DEMONSTRATION OF NEW SUBTYPES OF ADENOVIRUS 7 IN SOUTH AFRICA, AND PROBING OESOPHAGEAL CARCINOMA CELL LINES FOR EVIDENCE OF ADENOVIRUS OR OF OTHER ONCOGENIC VIRUSES

by

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JUNE 1987

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OESOPHAGEAL CARCINOMA - SEARCH FOR A POSSIBLE VIRAL AETIOLOGY

ACKNOWLEDGEMENTS

I wish to thank

Professor K.R. Dumbell, Dr. M.J. Renan and Dr. L.M. Steyn, for their guidance and valuable criticism in the supervision of this project.

Professor A.A. Forder, Head of the Department of Medical Microbiology, for making available the facilities of his Department for this study.

My colleague, Mrs. Jenny Kannemeyer, for her assistance and expert tuition in tissue culture.

Mrs. L. van Schalkwyk and Dr. L.M. Stannard for help with the electron microscopy.

Mrs. Peggy McDowell, for assistance with the photography.

Mrs. Joan Parsons, for her hard work in typing this thesis.
and, my friends and parents for their encouragement.

Special thanks to the Council for Scientific and Industrial Research for financial support.

ABSTRACT

This study was carried out in 2 parts:

1. Genome analysis of human adenovirus species 7;
2. Search for a possible viral aetiology in oesophageal carcinoma.

Sixteen laboratory isolates of adenovirus species 7, isolated in South Africa between 1975 and 1986, were characterized by restriction endonuclease analysis of their DNA genomes. Virus was propagated in human embryo fibroblast cells; genomic DNA, extracted and purified from cellular DNA extracts, was analyzed using 9 different restriction enzymes. Results of this study have demonstrated 2 new genome types of adenovirus 7c which have not previously been identified.

The 2 novel strains, designated as genome types Ad7c1 and Ad7c2, were shown to differ from prototype Ad7c according to restriction profiles with EcoRI; 2 new EcoRI sites were demonstrated in Ad7c1 and 1 in Ad7c2. The restriction sites were mapped on the viral genomes (at 3.68kb and 5.32kb from the left terminus) by double enzyme digestions, cloning of viral DNA, and nucleic acid hybridization using a cloned Ad7 probe.

Strains resembling the prototype Ad7c and Ad7p (Gomen) genome types were also identified in the 1985 and 1986 Ad7 isolates.

In order to investigate the possible role of a viral co-factor in the aetiology of oesophageal carcinoma, 18 probes, derived from potentially oncogenic viruses, were used to screen 3 human oesophageal carcinoma cell lines for the possible presence of integrated viral DNA. One of these, an Ad7 recombinant plasmid probe, was developed by cloning DNA from the transforming region of the Ad7c1 strain into the plasmid vector pUC19.

Cellular DNA, extracted from the 3 tumor lines HCU18, HCU33 and HCU39, was tested by means of both DNA dot hybridization and Southern blot hybridization for the presence of Epstein-Barr virus, human papillomavirus (types 1, 5, 6, 8, 11, 16, 18), human adenovirus (strains 5, 7, 12, 31) and human T-lymphotropic virus type I DNA. Both assays were demonstrated to be sensitive enough to detect 1 copy of viral DNA per cell. No hybridization between HPV, EBV, HTLV-I or adenovirus DNA probes, and the cellular DNA was detected. These findings indicate that the stable integration of these tumor viruses in host chromosomes did not play a role in the maintenance of the malignant phenotype of the 3 extensively passaged cell lines.

Cells of the 3 oesophageal tumor lines were further examined by transmission electron microscopy, but the presence of virus particles in these cells was not observed.

Parts of this thesis have been accepted for publication:

1. South African Medical Journal (Supplement), 71(6), 6-8, 1987.
2. Journal of Medical Virology, 1987 (in press).

ABBREVIATIONS

AD	Adenovirus
AIDS	Acquired immunodeficiency syndrome
ATV	Activated trypsin versene
BL	Burkitt's lymphoma
bp	Base pairs
BPV	Bovine papillomavirus
CIF	Cellular interfering factor
CIP	Calf intestinal alkaline phosphatase
CPE	Cytopathic effect
cm	Centimetre
dATP	Deoxy-adenosine triphosphate
dCTP	Deoxy-cytidine triphosphate
dGTP	Deoxy-guanosine triphosphate
DMSO	Dimethylsulphoxide
dpm	Disintegrations per minute
dTTP	Deoxy-thymidine triphosphate
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetra-acetic acid
EV	Epidermodysplasia verruciformis
H&E	Haematoxylin and eosin
HF	Human fibroblast
HPV	Human papillomavirus
HTLV	Human T-lymphotropic virus
kb	Kilobases
kV	Kilovolts
LB	Luria broth
LCL	Lymphoblastoid cell line
lmt	Low melting temperature
M	Molar
MEM	Minimum essential medium
mg	Milligram
mins	Minutes

ml	Millilitre
mM	Millimolar
mm	Millimetre
mu	Map units
mw	Molecular weight
NIV	National Institute of Virology
NIH	National Institute of Health
NPC	Nasopharyngeal carcinoma
PBS	Phosphate buffered saline
pg	Picogram
RE	Restriction endonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
T_m	Melting temperature of duplex DNA
Tris	Tris (hydroxymethyl) amino methane
UV	Ultra violet
v/v	Volume in volume
w/v	Weight in volume
µCi	Microcurie
µg	Microgram
µl	Microliter

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PART I

TWO NEW GENOME TYPES OF HUMAN ADENOVIRUS SPECIES 7

1. INTRODUCTION

The human adenoviruses, initially discovered by Rowe and colleagues in 1953 (Rowe *et al.*, 1953) are medium-sized viruses, containing linear double-stranded DNA genomes, 33-45 kilobase pairs (kb) in length. In 1962 it was discovered that certain species (formerly serotypes; Wigand *et al.*, 1982) are oncogenic when injected into newborn hamsters (Huebner *et al.*, 1967). Since then, these viruses have been used extensively as a model for studies on tumor induction *in vivo* and cell transformation *in vitro*.

1.1 CLASSIFICATION OF ADENOVIRUSES

Up to now 41 human adenovirus species have been recognized. These are divided into 7 subgenera (Table 1). Huebner (1967) originally classified the human adenoviruses into 3 subgenera on the basis of their oncogenicity in newborn hamsters. Subgenus A is highly oncogenic, inducing tumors with high frequency and after a short latency period; subgenus B is weakly oncogenic, causing tumors in a fraction of the injected animals after long latency periods; and subgenus C is nononcogenic. Subgenus C was later further subdivided into subgenera C, D and E on the basis of differences in the antigenicity of the T antigen, and GC content of the genome (McAllister *et al.*, 1969; Wadell *et al.*, 1980). All members of subgenera A-E are also capable of morphologically transforming rat or hamster cells *in vitro*. (The difference between the *in vivo* and *in vitro* properties is thought to be due to the different immunological responses elicited by the various subgenera). In addition, Wadell *et al.* (1980) have analyzed the internal polypeptides of the 41 human adenovirus species, and extended the number of subgenera to a total of 7. Through genome analysis, they have also shown that the number of SmaI restriction fragments of the adenovirus genomes was characteristic for each subgenus, and, with an exception for subgenus A (due to heterogeneity of their genomes) different species belonging to the same subgenus displayed several comigrating restriction fragments.

The antigenic determinants that define the human adenovirus species, localized on the viral hexons and fibers, are measured by neutralization and hemagglutination-inhibition tests,

respectively. Hexon and fiber antigens are coded for by only a minor fraction of the viral genome. Therefore these traditional serological techniques cannot accurately be used for studying relationships between different viruses, since the information derived would be representative of a few gene products only. This point is illustrated by the fact that Ad12, Ad18 and Ad31 cross-react serologically (Hierholzer et al., 1975; Wigand and Keller, 1978), while their viral genomes only exhibit 48-69% DNA homology (Green et al., 1979). Furthermore, within the group of human adenoviruses, a wide genetic variation is expressed, and serological intermediates are frequently encountered (Wadell et al., 1980).

TABLE 1 : Properties of Human Adenovirus Subgenera A to G

Subgenus	Species	DNA			Hemagglutination Patterns	Oncogenicity in Newborn Hamsters
		Homology[a] (%)	G+C (%)	Nr.of SmaI Fragments		
A	12,18,31	48-69 (8-20)	48	4-5	IV	High
B	3,7,11,14 16,21,34 35	89-94 (9-20)	51	8-10	I	Weak
C	1, 2, 5, 6	99-100 (10-16)	58	10-12	III	Nil
D	8,9,10,13,15 17, 19, 20 22 - 30 32, 33 36 - 39	94-99 (4-17)	58	14-18	II	Nil
E	4	(4-23)	58	16-19	III	Nil
F	40	n.d.[b]	n.d.	9	IV	Nil
G	41	n.d.	n.d.	11-12	IV	Nil

a: Percent homology within subgenus. Figures in brackets: homology with members of other subgenera.
b: n.d., not done

Adapted from data cited by Wadell (1984).
(Subdivision according to polypeptide patterns, not shown).

1.1.1 The Importance of Genome Analysis

The recent advent of restriction endonuclease analysis of adenovirus strains has proved a sensitive technique for the classification of these viruses, and is second only to nucleotide sequence analysis.

The original distinction of the accepted subgenera, has now been confirmed by restriction endonuclease analysis (Wadell et al., 1980) and furthermore, species distinctions within subgenera, based on neutralization tests, have also been reinforced. However, differences among strains of the same species cannot be detected by the traditional serological techniques (namely, hemagglutination inhibition or neutralization tests). In this regard DNA restriction analysis provides a powerful tool whereby distinct viral entities, designated genome types (or genotypes), can be defined. Different genome types within a species have been associated with different degrees of virulence and pathogenicity, and have also been found to vary in prevalence in human populations at different times (Wadell et al., 1981). Thus analysis of genome types is of clinical and epidemiological importance. It has further uses in:

1. the study of relationships between viruses at the genome level;

2. characterization of noncultivable viruses, and

3. tracing infection chains.

1.1.2 Classification of Adenovirus Species 7 Genome Types

Adenovirus type 7 (Ad7) is the adenovirus species that has been most frequently associated with severe disease. Li and Wadell (1986) have recently published an analysis of 40 selected strains of Ad7 from around the world, using 12 different restriction endonucleases. They have identified 15 different genome types, designated Ad7p, Ad7p1, Ad7a, Ad7a1 to Ad7a5, Ad7b, Ad7c, Ad7d, Ad7d1, Ad7e, Ad7f and Ad7g. This adds eight new types to the seven which had previously been identified by using restriction endonucleases BamHI and SmaI, namely Ad7p (Berge et al., 1955), the

prototype strain Gomen of Ad7, Ad7a (Rowe et al., 1958), the vaccine strain, Ad7b, Ad7c, Ad7d, Ad7e, and Ad7f (Wadell et al., 1981; Wadell et al., 1985; Wadell and Varsanyi, 1978; Wadell et al., 1980). They found that only four restriction endonucleases, BamHI, BclI, BglI and XbaI need to be used to distinguish all 15 Ad7 genome types.

Bailey and Richmond (1986) have since added a further three to this group, namely Ad7b1, Ad7b2 and Ad7b3. These variants of genome type Ad7b were identified after analysis of 44 strains isolated during an epidemic in England at the end of 1983. The variants occurred at approximately similar frequencies throughout the epidemic and were associated with similar clinical illnesses.

In essence, this classification of Ad7 is based on designating the prototype strain as p and variants on BamHI digestion patterns, where restriction fragment length polymorphism is limited to less than 3% of the fragment, being designated as a group with an alphabetic letter as a name (a, b, c, etc.). Through the use of the computer program "COMAP", initially described by Adrian and Heinrich (1986), which facilitates the pairwise analyses of co-migrating DNA restriction fragments, restriction fragment length polymorphism variants can be identified. Variants within individual groups - distinguished by use of additional restriction endonucleases, are designated by arabic numerals (1, 2, 3, etc.), in the order in which they are discovered.

1.2 EPIDEMIOLOGY OF ADENOVIRUS SPECIES 7 INFECTIONS

Restriction endonuclease analysis has proved to be invaluable in studies of the molecular epidemiology of adenoviruses. Wadell *et al*. (1985) have recently analyzed the distribution of the different Ad7 genome types among 314 isolates from patients and healthy shedders from around the world. The majority of isolates (90%) were represented by the Ad7b and Ad7c genome types, and these appeared to be mutually exclusive. In Europe Ad7c was prevalent from 1958 to 1969, while Ad7b has been prevalent since 1969. A similar shift from Ad7c to Ad7b occurred in Australia, but this was not detected until 1975. Ad7b genome types have predominated in Australia, Europe and North America during the last decade, but the reason for this shift from Ad7c to Ad7b as causative agents in Ad7 respiratory outbreaks remains unknown. Among 22 Ad7 strains isolated in Johannesburg (South Africa) during the period of 1967 and 1976, one isolated in April 1967 was genome typed as Ad7b, while the rest were identified as Ad7c.

Thus Africa, and Brazil and Japan (where Ad7e and Ad7p were detected, respectively) represent the only regions where Ad7b has not been detected in recent years.

1.3 PATHOGENICITY OF ADENOVIRUS SPECIES 7 INFECTIONS

Adenoviruses have been reported to be the causative agents in approximately 2% of all acute respiratory infections in non-hospitalised children, and in 4%-25% of infections in children hospitalised with such symptoms (van der Veen, 1963). Although Ad7 is also known to produce mild upper respiratory or gastrointestinal illness, it is, in most cases, associated with epidemic outbreaks of lower respiratory tract disease and relatively high mortality (Straube et al., 1983). Genome types 7b and 7c appear to be responsible for the most severe cases of Ad7 related disease, and for the community outbreaks that have been reported.

Although there have been few examples of nosocomial outbreaks, Straube et al. (1983) have described such an epidemic that occurred in a San Diego childrens' hospital in September 1980 causing the death of 4 out of 6 patients infected with adenovirus type 7b , and have demonstrated that infections with Ad7 can cause serious and potentially fatal disease in hospitalised patients and staff, even during times of minimal Ad7 activity in the larger community. Fee et al. (1983), who reported a similar outbreak in a Pennsylvania paediatric hospital, have suggested that more effective infection-control measures should be taken in order to prevent such infections.

The fact that supervening viral pneumonia - caused by adenovirus or herpesvirus infections - can follow in the wake of measles, is also well recognized. This is especially important in South Africa where measles is known to be an important cause of morbidity and mortality among poor children. Kaschula et al. (1983), who carried out a study of this phenomenon in the Red Cross Childrens' Hospital, Cape Town, have emphasised the importance of these supervening secondary infections as contributing factors to such morbidity and mortality.

1.4 AIM OF THIS WORK

In May 1986 a small outbreak of pneumonia occurred in a respiratory intensive care ward of the Red Cross Childrens' War Memorial Hospital, Cape Town. Virus was isolated from respiratory secretions from 4 children in our routine diagnostic virology laboratories and all were typed as adenovirus species 7 by routine serological techniques. Two of the 4 children had post measles pneumonia while the others had underlying respiratory problems, and appeared to acquire the adenovirus pneumonia in hospital. Two of the 4 children died: one with post measles pneumonia, and one other.

The purpose of this study was to type the viral isolates by restriction endonuclease analysis, and then to compare them both to other recent isolates of Ad7 from the local community, and to a similar group isolated in the Johannesburg area. A third aim was to develop a recombinant Ad7 probe, using DNA from the transforming region of one of the strains. This probe was then used as part of a survey which screened DNA from three human oesophageal carcinoma cell lines for the possible presence of integrated DNA tumor viruses (Part II of thesis).

2. MATERIALS AND METHODS

2.1 ORIGIN OF REFERENCE STRAINS AND PATIENT ISOLATES

Reference strains of adenovirus 7 were obtained as follows: Ad7a from National Institute of Health (NIH), Bethesda, USA; Ad7p, Ad7b, Ad7c, from Dr. G. Wadell, University of Umeå, Sweden.

Strains 2314, 2332, 2256, 2203 and 2182 were all patient isolates obtained in our routine diagnostic virology laboratories. The first four represent a group isolated in May 1986, from respiratory secretions of 4 children in a small outbreak of pneumonia in a respiratory intensive care ward of the Red Cross Childrens' War Memorial Hospital, Cape Town. Strain 2182 is a single lung isolate, initially isolated in 1975, and stored at $-20^{o}C$, from a young child who died of post measles pneumonia (from the same hospital). The 1985/1986 group of 11 isolates - Nrs. 2154, 2168, 2156, 2153, 1980, 1727, 2084, 274, 548, 702, and 1004 were obtained from Dr. A. Kidd at the National Institute of Virology (NIV) in Johannesburg.

2.2 INITIAL IDENTIFICATION OF VIRUS STRAINS

A detailed description of tissue culture media and methods used is given in Appendix I and II, respectively. All viral isolates were typed by standard methods, in our routine diagnostic virology laboratories, prior to restriction endonuclease analysis: Specimens were inoculated onto confluent monolayers of HeLa cells grown on 8 x 22 mm^2 glass coverslips, which were held in airtight, sterile, roller tubes (Kimax), and grown under rotation in a $37^{o}C$ incubator. The virus was allowed to grow for 1-2 days (at $37^{o}C$) after which the coverslips were removed, and stained with haematoxylin and eosin, according to standard procedure (Humason, 1972), such that a typical adenoviral cytopathic effect (CPE) could be observed. Species identification, as an adenovirus type-7, was routinely accomplished by neutralization with hyperimmune serum (NIH). Human embryo fibroblasts, derived from lung or whole embryo explant cultures in our laboratory, and grown in sterile plastic tubes (Falcon) under rotation at $37^{o}C$, were similarly infected with viral isolates and observed for signs of a typical

adenoviral CPE. When extensive cytopathic change was evident the growth medium of certain of these cultures was removed, and stored at -20°C, to serve as a stock of infectious virus for subsequent viral propagation and DNA extraction.

2.3 VIRUS PROPAGATION AND DNA EXTRACTION

Viral isolates, and reference strains, were propagated in monolayers of human embryo fibroblast (HF) cells. Harvests of 5-10ml of infected cell medium (stored at -20°C) were thawed and used to inoculate semi-confluent HF cell sheets grown in 75 cm^2 tissue culture flasks (Appendix II). After removal of the growth medium, the stock virus suspension was added and spread evenly over the surface of the cell culture. The culture flask was then incubated (in a horizontal position) for 1 hour at 37°C, to facilitate adsorption of the virus to the cells. Following this time period, the cell culture medium was made up to 50ml by the addition of 40-45ml of growth medium - Eagle's minimum essential medium, supplemented with 10% foetal calf serum (Appendix I). The cells were maintained in a standard CO_2 incubator at 37°C, and observed daily for evidence of typical cytopathic changes. When more than 75% of cells showed the characteristic CPE, usually within 4-6 days, DNA was extracted by a modification of the "Hirt" procedure (Eizuru *et al.*, 1984).

After removal of the medium the cells were washed twice with phosphate buffered saline (pH 7.0), and then lysed gently for 20 mins at 37°C, with a lysing solution (4ml per flask) consisting of 0.01M Tris (pH 8.0), 0.6% SDS. and 0.01M EDTA (pH8.0). Sodium chloride was added to a final concentration of 1M, and the mixture was kept at 4°C overnight to facilitate precipitation of high molecular weight cellular DNA. The "Hirt supernatant" (containing predominantly viral DNA) was then separated by centrifugation at 10 000 rpm for 60 mins (Sorvall RC-5 centrifuge) at 4°C. It was subsequently digested with proteinase K (Boehringer Mannheim) at 500µg/ml for 1 hour at 56°C, and then extracted twice with Tris-equilibrated phenol,and once with chloroform (chloroform : isoamylalcohol, 24:1). The aqueous and organic phases were separated, after each extraction, by centrifugation at 3 000 rpm

for 10 mins. Viral DNA was precipitated with 2.5 volumes of absolute ethanol and 300mM sodium acetate (NaAc) at -20°C overnight (or alternatively at -70°C for 10 mins), and pelleted at 10000 rpm for 60 mins. The precipitated DNA was then dissolved in 500µl of RNase buffer (0.1M NaCl, 0.01M Tris, pH8.0) and treated with 20µg of RNase A (Sigma) by incubation at 37°C for 60 mins. The RNase had been pre-heated to remove any DNase activity (Maniatis et al., 1982; pg 451). The digestion was terminated by extraction once with phenol, and once with chloroform, and viral DNA again precipitated by the addition of 2.5 volumes of absolute ethanol and 300mM NaAc, at -20°C overnight. After centrifugation at 12 000 rpm for 15 mins, the viral DNA pellet was washed with cold 70% ethanol, vacuum dried, and redissolved in 100µl of sterile distilled water. It could then be stored at 4°C until used for restriction endonuclease analysis.

2.4 RESTRICTION ENDONUCLEASE ANALYSIS

The concentrations of extracted viral DNA were determined by comparison with known concentrations of λ-phage DNA following agarose gel electrophoresis and ethidium bromide staining of the DNA: Electrophoresis was carried out in 1% agarose gels in TAE buffer (40mM acetate, 2mM EDTA, 40mM Tris, pH 7.6) at 80V for approximately 30 mins. The gels were stained with 500µg/ml ethidium bromide and photographed with ultra violet transillumination (300nm) using a Mamiya camera and Polaroid Land pack 667 film. The intensity of the UV light induced fluorescence of the viral DNA was compared with that of the λDNA standards.

Restriction endonuclease digestion was carried out according to the directions of the manufacturers (Amersham or Boehringer Mannheim) Aliquots containing 2µg of viral DNA in a total volume of 20µl were digested with 2 to 4 units of different restriction endonucleases (RE) and 2µl of the appropriate enzyme buffer. All reactions were carried out for 2 hours at 37°C. To identify the location of new RE recognition sites, double RE digests, with the enzymes SalI and EcoRI, were carried out in selected cases. In such instances the high salt buffer, specified by the manufacturer for use with EcoRI, was utilised and the reactions carried out in parallel. Digestions were terminated by the addition of 2µl of

STOP buffer (50% v/v glycerol, 100mM EDTA, 1% w/v SDS, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol) and subsequent heating at 65°C for 10 mins. The mixtures were then applied to horizontal 1.0% agarose gels, and the fragments separated by electrophoresis in TAE buffer at a constant voltage of 2V/cm for 16-18 hours. After staining the gels in a solution of 500ng/ml ethidium bromide in TAE buffer, for 15-30 mins, the bands could be visualized by UV transillumination. Patterns were recorded on Ilford FP4 photographic film.

In certain cases restriction enzyme profiles of Ad7 strains were transferred, after separation in 1% agarose gels, onto Hybond-N nylon membranes (Amersham) by using a modification of the method described by Southern (1975). In this event a ruler was placed next to the molecular weight (mw) markers, before photographing the gel. The reciprocal of the mobility of the mw markers was plotted against their known fragment sizes (in kilobases). This enabled the sizes of specific viral DNA fragments to be read from the graph after autoradiography (Southern, 1975).

Viral DNA was denatured and neutralized in situ prior to transfer: Initially the gel was washed twice for 5 mins in 0.25M HCl, to depurinate the DNA at random. The DNA was then denatured in 0.5M NaOH and 1M NaCl; the gel was washed twice in 300ml of the denaturing buffer for 20 mins each, rinsed in distilled water, and neutralized by two washes in 0.5M Tris (pH 7.5) and 3M NaCl.

2.5 TRANSFER OF DNA TO NYLON MEMBRANES

A sheet of Hybond-N nylon membrane (Amersham) was cut to the exact size of the gel. Two sheets of Whatman 3MM filter paper were also cut to the same size, and two additional sheets cut to approximately 40cm x 40cm. The two large filters were saturated in 20X SSC (3M NaCl, 0.3M Tri-sodium citrate, pH7.4) and placed on a piece of Saran wrap on a flat bench surface. Care was taken at all stages to prevent the trapping of bubbles between the different layers of gel, membrane and filter paper. If any were trapped they were removed by rolling a glass rod over the membrane/filters to force the bubble out. The pre-treated gel was then slid onto the centre of the filters and air-bubbles removed. The uncovered

filter paper was covered with Saran wrap in order to ensure diffusion of the 20X SSC through the gel and to prevent buffer evaporation during the transfer. It is not necessary to pre-soak the Hybond-N nylon membrane as it is hydrophilic and wets directly on contact with the gel. After lowering it carefully onto the surface of the gel, it was covered with the two filters of the same size, the first of which had been pre-soaked in 20X SSC. A stack of tissues were folded to the exact size of the gel, and placed on top of the filter papers. A glass plate and weight were placed on top of these and transfer was then allowed to proceed for 16-24 hours at room temperature. Saturated tissues were replaced with dry ones when necessary.

After transfer of the DNA, the positions of the wells in the gel were marked on the overlying nylon membrane. The membrane was then removed, rinsed in 1X SSC, and air-dried. The DNA was fixed onto the membrane by placing it, DNA-side downwards, on an ultraviolet transilluminator and exposing it to UV-illumination (300nm) for 15-20 mins. In this way DNA becomes covalently linked to the Hybond-N nylon membrane surface.

2.6 CLONING OF ADENOVIRUS SPECIES 7 EcoRI D FRAGMENT

2.6.1 Digestion of vector and viral DNA

One microgram of DNA from Adenovirus species 7 (strain 2203) and from the plasmid vector, pUC19, was digested with EcoRI according to standard procedures. An aliquot (one tenth volume) of each reaction volume was run on a 1% agarose minigel in order to check that the digestion had been complete. Reactions were terminated by a phenol, and subsequent chloroform extraction. The DNA was then precipitated with 2 volumes of ice-cold absolute ethanol in the presence of 300mM NaAc. After centrifugation (at 12 000 rpm for 10 mins) the resultant DNA pellets were washed with 70% ethanol and dried under vacuum. Vector and viral DNA were re-dissolved in 20µl and 10µl, respectively, of sterile distilled water. The two samples were then stored on ice prior to ligation.

2.6.4 Bacterial transformation and selection of recombinants

Competent E.coli JM107 cells were prepared by calcium chloride pretreatment (Maniatis et.al.,1982; pg 250). Cells were stored (resuspended in 50mM $CaCl_2$ and 10mM Tris, pH 8.0) at 4°C for 12-24 hours prior to transformation.

Ligation mixes (10µl) were added to 200µl aliquots of pretreated JM107 cells, mixed, and stored on ice for 30 mins. A 20ng aliquot of undigested pUC19 DNA was similarly added to 200µl of E.coli JM107 cells, to serve as a transformation control. The mixtures were then heated at 42°C for 2 mins, after which 1ml of Luria broth (LB medium; Maniatis et al., 1982, pg.68) was added to each and incubation continued at 37°C for 1 hour. The transformed JM107 cells were plated on Luria agar plates (Maniatis et al., 1982; pg.70) containing 50µl of X-GAL (2% w/v 5-bromo-4-chloro-3-indolyl-β-galactoside, in dimethylformamide), 20µl of IPTG (100mM Isopropropyl-β-D-thio-galactopyranoside, in distilled water) and 50µg/ml ampicillin per 20ml agar. Plates were incubated at 37°C for 16 hours. Blue colonies on control plates were then counted; 0-3 colonies on the CIP control, and 200-250 colonies on the ligation control, indicated efficient dephosphorylation and ligation reactions. The transformation efficiency was calculated from the number of colonies on the transformation control; an average efficiency of 0.6×10^6 transformants per µg of DNA was achieved in this, and subsequent transformation reactions (refer to part II, Section 2.3.1). White colonies, containing potential recombinant plasmids, were selected from experimental plates. Intact pUC19 plasmid encodes an IPTG inducible β-galactosidase enzyme which cleaves X-GAL to give a blue reaction product. However, if the β-galactosidase gene is mutated by inserted DNA, no enzyme is produced and thus colonies containing recombinant plasmids are white. Potential recombinants were screened by inoculation of 10ml of LB medium containing 50µg/ml ampicillin per millilitre. These cultures were incubated overnight at 37°C in a rotary incubator. Plasmid DNA was then extracted by a rapid "miniprep" method (Birnboium and Doly, 1979), and analyzed by restriction enzyme digestion, and subsequent electrophoresis in 1% agarose.

2.7 LONGTERM STORAGE OF SELECTED RECOMBINANT PLASMID IN 15% GLYCEROL

After selecting the required Ad7 recombinant plasmid a glycerol stock of the transformed JM107 cells was made for long-term storage at -70°C. An 850µl volume of E.coli JM107 cells, taken from a 10ml overnight culture, was mixed with 150µl of sterile glycerol, rapidly frozen on dry ice, and then stored at -70°C. Viable bacteria could be recovered by further inoculation of 10µl of the thawed suspension into 10ml of LB medium, containing 50µg/ml ampicillin.

2.8 PLASMID PREPARATION AND RADIOISOTOPE LABELLING OF VIRAL DNA INSERT

2.8.1 Amplification and isolation of plasmid DNA

E.coli JM107 carrying the required Ad7 recombinant plasmid was inoculated from the stock culture into 10ml LB medium, containing 50 µg/ml ampicillin. After overnight growth, at 37°C, 100µl of this starter culture was inoculated into 25ml of LB medium containing ampicillin (50µg/ml). Growth at 37°C was allowed to proceed, with constant rotation, until the OD_{600} of the medium was between 0.6 and 1.0 units. At this stage the bacterial cells were in the late logarithmic phase of growth. This late log culture (25ml) was then inoculated into 500ml of LB medium, containing ampicillin (50 µg/ml), and incubation at 37°C continued for a further 2.5 hours. At this point chloramphenicol (Sigma) was added to a final concentration of 170µg/ml. and the subsequent amplification allowed to proceed, with vigorous shaking, for 12-16 hours.

The bacterial cells were harvested by centrifugation at 6 000 rpm for 10 mins at 4°C, washed in 100 ml of ice-cold STE (0.1M NaCl, 10mM Tris [pH7.8], 1 mM EDTA), and then lysed according to the "alkaline lysis procedure" described by Maniatis et al. (1982; pg.90-91). This method is highly efficient for the isolation of small plasmids, less than 10 kilobases in length.

