THE ROLE OF HUMAN CYTOMEGALOVIRUS IN TRANSFORMATION AND IN THE DEVELOPMENT OF CERVICAL INTRAEPITHELIAL NEOPLASIA

by

Karen Fletcher

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Institute of Virology
Church Street
GLASGOW
G11 5JR

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

To my fiancé, Robert

SUMMARY

The aim of this study was to investigate the role of human cytomegalovirus (HCMV) in transformation and its possible involvement in the development of cervical intraepithelial neoplasia (CIN).

Although cell transformation by HCMV is well documented in the literature, the mode of transformation has not yet been elucidated. Nelson et al. (1982) identified a DNA sequence within the HindIII E fragment of the HCMV AD169 genome which when transfected into NIH 3T3 cells, can initiate colony formation in methylcellulose and tumourigenicity in nude mice. It was intended to extend these experiments to investigate the molecular basis for HCMV cell transformation. Repeated attempts to transform 3T3, human embryo lung (Helu) and rat embryo (RE) cells with the HCMV AD169 HindIII E fragment were unsuccessful. However, transformation of RE cells was repeatedly observed after infection of the cells with UV-irradiated HCMV AD169 virus. Southern blot analysis could not detect HCMV DNA in HCMV transformed cell and derived tumour cell lines. This suggested that the retention of viral DNA in the transformed cells may be transient and only necessary to initiate the transformation event. One mechanism by which HCMV DNA could initiate transformation is by activating a cellular proto-oncogene. Experiments were carried out

in collaboration with Ms. L. Clark and Dr. J.C.M. Macnab to see if the DNA from a rat tumour induced after inoculation with HCMV transformed cells contained a transforming gene. The rat tumour DNA was found to contain a transforming gene that could be transfected into 3T3 cells and could initiate tumourigenesis when the transfected cells were injected into nude mice. As viral DNA could not be detected in the original HCMV transformed cells, the transforming gene must have been of cellular origin. Preliminary experiments suggested that HCMV may have activated a ras oncogene in the transformed cells.

Past seroepidemiological studies have implicated HCMV as an oncogenic agent in the development of CIN and cervical carcinoma. The second aim of this study was to provide molecular evidence for an association between HCMV and CIN. The DNA from biopsies of 43 CIN patients in the West of Scotland was examined for the presence of HCMV DNA sequences by Southern blot analysis. Two biopsies, C2 and C17 were found to contain DNA sequences that hybridized to the HCMV AD169 HindIII E fragment. In C17 the hybridizable DNA sequences were only present at about 0.1 copy/cell and therefore in an amount too low to permit detailed sequence analysis. The hybridizable DNA sequences in C2 were present at about 20 copies/cell and were found to contain BamHI restriction fragments that comigrated with the BamHI P, W, c and e fragments of HCMV AD169.

The hybridizable CIN DNA sequences may represent HCMV sequences that were retained in the tissue after HCMV infection and may have instigated the development of CIN. Two other BamHI fragments of C2 DNA were detected using the HCMV AD169 HindIII E fragment as a probe and these may represent rearranged sequences in the retained HCMV DNA. Rearrangements could have occurred when the viral DNA became integrated into the cellular DNA of this patient. As only a small amount of DNA was available from the C2 biopsy, further analysis of the viral sequences retained and the nature of the rearrangements was only possible if the relevant sequences could be cloned. A λ library of the C2 DNA was constructed using the vector EMBL3 and three clones were isolated that contained C2 sequences that hybridized to the cloned HCMV AD169 HindIII E fragment. One of the clones was found to contain C2 sequences that hybridized to pAT vector sequences present in the probe. The other two clones contained C2 sequences that hybridized specifically to the HCMV AD169 HindIII E fragment but were unfortunately lost during the purification procedure after only 2 or 3 rounds of replication.

Since HSV-2 and HPV have also been implicated in the development of CIN and cervical carcinoma, the same 43 CIN biopsies were analysed for the presence of HSV-2 and HPV11 DNA sequences. Authentic HPV11 DNA was detected in two CIN biopsies (C13 and C19) but no

conclusive evidence of HSV-2 specific DNA could be found, although sequences that hybridized to vector pBR322 were detected.

As herpesviruses are important pathogens of the cervix, biological studies were carried out to determine the response of cervical cells to infection *in vitro* by HCMV and HSV-2. Ectocervical cells were found to be fully permissive for HSV-2 replication but nonpermissive for HCMV infection. HCMV infection of ectocervical cells did not produce any morphological alteration of the cells and did not result in the production of infectious virus. Immunofluorescence revealed that only about 10% of the infected cells contained HCMV specific antigens. Because latent infection is an important factor in the pathogenesis of herpesviruses, an investigation of latent infection of ectocervical and Helu cells was also undertaken. By incubating infected cultures at 42°C it was possible to establish HSV-2 infections during which infectious virus could not be detected. Superinfection of 42°C treated HSV-2 infected Helu cultures with HCMV AD169 and *ts* mutants of HSV types 1 and 2 resulted in reactivation of the virus. Furthermore, the reactivated virus was found to be capable of efficient replication. Superinfection of 42°C treated HSV-2 infected ectocervical cell cultures with HCMV did not reactivate virus and superinfection of the cultures with HSV *ts* mutants resulted in cell death. The

possible role of hormones in reactivating virus was also investigated in Helu cells. Exposure of 42°C treated HSV-2 infected Helu cells to 10^{-7}M or 10^{-9}M oestrogen, progesterone or dexamethasone did not result in virus reactivation. The reactivation of HSV-2 by HCMV from Helu but not from ectocervical cells may be related to the fact that ectocervical cells are epithelial cells and Helu cells are fibroblasts. Like the course of normal HCMV infection, the mechanism of reactivation may be different in the different cell types. These experiments illustrate that fibroblasts do not provide a suitable <u>in vitro</u> model system for studying the role of herpesviruses in the development of cervical neoplasia and emphasise the necessity for further studies on the response of cervical cells to herpesviruses.

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ABBREVIATIONS

ACC	adenocarcinoma of the colon
ACIF	anticomplement immunofluorescence
Ad	adenovirus
AIDS	aquired immune deficiency syndrome
ALV	avian leukosis virus
bp	base pairs
BPV	bovine papilloma virus
CF	complement fixation assay
CID	congenital cytomegalic inclusion disease
CIN	cervical intraepithelial neoplasia
CIS	carcinoma *in situ*
cm	centimetre
cpe	cytopathic effect
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dTTP	deoxythymidine triphosphate
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
ECMV	equine cytomegalovirus
EGF	epidermal growth factor
EHV	equine herpesvirus
ELISA	enzyme linked immunosorbent assay

g	grams
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HEF	hamster embryo fibroblast
Helu	human embryo lung
HPV	human papilloma virus
hr	hour
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HTLV	human T cell leukaemia virus
IE	immediate early
Ig	immunoglobulin
IHA	indirect haemaglutination assay
kb	kilobase
KS	Kaposi's sarcoma
LTR	long terminal repeat
M	mole
MCMV	murine cytomegalovirus
MHC	major histocompatibility complex
uCi	microcurie
ug	microgram
ul	microlitre
min	minute
mM	millimole
mm	millimetre
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
mu	map units

MW	molecular weight
NC	normal cervix
ng	nanogram
NIEP	noninfectious enveloped particle
nm	nanometre
ODC	ornithine decarboxylase
PAGE	polyacrylamide gel electrophoresis
PDGF	platelet derived growth factor
pfu	plaque forming units
pg	picogram
PRV	pseudorabies virus
RAV	Rous associated virus
RE	rat embryo
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RSV	Rous sarcoma virus
SV40	simian virus 40
ts	temperature sensitive
u	units
UC	ulcerative colitis
UV	ultraviolet light
V	volt
VZV	varicella-zoster virus
w/v	weight/volume

INTRODUCTION

GENERAL PROPERTIES OF HUMAN CYTOMEGALOVIRUS

Human cytomegalovirus (HCMV) is a member of the herpes virus group and is a very important pathogen responsible for a wide spectrum of diseases (Weller, 1971). Viral infection can be fatal in the foetus, newborn and immunodeficient. HCMV is a serious cause of congenital birth defects. The frequency of congenital HCMV infection approaches 1.5% of all births. Congenital cytomegalic inclusion disease (CID) is usually fatal and surviving infants suffer from extensive mental and motor dysfunction. HCMV also causes a type of infectious mononucleosis and interstitial pneumonia which can be fatal in organ and bone transplant patients (Betts, 1982, and Balfour, 1984). The virus is present in most body fluids and excretions including blood, saliva, urine and semen and transmission can occur congenitally, natally, venereally and through blood transfusions and organ and bone marrow transplants. Disease can also result from reactivation of latent virus, particularly in immunodeficient hosts. In addition, the virus has oncogenic potential and has been associated with a number of human neoplasias, including Kaposi's sarcoma, cervical carcinoma, prostatic carcinoma and adenocarcinoma of the colon. This thesis describes studies undertaken to investigate the oncogenic potential of HCMV and its possible involvement in the development of cervical intraepithelial neoplasia (CIN), the

precursor stage to cervical carcinoma.

Morphology

Cytomegalovirions are composed of a spherical core about 60nm in diameter, surrounded by a 90-110nm icosahedral capsid of 162 capsomeres (Smith and Rasmussen, 1963; Montplaisir et al., 1972). The virions are enveloped by 2 discrete membranes, an inner membrane derived from the nuclear membrane and an outer loose-fitting membrane derived from the cytoplasmic membrane of infected cells. The outer membrane is mainly composed of glycoproteins. A variable number of glycoproteins have been described ranging from 4 (Fiala et al., 1976; Gibson, 1983) 5 (Farrar and Oram, 1984) 7 (Kim et al., 1976; Nowak et al., 1984b) to 8 (Stinski, 1976). Antisera to the glycoproteins contain antibodies that neutralize viral infectivity and react with antigens in HCMV infected cells (Stinski, 1976). In addition to mature virions, dense bodies are observed in HCMV infected cells. These are spherical particles which vary in size but are usually about twice the diameter of mature enveloped virions. They are formed concurrently with cytomegalovirions by the envelopment of electron dense material by budding into cytoplasmic vacuoles in the golgi region of infected cells. The envelope is very similar, if not identical, to the outer membrane of the mature virion. Both membranes share common antigens as

determined by immune microscopy (Craighead et al., 1972) and both attach and fuse to the membrane of host cells. The electron dense material is thought to be composed of virion structural proteins. Nothing is known of the biological significance of dense bodies. They contain very little or no viral DNA and may represent the end products of aberrant assembly of HCMV structural envelope and tegument proteins.

Between 20 and 35 structural virus-specific polypeptides have been described in HCMV virions and dense bodies ranging in molecular weight (MW) from 11,000 to 290,000 daltons (Sarov and Abady, 1975; Fiala et al., 1976; Kim et al., 1976; Gupta et al., 1977; Stinski, 1976, 1977). A few polypeptides are present exclusively in either mature virions or dense bodies but the majority are shared by both. The major capsid protein is a protein of 150,000 daltons and the major matrix protein a 68,000 dalton protein (Stinski, 1977). These proteins are present in both virions and dense bodies.

Irmiere and Gibson (1983) have described a third type of virus particle produced in lesser amounts in HCMV infected cells. These have been called non-infectious enveloped particles (NIEP's). They are morphologically distinguished from virions only by their core structure. The NIEP capsid has a diameter 10-15% smaller than that of the virion and has a much less densely stained centre. It is enveloped, does not contain viral DNA, and is

consequently non infectious. The protein composition of NIEP's is very similar to that of the virion. It differs only in that NIEP's contain significantly less of the 74,000 and 69,000 dalton matrix proteins and seem to contain an additional 35,000 dalton protein not present in virions (Irmiere and Gibson, 1983;1985). The 35,000 dalton protein is the most abundant NIEP protein species and has properties in common with the 37,000 dalton B-capsid protein of Colburn CMV, a simian-like human CMV isolate (Gibson 1981a) and the 38,000-40,000 dalton B-capsid protein of herpes simplex virus (HSV) (Gibson and Roizman, 1972, 1974) suggesting that a protein of this nature may play a common role in the assembly pathway of herpesviruses. It has been proposed that this protein functions to mediate DNA packaging and/or nucleocapsid envelopment and in the normal course of events is modified and/or removed from the particle (Gibson and Roizman, 1972; Gibson 1981a). NIEP's are produced by all human CMV strains but not by Old World Monkey CMV infected cells or HSV. NIEP's are generally present in much lower amounts than virions. However, strain AD169 overproduces NIEP's by ten-fold and the additional NIEP protein of this strain has an apparently larger size (36,000 daltons) than the corresponding protein of other strains.

Genomic structure

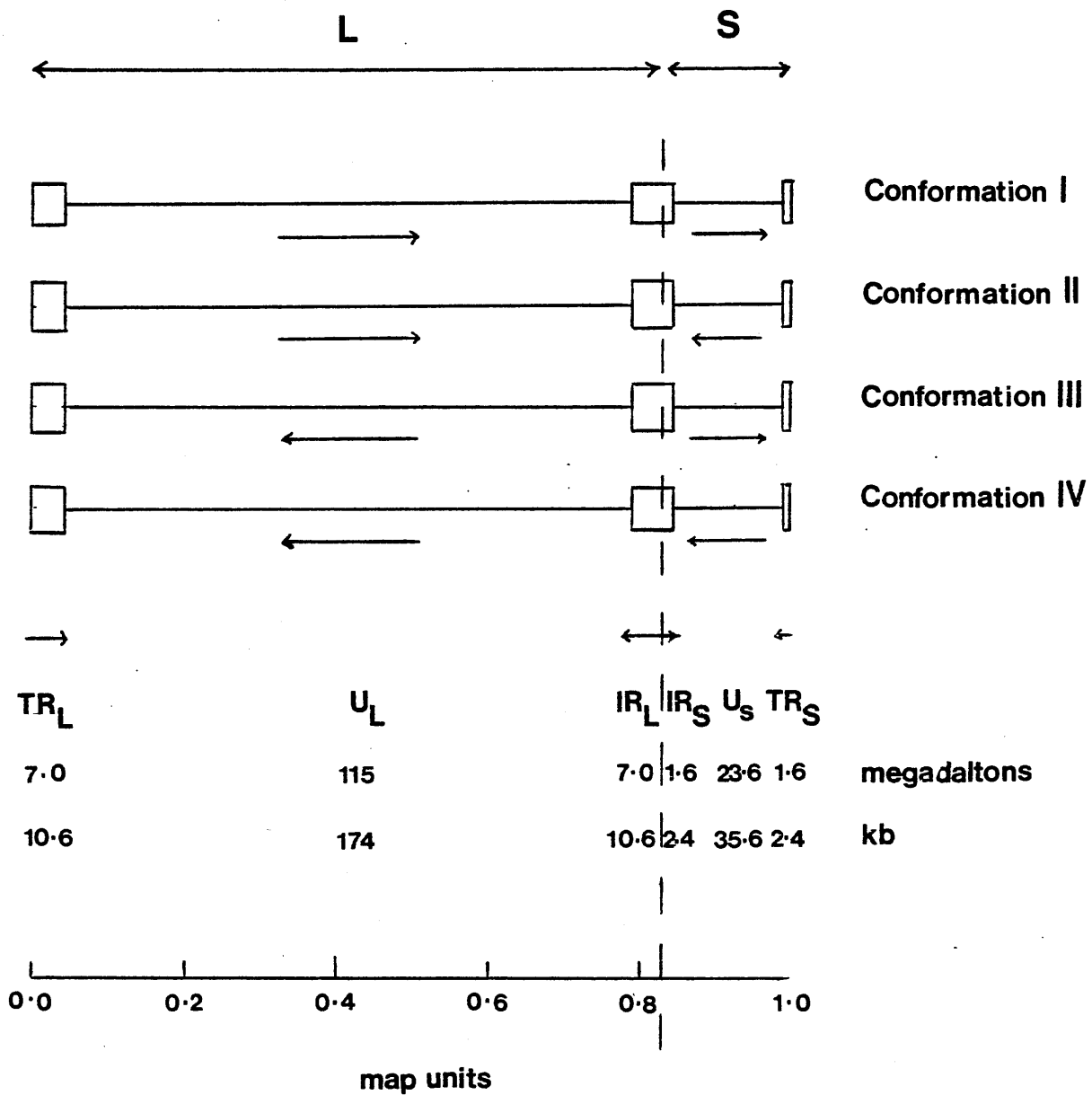
The HCMV genome is one of the largest among DNA viruses consisting of a linear double stranded DNA molecule of approximately 225kb. Reports vary as to the MW of HCMV DNA. Huang et al. (1973) estimated the MW to be $100x10^6$ daltons by cosedimentation with HSV DNA and Sarov and Friedman (1976) used length measurements to arrive at a MW of $107\pm2.7x10^6$ daltons. Geelen et al. (1978) reported a MW of $147\pm6.2x10^6$ daltons. Kilpatrick and Huang (1977), DeMarchi et al. (1978) and Stinski et al. (1979) all calculate the MW to be about $150x10^6$ daltons and this is now generally accepted as the correct value for full length HCMV DNA. Stinski et al. (1979) investigated size heterogeneity of HCMV DNA and concluded that serial high-multiplicity passage results in the production of defective cytomegalovirions which contain shorter DNA molecules of $100x10^6$ and $60x10^6$ daltons. This may explain some of the earlier observations. Alterations in the restriction enzyme profile eg defective-specific fragments of defective HCMV DNA suggests that deletions, substitutions or duplications may all be mechanisms by which defective DNA may be generated. The development of defective virions is associated with a decrease in infectivity but an increase in the particle/pfu ratio. Although defective virions have been identified for HSV, defectiveness in HSV is not associated with a decrease in the size of the virion DNA

(Frenkel et al., 1975, 1976). This suggests that the generation of HCMV DNA defective molecules is different from that of HSV.

HCMV DNA has a buoyant density of 1.716gcm^3 which corresponds to a G+C content of about 57%. Melting curve analysis reveals a bisigmoidal curve with 2 distinguishable melting transition points at 86°C and 94°C corresponding to a G+C content of 43% and 60% respectively (Kilpatrick and Huang, 1977).

The HCMV genome consists of a long unique (U_L) and short unique (U_S) region, each bound by inverted repeat sequences (IR_L/TR_L and IR_S/TR_S) (LaFemina and Hayward, 1980; Westrate et al., 1980). During virus replication the long and short unique regions become inverted with respect to each other resulting in HCMV DNA preparations of 4 genomic arrangements present in equimolar amounts (Figure 1). Restriction endonuclease digestion studies confirm this and demonstrate that all 4 forms are found in virus isolates and are capable of replication (Westrate et al., 1980; DeMarchi, 1981). The sequence arrangement in HCMV DNA is of the same general plan as in HSV DNA (Sheldrick and Berthelot, 1974; Hayward et al., 1975; Delius and Clements, 1976) but with regions of different relative and absolute size.

In HSV, intrastrain heterogeneity of viral DNA has been observed in the terminal and joint fragments (Wagner and Summers, 1978; Locker and Frenkel, 1979; Post et al.,


L
S
Conformation I
Conformation II
Conformation III
Conformation IV
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U$_L$
IR$_L$
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U$_S$
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7·0
115
7·0
1·6
23·6
1·6
megadaltons
10·6
174
10·6
2·4
35·6
2·4
kb
0·0
0·2
0·4
0·6
0·8
1·0
map units

FIGURE 1 Structure of the HCMV Genome

Schematic representation of the DNA sequence arrangement of the HCMV AD169 genome. Short (S) and long (L) segments are each framed by a pair of inverted repeats (IR_S/TR_S and IR_L/TR_L). Unique sequences of short and long segments (U_S and U_L) may be arranged in either orientation, resulting in four isomeric conformations.

(Figure from Fleckenstein et al., 1982)

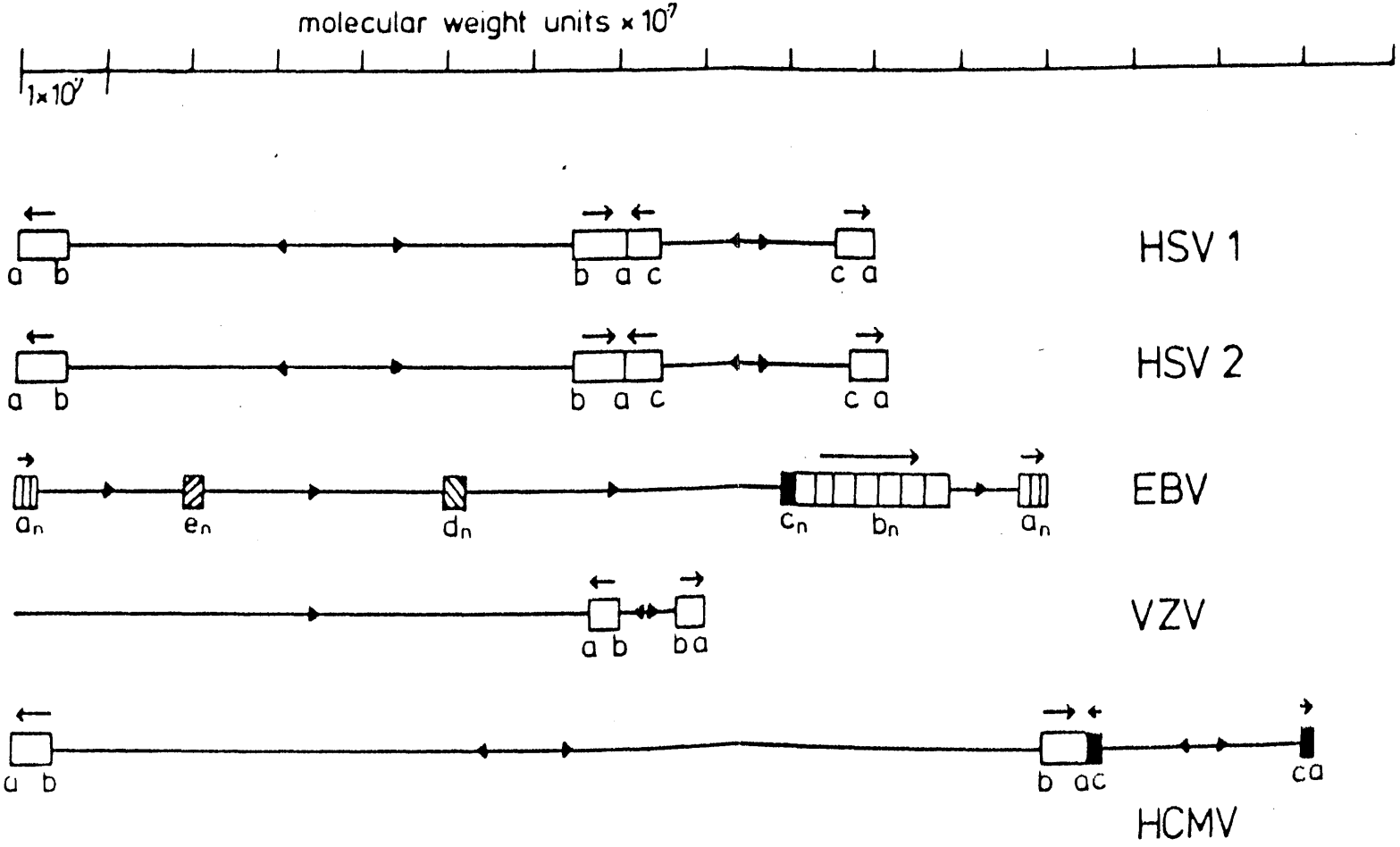
1980). These sequences have been designated the "a" sequences and are thought to play an important role in the isomerization of the viral genome (Mocarski et al., 1980; Smiley et al., 1981; Mocarski and Roizman, 1982). HCMV DNA presumably has a similar "a" sequence arrangement since Geelen and Westrate (1982) were able to find unit length circular DNA molecules after brief exonuclease III treatment followed by denaturation and reannealing of full length HCMV DNA. Preliminary data for the Towne strain of HCMV has revealed a 750bp sequence within the terminal and joint fragments that may be analogous to the HSV "a" sequence (LaFemina and Hayward, 1980, 1983). Similarly, Spector et al. (1982) have observed sequence heterogeneity in the terminal fragments bounding the L and S regions and in the repeats bounding the L region of strain AD169. This sequence variability may result from insertions and/or deletions of sequences within the repeated regions, as has been reported for HSV (Wagner and Summers, 1978).

The origin of replication and packaging signals have been localized for HSV (Stow, 1982) but have not yet been determined for HCMV.

Physical maps of HCMV strain AD169 (Westrate et al., 1980; Oram et al., 1982; Fleckenstein et al., 1982; Greenaway et al., 1982; Spector et al., 1982), Towne (LaFemina and Hayward, 1980) and Davis (DeMarchi, 1981) have been constructed after restriction by several

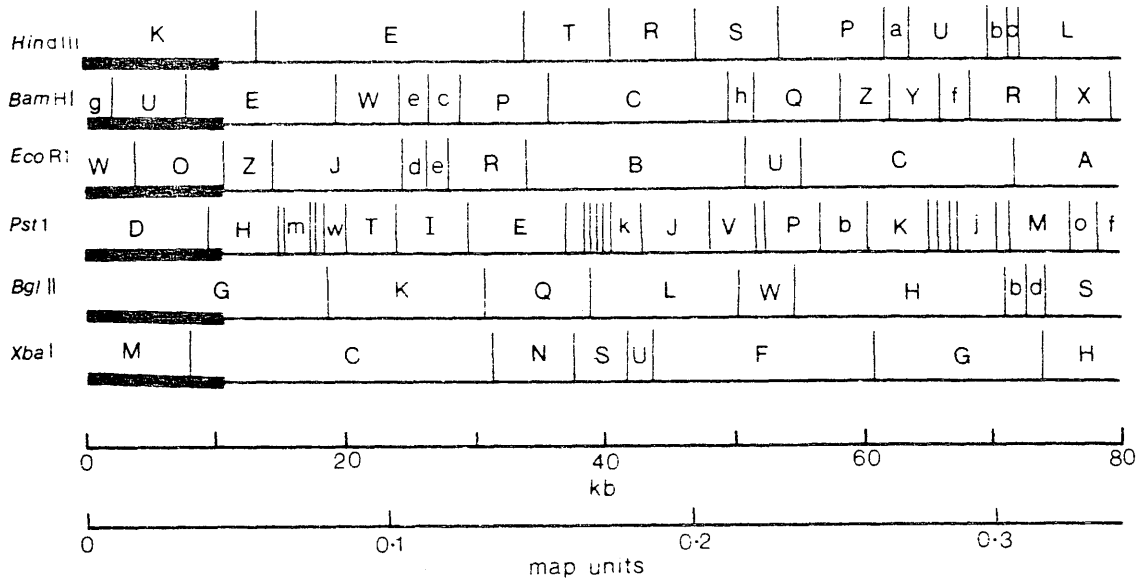
Structures of Human Herpesvirus Genomes

Linear genome structures of the 5 human herpesviruses are shown to scale. In each case, the observed orientations (filled arrowheads) of U_L and U_S are indicated. Repeated sequences are shown as rectangles, and their relative orientations by arrows and letters (a, b, c, a', b', c'), a_n-e_n signify multiple tandem repeats. The letters do not denote similarity of sequence or functions in those regions between different genomes.



different endonucleases. Physical maps for the digestion products of HindIII, BamHI EcoRI, PstI, BglII and XbaI of HCMV strain AD169 are given in Figure 2.

DNA restriction endonuclease analysis of different isolates of HCMV shows that there are a large number of comigrating fragments, suggesting extensive conservation of DNA sequence order among the various strains. There are a few unique DNA fragments present in epidemiologically unrelated isolates (Huang et al., 1976; Kilpatrick et al., 1976; Doerr et al., 1979; Westrate et al., 1983). Restriction endonuclease analysis of various HCMV isolates can yield information on the epidemiology of the virus. For example restriction enzyme profiles of HCMV isolates from sexual partners have been found to be identical suggesting that HCMV can be sexually transmitted (Chandler et al., 1984; Handsfield et al., 1985). HCMV isolates from mother and baby have also been found to be identical (Chandler et al., 1984) suggesting that HCMV can be congenitally or natally transmitted. Huang et al. (1976) demonstrated that 10 epidemiologically unrelated HCMV isolates shared 80% homology with the AD169 prototype strain by DNA-DNA reassociation kinetics. HCMV strains Towne and AD169 share about 90% DNA sequence homology (Pritchett, 1980). This is a striking contrast to the degree of homology that exists between the herpes simplex viruses. HSV-1 and HSV-2 share about 48% nucleic acid homology (Kieff et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PstI
BglII
XbaI
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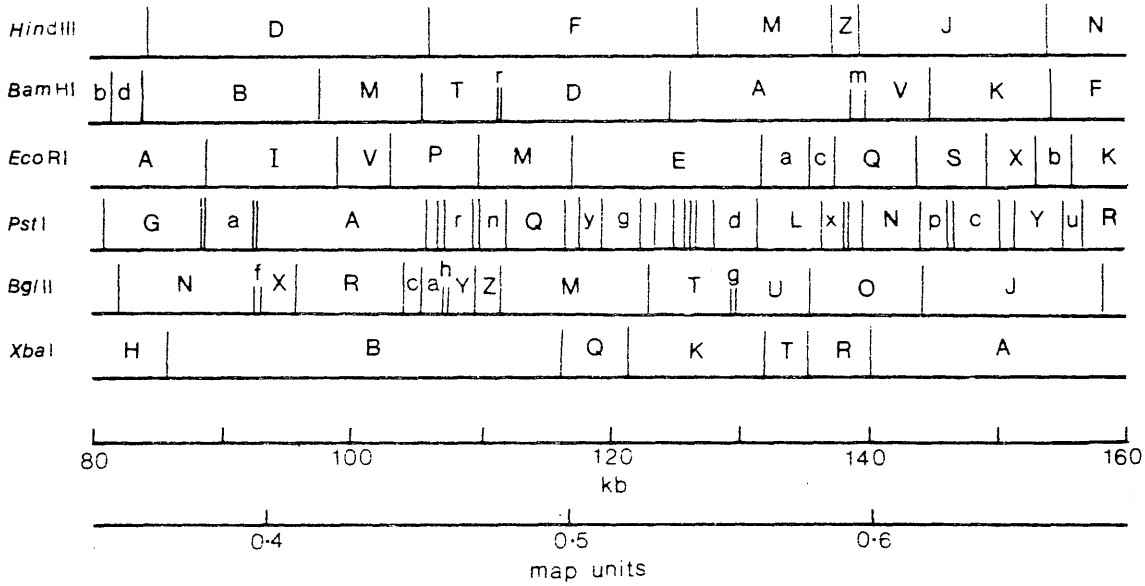
20
40
60
80
kb
0
0·1
0·2
0·3
map units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kb
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0·6
map units

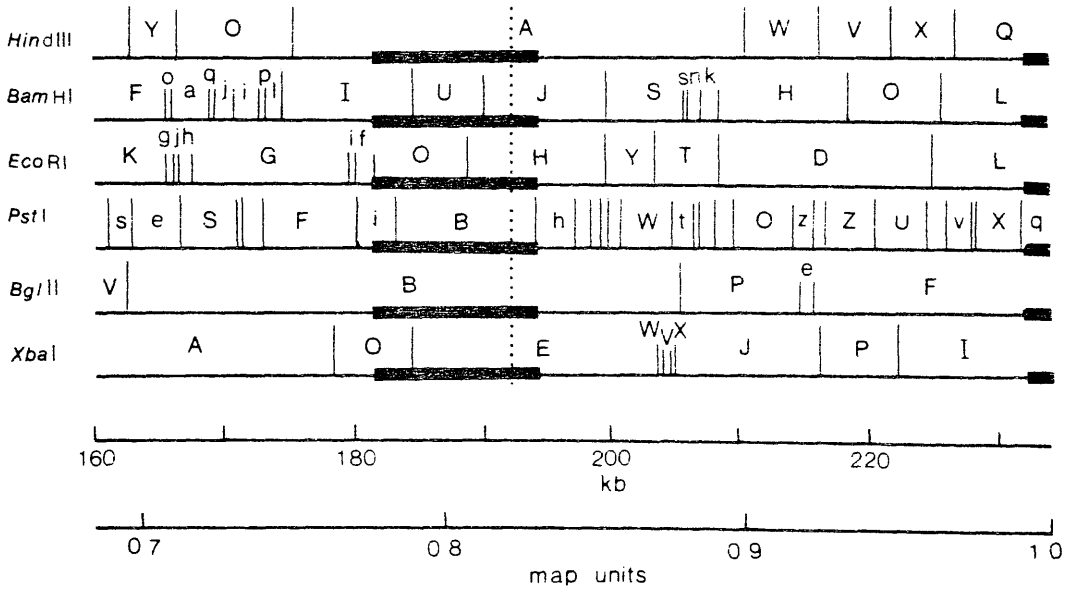

HindIII
BamHI
EcoRI
PstI
BglII
XbaI
160
180
200
220
kb
0 7
0 8
0 9
1 0
map units

FIGURE 2 Restriction endonuclease cleavage maps of HCMV DNA strain AD169

Map of the HCMV genome strain AD169 for the enzymes HindIII, BamHI, EcoRI, PstI, BglII and XbaI. Only the prototype orientation is shown. Repeated sequences are indicated by thick horizontal lines, the join between the long and short internally repeated sequences is indicated by the dotted line.

(Figure from Greenaway et al., 1982).

An unbound copy of this Figure can be found at the back of this thesis.

al., 1972). Most of the genetic variability and heterogeneity between CMV isolates exists within the repeat sequences (LaFemina and Hayward, 1980; Westrate et al., 1983).

The HCMV genome shares less than 5% nucleic acid homology with the DNAs of HSV-1, HSV-2, EBV or simian and murine CMV (Huang and Pagano, 1974). Recently, D'Aquila and Summers (1985) have reported homology between the HCMV Towne BamHI E fragment and the DNA polymerase loci of HSV-1 and EBV. Peden et al. (1982) and Rüger et al. (1984, 1985) have shown that AD169 DNA has sequence homologies to cellular DNAs of various origins. The homologous sequences have been localized to 4 regions of the long unique segment (EcoRI fragments R, 1 and b and HindIII fragment S) and one region in the terminal inverted repeats (EcoRI fragments 0 and H) and are not related to human Alu repeats or to cloned human c-myc sequences.

Productive infection

HCMV is highly species specific, consequently the interaction between HCMV and the infected cell differs between permissive and nonpermissive hosts. HCMV nonproductive infection and latency will be discussed later.

HCMV has a lengthy growth cycle. Viral DNA synthesis does not occur until approximately 24 hr post

infection (DeMarchi and Kaplan, 1976; Huang et al., 1973) and progeny virus may not appear until 72 hr post infection (Michelson-Fiske et al., 1977; St. Jeor and Rapp, 1973a). The replicative cycle is often even longer in cells infected with primary isolates of CMV. Furthermore, the cellular response to infection depends upon the physiological state of the cells (DeMarchi and Kaplan, 1977a) HCMV replication is enhanced by a number of chemicals including 5-iodo-2'-deoxyuridine (St. Jeor and Rapp, 1973b), dexamethasone (Tanaka et al., 1984a, 1984b), and cortisol (Koment, 1985).

Virus penetration occurs rapidly and virus capsids can be detected in close association with nuclear pores by 5 min after the initiation of penetration (Smith and DeHarven, 1974). Virus induced antigens can be detected early in infection. Geder (1976) detected HCMV induced nuclear antigens that resembled Epstein Barr virus nuclear antigen by anticomplement immunofluorescence (ACIF) within 3 hr post infection. Immediate early antigens have been detected in the nucleus within the first hour of infection by indirect immunofluorescence and ACIF (Michelson-Fiske et al., 1977; Reynolds, 1978).

The infected cell undergoes drastic changes during the early phase of HCMV infection. Alterations in chromatin conformation are detectable at the end of a 1 hour virus adsorption period (Kamata et al., 1978). This requires virus coded functions expressed immediately

after infection. By 18 hr post infection a novel chromatin pattern can be observed by electron microscopy (Kierszenbaum and Huang, 1978). In the first 24 hr of infection, HCMV stimulates host cell DNA, RNA, protein synthesis and the production of enzymes particularly those associated with the S phase of the cell cycle. These effects will be discussed in detail in a later section. Virus specific cytopathic effect (cpe) ie cell rounding, can be observed in infected cells by 6 hr post infection (Stinski, 1977).

Gene expression

Like other herpesviruses, HCMV has regulated phases of transcription (DeMarchi et al., 1980; Stinski et al., 1981; Wathen et al., 1981; McDonough and Spector, 1983) and translation (Stinski, 1977, 1978; Stinski et al., 1980; Wathen et al., 1981). In general viral transcription and translation can be divided into 3 phases; immediate early (IE), early and late.

IE transcription occurs in the absence of de novo protein synthesis and transcripts can be detected 2-4 hr post infection (DeMarchi et al., 1980). Major immediate early transcription of HCMV originates from the long unique region (DeMarchi, 1981; Wathen and Stinski, 1982; Stinski et al., 1983; McDonough and Spector, 1983; Jahn et al., 1984; Wilkinson et al., 1984). This differs from HSV and pseudorabies virus in which the major IE

transcripts originate from the long and short repeat sequences (Clements et al., 1977; Jones et al., 1977; Clements et al., 1979; Feldman et al., 1979; Costa et al., 1980). In HCMV strain AD169, IE transcription occurs predominantly from the HindIII E region (McDonough and Spector, 1983; Jahn et al., 1984; Wilkinson et al., 1984). The most abundant IE mRNA is a 1.95 kb transcript transcribed from left to right between map units (mu) 0.0764 to 0.0865 on the prototype molecule of AD169. The most abundant IE mRNA in cells infected with HCMV Towne (1.95 kb; Wathen and Stinski, 1982; Stinksi et al., 1983) and Davis (2.2 kb; DeMarchi 1981, 1983) maps in the same relative position of the long unique region as the 1.95 kb IE mRNA of strain AD169. Wilkinson et al. (1984) demonstrated by hybrid-selected translation that the abundant 1.95 kb mRNA encoded the major IE polypeptide with a MW of 75,000 daltons. The major IE polypeptides of strain Davis, Towne and AD169 each have slightly different MW's. (Cameron and Preston, 1981; Gibson, 1981b) but cross react immunologically (Goldstein et al., 1982). Transcription of two middle abundant polyadenylated mRNAs of 1.7 and 2.15 kb is initiated immediately downstream and in the same orientation as the IE 1.95 gene. These mRNAs differ in both size and orientation of transcription from those observed in IE coding region 2 immediately downstream of the major IE gene (coding region 1) of HCMV strain Towne (Stinski et

al., 1983). Other mRNAs are transcribed from the HindIII E region and these are summarized in Figure 3.

A second IE transcriptionally active area of the HCMV AD169 genome has been identified at 0.593-0.619 mu. 1.75, 3.8 and 4.8 kb mRNAs are transcribed from this region (Wilkinson et al., 1984). Additional minor IE transcription has also been observed from other regions of the HCMV genome and the data is presented in Figure 3.

Jahn et al. (1984) also identified a 5 kb IE transcript of HCMV AD169 of which only a small part is polyadenylated. This may correspond to the 5.2 kb IE mRNA identified by Wilkinson et al. (1984). The DNA coding for the 5 kb IE mRNA is transcribed in high quantities during the late phase of virus replication, unlike the other IE genes. This may represent an exception from the general rule of temporal regulation of herpesvirus transcription. It is noteworthy that the transforming region identified by Nelson et al. (1982) is included within the DNA coding region for this 5 kb transcript.

The major IE gene of HCMV AD169 has been sequenced (Akrigg et al., 1985) and appears to be structurally very similar to that of strain Towne (Stenberg et al., 1984). The gene encodes a spliced molecule of 1736 nucleotides made up of 4 exon sequences. A single open reading frame starting in the second exon extends for 491 amino acids corresponding to a protein of MW 64,000 daltons. This is

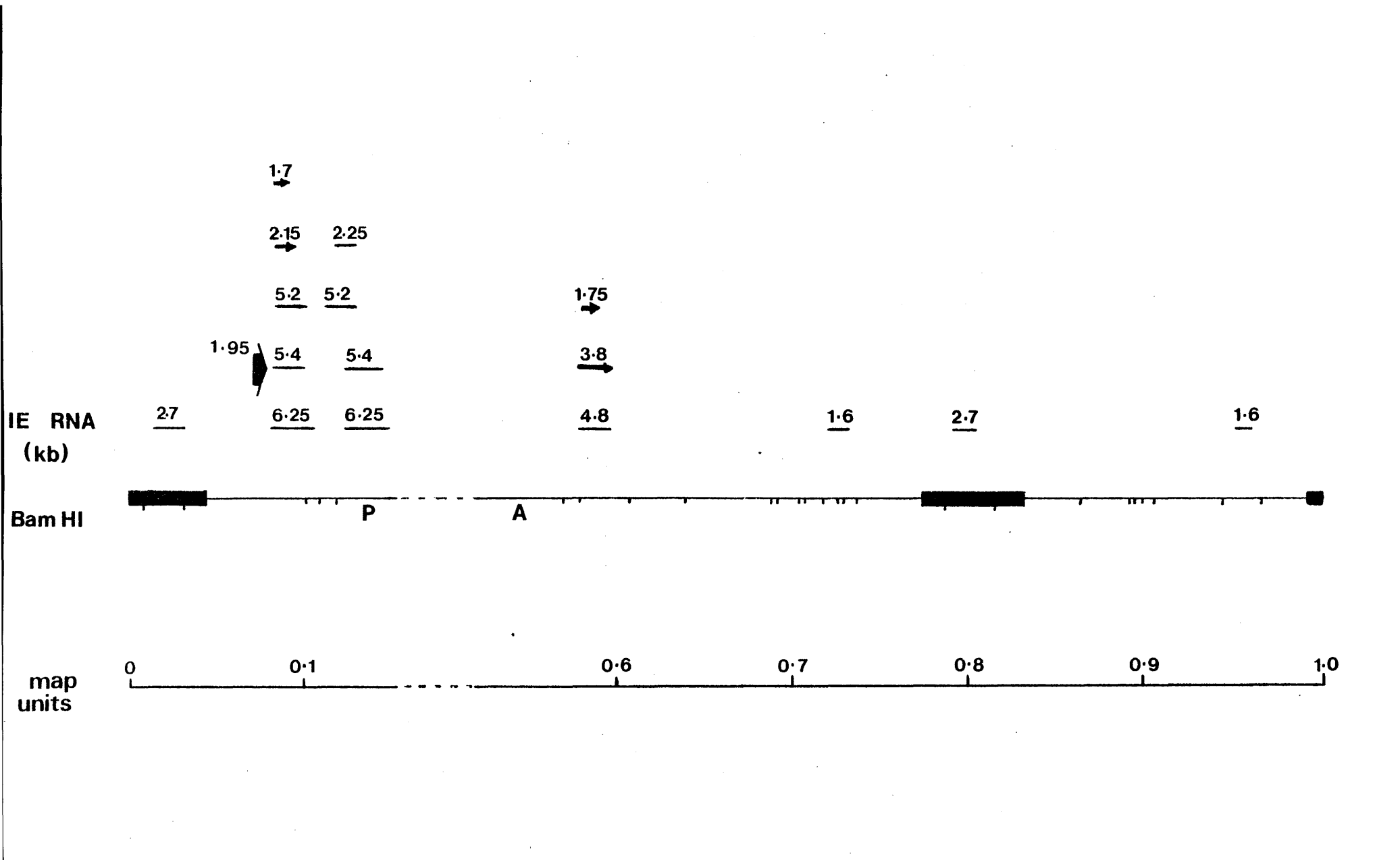

1·7
2·15
2·25
5·2
5·2
1·75
1·95
5·4
5·4
3·8
IE RNA (kb)
2·7
6·25
6·25
4·8
1·6
2·7
1·6
Bam HI
P
A
map units
0
0·1
0·6
0·7
0·8
0·9
1·0

FIGURE 3 Transcription of the HCMV Genome

Location of immediate early transcription on the HCMV genome. A summary showing size, map location and, in some cases, orientation of immediate early transcripts. The region of the genome omitted from this map between BamHI P and BamHI A shows no significant hybridization with cytomegalovirus polyadenylated RNA.

(Figure from Wilkinson et al., 1984)

of considerably lower MW than that estimated by SDS PAGE. This may be due to the high proline content which may disrupt part of the protein's helical structure leading to anomalous mobility on SDS PAGE. There were only 2 amino acid differences between the major IE proteins of AD169 and Towne which is surprising in view of the significant differences in their electrophoretic mobilities by SDS PAGE (Cameron and Preston, 1981; Gibson, 1981b). There is a putative promoter regulatory region extending 509 nucleotides upstream from the transcriptional start site (Akrigg et al., 1985). It contains typical TATA and CAAT boxes and several short direct and inverted repeat sequences of 16, 18, 19 and 21 nucleotides which may also influence the regulation of transcription. A promoter regulatory region 465 nucleotides upstream of the cap site for transcriptional initiation has also been described for HCMV strain Towne (Thomsen et al., 1984; Stinski et al., 1983). The Towne major IE gene is activated by cis-acting (enhancer) elements in the promoter-regulatory sequence and by virus-specific trans-acting components (Stinski and Roehr, 1985). There is evidence for the presence of enhancers and trans-acting components in the AD169 genome. Akrigg et al. (1985) have identified potential enhancer core sequences in the complementary strand of the IE promoter at -98 to -93, -157 to -161 and -412 to -417. Boshart et al. (1985) have recently identified

strong enhancer sequences located upstream of the transcription initiation site of the major IE gene between nucleotides -118 and -524. The enhancer is 3-5 fold more active than the SV40 enhancer. Galloway and Hill (1985) have also reported a transcriptional enhancer within the transforming region identified by Nelson et al. (1982) downstream of the major IE gene. In addition, Everett and Dunlop (1984) have reported trans-activation of the HSV-1 glycoprotein D and rabbit β globin promoters by HCMV AD169 which suggests that the AD169 genome also contains trans-activating elements.

Early transcription occurs in the absence of viral DNA synthesis between 4 and 24 hr post infection (DeMarchi et al., 1980). At early times, abundant transcription is shifted from the long unique to the long repeat and adjacent sequences in strains, Davis, Towne, and AD169 (DeMarchi, 1981; Wathen and Stinski, 1982; McDonough and Spector, 1983) and continues late into infection. Since early viral gene products are presumably involved in viral DNA replication, the continuous expression of the gene products from the long repeat sequences could be related to the extended phase of viral DNA replication which does not reach its peak until 72-96 hr post infection. Ten to 16 early polypeptides have been detected within the first 6 hr of infection (Stinski, 1978; Blanton and Tevethia, 1981). One of the early proteins has been identified as a 68,000

dalton phosphoprotein with protein kinase activity (Michelson et al., 1984; Mar et al., 1985). The protein can be detected by immunoprecipitation from 3 to 120 hr after infection and is induced by AD169, Towne and Davis strains but not by simian CMV's. The 68,000 dalton protein is of particular interest as it may be involved in transformation since some transformation-related gene products of both RNA (Collett and Erikson 1978) and DNA viruses (Griffin et al., 1979; Smith et al., 1979) described so far appear to be protein kinases.

Late transcription occurs after viral DNA synthesis from most regions of the HCMV genome. The proteins coded for by the late mRNA are structural proteins and at least 35 have been described (Sarov and Abady, 1975; Fiala et al., 1976; Kim et al., 1976; Gupta et al., 1977; Stinski 1976, 1977, 1978). Few late transcripts and their corresponding proteins have been studied to date. Davis et al. (1984) have mapped a major virion phosphorylated tegument protein of 67,000 daltons to an mRNA coded for by the long unique region between mu 0.37 and 0.39. The nucleotide sequence of this protein has been determined (Davis et al., 1985). The authors suggest that the protein may have important functions, such as protein kinase activity, DNA binding and possible transcriptional activation of IE genes. Nowak et al. (1984a) have identified the DNA sequences coding for a virion phosphoprotein of 71,000 daltons. An mRNA of the same

size, map coordinates and orientation is translated into the 65,000 dalton polypeptide. Other structural proteins have been analysed but not yet mapped (Farrar and Oram, 1984; Clark et al., 1984).

Genetics

Genetic analysis of temperature sensitive (ts) mutants of HSV has generated a wealth of information about the locations and functions of essential genes in the HSV lytic cycle. Recombination analysis has produced genetic linkage maps for HSV genomes (Brown et al., 1973; Subak-Sharpe, 1973; Timbury and Calder, 1976; Preston, 1981) and many polypeptides have been mapped by analysis of intertypic HSV-1 and HSV-2 recombination and by marker rescue (Marsden et al., 1978; Preston et al., 1978). Unfortunately, this approach to gene mapping and analysis of gene functions has met with little success when applied to HCMV. After mutagenesis by UV light, Ihara et al. (1978) isolated 15 ts mutants of HCMV which were unable to form plaques at 39°C but formed plaques at 34°C. Five mutants defective in viral DNA synthesis at 39°C were classified into 2 complementation groups according to whether they were normal or defective in inducing viral DNA polymerase. Yamanishi and Rapp (1979a) used DNA-negative HCMV ts mutants to induce host DNA synthesis and DNA polymerase in permissive and nonpermissive cells. They concluded that 4, and possibly

more, cistrons control the synthesis of HCMV DNA and that HCMV DNA is not required for the induction of host cell DNA synthesis. The ts mutants of HCMV are, however, unstable as they revert to wild type on recovery from the freezer (Ihara, personal communication). Further studies have not, therefore, been possible with ts mutants. Attempts to produce HCMV ts mutants in this Institute also proved to be unsuccessful (J. Cameron, personal communication).

Genetic mapping of the HCMV genome is more likely to be accomplished by sequencing. This approach has been successful with VZV (Davison, 1985). Most of the HCMV AD169 HindIII E fragment containing the transforming region identified by Nelson et al. (1982) and the major immediate early gene has already been sequenced (Kouzarides et al., 1983; Nelson et al., 1984; Akrigg et al., 1985) and the group in Cambridge are currently sequencing the entire HCMV AD169 genome (Kouzarides et al., 1985).

Nonproductive infection

During nonproductive infection, virus adsorption and penetration occur but there is no viral DNA synthesis. Viral cpe can sometimes be observed. For example, Fioretti et al. (1973) observed cpe when guinea pig cells were infected at high multiplicity. Some, but not all, early functions of HCMV are expressed in nonpermissive

cells. IE and early antigens have been detected in the nucleus and cytoplasm of HCMV infected nonpermissive cells (Fioretti et al., 1973; Michelson-Fiske et al., 1977) and also in HCMV-transformed nonpermissive cells (Albrecht and Rapp, 1973) by immunofluorescence. IE and early proteins, but not late proteins can also be detected in infected nonpermissive cells by SDS PAGE (Stinski, 1978). The viral genome can persist in nonpermissive cells for a long period of time and be recovered by fusion with permissive human cells (Boldogh et al., 1977). Stimulation of host cell macromolecule synthesis, thought to require early HCMV gene products, also occurs in HCMV infected nonpermissive cells (St. Jeor et al., 1974; Furukawa et al., 1975). DeMarchi (1985) observed that in nonpermissively infected rabbit kidney cells, only the earliest of the early transcripts are synthesised and progression of transcription into the later part of the early phase does not occur. The lack of expression of some early functions in these nonpermissively infected cells results, therefore, from a lack of transcription of some early virus genes. Transcription could be prevented by cellular events such as cell proteins binding to DNA and preventing further transcription.

Stinski et al. (1981) have proposed that the nonpermissive cell may favour persistence or latency of the viral genome. Latency occurs in the nonpermissive

cell when the genome does not proceed to replication. Physiological changes and the eventual expression of a required early viral gene may convert the nonpermissive cell to a permissive cell. As a consequence of this, the viral genome could move from the latent state to productive infection. This hypothesis is supported by the work of St. Jeor and Rapp (1973) which showed that it is possible to induce the replication of HCMV in nonpermissive cells pretreated with 5-iodo-2'-deoxyuridine. Possibly 5-iodo-2'-deoxyuridine interferes with the production of a cellular product inhibitory for HCMV replication or alternatively induces a cell product, not normally present, which aids or permits virus replication.

Latent and persistent infections

Most HCMV infection is subclinical (Weller, 1971). Primary infection is followed by persistent infection, during which virus continues to be shed sometimes for long periods of time. Even after virus shedding has ceased, the virus persists in a 'silent' state, probably for life. This latter condition is referred to as 'latency'. During latent infection, no infectious virus or late antigens can be detected.

The majority of the adult population is believed to harbour HCMV in a latent state. This is supported by the isolation from infected individuals of cell lines that

contain HCMV DNA but are free of detectable infectious virus or late antigens (Rapp et al., 1975; Joncas et al., 1975). Reactivation of latent virus can occur in response to an imbalance in the immunological integrity of the host. Clinical situations which allow for the reactivation of HCMV include pregnancy (Montgomery et al., 1972) organ and bone marrow transplants (Gadler et al., 1982; Craighead, 1969; Rinaldo et al., 1976) blood transfusions (Diosi et al., 1969; Perham et al., 1971) and immunosuppressive chemotherapy (Dowling et al., 1970). All these situations share two common features, a change in the immune system and the presence of foreign antigen.

The cell type in which HCMV persists or remains latent is unknown. There is evidence to suggest that lymphocytes (Pagano, 1975; St. Jeor and Weisser, 1977) or leukocytes (Fiala et al., 1975) may be involved. Joncas et al. (1975) have reported the presence of an unexpressed HCMV genome in an EBV-transformed human lymphoblastoid line. Other possible sites of HCMV latency include human skin fibroblasts (Williams et al., 1980) and human kidney epithelial cells (Pagano, 1975). HSV has been shown to be latent in the trigeminal ganglia (Bastian et al., 1972; Baringer and Swoveland, 1973; Warren et al., 1977). There is no evidence to suggest that HCMV is latent in ganglia. It would seem that HCMV may become latent in a variety of cell types.

Very little evidence is known about the state of the viral genome during latency. Gadler and Wahren (1983) presented evidence to suggest that HCMV DNA integrates into, or associates with a subset of cell DNA relatively rich in A-T content. They assume complete viral genomes persist in the cells but do not know whether they are fragmented or present as a continuous sequence.

