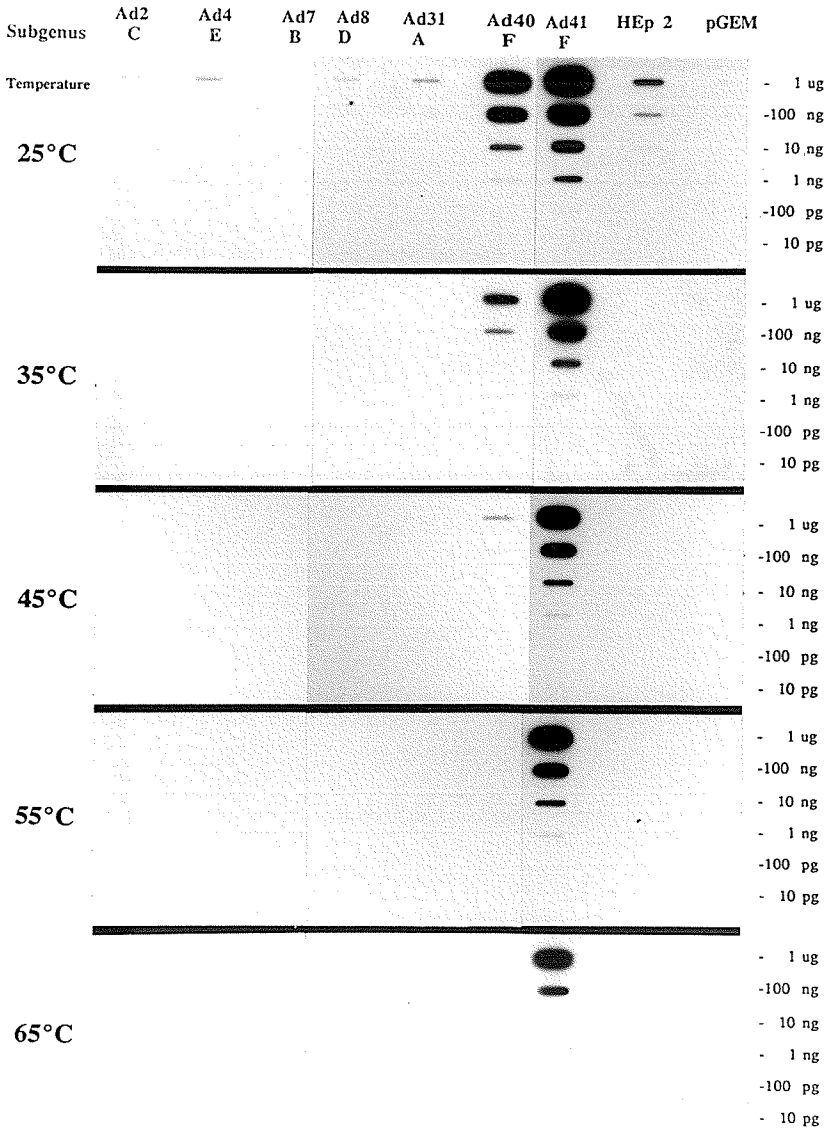
other than of Ad41 but the first part of the sequence can be used at higher melting temperatures and conditions of greater stringency. This L2 sequence was synthesized as two 40 base oligomers by sequential nucleotide addition by a commercial DNA Synthesizer and purified by high pressure liquid chromatography. These two oligomers, which normally form a nearly contiguous sequence coding for the L2 loop of the hexon, were designated Hex5A and Hex5B and have the following sequences;

Hex5A: GCAGCTACAGACACGTACTCTGGCATAAAGGCCAATGGCC

Hex5B: AACCTGGACTGCAGACGACAATTATGCCGACAGAGGGGCA

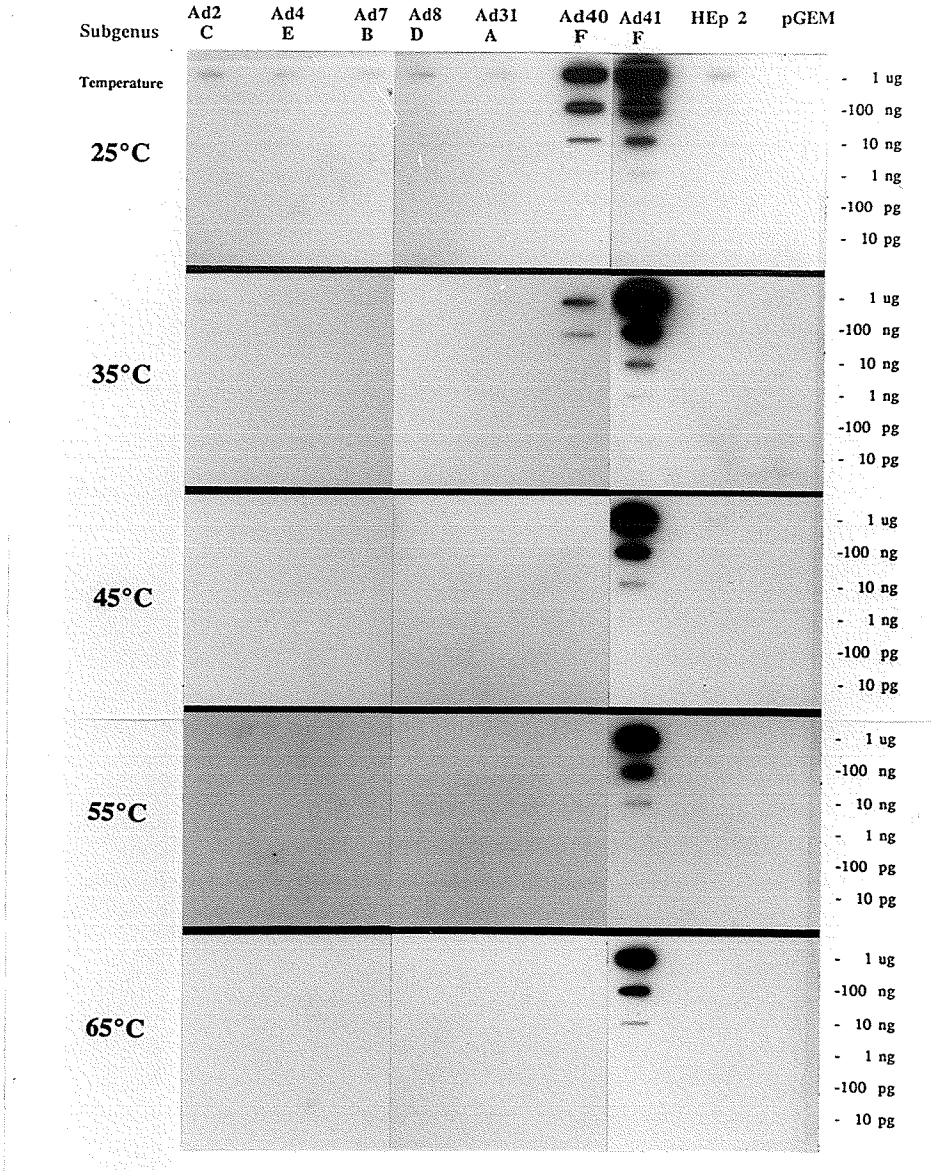
The melting temperature of Hex5A and Hex5B, calculated by best neighbour parameters, are 75.5°C and 79.7°C, respectively. The sensitivity of the two probes for Ad40 DNA and the DNA of other species was tested empirically by hybridizing the synthetic probes, radiolabelled by 5' end labelling, against membranes spotted with dilutions of adenovirus, λ phage and HEp 2 DNA in conditions of increasing stringency from 25°C to 65°C in solutions imitating 10°C increments. The results, shown in a series of panels in Figure 34 and Figure 35, are similar, despite a 22% difference in the identity of the sequences with Ad40 DNA, for Hex5A and Hex5B probes. At 25°C both probes react widely with the DNA of other adenovirus species and with the unrelated control DNA preparations. At 35°C the reaction is limited to Ad41 and Ad40 DNA. The effect of the greater proportion of shared nucleotides of the Hex5A sequence with other adenovirus DNA emerges in the greater intensity of hybridization of this probe with other subgenera DNA preparations at lower temperatures and the continued hybridization of Hex5A with Ad40 DNA at 45°C in Figure 34. Both probes are completely specific in the lower panels and

Figure 34. Hybridization of the Hex5A Probe with Subgenera DNA at Various Temperatures.



The synthetic probe was hybridized with membranes on 2 occasions, once at 25°C and once at 55°C. The other temperatures were simulated by the addition of formamide to the hybridization solution of separate membranes according to the estimation that 1% formamide lowers the melting temperature by 0.72°C (McConaughy, Laird and McCarthy, 1969). Each membrane was exposed to film for 48 hours.

Figure 35. Hybridization of the Hex5B Probe with Subgenera DNA at Various Temperatures.



The synthetic probe was hybridized with identical membranes at different conditions of stringency defined by temperature or the concentration of formamide in the reaction mixture which decrease the melting temperature of duplex DNA by 0.72°C for each % concentration. The membranes in each panel were exposed to film for 48 hours.

could be used as to differentiate between species of subgenus F at temperatures above 55°C.

5. Diagnosis of Adenovirus in Clinical Samples by Various Tests.

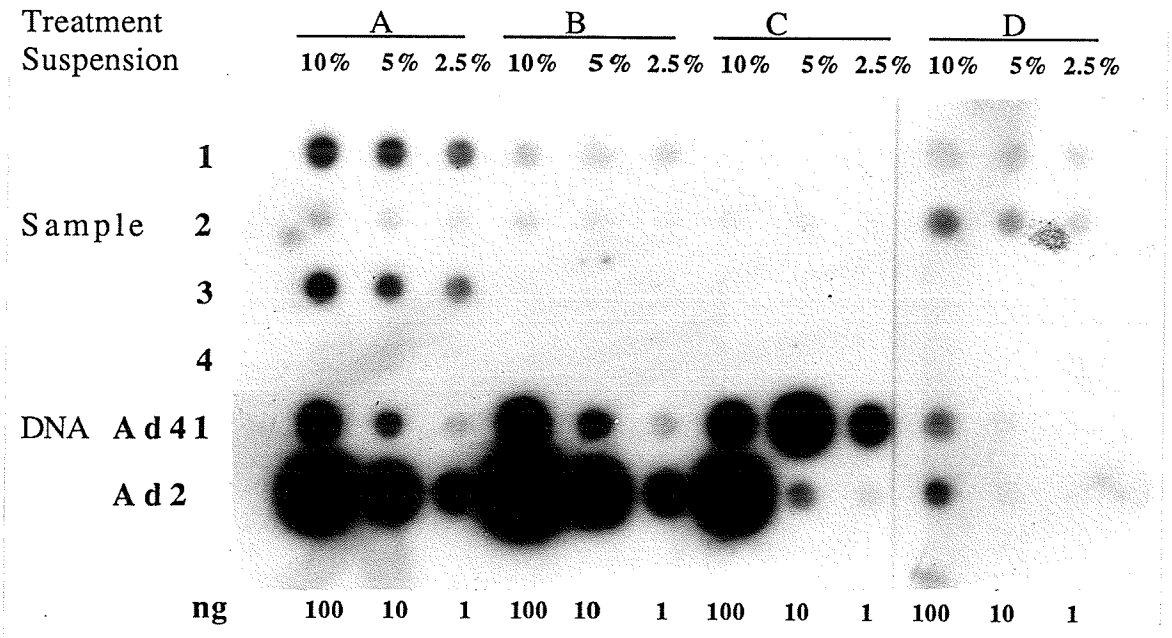
In order to assess the capabilities of the hybridization test with the probes developed for detection of all adenoviruses, subgenus F species and Ad41 specifically, a study was undertaken to compare hybridization with tests currently in routine use and the latest commercial kits for diagnosis of adenoviruses in paediatric gastroenteritis. For the period from July of 1990 to June, 1991 all stool samples from children 3 years old or less sent to the Cadham Provincial Laboratory, the reference centre for the province of Manitoba, were examined for the presence of adenoviruses by electron microscopy, tissue culture and enzyme immunoassay. Two commercial immunoassay kits were employed, a group reactive test utilizing a monoclonal antibody to a conserved internal hexon epitope that reacts with all human adenovirus species (Cepko *et al.*, 1983), and a kit using monoclonal antibodies specific for enteric adenovirus species. Tissue culture was performed with three cell lines, 293 cells, primary rhesus monkey kidney cells and HEp 2 cells. All samples were examined under the electron microscope, screened with immunoassays and inoculated into culture tubes. The clinical specimens which could be grown were processed further to identify as many species as possible among the isolates. Virus from specimens which grew in the conventional HEp 2 or rhesus monkey cells were subjected to the neutralization test with antisera to Ad1 through to Ad6. Electron microscopy positive specimens were cultured on 293 cells and the DNA extracted from the cell lysates for restriction analysis, if possible. A portion of those specimens that

proved uncultivable were subjected to a blocking immunoassay as a further means of identification. This assay used two monoclonal antibodies supplied by Jan de Jong, one specific to Ad40 and the other to Ad41, to bind the virus antigens in the to the specimen during incubation and inhibit binding of enteric specimens to a capture antibody, the monoclonal antibody to the common antigen on the internal aspect of the hexon (Cepko *et al.*, 1983) applied to plate wells. In this way over 1,000 specimens were examined. Adenovirus containing specimens were confirmed by several means. Most adenovirus isolates were identified to the level of species and an accurate assessment of the rates of detection of the different methods was compiled. A total of 200 of these specimens were tested with the HPII fragment as the common probe and a variety of Ad41 DNA probes to evaluate the sensitivity and specificity of the different probes and the method of hybridization in comparison to the other methods of adenovirus isolation and typing available. The best means of preparing and denaturing the DNA from clinical samples for probing were first investigated.

Four clinical stool samples positive for adenovirus by electron microscopy and a series of dilutions of Ad2 and Ad41 DNA as controls were treated by four different methods in common usage and spotted on duplicate membranes in 4 separate panels. Each specimen was suspended in phosphate buffered saline at approximately 1 gram of stool per 10 ml using a vortex mixer and clarified by two successive rounds of centrifugation at 1,500 rpm or 800 g for 10 minutes and then for 5 minutes at 15,000 g in a microcentrifuge. Doubling dilutions of each clarified specimen were made in phosphate buffered saline and 0.5 ml of each dilution denatured by one of three methods; boiling

for ten minutes, or by the addition of 166 μl of 3M NaOH or by spotting directly to the nylon membrane, detaching this panel of the membrane and denaturing the DNA by placing the panel over a piece of blotting paper saturated in 1M NaOH. Takiff *et al.*, 1985 have noted that prior extraction of protein from the clinical samples can improve the sensitivity of detection of DNA. To test this observation a further 0.5 ml aliquot of each specimen was treated with 50 μl of 10% sodium dodecyl sulphate and 125 μg of proteinase K, incubated at 60°C for 60 minutes, extracted with an equal volume of phenol saturated with phosphate buffered saline (PBS). The samples were then extracted with two volumes of chloroform, diluted and denatured by boiling. The boiled samples were cooled by immersion in ice water and 112.5 μl aliquots spotted on the membrane. All 4 panels of duplicate membranes were baked and hybridized together at 68°C with a HPII fragment probe. The autoradiograph of one of the membranes is shown in Figure 36 and contains protein extracted samples in panel A; 100 μl aliquots of the unextracted samples that were boiled and spotted in panel B of Figure 36; the NaOH denatured samples that were neutralized by the addition of an equal volume, 660 ul, of 2M ice cold ammonium acetate and spotted in aliquots of 264 ul, to compensate for the addition of reagents, in the third panel. The samples directly spotted to panel D of Figure 36 were neutralized by transferring the denatured membrane to a blotting paper saturated with 1M ammonium acetate for 15 minutes. Control DNA, Ad41 and Ad2 DNA in 3 log dilutions from an initial concentration of 10 ng/ml, were treated in the same manner as the samples on each panel. This experiment demonstrated that the prior protein extraction did improve the sensitivity of detection of dilutions of at least one of the clinical samples in panel A. The samples in the first panel formed more complete circles and it would

Figure 36. Trial of Four Methods for Preparation of Clinical Stool Samples for Hybridization.



10% stool suspensions in which adenovirus had been observed in the electron microscope were treated in four ways. Panel A, protein was removed with phenol after digestion with SDS and proteinase K, the samples boiled, cooled and applied to membrane; panel B, samples were boiled, cooled and spotted; panel C, samples were denatured in 1M NaOH, neutralized and spotted; panel D, samples were spotted directly, denatured by placement over paper soaked in 1M NaOH and then neutralized. Control Ad41 and Ad2 DNA dilutions were spotted after the same treatment as the samples in each panel. Membranes were hybridized under stringent conditions with the HPII probe.

seem that deproteination of the sample also reduces surface tension that impedes the flow of some samples and resulted in clearer definition of the hybridization spots.

Based on these results, each of the clinical samples, dissolved in PBS as a 10% suspension and clarified by centrifugation, was processed in 0.5 ml aliquots, treated first with 75 μl of 10% SDS and 200 μg of proteinase K and extracted with equal volumes of phenol and then chloroform. The samples were reextracted with phenol if a large interphase remained and the process repeated till the suspensions were clear. There was no appreciable difference between the test samples denatured by boiling or NaOH (Figure 36, panels B and C) and the more simple process of denaturation by boiling, after protein extraction, was used for all the clinical samples.

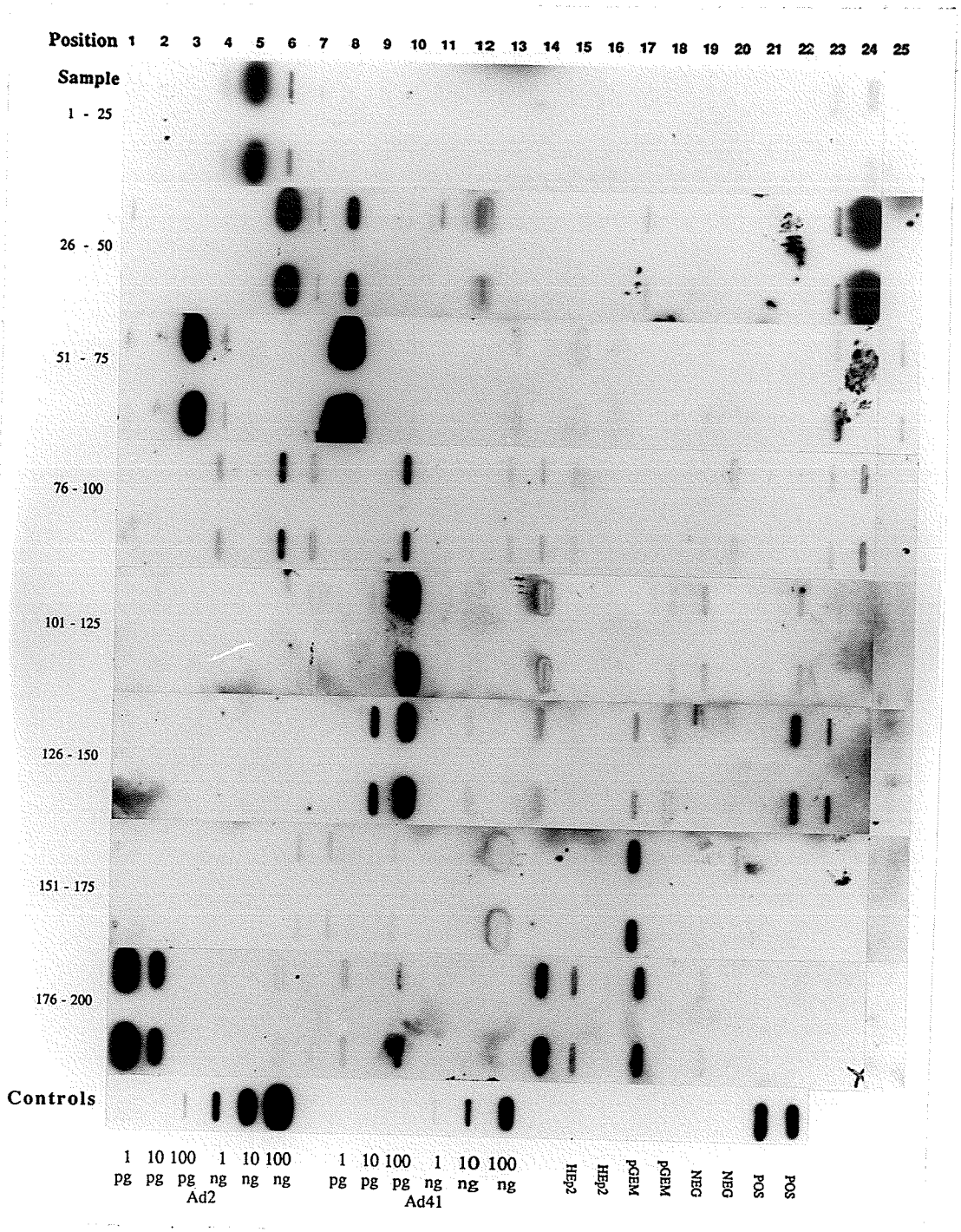
Two hundred clinical samples, coded and spotted in random order, were spotted in 150 μl aliquots for the probe test. 65 of the specimens were known positives in which the presence of adenovirus had been detected by at least one test. It was necessary to determine whether the treatment during evaluation by other diagnostic tests prior to hybridization had affected the quality of the specimens and 9 adenovirus positive samples were spotted twice to compare the 10% suspension used in prior evaluations and a fresh suspension made from frozen samples. 2 adenovirus positive specimens were from the same patient. 12 specimens contained rotavirus, 8 were positive for enterovirus, 6 had small round virus particles evident by electron microscopy and 1 contained coronavirus. The remaining 108 specimens had not had any virus detected by electron microscopy, enzyme immunoassay, or tissue culture. The manifold used to spot the samples had wells

arranged in 3 rows of 24 slots. 24 specimens were spotted in duplicate in the first two complete rows. A twenty fifth sample was spotted by the last 2 slots of the third row so that the 200 samples were distributed on 8 membranes. The remaining wells of the third row were used for controls. A dilution series of purified Ad41 and Ad2 DNA from 100 ng to 1 pg was spotted in wells 13 to 8 and 6 to 1, respectively, leaving a space after the largest concentration of DNA to avoid overlap, as a set of standards against which to approximate the amount of DNA in the samples. Duplicate 1 μg samples of plasmid DNA and cellular DNA and DNA of known positive and negative stool samples were also spotted with an intervening well in the third row to monitor the reaction of the probes. Several identical sets of the 8 membranes were made and each set was treated in identical conditions to make the results as comparable as possible. Each set of membranes were divided in half and sealed in two bags and both bags exposed to the same amount of different probes, 10^8 cpm of radiolabelled denatured probe DNA in each reaction mixture, and allowed to hybridize at 68°C for 16 hours. Each set of membranes was placed between film and two intensifying screens and placed at -70°C. Film was developed at regular intervals. With the exception of the HPII probe specimens, the intensity of probe reactions were comparable and film developed after 48 hours exposure to each membrane of each set was collected, photographed and the prints spliced for presentation of the reaction of the complete set of 200 samples at identical times under identical conditions. In each film the twenty fifth specimen has been cut from the third row and repositioned so that all the samples of each membrane can be presented in two rows of duplicates. A control row, minus the last two wells, is shown below the samples and demonstrates the reaction of the probe with the purified virus, cell and plasmid DNA

and positive and negative stool samples at the same time as the reaction with the samples was developed. The reaction of the HPII fragment probe is shown in the first composite representation of all 200 samples in Figure 37. Unlike the other membranes, the samples exposed to the HPII probe were not all kept with the film for the same length of time. The reaction of the HPII probe with the samples tended to be light compared to the other probes and some of the samples were kept with the film for prolonged times to ensure the reaction was clearly visible. The time lapse before the films were developed varied from 48 hours to 160 hours, as a result Figure 37 is composed of some panels in which the background, although not obscuring the samples, is marked. From the control lane it can be seen that the HPII probe reacted indiscriminately with adenovirus DNA, detecting 100 pg of homologous Ad2 DNA 1 ng of Ad41 DNA. The positive control specimen but no plasmid or cell DNA was detected. The HPII probe reacted with 60 samples altogether as summarized in Table 4.

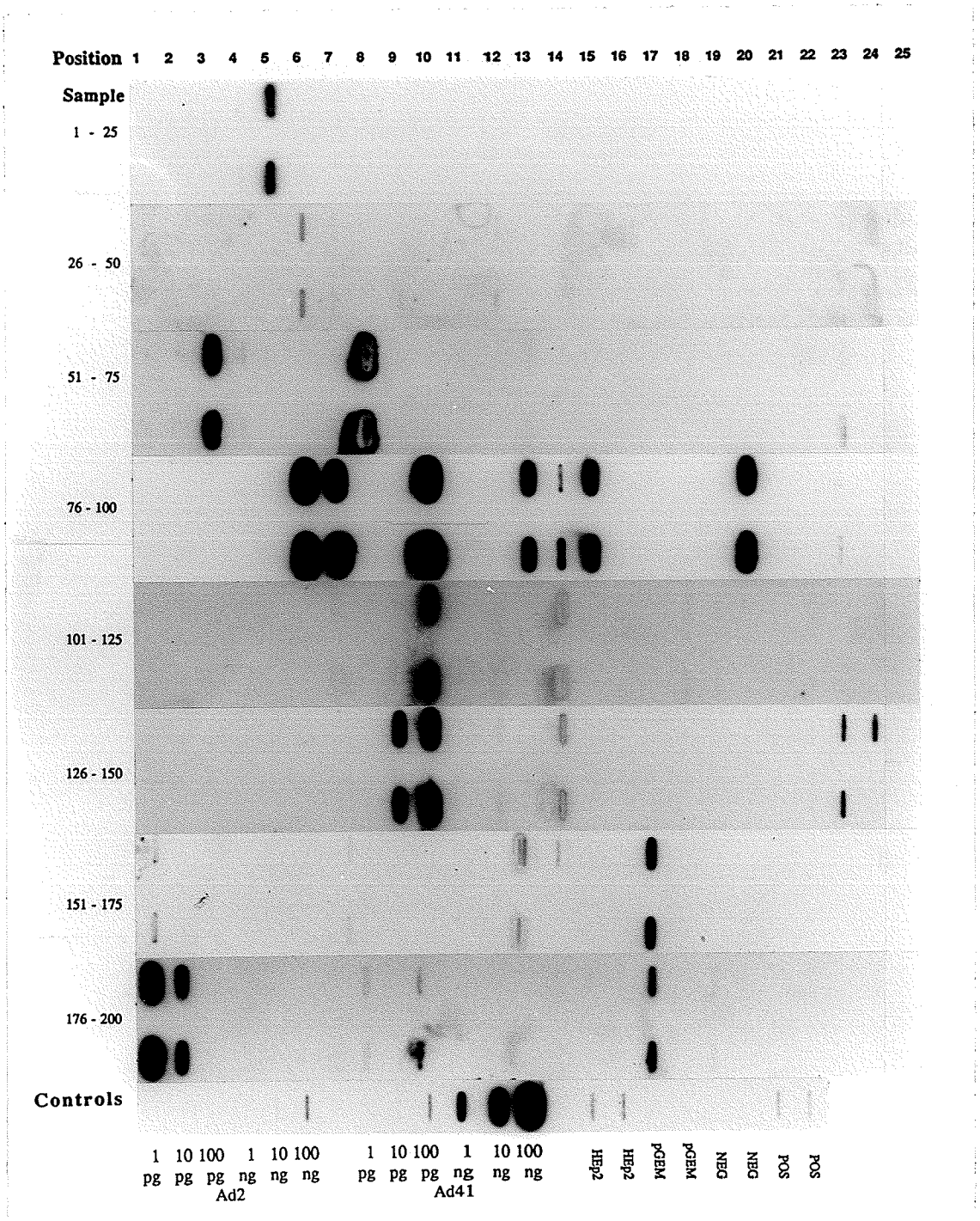
The reaction of a genomic Ad41 DNA probe is shown in Figure 38. The reactions of all of the samples in this and subsequent s were developed after exposure to film for 48 hours. The genomic Ad41 probe gave extensive cross-reaction with Ad2 control DNA but did not react with the plasmid or negative stool sample. A slight reaction with the cellular DNA control indicated some cellular contamination, relatively small in terms of absolute DNA content, in the genomic probe. 35 of the samples were detected with the genomic probe as summarized in Table 4. Most of the positive samples demonstrate, despite the shorter time interval of film exposure, a greater intensity of reaction than expressed with the HPII probe and the presence of larger amounts of viral DNA

Figure 37. Hybridization of Clinical Samples with an HPII Fragment Probe.



10% stool suspensions, deproteinated and boiled, were spotted on membrane, baked and hybridized with the HPII fragment as the cross-reactive probe. The film enclosed with the membranes were developed between 48 and 160 hours.

Figure 38. Hybridization of Clinical Samples with Ad41 Genomic DNA.



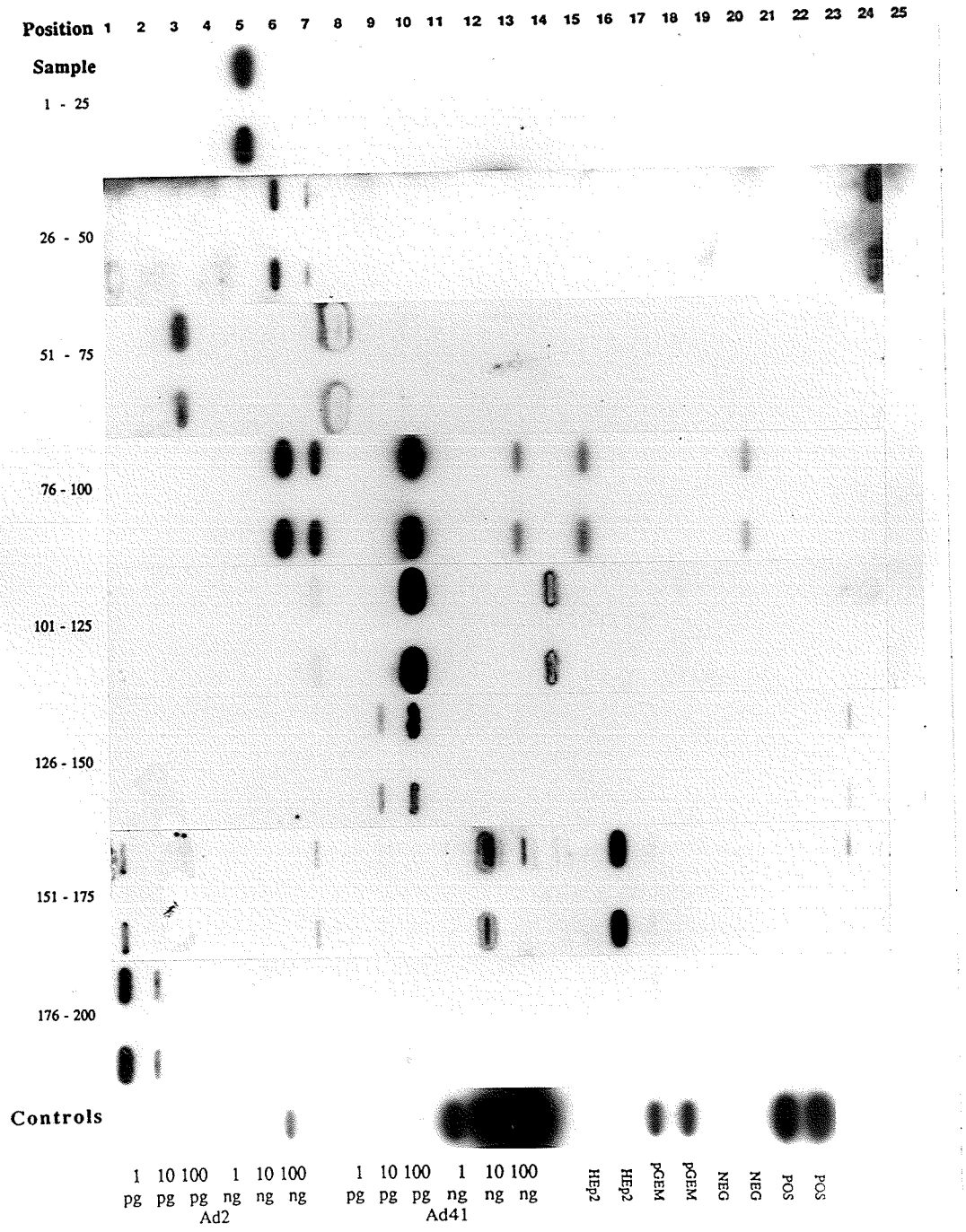
Duplicate membranes were hybridized with genomic Ad41 DNA. Film was kept with the membranes for 48 hours before developing.

homologous to the Ad41 probe. Comparison of intensity of sample spots with the control dilution series would indicate that the strongest samples would contain more than 100 ng of homologous DNA in the 150 μl of 10% suspension spotted.