After dissolving the final DNA pellet in 8ml of TE buffer (pH8.0) plasmid DNA was further purified from contaminating chromosomal DNA by centrifugation to equilibrium in cesium chloride (CsCl), ethidium bromide density gradients. Cesium chloride (Boehringer Mannheim) at a concentration of 1g/ml, was added to the DNA solution, dissolved, and then 0.8ml of a solution of ethidium bromide (10mg/ml in H_2O) added for every 10ml of CsCl solution. The final density of the solution should be 1.55g/ml (refractive index, 1.386). The refractive index of the solution was determined by using a refractometer (SCIEX), and adjusted to a reading of 1.386-1.390 by the addition of solid CsCl. The CsCl solution was then centrifuged at 50 000 rpm, for 20 hours, in a Beckman ultracentrifuge at 20°C (V Ti 65 rotor). The lower plasmid band was collected, and extracted 3-4 times with isoamylalcohol (Merck) in order to remove the ethidium bromide. The aqueous phase was dialyzed for 20-24 hours, at 4°C against four changes of TE (pH8.0). After one phenol extraction and a subsequent extraction with chloroform, plasmid DNA was precipitated by the addition of 2 volumes of absolute ethanol in the presence of 300mM NaAc. The precipitate was recovered by centrifugation, at 12 000 rpm for 10 mins, washed in a small volume of 70% ethanol and dried under vacuum. The DNA was redissolved in 0.2-0.4ml TE buffer (pH8.0), and the final DNA concentration was calculated by determining the absorbance at 260 nm: DNA concentration = (OD_{260} x 50 x dilution)µg/ml.

2.8.2 Isolation of viral DNA from the recombinant bacterial plasmid

The cloned viral DNA insert was excised from the recombinant bacterial plasmid prior to radioisotope labelling, so as to avoid any spurious hybridization of vector DNA sequences. The method used was a modification of that described by Dretzen *et al.* (1981).

The recombinant plasmid was digested with *Eco*RI, and the viral DNA insert was separated from the vector DNA by agarose gel electrophoresis (in 0.8% agarose in TAE buffer [pH8.0]). To isolate the viral DNA, DEAE-cellulose paper (Whatman), presoaked in

electrophoresis buffer, was placed in a slit in the gel immediately in front of the DNA band. Electrophoresis was then continued at 100V for 10-15 mins until the viral DNA had migrated onto the DEAE paper. As the gel contained ethidium bromide (1µg/ml) the migration of the DNA band could be monitored intermittently by UV illumination (300nm). DEAE paper was then removed, cut into a fine pulp with a razor blade and suspended in 300µl of elution buffer (20mM Tris [pH7.5], 1mM EDTA, 1.5 M NaCl). After incubation for two hours at 37°C, the sample was mixed on a vortex mixer and centrifuged at 12 000 rpm for 10 mins. The supernatant was collected and the paper pellet was mixed with a further 200µl of elution buffer. This suspension was centrifuged (12 000 rpm for 10 mins) and the resultant supernatant pooled with the former to make up the final volume of 500µl.

After extraction with an equal volume of phenol, and subsequent chloroform extraction, the DNA was precipitated with two volumes of absolute ethanol and 300mM NaAc at -20°C overnight. Insert DNA was then pelleted by centrifugation (12 000 rpm for 10 mins),washed with 70% ethanol, dried under vacuum and redissolved in 10 µl of TE buffer (pH8.0). The concentration of the purified viral DNA insert was determined by comparison with known concentrations of λ DNA following agarose gel electrophoresis and ethidium bromide staining of the DNA, as described in Section 2.4.

2.8.3 Radioisotope labelling of the excised viral DNA insert

Radioisotope labelling of viral DNA was carried out according to standard procedures, as specified by the manufacturer (Amersham) of the "Nick-Translation Kit" which was used. Insert DNA (0.5-1 µg) was incubated for 90 mins at 15°C in the presence of 100µCi α^{32}P-dCTP, of specific activity 400Ci/mmol (Amersham), and 20 µM/l dATP, dGTP and dTTP in a concentrated nick translation buffer solution. To this solution 5 units of DNA polymerase I and 100pg DNase I was added, and the final reaction volume made up to 100µl with sterile distilled water. The nick-translation reaction was terminated by loading the mixture directly onto a 4cm x 1cm column of sephadex G-50 suspended in a buffer containing 150mM NaCl, 10mM EDTA, 0.1% SDS and 50mM Tris(pH7.5). A 10µl volume of 10X STOP buffer was added to the sample prior to loading in order

to monitor the progress of the sample through the column. Once the dye front had reached the halfway mark, sixteen three-drop fractions were collected. Fractions containing radiolabelled DNA were detected with a Geiger-counter (mini-monitor type 5.10), and pooled. The radioactivity in a one hundredth volume aliquot of the pooled fractions was used for the accurate quantitation of the radioactivity by Cerenkov counting, and the specific activity (in dpm/µg) of the labelled DNA calculated. Specific activities of (0.5 - 1) x 10^8 dpm/µg were obtained. The radiolabelled DNA was denatured by heating to 100°C for 5 mins, followed by rapid cooling on ice, in order to prevent re-annealing of the DNA strands.

2.9 HYBRIDIZATION OF ^{32}P-LABELLED PROBE TO DNA BOUND TO HYBOND-N NYLON MEMBRANES

Hybridization was carried out according to the procedure of Johnson *et al.* (1984). The Hybond-N nylon membranes were prehybridized with a 10ml solution containing 0.25% w/v nonfat dry milk in 900mM NaCl, 90mM Na citrate (0.05X BLOTTO, 6x SSC) at 42°C for 4 hours. Radiolabelled, single-stranded probe DNA was then added to a further 10ml volume containing 0.05X BLOTTO, 6X SSC and 50% formamide. Membranes were transferred to this solution and hybridization was allowed to continue for 16-18 hours, at 42°C. Both the prehybridization and the hybridization reactions were carried out in plastic bags which were heat-sealed; care was taken to ensure that all air-bubbles had been removed. Membranes were then washed to remove any unbound and non-specifically bound probe. Initial washes were twice, for 30 mins each, in 0.05X BLOTTO, 2X SSC and 0.1% SDS, at room temperature. Two final high stringency washes were at 55°C in 0.1X SSC and 0.1% SDS (also for 30 mins each). Filters were then air-dried, covered with Saran wrap, and exposed to X-ray film (Kodak X-Omat MA) with intensifying screens. Autoradiography was allowed to proceed for 16-24 hours, at -70°C, prior to photographic development.

3 RESULTS

3.1 VIRUS PROPAGATION AND NUCLEIC ACID EXTRACTION

3.1.1 Initial Identification of Laboratory Isolates of Ad7

All Ad7 virus isolates studied were routinely typed by standard neutralization tests (either in our routine diagnostic virology laboratories, or in the laboratory from which they were sent) prior to restriction endonuclease analysis. While these results classified them as adenovirus species 7 (subgenus B) isolates, precise information on their specific genome types remained unknown.

The Ad7 isolates were also shown to exhibit a typical adenovirus cytopathic effect when grown in monolayers of HeLa cells, or of human embryo fibroblast cells. This was observed after 1-2 days of growth at 37°C: HeLa cells, stained with haematoxylin and eosin (H&E), showed inclusions typical of adenovirus (Figure 1).

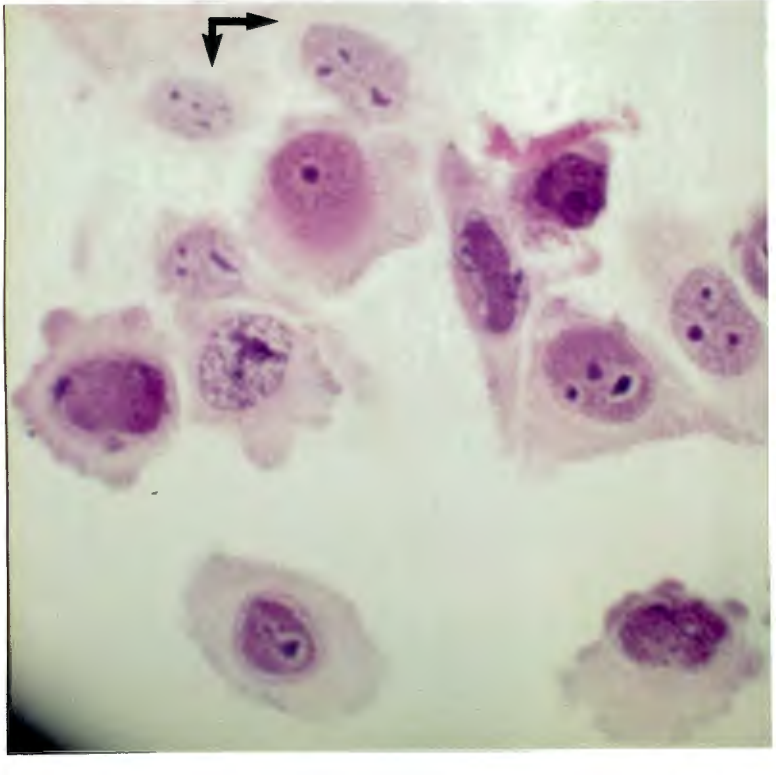


FIGURE 1 :

Cytopathic effect of adenovirus in HeLa cells. The typical basophilic intranuclear inclusions are displayed in cells showing various stages of infection. Uninfected cells are indicated. Magnification : 240X. Stain: H&E.

3.1.2 DNA Extraction

Virus was grown in human embryo fibroblast cells in sterile 75cm^2 tissue culture flasks, and was extracted when 75% of the cells exhibited a typical CPE - usually after 5-6 days. Optimum nucleic acid yields were achieved when semi-confluent, as opposed to confluent, cell sheets were inoculated with virus. At this stage the cells are presumably more permeable, and easily accessible to the virus than when they are densely packed in a confluent cell sheet. An average yield of 50 µg of viral DNA* per 75cm^2 tissue culture flask was achieved, and this figure was 1.5 to 2 fold higher than that obtained when confluent cell sheets were infected.

3.2 RESTRICTION ENDONUCLEASE ANALYSIS

3.2.1 Introduction

Li and Wadell (1986) have shown that the restriction enzyme BamHI can distinguish the 10 major genome types of Ad7 that have so far been identified (i.e. Ad7p, Ad7p1, Ad7a, Ad7a1, Ad7b, Ad7c, Ad7d, Ad7e, Ad7f, Ad7g). Five of the 8 additional subtypes (Ad7d1, Ad7a2-5) can be distinguished with the enzymes XbaI, BclI and BglI. Three further subtypes of Ad7b (Ad7b1, Ad7b2, Ad7b3), recently identified by Bailey and Richmond (1986), can be distinguished by digestion with PvuII, BglI and SstII.

In order to determine the genome types of 4 viral isolates (2314, 2203, 2256, 2332), from 4 children in a small outbreak of pneumonia at the Red Cross Childrens' Hospital (Cape Town), restriction enzyme profiles of the 4 isolates were compared, firstly to one another, using 9 different enzymes, and secondly to those of 4 Ad7 reference strains (Ad7p, Ad7a, Ad7b, Ad7c) using the enzymes BamHI, SmaI and EcoRI. These 3 enzymes were used by Wadell *et al.* (1981) in the initial identification and discrimination of the 4 above reference strains. One further local 1975 Ad7 isolate was similarly typed by genome analysis, for comparative purposes.

3.2.2 Analysis of "1986 May" Ad7 Isolates (2314, 2203, 2256, 2332)

DNA extracted from the 4 Ad7 isolates, 2314, 2203, 2256 and 2332, designated "1986 May" isolates, was analyzed with nine different restriction enzymes, namely BamHI, SmaI, EcoRI, XbaI, HpaI, HindIII, BglI, BglII and SalI. Resultant profiles for the first 3 of these enzymes are shown in Figures 2A,B and C respectively. Reference strain Ad7a DNA was similarly digested, and run as a control in lane 5 of each gel. All 4 "1986 May" isolates were found to give identical patterns when digested with each of these 9 restriction enzymes. Thus it would appear that the outbreak of respiratory infection, associated with these isolates, was caused by 1 particular virulent strain of adenovirus species 7. In addition, by comparison with published Ad7 restriction maps (Li, 1986), patterns derived with each of these restriction enzymes, excluding EcoRI, appeared identical to those reported for Ad7c.

3.2.3 Analysis of Ad7 Reference Strains, and One 1975 Isolate

In order to determine the particular Ad7 genome type represented by the "1986 May" isolates, DNA from reference strains Ad7p, Ad7a, Ad7b and Ad7c was digested with BamHI, SmaI and EcoRI, and their resultant restriction enzyme profiles, as shown in Figure 3 A, B and C, respectively, compared with those of the "1986 May" isolates (lane 6 of Figure 3A, B and C). Wadell et al. (1981) have described the use of these 3 enzymes in the initial identification of Ad7c. This genome type can be distinguished from the other 3 above reference strains by cleavage either with BamHI, or with EcoRI and SmaI. Patterns obtained with EcoRI are identical for Ad7c and Ad7b, and those obtained with SmaI identical for Ad7a and Ad7c.

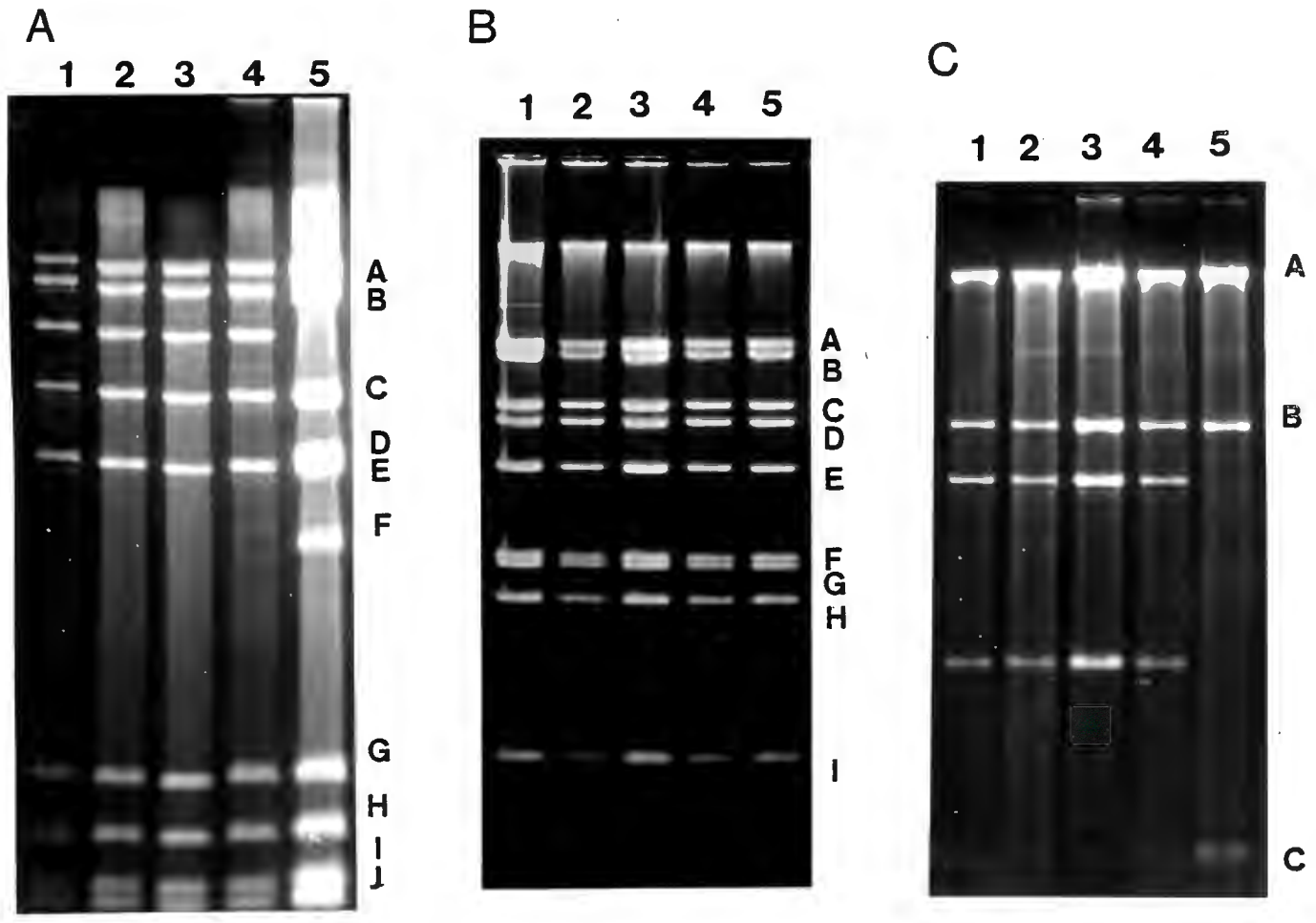


FIGURE 2

DNA restriction patterns after digestion of DNA from adenovirus "1986 May" isolates with (A) BamHI, (B) SmaI and (C) EcoRI. Two micrograms of DNA from each of the following isolates, were digested and separated as described in the text:

Lane 1 : Ad7 - 2314
Lane 2 : Ad7 - 2203
Lane 3 : Ad7 - 2256
Lane 4 : Ad7 - 2332
Lane 5 : Adenovirus 7a reference strain
RE fragments are indicated

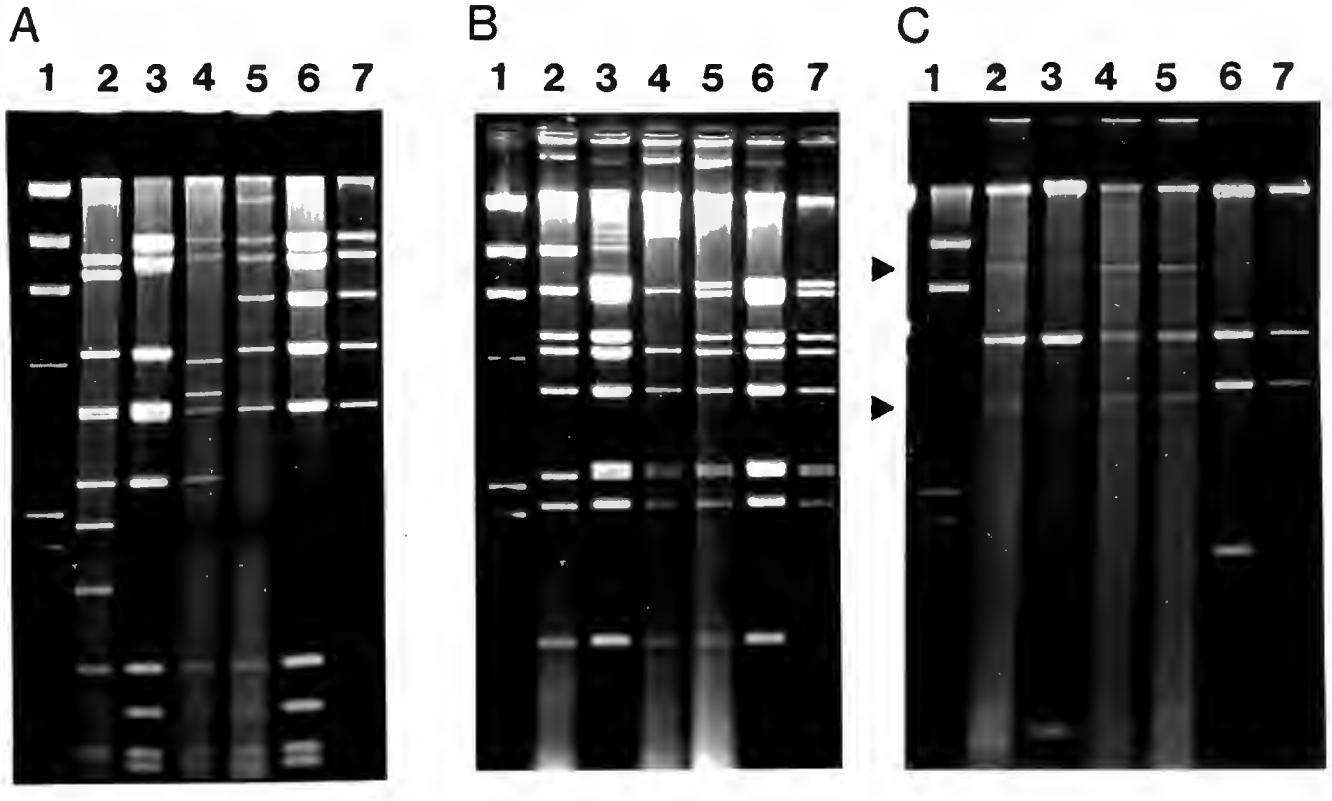


FIGURE 3

DNA restriction patterns after digestion of DNA from adenovirus reference strains, and laboratory isolates, with (A) BamHI, (B) SmaI and (C) EcoRI :

Lane 1 : Lambda (λ) phage DNA cleaved with HindIII, as molecular weight reference.
Lane 2 : Ad7p
Lane 3 : Ad7a
Lane 4 : Ad7b
Lane 5 : Ad7c
Lane 6 : Ad7-2203 (1986, May)
Lane 7 : Ad7-2182 (1975)
Cellular (HF) bands are indicated in Figure 3C.
(λ HindIII restriction fragments (kb), in order of descending size: 23.13; 9.42; 6.68; 4.36; 2.32; 2.03; 0.56)

One 1975 adenovirus species 7 isolate - Nr.2182 - was similarly analyzed, and is represented in lane 7 of each gel. This specimen was originally isolated from a patient in the same ward at the Red Cross Hospital as the "1986 May" group, and was included for comparative purposes, since a similar 1975 isolate, from this ward, was typed in 1983 by Dr. G. Wadell (University of Umeå, Sweden) as an Ad7c genotype (Wadell et al., 1983).

The patterns derived for all Ad7 reference strains tested (Ad7p, Ad7a, Ad7b, Ad7c) correspond exactly to maps published for these strains, with the enzymes BamHI, SmaI, and EcoRI, as is illustrated in Figure 4.

It is clear from Figures 3A (BamHI) and 3B (SmaI) that both the "1986 May" (lanes 6) and the 1975 (lanes 7) isolates yield the same pattern as reference Ad7c (lanes 5) for both enzymes (as well as to reference Ad7a (lanes 3) for SmaI). Similarly, when RE profiles of the "1986 May" isolates, digested with the enzymes SalI, XbaI, HpaI, HindIII, BglI and BglII, were compared with published maps of Ad7c for these enzymes (Li, 1986) patterns were found to be identical in all cases (results not shown). The 1975 Ad7 isolate also appeared identical to reference Ad7c by patterns of SalI, BglI and BglII (results not shown). However, after digestion with EcoRI (Figure 3C) the "1986 May" and 1975 isolates (lanes 6 and 7, respectively) produced patterns that were different both to one another and to those of all Ad7 reference strains shown (lanes 2-5). When compared to the reference Ad7c, in addition to EcoRI fragments A and B of this strain, the 1975 isolate has 1 extra EcoRI fragment, while the "1986 May" isolate has 2 further fragments: 1 similar in size to that of the 1975 isolate, and 1 smaller fragment. Since these novel EcoRI restriction fragments appear in both Ad7 strains, together with a conserved EcoRI B fragment, and a largely intact EcoRI A fragment, it follows that 2 extra EcoRI restriction sites, in the case of the "1986 May" isolate, and 1 extra site for the 1975 isolate, must exist in close proximity either to the left hand end, or to the right hand end of the EcoRI A fragment.

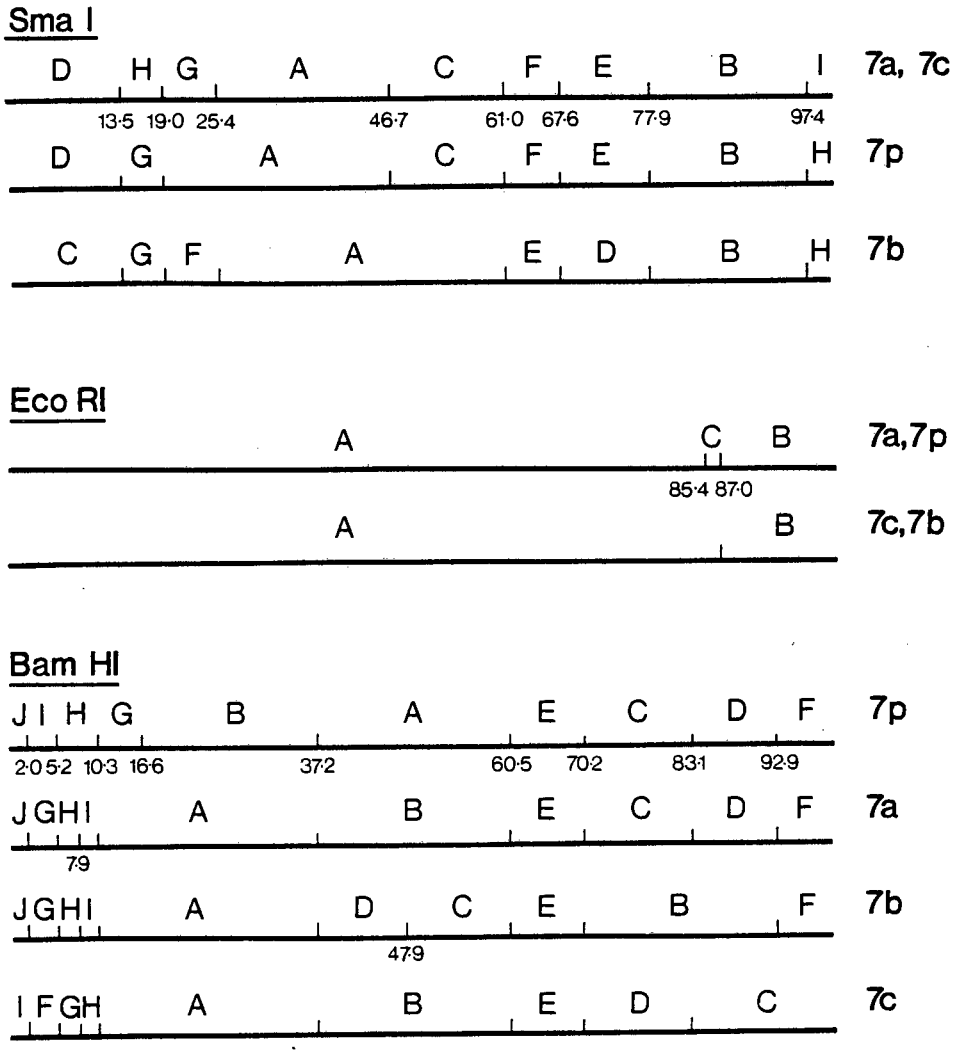


FIGURE 4 :

Restriction site maps (BamHI, SmaI, EcoRI) of the genomes of Ad7p, Ad7a, Ad7b and Ad7c, given in map units (1 mu = 0.36 kb) - Adapted from Wadell et al., 1981.

3.2.4 Mapping of EcoRI Restriction Sites by Double Digestion with EcoRI and SalI

In order to map the positions of the new EcoRI cutting sites in the genomes of "1986 May" and 1975 isolates of adenovirus species 7, 2µg of viral DNA from each of isolates 2203 ("1986 May") and 2182 (1975) was double-digested with EcoRI and SalI. The resultant cleavage products were analyzed by electrophoresis in a 1% agarose gel (Figure 5). A SalI/EcoRI digest of Ad7c DNA (2µg) was similarly analyzed for comparison (lane 2).

Both new strains of adenovirus species 7 - the 1975 and "1986 May" isolates - were found to exhibit the same total size of genome (~36 kb), and novel cutting by EcoRI only in SalI fragment C, at the left hand end of the published restriction map for Ad7c (refer to Figure 8). The "1986 May" isolate (lane 4) showed 3 new fragments (1.64 kb, 3.68 kb and 1.01 kb) when compared to the Ad7c control (lane 2), clearly derived from the 6.34 kb Sal C fragment. One EcoRI cutting site must be fixed 3 map units (1.01 kb) to the left of the published SalI site at 17.5 map units (6.33 kb) which would leave 2 possibilities for the second additional EcoRI site: 1.64kb or 3.68kb further to the left of this established restriction site.

Similarly, 2 new fragments of 3.68 kb and 2.65 kb, corresponding to the Ad7c 6.34 kb Sal C fragment, were evident in the 1975 isolate (lane 3). This established the position of the new EcoRI restriction site in this strain : 3.68 kb from the left hand end of the SalI fragment C.

In order to determine the lengths of unknown Ad7 restriction fragments, λ phage DNA (2µg) was digested with the restriction enzyme PstI, and the resultant characteristic fragments separated by electrophoresis in parallel with the Ad7 digests (Figure 5, lane 1). PstI, in contrast to HindIII, generates several distinct λ DNA fragments, in the range of 0.45 - 4.51kb. Thus relatively small restriction fragments can be more accurately sized by using this enzyme. Fragment lengths of Ad7-2203 and Ad7-2182 DNA, determined after single and double digestions with EcoRI and SalI, are indicated in Table 2.

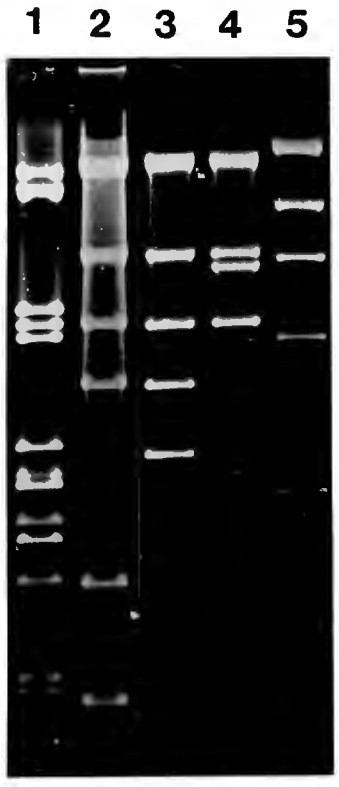


FIGURE 5

Mapping of EcoRI restriction sites in the genomes of "1986 May" and 1975 Ad7 isolates. The figure shows electrophoresis of fragments obtained after double digestions with EcoRI and Sa1I:

Lane 1 : λ PstI digest.
Lane 2 : Ad7-2203 (1986, May)
Lane 3 : Ad7-2182 (1975)
Lane 4 : Ad7c (reference strain)
Lane 5 : λ HindIII digest

(λ PstI restriction fragments (kb), in order of descending size: 11.49; 5.08; 4.75; 4.51; 2.84; 2.56; 2.46; 2.44; 2.14; 1.99; 1.70; 1.16; 1.09; 0.81; 0.51; 0.47; 0.45)

TABLE 2 : Ad7 Restriction Fragments (in kb) Generated by Digestion with Sa1I and EcoRI

	EcoRI	Sa1I	EcoRI/Sa1I
I : Ad7c[a] (reference strain)	A : 31.49 B : 4.71	A : 18.14 B : 11.73 C : 6.34	18.14 7.02 6.34 4.71
II : Ad7-2203	A : 26.17[b] B : 4.71 C : 3.68 D : 1.64	A : 18.14 B : 11.73 C : 6.34	18.00 7.02 4.71 3.68 1.64 1.01
III : Ad7-2182	A : 27.80[b] B : 4.71 C : 3.68	A : 18.14 B : 11.73 C : 6.34	18.00 7.02 4.71 3.68 2.65

a : Ad7c EcoRI and Sa1I fragment lengths adapted from Wadell et al (1981), and Li and Wadell (1986).
b : EcoRI A fragment sizes calculated on the basis of a 36.2 kb Ad7 genome.