A number of in vitro model systems for studying HCMV latency have been developed. Some groups have utilized inhibitors of viral replication such as cytosine-arabinoside (Gönczöl and Vaczi, 1973), phosphonoformic acid (Gadler and Wahren, 1983) and human leucocyte interferon and acyclovir (Bucher et al., 1983) to establish latency in cells normally permissive for HCMV. The value of acyclovir in the model system used by Bucher et al. (1983) is questionable, since acyclovir is not inhibitory to HCMV replication. Li and Albrecht (1982) established nonproductive persistent HCMV infections in human embryo lung (Helu) cells by using supra-optimal temperatures to restrict HCMV replication. Mocarski and Stinksi (1979) established persistent HCMV infections in human fibroblasts by high multiplicity infection and long term tissue culture.

Murine cytomegalovirus (MCMV) has also been used in model systems for studying latency. MCMV is latent in spleen cells (Mayo et al., 1978; Wise et al., 1979; Hudson et al., 1979; Mercer and Spector, 1985), salivary

gland tissues (Cheung and Lang, 1977; Cheung et al., 1980) and prostate gland tissue (Cheung and Lang, 1977). Like HCMV, latent MCMV can be reactivated by immunosuppression, (Jordan et al., 1977; Mayo et al., 1978) and during pregnancy (Gould and Mims, 1980). No MCMV specific antigens or virus particles can be detected in latently infected cells but nucleic acid hybridization studies indicate the presence of viral DNA. Cheung et al. (1980) were able to detect 45 copies per cell of MCMV specific DNA in cultures of salivary gland cells and 0.2 copies per cell in prostate gland cells.

It is hoped that using the model systems described will help to determine how CMV exists during its latent state and define more precisely the stimuli involved in reactivation.

ALTERATIONS IN HOST MACROMOLECULAR SYNTHESIS AFTER INFECTION BY HCMV

Stimulation of host cell nucleic acid synthesis

HCMV is able to induce cellular DNA synthesis in both permissive Helu cells and nonpermissive Vero cells (St. Jeor et al., 1974). AD169, Davis and Colburn (C-87) strains have been found to stimulate host cell DNA synthesis which suggests that this stimulation may be characteristic of the cytomegalovirus group. The DNA synthesis induced by HCMV infection appears to represent

normal semiconservative replication as opposed to repair synthesis (St. Jeor et al., 1974; DeMarchi and Kaplan, 1976). It has been suggested that HCMV replication and synthesis of new virus is dependent upon cell DNA synthesis (St. Jeor and Hutt, 1977). It appears that viral DNA synthesis is not required for the stimulation of host cell DNA synthesis as maximum stimulation occurs before the onset of viral DNA replication (St. Jeor et al., 1974). Certain HCMV ts mutants defective in viral DNA synthesis retain their ability to induce cellular DNA synthesis at both the permissive and nonpermissive temperatures in both permissive and nonpermissive cells. (Ihara et al., 1977; Yamanishi and Rapp, 1979a). This suggests that an early gene product of HCMV may be responsible for stimulation.

The ability of infectious virions to induce the synthesis of cellular DNA increases if the virions are irradiated with low doses of UV (DeMarchi and Kaplan 1977; Boldogh et al., 1978). After high doses of UV no stimulation is observed (St. Jeor et al., 1974; Furukawa et al., 1975).

DeMarchi and Kaplan (1977b) have found that defective virions play an important role in the stimulation of host cell DNA synthesis. More recently, defective virions have been found to induce a cytomegalovirus growth factor that enhances DNA synthesis and mitotic activity in target cells (Gönczöl and

Plotkin, 1984).

Furukawa et al. (1976) reported that much of the stimulation of cellular DNA synthesis is due to an increased rate of mitochondrial DNA synthesis. HCMV stimulation of mitochondrial DNA synthesis is not mediated through a stimulation of nuclear DNA synthesis as in polyoma and SV40 infection (Levine, 1971; Vesco and Basilico, 1971; Radsak and Albring, 1974).

In addition to stimulating host cell DNA synthesis, HCMV infection also stimulates host cell RNA synthesis in permissive and nonpermissive cells (Tanaka et al., 1975; Furukawa et al., 1975). Stimulation of RNA synthesis in infected cells precedes the synthesis of viral DNA and progeny virus by approximately 24 hr and the RNA species synthesised include ribosomal 28S and 18S, and 4S transfer RNA. The stimulation of host cell ribosomal RNA seems to be a characteristic feature of HCMV infections. Oncogenic viruses like polyoma and SV40 have been shown to enhance host cell RNA species but not host cell ribosomal and transfer RNA synthesis (Oda and Dulbecco, 1968). Herpesviruses inhibit the synthesis of cellular RNA and polysomes early in infection (Flanagan, 1967; Hay et al., 1966; Tamara et al., 19721 Wagner and Roizman, 1969). Recently, HSV has been shown to stimulate certain cellular RNA species (D. Latchman, personal communication). Tanaka et al. (1975) suggest that HCMV induction of host cell RNA synthesis is dependent upon a

protein(s) that is synthesised during the early stages of infection.

Stimulation of host cell protein synthesis

HCMV stimulates host cell protein synthesis on infection. During the early phase of HCMV infection, 70-90% of the total proteins synthesised are host specific (Stinksi, 1977). Although host protein synthesis decreases at the onset of viral DNA synthesis, it still accounts for 40-50% of the total proteins synthesised during the late phase of infection. This distinguishes HCMV from HSV which only induces a small number of cellular proteins on infection (LaThangue et al., 1984 and personal communication) and subsequently specializes in viral protein synthesis. Selective suppression of host cell protein synthesis by the hypertonic conditions indicates that HCMV-induced proteins are synthesised in a cyclic manner (Gupta and Rapp, 1978). It is not known how HCMV stimulates host cell protein synthesis but as stimulation can occur before the onset of viral DNA replication it is possible that an early virus coded protein may be responsible.

The cellular proteins induced by HCMV infection have been poorly studied. Weder and Radsak (1983) described the induction of a host specific chromatin-associated glycopolypeptide which possibly mediates the stimulation of host cell DNA synthesis. More recently, Radsak et al.

(1985) described three host derived glycopolypeptides one of which exhibits immunological relatedness to fibronectin.

Stimulation of host cell enzyme synthesis

In HCMV infected cells the stimulation of host cell nucleic acid synthesis is usually preceded by enhanced enzyme activities. These include thymidine kinases, DNA polymerases, DNA-dependent RNA polymerases, exonuclease and topoisomerases, ornithine decarboxylase and plasminogen activator. Enhanced enzyme activity occurs after the derepression of host cell chromatin. HCMV induces at least two chromatin associated factors immediately after infection which activate template activity of chromatin (Kamata et al., 1978).

HCMV infection stimulates the production of cellular thymidine kinases within 12 hr after infection. In HCMV infected fibroblasts there is rapid high level stimulation of cytoplasmic cellular thymidine kinase activity (Zavada et al., 1976) and mitochondrial thymidine kinase activity (Estes and Huang, 1977). Characterization of these enzymes with respect to phosphate donor specificity, optimal pH, thermostability, salt inhibition and electromobility shows that the postulated virus stimulated thymidine kinase activities in HCMV-infected cells are actually all of cellular origin. HCMV does not code for its own thymidine kinase,

a feature that distinguishes it from HSV and also accounts for the fact that antiviral drugs, such as acyclovir, that act by inhibiting the production of viral thymidine kinases are not effective against HCMV. HCMV infection also stimulates host cell synthesis of α and β DNA polymerases (Hirai, et al., 1976; Hirai and Watanabe, 1976). These enzymes may be associated with the stimulation of host cell DNA synthesis. It is not known whether they are also involved in the initiation or elongation of viral DNA synthesis or act as core enzyme for the formation of viral-specific holoenzyme for viral DNA synthesis. In addition, HCMV induces a novel virus-specific DNA polymerase in infected cells that can be distinguished from the cellular DNA polymerases by its distinct chromatographic behaviour, template primer specificity, sedimentation property and salt requirement for maximal activity (Huang 1975; Hirai et al., 1976; Nishiyama et al., 1983). Tanaka et al. (1978) also found that HCMV infection induces three major classes of DNA-dependent RNA polymerases in human diploid cells. There is also evidence that the levels of topoisomerases are greatly enhanced upon infection with HCMV (Huang et al., 1984). These enzymes reduce the helical turn numbers of supercoiled DNA molecules which could facilitate DNA mutagenesis by an oncogenic virus. Isom (1979) found that HCMV markedly stimulated the activity of ornithine decarboxylase (ODC) and that the increase in

activity is apparent 12 hr post infection. ODC is the first enzyme and rate-limiting step in the biosynthesis of polyamines and increases in ODC activity are found to accompany and precede rises in DNA synthesis. Levels of the enzyme are low in quiescent cells but high in tumour tissue and transformed cells and also increase after infection with tumour viruses. The ability of HCMV to stimulate ODC activity is dependent upon the multiplicity of infection (moi). Stimulation is not inhibited by the addition of polyamine although it is reversibly inhibited by the addition of phosphonoacetic acid.

HCMV infection has also been found to stimulate the production of plasminogen activator in both permissive and nonpermissive cells (Yamanishi and Rapp, 1979b). Plasminogen activator is another enzyme closely associated with malignant transformation (Unkeless et al., 1973; Ossowski et al., 1974). It is a protease that converts a serum plasminogen to plasmin which in turn hydrolyses fibrin. HCMV stimulation of plasminogen activator is apparent 24 hr post infection. It does not require viral DNA replication suggesting that the stimulation of plasminogen activator is controlled by an early gene function of HCMV.

Stimulation of Glucose uptake

It has been known for sometime that enhanced glucose uptake is associated with malignant transformation

(Hatanaka, 1974). HCMV infection causes a two-fold increase in the uptake of glucose by infected cells 20 hr post infection and an even greater increase later in the viral replication cycle (Landini, 1984). The selective increase in glucose uptake in HCMV infected cells could be the result of virus-induced stimulation in cellular macromolecule synthesis producing a demand for energy yielding reactions. This could lead to a rapid depletion of glucose from the medium and an increase in the number of glucose receptors on the plasma membrane. However, the stimulation of glucose uptake appears to depend upon early transcription of the HCMV genome and de novo protein synthesis, events not required for HCMV stimulation of host cellular macromolecule synthesis. Furthermore, at least part of the enhanced glucose uptake seems to be independent of the sugar level in the medium. This would suggest that there may be a specific virus-induced modification of the infected cell plasma membrane.

ONCOGENIC TRANSFORMATION OF CELLS BY HCMV

HCMV can transform both permissive and nonpermissive cells in vitro and these transformed cells are tumourigenic when introduced into experimental animals. Furthermore, the oncogenicity of the cells is dramatically increased after animal passage and the

tumours frequently metastasize.

Oncogenic transformation was first demonstrated by Albrecht and Rapp (1973) in hamster embryo fibroblasts (HEF) after exposure to UV-irradiated HCMV. The HCMV-transformed HEF cells are tumourigenic in weanling hamsters and express HCMV-induced cytoplasmic and membrane antigens. No infectious virus can be isolated or virions detected in the cells. Isom et al. (1983) have shown that UV-irradiated HCMV will also transform guinea pig fibroblasts and that the transformed cells produce tumours when inoculated into athymic nude mice. In this laboratory we have successfully transformed rat embryo (RE) cells using UV-irradiated HCMV AD169 and these transformed cells are again tumourigenic in Hooded Lister rats. They express antigens apparently induced by HCMV infection but lack of detectable HCMV virus DNA sequences (Fletcher, this thesis) suggests that such antigens are not HCMV-specific but probably of cellular origin.

HCMV has also been shown to transform human cells in vitro. Rapp et al. (1975) isolated a strain of HCMV (designated Mj) from human cells of prostatic origin. After continuous passaging these cells demonstrated non-contact-inhibited growth which suggests that the cells are either chronically infected or have been transformed in vitro by HCMV. Geder et al. (1976) further investigated the latter possibility by

establishing persistent infections in Helu cells using the HCMV Mj strain. After a crisis period, foci of transformed cells appeared and two transformed cell lines were subsequently established. These transformed cells are tumourigenic in athymic nude mice (Geder et al., 1976, 1977) and have detectable HCMV-induced membrane and nuclear antigens (Geder et al., 1976; 1977; Geder and Rapp, 1977). Microcytotoxicity tests reveal that the transformed cells share a common membrane antigen with HCMV-transformed HEF cells. Lack of published evidence for the retention of HCMV DNA sequences and our own experience, cast doubt on the presence of HCMV coded antigens and suggest that the antigens recognised in HCMV-transformed cells are cellular in origin and may result from polypeptides switched on by HCMV infection perhaps by transactivation and subsequently upregulated continuously. The studies do, however, provide further evidence for the possible oncogenic potential of HCMV in its natural host.

More recently, transformation studies using restriction endonuclease fragments of the HCMV genome have led to the identification of the DNA sequences involved in initiating transformation. Nelson et al. (1982) transfected NIH 3T3 cells with restriction DNA fragments of HCMV AD169 and obtained transformation with the HindIII E and XbaI N fragments. The transformed cells exhibit anchorage-independent growth in

methylcellulose and tumourigenicity in Balb/c nude mice. No viral DNA has been detected in the transformed cells at a sensitivity of 0.5 copies per cell. However, Buonaguro et al. (1985) have generated transformants which retain viral DNA by cotransfecting the transforming fragment with a dominant marker (pSV2-neo). Characterisation of the retained viral sequence is in progress. The frequency of transformation is lower than that of transformation by avian sarcoma and leukosis viruses but comparable to that obtained with adenovirus or with HSV-2 DNA fragments. Nelson has revealed the transforming region to be a 2.9kb sequence located within the HindIII E fragment between mu 0.123 and 0.14 (Nelson et al., 1982). Later experiments have shown that this fragment can be reduced to about 558 bp and still initiate transformation (Nelson et al., 1983).

The transforming region of AD169 has been sequenced (Nelson et al., 1983; Kouzarides et al., 1983). The possibility of the sequence coding for a protein that could be involved in transformation has been considered. The sequence contains no open reading frames of significant size that could code for such a protein and further investigations have ruled out the possibility of RNA splicing (Nelson et al., 1983). The transforming activity is sensitive to EcoRI digestion suggesting that the nucleotide sequence at the EcoRI site must be essential for transformation. Kouzarides et al. (1983)

noted that the EcoR1 site falls within an 8 bp inverted complementary repeat that could be part of a promoter sequence. This suggestion is also supported by the fact that the transforming region is A-T rich, a feature characteristic of promoter sequences. The sequence is bounded by suitable transformation signals and could encode a polypeptide of 80 amino acids if a single donor and acceptor splice site were used. Galloway and Hill (1985) have since presented evidence to suggest that the transforming region is a transcriptional activator that could initiate transformation by altering cellular gene expression.

Spector and Vacquier (1983) and Gelmann et al. (1983) detected DNA sequences homologous to v-myc in the AD169 HindIII E fragment by Southern analysis. However, Rasmussen et al. (1985) have reported that homologous regions between the HCMV genome and v-myc are limited to short G+C rich regions in each genome and that the HCMV genome shares little or no homology with the human c-myc gene. Kouzarides et al. (1983) could find no evidence of homology with viral oncogenes within the transforming region itself.

Analysis of the transforming sequence has revealed one interesting feature. There is a stem-loop structure located near the HindIII site with an EcoRI site in the stem of the molecule. This is homologous with a stem-loop structure found in the BglII N transforming fragment of

HSV-2 (Galloway et al., 1984).

Clanton et al. (1983) have described a second transforming region of the HCMV genome. This Towne XbaI E fragment is homologous to the HSV-2 BglII C fragment previously described to be capable of transforming normal diploid HEF cells (Jariwalla et al., 1980). It is also homologous to a sequence located adjacent to the AD169 transforming region described by Nelson et al. (1982). The XbaI E fragment of Towne transforms normal HEF cells in the continuous passage assay and NIH 3T3 cells in the focal transformation assay. The transformed cells are tumourigenic in athymic nude mice. El-Beik et al. (1984) localized the transforming activity within the two terminal XbaI/Bam HI subfragments EJ (7.6 kb) and EM (3.0 kb). Razzaque et al. (1985) reported the presence of EM sequences in cell lines transformed with EM alone and the lack of EJ sequences in cell lines transformed with EJ alone. Jariwalla et al. (1985) have shown that metastasis in rats is mediated by the co-transfer of EM and EJ DNA fragments. Southern analysis of genomic DNA from EJ + EM -induced rat tumours revealed the presence of EM but not EJ sequences. Northern analysis of total RNA from the same tumours exhibited complete shut-off in the synthesis of cytoskeletal alpha-actin, a transformation-sensitive marker in rat fibroblasts.

Gelmann et al. (1983) have identified myc homologous sequences located in the 2 Bam HI subfragments of the

Towne XbaI E fragment. However, since homology is restricted to the 5' half of *myc* it is unlikely that the homologous sequences in HCMV represent specific functional genes.

A comparison between transformation by HCMV and HSV-2 reveals several features common to both. Both viruses have more than one location of sequences involved in transformation and viral DNA is not retained in the transformed cells in most cases. One transforming sequence of HCMV DNA is similar to one of HSV-2 in possessing a stem-loop structure. Despite these similarities, it is unlikely that both viruses have a similar mode of transformation. Recent evidence suggests that HSV-2 induces mutations of cellular genes (Brandt *et al.*, 1985) HCMV has not been shown to have similar mutagenic capacity. The possible modes of HCMV transformation are discussed more fully later in this thesis.

In addition to the human cytomegaloviruses, equine cytomegalovirus (ECMV) has oncogenic potential and can transform cells *in vitro* (Staczek *et al.*, 1984). High multiplicity infection of primary LSH hamster embryo cells with ECMV results in the coestablishment of persistent infection and oncogenic transformation. ECMV-transformed cells are tumourigenic in LSH hamsters, resistant to ECMV and equine herpesvirus type 1 (EHV-1) superinfection and express ECMV-specific proteins.

Transformed cells have also been found to contain small amounts of a limited portion (<0.05%) of the ECMV genome. However, no evidence exists that ECMV's are associated with neoplastic disease in the horse - an uncommon state.

THE ASSOCIATION OF HCMV WITH VARIOUS HUMAN NEOPLASIAS

It is very difficult to prove that viruses are involved in the development of human cancers. Causal associations have to be deduced from sero or molecular epidemiological surveys and the results of such surveys must be interpreted with caution. HCMV is a ubiquitous pathogen and exhibits a complex epidemiology. Seroepidemiological studies are complicated by the naturally high level of antibodies to HCMV in the population. The overall frequency of antibodies to HCMV in a normal population for all ages is thought to be approximately 54% (Stern and Elek, 1965) but is higher among the newborn and older members of the population (Embil *et al.*, 1969). Fluctuations of antibody levels occur in individuals due to blood transfusion, immunosuppression (particularly as a result of chemotherapy) and pregnancy. Although molecular epidemiological studies may reveal the presence of virus or viral information in tumour tissue, it is not conclusive of a causal relationship. As HCMV can infect a wide range of cells and tissues and be present in

almost all body fluids, the presence of virus in tumour tissue could be fortuitous. Cancer patients are immunosuppressed and ideal targets for HCMV infection. Latent virus could reactivate or secondary infection occur during malignancy.

These problems should be borne in mind when evaluating the evidence presented here for a causal association between HCMV and various human neoplasias.

Cervical carcinoma

Several epidemiological studies (Rawls et al., 1968; Nahmias et al., 1970) and molecular studies (Eglin et al., 1981; Galloway and McDougall, 1983; Park et al., 1983; Macnab et al., 1985) have linked cervical neoplastic disease to infection by HSV-2. However, in some geographical areas such an association is not observed and not all women with cervical carcinoma have serologic evidence of past HSV-2 infection (Vonka et al., 1984). These factors indicate a possible multifactorial etiology of cervical carcinoma and it has been suggested that HSV-2 may interact with other agents that infect the cervix (Zur Hausen, 1982). Human papillomaviruses (HPV) have been strongly implicated in cervical carcinoma. Koilocytes and antigens characteristic of HPV infection can be detected in neoplastic tissue (Meisels et al., 1979; Shah et al., 1980). DNA from HPV6 and 11 has been detected in CIN tissue (Gissmann et al., 1983; McCance et

al., 1983) and DNA from HPV16 and 18 has been detected in carcinoma tissue (Dürst et al., 1983; 1985; Boshart et al., 1984). Evidence is presented in this thesis for considering HCMV as a possible oncogenic agent in the development of cervical cancer.

HCMV has been isolated from semen, prostate, uterus and cervix and can be sexually transmitted. It can persist for many months in asymptomatic carriers and has been associated with other sexually transmitted diseases. In a study by Jordan et al. (1973), HCMV was more frequently isolated from the cervices of women with suspected venereal disease than HSV-2. HCMV has also been isolated from the genital tract and urine of sexual partners (Chretien et al., 1977; Chandler et al., 1984; Handsfield et al., 1985). In addition, HCMV has been identified as a common cause of cervicitis and urethritis following venereal transmission (Evans, 1976).

It is now generally accepted that cervical carcinoma is a progressive disease. The first signs of the disease are characterised by the presence of abnormal epithelial cells in the cervix and are most often graded as dysplasia or CIN. Severe dysplasia is usually referred to as carcinoma in situ (CIS). When the disease has progressed to the stage where neoplastic cells breach the basement membrane it is termed invasive carcinoma.

There have been relatively few seroepidemiological studies investigating an association between HCMV and

cervical carcinoma. All stages of the disease have been investigated but the studies so far have yielded controversial results. Fucillo et al. (1971) found that indirect haemaglutination (IHA) antibody titres to HCMV were not significantly elevated among patients prior to the development of CIS or during the period that CIS was present when compared to matched controls. Sprecher-Goldberger et al. (1971) looked at complement fixing (CF) antibodies to adenovirus associated virus, adenoviruses, HCMV and HSV in patients with various types of cancer and in control individuals but found no association between elevated HCMV antibodies and cervical carcinoma. Kumar et al. (1980) studied the humoral and cellular immune responses in patients with cervical carcinoma and healthy controls. They found no significant differences between CF and IHA antibodies to HCMV and lymphocyte stimulation in patients with cervical carcinoma and healthy controls. Similarly Hart et al. (1982b) found no correlation between antibody titres to HCMV tested by ELISA, ACIF and neutralization methods and either cervical dysplasia or CIS. Best et al. (1983) found no significant difference in the prevalence or titres to HCMV IgG and IgA antibodies in patients with cervical dysplasia or carcinoma and in matched controls from England, Sri Lanka, Malawi and Sudan.

By contrast, some studies have correlated elevated antibody levels to HCMV with cervical dysplasia and

carcinoma. Vestergaard et al. (1972) reported a significantly higher level of CF antibodies to HCMV in patients with cervical carcinoma when compared with matched controls. There appeared to be no correlation between the incidence of antibodies and clinical stage of the disease. Pacsa et al. (1975) detected CF antibodies to HCMV more frequently in the sera of women with cervical atypia than in the sera of women with cervical disorders other than atypia or healthy controls. Stoian et al. (1982) looked at the prevalence of HCMV antibodies in patients with various cancers and reported a higher incidence of CF antibodies to HCMV in patients with breast cancer, uterine cervical carcinoma and ovarian neoplasms. This study, however, was not suitably controlled. More recently there has been a seroepidemiological study of patients attending a colposcopy clinic at the Western Infirmary, Glasgow (Walkinshaw, Roberts, and Cordiner, in preparation). This study was carried out between 1982 and 1984 and included both patients with CIN and patients with no evidence of dysplasia. Exposure to HCMV and HSV-2 was determined by the presence of CF antibodies to the viruses and HPV infection was diagnosed by histological examination of colposcopy specimens. Initially the data showed no significant difference between CIN patients and controls for previous infection with a single agent (HCMV, HSV-2, HPV or Chlamydia) suggesting that single

agent infection is not a significant risk factor. However, significantly more patients with CIN had evidence of prior HCMV and HSV-2 or HCMV, HSV-2 and HPV combined infections when compared with the controls. This may represent evidence for interaction between these viruses or the presence in the study population of a small group exposed to multiple infectious agents.

Further evidence for an association comes from a study by Melnick et al. (1978). They isolated HCMV from cell cultures derived from 2 out of 10 cervical cancer biopsies from patients in an advanced state of the disease. The patients demonstrated CF antibodies to HCMV and 6 out of 8 patients tested also had HSV-2 antibodies. Huang et al. (1983) performed a molecular epidemiological survey of HCMV and HSV-2 DNA in the normal cervix and cervical carcinomas from various geographic regions by DNA-DNA reassociation kinetic analysis. They found no significant difference between the frequency of HCMV DNA in African patients with normal cervices compared with those with carcinoma of the cervix, but among patients from Taiwan HCMV DNA was detected more frequently in cervical carcinoma specimens than in normal cervices. The HCMV DNA positive rate was found to be significantly lower in cervical carcinoma specimens from Finland and the USA. From my own work described in this thesis I have detected HCMV DNA in 2 of 43 CIN biopsy specimens from patients in the West of Scotland. These patients

were also included in the epidemiological study by Walkinshaw, Roberts and Cordiner mentioned previously.

It is very difficult to postulate a causal association between cervical carcinoma and HCMV alone. It is far more likely that more than one virus and probably other environmental factors are involved in the etiology of this disease. These possibilites are discussed later in this thesis.

Prostatic carcinoma

HCMV has been considered as a possible oncogenic agent in the development of this disease for several reasons. HCMV can be sexually transmitted and the prostate appears to be a repository for HCMV (Lang and Kummer, 1972; Lang et al., 1975). As mentioned previously, Rapp et al. (1975) isolated a strain of HCMV, CMV-Mj, from normal prostatic tissue. Furthermore, this strain has been shown to be oncogenic. The prostatic cells infected in vivo by CMV-Mj grew to passage levels higher than those routinely attained by normal cells and exhibited properties characteristic of transformed cells. Geder et al. (1976) transformed Helu cells in vitro with CMV-Mj and established a transformed cell line designated CMV-Mj-HEL-2. When these cells were injected into athymic nude mice, tumours were produced from which another cell line, CMV-Mj-HEL-2, T-1, was established. Some of the evidence for an association of HCMV with

prostatic carcinoma comes from immunological data involving these transformed cells.

Patients with prostatic carcinoma show a significant humoral immune response to HCMV. Antibody titres against CMV-Mj-HEL-2, T-1 have been detected in patients sera by indirect immunofluorescence (Sanford et al., 1977b; Sandford et al., 1978). Studies have also indicated that patients with prostatic carcinoma demonstrate a cellular immune response to HCMV. Lymphocyte microcytotoxicity assays using CMV-Mj-HEL-2 and CMV-Mj-HEL-2, T-1 have revealed that a high percentage of patients with prostatic carcinoma show significant cytotoxicity to both these transformed cell lines (Sandford et al., 1977a; Sandford et al., 1978; Dagen et al., 1978). Control patients with benign prostatic hyperplasia and other urinary tract tumours showed significant lymphocyte cytotoxicity to CMV-Mj-HEL-2, T-1 but not CMV-Mj-HEL-2 transformed cells. The high degree of in vitro cytotoxicity to CMV-Mj-HEL-2, T-1, may reflect the ubiquity of HCMV infection in the general population. The fact that significantly more patients with prostatic carcinoma show cytotoxicity to CMV-Mj-HEL-2 suggests the presence of multiple antigens on the target cell membrane. Patients with prostatic carcinoma may recognise a tumour specific antigen as well as a viral specific antigen induced by HCMV. However, it must be stressed that the assays used in the studies mentioned

are not specific for HCMV, so there is a strong possibility that the antigens recognised are cellular in origin either casting doubt on the involvement of HCMV in prostatic cancer or suggesting that the mechanism of involvement may be obtuse.

HCMV antigens have also been detected in cultures derived from prostatic cancer cells. Geder et al. (1977) found 2 out of 34 prostatic cancer cell lines reacted with HCMV-specific human convalescent sera in indirect immunofluorescence tests.

More recently, Boldogh et al. (1983) have analysed normal prostate, benign prostate hypertrophy and prostatic adenocarcinoma biopsy specimens for HCMV and HSV-2 specific nucleic acids. They detected HCMV DNA in all 3 tissue groups and HCMV RNA in benign prostate hypertrophy and prostatic adenocarcinomas but not in normal prostate. It is possible that normal prostatic tissue is a site of latent HCMV infection and that the virus is reactivated during conversion of cells to malignancy. HSV-2 DNA was detected at a much lower frequency and HSV-2 RNA was only detected in one specimen. HCMV-induced antigens were demonstrated in benign prostate hypertrophy and prostatic adenocarcinoma sections by ACIF.

In conclusion, the studies so far indicate a very tenuous link between prostatic carcinoma and HCMV infection.

Adenocarcinoma of the colon

Little is known about the etiology of this disease, although epidemiological studies have implied that environmental and genetic factors may be involved. Another possibility is that there may be a viral association with the disease. HCMV has been found in the gastrointestinal tract of patients with ulcerative colitis (UC), a disease which predisposes to later malignancy (Powell et al., 1961) Levine et al., 1964; Henson, 1972; Farmer et al., 1973) and also in cell cultures derived from biopsy samples of patients with adenocarcinoma of the colon (ACC) (Hashiro et al., 1979). However, Avni et al. (1981) looked at antibody pattern to HCMV in patients with ACC using immunoperoxidase antibody to membrane antigen and CF techniques. They found significantly elevated antibody titres to HCMV in the sera of ACC patients undergoing chemotherapy but not in the sera of untreated ACC patients. A more recent study by Hart et al. (1982a) failed to detect HCMV in frozen sections of ACC gut mucosa using amplified direct immunofluorescence.

A number of studies have been undertaken to determine the presence of HCMV DNA in UC and ACC patients. These again have yielded controversial results. Using cRNA-DNA membrane hybridization which can detect 2 or more genome equivalents of HCMV DNA per cell,

Roche and Huang (1977) could only detect HCMV DNA in 1 out of 9 cases of UC and in no cases of Crohn's disease. In a later study they detected HCMV DNA in 4 out of 7 specimens of ACC, 1 out of 2 patients with familial polyposis and 1 out of 3 patients with UC. The techniques of DNA-DNA hybridization and reassociation kinetics are more sensitive and can detect 0.1 genome equivalents of HCMV per cell. Using a reassociation kinetics test Brichacek et al. (1980) failed to detect HCMV DNA in ACC biopsies. Roche et al. (1981) detected HCMV DNA in the majority of patients with ACC but also in histologically normal and diseased but non-neoplastic tissue. They, therefore, concluded that the HCMV detected was not specifically cancer associated. Hart et al. (1982a) also failed to detect HCMV mRNA in frozen tissue sections from patients with ACC by in situ hybridization. In considering the results of the studies to date it seems unlikely that HCMV plays a direct causative role in ACC unless as an initiation of some event in which retention of the HCMV genome is not required.

Kaposi's sarcoma

This is a multiple pigmented haemangiosarcoma occurring on the skin, common in equatorial Africa with a distribution strongly reminiscent of Burkitt's lymphoma. In Europe, it has been reported to occur with greater

frequency in Eastern countries and in certain parts of Italy, and is common among Ashkenazic Jews and homosexuals. Giraldo et al. (1972a) were the first to observe herpesviruses in cell cultures derived from biopsies taken from patients with Kaposi's sarcoma (KS). Immunological studies demonstrated a common precipitating soluble antigen in cell extracts of these virus-carrier lines tested against HCMV and EBV sera. A herpesvirus was subsequently isolated from one of these cell lines (Giraldo et al., 1972b) which was later confirmed to be a strain of HCMV by Glaser et al. (1977).

Seroepidemiological studies have shown that European KS patients (mainly regressors ie patients in which KS regresses rather than proceeds to malignancy) have elevated antibody titres to HCMV with a significant increase in the geometric mean over melanoma patients and healthy matched controls (Giraldo et al., 1975). No significant serologic association was observed with EBV, HSV-1 or HSV-2. African KS patients (mainly progressors) showed no serological relationship with any of these viruses. These studies were extended to larger groups of patients and a specific serological association of HCMV with American but not African KS patients was found (Giraldo et al., 1978).

Molecular epidemiological studies have identified HCMV-specific nucleic acids in KS biopsies and cell cultures (Giraldo et al., 1980; Boldogh et al., 1981;

Drew et al., 1982; Fenoglio et al., 1982; Spector et al., 1983). CMV-specific antigens have also been demonstrated to varying degrees.

Kaposi's sarcoma is frequently associated with acquired immunedeficiency syndrome (AIDS). Downing et al. (1984) reported that African KS patients have immunological and virological profiles similar to those seen in American patients with AIDS. Individuals within the high risk groups for KS and AIDS generally have evidence of prior or current HCMV infection. However, they also show evidence of previous infection with other viruses including EBV, HBV and HTLV. This makes it very difficult to determine whether HCMV plays any role in the etiology of this disease. Possibly the factors that predispose these individuals to virus infection also predispose them to KS. KS in AIDS patients never regresses and the multifocal lesions are metastatic. The disease results in eventual death.

Nasopharyngeal carcinoma

Desgranges et al. (1983) isolated HCMV from cell cultures derived from 2 out of 11 EBV-associated nasopharyngeal carcinoma biopsy specimens from N African patients. The authors believe that the cultures were persistently infected with HCMV and propose that an interaction between EBV and HCMV could favour co-carcinogenesis. HCMV could facilitate fusion between

epithelial cells and infiltrating EBV genome carrying B lymphocytes, thus promoting the transfer of the EBV genome to nasopharyngeal epithelial cells.

More recently, however, EBV has been found to infect differentiating epithelial cells (Sixbey et al., 1983).

Neuroblastoma and Wilm's Tumour

Neuroblastomas and probably Wilm's tumours arise from cells derived from the neural crest. As herpesviruses preferentially infect and persist in nervous tissue, Wertheim and Voute (1976) undertook a study to investigate whether herpesviruses may be associated with these tumours. They found a significant proportion of children with these tumours to have CF antibodies to HCMV. They propose that the children may have been infected transplacentally and that the virus persisted in the neural crest derivatives, eventually transforming the cells and giving rise to the tumour after a latent period. However, these studies do not seem to have been followed up and more recently these tumours have been linked with chromosomal aberrations, specifically a deletion of band p13 of chromosome 11 (Solomon, 1984).

Chromosome aberrations have, however, been associated with Burkitt lymphomas. Translocations between the c-myc gene on chromosome 8 and the immunoglobulin heavy chain genes on chromosome 14 (Taub et al., 1982); the k light chain genes on chromosome 2 (Erikson et al., 1983) or the λ light chain genes on chromosome 22 (Lenoir et al., 1982) are characteristic of Burkitt lymphomas. EBV

is thought to be responsible for these translocations and we cannot preclude a similar mechanism for HCMV in Wilm's tumours.

ONCOGENES

Several mechanisms of oncogenesis have been identified; transformation by virus-encoded oncogenes (reviewed by Tooze, 1981; Weiss et al., 1982; Bishop, 1985) and activation of cellular proto-oncogenes (reviewed by Bishop, 1983; Klein, 1983; Land et al., 1983).

Viral Oncogenes

The oncogenicity of several small DNA tumour viruses has been found to involve the coordinated expression of more than one viral oncogene eg adenoviruses, papovaviruses. Human adenoviruses were the first human viruses found to be capable of transforming cells in culture, although neither adenovirus DNA nor gene products have been associated with naturally occurring tumours. Only the members of the subgenera A and B (eg Ad12 and Ad7, respectively) can cause tumours in rodents but all human adenoviruses studied so far are capable of transforming cells in vitro (Gallimore et al., 1977). Varying amounts of adenovirus DNA sequences are integrated randomly into cellular DNA but a region encompassing the left hand end

of the adenovirus genome is common to all adenovirus transformed cell lines (Gallimore et al., 1974; Sambrook et al., 1974). In Ad12 transformed cells the entire genome is often present. The left hand 11% of the adenovirus genome corresponds to early region 1 (E1) which consists of two transcriptional units E1a and E1b. In transformed cells, E1a codes for two coterminal RNAs (12S and 13S) that differ only in the amount of RNA removed internally by splicing. The RNAs code for two polypeptides (26,000 and 32,000 daltons) that are structurally related. Region E1b codes for one major RNA species of 22S which specifies two unrelated polypeptides of 20,000 and 55,000 daltons.

Rat cells transformed by E1 of Ad5 or Ad12 are indistinguishable from cells transformed by intact DNA or virions, indicating that all transforming functions are located in E1. DNA fragments comprising certain parts of E1 may still contain transforming activity, although the resulting transformed cells show abnormal phenotypes. The E1a region of Ad2 and Ad5 is responsible for immortalizing cells. Cells transformed by E1a and the adjoining 50% of E1b of Ad2 and Ad5 appear completely transformed although they are not tumourigenic in nude mice (Jochemsen et al., 1982). Both E1a products seem to be required for the fully transformed phenotype. The 26,000 dalton protein appears to be the most important for anchorage independent growth. The 32,000 dalton

protein may stimulate transcription of cellular genes perhaps by binding to and altering the properties of host cell RNA polymerase. The E1a products of Ad12 inhibit expression of class 1 MHC antigens by transformed cells (Tanaka et al., 1985). In this way Ad12 transformed cells inoculated into animals may manage to evade the host immune system and establish tumours with high frequency.

The E1b region cannot transform cells in the absence of E1a products but nevertheless is required for the conversion of cells to the fully transformed phenotype. Introduction of a mutation in one of the Ad12 E1b products results in loss of oncogenicity (Jochemsen et al., 1982) and transplantation studies using cells transformed by Ad5/Ad12 hybrid E1 regions consisting of E1a of nononcogenic Ad5 and E1b of oncogenic Ad12 (and vice versa) have shown that the high oncogenic potential of Ad12 transformed cells in nude mice is determined by the 55,000 dalton E1b protein (Bernards et al., 1983). The 55,000 dalton E1b protein can form a complex with the p53 cellular tumour antigen (Sarnow et al., 1982) and this complex might be important for the transforming function of this E1b protein.

Transformation by polyoma virus requires the coordination of three distinct oncogenes which become integrated into cellular DNA and are continually expressed in virus-transformed and tumour cells (reviewed

by Ito, 1980; Cuzin et al., 1984). The products of these viral oncogenes constitute the viral T antigens that are immunoprecipitated by antibodies present in the serum of tumour-bearing animals and are known as large T (MW 105,000 daltons) middle T (56,000) and small T (22,000). During the lytic cycle of the virus in permissive cells, large T is a nuclear protein required for the initiation of viral DNA synthesis, middle T is a membrane protein associated with tyrosine kinase activity which is likely to correspond to the c-src gene product and small T is found in the cytosol and plays some role in the maintenance of the cell cytoskeleton. During tumourigenesis, large T is responsible for immortalizing cells and for reduced serum requirement and middle T is responsible for the remainder of the transformed phenotype. Small T may cause disruption of actin cables and reduce the tightness of cell adhesion to substratum. All three T antigens are required to produce the fully transformed state. The functions of the polyoma large and middle T antigens are combined in the large T antigen of simian virus 40 (SV40) which transforms primary cells (Kriegler et al., 1984). SV40 small T antigen may be required exclusively to initiate transformation of resting cells and has a structure reminiscent of, and might mimic the action of certain growth factors (Martin, 1981). Like the adenovirus E1b protein, SV40 large T antigen forms a complex with the cellular p53 protein

(Sarnow et al., 1982) which suggests some common features in the mode of action of these viral proteins.

Papillomaviruses can transform cells in vitro but no connection has yet been made between this property and tumourigenesis in the natural host. Transforming domains have so far been mapped only in the genome of bovine papillomaviruses (BPV) (Yang et al., 1985). Two have been found, one that carries four complete open reading frames and another that carries only one. The products of these domains and the mechanisms by which they act are not known. Each domain can independently transform cells in culture and when the two domains are used together their effects are additive.

Among the herpesviruses, EHV-1 may have a mode of transformation similar to that of adenoviruses and papovaviruses in that a small amount of DNA (about 6% of the total genome length lying between map units 0.32 and 0.38) appears to be retained and integrated into the cellular DNA of transformed hamster cells (O'Callaghan et al., 1983). In cells transformed by most other herpesviruses, integration of specific DNA regions cannot be detected.

Herpesvirus saimiri (HVS) will transform T cells in culture and produce tumours in primate hosts. HVS DNA appears to be present in transformed and tumour cells in multiple copies existing as non integrated circular molecules. Studies using non oncogenic deletion mutants

have identified a 2kb region of the genome that is required for transformation and oncogenicity (Desrosiers et al., 1985).

Epstein Barr Virus (EBV) is another herpesvirus capable of transforming lymphocyte cells in vitro. The presence of antibodies to EBV in patients with Burkitt's lymphoma and nasopharyngeal carcinoma has suggested that the virus is also associated with these tumours. The viral genome is maintained in transformed and tumour cells mainly as a circular episome present usually in high copy number. All lymphoblastoid cell lines examined this far that contain EBV DNA express a nuclear antigen termed EBNA which can be detected with antibody from the serum of patients recovering from infectious mononucleosis. The size of the EBNA component varies between different strains of EBV. To date three EBNA proteins have been described; EBNA1, EBNA2 and EBNA 3 of MW's 65,000 (Strnad et al., 1981) 82,000 (Hennessy and Kieff, 1983) and 140,000 daltons (Hennessy et al., 1985) respectively. MW's vary according to the gel systems used. Hearing and Levine (1985) have demonstrated that the EBV Bam HI K fragment encodes an 88,000 MW EBNA protein. An EBNA protein has been found to be associated with the cellular p53 protein in cells transformed by EBV (Crawford et al., 1981).

Transfection studies using restriction fragments of DNA have identified specific viral transforming genes in

the human herpesviruses HSV-1, HSV-2 and HCMV. In HSV-1 the transforming region occurs within the Bgl II i fragment and in HSV-2 in the Bgl II n and c fragments. There is no evidence that these sequences are retained and integrated into cellular DNA in transformed and tumour cells.

A large number of viral oncogenes have been identified among the 'acute' transforming retroviruses (Bishop and Varmus, 1982). These RNA viruses replicate by way of a DNA provirus inserted into cellular DNA (Figure 4). They are assumed to have evolved by recombination between the genome of a non-transforming virus and transduced cellular proto-oncogenes. The genes exert their oncogenic effect when recombined into the viral genome where they become governed by a strong promoter in the long terminal repeat region of the proviral genome.* At first it was thought that transformation by retroviral oncogenes was self-sufficient but now it appears that transformation may be assisted by other factors. For example, tumourigenesis by v-abl may be assisted by cellular genes (Ohno et al., 1984); v-src encodes a second small protein that could make its own independent contribution to tumourigenesis (Mardon and Varmus, 1983) and several retroviruses may act in concert to produce tumours (Graf and Beug, 1983; Kan et al., 1984). The human T cell leukaemia viruses, HTLV-1 and HTLV-11 are unique among

* See Figure 4.

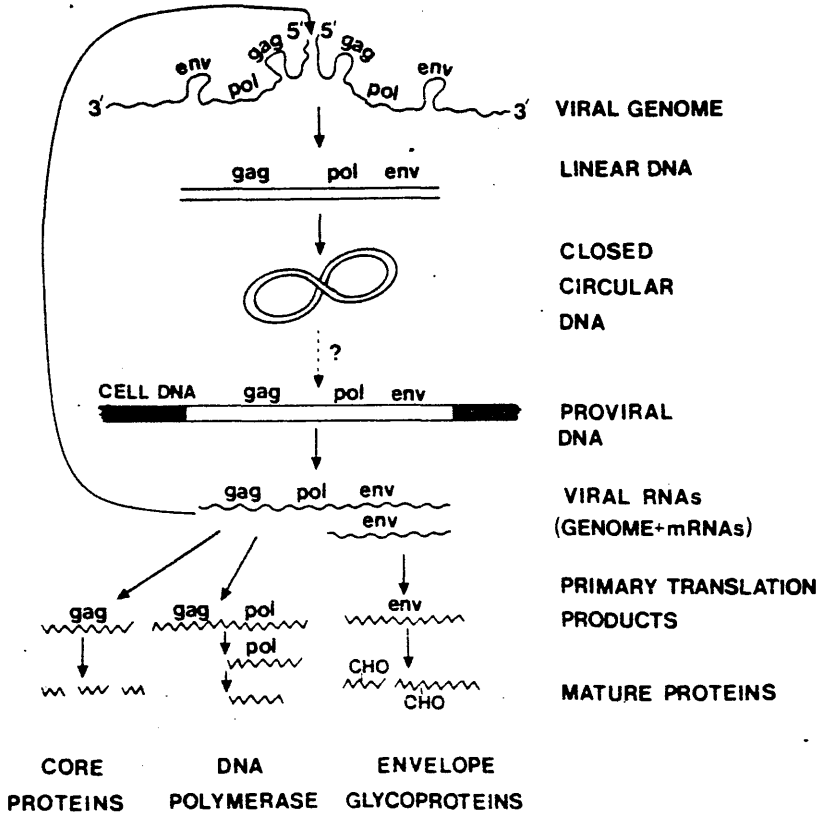

5'
5'
gag
env
pol
pol
env
3'
3'
VIRAL GENOME
gag pol env
LINEAR DNA
CLOSED
CIRCULAR
DNA
?
CELL DNA
gag pol env
PROVIRAL
DNA
gag pol env
env
VIRAL RNAs
(GENOME+mRNAs)
PRIMARY TRANSLATION
PRODUCTS
gag
gag pol
pol
env
CHO
CHO
MATURE PROTEINS
CORE
PROTEINS
DNA
POLYMERASE
ENVELOPE
GLYCOPROTEINS

FIGURE 4 Replication of retroviruses

Schematic representation of the replication of retroviral genomes. The viral RNA genome is diploid and composed of two single-stranded, identical subunits of about 5-10kb. The RNA is reverse transcribed in the cytoplasm of infected cells into a linear duplex molecule by an enzyme encoded by the retroviral pol gene. Some of the linear DNA migrates to the host cell nucleus where a fraction of it is converted to a closed circular species, the function of which is unknown. The viral DNA is then integrated into the host genome and the viral genes transcribed and translated. The pol gene encodes the DNA polymerase, the gag gene encodes the core proteins and the env gene encodes the envelope glycoproteins.

Symbols: ~~~~ RNA

———— DNA

^^^^^ Protein

* Retroviruses that have incorporated cellular genes into their genomes by a recombination event are defective and require helper viruses in order to replicate. No such helper viruses have, however, been identified in humans.

the transforming retroviruses in that they do not have a viral oncogene with a normal cellular homologue, nor do they integrate at any preferential site in tumours or transformed cells. These viruses are closely associated with specific human T cell leukaemias and lymphomas and can transform normal T lymphocytes *in vitro*. In addition to the characteristic retroviral *gag*, *pol* and *env* genes, they have an extra region of the genome designated X or LOR. The region has no definite assigned function but is believed to participate in HTLV-induced cellular transformation. The region has been shown to contain a gene encoding a protein of 40,000 in HTLV-1 and 37,000 daltons in HTLV-11 (designated $p40^{x-1}$ and $p37^{x-11}$ respectively) (Slamon *et al.*, 1984). It is possible that these proteins may function in a manner analogous to the papovavirus T antigens or the adenovirus Ela proteins.

Cellular oncogenes

Cellular proto-oncogenes are normal cellular genes potentially capable of inducing oncogenic transformation. More than 20 such genes have been identified by homology to the transforming genes of retroviruses and by the biological activity of cellular DNAs in transfection assays (reviewed by Cooper, 1982). Proto-oncogenes have been highly conserved throughout evolution and appear to represent single-copy sequences in cellular DNA, with the exception of the *ras* genes which constitute a multigene

family. The precise function of the proto-oncogenes is unknown, although it has been assumed that they participate in the regulation of cell proliferation. Oncogenes have been identified in a large number of human and animal tumour cells and it is presumed but not proven that each oncogene was instrumental in generating the tumour from which it was isolated.

Proto-oncogenes can be activated to become oncogenes in a number of ways. A proto-oncogene may undergo mutation, so that the protein encoded by the mutant oncogene is structurally different from the corresponding normal protein. Alternatively, oncogenesis may involve a "dosage-effect", and mutation could produce a tandem duplication of a proto-oncogene. Gene amplification could then occur by successive unequal sister chromatid exchanges in different cell cycles until sufficient protein is produced to transform the cell (Pall, 1981). Proto-oncogene activation can also occur by promoter insertion (Hayward et al., 1981) or enhancer insertion (Weber and Schaffner, 1985). In either case the expression of the proto-oncogene is upregulated resulting in over-production of the gene product. Chromosomal aberrations such as translocations and deletions can also result in activation of proto-oncogenes (reviewed by Rowley, 1983). For example, in some Burkitt's lymphomas the c-myc proto-oncogene has been translocated into the immunoglobulin heavy chain locus (Taub et al., 1982)

where its expression is upregulated by enhancer elements present in the heavy chain locus (Boss, 1983). Evidence is also accumulating for the involvement of multiple cooperating independently-activated oncogenes in transformation eg Ela and ras (Land et al., 1983; Ruley, 1983) providing confirmation at the molecular level that carcinogenesis is a multistep process.

Whatever the mechanism of activation, an oncogene must finally have its effect by way of the protein it encodes. The oncogenic protein almost invariably has a function similar to that of its cellular counterpart. The oncogenic proteins studied so far have varied functions and are characterised in Table 1.

Many of the oncogenic proteins studied so far have been found to be, or to be associated with, protein kinases. The protein products of the src, yes, fgr, abl, fps, fes and ros oncogenes are all tyrosine-specific protein kinases. Since tyrosine phosphorylation may be intimately involved in the complex regulatory systems that maintain cellular shape and control the growth of cells, subtle alterations in these systems could give rise to the cancerous state. Potential target proteins for these protein kinases in the normal cell and the possible effects of tyrosine phosphorylation are given in Table 2. The nucleotide sequences of the tyrosine-kinase associated oncogenes as well as proto-oncogenes are all related. The homology is strongest in the parts of the

TABLE 1 Oncogenes and the characterization of their products

ONCOGENE	VIRAL ORIGIN	VIRAL GENE PRODUCT	CELLULAR HOMOLOGUE	ACTIVITY	SUBCELLULAR LOCATION	HUMAN NEOPLASIA ASSOCIATED
src	Rous sarcoma virus	$pp60^{v\text{-}src}$	$pp60^{c\text{-}src}$	Tyrosine kinase	Plasma membrane	
yes	Y 73 avian sarcoma virus	$P90^{gag\text{-}yes}$		Tyrosine kinase	Plasma membrane?	
fps	Fujinami sarcoma virus	$P140^{gag\text{-}fps}$	$P80^{c\text{-}fps}$	Tyrosine kinase	Cytoplasm Plasma membrane	
fes	Snyder-Theilen feline sarcoma virus	$P85^{gag\text{-}fes}$	$P92^{c\text{-}fes}$	Tyrosine kinase	Cytoplasm (Cytoskeleton?)	Leukaemia
ros	UR2 avian sarcoma virus	$P68^{gag\text{-}ros}$		Tyrosine kinase	?	
abl	Abelson murine leukaemia virus	$P120^{gag\text{-}abl}$	$P150^{c\text{-}abl}$	Tyrosine kinase	Plasma membrane	Leukaemia
erb-A	Avian erythro-blastosis	$p75^{gag\text{-}erbA}$	Human oestrogen receptor	Activates oestrogen protein kinase domain	Cytoplasm	
fms	Feline sarcoma virus McDonough strain	$gp^{120}fms$	Receptor for macrophage stimulating factor (CSF-1)	Activates CSF-1 receptor's protein kinase domain	Plasma membrane endoplasmic reticulum Golgi apparatus	
mil	Avian sarcoma virus MH2	$p^{100}gag\text{-}mil$		Protein-serine/ threonine kinase	Cytoplasm	
raf	Murine sarcoma virus			Potential protein kinase	Cytoplasm	
rel	Reticuloendo-theliosis virus strain T			?	Ctytoplasm	
fos	Murine sarcoma virus			?	Nuclear	
ski	Avaian sarcoma virus			?		
ets	Avian leukaemia virus			?		
kit	Feline leukaemia virus. Hardy Zuckerman strain			?	Nucleus (fused with product of v-myb)	

ONCOGENE	VIRAL ORIGIN	VIRAL GENE PRODUCT	CELLULAR HOMOLOGUE	ACTIVITY	SUBCELLULAR LOCATION	HUMAN NEOPLASIA ASSOCIATED
fgr	Gardner-Rasheed feline sarcoma virus	$P70^{gag-fgr}$		Tyrosine kinase	?	
erb-B	Avian erythro-blastosis virus	$gp65^{erb-B}$	Truncated EGF receptor	Activates EGF receptor's tyrosine-specific protein kinase domain	Plasma membrane	
mos	Moloney sarcoma virus	$P37^{mos}$		? Potential protein kinase	Cytoplasm	Leukaemia
sis	Simian sarcoma virus	$p28^{sis}$	PDGF B-chain	PDGF agonist	Cytoplasm	
myc	Avian myelocyto-matosis virus MC29	$P110^{gag-myc}$	$P58^{c-myc}$	Binds DNA	Nucleus	Lymphomas
myb	Avian myelo-blastosis virus			Binds DNA?	Nucleus	Leukaemia Lymphoma Ovarian carcinoma

ONCOGENE	VIRAL ORIGIN	VIRAL GENE PRODUCT	CELLULAR HOMOLOGUE	ACTIVITY	SUBCELLULAR LOCATION	HUMAN NEOPLASIA ASSOCIATED
H-ras	Harvey murine sarcoma virus	$p21^{v\text{-}H\text{-}ras}$	$p21^{c\text{-}H\text{-}ras}$	Threonine kinase binds GDP or GTP	Plasma membrane	Carcinoma
K-ras	Kirsten murine sarcoma virus	$p21^{v\text{-}k\text{-}ras}$	$p21^{c\text{-}k\text{-}ras}$	Threonine kinase binds GDP or GTP	Plasma membrane	Carcinoma leukaemia, sarcoma
N-ras			$p21^{N\text{-}ras}$	Threonine kinase binds GDP or GTP	Plasma membrane	Leukaemia Carcinoma

TABLE 2 Potential target proteins for tyrosine-specific protein kinases in the normal cell and the possible effects of tyrosine phosphorylation

TARGET PROTEIN	CELLULAR LOCATION	NORMAL FUNCTION	POSSIBLE EFFECT OF TYROSINE PHOSPHORYLATION
Vinculin	Adhesion plaques	Links actin to a hypothetical anchor protein in cell membrane	Disruption of the vinculin link causing disorganization of actin bundles
p36	Inner face of plasma membrane of some cell types	Structural functions eg in intestinal columnar cells, anchors actin filaments to the terminal web of microvilli	Cytoskeletal changes
Enolase Phosphoglycerate mutase Lactate dehydrogenase	Mitochondria	Enzymes in glycolytic pathway	Increase in glycolysis(?)

