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REGULATION OF HUMAN CYTOMEGALOVIRUS STRAIN AD169 GENE EXPRESSION

by

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To Maria and Euan

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DECLARATION

The work described in this thesis was performed by the author and has not been submitted for examination elsewhere. For ease of reference the results section contains details of plasmid constructions performed by others; where this occurs it is acknowledged in the text.

ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ATP	Adenosine triphosphate
BAP	Bacterial alkaline phosphatase
bp	Base pairs
CAT	Chloramphenicol acetlytransferase
CCV	Channel catfish virus
C.F.	Complement-fixing
CID	Cytomegalic inclusion disease
CMV	Cytomegalovirus
CMV-GF	Cytomegalovirus growth factor
c.p.e.	Cytopathic effect
CTL	Cytotoxic T lymphocyte
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddATP	Dideoxyadenosine triphosphate
ddCTP	Dideoxycytidine triphosphate
ddGTP	Dideoxyguanosine triphosphate
ddTTP	Dideoxythymidine triphosphate
DFMO	Difluotomethylornithine
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dTTP	Deoxythymidine triphosphate
EBV	Epstein Barr virus
EC	Human embryonal carcinoma cells
EDTA	Ethylenediaminetetra-acetic acid
EHV-1	Equine herpesvirus type 1
Fc	Constant fragment of Ig
FITC	Fluorescein isothiocyanate
g	Glycoprotein (HSV-1)

gp	Glycoprotein (HCMV)
HCMV	Human cytomegalovirus
HEF	Human embryonic fibroblast
HIV	Human immunodeficiency virus
hsp	Heat shock protein
HSV-1	Herpes simplex type 1
HSV-2	Herpes simplex type 2
HST	Hybrid-selected translation
HVS	Herpesvirus saimiri
ICSP	Infected cell-specific polypeptide
IE	Immediate early
Ig	Immunoglobulin
IPTG	Iso-propyl-β-d-thio-galactopyranoside
IR	Internal repeat
K	Thousand daltons
kb	Kilobase or kilobase pairs
L	Long component of the genome
LTR	Long terminal repeat
m.o.i.	Multiplicity of infection
MOPS	Morpholinopropanesulphonic acid
mRNA	Messenger ribonucleic acid
n	nucleotides
NIEPs	Non-infectious enveloped virus particles
NF-1	Nuclear factor 1
NP40	Nonidet P40
NK	Natural killer
O.D.	Optical Density
Oligo(dT)	Oligodeoxythymidilic acid
ORF	Open reading frame
PAA	Phosphono acetic acid

PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocyte
PEG	Polyethylene glycol
PFA	Phosphonoformic acid
p.f.u.	Plaque forming unit
p.i.	Post infection
PIPES	Piperazine-N,N'-bis[2-ethanesulphonic acid]
Poly(A)	Polyadenylic acid
PRV	Pseudorabies virus
p.t.	Post transfection
R	Purine
RIT	HCMV-induced repressor of IE transcription
RNA	Ribonucleic acid
RSV	Rous sarcoma virus
S	Short component of the genome
S	Svedberg unit
SDS	Sodium dodecyl sulphate
ssDNA	Single-stranded deoxyribonucleic acid
TEMED	N,N,N',N'-Tetramethylethylene diamine
TIF	Virion *trans*-inducing factor
TK	Thymidine kinase
TLC	Thin layer chromatography
TR	Terminal repeat
Tris	Tris(hydroxymethyl)aminoethane
tRNA	Transfer ribonucleic acid
U_L	Long unique region of genome
UTP	Uridine triphosphate
U_S	Short unique region of genome
u.v.	Ultra violet light
Vmw	Viral protein, molecular weight x 10^{-3} (HSV-1)

v/v Volume/volume

VZV Varicella zoster virus

w/v Weight/volume

X-gal 5-bromo-4-chloro-3-indoyl-β-galactoside

Y Pyrimidine

SUMMARY

Human cytomegalovirus (HCMV) strain AD169 encodes a single abundant 1.95kb immediate early (IE) mRNA and a single abundant 2.7kb early RNA. The major IE gene (0.756-0.745 map units) was shown in nuclease protection experiments to encode a spliced molecule of 1,736 nucleotides (excluding the poly (A) tail) consisting of four exons of 121, 88, 185 and 1,342 nucleotides. Three introns of 827, 114 and 170 nucleotides were located near the 5' end of the gene. The structural analysis of the major IE gene enabled the amino acid sequence of the major IE polypeptide to be deduced from the DNA sequence. The major early gene, which is contained in both copies of the HCMV long repeat, was found not to be spliced. A translation product of the 2.7kb early RNA has yet to be identified. Reporter genes were used in transient DNA transfection experiments to monitor expression from HCMV and other viral promoters. HCMV infections *trans*-activated expression from the transfected SV40 early, Rous Sarcoma virus, HSV-1 thymidine kinase (TK), the HCMV major IE and the HCMV major early promoters. Expression from both the HCMV IE and the HSV-1 TK promoters was stimulated much more gradually by HCMV than by HSV-1 infections. Experiments performed using u.v.-irradiated virus and inhibitors of HCMV replication indicated that the transfected IE promoter was stimulated primarily by a *de novo* synthesised HCMV-encoded gene product(s). When the concentration of the plasmids IEP1catIEterm and AccHincat transfected into cells was lowered sufficiently, HCMV infection was observed to repress expression from the transfected IE promoter. A sequence in the HCMV major IE gene between -299 and +69 apparently contains a *cis*-acting signal which responds to an HCMV-induced repressor. Competitive co-transfection experiments indicated that an HCMV-induced repressor of IE transcription interacts with at least three distinct regions within the IE promoter.

INTRODUCTION

1:1 Classification

Human Cytomegalovirus (HCMV) is a member of the family Herpesviridae. The basic morphology of herpesvirions has been conserved throughout the family and is a basis for classification. Herpesvirions contain a single icosadeltahedral capsid (110-120nm in diameter), consisting of 162 capsomeres, enclosed in a condensed protein coat or tegument of variable size and an outer lipoprotein envelope (Wildy et al., 1960). The capsid contains a linear double-stranded DNA molecule. Characteristically, replication of herpesvirus genomes and capsid assembly takes place in the nuclei of infected cells.

Herpesviruses have been observed to infect a wide range of host species including fungi (Kazama and Schornstein, 1972), crustaceans (Farley et al., 1972), fish (Wolf and Darlington, 1971), amphibians (Lunger, 1964), birds (Churchill and Biggs, 1967) and numerous mammals (Roizman et al ., 1981). There are at least five human herpesviruses (HSV-1, HSV-2, VZV, HCMV and EBV) all of which cause clinically important diseases.

The Herpesviridae has been divided into three subfamilies primarily on the basis of the biological properties of the viruses in vitro and in vivo:

(a) Alphaherpesvirinae: In vivo alphaherpesviruses usually infect epithelial cells giving rise to vesicular lesions. Following primary infection alphaherpesviruses frequently persist in a latent form in the neuronal cells of ganglia. The latent virus may periodically reactivate giving rise to secondary infections. The alphaherpesviruses have a relatively short growth cycle (less than 18h) and can exhibit a narrow or wide host range both in vitro and in vivo. The genomes of alphaherpesviruses range in size between 130kb (channel catfish virus; Chousterman et al., 1979) and 151kb (Saimiriine herpesvirus 1; Roizman et al., 1981) and typically have a high

G+C content although values vary from 32% (Canid herpesvirus 1; Plummer et al., 1969) to 74% (PRV; Honess and Watson, 1977). DNA hybridisation experiments have shown that HSV-1, HSV-2, EHV-1, PRV and VZV all share significant DNA sequence homology and that homologous regions are arranged in a similar order ("essentially colinear") on the genomes of these alphaherpesviruses (Davison and Wilkie, 1983).

(b) Betaherpesvirinae: The betaherpesvirinae contains the cytomegaloviruses of man, monkeys, guinea pigs, mouse and other mammals. Characteristically, the betaherpesviruses are slow growing, have a restricted host range (in vitro and in vivo) and produce both nuclear and cytoplasmic inclusions in infected cells. The betaherpesviruses have relatively large genomes, ranging in size between 144kb (bovine CMV; Ehlers et al., 1985) and 240kb (murine CMV; Mercer et al., 1983) and have a G+C content of between 50% to 60% (Honess, 1984).

(c) Gammaherpesvirinae: The gammaherpesviruses have a restricted host range and usually replicate in lymphoblastoid cells, although some can lytically infect fibroblast and epithelial cells. Gammaherpesviruses can persist in cell populations as episomal DNA elements exhibiting restricted gene expression. The genomic DNA of this subfamily ranges in size from 105kb (Bovid herpesvirus 4; DeVilliers, 1979) to 166kb (Mareks disease virus; Lee et al., 1971). The G+C content of gammaherpesvirus DNA, with the exception of the EBV-like viruses of old world monkeys (approx. 59%), is relatively low (Honess, 1984).

1:2 Historical Perspective

The earliest identification of a HCMV infection has been attributed to Ribbert (Ribbert, 1904; Ho, 1982) who in 1881 identified 'protozoan-like' cells in the kidney of a still-borne foetus. Ribbert appreciated his

observations were significant when Jesionek and Kiolenenoglou (1904) described the presence of large cells containing intranuclear bodies in the kidneys, lungs and liver of an eight month foetus. The 'protozoan-like' cells identified by Jesionek and Kiolemenoglou contained an eccentrically positioned nucleus with intranuclear inclusions. It is now appreciated that these early studies describe fatal cases of congenitally acquired cytomegalic inclusion disease (CID). At the time, however, the enlarged cells were mistaken for protozoa.

Goodpasture and Talbot (1921) first coined the term 'cytomegalia' to describe the enlarged cells isolated from the kidneys, lungs and liver of a six week old infant at post mortem. The presence of cytomegalic cells in more than one organ was interpreted as indicating that the pathogen was carried in the blood stream. Goodpasture and Talbot believed that the cytomegalia was similar to the effect observed in VZV infected cells and was not due to the presence of a protozoan. Von Glahn and Pappenheimer (1925), describing the first report of a cytomegalia in the post mortem of an adult, suggested that the presence of nuclear inclusions indicated a nuclear infection, possibily caused by an agent related to the herpesvirus group.

Jackson (1920) observed that the salivary glands and kidneys of apparently healthy laboratory guinea pigs frequently contained cytomegalic cells. Extracts prepared from cells exhibiting a cytomegalia were injected into the salivary glands of young guinea pigs and cells containing typical nuclear inclusion detected in the animals 7 to 15 days post inoculation (Cole and Kuttner, 1926). The causative agent of the infection was shown to be a virus by its ability to pass through the appropriate filters. When extracts containing the virus were injected into animals other than guinea pigs nuclear inclusions were not observed (Cole and Kuttner, 1926); this was an early indication of the species-specificity exhibited by members of the CMV (betaherpesvirinae) group.

In vitro cultivation of guinea pig CMV was first achieved in minced fragments of guinea pig tissue which were being maintained in Maitland's medium (Andrewes, 1930). Infected cells containing a single nuclear inclusion, initially detected between 48 to 72h p.i., were observed to increase in size during the course of an infection. Although the *in vitro* neutralisation of guinea pig CMV by immune sera was demonstrated by Andrewes (1930) serial subculture of the virus *in vitro* was not achieved at this time.

In the 1950's Dr Margaret Smith, responding to the high incidence of CID being reported in infants and young children at post mortem, systematically set out to propogate CMV *in vitro* (reviewed Weller, 1970). *In vitro* serial subculture of a cytomegalovirus was first achieved with the murine Salivary Gland Virus (Smith, 1954). An extract was prepared from the salivary glands of an infected mouse and added to murine embryonic tissue being cultured in a complex serum-rich medium which contained antibiotics. Large, rounded, refractile cells were first detected in the monolayer between 6 to 7 days p.i. and in the following 2 to 3 days the c.p.e. spread throughout the cell culture. Murine CMV was serially passaged *in vitro* and then reinoculated into mice where the virus again produced characteristic cytomegalic inclusions. Virus titration demonstrated that there was a large increase in the number of infectious particles during *in vitro* passage. A similar protocol was then used successfullly to subculture two HCMV isolates in human uterine wall tissue (Smith, 1956).

Working independently two other groups isolated and subcultured HCMV shortly after Smith (Weller, 1970). Rowe and co-workers, while culturing adenoid tissue removed from young children during surgery, fortuitously detected cytomeglia in three of the cultures after incubation periods of between 22 to 51 days; one of the isolates (strain AD169) obtained by Rowe *et al.* (1956) was used in experiments described in this thesis. Weller *et al.*, (1957) fortuitously isolated HCMV strain Davis using a procedure which

was originally designed to culture toxaplasma from lung biopsies. The similar c.p.e.'s produced by strains AD169 and Davis in tissue culture cells were sufficiently distinctive to differentiate the HCMV infections from those of VZV or other human herpesviruses (Weller et al., 1957).

The three most commonly used laboratory strains of HCMV are strains AD169, Davis and Towne. The Towne strain was isolated in 1970 from a two month old infant who survived microcephaly and hepatosplenomegaly caused by a congenital infection (Plotkin et al., 1975).

1:3 Epidemiology

The ability to serially culture HCMV *in vitro* facilitated the development of diagnostic techniques necessary to investigate HCMV epidemiology. The detection of antibodies directed against HCMV in an individual's blood, using techniques such as complement fixation, is evidence of an individual having experienced an infection. Serological surveys were undertaken to measure the level of circulating antibody to HCMV in different age groups and communities.

An early epidemiological survey performed in Bethesda (USA) by Rowe and co-workers (Rowe et al., 1956) found that the proportion of the population with C.F. antibody to HCMV increased with age from 14%, in the 6 to 23 month age group, to 81%, in the over age 35 years group (table 1:1). The highest rate of seroconversion was seen in babies and infants under age 4 years. More recent surveys have produced similar results (Stern and Elek, 1965; Carlstrom and Jalling, 1970). During pregnancy maternal antibody is transferred across the placenta into foetal blood. Passive immunity conferred by maternal antibody declines during the first six months after birth. Passively acquired maternal antibody accounts for the high proportion of samples from cord blood (71%) and neonates (29%) which contained HCMV-specific CF antibody (Table 1:1; Rowe et al., 1956).

Sample (age group)	No. Positive/No. Tested
Cord Blood	12/17 (71%)
6 weeks	5/17 (29%)
6-23 months	3/21 (14%)
2-4 years	11/36 (31%)
5-9 years	11/33 (33%)
10-15 years	22/49 (45%)
18-25 years	52/98 (53%)
>35 years	42/52 (81%)

Table 1:1: Correlation beteen age and the incidence of complement-fixing antibody to HCMV in Bethesda, U.S.A. (Rowe et al., 1956).

A W.H.O. co-ordinated survey, using standardised reagents and conditions, monitored the level of CF antibody in 20 to 40 year old healthy blood donors in different parts of the world (Krech, 1973). The results showed that HCMV is endemic worldwide (Table 1:2). The high incidence of HCMV in countries from the equator to the arctic indicates that climatic conditions do not significantly affect HCMV transmission. Immunity to HCMV is, however, less prevalent in more affluent societies. This is, perhaps, most clearly illustrated in Johannesburg where 100% of Bantu negros were seropositive as compared with 70% of the white population. In a similar study, performed in London, 90% of women were seropositive in a then relatively less prosperous immigrant Asian community compared with 58% of native women (Stern and Elek, 1965).

Alford et al. (1981) investigated the relationship between socioeconomic status and the age distribution of primary infections. The study, carried out in Birmingham, USA, found that by child-bearing age 75% of women in a low income group were seropositive compared with 35% of women

Country (Town)	Sample Size	% Seropositive
France (Lyon)	98	40
W. Germany (Freiburg)	89	42
Switzerland (St Gallen)	105	45
USA (Albany)	98	45
Australia (Melbourne)	99	54
USA (Honolulu)	145	67
South Africa (Johannesburg, whites)	96	75
Argrentinia (Buenos Aries)	43	81
Czechoslovakia (Bratislava)	100	83
Trinidad (Port of Spain)	99	86
Mauritius	93	89
USA (Anchorage)	100	94
Hong Kong	99	94
Japan (Sendai)	99	96
Greenland	90	98
Tanzania (Dar Es Salaam)	117	98
Morocco	109	98
Fiji Islands	95	100
Uganda (Entebbe)	143	100
Nigeria (Ibadan)	95	100
South Africa (Johannesburg, Bantu Negroes)	112	100
Philippines (Manila)	89	100
India (Chandigarh)	68	100

Table 1:2: Incidence of complement-fixing antibody to HCMV in healthy blood donors, age 20-40 years, world-wide (from Krech, 1983).

in a middle to high income group. A higher proportion of women in the middle to high income group were, therefore, susceptible to primary infection during pregnancy. 0.5% of the low income group and 0.9% of the higher income group experienced primary infection during pregnancy (Alford et al., 1981). Symptomatic congenital HCMV infections are known to occur more frequently in the children of mothers experiencing a primary infection than in those experiencing a reactivation or secondary infection (see section 1:4). With the improvement in socioeconomic conditions seen in industrialised countries there has been a concomitant change in the epidemiology of HCMV. In more developed communities, on average, individuals are exposed to HCMV infection later in life and consequently a higher proportion of mothers in the more developed communities experience a primary infection during pregnancy.

1:4 Congenital Infection

The most frequently diagnosed disease caused by HCMV is congenitally-acquired Cytomegalic Inclusion Disease (CID). HCMV can be transmitted, via the placenta, to the foetus from a mother experiencing either a primary or secondary infection. Histopathological examination of infants with CID invariably reveals the presence of pathognomic cytomeglic epithelial cells in the liver, lungs, kidneys, pancreas and/or salivary glands. Symptoms associated with CID include petechia, hepatosplenomegaly, jaundice, microcephaly, low birth weight, prematurity, inguinial hernias and chorioretinitis (Stagno et al., 1984).

Estimates of the rate of congenital HCMV infection range between 0.28% and 2.2% of total births (Peckam et al., 1983). In general the incidence of

City	HAMILTON	LONDON
Size of Cohort	15,212	14,789
Congenitally Infected	64 (0.42%)	42 (0.28%)
Diagnosed CID from Overt Symptoms	4/64	1/42
Profound Bilateral Deafness	2/41	
Profound Unilateral Deafness	3/42	
Mild Bilateral Deafness	1/42	
Mild Unilateral Deafness	1/42	
Total with Sensorineural Hearing Loss	7/42	4/42
Additional Serious Sequelae Attributed to Congenital HCMV Infection	1/42	3/42
Serpositive Mothers in the Community	44%	56%

Table 1:3 Incidence of congenital HCMV infection and HCMV-related symptoms in prospective studies carried out in Hamilton, Canada (Larke et al., 1980; Saigal et al., 1982) and London (Peckham et al., 1983).

congenital infection is directly related to the proportion of seropositive mothers in the population. A series of prospective long-term follow-up studies, in which large numbers of neonates were screened, has been undertaken to evaluate the full consequeces of CID. The presence of a viruria at birth is diagnostic of a congenital infection. Table 1:3 summarizes the results of two surveys performed in populations where there was a relatively low ratio of seropositive mothers. Only 5/1061 congenitally infected neonates were diagnosed as having symptoms of CID. Of these five symptomatic cases one (from the London study) developed microcephaly, spastic quadroplegia, optical atrophy and bilateral hearing loss; a second had profound bilateral deafness and the remaining three cases experienced no long term handicap. Previous studies, which had been concerned primarily with patient referrals, had found a 20% to 30% fatality rate in symptomatically infected neonates with between 90% to 95% experiencing some long term complication (reviewed by Stagno et al., 1984). The improved prognosis suggested by the Canadian study was thought to reflect the efficient diagnosis of neonates exhibiting milder symptoms of the disease.

Two out of 101 neonates who were asymptomatic at birth subsequently developed quadraplegic cerebal palsy, hearing loss and additional sequelae. The principal finding of the long-term follow-up study was that there was a high incidence of sensorineural hearing loss in apparently asymptomatically infected children. The Canadian group monitored the health of most of the congenitally infected children up to age 5 years; 7 out of 41 children in this group exhibited sensorineural hearing loss (Table 1:3). Extrapolating from these figures Hanshaw (1982) estimated there are 100,000 individals in the USA alone with impaired hearing resulting from congenital exposure to HCMV. Sensorineural deafness had previously been shown to be associated with symptomatic congenital HCMV infections (Medearis, 1964; Williamson et al., 1982). HCMV can establish widespread infections in the epithelial cells of the inner ear and infectious virus has been isolated from perilymph removed

from the inner ear (Myers and Stool, 1968; Davis, 1969).

Transplacental HCMV infection takes place in the presence of maternal antibody, in a number of cases congenital infections have been detected in consecutive pregnancies (Embil et al., 1970; Stagno et al., 1973). Although pre-existing maternal immunity does not prevent congenital infection it does moderate the pathogenic consequences of the disease. A prospective survey undertaken in Birmingham, USA, found that 5/33 congenitally infected babies born to mothers experiencing a primary infection exhibited overt symptoms of CID as compared with 0/27 congenitally infected children born to mothers experiencing a reactivation or secondary infection (Stagno et al., 1982). Stagno et al., (1982) observed that women of higher socioeconomic status were more susceptible to primary infection during pregnancy than women of lower socioeconomic status, who were predominantly seropositive, and that their children were consquently more likely to experience overt symptoms of CID.

In Chile, where 98% of the population is seropositive and the rate of congenital infection is 1.7%, Stagno et al (1984) were unable to identify a single case of CID in autopsy reports or in neonatal referals exhibiting potential symptoms of the disease. CID therefore may be a disease found only in socioeconomically advanced communities.

1:5 Perinatal Infection

Neonates who experience a perinatal infection do not begin to secrete virus in their urine until age 1 to 2 months. The incidence of perinatal infection ranges between 8% (Birmingham, USA) to 56% (Japan) in different communities (Stagno et al., 1980). The mother appears to be the principal source of virus in perinatal infections. In France it was observed in a survey of 10 month old children born to seronegative mothers none seroconverted (Cabau at al., 1979).

Breast milk is a significant source of virus in perinatal infections.

The highest rates of perinatal infection are observed in communities which favour breast-feeding, e.g. Japan and Finland. The babies of 28 mothers who secreted virus in their milk were studied. In the survey 0/9 babies that were bottle fed experienced an HCMV infection as compared with 11/19 that were breast fed.

The second major potential source of perinatal infection is from maternal secretions as the baby passes through the birth canal. It is estimated that between 11% and 28% of mothers secrete HCMV from the cervix at the time of birth (Reynolds et al., 1973). It is difficult to determine the rate at which HCMV is transmitted during birth, as opposed to immediately before or after, with any certainty. A study performed in Birmingham, USA, however, provides indirect evidence that there may be an association. The study found that 40% of children born to women secreting HCMV from the cervix subsequently went on to develop a neonatal infection (Reynolds et al., 1973).

Although HCMV may occasionally cause pneumonitis, long-term studies have yet to identify any other disease associated with perinatal infection (Granstrom, 1980). Congenital and perinatal HCMV infections frequently give rise to chronic infections, in more than 50% of cases virus was still being secreted in urine at age 6 years (Stagno et al., 1984). These chronic infections are normally asymptomatic and do not appear to affect normal development.

1:6 Infectious Mononucleosis

HCMV is the causative agent of approximately 7% of all cases of infectious mononucleosis (Klemola, 1973; Horwitz et al., 1977). Infectious mononucleosis is the only disease of immunologically competent adults which has been attributed to HCMV. HCMV mononucleosis is characterised by a persistant fever which lasts for 2 to 5 weeks, peripheral blood lymphocytosis, abnormal liver functions, mild hepatitis and a

heterophile-negative antibody response (Klemola and Kaariniainen, 1965). The majority of HCMV mononucleosis cases occur in individuals over age 15 years, the syndrome is not normally associated with CID in neonates.

Humoral antibody production and NK cell function remain intact throughout the infection (Rinnaldo et al., 1977). In vitro measurements, however, show that the cytotoxic T-lymphocyte response to stimulation with concanavalin A, pokeweed mitogen, specific (HSV-1) antigens and allogenic cells is diminished (Rinnaldo et al., 1977). Recovery from the disease has been correlated with reparation of the CTL blastogenic response (Carney et al., 1983).

A large absolute increase in the number of $CD8^+$ ($OKT8^+$ or cytotoxic suppressor) T-cells and a small absolute decrease in the number of $CD4^+$ ($OKT4^+$ or cytotoxic helper) T-cells is observed in lymphocyte preparations from HCMV mononucleosis patients. This causes an inversion of the helper:suppressor T-cell ratio (Rinnaldo et al., 1983; Hirsh, 1984). An invertion in the helper:suppressor cell ratio is associated with a reduced level of cellular immunity in AIDS patients, although in AIDS patients the invertion appears to be caused by a reduction in $CD4^+$ cell numbers.

The observed increase in suppressor T-cell numbers may not be the sole cause of the depressed CTL response. If plastic-adherent monocytes are extracted from the PBL cells of HCMV mononucleosis patients a recovery in the CTL blastogenic response is observed. The re-addition of fresh monocytes from HCMV mononucleosis patients, although not from normal donors, to the cell culture resulted in the CTL response being suppressed again (Rinaldo et al., 1980). The interaction between HCMV and monocytes may induce the synthesis of a factor which suppresses the CTL response (Hirsch, 1984).

A small proportion of PBL's infected in vitro (1-15%) will express the major IE polypeptide. Monocytes were infected most efficiently (10% of the monocyte population) with NK cells (6.3%), B-lymphocytes (1.0%), $CD4^+$ (1.6%) and $CD8^+$ (3%) cells also being susceptible (Rice et al., 1984). The

expression of late virus antigens was, however, not detected which indicated that the infection was abortive. The majority of large atypical lymphocytes observed in mononucleosis patients belong to the $CD8^+$ grouping and cannot be stimulated by mitogens (Hirsch, 1984). HCMV has recently been shown to replicate in a subpopulation of $CD8^+$ cells which have been stimulated by either interleukin 2 or allogeneic lymphocytes (Braun and Reiser, 1986; section 1:24).

1:7 Iatrogenic Infections

Following the discovery that HCMV was a causative agent of infectious mononucleosis the association was made between HCMV and the post perfusion syndrome (Kaariainen et al., 1966). The post perfusion syndrome develops 2 to 7 weeks after open heart surgery or treatment which involves the transfusion of fresh blood. The disease is clinically similar to infectious mononucleosis and is accompanied by a rise in titre of antibody to HCMV (Kaariainen et al., 1966).

The transmission of HCMV by blood transfusion can be averted by storing blood for 48h (Paloheimo et al., 1968). Ho (1982) compiled statistics from 12 prospective studies on the incidence of HCMV infection following blood transfusion. HCMV infection were detected in 14% of patients who received transfusions with 4%, predominantly seronegative, manifesting symptoms. The risk of infection was calculated to be approximately 3% per unit of blood transfused. HCMV viraemia was extremely rare being detected primarily in patients exhibiting clinical symptoms of infection (Cheeseman et al., 1979; Rinnaldo et al., 1980; Macher et al., 1983).

HCMV cannot easily be detected in transfused blood; out of a total of 1448 units of transfused blood collected in the USA and the UK none yielded infectious HCMV (compiled by Ho, 1982). Attempts to isolate HCMV from lymphocytes of healthy, seropositive blood donors have also been unsuccessful (Rinnaldo et al., 1978). Transmission of HCMV in transfused

blood may depend on the reactivation of latent virus being harboured in lymphocytes. The incidence of infection is reduced when lymphocytes are removed from the transfused blood (Lang et al., 1977) or the blood is stored in a manner which reduces lymphocyte viability (Tolkoff-Rubin et al., 1978). An exceptionally high incidence of HCMV infection has been observed in individuals who received transfusions of purified leukocytes (Winston et al., 1980).

Almost exclusively congenital and neonatal infections are acquired from the mother. Neonates who are transfused with seropositive blood are an exception. A prospective study found that 13.5% of seronegative neonates who received transfusions of seropositive blood subsequently secreted HCMV in urine (Yeager et al., 1981). 40% (4/10) of infected seronegative neonates died and 10% (1/10) experienced severe symptoms of a systemic HCMV infection (Yeager et al., 1981). Passive protection by maternal antibody is important in protecting the foetus and newborn children from a potentially life threatening HCMV infection.

HCMV emerged as a major complication in surgical operations following the advent of immunosuppressive drug therapy around 1960. Pass et al. (1980) estimated that 90% of renal transplant recipients experienced infection by HCMV. Approximately 35% of renal transplant recipients experienced a febrile self-limiting disease between 30 to 90 days post transplantation but a further 2.3% suffered a disseminating fatal infection (Glen, 1981). In recent years a combination of using less intensive immunosuppressive therapy and reducing the level of immunosuppression in response to HCMV infections has made HCMV less of a problem for renal transplant operations (Betts, 1982)

HCMV infections continue to be a major hazard in bone marrow transplant operations. Approximately 80% of bone marrow recipients experience an overt HCMV infection with 20% experiencing an HCMV-associated pneumonia. There is an 80% mortality rate for bone marrow recipients who develop the

HCMV-associated pneumonia (Meyers _et al._, 1980). Since the level of immunosuppression cannot be modulated in bone marrow transplant recipients in response to infection, treatment of the HCMV-associated pneumonia appears to be dependent on the development of effective chemotherapy. A promising candidate HCMV anti-viral agent is the acyclovir analogue BW B759U (Biron _et al._, 1985).

In a prospective study of bone marrow recipients Quinnen _et al._ (1981; 1982) observed that failure to survive infection was correlated with: (a) an absence of the NK cell response at the onset of the infection and (b) a subsequent failure to activate the HLA restricted CTL response. A reduced helper:suppressor T-cell ratio was observed in both fatal and non-fatal HCMV infections (Schroff _et al._, 1982; Quinnan and Rook, 1982). An effective CTL response is also known to be important in renal transplant patients experiencing an infection. HCMV infected renal transplant recipients who fail to develop a CTL response experience a systematic disease and a viraemia which, although rarely fatal, can lead to graft rejection (Schooley _et al._, 1983; Quinnan and Rook, 1984).

1:8 HCMV and AIDS

The term AIDS (acquired immunodeficiency syndrome) is used to describe patients suffering from at least one life threatening infection or a Kaposi sarcoma resulting from HIV-(human immunodeficiency virus, the causative agent of AIDS) induced immunosuppression. HIV lytically infects $CD4^+$ cells which have a "helper" function in stimulating the CTL response. AIDS severely compromises the cellular immune response which normally copes efficiently with HCMV infections. Almost all AIDS patients experience overt symptoms of an HCMV infection. Rook _et al._ (1985) isolated infectious HCMV from 54/56 AIDS patients and in 37/56 cases the virus was associated with buffy coat cells. Typically severe HCMV infection is seen at a late stage in AIDS with a progressive HCMV viraemia being associated with a concomitant

decrease in the CTL and NK responses. In a histological study performed at autopsies on 15 patients who had succumbed to AIDS there were extensive HCMV lesions in both the lungs and adrenal glands in 14 cases (Macher et al., 1983). Lesions produced by disseminating HCMV infection have been described in a wide variety of tissues and organs (Reichert et al., 1985). HCMV-induced pneumonia has been reported to be the most frequent cause of death in AIDS patients (Reichert et al., 1985). It is not clear if the immunosuppressive properties of HCMV infection contributes towards the syndrome or if HCMV is purely an opportunistic pathogen.

1:9 Oncogenicity

HCMV is able to morphologically transform cells in culture. Albrecht and Rapp (1973) observed that foci of non-contact inhibited cells were produced when hamster embryo fibroblasts were infected with uv-irradiated virus. Similarly, infection of HEF cells with the slow-growing genital HCMV isolate, strain Mj, induced the production of morphologically transformed cells (Geder et al., 1976). In both cases cultured cell lines expressed HCMV antigens and were found to induce tumour formation when injected into animals. Continuous passage of the human cell line resulted in a decrease in both the expression of virus antigen and the tumorogenicity of the cells.

In DNA transfection experiments Nelson et al. (1982) were able to reproducibly transform murine NIH 3T3 cells with restriction endonuclease digested HCMV strain AD169 genomic DNA and cloned subgenomic fragments. 11/12 of these transformed cell lines were shown to be able to induce tumour formation when injected into BALB/c nude mice (Nelson et al., 1982). The major transforming region was mapped to a 490bp DNA fragment in the genome of strain AD169 (Nelson et al., 1984). RNA transcripts of 5.4kb and 6.25kb are encoded by this region during the IE, early and late phases of infection (Jahn et al., 1984; Wilkinson et al., 1984). However, the longest ORF associated with the transforming sequence would encode a polypeptide of only

41 amino acids (Kouzarides _et al._, 1983; Nelson _et al._, 1984). Additionally, S-1 nuclease mapping experiments indicate that transcripts derived from the transforming region are not spliced (Nelson _et al._, 1984). The mechanism by which the 490bp DNA fragment induces transformation is not known. Attempts to detect the integrated HCMV sequences in DNA purified from transformed cells have so far been unsuccessful (Nelson _et al._, 1982).

In a different series of experiments Clanton _et al._ (1983) identified regions within the HCMV strain Towne genome which appeared to share homology with transforming regions of HSV-2 DNA. Transfection of normal syrian hamster cells with the cloned strain Towne DNA _XbaI_ fragment E (which showed limited homology with the HSV-2 _BglII_ fragment C) resulted in the production of refractile rapidly-growing, immortalised cell colonies. These transformed cell lines also induced tumour formation when injected into athymic mice. The transforming regions identified within strains AD169 and Towne mapped to adjacent fragments on the genomes of their respective viruses but were distinct. HCMV strain Towne _XbaI_ fragment E is abundantly transcribed during the IE phase of gene expression (Wathen and Stinski, 1982).

It has been reported that a number of regions of the HCMV genome shares homology with the oncogene v-_myc_ (Gelmann _et al._, 1983; Spector and Vacquier, 1983). Nucleotide sequence analysis has shown, however, that the hybridisation between HCMV and _myc_ sequences under low stringency conditions was not due to sequence homology, merely nonspecific annealing of GC-rich DNA fragments (Kouzarides _et al._, 1983; Nelson _et al._, 1984; Rasmussen _et al._, 1985).

HCMV transforms cells _in vitro_ relatively inefficiently, as compared with avian sarcoma virus and avian leukosis virus (Nelson _et al._, 1982), but the transformed cell lines which are produced are usually tumorogenic. The Herpesviridae contains a number of viruses which cause or are strongly associated with specific cancers. HCMV or elevated levels of HCMV specific

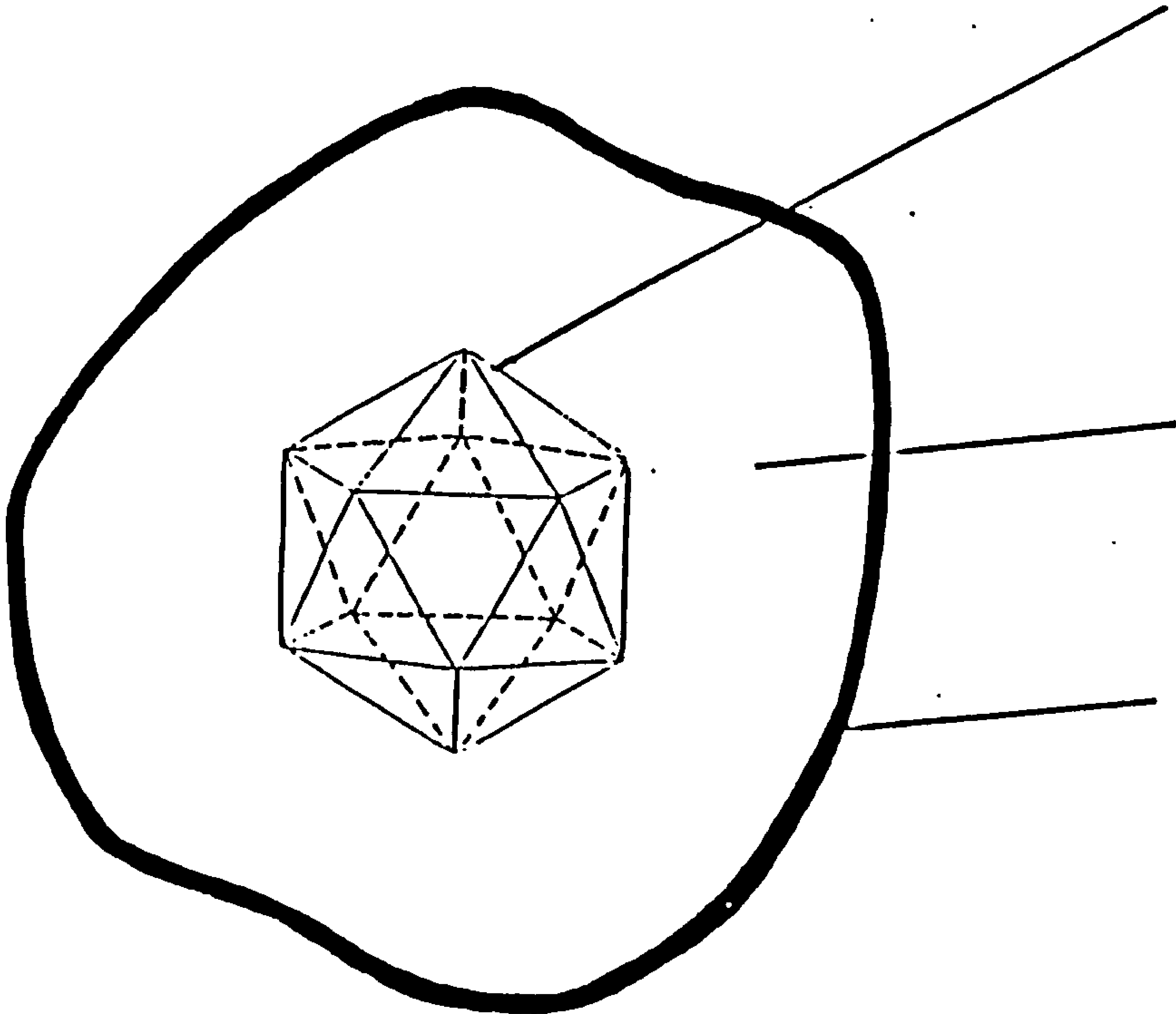


Fig 1:1 : Schematic Reprentation of the HCMV Virion.

antibody has been associated with prostatic adenocarcinomas (Kunkel et al., 1980; Boldogh et al., 1981), cervical cancer (Melnick et al., 1981), colonic adenocarcinomas (Hashiro et al., 1979; Roche et al., 1981) and Kaposi's sarcoma (Giraldo et al., 1972; 1975; 1978; Irew et al., 1982). The clinical evidence is, however, circumstantial and it has yet to be demonstrated that HCMV either causes or contributes directly or indirectly towards tumour production in vivo.

1:10 The Virion

The basic morphology of herpesvirions has been conserved throughout the group. All herpesvirions contain four distinct structural elements: a core containing DNA, an icosohedral capsid (110-120nm in diameter) consisting of 12 pentameric and 150 hexameric capsomeres, an electron-dense tegument region surrounding the capsid and an outer lipoprotein envelope (Fig 1:1; Wildy et al., 1960). The size of herpesvirions can vary significantly depending on the amount of tegument protein taken up, HSV-1 virions have been observed to vary between 120nm to 180nm in diameter (McCombs et al., 1971).

A number of features distinguish the virions of HCMV and HSV-1. The HCMV core was observed by electron microscopy to be comprised of seven or more globular subunits whereas the HSV-1 core was a single continuous structure (Smith and DeHarven, 1973). It has recently been reported that HCMV may possess a second loose-fitting outer envelope which is easily removed by osmotic shock (Farrar and Oram, 1984). Surface projections, or spikes, are present on the outer surface of HSV-1 envelopes but they have yet to be detected on HCMV virions.

Three different types of enveloped particle are produced during an HCMV infection: (a) dense bodies; non-infectious, spherical, electron-dense particles which contain large amounts of tegument protein (Sarov and Abady, 1975; Gibson, 1981a), (b) non-infectious enveloped particles (NIEPs) which

Simian CMV (Kdaltons)	HCMV (Kdaltons)	Comments
205	212	virion protein
145	153	major capsid protein
119	149	abundant basic phosphoprotein (tegument)
129	140	non-virion
	122	minor virion phosphoprotein
112	115	virion
	110	minor virion phosphoprotein
	94	minor virion phosphoprotein
	88	minor virion phosphoprotein
78	80	minor virion phosphoprotein
	75	minor virion phosphoprotein
69	74	abundant tegument phosphoprotein (upper matrix protein) maps to HindIII L, c and b (a)
66	69	most abundant tegument phospho-protein, (lower matrix protein) maps to HindIII L, c and b (a)
	67	abundant tegument phosphoprotein protein kinase, maps to HindIII D (b)
	53	minor virion phosphoprotein
51	52	non-virion, nuclear, DNA binding protein, maps to HindIII M (c)
	43	minor virion phosphoprotein
40	42	virion
38	39	virion
37	35	abundant non-virion, assembly protein, present in B-capsids and NIEPs
34	34	minor capsid protein
32	32	minor virion
	29	virion phosphoprotein, cytoplasmic
27		minor virion
	24	minor virion phosphoprotein
		Envelope Glycoproteins
	250	
	130	in complex with gp52
	95	in complex with gp52
	67	
	52	in complex with gp52 and gp130 homology with gB of HSV-1 and EBV, maps to HindIII F (d)

Table 1:4: Summary of Simian CMV strain Colburn and HCMV late ICSPs modified from Gibson (1983). Sizes of the glycoproteins was from HCMV strain AD169 (Farrar and Oram, 1985). Sizes of the unglycosylated HCMV protein were from strain 751 (Gibson, 1983) except for the 24, 43, 53, 75, 88, 94, 110 and 122 K minor phosphoproteins (strain AD169; Roby and Gibson, 1986), the 29 K phosphoprotein (strain AD169; Nowak et al., 1984a) and the 67 K protein kinase (strain Towne; Davis et al., 1984). The map location of the genes encoding the polypeptides was described in (a) Nowak et al., 1984b, (b) Davis et al., 1984, (c) Mocarski et al., 1985b and (d) Cranage et al., 1986.

contain the same proteins as infectious virions plus an additional 35K protein (the assembly protein, see section 1:22) but no DNA (Irmiere and Gibson, 1983) and (c) complete virions.

It has been estimated that HCMV virions contain between 33 to 35 polypeptides with molecular weights ranging from less than 10K to greater than 200K (Sarov and Abady, 1975, Fiala et al., 1976; Kim et al., 1976; Stinski, 1976; Gupta et al., 1977; Stinski, 1977; Gibson, 1981a; Irmiere and Gibson, 1983). The composition of different HCMV isolates has been shown to be almost identical with respect to the size and number of the constituent protein species (Gupta et al., 1977). HCMV polypeptides are usually identified by their apparent molecular weights determined by SDS-PAGE. Unfortunately this system creates a degree of confusion as the same protein species is given a different designation by different groups. The terminology adopted in Table 1:4 will be sustained throughout this thesis.

Four enzymic activities have been detected in isolated virions: a DNA nicking activity (Landini et al., 1982), a DNA polymerase (Mar et al., 1981) and two distinct protein kinase activities (Mar et al., 1981; Roby and Gibson, 1986). One of the protein kinases specifically phosphorylated tegument proteins in an in vitro assay (Mar et al., 1981; Roby and Gibson, 1986) while the other was able to phosphorylate an exogenous (casein) substrate (Roby and Gibson, 1986). Both protein kinase activities were found to be associated with the virion tegument fraction and were biochemically distinct from the protein kinase which is induced in HCMV infected cells during the early phase (Michelson et al., 1984). An abundant 67/68K tegument phosphoprotein has been shown to be responsible for one of the virion protein kinase activities (Davis and Huang, 1985; Britt and Auger, 1986). The gene encoding this protein has been mapped on the virus genome (Davis et al, 1984) and its DNA sequenced (Davis and Huang, 1985).

The majority of HCMV tegument proteins, like those of HSV-1, are phosphorylated (Table 1:4; Lemaster and Roizman, 1980). Interest has

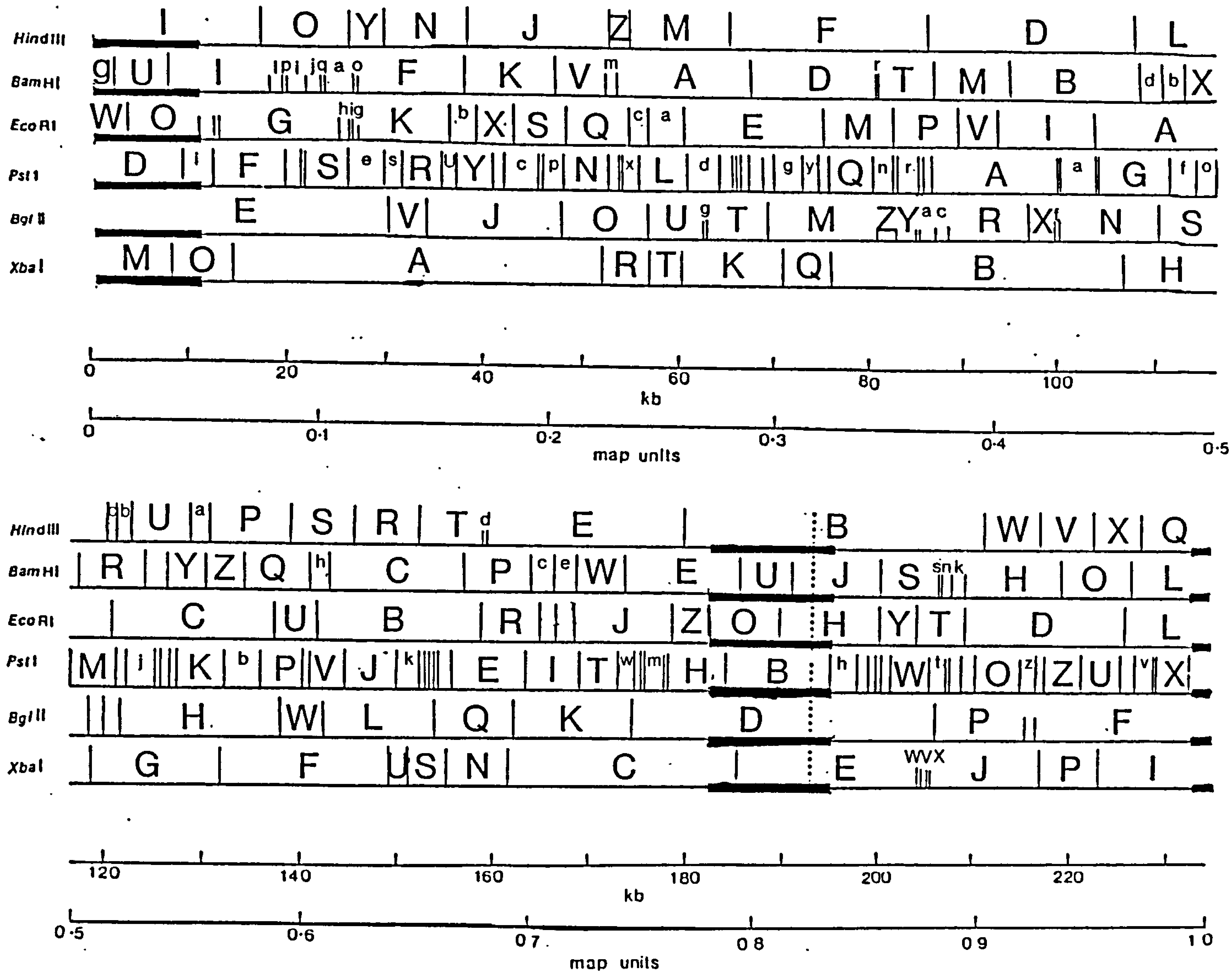


Fig 1:2: Restriction endonuclease cleavage map of HCMV strain AD169, adapted from Greenaway *et al.* (1982).

recently focused on the herpesvirus tegument proteins following the demonstration that the HSV-1 Vmw65 tegument protein stimulates transcription from HSV-1 IE promoters (Campbell et al., 1984). It is not yet clear whether HCMV encodes a similar trans-acting virion component (Spaete and Mocarski, 1985b; Stinski and Roehr, 1985).

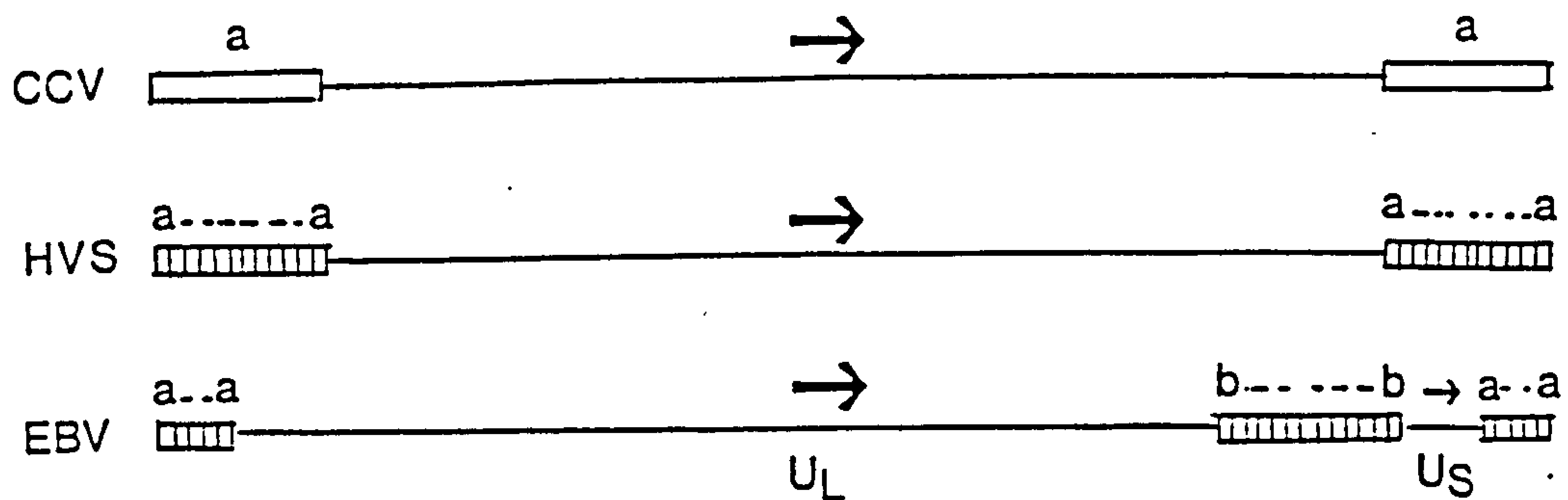
1:11 The Virus Genome

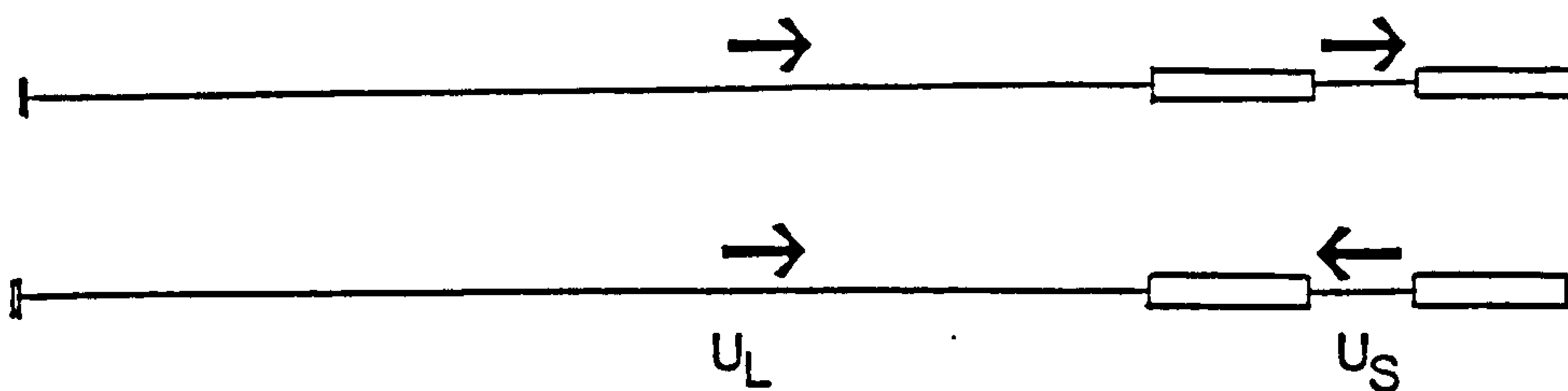
The molecular cloning of the HCMV DNA has been crucial in studies of genome structure and function. Restriction endonuclease cleavage maps have been constructed for strains AD169 (Fleckenstein et al., 1982; Greenaway et al., 1982; Oram et al., 1982; Spector et al., 1982; Westrate et al., 1980), Davis (DeMarchi, 1981), SG (Westrate et al., 1983) and Towne (LaFemina and Hayward, 1980; Thomsen and Stinski, 1981). A detailed restriction endonuclease map of strain AD169 is shown in Fig 1:2. The HindIII cloned subgenomic DNA fragments used in the construction of this map were also used in experiments performed for this thesis.

The HCMV genome, contained in infectious virions, is a linear double stranded DNA molecule of approximately 235kb or 150 x 10^6 daltons (Kilpatrick and Huang, 1977; Demarchi et al., 1978; Geelen et al, 1978; Lakeman and Osborn, 1979, Stinski et al., 1979) with an overall G+C content of 58% (Crawford and Lee, 1964; Huang et al., 1973). Purified full length HCMV DNA is infectious when transfected into human embryonic lung cells; HCMV replication, therefore, is not absolutely dependent on any enzyme which may be carried in infecting virions (Geelen et al., 1978).

Herpesvirus genomes can be divided into three different classes on the basis of their structural organisation (Fig 1:3; Honess and Watson, 1977).