3.2.5 Conclusion

Four "1986 May" isolates of adenovirus species 7 (from a small hospital outbreak of pneumonia) examined by genome analysis with 9 different enzymes, were shown to be identical to one another, and to the reference Ad7c strain, for all enzymes excluding EcoRI. A 1975 Ad7 isolate, similarly examined, also differed from reference Ad7c only on EcoRI digestion. While this isolate had 1 additional EcoRI site, the "1986 May" isolates had 2 further EcoRI cutting sites, located at the left hand end of the viral genome in SalI fragment C. Thus, according to the classification system of Li and Wadell (1986), which divides different adenovirus species 7 genome types into separate groups on the basis of their BamHI restriction patterns, these results have identified two new variants of adenovirus genome type 7c, which are named "Ad7c1" and "Ad7c2". One isolate, from the "1986 May" group (Nr 2203), was recently sent to Dr. G. Wadell (University of Umeå, Sweden) for identification, and was typed as "Ad7c1" (1986, personal communication). This nomenclature is thus adopted for this strain, and "Ad7c2" for the second novel genotype, isolated in 1975.

3.3. MAPPING OF ADDITIONAL EcoRI RESTRICTION SITES BY DNA HYBRIDIZATION

3.3.1 Introduction

By double digestion with the restriction enzymes EcoRI and SalI, it was shown that 1 of the additional EcoRI sites of Ad7c1 ("1986 May" isolates) could be located on the viral genome in 1 of 2 possible positions (Section 3.2.4). In order to confirm this location, the smaller of the two additional EcoRI restriction fragments (EcoRI D) from Ad7c1 (Nr 2203) was cloned into the plasmid vector pUC19, and further used as a radiolabelled probe for hybridization to particular restriction enzyme profiles (SmaI and EcoRI) of Ad7 DNA immobilised on Hybond - N nylon membranes Cloning of this fragment enabled it to be amplified at high efficiency in the form of recombinant plasmid DNA. It also served to eliminate possible contamination from remaining viral sequences (or from HF DNA) prior to hybridization analysis.

3.3.2 Cloning of Ad7c1 EcoRI D Fragment

Two new EcoRI restriction fragments, EcoRI C and D, were demonstrated in Ad7c1. Of these, one was located at the extreme left hand end of the viral genome and one alongside this and to the left of EcoRI fragment A. However, the orientation of these restriction fragments with respect to one another remained unknown. The cloning strategy adopted was based on the assumption that restriction fragments at the extreme ends of the viral genome, and thus bordered by only one restriction enzyme site would not be cloned. This shotgun approach involved ligation by combining total viral DNA (Ad7c1 cleaved with EcoRI) with single stranded vector DNA (pUC19 cleaved with the same enzyme) assuming that a more centrally located Ad7c1 EcoRI fragment would be selectively cloned (Section 2.6). One would not expect to clone EcoRI fragment A of Ad7c1 as this is 26 kb in length, and thus too large for the plasmid vector of choice, namely pUC19. This vector can only be used efficiently for cloning relatively small DNA inserts, less than 10 kb in length, but would be suitable for a putative 3.68 kb or 1.64 kb Ad7c1 viral insert.

From 2 experimental ligation reactions, using 100ng of pUC19 DNA, and an equimolar ratio of insert and vector DNA, 20 white colonies containing potential recombinant plasmids were derived. Twelve of these colonies, randomly selected, and screened using a rapid "miniprep" method of plasmid preparation (Birnboium and Doly, 1979) and subsequent EcoRI digestion, were all found to harbour an identical recombinant plasmid - pUC19 (2.6 kb) containing a 1.64 kb Ad7c1 viral insert, cloned at the EcoRI restriction site. One of these 12 was again selected and the recombinant plasmid, named pAd7c1EcoD, was amplified on a large scale by growth in E.coli JM107, and purified in a CsCl density gradient.

The selective cloning of this 1.64 kb Eco D fragment indicates that the linear order of the EcoRI fragments on the Ad7c1 genome, should be CDAB. This was confirmed by subsequent hybridization analysis (Section 3.3.3).

3.3.3 Hybridization Using the Cloned Ad7c1 EcoRI D Fragment

The cloned Ad7c1 EcoRI D fragment (1.64 kb) excised by EcoRI cleavage from the purified recombinant plasmid pAd7c1EcoD (4.24 kb) is indicated in Figure 6A. The viral DNA insert was purified from vector DNA sequences by digestion of the purified plasmid DNA (4-5 µg) with EcoRI, and subsequent preparative electrophoresis in a 0.8% agarose gel. Recovered insert DNA (1-2 µg) could then be radiolabelled by nick-translation in the presence of α^{32}P dCTP (to a specific activity of 1×10^8 dpm/µg DNA). EcoRI restriction enzyme profiles of the 3 Ad7 genotypes (Figure 6B), Ad7c (reference strain), Ad7c1 and Ad7c2, immobilised on a hybond-N nylon membrane, were probed with this radiolabelled EcoRI D fragment (Figure 6C). Hybridization to the large EcoRI A fragment is clearly shown in lane 3, for reference Ad7c, and also in lane 1, for Ad7c2, but only to EcoRI D in lane 2 for control Ad7c1. This verifies the location of the novel EcoRI restriction site in Ad7c2, at 10.2 map units, or 3.68 kb from the left hand end of the viral genome.

In order to confirm the predicted EcoRI restriction map for Ad7c1 (Section 3.3.2), a SmaI restriction enzyme profile of Ad7c1 DNA, transferred onto Hybond-N nylon membrane, was probed with a similar radiolabelled Ad7c1 EcoRI D probe (Figure 7A). This identified 2 SmaI restriction fragments, D and H, which must overlap with EcoRI fragment D. As the leftmost SmaI restriction site of Ad7c1, (separating fragments D and H) is located at 13.5 map units, this indicates that the 2 EcoRI restriction sites, bordering the cloned 1.64 kb fragment, must be located on either side of this map position: at 14.7 map units, and 1.64 kb to the left of this site at 10.2 map units (Figure 7B).

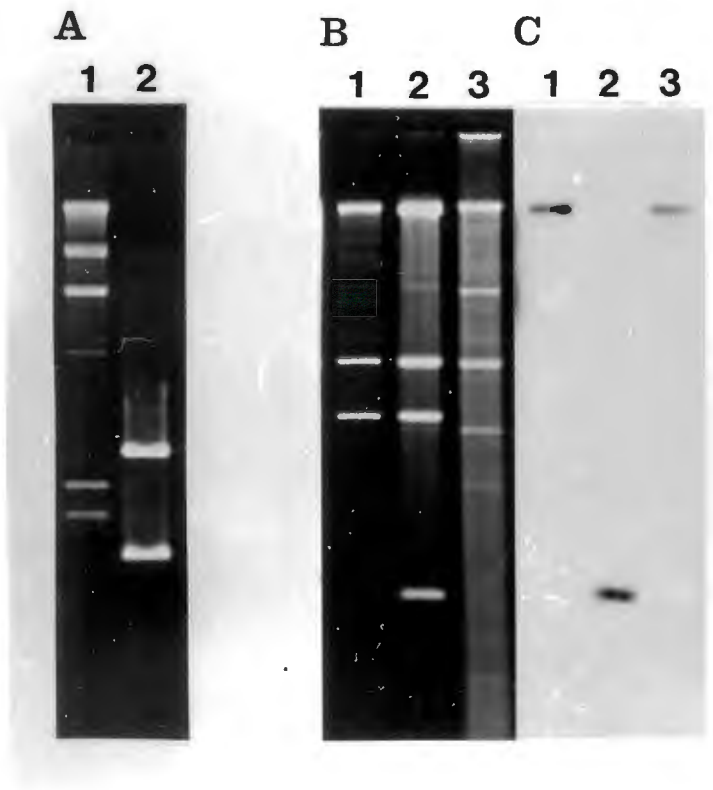


FIGURE 6

Hybridization of Ad7c1 Eco D fragment to Ad7 DNA, digested with EcoRI. The cloned Eco D fragment (1.64 kb), released from pUC19 vector DNA (2.6 kb) by cleavage with EcoRI, is indicated in Figure 6A, lane 2. (λ DNA digested with HindIII is shown in lane 1, as a molecular weight reference).

Figure 6B shows EcoRI restriction enzyme profiles of Ad7 strains analyzed by agarose gel electrophoresis; an autoradiograph of this gel, probed with the radiolabelled EcoRI fragment D, is illustrated in Figure 6C.

Lane 1 : Ad7c2 (Nr.2182)
Lane 2 : Ad7c1 (Nr.2203)
Lane 3 : Ad7c (reference strain)

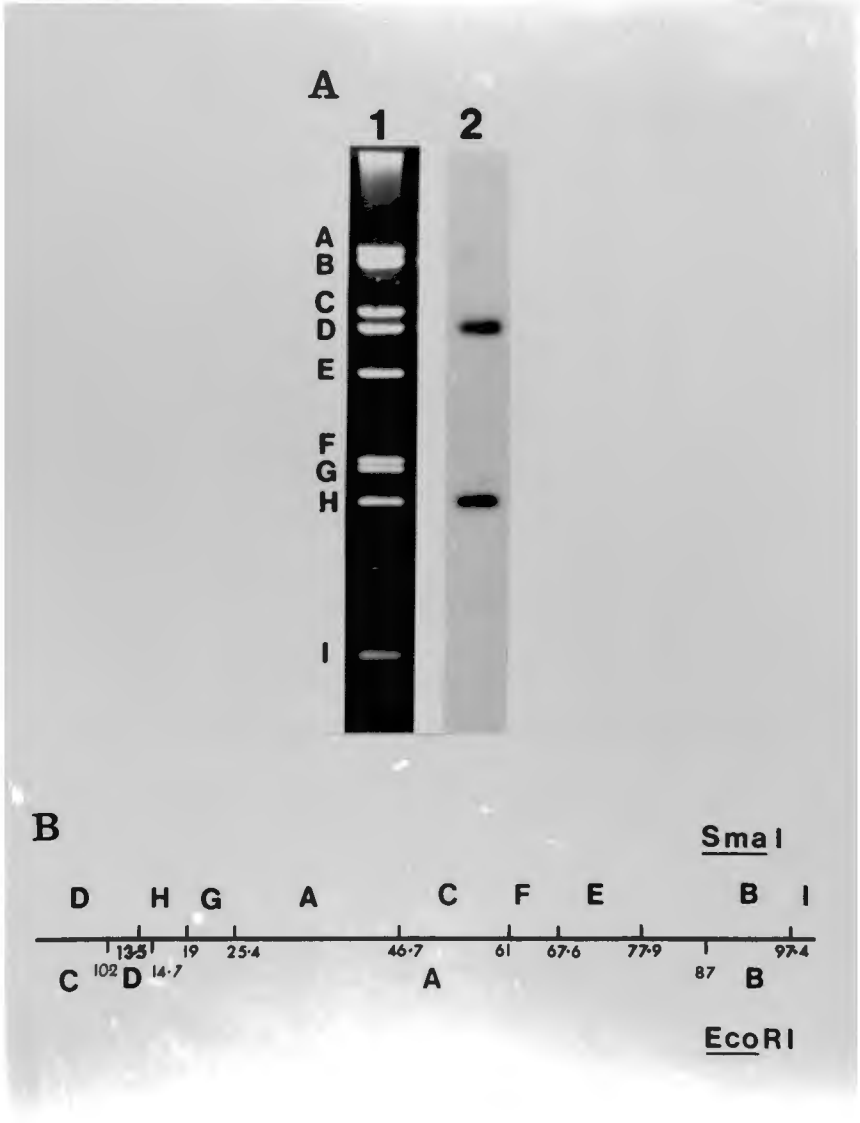


FIGURE 7

Hybridization of Ad7c1 Eco D fragment to Ad7c1 DNA, digested with SmaI. Figure 7A shows a SmaI profile of Ad7c1 DNA, analyzed by agarose gel electrophoresis (lane 1), and by hybridization to a radiolabelled Eco D probe (lane 2). SmaI and EcoRI restriction site maps of the Ad7c1 genome (in map units) are indicated in Figure 7B.

3.3.4 Conclusion

In conclusion, the positions of new EcoRI restriction sites in the genomes of 2 new variants of adenovirus genome type 7c, designated Ad7c1 and Ad7c2, have been determined. Resultant EcoRI (and SalI) restriction maps are illustrated in Figure 8.

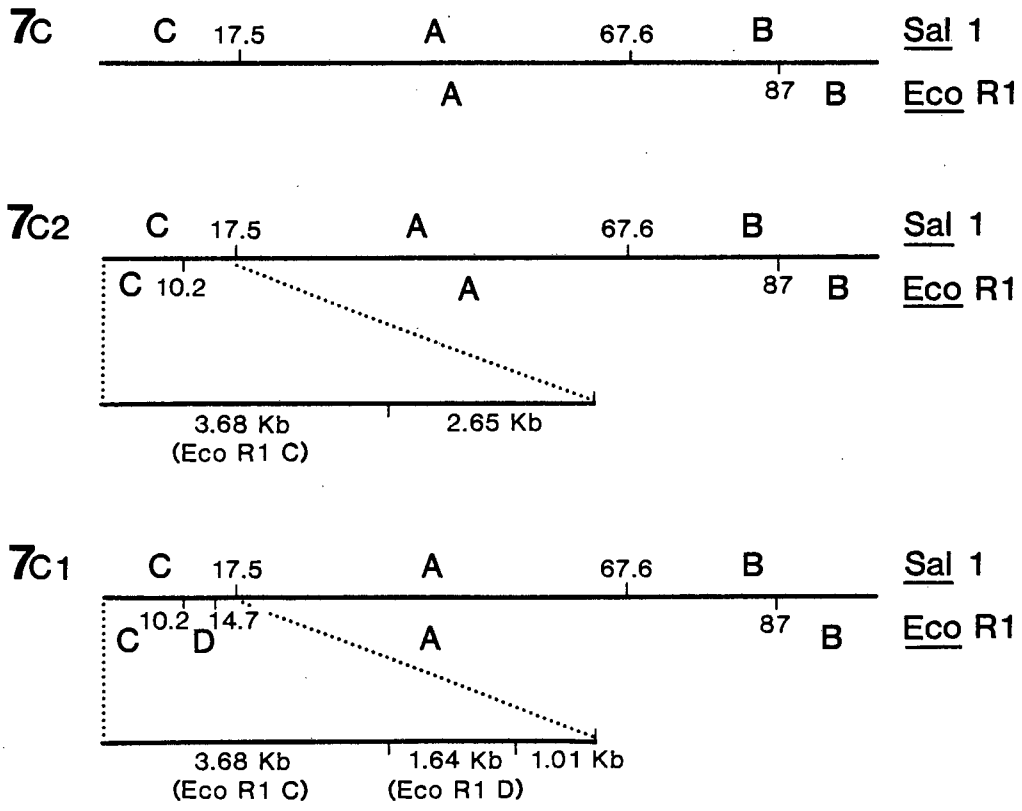


FIGURE 8

Restriction site maps (SalI, EcoRI) of the genomes of Ad7c, Ad7c2 and Ad7c1, given in map units. An expanded scale, in kilobases, is shown for the SalI C fragments of Ad7c2 and Ad7c1. This illustrates the location of the additional EcoRI cutting sites at 3.68 and 5.32 kb (14.7 mu) of Ad7c1 and at 3.68 kb of Ad7c2. (EcoRI and SalI maps of Ad7c taken from Wadell *et al.*, [1981] and Li and Wadell [1986]).

3.4. COMPARISON TO OTHER LOCAL AND JOHANNESBURG ISOLATES OF ADENOVIRUS SPECIES 7

In order to compare these newly identified genome types of Adenovirus species 7 to other Ad7 isolates, a further group of 19 isolates, from both Cape Town and Johannesburg, were identified by restriction endonuclease analysis, using BamHI, SmaI and EcoRI. The results of this survey are summarized in Table 3.

The first group of 11 Johannesburg isolates, obtained from Dr. A. Kidd at the National Institute for Virology (Johannesburg), were recent isolates from 1985 (7) and 1986 (4). From the 1985 group, 1 was shown to be an adenovirus prototype 7c (Nr 2084), while the remaining 6 were all identical to our new variant Ad7c2 (Nrs 2154, 2168, 2156, 2153, 1980 and 1727) with regard to restriction enzyme profiles with BamHI, EcoRI and SmaI. Of the 1986 isolates, two were adenovirus prototype 7c genotypes (Nrs 274 and 548), while the last two (Nrs 702 and 1004), apparently unlike any previously identified Ad7 genome type, were identified as adenovirus type 34 (subgenus B:2) genotypes, by comparison with previously published restriction maps for the enzymes BamHI and SmaI (Adrian, 1986). Representative restriction enzyme profiles of each of these identified genome types are illustrated in Figure 9. The strain shown in lanes 4 is an Ad7b reference strain, which was included as a control.

In concurrent studies in our laboratory, 8 additional Cape Town isolates of Ad7 have been grown and identified, after genome analysis with the same 3 enzymes - BamHI, EcoRI and SmaI. One of these, a 1976 Ad7 isolate (Nr 655) was found to be identical to our previously identified 1975 isolate (Nr 2182), and was typed as an Ad7c variant, Ad7c2. Both of these were single lung isolates from young children from the same ward at the Red Cross Hospital as the "1986 May" group, who died of post measles pneumonia. Four 1985 Cape Town isolates (AK1, AK2, AK3 and AK4) obtained from Dr. A. Kidd (NIV, Johannesburg) were similarly identified as Ad7c2 genome types. These were associated with a small outbreak of acute respiratory infection in 4 children with post measles pneumonia. Two further 1985 Ad7 isolates from our laboratory - 1 from a child with post measles pneumonia (Nr 4822) and 1 from a child with

myocarditis (Nr 3383), both of whom died - were both typed as adenovirus 7 prototype (Ad7p) genotypes. Restriction enzyme profiles of these strains are indicated in lanes 1 and 2 of Figure 9. One recent October 1986 Ad7 isolate (Nr 4412) was from a child with post measles pneumonia, in the same ward at the Red Cross Childrens' Hospital as our previously identified 4 "1986 May" isolates (identified as Ad7c1 genotypes). This isolate, however, was shown to be an adenovirus "reference-like" 7c (Ad7c) strain.

TABLE 3 : Genome Types of Ad-7 Isolated During the Period 1975-86

Specimen Nr.	Isolation Date	Genome Type
I : CAPE TOWN ISOLATES		
2182	1975	Ad7c2
655	1976	Ad7c2
AK1	1985	Ad7c2
AK2	1985	Ad7c2
AK3	1985	Ad7c2
AK4	1985	Ad7c2
3383	1985	Ad7p
4822	1985	Ad7p
2256	1986, May	Ad7c1
2203	1986, May	Ad7c1
2314	1986, May	Ad7c1
2332	1986, May	Ad7c1
4412	1986, October	Ad7c
II : JOHANNESBURG ISOLATES		
2154	1985	Ad7c2
2168	1985	Ad7c2
2156	1985	Ad7c2
2153	1985	Ad7c2
1980	1985	Ad7c2
1727	1985	Ad7c2
2084	1985	Ad7c
274	1986	Ad7c
548	1986	Ad7c
702[a]	1986	Ad34
1004[a]	1986	Ad34

a: Specimens 702 and 1004 were typed as adenovirus species 7 by serum neutralization.

FIGURE 9

Representative restriction enzyme profiles (BamHI, SmaI and EcoRI) of Ad7 strains, isolated in Cape Town and Johannesburg, in 1985 and 1986.

Lanes 1 : Ad7 - 3383 (1985)
Lanes 2 : Ad7 - 4822 (1985)
Lanes 3 : Ad7 - 1980 (1985)
Lanes 4 : Ad7b reference strain
Lanes 5 : Ad7 - 702 (1986)
Lanes 6 : Ad7 - 548 (1986)
Lane 7 : λ HindIII digest, as molecular weight reference.

4 DISCUSSION

To date 41 human adenovirus species, divided into 7 subgenera, have been recognized. Of these, members of subgenus B, and in particular, adenovirus 7, is most frequently associated with severe disease and epidemic outbreaks of systemic infections. In a recent survey of the worldwide distribution of Ad7 genome types, Wadell et al. (1985) have demonstrated a preponderance of Ad7c and Ad7b in both Europe and Australia. Ad7c was the dominant genome type of adenovirus 7 in the years before 1970-1975, in both Europe and Australia. Thereafter a shift from Ad7c to Ad7b was shown to occur, and in the last decade this genome type (Ad7b) has predominated in Europe, Australia and North America. Twenty-two South African Ad7 strains isolated during the period of 1967 to 1976, and obtained from Dr. B.D. Schoub (NIV, Johannesburg), were also analyzed in this survey. The earliest strain was identified as Ad7b while all the rest were Ad7c genome types.

We have analyzed a total of 24 different isolates of adenovirus species 7, 9 of which were isolates from our laboratories, while 15 were obtained from Dr. A. Kidd at NIV, Johannesburg. In this study 2 new genome types of adenovirus 7 have been identified, and according to restriction enzyme patterns with EcoRI (and based on the classification system of Li and Wadell [1986]), these are variants of adenovirus genome type 7c. The Ad7c strains reported here from 1975 and 1976 (Nr 2182 and Nr 655) were identical to one another, but had 1 extra EcoRI restriction site (at 10.2 map units) when compared to reference Ad7c. A second novel Ad7c variant isolated in 1986 (Nrs 2256, 2203, 2314 and 2332) exhibited a second additional EcoRI cutting site at 14.7 map units. A total of 18 different genome types of Ad7 have recently been described (Li, 1986; Bailey, 1986), but no variants of Ad7c have yet been reported. One of our isolates, from the "1986 May" group (Nr 2203), has been identified by Dr. G. Wadell (University of Umea, Sweden) as "Ad7c1" (personal communication). This nomenclature was therefore adopted for the "1986 May" strain, and "Ad7c2" for the genotype isolated in 1975 and 1976 (and subsequently in 1985). There did not appear to be a difference in the virulence of these

genotypes when compared to prototype Ad7c. The majority of isolates were associated with cases of post measles pneumonia in children less than 12 months of age.

While analyses of Cape Town Ad7 isolates have identified the existence of Ad7c2 during the period of 1975-1985, and Ad7c1 in May 1986, the recent identification of the prototype Ad7c strain (Nr 4412), isolated from the same ward as the four "1986 May" strains, must indicate the continued co-circulation of different genome types of adenovirus species 7. Similarly, comparison with Johannesburg Ad7 strains has revealed the existence of Ad7c2 in 1985 isolates, and the prototype Ad7c genotype in 1985 and 1986. While the Ad7c genome types are clearly still prevalent in South Africa, it has been suggested that Ad7b and Ad7c alternate over the decades as the aetiological agents in Ad7 respiratory outbreaks (Wadell, 1981).

We have also identified the Ad7 prototype (Rowe et al., 1958) in 2 1985 Cape Town isolates (Nr 3383 and Nr 4822). This genome type is infrequently isolated, and together with Ad7a represents the major portion of the Ad7 strains isolated from healthy carriers (Wadell et al., 1985). While Ad7p is thought to be less virulent than the Ad7b and Ad7c genome types (Wadell et al., 1985), it is noteworthy that both of our 1985 Ad7p isolates were associated with symptoms of severe Ad7 infection, resulting in death. This genotype has also recently been identified in a number of 1987 isolates from our laboratory.

Restriction endonuclease analyses of 2 of the Johannesburg isolates (Nr 702 and Nr 1004) revealed their identities as Ad34, and not Ad7 genome types. Both isolates had previously been typed as Ad7 strains by neutralization. This result was surprising in view of the fact that prototype Ad34 and Ad7 strains have been reported to exhibit no cross-reaction in standard neutralization and hemagglutination - inhibition reactions (Wigand et al., 1985). The 8 species of human adenoviruses grouped into subgenus B have been separated into 2 clusters of DNA homology (Wadell et al., 1980), the first (B:1) containing Ad3, Ad7, Ad16 and Ad21, and the second (B:2) containing Ad11, Ad14, Ad34 and Ad35. Since subgenus B:2 members are rarely isolated, accounting for less than 1% of all reported adenovirus isolated (Schmitz et al., 1983), the reported

frequent shedding of Ad34 and Ad35 in patients with acquired immunodeficiency syndrome (AIDS) has stimulated much interest (de Jong et al., 1983). These 2 species are characteristically isolated from immunocompromised patients, renal and bone marrow transplant patients, and AIDS patients (Wigand, 1985). De Jong et al. (1983) have emphasized the importance of characterizing adenovirus isolates, often found in the urine of AIDS patients, both serologically and by restriction endonuclease analysis. They were able to demonstrate the existence of new Ad-35-like viral isolates, derived from the recombination between a small part (40%) of the Ad7 genome, coding for the fiber polypeptide, and greater than 90% of the Ad35 genome. (The two kinds of type specific determinants on the adenovirus particle, which are measured by neutralization assays, are carried by hexons and fibers [Wadell et al., 1980]). Thus, it is possible that the "Ad34" strains (Nr 702 and Nr 1004) reported here are similar Ad7/Ad34 recombinants that neutralize with Ad7 antiserum, but show restriction patterns identical to Ad34. This hypothesis would need to be tested, by analysis with additional restriction enzymes that cut differentially near the right end of the viral genome, in the regions encoding the fiber (86-91 mu) and hexon (50-59 mu) polypeptides (de Jong et al., 1983).

Due to the large number of adenovirus species (41 at present), and the variability in restriction patterns within each species, restriction endonuclease analysis is not generally employed for the routine typing of adenovirus isolates. However, methods have been developed to facilitate the rapid analysis of large numbers of isolates (Lehtomaki et al., 1986; Suzuki et al., 1981; Brown et al., 1984; Fife et al., 1985) and the unique advantages (such as speed and accuracy) of this form of virus typing over standard neutralization techniques have been reported. In conclusion, restriction endonuclease analysis has allowed us to identify and to characterize 2 new adenovirus species 7 genome types, and further to assess their importance with regard both to epidemiology and to pathogenicity.

PART II

OESOPHAGEAL CARCINOMA - SEARCH FOR A POSSIBLE VIRAL AETIOLOGY

1. INTRODUCTION

Cancer of the oesophagus is presently the leading cause of cancer deaths among the black population of Southern Africa (Bradshaw and Harington, 1986). The incidence of this disease shows a striking geographical localization, and in certain regions of the country rates have been reported to be higher than any other known cancer anywhere in the world (Gillis and Hole, 1978).

Although oesophageal carcinoma has been common in certain sharply defined areas of China (Yang, 1980) and Iran (Hormozdiari et al., 1975) for centuries, its incidence among the black population of Southern Africa remained insignificant until the late 1940's. Since then, however, it has reached epidemic proportions, particularly in the Transkei, and it appears to be increasing in other parts of the country among both the urban and the rural population (Kneebone and Mannell, 1985). Even within the Transkei, a marked degree of regional variation in incidence has been reported, ranging from 2 cases per 100 000 in the Bizana district in the north to 116 cases per 100 000 in the Butterworth area in the south (Morris and Price, 1986).

1.1 AETIOLOGY

The aetiology and pathogenesis of carcinoma of the oesophagus are the subjects of extensive investigation. While several factors have been implicated as aetiological agents, no common cause has been described for all geographical areas associated with a high incidence of this tumor, and the involvement of multiple factors with the disease has been suggested. Certain of the more favoured aetiological agents are briefly discussed in the following sections.

1.1.1 Dietary Pattern

Since all ingested substances have contact with the oesophagus, diet is clearly of importance when investigating tumors of the oesophagus. A dietary effect could be brought about either by the ingestion of carcinogenic agents such as nitrosamines and fungal toxins, or by some dietary deficiency. Extensive studies have been

carried out to compare the staple diet of populations at high or low risk for oesophageal cancer (van Rensburg, 1981). In all high risk areas the dietary staple was found to be maize and/or wheat. An examination of the chemical composition of these foods revealed that such a "high risk" diet was deficient in respect of 2 B vitamins, nicotinic acid and riboflavin, and also 2 essential minerals, magnesium and zinc. A combined deficiency in the first 2 vitamins could damage the oesophagus and perhaps predispose to malignancy (Warwick and Harington, 1973). Such "promoting" damage to the oesophageal mucosa, which can also be brought about by mineral deficiencies, would appear to be an early event in the onset of oesophageal carcinoma, followed by specific carcinogenic induction (Bradshaw and Harington, 1986).

1.1.2 Potential Carcinogens or Co-Carcinogens

1.1.2.1 Alcohol and tobacco

While alcohol and tobacco have been implicated as risk factors in South Africa (van Rensburg, 1985) they are thought to be unimportant in the high risk regions of Iran (Hormozdiari et al., 1975). The use of alcohol is known to increase the requirements of the abovementioned nutrients, and it could thus mediate its effect via nutritional deficiencies. Alternatively, it may have a dehydrating effect on the oesophagus, and in this way damage it (Warwick and Harington, 1973). There has been shown to be a correlation between carcinoma of the oesophagus and the smoking of unprocessed, sundried tobacco (Warwick and Harington, 1973). Transkei tobacco was found to be particularly mutagenic and to contain a higher than usual content of nitrate and nitrosamines (Rose, 1982).