The cellular proteins listed have been found to contain phosphotyrosine in transformed cells, and could therefore be potential target proteins for tyrosine-specific phosphokinases. Less than 0.1% of the protein-bound phosphate is attached to tyrosine in a normal cell.

genes that are believed to code for the kinase domains of the protein products, ie the 3' end for *src*, *yes*, *fes*, *fps* and *fgr* and the 5' end for *abl*. In this part of the molecules, homologies have been found also to the oncogenes *mos*, *rel*, *raf*, *mil*, *fms* and *erbB*. We surmise that the products of these oncogenes have functions similar in some sense to protein kinases.

Some oncogenes may code for the production of a growth factor (reviewed by Heldin and Westermark, 1984). For example, the predicted sequence of the transforming protein p28*sis* of simian sarcoma virus is almost identical to that of platelet-derived-growth factor (PDGF; Waterfield *et al*., 1983; Doolittle *et al*., 1983). It is assumed that the p28*sis* product functions by stimulating cell replication by interacting with the PDGF receptor.

Oncogenic proteins may also function by switching on the production of a cellular growth factor (reviewed by Heldin and Westermark, 1984). The oncogenic products of Moloney murine sarcoma virus (p37*mos*) and Kirsten murine sarcoma virus (p21*kras*) directly or indirectly regulate the synthesis of a transforming growth factor (TGF) which probably functions by blocking EGF receptors.

There are structural and functional homologies between growth factor receptors and tyrosine-specific protein kinases. Recently, the EGF receptor has been shown to have strong homology with gp65*erbB*, the

transforming protein of avian erythroblastosis virus (Downward *et al.*, 1984). The EGF receptor has an extracellular domain that binds EGF and a tyrosine-specific protein kinase domain inside the cell. The binding of an EGF molecule presumably changes the receptor's conformation so that the intracellular domain catalyses the phosphorylation of a cellular substrate protein. gp65*erbB* may correspond to a truncated EGF receptor. The absence of the EGF binding domain might remove the control by EGF molecules binding and the result could be the continuous generation of a signal equivalent to that produced by EGF causing cells to proliferate rapidly.

The most frequently isolated tumour oncogenes are the cellular homologues of the retroviral oncogenes *ras* and *myc*. The p21*ras* proteins encoded by the *ras* oncogene are localized in the plasma membrane. They are GTP-binding proteins that may act as coupling factors in cell reactions and transmit a continuous signal rather than a regulatory one. The proteins encoded by the *myc*, *myb* and *fos* oncogenes are localised in the nucleus and are probably DNA binding proteins. The presence of large amounts of these proteins may establish the transition of a normal cell to one with infinite growth potential.

To summarise, the products of proto-oncogenes from which oncogenes are derived seem to have roles that must be crucial in the control of cell growth and

differentiation and in embryonic development. Transforming proteins may have their profound effects on cells because they perturb these fundamental cellular processes. As our knowledge of the functions of the various transforming proteins increases, we become progressively nearer to understanding at least some stages in the multistep process of carcinogenesis.

MATERIALS

1. VIRUSES

The following viruses were used in this study:-

(a) Human cytomegalovirus strain AD169 (HCMV AD169) (Rowe et al., 1956). Plaque-purified virus was supplied by Dr. J.D. Oram.

(b) Herpes simplex virus type 2 strain HG52 (HSV-2 HG52)
(Timbury, 1971) provided by Mrs. M. Murphy.

(c) Temperature-sensitive (ts) mutants of HSV-2 HG52 (Timbury, 1971) and of herpes simplex virus type 1 strain 17 syn+ (Brown et al., 1973; Crombie, 1975) were used for virus reactivation studies and were also supplied by Mrs. M. Murphy.

2. TISSUE CULTURE CELLS

(a) Cell lines

Helu, a continuous cell line established from human embryo lung cells was purchased from Flow Laboratories, Irvine, Ayrshire, Scotland. Helu cells were used to prepare HCMV AD169 virus stocks, to titrate virus and for virus reactivation studies where stated. Human embryo cells were also established from abortis material.

BHK21/C13, a continuous cell line established from baby hamster kidney cells by Macpherson and

Stoker(1962) maintained in this Institute. BHK21 cells were used to titrate HSV-2 HG52 and HSV ts mutants.

3T3, a continuous cell line established from mouse fibroblasts. 3T3 cells were provided by M. Freshney (Beaston Institute, Glasgow), G. van de Woode (National Cancer Institute, Bethesda), G.M. Cooper (Harvard University, Boston) and C. Marshall (Chester Beatty Institute, England) and were used for transformation studies.

(b) Primary cells

Rat embryo (RE) cells were prepared from a single litter of approximately 20 day old embryos from an inbred Hooded Lister rat colony maintained in this Institute.

Cervical cells were prepared from the cervices of routine hysterectomy specimens of patients with no history of, or colposcopic evidence of CIN or carcinoma.

(c) HCMV transformed and tumour cell lines

Primary RE cells were transformed using UV-irradiated HCMV AD169 by A. Bunce in this Institute. Tumour cell lines were derived from tumours induced in Hooded Lister rats by the inoculation of HCMV transformed cells. All HCMV

transformed and tumour cell lines were obtained from Dr. J.C.M. Macnab.

3. TISSUE CULTURE MEDIA AND SOLUTIONS

Helu, BHK21, RE and HCMV transformed and tumour cell lines were grown in BHK21 Glasgow modification of Eagle's medium (Gibco Laboratories, Paisley, Scotland) supplemented with 2mM L-glutamine, 100ug/ml streptomycin and 100u/ml penicillin. The following tissue culture media have been used:-

ECX Eagle's medium containing X% calf serum

ETCX Eagle's medium containing X% calf serum and 10% (v/v) Difco tryptose phosphate broth (T.P.)

EFX Eagle's medium containing X% foetal calf serum

EHu2 Eagle's medium containing 2% pooled human sera

Ec2-Pi Phosphate-free Eagles medium containing 2% calf serum.

3T3 cells were grown in Dulbecco's modified Eagle's medium (D) (Flow Laboratories, Irvine, Ayrshire, Scotland) supplemented with 2mM L-glutamine, 100ug/ml streptomycin, 100u/ml penicillin and X% foetal calf serum.

Cervical cells were grown in Dulbecco's modified Eagle's medium supplemented with the following:-

2mM L-glutamine

100u/ml penicillin

100ug/ml streptomycin

10% (v/v) foetal calf serum

10^{-10}M cholera toxin

0.5ug/ml hydrocortisone acetate

10ng/ml epidermal growth factor (EGF)

This medium has been abbreviated CM in the text.

Versene solution consisted of 0.006M EDTA in PBS containing 0.0015% (w/v) phenol red.

Trypsin was prepared as a 0.25% solution supplemented with 0.0015% (w/v) phenol red.

Agar medium

Agar overlay medium for HCMV AD169 titrations was prepared at 45°C to a final concentration of 0.3% SeaKem LE agarose and 1xEagle's medium with the sodium bicarbonate content increased to 8mM and supplemented with 2% foetal calf serum.

Noble agar (Difco Bacto) was used for plating cells in soft agar to assay for anchorage independent growth. A 3.2% stock of Noble agar was prepared in distilled water then sterilized. 4ml of agar underlay was added to 50mm petri dishes and the cell suspension added in a 2ml overlay medium.

Agar underlay: prepared at 45°C to a final concentration of:

0.6% agar

1xEagle's medium containing 100u/ml of penicillin and 100ug/ml of streptomycin

10% foetal calf serum

10% TP

overlay: prepared at 45°C to a final concentration of:-

0.3% agar

1xEagle's medium

10% foetal calf serum

10% TP

4. BACTERIA AND BACTERIOPHAGE CULTURE MEDIA AND SOLUTIONS

Liquid media

Bacteria were propagated in L-Broth (LB) which consisted of 1% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco) and 0.17M NaCl with the pH adjusted to 7.5.

Bacteriophage were stored and diluted in SM buffer which was prepared from 10mM NaCl, 10mM $MgSO_4 . 7H_2O$, 10mM Tris-HCl (pH7.5) and 0.01% (w/v) gelatin.

Media containing agar or agarose

Agar plates were prepared from LB containing 1.5% (w/v) Bacto-agar and sterilized by autoclaving.

Top agarose for plating bacteriophage was prepared by adding 0.7% (w/v) agarose (type-1, low EEO) to LB medium and sterilized by autoclaving.

5. CLONED VIRAL DNA AND PLASMID VECTORS

The Hind III DNA fragments of HCMV AD169 were cloned into plasmid vector pAT153 at the single Hind III site and propagated in E. coli HB101 or C292 (Oram et al., 1982). The Hind III/Bam HI subclone (EHB3) was cloned into the Bam HI site of pAT153. All cloned HCMV DNA fragments were provided by Dr. J.D. Oram (Public Health Laboratory, Porton Down, Salisbury, England).

The Bam HI t fragment of HSV-2 HG52 was cloned into the Bam HI site of plasmid vector pAT153 and was supplied by Dr. J.C.M. Macnab.

HPV 11 DNA was cloned into phage λ 47 and subcloned in pBR322 together with 69% of the BPV-1 genome (Gissmann et al., 1982, 1983).* Recombinant plasmid was propagated in C292 cells and was provided by Dr. J.C.M. Macnab.

6. CLONING VECTOR AND HOST BACTERIA

Bacteriophage vector EMBL3 (Frischauf et al., 1983) was used to construct a genomic library of CIN DNA. Bacterial host strains Q358 and Q359 (Karn et al., 1980) were used. Both recombinant and non-recombinant phage will grow on Q358 but only recombinant phage will grow on Q359. EMBL3 arms, host strains and packaging extract were purchased from

* This plasmid was kindly provided by Zur Hausen. A plasmid containing HPV11 DNA but no BPV DNA was not available at the time this work was commenced.

Vector Cloning Systems, 3770 Tansy St., San Diego, C.A. 92121, USA,

Host bacteria were propagated in LB supplemented with 10mM $MgSO_4$ and 0.2% maltose. Host strain K803 (Wood, 1966) was provided by Dr. I. Kennedy and was used for propagating recombinant bacteriophage for DNA extraction.

7. STANDARD BUFFER SOLUTIONS

PBS	170mM NaCl, 3.4mM KCl, 1mM Na_2HPO_4, 2mM KH_2PO_4, pH7.2
HBS	(Hepes buffered saline) 140mM NaCl, 0.75mM Na_2HPO_4, 25mM Hepes(N-2-hydroxyethylpiperazine N-2 ethane sulphonic acid) pH7.1
TE	1mM EDTA, 10mM Tris-HCl
TNE	5mM EDTA, 50mM Tris-HCl (pH7.4), 150mM NaCl
SSC	150mM NaCl, 15mM Tri-sodium citrate, pH7.4
SSPE	180mM NaCl, 10mM NaH_2PO_4 (pH7.4) 1mM EDTA (pH7.4)
1xE	30mM NaH_2PO_4, 1mM EDTA, 36mM Tris-HCl (pH7.8)
1xTBE	89mM Tris-borate, 89mM boric acid
Dye Ficoll	150mM Na_2HPO_4, 10mM EDTA, 180mM Tris (pH7.8) 10% (w/v) Ficoll, 0.05% (w/v) bromophenol blue
Cell lysis buffer	50mM Tris-HCl, 10mM EDTA, 100mM NaCl, 0.4% SDS, pH8.0.

Triton lysis buffer	62.5mM EDTA, 50mM Tris-HCl (pH8.0) 2% Triton -X-100
NTB	Nick translation buffer, 50mM Tris-HCl (pH7.8), 5mM $MgCl_2$, 1mM dithiothreitol (DTT)
Denhardt's solution	0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrollidone, 0.02% bovine serum albumin (BSA)
DNase dilution buffer	50% glycerol, 10mg/ml BSA, 5M NaCl, 1M Tris-HCl (pH7.5), 250mM EDTA, 1M DTT
Ligation buffer	50mM Tris-HCl (pH7.8), 10mM $MgCl_2$, 20mM DTT 1mM ATP, 50ug/ml BSA

8. CHEMICALS

Agarose, trizma base (tris hydroxymethylaminomethane) salmon sperm DNA, bovine serum albumin, polyvinylpyrollidone, Triton-X-100, cholera toxin (from Vibrio cholera), hydrocortisone acetate and epidermal growth factor were obtained from Sigma (London) Chemical Company Ltd., Kingston-Upon-Thames, London, UK.

Trichloroacetic acid (TCA), 2, 5-diphenyloxazole (POP) and caesium chloride were purchased from Koch-Light Laboratories Ltd, Colinbrook, Bucks, UK.

Ficoll (average molecular weight 400,000) and Sephadex G50 were purchased from Pharmacia Fine

Chemicals, Uppsala, Sweden.

SeaKem LE agarose was obtained from FMC Corporation, Marine Colloids Division, Rockland, ME 04841 USA.

Difco agar Noble, Difco bacto agar and Difco yeast extract were purchased from Difco Laboratories, Detroit, Michigan, USA.

Ethanol (analytical grade) was purchased from James Burroughs Ltd, London, UK.

All other chemicals were purchased from BDH Chemicals Ltd, Poole, Dorset, UK and were of analytical or reagent grade.

9. FLUORESCENCE REAGENTS

Goat anti-rat antibody conjugated with fluorescein isothiocyanate was obtained from Nordic, Maidenhead, Berks, UK.

Swine anti-human IgG antibody conjugated with fluorescein isothiocyanate was obtained from Wellcome Laboratories, Temple Hill, Dartford, Kent UK.

Hoechst 33258 stain was a gift of Hoechst.

RADIOCHEMICALS

All radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks, UK. The

specific activities of the 5'-α-^{32}P deoxynucleoside triphosphates were 3,000 Ci/mmol. (^{32}P) orthophosphate was carrier free.

ENZYMES

All restriction endonucleases and DNA polymerase 1 were purchased from Bethesda Research Laboratories, Rockville, Maryland, USA.

Deoxyribonuclease (bovine pancreas) and ribonuclease A (bovine pancreas) were obtained from Sigma (London) Chemical Company Ltd., Kingston-Upon-Thames, London, UK.

Proteinase K and calf intestinal phosphatase were purchased from Boehringer Mannheim, GmbH, W. Germany.

Ligase was purchased from New England Biolabs Inc. (NEB) Beverly, MA, USA.

MISCELLANEOUS MATERIAL

Kodirex X-ray film, X-omat S film, Dx80 developer:-

Kodak Ltd., London, UK

Amfix:-

May and Baker Ltd., Dagenham, UK

Dupont Cronex Lighting Plus intensifying screens:-

Dupont, Newton, Connecticut, USA.

Plastic centrifuge tubes:-

Falcon Incorporated, Oxhard, California, USA.

Visking dialysis membrane:-

Medical International Ltd., London, UK

Millex filters 0.22 and 0.45 um:-

Millipore UK Ltd., Abbey Road, London, UK.

Whatman chromatography paper, 3mm sheets and 2.5cm circles:-

Whatman Ltd., Maidstone, England, UK

Biodyne transfer membranes:-

Pall Ultrafine Filtration Corporation, Glen Cove, N.Y. 11542.

Gene Screen Plus ^{TM} Hybridization transfer membrane sheets NEF-976:-

New England Nuclear, Dupont UK Ltd. NEN Products Division, Stevenage, Herts, UK.

Nitrocellulose membrane filters 0.45um, 82mm diameter BA85/21:-

Schleicher and Schuell, D3354, Dassel, W. Germany.

Plastic tissue culture flasks and 35mm petri dishes:-

Inter Med (Nunc) A/S Nunc, Kamstrupvej 90, Kamstrup, DK-4000, Roskilde, Denmark.

Plastic 90mm petri dishes and 50mm plastic tissue culture petri dishes:-

Sterilin Ltd., Teddington, Middlesex, England, UK.

METHODS

1. CELL CULTURE

(a) Cell lines

BHK21/C13 cells were cultured in rotating 80oz bottles seeded at $4x10^7$ cells in 200ml of ETC10. 50mm diameter plastic petri dishes were seeded at a density of $2.5x10^6$ cells per dish in 4ml of ETC5.

Helu cells were cultured in 800ml plastic tissue culture flasks seeded at $2x10^7$ cells in 30ml of EF5 + 1% non essential amino acids. 35mm diameter petri dishes were seeded at a density of $4x10^5$ cells per dish in 2ml of EF5 + 1% non essential amino acids and 50mm diameter petri dishes at a density of $1x10^6$ cells per dish in 4ml of medium.

HCMV-transformed and tumour cell lines were propagated in EF5 seeded at a density of $2x10^7$ cells per 800ml tissue culture flask. 3T3 cells were cultured in DF10 seeded at $1x10^6$ per 800ml tissue culture flask or $2x10^5$ cells per 50mm petri dish. Cells were never allowed to become completely confluent.

All cells were harvested by washing twice with trypsin-versene, incubating at 37°C for a few minutes and resuspending at the required concentration in the appropriate medium. Cells were grown in an atmosphere of 5% CO_2 at 37°C.

Helu cells were routinely checked for mycoplasma

infection as follows. Cells were grown on glass coverslips for 48 hrs, washed in PBS, and then fixed in methanol: acetone (3:1) cooled to -20°C for 4-5 min. After air drying the cells were stained with Hoechst stain (10ul of stain diluted to 0.05ug/ml) for 10 min at room temperature. The coverslips were then washed in 3 changes of PBS and mounted in 20% glycerol-PBS on glass slides. The cells were examined under the UV microscope for evidence of mycoplasma infection. Any mycoplasma present on the cell surface could be identified by fluorescence.

(b) Human embryo lung cells

Human embryo lung cells were prepared from a 15 wk old aborted foetus. The lungs were aseptically removed and left for 30 min in EF5 with a high concentration of antibiotics. The tissue was then washed six times in trypsin to remove red blood cells and finely minced with sterile scissors in about 5-10ml of trypsin. Fragments of tissue were dissociated by two separated trypsinization procedures at 37°C in 0.25% trypsin each of 20 min duration. Single cells were pelleted at 1000 rpm for 10 min at 4°C, counted in a haemocytometer and seeded at $2x10^7$ cells per 800ml tissue culture flask in EF5. After 24 hr the medium plus any floating cells was removed and poured into a fresh flask and fresh medium was added to the cultures. The remaining

fragments of tissue were seeded in EF5 in 50ml tissue culture flasks as explants and left for 2-3 days at 37°C before changing the medium. Once confluency was attained, the cells were trypsinized and reseeded as previously described for Helu cells.

(c) Primary rat embryo cells

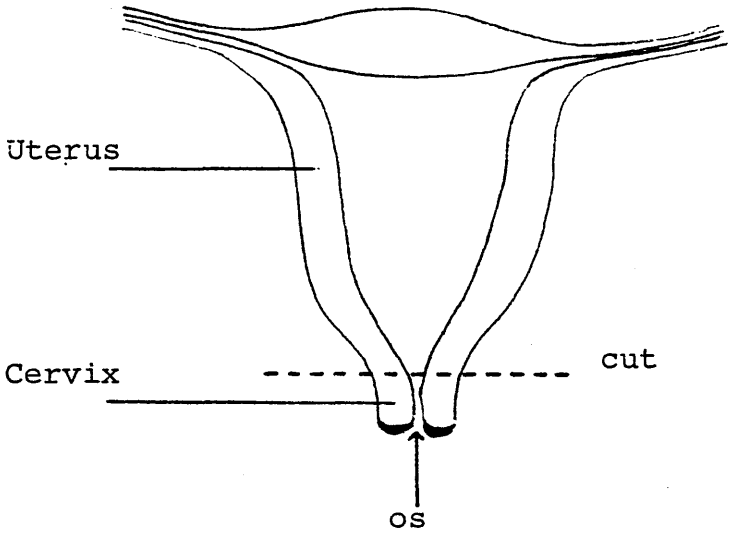
Rat embryo cells were prepared from 20 day old embryos from an inbred Hooded Lister rat from the colony maintained in this Institute. The embryos were aseptically removed from the rat and washed in six changes of versene. After evisceration the embryos were finely minced and washed in PBS to remove red blood cells. The tissue fragments were dissociated by two separate trypsinization procedures as described above for human embryo lung cells. Single cells were pelleted, counted and $1x10^8$ cells were seeded into rotating 80oz roller bottles in EF10. For use in assays RE cells were treated as BHK21/C13 cells but seeded in EF10.

(d) Cervical cells

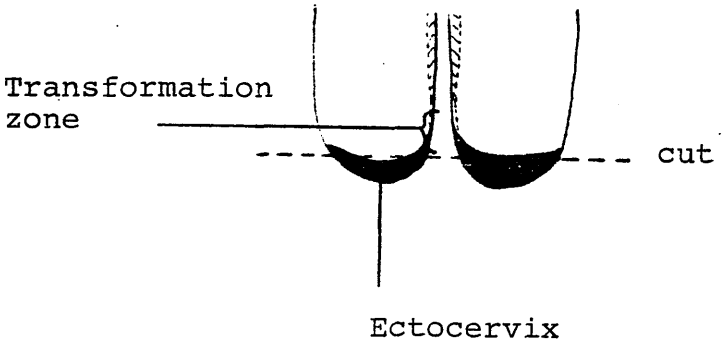
Cervical cells were prepared from hysterectomy specimens from patients with no evidence of CIN or carcinoma. Cells prepared from the cervices of women who were in the first 14 days of the menstrual cycle usually grew more efficiently in cell culture. For

DISSECTION OF THE CERVIX TO SEPARATE ECTO- AND ENDOCERVICAL TISSUE

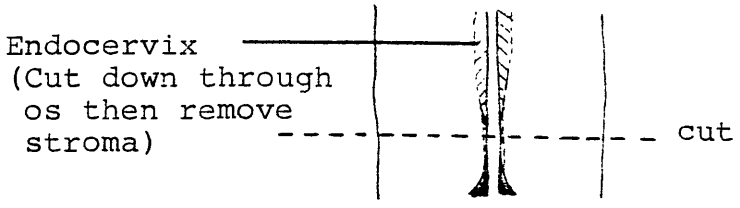
Excision of cervix from hysterectomy specimen



Excision of ectocervix



Excision of endocervix



this reason, premenopausal cervices were used wherever possible. The cervix was excised from the uterus and vagina and washed three times in PBS to remove red blood cells. The ecto - and endocervix were removed by cutting 2mm above and below the transformation zone (see diagram). Tissue from the transformation zone was discarded as it contains a mixture of both cell types. Ecto - and endocervical explants were prepared separately. Muscle and stroma were carefully removed using a sharp scalpel until a thin epithelium of cells remained. If the cervix had been stained blue by the surgeon (a technique used in surgery to identify the ectocervix and vaginal epithelium) the blue dye was gently scraped off in PBS. The epithelium was then finely minced using 3-4" curved iris scissors. A few drops of PBS were added to prevent the tissue from drying out. About 10-20 small tissue fragments were seeded per 35mm plastic tissue culture petri dish and just covered with a small volume of foetal calf serum. The explants were incubated at 37°C in an atmosphere of 5% CO_2, overnight, to allow them to anchor to the plates. Next morning 2ml of CM without EGF was added to each plate and the explants incubated for a further 2-3 days without any disturbance. After this time, any floating explants were removed and fresh complete CM added to the plates. The medium was changed twice weekly. Ectocervical cells formed a confluent

monolayer in 2-3 weeks. Endocervical cells would not form confluent monolayers and if the cells were cultured for more than three weeks, the cells tended to lift off the plates. For this reason, endocervical cells were used as soon as no further appreciable increase in size of the monolayer could be detected ie usually after 7-10 days in culture.

2. PRODUCTION OF AD169 VIRUS STOCKS

HCMV AD169 virus stocks were prepared by infecting just subconfluent Helu cells in 800ml plastic tissue culture flasks at a moi of 0.01pfu/cell. The virus was absorbed for 90 min at 37°C and the cells overlaid with 30ml of EF5. After 4 days, the medium was discarded and 10ml of fresh medium added. Infected cells were incubated at 37°C for a total of 10-11 days. Medium was collected daily and 10ml of fresh medium added from day 6 post infection. On day 10 or 11, any remaining cells were shaken into the medium before harvesting. The collected medium from each day was divided into 1ml aliquots in sterile black capped vials and stored at -70°C. The virus was sonicated in a Cole-Palmer ultrasonic cleaning bath before use. Sterility checks were routinely performed by streak-plating virus stocks onto blood agar plates. Virus stocks were also checked for mycoplasma contamination by infecting Helu cells on

glass coverslips and staining with Hoechst as previously described for the detection of mycoplasma in cell cultures.

3. TITRATION OF VIRUS

(a) HCMV AD169

Virus stocks were plaqued by the method of Wentworth and French (1970). Serial ten-fold dilutions of virus were made in 2xEF2. 100ul inocula were added to almost confluent monolayers of Helu cells in 50mm plastic tissue culture petri dishes. After 90 min absorption at 37°C, 5ml of overlay agar medium was added to each plate and the monolayers incubated for 7 days at 37°C in 5% CO_2. After this period a second agar overlay of 5ml was added as a feeder layer and the monolayers incubated for a further 7 days. Monolayers were fixed by adding 2ml of 10% formalin to each plate. The agar overlays were discarded after 10 min at room temperature and the fixed monolayers washed with tap water and stained with Giemsa (10 min at room temperature). Plaques were counted using a dissecting microscope.

HCMV was also plaqued by a much quicker method that did not involve the use of agar overlays. Virus was diluted in EF2 and 100ul inocula added to monolayers of Helu cells as previously described.

After virus adsorption, 4ml of EF2 were added and the infected monolayers incubated at 37°C for 10 days. Monolayers were then stained with Giemsa and scored for plaques. Titres obtained using this method did not differ signficantly from those obtained using the method of Wentworth and French (1970). Since the method of Wentworth and French (1970) is quoted in the literature as being routinely used, this method was preferred for titrating virus stocks.

(b) HSV

Serial ten-fold dilutions of virus were made in PBS + 2% calf serum and 100ul inocula added to BHK/C13 cell monolayers. The virus was absorbed for 45 min at 37°C, after which time 4ml of EHu2 were added. Cells infected with wild type virus were incubated at 37°C for 2 days. ts mutants were titrated at both the permissive (31°C) and nonpermissive temperature (38.5°C) for 3 and 2 days respectively. Monolayers were stained for 10 min at room temperature with Giemsa and plaques counted using a dissecting microscope.

4. ESTABLISHMENT OF LATENTLY INFECTED CELL CULTURES

Latently infected cell cultures were established at supra-optimal temperatures by the method described by E. Notarianni (submitted for publication) and Russell and Preston (1986). Cell

monolayers were infected at a moi of 0.003 pfu/cell with HSV-2 HG52. After 1 hr absorption at 37°C, the monolayers were overlaid with prewarmed medium and incubated at 42°C. After a period of 6 days the cultures were shifted down to 37°C and incubated for a minimum of 3 days. During this time the cultures were carefully examined for any signs of virus reactivation. If no spontaneous reactivation was apparent, some of the latently infected cultures were superinfected with either HCMV AD169 or ts mutants of HSV as described below.

5. SUPERINFECTION OF LATENTLY INFECTED CELLS

Latently infected cells were superinfected with 5 pfu/cell of a ts mutant or between 0.5 and 5 pfu/cell of HCMV AD169. HCMV AD169 was absorbed for 1 hr at 37°C and the superinfected cultures incubated at 37°C for 3 days. Duplicate plates of latently infected cells were superinfected at both 37°C and 38.5°C with HSV ts mutants and incubated for 3 days at both temperatures. At the end of the incubation period, the cells were scraped off into the medium, sonicated and then titrated on monolayers of BHK Cl3 cells for reactivated virus. Those cultures superinfected with ts mutants were titrated at both the permissive (31°C) and nonpermissive temperature (38.5°C) for 3 and 2 days

respectively. The yield of virus from each plate was calculated.

6. IMMUNOFLUORESCENCE

Cells were grown on sterile coverslips in 50mm petri dishes and subconfluent monolayers were infected with virus. After the appropriate incubation period, the cells were washed 6 times in PBS and then fixed for 4 min in methanol: acetone (3:1) at -20°C. After air drying the cells were dipped in PBS and incubated with 100ul of the appropriate primary antibody for 30 min at 37°C in a humid atmosphere. The cells were then washed three times in PBS and incubated for 30 min at 37°C with 100ul of the secondary antibody (an antibody raised against the IgG of the primary antibody conjugated with fluoroscein isothiocyanate - FITC). Finally, the cells were washed three times in PBS and mounted on glass slides in glycerol.

The various antibodies used included the following:-

Primary antibodies:

(a) Human antiserum to HCMV obtained from the serum of a patient submitted to the Diagnostic Unit for antibody analysis. The serum has an HCMV CF antibody titre of >1:512 but an HSV-2 CF antibody titre of <1:8 and was diluted 1:10 prior to use.

(b) Rat antiserum to the IE polypeptides of HCMV prepared in Hooded Lister rats maintained in this Institute. Primary RE cells were infected with 2.5 pfu/cell of HCMV AD169 for 11-12 hr in the presence of 50ug/ml of cycloheximide and released for 2-3 hr in 10ug/ml of Actinomycin D. The cells were scraped off the plates and injected either subcutaneously or intradermally into rats with Freunds complete adjuvant. Rats were injected for three consecutive weeks, boosted 10-14 days later and finally bled after 10 days. The antiserum was checked for specificity against HCMV AD169 IE polypeptides by reacting the antibody with proteins extracted from HCMV AD169 infected RE cells in an immunoprecipitation assay. The antiserum was diluted 1:10 prior to use.

(c) Rat antiserum to the α and β IE polypeptides of HSV-2 HG52 prepared in Hooded Lister rats. Primary RE cells were infected with 10 pfu/cell of HSV-2 HG52 in the presence of 50ug/ml of cycloheximide for 7 hr and released for 3 hr in the presence of 10ug/ml of Actinomycin D. Rats were injected as described above and the specificity of the antiserum checked by immunoprecipitation assay. The antiserum was diluted 1:10 prior to use.

Secondary antibodies:

(a) Swine anti-human IgG FITC. This was used as the second antibody to human antiserum to HCMV and was diluted 1:20 before use.

(b) Goat anti-rat FITC. This was used as the second antibody to rat antisera to HCMV and HSV-2 and was diluted 1:20 before use.

8. TRANSFORMATION OF RE CELLS BY UV-IRRADIATED VIRUS

HCMV AD169 virus was irradiated using an Englehard Hanovia Lamp model 16 with a 12" low pressure mercury vapour discharge tube, operated at 15 watts and 30mA. 0.6-0.8ml of virus (suspended in PBS) was gently stirred in a 35mm petri dish for 20 min under the UV lamp placed 27.5 cm above the dish. At this height the emission of radiation from the lamp was 17 ergs/mm^2/sec (Macnab, 1976). UV-irradiated virus was stored at -70°C until required.*

RE cells were seeded at $2x10^5$ cells per 50mm petri dish and incubated from 16-24 hr at 37°C until just subconfluent. The medium was removed and the cells infected with 1-2 pfu/cell (initial titre) of UV-irradiated HCMV AD169. After 90 min absorption, the virus was removed, the cells washed three times in EF10 and then overlaid with 4ml of medium. The medium was changed after 10 min and washing was continued if the cells were detaching from the plate (a cytotoxic effect in response to the high multiplicity of virus infection). 20ul of human

* UV - irradiated virus was checked for infectivity before use. No plaques were produced when cells were infected at 37°C with 1-2pfu/cell (initial titre) of UV-irradiated virus.

antiserum to HCMV (see above) was added to each plate. The medium was changed 4 or 8 hr post infection and after 2 days the cells were trypsinized and split 1:3. Once confluent, the monolayers were maintained in EF2 and regularly examined for the appearance of foci of transformed cells.

9. TRANSFECTION OF CELLS WITH CLONED DNA FRAGMENTS

Cloned DNA fragments were introduced into cells using the calcium phosphate transfection technique. There have been a number of modifications made to the original calcium phosphate technique described by Graham and van der Eb (1973) aimed at increasing the efficiency of uptake and subsequent expression of exogenous DNA. Two different transfection procedures were used in the studies described in this thesis. For either procedure, $2x10^5$ cells were seeded per 50mm petri dish and incubated for 16-24 hr at 37°C until the monolayers were just subconfluent for transfection.

Transfection procedure 1

The appropriate amount of donor DNA and carrier DNA (if required) were dissolved in 210ul of TE buffer, pH7.5. 30ul of 2M $CaCl_2$ were added to the DNA solution with gentle agitation. After mixing well, the DNA-$CaCl_2$ solution was added dropwise to a tube

containing 240ul of 2xHBS, pH7.1, whilst gently vortexing. The calcium phosphate - DNA co-precipitate was allowed to form at room temperature for 30 min before being added dropwise to monolayers of cells in 4ml of growth medium. The calcium phosphate - DNA co-precipitate was incubated with the cells for 24hr at 37°C. The medium was then removed, the cell monolayers washed and overlaid with 4ml of fresh medium. If RE or Helu cells were used for transfection, the cells were boosted for 4 min by the addition of 1-2ml of 15% glucose, 10% glycerol in 1xHBS. The monolayers were washed three times to remove the glycerol and overlaid with fresh medium. 3T3 cells were not boosted as the procedure resulted in the cells detaching from the plates.

Transfection procedure 2

The second transfection procedure used was that described by Spandidos and Wilkie (1984a). 500ul of solution of donor DNA plus carrier DNA (if required) was prepared in 0.1mM EDTA, 1.0mM Tris-HCl, pH8.0, to a final concentration of 80ug/ml. 400ul of 0.1mM EDTA, 1.0mM Tris-HCl pH8.0, and 100ul 2.5M $CaCl_2$ were added to the DNA solution and mixed well. The DNA-$CaCl_2$ solution was slowly added with continuous mixing to 1ml of 2xHBS in a second tube. The calcium phosphate-DNA co-precipitate was allowed to form at room temperature

for 30min. After incubation, 500ul of the calcium phosphate-DNA suspension were added dropwise to monolayers of cells in 5ml of growth medium. The cells were incubated at 37°C for 24hr to allow absorption of the calcium phosphate-DNA precipitate. If selectable markers (eg the aminoglycoside 3' phosphotransferase gene) were used in either transfection procedure, the appropriate selective medium was added after the 24 hr incubation period with the calcium phosphate-DNA co-precipitate.

All cultures were trypsinized and passaged 1:4, 24 hr after being exposed to fresh medium. Cells were examined regularly for the appearance of transformed foci.

10. ASSAY FOR ANCHORAGE INDEPENDENT GROWTH

To assay for anchorage independent growth, cells were plated in soft agar at a density of $2\text{-}4\text{x}10^5$ cells per 50mm petri dish. Petri dishes were prepared with 4ml of agar underlay as in Methods. Transfected cells were trypsinized, pelleted at 1000rpm for 10 min at 4°C and resuspended in EF10 to give a final concentration of $1\text{-}2\text{x}10^6$ cells/ml. 0.2ml of the cell suspension was added to 2ml of agar overlay maintained at 42°C and gently pipetted onto the agar underlay. The agar was allowed to set at room temperature. The cells were

then incubated at 37°C and 1ml of EF10 was added every 3 days. Colonies should appear within 10-30 days.

11. ASSAY FOR TUMOURIGENICITY

Transfected 3T3 cells were assayed for their ability to produce tumours in nude mice using a modification of the method described by Fasano et al. (1984). 3T3 cells were transfected as described previously. The transfected cells were trypsinized and split into four 50mm petri dishes 48 hr after transfection. Once the monolayers had just reached confluency, the cells from all four plates were trypsinized, pooled, pelleted at 1000rpm for 10min at 4°C, and resuspended in 0.2ml of DF10. Approximately 10^7 cells were injected subcutaneously over the right shoulders of athymic, 4-8 wk old, female nude mice. Tumour growth was monitored weekly. Before death from tumour growth, the mice were sacrificed and the tumours removed. Some of the tumour tissue was snap frozen in liquid nitrogen to obtain DNA at a later time and some was further passaged in nude mice.

12. EXTRACTION OF HCMV AD169 DNA

Monolayers of Helu cells were prepared in sixty 800ml plastic tissue culture flasks and infected with

HCMV AD169 at a moi of 0.01pfu/cell. The overlay medium was collected daily from day 7 to day 11 post infection for the preparation of virion DNA. Cell debris was removed from the medium by centrifugation at 2000rpm for 10 min at 4°C. The virus was then pelleted from the supernatant by centrifugation at 13,000rpm for 90 min, resuspended in TNE buffer and treated with 50ug/ml DNase for 30 min in the presence of 10mM $MgCl_2$. EDTA was then added to a final concentration of 50mM and the virus again pelleted, resuspended in TNE plus 0.2% SDS and digested overnight with 200ug/ml proteinase K. Viral nucleic acids were extracted 3 times with TNE saturated phenol, once with chloroform and then dialysed overnight at 4°C against 3 changes of 0.1xSSC. The nucleic acids were then ethanol precipitated, resuspended in 10mM Tris-HCl pH7.5 10mM NaCl and 100mM EDTA and treated with 100ug/ml RNase for 2 hr at 37°C. Viral DNA was subsequently re-extracted with phenol and chloroform, dialysed, ethanol precipitated and finally resuspended in TE pH7.5. DNA preparations were aliquoted and stored at -20°C.

13. EXTRACTION OF CELLULAR DNA

Confluent monolayers of cells grown in 80oz roller bottles were washed three times in cold PBS and then scraped off in a small volume of PBS. The cells were

pelleted at 1000rpm for 10 min at 4°C, resuspended in cell lysis buffer and digested with 200ug/ml proteinase K for 3 hr at 37°C. The viscous DNA preparation was then diluted with TE buffer and gently extracted twice with TE saturated phenol and once with chloroform. The aqueous phase was made 0.2M sodium acetate and the DNA precipitated in 2.5 volumes of ethanol at -20°C overnight. The nucleic acids were then pelleted at 2500rpm for 20 min at 4°C and resuspended in TE plus 100ug/ml RNase A for 3 hr at 37°C. After RNase digestion, the DNA was extracted twice with TE saturated phenol and once with chloroform and then dialysed extensively against 0.1xSSC at 4°C. Stock DNA solutions were adjusted to a concentration of 1mg/ml where possible and stored in aliquots at -20°C.

14. EXTRACTION OF DNA FROM CIN TISSUE

CIN tissue was washed in cold PBS, finely minced and then digested with 200ug/ml proteinase K in cell lysis buffer for 2hr at 37°C on a rotator. The preparation was extracted once with TE saturated phenol and chloroform, dialysed overnight at 4°C against 0.1xSSC and ethanol precipitated in the presence of 0.2M sodium acetate. To reduce the number of manipulations and consequent loss of DNA because the yield of nucleic acids was very low (about

10-100ug/sample), the samples were not treated with RNase. RNA which migrates ahead of DNA on gel electrophoresis, was cut from the gel prior to blotting.

15. PREPARATION OF DENATURED SALMON SPERM DNA

Salmon sperm DNA was dissolved in sterile distilled water by gently rotating at room temperature for several hours. After phenol and chloroform extractions, the DNA was ethanol precipitated at -20°C overnight in the presence of 0.2M sodium acetate and resuspended to give a final concentration of 10mg/ml. The DNA was then boiled for 10 min and sonicated using a sonic probe. Small aliquots were stored at -20°C.

16. ESTIMATION OF DNA CONCENTRATION

The concentration of high MW DNA was rapidly estimated by agarose gel electrophoresis in mini gels. Small samples of DNA were electrophoresed with known standard concentrations of λ DNA on 0.6% (w/v) agarose gels in TBE electrophoresis buffer containing ethidium bromide. The DNA was visualised by UV illumination of the gels and the concentration determined by visual comparison of the sample fluorescence with that of the standards.

The concentration of low MW DNA eg carrier salmon

sperm DNA was estimated by optical density (OD) measurement at 260nm. The absorption at 260nm of various dilutions of the DNA sample was measured using quartz microcuvettes in a Perkin Elmer double beam R4 spectrophotometer. The concentration was estimated assuming that 1 optical density unit is equal to 40ug/ml of single stranded DNA. The purity of the DNA could be assessed by calculating the ratio between the readings at 260nm and 280nm (OD_{260}/OD_{280}). For pure preparations of DNA this ratio is 1.8.

17. AGAROSE GEL ELECTROPHORESIS

Agarose gel concentrations between 0.4% and 1% (w/v) were employed in this study. The agarose was dissolved by boiling, cooled to about 50°C and 0.5ug/ml ethidium bromide added before pouring. Horizontal gels run in E buffer were routinely used. The DNA samples were loaded with 1/10 volume Dye-Ficoll and electrophoresed at room temperature at about 2 V/cm. Gels were photographed under either long wave or short wave UV irradiation using Polaroid type 665 or 667 film.

18. RESTRICTION ENDONUCLEASE DIGESTION OF DNA

All restriction endonuclease digestions were

performed in the buffers recommended by the suppliers for each enzyme. Digestion of high MW cellular DNA was accomplished with an estimated 4-fold excess of enzyme overnight at 37°C. Most other digests were performed at 37°C for 3 hr with sufficient enzyme to produce a limit digest unless otherwise stated in the text. Reactions were terminated by the addition of 1/10 volume of Dye-Ficoll and the products of digestion separated by agarose gel electrophoresis and visualised by UV illumination.

19. PREPARATION OF COMPETENT E. coli AND TRANSFORMATION BY PLASMID DNA

Adapted from the procedure described by Dagert and Ehrlich 1979

One litre of LB was inoculated with 20ml of an overnight bacterial culture and grown with vigorous shaking at 37°C to an optical density of 0.2 at 650nm. The culture was then chilled on ice for 10 min and centrifuged at 6000rpm for 10 min at 4°C. The pellet was drained well and resuspended in 400ml of ice-cold sterile 0.1M $CaCl_2$. The cell suspension was left on ice for 20 min, repelleted, resuspended in 10ml of ice-cold sterile 0.1M $CaCl_2$ and left for a minimum of 6 hr on ice. 20-50ng of DNA in a volume of 1-10ul were added to 100ul of the $CaCl_2$ treated bacteria, carefully mixed and left on ice for 10 min. The bacteria were

then heat shocked for 1 min at 37°C in a water bath and 500ul of prewarmed LB plus 10mM $MgCl_2$ added. The preparation was finally incubated at 37°C on a shaker for 50 min and 100ul aliquots plated directly onto media selective for the appropriate plasmid resistance gene.

20. PREPARATION OF PLASMID DNA

Based on the cleared lysate method described by Clewell and Helinski (1970).

An overnight culture was prepared from seed stocks and used to inoculate 2 litres of LB plus 100ug/ml ampicillin. The bacteria were grown with vigorous shaking to an OD of 0.8 at 630nm. Chloramphenicol was then added to a final concentration of 100ug/ml (to stop the bacteria from multiplying and increase the number of plasmids per cell) and the cultures reincubated for 16-24 hr at 37°C. The bacterial cells were pelleted at 8000 rpm for 15 min at 4°C and resuspended in 16ml of cold sucrose-tris (25% sucrose in 50mM Tris-HCl pH8.0). The bacterial suspension was distributed between 4 tubes chilled to 4°C. Each tube was treated with 2ml of lysozyme (5mg/ml) for 5 min at 4°C, followed by 3ml of 250mM EDTA pH8.0 for 5 min at 4°C and then 9ml of Triton lysis mix for 20 min at 4°C. Cell debris was removed by centrifugation at 15,000 rpm at 4°C. The lysate was extracted once with phenol saturated with TE pH8.0 and dialysed against TE buffer

at 4°C with at least 2 changes. The nucleic acids were precipitated with isopropanol at room temperature and the precipitate resuspended in TE. The plasmid DNA was purified by centrifugation to equilibrium in caesium chloride ethidium bromide gradients. Gradients were prepared to give a final density of 1.55g of caesium chloride per ml with 500ug/ml of ethidium bromide and centrifuged in a Sorvall TV865B rotor on an OTD 50 ultracentrifuge for a minimum of 16 hr at 15°C. The DNA bands were visualised by fluorescence in long wave UV and the plasmid DNA collected by tube puncture. Ethidium bromide and caesium chloride were removed by extracting three times with isopropanol saturated with caesium chloride solution followed by extensive dialysis against TE buffer at 4°C. The DNA was ethanol precipitated and resuspended in TE to give a final concentration of 1mg/ml.

21. RECOVERY OF DNA FROM AGAROSE GELS

Cloned HCMV DNA sequences were excised from vector sequences and purified twice by agarose gel electrophoresis. After the relevant restriction enzyme digestion, the fragments were separated on a 1% (w/v) agarose slab gel containing 0.5ug/ml ethidium bromide and then visualised by long wave UV illumination. The required DNA band was cut from the gel and the DNA

electroeluted using a microelution kit (ISCO Ltd). The agarose containing the DNA was cut into small fragments and added to the "sample well". The DNA was electroeluted in TBE electrophoresis buffer at 100V until all the DNA was concentrated on the dialysis membrane over the smaller "collection well". The DNA was collected in a small volume, re-electrophoresed through a 1% (w/v) agarose gel and electroeluted a second time. The ethidium bromide was removed from the DNA by passing through a Dowex AG-50W-X8 column suspended in TNE and the collected DNA precipitated with two volumes of ethanol and resuspended in TE to give a final concentration of 1mg/ml.

22. In vivo LABELLING OF DNA

DNA was labelled in vivo with ^{32}P after Lonsdale (1979). Linbro wells (1.5cm diameter) were seeded with $2x10^5$ Helu cells in 1ml of Ec2-Pi and incubated at 37°C for 24 hr. Medium was removed and the cells infected with 2pfu/cell of HCMV. The virus inoculum was removed after 90 min, the cells washed twice and then overlaid with 1ml of Ec2-Pi. The infected cells were incubated at 37°C until the first signs of cpe were apparent. The medium was then removed and replaced with 0.5ml of Ec2-Pi containing 50uCi/ml of ^{32}P orthophosphate (carrier free). Incubation was continued until 70-100%

cpe was observed.

Cells were harvested by the addition of 0.5ml of 5% (w/v) SDS to each well and mixed with an equal volume of phenol saturated with saline EDTA (150mM NaCl, 100mM EDTA, pH8.0) and placed on ice for 10 min. The phases were separated by centrifugation at 2000 rpm for 10 min. Two volumes of ethanol were added to the aqueous phase and the nucleic acids pelleted by centifugation at 2000 rpm for 10 min at 4°C. The pellet was air dried and resuspended for 2 hr at 37°C in 200ul of distilled water containing 100ug/ml of RNase-A. 5ul aliquots were TCA precipitated and counted in scintillant (see below). Samples were stored at -20°C.

23. LABELLING OF DNA PROBES BY NICK TRANSLATION

DNA probes were labelled *in vitro* by nick translation essentially as described by Rigby *et al*. (1977) HCMV and HSV-2 DNA fragments which are G-C rich were labelled with [α-^{32}P] dGTP and [α-^{32}P] dCTP, and HPV 11 DNA, which is A-T rich, with [α-^{32}P]dATP and [α-^{32}P] dTTP. 0.25ug DNA were nick translated in a final volume of 50ul containing 0.01% BSA, 1xNTB, 0.04mM cold dATP and 0.04mM cold dTTP (cold dGTP and dCTP were used when nick translating HPV 11 DNA). $2x10^{-5}$mg/ml of DNase was

added and the tube incubated for a few minutes at 37°C and then placed on ice. 30uCi of each [α-^{32}P] deoxynucleoside triphosphate and 2u of DNase polymerase 1 were added and the mixture incubated at 15°C for about 90 min. Isotope incorporation was measured by spotting duplicate filter paper discs with 1ul of the reaction mixture. The DNA was precipitated and the unbound counts removed from one filter paper disc by three washes of 5 min in 5% (w/v) TCA at 4°C. The disc was then rinsed in ethanol and dried. Both the washed and unwashed discs were counted for ^{32}P in scintillant fluid (5g PPO per litre of toluene) and the percentage isotope incorporation calculated. Unincorporated deoxynucleoside triphosphates were removed by passing the DNA mixture through a 10ml Sephadex G50 column. The radiolabelled DNA was collected from the first peak in a volume of approximately 1-2ml. 1ul of the DNA probe was added to 500ul of water and counted for ^{32}P. The probe efficiency was calculated. Probes with efficiencies of 1-5x10^{8}cpm/ug DNA were used for blot hybridizations.

24. DNA BLOT HYBRIDIZATIONS

The method used for DNA blot hybridization was essentially that described by Southern (1975). High MW DNA was digested with an excess of restriction

endonuclease and the products of digestion fractionated by electrophoresis through 0.6% agarose gels. The DNA fragments were depurinated by gently shaking the gels in 0.2M HCl at room temperature for 30 min. The gels were then washed three times in deionized water and the DNA denatured by gently shaking the gels in Gel Soak 1 (0.2M NaOH, 0.6M NaCl) for 1 hr. The gels were then neutralized in Gel Soak 2 (1M Tris; 0.6M NaCl, pH7.5) for 30-45 min. Each gel was then transferred to a sheet of Whatman 3MM paper on a glass plate supported in a tray of 10xSSC. A sheet of presoaked Biodyne or Gene Screen Plus membrane was laid over the gel followed by 6 pieces of whatman 3MM filter paper cut 2mm smaller than the gel size. A weighted stack of dry paper towels was placed on top. The blot was left for 16-24 hr at room temperature to allow complete transfer of the single stranded DNA to the membrane. After transfer, Biodyne blots were washed for 30 min in 2xSSC and Gene Screen Plus blots immersed in an excess of 0.4MNaOH for 30-60 seconds to ensure complete denaturation of immobilized DNA and then neutralized in 0.2M Tris-HCl (pH7.5), 2xSSC. The blots were allowed to dry at room temperature. Biodyne blots were baked at 80^{o}C in a vacuum oven for 2 hr to permanently fix the DNA to the membrane. This procedure was not required for Gene Screen Plus blots. The gels were stained in a solution of 0.5ug/ml of ethidium bromide

for 1-2 hr and examined by long wave UV illumination to check that complete transfer of DNA had occurred.

The conditions for DNA hybridization were determined using the equation of Schildkraut and Lifson (1965). Probes were denatured in 0.2M NaOH, boiled for 20 min, then cooled quickly on dry ice/ethanol at -70°C, taking care not to freeze the probe. The probe was neutralized by the addition of an appropriate volume of HCl to the total hybridization solution containing the probe.

Blots probed with HCMV and HSV-2 were hybridized in aqueous conditions. The blots were prehybridized at 72°C for a minimum of 6hr in 3xSSC, 10xDenhardt's solution, 0.5% SDS and 10ug/ml of denatured salmon sperm DNA. Hybridization was carried out in a similar solution in which the denatured salmon sperm DNA was replaced by the radiolabelled denatured probe. Blots were hybridized at 72°C for 16-24hr. Prehybridization and hybridization were carried out in a total volume of 10ml in sealed polythene bags submerged in a plastic box placed in a shaking water bath.

Blots probed with HPV 11 DNA were hybridized in the presence of formamide. The blots were prehybridized in 50% formamide, 5xSSC, 5xDenhardt's solution, 0.05M HEPES, 0.5% SDS and 0.5mg/ml of denatured salmon sperm DNA for 6 hr at 42°C. Hybridization was carried out for 3 days at 42°C in a

similar solution, substituting the denatured radiolabelled probe for the denatured salmon sperm DNA.

After hybridization, Biodyne blots were washed for 30 min at room temperature in 10xDenhardt's solution, 3xSSC followed by three 30 min stepwise decreasing washes of 3-0.1xSSC, 0.1% SDS plus 10mM sodium pyrophosphate at the hybridization temperature. Gene Screen Plus blots were washed twice in 2xSSC at room temperature for 5 min, then twice in 2xSSC, 1.0% SDS at the hybridization temperature for 30 min and finally twice in 0.1xSSC, at room temperature for 30 min. Neither membrane type was allowed to dry out at any time to avoid irreversible binding of the DNA probe to the blot. The blots were placed between two layers of cling film and autoradiographed by exposure at -70°C to flashed Kodak X-Omat H film used in conjunction with a Dupont phototungstate intensifier screen.

Before rehybridization, probes were removed by washing the membranes according to the procedure recommended by the suppliers. Biodyne membranes were washed for 1 hr in 50% formamide, 10mM sodium phosphate (pH6.5) at 65°C and then in 2xSSC, 0.1% SDS for 15 min at room temperature. Gene Screen Plus membranes were washed for 30 min in 0.4M NaOH at 42°C and then in 0.1xSSC, 0.1% SDS, 0.2M Tris-HCl (pH7.5) at 42°C for 30 min with gentle agitation.

25. CONSTRUCTION OF A CIN DNA LIBRARY IN BACTERIOPHAGE VECTOR EMBL 3

The cloning strategy used in this study was to construct a complete library of CIN DNA and then to identify by hybridization those recombinant bacteriophage that contained DNA sequences homologous to HCMV DNA. Large DNA fragments of 15-20 kb were generated by partial digestion of CIN DNA with Sau 3A restriction endonuclease and were cloned into the lambda vector EMBL 3 (Frischauf et al., 1983). The methods employed are described below:-

Preparation of 15-20 kb fragments of CIN DNA

DNA fragments of 15-20 kb were prepared essentially as described by Maniatis et al. (1978). A reaction mixture was prepared with 10ug of CIN DNA and restriction enzyme buffer in a final volume of 250ul. 50ul of the reaction mixture were dispensed into one Eppendorf tube and 25ul into tubes 2-9. Three units of Sau 3A enzyme were added to tube 1, mixed, and 25ul transferred to tube 2. The 2-fold serial dilution was continued through to tube 8. The reactions were incubated for 30 min in a water bath at 37°C. The tubes were chilled on ice and EDTA added to a final concentration of 20mM to stop the reaction. The samples were analysed by electrophoresis through a 0.4%

agarose gel with λ DNA size markers in the 2 outside lanes. The λ DNA was digested with HindIII and XhoI enzymes to generate accurate markers in the 15-20 kb size region, heated at 68°C for 15 min and chilled quickly on ice before use. Electrophoresis was carried out overnight at 30-35V. The gel was photographed and the amount of enzyme needed to produce the maximum amount of fluorescence in the 15-20 kb region was determined. This was accomplished by blocking off all regions of the gel on the photograph not containing the DNA of the desired size and visually comparing the intensity of fluorescence in the various gel tracks. As the intensity of fluorescence is related to the mass distribution of DNA, half the amount of enzyme that produced the maximum amount of fluorescence was required to obtain the maximum number of molecules of 15-20 kb. Using these optimized conditions, a large scale digest of 15ug of CIN DNA was prepared. The enzyme concentration, time, temperature and DNA concentration were identical to those used in the pilot reactions. The reaction was stopped by chilling on ice and the addition of EDTA to a final concentration of 20mM. The DNA was then extracted twice with phenol/chloroform (1:1 v/v), ethanol precipitated and resuspended in 50ul of distilled water.