The hybridization of the plasmid p41EC, containing an insert of the *Eco*RI C fragment, is shown in Figure 39. 32 of the samples, not always the same ones as reacting with the genomic DNA, are detected by the p41EC probe (Table 4). The plasmid control DNA reacted strongly with p41EC and may account for some of the differences in reactions. The *Bgl*II D fragment, purified from the plasmid vector by a single passage through agarose, has been hybridized with the samples pictured at 48 hours after enclosure with film in Figure 40. The reaction of the *Bgl*II D probe with the Ad2 dilution series is approximately 10,000 fold less than with the Ad41 DNA concentrations and is consistent with the differential sensitivity for homologous and heterologous DNA observed in the adenovirus subgenera sensitivity tests. Electroelution of the fragment after separation on agarose gel does not entirely separate it from the plasmid DNA and some reaction of the p41EC probe with the pGEM control was evident, although greatly reduced. 30 clinical stool samples (Table 4) were reactive with the *Bgl*II fragment probe, the majority of the most responsive samples were in agreement with Ad41 and p41EC detection, although several of the samples nearer the limits of detection were not shared with other probes.

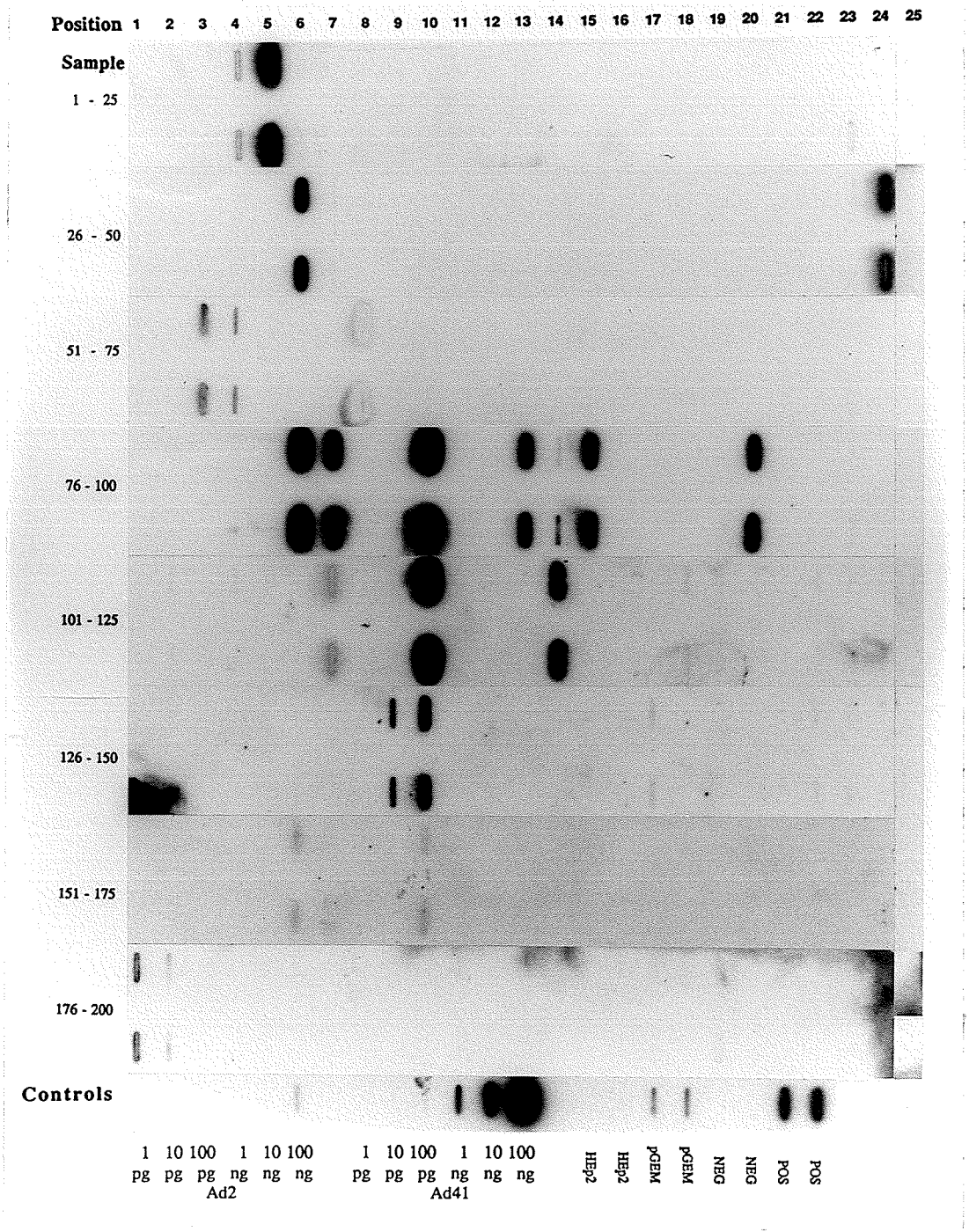
The reactions of the samples at 48 hours with the synthetic Hex5B probe have been collated in Figure 41. This probe, in accordance with the results of the subgenera DNA sensitivity tests, reacted in a highly specific manner, reacting only with adenovirus

Figure 39. Hybridization of Clinical Samples with Plasmid p41EC.



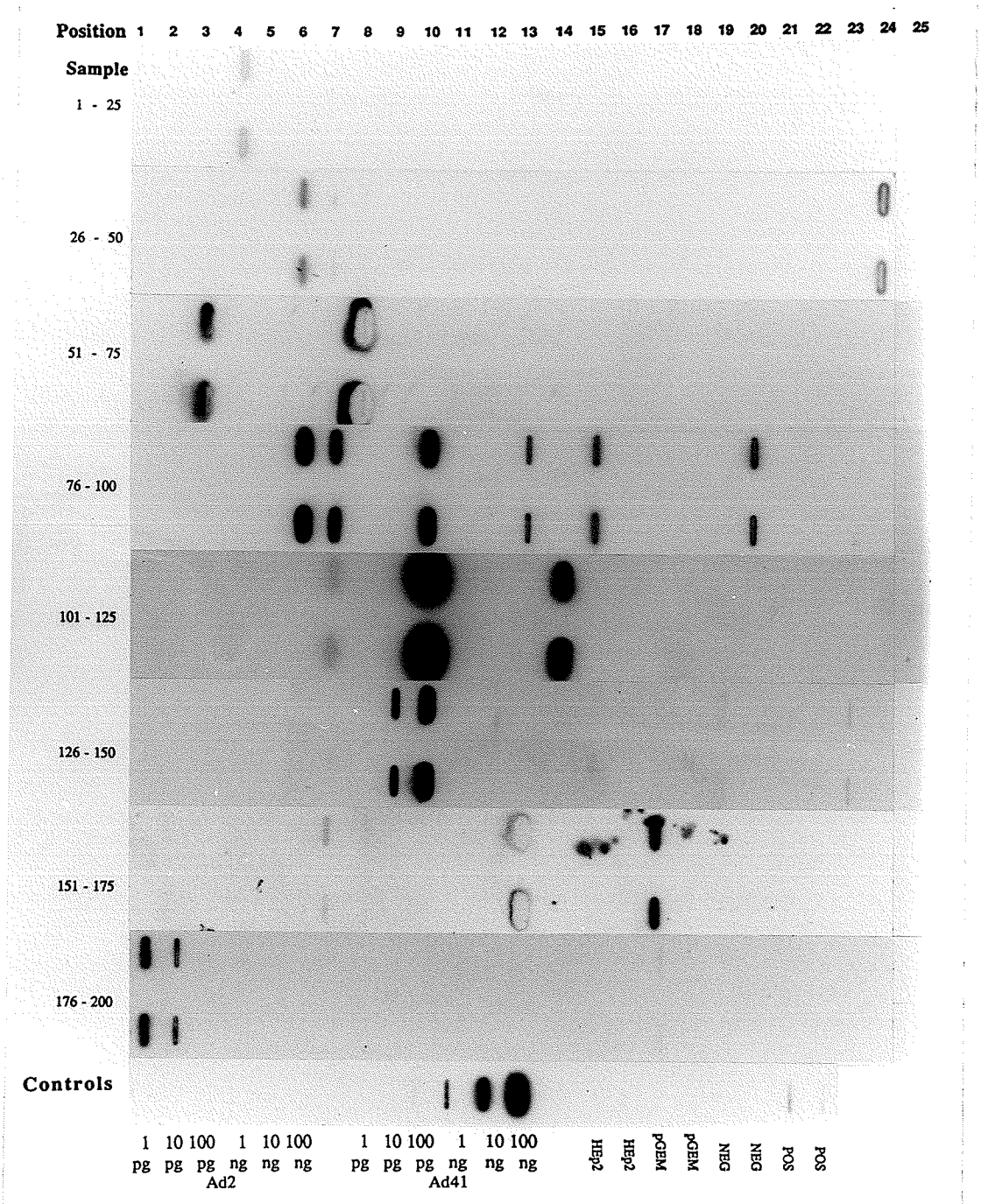
The clinical samples, spotted on membranes, were hybridized with p41EC plasmid which contains the *Eco*RI C fragment of Ad41. Films were developed after 48 hours.

Figure 40. Hybridization of the Clinical Samples with the *Bgl*II D Fragment.



Clinical samples spotted on nylon membrane were hybridized with the *Bgl*II D fragment of Ad41 as the subgenus F specific probe. Films were developed after 48 hours.

Figure 41. Hybridization of Clinical Samples with the Synthetic Hex5B Probe.



The clinical samples were hybridized at 65°C with 10^8 cpm of the Ad41 specific synthetic Hex5B sequence. A 48 hour autoradiograph is shown.control samples and

detecting quantities of Ad41 DNA with a greater than 1,000 fold greater absolute sensitivity than the Ad2 DNA. The intensity of reaction of this probe measured in the same 48 hour time of exposure of the film exposed to the hybridized membrane was appreciably less than the other probes of Ad41 DNA. 25 of the clinical samples were detected with the synthetic probe as shown in Table 4.

The hybridization reaction of the 200 clinical samples with each of the probes, in the order spotted on the duplicate membranes, have been compiled in Table 4 and compared with the results of the other diagnostic tests performed on the samples. An arbitrary scale from 1+ to 5+ has been used to grade the hybridization reaction of each specimen to evaluate the intensity of hybridization with each probe. Reactions at the limit of detection have been marked $\pm$.

The reactions of the probes concur with the results of the other diagnostic tests except in a few instances; no adenovirus was found in specimens 6828, 6783 and 6575, in positions 102, 104 and 160, respectively, other than by hybridization with probes derived from plasmids. Therefore, the reaction of the p41EC and *Bgl*II D probes with these specimens was considered spurious. Two specimens, numbers 10165 and 3887, in positions 49 and 177, reacted with Ad41 DNA probes although only species Ad2 and Ad5 had been detected in these specimens. These specimens probably contained both a subgenus C and an enteric adenovirus species in concurrent infection. The HPII probe detected all of the specimens that reacted with the Hex5B probe, with the exception of one, and generally responded with a similar but consistently lower degree of

Table 4. Detection and Identification of Adenovirus in Clinical Samples by Various Tests.

SAMPLE NUMBER	CLINICAL SPECIMEN	ELECTRON MICROSCOPY	GROUP EIA	CULTURE NEUTRALIZ.	SUBG. F EIA	RESTRICTION ANALYSIS	HYBRIDIZATION PROBE 1	2	3	4	5
4	12566	+	+	UId	+	Ad41A	±	±	+	±	+
5	13690	-	+	NG	+	NG	3+	2+	4+	3+	-
6	6572	-	-	UId	-	Ad2	+	-	-	-	-
7	3025	-	-	Ad5	nd	nd	±	-	-	-	-
16	12991	-	-	Ad4	nd	nd	-	-	-	-	-
24	12557	-	-	Ad2	nd	nd	±	-	-	-	-
31	1974	+	+	UId	+	Ad41A	4+	+	2+	+	+
32	11365	+	+	NG	+	NG	+	±	-	+	+
33	6572	-	-	Ad2	-	nd	2+	-	-	-	-
37	12347	+	+	Ad4	nd	nd	+	±	-	-	-
42	3489	-	-	Ad2	nd	nd	+	-	-	-	-
48	13704	-	+	Ad2	nd	nd	+	-	-	-	-
49	10165	+	+	Ad2	nd	nd	5+	±	2+	2+	+
53	5183	+	+	UId	+	Ad41A	4+	3+	+	2+	2+
54	13347*	+	+	Ad4	nd	nd	+	±	+	-	-
58	825	+	+	UId	+	Ad41A	5+	4+	2+	3+	4+
63	13602	-	-	Ad5	nd	nd	±	-	-	-	-
65	18	-	+	Ad2	nd	nd	±	-	-	-	-
69	5607	-	-	UId	-	Ad1v	±	-	-	-	-
73	13002	+	+	UId	-	Ad3v	+	+	-	-	-
75	6843	+	+	UId	nd	Ad2v	+	-	-	-	-
79	13257	-	+	Ad3	nd	nd	+	-	-	-	-
81	2884	+	+	NG	+	NG	2+	5+	4+	3+	3+
82	3054	+	+	UId	+	Ad41A	+	5+	3+	2+	2+
85	2884	+	+	NG	+	NG	2+	5+	5+	4+	3+
88	12779	+	+	UId	+	Ad41A	+	3+	2+	+	+
89	4504	+	-	Ad5	nd	nd	+	2+	+	-	-
90	12780	+	+	UId	+	Ad41A	±	3+	2+	+	+
95	12779	+	+	UId	+	Ad41A	+	3+	2+	+	+
98	2859	+	-	Ad2	nd	nd	+	±	±	-	-
99	13266	-	+	Ad6	nd	nd	+	-	-	-	-
100	2859	+	+	Ad2	nd	nd	±	-	-	-	-
102	6828	-	-	NG	nd	nd	-	-	±	±	-

104	6783	-	-	NG	nd	nd	-	-	±	±	-
107	1296	-	+	UId	+	Ad41A	±	-	+	+	±
110	2115	+	+	UId	+	Ad41A	4+	2+	5+	5+	5+
114	12458	+	+	UId	+	Ad41A	2+	+	2+	+	3+
118	13348*	+	+	Ad4	nd	nd	±	±	±	-	-
119	2286	-	+	UId	-	Ad2v	+	-	-	-	-
122	12783	-	+	Ad2	nd	nd	+	-	±	-	-
134	701	+	+	UId	+	Ad41A	2+	2+	+	+	2+
135	11861	-	+	NG	+	NG	3+	4+	2+	2+	3+
137	4293	+	+	UId	nd	Ad12	±	±	-	-	-
139	4504	+	-	Ad5	nd	nd	+	+	-	-	-
142	12783	-	+	Ad2	nd	nd	+	-	+	-	-
143	12533	-	+	NG	+	NG	±	-	-	-	±
147	1564	-	+	Ad2	nd	nd	2+	-	±	-	-
148	11943	+	+	UId	+	Ad41A	+	+	±	+	+
151	2823	-	+	NG	-	NG	-	±	-	+	-
156	12533	-	+	NG	+	NG	±	-	±	-	±
157	4389	+	-	UId	-	Ad41A	±	±	-	+	-
159	4011	+	-	NG	-	NG	±	-	-	±	-
160	6575	-	-	NG	nd	nd	-	-	±	-	-
162	12480	+	+	UId	+	Ad41A	2+	+	-	2+	2+
166	13258	+	+	NG	+	NG	2+	2+	-	2+	2+
167	5154	+	-	NG	-	NG	-	-	-	±	-
171	9234	-	-	Ad1	nd	nd	-	-	-	-	-
176	12477	+	+	UId	+	Ad41A	5+	4+	+	2+	2+
177	3887	+	+	Ad5	nd	nd	3+	2+	±	+	+
181	2465	-	-	Ad2	nd	nd	±	-	-	-	-
183	4292	+	+	Ad2	nd	nd	+	+	-	-	-
185	13257	-	+	Ad3	nd	nd	+	-	-	-	-
189	3200	-	-	Ad1	nd	nd	2+	-	-	-	-
190	2551	-	-	Ad2	nd	nd	+	-	-	-	-
192	12480	+	+	UId	+	Ad41A	2+	2+	-	+	±
194	13347*	+	+	Ad4	nd	nd	±	±	±	-	-
200	13621	-	-	Ad2	nd	nd	3+	-	-	-	-

NG = no growth. UId = unidentified. nd = not done. * samples from same patient. Probe 1 = HPII, 2 = Ad41, 3 = p41EC, 4 = *Bgl*II D, 5 = Hex5B.

hybridization intensity. The sensitivity and specificity results of the various tests and each hybridization probe are compared in Table 5. Discounting the duplicate samples of the same specimen and the second specimen from the same patient, electron microscopy and the adenovirus group immunoassay detected adenovirus in 30 and 34 or 54.6% and 61.8% of the 55 unique samples respectively. An adenovirus was grown from 45 of the unique samples in 293, HEp 2 or rhesus monkey kidney cells and 25 or 45.5% of these isolates were typed by neutralization with antisera to Ad1, Ad2, Ad3, Ad4, Ad5 or Ad6. Compared to these methods the sensitivity of hybridization rated well. The reaction of the HPII probe, allowed to develop for extended times, detected 51 of the 55 total specimens or 92.7%. Detection of enteric specimens with all the Ad41 DNA probes was high despite the shorter interval of exposure of the hybridized membranes to film. The high concentration of virus particles in enteric specimens probably enabled the reaction of the samples to develop on film in a relatively short time in comparison to the HPII probe. This high concentration of virus particles is also reflected in the improved rate of detection of enteric adenovirus isolates by enzyme immunoassay. Of the 26 unique specimens of enteric adenovirus on which the subgenus F immunoassay test was performed all but 4 were found positive, a sensitivity rate of 84.6%. There were 28 unique enteric adenovirus containing specimens in all, 4 of which went undetected with a genomic Ad41 DNA probe, 10 with probe p41EC, 2 with the *Bgl*II D fragment and 4 using the synthetic hex5B sequence, resulting in sensitivity rates for these probes of 85.7%, 64.3%, 92.9% and 85.7%, respectively. The high sensitivity of some of the probes was somewhat offset by their lack of specificity. The complete Ad41 genome, with many sequences in common with other species, reacted with 9 specimens identified

Table 5. Sensitivity and Specificity of Various Diagnostic Tests.

DIAGNOSTIC METHOD		SENSITIVITY	SPECIFICITY
ELECTRON MICROSCOPY		**54.6%** 30/55	**100%** 30/30
CULTURE & NEUTRALIZATION		**45.5%** 25/55	**100%** 25/25
GROUP ENZYME IMMUNOASSAY		**61.8%** 34/55	**100%** 34/34
SUBG. F ENZYME IMMUNOASSAY		**84.6%** 22/26	**100%** 26/26
HYBRIDIZATION	HPII Probe	**92.7%** 51/55	**100%** 51/51
	Ad41 Probe	**85.7%** 24/28	**72.7%** 24/24+9
	p41EC Probe	**64.3%** 18/28	**62.1%** 18/18+11
	*Bgl*II D Probe	**92.9%** 26/28	**92.8%** 26/26+2
	Hex5B Probe	**85.7%** 24/28	**100%** 24/24

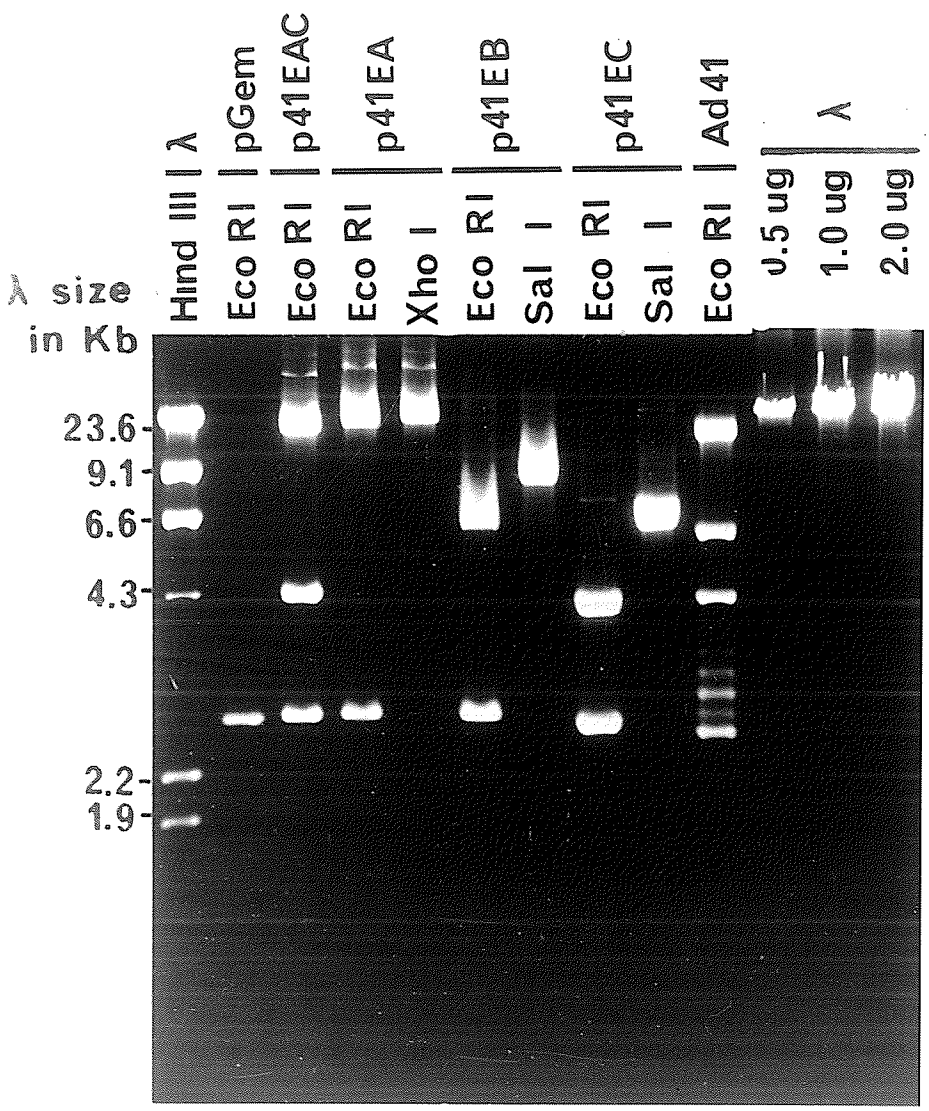
as containing non-enteric adenovirus species, a specificity rating of just 72.7%. The p41EC probe had two sources of false positive reactions, hybridizing with some specimens containing other species of adenovirus because of shared sequence homology and detecting plasmid DNA presumably released from endogenous bacteria. The specificity of the p41EC probe, with 11 false positives and 18 true positives, was just 62.1%. The *Bgl*II D fragment probe, with some of the associated plasmid DNA removed by electroelution, had reduced reaction with bacterial plasmid containing specimens and with only 2 false positives had a specificity of 92.8%. The synthetic Hex5B probe, unique to Ad41, did not give any spurious reactions with the specimens and was rated with 100% specificity. In summary, a markedly increased detection rate of adenovirus isolates was achieved by hybridization in comparison to electron microscopy and tissue culture, methods presently in widespread use. The high concentration of viral particles in enteric adenovirus containing specimens enabled the subgenus F specific immunoassay to attain similarly rates of detection.

6. Restriction Mapping of the Prototype Strain of Adenovirus Species 41

A) Characterization of the Plasmids Containing Cloned *Eco*RI Fragments of Ad41.

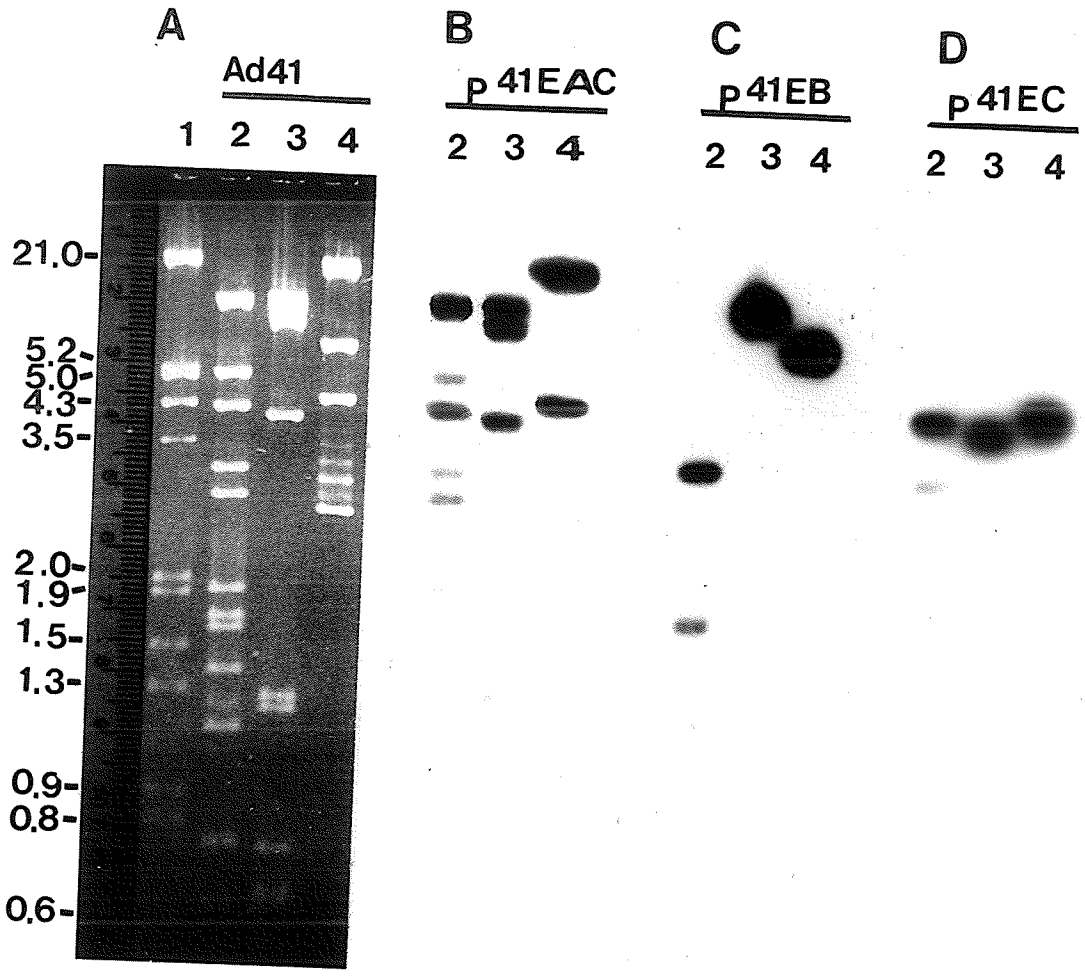
Acquisition of sufficient pure genomic DNA of Ad41 was complicated by the fastidious growth characteristics of the enteric adenovirus species. In order to circumvent this complication 84% of the Ad41 genome was cloned in the plasmid pGem 3Z in 3 *Eco*RI fragments, A, B and C which spanned from 8 to 61 map units, 74 to 92 map units and 61 to 74 map units, respectively. These fragment containing plasmids were named p41EAC, p41EA, p41EB, p41EC according to the size of the *Eco*RI fragment(s) contained. When digested with *Eco*RI and electrophoresed in agarose, as in Figure 42, the Ad41 DNA fragments were separated from the plasmid, forming bands that corresponded to the *Eco*RI fragments released by digestion of genomic Ad41 DNA in lane 10. The plasmids were characterized by their reaction as probes with blotted fragments of Ad41 genomic DNA transferred from agarose gels to nylon membranes. Plasmid DNA purified by centrifugation through CsCl density gradients was radiolabelled and hybridized with the target Ad41 genomic DNA digested with *Eco*RI, *Bam*HI and *Hin*dIII from the gel shown in panel A of Figure 43. The probe reactions of plasmids p41EAC, p41EB and p41EC are shown in panels B, C and D respectively and disclosed discrepancies in the positioning of fragments in the *Bam*HI and *Hin*dIII restriction maps proposed for the prototype strain Tak of Ad41 (Broker, Keller and Roberts, 1985; Akusjarvi and Wadell, 1987). The p41EAC plasmid, containing both A and C Ad41 *Eco*RI fragments, hybridized with *Bam*HI fragments A, C, D, E and F (Figure 43 B, lane 3), *Hin*dIII fragments A, B, C, D, E, F and I (Figure 43 B, lane 2) and fragments

Figure 42. Ad41 *Eco*RI Fragment Containing Plasmids.



Plasmids containing *Eco*RI fragment inserts were extracted from large plasmid preparations, purified in isopycnic density gradients and applied to an 0.8% agarose gel in digested aliquots of 2 μg. The gel was flanked by λ DNA size standards and genomic Ad41 DNA digested with *Eco*RI. Each plasmid was digested with *Eco*RI to liberate the inserts for comparison to the genomic DNA fragments or linearized with *Xho*I or *Sal*I.

Figure 43 A to E. Hybridization of Ad41 *Bam*HI, *Eco*RI and *Hin*dIII DNA Fragments with Cloned *Eco*RI Fragments.



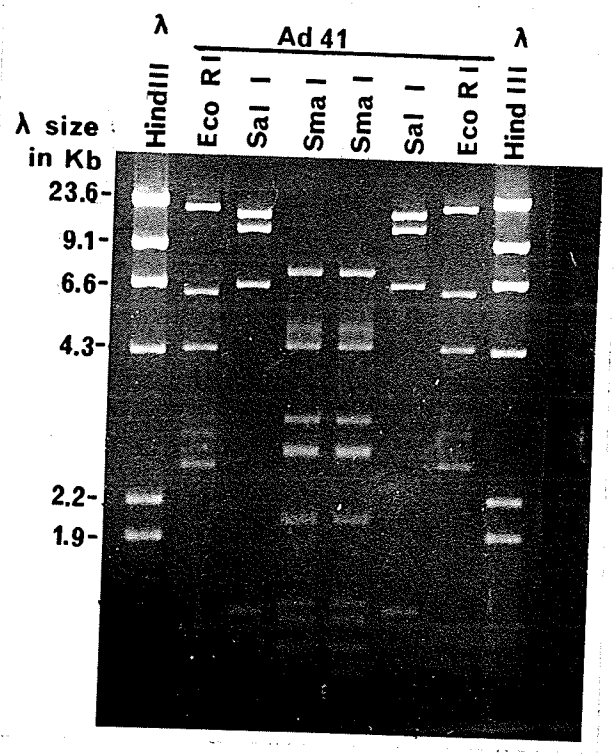
A: 1 μg aliquots of Ad41 DNA, purified by density gradient centrifugation and digested with *Hin*dIII, lane 2; *Bam*HI, lane 3; and *Eco*RI, lane 4, were run in a duplicate series in 0.8% agarose gel electrophoresis. 1 μg aliquots of λ DNA digested with *Hin*dIII and *Eco*RI were run alongside. Half of the gel is shown.