The class 1 herpesviruses are the most straightforward; their genomes contain direct repeats of DNA elements but are not subject to rearrangement, i.e. segment inversion, during DNA replication and therfore consist of a single isomeric form of DNA (Fig 1:3). Three different types of





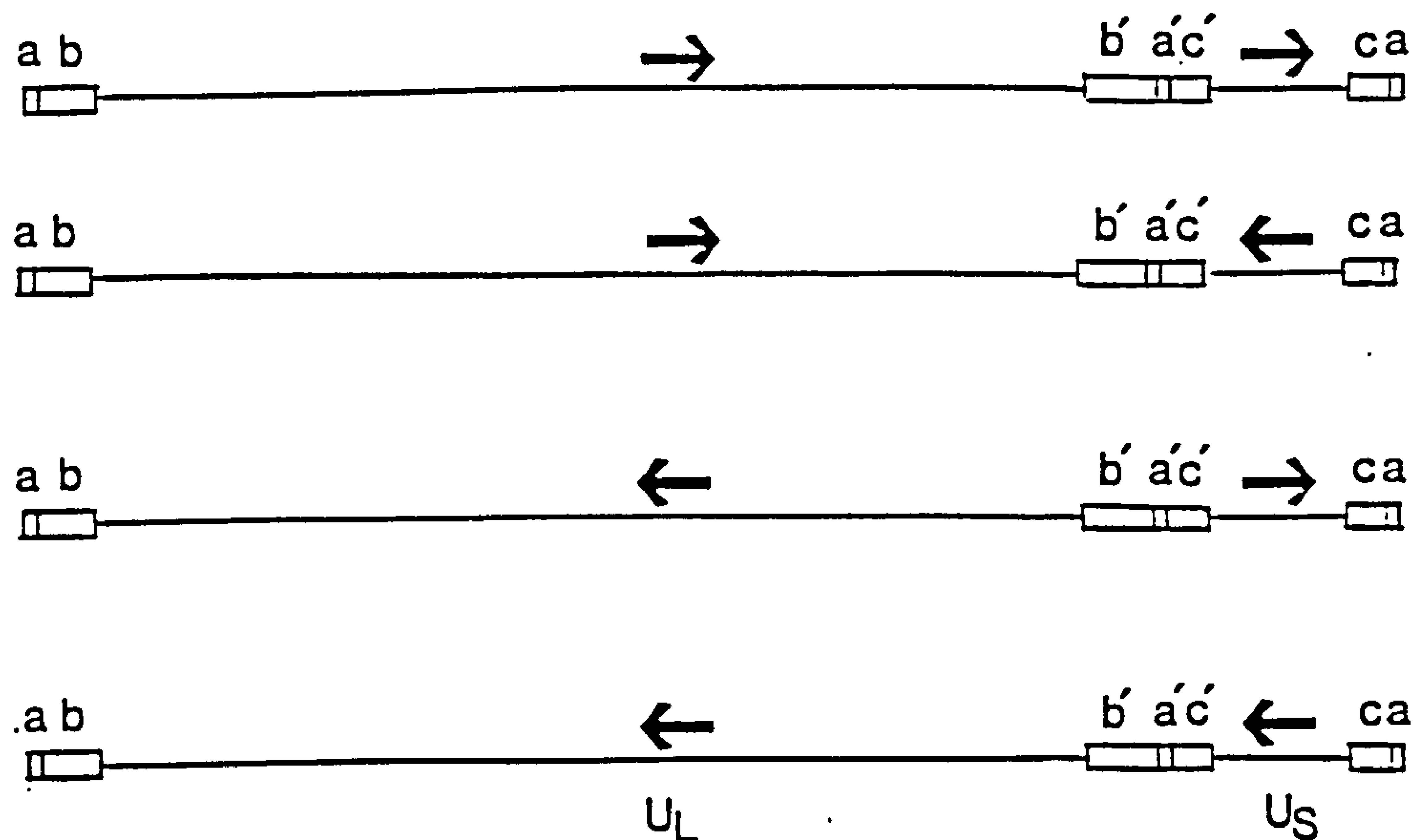


Fig 1:3: There are three classes of herpesvirus genomes. (a) class 1 genomes exist in a single isomeric form even though they can contain a variety of repeated DNA elements as is illustrated for the genomes of CCV, HVS and EBV. The GC-rich TR region of HVS is known as H-DNA. (b) Class 2 herpesvirus genomes exist in two isomeric forms: the prototype (P) orientation and with an inverted short (S) region (the I_S conformation). (c) Both the L (long) and S components of class 3 herpesviruses can be inverted relative to each other so that their genomes exist in four equimolar isomeric forms: the prototype (P), with an inverted short region (I_S), with an inverted long region (I_L) and with both the long and short regions inverted (I_{LS}). The a sequence is present in at least one copy at both termini and, in an inverted orientation, at the L-S junction. The remainder of the long (TR_L and IR_L) and short (TR_S and IR_S) repeats are made up of a sequence designated b or c respectively. The prime sign indicates the sequence is inverted.

class 1 genomes have been identified and are represented in figure 1:3 by channel catfish virus (CCV), herpesvirus saimiri (HVS) and EBV. The CCV genome consists of a long unique (U_L) region flanked by a repeated sequence, both copies of the terminal repeat (TR) being in the same orientation (Chousterman et al., 1979). The second type of class 1 genome structure is similar to that of CCV except that the TR sequences (H-DNA in Fig 1:3) comprise multiple tandem reiterations of the same short sequence, in the case of HVS the repeats are 1.4kb long (Fleckenstein and Desrosier, 1982). EBV, representing the third type of class 1 genome, has terminal repeats containing reiterated sequences similar to those of HVS plus an additional internally repeated element (IR) comprised of multiple reiterations of a different repeat. An analysis of the nucleotide sequence of EBV has identified further less extensive repeated elements elsewhere on the genome (Baer et al., 1984).

Class 2 herpesvirus genomes contain a U_L and a short unique (U_S) region with the U_S region being flanked by inverted repeats (IR_S and TR_S). The short component (S) of the genome can become inverted, relative to the long component (L), during replication thus generating two equimolar isomeric forms of genomic DNA (Fig 1:3). A small proportion of purified virion VZV DNA (5%) also has an inverted L component. This subpopulation, however, is thought to be generated during the process of DNA packaging rather than a structural rearrangement of the VZV genome caused by recombination during DNA replication (Davison, 1984).

The class 3 herpesvirus genomes contain U_L and U_S regions each flanked by a different inverted repeat (TR_L, IR_L ; IR_S, TR_S). The L and S components of the genome can invert, relative to each other, during replication which results in there being four different isomeric forms of virus DNA (Fig 1:3). The four different isomers are present in equimolar concentrations in virion DNA preparations. HCMV, like HSV-1, has a class 3 genomic structure.

Restriction endonuclease digestion of HCMV virion DNA generates a

complex mixture of molar and submolar (derived from the termini and internal repeats of the different isomers) DNA fragments. The submolar DNA fragments derived from the L-S junctions and both termini exhibit a stepped heterogeneity in size which is seen as a DNA 'ladder' following agarose gel electrophoresis (LaFemina and Hayward, 1980; Tamashiro et al., 1982). A similar size heterogeneity in the TR_L and junction fragments of HSV-1 DNA is due to the presence of multiple copies of the a sequence; the HSV-1 a sequence is a 265bp DNA element which is present in the same orientation at both termini of the HSV-1 genome (Wagner and Summers, 1978) and in the inverse orientation at the L-S junction (Davison and Wilkie, 1981). The a sequence is involved in both the inversion of L and S segments during virus DNA replication (Chou and Roizman, 1985) and in the packaging of virus DNA into capsids (Mocarski et al., 1985a).

Hybridisation experiments demonstratated that the DNA sequence which spans the HCMV L-S junction is also present, in an inverted orientation, at both termini. The HCMV genome therefore contains a terminal redundancy similar to the HSV-1 a region (LaFemina and Hayward, 1983; Tamashiro et al., 1984). The 553bp a sequence of strains AD169 (Tamashiro et al., 1984) and the 762bp a sequence of strain Towne (Spaete and Mocarski, 1985a) was determined by DNA sequence analysis of their L-S junctions. In addition to straightforward duplication of the a sequence the genome of strain AD169, and to a lesser extent also strain Towne, contains a complex series of a variants which appear to have been produced by a combination of deletion and recombination events (LaFemina and Hayward, 1983; Tamashiro et al, 1984; Tamashiro and Spector, 1986; Spaete and Mocarski, 1985a).

In spite of their size difference the a component of strains AD169 and Towne share regions of extensive sequence homology. The a sequences of HCMV strains AD169 and Towne both contain direct repeats which are analogous to, but do not share homology with, repeats found in the HSV-1 a sequence (Davison and Wilkie, 1981). There is, however, a 26bp sequence (CGGGGGGGTGT-

TTTTAGCGGGGGGGGGGT) which is identical in strains AD169 and Towne and shares significant homology with a sequence contained within the a components of HSV-1, HSV-2, VZV, EBV and HVS (Tamashiro et al., 1984; Spaete and Mocarski, 1985a; Tamashiro and Spector, 1986). The conserved sequence (the herpes pac-2 homology; Deiss et al., 1986) is found between 25 to 35 bases from the end of herpesvirus genomes and is thought to represent one of the cis-acting signals which direct the cleavage of concatemeric herpesvirus DNA into unit length and packaging into capsids (Spaete and Mocarski, 1985a; Tamashiro and Spector, 1986).

Spaete and Mocarski (1985a) have demonstrated that the 966bp KpnI/XbaI fragment from the HCMV strain Towne L-S junction can functionally complement in cis a deletion of the HSV-1 a sequence in an HSV-1 amplicon. HSV-1 amplicons are eukaryotic plasmid vectors containing an HSV-1 origin of replication and a cleavage/packaging (the HSV-1 a sequence) signal. With the aid of functions provided by HSV-1 helper virus, the amplicon is replicated and packaged into virions as long tandemly repeated molecules of concatemeric DNA. The ability of the HCMV a sequence to replace the functions provided by the HSV-1 a sequence in HSV-1 amplicons has a number of interesting implications. The result implies that (a) the limited homology which exists between the a sequences of HSV-1 and HCMV may be sufficient to delineate the cis-acting herpesvirus cleavage-packaging signal, (b) the processes involved in the packaging HSV-1 and HCMV DNA have been conserved from a common ancestral herpesvirus and (c) HCMV may contain additional genes with functional equivalents on the HSV-1 genome (Spaete and Mocarski, 1985a).

In spite of the functional conservation of cleavage-packaging signals between HCMV and HSV-1 DNA hybridisation experiments have shown that HCMV shares little homology with HSV-1, HSV-2, EBV and nonhuman members of the betaherpesvirinae (simian CMV strain Colburn and murine CMV; Huang and Pagano, 1974). An analysis of the nucleotide sequence of the HCMV strain

AD169 S region was unable to detect any DNA or protein sequence homology with other characterised human herpesviruses and indicates that organisation of genes within the HCMV U_S region is different to that of HSV-1 and VZV (Weston and Barrell, 1986). Although the level of sequence homology between HCMV and HSV-1 is limited, cross-hybridisation can be detected in conditions of low stringency (Clanton et al., 1983). Recently an analysis of the nucleotide sequence of HCMV HindIII fragment F has revealed homology at the amino acid sequence level between both a glycoprotein (designated gB in HSV-1) and the DNA polymerase genes of HSV-1, VZV, EBV and HCMV (Cranage et al., 1986; Kouzarides at al., 1987a).

A comparison of the restriction endonuclease cleavage patterns of clinical isolates (Kilpatrick and Huang, 1977; Huang et al., 1980) and the available restriction endonuclease maps of laboratory strains indicates that the HCMV genome has been highly conserved. DNA/DNA reassociation experiments have shown that the genomes of strains AD169 and Towne are approximately 90% homologous and that the differences are concentrated in defined areas, notably the XbaI fragments A, C, G and J of strain AD169 (Pritchett, 1980). A region of significant interstrain variability has been mapped within and adjacent to the long inverted repeats (LaFemina and Hayward, 1980). In strain AD169 the TR_L ad IR_L elements are approximately 11.5kb and the IR_S and TR_S elements approximately 2.5kb (Spector et al., 1982). The TR_L/IR_L elements of strain Towne are a similar size (LaFemina and Hayward, 1980), but those of the Davis (less than 4kb; Demarchi, 1984) and SG (approx. 3kb; Westrate et al., 1983) strains are significantly smaller. It is relevant to note that HCMV is the only class 3 betaherpesvirus which has been identified; bovine (Ehlers et al., 1985), guinea pig (Gao and Isom, 1984), murine (Mercer et al., 1983) and simian strain Colburn (LaFemina and Hayward, 1980) CMV's all do not contain inverted internal repeats. In evolutionary terms, the HCMV genome may have acquired its internal repeats relatively recently. Honess (1984) proposed that the variation in size of

the repeats in different HCMV strains implies that their length is not efficiently constrained by selective pressure; in HSV-1 a number of IE genes essential for efficient virus replication map in the region of inverted repeats and may be instrumental in controlling their sizes (Whitton and Clements, 1984).

Recently strain AD169 DNA was shown to share limited homology with cellular DNA of human, owl monkey, chinese hamster but not murine origin. The homologous sequences were mapped on the HCMV genome to four distinct parts of the U_L region and one within the inverted repeats (Ruger et al., 1984). Simian CMV strain Colburn also hybridises with cellular DNA. The cross-hybridising region of strain Colburn has been sequenced and was shown to be caused by repeats of the dinucleotide CA (Jeang and Hayward, 1983). The strain Colburn CA-rich sequence is in the same relative position on the simian CMV genome as the L-S junction is in HCMV. The significance of the homologies between HCMV or simian CMV and cellular DNA is not clear.

1:12 Virus Entry Into Permissive Cells

While characteristically cytomegaloviruses have a relatively slow growth cycle, the initial events in an HCMV infection proceed rapidly. β_2 Microglobulin bound to the surface of HCMV virions (McKeating et al., 1987) is thought to enable the virion to attach to HLA class 1 molecules on the cell surface (Grundy et al., 1987). HCMV enters the cell by fusion of the virion envelope with the outer cell membrane (Iwasaki et al., 1972; Smith and DeHarven, 1974). Nucleocapsids stripped of their envelopes migrate rapidly to the nucleus where they can be detected, adjacent to the nuclear pores, 5 min p.i. (Smith and DeHarven, 1974). Nucleocapsids are not detected inside the nucleus at this time, which implies that DNA may be 'injected' directly from the capsid into the nucleus. Virus DNA is detected in the nucleus 20 min p.i. (Wahren et al.; 1984). Virus infection has an immediate effect the cellular morphology; microvilli and large "vesicular

protuberances" develop on the cell surface (Garnet, 1979). Stimulation of chromatin activity (Kamata et al., 1978) and the expression of nuclear antigen (Michelson-Fiske et al.,1977; Reynolds, 1978) are detected within 1h p.i.

1:13 Development of the C.P.E.

HCMV was named after the c.p.e. it produces. The c.p.e. which first appears during the early phase, however, is not a cytomegalia. Between 5-12h p.i. permissive cells become increasingly refractile, round up and there is a significant contraction in size (Stinski, 1977). At 5h p.i. the nucleus becomes marginated, irregular in shape and develops a single ring-shaped granular inclusion made of a fibrillar material (Calvallo et al., 1981). The onset of the early c.p.e. coincides with an influx of Ca^{2+} ions into cell (Albrecht et al., 1984). The increase in Ca^{2+} concentration inside the cell appears to be essential for virus replication. Inhibitors of Ca^{2+} transport, such as Verapamil and Papaverine, are able to block the development of the early c.p.e. and virus replication (Albrecht et al., 1984).

During the course of the early phase infected cells continue to contract and the nuclear inclusion becomes larger and better defined. By 24h p.i. the nucleus has adopted a regular reniform shape and is marginated (Cavallo et al., 1981). A large juxtanuclear body, which takes up a lipid-staining dye (Sudan IV) can be observed, at this time, with a light microscope. When the cells were stained with acridine orange, a nucleic acid staining dye, the cytoplasmic vesicle was unstained but was encircled by an orange halo of RNA (McAllister et al., 1963). The cytoplasmic vesicle itself is composed mainly of golgi apparatus (Iwasaki et al., 1973; Smith and DeHarven, 1973).

The cytoplasmic networks of actin and intermediate filaments dissociate during the first 24h p.i. and do not reform during the late phase of infection (Losse et al., 1982; Albrecht et al., 1984). Actin, which

continues to be synthesised, becomes associated with the perinuclear region (Losse et al., 1982) whereas protein from the intermediate filaments migrates into the nucleus where it appears to become a major component of the nuclear inclusion (Albrecht et al., 1984).

After 24h p.i. infected cells show a gradual but large increase in size and the characteristic late phase 'cytomegalia' developes. Infected cells become less rounded and by 72-144h p.i. taking on an epithelial shape. Both the nucleus and nucleolus expand in line with the rest of the infected cell. The fibrillar network of ring-shaped nuclear inclusions spreads through the whole of the expanded nucleus as the infection proceeds encircling electron translucent areas (Calvallo et al., 1981).

1:14 Three Phases of HCMV Gene Expression

Transcription and replication of the virus genome takes place in the nucleus. HCMV operates a cascade system of gene regulation in which an increasing number of virus encoded RNA and protein species are synthesised during the course of a productive infection (Stinski, 1977; Stinski 1978; Wathen et al., 1981). The HCMV infectious cycle is conventionally divided into three phases: immediate early (IE or α), early (or β) and late (or γ).

The IE phase encompasses the events occuring after virus infection but prior to de novo virus-encoded protein synthesis. Although IE gene expression is almost completely dependent on unmodified cellular transcriptional, RNA-processing and translational systems, a HSV-1 tegument protein, taken up by cells during virus adsorption, has been shown to stimulate HSV-1 IE transcription (Campbell et al., 1984). It has recently been proposed that HCMV virions contains a similar function (Spaete and Mocarski, 1985b; Stinski and Roehr, 1985). The transition from IE to early phase gene expression occurs rapidly (Blanton and Tevethia, 1981). In order to study the IE phase gene products (RNA and protein) cells are infected and maintained in the presence of an inhibitor of protein synthesis (such as

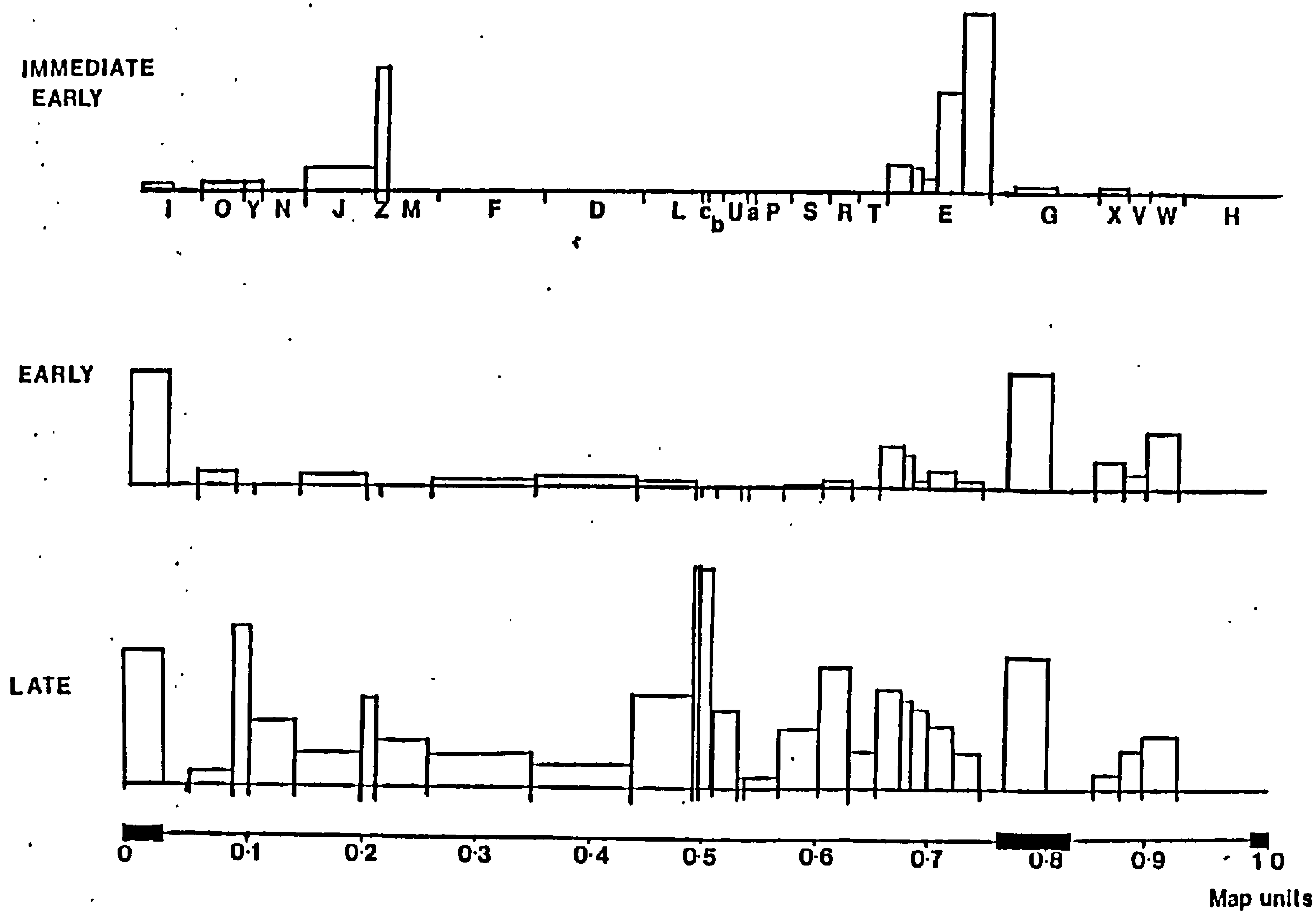


Fig 1:4: Histogram showing levels of hybridisation beteen IE, early and late phase cytoplasmic poly $(A)^+$ RNA and different regions of the HCMV genome (from Wilkinson, 1983). The hybridisation was performed against dot blots of cloned HCMV HindIII fragments except for the inverted repeat regions where the PstI fragment B was used. The level of hybridisation, determined by densitometry, was divided by the molecular weight of the cloned fragment to give the value plotted. Transcription from sequences adjacent to the inverted repeats was not investigated in this experiment. The position of HindIII fragments is shown.

cycloheximide) for 1h before and up to 12h after infection. Cycloheximide prevents the transition from the IE to the early phase by inhibiting _de novo_ virus-encoded protein synthesis. Treatment with an inhibitor of protein synthesis enhances IE transcription and thereby facilitates studies of IE gene expression. Enhanced IE protein synthesis is obtained by releasing infected cells from the translational (cycloheximide) block in the presence of an inhibitor of transcription, such as actinomycin D.

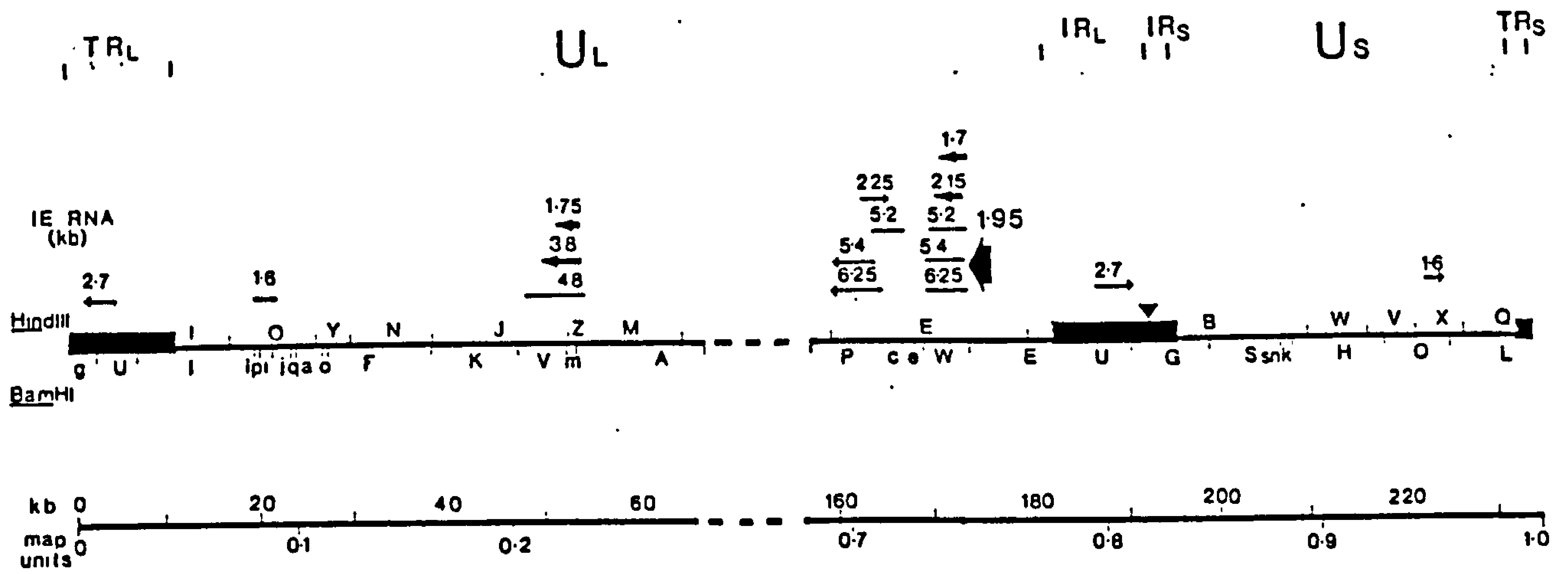
Early phase gene expression extends from the onset of virus encoded protein synthesis up until the initiation of virus DNA replication. Early RNA is routinely purified from cells grown in the presence of an inhibitor of the virus DNA polymerase (such as PFA) up to 24h p.i.

Late genes are defined as those expressed after the initiation of virus DNA replication. The transition from early to efficient late phase gene expression takes place by 96h p.i.

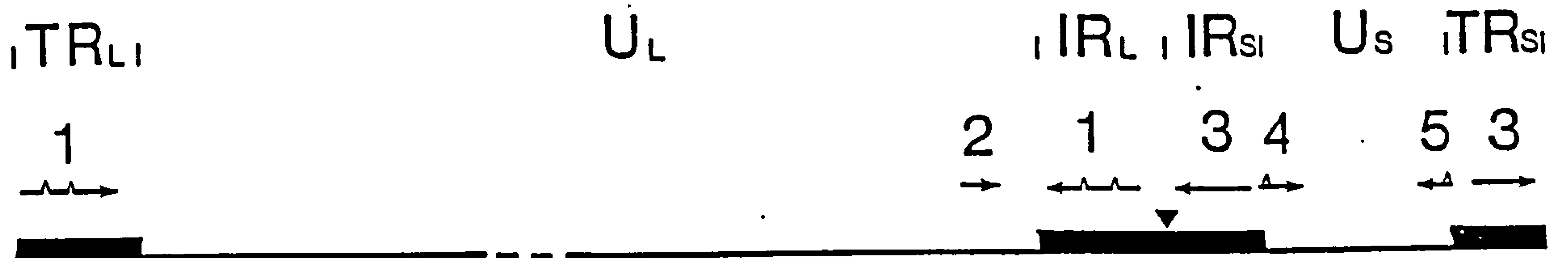
The use of metabolic inhibitors helps to delineate the bounderies between the different phases of gene expression. Gene products synthesised during one phase may continue to be synthesised during subsequent phases. Additionally, the division of the HCMV replicative cycle into the IE, early and late phases does not exclude the possibility that specific or co-ordinated changes in gene regulation occurs at other stages of infection.

1:15 IE RNA Transcripts

An estimated 90% of cytoplasmic poly $(A)^+$ IE RNA is derived from two regions of the virus genome; the 20.35kb _Hind_III fragment E and 6.6kb of DNA at the junction of _Hind_III fragments J and Z (Fig 1:4; DeMarchi, 1981; Wathen and Stinski, 1982; DeMarchi, 1983a; McDonough and Spector, 1983; Wilkinson, 1983). The size, number, and in some cases, the orientation of transcription of IE RNA species has been determined in experiments in which cloned subgenomic DNA fragments were hybridised with 'Northern' transfers



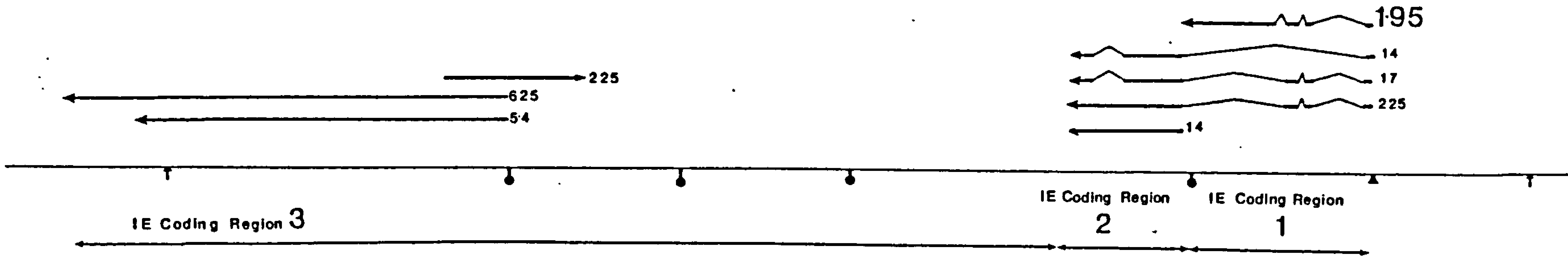
a) HCMV

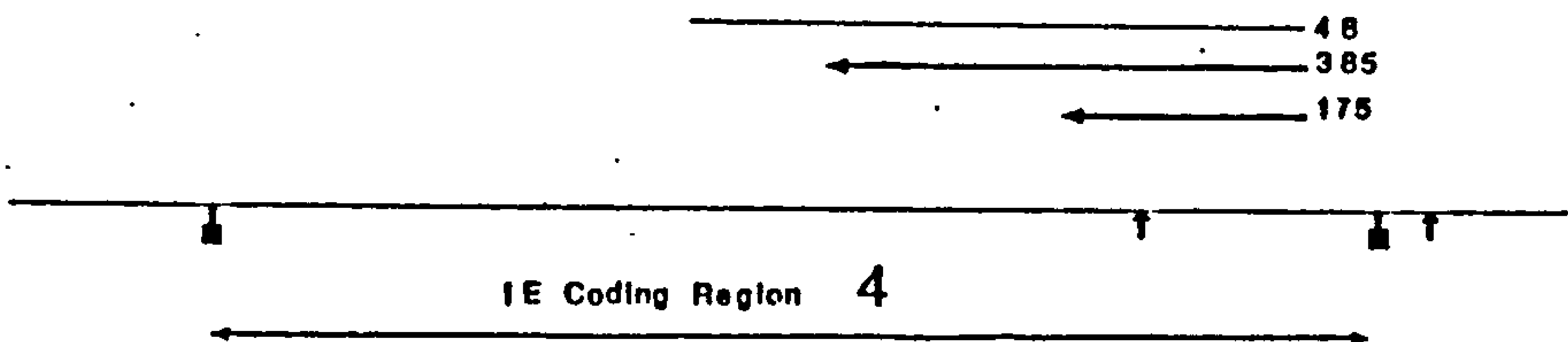


b) HSV-1

Fig 1:5: The pattern of IE transcription from (a) the HCMV strain AD169 genome (Wilkinson et al., 1984) and (b) the HSV-1 genome (Rixon and Clements, 1982; Perry et al., 1986). HCMV IE RNAs are identified by their sizes (kb) whereas HSV-1 IE RNAs have been given their agreed designations, 1-5. The positions of introns (Λ) in HSV-1 transcripts are shown.

Fig 1:5; Wathen et al., 1981; Wathen and Stinski, 1982; DeMarchi, 1983c; Wilkinson, 1983; Jahn et al., 1984; Wilkinson et al., 1984). HCMV strain AD169 encodes at least one major, four middle abundant and ten minor size classes of IE RNA (Fig 1:5). For ease of reference HindIII fragment E has been subdivided into IE coding regions 1, 2 and 3 as described in Figs 1:6 (Stinski et al., 1983) and the HindIII J/Z junction has been designated IE coding region 4.


a.
0682 Map Units
Hind III fragment E
0768 Map Units
490bp Transforming Fragment
1·95
14
17
225
14
225
625
5·4
IE Coding Region 3
IE Coding Region 2
IE Coding Region 1


b.
← Hind III J
0228 Map Units
Hind III Z
0236 Map Units
0207 Map Units
EcoRI fragment Q
0234 Map Units
4·8
3·85
1·75
IE Coding Region 4
2 kb

IE Coding Region 1: The most abundant IE mRNA encoded by HCMV strains AD169 (1.95kb; Wilkinson, 1983; Jahn et al., 1984), Davis (2.2kb; DeMarchi, 1983c) and Towne (1.95kb; Wathen and Stinski, 1982) map to the same relative position, within the U_L region, of each genome (IE coding region 1; Fig 1:6). The 1.95kb IE transcripts of strains AD169 and Towne have been precisely mapped by DNA/RNA hybridisation experiments between 0.762 and 0.745 map units on their respective virus genomes. Within the IE genes of strains AD169 and Towne the orientation of transcription is the same, the relative positions of the BamHI, Pstl and Sstl restriction endonuclease cleavage sites are similar and the transcriptional initiation sites, identified by in vitro transcription experiments, are identical (Stinski et al., 1983; Wilkinson, 1983; Thomsen et al., 1984). The strain AD169 and Towne major 1.95kb IE mRNAs were shown to be transcribed from at least 2.85kb of genomic DNA; indicating that in both strains AD169 and Towne at least 0.9kb of the initial transcript was excised by splicing. Work performed for this thesis (Akrigg et al., 1985) and by Stenberg et al. (1984) has precisely mapped the positions of three introns in the major IE genes of strains AD169 and Towne (Fig 1:6).

Hybrid selected translation (HST) experiments have been used to identify and map some of the products of IE gene expression. In HST experiments RNA encoded by a specific gene is selected by hybridisation with the appropriate cloned subgenomic DNA fragments immobilised on a solid

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Fig 1:6: HCMV IE coding regions 1 to 4. (a) HindIII fragment E contains IE coding regions 1 to 3 and (b) IE coding region 4 goes across the junction of HindIII fragments J and Z. The splicing pattern for the major 1.95 kb IE RNA of strains AD159 and Towne are identical (this thesis; Stenberg et al., 1984). The transcriptional data for RNAs derived from IE coding region 2 is from Stenberg et al., 1985). Although detailed transcriptional mapping data for IE coding region 2 of strain AD169 is not available it is known to encode IE RNAs of 1.7 kb and 2.25 kb but not 1.4 kb (Wilkinson et al., 1984). Data for IE coding regions 3 and 4 is for strain AD169 (Wilkinson et al., 1984). The position of some BamHI (●), EcoRI (■), HindIII (↓) and SstI (▲) sites are shown.

matrix (e.g. a nitrocellulose membrane). Following extensive washing, to remove all non-homologous RNA, the annealed mRNA is eluted and translated *in vitro* in a rabbit reticulocyte lysate. The results of experiments performed using strains AD169 and Towne IE RNA are detailed in Table 1:5. HST experiments have shown that the major IE polypeptide is the product of IE coding region 1 in strains AD169 (Wilkinson *et al.*, 1984), Davis (DeMarchi, 1983) and Towne (Stinski *et al.*, 1983).

IE Coding Region 2: The pattern of transcription from coding region 2 is complex (Figs 1:5 & 1:6). The nucleotide sequence region 2 in the Towne strain has been determined and a partial characterisation of RNA splicing accomplished (Stenberg *et al.*, 1985). At least five RNA species (1.1, 1.4, 1.7, 1.7 and 2.25kb) have been mapped to IE coding region 2 of strain Towne (Stinski *et al.*, 1983; Stenberg *et al.*, 1985). The 1.4kb, the spliced 1.7kb

Virus strain		Polypeptides (Kdaltons)										
Towne	[a]Coding region 1 (HST)			75				39				
Towne	[a]Coding region 2 (HST)		75			56	42				21	16.5
Towne	[b]Coding region 2 (seq)					51	48		30	27		
Towne	[c]Total (Immunoppt)		75	72	68	59		39			21	19
AD169	[d]Total (Immunoppt)	78	77	73					31			
AD169	[e]Coding region 1 (HST)			75								
AD169	[e]Coding region 2 (HST)	78.5	78			62	59					
AD169	[e]Coding region 4 (HST)						51	47	40			

[a] Stinski *et al.*, 1983.
[b] Stenberg *et al.*, 1985.
[c] Stinski, 1978.
[d] Blanton and Tevethia, 1981.
[e] Wilkinson *et al.*, 1984.

Table 1:5: A comparison of IE polypeptides identified by immunoprecipitation, hybrid selected translation (HST) and predicted from nucleic acid sequencing/nuclease protection studies (seq). The existance *in vivo* of proteins predicted from nucleotide sequence/nuclease protection data has yet to be confirmed experimentally (Stenberg *et al.*, 1985). The major IE polypeptides are shown in bold type. The major IE coding regions 1 and 2 are in the same relative positions on the genomes of strains AD169 and Towne as is described in Fig 1:3.

and the 2.25kb mRNAs respectively were shown to contain 2, 4 and 3 introns and to encode polypeptides of 27K, 48K and 51K. Transcription of these three spliced mRNA species is thought to be initiated from the major IE gene promoter in IE coding region 1 (Stenberg et al., 1985). Stenberg et al. (1985) proposed that the translation products of the spliced 1.7kb and 2.25kb mRNAs both contain a short sequence in common with the amino terminus of the major IE polypeptide. Transcription of a second 1.7kb mRNA, which is thought to encode a 30K polypeptide, was initiated immediately downstream from the major IE gene; using a promoter which overlaps the 3' end of the major 1.95kb mRNA coding region.

In HCMV strain AD169 two middle abundant size classes of IE RNA are transcribed from within 0.7kb downstream from the terminus of the major IE gene and in the same orientation (Wilkinson et al., 1984); equivalents of the strain Towne 1.1kb and 1.4kb IE mRNAs transcribed from coding region 2 (Fig 1:6) have, however, not been detected (Jahn et al., 1984; Wilkinson et al., 1984). The strain AD169 IE coding region 2 encodes at least four size classes of IE protein (Table 1:5) but only two size classes of middle abundant RNA; the abundance of the minor 5.2kb, 5.4kb and 6.25kb RNAs was too low to be expected to produce detectable products in HST experiments. It is possible that more than one IE mRNA of 1.7kb and/or 2.25kb is transcribed from strain AD169 IE coding region 2.

IE Coding Region 3: IE coding region 3 of strain AD169 has been studied in detail primarily because it contains a 490bp DNA fragment which has been shown to be capable of transforming cells in vitro (Fig 1:6; Nelson et al., 1984). The minor 5.4kb and 6.25kb IE RNAs which are transcribed through the transforming region (Fig 1:6) have been shown not to be spliced in the region of the transforming DNA fragment (Nelson et al., 1984). The longest ORF within the transforming region, as predicted by an analysis of the nucleotide sequence (Kouzarides et al., 1983; Nelson et al., 1984), would

however encode a polypeptide of only 41 amino acids. The transformation process mediated by this region therefore may not be caused by a HCMV-encoded protein.

IE Coding Region 4: In strain AD169 two middle abundant (1.75kb and 3.8kb) and one minor (4.8kb) RNA species encoded by IE coding region 4 hybridise with DNA probes from both *Hind*III fragments J and Z (Fig 1:5). The two middle abundant RNAs are transcribed in right to left direction with respect to the prototype orientation of the genome (Fig 1:5; Wilkinson *et al.*, 1984).

1:16 Post-transcriptional Control Over Processing of IE RNA

Following transcription a poly (A) tail is added to the 3' ends of most IE RNAs and in some cases, at least, introns are removed from primary transcripts by RNA-splicing (see section 1:15) before they are transported to the cytoplasm. Some IE transcripts, however, have been shown to be preferentially retained in the nucleus. The 5.4kb and 6.25kb IE RNAs from IE coding region 3 are present in low abundance in IE cytoplasmic poly $(A)^+$ RNA but in high abundance in early and late RNA preparations. DeMarchi (1983a) observed that RNAs of similar sizes (4.8kb and 5.2kb) encoded by the equivalent region of strain Davis were efficiently transcribed under IE conditions but were not transported to the cytoplasm until the early phase. RNA synthesised from a region between 0.483-0.553 map units of strain Davis, which accounted for 9% of total IE transcription was also not transported to the cytoplasm under IE conditions (DeMarchi, 1983a). Since it has yet to be determined whether any of the RNAs confined to the nucleus during the IE phase encode proteins it is not clear if this phenomenon represents post-transcriptional regulation of HCMV gene expression.

1:18 IE Gene Expression

The HCMV major IE antigen produces a generalised staining pattern over the whole of the cell nucleus in immunofluorescence studies (Goldstein et al., 1982). Although the major IE polypeptide is associated with the nucleus, when infected cells are lysed with the detergent NP40 (which solubilises the plasma membrane), between 60% to 90% of the major IE polypeptide partitions with the cytoplasmic fraction (Jeang and Gibson, 1980; Gibson, 1981b). Immune electron microscopy studies have shown that much of the major IE antigen is associated with the outside of the nuclear membrane and the perinuclear region (K. Blake, personal communication) however some of the major IE polypeptide may penetrate the nuclear membrane as it has been reported to be associated with cellular chromatin (S. Michelson quoted by Stinski et al., 1983).

Comparisons made by SDS-PAGE of ICSP's produced using different isolates indicates that there is little inter-strain variation in the sizes of HCMV proteins (Gupta et al., 1977), additionally the protein profiles of HCMV isolates and simian CMV strain Colburn are also very similar (Gibson, 1983; Table 1:4). The major IE polypeptide is a noticable exception. Gibson (1981b) observed that when directly compared in the same polacrylamide gel the major IE polypeptides of strains Colburn, AD169, Davis and Towne had apparent molecular weights of 94K, 79K, 78K and 76K respectively. The observed differences in the electrophoretic mobilities of these proteins were not thought to be caused by post-translational processing as they were also apparent when the proteins were translated in vitro (Cameron and Preston, 1981).

Although the apparent molecular weights of the strains AD169 and Towne major IE polypeptides differ by 3K their genes are similar (see section 1:16) and in other respects the proteins have similar properties. The major IE polypeptides of strains AD169 and Towne are both phosphorylated (Gibson, 1981b), share the same isoelectric point and are antigenically related; both

proteins are recognised by a monoclonal antibody produced by Goldstein et al. (1982).

During the course of a productive infection the characteristic nuclear staining pattern of the major IE polypeptide can be detected by immunofluorence 20-60 min p.i. (Michelson-Fiske et al., 1977). Experiments in which infected cell proteins were pulse labelled with [^{35}S]-methionine showed that the major IE polypeptide is efficiently expressed only during the first few hours of a permissive infection (Stinski, 1978; Blanton and Tevethia, 1981). The major IE mRNA was present in only trace amounts at 2h p.i., is relatively abundant at 5h p.i. but by 12h p.i. its abundance has declined 10 fold to a level which is maintained until the late phase (Stenberg and Stinski, 1985). The repression of major IE gene expression during the early and late phases is exerted at the level of transcriptional initiation (Nelson and Groudine, 1986). Recently DNA transfection studies have indicated that the major IE polypeptide may directly or indirectly cause transcription from its own gene to be repressed; the major IE gene may therefore autoregulate its own expression (Stenberg and Stinski, 1985).

Because cycloheximide prevents both the shut-off of IE gene expression and the transition to early phase it is used experimentally to artificially increase the quantity of IE gene products in infected cells. Cycloheximide treatment, however, results in a 90% reduction in the yields of total poly $(A)^+$ RNA obtained from human embryo fibroblasts (MRC5 cells; Wilkinson, 1983). DeMarchi (1983c) has shown that the 2.2kb major IE mRNA of strain Davis is much more stable in cycloheximide treated cells than other HCMV transcripts. Consequently, cycloheximide treatment can be expected to significantly enhance the concentration of the major IE mRNA relative to that of other host and virus-coded species in infected cells.

Convalescent immune sera immunoprecipitated three polypeptides with molecular weights of 73K, 77K and 78K in similar abundance from extracts of strain AD169 infected cells prepared 1.5h p.i. (Blanton and Tevethia, 1981).

The 77K and 78K proteins may correspond to the strain AD169 78K and 78.5K IE polypeptides mapped to IE coding region 2 by HST (Table 1:5). Cycloheximide treatment for 5h p.i. resulted in the subsequent overproduction of the 73K (major) IE polypeptide, following cycloheximide release, but did not significantly enhance the synthesis of the 77K and 78K polypeptides (Blanton and Tevethia, 1981). Michelson et al. (1979) also detected three IE polypeptides of similar size (76K and a doublet of 82K) in HCMV strain Mira-infected cells between 1h to 3h p.i. The 76K strain Mira protein was present in both nuclear and cytoplasmic fractions, which is typical of the major IE polypeptide, but interestingly the two 82K proteins were detected only in the nuclear fractions (Michelson et al., 1979). The largest ORF identified so far from the nucleotide sequence of IE coding region 2 (strain Towne) would encode a protein of only 51K, synthesised from the spliced 2.25kb IE mRNA (Fig 1:6; Stenberg et al., 1985). There is some evidence, however, from HST experiments that strain Towne coding region 2 also encodes a 75K polypeptide which is distinct from the major IE polypeptide (Stinski et al., 1983).

HST showed that the three RNA species mapped to IE coding region 4 encoded at least three polypeptides with molecular weights of 40K, 47K and 51K (Table 1:5). IE-1.75 which is derived largely from HindIII Z probably encodes the 40K protein whereas IE-3.8 which hybridises more strongly with HindIII J may encode the 47K protein (Wilkinson et al., 1984).

The translation products of minor IE RNAs transcribed from IE coding region 3 and other regions of the HCMV genome have yet to be identified.

1:18 Comparison Betweeen HCMV and HSV-1 IE Transcription

The patterns of HCMV and HSV-1 IE transcription is compared in Fig 1:5. In both viruses IE gene expression is restricted to a relatively small portion of the virus genome. Four of the five HSV-1 IE genes are clustered around the inverted repeats; HSV-1 IE mRNAs 1 and 3 are encoded entirely

from within the long and short repeat respectively and their genes are therefore present in two copies per HSV-1 genome. In contrast, the principal HCMV IE genes all map within the U_L region. The available information (see section 1:15) indicates that the structural organisation of the HCMV IE genes is complex, transcription initiated from the major IE promoter alone may encode four or more mRNA species (Stenberg et al., 1984; Akrigg et al., 1985; Stenberg et al., 1985). Differential RNA splicing is employed by HCMV in IE coding regions 1 and 2 to generate a complex series of related polypeptide encoded by a combination of different exons. Gene expression from HCMV IE coding regions 3 and 4 is not yet well characterised but it is apparent that in both regions more than one size class of transcript is synthesised from the same strand either by an alternative splicing mechanism and/or by using more than one transcriptional initiation or polyadenylation site (Table 1:6).

IE mRNAs-1, -4 and -5 are the only HSV-1 IE transcripts which have been shown to be spliced (Perry et al., 1986; Rixon and Clements, 1982). HSV-1 IE gene 1 contains two introns of 767 bases and 136 bases both of which map within the coding region for the IE110 protein (Fig 1:5; Perry et al., 1986). IE mRNAs -4 and -5, which are transcribed from the HSV-1 short repeats into opposite ends of the U_S component (Fig 1:5), both contain a single intron in their 5' leader sequences. The promoter, leader and the intron sequences of IE mRNAs -4 and -5 are essentially identical. Since the HSV-1 intron common to both IE mRNAs -4 and -5 does not contain the translational initiation codon 'AUG' and is not always excised during RNA processing, its removal may not be essential for gene expression (Rixon and Clements, 1982).

The HCMV IE genes differ from those of HSV-1 in (a) their relative locations on the virus genomes and (b) the HCMV IE genes, at least in coding regions 1 and 2, use alternative RNA splicing pathways to make more elaborate use of the available coding capacity.

1:19 Early Phase Gene Expression

The early phase of the HCMV replicative cycle is long relative to that of HSV-1. Following a high m.o.i. infection, replication of HCMV DNA is not detected until 12h p.i. and does not reach a maximum until 72-96h p.i. (Huang et al., 1973). The efficient expression of both early and late HCMV genes is dependent on transcriptional activation by (a) de novo synthesised IE gene product(s). The transition from IE to early phase gene expression occurs relatively rapidly. By 4-6h p.i. IE protein synthesis is already in decline and the synthesis of early proteins can be detected (Blanton and Tevethia, 1981). DNA/RNA hybridisation experiments have also detected early phase transcription at 4h p.i. (DeMarchi et al., 1980).

The general pattern of transcription observed at 4h p.i. is maintained until 24h p.i. (DeMarchi et al., 1980). Cytoplasmic poly $(A)^+$ RNA isolated from strain AD169-infected, PFA-treated cells 24h p.i. hybridised weakly with most regions of the virus genome but strongly with the long repeat (56% of early RNA hybridisation was with TR_L/IR_L), HindIII W (9.5%) and HindIII V (3.1%), (Fig 1:4; Wilkinson, 1983; McDonough and Spector, 1983). HCMV encodes a single abundant 2.7kb early RNA from the long repeat region (McDonough et al., 1985; this thesis), however the virus does not encode a correspondingly abundant virus-encoded early polypeptide. A translation product for the abundant 2.7kb early RNA has not been identified.

Hybridisation experiments performed using whole cell early phase RNA have shown that, as during the IE phase, some transcripts encoded by the U_L component are retained in the nucleus until later in the infectious cycle (Wathen and Stinski, 1982; DeMarchi, 1983a). The HCMV major tegument and DNA-binding proteins which are synthesised only in trace amounts during the early phase are major components of the cell in the late phase. Geballe et al. (1986a) observed that although the genes for these proteins were not transcribed under IE conditions they were transcribed at 4h p.i. The genes

encoding the HCMV major tegument and DNA-binding protein are transcribed during the early phase but, possibly due to post-transcriptional regulation, they are expressed as conventional late phase genes.

1:20 Late Phase Gene Expression

The pattern of HCMV gene expression increases in complexity as the infection progresses. Hybridisation experiments between cytoplasmic poly $(A)^+$ RNA and cloned HCMV DNA fragments indicate that during the late phase most regions of the virus genome are being expressed (Fig 1:4; DeMarchi et al., 1980; DeMarchi, 1981; Wathen and Stinski, 1982; McDonough and Spector, 1983; Wilkinson, 1983). A systematic experiment in which cloned XbaI DNA fragments representing the complete strain Towne genome were hybridised with Northern transfers of late RNA identified more than 85 virus-encoded RNA transcripts (Wathen and Stinski, 1982). HCMV is known to encodes a large number of late proteins (Table 1:3). The potential coding capacity of the 235kb HCMV genome is considerable but recently some progress has been made towards unravelling the complexities of late phase gene expression.

The cloning vector λgt11 was designed to select for the expression of short open reading frames (ORFs) cloned upstream from the bacterial β-galactosidase gene. The inserted short ORF is expressed as a fused protein covalently linked to β-galactosidase. Mocarski et al. (1985b) shotgun cloned short sonicated fragments of HCMV DNA into λgt11. One of the clones generated expressed a protein recognised by the monoclonal antibodies CH 13-2 and CH 16-1 (Pereira et al., 1982). The cloned DNA fragment was shown to be derived from HCMV HindIII M (strain AD169). HST experiments demonstrated that a 5kb mRNA transcribed from HindIII M encoded a 50K polypeptide which was immunoprecipitated by a mixture of CH 13-2 and CH 16-1 (Mocarski et al., 1985b). The 50K polypeptide was identified as being the (52K) DNA-binding protein characterised by Gibson (1983; Table 1:4).

Nowak et al. (1984b) used a series of overlapping cosmid clones

spanning the entire strain AD169 genome in HST experiments. Immunprecipitation of the *in vitro* translation products with monoclonal antibodies enabled the genes encoding the 69K (65K according to Nowak *et al.*, 1984b) and 74K (71K) major tegument, or lower and upper matrix, proteins to be mapped. Both proteins were encoded by (a) 4kb mRNA(s) transcribed in a left to right orientation (with respect to the prototype orientation of the genome; see Fig 1:2) from *HindIII* fragments L, c and b. The mechanism by which the two tegument proteins were encoded by the same region of DNA was not ascertained. The proteins, however, were antigenically distinct which suggests that they do not share extensive amino acid sequence. Nowak *et al.* (1984b) also identified the approximate map locations of a large number of additional polypeptides, some of which co-migrated wth virion components, in these HST experiments. It was not possible, however, to definitively identify additional translation products from their size alone.

Davis *et al.* (1984) mapped an abundant 1.9kb late mRNA within the strain Towne *Eco*RI fragment G (in strain AD169 the transcript maps to within *HindIII* D and *BglII* X). The late gene was cloned into a eukaryotic expression vector (based on the SV40 origin of replication) and transfected into COS cells. The expression of a 67K phosphoprotein was detected in the transfected cells using a monoclonal antibody (Davis *et al.*, 1984). The 67K phosphoprotein was identified as a component of the virion tegument and co-purified with a protein kinase activity. The nucleotide sequence of this gene was determined (Davis and Huang., 1985).

Mach *et al.* (1986) recently detected the expression of a short fragment of the gp52 structural gene cloned into λgt11 using a polyvalent antiserum raised to gp52 in rabbit. The gp52 structural gene was thereby shown to be located within *HindIII* fragment F. An analysis of the nucleotide sequence of *HindIII* F identified an ORF for a potential glycoprotein which shared a significant level of amino acid homology with the glycoprotein B's of HSV-1

and EBV (Cranage et al., 1986). Gp52 is produced by the proteolytic cleavage of a larger precursor molecule and shares amino acid sequence homology with glycoproteins encoded by other herpesviruses.

The nucleotide sequence of HindIII F also predicted an ORF which exhibited significant homology with the DNA polymerase genes of other sequenced herpesviruses (Kouzarides et al., 1987b). The entire HCMV genome is currently being sequenced. Many of the proteins involved in the mechanics of virion replication (e.g. the DNA polymerase, DNA cleavage and packaging enzymes, capsid and other virion proteins) appear to have been conserved across the herpesvirus group. A comparison of the HCMV DNA sequence with the known sequences of HSV-1, VZV and EBV may identify further conserved herpesvirus proteins and assist in determining the functions of additional HCMV genes.

1:21 HCMV DNA Replication

HCMV encodes its own DNA polymerase (Huang, 1975) which has been mapped on the genome of strain AD169 within the HindIII fragment F (Kouzarides et al., 1987b). In biochemical tests the HCMV DNA polymerase has similar properties to the cellular α-DNA polymerases which are involved in DNA replication as opposed to repair (Hirai and Watanabe, 1976). The HCMV DNA polymerase is PAA and aphidocolin sensitive, resistant to high concentrations of 2',3' ddTTP and functions optimally in high salt concentrations (Huang, 1975a; 1975b; Nishiyama et al., 1983). A 3' to 5' exonuclease activity, which is also PAA sensitive, is associated with the virus DNA polymerase (Nishiyama et al., 1983).

HCMV DNA replication is first detected 12h p.i., increases to reach an initial peak of activity at 24h p.i., then declines and rises again to its maximum level during the late phase, 72-96h p.i. (Stinski, 1978). Although the replicative cycle of HSV-1 and HCMV in human fibroblasts takes 8h and 4 days respectively (Smith and DeHarven, 1973) many of the processes involved

in virus DNA replication and encapsidation appear to be very similar. During the early stages of a permissive or semi-permissive infection the HCMV genome circularises (LaFemina and Hayward, 1983) due to an interaction between the complementary a sequences present as direct repeats at each terminus of the genome (Fig. 1:3). Circularisation of the HCMV genome, a property shared with other herpesviruses, is PAA-sensitive and may therefore be dependent on a function of the virus-encoded DNA polymerase.