1.1.2.2 Nitrosamines

Nitrosamines are site specific carcinogens in many experimental animals (Warwick and Harington, 1973). Traces of some nitrosamines which are implicated in liver cancer, and others which are involved in oesophageal cancer in animals were found in many food and beer samples from the Transkei, although there were no striking differences in the absolute levels between low and high incidence districts (Rose, 1982). At present there is not enough

evidence to support a role for these compounds as a major cause in the genesis of oesophageal carcinoma.

1.1.2.3 Mycotoxins

Van Rensburg (1985) has proposed that the only possible source of the carcinogens and promoters common to all endemic regions originates from heavy fungal disease of food crops. In the Transkei maize is the monostaple food and the base for much of the beer brewed in great quantities. This crop has frequently been found to be infected with the fungus, *Fusarium moniliforme*, both in the Transkei and in China, while in Iran frequent infection of wheat by *Alternaria alternata* has been described (Marasus, 1982). These fungal contaminants were shown to exhibit a higher incidence in high as opposed to low rate areas of oesophageal cancer. While the nature of the carcinogenesis-enhancing substances in the fungal cultures are not known, both of these fungi were found to enhance tumor development when fed to laboratory rats which had received small doses of cancer-inducing nitrosamines (van Rensburg, 1985; Jaskiewicz, 1987). Much of this recent evidence points to a significant role for mycotoxic activity in the aetiology of oesophageal carcinoma.

1.2 THE POSSIBLE ROLE OF ONCOGENIC VIRUSES

While the roles of the abovementioned factors (nutritional deficiency, dietary habit and carcinogenic exposure) in the aetiology of oesophageal cancer cannot be excluded, they are believed to be insufficient to explain the striking regional variations in the incidence of this neoplasm, or the increase in it's incidence in the last 25 years. A high incidence of particular malignant tumors, in well defined geographical areas, is often associated with viral aetiological factors. For example, there is a statistical correlation between infection with Hepatitis B virus and the development of primary liver cancer, especially in Taiwan and parts of Africa. Furthermore, Epstein-Barr virus has been epidemiologically linked to Burkitt's lymphoma in central Africa and to nasopharyngeal carcinoma in South-East Asia. The retrovirus human T-lymphotropic virus (HTLV-I) has been implicated in the pathogenesis of adult T-cell leukemia, especially in the Southern Islands of Japan. The association of carcinoma of the oesophagus with certain geographical areas thus suggests a possible viral aetiology for this tumor.

Possible oncogenic DNA viruses that could be involved in the aetiology of oesophageal carcinoma are discussed in the following sections. These viruses are: adenoviruses, papillomaviruses, and Epstein-Barr virus. Probes from these viruses were used in the survey of oesophageal tumor cells described in later sections of this work.

1.2.1 Human Adenoviruses

The human adenoviruses are medium-sized viruses containing a linear double-stranded DNA genome, 33-45 kilobases in length. Forty-one serotypes of human adenoviruses have thus far been identified. These are classified into 7 subgenera, A-G (refer to Part I, Table 1), the members of subgenus A (Ad12, Ad18, Ad31) being highly oncogenic in animals, and those of subgenus B (Ad3, Ad7, Ad14, Ad16, Ad21, Ad34, Ad35) weakly oncogenic; the members of subgenera C to E are nononcogenic but together with those of subgroups A and B, will readily transform rodent cells in culture to a neoplastic phenotype.

1.2.1.1 Association with human disease

Most humans are infected early in life by several of the common human adenoviruses. Infections with subgenus A members are relatively common and are mainly associated with gastrointestinal disease in young children. While subgenus B:2 members (Ad11, Ad14, Ad34, Ad35) are relatively rarely isolated, the members of subgenus B:1 (Ad3, Ad7, Ad16, Ad21) appear in epidemic outbreaks of conjunctivitis and/or respiratory disease, and can cause fatal infections in children under 2 years of age (Part I, Section 1.3). The subgenus C adenoviruses are the most prevalent. They cause predominantly mild infections of the upper respiratory tract and readily form latent infections in lymphoid tissues. In contrast to these, and the fastidious adenoviruses of subgenera F and G, (isolated in large quantities from cases of infantile diarrhea), subgenus D and E members are very rarely isolated (Wadell, 1986).

The fact that adenovirus species of subgenera A,B:2 and C, with predilections for persistent infections, can cause severe or lethal infections in immunocompromised patients is well known. Although to date extensive studies have failed to show an association between adenoviruses and any type of human cancer (Green and Mackey, 1977; Green *et al.*, 1980), these viruses do have oncogenic properties and it is therefore possible that they could play some role in human cancer. The reported frequent shedding of Ad34 and Ad35 in the urine of patients with acquired immunodeficiency syndrome (AIDS), may be significant (de Jong *et al*, 1983). In this study, adenovirus was isolated at a much higher rate from AIDS patients than from a comparable group of renal transplant patients, and one particular genotype - Ad35 - was represented. This is difficult to explain simply in terms of the reactivation of latent virus as a consequence of immunosuppression.

1.2.1.2 Cell transformation

Human adenoviruses can malignantly transform a variety of rodent cells grown in culture, although these cells are either semipermissive, or completely nonpermissive to viral replication. Human cells, which are fully permissive to adenovirus replication, are extremely difficult to transform. In fact, only 2 transformed

human cell lines have been isolated to date: an Ad5 transformed embryonic kidney cell line, the 293 line, and an Ad12 transformed human retinoblast line (van der Eb and Bernards, 1984). The left hand (5') 15% of the viral genome is known to contain the early region E1, transcribed in the early phase of lytic infection, and to encode the transforming genes of nononcogenic Ad2 and Ad5, weakly oncogenic Ad3 and Ad7, and highly oncogenic Ad12 and Ad31 (van der Eb and Bernards, 1984). The respective E1 regions of these different species are organized in a very similar way, as shown in Figure 10. They comprise two adjacent transcriptional units, E1A (1.3-4.5%) and E1B (4.6-11.5%), encoding proteins involved in transformation.

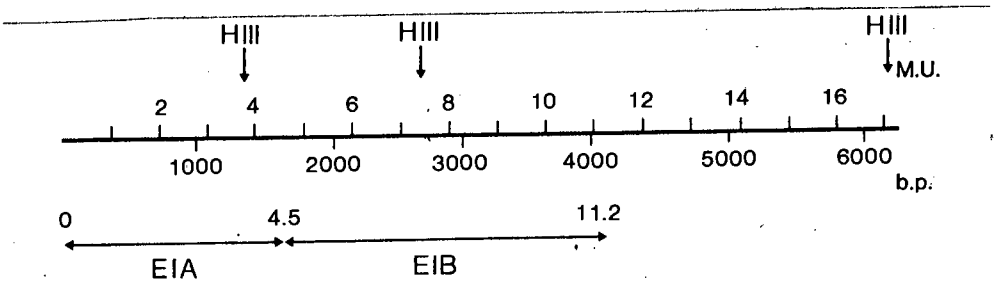


FIGURE 10

Organization of the transforming region E1 of human adenovirus 7. The two subregions E1A and E1B are indicated. The figure shows the leftmost 17% of the Ad7 Grider strain DNA, including the HindIII IJ region (0-2.72 kbp) with transforming activity. Adapted from Fujinaga et al. (1984).

Although transfection of cells with E1A sequences alone will result in partial transformation, these cells are not tumorigenic. In contrast, cells transformed by the E1 region are both fully transformed and tumorigenic (Berk, 1986). Thus E1A function is central to adenovirus induced transformations although the exact mechanism involved is not yet fully understood.

While proteins encoded by region E1B play a cooperative part in the transformation process, they cannot alone induce alterations in cell growth or morphology. Two major protein products, 55K and 19K proteins, are encoded by the E1B region. The 19K product is essential for adenovirus mediated cell transformation, and is thought to be the major transforming protein. However, the 55K protein does influence the frequency of tumorigenicity of the transformed cells, in a species specific manner (Stillman, 1986). As for the E1A proteins, the exact role these 2 proteins play in transformation by adenoviruses is not yet fully understood.

1.2.1.3 Integration of the viral genome

In most tumors and transformed cell lines induced by adenovirus, multiple copies of viral DNA and/or viral DNA fragments are found integrated into the host cell genome, and are present in non-stoichiometric amounts. There is no evidence that specific host or viral DNA sequences are involved in this process. Continuous expression of viral transforming DNA sequences seems to be required for the maintenance of the transformed state, but it is also not clear whether specific integration(s) is required for the the establishment and/or maintenance of transformed phenotypes (Fujinaga et al., 1984).

1.2.2 Epstein-Barr Virus

Epstein-Barr virus (EBV) was originally described by Epstein et al. (1964) who discovered the virus in cultured lymphoblasts derived from African patients with the B-cell lymphoma, Burkitt's lymphoma (BL). This large virus, a member of the human herpesvirus group, contains a linear double-stranded DNA genome, 172kb in length. Studies on EBV have been seriously hindered by the lack of a tissue culture system that will allow lytic infection.

1.2.2.1 Association with human malignant diseases

EBV has been strongly associated with two malignant diseases in man, Burkitt's lymphoma and nasopharyngeal carcinoma (NPC), and in 1968 it was shown to be the causative agent of a third common disease,infectious mononucleosis (Ernberg and Kallin, 1984).

Burkitt's lymphoma is a highly malignant tumor which has a high incidence both in tropical Africa and in New Guinea. It occurs with much less frequency, not always linked to EBV, in other parts of the world. All malignant B-lymphocytes, whether associated with EBV infection or not, show a reciprocal 8 to 14 chromosomal translocation, or a variant translocation of chromosome 8 to chromosome

2 or 22. The fact that in all translocations the c-*myc* oncogene has been identified at or near the breakpoint of the chromosomal translocation suggests that the activation or deregulation of this gene could be the key event leading to the malignant phenotype (Lenoir, 1986).

In contrast to uniclonal BL cell lines, polyclonal lymphoblastoid cell lines (LCL's), that are derived after EBV infection of human B lymphocytes *in vitro*, are neither tumorigenic in immunosuppressed animals, and nor do they exhibit these non-random chromosomal translocations (Lenoir 1986).

Thus, while it is almost certain that EBV plays a causative role in endemic BL, it is clearly not absolutely necessary nor sufficient in itself for malignant degeneration. The fact that every cell in African Burkitt's tumors is infected with EBV strongly suggests that viral infection preceeds malignant proliferation. It has been proposed that EBV primes the cell for malignant transformation by promoting proliferation, and that the influence of some co-carcinogenic factor further promotes the emergence of the malignant B-cell (Kieff *et al.*, 1982). Since the high incidence areas of BL are also the only areas in the world where malaria is hyperendemic, a chronic malaria infection has long been thought to represent this co-factor (Epstein, 1984). Malaria is not only immunosuppressive, but it is also able to stimulate B-cell proliferation. A second hypothesis is that African EBV isolates differ from other EBV isolates, and in analogy to human papillomavirus (Section 1.2.3), specific subtypes are associated with the malignant disease and others associated with nonmalignant conditions induced by the virus. However, Bornkamm *et al.* (1984) have addressed this question and found no apparent difference in the EBV genomes carried in Burkitt tumor cells and those carried in nonmalignant lymphoblastoid cells from the same individuals.

The second EBV associated human malignancy, nasopharyngeal carcinoma, occurs infrequently in most populations, but has a high

incidence in southern China. EBV DNA has been demonstrated in the epithelial carcinoma cells of these tumors, and this provides definitive evidence that the host range of the virus in humans is broader than B-lymphocytes. Both environmental and genetic factors have been implicated as aetiological agents in the pathogenesis of the disease (Kieff et al., 1982).

1.2.2.2 The intracellular EBV genome

It has not yet been determined whether integration of EBV into the host cell genome is an important primary event during tumorigenesis. Each BL-derived cell line has been shown to carry a constant number of copies of the EBV genome from one generation to another, and the majority of these are episomal. However, direct evidence in favour of EBV integration does exist in the case of the long established BL cell lines Raji (Anvret et al., 1984) and Namalwa (Matsuo et al., 1984). In contrast, Harris et al. (1985) have analyzed three recently isolated BL derived cell lines, and have demonstrated the close association of EBV DNA with metaphase chromosomes of the host cell. However, this association did not involve covalent linkage, suggesting that EBV integration might not be of primary importance in the genesis of the tumor.

1.2.2.3 Cell transformation and antigen expression

In contrast to cells transformed by adenoviruses or papovaviruses, the entire EBV genome is present in cells transformed by this virus. These infected cells regularly express a restricted set of viral genes, some of which must be responsible for the induction and maintenance of cell proliferation. In analogy to the papova and adenoviruses, these genes are likely to be continually expressed in the proliferating cell.

The linear EBV genome (172kb) is divided into 5 domains of unique sequence complexity, U1-U5, bounded by direct tandem copies of repeated DNA sequences located at the termini, TR, and at internal

sites within the molecule, IR1-IR4 (Figure 11). Functions in cellular transformation have been proposed for proteins encoded by 4 different viral genes (LT1-LT4), but the exact roles these proteins play have not yet been elucidated (Dambaugh et al., 1986).

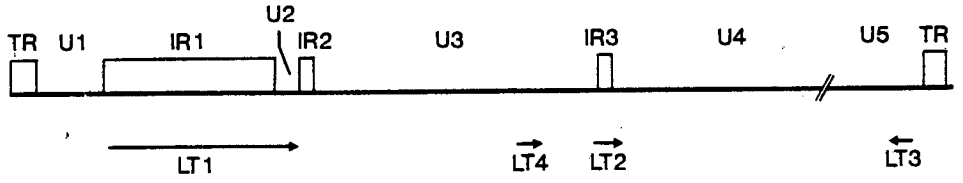


FIGURE 11

Schematic diagram of linear EBV DNA (172kb) showing the organization of unique (U1-5) and major tandem repeat (IR1-3) DNA domains. The locations of four major transcripts (LT1-4) expressed in latent, immortalizing infection are shown. The discontinuity in the genome reflects a deletion in the B95-8 strain of EBV. Adapted from Dambaugh et al. (1986).

1.2.2.4. EBV infection of epithelial cells

While EBV has been classically regarded as a strictly B-lymphotropic agent, there is now a growing body of evidence suggesting that epithelial cells may be infected by this virus. Although its association with malignant cells of NPC has long been known, more recent studies have reported the detection of the viral genome in lymphoepitheliomas of the salivary glands and of the thymus (Young *et al.*, 1986). Furthermore, evidence for the existence of functional EBV receptors on human epithelial cells lining the mouth and the pharynx (as well as on ectocervical epithelial cells) has recently been reported (Young *et al*, 1986). Sixbey *et al*. (1983) have also demonstrated that certain human epithelial cells infected *in vitro* will sustain a productive EBV infection, unlike the LCL's which can seldom be induced into a virus productive cycle. These results suggest that infection of epithelial cells could be crucial both to the events of primary infection and to the mechanism of virus persistence in the immune host.

It would, however, appear that the mechanism of cellular transformation by EBV is not the same for epithelial cells and for B-lymphocytes. Griffin and Karran (1984) have recently described the immortalization of monkey kidney epithelial cells by cloned viral DNA derived from the right hand (3') end of the viral genome. This region did not include any of the 4 regions of EBV DNA shown to be expressed in virus-immortalized B-cells (Section 1.2.2.3).

TABLE 4 : Papillomavirus Types and Associated Lesions

HPV Types	Associated Lesions	Observed Malignant Conversion (in vivo)
HPV1a,b,c; HPV2a-e; HPV3a,b; HPV4; HPV7; HPV10; HPV41	Skin warts	---
HPV6a-f; HPV30; HPV11a,b; HPV40; HPV16	Laryngeal warts	++
HPV13; HPV32; HPV6a; HPV2; HPV11; HPV16	Oral warts	+
HPV5a,b; HPV3a; HPV8; HPV9; HPV12; HPV10; HPV17a,b; HPV19-29	Epidermodysplasia Verruciformis (EV)	+++
HPV6a-f; HPV11a,b; HPV18; HPV16	Genital warts	++
HPV16; HPV18; HPV31; HPV35	Cervical tumors	+++
HPV36; HPV39	Skin lesions	---
HPV33; HPV34; HPV37; HPV38	Small numbers of genital and skin carcinomas	

Adapted from data cited by McCance (1986), and Tooze (1981).

1.2.3 The Human Papillomaviruses

Papillomaviruses were the first DNA tumor viruses to be discovered. They are relatively small viruses containing a circular double-stranded DNA genome, approximately 8 kilobases in length. They have not been studied as extensively as the other members of the family Papovaviridae (namely the polyoma viruses and simian virus 40) largely due to the lack of a suitable cell culture system for virus propagation.

Papillomaviruses are the aetiological agents of papillomas, or warts, in a wide range of animals including man. To date 41 different types of human papillomavirus (HPV) have been recognized and these are associated with a variety of lesions on many squamous epithelial surfaces (Table 4). Although usually benign, these squamous cell tumors can undergo malignant transformation. While both epidermodysplasia verruciformis (EV) and the cervical tumors exhibit a high rate of malignant transformation, genital and laryngeal papillomas have also been observed to undergo malignant transformation, but at lower frequencies. Factors that can influence the malignant transformation of the tumors include: the genetic make-up of the host; deficiency in the host's immune system; and co-carcinogens in the environment (Orth et al, 1977).Only certain papillomavirus types are involved in the development of malignant tumors, but the degree of involvement of the virus and the mechanism of this transformation to malignancy are unclear.

The accepted system of division of the genus into different subtypes is based on the identification of a new subtype as one that shows <50% DNA sequence homology (percentage cross-hybridization) with previously identified HPV's (McCance, 1986).

1.2.3.1 Association with malignant and pre-malignant diseases

While some papillomavirus types are associated with benign papillomas, the DNA of other types can be identified in a high percentage of malignant tumors. This is particularly evident in carcinomas developing in EV patients, and for genital cancer, and suggests a different oncogenic potential of the different HPV's.

1.2.3.1.1 Epidermodysplasia verruciformis (EV)

EV is a very rare disease with lifelong persistence. The patients usually have congenital defects of cell-mediated immunity, and about one third of cases develop malignant carcinomas on an average of 24 years after the onset of the disease (Gissmann, 1984). These carcinomas develop from flat skin warts, particularly at sun-exposed sites, suggesting UV light as a co-factor in the development of the disease (Gissmann, 1984). A large group of HPV types have been found associated with these lesions, but only HPV5 and HPV8 DNA has been demonstrated in carcinomas present in these patients (Smith et al., 1984).

1.2.3.1.2 Genital and cervical cancer

There is a growing body of evidence implicating papillomaviruses in the causation of human genital cancer (Crawford, 1984). This evidence includes demonstrations that the majority of the tumor cells contain viral DNA. HPV16 is most frequently found occurring in about 60-90% of cervical cancers (McCance et al., 1986; Durst et al., 1983). Next most commonly found is HPV18, which is present in 15-36% of the tumors, as well as in cervical cancer cell lines such as HeLa, which has been extensively passaged in culture. However, this HPV type is absent from benign tumors and premalignant lesions (Boshart et al., 1984). HPV16 DNA is also found in a small number of benign genital tumors, often in conjunction with the more usual HPV6 and 11 (Durst et al, 1983). It is isolated at an increasing rate as the disease increases in severity, and is

also the type most frequently found in genital malignancies, other than cervical carcinoma (McCance, 1986; McCance et al., 1986).

It appears that integration of the viral DNA into the cellular genome is important in the generation of genital cancer. In the majority of malignant lesions such integration occurs, and even in commonly used cervical cancer cell lines, such as HeLa, HPV18 DNA sequences are present in the form of defective integrated copies, and amplified to a high copy number (Schwarz et al., 1985). In benign and premalignant lesions, however, HPV DNA is found as free viral monomers, often also in the presence of infectious virus which cannot be produced in the undifferentiated cells of malignant lesions. While integration into the host chromosome has been shown to occur at several random sites, there does appear to be some specificity with regard to the break site in the viral genome (McCance, 1986). This leads to an interruption of a region containing the E1 and E2 early genes (Section 1.2.3.2).

Since not all advanced stages of cervical intraepithelial neoplasia lead to invasive disease, it is clear that other factors must be involved in the progressive disease. Zur Hausen (1982) has proposed that "initiating events" could interact synergistically with a promoting papillomavirus infection and result in the transformation of a premalignant lesion to a malignant carcinoma. In analogy to UV-light in EV patients, and chemical carcinogens in oesophageal papillomatosis of cattte (Section 1.2.3.1.4), these initiating events could be associated with herpes simplex virus infections, or smoking, in the case of genital cancer (Prakash et al., 1985).

1.2.3.1.3 Carcinomas of the larynx and oral cavity

Carcinomas of the oral cavity are associated with HPV2, 11 and 16 (McCance, 1986; de Villiers et al., 1985). While carcinomas of the larynx are associated with HPV16 and HPV30 (Brandsma et al., 1986; Kahn et al., 1986), HPV6 and 11 are also found in benign laryngeal

papillomas (Gissmann et al., 1983), which have been shown to convert to malignancy under the influence of X-radiation.

1.2.3.1.4 Carcinoma of the alimentary canal in cattle

The synergism between papillomavirus infections and other co-carcinogens has been well-studied in various animal model systems. In Scotland papillomas of the alimentary canal of cattle, induced by bovine papillomavirus 4, were shown to undergo a high rate of malignant transformation in animals feeding on bracken fern (Gissmann, 1984). These papillomavirus induced lesions are common in the bovine oesophagus (Jarrett, 1973; Plowright, 1971), but in contrast to the situation observed with genital cancer, oesophageal cancer cells were not found to harbour viral DNA. This suggests that the presence of BPV-4 DNA is not required for the progression to, or the maintenance of the transformed state, but may be required for the initiation stage (Campo et al., 1985).

1.2.3.2 Genomic organization and transformation of papillomaviruses

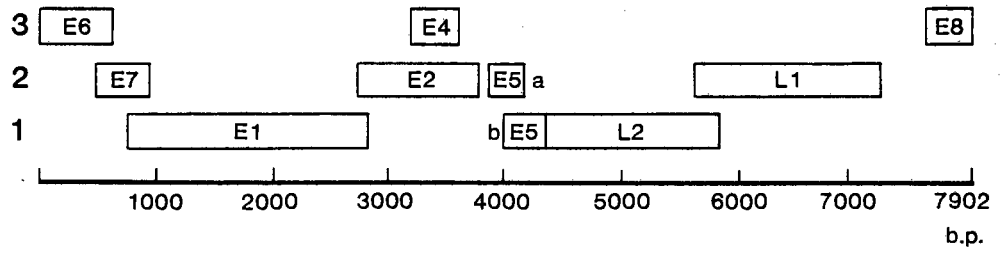


FIGURE 12

The organization of early and late genes in three possible reading frames of the HPV6 genome (7.9kb).
Adapted from McCance (1986).

The viral genomes of 6 human papillomavirus types, HPV1a, HPV6, HPV16, HPV8 and HPV11, as well as bovine papillomavirus type 1 (BPV1) have been completely sequenced (McCance, 1986). The organization of the genomes is well conserved between these types

and has a number of open reading frames encoding both early and late proteins (Figure 12).

The difficulty in replicating HPV DNA *in vitro* has severely hindered the study of mechanisms of transformation by this virus. However, mouse cell transformation with cloned HPV DNA has been reported (Watts *et al.*, 1984; Yasumoto *et al.*, 1986) and more recently the transformation of heterografted human skin tissue by infection with HPV11 was demonstrated (Kreider *et al.*, 1986). This should facilitate the future study of factors permitting the neoplastic progression of HPV-induced lesions in human tissues.

BPV-type 1 is not subjected to the same *in vitro* growth restrictions seen with HPV DNA, since it is maintained as a stable plasmid in transformed fibroblasts. The E2 gene of this virus participates in gene control and has been shown to be inactivated when BPV1 transforms cells to the cancerous state (Marx, 1986). Furthermore, recent studies have identified the existence of a transcriptional enhancer element in the non-coding region of the viral genome, which is transactivated by the E2 gene product, and may control the efficiency of replication and transformation (McCance, 1986). The bovine E2 gene is in a region that may be analogous to that disrupted by integration of the HPV DNA (Marx, 1986). This integration has shown a remarkable specificity for opening circular viral DNA within the E1-E2 open reading frames (Schwarz *et al.*, 1985). A recent analysis of HPV16 integration in a cervical carcinoma has demonstrated the deletion of the entire E2 gene and the greater part of the E1 gene (Matsukura *et al.*, 1986). Similarities between the HPV E1 gene product and the polyoma virus large T antigen, a product necessary for the initiation of viral DNA replication, have revealed a possible role for the E1 protein in the control of eukaryotic DNA replication (Clertant *et al.*, 1984).

The HPV E6-E7 open reading frames are specifically transcribed in cervical cancer cell lines (Schwarz *et al.*, 1985), and although no

protein product has, as yet, been identified, it is significant that a corresponding region in BPV1 DNA has transforming potential (Schiller et al., 1984; Schiller et al., 1986). Based on recent studies of E6 and E7 gene expression in the HPV18 containing HeLa cell line, zur Hausen (1986) has proposed a model whereby the development of human cancer is viewed as a failure of host-cell control of persisting viral genes. A cellular interfering factor, or CIF, is thought to negatively control HPV expression in infected cells. During cell differentiation, however, these genes are switched off permitting the transcription and replication of viral DNA. This would explain the difficulty in replicating HPV DNA in vitro in terms of the dependence of viral replication on certain stages in squamous epithelial cell differentiation. A similar interruption in this cell-mediated control could be brought about by the action of synergistic co-factors. This could involve either the modification of the persisting viral genome such that it does not respond to CIF control, or alternatively a modification of the CIF genes, or CIF activating genes, to result in a non-functioning CIF. This model provides a ready explanation for the long latency periods between primary infection and tumor appearance, observed in many virus-associated human cancers.

1.2.3.3 Human papillomavirus infection and oesophageal carcinoma

Morphological evidence of papillomavirus infection of the oesophagus has recently been published (Winkler et al., 1985; Hille et al., 1985; Hille et al., 1986; Syrjanen, 1982). Isaacson and co-workers observed histological changes indicative of such an infection in 8 out of 24 biopsy specimens from South African patients with carcinoma of the oesophagus (Hille et al., 1985) and in a further study in 23 of a total of seventy specimens (33%) (Hille et al., 1986). Syrjanen has reported similar changes in 24 out of 60 carcinomas studied in Finland (40%) (Syrjanen, 1982). Further evidence has been provided by immunohistochemical studies: HPV antigens have recently been demonstrated in oesophageal biopsy specimens showing morphological changes indicative of HPV

infection. Hille et al (1986) have shown 7 of a total of 23 (30%) tumor specimens to be positive for HPV antigens by an immunoperoxidase technique. In a similar study Winkler et al (1985) showed 31% of the tumors to be positive for HPV antigen.

Morris and Price (1986) have recently described a hypothesis linking the concept of mucosal immune surveillance with the above morphological evidence, in a sequence of events leading to squamous dysplasia and invasive carcinoma. They suggest that in the presence of inadequate vitamin nutrition as well as heavy or recurrent virus exposure (which is common in close living conditions) certain cells of the immune system, responsible for the detection and erradication of exogenous antigenic material, are functionally overwhelmed. This would facilitate the localized colonization of the oesophageal mucosa and the development of HPV-induced flat warts. Further progression of the disease could occur under the influence of persistent infection with a particularly oncogenic HPV subtype, nutritional deficiency and additional initiating co-factors, such as aflatoxin and alcohol. This could finally lead to the development of invasive carcinoma in a multi-step sequence of events. The hypothesis explains both the regional variation in the incidence of oesophageal carcinoma, and the sharp rise in the incidence of the disease in the black population of South Africa. On the basis of this model, the presence of persisting viral genes would not be essential for the maintenance of the malignant phenotype, only for it's initiation.

Histology and immunoperoxidase methods alone are not entirely conclusive of HPV infection, and absolute confirmation through electron microscopy and molecular hybridization techniques is required. To date there has only been one report of the detection of HPV DNA in oesophageal carcinoma tissue (Kulski et al., 1986). Five out of a total of 10 specimens analyzed by hybridization to a mixed probe of HPV types 11, 13, 16 and 18 were found to be positive. Since these HPV-positive oesophageal carcinomas were identified in Western Australia, a low risk area, there is now an

urgent need to examine oesophageal carcinoma cell lines and biopsy specimens from high risk areas in South Africa for evidence of HPV DNA, and further to elucidate the possible role of this DNA tumor virus in the aetiology of oesophageal carcinoma.

1.3 AIM OF THIS WORK

Dr. K. Robinson, at the University of Natal (Durban) established 10 continuous oesophageal carcinoma cell lines, from oesophageal tumors of varying differentiation. *In vitro* and *in vivo* studies of these tumors have revealed that properties such as the degree of malignancy of the source tumors could be well correlated with their ability to become successfully established and to grow for lengthy periods *in vitro*. However, there was little correlation between tumor differentiation and *in vitro* potential (Robinson, 1981).

In order to investigate the possible role of a viral co-factor in the aetiology of oesophageal carcinoma, I have screened three of these oesophageal cell lines by DNA/DNA hybridization, for the presence of stable, integrated viral sequences. The three cell lines analyzed were HCU18, HCU39 and HCU33: HCU18 was grown from a poorly differentiated tumor specimen, HCU39 from a moderately differentiated tumor, and HCU33 from a well differentiated tumor. Cellular DNA was screened for evidence of the following human DNA tumor viruses: papillomaviruses (types 1,5,6,8,11,16,18), adenoviruses (species 5,7,12,31) and Epstein-Barr virus, as well as for the RNA virus, human T-lymphotropic virus, HTLV-I.

The human papillomaviruses would appear to be the most likely of the different viral candidates, since there has been much recent evidence of papillomavirus infection of the human oesophagus both from morphological and from immunohistochemical studies (Section 1.2.3.3).

Although Epstein-Barr virus is traditionally regarded as a B-cell lymphotropic agent, evidence now indicates that this virus can also infect epithelial cells (Section 1.2.2.4). Similarly, while HTLV-I is strictly a T-lymphotropic agent, it has been shown that T-lymphocytes can infiltrate tissue such as the liver and the oesophagus, especially as a consequence of papillomavirus infection (Campbell, 1987, personal communication).

