AN INVESTIGATION OF THE CELLULAR STRESS RESPONSE IN CELLS INFECTED WITH HERPES SIMPLEX VIRUS.

By

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SUMMARY.

This study entailed an investigation of the cellular stress response in secondary chick embryo fibroblasts (CEF) during infection by HSV. It was established that infections at a †NPT with temperature-sensitive mutants of HSV-1 which are defective in immediate-early viral polypeptide Vmw IE 175 (i.e. tsD, tsK and tsT) cause the stress response to be induced, as manifest by a marked stimulation of synthesis of stress proteins. Induction by tsK was shown to be dependent upon the synthesis of immediate-early viral polypeptides. Infections with other mutants of HSV-1 (tsl201, tsB, tsE, tsG and MDK/2) at a NPT and, to a lesser extent, the revertant of tsK, $ts^{+}K$, or with wt HSV-1 or wt HSV-2, all of which are non-defective in Vmw IE 175 or the HSV-2 equivalent, Vmw IE 182, cause synthesis of stress proteins to be increased. To account for these observations a hypothesis was advanced, that cells are subjected to stress during infection with wt or mutant HSV, owing to the expresssion of viral functions, and that induction of the stress response is subsequently inhibited depending upon the characteristics of the infecting virus: inhibition is most effective in cells infected with wt viruses, and least effective in cells infected at a NPT with temperature-sensitive mutants of HSV-1 defective in Vmw IE 175.

The responsiveness of cells to treatment with the stress-inducing reagent, disulfiram, was inhibited during infections by HSV-1. The immediate-early class of viral polypeptides was implicated in causing this inhibition: Vmw IE 175, in particular, was found to contribute to the effect, but only during an early stage of infection. Results were consistent with the hypothesis that a virus-induced stress response may be prevented early in infection by wt HSV-1, but not by mutants defective in Vmw IE 175.

Synthesis of viral polypeptides and processing of Vmw IE 68 in infected cells was adversely affected by pre-treatment of the cells with stress-inducing reagents. Therefore the stress response may modify the cytopathogenicity of HSV-1 and the cell's susceptibility to productive infection.

Vmw IE 175 migrated from the cytoplasmic to the nuclear

† Non-permissive temperature

fractions of wt HSV-1-infected or tsK-infected cells incubated at either a permissive or a non-permissive temperature, and was detectable in nuclear fractions as forms with decreased electrophoretic mobility. The processing of nuclear Vmw IE 175, and also of cytoplasmic Vmw IE 110, was defective in cells infected with tsK at a NPT, compared with wt HSV-1-infected cells.

Cytoskeletons were extracted from HSV-1-infected or disulfiram-treated CEF and their polypeptide components compared. Stress proteins, whether their synthesis had been induced by treatment of cells with disulfiram or by infection of cells with tsK at a NPT, partitioned similarly between the cytoskeleton-containing fractions and respective supernatants. Infection of cells with wt HSV-1 or tsK and treatment of cells with disulfiram caused reduction in the abundance of radiolabelled polypeptides recovered with cytoskeletons. Viral polypeptides were detected in the cytoskeleton-containing fraction from HSV-1-infected cells; and Vmw IE 68 appeared to be recovered exclusively in this fraction.

To investigate the possible involvement of the stress response in the previously-documented abortive infections by HSV at hyperthermic temperatures, low-multiplicity infections of human embryonic lung cells with wt HSV-1 and wt HSV-2 at 41.5-42 ^{o}C were examined. The persistence of infectious virus in the presence of 0.5% or 2% of serum, and the suppression of productive infection by 10% serum indicated the importance of cellular factors in abortive infections at such temperatures. Following infection at a multiplicity of 0.01, using 10% serum and incubation at 41.5-42 ^{o}C for not less than 6 days, cultures were found to survive restoration to 37 ^{o}C for 12-17 days before succumbing to productive infection. Elevating the concentration of serum from 0.5% or 2% to 10% at 41.5-42 ^{o}C caused suppression of productive infection; and surviving (carrier) cultures were propagated at 37 ^{o}C in excess of 47 days post-infection without undergoing spontaneous reactivation. Recovery of cells from abortive infection was suggested by the cessation of synthesis of viral polypeptides during incubation at 41.5-42 ^{o}C, and by the loss of specific viral antigens from carrier cultures during propagation at 37 ^{o}C. The cellular stress response was activated in infected cells by incubation at 41.5-42 ^{o}C, and may have been involved in the decrease in synthesis

of viral polypeptides.

Infecting viruses were reactivated from surviving cultures by superinfection with temperature-sensitive mutants at a NPT. DNA extracted from reactivated HSV-2 isolates differed from the prototype in that the restriction fragment, BamHI g, demonstrated variability in electrophoretic mobility.

Viral information was not detectable in passaged carrier cultures by titration, by immunofluorescence using monoclonal antibodies to HSV-1 polypeptides, by Southern blot analysis (at the level of 1 viral genome per cell), or by transfection of cellular DNA (at the level of two viral genomes per cell). The inability to detect infectious virus in cell extracts may have reflected an insensitivity of the titration assay in detecting a chronic infection. Evidence was not obtained that the suppression of lytic infection at 41.5-42 ^{o}C was mediated by a short-lived, repressor protein, since attempts to induce reactivation by treatment of infected cultures with cycloheximide were unsuccessful. Consequently, the data do not allow to discriminate whether cultures infected at 41.5-42 ^{o}C using EF10, and carrier cultures that were derived from these infections, contained virus in a non-replicating or a persistently replicating state.

Results of Southern blot hybridizations indicated that HSV-1-virion DNA and plasmid DNA containing HSV-1-specific sequences are homologous with sequences in the DNA of uninfected human cells. The stringency of the hybridization and washing conditions indicated that these cellular sequences are (G+C) rich, and either highly homologous or partially homologous but highly reiterated. Hybridization was increased to DNA from cultures abortively infected with wt HSV-1 at 41.5-42 ^{o}C, compared with DNA from similarly-treated, mock-infected cells, and may have reflected virus-induced amplification or rearrangement of repetitive cellular sequences.

ABBREVIATIONS.

ara-C ..... cytosine arabinoside
ATP ..... adenosine triphosphate

BHK-21 C13 .... baby-hamster kidney (cells), clone 13
bp ..... base pair(s)
Bq..... bequerels
BSA ..... bovine serum albumin

CEF ..... chick-embryo fibroblasts
Ci ..... curie
c.p.e. ..... cytopathic effect
c.p.m. ..... counts per minute

d ..... day(s)
dATP ..... 2´-deoxyadenosine-5´-triphosphate
dCTP ..... 2´-deoxycytidine-5´-triphosphate
dGTP ..... 2´-deoxyguanosine-5´-triphosphate
dTTP ..... 2´-deoxythymidine-5´-triphosphate
DATD ..... N,N´-diallyltartardiamide
DF ..... Dye-Ficoll
DIS ..... disulfiram (antabuse; bis (diethyl dithio carbamate) disulphide)
DMSO ..... dimethyl sulphoxide
DNA ..... deoxyribonucleic acid
DNase ..... deoxyribonuclease
DTT ..... dithiothreitol

EDTA ..... ethylene diaminetetra-acetic acid

FC ..... filament caps

G+C ..... moles per cent deoxyguanosine plus deoxycytosine moieties
GS1 ..... gel soak 1
GS2 ..... gel soak 2

h ..... hour(s)

ABBREVIATIONS

HeBS ..... HEPES-buffered saline
HeLu ..... human-embryo lung (cells)
HEPES ..... N-2-hydroxyethylpiperazine N´-2-ethane sulphonic acid
HSV ..... herpes simplex virus
HSV-1 ..... herpes simplex virus type 1
HSV-2 ..... herpes simplex virus type 2

IE ..... immediate early

k ..... $x10^3$
kb ..... kilobase(s)
kbp ..... kilobase pairs
KTS ..... kethoxal bis(thiosemicarbazone)

min ..... minute(s)
m.o.i. ..... mutiplicity of infection
mRNA ..... messenger RNA
MTR1 ..... morphological transforming region of HSV-1.
MTR2 ..... morphological transforming region of HSV-2.
m.wt. ..... molecular weight

NPT ..... non-permissive temperature

OD ..... optical density
oz ..... ounce

PAGE ..... polyacrylamide gel electrophoresis
PBS ..... phosphate-buffered saline
p.f.u. ..... plaque forming units
p.i. ..... post-infection
p.m.i. ..... post-mock-infection
PPO ..... 2,5 diphenyloxazole
PT ..... permissive temperature

REF ..... rat-embryo fibroblasts
RNA ..... ribonucleic acid
RNase ..... ribonuclease
rRNA ..... ribosomal RNA

ABBREVIATIONS

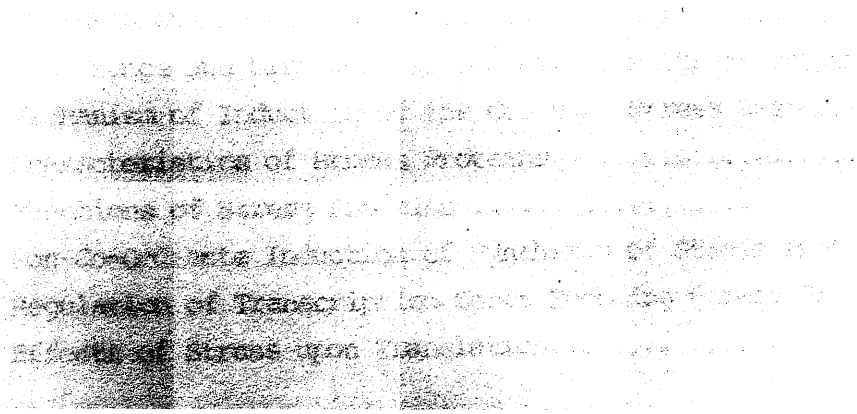
r.p.m. ..... revolutions per minute
RSV ..... Rous sarcoma virus

SDS ..... sodium dodecyl sulphate (sodium lauryl sulphate)
SP ..... stress protein
syn ..... syncytial
syn^+ ..... non-syncytial

TCA ..... trichloro-acetic acid
TEMED ..... N,N,N´,N´-tetramethylethylenediamine
TK ..... thymidine kinase
TP ..... tryptose phosphate broth
Tris ..... tris (hydroxymethyl) aminomethane (trizma base)
ts ..... temperature-sensitive

U ..... units
u.v. ..... ultraviolet
(v/v) ..... volume:volume (ratio)

wt ..... wild-type
(w/v) ..... weight:volume (ratio)



INDEX OF INTRODUCTION.

INTRODUCTION.

The first section of the Introduction describes the biology of herpesviruses, with particular emphasis upon herpes simplex virus types 1 and 2, which were employed in this study. The two subsequent sections briefly review those areas which are of particular interest to the experimental results: firstly, latent infection by HSV *in vivo*, and *in vitro* systems which may be relevant to this phenomenon; and secondly, the cellular response to metabolic stress.

A. BIOLOGY OF THE HERPESVIRUSES.

Classification of Herpesviruses.

The herpesviruses form part of a homogeneous grouping, the *Herpesviridae*, having a common morphology and a size of molecule of double-stranded DNA ($80-150 \times 10^6$), but displaying diverse growth characteristics (Fenner, 1976; Matthews, 1982) and large variation in the deoxyguanosine plus deoxycytidine (G+C) content (37-75%) and in the extent of homology between the viral genomes (Honess & Watson, 1977a). The *Herpesviridae* are divided into three sub-families, the *alphaherpesvirinae*, *betaherpesvirinae* and *gammaherpesvirinae*, on the basis of size of the viral genome, host range, duration of the lytic cycle, cytopathogenicity and characteristics of latent infection (Matthews, 1982).

Alphaherpesvirinae are characterized by a wide host-range *in vitro*, a short lytic cycle (<24 h) and the ability to spread rapidly in cell culture. These viruses may establish latent infections in the ganglia, and are typified by herpes simplex virus type 1 (HSV-1). The sub-family also includes herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), pseudorabies virus (PRV), equine herpesviruses types 1, 2 and 3 (EHV-1, -2 and -3) and bovine mammillitis virus (BMV). The m.wt. of the viral DNA is $85-110 \times 10^6$.

In comparison with *alphaherpesvirinae*, *betaherpesvirinae* have

a larger genome (m.wt. 130-150x10^6) and a longer lytic cycle (>24 h). The host range in vitro is restricted to fibroblasts. These viruses cause enlargement of infected cells (cytomegalia) both in vivo and in vitro, and may become latent in lymphoreticular tissues. Carrier cultures are easily established. The sub-family is typified by human cytomegalovirus (HCMV).

Gammaherpesvirinae possesses a genome of similar size to that of alphaherpesvirinae (m.wt. 85-110x10^6). The duration of the lytic cycle is variable. Viruses of this sub-family are usually considered to be lymphotrophic and to have a narrow host range in vitro; all viruses replicate in lymphoblastoid cells and are specific for either B- or T-lymphocytes, in which they may cause either lytic or latent infection. However, certain viruses, such as the type-species, Epstein Barr virus (EBV: Sixbey et al., 1983), and Marek's disease virus (MDV: Payne et al., 1976), also replicate lytically in epithelial cells in vivo or in vitro. This sub-family includes herpesvirus saimiri (HVS).

Pathogenicity of Herpesviruses.

Herpesviruses are transmitted by contact between moist mucosal surfaces. The five human herpesviruses, HSV-1, HSV-2, VZV, EBV and HCMV, are prevalent in all populations, and frequently persist for the life-time of the infected individual, primary or reactivated infection causing acute systemic or neurological diseases.

HSV-1 and HSV-2, although related genetically and serologically, are distinguishable by their different pathogenicities (Dowdle et al., 1967). HSV-1 is predominantly associated with oral and facial lesions (gingivostomatitis and 'cold sores': Fiddian et al., 1983), the primary infection occurring usually during childhood (Buddingh et al., 1953). HSV-2 is associated with genital lesions (Dowdle et al., 1967) and is the second most common agent of venereal disease. In addition to the common clinical manifestations, these viruses are responsible for several more serious diseases, such as ocular keratitis (Falcon, 1983), nonepidemic fatal encephalitis (Davis & Johnson, 1979)

and disseminated infection in neonates (Whitley et al., 1980). Following the primary infection, both HSV-1 and HSV-2 have the capacity to persist in the individual in the absence of clinical symptoms. Repeated reactivation of infection may occur at intervals to produce either recrudescence of clinical disease (see section B) or asymptomatic shedding of virus into various secretions (Douglas & Couch, 1970).

Several herpesviruses are known to be tumorigenic in their natural hosts: Lucké herpes virus (LHV; Granoff, 1983), MDV (Payne et al., 1976) and possibly EBV. EBV is a causative agent of infectious mononucleosis, and is the virus which is linked most convincingly to human neoplasms, consistently being recoverable from the cells of patients with two forms of cancer, Burkitt's lymphoma and nasopharyngeal carcinoma (Epstein et al., 1964, 1965; Biggs et al., 1972). HSV-2 has been implicated in carcinoma of the cervix by epidemiological (Naib et al., 1973; Rawls et al., 1973), immunological (Aurelian et al., 1971; Pacsa et al., 1976; Dreesman et al., 1980; Gilman et al., 1980) and molecular hybridization (Eglin et al., 1981; Galloway & McDougall, 1983; Park et al., 1983) studies. HSV-2 is claimed to be oncogenic in newborn hamsters (Nahmias et al., 1970b); and in cell culture, both HSV-1 and HSV-2 can convert cell strains into continuous cell lines, which may cause invasive tumours in the appropriate experimental hosts (Duff & Rapp, 1971, 1973; Darai & Munk, 1976; Macnab et al., 1980). A single region of the HSV-1 genome has been implicated in causing morphological transformation, between 0.31-0.41 map units (restriction fragment BglII i, or MTR1; Reyes et al., 1979), whereas two regions have been implicated for HSV-2, between 0.41-0.58 (BglII c; Jariwalla et al., 1980) and 0.58-0.62 map units (BglII n, MTR2; Reyes et al., 1979; Cameron & Macnab, 1980; Galloway & McDougall, 1981). The significance of this difference between the two viruses, and the mechanism of transformation by HSV, remain to be elucidated.

Structure of the Herpesvirus Virion.

The herpesvirus virion is 120-200 nm in diameter, and is composed of:

(i) a central, electron-dense core containing the viral DNA (Furlong et al., 1972) wrapped about an electron-translucent, proteinaceous spool (Chai et al., 1971).

(ii) an icosahedral capsid, 100-110 nm in diameter, consisting of 162 capsomers (Wildy et al., 1969). The capsomers are elongated prisms, which are hexagonal and hollow in cross-section; and intercapsomeric fibrils have been observed (Wildy et al., 1969; Vernon et al., 1974).

(iii) the tegument, an amorphous, fibrous layer surrounding the capsid (Roizman & Furlong, 1974).

(iv) the envelope, a bilayer membrane with surface projections, which surrounds the tegument and is impervious to negative stain.

Thirty-three structural viral polypeptides, some of which are phosphoproteins or glycoproteins, have been identified in purified, mature virions of HSV-1 (Spear & Roizman, 1972; Heine et al., 1974; Gibson Roizman, 1974; Marsden et al., 1976); and these show a close correspondence with the structural viral polypeptides of HSV-2 (Cassai et al., 1975; Killington et al., 1977). Most of the glycoproteins appear to be located in the envelope (Spear and Roizman, 1972). [Two main systems of nomenclature exist for the polypeptides of HSV-1 and HSV-2: the first (Marsden et al., 1976), which is used throughout this study, employs the prefix, Vmw, followed by the m.wt. of the polypeptide $x10^{-3}$; the second is a numerical system, and employs the prefix ICP (infected cell polypeptide; Honess & Roizman, 1973). Alternative designations for a viral polypeptide are given where appropriate. Viral glycoproteins are designated according to the nomenclature adopted at the Eighth International Herpesworkshop (1983)]. Seven major capsid polypeptides have been identified in HSV-1, ranging in m.wt. from 12,000 to 155,000 (Cohen et al., 1980); Vmw 155 (or ICP 5) is the major capsid polypeptide (Gibson & Roizman, 1974), and Vmw 43 (also termed VP21) may represent the core protein (Gibson & Roizman, 1972). Although Vmw 40 is not a constituent of the mature virus particle, this polypeptide has been proposed to undergo modification (possibly cleavage or phosphorylation) to form VP22, a virion

polypeptide that is loosely bound to the capsid (Gibson & Roizman, 1974). An HSV-1 mutant, tsl201, fails to encapsidate viral DNA at the NPT (Preston et al., 1983) as a result of a temperature-sensitive (ts) defect in the processing of Vmw 40.

Structure of the Genome of HSV.

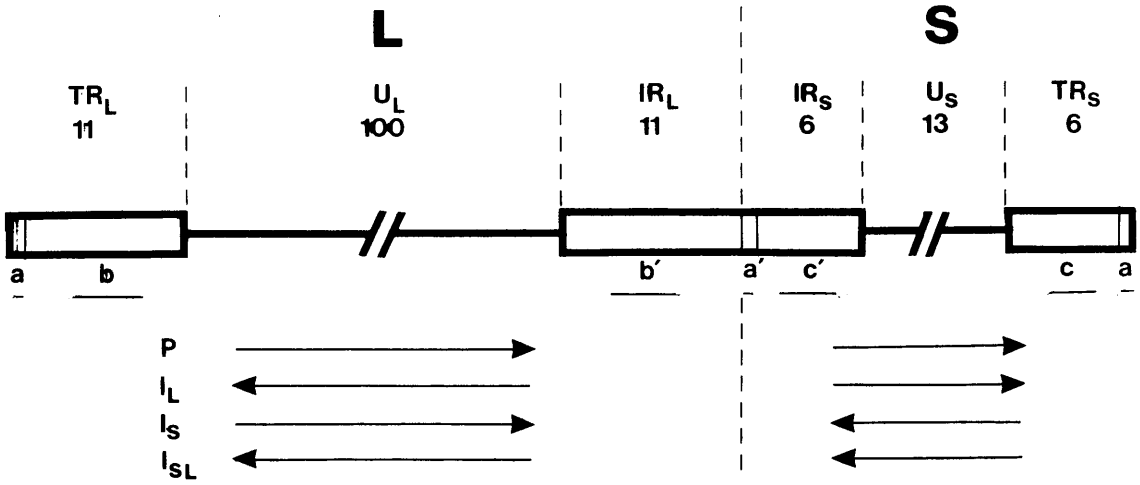
The genomes of HSV-1 and HSV-2, shown schematically in **Fig. i**, are linear, double-stranded molecules of DNA of approximately 150 kilobase pairs, and whose m.wt.'s have been determined by various methods to be approximately $100x10^6$ (Ben-Porat & Kaplan, 1962; Russell & Crawford, 1964; Kieff et al., 1971; Wilkie, 1973; Grafstrom et al., 1974; Frenkel & Roizman, 1971; Clements et al., 1976; Wilkie, 1976). The G+C contents of the genomes of HSV-1 and HSV-2 are 67% (Kieff et al., 1971) and 69% (Halliburton, 1972), respectively. Although the CpG dinucleotide is present at a reduced frequency in mammalian cell DNA, this feature is not shared by the DNA of HSV (Subak-Sharpe et al., 1966). The genomes of HSV-1 and HSV-2 show least 47% sequence homology, but differ in the location of many restriction enzyme cleavage sites (Kieff et al., 1972; Morse et al., 1977; Cortini & Wilkie, 1978). The genomes are colinear, as revealed by intertypic recombination and complementation (Esparza et al., 1976), by hybridization studies (Davison & Wilkie, 1983a), and by mapping of genes encoding specific viral functions (see below).

The DNA molecules of HSV-1 and HSV-2 possess alkali-labile regions, and centrifugation on alkali sucrose gradients provides populations with short, single-stranded breaks (Kieff et al., 1971; Wilkie, 1973). This efect has been suggested to reflect the presence of ribonucleotides in the DNA molecule (Muller et al., 1979), or of nicks at specific sites (Wilkie et al., 1974).

The genome of HSV consists of a long (L) and a short (S) segment, comprising 82% and 18% of the viral DNA, respectively, and each of which includes a unique region (U_L or U_S), which is bounded by inverted repeats (TR_L and IR_L for the long segment, and TR_S and IR_S for the short segment) (Hayward et al., 1975; Sheldrick & Berthelot,

Fig. i. The structure of the genome of HSV.

The viral genome has a m.wt. of 100×10^6 and contains approximately 150 kbp. The molecule consists of a long (L) and a short (S) segment, each of which comprises a unique region (U_L or U_S) bounded by inverted repetitions (TR_L and IR_L; TR_S and IR_S). Fractional genome units (or map units), and the postitions and relative orientations of repeated sequences (a, b and c; and a′, b′ and c′) are indicated. During viral replication, the L and S segments invert; consequently, the genomes of viral progeny consist of equimolar amounts of four isomers (P, I_L, I_S and I_{SL}) differing only in the relative orientation of the two segments.


L
S
TR_L
11
U_L
100
IR_L
11
IR_S
6
U_S
13
TR_S
6
a
b
b´
a´
c´
c
a
P
I_L
I_S
I_SL

1975; Delius & Clements, 1976; Roizman, 1979). Inverted repeats flanking the U_L sequences have been designated ab and b´a´, those flanking U_S, a´c´ and ca (Wadsworth et al., 1975). The a sequence was defined originally as that terminal sequence required to be exposed by a processive exonuclease for optimal circularization of HSV DNA to occur (Wadsworth et al., 1976). This sequence is present in the same orientation at the ends of the molecule, and in an inverted orientation at the L-S junction (Grafstrom et al., 1974, 1975; Wagner & Summers, 1978; Davison & Wilkie, 1981; Mocarski & Roizman, 1981). The a sequence is the only known sequence that is common to both segments, and it is generally assumed that the terminal and joint a sequences are identical. The a sequences of HSV-1 (strains 17 and USA 8) and HSV-2 (HG52) are approximately 400bp and 250bp in length, respectively, and have high G+C contents (83% and 84%), but show little sequence similarity (Davison & Wilkie, 1981).

Sheldrick & Berthelot (1974) suggested that such an organization of the viral genome could lead, through recombination, to inversion of the L and S segments; this would result in a population of molecules with an equimolar concentration of each of four conformations of DNA, differing only in the relative orientation of the two segments. This prediction has been confirmed by restriction endonuclease analysis of HSV DNA (Hayward et al., 1975; Wilkie & Cortini, 1976; Skare & Summers, 1977) and by partial denaturation analysis (Delius & Clements, 1976). One of these conformations has been designated as the prototype (P), and the others are defined by inversion of the S segment (I_S), of the L segment (I_L), or by inversion of both the L and S segments (I_{LS}), as depicted in Fig. i (Roizman et al., 1974; Hayward et al., 1975). It has been established that the a sequence of HSV-1 contains the signals which direct inversion of the L and S segments (Mocarski et al., 1980; Smiley et al., 1981; Mocarski & Roizman, 1982a, b), as well as the cleavage of unit-length DNA molecules from concatemers (Mocarski & Roizman, 1982b) and the encapsidation of viral DNA (Stow et al., 1983).

Lytic Cycle of HSV.

Infection of cultured cells by HSV is, in general, productive and cytocidal (Wagner & Roizman, 1969; McCormick, 1978). The lytic cycle of HSV *in vitro* is rapid, progeny virions appearing in the nucleus as soon as 6 h post-infection (p.i.). Although the ultimate yield of virions may be in excess of 10^4 particles per infected cell, only a small fraction of these will score as plaque-forming units.

The probable manner in which HSV initiates cellular infection, as suggested by Morgan *et al.* (1968) from studies using electron microscopy (e.m.), involves five stages: adsorption of virus to the cell membrane, digestion of the envelope, digestion of the juxtaposed area of the cell membrane, passage of the capsid into the cytoplasm, and digestion of the capsid with release of the core. Evidence supporting this sequence of events is accumulating, including demonstrations that the viral envelope is required for infectivity (Smith, 1964; Spring & Roizman, 1968; Rubinstein *et al.*, 1972; Stein *et al.*, 1979), and that receptors for virus are present on the cell membrane (Hochberg & Becker, 1968; Blomberg, 1979; Vahlne *et al.*, 1979). Electrostatic interactions may be important in the initial attachment of HSV to cells, perhaps requiring divalent cations (Spivack *et al.*, 1982). The probable temperature-dependence of the second stage of infection, the digestion of the viral envelope, indicates the involvement of an enzymatic reaction (Farnham & Newton, 1959; Holmes & Watson, 1963; Huang & Wagner, 1964). The viral glycoprotein, gA/gB, may be involved in the penetration of the capsid into the cell (Manservigi *et al.*, 1977; Sarmiento *et al.*, 1979; Little *et al.*, 1981; Spivack *et al.*, 1982). A DNA-protein complex is translocated to the nucleus by an unknown mechanism (Hochberg & Becker, 1968; Hummeler *et al.*, 1969). (An HSV-1 mutant, tsB7, has been isolated which is defective in uncoating (Knipe *et al.*, 1981) and which causes viral capsids containing viral DNA to accumulate at the nuclear pores (Batterson & Roizman, 1983).) On reaching the nucleus, the viral genome is expressed and viral replication is initiated.

The sequence of development of viral particles in infected cells has been well characterized by e.m. (Morgan *et al.*, 1959; Nii *et al.*, 1968a; Miyamoto & Morgan, 1971; Smith & de Harven, 1973) complemented with the use of inhibitors of viral replication (Nii *et al.*,

An HSV-1 mutant, ts 1204, has been characterised which has a ts defect in penetration into cells and assembly of nucleocapsids (Addison *et al.*, 1984).

1968b; Friedman et al., 1975; Marciano-Cabral et al., 1978) or virus mutants (Cabral & Schaffer, 1976; Atkinson et al., 1978; Dargan & Subak-Sharpe, 1983). Characteristic features of infection by HSV are the condensation and margination of chromatin, and the distortion and reduplication of the nuclear membrane. Intranuclear, hollow viral particles become apparent by 5 h p.i., and dense, enveloped particles by 6 h p.i.. Later in infection (by about 24 h p.i.), nuclei appear to contain crystals of capsids and accumulations of amorphous, granular material. Rarely, bizarre forms are evident, including dense filaments and tubular structures, which have been shown by kinetic studies to be intermediates in virus assembly (Iwasaka et al., 1979; Oda et al., 1979). Dargan & Subak-Sharpe (1983) reported additional morphological alterations to cells, resulting from infections with ts mutants of HSV at a NPT: namely, a modification of the rough endoplasmic reticulum, and the intranuclear accumulation of enveloped virus particles and ring-like particles. After assembly in the nucleus, the capsid migrates to the nuclear membrane, from which it acquires an envelope. Only particles containing a dense core are selected for envelopment. The envelopment ensues from a budding process which leaves the nuclear membrane intact; and the resulting enveloped particles are enclosed, either singly or in pairs, in vesicles derived either from the outer lamella of the perninuclear cisterna or from a lamella of the endoplasmic reticulum, which are continous structures (Falke et al., 1959; Epstein, 1962b; Darlington & Moss, 1968; Nii, 1971; Gibson & Roizman, 1972). The enclosed, enveloped particles subsequently migrate to the plasma membrane, where the vesicle opens, releasing the enveloped particle and maintaining the continuity of the plasma membrane, by a process tantamount to the reverse of phagocytosis (Darlington & Moss, 1968). Alternatively, virions may exit the cells via cytoplasmic ducts (Schwartz & Roizman, 1969; Nii, 1971). Viral glycoproteins are situated in the membranes of the infected cell, thereby providing virus-specific envelopes (Roizman & Spear, 1971).

The transmission of infectious virus occurs through the medium or by intercellular passage (Stoker, 1958). Infection causes alteration to the gross morphology of the cell; strains of wt virus produce cell rounding, whereas some mutant strains, termed 'syn' strains, cause the recruitment of nuclei into giant cells, or syncytia (Gray et al.,

1958; Hoggan & Roizman, 1959).

Inhibition of Synthesis of Host Protein in HSV-Infected Cells.

The lytic cycle of HSV proceeds at the expense of synthesis of cellular DNA (Cohen et al., 1971), RNA and rRNA (Wagner & Roizman, 1969; Sasaki et al., 1974; Roizman et al., 1979), and protein (Sydiskis and Roizman, 1966). Current understanding of the effects of infection by HSV on the synthesis of host macromolecules has been reviewed by Fenwick (1984).

The suppression of synthesis of host protein during infection by HSV is a rapid process, and two stages have been discerned to date:

(i) An initial "shut-off" is mediated by a heat-labile component of the virion, and entails initially the disaggregation of host polysomes (Sydiskis & Roizman, 1966; Nishioka & Silverstein 1977, 1978; Fenwick & Walker, 1978; Fenwick & Clark, 1982; Read & Frenkel, 1983). This is accompanied by virion-induced inhibition of the initiation of translation (Newton & Rasouly, 1983), and also by virion-induced inactivation of the template activity of existing cellular mRNA (Inglis & Newton, 1981; Fenwick & McMenamin, 1984; Newton & Rasouly, 1983). Following their dissociation, the polysomes subsequently reassociate, and, although cellular mRNA is synthesized at a low level throughout infection, 90% of the mRNA which is associated with the polysomes late in infection is virus-specific (Stringer et al., 1977). Roughly half of the polyadenylated RNA in the cytoplasm of infected cells is cellular (Stenberg & Pizer, 1982). However, in in vitro assays, viral mRNA is found to be translated less efficiently than cellular mRNA (Inglis & Newton, 1981), and the mechanisms of discrimination in vivo between cellular and viral mRNA remain to be determined.

Since virion-associated shut-off occurs more rapidly in cells following infection with certain strains of HSV-2 than with HSV-1, the use of intertypic recombinants has allowed the HSV-2 functions controlling the virion-associated shut-off (Morse et al., 1978; Fenwick et al., 1979) and the inhibition of synthesis of cellular

DNA (Fenwick et al., 1979) to be located in the viral genome at 0.52-0.59 map units. However, Halliburton (1980) mapped the HSV-2 function controlling the synthesis of host protein to a different region, at 0.69-0.75 map units. And in HSV-1, functions for the control of synthesis of cellular DNA and protein appear to be dissociable (Epstein & Jacquemont, 1983). Therefore, more than one locus in the viral genome may be responsible for the inhibition of synthesis of host DNA and protein in HSV-infected cells.

(ii) When virion-associated shut-off is absent, synthesis of host protein is inhibited by a process which is dependent upon the expression of viral genes (Fenwick & Clark, 1982; Read & Frenkel, 1983). Such expression-dependent shut-off requires a viral factor which is produced early or late in infection, and which is distinct from the factor responsible for virion-associated shut-off (Read & Frenkel, 1983). It has been proposed that expression-dependent shut-off and regulation of synthesis of IE viral polypeptides (see below) in infected cells are related phenomena (Silverstein & Engelhardt, 1979; Read & Frenkel, 1983). (Expression- dependent shut-off occurs also in cells infected with other herpesviruses, including PRV, EBV, HVS and HCMV (Fenwick, 1984).) There is evidence to suggest that expression-dependent shut-off involves the degradation of cellular mRNA. For example, in Friend cells, by 2 h p.i. with HSV-1, mRNA encoding globin is degraded by a process which depends upon the synthesis of protein post-infection, and which may reflect the degradation of the total population of cellular mRNA (Nishioka & Silverstein, 1978). Similarly, in polyoma-transformed BHK cells, levels of polyoma RNA are decreased by infection with HSV-1 (Pizer & Beard, 1978). Hybridization studies have shown that infection by HSV-1 causes a reduction in the level of moderately abundant cytoplasmic mRNA's, and may therefore cause selective degradation of cellular mRNA sequences (Nakai et al., 1982).

The suppression of synthesis of host protein in HSV-infected cells is likely to be a complex process, as cells that are infected at a NPT with ts mutants of HSV-1 demonstrate various degrees of inhibition (Marsden et al., 1978). Additional virus-induced effects have been reported: infection of adenovirus-transformed rat cells with HSV-1 causes inhibition of transport of adenovirus-specific RNA from the

nucleus to the cytoplasm (Spector & Pizer, 1978); and the expression of IE viral genes in HSV-1-infected, adenovirus-transformed human cells decreases the transcription of adenovirus-specific RNA (Stenberg & Pizer, 1982). Furthermore, the activity of RNA polymerase II in mouse (L) cells declines following infection with HSV-1 (Preston & Newton, 1976). During infection of cells by HSV-1, there is a loss in the permeability barrier of the cell membrane to small molecules (Benedetto et al., 1980), correlating with inhibition of synthesis of host protein; and this observation supports the hypothesis that inhibition may result from a general mechanism involving alterations in the intracellular concentration of inorganic ions.

Replication of Herpesvirus DNA.

The replication of herpesvirus DNA has been studied most extensively using HSV and PRV, but the precise mode of replication remains to be elucidated. Viral DNA is replicated semi-conservatively in the nucleus of infected cells (Newton & Stoker, 1958; Munk & Sauer, 1964; Kaplan & Ben Porat, 1964). Several viral polypeptides are required for DNA synthesis, including the DNA polymerase and the major DNA binding protein (see below). The majority of infecting PRV genomes (Jean & Ben-Porat, 1976), but only a small proportion of infecting HSV genomes (Jacob & Roizman, 1977), undergo replication. Viral DNA synthesis is initiated at about 3 h p.i., peaks at 4-6 h p.i., and is terminated by 12 h p.i. (Roizman, 1969). Using e.m., Rixon (1977) observed that early in infection by HSV, synthesis of viral DNA occurs at a number of discrete sites on the perimeter of the nucleus, and eventually encompasses the entire nuclear area.

The DNA of infecting HSV virions contains random, single-stranded nicks of unknown function, and which are repaired during DNA replication (Kieff et al., 1971; Gordin et al., 1973; Hyman et al., 1977; Nishiyama et al., 1983). Upon infection, the DNA of both PRV and HSV acquires single-stranded ends (Ben-Porat, 1976; Jacob & Roizman, 1977), which are considered to participate in the circularization of the molecule by a mechanism suggested, for HSV, to involve the a sequence (Roizman, 1979). E.m. of nascent viral DNA has revealed that early in infection, unit-sized linear and circular molecules, larger 'Y'-shaped

molecules, and molecules with terminal loops are present; larger and more complex linear or circular structures, with internal eyes and forks, are evident at later times in infection (Shlomai et al., 1976; Ben-Porat et al., 1976; Friedman et al., 1977; Jacob & Roizman, 1977; Rixon, 1977; Hirsch et al., 1977; Becker et al., 1978). Meanwhile, there is a decrease in the number of terminal fragments detectable in viral DNA by restriction endonuclease analysis (Jacob et al., 1979). These observations have been interpreted as indicating that herpesvirus DNA is replicated bi-directionally, by a rolling-circle mechanism, to yield head-to-tail concatamers (Jean & Ben Porat, 1976; Jean et al., 1977; Ben Porat & Rixon, 1977; Jacob et al., 1979).

Origins of viral replication have been detected at similar positions in the nascent DNA of HSV and PRV, close to the centre of U_L (Spaete & Frenkel, 1982), and within a sequence common to IR_S/TR_S, the inverted repeats of the short segment (Shlomai et al., 1976: Friedmann et al., 1977; Hirsch et al., 1977; Jean et al., 1977; Mocarski & Roizman, 1982; Stow, 1982). Stow & McMonagle (1983) have identified a 90bp segment in the TR_S/IR_S of HSV-1 which contains all of the cis-acting sequences necessary for activity as an origin of replication of viral DNA.

Defective Interfering Particles of HSV.

In common with other viruses, HSV gives rise to defective virus particles during serial, undiluted propagation of virus stocks, causing interference with the infectivity of standard virus (reviewed by Frenkel, 1981). The DNA molecules of these particles are equivalent in size to those present in mature virions, but represent derivatives of standard viral DNA in which deletions are substituted by multiple reiterations of low-complexity sequences in head-to-tail array. Defective particles arise at late times in infection (Becker et al., 1978), and are believed to be produced by the same mechanisms that are involved in the concatameric replication of standard viral DNA. This is supported by the observations that replication of defective particles is inhibited by phophonoacetate (PAA), an inhibitor of the viral DNA

polymerase (Frenkel, 1981), and that populations of defective genomes consist of homopolymers with a single type of repeated unit, rather than recombinant products of variable size (Frenkel et al., 1975). Thus, studies of the selective evolution of defective genomes are relevant to an understanding of the replication of the more complex, nondefective genome.

In general, defective HSV genomes may be divided into two classes. Class I defective genomes contain reiterations of sequences arising either exclusively from, or in direct continuity with, either end of the S segment of the standard DNA (Frenkel et al., 1975; Hayward et al., 1975; Frenkel et al., 1976; Graham et al., 1978; Locker & Frenkel, 1979; Vlazny & Frenkel, 1981). Class II defective genomes contain DNA sequences derived from two non-contiguous regions of the standard DNA, within the S and L segments (Murray et al., 1975; Frenkel et al., 1980; Kaerner et al., 1981; Locker et al., 1982; Knopf et al., 1983). Only two cis-acting signals are necessary for the maintenance of defective genomes within a virus stock: an origin of replication and the site specifying the cleavage of concatamers, the a sequence (Stow et al., 1983).

The delay that is observed in the replication of standard virus from stocks rich in defective particles has been suggested to arise from competition between specific sequences within the defective and standard genomes for factors involved in DNA replication (Frenkel, 1981; Schroder et al., 1984). Stow (1985) obtained evidence that the phenomenon of interference arises from the replication of defective genomes, since, in transfection assays using plasmids containing a viral origin of replication, a functional origin is required for interference with the infectivity of standard DNA to occur. However, he was unable to discriminate whether interference arose from amplification of the origin, or from amplification of adjacent but distinct sequences. Linkage of the a sequence to the origin enhanced the interfering capacity of the plasmids; and it was suggested that the role of the a-sequence in interference is to permit the encapsidation of defective genomes and the intercellular transmission of defective particles.

Transcription of HSV DNA.

The genome of HSV is transcribed in the nucleus by host RNA polymerase II (Alwine et al., 1974; Ben Ze'ev et al., 1976; Costanzo et al., 1977). The unmodified host polymerase may initiate transcription of the viral genome, as purified viral DNA itself is infectious (Graham et al., 1973). During infection, modifications may occur to the cellular polymerase (Ben Ze'ev et al., 1976; Preston & Newton, 1976; Spector & Pizer, 1978), perhaps causing preferential recognition of viral DNA sequences. Novel RNA polymerases have not been detected in HSV-infected cells (Lowe, 1978). Viral mRNA migrates from the nucleus to the cytoplasm within 15 min of infection, and becomes associated with the polysomes (Wagner & Roizman, 1969b; Stringer et al., 1977). At early and late times in infection, both polyadenylated and non-adenylated species of viral RNA are detectable in the nucleus and cytoplasm (Stringer et al., 1977). Nuclear viral RNA is larger than that associated with the polysomes (Wagner & Roizman, 1969b), but a precursor-product relationship has not yet been demonstrated.

The synthesis of viral mRNA is controlled in a sequential manner, and three phases, termed immediate-early (IE, or alpha), early (beta) and late (gamma) have been identified (Frenkel & Roizman, 1972; Swanstrom & Wagner, 1974; Clements et al., 1977; Jones & Roizman, 1979). Upon infection of the host cell, limited regions of the viral genome are transcribed to give IE mRNA's. Some or all of the products of translation of IE mRNA, the IE viral polypeptides, alter the transcription of the viral genome so that the early class of mRNA's is synthesized. Early viral polypeptides permit the replication of viral DNA.

Studies using inhibitors of protein synthesis (Rakusanova et al., 1971; Kozak & Roizman, 1974; Clements et al., 1977; Jones & Roizman, 1979) have shown that synthesis of IE mRNA occurs in the absence of synthesis of viral polypeptides. Five major species of IE mRNA's of HSV-1 and HSV-2 have been identified, together with their polypeptide products, and their transcription maps have been determined (**Fig. ii:** Clements et al., 1979; Watson et al., 1979; Easton & Clements, 1981; Whitton, 1984). It has been established that synthesis

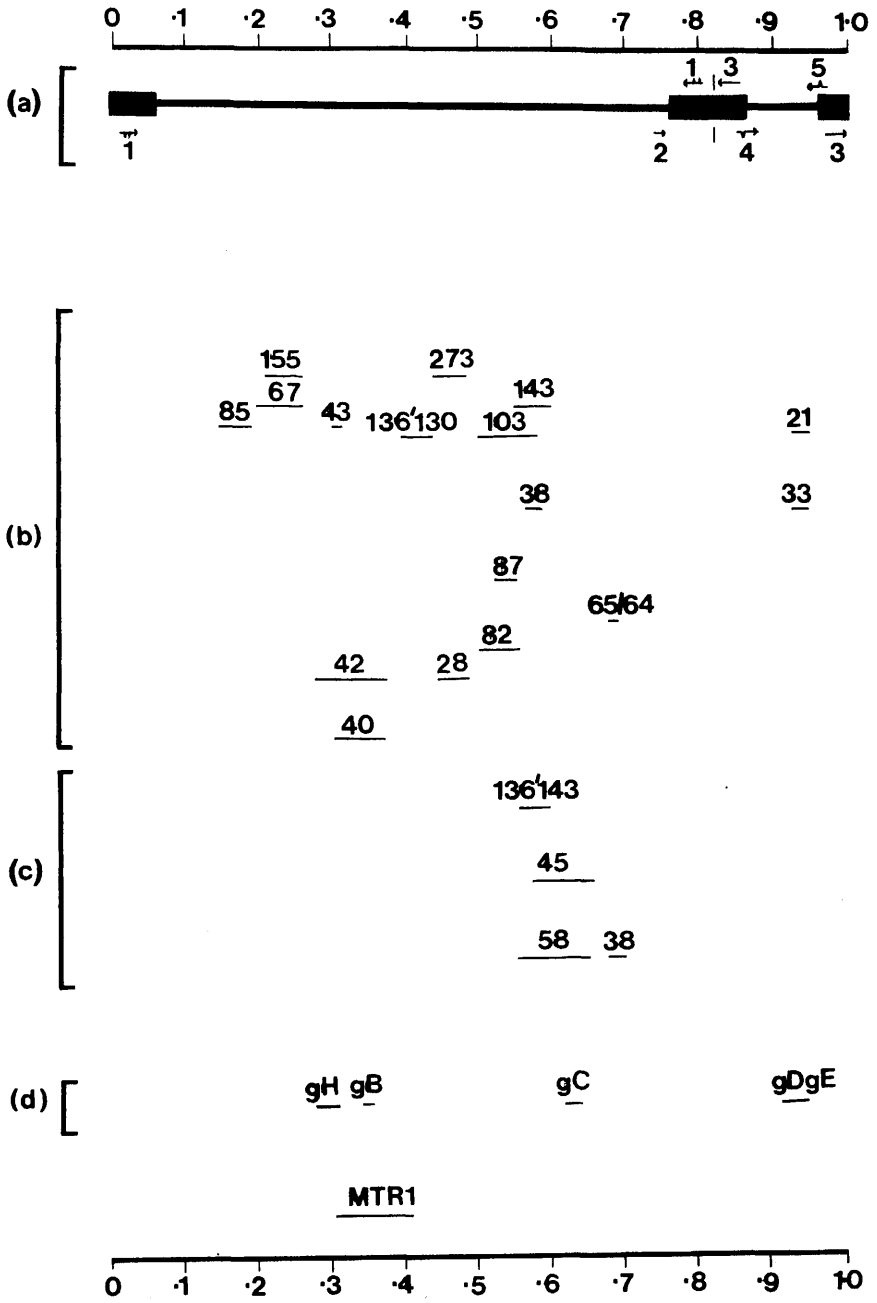
of the polypeptide product of IE mRNA 3 (Vmw IE 175), at least, is required throughout infection for the synthesis of early and late viral RNA to occur (see below).

In cells biochemically transformed with chimaeric HSV-1 DNA consisting of the promoter (or site at which RNA polymerase binds to initiate transcription) and regulatory regions of IE genes linked to the early gene for thymidine kinase, transcription of the viral DNA is regulated as an IE gene and is stimulated by a virion-associated factor (Post et al., 1981) in a type-independent manner (Cordingley et al., 1983). Those sequences which are necessary for the stimulation of transcription to occur have been located in the upstream regions of IE genes (Mackem & Roizman, 1982a, b and c; Cordingley et al., 1983; Lang et al., 1984; Whitton, 1984); and a late, structural polypeptide of HSV-1, of m.wt. 65,000 and mapping at 0.67-0.69, has been identified as the virion-associated, stimulating factor (Campbell et al., 1984).

During the course of infection, the complexity of virus-specific mRNA increases as transcription progresses from the IE to the early and late patterns, where viral mRNA's are found to correspond to all regions of the viral genome (Wagner et al., 1972; Kozak & Roizman, 1974; Swanstrom & Wagner, 1974; Clements et al., 1977; Jones & Roizman, 1979). By definition, early mRNA's are produced prior to, or in the absence of, synthesis of viral DNA, whereas late mRNA's are produced after synthesis of viral DNA has been initiated (Wagner et al., 1972; Swanstrom et al., 1975; Clements et al., 1977; Holland et al., 1980). However, it is unclear whether the distinction between early and late mRNA's merely reflects a difference in their relative abundance during infection (Swanstron, 1975), or whether their transcription is controlled in a sequential manner. Synthesis of certain mRNA's, at least, appears to be dependent upon the synthesis of viral DNA, and these species may therefore represent a true 'late' class of viral transcripts (Holland et al., 1980). Evidence has been obtained to suggest that the major DNA-binding protein of HSV-1, Vmw 136'130 (or ICP 8) represses the expression of viral genes, by decreasing the abundance of cytoplasmic viral mRNA (Godowski & Knipe, 1983).

Detailed transcription maps have been obtained for several species of early and late transcripts of HSV-1, including the mRNA for thymidine kinase (McKnight & Gavis, 1980; Smiley et al., 1980; Preston & McGeoch, 1981), glycoprotein C (Frink et al., 1981, 1983), glycoprotein D (Watson et al., 1982, 1983) and alkaline exonuclease (Costa et al., 1983). Furthermore, Rixon & McGeoch (1984) have identified three overlapping, 3´-coterminal mRNA´s of HSV-1 mapping in TR_S/U_S and encoding unique polypeptides. The longest mRNA, IE mRNA 5, encodes Vmw IE 12, and the remaining mRNA´s are late species encoding polypeptides of m.wt. 33,000 and 21,000. McLauchlan & Clements (1982, 1983) have located two overlapping mRNA´s from MTR1 and MTR2, coding for the two subunits of the viral ribonucleotide reductase, Vmw 136´143 and Vmw 38 (Dutia, 1983). Preliminary transcription maps have been constructed for HSV-1, including eighteen early and forty late mRNA´s (Anderson et al., 1979, 1980; Holland et al., 1979, 1980). The unique region of the short segment (U_S) has been sequenced in its entirety, and shown to contain twelve genes (McGeoch et al., 1985).

HSV mRNA´s have been shown to share features in common with eukaryotic mRNA´s, including internal methylation (Moss et al., 1977), terminal methylation with characteristic cap structure (Bartkoski & Roizman, 1976; Moss et al., 1977), and frequently a polyadenylic tract at the 3´ end (Bachenheimer & Roizman, 1972; Silverstein et al., 1976; Stringer et al., 1977). Sequence analysis of several HSV-1 genes has revealed the presence of the usual sequence (AATAAA) which signals polyadenylation (McKnight, 1980; Easton, 1981; Wagner et al., 1981; Clements, 1982). A consensus sequence has been located (downstream from the polyadenylation signal) which is required for the formation of 3´ termini of viral mRNA (McLauchlan et al., 1985). Certain HSV mRNA´s have been demonstrated to undergo splicing. For HSV-1, mRNA IE4 and IE5 have common 5´-terminal leader sequences derived from IR_S and TR_S, respectively, spliced to sequences derived from U_S (see **Fig. ii:** Watson et al., 1981a; Rixon & Clements, 1982); and sequence analysis (Murchie & McGeoch, Watson & Vande Woude, 1982) has revealed the presence of splice donor/acceptor sites in the viral DNA as designated by Breathnach and Chambon (1981). Whitton & Clements (1984) confirmed that similar splicing occurs in the corresponding IE mRNA´s of HSV-2. mRNA IE1 of HSV-1 has recently been found to contain


0 ·1 ·2 ·3 ·4 ·5 ·6 ·7 ·8 ·9 1·0
(a)
1
3
5
1
2
4
3
(b)
155
67
85
43
273
136
130
143
103
21
38
33
87
65
64
82
42
28
40
(c)
136
143
45
58
38
(d)
gH
gB
gC
gDgE
MTR1
0 ·1 ·2 ·3 ·4 ·5 ·6 ·7 ·8 ·9 1·0

two introns (Miss. L. Perry, personal communication). A family of 5′- and 3′-coterminal late mRNA′s of HSV-1 includes an unspliced species, which encodes glycoprotein C, and at least three minor, spliced forms; although splicing alters the coding potential of these mRNA′s, the functional significance of this processing is unknown (Frink 1983).

Synthesis of HSV Polypeptides.

More than 50 virus-specified polypeptides have been detected in productively HSV-1-infected cells by one-dimensional electrophoresis (Honess & Roizman, 1974; Powell & Courtey, 1975; Marsden et al., 1976), and 230 species, some of which may be related by post-translational modification, by higher resolution, two-dimensional electrophoresis (Haarr & Marsden, 1981). Viral polypeptides are synthesized during immediate-early (IE), early and late stages of infection, in consequence of the regulated transcription of viral mRNA.

The sequential control of synthesis of viral polypeptides was first suggested by Honess & Roizman (1974), from results of experiments in which infected cells were treated with an inhibitor of protein synthesis. Following removal of the inhibitor, different groups of viral polypeptides were synthesized, depending upon the time post-infection at which the inhibitor had been added. These data were interpreted as indicating the existence of three classes of viral polypeptides - alpha, beta and gamma - each of which induces the synthesis of, and is inhibited in its own synthesis by, the subsequent class, this sequence occuring independently of the synthesis of viral DNA.

Alpha - or immediate-early (IE) - polypeptides are produced immediately after infection, their rate of synthesis reaching a maximum between 2 and 4 h p.i. and then declining (Honess & Roizman, 1974; Pereira et al., 1977; Wilcox et al., 1980). Five major species of IE polypeptides have been recognized: Vmw IE 175, Vmw IE 110, Vmw IE 68, Vmw IE 63 and Vmw IE 12 (Preston et al., 1978; Fenwick et al., 1980; MacDonald, 1980). In addition, several minor species are synthesized depending upon the host-cell type; Vmw IE 136′143, Vmw IE 28, Vmw IE 25, Vmw IE 23, Vmw IE 16.5 and Vmw IE 12.5 (MacDonald, 1980).

At least one IE viral polypeptide, Vmw IE 175, is required to initiate and maintain the synthesis of beta - or early - viral polypeptides (see below). Synthesis of early viral polypeptides, as exemplified by Vmw 136´130, reaches a maximum between 5 and 7 h p.i. (Honess & Roizman, 1975). The gamma - or late - viral polypeptides, for example Vmw 155, Vmw 117, Vmw 82 and Vmw 65, are largely, though not exclusively, made after replication of viral DNA has begun, increasing in rate until 12 to 15 h p.i..

Experimental evidence has accumulated which indicates that the synthesis of non-IE viral polypeptides is not stringently controlled by the expression of IE genes, and that the original scheme of Honess & Roizman (1974) requires revision. For example:

(i) Treatment of HSV-infected cells with amino-acid analogues arrests the lytic cycle at the IE phase; and this is believed to arise from incorporation of the analogues into IE viral polypeptides, thereby disrupting their function (Honess & Roizman, 1975; Pereira et al., 1977). However, several non-IE viral polypeptides also are synthesized under these conditions (Pereira et al., 1977).

(ii) In BHK cells infected at a NPT with tsK, an HSV-1 mutant with a ts defect in Vmw IE 175, synthesis of non-IE viral polypeptide Vmw 117 is detectable (MacDonald, 1980).

(iii) The major DNA-binding protein, Vmw 136´130 (or ICP 8), is an early viral polypeptide, and its synthesis should therefore depend upon the expression of IE genes. However, Locker et al. (1982) showed that Vmw 136´130 was produced abundantly in cells infected with virus stocks containing class II defective particles of tsLB2 (a ts mutant which is defective in Vmw IE 175), at a NPT for the helper virus.

(iv) When infected cells are treated with inhibitors of the synthesis of viral DNA, there is a reduction in the synthesis of certain late viral polypeptides (Powell et al., 1975; Ward & Stevens, 1975; Wolf & Roizman, 1978); and from this it was suggested that late viral polypeptides may be sub-divided into two classes, one of which requires the synthesis of progeny DNA for their production. The block in

production of this latter class is partially overcome by increasing the m.o.i.; thus, there may exist a class of late viral polypeptides which depend upon a high copy number of viral genomes to stimulate their synthesis (Wolf & Roizman, 1978).

(v) Analysis of viral polypeptides that are synthesized in cells infected with ts mutants of HSV at a NPT (Marsden et al., 1976; DeLuca et al., 1984) indicates that the production of viral polypeptides may involve a more complex, inter-dependent sequence of events than that proposed by Honess & Roizman (1974).

Functions of IE Viral Polypeptides of HSV-1.

Functional IE viral polypeptides and continuing transcription are required for the expression of non-IE viral genes (Honess & Roizman, 1975). Studies of the function of IE viral polypeptides have relied upon the use of metabolic inhibitors (Honess & Roizman, 1975; Pereira et al., 1977) or of conditional lethal mutants possessing lesions in genes encoding IE viral polypeptides. To date, mutations of this type have been demonstrated only in the gene encoding Vmw IE 175 (or ICP 4) in HSV-1 (Marsden et al., 1976; Dixon & Schaffer, 1980; DeLuca et al., 1984), and studies of the corresponding mutants have demonstrated that this viral polypeptide is essential for productive infection to occur. The known function of Vmw IE 175 is to activate the transcription of early and late viral genes (Watson & Clements, 1978, 1980; Preston, 1979a, b); and cells infected at a NPT with mutants which are ts for Vmw IE 175 produce abundant IE viral polypeptides and reduced or undetectable amounts of early and late viral products (Courtney & Powell, 1975; Marsden et al., 1976; Preston, 1979a; Dixon & Schaffer, 1980; DeLuca et al., 1984). The degree of restriction of viral gene expression in certain mutants has been shown to vary with the site of amino-acid substitution in Vmw IE 175 (Marsden et al., 1976; Preston, 1981). One such mutant, tsK, which was isolated by Crombie (1975), features prominently in these studies; and the infection of cells by tsK at a NPT is effectively arrested at the IE stage (Marsden et al., 1976; Preston, 1979a). The mutation in tsK results from the substitution of alanine with valine at amino-acid residue 575 (Murchie,

1982). DeLuca et al. (1984) have isolated HSV-1 mutants which are ts for Vmw IE 175 and for virus growth, but which allow the expression of several early genes and the synthesis of viral DNA in cells infected at a NPT; and it was conseqeuently suggested that Vmw IE 175 plays a multifunctional role in the regulation of expression of viral genes. The activity of Vmw IE 175 is known to be required throughout infection by HSV-1, since raising the temperature of incubation of tsK-infected cells from a PT to a NPT causes reversion to the IE pattern of transcription of viral mRNA (Watson & Clements, 1980). However, the precise mechanism of action of Vmw IE 175 remains unknown.

Vmw IE 110 also may play an important role in virus replication in cell culture: Brown et al. (1984) observed that a certain mutation in the gene encoding this polypeptide mapping within TR_L was absent from the gene mapping within IR_L, suggesting that the mutation may not be tolerated in a homozygous condition. In addition, a mutant was isolated which lacks the internal repeat region but contains a recombinant terminal repeat region; the replication of this mutant is significantly impaired relative to wt virus.

Intact Vmw IE 68 appears to be dispensable for normal growth in culture, since deletion of a large part of the coding region for this polypeptide causes the appearance of a truncated species, m.wt. 23,000, whilst the virus yield is unaffected (Post & Roizman, 1981). However, virus yields depend upon the host cell type, and correlate with the presence of the truncated polypeptide (Halliburton et al., 1983).

In vitro assay systems have been developed with the aim of elucidating the regulation of transcription of viral genes by IE viral polypeptides; these involve the co-transfection into cells of chimaeric genes (containing promoters and regulatory regions of early viral genes linked to either viral or non-viral genes) with genes encoding IE viral polypeptides. This approach has lead to the identification of IE viral genes which, individually or in combination, cause activation or modulation of expression from IE (Campbell et al., 1984; see above) or early (Everett, 1984; O'Hare & Hayward, 1985) viral promoters. Everett (1984) examined the effect exerted by IE viral genes upon expression from the promoter of the early viral gene encoding

glycoprotein D. Co-transfection of the gene encoding Vmw IE 175 caused activation of transcription from the early viral promoter as well as from cellular promoters, whereas the genes encoding the four remaining major IE viral polypeptides (Vmw IE 110, Vmw IE 68, Vmw IE 63 and Vmw IE 12) were ineffective. However, the combination of genes encoding Vmw IE 175 and Vmw IE 110 caused enhanced activation, compared with the gene encoding Vmw IE 175, alone. A functional promoter was the only requirement for activation in this system. Furthermore, co-transfection of IE viral genes of HCMV, VZV and PRV caused similar activation of transcription from the early viral or cellular promoters; and it was suggested that the products of IE viral genes of HSV-1 and other herpesviruses may activate transcription by a common mechanism. O'Hare & Hayward (1985) similarly investigated the control of transcription from the promoters of early (termed 'delayed-early') viral genes encoding HSV-1 thymidine kinase and HSV-2 MTR2, by IE viral polypeptides of HSV-1. In these experiments, transcription from the early viral promoters was activated by co-transfection of genes encoding Vmw IE 175 and Vmw IE 110, which were effective independently. No activation was detected for Vmw IE 68 and Vmw IE 63, although Vmw IE 12 appeared to augment the activity of Vmw IE 175 and Vmw IE 110. Evidence was obtained to suggest that the presence of a promoter is insufficient for the response to IE viral polypeptides, and that *cis*-acting regulatory sequences are involved in the activation. The authors suggested that a defect in Vmw IE 175 may be dominant in cases where Vmw IE 110 is functional, to account for the observation that in cells infected with mutants in Vmw IE 175 the pattern of transcription is arrested at the IE stage. Thus, the results of Everett (1984) and O'Hare & Hayward (1985) indicate that Vmw IE 175 may act independently to stimulate transcription from promoters of early viral genes, suggesting a direct role for the polypeptide in the regulation of transcription. The possibility also arises of functional interaction between IE viral polypeptides, such as Vmw IE 175 and Vmw IE 110.

Post-Translational Modification of HSV Polypeptides.

Many HSV polypeptides undergo post-translational modification in infected cells (Preston, 1977; Morse *et al.*, 1978; Marsden *et al.*,

1982), most commonly by (i) cleavage, (ii) conversion to species with altered electrophoretic mobilities, (iii) phosphorylation, (iv) glycosylation, or (v) sulphation.

(i) Cleavage:
MacDonald (1980) demonstrated that IE viral polypeptides of HSV-1 and HSV-2 are unstable during prolonged incubation of infected cells; in particular, Vmw IE 136'143 is processed to species of m.wt.'s 100,000 and 90,000, and Vmw IE 63 to a species of m.wt. 26,000. Processing is blocked by the inhibitor of proteolysis, tosylphenylchloromethyl ketone (TPCK), indicating that it involves specific proteolysis.

(ii) Alteration in Electrophoretic mobility:
Several viral polypeptides undergo modification which results in an alteration in their electrophoretic mobility. Such modification has, in some cases, been correlated with the function of the polypeptides, although the exact nature of the modification is unknown.

The precursor of Vmw IE 175 (ICP 4) is converted to slower-migrating forms in HSV-1-infected cells (Pereira et al., 1977; Preston, 1979b; MacDonald, 1980; Wilcox et al., 1980), and such modification occurs on entry into the nuclear fraction (Fenwick et al., 1978). Wilcox et al. (1980) identified three forms of Vmw IE 175 with different electrophoretic mobilities, which were designated ICP's 4a, 4b and 4c. ICP's 4a and 4c were detected by pulse-labelling on removal of a cycloheximide-block in protein synthesis. The mobility of ICP 4a was described as gradually decreasing (between 2 and 10 h) to that characteristic of ICP 4b. (These authors also detected three forms of ICP4 (or Vmw IE 182) in HSV-2-infected cells.) In cells infected at a NPT with tsK (Preston, 1979b; MacDonald, 1980) and other ts mutants with lesions in Vmw IE 175 (MacDonald, 1980), processing of Vmw IE 175 is blocked in an intermediate form.

Vmw IE 110, Vmw IE 68 and Vmw IE 63 also undergo modification to slower-migrating forms in HSV-1-infected cells (Pereira et al.; 1977; Fenwick et al., 1980; MacDonald; 1980). Vmw 40 is processed to forms of lower electrophoretic mobility in HSV-1-infected cells, and this processing has been shown to be essential for the encapsidation of

viral DNA to occur (Preston et al., 1983).

(iii) Phosphorylation:
Pereira et al. (1977) and Marsden et al. (1978) have described the incorporation of [^{32}P]orthophosphate into HSV polypeptides. Using intertypic recombinants, Marsden et al. (1978) identified and characterized sixteen major HSV-1 phosphoproteins (Vmw IE 175, Vmw IE 136'143, Vmw 117, Vmw 87, Vmw 85, Vmw 82, Vmw 68, Vmw 65'65, Vmw 58, Vmw 65'55, Vmw 45, Vmw 38, Vmw 36, Vmw 32, Vmw 28 and Vmw 21) and eighteen major HSV-2 phosphoproteins (which include counterparts of the HSV-1 species). Of the five major HSV-1 IE polypeptides, all but Vmw IE 12 are phosphorylated (Pereira et al., 1977; Marsden et al., 1978; Fenwick & Walker, 1979; Marsden et al., 1982), by a process which is independent of the expression of later viral functions (Fenwick & Walker, 1979).

Wilcox et al. (1980) demonstrated that the three forms of ICP 4 (Vmw IE 175) having different electrophoretic mobilities are phosphorylated in HSV-1-infected cells; ICP's 4a and 4c are phosphorylated concomitantly with their synthesis and cycle rapidly between phosphorylated and non-phosphorylated states, whereas ICP 4b remains more stably phosphorylated. The three electrophoretic forms of ICP 4 in HSV-2-infected cells also undergo phosphorylation, but only ICP 4c is stably phosphorylated. In addition, ICP's 6, 11, 22 and 27 (or Vmw 136'143, Vmw 117, Vmw IE 68 and Vmw IE 63) cycle between phosphorylated and non-phosphorylated forms in HSV-1-infected cells. Unlike ICP's 4a and 4c, IE viral polypeptides ICP's 22 and 27 are not phosphorylated immediately after their synthesis. Although phosphorylation of viral polypeptides does not correlate with their intracellular location, phosphorylation of the DNA-binding proteins ICP's 6 and 29 (Vmw 138'144 and Vmw 50) decreases and increases, respectively, the affinity of these species in HSV-2-infected- cell extracts for DNA in an in vitro assay.

(iv) Glycosylation:
HSV specifies several major glycoproteins, designated gB, gC, gD and gE; and the HSV-1 and HSV-2 counterparts of these glycorpoteins are antigenically and functionally related (Marsden et al., 1976, 1978;

Spear, 1976; Baucke & Spear, 1979; Eisenberg et al., 1979: Pereira et al., 1982; Zezulak & Spear, 1983). (HSV-1 glycoproteins gA and gB are derived by proteolysis (Pereira et al., 1982).) In addition, Buckmaster et al. (1984) have characterized a further HSV-1 glycoprotein, m.wt. 120,000, designated gH; and Marsden et al. (1984) have identified an HSV-2 glycoprotein of approximate m.wt. 92,000 ('g92K') for which no HSV-1 counterpart has yet been recognized, and which may correspond to an HSV-2 glycoprotein, gG, detected by Roizman et al. (1984). (Certain viral glycoproteins were detectable in the samples analysed in this study: gB corresponds to Vmw 122 (precursor) and Vmw 117, and gD to Vmw 51, in HSV-1-infected cells; and gB to Vmw 121 (precursor) and Vmw 118, and gD to Vmw 50, in HSV-2-infected cells.)

Glycosylation of the viral polypeptides is known to occur in discrete steps, involving initially the addition of a core oligosaccharide, containing N-acetylglucosamine and mannose, to an asparagine residue (Honess & Roizman, 1975; Wenske et al., 1982; Haarr & Marsden, 1981; Palfreyman et al., 1983). The majority of the carbohydrate chains are linked to the peptide back-bone through N-glycosidic bonds, although gC of HSV-1 and HSV-2 also contains O-glycosidically-linked sugar chains (Pizer et al., 1980; Hope & Marsden, 1983; Johnson & Spear, 1983; Zezulak & Spear, 1983).

Viral glycoproteins are found in the plasma membrane of infected cells by 4 h p.i. (Norrild et al., 1980), but the route of transport to the plasma membrane is unknown. Norrild et al. (1983), using immunofluorescent techniques, have localized gD to the vinculin-containing junctional areas, focal adhesion sites and ventral surface of HSV-1-infected fibroblasts. The authors obtained evidence that gD interacts with components of the cytoskeleton and affects the adhesion and cell-to-cell junctional areas of infected cells. Occasionally gA/gB, but not gC, was detected in the focal adhesion sites. gA/gB and gC appear to be non-essential components of the virion (Manservigi et al., 1977; Sarmiento et al., 1979); virions lacking gC are infectious, but virions produced under conditions where synthesis of gA/gB is impaired are non-infectious (Ruyechan et al., 1979; Sarmiento et al., 1979). gB may play important roles in the

penetration of virus and in virus-induced cell-fusion (Spivack et al., 1982). HSV glycoproteins serve as targets for neutralizing antibodies (Powell et al., 1974; Cohen et al., 1978) and immunocytolysis (Norrild et al., 1980).

(v) HSV glycoproteins are sulphated, the major sulphated glycoprotein being gE, and the major part of the sulphate being attached to N-linked oligosaccharide (Hope et al., 1982).

In addition to modifications (i) to (iii), Vmw IE 175 has been found to undergo poly(ADP ribosyl)ation (Preston & Notarianni, 1983), which correlates with activity of the polypeptide in infected cells.

HSV-Specified Enzymes.

The activites of numerous virally-specified enzymes have been identified and characterized in HSV-infected cells, including:

(i) Alkaline deoxyribonuclease (Morrison & Keir, 1968), which copurifies with a polypeptide with a m.wt. of 85,000 (Banks et al., 1983). This enzyme is designated Vmw 87.

(ii) deoxynucleoside pyrimidine kinase (Vmw 43), or thymidine kinase (Kit & Dubbs, 1963), which catalyzes the phosphorylation of thymidine and deoxycytidine (Jamieson & Subak-Sharpe, 1974), and which consists of a dimer, with subunits of m.wt. 42,000 (Honess & Watson, 1974; Jamieson & Subak-Sharpe, 1978).

(iii) DNA polymerase (Vmw 145) (Keir et al., 1966; Mao et al., 1975), whose activity copurifies with polypeptides of m.wt.'s of 144,000, 74,000, and 29,000 (Knopf, 1979).

(iv) Ribonucleotide reductase (Dutia, 1983; Frame et al., 1985).

(v) Nucleoside phosphotransferase (Jamieson et al., 1974).

(vi) Deoxyuridine triphosphate nucleotidylhydrolase (Caradonna & Cheng, 1981).

(vii) Cyclic AMP-dependent protein kinase (Blue & Stobbs, 1981).

(viii) Topoisomerase (Leary & Francke, 1984).

HSV DNA-binding proteins:

Bayliss et al. (1975) have identified sixteen HSV-1 polypeptides having DNA-binding activity, including Vmw 145 (DNA polymerase), Vmw 136'130 (major DNA-binding protein), Vmw 87 (exonuclease) and Vmw 43 (thymidine kinase). Powell & Purifoy (1976) reported that the DNA-binding proteins of HSV-1 and HSV-2 have comparable m.wt.'s. The major capsid polypeptide, Vmw 155, also has been shown to have a low DNA-binding activity (Powell & Purifoy, 1976; Hay, 1979). Vmw IE 175 is considered to bind DNA in the presence of (unidentified) host factors (Freeman & Powell, 1982).