To prevent reannealing, the DNA fragments were treated with phosphatase in 50mM Tris-HCl pH8.0, 0.1mM EDTA pH8.0

in a final volume of 100ul at 37°C for 2 hr. Proteinase K, SDS and EDTA were added to final concentrations of 100ug/ml, 0.2% and 10mM respectively, in a final volume of 100ul and the reaction mixture incubated at 55°C for 1 hr. The DNA was then extracted 3 times with phenol/chloroform, ethanol precipitated and resuspended in 20ul of distilled water.

Ligation and packaging reactions

An equal molar ratio of ligatable CIN DNA and EMBL 3 arms was required for efficient ligation. Therefore, assuming the EMBL 3 arms were 30 kb in length and the CIN DNA insert 20 kb, 0.4ug of CIN DNA were required for every lug of arms. Ligation was carried out in the buffer recommended by NEB, the suppliers of the T4 DNA ligase, at 15°C overnight. The *in vitro* packaging reaction was carried out using the packaging extract purchased from Vector Cloning Systems. The ligated CIN DNA was mixed with the packaging extract according to the instructions provided. The reaction was stopped by the addition of 500ul of SM and 20ul of chloroform and the packaged DNA stored at 4°C until required.

The cloning efficiency was calculated by titrating the λ library on host strains Q358 and Q359 (Karn *et al.*, 1980). Both recombinant and non recombinant phage grew on Q358 but only recombinant phage grew on Q359.

26. PREPARATION OF PLATING BACTERIA

10ml of LB supplemented with 10mM $MgSO_4$ and 0.2% (w/v) maltose were inoculated from glycerol stocks of host bacteria and incubated overnight at 37°C. The overnight culture was used to inoculate 200ml of LB plus 10mM $MgSO_4$ and 0.2% (w/v) maltose and grown to an optical density of 1 at 630nm. The bacteria were pelleted at 6000 rpm for 10 min at 4°C and resuspended in 20ml of sterile 10mM $MgSO_4$ to give approximately $1x10^9$ bacterial cells per ml. The bacteria were stored at 4°C and were used up to about 7 days.

27. TITRATION OF BACTERIOPHAGE

Serial ten-fold dilutions of bacteriophage were prepared in SM and 100ul of each dilution added to a sterile 4x0.5" glass tube containing 100ul of plating bacteria. Phage and bacteria were incubated at 37°C for 20 min to allow the phage particles to absorb to the bacteria. 3ml of melted 0.7% agarose overlay medium were then added to each tube and immediately poured onto fresh LB plates prewarmed to 37°C. The suspension was quickly swirled to ensure even distribution of bacteria and agarose. Once the top agarose had set the plates were inverted and incubated at 37°C for 12-16 hr.

28. IDENTIFICATION OF RECOMBINANT CLONES

Plaque hybridization was carried out as described by Benton and Davis (1977). Aliquots of packaging mixture containing approximately 10,000 pfu of phage were mixed with 100ul of plating bacteria (Q359) and incubated at 37°C for 20 min. 3ml of molten top agarose (0.7%) were added and immediately poured onto dried LB plates prewarmed to 37°C. The plates were incubated inverted at 37°C for 12 hr or until the plaques were clearly visible but not showing confluent lysis. The plates were chilled at 4°C for 2 hr to allow the agarose to harden. An asymmetrically marked nitrocellulose filter was applied to the surface of each plate so there was direct contact between the plaques and the filter. The plate was marked according to the position of the filter marks and for more accurate alignment each plate was stabbed 3 times with an 18 gauge needle through both filter and agar. The filter was left in contact with the plaques for 30 sec to allow molecules of unpackaged bacteriophage DNA present in the plaques to bind to the filter. The filter was then carefully peeled off using blunt-ended forceps and immersed DNA side up in a shallow tray of denaturing solution (1.5M NaCl, 0.5M NaOH) for 30-60 sec. The filter was transferred to neutralizing

solution (1.5M NaCl, 0.5M Tris-HCl pH8.0) for 5 min, rinsed in 2xSSPE and air dried on Whatman 3MM paper.

A second replica filter was taken from the same plate. A small amount of ink was added to the holes made in the agar from the needle stabs and the second filter placed on the surface of the plate aligning the asymmetric marks as closely as possible. The filter was stabbed where the ink marks showed through and was left for 60 sec. The DNA was denatured and neutralized as described above.

Reconstructions were prepared by spotting DNA from HCMV AD169 (7.4, 3.7, 0.74ng), EMBL3 (1.7, 0.85, 0.17ng) and pAT (100, 50, 10pg) onto a nitrocellulose filter and denaturing and neutralizing the DNA as described above.

The filters were baked at 80°C for 2 hr in a vacuum oven to permanently fix the DNA to the nitrocellulose.

Before hybridization, the filters were soaked for 5 min in 6xSSC and then transferred to a litre glass beaker containing 100ml of prewashing solution (50mM Tris-HCl, pH8.0, 1M NaCl, 1mM EDTA and 0.1% SDS). The filters were gently shaken at 42°C for 1-2 hr to remove any absorbed medium, agarose or bacterial debris. The prewashing solution was removed and 60ml of prehybridization solution added (5xSSPE, 5xDenhardt's solution, 0.1% (w/v) SDS and 100ug/ml of denatured

salmon sperm DNA). Prehybridization was carried out at 65°C for 4-6 hr. The HCMV DNA probe (0.25ug) was prepared by *in vitro* nick translation and denatured as previously described. Hybridization was carried out in 25-30 ml (for up to 15 filters per beaker) at 65°C for 24 hr in a solution identical to the prehybridization solution but without denatured salmon sperm DNA.

After hybridization the filters were given 3-4 washes of 5 min in 500ml of 2xSSC and 0.1% (w/v) SDS at room temperature followed by two 90 min washes in 500ml of 1xSSC, 0.1% (w/v) SDS at 65°C. The filters were air dried, placed between 2 sheets of cling film with radioactive ink markers and exposed to flashed Kodak X-Omat H film with a Dupont phototungstate intensifier screen at -70°C overnight.

After development the films were aligned with the filters using the radioactive ink markers. The positions of the asymmetric marks and stab holes were marked on the film and aligned with those present on the original agar plates. Positive plaques were identified by hybridization and picked using a sterile pasteur pipette equipped with a rubber bulb. The pipette was used to stab through the chosen plaque and underlying agar and mild suction applied so that the plaque and agar were drawn into the pipette. The fragments of agar were flushed out in 1.0ml of SM containing a drop of chloroform and left to stand for

1-2 hr at room temperature. The phage suspension was stored at 4°C until required.

29. PREPARATION OF PLATE LYSATE STOCKS OF BACTERIOPHAGE

$8.5x10^5$ pfu of bacteriophage were mixed with 100ul of plating bacteria (approximately $1x10^8$ cells) and incubated at 37°C for 20 min. $8.5x10^5$ pfu was the number of bacteriophage required to obtain confluent lysis on a 90mm plate after 12 hr incubation on Q359 host bacteria. The phage/bacteria suspension was mixed with 3ml of top agarose (0.7% w/v) and poured onto 30ml of fresh LB agar. The plate was incubated for 8-12 hr at 37°C until lysis was confluent. The plate was kept as moist as possible and not inverted during incubation. After incubation, 5ml of SM were added and the plate stored at 4°C for a minimum of 4 hr with intermittent gently shaking. The SM was harvested using a pasteur pipette and 1ml of fresh SM added to the plate which was then stored in a tilted position for 15 min to allow the SM to drain into one area. The SM was removed and combined with the first harvest. 100ul of chloroform were added, the solution vortexed and centrifuged at 4000g for 10 min at 4°C. The supernatent was recovered, one drop of chloroform added and the stock stored at 4°C.

30. PURIFICATION OF BACTERIOPHAGE

Approximately $2x10^7$ pfu of recombinant bacteriophage in a volume of 50ul (made up with SM if necessary) were mixed with 50ul of fresh plating bacteria (approximately $5x10^7$ cells) host strain K803. The phage were absorbed for 15 min at 37°C, then added to 350ml of LB supplemented with 10mM $MgSO_4$ and 0.2% maltose and incubated overnight at 37°C with vigorous shaking. Concomitant growth of bacteria and phage should occur resulting in lysis. If lysis were apparent (indicated by the presence of a precipitate of bacterial debris) 4ml of chloroform were added and incubation continued for a further 30 min. Bacteriophage particles were purified essentially by the method of Yamamoto et al. (1970). The culture was chilled to room temperature and pancreatic RNase and DNase added, both to a final concentration of lug/ml. After 30 min at room temperature, solid sodium chloride was added to a final concentration of 1M, dissolved by gently swirling and the culture left on ice for 1 hr. Bacterial debris was pelleted by centrifugation at 8200 rpm for 10 min at 4°C and solid polyethylene glycol (PEG 6000) added to the supernatant to a final conentration of 10% (w/v). The PEG was dissolved by gently stirring on a magnetic stirrer at room

temperature. Bacteriophage were precipitated by standing the preparation on ice for 2 hr followed by centrifugation at 8200 rpm for 10 min at 4°C. The pellet was drained well and resuspended in 1.5-2.0ml of SM. An equal volume of chloroform was added to the bacteriophage suspension, vortexed for 30 secs and then centrifuged at 11,500 rpm for 15 min at 4°C. The aqueous phase was recovered and 0.5g/ml of solid caesium chloride added and dissolved. The bacteriophage/caesium chloride solution was then layered onto caesium chloride step gradients. Caesium chloride solutions with densities of 1.7g/ml, 1.5g/ml and 1.45g/ml were prepared in SM and sequentially pipetted into cellulose nitrate tubes. The gradients were centrifuged at 22,000 rpm for 2 hr at 4°C in a Sorvall AH650 rotor on an OTD50 ultracentrifuge. The bacteriophage particles formed a bluish-grey band which was removed by tube puncture with a 21 gauge needle. Sufficient caesium chloride solution (1.5g/ml in SM) was added to the bacteriophage to fill a cellulose nitrate tube for a Sorvall TST41 rotor. The gradient was centrifuged to equilibrium at 35,000 rpm for 48 hr at 4°C. The band of bacteriophage particles was collected as before and stored in caesium chloride solution in a tightly capped tube at 4°C until required.

31. EXTRACTION OF BACTERIOPHAGE DNA

DNA was prepared from purified bacteriophage preparations as follows. The caesium chloride was removed by dialysis at room temperature against 1 litre of 10mM NaCl, 50mM Tris-HCl, pH8.0, and 10mM $MgCl_2$ for 2 hr with one change of buffer. The bacteriophage were then lysed in 20mM EDTA, pH8.0, and 0.5% SDS and treated with 50ug/ml of proteinase K at 37°C for 1 hr. The DNA was extracted once with phenol equilibrated in TE, once with phenol/chloroform (1:1 v/v) and once with chloroform. The preparation was then dialysed extensively against TE buffer pH8.0 at 4°C overnight with 3 changes of buffer and stored at 4°C. The concentration of the DNA was determined by agarose gel electrophoresis.

RESULTS

SECTION A

TRANSFORMATION STUDIES WITH HCMV AD169

The ability of HCMV to transform cells in culture has been well documented in the literature. There have been several studies describing morphological transformation by HCMV of various cell types including HEF cells (Albrecht and Rapp, 1973), human prostatic cells (Rapp et al., 1975), Helu cells (Geder et al., 1976) and guinea pig fibroblasts (Isom et al., 1983). In addition, RE cells have been successfully transformed by UV-inactivated HCMV AD169 in this laboratory. There have, however, only been 2 reported studies investigating transformation by HCMV at the molecular level. Nelson et al. (1982) transfected NIH 3T3 cells with restriction endonuclease HCMV AD169 DNA fragments in order to identify the transforming region(s). Anchorage-independent growth was used as the criterion for transformation. They showed that the HindIII E and Xbal N DNA fragments of HCMV AD169 could transform NIH 3T3 cells in culture. Clanton et al. (1983) performed similar experiments with the Towne strain in HCMV and found that the Xbal E fragment could initiate transformation of HEF and NIH 3T3 cells. The aim of the experiments in this section was to test the repeatability of the transformation assays and to extend

the work of Nelson et al. (1982) to further investigate a molecular basis for cell transformation.

Quality of current virus stocks

Before any experiments were undertaken, it was necessary to check the two HCMV AD169 stocks available in the laboratory. The Lonsdale method for in vivo ^{32}P labelling and extraction of viral DNA was used. This method is quick and did not require large scale preparation of viral DNA which tends to be difficult due to the fact that HCMV only grows to low titre. Aliquots of DNA from each of the HCMV virus stocks (labelled 1 and 2) were digested with HindIII and BglII and the products of digestion separated by electrophoresis through 0.6% agarose gels. The gels were then dried and autoradiographed. The restriction profiles of the two virus stocks differed both from each other and also from those published by Westrate et al. (1980), Oram et al. (1982) and Spector et al. (1982) (Figure A.1). The reason for this is unclear. Stock one was found to be heavily contaminated with mycoplasma. Mycoplasma DNA has a molecular weight of approximately $5x10^8$ daltons - larger than that of HCMV. It is possible that mycoplasma contaminating the virus stock incorporated sufficient ^{32}P into its DNA during replication to be detected on the autoradiograph after digestion with restriction endonucleases. A more likely explanation, however, is

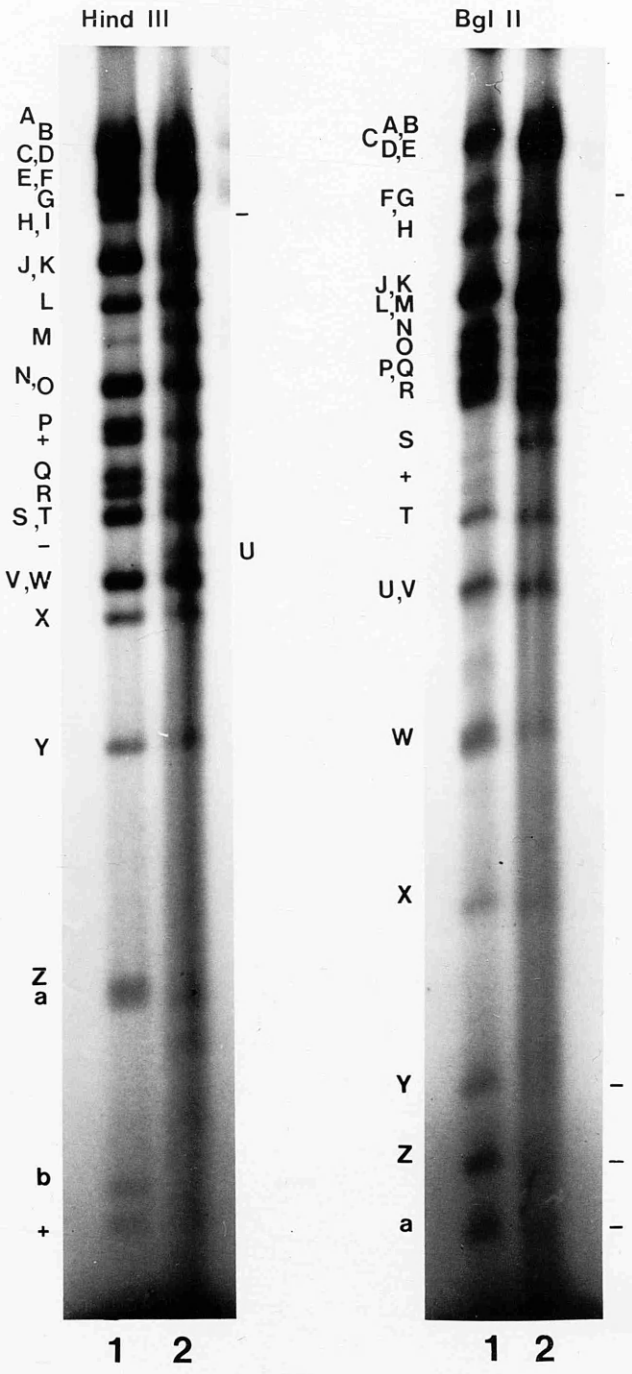

Hind III
Bgl II
A
B
C,D
E,F
G
H,I
J,K
L
M
N,O
P
+
Q
R
S,T
–
V,W
X
Y
Z
a
b
+
U
C
A,B
D,E
F,G
H
J,K
L,M
N
O
P,Q
R
S
+
T
U,V
W
X
Y
Z
a
1
2
1
2

FIGURE A.1 Restriction enzyme analysis of two separate stocks of HCMV AD169

Virus from two stocks (1 and 2) available were labelled *in vivo* with ^{32}P, the DNA digested with HindIII and BglII and separated on a 0.6% agarose gel. The letters refer to specific HCMV AD169 DNA fragments, the physical map locations of which are given in Figure 2.

Symbols: + novel restriction fragments

\- missing restriction fragments

that the novel restriction fragments detected represent defective HCMV DNA generated by high serial passage of the virus.

To avoid any complications that might have arisen in later experiments through the use of these HCMV AD169 virus stocks, and also because the DNA profiles of these viruses were not consistent with the HCMV DNA clones that were to be used in this study, it was decided to obtain a fresh stock of virus. A plaque-purified stock of HCMV AD169 was kindly provided by Dr. J.D. Oram. When DNA from the new stock was analysed, it was found to have restriction enzyme profiles that corresponded to those published previously (Oram et al., 1982). This virus stock was used throughout this study.

Characterization of cloned HCMV AD169 DNA restriction fragments

Cloned restriction fragments of HCMV AD169 DNA were obtained from Dr. J.D. Oram. HindIII fragments of HCMV AD169 DNA were cloned into the HindIII site of vector pAT153 and initially propagated in E. coli strain HB101 (Oram et al., 1982). This host strain does not grow very quickly so the cloned fragments were transfected into C292 cells, a faster growing strain of E. coli by the procedure described in Methods. It was then

necessary to check the clones to make sure that the cloned DNA inserts had not become rearranged in anyway during the transfection procedure. 0.5ug of DNA from each plasmid clone were digested with HindIII and electrophoresed through 0.7% agarose gels plus 0.5ug/ml of ethidium bromide. HindIII fragments of λ DNA were chosen as MW markers and were included in the outside lanes on the gels. Photographs of the gels are presented in Figure A.2. There was no evidence of any rearrangements that might have occurred during transfection of the majority of the various cloned DNAs. The DNA insert in clone G appeared to be approximately half the molecular weight to that reported by Oram et al. (1982). The insert should have had a molecular weight of about 13.6×10^6 daltons. However, this clone contained the K and Q HindIII fragments which contain repeat sequences and was not used in these studies as explained later. The DNA insert in clone Z appeared to be of a slightly higher molecular weight to that reported for the HindIII γ fragment by Oram et al. (1982). The digestion of clone Z did not proceed to completion resulting in additional bands on the gel representing partially digested DNA.

Transfection of cells with cloned restriction fragments of HCMV AD169 DNA

Mouse 3T3 cells were used in the initial

Designation of clone.

C D E F G J L M Y O P

kb

Molecular weight in kilobases

23·1

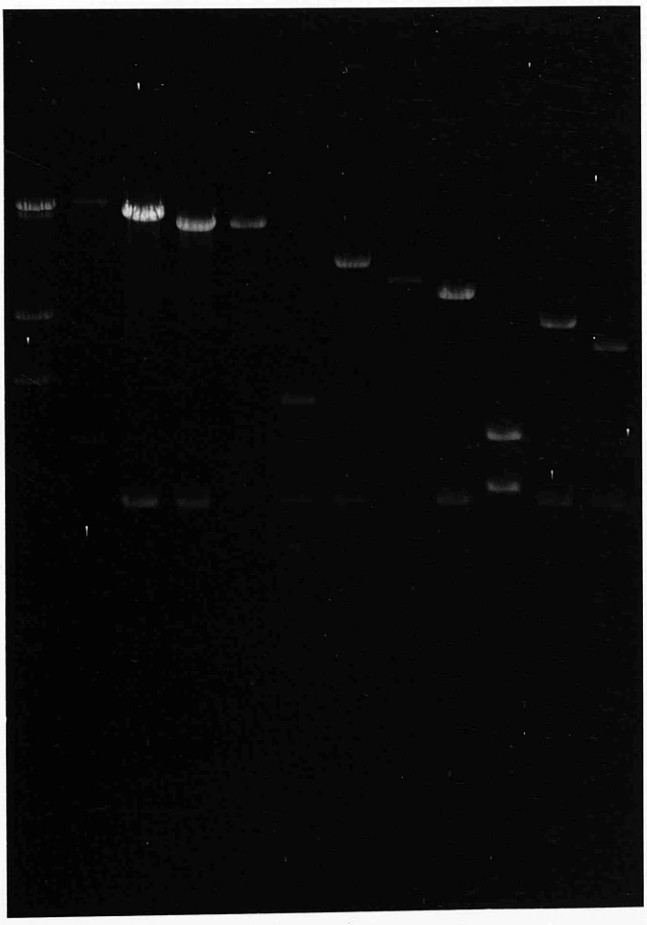
9·4

6·7

4·4

2·3

2·0



R S T U V W X Z N a b c

kb

23·1

9·4

6·7

4·4

2·3

2·0

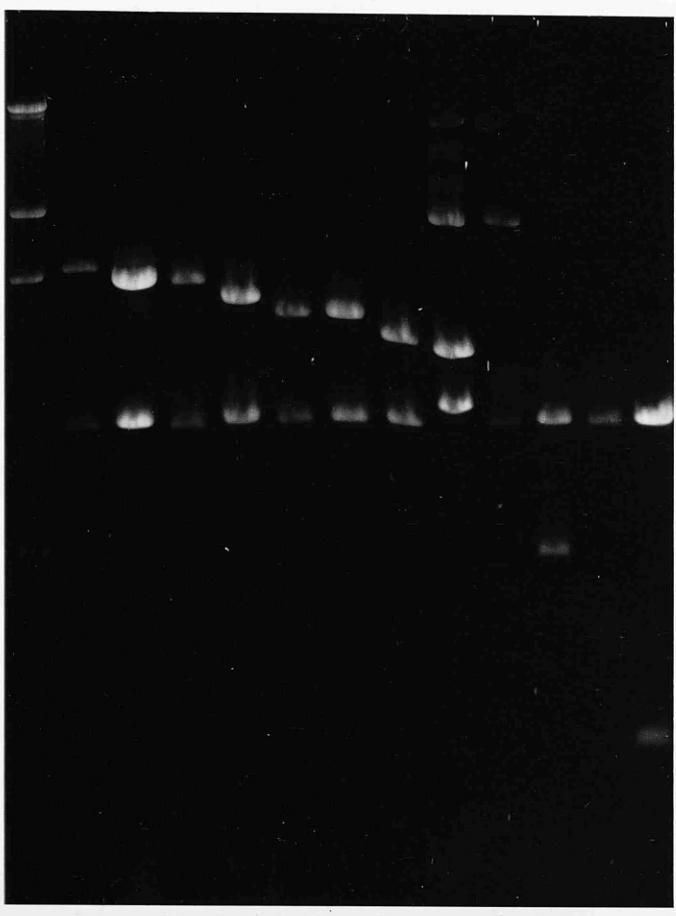


FIGURE A.2 Restriction enzyme analysis of cloned HCMV AD169 HindIII fragments after transfection into E.coli C292

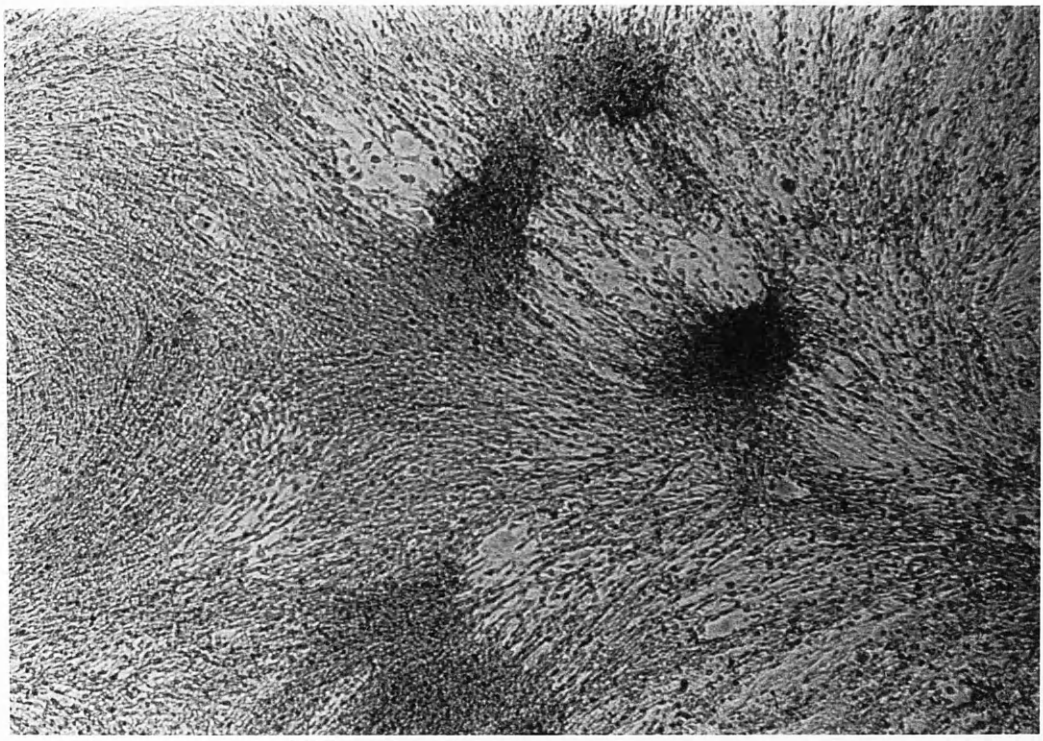
0.5ug of plasmid DNAs were digested with HindIII and the restriction fragments separated on 0.6% agarose gels. Size markers were provided by HindIII fragments of λ DNA.

transfection experiments. They are an established cell line frequently used in transfection experiments for the efficient uptake of exogenous DNA. Stock 3T3 cells were never allowed to reach confluency to prevent the formation of spontaneous transformants in the cell line.

Approximately $2x10^5$ 3T3 cells in 50mm plates were transfected with 10-20ug of the cloned HindIII E fragment of HCMV AD169 by procedure one described in Methods. In line with other workers (Galloway and McDougall, 1981; Nelson et al., 1982) the plasmid was cleaved with HindIII to linearize the DNA prior to transfection. Control cells were transfected with either 10ug of linearized pAT153 plasmid, 500ul of HBS or 200ng of plasmid pH06T1 which contains a cloned insert of the T24 Ha-ras1 gene (Spandidos and Wilkie, 1984b) plus 10ug of carrier RE DNA. 3T3 cells were not boosted during the transfection procedure as the cells detached from the plates within 30 seconds of the 15% glycerol - HBS solution having been added. The cells were split 1:4 48 hr post transfection and then maintained in DF2 at 37°C for as long as possible.

Foci of transformed cells appeared 14-17 days post transfection with pH06T1. Approximately 5 foci were obtained per 10^5 cells. Figure A.3. illustrates the type of foci obtained. No foci were obtained after transfection of cells with the cloned HCMV AD169 HindIII E fragment, pAT153 or 1xHBS.

FIGURE A.3 Transformation of 3T3 cells by the T24 Ha-ras1 oncogene



Photograph of transformed foci observed after transfection of 3T3 cells with plasmid pH06TI which contains the T24 Ha-ras1 oncogene. Foci appeared 14-17 days after transfection at a frequency of about 4-5 foci per 10^5 cells.

Magnification X 320.

The 3T3 cells used in these experiments were obtained from M. Freshney (Beatson Institute, Glasgow) and had been in culture for some time. It was therefore decided to repeat the transfection experiments using 3T3 cells from different sources. 3T3 cells were kindly provided by G.M. Cooper (Harvard University, Boston, USA), G. van de Woode (National Cancer Institute, Bethesda, USA) and C. Marshall (Chester Beatty Institute, London). Again, no morphological transformation could be obtained using the cloned HCMV AD169 HindIII E fragment with any of the 3T3 cell stocks.

Transfection experiments were also carried out using a different transfection procedure (procedure 2, see Methods) and a dominant selectable marker, the prokaryotic aminoglycoside 3' phosphotransferase gene which confers resistance to neomycin. The HindIII E fragment was cotransfected with the pSr-neo hybrid plasmid (P. Berg, Standford University Medical Centre, California) which contains the pBR322 origin of replication and the ampicillin resistance gene, the 1.4kb bacterial aminoglycoside phosphotransferase gene and the the SV40 origin of replication and early promoter region. Cells transformed for neomycin resistance were selected for by the addition of 100-500ug/ml of neomycin to the growth medium. Again, no transformation was obtained using the HCMV AD169

HindIII E fragment. Morphological transformation of 3T3 cells by the HCMV AD169 HindIII E fragment also could not be demonstrated in experiments undertaken by T. Kouzarides and C. Marshall (personal communications).

The HindIII E fragment was identified as a transforming region of HCMV AD169 using anchorage independent growth as the criterion for transformation (Nelson et al., 1982). It is possible that transformation by HCMV requires the coordinated expression of more than one transforming gene for conversion of cells to the fully transformed phenotype. For example, in cells transformed by polyoma virus, anchorage independent growth is determined by the expression of the small T gene but expression of middle T gene is required for morphological transformation. Possibly the HindIII E fragment of AD169 contains the gene responsible for anchorage independence but another, as yet unidentified gene is necessary for morphological transformation. To test this hypothesis, 3T3 cells were transfected as previously described and then plated out in soft agar at a concentration of $2x10^5$ and $4x10^5$ cells per 50mm plate. The cells were maintained in soft agar for one month during which time no sign of any colonies of transformed cells were observed.

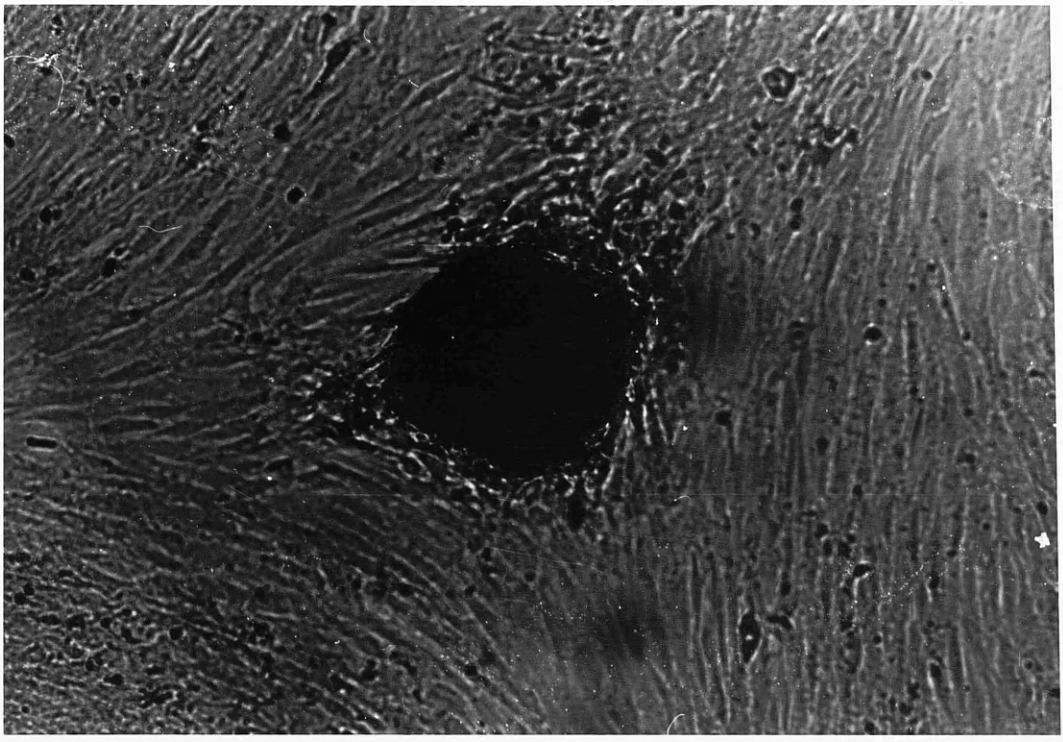
The transfection experiments were also repeated in both primary RE cells and Helu cells, but on no occasion was morphological transformation or anchorage

independent growth observed after transfection with the HCMV AD169 HindIII E fragment.

Transformation of RE cells by UV-irradiated HCMV AD169

Since repeated attempts to transform cells with the HCMV AD169 HindIII E fragment were unsuccessful, it was decided to follow up the earlier experiments carried out in this Institute on the transformation of RE cells by UV-irradiated HCMV AD169.

Subconfluent monolayers of primary RE cells were infected with 1 and 2pfu/cell of UV-irradiated HCMV AD169 as described in Methods. Once the monolayers were confluent, the cells from each plate were split 1:3 and again grown to confluency. The plates were maintained at 37°C with regular medium changes in EF2. Transformed cells could be discerned approximately three weeks after infection and appeared to be of three distinct morphological types. These are shown in Figures A.4 and 5. The most common type of transformed cell (type 1) formed large round foci which were very prominent in the cell monolayers (Figure A.4). These foci occurred at a frequency of about 4-5 foci per 10^6 cells - a frequency higher than that of transformation by UV-irradiated and ts mutants of HSV. The second type of HCMV transformed cell (type 2) also formed foci but at a lower frequency than type 1 HCMV transformed cells. The type 2 HCMV transformed cells were rounded in appearance and



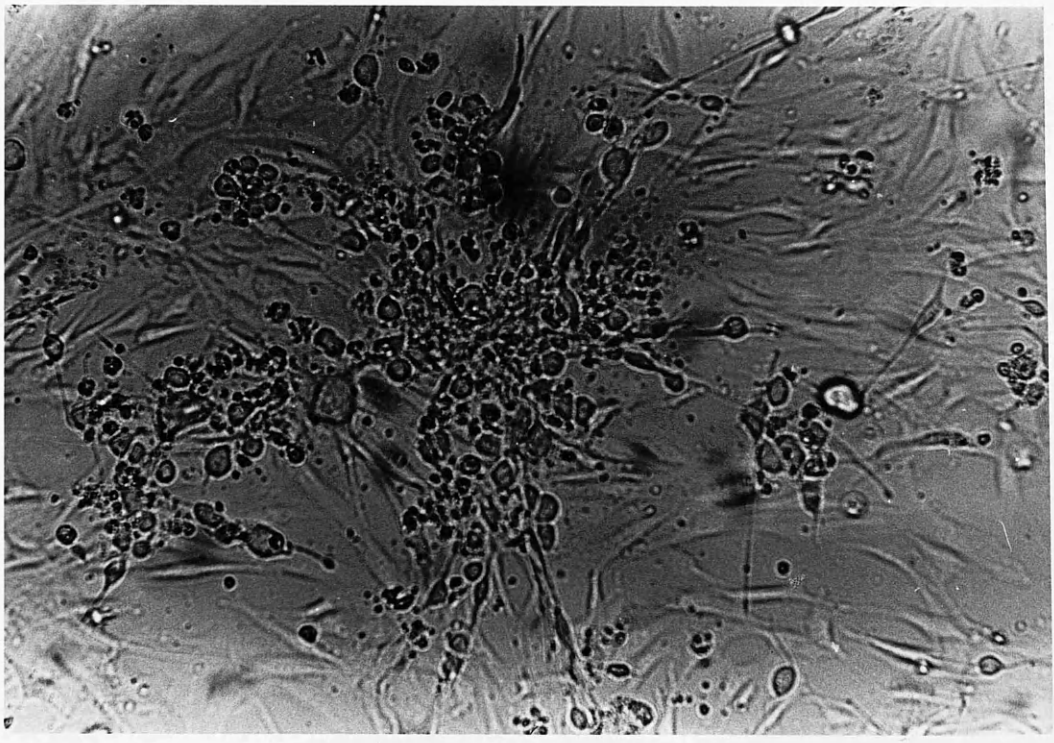


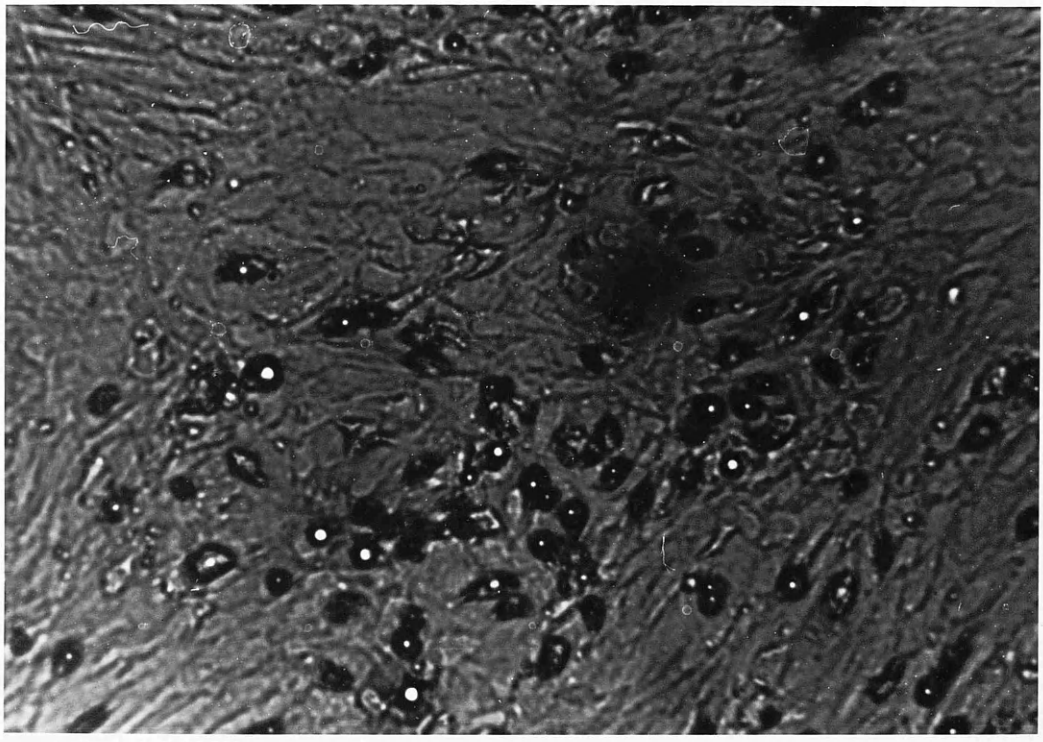
FIGURE A.4 Transformation of RE cells by UV-irradiated HCMV AD169

Type 1 focus formed by HCMV transformed cells

Type 2 focus formed by HCMV transformed cells

FIGURE A.5 Transformation of RE cells by UV-irradiated HCMV AD169

Type 3 HCMV transformed cells



infiltrated the surrounding normal RE fibroblasts at the outer edge of the foci. Unlike the foci formed by type 1 HCMV transformed cells, the foci formed by type 2 HCMV transformed cells had no regular shape (Figure A.4). The third type of HCMV transformed cell (type 3) did not form foci. Type 3 HCMC transformed cells were rounded, highly refractile and tended to occur in clusters. These cells were observed alongside the two other types of HCMV transformed cells (Figure A.5).

At first it was thought that the three morphological types of HCMV transformed cells might represent different stages in the transformation process, but close observation of the cells over a period of two more weeks revealed no further morphological changes. The type 3 HCMV transformed cells did not form foci and the foci of types 1 and 2 only increased in size up to a point. These experiments proved that it was possible to repeat the earlier transformation experiments using UV-irradiated HCMV AD169.

Some of the RE cells infected with UV-irradiated HCMV AD169 were plated into soft agar 48 hr after infection to assay for anchorage independent growth. Cells were plated in soft agar at $2x10^5$ and $4x10^5$ cells per 50mm plate and maintained at 37°C for about 2 months. No colonies of transformed cells were observed in this experiment. However, some of the cells appeared

viable and highly refractile when examined under the microscope and were similar to the type 3 HCMV transformed cells observed in the cell cultures maintained at 37°C in EF2.

The transformation studies were continued by analysing HCMV transformed and tumour cell lines established in the earlier experiments using UV-irradiated HCMV AD169. Transformed cell lines 1c and 0.01c were established by A. Bunce after transformation of RE cells with 1 and 0.01 pfu/cell respectively, of UV-irradiated HCMV AD169. Tumour cell lines CMT-1 and CMT-5 were derived from tumours induced in Hooded Lister rats after inoculation with approximately $5x10^7$ cells from the 1c transformed cell line.

Southern blot analysis of DNA from HCMV transformed and tumour cell lines

Analysis of cells transformed by SV40 and polyoma virus has revealed that viral DNA becomes integrated into the cellular genome and that continued expression of specific viral genes is necessary for maintenance of the fully transformed state (Ketner and Kelly, 1976; Botchan et al., 1976; Cuzin et al., 1984). The integration of viral DNA into the cellular genome has also been found to be a crucial event in transformation by adenoviruses (Sutter et al., 1978; Sambrook et al., 1979;) and retroviruses (Duesberg, 1979; Bishop and

Varmus, 1982). By analogy it would seem reasonable to suppose that HCMV DNA is also integrated and retained in HCMV transformed and tumour cells. Southern blot analysis was used to provide direct evidence for the presence of HCMV DNA within the HCMV transformed and tumour cell lines established in this Institute.

Southern transfers were prepared of HindIII digested DNA from the HCMV transformed and tumour cell lines plus appropriate reconstructions and controls. The DNAs were hybridized to various cloned HindIII restriction fragments of HCMV AD169. Three probes were used in the hybridization reactions:-

1. The HCMV AD169 HindIII E fragment reported by Nelson et al. (1982) to initiate transformation of NIH 3T3 cells.
2. EHB3, a HindIII/BamHI subclone of the HCMV AD169 HindIII E fragment containing the transforming sequence (Wilkinson et al., 1984).
3. A composite probe of HindIII fragments encompassing the entire HCMV AD169 genome. The HindIII fragments were nick translated as a mixture in which each fragment was present in the correct molar ratio. Fragments containing the inverted repeat sequences were omitted from the probe because they have been reported to contain sequences which share homology with cellular DNA (Peden et al., 1982). This probe was used in preference to intact total HCMV AD169 DNA as it could be nick translated to a higher specific activity.

The genomic locations of the cloned HCMV AD169 restriction fragments used as probes are represented diagramatically in Figure A.6.

No specific hybridization was obtained between any of the HCMV AD169 probes and the DNAs from the HCMV transformed and tumour cell lines analysed. Hybridization reactions could detect 1 copy/cell or more of viral sequences present in cellular DNA. Figure A.7 shows the results of the hybridization of the HCMV AD169 HindIII E probe to the HCMV transformed and tumour cell lines. Weak hybridization was detected to a fragment of about 3.6kb in the CMT-1 tumour cell line. However, this was found to represent hybridization of vector sequences to cellular DNA, since the fragment could not be detected when the DNA was rehybridized with an HCMV AD169 HindIII E probe from which vector sequences had been removed.

There are two possible explanations for the negative hybridization results obtained. The first is that the technique of Southern blot analysis is not sufficiently sensitive to detect very small viral DNA sequences present in low copy number. Nelson et al. (1983) have reported that the ability of HCMV AD169 to initiate transformation of NIH3T3 cells can be attributed to a DNA sequence of only 500bp. If this sequence was retained in transformed cells at low copy number it would escape detection by Southern blot analysis. Alternatively, retention of viral DNA in HCMV transformed cells may be transient and only necessary

HindIII - CLEAVAGE MAP OF HCMV STRAIN AD169

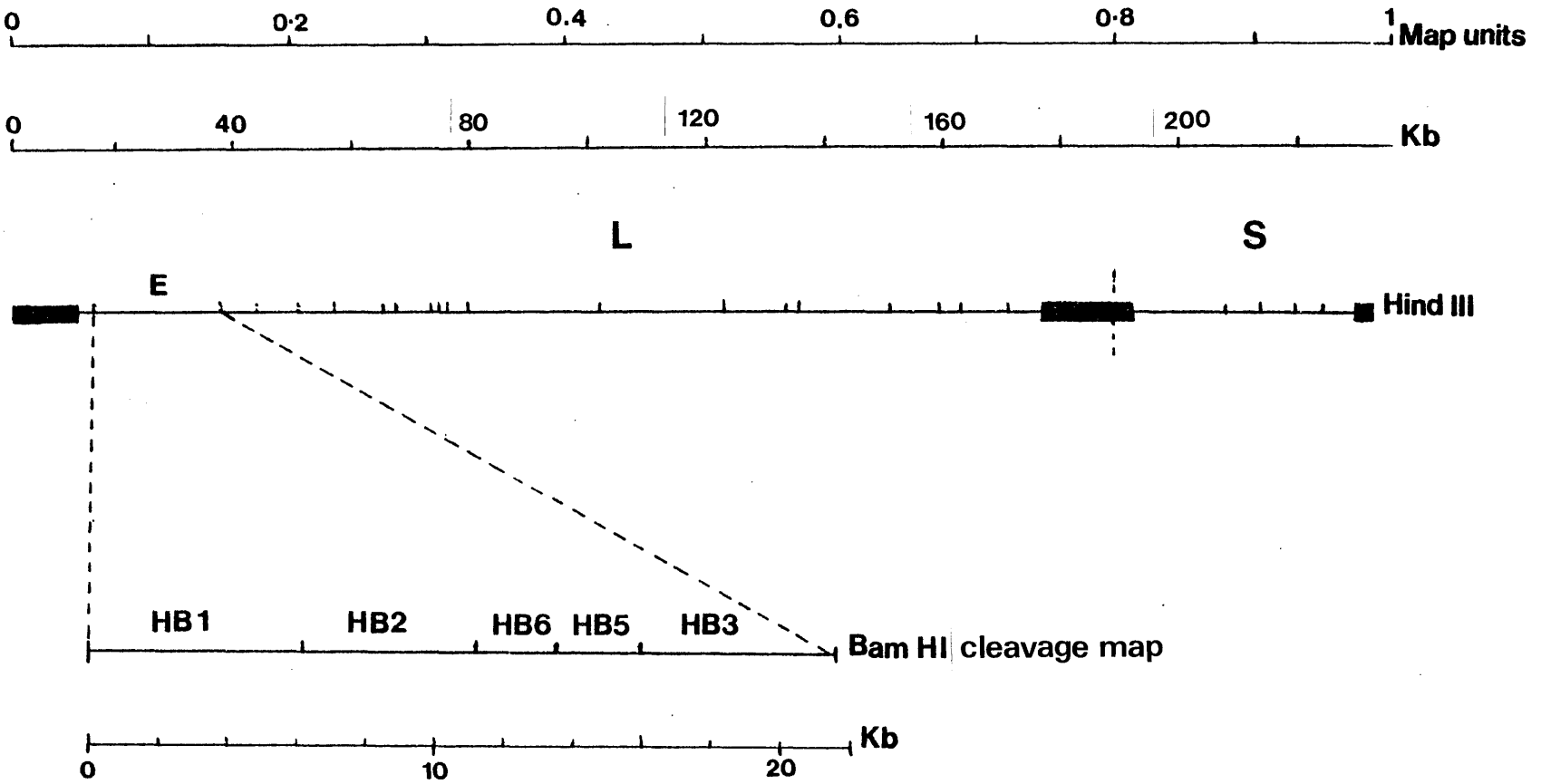


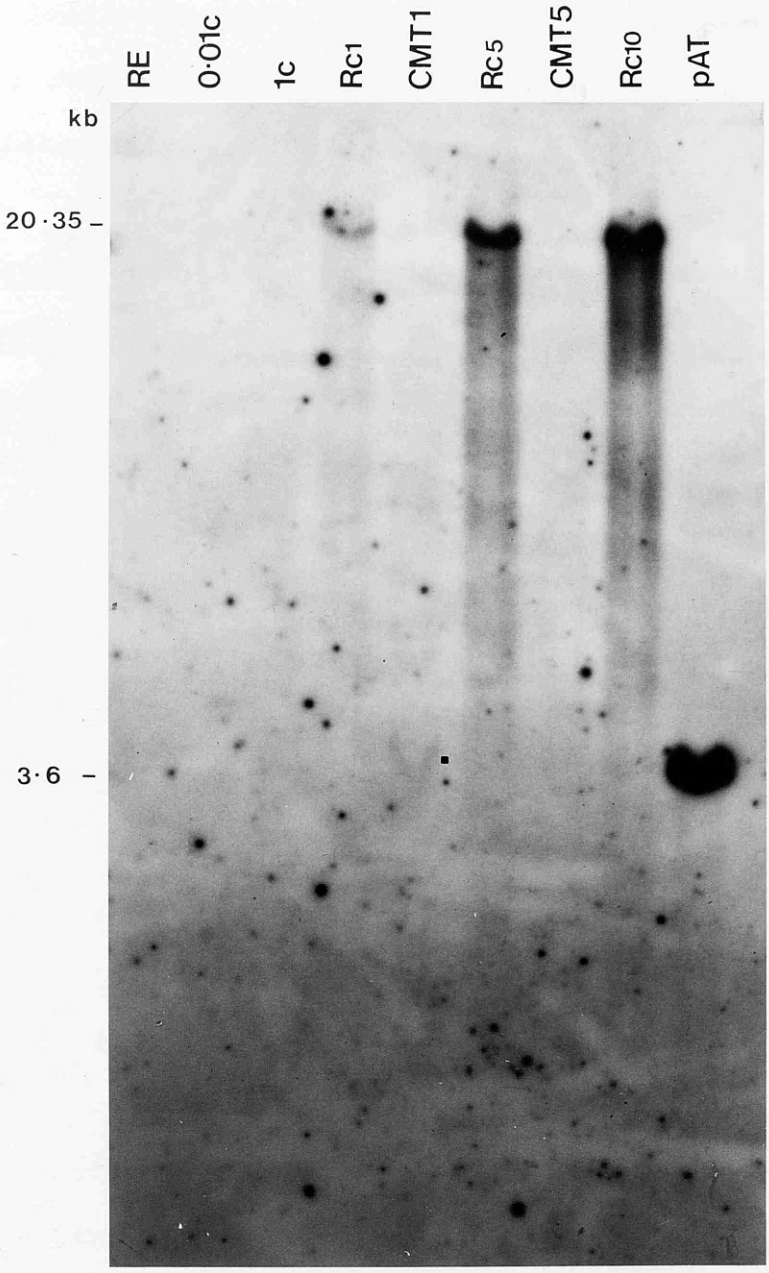
FIGURE A.6 Cloned HCMV AD169 restriction fragments used as probes

Diagram of the HCMV AD169 genome showing the location of the cloned restriction fragments used as probes in blot hybridizations. Solid boxes represent terminal and internal repeat sequences which bind the long (L) and short (S) unique components of the genome. Nomenclature taken from J.D. Oram et al. (1982).

An unbound copy of this diagram can be found at the back of this thesis.

FIGURE A.7 Southern blot analysis of HCMV transformed and tumour cell lines for the presence of specific HCMV DNA sequences

High molecular weight DNA (20ug) from control rat embryo (RE) and from HCMV transformed and tumour cell lines 1c, 0.01c, CMT-1 and CMT-5, was digested with a four-fold excess of HindIII. The products of digestion were separated on a 0.6% agarose gel, transferred to Biodyne membrane and hybridized at 70°C in 6xSSC with a ^{32}P labelled probe containing the HCMV AD169 HindIII E fragment of specific activity $2.2x10^8$cpm/ug DNA. Reconstruction tracks Rc1, Rc5 and Rc10 contain 20ug of RE DNA plus 1 copy/cell (0.74ng), 5 copies/cell (3.7ng) and 10 copies/cell (7.4ng) of HCMV AD169 DNA, respectively, and were treated as described above. pAT represents a control reconstruction containing 20ug of RE DNA plus 50pg (approximately equivalent to 5 copies/cell) of cloned vector DNA.



Hind III

to initiate the transformation event. If this is the case then it would appear that HCMV transforms cells by a different mechanism to that described for SV40, polyoma and adenoviruses. One possible mechanism by which HCMV could initiate cell transformation is by activation of a cellular proto-oncogene.

Transfection of 3T3 cells with DNA from a rat tumour induced after inoculation with HCMV transformed cells

Experiments were carried out in collaboration with Miss L. Clark and Dr. J.C.M. Macnab to see if the DNA from a rat tumour induced after inoculation with HCMV transformed cells contained a transforming gene capable of inducing tumour formation in athymic nude mice.

Tumours were induced in Hooded Lister rats by the inoculation of approximately $5x10^7$ cells from the 1c HCMV transformed cell line and the DNA extracted as in Methods. This tumour DNA is referred to as CMV-T DNA. Approximately $2x10^5$ 3T3 cells per 50mm petri dish were transfected with 10ug of CMV-T DNA plus carrier RE DNA. Control cells were transfected with 10ug of carrier RE DNA and 200ng of the pH06T1 plasmid plus 10ug of carrier RE DNA. The transfected cells were assayed for tumourigenicity in athymic nude mice as described in Methods.

Nude mice inoculated with 3T3 cells transfected with the pH06T1 plasmid developed fibrosarcomas within 8-10 days post inoculation. Nude mice inoculated with 3T3 cells

transfected with CMV-T DNA developed tumours 6-8 weeks after inoculation. Tumour induction was not observed in nude mice inoculated with 3T3 cells transfected with RE DNA. A small amount of tumour tissue was taken from a nude mouse inoculated with transfected CMV-T DNA and passaged in a second nude mouse. The passaged tumour was later removed and the DNA extracted for use in later experiments. This DNA was designated NMT DNA.

These experiments showed that the CMV-T DNA contained a transforming gene which could be transfected and could initiate tumour formation in nude mice. As Southern blot analysis could not detect HCMV DNA in the HCMV transformed and tumour cell lines, it was unlikely that the transfected gene in these experiments was a viral transforming gene. The transfected gene was more likely to have been of cellular origin. These experiments thus provide support for the hypothesis that HCMV initiates transformation by activating a cellular proto-oncogene.

Southern blot analysis of HCMV transformed cell and tumour DNAs for the presence of ras genes

The ras genes are the most frequently isolated tumour oncogenes so experiments were undertaken to try to determine if HCMV had amplified a ras oncogene in the HCMV transformed and tumour cells. A Southern transfer was prepared of 20ug of PvuII digested DNA from the 1c transformed cell and CMT-1 tumour cell lines and of CMV-T and NMT DNAs.