The human adenoviruses have not been associated with any known human cancer. They are, nevertheless, oncogenic, and naturally cause severe infections of the respiratory and gastrointestinal tract in humans, and were therefore also included in this survey.

2. MATERIALS AND METHODS

2.1 ORIGIN OF CELL LINES AND PLASMID PROBES

The three oesophageal tumor cell lines, HCU18, HCU33 and HCU39, established in culture by Dr. K. Robinson, were provided by Dr. C. Albrecht.

Human papillomavirus probes were provided by Dr. Campo (Glasgow) and Dr. E.M. de Villiers (Heidelberg). Epstein-Barr virus probes were provided by Dr. B. Griffin (London), and adenovirus probes by Dr. J. Bos (Leiden). An HTLV-I probe was provided by Dr. S. Josephs (Bethesda). Virus positive and negative control DNA was provided by colleagues of Dr. M. Renan at the Institute of Cancer Research, London. These control DNA's were: HPB-ALL, (acute lymphocytic leukemia, non-viral; negative control), Namalwa (Burkitt's lymphoma B-cell line, with integrated EBV genome; positive control) and C91/PL (an HTLV-I producer T-cell line; positive control). The positive control cell lines, Raji (Burkitt's lymphoma B-cell line, containing multiple copies of the EBV genome), and HeLa (cervical carcinoma cell line, containing multiple copies of HPV18), are routinely maintained in our tissue culture laboratory. The human embryo fibroblast strains, HF1/81 and 4/81, were established in this laboratory, and were used as negative controls.

2.2 TRANSMISSION ELECTRON MICROSCOPY (TEM)

2.2.1 Preparation of cell cultures for embedding in resin

The reagents used in the preparation of cell cultures for TEM are listed in Appendix I.

Cell cultures of oesophageal tumor lines HCU18, HCU33 and HCU39, grown in 75cm^2 Falcon tissue culture flasks, were harvested for electron microscopy when the cell monolayers reached confluence. The monolayers were washed with physiological saline, and subsequently with 0.1M sodium phosphate buffer (pH 7.2). They were removed from the flasks by mechanical scraping, using a glass rod with rubber attachment. This serves to avoid the possible damaging

effects of trypsin on the cells. After resuspension in 8-10ml of 0.1M sodium phosphate buffer, the cells were centrifuged to a pellet (1 500 rpm for 10 mins), and further resuspended in 8-10ml of 3% (v/v) gluteraldehyde. Cells were fixed in this solution by incubation at 4° for 30 mins. Thereafter the gluteraldehyde was removed by centrifugation (1500 rpm for 10 mins) and subsequent washing of the pellets in 0.1M sodium phosphate buffer (30 mins at 4°C). After two further 30 minute washes the cells were incubated in the same buffer at 4° overnight. The suspended cells were then pelleted by centrifugation at 4000 rpm for 20 mins, and the intact pellet retrieved and post-fixed for 1 hour in a 1% OsO_4 solution (Palades fixative). Tissue blocks were washed three times (for two mins each) in sterile distilled water and subsequently stained in 2% uranyl acetate for 15 mins at room temperature. Excess stain was removed by 3 further 2 minute washes. Specimens were then dehydrated by incubation in ethanol, as follows:

- 10 mins in a 60% solution of ethanol
- 10 mins in a 80% solution of ethanol
- 3 times, for 10 mins each, in absolute ethanol.

2.2.2 Embedding and trimming of tissue blocks

After fixation and dehydration the tissue blocks were embedded in L.R. White acrylic resin. The final dehydrating solution of ethanol was discarded and the resin added, and allowed to stand at room temperature for 1 hour. To remove any remaining alcohol, the resin was replaced with fresh embedding medium, and left to stand for a further hour at room temperature. This last step was repeated once, after which tissue blocks were removed, and cut, using a sharp blade, into blocks of 0.5-1.0mm dimensions. Blocks were placed inside Beem capsules, which were filled with resin. Polymerization was carried out in an atmosphere of nitrogen at 60°C overnight, and the tissue blocks were subsequently trimmed under a binocular dissecting microscope (Olympus).

2.2.3 Preparation of thin sections

Glass knives prepared using an LKB knifemaker and 6mm thick plate glass (Plate Glass, Cape Town), were carefully examined for any

defects, prior to use. A waterbath was attached to the knife with waterproof pressure tape, and sealed at its base with Ernest Fullum boatsealer.

Ultrathin sections were cut from the embedded tissue blocks by using a Sorval model MT-2 ultramicrotome. The thickness of the sections produced were judged from the interference colours shown by the sections floating in the waterbath (Peachey, 1958). Sections of 60-90nm, and silver in colour, were selected and transferred to 300 mesh 3mm diameter copper grids for staining and subsequent examination by electron microscopy.

2.2.4 Staining and examination of thin sections

A single drop of 2% uranyl acetate, and 0.2% lead citrate were placed separately on parafilm on the bottom of a petri dish. Grids were placed, with the sections downwards, on the uranyl acetate for 2 mins, and subsequently on the lead citrate for 2 mins. The grids were washed with sterile distilled water after each stain, and were finally placed on filter paper to dry.

Stained specimens were examined in a Siemens Elmiskop 1A electron microscope, at 60kV.

2.3 PLASMID PREPARATION AND PURIFICATION

2.3.1 Bacterial transformation

Purified recombinant bacterial plasmids were initially used to transform recipient bacteria for the purpose of subsequent amplification and isolation of plasmid DNA. Competent *E.coli* HB101 cells were prepared by calcium chloride pretreatment (Maniatis *et al.*, pg.250). Bacterial cell transformation was carried out as described in Part I, Section 2.6.4. Ampicillin resistant transformants were selected by plating on Luria agar plates containing 50µg/ml ampicillin.

2.3.2 Preparation of stock cultures of recombinant plasmids

Overnight 10ml cultures of E.coli HB101, carrying specific recombinant plasmids, and grown from a single ampicillin resistant transformant, were used to inoculate Luria agar plates (containing 50µg/ml ampicillin). After growth for 16-18 hours at 37°C, plates could be stored at 4°C, and viable bacteria recovered for up to a month after inoculation. Long-term storage of selected strains was achieved by incubation at -70°C in 15% glycerol (Part I, Section 2.7).

2.3.3 Amplification and isolation of plasmid DNA

E.coli HB101-derived strains carrying recombinant plasmids were inoculated from stock cultures into 10ml LB medium, containing 50µg/ml ampicillin. Amplification, and subsequent isolation of plasmid DNA, was generally achieved according to the procedure described in Part I, Section 2.8.1. However, this "alkaline lysis" method is only efficient for the isolation of small plasmids, less than 10kb in length. Therefore certain of the signifcantly larger plasmids were more efficiently isolated by using an alternative "cleared lysate" method, as follows:

The 10ml overnight culture was inoculated into 300ml of pre-warmed LB medium (containing 50 µg/ml ampicillin). This culture was incubated at 37°C, shaking vigorously, for 12-16 hours. Bacterial cells, harvested by centrifugation (6000 rpm, 10 min., 4°C) were resuspended in 5.2ml of 25% sucrose in 0.05M Tris (pH8.0). A 0.8ml volume of freshly prepared lysozyme solution (20mg/ml in 0.25M EDTA, pH8.0) was added to the cell suspension, and the mixture was swirled on ice for 5 mins. After the addition of 5.2ml of 0.25M EDTA (pH8.0), swirling was continued on ice for a further 5 mins. An 8ml volume of Brij/DOC solution (1% Brij 58, 0.4% sodium deoxycholate in 0.01M Tris, 0.001M EDTA, pH8.0) was then added by rapid expulsion from a 10ml pipette, mixed, and left on ice for 20-30 mins. During this period the cells lysed to form a viscous mass. The cell debris and much of the chromosomal DNA was subsequently pelleted by centrifugation, at 15 000 rpm for 45 mins at 4°C. The resultant straw-coloured supernatant was then

decanted, and prepared for centrifugation in a CsCl density gradient, as described in Part I, Section 2.8.1.

2.4 ISOLATION OF VIRAL SEQUENCES FROM RECOMBINANT BACTERIAL PLASMIDS

2.4.1 Isolation by electrophoresis onto DEAE-cellulose paper

Cloned viral DNA inserts were excised from recombinant bacterial plasmids prior to radioisotope labelling. This precluded spurious hybridization of vector DNA sequences to any plasmid DNA which might have contaminated the human DNA samples. The method used, a modification of that described by Dretzen *et al*. (1981), is described in Part I, Section 2.8.2.

2.4.2 Extraction from low-melting temperature (lmt) agarose

Most grades of agarose are contaminated with sulfated polysaccharides, which are potent inhibitors of many of the commonly used enzymes. Since these are extracted from the gel together with the DNA, they can cause problems in subsequent cloning reactions. Therefore, to isolate a particular Ad7c1 DNA restriction fragment for cloning into pUC19, a highly purified low-melting temperature agarose (Sea Plaque from FMC Bioproducts) was used. Digested DNA was separated by agarose gel electrophoresis (in 0.8% lmt agarose in TAE, pH8.0) and the required restriction fragment eluted from the gel, according to the method described by Maniatis *et al*. (1982, pg.170).

2.5 ISOLATION OF TOTAL HUMAN DNA FROM CELL LINES GROWN IN CULTURE

Cell cultures, grown in sterile $75cm^2$ tissue culture flasks were harvested for DNA extraction when the cell monolayers reached confluence. The cells were washed twice with 4-5ml of sterile physiological saline and then detached by trypsinization. The resultant cell suspension was pelleted, at 1 500 rpm for 5 mins, washed a further 2 times with physiological saline, and pelleted (1 500 rpm, 5 mins). Cells were resuspended in 10ml of 0.1M Tris, 0.1M EDTA (pH8.0), and a 0.1ml aliquot counted using a haemocytometer (Bausch and Lamb). The concentration of cells was

then determined, and adjusted with 0.1M Tris, 0.1M EDTA (ph8.0) to a final value of (2-4) x 10^6 cells/ml. Proteinase K was added at 100µg/ml, and SDS at a concentration of 2%. The suspension was mixed gently for 5 mins at room temperature, and then incubated at 56°C for 1 hour. Two phenol extractions, using an equal volume of Tris-equilibrated phenol, were carried out, and the aqueous and organic phases separated by centrifugation at 3 000 rpm for 10 mins. After 4 further extractions, twice with phenol/chloroform (1:1) and twice with chloroform, the nucleic acid was precipitated from the aqueous phase by the addition of 2 volumes of ice-cold absolute ethanol (in the presence of 300mM NaAc) and incubation at -20°C overnight. Cellular DNA was then pelleted by centrifugation at 4 000 rpm for 15 mins, washed in 70% ethanol, and pelleted again (4 000 rpm., 15 mins). The DNA pellet was dried under vacuum and re-dissolved in one fifth of the original volume of TE buffer (pH8.0). The resultant suspension was digested with heat-treated RNase (100µg/ml) by incubation at 56°C for 2-3 hours. After 4 further extractions with phenol (2x), phenol/chloroform (1x) and chloroform (1x), DNA was again precipitated by the addition of 2 volumes of ice-cold absolute ethanol (in the presence of 300 mM NaAc) and incubation at -20°C overnight (or alternatively at -70°C for 1 hour). The purified cellular DNA, recovered by centrifugation at 4 000 rpm for 15 mins, was washed in 70% ethanol to remove any remaining salt. It was then dried under a vacuum and redissolved in one-tenth of the original volume of sterile distilled water, by gentle mixing and incubation at 4°C overnight.

The final DNA concentration was determined by comparison with known concentrations of λ-phage DNA following agarose gel electrophoresis and ethidium bromide staining of the DNA, as described in Part I, Section 2.4. The yield of pure DNA isolated from 1 x 10^8 cells ranged from 200 to 400µg. The DNA samples were stored at 4°C until further use.

2.6 RESTRICTION ENDONUCLEASE DIGESTION AND AGAROSE GEL ELECTROPHORESIS OF TOTAL HUMAN DNA

Twenty micrograms of DNA, extracted from established human cell lines, was digested with 60 units of restriction endonuclease

(Boehringer-Mannheim or Amersham) in the presence of the appropriate enzyme buffer. Final digestion volumes were made up to a total of 200µl with sterile, distilled water. Incubation was carried out at 37°C for 12-16 hours in order to ensure that digestion was complete. Reactions were then terminated by heating the samples at 65°C for 5 mins. The DNA was precipitated by the addition of 2 volumes of absolute ethanol (in the presence of 300mM NaAc) and subsequent incubation at -70°C for 1 hour. Precipitated DNA was recovered by centrifugation, washed in 70% ethanol and dried under vacuum. It was then re-dissolved in 50µl of sterile distilled water.

Prior to the final separation of restriction fragments, 3µl aliquots of each sample were analyzed by electrophoresis in 0.8% agarose (in TAE, pH8.0), initially to ensure that the digestion had been complete, and subsequently to adjust all of the samples to the same DNA concentration. Aliquots of each sample, containing 10µg of DNA, were then made up to a final volume of 40µl with sterile distilled water, and 4µl of loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 15% w/v Ficoll). Electrophoresis was performed in 0.8% agarose gels (in TAE, pH8.0) at 40V (2V/cm) for 16-18 hours.

When electrophoresis was complete, the gels were stained in a solution of 500µg/ml ethidium bromide (in TAE buffer), viewed by UV transillumination, and photographed. Prior to transfer of the separated DNA restriction fragments onto Hybond-N nylon membranes, the DNA was denatured and neutralized in situ, as described in Part I, Section 2.4.

2.7 TRANSFER OF DNA TO NYLON MEMBRANES

Cellular DNA, which had been digested and separated by electrophoresis, was transferred onto nylon membrane according to a modification of the method of Southern (1975), as described in Part I, Section 2.5. Alternatively, total, undigested DNA was spotted onto the nylon membrane, as follows:

Aliquots of sterile, distilled water containing 0.5, 1.0, 2.0 and 4.0 µg of cultured oesophageal tumor cell DNA, and similar aliquots, containing positive and negative DNA's, were filtered through a Hybond-N-membrane, using a Bio-Rad Dot-Blot apparatus. Recombinant bacterial plasmid DNA (10-80 pg) was also filtered onto the membrane as positive controls. The membrane was prewetted in 20X SSC prior to filtration. Excess SSC was blotted off onto sterile 3MM filter paper (Whatman). After filtration was complete the membrane was removed from the apparatus and placed DNA-side downwards on a piece of 3MM filter paper, saturated with denaturing solution (1.5M NaCl; 0.5M NaOH) for 5 mins. It was then transferred to a second piece of filter paper, saturated with neutralizing solution (3M NaAc, pH5.2) for a further 5 mins. After air-drying the membrane the DNA was covalently bound to its surface by UV-illumination (300nm) for 2-5 mins.

2.8 HYBRIDIZATION OF ^{32}P-LABELLED PROBES TO DNA BOUND TO NYLON MEMBRANES

2.8.1 Southern blot hybridization

Prehybridization and hybridization was carried out in the presence of Denhardts solution, and denatured, sonicated salmon sperm DNA. Denhardts solution was prepared as a 50X concentrated stock containing 1% w/v Ficoll, 1% w/v polyvinylpyrrolidone (type 360) and 1% w/v bovine serum albumin fraction 5, dissolved in sterile distilled water. It was stored frozen, at -20°C, in 5ml aliquots, and was thawed at 37°C prior to use. Both prehybridization and hybridization reactions were carried out in heat-sealed plastic bags, as described in Part I, Section 2.9. Viral DNA probes of high specific activity ([5-15] x 10^7 dpm/µg DNA) were prepared by nick translation in the presence of α^{32}P dCTP (Part I, Section 2.8.3).

The membranes were prehybridized with a 10ml solution containing 5X SSC, 50mM Na_2PO_4, 5X Denhardts solution, 50µg/ml denatured, sonicated salmon sperm DNA, and 50% deionised formamide, for 2-4 hours at 42°C. Hybridization of radiolabelled single-stranded probe DNA was performed in a further 10ml solution, containing 3XSSC, 20mM Na_2PO_4, 1X Denhardts solution, 50µg/ml denatured salmon sperm DNA, 50% formamide and 10% w/v dextran sulphate. This

reaction was carried out at 42°C for 16-18 hours. Unhybridized probe DNA was removed by stringent washing of the membrane as follows: twice for 30 mins at room temperature with 2XSSC containing 0.1% SDS, followed by two washes at 60°C for 30 mins each in 0.1XSSC containing 0.1% SDS. The membranes were then autoradiographed, with intensifying screens, on X-ray film (XOmat MA, Kodak) for an average of 10 days at -70°C.

Low stringency hybridization to HPV DNA was performed as described above, but at a formamide concentration of 20%, and in the presence of 6XSSC (T_m-42°C). The membrane was washed 3 times in 2XSSC with 0.1% SDS at room temperature, and for 1 hour at 50°C (T_m-40°C). After overnight autoradiography, the membrane was washed twice at high stringency, in 0.5XSSC plus 0.1% SDS, at 55°C for 1 hour (T_m-25°C), and autoradiographed for 1 day to 10 days.

The melting temperature, T_m, measures the thermal stability of DNA hybrids, and is calculated as follows (Anderson and Young, 1986):

$$T_m = 81.5 + 16.6 \text{ (log M)} + 0.41 \text{ (\%G+C)} - 0.72 \text{ (\% formamide)}$$

(where M, the molarity of the monovalent cation, is 1 in a 6XSSC solution, and % G+C is 41% for HPV DNA [Lancaster and Olson, 1982]).

Hybridizations should be carried out at an incubation temperature that is 20-25° below T_m, to achieve a maximum rate of DNA-DNA annealing. Mismatching of DNA duplexes has the effect of lowering the T_m. An increase in formamide concentration also lowers the T_m by 0.7°C for every 1% increase in the concentration (Maniatis *et al.*, 1982; pg.389). Thus, by holding the incubation temperature constant and lowering the formamide concentration, conditions can be optimised to detect mismatched duplexes. The stringency of the reaction can also be altered by varying the salt concentration, and this should be kept fairly high to detect mismatched sequences.

2.8.2 Dot blot hybridization

Dot blot hybridization tests were carried out, in most cases, according to the procedure of Johnson et al. (1984), as described in Part I, Section 2.9. This is a more simple method that uses an incubation cocktail, termed "BLOTTO", containing nonfat dry milk as a blocking agent (in place of Denhardts solution and sheared salmon sperm DNA). Johnson et al. (1984) have demonstrated that this procedure is as efficient as conventional Southern blot analyses, and is capable of detecting single copy sequences in mammalian genomes. The "BLOTTO" method was thus chosen as a more suitable alternative for the rapid analysis of a large number of samples.

2.9 REMOVAL OF PROBE AND RE-USE OF DNA BLOTS

Hybond-N membranes which were to be used more than once were kept moist, wrapped in Saran Wrap, and stored at 4°C in the dark. Probe DNA was removed from the membrane by incubation at 45°C in 0.4M NaOH for 30 mins, followed by a further 30 minute incubation (at 45°C) in a solution containing 0.1XSSC, 0.1% SDS and 0.2M Tris (pH 7.5). The membrane was then autoradiographed to ensure that removal of the probe had been successful.

3. RESULTS

3.1 ULTRASTRUCTURAL ANALYSIS

3.1.1 Introduction

The 3 oesophageal carcinoma cell lines HCU18, HCU33 and HCU39 were established in culture during the period 1976-1980. These, together with 7 others, initiated during the same period, have been extensively examined by electron microscopy (Robinson, 1981). Several features observed in certain of these cell lines were suggestive of a possible viral involvement with carcinoma of the oesophagus. These included:

1. Prominent 20-30nm intranuclear particles in 1 cell line;

2. Bundles of 8-14nm cytoplasmic rods in 4 cell lines including HCU18 and HCU33;

3. Aggregates of electron dense 40nm cytoplasmic rods in 3 cell lines, including HCU33;

4. Well developed annulate lamellae in 5 cell lines including HCU18 and HCU33;

5. Well developed rough endoplasmic reticulum and golgi complexes in several specimens (notably HCU18), suggestive of increased protein synthesis.

Virus particles were not detected in the 3 cell lines HCU18, HCU33 and HCU39. A possible viral infection could, however, involve the persistence of viral DNA in the tumor cells without the production of virus particles. Latently infected cells could be non-permissive of viral replication, or alternatively semi-permissive, inducing the production of virus at a low frequency. Since the 3 cell lines have been extensively passaged in tissue culture subsequent to these initial studies, the tumor cells could well have become altered both in genotype and phenotype during adapta-

tion to an *in vitro* environment. These cell lines were briefly re-examined in this study for the possible presence of virus particles in the tumor cells.

3.1.2 In Vitro Characteristics of Cell Lines HCU18, HCU33 and HCU39

A coverslip culture of oesophageal tumor cells from line, HCU18 is shown in Figure 13. Cells of the 3 lines could be distinguished morphologically, and their features corresponded to those originally described for these 3 cell lines (Robinson, 1981). HCU33 cells varied in size and shape and were characterized by their rapid growth in tissue culture; HCU18 cells were angular in shape, while HCU39 cells appeared to be more rounded. Particularly large cells containing 3-4 nuclei were also evident in all 3 cell lines. It has been suggested that these giant cells could have arisen as a result of a virus-induced fusion process (Robinson, 1981). Cells of all 3 lines were shown to exhibit a typical piling effect characteristic of tumor cells grown in culture.

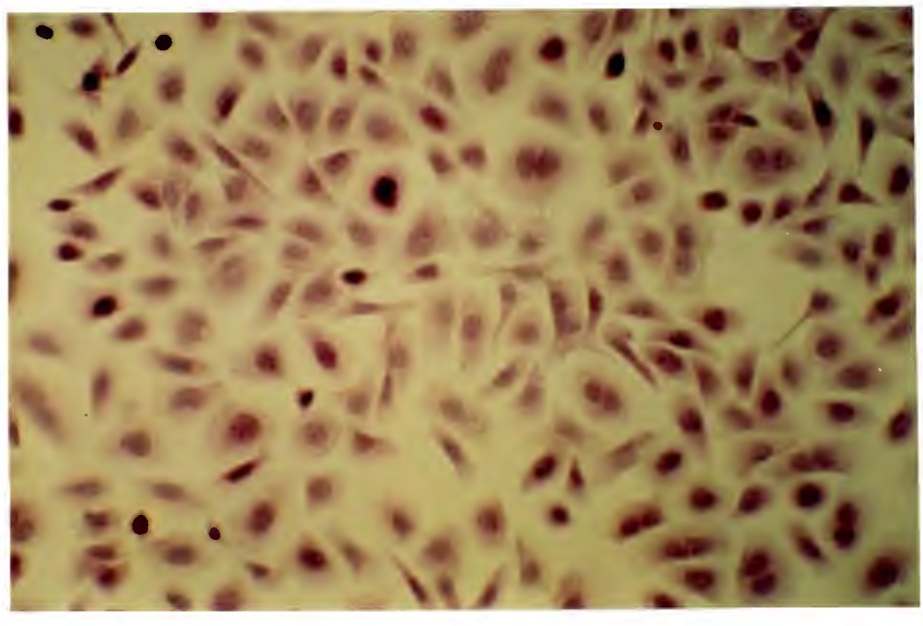


FIGURE 13

HCU18 cells stained with haematoxylin and eosin.
The cells show a typical epithelial morphology.
Magnification : 60X.

3.1.3 Transmission Electron Microscopy

Electron micrographs of each of the 3 tumor cell lines are shown in Figures 14, 15, 16 and 17. Although the presence of obvious virus-like structures could not be detected in any of the 3 cell lines, several features that have been previously described (Robinson, 1981), and that could be indicative of a viral association with these cells, were observed in the present work!

1. The extensive presence of rough endoplasmic reticulum was noted in HCU18 and HCU33 cells (Figures 14 and 15). This was occasionally dilated, and appeared to be particularly well developed in line HCU18.

2. Mitochondria and golgi bodies were apparent in all 3 lines, and were particularly prominent in HCU18 cells (Figure 14). The mitochondria were round in shape with clearly defined cristae.

3. Dense lamellar structures, or myelinoid bodies were present in all 3 cell lines. They were found in the cytoplasm of the tumor cells, and varied both in size and shape (Figure 16).

Regular nuclei, with densely staining nucleoli, were also observed in all 3 cell lines (Figures 15, 16). Numerous lipid-containing vacuoles were characteristically present in the cytoplasm of HCU39 cells (Figure 16).

A particularly interesting feature of the HCU33 line was the presence of regular arrays, observed frequently in the cytoplasm of these cells (Figure 17). They were found in association with mitochondria, and were composed of a repeated subunit structure, approximately 50nm in diameter. Paracrystalline bodies have previously been observed in the cytoplasm of HCU33 cells, in close proximity to mitochondria. These consisted of parallel arrays of fine filaments which varied in diameter (Robinson, 1981). The subunit structures demonstrated in this study appeared to be hexagonal in shape, and bounded by a double-layered membrane. Longitudinal filamentous structures were not observed.

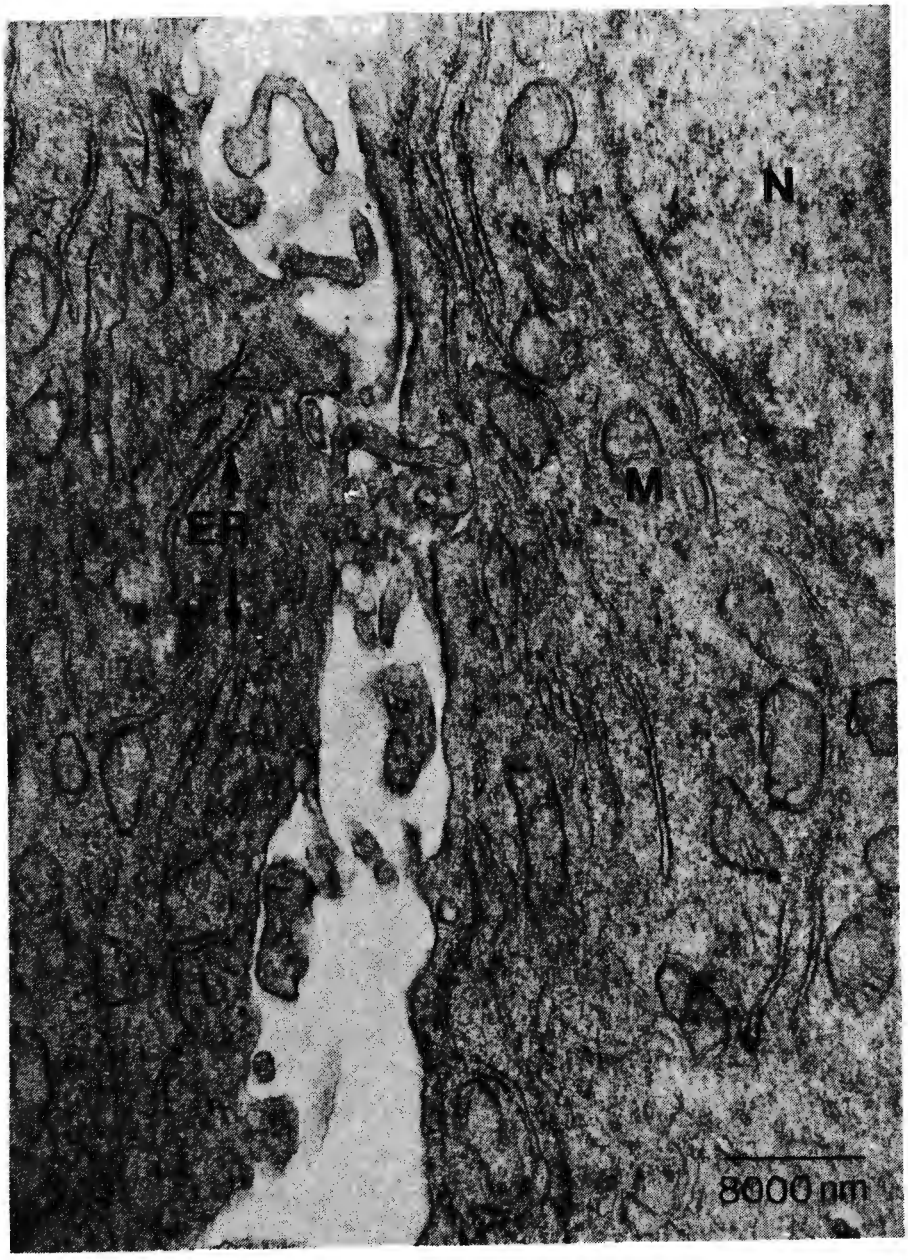


FIGURE 14

Transmission electron micrograph showing two adjacent cells of the HCU18 line. The extensive presence of well developed rough endoplasmic reticulum (ER), with attached ribosomes, is evident. Numerous mitochondria (M) are also seen distributed within the cytoplasm, and a single nucleus (N) bounded by a double nuclear membrane, is present.