Vmw 136'130 is the best-characterized DNA-binding protein. It has a higher affinity for single-stranded DNA than for native DNA (Powell & Purifoy, 1976), and has the capacity to maintain an extended conformation in single-stranded DNA (Ruyechan, 1983), and to de-anneal a polydeoxyadenylic-acid/polydeoxythymidylic-acid helix (Powell et al., 1981). Synthesis of viral DNA is inhibited by monospecific antiserum against Vmw 136'130 (Powell et al., 1981), and cells that are infected with mutants which are ts for this polypeptide cease to synthesize viral DNA on incubation at a NPT (Littler et al., 1983). Consequently, a role for the major DNA-binding protein in the synthesis of viral DNA has been suggested. Mutation in the gene encoding the major DNA-binding protein causes de-stabilization of viral DNA polymerase and alkaline deoxyribonuclease activities (Littler et al., 1983). An additional function has been proposed for the major DNA-binding protein by Godowski & Knipe (1983); that of a repressor of the expression of viral genes.

Electron microscopy of HSV-1 DNA has revealed the association of protein with the termini and joint region of the viral genome (Wu et al.,

1979); and four polypeptides, m.wt.'s 71,000, 50,000, 20,000 and 16,000, are bound to the 3' termini of detergent-extracted, viral DNA (Hyman, 1980). Dalziel and Marsden (1984), using a DNA-binding assay, have identified a late HSV-1 polypeptide, m.wt. 21,000, which interacts specifically with the a sequence of HSV-1 DNA. In view of the involvement of the a sequence in the cleavage and encapsidation of viral DNA, this finding may have important implications concerning the mechanism of viral replication. The 21,000-m.wt. polypeptide maps within U_S, and the DNA sequence indicates that the polypeptide is highly basic in character, with a reiterated segment of the amino-acid triplet, X-Pro-Arg, where X may represent any amino acid, near the carboxy terminus (Rixon & McGeoch, 1984).

Physical Mapping of HSV Polypeptides.

Timbury and Subak-Sharpe (1973) first demonstrated that intertypic complementation and recombination can occur between HSV-1 and HSV-2, in mixed infections of cells with ts mutants at a NPT. Consequently, intertypic recombinants have been used to great effect in the mapping of viral polypeptides, functions and markers, and in demonstrating that the genomes of HSV-1 and HSV-2 are colinear (Timbury & Subak-Sharpe, 1973; Morse et al., 1977; Preston et al., 1978). Intertypic recombinants also have been isolated by using resistance to the inhibitor of viral DNA synthesis, phosphonoacetic acid (PAA) (Morse et al., 1977; Preston et al., 1978; Chartrand et al., 1979, 1980), or plaque morphology (Halliburton et al., 1977; Preston et al., 1978) as selection pressures, or have been generated by intertypic marker rescue (Knipe et al., 1978; Stow, 1978). The mapping of HSV polypeptides involves identification of the polypeptide by SDS-PAGE, assignation to type 1 or type 2, and correlation with physical maps of the viral genomes, obtained by restriction enzyme analysis (Morse et al., 1978a; Marsden et al., 1978). Identification of the polypeptides specified by recombinants relies mainly on the fact that many corresponding HSV-1 and HSV-2 polypeptides differ sufficiently in their electrophoretic mobilities in SDS-PAGE to be discriminated (Cassai et al., 1975; Halliburton et al., 1977).

More recently, molecular techniques have allowed more precise location of regions of the viral genomes encoding viral functions (Murchie & McGeoch, 1982; Hope et al., 1982; Frink et al., 1983; Watson et al., 1982, 1983; Rixon & McGeoch, 1984), and also the identification of additional products of these regions. For example, the gene encoding thymidine kinase (Vmw 43) was mapped, using intertypic recombinants, to 0.3-0.309 map units (Halliburton et al., 1980). Preston & McGeoch (1981) have shown that mRNA's corresponding to this region of the viral genome are translated in vitro to give two polypeptides of m.wt.'s 43,000 and 39,000. And Marsden et al. (1983) reported that three polypeptides of m.wt.'s 43,000, 39,000 and 38,000 are translated from a single mRNA, from three initiation codons having the same phase. (Whether the 39,000- and 38,000-m.wt. species have enzymatic activity has not been demonstrated.)

The map positions of a number of HSV-1-specified polypeptides and functions are shown in **Fig. ii.**

B. LATENT INFECTION BY HSV *IN VIVO*, AND RELATED *IN VITRO* SYSTEMS.

Latent Infection by HSV *in Vivo*.

"Latency" is the term applied to the enduring existence of HSV genomes *in vivo* without clinical signs of infection. Latent infection is established in man and animals following overt, peripheral infection with either HSV-1 or HSV-2, and is disclosed by periodical recurrences of infectious virus, or by the recovery of virus following explantation of autonomic ganglia, sensory ganglia or tissues from the central nervous system (Baringer, 1975; Stevens, 1975; Klein, 1982). It has been established that the immune responses of the host are involved in latent infections by HSV, specifically in controlling the outcome of primary infection, and in recrudescence and recurrence of infection (Wildy *et al.*, 1982).

To account for the state of HSV during latency, Roizman (1965) proposed that either the viral genome is maintained in a non-replicating form, or infectious virus persists, infection being contained by the immune system of the host. To date, evidence has been obtained to support both hypotheses. Evidence supporting the static hypothesis includes the inability to detect infectious virus in homogenates of tissue explants (Baringer, 1975; Stevens, 1975; Klein, 1982), and the inability to detect viral structures or antigens (Stevens, 1975), or viral mRNA (Puga *et al.*, 1978), in trigeminal ganglia explanted from latently infected animals. Evidence for the dynamic hypothesis includes the detection of virus in various secretions from asymptomatic individuals (Buddingh *et al.*, 1953; Douglas & Couch, 1970), and observations that the titre of neutralizing antibody usually is maintained for long periods, irrespective of recurrent lesions, suggesting frequent antigenic stimulus (Douglas & Couch, 1970). Furthermore, Baringer & Swoveland (1974) obtained morphological evidence that virus is produced continually in the trigeminal ganglia of infected rabbits. Thus, the nature of latency remains to be defined.

Several studies have shown that during latency the genome of HSV-1 resides in sensory and autonomic ganglia (Stevens & Cook, 1971;

Baringer & Swoveland, 1973; Baringer, 1974; Cook & Stevens, 1976; Puga et al., 1978; Rock & Fraser, 1983) and in the central nervous system (Knotts et al., 1973; Sequiera et al., 1979; Cabrera et al., 1980; Rock & Fraser, 1983) of humans and experimentally infected animals; and evidence suggests the persistence of viral genomes as non-linear (perhaps circular or concatameric) molecules in the ganglia of latently infected animals (Rock & Fraser, 1983). Viral-specific thymidine kinase was detected in chronically infected ganglia up to 8 weeks p.i. (Yamamoto et al., 1977).

There is evidence supporting the view that latent HSV is confined to neurons (Baringer, 1975; Cook & Stevens, 1976; Galloway et al., 1979; McLennan & Darby, 1980; Kennedy et al., 1983). However, Schwartz & Elizan (1973) consider glial cells more likely to be the site of latency since, during acute infections, these cells appear to be abortively infected while neurons are permissive for viral replication (Dillard, 1972; Hill & Field, 1973; Vahlne et al., 1979; McLennan & Darby, 1980). In addition to tissues of the nervous system, infectious HSV-1 and HSV-2 have been detected in (non-homogenized) explants of skin taken from the site of inoculation, the foot-pad, in asymptomatic, latently-infected mice and guinea-pigs (Hill, 1980; Donnenberg et al., 1980; Scriba, 1976, 1977 and 1981; Al-Saadi et al., 1983), and also in† homogenized explants (Scriba, 1976; Donnenberg, 1980). These findings raise the possibilities that HSV is capable of establishing latent or persistent infections in the skin, independently of latent ganglionic infections (Donnenberg et al., 1980; Hill et al., 1980; Scriba, 1981; Al-Saadi et al., 1980).

HSV Functions Involved in Pathogenesis.

Temperature-sensitive mutants of HSV-1 and HSV-2 have been used to examine the mechanism of latency, and differences in the ability of mutants to be recovered from brains and ganglia of infected animals have been described (Lofgren et al., 1977; Watson et al., 1980; Clements & Subak-Sharpe, 1983; Al-Saadi et al., 1983). Six ts mutants of HSV-1 produce latent infections in mice at reduced frequency, from which it was concluded that at least one IE viral function, that of

† from guinea-pigs

Vmw IE 175, and one or more later viral functions may be necessary for the establishment or maintenance of latency in mouse brains (Watson et al., 1980). The ability of ts mutants to establish latent infection appears to be independent of their capacity to induce synthesis of viral DNA (Watson et al., 1980; Al-Saadi et al., 1983) In addition, the activity of viral thymidine kinase may be necessary for acute and latent infection of the trigeminal ganglia of guinea pigs and mice (Field & Wildy, 1978; Tenser & Dunstan, 1979; Tenser et al., 1979).

In Vitro Systems of Possible Relevance to Latent Infection by HSV.

There are four classes of in vitro systems which circumvent the cytocidal properties of HSV, and which may have some bearing on latent or persistent infections in man.

The first class attempts to simulate the static model of herpes virus latency, according to which the viral genome is maintained in a non-replicating form (Roizman, 1965): productive infection is suppressed by the use of u.v.-irradiated virus or by treatment of cells with inhibitors of viral replication, followed by incubation of HSV-infected cultures at hyperthermic temperatures (see below) of 40-40.5 ^{o}C (O'Neill, 1977; Colberg-Poley et al., 1979; Nishiyama & Rapp, 1981; Wigdahl et al., 1981, 1982a, b; Wigdahl et al., 1983a, b; Wigdahl et al., 1984).

The second class consists of persistent infections established and maintained using various supportive measures, including: anti-viral antibody in the medium (Hoggan & Roizman, 1959; Hinze & Walker, 1961); suboptimal incubation temperatures (Coleman & Jawetz, 1961); metabolic arrest induced by the use of medium deficient in certain nutrients (Pelmont & Morgan, 1959); and the presence of a second virus which stimulates the production of interferon, or the addition of interferon to the medium (Glasgow & Habel, 1963), in order to inhibit the replication of HSV. On correction of the particular deficiencies or on removal of the supportive agent, viral synthesis resumes and the cultures succumb; thus, the cultures acquire temporary protection from the several supportive measures, but remain inherently susceptible to the cytopathic effects of HSV.

In contrast, the third class consists of persistent HSV infections established in cells of various types (neuronal, glial, epithelial, fibroblast or lymphoid), requiring no external supportive measures for their maintenance, and which may undergo periods of quiescence or exacerbation of infection (Hampar & Burroughs, 1969; Nii, 1969; Robey et al., 1976; Vahlne & Lykke, 1977; Doller et al., 1979; Rice et al., 1979; Levine et al., 1980; Dawson et al., 1983). For systems in this class, it has been suggested that the establishment of persistence depends upon an initial abortive infection (Nii, 1969; Levine et al., 1980; Dawson et al., 1983).

Abortive infection results also from infection of permissive cells with HSV-1 or HSV-2 at temperatures at or above 39 ^{o}C (Farnham & Newton, 1959; Crouch & Rapp, 1972a, b; Darai et al., 1975; Melvin & Kucera, 1975; Marcon & Kucera, 1976). Thus, a fourth class of in vitro systems consists of cultures surviving abortive infection by HSV-1 or HSV-2 at the hyperthermic temperature of 42 ^{o} C, and such models have been used to study conversion of cells to an oncogenic phenotype (Darai & Munk, 1973; Darai et al., 1975; Darai & Munk, 1976; Schroder et al., 1977; Cameron, 1982). The adsorption of virus and the eclipse period are unaffected by supraoptimal temperatures (Gharpure, 1965; Darai et al., 1975; Marcon & Kucera, 1976), but synthesis of viral DNA is progressively inhibited (Crouch & Rapp, 1972a; Darai et al., 1975; Melvin & Kucera, 1975; Marcon & Kucera, 1976). However, Crouch & Rapp (1972a, b) demonstrated that the temperature-sensitive event in the production of infectious virus at 39^{o}C is cell-dependent and separable from the inhibition of synthesis of viral DNA. Further evidence for the role of cellular factors in the temperature-dependent inactivation of HSV was provided by Gharpure (1965), who showed that subjecting BHK cells to a brief heat-shock prior to their infection reduces their ability to support viral replication.

C. THE CELLULAR STRESS RESPONSE.

Occurrence and Discovery of the Cellular Stress Response.

Most organisms and cell types which have been examined to date, from bacteria to protozoans and higher plants, from Drosophila to avian and mammalian cells, react similarly to metabolic stress, such as imposed by incubation at hyperthermic temperatures (or heat shock), with an alteration in the pattern of proteins which they synthesize (reviewed by Schlesinger et al., 1982b). This response is rapid and complex, involving alterations in transcription and translation: but, in general, synthesis of most RNA's and proteins is suppressed whilst discrete species of mRNA are preferentially transcribed and translated, yielding new proteins and increasing the concentration of several others. The following brief description, the general terms, "stress response" and "stress protein", refer to the phenomenon when induced in cells by treatment with whatever stress-inducing agent; the terms "heat-shock response" and "heat-shock protein" refer to the phenomonenon when induced specifically by incubation at a hyperthermic temperature.

The heat-shock response was first discovered in the salivary glands of Drosophila, where elevation of the incubation temperature (from, e.g., 25 ^{o}C to 36-37 ^{o}C), or treatment with any of a variety of inhibitors of respiration, induces (within 1 minute) altered puffing in the chromosomes (Ritossa, 1962, 1964). It was subsequently discovered that another aspect of the heat-shock response involves the induction of synthesis of heat-shock proteins (Tissieres et al., 1974), and biochemical and molecular studies have provided much information concerning the heat-shock response and the organization of heat-shock genes in this organism (reviewed by Ashburner & Bonner, 1979; Pelham, 1985). In essence, the response involves: induction of synthesis of heat-shock RNA's, some of which are rapidly translated (within 10 min) into heat-shock proteins (Mirault et al., 1978); a rapid disappearance of polysomes (within 15 min), which is followed by their reappearance (McKenzie et al., 1975); suppression of synthesis of most other RNA's, apart from those encoding histones or those encoded by the mitochondria (Ashburner & Bonner, 1979; Ballinger & Pardue, 1983);

and disruption of the normal processing of rRNA (Lengyel & Pardue, 1975; Ballinger & Pardue, 1983). These changes are marked by the migration of RNA polymerase II and other chromosomal proteins to the heat-shock puffs, and this migration is inhibited by inhibitors of RNA synthesis, such as actinomycin D (Ashburner & Bonner, 1979).

Mechanism of Induction of the Cellular Stress Response.

Numerous agents and treatments are known to induce the synthesis of stress proteins in cells of various types, but whether they act by the same or by independent pathways, and precise mechanisms of induction, remain to be determined (Ritossa, 1962; Ashburner & Bonner, 1979; Johnston et al., 1980; Schlesinger et al., 1982b). Two main hypotheses have been proposed to account for induction of the stress response, based on the common effects exerted on cells by the various stress-inducing treatments.

According to one hypothesis, recovery from anoxia (Leenders and Berendes, 1972) and treatments with oxidizing agents (Leenders and Berendes, 1972), uncouplers of oxidative phosphorylation, inhibitors of electron transport, hydrogen acceptors or inhibitors of specific enzymes (Ashburner & Bonner, 1979) may induce the response by perturbing the intracellular environment (Ashburner & Bonner, 1979); and it has been proposed (Leenders et al., 1974b) that the mitochondria - and oxidative phosphorylation in particular - may be the common target for these stress-inducing agents and treatments. However, induction in response to some of these treatments appears to involve neither alterations in the pool of ATP nor in the ATP/ADP ratio (Leenders et al., 1974a).

More recently, genetic analysis of the stress response has been made possible by the characterization of mutations affecting the response in Escherichia coli (Neidhardt & Van Bogelen, 1981; Yamamori and Yura, 1982), in yeast (Iida & Yahara, 1984), Dictyostelium (Loomis & Wheeler, 1982) and in Drosophila (Hiromi & Hotta, 1985). In the case of E. coli, primary products of the mutant genes have been identified and progress has been made in elucidating one possible mechanism of induction in this organism: it has been reported

(Goff et al., 1984) that one of the seventeen stress proteins of E. coli is the ATP-dependent protease, La, which catalyzes the initial step in the degradation of abnormal proteins in vivo (Goldberg et al., 1982). Protease La is encoded by the lon gene, and transcription of this and other heat-shock genes is regulated in a positive manner by a sigma factor which is the product of the htpR gene (Grossman et al., 1984). Since htpR mutants show decreased transcription of lon, lower activity of ATP-dependent protease and decreased capacity to dispose of abnormal proteins, even during rapid growth under permissive conditions, it has been suggested that the activity of La may be increased during stress in order to dispose of abnormal proteins which may accumulate under these conditions (Goff et al., 1984). This is supported by the observation that the production of abnormal polypeptides in E. coli (by treatment with canavanine, puromycin, or streptomycin) stimulates transcription of lon and other heat-shock genes (Goff & Goldberg, 1985). Furthermore, transcription of lon is induced in E. coli cells which synthesize a single, unfolded polypeptide, such as is expressed from the (cloned) gene for human tissue plasminogen activator, or from genes for human serum albumin. Thus, abnormal polypeptides may be the trigger for induction of the stress response in E. coli. The stress response is induced by altered molecules also in Drosophila, since mutations in the structural gene for actin III, which affect the structure of the isoform in the indirect flight muscles, cause constitutive expression of heat-shock proteins in this tissue (Hiromi & Hotta, 1985). And it may be pertinent that inducing agents such as heavy-metal ions (Levinson et al., 1978a, b, 1979), sulphydryl reagents (Levinson et al., 1978, 1979), sodium arsenite (Johnston et al., 1980), amino-acid analogues (Kelley & Schlesinger, 1978; Hightower, 1980), puromycin (Hightower, 1980), oxidising agents (Leenders and Berendes, 1972), hydrogen peroxide and hyperthermic incubation (Ashburner & Bonner, 1979) are all potent denaturants for proteins, and could lead to the synthesis of abnormal proteins.

Characteristics of Stress Proteins.

The pattern of stress proteins that is revealed by SDS-PAGE varies

between different organisms, but usually includes species having m.wt.'s in the ranges of 15-30,000, 60-70,000 and 80-90,000 (Schlesinger et al., 1982a). The major heat-shock proteins of Drosophila, for example, have m.wt.'s of 22,000, 23,000, 26,000, 27,000, 36,000, 68,000, 70,000 (which is related to the 68,000-m.wt. species) and 82,000 (Ashburner & Bonner, 1979). Cultured chick embryo fibroblasts (CEF; which were used extensively in experiments described in this study) are induced to produce stress proteins with m.wt's approximately 90,000, 70,000, 35,000 and 25,000 (Hightower & Smith, 1978; Kelley & Schlesinger, 1978; Levinson et al., 1978a, b, 1980; Johnston et al., 1980; Kelley et al., 1980; Wang et al., 1981; Collins & Hightower, 1982; Schlesinger et al., 1982a, b). Stress proteins with m.wt.'s of 25, 000, 70,000, 90,000 and 110,000 have been detected also in CEF in the absence of any stress-inducing agent (Hightower & Smith, 1978; Wang et al., 1981; Kelley et al., 1980). In this description, stress proteins will be designated by their m.wt.'s ($x10^{-3}$), suffixed by 'SP'; and heat-shock proteins by their m.wt.'s ($x10^{-3}$), prefixed by 'hsp'.

Using high-resolution, two-dimensional gel systems, the pattern of proteins synthesized by stressed cells are found to be very complex. In Drosophila, charge-heterogeneity and size polymorphism may result from protein modification as well as from transcriptional differences (Mirault, 1978). And some of the protein variants in CEF have been shown to arise by post-translational modification by phosphorylation (Kelley & Schlesinger, 1978; Brugge et al., 1981; Oppermann et al., 1981) and methylation (Wang et al., 1981).

Analysis of cloned genes from Drosophila, yeast, Xenopus and E. coli indicates that the sequences encoding hsp83 and hsp70 have been conserved in evolution (Ingolia et al., 1982; Bardwell & Craig, 1984; Bienz, 1984; Farrelly & Finkelstein, 1984). The structure of the two major stress proteins also is conserved, since antibodies raised against 70SP and 90SP from CEF cross-react with proteins with similar m.wt.'s from organsims as diverse as protozoans, Drosophila, plants, mammals and amphibians (Kelley & Schlesinger, 1978), and since proteins from avian and mammalian cells demonstrate similar isoelectric points and peptide maps (Wang et al., 1981).

90SP in CEF is located in the cytoplasm, and may be isolated as aggregates of m.wt. 560,000; it is a major phosphorylated protein in the unstressed cell, being phosphorylated on serine residues (Schlesinger, 1980a, b). And in CEF transformed by Rous sarcoma virus (RSV), this protein is present in cytoplasmic complexes containing the protein kinase which is encoded by the transforming gene, src, of RSV (Courtneidge & Bishop, 1982).

The most abundant and ubiquitous stress protein is 70SP. It is produced in substantial amounts in unstressed cells of various types (Kelley & Schlesinger, 1982; Schlesinger et al., 1982; Bardwell & Craig, 1984), and is present as phosphorylated and unphosphorylated forms in unstressed and stressed CEF (Schlesinger et al., 1982a). Eukaryotes frequently possess multiple copies of the gene encoding this protein (Ashburner & Bonner, 1979; Ingolia et al., 1982; Bienz, 1984). In yeast, constitutive synthesis of 70SP may involve expression of genes that are similar to, but distinct from, those expressed following heat shock (Ingolia et al., 1982). Wang et al. (1981) reported that 70SP (referred to as HSP 68, or "thermin") contains two components, one of which copurifies with a component of the microtubules, and which remains associated with the intermediate-filament-enriched Triton/KCl cytoskeletons (Wang et al., 1980). Furthermore, 70SP is bound to the microfilaments from heat-shocked CEF (Schlesinger et al., 1982a, b), suggesting a role for 70SP in the organization of the cytoskeleton. In heat-shocked Drosophila cells, however, most of this protein is recovered in the nucleus (Velazquez & Lindquist, 1984).

25SP in CEF may also be associated with the cytoskeleton (Kelley & Schlesinger, 1978), and antibodies to the induced protein cross-react with a 22,000-m.wt. species that is present in all normal avian muscle tissue and decorates the microfilaments (Atkinson, 1981). Stress proteins in the 15-30,000-m.wt. region are found in the nuclei of Drosophila and slime moulds (Levinger and Varshavsky, 1981; Ingolia & Craig, 1982), and the sequences of the DNA encoding proteins demonstrate about 40% homology with each other and with the sequences for the alpha-crystallins, the major components of the vertebrate eye

lens (Ingolia & Craig, 1982; Schlesinger et al., 1982).

Functions of Stress Proteins.

To date, stress proteins have been proposed to perform several physiological functions, as summarized below:

i) The "heat-shock" or "stress" response has been suggested to be homeostatic in function, correcting the defects or damage imposed by experimental conditions (Ashburner & Bonner, 1979; Schlesinger et al., 1982a, b). Indeed, evidence is accumulating that stress-proteins, by whichever way they are induced, confer thermotolerance (Peterson & Mitchell, 1981) and protect cells from the lethal effects of certain stress-inducing agents, such as ethanol (Li, 1983).

ii) It has also been suggested that stress proteins play a role in terminating the stress response, by repressing the synthesis of their own mRNA (Ashburner & Bonner, 1979); and quantitative data has been obtained in support of this hypothesis (DiDomenico et al., 1982). In E. coli, the heat-shock response is modulated by hsp70, which is the product of the dnaK gene (Grossman et al., 1984).

iii) The findings that in CEF, 90SP is associated with the transforming protein of RSV (Oppermann et al., 1981), and that in mammalian cells synthesis of 70SP is induced by the product of a transforming gene of adenovirus (Nevins, 1982), suggest an involvement of stress proteins in cellular transformation. In this connection it may be relevant that the mouse c-myc oncogene regulates the expression of chimaeric genes containing the promoter for the gene for hsp70 from Drosophila (Kingston et al., 1984).

iv) The expression of certain stress proteins appears to be regulated in a complex manner, and may depend upon the stage in development or differentiation of the cell. For example, the patterns of heat-shock proteins that are produced in Drosophila cells (Tissieres et al., 1974; Lewis et al., 1975) and in chick embryos (Voellmy & Bromley, 1982) appear to be tissue-specific; and stress proteins are found to be

expressed selectively in Drosophila at certain stages of normal development, and can be induced by the hormone ecdysone (Mason et al., 1984; Vitek & Berger, 1984). Bensaude et al. (1983) demonstrated that 70SP is the first major product of the activated genome of the embryonic mouse, and suggested that 70SP is required for the onset of embryonic gene expression. Stress-inducing treatments induce parthenogenetic activation of mouse oocytes (Kaufman, 1983), and in Drosophila pupae, heat shock induces phenocopy formation, i.e. specific phenotypic abnormalities (Mitchell et al., 1979). Morange et al. (1984) reported an absence of a normal heat-shock response in mouse teratocarcinoma (PCC4-Aza-RI) cells when in the undifferentiated state, but responsiveness was recovered by the cells following retinoic-acid-induced differentiation. Similarly, Singh & Yu (1984) reported an accumulation of 70SP during hemin-induced differentiation of the human erythroid cell line, K562.

v) Since the studies that are described in this thesis were carried out, it has been reported that the stress response is induced during infections of cells of various types by several viruses, which suggests that the response is a general feature of the interaction between host and virus:

a) In E. coli, synthesis of proteins groE and dnaK, both of which have been identified as heat-shock proteins, is induced by infection with bacteriophage lambda (Drahos & Hendrix, 1982; Kochau & Murialdo, 1982; Yamamori & Yura, 1982).

b) Levels of mRNA for stress proteins are elevated in CEF by infection with either virulent or avirulent strains of Newcastle disease virus, but synthesis of stress proteins is induced only by avirulent strains (Collins & Hightower, 1982).

c) Synthesis of a 86,000-m.wt., putative stress protein is induced in CEF by infection with paramyxoviruses Sendai and simian virus 5 (SV5) (Peluso et al., 1978).

d) Simian virus 40 (SV40) and polyoma virus induce the synthesis of 90SP and 70SP in productively infected monkey and mouse cells, respectively

(Khandjian & Turler, 1983).

e) Infection of HeLa cells with adenovirus causes induction of synthesis of 70SP. Induction requires the expression of the E1A gene, since virus mutants in which the gene is deleted fail to induce the response in infected cells, and since human enbryonic kidney cells that are transformed with a fragment containing this viral gene constitutively synthesize the stress protein, as well as the product of the E1A gene (Nevins, 1982).

f) LaThangue et al. (1984) reported that a protein which accumulates in heat-shocked or disulfiram-treated cells accumulates also in cells infected with HSV-2: that is, infection by HSV induces changes associated with stress-inducing agents.

Non-Co-Ordinate Induction of Synthesis of Stress Proteins.

An important feature of the stress response is the non-co-ordinate nature of induction of synthesis of individual stress proteins. In the case of Drosophila, this is considered to arise through control of levels of mRNA (Ashburner and Bonner, 1979). The gene for hsp83 is expressed constitutively in unstressed Drosophila cells, and this has been equated to the presence of an extended heat-shock-element with high affinity for transcription factor, described above (Pelham, 1985). In CEF also, induction of synthesis of the four stress proteins in CEF is discoordinate as a function of the concentration of the inducing reagent (Levinson et al., 1978a; Johnston et al., 1980), and as a function of the duration of treatment (Kelley & Schlesinger, 1978; Levinson et al., 1978a, b; Johnston et al., 1980; Levinson et al., 1980). Published data concerning the dosage effects of stress-inducing agents and kinetics of induction of synthesis of stress proteins in CEF are considered further in chapter 2.

Regulation of Transcription Genes Encoding Stress Proteins.

In general, stress proteins are produced by translation of novel mRNA's,

and most studies of the stress response have analysed the regulation of transcription of such messages (Kelley & Schlesinger, 1978; Slater et al., 1981; Hickey & Weber, 1982). However, examples exist of the control of translation rather than transcription of mRNA encoding stress proteins, such as occurs in Xenopus oocytes (Bienz & Gurdon, 1982), where hsp70 is synthesized from pre-existing mRNA during incubation at an increased temperature. Another example is the synthesis in HeLa cells of "prompt" heat-shock proteins, also due to translation of pre-existing mRNA; at least 50 of these proteins are detectable in HeLa cells, and are recovered exclusively with a complex formed between the nuclear-matrix and intermediate filaments (Reiter & Penman, 1983).

However, in cells of most types, the stress response involves activation of transcription of specific genes. The mechanism controlling activation of these genes has been conserved in evolution, since Drosophila genes for hsp70, when introduced into cells of various other species (mouse, monkey, Xenopus oocytes), are expressed only under conditions of stress (Schlesinger et al., 1982b; Bienz, 1984; Cohen & Meselson, 1984). Molecular studies have identified and located a sequence regulating the expression of the gene for hsp70 of Drosophila. This sequence lies upstream of the gene for hsp70, 20 bp upstream from the TATA box (a conserved element common to all eukaryotic promoters); and comparison with promoters sequences of other genes for heat-shock proteins in Drosophila, and of genes for heat-shock proteins from other species, has revealed the presence of a homologous sequence, termed the "heat-shock element", consisting of the mimimum consensus sequence, C--GAA--TTC--G (Pelham, 1985). Linkage of such a sequence with the promoter for the HSV thymidine kinase gene renders expression of thymidine kinase heat-inducible in biochemically-transformed cells and Xenopus oocytes (Pelham & Bienz, 1982).

In Drosophila, DNA in the region of promoters of genes for hsp70 and hsp83 is exposed in the chromatin, and nucleosomes are excluded from these regions (Wu, 1984a). DNase protection experiments have shown that transcription of the heat-shock element correlates with the binding of proteins to this sequence during heat-shock (Wu, 1984a, b), and two protein fractions have been partially purified having the ability to bind to the heat-shock element of hsp70 and to permit

transcription *in vitro* of heat-shock genes (Parker & Topol, 1984).

Effects of Stress upon Translation.

Within 10 min of incubation of Drosophila cells at 37°C, the polysomes dissociate; and by 30-40 min, the polysomes begin to reassociate, reaching a plateau 1-3 h after heat shock (McKenzie *et al.*, 1975). Heat-shocked *Drosophila* cells contain, in addition to heat-shock mRNA, mRNA which was synthesized prior to heat shock (McKenzie, 1976) and which remains translatable *in vitro* (Mirault *et al.*, 1978; Storti *et al.*, 1980). Such non-heat-shock mRNA has been recovered from polysomes of heat-shocked *Drosophila* cells (Kruger & Benecke, 1981) and HeLa cells treated with amino-acid analogues (Thomas & Matthews, 1982). Thus, the stress response must involve the selective translation of heat-shock mRNA. Once induced, the mechanism of discrimination appears to be stable, since it continues to operate in cell-free lysates from heat-shocked cells (Storti *et al.*, 1980; Kruger & Benecke, 1981; Scott & Pardue, 1981). Neither preparations of mRNA nor lysates from heat-shocked or unheated cells show the type of dominance effects that would be expected due to the presence of either inhibitors or activators of translation of specific classes of mRNA's (Storti *et al.*, 1980; Scott & Pardue, 1981). Rather, results are compatible with the absence, from lysates of heat-shocked cells, of a factor which is required for the translation of non-heat-shock mRNA but not for the translation of heat-shock mRNA, as the crude ribosomal pellet of control lysates is able to rescue the synthesis of non-heat-shock mRNAs in heat-shocked lysates (Scott & Pardue, 1981). Furthermore, as preparations of RNA that is supplied to lysates are free from protein, the mechanisim of selection of heat-shock mRNA may involve features of its primary or secondary structure. In heat-shocked *Drosophila* cells, the polyadenylic tracts are lost from the 3' end of non-heat-shock mRNA, but this occurs as a result, rather than a cause, of heat shock (Storti *et al.*, 1980). The mechanism of discrimination between the translation of heat-shock and non-heat-shock mRNA in *Drosophila* cells may also involve differential rates of polypeptide elongation, as the rates of both elongation and initiation of translation are 15-30 times slower on non-heat-shock mRNA than on heat-shock mRNA in heat-shocked cells

(Ballinger & Pardue, 1983).

Yeast cells, in contrast to Drosophila cells, lose their normal mRNA's from polysomes during stress, and these species are not translatable in vitro (Lindquist, 1981). For CEF, brief heat-shock causes no substantial inhibition of protein synthesis; non-heat-shock mRNA's are not translatable in vitro, and therefore may be degraded or modified during heat shock (Schlesinger et al., 1982a).

A feature of the stress response which is common to all organisms is the recovery, after removal of the stress-inducing agent, of the normal pattern of protein synthesis. The mRNA's for heat-shock proteins have half-lives of 2-4 h, in contrast with 20 h for most other eukaryotic mRNA's (Schlesinger et al., 1982b). In Drosophila, transcription of mRNA for heat-shock proteins is terminated as soon as incubation is resumed at the normal growth temperature, and transcription of pre-existing non-heat-shock mRNA is resumed. In CEF (Schlesinger et al., 1982a) and yeast (Lindquist, 1981), however, recovery from heat shock requires the synthesis of novel RNA and protein, suggesting that transcription is required to displace heat-shock mRNA from polysomes.

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MATERIALS AND METHODS.

1. Viruses.

The wild-type (wt) viruses that were used in this study were HSV-1, Glasgow strain 17 syn^+ (Brown et al., 1973) and HSV-2, strain HG52 (Timbury, 1971).

A strain of HSV-1 which is of non-syncytial plaque morphology, HSV-1 17 syn^+, was originally isolated by Dr. C. A. P. Ross at the Regional Virus Laboratory, Ruchill Hospital, Glasgow. From this strain was obtained a single-step mutant of syncytial plaque morphology, HSV-1 17 syn, the parental virus from which were derived the ts mutants of HSV-1 that were used here (Brown et al., 1973; Crombie, 1975; Marsden et al., 1976). These mutants were of the non-syncytial (syn^+) morphology unless otherwise indicated, most having been converted from the syn to the syn^+ form by spontaneous reversion (which process occurs at a frequency of less than $1x10^6$; Brown et al., 1973); tsK and tsT, however, were converted to syn^+ by Dr. V. G. Preston and Dr. I. K. Crombie, respectively, by back-crossing with wt HSV-1 17 syn^+. The spontaneous revertant of tsK, ts^+K^+, which was isolated by Dr. D. Dargan, induces the synthesis of wt patterns of viral polypeptides in infected cells and shows wt growth rates at 38.5 °C, the NPT for tsK.

HSV-2 HG52 was isolated by Dr. J. Peutherer at the Virus Laboratory, Department of Bacteriology, University of Edinburgh. From this strain were derived (Timbury, 1971) the ts mutants of HSV-2 that were used here. (Note:- tsl3 and ts9, in the current nomenclature, correspond to mutants numbered x and 25 in Timbury & Subak-Sharpe, 1973.)

Seed stocks of wt and mutant viruses were provided by Dr. V. G. Preston. HSV-1 mutants tsK TK^- and MDK/2 and stocks of u.v.-irradiated tsK were obtained from Dr. C. M. Preston, and the HSV-2-mutant, tsl3, from Dr. H. Rixon.

Mutant MDK/2 was derived from the Kit strain of HSV-1

2. Tissue-Culture Cells.

From Dr. V. G. Preston were obtained low-passage cultures of the continuous, baby-hamster-kidney-cell line, BHK-21 C13, which had been established by Macpherson & Stoker (1962). These cultures were used to propagate and to titrate virus stocks.

Secondary cultures of chick-embryo fibroblasts (CEF) were prepared from ten-day-old fertilized eggs that had been supplied to the department.

Secondary cultures of human-embryo lung (HeLu) cells were obtained at about the 17th. passage from Flow Laboratories, Irvine, Ayrshire.

Primary and secondary cultures of rat-embryo fibroblasts (REF), prepared from inbred Hooded Lister rats maintained in the department, were obtained from Miss A. Bunce and Dr. M. Park.

3. Tissue-Culture Media and Solutions.

Cells were grown in Glasgow modified medium (Busby et al., 1964) supplemented with 100 U of penicillin/ml, 100 ug of streptomycin/ml and 0.002% (w/v) phenol red, and to which serum and tryptose phosphate broth were added in various proportions:

EC10 Eagle's medium containing 10% calf serum.

ETC10 Eagle's medium containing 10% calf serum and 10% tryptose phosphate broth.

EFX Eagle's medium containing X% foetal calf serum.

EHu2 Eagle's medium containing 2% pooled human serum.

EF2-Pi Phosphate-free Eagle's medium containing 2% foetal calf serum.

Emet/5C2 Eagle's medium containing one-fifth of the normal concentration of methionine and 2% calf serum.

Calf serum was prepared in the department from the blood of animals slaughtered either at the Thornhill Abattoir, Dumfriesshire.

Foetal calf serum was obtained from Gibco-Biocult Laboratories Ltd., Glasgow, and was heat-inactivated in order to kill possible contaminating viruses or bacteria, which might have inhibited the replication of HSV.

4. Agar Medium for Assaying the Anchorage-Independent Growth of Cells.

Noble agar (Difco, Bacto) was made up as a 3.2% (w/v) stock in distilled water and sterilized by autoclaving. For assaying the growth of cells in soft agar, underlay and overlay media were prepared and maintained at 45 ^{o}C until use: underlay medium was EF10 with 0.6% (w/v) Noble agar; and overlay medium was EF10 with sufficient Noble agar to give a final concentration of 0.3% (w/v) upon addition of the cell suspension.

5. Agar Medium for the Plaque-Purification of Viruses.

For the plaque-purification of viruses under agar, an overlay medium was prepared at 45 ^{o}C, composed of Eagle's medium minus phenol red, with 2% calf serum and 2% (w/v) Noble agar.

6. Standard Buffers and Solutions.

Denhardt's solution
0.02% (w/v) Ficoll 400, 0.02% (w/v) polyvinylpyrrolidone, 0.2% BSA.

Electrophoresis sample buffer
2% (w/v) SDS, 5% (v/v) 3-mercaptoethanol, 10% (v/v) glycerol, 0.05 M Tris.HCl, 0.1% (w/v) bromophenol blue, pH 6.8.

Gel Destain

methanol : water : acetic acid, 5:5:1 (v/v).

Gel Fix

methanol : water : acetic acid, 9:10:1 (v/v).

Gel stain

methanol : water : acetic acid, 5:5:1 (v/v), with 0.25% (w/v) Coomassie brilliant blue.

Gel-tank buffer

0.63% (w/v) trizma base, 0.4% (w/v) glycine, 0.1% (w/v) SDS.

Gentamycin

Final concentration: 500 ug of gentamycin per ml of growth medium.

Giemsa stain

A 1.5% (v/v) suspension of Giemsa in glycerol heated at 56 ^{o}C for 90-120 min and diluted with an equal volume of methanol.

HeBS

8g/l NaCl, 0.37g/l KCl, 0.1g/l Na_2HPO_4, 1g/l D-glucose, 5g/l HEPES, pH 7.05.

Mycostatin (Squibb, Nystatin)

Final concentration: 50 U mycostatin per ml of growth medium.

NTE

10 mm Tris.HCl, 10 mM NaCl, 1 mM EDTA, pH 7.5.

PBS (Phosphate-buffered saline)

0.17M NaCl, 3.4 mM KCl, 10 mM $Na_2 HPO_4$, 2 mM $KH_2 PO_4$, pH 7.4.

Resolving gel buffer

0.632% glycine, 0.4% (w/v) SDS, 1.5 M Tris.HCl, pH 8.9.

Saline EDTA

0.15 M NaCl, 0.1 M EDTA, pH 8.0.

Scintillation fluid

5g PPO per litre of toluene.

SSC

150 mM NaCl, 15 mM trisodium citrate, pH 7.4.

Stacking gel buffer

0.4% (w/v) SDS, 0.5 M Tris.HCl, pH 6.7.

Sucrose Buffer

10 mM Tris.HCl, 2 mM $MgCl_2$, 10 mM NaCl, 0.32 M sucrose, pH 7.5.

TE

10 mM Tris.HCl, 1 mM EDTA, pH 7.5.

Tris saline

8g NaCl, 2g KCl, 0.1g $Na_2 HPO_4$, 1g dextrose, 3g trizma base and 1.5 ml of 1% phenol red were dissolved in 1 litre of distilled water at room temperature, pH 7.4.

Trypsin

0.25% (w/v) Difco trypsin dissolved in tris saline.

Versene

0.6 mM ethylene diamine tetra acetic acid disodium salt dissolved in PBS and containing 00.0015% (v/v) phenol red.

7. Commercial Suppliers of Chemical Reagents.

Chemical reagents of analytical grade were supplied by:

BDH Chemicals Ltd., Poole, Dorset.
Bio-Rad Laboratories, Richmond, California. U.S.A..
Difco Laboratories, Detroit, Michigan, U.S.A..
James Burroughs Ltd., London.
Koch-Light Laboratories Ltd., Colinbrook, Bucks..
Pharmacia Fine Chemicals, Uppsala, Sweden.
Sigma (London) Chemical Company, Kingston-upon-Thames, London.

In particular, DATD was obtained from Aldrich Chemical Company Inc., Milwaukee, Wisconsin, U.S.A.; disulfiram from Sigma (London) Chemical Company; Gentamicin Reagent Solution from Schering Corporation, U.S.A.; Lyovac Cosmegen (actinomycin D) from Merck, Sharp and Dohme International, Merck and Co. Inc., N.J., U.S.A.; and Mycostatin from E.R.Squibb and Sons, N.Y., U.S.A..

8. Sources of Other Materials.

Du Pont, Newtown, Connecticut, U.S.A.: Dupont Cronex Lighting Plus intensifying screens.
Flow Laboratories, Irvine, Ayrshire: plastic tissue-culture dishes and flasks; 24-well "Linbro" trays; tissue-culture media.
Gibco Europe Ltd., Renfrewshire: foetal calf serum.
Kodak Ltd., London: Kodirex X-ray film; X-omat S (XS) film; DX-80 developer.
May and Baker Ltd., Dagenham: Amfix.
Miles Laboratories Ltd., P.O. Box 37, Stoke Poges, Slough: rabbit fluorescein-isothiocyanate-conjugated anti-mouse immunoglobulin G.
Schleicher and Schull, GmbH, Dassel, West Germany: nitrocellulose membrane filters, BA85.

9. Radiochemicals.

Radioisotopes were supplied by the Radiochemical Centre, Amersham, Bucks.: [^{32}P]orthophosphate (PBS11) was carrier-free; the specific activities of [α-^{32}P]deoxynucleoside triphosphates (dCTP, PB10205; dGTP, PB10206) were 2,000-3,000 Ci/mmol; and the specific activity of L-[^{35}S]methionine (SJ104) was 600 Ci/mmol. (1 Ci = 3.7×10^{10} Bq.)

10. Enzymes.

Polymerase I and restriction endonucleases BamHI and HpaI were supplied by Bethesda Research Laboratories Inc., Rockville, Maryland, U.S.A.. Deoxyribonuclease I (bovine pancreas), ribonuclease A (bovine pancreas),

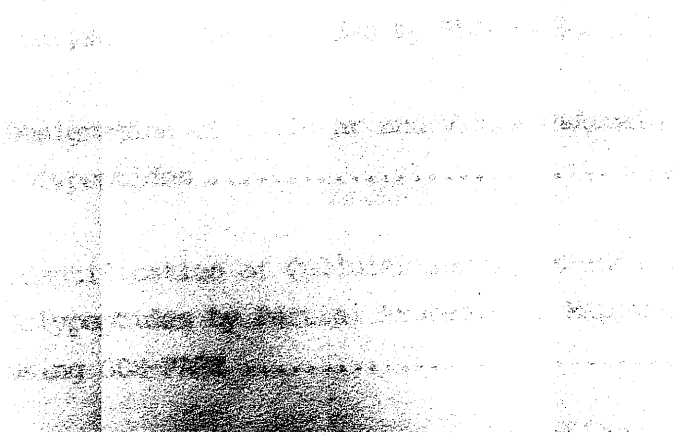
and various proteolytic enzymes (pronase, proteinase K, chymotrypsin and Staphylococcus Aureus V8 protease) were obtained from Sigma (London) Chemical Company Ltd., Kingston-Upon-Thames, London. Restriction endonuclease EcoRI had been prepared in the Department by Mr. M. Dunlop.

11. Recombinant Plasmids and Virion DNA.

The recombinant plasmids used in this study, pBam f and pBam k, contained the BamHI f and BamHI k fragments of HSV-1, respectively, inserted into the BamHI site of the plasmid vector, pAT153. These plasmids had been propagated by Dr. C. M. Preston and Miss M. E. Campbell, from seed stocks supplied by Dr. V. G. Preston. Dr. C. M. Preston also provided HSV-1 virion DNA which had been purified by isopycnic centrifugation on caesium chloride gradients.

12. Monoclonal Antibodies and Antiserum.

Mouse monoclonal antibodies and control ascites fluid were provided by Dr. J. Palfreyman. Rabbit antiviral antiserum and pre-immune serum were provided by Dr. J. Macnab.



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1. Tissue Culture.

(a) Baby-hamster kidney (BHK) cells were seeded in 80 oz bottles, at a density of $4x10^7$ cells in 200 ml of ETC10 per bottle and in an atmosphere of 5% CO_2 in air (v/v), and were cultured by rotation at 37 °C; confluent monolayers comprising $2x10^8$ cells per bottle were formed from 2 to 4 d later. The cells were harvested from the surfaces of the bottles and dissociated by two washings with a mixture of trypsin and versene (1:3 (v/v)), and were resuspended in fresh medium at the higher density of $5x10^5$ cells/ml for re-seeding onto plastic petri dishes: 50-mm dishes received 4 ml of this suspension; 35-mm dishes, 2 ml; and 15-mm "Linbro" wells, 1 ml. Reseeded thus, the cells formed confluent monolayers after incubation overnight at 37 °C.

(b) Chick-embryo fibroblasts (CEF) were prepared from ten-day-old fertilized eggs. Each egg was first checked, by candling, for embryo development; and the shell was cleaned with methanol and broken at a point above the air-space to allow the embryo to be removed. Embryos were decapitated, eviscerated, washed twice in several changes of PBS and minced finely with scissors; the tissue was dissociated in a solution of 0.25% trypsin, stirred at 37 °C for 10 min. Cells were collected from suspension by centrifugation at 1,000xg and 4 °C for 30 min, and washed repeatedly by resuspending the pellet in PBS followed by recentrifugation. The cells were finally resuspended in EC10, using either $2x10^8$ cells in 200 ml of medium for seeding in 80 oz bottles, or $1x10^6$ cells/ml for seeding onto plastic petri dishes. Confluent populations of cells were obtained after incubation of the primary cultures overnight at 37 °C; most of the cells were fibroblastic in appearance. To passage secondary cultures, monolayers of primary cells were dissociated with trypsin/versene and re-seeded in fresh medium at a third of their density at confluence; the cultures re-attained confluence from 3 to 5 days later. CEF were used for experimentation between their 3rd. and 10th. passages.

(c) Human-embryo lung (HeLu) cells and rat-embryo fibroblasts (REF) were sub-cultured using the procedures described above for CEF, excepting that the growth medium was EF10 rather than EC10, and was supplemented with 1% non-essential amino acids. A confluent monolayer of HeLu cells or REF in an 80 oz bottle contained about $1x10^8$ cells, sufficient to

seed three more bottles, which would reach confluence 3 d later. HeLu cells were cultured between the 17th. and 25th. passages.

2. Propagation of Virus Stocks.

Monolayers of BHK cells in 80 oz bottles were infected, when barely confluent, with 0.005 p.f.u. of virus/cell in an inoculum of 50 ml of ETC10 per bottle. The infected cultures were incubated at 31 °C for several days to allow an extensive viral c.p.e. to develop; the cells were dislodged into the medium by shaking the bottles, and pelleted from the medium by centrifugation at 1,500xg and 4 °C for 20 min. The pellet of infected cells was sonicated vigorously, using a Cole-Palmer ultrasonic cleaning bath, to release the cell-associated virus (CAV), and the resulting suspension was cleared of cellular debris by centrifugation at 1,5000xg and 4 °C for 10 min, aliquoted in glass vials and stored at -70 °C. When infections were with non-syncitial HSV-1, the supernatant fraction would contain cell-released virus (CRV) at high titres. This source of virus was harvested by centrifugation of the supernatant in a GSA rotor at 10 k r.p.m. and 4 °C for 2h; the pellet of virus was resuspended in ETC10, dispersed by brief sonication, aliquoted and stored.

Virus stocks were tested for sterility by inoculation onto blood agar plates, which were subsequently incubated at 31 °C and 37 °C for several days and observed for the growth of contaminating microorganisms.

3. Titration of Virus Stocks.

Serial dilutions of virus stocks were made in PBS containing 2% calf serum, and 0.1 ml aliquots were used to inoculate monolayers of BHK cells in 50-mm dishes. The inoculated cultures were incubated at 31 °C for 1 h while absorption of virus occurred, overlaid with 4 ml of EHu2 and incubated for 2 d at either 31 °C or 38.5 °C (the PT and NPT, respectively, for ts mutants), or until virus plaques were observable with a Wild M7A stereoscopic dissecting microscope. The monolayers were stained with Giemsa and virus plaques were counted.

4. Plaque Purification of Viruses.

(a) Under Liquid Medium.

Infected monolayers were overlaid with EHu2 and incubated at the appropriate temperature until well-isolated plaques had developed. The monolayers were washed with PBS to remove human serum, and those infected cells that formed the perimeter of a single plaque were collected using a finely-drawn Pasteur pipette and transferred to a glass vial containing 0.5 ml of EC2. The samples were sonicated briefly, titrated and stored at -70 ^{o}C.

(b) Under Agar Medium.

Infected monolayers were overlaid with 4 ml of liquid agar medium, which was then allowed to set at 4 ^{o}C for 5 min. Incubation of the cells was resumed at the appropriate temperature until well-isolated plaques could be discerned. The cells that formed a single plaque and the overlying agar medium were removed, and the samples were treated as described in (a).

For both procedures, virus plaques were viewed using a Wild M7A stereoscopic dissecting microscope. The viruses so isolated were propagated in BHK cells to provide seed stocks.

5. Chemical Induction of the Stress Response in Cultured Cells.

The procedures for induction of the stress response (i.e., the synthesis of stress proteins) were the same for all cell types tested. Experiments employed confluent monolayers of cells in 35-mm dishes.

When the response was to be induced chemically, the growth medium was replaced with fresh medium containing either of the stress-inducing agents, sodium arsenite or disulfiram. For induction by sodium arsenite, a stock solution at 50 mM in distilled water was diluted one-thousand-fold in fresh growth medium, giving a final concentration of 50 uM. For induction by disulfiram, a stock solution at 30 mM in DMSO was serially diluted, first in distilled water and finally

in fresh growth medium, to give a final concentration of 0.3 uM disulfiram with 0.003% (v/v) DMSO. The solvent, DMSO, at its final concentration was ineffective in inducing the stress response.

6. Induction of the Stress Response in Cultured Cells by Heat Shock.

Heat shock was performed by continuous incubation of cells in an incubator maintained at hyperthermic temperatures of 41.5-42^{o}C, in an atmosphere of 5% CO_2 in air, without recourse to additional buffering of the growth medium.

7. In Vivo Radio-Isotopic Labelling of Cellular and Virus-Induced Polypeptides with L-[^{35}S]Methionine.

(a) Pulse-Labelling of Polypeptides.
Cells were washed twice during 5 min with pre-warmed PBS to remove traces of methionine-containing growth medium, and overlaid with a radioactive solution containing, unless otherwise indicated, 100 uCi of [^{35}S]methionine per ml of PBS, using 0.2 ml of this solution per $2x10^6$ cells. Incubation of the cells was resumed at a specified temperature for a period of pulse-labelling of 30 min. The radioactive solution was removed, and the cells were washed quickly with ice-cold PBS and harvested by solubilization in one-third-strength electrophoresis sample buffer. The samples were stored at -70 ^{o}C pending further analysis.

(b) Pulse-Chasing of Polypeptides.
At the end of the period of pulse-labelling, the radioactive medium was removed, and the cells were washed twice and overlaid with medium containing the normal concentration of methionine. Incubation of the cells was resumed for the duration of the chase under specified conditions. The cells were harvested and the samples stored as described in (a).

(c) Continuous (Long) Labelling of Infected-Cell Polypeptides.
Monolayers of cells in 15-mm "Linbro" wells were infected with 20 p.f.u. of virus/cell, and incubated at 31 ^{o}C for 1 h in order for absorption

of virus to occur. The infected monolayers were washed twice with Emet/5C2 and overlaid with 0.5 ml of this medium, and incubation was continued at a specified temperature. At 2 h p.i. [^{35}S]methionine (5 uCi/ml) was added to the medium, and at 24 h p.i. the cells were harvested as described in (a).

(d) Pulse-Labelling and Pulse-Chasing of Immediate-Early Viral Polypeptides.

Monolayers were infected with 20 p.f.u. of virus/cell in the presence of cycloheximide, using 200 ug of cycloheximide/ml of medium for BHK cells, but only 20 ug/ml for CEF (since the latter cell type is more sensitive to the cytotoxic effects of cycloheximide than the former), and incubated at 31 ^{o}C for 1 h. To remove the inoculum, the monolayers were washed and overlaid with pre-warmed medium containing cycloheximide, and incubation was continued at a specified temperature. At 3 h p.i., the medium was removed, the monolayers were washed with pre-warmed, cycloheximide-free medium, and polypeptides were pulse-labelled for 20 min as in (a), or pulse-chased as in (b).

8. Analysis of Polypeptides by SDS-PAGE.

Polyacrylamide gel electrophoresis was executed according to the principles of Laemmli (1970). Single concentration gels (19 cm x 16 cm x 0.1 cm) were used, unless otherwise stated, and these were composed of a resolving gel of 12% polyacrylamide and a stacking gel of 4.5% polyacrylamide, both cross-linked with DATD in the ratio of 1:40 (w/w) DATD:polyacrylamide. This gel system was chosen the better to resolve individual immediate-early viral polypeptides into components of different electrophoretic mobilities (Preston, 1979b). Electrophoresis was conducted at 40 milliamps at room temperature. The gels were dried under vacuum onto Whatman 3MM paper using a Bio-Rad heated gel drier, and subjected to direct autoradiography using Kodak Kodirex, pre-flashed Kodak XS or Agfa films. For quantitation of polypeptide synthesis, the autoradiograms were scanned using a Joyce-Loebl microdensitometer.

9. Designation of Cellular and Virus-Induced Polypeptides.

Polypeptides were labelled in accordance with published designations, where available (Marsden et al., 1976; Marsden et al., 1978), or according to their apparent molecular weights on polyacrylamide gels. The following nomenclature was used: a virus-induced polypeptide of m.wt. X,000 is designated "Vmw X", except for an immediate-early polypeptide, which is specified as "Vmw IE X"; a stress-induced, cellular polypeptide of m.wt. X,000 is designated "XSP".

10. Identification of Cellular and Virus-Induced Polypeptides by Partial Proteolysis Mapping Using SDS-PAGE.

Partial proteolysis (or peptide) mapping was used as a test of the identity between two polypeptide bands of the same electrophoretic mobility (Anderson et al., 1973; Cleveland et al., 1977; Cash et al., 1979).

Polypeptides were pulse-labelled in vivo using 700 uCi of [^{35}S]methionine/ml, and resolved by SDS-PAGE on 12%-polyacrylamide, DATD-cross-linked gels. Each wet gel was sealed with cling-film and subjected to direct autoradiography at 4 ^{o}C using pre-flashed Kodak XS film. The resulting autoradiogram was aligned with the gel, which had been placed on a light-box, and the polypeptide bands of interest were excised from the gel with a scalpel blade. Proteins in the bands were eluted electrophoretically at 4 ^{o}C; elution was performed by packing the gel slice between plugs of cotton wool, in sections of a glass pipette which was capped at the anode end with a dialysis bag. The electro-elution buffer (pH 8.0) consisted of 0.3% (w/v) trizma base, 0.144% (w/v) glycine, 0.0074% (w/v) EDTA, 0.1% SDS. The radioactive counts recovered were sufficient to make the precipitation of protein unnecessary.

Eluted proteins were partially digested with Staphylococcus Aureus V8 protease or chymotrypsin, in a 50ul reaction mixture containing 10 ug of BSA, 0.5 M Tris.HCl (pH 6.7), and 0.4% SDS, at 37 ^{o}C for 30 min. These proteases remain active in the presence of this concentration of SDS. The reactions were terminated by addition of 10 ul of SGM buffer. (SGM buffer: 60% (v/v) glycerol, 4% (w/v) SDS and

20% (v/v) 3-mercaptoethanol.) The samples were heated to 100 °C for 2 min, and resolved by SDS-PAGE through 15%- polyacrylamide, DATD-cross-linked gels. The patterns of digestion were visualized by fluorography at -70 °C using flashed Kodak XS film. Several digestions were performed, using a constant amount of each polypeptide but different amounts of protease, to find an enzyme concentration which would generate fragments of a wide range of sizes, with a residual band of the original polypeptide.

11. Radio-isotopic Labelling of DNA with [^{32}P].

(a) In Vivo (Lonsdale, 1979).

Monolayers of BHK cells in 15-mm "Linbro" wells were pre-incubated overnight in the presence of EF2-Pi, and infected with 5 p.f.u. of virus/cell at 31 °C. At 3 h p.i., each well received 100 uCi of [^{32}P]-orthophosphate, and incubation of the monolayers was continued at 31 °C until extensive viral c.p.e. had developed, between 36 and 48 h p.i., when radiolabelled DNA was extracted from the infected cells according to the method of Lonsdale (1979).

(b) In Vitro, by Nick Translation.

HSV virion DNA and DNA from recombinant plasmids was radiolabelled, for use in hybridization reactions, to specific activities of approximately $1x10^8$ cpm/ug by the incorporation of [-^{32}P]deoxynucleoside triphosphates dGTP and dCTP during "nick translation" reactions (Rigby et al., 1977; Maniatis et al., 1975) with DNA polymerase I. The reaction mixtures consisted of 0.25 ug of DNA to be radiolabelled in nick translation buffer (NTB) with 2 uM dATP and 2 uM dTTP present, in a final volume of 50 ul. (NTB: 50 mM Tris.HCl, 5 mM $MgCl_2$, 50 ug of BSA/ml and 1 mM DTT.) Unincorporated nucleotides were removed by three precipitations using iso-propanol.

Prior to radiolabelling, plasmid DNA was reacted briefly with DNase I in order to introduce "nicks" or sites at which polymerization could later be initiated into otherwise intact, double-stranded DNA. When virion DNA was to be radiolabelled this preliminary step was unnecessary, since virion DNA is known to be extensively nicked.

Immediately prior to its use, the radiolabelled DNA was suspended in 0.5 ml of TE buffer containing 0.2 M NaOH, transferred to a glass vial and denatured by heating to 90 °C for 2 min; the vial was placed in an ice-bath to ensure rapid cooling, and the solution was neutralized with concentrated HCl.

12. Agarose-Gel Electrophoresis.

Restriction fragments of DNA were fractionated according to size, by electrophoresis through horizontal agarose slab gels (0.5 x 15 x 25 cm; 0.6%-1.2% (w/v) agarose) in E buffer (1xE buffer: 30 mM Na_2PO_4, 1 mM EDTA and 36 mM Tris.HCl, pH 6.8. The buffer contained, unless otherwise indicated, 0.5 ug of ethidium bromide/ml.) Samples were electrophoresed at 50 volts until the tracking-dye (bromophenol blue) had migrated 20 cm from the origin. The DNA in the (ethidium-bromide-stained) gels was made visible by illumination with long-wave (365 nm) u.v. light, and was photographed using Polaroid film. Gels that were intended for direct autoradiography were dried under vacuum onto Whatman 3MM paper, using a Bio-Rad heated gel drier.

13. Restriction Endonuclease Reactions.

Restriction endonuclease reactions were performed under the conditions stipulated by the supplier, using an excess of enzyme in reaction volumes of 50 ul, and for an incubation period of 3 h. Reactions were terminated by addition of 10 ul of DF per 50 ul sample. (DF: 5xE buffer, 100 mM EDTA, 10% (w/v) Ficoll 400, 0.1% (w/v) bromophenol blue.)

During reactions with large amounts (10 ug or more) of high-molecular-weight, cellular DNA, the extent of digestion was estimated by comparing, under u.v. illumination, the migration of digested and undigested samples through ethidium-bromide-stained, agarose mini-gels in TBE buffer. (TBE buffer: 90 mM Tris.HCl, 90 mM boric acid, 1 mM EDTA, pH 8.3.)

14. Designation of Restriction Fragments of HSV-DNA.

Fragments generated by cleavage of HSV-1 and HSV-2 DNA with restriction endonucleases BamHI and EcoRI were labelled in accordance with the designations of Cortini et al. (1978) and Davison et al. (1981): i.e., alphabetically, and in order of apparent size.

15. Extraction and Purification of (High-Molecular-Weight) HeLu-cell DNA (Park, 1983).

HeLu cells were cultured in flasks or 80 oz bottles until confluent. The growth medium was removed, and the cells were washed twice with ice-cold PBS and harvested in a small volume. The cells were pelleted by centrifugation at 2,000xg and 4 ^{o}C for 10 min, and resuspended in lysis buffer A. (Lysis Buffer A: 50 mM Tris.HCl, 100 mM NaCl, 10 mM EDTA, 0.4% SDS, pH 8.0.) Proteinase K was added to give a concentration of 100 ug/ml, and the lysate was incubated at 37 ^{o}C for 3 h. The lysate was extracted twice with buffer-saturated phenol and once with chloroform. Sodium acetate was added to give a final concentration of 0.2 M, followed by 2.5 volumes of ethanol, and the sample was stored at -20 ^{o}C for 2 h. Nucleic acid was pelleted by centrifugation at low speed, and the pellet was washed with ethanol, air-dried, and resuspended in TE buffer containing 100 ug of RNAse A/ml; the reaction mixture was incubated at 37 ^{o}C for 3 h. The sample was re-extracted twice with buffer-saturated phenol and once with chloroform, and dialysed overnight at 4 ^{o}C against 0.1xSSC. The concentration of DNA in the sample was assayed by absorption spectrophotometry and adjusted to 1 mg/ml, assuming that an absorption of 1 OD unit is equivalent to 40 ug of DNA/ml.

16. Transfer of DNA to Nitrocellulose Membranes (Southern, 1975).

Restriction fragments of DNA were resolved by electrophoresis through 0.6%-agarose gels, and transferred to nitrocellulose membranes according to the method of Southern (1975). The preparative stages of the procedure included the modification of Wahl et al. (1979), whereby the DNA in the gel is partially depurinated prior to being denatured in alkali.

The gel was submersed in 0.2 M HCl for 30 min at room temperature, with shaking, to cause depurination of the DNA within the gel, washed with distilled water to remove residual acid, and submersed in Gel Soak 1 (0.2 M NaOH, 0.6 M NaCl) for 1 h to cause alkali denaturation of the DNA. Alkali in the gel was neutralized by covering the gel in Gel Soak 2 (1 M Tris.HCl, 0.6 M NaCl, pH 7.5) for 30 min, after which the gel was ready for blotting onto a nitrocellulose membrane as described by Maniatis et al. (1982). The transfer of DNA from the gel to the membrane was allowed to occur at room temperature for 16-24 h. The membrane was washed in 6xSSC at room temperature for 5 min, air-dried and baked under vacuum at 80 ^{o}C for 2 h. The gel was rehydrated with a solution of 0.5 ug of ethidium bromide/ml of distilled water, and inspected by u.v. illumination to confirm that the transfer of DNA had been total.

17. Hybridization of Radiolabelled DNA to Nitrocellulose-Bound DNA.

Radiolabelled DNA was hybridized to nitrocellulose-bound DNA using the method developed by Park (1983); this method is based on the procedures of Southern (1975) and Denhardt (1966), and prescribes hybridization at high temperature in aqueous solution. The pre-hybridization and hybridization reactions were conducted in heat-sealed, polythene bags in a shaking water bath that was maintained at 74 ^{o}C throughout. The hybridized membranes were washed using a modified procedure.

A baked membrane was submersed in 6xSSC at room temperature for 2 min and placed in a polythene bag. Sufficient pre-hyridization mix was introduced to cover the membrane completely (about 10 ml, or 0.2 ml/cm^2 of membrane), the bag was sealed, and the reaction mixture was incubated for 2 h. (Pre-hyridization mix: 10xDenhardt's solution (Denhardt, 1966), 3xSSC, 0.1% (w/v) SDS and 10 ug of sonicated salmon sperm DNA/ml of solution.) The pre-hyridization mix was drained and replaced with the same volume of hyridization mix, and the reaction mixture was incubated for 16 to 24 h. (Hyridization mix: 10xDenhardt's solution, 6xSSC, 0.1% (w/v) SDS, 10 mM EDTA, 10% (w/v) dextran sulphate (Wahl et al., 1979), and 25 ng/ml denatured, radiolabelled DNA with a specific activity of about 1x10^8 c.p.m./ug

of DNA.) The final activity of the radiolabelled DNA was $1\text{-}5 \times 10^7$ c.p.m./ml of hybridization mix.

The hybridized membrane was washed with gentle shaking in plastic boxes containing 1 litre of solution, using the following procedure: 5 min at room temperature in 2xSSC and 0.5% (w/v) SDS; 15 min at room temperature in 2xSSC and 0.1% (w/v) SDS; one wash of 30 min, and one of 1 h, at 74 °C in 0.1xSSC, 0.5% (w/v) SDS and 10 mM sodium decahydrate pyrophosphate. The washed membrane was rinsed briefly in distilled water, air-dried and submitted to indirect autoradiography for 1 to 10 d at -70 °C using pre-flashed Kodak XS film.

18. Fractionation of Cells into Nuclei and Cytoplasms (Preston, 1979b).

After polypeptides were pulse-labelled with [^{35}S]methionine *in vivo*, cells were washed and scraped into a small volume of ice-cold PBS, pelleted by centrifugation at 2,000xg for 10 min, and resuspended in 200 ul of lysis buffer B per 2×10^6 cells. (Lysis buffer B: 10 mM Tris.HCl (pH 7.5), 2 mM $MgCl_2$, 10 mM NaCl, 5 mM 3-mercaptoethanol and 0.5% (v/v) NP40.) The suspension was mixed vigorously over 5 min, and centrifuged at 2,000xg for 2 min to give a pellet of nuclei and a supernatant containing the cytoplasmic lysate. The supernatant was added to 100 ul of electrophoresis sample buffer. The nuclei were washed by resuspending and repelleting in 1 ml of sucrose buffer (sucrose buffer: 10 mM Tris.HCl (pH7.5), 2mM $MgCl_2$, 10 mM NaCl and 0.32 M sucrose), and solubilized in 150 ul of one-third-strength electrophoresis sample buffer. The nuclear and cytoplasmic samples were analysed by SDS-PAGE.

19. Detergent Extraction of CEF to Expose a Cytoskeleton (Brown *et al.*, 1976).

CEF were treated according to the method of Brown *et al.* (1976), by which approximately 80% of the total cell protein is extracted, leaving a cytoskeleton that retains part of its integrity and which is composed of fibronectin, actin filament bundles and intermediate-sized

filaments.

Cells were washed once with pre-warmed PBS and twice with TGMC buffer, overlaid with a solution consisting of 0.5% Triton X-100 (v/v) in TGMC buffer, using 1 ml of solution/$2x10^6$ cells, and incubated at room temperature for 10 min. (TGMC buffer: 137 mM NaCl, 5 mM KCl, 5 mM glucose, 25 mM Tris.HCl (pH7.4), 0.5 mM Mg/Cl_2 and 0.025 mM $CaCl_2$.) The supernatant was was added to one-third-strength electrophoresis sample buffer, and the material adhering to the dishes was rinsed with 5 ml of TGMC buffer and solubilized in one-third-strength electrophoresis sample buffer. Samples analysed by SDS-PAGE, and gels were stained and destained before being dried and submitted to direct autoradiography.

20. Immunofluorescence.

A range of mouse monoclonal antibodies that recognize HSV-1 polypeptides has been isolated and characterised in the department by Dr. J. Palfreyman. These antibodies, and a rabbit antiviral antiserum provided by Dr. J. Macnab, were used to detect viral antigens in preparations of permeabilized, infected cells by indirect immunofluorescence.

(a) Preparation of HeLu Cells for Immunofluorescence Studies.
Uninfected or mock-infeted cells were seeded sparsely onto no. 1 (13 mm diameter) microscope glass coverslips placed in 15-mm "Linbro" wells; $4x10^4$ cells at most were seeded in 200 ul of EF10 per coverslip to ensure a high quality of immunostaining. The cells were incubated at 37 ^oC for 2 h in this small volume of medium to encourage their settling, overlaid with a further 1 ml of EF10, and their incubation was continued overnight. The cells were then ready for chemical fixation.

In order to examine patterns of immunofluorescence in productively infected cells, uninfected cells were infected at this point with 5 p.f.u. of HSV-1 or HSV-2/cell in 100 ul of medium per well, and incubated at 31 ^oC for 1 h to allow absorption of virus to occur; the inoculum was removed and replaced with 1 ml of fresh

medium, and incubation of the cells was continued at 37 °C until 6 h p.i., when the cells were subjected to chemical fixation.

(b) Permeabilization of Cells by Chemical Fixation.

Localization of intracellular antigens requires cell membranes to be rendered permeable to antibodies. This was achieved by fixing the cells in a 3:1 mixture (v/v) of methanol and acetone, a "precipitant fixative", which removes lipid and causes the precipitation of protein. This method of fixation preserves general cellular morphology and gives a low background of fluorescence.

Cells that had been cultured on coverslips were washed in warm PBS by gentle dipping to remove traces of serum, and immersed in fixative at -20 °C for 10 min. The coverslips were air-dried and stored at -20 °C in sealed, plastic petri dishes containing silica gel, at -20 °C until required.