Like other herpesviruses (Spear and Roizman, 1980), the HCMV genome is probably replicated according to the rolling circle model which would result in virus DNA being synthesised in long concatemers. HCMV DNA purified from late phase infected cells has been shown to contain a high concentration of L-S joint fragments and relatively few terminal fragments which implies that the genome is replicated either in long concatemers or DNA circles (LaFemina and Hayward, 1983). Additionally the HCMV a sequence can substitute for the HSV-1 a sequence function in HSV-1 amplicons (Spaete and Mocarski, 1985a; section 1:11). The a sequence provides the cis acting signal which directs the cleavage of concatemeric DNA into unit lengths which occurs during packaging into capsids.

Replication of cellular DNA is inhibited in permissive cells by 10h p.i., even in the presence of PAA (DeMarchi, 1983b). This result is apparently in conflict with a previous observation that HCMV infections stimulate cellular DNA replication (St Jeor et al., 1974). DeMarchi and Kaplan (1977) have suggested that HCMV may stimulate cellular DNA replication only in cells abortively infected by defective virions.

1:22 Virus Maturation

The continued synthesis of host cell proteins throughout the HCMV replicative cycle makes the identification of virus encoded proteins more difficult. The majority of late phase ICSPs, which have been identified, co-migrate in SDS-polyacrylamide gels with components of purified virions

(Stinski, 1977). Late phase virus-encoded protein synthesis is, therefore, believed to be primarily associated with virion production. The initial stages in the construction of HCMV virus particles take place in the nucleus.

In detailed studies of the simian CMV strain Colburn replicative cycle Gibson (1981a) identified two forms of immature virus particle in infected cell nuclei. The 'type A' particles are empty virus capsids containing 28K, 34K and 145K virion proteins in a ratio of 1:3:7 respectively (Gibson, 1981a). 'Type B' particles contain all the A-capsid proteins plus two additional phosphoproteins of 37K and 45K and also virus DNA. Since the 37K and 45K phosphoproteins are not processed into mature virions, it has been proposed that these "assembly proteins" play a role in nucleocapsid maturation (Gibson, 1981a). Tryptic hydrolysis of the 37K and 45K proteins indicates that the two proteins are very similar and probably share amino acid sequences (Gibson, 1981a). The CMV assembly proteins and the HSV-1 38K (VP22a) capsid-associated protein are unusual in that they fluoresce pink when stained with coomassie brilliant blue. The cause of the fluorescence is not understood but its existence suggests there may be a functional relationship between the CMV and HSV-1 capsid-associated proteins (Gibson, 1981a). A temperature-sensitive HSV-1 mutant (17tsVP1201) contains a defect which prevents the processing of VP22a (38K) from a 40K precursor protein. In HSV-1 17tsVP1201-infected cells, at the non-permissive temperature, viral DNA is replicated and nucleocapsids are produced but the nucleocapsids do not package the viral genome; a functional VP22a protein is therefore required for packaging HSV-1 DNA (Preston et al., 1983).

Immediately following the initiation of virus DNA replication simian CMV strain Colburn induces the synthesis of a 51K DNA-binding protein (Gibson et al., 1981a). The DNA-binding protein is phosphorylated, positively charged and localised in the nucleus of infected cells. In certain circumstances the 51K protein can be found associated with B-capsid

particles, which suggests it is involved in virus maturation. However, the 51K DNA-binding protein, like the assembly proteins, is not incorporated into mature virions (Gibson et al., 1981a). Since both empty and filled capsids are associated with nuclear inclusions in infected cells, the packaging of HCMV DNA into nucleocapsid particles is thought to take place at the nuclear inclusions (Becker et al., 1965; McGavran and Smith, 1965; Cavallo et al., 1981). HCMV DNA preparations made from virus stocks enriched for defective particles contain a high proportion of subgenomic DNA molecules, the majority of which were approximately 60 x 10^6 and 100 x 10^6 daltons (Stinski et al., 1979). The presence of these relatively short DNA molecules in mature virions suggests that the mechanism responsible for cleaving and packaging concatenated virus DNA is not be strictly dependent on HCMV capsid particles being filled with genomic length units of DNA.

The mechanism involved in packaging viral DNA may have been conserved throughout the Herpesviridae. HSV-1 also manufactures A- and B-capsid particles in infected cells with a similar size and protein composition to those of simian CMV (Gibson and Roizman, 1972; Gibson, 1981a). The capsid-associated proteins of HSV-1 and CMV have similar properties (Gibson, 1981a), there is a region of DNA sequence homology within the cleavage and packaging signals (a sequences) of HCMV and HSV-1 (see section 1:11; Tamishiro et al., 1984) and the HCMV L-S junction can complement the cis-signal (contained in the a sequence) for cleavage and packaging DNA in an HSV-1 amplicon (see section 1:11; Spaete and Mocarski, 1985a).

Nucleocapsids become coated with tegument protein in passing from the nuclear inclusion to the nuclear envelope (McGavran and Smith, 1965). The most abundant HCMV polypeptide, both in infected cells, and purified virions is the 69K major tegument, or lower matrix, protein (Stinski, 1976). The 69K tegument protein also accounts for 95% of the mass of dense bodies (Irmiere and Gibson, 1983). Immunofluorescence studies show that the 69K protein is found in the nucleoplasm of infected cells but it is not associated with the

nuclear inclusions (Weiner et al., 1985). This observation is consistant with nucleocapsids acquiring their coating of tegument protein after leaving the nuclear inclusion. In infected cells both the 69K tegument protein and the 153K major capsid protein are associated with the nuclear skeleton, or matrix (Yamauchi et al., 1985). The early stages of virion maturation appear to involve an ordered interaction between virion proteins and the fibrillar protein networks contained in the infected cell nucleus.

There are three major (including the 69K tegument protein) and nine minor phosphoproteins in mature virions, none of which are components of the nucleocapsid (Table 1:4; Roby and Gibson, 1986). The extensive phoshorylation of tegument proteins may be related to virion maturation. The tegument of mature HCMV virions contains two distinct protein kinase activities, one of which specifically phosphorylates tegument proteins in vitro (Mar et al., 1981; Roby and Gibson, 1986). Recent reports have shown the protein kinase to be a 67K/68K virion protein which, although abundant, is distinct from the 69K major tegument protein (Davis and Huang, 1985; Britt and Auger, 1986).

HCMV virions have been shown to contain the polyamines spermine and spermidine (Gibson et al., 1984). Treatment of infected cells with difluoro-methylornithine (DFMO), an anologue of ornithine which inhibits polyamine synthesis, causes a reduction in the yields of both infectious virions and NIEPs (Gibson et al., 1984). Since NIEPs do not contain DNA it was proposed that polyamines have a role in the maturation of HCMV enveloped particles other than neutralising the charge on DNA; polyamines may possibly be required to neutralise charged phosphoproteins during the assembly of the HCMV tegument (Roby and Gibson, 1986). While spermine and spermidine are also both present in HSV-1 virions, only spermine is found in the nucleocapsid where it is presumed to be involved in neutralising the charged phosphate groups on tightly packaged virus DNA. Spermidine, consequently, must be associated either with the HSV-1 tegument region or the envelope

(McCormick, 1978).

Immature virus particles bud through the nuclear membrane into the cytoplasm (Smith and DeHarven, 1973; Severi et al., 1979). According to the model proposed by Severi et al. the passage of the virus particle out of the nucleus occurs in two stages. Initially the virus particles bud through the inner nuclear membrane acquiring a temporary envelope in the process. The envelope, containing the virus particle, then fuses with the outer nuclear membrane and the naked particle is ejected into the cytoplasm (Severi et al., 1979). The presence of non-enveloped virus particles within the cytoplasm (Smith and DeHarven, 1973; Severi et al., 1979) was interpreted as indicating that the mature virion does not obtain an envelope from the nucleus. This model, however is not universally accepted (Gibson, 1981a; Farrar and Oram, 1984). Farrar and Oram (1984) proposed that the HCMV virion contains two envelopes and that the inner envelope is probably derived from the nuclear membrane.

The final phase of virion maturation takes place in the cytoplasmic vesicle which is composed mainly of golgi apparatus (McAllister et al., 1963; Smith and DeHarven, 1973). The proteins associated with the virion envelopes are predominantly glycosylated (Stinski, 1977). The five glycoproteins which have been identified in the strain AD169 outer virion envelope have apparent molecular weights of 52K, 67K, 95K, 130K and 250K (Table 1:4; Farrar and Oram, 1984). In purified extracts, the 52K glycoprotein was found to be linked by disulphide bonding to the 95K or the 130K glycoproteins to produce a series of high molecular weight protein complexes (Farrar and Greenaway, 1986). Polyvalent sera and monoclonal antibodies have been raised against HCMV glycoproteins. It is clear from immunofluorescence studies that the glycoproteins are predominantly associated with the cytoplasmic vesicle and the plasma membrane (Pereira et al., 1982; Nowak et al., 1984; Law et al., 1985).

HCMV acquires its outer envelope, containing the membrane

glycoproteins, by budding through the membranes of the golgi and the plasma membrane (Farrar and Oram, 1984). A 28K/29K unglycosylated virion phosphoprotein was shown, by immunofluorescence, to be found associated with the cytoplasmic inclusion but not the nucleus of infected cells (Nowak et al., 1984; Re et al., 1985). The 28K/29K phosphoprotein is not a surface component of the virion (Nowak et al., 1984). In the maturation process which takes place at the cytoplasmic vesicle the virion acquires at least one unglycosylated protein.

Numerous glycoprotein species have been detected in infected cells in addition to the five shown to be contained in the mature virion (Pereira et al., 1982; Pereira et al., 1984; Sullivan-Tailyour and Garnett, 1986). Many of these glycoprotein species are generated by varying degrees of glycosylation combined with proteolytic cleavage of four antigenically distinct precursor molecules (Pereira et al., 1984). A monoclonal antibody raised to the 52K glycoprotein (gp52) recognises at least seven size classes of protein in infected cells (Pereira et al., 1984). While the in vitro translation product of gp52 mRNA has an apparent molecular weight of 95K, the size of the protein in infected cells is both increased due glycosylation and reduced as a consequence of proteolytic cleavage to ultimately generate a protein with an apparent molecular weight of 52K (Pereira et al., 1984; Cranage et al., 1986; Mach et al., 1986).

The induction of an immune response directed against the virion surface glycoproteins may be important in eliciting protection against HCMV infections in vivo (Gibson and Irmiere, 1984). Polyvalent sera and a number of the monoclonal antibodies raised to HCMV glycoproteins have been shown to be capable of neutralising HCMV infections in vitro (Pereira et al., 1982; Furukawa et al., 1984; Rasmussen et al., 1985; Cranage et al., 1986). Furukawa et al. (1984) observed that crude extracts of HCMV envelopes elicited both the humoral and cell-mediated immune response in guinea pigs. It may be possible to develope an effective subunit vaccine based on the

glycoproteins contained in HCMV outer envelope (Gibson and Irmiere, 1984).

1:23 Effects of HCMV Infection on Host Cell Metabolism

Chromatin: On entering the nucleus HCMV DNA becomes associated with chromatin (St Jeor *et al.*, 1982). Kamata *et al.* (1978) detected rapid changes in chromatin structure in HCMV-infected cells by measuring the circular dichroism and ethidium bromide binding capacity of purified chromatin. A small increase in ethidium bromide binding to chromatin was detected within 1h p.i. As the infection proceeds there is a steady increase in the level of ethidium bromide binding up to 24h p.i. after which time the level gradually declines. The circular dichroism measurements of chromatin, in contrast, had declined by 1h p.i. but returned to the original level by 12h p.i. and continued to increase up until 48h p.i. Experiments using u.v.-inactivated virus and an inhibitor of protein synthesis indicated that the observed effects were dependent on *de novo* virus-encoded protein synthesis. Since a change in chromatin structure was detected by 1h p.i. an IE gene product is probably involved.

In order to identify the factors responsible for inducing the observed effects protein was stripped off chromatin purified from infected cells, fractionated on DNA-cellulose and added back to chromatin extracted from uninfected cells. The virus-induced factors responsible for increasing ethidium bromide binding and affecting changes in circular dichroism measurements were eluted from DNA-cellulose in different fractions, chromatin structure in HCMV-infected cells is therefore affected by at least two distinct factors. The magnitude of the observed effects suggested that HCMV induces a change in total cellular chromatin and not merely that portion associated with virus DNA (Kamata *et al.*, 1978). In support of these observation Kierszenbaum and Huang (1978), in electron microscopy studies, have identified abnormal nucleosome structures in HCMV-infected cells 18h p.i.

Cell DNA Synthesis HCMV can stimulate semiconservative DNA replication in both permissive human embryo fibroblasts (HEF) and non-permissive Vero cells whose growth has been arrested using media with a low serum content (St Jeor et al., 1974). Additionally, cellular DNA replication is stimulated during the non-productive infection of rabbit kidney cells which express early HCMV antigens (DeMarchi, 1983b). In actively growing HEF cells, however, cellular DNA replication is stimulated efficiently only in cells which do not express HCMV antigens (DeMarchi and Kaplan, 1977). DeMarchi (1983b) suggests that the stimulation of cellular DNA in productively infected HEF cells may be repressed by an early gene function which is not expressed during the non-productive infection of rabbit kidney cells.

Gonczol and Plotkin (1984) have identified a growth factor (CMV-GF) which is released from either permissive or non-permissive cells exposed to infection by HCMV. CMV-GF is synthesised by cells infected with u.v.-inactivated virus and is therefore thought to be encoded by the host cell. Microtubule depolymerisation occurs in cells during the early phase of HCMV infections (section 1:13) and Taxol, a compound which stabilises microtubules, has been shown to inhibit both the stimulation of cellular DNA replication in HCMV-infected cell monolayers and mitogenic stimulation by CMV-GF (Albrecht et al., 1984; Gonczol and Plotkin, 1984). The mechanism by which infection with HCMV and CMV-GF both stimulate cellular DNA synthesis involves the disruption of microtubules and consequently may possibly be related.

Transcriptional Stimulation: There is a transient reduction of up to 50% in the the level of newly synthesised RNA entering the cytoplasm between 1 to 10h p.i. (Garnett, 1979). However, the rate of total cellular RNA synthesis is increased by 18h p.i. and reaches a level 3 times higher than uninfected controls by 60h p.i. (Tanaka et al., 1975). The concentrations of the 4S, 18S and 28S rRNAs is 10 fold higher and cytoplasmic poly $(A)^+$ RNA only 2.4

fold higher in late phase HCMV-infected cells than in uninfected controls (Tanaka et al., 1975; Wilkinson, 1983). Hybridisation of pulse-labelled RNA with HCMV DNA has shown that only 0.6% of RNA synthesised between 0-2h p.i. and 1.8% of RNA synthesised between 28-30h p.i. is virus-encoded (Chua et al., 1981).

Infection with HCMV stimulates the activity of the cellular RNA polymerases I, II and III (in an in vitro assay) by factors of six-, sixteen- and three-fold respectively (Tanaka et al., 1978). The stimulation of endogenous RNA polymerase activities must be induced by a protein synthesised during the first 6h p.i. since the transcriptional activation induced by HCMV infection is inhibited if cells are treated during the first six hours of an infection with either cycloheximide or actinomycin D (Tanaka et al., 1978). Actinomycin D treatment of early infected cells also inhibits the subsequent replication of the virus (Landini et al., 1979).

The activation of cellular transcriptional processes is an unusual and distinctive feature of HCMV infections.

Protein Synthesis: Cellular encoded polypeptides continue to be synthesised throughout the HCMV replicative cycle. Only 10-30% of early and 50-60% of late phase protein synthesis is attributable to virus-encoded polypeptides (Stinski, 1977). There is a transient reduction of greater than 50% in the level of total protein synthesis in infected cells during the first 20h p.i. (Stinski, 1977; Garnett, 1979), followed by an increase from 22h p.i. to reach the peak rate of synthesis between 30-32h p.i. Translational activity then declines but is subsequently re-stimulated and a second peak is seen between 42-60h p.i. (Stinski, 1977). Although transcription is greatly enhanced during the late phase of HCMV infections, suprisingly even at peak levels (32h and 60h p.i.) protein synthesis is only slightly greater in infected cells than in uninfected controls (Stinski, 1977). Experiments performed in parallel with inactivated virus (irradiated with u.v. light or

neutralised with antibody) indicated that de novo HCMV gene expression was not required for the suppression of early phase protein synthesis but it was necessary for the subsequent translational activation observed during the late phase.

HCMV has been shown to stimulate either the synthesis or the activity of a number of specific cellular proteins (Table 1:6). Although HCMV does not encode a thymidine kinase enzyme (TK), virus infection efficiently stimulates the activity of both cellular (cytosol and mitochondrial) TK enzymes. The stimulation of TK activity in the cytosol can be detected by 12h p.i. and reaches a maximum level (10-12 times that of uninfected control

Activated Polypeptide or Polyamine	Host Encoded	References
TK	nucleus & mitochondria	Zavada et al., 1976 Estes & Huang, 1977
Plasminogen Activator Factor	Yes	Yaminishi & Rapp, 1979
Ornithine Decarboxylase	Yes	Isom, 1979
Spermine & Spermidine	Yes	Tyms & Williamson, 1980
RNA polymerases I, II, & III	Yes	Tanaka et al., 1978
Ig G binding protein	?	Sakuma et al., 1977 Falcieri et al., 1980 Gonczol et al., 1981 Murayma et al., 1986
CMV-GF (Growth Factor)	Yes	Gonczol and Plotkin, 1984
Repressed Polypeptide		
Fibronectin	Yes	Ihara et al., 1982

Table 1:6: Effect of HCMV infection on expression of specific cellular proteins and polyamines.

cells) at 48h p.i. (Estes and Huang, 1977). In uninfected cells regulation of TK expression is dependent on the cell cycle. TK activity is stimulated late in the G1 phase immediately prior to cellular DNA replication (Liu et al., 1985). Ornithine decarboxylase (Table 1:6) is also normally induced during the G1 phase in uninfected cells (Isom, 1979). The induction of enzymes associated with the cellular late G1/S phase may be essential for HCMV replication. Inhibiting host cell transcription by treatment with actinomycin D prior to infection has been shown to block HCMV, but not HSV-1, replication in early phase gene expression, this result suggests that HCMV replication is particularly dependent on host cell functions (Landini et al., 1979).

It has yet to be determined whether the HCMV induced IgG Fc receptor protein is virus or host cell encoded. The Fc receptor is a 42K glycoprotein (Sakuma et al., 1977; Gonczol et al., 1981) which is synthesised at both the early and late phases during infection (Falcieri et al., 1980).

One cellular protein whose synthesis is known to be inhibited by HCMV is fibronectin. Fibronectin is a large structural polypeptide present on the cell surface which is important in determining cellular morphology. Inhibition of fibronectin synthesis requires virus-encoded protein synthesis and is detected by 6h p.i. Immunofluorescence studies show that by 6h p.i. the fibronectin lattice on the cell surface on the cell surface has already become distorted. Inhibition of fibronectin synthesis may be involved in the production of the early c.p.e. (Ihara et al., 1982).

1:24 HCMV Infection in Permissive, Semi-Permissive and Non-Permissive Cells

Characteristically cytomegaloviruses replicate efficiently only in cells of the normal host or a closely related species (Table 1:7). For example murine Balb/c-3T3 cells (LaFemina and Hayward, 1983), mink lung fibroblasts (Hart and Norval, 1981) and bovine embryonic fibroblasts (Waner and Weller, 1974) are totally non-permissive to HCMV replication. The host

cell specificity of the betaherpesvirinae is, however, not absolute. Limited HCMV replication and maturation is tolerated by certain non-human cell lines; these are classed as a semi-permissive infections. Rabbit lung fibroblasts (Farber et al., 1979; Hart and Norval, 1981) and simian vero cells (Waner and Weller, 1974) have been shown to be semi-permissive for HCMV replication (Table 1:7). Yields of infectious virus obtained from rabbit fibroblasts are significantly less (10,000 fold) than those from human fibroblasts (Hart and Norval, 1981).

The progress of virus infection in semi- and non-permissive cells has been investigated. In non-permissive murine Balb/c-3T3 cells the HCMV genome fails to circularise following infection and virus-encoded protein synthesis is largely restricted to overproducing the major IE polypeptide (LaFemina and Hayward, 1983). RNAs purified from baby mouse kidney, rabbit kidney, African green monkey and Vero cells 36h p.i. were hybridised with Southern transfers (Southern, 1975) of restriction endonuclease digested HCMV genomic DNA (DeMarchi 1983a; 1983c; 1984). The hybridisation results indicated that in both non- and semi-permissive cell types although virus transcription

	Permissive	Semi-permissive	Non-permissive
Non-human	None	Rabbit Fibroblasts Vero cells	293 cells Murine Balb/c-3T3 Mink Lung Fibroblasts Bovine Embryonic Fibroblasts
Human	Fibroblasts Epithelial cells Differentiated EC cells Some Stimulated T-lymphocytes	Persistantly-infected fibroblasts	EC cells Most PBLs Hela cells

Table 1:7: Susceptibility of different cell types to infection with HCMV

progresses to the early phase the region encoding the major IE gene, normally repressed at this stage of a permissive infection, was still being expressed. Since the pattern of HCMV transcription in each non-human cell line at 36h p.i. was distinctive, DeMarchi (1984) suggested that the progress of the sequential cascade activation of HCMV gene expression may be dependent on many factors encoded by the host cell.

Not all human cells support HCMV replication. Human embryonal carcinoma (EC) cell lines fail to express even the major IE polypeptide following infection (Gonczol et al., 1984). Since HCMV DNA has been shown to enter the nuclei of infected EC cells, the infection would appear to be blocked at the level of IE gene expression (Gonczol et al., 1985). Retinoic acid can be used to induce EC cells to differentiate into a variety of different cell types. Following infection, differentiated EC cells are able both to express the HCMV major IE polypeptide and to support virus replication (Gonczol et al., 1984). Undifferentiated EC cells have been shown to contain a factor which can complement mutations in the adenovirus Ela gene (Imperiale et al., 1984). In addition to stimulating transcription from adenovirus promoters, the Ela gene product is known to repress transcription from promoters containing enhancer elements (Borrelli et al., 1984). The Ela gene product and a repressor present in EC cells appear to negatively regulate enhancer activity in an identical fashion (Hen et al., 1986). Since the HCMV major IE promoter is known to contain an enhancer element (Boshart et al., 1985), the Ela-like factor present in EC cells may be responsible for inhibiting HCMV IE gene expression.

Hela cells also do not support expression of the HCMV major IE polypeptide following infection although the major IE antigen is expressed if its gene is introduced into the cell by DNA transfection (Fig 3:7). Hela is an immortalised cell line which has been selected for efficient growth in vitro. It is not known at what stage the infection of Hela cells is blocked; during virus adsorption, transport of the virus genome into the nucleus or

the expression of the IE gene itself.

HCMV replicates most rapidly and gives the highest yields when cultured in human embryonic fibroblasts (HEF's), consequently in the laboratory HCMV is invariably isolated and propagated in HEF cells. In vivo, however, the cytomegalic inclusions characteristic of HCMV infections are most frequently observed in cells of epithelial origin (Knowles, 1976). Cultivation of HCMV in vitro in epithelial cells has been described but replication is slower and less efficient than in fibroblasts (St. Jeor and Rapp, 1973; Knowles, 1976; Vonka et al., 1976).

1:25 Latency

HCMV, like other herpesviruses, can reactivate after long periods of apparent latency (Huang et al., 1980). The mechanism by which HCMV establishes and is subsequently re-activated from the latent state is not known. However, it has been shown that HCMV infections of certain human cell types are non-productive (Table 1:7). These non-productive infections represent a possible mechanism by which the virus may persist in infected cells undetected by immune surveillance in vivo.

The relationship between HCMV and the cells of the immune system is of great clinical importance. Mononuclear cells taken from asymptomatic, seropositive individuals have been shown to contain HCMV IE RNA (Schrier et al., 1985) and infectious virus can be isolated from the buffy coat material of patients experiencing an overt infection. In vitro, peripheral blood lymphocytes (PBL) can be infected more efficiently with recent HCMV isolates than with high passage, laboratory-adapted strains (Rice et al., 1984). However, even using recent HCMV isolates, less than 15% of PBLs exposed to infection express the major IE antigen; expression of the major IE antigen was detected in monocytes, B-lymphocytes, natural killer, $CD4^+$ and $CD8^+$ cells (Rice et al., 1984; Schrier et al., 1985). The in vitro infection of PBLs observed by these workers appeared to be blocked at an early stage of

the HCMV replicative cycle since neither late antigen expression nor infectious virus was detected. More recently it has been shown that a small proportion (approximately 0.05%) of cells in a mixed lymphocyte population could produce infectious HCMV when they were stimulated by either interleukin 2 or allogeneic mixed lymphocytes (Braun and Reiser, 1986). Immunofluorescence studies indicated that the majority of HCMV replication was taking place in $CD8^+$ (cytotoxic suppressor T) cells (Braun and Reiser, 1986). Since mitogenic stimulation by phytohaemagglutinin did not induce the permissive state in T lymphocytes some factor other than rapid cell growth appears to be required, possibly some factor involved in cellular differentiation. Reiser et al. (1986) have also recently shown that bone marrow stem cells can support HCMV replication if they are cultivated in vitro for 3 weeks prior to infection, possibly during growth in vitro the cells undergo a differentiation step which permits virus replication. In vivo, lymphocytes may support a level of virus replication and may have an important role in both the long-term maintenance (latency) and in the dissemination of infection.

Mocarski and Stinski (1979) observed that following productive, cytolytic infection of human fibroblasts a small number of cells survived. These cells were cultured. All the resulting cell lines produced were observed to contain multiple copies of the HCMV genome and 30% of the cell lines secreted infectious virus. Interestingly, cultures which initially produced virus could be converted to a non-producing state by adding anti-HCMV sera to the growth media. When these cells were further subcultured in the absence of antisera they reverted to producing virus. The mechanisms involved in HCMV persistance in a sub-population of infected fibroblasts and the suppression of virus replication by humoral antibody is not understood.

The identification and characterisation of the HCMV gene products involved in regulating gene expression may ultimately be important in

understanding how latency is modulated.

1:26 Gene Regulation in Herpesviruses

The regulation of gene expression has been studied more intensively and is better characterised in cells infected with HSV-1 than with any other herpesvirus. HSV-1 exerts its earliest effects on gene expression before the virion enters the cell. Kemp et al. (1986) demonstrated that the binding of the virion to its receptor on the cell surface was sufficient to stimulate transcription from at least two cellular genes. An additional subset of cellular genes are stimulated following virus absorption in the presence of an inhibitor of protein synthesis (Kemp et al., 1986). During the course of productive infections even though a small number of cellular genes are either induced or continue to be expressed (Notarianni and Preston, 1982; LaThangue et al., 1984) the vast majority of cellular macromolecular synthesis is repressed (Roizman et al., 1965); this situation in HSV-1 infected cells is in contrast to the marked stimulation of cellular RNA and protein synthesis observed during HCMV infections (section 1:23).

In the absence of de novo virus-encoded protein synthesis only five HSV-1 IE genes are transcribed (Fig 1:5); expression from the promoter of IE gene 3 has been studied in greatest detail. The IE gene 3 promoter contains an upstream regulatory region which, like an enhancer element, stimulates transcription in cis from heterologous promoters relatively independently of orientation or distance but, unlike conventional enhancers, does not function effectively when inserted downstream of the promoter (Cordingley et al., 1983; Lang et al., 1984; Preston and Tannahill, 1984). More detailed analysis of the IE gene 3 enhancer-like element demonstrated that at least three seperable, non-homologous elements contribute towards its activity (Bzik and Preston, 1986).

Transcription from HSV-1 IE genes is also stimulated in trans by a structural component of the virion, a 53,342 dalton (Vmw 65) tegument

phosphoprotein (Batterson and Roizman, 1983; Cordingley et al., 1983; Campbell et al., 1984; Dalrymple et al., 1985; Pellet et al., 1985). The presence in cis of the consensus sequence TAATGARAT, which is present in at least one copy in all nine sequenced HSV-1 and HSV-2 IE promoters, by itself can be sufficient to confer on a heterologous promoter the capability for its expression to be induced by the virion trans-activator. The level of stimulation is, however, greater when a GA-rich sequence, adjacent to the TAATGARAT consensus in the IE gene 3 promoter, is also present (Gaffney et al., 1985; Bzik and Preston, 1986). At present it is not clear whether HCMV virions contain a trans-activator of IE transcription; at least one alphaherpesvirus, PRV, has recently been shown not to (Campbell and Preston, 1987).

A function of herpesvirus IE gene expression in permissive cell systems is the sequential transcriptional activation of early genes. Transient DNA transfection studies have identified two HSV-1 IE genes involved in stimulating early gene expression. In co-transfection experiments cloned DNA fragments, containing IE gene 3, stimulated expression from the HSV-1 early promoters for the gD, TK and Vmw 38 structural genes but repressed expression from its own promoter and that of IE gene 1 (Everett, 1984; O'Hare and Hayward, 1985a; 1985b). In similar experiments IE gene 1 alone also stimulated expression from HSV-1 early promoters and, in transfections using IE genes 1 and 3 together, the two trans-activators acted synergistically. Unlike IE gene 3, however, IE gene 1 stimulates expression from HSV-1 IE promoters (O'Hare and Hayward, 1985b). HSV-1 IE gene expression is therefore stimulated by the virion component, Vmw65, the product of IE gene 1 (Vmw110) and repressed by the product of IE gene 3 (Vmw175).

Recent studies have shown that IE gene 2, which encodes Vmw63, is essential for virus growth; temperature-sensitive mutants in IE gene 2 permit virus DNA replication but are restricted in late phase gene

expression (Sacks et al., 1985). In DNA transfection experiments the product of IE gene 2 (Vmw63) was shown to stimulate expression from the promoter for the HSV-1 late gene encoding the major capsid protein, but not from the early promoters of the gD and TK genes, and only when used in the combination with HSV-1 IE genes 1 and 3 (Everett, 1986).

A number of temperature-sensitive mutants of IE gene 3 have been produced, the best characterised of which is probably HSV-1 TsK. TsK contains a single base substitution in the coding region of Vmw175 which, at the nonpermissive temperature, causes IE RNAs and IE proteins to be overproduced in infected cells and prevents the transition to early and late phase gene expression (Marsden et al., 1976; Watson and Clements, 1978; Davison et al., 1984). While expression of a functional Vmw175 protein is essential for virus replication, the expression of Vmw110 without a functional Vmw175 is insufficient to activate significant early phase transcription (DeLuca et al., 1985). During productive infections Vmw175 associates with foci of replicating HSV-1 DNA in the cell nucleus (Randall and Dinwoodie, 1986). Recent evidence indicates that Vmw175 binds directly, or indirectly in combination with a cellular intermediate, specifically to DNA sequences in HSV-1 IE promoters to suppress expression and to early promoters to stimulate expression (Beard et al., 1986; Faber and Wilcox, 1986; Kristie and Roizman, 1986a; 1986b). Recently, a mutant with a deletion in both copies of IE gene 1 has been constructed which is able to replicate without helper functions but it produces relatively low yields of infectious virus which have a high particle to p.f.u. ratio (Stow and Stow, 1986). Stow and Stow (1986) suggest that at low m.o.i. Vmw110-mediated stimulation of IE gene 3 transcription may be required to induce sufficient production of Vmw175 to activate early phase gene expression.

Expression from the HSV-1 gD and β-globin promoter in transfected cells can be stimulated by infection with HSV-1, HSV-2, VZV, HCMV, Equine herpesvirus-1 and adenovirus-2 and in co-transfection experiments by IE

coding regions from HSV-1, HSV-2, PRV and HCMV (Everett and Dunlop, 1984; Everett, 1984). The HCMV strain AD169 genome trans-acting function was encoded by HindIII E, which contains IE coding regions 1, 2 and 3 (Fig 1:6); when IE coding region 1 was used by itself however the level of transcriptional activation from the gD promoter was "scarcely detectable" (Everett, 1984). Spector and Tevethia (1986) have also shown that an HCMV DNA fragment containing IE coding regions 1 and 2 can complement an adenovirus-5 mutant with a deleted E1a gene sufficiently to permit adenovirus replication. HCMV IE coding region 1 and 2, but not IE coding region 1 alone, therefore encodes at least one trans-activating function which stimulates transcription in an apparently non-specific fashion.

1:27 Objectives

The primary objective of this study was to perform a detailed structural analysis on the major IE gene and the abundantly transcribed early gene, which maps within TR_L/IR_L, of strain AD169 as a basis for investigating HCMV gene regulation. Reporter genes were cloned downstream and under the control of the IE and early promoters and the novel constructs used to investigate (a) activation of expression from the IE promoter by a virion component, (b) the negative regulation of the IE promoter which occurs during the early and late phases and (c) the delayed activation of early gene expression.

MATERIALS AND METHODS

2:1 Cells, Cell Culture and Infection With Virus

MRC5 is a well-characterised human diploid embryo lung fibroblastic cell line which supports efficient HCMV replication. MRC5 cells were generously provided by Mr P. Wilton-Smith and were used between passage levels 18 to 28. In contrast Hela cells are an established human epithelial cell line which do not support HCMV replication. The strain of Hela cells (kindly supplied by Dr R. Everett, originally from Dr W. Schaffner) used can be efficiently transfected with DNA.

Cells were routinely maintained in 75 cm^2 and 175 cm^2 plastic flasks. Tissue culture media, used throughout, contained 6% foetal bovine serum (Imperial Laboratories), 100 μg/ml kanamycin sulphate (Winthrop Laboratories) and 3.7 g/l $NaHCO_3$ in Dulbecco's modified Eagle's medium (Imperial Laboratories). Cells were passaged by washing once with PBS (1.8 g/l NaCl; 0.22 g/l KCl; 1.14 g/l Na_2HPO_4; 0.27 g/l KH_2PO_4, pH 7.4), and removed from solid support in 2% (w/v) trypsin; 5% (w/v) $NaHCO_3$; 5% (w/v) EDTA in PBS. Unless otherwise stated, MRC5 and Hela cells were passaged at a ratio of 1:3 and 1:10 respectively. Cell cultures were gassed with 5% CO_2 in air prior to incubation at 37°C. Plastic tissue culture roller bottles, flasks and petri dishes were supplied by Corning Glass Works and Nunc. Tissue culture cells were enumerated using a haemacytometer (Weber Scientific, Lancing) following dilution in a solution containing Trypan blue (0.02%) in saline (0.85% w/v NaCl) as a vital stain.

HCMV strain AD169 was provided by Dr J. Booth, St Georges Hospital, London and HSV-1 TK^- (strain MDK5M) by Dr A.C. Minson, Cambridge University. To produce virus stock, MRC5 cells were infected with HCMV (>0.01 p.f.u./cell) when 80% confluent, media was replaced at two weeks p.i. and virus harvested from 3 weeks p.i., depending on the development of the characteristic HCMV c.p.e. Since strain AD169 virions are efficiently released into the supernatant, virus was harvested in the culture media.

Usually two or more harvests were taken at two to three day intervals. Cellular debris was removed by centrifugation (1000 rpm for 10 min in a Beckman TJ6 centrifuge) and the virus stored in culture supernatant at $-70^{\circ}C$.

Hela cells (80% confluent) were infected with HSV-1 TK^{-} at a m.o.i. of 0.1 p.f.u./cell. Cells and media were removed 2-3 days p.i. at which time 100% of cells exhibited c.p.e. The suspension was treated to three rounds of freezing ($-70^{\circ}C$) and thawing to lyse the infected cells. Cellular debris was removed by centrifugation (10 min at 1000 rpm in a Beckman TJ6 centrifuge) and virus stored in culture supernatant at $-70^{\circ}C$.

HCMV and HSV-1 were both titrated in MRC5 cells. 200 µl aliquots of ten-fold serial dilutions of virus were was added in parallel to 10 wells (15 mm diameter; Nunc) containing 80% confluent MRC5 cells. Virus was left for 1h at $37^{\circ}C$ to be adsorbed by the cells, the inoculum was removed, cells washed once with 2 ml culture medium and 2 ml of medium was then added to the cells. HSV-1 infected cells were incubated for 1 week p.i. and HCMV-infected cells for 4 weeks p.i. Culture media was changed on HCMV-infected cells 2 weeks p.i. Cells were examined for the presence of a c.p.e. and virus infectivity calculated by a Spearman-Karber endpoint determination (Finney, 1971).

U.V.-irradiation of HCMV stocks was performed using a purpose-built apparatus. 10 ml of virus was pipetted into a 100 mm petri dish which was placed on a rocking table situated 300 mm from the u.v. light source. The gentle rocking motion of the table ensured that the virus suspension was evenly irradiated. The u.v.-light source delivered 1.77 mW/cm^2 which is equivalent to 17.7 $\mu J/mm^2$ per second. Samples from the same HCMV stock (titred at 2×10^7 p.f.u./ml) was irradiated for 0s, 10s (177 $\mu J/mm^2$), 30s (531 $\mu J/mm^2$), 90s (1.59 mJ/mm^2), 5 min (5.31 mJ/mm^2), 15 min (15.93 mJ/mm^2) and 45 min (47.7 mJ/mm^2). 2 ml of each irradiated virus sample was innoculated on to two 50 mm dishes containing MRC5 cells. One dish was

incubated for up to 4 weeks as a test for virus infectivity. Cells in the second dish were tested 6h p.i. by immunofluorescence for the expression of the major IE antigen.

2:2 Immunofluorescence

Cells used for immunofluorescence experiments were grown on 6 mm x 22 mm coverslips. At the appropriate time coverslips were taken from the tissue culture dish and placed in a purpose-built rack which was immersed in PBS for 30s to remove surplus media. Cells were then fixed on to the coverslips by immersion in acetone (-20°C) for 5 min. Coverslips were occasionally stored at -20°C in this state.

Antibodies used in the antigen recognition stage included a monoclonal antibody against the major IE antigen (L14, kindly provided by Dr J. Nelson), a pool of 10 human convalescent sera which recognised the IE nuclear antigen (kindly provided by Ms K. Blake) and hyperimmune guinea pig sera raised to the lassa virus nucleocapsid protein (kindly provided by Dr G. Lloyd). The monoclonal antibody L14, which was derived from ascitic fluid was used at a dilution of 1:500 (with PBS), other antibodies were diluted 1:20 with PBS before use. In the first stage of antigen recognition 100 μl of the antibody was applied to the fixed cells and incubated for 30 min at 37°C in a moist chamber. The coverslips were then washed in 250 ml of PBS for 30 min at 37°C, drained, and 100 μl of the second (fluorescent tagged) antibody applied. Antibodies used in this stage included rhodamine-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-guinea pig and FITC-conjugated goat anti-human IgG (ICN Biomedicals). The conjugated antibodies were all diluted 1:20 with PBS before use.

In double-immunofluorescence experiments, the monoclonal antibody L14 and the guinea pig anti-lasssa sera were mixed and used in parallel during the antigen recognition stage. Likewise the rhodamine-conjugated anti-mouse and the FITC-conjugated anti-guinea pig sera were used together in the

second stage of the reaction.

Finally, coverslips were washed for 30 min in PBS at 37°C and dried before being mounted on glass slides in 90% glycerol. Photographs were taken using a Leitz Dialux 20EB microscope with appropriate filters and attachments.

2:3 RNA Purification

In order to minimise degradation of RNA by ribonuclease during extraction all glassware, centrifuge tubes and, where practical, reagents were thoroughly cleaned and autoclaved. All steps, except for phenol/chloroform extractions, were carried out between 0-4°C with all reagents and apparatus being precooled before use.

The protocol used was based on an extraction method described by Dunn and Sambrook (1980). Media was decanted from the cell monolayer which was then washed once with PBS. Cells were harvested in PBS by rolling 3 mm diameter glass beads over the surface and centrifuged at 1000 rpm for 10 min (Sorvall GSA rotor, Sorvall RC5B centrifuge). The pellet resuspended in 10 volumes TNE buffer (0.15M NaCl; 1mM EDTA; 0.1M Tris-HCl, pH 7.5), containing 20 mg/ml placental ribonuclease inhibitor (Sigma), and left for 10 min to equilibrate. Cells were lysed by the addition of Nonidet P40 (NP40) to a final concentration of 1% (v/v) and vortexing vigorously for 30s. The extract was cleared of nuclei and cellular debris by centrifugation at 10,000 rpm for 20 min (Sorvall SS34 rotor). The supernatant was then adjusted to 0.2% (w/v) with SDS and to 1mM with EDTA followed by extraction with phenol (BDH chromatography grade phenol equilibrated with 1mM EDTA; 0.5M Tris-HCl, pH 8.0). Phenol extraction was repeated twice more followed by extraction twice with chloroform alone. Total cytoplasmic RNA was precipitated by the addition of 2 volumes of ethanol, stored overnight at -20°C and recovered by centrifugation at 12,000 rpm for 20 min in a Sorvall SS34 rotor. The RNA pellet was dried and resuspended in distilled water

containing 2.5 μg/ml placental ribonuclease inhibitor.

In some cases poly $(A)^+$ RNA was purified from total cytoplasmic RNA by chromatography on oligo(dT) cellulose columns. Total cytoplasmic RNA was dissolved in binding buffer (0.5M NaCl; 1mM EDTA; 0.5% (w/v) SDS; 10mM Tris-HCl, pH 7.5) and passed three times through a column (70 mm x 90 mm diameter) containing oligo(dT) cellulose (Collaborative Research). The column was washed with 10 ml of binding buffer and poly$(A)^+$ RNA eluted in 1mM EDTA; 0.05% (w/v) SDS; 10mM Tris-HCl, pH 7.5.

The concentration of RNA solutions was determined using a scanning spectrophotometer (Beckman DU8), 40 μg/ml of RNA has an absorbance at 260 nm of approximately one unit.

2:4 DNA Transfection of Eukaryotic Cells

The $CaPO_4$ DNA transfection methodology was developed by Graham and Van der Eb (1973). 20 μg of plasmid DNA was dissolved in 420 μl of TEP (0.5mM EDTA; 1mM Tris, pH 7.5) and pipetted into a 10 ml plastic Falcon tube. The Falcon tube was gently vortexed as 60 μl of 2M $CaCl_2$ was added dropwise to the DNA solution. The resulting mixture was then added dropwise to a Falcon tube containing 480 μl of 2 x HBS (280mM NaCl; 50mM HEPES; 1.5mM Na_2HPO_4, pH 7.12) with gentle vortexing. The precipitate was allowed to form for 30 min before being added to cells.

Cells were seeded in petri dishes 24h before transfection to produce monolayers which were 80% confluent. The amount of the transfection mixture added to the cells was dependent on the size of the petri dish used. 20 μg of DNA was added to 100 mm diameter dishes, 5 μg to 50 mm diameter dishes and 2 μg to 35 mm diameter dishes. When a large number of identical transfections were performed in parallel the transfection mixtures were produced using the same quantities of reagents as described above, although in order to standardise the experiments the mixtures were pooled before being distributed to the cells.

The precipitate was applied directly to the tissue culture supernatant and left 18h for the DNA to be taken up by the cells after which the media was discarded, the monolayers washed once with TBS buffer (137mM NaCl; 5mM KCl; 0.7mM $CaCl_2$; 0.6mM Na_2HPO_4; 25mM Tris-HCl, pH7.4), once with complete media, then complete media was added and the cells incubated further. In experiments in which transfected cells were subjected to virus infection this was performed 6h after the media had been replenished, i.e. 24h post transfection.

2:5 Bacterial Strains, Plasmids and Media

The bacterial strains used were:

E. coli K12 JM101: F', Δ(lac, pro), thi, supE, traD36, proAB, lacIq, ZAM15, (Messing et al., 1981).

E. coli K12 HB101: F^-, hsdS20 (r^-,m^-), recA13, ara-14, proA2, lacY1, galK2, rspL20 (Sm^r), xyl-5, mtl-1, supE44, (Boyer et al., 1969).

E. coli strain HB101 was used preferentially for propagating plasmids, the recA13 genotype makes it less likely for plasmids to be involved in a recombination event in this host. E. coli JM101 was used for growth of the 'male-specific' M13 phage. Additionally, the defective phenotype of the lac gene in E. coli JM101 made it a suitable host for use in DNA cloning experiments which involved insertional inactivation of a plasmid or phage bourne lacZ gene to identify recombinants.

Details of the HCMV recombinant DNA gene bank and its construction are given in Oram et al. (1982) and Greenaway et al. (1982). The plasmid cloning vectors pUC8, pUC18 and pUC19 were obtained from BRL. pMTL21b, a plasmid based on the 'pUC' series of vectors but containing additional unique cloning sites, was a kind gift of Dr N. Minton. pCAT and pSV2cat (Gorman et al., 1982a) were obtained courtesy of Dr D. Gaffney, and pRSVcat (Gorman et al., 1982b) obtained courtesy of Dr A. Lehmann. pMD102 (Everett et al., 1983) and IEP1lassaterm (unpublished) were provided by

Drs R.D. Everett and C. Clegg respectively. IEP1catIEterm, AccHincat, SV2catIEterm, pVUIITK1, 0.7TK1, 1/34TK1 and 0.7-1/34TK1 were all provided by Dr A. Akrigg. The plasmid pT23 contains the HCMV PstI fragment B tailed into the Pstl site of pAT153 (Akrigg and Wilkinson, unpublished).

The M13 phage vectors M13mp8 and M13mp11 were obtained from BRL. DNA fragments subcloned from the major IE gene into M13mp8 which were used in nuclease protection studies on the HCMV major IE gene (Table 3:1) were provided by Dr A. Akrigg. M13mp11(1/39) was provided by Dr P.J. Greenaway.

Bacteria were grown either in L-broth (10 g bacto-tryptone, 5 g bacto-yeast extract and 10 g NaCl per litre, pH 7.5) or YT-broth (10 g bacto-tryptone, 10 g bacto-yeast extract and 5 g NaCl per litre, pH 7.5). For L-agar and YT-agar 15g per litre of bacto-agar was added to L-broth and YT-broth respectively. M13 phage plaques were grown on a bacterial lawn in H-top agar (10 g bacto-tryptone, 8 g NaCl and 8 g agar per litre) overlays; the overlays were poured on plates containing YT-agar.

In antibiotic selection experiments ampicillin was used at a final concentration of 50 μg/ml when recombinants were being selected following transformation but at 100 μg/ml at all subsequent stages. Tetracycline was used at a concentration of 20 μg/ml. The chromogenic substrate X-gal (5-bromo-4-chloro-3-indoyl-β-galactoside), which turns blue when hydrolysed by β-galactosidase, was used at a final concentration of 0.02% (w/v) together with 50μM IPTG (iso-propyl-β-D-thio-galactopyranoside).

2:6 Restriction Endonuclease Digestion and The Electrophoresis of DNA in Agarose and Polyacrylamide Gels

DNA was digested with restriction endonucleases using buffers and conditions recommended by the manufacturers (BCL or BRL). The products of restriction endonuclease digestion were analysed by electrophoresis in an appropriate gel system. 1% horizontal agarose gels (100 mm x 100 mm x 10 mm) prepared in 1 x TBE buffer (0.089M Tris base; 0.089M boric acid;

0.002M EDTA, pH 8.0) were used routinely for rapid analysis. When necessary, 1% vertical agarose gels (200 mm x 200 mm x 15 mm) were used to resolve closely migrating fragments or to obtain more accurate molecular weight determinations. Vertical agarose gels were prepared in 1 x E buffer (20mM sodium acetate; 1mM EDTA; 40mM Tris-HCl, pH 8.2).

DNA fragments less than 200bp long were analysed using a vertical 6% polyacrylamide gel (200 mm high x 150 mm wide x 3 mm thick). 6% (w/v) acrylamide; 0.16% (w/v) N,N'-bis-methylene acrylamide in 1 x F buffer (20mM MOPS; 1mM EDTA; 5mM sodium acetate, pH 7.0) was degassed under vacuum and polymerised by the addition of 0.025% TEMED and 0.2% (w/v) ammonium persulphate immediately before the gel was poured.

Samples were applied to wells in 5% loading buffer (0.025% bromphenol blue; 0.05% xylene cyanol FF in the appropriate electrophoresis buffer and electrophoresis performed in 1 x TBE (horizontal agarose gels) for 1h at 60 volts, 1 x E (vertical agarose gels) for 16 h at 20 volts or 1 x F buffer (vertical acrylamide gels) for 2.5h at 80 volts. DNA was stained by immersing the gels for 20 min in a solution containing 1 μg/ml ethidium bromide dissolved in the appropriate running buffer. DNA stained with ethidium bromide was visualised using a u.v. (302 nm) transilluminator. HindIII digested λ virus DNA (BRL) and HaeIII digested ϕX174 virus DNA (BRL) were used as molecular weight standards.

2:7 Recovery of DNA Fragments From Gels

Individual DNA fragments were excised from both agarose and acrylamide gels using a scalpel. Each gel fragment was placed into a dialysis bag containing 0.3 ml of 1 x TBE buffer which was then sealed and put in a electrophoresis tank, submerged in 1 x TBE and subjected to electrophoresis at 40 volts for 20 min. DNA electroeluted from the gel is unable to penetrate the membrane of the dialysis bag. Electrophoresis buffer containing eluted DNA was removed from dialysis bags, extracted

twice with phenol, twice with chloroform and precipitated with two volumes of ethanol. Precipitated DNA was recovered by centrifugation, dried and dissolved in TE buffer (10 mM Tris-HCl; 1mM EDTA, pH 7.5).

2:8 Phosphatase Treatment of DNA Fragments

Restriction endonuclease cleaved DNA fragments were treated with phosphatase to remove 5' terminal phosphate groups. In recombinant DNA experiments restriction endonuclease digested plasmid vector DNA was invariably treated with phosphatase. The removal of both terminal phosphate groups makes circularisation of the vector dependent upon its 3' terminal hydroxl groups forming phosphodiester bonds with the 5' phosphate groups of a foreign DNA fragment. Phosphatase treatment thereby increases the proportion of plasmids generated in a cloning experiment which contain inserts. DNA fragments destined to be 5' end-labelled with $[\gamma-^{32}P]ATP$ using polynucleotide kinase were also treated with phosphatase.

Following digestion with restriction endonucleases DNA was precipitated with ethanol, recovered by centrifugation, dried and dissolved in 1mM $MgCl_2$; 0.1mM $ZnCl_2$; 1mM spermidine; 50mM Tris, pH 9.0. Bacterial alkaline phoshatase (BAP; BRL) was added at a concentration of 75 units per µg of DNA and the reaction incubated at 65°C for 30 min. The reaction products were subjected to electrophoresis in agarose gels and the desired DNA fragments recovered by electroelution. When phosphatased DNA fragments were not subjected to electrophoresis, BAP was inactivited by extracting the sample three times with phenol and twice with chloroform. The DNA fragment was then recovered by ethanol precipitation.

2:9 Ligation of DNA Fragments

Linearised vector DNA (plasmid or M13 double stranded DNA) was covalently linked at both ends to the DNA fragment being cloned resulting in the formation of circular DNA molecules which are capable of being

replicated in E. coli. The enzyme used to catalyse the formation of phosphodiester bonds between the vector and the insert was T4 DNA ligase (BCL). A 1:1 ratio of insert to vector molecules was used unless the vector was not phosphatased in which case the ratio was 2:1, or blunt ended DNA fragments were being used in which case the ratio was 4:1. The total DNA concentration in ligation reactions was kept below 20 µg/ml to minimise the formation of concatemeric structures. DNA was dissolved in 21.5 µl TE buffer and 2.5 µl of 10 x ligase buffer (10mM ATP; 50mM $MgCl_2$; 50mM dithiotreitol; 50mM Tris-HCl, pH 7.5) added. The reaction was started by the addition of 1 µl (1 unit) of T4 DNA ligase and incubated overnight at 16°C.

2:10 Transformation of Escherichia coli

E. coli was transformed with plasmid DNA according to the method of Kushner (1978). Overnight cultures of E. coli were diluted 1:100 in L-broth and incubated with shaking until they reached an OD_{650} of 0.2. Bacteria from 1.5 ml of culture were pelleted by centrifugation in a microfuge (3 min) and resuspended in 1.0 ml of 10mM $RbCl_2$; 10mM MOPS, pH 7.0, (0°C). The suspension was immediately centrifuged for 3 min in an eppendorf microfuge, the supernatant discarded and the bacterial pellet resuspended in 1 ml of 10mM $RbCl_2$; 50mM $CaCl_2$; 100mM MOPS, pH 6.5, (Buffer B). E. coli were maintained in suspension (on ice) for 30 min before being pelleted and resuspended in 0.2 ml of Buffer B (0°C). 0.2 µg, or less, of DNA was added and the bacterial suspension incubated on ice for 30 min and then given a heat shock (43.5°C for 30s). 1 ml of L-broth was added and the culture incubated at 37°C for 1h to permit expression of the antibiotic resistance markers encoded by transforming plasmids. Aliquots of the transformed culture were spread on L-agar plates containing the appropriate antibiotic (50 µg/ml of ampicillin or 20 µg/ml of tetracycline). Individual colonies of transformed E. coli were obtained by

incubating the plates at $37^{o}C$ overnight.

2:11 Plasmid Purification From Small Culture Volumes

Purification of plasmid DNA on a small scale was performed according to the method of Birnboim and Doly (1979). Plasmid-containing E. coli, picked from a single colony, was grown in 5 ml of L-broth (containing appropriate antibiotics) overnight at $37^{o}C$ in a shaking incubator. 1.5 ml of the culture was centrifuged for 3 min in a microfuge, the bacterial pellet resuspended in 100 µl solution of 2 mg/ml lysozyme (Sigma, 10mM EDTA; 50mM glucose; 25mM Tris-HCl, pH 8.0 and incubated for 30 min at $0^{o}C$. Following bacterial lysis, DNA was denatured by adding 200 µl of 0.2M NaOH; 1% (w/v) SDS (5 min at $0^{o}C$). The addition of 150 µl 3M sodium acetate (pH 4.8) for 60 min at $0^{o}C$ results in plasmid 'snap-back' into a DNA duplex whereas mismatch during renaturation E. coli chromosomal DNA results in it being precipipitated. Precipitated chromosomal DNA was pelleted by centrifugation (5 min in an Eppendorf microfuge). Plasmid DNA in the supernatant was precipitated with three volumes of ethanol at $-20^{o}C$ for 30 min, recovered by centrifugation, dissolved in 200 µl 0.3M sodium acetate, pH 4.8, and precipitated again with three volumes of ethanol. Finally the dried ethanol precipitate was dissolved in 100 µl of 0.1mM EDTA; 10mM Tris-HCl, pH 7.5.

Small scale plasmid preparations were used primarily in screening bacteria for the presence of cloned inserts in recombinant DNA experiments. DNase-free RNase was routinely added during restriction endonuclease cleavage of these DNA preparations to digest contaminating RNA which otherwise would conceal DNA fragments less than 1kb.