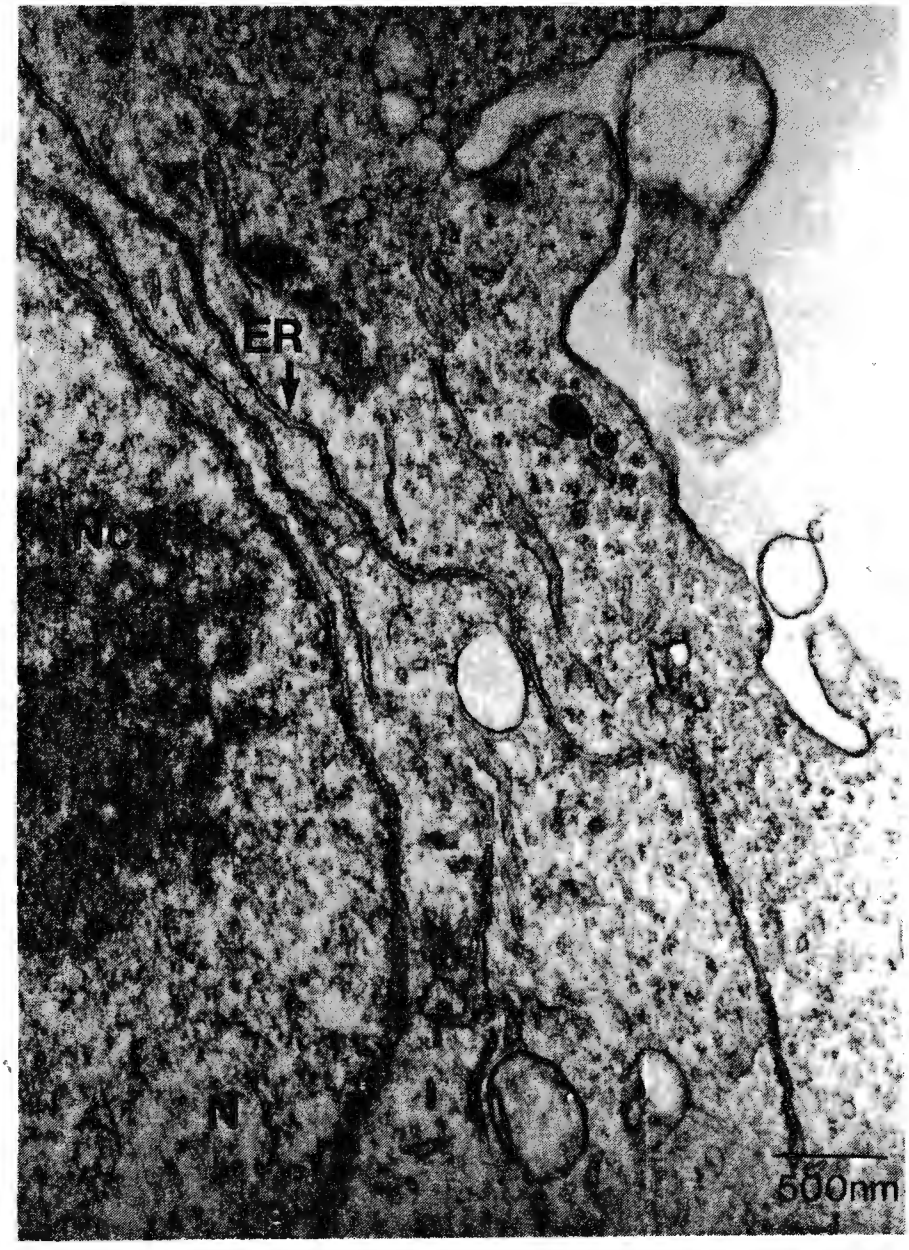


FIGURE 15

Transmission electron micrograph showing the presence of well-developed rough endoplasmic reticulum (ER) in the cytoplasm of an HCU33 cell. The nucleolus (Nc) is seen as an electron dense body, within the nucleus (N).

FIGURE 16

Transmission electron micrograph of an HCU39 cell. Electron dense lamellar structures, or myelinoid bodies (My) are present; mitochondria (m) and numerous vacuoles, some containing lipid (LV) are also evident. The large nucleus (N) contains an electron dense nucleolus (Nc).

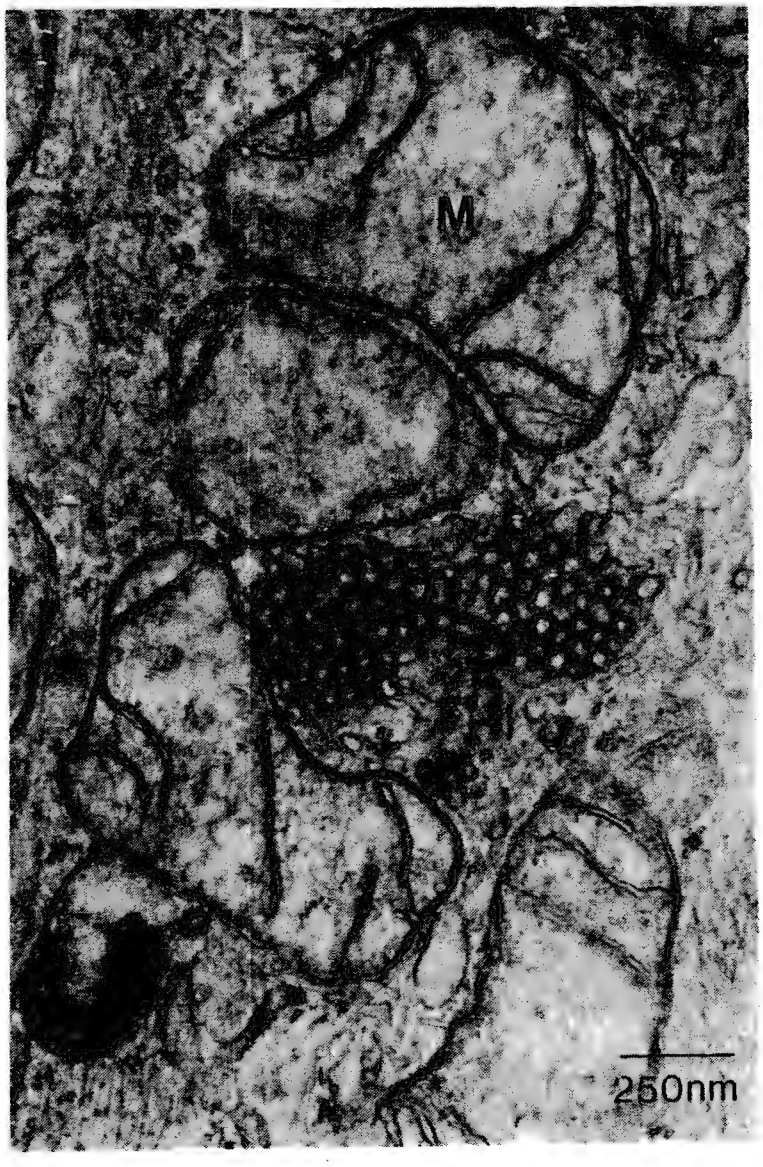


FIGURE 17

Transmission electron micrograph of a regular array, in close proximity to mitochondria (M), in the cytoplasm of an HCU33 cell. The hexagonal subunit structure (50nm in diameter), bounded by a double-layered membrane, is clearly evident.

3.1.4 Conclusion

The 3 oesophageal tumor cell lines, HCU18, HCU33 and HCU39, were shown to retain many of the phenotypic characteristics originally described for these cells (Robinson, 1981). Examination by light microscopy revealed that the cells of each line could be distinguished morphologically, and several features, characteristic of the individual lines, were observed by transmission electron microscopy.

These studies did not reveal the presence of virus-like structures in the oesophageal tumor cells. However, several of the morphological features observed could be associated with a viral activity in these cells, as follows:

1. The prominent presence of rough endoplasmic reticulum in HCU18 and HCU33 cells could reflect an increase in cellular protein synthesis due to virus replication.

2. Well-developed mitochondria and golgi bodies could also be indicative of an increased energy requirement and transport activity induced by the presence of virus.

3. The myelinoid-like bodies observed in the three tumor lines have previously been described in virus infected cells (Miyai *et al.*, 1963).

Each of these features may alternatively be related to the rapid growth and differentiation of the tumor cells.

Groups of hexagonal structures were observed in the cytoplasm of HCU33 cells. The regular subunit structure of these arrays suggests that they may represent deranged viral capsid production. They could, alternatively, be an artifact of tissue culture, or be associated with abnormal protein metabolism.

3.2 PREPARATION OF VIRAL DNA PROBES

3.2.1 Introduction

All recombinant plasmid probes were expanded in the *E.coli* HB101 (or JM107) host strain, and purified in CsCl density gradients. The viral DNA inserts were subsequently excised by digestion with the appropriate restriction enzyme, and purified for radiolabelling and hybridization analysis (Section 3.3). A total of 18 different plasmid probes were used (Table 5). These encoded viral DNA sequences from different segments of Epstein-Barr virus, human papillomavirus (types 1,5,6,8,11,16 and 18), human adenovirus (species 5,7,12 and 31), and human T-lymphotropic virus type 1.

3.2.2 Human Papillomavirus

Increasing evidence, as discussed in Section 1.2.3, points to a role for specific types of HPV in certain types of human cancer. Plasmid probes, developed from 7 different HPV types (HPV 5,8,6,11,16,18 and 1) were used. Six of these have been identified at relatively high frequency, particularly in association with specific squamous cell carcinomas (HPV 5,8,16 and 18), or in association with lesions that frequently convert to malignancy *in vivo* (HPV6 and 11).

Since the HPV genome is only on average 7.8kb in length, the entire sequence has been cloned into pBR322 for each of the different HPV types (Table 5). However, HPV5, HPV8 and HPV6 were all cloned as two separate fragments, and each of these fragments was used in this work as a separate radiolabelled HPV specific probe in hybridization tests. Viral DNA inserts released from pBR322 sequences by cleavage with *Bam*HI and/or *Eco*RI and subsequent electrophoresis in 1% agarose gels, are shown in Figure 18. Figure 18A shows HPV1 recombinant plasmid, Figure 18B, the genital HPV plasmids, and Figure 18C, the EV-associated HPV plasmids. The HPV18 DNA insert is cleaved by *Eco*RI and *Bam*HI into 3 separate fragments, 4.2kb, 2.4kb and 1.1kb in length. HPV11 has one internal *Eco*RI restriction site, and is cloned at the *Bam*HI site of pBR322. For hybridization analysis, as for all other HPV's, the entire HPV 11 insert (7.7kb) was excised and radiolabelled.

TABLE 5 : Recombinant Bacterial Plasmids & Associated Viral DNA Inserts

	Plasmid Designation	Vector	Insert	Source	Reference
I PAPILLOMA-VIRUS	pHPV 6Amp2	pBR322	5.4kb (BamHI/EcoRI)	Dr.Campo (Glasgow)	de Villiers et al.,1981
	pHPV 6K21	pBR322	2.5kb (BamHI/EcoRI)	Dr.Campo (Glasgow)	de Villiers et al.,1981
	pHPV 16	pBR322	7.2kb (BamHI/EcoRI)	Dr.Campo (Glasgow)	DÜrst et al., 1983
	pHPV 18	pBR322	7.85kb(BamHI/EcoRI)	Dr.Campo (Glasgow)	Boshart et al., 1984
	pHPV 5/48	pBR322	1.9kb (EcoRI)	Dr.de Villiers (Heidelberg)	Kremsdorf et al., 1982
	pHPV 5/9	pBR322	5.9kb (EcoRI)	Dr.de Villiers (Heidelberg)	Kremsdorf et al., 1982
	pHPV 8/4	pBR322	5.7kb (BamHI/EcoRI)	Dr.de Villiers (Heidelberg)	Pfister et al, 1981
	pHPV 8/3	pBR322	2.0kb (BamHI/EcoRI)	Dr.de Villiers (Heidelberg)	Pfister etal., 1981
	pHPV 11	pBR322(L47)[a]	7.7kb (BamHI)	Dr.Campo (Glasgow)	Gissman et al., 1982
	pHPV 1	pBR322	7.7kb (BamHI)	Dr.Burnett (Birmingham)	Heilman et al., 1980
II EPSTEIN-BARR VIRUS	pBamHIF	pBR322	7.4kb (BamHI)	Dr.Griffin (London)	Arrand et al., 1981
	pBAMHIW	pBR322	3.07kb(BamHI)	Dr.Griffin (London)	Arrand et al., 1981
	pEcoRIC	HomerI	17.17kb(EcoRI)	Dr.Griffin (London)	Arrand et al., 1981
III ADENOVIRUS	pAd12RIC	pAT153	5.5kb (EcoRI)	Dr. Bos (Leiden)	Bos et al., 1981
	pAd31	pAT153	5kb (BamHI)	Dr. Bos (Leiden)	Bos, pers. communic.
	pAd5XhoIC	pAT153	5.71kb(EcoRI/HindIII)[b]	Dr. Bos (Leiden)	Bernards et al., 1982
	pAd7clHindJ	pUC19	1.34kb(HindIII)	---	---
IV HTLV-I	pMT2	pUC12	8.5kb (SacI)	Dr.Josephs (Bethesda)	Josephs, personal communication.

a. HPV11 was first cloned into lambda phage (L47), and subsequently into pBR322. (Gissman et al., 1982a).

b. The complete 5.71kb Ad5 insert cannot be excised from pAd5 XhoIC; the 3kb EcoRI/HindIII fragment was used as a probe (Section 3.2.4.3).

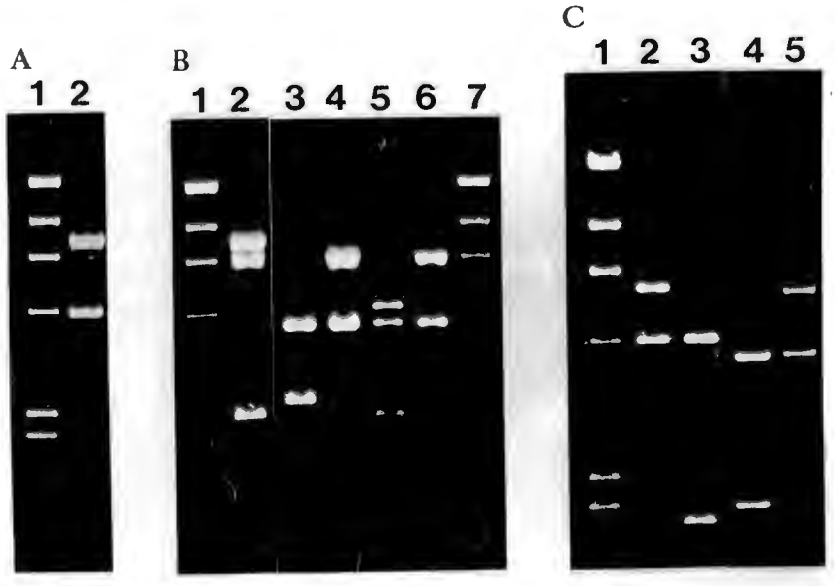


FIGURE 18

Human papillomavirus recombinant plasmid probes. Viral DNA sequences were released from pBR322 vector DNA by cleavage with BamHI and/or EcoRI (as indicated in Table 5 [pHPV11 is digested with BamHI and EcoRI]), and separated by electrophoresis in 1% agarose. Figure 18A shows pHPV1 (lane 2), Figure 18B, the genital HPV's (pHPV11, lane 2; pHPV6K21, lane 3; pHPV6Amp2, lane 4; pHPV18, lane 5; pHPV16, lane 6), and Figure 18C the EV-associated HPV's (pHPV5/9, lane 2; pHPV5/48, lane 3; pHPV8/3, lane 4; pHPV8/4, lane 5) λ DNA, digested with HindIII is indicated in lane 1 of each Figure (and lane 7 of 18B).

3.2.3 Epstein-Barr Virus

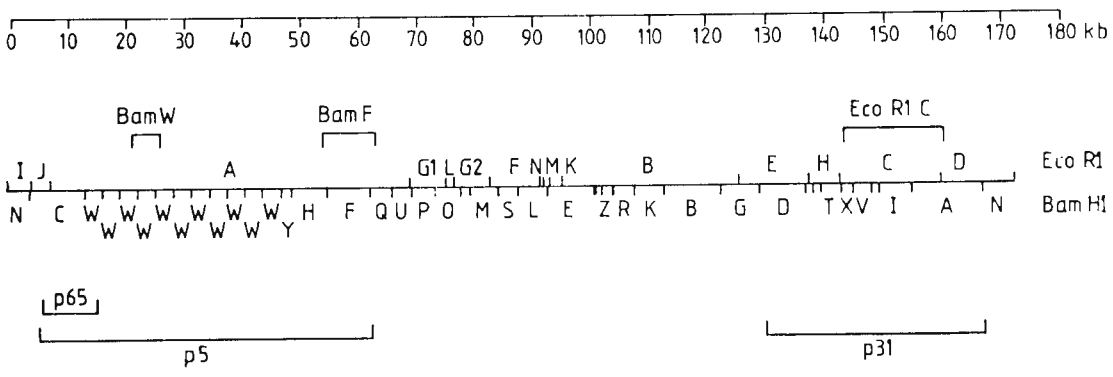


FIGURE 19

Restriction endonuclease cleavage maps (BamHI, EcoRI) of the B95-8 strain of EBV DNA. The BamHI and EcoRI restriction fragments, used as radiolabelled probes, are indicated (BamW, BamF, EcoRIC). Regions p65, p5 and p31 represent viral sequences encoding putative B-lymphocyte (p65,p5), and epithelial cell (p31) immortalizing proteins.

Three EBV viral probes were used: the BamHI W (BamW), BamHI F (BamF), and EcoRI C (EcoC) restriction fragments of the EBV genome. The genome of Epstein-Barr virus is composed of unique DNA interspersed with repetitive DNA sequences. The 3.1kb large internal repeat, or BamHIW repeat, occurs approximately 11 times in the B95-8 isolate of Epstein-Barr virion DNA (Arrand et al., 1981). This fragment, and the 7.4kb BamHI F fragment of the B95-8 EBV strain have been cloned in pBR322 at the BamHI restriction site (Arrand et al., 1981). The significantly larger 17.2kb EcoRI C fragment of EBV B95-8, has been cloned into the cosmid vector, Homer I (Arrand et al., 1981). The relative positions of these 3 fragments on the restriction enzyme cleavage map of EBV B95-8 are shown in Figure 19. The 3 clones designated p65, p5 and p31 represent EBV DNA sequences that have been cloned, and analyzed for their ability to transform cells in vitro (Griffin and Karran, 1984). While p5, which encompasses BamF and BamW DNA sequences, is immortalizing for B-lymphocytes (Griffin, personal communication) neither this clone nor the p65 clone will immortalize epithelial

cells *in vitro*. However, the clone designated p31, which includes the sequence encoded by *Eco*RI fragment C, was found to transform both monkey and human epithelial cells *in vitro* (Griffin and Karran, 1984).

Furthermore, the transforming region of p31 does not overlap with any of the 4 regions (LT1 → LT4) which are expressed in EBV immortalized B lymphocytes (Section 1.2.2.3). The protein encoded by LT1 (map position 13 → 50kb) is specifically regarded as essential for EBV induced cell transformation (Kieff *et al*, 1982).

3.2.4 Human Adenovirus

3.2.4.1 Subgenus A probes

Recombinant plasmid probes from 2 of the members of highly oncogenic subgenus A, Ad12 and Ad31, were provided by Dr. H. Bos (Leiden). Each of these plasmids encodes sequences from the entire E1 transforming region of the different adenovirus strains. Ad12 and Ad31 DNA inserts, used as labelled specific probes in this work, are shown in Figure 20A (lanes 2 and 3, respectively), separated from their pAT153 vector sequences by electrophoresis in 1% agarose.

3.2.4.2 Subgenus B probe

No probes were available to us to analyze the oesophageal tumor cells for the presence of integrated copies of the weakly oncogenic (subgenus B) adenoviruses, such as Ad7. A laboratory isolate of Ad7 (Nr2203) was therefore used as the source of a specific DNA probe. This strain, isolated from a patient with post measles pneumonia, at the Red Cross Childrens' Hospital (Cape Town), has been designated as an Ad7c1 genome type (refer to Part I).

The transforming genes of Ad7 reside in the (5') leftmost 7.8% of the viral genome, within the early region E1 (Fujinaga *et al*., 1984). This region is represented by the *Hind*III I.J restriction fragments. In this work the 1.34kb *Hind*III J fragment was cloned

into the plasmid vector pUC19 at the HindIII restriction site, and subsequently amplified for use as an Ad7 specific probe. This HindIII J fragment encodes a short segment of the E1A region and a large portion of E1B (refer to Figure 10). It was regarded to be suitable for use as a probe since the expression of both the E1A and the E1B genes is required for the oncogenicity of Ad7.

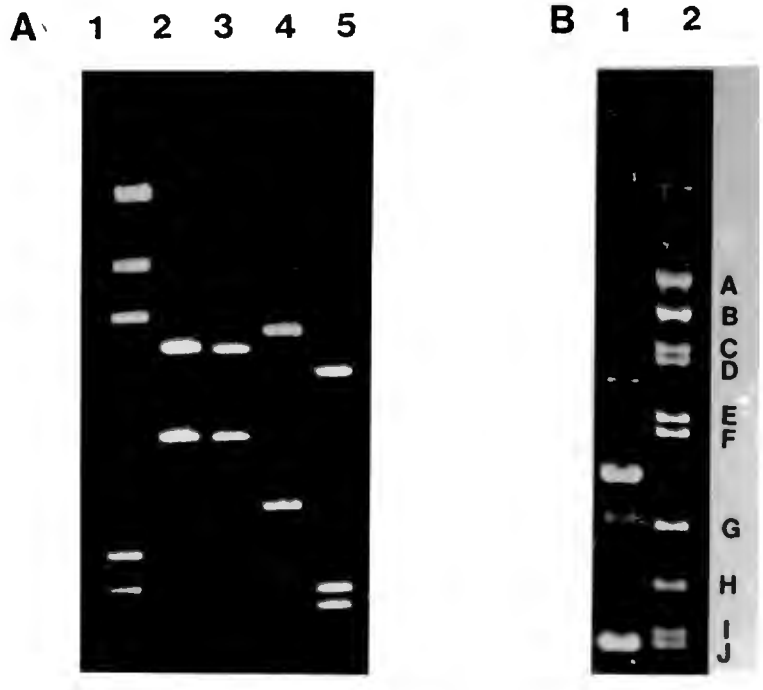


FIGURE 20

Human adenovirus recombinant plasmid probes.

A: Ad12, Ad31 (subgenus A) and Ad5 (subgenus C) recombinant plasmids, analyzed by electrophoresis in 1% agarose.
Lane 1 = λ HindIII digest; 2 = pAd12RIC, EcoRI digest; 3 = pAd31, BamHI digest; 4 = pAd5XhoIC, EcoRI/HindIII digest; 5 = pAd5XhoIC, SacI digest.

B: Plasmid pAd7c1HindJ, digested with HindIII and separated by electrophoresis in 1% agarose (lane 1). Lane 2 shows a HindIII digest of Ad7-2203 (subgenus B); fragment sizes (kb) in decreasing order of molecular weight are: 7.53 (A), 5.97 (B), 4.85 (C), 4.56 (D), 3.44 (E), 3.33 (F), 2.10 (G), 1.70 (H), 1.38 (I), 1.34 (J).

The procedure used in this study for cloning the Ad7c1 HindIII J fragment into pUC19, was essentially as described in Part I, Section 2.6 for the cloning of Ad7c1 EcoRI D. However, in this case the HindIII J fragment was first purified from the remaining viral sequences prior to the ligation of vector and insert DNA. This was done by electrophoretic separation and isolation in a low

melting temperature agarose gel. Thirty-six recombinant bacterial clones were derived from the ligation of 75 -100ng of insert DNA - Ad7c1 HindIII J fragment - and an equimolar quantity of pUC19 DNA (150-200ng). Twelve of these clones were screened, and found to harbour an identical plasmid (3.94kb) comprising pUC19 DNA (2.6kb) and a 1.34kb Ad7c1 insert (HindIII J fragment) cloned at the HindIII restriction site (Figure 20B). This plasmid, which we designate pAd7c1HindJ, was further amplified in E.coli JM107, and purified for use in subsequent hybridization tests.

3.2.4.3 Subgenus C probe

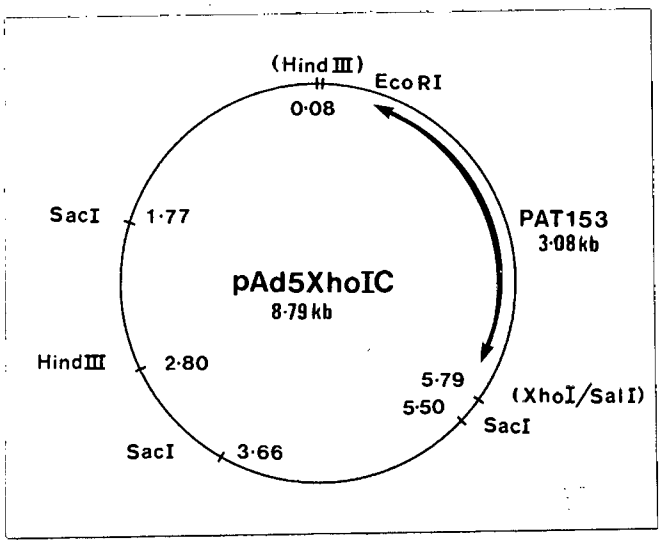


FIGURE 21

The Ad5 recombinant plasmid, pAd5XhoIC. The XhoIC fragment (0.08-5.79kb) of Ad5 was cloned into the 3.08kb SalI/HindIII fragment of pAT153 (Bernards et al., 1982). Positions of restriction sites in the Ad5 genome are indicated (in kb) by the position of the site relative to the left end of the viral DNA.

The recombinant plasmid, pAd5XhoIC, provided by Dr. H. Bos (Leiden), is shown in Figure 21. The XhoIC fragment of Ad5, cloned into the plasmid vector pAT153, encodes the entire E1 region, which falls within the first 4 kilobases of the viral genome. The restriction enzyme sites indicated in brackets represent cutting sites that were destroyed in the procedure used for cloning this fragment (Bernards et al., 1982). Digestion with SalI and XhoI will yield cohesive termini that can be ligated together, but form

a hybrid site that is not recognized by either enzyme. While incubation with the enzymes SalI and XhoI had no effect on the intact plasmid, digestion with HindIII served to linearize the 8.79kb plasmid (results not shown). This intact HindIII cutting site clearly represents that site at map position 2.8kb on the viral genome (and not at 0.08kb) since double digestion with EcoRI and HindIII gave rise to two fragments 5.7kb and 3.0kb in length (Figure 20A, lane 4). The smaller of these EcoRI/HindIII fragments essentially encodes the Ad5 HindIII G restriction fragment, which will transform cells *in vitro* (van der Eb and Bernards, 1984), and a small 0.03kb overlap of pAT153 DNA. This sequence contains region E1A and about half of region E1B, and was used in this study as a labelled specific Ad5 probe for hybridization to oesophageal tumor cell DNA.

3.2.5 Human T-lymphotropic virus type I

Although the retrovirus HTLV-I is strictly regarded as a T-lymphotropic agent, T-lymphocytes have been reported to infiltrate oesophageal tissue, especially as a consequence of papillomavirus infection (Campbell, 1987, personal communication). The pMT2 clone is a nearly full length provirus cloned into the plasmid vector pUC12 at the SacI restriction site. This complete sequence was excised and used as a radiolabelled DNA probe, to screen the 3 tumor lines for integrated HTLV-I DNA.

3.3 HYBRIDIZATION ANALYSIS

3.3.1 Introduction

The 10 different HPV specific probes that were used were derived from 7 of the 41 HPV types that have thus far been identified. All of these probes were tested in dot blot hybridization tests, under conditions of high stringency. The human papillomaviruses are a remarkably heterogeneous group characterized by a lack of nucleic acid homology - the DNA's of different types generally show only a few percent cross-hybridization under stringent hybridization conditions (McCance, 1986). However, all of the HPV's identified to date will cross-hybridize under conditions of reduced stringency (Kahn et al., 1986). It is this property that has facilitated the recent identification of new HPV types in tissue samples and cell lines (Kawashima et al., 1986; Lorincz et al., 1986; Brandsma et al., 1986; Ostrow et al., 1987; Boshart et al., 1984). By using high specific activity probes, developed from cloned HPV DNA, under conditions of reduced stringency, DNA from one HPV type can be used to detect another, allowing it in turn to be cloned and subsequently used at high stringency. Therefore, to detect the possible presence of a further, and possibly as yet unidentified HPV type, in the oesophageal tumor cell DNA, an HPV18 probe was used in Southern blot hybridization tests under conditions of reduced stringency. Both HPV18 and HPV16 are regarded as particularly oncogenic, and represent the only 2 HPV types whose viral DNA is frequently found integrated in the host cell genome in malignant tumors. Both types, but predominantly HPV18, are also found in an integrated state in cell lines derived from cervical carcinomas (McCance, 1986).

The 3 EBV probes that were used (BamW, BamF and EcoC) were derived from the B95-8 strain of EBV. Although EBV isolates from different populations have been found to differ, these strain differences are largely insignificant (Dambaugh et al., 1986). Therefore, each of the probes, tested under conditions of high stringency, should be specific for any particular EBV isolate that may be present.

Within the human adenovirus group, 41 different species have been identified to date. However, in contrast to the 41 HPV types, the different members of each subgenus show a large degree of nucleic acid homology (Part I, Table 1). Therefore DNA probes from Ad12 and Ad31 (subgenus A), Ad7 (subgenus B), and Ad5 (subgenus C) were used in this work as representative adenovirus probes for their respective subgenera, and were tested at high stringency in DNA dot blot and Southern blot hybridization tests. The HTLV-I specific DNA probe was also tested by dot blot hybridization at high stringency.

3.3.2 Human Papillomavirus

3.3.2.1 Dot blot analysis

Cellular DNA, from tumor cell lines HCU18, HCU33 and HCU39, was screened by dot blot hybridization (at high stringency) to each of 10 HPV specific probes (Table 5). The viral DNA inserts, excised from their respective recombinant plasmids, were labelled to a high specific activity, in the range of (5-15) x 10^7 dpm/μg DNA. Dilutions of cellular DNA in the range 0.5 to 4.0 μg, were tested. Three negative controls were also included. These were cellular DNA from two laboratory cell lines, HeLa and HF1/81 (a human embryo fibroblast cell strain), and human placental DNA or foetal liver DNA. For HPV18, HeLa represented a positive control since this cell line contains HPV18 integrated into the cellular genome at 10-50 copies per cell (Schwarz et al., 1985). The recombinant plasmid, encoding the particular viral insert used as a radiolabelled probe, was spotted onto the membrane (in the range 10-80pg) as a positive control in each case.

Results obtained for each of the 10 HPV probes after exposure to X-ray film for 10-14 days, are illustrated in Figure 22A,B and C. These results clearly indicate the absence of human papillomavirus DNA, specifically of types 1,5,6,8,11,16 and 18, from the cellular DNA of the 3 tumor cell lines HCU18, HCU33 and HCU39.