Reconstructions were prepared from 20ug of PvuII digested calf thymus (CT) DNA plus 10 copies/cell (220pg) and 1 copy/cell (22pg) of PvuII digested DNA from the ras containing plasmid pH06T1. PvuII digested DNA from a transformed cell line established after transfection of 3T3 cells with 200ng of pH60T1 (ras-TC) was also included as a positive control. The DNAs were hybridized with 0.25ug of ^{32}P-labelled pH06T1 DNA for 3 days at 42^{o}C in 50% formamide, 5xSSC, 1xHEPES, 5xDenhardt's and 1% SDS.

Hybridization was obtained to 1c and CMT-1 DNA, ras-TC and the reconstruction tracks but not to CMV-T or NMT DNAs (Figure A.8). If an activated ras gene was involved in transformation, the probe would be expected to hybridize to all of the HCMV transformed cell and tumour DNAs. The pH06T1 plasmid contains rat repetitive sequences so it is possible that the probe hybridized to rat cellular sequences in the 1c and CMT-1 cell lines. Unfortunately, CT DNA was used as the cellular DNA in the reconstructions and RE control DNA was not included on the blot. The DNAs need to be rehybridized on a blot including control RE DNA and with a ras probe from which the rat repetitive DNA sequences have been removed. This will determine if the probe has hybridized to rat cellular DNA sequences and whether the HCMV transformed 1c cells and CMT-1 tumour cells contain activated ras genes.

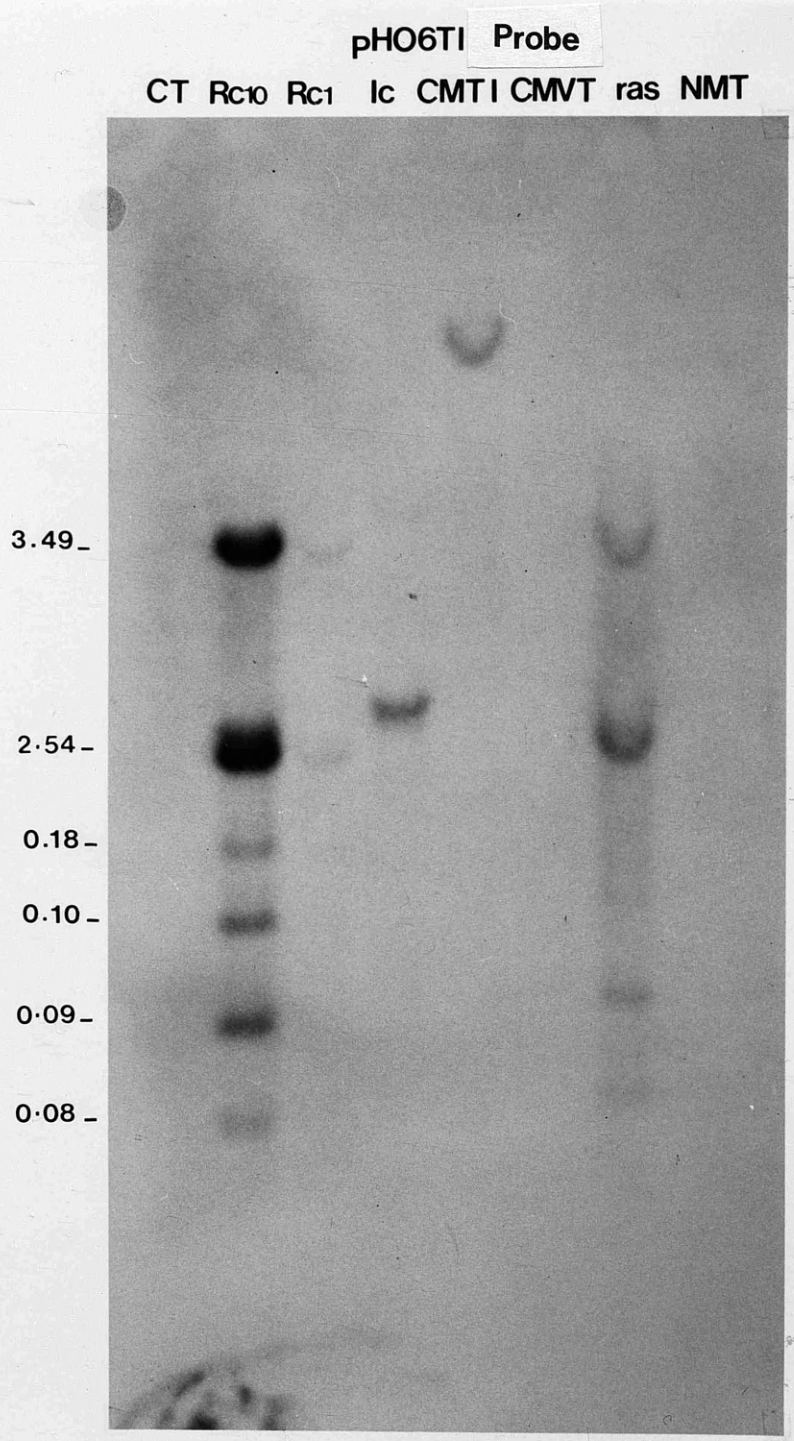

pHO6TI Probe
CT
Rc10
Rc1
Ic
CMTI
CMVT
ras
NMT
3.49
2·54
0.18
0.10
0·09
0·08
Pvu II

FIGURE A.8 Southern blot analysis of HCMV transformed cell and tumour cell DNA for the presence of ras oncogenes

An autoradiograph of a Southern transfer containing PvuII digested DNA (20ug/track) from HCMV transformed cell line 1c, tumour cell line CMT1, ras transformed cell line ras-TC (labelled ras) and tumour DNAs CMV-T and NMT. Reconstructions Rc1 and Rc10 were prepared from 20ug of calf thymus (CT) DNA plus 1 copy/cell (22pg) and 10 copies/cell (220pg), respectively, of pH06T1 DNA digested together with PvuII. The DNAs were hybridized for 3 days at 42°C in the presence of formamide with pH06T1 DNA labelled to a specific activity of 5.3×10^7cpm/ug DNA.

SECTION B

SOUTHERN BLOT ANALYSIS OF DNA FROM HUMAN CERVICAL PRENEOPLASTIC TISSUE FOR THE PRESENCE OF HCMV, HSV-2 AND HPV SPECIFIC SEQUENCES

HCMV, HSV-2 and HPV have all been implicated as possible oncogenic agents in the development of cervical neoplastic disease. Southern blot analysis was used as an approach to screen DNA extracted from CIN tissue for the presence of HCMV, HSV-2 and HPV specific sequences.

The sensitivity of the Southern blot hybridization technique

Past attempts to analyse cervical carcinoma biopsy specimens for the presence of HSV-2 DNA showed that in cases where viral DNA was detected, HSV-2 sequences were present in low copy number (Galloway and McDougall, 1983; Park *et al.*, 1983). Since HCMV, HSV-2 and HPV DNA may be present at low copy number in CIN tissue, every attempt was made to increase the sensitivity of the Southern blot hybridization technique, such that it would detect viral fragments present at between 0.2 and 1 copy/cell within cellular DNA.

A greater probability of detecting viral DNA present at low copy number could be obtained by transferring large amounts of DNA to the transfer membranes. 20ug of digested cellular DNA were routinely transferred in these experiments. To increase the efficiency of transfer of high

molecular weight (MW) fragments, the DNA was 'acid nicked' (depurinated) by gently shaking the gels in 0.25M HCl for 30 min at room temperature prior to alkaline treatment. This procedure partially cleaved the high MW DNA into smaller fragments which bind more efficiently to the membrane. However, the hydrolysis reaction was not allowed to proceed too far and produce fragments of less than 300 bp which are too short to bind efficiently to the membrane. The sensitivity was also increased by labelling the cloned DNA fragments used as probes in the hybridization reactions to high specific activities with α-^{32}P deoxynucleoside triphosphates. Probes were only used if they had a minimum specific activity of $1x10^8$cpm/ug DNA and would routinely detect 0.2 copies/cell of a viral fragment in 20ug of cellular DNA.

Selection of hybridization transfer membranes

A major problem throughout this study was the limited amount of CIN DNA available for Southern blot analysis. CIN DNA was extracted from punch biopsies. These were no more than a few mm in diameter and yielded approximately 50-100ug of DNA. Only one biopsy was available from each patient. If possible, it was hoped to analyse each CIN specimen for the presence of HCMV, HPV and HSV-2 specific DNA sequences. This was accomplished by using reprobable nylon-based transfer membranes for Southern blot analysis. Although nitrocellulose membranes are reprobable, they have a

tendency to become brittle and to break up during the washing procedure prior to rehybridization. The nylon-based membranes are more durable, remain intact during washing procedures even at high temperatures and can be successfully reprobed a number of times. Use of these membranes thus reduced the amount of CIN DNA required for Southern analysis and the number of Southern transfers made. The efficiency of the nylon-based membranes was found to be comparable with that of nitrocellulose. Initially Biodyne transfer membranes were used. However, the same level of sensitivity could not be achieved using later batches purchased from the same supplier so an alternative transfer membrane was sought. Gene screen plus ^{TM} hybridization transfer membranes were chosen as the manufacturers claim that these membranes have been designed to increase the efficiency of transfer of high MW DNA fragments from electrophoresis gels. The Gene screen plus transfer membranes were very successful for Southern blot analysis although they tended to give a higher background compared to the Biodyne membranes. Background hybridization was reduced by increasing the amount of SDS in the prehybridization and hybridization solutions from 0.1% to 0.5% as suggested by the manufacturers.

Conditions for Southern blot hybridizations

Hybridizations of HCMV DNA probes to Southern transfers were performed in aqueous conditions (6xSSC, 10xDenhardt's

solution and 0.5% SDS) for 16-24 hr at 70°C. These conditions are moderately stringent, being about 35°C below the hybrid melting temperature (Tm) and allowing for 35% mismatch of base pairs. The Tm was calculated using the equation derived by Schildkraut and Lifson (1965) assuming the average G+C content of HCMV to be 57%. Moderately stringent conditions were chosen to allow for the complexity and wide variation in the G+C content of the HCMV genome (from 43% to 60%) and also to allow for the possibility that HCMV present in CIN may contain some host cell DNA sequences of unknown G+C content.

Hybridization of HSV-2 DNA to Southern transfers was performed in 3xSSC, 10xDenhardt's solution and 0.5% SDS for 16 to 24 hr at 72°C. These conditions were slightly more stringent than those used for HCMV hybridization reactions, being about 30°C below Tm and allowing for 30% mismatch of base pairs.

Hybridization of HPV DNA to Southern transfers was performed in the presence of 50% formamide and 5xSSC at 42°C for 3 days. The presence of every 1% formamide in the hybridization solution reduces the Tm by 0.6°C. The conditions for the hybridization of HPV11 were of a level of stringency comparable to those for HSV-2.

Cloned DNA fragments used in hybridization reactions

The HCMV DNA cloned probes used in the hybridization reactions were the same as those previously described in

Section A. The HCMV HindIII E fragment and the HCMV DNA insert in EHB3 were liberated and purified from vector sequences. This made the fragments more suitable for nick translating to high specific activities and helped to alleviate the problem of non specific hybridization of vector sequences to human DNAs (discussed later).

The Bam HI t fragment of HSV-2 HG52 was chosen as the HSV-2 DNA probe because it contains one of the transforming regions identified in HSV-2 that is capable of initiating morphological transformation (Galloway and MacDougall, 1983).

The CIN DNAs were also probed with HPV11 DNA. The DNA was initially cloned into λ 47 and then subcloned in pBR322 (Gissmann et al., 1982, 1983). HPV11 was chosen as a probe because DNA from CIN tissue was initially found to hybridize to HPV6 and HPV11 (Gissmann et al., 1983; McCance et al., 1983).

Human CIN tissue and controls

CIN tissue was chosen for analysis because CIN is thought to represent the precursor stage to cervical carcinoma. If HCMV DNA is involved in the initiation of transformation and is not required for maintenance of the transformed state as previous results have suggested, viral DNA would only be detectable during the initial stages of malignancy.

Punch biopsies were obtained from patients with CIN in

the West of Scotland who were referred to a colposcopy clinic in the Western Infirmary (Glasgow) with abnormal cervical cytology observed on examination of cervical smears. The biopsies (taken prior to laser therapy) were kept on ice and the DNA extracted within 2 hr of the specimens having been taken. The same CIN patients were included in an epidemiological study by S. Walkinshaw, (personal communication). Sera from the patients were examined for the presence of antibodies to HSV-2 and HCMV using the complement fixation (CF) assay. The patient was considered positive for CF antibodies to either virus if the CF titre was 8 or greater. The patients were also screened for HPV infection by evidence of koilocytes seen on histological examination of stained sections from colposcopy specimens. The serological and histological data plus the ages of the patients from whom biopsies were taken are summarized in Table B.1. The CIN biopsies have been designated C1 to C43 for convenience according to the order in which they were obtained. High molecular weight DNA was extracted from the CIN biopsies as described in Methods.

Two types of control tissue were used:- rat embryo (RE) DNA which was extracted from approximately 20 day old embryos from an inbred Hooded Lister rat colony maintained in this Institute, and normal cervix (NC) DNA which was prepared from the ectocervix of hysterectomy specimens from patients with no evidence or history of malignant disease.

TABLE B.1 Serological and histological data available for the CIN patients involved in this study

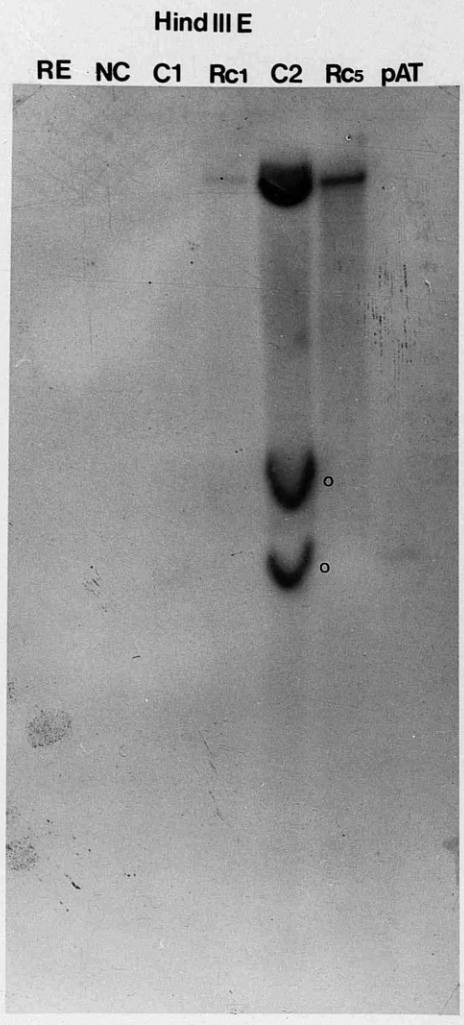
CIN biopsy	Age of patient	Grade CIN	Antibody titre to HCMV	Antibody titre to HSV	HPV infection
C1	28	II	128	16	+
C2	29	III	<8	<8	
C3	26	III	32	64	
C4	21	II	<8	16	
C5	48	II	8	16	
C6	39	III	<8	32	+
C7	30	III	<8	<8	
C8	26	II	ND	ND	+
C9	35	III	<8	ND	
C10	43	III	8	16	
C11	38	III	8	<8	+
C12	30	II	16	16	+
C13	30	III	16	8	
C14	30	III	<8	<8	
C15	26	II	<8	<8	
C16	28	II	8	8	
C17	25	II	8	8	+
C18	33	III	<8	8	
C19	35	III	<8	16	+
C20	28	II	16	16	
C21	37	II	16	8	+
C22	32	II	8	<8	+
C23	29	II	<8	8	+
C24	24	III	<8	16	
C25	32	III	<8	<8	
C26	33	II	8	16	+
C27	33	I	<8	8	
C28	28	II	<8	8	+
C29	32	II	8	8	+
C30	36	II	16	8	+
C31	35	III	<8	16	
C32	28	II	<8	16	+
C33	30	III	8	8	
C34	27	I	<8	<8	
C35	28	III	32	16	
C36	31	III	16	<8	
C37	28	II	<8	ND	
C38	29	II	8	<8	+
C39	28	II	<8	<8	
C40	31	II	16	16	
C41	39	III	<8	<8	
C42	22	I	<8	32	
C43	33	III	<8	32	

ND = Not determined

Antibody titres determined by the Diagnostic Laboratory, Institute of Virology, Glasgow.

Hybridization of HCMV DNA probes to CIN DNA

The CIN DNAs, RE and NC control DNAs (20ug of each) were digested with an excess of HindIII enzyme. Reconstructions were prepared from 20ug of RE DNA plus 0.2, 1, and 5 copies/cell (0.15ng, 0.74ng and 3.7ng, respectively) of HCMV AD169 DNA and digested together with an excess of HindIII. The digested DNAs were then electrophoresed through 0.6% agarose gels and transferred to either Biodyne or Gene Screen Plus membranes as described in Methods. The Southern transfers were then hybridized at different times to the three HCMV probes previously described in Section A, namely, the HCMV AD169 HindIII E fragment, EHB3 probe and the HCMV AD169 composite probe (a probe containing HindIII fragments encompassing most of the HCMV AD169 genome). These probes are diagrammatically represented in Figure A.6, an unbound copy of which can be found at the back of this thesis. Hybridization was observed with the HCMV DNA probes to only 2 of the 43 CIN DNAs analysed, C2 and C17 (Figures B.1 and B.3). All other CIN DNAs were negative for hybridization at levels of sensitivity which would detect 0.2 copies/cell of an HCMV DNA fragment in 20ug of cellular DNA. Figures B.5 and B.6 show two representative blots (probed with the EHB3 and HCMV AD169 composite probes) of HindIII fragments of CIN DNAs that were negative for sequences that hybridize to HCMV. No non specific hybridization was observed to either RE or NC control DNAs.


Hind III E
RE NC C1 Rc1 C2 Rc5 pAT
kb
20·35
3.6

Hind III


EHB3
RE NC C1 Rc1 C2 Rc5 pAT
kb
20.35

FIGURE B.1 Hybridization of HCMV HindIII E and EHB3 probes to a Southern transfer containing CIN DNA

Autoradiographs of the same Southern transfer containing HindIII digested DNA (20ug/track) from human CINs C1 and C2 hybridized at different times with HCMV HindIII E and EHB3 probes. Reconstruction tracks Rc1 and Rc5 were prepared from 20ug of rat embryo DNA plus 1 copy/cell (0.74ng) and 5 copies/cell (3.7ng), respectively, of HCMV AD169 DNA and digested together with an excess of HindIII. pAT represents a control reconstruction containing 20ug of rat embryo DNA plus 50pg of cloned vector DNA both digested with HindIII. RE and NC represent control tracks of 20ug of HindIII digested rat embryo and normal cervix DNA, respectively.

Both probes hybridized to the 20.35kb HindIII E fragment in the virus reconstruction tracks and to a similar sized fragment in C2. The HindIII E probe also hybridized to two fragments of C2 DNA of about 3 and 5kb (labelled with open circles in the photograph). The DNA insert in the EHB3 probe was purified from vector sequences.

Figure B.1 shows an autoradiograph of a Southern transfer of HindIII digested C2 DNA hybridized to the HCMV HindIII E probe. In this particular hybridization, the vector sequences had not been removed from the HCMV HindIII E probe. The probe hybridized to 3 fragments of C2 DNA, one of which comigrated with the HindIII E fragment of HCMV AD169 present in the reconstruction tracks. The other two bands of lower MW did not hybridize to the HCMV HindIII E fragment after it had been purified from vector sequences (not shown). Thus, hybridization of the cloned DNA probe to these lower MW bands presumably represents hybridization to sequences with homology to vector pAT153 sequences. Figure B.1 also shows the same Southern transfer hybridized to the HCMV EHB3 probe. This DNA probe had been purified from vector sequences and also hybridized to the fragment of C2 DNA that comigrated with the HCMV AD169 HindIII E fragment.

The nature of the hybridization of C2 DNA sequences to vector sequences is unknown. It is possible that the cervix of the patient from whom the biopsy was taken may have been contaminated with bacteria containing a plasmid population with which pAT153 shares homology. Data from a bacteriological study of this group of CIN patients at the Western Infirmary revealed a higher degree of cervical infection with coliform bacteria in CIN patients when compared with controls (W. Roberts, personal communication)

In a separate investigation, cervical swabs were examined for coliform bacteria and the bacterial DNAs

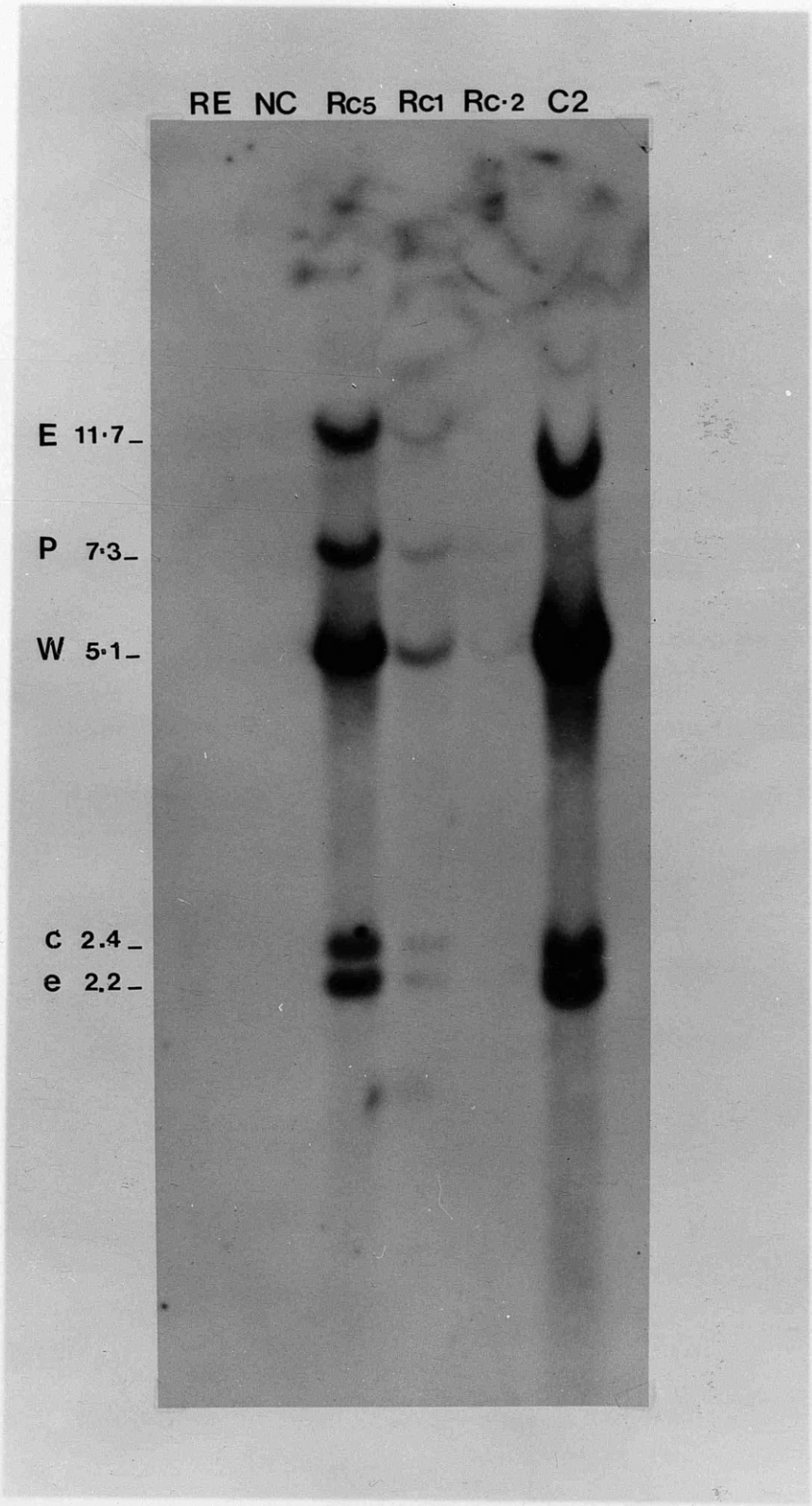

RE
NC
Rc5
Rc1
Rc·2
C2
E 11·7
P 7·3
W 5·1
c 2.4
e 2.2

FIGURE B.2 Hybridization of the HCMV HindIII E probe to a Southern transfer containing C2 DNA

Autoradiograph of a Southern transfer containing normal cervix (NC), rat embryo (RE) and C2 DNA (20ug/track) digested with BamHI and hybridized to the purified HCMV AD169 HindIII E fragment. Reconstructions Rc.2, Rc1 and Rc5 were prepared from 20ug of RE DNA plus 0.2 copies/cell (0.15ng), 1 copy/cell (0.74ng) and 5 copies/cell (3.7ng), respectively, of HCMV AD169 DNA and digested together with BamHI.

The HCMV AD169 HindIII E fragment hybridized to 5 BamHI fragments, E, P, W, c and e in the reconstruction tracks. The same probe hybridized to DNA sequences in C2 that comigrated with BamHI fragments P, W, c and e and also to two additional fragments, one of a higher molecular weight and one of a lower molecular weight than the BamHI E fragment of AD169.

analysed by Southern blotting. A significant proportion of the bacterial DNAs were found to hybridize to pBR322 sequences (R. Thompson, personal communication). Several plasmids share sequence homology at and adjacent to their origins of replication (Selzer et al., 1983) and it is probable that the hybridization of pAT153 DNA to human CIN DNA is through common sequences within this region, as described by Park (1983).

The C2 DNA was further analysed by digestion with BamHI and hybridization to the purified HindIII E fragment (Figure B.2). The HindIII E fragment hybridizes to 5 BamHI fragments, E, P, W, c and e of the HCMV AD169 genome. The HindIII E probe hybridized to DNA sequences in C2 that comigrated with HCMV AD169 BamHI fragments W, P, c and e. The probe hybridized to 2 additional fragments, one of a higher MW and one of a lower MW than the BamHI E fragment of AD169. These bands could represent rearrangement of viral DNA sequences within the CIN DNA. An alternative explanation could be that the biopsy had been infected in vivo with an isolate of HCMV that has a different restriction pattern to AD169 in this particular region of the genome. However, this possibility was eliminated when the Southern transfer containing the BamHI digested C2 DNA was rehybridized to the AD169 composite probe. No additional sequences within the C2 DNA were found to hybridize specifically to HCMV AD169 DNA (data not shown). If the biopsy had been infected with HCMV, other regions of

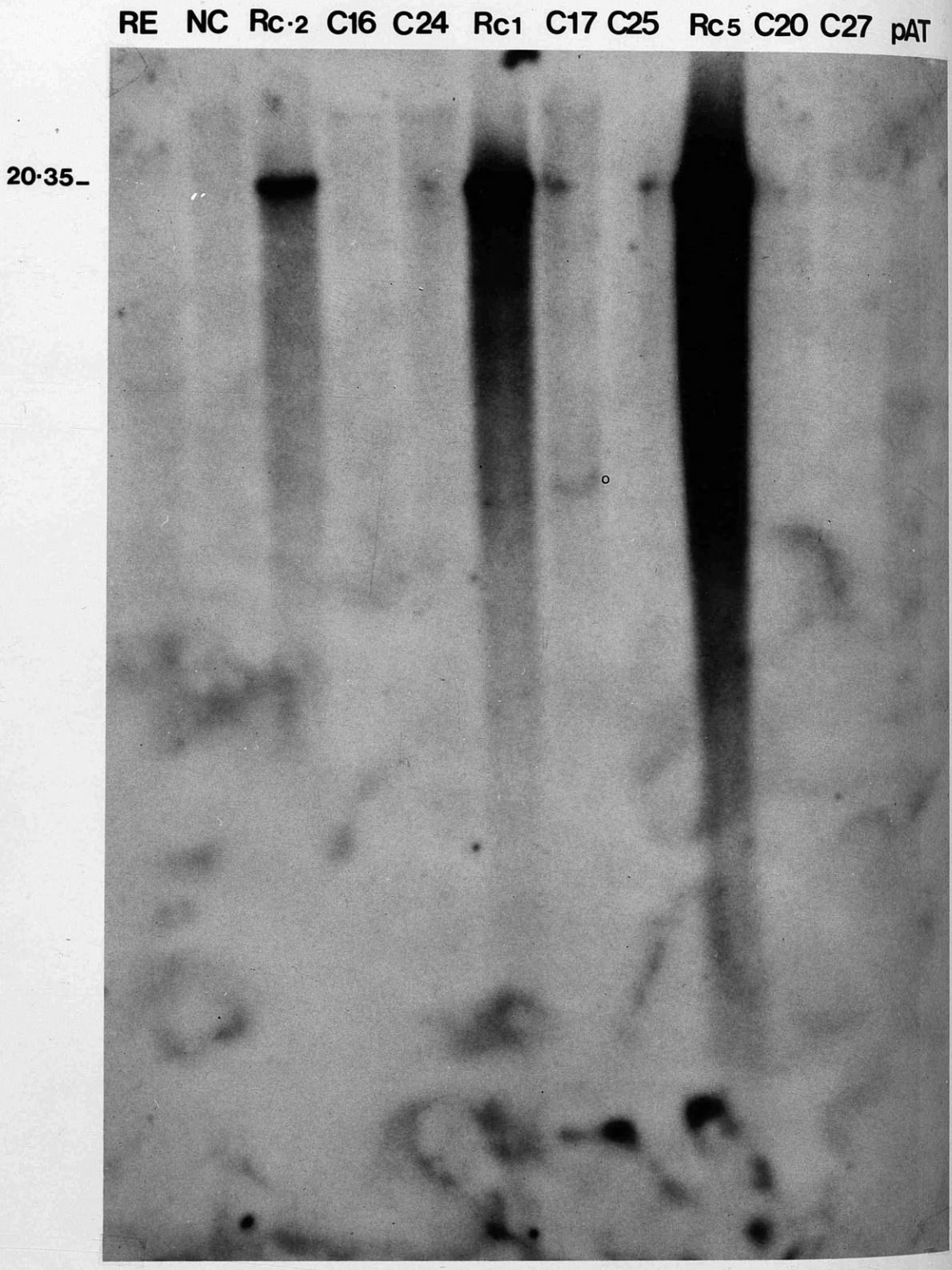

RE
NC
Rc·2
C16
C24
Rc1
C17
C25
Rc5
C20
C27
pAT
20·35

FIGURE B.3 Hybridization of the HCMV EHB3 probe to a Southern transfer containing CIN DNAs

Autoradiograph (9 day exposure) of a Southern transfer of normal cervix (NC), rat embryo (RE) and CIN DNAs 16, 17, 20, 24, 25 and 27 digested with HindIII and hybridized with the HCMV EHB3 probe. Reconstructions Rc.2, Rc1 and Rc5 were prepared from 20ug of RE DNA plus 0.2 copies/cell (0.15ng), 1 copy/cell (0.74ng) and 5 copies/cell (3.7ng), respectively, of HCMV AD169 DNA and digested together with HindIII. pAT represents a control reconstruction containing 20ug of HindIII digested RE DNA plus 50pg of vector DNA.

The EHB3 probe hybridized to the 20.35kb HindIII E fragment in the reconstruction tracks and to a DNA fragment of about 6kb in C17 (marked with an open circle).

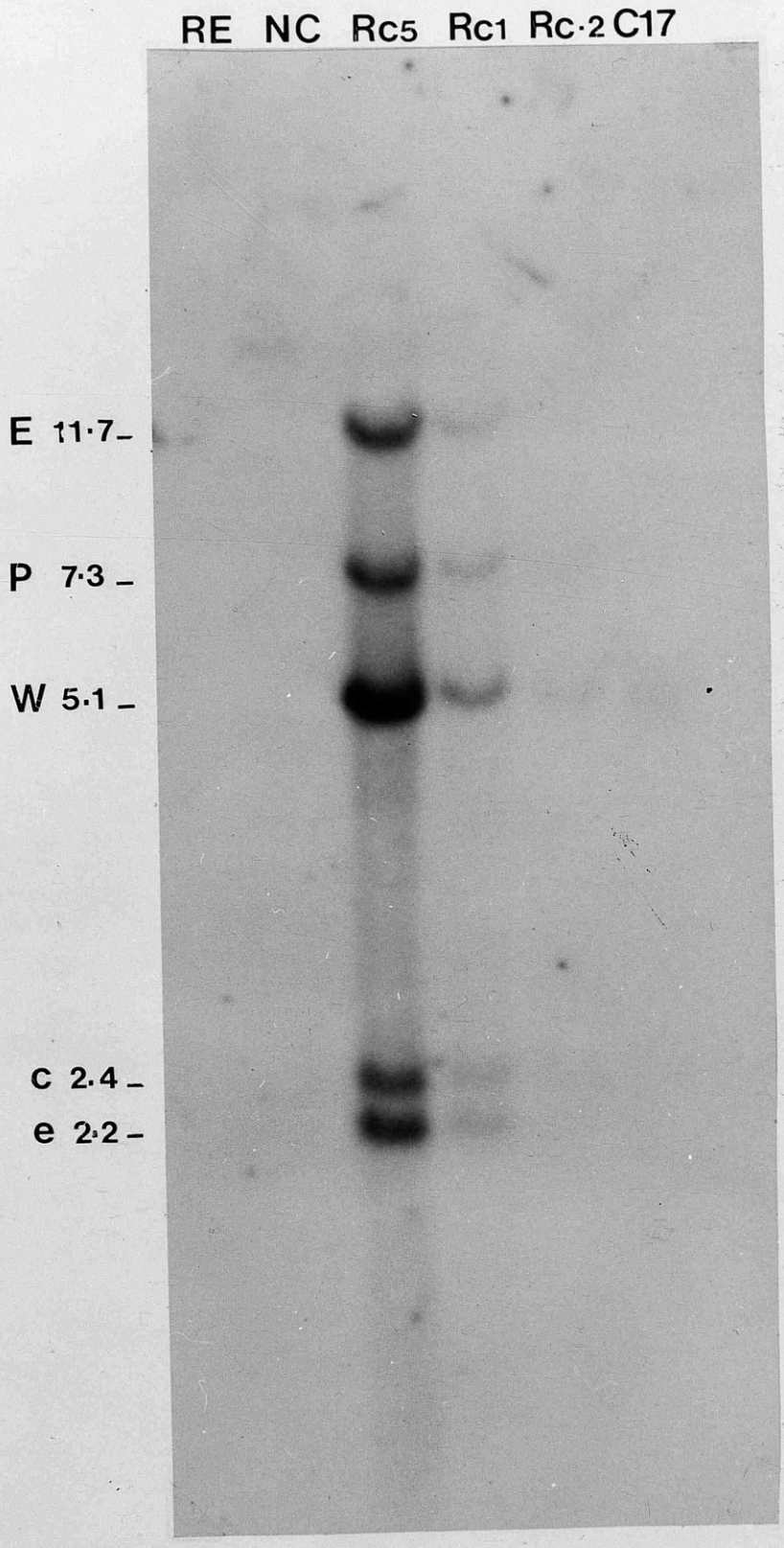

RE
NC
Rc5
Rc1
Rc·2
C17
E 11·7
P 7·3
W 5·1
c 2·4
e 2·2

FIGURE B.4 Hybridization of the HCMV HindIII E probe to a Southern transfer containing C17 DNA

Autoradiograph of a Southern transfer containing normal cervix (NC), rat embryo (RE) and C17 DNA (20ug/track) digested with BamHI and hybridized to the purified HCMV AD169 HindIII E fragment. Reconstructions Rc.2, Rc1 and Rc5 were prepared from 20ug of RE DNA plus 0.2 copies/cell (0.15ng), 1 copy/cell (0.74ng) and ~5 copies/cell (3.7ng), respectively, of HCMV AD169 DNA and digested together with BamHI.

The HindIII E probe hybridized to 5 BamHI fragments E, P, W, c and e in the reconstruction tracks and very weakly to a DNA fragment in C17 that comigrated with the BamHI W fragment of HCMV AD169 (labelled with a black dot on the photograph).

the genome would have been represented in equimolar amounts in the sample. Furthermore, antibodies to HCMV could not be detected in the sera of the patient from whom the C2 biopsy had been taken (Table B.1). It would thus seem unlikely that hybridization to the HCMV AD169 HindIII E fragment represented a current infection of the C2 cervical biopsy with HCMV.

Figure B.3 shows a 9 day exposure autoradiograph of a Southern transfer containing HindIII digested C17 DNA hybridized to the EHB3 probe. The DNA insert in the EHB3 probe had been purified from vector sequences and hybridized to one HindIII fragment of C17 DNA. The hybridizable DNA sequences in C17 were present at less than 0.1 copy/cell by comparison with the reconstruction tracks and were only visible after a 9 day exposure. The C17 DNA was further analysed by BamHI digestion and hybridized to the purified HindIII E fragment. The probe hybridized very weakly to a band colinear with the BamHI W fragment of HCMV AD169, indicating that the C17 DNA does share restriction sites with AD169 (Figure B.4). Either BamHI digestion of C17 DNA cleaved the hybridizable DNA into fragments too small and present in too low copy number to be detected with the HindIII E probe, or else not all the fragments present in virion DNA were equally represented in the viral insert in C17.

The hybridizable DNA in C17 was present in too low copy number to permit further analysis of sequence arrangement.

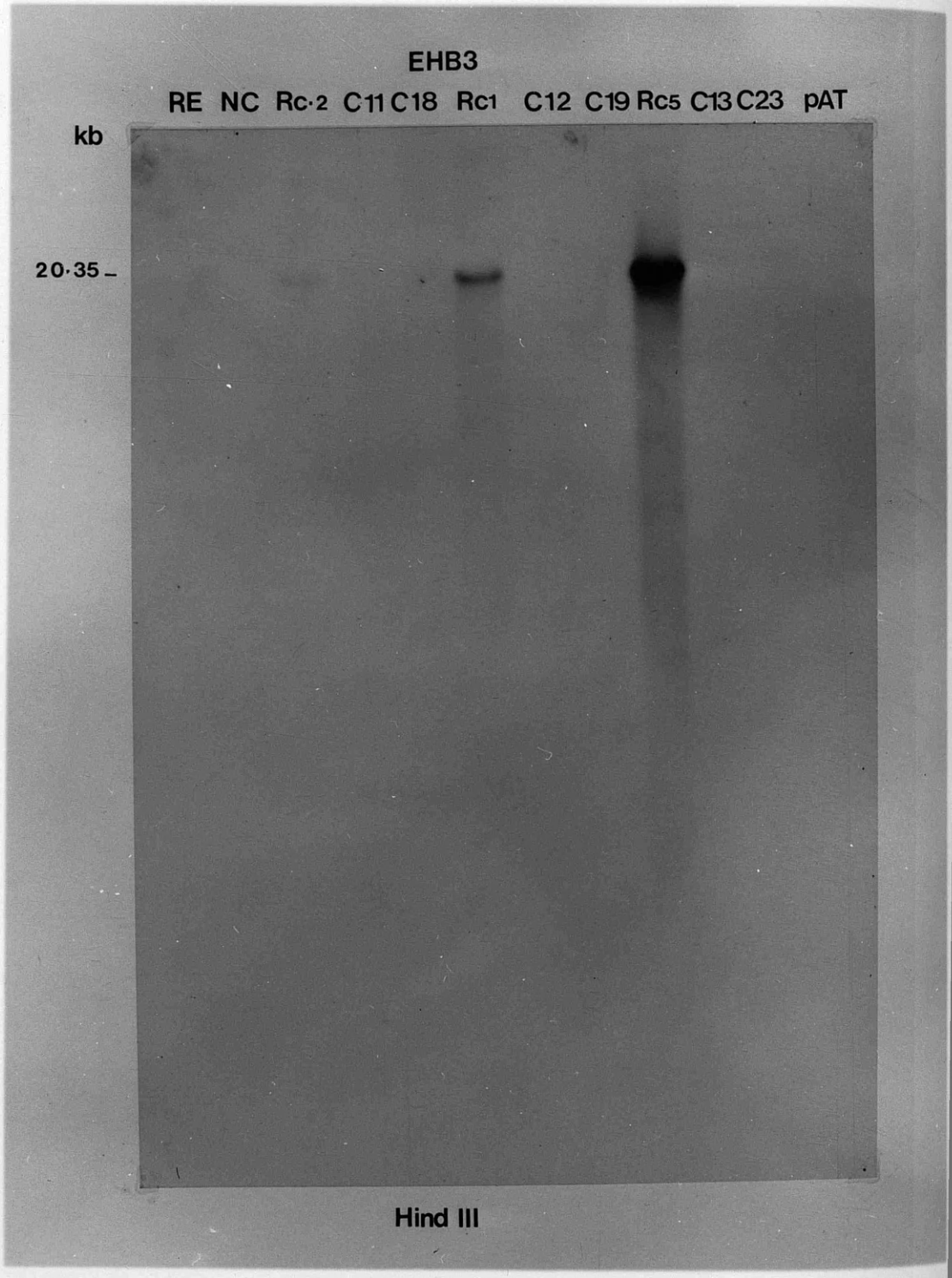

EHB3
RE NC Rc·2 C11 C18 Rc1 C12 C19 Rc5 C13 C23 pAT
kb
20·35
Hind III

FIGURE B.5 Hybridization of the HCMV EHB3 probe to a Southern transfer containing CIN DNAs

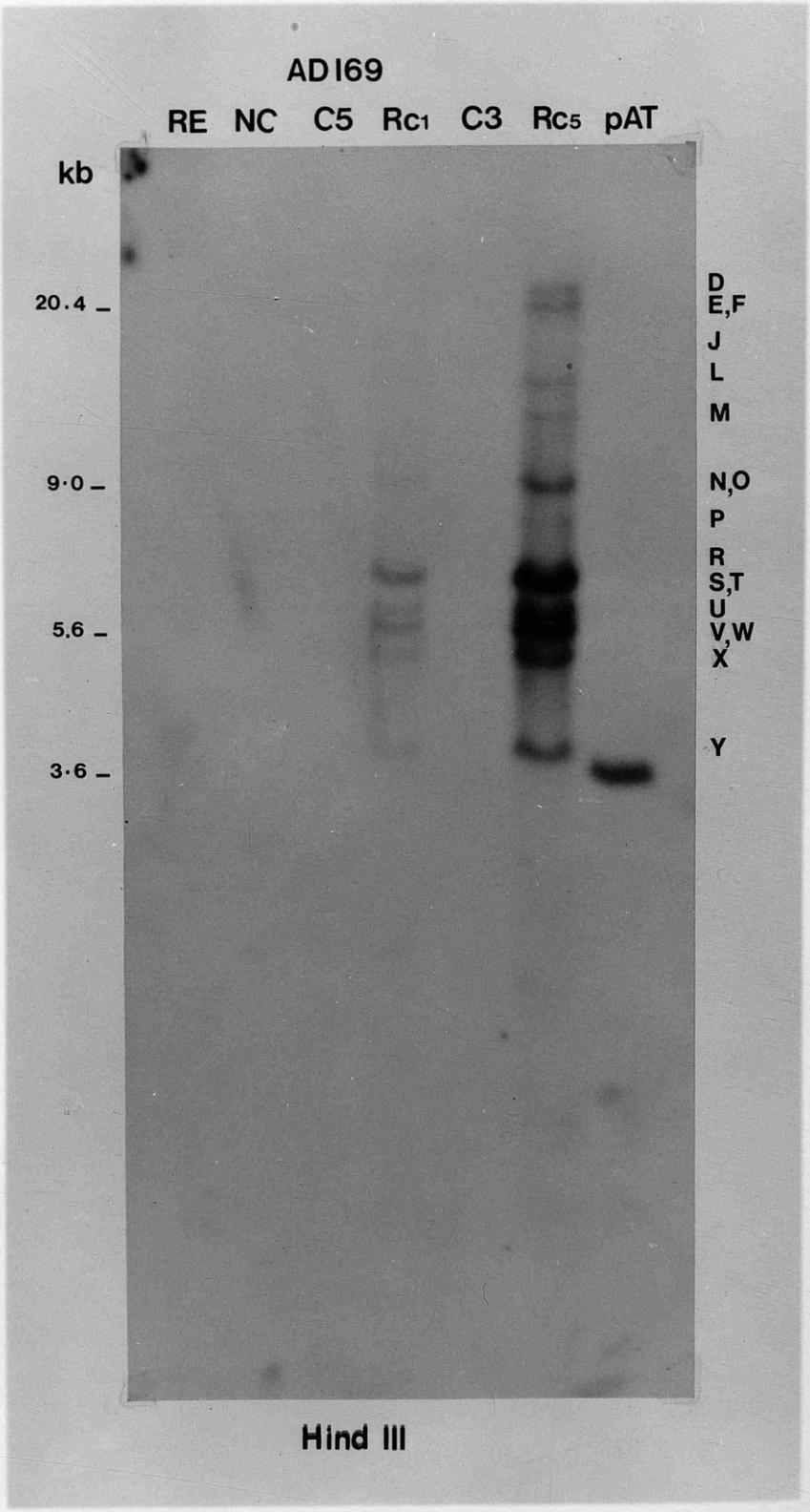
Autoradiograph of a Southern transfer containing normal cervix (NC), rat embryo (RE) and CIN DNAs 11, 12, 13, 18, 19 and 23 (20ug/track) digested with HindIII and hybridized to the HCMV AD169 EHB3 probe. Reconstructions Rc.2, Rc1 and Rc5 were prepared from 20ug of RE DNA plus 0.2 copies/cell (0.15ng), 1 copy/cell (0.74ng) and 5 copies/cell (3.7ng) respectively, of HCMV AD169 DNA and digested together with HindIII. pAT represents a control reconstruction of 20ug of HindIII digested RE DNA plus 50pg of vector DNA.

This blot is representative of one that did not show any hybridization of CIN DNA to HCMV sequences.

FIGURE B.6 Hybridization of the HCMV AD169 composite probe to a Southern transfer containing CIN DNAs

Autoradiograph of a Southern transfer containing normal cervix (NC), rat embryo (RE) and CIN DNAs C5 and C3 (20ug/track) digested with HindIII and hybridized to the HCMV AD169 composite probe. Reconstructions Rc1 and Rc5 were prepared from 20ug of RE DNA plus 1 copy/cell (0.74ng) and 5 copies/cell (3.7ng) respectively, of HCMV AD169 DNA and digested together with HindIII. pAT represents a control reconstruction of 20ug of HindIII digested RE DNA plus 50pg of vector DNA.

This blot is representative of one that did not show any hybridization of CIN DNA to HCMV sequences.


AD169
RE
NC
C5
Rc1
C3
Rc5
pAT
kb
20.4
9·0
5.6
3·6
D
E,F
J
L
M
N,O
P
R
S,T
U
V,W
X
Y
Hind III

However, it was attempted to clone the hybridizable DNA sequences in C2 in lambda vector EMBL3 to eventually characterize the viral insert. These experiments are described in Section C.

Hybridization of HPV11 and HSV-2 DNA probes to CIN DNA

The Southern transfers prepared of the CIN DNAs were rehybridized with the HPV11 DNA and HSV-2 DNA Bam HI probes. The blots were washed prior to rehybridization and autoradiographed to ensure that the previous probe had been completely removed. Reconstructions for the hybridizations were prepared from 20ug of RE DNA plus 1 copy/cell (0.5ng) and 5 copies/cell (125pg) of HPV11 DNA or 1 copy/cell (0.5ng) and 5 copies/cell (2.5ng) of HSV-2 HG52 DNA and were digested together with HindIII. The fragments were separated on 0.6% agarose gels and transferred to Gene screen plus membranes as described in Methods. Small strips of membrane containing the appropriate reconstructions were hybridized in the same polythene bag as the Southern transfer containing the CIN DNA.

Hybridization was obtained with the HPV11 DNA probe to the following CIN DNAs; C8, C13, C16, C17, C18, C19, C20 and C40 (Figures B.7 and B.8). When the blots were later hybridized to the HSV-2 Bam HI probe, hybridization was obtained to CIN DNAs C16, C17, C18, C19 and C20, (Figure B.9). On aligning the autoradiographs obtained from the blots concerned, it was noticed that the HPV11 and HSV-2 Bam HI

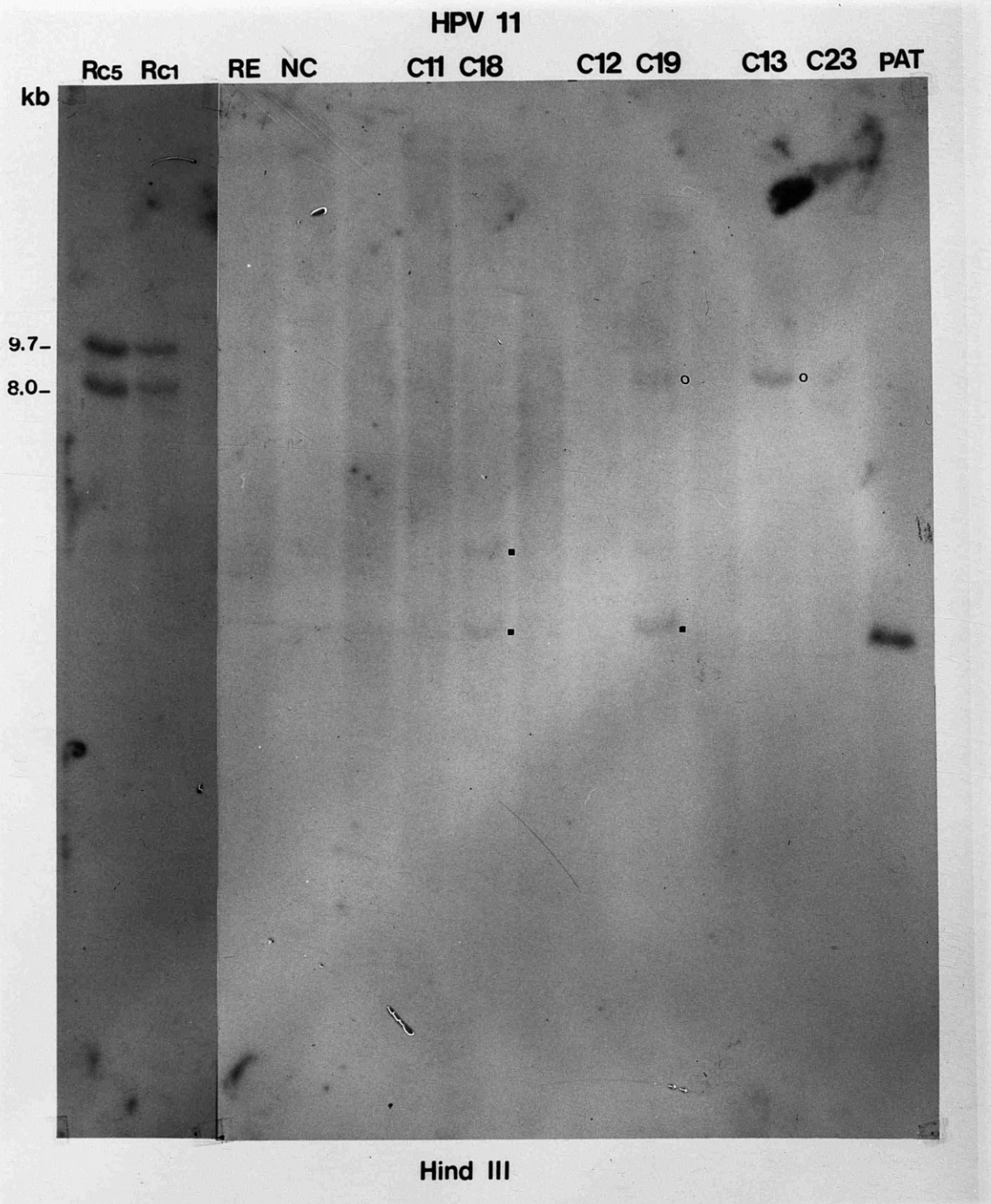

HPV 11
Rc5
Rc1
RE
NC
C11
C18
C12
C19
C13
C23
PAT
kb
9.7
8.0
Hind III

FIGURE B.7 Hybridization of the HPV11 probe to a Southern transfer containing CIN DNAs

Autoradiograph of a Southern transfer containing normal cervix (NC), rat embryo (RE) and CIN DNAs C11, C12, C13, C18, C19 and C23 (20ug/track) digested with HindIII and hybridized to the HPV11 probe. pAT represents a control reconstruction of 20ug of HindIII digested RE DNA plus 50pg of vector DNA. Unlabelled tracks represent the reconstructions containing HCMV AD169 DNA that were transferred along with the CIN DNAs. The reconstructions with HPV11 DNA were transferred separately onto strips of Gene Screen Plus membrane and hybridized to the probe together with the Southern transfer containing the CIN DNAs. Reconstructions Rc1 and Rc5 were prepared from 20ug of RE DNA plus 1 copy/cell (25pg) and 5 copies/cell (125pg) respectively, of the cloned HPV11 DNA.

The HPV11 probe consisted of HPV11 DNA and 69% BPV-1 DNA sequences fused to pBR322. A HindIII digest of this recombinant plasmid gives rise to two fragments, one of 9.7kb which represents the BPV-1 and pBR322 sequences, and the other of 8kb which represents HPV11 DNA. The HPV11 probe hybridized to both the 9.7 and 8kb fragments in the reconstruction tracks and to DNA fragments of about 8, 5 and 3.6kb in CIN DNAs C13, C18 and C19. The 5 and 3.6kb fragments in CINs C18 and C19 (marked with black dots) were also found to hybridize to the HSV-2 Bam$\overset{HI}{\wedge}$t probe (not shown) and probably represent sequences homologous to pBR322 DNA. The 8kb fragments in C13 and C19 (marked with open circles) represent authentic HPV11 DNA of unit length.