2:12 Plasmid Purification From Large Culture Volumes

Large scale purification of plasmid DNA was performed according to the method of Clewell and Helinski (1969). Plasmid-containing E. coli

picked from individual colonies were grown in 5 ml of L-broth (containing the appropriate antibiotic) at $37^{o}C$ overnight in a shaking incubator. The overnight cultures were used to inoculate 200 ml of L-broth, also containing the appropriate antibiotic, which was then incubated at $37^{o}C$ with shaking until the OD_{650} reached 1.0, at this stage chloramphenicol was added to a concentration of 150 µg/ml. After a further 12h incubation bacteria were harvested by centrifugation at 7000 rpm, $4^{o}C$ for 10 min in a Sorvall GSA rotor and resuspended in 2 ml of 25% (w/v) sucrose; 50mM Tris-HCl, (pH 8.0). 0.3ml of 20 mg/ml lysozyme (Sigma) in 0.25M EDTA was added and the suspension left for 5 min at $0^{o}C$ after which 2 ml of 0.25M EDTA was added and the suspension left a further 5 min at $0^{o}C$. The cells were then lysed by the addition of 3 ml 1% (w/v) Brij 58 (Atlas Chemical Industries); 0.5% (w/v) sodium deoxycholate; 1mM EDTA; 10mM Tris-HCl (pH 8.0) and pipetting vigorously up and down three times in a 10 ml pipette. The lysate was left on ice for 20 min and then centrifuged at 15,000 rpm, $4^{o}C$ for 45 min (Sorvall SS34 rotor). The supernatant was decanted, its volume measured and 0.95 g/ml CsCl and 200 µg/ml ethidium bromide added. Plasmid was separated from chromosomal DNA by equilibrium gradient centrifugation at 38,000 rpm for 60h (Beckman L5-65B ultracentrifuge, Ti 50 rotor). After centrifugation tubes were examined under u.v. light (wavelength 366 nm) and the lower fluorescent band containing plasmid DNA was harvested using a 2 ml syringe with a 3/8 inch 21G needle. The upper band contains mainly chromosomal DNA.

Plasmid DNA was extracted three times with propan-2-ol (saturated with CsCl and H_2O) to remove the ethidium bromide and dialysed three times for 4h against 200 volumes of TE buffer. The absorbance of plasmid preparations was measured at 260 nm (Pye Unicam SP6-550 spectrophotometer) to determine the DNA concentration (50 µg/ml of DNA has an absorbance at 260 nm of approximately 1.0).

2:13 Nick Translation of DNA

High specific activity radiolabelled DNA hybridisation probes were produced *in vitro* by nick translation (Rigby *et al.*, 1977).

1 μg of DNA was dissolved in a 50 μl reaction mixture containing 10 μCi [α-^{32}P] dATP (410 Ci/mmol; Amersham International); 15μM dCTP; 15μM dGTP; 15μM dTTP; 50mM NaCl; 10mM $MgCl_2$; 100mM Tris-HCl, pH7.5). Bovine pancreatic deoxyribonuclease (Sigma) was added to a final concentration of 1 pg/ml to plasmid DNA (although not to HCMV DNA) to introduce single stranded nicks which are required for DNA polymerase 1 to initiate replication; purified HCMV DNA is already nicked. After being held for 10 min at room temperature 5 units of DNA polymerase 1 (BRL) was added and the reaction incubated at 15°C for 2h. Unincorporated isotope was removed by chromatography through a Sephadex G-50 column. Radiolabelled DNA was eluted in the void volume.

2:14 Formaldehyde-Agarose Gel Electrophoresis and The Transfer of RNA to Nitrocellulose Membranes

RNA samples were dissolved in 1 x F buffer containing 2.2M formaldehyde and 50% recrystalised formamide (Casey and Davidson, 1977) and heated at 60°C for 5 min to fully denature RNA. After cooling the sample was mixed at a ratio of 5:1 with sample buffer (15% (w/v) Ficoll; 0.25% (w/v) bromophenol blue) and applied to a vertical denaturing agarose-formaldehyde gel. 1% agarose gels were prepared in 1 x F buffer and 2.2M formaldehyde, formaldehyde being added when the melted agarose solution had cooled to 60°C (Lehrach *et al.*, 1977). Molecular weight markers included *E. coli* 16S and 23S rRNA (1541 and 3000 bases respectively, Brosius *et al.*, 1978; supplied by BDH) and human 18S and 28S rRNA (1740 and 4850 bases each; Loening *et al.*, 1969; Welleaur and Dawid, 1973) prepared from MRC5 cells. 1 x F buffer, without formaldehyde, was used as the running buffer and electrophoresis carried out at 50 volts for

16h. RNA was stained by immersing the in the gel for 1h in 1 µg/ml ethidium bromide dissolved in 1 x F buffer and visualised using a u.v. transilluminator.

RNA was transferred from formaldehyde agarose gels to nitrocellulose according to the method of Thomas (1980), this procedure is commonly referred to as a 'Northern Transfer'. After being destained the gel was placed horizontally on a wick (four sheets of Whatman 3MM paper) connected to a reservoir containing 20 x SSC (3M NaCl; 0.3M sodium citrate, pH 7.0). A nitrocellulose membrane (Schleicher and Schuell) was wetted with H_2O, equilibrated in 20 x SSC and carefully placed on the gel. A 10 cm stack of blotting paper was placed on top of the nitrocellulose and held in place with a weight. The apparatus was left for 48h for the RNA to be blotted on to the membrane. The membrane was then removed, dried and baked for 2h under vacuum at 80°C.

2:15 Filter Hybridisation Conditions

Hybridisation of radiolabelled DNA probes with RNA immobilised on nitrocellulose membranes was performed using conditions described by DeMarchi (1981). Nitrocellulose membranes were immersed in 50% (v/v) formamide in 2 x SSC and then placed in plastic bags containing 20 ml of hybridisation buffer (43% (v/v) formamide; 0.5 mg/ml denatured salmon sperm DNA (Sigma); 2 x SSC; 0.5% (w/v) SDS). The bags were sealed and the membranes prehybridised for 4h at 45°C. When necessary hybridisation probes were denatured by boiling for 5 min, cooled rapidly on ice and 10^6 c.p.m. (Cerenkov) of radiolabelled DNA added to make up a final volume of 5 ml 1 x hybridisation buffer. The prehybridisation buffer was replaced with the hybridisation solution containing the probe, the bag was sealed and hybridisation performed at 42°C for 16h. Membranes were then washed (at room temperature) three times in 50% (v/v) formamide; 2 x SSC, twice in 2 x SSC, dried and subjected to autoradiography.

2:16 End-labelling DNA Fragments

DNA fragments used in nuclease protection experiments were 5' end-labelled with [γ-^{32}P]ATP and polynucleotide kinase or 3' end-labelled with [α-^{32}P]dATP and the Klenow DNA polymerase. All DNA fragments were isolated from agarose gels by electroelution before being end-labelled.

DNA fragments destined to be 5' end-labelled were treated with phosphatase (section 2:8) prior to agarose gel electrophoresis. Following electroelution DNA fragments were recovered by ethanol precipitation, dissolved in 25 µl TE buffer and desalted by chromatography through a small (100 µl) Sephadex G50 column to remove inhibitors of polynucleotide kinase. DNA in the void volume from the column was recovered by ethanol precipitation and dissolved in a 20 µl solution containing 10mM $MgCl_2$; 0.1mM EDTA; 0.1mM spermidine; 5mM dithiothreitol, 50mM Tris-HCl (pH 7.5) and 50 µCi [γ-^{32}P]ATP (3000 Ci/mmol; Amersham International). 10 units of T4 polynucleotide kinase (BRL) were added and the reaction incubated at 37°C for 1h.

DNA fragments were 3' end-labelled by using the Klenow polymerase to synthesise DNA complementary to the short protruding single-stranded 5' sequence generated during cleavage of DNA with some restriction endonucleases. DNA fragments, recovered from gels, were dissolved in a 20 µl solution containing 15µM dCTP; 15µM dGTP; 15µM dTTP; 10mM $MgCl_2$; 50mM NaCl; 50mM Tris-HCl (pH 7.5) and 10 µCi [α-^{32}P]dATP (410 Ci/mmol; Amersham International). 1 unit of Klenow DNA polymerase (BCL) was added and the reaction incubated at 20°C for 30 min.

Unincorporated label was removed from end-labelled DNA by Sephadex G50 chromatography.

2:17 Preparation of M13 Phage ssDNA Templates

50 µl from an overnight culture of E. coli JM101 was added to 5 ml of YT-broth and infected with M13 phage picked from a single plaque.

Following incubation for 6h at 37°C 1.5 ml of the phage-infected culture was centrifuged for 5 min in an Eppendorf microfuge. The culture supernatant, containing mature M13 phage released during the infection, was removed to a fresh 1.5 ml Eppendorf tube and the pellet containing infected bacterial cells discarded. 150 µl of 20% (w/v) PEG; 2.5M NaCl was added to the supernatant, incubated at 20°C for 10 min and the phage, precipitated by PEG, was recovered by centrifugation (5 min in an Eppendorf microfuge). Care was taken to ensure complete removal of the supernatant before the pellet was resuspended in 100 µl of 0.3M Na Acetate (pH 4.8). The suspension was then extracted twice with phenol (50 µl), twice with chloroform (100 µl) and finally precipitated with 0.5 ml of ethanol (-70°C for 30min). M13 phage ssDNA was then recovered by centrifugation, dried and dissolved in 30 µl of TE buffer.

2:18 DNA Sequencing Reaction

ssDNA templates prepared from M13 phage were sequenced by the dideoxynucleotide chain termination method of Sanger et al. (1977). Prior to the sequencing reaction a synthetic oligonucleotide primer is annealed to a complementary sequence on the M13 ssDNA template immediately 5' to the clustered cloning sites. A mixture of 5 µl template DNA (see section 2:17), 1 µl 17n oligonucleotide primer (0.2pM, Collaborative Research), and 1 µl TM buffer (50mM $MgCl_2$; 100mM Tris-HCl, pH 8.5) were made up to a final volume of 10 µl with water. The sample was incubated in a 60°C oven for 1h to permit the efficient hybridisation of the primer to the template.

Four different reactions are involved in sequencing each template, one for each nucleotide in DNA. Four stock solutions of nucleotide triphosphates were prepared, each containing a different dideoxynucleotide. A' contained 125µM dCTP; 125µM dGTP; 125µM dTTP; 15µM ddATP, C' contained 6.25µM dCTP; 125µM dGTP; 125µM dTTP; 40µM ddCTP, G'

contained 125μM dCTP; 6.25μM dGTP; 125μM dTTP; 80μM ddGTP, and T' contained 125μM dCTP; 125μM dGTP; 6.25μM dTTP; 250μM ddTTP.

Immediately before use 1 μl of the Klenow polymerase (1 unit, BRL) and 1 μl (10 μCi) of [α-^{32}P]dATP (410 Ci/mmol; Amersham International) was added to 10 μl of the hybridised template-primer solution at 0°C. 2 μl of the resulting mixture was pipetted inside and near the top of four 1.5 ml Eppendorf tubes. 2 μl of solution A', C', G' and T' were each added to one of the tubes which were then centrifuged (5s) in an Eppendorf microfuge. The sequencing reaction begins when the solutions mix at the bottom of the tubes. Tubes were incubated for 15 min (20°C) and then 2 μl of a nucleotide triphosphate chase solution (0.25mM dATP; 0.25mM dCTP; 0.25mM dGTP; 0.25mM dTTP) was added and the reaction incubated for a further 10 min (20°C). 2 μl of sample buffer (2 ml 0.5M EDTA, 0.1 g bromophenol blue, 0.1 g xylene cyanol FF added to 100 ml of deionised formamide) was added to each tube and the samples boiled for 3 min, to denature DNA, before being applied to a sequencing gel.

Electrophoresis was carried out in a 0.3 mm (thick) x 200 mm (wide) x 400 mm (high) vertical 6% polyacrylamide gel. 1 litre of sequencing gel solution contains 150 ml of a 40% acrylamide stock solution (38% acrylamide; 2% bis-acrylamide), 460 g of urea and 100 ml of 10 x TBE buffer. 70 μl of ammonium persulphate and 70 μl of TEMED were added to 50 ml of (degassed) sequencing gel solution immediately before the gel was poured. Sequencing gels were electrophoresed at 1200 volts for approximately 2h. The electrophoresis apparatus was supplied by Raven Scientific and the power supply was an LKB model 2197.

Following electrophoresis the gels were immersed in fixing solution (10% acetic acid; 10% methanol; 80% water) and dried by gently blotting with paper towels. The gel was covered with Saran Wrap before being subjected to autoradiography at room temperature.

2:19 Mapping of RNA Transcripts in Nuclease Protection Studies

Structural analysis of the major IE mRNA was performed using the nuclease mapping technique of Berk and Sharp (1977) as modified by Weaver and Weissman (1979). When this technique was used DNA was either 5' end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase or 3' end-labelled with [α-^{32}P]dATP using the Klenow DNA polymerase (section 2:16).

Nuclease mapping studies were also carried out with ssDNA probes prepared from M13 subclones (Farrell et al. (1983). Radiolabelled DNA complementary to the M13 cloned insert was synthesised using essentially the same protocol described for the DNA sequencing reaction (section 2:18) except that dideoxynucleotides were not used. The nucleotide triphosphate solution used comprised 125µM dCTP; 125µM dGTP; 125µM dTTP and 20 µCi [α-^{32}P]dATP (410 Ci/mmol, Amersham International). The nucleotide triphosphate chase solution was added as in the DNA sequencing reaction (section 2:18). Following the in vitro DNA radio-labelling reaction the mixture was diluted with distilled water (15 µl) and heated to 100°C for 5 min to denature the partial DNA duplex. The strands were separated by electophoresis in a 1% agarose gel and the faster-migrating radiolabelled strand recovered by electroelution (section 2:7).

Radiolabelled DNA probes were mixed with the appropriate RNA samples and precipitated with ethanol. The precipitate was resuspended in 20 µl 80% (v/v) recrystalised formamide; 0.4M NaCl; 0.001M EDTA; 0.04M PIPES (pH 6.4) (H-buffer), heated to 70°C for 5 min and the solution incubated for 4 or 16h at 42°C, unless otherwise stated in the text. The solution was then diluted 15 fold with mung bean nuclease buffer (0.03M Na acetate (pH 4.6); 0.05M NaCl; 0.001M $ZnCl_2$) or exonuclease VII buffer (0.067M KPO_4 pH7.9); 0.083M EDTA). Mung bean nuclease (100 units, P.L. Biochemicals) or exonuclease VII (2 units, BRL) was added and the reactions incubated at 37°C for 60 min. When practical exonuclease VII treated samples were

desalted by chromatoraphy through a Sephadex G50 column. Nucleic acids were precipipated with ethanol, recovered by centrifugation, dried, dissolved in TE buffer and analysed by electrophoresis either in denaturing formaldehyde-agarose gels or DNA sequencing gels.

2:20 Primer Extension Analysis of RNA Transcripts

The transcriptional start site of the HCMV major early gene was identified in a primer extension experiment. DNA complementary to the cloned insert in M13mp11(1/39) was synthesised using essentially the same protocol described for the DNA sequencing reaction (section 2:18) except that dideoxynucleotides were not used. The nucleotide triphosphate solution used comprised 125µM dCTP; 125µM dGTP; 125µM dTTP and 10 µCi [α-^{32}P]dATP (410 Ci/mmol, Amersham International). The chase nucleotide solution was added, as in the DNA sequencing reaction, to obtain maximum extension of the newly synthesised strand. The synthesised DNA duplex was digested with XmaIII, denatured by boiling in the presence of 50% (v/v) formamide (3 min) and single-stranded DNA fragments separated by electrophoresis in a 6% acrylamide-urea sequencing gel. The radiolabelled 103n ssDNA primer was detected by autoradiography and recovered by electroelution (section 2:7).

10 µg of total cytoplasmic RNA (or tRNA) was hybridised for 4h at 40°C with 12,000 cpm (Cerenkov) of primer DNA in 30 µl of 80% (v/v) formamide; 0.4M NaCl; 1mM EDTA; 0.04M PIPES (pH 6.4). Nucleic acid was precipitated with ethanol and redissolved in a 20 µl solution containing 2.5 units/ml placental ribonuclease inhibitor (Sigma); 1mM dATP; 1mM dCTP; 1mM dGTP; 1mM dTTP; 50mM KCl; 10mM $MgCl_2$; 10mM dithiothreitol; 50mM Tris-HCl (pH 8.0). Reverse transcriptase (25 units, Anglian Biotechnology) was added and the reaction incubated at 42°C for 30 min. The reaction products were recovered by ethanol precipitation, separated by electrophoresis in a DNA sequencing gel. Autoradiography was performed at

room temperature for 16h.

2:21 Autoradiography

Autoradiography was performed by placing Kodak X-Omat RP film against the sample in a light-tight box at room temperature for the period of time necessary for optimal exposure of the film. In DNA/RNA hybridisation experiments a Dupont Cronex Lightning plus intensifying screen was also placed on top of the film and in this case the film was exposed at $-70^{\circ}C$. The film was developed in Kodak LX 24 developer (5 min at $20^{\circ}C$), rinsed in water for 1 min, immersed in Kodak FX 40 fixer (5 min at $20^{\circ}C$), washed in running water (30 min) and dried.

2:22 Assay for Thymidine Kinase Activity in Transfected Cells

Thymidine kinase (TK) activity in transfected cells was measured essentially according to the method described by Cordingley et al. (1983). Cells monolayers were rinsed once with 1 x PBS and then scraped off 35 mm or 50 mm diameter petri dishes using the flexible end of a fine-tipped pastette directly into either 300 µl or 400 µl of TK extraction buffer (1% (v/v) NP40; 10mM KCl; 1mM $MgCl_2$; 1mM mercaptoethanol; 10mM $MgCl_2$; Tris-HCl, pH 7.5) Cells were lysed by vortexing vigorously for 30s and then nuclei and cellular debris was removed by centrifugation in a MSE microfuge (high speed for 5 min). The cellular extract was stored at $-80^{\circ}C$ until assayed.

The TK enzyme assay consisted of 10 µl of cell extract; 5mM ATP; 50µM dTTP; 10 µCi of [5'-^{3}H]thymidine (40 Ci/mmol; Amersham International); 10mM $MgCl_2$; 100mM $NaPO_4$, pH 6.0 in a final volume of 50 µl. The reaction was incubated at $30^{\circ}C$ for 4h before being stopped by the addition of 10 µl of 2 mM thymidine and heating to $100^{\circ}C$ for 2 min. 45 µl of the reaction mixture was spotted on to 15 mm square piece of Whatman DE81 paper. The filter paper was washed three times in 4 mM ammonium formate, pH 4.0, at

37^oC, twice in ethanol, twice in ether and dried. The amount of phosphorylated [^3H]thymidine bound to Whatman DE81 paper was counted in 5 ml of scintillation fluid using a Hewlett Packard Tri Carb 8000 scintillation counter.

2:23 Assay for Chloramphenicol Acetyltransferase Activity in Transfected Cells

Chloramphenicol acetyltransferase (CAT) in transfected cells was measured according to the method described by Gorman et al. (1982a). Monolayers were rinsed once with 1 x PBS and the cells scraped off the petri dish, using the flexible end of a fine-tipped pastette, into 0.5 ml of PBS. Cells were pelleted by low speed centrifugation in a MSE microfuge (1 min) and resuspended in 100 µl of 0.25M Tris-HCl (pH 7.5). The cells were lysed by the addition of NP40 to a final concentration of 1%, vortexing vigorously for 30s and exposing to one round of freezing (-80^oC) and thawing. Nuclei and cellular debris were removed by high speed centrifugation in a MSE microfuge (3 min). Cellular extracts were stored at -80^oC until assayed.

The CAT enzyme assay contained 20 µl of cell extract; 0.5 µCi of [^{14}C]chloramphenicol (50 mCi/mmol; Amersham International) and 100 µl of 0.25M Tris-HCl (pH 7.5) in a final volume of 160 µl. The reaction was started by the addition of 20 µl 4mM acetyl coenzyme A (Sigma) and incubated for between 20 min to 2h at 37^oC. The reaction was stopped by adding 0.7 ml of ethyl acetate (-20^oC) and vortexing vigorously; ethyl acetate also serves to extract chloramphenicol from the aqueous phase. The organic phase was removed to an eppendorf tube and dried down in a vacuum desiccator. The dried product was redissolved in 20 µl of ethyl acetate and spotted on to thin-layer silica gel chromatography (TLC) plates (Merck). The TLC plates were suspended above 100 ml of 95% chloroform; 5% methanol in a chromatography tank for 10 min, to allow the silica gel to equilibrate. The foot of the TLC plate was then placed in the solvent

which was allowed to ascend the plate by capilliary action for 45 min. Choramphenicol and its three acetylated forms (the products of the reaction) were visualised by autoradiography of the TLC plate. Quantitative results were obtained by scraping the silica gel off relevant regions of the plate into scintillation fluid and measuring their radioactivity using a Hewlett Packard Tri Carb 8000 scintillation counter.

2:24 Assay of Protein Concentration

Values for the protein concentrations of cellular extracts were obtained using the method of Bradford (1976). 100 mg of Coomassie Brilliant Blue G-250 (BDH) was dissolved in 50 ml of methanol and filtered through Whatman 3MM paper. 100 ml of 95% orthophosphoric acid and 200 ml of water was added to the filtrate. This concentrated stock solution was then diluted 1:5 for use. 100 µl of each sample asssayed was added to 5 ml of the diluted dye and gently mixed. After 10 min the increase in absorbance at 595 nm was measured. The protein concentration of samples were calculated by comparing the OD_{595} with values derived from a standard. A standard curve was prepared for each assay performed using bovine serum albumin at varying concentrations between 25 µg/ml to 2 mg/ml.

RESULTS

3:1 Structural Analysis of the Major IE Gene

HCMV encodes a highly abundant 1.95kb IE mRNA which is transcribed from between 0.756–0.745 map units with respect to the prototype orientation of the strain AD169 genome (Figs 1:5, 1:6 and 3.1). The coding region for the 1.95kb IE RNA has been shown by RNA/DNA hybridisation experiments to extend for at least 2.9kb; this result was interpreted as indicating that the major IE gene may contain in excess of 0.95kb of intron sequence (Wilkinson, 1983; Wilkinson et al., 1984). In order to precisely map the 5' and 3' ends of the major IE gene and identify the locations of splice sites nuclease protection studies were performed using the technique of Berk and Sharp (1977) as modified by Weaver and Weissman (1979) and Farrell et al. (1983). The structural analysis of the major HCMV IE gene was carried out in combination with the determination of its nucleotide sequence, which was performed by Dr A. Akrigg. The complete nucleotide sequence of the strain AD169 major IE gene has now been ascertained and is shown in appendix 2 (Akrigg et al., 1985).

Mapping the 3' Terminus: Previous studies had suggested that transcription of the 1.95kb major IE mRNA extends a short distance past the BamHI site at 0.745 map units (Fig 3:1); the BamHI/PstI fragment BP-4 (0.742–0.745 map units, Fig 3:1) hybridises weakly with a 1.95kb RNA which co-migrates with and is transcribed from the same strand as the major IE RNA (Wilkinson et al., 1984). Additionally, HST experiments showed that a polypeptide, which co-migrated with the major IE protein on SDS-PAGE, is translated from an IE mRNA selected by the recombinant plasmid EHB2, EHB2 contains sequences between 0.726–0.745 map units (Fig 3:1; Wilkinson et al., 1984). Mung bean nuclease protection experiments were therefore performed to determine how far downstream of the BamHI site the major IE gene extends.

The plasmid EHB2 was digested with both BamHI and PstI and electrophoresed in a 1% agarose gel. The 675bp DNA fragment BP-4 (Fig 3:1) was electroeluted from the gel and 3' end-labelled using [α-^{32}P]dATP and the Klenow DNA polymerase. 5 μg of either total cytoplasmic IE RNA or tRNA was mixed with the radiolabelled DNA probe and precipitated with ethanol. The dried precipitates were then dissolved in 120 μl of H buffer and divided into six 20 μl aliquots. The samples were incubated first at 65°C for 5 minutes to denature the double-stranded DNA probe and then for 4h at the temperatures detailed in Fig 3:2 to permit the formation of DNA:RNA hybrids. After treatment with mung bean nuclease the samples were electrophoresed in a denaturing acrylamide gel which was then subjected to autoradiography. A

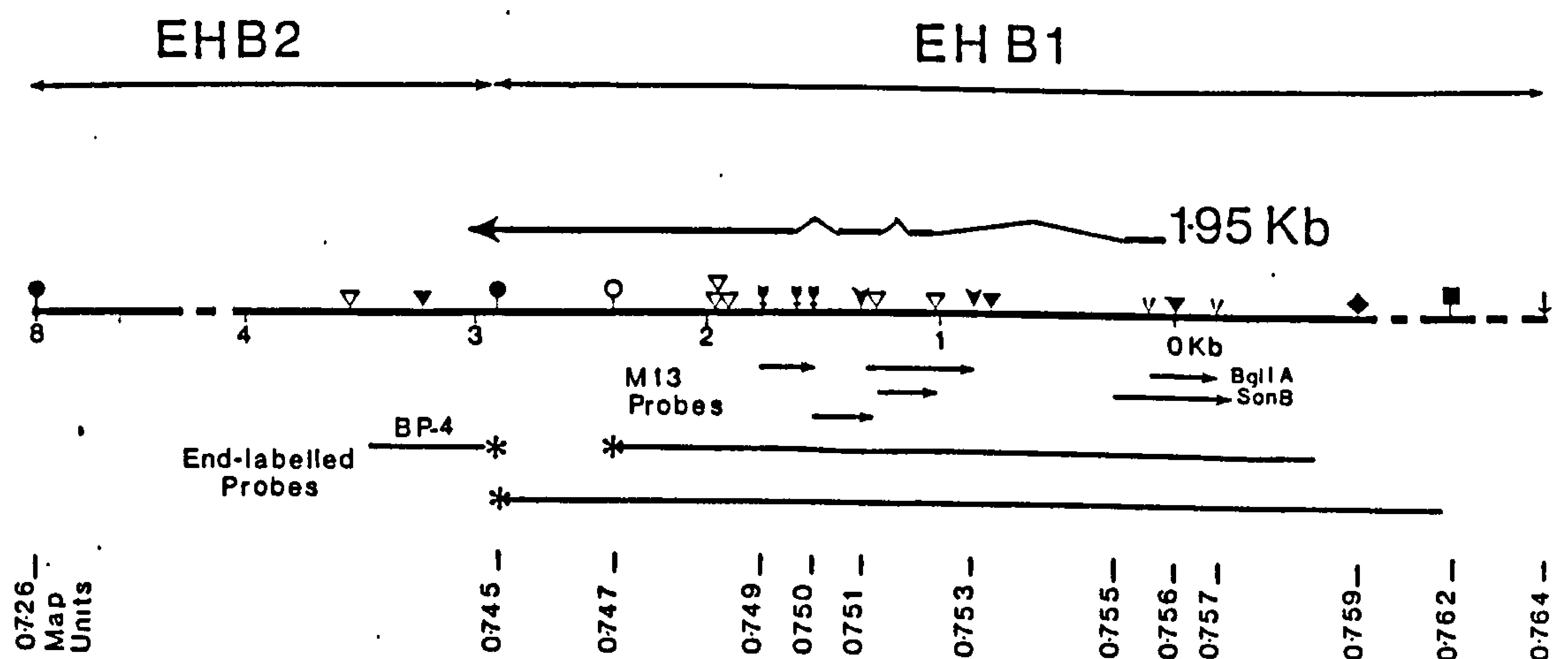


Fig 3:1: The map location of hybridistion probes used in nuclease protection studies on the 1.95kb major IE RNA. End-labelled probes (*—) were prepared from the recombinant plasmids EHB1 and EHB2 (Greenaway et al., 1982). EHB1 contains the HindIII/BamHI fragment (0.745-0.764 map units) and EHB2 the BamHI fragment (0.726-0.745 map units) cloned into the appropriate sites in the vector pAT153. The relative location on the HCMV genome of ssDNA probes prepared from recombinant M13 phage (➡) is indicated as are the positions of relevant BamHl (●), BglI (V), BglII (♀), ClaI (◆), EcoRI (■), HindIII (↓), PstI (▽), PvuII (Y), SstI (▼) and TaqI (Ϫ) restriction endonuclease cleavage sites.

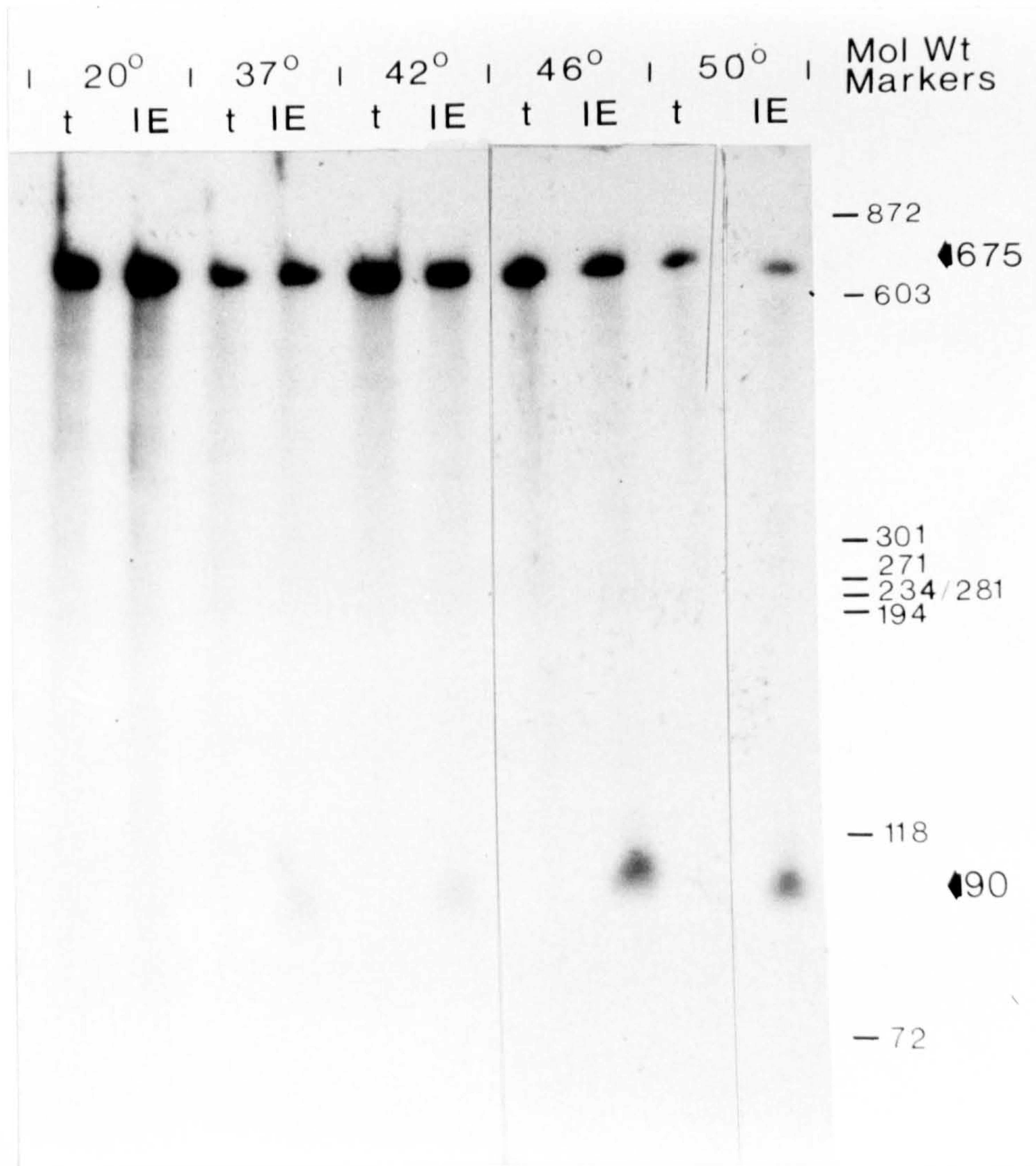


Fig 3:2: Mapping the 3' end of the major IE gene. Total cytoplasmic IE RNA (IE) or tRNA (t) were hybridised in H buffer with the 675 bp 5' end-labelled DNA fragment BP-4 at 20°C, 37°C, 42°C, 46°C and 50°C as indicated. Following digestion with mung bean nuclease samples were electrophoresed in a 7M urea, 6% acrylamide denaturing gel. The top band (675n) was produced by reassociated DNA duplex molecules which are resistant to mung bean nuclease digestion. The lower band (90n) is produced by the 1.95kb IE RNA hybridising to the 3' end-labelled DNA strand and thereby protecting it against nuclease digestion.

small radiolabelled nuclease-protected fragment of approximately 90n was observed in the samples incubated at 37, 42, 46, and 50°C. In order to obtain a more accurate evaluation of the size of the nuclease-protected fragment the experiment was repeated (with a hybridisation temperature of 46°C) and the sample electrophoresed in a DNA sequencing gel (section 2:18). IE mRNA protected an end-labelled DNA fragment of precisely 90n (Fig 3:3). This result located the 3' end of the major IE gene as being 90n downstream from the BamH1 site (0.745 map units). A consensus polyadenylation signal (AAUAAA; Proudfoot and Brownlee, 1976) is present in the characteristic position 22-28n upstream from the 3' end of the transcript (Akrigg et al., 1985).

Identification of the Transcriptional Start Site: The 4.2kb BamHI/EcoRI DNA fragment between 0.745-0.762 map units was excised from the plasmid EHB1 (Fig 3:1), radiolabelled at its 5' ends (using [γ-^{32}P]ATP and polynucleotide kinase) and hybridised with IE RNA in H buffer as described in section 2:16 and 2:19. Samples containing the RNA:DNA hybrids were divided into two equal aliquots and treated with either exonuclease VII or mung bean nuclease. The nuclease digested samples were then electrophoresed in a denaturing agarose gel (Fig 3:4). Protected fragments of 1.4, 1.7, 1.9 and 2.9kb were detected following digestion with exonuclease VII whereas a single fragment of 1.4kb was detected in the mung bean nuclease treated sample (Table 3:1).

Since the BamHI site lies within the coding region of the gene the 5' radiolabelled end of the complementary DNA probe is protected from mung bean nuclease and exonuclease VII digestion by hybridisation with the major IE RNA. Exonuclease VII progressively digested the ssDNA component of RNA:DNA hybrids starting from the 3' unlabelled end of the probe. Exonuclease VII digestion was expected to generate a single size class of end-labelled DNA fragments which would define the distance between the transcriptional start

Fig	Lane	DNA Fragment	Map Units	^{32}P-Radiolabel	Enzyme	Size of Protected Fragment
3:4	1	BamHI-EcoRI	0.745-0.762	5' end	Exonuclease VII	2.9, 1.9, 1.7, 1.4 kb
3:4	3	"	"	"	Mung Bean Nuclease	1.4 kb
3:4	5	BglII-ClaI	0.747-0.759	5' end	Exonuclease VII	2.4, 1.4, 1.2, 0.8 kb
3:4	7	"	"	"	Mung Bean Nuclease	0.8 kb
3:3& 3:2	1 -	Pstl-BamHI (PB-4)	0.742-0.745	3' end	Mung Bean Nuclease	90n
3:3	2	BglI-BglI (BglI A)	0.756-0.757	Primer Extension	Mung Bean Nuclease	69n
3:3	3	Sonicated (SonB)	0.755-0.757	Primer Extension	Mung Bean Nuclease	121n
3:3	8	PvuII-PvuII	0.751-0.753	Primer Extension	Exonuclease VII	275n
3:3	4	"	"	Primer Extension	Mung Bean Nuclease	88, 73n
3:3	9	PstI-PstI	0.751-0.752	Primer Extension	Exonuclease VII	242n
3:3	5	"	"	Primer Extension	Mung Bean Nuclease	88, 40n
3:3	6	TaqI-PstI	0.750-0.751	Primer Extension	Mung Bean Nuclease	151n
3:3	7	TaqI-TaqI	0.749-0.750	Primer Extension	Mung Bean Nuclease	166n

Table 3:1: Protected fragments obtained by nuclease digestion of IE RNA/DNA hybrids.

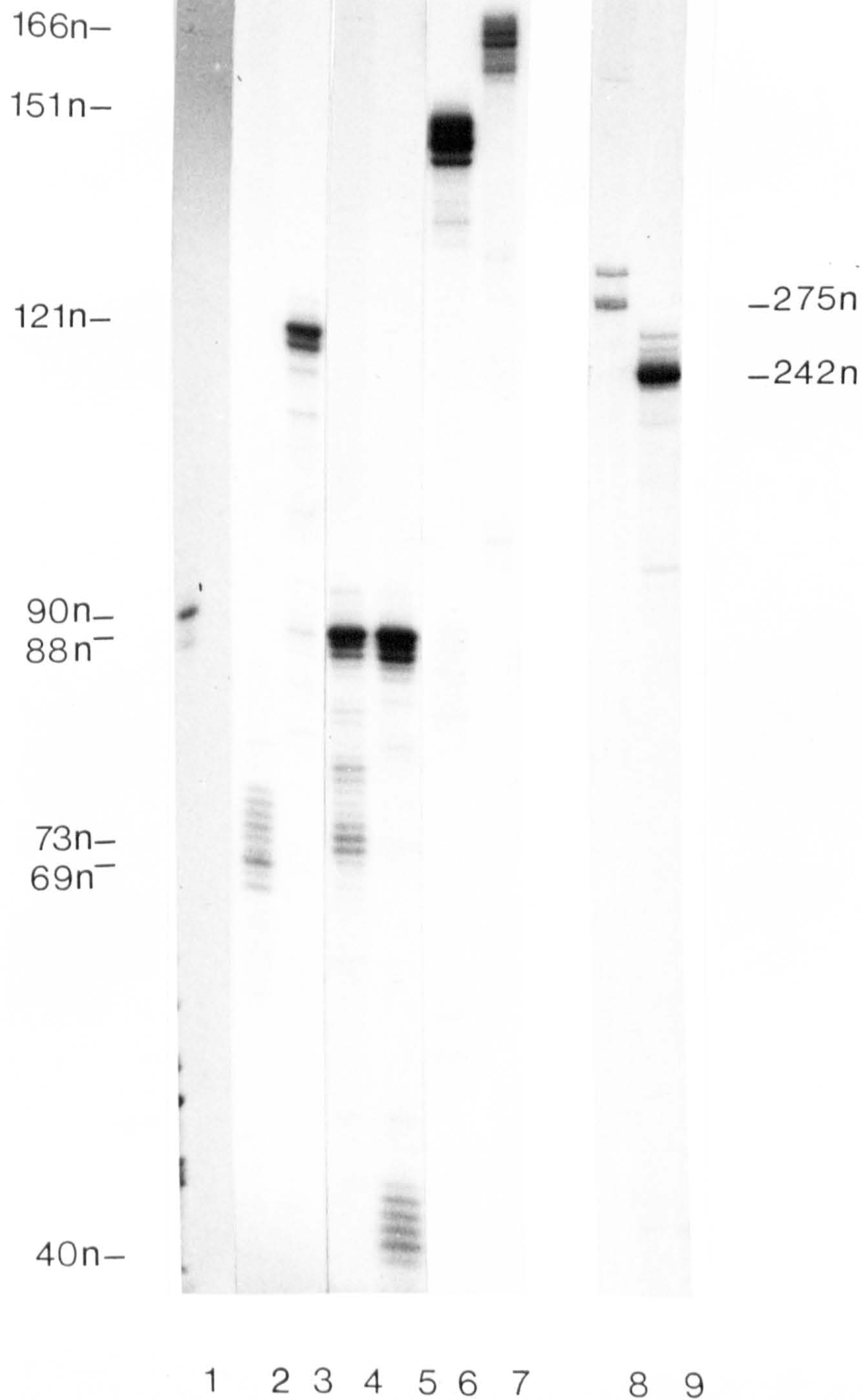


Fig 3:3: Fine mapping of the major IE gene in nuclease protection experiments. Table 3:1 gives details of DNA fragments, the method by which each fragment was radiolabelled and the nuclease used to digest DNA/RNA hybrids for lanes 1-9. Samples were electrophoresed in a denaturing 6% acrylamide DNA sequencing gel. The figure is a composite of results obtained using more than one gel. Molecular weight estimates were obtained from DNA sequencing tracks run in parallel. Nuclease protected fragments were not detected in control samples in which total cytoplasimic RNA from uninfected MRC5 cells was used.

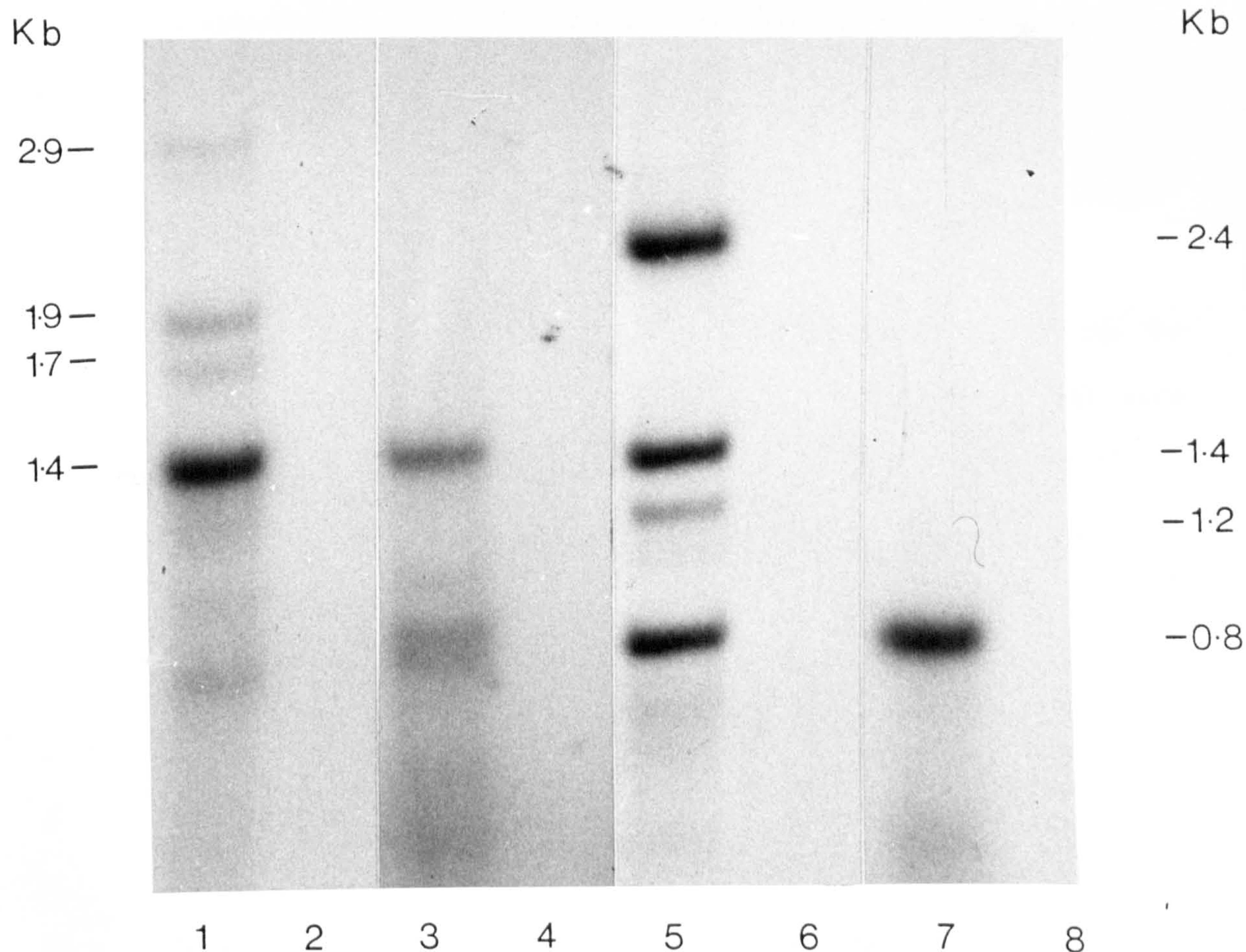


Fig 3:4: Mapping the 5' end of exons within the major IE gene. RNA and end-labelled DNA fragments were hybridised at 42°C in H buffer before being treated with either mung bean nuclease or exonuclease VII and electrophoresed through a 1% formaldehyde-agarose gel. Total cytoplasmic IE RNA was used in lanes 1, 3, 5 and 7, and total cytoplasmic RNA from uninfected MRC5 cells was used in lanes 2, 4, 6 and 8. Details of the DNA fragments used are given in Table 3:1 and Fig 3:1. Molecular weight estimates were made from HindIII digest of bacteriophage λ DNA.

site and the BamHI site. The ability of exonuclease VII digestion to generate four discrete bands (Fig 3:4) may be caused either by a low level of contaminating endonuclease activity nicking the single stranded DNA loops of intron sequences generated in the hybrid molecules or alternatively there may be some instability in RNA/DNA hybrids involving short exons. The data indicates that intron/exon junctions exist 1.4, 1.7 and 1.9kb upstream from the BamH1 site (0.745 map units) and that transcription is initiated 2.9kb upstream.

When the BglII/Cla1 fragment (0.747-0.762 map units) was used for this analysis exonuclease VII protected fragments of 0.8, 1.2, 1.4 and 2.4kb were generated (Fig 3:4; Table 3:1). Since the BglII site is 475bp upstream of the BamHI site, the positions of the transcriptional initiation site and intron/exon junctions calculated using probes labelled at the BglII and BamHI sites are in good agreement. In vitro transcription experiments have also mapped the transcriptional start site to approximately 2.5kb upstream of the BglII site (0.747 map units), (Wilkinson, 1983).

Fine Mapping of Exons: The positions of exons in the major IE gene did not coincide with suitable restriction endonuclease cleavage sites which would facilitate their being mapped using the technique of Weaver and Weissman (1979), as used above. The nucleotide sequence analysis of the major IE gene generated a large number of characterised recombinant M13 clones containing DNA fragments produced using a variety of restriction enzymes and by sonication. The preparation of radiolabelled ssDNA probes from recombinant M13 clones for use in nuclease protection experiments is relatively straightforward (section 2:19). Fig 3:1 shows the relative postions on the HCMV genome of DNA fragments, cloned into M13 phage, which were used to the exons in the major IE gene.

The first (nearest the promoter) exon was mapped using the overlapping cloned DNA fragments BglI A (0.756-0.757 map units) and SonB (0.755-0.757

map units). Single major nuclease protected fragments of 69n and 121n were obtained with BglI A and SonB respectively (Fig 3:3; Table 3:1). Since the DNA sequence in BglI A is also completely contained within SonB, it follows that that transcription is initiated 69bp upstream from the end of BglI A. Transcription was therefore shown to be initiated 15bp downstream from from the SstI site at 0.756 map units at a base now designated +1 (Fig 3:1). With respect to this transcriptional start site SonB and BglI A extend from bases -320 to +147 and -299 to +69 respectively. The first exon, which is completely contained in SonB, is therefore 121bp long. A typical consensus TATA box homology is found 23n upstream from the transcriptional start site (Akrigg et al., 1985).

The positions of the second and third exons were mapped using the 0.4kb PvuII fragment (0.751-0.753 map unts) and its 242bp component PstI fragment (0.751-0.752; Fig 3:1). Hybridisation followed by mung bean nuclease or exonuclease VII digestion produced the protected bands seen in Fig 3:3 lanes 4, 5, 8 and 9 (see also Table 3:1). The presence of minor bands, particularly with smaller protected fragments, was probably due to 'breathing' at the ends of hybrid molecules. The size of the DNA fragments protected by exonuclease VII digestion differed in size by 33n with the two probes as did the smaller protected fragments in the mung bean nuclease digests (Table 3:1). Since the distance between the downstream ends of the short PstI and PvuII fragments is also 33bp, the position of the start of the second exon was located by the exonuclease digests to 242n and 275n upstream of the PstI and PvuII (0.751 map units) sites respectively at +948. The length of the second exon was estimated from the mung bean nuclease digest (larger fragments) to be 88n and the start of the third exon located approximately 40n and 73n upstream from the PstI and PvuII (0.751 map units) sites respectively (Fig 3:1).

The position of the remaining intron was deduced using the TaqI-PstI fragment (0.750-0.751 map units) and the TaqI-TaqI fragment (0.749-0.750 map

units) which was produced by partial DNA digestion (Fig 3:1); digestion with mung bean nuclease gave single major protected fragments of 151n and 166n respectively (Fig 3:3; Table 3:1). It follows, therefore, that the TaqI-PstI fragment contains 151n of exon three and the TaqI-TaqI fragment 166n of exon four.

The relative position and sequences surrounding the splice sites is illustrated in Fig 3:5. The sequences at the intron donor and acceptor splice sites were found to conform to the consensus sequences identified by Mount (1982). The exact position of the splice sites was subsequently confirmed by sequencing an almost full length cDNA clone derived from the 1.95kb major IE RNA (Akrigg et al., 1985).

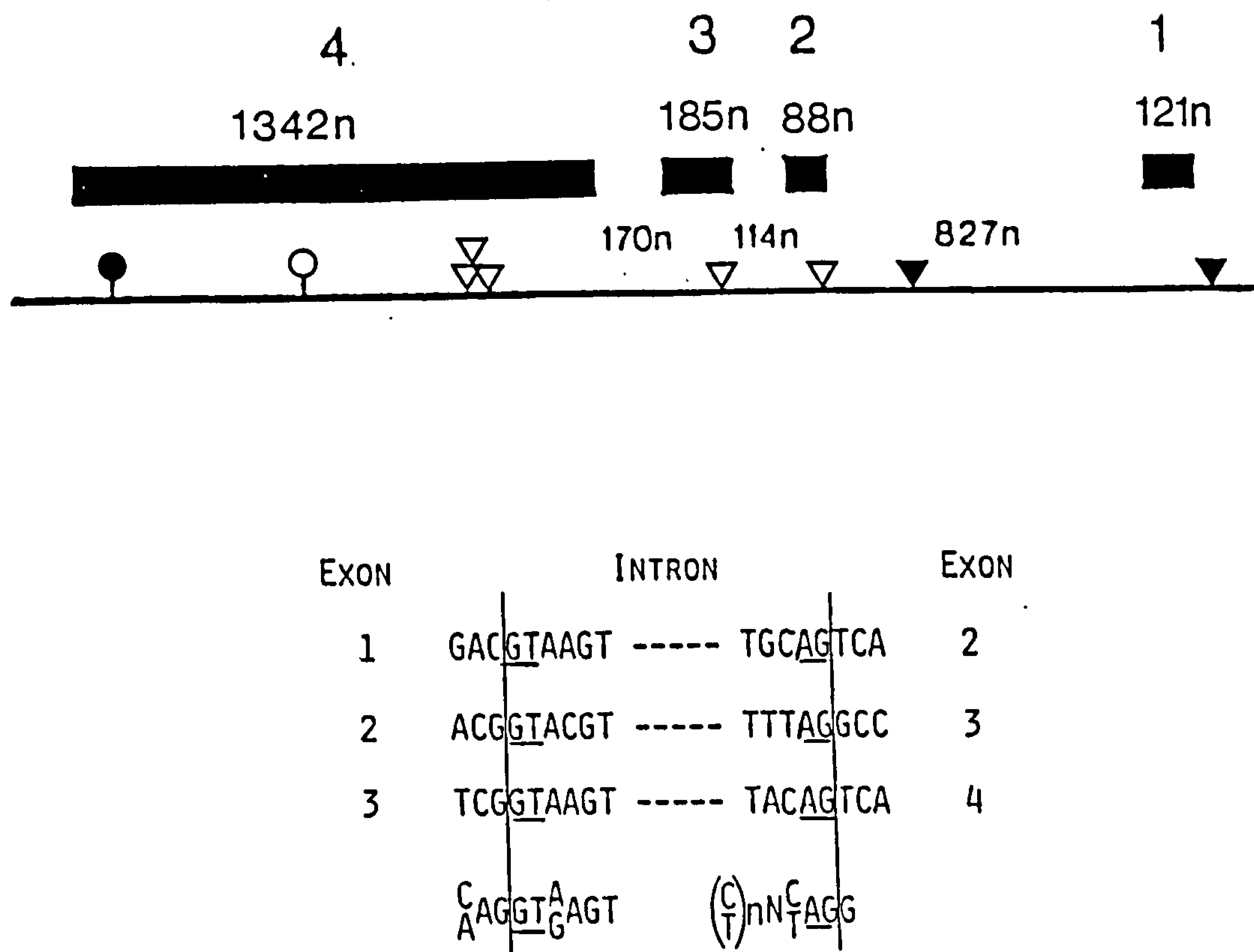


Fig 3:5: The lengths of the four exons (thick bars) and three introns of the major IE gene are shown in the above abbreviated physical map. The sequences surrounding the splice junctions are compared with the consensus sequences of Mount (1982). BamHI (●), BglII (○), PstI (▽) and SstI (▼) restriction endonuclease cleavage sites are shown.

3:2 IE Gene Expression in Transfected Cells

The DNA sequence and structural analysis ot the HCMV major IE gene was performed primarily to identify the amino acid sequence of the major IE protein, but also to identify the IE promoter with a view to investigating IE transcriptional regulation. The nucleotide sequence of the IE promoter contains a number of interesting features (Akrigg et al., 1985). Within 513bp upstream of the transcriptional start site, in addition to the consensus TATA box, there are two copies of a GC-rich 21bp repeat, a 18bp and 19bp repeat are represented four times each and a 16bp repeat is present three times. Each of the 19bp repeats contains a copy of a CAAT box homology and includes palindromic sequences (Thomsen et al., 1984; Akrigg et al., 1985). An interaction between the two 19bp repeats nearest to the TATA box could hypothetically form a stem loop structure with a stem of 18-19bp and a loop of 51n (Thomsen et al., 1984). Potential enhancer core sequences (TGG$\frac{AAA}{TTT}$; Weiher et al., 1983) exist in the complementary strand of the promoter at -93 to -98, -156 to -161 and -412 to -417 (Akrigg et al., 1985). The IE promoter of strain AD169 has recently been shown experimentally to contain the most powerful 'enhancer' element so far identified, some three to five fold stronger than the SV40 enhancer (Boshart et al., 1985). The IE promoter also contains a number of potential Sp-1 factor binding sites (GGGCGG; Gidoni et al., 1984) and has recently been shown experimentally to bind nuclear factor 1 (Hennighausen and Fleckenstein, 1986).