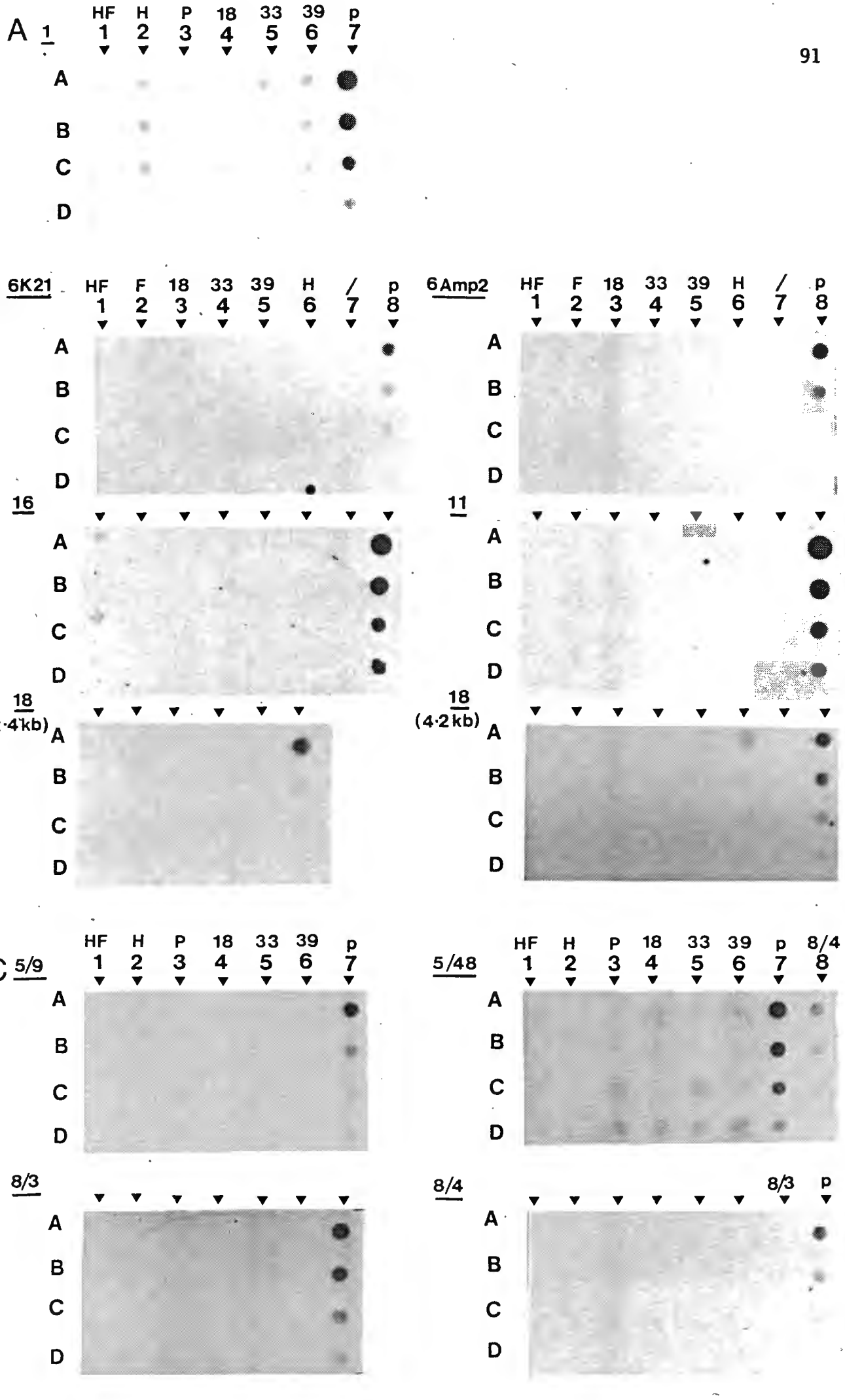
Weak signals were evident in certain lanes for HPV5 and HPV1, but since these were significantly weaker than the respective plasmid

FIGURE 22

Autoradiographs of dot blot hybridization tests for the presence of HPV DNA in tumor and control DNA samples. Figures 22A-C show results obtained for HPV1(A), the genital HPV's, HPV 6, 11, 16 and 18(B), and EV-associated HPV5 and 8(C). Specific viral DNA probes (described in Section 3.2.2) are indicated alongside each figure.

Serial twofold dilutions of the following human DNA samples, starting with 4µg, are represented in rows A-D: human fibroblast, strain 1/81, (HF), foetal liver (F), human placenta (P), HeLa (H), and HCU18, HCU33 and HCU39 (18, 33, 39). A maximum of 80pg, and serial twofold dilutions (rows A-D) of the respective homologous HPV plasmids (p) are represented in lanes 7 and 8, as indicated. Heterologous HPV8 plasmid DNA (8/3 or 8/4) is similarly represented in lanes 7 and 8 of Figures 22C-8/4 or 5/48. Lanes that were left open are indicated by /.

Each of the probes was labelled to a specific activity of (5-15) x 10^7 dpm/µg DNA. Exposure to X-ray film (Kodak XOmat MA) was for 10-14 days.


A 1
HF 1
H 2
P 3
18 4
33 5
39 6
p 7
A
B
C
D
B 6K21
HF 1
F 2
18 3
33 4
39 5
H 6
/ 7
p 8
16
18 (2·4kb)
6Amp2
11
18 (4·2kb)
C 5/9
5/48
8/4
8/3

controls, and did not show a clear dilution in the signal from 4 to 0.5ng, they were interpreted as non-specific background hybridization of the probe DNA.

HPV8 recombinant plasmid DNA (10-80pg) was included in lane 7 of Figure 22C-8/4 (HPV8/3) and in lane 8 of Figure 22C-5/48 (HPV8/4). Positive signals shown in the latter are thus a result of cross-hybridization between these closely related viruses, which have 17% of their nucleotide sequences in common (Kremsdorf et al, 1982).

Control HeLa DNA was strongly positive when the 2.4kb EcoRI/BamHI fragment of HPV18 was used as a probe (Figure 22B). When the 4.2kb EcoRI/BamHI fragment was used the signal was significantly weaker, since only a small fraction of this HPV18 DNA fragment is integrated in the HeLa cell genome (Schwarz et al., 1985).

3.3.2.2 Southern blot analysis

Although the 3 tumor cell lines HCU18, HCU33 and HCU39, were clearly negative for HPV18 when tested by dot blot hybridization, they were also tested by Southern blot hybridization to HPV18 DNA, but at low stringency (T_m-42°) in order to detect the possible presence of a related HPV type that would not cross-hybridize under conditions of high stringency.

Cellular DNA was extracted from the 3 cell lines, and from positive and negative controls. These included the HeLa line, the human embryo fibroblast strains HF1/81 and HF4/81, and human placenta. DNA (10ng), was digested with EcoRI and BamHI, and separated in a 0.8% agarose gel, as shown in Figure 23A. The presence of fast migrating satellite bands, derived from repetitive human DNA sequences, clearly shown in the figure, are indicative of complete digestion by the enzyme(s).

The DNA was subsequently transferred to Hybond-N nylon membrane, and probed at low stringency (T_m-42°C) with linear HPV18 DNA. A mixture of two subgenomic fragments of HPV18 DNA, the 2.4kb BamHI/EcoRI fragment and the 1.1kb BamHI fragment, was used as a

radiolabelled probe. These complete fragments are both integrated in the HeLa cell genome. While the smaller of the two represents a noncoding regulatory region of HPV18 DNA, the 2.4kb fragment includes open reading frames E1, E6 and E7, which are thought to encode HPV transforming functions (Section 1.2.3.2). The membrane was hybridized and washed at low stringency (T_m-40°C), and exposed to X-ray film overnight. Strong signals were evident in each of the 7 DNA lanes but distinct bands could only be detected in the HeLa control (results not shown). Since the background hybridization was especially strong, the membrane was re-washed a further 2 times at higher stringency (T_m-25°C). After exposure to X-ray film for 1-10 days 3 bands became clearly visible in HeLa DNA, but none were observed in lanes 2-7, even after longer exposure times (Figure 23B). The 2 HPV18 DNA fragments used as probes are indicated by arrows at the respective cellular bands which they recognize. This result corresponds to the pattern derived by Schwarz et al. (1985) for the integration of HPV18 DNA into the HeLa cell genome.

These results cannot conclusively exclude the presence of HPV18-related sequences in the tumor DNA. The background hybridization was particularly strong in lanes 5-7 and HPV specific signals could well have been obscured in this region. A weakly homologous (and possibly low copy number) sequence may initially have been masked by background hybridization, and further not detected after the second wash due to probe dissociation at this higher stringency (T_m-25°C). This work could have been improved by including additional controls: standard reconstructions, representing various copy numbers per cell of a heterologous HPV type, would have facilitated the determination of detection limits at different stringencies.

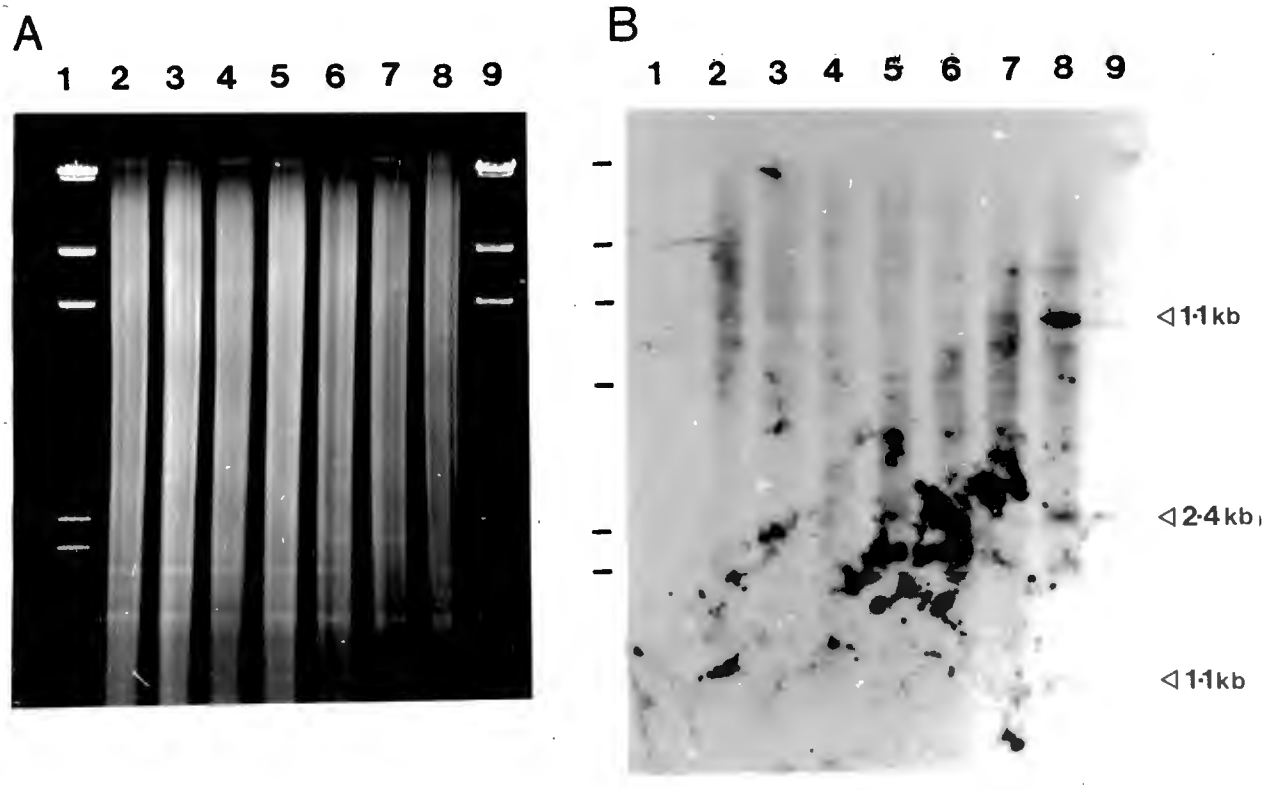


FIGURE 23

Low stringency hybridization tests using HPV18 DNA probes. Control and oesophageal tumor cell DNA was cleaved with EcoRI and BamHI and subjected to electrophoresis in 0.8% agarose (A). The DNA was transferred to Hybond-N nylon membrane, and probed with a mixture of two HPV18 specific DNA fragments (2.4kb and 1.1kb BamHI/EcoRI restriction fragments), at low stringency (T_m -42°C). The membrane was washed initially at T_m -40°C, and subsequently at T_m -25°C and exposed to X-ray film for 10 days (B). The specific activity of the HPV18 probe was 8 x 10^7 dpm/µg. Subgenomic BamHI/EcoRI fragments of HPV18 DNA used as labelled specific probes are indicated by arrows at the respective cellular bands which they recognize.

Lane 1 = λ HindIII digest; 2 = HF4/81; 3 = foetal liver DNA; 4 = placental DNA; 5 = HCU18; 6 = HCU33; 7 = HCU39; 8 = HeLa; 9 = λ HindIII digest.

3.3.2.3. Conclusion

Dot blot hybridization tests have clearly indicated that DNA from the particular human papillomavirus strains tested, namely HPV1, HPV5, HPV6, HPV8, HPV11, HPV16 and HPV18, is not present in integrated, or episomal form in the cellular DNA of the three oesophageal tumor cell lines HCU18, HCU33 and HCU39. Hybridization tests for Epstein-Barr virus (refer to Section 3.3.3.1) have shown that a single copy per cell of integrated EBV DNA can be detected (by Southern or dot blot hybridization) at an estimated level of 3pg per microgram of cellular DNA, by using a 7.4kb viral probe.For the HPV assays, single copy positive control DNA was not available. However, under similar conditions of stringent hybridization, one should also detect a single copy of HPV DNA, 7-8kb in length, and present at 3pg/µg of cellular DNA. The plasmid positive controls for each of these dot blot tests have clearly demonstrated the detection of viral DNA inserts in the range of 5-50pg. Therefore, the presence of 1 copy of HPV DNA (of 7-8kb) should be within the limits of detection of this assay. However, the presence of a partially integrated genome cannot be excluded; a single copy, less than 3kb in length, would not be detected at less than 1pg per microgram of cellular DNA.

Southern blot hybridization, at low stringency, to an HPV18 specific probe, has further failed to demonstrate the presence of viral DNA, either of this HPV type, or of any related HPV strain. Since HPV18 does exist in an integrated state in HeLa cell DNA, these results also indicate that the 3 oesophageal cell lines examined in this study are not contaminated with HeLa cells.

3.3.3 Epstein-Barr Virus

For the EBV series of tests positive controls were provided by cellular DNA extracted from two well-known lines, Raji and Namalwa. Both of these lines were established from patients with African B-cell (Burkitt's) lymphoma. The Raji cells contain multiple copies of the EBV genome, present largely in the form of extrachromosomal episomal DNA (Anvret *et al.*, 1984). Namalwa cells, however, contain a single copy of the viral DNA which

exists exclusively integrated within the host cell genome (Matsuo et al., 1984). Human placental DNA, and DNA extracted from the laboratory HeLa cell line, were included as negative controls.

3.3.3.1 Hybridization to EBV BamHI F and BamHI W restriction fragments

3.3.3.1.1 Southern blot hybridization

The BamW and BamF probes were tested by hybridization to a Southern blot of BamHI-digested human DNA extracted from the 3 oesophageal tumor cell lines (HCU18, HCU33 and HCU39) and from the positive and negative controls described above. The different DNA samples (10µg), digested with BamHI, were separated by electrophoresis in 0.8% agarose. Forty picograms of BamHI digested EBV recombinant plasmids, pBamHIF and pBamHIW were included in lanes 10 and 11 of the gel as additional controls.

Following stringent hybridization of a Southern blot of this gel to the BamF probe (Figure 24), no hybridization to the DNA of cell lines HCU18 (lane 5), HCU33 (lane 6), or HCU39 (lane 7), was seen. In contrast, strong signals were obtained from EBV-positive Raji and Namalwa cells (lanes 2 and 3, respectively) and from the plasmid BamHIF restriction fragment in lane 10. Hybridization to lambda phage DNA, evident in lanes 1 and 9 is thought to be due to lambda DNA contamination of the probe, which could have occurred in the process of purifying the BamF fragment from vector sequences, prior to radiolabelling.

Based upon a human genome of 3×10^9 base pairs, 1 copy of EBV DNA per diploid genome is therefore equivalent to 25pg of EBV BamHI F (7.4kb) in 10µg of cellular DNA. Since 40pg of recombinant plasmid pBamHIF contains 25pg of EBV BamHI F restriction fragment, one should detect this sequence at a similar level in Namalwa DNA (10µg) and the plasmid control. Signals shown in lanes 3 and 10 (Figure 24) thus correlate well with the predicted results, and furthermore clearly indicate that the minimum level of detection that we can achieve using this system is 1 copy, or less than 1 copy of viral DNA per cell.

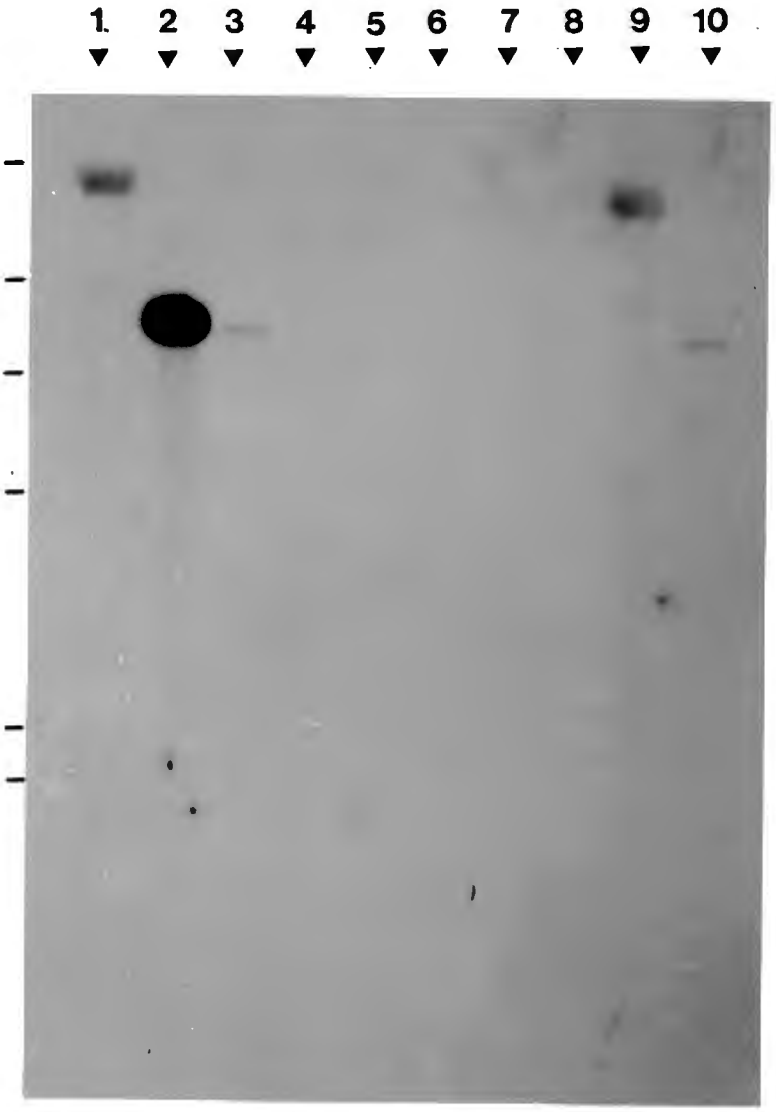


FIGURE 24

Autoradiograph of Southern blot hybridization tests for the presence of EBV (BamHI F fragment) in tumor and control DNA samples. Cellular DNA (10µg) digested with BamHI was separated by electrophoresis in 0.8% agarose. After transfer to Hybond-N nylon membrane, it was hybridized to 1µg of radiolabelled EBV - BamF DNA (specific activity, 8 x 10^7 dpm/µg) and exposed to X-ray film for 10 days.

Lane 1 = λ HindIII digest; 2 = Raji; 3 = Namalwa; 4 = HeLa; 5 = HCU18; 6 = HCU33; 7 = HCU39; 8 = human placental DNA; 9 = λ HindIII digest; 10 = 40pg of pBamHIF; digested with BamHI. The positions of λ HindIII restriction fragments are indicated.

Probe DNA was subsequently removed from the membrane by stringent washing, as described in Section 2.9. Further stringent hybridization of the BamW probe again revealed no significant signal for any of the 3 oesophageal tumor cell lines HCU18, HCU33 and HCU39 (Figure 25, lanes 5,6 and 7, respectively). Strong signals were obtained for Raji (lane 2), Namalwa (lane 3) and pBamHIW (lane 11) controls. However, these bands in lanes 2 and 3 were much stronger than those detected in Figure 24, due to the fact that the BamHIW restriction fragment occurs as a repeated sequence in the EBV genome; it is repeated 11 times in the B95-8 strain of EBV. (Baer et al., 1984). Strong hybridization of BamW to flanking BamHI fragments C (9.22kb) and Y (1.84kb) was also observed in Raji DNA, and in Namalwa DNA, after a longer exposure time. This could result from strain differences in this region of the EBV genome; the BamHI WYH and EcoRI C sequences represent two regions where such differences have been noted (Baer et al., 1984). Additional bands evident in lane 2 are due to incomplete digestion by the enzyme; one of these (indicated by an arrow), however, represents hybridization to the BamF probe, which was not completely removed in the previous washing step.

The very faint bands apparent in lanes 5 (~ 4.2kb), for HCU18 DNA, and 8 (~ 2.7kb), for human placental DNA, are not regarded as significant since they do not migrate at, or near, the 3.07kb position and are much weaker than any of the controls in lanes 2, 3 or 11. The EBV genome has been reported to exhibit a limited degree of homology with human DNA, and the BamW fragment, itself a repetitive sequence, was shown to be homologous to an Alu repetitive sequence in mammalian chromosomal DNA (Arrand et al., 1983). These results can thus be explained in terms of the non-specific binding of the BamW probe to HCU18 and placental DNA sequences.

Hybridization to λ DNA sequences, presumably, as for BamF, a result of λ contamination of the probe DNA, was again detected (lanes 1 and 9).

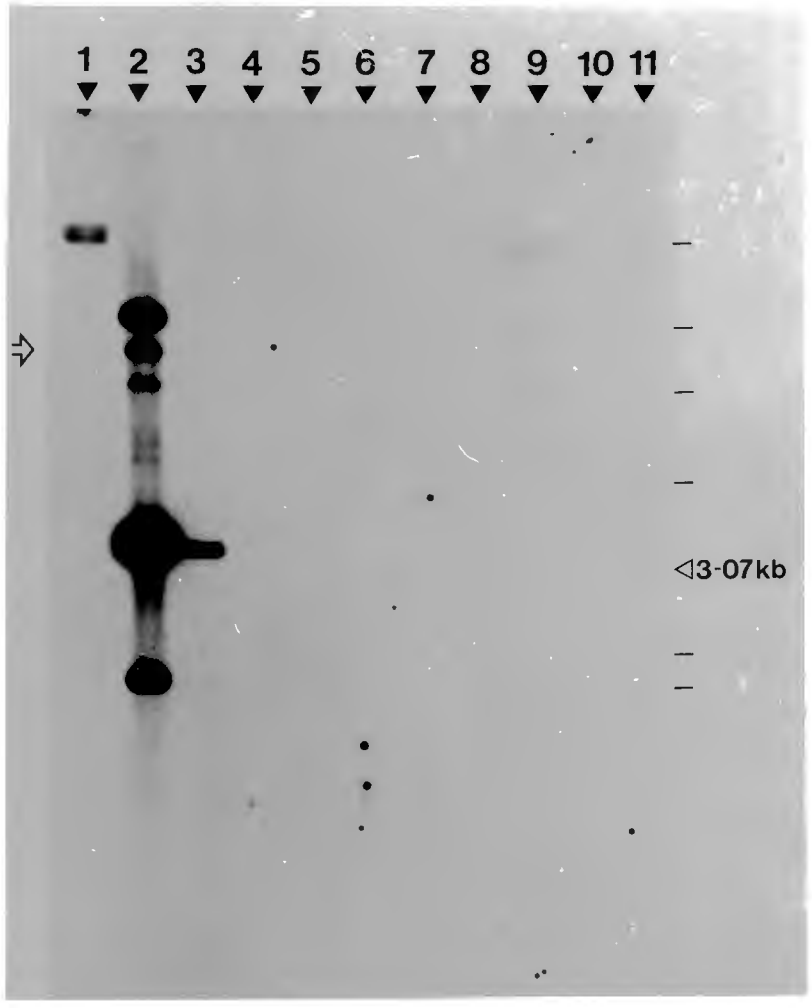


FIGURE 25

Autoradiograph of Southern blot hybridization tests for the presence of EBV (BamHI W fragment) in tumor and control DNA samples. The Southern blot, shown in Figure 24, was re-probed with 1ng of radiolabelled EBV - BamW DNA (specific activity, 1 x 10^8 dpm/ng), and exposed to X-ray film for 5 days.

Lane 1 = λ HindIII digest; 2 = Raji; 3 = Namalwa; 4 = HeLa; 5 = HCU18; 6 = HCU33; 7 = HCU39; 8 = human placental DNA; 9 = λ HindIII digest; 10 = pBamHIF; 11 = 40pg of pBamHIW, digested with BamHI.
λ HindIII size markers are shown. Hybridization to residual EBV BamF probe, in lane 2, is indicated by an arrow.

3.3.3.1.2 Dot blot hybridization

The tumor cells were further tested by dot blot hybridization to the EBV BamF probe. The detection of a single copy viral genome may be inhibited in this assay due to masking by surrounding cellular DNA. In Southern blot hybridization tests, where DNA fragments are separated by electrophoresis, this restriction should not be as apparent.

Figure 26 (lane2) clearly demonstrates the detection of single copy EBV BamHI F at a level of 3-4pg per microgram of cellular DNA: the intensity of hybridization to 4µg of Namalwa DNA (lane 2, A) is similar to that of 20pg of plasmid control (lane 10, C). No hybridization to oesophageal tumor DNA (lanes 7-9), or negative control DNA (lanes 3-6) was observed. Thus, from this result, it would appear that the sensitivity of dot blot hybridization is comparable to that of Southern blot hybridization (Section 3.3.3.1.1) for the detection of single copy DNA in mammalian genomes.

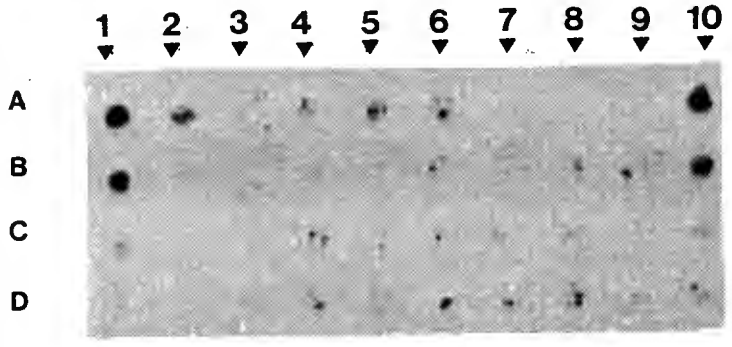


FIGURE 26

Dot blot hybridization of tumor and control DNA's to an EBV BamF probe. Serial twofold dilutions of human DNA (4-0.5µg) and pBamHIF (80-10pg) were applied to wells A-D.

Lane 1 = Raji; 2 = Namalwa; 3 = HPB-ALL; 4 = human fibroblast DNA (4/81); 5 = HeLa; 6 = foetal liver DNA; 7 = HCU18; 8 = HCU33; 9 = HCU39; 10 = pBamHIF.

3.3.3.2 Hybridization to EBV EcoRI C restriction fragment

Cellular DNA extracted from the 3 cell lines, and control specimens, was digested to completion with EcoRI, and separated by electrophoresis in 0.8% agarose. A Southern blot of this gel was probed at high stringency with the EBV EcoC probe, and the result, as shown in Figure 27, demonstrates significant hybridization of the probe only in lanes 2,3 and 10, for Raji DNA, Namalwa DNA, and the homologous fragment of pEcoRIC (40pg). The faster migration of this plasmid band in lane 10, when compared to the respective sequences in Raji or Namalwa DNA can be explained by the fact that a deletion of ~ 13.6kb is present in the EcoRI C fragment of EBV B95-8, at map position 152kb (Baer et al., 1984). A very faint band was detected in lane 5 (HCU18) co-migrating with the EcoRI C sequence of EBV B95-8 (lane 10). This, however, was regarded as insignificant, and was not analyzed further, for the following reasons:

1. Faint hybridization was only detected in 1 of the 3 cell lines; the presence of this sequence is thus not likely to play a role in the genesis of malignancy.

2. No hybridization to BamF or BamW probes was detected (Section 3.3.3.1), thus excluding the presence of episomal EBV DNA in this line. The whole EBV genome is normally detected in transformed cell lines and tumor tissue, whether present in episomal or in integrated form (Dambaugh et al., 1986).

Although the EBV EcoRI C fragment (17.17kb) is much larger than the BamHI F fragment (7.4kb), the signal shown for the Namalwa control is much weaker than that obtained with the BamF probe (Figure 24, lane 3). This can be explained by the lower efficiency, both in transfer and in radiolabelling, of relatively large restriction enzyme fragments. (The specific activity of the EcoC probe was 1.0×10^7 dpm/µg DNA). The strong hybridization to λ DNA in lanes 1 and 9 could be a result of contaminating λ DNA, and/or cosmid vector DNA sequences, in the EcoC probe.