B to E: The gel was transferred to nylon membrane, divided in half and hybridized with plasmid probes p41EAC in panel B; p41EB in panel C; p41EC in panel D.

A and C ofthe *Eco*RI digest in lane 4. p41EB, the *Eco*RI B fragment containing plasmid, hybridized with *Bam*HI fragment B and *Hin*dIII fragments D, J, G and H. Plasmid p41EC reacted with the fragments D and F of the *Bam*HI digest and C and D of the *Hin*dIII digest. According to the present form of prototype strain Ad41 restriction maps, drawn by Akusjarvi and Wadell (1987), plasmid p41EB should have reacted with *Hin*dIII fragment I and p41EA with fragment H, not visa versa. The plasmids reacted with fragments overlapping the span of the *Eco*RI inserts and it was possible to ascertain that complete *Eco*RI fragments had been cloned. Thus, the most plausible explanation to account for the discrepancies was that the published Ad41 restriction maps were flawed. To endorse this explanation, errors were also found in the *Bam*HI restriction map. Whilst the order of *Bam*HI fragments in the area of the genome covered by the *Eco*RI fragments A, B and C did conform to the present restriction maps, analysis of the E1 gene sequence (van Loon *et al.*, 1987) for *Bam*HI restriction sites indicated that the small fragments at the left terminus had been incorrectly ordered.

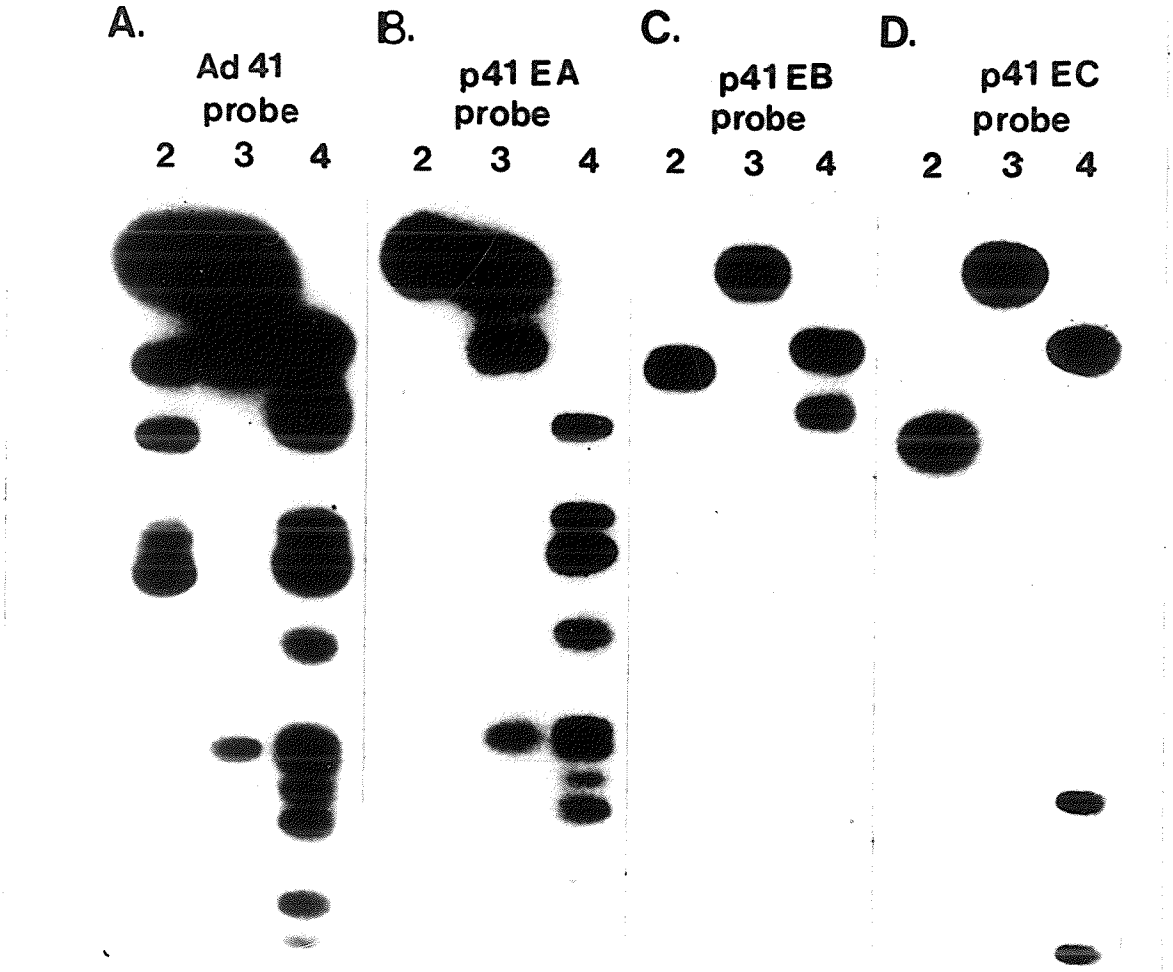
In a further experiment the plasmids were used as probes against genomic Ad41 DNA digested with *Eco*RI, *Sal*I and *Sma*I blotted from the gel in Figure 44. The DNA fragments which hybridized with the plasmid probes are shown in the autoradiographs in Figure 45 compared to a genomic Ad41 DNA probe. The *Eco*RI and *Sal*I fragments overlapping the *Eco*RI cloned fragments reacted positively with the appropriate probe, but *Sma*I fragments hybridized in a pattern inconsistent with the published *Sma*I map. The reaction of the various plasmid probes with the genomic *Eco*RI, *Bam*HI, *Hin*dIII, *Sal*I and *Sma*I fragments, portrayed in the order published by Akusjarvi and Wadell

Figure 44. Gel Electrophoresis of Ad41 *Eco*RI, *Sal*I, *Sma*I DNA Fragments.



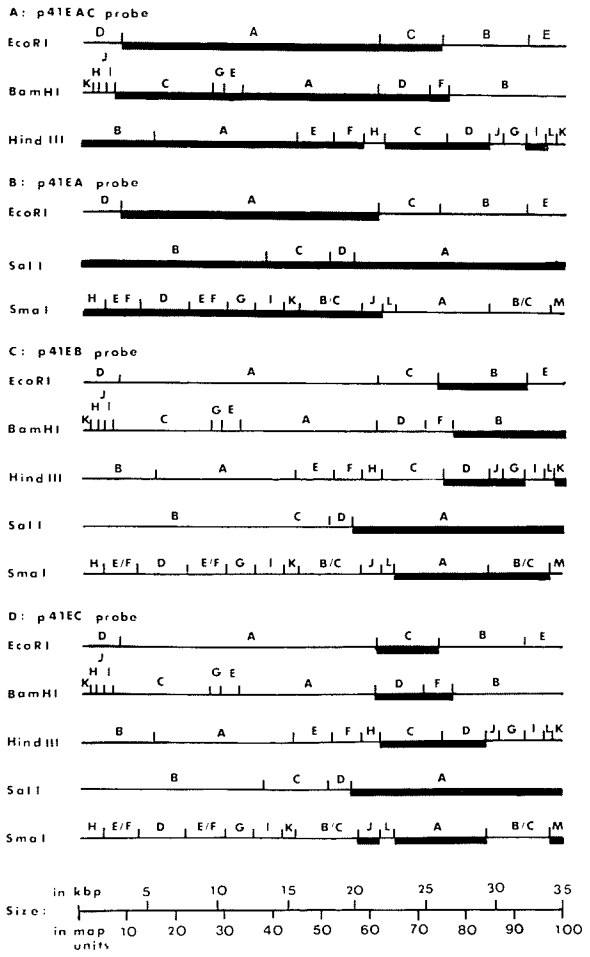
0.5 μg aliquots of Ad41 DNA, prepared from virions banded in CsCl density gradients, were digested with *Eco*RI, *Sal*I and *Sma*I restriction enzymes and run in duplicate in an 0.8% agarose gel. 0.75 μg of λ DNA digested with *Hind*III was run in outside wells as molecular weight standards.

Figure 45. Ad41 *Eco*RI, *Sal*I, *Sma*I DNA Fragments Hybridized with *Eco*RI Fragment Containing Plasmids.



The gel in 45 was blotted to a nylon membrane. The DNA was hybridized with a genomic Ad41 DNA probe to exhibit all the Ad41 fragments in panel A; and cloned Ad41 fragment probe p41EA in panel B; p41EB in panel C; p41EC in panel D. Ad41 DNA digests with EcoRI, SalI and SmaI were blotted in lanes 2, 3 and 4 respectively.

Figure 46. Reaction of the *Eco*RI Fragment Containing Plasmid Probes with Ad41 DNA According to the Present Restriction Maps.



*Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I and *Sma*I fragments of genomic Ad41 DNA which hybridized with cloned *Eco*RI A and C, A alone, B and C fragment containing plasmids p41EAc, p41EA, p41EB, p41EC respectively in Figures 44 and 45 have been underlined. The restriction maps were taken from Akusjarvi and Wadell (1986).

(1987), is shown in Figure 46. Hybridizing fragments are underlined and discrepancies between the results and the maps are evident as gaps in areas of the genome covered by *Eco*RI fragments contained in the plasmid probes or as underlined areas outside of the area of the genome cloned. The reaction of Ad41 DNA in the gel in Figure 44, blotted and hybridized with the *Eco*RI plasmids shown in Figure 42, was compared to the genomic probe hybridization in the panel A of Figure 45. *Eco*RI A fragment in plasmid p41EA evidently overlaps *Sma*I C, D, both E/F, G, H, I, J and K fragments (Figure 45, panel B). Fragment J hybridized relatively lightly which may indicate cleavage by *Eco*RI. A positive reaction from *Sma*I fragment H would not be expected as it is reported to lie outside of the area of Ad41 cloned with *Eco*RI (Figure 46, panel B). Plasmid p41EB reacted with fragments of *Eco*RI and *Sma*I digested DNA derived from the right end of the genome. Plasmid p41EC, however, reacted specifically with *Sma*I fragments A, J and M (Figure 45, panel C). The reported position of *Sma*I fragment M does not correspond to the *Eco*RI C fragment position and fragment M would not be expected to hybridize with a p41EC probe. *Sma*I fragment L was the only fragment that did not appear to hybridize with either p41EA, p41EB or p41EC although it is presently positioned at approximately 61 to 65 maps units, within the parameters of the *Eco*RI C fragment (Figure 46, panel D).

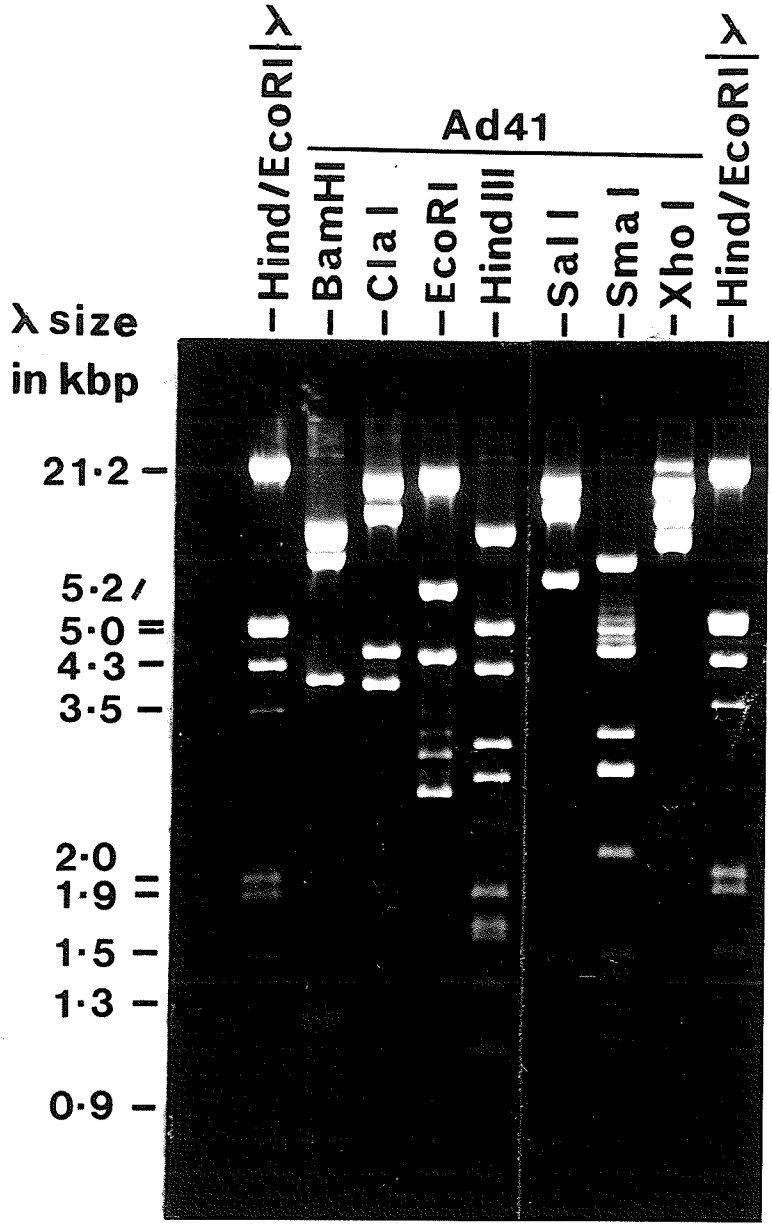
No anomalies were discovered in the order of *Sal*I fragments. The *Sal*I restriction map, however, is relatively simple and has been published before (van Loon *et al.*,1985). All of the more complicated, novel maps presented by Akusjarvi and Wadell (1987) examined in the two preceding experiments were found to harbour errors. More accurate

Ad41 restriction maps are required for examination of genomic variants in the analysis of the epidemiology of adenoviral gastroenteritis. Therefore, a study to ascertain the correct version of Ad41 prototype strain Tak restriction maps was undertaken.

Genomic Ad41 DNA, digested with restriction endonucleases *Bam*HI, *Cla*I, *Eco*RI, *Hind*III, *Sma*I and *Xho*I as in Figure 47, produces characteristic banding patterns when subjected to gel electrophoresis. The banding patterns of enzymes *Bgl*II and *Pst*I are shown in Figure 55. The fragment sizes were determined by comparison to lambda phage DNA standards flanking each gel. The size of large *Eco*RI, *Sal*I and *Xho*I fragments were estimated by addition of the lengths of subfragments created by digestion with two enzymes. The sizes of discernible fragments in base pairs are presented in Table 6. Restriction fragments of most digests combined to form an estimated genomic length of between 34.4 and 35K, averaging 34,678 base pairs. The total size of *Hind*III fragments was considerably less and may indicate the existence of several additional restriction sites within several hundred base pairs of each other.

The compatibility of the *Sma*I restriction endonuclease map with the restriction maps for other restriction endonucleases was tested by electrophoresis of Ad41 DNA digested with *Sma*I and an other restriction enzyme in dual digests (Shinagawa *et al.*, 1983). The Ad41 *Sma*I restriction pattern in lane 2 of the electrophoresis gel shown in Figure 48, corresponds to the *Sma*I pattern of Adrian *et al.*, (1986) except that a characteristically smeared but distinguishable B band is present. Information on the position of *Sma*I fragments can be deduced from their cleavage by the second restriction enzyme. In lane

Figure 47. Restriction Patterns of Ad41 Prototype Strain Tak.

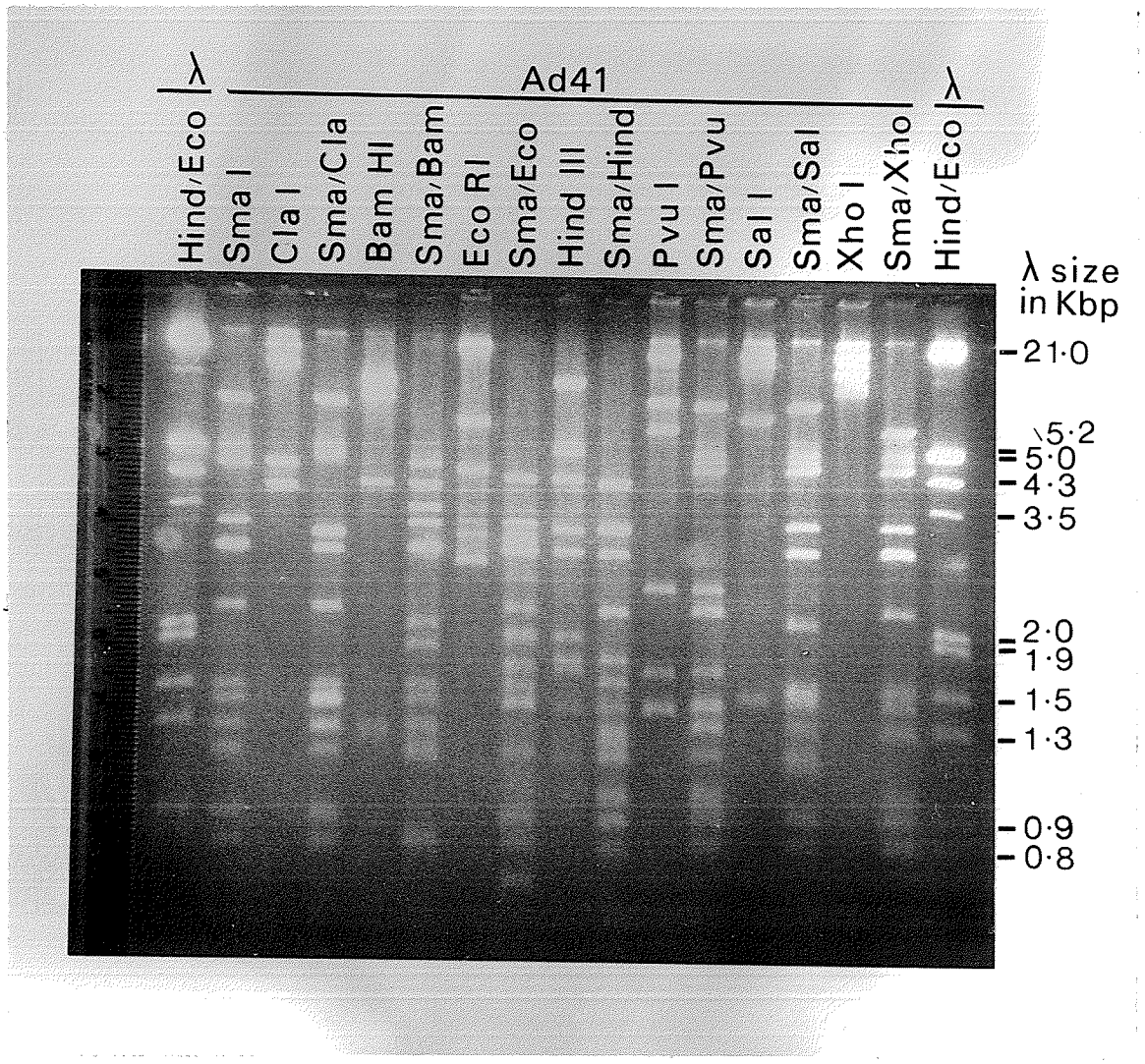


1 μg aliquots of purified Ad41 DNA, digested with restriction enzymes *Bam*HI, *Cla*I, *Eco*RI, *Hind*III, *Pst*I, *Sal*I, *Sma*I and *Xho*I, were electrophoresed in 0.8% agarose. λ DNA molecular weight standards were run in flanking wells.

Table 6. Size of Each Ad41 Restriction Fragment.

Fragment	Estimated sizes of fragments in base pairs with:								
	*Bam*HI	*Bgl*II	*Cla*I	*Eco*RI	*Hin*dIII	*Pst*I	*Sal*I	*Sma*I	*Xho*I
A	9,100	6,450	15,330	18,830	9,400	7,245	15,140	7,330	15,590
B	8,850	5,010	10,970	6,440	5,050	6,680	11,400	4,500	10,940
C	7,560	4,680	4,575	4,370	4,200	4,730	6,650	4,300	8,425
D	4,200	3,890	3,940	2,630	3,150	4,365	1,455	3,300	
E	1,320	3,890		2,550	2,800	3,850		2,680	
F	1,305	3,125			1,975	2,540		2,680	
G	750	2,940			1,780	2,425		2,190	
H	620	2,290			1,680	1,500		1,620	
I	590	1,950			1,340	1,110		1,480	
J	486	440			1,200	525		1,330	
K	160				750			1,225	
L					570			950	
M								815	
	34,941	34,665	34,815	34,820	33,895	34,970	34,645	34,400	34,955

Figure 48. Dual Enzymatic Digestion of Ad41 DNA.



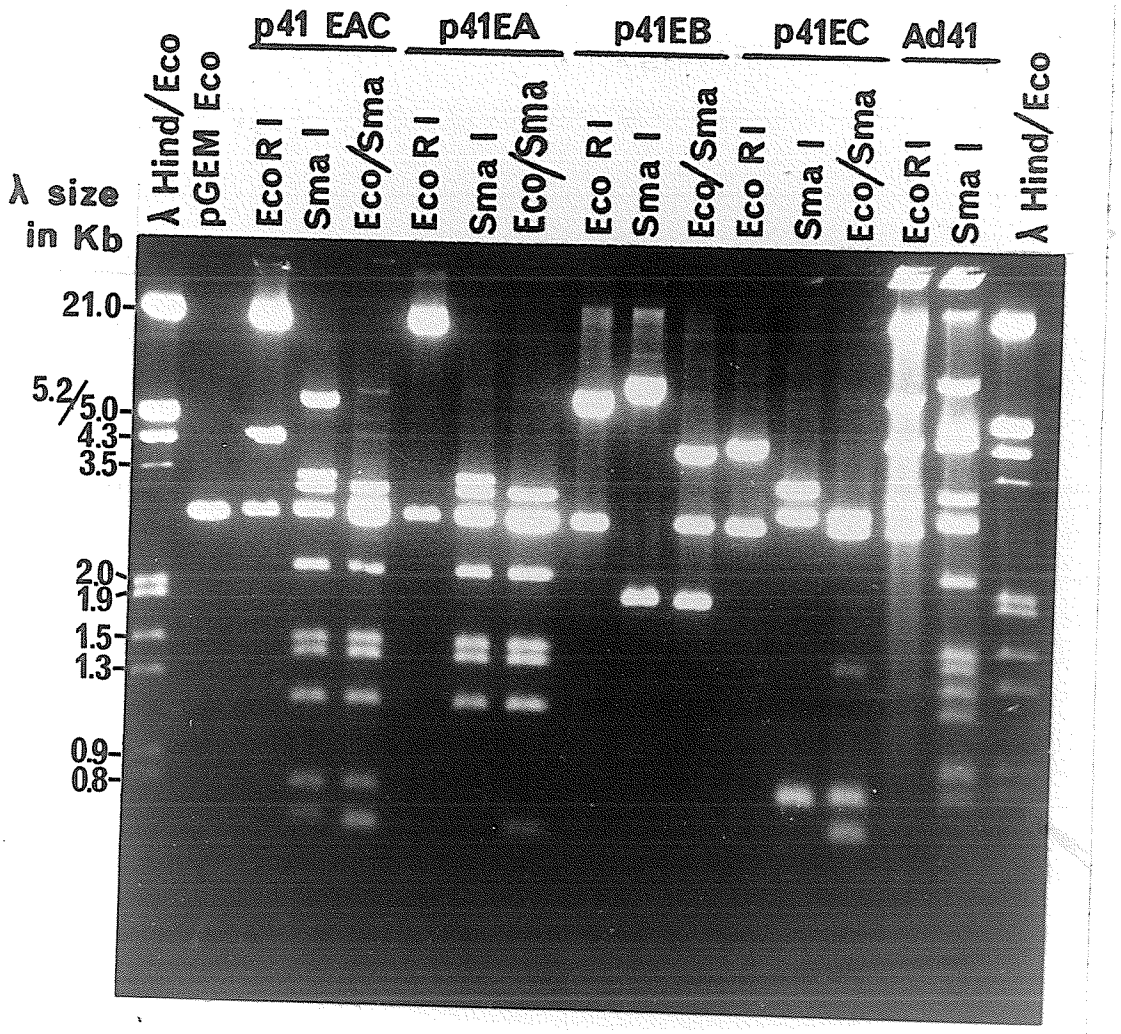
1 μg DNA samples were electrophoresed at 25 v overnight in a 0.8% agarose gel. Ad41 genomic DNA, prepared by the method of Shinagawa *et al.*, (1985), was digested with single restriction enzymes followed by dual enzyme digests with *Sma*I.

4 of Figure 48, further digestion of *Sma*I fragments of Ad41 DNA with *Cla*I reduced *Sma*I fragments C, D, E/F, which placed these fragments at the defined *Cla*I restriction sites at 11, 24, and 55 map units (van Loon *et al.*, 1985). In the *Bam*HI and *Sma*I double digest in lane 6 of Figure 48, only six of the *Bam*HI incision sites could be accounted for in the cleavage of *Sma*I fragments A, B, E/F, G, J and L. However, an intact *Sma*I B fragment and a cleaved *Sma*I H fragment would have been expected from intersection of the present restriction endonuclease maps (Figure 46). The *Eco*RI digestion of Ad41 *Sma*I fragments in Lane 8 of Figure 48 is difficult to interpret due to the number of small fragments liberated. Three of the four *Eco*RI restriction sites occur in *Sma*I fragments A, B, and J; the fourth could occur in the *Sma*I C fragment. This positioned one or other of fragments C and J at positions 8 and 61 map units, in conflict with the present placement. The cleavage of the *Sma*I J fragment by *Eco*RI corroborated the earlier result in which *Sma*I fragment J hybridized with both p41EA and p41EC probes and evidently overlapped both *Eco*RI A and C fragments. Thus, *Sma*I fragment J was positioned at 61 map units and fragment C at 8 map units. *Hin*dIII intersected *Sma*I fragments A, B, C, E/F, H and possibly some lower molecular bands (lane 10, Figure 48). When Ad41 DNA was doubly digested with *Pvu*I and *Sma*I in lane 12, *Sma*I D, both E/F and H fragments were intersected. *Sma*I fragment H would not be expected to be dissected by either *Hin*dIII or *Pvu*I and must have a central position in the genome where the restriction sites of these two enzymes coincide. The H fragment did hybridize with the p41EA probe, contradicting its present placement at the left terminus of the genome. The *Sal*I digest of *Sma*I fragments in lane 14 intersected one of the *Sma*I E/F fragments and *Sma*I G which is again at variance with the *Sma*I map. Finally, in lane 16, the *Sma*I

fragments cut by *Xho*I restriction sites were A and K, a result which corresponded exactly with the pattern expected from present restriction enzyme maps. Nevertheless, the present *Sma*I map does not give the expected fragment pattern in dual enzyme digest with most other restriction enzymes. Repeated misalignment at different points on the genome indicated that several of the *Sma*I fragments are out of position in the present form of the *Sma*I restriction map.

The *Sma*I restriction map was further investigated by identification of the *Sma*I fragments contained within the *Eco*RI fragments of the cloned plasmids. Ad41 *Eco*RI clones p41EAC, p41EA, p41EB and p41EC were digested with *Eco*RI to release the insert and with *Sma*I and *Eco*RI together to compare the resultant fragments to *Sma*I digest of Ad41 genomic DNA in lane 16 of the electrophoresis gel in Figure 49. Plasmid p41EAC released *Sma*I fragments D, both E/F fragments, G, H, I and K fragments (Figure 49, lane 8), while p41EC in lane 14 contained fragment M, part of a large fragment that could only be fragment A, and a small fragment of some 775 base pairs in size shared by p41EA and p41EAC. This shared fragment probably represented part of fragment J, corroborating previous results in which both p41EA and p41EC as probes hybridized at reduced strength with the *Sma*I fragment J. The placement of fragment J at the *Eco*RI restriction site at 61 map units between *Eco*RI A and C fragments is in accordance with the results of the Ad41 double digestion patterns (Figure 48, lanes 6 and 8) where *Bam*HI and *Eco*RI restriction enzyme sites coincided and intersected *Sma*I fragment J. Plasmid p41EB released two partial fragments that are identified by the hybridization of p41EB with *Sma*I fragments A and B (45, panel C). The overlap of the *Eco*RI B fragment with

Figure 49. Digestion of the *Eco*RI Fragment Containing Plasmids with *Eco*RI and *Sma*I.



The plasmids containing the *Eco*RI fragments were digested with *Sma*I and or *Eco*RI and run on a 0.8% agarose electrophoresis gel. *Sma*I digestion liberated the *Sma*I fragments contained within each of the *Eco*RI fragments whilst the dual enzyme digestion released the partial *Sma*I fragments at the termini of the *Eco*RI fragments. 2 μg of λ DNA as molecular weight standards, 0.5 μg of the linearized plasmid vector and 3 μg aliquots of Ad41 genomic DNA, digested to show the complete *Eco*RI and *Sma*I restriction patterns, were included in different lanes of the gel.

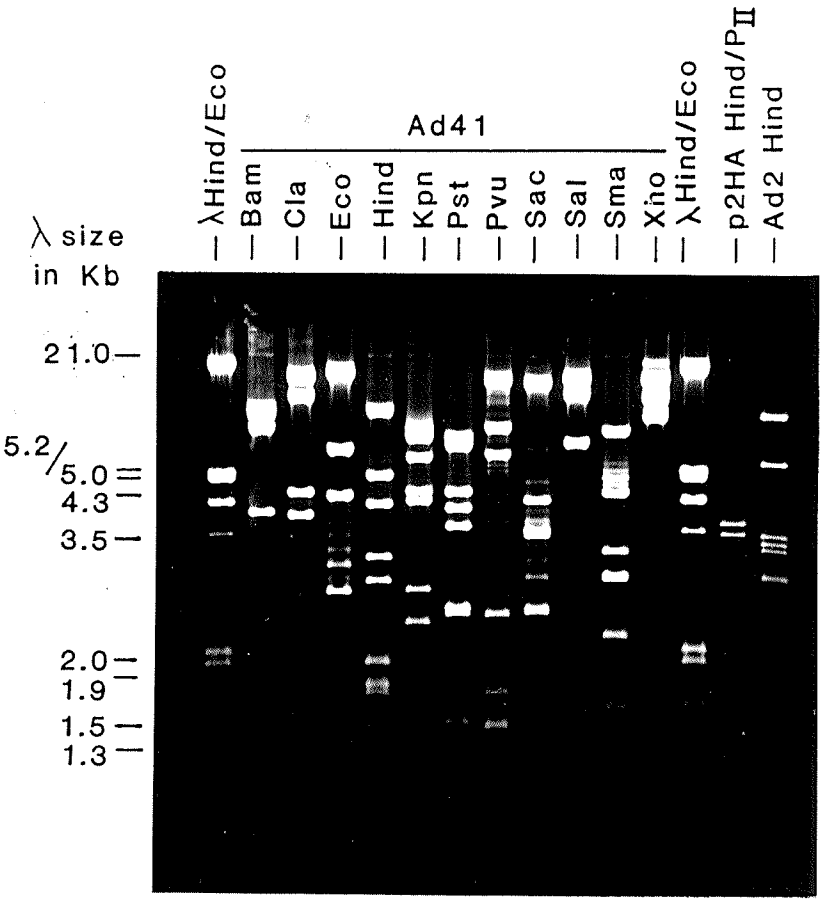
*Sma*I fragments A and B is in keeping with the existing *Sma*I map (Akusjarvi and Wadell, 1986). *Sma*I fragment L is the only fragment missing entirely from the 84% of the genome contained in the *Eco*RI clones, implying that its position is at one or other terminus. Dual digestion with *Sma*I and *Hin*dIII, which creates a number of small fragments from the right terminus of the Ad41 genome, leaves *Sma*I fragment L intact (Figure 48, lane 10) while restriction enzyme *Bam*HI, with several close restriction sites near the left terminus is the only enzyme to intersect fragment J in dual digest with *Sma*I (Figure 48, lane 6). Therefore, the position of *Sma*I L fragment was assigned to the left terminus of the Ad41 genome. The *Sma*I fragment C is only partially contained in plasmids p41EAC and p41EA from where it was released by *Eco*RI digestion as a partial band that comigrated with E/F (Figure 49, lanes 5 and 8). The proximity of *Cla*I, *Eco*RI and *Hin*dIII restriction sites, which all intersected *Sma*I fragment C (Figure 48), occurs between 8 and 14 map units, localizing the C fragment toward the left terminus also. The size of the *Sma*I C fragment precludes the L fragment following the C fragment and remaining outside the area of the genome cloned. Thus, the first *Sma*I fragment at the left terminus must be L and the second C.