The mechanisms involved in the control of eukaryotic gene expression can be readily investigated *in vitro* in transient DNA transfection experiments. Using the transfection technique described in section 2:4 any DNA fragment can be introduced into tissue culture cells and the expression of specific gene products obtained in a proportion of cells in a transfected monolayer. In a preliminary experiment to test whether the HCMV IE promoters would function in transfection experiments recombinant plasmids containing the IE genes were transfected into cells and the expression of virus

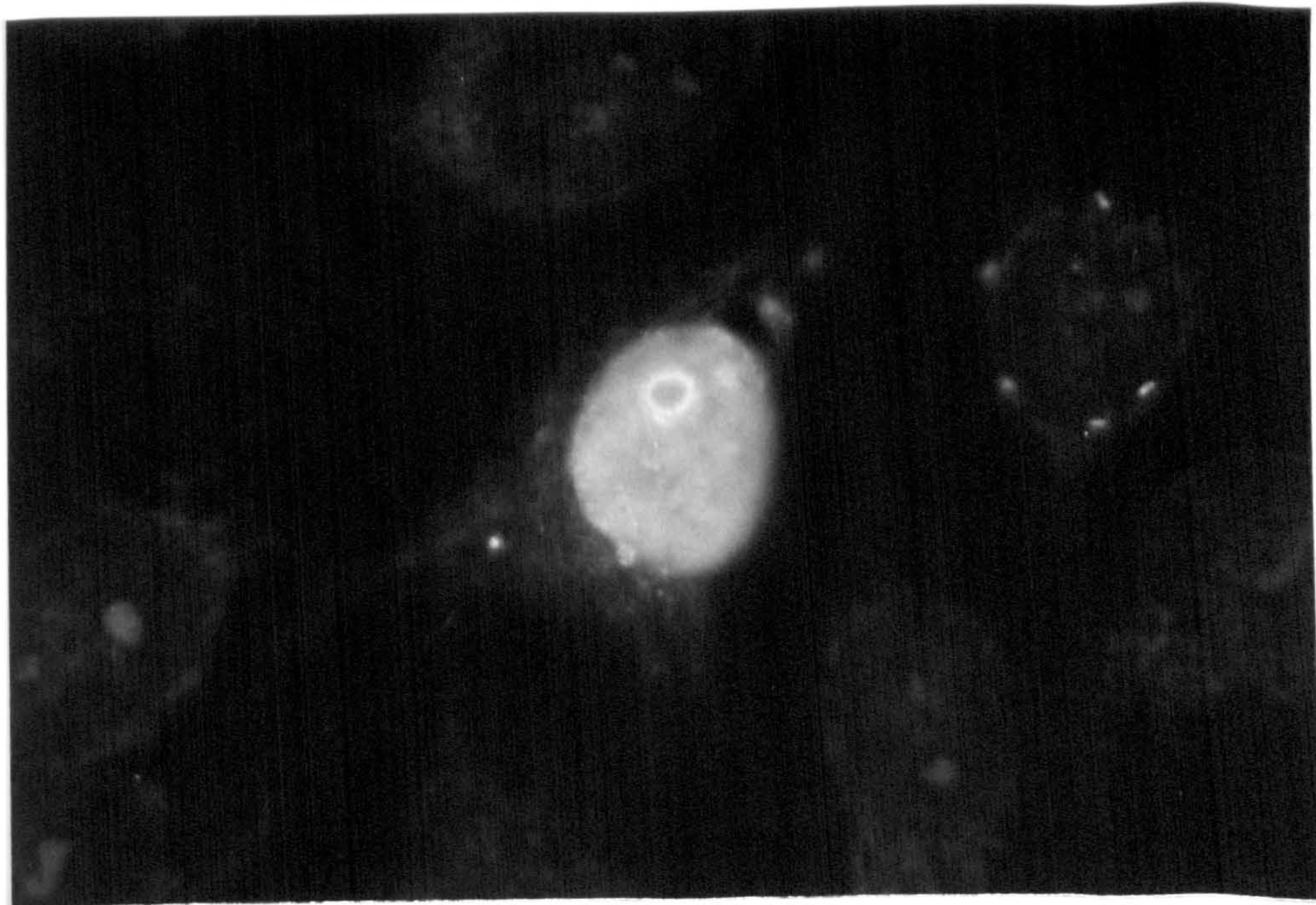
antigens assayed by immunofluorescence.

The plasmid EHB1 (Fig 3:1), which contains all but the last 90 bp of the major IE gene (IE coding region 1; Fig 1:6), was transfected into Hela cells. Forty-eight hours post transfection the cell were assayed by immunofluorescence using convalescent sera. Fig 3:6a shows the nuclear fluorescence detected in EHB1-transfected cells which is similar to the characteristic pattern produced by HCMV IE antigen during productive infections (Michelson-Fiske et al., 1977).

The HCMV DNA fragment BglII K contains all of IE coding region 2 but does not include the major IE promoter (Fig 1:6). BglII fragment K was excised from the recombinant clone containing HindIII E and recloned into the unique BamHI site in pSV2neo (Southern and Berg, 1982) to generate the plasmid pBGK. When transfected into Hela cells the plasmid pBGK induced the synthesis of a compartmentalised nuclear-associated antigen which is manifested as the unusual doughnut-shaped fluorescence seen in Fig 3:7b. Expression was not detected from transfected DNA when plasmids containing IE coding regions 3 (EHB3; Greenaway et al., 1982) and 4 (EcoRI Q; Oram et al., 1982) were used.

The regulation of expression from the major IE promoter may be important in determining the fate of HCMV infections. The main objective of the experiment was to test whether IE promoters would function when transfected into cells. The positive result obtained with the plasmid EHB1 provided a basis for going on to use the DNA transfection technique to study transcriptional regulation of the major IE promoter. In addition to the major 1.95kb IE RNA, the transcription of many of the RNAs derived from IE coding region 2 has been shown to be initiated from the major IE promoter (Stenberg et al., 1985). The detection of antigen expression in cells transfected with HCMV BglII fragment K implies that there is also an efficient constitutive promoter within IE coding region 2 of strain AD169.

a)



b)

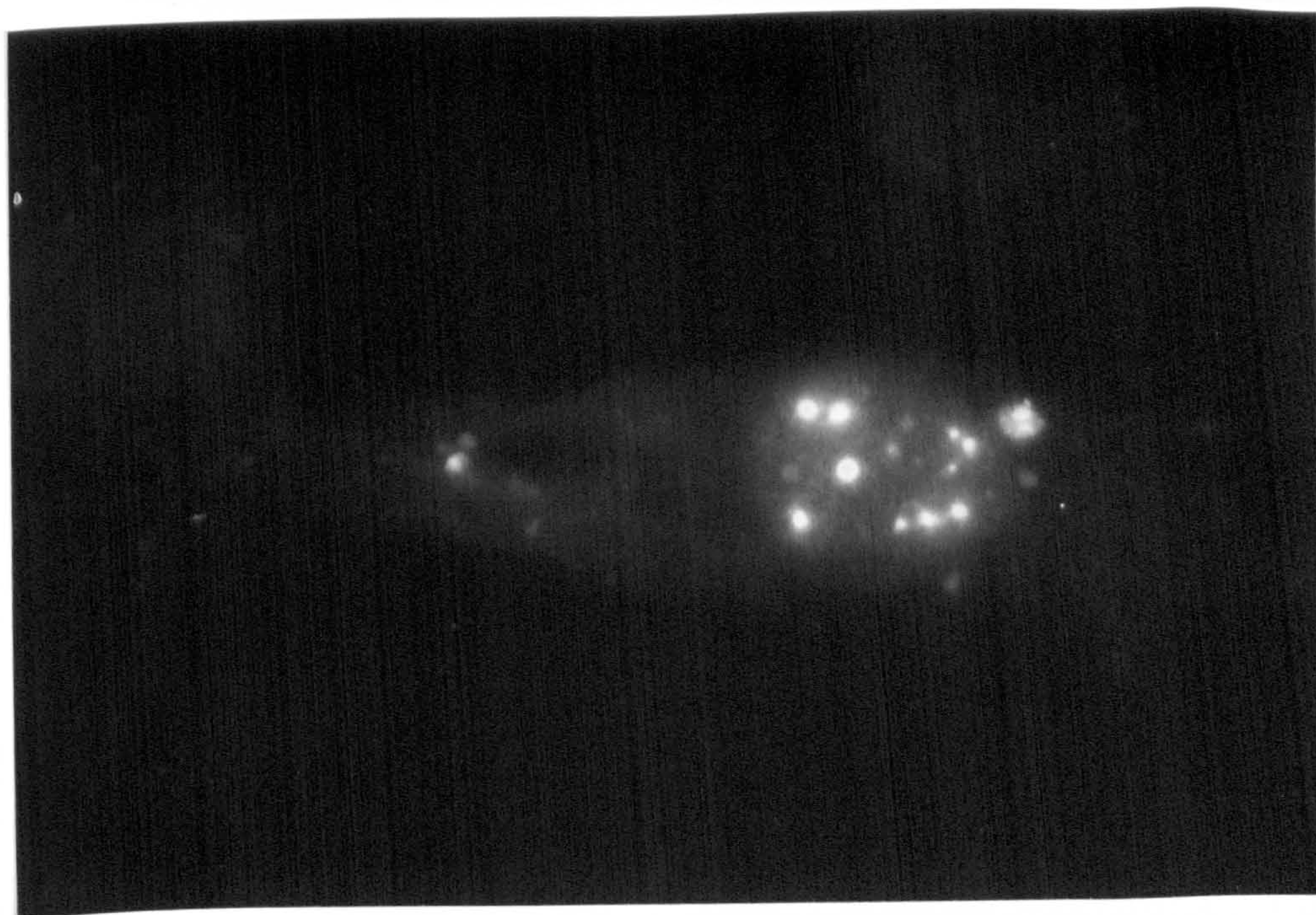


Fig 3:6: Hela cells transfected with (a) EHB1 and (b) pBGK. Indirect immunofluorescence was performed using human convalescent antisera as the first antibody and FITC conjugated goat anti-human sera as the second antibody.

3:3 Construction of IEP1 and IEPTK1

The ability of the HCMV IE promoter to direct transcription in DNA transfection experiments provides a convenient system for the detailed investigation of IE transcriptional regulation. The level of transcription from eukaryotic promoters can be measured indirectly in transient DNA transfection studies if the structural gene for an assayable enzyme, such as thymidine kinase (TK) or chloramphenicol acetyl transferase (CAT), is cloned in the appropriate orientation and position downstream of the promoter.

The recombinant bacteriophage M13mp8(BglI A), which was used in nuclease-protection experiments (section 3:1), contains IE promoter sequences between bases -299 to +69 (Fig 3:1). BglI fragment A contains most, although not all, of the repeated elements present in the IE promoter-regulatory region, the TATA box consensus, the transcriptional start site and 69n of leader sequence (Akrigg et al., 1985). The 5' proximal translational initiation codon is contained in the second exon and, therefore, is not in BglI A (Akrigg et al., 1985). The BglI fragment A was excised from M13mp8(BglIA) by cleavage of the flanking EcoRI and HindIII sites and recloned between the unique EcoRI and HindIII sites in pUC8 as described in Fig 3:7a to generate the plasmid IEP1. IEP1 is a convenient vector for use in eukaryotic expression studies. The series of unique restriction endonuclease cleavage sites downstream of the promoter fascilitates the cloning of heterologous structural genes under its control, while the unique upstream EcoRI site readily allows the IE promoter to be excised from IEP1 and recloned into a different environment.

In order to assay expression from the IE promoter in transfection experiments the HSV-1 TK structural gene was excised from the plasmid M2 (Wilkie et al., 1979) on a BglII/BamHI fragment and was inserted into the BamH1 site in IEP1 to generate the plasmid IEPTK1 (Fig 3:7b). The BglII site is situated within the leader sequence of the TK gene so it is envisaged that translation of the enzyme should be initiated naturally. HCMV

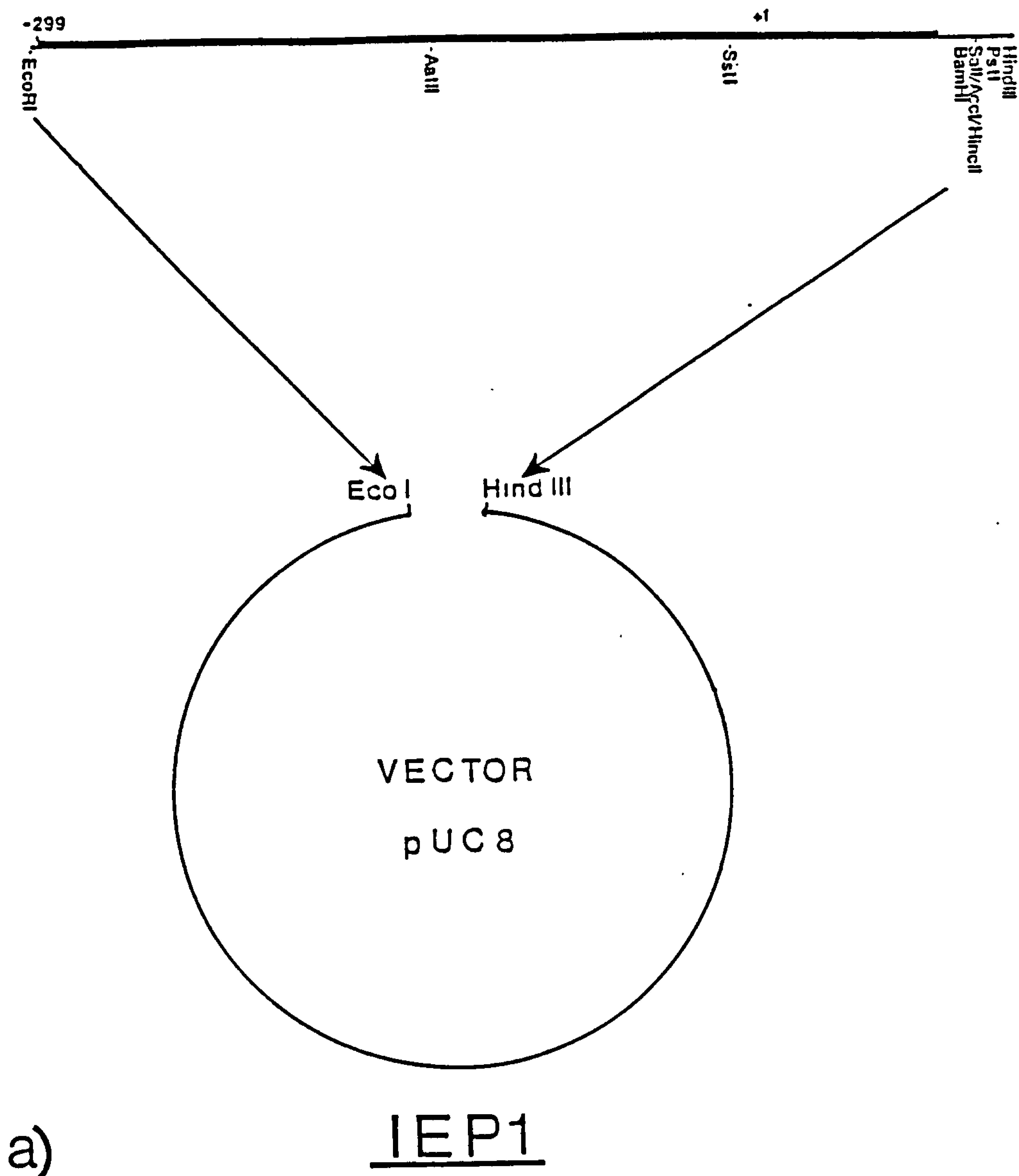
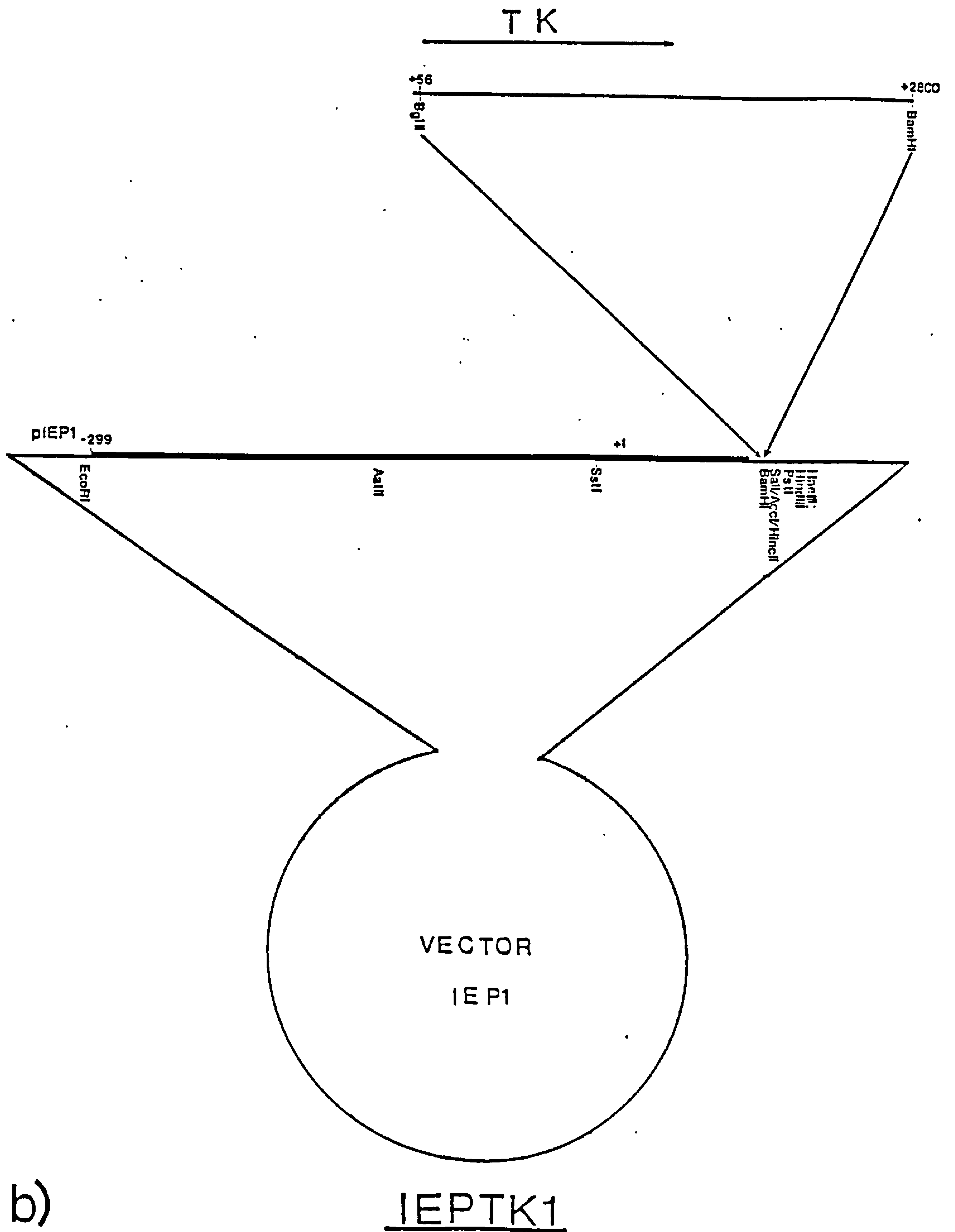


Fig 3:7: The construction of plasmids IEP1 and IEPTK1. (a) IEP1. M13mp8(BglI A) and pUC8 were cleaved with EcoRI and HindIII and fractionated on a 1% agarose gel. The 398bp fragment containing BglI A and the larger (approximately 2.7kb) fragment were recovered from M13mp8(BglI A) and pUC8 digested DNA, respectively, by electroelution. The two purified fragments were ligated and transformed into *E. coli* JM101. (b) IEPTK1. The TK structural gene was excised from the plasmid pM2 (Wilkie *et al.*, 1979) on a 2.75kb (approximately) DNA fragment and ligated with BamHI-cleaved, phosphatase-treated IEP1 DNA. The ligated DNA was transformed into *E. coli* HB101 and plasmid DNA produced from transformants according to the method of Birmboim and Doly (1979; section 2:11). Plasmid DNA was cleaved with restriction endonucleases to screen for a recombinant plasmid containing the TK gene inserted in the appropriate orientation, as shown above.

does not appear to encode its own TK gene although virus infection does stimulate the expression of cellular TKs. The activity of the cellular enzymes can be specifically inhibited in enzyme assays by including dTTP in the reaction.



b) IEPTK1

3:5 Expression From IEPTK2 in Transfected Cells

While the IE promoter-regulatory sequence present in IEPTK1 is sufficient to ensure constitutive expression of the TK structural gene, it extends only to 299bp upstream from the transcriptional start site. IEPTK1 may therefore lack important regulatory elements, e.g. the plasmid contains neither a complete copy of the promoter-associated 21bp repeat (Akrigg et al., 1985) nor the far upstream sequences which may be associated with the induction of transcription in differentiating EC cells (Nelson and Groudine, 1986).

To investigate the possibility that promoter sequences upstream of position -299 are necessary for the transcriptional repression of the major IE promoter during the early and late phases of the HCMV infectious replicative cycle the plasmids IEP2 and IEPTK2 were constructed as described in Fig 3:9. IEPTK2 is identical to IEPTK1 except that it contains an additional approximately 1.55kb of sequence upstream from the IE promoter.

The presence of the additional upstream promoter sequence in IEPTK2, relative to IEPTK1, did not significantly influence the temporal regulation of expression from the IE promoter (Table 3:3; Fig 3:10). Using an input m.o.i. of 1 p.f.u./cell HCMV infection did not affect the level of TK expression from either IEPTK1 or IEPTK2 appreciably at 24h p.i. (Figs 3:8 & 3:10). In uninfected cells the level of expression from both plasmids gradually decreased with time during the later stages of the experiments, whereas in cells infected by HCMV expression from IEPTK1 and IEPTK2 was stimulated reaching a maximum level between 72-96h p.i. after which point expression again declined.

In the context of the conditions used in these experiments the presence of the additional 5' promoter-regulatory sequence in IEPTK2 relative to IEPTK1 did not confer on the transfected IE promoter the ability to be repressed by infection with HCMV.

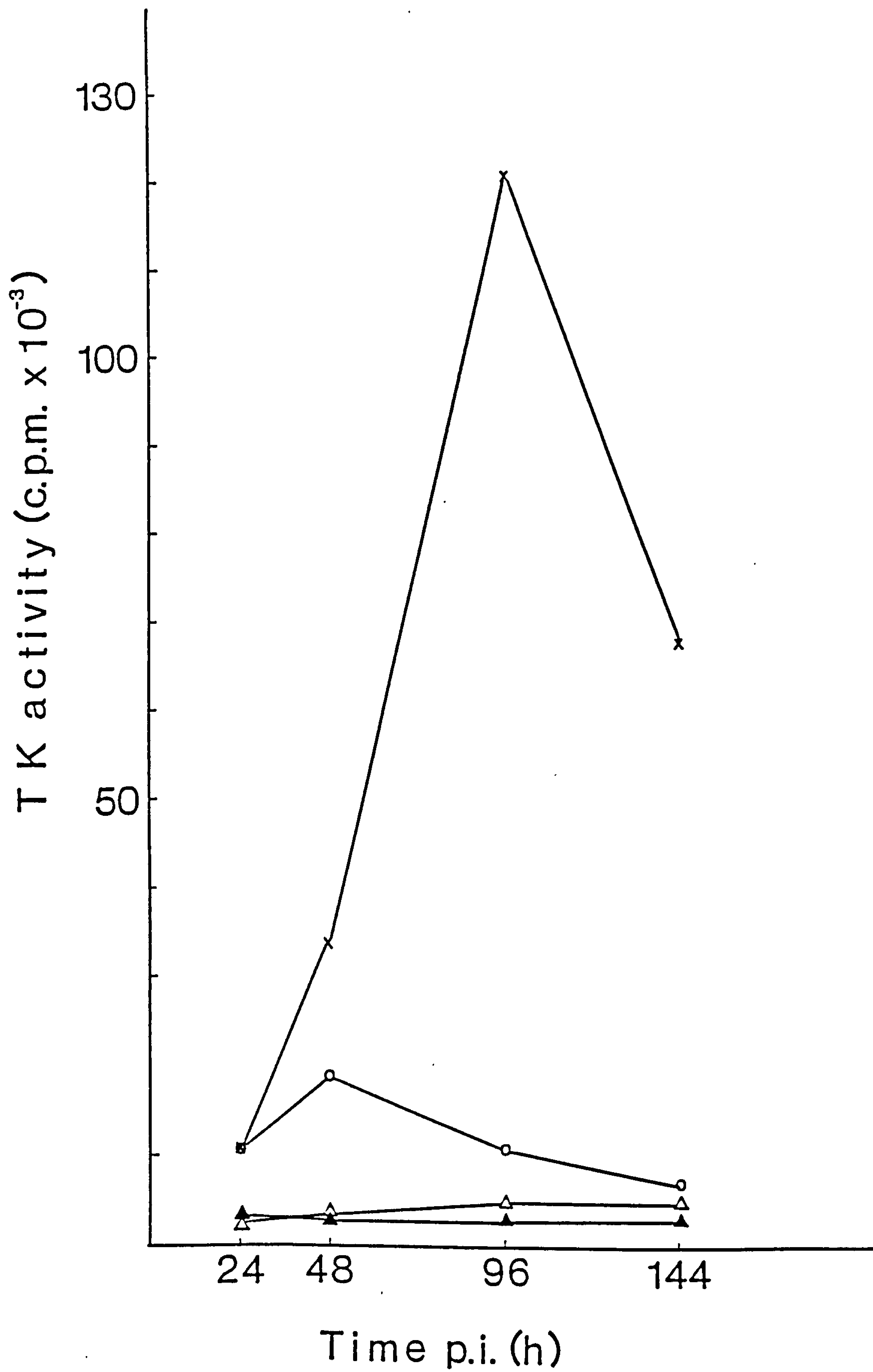


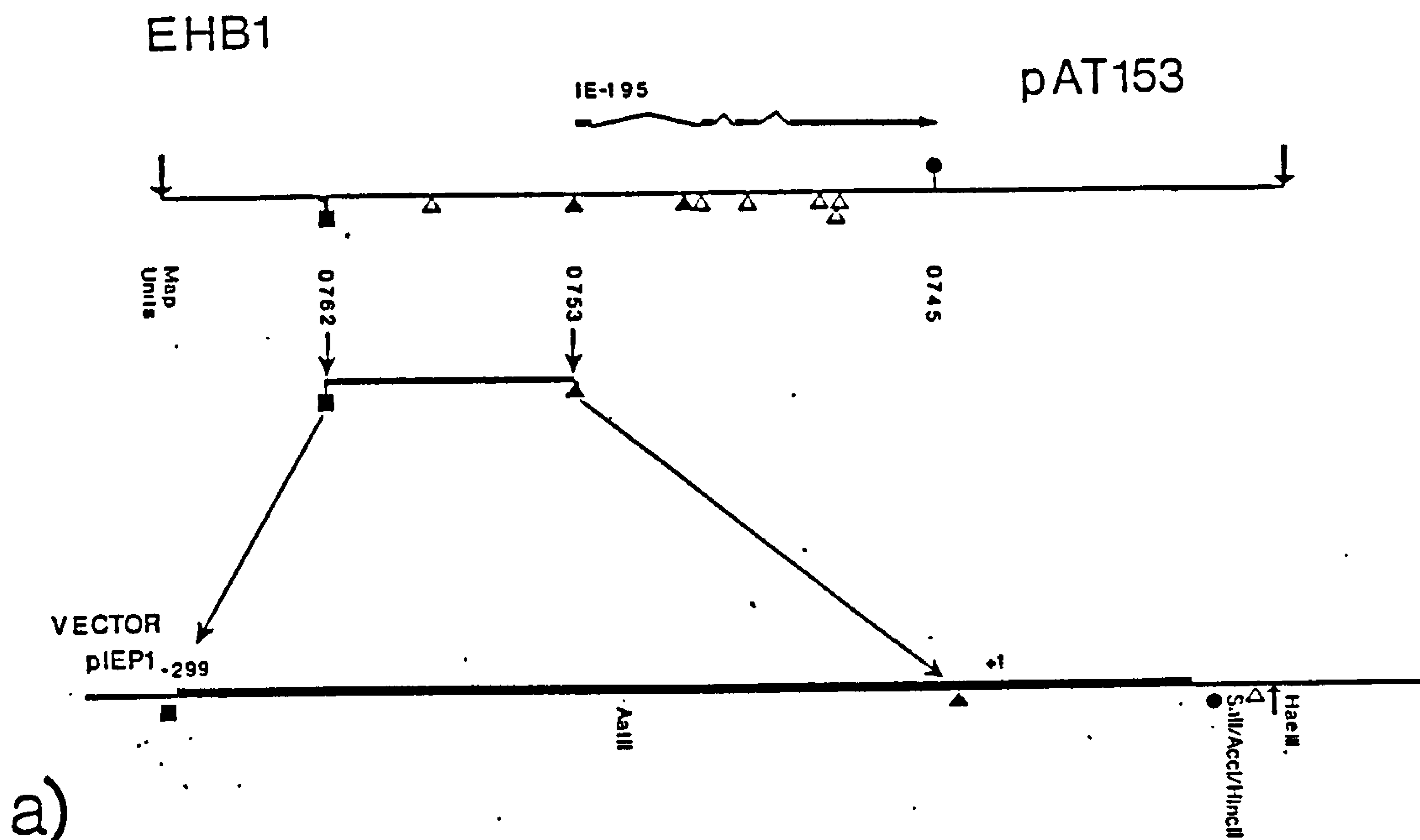
Fig 3:8: Effect of HCMV infection on expression from IEPTK1. △, IEP1-transfected cells; ▲, IEP1-transfected, HCMV-infected cells; O, IEPTK1-transfected cells; X, IEPTK1-transfected, HCMV-infected cells.

be immune to the effects of the repressor(s) which normally negatively regulates transcription of the major IE gene during the early and late phases of productive HCMV infections and to be stimulated by virus-induced *trans*-activating functions. The increase in TK enzyme activity in IEPTK1-transfected, HCMV-infected cells was clearly not due to virus infection stimulating the expression of cellular TK genes; this was shown by the low levels of TK activity detected in control cells which were transfected with vector DNA (IEP1) and infected with HCMV (Table 3:2; Fig 3:8).

The inability of infection with HCMV to repress transcription from IEPTK1 in transfected cells could be explained if the IE promoter-regulatory element contained in IEPTK1 did not containing the *cis*-acting signal required for transcriptional repression.

Time (h) p.t.	p.i.	IEP1	IEP1+ HCMV	IEPTK1	IEPTK1+ HCMV
48	24	2,496	2,525	10,681	10,631
72	48	3,496	3,429	18,869	33,885
120	96	5,194	2,939	10,615	120,147
168	144	4,909	3,192	7,076	68,814

Table 3:2: Assay of TK enzyme activity in IEPTK1-transfected cells. Each sample was assayed three times, the average calculated and the value obtained by assaying TK extraction buffer (5,646) subtracted. Results are given as c.p.m./10μl of cell extract. HCMV infections were performed at a m.o.i. of 1 p.f.u./cell.



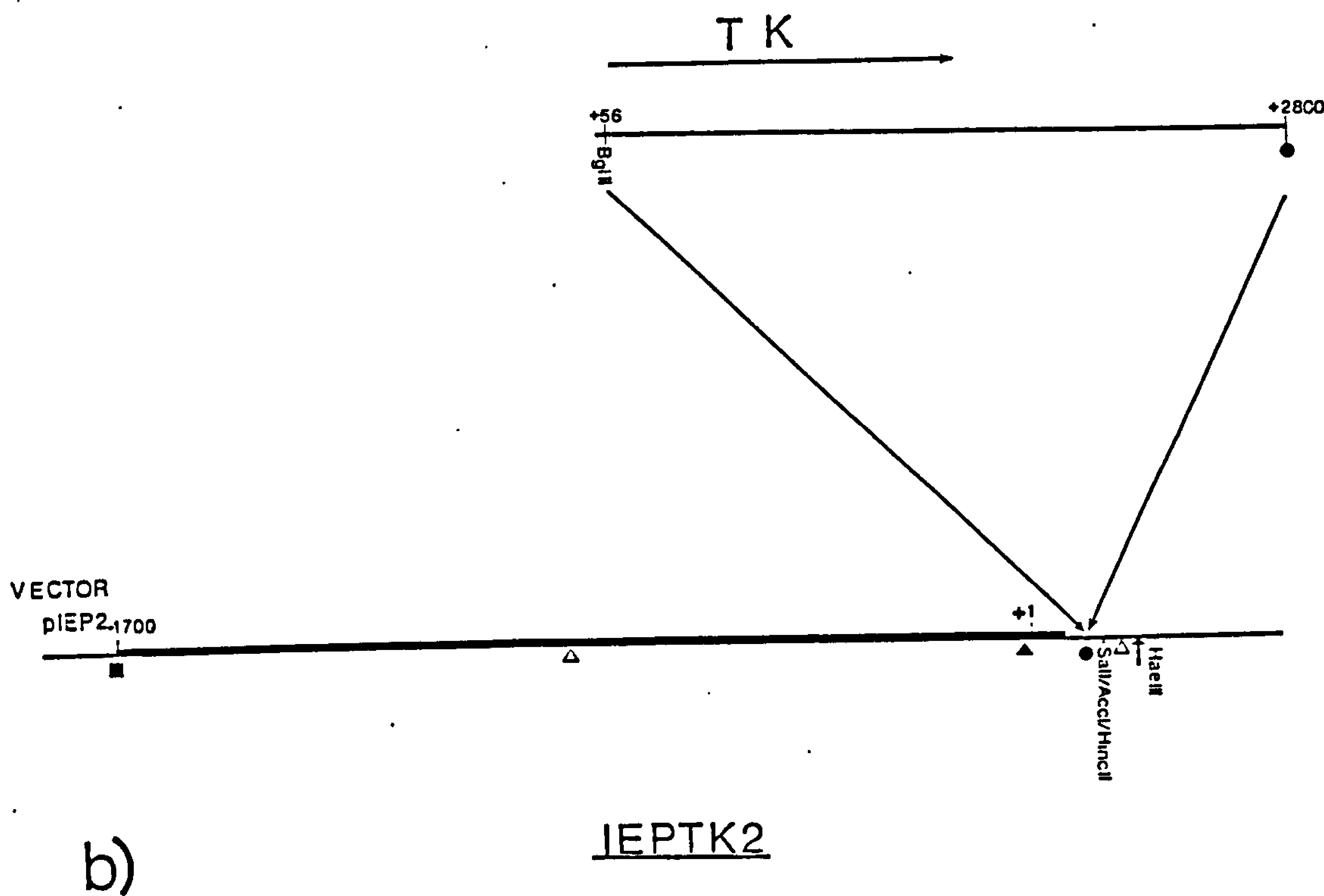


Fig 3:9: Construction of IEP2 and IEPTK2. (a) IEP2. EHB1 and IEP1 were each digested with both EcoRI and SstI and DNA fragments generated fractionated by electrophoresis in a 1% agarose gel. The 1.85kb EcoRI/SstI fragment (0.753-0.762 map units) was recovered from EHB1 digested DNA and the 2.7kb EcoRI/SstI fragment recovered from IEP1 digested DNA by electroelution. The two fragments were ligated and transformed into E. coli HB101. The net effect of this cloning was to extend the 5' IE promoter-regulatory sequence in IEP1 an additional 1.55kb (approximately). (b) The same methodology was used to insert the HSV-1 TK gene into IEP2 as was used to insert it into IEP1 (see Fig 3:7b). BamHI (●), EcoRI (■), HindIII (↓), PstI (△) and SstI (▲) sites are shown.

3:5 Expression From IEPTK2 in Transfected Cells

While the IE promoter-regulatory sequence present in IEPTK1 is sufficient to ensure constitutive expression of the TK structural gene, it extends only to 299bp upstream from the transcriptional start site. IEPTK1 may therefore lack important regulatory elements, e.g. the plasmid contains neither a complete copy of the promoter-associated 21bp repeat (Akrigg et al., 1985) nor the far upstream sequences which may be associated with the induction of transcription in differentiating EC cells (Nelson and Groudine, 1986).

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In the context of the conditions used in these experiments the presence of the additional 5' promoter-regulatory sequence in IEPTK2 relative to IEPTK1 did not confer on the transfected IE promoter the ability to be repressed by infection with HCMV.

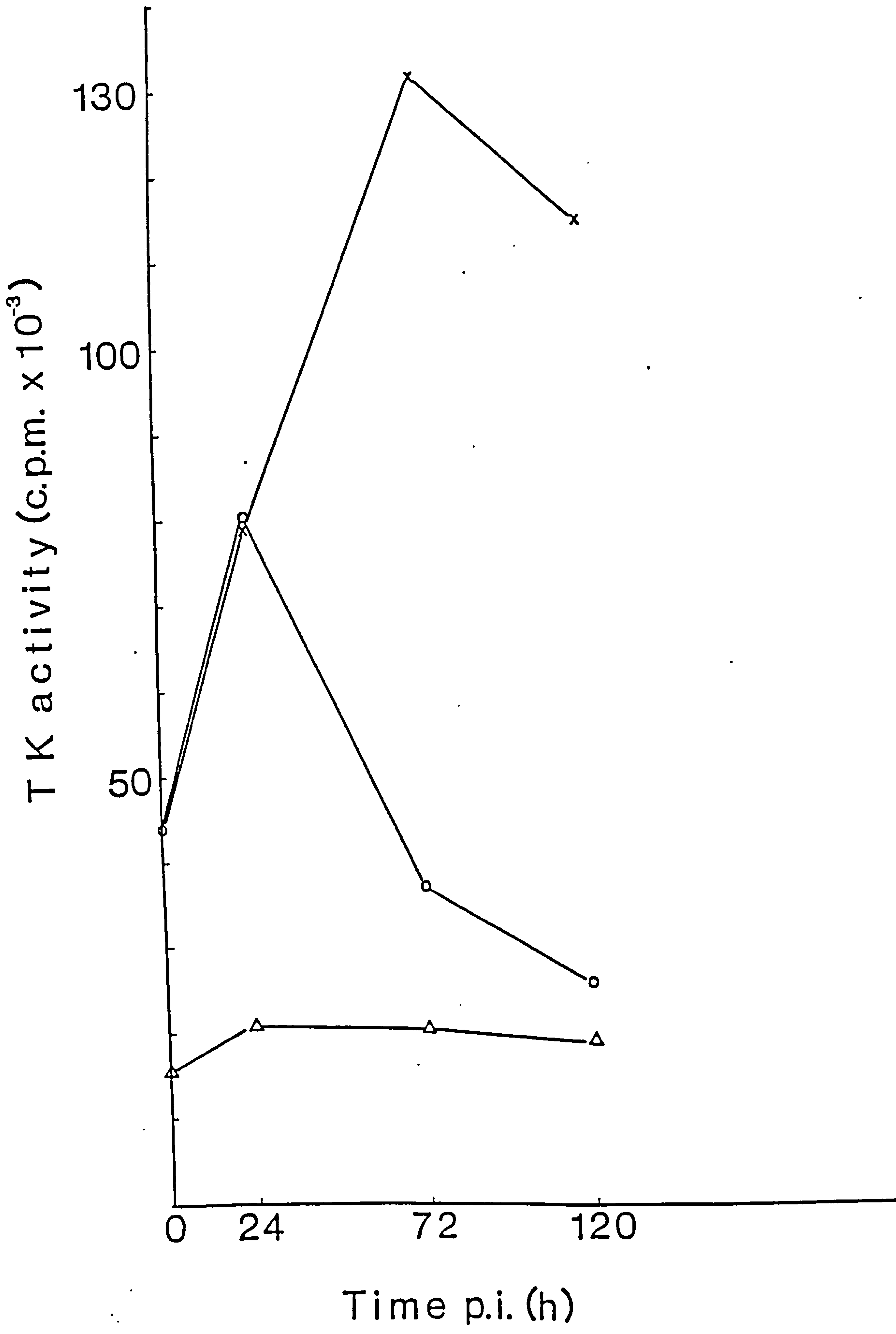


Fig 3:10: Effect of HCMV infection on expression from IEPTK2. △, IEP1-transfected cells; O, IEPTK2-transfected cells; X, IEPTK2-transfected, HCMV-infected cells.

Time (h) p.t.	p.i.	IEP1	IEPTK2	IEPTK2+ HCMV
24	0	15,787	44,237	-
48	24	22,446	81,320	79,453
96	72	21,892	37,194	132,748
144	120	19,951	25,768	115,705

Table 3:3: Assay of TK enzyme activity in IEPTK2-transfected cells. Each sample was assayed three times, the average calculated and the value obtained by assaying TK extraction buffer (19,361) subtracted. Results are given as c.p.m./10 µl of cell extract. HCMV infections were performed at a m.o.i. of 1 p.f.u./cell.

3:6 The Effect of U.V.-irradiated HCMV on Expression From IEPTK1

The transcription of a number of HSV-1 IE genes has been shown to be stimulated by a 65K phosphoprotein component of the tegument (Campbell *et al.*, 1984) which enters the cell during the infectious process. The HSV-1 and HCMV virions contain proteins with similar molecular weights and biochemical properties (section 1:10 & 1:22). In order to investigate whether the observed stimulation of IEPTK1 expression induced by HCMV infection of transfected cells was caused by a virion component or a *de novo* synthesised virus-encoded gene product IEPTK1 transfected cells were infected in parallel with either viable HCMV or virus which had been inactivated by irradiation with u.v.-light. At low dosage, u.v.-light cross-links thymidine dimers in the virus genome which prevents both HCMV replication and transciption but not virus adsorption into cells (Hirai *et al.*, 1977).

As described in section 2:1, 10 ml aliquots of a stock solution of virus were irradiated at 1770 $\mu W/cm^2$ with u.v.-light for 0s, 10s (177 $\mu J/mm^2$), 30s (531 $\mu J/mm^2$), 90s (1.59 mJ/mm^2), 5 min (5.31 mJ/mm^2), 15 min (15.9 mJ/mm^2) and 45 min (47.7 mJ/mm^2). Stocks of irradiated virus were then

stored in 1 ml volumes at -70 ^{o}C until required. The virus sample which had not been exposed to irradiation (0s) was titrated and shown to have an infectivity of 2 x 10^{7} p.f.u./ml. Each of the irradiated virus stocks (2 ml) was used to infect monolayers of MRC5 cells (in 50 mm petri dishes) which were then monitored for both plaque formation and the expression of the major IE polypeptide. Virus samples receiving less than 5.31 mJ/mm^{2} all produced a gross c.p.e. in MRC5 cells by 2 weeks p.i. Virus samples receiving 5.31 mJ/mm^{2} or more were unable to induce plaque formation by 4 weeks p.i. Expression of the HCMV IE antigen (6h p.i.) was detected in immunofluorescence experiments using a monoclonal antibody to the major IE protein (section 2:2). Nearly 100% of cells in monolayers infected with virus exposed to 0, 177 and 531 μJ/mm^{2} of u.v. light expressed the nuclear antigen compared to 20% of cells infected with virus exposed to 1.59 mJ/mm^{2} and 0% of cells infected with virus exposed to 5.31 mJ/mm^{2} or more. Irradiation with u.v. light for 5 min was sufficient to destroy the ability of the virus inoculum to induce either plaque formation or the synthesis of the major IE antigen. Since the object of irradiating the virus was to inhibit _de novo_ virus-encoded gene expression the inactivation of IE antigen expression is more directly relevant to the experiment than the loss of virus infectivity.

In order to maximise the transcriptional stimulation from the IE promoter high m.o.i.'s (2 or 20 p.f.u./cell), or the equivalent volume of irradiated virus, were used to infect cells transfected with IEPTK1. In this series of experiments stimulation of IEPTK1 expression by HCMV infection was apparent by 24h p.i.; expression being stimulated more strongly at this time with the higher m.o.i. (Table 3:4; Fig 3:11). The peak levels of IEPTK1 expression produced using a m.o.i. of 2 or 20 p.f.u./cell were similar and occurred during the late phase (72h p.i.) of the infection (Fig 3:11).

Irradiating virus with u.v.-light for 5 min (5.3 mJ/mm^{2}) prior to infection significantly reduced the stimulation of IEPTK1 expression,

Sample	Time (h p.i.) 0	24	48	72	144
IEP1	-	-	736	-	2,304
IEPTK1	266,496	123,051	32,308	31,641	18,558
IEPTK1 + 2 p.f.u./cell HCMV	-	295,643	334,007	705,099	320,267
IEPTK1 + 20 p.f.u./cell HCMV	-	447,384	695,152	829,345	269,551
IEPTK1 + 2 "p.f.u."/cell HCMV 5.31 mJ/mm^2	-	173,674	55,811	43,268	11,036
IEPTK1 + 20 "p.f.u."/cell HCMV 5.31 mJ/mm^2	-	240,746	89,360	66,782	41,021
IEPTK1 + 20 "p.f.u."/cell HCMV 47.7 mJ/mm^2	-	97,942	24,793	24,392	15,734

Table 3:4: Assay of TK enzyme assay in IEPTK1-transfected cells, effect u.v.-irradiated HCMV on expression from the IE promoter. Duplicate samples were each assayed three times, the averages calculated and the value obtained by assaying TK extraction buffer (14,983) subtracted. Results are given as an average value of TK activity in cpm/10 µl of cell extract. Samples giving values over 200,000 cpm were diluted and re-assayed to be brought into the linear range of the reaction. The amount of virus added to different samples is indicated. Where u.v.-treated HCMV was used the inoculum is described in terms of the infectivity of the equivalent volume of non-irradiated virus ("p.f.u."/cell) and the extent of exposure to u.v. light indicated.

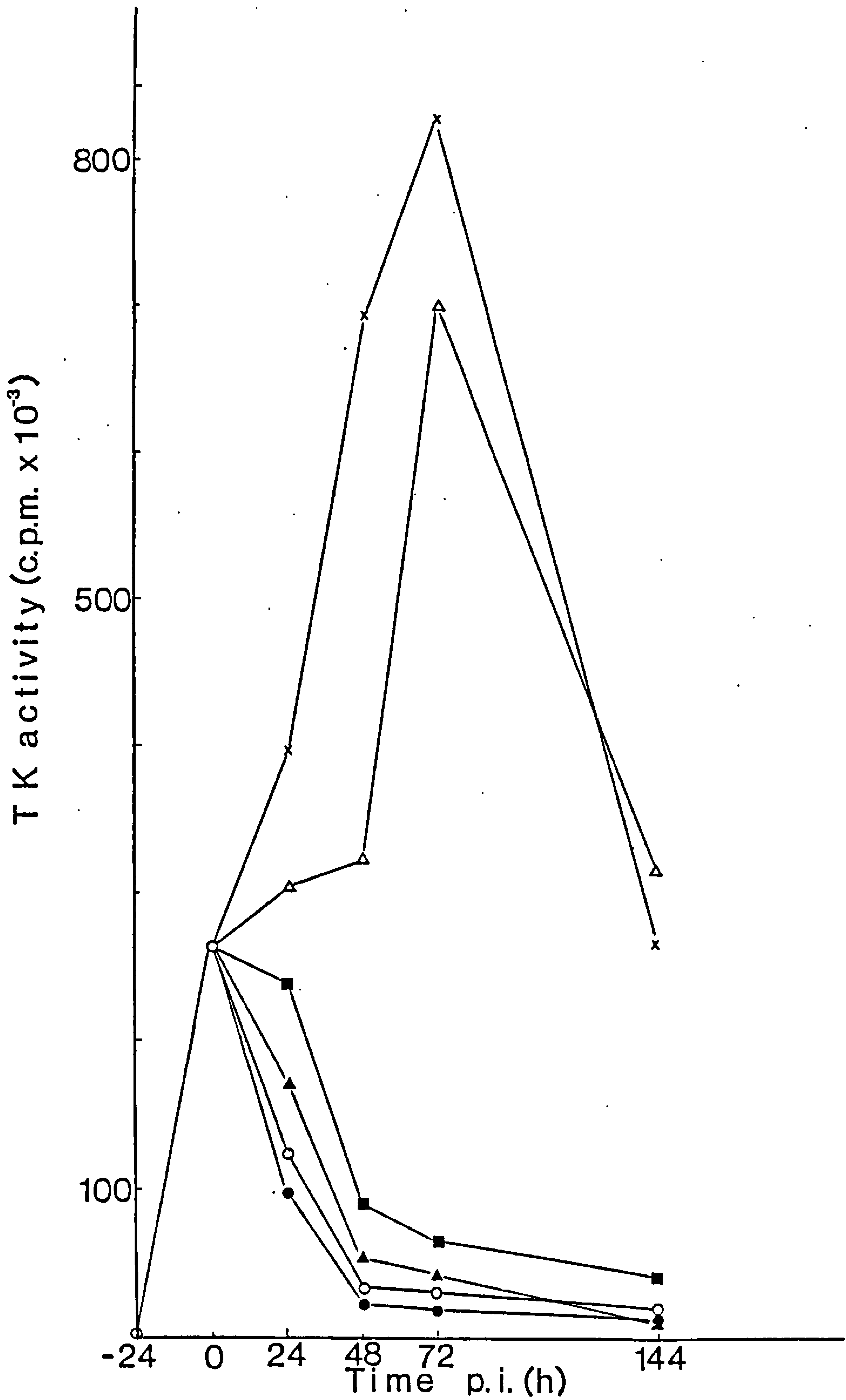


Fig 3:11: Effect of infection with u.v.-irradiated HCMV on expression from IEPTK1. All samples were from IEPTK1-transfected cells. ○, uninfected; △, infected with 2 p.f.u./cell of HCMV; X, infected with 20 p.f.u./cell; ▲, infected with the equivalent of 2 p.f.u./cell inactivated with 5.31 mJ/mm^2 of u.v. light; ■, infected with the equivalent of 20 p.f.u./cell inactivated with 5.31 mJ/mm^2; ●, infected with the equivalent of 20 p.f.u./cell inactivated with 47.7 mJ/mm^2.

especially during the later stages (from 48h p.i.) of the experiment (Fig 3:11). Infection with u.v.-irradiated (5.3 mJ/mm^2) virus did, however, produce a small (up to three fold) dosage-dependent increase in expression (Table 3:4; Fig 3:11). The infection of IEPTK1-transfected cells with virus which been irradiated with u.v.-light for 45 min (47.7 mJ/mm^2) did not detectably stimulate expression from the IE promoter; prolonged exposure to u.v.-light may prevent virus adsorption/infection by damaging components of the virion other than DNA.

The observed results are consistent with there being: (a) a (u.v.-resistant) structural component of the HCMV virion which stimulates the IE promoter and (b) a *de novo* synthesised HCMV-encoded *trans*-activator which stimulates the IE promoter in transfected cells. Since most of the stimulation in IEPTK1 expression was destroyed by u.v.-irradiating the virus prior to infection inactivated, the *trans*-activator would appear to exert the greater effect on the transfected IE promoter.

Although the expression of the IE polypeptide was not detected in cells infected with u.v.-irradiated (5 min) virus it is possible that limited undetected virus-encoded gene expression may have resulted in the *de novo* synthesis of a *trans*-activator function identical to that produced by infection with non-irradiated virus. The level of stimulation produced by u.v.-irradiated virus, however, remains constant during the time course (between 2-3 fold with the higher concentration of inactivated virus) and does not increase during the late phase as is the case with non-irradiated virus (Fig 3:11). The stimulation produced by irradiated virus was not caused by the presence in the inoculum of the HCMV-induced growth factor (Gonczol and Plotkin, 1984; see section 3:8).

During permissive HCMV infections transcription from the IE promoter is normally repressed after 6h p.i. The stimulation of IEPTK1 expression during the late phase of infection suggests that the transfected IE promoter is not efficiently repressed by the HCMV-induced repressor of IE transcription.

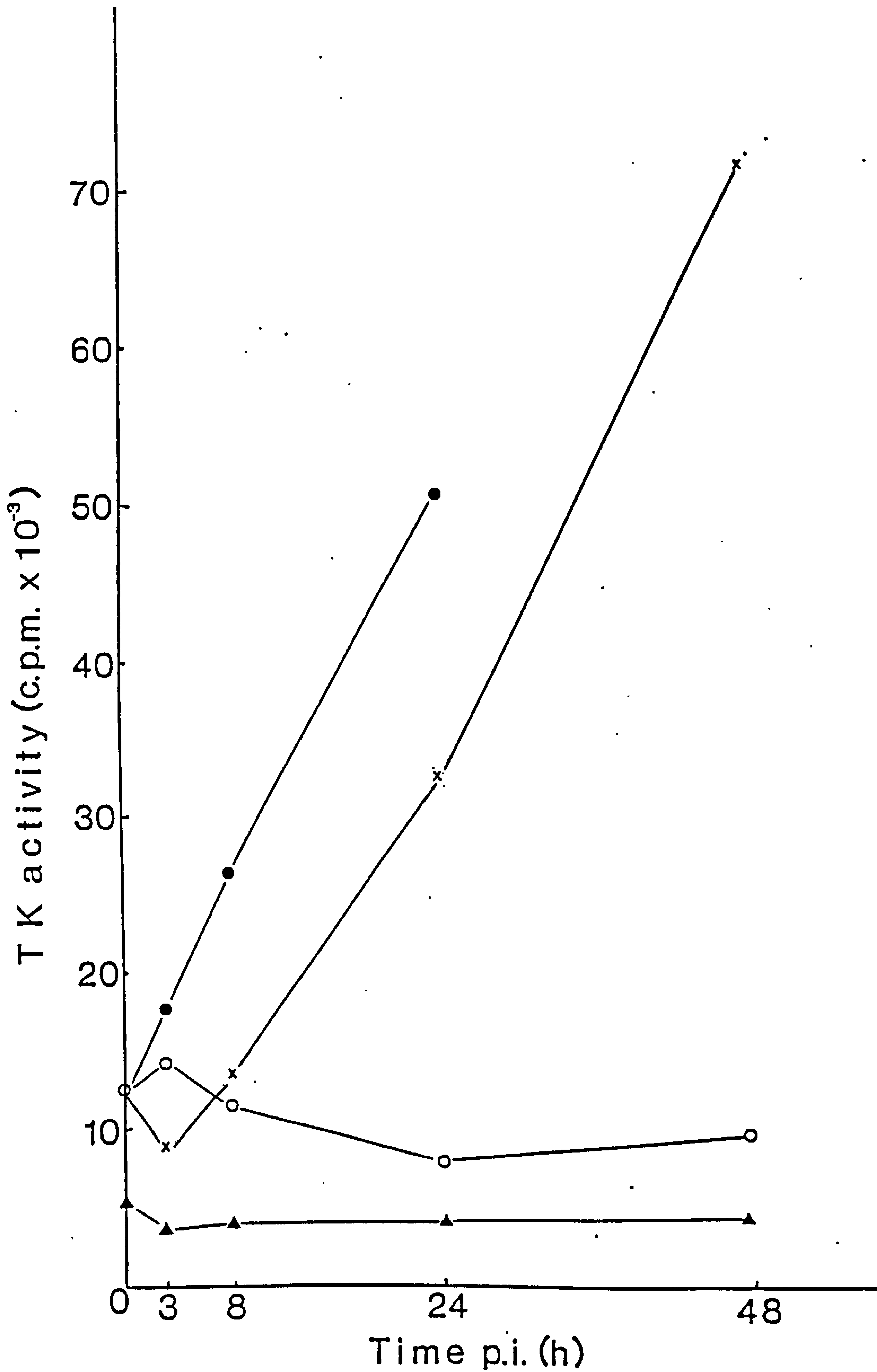


Fig 3:12: Effect of HSV-1 TK^{-} infection on expression from IEPTK1. ▲, pVUTK1-transfected cells; ○ , IEPTK1-transfected cells; X , IEPTK1-transfected, HCMV-infected cells; ●, IEPTK1-transfected, HSV-1 infected cells.