(c) Immunostaining of Fixed preparations.

All of the stock solutions for immumo-staining were diluted 1 in 20 in PBS, and spun at 13,000xg for 2 min to remove high-molecular weight debris and aggregated protein. Coverslips carrying chemically fixed cells were mounted on microscope glass slides using a chemical adhesive. The samples were washed by dipping the slides in PBS at room temperature, overlaid with 50 ul of a solution that contained either pre-immune serum or control ascites fluid, and pre-incubated in a humidified box at 37 °C for 30 min. The procedure was repeated twice, once using as the overlay a solution of the monoclonal antibody to be tested, and once using a solution of a fluorescent conjugate, specific for the animal (mouse) from which the monoclonal antibodies had been derived. The samples were washed in copious PBS and distilled water, coated with a 1:1 mixture (v/v) of PBS and glycerol, and overlaid with fresh coverslips.

(d) Fluorescence Microscopy.

Preparations were examined using a Leitz-Orthoplan microscope, which received incident light from a mercury vapour source and operated with a KP500 interference filter. Cells were viewed with the microscope objective focussed on the upper (dorsal) surfaces.

(e) Photographing Immuno-Stained Preparations.

Fluorescence in fixed cells was photographed using a Leitz-Orthoplan microscope fitted with Ortholux automatic camera attachments and Ilford HP5 film.

21. Photographing Cells in Culture.

Cells in culture were photographed using Pentax photographic equipment and Ilford FP4 film.

22. Electron Microscopy.

Dr. F. Rixon and Mr. J. Aitken of the Laboratory of Electron Microscopy prepared and photographed the thin sections presented in this dissertation.

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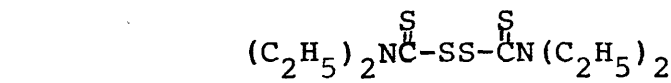
RESULTS.

The IE viral polypeptide, Vmw IE 175, plays an essential role in productive infection of cultured cells by HSV-1 (Courtney & Powell, 1975; Marsden et al., 1976; Preston, 1979a; Dixon & Schaffer, 1980). A known function of this polypeptide is to suppress the expression of IE genes while activating transcription of early and late viral genes (Preston, 1979a, b; Watson & Clements, 1980); and this prompted an investigation of the possible regulation of the expression also of cellular genes by Vmw IE 175 in HSV-1-infected cells.

The cellular stress response is an inducible, genetic response that is manifest by abundant synthesis of so-called "stress proteins". Induction results from an increase in the amount of mRNA specific for these proteins. The possibility of HSV affecting the stress response was indicated by the observation of Fenwick et al. (1980) that in Vero cells infected with HSV-1 (F) and treated with the stress-inducing agent, azetidine (an analogue of proline and hydroxyproline), IE viral polypeptides accumulated but synthesis of stress proteins failed to be induced. To examine the effects of infection with HSV upon the cellular stress response, secondary CEF were used, as their response to stress-inducing agents is well documented (Hightower & Smith, 1978; Kelley & Schlesinger, 1978; Levinson et al., 1978a, b; Johnston et al., 1980; Kelley et al., 1980; Levinson et al., 1980; Wang et al., 1981; Collins & Hightower, 1982; Schlesinger et al., 1982a, b). Mutant tsK, which possesses a temperature-sensitive lesion in Vmw IE 175, was employed especially to study the stress response in infections arrested in the IE phase (Marsden et al., 1976; Preston, 1979a).

Levinson et al. (1978a, 1978b and 1980) characterized the response of secondary CEF to a variety of stressful stimuli, including treatment with disulfiram, and assigned apparent m.wt.'s of 100,000, 70,000, 35,000 and 25,000 to the four major induced proteins. Synthesis of the 35,000-m.wt. species was found to be most variable, depending on the method of induction (Levinson et al., 1980). A closer estimate for the m.wt. of the largest of the major stress proteins is 90,000, and this modified value is adopted here: the four major stress proteins in

Disulfiram (or bis(diethylthiocarbamoyl)disulphide):



m. wt., 297.

CEF are designated 90SP, 70SP, 35SP and 25SP. Concentrations of disulfiram between 0.12 uM and 0.32 uM are sufficient to cause the simultaneous induction of synthesis of all four stress proteins in CEF incubated at 38 °C; when CEF are treated with the higher concentration of disulfiram for 1 h, synthesis of DNA and RNA is reduced by 80% and 50%, respectively, whereas the overall rate of protein synthesis is unaffected (Levinson et al., 1978b).

1. THE STRESS RESPONSE IS ACTIVATED FOLLOWING INFECTION OF SECONDARY CEF WITH TSK.

1.1. Infection with TsK at a NPT Causes Induction of Synthesis of Polypeptides that Comigrate in SDS-PAGE with Stress Proteins.

Fig. 1.1. (tracks 3 to 6) shows a time-course of polypeptide synthesis in secondary CEF infected with tsK at the NPT of 38.5 °C. There was induction of synthesis of IE viral polypeptides Vmw IE 175, Vmw IE 136'143, Vmw IE 110 and Vmw IE 63; functional Vmw IE 175 is thus required for the transition to later viral functions to occur in CEF as well as in BHK cells (Marsden et al., 1976; Preston, 1979a). Meanwhile, synthesis was induced of other polypeptides, previously unidentified in tsK-infected cells: these comigrate with the major (90SP, 70SP, 25SP, and to a lesser degree, 35SP) and minor (m.wt.'s 20-23,000) stress proteins which were induced by treatment with disulfiram at a concentration of 0.3 uM (track 2). Synthesis of these species was not induced in wt HSV-1-infected cells under these conditions, nor by tsK-infected cells incubated at a PT (chapter 2). The results suggested that infection of CEF with tsK at a NPT stimulated activation of cellular genes encoding stress proteins, and the effect was investigated further.

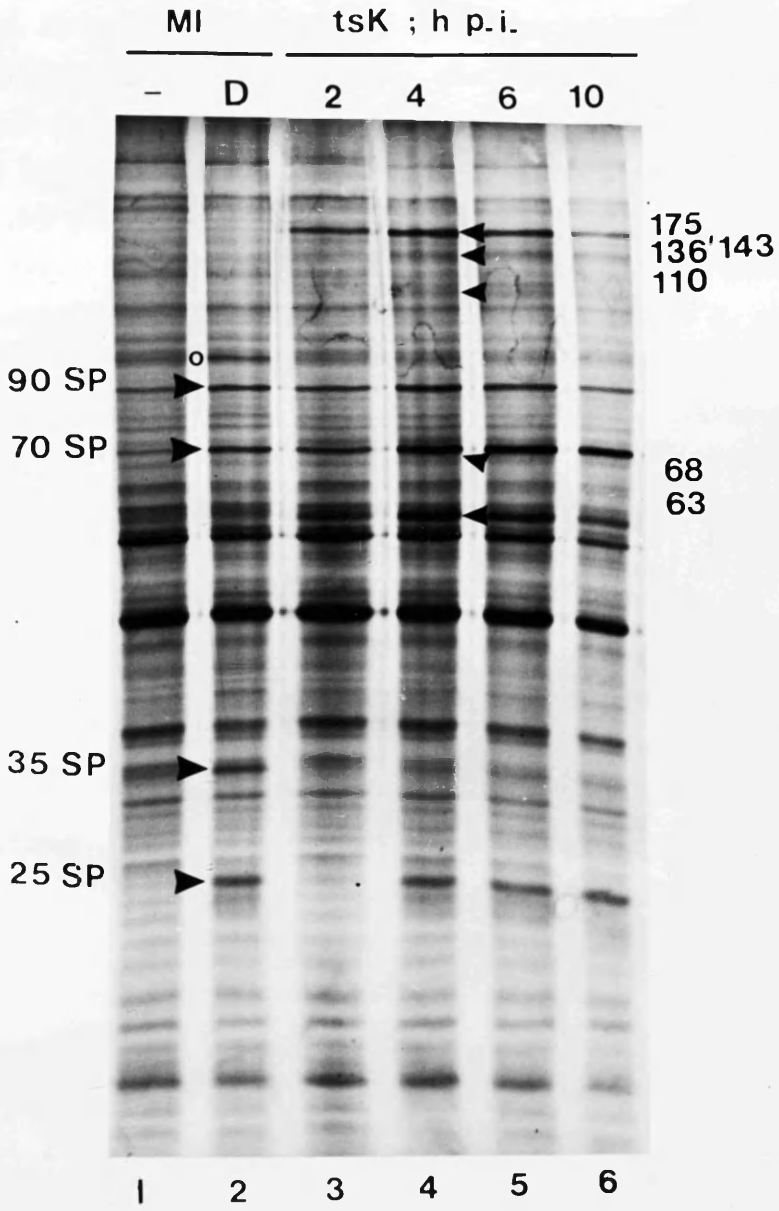
1.2. Identification, by Partial Proteolysis Mapping, of Stress Proteins Synthesized in TsK-Infected CEF.

To determine their relatedness (in terms of amino-acid sequences), the polypeptides whose syntheses were stimulated in CEF following infection with tsK or in reponse to treatment with disulfiram were submitted to

Fig. 1.1. Time-course of polypeptide synthesis in secondary CEF infected with tsK at a NPT.

Secondary CEF mock-infected or infected with tsK (20 p.f.u./cell) at 38.5 °C. Cultures were pulse-labelled for 0.5h with [^{35}S]methionine at these times p.i.: 2 h (track 3), 4 h (track 4), 6 h (track 5) and 10 h (track 6). Mock-infected cultures were pulse-labelled at 10 h p.m.i. (track 1), or after treatment with disulfiram (0.3 uM) for 4 h (track 2), at 38.5 °C. Radiolabelled polypeptides were resolved by SDS-PAGE through a 12%-polyacrylamide, DATD-cross-linked gel. Major stress proteins are labelled to the left of track 1, and IE viral polypeptides to the right of track 6. An additional, cellular polypeptide, apparent m.wt. approximately 100,000, whose synthesis is induced infrequently by disulfiram, is labelled to the left of track 1 (o).

Levels of synthesis of stress proteins in this and other experiments usually were compared by inspection of the relative density of bands in the autoradiograms, and were quantitated by densitometry only where indicated.


MI
tsK ; h p.i.
–
D
2
4
6
10
175
136
143
110
90 SP
70 SP
68
63
35 SP
25 SP
1
2
3
4
5
6

peptide mapping by limited proteolysis, a technique that provides a stringent test of identity (Anderson et al., 1973; Cleveland et al., 1977; Cash et al., 1979). In **Fig. 1.2** are shown peptide maps representative of experiments using a wide range of concentrations of proteases. Patterns of fragments obtained from 90SP, 70SP and 25SP synthesized after treatment with disulfiram corresponded closely to patterns derived from the 90,000-, 70,000- and 25,000-m.wt. counterparts synthesized in tsK-infected cells, the major peptides being represented in each of the paired digests. Thus, the polypeptides synthesized in tsK-infected cells were authentic, major stress proteins. As noted previously by Levinson et al. (1980) for disulfiram-induced stress proteins, the peptide maps for tsK-induced stress proteins were non-identical, signifying that these polypeptides were distinct. Although variants of 90SP and 70SP are known to arise by post-translational modification (Brugge et al., 1981; Oppermann et al., 1981; Slater et al., 1981; Wang et al., 1981), no differences could be detected between the peptide maps of stress proteins synthesized in disulfiram-treated cells compared with tsK-infected cells.

1.3 Protein Synthesis is Required for the Stress Response to Be Induced in CEF during Infection by TsK at a NPT.

The stress response that was observed in tsK-infected CEF might have been stimulated by the contents of the viral inoculum, which included virions and cell debris, or by IE polypeptides synthesized during infection at the NPT. This and the following two experiments (described in section 1.4) were performed to ascertain whether either of these potential sources contained the stress-inducing agent.

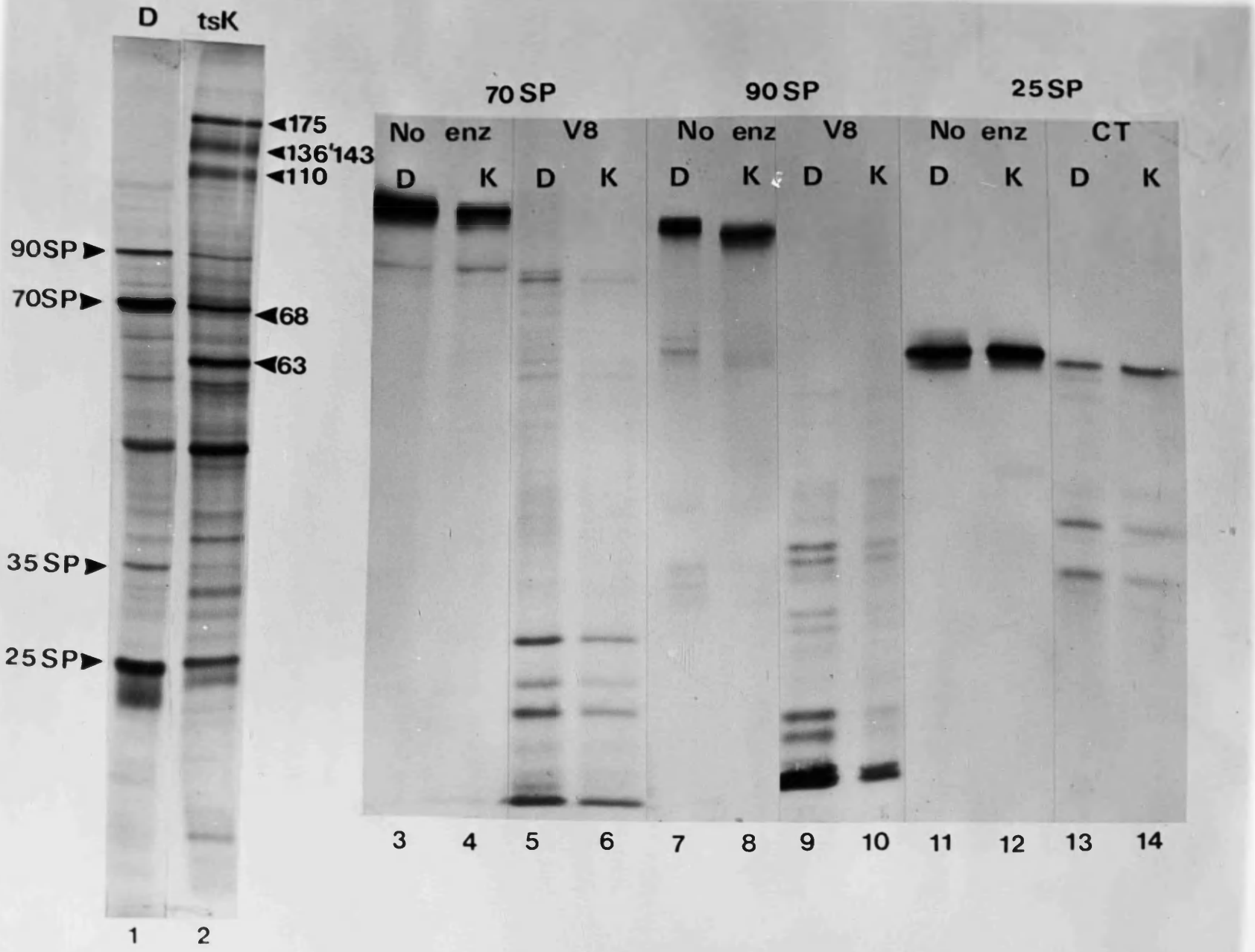
The stress response may be activated in CEF treated simultaneously with cycloheximide and stress-inducing reagents (or heat shock) so that synthesis of stress proteins is induced on removal of cycloheximide (Kelley & Schlesinger, 1978). Therefore, the first experiment employed the inhibitor, cycloheximide, in order to determine whether protein synthesis was required for activation of the stress response in CEF during infection by tsK. Secondary CEF were mock-infected or infected with wt HSV-1 or tsK at 38.5 ^{o}C;

Fig. 1.2. Identification, by partial proteolysis mapping, of stress proteins synthesized in tsK-infected secondary CEF at a NPT.

Secondary CEF were infected with tsK (40 p.f.u./cell) for 8 h at 38.5 ^{o}C, or were mock-infected and treated with disulfiram (0.3 uM) for 3 h at that temperature. Cultures were pulse-labelled with [^{35}S]methionine (700 uCi/ml), and radiolabelled polypeptides were resolved by SDS-PAGE through a 12%-polyacrylamide, DATD-cross-linked gel. The wet gel was subjected to direct autoradiography (tracks 1 and 2), and the 90,000-, 70,000- and 25,000-m.wt. regions were excised from duplicates of tracks 1 and 2. Proteins were electro-eluted from the bands and partially digested with either Staphylococcus Aureus V8 protease or chymotrypsin for 30 min at 37 ^{o}C. Partial digests were resolved by SDS-PAGE through a 15%-polyacrylamide, DATD-cross-linked gel, and the gel was submitted to fluorography. The resulting fluorogram is shown in tracks 3 to 14.

Track 1: polypeptides synthesized in disulfiram-treated cells. Track 2: polypeptides synthesized in tsK-infected cells. Tracks 3, 5, 7, 9, 11 and 13: peptide maps of stress proteins synthesized in disulfiram-treated cells (D). Tracks 4, 6, 8, 10, 12 and 14: peptide maps of polypeptides synthesized in tsK-infected cells (K).

Tracks 3 to 6: 70,000-m.wt. counterparts, mock-digested (tracks 3 and 4); digested with 150 ug of Staphylococcus Aureus V8 protease/ml (V8; tracks 5 and 6). Tracks 7 to 10: 90,000-m.wt. counterparts, mock-digested (tracks 7 and 8); digested with 50 ug of Staphylococcus Aureus V8 Protease/ml (V8; tracks 9 and 10). Tracks 11 to 14: 25,000-m.wt. counterparts, mock-digested (tracks 11 and 12); digested with 150 ug of chymotrypsin/ml (CT; tracks 13 and 14).


D
tsK
175
136'143
110
68
63
90SP
70SP
35SP
25SP
1
2
70SP
90SP
25SP
No enz
V8
No enz
V8
No enz
CT
D
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cycloheximide was added to the medium, with or without disulfiram, either at the time of infection or at 4 h p.i., and incubation of the cells was continued for 3 h. Cycloheximide and disulfiram were removed, the cultures were pulse-labelled with [^{35}S]methionine, and radiolabelled polypeptides were resolved by SDS-PAGE.

Several features are illustrated by the results of this experiment, shown in **Fig. 1.3:**

1) The stress response was activated in mock-infected cells by treatment with disulfiram in the presence of cycloheximide (track 2); on removal of these reagents, synthesis of 90SP, 70SP and 25SP was strongly induced, while synthesis of 35SP was less affected. This observation is considered further in the Discussion.

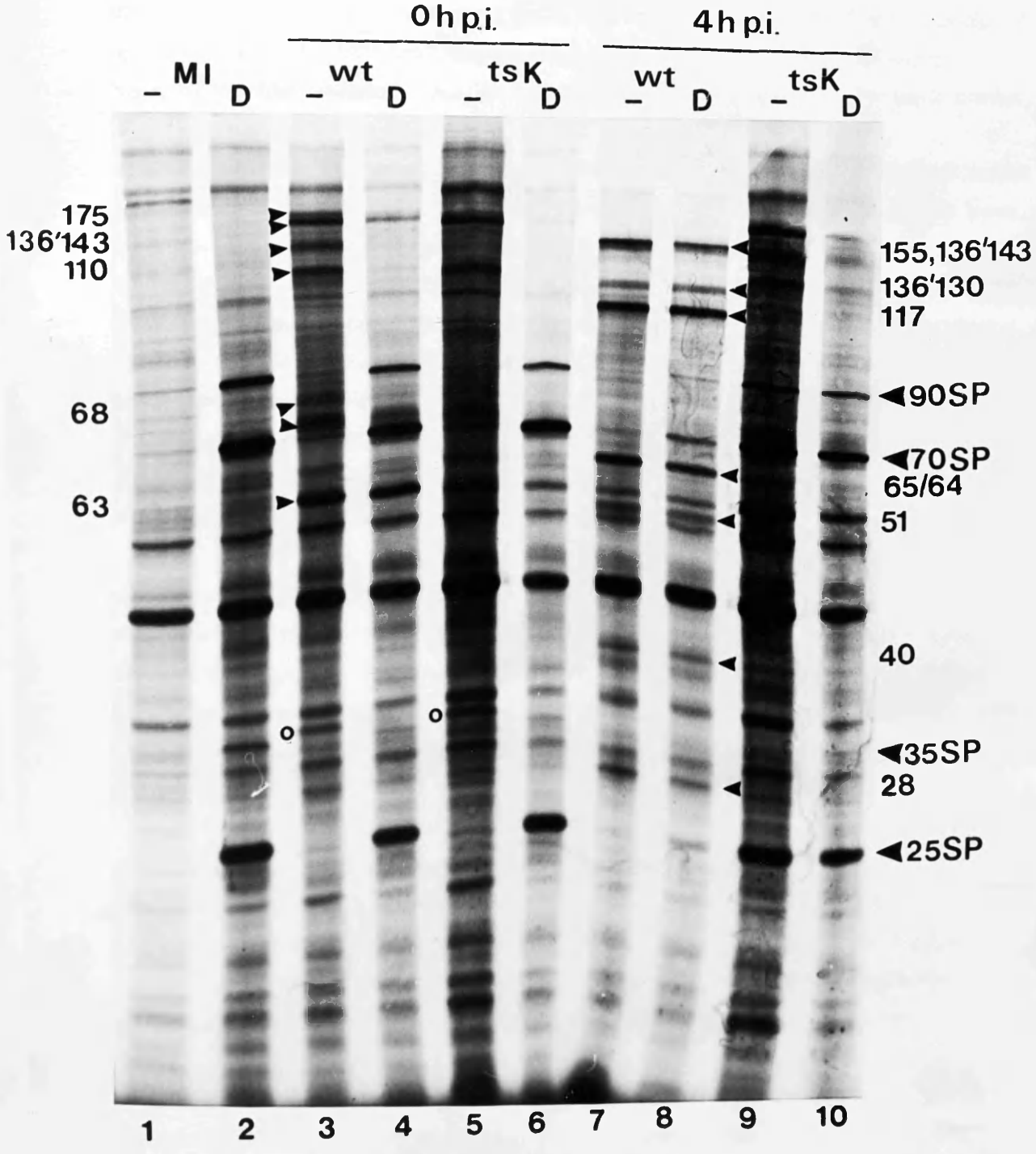
2) In cells that were infected with wt HSV-1 in the presence of cycloheximide (track 3), the removal of cycloheximide was followed by the production of IE viral polypeptides Vmw IE 175 (with 2 forms apparent), Vmw IE 136´143, Vmw IE 110 and Vmw IE 68 (with 2 forms apparent), and, already, traces of later viral polypeptides, Vmw 65/64. Synthesis of stress proteins remained at constitutive levels (track 1).

3) In cells that were infected with wt HSV-1 in the presence of both cycloheximide and disulfiram (track 4), the removal of the reagents was followed by induction of synthesis of 90SP, 70SP and 25SP at levels approaching those in the similarly-treated, mock-infected cells (track 2). Synthesis of most viral polypeptides (Vmw IE 136´143, Vmw IE 110 and Vmw 65/64 especially) was reduced, although synthesis of Vmw IE 63 was undiminished; and processing of Vmw IE 68 to a form of lower electrophoretic mobility was noticeably inhibited.

4) When wt HSV-1-infected cells were treated at 4 h p.i. with cycloheximide, either with (track 8) or without (track 7) disulfiram, the range of viral polypeptides that subsequently was synthesized resembled that in untreated, wt HSV-1-infected cells (results not shown), and included Vmw 155, Vmw 136´130, Vmw 117, Vmw IE 63, Vmw 51, Vmw 40 and Vmw 28. [A polypeptide species which co-migrated with the IE viral polypeptide, Vmw IE 63, or the HSV-2-equivalent, was observed consistently to be produced throughout infection of CEF with various mutants of HSV-1, with wt HSV-1 or with wt HSV-2 (see also **Fig.´s 2.1**

Fig. 1.3. Protein synthesis is required for the stress response to be induced in secondary CEF by infection with tsK at a NPT.

Secondary CEF were infected with wt HSV-1 (20 p.f.u./cell; tracks 3, 4, 7 and 8) or tsK (20 p.f.u./cell; tracks 5, 6, 9 and 10), or mock-infected (tracks 1 and 2), at 38.5 ^{o}C. At either the time of infection (tracks 3 to 6) or 4 h p.i. (tracks 7 to 10), cycloheximide (20 ug/ml) was added to the medium, with (tracks 2, 4, 6, 8 and 10) or without (tracks 3, 5, 7 and 9) disulfiram (0.3 uM), and incubation was continued for 3 h. The reagents were removed, and the cultures were washed with cycloheximide-free medium and pulse-labelled. IE viral polypeptides are indicated to the left of track 3, and identified to the left of track 1; and non-IE viral polypeptides are indicated to the right of track 8, and identified to the right of track 10. An unidentified species, apparent m.wt. 36,000, synthesized by cycloheximide-treated wt-HSV-1-infected or tsK-infected cells, is indicated (o).


0h p.i.
4h p.i.
– MI D
– wt D
– tsK D
– wt D
– tsK D
175
136'143
110
68
63
155,136'143
136'130
117
90SP
70SP
65/64
51
40
35SP
28
25SP
o
o
1 2 3 4 5 6 7 8 9 10

and 2.2).] Treatment of wt HSV-1-infected cells with cycloheximide and disulfiram (track 8) caused an increase in synthesis of 70SP and 25SP to levels that were intermediate between induced (track 2) and constitutive (track 1) levels.

5) In cells that were infected with tsK in the presence of cycloheximide alone (track 5), IE viral polypeptides were synthesized following removal of the reagent: Vmw IE 175, Vmw IE 110, Vmw IE 68 (with 2 forms apparent) and Vmw IE 63. Synthesis of stress proteins was not stimulated, but remained at the levels in untreated, mock-infected cells (track 1). In cells that were infected with tsK in the presence of both cycloheximide and disulfiram (track 6), and following removal of these reagents, synthesis of stress proteins was stimulated whereas synthesis of viral polypeptides (Vmw IE 110 and Vmw IE 68 especially) was reduced or absent. But in tsK-infected cells that were treated with cycloheximide and disulfiram at 4 h p.i. (track 10), synthesis of viral polypeptides was not detectably affected; and in the presence (track 10) or absence (track 9) of disulfiram, stress proteins were synthesized at the same levels following the removal of cycloheximide.

6) An unidentified polypeptide species, apparent m.wt. 36,000, was synthesized in cells infected with wt HSV-1 (track 3) and tsK (track 5) in the presence of cycloheximide, but not in cells infected in the presence of cycloheximide and disulfiram (tracks 4 and 6). This species may correspond to a cleavage product of an IE viral polypeptide (Gerdes et al., 1979).

It is concluded that protein synthesis was necessary for activation of the stress response to occur in cells post-infection with tsK. In contrast, in disulfiram-treated, wt HSV-1-infected and tsK-infected cells the response was activated in the absence of protein synthesis, and therefore was unimpaired by the experimental manipulations.

Wild type-HSV-1-infected and tsK-infected cells were responsive to treatment with disulfiram added (together with cycloheximide) at the time of infection, but less responsive at 4 h p.i.. This observation concurs with results to be described in chapter 5, which show that during infection by wt HSV-1 or tsK, cells become unresponsive to treatment with disulfiram. Effects of treatment of infected cells with

disulfiram upon the synthesis and processing of IE viral polypeptides are considered further in chapters 6.5 and 7.

1.4. The Stress Response Is Not Induced by Exposure of CEF to a Viral Inoculum.

Results of the previous experiment showed that activation of the stress response in CEF by infection with tsK at 38.5 °C depends upon protein synthesis post-infection. It would have been in keeping with the results if a constituent of the inoculum (for example, a virion component) had been indirectly responsible, by interacting with - or by stimulating the synthesis of - a short-lived, cellular protein able to activate the stress response more directly. Other factors, absent from tsK-infected cells, might have inhibited the response in wt HSV-1-infected cells, where induction of synthesis of stress proteins is not usually apparent. Therefore, it was necessary to determine whether activation of the stress response was conditional upon the synthesis of viral or cellular polypeptides; and so the following two experiments were designed to investigate the synthesis of stress proteins in HSV-infected cells in which synthesis of viral polypeptides was suppressed.

The viral genome may be inactivated by irradiation of the virion with u.v. light, the processes of adsorption, penetration and uncoating of the virion remaining unaffected (Eglin et al., 1980). Therefore, the effects of u.v. irradiation of tsK upon induction of the stress response in cells infected at the NPT were examined. Stocks of tsK were obtained (from Dr. C. M. Preston) which had been exposed to increasing doses of u.v. irradiation, and Fig. 1.4.1 shows the titres of these stocks in BHK cells at 31 °C (Dr. C. M. Preston, personal communication). Secondary CEF were "infected" with u.v.-irradiated tsK at 38.5 °C (using the equivalent of 40 p.f.u./cell of non-irradiated virus), and polypeptide synthesis in infected cells was analysed 8 h later. Fig. 1.4.2 shows that synthesis of IE viral polypeptides and stress proteins was decreased with increasing dose of u.v. irradiation of the virus, so that in cells that were "infected" with the most heavily-irradiated virus, synthesis of IE viral polypeptides was undetectable (track 8) while synthesis of stress proteins remained at constitutive levels (track 2).

Fig. 1.4.1. Inactivation curve of tsK with increasing dose of u.v. irradiation. The graph shows the logarithm of titres of virus stocks in BHK cells at 31 ^{o}C (Dr. C. M. Preston, personal communication).

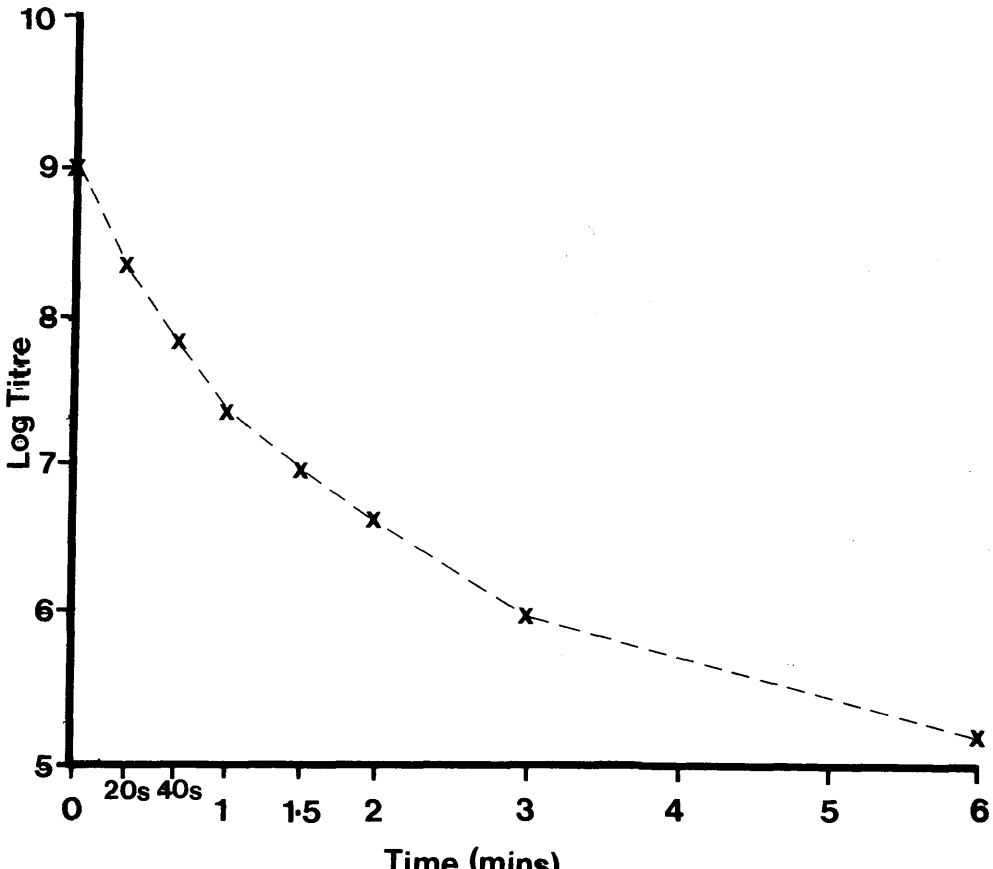
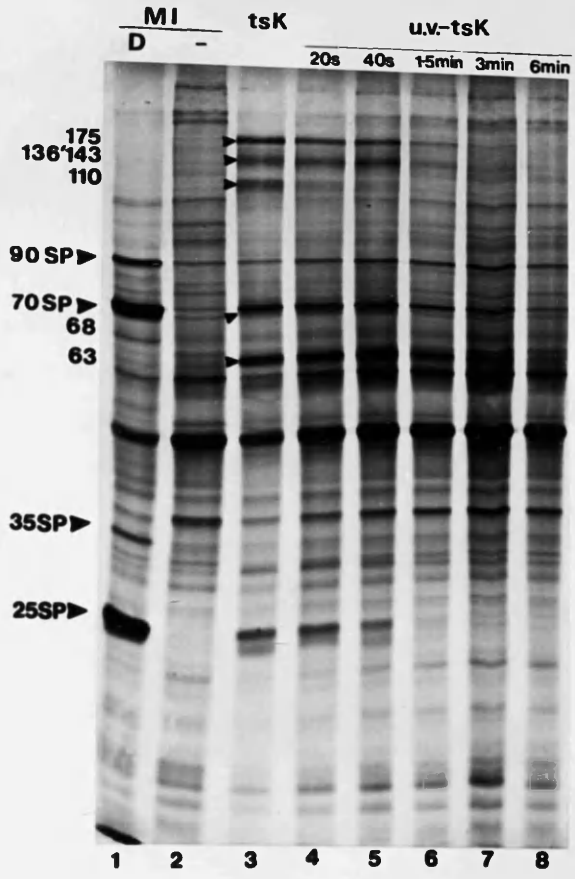

10
9
8
7
6
5
Log Titre
0
20s
40s
1
1·5
2
3
4
5
6
Time (mins)

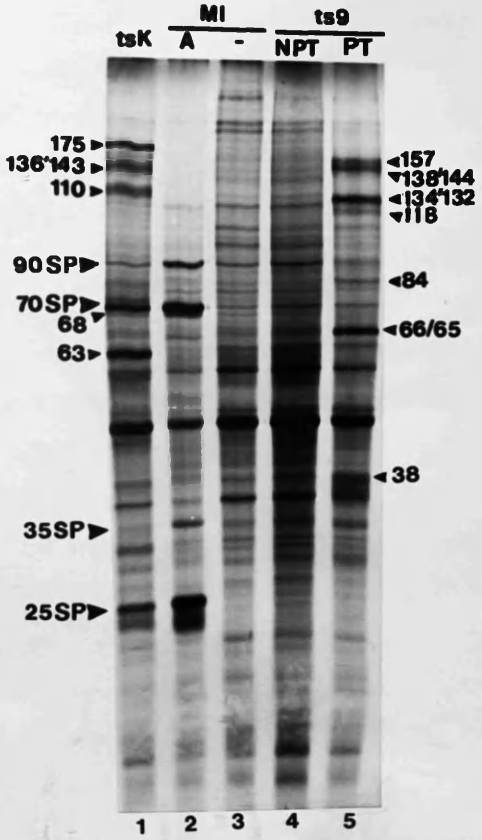
Fig. 1.4.2. Polypeptide synthesis in secondary CEF "infected" with u.v.-irradiated tsK at a NPT.

Secondary CEF were mock-infected (tracks 1 and 2) and treated with disulfiram (0.3 uM) for 3 h (track 1), or infected with either non-irradiated (track 3) or u.v.-irradiated tsK (tracks 4 to 8), using the equivalent of 40 p.f.u. of non-irradiated virus/cell, at 38.5 ^{o}C. The times of u.v. irradiation of the virus stocks were: 20 s (track 4), 40 s (track 5), 1.5 min (track 6), 3 min (track 7), 6 min (track 8). Cultures were pulse-labelled at 8 h p.i..

Fig. 1.5. Polypeptide synthesis in secondary CEF infected with ts9 at a NPT.

Secondary CEF were mock-infected (track 3) or infected with ts9 (40 p.f.u./cell; tracks 4 and 5) or tsK (40 p.f.u./cell; track 1), and incubated for 1 h at 31 ^{o}C to allow the absorption of virus to occur. Incubation of the cultures was continued at either a NPT (38.5 ^{o}C; tracks 1, 2, 3 and 4) or a PT (31 ^{o}C; track 5) for 7 h, after which the cultures were pulse-labelled. An additional, mock-infected culture was treated with sodium arsenite (A; 50 uM) for 3 h (track 2).


MI
D
-
tsK
u.v.-tsK
20s
40s
1·5min
3min
6min
175
136'143
110
90SP
70SP
68
63
35SP
25SP
1
2
3
4
5
6
7
8


MI
tsK
A
-
ts9
NPT
PT
175
136'143
110
90SP
70SP
68
63
35SP
25SP
157
138'144
134'132
118
84
66/65
38
1
2
3
4
5

The second experiment employed ts9, an HSV-2 mutant that may be defective in uncoating, and which fails to induce detectable synthesis of viral polyeptides in BHK cells infected at the NPT (Dr. C. M. Preston, personal communication). (An HSV-1 equivalent of this mutant was not then available for comparison). **Fig. 1.5** shows that CEF infected with ts9 at the NPT neither synthesized viral polypeptides nor demonstrated the stress response (track 4). Cells infected with this mutant at the PT (track 5) produced a typical range of late viral polypeptides and synthesized stress proteins only at constitutive levels (track 3).

The viruses that were used in these experiments, u.v.-irradiated tsK and ts9, were competent in the processes of adsorption and penetration (irradiated tsK was also competent in uncoating), but defective in the expression of viral functions: suppression of synthesis of viral polypeptides appeared to be total in ts9-infected cells incubated at the NPT, but varied in degree between cells infected with different stocks of u.v.-irradiated tsK. If, as was proposed earlier in this chapter, a constituent of the viral inoculum interacts with a cellular factor to activate the stress response (which may be inhibited during productive infection), then infection with fully-inactivated tsK or with ts9 should cause such activation to occur. However, infection with neither virus stimulated the synthesis of stress proteins, and so the proposed mechanism of indirect activation of the response by constituents of the viral inoculum may be discounted.

It must be concluded that the stress response which was observed in cells post-infection with tsK at the NPT was caused by the synthesis of viral polypeptides - more precisely, IE viral polypeptides, the only class of viral polypeptides to be produced under these conditions. Two further observations agree with this conclusion: in cells infected with u.v.-irradiated tsK, levels of synthesis of IE viral polypeptides and of stress proteins were co-ordinate (**Fig. 1.4.2**); and in cells infected with non-irradiated tsK at the NPT, synthesis of stress proteins increased to fully-induced levels while IE viral polypeptides accumulated (section 1.1).

2. INVESTIGATION OF THE STRESS RESPONSE IN CEF INFECTED WITH TS-MUTANT HSV-1, WT HSV-1 OR WT HSV-2.

A series of seven experiments was performed to determine whether infection of CEF by any of a number of ts mutants of HSV-1 (tsl201, tsB, tsD, tsE, tsG, tsK syn, tsT and MDK/2) at the NPT, by the revertant of tsK syn$^+$, ts$^+$K syn$^+$, or by wt HSV-2 would cause induction of synthesis of stress proteins in the manner that is observed with tsK; and whether such induction could be attributed to specific viral functions. In each experiment, infected cultures were pulse-labelled with [^{35}S]methionine at 9 h p.i. at either the PT or a NPT, a time when infections by wt viruses would have progressed to a late stage, and radiolabelled polypeptides were analysed by SDS-PAGE. Polypeptides that comigrated in SDS-PAGE with the major stress proteins (whose syntheses were induced in cells treated with disulfiram or infected with tsK syn$^+$) were assumed to be identical with stress proteins, and are referred to as such.

The known characteristics of the mutants that were tested may be summarized as follows:

TsD, tsK and tsT are members of the same complementation group, their ts lesions residing in the diploid gene that encodes Vmw IE 175 (Preston, 1981; Davison *et al.*, 1984). The mutations in tsD and tsT result in amino-acid substitutions that are situated closer to the carboxy terminus of Vmw IE 175 than is the lesion in tsK (Preston, 1981). BHK cells that are infected with tsD or tsT, as with tsK, continue to produce IE viral polypeptides during incubation at the NPT, and synthesize forms of Vmw IE 175 with increased electrophoretic mobility (MacDonald, 1980); unlike tsK-infected cells, however, they demonstrate synthesis of some early and late polypeptides (Vmw 155, Vmw 117 and Vmw 65/64) at the NPT (Marsden *et al.*, 1976; Gerdes *et al.*, 1979; Preston, 1981). In experiments reported here, tsD and tsT were found to be even less restricted in CEF than in BHK cells, causing production of apparently normal amounts of many early and late viral polypeptides simultaneously with IE viral polypeptides at temperatures as high as 40 ^{o}C (results not shown). This suggests that differences exist in the functions of Vmw IE 175 between the two infected-cell

types. Cells infected with tsB and tsE fail to synthesize viral DNA at the NPT and the lytic cycle is arrested at an early (but later than IE) stage (Brown et al., 1973). Tsl201 (or 17tsVPl201) is defective in the gene encoding Vmw 40 (or p40), a polypeptide that is involved in the encapsidation of viral DNA; and cells infected with this mutant produce viral DNA at the NPT (Preston et al., 1983). Mutant MDK/2 was not isolated in the Department, but was obtained from Dr. S. Kit, and is TK^-.

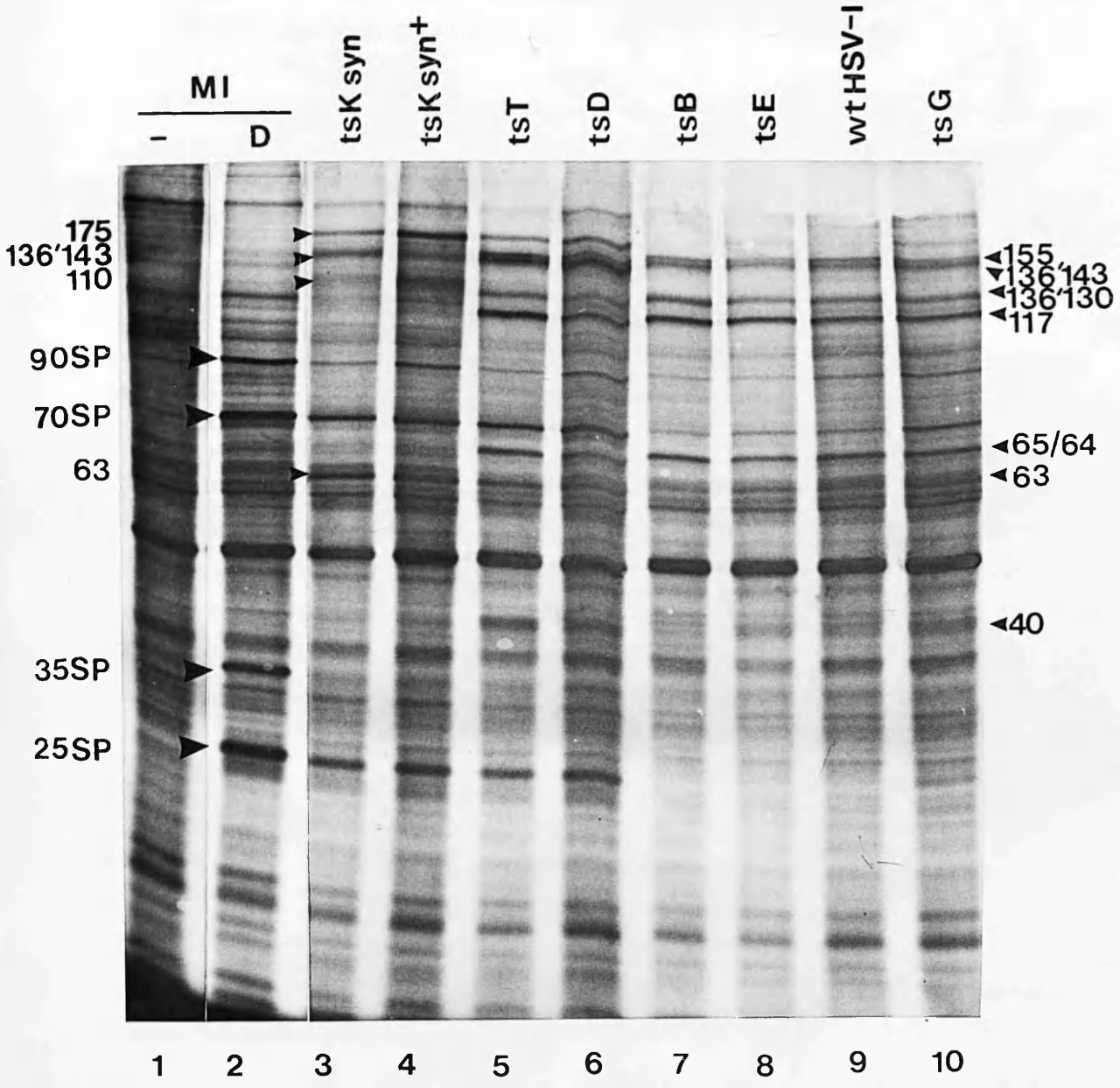
Some of these mutants of HSV-1 have been classified by Dargan and Subak-Sharpe (1983), using the system of Atkinson et al. (1978), according to the ability of infected cells to produce nucleocapsid-related structures; and by Dargan & Subak-Sharpe (1983) according to the estimated times post-adsorption at which the cycle of viral replication is blocked: tsK, tsD and tsT are class I mutants, blocked at stages corresponding to 0-1 h post-adsorption; tsE is a class II mutant, blocked at 0-1 h; tsB is a class II mutant, blocked at 2.2 h; and tsG is a class III mutant, blocked at 1.5 h. In cells infected with class I mutants, nucleocapsid-related structures are absent; with class II mutants, structures are evident, but are empty or partially-filled; and with class III mutants, fully-formed nucleocapsids are detected. Tsl201 also may be classified as a class II mutant, since the nuclei of cells infected with this mutant at NPT contain empty nucleocapsids (Preston et al., 1983). Dargan & Subak-Sharpe (1983) considered that the block in the lytic cycle of tsG is unusually early, since tsG-infected cells support the replication of viral DNA at the NPT, and suggested that the presence of a further mutation might account for the discrepancy. Similarly, the observations that tsB and tsE are members of the same complementation group, and yet have different block-times and cause different patterns of morphological alterations in infected cells, could be reconciled by the existence of additional mutations in both tsB (Dr. D. Dargan, unpublished results) and tsE (Stow, 1978).

Fig. 2.1 shows representative patterns of polypeptide synthesis in secondary CEF infected with selected viruses at 38.5 °C (see also **Fig. 5.2.1**), in which the following features are apparent:

1) In mock-infected cells, levels of constitutive synthesis of 90SP and 70SP were fairly high and of 35SP and 25SP were less apparent (track 1).

Fig. 2.1. Polypeptide synthesis in secondary CEF infected with wt HSV-1 and ts mutants of HSV-1 at a NPT.

Secondary CEF were mock-infected (tracks 1 and 2) or infected with wt HSV-1 or ts mutants of HSV-1 (20 p.f.u./cell: track 3, tsK syn; track 4, tsK syn^+; track 5, tsT; track 6, tsD; track 7, tsB; track 8, tsE; track 9, wt HSV-1; track 10, tsG) at the NPT of 38.5 ^oC. A mock-infected culture was treated with disulfiram for 3 h prior to pulse-labelling (track 2). The cultures were pulse-labelled at 9 h p.i..


MI
I
D
tsK syn
tsK syn+
tsT
tsD
tsB
tsE
wt HSV-I
tsG
175
136'143
110
90SP
70SP
63
35SP
25SP
155
136'143
136'130
117
65/64
63
40
1
2
3
4
5
6
7
8
9
10

Synthesis of the four major stress proteins was strongly induced following treatment with disulfiram (track 2).

2) Infection of cells with either tsK syn (track 3) or tsK syn^+ (track 4) caused synthesis of 90SP, 70SP and 25SP to be induced, while synthesis of 35SP was unaffected.

3) With tsT (track 5) and tsD (track 6), synthesis of 70SP and 25SP was induced, while synthesis of 90SP and 35SP was unaffected.

4) With tsB (track 7), tsE (track 8) and wt HSV-1 (track 9), synthesis of stress proteins was uninduced.

5) With tsG (track 10), synthesis of 70SP was induced, and synthesis of 90SP and 25SP was increased compared with constitutive levels.

Cells infected with tsK syn or tsK syn^+ synthesized only the IE class of viral polypeptides (Vmw IE 175, Vmw IE 136´143, Vmw IE 110 and Vmw IE 63), whereas cells infected with tsT and tsD synthesized IE and non-IE viral polypeptides Vmw IE 175, Vmw 155, Vmw IE 136´143, Vmw 136´130, Vmw 117, Vmw 65/64, Vmw IE 63 and Vmw 40. The latter group of viral polypeptides was synthesized also in cells infected with tsB (track 7), tsE (track 8), wt HSV-1 (track 9) and tsG (track 10), although the species migrating as IE 63 was synthesized in reduced amounts (see also chapter 1.3) and synthesis of Vmw IE 175 was not detectable in cells infected with either tsE or wt HSV-1.

Throughout this series of experiments, synthesis of stress proteins was not induced by infection at 31 ^{o}C with any of the viruses tested (results not shown).

Owing to the method of isolation of tsK syn^+, by back-crossing the syn form of the original mutant with wt HSV-1 17 syn^+, the stock of tsK^+ that was used in these studies might have carried portions of the genome of wt HSV-1 17 syn^+ which, rather than the defective locus for Vmw IE 175, were responsible for the observed activation of the stress response in tsK-infected cells. This possiblity was discounted by the observation of induction of the stress response following infection with a stock of tsK of the syn plaque morphology (Fig. 2.1, track 3).

In this series of experiments, infection by ts mutants of HSV-1 caused broadly reproducible but non-coordinate effects upon levels of synthesis of stress proteins. These observations are best understood by consideration of published data on the dosage effects and kinetics of induction of synthesis of stress proteins in CEF in response to stress-inducing agents:

Induction of synthesis of the four stress proteins in CEF is discoordinate as a function of the concentration of the inducing reagent (Levinson et al., 1978a; Johnston et al., 1980), and as a function of the duration of treatment. The kinetics of induction in CEF have been described for disulfiram (Levinson et al., 1978b), KTS (Levinson et al., 1978a), sodium arsenite (Johnston et al., 1980), canavanine (Kelley & Schlesinger, 1978) and heavy metal ions (Levinson et al., 1980), and comparison shows that the following sequence of events occurs in response to treatment with these reagents at non-cytotoxic concentrations (e.g., 0.12 uM disulfiram, 0.12 uM KTS, 25 uM sodium arsenite, 2 uM canavanine or 0.2 mM copper sulphate):

i) 35SP is the first stress protein whose synthesis is induced, and the first whose synthesis is depressed (between 8 h and 24 h of treatment).

ii) Induction of synthesis of 90SP, 70SP is retarded relative to 35SP, but after 1 h of treatment, synthesis of 70SP proceeds at the fastest rate.

iii) Synthesis of 25SP is the last to be induced, and continues after 24 h of treatment while synthesis of the other stress proteins subsides.

(Time-courses of induction of synthesis of stress proteins in CEF treated with 0.3 uM disulfiram were found to be in keeping with these observations (chapter 6).) At higher concentrations of inducing reagents, synthesis of all four stress proteins is induced apparently simultaneously; and at higher concentrations still, synthesis of first 35SP, then 90SP, and, finally, 70SP is depressed relative to 25SP. Total protein synthesis (and the synthesis of actin in particular) is inhibited with increasing concentration of the inducing-reagent. With cytotoxic concentrations of reagents protein synthesis is gradually and

permanently reduced.

It is therefore apparent that levels of synthesis of stress proteins in CEF are not proportional to the severity of the stress imposed. Rather, it is proposed that the degree of stress to which a culture is subjected may be estimated, compared with a control culture, from the relative levels of synthesis of stress proteins together with any alteration in the rate of synthesis of host polypeptides. For example, increased synthesis of 35SP, with no accompanying change in the rate of total protein synthesis, would indicate the initial stages of a stress response or, alternatively, a response to mild stress. Pronounced synthesis of 70SP and 25SP, with a reduction in total protein synthesis, would indicate later stages of a response, or a response to stronger stress. Induction of synthesis of 25SP alone would indicate a late stage in the response to prolonged stress, or a response to more severe stress. Thus, the results of this series of experiments may be summarized and interpreted according to the degree of stress to which infected cells were subjected, from the features of the response that was induced. The viruses are listed below in order of decreasing intensity of the stress response which developed in infected cells at a NPT (38.5 ^{o}C or 40 ^{o}C):

1) Infection by tsT or tsK syn caused a stress response that resembled, for example, the response of uninfected cells to treatment with high concentrations of the potent stress-inducing reagent, sodium arsenite (Johnston et al., 1980), in that synthesis of 70SP and 25SP was induced most strongly, and overall synthesis of host protein was reduced.

2) Infection by tsK syn^{+} and tsD caused induction of synthesis of 90SP, 70SP and 25SP, with synthesis of 35SP occasionally observed in tsD-infected cells. Induction of 35SP indicates that the response was less intense than in tsT-infected and tsK-infected cells.

3) Infection by tsG invariably caused induction of synthesis of 70SP (**Fig. 2.1**) and increased the synthesis of the other three stress proteins above constitutive levels. With tsG, tsB, tsE, tsl201, MDK/2 and ts^{+}K syn^{+}, the increase in synthesis of 90SP, 70SP and 25SP resembled the response of uninfected cells to milder stress.

4) With wt HSV-1 and HSV-2, infection caused overt induction of synthesis of stress proteins only when the levels of constitutive synthesis of stress proteins were high (see below); otherwise, levels of constitutive synthesis were maintained or reduced after infection (especially in wt HSV-2-infected cells).

It is therefore concluded that the stress response may be a general feature of infection by mutants of HSV-1, which is not attributable to a particular viral function. Infections by viruses which either induced (tsl201, tsG and ts^+) or failed to induce (tsB and tsE) synthesis of viral DNA elicited a stress response of comparable intensity. However, the stress response was induced most strongly in infections at NPT by mutants which are defective in Vmw IE 175. The intensity of the response correlated also with the classification of viruses according to the degree of morphological development of nucleocapsids in infected cells (Atkinson et al., 1978): strong responses were induced by class I mutants (tsD, tsK and tsT), weaker responses by class II (tsB, tsE, tsl201) and class III (tsG) mutants, and no detectable response by wt HSV-1 or wt HSV-2.

Polypeptide synthesis in each of the seven cultures employed in these experiments was examined closely also in order to assess (a) levels of constitutive synthesis of stress proteins, (b) the responsiveness of cultures to treatment with disulfiram, (c) alterations in levels of synthesis of stress proteins following infection with ts mutants of HSV-1 and wt viruses, and (d) the ability of infected cultures to support synthesis of viral polypeptides. The following points emerged:

i) There was variation in the responsiveness of cultures to treatment with disulfiram, which correlated with variation in the levels of constitutive synthesis of stress proteins: when constitutive levels were high (**Fig. 2.1**), induction in response to treatment with disulfiram was strong; and when constitutive levels were low (see **Fig. 5.2.1**), induction was weaker.

ii) There was variation in the synthesis of late viral polypeptides between cultures infected with wt HSV-1, which correlated with variation

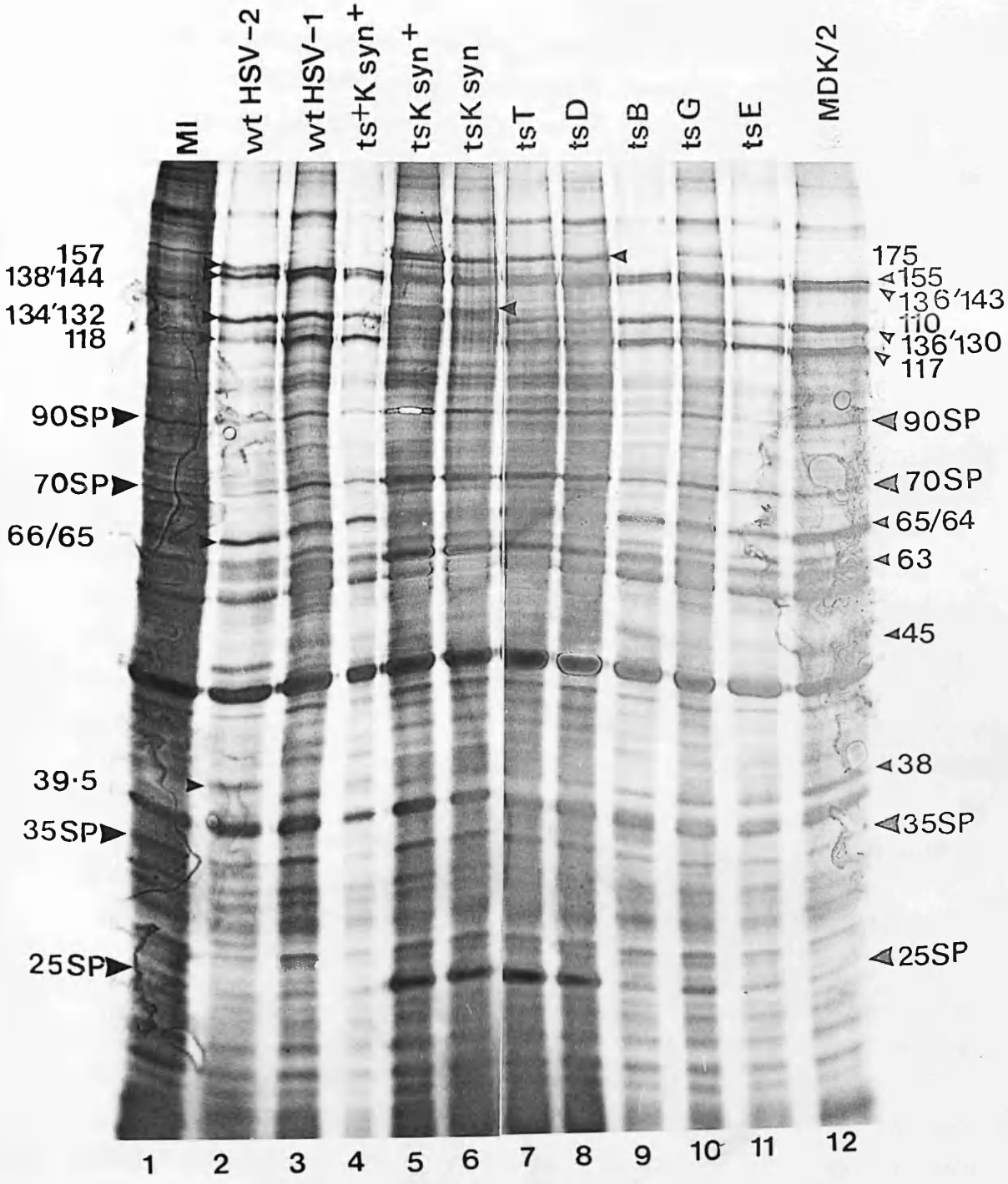
in the levels of constitutive synthesis of stress proteins: synthesis of certain late viral polypeptides was low (Vmw 51 and Vmw 28) or absent (Vmw 87, Vmw 85, Vmw 82 and Vmw 67) when levels of constitutive synthesis of stress proteins were high.

iii) Whether infection by less-strongly inducing viruses such as tsB, tsE, ts^+K, wt HSV-1 or wt HSV-2 (see points 3 and 4, above) caused the levels of synthesis of stress proteins to be increased in infected cultures appeared to depend upon the levels of constitutive synthesis: cultures with higher constitutive levels were most affected by infection; and cultures with lower constitutive levels were less affected.

Although the levels of constitutive synthesis of stress proteins in secondary CEF were not observed to vary according to the passage number of the cultures, the primary culture of CEF showed higher levels of constitutive synthesis than any of the secondary cultures tested; these levels (**Fig. 2.2**, track 1) were sustained following infection with wt HSV-2 (track 2), wt HSV-1 (track 3) or the revertant, ts^+K (track 4), and were increased after infection with mutants tsK syn^+ (track 5; 90SP, 70SP and 25SP), tsK syn, tsT, tsD (tracks 6 to 8; 70SP and 25SP), tsB, tsG, tsE and MDK/2 (tracks 9 to 12; all four major stress proteins, 70SP and 25SP especially with tsG). Furthermore the range of late viral polypeptides that was synthesized appeared to be restricted in the primary cells infected with wt HSV-1 or HSV-2: for example, synthesis of late viral polypeptides Vmw 87 and Vmw 67 and the equivalent HSV-2 polypeptides were undetectable. These observations suggest that the primary culture was more susceptible to induction of the stress response by infection with HSV and, correspondingly, less able to support the synthesis of viral polypeptides than the secondary cultures.

Fig. 2.2. Polypeptide synthesis in primary CEF infected with wt HSV-1, wt HSV-2 or ts mutants of HSV-1 at a NPT.

Primary CEF were mock-infected (track 1) or infected with wt HSV-2, wt HSV-1 or ts mutants of HSV-1 (40 p.f.u./cell: track 2, wt HSV-2; track 3, wt HSV-1; track 4, $ts^{+}K\ syn^{+}$; track 5, $tsK\ syn^{+}$; track 6, tsK syn; track 7, tsT; track 8, tsD; track 9, tsB; track 10, tsG; track 11, tsE; track 12, MDK/2) at the NPT of 38.5 ^{o}C. The cultures were pulse-labelled at 9 h p.i.. HSV-2 polypeptides are labelled to the left of track 1, and HSV-1 polypeptides to the right of track 12.


MI
wt HSV-2
wt HSV-1
ts+K syn+
tsK syn+
tsK syn
tsT
tsD
tsB
tsG
tsE
MDK/2
157
138/144
134/132
118
90SP
70SP
66/65
39·5
35SP
25SP
175
155
136/143
110
136/130
117
90SP
70SP
65/64
63
45
38
35SP
25SP
1
2
3
4
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11
12

3. COMPARISON OF THE RESPONSE OF CEF TO TREATMENT WITH DISULFIRAM ON SUCCESSIVE DAYS OF INCUBATION UNDER STANDARD CONDITIONS.

The aims of this experiment were to determine whether, during incubation of secondary CEF for several days under standard conditions, there exist variations in (a) the responsiveness of cells to treatment with disulfiram, or (b) the capacity of cells to support the synthesis of viral polypeptides following infection with HSV-1. Confluent monolayers of secondary CEF were incubated at 37 ^{o}C for 5 d without replenishment of the medium. On consecutive days, cultures were mock-infected or infected with wt HSV-1 or tsK, and either incubated at 37 ^{o}C or were treated with disulfiram at 38.5 ^{o}C for 3 h. The cultures were pulse-labelled with [^{35}S]methionine, and radiolabelled polypeptides were resolved by SDS-PAGE.

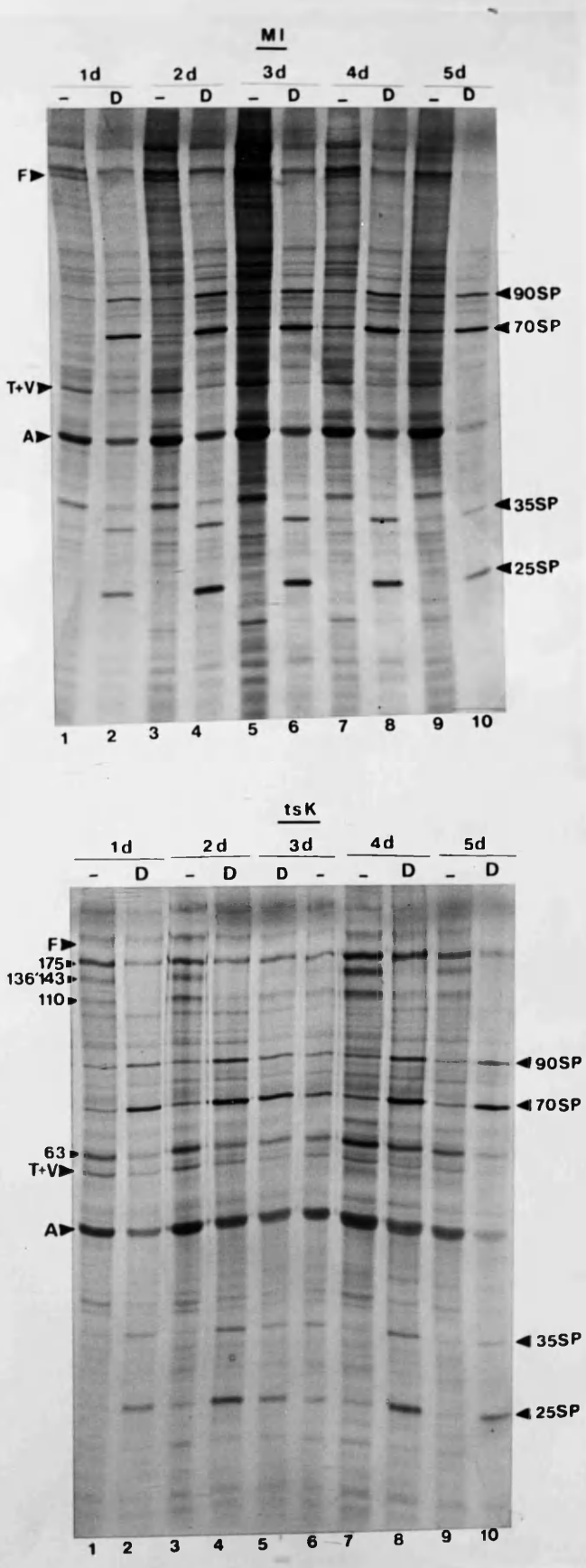
Fig. 3.1 shows that on each consecutive day of incubation at 37 ^{o}C, cells were equally responsive to treatment with disulfiram at 38.5 ^{o}C, and synthesis of the four major stress proteins was strongly induced (tracks 2, 4, 6, 8 and 10). In addition, treatment of cells with disulfiram suppressed the synthesis of actin (m.wt. 43,000) and of tubulin plus vimentin (cytoskeletal proteins of m.wt.'s 52-55,000, which co-migrated in this gel system), and caused the amount of radiolabelled fibronectin (m.wt. 230,000) that was recovered in cell extracts to be reduced, which might have reflected alteration in the rate of synthesis, processing, secretion or degradation of this membrane glycoprotein (Yamada et al., 1977; Olden, et al., 1978). It is apparent that throughout the period of incubation at 37 ^{o}C (tracks 1, 3, 5, 7 and 9), cells constitutively produced stress proteins at detectable levels, which increased during incubation under culture conditions, reaching maxima by day 3 (track 5). Reports were cited in the Introduction that cultured cells constitutively produce stress proteins when incubated at physiological temperatures. It is particularly relevant that Hightower & Smith (1978), using secondary cultures of CEF, have proven the identity between canavanine-enhanced proteins, which they designated P88, P71,72 (doublet) and P23, and proteins of similar electrophoretic mobility that were isolated from cultures maintained under standard conditions (corresponding to 90SP, 70SP and 25SP, by our notation). To date, all stress proteins with m.wt.'s between 70,000 and 110,000 have been

Fig. 3.1. Polypeptide synthesis in mock-infected secondary CEF incubated at 37 °C.

Confluent monolayers of secondary CEF were incubated at 37 °C for 1 d (tracks 1 and 2), 2 d (tracks 3 and 4), 3 d (tracks 5 and 6), 4 d (tracks 7 and 8) or 5 d (tracks 9 and 10). On consecutive days, the cultures were mock-infected and either incubated at 37 °C for 3 h (tracks 1, 3, 5, 7, and 9), or treated with disulfiram (0.3 uM) at 38.5 °C for 3 h (tracks 2, 4, 6, 8 and 10), after which the cultures were pulse-labelled. Fibronectin (F), tubulin plus vimentin (T + V) and actin (A) are indicated.

Fig. 3.2. Polypeptide synthesis in secondary CEF infected with tsK, in the presence or absence of disulfiram, at 38.5 °C.

Monolayers were incubated at 37 °C, as described in the legend to **Fig. 3.1**, for 1 d (tracks 1 and 2), 2 d (tracks 3 and 4), 3 d (tracks 5 and 6) 4 d (tracks 7 and 8) or 5 d (tracks 9 and 10). On consecutive days, the cultures were infected with tsK (40 p.f.u./cell) in the presence (tracks 2, 4, 5, 8 and 10) or absence (tracks 1, 3, 6, 7 and 9) of disulfiram (0.3 uM), and incubated at 38.5 °C for 3 h, and pulse-labelled.