*Sal*I restriction enzyme cuts only *Sma*I fragments E/F and G which served to place these fragments at positions 38 or 51 to 56 map units according to the existing *Sal*I map (van Loon *et al.*, 1985). *Cla*I and *Hin*dIII also intersect *Sma*I fragment E/F and share a restriction site near 55 map units (van Loon *et al.*, 1985), placing one E/F fragment in a central position and fragment G at 38 map units. *Sma*I fragment D was placed provisionally at about 21 map units by the intersection of both *Pvu*I and *Cla*I restriction

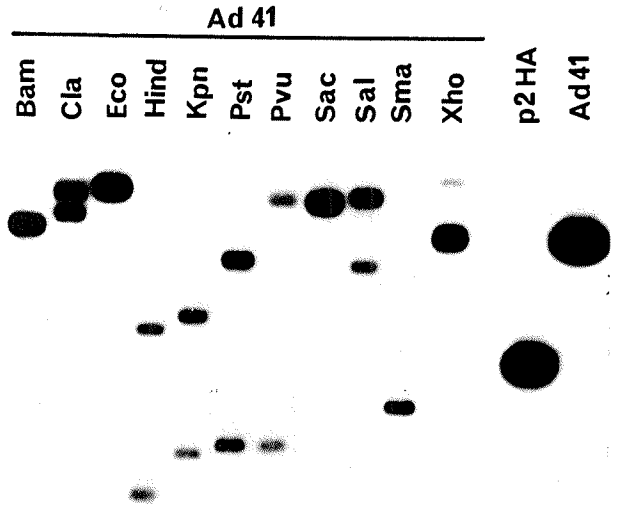
sites. The *Pvu*I restriction map has three restriction sites close together at the left end of the genome which intersect *Sma*I fragments D and E/F. The *Bam*HI restriction map similarly has three restriction sites to the right of those of *Pvu*I which intersected E/F and G fragments. This suggested that the order of SmaI fragments was D, followed by E/F, then G in this region. If the *Sma*I fragments L, C, D, E/F and G are placed together from the left terminus their combined molecular weight, estimated by graph from the lambda phage DNA standards, totals 39% of the genome. The placement of the G fragment can also be substantiated from the *Sal*I restriction map with a restriction site positioned at map unit 38 (van Loon *et al.*, 1985) which, with this arrangement of fragments, would cleave G to the right of D and E/F, as did occur. Central fragments of the Ad41 *Sma*I restriction map were determined from a variety of sources. The second E/F fragment was cleaved twice by *Sal*I restriction sites at 51 and 56 map units and by *Cla*I at 55 map units (van Loon *et al.*, 1985). The Ad41 hexon sequence (Toogood and Hay, 1988), searched by computer for restriction site recognition sequences, contains two *Sma*I restriction sites at precisely the right distance apart to accommodate an E/F fragment. The central position of one of the E/F fragments was also confirmed by the hybridization of Ad41 DNA with an HPII fragment probe containing the Ad2 hexon gene sequence. In panel A of Figure 50, Ad41 DNA, digested with a variety of enzymes, is shown after overnight electrophoresis. The Ad41 DNA was transferred to a nylon membrane and hybridized with the HPII probe under stringent conditions. The autoradiograph in panel B of Figure 49 shows those Ad41 fragments that contain hexon sequences homologous with the Ad2 gene. These fragments are highlighted in 51 on the present versions of the Ad41 restriction maps. Some of the internal fragments of the

Figure 50 A and B. Hybridization of Ad41 DNA with an Ad2 HPII Fragment Probe.

A:



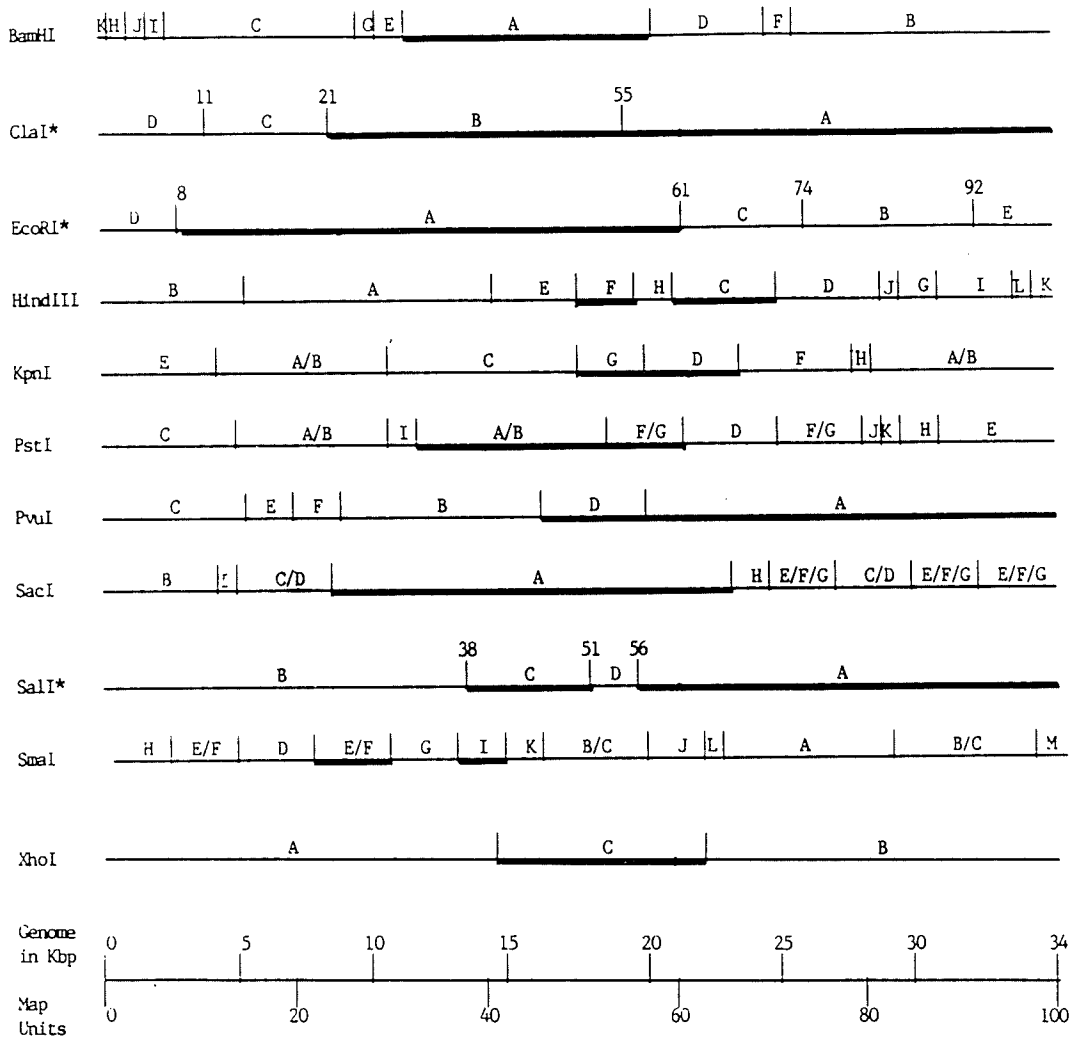
B:



A: Purified Ad41 DNA was digested in 1 μg samples with a variety of restriction enzymes and run on a 0.8% agarose gel with λ, Ad2 and plasmid p2HA DNA. B: The gel was blotted and hybridized with an HPII fragment probe at 68°C. 48 hr film is shown.

Ad41 hexon gene, the *Hin*dIII I fragment and the *Sal*I D fragment, demonstrate that the Ad2 and Ad41 hexon genes do not share a high degree of homology throughout their length. The *Sma*I fragments covering the hexon gene are evidently the E/F and H fragments (Figure 50, panel B, lane 10), although these are currently displaced toward the left terminus of the genome in the present map (Figure 51). Fragment K was cleaved in Figure 48 only once, by *Xho*I which also intersected *Sma*I fragment A and has restriction sites around 44 and 68 map units, as estimated from the fragment size in comparison to λ standards. *Sma*I fragment A, by its hybridization with p41EC, can be placed over the *Xho*I restriction site at 68 map units, indicating that *Sma*I K is cleaved at 44 map units. Placement of the H fragment adjacent to E/F incorporates a calculated 53% of the genome from the right terminus with the fragments A, B, J and M that hybridize with p41EC and p41EB probes and signifies that the next fragment must cover the position of the first *Xho*I restriction site. Placement of *Sma*I fragment K adjacent to H in the centre of the genome leaves a space of approximately 4 map units between *Sma*I K and the placement of *Sma*I fragment G which could accommodate fragment I. The dual digest of Ad41 with *Sma*I and *Hin*dIII (Figure 48, lane 10) which has four restriction sites in the central area of the genome at distances that could intersect *Sma*I fragments G, H, I and J, as observed, and leave fragment K intact. Of the remaining *Sma*I fragments A, B, J and M; the J fragment has been positioned at the junction of the *Eco*RI A and C fragments at 61 map units (van Loon *et al.*, 1985). The combination of L, C, D, E/F, G, K, H, I and E/F fragments totals approximately 59% of the genome and signifies that fragment J must proceed M to straddle the *Eco*RI restriction site. Fragment M hybridized with the p41EC probe (Figure 45, panel D), containing the *Eco*RI C

Figure 51. Reaction of the Ad2 Hexon Fragment Probe HPII with Restriction Digests of Ad41 DNA.

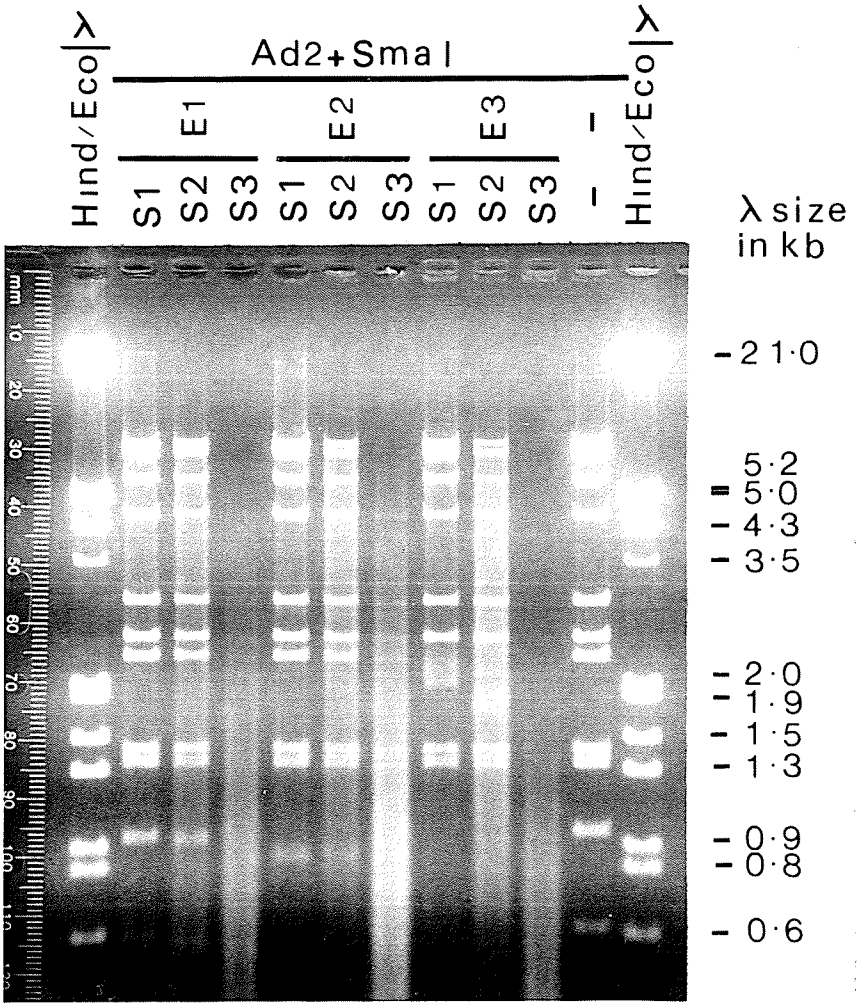


Those fragments of Ad41 hybridizing with the HPII probe in panel B of Figure 50 are underlined. The restriction maps were taken from Akusjarvi and Wadell (1986) or ★ from van Loon *et al.*, (1985).

fragment, and was placed immediately to the right of fragment J. Lastly, the order of the *Sma*I fragments A and B, that hybridize with probe p41EB at the right end of the genome, was defined by the cleavage of fragment A by *Xho*I and *Bam*HI enzymes which have restriction sites between 65 and 80 map units. The complete order of *Sma*I fragments from the left to right termini of the Ad41 genome was thereby ascertained as L, C, D, E/F, G, K, H, I, E/F, J, M, A, B.

Hybridization of fragments of genomic Ad41 DNA with the *Eco*RI fragment containing plasmids had indicated that there were errors in the *Hin*dIII restriction map also. The hybridization of *Hin*dIII fragment I with plasmid p41EA and fragment H with a p41EB probe suggested that the placement of these fragments had been reversed. The central position of *Hin*dIII fragment I was corroborated by its reduction in the dual digestion of Ad41 DNA with *Hin*dIII and *Sma*I (Figure 48, lane 10). *Sma*I, with several central restriction sites but few sites toward the right terminus, cleaved *Hin*dIII fragment I and did not affect fragment H. Ordering fragments at the right end of the Ad41 *Hin*dIII restriction map proved difficult as few enzymes had restriction sites in that area that would yield information by dual enzyme digest. To cover this deficiency a novel method was developed in which the *Hin*dIII fragments were ordered by sequential loss in exonuclease III and S1 nuclease digestion. Firstly, Ad2 DNA which was obtainable in quantity was used to find useful concentrations of exonuclease III and S1 nucleases. The Ad2 genomic DNA in the agarose gel shown in Figure 52 has been treated with a range of concentrations of exonuclease III and SI nucleases, prior to digestion with *Sma*I and electrophoresis, so as to establish the concentrations that give a moderate digestion rate.

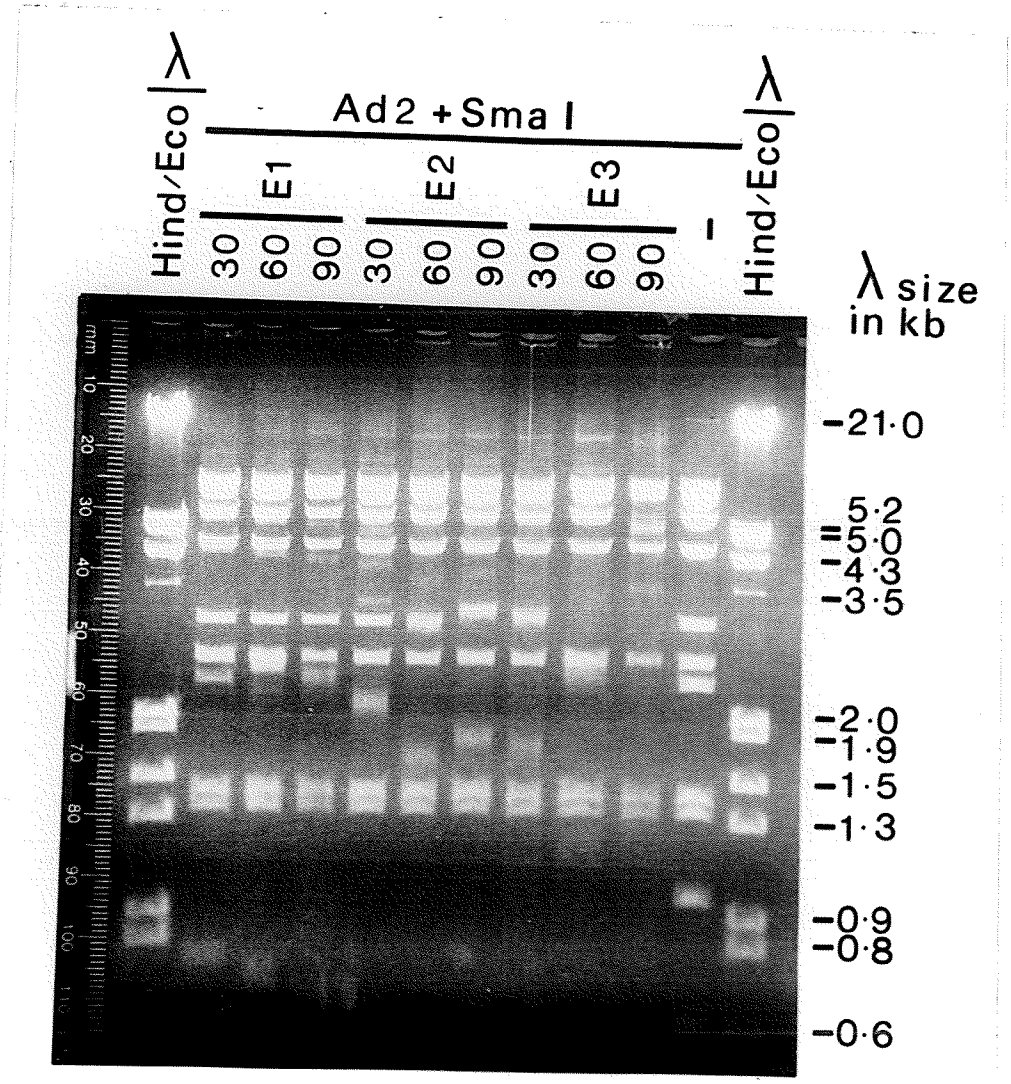
Figure 52. Reduction of Ad2 DNA by Exonuclease III and S1 Nuclease.



3 aliquots of 6 μg of purified Ad2 genomic DNA were exposed to 0.08, 0.2 or 0.8 units of exonuclease III in E1, E2 and E3 respectively for 30 minutes, divided into 3 and exposed for a further 30 minutes to 1, 10 or 100 units of S1 nuclease in lanes marked S1, S2 and S3 respectively. The DNA was then extracted with phenol and chloroform, precipitated and resuspended in medium buffer with 10 units of *Hin*dIII restriction enzyme. After 3 hour incubation at 37°C the aliquots were run in a 0.8% agarose gel with *Sma*I digested genomic Ad2 DNA and λ DNA molecular weight standards.

The first concentration of exonuclease III had no discernible effect on the size of the *Sma*I fragments, the second concentration caused a decrease in the outermost *Sma*I fragments K and J, the smallest fragments of the Ad2 digest, of about 50 base pairs and the third concentration affected *Sma*I G, the next fragment in from the terminus. The two higher concentrations of S1 nuclease caused smearing by extensive degradation of the DNA and the lowest value of 1 unit/μg DNA was adopted to reduce the single stranded tails created by exonuclease III digestion. A rate of digestion of genomic adenovirus DNA in base pairs per unit of exonuclease III per minute was derived from the extent of digestion of the genomic samples of Ad2 DNA shown in Figure 53. A decrease of some 250 base pairs in the size of fragment J at 30 minutes with 0.5 units per μg of DNA, a reduction of approximately 300 base pairs at 60 minutes and 325 at 90 minutes (Figure 53, lanes 2, 3 and 4) was estimated from the λ DNA standards, equivalent to an initial rate of about 17 bp/u/min. With 1 unit per μg (Figure 53, lanes 5, 6 and 7) about 100 base pairs had been trimmed from fragments G inside the 575 base pair K fragment at the right terminus after 30 minutes, equivalent to a reduction rate of 23 bp/u/min. With 2 units of exonuclease III the G fragment was reduced by some 500 base pairs and fragment E inside the 1006 base pair J fragment attacked (Figure 53, lane 8), a reduction rate of about 18 bp/u/min. The same dramatic slowing of reduction rate was apparent in the next 30 minutes with only a further 50 base pair loss in the size of fragment E. Although the rate of digestion with exonuclease III is exponential both with time and concentration, evaluations of these factors can be made from this simple experiment. The average digestion rate of the Ad2 genome by exonuclease III over the first thirty minutes of the reactions was 19 bp/u/min.

Figure 53. Timed Reduction of Genomic Ad2 DNA by Exonuclease III.

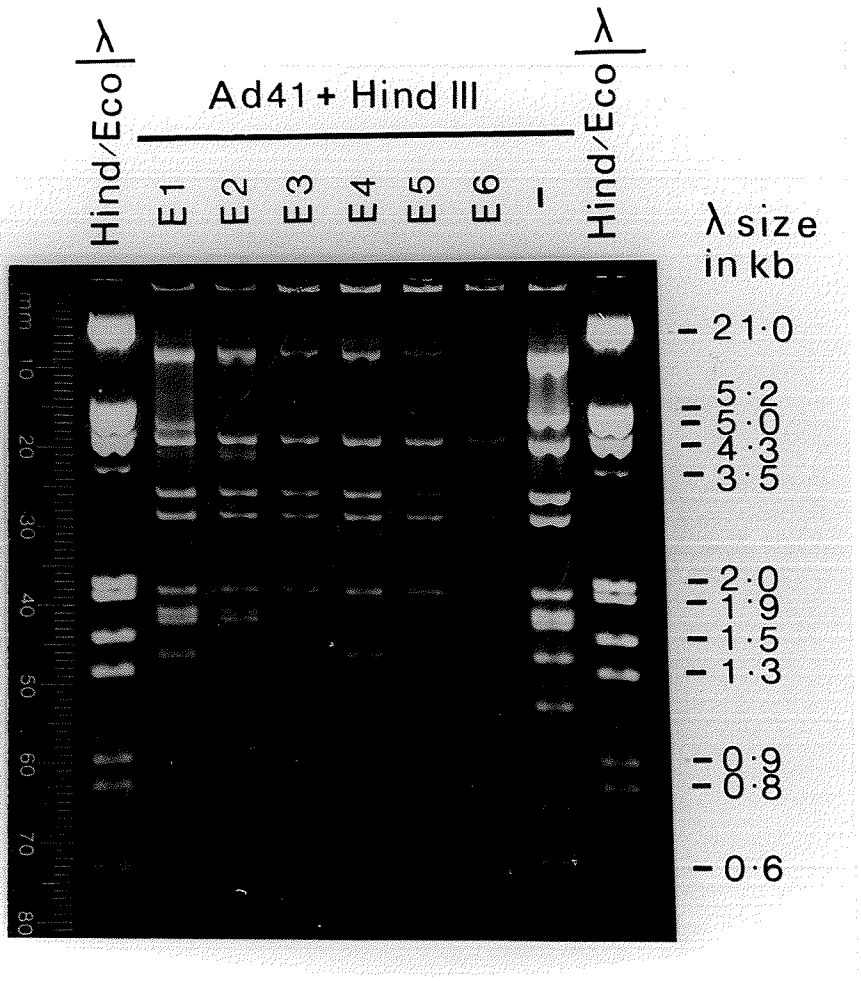


3 aliquots of 6 μg of Ad2 genomic DNA were incubated at 37°C with 1, 2 or 4 units of exonuclease III in E1, E2 and E3 respectively. At 30, 60 and 90 minutes a third of the reaction volume was removed and the reaction terminated by the addition of S1 nuclease buffer containing 330 mM sodium acetate. At the end of 90 minutes 1 unit of S1 nuclease enzyme were added and each sample incubated for 30 minutes and the DNA extracted. Resuspended samples were digested with *Sma*I and run in a 0.8% agarose gel.

In the final experiment in this series a range of increasing concentrations of exonuclease III was used on genomic Ad41 DNA prior to digestion with *Hin*dIII. The agarose gel in Figure 54 shows progressive reduction of Ad41 *Hin*dIII fragments B then A at one terminus and L followed by, in order, G, H, K, J and D from the other terminus in increasing concentrations of exonuclease III. The digestion rate, estimated from the degradation of fragment G, inside fragment L of 570 base pairs, with 1 unit of exonuclease III (Figure 54, lane 3), degradation of fragment H in lane 5 and loss of both J and K fragments in lane 6 and inroads upon fragment D, 5,980 base pairs from the terminus, in the seventh lane of Figure 54, is quite consistent at approximately 20 bp/u/min. With the order of internal fragments established by dual enzyme digestion, the complete sequence of fragments from the left terminus was determined to be B, A, E, F, I, C, D, J, K, H, G , L in the Ad41 *Hin*dIII restriction map.

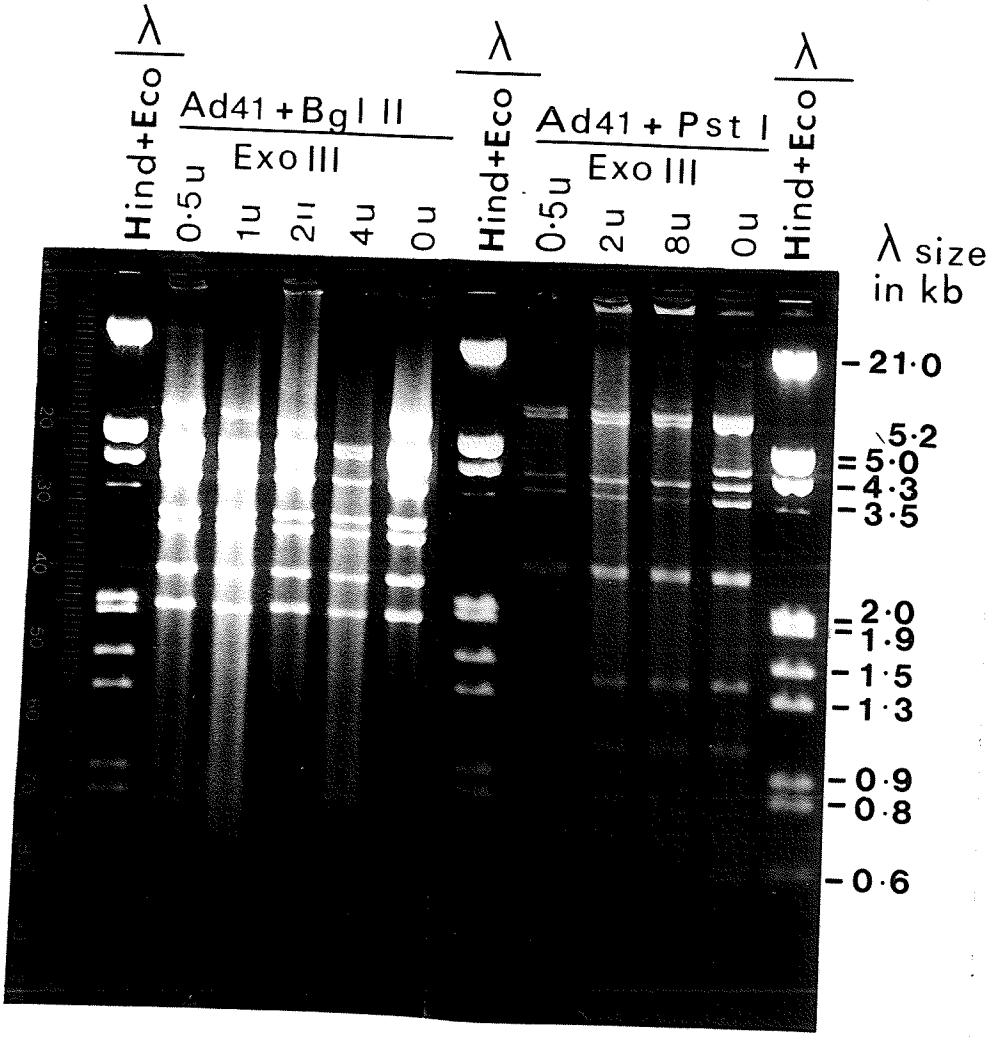
Exonuclease III reduction was subsequently used to order fragments in the *Bgl*II and *Pst*I restriction maps. The first series of lanes in the agarose gels spliced in Figure 55 shows the concurrent reduction of fragments A and D/E at opposite ends of the Ad41 genome after digestion with the enzyme *Bgl*II. With increasing concentrations of exonuclease III the second D/E fragment of the double band was also attacked whilst fragment A was further degraded. Ordering of the internal fragments of the *Bgl*II restriction map was achieved by a combination of previously used techniques with the *Eco*RI fragment containing plasmids in the agarose gel shown in Figure 56. Digestion of the *Eco*RI B fragment containing plasmid, p41EB, with *Bgl*II (Figure 56, lane 13) released a single whole *Bgl*II fragment, D/E. This served to fix the A fragment at the left terminus and

Figure 54. Analysis of Ad41 *Hind*III Restriction Fragments with Exonuclease III.