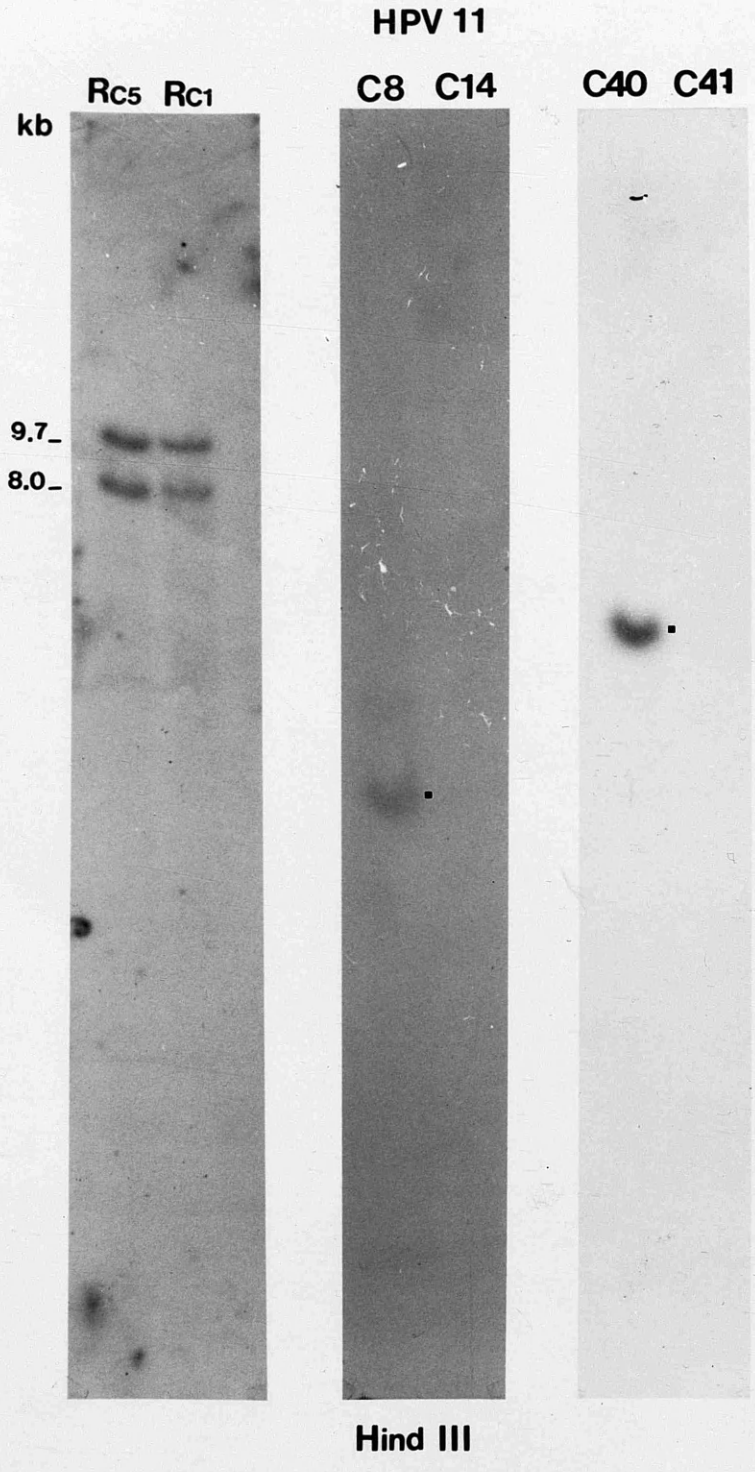
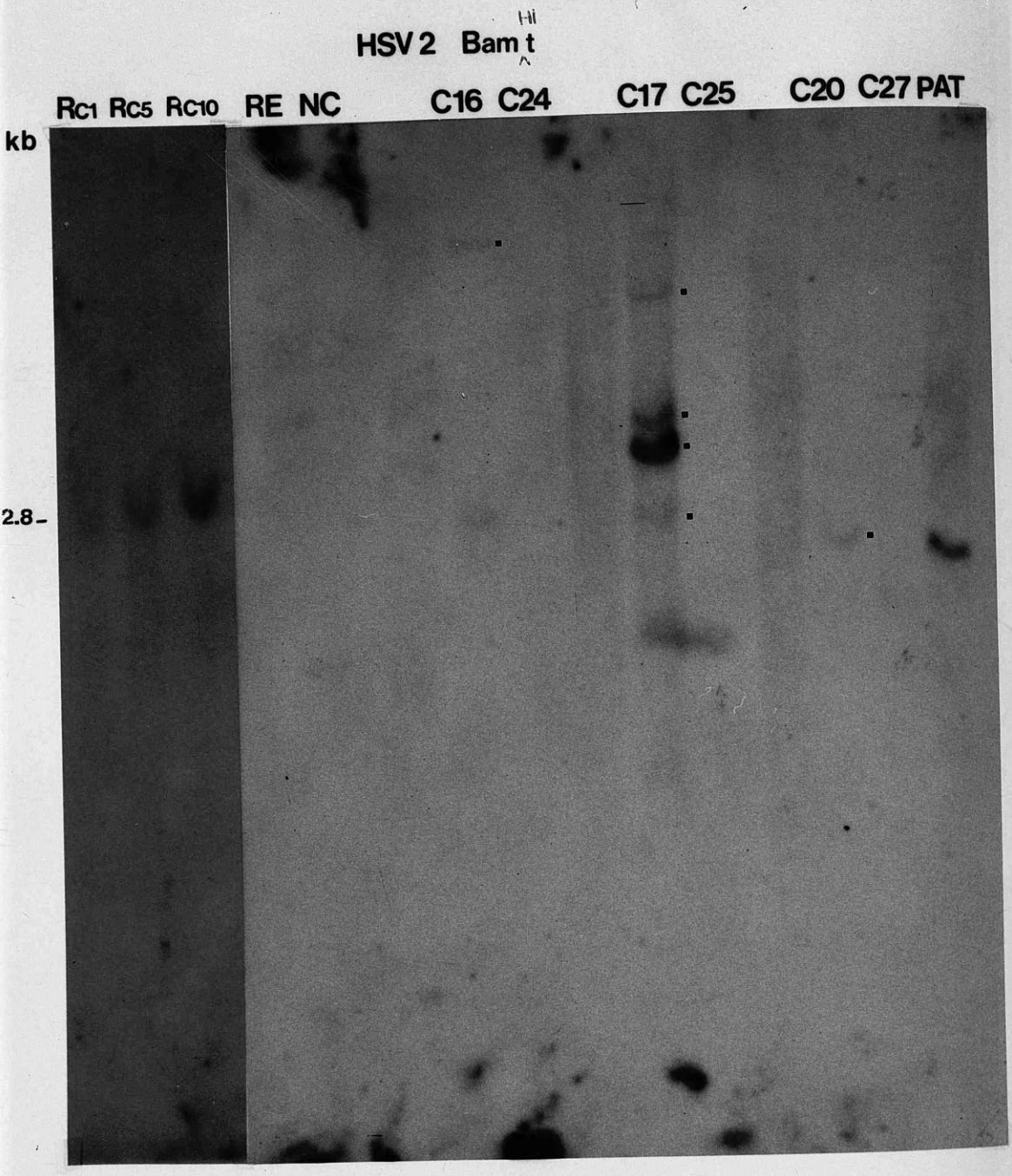

HPV 11
Rc5 Rc1
C8 C14
C40 C41
kb
9.7
8.0
Hind III

FIGURE B.8 Hybridization of the HPV11 DNA probe to Southern transfers of CIN DNAs C8, C14, C40 and C41

Autoradiographs of Southern transfers containing CIN DNAs C8, C14, C40 and C41 (20ug/track) digested with HindIII and hybridized to the HPV11 DNA probe. Reconstructions Rc1 and Rc5 were prepared from 20ug of RE DNA plus 1 copy/cell (25pg) and 5 copies/cell (125pg) respectively, of HPV11 DNA and digested together with HindIII.

In the reconstruction tracks, the HPV11 probe, hybridized to the 9.7kb fragment (representative of the BPV-1 and pBR322 DNA sequences) and the 8kb fragment (representative of HPV11 DNA). The HPV11 probe also hybridized to two fragments of about 6 and 4kb in CIN DNAs C40 and C8, respectively. It is unlikely that these fragments represent authentic HPV11 DNA sequences as where HPV11 DNA has been detected in CIN tissue, it has been found to be of 8kb unit length. These fragments therefore probably hybridized to vector sequences in the probe.



Hind III

FIGURE B.9 Hybridization of the HSV-2 Bam$\hat{H}$I probe to a Southern transfer containing CIN DNAs

Autoradiograph of a Southern transfer containing normal cervix (NC), rat embryo (RE) DNA and CIN DNAs C16, C17, C24, C25, and C27(20ug/track) digested with HindIII and hybridized to the HSV-2 Bam$\hat{H}$I probe. pAT represents a control reconstruction of 20ug of HindIII digested RE DNA plus 50pg of vector DNA. Unlabelled tracks represent the reconstructions containing HCMV AD169 DNA that were transferred along with the CIN DNAs. The reconstructions with HSV-2 DNA were transferred separately onto strips of Gene Screen Plus membrane and hybridized to the probe together with the Southern transfer of the CIN DNAs. Reconstructions Rc1, Rc5 and Rc10 were prepared from 20ug of RE DNA plus 1 copy/cell (0.5ng), 5 copies/cell (2.5ng) and 10 copies/cell (5ng) respectively, of HSV-2 HG52 DNA digested together with BamHI.

The HSV-2 Bam$\hat{H}$I probe hybridized to DNA sequences present in C16, C17 and C20 (marked with black dots on the photograph) and to a 2.8kb fragment in the reconstruction tracks. Since the same sequences in C16, C17 and C20 also hybridized to the HPV11 DNA probe (not shown) and both probes contained pBR322 sequences, it was concluded that the hybridizable sequences in these CINs were homologous to pBR322 DNA and not to HSV-2 DNA.

t probes had hybridized to identical fragments in CIN DNAs C16, C17, C18, C20 and to one band of about 3.6kb in C19. This was known not to have resulted from residual probe from the first hybridization because the blots had been checked by autoradiography prior to rehybridization. The only explanation for the identical hybridization results was that the CIN DNAs had hybridized to vector sequences present in the probes. Both probes contained pBR322 sequences. The possible nature of this hybridization has already been discussed. The HPV11 probe hybridized to two 8kb fragments, one in C13 and the other in C19, that were not detected using the HSV Bamt probe. These probably represent authentic HPV sequences. They could represent either HPV11 or HPV6 DNA, since HPV11 DNA shares approximately 82% homology with HPV6 DNA (Dartmann et al. submitted for publication). An HPV11 DNA probe could therefore detect HPV6 sequences under hybridization conditions of high stringency. There was no evidence from the hybridization reactions that the hybridization obtained with the HPV11 DNA probe to CIN DNAs C8 and C40 could be attributed to pBR322 sequences. However, HPV sequences detected in CIN and carcinoma tissue and in cell lines derived from cervical carcinomas have been reported to be of around unit 8 kb length, and the sequences detected in C8 and C40 appear to be of a much lower MW. This raises the possibility that these CINs may after all contain sequences that hybridize to pBR322 sequences. The result of these hybridizations

highlight the problems of plasmid vector sequences hybridizing to human cellular DNAs and emphasize the caution necessary in interpreting hybridization results and the necessity to use multiple digests to verify authentic viral sequences within cellular DNAs.

No specific hybridization to HSV-2 DNA was obtained with any of the CIN DNAs. There could be two explanations for the lack of detection of HSV-2 and very low level of detection of HPV11 specific DNA sequences in CIN DNAs. Firstly, by this stage the membranes had already been probed at least three times. The washing procedure prior to each rehybridization may have removed some of the CIN DNA. If viral sequences were present in the CIN DNAs at very low copy numbers there may have been insufficient CIN DNA remaining on the blots after washing for the viral sequences to be detected. This would not have affected hybridization to the reconstructions because they had been transferred on separate strips of nylon membrane. Secondly, the results may be genuine and HPV11 and HSV-2 DNA sequences are not present in the majority of CIN samples analysed. This does not necessarily mean that the viruses do not play some role in the development of cervical neoplastic disease as will be discussed later.

SECTION C

CLONING OF C2 DNA SEQUENCES IN BACTERIOPHAGE LAMBDA

Southern blot analysis of the DNA from CIN biopsy C2 revealed the presence of sequences that hybridized to the HindIII E fragment of HCMV AD169. These sequences were present at about 20 copies/cell. In addition, C2 DNA also contained sequences that hybridized to pAT153. As only a small amount of DNA was available from this CIN biopsy, further analysis of these hybridizable sequences could only be accomplished if the sequences were successfully cloned. This section describes the strategy used for cloning the C2 DNA in bacteriophage lambda.

Choice of cloning vector and preparation of C2 DNA for cloning

The fragment of C2 DNA that hybridized to HCMV was about 20kb, so a vector capable of accepting a fragment of this size was required. EMBL3 was chosen as a suitable cloning vector for the C2 DNA. This is a lambda replacement vector with a nominal cloning capacity of approximately 9 to 23kb (77 to 105% of wild type lambda). EMBL3 also carries the polylinker sequences SalI, BamHI, EcoRI replacing the BamHI sites carried by lambda 1059 (Frischauf et al., 1983). Foreign DNA sequences can be cloned into the BamHI site and the entire DNA insert excised from the vector by digestion

with SalI. The efficiency of cloning is sufficiently high to allow the recovery of essentially complete libraries of mammalian genomes from microgram amounts of DNA and rapid high screening techniques make this lambda replacement vector a very convenient system to use.

The C2 DNA was partially digested with Sau3A enzyme to generate fragments of 15-20kb for cloning into the BamHI site of EMBL3. Sau3A was used to partially digest the high MW DNA because it cleaves the DNA more frequently than BamHI. Sau3A is an isoschizomer of BamHI and recognises the tetranucleotide sequence GATC. BamHI recognises the hexanucleotide sequence GGATCC. Assuming that restriction endonuclease sites are distributed randomly along DNA and that the DNA has a G+C content of 50% the tetranucleotide target for Sau3A will occur on the average once every 4^4 (ie 256) nucleotides, whereas the hexanucleotide target for BamHI will occur once every 4^6 (ie 4096) nucleotides. Since Sau3A is an isoschizomer of BamHI the fragments generated by partial digestion of eukaryotic DNA with Sau3A can still be cloned into the BamHI site of EMBL3. Microgram aliquots of C2 DNA were digested with varying amounts of Sau3A and analysed by electrophoresis using a series of λ DNA fragments of known MW as markers. By comparison of the intensities of fluorescence, it was found that 0.094u of Sau3A per ug of C2 DNA were required to produce the maximum number of DNA molecules of size 15-20kb in 30min at 37°C.

The C2 DNA fragments were treated with phosphatase as described in

Methods. This procedure removed the 5' terminal phosphate groups so preventing reannealing and the formation of dimers which would be too large to be packaged into the lambda phage heads.

The lambda library of C2 DNA

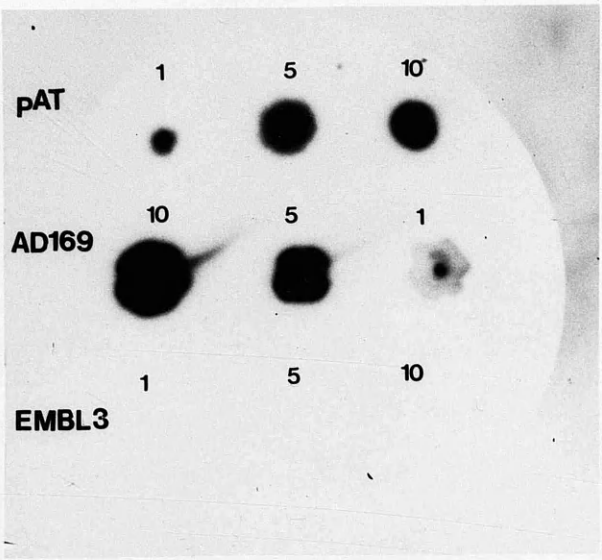
After ligation of the C2 DNA fragments to the EMBL3 arms and packaging of the ligated DNA, the recombinant phage were titrated on Q358 and Q359 host bacteria. Approximately $1.4x10^7$ plaques per ug of EMBL3 arms were obtained on Q358 bacteria and approximately $9x10^4$ plaques per ug EMBL3 arms were obtained on Q359 bacteria. Since only recombinant phage containing a C2 DNA insert could grow on Q359 bacteria, $9x10^4$ represents the number of recombinant phage in the lambda library. The plaques obtained on Q359 bacteria were very tiny and quite difficult to see. Plaque formation also took longer than 8hr, the majority of plaques were not visible in under 16hr. The plaques formed on Q358 bacteria were much larger.

To detect one copy/cell of a specific fragment of DNA it is necessary to screen approximately $1.5x10^6$ plaques. Since the C2 DNA fragment that hybridized to HCMV was present at 20 copies/cell, it was theoretically possible to identify this fragment in a library of only $7.5x10^4$ plaques. The number of recombinant phage for screening could have been increased by amplifying the library. However, it was decided not to amplify because most of the recombinant phage

grew poorly and might have been heavily underrepresented in the amplified library. Such recombinants, even those that form minute plaques, can sometimes be isolated if the amplification step is omitted.

Screening recombinant phage

The entire lambda library was plated out on Q359 bacteria in ten 90mm petri dishes to give approximately 9000 plaques per dish. Impressions were taken as described in Methods and the filters hybridized to the HCMV AD169 HindIII E probe labelled to a specific activity of $1.5x10^8$cpm/ug DNA. The pAT153 vector sequences had not been removed from this probe. It was hoped by using this probe to isolate the DNA sequences present in C2 that hybridize to pAT153 as well as those that hybridize to the HCMV AD169 HindIII E fragment. The filters were hybridized under conditions of relatively low stringency (5xSSPE, and 0.1% SDS at 65°C) to increase the probability of detecting positive clones. After the first screening the autoradiographs showed the presence of positive clones on one of the ten plates (Figure C.1). The positive clones were clustered in one small area of the plate. The large number of plaques present made it impossible to pick one isolated plaque so three clusters of plaques were picked and labelled 1, 2 and 3 as indicated in Figure C.1. 100ul aliquots of undiluted, and 1:10 and 1:100 dilutions of each of these plaque stocks were plated out on Q359 bacteria. After overnight incubation suitable numbers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10
AD169
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EMBL3
1
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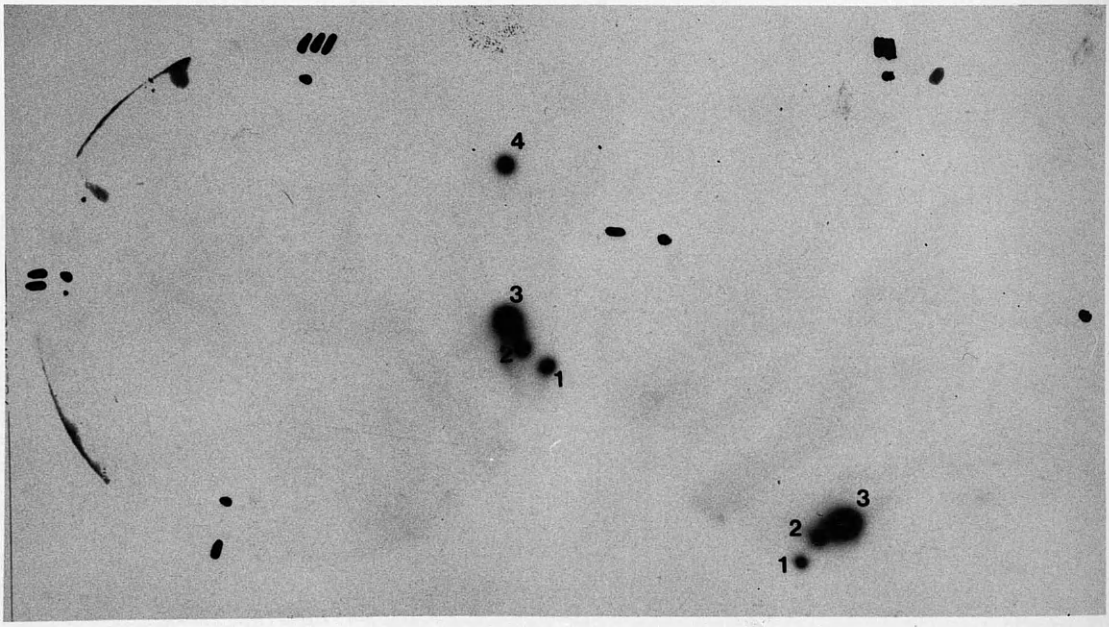

4
3
2
1
3
2
1

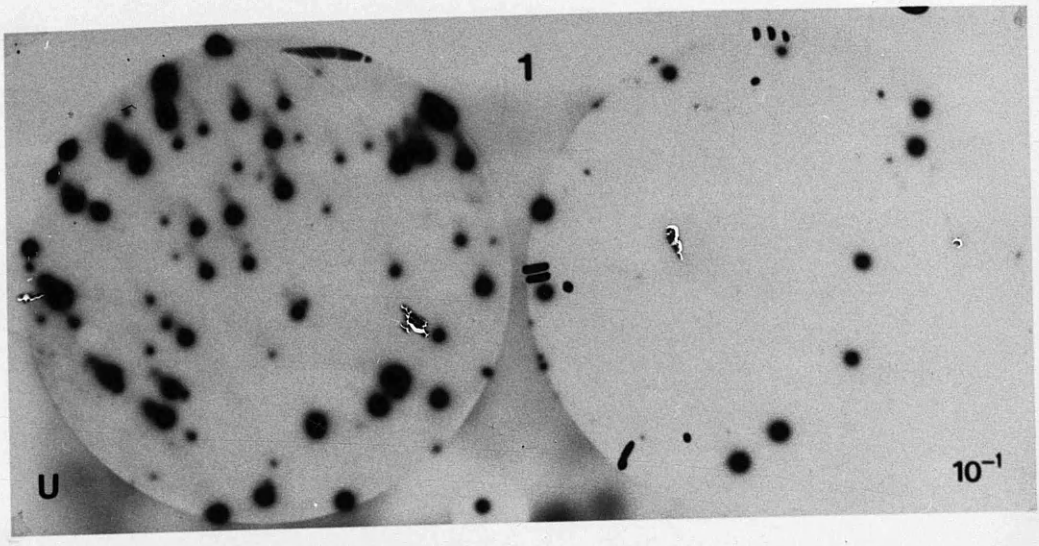
FIGURE C.1 Isolation of positive clones containing C2 DNA sequences that hybridized to the HCMV AD169 HindIII E probe

Top: An autoradiograph of a reconstruction filter hybridized to the HCMV AD169 HindIII E probe which contained pAT vector sequences. Reconstructions include 10, 50 and 100pg of pAT DNA, 0.74, 3.7 and 7.4ng of HCMV DNA and 0.17, 0.85 and 1.7ng of EMBL3 DNA. The amounts of DNA used in the reconstructions correspond to the amounts that would be required for 1, 5 and 10 copies/cell of the DNA with 20ug of cellular DNA.

Bottom: An autoradiograph of duplicate filters from a plate containing plaques which hybridized to the HCMV AD169 HindIII E probe. Each positive plaque was picked and numbered as shown. Plaques 1 and 2 were later found to contain C2 sequences that hybridized to HCMV DNA. Plaque 3 was later shown to contain C2 sequences that hybridized to pAT DNA. Plaque 4 did not hybridize to the HCMV DNA probe in subsequent experiments and since it was only present on one of the filters it must have been a hot spot.

of plaques were obtained on the plates containing the undiluted and 1:10 dilutions. Impressions were taken from these plates and hybridized to the HCMV AD169 HindIII E probe labelled to a specific activity of $1.9x10^8$ cpm/ug DNA. The results are shown in Figure C.2.

Hybridization was obtained to plaques on all of the plates indicating that the positive plaques had been successfully picked. The hybridization to plaque 3 was particularly strong and approximately 80% of the plaques on the plate were positives. The hybridization to plaques 1 and 2 was less marked, still less than 50% of the total number of plaques per plate were positive. A selection of positive plaques was picked from the plates. In this second round of purification, the picked plaques were labelled using the number of the original plaque followed by a letter. These plaque stocks were plated out onto Q359 bacteria. However, relatively few plaques were obtained (about 1000 plaques per single picked plaque) and impressions had to be taken from the plates of undiluted stocks. These were hybridized to an HCMV AD169 HindIII E probe from which the pAT153 sequences had been removed to determine which, if any, of the positive clones contained sequences that hybridized to pAT. The results of the hybridization are presented in Figure C.3. No hybridization was obtained to any of the plaques picked from stock 3 (not shown). Thus, the clones derived from plaque 3 contained C2 DNA sequences that hybridize to pAT153. Since single picked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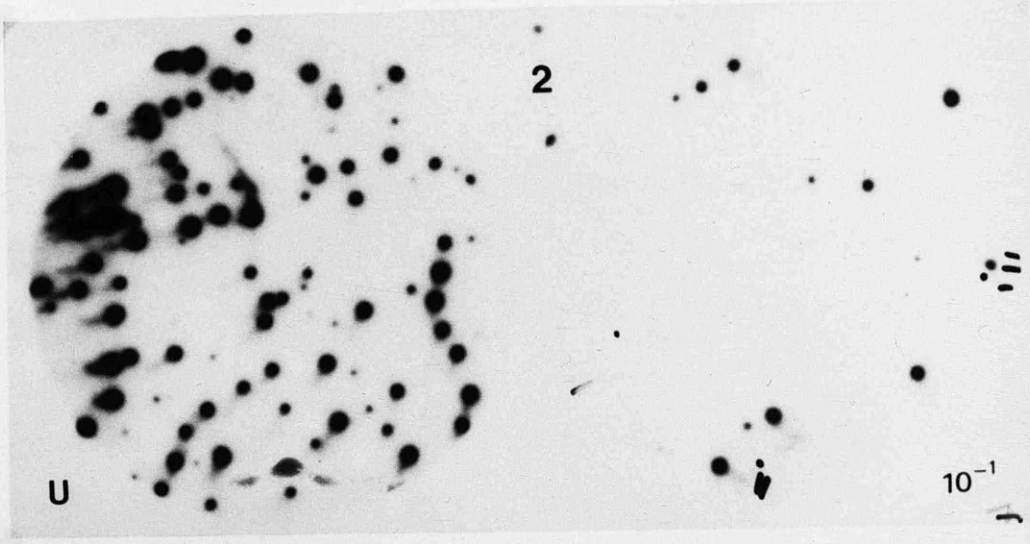
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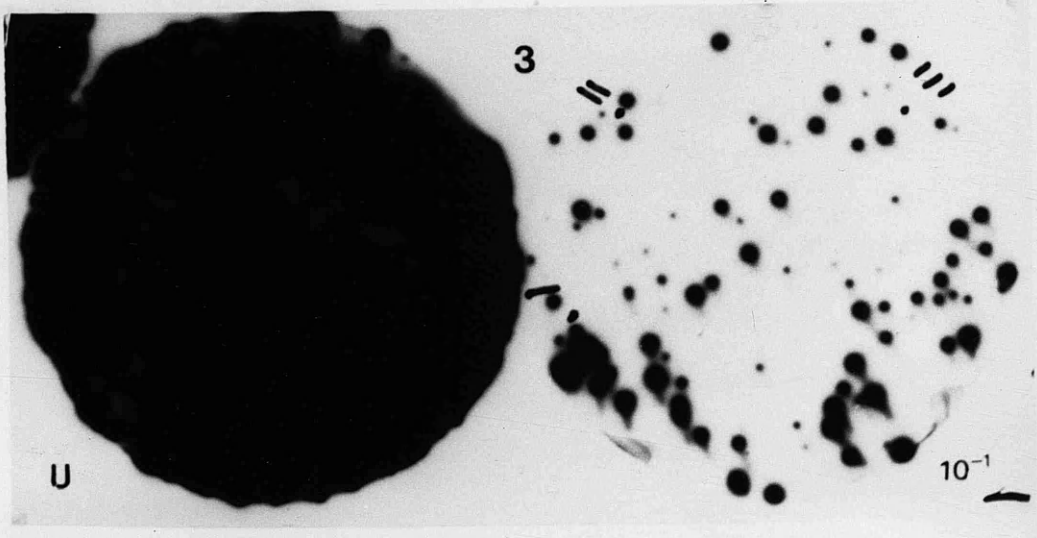
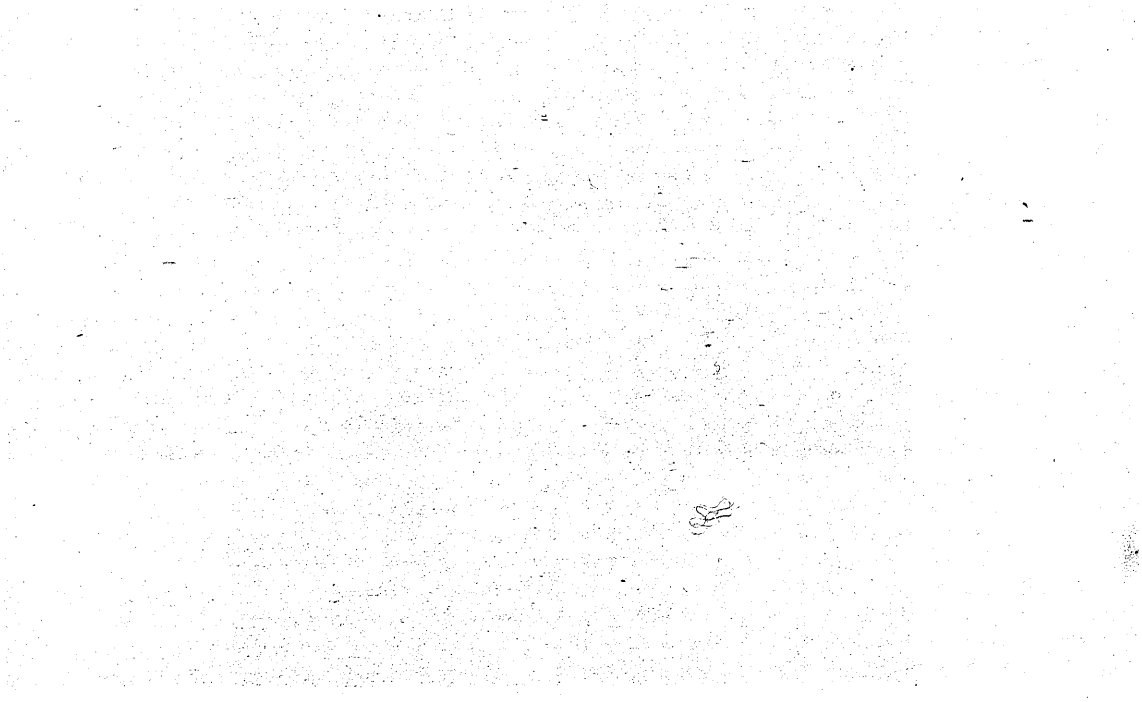
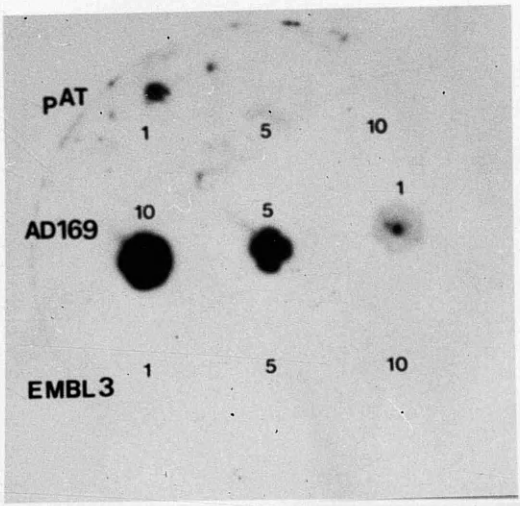

3
U
10^{-1}

FIGURE C.2 Hybridization of positive clones 1, 2 and 3 to the HCMV AD169 HindIII E probe

Plaque stocks were prepared from the positive clones isolated from the library. Aliquots of undiluted (U) and 1:10 diluted (10^{-1}) stocks were plated out on Q359 bacteria. Duplicate filters were prepared from the plates and hybridized to the HCMV AD169 HindIII E probe. The autoradiographs of the filters are shown here. All three stocks contained clones that hybridized to the HCMV AD169 HindIII E probe.

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AD169
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EMBL3
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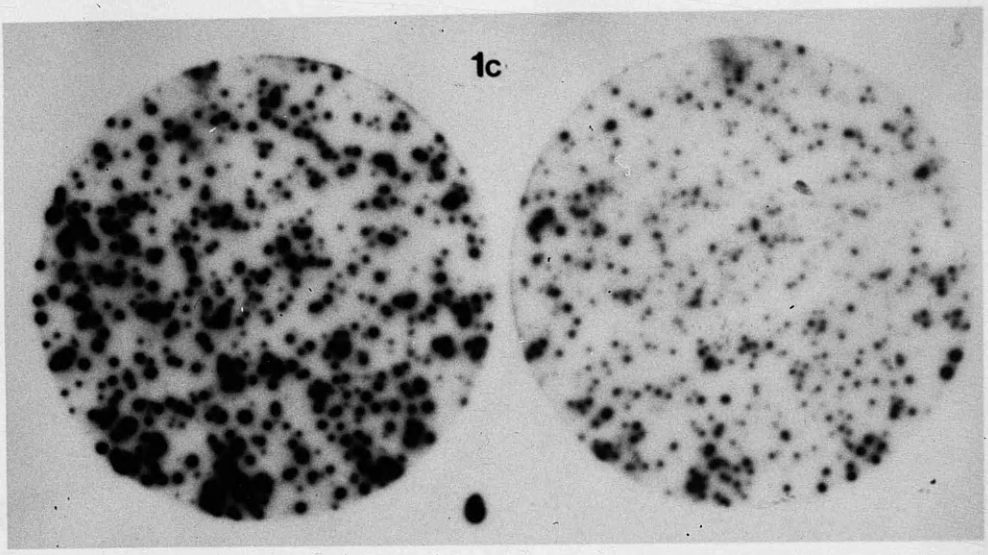
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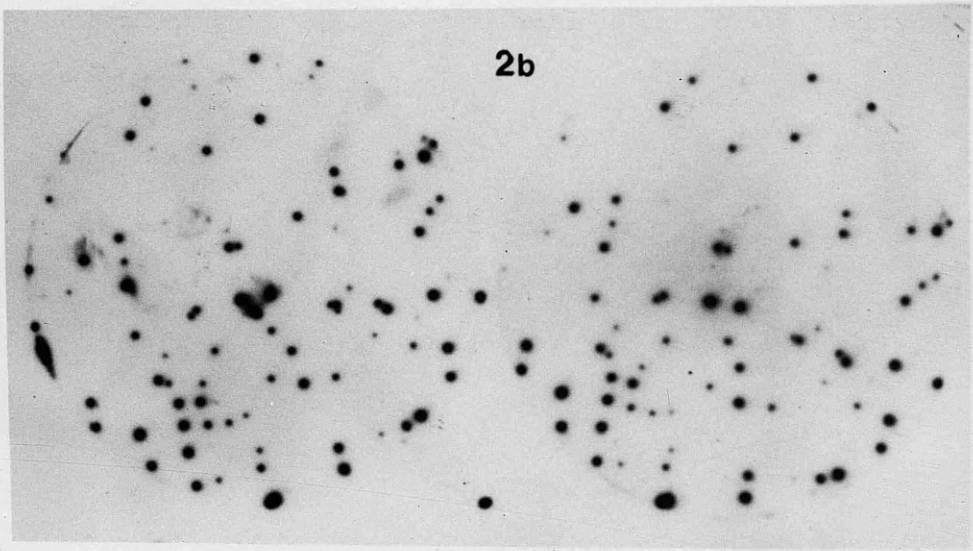
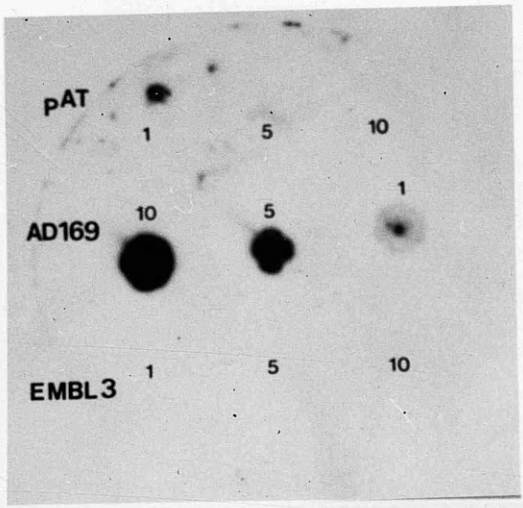

2b

FIGURE C.3 Purification of clones 1 and 2 that contained C2 DNA sequences that hybridized to HCMV

Top: An autoradiograph of a reconstruction filter hybridized to the HCMV AD169 HindIII E fragment (purified from vector sequences). Reconstructions include 10, 50, and 100pg of pAT DNA, 0.74, 3.7 and 7.4ng of HCMV DNA and 0.17, 0.85 and 1.7ng of EMBL3 DNA. The amounts of DNA used in the reconstructions correspond to the amounts that would be required for 1, 5 and 10 copies/cell of the DNA with 20ug of cellular DNA.

Bottom Two: Autoradiograph of duplicate filters prepared from undiluted twice plaque-purifed stocks of clones 1 and 2 and hybridized to the HCMV AD169 HindIII E fragment. At this stage approximately 75% of the plaques present on the plates were found to be positive.


pAT
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AD169
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EMBL3
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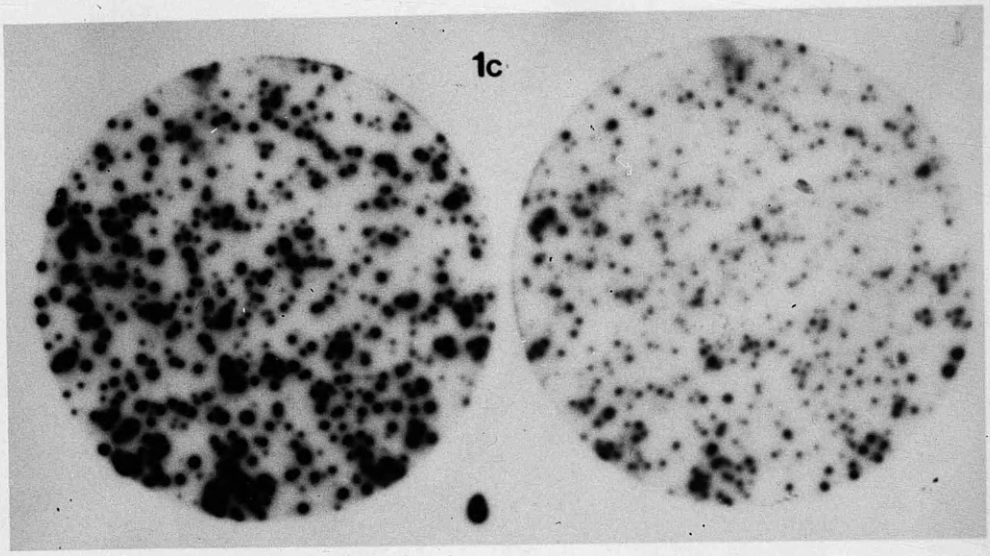
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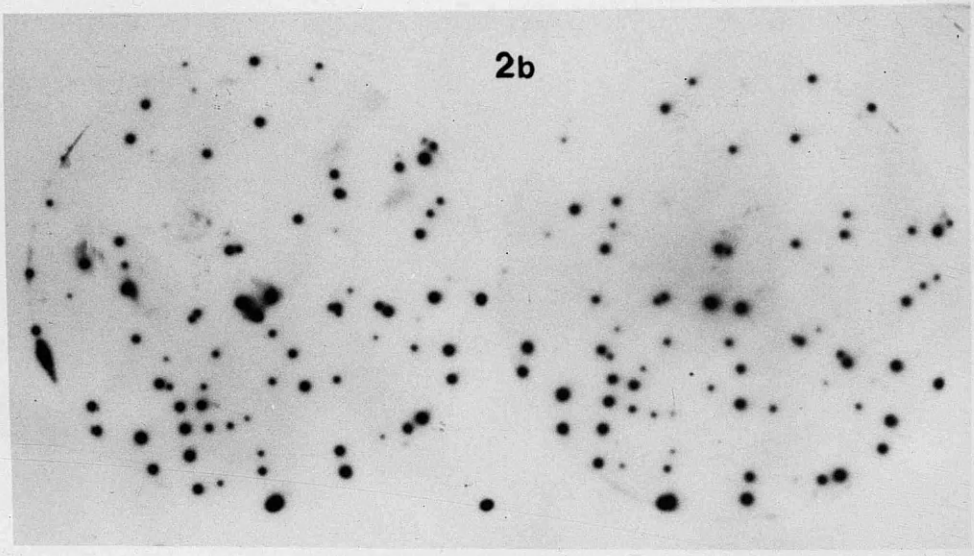

2b

FIGURE C.3 Purification of clones 1 and 2 that contained C2 DNA sequences that hybridized to HCMV

Top: An autoradiograph of a reconstruction filter hybridized to the HCMV AD169 HindIII E fragment (purified from vector sequences). Reconstructions include 10, 50, and 100pg of pAT DNA, 0.74, 3.7 and 7.4ng of HCMV DNA and 0.17, 0.85 and 1.7ng of EMBL3 DNA. The amounts of DNA used in the reconstructions correspond to the amounts that would be required for 1, 5 and 10 copies/cell of the DNA with 20ug of cellular DNA.

Bottom Two: Autoradiograph of duplicate filters prepared from undiluted twice plaque-purifed stocks of clones 1 and 2 and hybridized to the HCMV AD169 HindIII E fragment. At this stage approximately 75% of the plaques present on the plates were found to be positive.

plaques only gave rise to about 1000 plaques, between 10 and 20 positive plaques were picked from the plates to give a single stock. Undiluted aliquots from these plaque stocks were plated out on Q359 bacteria, impressions taken and the filters hybridized to the HCMV AD169 HindIII E fragment. No hybridization was obtained to the plaques purified from the original plaque 2. More positive plaques were picked and impressions taken but again the plaques did not hybridize with the HCMV AD169 HindIII E fragment. Faint hybridization was obtained to plaques picked from 1c. However, the autoradiographs showed a large number of positive spots which could not be aligned with plaques on the plates. These were definitely not hot spots on the filters because they were present in the same place on both the impressions taken from the same plate. The positive clones in these cases must grow poorly and the plaques formed were too small to be seen with the naked eye. This made it very difficult to pick isolated positive plaques. About 20 positive plaques were picked from the plate and a plate lysate stock prepared from these as described in Methods.

Attempts to characterize the DNA sequences in C2 that hybridized to HCMV DNA

The plate lysate stock was used to infect K803 bacteria. Bacteriophage particles were purified and DNA prepared as described in Methods. About 10ug of the phage DNA was digested with an excess of SalI enzyme to excise the

C2 DNA insert from the vector. Small aliquots of the SalI digested phage DNA were digested separately with HindIII, BamHI, EcoRI and PstI. Small aliquots of the HCMV AD169 HindIII E fragment were also digested with the above enzymes (but not SalI) to provide controls. It was hoped that by comparing the restriction profiles of the digested phage DNA with the corresponding restriction profiles of the HCMV AD169 HindIII E fragment, it would be possible to map the hybridizable sequences in C2. The digested DNAs were electrophoresed through a 0.7% agarose gel and examined by UV-illumination. HindIII fragments of λ were used as molecular weight markers. The SalI digest of the phage DNA produced 3 bands, an upper and lower band representing the EMBL3 arms and a middle band representing the cloned C2 DNA insert. The insert appeared to be about 10kb in size. The DNA was then transferred to Gene Screen Plus membrane by the procedure of Southern and hybridized to the HCMV AD169 HindIII E fragment. No hybridization was obtained to the digested phage DNA, although the probe hybridized strongly to the control digested HindIII E DNA. The reason why the isolated recombinant phage did not hybridize is unknown. The most likely explanation is that the cloned C2 DNA sequences that hybridized to HCMV were in some way detrimental to phage growth. They may be analogous to the 'poison sequences' of pBR322. The positive clones certainly grew very poorly as they produced minute plaques and had a very low plaquing efficiency. Possibly when the plaques

were picked to make a plate lysate stock, one or two of the clones may not have been positives and outgrew the clones containing the HCMV insert during the preparation of the phage DNA. The extracted DNA would not then have hybridized to the HCMV AD169 HindIII E fragment.

There is also the possibility that the sequences within the C2 DNA that hybridized to HCMV contained an unstable gene that was subsequently lost from the phage. This would help to explain why the HCMV DNA inserts in both the 1c and 2b clones were lost from the library. It has also been reported that the presence of tandemly repeated sequences in eukaryotic DNA can lead to deletions by recombination during phage propagation (Arnheim and Kuehn, 1979; Fritsch et al., 1980; Lauer et al., 1980). Complete sequence data for the HCMV AD169 HindIII E fragment is not yet available but since the HindIII E fragment has been successfully cloned (Oram et al., 1982) it is unlikely that the fragment contains large tandem repeats that would be deleted during phage propagation. Thus it seems improbable that the homologous C2 sequences could be lost in this manner.

SECTION D

STUDIES ON THE INFECTION OF CERVICAL CELLS BY HERPESVIRUSES

HCMV and HSV-2 are very common infectious agents of the human cervix. HSV-2 produces characteristic cytological changes visible in cervical smears whereas HCMV causes mostly inapparent infections. Although these herpesviruses are important pathogens, there have been very few studies investigating the nature of herpesvirus infections in cervical cells. The experiments described in this section aimed to investigate:

1. the cellular alterations induced by HCMV and HSV-2 infection of cervical cells
2. the production of infectious virus and antigens in cervical cells
3. the ability of the viruses to establish latent infections in cervical cells
4. the possible stimuli capable of reactivating latent virus.

Histology of the normal cervix

The portio surface of the human cervix is covered by a non-keratinizing stratified squamous epithelium known as the ectocervix (Figure D.1A). The inner surface of the cervix surrounding the os is covered by a single layer of columnar epithelial cells known as the endocervix (Figure D.1B). The

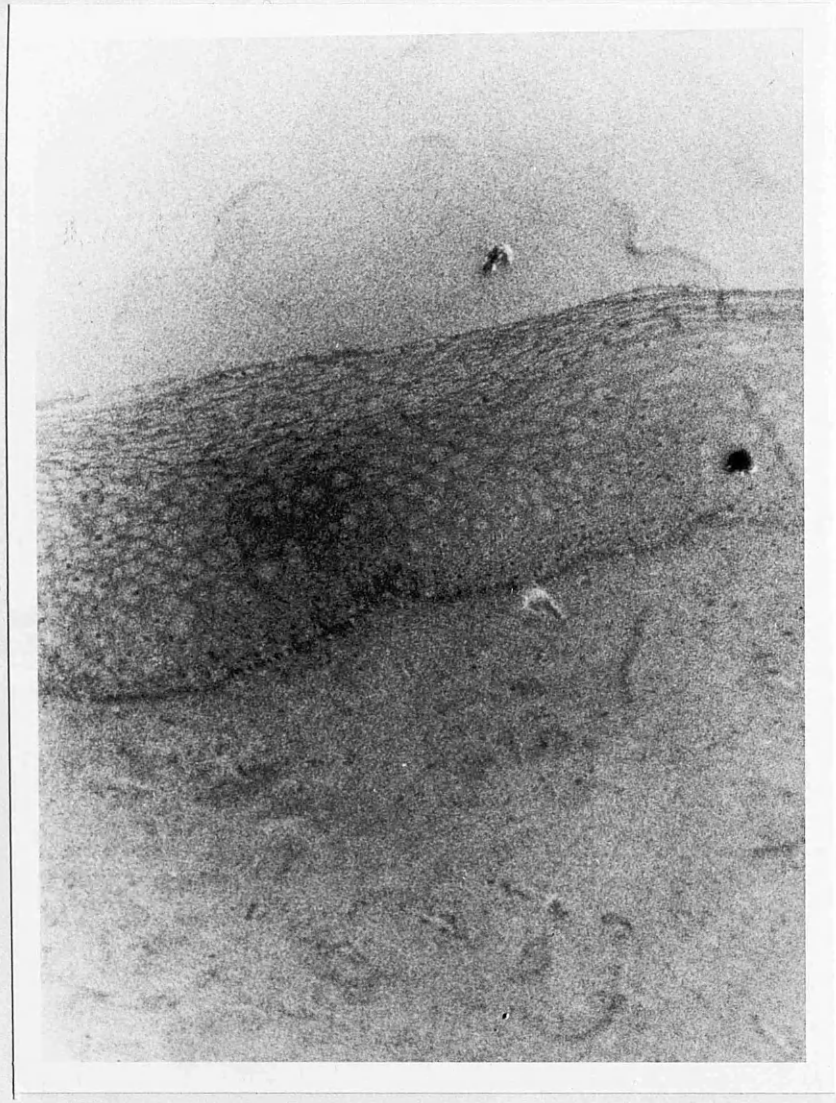
FIGURE D.1 Histology of the normal cervix

A. Section through the human cervix showing the stratified squamous epithelium of the ectocervix.
Magnification x320.

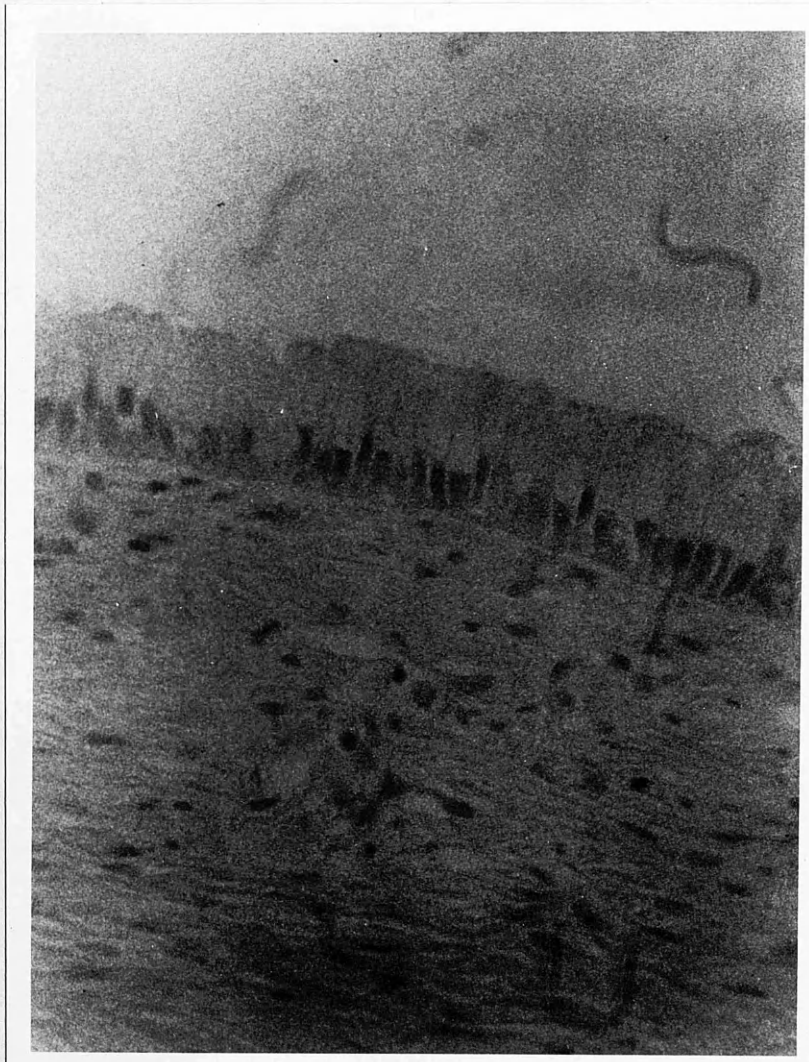
B. Section through the human cervix showing the columnar epithelium of the endocervix.
Magnification x320.

Sections kindly provided by the Pathology Department,
Western Infirmary, Glasgow.

A



B

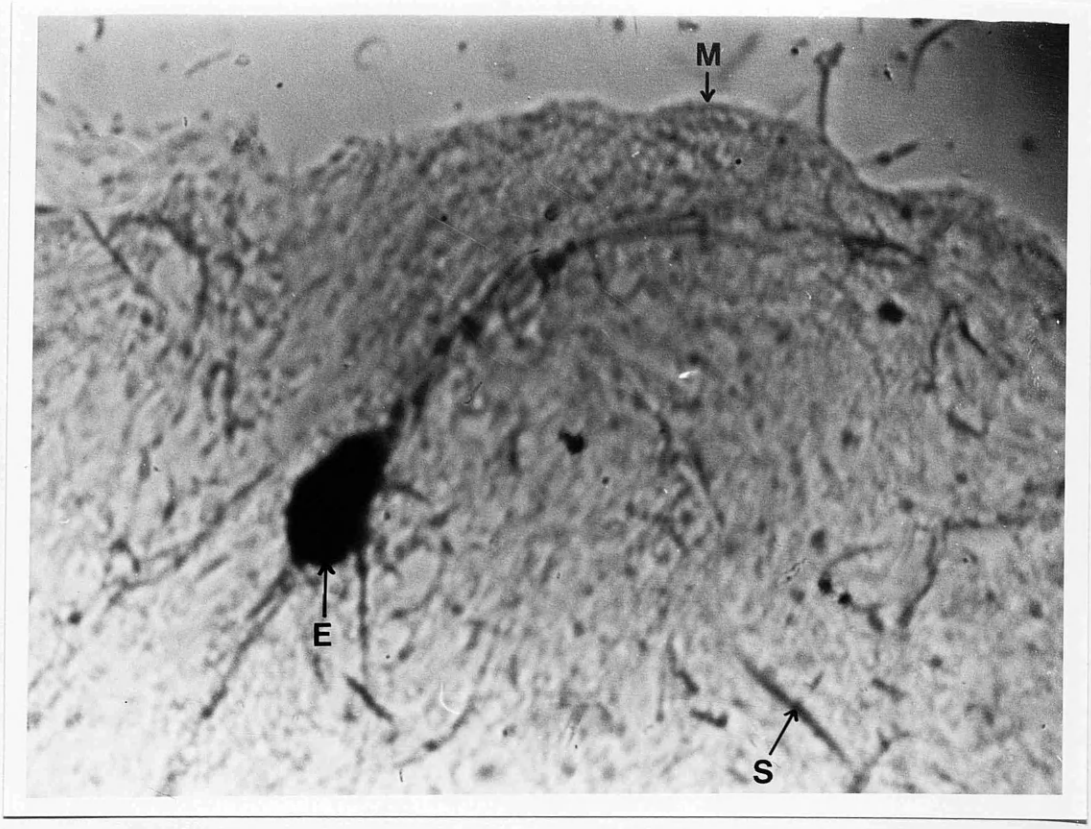


endocervical epithelium forms villi and long ridges with intervening clefts that extend several mm deep from the surface. Endocervical cells are mucous-producing cells and also line glands present in the underlying stroma. Both the ecto- and endocervical cells are exposed to injury and infection and form a protective layer around the cervix.