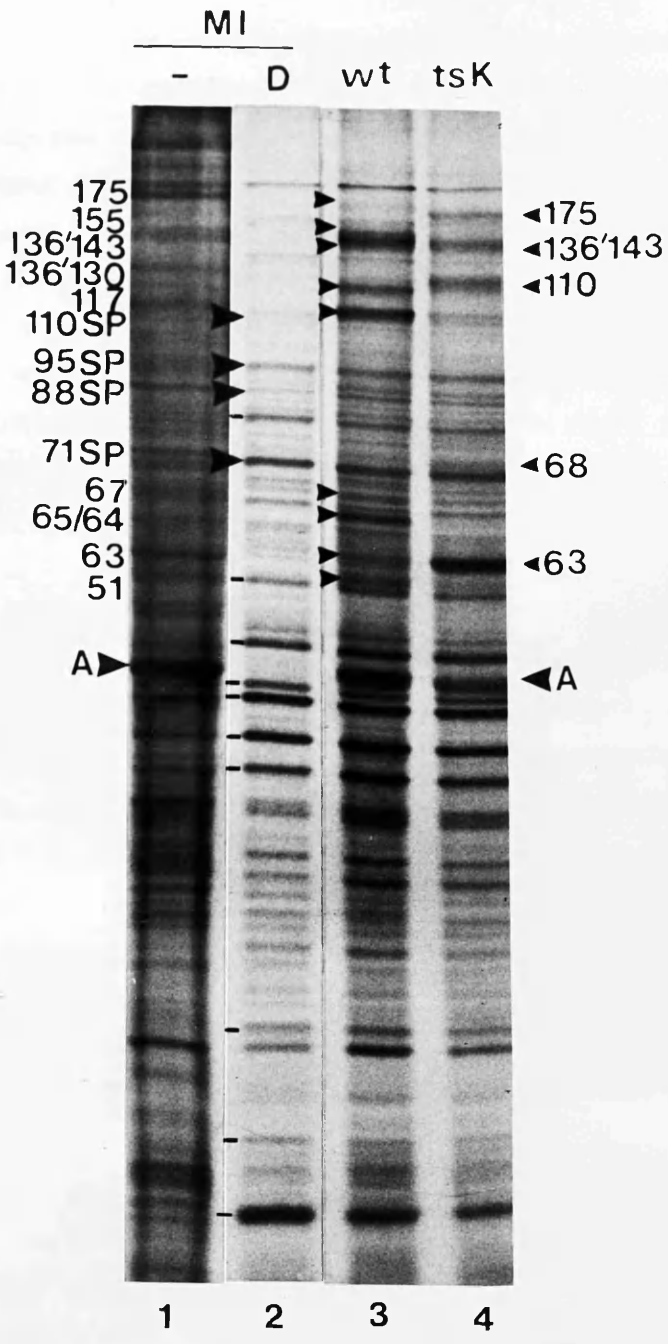

MI
1d
2d
3d
4d
5d
– D – D – D – D – D
F
T+V
A
90SP
70SP
35SP
25SP
1 2 3 4 5 6 7 8 9 10
tsK
1d
2d
3d
4d
5d
– D – D D – – D – D
F
175
136
143
110
63
T+V
A
90SP
70SP
35SP
25SP
1 2 3 4 5 6 7 8 9 10

detected in normal CEF (Wang et al., 1981; Kelley et al., 1980).

Fig. 3.2 shows polypeptide synthesis in cells that were infected with tsK at 38.5 ^{o}C either in the presence (tracks 2, 4, 5, 8 and 10) or absence (tracks 1, 3, 6, 7 and 9) of disulfiram. Treatment of cells with disulfiram caused an increase in synthesis of stress proteins and a reduction in synthesis of viral polypeptides; Vmw IE 136'143 and Vmw IE 110 were especially affected. Throughout this experiment, the pattern of viral polypeptides synthesized by wt HSV-1-infected cells was similar, and synthesis of stress proteins in wt HSV-1-infected cells remained at or below constitutive levels (results not shown).

Fig. 4.1. Polypeptide synthesis in primary REF treated with disulfiram or infected with wt HSV-1 or tsK at 38.5 ^{o}C.

Primary REF were mock-infected in the presence (track 2) or absence (track 1) of disulfiram (0.3 uM) at 38.5 ^{o}C for 3 h, or were infected with wt HSV-1 (40 p.f.u./cell; track 3) or tsK (40 p.f.u./cell; track 4) at 38.5 ^{o}C for 6 h, and pulse-labelled. Stress proteins which were identified previously by Johnston et al. (1980) are labelled to the left of track 1. Additional stress proteins (-) and actin (A) also are indicated.


MI
-
D
wt
tsK
175
155
136'143
136'130
117
110SP
95SP
88SP
71SP
67
65/64
63
51
A
175
136'143
110
68
63
A
1
2
3
4

4. THE STRESS RESPONSE IS INDUCED IN PRIMARY REF BY INFECTION WITH WT HSV-1.

The observation of higher levels of constitutive synthesis of stress proteins in primary CEF compared with secondary CEF (chapter 2.2) prompted a comparison of the stress response in a primary and secondary cultures of another cell type, rat embryo fibroblasts (REF). Although the stress response of cultured rat embryo cells is less well characterized, it appears to be analogous to the response of CEF in that synthesis of stress proteins is induced by a process requiring the synthesis of mRNA (Hightower & White, 1981). Treatment of cultured REF with canavanine causes induction of synthesis of a major stress protein (m.wt. 71,000) and several minor species (m.wt.'s 78,000, 88,000, 95,000 and 110,000), to degrees that vary between cultures (Hightower & White, 1981).

Two experiments were performed to investigate the stress response in REF infected with HSV-1. The first employed primary cells, which had not yet assumed a typical fibroblastic appearance but were small and epithelial-like; and the second employed secondary cells, which displayed a typical fibroblastic morphology.

Primary REF were infected with wt HSV-1 or tsK for 6 h, or mock-infected and treated with disulfiram for 3 h, at 38.5 ^{o}. The cultures were pulse-labelled with [^{35}S]methionine, and radiolabelled polypeptides were analysed by SDS-PAGE, as shown in **Fig. 4.1.**

The pattern of polypeptides which were synthesized in uninfected cells (track 1) was altered greatly by treatment with disulfiram (track 2): the complement of polypeptides whose synthesis was induced or increased (and which are "stress proteins" by definition) included not only the major (71SP) and minor (110SP, 95SP and 88SP) species reported by Hightower & White (1981), but also many other species, some of which are indicated. The induction of synthesis of so large a number of stress proteins and the suppression of synthesis of actin are features which, in CEF, would be symptomatic of a response to a high and cytotoxic concentration of a stress-inducing reagent (Johnston *et al.*, 1980). Indeed, in this experiment disulfiram-treated cells displayed a more

severe cytotoxic effect than wt HSV-1-infected cells at 6 h p.i.. TsK-infected cells demonstrated no c.p.e. at this time.

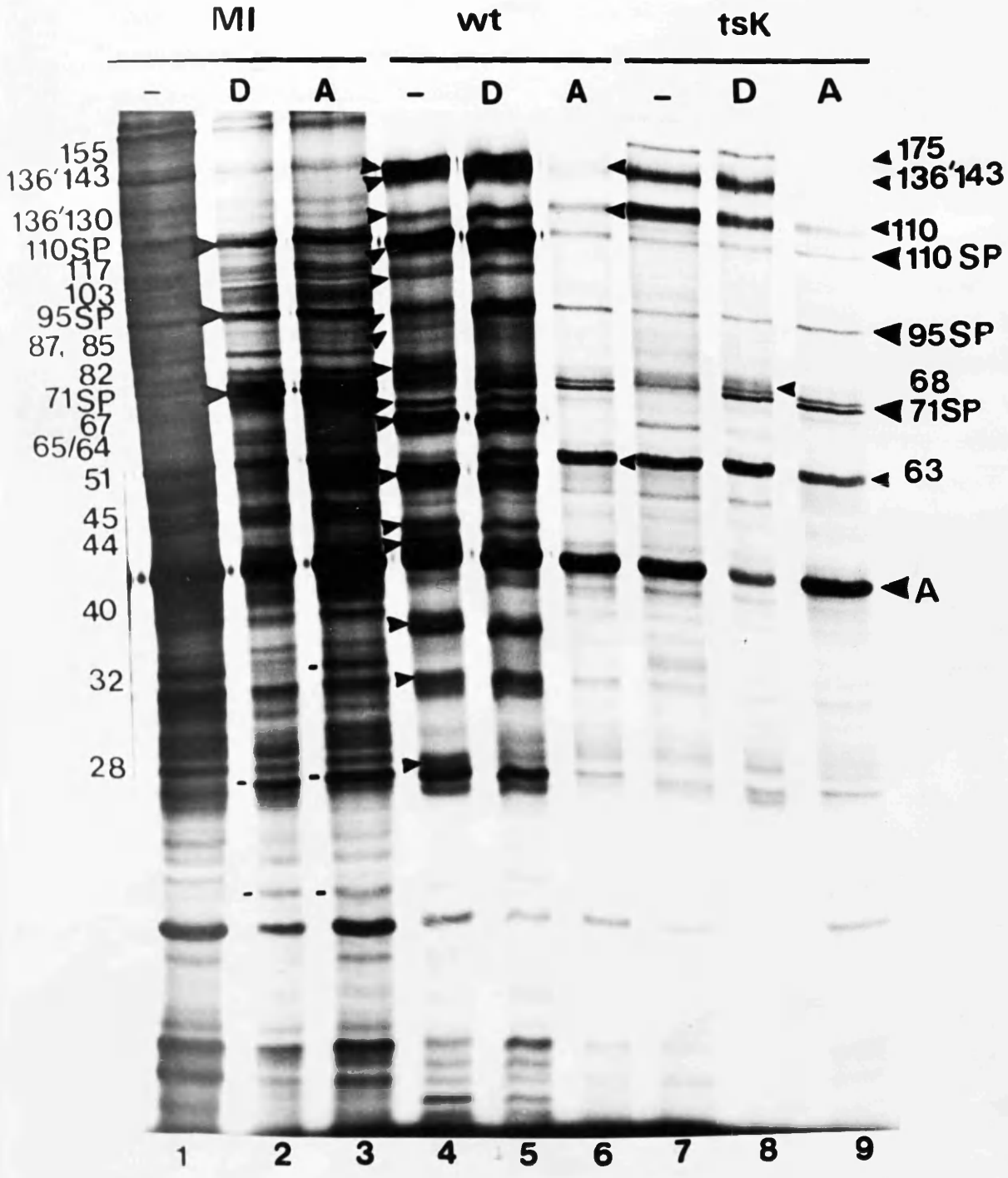
TsK-infected cells (track 4) produced IE viral polypeptides Vmw IE 175, Vmw IE 136'143, Vmw IE 110, Vmw IE 68 and Vmw IE 63. Wt HSV-1-infected cells (track 3) produced viral polypeptides of different temporal classes: IE polypeptides Vmw IE 136'143 and Vmw IE 63, and a polypeptide species which may correspond to a slow-migrating, processed form of Vmw IE 175 (see chapter 7); early polypeptide Vmw 136'130; and late viral polypeptides Vmw 155, Vmw 117, Vmw 67, Vmw 65/64 and Vmw 51. Many late viral polypeptides were not detectable (for example, Vmw 87, Vmw 85, Vmw 82, Vmw 45 and Vmw 40), suggesting that the primary REF were not fully permissive for wt HSV-1. However, the yields of infectious virus in primary REF were not examined.

The pattern of host polypeptides which were synthesized in wt HSV-1-infected (track 3) and tsK-infected cells (track 4) was identical to that in uninfected, disulfiram-treated cells (track 2) - except for the continued synthesis of actin in infected cells - and comprised the full complement of stress proteins. This indicates that the stress response was induced in primary REF by infection with wt HSV-1 or tsK, and indicates, furthermore, that synthesis of host proteins in primary REF may be affected in a similar manner following treatment with disulfiram or infection with HSV-1.

Fig. 4.2 shows that the response of secondary REF to treatment with disulfiram (track 2) or sodium arsenite (track 3) involved induction of synthesis of a more limited array of stress proteins, including 110SP, 95SP and 71SP (which was resolved into two distinct components), while actin continued to be synthesized. Sodium arsenite appeared to be a more potent stress-inducing agent than disulfiram under the experimental conditions (although the effects of a range of concentrations of the reagents were not compared). These responses were typical of secondary cells and broadly reproducible between cultures (results not shown). Secondary cells that were treated with sodium arsenite demonstrated a more severe cytotoxic effect than secondary cells that were treated with disulfiram, but less than that incurred by the culture of primary cells following treatment with disulfiram.

Fig. 4.2. Polypeptide synthesis in secondary REF infected with wt HSV-1 or tsK at 38.5 ^{o}C in the presence or absence of stress-inducing reagents.

Secondary REF were mock-infected (tracks 1 to 3) and treated with disulfiram (0.3 uM; track 2) or sodium arsenite (50 uM; track 3) at 38.5 ^{o}C for 3 h, or were infected with wt HSV-1 (40 p.f.u./cell; track 4) or tsK (40 p.f.u./cell; track 7) at 38.5 ^{o}C for 6 h, and pulse-labelled. Additional cultures were treated with either disulfiram or sodium arsenite from 1 h prior to and during infection with wt HSV-1 (tracks 5 and 6, respectively) or tsK (tracks 8 and 9, respectively).


MI
wt
tsK
–
D
A
–
D
A
–
D
A
155
136'143
136'130
110SP
117
103
95SP
87, 85
82
71SP
67
65/64
51
45
44
40
32
28
175
136'143
110
110 SP
95SP
68
71SP
63
A
1
2
3
4
5
6
7
8
9

Untreated, wild-type HSV-1-infected cells did not display the stress response (track 4), but in tsK-infected cells (track 7) levels of synthesis of stress proteins were more pronounced relative to levels in mock-infected cells, in keeping with the induction of the stress response in secondary CEF by infection with tsK at the NPT (chapters 1 and 2). Wt HSV-1-infected cells appeared to synthesize a wider range of late viral polypeptides (track 4) than wt HSV-1-infected primary REF, including Vmw 82, Vmw 45 and Vmw 40, but synthesis of IE viral polpeptides was undetectable.

When secondary REF were treated with stress-inducing reagents from 1 h prior to and during infection by wt HSV-1 or tsK, the stress response was induced and synthesis of viral polypeptides was adversely affected. In wt HSV-1-infected cells treated with disulfiram (track 5), IE viral polypeptide Vmw IE 63 was synthesized simultaneously with late viral polypeptides, while synthesis of specific late viral polypeptides was reduced (Vmw 44) or undetectable (Vmw 82); this indicates that viral replication was impaired to some extent. Treatment of wt HSV-1-infected cells with sodium arsenite caused much more pronounced effects upon the synthesis of viral polypeptides (track 6): synthesis of only the IE class of viral polypeptides (Vmw IE 136'143, Vmw IE 110 and Vmw IE 63) was detectable, and tsK-infected cells treated with sodium arsenite (track 9) gave a very similar result. Treatment of tsK-infected cells with disulfiram caused no detectable effect on the synthesis of viral polypeptides (track 8).

Results presented in this chapter show that there exists between cultures of REF variability in (a) the response of cells to reagent-mediated stress, (b) the levels of synthesis of stress proteins following infection with wt HSV-1, and (c) the ability to support synthesis of viral polypeptides. These features will be discussed in turn:

(a) That the primary cells were differently sensitive than the secondary culture to treatment with disulfiram was evidenced by the induction of synthesis of a wider range of stress proteins, by the reduction in synthesis of actin, and by the incursion of greater cytotoxic effects. Hightower & White (1981) observed that a family of stress proteins, m.wt.'s 71,000-73,000, is produced in incubated slices of rat organs, and speculated that the stress response might have been induced in

reaction to tissue damage or physiological trauma. By analogous reasoning, it is suggested that the recently established, primary culture that was used in the first experiment was particularly sensitive, or gave a heightened response, to treatment with disulfiram owing to the trauma involved in the isolation of rat-embryo cells. The secondary cultures, in comparison, might have adapted to conditions in culture, and therefore demonstrated more limited and standard responses to stress-inducing reagents. The difference in morphology that was noted between the primary and secondary cultures might have reflected a variable condition (for example, the degree of differentiation or adaptation to culture conditions) which predetermines the responsiveness of cells to stress.

(b) A notable result of the first experiment was the activation of the stress response in primary REF by infection with wt HSV-1 as well as by tsK. It is in keeping with observations described for primary and secondary CEF (chapter 2) that the primary culture of REF showed increased synthesis of stress proteins during infection with wt HSV-1, whereas secondary cultures did not. It is therefore suggested that infection by wt HSV-1 (or tsK) may cause primary or secondary CEF or REF to be subjected to stress, but whether the stress response is induced following infection depends upon the sensitivity of the cultures to stress-inducing treatments.

(c) Wt HSV-1-infected primary REF did not support the synthesis of late viral polypeptides as well as wt HSV-1-infected secondary REF; and this difference also may be connected with the induction of the stress response in primary cells during infection, since treatment of secondary REF with stress-inducing reagents prior to infection with wt HSV-1, adversely affeted the synthesis of late viral polypeptides (**Fig. 4.2**). The effects of inducing the stress response in HSV-1-infected cells upon the synthesis of viral polypeptides were investigated further, as described in chapters 5 and 6.

Given these differences in the behaviour of primary and secondary REF, it is further suggested that primary cells show features in connection with the stress response which may be relevant to natural infections by HSV-1.

5. THE RESPONSIVENESS OF CEF TO TREATMENT WITH DISULFIRAM DECREASES DURING INFECTION BY HSV-1.

Results that were presented in chapter 4 indicated that treatment of secondary REF with stress-inducing reagents prior to and during infection by wt HSV-1 or tsK caused the stress response to be induced and synthesis of viral polypeptides to be inhibited. Experiments to be described in this and the following chapter were intended to investigate the possibility of similar effects occurring also in HSV-1-infected secondary CEF.

5.1. Testing the Responsiveness of CEF to Treatment with Disulfiram at Different Stages in Infection by Wt HSV-1 or TsK.

The responsiveness of wt HSV-1-infected and tsK-infected CEF to treatment with disulfiram was tested between the time of infection and a late stage, 5 h p.i. at 38.5 ^{o}C; disulfiram and cycloheximide were added simultaneously to the medium, and polypeptide synthesis in the cultures was analysed 3 h later. Cycloheximide was added during treatment of the cells with disulfiram so that even short-lived changes in polypeptide synthesis would be detectable during the short period (20 min) of pulse-labelling with [^{35}S]methionine that followed removal of these reagents.

Fig. 5.1.1 shows a time-course of polypeptide synthesis in cultures infected at 38.5 ^{o}C: wt HSV-1-infected cells (tracks 2 to 6) synthesized a number of non-IE viral polypeptides at detectable levels by 2 h p.i. (track 3) and at increasing rates thereafter; Vmw 155, Vmw 136´130, Vmw 117, Vmw 67, Vmw 65/64, Vmw 51, Vmw 40, Vmw 38 and Vmw 28. Immediate-early polypeptides Vmw IE 136´143 and Vmw IE 63, continued to be produced throughout (see chapter 1.3), while synthesis of stress proteins remained undetectable (35SP and 25SP) or at constitutive levels (90SP and 70SP; track 1). TsK-infected cells (tracks 7 to 11) produced only the IE class of viral polypeptides; Vmw IE 175, Vmw IE 136´143, Vmw IE 110 and Vmw IE 63, but synthesis of Vmw IE 68 was not detectable. Synthesis of 90SP and 70SP increased gradually during infection by tsK.

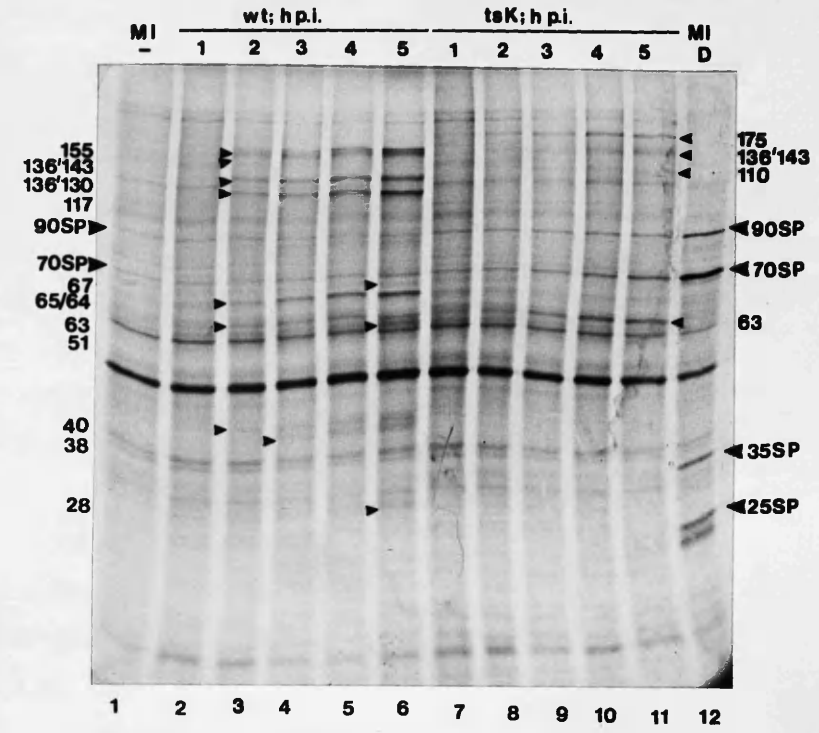
Fig. 5.1.2 shows polypeptide synthesis in wt HSV-1-infected

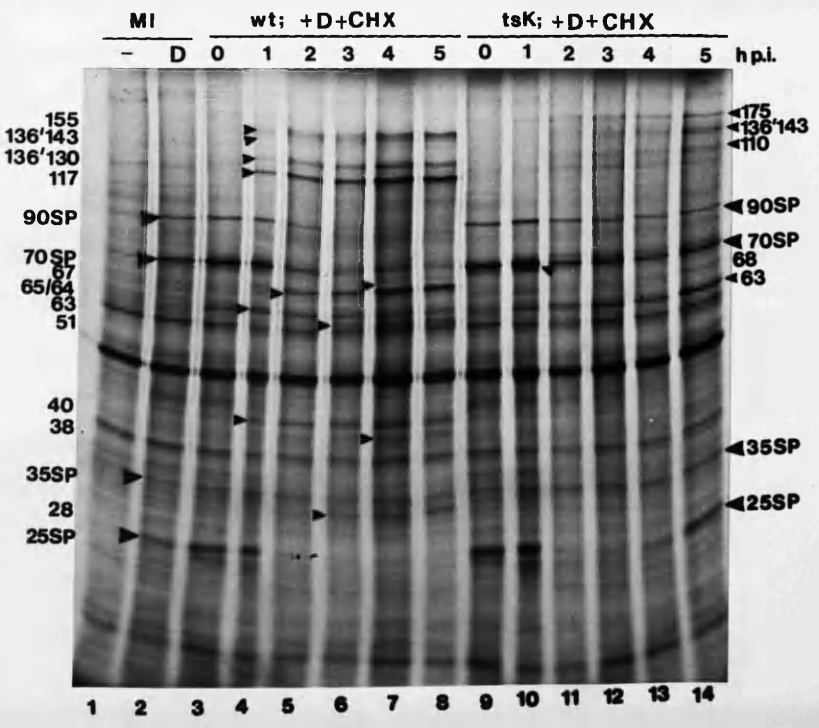
Fig. 5.1.1. Time-course of polypeptide synthesis in secondary CEF infected with wt HSV-1 or tsK at 38.5 °C.

Secondary CEF were mock-infected (tracks 1 and 12) or infected with wt HSV-1 (20 p.f.u./cell; tracks 2 to 6) or tsK (20 p.f.u./cell; tracks 7 to 11) at 38.5 °C. The cultures were pulse-labelled at these times p.i. or p.m.i.: 1 h (tracks 2 and 7); 2 h (tracks 3 and 8); 3 h (tracks 4 and 9); 4 h (tracks 5 and 10); and 5 h (tracks 1, 6 and 11). At 5 h p.m.i., a culture was treated with disulfiram (0.3 uM) for 3 h and then pulse-labelled (track 12).

Fig. 5.1.2. Polypeptide synthesis in wt-HSV-1-infected and tsK-infected secondary CEF following treatment with disulfiram and cycloheximide at 38.5 °C.

Secondary CEF were mock-infected (tracks 1 and 2) or infected with wt HSV-1 (20 p.f.u./cell; tracks 3 to 8) or tsK (20 p.f.u./cell; tracks 9 to 14) at 38.5 °C. Disulfiram (0.3 uM) and cycloheximide (20 ug/ml) were added to the growth medium at: the time of infection (tracks 3 and 9); 1 h p.i. (tracks 4 and 10); 2 h p.i. (tracks 5 and 11); 3 h p.i. (tracks 6 and 12); 4 h p.i. (tracks 7 and 13), and 5 h p.i. (tracks 8 and 14). At 5 h p.m.i., a culture was treated with disulfiram and cycloheximide (track 2). The cultures were incubated in the presence of these reagents for 3 h; the reagents were removed, and the cultures were pulse-labelled.


MI
wt; h p.i.
tsK; h p.i.
MI
–
1 2 3 4 5 1 2 3 4 5
D
155
136'143
136'130
117
90SP
70SP
67
65/64
63
51
40
38
28
175
136'143
110
90SP
70SP
63
35SP
25SP
1 2 3 4 5 6 7 8 9 10 11 12


MI
wt; +D+CHX
tsK; +D+CHX
– D 0 1 2 3 4 5 0 1 2 3 4 5 h p.i.
155
136'143
136'130
117
90SP
70SP
67
65/64
63
51
40
38
35SP
28
25SP
175
136'143
110
90SP
70SP
68
63
35SP
25SP
1 2 3 4 5 6 7 8 9 10 11 12 13 14

cells (tracks 3 to 8) and tsK-infected cells (tracks 9 to 14) following combined treatment with disulfiram and cycloheximide at times ranging between 1 and 5 h p.i.. The following features are apparent:

i) Comparison of polypeptides synthesized in mock-infected cells after treatment with disulfiram and cycloheximide (track 2) with polypeptides synthesized after treatment with disulfiram alone (**Fig. 5.1.1**, track 12) shows that induction of synthesis of 90SP, 70SP and 25SP was unaffected by the presence of cycloheximide, whereas synthesis of 35SP was less effectively induced (in agreement with the findings described in chapter 1.3), as was synthesis of minor stress proteins with m.wt.'s 20-23,000.

ii) Cells responded to treatment with disulfiram and cycloheximide when the reagents were applied prior to 2 h p.i. with wt HSV-1 (tracks 3 and 4) or tsK (tracks 9 and 10) - synthesis of 90SP, 70SP and 35SP was induced to levels as high as, or higher than, levels in treated, mock-infected cells (track 2).

iii) Cells became markedly less responsive to treatment with disulfiram between 1 and 2 h p.i. with either wt HSV-1 or tsK: in wt HSV-1-infected cells treated later than 2 h p.i. (tracks 6 to 8), synthesis of 90SP, 70SP, 35SP and 25SP remained at or below constitutive levels (track 1); and in tsK-infected cells treated at 5 h p.i. (track 14), synthesis of 90SP and 70SP remained at the levels in untreated, tsK-infected cells (**Fig. 5.1.1**, tracks 7 to 11), although slight induction was detectable for 35SP and 25SP. Wt HSV-1-infected and tsK-infected cells remained unresponsive to treatment until at least 11 h p.i. (results not shown). Synthesis of viral polypeptides in wt HSV-1-infected cells and tsK-infected cells was unaffected by treatment later than 2 h p.i., apart from an increase in synthesis of Vmw IE 68 in tsK-infected cells - an effect that is usually observed in HSV-1-infected cells on removal of a cycloheximide-block in protein synthesis.

Thus, cells became less responsive to treatment with disulfiram plus cycloheximide during infection with wt HSV-1 or tsK at 38.5 ^{o}C. Fully-functional Vmw IE 175 may not be implicated in this effect, since the polypeptide is defective in tsK-infected cells incubated at this temperature (Preston, 1979a and b).

5.2. Mutant- or Wt HSV-1-Infected CEF Are Unresponsive to Treatment with Disulfiram Several Hours Post-Infection at a NPT.

In a series of four experiments, secondary CEF were infected with wt HSV-1, wt HSV-2 or mutants of HSV-1 at 38.5 ^{o}C, in the presence or absence of disulfiram from 6 to 9 h p.i., and polypeptide synthesis in treated and untreated cultures was analysed and compared. (The viruses that were employed were tsB, tsD, tsE, tsG, tsT, tsK syn^{+}, tsK syn, ts^{+}K syn^{+}, MDK/2 tsl201, wt HSV-1 and wt HSV-2.) The results of one experiment are presented in Fig.'s 5.2.1 and 5.2.2.

Fig. 5.2.1 shows polypeptide synthesis in untreated secondary CEF, at 9 h p.i. with wt HSV-1 or mutants of HSV-1 at the NPT of 38.5 ^{o}C. In mock-infected cells (track 1), constitutive synthesis of stress proteins was barely detectable at this time, but synthesis of 90SP, 70SP and 35SP was stimulated by treatment with disulfiram for 3 h (track 11). In keeping with the general observations described in chapter 2, levels of synthesis of stress proteins were increased following infection by all mutants of HSV-1, including the revertant, tsK^{+} syn $^{+}$, but not by wt HSV-1:

i) Wt HSV-1-infected cells (track 2) synthesized stress proteins at constitutive levels (track 1), and a broad range of viral polypeptides (including Vmw 87, Vmw 67, Vmw 65/64, Vmw 51, Vmw 40 and Vmw 28), but little of the IE viral polypeptide Vmw IE 63, indicating that infection had progressed to a late stage.

ii) Infection by tsT (track 5) induced synthesis of 70SP and 25SP; while tsK syn^{+} (track 3) or tsD (track 6) strongly induced 90SP, 70SP and 25SP.

iii) Synthesis of stress proteins was increased to varying degrees by infection with ts^{+}K syn^{+}(track 4; 90SP and 70SP), tsB (track 7; 90SP, 70SP and 25SP), tsE (track 8; 70SP and 25SP), tsl201 (track 9; 90SP and 70SP) and MDK/2 (track 10; 90SP and 70SP).

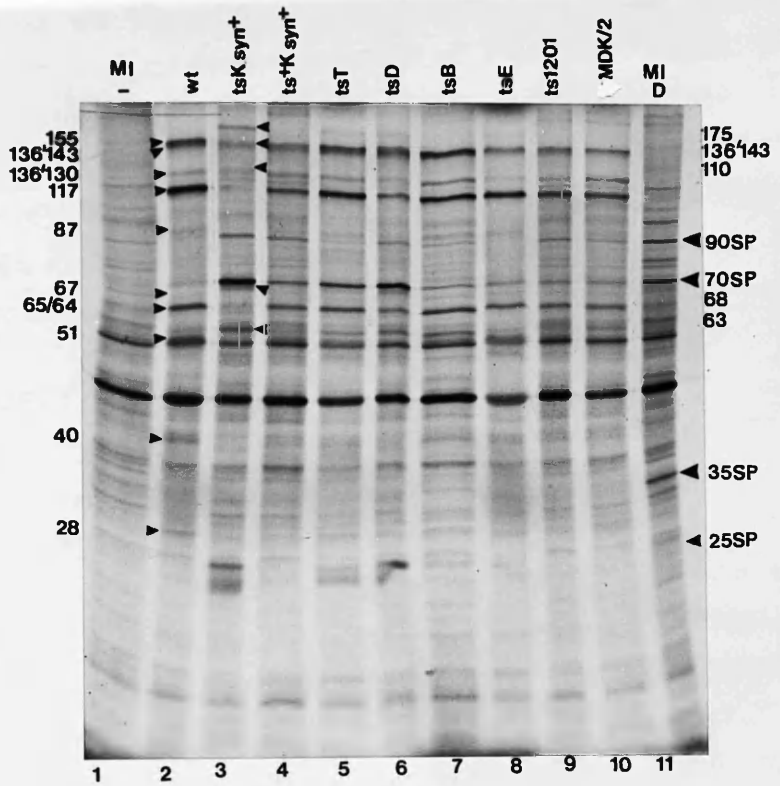
Fig. 5.2.2 shows polypeptide synthesis in infected cells

Fig. 5.2.1. Polypeptide synthesis in secondary CEF infected with wt HSV-1 and ts mutants of HSV-1 at 38.5 °C.

Secondary CEF were mock-infected (tracks 1 and 11) or infected with wt HSV-1 or ts mutants of HSV-1 (20 p.f.u./cell: track 2, wt HSV-1; track 3, tsK syn^+; track 4, ts^+K syn^+; track 5, tsT; track 6, tsD; track 7, tsB; track 8, tsE; track 9, ts1201; track 10, MDK/2) at 38.5 °C. A mock-infected culture was treated with disulfiram for 3 h prior to pulse-labelling (track 11). The cultures were pulse-labelled at 9 h p.i..

Fig. 5.2.2. Polypeptide synthesis in secondary CEF infected with wt HSV-1 and ts mutants of HSV-1 and treated with disulfiram at 38.5 °C.

Secondary CEF were mock-infected or infected with wt HSV-1 or ts mutants of HSV-1, as described in the legend to **Fig. 5.2.1.** Infected cultures were treated with disulfiram (0.3 uM) from 6 h to 9 h p.i. at 38.5 °C. Cultures were pulse-labelled at 9 h p.i..


MI
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tsK syn+
ts+K syn+
tsT
tsD
tsB
tsE
ts1201
MDK/2
MI
D
155
136+143
136+130
117
87
67
65/64
51
40
28
175
136+143
110
90SP
70SP
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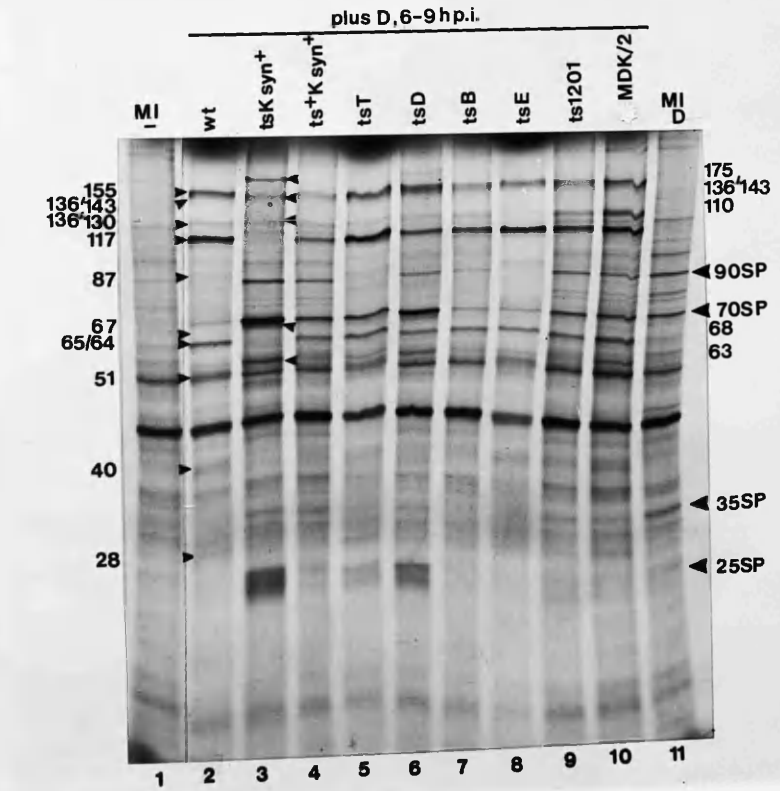
63
35SP
25SP
1 2 3 4 5 6 7 8 9 10 11


plus D, 6-9 h p.i.
MI
wt
tsK syn+
ts+K syn+
tsT
tsD
tsB
tsE
ts1201
MDK/2
MI
D
155
136+143
136+130
117
87
67
65/64
51
40
28
175
136+143
110
90SP
70SP
68
63
35SP
25SP
1 2 3 4 5 6 7 8 9 10 11

following treatment with disulfiram from 6 h p.i. to 9 h p.i. at 38.5 °C. The only detectable effects of the treatment were that synthesis of Vmw 40 and Vmw 28 was reduced slightly in wt HSV-1-infected cells (track 2), and that synthesis of 90SP, 70SP and 35SP was increased slightly in tsl201-infected (track 9) and MDK/2-infected (track 10) cells. In the remaining experiments of the series, which employed these and other viruses (tsG, tsK syn and wt HSV-2), differences were not detectable in the synthesis of viral polypeptides nor in the levels of synthesis of stress proteins between treated and untreated, infected cells.

These results therefore extend the observations made for wt HSV-1-infected and tsK-infected cells, described in chapter 5.1: secondary CEF were unresponsive to treatment with disulfiram several hours p.i. with wt HSV-1, wt HSV-2 and mutants of HSV-1 (tsB, tsD, tsE, tsG, tsT, tsK syn^+, tsK syn, ts^+K syn^+, tsl201 and MDK/2) at 38.5 °C; and although the stress response was induced in mutant-infected cells, treatment with disulfiram at this time did not produce an effect upon existing levels of synthesis of stress proteins, except in the cases of tsl201 and MDK/2.

5.3. Functional Vmw IE 175 Decreases the Response of wt HSV-1-Infected Cells to Treatment with Disulfiram Early in Infection.

As wt HSV-1-infected and tsK-infected cells become unresponsive to treatment with disulfiram from about 2 h p.i. at 38.5 °C, and since tsK-infected cells - but not wt HSV-infected cells - show gradual induction of synthesis of stress proteins following infection, it may be argued that infection with either virus causes cells to be subjected to increasing stress, the stress response becoming inhibited at an early stage in wt HSV-1-infected cells but not in tsK-infected cells. This hypothesis was tested by comparing the responsiveness of wt HSV-1-infected and tsK-infected CEF to treatment with disulfiram at various times during infection. In particular, the possibility was considered that differences in the responsiveness of wt HSV-1 and tsK-infected cells could be correlated with the synthesis of functional or non-functional Vmw IE 175.

Secondary CEF were infected with wt HSV-1 or tsK and incubated at 31 °C. At 3 h, 5 h and 7 h p.i., disulfiram and cycloheximide were added to the growth medium of replicate infected cultures, and the temperature of incubation raised to 38.5 °C so that previously synthesized Vmw IE 175 would be inactivated in tsK-infected cells. The purpose of cycloheximide was to prevent activation of the stress response by an additional means: that is, due to the accumulation of IE viral polypeptides which would otherwise occur in tsK-infected cells during incubation at the NPT. Incubation of the cultures was continued at 38.5 °C for 3 h. Polypeptide synthesis in replicate cultures was analysed either prior to (**Fig. 5.3.1**) or following treatment (**Fig. 5.3.2**) with disulfiram.

That infections by wt HSV-1 and tsK progressed at comparable rates at 31 °C was indicated by identical patterns of synthesis of viral polypeptides in infected cells (**Fig. 5.3.1**, tracks 3 to 8); by 3 h p.i., late viral polypeptides were produced (tracks 3 and 4) and synthesis of stress proteins was at or below constitutive levels (track 1).

Synthesis of major stress proteins 90SP, 70SP and 25SP was strongly induced in mock-infected cells in response to treatment with disulfiram in the presence of cycloheximide (**Fig. 5.3.2**), but the response of infected cells depended upon the time p.i. at which the reagents were applied and the temperature of incubation was raised to 38.5 °C:

i) The response of wt HSV-1-infected cells to treatment at 3 h p.i. (track 3) was reduced compared with the response of similarly-treated, mock-infected cells; and the response was further reduced in wt HSV-1-infected cells that were treated at 5 h, 6 h or 7 h p.i. (tracks 5, 7 and 9). It is also notable that in wt HSV-1-infected cells, synthesis of Vmw IE 175 was resumed and of Vmw IE 63 was increased following treatment at these times.

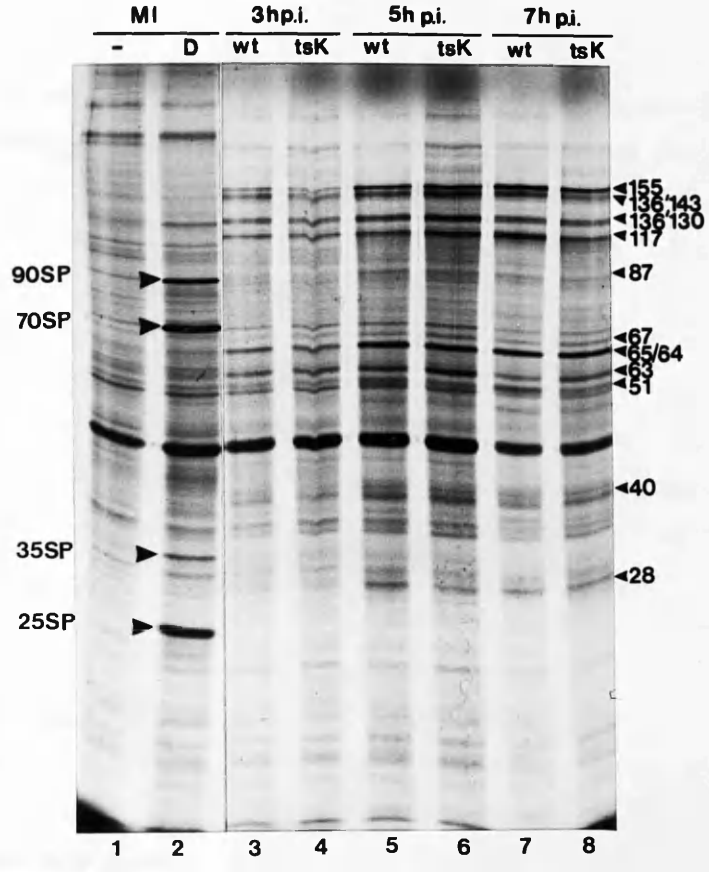
ii) TsK-infected cells responded strongly when treated at 3 h p.i. (track 4), but thereafter their response became reduced (tracks 6, 8 and 10), by 7 h approaching levels obtained in similarly-treated, wt

Fig. 5.3.1 Time-course of polypeptide synthesis in secondary CEF infected with wt HSV-1 or tsK at 31 °C.

Secondary CEF were mock-infected (track 1) or infected with wt HSV-1 (20 p.f.u./cell; tracks 3, 5 and 7) or tsK (20 p.f.u./cell; tracks 4, 6 and 8) at 31 °C. The cultures were pulse-labelled at these times p.i.: 3 h (tracks 3 and 4); 5 h (tracks 5 and 6); and 7 h (tracks 7 and 8). A mock-infected culture was treated with disulfiram (0.3 uM) for 3 h at 38.5 °C (track 2).

Fig. 5.3.2. Polypeptide synthesis in secondary CEF infected with wt HSV-1 or tsK at at 31 °C, following treatment with disulfiram and cycloheximide at 38.5 °C.

Cultures were mock-infected (tracks 1 and 2) or infected with wt HSV-1 (tracks 3, 5, 7 and 9) or tsK (tracks 4, 6, 8 and 10) at 31 °C, as described in the legend to **Fig. 5.3.1.** Disulfiram (0.3 uM) and cycloheximide (20 ug/ml) were added to the medium at these times p.i.: 3 h (tracks 3 and 4); 5 h (tracks 5 and 6); 6 h (tracks 7 and 8); and 7 h (tracks 9 and 10). Incubation of the cultures was continued at 38.5 °C for 3 h, after which the cultures were pulse-labelled.


MI
3hp.i.
5h p.i.
7h p.i.
-
D
wt
tsK
wt
tsK
wt
tsK
155
136'143
136'130
117
87
67
65/64
63
51
40
28
90SP
70SP
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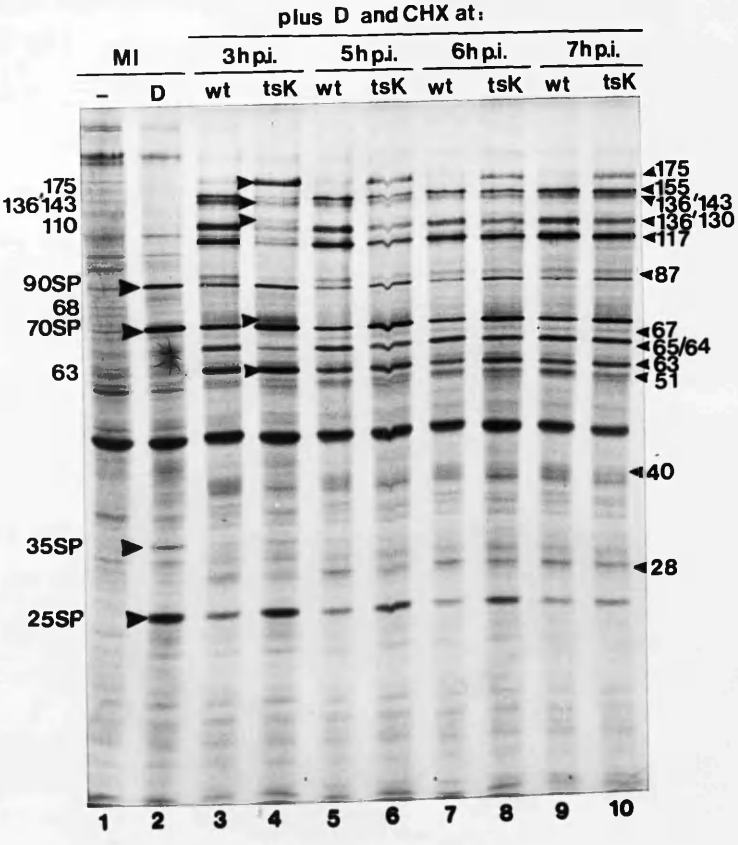
plus D and CHX at:
MI
3hp.i.
5hp.i.
6hp.i.
7hp.i.
-
D
wt
tsK
wt
tsK
wt
tsK
wt
tsK
175
136'143
110
90SP
68
70SP
63
35SP
25SP
175
155
136'143
136'130
117
87
67
65/64
63
51
40
28
1
2
3
4
5
6
7
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9
10

HSV-1-infected cells. The continued synthesis of non-IE viral polypeptides by tsK-infected cells at the NPT was due to the translation of persisting non-IE viral transcripts, since upshift of tsK-infected cells to a NPT at 7 h p.i. prevents transcription of early and late viral genes (Watson & Clements, 1980).

Thus, wt HSV-1-infected cells and tsK-infected cells became less responsive to treatment with disulfiram and cylcoheximide during the course of infection at 31 ^{o}C. This effect was dependent upon the expression of viral functions, since infection with wt HSV-1 or tsK in the presence of cycloheximide caused cells to remain as susceptible as mock-infected cells to induction of the stress response by treatment with disulfiram (see **Fig.'s 1.3** and **6.5**). The response of mock-infected and infected cells to treatment at the first time-point was quantitated by densitometry of tracks 1 to 10, using exposures that were within the linear response range; peak heights in the densitometer tracings were measured, normalised to actin and corrected for constitutive synthesis. The values which were derived, shown in **Table I**, express the increase in synthesis of stress proteins in wt HSV-1-infected and tsK-infected cells relative to a value of 100 for similarly-treated, mock-infected cells. The data indicate that following treatment at 3 h p.i., wt HSV-1-infected and tsK-infected cells synthesized stress proteins at lower levels than mock-infected cells, synthesis of 35SP being especially affected; the reduction in wt HSV-1-infected cells was approximately double that in tsK-infected cells for 90SP, 70SP and 25SP. Thus, the response of cells to treatment with disulfiram (in the presence of cylcoheximide) may be inhibited during infection by HSV-1, functional Vmw IE 175 being partly responsible at an early stage of infection. Synthesis of stress proteins was increasingly inhibited in wt HSV-1-infected and tsK-infected cells following treatment at times later than 3 h p.i..

5.4. IE Viral Polypeptides Inhibit the Response of HSV-1-Infected CEF to Treatment with Disulfiram.

To investigate the extent to which IE viral polypeptides could affect the response of secondary CEF to treatment with disulfiram, cells were

Table I: Levels of induction of synthesis of stress proteins in infected CEF relative to mock-infected cells, following infection at 31° for 3 h and treatment with disulfiram and cycloheximide at 38.5° for 3 h.

	MI	wt HSV-1	tsK
3 h p.i. at 31 °C:			
90SP	100	27	71
70SP	100	47	80
35SP	100	8	21
25SP	100	33	75
5 h p.i. at 31 °C:			
90SP	100	18	49
70SP	100	35	72
35SP	100	8	7
25SP	100	21	51
6 h p.i. at 31 °C:			
90SP	100	13	44
70SP	100	34	70
35SP	100	8	6
25SP	100	20	40
7 h p.i. at 31 °C:			
90SP	100	14	43
70SP	100	35	59
35SP	100	8	13
25SP	100	18	41

infected with wt HSV-1 or tsK in the presence of cycloheximide and incubated at 38.5 °C for 3 h; and cycloheximide was withdrawn from the cultures for an interval of 20 min, during which time only the IE class of viral polypeptides was synthesized in infected cells to detectable levels (results not shown; Preston, 1979a). Cycloheximide was restored to the medium, disulfiram was added, and incubation of the cultures was continued at 38.5 °C for 3 h. Polypeptide synthesis in the cultures was analysed on removal of these reagents, and is shown in Fig. 5.4. Early and late polypeptides were synthesized by wt HSV-1-infected cells, indicating that non-IE viral genes were transcribed during or after release from the cycloheximide-block in protein synthesis.

Table II shows the increase in synthesis of stress proteins in infected cells relative to mock-infected cells following treatment with disulfiram and cycloheximide. Induction was substantially reduced in tsK-infected cells, and even more so in wt HSV-1-infected cells, compared with mock-infected cells; and the reduction may be attributed to the production of IE viral polypeptides by infected cells prior to treatment with disulfiram. The further reduction observed in wt HSV-1-infected cells would be caused either by functional Vmw IE 175 directly, or by low levels of non-IE viral polypeptides which may have been synthesized during the period of release from cycloheximide. Inhibition of the stress response in wt HSV-1-infected cells was more effective here than in the previous experiment (Fig. 5.3.1), where cells were treated at 3 h p.i. at 31 °C, by which time many non-IE viral polypeptides were produced at high levels. Therefore, in the experiment of Fig. 5.3.1, IE viral polypeptides, rather than low levels of non-IE viral polypeptides, must be mainly responsible for the inhibition of the response of wt HSV-1-infected cells to treatment with disulfiram.

Fig. 5.4. Effects of IE viral polypeptides upon induction of synthesis of stress proteins in secondary CEF in response to treatment with disulfiram.

Secondary CEF were mock-infected (tracks 1 and 2) or infected with wt (20 p.f.u./cell; track 3) or tsK (20 p.f.u./cell; track 4) at 38.5 ^{o}C in the presence of cycloheximide (20 ug/ml) for 3 h. Cycloheximide was removed, and the cultures were incubated in cycloheximide-free medium for 20 min. Cycloheximide was restored to the medium, disulfiram (0.3 uM) was added (tracks 2 to 4), and incubation of the cultures was continued at 38.5 ^{o}C for 3 h. The cultures were pulse-labelled for 20 min only.

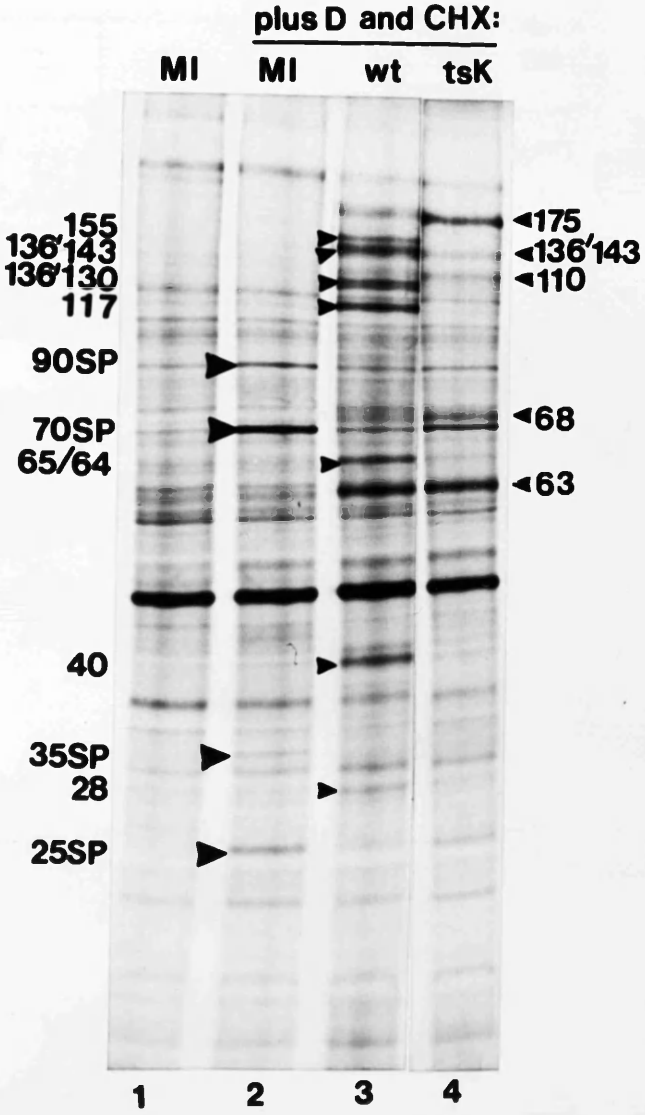

plus D and CHX:
MI
MI
wt
tsK
155
136/143
136/130
117
90SP
70SP
65/64
40
35SP
28
25SP
175
136/143
110
68
63
1
2
3
4

Table II: Levels of induction of synthesis of stress proteins in infected cells relative to mock-infected cells, following release from a cycloheximide-block in protein synthesis and treatment with disulfiram and cycloheximide at 38.5^{o} for 3 h.

	MI	wt HSV-1	tsK
90SP	100	0	25
70SP	100	6	27
35SP	100	0	0
25SP	100	16	33

6. INDUCING THE STRESS RESPONSE IN CEF AND HELU CELLS ADVERSELY AFFECTS LYTIC INFECTION BY WT HSV-1.

Results obtained using secondary REF (**Fig. 4.2**) and secondary CEF (shown in **Fig.'s 5.1.2** and **5.2.2**) indicated that treatment of cells with stress-inducing agents early in infection by wt HSV-1 caused synthesis of certain IE viral polypeptides (Vmw IE 175 and Vmw IE 63) to be sustained and synthesis of other IE and non-IE viral polypeptides to be reduced. This chapter investigates the extent to which activation of the stress response in CEF and other cell types, BHK cells and human embryonic lung (HeLu) cells (by treatment with stress-inducing reagents) may influence synthesis of viral polypeptides during infection by HSV.

6.1. Pre-Treatment of CEF with Disulfiram Affects the Synthesis of Viral Polypeptides following Infection with Wt HSV-1 or TsK.

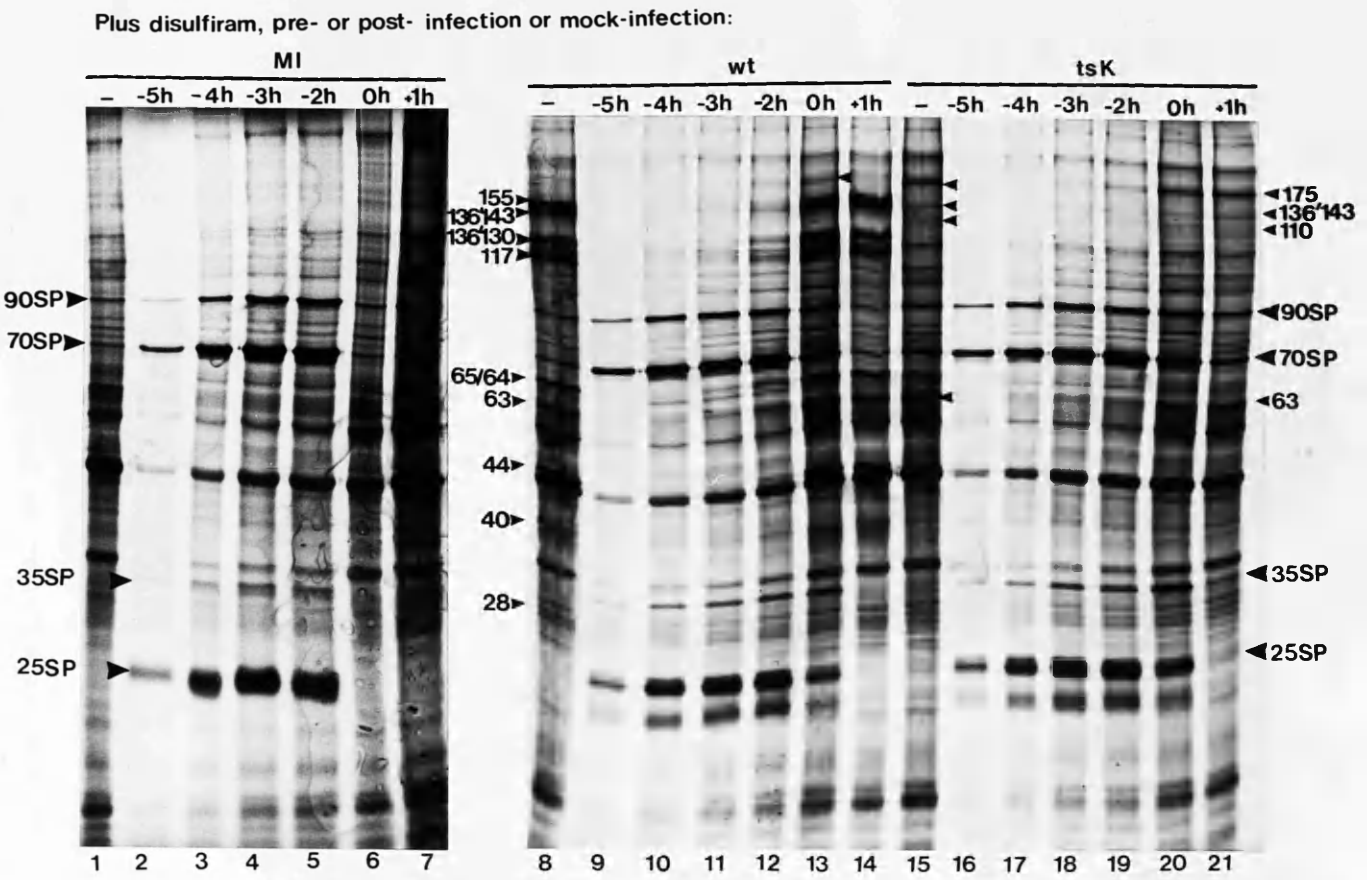
The effects of pre-treatment of CEF with disulfiram upon the synthesis of viral polypeptides and stress proteins following infection with wt HSV-1 or tsK at 38.5 ^{o}C were examined. Disulfiram was added to the medium of cultures at times ranging from 5 h pre-infection to 1 h post-infection with wt HSV-1 or tsK (at which time cells remain responsive to treatment with disulfiram; chapter 5), and incubation was continued until 5 h p.i., when polypeptide synthesis in the cultures was analysed, as shown in **Fig. 6.1.**

Several important observations may be drawn from the results:

(i) Treatment of mock-infected or infected cells with disulfiram for 7 h (tracks 5, 12 and 19) caused induction of synthesis of the four major stress proteins; by 10 h of treatment (tracks 2, 9 and 16), 70SP and 25SP were mainly synthesized. During prolonged exposure of cells to disulfiram (tracks 2 and 3; 9 and 10; 16 and 17), the overall rate of protein synthesis (as assayed by the incorporation of radioactivity into TCA-precipitable material) and the levels of synthesis of stress proteins were considerably reduced. [Similar time-dependent kinetics of induction and reduction in the rate of total protein synthesis have been reported for CEF treated continuously with sodium arsenite (Johnston

Fig. 6.1. Polypeptide synthesis in secondary CEF treated with disulfiram prior to or following infection with wt HSV-1 or tsK at 38.5 °C.

Secondary CEF were mock-infected (tracks 1 to 7), or infected with wt HSV-1 (20 p.f.u./cell; tracks 9 to 14) or tsK (20 p.f.u./cell; tracks 16 to 21) for 5 h at 38.5 °C. The cultures were treated continuously with disulfiram (0.3 uM) from these times prior to infection or mock-infection: 5 h (tracks 2, 9 and 16), 4 h (tracks 3, 10 and 17), 3 h (tracks 4, 11 and 18), 2 h (tracks 5, 12 and 19): or from the time of infection or mock-infection (tracks 6, 13 and 20), or from 1 h post-infection or mock-infection (tracks 7, 14 and 21). Additional cultures were mock-infected (track 1), or infected with wt HSV-1 (track 8) or tsK (track 15) in the absence of disulfiram. The cultures were pulse-labelled at 5 h p.i. or p.m.i..


Plus disulfiram, pre- or post- infection or mock-infection:
MI
wt
tsK
– -5h -4h -3h -2h 0h +1h
90SP
70SP
35SP
25SP
155
136'143
136'130
117
65/64
63
44
40
28
175
136'143
110
90SP
70SP
63
35SP
25SP
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

† Treatment with disulfiram has been found to cause a large reduction in the production of infectious virus in wt HSV-1-infected cells (Dr. C. M. Preston, personal communication).

et al., 1980).]

(ii) Synthesis of viral polypeptides also was reduced in wt HSV-1-infected and tsK-infected cells by 2 h or longer pre-treatment with disulfiram. (Synthesis of Vmw IE 175 was detectable in wt HSV-1-infected cells that were treated with disulfiram from 3 h or 2 h prior to infection and from the time of infection (tracks 11 to 13).)

(iii) The response of wt HSV-1-infected and tsK-infected cells to treatment with disulfiram at the time of infection (tracks 13 and 20) was increased (or "superinduced") relative to mock-infected cells (track 6). However, when cells were treated from 2 h or longer prior to infection (tracks 9 to 12, and 16 to 19), resulting levels of synthesis of stress proteins were similar to those in mock-infected cells (tracks 2 to 5). To explain these observations, it is suggested that either: (a) the stress response was stimulated maximally by prolonged treatment of cells with disulfiram, so that no further increase was caused by infection with wt HSV-1 or tsK; or (b), since protein synthesis is required post-infection with wt HSV-1 or tsK for the stress response to be superinduced in reagent-treated, infected cells (chapter 1.3), the shut-down in protein synthesis during prolonged exposure to disulfiram may have precluded a stimulation of the rates of synthesis of stress proteins following infection by wt HSV-1 or tsK.

(iv) Pre-treatment of cells with disulfiram was observed to reduce the c.p.e. incurred following infection with wt HSV-1, perhaps reflecting the reduction in synthesis of viral polypeptides under these conditions.

6.2. Superinduction of the Stress Response in Disulfiram-Treated CEF by Infection with Wt HSV-1 or TsK, and Effects upon the Synthesis of Viral Polypeptides.

The superinduction of the stress response in reagent-treated secondary CEF was found to be a reproducible effect of infection by wt HSV-1 or tsK, which was maximal when cells were treated from 1 h prior to infection. This is illustrated by the results of an experiment in which cells were treated continuously with disulfiram from 1h prior to

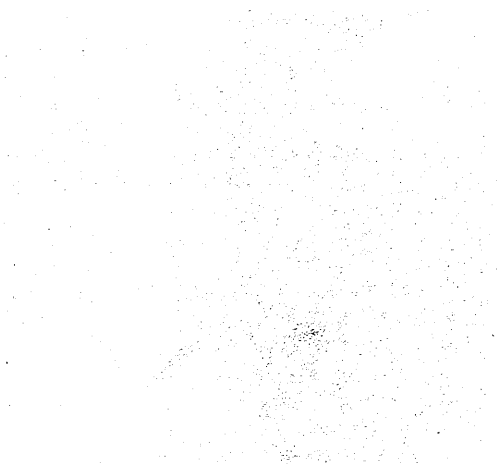
infection, or 1 h p.i., with wt HSV-1 or tsK at 38.5 ^{o}C. Additional mock-infected and infected cultures were incubated at 38.5 ^{o}C in the absence of disulfiram.

Fig. 6.2.1 shows the time-course of induction of synthesis of stress proteins in mock-infected CEF during continuous treatment with disulfiram: in untreated cells (track 1), synthesis was detectable of 90SP and 70SP, but not of 25SP or 35SP. By 3 h of treatment (track 3), synthesis of 90SP, 70SP and 35SP was induced to high levels, and synthesis of 25SP was increased; by 5 h (track 4), synthesis of 35SP had declined, while synthesis of the 90SP, 70SP and 25SP was maintained; and by 7 h (track 5), synthesis of 90SP, 70SP and 35SP was restored to uninduced levels, but synthesis of 25SP continued and was still detectable at 9 h (track 6) and 11 h (track 7). The rate of total protein synthesis was progressively diminished, and almost negligible by 11 h of treatment. These kinetics were typical of a response of CEF to continuous treatment with a stress-inducing reagent, as described previously by other workers (see chapter 2; Levinson et al., 1978a and 1978b; Johnston et al., 1980).

Fig.'s 6.2.2 and **6.2.3** show time-courses of polypeptide synthesis in CEF infected at 38.5 ^{o}C with wt HSV-1 or tsK, respectively, and treated with disulfiram from 1 h p.i. (tracks 3 to 7, both figures) or from 1 h prior to infection (tracks 8 to 12). Both wt HSV-1-infected cells and tsK-infected cells were responsive to treatment with disulfiram when the reagent was applied from either 1 h prior to infection or 1 h p.i., and synthesis of stress proteins was strongly induced. However, treatment of cells with disulfiram prior to infection with wt HSV-1 or tsK evoked a much stronger response than treatment post-infection, resulting levels of synthesis of stress proteins greatly exceeding the levels induced in similarly treated, mock-infected cells: compare, for example levels of synthesis of stress proteins in wt HSV-1-infected and tsK-infected cells treated with disulfiram from 1 h prior to infection (track 8, both figures) with levels in mock-infected cells treated from 1 h prior to mock-infection (**Fig. 6.2.1**, track 3). Concerning the time-course of synthesis of stress proteins in wt HSV-1-infected and tsK-infected cells, levels of synthesis were maximal at 4-6 h p.i. when disulfiram was added 1 h p.i. (tracks 4 and 5), and at 2-4 h p.i. when disulfiram was added 1 h prior to infection;

Fig. 6.2.1. Time-course of polypeptide synthesis in disulfiram-treated secondary CEF at 38.5 °C.

Secondary CEF were mock-infected and incubated at 38.5 °C in the absence (track 1) or presence of disulfiram (0.3 uM) for 1 h (track 2), 3 h (track 3), 5 h (track 4), 7 h (track 5), 9 h (track 6) or 11 h (track 7), and then pulse-labelled.



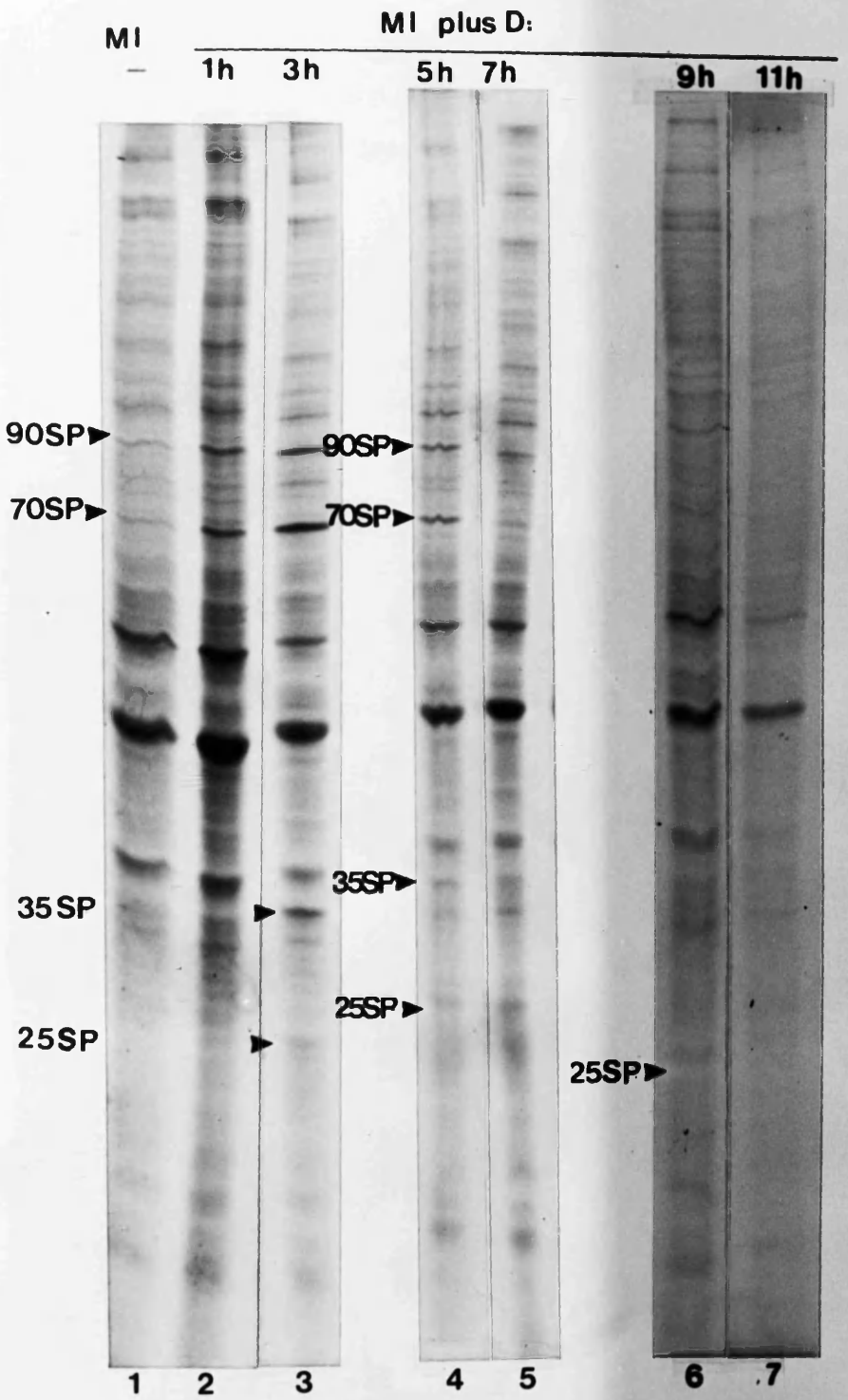
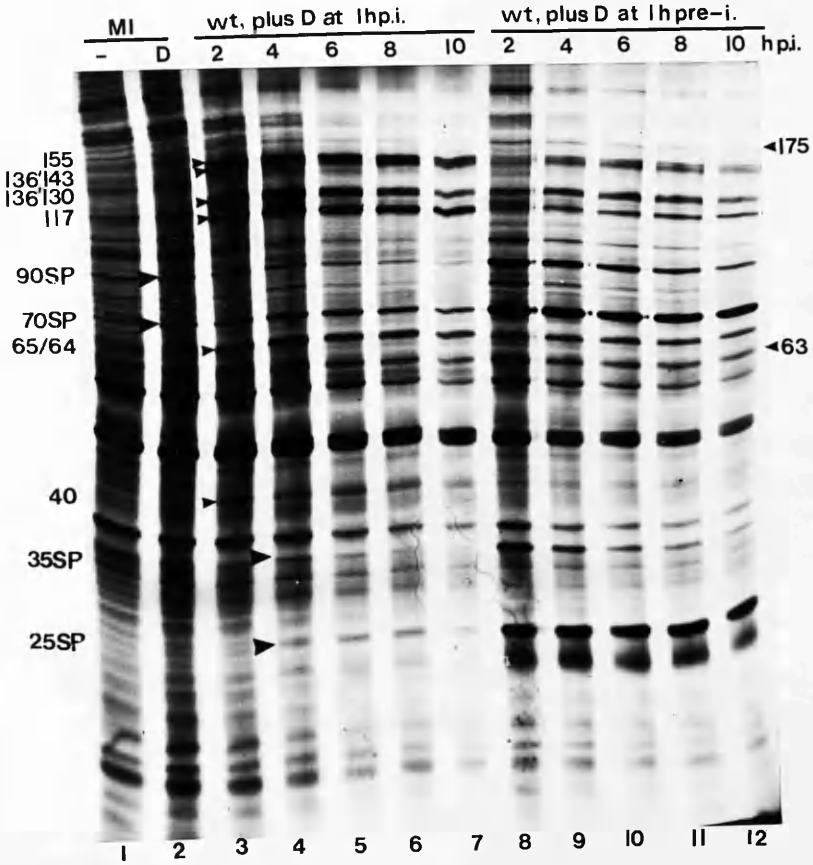
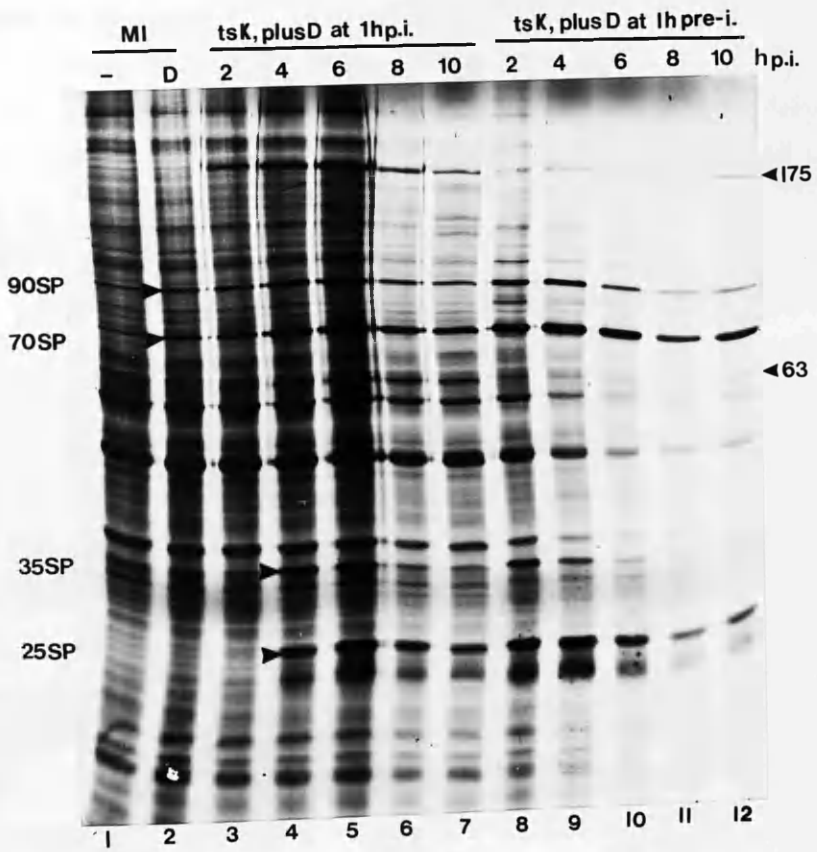

MI
MI plus D:
—
1h
3h
5h
7h
9h
11h
90SP
70SP
35SP
25SP
90SP
70SP
35SP
25SP
25SP
1
2
3
4
5
6
7

Fig.'s 6.2.2 and 6.2.3. Polypeptide synthesis in secondary CEF treated with disulfiram from either 1 h p.i. or 1 h prior to infection with wt HSV-1 or tsK at 38.5 ^{o}C.