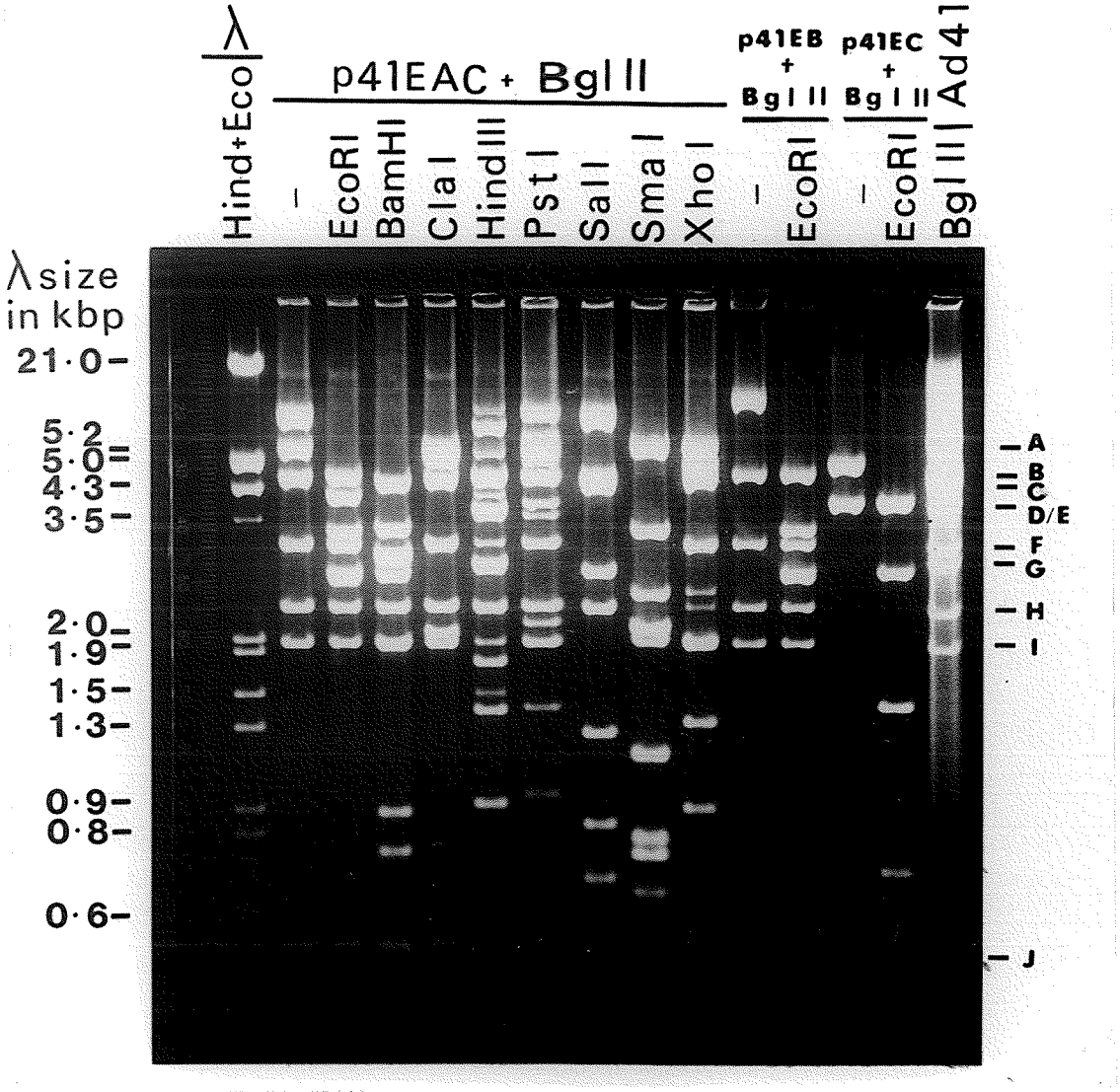
1 μg aliquots of genomic Ad41 DNA were incubated with increasing concentrations of exonuclease III for 30 minutes; 0.5 units, 1, 2.5, 5, 10, and 15 units in E1, E2, E3, E4, E5 and E6, respectively. Each aliquot was then digested with 1 unit of S1 nuclease for 30 minutes, extracted with phenol and chloroform, and precipitated. Aliquots were digested for 3 hours with 10 units of *Hind*III and electrophoresed overnight in a 0.8% agarose gel. A *Hind*III digest of Ad41 genomic DNA and λ DNA size standards were included in the gel.

Figure 55. Analysis of *BglII* and *PvuI* Restriction Maps with Exonuclease III.



1 μg aliquots of genomic Ad41 DNA were incubated with doubling concentrations of exonuclease III for 30 minutes. Each aliquot was then digested with 1 unit of S1 nuclease for 30 minutes, extracted with phenol and chloroform, and precipitated. Washed aliquots were digested for 3 hours with 10 units of *BglII* or *PvuI* and electrophoresed overnight in a 0.8% agarose gel. Ad41 genomic DNA digested with *BglII* or *PvuI* was run adjacent to the treated aliquots and λ DNA size standards were included in the gel.

Figure 56. Analysis of the Ad41 *Bgl*II Restriction Map with *Eco*RI Fragment Containing Plasmids.



1.5 μg aliquots of plasmids p41EAc, p41EA and p41EB containing the A and C, A or C *Eco*RI fragments, respectively, of the Ad41 genome were digested with *Bgl*II alone or in combination with a variety of common restriction enzymes. Digested aliquots were run in 0.8% agarose with λ DNA standards and Ad41 genomic DNA digested to give the full *Bgl*II restriction pattern.

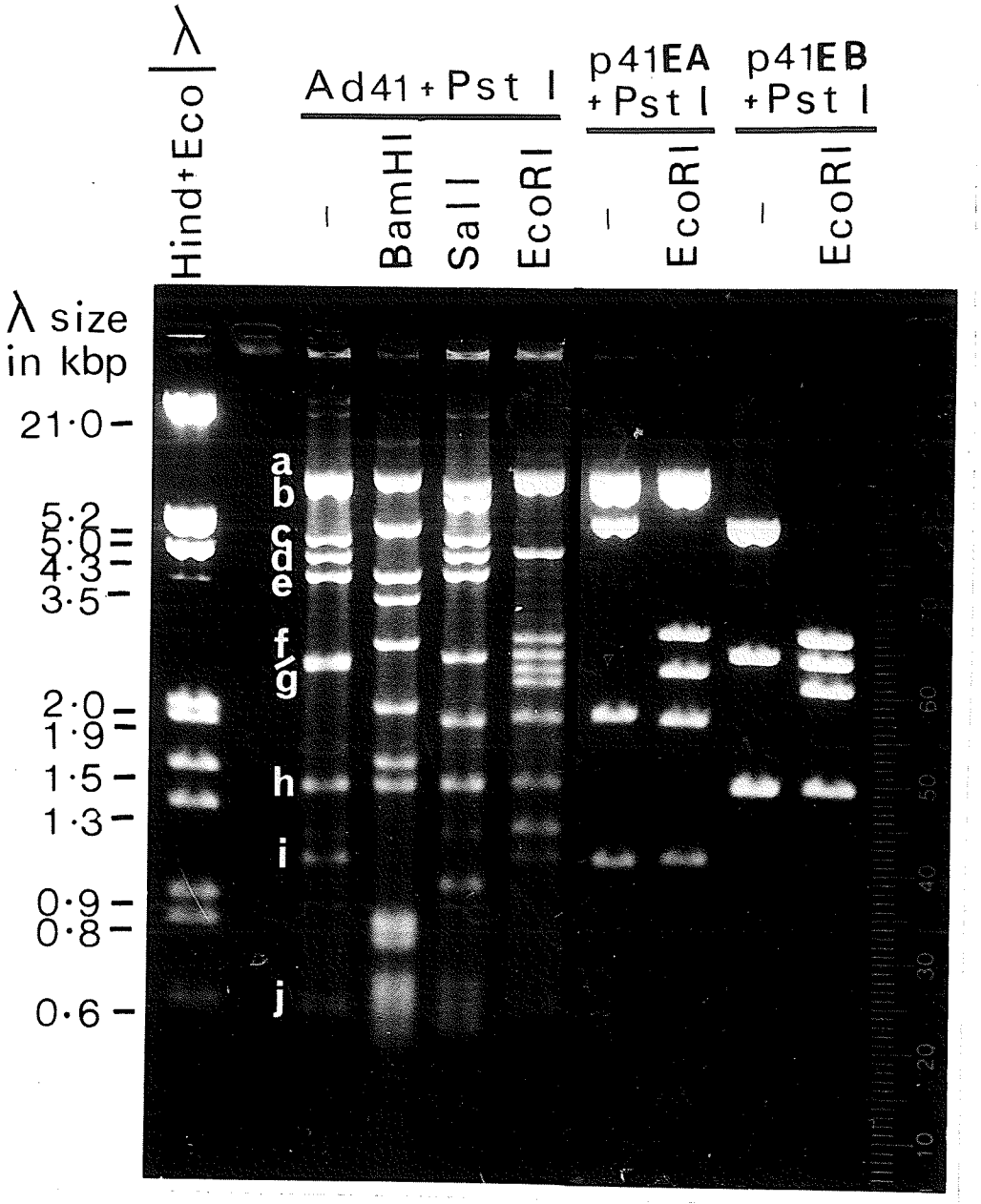
the two D/E fragments at the right terminus of the *Bgl*II restriction map. The fragments released by *Bgl*II digestion of plasmid p41EAC in Figure 56 were C, F, H, I and J. Fragment G is also included within the cloned *Eco*RI A and C fragments but due to the ligation of the *Eco*RI C fragment to the A fragment in reversed orientation in the cloning of this plasmid the G fragment is retained in a 5,430 base pair band that travelled below the uppermost band containing the plasmid and the tail fragments. This second band of the p41EAC pattern was the only one missing from the plasmid p41EA digest (Figure 55, lane 11) and was intersected by dual enzymatic digestion of *Bgl*II with *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I and *Sma*I, fixing its position at the right end of the plasmid insert. The *Bam*HI and *Bgl*II enzyme combination also reduces the plasmid band and trims small fragments from both *Bgl*II C and F bands, positioning C and F next to each other towards the left terminus. Digestion with *Hin*dIII cleaved fragment F but left *Bgl*II fragment C intact, placing the *Bgl*II C fragment between the spaced restriction sites of the *Hin*dIII A fragment and prior to F from the left terminus. The H fragment was then positioned next to *Bgl*II F by cleavage with *Xho*I. Positioning of the J fragment adjacent to I within the hexon gene by analysis of the sequence for *Bgl*II recognition sites was supported by intersection of fragment I with *Sal*I. The complete *Bgl*II fragment order from the left terminus was determined as A, C, F, H, J, I, G, B, D/E, D/E.

The first *Pst*I fragments to be affected in lanes 8, 9, and 10 of 55, are the J and C fragments and the disappearance of J is followed by the reduction of fragment E. Again, the order the *Pst*I fragments was ascertained by double digestion and comparison of genomic and plasmid fragments. Dual digestion of genomic DNA with *Pst*I and *Bam*HI

(Figure 57, lane 4) with numerous restriction sites near the left terminus, cleaved fragment C but neither J nor E, establishing the terminal fragments. The *Eco*RI A fragment containing plasmid, p41EA, released *Pst*I fragments A, B, two plasmid bands, and fragment I (Figure 57, lane 7). Fragment A was placed in the middle of the genome, left of fragment F/G, by the hybridization of these two fragments with the HPII probe (Figure 50). Fragment I was placed to the left of A by the intersection with *Bam*HI and *Sal*I, and fragment B left of I. Finally, *Pst*I fragment D was placed in the gap between fragment F/G hybridizing with the Ad2 hexon gene and the H and F/G fragments released from plasmid p41EB and the latter two fragments ordered by the intersection of H by *Sma*I. The final order of the *Pst*I fragments in the complete restriction map was defined as C, B, I, A, F/G, D, F/G, H, E, J.

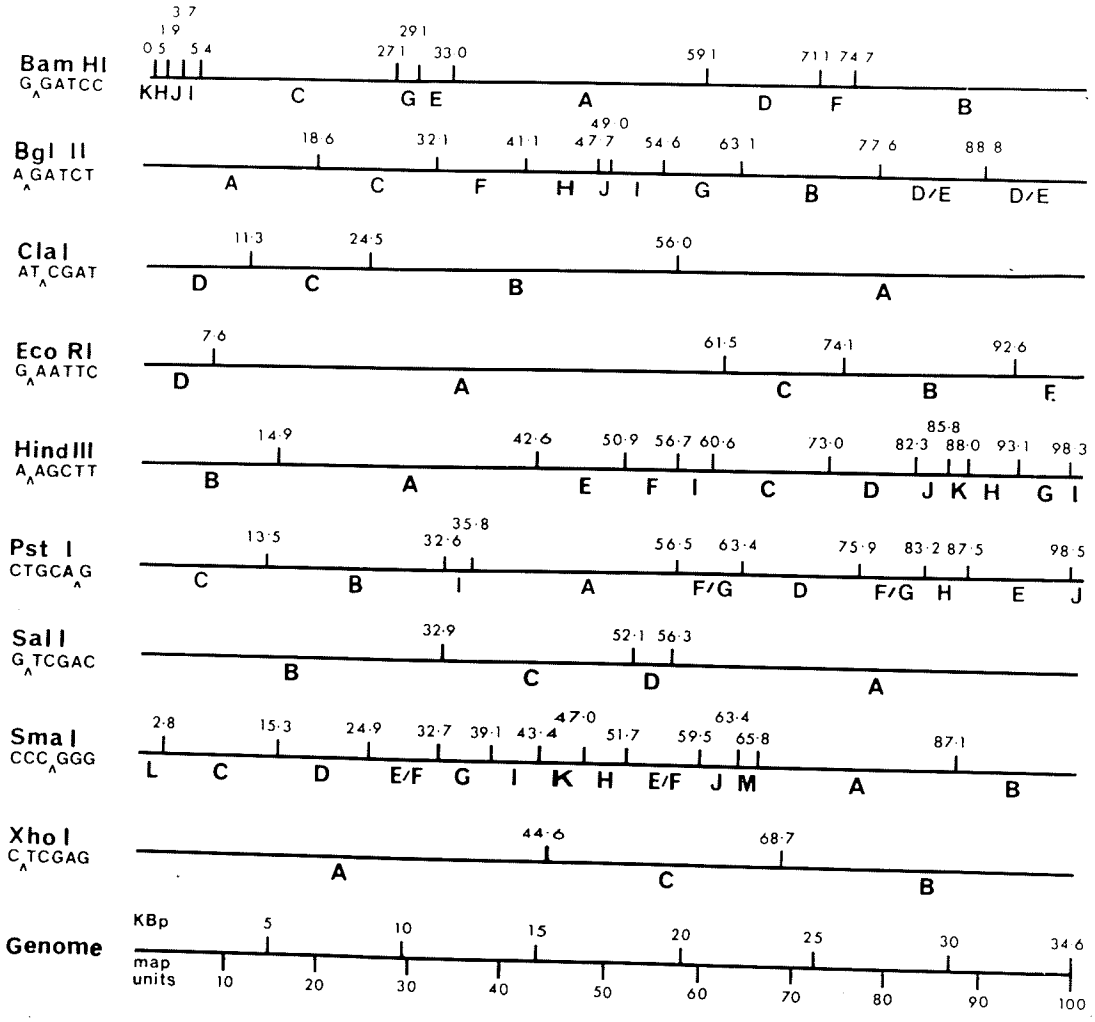
The revised Ad41 prototype strain restriction maps, for enzymes *Bam*HI, *Bgl*II, *Cla*I, *Hin*dIII, *Pst*I, *Sal*I, *Sma*I and *Xho*I are presented together in Figure 58, complete with restriction sites demarcated in map units. The *Bam*HI restriction map has been revised at the left terminus where errors in the order of the fragments K, H, I and J were indicated by analysis of the E1 gene nucleotide sequence. Fragments of the *Hin*dIII map were corrected both in the center of the genome and in the order of the small fragments at the right terminus. The *Sma*I restriction map was extensively reformulated and *Pst*I fragments J and K repositioned or omitted. The positioning of fragments of *Cla*I, *Sal*I and *Eco*RI restriction maps, with relatively few fragments, do agree very closely, within 1 or 2 map units in most instances, with the placement of van Loon *et al.* (1985). The *Bgl*II restriction map has not been presented before and the map units of *Xho*I restriction

Figure 57. Analysis of the *Pst*I Restriction Map Using *Eco*RI Fragment Containing Plasmids



Ad41 genomic DNA and plasmids p41EA and p41EB were digested in 1 μg aliquots with *Pst*I alone or in dual digest with another restriction enzyme. Ad41 DNA was electrophoresed with *Hin*dIII and *Eco*RI digested λ DNA molecular weight standards in an 0.8% agarose gel.

Figure 58. Amended Restriction Maps of the Prototype Strain Tak of Adenovirus Species 41.



The size of Ad41 DNA fragments as estimated in Table 6 and ordered according to data in Figures 43, 45, 48, 49, 50, 54 and 55 was used to reformulate the Ad41 restriction maps. Map unit value were calculated as a proportion of the average total of base pairs in the summed restriction fragments.

sites have not previously been defined.

7. Identification of Virus Isolates Causing Gastroenteritis in Manitoba.

A) Isolation and Identification of Clinical Isolates.

Over 1,000 stool specimens have been received annually in recent years for viral diagnosis at the Cadham Provincial Laboratory. These specimens were suspended in phosphate buffered saline, examined negative contrast electron microscopy and inoculated on a series of cell monolayers chosen to grow a wide range of viruses. When compared to data for other viruses taken from Cadham Provincial Laboratory records, rotavirus, which grew poorly if at all in conventional tissue culture and was confirmed by application of a commercial ELISA kit to 10% stool suspensions, was detected with the higher frequency. Enteroviruses grew well in a number of cell lines and consistently formed about a quarter of the total number of virus diagnosed. Coronaviruses have been detected from their characteristic morphology as seen by the electron microscope and indeterminate small round enteric virus particles of about 40 nm or less in diameter have been seen in about a tenth of the specimens in which viral particles were identified, as shown in Table 7. The number of adenovirus isolates diagnosed per year has remained fairly constant in recent years, with marginally over 100 specimens detected annually on average, approximately a third of the total number of viral agents diagnosed in gastroenteritis. The proportion of adenovirus as a cause of gastroenteritis in the province of Manitoba averaged 32% of the total number viral pathogens detected over the period 1987 to 1992, an isolation rate only fractionally smaller than rotavirus, the principal cause of gastroenteritis detected in the Cadham Provincial Laboratory.

Table 7: Detection of Viruses from Stool Specimens At the Cadham Provincial Laboratory.

YEAR	ADENOVIRUS	CORONAVIRUS	ENTEROVIRUS	ROTAVIRUS	SRV	TOTAL
1987	112 (33.9%)	-	76 (23.0%)	115 (34.9%)	27 (8.2%)	330
1988	95 (29.2%)	8 (2.5%)	83 (25.5%)	118 (36.3%)	21 (6.5%)	325
1989	106 (32.8%)	2 (0.6%)	65 (20.1%)	123 (38.1%)	27 (8.4%)	323
1990	102 (32.6%)	5 (1.6%)	77 (24.6%)	93 (29.7%)	36 (11.5%)	313
1991	99 (31.2%)	3 (1.0%)	92 (29.0%)	81 (25.6%)	42 (13.3%)	317
MEAN	103 (31.9%)	4 (1.1%)	77 (24.4%)	106 (32.9%)	31 (9.6%)	321.6

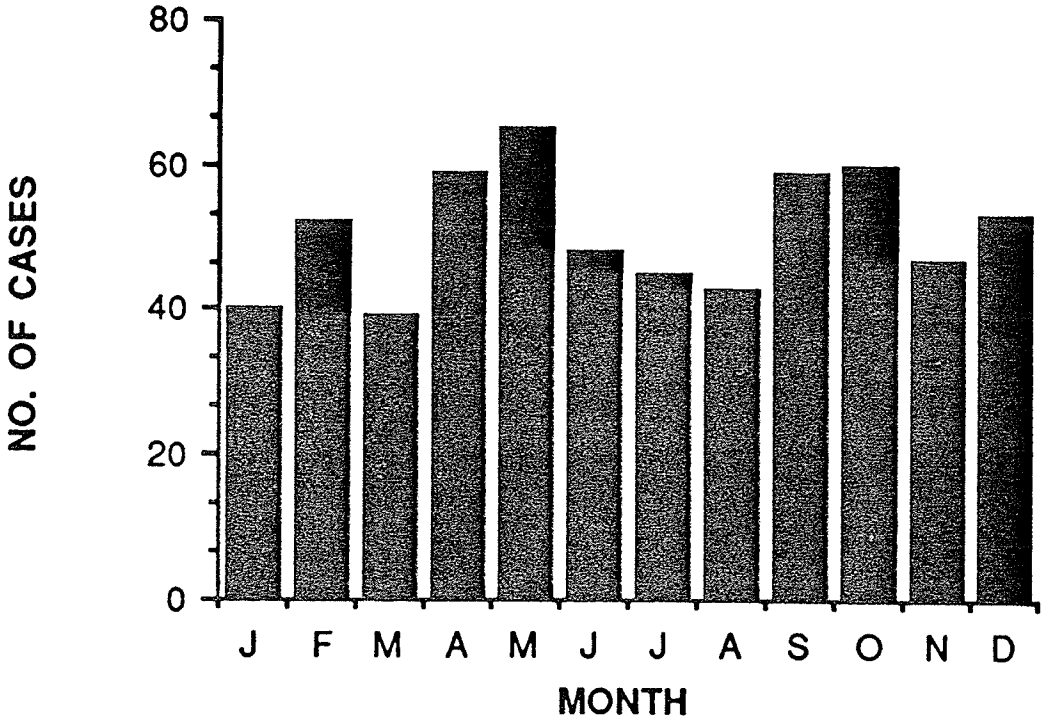
B) Seasonal Distribution of Adenovirus Isolates and Analysis of the Sex and Age of Gastroenteritis Patients.

The average number of adenovirus containing stool specimens detected at the Cadham Provincial Laboratory for each month from the last five years was plotted in 59 and showed no consistent association with the time of year. The isolates show two peaks of increased shedding in the spring and fall but the variation was not significant by a multiple comparison analysis ($p = 0.12$). The age of patients was not reported on many requisitions and the exact age in months was not defined on many more and complete analysis of the age of adenovirus infected patients was not possible. The proportions of approximately three quarters of the total patients where age in months was given have been grouped in six monthly classes in Figure 60. 94% of the patients diagnosed with adenovirus gastroenteritis whose age was known were under 3 years of age. The peak incidence occurred in the 6 to 12 month bracket. Only 4 patients were described as older than 4 years of age and the oldest documented patient was 6. Males and females were equally almost affected in each age group analyzed. 52% of the total 514 patients diagnosed with adenovirus gastroenteritis during the entire five years were male and 48% female.

C) Identification of the Species of Adenovirus Isolates.

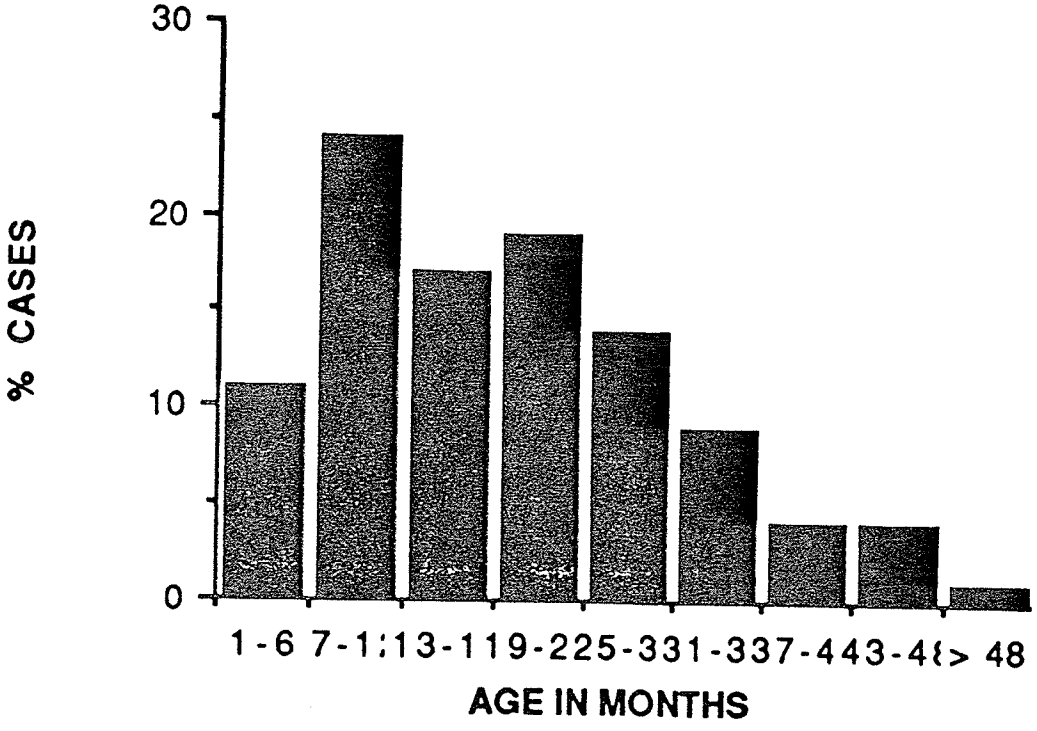
From 1989 onwards attempts were made to identify all of the adenovirus isolates detected at the Cadham Laboratory by species so as to determine the prevalence of individual species in gastroenteritis and follow changes in the form and quantity of species that

Figure 59. Monthly Distribution of Adenovirus Detection in Stool Samples.



The numbers of adenoviruses diagnosed in each month between 1987 and 1992 in stool specimens were summed and presented as the total number of patients detected with adenovirus over the five year period.

Figure 60. Age Distribution of Patients with Acute Adenovirus Gastroenteritis.



The ages of patients were compiled from Cadham Provincial Laboratory records and the proportions of patients in six monthly age brackets are presented.

would contribute to knowledge of the epidemiology of adenoviruses. Stool specimens containing sufficient sample of unidentified adenovirus isolates from years 1987 and 1988 were thawed from storage at -70°C and analyzed retrospectively. Isolates that grew in conventional culture were subjected to a neutralization test with antisera to Ad1, Ad2, Ad3, Ad4, Ad5 and Ad6. Isolates whose growth, after incubation with a specific reference antiserum, was inhibited so that more than half of the cells in the culture remained uninfected relative to a non-neutralized control culture were deemed typed. Where there was a difference in the infected cell proportion of less than a complete 50% between the control and a neutralized sample the test was repeated or the isolate identified by other means. Most isolates whose growth was not distinctly retarded by the antisera were tested by an EIA test specific for the enteric adenovirus species Ad40 and Ad41. Unexpectedly, most fastidious adenovirus faecal specimens, with virus evident by electron microscopy but growing poorly in conventional epithelial cell culture, failed to react with the enteric EIA test, and an analysis of these untyped specimens with restriction endonucleases was initiated. All unneutralized isolates were cultured in conventional cell lines, where possible, or 25 cm^2 flasks of semiconfluent 293 cells if fastidious, and treated by the methods of Hirt (1967) or Shinagawa *et al.*, (1985) to prepare viral DNA for restriction analysis. Fastidious isolates that failed to yield sufficient DNA from a single flask of 293 cells were regrown at a high multiplicity and passaged with fresh A549 cells by cocultivation. The recent isolates of 1991 that failed to grow in cocultivated cells were further tested by blocking assay using monoclonal antibodies supplied by Jan de Jong. The isolates were first analyzed with restriction enzymes *Bam*HI and *Hind*III and the species identified by comparison of the pattern to

the restriction digests of the first 41 species drawn by Adrian *et al.*, (1986). Isolates with *Bam*HI or *Hin*dIII patterns that did not conform exactly to the restriction patterns of prototype strains of species Ad1 to Ad41 were further analyzed with enzymes *Bgl*II and *Sma*I, for which the prototype patterns were also available, and, on occasion, *Eco*RI or *Sal*I which give distinctive patterns with Ad41 DNA. The results of this program to speciate adenovirus isolates are compiled in Table 8 and compared with the numbers of various adenovirus strains found by restriction analysis in Manitoba during an earlier study (Hammond *et al.*, 1985) from 1980 to 1983. The comparison between the first and last years of the decade shows a much greater diversity of strains of a number of prominent species currently circulating as a cause of adenovirus gastroenteritis. The proportion of variant strains is lowest in the earlier years of the 1980s, where only 2 of the 30 isolates identified by restriction analysis, or 6.7% of the isolates, differed from the most prominent strain of each species. The amount of variant strains increased successively in more recent years with 13% and 15% of the total isolates showing variations from the restriction patterns of the prototype of species. In 1991 the proportion of genomic variant strains found was more than twice the proportion found in isolates from ten years before. Variant strains of species Ad3 and Ad2 account for most variant isolates. Ad1, Ad5 and Ad31 variants strains were also identified. Restriction analysis was performed on those isolates that were not identified with neutralizing antibodies to prototype strains of the first seven species. A notable trend evident in Table 8 was that an increasing proportion of the isolates identified by restriction analysis were variants of the first seven species. These variant strains of Ad1, Ad2, Ad3, Ad5 and Ad7 were not neutralized by species specific antibodies that did neutralize all but 2 isolates of the first

Table 8: Frequency of Isolation of Adenovirus Species and Prevalence (%) of Strains in Acute Gastroenteritis in Manitoba.

Species by Neutraliz.	1980-83		1987		1988		1989		1990		1991	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Ad1	33	10%	19	17%	13	14%	12	11%	11	11%	13	13%
Ad2	71	23%	21	19%	16	17%	18	17%	26	16%	25	25%
Ad3	45	14%	7	6%	7	7%	2	2%	4	4%	3	3%
Ad4	1	<1%	-	-	-	-	-	-	-	-	3	3%
Ad5	6	2%	1	1%	3	3%	1	1%	4	4%	5	5%
Ad6	2	<1%	2	2%	1	1%	1	1%	-	-	2	2%
Ad7	13	4%	2	2%	4	4%	3	3%	5	5%	-	
TYPED	172	55%	52	46%	44	46%	37	35%	41	40%	41	41%
UNTYPED	142	45%	60	54%	51	54%	69	65%	61	60%	58	59%
TOTAL	314		112		95		106		102		99	

Species by RE Analysis	1980-83		1987		1988		1989		1990		1991	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Ad1	-	-	1	4%	-	-	1	1%	3	3%	3	3%
Ad2	2	3%	-	-	-	-	2	3%	1	1%	3	3%
Ad2v*	-	-	-	-	2	11%	9	13%	4	4%	7	7%
Ad3	-	-	-	-	-	-	1	1%	-	-	-	-
Ad3v	-	-	1	4%	1	5%	4	6%	-	-	1	1%
Ad3v2*	-	-	-	-	-	-	2	3%	3	3%	2	2%
Ad4	-	-	-	-	-	-	2	3%	-	-	-	-
Ad5	-	-	1	4%	1	5%	3	4%	-	-	-	-
Ad5v	-	-	1	4%	-	-	1	1%	3	3%	2	2%
Ad6	-	-	-	-	-	-	-	-	1	1%	-	-
Ad12	-	-	-	-	-	-	-	-	-	-	-	-
Ad15	1	2%	-	-	-	-	-	-	-	-	-	-
Ad31	5	8%	-	-	1	5%	3	4%	3	3%	-	-
Ad31v	1	2%	-	-	-	-	-	-	-	-	1	1%
Ad40	-	-	-	-	-	-	1	1%	-	-	-	-
Ad40v	9	15%	1	4%	-	-	-	-	-	-	-	-
Ad41	1	2%	-	-	-	-	-	-	-	-	-	-
Ad41v	11	18%	5	21%	5	27%	18	25%	38	37%	34	34%
TYPED	30		13		10		47		56		53	
UNTYPED	5		47		85		22		5		5	
TOTAL	35		60		95		69		61		58	

* v and v2 designate genomic variant strains.