The ectocervix and endocervix are separated by the squamo-columnar junction. In a young female, the normal squamo-columnar junction is sharp; the columnar epithelium ends and native squamous epithelium begins. However, after puberty, acid secretions from the vagina initiate the process of squamous metaplasia. The endocervical villi fuse producing a new flat squamous epithelium punctuated by gland openings and bordered by native columnar epithelium in the os and native squamous epithelium on the ectocervix. The area over which this occurs is often referred to as the 'transformation zone', and is the area in which CIN usually develops.

Culture of human ecto- and endocervical epithelial cells

In order to investigate viral infection of cervical cells it was necessary to establish a suitable method for the *in vitro* culture of these epithelial cells. As ecto- and endocervical cells may vary in their susceptibility to herpesviruses the two different cell types were cultured separately. It was necessary to exercise great care during the dissection of the cervix to ensure that no tissue was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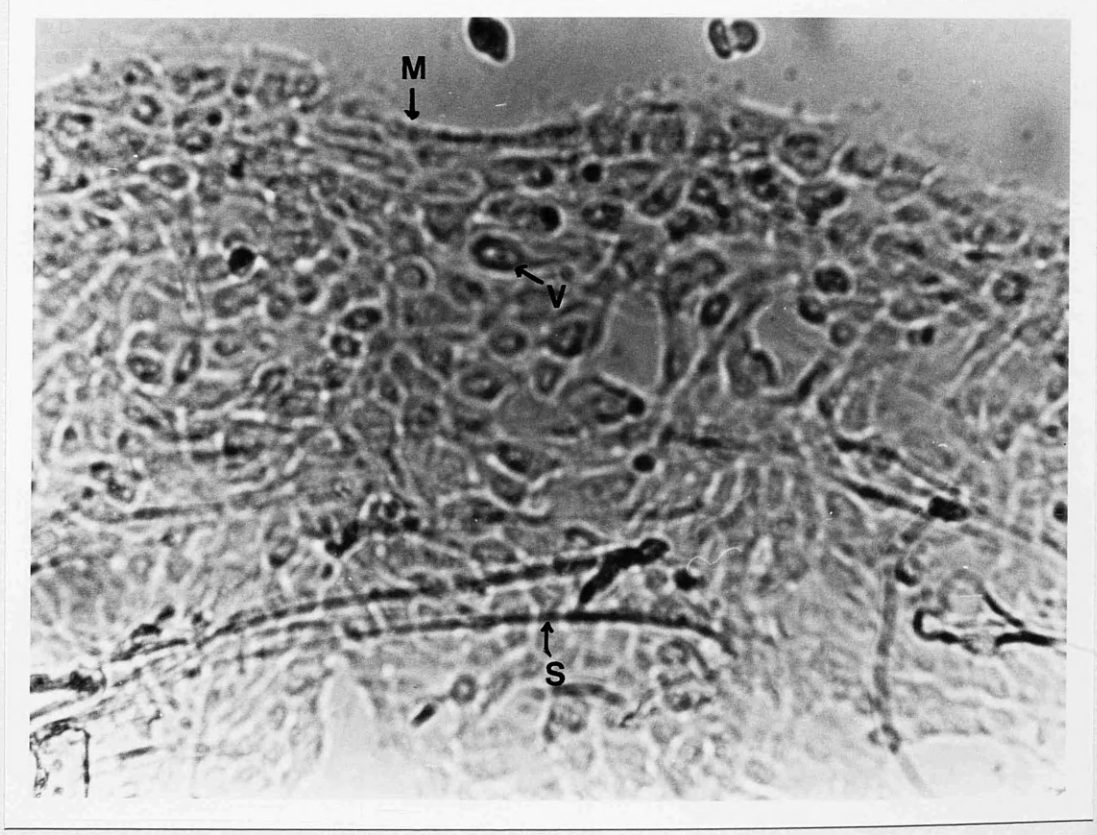
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FIGURE D.2 In vitro growth properties of ectocervical cell

Photographs of a 10 day old explant culture of ectocervical cells. Magnification x80 (top) and x320 (bottom).

Arrows M Outer margin of proliferating cells

S Sloughing of cells

E Explant

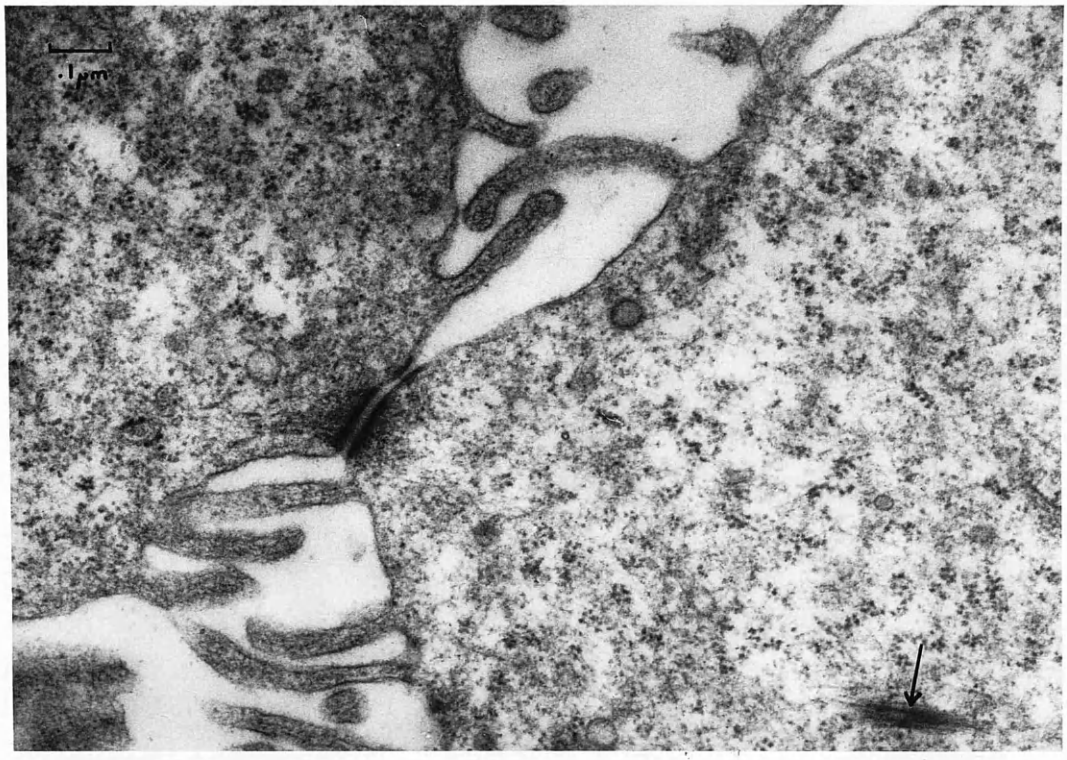
V Vacuolated cell

in Methods. The growth characteristics of the ecto- and endocervical cells were found to differ and are described below.

Growth properties of ectocervical cells

The attachment of successful explants took place within 3 to 7 days. Roughly a third of fragments did not attach and were removed with a pipette. From the third day onwards growing monolayers could be seen to surround the edges of the tissue fragments. The proliferating cells appeared to be at the outer edges of the monolayers. The ectocervical cells grew in a pavement-like mosaic pattern and formed organised monolayers with an even margin. Confluent monolayers covering the entire surface of a petri dish were obtained by seeding several explants in the same dish. The outer margins of the monolayers from each explant were visible even after the monolayers had coalesced. After about 2 weeks, the cell processes began to overlap and fragments began to slough into the medium. Confluent monolayers could be maintained for no longer than 7-10 days before the monolayers started to deteriorate. For this reason subconfluent monolayers showing no signs of sloughing were preferably used in experiments.

The ectocervical cells were also examined by electron microscopy. The presence of desmosomes and tonofilaments (Figure D.3) confirmed the epithelial nature of the cells.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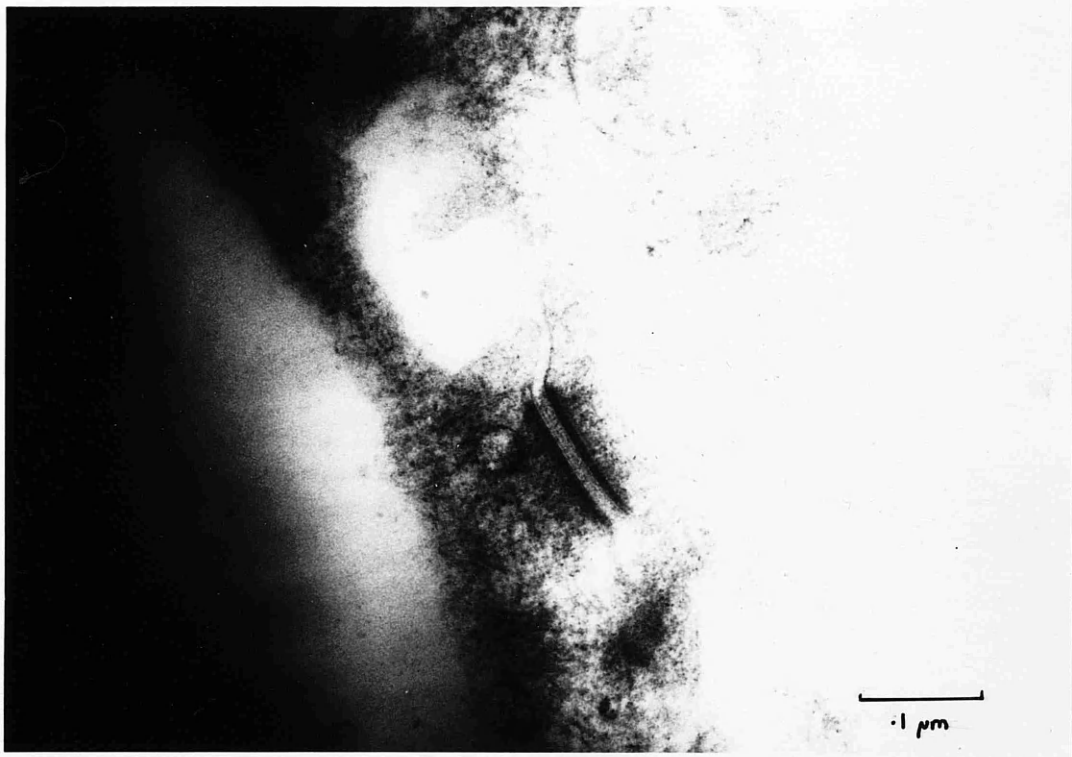

.1 μm

FIGURE D.3 Electron microscopy of ectocervical cells

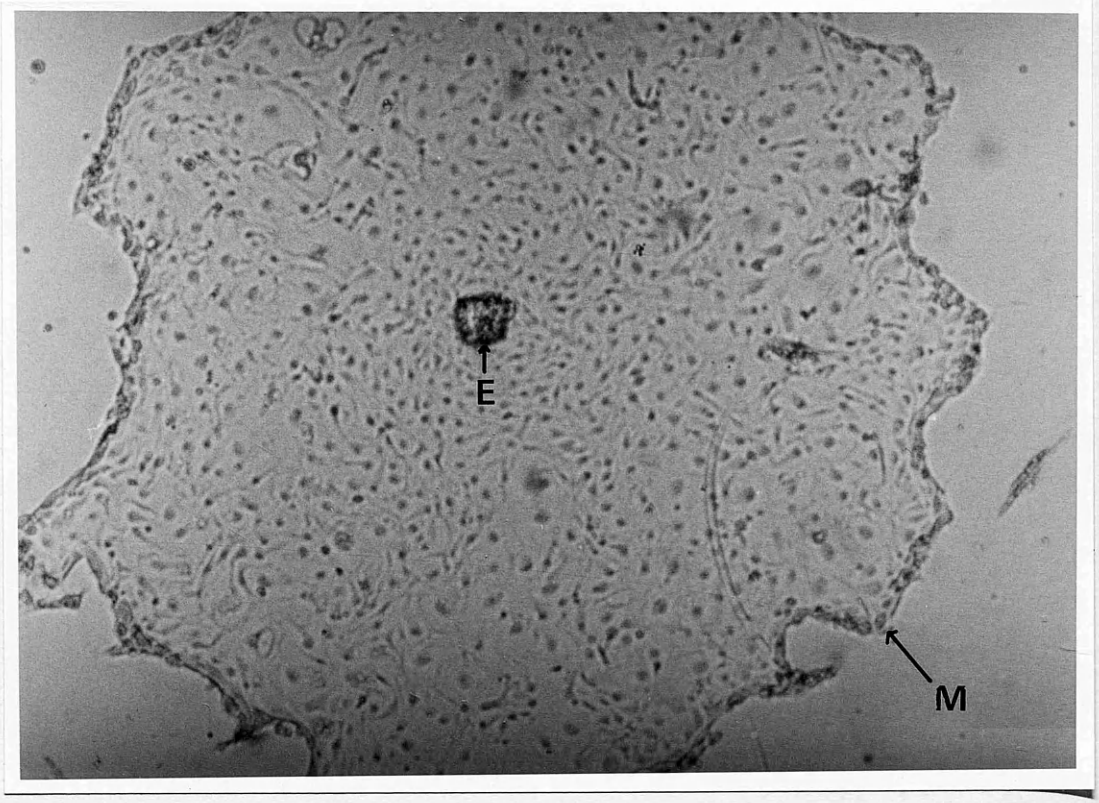
Electron micrographs of ectocervical cells showing the presence of desmosomes and tonofilaments. Magnification x45,000 (top) and x90,000 (bottom). Arrow indicates the location of the tonofilaments.

I thank J. Aitken for carrying out the electron microscopy.

Growth properties of endocervical cells

Endocervical cells were very difficult to culture. The attachment of explants to the plates was often hampered by the large amount of mucus produced by the endocervical cells. Few explants grew successfully in culture. The endocervical cells did not usually form regular organized monolayers like the ectocervical cells. The cells tended to grow in an irregularly whorled pattern and were relatively loosely attached to each other. They were mainly pear-shaped with the narrow end often forming a long curled process. Around small epithelium fragments tighter epithelial groups were observed (Figure D.4). Satellite cells, single or in clusters were occasionally noted. These cells were more rounded in appearance. After about 10-14 days in culture the endocervical cells ceased proliferating and started to detach from the plates.

As the explant technique did not appear to be very successful for culturing endocervical cells, a second technique employing the use of a fibroblast feeder layer was tried. Swiss 3T3 cells (American Type Culture Collection, Rockville, Maryland, USA) were γ-irradiated with 6000 rads from a cobalt-60 source and seeded at a density of 2×10^5 cells per 50mm petri dish. Fragments of endocervical epithelium were disaggregated into single cells by two trypsinization procedures each of 20 min duration. Approximately 10^5 cells were plated out onto each feeder layer. Using this method small clusters of cells attached


E
M

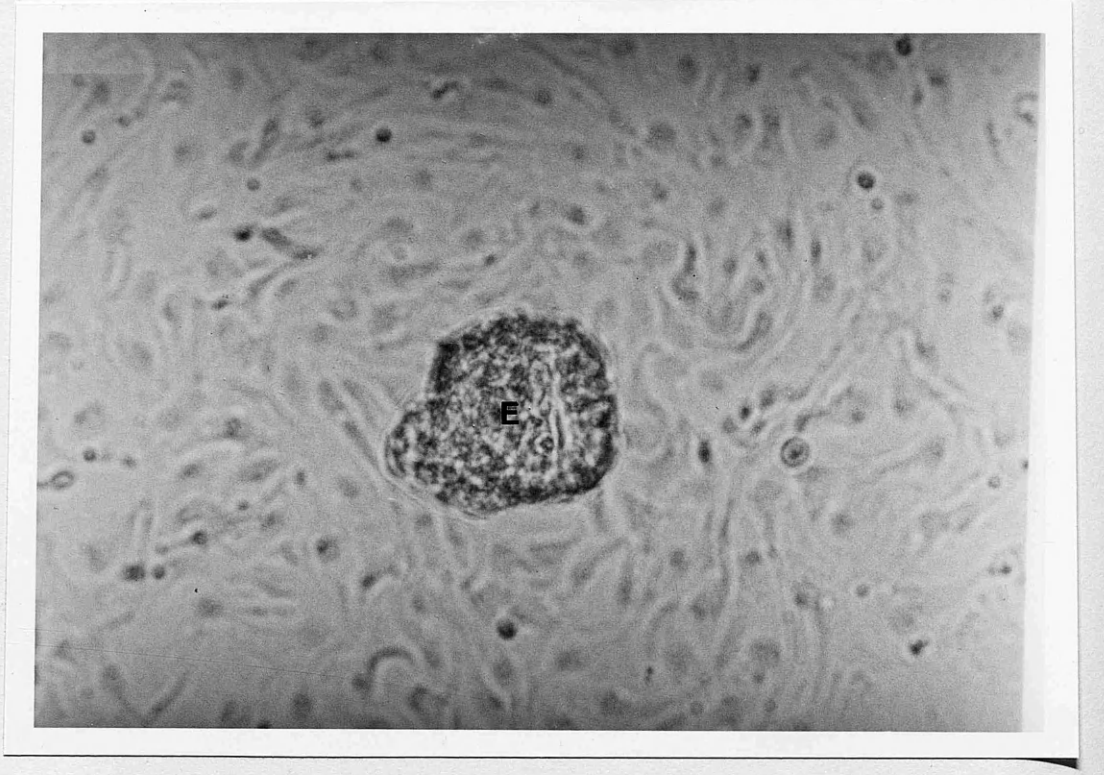

E

FIGURE D.4 In vitro growth properties of endocervical cells

Photographs of a small explant culture of endocervical cells. Magnification x80 (top) and x320 (bottom).

Arrows E Explant

M Outer margin of proliferating cells

Infection of ectocervical cells by HCMV AD169

It is possible that fresh HCMV isolates may have infected ectocervical cells better than the laboratory strain AD169. However, in previous studies undertaken to characterise HCMV isolates, it was found that fresh isolates grew very slowly and cpe was only apparent 3 weeks or more after infection. It is unlikely that ectocervical cells could be maintained for this length of time in culture and there was not sufficient time during this studentship to test a number of isolates to select for faster growing strains.

to the plates but the cultures still degenerated after 10 days. Fibroblast contamination became a problem using this method, whether this was due to fibroblasts that had escaped irradiation or arose from stromal material that had not been completely removed from the epithelium is open to speculation.

Due to the great difficulty in culturing endocervical cells and their limited life time in culture, these cells were of limited use in the following experiments.

Infection of ectocervical cells by HCMV AD169

Monolayers of ectocervical cells were infected with 0.5, 1, 2, and 5pfu/cell of HCMV AD169 when the cell monolayers were about 80% confluent. The number of cells was estimated assuming that $5x10^5$ cells gave 100% coverage of a 35mm petri dish. The infected cells were observed over a period of two weeks during which time no morphological alterations of the cells were apparent when compared with uninfected cultures. The growth medium from the HCMV infected cultures was added to just subconfluent monolayers of Helu cells to assay for infectious virus. No viral cpe was observed in the Helu cells after 3 weeks. The HCMV infected ectocervical cells were scraped off the plates in about 400ul of PBS, sonicated and titrated on just subconfluent monolayers of Helu cells. Again no viral cpe or plaque formation was observed after 3 weeks. In case HCMV was present at very low titre, the titrated monolayers

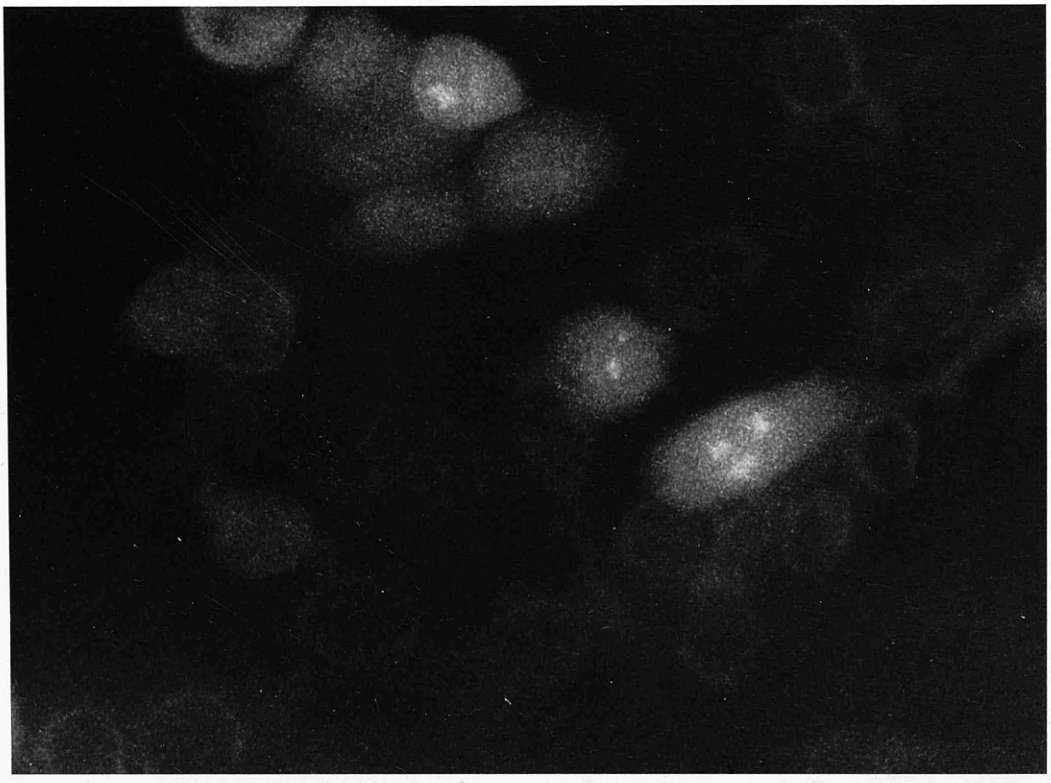
were trypsinized and reseeded at a density of $2x10^5$ cells per 50mm petri dish. It was hoped that by reseeding the cells, any virus present at low titre would have a better chance of replicating and producing cpe. However, no evidence of viral infection was observed. These experiments suggested that either infectious HCMV is not produced in ectocervical cells or that the virus was present at a titre too low to be detected by the above methods.

Low titre virus can sometimes be detected by cocultivation of the infected cells with different cells permissive for virus replication. Infected ectocervical cell cultures were incubated for 10 days and then approximately $1x10^5$ Helu cells were added to the cultures. The Helu cells eventually overgrew the ectocervical cells but showed no signs of viral cpe.

Electron microscopy of HCMV infected ectocervical cultures did not reveal any virus particles within the cells. However, it should be noted that electron microscopy only successfully detects virus particles if a high proportion of the cells have been infected. It can be concluded from these experiments that either HCMV AD169 cannot replicate in ectocervical cells <u>in vitro</u> or that the virus can only replicate to a very limited degree.

HCMV AD169 infected ectocervical cells were also studied by immunofluorescence to determine the presence of HCMV specific antigens. Immunofluorescence was carried out using both rat antiserum which would detect HCMV IE

A



B

FIGURE D.5 Infection of ectocervical cells by HCMV AD169

Immunofluorescence of HCMV infected (A) and mock infected (B) ectocervical cells. Ectocervical cells were infected with 2 pfu/cell of HCMV AD169 or mock infected with growth medium and incubated for 3 days. Rat antisera to HCMV AD169 IE polypeptides was used as the primary antibody and goat anti-rat conjugated with fluoroscein isothiocyanate as the secondary antibody. Nuclear fluorescence was observed in about 10% of the HCMV infected cells. Only background fluorescence was observed in the control mock infected cells.

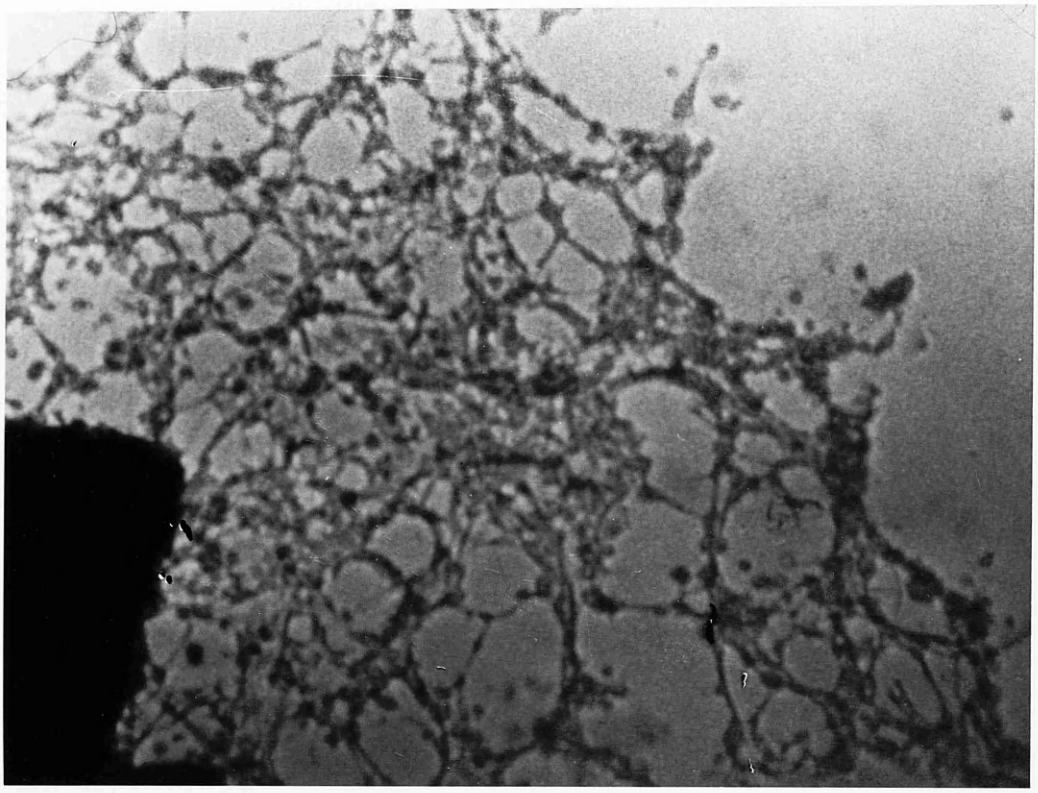
polypeptides, and human antiserum which would detect HCMV IE, early and late polypeptides. Nuclear fluorescence was observed in a small proportion (about 10%) of the cells to both antisera (Figure D.5). Thus a small proportion of ectocervical cells were presumably abortively infected with HCMV.

Infection of ectocervical cells by HSV-2 HG52

Subconfluent monolayers of ectocervical cells were infected with 0.5, 1 and 2pfu/cell of HSV-2 HG52. The number of cells was estimated assuming that $5x10^5$ cells gave 100% coverage of a 35mm petri dish. Cell rounding was observed 24hr post infection. After two days the cell processes began to retract and the cells began to pull away from one another (Figure D.6A). The rounded cells then detached from the plate. The infected cells were scraped off into the medium, sonicated and 100ul aliquots titrated on BHK/C13 cells. The titration results are given in Table D.1. The HSV-2 titres obtained in ectocervical cells were comparable with those obtained in similarly infected cultures of BHK/C13 or Helu cells, indicating that the ectocervical cells are equally susceptible to infection by HSV-2 HG52. At low moi, HSV-2 produces characteristic plaques in ectocervical cells (Figure D.6B).

IE HSV-2 specific antigens could be detected in infected ectocervical cells by immunofluorescence. Both nuclear and cytoplasmic fluorescence were observed using rat

A



B

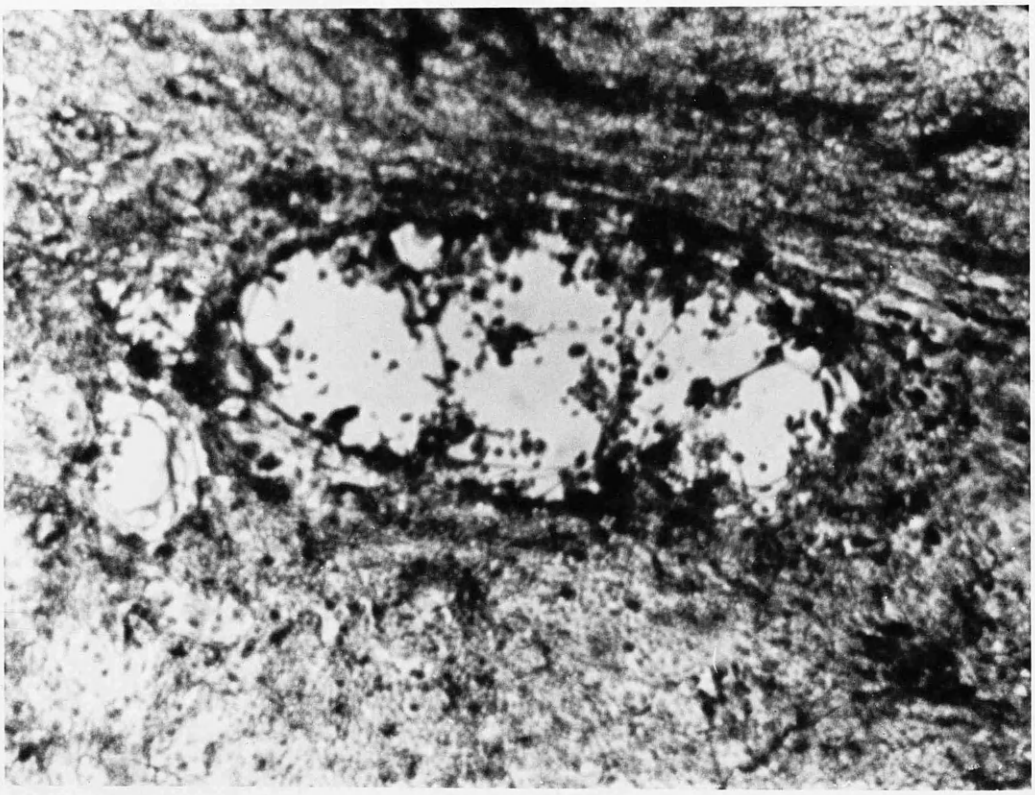


FIGURE D.6 Infection of ectocervical cells by HSV-2 HG52

A. Typical cpe observed in an ectocervical explant culture infected with 1 pfu/cell of HSV-2 HG52, 2 days after infection. Magnification x80.

B. A characteristic plaque produced after infection of ectocervical cells with 0.1 pfu/cell of HSV-2 HG52. Monolayer has been stained with Giemsa. Magnification x320.

TABLE D.1. Infection of ectocervical cells by wild type HSV-2 HG52

M.O.I	No. of plaques on BHK/C13 cells after 2 days						Titre (Average) pfu/ml
	Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
Mock infected	0	0	0	0	0	0	-
0.5pfu/cell	>100	>100	>100	100	26	2	2.3×10^6
1 pfu/cell	>100	>100	>100	100	15	7	4.25×10^6
2 pfu/cell	>100	>100	>100	123	36	7	5.35×10^6

antisera prepared principally against the α and β IE and early polypeptides of HSV-2.

Establishment of latent HSV-2 infections in cell cultures

Herpesviruses are renowned for their ability to establish infections in the natural host. There is the possibility that herpesviruses could initiate oncogenesis during reactivation of latent virus rather than as a direct result of primary infection. HSV remains latent in neurological tissue. In relation to genital herpes infections, the potential sites of virus latency include sacral and lumbar dorsal root ganglia, Frankenhauser's sacral nerve plexus and ganglia of the pelvic autonomic system. HSV-2 has been reactivated from human sacral dorsal root ganglia (Baringer, 1974) and from human uterosacral ligaments (Eglin et al., 1982). There have, however, been no published attempts to develop an in vitro system for studying HSV-2 latent infections in cervical explants.

To establish a state in which infectious HSV-2 could not be detected, subconfluent cell monolayers were infected with 0.003pfu/cell and the cultures immediately incubated at a supraoptimal temperature of 42°C. Cultures were maintained at this temperature for 6 days. Experiments were set up using both Helu and ectocervical cells. The ectocervical monolayers had to be infected when they were about 75% confluent so that the experiments could be completed before the monolayers started to deteriorate.

Control uninfected Helu and ectocervical cells were maintained at 42°C for 6 days and the infected with 0.003 pfu/cell of HSV-2 at 37°C. Plaques were produced within 3 days. Virus replication was not therefore inhibited by heat shock proteins or other inhibitory effects produced in the cells at 42°C.

Both Helu and ectocervical cells continued to grow at 42°C and the cell monolayers usually attained confluency before the end of the 42°C incubation period.

After 5-6 days at 42°C, some of the infected Helu and ectocervical cell cultures were assayed for the presence of infectious virus. The cells were scraped off into the growth medium, sonicated and titrated on BHK/C13 cells. No viral cpe or plaque formation was observed indicating that virus replication had probably been restricted at the supraoptimal temperature.

The remaining infected Helu and ectocervical cultures were shifted down to 37°C. HSV-2 infected Helu cells could be successfully maintained at 37°C for up to 2 weeks and no evidence of virus replication was observed during this time. Helu cultures infected with 0.003pfu/cell of HSV-2 HG52 but not incubated at 42°C, could not be maintained for longer than 3-4 days and viral cpe was evident within 2 days post infection. HSV-2 infected ectocervical cells could only be maintained at 37°C for about 7 days following incubation at 42°C. After this time the monolayers started to deteriorate and cells detach from the plate. This was a function of the age of the ectocervical cell cultures and not cpe resulting from reactivated virus, as uninfected cultures deteriorated in a similar fashion after the same period of time. Ectocervical cultures infected with 0.003pfu/cell of HSV-2 HG52 but not incubated at 42°C, showed signs of viral cpe within 2 days post infection and could not be maintained for

more than 3-4 days.

These experiments showed that by incubating infected cultures at 42°C, it was possible to establish HSV-2 infections during which infectious virus could not be detected in both Helu and ectocervical cells. Incubation of infected cultures at the supraoptimal temperature of 42°C must, therefore, result in restriction of the viral replicative cycle. Either the production of infectious virions is delayed under these conditions or the virus has established a latent infection *in vitro*. This model system has great potential for studying the effects of various stimuli that could be involved in reactivating latent viral infections of the cervix.

Reactivation of latent HSV-2 by HCMV AD169 and HSV ts mutants

The cervix is exposed to effects of natural female hormones, synthetic hormones such as those used in the contraceptive pill, sperm and various sexually transmitted organisms. All of these are potential stimuli for the reactivation of latent virus in cervical cells. The following experiments were carried out to determine if HCMV AD169 and *ts* mutants of HSV types 1 and 2 could reactivate HSV-2 latent in cervical cells *in vitro*.

Helu and ectocervical cells were infected with 0.003pfu/cell of HSV-2, maintained at 42°C for 6 days and then shifted to 37°C as previously described. After

TABLE D.2 Reactivation of HSV-2 from a quiescent state by HCMV AD169

Helu and ectocervical cells were infected with $3x10^3$ pfu of HSV-2, incubated at 42°C for 6 days and then shifted to 37°C. After 3 days at 37°C, the cultures were infected with varying amounts of HCMV AD169 at 37°C and then maintained at 37°C for 3 days. The cells were then harvested, sonicated and titrated on BHK/C13 cells at 37°C to assay for the presence of reactivated virus.

M.O.I.	Average yield of reactivated virus from Helu cells (pfu)	Average yield of reactivated virus from ectocervical cells (pfu)
0.1	$1.2x10^5$	0
0.5	$4.0x10^5$	0
1.0	$9.4x10^5$	0
5.0	$4.0x10^4$	0
Mock infected	0	0

incubation of the infected cultures at 37°C for 3 days, the monolayers were superinfected with either HCMV AD169 or HSV ts mutants. Monolayers were superinfected with 0.1, 0.5, 1.0 and 5.0pfu/cell of AD169 at 37°C and then maintained at this temperature for 3 days. Control monolayers were mock infected with 100ul of growth medium at 37°C and maintained at this temperature for 3 days. The cells were then harvested in the medium, sonicated and titrated on BHK/C13 cells at 37°C for 3 days to assay for infectious virus. The results of the titrations are given in Table D.2. The remaining monolayers were superinfected with 5pfu/cell of either tsG or tsl* (ts mutants of HSV-1 and HSV-2, respectively) at both 37°C and 38.5°C and maintained at these temperatures for 3 days. Control monolayers were mock infected with 100ul of growth medium at both 37°C and 38.5°C and maintained at these temperatures for 3 days. The cells were then harvested, sonicated and titrated on BHK/C13 cells at both 31°C and 38.5°C for 3 days. The results of these titrations are given in Table D.3.

The results showed that infectious virus could be detected in Helu cells that had been superinfected with HCMV AD169 but not in ectocervical cells that had been superinfected with HCMV AD169. Since BHK/C13 cells are nonpermissive for HCMV replication, the infectious virus detected must have been HSV-2. No infectious virus was detected in either Helu or ectocervical cells that had been mock infected with growth medium. This suggests that

*

All ts mutants were checked for reversion before use.

TABLE D.3 Reactivation of HSV-2 from a quiescent state by ts mutants of HSV-1 and HSV-2

Helu and ectocervical cells were infected with $3x10^3$pfu of HSV-2, incubated at 42°C for 6 days, and then shifted to 37°C. After 3 days at 37°C, the cultures were superinfected with 5pfu/cell of either ts G or ts1 at both 37°C and 38.5°C and maintained at these temperatures for 3 days. Control monolayers were mock infected with 100ul of growth medium at both 37°C and 38.5°C and maintained at these temperatures for 3 days. The cells were then harvested, sonicated and titrated on BHK/C13 cells at both 31°C and 38.5°C for 3 days.

ts mutant	Superinfection Temperature	Titre of yield after titration on BHK/C13 cells (pfu)			
		Helu cells		Ectocervical cells	
		38.5°C	31°C	38.5°C	31°C
tsG	38.5°C	$7.7x10^3$	$3.2x10^2$	0*	0*
tsG	37°C	$1.2x10^4$	$3.0x10^2$	0*	0*
ts1	38.5°C	$2.6x10^3$	$3.0x10^2$	0*	0*
ts1	37°C	$1.4x10^5$	$2.0x10^2$	0*	0*
Mock infected	37°C	0	0	0	0
	38.5°C	0	0	0	0

* Cells had detached from the plates during the 3 day superinfection period

incubation at 42°C induced latent infection of HSV-2 and that HCMV AD169 is capable of reactivating latent HSV-2 *in vitro* from Helu cells. Reactivation of HSV-2 from a quiescent state by HCMV has also been demonstrated by Coleberg-Poley *et al.* (1979) and Russell and Preston, 1986). The same researchers later showed that reactivation could be attributed to an early HCMV function (Coleberg-Poley, *et al.*, 1981). Superinfection with 1pfu/cell of HCMV AD169 produced the greatest yield of reactivated virus. Superinfection with 5pfu/cell of HCMV AD169 actually reduced the yield of reactivated virus compared with lower multiplicities of infection. The Helu cells were initially infected with $3x10^3$pfu of HSV-2. The yields of reactivated HSV-2 were 10-100 fold higher than the amount of input virus, indicating that the virus had not only reactivated but was also replicating efficiently. The virus yields obtained after superinfection with HCMV AD169 were tenfold higher than those obtained after superinfection with HSV *ts* mutants.

Superinfection of HSV-2 latently infected ectocervical cells with HCMV AD169 did not reactivate virus. The experiments were repeated several times and the superinfected cultures maintained at 37°C for as long as possible before deterioration of the monolayers occurred through age. No viral cpe was detected during this time. Some of the superinfected cultures were titrated on BHK/C13 cells. On close examination, some parts of these monolayers

looked as if they may have been developing syncytia. The titrated BHK/C13 cells were then trypsinized and reseeded in 260ml tissue culture flasks. It was hoped that by reseeding the cells thinly, any virus present would have a greater chance of replicating and producing cpe. However, no plaques developed. Approximately $1x10^5$ BHK/C13 cells were added to the remaining superinfected ectocervical explants and the cultures incubated at 37°C for as long as possible. No evidence of viral cpe or plaque formation was observed.

The reason for the ability of HCMV to reactivate latent HSV-2 from Helu cells but not from ectocervical cells may be related to the different nature of the cells. Ectocervical cells are epithelial cells whereas Helu cells are fibroblasts. Virus-cell interactions must be important in establishing and/or reactivating latent virus infections and will be different in fibroblasts and epithelial cells.

Infectious virus was detected in Helu cells that had been infected with HSV-2, incubated at 42°C for 6 days, then shifted to 37°C for 3 days and finally superinfected with ts mutants of HSV-1 and 2. The titrations were carried out using BHK/C13 cells and at both 31°C and 38.5°C.

The results of these experiments were totally unexpected. Higher titres of virus should have been detected at 31°C and not at 38.5°C, since both ts mutants and wild type HSV-2 can replicate at 31°C whereas only wild type HSV-2 can replicate at 38.5°C. Despite these results, the experiments suggest that

incubation at 42°C induced latent infection of HSV-2 and that HSV ts mutants are capable of reactivating latent HSV-2 in vitro from Helu cells.

After superinfection of HSV-2 latently infected ectocervical cells, the cells detached from the plates within 3 days. Since repeated attempts to detect infectious virus in these cultures by titration on BHK/C13 cells yielded negative results, it was assumed that the inoculum of superinfecting virus was cytotoxic to the ectocervical cells. Unfortunately, due to the difficulty in obtaining suitable cervical tissue for these experiments at this time, it was not possible to repeat the experiments using a lower superinfecting inoculum.

Attempts to reactivate latent HSV-2 by hormone treatment

The following experiments were carried out to investigate the potential of the female hormones oestrogen and progesterone, and the synthetic steroid dexamethasone to reactivate latent HSV-2.

Helu cells were infected with 0.003pfu/cell of HSV-2, incubated at 42°C for 6 days and then shifted to 37°C as previously described. After the cultures had been incubated

at 37°C for 3 days, the medium was replaced with fresh medium containing hormone-free foetal calf serum. The hormones were removed from the foetal calf serum by treating the serum with charcoal, pelleting the charcoal and filter sterilizing. The cultures were incubated at 37°C for 24hr after which time the medium was replaced with fresh medium containing 10^{-7} and 10^{-9}M concentrations of either oestrogen, progesterone or dexamethasone. 10^{-9}M represented the normal physiological concentration of hormone in the body. The cultures were assayed for reactivated virus after 3 days at 37°C. No infectious virus was detected in any of the hormone treated cultures. Control monolayers were either mock infected with 100ul of growth medium or superinfected with 1pfu/cell of HCMV AD169. No infectious virus was detected in the mock infected cultures. Approximately 10^5pfu of infectious virus was detected in the culture superinfected with HCMV AD169, indicating that HSV-2 was present in the monolayers in a quiescent state and could be reactivated by HCMV. The experiments were repeated and the cultures assayed for the presence of infectious virus after 7 and 14 days. Again no evidence of viral cpe or of plaque formation was observed. Unfortunately, insufficient cervical cells were obtained during the period in which these studies were undertaken to repeat the experiment to determine if hormone treatment can reactivate HSV-2 latent in ectocervical cells.

DISCUSSION

An infectious agent is identified as the causative agent of a disease if it fulfils the following criteria:

1. The agent can be regularly isolated from the disease.
2. It independently causes the disease.
3. Vaccination against the infectious agent prevents the disease.

However, in the case of an oncogenic virus, these criteria cannot always be assessed, particularly since malignant diseases tend to be of a multifactorial nature. Thus, a different set of parameters need to be used to define the role of an oncogenic virus in causing a malignant disease. Firstly, it is necessary to investigate the epidemiological evidence for an association of the virus with the disease. This involves both the detection of antibodies to the virus in the sera of patients with the disease, and the detection of virus-coded information such as DNA, RNA and proteins, within the diseased tissue. The ability of the virus to induce oncogenic changes within the cells needs to be investigated <u>in vitro</u> using a model transformation system and also <u>in vivo</u> using an animal model. This thesis describes work undertaken to investigate the possible role of HCMV in the development of CIN, the precursor stage to invasive carcinoma of the cervix.

Although some seroepidemiological studies have

revealed the presence of antibodies to HCMV in patients with CIN and cervical carcinoma (as previously discussed and cited on p40-42), prior to this thesis, only one molecular epidemiological study had been carried out. This study detected HCMV DNA in cervical carcinoma specimens from some geographical regions but made no investigation of CIN tissue (Huang et al., 1983). It was therefore decided to investigate the association of HCMV with CIN by analysing CIN tissue from patients in the West of Scotland for HCMV DNA sequences by Southern blot analysis. Since HPV and HSV-2 have also been implicated in the development of cervical neoplasia, the same CIN specimens were analysed for HPV and HSV-2 DNA sequences.

Various *in vitro* transformation systems have been described for HCMV but as yet the mechanism of transformation remains unknown. In this thesis two different *in vitro* transformation systems were investigated as potential models for studying the mechanism of transformation by HCMV. These were, transfection of cells with the HCMV AD169 HindIII E fragment using the calcium phosphate transfection technique, and infection of RE cells with UV-inactivated HCMV. Cell transformation was not accomplished using the HindIII E fragment but treatment of RE cells with UV-inactivated virus proved to be a successful and reproducible transformation system. This system was then used to try and identify a possible mechanism of transformation by HCMV.

Although the model system described is useful for investigating transformation and tumourigenesis by HCMV, it does not simulate the environmental conditions to which the virus would be exposed within the cervix. The rat embryo cells employed in the *in vitro* transformation system are fibroblasts whereas the cells exposed to viral infection in the cervix are epithelial in nature. These two different cell types may differ in their susceptibility to viral infection and the course of infection may be slightly different in cervical cells and important in inducing neoplastic change. It was therefore considered necessary to investigate the interaction of HCMV with the target cervical cells *in vitro*. Cervical cells were grown from explants of cervical epithelium obtained from hysterectomy specimens and were infected with HCMV or HSV-2. The cellular alterations induced by viral infection and the production of infectious virus and viral antigens in the cells were investigated. The ability of HSV-2 to establish latent infections in the cervical cells was also studied and experiments were carried out to determine if HCMV or steroid hormones could function as reactivating agents.

Unfortunately, time did not permit an investigation of the association of HCMV with the development of CIN using an animal model. It would have been interesting to treat the cervices of experimental

mice with formalin - inactivated or UV-inactivated HCMV to see if prolonged exposure to inactivated virus could induce premalignant and malignant lesions, as has already been done with HSV-2 (Wentz et al., 1981).

The results of the experiments presented in this thesis are summarized and discussed on the following pages.

TRANSFORMATION STUDIES

Transformation of RE cells by UV-irradiated HCMV proved to be a successful and reproducible model system for in vitro transformation. Three types of transformed cells were observed in treated cultures. Different regions of the HCMV genome may have been responsible for initiating the different types of transformed cells. This transformation system has also been used successfully in the past within this Institute. A former student, Ann Bunce, established a number of cell lines from the foci of cells transformed by UV-irradiated HCMV AD169 and from tumours induced in rats after inoculation with HCMV transformed cells. These transformed and tumour cell lines were analysed for the presence of HCMV DNA sequences to try to elucidate a mechanism of transformation by HCMV. HCMV specific DNA sequences could not be detected in any of the HCMV transformed or tumour cell lines examined by

Southern blot analysis at a level of sensitivity that could detect 1 copy/cell of viral DNA within cellular DNA. Only a very small sequence of viral DNA may have been retained in low copy number in the transformed cells. The Southern blot technique would not be sufficiently sensitive to detect such small amounts of DNA. Alternatively the retention of viral DNA in the cell may be transient and only necessary to initiate the transformation event.

Experiments showed that the DNA from a rat tumour induced by the inoculation of HCMV transformed cells contained a transforming gene that could be transfected and could initiate tumour formation in athymic nude mice. Since reasonably low levels of viral DNA could not be detected within the original transformed cells, the transfected gene was probably of cellular origin. Further experiments suggested that the transfected gene may be an activated ras gene, although additional verification of these experiments is necessary as has been previously explained. These experiments raise the possibility that HCMV may initiate transformation by activating a cellular oncogene. This would seem a reasonable proposal since HCMV does influence the expression of cellular genes in infected cells causing an increase in the synthesis of cellular DNA, RNA proteins and enzymes. The possible mechanisms of transformation by HCMV will be discussed later.

Repeated attempts to transform 3T3 cells with the HindIII E fragment of HCMV AD169 were unsuccessful in these experiments. This may have been partly due to technical difficulties involved with the calcium phosphate transfection technique. In the transfection experiments, 10ug of cloned HCMV DNA were used. Cells take up only a small amount of exogenous DNA on transfection. On reflection, it may have been better to have purified the HCMV HindIII E fragment from vector sequences prior to transfection so that what DNA was taken up by the cell contained HCMV and not plasmid vector sequences. The HindIII E fragment is also quite large ($\sim$ 20kb) and transfection may have been more successful using a smaller fragment containing the transforming sequence such as the BamHI P fragment. Other factors affecting transformation may have been differences in the cells and growth medium. Unfortunately, it was not possible to obtain the original NIH 3T3 cells used by Nelson et al. (1982) in their pioneering studies. Nelson et al. (1982) used Hyclone serum from Utah whereas the foetal calf serum used in the growth medium in these experiments was purchased from Gibco Laboratories, Paisley, Scotland. Differences in foetal calf serum have been found to affect the efficiency of transfection (J. Nelson, personal communication).

In the original transfection studies by Nelson et

al. (1982), anchorage independent growth was used as the criterion for transformation. It is possible that the HCMV AD169 genome contains two transforming regions, one responsible for morphological transformation and one for inducing anchorage independent growth. The gene coding for morphological transformation may not lie within the HCMV AD169 HindIII E fragment. This would explain why morphological transformation of different cell types could not be obtained using the HindIII E fragment. Transformation of RE cells by UV-irradiated HCMV was possible because all regions of the viral DNA were present. However, it is difficult to explain why the HindIII E fragment did not induce anchorage independent growth of transfected cells. Methylcellulose was used by Nelson et al. (1982) to assay for anchorage independent growth whereas soft agar was used in these experiments. The selective efficiency of these media is usually comparable but it may have been worthwhile repeating the transfections and assaying for anchorage independence by growth in methylcellulose.

POSSIBLE MECHANISMS OF TRANSFORMATION BY HCMV

HCMV may transform cells by a number of mechanisms. For example, HCMV may activate a cellular proto-oncogene. Activation could be accomplished by promoter or enhancer insertion or by the integration of

an insertion sequence (IS)-like structure into the host cell DNA. In some human cancers activation of a cellular proto-oncogene has been associated with specific chromosomal aberrations and gene rearrangements. In addition cellular genes can be transactivated by some viral gene products. Other possible mechanisms of transformation include mutagenesis and changes in the DNA methylation pattern within cells. These mechanisms are discussed on the following pages.

Activation of a cellular proto-oncogene by promoter or enhancer insertion

Activation of a cellular proto-oncogene by promoter insertion was initially proposed to explain the induction of B-cell lymphomas by avian leukosis viruses (ALV's). These retroviruses induce bursal lymphomas in infected birds only after a latent period of 4-12 months. Integration of an ALV provirus into the host DNA results in the viral coding sequence being flanked by two long terminal repeats (LTRs). The LTR contains a putative promoter sequence (Ju and Skalka, 1980). Initiation of normal viral RNA synthesis occurs within the left LTR but because this sequence is repeated at the right end of the provirus, initiation could also occur within the right LTR. The 'promoter insertion' theory predicts that the provirus integrates adjacent to

a cellular proto-oncogene and that transcription is initiated from the viral promoter within the LTR generating hybrid RNA molecules containing both cellular and viral sequences (Neel et al., 1981). Such tumour specific RNAs can be detected in ALV-induced lymphomas and are expressed at high levels (Neel et al., 1981; Payne et al., 1981). Hayward et al. (1981) provided strong evidence that in about 85% of lymphomas the c-myc gene has been activated. The resultant enhanced expression of this cellular gene has presumably led to neoplastic transformation. Integration of the provirus at a site which could activate the c-myc gene is presumably a rare event, since integration is known to occur at multiple sites in the host genome.

In ALV-induced lymphomas in which the provirus has been integrated upstream of the c-myc gene in the same orientation as c-myc, the upstream 5' LTR has been deleted from the provirus. Cullen et al. (1984) have presented evidence that viral mRNA transcription from the left LTR extending into the downstream LTR of a provirus-like construct restricts the ability of this LTR to function as an efficient promoter. They suggest that spontaneous deletion of the upstream left LTR may be necessary to allow c-myc transcription promoted by the remaining right LTR to increase to levels sufficient to cause malignant transformation.

Oncogenesis by slow transforming retroviruses

cannot always be explained by the hypothesis that a cellular proto-oncogene is activated by promoter insertion. For example, in a small number of ALV-induced B lymphomas, the provirus has been found to be integrated downstream of the c-myc gene but in the same orientation or upstream of the c-myc gene in the opposite orientation (Payne et al., 1982). These two alternative configurations do not allow the direct use of the LTR as the promoter for c-myc expression as predicted by the promoter insertion model. Similarly, in murine T cell lymphomas induced by murine leukaemia viruses, the retroviral inserts are not aligned in a manner compatible with activation of c-myc by promoter insertion (Corcoran et al., 1984). The avian retroviruses, Rous sarcoma virus (RSV), Rous associated viruses (RAVs) and the endogenous RAV-0 all have functional LTR promoter elements but RSV and RAV are oncogenic in chickens but RAV-0 is not. RAV-0 does, however, lack the LTR enhancer elements (Weber and Schaffner, 1985). The above mentioned workers propose that oncogenesis by these retroviruses is a function of activation of a cellular proto-oncogene by enhancer insertion. Because enhancers are able to stimulate a nearby gene from a 5' or a 3' position, in either orientation and over relatively large distances, they have all the properties required to explain the activation of a cellular proto-oncogene by LTR enhancer

sequences.

Very strong promoter and enhancer sequences are located upstream from the transcription initiation site of the major IE gene of HCMV (Akrigg et al., 1985; Boshart et al., 1985; Stinski and Roehr, 1985). The HCMV enhancer is of particular interest because it seems to have little cell type or species preference and is 3-5 fold more active than the SV40 enhancer (Boshart et al., 1985). It has been shown to enhance transcription of the rabbit β globin gene (Boshart et al., 1985) and does, therefore, have the ability to enhance transcription of cellular as well as viral genes. Both the HCMV promoter and enhancer are contained within the HindIII E fragment of HCMV AD169. The IE promoter is about 509 bp and the enhancer about 406 bp (although smaller sequences retain enhancer activity). It is possible that either of these sequences may become inserted into cellular DNA within the transformed cells but such small sequences could not be detected by Southern blot analysis with the size of probe used in this study. In addition, such sequences may only be required to initiate some cellular transcription event and may not then be continuously required. In such a case detection of this sequence would be purely fortuitous.