3:7 HSV-1 Infection Rapidly Stimulates IEPTK1 Expression

HSV-1 mutants with a defective TK gene have been shown to replicate efficiently in actively growing tissue culture cells (Jamieson et al., 1974). The availabilty of such mutants made it possible to investigate the effects of HSV-1 infection on expression from IEPTK1 in transfected cells.

In this series of experiments the plasmid pVUTK1 (kindly provided by Dr A. Akrigg) was included as a negative control. pVUTK1 contains the HSV-1 TK gene, under the contol of its own promoter, excised from the recombinant plasmid M2 (Wilkie et al., 1979) on a PvuII-generated DNA fragment and inserted into the unique HincII site in pUC19. The level of TK enzyme activity detected in extracts from pVUTK1-transfected cells was slightly above the background levels of the assay (Table 3:5). HSV-1 TK$^-$ infection of IEPTK1-transfected cells induced a slight increase in expression from the HCMV IE promoter as early as 3h p.i., an approximately two-fold stimulation at 8h p.i. and an approximately six-fold stimulation at 24h p.i. By 48h

Time (h p.i.)	pVUTK1	IEPTK1	IEPTK1 + HCMV	IEPTK1 + HSV-1
0	5,007	12,093	-	-
3	3,648	14,315	8,020	17,725
8	4,048	11,336	13,433	26,924
24	3,510	8,001	32,525	46,785
48	4,419	9,803	72,779	-

Table 3:5 Assay of TK enzyme activity in IEPTK1-transfected cells infected with either HCMV or HSV-1. Duplicate samples were assayed three times each, the average calculated and the value obtained by assaying TK extraction buffer (4,002) subtracted. Results are expressed as an average value of TK activity in cpm/10 µl of cell extract. An input m.o.i. of 4 p.f.u./cell was used for both viruses.

p.i. the HSV-1 infection had destroyed the cell monolayer so it was not possible to take meaningful samples. The HCMV IE promoter in transfected cells is stimulated by HSV-1 encoded trans-activating proteins and appears to escape negative regulation by the repressors of HSV-1 IE gene expression.

In an experiment performed in parallel, IEPTK1-transfected cells were infected with HCMV using a similar m.o.i. HCMV-induced stimulation of expression from the HCMV IE promoter was not detected, however, until 24h p.i. (Table 3:5; Fig 3:13), indeed at 3h p.i. HCMV infection produced a transient reduction in IEPTK1 expression. Although HCMV stimulated expression from IEPTK1 more slowly than HSV-1 TK^- higher levels of IEPTK1 expression were detected in HCMV-infected cells.

3:8 Stimulation of IEPTK1 Expression: The Effects of Metabolic Inhibitors and Heat Treatment of Virus

2-Deoxy-D-glucose and phosphonoformate (PFA) inhibit the growth of HCMV during the early phase of the replicative cycle. 2-Deoxy-D-glucose, a sugar analogue, inhibits HCMV DNA replication although not the synthesis of the viral DNA polymerase (Weder and Radsak, 1981) and PFA specificly inhibits the activity of the viral DNA polymerase (Wahren and Oberg, 1979). Experiments described above have shown that the large stimulation of IEPTK1 activity in DNA transfection studies is primarily associated with the late phase of the HCMV replicative cycle. These metabolic inhibitors were used to investigate at which phases the factor which stimulates expression from the transfected IE promoter was synthesised.

Even in the presence of PFA, HCMV infection induced a large stimulation of expression from the transfected IE promoter at 72h p.i. (Table 3:6). PFA did, however, suppress the HCMV-induced stimulation of expression from IEPTK1 by approximately 50% (Table 3:6) which indicates that although the virus-encoded trans-activator is produced during the early phase its activity is further enhanced after virus DNA replication. 2-Deoxy-D-glucose

appeared to inhibit the stimulation of expression from IEPTK1 by HCMV infection to a greater extent than PFA; it should be noted, however, that 2-deoxy-D-glucose had a marked cytotoxic effect on the infected cells.

The growth factor induced by HCMV infection of fibroblasts has been shown to be heat stable (Gonczol and Plotkin, 1984). The data contained in Table 3:6 shows that pre-incubating the HCMV seed virus at 56°C for 30 min prior to infection abolishes the stimulation of IEPTK1 expression in transfected cells. This result indicates that the stimultion of IEPTK1 expression is was not caused by the HCMV-induced growth factor.

Treatment	TK activity (cpm)
-	6,809
HCMV	101,142
HCMV + PFA (100 μg/ml)	47,818
HCMV + Deoxyglucose (10mM)	13,766
HCMV preincubated at 56 °C for 30 min	5,620

Table 3:6: The effect of various treatment on the ability of HCMV infection to stimulate expression from IEPTK1 in transfection experiments. Duplicate samples were assayed three times each, the average calculated and the value obtained from assaying TK extraction buffer (8,466) subtracted. Results are expressed as an average of TK enzyme activity in cpm/10 μl of cell extract. HCMV infections were performed at a m.o.i. of 1 p.f.u./cell and samples harvested 72h p.i.

3:9 HCMV IE Gene Expression in Cells Transfected With IEP11assaterm

It has been reported that the major IE gene product, either directly or indirectly, represses transcription from its own promoter (Stenberg and Stinski, 1985). The proposed autoregulation of the major IE gene during productive infections provides a possible mechanism to explain why HCMV infection stimulates expression from the major IE promoter (in IEPTK1) in transfected cells (section 3:4). In transfection experiments, DNA associated with precipitated calcium phosphate crystals was applied to cell monolayers (section 2:4). A feature of this methodology is that when a single plasmid is used the transfected cells receive a very large number of copies of that plasmid (Loyter et al., 1982). It is possible that the large copy number of the transfected IE promoter may saturate essential cellular transcription factors and thus inhibit expression from the HCMV major IE gene following virus infection. Hypothetically, in the absence of repressor synthesis, expression from the transfected IE promoter could be stimulated by HCMV-encoded trans-activators.

In order to monitor expression from transfected plasmids in individual cells a reporter gene was used which could be detected immunologically. IEP11assaterm, kindly provided by Dr C. Clegg, consists of the structural gene of the lassa virus nucleocapsid protein expressed under the control of the HCMV IE promoter (identical to the promoter element in IEPTK1) plus a 3' polyadenylation signal, also derived from the HCMV major IE gene, inserted into a pUC7 based vector (Fig 3:13). To determine whether the HCMV major IE gene is expressed in cells containing high concentrations of the IE promoter the plasmid IEP11assaterm was transfected into MRC5 cells which were then infected with HCMV. Immunofluorescence was used to assay for the expression of both the lassa virus nucleocapsid protein and the HCMV major IE protein in the same cell. Expression of the lassa virus nucleocapsid protein was detected using a specific hyperimmune sera raised in guinea pig as the first antibody and fluorescein-conjugated goat anti-guinea pig IgG as the second

antibody; this produces a green cytoplasmic fluorescence in IEP1lassaterm transfected cells as shown in Fig 3:14a. The nucleocapsid protein is also detected in the cytoplasm of cells infected with lassa virus. Expression of the HCMV major IE polypeptide in virus-infected cells was detected using the monoclonal antibody L-14 (Rice et al., 1984) as the first antibody and rhodamine-conjugated goat anti-mouse antibody as the second; expression of the IE antigen produces the red nuclear fluoresence in HCMV infected cells seen in Fig 3:14b.

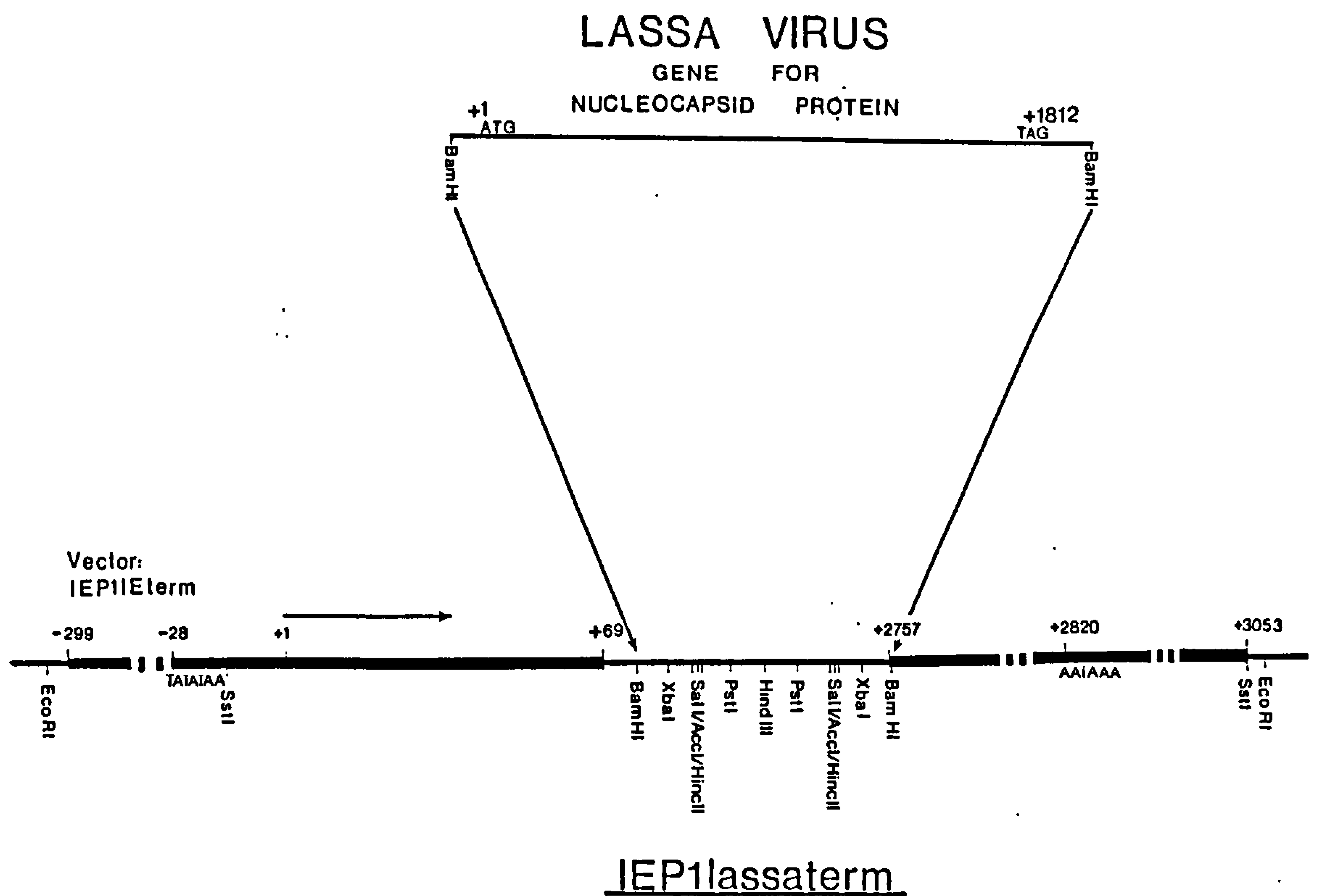
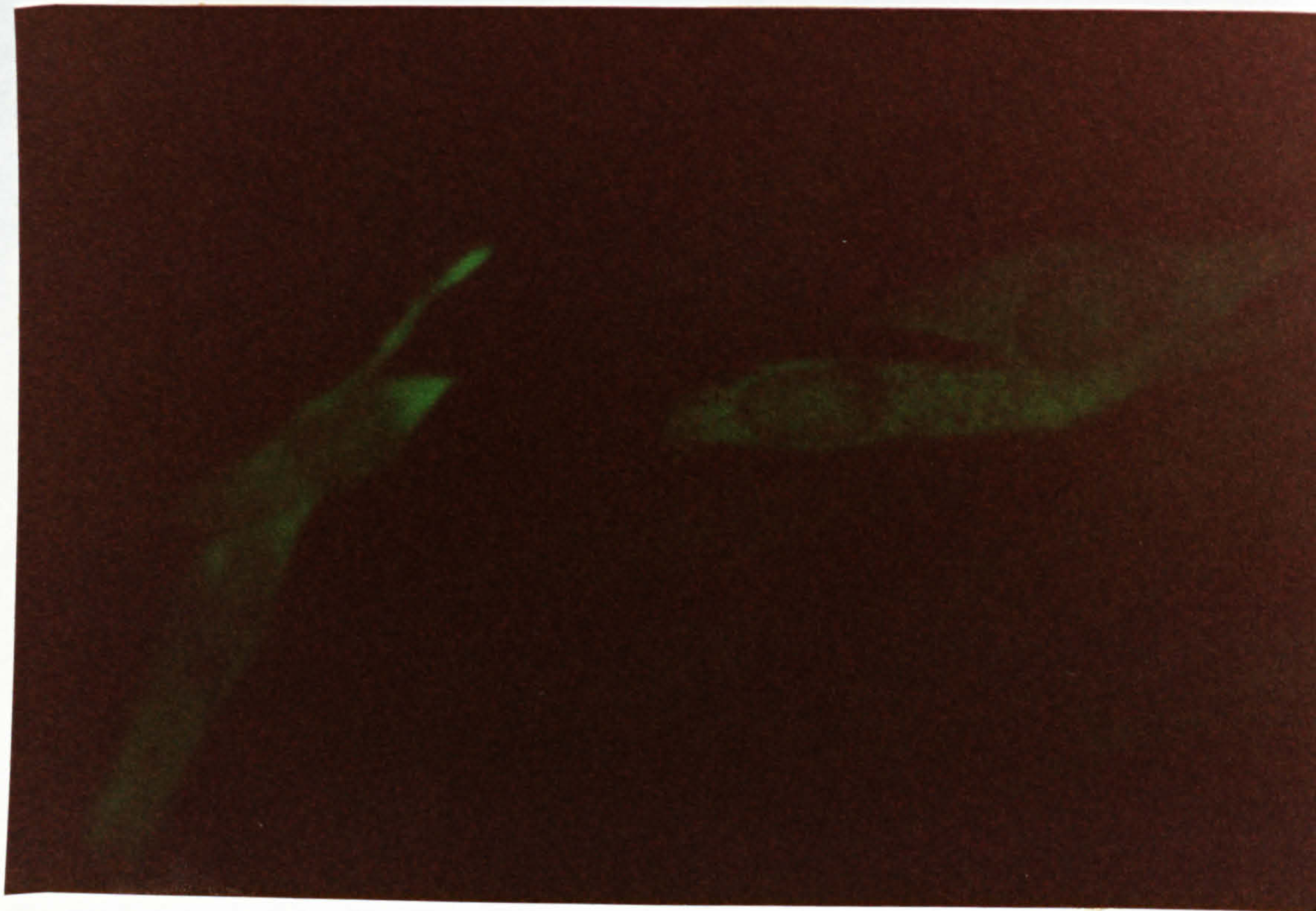
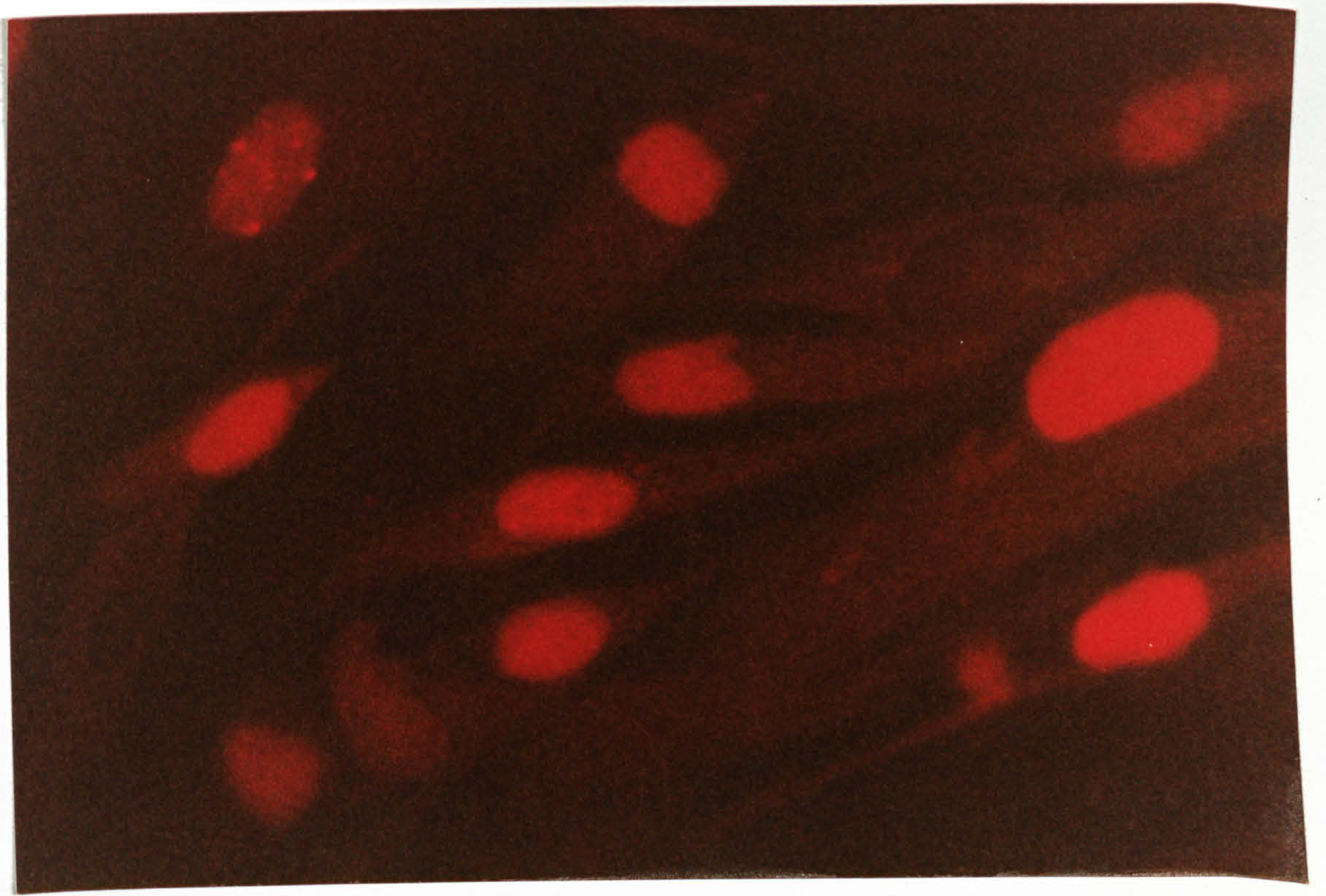


Fig 3:13: Construction of IEP1lassaterm. The vector IEP1IEterm was kindly provided by Dr A. Akrigg. The polyadenylation signal (+2757 to +3053 with respect to the transcriptional start site) was excised from EHB2 (see Fig 3:1) with BamHI/Sstl and cloned into the same unique sites in M13mp11. IEP1IEterm was produced by excising the IE promoter from IEP1 and the IE polyadenylation signal from the M13mp11 recombinant both on EcoRI/HindIII fragments and ligating them together into pUC7. IEP1lassaterm, kindly provided by Dr C. Clegg, was produced by excising the lassa virus nucleocapsid gene from pLS120 (Clegg and Oram, 1985) on a BamHI fragment and cloning it between the BamHI sites in IEP1IEterm as shown. The BamHI fragment from pLS100 contains approximately 100bp of sequence 5'to the translation initiation codon and approximately 40bp of sequence 3' of the translation termination codon.

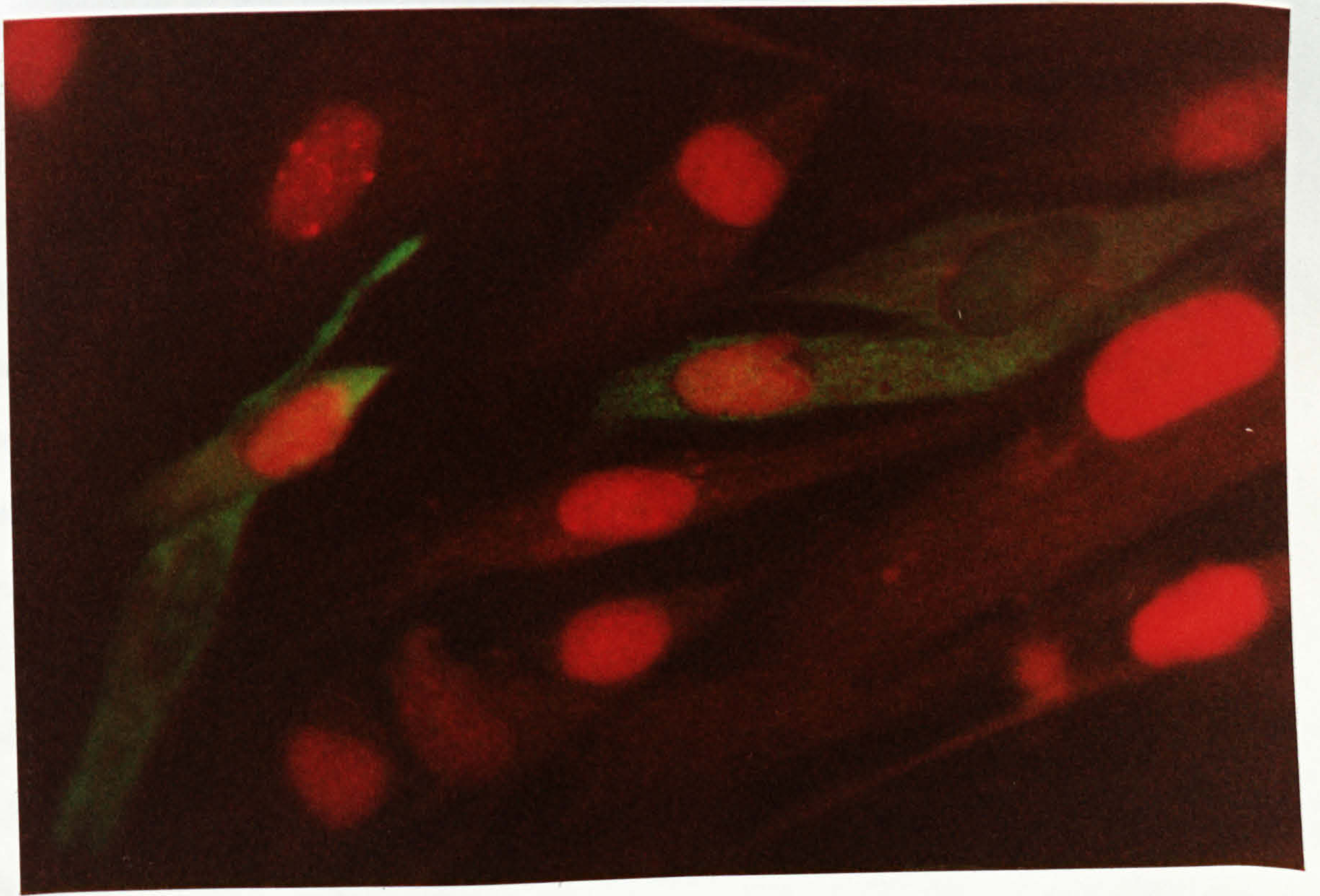


a

Fig 3:14: HCMV IE gene expression in cells transfected with IEP1lassaterm DNA. MRC5 cells in 50 mm diameter petri dishes were transfected with 5 µg of IEP1lassaterm DNA. 18h post transfection the cells were washed once with TBS, once with complete media and incubated a further 6h. The monolayer was then infected with HCMV at a m.o.i. of 10 p.f.u./cell. Cells, grown on glass coverslips, were removed 6h p.i. and immunofluorescence carried out as described in the text and in section 2:2. The figure illustrates the same field photographed (a) with a green filter to show the cytoplasmic fluorescence produced by the lassa virus nucleocapsid protein, (b) with a red filter to show the nuclear fluorescence produced by the HCMV major IE antigen and (c) a double exposure with first the red and then the green filter which shows the same cell can express both antigens.



b



c

Cells transfected with IEP11assaterm and infected with HCMV can express both the lassa virus cytoplasmic antigen and the HCMV nuclear IE antigen simultaneously (Fig 3:14c). Clearly the HCMV major IE gene product is expressed in cells transfected with IEP11assaterm and, therefore, presumably also in cells transfected with IEPTK1. Consequently the stimulation of expression from the IE promoter in transfected cells by HCMV infection cannot be attributed to the major IE polypeptide, the proposed repressor of the IE promoter, not being synthesised.

3:10 The Relationship Between The Concentration of The IE Promoter in Transfected Cells and its Stimulation by Infection With HCMV

When introduced into cells by DNA transfection, expression from the IE promoter was unexpectedly found to be stimulated between 24-72h p.i. by a *de novo* synthesised, HCMV-encoded *trans*-activator (sections 3:4, 3:5 and 3:6). Stenberg and Stinski (1985) recently proposed that the HCMV major IE polypeptide itself is involved in negatively regulating IE transcription. Immunofluorescence studies (section 3:9) have shown that the major IE polypeptide is expressed following HCMV infection of cells transfected with the IE promoter. The high copy numbers of the IE promoter in transfected cells, therefore, does not completely saturate transcription factors necessary for the expression of the major IE gene product from the viral genome. It is possible, however, that high concentrations of the transfected IE promoter may be sufficient to saturate the HCMV-induced repressor of IE transcription (RIT). The copy number of the IE promoter in transfected cells can be lowered simply by reducing the concentration of the plasmid containing the IE promoter in transfection mixtures. The relationship between the concentration of the IE promoter in transfected cells and its stimulation by infection with HCMV was therefore investigated.

Lowering the concentration of IEPTK1 DNA used in transfection mixtures produced a concomitant decrease in the level of constitutive expression

detected from the IE promoter in transfected cells (Table 3:7). The direct relationship between the concentration of the IEPTK1 plasmid and the level of expresson from the reporter gene supports the observation that the IE promoter was not completely saturating essential transcription factors in transfected cells within the range of concentrations used (section 3:9). However, three-fold increases in the concentration of IEPTK1 DNA transfected into cells resulted in only an approximately two-fold increases in expression from the IE promoter. In uninfected cells the rate of expression from the IEPTK1 would appear to be dependent not only on the concentration of the IE promoter but also on some constraint exerted on the availibility of cellular factors required for efficient expression.

Amount of IEPTK1 in Transfection mix (µg DNA)	IEPTK1	IEPTK1 + HCMV	Stimulation by HCMV
10	18,371	840,979	x 45
3	8,840	205,534	x 23
1	4,013	56,685	x 14
0.3	2,497	23,093	x 9
0.1	2,023	3,866	x 1.9
0.03	2,239	4,049	x 1.8
0	736	-	-

Table 3:7 Relationship between the concentration of IEPTK1 DNA in transfections and stimulation of expression from the IE promoter by HCMV infection. Duplicate samples .were assayed three times each, the average calculated and the value obtained by assaying TK extraction buffer (3051) subtracted. Results are expressed as cpm/10 µl of cell extract. Samples giving values over 100,000 cpm were diluted and reasssayed to be brought into the linear range of the reaction. Each transfection mixture containing 20 µg of DNA, the remainder being made up by the pUC8, was split into four equal portions. 5 µg of DNA was applied to each 50 mm diameter petri dish containing MRC5 cells. HCMV infection was performed using a m.o.i. of 4 p.f.u./cell and samples harvested 72h p.i. (96h p.t.).

Although the results of this experiment again showed that expression from the transfected HCMV IE promoter was stimulated by infection with HCMV, as the concentration of IEPTK1 DNA per transfection was lowered so was the relative stimulation of expression from the IE promoter by HCMV infection (represented by the stimulation index in Table 3:7). The level of stimulation was 45-fold with 10 μg IEPTK1/transfection, 23-fold with 3 μg and 14-fold with 1 μg. At a concentration of IEPTK1 DNA per transfection of 0.3 μg or lower, the level of TK enzyme activity in transfected cells was not sufficiently above background values for the enzyme assay to be significant. It was noticable that three-fold increases in the amount of IEPTK1 DNA transfected also resulted in approximately three-fold increases in the level of IE gene expression in HCMV-infected cells. Expression from the transfected IE promoter, in infected cells, therefore appeared to be limited by the concentration of the DNA template.

The results obtained were consistent with the hypothesis that high concentrations of the transfected IE promoter were saturating the RIT function in HCMV-infected cells. However, due to the limited sensitivity of the system used to assay expression from the IE promoter it was not possible to determine whether HCMV infection would repress expression from the IE promoter if the concentration of IEPTK1 DNA used in transfections was reduced sufficiently. In an endeavour to provide a more sensitive assay for IE promoter activity the experiment was repeated, this time using CAT in place of TK as the reporter gene. The bacterial CAT gene is widely used to monitor expression from eukaryotic promoters because an extremely sensitive assay has been devised to measure its activity (section 2:23) and, since eukaryotic cells do not encode an equivalent of CAT, the background levels in the assay are extremely low. The plasmid IEP1catIEterm, provided by Dr A. Akrigg, contains the CAT structural gene under the control of the HCMV IE promoter (identical to the IE promoter element in IEPTK1) and a 3' polyadenylation signal also obtained from the HCMV major IE gene (Fig 3:15).

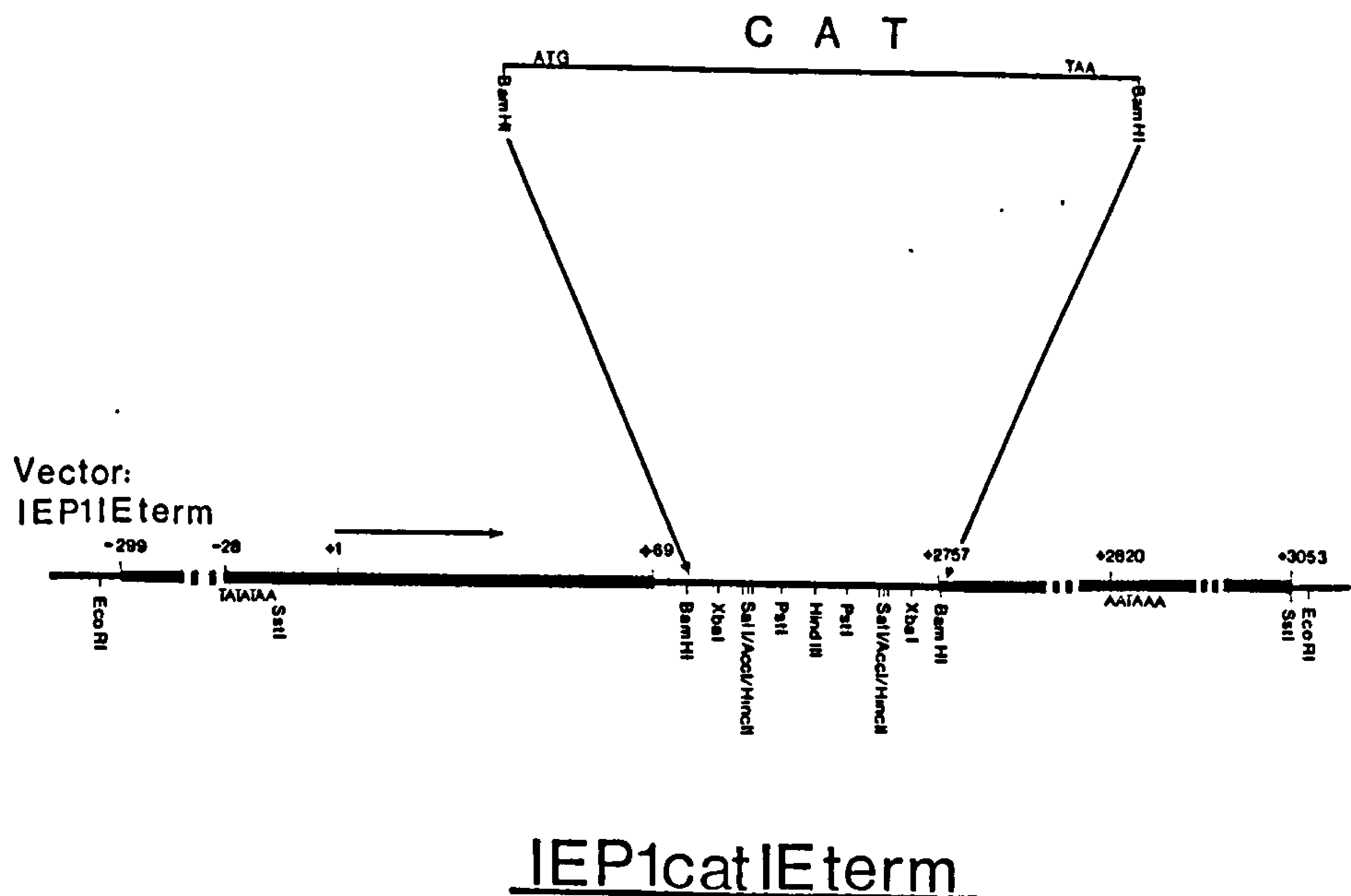
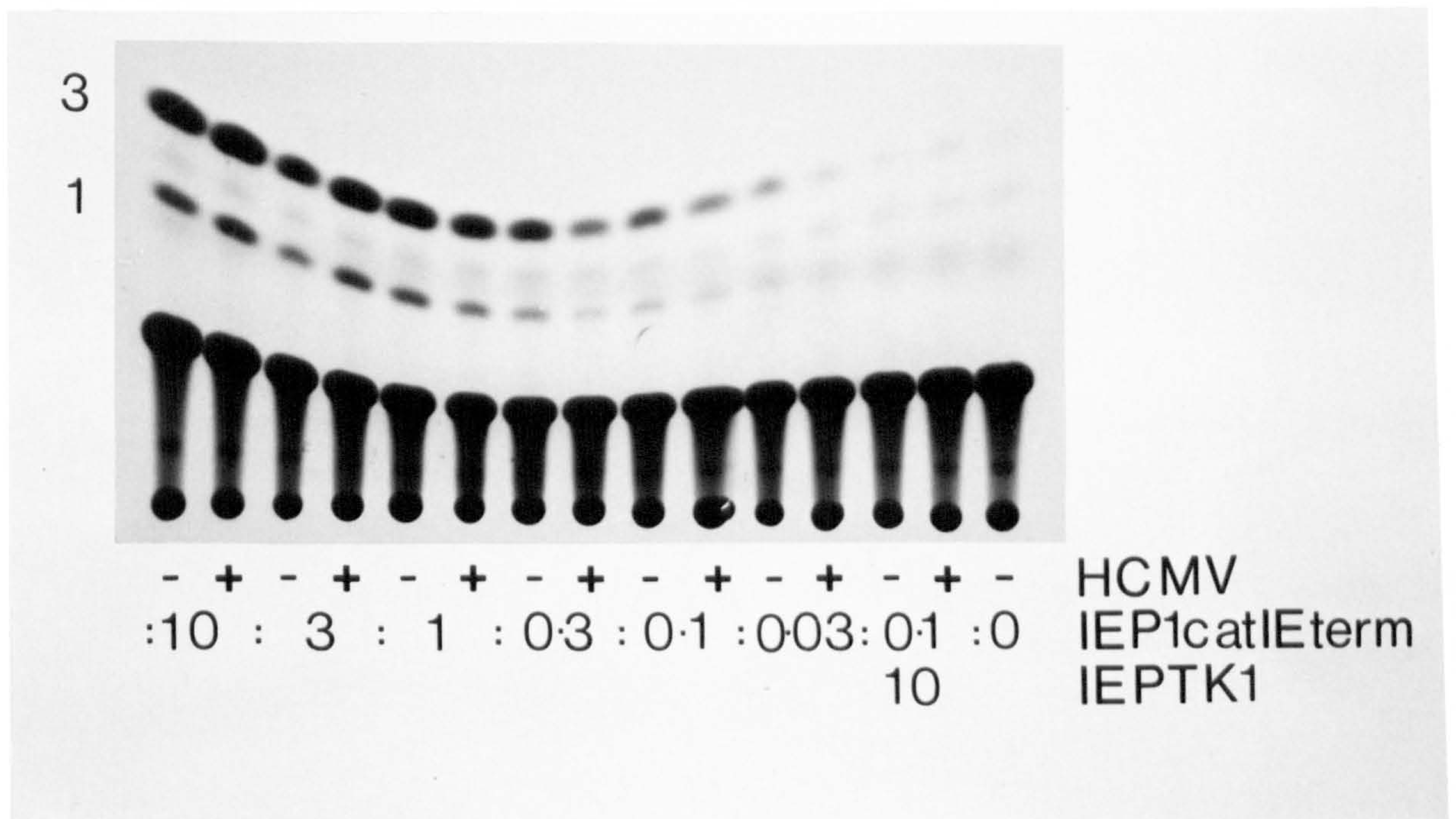


Fig 3:15: Construction of IEP1catIEterm. The plasmid IEP1catIEterm, provided by Dr A. Akrigg, was produced by excising the CAT structural gene from the plasmid pCAT (kindly provided by Dr D. Gaffney) on a BamHI fragment which was then cloned between the BamHI sites in the vector IEP1IEterm as shown in the diagram. The construction of IEP1IEterm is described in Fig 3:13.

The results of the CAT assay shown in Fig 3:16 indicate that at high DNA concentrations (10 and 3 μg per transfection) expression from IEP1catIEterm was stimulated 72h p.i. with HCMV. At a concentration of 1 μg IEP1catIEterm DNA per transfection HCMV infection did not appreciably affect the level of expression and at lower concentrations (0.3, 0.1 and 0.03 μg of IEP1catIEterm DNA per transfection) infection with HCMV caused a significant reduction in expression from the IE promoter. When 10 μg of IEPTK1 DNA and 0.1 μg of IEP1catIEterm were co-transfected into MRC5 cells infection with HCMV no longer repressed but stimulated expression from IEP1catIEterm (Fig 3:16). The results of these experiments indicate that when the IE promoter is introduced into MRC5 cells in low copy numbers it is susceptible to an HCMV-induced repressor of IE gene expression, but when it is present in high copy numbers the transfected IE promoter is no longer repressed by virus infection but is stimulated by an HCMV-induced



IEP1catIEterm (µg DNA)	IEPTK1 (µg DNA)	HCMV	CAT ACTIVITY (c.p.m./µg protein)	CAT ACTIVITY - CONTROL (c.p.m./µg protein)
10	0	-	506.5	503.0
10	0	+	832.1	828.6
3	0	-	247.7	244.2
3	0	+	415.0	411.5
1	0	-	286.7	283.2
1	0	+	231.7	228.2
0.3	0	-	220.5	217.0
0.3	0	+	70.2	66.7
0.1	0	-	97.5	94.0
0.1	0	+	49.8	46.3
0.03	0	-	48.8	45.3
0.03	0	+	14.0	10.5
0.1	10	-	7.1	3.6
0.1	10	+	22.9	19.4
0	0	-	3.5	

Fig 3:16: Titration of the concentration of IEP1catIEterm DNA in transfection experiment; the effect of HCMV infection on CAT expression. Transfection mixtures containing 10, 3, 1, 0.3, 0.1 or 0.03 µg of IEP1catIEterm DNA were added to MRC5 cells as indicated in the figure. One transfection mixture also contained 10 µg of IEPTK1 DNA. The DNA content in each transfection mixture was made up to 20 µg by the addition of pUC18 vector DNA. 5 µg of DNA from each transfection mixture was added to two 50 mm diameter petri dishes, one of which was subsequently infected with HCMV at a m.o.i. of 4 p.f.u./cell. Cells were harvested 72h p.i. (96h p.t.). - indicates samples taken from uninfected cells and + samples taken from infected cells. The acetylated products of the reaction, 1-acetyl chloramphenicol (1) and 3-acetyl chloramphenicol (3), are labelled. The sample containing 0 IEP1catIEterm was a negative control performed using an extract prepared from untransfected MRC5 cells. In quantifying the results the value obtained for this control was subtracted from all others.

trans-activator. Although infection with HCMV was able to reduce the level of expression from expression from IEP1catIEterm DNA, in none of the samples was expression from the transfected IE promoter totally repressed (Fig 3:16). It was observed in immunofluorescence experiments, similar to those described in section 3.9, that following a high m.o.i. with HCMV only 52% of MRC5 cells transfected with the plasmid IEP1lassaterm expressed the HCMV major IE antigen 6h p.i. as compared with 78% of untransfected control cells. In view of the inefficiency with which HCMV infects cells exposed to DNA transfection, it is perhaps not surprising that only limited repression of expression from IEP1catIEterm could be detected.

While the repression of expression from IEP1catIEterm produced by infection with HCMV was reproducible, the concentration of IEP1catIEterm per transfection at which repression could be detected was dependent on the efficiency of transfection. At a concentration of 0.3 μg per transfection, expression from the plasmid IEP1catIEterm is inhibited by infection with HCMV whereas at the same concentration IEPTK1 expression was stimulated nine-fold by virus infection (Fig 3:16; Table 3:7). The different results obtained in these two experiments may be attributable simply to a difference in transfection efficiency. However, although the promoter elements of the two plasmids are identical IEP1catIEterm contains an additional 233bp of sequence from the 3' end of the major IE gene. The presence of this downstream element is known to affect expression from the IE promoter. The plasmids IEP1catIEterm and AccHincat are essentially identical except that the polyadenylation signal in AccHincat is derived from SV40 (Fig 3:17a), but in transient DNA transfection experiments expression from IEP1catIEterm is consistently five-fold higher than that from AccHincat (A. Akrigg, personal communication). The mechanism by which the the 3' end of the IE gene stimulates expression has yet to be determined. The possiblity that the HCMV IE gene downstream sequence may be involved in negatively regulating expression from the IE promoter was therefore investigated.

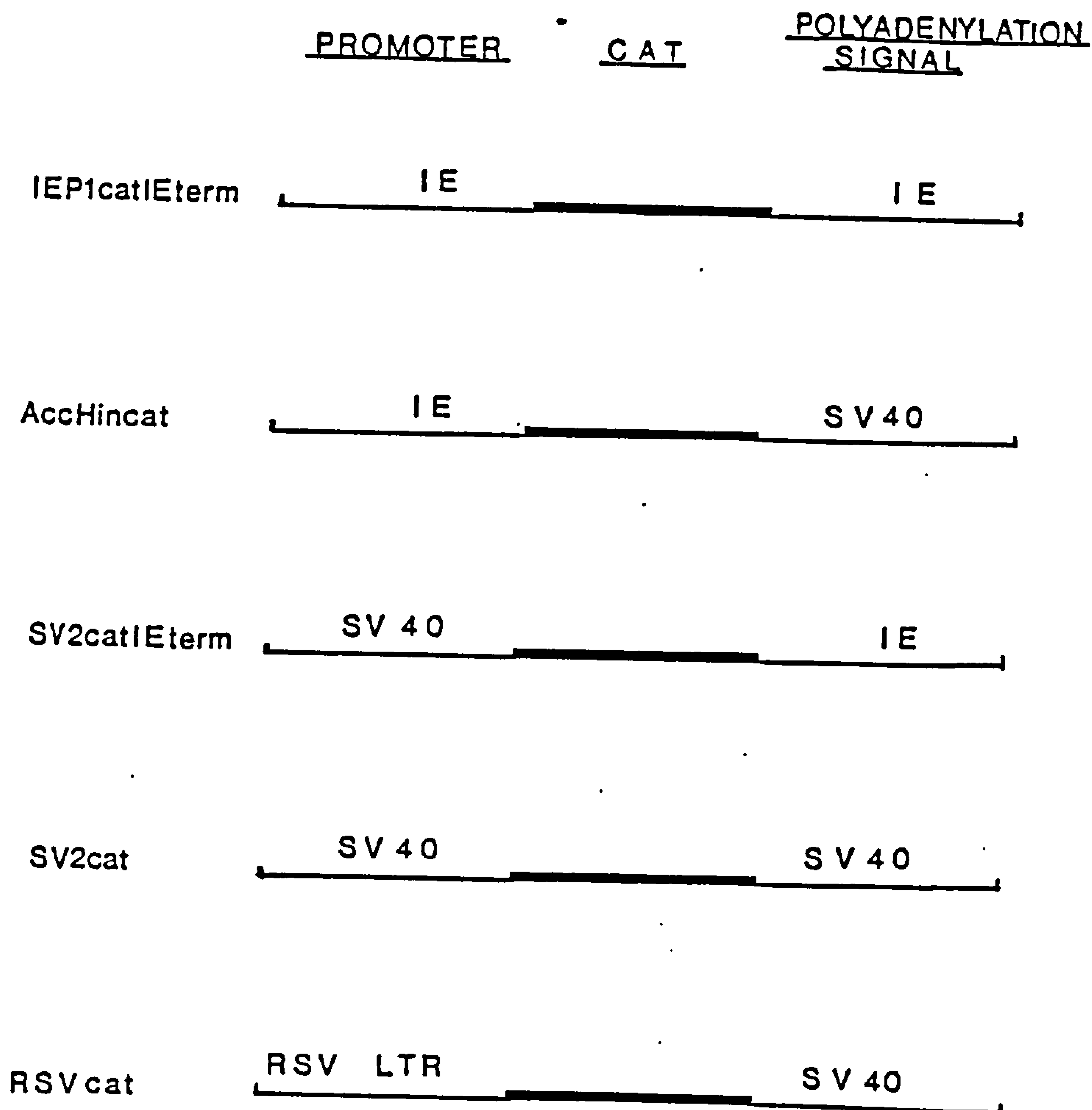
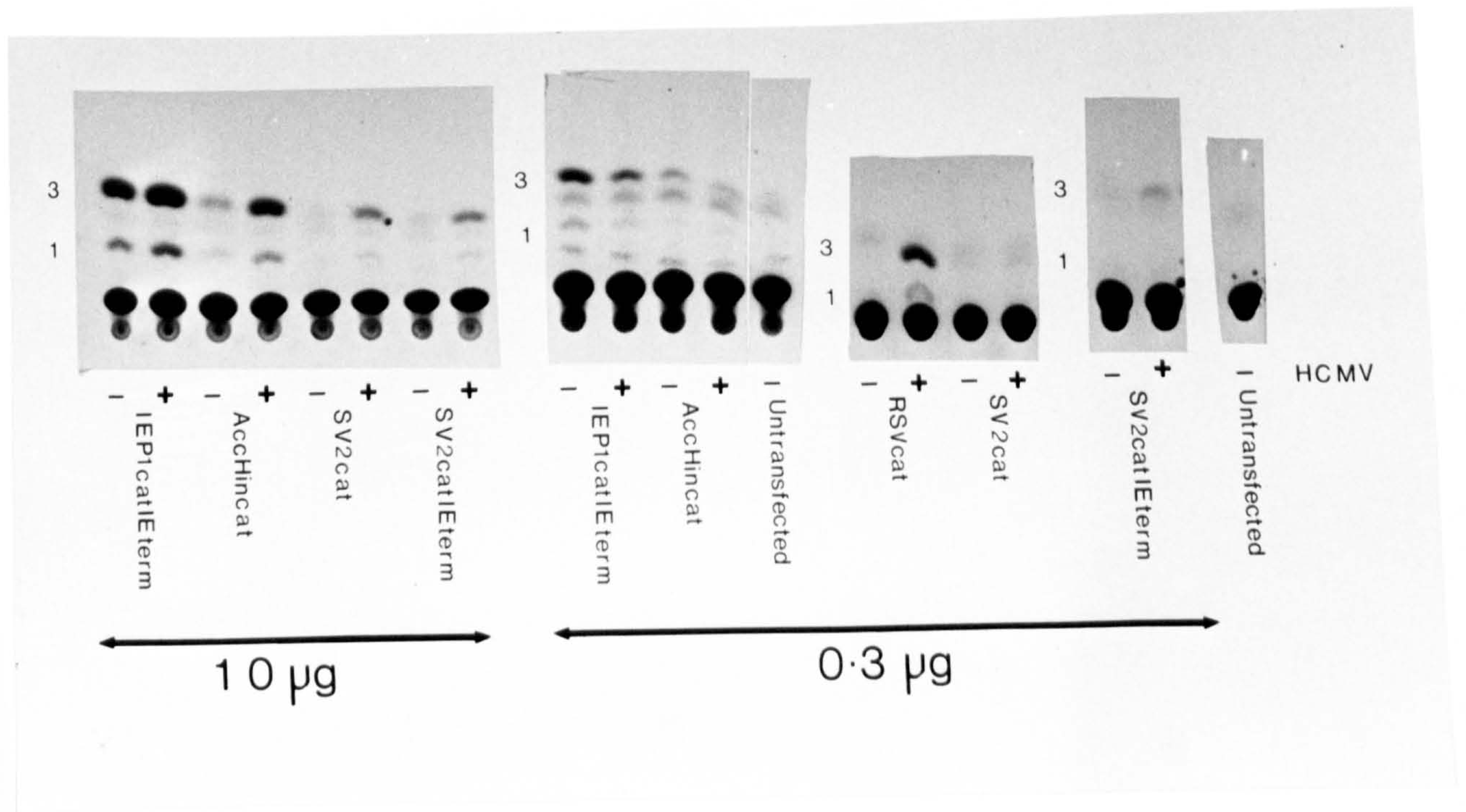


Fig 3:17: Effect of HCMV infection on expression from the plasmids IEP1catIEterm, AccHincat, SV2cat, SV2catIEterm and RSVcat in transfection experiments. (a) Schematic representation indicating the relevant details of the plasmids used. IEP1catIEterm, AccHincat and SV2catIEterm were kindly provided by Dr A. Akrigg, The construction of IEP1catIEterm is described in Fig 3:15, AccHincat was produced by replacing the SV40 promoter from SV2cat (excised by digestion with *AccI* and *HindIII*) with the IE promoter from IEP1 (on an *EcoRI*/*HindII* fragment). SV2catIEterm was produced by replacing the SV40 polyadenylation site in SV2cat with the HCMV IE polyadenylation signal in IEP1catIEterm using convenient EcoRI site in both plasmids. Details of the construction of SV2cat and RSVcat is given by Gorman *et al.* (1982a; 1982b). (b) Transfection mixtures containing 10 µg or 0.3 µg of IEP1catIEterm, AccHincat, SV2cat, SV2catIEterm or RSVcat DNA were prepared; the final DNA content being made up to 20 µg with pUC18 DNA. For each transfection 5 µg of DNA was added to two 50 mm diameter petri dishes containing MRC5 cells, one of which was infected with HCMV (4 p.f.u./cell) 24h later. Cells were harvested 72h p.i. and assayed for CAT activity. Enzymes assays for cells transfected with 0.3 µg SV2cat, SV2catIEterm and RSVcat were incubated at 37°C for 2h, other samples were incubated at 37°C for 30 min. Films were exposed for different times (16h or 5 days) during autoradiography in order to optimise the detection of reaction products. Negative controls were prepared from untransfected MRC5 cells. In quantifying the results the value obtained for this control was subtracted from all others. N.D. indicates no detectable enzyme activity.



b)

PLASMID	AMOUNT PER TRANSFECTION (µg DNA)	HCMV	CAT ACTIVITY (c.p.m./µg protein)	CAT ACTIVITY - CONTROL (c.p.m./µg protein)
IEP1catIEterm	10	-	2481	2466
IEP1catIEterm	10	+	4319	4309
AccHincat	10	-	608	593
AccHincat	10	+	2695	2680
SV2cat	10	-	44	29
SV2cat	10	+	642	627
SV2catIEterm	10	-	106	91
SV2catIEterm	10	+	630	615
IEP1catIEterm	0.3	-	557	542
IEP1catIEterm	0.3	+	534	519
AccHincat	0.3	-	86	71
AccHincat	0.3	+	73	58
None	-	-	15	
RSVcat	0.3	-	80	ND
RSVcat	0.3	+	1521	1436
SV2cat	0.3	-	106	21
SV2cat	0.3	+	243	158
SV2catIEterm	0.3	-	135	50
SV2catIEterm	0.3	+	237	152
None	-	-	85	

The relevant features of the plasmids IEP1catIEterm, AccHincat, SV2catIEterm, SV2cat and RSVcat are described in Fig 3:17a. At a concentration of 10 µg of each plasmid per transfection HCMV infection stimulated expression from IEP1catIEterm, AccHincat, SV2catIEterm and SV2cat while a concentration of 0.3 µg per transfection HCMV infection stimulated expression from RSVcat, SV2cat and SV2catIEterm but not from IEP1catIEterm or AccHincat. HCMV encoded trans-activator functions therefore stimulated expression from the SV40 early promoter and the RSV promoter, both of which contain enhancer elements, when they were transfected at either high or low DNA concentration.

At a concentration of 10 µg per transfection mixture, constitutive expression from IEP1catIEterm was more than four-fold higher than that from AccHincat. HCMV infection, however, had a relatively stronger stimulatory effect on expression from AccHincat than from IEP1catIEterm. When used at a concentration of 0.3 µg per transfection mixture, a small reduction in expression from IEP1catIEterm and AccHincat was detected following infection with HCMV. This effect was reproducible. It is not clear from these experiments, in which CAT was used as the reporter gene, why infection with HCMV did not repress expression from IEPTK1 when used at a concentration of 0.3 µg per transfection. It is possible that a regulatory region within the TK structural gene was responding to stimulation by HCMV-encoded trans-activating functions. Alternatively, transfections with IEPTK1 DNA may have been exceptionally efficient so that, even when its concentration was reduced in transfection mixtures, the copy number of the plasmid taken up by cells was still high enough to prevent HCMV-induced transcriptional repression.

The results of this and similar experiments suggest that an IE promoter-regulatory region (between bases -299 to +72) present in IEP1catIEterm and AccHincat was necessary for HCMV-induced repression of IE promoter activity whereas the IE gene downstream sequence, contained in

IEP1catIEterm and SV2catIEterm but not AccHincat and SV2cat, was not essential for repression.

3:11 Competitive Co-transfection Experiments to Investigate Repressor-Binding By The Major IE Promoter

The results of experiments described in section 3:10 indicated that the repressor of IE gene expression, which is active during the early and late phases of the HCMV replicative cycle, interacts either directly or indirectly with the IE promoter-regulatory element between bases -299 to +69 (section 3:10). When cells were transfected with high concentrations of the IE promoter HCMV infection was unable to negatively regulate expression from the IE promoter, indeed expression was stimulated by HCMV-induced trans-activators (section 3:10). These results were interpreted as indicating that high concentrations of the IE promoter in transfected cells may be saturating the RIT function during HCMV infection. When 0.1 µg of IEP1catIEterm DNA was co-transfected with 10 µg of IEPTK1 DNA then HCMV infection was observed to stimulate expression from IEP1catIEterm (Fig 3:16), co-transfection with large amounts of IEPTK1 DNA therefore prevented HCMV infection repressing expression from IEP1catIEterm. This apparent ability to compete out the HCMV-induced repressor in co-transfection experiments potentially provided an experimental system to identify sequences within the IE promoter-regulatory region, or elsewhere, which interact with the repressor.