Secondary CEF were incubated at 38.5 ^{o}C, and treated with disulfiram (0.3 uM) continuously from either 1 h p.i. (tracks 3 to 7, both figures) or 1 h prior to infection (tracks 8 to 12, both figures) with wt HSV-1 (20 p.f.u./cell; **Fig. 6.1.2**, tracks 3 to 12) or tsK (20 p.f.u./cell; **Fig. 6.1.3**, tracks 3 to 12). Cultures were pulse-labelled at these times p.i.: 2 h (tracks 3 and 8, both figures), 4 h (tracks 4 and 9), 6 h (tracks 5 and 10), 8 h (tracks 6 and 11) and 10 h (tracks 7 and 12). Mock-infected cultures were pulse-labelled 10 h p.m.i. (track 1), or after treatment with disulfiram for 2 h (track 2).


MI
wt, plus D at 1hp.i.
wt, plus D at 1h pre-i.
– D 2 4 6 8 10 2 4 6 8 10 hp.i.
155
136'143
136'130
117
90SP
70SP
65/64
40
35SP
25SP
175
63
1 2 3 4 5 6 7 8 9 10 11 12


MI
tsK, plusD at 1hp.i.
tsK, plus D at 1h pre-i.
– D 2 4 6 8 10 2 4 6 8 10 hp.i.
90SP
70SP
35SP
25SP
175
63
1 2 3 4 5 6 7 8 9 10 11 12

thereafter relative levels of synthesis of stress proteins were maintained while the rate of total protein synthesis decreased.

Fig. 6.2.4 compares polypeptide synthesis in treated and untreated cells, 4 h p.i. with wt HSV-1 or tsK. Treatment of infected cells with disulfiram from 1 h p.i. had little effect upon the pattern of viral polypeptides produced (tracks 5 and 8). However, treatment of wt HSV-1-infected (track 6) and tsK-infected cells (track 9) from 1 h prior to infection caused synthesis of Vmw IE 175 to be sustained while causing synthesis of other viral polypeptides to be reduced.

6.3. Comparison of the Effects of Temporary and Continuous Treatment of Wt HSV-1-Infected or TsK-Infected CEF or BHK cells with Stress-Inducing Reagents upon the Synthesis of Viral Polypeptides.

An experiment was performed to determine whether the presence of disulfiram is required continuously, or whether temporary treatment of cells for 1 h immediately prior to infection is sufficient, to sustain changes in the pattern of synthesis of viral polypeptides in secondary CEF following infection with wt HSV-1 or tsK at 38.5 ^{o}C. Replicate infected cultures were treated with sodium arsenite. Polypeptide synthesis in the cultures was analysed at 5 h p.i., and the results are shown in **Fig. 6.3.1.**

Treatment of mock-infected cells with disulfiram for 6 h in total (track 4) caused synthesis of the major stress proteins - especially 25SP - to be induced; but treatment with sodium arsenite for 6 h (track 5) caused much stronger induction of synthesis of 90SP, 70SP and 25SP, to levels exceeding the rate of actin. However, when cells were treated with either reagent for 1 h only, prior to mock-infection, the stress response 5 h later was not as strongly induced: disulfiram-treated cells (track 2) showed increased synthesis of only 25SP (compared with untreated cells, track 1); and sodium-arsenite-treated cells (track 3) showed increased synthesis of 90SP, 70SP, 35SP and especially 25SP. (That preferential synthesis of 25SP occurs in CEF following removal of the stress-inducing reagent and during the return to the uninduced pattern of polypeptide synthesis was shown by Levinson *et al.* (1978a).)

Fig. 6.2.4. Polypeptide synthesis in untreated and disulfiram-treated, wt-HSV-1-infected and tsK-infected secondary CEF.

Samples from cultures which were mock-infected or infected with wt HSV-1 or tsK and treated with disulfiram and pulse-labelled at 4 h p.i. or p.m.i., as described in the legend to **Fig.'s 6.2.2** and **6.2.3**, were analysed by SDS-PAGE.

Tracks 1 to 3; mock-infected cells. Tracks 4 to 6; wt-HSV-1-infected cells. Tracks 7 to 9; tsK-infected cells. Cultures were incubated in the absence of disulfiram (tracks 1, 4 and 7), or were treated with disulfiram either from 1 h p.i. or p.m.i. (tracks 2, 5 and 8), or from 1 h prior to mock-infection or infection (tracks 3, 6 and 9).

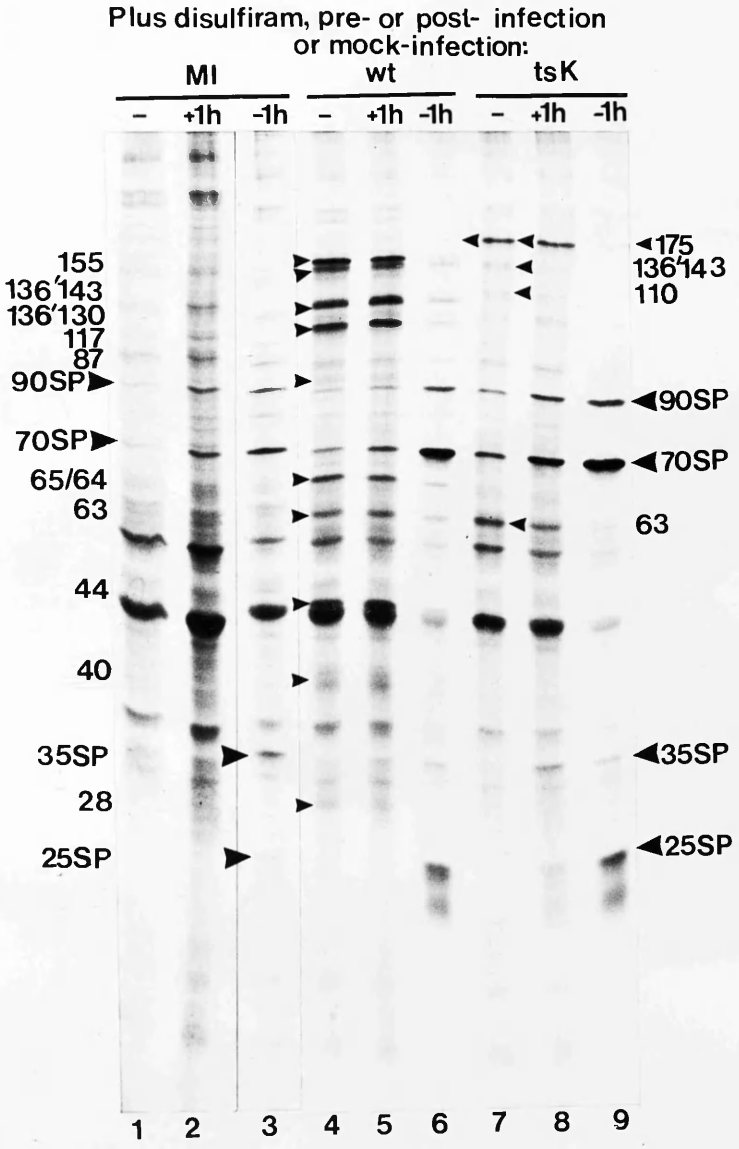
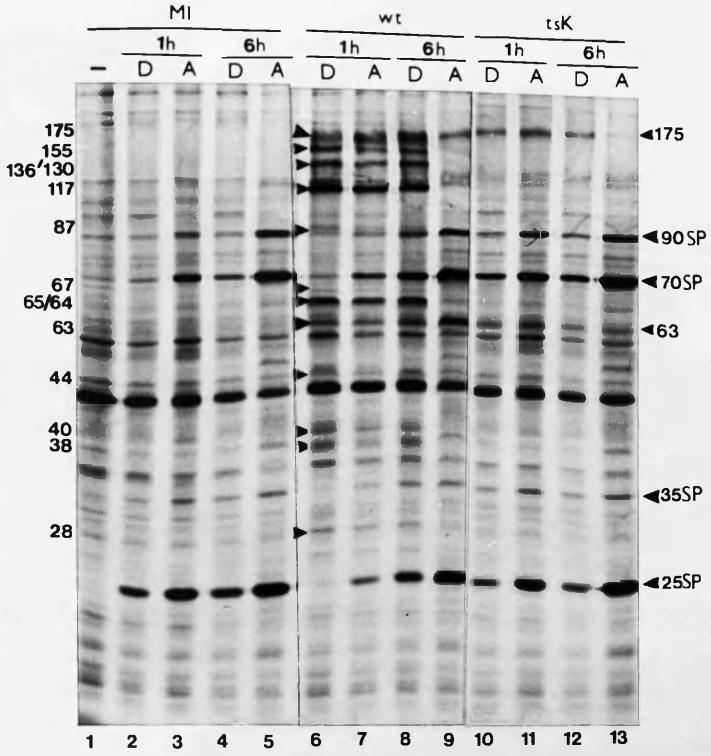

Plus disulfiram, pre- or post- infection
or mock-infection:
MI
wt
tsK
–
+1h
-1h
–
+1h
-1h
–
+1h
-1h
155
136'143
136'130
117
87
90SP
70SP
65/64
63
44
40
35SP
28
25SP
175
136'143
110
90SP
70SP
63
35SP
25SP
1
2
3
4
5
6
7
8
9

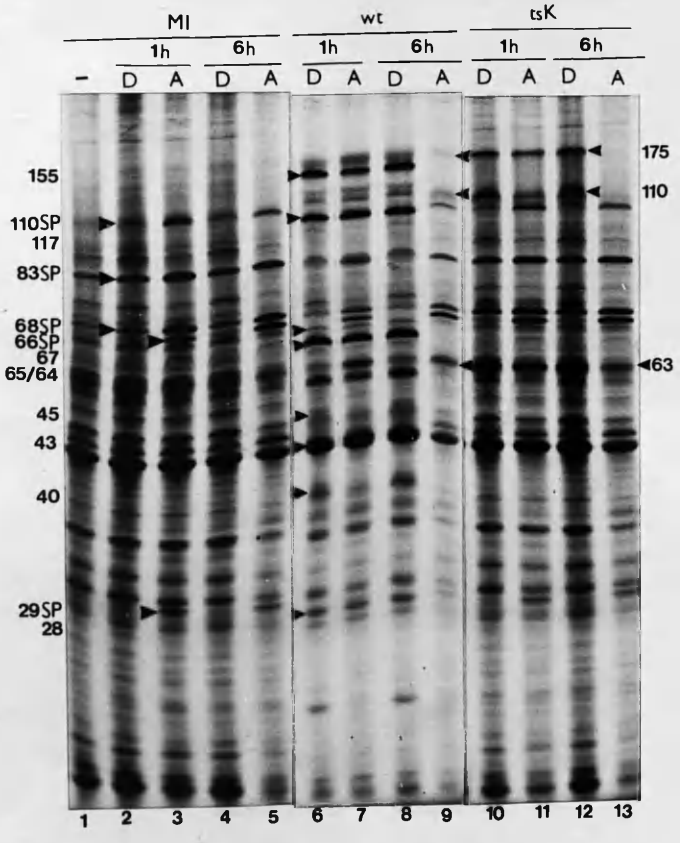
Fig. 6.3.1. Polypeptide synthesis in secondary CEF treated with stress-inducing reagents prior to, or during, infection with wt HSV-1 or tsK at 38.5 ^{o}C.

Secondary CEF were treated with either disulfiram (0.3 uM; tracks 2, 4, 6, 8, 10 and 12) or sodium arsenite (50 uM; tracks 3, 7, 9, 11 and 13) at 38.5 ^{o}C from 1 h prior to mock-infection (tracks 2 to 5) or infection with wt HSV-1 (20 p.f.u./cell; tracks 6 to 9) or tsK (20 p.f.u./cell; tracks 10 to 13). The reagents were withdrawn either 1 h later (at the time of mock-infection (tracks 2 and 3) or infection with wt HSV-1 (tracks 6 and 7) or tsK (tracks 10 and 11)), or 6 h later (at 5 h p.m.i. (tracks 4 and 5) or p.i. with wt HSV-1 (tracks 8 and 9) or tsK (tracks 12 and 13)). An additional, mock-infected culture was incubated at 38.5 ^{o}C in the absence of either disulfiram or sodium arsenite (track 1). The cultures were pulse-labelled at 5 h p.i. or p.m.i..

Fig. 6.3.2. Polypeptide synthesis in BHK cells treated with stress-inducing reagents prior to, or during, infection with wt HSV-1 or tsK at 38.5 ^{o}C.

Legend as for **Fig. 6.3.1**, except that BHK cells were used.


MI
wt
tsK
1h
6h
–
D
A
175
155
136/130
117
87
67
65/64
63
44
40
38
28
175
90SP
70SP
63
35SP
25SP
1 2 3 4 5 6 7 8 9 10 11 12 13


MI
wt
tsK
1h
6h
–
D
A
155
110SP
117
83SP
68SP
66SP
67
65/64
45
43
40
29SP
28
175
110
63
1 2 3 4 5 6 7 8 9 10 11 12 13

In cells that were infected with wt HSV-1 after treatment with either disulfiram or sodium arsenite for 1 h (tracks 6 and 7, respectively), synthesis of stress proteins was reduced, compared with similarly-treated, mock-infected cultures (tracks 2 and 3) - in disulfiram-treated cells to constitutive levels - and Vmw IE 175 was produced. In cells infected with tsK after treatment for 1 h with either disulfiram (track 10) or sodium arsenite (track 11), levels of synthesis of 90SP and 70SP were increased, compared with similarly treated, mock-infected cells. In short, the response of cells to treatment for 1 h with stress-inducing agents was subdued by infection with wt HSV-1 but not with tsK.

In cells that were treated for 6 h with disulfiram or sodium arsenite (tracks 8 and 9, respectively), synthesis of stress proteins was induced to the same (track 9) or greater (track 8) levels than in similarly treated, mock-infected cells (tracks 4 and 5). Synthesis of Vmw IE 175 was detectable in wt HSV-1-infected cells treated continuously with either reagent, but synthesis of other viral polypeptides was inhibited by continuous treatment with sodium arsenite (track 9). And in cells that were were treated for 6 h with disulfiram or sodium arsenite (tracks 12 and 13, respectively), synthesis of stress proteins was induced to the same or greater (superinduced) levels than in similarly-treated, mock-infected cells, but synthesis of viral polypeptides was reduced. Thus, 6 h continuing treatment of cells with stress-inducing reagents was more effective than 1 h of pre-treatment in suppressing synthesis of viral polypeptides following infection.

It is deduced, as in chapter 6.1, that either (a) the stress response was stimulated maximally by continuous treatment of cells with sodium arsenite, so that no further increase was caused by infection with wt HSV-1 or tsK, or (b) superinduction of the stress response by infection with wt HSV-1 or tsK is related to levels of synthesis of viral polypeptides: since synthesis of viral polypeptides was reduced by continuous treatment with sodium arsenite, the stress response was not reinforced.

The experimental procedure was repeated using BHK cells instead of CEF, and similar results were obtained (Fig. 6.3.2). Sodium

arsenite was found to be a more effective in inducing the stress response in mock-infected and infected BHK cells than disulfiram, and caused a general inhibition of protein synthesis when added for 6 h (tracks 5, 9 and 13). Treatment of cells with sodium arsenite for 1 h prior to infection with wt HSV-1 (track 7) caused synthesis of Vmw IE 175, Vmw IE 110 and Vmw IE 63 to be prolonged while synthesis of stress proteins was induced (including species of m.wt. 83,000, 68,000 and 66,000, previously identified by Wang et al., 1981). These effects were accentuated, and synthesis of non-IE viral polypeptides was inhibited, when the reagent was present throughout infection by wt HSV-1 (track 8). Continuous treatment of BHK cells with sodium arsenite from 1 h prior to infection with tsK also caused a reduction in synthesis of most viral polypeptides, synthesis of Vmw IE 63 alone being detectable.

6.4. Effects of Continuous Treatment of Wt HSV-1-Infected or TsK-Infected HeLu Cells with Sodium Arsenite upon Synthesis of Viral Polypeptides.

HeLu cells were treated continuously with sodium arsenite from 1 h prior to and during infection with wt HSV-1 or tsK at 38.5 ^{o}C, and polypeptide synthesis was analysed at 4 h p.i.. Hightower (1980) identified stress proteins synthesized in human (FS-4) cells in response to treatment with amino-acid analogues, and assigned m.wt's of 110,000, 88,000, 70-73,000, (one or two species), which are employed here.

Fig. 6.4 shows that untreated, mock-infected cells constitutively synthesized stress proteins 110SP, 88SP and 70-73SP at detectable levels. Treatment with sodium arsenite for 3 h in total (track 2) caused increased synthesis of not only the stress proteins identified by Hightower (1980), but also species of m.wt.'s 24,000-25,000; and treatment of cells for 5 h (track 3) caused similar synthesis of the major stress proteins, accompanied by greater overall reduction in the synthesis of proteins including actin.

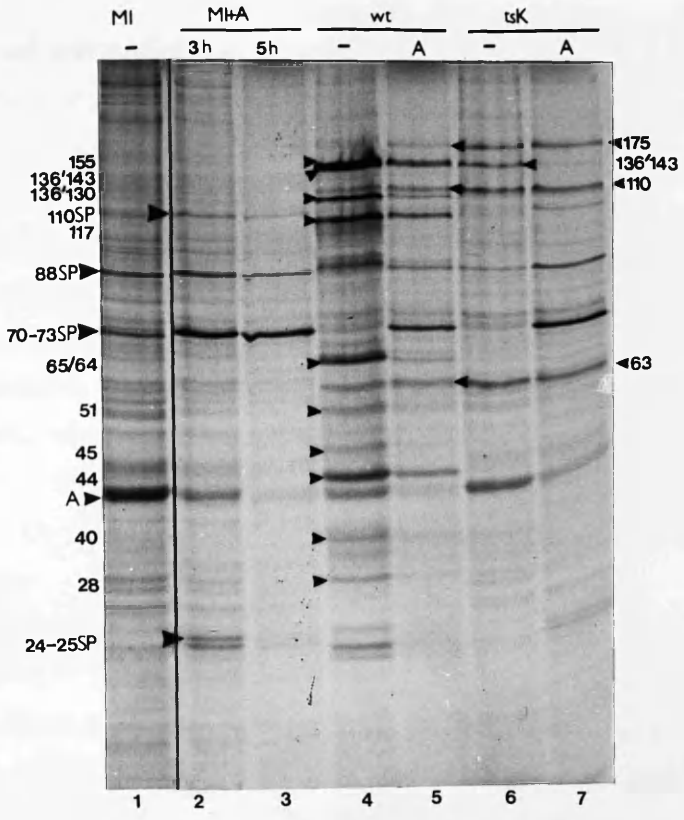
At 4 h p.i. with wt HSV-1 (track 4), infected cells synthesized a range of late viral polypeptides. Treatment of cells with sodium arsenite from 1 h prior to infection with wt HSV-1 (track 5) caused strong induction of synthesis of stress proteins and reduction in synthesis of late viral polypeptides, while synthesis of Vmw IE 175, Vmw

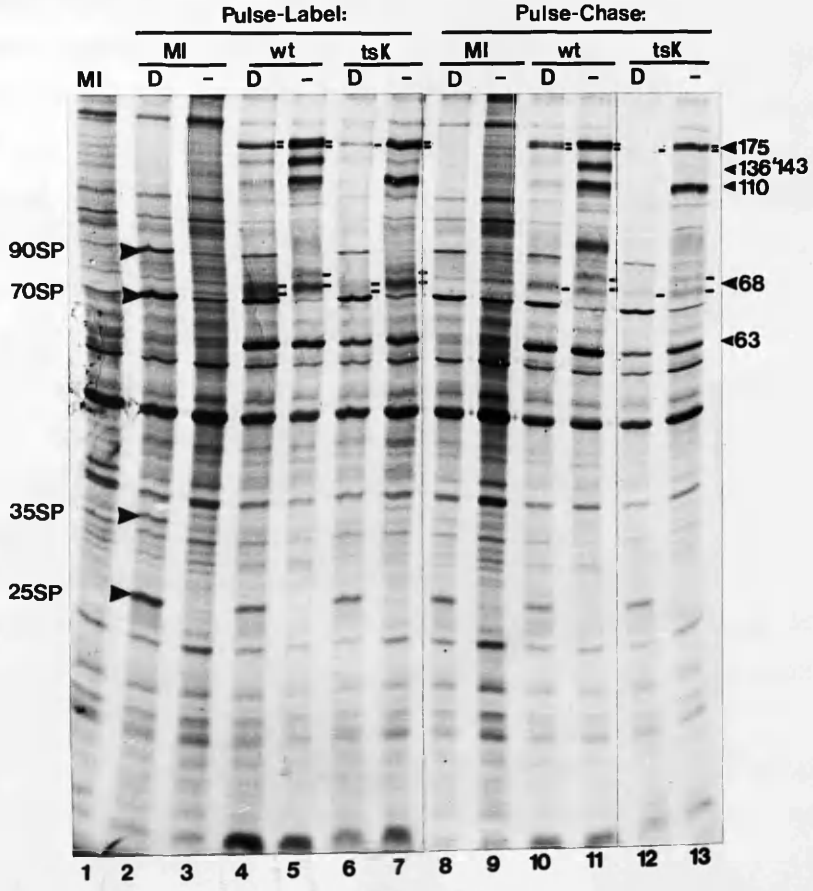
Fig. 6.4. Polypeptide synthesis in HeLu cells treated with sodium arsenite prior to and during infection with wt HSV-1 or tsK at 38.5 °C.

HeLu cells were treated continuously with sodium arsenite (50 uM) at 38.5 °C from 1 h prior to and during mock-infection (tracks 2 and 3) or infection with wt HSV-1 (20 p.f.u./cell; track 5) or tsK (20 p.f.u./cell; track 7). Additional cultures were mock-infected (track 1) or infected with wt HSV-1 (track 4) or tsK (track 6) in the absence of sodium arsenite. At 4 h or p.i. or p.m.i., cultures were pulse-labelled; a treated culture was pulse-labelled at 2 h p.m.i. (track 2). Actin is indicated (A).

Fig. 6.5. Synthesis and processing of IE viral polypeptides in wt-HSV-1-infected and tsK-infected CEF treated with disulfiram.

Secondary CEF were mock-infected (tracks 2, 3, 8 and 9) or infected with wt HSV-1 (tracks 4, 5, 10 and 11) or tsK (tracks 6, 7, 12 and 13) at 38.5 °C in the presence of cycloheximide (20 ug/ml), with (tracks 2, 4, 6, 8, 10 and 12) or without (tracks 3, 5, 7, 9, 11 and 13) disulfiram (0.3 uM). At 3 h p.i. or p.i.m. the reagents were removed, and the cultures were radiolabelled for 20 min: duplicate cultures were harvested either immediately (pulse-label; tracks 2 to 7), or after further incubation for 2 h in the presence of cycloheximide plus or minus disulfiram, as before (pulse-chase; tracks 8 to 13). An additional, mock-infected culture is also shown (track 1).


MI
MI+A
wt
tsK
–
3h
5h
–
A
–
A
155
136'143
136'130
110SP
117
88SP
70-73SP
65/64
51
45
44
A
40
28
24-25SP
175
136'143
110
63
1
2
3
4
5
6
7


Pulse-Label:
Pulse-Chase:
MI
wt
tsK
MI
wt
tsK
MI
D
–
D
–
D
–
D
–
D
–
D
–
90SP
70SP
35SP
25SP
175
136'143
110
68
63
1
2
3
4
5
6
7
8
9
10
11
12
13

IE 110 and Vmw IE 63 was sustained. TsK-infected cells (track 6) synthesized 88SP and 70-73SP below constitutive levels. And treatment of cells with sodium arsenite from 1 h prior to infection with tsK (track 7) caused synthesis of Vmw IE 136'143 to be reduced.

Thus, the results were in reasonable agreement with those obtained using secondary CEF or BHK cells in that treating cells with a stress-inducing reagent prior to and during infection with wt HSV-1, and to some extent with tsK, caused synthesis of most viral polypeptides to be reduced, while synthesis of certain IE viral polypeptides was maintained. These observations indicate that the viral lytic cycle was inhibited at a very early stage in cells of various types by these treatments.

6.5. The Synthesis and Processing of Immediate Early Viral Polypeptides in Wt HSV-1-Infected and TsK-Infected CEF Are Affected by Treatment of the Cells with Disulfiram.

Preston (1979a) reported that in cells infected with wt HSV-1 for several hours in the presence of cycloheximide, and during a period of 25 min following the removal of cycloheximide, only the IE class of viral polypeptides is produced at detectable levels. By using this protocol it was therefore possible to examine the effects of treatment of HSV-1-infected CEF with disulfiram upon the synthesis of IE viral polypeptides.

Secondary CEF were infected with wt HSV-1 or tsK at 38.5 ^{o}C in the presence of cycloheximide, plus or minus disulfiram. At 3 h p.i., cycloheximide and disulfiram were removed, and the cultures were radiolabelled for 20 min with [^{35}S]methionine. Duplicate cultures were harvested either immediately (pulse-label), or after further incubation for 2 h in the presence of cylcoheximide plus or minus disulfiram (pulse-chase). The radiolabelled polypeptides were resolved by SDS-PAGE.

Comparison of pulse-labelled and pulse-chased samples (**Fig.** 6.5) reveals the fate of radiolabelled polypeptides during continued incubation in the presence of cycloheximide; i.e. in the absence of further protein synthesis. When cells were infected with wt HSV-1 (track

5) or tsK (track 7) in the presence of cylcoheximide (and in the absence of disulfiram), mainly the IE class of viral polypeptides were synthesized during the period of radiolabelling. Preston (1979a) reported that the complement of viral polypeptides that was synthesized in tsK-infected BHK cells at the NPT included Vmw IE 136'143, whereas synthesis of this polypeptide was less abundant after release from a cycloheximide-block; and suggested that synthesis of Vmw IE 136'143 is stimulated by IE viral functions that are distinct from Vmw IE 175. However, the data in **Fig. 6.5** indicate that synthesis of Vmw IE 136'143 was greater in wt HSV-1-infected cells than in tsK-infected cells during the period of radiolabelling, and suggests rather that synthesis of Vmw IE 136'143 was increased by the presence of non-defective Vmw IE 175. It is also apparent that Vmw IE 68 synthesized in wt HSV-1-infected cells (tracks 5 and 11) and in tsK-infected cells (tracks 7 and 13) was processed to slower-migrating forms during the pulse-chase (Pereira et al., 1977; Fenwick et al., 1980; MacDonald, 1980).

Treatment of wt HSV-1-infected or tsK-infected cells with disulfiram prior to radiolabelling caused induction of synthesis of stress proteins and reduction in synthesis of viral and cellular polypeptides, and altered the relative levels of synthesis of viral polypeptides: synthesis of Vmw IE 175 and Vmw IE 63 was sustained whereas synthesis of Vmw IE 110 and Vmw IE 136'143 was strongly reduced (tracks 4 and 6). Furthermore, processing of Vmw IE 68 was blocked by treatment with disulfiram prior to pulse-labelling (tracks 4 and 6) and during the pulse-chase (tracks 10 and 12). (See also chapter 1.3).

Comparing the electrophoretic mobilities of Vmw IE 175 in different samples, two - or possibly three - component forms of this polypeptide are discernible. During the pulse-label, wt HSV-1-infected cells synthesized two forms (track 4), which were visible as distinct bands in the autoradiogram; and the mobility of the faster-migrating component appears decreased during the pulse-chase (track 11). TsK-infected cells synthesized mainly a faster-migrating form, and a small quantity of a slower-migrating form, during pulse-labelling (track 6); and the mobility of the former component appears decreased during the pulse-chase (track 13). In both wt HSV-1-infected cells (track 4) and in tsK-infected cells (track 6), the presence of disulfiram prior to

pulse-labelling reduced incorporation of radioactivity into the slower-migrating component; and the presence of disulfiram during the pulse-chase retarded the conversion of the faster- to the slower-migrating forms (tracks 10 and 12). Further analysis (described in chapter 7) using gels of greater resolving capacity revealed that the form of Vmw IE 175 accumulating in disulfiram-treated CEF co-migrated with - and may correspond to - the precursor, 'a-form' of Vmw IE 175 that was detectable in the cytoplasmic fractions of wt HSV-1-infected and tsK-infected BHK cells and CEF, and which normally is converted to slower-migrating forms in the nuclear fraction (Preston, 1979b; Wilcox et al., 1980).

Comparing the electrophoretic mobilities of Vmw IE 175 in these samples, the polypeptide appeared to be processed to slower-migrating forms during pulse-chasing in both wt HSV-1-infected (tracks 5 and 11) and tsK-infected (tracks 7 and 13) cells; and the presence of disulfiram during the pulse-chase appeared to retard this conversion (tracks 10 and 12).

It cannot be excluded that disulfiram may act upon the processing of viral polypeptides directly, rather than through induction of the stress response.

7. INVESTIGATION OF FORMS OF Vmw IE 175 WITH DIFFERENT ELECTROPHORETIC MOBILITIES: FRACTIONATION AND ELECTROPHORETIC SEPARATION.

Results obtained using secondary REF (chapter 4.2), HeLu cells (chapter 6.4) and secondary CEF (chapters 5.3, 6.1, 6.2 and 6.3) indicated that continuous treatment of cells with stress-inducing reagents from early times in infection by wt HSV-1 caused synthesis of IE viral polypeptides, Vmw IE 175 and Vmw IE 63 in particular, to be sustained at detectable levels during infection. Furthermore, results presented in chapters 1.3 and 6.5 indicated that the electrophoretic mobilities - and therefore the processing - of Vmw IE 175 and Vmw IE 68 were affected by treatment of wt HSV-1-infected and tsK-infected CEF with disulfiram. Consequently, the following series of experiments was undertaken to investigate: firstly, forms of Vmw IE 175 with different electrophoretic mobilities, and their intra-cellular distribution in BHK cells and CEF infected with wt HSV-1 or tsK at a NPT; and, secondly, the electrophoretic mobility of Vmw IE 175 in disulfiram-treated, wt HSV-1-infected and tsK-infected CEF.

In various types of cells infected with HSV-1 (F), three forms of [^{35}S]methionine-labelled Vmw IE 175 (or ICP 4) have been detected and separated according to their different electrophoretic mobilities (Pereira *et al.*, 1977; Fenwick *et al.*, 1978; Wilcox *et al.*, 1980). MacDonald (1980) detected three forms of Vmw IE 175 in infected BHK cells infected with HSV-1 strain 17; and in cells infected with mutants tsD, tsK and tsT at a NPT, processing of Vmw IE 175 was blocked in an intermediate form. Preston (1979b) reported that Vmw IE 175 in the nuclei of wt HSV-1- infected BHK cells is processed to a form with slightly increased electrophoretic mobility, and that such processing is defective in tsK-infected cells incubated at a NPT. The different forms of Vmw IE 175 had been identified using either single-concentration or gradient polyacrylamide gels which were cross-linked with DATD. Similar gel systems were employed in the following experiments to resolve forms of Vmw IE 175 synthesized at a NPT in BHK cells and CEF infected with wt HSV-1 or tsK at a NPT.

7.1. Partitioning of IE Viral Polypeptides between Cytoplasmic and

Nuclear Fractions of Wt HSV-1-Infected and TsK-Infected BHK cells.

BHK cells were infected with wt HSV-1 or tsK, in the presence of cylcoheximide, and incubated at 38.5 °C for 3 h. Cycloheximide was withdrawn, cultures were radio-labelled for 30 min with [^{35}S]methionine, and harvested either immediately (pulse) or after further incubation for 2 h in the presence of cycloheximide (pulse-chase), at either 31 °C or 38.5 °C. The cells were then fractionated into nuclear and cytoplasmic preparations according to the method of Preston (1979b), and radiolabelled polypeptides were resolved by SDS-PAGE.

Fig. 7.1 demonstrates the partitioning of pulse-labelled and pulse-chased IE viral polypeptides between the nuclear and cytoplasmic fractions. By the end of the period of pulse-labelling and pulse-chasing, the greater proportion of IE viral polypeptides Vmw IE 175, Vmw IE 110, Vmw IE 68 and Vmw IE 63 had migrated to the nuclear fractions wt HSV-1-infected or tsK-infected cells (tracks 2 to 7): the quantity of Vmw IE 175, relative to other IE viral polypeptides, that was present in the cytoplasmic fractions of infected cells after pulse-labelling (tracks 8 and 9) declined during the subsequent pulse-chase at either 38.5 °C or 31 °C (tracks 10 to 13). Vmw IE 68 was detectable only in the nuclear fractions, but Vmw IE 136'143 mainly in the cytoplasmic fractions, of infected cells.

Vmw IE 175 in the cytoplasmic fractions of wt HSV-1-infected and tsK-infected cells (tracks 8 to 13) suggested faster electrophoretic mobility than the forms in the nuclear fractions (tracks 2 to 7). Pulse-labelled Vmw IE 175 in the nuclear fraction of wt HSV-1-infected cells (tracks 2) migrated as a broad band; and after the pulse-chase at either 38.5 °C or 31 °C, the migration of the leading-edge of the band was retarded, indicating that modification of the polypeptide had occurred to slower-migrating forms (tracks 4 and 5). Pulse-labelled Vmw IE 175 in the nuclear fraction of tsK-infected cells (track 3) migrated as a narrower band than Vmw IE 175 in the nuclear fractions of wt HSV-1-infected cells (track 2); and after the pulse-chase at either 38.5 °C or 31 °C, the mobility of the trailing edge of the band was retarded, and the band was broader, indicating that modification had occurred to slower-migrating forms (tracks 6 and 7). The retardation in the

Fig. 7.1. Partitioning of IE viral polypeptides between cytoplasmic and nuclear fractions of wt HSV-1-infected and tsK-infected BHK cells.

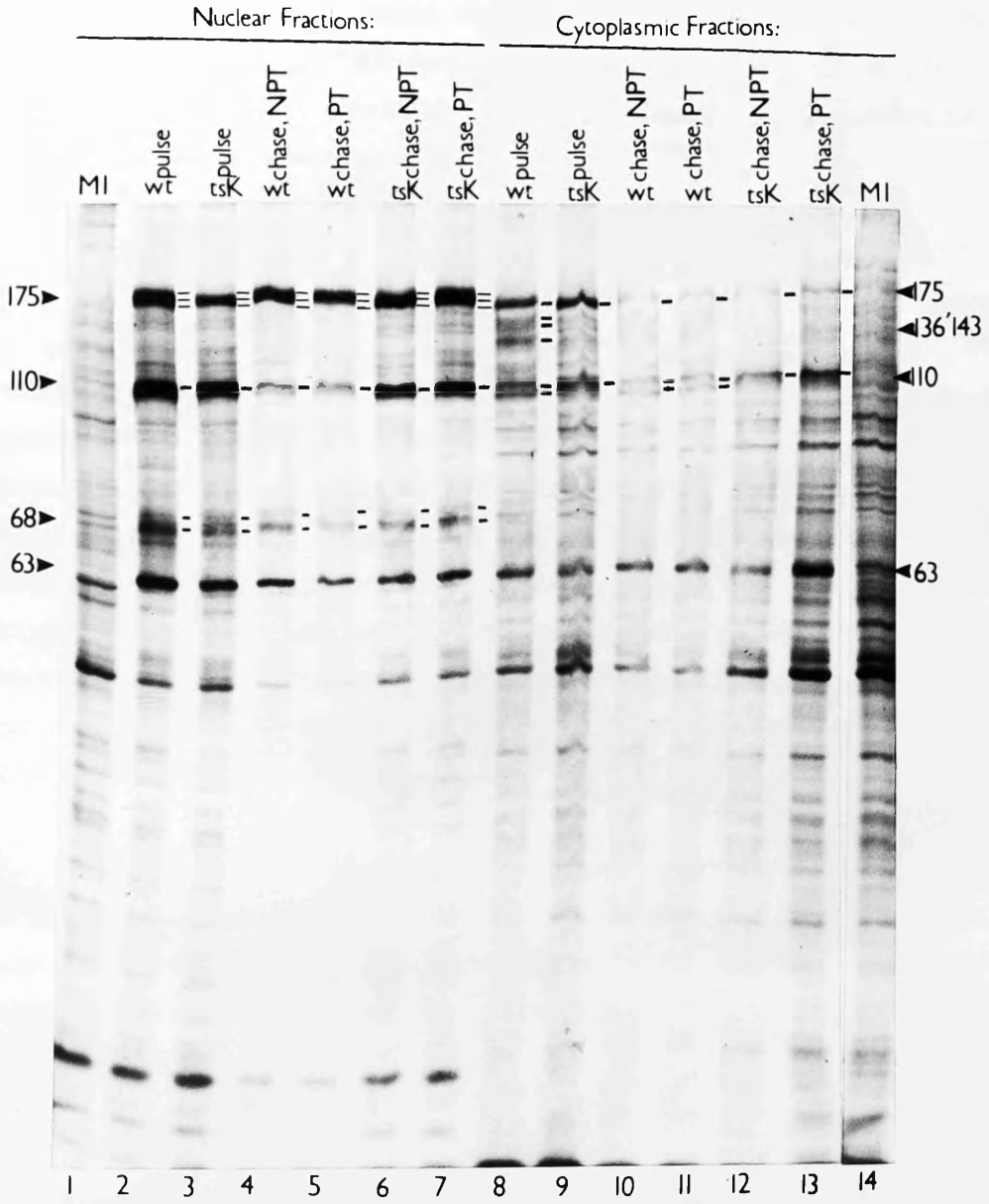
BHK cells were mock-infected or infected with wt HSV-1 or tsK (20 p.f.u./cell) in the presence of cycloheximide (200 ug/ml), and incubated at 38.5 ^{o}C for 3 h. Cycloheximide was withdrawn, and the cultures were radiolabelled for 30 min with [^{35}S]methionine. The cultures were harvested either immediately (pulse), or after further incubation (chase) for 2 h in the presence of cycloheximide at either 38.5 ^{o}C (NPT) or 31 ^{o}C (PT), and the cells were fractionated into nuclear and cytoplasmic preparations according to the method of Preston (1979b). Equal proportions of total samples were analysed by SDS-PAGE through a 9%-polyacrylamide, DATD-cross-linked gel.

Tracks 1 to 7 show nuclear fractions, and tracks 8 to 14 show cytoplasmic fractions of mock-infected, wt HSV-1-infected and tsK-infected cells.

Tracks 1 and 14 show nuclear and cytoplasmic fractions, respectively, of mock-infected cells.

Tracks 2, 4, 5, 8, 10 and 11 show fractions derived from cultures infected with wt HSV-1: track 2, nuclear fraction, pulse-label; 4, nuclear fraction, pulse-chase at 38.5 ^{o}C; 5, nuclear fraction, pulse-chase at 31 ^{o}C; 8, cytoplasmic fraction, pulse-label; 10, cytoplasmic fraction, pulse-chase at 38.5 ^{o}C; 11, cytoplasmic fraction, pulse-chase at 31 ^{o}C.

Tracks 3, 6, 7, 9, 12 and 13 show samples derived from cultures infected with tsK: track 3, nuclear fraction, pulse-label; 6, nuclear fraction, pulse-chase at 38.5 ^{o}C; 7, nuclear fraction, pulse-chase at 31 ^{o}C; 9, cytoplasmic fraction, pulse-label; 12, cytoplasmic fraction, pulse-chase at 38.5 ^{o}C; 13, cytoplasmic fraction, pulse-chase at 31 ^{o}C.


Nuclear Fractions:
Cytoplasmic Fractions:
M1
wt pulse
ts pulse
wt chase, NPT
wt chase, PT
ts chase, NPT
ts chase, PT
wt pulse
ts pulse
wt chase, NPT
wt chase, PT
ts chase, NPT
ts chase, PT
M1
175
110
68
63
75
136'143
110
63
1 2 3 4 5 6 7 8 9 10 11 12 13 14

migration of Vmw IE 175 appeared greater after pulse-chase of tsK-infected cells at 31 °C (track 7) compared with 38.5 °C (track 6). These observations indicate that Vmw IE 175 migrated to the nuclear fractions of wt HSV-1-infected and tsK-infected cells at either 38.5 °C or 31 °C, and underwent processing to forms with slower electrophoretic mobilities. Other experiments employing cells infected with either wt HSV-1 or tsK demonstrated that some modification of Vmw IE 175 to slower-migrating forms occured within 10 min of its migration to the nuclear fraction (results not shown).

Vmw IE 110 in the cytoplasmic fractions of infected cells also was resolved into forms of different electrophoretic mobilities: the cytoplasmic fractions of wt HSV-1-infected cells contained two forms (tracks 8, 10 and 11), but the corresponding fractions of tsK-infected cells contained mainly a slower-migrating form (tracks 9, 12 and 13), which comigrated with Vmw IE 110 in the nuclear fractions of cells infected with either wt HSV-1 or tsK (tracks 2 to 7). These observations provide the first indication of aberrant processing of an IE viral polypeptide other than Vmw IE 175 in tsK-infected cells at the NPT. (MacDonald (1980) previously reported the detection of two processed forms of Vmw IE 110 in BHK cells infected with mutants tsK, tsD and tsT, or with ts^+ virus.)

Vmw IE 136´143 that was detectable in the cytoplasmic fraction of pulse-labelled, wt HSV-1-infected cells was resolved into three forms with different electrophoretic mobilities. Two forms of this species have been described previously by Pereira et al. (1977) and MacDonald (1980).

7.2. Electrophoretic Separation of Forms of IE Viral Polypeptides in the Nuclear and Cytoplasmic Fractions of Wt HSV-1-Infected and TsK-Infected BHK Cells and CEF.

BHK cells and CEF were infected with wt HSV-1 or tsK in the presence of cycloheximide, radiolabelled with [^{35}S]methionine, and pulse-chased in the presence of cycloheximide, at 38.5 °C. Additional cultures of CEF were infected with wt HSV-1 or tsK in the presence of disulfiram at 38.5 °C for 3 h. Forms of Vmw IE 175 that were present in the

cytoplasmic and nuclear fractions of pulse-chased, infected cells and in total cell extracts of disulfiram-treated, infected cells were separated by SDS-PAGE using gels of various concentrations of polyacrylamide, cross-linked with either DATD or bis-acrylamide, and of longer length than usual (**Fig. 7.2.1**).

Extracts of CEF that were infected at 38.5 ^{o}C with tsK in the absence of disulfiram (track 13), or infected with tsK (tracks 1 and 11) or wt HSV-1 (track 2 and 12) in the presence of disulfiram, contained Vmw IE 175 which migrated faster than Vmw IE 175 present in the nuclear fractions of untreated, wt HSV-1-infected or tsK-infected CEF (tracks 9 and 10) or BHK cells (tracks 7 and 8); and this faster form, designated the 'a-form', is assumed to be the precursor to the slower-migrating forms in the nuclear fractions (Preston, 1979b; MacDonald, 1980; Wilcox et al., 1980). Extracts of wt HSV-1-infected CEF that were pulse-labelled at 3 h p.i. contained barely detectable amounts of fast- and slow-migrating components (track 14) of Vmw IE 175, in conflict with reports that synthesis of Vmw IE 175 is detectable early in infection, reaches a maximum from 2 to 4 h p.i. and then declines (Wilcox et al., 1980).

Tracks 3 to 6 show the partitioning of Vmw IE 175 between the cytoplasmic and nuclear fractions of wt HSV-1-infected and tsK-infected BHK cells. At the end of the pulse-chase, the a-form of Vmw IE 175 was barely detectable in the cytoplasmic fractions of tsK-infected and wt HSV-1-infected cells; and processed forms in nuclear fractions (tracks 5 and 6), in keeping with the migration of this polypeptide to the nuclear fraction of wt HSV-1-infected and tsK-infected cells during pulse-chase at 38.5 ^{o}C (chapter 7.1). Vmw IE 175 in the nuclear fraction of tsK-infected cells (track 5) migrated as a diffuse band (designated 'b-form') - possibly a doublet; but in the nuclei of wt HSV-1-infected cells (track 6), most Vmw IE 175 migrated more slowly, appearing as a slightly narrower band ('c-form') with barely-detectable traces of an intermediate (b-) form.

Other nuclear preparations were obtained from tsK-infected and wt HSV-1-infected BHK cells and CEF in a different fractionation experiment, and these are shown in tracks tracks 7 to 10. Here too, Vmw IE 175 in the nuclear fraction of tsK-infected BHK cells (track 7)

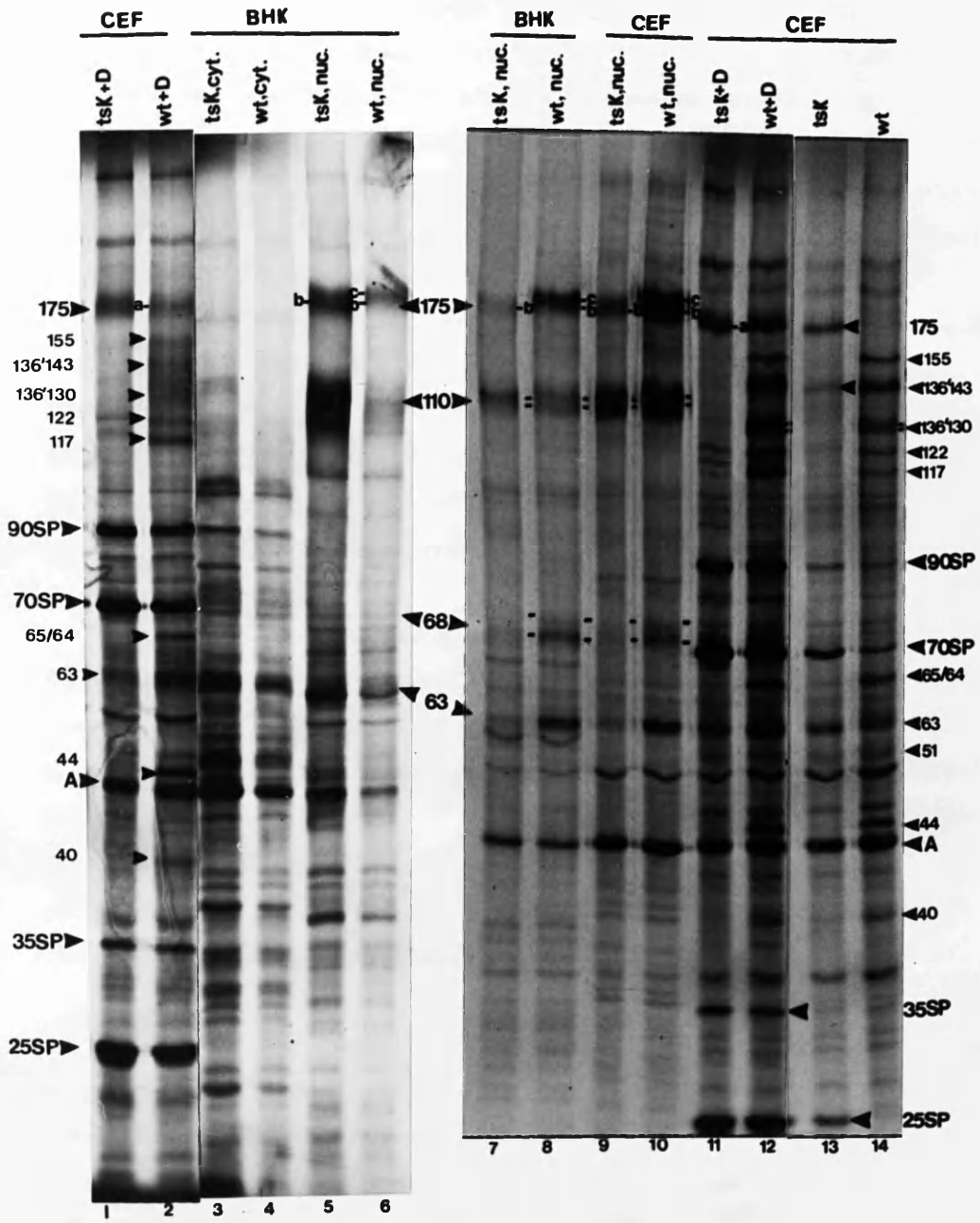
Fig. 7.2.1. Electrophoretic Separation of forms of Vmw IE 175 in total extracts or sub-celllular fractions of wt HSV-1-infected and tsK-infected BHK cells and CEF, which were untreated or treated with disulfiram.

BHK cells or secondary CEF were infected with wt HSV-1 or tsK (20 p.f.u./cell) in the presence of cycloheximide (200 ug/ml for BHK cells, 20 ug/ml for CEF), and incubated at 38.5 ^{o}C for 3 h. Cycloheximide was withdrawn, and the cultures were radiolabelled with [^{35}S]methionine for 30 min and pulse-chased, in the presence of cycloheximide, at 38.5 ^{o}C for 3 h. The cells were harvested and fractionated into nuclear and cytoplasmic preparations (Preston, 1979b), and equal proportions of total samples were analysed by SDS-PAGE through single concentration, 12%- (tracks 1 to 6) or 9%-polyacrlyamide (tracks 7 to 14) gels, each cross-linked with DATD and 220 mm in length. Electrophoresis was for 12 h (12% gel) or 20 h (9% gel) with a current of 40 milliamps.

Tracks 3 and 5 show radiolabelled polypeptides in the cytoplasmic and nuclear fractions, respectively, of tsK-infected BHK cells; tracks 4 and 6, cytoplasmic and nuclear fractions of wt HSV-1-infected BHK cells. Tracks 7 and 8, nuclear fractions of tsK-infected and wt HSV-1-infected BHK cells, respectively; tracks 9 and 10, nuclear fractions of tsK-infected and wt HSV-1-infected CEF.

Additional samples were analysed, consisting of total cell extracts of CEF, harvested 3 h p.i. with tsK (tracks 1, 11 and 13) or wt HSV-1 (tracks 2, 12 and 14) in the presence (tracks 1, 2, 11 and 12) or absence (tracks 13 and 14) of disulfiram (0.3 uM) at 38.5 ^{o}C.

Note:- samples shown in tracks 3 to 6, and samples shown in tracks 7 and 8 were derived from independent fractionation experiments. However, samples shown in tracks 1 and 2 are shown also in tracks 11 and 12. Actin also is indicated (A).


CEF
BHK
tsK+D
wt+D
tsK.cyt.
wt.cyt.
tsK.nuc.
wt.nuc.
BHK
CEF
CEF
tsK.nuc.
wt.nuc.
tsK.nuc.
wt.nuc.
tsK+D
wt+D
tsK
wt
175
155
136'143
136'130
122
117
90SP
70SP
65/64
63
44
A
40
35SP
25SP
175
110
68
63
175
155
136'143
136'130
122
117
90SP
70SP
65/64
63
51
44
A
40
35SP
25SP
1
2
3
4
5
6
7
8
9
10
11
12
13
14

appeared to have a faster average electrophoretic mobility than Vmw IE 175 in the nuclear fraction of wt HSV-1-infected BHK cells (track 8). The nuclear fraction of wt HSV-1 infected BHK cells contained the slowest-migrating component (c-form) together with a quantity of the intermediate (b-) form, which was poorly resolved from the slowest component. (Classifications of Vmw IE 175 as c-form or b-form refer only to the electrophoretic mobilities of nuclear forms of Vmw IE 175 compared with the cytoplasmic precursor, or a-form; and it is not implied, for example, that the b-form in tsK-infected cells corresponds precisely to the b-form in wt HSV-1-infected cells.) In nuclear fractions prepared from pulse-chased, tsK-infected (track 9) or wt HSV-1-infected CEF (track 10) under identical conditions, the electrophoretic mobilities of forms of Vmw IE 175 appeared similar to those in corresponding fractions of infected BHK cells. These results are in agreement with the observations of MacDonald (1980) that processing of Vmw IE 175 is blocked in an intermediate form in tsK-infected cells incubated at a NPT.

Vmw IE 110 and Vmw IE 68 that were present in the nuclear fractions of wt HSV-1-infected and tsK-infected BHK cells demonstrated heterogeneous mobility through the 12%-polyacrylamide gel (tracks 5 and 6). And these polypeptides in the nuclear fractions of wt HSV-1-infected BHK cells (track 8) and CEF (track 10) were each resolved into a doublet by electrophoresis through the 9%-polyacrylamide gel. Vmw 136´130 in extracts of disulfiram-treated (track 12) and untreated (track 14), wt HSV-1-infected CEF was resolved into two components.

Fig. 7.2.2 shows analysis of nuclear fractions and total extracts using gradient, 9-15%-polyacrylamide, DATD-cross-linked gels. (The samples shown in tracks 1 to 6 correspond to those in **Fig. 7.2.1.**) Here, Vmw IE 175 in the nuclear fraction of tsK-infected cells (track 5) demonstrated faster electrophoretic mobility than in wt HSV-1-infected cells (track 6), and was clearly resolved into two components (b1 and b2). Vmw 136´130 in extracts of disulfiram-treated wt HSV-1-infected CEF (track 2) also was resolved into two components.

Tracks 1 to 6 of **Fig. 7.2.3** show samples (which were shown also in tracks 7 to 12 of **Fig. 7.2.1**) electrophoresed through a 6%-polyacrylamide, DATD-cross-linked gel. Vmw IE 110 in the nuclear

Fig. 7.2.2. Separation of forms of Vmw IE 175 by electrophoresis through a gradient polyacrylamide gel.

Nuclear and cytoplasmic fractions of infected BHK cells, obtained as described in the legend to **Fig. 7.2.1**, were electrophoresed through a gradient, 9-15%-polyacrylamide gel that was cross-linked with DATD. The samples in tracks 1 to 6 correspond to those in tracks 1 to 6 of **Fig. 7.2.1.**

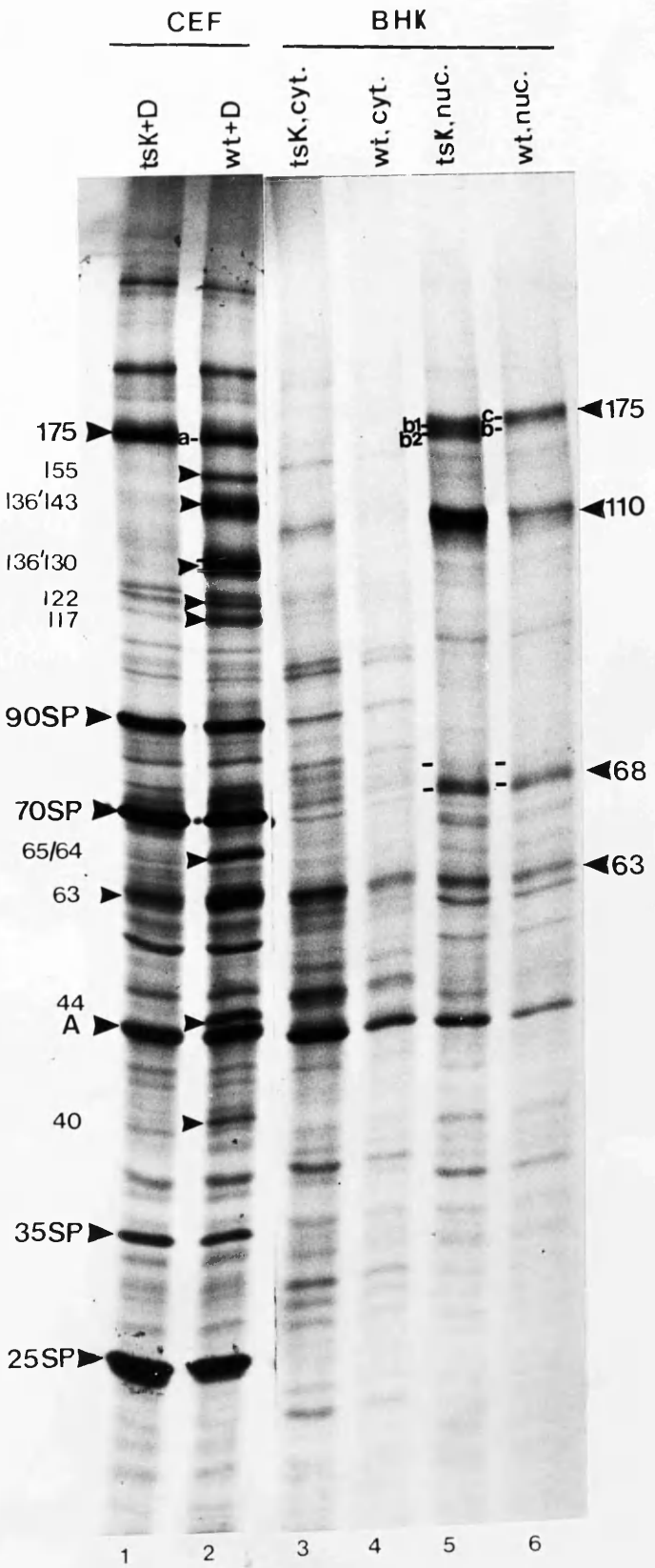
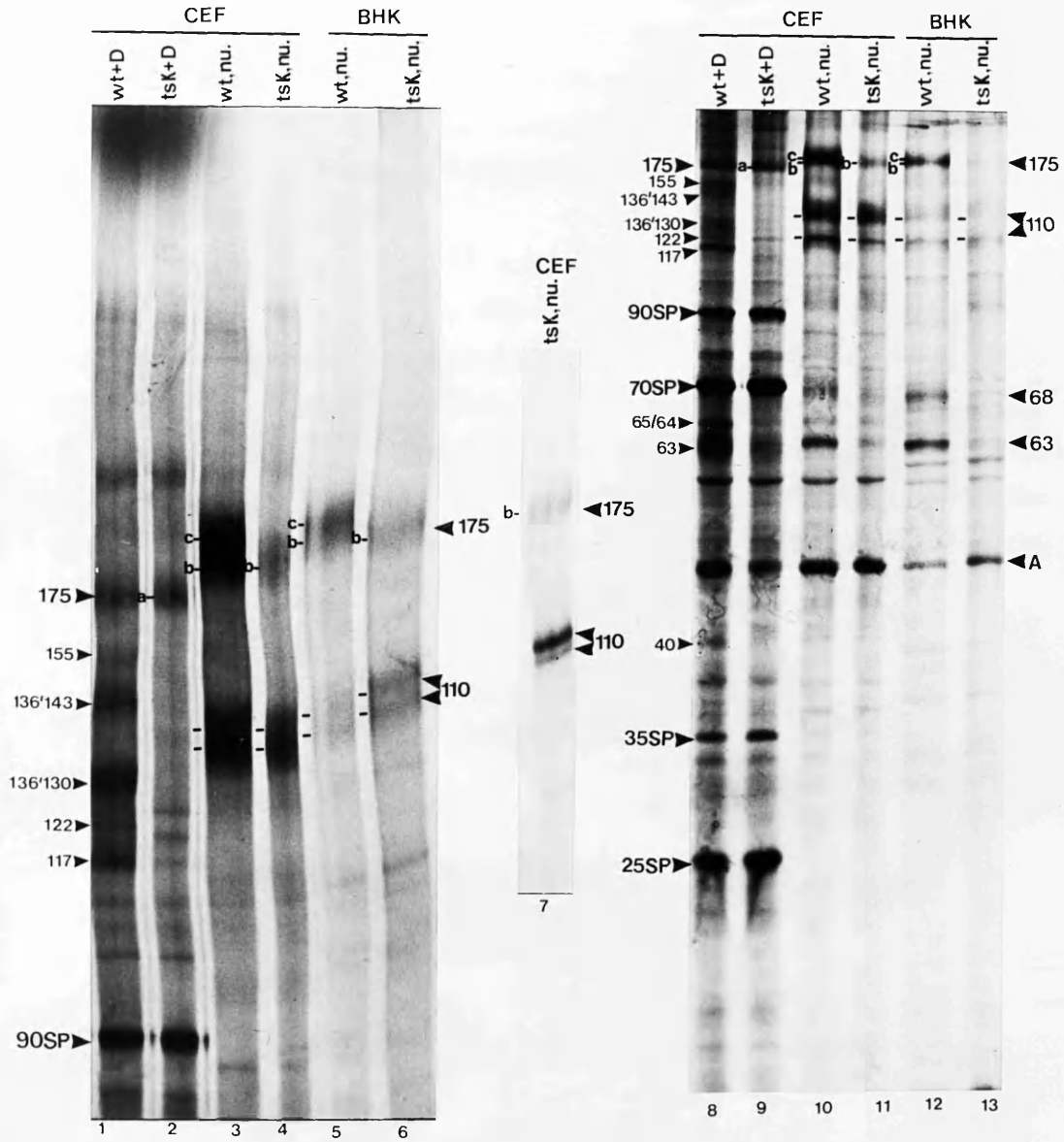

CEF
BHK
tsK+D
wt+D
tsK.cyt.
wt.cyt.
tsK.nuc.
wt.nuc.
175
155
136'143
136'130
122
117
90SP
70SP
65/64
63
44
A
40
35SP
25SP
a-
b1
b2
c
175
110
68
63
1
2
3
4
5
6

Fig. 7.2.3. Electrophoretic separation of forms of Vmw IE 110 using different resolving gel systems.

Samples that were obtained as described in the legend to **Fig. 7.2.1** were electrophoresed through a single-concentration, 6%-polyacrylamide, DATD-cross-linked gel (tracks 1 to 6); a gradient, 6-10% polyacrylamide, DATD-cross-linked gel (track 7); and a single-concentration, 8%-polyacrylamide gel that was cross-linked with bis-acrylamide (tracks 8 to 13). The samples correspond to those in tracks 7 to 12 of **Fig. 7.2.1.**

Tracks 1 and 8; extract of wt HSV-1-infected CEF treated with disulfiram at 38.5 ^{o}C. Tracks 2 and 9; extract of tsK-infected CEF treated with disulfiram at 38.5 ^{o}C.

Tracks 3 and 10; nuclear fraction of wt HSV-1-infected CEF. Tracks 4, 7 and 11; nuclear fraction of tsK-infected CEF. Tracks 5 and 12; nuclear fraction of wt HSV-1-infected BHK cells. Tracks 6 and 13; nuclear fraction of tsK-infected BHK cells.


CEF
BHK
wt+D
tsK+D
wt,nu.
tsK,nu.
wt.nu.
tsK,nu.
175
155
136'143
136'130
122
117
90SP
70SP
65/64
63
40
35SP
25SP
110
68
A
a-
b-
c-
1
2
3
4
5
6
7
8
9
10
11
12
13

fractions of wt HSV-1-infected and tsK-infected CEF (tracks 3 and 4, respectively) and BHK cells (track 5 and track 6) was resolved into two diffusely-migrating components. Using a gradient, 6-10%-polyacrylamide, DATD-cross-linked gel (track 7), clear resolution of two forms of Vmw IE 110 (in the nuclear fraction of tsK-infected CEF) was achieved. Tracks 8 to 13 show the same samples electrophoresed through a single-concentration, 8%-polyacrylamide gel that was cross-linked with bis-acrylamide. (The order of samples shown in tracks 8 to 13 is the same as that in tracks 1 to 6 of this figure). The resolution of forms of Vmw IE 175 in nuclear fractions was poor, but two forms of Vmw IE 110 were clearly separated.

It is apparent from the figures presented in this chapter that the various nuclear preparations remained contaminated with cytoskeletal material after fractionation: most noticeably with actin and the intermediate filament proteins in the 40,000 - 60,000 range of m.wt.'s. The experiments described in chapter 8 therefore investigated the possible interaction of viral polypeptides with components of the cytoskeleton.

8. EXTRACTION OF MOCK-INFECTED, WT HSV-1-INFECTED, TSK-INFECTED, OR DISULFIRAM-TREATED CEF TO GIVE A DETERGENT-RESISTANT CYTOSKELETON.

In the cytoplasm of cells of all types studied to date there exists an extensive, interconnected network, the "cytoskeleton", consisting of three major filamentous systems; microfilaments, intermediate filaments and microtubules (Schliwa & Blerkom, 1981). The cytoskeleton is resistant to extraction of the cell with detergent, the degree of preservation of the cytoskeleton varying with the composition of the extraction buffer, which must be of physiological pH and ionic strength. Schliwa & Blerkom (1981) demonstrated, by high-voltage electron-microscopy of whole-mount preparations, that treatment of CEF with detergent results in gradual removal of the plasmalemma and partial release of "cytosolic" proteins from the cytoplasmic ground substance. This supports the theory of Wolosewick & Porter (1979) that the cytoskeleton forms a structural framework with which a large number of proteins of the cytoplasmic ground substance associate to form the "microtrabecular lattice".

The method of Brown et al. (1976), originally developed to study actin bundles (microfilaments) formed in CEF in vivo, provides a quick and quantitative way of exposing a cytoskeleton which remains attached to the substratum while retaining the original shape of the cell. (Nuclei also remain in their original disposition following extraction.) By this method, using a concentration of the weak, non-ionic detergent, Triton X-100 of 0.5%, maximum extraction of 80% of the total cellular protein is achieved. The Triton-resistant cytoskeleton consists largely of fibronectin, intermediate filaments and bundles of microfilaments (microtubules are not retained during this extraction), as identified by SDS-PAGE in conjunction with phase contrast light microscopy and electron microscopy. Three major proteins are evident, and are unextractable by increasing the ionic strength by addition of NaCl to 0.7 M. One component comigrates in SDS-PAGE with muscle actin (m.wt. 42,000), and would account for half of the total cellular actin in forming the microfilaments. The other two components, m.wt.'s 52,000 and 230,000, remain quantitatively associated with the cytoskeleton during extraction and are not released into the supernatant; the former corresponds to vimentin and variants of desmin

that are found in the intermediate filaments of fibroblasts (Gard et al., 1979), and the latter to fibronectin. Fibronectin has been postulated to function in adhesion, and its retention in the Triton-resistant cytoskeleton supports this view. A minor component of m.wt. 200,000 comigrating with muscle myosin was also noted in this fraction (Brown et al., 1976).

The following experiments were performed to investigate the distribution of viral polypeptides between supernatants and Triton-resistant cytoskeletons of HSV-1-infected CEF, and to compare the distribution of stress proteins between the same fractions of tsK-infected or disulfiram-treated cells. CEF were employed rather than BHK cells, being more amenable to detergent-extraction while adhering to the substratum (owing, perhaps to the greater abundance of fibronectin in CEF than in BHK cells (Brown et al., 1976)).