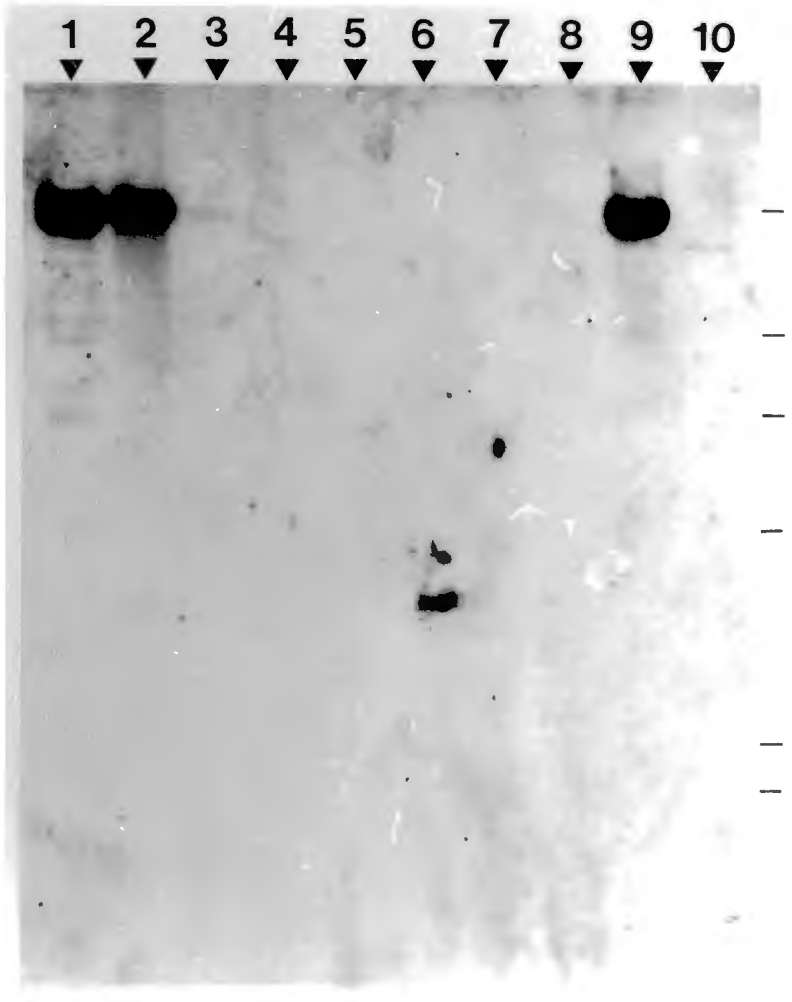


FIGURE 27

Autoradiograph of Southern blot hybridization tests for the presence of EBV (EcoRI C fragment) in tumor and control DNA samples. Sample DNA (10μg) digested with EcoRI, was separated by electrophoresis in 0.8% agarose and immobilized on Hybond-N nylon membrane. The membrane was probed with an EBV EcoC probe (specific activity 1 x 10^7 dpm/μg) and exposed to X-ray film for 14 days.

Lane 1 = λ HindIII digest; 2 = Raji; 3 = Namalwa; 4 = HeLa; 5 = HCU18; 6 = HCU33; 7 = HCU39; 8 = human placental DNA; 9 = λ HindIII digest; 10 = 40pg of pEcoRIC, digested with EcoRI. λ HindIII size markers are indicated.

3.3.3.3 Conclusion

These results have shown that the EBV genome is not stably integrated (or present episomally) in the cellular DNA of the 3 oesophageal tumor cell lines, HCU18, HCU33 and HCU39. Probes derived from 3 EBV restriction fragments were tested: BamHI W and BamHI F include sequences represented by the p5 region of the EBV genome, encoding putative B-lymphocyte immortalizing protein(s), while EcoRI C includes sequences represented by p31 which is immortalizing for epithelial cells (Griffin and Karran, 1984). Although hybridization to the EcoC probe was detected in line HCU18, the possible presence of this fragment in only 1 of the 3 tumor cell lines was not regarded as significant.

Since EBV exists as a single copy integrated in the Namalwa cell genome, these results have further shown that the detection limit of our system (under conditions of high stringency hybridization) is 1 copy, or less than 1 copy, of viral DNA per cell.

3.3.4 Human adenovirus

The 3 oesophageal tumor lines (HCU18, HCU33, HCU39) were analyzed by dot blot hybridization to 4 different adenovirus probes. The results, as shown in Figure 28A-C were interpreted as follows:

1. Subgenus A : Ad12 (EcoRI C probe) and Ad31 (BamHI probe) demonstrated hybridization only in lanes 1 for their respective plasmid controls.

2. Subgenus B : Ad7 (HindIII J probe) showed significant hybridization for positive control - Ad7 viral DNA (strain 2203) diluted in the range 50 - 400 pg - in lane 1. Weak signals were also apparent in lanes 2-6, for tumor and control DNA samples.

3. Subgenus C : Ad5 (HindIII G probe) hybridized strongly to the plasmid control in lane 1. Weak signals were also detected for HCU18 (lane 4) and HCU39 (lane 2) DNA, and for negative controls (HF, HeLa) in lanes 6 and 7.

In order to confirm the fact that the inconclusive results, as shown in Figures 28B and C, were negative, the 3 tumor lines were further tested by Southern blot hybridization to Ad5 and Ad7 specific probes. Cellular DNA (10µg), from the same control and test specimens, was digested with SacI and transferred to Hybond-N nylon membrane. Hybridization to an Ad5 HindIII J probe showed no signal for tumor and control human DNA's (Figure 28D, lanes 2-7) and strong signals only in lane 1 for the plasmid pAd5XhoIC control (100pg). Similar probing of this membrane with the Ad7 HindIII J probe showed no hybridization, even after a 3 week exposure time (results not shown).

Since the human adenoviruses have been reported to exhibit some homology to human DNA (Arrand et al., 1983), the weak signals observed in Figures 28B and C are accepted to represent non-specific hybridization of the Ad5 and Ad7 DNA sequences. Arrand et al (1983) have demonstrated that specific regions of the adenovirus genome have close homology to human DNA sequences. It is of interest that subgenus B and C, but not subgenus A, members

exhibited such homology within their respective transforming regions.

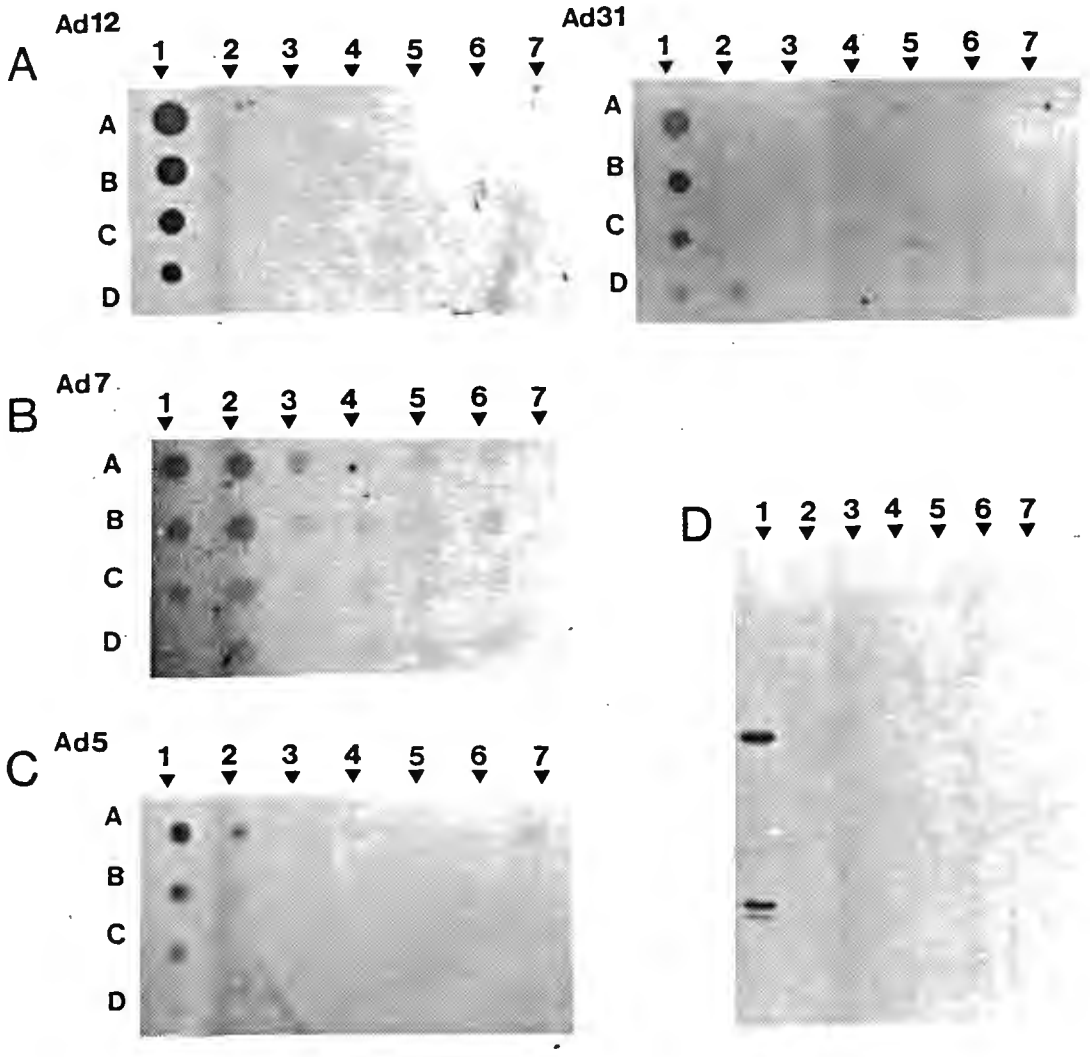


FIGURE 28

Autoradiographs of dot blot (A-C) and Southern blot (D) hybridization tests for the presence of adenovirus DNA in tumor and control DNA samples. Probes from Ad12 and Ad31, of subgenus A (A), Ad7 of subgenus B (B) and Ad5 of subgenus C (C and D) were used, as described in the text. Serial twofold dilutions (4-0.5ng) of the following human DNA samples are represented in rows A-D: HCU39 (lanes 2), HCU33 (lanes 3), HCU18 (lanes 4) human placental DNA (lanes 5), HeLa (lanes 6), HF 4/81 (lanes 7).

A maximum of 80pg and serial twofold dilutions (rows A-D) of recombinant plasmid DNA's are represented in lanes 1 of Figures 28A and C. Similar dilutions of Ad7 DNA (strain 2203), in the range 50-400pg are represented in lane 1 of Figure 28B. SacI digested pAd5XhoIC DNA (100pg) is shown in lane 1 of Figure 28D, as a positive control.

Probes were labelled to a specific activity of (5-10) x 10^7 dpm/ng DNA. Exposure to X-ray film was for 10-14 days.

3.3.4.1 Conclusion

The 3 oesophageal cell lines (HCU18, HCU33, HCU39) were screened for transforming DNA sequences from Ad12 and Ad31 of highly oncogenic subgenus A, Ad7 of weakly oncogenic subgenus B and Ad5 of nononcogenic subgenus C. The results as shown failed to demonstrate the presence of viral DNA within these cells.

Human adenoviruses within each subgenus have homologous transforming regions, specific to that subgenus (Green et al., 1979). Therefore hybridization studies using a given strain are applicable to other strains within the same subgenus. Based on a human genome of 3×10^9 base pairs, one should detect the presence of 1 copy of adenovirus DNA per cell at an approximate level of 2pg/µg cellular DNA, using a 5-6 kb probe. Thus, according to the same criteria discussed in Section 3.3.2.3, this system should detect members of subgenera A and C at such a level, and the negative results shown for Ad12, Ad31 and Ad5 can be interpreted to exclude the presence of members of subgenera A and C in the three tumor cell lines. Although the 1.34kb Ad7 probe may not detect the presence of one copy of viral DNA per cell, Southern blot hybridization to this probe was clearly negative, after long exposure to sensitive X-ray film (Kodak XOmat AR5). Furthermore, most adenovirus transformed cells have been found to contain multiple copies of the left-hand transforming region of the virus (Fujinaga et al., 1984). Therefore, in addition to groups A and C, we can, with some certainty, exclude a group B-adenovirus involvement in the oesophageal tumor cells.

3.3.5 Human T-lymphotropic Virus Type-I

Cellular DNA from tumor lines HCU18, HCU33 and HCU39 was shown to be negative when screened for the presence of integrated HTLV-I DNA: A dot blot probed with the HTLV-I specific DNA probe is shown in Figure 29. The two positive controls, plasmid pMT2 DNA (lane 1) and DNA from the HTLV-I producer line, C91/PL (lane 7), are strongly positive. However, oesophageal tumor DNA (lanes 2-4) and negative control DNA (lanes 5 and 6) are clearly negative for the presence of HTLV-I.

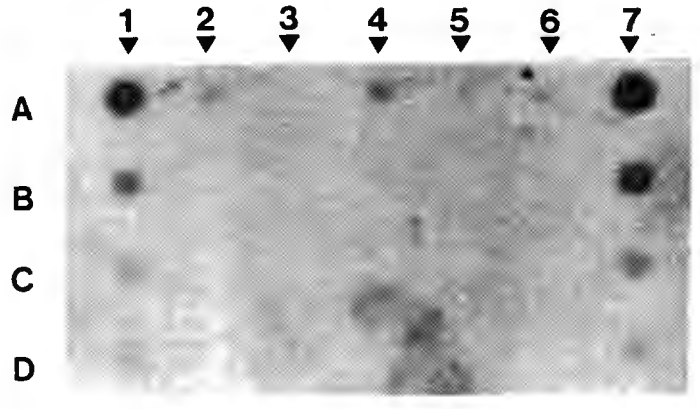


FIGURE 29

Dot blot hybridization of tumor and control DNA's to an HTLV-I DNA probe. Serial twofold dilutions of human DNA (4-0.5ng) and plasmid pMT2 (80-10pg) were applied to wells A-D.

Lane 1 = pMT2; 2 = HCU39; 3 = HCU33; 4 = HCU18; 5 = human placental DNA; 6 = HF 4/81; 7 = C91/PL.

4. DISCUSSION

The aetiology of oesophageal cancer is at present the subject of intense investigation. There are two major unexplained observations relating to this neoplasm:

1. the striking regional variation in the incidence of the disease; and

2. the sharp rise in the incidence in South Africa in the last 25 years.

Both of these observations can be explained, although not exclusively, on the basis of a viral aetiology. While there is very little direct evidence to support such an aetiology, there have been several reports describing morphological changes indicative of papillomavirus infection of the oesophagus (Syrjanen et al., 1982; Winkler et al., 1985; Hille et al., 1985; Hille et al., 1986). In these studies, infected epithelia were identified close to, or adjacent to, invasive squamous carcinomas. HPV antigens have also been demonstrated in certain of the specimens showing such morphological changes (Hille et al., 1986; Winkler et al., 1985). A number of workers have consequently proposed that, in analogy to cervical carcinoma, HPV may be involved in the development of squamous carcinoma of the oesophagus.

The presence of an oncogenic HPV subtype in certain localized communities could explain why striking differences in cancer incidence occur. In addition to the escalating incidence of oesophageal carcinoma, the incidence of HPV infections in the black population of South Africa has also reached epidemic proportions (Morris and Price, 1986). The close-living conditions found in certain isolated communities, and those that are also prone to malnutrition, would favour the spread of virus. The disease is reported to be particularly common in communities where malnutrition is likely to be found (Section 1.1.1). It is of interest that carcinoma of the mouth, pharynx and bronchus have been found to be associated with oesophageal cancer (Fitzpatrick etal., 1985). Furthermore, a high incidence of HPV-induced lesions of the oral cavity has been reported in close-living, isolated communities in South Africa (Van Wyk, 1977).

Evidence for the possible role of a viral factor in the aetiology of oesphageal carcinoma is perhaps best provided by the demonstration of viral particles in the tumor cells. While histology and immunoperoxidase methods alone are not entirely conclusive of a viral infection, absolute confirmation could be provided by electron microscopy and molecular hybridization techniques. Three oesophageal carcinoma cell lines, HCU18, HCU33, and HCU39, were examined in this study by transmission electron microscopy. While several morphological features suggestive of a possible viral activity were noted, the reported absence (Robinson, 1981) of virus particles from these cells was confirmed in this study.

In general, cellular transformation by DNA and RNA viruses is accompanied by integration of viral sequences required for the maintenance of transformation. Although the HPV's were originally believed to be an exception to this rule (Pfister, 1984) recent studies have demonstrated the integration of HPV18 and HPV16 DNA in genital carcinomas (Schwarz et al., 1985; Choo et al., 1987; Pater et al., 1986; Durst et al., 1983, 1985). These two HPV types are mainly found in an integrated form in carcinoma tissue, and cell lines derived from such tissue, but in an episomal form in benign tumors. It has been postulated that integration into the host genome occurs during transition from a noninvasive to an invasive stage of tumorigenesis (Pater et al., 1986). Such an event may thus be important in the induction and/or the maintenance of malignancy, and may also pertain to other squamous cell carcinomas such as oesophageal carcinoma. DNA hybridization tests, described in the present study have, however, shown that human papillomavirus (types 1,5,6,8,11,16 and 18) are not present in the cellular DNA of the 3 oesophageal cell lines HCU18, 33 and 39. Similar negative results were demonstrated for Epstein-Barr virus, human adenoviruses (species 12,31,7 and 5) and HTLV-I. These results indicate, at least for the particular virus types tested, that stable integration of the viral genome is not essential for the maintenance of the malignant phenotype.

The HPV probes that were used included the putative oncogenic types 5,8,16 and 18, as well as HPV6 and 11, frequently identified in carcinomas of the larynx and the oral cavity. Results obtained with these specific probes cannot exclude the possible involvement of HPV in the 3 cell lines. The number of identified virus types

is steadily increasing at the moment; 41 types have been identified to date and this is almost twice the number that were known 2 years ago. These different types are remarkably heterogeneous, and all show less than 50% cross-hybridization. It is possible that HPV DNA, from a type not yet identified, is present in an integrated form in the oesophageal tumor cells. However, low stringency hybridization (T_m-42°C), with an HPV18 probe failed to detect the presence of viral DNA in these cells. All 41 HPV types identified to date have been shown to cross-hybridize under relaxed conditions of hybridization (Kahn et al., 1986), and Ostrow et al. (1987) have recently demonstrated that under these conditions 'heterologous' types can be detected at a level of less than 1 copy per diploid cell. It therefore seems unlikely that viral DNA from any of the 36 HPV types that were not tested is present within these cells. Furthermore, these negative results were recently confirmed by those of de Villiers and coworkers, who have analyzed 10 of the South African oesophageal carcinoma cell lines (including about 35 biopsy specimens) at varying stringencies, and with a variety of different HPV probes (de Villiers, 1986, personal communication).

The adenovirus and EBV probes that were used in this study were all derived from transforming regions of these viruses. For the human adenoviruses this E1 region is well-defined and homologous for different members of each subgenus (Green et al., 1979). Since the integration and expression of E1 sequences is essential for the oncogenicity of adenoviruses, the negative results demonstrated for these viruses indicate that subgenus A, B or C members are not involved in the 3 oesophageal tumor lines.

In contrast to the adenovirus E1 region, the transforming region of EBV has not been well defined. Protein products encoded by 4 different regions of the viral genome (LT1 → LT4) have been implicated in EBV induced B-lymphocyte transformation (Section 1.2.2.3). The expression of LT1 (encoded by BamHIW sequences) is specifically regarded as essential for transformation by this virus (Kieff et al., 1982). However, recent studies by Rowe et al. (1986) have revealed that the 2 protein products encoded by LT3 and LT1 are not expressed in certain newly established EBV containing BL cell lines. This suggests that neither of these products is required for continuous growth of EBV-positive BL cell

the EBV genome, not represented by the three probes used in this study (BamW, BamF and EcoC), could encode such functions and exist in an integrated form in the 3 tumor lines. However, this is unlikely in view of the fact that in most BL derived cell lines EBV exists in an episomal state, and furthermore the entire viral genome is always present even when it is integrated, as in Namalwa DNA (Matsuo et al., 1984).

The absence of integrated (or episomal) viral DNA from cell lines HCU18, HCU33 and HCU39 may be interpreted in several ways:

1. Carcinomas generally consist of a mixture of heterogeneous cells. The oesophageal tumor lines tested in this survey may thus not be representative of the cells initially transformed in vivo. However, extensive in vivo and in vitro studies have indicated that several properties of these, and other oesophageal carcinoma cell lines (initiated during the same period), correlate well with those of the source tumors in vivo: lines developed from poorly differentiated tumors showed similarities to one another, as did those from well differentiated tumors (Robinson, 1981).

2. The oesophageal tumor cells in this survey have been extensively passaged, and the fact that cells may become altered, both in genotype and in phenotype, during adaptation to an in vitro environment must be considered. Since the cells would be subject to different genetic constraints, depending on their environment, the expression of specific viral proteins in these cells may be essential for the maintenance of malignancy in vivo, but not required for malignant growth in vitro. It is thus conceivable that the presence of the viral genome is no longer required, even if persistence of the virus was necessary for the initial proliferation (and/or maintenance) of tumor cells in vivo.

3. A "hit and run" mechanism, similar to that suggested for herpes simplex virus (Galloway et al., 1984) may pertain to the oesophageal carcinomas currently under investigation. In terms of this mechanism, transient infection of the host cell is sufficient to mutate or deregulate crucial transcriptional units and to initiate transformation; continuous presence of

viral DNA is, however, not required to maintain the malignant phenotype (Campo *et al.*, 1985). In support of such a model, Jarrett *et al.* (1986, personal communication) have recently demonstrated that transformed cells from bovine papillomavirus-induced papillomas, can be cultured and shown to induce tumors in nude mice. However, these cells, which can only be grown out in the very early stages of tumor induction, do not contain papillomavirus genome, either episomally or integrated. While it is thought that such a mechanism might also apply to human viruses, the nature of this "hit and run" effect remains unknown.

In conclusion, it is clear that more extensive studies, particularly using fresh tumor material, need to be carried out in order to further elucidate the possible role of a viral co-factor in the aetiology of oesophageal carcinoma.

APPENDIX I - MATERIALS

1.1 TISSUE CULTURE

1.1.1 Miscellaneous Solutions

(a) Physiological saline

0.85% (w/v) NaCl in distilled H_2O.

(b) Trypan blue

0.5% (w/v) trypan blue in physiological saline.
Filter through paper prior to use.

(c) PSN solution

To make 100 ml:
Dissolve 2 x 10^6 units penicillin (2 bottles), 2g streptomycin (2 bottles), 2g neomycin (4 bottles), in 100 ml physiological saline.
Filter sterilize, dispense into 3ml aliquots, and store at $-20^{\circ}C$.

(d) Phenol red (0.4%)

To make 100ml:
Dissolve 0.4g phenol red in 60ml 50mM NaOH, and make up to 100ml with distilled H_2O.
Sterilize by filtration.

(e) 5% sodium bicarbonate solution

5% (w/v) $NaHCO_3$, 0.1% (v/v) phenol red solution (0.4%) in distilled H_2O. CO_2 is bubbled through the solution; it is then filter sterilized, dispensed in bijou bottles (leaving no air space), and stored at $4^{\circ}C$.

1.1.2 Cell culture media

(a) 10% MEM

Eagle's minimum essential medium (MEM) (Gibco Europe), with Earle's salts, L-Glutamine and non-essential amino acids, was used as a growth medium. It was supplemented with 10% (v/v) foetal calf serum (State Vaccine Institute, South Africa), 1% (v/v) sodium bicarbonate solution, and 0.5% (v/v) PSN solution. The pH was maintained at approximately 7.0. Foetal calf serum was sterilized by filtration and UV irradiation, and heat-inactivated at 56°C for 30 mins.

(b) 4% MEM

Eagle's MEM, with Earle's salts, L-Glutamine and non-essential amino acids, was used as a maintenance medium. It was supplemented with 4% (v/v) foetal calf serum, 2% (v/v) sodium bicarbonate solution, and 0.5% (v/v) PSN solution.

(c) 10% RPMI

RPMI 1640 medium (Gibco Europe), with L-Glutamine was used as a growth medium for the Raji cell line. It was supplemented with 10% (v/v) foetal calf serum, 1% (v/v) sodium bicarbonate solution, and 0.5% (v/v) PSN solution.

Cell culture media were sterilized by filtration prior to use.

1.1.3 Buffers

(a) Phosphate buffered saline (PBS)

NaCl	8g
KCl	0.2g
Na_2HPO_4	0.91g
KH_2PO_4	0.12g

in 1 litre of distilled H_2O.
Adjust the pH to 7.0 with HCl or NaOH; sterilize by autoclaving (115°C; 20 min).

(b) Hepes buffer

To make 100 ml of 1M stock solution:
Dissolve 23.8g Hepes in 80 ml of 0.3N NaOH.
Adjust the pH to 7.2 (with 1N NaOH or 1N HCl).
Make up to 100ml with distilled H_2O.
Sterilize by filtration.

(c) Freezing medium

100ml of 10% MEM containing 0.5% (v/v) PSN solution, 7.5% (v/v) dimethylsulphoxide (DMSO) and 1.5% (v/v) Hepes buffer.

(d) Trypsin base

To make 1 litre of a 10X stock solution:

NaCl	80.0g
KCl	2.0g
KH_2PO_4	1.2g
Na_2HPO_4 (anhydrous)	9.1g
Phenol Red (0.4%)	25.0ml
Glucose	5.0g

Dissolve in about 700ml of distilled H_2O and adjust to pH7.8.
Add 2g EDTA in 100ml distilled H_2O, and adjust to pH7.8 with 1N NaOH.
Make up to 1000ml with distilled H_2O.
Filter sterilize, and store at $-20^{o}C$ in 20 ml aliquots.

(e) Trypsin

5% trypsin stock solution:
Acidify 100ml of trypsin base (1X in distilled H_2O) to bright yellow with 1N HCl.
Dissolve trypsin powder in 1X trypsin base without agitation, at room temperature.
Incubate at $4^{o}C$ overnight.
Stir for 30 mins at room temperature.
Centrifuge at 9000 rpm for 45 mins ($4^{o}C$; Sorvall RC-5 centrifuge).

Distilled H_2O 1.25ml

APPENDIX II - TISSUE CULTURE METHODS

2.1 Recovery of Cells from Ampoules Stored in Liquid Nitrogen

Ampoules, containing the cells, were removed from storage in liquid nitrogen and placed in a beaker of water at 37°C, to thaw the contents rapidly and thus reduce cell damage. The cell suspension was diluted 8-10 fold with 10% MEM, or with 10% RPMI in the case of Raji cells. Cells were then pelleted, in a bench-top centrifuge at 1500 rpm for 5 mins, washed with sterile, physiological saline to remove any remaining DMSO, and resuspended in 10ml of 10% MEM (or RPMI, for Raji cells). After counting the cells (Section 2.4), they were seeded, in the appropriate culture medium, at a concentration of 1×10^5 cells/ml. This seeding rate was applicable to HCU18, HCU33, HCU39, HeLa and Raji cell lines. Human fibroblast cells were seeded at a concentration of 2×10^5 cells/ml.

2.2 Passage of Cells

Cell cultures were passaged when monolayers reached confluence, usually once or twice per week for oesophageal tumor cell lines. The growth medium was discarded, and cell sheets were rinsed with 4-5ml of sterile physiological saline to remove any remaining medium. A thin layer of activated trypsin versene (ATV), prewarmed to 37°C, was pipetted over the surface of the cells. After incubation at 37°C for 15 mins, cells were dislodged from the growth surface, and a small volume of culture medium was added in order to inactivate the trypsin. The resultant suspension of cells was pelleted (1500 rpm for 5 mins), resuspended in 10ml of culture medium (10% MEM) and counted (Section 2.4). Cells were then diluted and seeded at appropriate concentrations. After 24 hours, when monolayers were established, the culture medium was replaced with fresh 10% MEM. Alternatively, if rapid cell growth was not required, a maintenance medium, or MEM supplemented with 4% foetal calf serum, (4% MEM) was used.

Raji cells are grown in suspension in 10% RPMI medium. Cells were therefore separated from the culture medium by centrifugation (1500 rpm for 5-10 mins), resuspended, and seeded as described above.

All cell lines, apart from HeLa, were incubated in a CO_2 incubator at 37°C. The bicarbonate in the culture medium was thus made up to a final concentration of 0.25%, in order to maintain the pH at a level of pH6-pH7.

2.3 Storage of Oesophageal Tumor Cells (HCU18, HCU33, HCU39) in Liquid Nitrogen at -180°C

The cell culture medium was removed and confluent cell monolayers were washed with 4-5ml of sterile physiological saline, and harvested using ATV. The resultant cell suspension was pelleted at 1500 rpm for 5 mins, and then resuspended in 2ml of storage buffer (per 75 cm^2 flask). Cells were diluted in storage buffer to a final concentration of $(1-2)\times10^6$ cells/ml, and dispensed into ampoules containing 1ml each. Ampoules were frozen in a controlled rate freezing machine to minimize cell damage. They were then stored in liquid nitrogen at -180°C.

2.4 Determination of Cell Concentration

Cells were counted using a haemocytometer (Bausch and lamb), as follows:
The cell suspension to be counted was made up to a volume of 10ml with culture medium. A 0.1ml aliquot of the suspension was diluted with an equal volume of trypan blue solution, and applied to the graduated section of a haemocytometer such that the surface was just covered. By viewing under the microscope (Wild Heerbrugg) the average cell count per square, $0.001mm^3$ in volume, in a total of four squares, was determined. The cell concentration could then be calculated:

$$\text{Cell concentration} = \frac{\text{Total No. of cells}}{4} \times 2 \times 10^4 \text{ cells/ml} .$$

To determine the total cell count, this value was multiplied by the total volume of cell suspension.

2.5 Growth of Cells on Coverslips and in Roller Tubes

Cells that were to be stained for light microscopy were grown on glass coverslips, lying free within airtight, sterile, glass roller tubes (Kimax). Human fibroblasts (HF) cells, which will not

grow on glass surfaces, were cultured on the walls of airtight, sterile, plastic roller tubes (Falcon). Infection of these HF cells with various adenovirus isolates was used for the initial identification of laboratory Ad. strains, as well as for the purpose of virus propagation.

Cells grown in tissue culture flasks were detached by trypsinization, resuspended in fresh 10% MEM, and then diluted to a final concentration of (2-4) x 10^5 cells/ml. Aliquots (1ml) of this suspension were pipetted into airtight, sterile roller tubes. These tubes were incubated, at an angle, in a 37^{o}C incubator for 24 hours. After this time the culture medium was removed and replaced with 2ml of 4% MEM. Incubation was continued, rolling the tubes, until cell monolayers were confluent, or in the case of infected cell cultures, until a visible cytopathic change was evident. The movement reduces the amount of debris that settles on the culture, resulting in a clearer evaluation of cytopathology. Coverslip cultures could then be removed, stained with haematoxylin and eosin according to standard procedure (Humason, 1972), and mounted on glass slides for viewing under a light microscope (Wild Heerbrugg).

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