A series of recombinant plasmids containing short segments of the IE promoter were generated as decribed in Fig 3:18a. HCMV HindIII fragment Z, in the plasmid H1D2, contains the IE promoter in IE coding region 4 (Oram et al., 1982; Wilkinson et al., 1984; Fig 1:6) and pMD102 contains the SV40 early promoter (Everett et al., 1983). In each transfection 19.7 µg of the test plasmid was mixed with 0.3 µg of IEP1catIEterm DNA and 5 µg of DNA applied to two 50 mm diameter dishes containing MRC5 cells. The effect of infection with HCMV on IEP1catIEterm expression was studied (Fig 3:18b). The

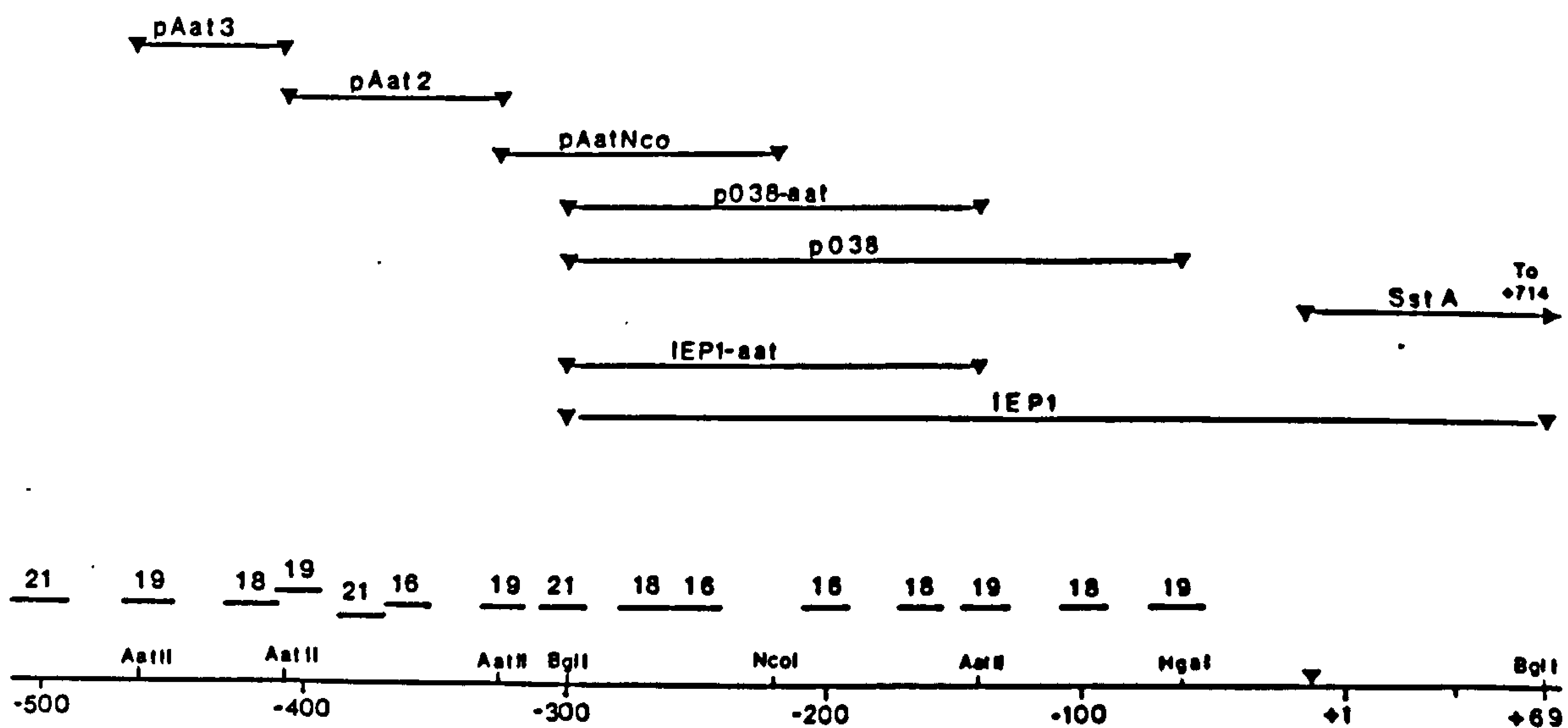


Fig 3:18a: Inactivating repression of the the major IE promoter in co-transfection experiments. The map locations of cloned DNA fragments derived from the major IE promoter-regulatory region which were used in co-transfection experiments are shown. The vector IEP1 is described in Fig 3:7. p0.38, kindly provided by Dr A.Akrigg, contains the BglI/HgaI fragment (position -299 to -63) cloned into the SmaI site in pUC18. pAat2 and pAat3 were produced by cloning AatII fragment (-321 to -404 and -404 to -457) into the unique AatII site in pMTL21b. IEP1-aat was generated by religating AatII cleaved IEP1 which deleted sequences between -135 to +69 and a small portion of pUC8 vector DNA. Similarly p0.38-aat was produced by religating AatII cleaved p0.38; the HCMV cloned inserts in IEP1-aat and p0.38-aat are identical. pAatNco contains the AatII/NcoI fragment (-321 to -222) cloned between the unique AatII and NcoI sites in pMTL21b and SstA contains the SstI fragment (-15 to +714) cloned into the unique SstI site in pUC19.

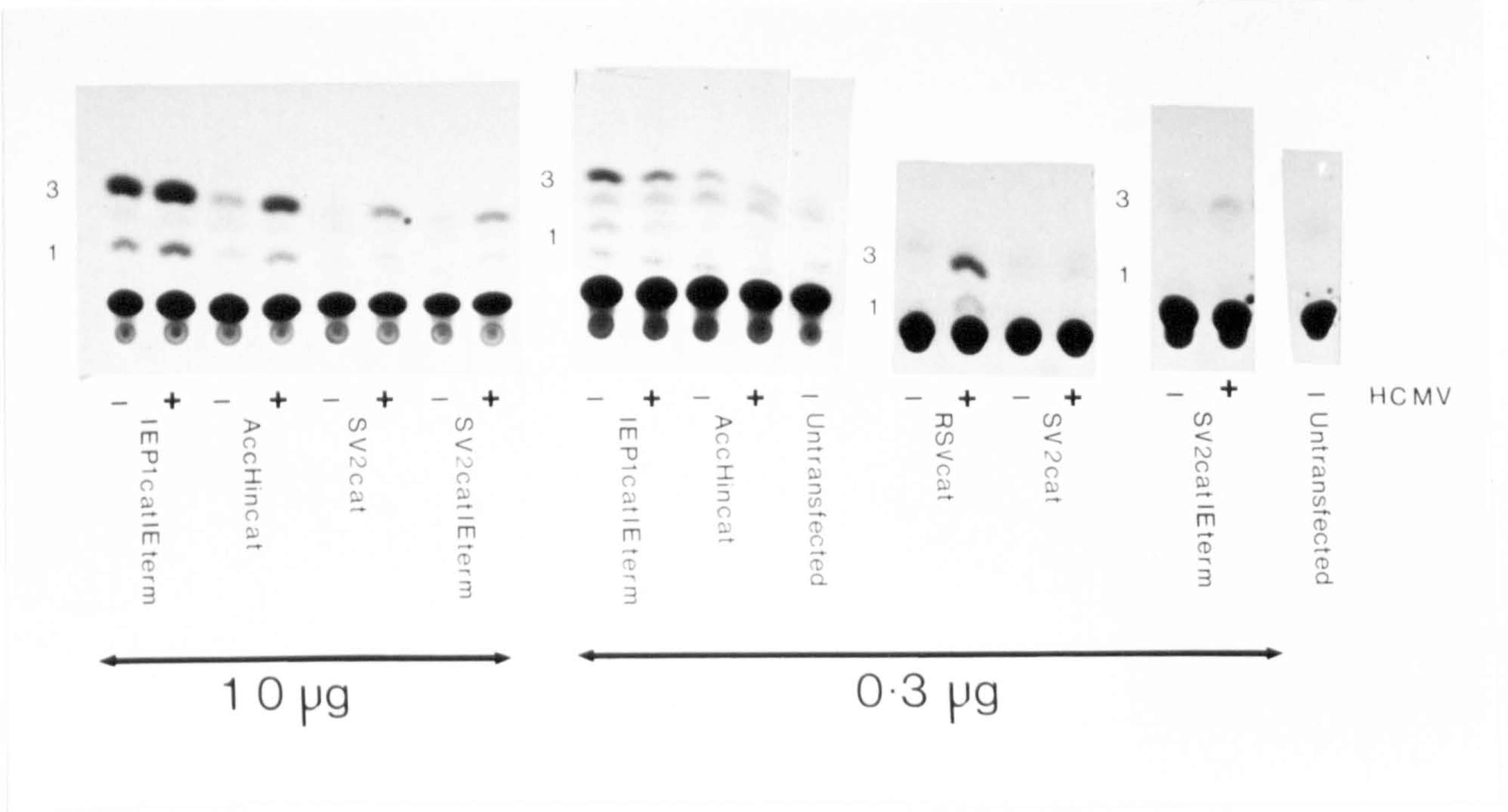


Fig 3:18b: Inactivating repression of the major IE promoter in co-transfection experiments. 0.3 µg of IEP1catIEterm DNA and 19.7 µg of the competing plasmid were used in each transfection. As before, for each transfection 5 µg of DNA was added to two 50 mm diameter petri dishes containing MRC5 cells, one of which was subsequently infected with HCMV (4 p.f.u./cell). Cells were harvested at 72h p.i. and assayed for CAT activity. 20 µl of cell extract was added to the CAT assay which was incubated for 2h at 37°C except for the cells transfected with 20 µg of IEP1catIEterm DNA in which case 5 µl of cell extract was used and the assay incubated at 37°C for 40 min. Negative control samples were prepared from untransfected MRC5 cells. In quantifying the results the relavent value obtained for this control was subtracted.

COMPETING PLASMID	HCMV	CAT ACTIVITY (c.p.m./μg protein)	CAT ACTIVITY - CONTROL (c.p.m./μg protein)
pUC18	-	524	480
pUC18	+	340	301
IEP1	-	54	15
IEP1	+	303	264
p0.38	-	178	139
p0.38	+	313	274
pAat2	-	43	4
pAat2	+	131	92
pAat3	-	122	84
pAat3	+	347	308
IEP1-aat	-	90	51
IEP1-aat	+	214	175
p0.38aat	-	86	47
p0.38aat	+	169	130
pAatNco	-	240	201
pAatNco	+	589	550
SstA	-	363	324
SstA	+	308	269
HindIIIZ	-	205	166
HindIIIZ	+	154	115
pMD102	-	155	116
pMD102	+	120	81
pAT153	-	892	853
pAT153	+	631	592
None	-	39	
IEP1catIEterm	-	192	185
IEP1catIEterm	+	819	812

Fig 3:18b: Continued.

results obtained with the control samples were consistent with previous observations; HCMV infection stimulated expression from the reporter gene when IEP1catIEterm comprised 100% (20 µg/20 µg) of transfected DNA and resulted in a small reduction in expression when IEP1catIEterm comprised only 0.3 µg/20 µg of transfected DNA. Constitutive expression from IEP1catIEterm was higher when it was co-transfected with the plasmid vectors, pUC18 and pAT153, than it was when co-transfected with any of the plasmids containing cloned eukaryotic promoter-regulatory sequences (Fig 3:18b). While transfection frequencies do vary with different DNA preparations, the magnitude of the differences observed in this experiment suggests that the co-transfecting DNA fragments were competing with IEP1catIEterm for cellular transcription factors.

Expression from IEP1catIEterm was stimulated by virus infection when co-transfected with IEP1, p0.38, pAat2, pAat3, IEP1-aat, p0.38-aat and pAatNco (all of which contain sequences upstream of the transcriptional start site) but not when co-transfected with Sst A, HindIII Z or pMD102 (Fig 3:18). Co-transfection with three distinct non-overlapping DNA fragments, delineated by the AatII sites in the IE promoter-regulatory region at -135, -321, -404 and -457, caused expression from the co-transfected IE promoter to be induced by virus infection. The 53bp (in pAat3) and the 83bp (in pAat2) AatII fragments are particularly notable because neither sequence is present in pIEP1catIEterm. The IE promoter-regulatory region contains a complex series of 16, 18, 19 and 21bp direct repeats but none of these repeats is present in all of the fragments which conferred inducibility on the IE promoter in the co-transfection experiments (Fig 3:18a). Boshart et al. (1985) observed that a series of non-overlapping DNA fragments in the HCMV IE promoter between -118 to -524 possessed enhancer activity. The reduction in the level of constitutive IEP1catIEterm expression when co-transfected with fragments from the IE promoter-regulatory region may be due to competition by these fragments for enhancer-binding proteins.

The possiblity that during productive infections transcriptional repression of the major IE gene may be mediated through its enhancer elements will be considered in the discussion. It is relevant to note, however, that although co-transfection with HindIII Z or the SV40 early promoter (pMD102; which contains an enhancer element) reduced the constitutive level of expression from IEP1catIEterm, it did not result in expression from IEP1catIEterm being stimulated by virus infection.

As in cells transfected with a high copy number of IEPTK1 DNA (section 3:10), in these co-transfection experiments HCMV infection is probably stimulating expression from the reporter gene by providing an excess of transcription factors to the IE promoter. It is notable that in none of the co-transfection experiments, in which HCMV infection stimulated expression from IEP1catIEterm, was the level of CAT activity significantly higher than that detected in pUC18- or pAT153-IEP1catIEterm transfected cells in which expression had been repressed by virus infection.

It has recently been proposed that the HSV-1 Vmw 175 polypeptide represses transcription from HSV-1 IE gene by binding directly to a sequence close to the transcriptional start site. Since co-transfection with SstA did not result in HCMV infection stimulating expression from IEP1catIEterm, sequences downstream of position -15 do not appear to be important in the transcriptional repression of the HCMV major IE promoter.

3:12 Characterisation of the Abundant HCMV Early Transcript

In excess of 60% of HCMV-encoded cytoplasmic poly $(A)^+$ RNA isolated from infected cells during the early phase hybridises with the HCMV PstI fragment C, a DNA fragment which contains most of both the IR_L and IR_S regions (Fig 3:19). More detailed experiments, in which early RNA was hybridised with Southern transfers of the HCMV cloned gene bank digested with restriction endonucleases, indicated that abundant early transcription was localised in the region of a 1.5kb EcoRI/BamHI fragment (0.008–0.014 and 0.812–0.818 map units; Fig 3:19) present in both the TR_L and IR_L (Wilkinson, 1983).

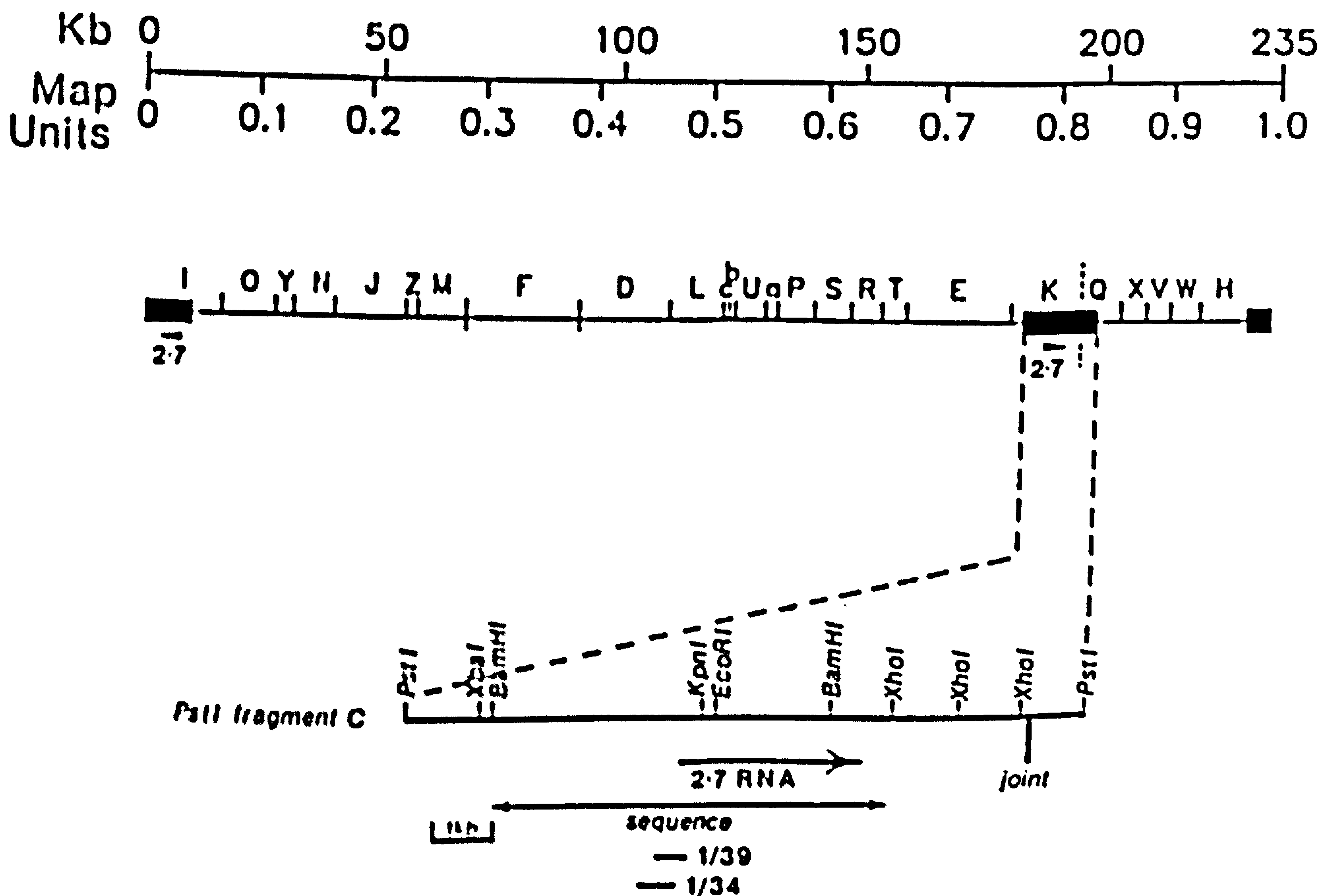
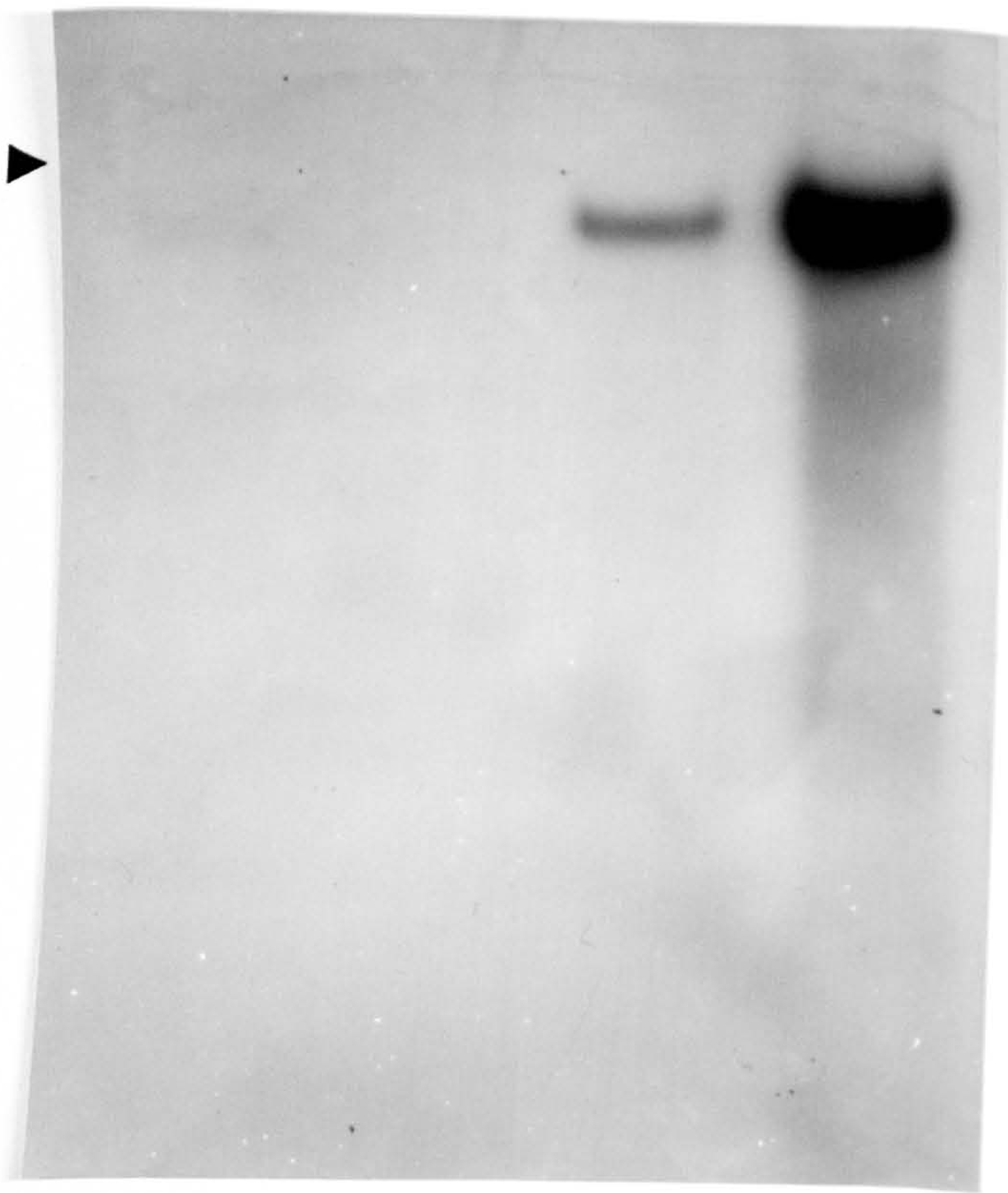
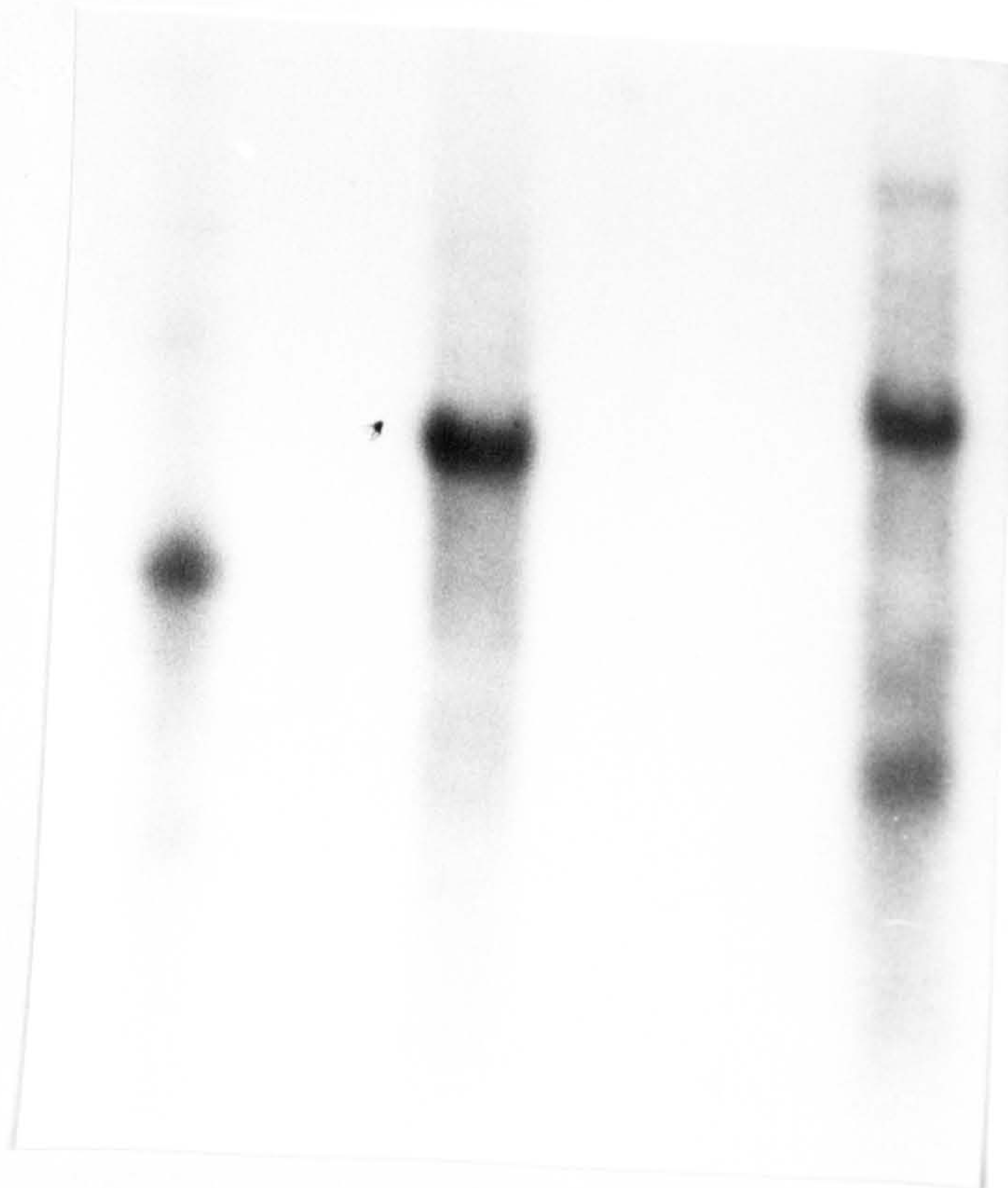


Fig 3:19: Physical map of the HCMV genome showing the locations of HindIII restriction endonuclease cleavage sites (Oram et al., 1982) and the region within PstI C encoding the 2.7kb early RNA. The region of DNA which has been sequenced (Greenaway and Wilkinson, 1987) and the map location of M13mp11(1/39) and M13mp11(1/34) are indicated.


2·7
a
IE
2h
Early
72h


2·7
1·95
b
IE
Early
72h

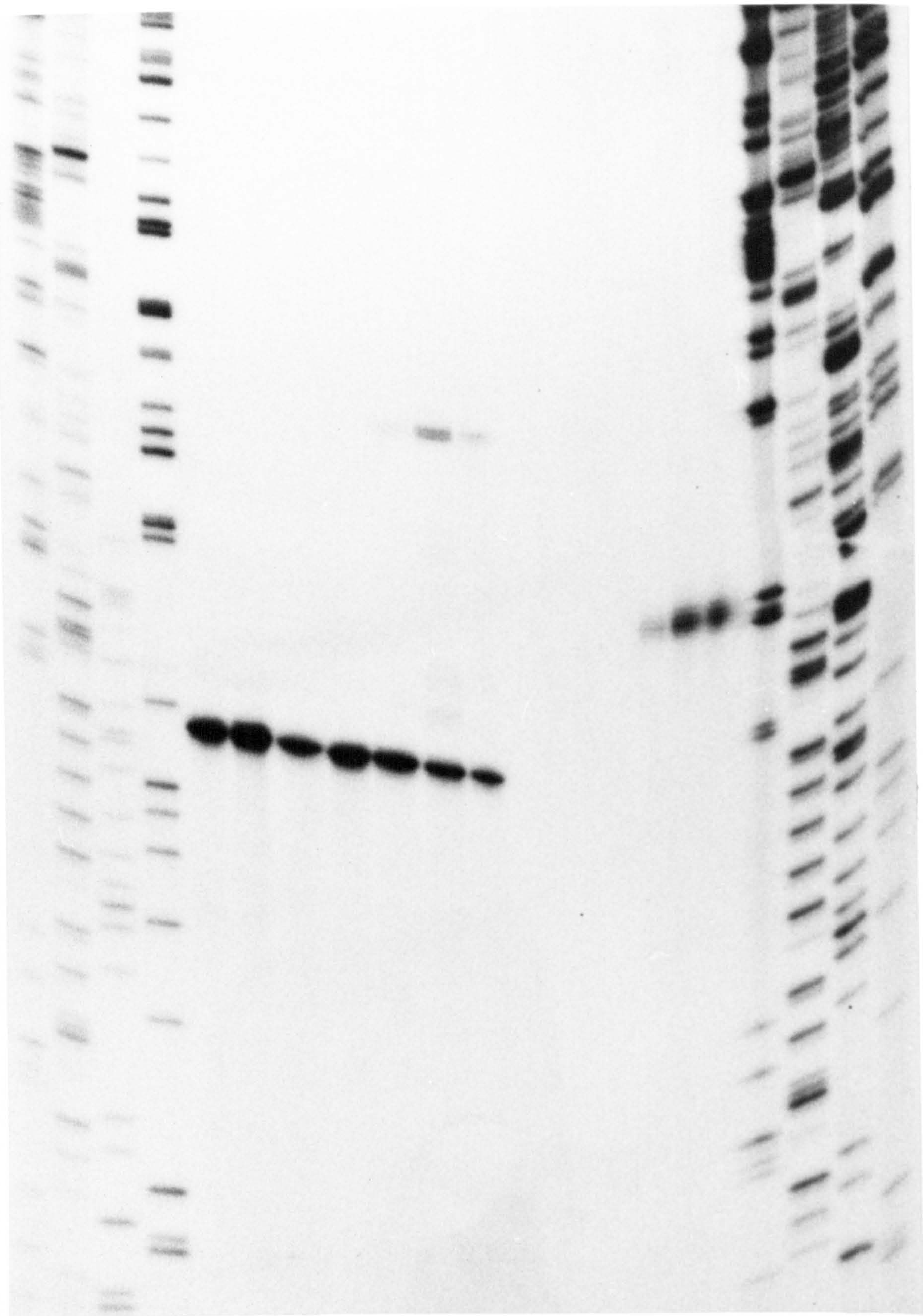
Cytoplasmic poly $(A)^+$ RNA was isolated from HCMV infected MRC5 cells 2h and 72h p.i. and under IE and early phase conditions (section 2:3). The purified RNA preparations were electrophoresed in a formaldehyde-agarose gel and transferred to nitrocellulose membranes (Thomas, 1980). Fig 3:20a shows that when the Northern transfer was probed with a recombinant clone containing PstI fragment C an RNA species of 2.7kb was detected in IE, early and late phase RNA preparations but not in RNA prepared 2h p.i. The 2.7kb transcript, which is synthesised in very small amounts under IE conditions (Fig 3:20a), had previously been identified as a minor IE RNA (Wilkinson, 1983). It is possibe that the 2.7kb RNA is transcribed during the IE phase as a consequence of incomplete inhibition of protein synthesis by cycloheximide resulting in a limited level of early phase transcription. When total HCMV DNA was used as the hybridisation probe the 2.7kb RNA was found to be the major early phase cytoplasmic transcript (Fig 3:20b). Although the concentration of the 2.7kb RNA in infected cells increases considerably during the transition from the early to the late phase of gene expression it is no longer the predominant transcript because of the efficient expression of HCMV late genes (Fig 3:20b).

The mapping and structural analysis of the 2.7kb early RNA was supported by the determination of the nucleotide sequence of the gene (performed by Dr P.J. Greenaway). The complete nucleotide sequence of the strain AD169 major early gene is contained in appendix 3 (Greenaway and Wilkinson, 1987).

Fig 3:20: Analysis of HCMV transcription at different stages of the replicative cycle. Cells were infected with HCMV and cytoplasmic poly $(A)^+$ RNA isolated after infection for 12h in the presence of 100 μg/ml cycloheximide, 2h p.i., after infection for 24h in the presence of 100 μg/ml PFA and 72h p.i. RNA samples (1 μg) were electrophoresed in 1% agarose-formaldehyde gels and the fractionated material transferred to nitrocellulose membranes. Membranes were hybridised with radioactive probes prepared from (a) the plasmid pT23 (containing HCMV PstI fragment C) and (b) HCMV DNA.

McDonough et al. (1985) showed that between a region 0.63kb upstream and 1.85kb downstream of the EcoRI site (Fig 3:19) the major early gene is not spliced. These experiments have been repeated and their results confirmed (results not shown). Problems were encountered in nuclease protection experiments due to artifactual but reproducible cleavage of RNA/DNA hybrids at distinct points. The nucleotide sequence data indicates that these artifactual cleavage sites occur in sequences containing long tracts of A and T(U) residues, e.g. between bases 5004 to 5035 on the sequence presented in appendix 4 (Greenaway and Wilkinson, 1987). The 3' terminus of the gene was mapped to 265bp downstream from the BamHI site detailed in Fig 3:19. A consensus polyadenylation signal (AATAAA; Proudfoot and Brownlee, 1976) is present in the appropriate position upstream (bases 5742 to 5747; appendix 4) from the proposed 3' terminus of the gene. The position of the 5' end of the gene was initially less clear. Although nuclease protection experiments indicated transcription was initiated 0.6kb upstream of the EcoRI site (Fig 3:19) the possibility of an undetected upstream exon was suspected for a number of reasons. Firstly, if translation was initiated at the first 5' proximal AUG codon the proposed RNA would encode a polypeptide of only seven amino acids. Secondly, there is a reasonable homology to the consensus splice acceptor sequence (Mount, 1982) close to the proposed transcriptional start site (CTTCCCCAGA between bases 3276 to 3285 in appendix 4). Finally, the sequence upstream of base 3277 did not contain obvious consensus promoter elements.

To test for the presence of an additional upstream exon in the major early gene a primer extension experiment was performed. The recombinant M13 clone M13mp11(1/39), provided by Dr P.J. Greenaway, spans the transcriptional start site and contains approximately 0.5kb of HCMV nucleotide sequences upstream from base 3403. A 106n radiolabelled ssDNA primer complementary to the 2.7kb early RNA, extending from the Xma III cleavage site at base 3330 to base 3403 (plus 33n of M13 vector sequence),



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

was prepared from M13mp11(1/39) as described in section 2:20 and hybridised with early and late phase total cytoplasmic RNA. Reverse transcriptase extended the primer only an additional 49n (Fig 3:21) to base 3281. It was concluded that the major HCMV early gene does not contain an upstream exon. The 2.7kb early RNA is therefore not spliced and its trancriptional initiation site is near base 3281.

The transcriptional initiation site was precisely mapped in S1 nuclease protection experiments. A radiolabelled single stranded DNA hybridisation probe spanning the transcriptional start site was prepared as described in section 2:19 using the M13 clone M13mp11(1/39); the same M13 clone used for the primer extension experiment. Early and late phase RNA both protected a 126n S1 resistant fragment (Fig 3:21) and it was therefore concluded that transcription of the 2.7kb RNA was initiated 126bp upstream from the end of the insert cloned into M13 (base 3403) at base 3277.

Twenty-seven base pairs upstream of the designated transcriptional initiation site at base 3277 (+1) is the sequence which most closely resembles the consensus for a TATA box; ATATAAAA (base 3251 to 3258). An analysis of the HCMV early promoter revealed a 17bp sequence homology, centered on the TATA box, with the corresponding regions of the HSV-1 gD

Fig 3:21: Primer extension and S-1 nuclease analysis using the recombinant clone M13mp11(1/39). Tracks 1-4 and 18-21 are nucleotide sequence tracks for M13mp11(1/39) run as molecular weight standards. Tracks 5-11 are primer extension analyses from the XmaIII cleavage site at base 3330 using 10 μg of *E. coli* tRNA (track 5), total cytoplasmic RNA from uninfected MRC5 cells (tracks 6-8), early phase RNA (track 9) and late phase RNA (tracks 10,11) from HCMV-infected cells. The major band common to all tracks is the single-stranded primer molecule. The extended fragment of 49n can only be observed in tracks 9-11. Tracks 12-17 are S-1 nuclease protection analyses in which ssDNA complementary to the entire cloned insert in M13mp11(1/39) was hybridised with 10 μg of total cytoplasmic RNA from uninfected Hela cells (track 12), uninfected MRC5 cells (tracks 13,14), and early phase (track 15) and late phase (tracks 16,17) from infected cells. The nuclease protected fragment can only be observed in tracks 15-17.

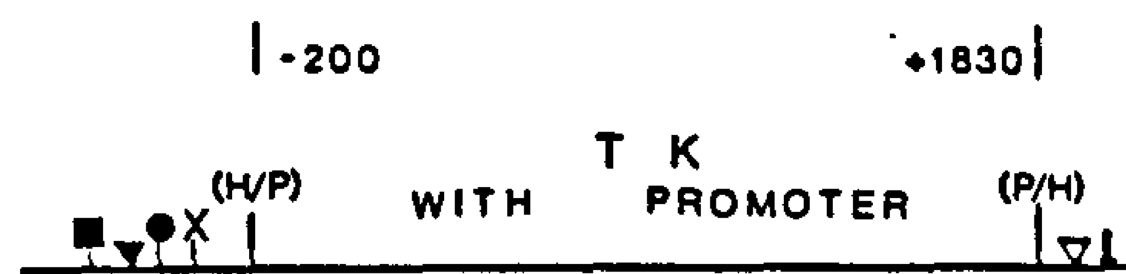
(Everett, 1983) and a human heat shock protein (hsp 70; Wu, 1984) gene promoters (Table 3:8). The 2.7kb early promoter does not contain any of the direct or inverted repeats associated with the IE gene nor does it contain the consensus core sequence for an enhancer ($TGG\frac{AAA}{TTT}$; Weiher et al., 1983) or the binding site of the transcription factor Sp-1 (GGGCGG). One imperfect 13 bp repeat (TCAGTCCGAATGA) was present between bases 2983 to 2995 and 3003 to 3014 and one perfect 9bp direct repeat (AAACCGTAT) was present between bases 3131 to 3139 and 3147 to 3155. The latter repeat is also part of a longer 15bp imperfect repeat. The GC and AC-rich regions which are present upstream from the TATA box of the HCMV major early gene have also been identified in the same relative position in other herpesvirus early promoters (Everett, 1983); no direct sequence homology has been identified however. The GC-rich sequence between bases 3222 to 3246 may possibly be functionally important as the analogous region of the HSV-1 gD promoter has been shown experimentally to be required for efficient expression (Everett, 1984).

An analysis of the nucleotide sequence of the HCMV early gene shows the major early RNA to contains multiple stop codons in all three reading frames. Assuming that an AUG is required to initiate translation the longest ORF would encode a polypeptide of only 170 amino acids. It is possible that the HCMV major early RNA transcript does not encode a polypeptide (Greenaway and Wilkinson, 1987).

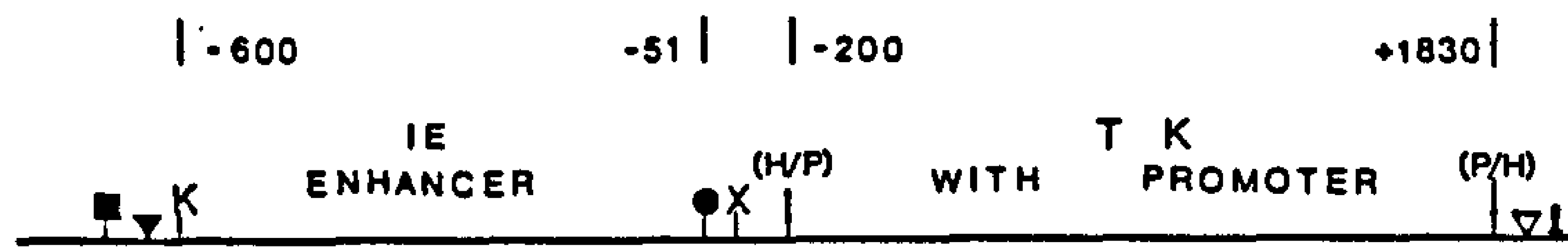
Promoter	Sequence
Human hsp70 promoter	T A T A A A A - C G CcA G G G G (-30 … -15)
HCMV 2.7 kb early promoter	T A T A A A A A C G C A G G G G T T T A G (-26 … -6)
HSV-1 gD promoter	T A A A A A - G C A G G G G T - T A G (-23 … -7)

Table 3:8: Corresponding homologous regions in the human hsp 70, HCMV 2.7kb early and the HSV-1 gD gene promoters.

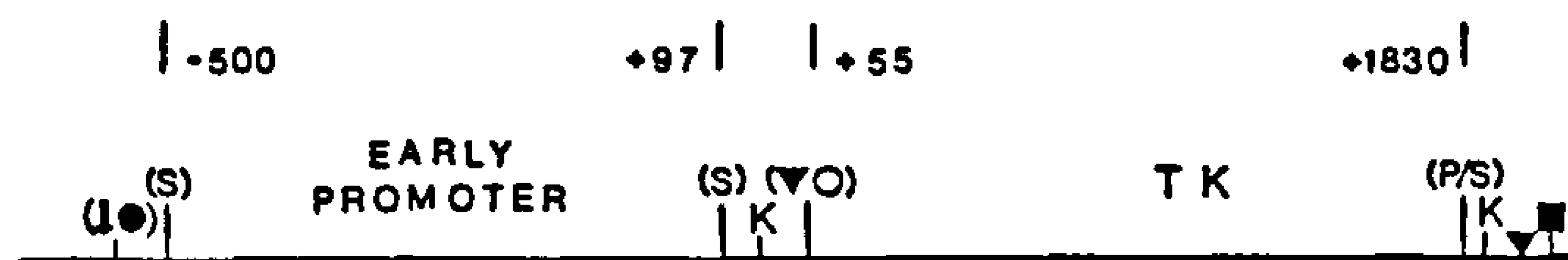
Fig 3:22: The construction of the plasmids pVUTK1, 0.7TK1, 1/34TK1 and 0.7-1/34TK1. (a) pVUTK1 was constructed by excising the complete HSV-1 TK gene from the plasmid pM2 (Wilkie *et al.*, 1979) on a *Pvu*II fragment (approximately 2kb) and inserted into the unique *Hinc*II site in pUC19. (b) 0.7TK1. The *Hinc*II/*Hga*I fragment (position -600 to -63) was excised from the IE promoter in IEP2, blunt-ended by treatment with T4 polymerase and inserted into the *Sma*I site in pUC18 to generate the plasmid p0.7. The TK gene was excised from pVUTK1 by digestion with *Kpn*I/*Xba*I, the DNA fragment blunt-ended using T4 polymerase and inserted into the *Hinc*II site in p0.7. The enhancer element in 0.7TK1 is inverted relevant to its orietation in the major IE gene. (c) 1/34TK1. The HCMV DNA fragment 1/34 produced by DNA sonication was cloned into the *Sma*I site in M13mp11 (Dr P.J. Greenaway, personal communication). A DNA fragment containing 1/34 was excised from M13mp11(1/34) by digestion with *Bam*HI/*Eco*RI and cloned between the *Bam*HI and *Eco*RI sites in pUC18 generating the plasmid p1/34. The plasmid pVUTK5 is identical to pVUTK1 except that the TK gene was inserted in the opposite orientation into pUC19. The TK structural gene was excised from pVUTK5 by digestion with *Bgl*II/*Eco*RI and inserted between the *Eco*RI and *Sst*I sites in p1/34 generating the plasmid 1/34TK1*. Finally restriction endonuclease cleavage sites upstream of the HCMV early promoter were removed by digestion with *Hind*II/*Bam*HI, blunt-ending using the Klenow polymerase and religating to produce the plasmid 1/34TK1. (d) 0.7-1/34TK1. 1/34TK1* was digested with *Bam*HI/*Hind*III and p0.7 digested with *Hind*III/*Sst*I. The *Bam*HI site upstream of the enhancer was ligated to the *Bam*HI site of the early promoter. The *Hind*III and *Sst*I sticky ends were then blunt-ended using T4 polymerase and ligated together to generate the plasmid 0.7-1/34TK1. The majority of work involved in constructing these plasmids was performed by Dr A. Akrigg. *Bam*HI (●), *Bgl*II (♀), *Eco*RI (■), *Hga*I (Hg), *Hinc*II (H), *Hind*III (↓), *Kpn*I (K), *Pst*I (▽), *Pvu*II (P), *Sma*I (S), *Sst*I (▼) and *Xba*I (X) restriction endonuclease cleavage sites are shown.



a) pVUTK1



b) 0·7TK1



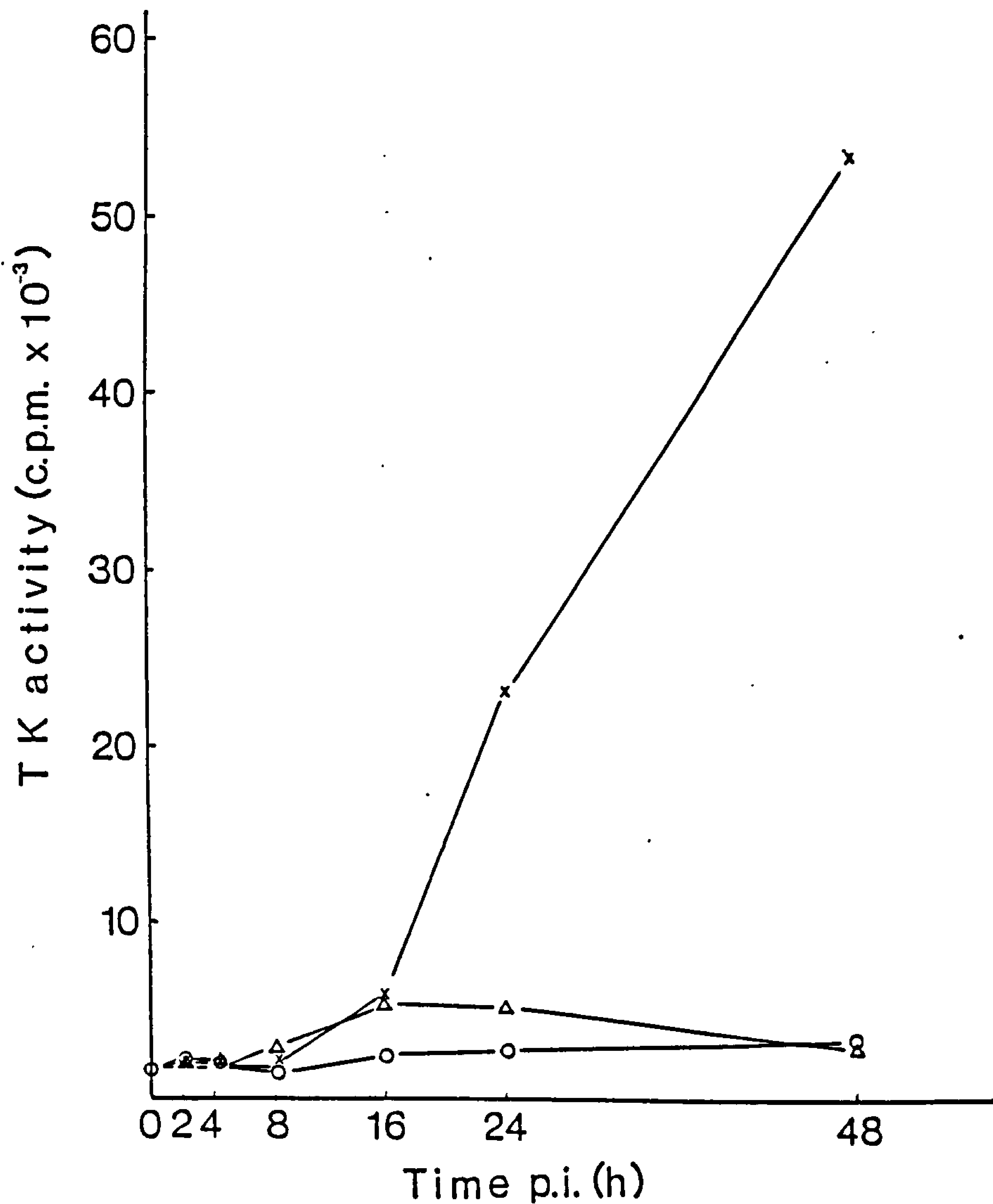
c) 1/34TK1

d) 0·7-1/34TK1

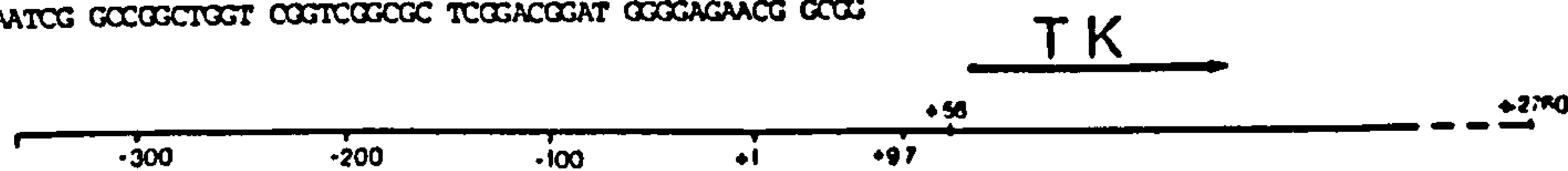
3:13 Activation of Expression From The HCMV Early and The HSV-1 TK Promoters By Virus Infection

The characterisation of the HCMV major early gene provided a basis from which to devise further experiments to study the transition from HCMV IE to early phase gene expression. In particular, a knowledge of the DNA sequence of the early gene facilitated the cloning of reporter genes downstream and under the control of the early gene promoter. The recombinant phage M13mp11(1/34), generated during the nucleotide sequence analysis of the HCMV major early gene (Greenaway, personal communication), contains a cloned insert extending from approximately -500 to precisely +97 (base 3377; appendix 3) with respect to the transcriptional start site of the 2.7kb early RNA as determined in section 3:12. The plasmid 1/34TK (constructed by Dr A. Akrigg) contains the HCMV early promoter excised from M13mp11(1/34) and inserted upstream from the TK structural gene in a pUC19 based plasmid as described in the legend to Fig 3:22c. It should be noted that the HCMV leader sequence cloned into 1/34TK1 contains a potential translational initiation codon at +89 which may influence TK expression. The promoter sequence in 1/34TK1 is shown in Fig 3:23.

The level of TK enzyme activity detected in uninfected MRC5 cells, transfected with the plasmid 1/34TK1, was similar to background levels of the assay which indicates that there was little constitutive expression from the early promoter. However, expression from 1/34TK1 was stimulated significantly by infection with HCMV (Table 3:9; Fig 3:23). During the course of the infection expression from 1/34TK1 increased gradually to reach its maximum level during the late phase (Table 3:9; Fig 3:23). There was a significant delay of between 8 to 12h following infection before an increase in expression from the transfected promoter was detected. This lag period could not overcome by increasing the m.o.i. of HCMV (results not shown). The HCMV early promoter appears to be regulated in a similar manner in infected cells whether introduced by DNA transfection or virus infection. In HCMV



CAGCAGACTG ATTCGTTGTA CGGAGTGGGT CATCGGTTGA ATGACGAAGA AGATCGTGAA
CTGTGGAGAG TTTCGGTTTC TTAATAATCC CATACGACAT GTGTTCATTT ATATCTAATT
TTAGGATGAT GACTATAGTA TAACTCTGGG GAACAAATAT CATACGTTAA TCACTTTAAG
TTACGCCGTT AGGAAAAGAA AATCAGTCCG AATGAAGCAT AGTCAGCCGA ATGATACAGC
AATAGCTTGT TTACAACGTG TTCTTTTTTA CATTATGAAC GTGCCTTGCT TTTTATACAC
ACATGGAGAC AGAGGTCCCT CAGCCCTTGT CACGACAACT CCCTTTTTCT AAACCGTATG
TGCTCCAAAC CGTATCTCCT CATCGTCACG TGAAATACCA TGGGACCCCT TTTCGTCACA
CACGTCTTTC CGCTTACCCA ACGCGTCAGC CCGCGCTCGG CAGAGCTACC ATATAAAAAC
+1
GCAGGGGTTT AGCAGCTTCC CCAGATCGCT GCTGCCCCGG CGTTCTCCAG AAGCCCCGGC
GGGCGAATCG GCCGGCTGGT CGGTCGGCGC TCGGACGGAT GGGGAGAACG GCGG



HCMV 2·7kb RNA Early Promoter

Fig 3:23: Stimulation of expression from the transfected HCMV major early promoter by infection with HCMV and HSV-1. The nucleotide sequence of the promoter-regulatory region (from approximately -500 to precisely +97) cloned into 1/34TK1 and its position relative to the TK structural gene are shown. O, 1/34TK1 transfected cells; X, 1/34TK1 transfected HCMV-infected cells; Δ, 1/34TK1 transfected HSV-1 infected.

infected cells, measurements of the levels of the 2.7kb RNA in the cytoplasm were unable to detect the transcript at 2h p.i., show it to be present in only trace amounts under IE conditions and at 8h p.i. but in increasing abundance subsequently (section 3:12; McDonough et al., 1985).

A short region of DNA sequence homology is shared by the HCMV early and the HSV-1 gD promoters (Table 3:8). In view of this homology, an attempt was made to activate expression from the HCMV early promoter by infecting 1/34TK1-transfected cells with HSV-1 TK$^-$. Expression from the transfected HCMV early promoter was not significantly enhanced by HSV-1 infection, although there was a very small transient increase in expression between 12 and 24h p.i. (Table 3:9; Fig 3:23). The inability of HSV-1 infections to stimulate expression from 1/34TK1 is unlikely to be a property of the reporter gene, since it was derived from HSV-1. Consequently, it would

Time (h p.i.)	1/34TK1	1/34TK1 + HSV-1	1/34TK1 + HCMV
0	1,674	-	-
2	2,280	2,176	2,219
4	2,131	2,197	1,865
8	1,622	2,841	1,885
16	2,369	5,183	6,119
24	2,782	5,211	23,053
48	3,571	2,810	53,178

Table 3:9: Effect of virus infection on expression from the transfected HCMV early promoter. All samples were taken from 1/34TK1 transfected cells. Results of the TK enzyme assay are expressed as c.p.m./10 µl cell extract. Duplicate samples were assayed three times each, the average value calculated and the value obtained by assaying TK extraction buffer (2,679) subtracted. Cells were infected with HCMV or HSV-1 at a m.o.i. of 5 p.f.u./cell.