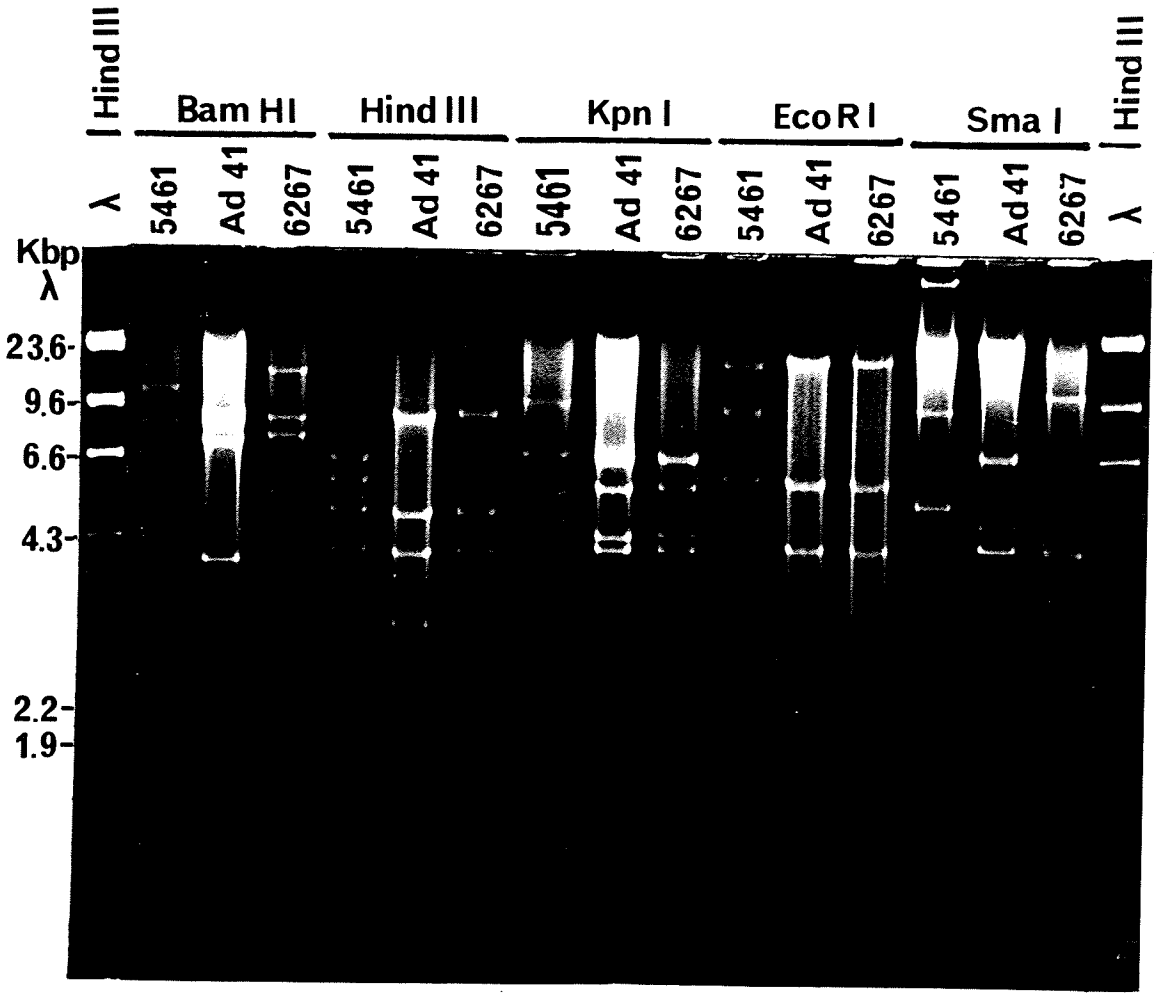
seven species between 1980 and 1983.

The quantification of strains in Table 8 also indicated that there has been rapid changes in the frequency and composition of causative species in adenovirus gastroenteritis. In the earlier years of the 1980s, the two fastidious species Ad40 and Ad41 were approximately equally prevalent and were but two of several prominent species that together formed composite causal agents of adenovirus gastroenteritis. The prevalent strain of Ad40 was a genomic variant with *Kpn*I and *Bam*HI restriction patterns that do not conform to those of the prototype strain (Hammond *et al.*, 1985). This variant has apparently disappeared from circulation in the intervening years between 1983 and 1987 and, apart from a single isolate of the prototype strain of Ad40 in 1989, the involvement of Ad40 in adenoviral gastroenteritis has decreased dramatically in the latter part of the decade. In contrast, the Ad41 variant has increased in relative importance so that since 1983 it has been the single most prevalent strain in adenovirus gastroenteritis, gaining in prevalence so that currently this variant strain is alone responsible for more than a third of the total annual isolates in recent years.

D) Characterization of Enteric Adenovirus Strains in Manitoba.

The enteric adenovirus isolates exhibited only three combinations of restriction patterns throughout the period of study, as shown in Figure 61. A single isolate, cultured in 1989, revealed restriction patterns that corresponded with those defined for the strain Hovi-X of species Ad40 (Kidd *et al.*, 1984). The single faecal sample, assigned a laboratory number of 5461, was the only specimen found to contain any strain of Ad40, among a

Figure 61. DNA Restriction Patterns of Enteric Adenovirus Isolates.



Single 25 cm^2 flasks of 293 cells, infected with 1 ml of a 10% suspension of clinical stool samples 5461 and 6267 or culture supernatant of Ad41 reference strain Tak, were harvested and the cells digested with proteinase K and extracted by the method of Hirt (1967). 10 μl aliquots of 50 μl DNA suspensions were digested for three hours with restriction enzymes and run in an 0.8% agarose gel. Lambda DNA, as molecular weight markers, has been spliced to outside lanes.

sum total of 100 enteric isolates cultured between 1987 and 1992. The electrophoresis of restriction fragments of this isolate adjacent to DNA of Ad41 prototype strain Tak, extracted from a reference strain, and the DNA of a clinical specimen containing an isolate of the prevalent Ad41 variant in Figure 61 demonstrates that the restriction maps of Ad40, with very few comigrating bands in common, are completely different from those of Ad41. This isolate of Ad40 strain Hovi-X and the Ad41 strain Tak specimen did react positively with the enteric adenovirus specific EIA. However, stools containing virus with Ad41 variant DNA patterns were completely unresponsive with the monoclonal antibody test. The limitations of the enteric EIA test were further examined with four specimens containing isolates of an Ad40 genomic variant strain, recultured from the previous study (Hammond *et al.*, 1985), and culture grown supernatants of reference strains of prototypes Ad41 Tak and Ad40 Dugan, all of which tested positive in the enteric test. The identification of the variant isolate stool specimens was confirmed by the reaction of all the specimens tested with a group reactive EIA test using a monoclonal antibody to an epitope present on the internal aspect of the hexon present in all Mastadenoviruses (Cepko, Whetstone and Sharp, 1983). The results with both enteric and group reactive EIAs are compared to restriction analysis results in Table 9 which indicate that the Ad41 specific monoclonal antibody utilised in the test (Herrmann, Peron-Henry and Blacklow, 1987) failed to react with the prevalent enteric adenovirus strain in Manitoba.

The vast majority of enteric isolates revealed variant restriction patterns similar but not identical to those of the Ad41 prototype. These restriction patterns were first described

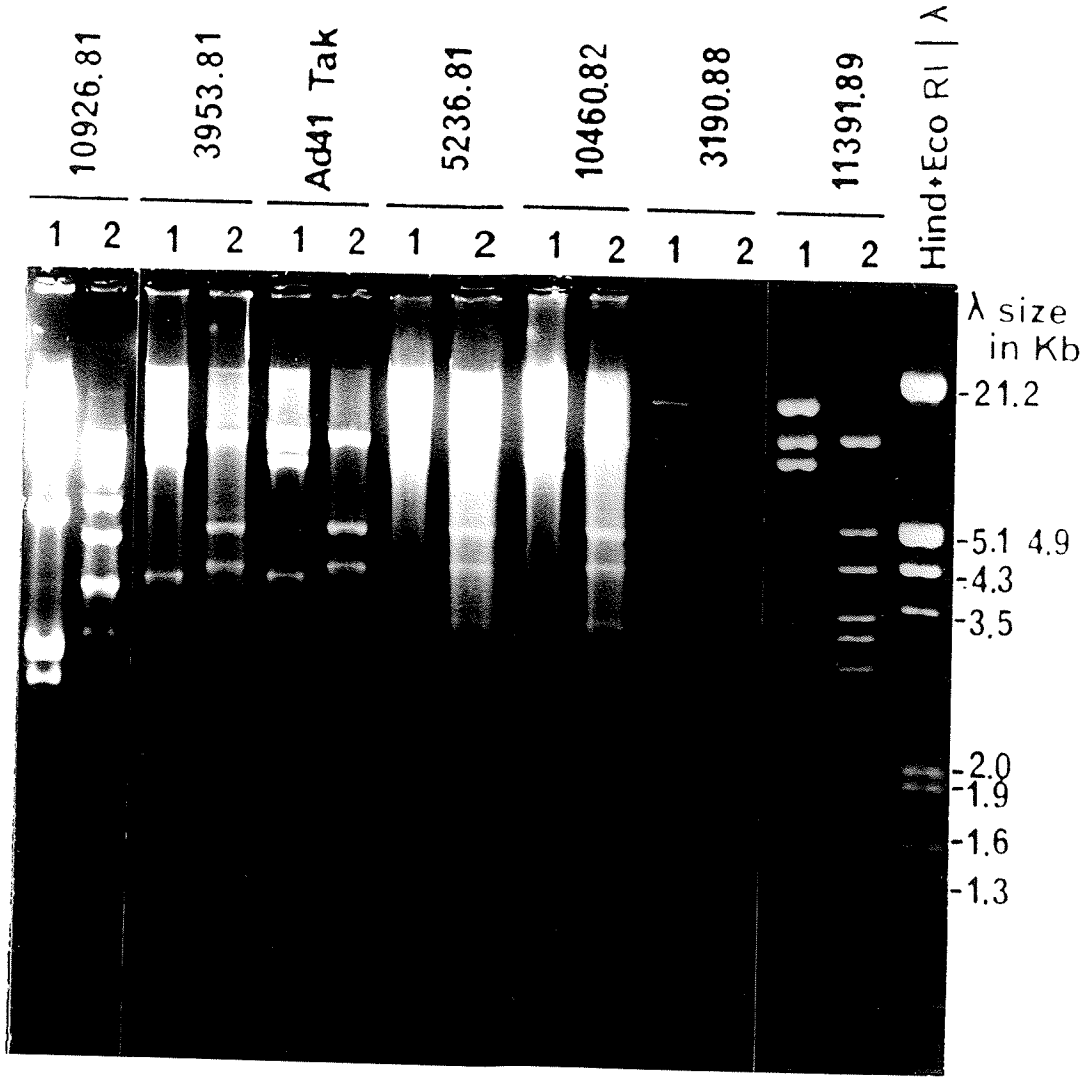
Table 9: Detection of Enteric Adenoviruses by Specific and Group Reactive EIAs.

Adenovirus type by Restriction Analysis	No. of isolates detected by: Enteric EIA		Group Reactive EIA	
	Positive	Negative	Positive	Negative
Clinical Isolates;				
Ad41 strain Tak	1	0	1	0
Ad40 strain Hovi-X	1	0	1	0
Ad41 genomic variant	0	15	15	0
Ad40 genomic variant	4	0	4	0
Reference Strains;				
Ad41 stain Tak	1	0	1	0
Ad40 strain Dugan	1	0	1	0

with DNA from virus cultured locally between 1981 and 1983 during a previous epidemiological study (Hammond *et al.*, 1985). The agarose gel in Figure 62 shows the *Bam*HI and *Hin*dIII restriction patterns of various clinical specimens from 1981 and 1982 electrophoresed with isolates from more recent years. The two specimens in lanes 1 and 2 and lanes 3 and 4 from 1981 represent the last occasion that strains of Ad40 Hovi-X and Ad41 strain Tak were found in Manitoba prior to their recent isolation in 1989. Comparison of the clinical isolates 5236 and 10460 from 1981 and 1982 with 3190 and 11391 from 1988 and 1989, respectively, illustrate that the *Bam*HI and *Hin*dIII restriction patterns of the Ad41 variant strain have not altered over an 8 year period. A small proportion of variant isolates, including 6267 in Figure 61, manifested a minor dissimilarity in the *Eco*RI restriction pattern involving the D fragment. This variation occurred in a few isolates cultured early in the study and was not found in later isolates. Despite frequent analysis of enteric isolates with a battery of restriction enzymes, no other variant strain of enteric adenovirus or restriction fragment abberation was observed.

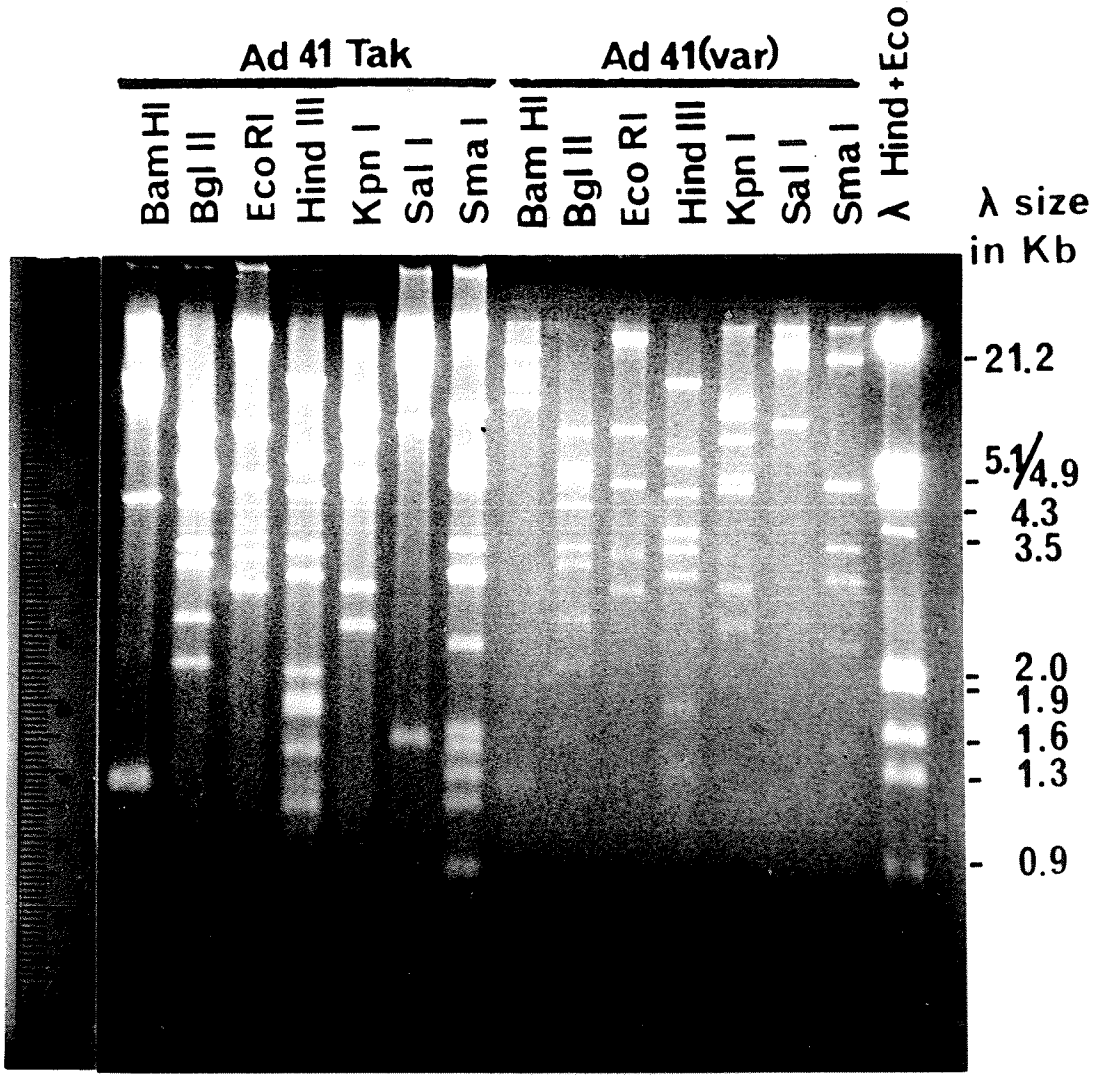
Comparison of the restriction fragment patterns generated by electrophoresis of genomic DNA of prototype Ad41 Tak and the variant strain digested with frequently used enzymes in Figure 63 shows that the *Bgl*II, *Kpn*I and *Sal*I maps are identical for the two strains, whilst *Bam*HI, *Hin*dIII and *Sma*I enzymes produced differences in two fragments or more. The *Bam*HI A fragment of the variant digest was elevated relative to that of strain Tak, and the D band was missing from the variant digest. With enzymes *Hin*dIII and *Sma*I, the genomic variant restriction patterns likewise demonstrated both elevated and absent bands. The position of restriction site disparities between the two Ad41 strains

Figure 62. *Bam*HI and *Hind*III Digests of Clinical Isolates from 1981 to 1989.



DNA extracted from flasks of 293 cells infected with clinical isolates or Ad41 strain Tak was digested with *Bam*HI (1) or *Hind*III (2) and electrophoresed in 0.8% agarose with lambda phage DNA. The year of isolation is indicated after the clinical number. Samples from 1981 and 1982 were processed by the method of Hirt (1967), 1988 and 1989 samples by the method of Shinagawa *et al.*, (1983).

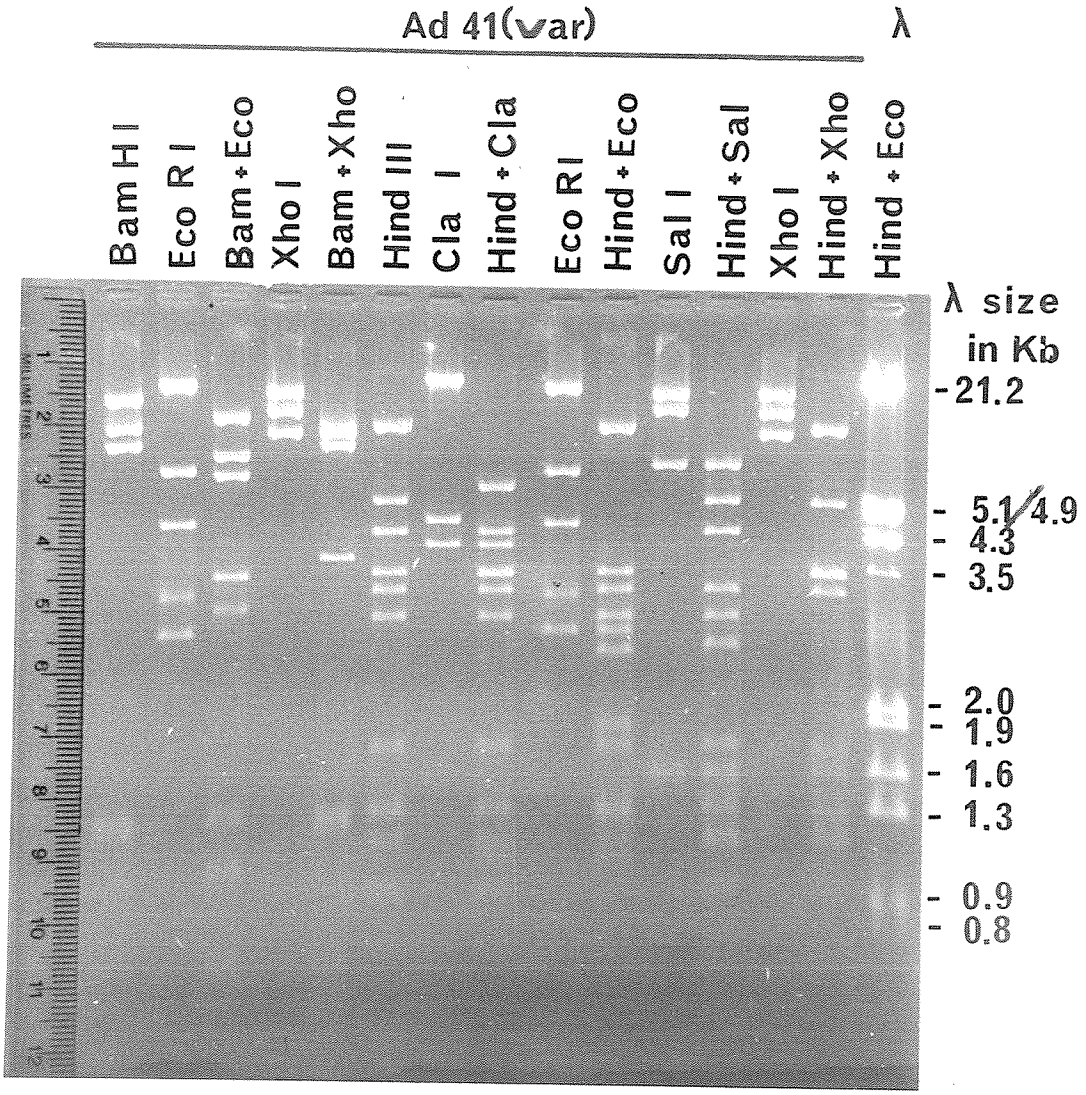
Figure 63. Comparison of Restriction Patterns of Ad41 Prototype Strain Tak and the Prevalent Ad41 Variant.



1 μg aliquots of prototype and variant strain DNA preparations (Shinagawa *et al.*, 1983) were digested with a range of the same restriction enzymes and run in parallel order in a 0.8% agarose gel. Lambda DNA, digested with *Hin*dIII and *Eco*RI, was placed in the outside lane as molecular weight markers.

was investigated by dual digestion of variant DNA and electrophoresis in the agarose gel in Figure 64. The uppermost *Bam*HI band of the variant was digested by *Eco*RI and *Xho*I, both of which subdivided the *Bam*HI D fragment of the Tak strain DNA in dual digest (Figure 48). This finding indicates that the *Bam*HI A and D, contiguous in the prototype strain, are combined, implying a mutation disguising the restriction site at map unit 59.3 (Figure 58), in the variant genome. Fragments F and I of the *Hin*dIII digest of Tak strain DNA are similarly combined in the variant genome, as was evident from the reduction of the novel band replacing these two fragments in the variant DNA digest (Figure 64, lane 6) by dual digestion with *Sal*I (Figure 64, lane 12), which has restriction sites in the centre of the genome at 52.1 and 56.3 map units. The enzyme *Cla*I, used for dual digestion because of its simple four fragment pattern with prototype strain DNA, also showed a missing restriction site between fragments A and B at 55 map units and consequently failed to reduce the novel *Hin*dIII band in dual digest of the variant DNA (Figure 64, lane 8). The *Sma*I variant restriction pattern displays two novel bands and four missing fragments relative to the prototype DNA digest. The A and B fragments, contiguous in the Ad41 strain Tak *Sma*I restriction map, have been combined by the absence of the restriction site separating the fragments at 85.4 map units in prototype DNA. The second adjacent pair of missing fragments, one of the *Sma*I E/F fragments and the H fragment of the Tak strain, of 2,680 and 1,620 base pairs respectively, have combined to form a 4,300 base pair band that comigrates with the C fragment in the variant DNA digest (Figure 63, lane 14). The mutated restriction site between fragments E/F and H is situated at 53.7 map units. Four restriction site differences between the prototype and variant genomes, therefore, occur between 51 and 60 map units in the

Figure 64. Dual Restriction Enzyme Digestion of the Prevalent Ad41 Variant Strain.

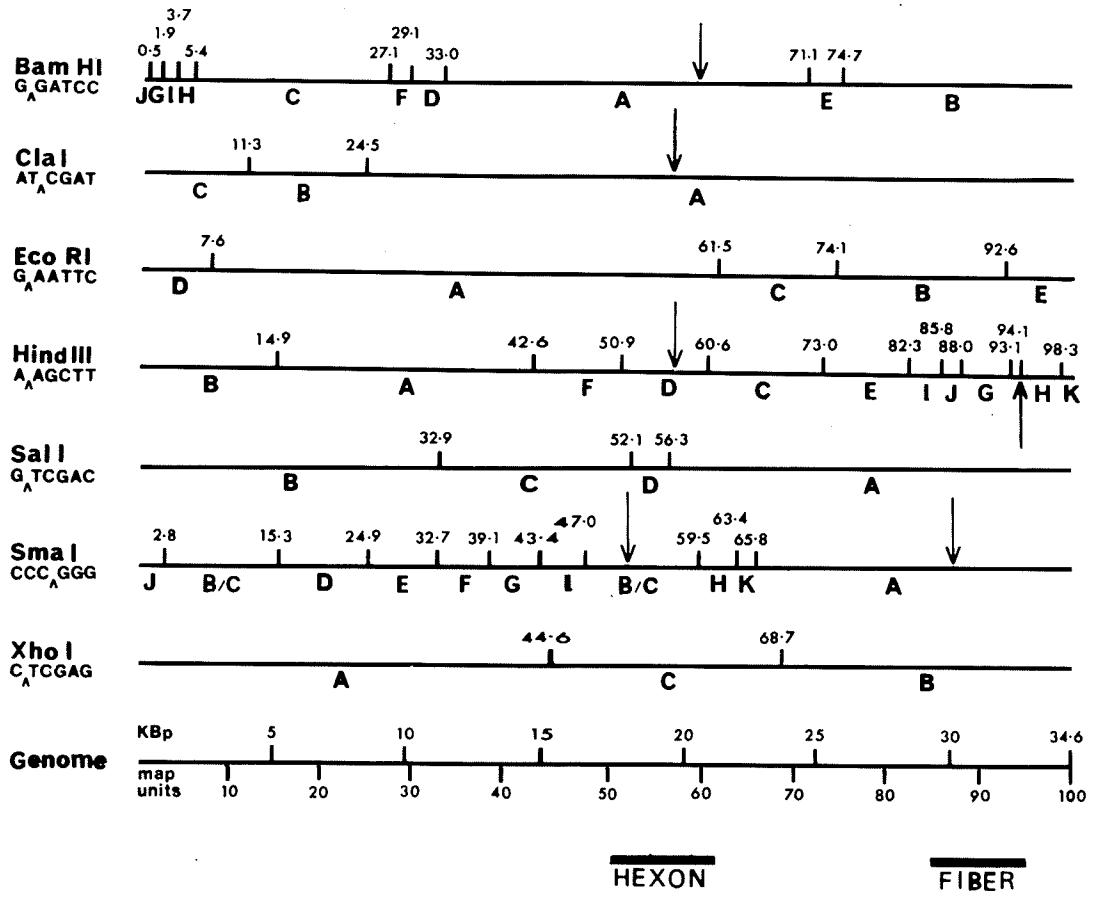


1 μg DNA samples of genomic Ad41 variant strain DNA, prepared by the method of Shinagawa *et al.*, (1983), was digested with single restriction enzymes or two enzymes together in dual enzyme digests to investigate the restriction maps of the variant strain. Digested samples were electrophoresed at 25 v overnight in a 0.8% agarose gel with lambda phage DNA as molecular weight markers.

centre of the genome. Another two discrepancies between the strains, a novel variant restriction site cleaving the prototype *Hin*dIII G band into two fragments and the mutation abrogating the restriction site between the prototype *Sma*I A and B fragments, occur between 85 and 90 map units. These discrepancies between the restriction patterns of the variant and prototype strains of adenovirus species Ad41 have been denoted on the prototype restriction maps in Figure 65. The positions of the hexon and fiber genes of Ad2 have been marked in the bottom panel of the and it is evident that the mutations conferring different restriction patterns on the variant strain occur in two clusters within the parameters of these genes.

In summary, the prevalent adenovirus strain in gastroenteritis in Manitoba, alone responsible for a third of the total cases, is a genomic variant of species Ad41. In an opposite trend to species Ad40 in Manitoba, the variant Ad41 strain has been increasing in relative importance as an agent of local gastroenteritis in the last ten years. The variant was not detected by a commercial EIA specific to enteric adenoviruses and has a number of restriction pattern differences to the prototype strain Tak of Ad41. These restriction site mutations were mapped to two genes, coding for the hexon and fiber capsomeres (Scott-Taylor *et al.*, 1990).

Figure 65. Restriction Site Differences Between the Restriction Enzyme Maps of Ad41 Prototype Strain Tak and the Prevalent Ad41 Variant Strain.



Mutations abrogating prototype restriction sites or creating novel sites are indicated on the restriction maps of the prototype strain. Restriction sites absent in the variant DNA are denoted by a down arrow, novel restriction sites by an up arrow. The parameters of the hexon and the fiber genes on the Ad2 genome have been indicated by black line below the maps.

DISCUSSION

Much of the time and effort invested in this project was spent in the purification of DNA preparations of adequate quality and quantity of the prototype strains of one species of each subgenus of human adenoviruses. These difficulties will be reviewed. Species Ad41 and Ad40, in particular, failed to establish repeated cycles of replication even in the more permissive 293 cell line. Species Ad8 and Ad12, of subgenera other than the traditionally fastidious subgenus F, gave a poor yield of DNA despite widespread cytopathic effect in the cell culture. The subgenus A species Ad12 was replaced in the study with species Ad31 which was found to be more cooperative in culture and more productive of viral DNA. Species other than Ad8 of subgenus D have not been well characterized. Although intense interest in tracing adenovirus movement in the epidemiology of epidemic keratoconjunctivitis has provided an abundance of restriction fragment patterns of subgenus D species in agarose electrophoresis (Adrian and Wigand, 1989; de Jong *et al.*, 1988; Ishii *et al.*, 1987; Noda *et al.*, 1988), restriction maps have not been produced. Restriction maps of clinical, non-prototype strains of species Ad9 and Ad19 have been published (Gomes and Niel, 1987) but these isolates were not widely available. In efforts to overcome the deficiencies in the quality of Ad8 DNA, a number of possible causes of reduced viral yield were investigated. Less harsh methods of lipid extraction than blending the cell lysate with freon did not improve the viral bands in density gradients. Loss of virus through disruption during repeated freeze-thawing or by proteolytic digestion after fracture of infected cells were both discounted as possibilities to account for reduced viral yield. Pieniazek *et al.* (1990) suggested that the common practise of

alternate cycles of freezing and thawing infected cells to release cell associated virions could damage the infectivity of enteric adenoviruses. Extrapolation of this explanation to Ad8 is unlikely given that the titre of Ad8 preparations did not consistently decrease when measured over ten completed cycles of freeze-thaw. In fact, the high proportions of incomplete virions observed in final preparations of the more difficult to grow virus species did not seem to be enhanced at any stage of the purification procedure. Examination of the relative proportions of damaged capsids under the electron microscope failed to endorse the notion that low yields of virus were due to progressive degradation of unstable virus particles during purification. The amount of damaged virions observed in the preparation of either Ad8 or Ad41 did not increase substantially and the high degree of incomplete particles appeared to be an intrinsic quality of the normal growth of both these species. It has been suggested that enteric adenoviruses produce a large quantity of non-infectious virions (Brown, 1985). Subgenus D viruses in general have been found to generate a large proportion of non-infectious particles (Green *et al.*, 1979), evaluated at a ratio of up to 2,300 total particles to 1 infectious virion, much higher than the ratios known of other subgenera. Ad8 may have an even lower infectious particle ratio than other subgenus D viruses tested, in the order of 27,000 particles counted by electron microscope per single fluorescent focal unit (Wigand *et al.*, 1983). This large production of defective particles may well interfere with the growth of the virus. The cytopathic effect observed with Ad8 culture in this study was often rapid and extreme but bore little relation to the final yield of DNA. Ad8 has been reported to cause cellular clumping and retraction as a distinguishing feature from other subgenus D viruses which induce only isolated rounded cells in tissue monolayers

(Wigand *et al.*, 1983). The low yield and cytopathic effect may be related to the relatively high production of toxic pentons (Doring, Nguyen and Wigand, 1972). Other studies attempting to improve Ad8 growth (Hanna and Jawetz, 1962; Wigand *et al.*, 1983) have failed and low infectivity titre was considered an inherent characteristic of Ad8 strain Trim. Prototype Trim of Ad8 is recognized as particularly refractive to *in vitro* tissue culture (Hanna and Jawetz, 1962) and it has been suggested that the prototype ought to be substituted in favour of strain 1127 or other more cultivable strains (Wigand *et al.*, 1983). A single prototype strain of one species from each subgenus was utilized in this study as properly representative of the human adenovirus species, therefore the prototype strain of Ad8 was retained.