Fig. 8.1 shows radiolabelled polypeptides in the supernatants (tracks 1 to 3) and Triton-resistant cytoskeletons (tracks 4 to 6) of mock-infected (track 1), disulfiram-treated (tracks 2 and 5) and tsK-infected (tracks 3 and 6) cells. Both the stained gel (not shown) and the autoradiogram revealed that fibronectin was recovered quantitatively in the Triton-resistant cytoskeleton of each culture, whereas myosin and actin were recovered in both the Triton-resistant cytoskeletons and the supernatants. Vimentin also was recovered in the Triton-resistant cytoskeletons; tubulin in the supernatants comigrated with the intermediate filament proteins in this gel system. Numerous minor, radiolabelled species were detected in the Triton-resistant cytoskeletons, owing perhaps to the association of newly synthesized polypeptides with intermediate filaments (Howe & Hershey, 1984). Stress proteins, whose syntheses were induced either by treatment of the cells with disulfiram or by infection with tsK, were distributed similarly between fractions from either culture, the four major species (90SP, 70SP, 35SP and 25SP) being recovered in both Triton-resistant cytoskeletons and supernatants. Certain minor species, which may correspond to the 88,000 doublet of stress proteins synthesized in REF (chapter 4.1; Johnston et al., 1980), were detectable only in the Triton-resistant cytoskeleton of disulfiram-treated or tsK-infected cells.

Fig. 8.1. Radiolabelled polypeptides in Triton-resistant cytoskeletons and supernatants extracted from mock-infected, disulfiram-treated and tsK-infected CEF.

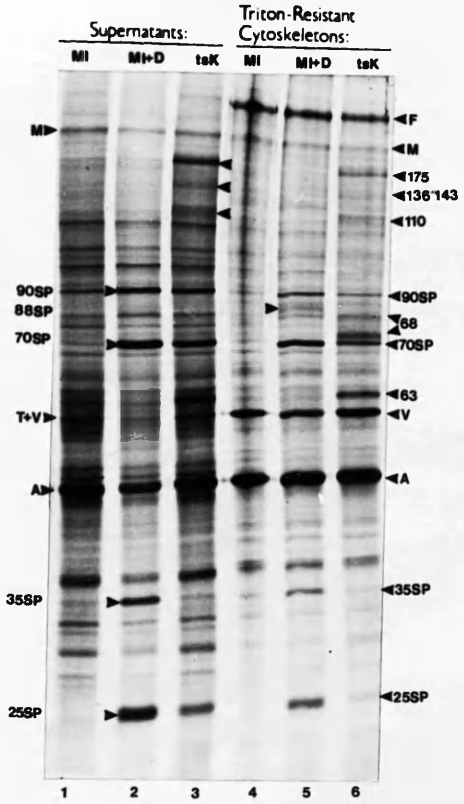
Secondary CEF were mock-infected in the presence or absence of disulfiram (0.3 uM), or infected with tsK (40 p.f.u./cell), at 38.5 ^{o}C for 4 h. The cultures were radiolabelled with [^{35}S]methionine for 30 min, and pulse-chased for 1 h in the presence or absence of disulfiram, as before. The cells were extracted according to the method of Brown et al. (1976), using a solution containing 0.5% Triton X-100. Radioactivity in the supernatants and residual Triton-resistant cytoskeletons (or residues) was equalized, and polypeptides in the samples were resolved by SDS-PAGE. The gel was stained and de-stained before being submitted to autoradiography.

Tracks 1 to 3; supernatants from mock-infected cells (track 1), disulfiram-treated, mock-infected cells (track 2) and tsK-infected cells (track 3). Tracks 4 to 6; Triton-resistant cytoskeletons from mock-infected cells (track 4), disulfiram-treated, mock-infected cells (track 5) and tsK-infected cells (track 6). Fibronectin (F), myosin (M), tubulin (T), vimentin (V) and actin (A) are indicated.

Fig. 8.2. Radiolabelled polypeptides in Triton-resistant cytoskeletons and supernatants extracted from mock-infected and wt HSV-1-infected CEF.

Secondary CEF were mock-infected or infected with wt HSV-1 (40 p.f.u./cell) at 38.5 ^{o}C for 5 h, radiolabelled with [^{35}S]methionine and pulse-chased for 1 h. The cells were harvested, and extracted according to the method of Brown et al. (1976). Duplicate cultures were radiolabelled to provide total cell extracts. Polypeptides in the samples were resolved by SDS-PAGE. The proportions of total cell extracts, superanatants and Triton-resistant cytoskeletons (or residues) that were analysed were in the ratio of 1 : 1 : 10. The gel was stained and de-stained before being submitted to autoradiography.

Tracks 1 and 4; total extracts of mock-infected cells and wt HSV-1-infected cells, respectively. Tracks 2 and 5; Triton-resistant cytoskeletons from mock-infected cells and wt HSV-1-infected cells. Tracks 3 and 6; supernatants from mock-infected cells and wt HSV-1-infected cells. Fibronectin (F), myosin (M), tubulin (T), vimentin (V) and actin (A) are indicated. In addition, larger arrowheads indicate two species in the Triton-resistant cytoskeleton of wt HSV-1-infected cells which co-migrated with Vmw 38 in the corresponding total extract and supernatant.


Supernatants:
Triton-Resistant Cytoskeletons:
MI
MI+D
tsK
MI
MI+D
tsK
M
90SP
88SP
70SP
T+V
A
35SP
25SP
F
M
175
136-143
110
90SP
68
70SP
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V
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35SP
25SP
1
2
3
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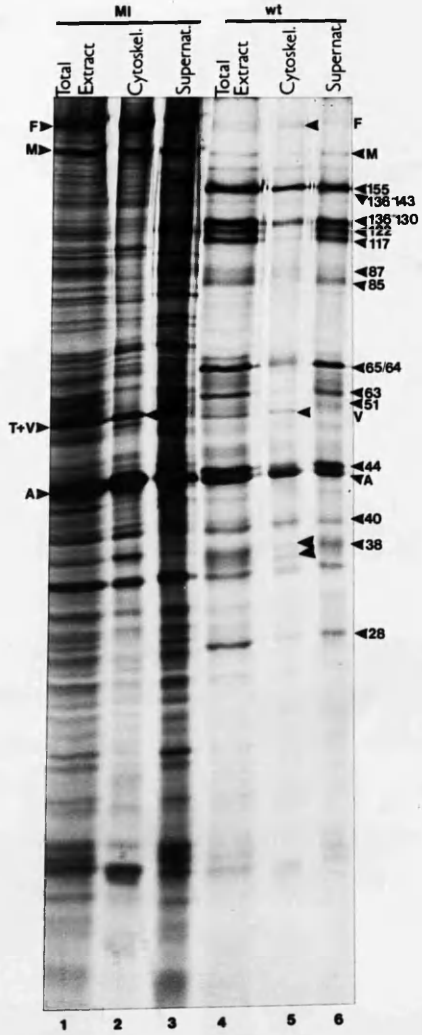
5
6


MI
wt
Total Extract
Cytoskel.
Supernat.
Total Extract
Cytoskel.
Supernat.
F
M
T+V
A
F
M
155
136-143
136-130
122
117
87
85
65/64
63
51
V
44
A
40
38
28
1
2
3
4
5
6

Vmw IE 175, Vmw IE 136'143 and Vmw IE 63 were recovered in both the supernatant and Triton-resistant cytoskeleton of tsK-infected cells, with the greater proportion of each in the supernatant. However, Vmw IE 68 appeared to be recovered exclusively in the Triton-resistant cytoskeleton, and two forms of different electrophoretic mobilities were detected.

Fig. 8.2 shows radiolabelled polypeptides in total cell extracts (tracks 1 and 4), Triton-resistant cytoskeletons (tracks 2 and 5) and supernatants (tracks 3 and 6) from mock-infected (tracks 1 to 3) and wt HSV-1-infected (tracks 4 to 6) cells. (Substantial c.p.e. were evident in wt HSV-1-infected cells at the time of harvesting.) The abundance of radiolabelled fibronectin, vimentin, and actin recovered in the Triton-resistant cytoskeleton of wt HSV-1-infected cells was reduced compared with mock-infected cells. Most of the viral polypeptides that were synthesized by wt HSV-1-infected cells were detectable in the Triton-resistant cytoskeleton, but only a few in substantial amounts: Vmw IE 136'143, Vmw 136'130 and Vmw 65/64. In the Triton-resistant cytoskeleton of wt HSV-1-infected cells were detected two species which co-migrated with Vmw 38 in the corresponding total extract and supernatant, and which may correspond to processed forms of Vmw 40 (Preston et al., 1983). However, it cannot be excluded that these viral polypeptides were contaminants in the cytoskeleton preparations.

In this and the previous experiment, roughly equal numbers of TCA-precipitable counts were recovered in total extracts or supernatants of each culture; but whereas 10% of the total counts were recovered in the Triton-resistant cytoskeleton of mock-infected cells, less than 1% was recovered in the equivalent fraction of wt HSV-1-infected, tsK-infected or disulfiram-treated cells. The amount of protein appearing in the stained gels was similar for the corresponding fractions of each culture. It is therefore inferred that radiolabelled proteins were less abundant in the Triton-resistant cytoskeleton of wt HSV-1-infected, tsK-infected or disulfiram-treated cells compared with mock-infected cells, reflecting either a lower degree of association between recently-synthesized proteins and the Triton-resistant cytoskeleton under the conditions of extraction, or decreased synthesis or availability of the class of proteins that is recovered preferentially in this fraction. In this respect, therefore, the

infection of CEF by wt HSV-1 or tsK, and treatment of CEF with disulfiram caused the Triton-resistant cytoskeleton to be affected similarly. It may be relevant that polysomes are preferentially associated with the detergent-resistant framework of the cytoskeleton (Howe & Hershey, 1984), and that levels of polysomes are decreased following either heat shock (Howe & Hershey, 1984) or infection by HSV-1 (Sydiskis & Roizman, 1966; Newton & Rasouly, 1983).

9. SUPPRESSION OF PRODUCTIVE INFECTION IN WT HSV-1-INFECTED HELU CELLS BY TREATMENT WITH ARA-C FOLLOWED BY HYPERTHERMIC INCUBATION.

Results described in chapters 4 to 6 showed that continuous treatment of wt HSV-1-infected cells (REF, CEF or HeLu cells) with stress-inducing reagents early in infection adversely affected the synthesis and processing of viral polypeptides; such treatment also reduced c.p.e. otherwise incurred following infection of cells with wt viruses or the more cytopathic temperature-sensitive mutants (chapters 2 and 6.2). These results raised the possibility that activation of the stress response in wt HSV-infected cells inhibits productive infection, and experiments were undertaken to investigate this hypothesis further.

As a preliminary study, the system of O'Neill was reproduced, in which HSV-infected cells are treated with an inhibitor of DNA replication, cytosine arabinoside (ara-C), and incubated at a hyperthermic temperatures in order to simulate the static model of herpesvirus latency (O'Neill, 1977). Human embryonic lung (HeLu) cells were infected with wt HSV-1 and treated as described by O'Neill (1977: for human foetal fibroblasts infected with HSV-2 strain 316-D) with minor modifications. Confluent, replicate cultures of HeLu cells in 35-mm-diameter dishes were pre-treated with 25 ug of ara-C/ml of EF2 at 37 ^{o}C for 24 h, and mock-infected or infected with 0.01 to 1 p.f.u. of wt HSV-1/cell. The cultures were treated with the inhibitor at 37 ^{o}C for 6 d further, the medium being changed daily. At 6 d p.i., the cultures were washed and overlaid with inhibitor-free medium (EF2), and the temperature of incubation was either increased to 41 ^{o}C or was maintained at 37 ^{o}C. The medium was changed on alternate days, and cultures were incubated at 41 ^{o}C for up to 18 d in total. (The hyperthermic temperature used by O'Neill (1977) was 39.5-40 ^{o}C.) Replicate samples were either harvested immediately or restored to 37 ^{o}C until c.p.e. became evident, when they were harvested. Cell extracts and medium were assayed for the presence of infectious virus by titration on BHK cells at 31 ^{o}C.

The results were in agreement with data published by O'Neill (1977) in these respects:

i) For infected cells that were incubated at 37 °C throughout, treatment with ara-C did not prevent development of c.p.e. on removal of the inhibitor. However, infectious virus was not detected in infected cultures incubated at 41 °C, throughout the 18 d of incubation at that temperature.

ii) Multiplicities of infection above 0.1 resulted in the degeneration of infected monolayers.

iii) When infected cultures were restored to 37 °C, infectious virus reappeared after a delay of 11 to 21 d, when a few focal lesions became apparent (2 or 3 per 10^6 cells), and final yields were as high as in untreated cells, approaching 10^8 p.f.u./ml. O'Neill (1977) found a latent period for HSV-2 of at least 11 d, and interpreted similar observations of focal lesions as indicating that very few cells harboured virus in a form that could be reactivated.

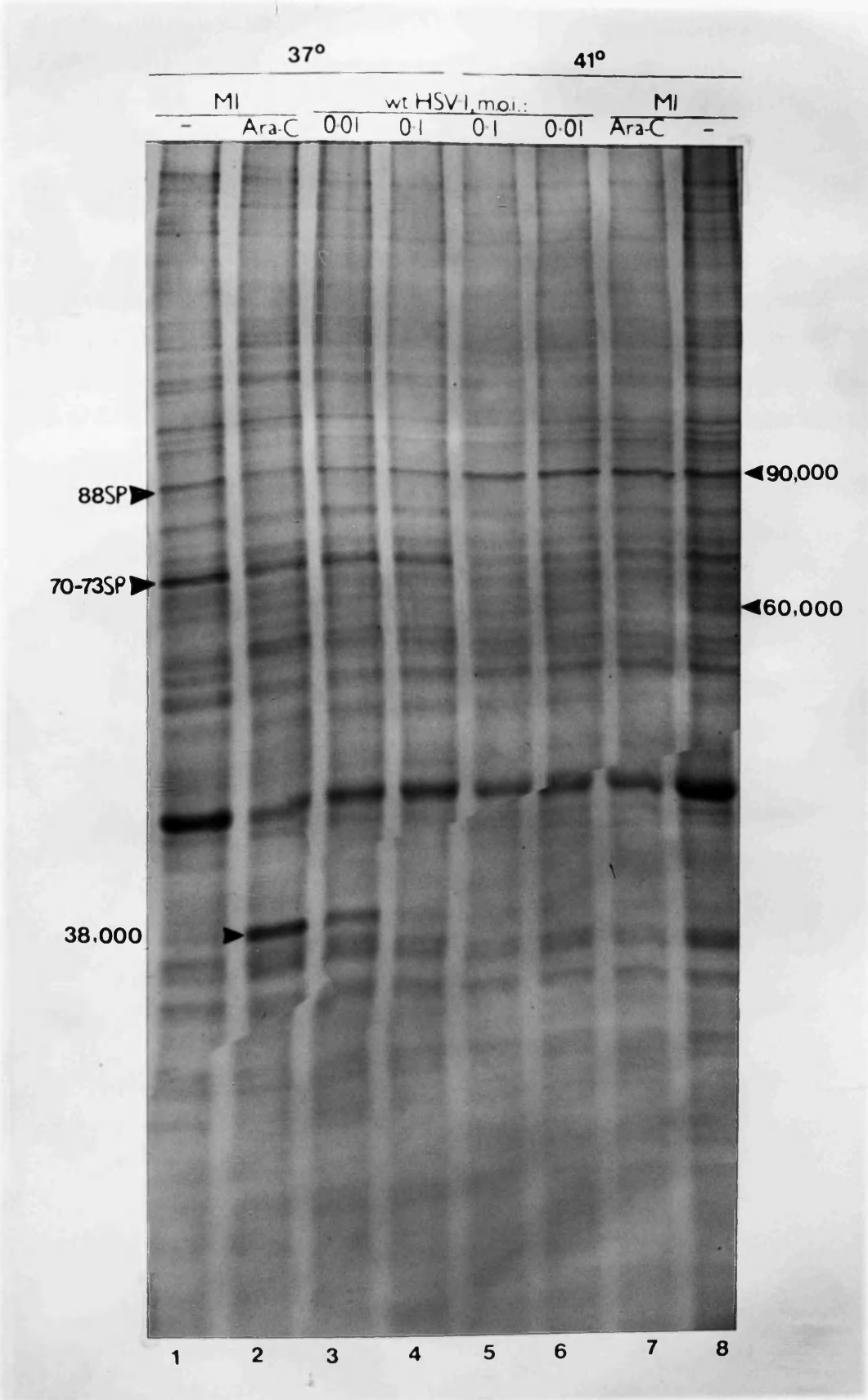
(Note:- features ii) and iii) were found to be common to the experimental system involving abortive infection of HeLu cells with wt HSV-1 or wt HSV-2 at hyperthermic temperatures, to be described.)

Ara-C-treated cells were pulse-labelled with [^{35}S]methionine, and radiolabelled polypeptides were resolved by SDS-PAGE (**Fig. 9**). Treatment of mock-infected (track 2) or infected (tracks 3 and 4) cells with ara-C at 37 °C was found reproducibly to decrease the rate of incorporation of radioactivity into TCA-insoluble material, compared with untreated cells (track 1), and to cause induction of synthesis of a polypeptide, m.wt. approximately 38,000. Synthesis of this species was lower in infected cells than in mock-infected cells, the reduction being almost total with a m.o.i. of 0.1 (track 4). (The particle/infectivity ratio of the virus stock was not determined). These effects were apparent also in cultures analysed 1 d p.i. (data not shown), and synthesis of viral polypeptides was not detected in cells infected at a multiplicity of up to 20, after several days of incubation at 37 °C (not shown). Following removal of the inhibitor and during incubation of cultures at 41 °C, the patterns of polypeptides synthesized by mock-infected (track 7) and infected (tracks 5 and 6) cells were identical, differing from that of mock-infected (track 1) and infected (tracks 2 and 3) cells maintained at 37 °C in the pronounced synthesis

Fig. 9. Polypeptide synthesis in ara-C-treated, wt HSV-1-infected or mock-infected HeLu cells.

HeLu cells were incubated in the presence of ara-C (25 ug/ml of EF2) at 37 °C for 1 d, mock-infected or infected with wt HSV-1 at a multiplicity of 0.01 or 0.1, and incubated further in the presence of ara-C at 37 °C. At 6 d p.m.i. or p.i. ara-C was withdrawn and the temperature of incubation was raised to 41 °C. The cultures were pulse-labelled for 1 h with [^{35}S]methionine at either 6 d p.m.i. or p.i. at 37 °C (tracks 1 to 4) or after further incubation at 41 °C for 6 d (tracks 5 to 8), and radiolabelled polypeptides were resolved by SDS-PAGE.

Track 1, untreated, mock-infected cells incubated at 37 °C. Track 2, cells treated with ara-C and mock-infected at 37 °C. Tracks 3 and 4, cells treated with ara-C and infected at a multiplicity of either 0.01 or 0.1, respectively, at 37 °C. Tracks 5 and 6, cells treated with ara-C, infected at a multiplicity of 0.1 or 0.01, respectively, at 37 °C, and incubated at 41 °C. Track 7, cells treated with ara-C, mock-infected at 37 °C, and incubated at 41 °C. Track 8, mock-infected cells, untreated with ara-C, and incubated 41 °C. Unidentified species with m.wt.'s of approximately 38,000, 60,000 and 90,000 are indicated.


37°
41°
MI
wt HSV-1, m.o.i.:
MI
-
Ara-C
0·01
0·1
0·1
0·01
Ara-C
-
88SP
70-73SP
38,000
90,000
60,000
1
2
3
4
5
6
7
8

of a 90,000-m.wt. species, and in the barely detectable synthesis of a 60,000-m.wt. species. These species were synthesized also in untreated, mock-infected cells incubated at 41 ^{o}C (track 8). Synthesis of stress proteins appeared to be uninduced in mock-infected and infected cultures after 6 d of incubation at 41 ^{o}C.

Thus, the role of hyperthermic incubation temperatures in suppressing productive infection in this experimental system remained unexplained, and subsequent experiments were intended to investigate more directly the possible involvement of the stress response in the abortive infection of cells by HSV at hyperthermic temperatures.

10. EFFECTS OF PROLONGED TREATMENT OF HSV-INFECTED CELLS WITH STRESS-INDUCING AGENTS UPON PRODUCTIVE INFECTION.

10.1. Prolonged Treatment of Wt HSV-1-infected HeLu cells with Sodium Arsenite.

Results of experiments described in chapters 5 and 6 indicated that up to at least 11 h p.i., synthesis of viral polypeptides was retarded in cells that were demonstrating the stress response. It was therefore attempted to extend the period of infection under investigation, to determine whether such retardation of viral replication could be sustained in stressed cells. Consequently, cytotoxic effects were reduced by lowering the m.o.i. from 20 p.f.u./cell to 0.01 p.f.u./cell, and by varying the concentration of the stress-inducing agent, sodium arsenite, in the medium. The effects of high or low concentrations of serum also were examined. HeLu cells were used in the remainder of this study, as this fibroblastic cell type frequently has been used in studies of abortive HSV-infections (Darai & Munk, 1973, 1976; Darai et al., 1975; Melvin & Kucera 1975; Marcon & Kucera, 1976; Nishiyama & Rapp, 1981).

HeLu cells grown to confluence in 35-mm-diameter dishes were infected with wt HSV-1 at a m.o.i. of 0.01, using either preconditioned EF0.5 or fresh EF10, at 37 ^{o}C. Sodium arsenite was added to the medium at 1 h p.i. to give final concentrations from 1 to 50 uM.

By 4 d of treatment, all cultures, whether infected or mock-infected, had degenerated. But up to 3 d of treatment, the cytotoxic effects of the reagent were reduced when fresh medium containing 10% serum (EF10) was used rather than preconditioned medium containing 0.5% serum (EF0.5); this difference may have resulted either from adsorption of the reagent by serum components, or from serum-dependent, cellular effects.

Infected cultures degenerated by 2 d p.i., whether infected in the presence or absence of sodium arsenite. At 1 d p.i., however, the least c.p.e. were demonstrated by cultures treated with intermediate concentrations of sodium arsenite (5-10 uM); and at 3 d p.i., seemingly healthy cells were observed within areas of degeneration. Meanwhile, no

viable cells were apparent in untreated, infected cultures. Those cells in treated, infected cultures which appeared viable at 3 d p.i. degenerated soon afterwards.

It was concluded that treatment of infected cultures with sodium arsenite could reduce the cytotoxic effects of infection, preventing total destruction of infected cultures, and could increase the survival of a small proportion of infected cells for a short period. The duration of these experiments was limited by the cytotoxic effects of sodium arsenite, and so an alternative means of inducing the stress response was investigated which might avoid this limitation: namely, induction by incubation of cells at hyperthermic temperatures. It might therefore be possible to study not only the effects of the stress response on lytic infection, but also the possible involvement of the stress response in the cell-specific, thermal inactivation of HSV (Gharpure, 1965; Crouch & Rapp, 1972a, b). Results of experiments described in chapter 6 indicated that continuous treatment of cells with stress-inducing reagents during infection was more effective than temporary treatment prior to infection in inhibiting synthesis of viral polypeptides. Thus, the effects of continued incubation of HeLu cells at the hyperthermic temperatures of 41.5-42 ^{o}C were examined.

10.2. Comparison of the Effects of Hyperthermic Incubation and Treatment with Sodium Arsenite upon Polypeptide Synthesis in HSV-infected HeLu cells.

Preliminary experiments showed that the incubation of mock-infected, wt HSV-1-infected and wt HSV-2-infected HeLu cells at 41.5-42 ^{o}C (**Fig. 10**, tracks 4, 8 and 12) was tantamount to the continuous treatment of cells with 10 uM sodium arsenite at 37 ^{o}C (tracks 3, 7 and 11) as regards the levels of induction of synthesis of stress proteins 88SP and 70-73SP in mock-infected cells (tracks 1 to 4), and the elevated synthesis of stress proteins and small reduction in synthesis of viral polypeptides in cells infected with a m.o.i. of 10 of wt HSV-1 (tracks 7 and 8) or wt HSV-2 (tracks 11 and 12). Consequently, 41.5-42 ^{o}C was considered to be a satisfactory incubation temperature for HeLu cells, for the purposes of these experiments.

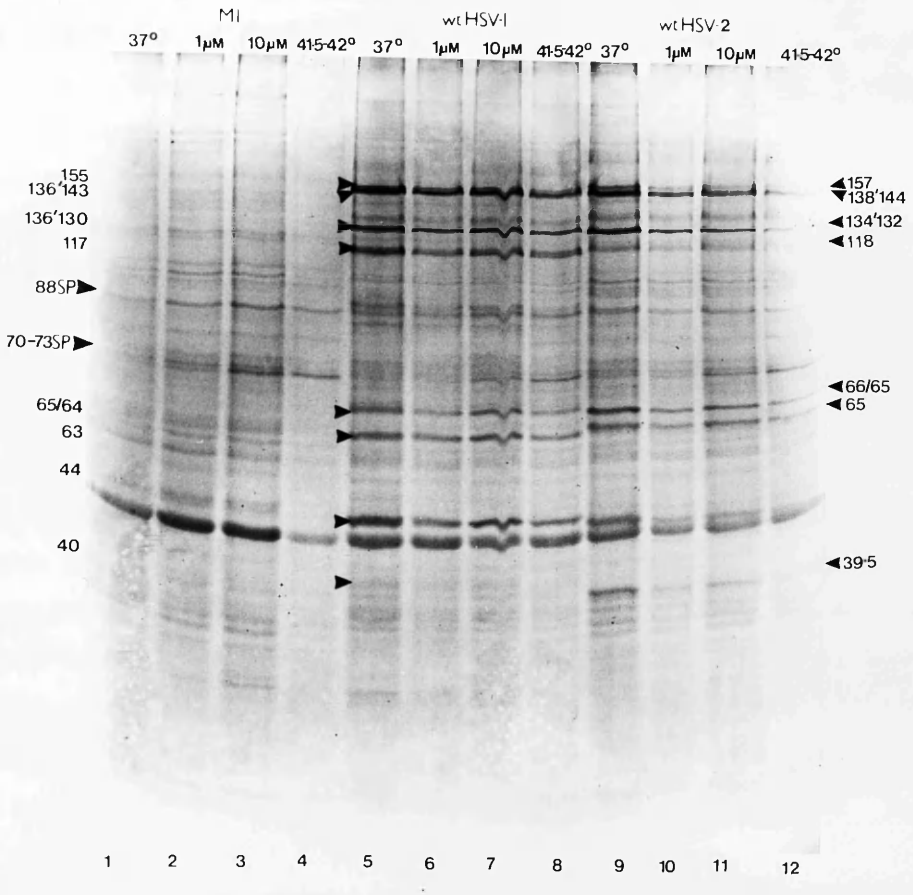
Fig. 10. Polypeptide synthesis in mock-infected, wt HSV-1-infected or wt HSV-2-infected HeLu cells, incubated at 37 °C or 41.5-42 °C, or treated with sodium arsenite at 37 °C.

HeLu cells were mock-infected or infected with wt HSV-1 or wt HSV-2 (10 p.f.u./cell), in the presence or absence of sodium arsenite (either 1 uM or 10 uM), and incubated at 37 °C for an absorption period of 1 h. Incubation was continued at either 37 °C or 41.5-42 °C for untreated cultures, or at 37 °C for sodium-arsenite-treated cultures. At 6 h p.i. the cultures were pulse-labelled with [^{35}S]methionine, and radiolabelled polypeptides were resolved by SDS-PAGE.

Tracks 1 to 4, mock-infected cultures: track 1, incubated at 37 °C; track 2, treated with 1 uM sodium arsenite; track 3, treated with 10 uM sodium arsenite; track 4, incubated at 41.5-42 °C.

Tracks 5 to 8, wt HSV-1-infected cultures: track 5, incubated at 37 °C; track 6, treated with 1 uM sodium arsenite; track 7, treated with 10 uM sodium arsenite; track 8, incubated at 41.5-42 °C.

Tracks 9 to 12, wt HSV-2-infected cultures: track 9, incubated at 37°; track 10, treated with 1 uM sodium arsenite; track 11, treated with 10 uM sodium arsenite; track 12, incubated at 41.5-42 °C.


MI
wt HSV-1
wt HSV-2
37°
1μM
10μM
41·5-42°
155
136'143
136'130
117
88SP
70-73SP
65/64
63
44
40
157
138'144
134'132
118
66/65
65
39·5
1
2
3
4
5
6
7
8
9
10
11
12

11. INFECTION OF HELU CELLS AT 41.5-42 °C.

11.1. Factors Determining the Yields of Wt HSV-1 at 41.5-42 C: Serum Concentration, M.O.I. and Pre-Incubation of Cells at 41.5-42 C.

The production of infectious virus by wt HSV-1-infected and wt HSV-2-infected HeLu cells at hyperthermic temperatures of 41.5-42 °C was examined, varying the m.o.i. (0.001, 0.01, 0.1 or 1.0), the concentration of foetal calf serum in the medium (0.5%, 2% or 10%, v/v), and the time of pre-incubation of cells at 41.5-42 °C. Confluent monolayers of cells were infected and incubated at 37 °C for an absorption period of 1 h, washed and overlaid with fresh EF0.5, EF2 or EF10, and incubated at 41.5-42 °C. Replicate cultures were harvested periodically and crude extracts, comprising disrupted infected cells and overlying medium, were titrated on BHK cells at 37 °C, the limit of detection of infectious virus being 5 p.f.u./ml. Results of a typical experiment, employing HSV-1 at a m.o.i. of 0.01, and from which surviving cultures were derived (cultures AI/1, AI/2 and AI/3; chapter 12), are shown in **Tables III** and **IV**. The results and observations drawn from this experiment were representative of a series of 12 experiments employing either wt HSV-1 or wt HSV-2 and, in total, about three-hundred monolayers. The outcome of infection by wt HSV-1 or wt HSV-2 at 41.5-42 °C was found to be determined by the (1) concentration of serum in the medium, (2) the m.o.i. and (3) the time of pre-incubation of cells at 41.5-42 °C:

(1) The concentration of serum controlled the production of detectable infectious virus: when 0.5% or 2% serum was used, productive infections developed, although yields were lower than at 37 °C: average yields of wt HSV-1 at 1 d p.i. at 37 °C were $2x10^7$ and $3x10^7$ p.f.u./ml using 10% and 0.5% serum, respectively, whereas the maximum average titre detected at 41.5-42 °C was $1.7x10^6$ p.f.u./ml (average of $1.29x10^6$ and $1.95x10^6$) at 5 d p.i. using 0.5% serum. (It was observed for monolayers incubated at 37 °C that plaques grew faster in the presence of 10% serum compared with 0.5% serum). But when 10% serum was used, infectious virus was undetectable from 2 h p.i. until at least 33 d p.i. at 41.5-42 °C (results not shown). Production of infectious virus was not detected at 41.5-42 °C in any of the

Table III. Yields of virus (p.f.u./ml) from wt HSV-1-infected HeLa cells incubated at 37 °C or 41.5-42 °C, using medium supplemented with 0.5%, 2% and 10% foetal calf serum (average of duplicate samples).

temp.	37 °C		41.5-42 °C,		
serum	0.5%	10%	0.5%	2%	10%
days p.i.*					
1	$2x10^7$	$3x10^7$	260	5	<5
2	-	-	nt	nt	<5
3	-	-	$7.5x10^3$	20	<5 (CHX)
4	-	-	nt	nt	<5
5	-	-	†$1.7x10^6$	<5	<5 (CHX)
6	-	-	-	<5	<5
7	-	-	-	230	<5
8	-	-	-	280	<5
9	-	-	-	90	<5

*m.o.i. = 0.01.

Cells and overlying medium were sonicated before titration.

nt: signifies not tested.

† 11 to 80 plaques were counted per monolayer, with an average of 35.

(CHX) signifies that replicate cultures were treated with 20-200 ug of cycloheximide/ml at 41.5-42 ° for 3 h, and incubated at either 37 °C or 41.5-42 °C for 5 d. No c.p.e. was observed at either temperature during this time, and no infectious virus was detected in medium and extracts before or after treatment.

Table IV. Effects of varying the time of pre-incubation of HeLu cells at 41.5-42 °C, and the m.o.i. with wt HSV-1 upon virus yields and c.p.e. at that temperature.

	Preincubation:					
	2 d		1 d		0 d	
d p.i. m.o.i.	p.f.u. /ml.	c.p.e.	p.f.u. /ml	c.p.e.	p.f.u. /ml	c.p.e.
1 d 10^{-3}	50	plaques	5	plaques	125	no c.p.e.
10^{-2}	300	plaques	145	plaques	5	no c.p.e.
10^{-1}	1285	c.p.e.	200	c.p.e.	125	no c.p.e.
3 d 10^{-3}	nt	plaques	50	plaques	5	no c.p.e.
10^{-2}	nt	plaques	245	plaques	20	c.p.e.
10^{-1}	1500	total c.p.e.	1000	total c.p.e.	50	c.p.e.
5 d 10^{-3}	nt	plaques	nt	plaques	nt	no c.p.e.
10^{-2}	nt	total c.p.e.	nt	plaques	nt	no c.p.e.
10^{-1}	-	-	-	-	nt	c.p.e.
6 d 10^{-3}	0	plaques	0	plaques	nt	no c.p.e.
10^{-2}	-	-	0	c.p.e.	nt	no c.p.e.
10^{-1}	-	-	-	-	nt	c.p.e.
8 d 10^{-3}	0	no c.p.e.	0	no c.p.e.	nt	no c.p.e.
10^{-2}	-	-	0	c.p.e.	nt	no c.p.e.
10^{-1}	-	-	0	-	0	total c.p.e.
14 d 10^{-3}	0	no c.p.e.	0	no c.p.e.	-	-
10^{-2}	-	-	70	No c.p.e.	-	-
10^{-1}	-	-	-		-	-

nt: signifies not tested.

Cells and overlying medium were sonicated before titration.

several experiments which used a concentration of serum of 10% or higher. Concentrations of serum higher than 10% caused cells incubated at 41.5-42 °C, whether infected or mock-infected, to detach from the monolayer into the medium. Thus, in succeeding experiments 10% serum was employed as the maximum concentration of serum which would suppress yields of infectious virus at 41.5-42 °C without causing loss of cells from the monolayers.

Incubation of wt HSV-1-infected or wt HSV-2-infected cells (m.o.i. of 0.01) at 39.5 °C using 10% serum failed to suppress productive infection (data not shown).

(2) The m.o.i. controlled the degree of c.p.e. incurred by infected cultures. When the m.o.i. exceeded 0.01, cultures rapidly degenerated although little or no detectable infectious virus was produced, depending upon the concentration of serum in the medium. (Cytotoxic effects of HSV-2 without production of detectable virus have been noted in hamster embryo fibroblasts infected with m.o.i. >1 at 39 °C (Crouch & Rapp, 1972b).) A m.o.i. less than 0.1 caused no apparent destruction of monolayers during incubation at 41.5-42 °C when the concentration of serum was 10%: but when 0.5% or 2% serum was used, both wt HSV-1 and wt HSV-2 caused plaques slowly to develop (see chapter 12, B and C series of cultures). (The ability of HSV-2 to induce plaques in human embryo fibroblasts at 40 °C despite reduced yields has been noted previously (O'Neill, 1977).) There was variability in the number of plaques forming in replicate monolayers: 11 to 80 plaques, with an average of 35, were noted at 5 d p.i. in wt HSV-1-infected cultures that were supplemented with 0.5% serum (**Table III**). Total c.p.e. was not observed when the m.o.i. was less than 0.1, even though the development of c.p.e. was accompanied by production of detectable infectious virus.

(3) The effects of pre-incubation of cells at 41.5-42 °C upon productive infection with wt HSV-1 were investigated. Replicate infected cultures were overlaid with fresh EF2, and either pre-incubated at 41.5-42 °C for 1 d or 2 d, or maintained at 37 °C prior to infection with wt HSV-1 at m.o.i. of 0.001, 0.01 or 0.1. The infected cultures were incubated at 37 °C for 1 h, and at 41.5-42 °C thereafter. A concentration of 2% serum was chosen in order to induce conditions that

would verge on the suppression of virus yields (from **Table III**). Plaques and c.p.e. were observed to develop faster - and virus yields were higher - in replicate cultures pre-incubated at 41.5-42 °C compared with cultures maintained at 37 °C prior to infection. The differences were especially marked for cultures pre-incubated at 41.5-42 °C for 2 d. By 3 d p.i., for example, non-pre-incubated cultures infected with m.o.i. of 0.001 or 0.01 showed no c.p.e. while the pre-incubated cultures contained plaques (**Table IV**). Thus, the suppression of productive infection at 41.5-42 °C was less effective in pre-incubated cultures, and it is suggested that the ability of cultures to support viral replication was regained to some degree during adaptation of the cells to prolonged incubation at a hyperthermic temperature.

In keeping with previous results, cultures infected with m.o.i. less than 0.1 in the presence of EF2 or EF0.5 developed c.p.e. in the form of focal lesions, and produced detectable infectious virus. However, cultures infected with m.o.i. of 0.1 in the presence of EF2 or EF10 developed widespread c.p.e., rather than focal lesions, and produced little (EF2) or no (EF10) detectable infectious virus.

11.2. Polypeptide Synthesis in HeLu Cells Infected with Wt HSV-1 at 41.5-42 °C .

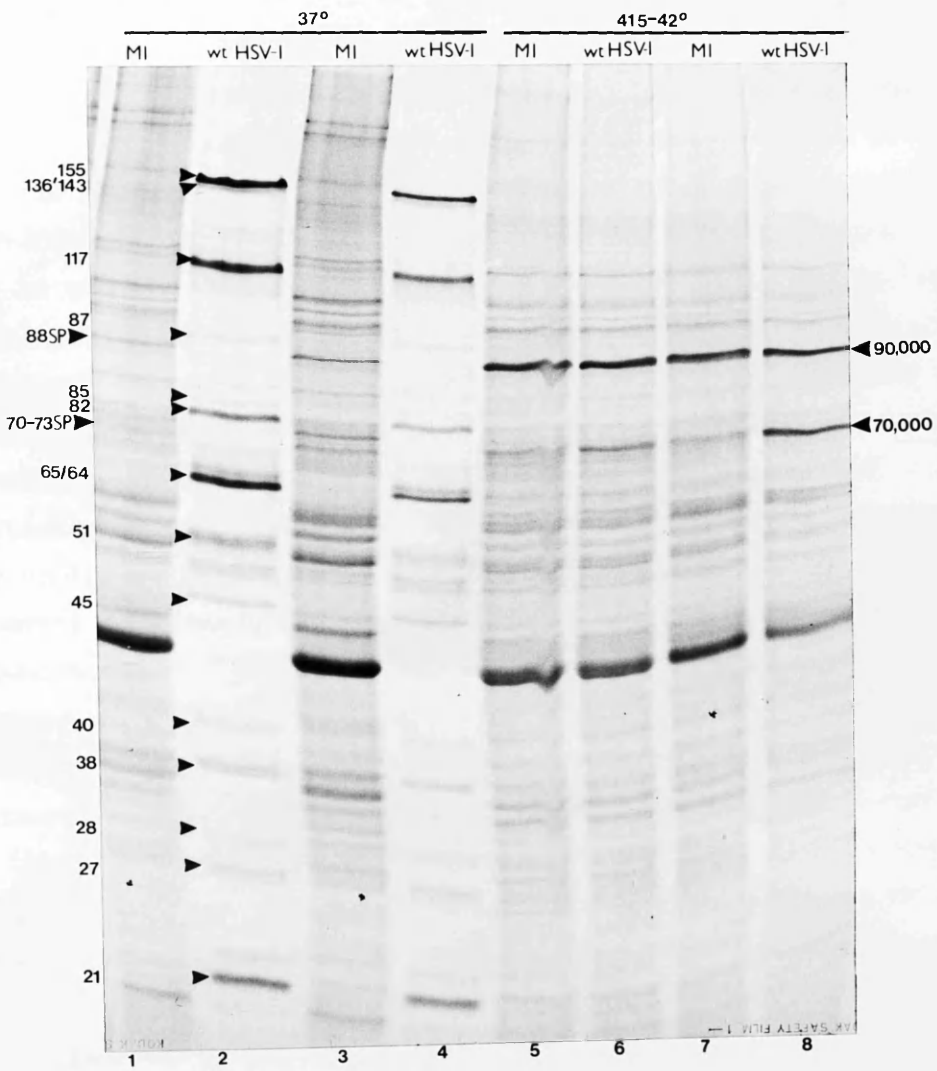
Helu cells were infected with 0.01 p.f.u. of wt HSV-1/cell at 41.5-42 °C using EF10, and pulse-labelled with [^{35}S]methionine at 1 d p.i.. Radiolabelled polypeptides in extracts of infected cells are shown in **Fig. 11.1**. Two independent cultures were analysed in this experiment, monolayers from one culture (tracks 1, 2, 5) being replicate to those used in the experiments summarised in **Tables III** and **IV**.

Synthesis of viral polypeptides was undetectable in extracts of infected cells at 1 d p.i. at 41.5-42 °C (tracks 6 and 8), reflecting the suppression of productive infection at this temperature compared with 37 C (tracks 2 and 4; and see **Table III**). As noted in chapter 10, a species with a m.wt. of approximately 90,000 was synthesized prominently by mock-infected and infected cells at 41.5-42 °C; and it is suggested that this species may correspond to a protein whose

Fig. 11.1. Polypeptide synthesis in mock-infected and wt HSV-1-infected HeLu cells incubated at 41.5-42 °C.

Monolayers were seeded from two separate cultures of HeLu cells. Cells were mock-infected or infected with 0.01 p.f.u. of wt HSV-1 per cell, and incubated at 37 °C for an absorption period of 1 h. Incubation was continued for 1 d at either 37 °C or 41.5-42 °C. The medium was supplemented with EF10. Cultures were pulse-labelled with [^{35}S]methionine, and radiolabelled polypeptides were resolved by SDS-PAGE.

Samples shown in tracks 1, 2, 5, and 6, and samples shown in tracks 3, 4, 7 and 8, were derived from separate cultures. Tracks 1 and 3, mock-infected cells incubated at 37 °C; tracks 2 and 4, infected cells at 37 °C; tracks 5 and 7, mock-infected cells at 41.5-42 °C; tracks 6 and 8, infected cells at 41.5-42 °C. Unidentified species with m.wt.'s of approximately 90,000 and 70,000 are indicated.


37°
415–42°
MI
wt HSV-1
MI
wt HSV-1
MI
wt HSV-1
MI
wt HSV-1
155
136/143
117
87
88SP
85
82
70–73SP
65/64
51
45
40
38
28
27
21
90,000
70,000
1
2
3
4
5
6
7
8

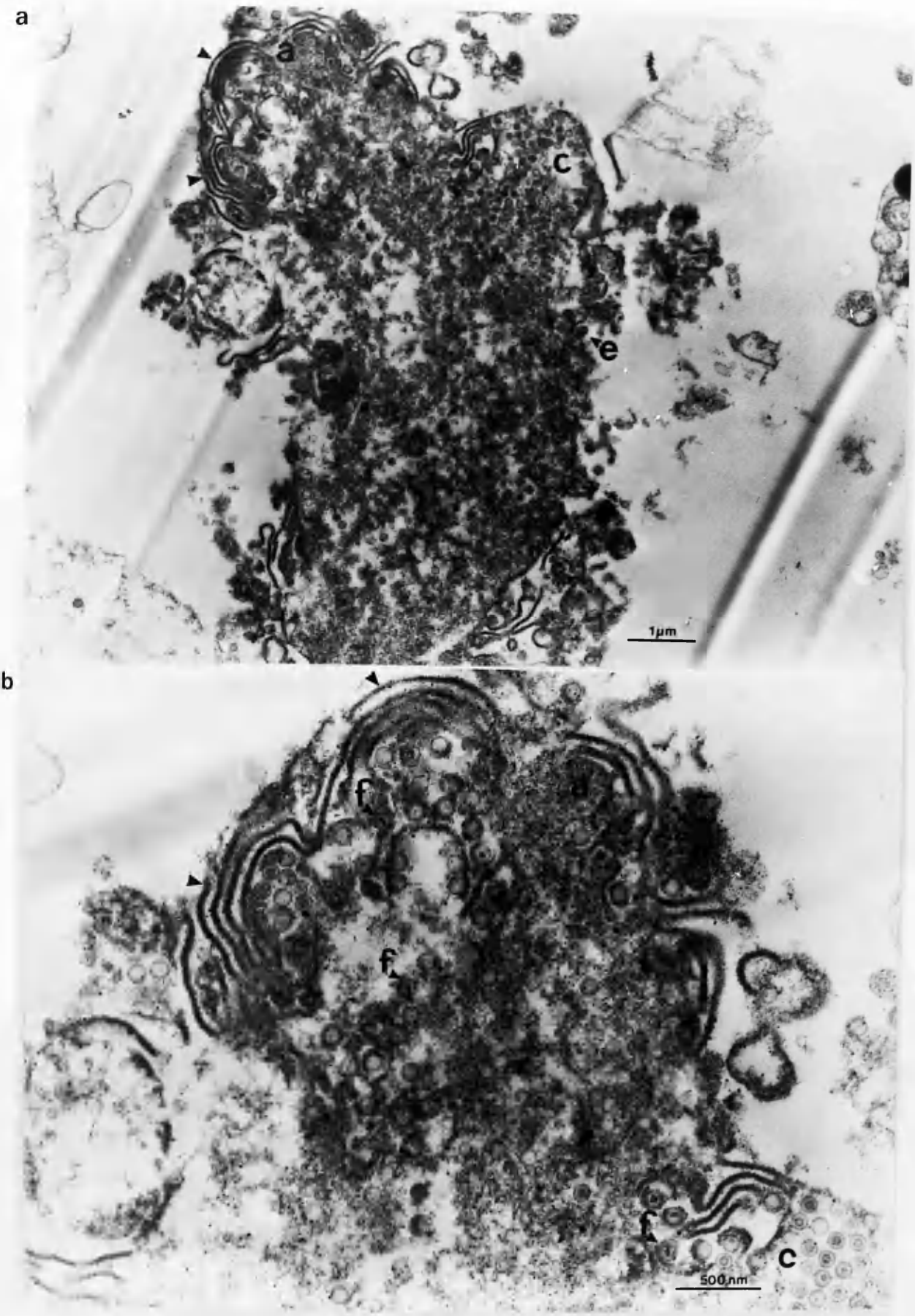
synthesis is induced by depletion of essential nutrients from the medium (Kasambalides & Lanks, 1981). It is notable that the infection of cells derived from either culture caused induction of synthesis of a polypeptide with a m.wt. of approximately 70,000, which comigrated with 70-73SP. In further experiments using EF2 or EF0.5 and a m.o.i. of 0.01, induction of synthesis of such a species was not observable in wt HSV-1-infected cells during incubation at 41.5-42 ^{o}C.

Cells infected with wt HSV-2 (m.o.i. 0.01) using EF10 and incubated at 41.5-42 ^{o}C were examined at 7 d p.i. (when infectious virus was not detectable in cell extracts) by electron microscopy by Dr. F. Rixon and Mr. J. Aitken. **Figs. 11.2a** and **b** show an effete nucleus which was observed in a field of roughly fifty cells from the wt HSV-2-infected culture (Dr. F. Rixon, personal communication), and which was in contact with seemingly uninfected cells. This nuclear remnant displayed reduplicated nuclear membranes (larger arrow-heads), and contained amorphous, electron-dense material (**a**) interspersed with scattered and semi-crystalline arrays of viral capsids (**c**), most of which were hollow, having no dense core of DNA, and some of which were partially filled (**f**). Amorphous accumulations have been described for the nuclei of productively HSV-infected cells (Nii et al., 1968c; Schwartz & Roizman, 1969; Dargan & Subak-Sharpe, 1983), and are immunoreactive with rabbit antiviral sera (Nii et al., 1968c). The occurrence of empty viral capsids in the nuclei of HSV-infected cells previously has been interpreted to signify defective viral replication (Schwartz & Roizman, 1969). Nii et al. (1968b) examined the effects of inhibiting synthesis of viral DNA (by treatment of infected cells with hydroxyurea) upon the morphogenesis of HSV: synthesis of viral capsids and virus-stimulated reduplication of nuclear and cytoplasmic membranes were unaffected, but the formation of dense cores and envelopment of viral capsids were inhibited. Thus, the condition of the nucleus in **Fig.'s 11.2a** and **b** indicates that an abortive infection occurred, where virus replication was restricted.

The effects of incubation of wt HSV-1-infected cells at 41.5-42 ^{o}C upon the synthesis of viral polypeptides was examined at higher m.o.i. of 0.01, 0.1, 1.0 or 10, using EF2. At 24 h p.i., cultures that were infected at 41.5-42 ^{o}C showed less c.p.e. than cultures infected and maintained at 37 ^{o}C. Polypeptide synthesis was analysed

Fig.'s 11.2a and b. Electron micrographs of an effete nucleus in a culture of wt HSV-2-infected cells, 7 d p.i. at 41.5-42 ^{o}C.

Note: reduplicated nuclear membranes (larger arrow-heads); amorphous, electron-dense material (a); semi-crystalline arrays of hollow viral capsids (c); partially-filled nucleocapsids (f); enveloped nucleocapsid (e).


a
a
c
e
1µm
b
f
a
f
f
c
500 nm

at 6 and 24 h p.i. or p.m.i., as shown in **Fig. 11.3.** That the stress response was activated in mock-infected and infected cells by incubation at 41.5-42 °C for 6 h was evidenced by the induction of synthesis of major stress proteins, m.wt.'s 88,000 and 70-73,000 (tracks 1 to 5). Levels of induction were reduced in cells infected with m.o.i. of 10 (track 5) compared with mock-infected cells (track 1) - inhibition of the stress response by infection with wt HSV-1 was noted in previous chapters. At 24 h p.i. or p.m.i. at 41.5-42 °C, synthesis of stress proteins was less apparent in wt HSV-1-infected and mock-infected cells, and incorporation of radioactivity into the samples was depressed (tracks 11 to 15).

At 6 h post-infection, synthesis of viral polypeptides was reduced in cells infected at 41.5-42 °C (tracks 4 and 5) compared with 37 °C (tracks 9 and 10), although synthesis of several non-IE viral polypeptides was detected in cultures infected with m.o.i. of 1 or 10 at 41.5-42 °C (including Vmw 155/Vmw IE 136'143, Vmw 117, Vmw 85, Vmw 82, Vmw 65/64, Vmw 45 and Vmw 40); in particular, synthesis of Vmw 87 and Vmw 65/64 was reduced at 41.5-42 °C (track 5) compared with 37 °C (track 10). This indicates that viral replication was restricted at the hyperthermic temperature. At 24 h post-infection at 41.5-42 °C, synthesis of viral polypeptides was undetectable in infected cultures (tracks 12 to 15), and the patterns of polypeptides synthesized by infected and mock-infected (track 11) cultures were indistinguishable; severe c.p.e. was incurred by the culture infected with a m.o.i. of 10 (track 15), and was reflected by low incorporation of radioactivity by this sample. Reduction in the rate of protein synthesis has been noted to occur in cells subjected to cytotoxic concentrations of stress-inducing reagents (Levinson *et al.*, 1978b) and intense heat shock (Ashburner & Bonner, 1979); infection of cells with m.o.i. of 10 at 41.5-42 °C may therefore have caused the response to incubation at 41.5-42 °C to be reinforced. (Results presented in chapters 4 to 6 showed that the response of CEF to stress-inducing treatments may be potentiated by infection with wt HSV-1.)

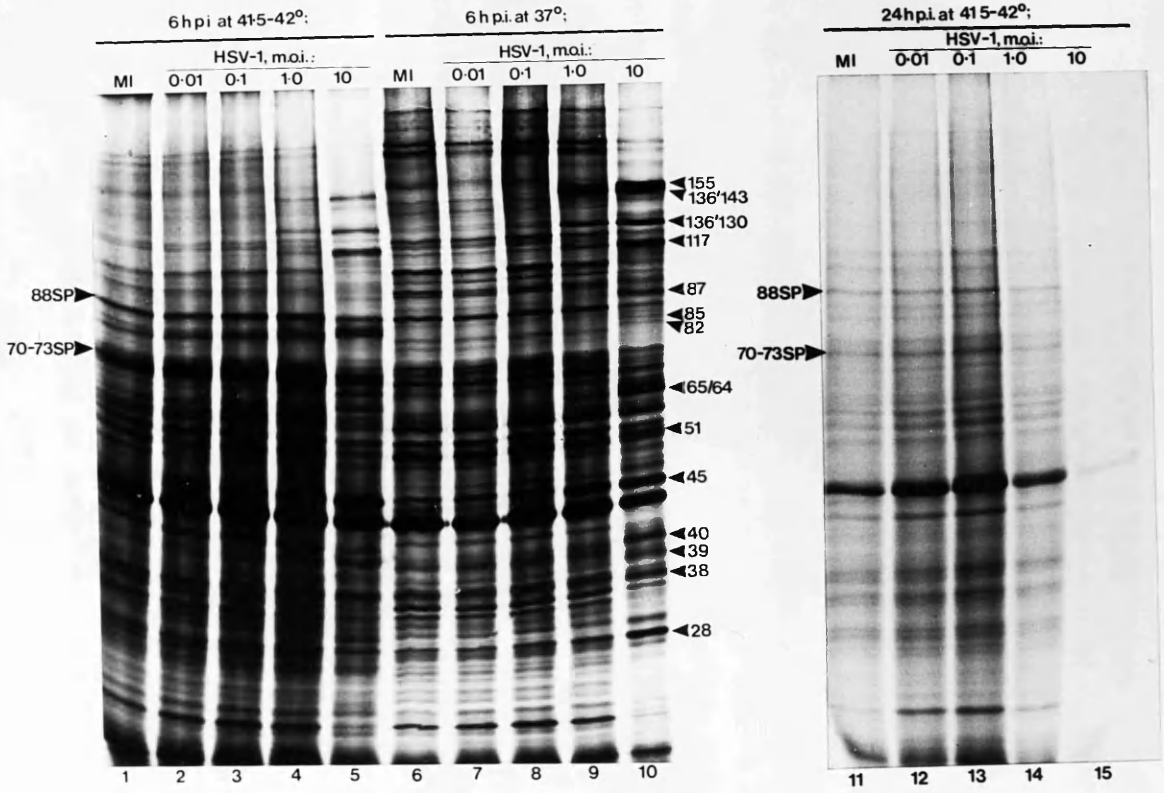
It is concluded that the stress response was activated in wt HSV-infected HeLu cells by incubation at 41.5-42 °C while synthesis of viral polypeptides was largely inhibited; and that the c.p.e. incurred by cells infected at a multiplicity greater than 0.01 at 41.5-42 °C

Fig. 11.3. Polypeptide synthesis in HeLu cells infected with wt HSV-1 at 37 °C or 41.5-42 °C.

HeLu cells were mock-infected or infected with HSV-1 at m.o.i. of 0.01 to 10, using medium supplemented with 2% foetal calf serum (EF2). Incubation was at 37° for an absorption period of 1 h, and at either 37 °C or 41.5-42 °C thereafter. At 6 h p.i. or 24 h p.i. cultures were pulse-labelled for 30 min with [^{35}S]methionine, and equal proportions of total extracts were the analysed by SDS-PAGE.

Tracks 1 to 10, cultures radiolabelled at 6 h p.i.: track 1, mock-infected cells at 41.5-42 °C ; tracks 2 to 5, cells infected with m.o.i. of 0.01 (track 2), 0.1 (track 3), 1.0 (track 4) or 10 (track 5) at 41.5-42 °C; track 6, mock-infected cells at 37 °C; tracks 7 to 10, cells infected with m.o.i. of 0.01 (track 7), 0.1 (track 8), 1.0 (track 9) or 10 (track 10) at 37 °C.

Tracks 11 to 15, cultures radiolabelled at 24 h p.i.: track 11, mock-infected cells at 41.5-42 °C; tracks 12 to 15, cells infected with m.o.i. of 0.01 (track 12), 0.1 (track 13), 1.0 (track 14) or 10 (track 15) at 41.5-42 °C.


6 h p.i. at 41·5–42°;
6 h p.i. at 37°;
24 h p.i. at 41·5–42°;
HSV-1, m.o.i.:
MI
0·01
0·1
1·0
10
88SP
70-73SP
155
136/143
136/130
117
87
85
82
65/64
51
45
40
39
38
28
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

may not have been caused by continuing synthesis of viral polypeptides, but rather may have resulted from the toxic combination of infection with wt HSV-1 and incubation at 41.5-42 °C.

11.3. Reactivation of Productive Infection in HeLu Cells Infected with Wt HSV-1 at 41.5-42 C, by Restoration to 37 °C.

When cultures of HeLu cells were infected with wt HSV-1 or wt HSV-2 (m.o.i. of 0.01), using EF10, and incubated at 41.5-42 °C, development of c.p.e. and production of infectious virus were suppressed (**Table III**). If such cultures were restored up to 5 d p.i. to 37 °C, infection would be reactivated within 3 d. (The reactivation of infection refers to a rise in titre of infectious virus in cell extracts from an undetectable level of less than 5 p.f.u./ml.) However, restored at or after 6 d p.i., there followed a considerable delay of 12-17 d before cultures showed reactivation of infection: thus, by 6 d of incubation at 41.5-42 °C, a transition had occurred which caused infections to be temporarily inapparent at 37 °C. Reactivation was subsequently manifest by the development of one or two plaques per culture, and titres approached 10^8 p.f.u./ml in cultures harvested 48 h later.

A similar transition was indicated by the studies of Darai *et al.* (1975): incubation of HSV-1 (ANG)-infected HeLu cells (m.o.i. 1 to 10) at 42 °C for up to 6 d did not destroy the ability of the cells to support synthesis of viral progeny; but after 8 d at 42 °C, the cells were unable to produce infectious virus within 72 h of incubation at 37 °C, even though viral DNA was detected in nuclei of infected cells 9 d p.i. at 42 °C. However, cloned cell lines, which were derived from infected cultures incubated at 42 °C for 8 d, spontaneously produced infectious virus during sub-culture at 37 °C (Darai and Munk, 1973; Darai *et al.*, 1975).

11.4. Attempts to Reactivate Productive Infection by Treatment of Cultures with Cycloheximide.

Experiments were performed to test the possibility that replication of virus was inhibited in cultures infected at the hyperthermic

temperature, due to the activity a repressor protein. At 3 to 5 d p.i. at 41.5-42 ^{o}C using EF10, wt HSV-1-infected cultures (m.o.i. 0.01) were treated with 20-200 ug/ml of cycloheximide at 41.5-42 ^{o}C for 3 h, and incubated at 37 ^{o}C for 1 d or at 41.5-42 ^{o}C for 5 d (corresponding to the A-series of cultures; **Table III**). Infectious virus was detected neither in the medium assayed prior to treatment, nor in infected-cell extracts assyed after treatment. Thus, evidence was not obtained for the suppression of lytic infection by a short-lived repressor protein. O'Neill (1977) similarly failed to reactivate the production of infectious HSV-2 from infected human embryo fibroblasts (which had undergone treatment with ara-C prior to incubation at 39.5-40 ^{o}C) by use of cycloheximide.

11.5. Growth of Abortively Infected Cells in Soft Agar.

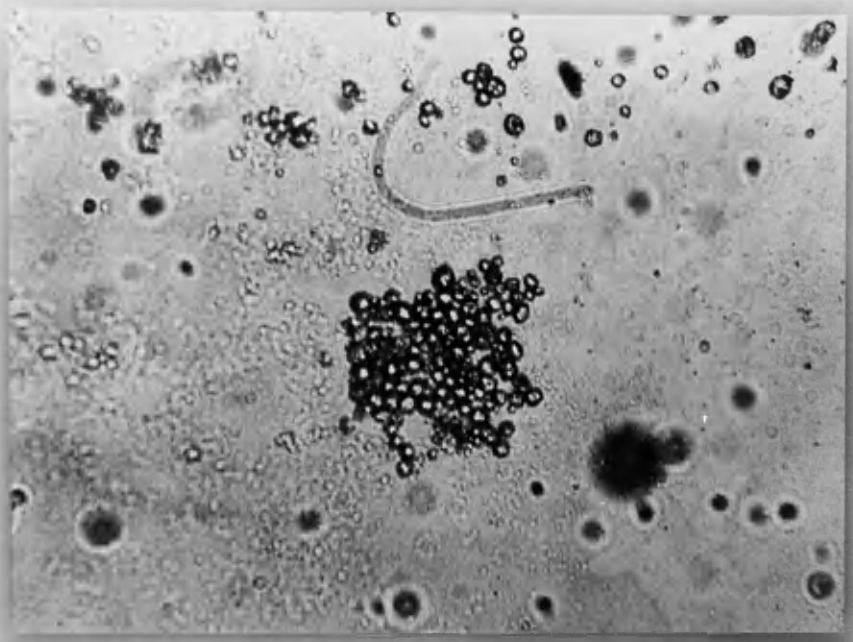
Cultures of HeLu cells were infected with m.o.i. of 0.01 of wt HSV-1 or wt HSV-2 or mock-infected at 41.5-42 ^{o}C, using EF10 (C-series of cultures, chapter 12). At 8 d p.i., the cells were tested for growth in soft agar; only cells from HSV-2-infected cultures were formed colonies (**Fig. 11.4**), and this occurred at an efficiency of $5x10^{-5}$, i.e. $5x10^{-3}$ per infecting p.f.u..

Fig. 11.4. Light micrograph showing cells, derived from an abortive infection with wt HSV-2 at 41.5-42 ^{o}C, growing as colonies in soft agar.

Fig. 12.1. Light micrograph of an abortively wt HSV-2-infected culture undergoing spontaneous reactivation, at 36 d p.i..

Fig. 12.2. Light micrograph of mock-infected cells, cultured in parallel with infected cells shown in **Fig. 12.1.**, 36 d p.m.i..

11.4



12.1

12. PROPAGATION AND CHARACTERIZATION OF CULTURES SURVIVING ABORTIVE INFECTION WITH WT HSV-1 OR WT HSV-2 AT 41.5-42 C.

Cultures were derived from infections of HeLu cells with wt HSV-1 or wt HSV-2 at 41.5-42 °C and propagated at 37 °C for considerable periods in the absence of c.p.e. and detectable infectious virus. The conditions at establishment and characteristics of these cultures are summarized in this chapter and in **Table V**, and the experiments that were performed upon them are summarized in **Fig.'s 12.3.1** to **12.3.3.** Although a m.o.i. with wt HSV-1 or wt HSV-2 of 0.01 was used throughout, the concentration of serum in the medium and the period of incubation at 41.5-42 °C were varied. These cultures may be classified into two types, the first being represented by the A series of cultures, which were derived from infections with wt HSV-1 using EF10 from the time of infection and an incubation period at 41.5-42 °C of at least 6 d, as described above.

Since a m.o.i. greater than 0.01 proved cytocidal, the finding that productive infections occurred at 41.5-42 °C in the presence of 0.5% or 2% serum was utilized in devising a protocol which might ensure the exposure of all cells in infected cultures to virus, whilst maximising the proportion of cells surviving infection. Cultures of the second type (B and C series) were established by this protocol: cultures were infected with wt HSV-1 or wt HSV-2 (m.o.i. 0.01) using EF0.5 or EF2 and incubated at 41.5-42 °C for 7 or 8 d, or until the developing plaques had destroyed approximately half of each monolayer; the medium was then changed to EF10, and incubation at 41.5-42 °C was continued. Three or four days later, when monolayers showed signs of regeneration and infectious virus was no longer detectable in the medium, the cultures were restored to 37 °C and propagated for periods sometimes in excess of 50 d p.i. before infection reactivated spontaneously. These infected cultures differed in their cellular morphology from similarly treated, mock-infected cells by exhibiting a more angular or spindle-shaped morphology, a lack of contact inhibition, and a tendency to aggregate and detach into the medium. These differences were especially apparent at the end of the period of incubation at 41.5-42 °C. Plaques developing spontaneously in these cultures remained localized for several days, and failed to spread throughout the cultures at the rate usually observed in productively HSV-infected

Table V: Conditions at the establishment of wt HSV-1-infected and wt HSV-2-infected HeLa-cell cultures at 41.5-42 °C, and instances of spontaneous or induced reactivation of productive infection during propagation of cultures at 37 °C.

Culture	*Virus	Concentration of serum and period at 41.5-42 °C.	Spontaneous reactivation in sub-cultures.	Reactivation of Infection by Superinfection with Ts Mutants at NPT.
AI/1	wt HSV-1	10% for 10 d.	Not up to 32 d p.i..	32 d p.i..
AI/2	wt HSV-1	10% for 33 d.	Not up to 45 d p.i..	NA
AI/3	wt HSV-1	" " " ".	Not up to 50 d p.i..	41 d p.i.: no reactivation.
BI	wt HSV-1	0.5% for 8 d, and 10% for 4 d.	Not up to 54 d p.i..	28 and 54 d p.i..
BII	wt HSV-2	- do -.	Not up to 54 d p.i..	28 and 54 d p.i..
CI/1	wt HSV-1	2% for 7 d, and 10% for 3 d.	Not up to 47 d p.i..	32 and 47 d p.i..
CI/2†	wt HSV-1	- do -.	29 d p.i..	
CI/3	wt HSV-1	- do -.	Not up to 47 d p.i..	NA
CII/1	wt HSV-2	- do -.	Not up to 47 d p.i..	32 and 47 d p.i..
CII/2†	wt HSV-2	- do -.	21 d p.i..	
CII/3†	wt HSV-2	- do -.	32 d p.i..	

*m.o.i. with wt HSV-1 or wt HSV-2, 0.01.

NA = not attempted.

† remaining sub-cultures of cultures CI/2, CII/2 and CII/3 were propagated up to 47 d p.i., without showing spontaneous reactivation.

Fig.'s 12.3.1 to 12.3.3. Flow charts summarising experiments performed upon cultures surviving abortive infection with wt HSV-1 or wt HSV-2 at 41.5-42 ^{o}C. The m.o.i. was 0.01, and EF10 was the medium used to propagate cultures at 37 ^{o}C or 31 ^{o}C. The number of cultures that were established in parallel are given in parenthesis, as are the proportion of cultures undergoing spontaneous reactivation of productive infection during propagation at 37 ^{o}C. "IF" signifies that cells were chemically fixed in preparation for immunofluorescence.

Fig. 12.3.1.

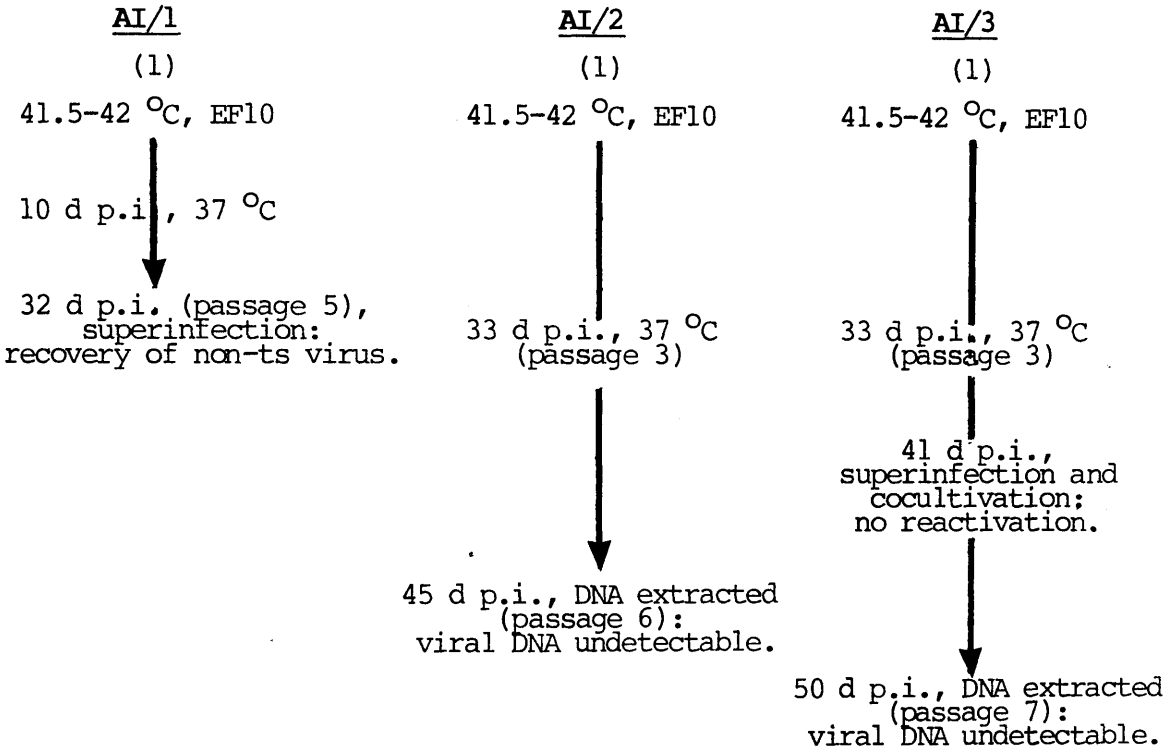

AI/1
(1)
41.5-42 °C, EF10
10 d p.i., 37 °C
32 d p.i. (passage 5), superinfection: recovery of non-ts virus.
AI/2
(1)
41.5-42 °C, EF10
33 d p.i., 37 °C (passage 3)
45 d p.i., DNA extracted (passage 6): viral DNA undetectable.
AI/3
(1)
41.5-42 °C, EF10
33 d p.i., 37 °C (passage 3)
41 d p.i., superinfection and cocultivation: no reactivation.
50 d p.i., DNA extracted (passage 7): viral DNA undetectable.

Fig. 12.3.2.