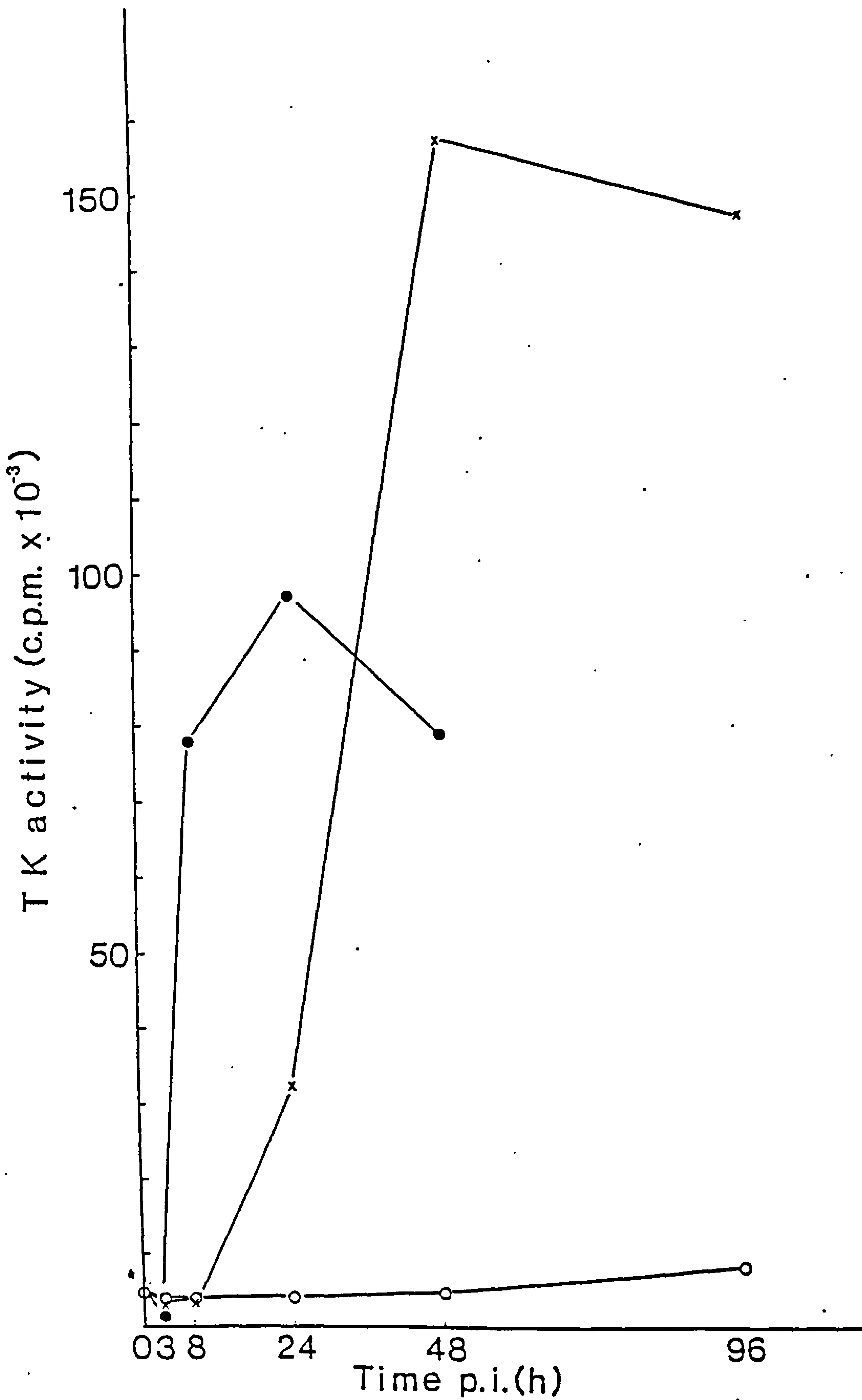


Fig 3:24: Stimulation of expression from the transfected HSV-1 TK gene promoter by infection with HCMV and HSV-1. O, pVUTK1 transfected cells; X, pVUTK1 tranfected HCMV-infected cells; ●, pVUTK1 transfected HSV-1 infected.

appear that either the HCMV early promoter does not respond to HSV-1 encoded _trans_-activators or that the short HCMV leader sequence is preventing expression.

In DNA transfection experiments, HSV-1 infections stimulated expression from the HCMV major IE promoter but not the HCMV major early promoter (section 3:4, this section). This inability of HSV-1 to activate expression from the HCMV major early promoter implied that the two herpesviruses may use different mechanisms to regulate early phase gene expression. It was therefore decided to investigate the effect of infection with HCMV on a HSV-1 early promoter. The plasmid pVUTK1 (kindly provided by Dr A. Akrigg) contains the HSV-1 TK gene under the control of its own early promoter (Fig 3:22a).

The levels of TK activity detected in pVUTK1-transfected and in untransfected cells were similar (Table 3:10), constitutive expression from the TK gene was therefore very low. Stimulation of expression from the

Time (h p.i.)	pVUTK1	pVUTK1 + HSV-1	pVUTK1 + HCMV
0	5,007	-	-
3	3,648	2,485	4,062
8	4,048	78,583	3,206
24	3,510	97,354	32,282
48	4,419	78,388	157,355
96	7,922	-	147,369

Table 3:10: Effect of virus infection on expression from the transfected HSV-1 TK promoter. All samples were taken from pVUTK1 transfected cells. Results of the TK enzyme assay are expressed as cpm/10 µl of cell extract. Duplicate samples were assayed three times each, the average calculated and the value obtained by assaying TK extraction buffer (4,002) subtracted. Cells were infected with HSV-1 TK^- or HCMV at a m.o.i. of 5 p.f.u./cell.

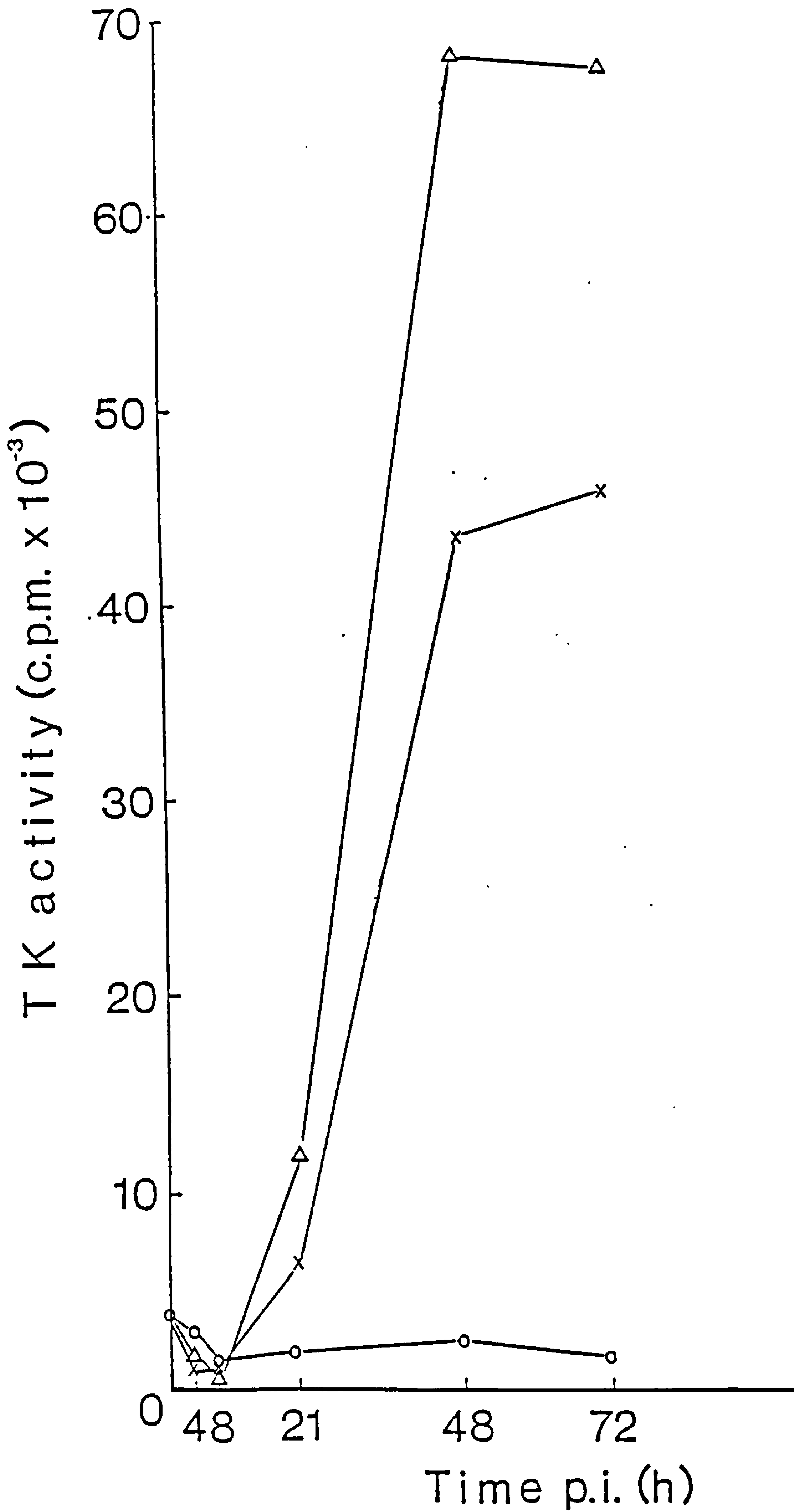


Fig 3:25: Effect of increased m.o.i. on HCMV stimulation of expression from the transfected HSV-1 TK promoter. All samples were from pVUTK1-transfected cells. O , uninfected; X , infected with 5 p.f.u./cell HCMV and Δ, infected with 35 p.f.u./cell HCMV.

transfected TK promoter by infection with HSV-1 TK⁻ was not detected 3h p.i. but at 8h, 24h and 48h p.i. TK enzyme activity in cell extracts was greatly enhanced (Table 3:10; Fig 3:24). By 96h p.i. the HSV-1 infection had destroyed the monolayer.

HCMV infection induced efficient expression from the transfected HSV-1 early promoter but not until 24-48h p.i. (Table 3:10; Fig 3:24). A similar lag period was also observed between HCMV infection and the virus-induced stimulation of expression from the transfected HCMV major IE (section 3:4) and major early (this section) promoters. In an attempt to overcome this lag period cells transfected with pVUTK1 were infected with a very high input m.o.i. of HCMV (Table 3:11; Fig 3:25). Although the level of TK expression at 21h, 48h and 72h p.i. was appreciably higher when the m.o.i. with HCMV was increased from 5 to 35 p.f.u./cell, there was no detectable stimulation of TK expression at 8h p.i. (Fig 3:25).

Time (h p.i.)	pVUTK1	pVUTK1 + 5 PFU/cell HCMV	pVUTK1 + 35 PFU/cell HCMV
0	3,513	-	-
4	2,768	1,396	1,821
8	998	1,159	55
21	1,355	6,527	11,848
48	2,470	43,816	67,964
72	1,621	46,289	20,258

Table 3:11: The effect on expression from the HSV-1 TK promoter of moderate and high m.o.i. with HCMV. All samples were from pVUTK1-transfected cells. Results of the TK enzyme assay are expressed in c.p.m./10 µl of cell extract. Duplicate samples were assayed three times each, the average calculated and the value obtained by assaying TK extraction buffer (4,490) subtracted.

In DNA transfection experiments while HCMV infection was able to stimulate expression from an HSV-1 early promoter, HSV-1 infection was unable to stimulate expression from the HCMV major early promoter. HSV-1 infection was, however, able to stimulate expression from the HCMV major IE promoter (section 3:7) and its own TK promoter (this section) appreciably more rapidly than HCMV in experiments performed in parallel, using the same plasmid constructs and the same cell system.

3:14 Activation of Expression From The HCMV Major Early Promoter in Cis By an Enhancer

The HCMV major IE gene contains one of the strongest characterised enhancer elements (Boshart et al., 1985). A 537bp DNA fragment, containing the enhancer region from the major IE gene, was inserted upstream of the HCMV major promoter in 1/34TK1 (as described in the legend to Fig 3:22d) to determine whether expression from the 1/34TK1 could be activated solely by stimulating transcription from the early promoter. The 537bp IE enhancer element was also inserted into the plasmid pVUTK1, upstream of the TK gene, as a positive control to confirm that the enhancer was functional (Fig 3:22). The plasmids 0.7-1/34TK1 and 0.7TK1 were constructed in collaboration with Dr A. Akrigg.

In order to maximise the transfection frequency, Hela cells, rather than MRC5 cells, were transfected with the plasmids pVUTK1, 0.7TK1, 1/34TK1, 0.7-1/34TK1 and IEPTK1. The presence of the 537bp enhancer element, in cis, stimulated expression from both the HSV-1 TK and HCMV early promoter (Table 3:12). Even when stimulated by the IE enhancer element, the level of expression from the HSV-1 and HCMV early promoters was significantly less than that obtained using the intact HCMV IE promoter (IEPTK1 in Table 3:12).

These results demonstrated that detectable levels of expression from 1/34TK1 can be obtained by stimulating transcription from the early promoter. In an attempt to map HCMV trans-activator(s), the plasmid 1/34TK1,

and analogous constructs containing the CAT reporter gene, have been co-transfected with recombinant plasmid containing HCMV IE genes. These experiments have been unable to demonstrate trans-activation of the HCMV early promoter (results not shown). It has recently been suggested that the leader sequence of the 2.7kb early transcript may exert some form of post-transcriptional control over expression, possibly by inhibiting the passage of ribosomes through the leader (Geballe et al., 1986b). It is clear from the results in table 3:10 that expression from 1/34TK1 is not absolutely dependent on an HCMV early gene product inter-acting with the leader sequence to permit translation. Although the presence of the upstream initiation codon within the HCMV leader sequence did not prevent translation of the TK enzyme, it may act to attenuate the level of its expression.

Plasmid	TK Enzyme Activity
pVUTK1	1,836
0.7pVUTK1	109,756
1/34TK1	8,501
0.7-1/34TK1	57,025
IEPTK1	357,444

Table 3:12: Cis-activation of expression from the HSV-1 TK and the HCMV major early promoters by an enhancer element derived from the HCMV major IE gene. The plasmid constructions are described in Figs 3:3 and 3:22. Hela cells in 100 mm diameter petri dishes were transfected with 20 μg of plasmid DNA. Cells were scraped off the petri dishes in 0.5 ml of PBS, pelleted by low speed centrifugation and resuspended in 100 μl of TK extraction buffer. The results of the TK enzyme assays are expressed as cpm/10 μl of cell extract. Duplicate samples were assayed three times each, the average calculated and the value obtained by assaying an extract from untransfected cells (28,648) subtracted.

Discussion

The nuclease mapping technique of Berk and Sharp (1977) was used to precisely map the 5' and 3' termini of the HCMV major IE gene and the exact location of four exons. The structural mapping of the IE gene permitted the amino acid sequence of the major IE polypeptide to be deduced from the DNA sequence (Akrigg et al., 1985; appendix 2). Translation is initiated within the second exon 966 bases downstream of the transcriptional start site which, after making allowance for the 5' proximal intron, indicates the 1.95kb major IE mRNA has a leader sequence of 139n.

The molecular weight of the HCMV major IE polypeptide, as calculated from the predicted amino acid sequence, is 55,001 daltons (strain AD169) and not 64K as reported previously (Stenberg et al., 1984; Akrigg et al., 1985). The discrepancy was due to a failure to compensate for the loss of H_2O molecules during peptide bond formation. Since the polypeptide is phosphorylated its final molecular weight following post translational processing will be slightly greater than 55,001 daltons but still considerably less than molecular weight estimations made by SDS-PAGE (approximately 75K; Blanton and Tevethia, 1981; Cameron and Preston, 1981; Gibson, 1981a; Wilkinson et al., 1984). The presence of high concentrations of proline, an α-helix breaker, at both termini may contribute towards the protein's anomolous migration in SDS-PAGE; similar discrepencies in molecular weight estimations have also been reported for other proline-rich herpesvirus proteins (Watson et al., 1982; Frink et al., 1983; McGeoch et al., 1986; Perry et al., 1986). The major IE polypeptide is mainly hydrophilic, particularly at the glutamic acid/aspartic acid-rich carboxy-terminal end, and carries a net negative charge which is consistent with its experimentally determined isoelectric point of approximately pH 5.9 (Gibson, 1983; Stenberg et al., 1984).

The structural organisation of the major IE genes of HCMV strains AD169

and Towne are very similar (Stenberg et al., 1984: Akrigg et al., 1985; this thesis). The 5' ends of the mRNAs are apparently identical and the 3' terminus of the strain AD169 gene is located only 1bp downstream of that reported for strain Towne. There are only three nucleotide differences in the coding sequence of the two strains which produce two amino acid changes in the encoded proteins: the 68th amino acid is glutamine and arginine and the 394th amino acid is valine and alanine in strains AD169 and Towne respectively. The similarity between the IE polypeptide amino acid sequence of the two strains was unexpected since their electrophoretic mobilities on SDS-PAGE indicated a difference of approximately 3K in their apparent molecular weights (Cameron and Preston, 1981; Gibson, 1981a). The anomolous migration of the major IE polypeptides of strains AD169 and Towne in gels is probably caused by an SDS-resistant conformational property bestowed by the high proline content of the two proteins. The two amino acid differences between the proteins may produce conformational changes (in the presence of SDS) which disproportionately affect their electrophoretic migration.

Exonuclease protection experiments precisely mapped the 5' end of the IE gene and thereby identified the promoter-regulatory region of the major IE gene. The extensive nucleotide sequence homology between the IE genes of strains AD169 and Towne extends into the promoter where there are only 13 base differences in the reported sequences (Thomsen et al., 1984; Akrigg et al., 1985). The DNA sequence of the HCMV IE promoter contains a number of remarkable features. In addition to a consensus TATA box between bases -23 to -29 the promoter contains a complex mixed series of imperfect direct repeats which consist most notably of three copies of a 21bp repeat, four copies of a palindromic 19bp repeat, four copies of a 18bp repeat and three copies of a 16bp repeat. Due to the palindromic nature of the 19bp repeat there is potential for the formation of a variety of stem loop structures within the IE promoter (Thomsen et al., 1984; Akrigg et al., 1985).

The HCMV IE promoter has been shown experimentally to contain an

exceptionally strong enhancer which has been mapped between bases -118 to -524 (Boshart et al., 1985). Enhancer elements are defined as cis-acting regulatory elements which potentiate transcriptional initiation from nearby genes relatively independent of distance or orientation (Weiher et al., 1983). The activity of enhancer elements is mediated through their interaction with diffusable transcription factors (Scholer and Gruss, 1984). Deletion studies indicated that the HCMV major IE promoter contains a series of elements each of which independently exhibits enhancer activity in an SV40-based 'enhancer trap'. The efficiency with which the different sub-elements from the IE enhancer region stimulated expression was increased when they were combined (Boshart et al., 1985). The core enhancer consenus sequence TGG^{AAA}_{TTT} identified by Weiher et al. (1983), which is contained in three of the four copies of the 18bp repeat, was however shown not to be essential for enhancer activity (Boshart et al., 1985).

The IE promoter also contains three CAAT box homologies (in 3 of the 4 copies of the 19bp repeat), four potential binding sites for the transcription factor Sp-1 (GGCGGG or CCCGCC; Gidoni et al., 1984) and five experimentally-determined binding sites ($TGGN_5GCCAA$) for nuclear factor 1 (NF1). Hennighausen and Fleckenstein (1986) have shown that NF1 binds specifically (in vitro) to five sites surrounding the IE promoter, four are located far upstream between bases -620 to -725 but the binding site with highest affinity for NF1 is located inside the first intron at approximately base +350.

Following virus infection the HCMV major IE gene is expressed in differentiated but not in undifferentiated human embryonal carcinoma (EC) cells (Gonczol et al., 1984). In chromatin extracted from HCMV-infected undifferentiated EC cells Nelson and Groudine (1986) identified five DNAase hypersensitive sites within the enhancer region, one near the TATA box and two adjacent to the NF1-binding site in the first intron. In chromatin purified from differentiated EC cells additional DNAase hypersensitive sites

were detected near the transcriptional start site and between bases -650 to -975, the latter region contains the upstream region associated with NF1-binding (Nelson and Groudine, 1986). Although the binding of NF1 to sequences upstream from the IE promoter is associated with transcriptional induction in EC cells there is currently no evidence to indicate NF1 has a regulatory role in IE gene expression. Indeed, in transient DNA transfection experiments transcription and transcriptional repression of the IE promoter (e.g. in IEPTK1 and IEP1catIEterm) can take place in the absence of both the upstream and downstream NF1-binding sites.

The transcription of the 1.95kb major IE RNA and probably the majority of RNA species derived from IE coding region 2 is initiated from the major IE promoter (Stenberg et al., 1985). The efficient expression of this transcriptional unit during the first 6h p.i. is ensured by the presence in the IE promoter of the strongest enhancer element which has yet been identified (Boshart et al., 1985). The HCMV major IE gene contains an exceptionally efficient constitutive promoter, in transient DNA transfection experiments expression from IEP1catIEterm is approximately 20 fold and 40 fold stronger than that from similar constructs based on the RSV LTR promoter and the SV40 early promoter respectively (Akrigg and Wilkinson, unpublished data; section 3:11). A different version of the HCMV IE promoter, linked to a polyadenylation signal sequence derived from SV40, was found to be approximately four-fold more efficient than the RSV promoter in driving expression of bovine growth hormone (Pasleau et al., 1985). Expression from the HCMV IE promoter is similarly much more efficient than that from the SV40 and RSV promoters in established cell lines; indeed the HCMV promoter has been decribed as being the "system of choice for high-level expression with mammalian vector systems" (Foecking and Hofstetter, 1986).

In a series of transient DNA transfection experiments (using the plasmid constructs IEPTK1, IEPTK2, AccHincat and IEP1catIEterm) the level of

expression from the major IE promoter was found to be further enhanced by infection with HCMV. This virus-induced stimulation of expression from the IE promoter was not anticipated since during HCMV infections of permissive cells in tissue culture transcription from the IE promoter is repressed after 6h p.i. (Nelson and Groudine, 1986). Expression of HSV-1 IE genes is known to be stimulated by a transcriptional trans-activator which is carried in the virion (Campbell et al., 1984). Experiments were therefore performed to investigate whether the HCMV major IE promoter was also being stimulated by a component of the virion. The level of expression from the reporter gene was measured in cells transfected with IEPTK1 which were (i) mock-infected, (ii) infected with u.v.-inactivated HCMV and (iii) infected with viable HCMV. These experiments showed that u.v.-irradiation abolished the majority, but not all, of the virus-induced stimulation of expression from the IE promoter. Inhibitors of HCMV DNA replication (e.g. PFA) were also shown to reduce the extent HCMV stimulated expression from the transfected IE promoter; a result which is consistent with the contention that the transfected IE promoter was stimulated by a de novo synthesised HCMV-encoded gene product. Expression from the transfected HCMV IE promoter was therefore stimulated both by a de novo synthesised, virus-induced trans-activator and, to a much lesser extent, by a component of virus infection which was resistant to u.v. light.

Two other groups have recently shown that, in the enforced absence of virus-encoded protein synthesis, HCMV infections stimulate expression from the major IE promoter. Using a continuous cell line containing integrated copies of the HCMV major IE promoter (linked to the chicken ovalbumin gene), Stinski and Roehr (1985) observed that the amount of RNA transcribed from the IE promoter increased following infection with HCMV in the presence of anisomycin. In a similar experiment Spaete and Mocarski (1985b) transfected a plasmid containing the IE promoter linked to bacterial β-galactosidase (the reporter gene) into permissive cells which were then infected with

HCMV in the presence of cycloheximide. Expression from the reporter gene, measured following cycloheximide-release in the presence of actinomycin D, was found to be stimulated by HCMV infection.

Although these three independently-conducted experiments all indicated that HCMV infections stimulate expression from the IE promoter in the absence of de novo virus-encoded protein synthesis, it does not necessarily follow that the stimulation is caused by a virion trans-activator analogous to HSV-1 Vmw65. It is possible that events involved in virus adsorption and penetration may produce metabolic changes within cells which either enhance transcription or relieve transcriptional repression from the IE promoter. For example, by merely binding to its surface receptor HSV-1 stimulates transcription from a subset of cellular genes (Kemp et al., 1986). Results obtained in a number of recent studies are consistent with there being a labile cellular protein which interacts with the HCMV IE promoter to suppress its transcription. Boom et al. (1986) produced a rat cell line containing IE coding regions 1 and 2 of strain AD169 stabily integrated into the cellular genome. In immunofluorescence studies only approximately 1% of the transformed cell line was observed to express the major IE antigen at detectable levels. However, when the cell line was exposed for a short period (3h) to an inhibitor of protein synthesis or given a heat shock, synthesis of the major IE nuclear antigen was induced in up to 20% and 80% of cells respectively. Administering a heat shock to cells prior to infection with HCMV has also been shown to increase the percentage of Vero cells which express the IE antigen from 6.4% to 25.3% (Zerbini et al., 1985) and, in permissive cells, to both shorten the eclipse phase and increase the plaquing efficiency of the virus (Zerbini et al., 1986/87). It is possible that virus adsorption and penetration may induce a 'heat shock' or similar response which inactivates a cellular factor which represses HCMV IE transcription.

Campbell and Preston (1987) observed that while PRV apparently does not

encode a virion trans-inducing factor (TIF), expression from the PRV IE gene was stimulated by HSV-1 TIF. A 15bp direct repeat within the PRV IE regulatory region exhibits sequence homology with the 18bp repeat in the HCMV IE promoter (which contains the enhancer 'core' consensus sequence) and the HSV-1 TAATGARATTC sequence, which has been shown to be involved in TIF-mediated transcriptional stimulation (Bzik and Preston, 1986; Campbell and Preston, 1987), (Table 4:1). Campbell and Preston (1987) suggested that in HCMV and PRV the presence of multiple copies of the "positive control sequence" may promote enhancer activity whereas in HSV-1 efficient IE gene expression is dependent on TIF stimulating transcription. The enhancer 'core' consensus sequence which is contained in the HCMV 18bp repeat is also part of a sequence which is conserved between the SV40 enhancer (Boshart et al., 1985), an enhancer element in the kappa immunoglobulin gene (Sen and Baltimore, 1986) and the HIV LTR (Nabel and Baltimore, 1987), (Table 4:1).

It has recently been proposed that negative regulation of transcription from the HCMV major IE promoter, during virus infections, may be mediated

Sequence		
G N T A A T G A R A T T C	HSV-1	Consensus
G G C C A A T G A G A T T G T	PRV	-420 to -435
G G C C A A T G G G A T T T Y	PRV	Consenus
(C/G) C (A/T) (A/T) A C G G G ACT T T C C A A	HCMV 18 bp	Consensus
G G G ACT T T C C A	SV40	Enhancer
G G G ACT T T C C A	HIV	Enhancer
G G G ACT T T C C A	Kappa Ig	Enhancer

Table 4:1: Homology between the HCMV 18bp repeat and other promoter elements.

by the major IE gene product itself. Stenberg and Stinski (1985) transfected COS-1 cells with a plasmid containing the entire HCMV major IE gene and a mutated form of the same plasmid in which a DNA fragment encoding 145 amino acids at the carboxy-terminus of the major IE polypeptide had beeen deleted. The level of expression of both the major IE RNA and protein were observed to be appreciably less in cells transfected with the plasmid containing the wild-type IE gene than it was in cells transfected with the mutated plasmid. Furthermore, co-transfection with the wild-type IE gene resulted in a reduced level of expression from the mutated form. From these results it was deduced that the major IE polypeptide acts in _trans_ to suppress transcription from its own promoter and that this autoregulation involved the carboxy-terminal end of the major IE polypeptide. The major IE protein, however, does not appear to bind directly with DNA although it has been reported to associate "preferentially with chromatin" (Stinski _et al._, 1983). Immunofluorescence studies show the HCMV major IE antigen to be distributed evenly throughout the nuclei rather than being preferentially associated with replicating complexes of viral DNA, as has been reported for the autoregulatory HSV-1 Vmw175 IE polypeptide (Randall and Dinwoodie, 1986). Although the HCMV major IE protein is associated with the nucleus, the limited biochemical data available suggest it may not bind specifically to HCMV DNA (section 1:18).

The HCMV-induced stimulation of expression from the IE promoter in IEPTK1-transfected cells contrasts with the situation during productive infections where transcription from the IE promoter is repressed from 6h p.i. The anomolous behaviour of the IE promoter in transfected cells was the subject of a systematic investigation. The inability to repress expression from the transfected IE promoter implied that the HCMV-induced repressor of IE transcription (RIT) was not interacting effectively with its receptor. Since the IE promoter-regulatory region in IEPTK1 extends only from bases -299 to +69, it was hypothesised that the _cis_-acting regulatory signal

required for IE transcriptonal repression may not be contained in IEPTK1. The IE promoter-regulatory sequence was therefore extended to 1.7kb upstream of the transcriptional start site in the plasmid IEPTK2. HCMV infection, however, was still found to stimulate expression from the extended promoter. The result of this and similar experiments indicated that the inability of HCMV infections to repress expression from the transfected major IE promoter was not due to the absence of a repressor-binding sequence.

The second possibility considered was that the putative repressor, the major IE polypeptide, might fail to be synthesised in cells transfected with a plasmid containing the IE promoter. It was envisaged that high copy numbers of the transfected IE promoter may compete with low copy numbers of the major IE promoter on the HCMV genome (introduced by infection) for essential transcription factors and thus prevent expression of the major IE polypeptide. Hypothetically, in the absence of its repressor, the transfected IE promoter could then be stimulated by HCMV-encoded trans-activators. Cells transfected with the plasmid IEP1lassaterm, which contains the lassa virus nucleocapsid protein under control of the HCMV major IE promoter, were able to simultaneously express both the lassa virus antigen and, following HCMV infection, the HCMV major IE antigen. It was therefore demonstrated that the presence of high copy numbers of the IE promoter in transfected cells did not, following subsequent virus infection, prevent expression of the HCMV major IE antigen.

Although the presence in cells of high copy numbers of the transfected IE promoter did not prevent HCMV gene expression, following virus infection, it could still have been sufficient to saturate the RIT function and thereby permit expression from the transfected IE promoter to be stimulated by HCMV-encoded trans-activators. In order to test this hypothesis, the copy number of the IE promoter introduced into cells was lowered simply by serially reducing the concentration of IEPTK1 or IEP1catIEterm used in DNA transfections. As the concentration of IEPTK1 used per transfection was

lowered so was the relative stimulation of expression from the IE promoter induced by virus infection. However, a three-fold increase in copy number of the IE promoter in transfected cells produced only an approximately two-fold increase in TK activity detected in uninfected cells as compared with an approximately three-fold increase in infected cells. This indicated that although the level of TK activity detected in uninfected cells was proportional to the concentration of transfected IEPTK1 DNA, competition for a limiting cellular factor also appeared to be restricting expression as the concentration of IEPTK1 DNA was increased. In HCMV-infected cells, on the other hand, a linear relationship existed between the concentration of transfected IEPTK1 DNA and the amount of TK activity detected in transfected cells. HCMV infection could, therefore, apparently overcome the constraints imposed on expression by limiting cellular factors.

Although the relative stimulation of expression from IEPTK1 DNA by HCMV infection was less when lower copy numbers of the plasmid were used, HCMV infection was not observed to repress expression from IEPTK1. In similar serial dilution/transfection experiments performed with the plasmid IEP1catIEterm, HCMV infection did repress expression from the transfected IE promoter. The activity of an HCMV-induced repressor was clearly unmasked by reducing the concentration of IEP1catIEterm DNA used in transfections. The inability to detect HCMV-induced repression of IEPTK1 expression may be due to the assay for TK activity being less sensitive than that for CAT activity. Alternatively, the TK structural gene may in some way affect expression from the IE promoter in IEPTK1-transfected cells. The possibility that the polyadenylation site from the IE gene, which is present in IEP1catIEterm but not in IEPTK1, was required for repression of the IE promoter was tested using a series of plasmid constructs containing all four possible combinations of the HCMV major IE promoter or the SV40 early promoter with either the SV40 or HCMV IE polyadenylation signal driving expression of the CAT gene. While the DNA fragment containing the HCMV IE

polyadenylation signal specifically increased expression from the HCMV promoter (A. Akrigg, personal communication; Fig 3:17), it was not essential for repression of the IE promoter. A sequence in the HCMV IE gene between positions -299 and +69 therefore contains a cis-acting signal which responds to an HCMV-induced repressor.

The identity of the sequence within the IE promoter involved in transcriptional repression was further investigated in a series of competitive co-transfection experiments. The rationale behind these experiments was based on the assumption that when the IE promoter was present in low copy numbers in transfected cells RIT repressed its expression, but when the IE promoter was present in high copy number the RIT function was saturated and HCMV-encoded trans-activators stimulated its expression. Large amounts of subcloned DNA fragments from the IE promoter, and elsewhere, were therefore co-transfected with low concentrations of IEP1catIEterm DNA in order to distinguish the DNA fragments, which by binding with the repressor, would enable HCMV-encoded trans-activators to stimulate expression from the IE promoter. The situation in the co-transfected cells, however, transpired to be more complex.

When the plasmid IEP1catIEterm was co-transfected with IEP1, p0.38, pAat2, pAat3, IEP1-aat/0.38-aat, HindIII Z or pMD102 the level of CAT expression from the major IE promoter was appreciably reduced. An internal standard, i.e. a reporter gene under the control of different promoter, was not used in these experiments because of the possibility that it could, potentially, also compete for RIT. However, since the presence of the co-transfected plasmids reduced the level of expression from IEP1catIEterm both consistently and (in some cases) very substantially, it was considered extremely unlikely that the effect could be due entirely to variations in the transfection frequency. The observed reductions in constitutive IEP1catIEterm expression were probably caused by the co-transfected plasmids competing for enhancer-binding proteins. Using a similar DNA transfection

protocol, Scholer and Gruss (1984) observed that pSV2cat plasmid DNA became saturating for available transcription factors at a concentration of 1 μg per 25 μg of total DNA applied to 10 cm petri dishes of CV-1 cells. In competitive co-transfection experiments, using homologous and heterologous promoter elements, the cellular transcription factor which was most readily saturated by high concentrations of SV2cat DNA interacted with the enhancer region (Scholer and Gruss, 1984; Scholer et al., 1985). Serial dilution of IEP1catIEterm DNA in transfection experiments indicated that it also began to saturate available transcription factors when used at a concentration of between 0.3 to 1.0 μg per transfection.

The plasmids IEP1, p0.38 and pMD102 have all been shown experimentally to contain enhancer elements (results not shown; Everett et al., 1983). Since co-transfection with pAat2, pAat3, IEP1-aat/0.38-aat, pAat/Nco and HindIII Z also reduced constitutive expression from IEP1catIEterm, the result provides indirect evidence that these cloned fragments may also all contain enhancer elements. The Sst-A fragment, which probably does not contain an enhancer, was least effective in reducing constitutive expression. The smallest cloned DNA fragment which competed effectively for transcription factors was only 59bp (contained in pAat3) but included a complete copy of the 18bp repeat and two partial copies of the 19bp repeat. The result obtained with pAat3 supports the observation of Stinski and Roehr (1985) that the 18bp and 19bp repeats contribute towards efficient expression from the IE promoter. It is interesting to note that the SV40 promoter, which shares homology with the 18bp repeat (Fig 4:1), also competes with the HCMV IE promoter for transcription factors. The extent to which plasmids containing different promoter-regulatory elements competed with IEP1catIEterm DNA for transcription factors was observed to vary considerably. It is possible that more than one enhancer-binding protein may be present in limiting concentrations and/or that some of the competing plasmids have different affinities for the same transcription factor.

The original aim of the competitive co-transfection experiments was to identify cis-acting sequence elements involved in repressing rather than enhancing HCMV IE transcription. In competition experiments, HCMV infection stimulated expression only when IEP1catIEterm was co-transfected with plasmids containing upstream promoter elements derived from the HCMV major IE gene. Co-transfection with these fragments, however, also reduced the level of constitutive expression from the IE promoter. Indeed, the HCMV-induced levels of expression in these samples were lower than levels detected in pUC18 or pAT153/IEP1catIEterm co-transfected cells in which expression was repressed by HCMV infection. In cases where HCMV infection stimulated expression from IEP1catIEterm, it was probably accomplished by increasing the availability of transcription factors which had been saturated by the competing plasmid. In competitive co-transfection experiments, HCMV encoded trans-activators appeared to be unable to stimulate expression from IEP1catIEterm above its maximum constitutive level in uninfected cells.

Since expression from the SV40 early and RSV promoters (both of which contain enhancer elements) was stimulated by HCMV infection, even when they were transfected at a low DNA concentration (0.3 µg/20 µg of total DNA), the RIT function would appear to have some degree of promoter specificity in its action. Co-transfection with the plasmid pMD102 (which contains the SV40 promoter) reduced the level of constitutive expression from IEP1catIEterm, a result which implies that the SV40 promoter was competing with the HCMV IE promoter for enhancer-binding proteins. Although HCMV appears to increase the availability of transcription factors in infected cells, virus infection did not stimulate CAT expression in IEP1catIEterm/pMD102 co-transfected cells. It would appear that while upstream promoter sequences from the major IE gene are able to compete out both enhancer-binding proteins and the RIT function (thus making IEP1catIEterm inducible by HCMV infection), the SV40 promoter may compete only for enhancer-binding proteins.

During the course of productive HCMV infections in tissue culture cells transcription from the IE promoter is repressed from 6h p.i. which implies that the negative effect on transcription of RIT is dominant over the positive influences of the enhancer or HCMV-induced trans-activators. Even if the IE promoter is being expressed inefficiently the HCMV-induced repressor should dominate the effects of trans-activators, indeed this is the case in cells transfected with IEP1catIEterm and pMD102 or HindIII Z. The results of the co-transfection experiment are therefore consistent with RIT binding to and being saturated by sequence common to pAat2, pAat3, pAatNco, p0.38-aat/IEP1-aat and p0.38. Although the IE promoter contains a mixed series of tandem repeats, none are shared by all the fragments which conferred inducibility on the IE promoter. It is possible that RIT may interact, either directly or indirectly, with more than one sequence element. The competitive co-transfection experiments indicated that RIT recognises at least three discrete regions in the IE promoter between bases -135 and -457 delineated by the AatII sites at -321 and -401. It would be interesting now to chemically synthesise the 16, 18, 19 and 22bp repeats and experimentally determine the effect each repeat had on both constitutive and HCMV-induced expression from the IE promoter in competitive co-transfection experiments. Potentially, the results of this experiment could differentiate promoter elements involved in enhancing and repressing transcription from the IE promoter.

The adenovirus Ela gene products have been shown to specifically repress transcription from promoters containing enhancer elements and to activate transcription from adenovirus early promoters and some cellular genes (Borrelli et al., 1984; Svensson and Akusjarvi, 1984). Adenoviruses carrying deletions in the Ela gene are host range mutants which can replicate only in a few cell types including 293, Hela and teratocarcinoma cells (Imperiale et al., 1984). 293 cells contain an integrated copy of the Ela gene which provides a helper function in trans, while teratocarcinoma

cells, and possibly also Hela cells, are thought to constitutively express functions which have virtually identical properties to transcriptional activator and repressor activities induced by the Ela gene products (Imperiale et al., 1984; Hen et al., 1986). Although productive HCMV infections are restricted to cells of human origin most types of mammalian cells will, following infection, express IE and at least some early viral genes (DeMarchi, 1983c). Hela cells, 293 cells (LaFemina and Hayward, 1986), undifferentiated EC cells (Gonczol et al., 1984) and lymphocytic stem cells (Braun and Reiser, 1986; Reiser et al., 1986) are notable exceptions in which even HCMV IE genes are not expressed. HCMV DNA is taken into the nuclei of undifferentiated EC and 293 cells following infection but is not replicated (Gonczol et al, 1984; LaFemina and Hayward, 1986). However, 293 (unpublished results), Hela (section 3:2) and EC cells (J. Sinclair, personal communication) will all express the major IE gene when it is introduced by DNA transfection. In transfected cells high copy numbers of the IE promoter may be saturating the cellular (Ela-like) repressor. Although RIT may be more specific than the Ela gene product in its action, both repressors act on DNA elements associated with enhancer activity. HCMV and adenovirus may both negatively regulate transcription by activating similar cellular repressor functions. However, it is also possible that the HCMV major IE polypeptide interacts directly with its own promoter to suppress transcription.

Although the site of HCMV latency in vivo has yet to be established, recent clinical and laboratory studies suggest it may lie within a subpopulation of lymphocytes (section 1:25). In vitro HCMV replication in T lymphocyte was detected only after cell growth had been stimulated with interleukin 2 (Reiser et al., 1986) and in bone marrow stem cells only after they had been precultured. It is possible that lymphocytic stem cells may contain an enhancer-specific transcriptional repressor similar to the Ela-like function in EC cells. When lymphocytes are stimulated to grow in

vitro, they may also undergo a differentiation step in which the repressor of HCMV IE gene expression becomes inactive. In latently infected cells this may induce virus replication.

An alternative mechanism for the re-activation of HCMV from lymphocytes is suggested by recent work on HIV. Mitogenic activation of lymphocytes has been shown to stimulate the binding of a protein (NF-kappaB) to an enhancer sequence present in both the kappa immunoglobulin gene and the HIV LTR resulting in transcriptional stimulation (Sen and Baltimore, 1986; Nabel and Baltimore, 1987). The binding of NF-kappaB to the enhancer stimulates transcription from adjacent promoters and, in the case of HIV, this stimulation may be involved in reactivating virus from latently infected lymphocytes. The DNA sequence recognised by NF-kappaB is also contained in the 18bp repeat present in the HCMV IE enhancer region (Nabel and Baltimore, 1987; Table 4:1). By analogy, the induction of NF-kappaB activity could potentially also stimulate HCMV IE gene expression in latently infected lymphocytes and thereby reactivate virus infection.

The reactivation of HCMV infections in immunosuppressed individuals is an important clinical problem particularly in transplant recipients and AIDS patients. A greater understanding of the mechanisms involved in regulating HCMV IE gene expression may provide valuable insight into both how the latent infections are established and, also, how subsequently the virus is able to reactivate.

During the early phase of the replicative cycle HCMV encodes a single high abundance 2.7kb RNA and an unspecified large number of additional low abundance RNAs (Wathen and Stinski, 1982; unpublished results). The 2.7kb early RNA, which is not spliced (McDonough *et al.*, 1985; section 3:13), is transcribed from a gene contained within both copies of the strains AD169 and Towne long repeats but is present as a single copy in the U_L region of strain Davis (DeMarchi, 1981; Wathen and Stinski, 1982; Wilkinson, 1983). The relatively rapid and efficient synthesis of this transcript suggested

that it may encode an important function. The 2.7kb HCMV major early gene was therefore studied in more detail.

The 5' end of the 2.7kb early gene was identified by a combination of a primer extension and S1 nuclease analysis. Assuming translation is initiated at an AUG codon and that coding assignments are normal the longest ORF associated with the gene would encode a polypeptide of 170 amino acids. Translation of the predicted polypeptide would be initiated from the fourth AUG codon downstream from the transcriptional start site, the previous three initiation codons all being associated with very short ORFs. 'False' initiation codons have been shown to be present in the leader sequences of other herpesvirus genes, e.g. the thymidylate synthase gene of HVS (Bodemer et al., 1986). The predicted polypeptide would be basic, have an exceptionally high proline content (14%) and contain two potential O-glycosylation sites within hydrophilic domains. The early promoter plus either 135bp or 95bp of leader sequence has been cloned upstream of the reporter genes β-galactosidase and TK respectively (Spaete and Mocarski, 1985b; section 3:14). Efficient expression was obtained from both these constructs following HCMV infection of the transfected cells (Spaete and Mocarski, 1985b; section 3:14). Ribosomes must therefore recognise the 'leader sequence' of the 2.7kb RNA and remain attached to the RNA at least until after the first short ORF (8 amino acids). The sequence adjacent to the proposed translational initiation codon for the 170 amino acid ORF (CACCCAUGC), however, is not in good agreement with the consensus sequence (CC$^{A}_{G}$CCAUGG) identified by Kozak (1984) with respect to the key bases at positions -3 and +4. Attempts to identify the protein predicted by the 170 amino acid ORF in vitro by hybrid selected translation, by analysing [^{35}S]-methionine and [^{3}H]-proline pulse-labelled proteins in infected tissue culture cells by SDS-PAGE and in prokaryotic expression systems have been unsuccessful (Wilkinson and Greenaway, unpublished results). Additionally, an analysis of the 170 amino acid ORF using the "Testcode" algorithm devised

by Fickett (1982) indicated there is only a 40% probability of the ORF being used. The possibility that the proposed ORF is not used and that the 2.7kb early RNA does not encode a functional polypeptide cannot be ruled out.

A characteristic property of HCMV is its relatively long replicative cycle, in the same permissive cell system HSV-1 and HCMV have an eclipse phase lasting 6h and 4 days respectively (Smith and DeHarven, 1973). The initial events following infection, however, occur rapidly with the expression of HCMV nuclear antigens being detected within 1h p.i. (Michelson-Fiske et al., 1977). Blanton and Tevethia (1981) were able to detect the synthesis of early polypeptides between 4-6h p.i. in immunoprecipitation experiments and Geballe et al. (1986a) identified two mRNA encoding predominantly late phase proteins (the abundant 65K tegument protein and the 48K DNA-binding protein) as early as 4h p.i. The transition from IE to early phase gene expression therefore occurs by 4h p.i.

The nucleic acid sequence and structural analysis of the HCMV major early gene provided the basis to investigate the transition from IE to early phase gene expression. DNA/RNA hybridisation experiments showed that although the 2.7kb early RNA was synthesised in low abundance under IE conditions, it was not detected in cytoplasmic RNA extracted from cells 2h p.i. The 2.7kb RNA was synthesised in trace amounts by 8h p.i. but by 24h p.i. it was present in high concentrations and was the predominant viral transcript. Although classed as an early transcript the 2.7kb RNA continued to be synthesised in even higher abundance during the late phase.

Expression from the HCMV early promoter was measured in DNA transfection studies using the plasmid 1/34TK1 in which the 2.7kb major early gene promoter regulates expression from the HSV-1 TK structural gene. The results obtained using the HCMV early promoter in transfection experiments were consistent with the DNA/RNA hybridisation data. Although constitutive expression from 1/34TK1 is extremely low, efficient expression

was detected following infection with HCMV. Even when a high m.o.i. was used, however, activation of expression from the transfected early promoter was not detected until after 8h p.i. Although the transfection studies did not detect stimulation of early gene expression until 8h p.i. the transition from IE to early phase gene expression occurs earlier. While the TK enzyme assay is extremely sensitive, it was necessary in these experiments to use HCMV-permissive human fibroblasts which have a low transfection frequency relative to Hela cells. It may be that expression from the transfected early promoter was stimulated by HCMV-encoded trans-activators before 8h p.i. at a level too low to be detected above background.

HCMV infections were able to stimulate expression from all the transfected promoters which were tested; i.e. the HCMV major IE the SV40 early, the RSV LTR, the HSV-1 TK (early) and the HCMV major early promoters. Time course experiments showed that, as with the HCMV early promoter, trans-activation of the HCMV major IE (artificially) and the HSV-1 early promoters was not detected before 8h p.i. In experiments performed in parallel, however, infection with HSV-1 efficiently stimulated expression from the transfected HCMV major IE and HSV-1 early promoters by 8h p.i. HSV-1 infections were unable to significantly stimulate expression from the transfected HCMV early promoter. Spaete and Mocarski (1985b) have recently reported similar results with the HCMV IE and early promoters while O'Hare and Hayward (1984) have shown that infection with simian CMV stimulates expression from the HSV-1 TK promoter.

The lag period between infection and detectable stimulaton of expression from transfected promoters was approximately 8h longer with HCMV than with HSV-1 infections. This 'lag period' is neither a property of the cell, since MRC5 cells were used throughout these experiments, nor HCMV early promoters since the effect was also observed using the HCMV IE and HSV-1 early promoters. Although post-transcriptional regulation of HCMV RNA processing has been described (DeMarchi, 1983a; Geballe et al., 1986a)

hybridisation data using both whole cell and polysomal RNA suggest the control is primarily exerted at the transcriptional level (DeMarchi *et al.*, 1980; DeMarchi, 1984). The gradual activation of early phase and consequently the protracted nature of HCMV early phase gene expression appears to be a fundamental property of HCMV transcriptional activation. A HCMV *trans*-activator has been mapped within strain AD169 *Hin*dIII fragment E (Everett, 1984), which contains IE coding regions 1, 2 and 3. In co-transfection experiments, however, Everett (1984) described the transcriptional stimulation from the HSV-1 gD promoter by the major IE gene alone as being "barely detectable". The identification of HCMV *trans*-activator functions will be central to our understanding of HCMV gene regulation and ultimately also virus pathogenicity. The characterisation of the HCMV major early gene and the fusion of its promoter to suitable reporter genes, such as in 1/34TK1, permits the activity of HCMV *trans*-activators to be assayed, in co-transfection experiments, using a homologous system. This system should enable the HCMV *trans*-activator genes to be mapped and characterised in more detail.

It has recently been suggested that HCMV also exerts control over early phase gene expression at the translational level. Geballe *et al.* (1986b) cloned a sequence spanning the transcriptional start site of the 2.7kb early gene into the major IE leader sequence and showed that the cloned insert delayed the stimulation of expression from the transfected IE promoter following infection with HCMV. This result was interpreted by the authors as indicating that the "5' leader sequence" from the 2.7kb early RNA may contain a *cis*-acting signal which delayed translation and consequently, with respect to expression, changed the temporal class of the gene from IE to early. It has been shown in this study, however, that in HCMV-infected cells the stimulation of the transfected IE promoter, by *de novo* synthesised HCMV *trans*-activators, was artificial and that under such conditions the observed stimulation of expression from the IE promoter already exhibits kinetics

similar to those of an early promoter. Additionally, expression from the early promoter, together with the relevant portion of the 'leader sequence' can be stimulated in *cis* by the IE enhancer element in DNA transfection experiments; thus translation of the 2.7kb early RNA is therefore not absolutely dependent on any *de novo*-synthesised HCMV-encoded gene product. It is possible that the AUG codon within the 'leader sequence' of the early gene may be attenuating translation from the downstream reporter gene. A reduction in the level of total expression, by the effects of attenuation, could appear as a delay in HCMV-induced expression since the initial effects of the *trans*-activators may not be distinguishable above background levels in the assay.

Finally, the ability of HSV-1 to stimulate expression from the HCMV IE but not the HCMV early promoter was unexpected in view of the sequence homology identified between the HSV-1 gD and the HCMV early promoters. Mosca *et al.* (1987) have recently demonstrated that HSV-1 stimulates expression from the simian CMV (strain Colburn) IE promoter and showed that this stimulation was dependent neither on a component of the HSV-1 virion nor a function of Vmw175. The HSV-1 IE polypeptide Vmw110 can stimulate transcription from heterologous promoters in transfection experiments but is unable to extensively activate HSV-1 early phase gene expression in the absence of a functional Vmw175 protein during virus infections (see section 1:26). It has been suggested that the major function of Vmw110 in infected cells may be to stimulate the transcription of HSV-1 IE genes (O'Hare and Hayward, 1985b). It is therefore possible that in HSV-1 infected cells Vmw110 may be able to stimulate transcription from the transfected HCMV major IE promoter but not the HCMV major early promoter. The HSV-1 Vmw175 protein has been shown to bind specifically to sequences in HSV-1 IE genes to negatively regulate HSV-1 IE transcription (Faber and Wilcox, 1986). The apparent inability of HSV-1 Vmw175 to stimulate expression from the HCMV early promoter may reflect a degree of specificity in activator function of

Vmw175.

The basic morphology of herpesvirions is sustained throughout the group and has, historically, been the primary basis for their classification. In recent years the grouping of all herpesviruses into the single family, the Herpesviridae, has been supported by the results of DNA sequence analyses. Many of the genes involved in replication, cleavage and packaging of virus DNA as well as genes encoding structural virion polypeptides have been conserved, to varying extents, between different members of the Herpesviridae (Baer et al., 1984; Spaete and Mocarski, 1985a; Cranage et al., 1986; Davison and Scott, 1986; Kouzarides et al., 1987a; 1987b; and references within). The homologies which have been identified are consistent with the Herpesviridae being comprised of a single group of related viruses derived from a common ancestor.

This study has concentrated primarily on the IE and early phases of HCMV gene expression. Like HSV-1, HCMV operates a cascade system of gene regulation which is conventionally divided into three phases; IE, early and late. However, a more detailed analysis identifies differences in the way the two viruses regulate gene expression. It has been known for some time that while infection with HSV-1 suppresses cellular macromolecular synthesis, in HCMV-infected cells it is stimulated. The efficient expression of HSV-1 IE genes is dependent on transcriptional stimulation in trans by a component of the virion (TIF) and the IE polypeptide Vmw110 (synthesised de novo), whereas efficient expression of the HCMV major IE gene and its associated transcriptional unit appears to depend primarily on the stimulation of transcription in cis by an extaordinarily strong enhancer element (Boshart et al., 1985). Although infection with u.v.-inactivated HCMV produced a slight stimulation in expression from the major IE promoter in DNA transfection experiments, there is currently no firm evidence that HCMV virions contain an analogue of the HSV-1 TIF.

In both HCMV and HSV-1 infected cells, IE gene expression is negatively

regulated at the transcriptional level (Nelson and Groudine, 1986; O'Hare and Hayward, 1985b). The HSV-1 IE protein, Vmw175 has been shown to suppress transcription through its interaction with a conserved sequence adjacent to the transcriptional start site of HSV-1 IE genes (Pizer et al., 1986; Muller, 1987). While it has been proposed that the HCMV major IE protein also autoregulates its own expression (Stenberg and Stinski, 1985), results obtained in competitive co-transfection experiments suggested that negative regulation of the HCMV major IE promoter is mediated through cis-acting sequences associated with its enhancer elements.

The transition from the IE to the early phase of gene expression occurs by 4h p.i. in both HCMV and HSV-1 infected cells. However, the stimulation of early phase transcription is a much more gradual process in HCMV-infected cells. Results obtained in this study indicate that the relatively slow rate of HCMV early phase gene activation is due neither to a property of HCMV-permissive cells nor HCMV early promoters. Infection with HCMV was also observed to stimulate expression from a wider range of promoters in transfection experiments. The different properties of HCMV and HSV-1 trans-activators suggest that their mode of action may not be identical.

The Herpesviridae is divided into three sub-families primarily on the basis of the biological properties of its members, e.g. host range, growth rate and lesions produced in vivo (section 1:1). While HSV-1 IE genes share homology with IE genes in other alphaherpesviruses (Davison and Wilkie, 1983; Davison and Scott, 1986), the HCMV major IE gene has not been shown to exhibit homology with any of the characterised herpesvirus IE genes nor with the EBV genome. Herpesvirus IE gene products are generally associated with the regulation of gene expression. It is interesting to speculate that some of the characteristic properties of the herpesvirus sub-families may be attributable to their different mechanisms of regulating gene expression. Members of the betaherpesvirinae are charateristically slow-growing and have a restricted host range. The slow growth of HCMV may well be due to the very

gradual _trans_-activation of early and late gene expression, while its restricted host range appears to be related to an inability to efficiently activate transcription from the virus genome in non- and semi-permissive cell system (DeMarchi, 1984). The potential for the enhancer element in the major IE gene to be involved in the modulation of latency has already been discussed. The mechanisms involved in regulating herpesvirus gene expression may ultimately prove to be crucial not only in determining their biological properties but also as determinants of pathogenisis.

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