Activation of a cellular proto-oncogene by an IS-like element

The possible involvement of an IS-like element in the activation of a cellular proto-oncogene was first described in a mouse myeloma (Rechavi et al., 1982) in which the 5' end of the cellular c-mos gene had been substituted by a segment of DNA from the LTR of the intercisternal A particle gene (Kuff et al., 1983). Contained within the inserted fragment were sequences that could be drawn as a stem-loop structure flanked by direct repeats ie an IS-like element. DNA sequence analysis of the HCMV transforming fragment of AD169 identified by Nelson et al. (1982) has shown that the sequence can form an IS-like structure (Galloway et al. (1984). Whether this HCMV fragment is active in DNA transposition and can thus activate a cellular proto-oncogene remains to be seen. The mechanism of activation of a cellular proto-oncogene by an IS-like element is not known. The element could initiate transcription of the cellular proto-oncogene by introducing a strong promoter. Alternatively, it may modify the chromatin structure at the insertion site which could create a putative promoter within the adjacent cellular proto-oncogene available for transcription. The potential for an IS-like element to be involved in the initiation of transformation is not limited to activating the expression of a cellular

proto-oncogene. The IS-like element could act as a mutagen by integrating into, and perhaps excising imprecisely from, cellular genes at random. Further investigations are necessary to determine if the IS-like element of HCMV can mediate cell transformation and the precise mechanism involved.

Activation of a cellular proto-oncogene by gene rearrangements

Activation of a cellular proto-oncogene may also be accomplished by chromosomal rearrangements that bring the proto-oncogene under the control of a new promoter. In some Burkitt lymphomas, the c-myc gene has been translocated from its normal position on chromosome 8 to the immunoglobulin heavy chain locus on chromosome 14 (Taub et al., 1982). In Burkitt lymphomas carrying the translocation 2;8, the c-myc gene remains on chromosome 8 but the immunoglobulin light chain locus on chromosome 2 is translocated to a region distal to the c-myc gene (Erikson et al., 1983). Enhancer elements have been found within the immunoglobulin genes (Boss, 1983) and may be responsible for the increased expression of c-myc. Since many patients with Burkitt lymphomas have high antibody titres to EBV, and EBV DNA has been found in these tumours, EBV has also been implicated in the development of these tumours. Possibly EBV is involved in initiating the translocation event.

Consistent chromosomal abnormalities, particularly translocations, inversions or deletions are frequently observed in the malignant cells of a number of human cancers and leukaemias. In many cases a cellular proto-oncogene has been located at the chromosomal bands that are involved in the translocations or deletions (reviewed by Rowley, 1983). For example, the proto-oncogenes c-*mos* and c-*abl* have been located at the breakpoint in the 8;21 and 9;22 translocations associated with acute myeloblastic leukaemia and chronic myelogenous leukaemia, respectively. The proto-oncogenes c-*ras*^{H} and c-*myb* have been localised in the regions of the chromosomes involved in the deletions associated with Wilm's aniridia and acute lymphoblastic leukaemia, respectively. However, none of these malignancies have been linked to infection by a specific virus.

Lüleci *et al*. (1980) have reported chromosomal abnormalities in HCMV infected Helu cells. Chromosomes 2, 3, 4 and 21 appeared to show more anomalies than the other chromosomes. The number of anomalies was not proportional to the length of the chromosomes. Analysis of the rat embryo cell lines transformed by HCMV in this laboratory has shown that chromosomal alterations rarely involve changes in chromosome number and that abnormalities are common in chromosomes 1 and 3 (J.C.M. Macnab, personal communication). Changes in chromosomes

1 and 3 are frequently involved in rat transformed cell lines and may be species dependent. It is not known whether selective chromosomal damage induced by HCMV is important in the oncogenicity of the virus.

Transactivation

Another possible mechanism involved in transformation is transactivation of the cellular genes important in growth control by proteins encoded by viruses. The adenovirus Ela protein and 180,000 dalton IE protein of pseudorabies virus (PRV) can both induce transcription of early adenovirus genes (Jones and Shenk, 1979; Feldman et al., 1982) and various cellular genes including human (Green et al., 1983) and rabbit (Svensson and Akusjarvi, 1984) β-globin and rat preproinsulin 1 (Gaynor et al., 1984), when these genes are newly introduced into cells by DNA transfection or infection in a virus particle. The Ela protein can also transactivate the heat shock protein 70 (hsp 70) cellular promoter (Nevins, 1982). The function of the hsp 70 gene product is unknown, but is presumably important in the cell since the protein is highly conserved between bacteria and eukaryotic organisms. This cellular protein may be involved in cellular transformation since hsp 70 expression appears to be elevated in several transformed cell lines. The IE gene products of HSV can also transactivate genes. The

$V_{mw}175$ protein encoded by the IE gene 3 of HSV-1 is capable of transactivating the HSV-1 viral glycoprotein D promoter and the rabbit β-globin promoter (Everett, 1983; 1984) and is able to complement adenovirus Ela mutants. In none of the above cases can the regulation of transactivation be attributed to recognition of a specific sequence within the promoter regions. The mechanism of transactivation remains obscure. It is possible that the transactivating proteins recognise DNA which is not highly organised into chromatin and either modify its structure so that it becomes readily accessible for transcription or transport it to particular active regions of the nucleus.

Evidence is now accumulating that the IE gene proteins of HCMV also have the ability to transactivate gene expression. Like the herpesviruses PRV and HSV, HCMV AD169 can transactivate transcription of early adenovirus genes and complement an Ad5 Ela mutant for lytic growth (Tevethia and Spector, 1984). Everett and Dunlop (1984) have shown that HCMV AD169 can transactivate transcription from both the HSV-1 glycoprotein D and rabbit β-globin promoters. Stinski and Roehr (1985) and Spaete and Mocarski (1985) have also reported the presence of transacting components in the promoter regulatory region of the major IE gene of HCMV Towne strain. HCMV has, therefore, potential for upregulating the expression of cellular genes by

transactivation and the large increase in host cell DNA synthesis on infection could be at least partly due to transactivation of cellular genes. Transactivation could result in transformation if the upregulated genes were important in growth control.

Mutagenesis

Gene mutations are commonly induced by ionizing radiations and chemical carcinogens. The primary mutagenic effect produced by UV-irradiation is thymidine dimerization which causes distortion of the DNA helix and therefore interferes with normal replication. Acridine dyes appear to cause mutations by inserting between two neighbouring purine bases in a single DNA strand resulting in insertion or deletion of a single nucleotide during replication. Base analogues such as bromodeoxyuridine and 2-aminopurine, alkylating agents, nitrous acid and hydroxylamine all induce transition mutations in which pairing errors arise through the transition of the pairing relationship of one purine base into the pairing form of another purine or of one pyrimidine into that of another pyrimidine. The pairing errors are copied during DNA replication, resulting in point mutation of a gene. Many investigators believe that if viruses are involved in the development of certain human malignancies, they initiate tumourigenesis by mutagenizing cells in a manner similar to that of

chemical carcinogens. HSV-1 has been shown to have mutagenic activity (Schlehofer and zur Hausen, 1982). Possibly a viral replicative product has mutagenic properties. Prime candidates include the viral encoded alkaline exonuclease, DNA polymerase, ribonucleotide reductase or thymidine kinase. Such a mechanism of transformation need not involve the retention of viral DNA sequences. It has not yet been shown whether HCMV can induce mutations within the host cell genome.

Mutagenic agents have been found to induce DNA repair mechanisms in treated cells. Such repair mechanisms include photoreactivation, excision repair, and postreplication repair. Studies have shown that mutagens can enhance the survival of UV-irradiated viruses in treated cells (Sarasin and Hanawalt, 1978; Lytle et al., 1978). HCMV has been found to have a similar effect since Nishiyama and Rapp (1980) reported that infection of primary rat kidney cells and Vero cells with HCMV enhanced survival of UV-irradiated HSV. The enhancement process was found to be sensitive to caffeine and since caffeine is known to inhibit certain repair processes in mammalian cells, it was proposed that HCMV may induce a DNA repair pathway in host cells. Further investigations showed that HCMV enhanced DNA repair capacity in the host cells without producing detectable lesions in the cellular DNA and without inhibiting DNA synthesis (Nishiyama and Rapp, 1981).

The results of the study suggested that HCMV induced a postreplicative DNA repair mechanism in the cells. Based on the repair system in bacteria, it has been hypothesised that postreplication repair is an error-prone process leading to the production of somatic mutations and as a result cancer (Lehman, 1974). However, Nishiyama and Rapp (1981) reported that the repair system induced by HCMV appeared to be error proof for UV-damaged HSV DNA when tested with HSV thymidine kinase-negative mutants. Unless HCMV induces error-prone pathways for host cell DNA repair, it would seem unlikely that HCMV causes cellular mutations by the induction of DNA repair mechanisms in cells.

THE DETECTION OF VIRAL DNA WITHIN CIN TISSUE

Forty three CIN biopsies from patients in the West of Scotland were analysed for the presence of HCMV, HPV and HSV-2 DNA sequences. Two biopsies, C2 and C17, were found to contain DNA sequences that hybridized to the HCMV HindIII E fragment of AD169. In C17, these sequences were present at about 0.1 copies/cell and therefore in an amount too low to permit detailed analysis of the hybridizable sequences. In C2, the hybridizable DNA sequences were present at about 20 copies/cell. Further analysis showed that the C2 DNA contained BamHI fragments that comigrated with the Bam

HI, W, P, c and e fragments of HCMV AD169. In addition, two other Bam HI fragments were detected in C2 that hybridized to the HCMV AD169 HindIII E fragment. One was of a higher MW and the other of a lower MW than the BamHI E fragment of HCMV AD169. These additional bands may consitute rearranged viral sequences or fragments of viral DNA present in a chimeric form with cellular DNA. The hybridizable DNA sequences probably represent viral sequences that have been retained after an HCMV infection and have become integrated into the cellular DNA and thus may have been responsible for initiating the development of CIN in this patient. A λ library of the C2 DNA was prepared in an attempt to isolate clones containing these viral sequences. This would have permitted a more detailed analysis of the sequences present. The results of the cloning experiments were very disappointing. Two clones were isolated that contained C2 DNA sequences that hybridized to the HCMV AD169 HindIII E fragment. However, after a few rounds of replication, the purified plaques no longer contained inserts that hybridized to the HCMV probe. The possible explanations for this were discussed previously in Section C. It is possible to re-screen the plaque stocks prepared when the positive clones were originally isolated from the library (ie stocks 1 and 2) and to try to purify the positive clones once again. It may be beneficial to plate out the clones on a BBL agar base in

a BBL trypticase-based top agar to increase the plaque size. This would facilitate the purification procedure. Unfortunately, it was not possible to repeat the procedure within the time allocated for this MRC studentship.

DNA sequences that hybridized to the HPV 11 and HSV-2 Bam^{HI}t probes were detected in CIN tissue. CIN biopsies C13 and C19 were found to contain sequences that hybridized specifically to HPV DNA. However, the remaining sequences detected represented cellular sequences that were homologous to pBR322 or pAT vector sequences present in the probe, as has previously been explained. This stresses the importance of purifying the viral DNA from vector sequences before using it as a probe in hybridization reactions involving cellular DNA.

HPV types 6, 11, 16 and 18 have all been implicated in the development of cervical carcinoma. The type of papillomavirus DNA detected in CIN and carcinomatous tissue is subject to geographical variation. HPV 11 DNA is frequently detected in CIN and carcinomatous tissue from patients in Australia (S. Campo, personal communication) whereas HPV 16 is more frequently detected in tissue from patients in the USA (Prakash et al., 1985; Crum et al., 1984), Germany (Dürst et al., 1985) and the West of Scotland and Northern England (J. Murdoch and J.C.M. Macnab, unpublished results). HPV 11 only shares approximately

59% homology with HPV 16 (Dartmann et al., submitted for publication). Under the conditions of stringency used in this thesis, HPV 16 DNA would not have been detected in the CIN biopsies analysed using the HPV11 DNA probe. It would have been interesting to probe the CIN biopsies with HPV 16 DNA. Unfortunately, it was not worthwhile reprobing the Southern transfers of the CIN DNAs prepared in this study because the level of sensitivity decreased after each wash and was not high enough to detect low copy numbers of viral DNA. Apart from the fact that there was insufficient time, there was not sufficient DNA to make new transfers of CIN DNA to probe with HPV 16 DNA.

HPV sequences have also been detected in normal cervical tissue (J. Murdoch and J.C.M. Macnab, personal communication). The presence of HCMV, HPV or HSV-2 DNA sequences in normal cervical tissue could not be investigated because normal cervical biopsy material was not available from the patients included in this study.

Past investigators have detected HSV-2 DNA in invasive cervical cancer tissue but at a low frequency. HSV-2 DNA sequences have been detected by Southern blot hybridization in 3 of 9 tumours (Galloway and McDougall, 1983), 1 of 8 tumours (Park et al., 1983) and 2 of 13 tumours (Prakash et al., 1985). In the study by Prakash et al. (1985) HSV-2 DNA could not be detected in the 9 CIN biopsy specimens analysed. The absence of HSV-2 DNA

sequences in the CIN biopsies analysed in this study does not therefore come as a surprise.

THE INFECTION OF CERVICAL CELLS BY HERPESVIRUSES

The studies described in Section D demonstrated that cervical cells differed in their susceptibility to herpesvirus infection. Ectocervical cells were found to be fully permissive for HSV-2 replication but not for HCMV replication. No infectious virus could be detected in HCMV infected ectocervical cell cultures and HCMV-specific antigens could only be demonstrated in a small proportion of the infected cells. Thus, HCMV can only replicate to a limited degree within these cells. Stinski et al. (1981) have proposed that nonpermissive cells may favour latency of the viral genome. As ectocervical cells appear to be nonpermissive for HCMV replication, they may therefore be potential sites for latent HCMV infection. In cases where CIN arises within the squamous epithelium of the cervix, latent rather than productive HCMV infection may be involved.

Endocervical cells have been reported to be fully permissive for HCMV replication (Vesterinen et al., 1975) but nonpermissive for HSV-2 replication (Vesterinen, 1976). It was not possible to successfully investigate herpesvirus infection of endocervical cells in these studies due to the difficulties involved in

establishing cultures of endocervical cells.

These studies have shown that the course of viral infection is determined by the nature of the host cell. Fibroblasts are usually employed for investigating *in vitro* viral transformation. As the cells involved in the development of CIN are epithelial in nature, such model systems are not suitable for investigating the potential of a virus to induce cervical neoplasia. These experiments must be done using cervical cells.

REACTIVATION OF LATENT HSV-2 BY HCMV AND ts MUTANTS OF HSV

By incubating infected cultures at 42°C it was possible to establish HSV-2 infections during which infectious virus could not be detected in either Helu (fibroblast) or ectocervical (epithelial) cells. However, infectious virus could be detected in Helu, but not ectocervical cells, if the 42°C treated HSV-2 infected cultures were superinfected with HCMV or *ts* mutants of HSV. The results from Helu cells suggest that HSV-2 established latent infections within the cells at 42°C and that HCMV and the HSV *ts* mutants reactivated the latent virus. Latent infection did not appear to permanently affect HSV-2 replicative functions since the virus was found to replicate efficiently after reactivation. Presumably a viral gene product or

cellular factor induced on superinfection was responsible for reactivating latent HSV-2. Reactivation of HSV-2 from a quiescent state by HCMV was previously demonstrated by Coleberg-Poley et al. (1981) and was attributed to an early HCMV function. Everett and Dunlop (1984) have reported that HCMV can transactivate transcription from the HSV-1 glycoprotein D promoter. During latent infection, the production of infectious virus may be prevented by a block on transcription of early genes. It is a possibility that an IE or early gene product of HCMV can transactivate transcription from an early HSV-2 promoter blocked during latent infection, resulting in reactivation of latent virus.

The ability of HCMV to reactivate HSV-2 from Helu (fibroblast) but not from ectocervical (epithelial) cells may be related to the fact that ectocervical cells are not fully permissive for HCMV replication. The HCMV gene product responsible for reactivation may not be produced in nonpermissive cells where HCMV replication is blocked at an early stage. Alternatively, HCMV infection of permissive cells may induce the production of a host cell factor which is responsible for reactivation. This host factor may not be induced in cells nonpermissive for HCMV replication. Everett and Dunlop (1984) reported that HCMV did not appear to induce glycoprotein D transcription in HeLa cells which are not fully permissive for HCMV

replication. This raises the possibility that transactivation may be involved in reactivation of latent HSV-2 and emphasises again the importance of the cell type in determining which viral functions are expressed. The processes involved in the reactivation of latent HSV-2 from ectocervical cells may be different to those involved in virus reactivation from Helu cells and different stimuli may be required.

HORMONE - VIRUS INTERACTIONS

Cervical cells are continually exposed to various secretions and hormones in vivo and these will probably influence viral infection of the cells. Experiments were carried out to determine whether various hormones could reactivate latent HSV-2 from Helu cells. Neither oestrogen, progesterone or the synthetic steroid dexamethasone could reactivate latent HSV-2 at the physiological concentration of 10^{-9}M or at the higher concentration of 10^{-7}M. Unfortunately, lack of cervical tissue at this time made it impossible to repeat the experiments using ectocervical cells. HSV-2 latent in ectocervical cells may respond to different stimuli from HSV-2 latent in Helu cells. Helu cells have not been shown to possess any oestrogen or progesterone receptors so the hormones may not have been able to exert their effects and produce the physiological changes necessary

for virus reactivation. Ectocervical cells on the other hand do possess oestrogen and progesterone receptors and may therefore respond to the effects of the hormones and result in virus reactivation. It would be worthwhile repeating the experiments using MCF7 cells (a breast cancer epithelial cell line expressing oestrogen receptors) or some other cell line that is known to possess oestrogen and progesterone receptors. It would also be interesting to see if hormones present in the growth medium can affect the titre of HSV-2 reactivated from latently infected cells by HCMV and ts mutants of HSV.

Some hormones have been shown to enhance virus replication. For example, the replication of polyoma virus and HCMV in vitro is greatly enhanced by dexamethasone and cortisol but not by oestradiol or progesterone (Morhenn et al., 1973; Tanaka et al., 1984a, 1984b; Koment, 1985). A few studies have suggested that progesterone increases the incidence of HSV-2 genital infections in mice (Munoz, 1973; Nishiura and Nii, 1976; Baker and Plotkin, 1978; Baker et al., 1980). How the hormones increase viral replication is unknown. They may facilitate viral infection by inducing proliferation of cells permissive for viral replication or by suppressing the immune system. Alternatively, they may have a direct effect on viral transcription or translation. For example,

transcription from a unique site within the mouse mammary tumour virus left hand LTR has been found to be regulated by glucocorticoids (Ucker et al., 1983). Hormonal enhancement of viral replication may contribute to the development of CIN if the virus has oncogenic potential in the host. If the virus is capable of initiating oncogenesis, hormonal enhancement of viral replication would increase the probability of the initiation event occurring.

HORMONES AS COFACTORS IN THE DEVELOPMENT OF CIN

Hormones may function as cofactors in the development of CIN through their ability to stimulate proliferation of cells. Hormones influence the normal repair processes of reepithelialization within the cervix. Oestrogens specifically stimulate proliferation of the stratified squamous epithelium of the ectocervix and progestogens stimulate the columnar epithelium and underlying reserve cells of the endocervix. When a lesion develops in the external surface of the cervix, under oestrogenic stimulation it becomes reepithelialized mainly by the stratified squamous epithelium producing regenerative epithelium. Under progestogenic stimulation, the lesion is repaired by proliferation of the reserve cells beneath the endocervix resulting in reserve cell hyperplasia which

then undergoes maturation through the process of squamous metaplasia. The last stage of the healing process is normally signalled by the appearance of a so-called third mucosa. In a small percentage of cases, however, reepithelialization does not proceed smoothly to normal maturation of the overgrowing epithelium and is instead followed by the development of precancerous lesions of various grades. In these cases, the original lesion may have been exposed to the effects of viral infections and sperm proteins which could have initiated oncogenesis within the cervical cells. During the repair processes, these initiated cells may have been induced to proliferate by specific hormones resulting in the development of cervical dysplasia. From the appearance of the various forms of intraepithelial neoplasia, it is possible to determine the cells from which they originated. For example, dysplasias and CIS of the squamous type develop from regenerative epithelium derived from the stratified squamous epithelium of the ectocervix. On the other hand, mucoid dysplasias and CIS of the reserve cell type develop on the basis of reserve cell hyperplasia.

THE POSSIBLE ROLE OF HCMV IN THE DEVELOPMENT OF CIN

The development of a fully malignant tumour involves complex interactions between several factors,

both exogenous (ie. environmental) and endogenous (ie genetic, hormonal etc). Tumourigenesis is a multistep process but for convenience it is often considered to occur in three stages, initiation, promotion and progression. Initiation requires only a single exposure to a carcinogen and appears to involve genomic changes (eg gene mutation, rearrangement and/or amplification). Promotion involves multiple exposures to agents that do not usually damage DNA directly. Progression involves the conversion of tumours from benign to malignant and can be considered an open-ended process since tumours may continue to increase in their degree of malignancy and heterogeneity. CIN probably represents the progressive stage in the development of invasive carcinoma of the cervix. A number of factors have been implicated in the development of cervical neoplastic disease including viruses (HPV, HSV-2 and HCMV), sperm proteins (Reid, 1964), hormones, (Dallenbach-Hellweg, 1981), the contraceptive pill (Vessey et al., 1980) and smoking (Harris et al., 1983). Because of the multifactorial nature of tumourigenesis, it is probable that all of these factors play a role at some stage in the development of this disease.

The role of HCMV in the development of CIN is speculative. Although HCMV was only detected in 2 of 43 CIN biopsies, the work presented in this thesis suggests that where the virus is present it may have a role in

the development of CIN. Analysis of the viral DNA retained in the CIN tissue showed that it originated from the HindIII E region of HCMV genome and may therefore contain the HCMV promoter and/or enhancer sequences. It is possible that integration of these sequences into the cellular DNA initiated oncogenesis by activating a cellular proto-oncogene. This raises the problem of how the HCMV DNA became integrated into the cellular DNA as integration has not been reported to be a normal event during HCMV replication. Gadler and Wahren (1983) presented evidence to suggest that HCMV DNA integrates or becomes closely associated with a subset of cellular DNA during latent infection. Could latent HCMV infection of the cervix be involved in the development of CIN? Ectocervical cells may be potential sites for latent HCMV infection as has already been discussed. Initiation of the events leading to oncogenesis may occur during virus reactivation when part of the viral genome is still integrated in the cellular DNA. A number of factors could be responsible for reactivating latent HCMV. It is well documented that HCMV is reactivated during pregnancy (Montgomery et al., 1972). In addition, squamous metaplasia and reepithelialization and repair processes occurring within the cervix may bring about the physiological changes within the cervical cells necessary for virus reactivation. All the above mentioned processes are

influenced by hormones. Possibly other factors such as sperm proteins, smoking and the contraceptive pill may cause physiological changes in the cervical cells and lead to virus reactivation.

Alternatively, HCMV may function as a promoter or cofactor in the development of CIN. The consequences of an initiation event do not become apparent until the initiated cells have replicated. Influences which stimulate cell proliferation in tissues containing initiated cells often have promoter or cocarcinogenic action. HCMV stimulates the synthesis of cellular DNA within infected cells and thus has the potential to act as a cocarcinogen in tumour development. It has recently been shown that the tumour promoter 12-0-tetradecanoylphorbol-13-acetate (TPA) induces transformation of mouse epidermal preneoplastic cells by inducing transcription of a gene not normally transcribed in these cells (Lerman et al., 1984). HCMV could act in a similar fashion to TPA in the development of cervical disease.

HCMV may also interact with other viruses. For example, the papillomaviruses are known to initiate the formation of papillomas but these do not progress to malignant tumours unless they are exposed to a promoter. In the case of tumours induced by BPV, the promoting agent is quercetin (a chemical present in bracken leaves). HPV8 causes skin lesions which tend to become

malignant in the skin areas exposed to sunlight. In this case UV is thought to be the promoting agent. Similarly, HPV may initiate oncogenesis of cervical cells and HCMV act as a promoter in the development of cervical neoplasia. HCMV may induce a cellular factor that acts as a tumour promoter or an HCMV encoded early gene product may itself have potential as a promoting agent. HCMV IE gene products have been shown to have the ability to transactivate cellular and viral genes as previously described. Possibly HCMV early gene products can function in *trans* to stimulate transcription from other viral genes involved in tumourigenesis.

Lastly, HCMV may contribute to the development of CIN by changing the methylation pattern of cervical cells. Some, but not all, oncogenically transformed cells have been found to be hypomethylated relative to their normal counterparts. Alterations in DNA methylation of genes have also been reported in some human cancers, for example in colon cancers (Feinberg *et al.*, 1984). DNA methylation is thought to be an important element in the regulation of gene expression (Felsenfield and McGhee, 1982). Changes in the methylation pattern of cells by HCMV may alter transcription of cellular genes important in growth control and lead to cell transformation and tumourigenesis.

FUTURE PROSPECTS

Preliminary investigations with HCMV transformed and tumour cells have suggested that HCMV may initiate cell transformation by activating a cellular proto-oncogene. Identification of the putative cellular oncogene activated in HCMV transformed and tumour cells would provide strong evidence for this mechanism. The activated oncogene could be identified by Southern blot analysis of the HCMV transformed and tumour cell DNAs using various cloned v-onc probes. The next step would then be to try and identify the mechanism of oncogene activation. An interesting experiment would be to transfect a plasmid containing the HCMV HindIII E fragment into Helu cells together with a plasmid containing the putative c-onc gene and then assaying for transcription of the gene. This would help to determine if transactivation was involved in oncogenesis by HCMV. It would also be interesting to investigate the mutagenic potential of HCMV. For example, the ability of UV-irradiated HCMV to induce resistance to 8-azaguanine of infected cells indicating a mutation in the hypoxanthine-guanine-phosphoribosyltransferase gene could be investigated in vitro, as previously described for HSV-1 by Schlehofer and zur Hausen (1982).

The experiments described in this thesis have suggested that HCMV may be involved in the development of CIN. However, it must be stressed that

tumourigenesis is a multifactorial, multigenic process and HCMV will not be the only factor involved. Further investigations are, however, necessary to provide stronger evidence for an association of HCMV with CIN. It would be useful to determine if HCMV-specific RNA is present within CIN tissue and hence determine if HCMV DNA has been transcribed in neoplastic cells. It may also prove interesting to screen the DNA from other CIN biopsies for activated oncogenes. It would be very worthwhile to try again to purify the C2 clones that hybridized to HCMV DNA sequences. Such clones could provide valuable clues as to the possible role of HCMV in the development of CIN in this patient. Sequence analysis of the HCMV DNA retained in this CIN would determine if HCMV promoter, enhancer or IS-like sequences may have been involved in initiating neoplasia. The cloned C2 viral sequences could also be used in transfection experiments with other plasmid-borne genes to determine if the retained viral sequences have transactivating ability.

There is the possibility that the mechanism involved in the transformation of RE cells by UV-irradiated HCMV may be different to that involved in the development of CIN. It would be ideal if cervical cells could be used in in vitro transformation studies with HCMV, but unfortunately the limited lifespan of these cells precludes their use in long term experiments. However, it is important to further

investigate the infection of cervical cells by herpesviruses. The *in vitro* experiments described in this thesis for establishing latent HSV-2 infection in cervical cells provide a suitable system for investigating the interaction between different viruses and between hormones and viruses, as well as latency. Further experiments could be undertaken to determine the state of the viral genome during latency and to compare the DNA restriction profiles of reactivated virus with those of wild type virus, to see if any rearrangement of viral sequences occurred during latency. The reactivation of latent HSV-2 by HCMV could be further investigated by identifying the region of the HCMV genome involved in reactivation. Reactivation studies using UV-irradiated HCMV would determine if early functions are involved. However, precise identification of the viral genes involved would be difficult since HCMV *ts* mutants are unstable. It would also be interesting to investigate reactivation of latent HSV-2 by HCMV in a variety of cell lines to establish whether the infected cell must be permissive for HCMV replication before reactivation can occur. It was suggested in this study that transactivation may be involved in reactivation. This could be further investigated by carrying out experiments to see if IE HCMV gene products can transactivate the HSV-1 glycoprotein D promoter in ectocervical cells. It was found in this study that hormones could not reactivate

latent HSV-2 from Helu cells. However, the mechanism of reactivation may be different in Helu and ectocervical cells so it would be useful to see if latent HSV-2 can be reactivated by female hormones from ectocervical cells or from HeLa or MCF7 cells which contain female hormone receptors.

Lastly, it is essential to investigate the association of HCMV infection with the development of CIN in an animal model. This could be done by treating the cervices of experimental mice with formalin-inactivated or UV-inactivated HCMV to see if prolonged exposure to inactivated virus can induce premalignant and malignant cervical lesions. This was carried out successfully using HSV-2 by Wentz et al. (1981).

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DETECTION OF SEQUENCES THAT HYBRIDIZE TO HUMAN CYTOMEGALOVIRUS DNA IN CERVICAL NEOPLASTIC TISSUE

by

K. Fletcher[1], J.W. Cordiner[2], and J.C.M. Macnab[1]

1 Medical Research Council, Virology Unit, Instititute of Virology, University of Glasgow, Church St., Glasgow, G11 5JR, Scotland

2 Queen Mother's Hospital, Yorkhill, Glasgow, G12 8QQ, Scotland

ACCEPTED BY 'DISEASE MARKERS'.

ABSTRACT

Punch biopsies were taken from patients with cervical intraepithelial neoplasia and the DNA was extracted and examined for sequences which hybridized to human cytomegalovirus DNA by Southern blotting analysis. Two biopsies out of forty three were found to contain DNA which hybridized to human cytomegalovirus DNA. In one biopsy, DNA sequences were detected which contained four restriction sites colinear with those of the prototype strain AD169. Evidence of rearranged viral DNA sequences was also found.

INTRODUCTION

Several epidemiological studies (Rawls et al., 1968; Nahmias et al., 1970) and molecular studies (Eglin et al., 1981; Park et al., 1983; Macnab et al., 1985; Galloway and MacDougall, 1983) have linked cervical neoplastic disease to infection by herpes simplex virus (HSV) type 2. Although most studies reveal an association between HSV infection and cervical neoplasia, in some geographical areas such an association is not observed and not all women with cervical neoplasia have serologic evidence of past HSV infection (Vonka et al., 1984). These factors indicate a possible multifactorial etiology of cervical neoplasia and recently it has been suggested that HSV-2 may interact as a cocarcinogen with other agents that infect the cervix (zur Hausen, 1982). Human papillomaviruses (HPV) have been strongly implicated in cervical neoplasia. HPV DNA has been detected in DNA from biopsies of both cervical intraepithelial neoplasia (CIN) and invasive carcinomas (Gissmann et al., 1983; McCance et al., 1983; Dürst et al., 1983, 1985; and Prakash et al., 1985). Human cytomegalovirus is another infectious agent with carcinogenic potential and epidemiologic features compatible with cervical neoplasia as described below.

HCMV frequently infects the cervix, has been demonstrated in the uterus, prostate and semen and can be sexually transmitted. The virus has been isolated from the genital tract and urine of sexual partners (Chandler et al. personal communication; Chretien et al., 1977) and has been identified as a common cause of cervicitis and urethritis following sexual transmission (Evans, 1976). It has even been isolated more frequently than HSV-2 from women attending one venereal disease clinic (Jordan et al., 1973). Like HSV-2, HCMV possesses oncogenic potential. Primary rat embryo cells have been transformed with UV-irradiated HCMV AD169 and these transformed cells are

mourigenic when inoculated into Hooded Lister rats (our unpublished data). These results support the earlier observations by Albrecht and Rapp, 1973 Rapp et al., 1975; Geder et al., 1976; and Nelson et al., 1982. In a study by Melnick et al. (1978) HCMV was isolated from two cell cultures derived from cervical biopsies of patients with advanced carcinoma. Surprisingly, few studies have investigated the possible association between HCMV and cervical neoplasia. The few seroepidemiological studies that have been done have yielded controversial results. Vestergaard et al. (1972) reported significantly higher levels of complement fixing antibodies to HCMV in patients with cervical carcinoma compared to matched controls and Pacsa et al. (1975) detected complement fixing antibodies to HCMV more frequently in the sera of women with atypia than in women with cervical disorders other than atypia or healthy controls. Stoian et al. (1982) also reported a higher incidence of complement fixing antibodies to HCMV in patients with breast, cervical and ovarian neoplasms. In contrast to these studies, other groups have been unable to correlate elevated antibody levels to HCMV with cervical neoplasia (Fucillo et al., 1971; Sprecher-Goldberger et al., 1971; Kumar et al., 1980; Hart et al., 1982; Best et al. personal communication).

Possibly becuase of the general low correlation between the presence of HCMV antibodies and cervical neoplasia, little attention has been given to the detection of HCMV-specific nucleic acids in cervical preneoplastic tissue. To our knowledge there has only been one such study in which HCMV DNA was detected in both normal and neoplastic cervical tissue by DNA-DNA reassociation kinetic analysis (Huang et al., 1983). The results were, however, subject to geographical variation. We report below the presence of sequences that hybridize to HCMV DNA in 2 of 43 punch biopsies analysed from patients with CIN in the West of Scotland; these patients had all bee

[redacted]red to a colposcopy clinic in the Western Infirmary, Glasgow, [redacted]h abnormal cervical cytology observed on examination of cervical smears.

MATERIALS AND METHODS

Preparation of virus and viral DNA

Plaque-purified HCMV strain AD169 (supplied by Dr. J.D. Oram, PHLS, Porton Down, Salisbury) was propagated in Flow 2002 human foetal lung cells infected at a m.o.i. of 0.01pfu/cell. The supernatent was collected daily from day 7 to day 11 post infection and titrated according to the method of Wentworth and French (1970). To extract the viral DNA, the cell debris was removed from the medium by centrifugation at 2K and the virus pelleted at 13K for 90 min; the pellet was resuspended in TNE (0.05M Tris. HCl pH7.4, 0.005M EDTA, 0.15M NaCl) and treated with DNase (50ug/ml) for 30 min in the presence of 10mM $MgCl_2$; EDTA was then added to a final concentration of 50mM and the virus repelleted, resuspended in TNE plus 0.2% SDS and digested overnight with proteinase K (200ug/ml). The nucleic acids were extracted with phenol and chloroform and then digested with RNaseA (100ug/ml) in 0.01M Tris. HCl pH7.5 0.001M EDTA and 0.01M NaCl. The DNA was subsequently re-extracted with phenol and chlorofom, dialysed extensively against 0.1xSSC (1xSSC is 0.015M sodium citrate plus 0.15M NaCl) ethanol precipitated and finally resuspended in 0.01M Tris. HCl pH7.5 and 0.001M EDTA.

Preparation of cell and CIN DNA

Control DNA was prepared from cervical tissue of routine hysterectomy specimens from five women with no evidence or history of neoplastic disease. We did not obtain ethical approval to take normal cervical samples from the same patients. In a future study this control and also more biopsy samples from patients with no evidence of cervical disease would be obtained.

CIN DNA was extracted from punch biopsy specimens taken from patients attending a colposcopy clinic at the Western Infirmary,

sgow. All specimens were kept on ice and the DNA extracted within hrs. DNA was prepared as previously described by Varmus et al. (1973) and Park et al. (1983).

Cloned restriction DNA fragments used as probes

The cloned restriction fragments used as probes were kindly supplied by Dr. J.D. Oram, PHLS, Porton Down, Salisbury (Figure 1). HindIII restriction framgments of HCMV AD169 DNA has been cloned into the HindIII site of pAT153 and propagated in E.coli strain HB101 (J.D. Oram et al., 1982). HB3 contained a HindIII/BamHl subfragment of the HindIII E fragment containing the transforming region identified by Nelson et al. (1982) cloned into the BamHl site of pAT153. Plasmid propagation and purification were carried out as previously described by Davison and Wilkie (1983). Before use as probes, all the cloned HCMV DNA sequences were excised from vector sequences and purified twice by agarose gel electrophoresis: Rigorous purification was necessary to eliminate the possibility of vector sequences hybridizing to some human CIN DNAs.

Southern blot hybridization

High molecular weight DNA extracted from CIN or control tissue was digested with an excess of either HindIII or BamHI enzyme using the buffer conditions recommended by the suppliers. The products of digestion were fractionated by electophoresis through 0.6% agarose gels and transferred to either Biodyne (Pall) or Gene screen plus (New England Nuclear) nylon filters using the procedure of Southern (1975) HCMV DNA fragments were labelled in vitro with [α-^{32}P]dCTP and [α-^{32}P]dGTP by nick translation (Rigby et al., 1977) to specific activities of 1-5x10^{8} cpm/ug DNA. Probes were denatured by boiling for 15 min in 0.2M NaOH and hybridized overnight at 70^{o}C in 6xSSC, 0.5% SDS, 0.02% ficol, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin and 50ug/ml denatured salmon sperm DNA. Gene screen plus filters were washed twice in 2xSSC for 5 min at room temperature, twice in 2xSSC, 1% SDS for 30 min at 70^{o}C and then twice in 0.1xSSC, for 30 min at room temperature. Biodyne filters were washed at 70^{o}C

three 30 min stepwise decreasing washes of SSC from 3 x to 0.1.
e filters were exposed for autoradiography with Dupont Lighting plus
intensifier screens to X-Omat (Kodak) film for 2-9 days.

RESULTS

Analysis of CIN DNA for HCMV-related sequences

Hybridization of the HindIII/BamHI subclone HB3 of HCMV HindIII E AD169 to CIN DNAs digested with HindIII enzyme revealed that 2 biopsies, C2 and C17, contain sequences that hybridize to this probe (Figure 2a,b). By comparison with the reconstruction tracks, the hybridizable DNA sequences were present at about 20 copies per cell in C2 and at less than 0.1 copy per cell in C17. All other CIN biopsies were negative for hybridization at levels of sensitivity which would detect 0.2 copies per cell of HCMV DNA restriction fragment.

Analysis of the sequence arrangement in C2 and C17

C2 and C17 CIN DNAs were analysed further by digestion with BamHI and hybridization to the HindIII E fragment. The HindIII E fragment hybridizes to five Bam HI fragments E, W, P, c and e of the AD169 genome. The HindIII E probe hybridized to DNA sequences in C2 that comigrated with BamHI fragments W, P, c and e (Figure 3) and also to two additional fragments, one of a higher molecular weight and one of a lower molecular weight than the BamHI E fragment of AD169. This may reflect rearrangement of viral DNA sequences within the CIN DNA and also loss of restriction sites.

BamHI digestion of C17 CIN DNA and hybridization to HindIII E resulted in very faint hybridization to a band colinear with the BamHI W fragment indicating that the C17 DNA does share restriction sites with AD169. We conclude that BamHI digestion of C17 DNA either cleaved the hybridizable DNA into some fragments too small to be successfully detected with the HindIII E probe or else that not all the fragments present in virion DNA were equally represented in this viral insert in CIN17.

As there was the possibility that the C2 CIN biopsy had been

ected *in vivo* by an isolate of HCMV, we hybridized the BamHI igested C2 DNA to HindIII fragments encompassing the entire AD169 genome. No hybridization was obtained to any other regions of the HCMV genome (results not shown). Had the biopsy been infected with HCMV, other regions of the genome would have been represented in equimolar amounts in the sample. Furthermore, antibodies to HCMV could not be detected in the sera of the patient from whom the C2 biopsy had been taken. It would thus seem unlikely that the hybridization to the HindIII E fragment represented a current infection of the C2 cervical biopsy with HCMV.

DISCUSSION

We have detected sequences that hybridize specifically to HCMV DNA in two out of 43 CIN biopsies analysed. Both positive CIN biopsies contained sequences that hybridized to the HindIII E fragment of HCMV AD169. In one CIN biopsy (C2) these hybridizable sequences were present at about 20 copies per cell and shared several characteristic restriction sites with the prototype AD169. There was also evidence of hybridization which we interpret to be rearrangement of viral sequences having led to loss of restriction sites. The hybridizable DNA in the second CIN biopsy (C17) was not present in sufficient copy number to determine the sequence arrangement. The detected HCMV DNA sequences are thought to represent viral sequences that have been retained after an HCMV infection by integrating into the cellular DNA and information in these sequences may be responsible for initiating the development of CIN in these two patients.

The role of HCMV in the development of CIN is speculative although molecular evidence of the persistence of HCMV DNA sequences in some cases of CIN is compelling. Analysis of the viral DNA retained in the CIN tissue showed that it originated from the HindIII E region of the HCMV AD169 genome. This very region of the viral genome contains a DNA sequence which when transfected into NIH 3T3 cells, can initiate colony formation in methyl cellulose and tumourigenicity in nude mice (Nelson et al., 1982). It is possible that integration of this transforming region into the cellular DNA of this CIN patient (C2) initiated the development of neoplasia. There is no evidence that the transforming region of HCMV codes for a viral transforming protein or that it shares homology with any of the known retroviral oncogenes (Kouzarides et al., 1983; Nelson et al., 1984). The transforming region does, however, contain an insertion sequence like structure which could initiate transformation either by

tivating a cellular proto-oncogene, or through mutagenic action by nonspecifically integrating into, and perhaps excising imprecisely from, cellular genes at random (Galloway et al., 1984).

In addition to the transforming region, the HindIII E fragment of the HCMV genome contains both the regulatory promoter and enhancer sequences of the major IE gene (Akrigg et al., 1985; Boshart et al., 1985). The hybridization results have shown that DNA corresponding to this region of the AD169 genome appears to be rearranged within the C2 DNA. This could have occurred when the viral DNA became integrated into the cellular DNA in this patient. The rearrangements may not have occurred within the promoter or enhancer sequences but in the intervening sequences. A λ library of the C2 DNA has been constructed and clones that hybridize to the HCMV HindIII E fragment are currently being identified. Sequence analysis of these clones will determine the nature of the DNA rearrangement observed and whether HCMV promoter or enhancer sequences are present.

If the C2 DNA contains the HCMV promoter or enhancer sequences then their integration could have activated a cellular proto-oncogene and initiated cervical neoplasia. Activation of a proto-oncogene by promoter insertion was first proposed by Neel et al. (1981) to explain the induction of bursal lymphomas by avian leukosis virus. Activation by enhancer insertion was proposed to explain the induction of tumours by slow tranforming retroviruses in which the integration of the retroviral proviruses is not compatible with the promoteer insertion model (Payne et al., 1982). Because enhancers are able to stimulate the transcription of a gene from a 5' or a 3' position in either orientation and over relatively large distances, the enhancer sequences can insert into the cell DNA at random and still activate a cellular proto-oncogene.

Tumourigenesis is a multifactorial and probably a multigenic process. Clearly HCMV is not the only factor involved in the

velopment of CIN and invasive carcinoma of the cervix. Other factors implicated in the development of the disease include HSV-2, HPV, sperm proteins (Reid, 1964), the contraceptive pill (Vessey et al., 1983) and smoking (Harris et al., 1983). Cervical carcinoma or CIN may arise by different mechanisms determined by the agents to which the patient has previously been exposed. In some pateints HCMV may function as a promoter of cocarcinogen rather than as an initiator in the development of CIN. For example, HCMV is known to stimulate host cell macromolecular synthesis (St Joer et al., 1984; Tanaka et al., 1985). The virus may therefore induce the proliferation of already initiated cells and hence contribute to the development of neoplasia. Cell proliferation may also facilitate secondary infection of cervical cells with HSV-2 or HPV and these viruses could either initiate transformation or be involved at certain differentiation stages leading to carcinogenesis. We have screened the C2 and C17 biopsies for HSV-2 and HPV11 DNA sequences. No HSV-2 or HPV11 DNA could be detected in either of these biopsies. In other patients factors other than HCMV may be involved.

Cervical neoplasia is regarded as the end result of a complex chain of events probably involving a number of enviornmental agents. This study provides evidence that HCMV may play a role in the development of CIN, the precursor stage to cervical carcinoma.

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FIGURE LEGENDS

Figure 1

Diagram of the HCMV AD169 genome showing the location of the cloned restriction fragments used as probes in blot hybridizations. Solid boxes represent terminal and internal repeat sequences which bind the long (L) and short (S) unique components of the genome. Nomenclature taken from J.D. Oram et al. (1982). All cloned HCMV DNA sequences were excised from vector sequences and purified twice by agarose gel electrophoresis prior to labelling in vitro with [α-^{32}P]CTP and [α-^{32}P]GTP to specific activities of 1-5x10^{8}cpm/ug DNA by nick translation (Rigby et al., 1977).

(Figure A.6. in this thesis)

Figure 2

Detection of fragments of HCMV DNA in DNA from CIN biopsies (nos. 1,2,16,17,20,24,25,27). From each CIN punch biopsy 20ug of high molecular weight DNA were digested with an excess of HindIII, fractionated through 0.6% agarose gels, transferred to either Biodyne Pall (a) or Gene screen plus NEN (b) membranes by the procedure of Southern (1975) and hybridized to a ^{32}P-labelled HB3 probe excised from vector sequences. Blots were exposed for autoradiography with Dupont Lighting plus intensifier screens for 2 (a) and 9 (b) days. Lanes RE and NC are control tracks containing 20ug of HindIII digested rat embryo and normal cervix DNA respectively. Lanes Rc.2, Rc1 and Rc5 contain reconstructions of .2 copy (0.148ng) 1 copy (0.74ng) and 5 copies (3.7ng) respectively of HCMV AD169 DNA plus 20ug of rat embryo DNA digested together with HindIII. pAT represents a control reconstruction containing 5 copies per cell of cloned vector DNA plus 20ug of rat embryo DNA. The HB3 probe hybridized to DNA sequences present in C2 and C17 DNA. Size markers are given in kilobase pairs.

(Figures B.1 and B.3. in this thesis)

gure 3

Detection of fragments of DNA containing HCMV DNA sequences following cleavage of C2 DNA with BamHI. 20ug of C2 DNA were digested with an excess of BamHI, fractionated through a 0.6% agarose gel, transferred to Gene screen plus membrane and hybridized to a ^{32}P-labelled HCMV AD169 HindIII E probe. Lanes RE and NC are control tracks containing 20ug of BamHI digested rat embyro and normal cervix DNA respectively. Reconstruction tracks with 0.2 (Rc.2) 1 (Rc1) and 5 copies of HCMV AD169 (Rc5) are included on the blot. The HindIII E probe hybridizes to five HCMV AD169 DNA fragments generated after BamHI digestion. The probe hybridized to viral DNA sequences in C2 which are co-linear with the BamHI fragments W, P, c and e and also to two additional bands which probably represent viral sequences present in a rearranged form and in which some restriction sites are possibly deleted.

(Figure B.2. in this thesis)

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FIGURE A.6 HindIII - CLEAVAGE MAP OF HCMV STRAIN AD169

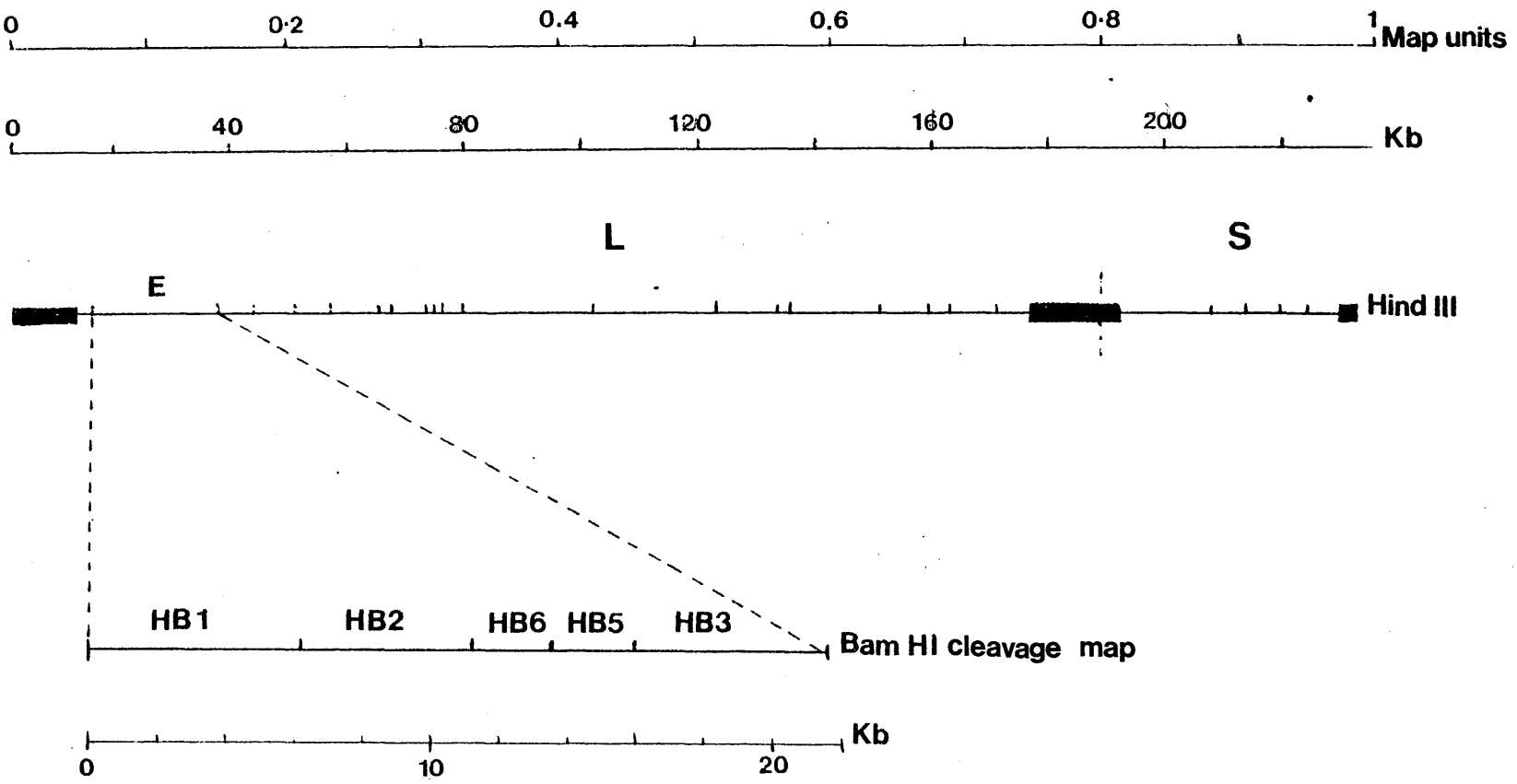
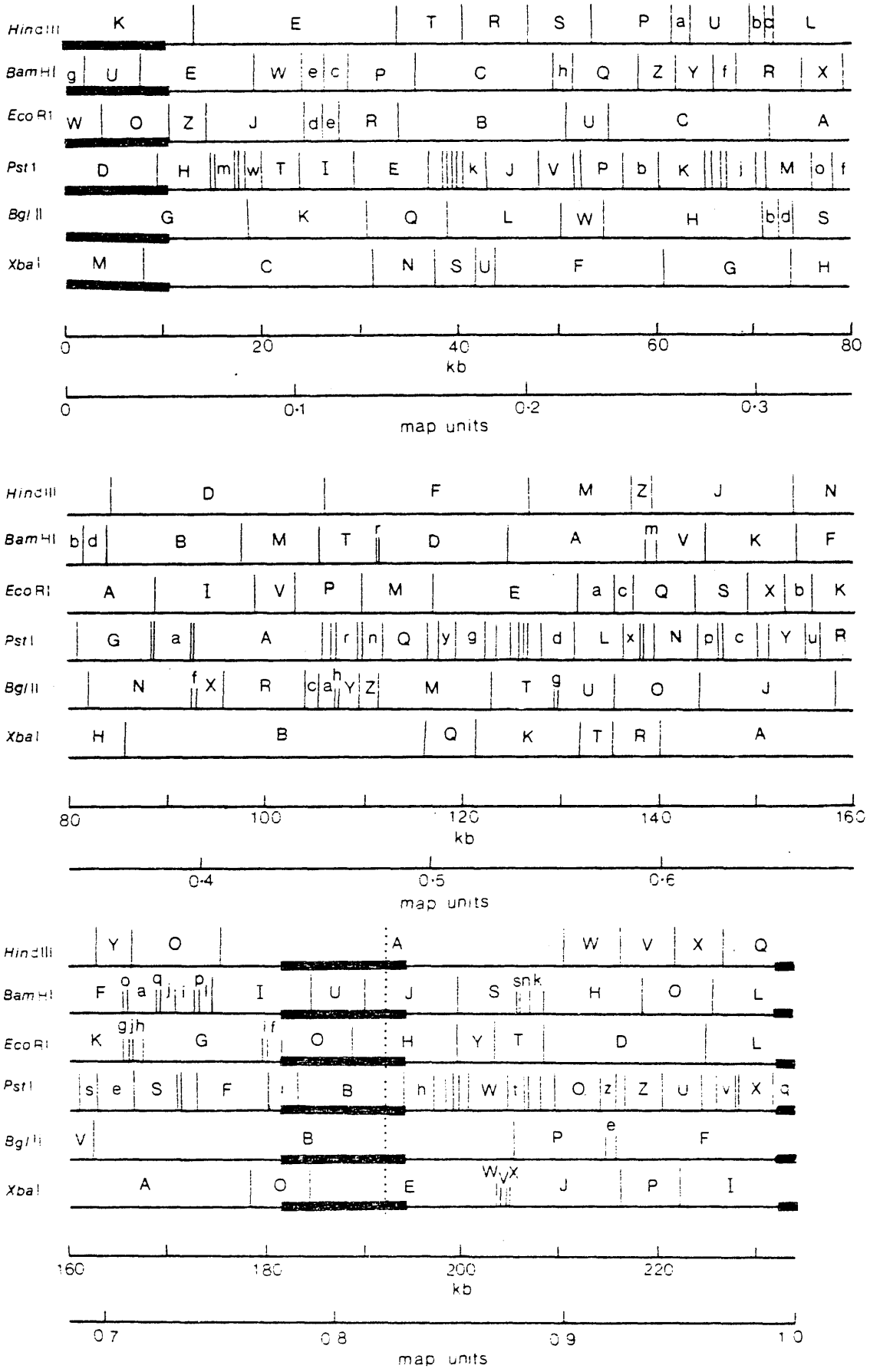


FIGURE 2


Hind III
BamHI
EcoRI
Pst I
Bgl II
Xba I
0
20
40
60
80
kb
0
0·1
0·2
0·3
map units
80
100
120
140
160
kb
0·4
0·5
0·6
map units
160
180
200
220
kb
0·7
0·8
0·9
1·0
map units