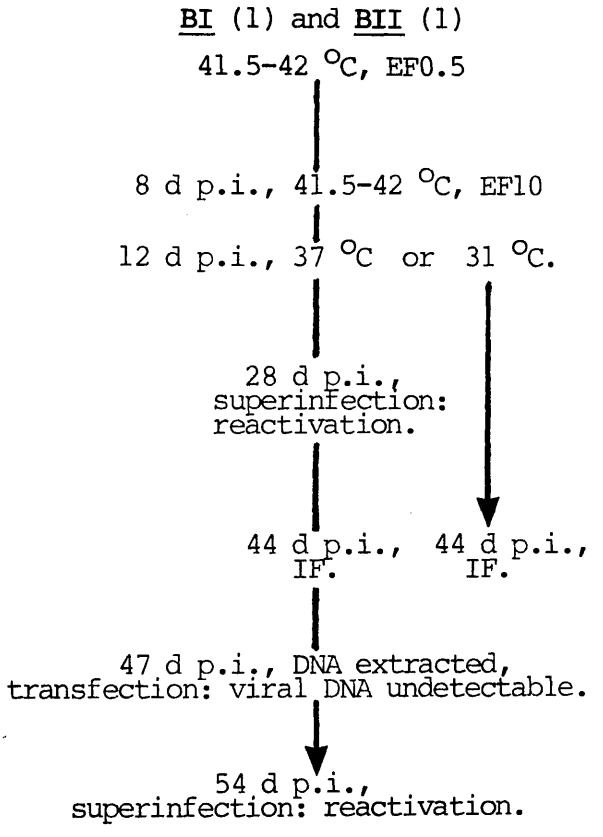
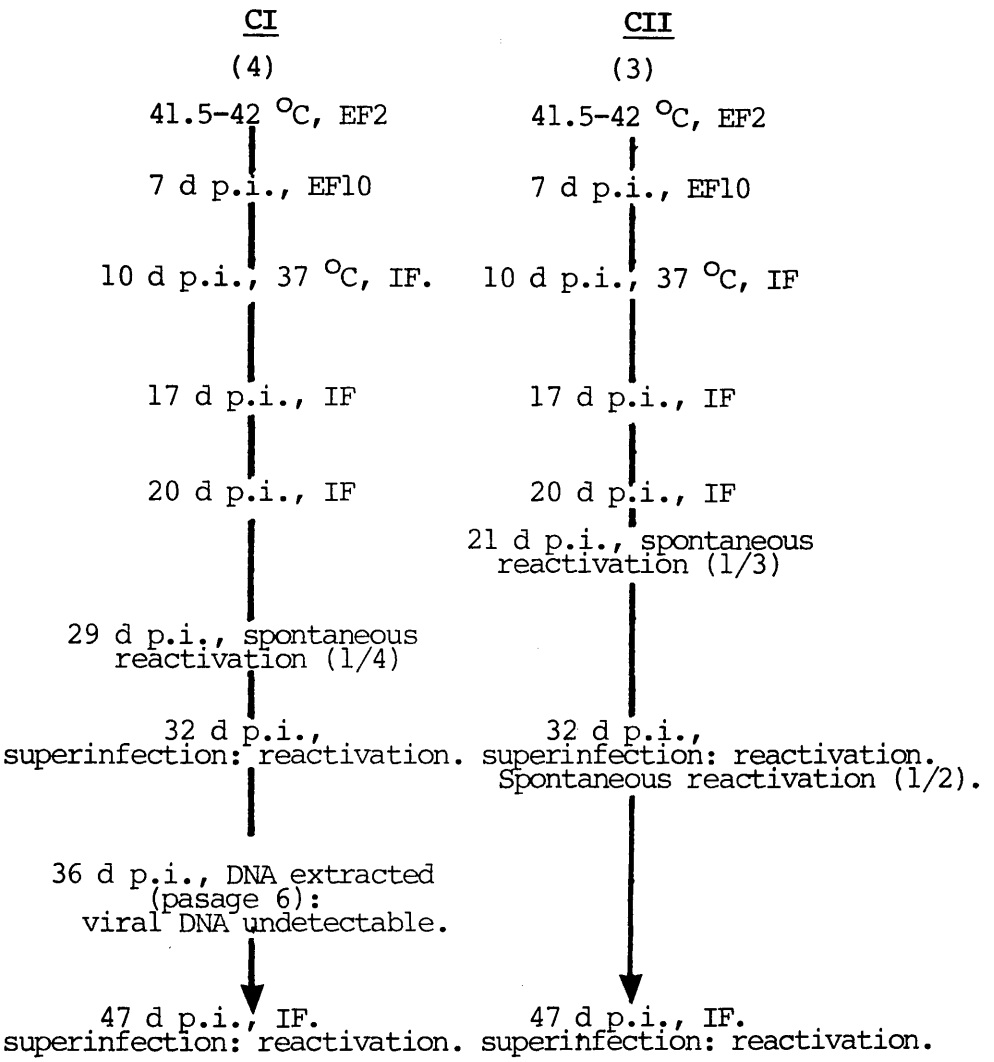

BI (1) and BII (1)
41.5-42 °C, EF0.5
8 d p.i., 41.5-42 °C, EF10
12 d p.i., 37 °C or 31 °C.
28 d p.i., superinfection: reactivation.
44 d p.i., IF.
44 d p.i., IF.
47 d p.i., DNA extracted, transfection: viral DNA undetectable.
54 d p.i., superinfection: reactivation.

Fig. 12.3.3.


CI
(4)
41.5-42 °C, EF2
7 d p.i., EF10
10 d p.i., 37 °C, IF.
17 d p.i., IF
20 d p.i., IF
29 d p.i., spontaneous reactivation (1/4)
32 d p.i., superinfection: reactivation.
36 d p.i., DNA extracted (pasage 6): viral DNA undetectable.
47 d p.i., IF. superinfection: reactivation.
CII
(3)
41.5-42 °C, EF2
7 d p.i., EF10
10 d p.i., 37 °C, IF
17 d p.i., IF
20 d p.i., IF
21 d p.i., spontaneous reactivation (1/3)
32 d p.i., superinfection: reactivation. Spontaneous reactivation (1/2).
47 d p.i., IF. superinfection: reactivation.

cultures. A proportion of cells within these lesions appeared to be unaffected, maintaining a flattened, fibroblastic morphology; meanwhile other cells would coalesce, detach from the substratum and remain in the medium for long periods (over a week), during which infectious virus was detectable in extracts of the detached cells and in the medium. These features are exemplified by the spontaneously reactivated, wt HSV-2-infected culture shown in Fig. 12.1; reactivation occurred at 30 d p.i., and cells were photographed at 36 d p.i. or p.m.i. (**Fig. 12.2**). At 42 d p.i., the medium from this infected culture contained 5 p.f.u./ml, while cell extract contained 45 p.f.u./ml.

Detectable infectious virus and c.p.e. were absent from all infected cultures at the time of restoration to 37 ^{o}C, and cells resumed division at rates comparable to those in mock-infected cultures, and were passaged 7 d after restoration to 37 ^{o}C, and every 3-4 d thereafter. EF10 was the medium used to propagate infected and mock-infected cells, although lowering the concentration of serum in the medium (or the temperature of incubation to 31 ^{o}C, or trypsinization of cells) did not appear to exacerbate infection. Inhibitors of viral replication or antiviral antisera were not employed to suppress productive infection in these cultures. Cultures were observed daily for the appearance of c.p.e., and at the commencement of each experiment, sonicated extracts (comprising 2.10^{6} cells) and overlying medium were assayed for the presence of infectious virus by titration on BHK cells (using one-tenth of the total sample per monolayer of BHK cells) in the absence of human serum.

That most cultures (culture AI/1, and B and C series) carried viral genetic information was indicated by subsequent reactivation of infection in sub-cultures, occurring either spontaneously or following superinfection with temperature-sensitive mutants at a NPT (**Table V**; and see chapter 13); these particular cultures will therefore be referred to as "carrier cultures". Cultures were terminated by use in experiments or due to the spontaneous liberation of detectable infectious virus at various stages during their propagation.

Cultures AI/1, AI/2 and AI/3 (Fig. 12.3.1).

Three cultures of wt HSV-1 infected HeLu cells were derived (along with corresponding mock-infected cultures) from the experiment summarized in **Tables III** and **IV**, using EF10. The first culture, AI/1, was derived from a monolayer of cells which was restored to 37 °C at 10 d p.i.. At 32 d p.i., after 5 passages, AI/1 cells were superinfected with 1 p.f.u. of tsK syn^+ or u.v.-inactivated tsK syn^+ per cell at the NPT of 38.5 °C (the technique of superinfection is described in chapter 13): c.p.e. became evident 1 d after superinfection with tsK syn^+; and c.p.e. (in the form of plaques and †syncitia) developed and receded twice in succession at 4 d and 7 d after superinfection with u.v.-inactivated tsK syn^+. Sonicated, superinfected cells and overlying medium were titrated on HeLu cells at 31 °C and 38.5 °C, and were found to contain non-temperature-sensitive virus:

- 1 d after superinfection with tsK syn^+, 1.4×10^3 p.f.u./ml at 31 °C and 20 p.f.u./ml at 38.5 °C.

- 6 d after superinfection with u.v.-inactivated tsK syn^+, 540 p.f.u./ml at 31 °C and 20 p.f.u./ml at 38.5 °C.

Control cells infected with tsK syn^+ at the NPT failed to produce virus detectable by titration at either 31 °C or 38.5 °C. Neither the reactivated virus progeny nor culture AI/1 were characterized further.

Cultures AI/2 and AI/3 were derived from monolayers which were trypsinized, reseeded and maintained at 41.5-42 °C until 33 d p.i., by which time the cells had undergone three passages. At 41 d p.i., in the absence of c.p.e. and detectable infectious virus, AI/3 cells were superinfected with 1 p.f.u. of tsK or u.v.-inactivated tsK per cell using EF10 or EF0.5, but no infectious virus was detected in any sample up to 3 d after superinfection. At 41 d p.i., cells were harvested from culture AI/3 and trypsinized, and aliquots of 10^5 cells were cocultivated with 2×10^6 BHK cells or HeLu cells in the presence of EF10 for for 7 d. Culture fluids were harvested after 1 d and 2 d, and cells and fluids were harvested after 7 d, but were not found to contain detectable infectious virus, by titration on BHK or HeLu cells in the absence of human serum at 37 °C for 7 d.

† comprising two or three cells.

Cellular DNA was extracted from AI/2 cells at the sixth passage (45 d p.i.), and from AI/3 cells at the seventh passage (50 d p.i.), and analysed for the presence of viral DNA sequences by Southern blot hybridizations with radiolabelled HSV-1 DNA or plasmids bearing viral sequences: HSV-1 fragments co-migrating with virion fragments were not detectable at the level of one copy per cell (chapter 16).

Cultures BI and BII (Fig. 12.3.2).

Cultures were infected with wt HSV-1 (BI) or wt HSV-2 (BII) using EF0.5 and incubated at 41.5-42 ^{o}C. At 8 d p.i., the medium was changed to EF10, and incubation of the cultures was continued at 41.5-42 ^{o}C until 12 d p.i.. The cultures were maintained at 37 ^{o}C or 31 ^{o}C for 6 weeks (until 54 d p.i.), during which time c.p.e. was not observed at either temperature.

At 28 d p.i., 37 ^{o}C-BI and -BII cells were superinfected with heterologous ts mutants (chapter 13), and infection by the original infecting viruses was reactivated from undetectable levels. And at 54 d p.i., BI and BII cells were superinfected with tsK syn and the original infecting viruses were reactivated, from the characteristic plaque morphologies and infected-cell polypeptides induced by the (non-temperature-sensitive) progeny viruses.

At 44 d.p.i., when c.p.e. and infectious virus were undetectable in cultures BI and BII at either 31 ^{o}C or 37 ^{o}C, BI and BII cells were fixed chemically in preparation for analysis by indirect immunofluorescence using rabbit antiviral antiserum and mouse monoclonal antibodies (chapter 14).

At 47 d p.i., DNA was extracted from cultures BI and BII which had been propagated at 37 ^{o}C, and was examined using the calcium phosphate transfection and enhancement procedure of Stow & Wilkie (1978): no viral plaques were obtained by transfection of this (unsonicated) DNA into BHK cells under conditions producing an average of 280 plaques per .05 ug of HSV-1 virion DNA per $2x10^{6}$ cells. That is, the infectivity of DNA from BI and BII cells was less than two viral

genomes per diploid cell. Alternatively, viral DNA which may have been present in BI and BII cells was in a form unable to infect under the conditions of the transfection assays.

Cultures CI and CII (Fig. 12.3.3).

Replicate cultures were infected with wt HSV-1 (CI) or wt HSV-2 (CII) at 41.5-42 ^{o}C using EF2. At 7 d p.i. the medium was changed to EF10, and at 10 d p.i., when no infectious virus was detectable, the single mock-infected-cell (CMI-cell) culture, four CI-cell and three CII-cell cultures were restored to 37^{o}C and propagated in parallel.

CI/1 and CII/1 and CMI cells were chemically fixed, in preparation for immunofluorescence, at 10 d, 17 d and 20 d p.i. or p.m.i. (chapter 14); infectious virus was not detectable in infected-cell-extracts at these times. Reactivation of infection occurred spontaneously in a sub-culture of CI/2-cells at 29 d p.i., and in sub-cultures of CII/2-cells and CII/3-cells at 21 d p.i. and 32 d p.i. (The culture fluid from the latter sub-culture contained, at 39 d p.i., $6x10^{3}$ p.f.u./ml.)

At 32 d p.i. and 47 d p.i., cells from unreactivated cultures CI/1 and CII/2 were superinfected with tsK syn and u.v.-inactivated tsK syn^{+} at the NPT. The original infecting viruses appeared to be reactivated by superinfection with tsK syn, from the characteristic plaque morphologies and infected-cell polypeptides induced by the (non-temperature-sensitive) progeny viruses. Superinfection with u.v.-inactivated tsK syn^{+} failed to induce production of virus detectable by titration at either the PT or the NPT.

At 36 d p.i., cellular DNA was extracted from the CMI-cell culture, from the unreactivated CI-cell culture, CI/1, and from the pooled unreactivated sub-cultures of CI/2-cells and CI/3-cells. This DNA was analysed for the presence of viral sequences by Southern blot hybridizations with radiolabelled HSV-1 DNA or plasmids bearing viral sequences: HSV-1 fragments co-migrating with virion fragments were not detectable at the level of one copy per cell (chapter 16).

In succeeding chapters are described experiments that were performed in order to investigate the nature of infection in these carrier cultures.

13. REACTIVATION BY SUPERINFECTION WITH TS MUTANTS OF HSV AT A NPT.

13.1. Superinfection of Cells from Carrier Cultures with Ts Mutants of HSV at the NPT.

The superinfection of cells with temperature-sensitive mutants of HSV at the NPT is a method which has allowed the detection of viral genetic information in HSV-transformed cells (Macnab & Timbury, 1976; Park et al.,1980; Cameron, 1982; Park & Macnab 1983) and in otherwise uninducible material from latently infected individuals (Brown et al., 1979; Lonsdale et al., 1980). The technique has been used also to reactivate infection in suppressed, HSV-infected cultures of human embryo cells (Colberg-Poley et al., 1979; Nishiyama & Rapp, 1981; Wigdahl et al., 1981; Wigdahl et al., 1982a; Wigdahl et al., 1982b); and it was possible to reactivate infectious virus in carrier cultures by the same means (chapter 12). Superinfections were with a syncytial stock of tsK and heterotypic viruses to permit unambiguous identification of viral progeny according to their characteristic plaque morphologies, their DNA restriction profiles, or their patterns of infected-cell polypeptides.

In one such experiment, cells from cultures BI and BII and mock-infected (BMI) cells were seeded in 35-mm-diameter dishes at a confluent density and superinfected, at 28 days p.i., with a m.o.i. 0.2 of ts viruses, whose characteristics are shown in Table VI. The medium used in these superinfections was EF2. Incubation was at 31 ^{o}C for an absorption period of 1 h, and at 38.5 ^{o}C (NPT) thereafter. Cells were harvested 3 and 6 d later, and extracts were titrated on BHK cells at 38.5 ^{o}C and 31^{o}C (PT). Virus yields, asccording to plaque morphologies, are shown in Table VII.

Infectious virus was undetectable in mock-superinfected cultures, and in BI, BII or BMI cells superinfected with u.v.-inactivated tsK syn^{+}, tsK TK^{-} or ts13. BMI, BI and BII cells that were superinfected with ts9 and ts1213 produced ts^{+}, i.e. revertant viruses, which either arose during superinfection or were present as contaminants in the original virus stocks. Plaques of the morphology of the original infecting viruses developed within 3 d of superinfection of BI cells with tsK syn or ts1213, and of BII cells with

Table VI: Characteristics of ts mutants employed in the superinfection of BI and BII cells.

Serotype	Mutant	Defect
HSV-1	tsK syn	Defective in immediate early viral polypeptide Vmw IE 175 (Watson & Clements 1980; Preston, 1979a & b), and of syncytial plaque morphology.
	tsK TK^-	Mutant tsK from which the gene encoding the enzyme thymidine kinase is deleted.
	u.v.-tsK	Ultraviolet-light-inactivated tsK.
	tsl213	Ts defect in uncoating (Personal communication, C. M. Preston).
HSV-2	tsl3	Lethal mutation affecting the stability of the virion and a second, non-lethal ts mutation in the viral exonuclease (Moss et al., 1979). (mutant x in Timbury & Subak-Sharpe, 1973)
	ts9	Ts defect in uncoating. (mutant 25 in Timbury & Subak-Sharpe, 1973)

Table VII: Titres†, according to plaque morphologies, of progeny viruses in superinfected-cell extracts.

	38.5 °C (p.f.u./ml)	31 °C (p.f.u./ml)
MI x tsK syn		
3 days	<5	<5
6 days	<5	<5
BI x tsK syn		
3 days	1.0×10^7 HSV-1 syn^+	1.0×10^7 HSV-1 syn^+ 0.6×10^5 HSV-1 syn
6 days	<5	<5
BII x tsK syn		
3 days	<5	<5
6 days	2.25×10^3 HSV-2 syn^+	2.25×10^3 HSV-2 syn^+
MI x ts9		
3 days	85 HSV-2 syn^+	45 HSV-2 syn^+
6 days	<5	<5
BI x ts9		
3 days	150 HSV-2 syn^+ 50 HSV-1 syn^+	100 HSV-2/HSV-1 syn^+
6 days	<5	<5
MI x tsl213		
3 days	<5	<5
6 days	1.9×10^5 HSV-1 syn^+	2.2×10^5 HSV-1 syn^+
BII x tsl213		
3 days	2.0×10^5 HSV-1 syn^+ 5.0×10^4 HSV-2 syn^+	3.2×10^5 HSV-1 syn^+ 5.0×10^5 HSV-2 syn^+
6 days	infectious virus was detected, but not characterized.	
MI x u.v.-tsK	<5, at 3 and 6 days.	<5, at 3 and 6 days.
BI x u.v.-tsK	- do -	- do -
BII x u.v.-tsK	- do -	- do -
MI x tsK TK^-	- do -	- do -
BI x tsK TK^-	- do -	- do -
BII x tsK TK^-	- do -	- do -
MI x tsl3	- do -	- do -
BI x tsl3	- do -	- do -

† Cell extracts were titrated on BHK C13 cells and incubated at 38.5 °C or 31 °C for 3 days

* virus plaques were observed to develop in the superinfected culture, but infectious virus was not detected.

tsK syn or ts9; the growth of plaques was restricted after about 4 d, when the cultures showed signs of regeneration. Infectious virus was detected only in one of duplicate BI-cell or BII-cell cultures superinfected with tsK syn, but in both duplicate BII-cell cultures superinfected with tsl213. One BI-cell culture that was superinfected with tsK syn demonstrated plaque-like lesions in the absence of detectable infectious virus; thus, the development of c.p.e. need not signify the presence of detectable infectious virus.

These results indicated that BI-cell and BII-cell cultures carried viral genetic information, and were accountable by two hypotheses: firstly, that recombination occurred between the genomes of superinfecting viruses and resident viral DNA, leading to the acquisition of the ts^+ character and altered plaque morphology by progeny viruses; and secondly, that superinfection caused reactivation of infection by the original infecting virus. In order to discriminate between these possibilities, progeny viruses recovered from intertypic superinfections of BI cells with ts9, and of BII cells with tsK syn and tsl213 were characterized. Cell extracts were re-titrated on BHK cells at 38.5 ^{o}C, the cells were overlaid with agar, and viruses were selected, as summarized below, according to their plaque morphologies. Virus isolates were propagated in BHK cells at 31 ^{o}C to provide stocks: HSV-2-like syn^+-morphology isolates gave an average yield of 10^7 p.f.u./ml (burst size 10 p.f.u./cell), and HSV-1-like, syn^+-morphology isolates 10^8 p.f.u./ml (burst size 100 p.f.u./cell).

(i) BI x tsK syn.
Fourteen HSV-1-like, syn^+-morphology plaques were propagated in BHK cells; yields ranged from $4x10^8$ to $3x10^8$ p.f.u./ml, with an average of $1.3x10^8$ p.f.u./ml.

(ii) BII x tsK syn.
Twenty-seven HSV-2-like, syn^+-morphology plaques were propagated in BHK cells; yields varied from $0.8x10^8$ to $2.4x10^7$ p.f.u./ml, with an average of $1.6x10^7$ p.f.u./ml.

(iii) BI x ts9.
One HSV-1-like, syn^+-morphology plaque yielded $4.6x10^7$ p.f.u./ml.

(iv) BII x tsl213.
Two HSV-2-like, syn^+-morphology plaques yielded 10^8 p.f.u./ml, and the isolate which was characterized further contained $0.3x10^8$ p.f.u. of HSV-1-like, syn^+ p.f.u./ml .

13.2. Classification of Serotypes of Virus Isolates from Analysis of Infected-Cell Polypeptides.

The virus isolates were classified as either HSV-1 or HSV-2 according to the pattern of viral polypeptides whose synthesis they induced in infected BHK cells (**Fig. 13.2**).

(i) BII x tsK syn.
Twenty-six of the twenty-seven HSV-2-like, syn^+-morphology isolates (tracks 4 to 9) induced synthesis of the HSV-2 pattern of viral polypeptides (track 3) in infected cells. The remaining isolate, however, induced syncytial c.p.e. and synthesis of mainly the HSV-1 pattern of viral polypeptides (track 10) in infected cells.

(ii) BI x ts9.
Cells infected with the HSV-1-like, syn^+-morpholgy isolate (track 13) induced the HSV-1 pattern of viral polypeptides (track 14).

(iii) BII x tsl213.
Cells infected with one HSV-2-like, syn^+-morphology isolate (track 11) synthesized mainly the HSV-2 pattern of viral polypeptides (with detectable quantities of HSV-1 polypeptides such as Vmw 117, Vmw 28, Vmw 22 and Vmw 21), whereas cells infected with the remaining isolate produced mainly HSV-1, and traces of HSV-2, polypeptides.

Thus it appeared that superinfection of BI and BII cells with heterologous ts mutants at the NPT had caused productive infection by the original infecting viruses to be reactivated.

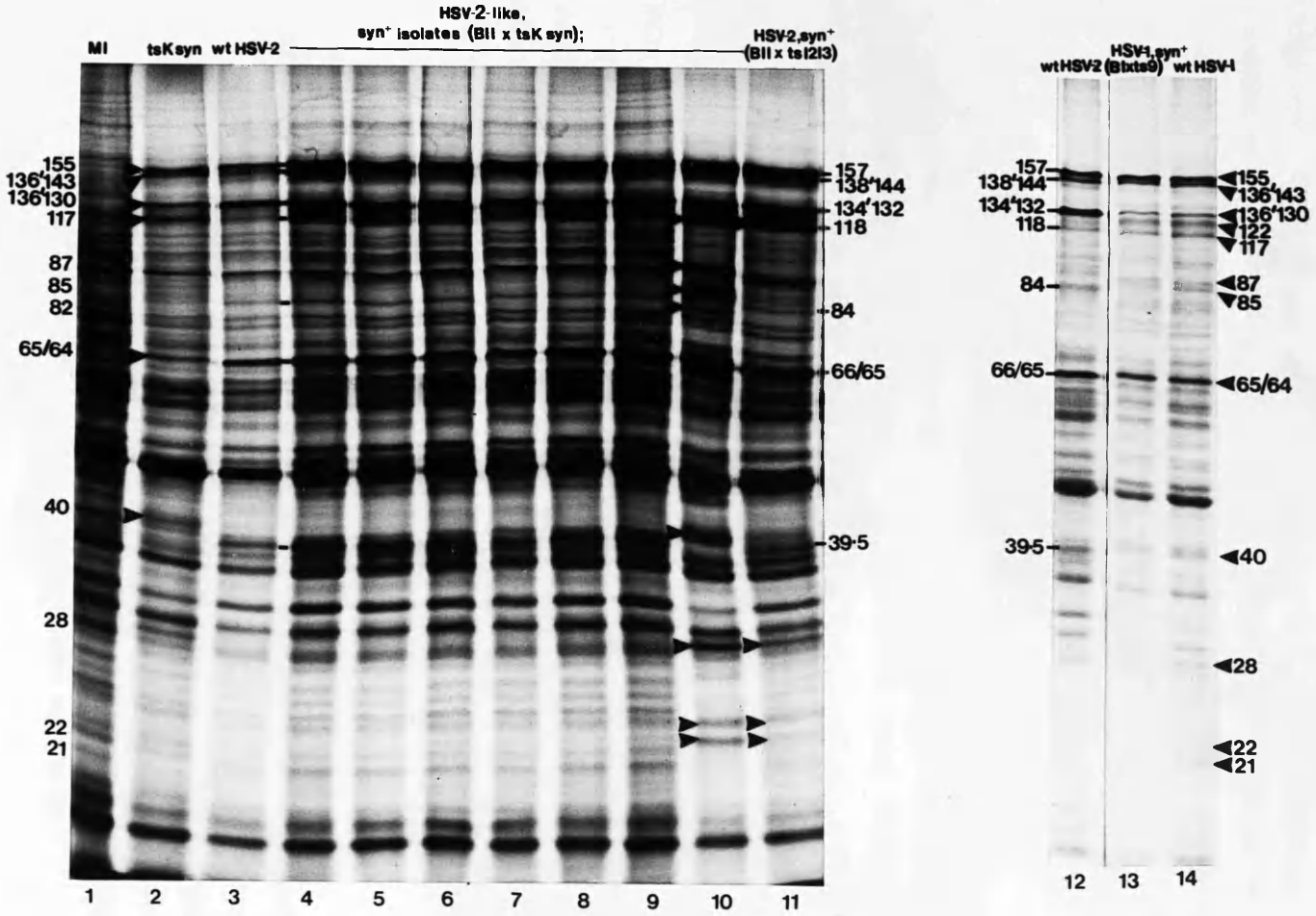
13.3. Analysis of Genotypes of Virus Isolates.

In order to confirm the serotypes of reactivated virus isolates, as

Fig. 13.2. Polypeptide synthesis in BHK cells infected with virus isolates obtained by superinfection of BI and BII cells with heterologous ts mutants at a NPT.

BHK cells were infected (5 p.f.u./cell) at 31 °C with the parental stock of wt HSV-2, tsK syn or virus isolates obtained at NPT following superinfection of BI with tsK syn or tsl213, or following superinfection of BI cells with ts9. At 6 h p.i., cultures were pulse-labelled with [^{35}S]methionine, and radiolabelled polypeptides in infected-cell extracts were resolved by SDS-PAGE.

Track 1, mock-infected culture; track 2, culture infected with tsK syn; track 3, infected with wt HSV-2; tracks 4 to 10, HSV-2-like, syn$^+$-morphology isolates from the superinfection of BII cells with tsK syn; track 11, HSV-2-like, syn$^+$-morphlogy isolate from the superinfection of BII cells with tsl213; track 12, wt HSV-2; track 13, HSV-1-like, syn$^+$-morphology isolate from the superinfection of BI cells with ts9; track 14, wt HSV-1. HSV-1 polypeptides are indicated by arrowheads, to the left of tracks 2, 10 and 11, and to the right of track 14. HSV-2 polypeptides are indicated by bars, to the right of tracks 3 and 11, and to the left of track 12.


MI
tsKsyn
wt HSV-2
HSV-2-like,
syn+ isolates (BII x tsK syn);
HSV-2,syn+
(BII x ts12I3)
155
136'143
136'130
117
87
85
82
65/64
40
28
22
21
157
138'144
134'132
118
84
66/65
39·5
1
2
3
4
5
6
7
8
9
10
11
HSV-1,syn+
wt HSV-2
(BIxts9)
wt HSV-I
157
138'144
134'132
118
84
66/65
39·5
155,
136'143
136'130
122
117
87
85
65/64
40
28
22
21
12
13
14

deduced from their plaque morphologies and the pattern of viral polypeptides whose synthesis they induced in infected cells, their genotypes were characterized by restriction enzyme analysis.

BHK cells were infected with virus isolates and radiolabelled with [^{32}P]inorganic phosphate, and total cellular DNA was purified from infected-cell extracts by the method of Lonsdale (1979). The radiolabelled DNA was digested with restriction enzymes BamHI, EcoRI or HpaII, and the restriction fragments generated were fractionated by agarose gel electrophoresis. The BamHI restrictions sites in the genomes of HSV-1 and HSV-2 are illustrated in **Fig. 13.3.1** (after Davison & Wilkie, 1981).

Fig. 13.3.2 shows BamHI restriction profiles of DNA from a random selection of HSV-2-like, syn$^+$ isolates obtained from the superinfection of BII cells with tsK syn (tracks 2 to 14). The restriction profile for the original, plaque-purified stock of wt HSV-2 is shown in track 1, and that of the superinfecting virus, tsK syn, in track 15. From the DNA of HSV-2-like, syn$^+$ isolates were obtained entire HSV-2 cleavage patterns within the limits of analysis, proving that progeny viruses were derived from the original infecting virus. The profiles for reactivated isolates were uncontaminated with HSV-1 fragments; material migrating ahead of fragments BamHI a,b may have represented products of partial digestion, or contaminating cellular DNA.

Concerning the mobilities of individual fragments in the restriction profiles for these reactivated HSV-2 isolates, it is notable that BamHI g and BamHI g´ were the only fragments to show variation in electrophoretic mobility, corresponding to an increase or decrease in size, relative to fragments for the parental stock, of approximately 400 bp. Fragment BamHI g spans the joint between the long and short regions of the viral genome (**Fig. 13.3.1**); minor populations of viral genomes in plaque-purified stocks of HSV-2 (HG52) possess reduplications of the a sequence within BamHI g (Davison & Wilkie, 1981), and such a population was represented by the slight band designated BamHI g´. In other words, the restriction profiles of viral DNA from these reactivated HSV-2 isolates were largely homogeneous with respect to fragment mobility, apart from variation in BamHI g. Examination of EcoRI

Fig. 13.3.1. The position of BamHI restriction fragments in the genomes of HSV-1 and HSV-2 (after Davison & Wilkie, 1981), and of EcoRI fragments in the genome of HSV-1 (after Wilkie et al., 1978). HSV-1 fragments are shown above the genome, and HSV-2 fragments below the genome. Fractional genome units also are indicated.

Fig. 13.3.2. BamHI restriction profiles of DNA from HSV-2-like, syn^+ isolates.

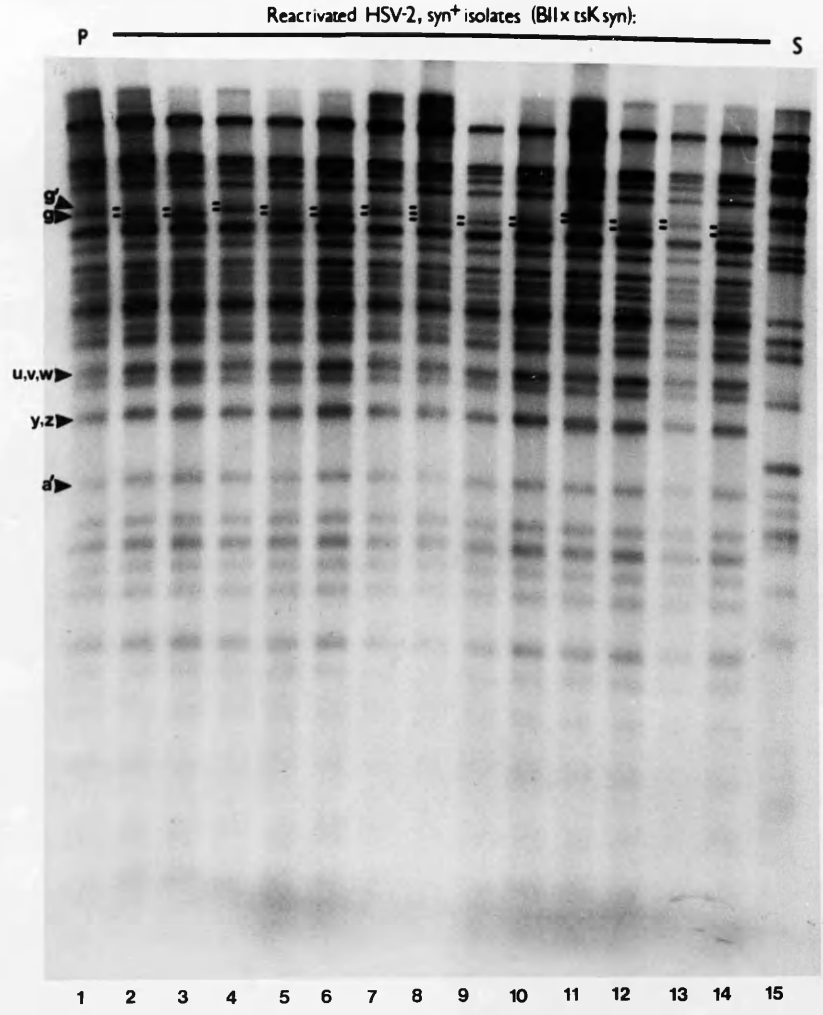
BHK cells were infected at 31 ^oC with the (plaque-purified) parental stock of wt HSV-2, with tsK syn, or with HSV-2-like, syn^+ isolates obtained from the superinfection of BII cells with tsK syn. Infected-cell DNA was radiolabelled with [^{32}P]inorganic phosphate (400 uCi/ml), and extracted according to the method of Lonsdale (1979). The radiolabelled DNA was digested with retriction enzyme BamHI, and restriction fragments were fractionated by electrophoresis through a 1.2%-agarose gel.

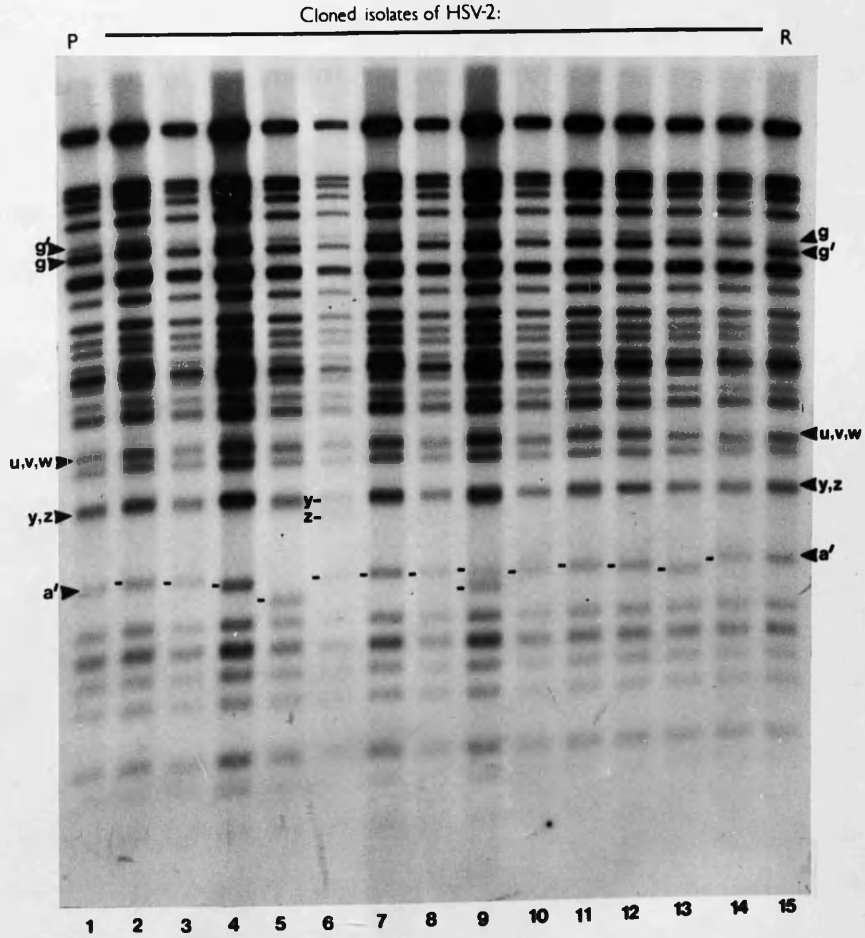
Track 1, parental stock (P) of wt HSV-2; tracks 2 to 14, HSV-2-like, syn^+ isolates; track 15, superinfecting virus (S), tsK syn.

Fig. 13.3.3. BamHI restriction profiles of DNA from isolates cloned from the parental stock of HSV-2.

Isolates were cloned from the parental stock of wt HSV-2 in BHK cells at 37 ^oC. Infections, radiolabelling, and restriction analysis of DNA were as described in the legend to **Fig. 13.3.2.**

Track 1, parental stock (P) of wt HSV-2; tracks 2 to 14, cloned isolates from the parental stock of wt HSV-2; track 15, a reactivated (R) HSV-2 isolate.


Reactivated HSV-2, syn+ isolates (BIIx tsK syn):
P
S
g'
g
u,v,w
y,z
a'
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


Cloned isolates of HSV-2:
P
R
g'
g
u,v,w
y,z
a'
y-
z-
g
g'
u,v,w
y,z
a'
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

and HpaII restriction profiles of the viral DNA revealed no differences in fragment mobility, perhaps owing to the large size of the restriction fragments generated by these enzymes, apart from a slight variation in EcoRI m (results not shown), a fragment which spans TR_S and a portion of U_S.

Fig. 13.3.3 shows BamHI restriction profiles for isolates cloned from the parental stock at 37 °C and propagated in parallel with the reactivated HSV-2 isolates (tracks 2 to 14), from the parental virus stock (track 1), and from a single reactivated HSV-2 isolate (track 15). In keeping with the findings of Davison and Wilkie (1981), it is apparent that between restriction for isolates cloned from the parental stock, there were variations in the mobilities of BamHI z (track 6) and BamHI a′ (which span the junctions between U_S and TR_S/IR_S), but not of BamHI g. Furthermore, when the parental virus stock was passaged once (m.o.i. 0.01) in HeLu cells at 37 °C prior to plaque purification and propagation in BHK cells (**Fig. 13.3.4**, tracks 2 to 14), BamHI g remained almost invariant (slight variation being observed only for the isolate shown in track 7) while pronounced variation occurred in BamHI u,v,w, BamHI z and BamHI a′.

Fig. 13.3.5, track 2 shows the BamHI restriction profile of DNA from the syncytial isolate obtained from the superinfection of BII cells with tsK syn. This profile consisted mainly of HSV-1 fragments (cf. track 3), with minor contamination from HSV-2 fragments (cf. track 1), indicating that the isolate was derived from the superinfecting virus. The HSV-1 fragment BamHI x displayed an increase in electrophoretic mobility, compared to the corresponding fragment for tsK syn, equivalent to a decrease in size of roughly 100 bp. BamHI x spans the junction between U_S and TR_S/IR_S, and shows size variation between cloned isolates of HSV-1 (Lonsdale et al., 1980; Davison & Wilkie, 1981). However, variation in BamHI x is usually minor compared to, for example, the variability demonstrated by terminal and joint fragments BamHI k, BamHI q and BamHI s (Davison & Wilkie, 1981), of which only BamHI s showed size heterogeneity in these restriction profiles (tracks 1 and 2). The variation in BamHI x that was observed for this syncytial isolate therefore may have arisen from recombination between this region of the genome of the superinfecting mutant, tsK syn, with the resident virus to generate an intratypic, ts^+ recombinant. During propagation

Fig. 13.3.4. BamHI restriction profiles of DNA from cloned isolates from the passaged parental stock of HSV-2.

The parental stock of wt HSV-2 was passaged once (m.o.i. 0.01) in HeLu cells at 37 °C prior to plaque purification and propagation in BHK cells. Infections, radiolabelling, and restriction analysis of DNA were as described in the legend to **Fig. 13.3.2.**

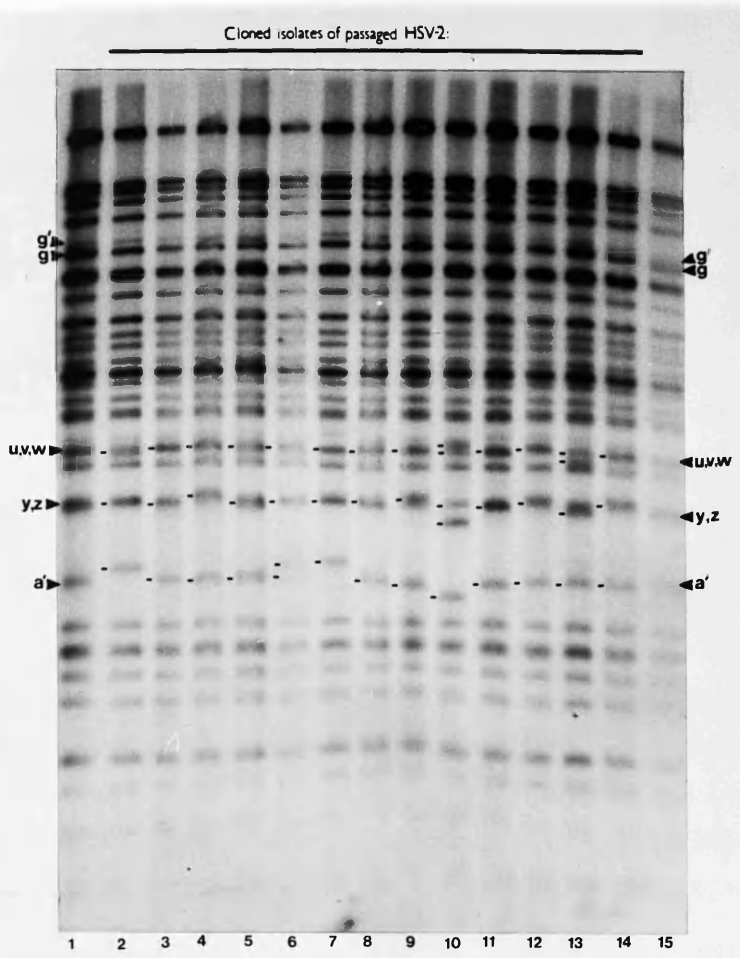
Track 1, parental stock of wt HSV-2; tracks 2 to 14, cloned isolates from the passaged parental stock of wt HSV-2; track 15, a reactivated HSV-2 isolate.

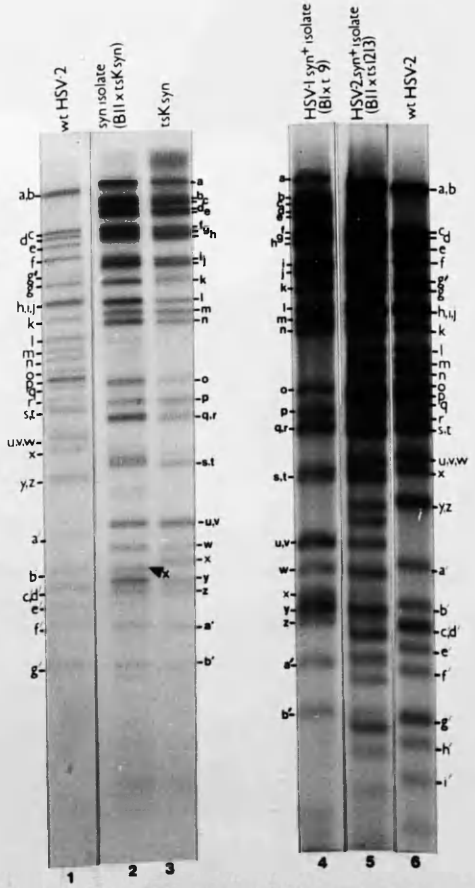
Fig. 13.3.5. BamHI restriction profiles of DNA from a syn isolate, and from HSV-2-like or HSV-1-like, syn^+ isolates.

Infections, radiolabelling, and restriction analysis of DNA were as described in the legend to **Fig. 13.3.2.**

Track 1, wt HSV-2; track 2, syn isolate obtained from superinfection of BII cells with tsK syn; track 3, tsK syn. Track 4, HSV-1-like, syn^+ isolate obtained from the superinfection of BI cells with ts9; track 5, HSV-2-like, syn^+ isolate obtained from the superinfection of BII cells with tsl213; track 6, wt HSV-2.

Restriction fragments of HSV-1 DNA are indicated to the right of track 3, and to the left of track 4; and restriction fragments of HSV-2 DNA are indicated to the left of track 1, and to the right of track 6. The restriction fragment corresponding to Bam HI x, in the DNA of the syn isolate, is indicated by an arrowhead to the right of track 2.


Cloned isolates of passaged HSV-2:
g'
g
u,v,w
y,z
a'
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


wt HSV-2
syn isolate (BII x tsK syn)
tsK syn
HSV1 syn+ isolate (BI x t 9)
HSV2 syn+ isolate (BII x ts123)
wt HSV-2
1 2 3 4 5 6

in BHK cells, such a recombinant may have outgrown the HSV-2-like, syn^+ virus originally selected. (The mutation in tsK has been identified as a single base alteration located in the coding sequences for Vmw IE 175, in BamHI y (Murchie, 1982), which is an adjacent fragment to BamHI x).

The DNA from the virus isolates obtained from the superinfection of BI cells with ts9 (track 4) demonstrated an HSV-1 BamHI restriction profile, in agreement with its classification based on plaque morphology and infected-cell polypeptides. Thus superinfection of BI cells with ts9 caused reactivation of infection by the original infecting virus. The isolate obtained from the superinfection of BII cells with ts1213 (track 5) demonstrated both HSV-1 and HSV-2 BamHI restriction fragments (with BamHI b, c, d, e and u,v from HSV-1, and BamHI 1, m and n from HSV-2), and may have represented a mixture of HSV-1 and HSV-2 genomes and/or a recombinant. Superinfection of BII cells with ts1213 therefore caused recovery of genetic information of the original infecting virus.

14. IMMUNOFLUORESCENCE STUDIES OF CARRIER CULTURES.

In order to investigate the possibility that cells in unreactivated carrier cultures contained viral antigens, samples of cells were taken at various times during sub-culture, seeded onto coverslips and fixed chemically in preparation for examination by indirect immunofluorescence (IIF). Each suspension was assayed in duplicate for the presence of infectious virus by titration of sonicated cell-extracts on BHK cells in the absence of human serum at 37 °C for 5 d, but infectious virus was not detected by this means in any of the samples tested.

14.1. Immunofluorescence Studies of BI and BII Cells, Using Rabbit Antiviral Antisera.

Cultures BI and BII had been established by infection with wt HSV-1 and wt HSV-2, respectively, and incubation at 41.5-42 °C until 12 d p.i., when the temperature of incubation was lowered to either 37 °C or 31 °C (chapter 12). BI and BII cells that were propagated at 37 °C (BI/37 °C and BII/37 °C cells), and which had undergone four passages, and BI cells that were maintained at 31 °C meanwhile (BI/31 °C cells), were processed at 44 d p.i.. Uninfected HeLu cells were used as controls.

A general anti-HSV antiserum and a pre-immune serum were provided by Dr. J. Macnab. The antiserum had been raised by immunizing New Zealand White rabbits with BHK cells that had been infected with the HSV-2 mutant, tsl, at a NPT for 24 h; this antiserum reacts with most HSV-2 and many HSV-1 polypeptides (Dr. J. Macnab, personal communication). In two experiments, which used 1/20 (**Fig. 14.1.1**) and and 1/10 (results not shown) dilutions of the stock solutions, BI/31 °C, BI/37 °C and BII/37 °C cells showed cytoplasmic fluorescence (**f**, **g** and **h**, respectively), compared with uninfected cells (**e**). It was noted that BII/37 °C cells were considerably more immunoreactive with preimmune rabbit serum than uninfected cells (**a**), showing strong nuclear fluorescence (**d**).

Table VIII: Mouse monoclonal antibodies.

Designation	Specificity (HSV-1)	Function of HSV-1-polypeptide	Equivalent HSV-2 polypeptide
Immediate early polypeptides:			
1098	Vmw IE 175	Permits transcription of non-IE viral genes (Preston, 1979a, b; Watson & Clements, 1980).	Vmw IE 182
1026	Vmw IE 136´143	Large subunit of ribonucleotide reductase (Dutia, 1983).	Vmw 138´144
1089	Vmw IE 63		
Early polypeptide:			
147*	Vmw 136´130	Major DNA binding protein (Godowski and Knipe, 1983.)	
Late polypeptides and virion components:			
91	Vmw 155	Major capsid polypeptide	
844	Vmw 65	Tegument polypeptide, (Campbell et al., 1984).	Vmw 66
60	Vmw 33		
Glycoproteins (Spear, 1976; Norrild et al., 1980; Pereira et al., 1982):			
1049	gA/B	Cell fusion (Manservigi et al., 1977).	
1001*	gC	"Social behaviour" of infected cells (Ruyechan et al., 1979).	
1140	gD	Cellular adhesion Norrild et al., 1983).	
These glycoproteins may function as a target for for immunocytolysis (Norrild et al., 1980).			

* Monoclonal antibodies which were found to be type-1-specific when tested against productively wt HSV-2-infected cells.

Table IX: Immunofluorescence in CI and CII cells stained with monoclonal antibodies against viral polyeptides.

	10 d p.i.	17 d p.i.	27 d p.i.	47 d p.i.
Control Ascites.				
CI	weak	neg.	neg.	neg.
CII	weak	neg.	neg.	-
Vmw IE 175.				
CI	perinuclear, cyto.	neg.	neg.	neg.
CII	perinuclear, cyto.	neg.	neg.	-
Vmw IE 63.				
CI	nuclear, cyto.	weak.	neg.	-
CII	nuclear, cyto.	neg.	neg.	-
Vmw 136´130.				
CI	nuclear	nuclear	neg.	neg.
CII	neg.	neg.	neg.	-
Vmw IE 136´143.				
CI	perinuclear, cyto.	neg.	neg.	-
CII	perinuclear, cyto.	neg.	neg.	-
Vmw 155.				
CI	nuclear, cyto.	neg.	neg.	-
CII	nuclear, cyto.	neg.	neg.	-
Vmw 65.				
CI	nuclear, cyto.	neg.	neg.	neg.
CII	nuclear, cyto.	cyto.	neg.	-
Vmw 33.				
CI	nuclear, cyto.	neg.	neg.	neg.
CII	nuclear, cyto.	neg.	-	-
gA/gB.				
CI	nuclear membrane	neg.	neg.	neg.
CII	weak	neg.	neg.	-
gC.				
CI	-	neg.	neg.	-
CII	-	neg.	neg.	-
gD.				
CI	plasma memb.	neg.	neg.	neg.
CII	plasma memb.	neg.	neg.	-

14.2. Immunofluorescence Studies of CI/1 and CII/1 Cells, using Mouse Monoclonal Antibodies against HSV-1 Polypeptides.

Cultures CI/1 and CII/1 had been established by infection with wt HSV-1 or wt HSV-2 and incubation at 41.5-42 °C until 10 d p.i., when the temperature of incubation was lowered to 37 °C (chapter 12). Cells were processed at 10 d p.i. (after the period of incubation at 41.5-42 °C), 17 d p.i., 27 d p.i. and 47 d p.i., and were examined for the presence of HSV-specific antigens by indirect immunofluorescence using mouse monoclonal antibodies against various HSV-1 polypeptides (**Table VIII**): immediate-early polypeptides Vmw IE 175, Vmw IE 136'143 and Vmw IE 63; early polypeptide Vmw 136'130; late polypeptides and virion components Vmw 155, Vmw 65 and Vmw 33; and glycoproteins gA/B and gD. The specificities of the antisera were tested by reaction with productively infected HeLu cells, processed 6 h p.i. with wt HSV-1 or HSV-2 (m.o.i. of 5) at 38.5 °C, and all but the antibody against Vmw 136'130 were found to be type common.

The results of immunofluorescent staining are summarized in **Table IX**, and representative results are shown in **Fig.'s 14.2.1** to **14.2.8.** Whereas mock-infected cells showed negligible or non-specific fluorescence throughout (**Fig. 14.2.1 a,** and **14.2.2, a**), all or most CI/1 cells and CII/1 cells processed at 10 d p.i. showed fluorescence approaching or exceeding the levels in productively HSV-infected cells for all of the viral polypeptides against which the tested monoclonal antibodies were directed. (Only the monoclonal antibody against Vmw 136'130 failed to cause fluorescence in CII/1 cells processed at 10 d p.i..)

The intracellular distributions of fluorescence were similar for all CI/1 and CII/1 cells processed at 10 d p.i., and resembled the distributions in productively infected cells when stained with antibodies against Vmw IE 175 (**Fig. 14.2.6**; nuclear and perinuclear), Vmw IE 63 (**Fig. 14.2.7**; nuclear and cytoplasmic), Vmw IE 136'143 (**Fig. 14.2.8**; nuclear and perinuclear), gA/B (not shown) and gD (**Fig. 14.2.5**; plasma membrane). The nuclear and perinuclear distribution of fluorescence for Vmw IE 175 was in keeping with observations that Vmw IE 175 in wt HSV-1-infected CEF and BHK cells is associated with nuclei or nuclear-bound structures (chapter 7); and that Vmw IE 175 may interact

Fig. 14.1. Indirect immunofluorescent staining of fixed BI and BII cells using rabbit anti-HSV antiserum. Uninfected HeLu cells (**a, e**), BI/31 °C cells (**b, f**), BI/37 °C cells (**c, g**) and BII/37 °C cells (**d, h**) stained using pre-immune serum (**a** to **d**) or rabbit anti-HSV antiviral antiserum (**e** to **h**). Magnifications: **f** and **g**, x400; **a** to **e** and **h**, x200.

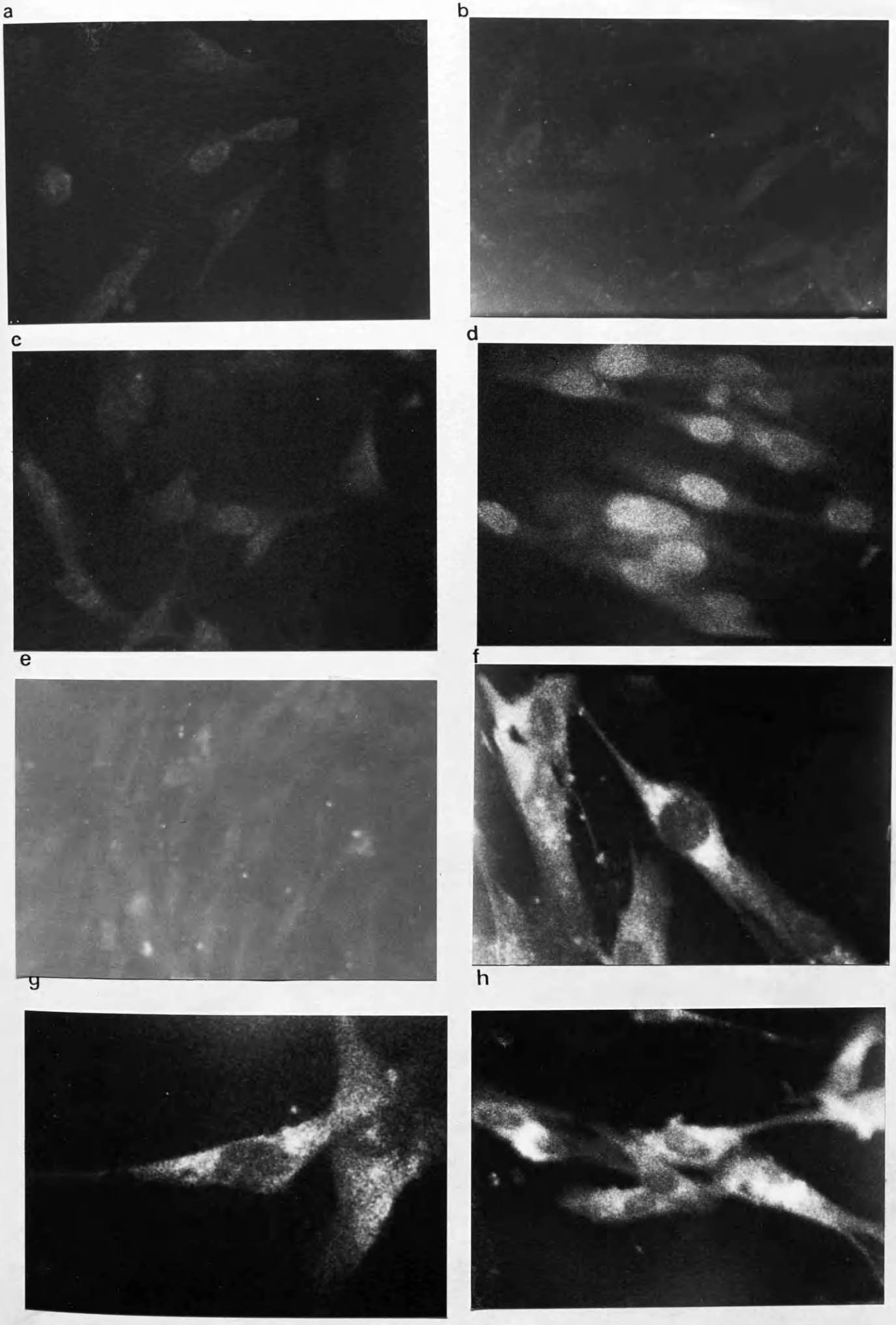
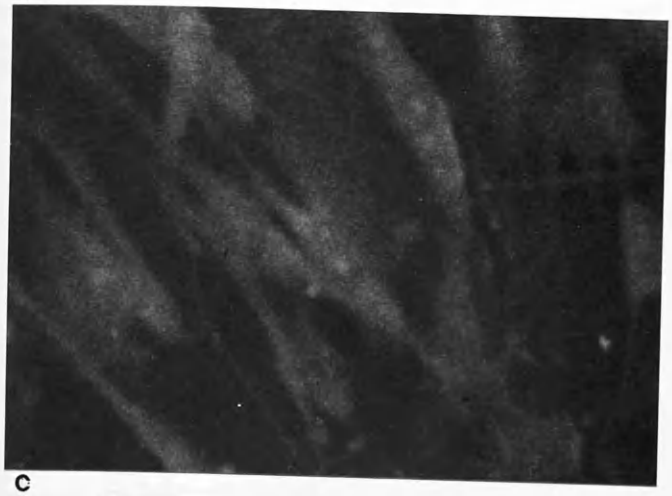

a
b
c
d
e
f
g
h

Fig. 14.2.1. Indirect immunofluorescent staining of fixed CI/1 and CII/1 cells with mouse monoclonal antibody against HSV-1 polypeptide Vmw 65: (a) mock-infected cells, processed at 10 d p.m.i.; (b) CI/1 cells, processed at 10 d p.i.; (c) CI/1 cells, processed at 17 d p.i.; (d) productively wt HSV-1-infected cells; (e) CII/1 cells, processed at 17 d p.i.. Magnifications: c x25; a to b and d to e, x100.

a



b

c

d

e

Fig. 14.2.2. Indirect immunofluorescent staining of fixed CI/1 cells with mouse monoclonal antibody against HSV-1 polypeptide Vmw 136/130: (a) mock-infected cells, processed at 10 d p.m.i.; (b) CI/1 cells, processed at 10 d p.i.; (c) CI/1 cells, processed at 17 d p.i.; (d) productively wt HSV-1-infected cells. Magnifications: c, x25; a, b and d, x100.

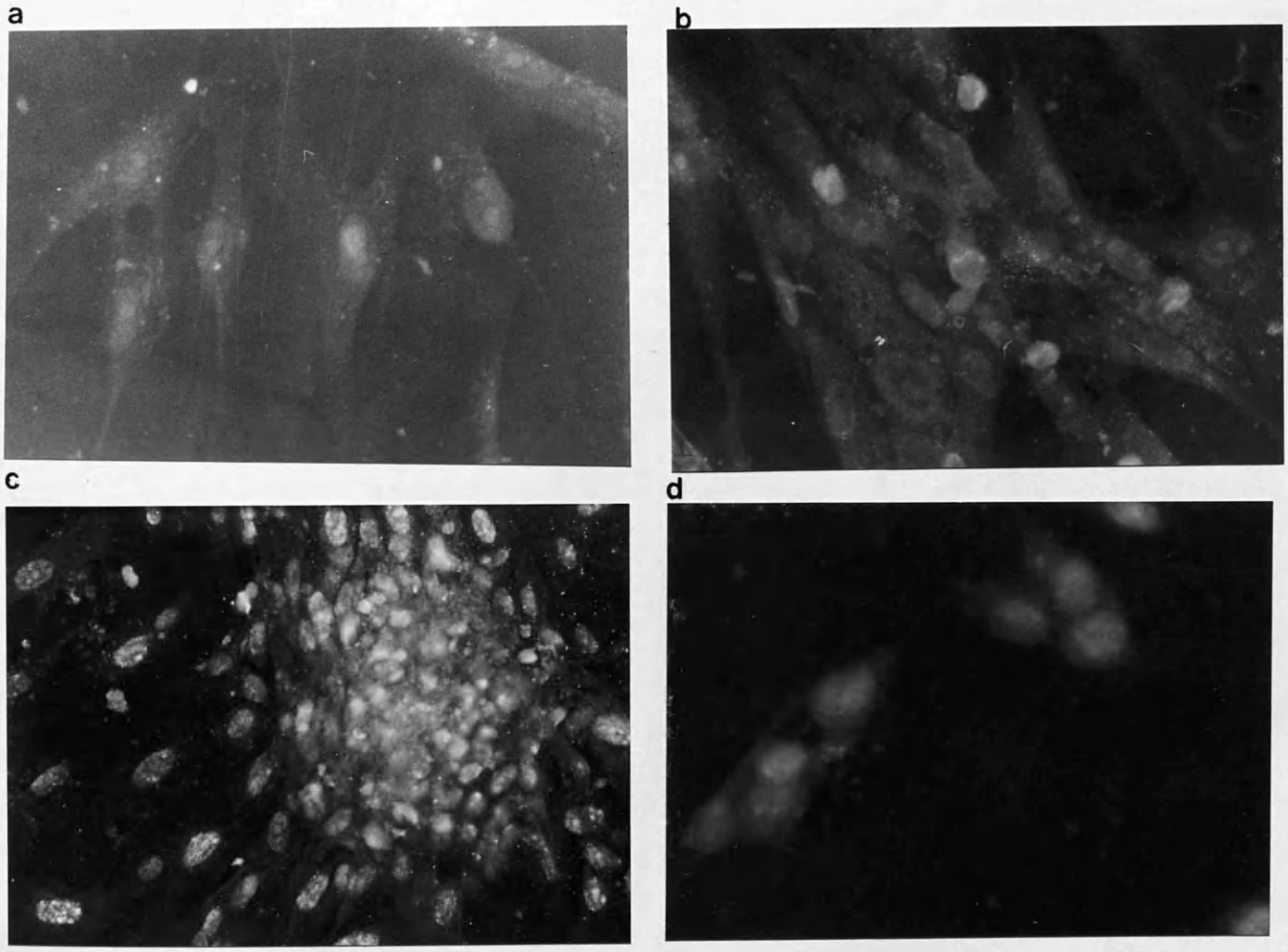

a
b
c
d

Fig. 14.2.3. Indirect immunofluorescent staining of fixed CI/1 cells with mouse monoclonal antibody against HSV-1 polypeptide Vmw 155: (a) CI/1 cells processed at 10 d p.i.; (b) productively wt HSV-1-infected cells. Magnifications: a, x100; b, x64.

a

b

Fig. 14.2.4. Indirect immunofluorescent staining of fixed CI/1 cells with mouse monoclonal antibody against HSV-1 polypeptide Vmw 33: (a) CI/1 cells processed at 10 d p.i.; (b) productively wt HSV-1-infected cells. Magnifications: x100.

a

b

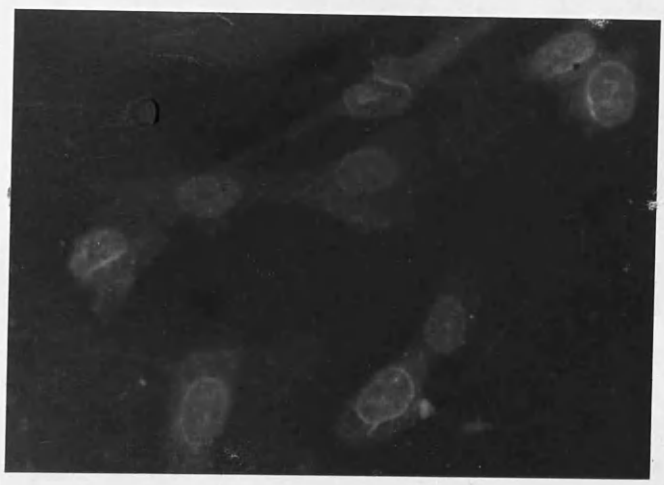


Fig. 14.2.5. Indirect immunofluorescent staining of fixed CI/1 cells with mouse monoclonal antibody against HSV-1 glycoprotein gD: (a) CI/1 cells processed at 10 d p.i.; (b) productively wt HSV-1-infected cells. Magnifications: a, x100; b, x64.

a

b

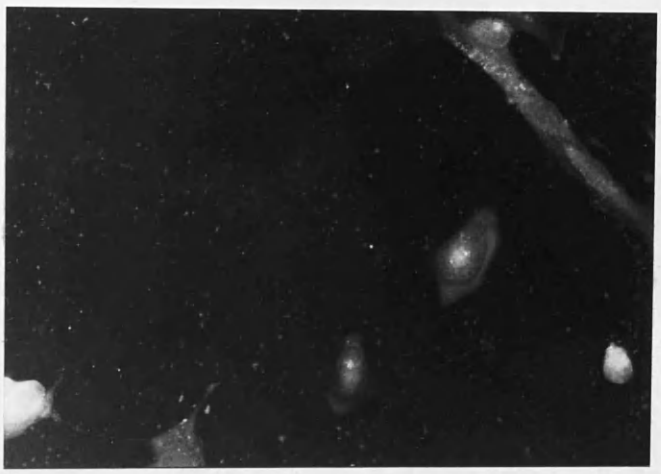


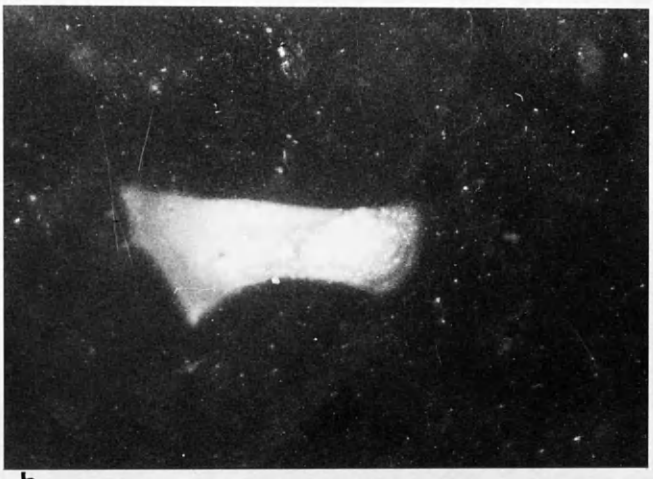
Fig. 14.2.6. Indirect immunofluorescent staining of fixed CII/1 cells with mouse monoclonal antibody against HSV-1 polypeptide Vmw IE 175: (a) CII/1 cells processed at 10 d p.i.; (b) productively wt HSV-2-infected cells. Magnifications: x100.

a

b

Fig. 14.2.7. Indirect immunofluorescent staining of fixed CII/1 cells with mouse monoclonal antibody against HSV-1 polypeptide Vmw IE 63: (a) CII/1 cells processed at 10 d p.i.; (b) productively wt HSV-2-infected cells. Magnifications: a, x100; b, x64.

a



b

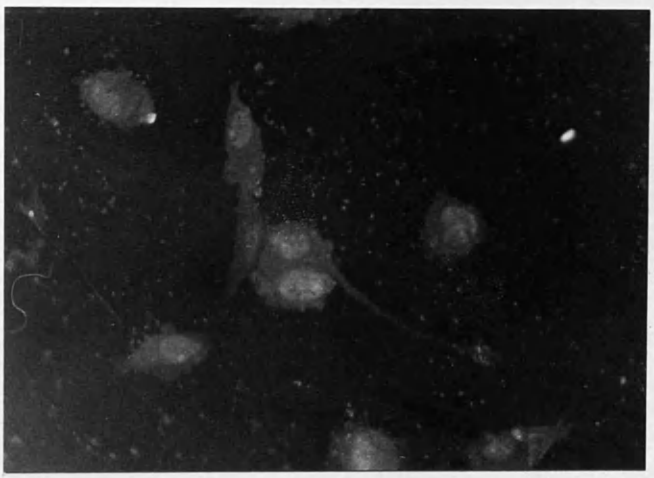
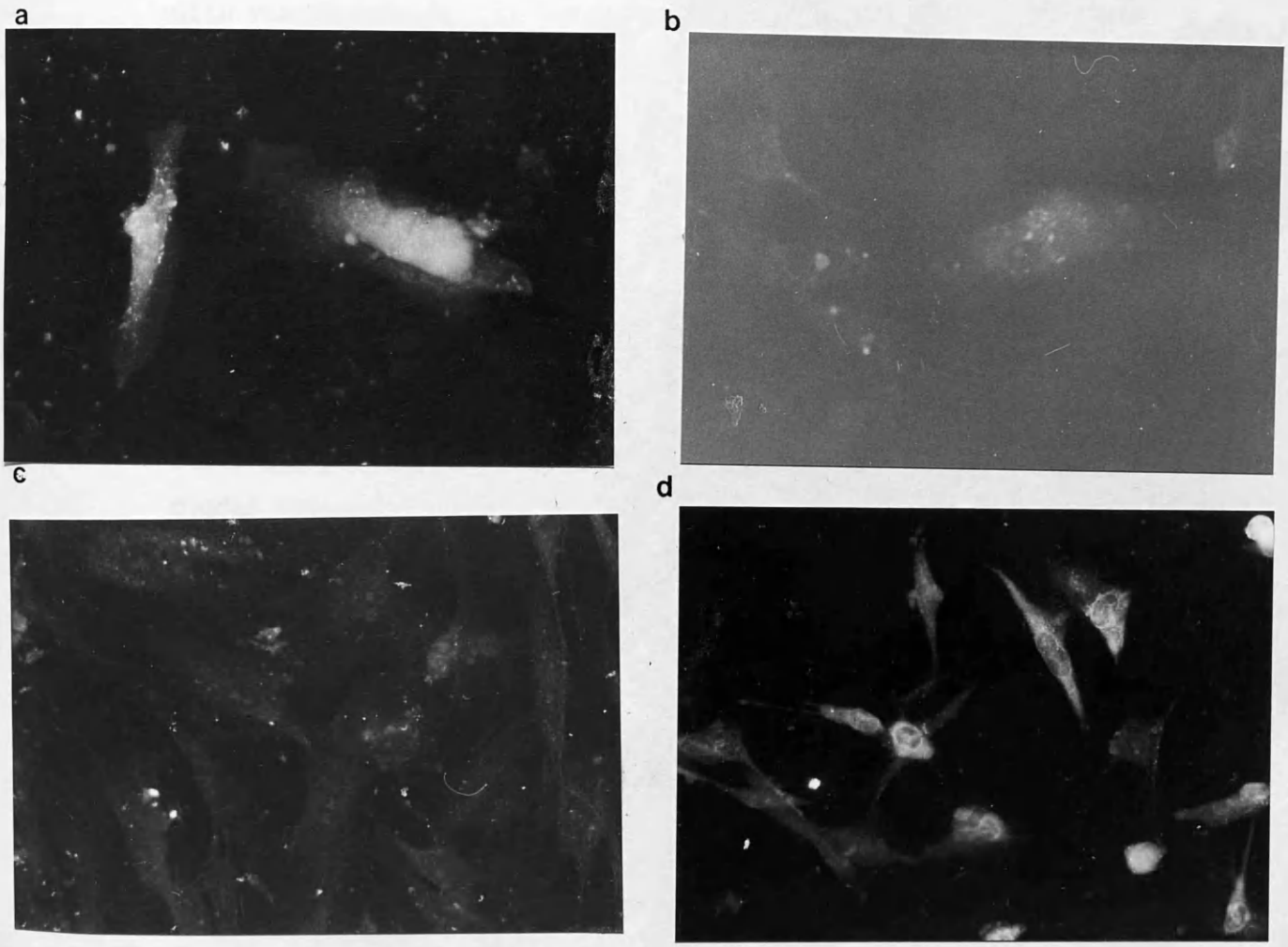


Fig. 14.2.8. Indirect immunofluorescent staining of fixed CII/1 cells with mouse monoclonal antibody against HSV-1 polypeptide Vmw IE 136´143: (a) CII/1 cells processed at 10 d p.i.; (b CII/1 cells processed at 17 d p.i.; (c) CII/1 cells processed at 27 d p.i.; (d) productively wt HSV-2-infected cells. Magnifications: a and b, x100; c and d, x64.


a
b
c
d

with elements of the cytoskeleton (chapter 8). (Fluorescence was observed on the nuclear as well as the plasma membrane of productively infected cells stained for gA/gB, contrary to reports in the literature (Norrild et al., 1980).) Fluorescence for gD was located at sites of contact between cells, at ventral surfaces and at extremities of the cytoplasm, in keeping with the data of Norrild et al. (1983), who showed that gD of HSV-1 (F)-infected Vero cells is located at the focal adhesion sites on the ventral surface of the cells, and co-distributes with vinculin at junctional areas of cells.