Modifications of the standard protocol (Wold, Green and McKay, 1978) for purification of Ad2 virions, employed in efforts to harvest greater yields from infected cells, compromised the quality of the DNA produced. Gentle methods of lipid extraction resulted in entrapment of virus particles in layers of cellular debris and gave yields of contaminated viral DNA preparations. Incomplete removal of the cell lipid prior to density gradient centrifugation, explored in attempts to adapt methods for preparation of other types of virus to adenovirus purification, made subsequent DNA purification difficult. Pelleted virus or preparations processed without separation of the component of incomplete virions by isopycnic centrifugation produced a smeared DNA product with a range of partial genome sizes. The original protocol of Wold, Green and McKay (1978) was ultimately adhered to with little alteration for the purification of most viral species as none of the alternative solvents or methods of extraction examined appeared to

enhance the proportion of intact virions or result in cleaner preparations of viral DNA.

The major improvement in virus yield was realized not with changes to purification methods but with modifications to standard cell culture techniques. The problem of fastidious adenovirus culture has promoted many attempts at improvement. Under the impetus to determine the nature of the fastidious adenoviruses observed in the stool of a high proportion of sick children (Appleton *et al.*, 1978; Brandt *et al.*, 1979; Madeley *et al.*, 1977) a number of semi-permissive cell lines for the growth of enteric adenovirus species were discovered. The suitability of Chang conjunctival cells and 293 cells, an HEK cell line transfected with the E1 genes of Ad5 (Graham *et al.*, 1977), were demonstrated (Kidd and Madeley, 1981; Takiff *et al.*, 1981) in the cultivation of some refractory isolates. The use of cynomologous monkey kidney cells led to the original isolation of another enteric adenovirus species, Ad40 strain Dugan (de Jong *et al.*, 1983). Although 293, Chang conjunctival and cynomologous monkey kidney cells have only moderate sensitivity to fastidious adenoviruses (de Jong *et al.*, 1983), they remain the cell lines of choice. There has been little improvement in the cultivation of fastidious adenoviruses other than a brief, uncorroborated report that a colonic tumour line, HT29, readily grew Ad41 (Uhnoo *et al.*, 1983). A549 cells, fibroblasts from a lung carcinoma (Lieber *et al.*, 1976), have been found to have a higher sensitivity to adenovirus and herpes simplex virus isolates from respiratory swabs than the traditionally used cell lines or the preferred primary human embryonic kidney cells (Smith *et al.*, 1986). A549 have since been applied with some success to the growth of conventional adenovirus species in stool samples (Woods, Yamamoto and Young, 1988). In this study the slower rate of

A549 cell multiplication prolonged the survival of an infected culture and enabled preparation of adequate quantities of DNA of species Ad8, Ad31, and some strains of Ad41, where previous attempts with HEp 2 or 293 cells had frequently failed.

A549 cells may also prove to benefit the cultivation of enteric adenovirus species. Prolonged stirring with infected cells was well tolerated by A549 cells which did produce the greatest titres of enteric adenoviruses in cocultivation. Cocultivated 293 cells always had a considerable number of stressed cells that made accurate assessment of CPE difficult. In a study comparing the growth of enteric adenovirus species in different cell lines, Witt and Bousquet (1988) found the highest yields of Ad40 were given by KB cells and A549 cells, although the titres were very low and the differences may not have been significant, while Hashimoto *et al.*, (1990) succeeded in producing plaques of Ad40 in A549 cells. However, the cultivation of enteric adenoviruses is a highly variable faculty in different laboratories. Uhnoo *et al.*, (1984), for example reported A549 cells to be non-permissive to enteric adenovirus infection while Hashimoto *et al.*, (1990) failed to grow Ad40 in KB cells. Similarly, the finding that enteric adenoviruses replicated efficiently in HEK cells cultured in media supplemented with low levels of serum (Pieniazek *et al.*, 1990) contradicts the experiences of other laboratories (Gary *et al.*, 1979; de Jong *et al.*, 1983). Some Ad40 and Ad41 isolates from clinical specimens have been shown to have better growth in HEp 2 cells than 293 cells (Perron-Henry, Herrmann and Blacklow, 1988) and fastidious growth characteristics are not unique to subgenus F adenoviruses (Brown, Petric and Middleton, 1984). In the present study 293 cells grown in media supplemented with low serum concentrations failed to thrive and

A549 cells did not produce substantial yields of prototype strains of enteric adenovirus species. The major improvement in yield of both Ad40 and Ad41 was realized by the mixing and cocultivation of virus infected cells with fresh A549 or 293 cells. This cocultivation, more than exposure to high concentration of stock virus in solution, facilitated infection of cells and promoted the spread of enteric adenoviruses. Cytopathic changes progressed much more rapidly in culture composed of infected cells cocultivated with equal quantities of fresh cells than in cultures exposed to the virus concentrated from the same passage of cells. Infected A549 and 293 cells, trypsinized and reseeded without mixing with fresh cells usually did not survive and did not yield significant quantities of virus. The exact nature of the mechanism was difficult to ascertain, but infection with enteric adenoviruses tends to spread to adjacent cells in infected foci (Retter *et al.*, 1979; Kidd and Madeley, 1981) and factors that promote cell contact could enhance the spread of cell associated virus. Immunofluorescent staining of non-permissive cells exposed to fastidious adenoviruses (Gary *et al.*, 1979; Retter *et al.*, 1979) showed isolated foci of infected cells and indicated that enteric adenoviruses were unable to initiate more than one cycle of replication via the culture medium. Similarly, pockets of infected cells were frequently observed in the culture of Ad40 and the early infection of 293 cell monolayers by Ad41 in this study. The infection of cells surrounding the initially infected cell in expanding foci suggests that a process involving membrane contact between cells may enhance the invasion of enteric adenoviruses. The mixing of cells improves the growth of other fastidious viruses (Benyesh-Melnick *et al.*, 1964) and is in routine practice in the cultivation of cytomegalovirus.

Despite the significant improvement to enteric adenovirus yields achieved with cocultivation, no virus band was ever seen in density gradients from Ad40 infected cells following any protocol and DNA preparations of this fastidious virus were made directly from lysed cells without purification of the virions. This species is evidently more difficult to grow than Ad41 as supported by the numbers of experienced workers (Chiba *et al.*, 1983; Kidd, Banatvala and de Jong, 1983; Uhnoo *et al.*, 1983; van Loon *et al.*, 1985) who profess having difficulty growing Ad40 even in 293 cells. In HT29 cells Ad41 apparently grew well but Ad40 remained isolated in single cells (Uhnoo *et al.*, 1983). The growth of enteric adenovirus species in 293 cells fostered the idea (Takiff, Straus and Garon, 1981) that the endogenous Ad5 sequences in these cells provided helper functions. Infection of cells transformed with small sequences of the adenovirus Ad2 or Ad5 E1 region localized the helper function to the E1B 55K gene (Mautner, MacKay and Steinthorsdottir, 1989). Studies on the *in vitro* growth of enteric adenoviruses have shown a correlation between the cellular transformation and susceptibility (Shinozaki *et al.*, 1987; Witt and Bousquet, 1988). The capability of Ad40 to propagate in A549 cells, which have no adenovirus E1 genes, corresponded to a much higher viral E1B expression in this cell line than non-permissive cells (Hashimoto *et al.*, 1991). The 55K protein product of the E1B gene has been implicated as aiding in the shutdown of host cell protein synthesis (Babiss and Ginsberg, 1984), transport of late mRNA (Leppard and Shenk 1989), and efficient synthesis of structural viral proteins (Lassam, Bayley and Graham, 1979). This importance of the E1B gene to late virus protein production may accord with the blockage to virus growth in non-permissive cells which Retter *et al.*, (1979), finding a paucity of adenovirus hexon gene transcripts, attributed to a late

blockage in replication.

There may be other factors involved in the relatively poor yield of Ad40. Brown (1985) observed that while production of Ad40 virions was 3 to 5 times less than other adenoviruses, the yield of infectious virus particles of this species was 100 to 1000 times less. The high proportion of non-infectious particles in the progeny of this species suggested a greater fragility of Ad40 virions, possibly related to a structural or functional difference of one of the capsomeres. In a possible connection with this suggestion, Pianiazek *et al.*, (1990) have found a loss of protein V in the capsids of Ad41 that corresponded with the failure of the virus to passage in 293 cells and explained the loss in infectivity as a defect in capsid assembly. Attempts to evaluate the effect of successive cycles of freeze-thawing on the titre of Ad40 virus prepared in this study, conducted in conjunction with Ad8, were not successful due to the low quantities of infectious virions in initial preparations. However, no dramatic loss of Ad40 titre was observed, that would substantiate the suggestion of exceptional fragility of Ad40 virions was observed. It was thought that, like Ad8, the quantity of incomplete Ad40 capsids was not aggravated by the fracture of cells.

Due to the limitations in cultivation, all Ad40 DNA and the Ad31 DNA used in the initial cross-hybridization experiments were prepared by a technique that used the adenovirus terminal protein to sequester the DNA in phenol (Shinagawa *et al.*, 1983). Banding of virions in CsCl density gradients in the original virus purification protocol separated the virus from cellular DNA which settled at a higher density than the virus

particles. In addition, the treatment of banded virus with DNAse to remove cellular nucleic acids could not be done with the methods of Shinagawa *et al.*, (1983) or Hirt (1967) where viral DNA is precipitated directly from cell lysate. The methods of Hirt (1967) and Shinagawa *et al.*, (1983) can improve viral DNA yield even of adenovirus isolates with fastidious culture characteristics by avoiding many purification steps. However, the Ad40 and Ad31 DNA prepared in these ways are less likely to be free of cellular contamination than DNA preparations of viruses purified according to the protocol of Wold, Green and McKay (1978). DNA used for probes, whether viral or plasmid, was always carefully purified by density gradient to eliminate hybridization of cellular DNA in the probe and target DNA.

Hirt extract is widely used to prepare DNA of many viruses, including adenovirus. The technique of Shinagawa *et al.*, (1983), applicable only to adenovirus and perhaps poliovirus which also has a protein attached to the genomic termini, is not extensively employed but has one distinct advantage over the method of Hirt (1967). DNA prepared by Hirt extraction commonly precipitates with such a large component of cellular DNA that the uppermost viral restriction enzyme bands in gel electrophoresis can be completely obscured. The method of Shinagawa *et al.*, (1983) was sometimes found to be less than reliable, at times resulting in no DNA precipitate from cells with extensive CPE. However, careful execution of this method could prepare viral DNA which digested readily with restriction enzymes to give patterns in which the large fragments were free of visible background DNA smears. Moreover, preparations of Ad8, Ad31, Ad40 and Ad41 DNA were made in this study by the method of Shinagawa *et al.*, (1983)

that gave little or no hybridization with cellular DNA probes. Hybridization of a dilution series of these DNA preparations demonstrated that less than 0.01% was cellular DNA. These DNA preparations were purified from A549 cell monolayers of up to 1,500 cm^2, an increase in scale of 60 times from the original description. This method could, therefore, supply a means of procuring large quantities of DNA as well as provide a useful alternative to the isolation of intact virions for preparation of pure viral DNA with ostensibly no cellular DNA content, for clear fragment patterns in restriction analysis or for use in hybridization studies.

Once purified, genomic adenovirus DNA preparations of one species of each human adenovirus subgenus were hybridized with an Ad41 genomic DNA probe under stringent conditions to locate genetic sequences common to all the human viruses of the Mastadenovirus genus. DNA of Ad2, Ad4, Ad7, Ad8 and Ad31, divided into genomic fragments by restriction enzymes according to the availability of restriction enzyme maps, was separated by gel electrophoresis and transferred to nylon membrane by Southern blot. The membranes were hybridized with genomic Ad41 probes under stringent conditions and fragments homologous to Ad41 DNA were detected by autoradiography and ascribed to their position in the genome by the restriction maps. The accuracy of definition of areas of cross-identity was determined by the availability of restriction maps, which ideally enabled the genomes to be subdivided in to multiple fragments that did not comigrate. The detail of knowledge acquired on each adenovirus species is roughly proportional to the incidence and cultivability of the species. Ad2, as a long established representative of adenoviruses in molecular studies has been

completely sequenced from one terminus to the other and restriction maps for every type I restriction endonuclease were generated by analysis of the filed genomic Ad2 sequence with the enzyme recognition sequences. Thus it was possible to choose restriction enzymes or combinations for Ad2 that released a wide range of discrete fragments and the conserved regions of the genome detected were most prescribed and localized with the Ad2 cross-hybridization. Consequently, the cross-reactive areas defined of species other than Ad2, with fewer restriction maps generated physically on less easily grown DNA, are broad and show some variation.

The present restriction maps for Ad8 were determined using a strain isolated in Hungary in 1962 (Takacs *et al.*, 1983) and have yet to be identified as belonging to the prototype strain Ad8 Trim (Kemp and Hierholzer, 1986). Two genotypic variants but no prototype isolates of Ad8 have been found in Japan (Fuji *et al.*, 1983) and a further two variants have been found in the United States over a 19 year period, one from Vietnamese refugees, (Kemp and Hierholzer, 1986). The Trim strain of Ad8 is still reported from USA and Europe and has managed to persist in the population while successive waves of epidemic keratoconjunctivitis caused by Ad19, Ad19A and Ad37 have been and gone (Kemp *et al.*, 1983). The *Bam*HI and *Kpn*I fragments of Ad8 Trim in this study differed from the patterns expected from the restriction maps of Takacs *et al.*, (1983). The restriction patterns of the prototype Trim in electrophoresis have more fragments when cleaved with enzymes *Kpn*I and *Sal*I (Kemp and Hierholzer, 1986) than drawn in the maps and it is likely that the Hungarian strain used by Takacs *et al.*, (1983) is a genotypic variant. DNA preparations of Ad8 reference stock at low passage produced the

same restriction fragment patterns whether purified from virions or from lysed cells. These restriction patterns did not correspond to those from the prototype strain produced by Kemp and Hierholzer (1986). Demarcation of the Ad8 fragments that hybridized with Ad41 DNA according to order in the Hungarian strain maps produced a patchwork of highlighted sequences which were frequently discontinuous and at extreme ends of the genome, showed little uniformity between different Ad8 digests and defied the placement of homologous sequences in the other species. Only the relatively simple *Bam*HI pattern of the variant, which differed from the pattern of strain Trim by a single extra fragment probably derived from a further restriction site in the A fragment of the prototype strain, gave an undistorted order of hybridizing fragments that corresponded with the other species. This indicated that the position of conserved sequences was consistent in each subgenus including subgenus D.

Three principle areas of the adenovirus genome, most clearly defined with the Ad2 hybridization with Ad41, were found in the reaction of the DNA of each subgenera. The first area of Ad2 cross-homology with Ad41, between 11.4 and 16.1 map units, contains the complete sequence of precursor IVa2 protein, the C terminus of the DNA polymerase, as well as a 12.2 kilobase open reading frame (ORF) (Persson and Philipson, 1982). Only the N terminus of the terminal protein is known to be coded in the second homologous area from 27.4 to 29.7 map units, although there is a 14.4 kilobase ORF. The central area of Ad2 hybridization begins in the middle of the V protein sequence at 47.5 map units and codes for a series of contiguous genes on the right strand from the pVI protein, the hexon to the 23K protease gene which overlaps

with part of the DNA binding protein gene sequence on the left strand. Three of the conserved genes, the DNA polymerase, the DNA binding protein and the terminal protein are all involved in DNA replication (Friefeld *et al.*, 1984). Four conserved structural genes are also intimately connected; protein VI, with protein III, binds directly to the hexon (Everitt, Lutter and Philipson, 1973) and is helped into position by the IVa2 protein, a scaffold protein involved in construction of the "light intermediate" capsid (Persson *et al.*, 1979). The marginally homologous 100K protein mediates the combination of three monomeric hexon polypeptides into the mature capsomere (Cepko and Sharp, 1983). The fact that over half of the hybridization intensity of each digest was attributed to the hexon gene alone emphasizes the importance of the hexon in virion morphogenesis and as a fundamental building block of the virus capsid.

The different species did show some variation in the position of hybridizing sequences. The left terminus of the Ad31 genome, unlike Ad2, hybridized with Ad41 DNA when isolated as a separate fragment. A fourth area of hybridization occurred at about 72 map units with some species. However, these differences from the Ad2 pattern of fragment hybridization generally appeared at low or marginal absorption and a repetitive pattern was preserved in the reactions of the major cross-reactive fragments of the other genomes. All of the Ad2 sequences of homology were contained within the broader cross-hybridizing areas of each of the other types. Moreover, the respective regions of the genomes reacted with a similar proportion of the total absorbance and a central fragment of each genome, between 50 and 60 map units and corresponding to the position of the hexon gene, was alone responsible for the majority of the absorbance of

each genome. This indicates that there is a shared pattern of gene conservation maintained through all subgenera of the human adenoviruses. Similar regions to the first and the central homologous areas of the Ad2 genome were found in common with mouse FL virus when cross-reaction between those two types was minutely mapped (Larsen, Margolskee and Nathans, 1979). All three regions of homology were represented in a comparison between bovine adenovirus 2 and human Ad2 (Hu, Hays and Potts, 1984), implying that the sequence conservation extends further than the human species and may include all Mastadenoviruses. This latter study was performed at a lower stringency and found sequence homology in a broad swathe of the genome excluding approximately 15% at either termini. The intensity of hybridization of fragments were ranked, however, and Ad2 fragments containing the hexon gene were evaluated as the most conserved between bovine and human adenovirus species. Part of the hexon was mapped as the single most homologous sequence shared by human Ad2 and the bird adenovirus CELO virus (Alestrom *et al.*, 1982) and the conservation of the hexon gene may continue in the Aviadenovirus genus and the hexon may comprise the most conserved sequence of all adenovirus species.

Comparison of the hexon sequences of Ad2, Ad5, Ad40 and Ad41 genomes (Akusjarvi *et al.*, 1984; Kinloch, MacKay and Mautner, 1984; Toogood and Hay, 1988; Toogood *et al.*, 1989) shows that there is a strict dichotomy between the sequences that form the epitopic loops and the highly conserved β-pleated sheets of the hexon structure (Roberts *et al.*, 1986). The first 400 and last 1,000 base pairs of the Ad41 or Ad40 hexon gene have over 80% nucleotide identity with the corresponding sequence in Ad2 or Ad5 and

are evidently responsible for the high degree of hybridization intensity of fragments enclosing map units 52 to 60. The Ad41 sequence to approximately 11 map units has also been sequenced (van Loon *et al.*, 1987). The initial part of the pIVa2 gene which coincides with the first anterior area of Ad2 cross-hybridization shows some 70% nucleotide identity with the corresponding Ad2 sequence, a level much greater than the 40 to 45% identity found in the preceding E1 genes. The genomic region coding for the 23K protease and the overlapping C terminus of the 72K DNA binding protein, with 74% nucleotide homology respectively to Ad2 proteins (Vos *et al.*, 1988) were visualized between 60 and 65 map units within the central area of cross-homology. The sequence of the 100K and 33K protein genes (Slemenda *et al.*, 1990), observed at the limit of sensitivity in some types at approximately 72 map units, indicates that the margin of detectable nucleotide identity between Ad2 and Ad41 under the conditions used approximates the 64% of nucleotides shared between the two sequences of these genes. The 46% of nucleotides shared by Ad41 and Ad2 in the code for the knob of the fiber (Pieniazek *et al.*, 1989), towards the right end of the genome, remain undetected. A hybrid of Ad2 and Ad41 strands, both of 52% G+C (Green *et al.*, 1979; van Loon *et al.*, 1985), would melt at approximately 102°C in 0.99 molar salt (Schildkraut and Lifson, 1965) if there were perfect sequence identity. Allowing that every 1% mismatch in sequence lowers the melting temperature by 1°C (Bonner *et al.*, 1973), hybridization at 68°C should enable Ad2 sequences of up to 34% mismatch or greater than 66% nucleotide identity with Ad41 to anneal. Therefore, theoretical expectations and the experimental conditions seem to concur closely.

The information gathered about the most conserved sequences of the genome was used to assess the performance of various portions of the Ad2 genome as probes in the detection of adenovirus DNA preparations of each subgenus in sensitivity tests. The subgenera DNA diluted from 1 μg to 1 pg in log dilutions on nylon membrane using a slot blot apparatus reacted to various levels with different probes. The relative difference between homologous and other subgenera DNA preparations was taken as an index of specificity. The slot blot has the advantage over dot blot apparati in that prozone effects, hollow circles presumably created by electrostatic forces in the cylindrical wells, are eliminated. The format of the slot blot, twenty rows of three slots in the air-tight Schlicher and Schuell model, was inconvenient for presentation and necessitated cutting each photograph to splice the wells in a row. The difference between probe reactions with homologous and heterologous adenovirus genomic DNA preparations should approach zero as the sequence identity of the probe with common target sequences increases. Accordingly, fragments from the termini of the Ad2 genome, with little sequence conservation, reacted with the subgenera DNA preparations in a highly specific manner similar to the reaction of the whole Ad2 genome as a probe, detecting quantities of Ad2 DNA 1,000 to 10,000 times as small as the amount of DNA detected of the other subgenera. Ad2 probes containing sequences of the first or second area homologous with Ad41 reacted with lower quantities of the heterologous species' DNA in a more uniform reaction. The p2HA plasmid, containing virtually the entire central area of homology in the *Hin*dIII A fragment of Ad2, reacted more evenly with lower quantities of the subgenera DNA preparations than any of the other *Hin*dIII clones or electroeluted fragments of the Ad2 genome assessed in the sensitivity tests. This results is in rough

agreement with similar dot blot experiments in which a plasmid equivalent to p2HA can be compared with Ad2 genomic DNA as a probe (Huang and Deibel, 1988). The *Hin*dIII A fragment probe in the previous study was relatively unreactive with subgenus A and B DNA samples. Indeed Ad12 DNA of subgenus A was apparently detected with less sensitivity by the p2HA equivalent probe than by the genomic DNA probe, although it has been shown here that the *Hin*dIII A fragment contains the major part of the most conserved sequences of the adenovirus genome. While it was found in this study that the Ad2 genomic DNA was less reactive with subgenus A DNA than DNA preparations of other subgenera, the inequality was lost with the cloning of conserved sequences in the p2HA probe. The major contrast between the present study and the previous report was with the difference in quantity of homologous and heterologous DNA detected by the genomic Ad2 probe. The genomic DNA probe reacted in Huang and Deibel's experiment with concentrations of DNA of some other subgenera equal to the Ad2 DNA concentrations detected concurrently, instead of the 3 to 4 log difference in reactivity observed here. The 10 to 20% level of shared DNA sequences between subgenera (Garon *et al.*, 1973; Green *et al.*, 1979) should only allow a maximum cross-reaction of 1 log lower for non-homologous DNA, if the homologous regions between Ad2 and other subgenera genomes were perfectly conserved. An additional detraction from optimal hybridization would be expected due to the large part of the genomic sequence with no identity and no binding capacity to DNA of other subgenera. The use of a smaller and more homologous region of conserved genes as a probe would be expected to show a broader reactivity than a probe sequence of variable homology, such as the whole Ad2 genome, on the principle that a greater and more even proportion of the probe should

react with other species while the sensitivity for homologous DNA should not be affected. The most equilateral reaction with the DNA of each subgenus in hybridization, in accordance with the position of the most conserved sequences in the adenovirus genome, was given by fragments containing central areas of the Ad2 genome between 50 and 60 map units. The HPII fragment reacted most evenly with all adenoviral types and detected the DNA of all other subgenera at a concentration of one to two log dilutions greater than the homologous reaction with Ad2, or quantities of DNA less than 100 times the amount of Ad2 DNA detected concomitantly. The electroeluted HPII fragment showed a 100 to 1000 fold increase in reactivity relative to a genomic probe and only about 10 fold lower sensitivity for DNA from non-homologous as opposed to homologous species. The lower sensitivity of HPII for the DNA of other species may be explained by the hexon's internal sequences of nucleotide divergence. It may be possible to optimize the cross-reactivity of a common probe by subcloning a region of the hexon corresponding to one of the P shell sequences. The first P1 sequence enclosed in a *Aval* or *Sau*3A fragment might be appropriate. The evaluation of the HPII fragment as the most cross-reactive probe sequence of the Ad2 genome indicates that it is possible to improve upon the cross-reactivity and sensitivity of genomic probes with definition of conserved sequences (Scott-Taylor and Hammond, submitted). The hexon gene, containing the most highly conserved adenovirus sequences, constitutes the best single sequence of the adenovirus genome for use as a cross-reactive probe for the diagnosis of all human adenoviral types by DNA hybridization.

The Ad41 genome was then examined for specific sequences so as to develop probes for

the detection of both enteric adenoviruses and to distinguish Ad41 specifically. The fastidious *in vitro* growth characteristics of enteric adenoviruses, which promoted the requirement for the diagnostic DNA probes, also hampered the production of sufficient amounts of DNA for analysis. The difficulty in supply of Ad41 DNA was alleviated by cloning the Ad41 *Eco*RI A, B and C fragments in the pGEM 3Z vector. The *Eco*RI restriction map with few large internal fragments and relatively small terminal fragments provided a simple means to clone and produce 85% of the Ad41 genome in quantity for restriction analysis. Two fragments E and D, 8% or about 2,700 bp of both termini were missing from the Ad41 clones, their ligation to the vector obstructed by remnants of the terminal protein. Characterization of the *Eco*RI clones by hybridization of the fragment containing plasmids with Southern blots of digested Ad41 DNA disclosed discrepancies with the restriction maps supposedly describing the prototype strain (Broker, Keller and Roberts, 1985; Akusjarvi and Wadell, 1987). These clones were initially characterized by reacting with *Eco*RI, *Sal*I and *Sma*I restriction enzyme digests of homologous genomic Ad41 DNA. The discrepancies could have resulted from either errors in the order of fragments in the restriction maps or the ligation of non-contiguous adenovirus sequences in the cloned plasmids. The repeated incompatibility of the fragment patterns of plasmids digested with dual enzymes supported the explanation that certain restriction maps were at fault and a study to correct the placement of fragments in the restriction maps of the Ad41 prototype strain Tak was undertaken.

Ordering of fragments towards the termini of adenoviruses can be problematical (Kitchingman, 1982). The terminal protein attached to the 5' terminal nucleotide of both

strands (Rekosh *et al.*, 1977) resists complete degradation with proteases and interferes with end labelling and cloning, the usual means of determining the terminal fragments. Gel retardation, a method utilising the intact terminal protein to prevent the entry of terminal fragments into an agarose gel, advocated as a method to overcome this problem (Kitchingman, 1982), was found to precipitate DNA that was difficult to redissolve and was prone to partial digestion. The order of the terminal fragments in the erroneous maps was eventually solved by the development of a novel method involving the treatment of genomic Ad41 DNA with the enzymes exonuclease III and S1 nuclease. Exonuclease III, a 3' to 5' specific exonuclease purified from E. coli, circumvents the terminal protein by degrading the 3' strand and exposing a single stranded sequence to S1 nuclease. S1 nuclease is an endonuclease with a 1,000 fold higher specificity for single stranded DNA which reduced the tailed genomic DNA to a blunt duplex strand with the terminal fragment(s) degraded by the combination of enzymes. Enzymatic reduction of sequential restriction fragments was found an effective, rapid and reproducible means of examining the order of terminal fragments. Furthermore, exonuclease III could reduce very large sequences of DNA at a steady rate and could be used to order restriction fragments in the entire genome. In all, restriction maps for enzymes *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, *Sma*I and *Xho*I were presented for the prototype strain Tak of species Ad41. The restriction maps for enzymes *Bam*HI, *Hin*dIII, *Pst*I and *Sma*I were reformulated (Scott-Taylor, Ahluwalia and Hammond, in press) the map for enzyme *Bgl*II, used in the preparation of specific probes, was newly devised. The amendments to the *Pst*I and *Bam*HI maps were in the placement of small fragments, difficult to cleave in the standard technique of dual enzyme digest. The enzymatic digestion of sequential

fragments with exonuclease III, not reliant on random cleavage sites, offers a solution to the tricky problem of locating small fragments. Fragments in the published versions of the *Hin*dIII and the *Sma*I map, in particular, were misplaced and have been extensively revised. Restriction maps for enzymes *Cla*I, *Eco*RI, *Sal*I and *Xho*I, with a relatively simple pattern of two, three of four restriction sites, have been published before (van Loon *et al.*, 1985). The positioning of fragments digested with these enzymes in this study concurs with previous placement to within a couple of map units.

The examination of the Ad41 genome for specific enteric adenovirus probes was carried out as before by assessing the reaction of DNA probes with dilutions of DNA preparations of each subgenus. The Ad41 *Eco*RI fragment containing plasmids were assessed first and, as could be expected from the span of the *Eco*RI A fragment from map units 8 to 61, enclosing all of the sequences found homologous with Ad2, the p41EA plasmid probe containing the *Eco*RI was the least specific. For the sake of completeness, however, the *Eco*RI A containing plasmid was subdivided and various electroeluted *Pvu*I fragments were tested in an unsuccessful attempt to locate areas of narrow species specificity between the conserved sequences. The *Pvu*I D fragment, enclosing the Ad41 hexon gene sequence, detected, like the counterpart Ad2 sequence, smaller quantities of the DNA of other species than both the parent plasmid and the whole genome as probes. There was a greater difference between the reaction with homologous and heterologous DNA preparations with the Ad41 hexon sequence containing probe than with the Ad2 HPII fragment probe, which may imply that the Ad41 hexon sequence has diversified further from species in other subgenera than has

Ad2.

Both Ad41 *Eco*RI B and C fragment containing plasmids detected Ad41 DNA with high specificity, reacting with concentrations 4 to 5 logs more dilute than non-homologous DNA, a 10,000 to 100,000 fold difference in sensitivity. The observation that the right hand portion of the Ad41 genome has little homology with Ad2 (Takiff *et al.*, 1985) or Ad5 (van Loon *et al.*, 1985) has been made before. Takiff *et al.*, (1985), investigating a cloned plasmid similar to p41EB, found that the specificity of the Ad41 *Eco*RI B containing plasmid mostly lay towards the right end in a *Bgl*II D subclone of this fragment. The specificity for the differential detection of homologous relative to heterologous DNA of the *Bgl*II D probe for Ad41 DNA was estimated at 5,000 times the level of detection by the genomic Ad2 DNA probe. This is comparable to the evaluation of the present study allowing for some variability between laboratories. The *Bgl*II D fragment in this study gave a more uniform reaction than the parent plasmid with concentrations of DNA of the other subgenera 5 log dilutions greater than the level of Ad41 DNA detected. The variability between laboratories derives from at least two parameters affecting sensitivity in hybridization are the specific activity of the probe and duration of exposure (Hyypia, 1984), that can confound comparison between studies using similar parameters and reagents. Autoradiography in this study was continued until the probe detected 1 pg of homologous genomic Ad41 DNA, equivalent to 2.5×10^4 genomes, and the difference in detection levels for homologous and heterologous DNA may be more apparent under such extended conditions. The *Bgl*II D fragment, with very low and even sensitivity for the DNA of other subgenera, and almost equal detection of

the DNA of both enteric adenovirus species, demonstrated ideal characteristics for use as a subgenus F specific probe.

Takiff *et al.*, (1985) found no increase in specificity for Ad41 DNA as opposed to Ad40 detection in subcloning the *Eco*RI B fragment. These authors shrewdly suggest that, due to the high level of homology observed between species of the same subgenus (Garon *et al.*, 1973), a strictly Ad41 specific probe may be difficult to segregate. Several Ad41 *Pvu*I fragment probes, excised from the *Eco*RI fragment containing plasmid, showed lower degree of hybridization with Ad40 than with Ad41. This was particularly observed for the *Pvu*I E fragment which reacted with Ad40 at a level equivalent to other non-homologous species. The differential sensitivity of *Pvu*I E for Ad41 DNA, however, was not optimal, detecting concentrations roughly 1,000 fold less than the DNA concentrations of other species including Ad40. Both the Ad41 *Eco*RI B and C fragment containing plasmids had moderate sensitivity to species Ad40, detecting Ad40 DNA concentrations between those of Ad41 and the other species, a reaction inappropriate for a specific or common probe. Ad40 and Ad41, however, are distinguishable by neutralization (de Jong *et al.*, 1983) indicating some divergence of hexon epitopes in subgenus F. Hybridization of Ad41 DNA with an Ad2 HPII probe showed that it was possible to isolate non-homologous, internal fragments of the hexon. The performance of hexon gene sequences isolated in *Sal*I D and *Hin*dIII I fragments in sensitivity tests did not conform to a consistent level with the DNA of other subgenera and the reaction with Ad40 DNA was neither close to or very different from that with Ad41 DNA. Examination of the hexon sequence shows that these two fragments contain elements of

the conserved P1 and P2 shells forming the body of the capsomere. Even cloning of the internal fragments of the hexon gene with restriction endonucleases with tetranucleotide recognition sequences did not eliminate P1 sequences which evidently detracted from the specificity of the electroeluted *Hin*dII fragment probe for Ad41 DNA alone.

Failing isolation of a probe specific for Ad41 DNA by cloning, synthetic probes unique to Ad41 were manufactured from sequences of the hexon gene known to code for surface epitopes responsible for the antigenic individuality of each species of adenovirus (Norrby, 1969). Two oligomeric probes were synthesised to reproduce the longest sequence of the Ad41 hexon gene with greater than 50% nucleotide diversity from the sequences of Ad40 and Ad2. The sequence coding for the L1 loop is the largest area of variability between the hexon gene sequences of different species (Toogood *et al.*, 1989). Alignment of the hexon gene sequences from Ad2, Ad40 and Ad41 demonstrated that the L1 sequence of variation was interrupted by sequences of homology important for the conformation of the loop (Toogood *et al.*, 1989) and the best sequence for use as probes was found in the sequence coding for the L2 loop. The 84 nucleotide length of sequence variation in the L2 loop was manufactured as two 40 nucleotide probe sequences, the first of which, with a more regular distribution of guanine and cytosine pairs, had the higher melting point calculated by nearest neighbour parameters (Breslauer *et al.*, 1986). These parameters are thermodynamic values for the stability of duplex DNA evaluated from the mean of free energies attributed to each successive nucleotide in a sequence according to the adjacent nucleotide and are more accurate than values simply based on the number of guanine and cytosine pairs. The higher melting temperature would have enabled the

synthetic sequence of the first 40 nucleotides to be used in conditions of greater stringency and possibly react with less false positive results with specimens at higher temperatures. However, a greater proportion of the first part of the sequence was shared with Ad40 DNA, in particular, and the reaction was less specific to Ad41 DNA in conditions close to those used for hybridization. A 40 nucleotide sequence should occur once every 4^{40} or 1.2×10^{24} base pairs, so the synthetic probes would react infrequently with foreign DNA without the need to invoke conditions of high stringency. Accordingly, the sequence of the second half of the L2 loop of the hexon gene, Hex5B, with better specificity for Ad41 DNA at lower temperatures, was used against clinical specimens in comparative tests of the accuracy and sensitivity of different methods of adenovirus detection.

Hybridization as a method compared well to more traditional means of detecting adenovirus in clinical specimens. The common probe, the HPII fragment reacted with all but two of the stool specimens considered positive by a combination of electron microscopy, tissue culture and enzyme immunoassay. No false positives were attributed to this probe. The sensitivity, when the reaction was allowed to develop for extended periods, was more than one and a half times that of other tests. The positive specimens tested by hybridization had been previously evaluated by the other diagnostic tests and were randomly allocated to the membrane with the negative specimens to make a blind study. Over 1,000 faecal specimens were examined by electron microscopy, immunoassay and culture between June 1990 and July 1991. Hybridization of over half of the positive specimens with relatively few negative specimens artificially enhanced the

sensitivity rating of the other tests. The sensitivities of electron microscopy and the adenovirus group enzyme immunoassay with the thousand specimens examined overall were evaluated at 35% and 45%, respectively, in contrast with the 55% and 62% found when the positive specimens were collected for comparison to hybridization. The true sensitivity of detection of adenovirus by the methods in current use is actually lower than found in the present study and the improvement in sensitivity possible by hybridization is greater than the results would indicate. The difference in sensitivity between the Ad41 DNA probes and other methods for detection of the enteric adenovirus isolates was not as dramatic as found with the common probe, probably because of the greater quantities of virus in enteric adenovirus specimens. The sensitivity of hybridization with the Hex5B, *Bgl*II D and even the whole genomic probe was, however, still superior to any other method, including the subgenus F specific enzyme immunoassay. Besides higher sensitivity, the hybridization method had two other distinct advantages over other methods. The use of multiple probes enabled the detection of dual infections. Additionally comparison of the intensity of specimen reaction to a dilution series of control DNA allowed an estimate of the quantity of DNA present in the sample. Dual adenovirus infections are probably not uncommon, judging from numbers of specimens that yield more than one virus upon careful culture (Brown, 1985; Brandt *et al.*, 1986; Kidd *et al.*, 1982; Wigand *et al.*, 1983) and at present none of the diagnostic methods in routine use are capable of identifying more than one isolate in a specimen.

A few of the most reactive of the enteric adenovirus specimens were defined by the control DNA dilution series as present in excess of 100 ng of viral DNA in the 150 μl

of 10% stool suspension. Although subjective evaluations of the relative concentrations of virus particles can be made from electron microscopy, no presently used method allows a quantitative definition of virus concentration. Since the Ad41 genome is comprised of 34,600 base pairs and weighs approximately 23 x 10^6 g/mole, it can be calculated using Avogadro's number that there are about 2.5 x 10^{10} molecules of the Ad41 genome per μg of DNA. In the 150 μl of stool suspension, containing 15 mg of stool and 0.1 μg of viral DNA, there are more than 2.5 x 10^9 genomes, therefore, and more than 1.7 x 10^{11} genomes or virus particles per gram of stool. While enteric adenoviruses are thought to produce a high proportion of non-infectious virions that may not have complete genomes, this calculation is in close agreement with the previous evaluation that enteric adenoviruses are usually excreted in concentrations of 10^{11} particles per gram of stool (Takiff, Straus and Garon, 1981) and demonstrates that the hybridization can define viral concentration with some reliability.

The specificity of some of the hybridization probes did not compare well to the other tests. The fact that known positives with sufficient remaining sample were used for the hybridization trial could have artificially enhanced the specificity of the other tests. However, no anomalous electron microscopy, tissue culture or enzyme immunoassay results were found in the evaluation of specimens not subjected to hybridization and 100% specificity ratings were attributed to each method overall. An attempt was made to confirm the positive subgenus F enzyme immunoassay test results of fastidious isolates, of which no DNA could be recovered for restriction analysis, by the blocking assay. While the full 50% reduction in binding, the criterion established by Jan de Jong

for positive identification, was not achieved with any of the fastidious isolates tested, fewer virions were able to attach to the capture antibody after incubation with the anti-Ad41 monoclonal antibody. Incomplete but measurable interaction with the specific antibody did occur and no contra-indications to the accuracy of reaction of the subgroup F enzyme immunoassay were found.

Only the synthetic probe gave no false positive reactions in the hybridization test. The specific detection of Ad41 with this probe ratified the initial hypothesis that probe sequences for the detection of both common and individual species would be found in the hexon gene. It would seem that the identity in DNA sequence between adenovirus species is sufficient to make it difficult to clone completely specific sequences for identification of individual species. The reaction of the synthetic probe was frequently less intense than the reaction of other Ad41 DNA probes to Ad41 containing specimens. One explanation for this could be that under optimal hybridization conditions the target DNA could become completely saturated with probe. In this case, a smaller probe sequence with less radionucleotide per copy may be limited by the quantity of target sequence available and may express less signal when a maximum amount of probe is bound. Different methods of radiolabelling used would contribute to the difference in signal. Endlabelling was found to work best with synthetic probes and the single radioisotope at the terminus of each copy of the manufactured probe is much less than the amount of radionucleotide incorporated into double stranded probes by DNA polymerase 1 reactions. The sensitivity of the synthetic probe was determined at a value below that of the *BglII* D fragment probe. However, the four specimens of enteric adenovirus with which the Hex5B did not

react all failed to grow in culture and the subgenus F virus present in all of these specimens were not identified. It is possible that some of these specimens were in fact of Ad40 which the *Bgl*II D probe, equally reactive with both enteric species, would detect. The Hex5B probe may not have reacted with some of the non-responsive specimens through specificity explicit to Ad41 and the sensitivity evaluation might be erroneously low.

The hybridization probes with poor specificity were the larger sequences with some homology to the DNA of other species of adenovirus. The genomic Ad41 DNA probe and the p41EC plasmid reacted with a number of non-enteric adenovirus containing specimens, decreasing the specificity rating of these probes. The genomic Ad41 probe, especially, correlated well with electron microscopy, detecting non-enteric adenoviruses whenever there were sufficient particles present to be evident visually. Some of the loss of specificity of some hybridization probes was very likely due to reaction with plasmid DNA in the samples, as several false positive specimens reacted only with cloned fragments. Electroelution of cloned fragments free from plasmid is advisable for probes for use in detecting viruses in stool samples, as reaction of vectors with plasmids present in alimentary flora has been seen (Huang and Deibel, 1988; Takiff *et al.*, 1985). The HPII fragment, carefully electroeluted several times through agarose, still reacted with large quantities of vector when cross-hybridized with Ad41 *Eco*RI plasmids. Many workers have similarly observed the difficulty of complete elimination of comigrating plasmid from insert probes. In the clinical specimens trials, however, no spurious reactions were attributed to the HPII probe although the *Bgl*II D fragment, cut out of the

vector and run once more through agarose, evidently still had sufficient associated plasmid DNA to detract from the specificity of the probe reactions. The experience with the HPII probe demonstrates that extensive electroelution can render a cloned probe sufficiently free of plasmid DNA contamination for specific use against clinical specimens. However, further purification from vector DNA other than a single separation through agarose is necessary for the use of probes derived from cloned DNA. This occurrence of false positives with cloned probes can be made more unlikely by employing another precaution in future; saturation of the probe with unlabelled pBR322 would interact with the plasmid DNA in the probe and prevent it from binding to the specimen.

Initially, the reaction of the HPII probe with the specimens was monitored by exposure to film for 48 hours, the same length of time as for other probes. Many of the specimens were barely visible at this time and it was necessary to prolong the duration with film to visualise the specimens with which the probe had reacted. The more rapid development of the reactions of the other probes composed of Ad41 DNA was not due to any procedural differences and probably reflects the excretion of enteric adenovirus isolates at much higher concentrations than other species (Takiff, Straus and Garon, 1981). This suggestion is endorsed by the high rate of detection of the enteric adenovirus positive specimens by electron microscopy and enzyme immunoassay. Electron microscopy is thought to have a low limit of sensitivity, failing with specimens containing less than about a million virus particles per gram of stool (Flewett, 1976). The particular value of alternative methods such as tissue culture and hybridization is in the ability to multiply

or detect those isolates present in the specimen at low levels. The 160 hours necessary for visualisation of the less responsive specimens is greater than the time generally allowed for isolates to grow in tissue culture and, although the considerable increase in sensitivity with hybridization may be worth the delay, the length of time required for accurate results by hybridization is a detracting factor.

Each of the methods evaluated, then, has advantages and disadvantages. The methods that give the more rapid results, such as electron microscopy and enzyme immunoassay, tend to detect mostly those specimens with large concentrations of virus. Electron microscopy cannot distinguish between different species of adenovirus, the facility is not widely available and is expensive to maintain. The methods that can detect low amount of virus, such as hybridization and tissue culture, are labour intensive and slow. Tissue culture, in addition, cannot deal with the high proportion of adenovirus isolates that are fastidious *in vitro* and allows the overgrowth of enteric adenovirus species in dual infection (Brown, 1985). Hybridization, until the procedure has been simplified and an alternative to radiolabelling established, is unsuitable for routine use. There are many points in the hybridization procedure where the time involved could be reduced. No work has yet been done to determine if the full length of prehybridization, for example, is necessary or could be avoided altogether with an alternative means of blocking the membrane. The largest impediment to routine adoption of hybridization is the use of radioactive probes. Chemiluminescence, a safe and attractive idea using the cloned product of luminescent bacteria, is likely to suffer the same drawbacks with the slow development of signal as radioisotopes. A better solution for a more rapid acting label may be tagging the probe

with an enzyme for a quicker chemical process of detection. Several commercial companies are emerging with non-radiolabelled nucleotide reagents that work well as substrates for DNA polymerase 1 in random prime or nick translation systems. The Boehringer Mannheim product, for example, utilises dioxygenin, a steroid that occurs in nature in the Digitalis family of plants, linked via an ester bond to the nucleotide base and detected after hybridization with a conjugated monoclonal antibody. It is apparently possible to detect 0.1 pg of homologous DNA in 50 ng of heterologous DNA with dioxygenin labelled probes (Boehringer Mannheim 1991 catalogue, page 208). The additional step with the enzyme conjugated antibody may amplify and increase the rate of development of the signal without losing any of the specificity of hybridization probes. Such relatively innocuous and stable reagents should make feasible to practise hybridization in the laboratory in safety without special precautions.

Adenoviruses were detected in a third, on average, of stool specimens from cases of acute gastroenteritis sent to the Cadham Provincial Laboratory from all points of the province of Manitoba during the last five years in which a viral agent was identified. Adenoviruses were isolated, as in other studies (Brandt *et al.*, 1983; 1985; Kidd *et al.*, 1982; Takiff *et al.*, 1981), at all times of the year without significant variation. A greater number of cases were detected in the spring and autumn months and some other studies have found an increase in isolation rate for the warmer months of the year (Kidd *et al.*, 1986; Vesikari *et al.*, 1981). This may be related to the structural integrity of adenoviruses as, unlike rotaviruses which are seasonal and tend to be isolated in the winter, adenovirus survive desiccation well. In one study infectious adenovirus could be

recovered from an external environment for up to 35 days after samples were spotted (Nauheim *et al.*, 1990), a hardiness which could enable transmission at all times of the year. Adenoviruses were detected only marginally less frequently than rotavirus and, far more common than bacterial and protozoan agents of infantile alimentary disease, rates as the second most important cause of paediatric gastroenteritis in Manitoba. This ranking of adenovirus behind only rotavirus in the aetiology of paediatric gastroenteritis is in agreement with other laboratories in the USA and Europe that have done an proportional assessment of the involvement of pathogenic agents (Brandt *et al.*, 1985; Uhnoo *et al.*, 1984; Vesikari *et al.*, 1981). The seasonal distribution of patient age upon adenovirus isolation do not take into account that the data from the Cadham Provincial Laboratory records was compiled with different methods and protocols in different years. The indications that there is little variation in isolation with the time of year and that the vast majority of adenovirus gastroenteritis patients are under two years of age are unlikely to have been affected by this variation. The oldest child from whom an adenovirus isolate was cultured was 6 years of age. Similarly, other studies have found adenoviruses to infect children up to age three, bit primarily children of two years or younger (Flewett *et al.*, 1975; Kidd *et al.*, 1982; Vesikari *et al.*, 1981) and the oldest infected child in a recent survey from Tokyo (Shinozaki *et al.*, 1991) was eight years old. Epidemic transmission of adenovirus infection is usually reported from paediatric wards (Flewett *et al.*, 1975; Chiba *et al.*, 1986; Yolken *et al.*, 1982B) and even when reported from a mixed community infection was limited to the youngest children (Richmond *et al.*, 1979). Flewett *et al.*, (1985), however, mention an adult case of adenovirus infection, a nurse in an orthopaedic ward, so the association of adenovirus

gastroenteritis is not totally exclusive to infants.

An Ad41 genomic variant first described by Hammond *et al.*, (1985) was found to predominate as the major fastidious isolate in Manitoba. Hammond *et al.*, (1987) notes that clinical isolates with a similar pattern have been designated as Ad41A by Kidd, Bavantala and de Jong (1983). These authors have included isolates with 15 different restriction enzyme patterns as a single genomic variant Ad41A according to a shared *Sma*I restriction pattern. This variant *Sma*I pattern was the single consistent feature in isolates collected from South Africa, Europe, and North America (Kidd, 1984). The establishment of a convention to name Ad41 according to *Sma*I restriction pattern would have some historical perspective in that Uhnoo *et al.*, (1983) originally separated Ad40 and Ad41 into two separate subgenera because the *Sma*I patterns have no comigrating fragments. This convention seems to have been followed since; Shinozaki *et al.*, (1988) described 11 restriction enzyme patterns in Ad41 serotypes from Japan but discussed these as four genome variants according to minor changes in the *Sma*I pattern. Buitenwerf, Louwerens and de Jong (1985) advised a rapid *Sma*I restriction analysis test for differentiation of Ad41 variants. The *Sma*I pattern does seem to be a fairly constant feature of Ad41 strains and could be very useful in distinguishing the relatedness of variant enteric isolates. Six variations in restriction pattern were described for the enzyme *Sma*I in 24 Ad41 genomic variant strains collected from all over the world (van der Avoort *et al.*, 1989), far less than the number of variations for other enzymes. Most of the *Sma*I variations were attributed to single specimens and most isolates conformed to either the Ad41A or prototype strain patterns. So far the *Sma*I pattern correlates with the

geographical variation of Ad41 and it may prove to clarify the epidemiology of this species. Despite the widespread use of restriction analysis to study the epidemiology of adenoviruses there has been no accepted practise of genomic variant description defined as yet. Many isolates of other adenovirus species with restriction pattern differences have been designated a single letter for a genome type where upon the relationship and divergence of these strains has been masked. It is agreed that a system for describing restriction pattern variation that preserves the relationship between genomic variants is needed so that the nature of changes in the prevalence and restriction patterns of strains is apparent. Adrian, Best and Wigand (1985) suggest cataloguing genome types in a code according to six common restriction enzymes. The prototype strain is designated 0 for all six enzymes and successive genomic variants attributed an increasing number for each restriction pattern that differs from previous isolates. The predominant enteric strain of Manitoba, as the first recognised Ad41 variant, could be designated strain 101010 for enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I, *Sma*I and *Xho*I.

Only the Ad41A *Sma*I pattern was found in isolates from the Newcastle area of England (Willcocks *et al.*, 1988) and Buitenwerf *et al.*, (1985) found two *Sma*I restriction fragment patterns, of the Ad41A and prototype Tak strains circulating in the Netherlands. There was an isolate with prototype strain restriction patterns described from Manitoba in the isolates from 1980 to 1983 by Hammond *et al.*, (1985). Ad41 strain Tak patterns have not been reported from countries other than Canada and the Netherlands where the strain was originally isolated in 1973 (de Jong *et al.*, 1983) and it could be that the prototype strain of Ad41 was once more widespread but now forms a small proportion

of enteric adenovirus strains in a limited distribution. A more recent characterization of strains (van der Avoort *et al.*, 1989) shows frequency of Tak strain isolates to have declined whilst a strain designated D12 that appears to equate with the restriction patterns of the Manitoban variant has risen to prominence and is now the most prevalent strain of Ad41 in the Netherlands. This study compared isolates collected from many laboratories in diverse locations but unfortunately did not define the sources of duplicate strains and it is not possible to determine the extent of distribution of the Ad41A strain. The *Sma*I pattern for Japanese isolates have been found to differ from both Ad41A and prototype Tak patterns. These results were given in diagrammatic form, without data, and the *Sma*I pattern of prototype Tak, at least, is misinterpreted. All other restriction enzyme patterns drawn for the Japanese isolates showed several variations which differed from both Tak and Ad41A patterns and it is possible that eastern Ad41 strains may well have diverged extensively. Certainly, the South African isolates of the 15 strains examined by Kidd (1984) were the most different, showing patterns for 4 of the 10 restriction enzymes used that were not matched by strains collected from Canada, Scotland, England and Germany, a phenomenon that Kidd attributed to geographical isolation and rapid transmission in crowded circumstances. The majority of the 15 strains from Europe and Canada, collected from Toronto in 1980 and 1981 (Blaskovic *et al.*, 1982) showed restriction patterns identical to those of the prototype strain Tak (Kidd, 1984). Four of the ten enzyme patterns examined, for *Kpn*I and *Eco*RI, *Bam*HI and *Sma*I, were shared by all strains and distinct from the prototype strain's patterns for these enzymes. Both the *Bam*HI and *Sma*I of the Manitoban variant strain were common to the 15 strains while the *Kpn*I and *Eco*RI patterns, identical to those of the prototype strain

Tak, differed from all 15 strains. The *Hin*dIII pattern of the Manitoban strain differed from all the patterns of the 15 strains as well as the prototype strain but was common to Dutch and Belgian isolates (van der Avoort *et al.*, 1989). The Manitoban strain, therefore, which has been the predominant enteric strain since at least 1980 and shows little variation over that time, may show restriction enzyme patterns intermediate between prototype Tak and strains prevalent in eastern Canada, Scotland, England and Germany. The greater similarity of Manitoban Ad41 restriction patterns to patterns described only from the Netherlands, Holland and Belgium and not from the intervening countries(Scott-Taylor *et al.*, 1990), is an interesting epidemiological puzzle that may unravel with more restriction pattern analysis.

The restriction patterns of the Manitoban variant strain vary from the prototype Tak strain for *Sma*I, *Bam*HI, *Cla*I, *Hin*dIII restriction enzymes. The differences are limited and result from the combination of some fragments due to absence of recognition sites in the variant DNA relative to the prototype strain and a single new restriction site in the variant genome with the enzyme *Hin*dIII. All of the mutations creating or abrogating restriction recognition sites in the variant genome occur with the hexon or fiber genes. These two genes code for capsomeres that form the major part of the external surface of the adenovirus capsid. Both of the capsomeres are neutralizable (Norrby, 1969) and thought to be involved in the process of host cell contact and invasion. The major part of the hexon sequence, coding for structural components fundamental to the construction of the entire capsid from the earliest light intermediate capsid formation (Persson *et al.*, 1979) to the completion of morphogenesis, is highly conserved. The hexon sequences

coding for external epitopes, the L1, L2 and L4 loops (Roberts *et al.*, 1986) are highly variable and responsible for the antigenic specificity of each species. The fiber structure also is composed of conserved repeat sequences that form the stalk whilst the sequence for the projecting knob is highly variable (Kidd and Erasmus, 1989). It is these epitopes, presented on the outside of the virus in repeating units, that would be exposed to the immune system of hosts and are likely to be prone to immunological selection pressure. The occurrence of all six restriction site differences between the prototype and the Manitoban variant strain of Ad41 in either of the two genes coding for external neutralizable epitopes probably reflects the selection of variant strains by an populace with antibody to surface epitopes of common strains of enteric adenovirus.

The existence of a selection pressure determining the prevalence of isolates contributing to adenoviral gastroenteritis is possibly also evident in the changes that were observed in the proportions of strains isolated in Manitoba between 1980 and 1990. No sign of the Ad40 genomic variant, observed at a much higher frequency than Ad40 strain Hovi-X on a par with Ad41 variant isolates between 1980 and 1983 (Hammond *et al.*, 1985), was seen after 1986. In analytical studies from other areas the restriction patterns of Ad40 tend to be relatively homogeneous and isolates of strain Hovi-X predominate (Kidd *et al.*, 1984; Takiff, Straus and Garon 1981; van der Avoort *et al.*, 1989). Enteric adenovirus specimens collected in Toronto in 1982 (Brown, Petric and Middleton, 1984) showed a preponderance of Ad40 isolates. Recently, however, the proportion of Ad40 among enteric isolates has diminished dramatically in the Newcastle area of England (Willcocks *et al.*, 1988), in the Netherlands (van der Avoort *et al.*, 1989) and in the Tokyo area of

Japan (Shinozaki *et al.*, 1991) and may very well be a general trend. Strain succession in ocular and respiratory infections of adenovirus have been documented Wadell *et al.*, 1985; Wadell, de Jong and Wolontis, 1981) and very probably have been witnessed in enteric adenovirus species in the decline of Ad40 prevalence in the last few years.

Initially, virtually all of the isolates of the first 6 species were neutralized by antibody reagents to the prototype strains. An increasing proportion of the isolates of Ad2, Ad3 and Ad5, in particular, have escaped neutralization in recent years and been identified by restriction analysis as belonging to these species. This suggests that selection of strains with altered surface epitopes is not limited to enteric adenoviruses but is a more general feature seen in all the more common agents of adenoviral paediatric gastroenteritis. 18% of the total isolates of 1991, almost a third of the isolates of the first 6 species, were strains of species Ad1, Ad2, Ad3 or Ad5 whose growth was not affected by incubation with antibodies to the prototype strains of those species. This increasing proportion of non-neutralized isolates indicates the evolution and increasing prevalence of forms of the virus whose surfaces no longer react with previously specific reagents and supports the notion that a selection pressure in adenoviral gastroenteritis is in effect.

Monoclonal antibodies prepared to the prototype strain for use as reagent in the first commercial immunoassay test kit to be marketed were not able to detect the Ad41 variant strain prevalent in Manitoba (Scott-Taylor *et al.*, 1990). These antibodies, devised by Herrmann *et al.*, (1987), were shown by radioimmunoprecipitation to react with the hexon. Thus, the inability of the kit to detect the variant can be ascribed to hexon gene

mutation underlying the variation in restriction endonuclease patterns. Similarly, the neutralizing activity of monoclonal antibodies produced by van der Avoort *et al.*, (1989) correlated with the presence of restriction sites at map units 52 and 56 in the hexon gene. The company producing this immunoassay test have since revised the reagents so that the kit is able to detect the majority of enteric adenovirus isolates in Manitoba. The nature of the improvement in reagents is not public information but apparently involves a cocktail of antibodies. Evidently the activity of highly specific monoclonal antibodies can fail due to variation in the external surface of variant strains and this solution of adding extra antibodies to patch defects in the activity of the test does not get at the root of the problem. The second and only other commercial immunoassay kit available (Adenoscreen, produced by Mercia Diagnostics Ltd., Guildford, UK) uses the monoclonal antibodies sent by Jan de Jong for the blocking assay. None of the local enteric adenovirus isolates incubated with these monoclonal antibodies were prevented from binding to the capture antibody by the 50% reduction established as a criterion for the identification of Ad41. The limited blocking of local enteric adenovirus isolates with these monoclonal antibodies probably reflects the partial identity of the local isolates with the prototype strain Tak and is evidence that the usefulness of this enzyme immunoassay may also be restricted in Manitoba and that it may be difficult to produce monoclonal antibodies with universal activity to Ad41 strains. Since few nucleotides were examined in the recognition sites of the restriction endonucleases, the concentration of mutations in the hexon and fiber genes, would indicate that these structures are not the ideal targets for reagent antibodies. The specificity of monoclonal antibodies, normally an asset, could be a drawback in the detection of an assortment of virus strains selected for the variations

in their outer surfaces. In this regard tests based on DNA detection, like the hybridization test developed in this study, may have advantages over monoclonal antibodies. It is evident that antigenic drift under the pressure of immunological selection is an ongoing process causing the diversification of adenovirus strains in respiratory (Wadell *et al.*, 1980B), ocular (Kemp *et al.*, 1983) and gastroenteric diseases (Scott-Taylor *et al.*, 1990). Immunologically selected point mutations have less detrimental effect on the efficacy of a DNA probe than single amino acid changes in the target epitope of an antibody so that the duration of usefulness of a DNA probe could be greatly extended in comparison to monoclonal antibody reagent to the same gene product. Additionally, sequences can be selected for hybridization probes from the genetic code of internal structures of the virion that may be less accessible to antibodies and less prone to immune selection. Further immunological selection of variant strains would promote the use of alternative methods of detection not subject to loss of activity through point mutation. It is expected from the results of the present study that DNA hybridization is a more viable long term means of detection than serological methods for the diagnosis of adenovirus in paediatric gastroenteritis.

APPENDIX

A: Dialysis Tubing

10-20 cm lengths of 23 mm diameter tubing (Spectrapor, 08-67A, Fischer Scientific) with a 8,000 mol. wt. pore size were boiled in 2% Na HCO_3/1 mM EDTA for 10 minutes,

rinsed with cold distilled water and boiled again in 1 mM EDTA. A plastic screen was placed over the tubing to keep the tubing submerged at all times. The boiled tubing was cooled and stored at 4°C. Tubing was handled with gloves and rinsed with distilled water prior to use.

B: Preparation of Phenol

Liquid phenol (A88I-1, Fischer Scientific) was distilled at 160°C in a thermostatically controlled heating mantle behind a fumehood. Aqueous impurities were evaporated first and after two to three hours of boiling the distillation of pure phenol, that crystallized on contact with collection bottles was initiated. 25 ml aliquots of phenol were collected and wrapped in tin foil and stored at -20°C. Stored phenol was melted in a water bath at 68°C and saturated with buffers for use within one month. 8 Hydroxyquinoline to a concentration of 0.1% was added and the phenol was shaken with an equal volume of 1 M Tris/HCl pH 8.0. The phases were allowed to separate and the extraction was repeated several times until the pH was greater than 7. The extraction was then repeated with 0.1 M Tris pH 8.0/1% mercaptoethanol and this solution left over the phenol. This preparation was stored in the dark at 4°C until use. Yellow or unfinished batches of saturated phenol older than one month were discarded.

C: Restriction Enzyme Buffers

(i) Low Salt Buffer:

10 mM Tris/HCl pH 7.6

10 mM $MgCl_2$

1 mM dithiothreitol

(ii) Medium Salt Buffer:

50 mM NaCl

10 mM Tris/HCl pH 7.6

10 mM $MgCl_2$

(iii) High Salt Buffer:

10 mM NaCl

50 mM Tris/HCl pH 7.6

10 mM $MgCl_2$

1 mM dithiothreitol

(iv) *Sma*I Buffer:

20 mM KCl

10 mM Tris/HCl pH 7.6

10 mM $MgCl_2$

1 mM dithiothreitol

D: Tracking Dye:

0.07% bromophenol blue

0.7% sodium dodecyl sulphate

33% glycerol

The solution was stored at 37°C and used at a dilution of one volume in three. The formula was taken from Meyers *et al.*, (1976).

E: Molecular Weight Standards in base pairs.

(i) λ phage DNA + *Hin*dIII:

Fragment Migration Order	Fragment Size in base pairs
A	23,130
B	9,416
C	6,557
D	4,361
E	2,322
F	2,027
G	564
H	125

(ii) λ phage DNA + *Hin*dIII and *Eco*RI:

Fragment Order	Size in base pairs
A	21,226
B	5,148
C	4,973
D	4,277
E	3,530
F	2,027

G	1,904
H	1,584
I	1,330
J	983
K	831
L	564
M	125

F: pBR322 Molecular Weight Standards

(i) pBR322 DNA + *Hin*fI:

Fragment Order	Size in base pairs
A	1,631
B	517
C	506
D	396
E	344
F	298
G	221
H	220
I	154
J	75

G: Ligation Buffer; 10x concentrate:

0.5 M Tris/HCl pH 7.4
0.1 M $MgCl_2$
0.1 M dithiothreitol

10 mM spermine

10 mM adenosine triphosphate

1 mg/ml bovine serum albumen

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