In productively infected cells stained for virion components Vmw 155 (**Fig. 14.2.3**), Vmw 65 (**Fig. 14.2.1**) and Vmw 33 (**Fig. 14.2.4**), fluorescence was in apposition with the nuclear membrane; but in CI/1 and CII/1 cells, fluorescence was located also in the cytoplasm. Since the nuclear membrane is the site of virus assembly in productively infected cells (Nii et al., 1968a) these results may be explained by aberrant assembly of virions in CI/1 and CII/1 cells. Fluorescence for Vmw 136'130 (**Fig. 14.2.2**) was nuclear in productively HSV-1 infected cells and CI/1 cells, but appeared, at high magnification, to be in closer apposition with the nuclear membrane in the nuclei of CI/1 cells.

That CI/1 and CII/1 cells processed at 10 d p.i. at 41.5-42 ^{o}C fluoresced for immediate-early and early viral polypeptides, which are not virion components, indicates that virions of the original infecting inoculum were not the only possible cause of the cells' immune-reactivity. And it is concluded that all cells in cultures CI/1 and CII/1, having survived infection at 41.5-42 ^{o}C, expressed viral antigens by the end of the 10 d period of incubation at 41.5-42 ^{o}C, in the absence of detectable infectious virus.

For CI/1 and CII/1 cells processed at 17 d, 27 d and 47 d p.i., however, fluorescence for viral polypeptides was at mock-infected levels (see **Fig. 14.2.8, b** and **c**), with two exceptions: (1) CI/1 cells processed at 17 d p.i. and stained for early viral polypeptide Vmw 136'130, or ICP8 (Morse et al., 1978), the major DNA-binding protein (Littler et al., 1981), all showed stronger fluorescence relative to the first time-point; and in both instances (at higher magnification, results not shown) the fluorescence outlined the nuclear membrane and was granular, rather than diffuse. (2) In CII/1 cells processed at 17 d

p.i. also and stained for Vmw 65 (Fig. 14.2.1 e), significant fluorescence was observed in the cytoplasm and outlining the nuclear membrane. These results may be explained by differential rates of elimination of viral antigens which had accumulated in CI/1 and CII/1 cells, or by the selective retention and/or expression of viral DNA sequences encoding Vmw 136'130 in CI/1 cells, and of sequences encoding the HSV-2 polypeptide equivalent to Vmw 65 in CII/1 cells.

Although functional Vmw IE 175 is required to permit the expression of non-IE viral functions (Preston, 1979a, b; Watson & Clements, 1980), it has been established that synthesis of ICP8 in Xenopus laevis oocytes does not require absolutely the expression of IE viral genes (Knopf et al., 1983).) A major class of defective particles of HSV-1 (ANG) encodes ICP8 (Knopf et al., 1983), and causes infected cells to produce this viral polypeptide in large quantities (Locker et al., 1982; Knopf et al., 1983). Thus, the fluorescence which was detected in CI/1 cells processed at 17 d p.i. and stained for Vmw 136'130 may have arisen from the prior synthesis of defective virus particles encoding this viral polypeptide, and the persistence and expression of the defective viral DNA during propagation of the abortively infected cells at 37 ^{o}C, in the absence of other detectable viral antigens.

Cells from cultures 37 ^{o}C/BI and 37 ^{o}C/BII, processed at 44 d p.i., also were negative when tested with monoclonal antibodies (not shown).

15. HYBRIDIZATION OF HSV-1-SPECIFIC SEQUENCES TO DNA OF UNINFECTED AND ABORTIVELY-INFECTED HELU CELLS.

DNA was extracted, according to the method of Park (1983), from cells in the AI and CI series of cultures. In five experiments this DNA was tested for the presence of sequences which would hybridize under stringent conditions to HSV-1-virion DNA, or to the DNA of the plasmids pBam f (provided by Miss M. E. Campbell) and pBam k (provided by Dr. C. M. Preston), which carry HSV-1-specific sequences derived from BamHI f and BamHI k, respectively, mapping at co-ordinates 0.646-0.696 and 0.81-0.852 of the viral genome in the prototypic orientation. (Vector-specific DNA was not removed from the HSV-1-specific fragments prior to use in hybridizations.)

Immediately before the extraction of cellular DNA, the AI and CI cultures were assayed for the presence of infectious virus by titration of sonicated cells and medium on BHK cells, without employing human serum: viral c.p.e. was undetectable after incubation of the BHK cells at either 37 ^{o}C or 31 ^{o}C for 5 d. Cultures AI/2 and A/3 were extracted at the sixth and and seventh passages, respectively; while culture CI/1, unreactivated, pooled sub-cultures of CI/2-cells and CI/3-cells, and a mock-infected culture (CMI), all of which had been established and cultured in parallel, were extracted at the sixth passages.

Cellular DNA and reconstruction mixtures (consisting of HSV-1-virion DNA, provided by Dr. C. M. Preston, and uninfected or mock-infected cellular DNA in various proportions) were digested with either restriction enzyme EcoRI (provided by Mr. M. Dunlop) or BamHI. BamHI and EcoRI restriction sites in the genome of HSV-1 are shown in **Fig. 13.3.1.** The digested DNA was fractionated by electrophoresis, transferred to nitrocellulose membranes and hybridized with radiolabelled DNA as described in Materials and Methods. Hybridization and washing of membranes were conducted under stringent conditions (Jeffreys and Flavell, 1977) of 6XSSC plus 10% (w/v) dextran sulphate and 0.1XSSC, respectively, at 74 ^{o}C; stable hybrids under such conditions would indicate either a high G+C content and a high degree of homology between the hybridizing sequences, or a high reiteration

frequency for less exactly homologous sequences (Schildkraut & Lifson, 1965). Maximal and stringent hybridization is equated with a temperature that is 25 ^{o}C below the hybrid melting temperature (Tm), as calculated by the equation of Schildkraut & Lifson (1965):

$$Tm = 16.6 \log M + 0.41(G+C) + 81.5$$ (where M represents the ion concentration).

The vector, pAT153, has a base sequence of 54% G+C; HSV-1 sequences BamHI f and BamHI k have base sequences of 67% (Mr. M. Dalrymple, personal communication) and 88% G+C (Dr. D. McGeoch, personal communication), respectively; and HSV-1 virion DNA, 67% (Kieff et al., 1981).

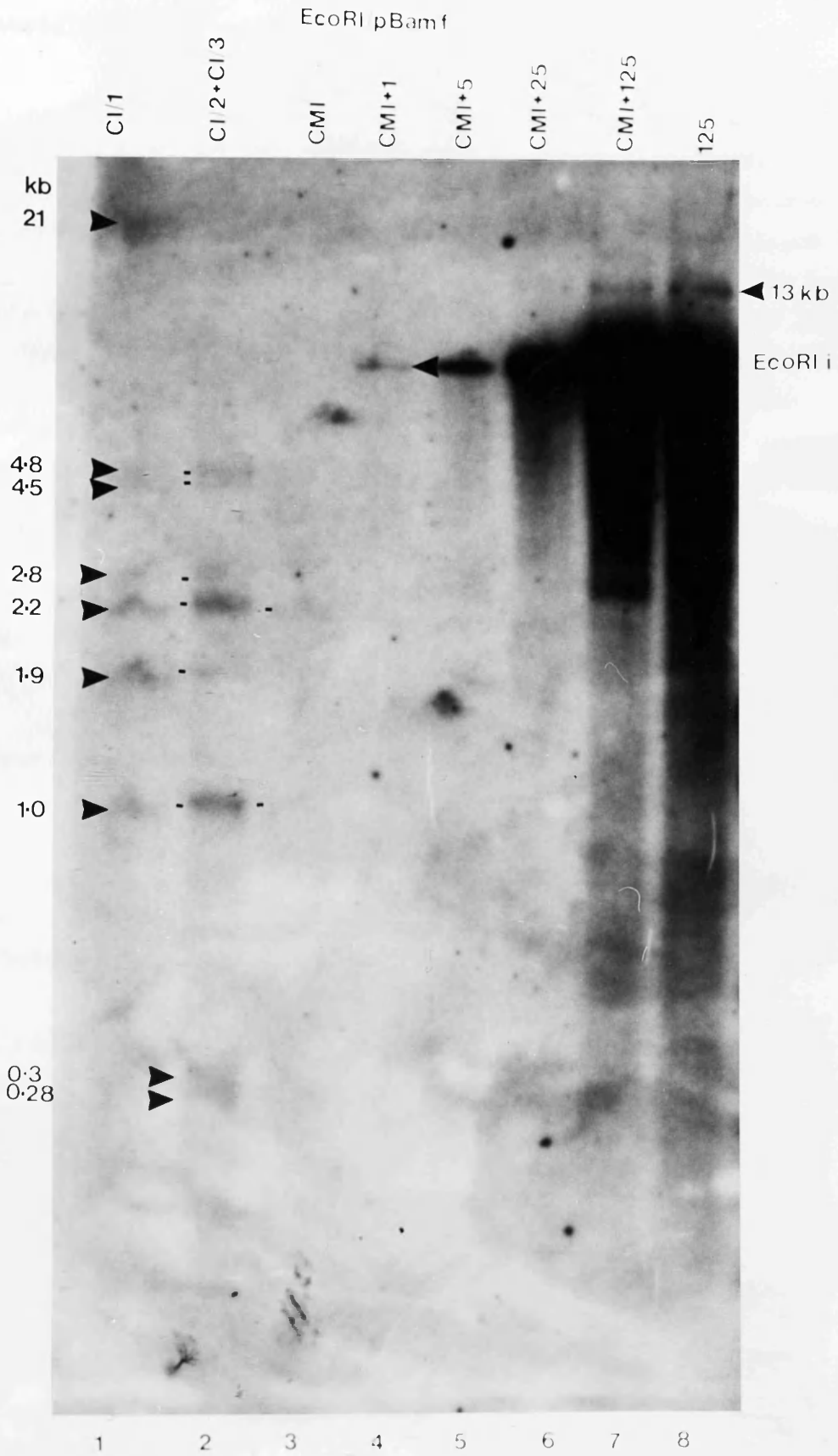
The main conclusion from the results of the hybridization experiments is that bands comigrating with restriction fragments of HSV-1 virion DNA were undetectable in digests of cellular DNA from the AI and CI series of abortively infected cultures, under conditions which readily allowed the detection of a viral fragment of 3.4 kb or greater present at 1 copy per cell, and of 0.95 kb present at 5 copies per cell. However, if extensive regions of the viral genome were present at less than 1 copy per cell, they would not have been identified under these conditions.

Hybridizable sequences which did not comigrate with virion fragments were readily detected in the DNA of uninfected and abortively-infected cultures at levels ranging from 1 to 25 copies per diploid-cell genome. This is demonstrated in **Fig. 15**, where radiolabelled pBam f was hybridized to EcoRI-digested DNA from CI cells, and to EcoRI-digested reconstruction mixtures containing DNA from mock-infected cells (CMI) with the equivalent of 0 to 125 copies of virion DNA per diploid-cell genome. DNA from pBam f hybridized in the reconstructions (tracks 4 to 8) to the homologous virion fragment, EcoRI i; and also, in those reconstructions containing the equivalent of 25 or 125 copies of HSV-1 virion DNA per diploid-cell genome (tracks 6 and 7), or HSV-1 virion DNA without CMI-cell DNA (track 8), to a band of 13 kb. This band was therefore viral in origin, and comigrated with virion fragments EcoRI a and b. The efficiency of detection of this band was substantially less than for a homologous sequence present in the

Fig. 15. Hybridization of radiolabelled pBam f to EcoRI-digested DNA from wt HSV-1-infected HeLu cells (CI/1, CI/2 and CI/3) and from mock-infected HeLu cells (CMI).

Cellular DNA (30 ug), plus or minus HSV-1 virion DNA, was digested with 100 U of EcoRI overnight. Restricted fragments were fractionated by electrophoresis through a 0.6%-agarose, ethidium-bromide- stained gel at 20 V for 36 h, blotted onto a nitrocellulose and hybridized to nick-translated, [^{32}P]-labelled pBam f (specific activity, 2.6×10^8 c.p.m./ug of DNA; 1.3×10^8 c.p.m. applied). The washed and dried filter was subjected to indirect autoradiography at -70° for 7 d.

Tracks 1 and 2; DNA from wt HSV-1-infected cells, culture CI/1 and pooled cultures CI/2 plus CI/3, respectively. Track 3; DNA from mock-infected HeLu cells, CMI. Tracks 4 to 7; CMI-cell DNA and the equivalent, for 30 ug of cellular DNA, of 1 (track 4), 5 (track 5), 25 (track 6) and 125 (track 7) copies of HSV-1-virion DNA per diploid-cell genome. Track 8; the equivalent of 125 copies of HSV-1-virion DNA, without CMI-cell DNA.


EcoRI pBamf
CI/1
CI/2+CI/3
CMI
CMI+1
CMI+5
CMI+25
CMI+125
125
kb
21
4·8
4·5
2·8
2·2
1·9
1·0
0·3
0·28
13 kb
EcoRI i
1
2
3
4
5
6
7
8

proportion of one copy per viral genome. However, the cause of this hybridization is unknown.

In longer exposures of the autoradiogram, faint bands of 4.8, 4.5, 2.2, 1.9, 1.0, 0.3 and 0.28 kb were detected in CMI-cell DNA (track 3) in the proportion of 1, or less than 1, copy of a sequence of maximum homology with BamHI f per diploid-cell genome. CI/1-cell (track 1) and CI/2- plus CI/3-cell DNA (track 2) showed, in addition to the bands detected in CMI-cell DNA, bands of 21.0, 4.5 and 2.8 kb, the intensity of hybridization corresponding with the presence of 1 or more copies of sequences homologous with pBam f per diploid-cell genome. Thus, the pattern of hybridization of pBam f was similar for both digests of CI-cell DNA, and the degree of hybridization overall was higher than for CMI-cell DNA. It is apparent from the reconstructions that the limit of detection of virus-specific sequences by hybridization with pBam f would have been 0.3-0.5 copies of molar fragment EcoRI i (8.4 kb) per diploid-cell genome.

Data which were derived from this and similar experiments (not shown) are summarized in **Tables X** to **XII**, where the sizes of bands detected in BamHI- or EcoRI-digested cellular DNA by hybridization with radiolabelled pBam f, pBam k or HSV-1 virion DNA are given. The stated copy numbers refer to sequences with maximum homology and are approximate, since less exact homologies, or different efficiencies of hybridization for fragments of different sizes (Park, 1983), would affect the intensities of hybridization. From the stringency of the hybridization and washing conditions, it is concluded that the hybrids were of a high G+C content and either well matched or highly reiterated (Schildkraut & Lifson, 1965). The possibility arises that the observed hybridization of pBamk and pBamf DNA to uninfected cellular DNA involved the vector-specific sequences. Although the DNA of the vector, pAT153, was reported to hybridize to DNA extracted from a human carcinoma, it fails to hybridize at a detectable level (very much less than 1 copy per cell) to normal human cell DNA, including HeLu-cell DNA (Park, 1983). The DNA of pBR322, from which pAT153 was derived, also fails to hybridize to human-cell DNA at detectable levels (Peden *et al.*, 1982). Thus, in these experiments, the hybridization of plasmids containing inserts of HSV-1 DNA to uninfected Helu-cell DNA may have involved the HSV-1-specific sequences. (It was not possible to repeat these

Tables X and XI: sizes and copy numbers of cellular sequences hybridizing with plasmid DNA containing HSV-1 inserts, or with HSV-1 virion DNA.

Table X (from Fig. 15).

Digest	EcoRI	
Hybridization	pBam f	
	Copy numbers	
DNA	(CI)	(CMI)
(kb)		
21.0	>1	ND
4.8	1	<1
4.5	1	<1
2.8	1	ND
2.2	1-5	1
1.9	1	<1
1.0	1-5	<1
0.3	1	<1
0.28	1	<1

'ND' signifies 'not detectable'.

'<1' signifies the detection of a hybrid at the level of less than 1 copy per diploid-cell genome.

Table XI.

Digest	BamHI	
Hybridization	pBam k	
DNA	Copy numbers (AI/2)	Uninfected
(kb)		
12.7	30	<3
10.0	ND	<3
9.3	<9	3
8.7	<9	3
7.6	<9	<3
5.0	9	<3
4.5	ND	<3
4.1	9	3
3.2	<9	<3
3.0	9	3

'ND' signifies 'not detectable'.

'<3' and '<9' signify the detection of a hybrid at the level of less than 3, and less than 9 but more than 3, copies per diploid-cell genome.

Table XII.: alignment of hybrids detected in EcoRI-digested cellular DNA using DNA from plasmids pBam k or pBam f, or with HSV-1 virion DNA. Bands were detected at a level of 1 to 25 copies per diploid-cell genome, assuming maximum homolgy between hybridizing sequences.

DNA Hybridization	CI HSV-1	CI pBam k	AI/3 pBam k	CI pBam f
	(kb)	(kb)	(kb)	(kb)
				*21
	11.0	11.0	11.8	
		10.0	10.0	
	8.3			
		7.9	7.9	
		*6.9	*6.9	
	6.3	6	5.6	
	5.0	4.8	4.6	*4.8
	4.2	*4.3	*4.1	*4.5
	3.2-3.3	3.5	3.6	
	2.0-2.6	2-2.8	2.4	2.2-2.8
		2.8		*2.8
		*2.3	2.4	*2.2
	2.0	2.1,2.0	2.0,1.95	
	1.6	1.7		*1.9
	1.4	1.4		
		1.2		
	1.05,0.9	*1.0		*1.0
		0.8		
	0.7	0.7		
		0.6		
	0.5			
				*0.3
		*0.25		*0.28

'*' denotes a four-fold or higher increase in hybridization relative to uninfected-cell or mock-infected-cell DNA.

hybridizations with HSV-1 sequences purified from the plasmids, or with pAT153-specific DNA alone, owing to the limited time which was available.) Several groups have found homology between human DNA and HSV-1 DNA, especially the repeated regions of the S component of the viral genome (Peden et al., 1982; Puga et al., 1982; Park, 1983). Park (1983) reported that the HSV-1 TaqI A fragment, consisting of 995 bp from within BamHI x (0.863-0.87 and 0.76-0.967 in IR_S and TR_S), hybridizes to bands of 4.5, 1.8 and 1.3 kb in BamHI digests of all human DNA analysed. It is suggested that the regions of hybridization of HSV-1-specific sequences to EcoRI-digested, human cellular DNA reported by Puga et al. (1982), i.e. 1-4 kb, 4-5 kb, 7-9 kb, 14 kb and 22 kb, correspond to regions of hybridization (including amplified bands) detected here using pBam f and pBam k, which would signify the existence of similar genomic arrangements of hybridizable sequences.

The patterns of hybridization of radiolabelled DNA from plasmids pBam k and pBam f, or of radiolabelled HSV-1 virion DNA, to DNA from uninfected cultures were similar to the patterns of hybridization to DNA from abortively-infected cultures. However, there was increased efficiency of hybridization to specific bands in digests of DNA from abortively- infected cultures compared with uninfected or mock-infected cultures, as summarized in **Tables X to XI**. This indicates that hybridizable cellular sequences were either rearranged or selectively amplified during or following the abortive infection of cells with wt HSV-1 under the conditions described in chapter 12.

When the patterns of hybridization of radiolabelled plasmid and virion DNA to EcoRI-digested cellular DNA from the AI and CI series of cultures were compared, it was noted that size classes of hybridization could be roughly aligned, as shown in **Table XII**. Furthermore, specific bands detected in CI-cell DNA by hybridization with pBam k or pBam f correlated with bands of more intense hybridization detected with HSV-1 virion DNA, particularly between the region from 2 to 4.3 kb (**Table XII**). Puga et al. (1982) also found similarities between the patterns of hybridization of HSV-1 virion DNA and a sub-fragment of BamHI k to EcoRI-digested DNA from human cells. The correlation of certain size classes of hybridization between pBamk and pBamf (**Table XII**) cannot relate to HSV-1-specific, common DNA sequences, but

suggests either (a) pAT153-specific hybridization, (b) fortuitous coincidence of HSV-1-specific size classes, or (c) HSV-1-specific hybridization owing to the high G+C contents of the cellular and viral sequences involved. This applies to bands of 4.8, 4.2, 2.0-2.8 and 1.0 kb. Although pBam k and pBam f hybridized to similar bands in cellular DNA, hybridization was more efficient using pBam k. This supports the hypothesis that hybridization involved viral-specific, rather than vector-specific, sequences (i.e. (b) or (c)). It is therefore suggested that the degree of homology with hybridizable cellular sequences was greater with pBam k than with pBam f, and that sequences present in BamHI k accounted for most of the hybridization obtained using HSV-1-virion DNA, as implied by Puga et al., (1982). BamHI f may represent a class of HSV-specific sequences which exhibit a lower degree of homology - perhaps owing to the lower G+C content of this fragment than of BamHI k (according to (c)) - and hybridize less efficiently, with these cellular sequences.

INDEX OF DISCUSSION.

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DISCUSSION.

The Cellular Stress Response is Induced by Infection of Cells with TsK.

The initial finding upon which subsequent experiments in this study were based was that infection of secondary CEF with tsK at the NPT causes the cellular stress response to be induced. This is a singular example of the activation of cellular gene expression by herpesvirus infection. An analogous system that has been investigated is the activation of synthesis of endogenous mouse xenotropic retrovirus in BALB/3T3 cells following inoculation of the cells with: u.v.-irradiated HSV-1 and HSV-2 (Hampar et al., 1976; Reed et al., 1976); DNA from human and non-human herpesviruses (Boyd et al., 1978); or defined fragments of HSV-1 DNA (Boyd et al., 1983).

The induction of the stress response in secondary CEF by infection with tsK at the NPT was characterized by these features:

i) Infection was followed by increasing synthesis of stress proteins, by several hours p.i. reaching levels of synthesis obtained in cells treated with stress-inducing reagents (chapter 1).

ii) 90SP, 70SP and 25SP, but not 35SP, were the stress proteins whose syntheses were thereby induced (chapter 1).

iii) Induction of the stress response was dependent upon synthesis of IE viral polypeptides, including defective Vmw IE 175, and accompanied their accumulation by infected cells (chapter 1).

iv) Up to at least 11 h p.i., relative levels of synthesis of stress proteins were sustained rather than following time-dependent kinetics such as are observed in cells treated continuously with disulfiram (chapter 6) and other stress-inducing agents (see Introduction).

In features i) and ii) the response in tsK-infected CEF resembles the response of uninfected CEF to treatment with canavanine (Kelley & Schlesinger, 1978); and in both cases activation of the response is

dependent upon protein synthesis (feature iii)). Also, induction of synthesis of 35SP is observed neither in response to treatment of CEF with canavanine nor following infection of CEF with tsK. According to the kinetics of synthesis of stress proteins observed in CEF (described in chapter 2), 35SP is the first species whose synthesis is induced and then repressed during continuous treatment of cells with stress-inducing agents (Johnston et al., 1980). Given that the stress response in canavanine-treated and tsK-infected CEF develops progressively from low levels, 35SP may therefore be synthesized at undetectable levels in CEF during the initial stages of these responses. At later times during treatment of cells with canavanine, as with other stress-inducing agents, synthesis of 70SP and 90SP predominates until 25SP replaces 70SP as the dominant protein synthesized (Kelley & Schlesinger, 1978); this contrasts with the absence of time-dependent kinetics for the response in tsK-infected cells (feature iv)).

A consistent and perhaps relevant observation was that in mock-infected cells treated with disulfiram and cycloheximide, and following removal of these reagents, synthesis of 35SP was uninduced, compared with cells treated with disulfiram alone (chapter 5.1). This effect may have been due to (a) specific inhibition by cycloheximide of induction of synthesis of 35SP, (b) the kinetics of induction of the stress response, by which mRNA for other major stress proteins would accumulate in the presence of cycloheximide and compete with mRNA encoding 35SP for translation on removal of the reagent, or (c) the relative instability of mRNA encoding 35SP. Hypotheses (b) and (c) are in keeping with the theory of control of synthesis of heat-shock proteins in Drosophila cells through the concentration of mRNA (Ashburner & Bonner, 1979).

The induction of the stress response by infection with tsK at the NPT is not peculiar to secondary CEF: the stress response appeared to be induced similarly in primary and secondary REF (chapter 4) and in BHK cells (results not shown)

Despite the requirement for synthesis of IE viral polypeptides by tsK-infected cells for induction of the stress response to occur (feature iii)), a system was described by Fenwick et al. (1980) in which accumulation of IE viral polypeptides by HSV-1-infected cells did

not appear to cause induction of synthesis of stress proteins: in HSV-1 (F)-infected Vero cells treated with the amino-acid analogue, azetidine, IE viral polypeptides were produced (ICP 4, ICP 6, ICP 0, ICP 22 and ICP 27, corresponding to Vmw IE 175, Vmw IE 136'143, Vmw IE 110, Vmw IE 68 and Vmw IE 63) while synthesis of stress proteins was suppressed; however, treatment of uninfected cells with azetidine caused the stress response to be induced. Therefore, the stress response was induced in infected cells neither by the accumulation of (probably) non-functional IE viral polypeptides nor by treatment of the cells with azetidine.

Incubation of HSV-infected cells with amino-acid analogues such as canavanine or azetidine arrests the lytic cycle at the IE phase; and it is believed that incorporation of the analogues into IE viral polypeptides disrupts the functioning of these polypeptides, thereby causing the observed inhibition of synthesis of non-IE viral polypeptides (Honess & Roizman, 1975; Pereira et al., 1977). Given the induction of the stress response in tsK-infected cells incubated at the NPT (chapter 1), and assuming that in the system of Fenwick et al. (1980) IE viral polypeptides accumulating in azetidine-treated, infected cells were non-functional, it is deduced that (A) certain IE viral polypeptides must be produced in a functional state in order to induce the stress response in HSV-1-infected cells, and (B) the stress response is not induced solely by the accumulation of non-functional IE viral polypeptides, including Vmw IE 175. The failure to induced the stress response in infected cells by treatment with azetidine (Fenwick et al., 1980) may have reflected the general inhibition of synthesis of cellular polypeptides which results from infection by HSV (Fenwick, 1984), since analogue-mediated induction of the stress response in CEF is known to be dependent upon the synthesis of cellular protein (Kelley & Schlesinger, 1978).

In experiments described in chapter 2, a range of ts-mutants of HSV-1, wt HSV-1 and wt HSV-2 were examined for the ability to induce the stress response in CEF. Infections with those viruses which are defective in Vmw IE 175 (tsD, tsT, tsK or tsK syn) resulted most markedly in induction of the stress response, despite synthesis in tsD-infected and tsT-infected cells of less defective Vmw IE 175 and, consequently, apparently normal amounts of many early and late viral polypeptides. And from this it was concluded that production of

defective Vmw IE 175 by infected cells leads to induction of synthesis of stress proteins. And this conclusion must be reconciled with deduction (B), above. One hypothesis to interpret these results is that the stress response is induced in infected cells by the accumulation of (functional) IE viral polypeptides, which is caused in turn by the production of defective Vmw IE 175. However, this hypothesis is discounted by observations (chapter 2) that infection of secondary CEF with any of a number of ts mutants of HSV-1 (tsl201, tsB, tsE, tsG and MDK/2) at a NPT, with the revertant of tsK, $ts^{+}K$, or with wt HSV-2, all of which are non-defective in Vmw IE 175 or the HSV-2 equivalent, Vmw IE 182, and which therefore do not cause overproduction of IE viral polypeptides at a NPT, resulted (to degrees that varied between cultures) in increased synthesis or induction of synthesis of stress proteins. (Furthermore, it may be concluded that the stress response was not induced specifically by overproduction of Vmw IE 63, since this IE viral polypeptide was produced at detectable levels in wt HSV-1-infected secondary CEF at late times in infection (see chapter 1), when the response appeared to be uninduced.)

Another hypothesis to account for these observations is that cells are subjected to stress during infection with wt or mutant HSV, owing to the expression of IE and perhaps also later viral functions, and that induction of synthesis of stress proteins is inhibited to degrees which depend upon the characteristics of the virus: inhibition is most effective in wt HSV-infected cells, and least effective in cells infected with mutants defective in Vmw IE 175. In other words, viral functions cause two opposing effects upon the stress response in HSV-infected cells, and the production of defective Vmw IE 175 by infected cells allows the response to become apparent. Dargan & Subak-Sharpe (1983) concluded that several ultrastructural alterations (margination of chromatin, intranuclear granular accumulations and nuclear membrane distortion) are very early events in the infection of cells with HSV-1 (occurring 0 to 1 h after adsorption) and may be consequences of the synthesis of IE viral polypeptides. Any of these changes in cellular morphology, of which all the mutant viruses tested were capable, may have been factors contributing to induction of the stress response in HSV-infected cells (chapter 2).

Synthesis of viral polypeptides was less restricted in

tsT-infected cells than in tsD-infected or tsK-infected cells, indicating that Vmw IE 175 is less defective for tsT than for tsD or tsK regarding the activation of expression of non-IE viral genes. However, the pattern and higher levels of synthesis of stress proteins in TsT-infected cells were interpreted as indicating a response to a higher degree of stress, compared with tsD-infected or tsK-infected cells; and the higher rate of synthesis of viral polypeptides, including non-IE viral polypeptides, may have been a factor which increased the intensity of stress in tsT-infected cells.

Evidence that cells are subjected to stress by infection with wt as well as mutant HSV-1 was provided by the observation of induction of the stress response in primary REF following infection with wt HSV-1 as well as with tsK (chapter 4). The primary culture was notable for its greater sensitivity to disulfiram than secondary cultures; and so infection by wt HSV-1 (or tsK) may subject REF to stress, but whether the stress response is induced following infection may depend upon the sensitivity of the cultures to stress-inducing agents.

It has been reported that virus isolates frequently demonstrate mutations in the gene encoding Vmw IE 175, resulting in overproduction of IE polypeptides at the NPT (Knipe et al., 1981). This implies, in view of the results presented in this study, that the cellular stress response may be involved in natural infections by HSV-1. Moreover, the stress response may also be involved in infections with stocks of HSV containing high proportions of defective virus particles. One class of defective particles of HSV-1 contains tandem repeats of DNA sequences including two complete IE genes, and cells infected with virus stocks containing these particles overproduce Vmw IE 175 (Frenkel et al., 1975; Murray et al., 1975). The coding potential of another class of defective particles of HSV-1 (ANG), termed dDNA 1, includes the major HSV-1 DNA binding protein but not IE viral polypeptides; and cells infected with a virus stock containing 20% dDNA 1 overproduce IE viral polypeptides as well as the major DNA binding protein (Knopf et al., 1983).

Superinduction of the Stress Response in HSV-1-Infected Cells.

When secondary CEF were continuously treated with disulfiram from a stage prior to or early in infection with wt HSV-1 or tsK at the NPT, the levels of synthesis of stress proteins then became "superinduced", i.e. increased relative to levels in similarly treated, mock-infected cells (chapter 5 and 6). Levels of superinduction increased with earlier treatment of CEF up to 1 h prior to infection, and were sustained up to at least 11 h p.i. (chapter 6.2); however, disulfiram treatment from a time earlier than 1 h prior to infection did not result in superinduction following infection, but in levels of synthesis of stress proteins approximating to those in similarly-treated, mock-infected cells (chapter 6.1). Under these conditions either treatment with disulfiram caused maximal stimulation of the stress response in mock-infected cells and infected cells, or the inhibition of synthesis of viral polypeptides precluded the superinduction of the response. It is notable (as deduced from results presented in chapter 1.3; and see also **Fig. 6.5**) that superinduction of the stress response in reagent-treated cells ocurred only when the cells were permitted to synthesize protein post-infection; and in experiments described in chapters 6.1 and 6.2, superinduction appeared to correlate with synthesis of viral polypeptides.

An effect similar to superinduction was observed in Drosophila cells by Di Domenico et al. (1982): when cells were treated with the amino-acid analogue, canavanine, during heat-shock, the stress response was higher than observed in untreated, heat-shocked cells. The authors considered this to indicate that inactivation of heat-shock proteins (by incorporation of the amino-acid analogue) causes the heat-shock response to be sustained, and that functional heat-shock proteins are involved in regulating their own synthesis. However, this explanation does not take into account the activity of canavanine as a stress-inducing agent (Kelley & Schlesinger, 1978). An alternative explanation is therefore suggested, which applies also to results presented in this study: that simultaneous treatment of cells with two stress-inducing agents (heat-shock and canavanine in Drosophila cells; disulfiram and infection by HSV-1 in CEF) results in an enhancement (i.e. superinduction) and prolongation of the heat-shock - or stress - response. This explanation is consistent with the notion that infection by wt HSV-1 causes cells to be subjected to stress.

Inhibition of Induction of the Stress Response in Infected Cells by Viral Functions.

Results presented in chapters 5 and 6 indicated that the responsiveness of wt HSV-1-infected and tsK-infected CEF to treatment with disulfiram was decreased during infection at the PT or NPT, and that three phases of responsiveness were detectable:

i) When infected cells were treated prior to, or up to 1 h p.i. at 38.5 ^{o}C, levels of synthesis of stress proteins were increased (superinduced), compared with similarly-treated, mock-infected cells (chapter 5 and 6). This effect was discussed above.

ii) Following 1 h p.i. at 38.5 ^{o}C (chapter 5.1) or 3 h p.i. at 31 ^{o}C (chapter 5.3), infected cells progressively became less responsive to treatment with disulfiram.

iii) By 3 to 5 h p.i. at 38.5 ^{o}C, infected cells were unresponsive to treatment with disulfiram (chapter 5.1). There was an added complication in tsK-infected cells incubated at the NPT, in the virus-induced synthesis of stress proteins. However, treatment of tsK-infected cells with disulfiram at or after 3 h p.i. did not produce superinduction of the stress response.

Like the effect of superinduction, the loss of the stress response in disulfiram-treated, infected cells ocurred only if the cells synthesized protein post-infection: when cells were infected in the presence of cycloheximide and disulfiram, and following removal of these reagents, levels of synthesis of stress proteins were comparable to those in similarly-treated, mock-infected cells (**Figs. 1.3** and **6.5**). Thus, both the superinduction and the inhibition of the stress response in infected cells may be considered to be dependent upon the expression of viral functions.

It was concluded that early in infection by wt HSV-1, Vmw IE 175 contributed to the decrease in the response of infected cells to treatment with disulfiram (chapter 5.3); and that IE viral polypeptides

in wt HSV-1-infected and tsK-infected cells could cause considerable reduction in the response of cells to treatment with disulfiram (chapter 5.5). Thus, a virus-induced stress response would be suppressed early in infection by wt HSV-1, with IE viral polypeptides, including functional Vmw IE 175, involved in inhibiting the response at this time. The need for functional Vmw IE 175 is supported by observations that mutants tsT and tsD, in CEF infected at the NPT, caused production of many early and late viral polypeptides, in addition to defective Vmw IE 175, in CEF infected at the NPT, and yet they caused induction of synthesis of stress proteins in a similar manner to tsK. Similarly, Epstein and Jacquemont (1983) observed that synthesis of a putative stress protein was uninhibited in rat XC cells infected at a NPT with a mutant of HSV-1 (13VB4tsC75), which is temperature-sensitive for ICP4 (or Vmw IE 175, by this notation).

The known function of Vmw IE 175 is to permit transcription of genes encoding early and late viral polypeptides (Preston, 1979a; Watson & Clements, 1980). And this function therefore must be responsible for the diminution (in wt HSV-1-infected cells compared with tsK-infected cells) of the response to treatment with disulfiram. Consequently, Vmw IE 175 may contribute indirectly to the inhibition of either a virus-induced or a reagent-induced stress response early in infection by wt HSV-1, by promoting the transcription and translation of viral mRNA at the expense of cellular mRNA (Stringer et al., 1977).

The unresponsiveness of infected cells to disulfiram developed several hours p.i., but required synthesis neither of functional Vmw IE 175 nor of early or late viral polypeptides, as tsK-infected cells did not respond to treatment during incubation at the NPT, when only IE viral polypeptides (including defective Vmw IE 175) were produced (chapter 5.1). Thus, synthesis of IE viral polypeptides was sufficient for the response to treatment with disulfiram to be inhibited. This unresponsiveness may be a general feature of infections by HSV, as CEF that were infected with a wide range of ts mutants of HSV-1 at the NPT or with wt HSV-1 or wt HSV-2 were found to be refractory to treatment with disulfiram at 6 h p.i. at 38.5 ^{o}C (chapter 5.2); and may be due to the virus-induced inhibition of synthesis of cellular RNA that occurs in HSV-infected cells (Wagner & Roizman, 1969a; Nakai et al., 1982; Stenberg & Pizer, 1982). Stenberg & Pizer (1982) demonstrated that when

adenovirus-transformed human cells are infected with HSV-1 strain 17, transcription of adenovirus-specific mRNA and cellular RNA becomes inhibited. This may be caused by IE viral polypeptides, as the synthesis of these other species of RNA is prevented (by 3 h p.i. at 39 ^{o}C) to the same extent by infection with tsK as with wt HSV-1. (Secondary effects were suggested to occur by 4 h p.i. to further inhibit the accumulation of adenovirus-specific RNA in the cytoplasm of wt HSV-1-infected cells, and the causative agent was considered to be absent from cells infected with mutants defective in Vmw IE 175.)

Results presented in chapter 2 indicated that the stress response was strongly induced in infections by tsD, tsK and tsT, where Vmw IE 175 was malfunctional, and also that synthesis of stress proteins was stimulated by infections with mutants tsB, tsE, tsG, MDK/2 and ts^{+}K. These findings were interpreted to signify that cells were responding to stress caused by infection with these viruses. Low levels of constitutive synthesis of stress proteins usually were maintained in wt HSV-1-infected cells. Any proposed mechanism for the inhibition of a virus-induced stress response therefore must take into account the maintenance, stimulation or induction of synthesis of stress proteins with the production of functional Vmw IE 175 during infections by particular viruses.

When either Drosophila cells or CEF are treated with either actinomycin D or cycloheximide whilst being subjected to stress, the stress response is prolonged as mRNA for stress proteins remains active (Di Domenico et al., 1982; Schlesinger et al., 1982a). As both the transcription of cellular mRNA and synthesis of cellular polypeptides are inhibited following infection by HSV (Fenwick, 1984), it is now suggested that pre-existing mRNA for stress proteins is stabilized in infected cells by mechanisms similar to those caused by the treatment of cells with actimomycin D and cycloheximide, so that levels of synthesis of stress proteins are maintained p.i.. Thus, if cells are subjected to virus-induced stress early in infection by HSV (before synthesis of host macromolecules is inhibited), then synthesis of stress proteins becomes fixed at charateristic levels: during infections by the most virulent mutants or wt HSV-1 (or by mutant viruses at the PT), the development of a cellular stress response is suppressed owing to the sequestration of the cells' synthetic resources

during the normal viral lytic cycle; but infections by mutant viruses in which the viral replicative cycle is retarded or arrested at an early stage are less effective in inhibiting induction of the stress response, and consequently stress proteins become synthesized at high levels. These proposals are supported by observations (1) that levels of synthesis of stress proteins are maintained in wt HSV-1-infected and tsK-infected cells, rather than following the time-dependent kinetics displayed by reagent-treated, mock-infected cells (chapter 6.2); and (2) that the intensity of the stress response induced following infection correlates with the classification of viruses according to Atkinson et al. (1978): strong responses were induced by class I mutants (tsD, tsK and tsT), weaker responses by class II (tsB, tsE, tsl201) and class III (tsG) mutants, and no response by wt HSV-1 or wt HSV-2.

Constitutive Synthesis of Stress Proteins and Infection by HSV.

Several observations support the notion that synthesis of viral polypeptides in HSV-1-infected cells may be influenced by cellular factors which are manifest by high levels of constitutive synthesis of stress proteins.

(i) Differences in levels of constitutive synthesis of stress proteins in cultures of secondary CEF appeared to be correlated with levels of synthesis of stress proteins attained following infection with ts mutants of HSV-1 and wt viruses, and with the ability of infected cultures to support synthesis of late viral polypeptides (chapter 2).

(ii) Although levels of constitutive synthesis of stress proteins did not vary in a predictable manner according to the passage number of CEF cultures (chapter 2), primary CEF and REF (chapter 4) showed certain common features: their levels of constitutive synthesis of stress proteins were higher than in secondary cultures, and were sustained or increased after infection with mutants of HSV-1, wt HSV-1 or wt HSV-2; and the range of synthesized late viral polypeptides appeared to be restricted in primary cells compared with secondary cells. Thus, primary cells appear to be more susceptible to activation of the stress response than secondary cells, and consequently to be less able to support lytic

infection by HSV. Possible reasons for the heightened responsiveness of primary REF to treatment with disulfiram were suggested in chapter 4 (namely, traumatic injury and metabolic stress), and these may apply also to primary CEF.

Effects of Induction of the Stress Response upon Infection by HSV.

Experiments revealed an additional feature of interaction between the stress response and infection by HSV-1: that treatment of cells (secondary REF, chapter 4.2; secondary CEF, chapter 6.1, 6.2 and 6.3; BHK cells, chapter 6.3; HeLu cells, chapter 6.4) with stress-inducing reagents prior to, or early in, infection by wt HSV-1 resulted in inhibition of synthesis of viral polypeptides. Similar effects were observed in HeLu cells subjected to heat shock (chapter 11.2). In addition, relative levels of synthesis of viral polypeptides were altered, so that synthesis of IE viral polypeptides Vmw IE 175 and Vmw IE 63 was sustained in reagent-treated CEF (chapters 5.3, 6.2 and 6.3), BHK cells (chapter 6.3) and HeLu cells (chapter 6.4). This effect may have reflected a general retardation of viral replication, or may have been in compensation for the lack of inhibition of expression of cellular genes under these conditions (in view of the inhibitory effect of IE viral polypeptides upon the stress response; chapter 5.3). It may be relevant that HSV-1 mutants defective in the virion-associated shut-off in host protein synthesis cause overproduction of IE viral polypeptides in infected cells, compared with wt virus (Read & Frenkel, 1983).

The same effects were exerted on the synthesis of viral polypeptides in cells infected with wt HSV-1 or tsK in the presence of disulfiram and cycloheximide, following the removal of the reagents (chapter 6.5): synthesis of viral polypeptides was reduced, and relative levels of synthesis of IE viral polypeptides were altered (synthesis of Vmw IE 175 and Vmw IE 63 was sustained, whereas synthesis of Vmw IE 110 and Vmw IE 136'143 was reduced). Thus, factors affecting the levels of synthesis of different IE viral polypeptides may have been activated in the absence of protein synthesis, during the period of treatment of infected cells with disulfiram and cycloheximide.

Treatments which provoked a stronger stress response were more effective in reducing synthesis of viral polypeptides: for example, treatment with 50 uM sodium arsenite was more effective in both respects than treatment with 0.3 uM disulfiram (chapters 4.2 and 6.3); and continuous treatment with either disulfiram or sodium arsenite during infection was more effective than temporary treatment prior to infection (chapter 6.3). Furthermore, the degree of inhibition of synthesis of viral polypeptides increased with the period of pre-treatment of cells with disulfiram, and correlated with the intensity of the stress response induced (chapter 6.1). This suggests that reagent-mediated induction of the stress response affects the synthesis of viral polypeptides directly, rather than through other, unrelated effects of the reagents.

It is concluded that the expression of HSV-1 genes, present at high copy numbers per infected-cell genome (the m.o.i. used was 20-40), was inhibited by induction of the cellular stress response. This may have been accompanied by the suppression of transcription and translation of mRNA not encoding stress proteins, which is part of the response of cells to stress (Ashburner & Bonner, 1979; Schlesinger et al., 1982). That the expression of amplified cellular genes also may be inhibited during the stress response was demonstrated by results (not shown) of an experiment, performed in collaboration with Dr. R. Wilson and Dr. P. Sanders, using variant Chinese hamster ovary cells (KG1MS and KG1MC4-0). These cells are characterized by high activities of the enzyme glutamine synthetase, arising from amplified sequences of DNA and elevated levels of mRNA encoding this enzyme (Wilson, 1981, 1982; Sanders & Wilson, 1984). Analysis of their polypeptide synthesis revealed that the overproduction of the glutamine synthetase polypeptide was inhibited by treatment of the variant cells with sodium arsenite, while the synthesis of stress proteins (previously identified in Chinese hamster ovary cells by Subjeck & Sciandra, 1982) was induced.

Processing of Viral Polypeptides in Disulfiram-Treated CEF.

Disulfiram inhibited the processing of Vmw IE 175 and Vmw IE 68 in wt HSV-1-infected and tsK-infected CEF (chapters 6.5 and 7.2). The form of Vmw IE 175 that was produced co-migrated with, and may correspond to,

the precursor form (detected in pulse-labelled, HSV-1-infected CEF (chapter 7.2), which normally is converted to slower-migrating forms in the nuclear fraction (chapter 7.1; Preston, 1979b; Wilcox et al., 1980). Vmw IE 68 is modified to slower-migrating forms (Pereira et al., 1977) in at least two steps (Fenwick et al., 1980), and also accumulates in the nuclear fraction (chapter 7.1; Fenwick et al., 1978).

Thus, treatment of HSV-1-infected cells with stress-inducing reagents may interfere with the functioning of Vmw IE 175 and Vmw IE 68, by inhibiting their processing. However, inactivation of Vmw IE 175, while expected to result in synthesis of a tsK-like profile of IE viral polypeptides in infected cells, would not account for the reduction in synthesis of all viral polypeptides - including IE viral polypeptides - observed under these conditions (chapter 6.5). And so, the inhibition of processing of Vmw IE 175 and Vmw IE 68 could be because of the reduction in synthesis of viral polypeptides during induction of the stress response. (The second step in the processing of Vmw IE 68 may depend upon non-IE, viral functions (Fenwick et al., 1980); thus, inhibition of synthesis of viral polypeptides would be predicted to affect the processing of at least this polypeptide.)

Inhibition of processing of viral polypeptides has been reported to occur also in NDV-infected cells treated with canavanine (Hightower & Smith, 1978), and in picornavirus-infected cells subjected to heat-shock (Moore et al., 1981). The possibility therefore arises that in all of these systems, interference with maturation of viral polypeptides arises from the metabolic effects of stress, rather than from unrelated effects of the various treatments. Such interference would have important implications for the morphogenesis of virions and for the cytopathology of infections in cells synthesizing stress proteins at high levels: cells from all species examined to date constitutively produce stress proteins in substantial amounts (Schlesinger et al., 1982b); and it has been established that the stress response is induced during infections by several other viruses (Nevins, 1982; Collins & Hightower, 1982; Drahos & Hendrix, 1982; Khandjian & Turner, 1983).

In conclusion, induction of the stress response causes modification of the cytopathogenicity of HSV-1, and may alter the

susceptibility of cells to productive infection. Studies on the inhibition of viral replication in stressed cells may therefore provide information relevant to the development of latent (Baringer, 1974) or transforming (Galloway & McDougall, 1983) infections by HSV.

Electrophoretic Separation of Forms of IE Viral Polypeptides.

The processing of IE viral polypeptides in wt HSV-1-infected and tsK-infected CEF and BHK cells was examined in experiments described in chapter 7. Results indicated that:

i) In wt HSV-1-infected and tsK-infected BHK cells, Vmw IE 175 that was synthesized on removal of a cylcoheximide-block in protein synthesis migrated to the nuclear fraction during further incubation in the presence of cycloheximide at either a PT or a NPT (chapters 7.1, 7.2.1).

ii) The gel systems that were employed allowed identification of three forms of [^{35}S]methionine-labelled Vmw IE 175 in wt HSV-1-infected cells, and three forms also in tsK-infected cells (**Fig.'s 7.1** and **7.2.1** to **7.2.2**). The cytoplasmic fractions of wt HSV-1-infected or tsK-infected BHK cells contained the fastest-migrating, precursor (a) form, which was clearly separated from forms in the nuclear fractions. (The a-form was present also in total extracts of wt HSV-1-infected or tsK-infected CEF that were treated with disulfiram.) The nuclear fraction of wt HSV-1 infected cells contained the slowest-migrating form (c), together with a variable quantity of an intermediate, poorly resolved (b) form. Vmw IE 175 in the nuclear fraction of tsK-infected cells (b) was clearly resolved into two forms (b1 and b2; **Fig. 7.2.2**), but the c-form of Vmw IE 175 was absent from the nuclear fraction of tsK-infected cells. These results are in keeping with findings that Vmw IE 175 is converted to slower-migrating forms in the nuclear fraction of HSV-1-infected cells (Fenwick *et al.*, 1978; Preston, 1979b; Wilcox *et al.*, 1980), and that modification of Vmw IE 175 is blocked in an intermediate form in tsK-infected cells incubated at a NPT (Preston, 1979b; MacDonald, 1980).

Wilcox *et al.* (1980) detected, in HEp-2 cells infected with HSV-1 (F) three forms of [^{35}S]methionine-labelled Vmw IE 175 with different

electrophoretic mobilities, which were designated ICP 4a, 4b and 4c. ICP 4a and 4c were detected by pulse-labelling on removal of a cycloheximide-block in protein synthesis. The mobility of ICP 4a was described as gradually decreasing (between 2 and 10 h) to that characteristic of 4b. The a-, b- and c-forms of Vmw IE 175 that were detected in wt HSV-1-infected cells in these experiments are considered to represent the three forms detected by Wilcox et al. (1980); the a-form corresponding to ICP 4a, b-form to 4b, and c-form to 4c. The diffuse migration of the b-form through single concentration polyacrylamide gels (**Fig.'s 7.2.1** and **7.2.3**) supports the notion that this form undergoes further processing, as described for ICP 4b by Wilcox et al. (1980).

The separation of b- and c-forms of Vmw IE 175 that were synthesized in cells infected with wt HSV-1 was not as effective as achieved by Wilcox et al. (1980), using similar resolving gel systems, for forms synthesized in cells infected with HSV-1 (F). This may reflect genetic differences between virus strains rather than the types of host cell, since poorer resolution of forms of Vmw IE 175 was obtained by Wilcox et al. (1980) using HSV-1 (HFEM) under similar conditions. Vmw IE 182 of wt HSV-2 (HG52) also was clearly separated into three nuclear, [^{35}S]methionine-labelled forms in experiments using BHK cells (results not shown), and appeared to correspond to forms detected by Wilcox et al. (1980) using HEp-2 cells infected with HSV-2 (G).

iii) Two forms of [^{35}S]methionine-labelled Vmw IE 110 with different electrophoretic mobilities were detected in the cytoplasmic fractions of wt HSV-1-infected BHK cells, but only one form was detected in the corresponding fraction of tsK-infected cells incubated at the NPT (chapter 7.1). This provides the first indication of aberrant processing of an IE viral polypeptide other than Vmw IE 175 in tsK-infected cells at a NPT: a finding which may be significant in view of evidence that Vmw IE 175 functions in conjunction with Vmw IE 110 to activate the transcription of early viral genes (Everett, 1984). Vmw IE 110 in the nuclear fractions of wt HSV-1-infected and tsK-infected BHK cells and CEF was further resolved into two components (**Fig. 7.2.3**).

Co-Fractionation of Viral Polypeptides with Components of the

Cytoskeleton.

Chapter 8 reports the effects of infection of CEF with wt HSV-1 or tsK and of treatment of CEF with disulfiram upon the polypeptide components of Triton-resistant cytoskeletons (Brown et al., 1976). Certain similarities emerged:

i) Whether their synthesis had been induced by treatment of cells with disulfiram or by infection of cells with tsK, stress proteins partitioned similarly between Triton-resistant cytoskeletons and the corresponding supernatants. The four major species (90SP, 70SP, 35SP and 25SP) and minor species (perhaps corresponding to the 88,000-m.wt. doublet of stress proteins detected in REF (chapter 4.2; Johnston et al., 1980) were recovered in Triton-resistant cytoskeletons.

ii) No changes were detectable, by inspection of stained gels, in the polypeptide components of Triton-resistant cytoskeletons from wt HSV-1-infected, tsK-infected or disulfiram-treated cells compared with untreated, uninfected cells. However, alterations occurred in the pattern of radiolabelled polypeptides which co-fractionated with pre-existing components of the Triton-resistant cytoskeletons. Radiolabelled polypeptides (including specific components of the cytoskeleton, e.g. fibronectin, myosin, vimentin and actin) were less abundant in Triton-resistant cytoskeletons from wt HSV-1-infected, tsK-infected or disulfiram-treated cells than from untreated, mock-infected cells. This may have reflected a decrease in protein synthesis, or a decrease in the association of newly-synthesized polypeptides with the cytoskeleton, as is usually observed in heat-shocked cells (Howe & Hershey, 1984). These alterations in the components of the cytoskeleton following infection may be related to the development of viral c.p.e.. [It may be relevant, in view of the potential of HSV to induce cellular transformation, that a reduction in the abundance of fibronectin at the surface of CEF is one of the observed phenotypic alterations associated with transformation induced by treatment with tumour promoters or by infection with Rous sarcoma virus (Driedger et al., 1977). And the disturbance of cytoplasmic structures, especially those containing actin and myosin, is another change which correlates with the transformation of many cell types (Rifkin et al., 1979).]

Immediate-early viral polypeptides (Vmw IE 175, Vmw IE 136'143, Vmw IE 110, Vmw IE 68 and Vmw IE 63) were recovered in Triton-resistant cytoskeletons from cells infected with tsK at a NPT; Vmw IE 68 appeared to be recovered exclusively in this fraction, being undetectable in the corresponding supernatant. However, as preparations of Triton-resistant cytoskeletons contain (in addition to microfilaments, intermediate filaments and fibronectin) contaminating nuclei or nuclear structures (Brown et al., 1976), it cannot be excluded that those viral polypeptides that were recovered in this fraction of tsK-infected cells (and wt HSV-1-infected cells) could have been associated with nuclear components (e.g., the nuclear matrix, chromatin or the nucleolus) rather than with elements of the cytoskeleton. Fenwick et al. (1978) isolated nuclei from HSV-1(F)-infected Vero cells, and observed that Vmw IE 175, Vmw IE 110 and Vmw IE 63, but not Vmw IE 68, were released by washing the nuclei in buffer containing 0.5 M NaCl. Thus, in my experiments IE viral polypeptides other than Vmw IE 68 may have been extracted from contaminating nuclei - or cytoskeletal structures associated with nuclei - owing to the presence of (137 mM) NaCl in TGMC buffer, which was used to lyse cells and to wash the Triton-resistant cytoskeletons.

In Triton-resistant cytoskeletons from wt HSV-1-infected cells, the abundance of Vmw 155/Vmw IE 136'143, Vmw 136'130 and Vmw 40 was increased relative to the remaining detectable viral polypeptides, and processed forms of Vmw 38 were detected. This suggests not only the interaction of viral polypeptides with components of the cytoskeleton, but also the involvement of the cytoskeleton - or of a complex formed between the cytoskeleton and the nuclear matrix, such as that described by Reiter & Penman (1983), - in virogenesis. However, the possiblility that the viral polyeptides represented contaminants in the cytoskeleton preparations cannot be excluded.

Outcome of Infection of HeLu Cells with HSV at Hyperthermic Temperatures.

The effects of m.o.i. and serum concentration upon the outcome of infection of HeLu cells by HSV-1 and HSV-2 at 41.5-42 °C were examined: cultures survived infection without signs of degeneration and no infectious virus was detectable provided that the m.o.i. was less than 0.1 and the serum concentration in the medium was 10%, and not 2% or less; after infection with m.o.i. of 0.01 using 10% serum and incubation at 41.5-42 °C for not less than 6 d, and on restoration to 37 °C, there was a delay of 12-17 d before reactivation of infection to detectable levels. This agrees with previous reports of a requirement for a minimum period of incubation at 42 °C ranging from 6 to 8 d for HSV-1-infected HeLu cells (Darai & Munk, 1973; Darai et al., 1975) or rat embryo cells (Darai & Munk, 1976), and of 10 d for HSV-2-infected rat embryo cells (Cameron, 1982) to survive restoration to 37 °C without succumbing immediately to productive infection; spontaneous reactivation was observed during propagation of these cultures also (Darai & Munk, 1973; Darai et al., 1975; Darai & Munk, 1976). O'Neill (1977) noted a delay of 11-16 d for infection to be reactivated in suppressed cultures of HSV-2-infected human embryo fibroblasts, established by treatment with ara-C and incubation at 39.5-40 °C, on restoration to 37°C. (In the system of O'Neill and the system here described, reactivation of infection coincided with the appearance of one or two focal lesions per culture.) It is deduced that the delay in reactivation at 37 °C has a similar underlying cause in these various systems.

The persistence of detectable infectious virus at 41.5-42 °C in the presence of low serum and the suppression of productive infection by high serum (**Tables III** and **IV**) indicate that the reduction in virus yields at 41.5-42 °C was due to serum-dependent effects. Synthesis of viral polypeptides was undetectable 24 h p.i. in infected cells at 41.5-42 °C using 2% serum (chapter 11.3), although some infectious virus was produced (**Table III**), indicating that the majority of cells in infected cultures were unable to support viral replication under these conditions. Thus, a concentration of 10% serum may serve to reduce the number of cells susceptible to productive infection. This deduction is supported by the observation that during

prolonged treatment of infected cultures with sodium arsenite at 37 °C, the viability of a proportion of the cells was increased temporarily in the presence of EF10 (chapter 10); high serum concentrations therefore may increase the tolerance of cells to the cytotoxic effects of infection and stress. It can be excluded that a population of resistant cells was selected during infection at 41.5-42 °C, because cell survival was multiplicity- and serum-dependent, with 10% serum eliminating the formation of infectious centres. And the suppression of productive infection cannot be explained solely by the production of interferon, since all of the cells should have been equally protected through the spreading of the substance through the medium.

The importance of serum in infections at hyperthermic temperatures has not been recognized before, but Coleman & Jawetz (1961) inferred a requirement for serum in the establishment of persistently HSV-infected cultures of Maben cells at the hypothermic temperature of 31 °C. A transition from a productive to an inapparent infection was produced by elevating the concentration of serum from low to high (**Table V**), which supports the role played by serum in abortive infection at 41.5-42 °C. All cells in cultures established in this manner absorbed virus and expressed viral antigens by the end of the period of incubation at 41.5-42 °C (chapter 14), when infectious virus was undetectable. Using a m.o.i. of 0.01 this experimental procedure allows a high proportion of cells (roughly 50%) to survive abortive infection at the hyperthermic temperature, compared with similar systems where higher multiplicities were used (Darai & Munk, 1973; Darai et al., 1975; Darai & Munk, 1976; Schroder et al., 1977).

Speculative Roles of Serum in Abortive Infections of HeLu Cells with HSV at Hyperthermic Temperatures.

The minimum and saturating concentrations for serum to stimulate density-arrested (G1-phase) cells to enter DNA synthesis are 1% and 10%, respectively (Clarke et al., 1970, Temin et al., 1971). Lytic infection by HSV is influenced by the cell cycle (Hampar & Ellison, 1964; Nahmias et al., 1964; Costa et al., 1974; Linnavuori & Hovi, 1983) and is not supported by cells in mitosis (Roizman, 1961).

Hampar and Ellison (1964) established that cells in the G1 and mitotic phases are less susceptible to the cytocidal effects of HSV than cells in the post-mitotic phase, and suggested that survival depended either on the ability of non-dividing cells to activate a repair mechanism, or on the limitation of cytocidal effects to cells in the later stages of the cell cycle. Since incubation of mammalian cells at hyperthermic temperatures of 41.5-42 ^{o}C delays or blocks progression into mitosis (Sapareto, 1980), an effect of incubation of HeLu cells at these temperatures may be to cause accumulation of cells in a pre-mitotic phase in which they are less susceptible to lytic infection. Serum may reduce the number of cells remaining in a susceptible state (perhaps a post-mitotic phase) by stimulating progression to a phase originating in G1 (perhaps the G1-S boundary) in which the growth of HSV would be restricted. Alternatively, serum may stimulate a cellular repair mechanism, of the type suggested by Hampar and Ellison (1964), causing infection to be abortive and allowing infected cells to recover. Indeed, in HSV-1-infected cells the uninfected pattern of protein synthesis was recovered during incubation at 41.5-42 ^{o}C (chapter 11).

Induction of the Cellular Stress Response during Thermal Inactivation of Wt HSV-1 in Infected HeLu Cells, and Recovery of Cells from Abortive Infection with Wt HSV-1 or Wt HSV-2 at 41.5-42 ^{o}C.

The cellular stress response was activated by incubation of infected cells 41.5-42 ^{o}C, at the same time as synthesis of viral polypeptides in HSV-1-infected cells was reduced (chapters 10 and 11). Treatment of infected HeLu cells with the stress-inducing agent sodium arsenite caused reduction in virus-induced c.p.e., and inhibition of synthesis of viral polypeptides (chapter 6.4); therefore, the stress response may have been one factor inhibiting viral replication in HeLu cells incubated at 41.5-42 ^{o}C compared with 37 ^{o}C. Thus, the stress response may underly the progressive, cell-dependent inactivation of HSV previously observed to occur at hyperthermic temperatures (Gharpure, 1965; Crouch & Rapp, 1972a, b). Virus yields and the ability of virus to form plaques in infected cultures increased with the time of pre-incubation of cultures at 41.5-42 ^{o}C (chapter 11): this suggests that cells regained suceptibility to infection during adaptation to hyperthermia.

Synthesis of 70,000-m.wt. polypeptide was induced in cultures of HeLu cells by infection at 41.5-42 ^{o}C, despite the low moi (0.01) which precluded the synthesis of viral polypeptides at detectable levels (chapter 10). Induction may have been serum-dependent, having been observed only when the medium used was EF10 and not EF2 nor EF0.5 (chapter 11). This species may have represented the major stress protein, m.wt. 70-73,000.

Electron micrographs shown in chapter 11 suggest that cells underwent an abortive infection with wt HSV-2 at 41.5-42 ^{o}C, where synthesis of viral DNA was inhibited. Similar observations have been made (Hill & Field, 1973) in ganglia of chick embryos, infected *in vitro* with fresh isolates of HSV-1 or HSV-2. Whereas fibroblasts and neurons appeared permissive for viral replication, few glial cells showed evidence of abortive infection, which included the presence of intranuclear, hollow viral capsids. Darai *et al.* (1975) were unable to detect synthesis of viral DNA in HSV-1-infected HeLu cells at 42 ^{o}C, although the presence of viral antigens in infected cells was demonstrated; and Marcon and Kucera (1976) reported that synthesis of viral DNA in HSV-2-infected cells was undetectable at temperatures above 40 ^{o}C.

Immunofluorescence Studies of Carrier Cultures.

Results from *in vivo* radiolabelling indicated that cells at 41.5-42 ^{o}C underwent abortive infection with wt HSV-1 from which they were able to recover the uninfected pattern of polypeptide synthesis (chapter 11). Further evidence was provided by the reduction in immunoreactivity of cultures CI/1 and CII/1 with monoclonal antibodies against viral polypeptides (chapter 14), indicating that viral antigens were lost during culture at 37 ^{o}C. This effect may be related to the well-documented, progressive loss of viral antigens and viral DNA from HSV-transformed cell lines with time in culture (Davis & Kingsbury, 1976; Minson *et al.*, 1976; Frenkel *et al.*, 1976; Reyes *et al.*, 1979; Galloway & McDougall, 1980). However, the lack of immunoreactivity of passaged cultures of the C and B series with monoclonal antibodies appears to be contradicted by the finding that passaged BI and BII

cells, when stained with rabbit antiviral antiserum, showed strong nuclear and cytoplasmic fluorescence. Of course the rabbit antiserum may have recognized viral antigens distinct from those against which the monoclonal antibodies were directed. An alternative explanation could be that the rabbit antiserum recognized cellular antigens which were present in increased abundance in abortively infected cells. That infection of cells with HSV causes induction of synthesis of cellular stress proteins was described in chapters 1, 2 and 4; and Levine et al. (1980) showed that rabbit anti-HSV antisera may react with a specific cellular determinant in common with pre-immune sera Thus, it would be possible in theory for rabbit antiviral antisera to recognise cellular antigens that are associated with infection by HSV. Cells from culture BII were more immunoreactive with pre-immune rabbit serum than uninfected cells, which suggests an enhancement of immunological reactions of a host-specific nature. Cameron (1982) found similarly that a minor proportion (6%) of cells in the transformed rat cell line, ICTI (which had been derived by abortive infection with HSV-2 (HG52) at 42^{o}C) showed strong nuclear fluorescence when stained with con serum, whereas uninfected cells showed no similar fluorescence; he suggested that transformation by HSV-2 had induced the synthesis of antigen in rat embryo cells, with which antibodies present in the serum of a control rat can react.

Several workers also have reported that passaged human and rat embryonic cell lines obtained by abortive infection at hyperthermic temperatures display immunoreactivity with rabbit antiviral antisera in immunofluorescence experiments (Darai & Munk, 1973; Darai & Munk 1976; Cameron, 1982). The reaction of rabbit antiviral antisera with non-virus-producing, HSV-1-transformed cell lines was diminished by pre-absorbing the antisera with lyophilised foetal calf serum and liver powder (Darai & Munk 1976). That is, antigens screened by the immunological reaction were present in cellular material. Attempts by Darai and Munk (1976) to reproduce the fluorescence using human serum instead of rabbit antiviral antiserum proved unsuccessful, and they concluded that human serum did not contain antibodies against what they presumed were viral antigens present in HSV-transformed cells. An alternative interpretation would be a lack of cross-reaction between antibodies in their human sera and rat-specific, transformation-associated antigens.

Possible Nature of Infection in Carrier Cultures.

Two observations led O'Neill (1977) to discount the possibility of a persistent infection - and to conclude that virus was maintained in a non-infectious form - in suppressed, HSV-2-infected cultures: the absence of detectable infectious virus during incubation at 39.5-40 ^{o}C, and the delay before spontaneous reactivation occurred in cultures restored to 37 ^{o}C (see chapter 9). Similar considerations apply also to cultures infected with wt HSV-1 or wt HSV-2 at 41.5-42 ^{o}C in the presence of 10% serum, and to carrier cultures derived from these infections (chapter 12). However, it must be cautioned that in both the system of O'Neill (1977) and the system here described the inability to detect infectious virus in cell extracts may have reflected the insensitivity of titration assays in detecting a chronic infection: in neither case were reconstruction experiments performed to test the sensitivity of the assay used. Consequently, the data do not allow to discriminate whether cultures infected at 41.5-42 ^{o}C using EF10 and carrier cultures derived from these infections contained virus in a non-replicating or a persistently replicating state.

Evidence was not obtained that the suppression of lytic infection at 41.5-42 ^{o}C was mediated by a short-lived, repressor protein, since attempts to reactivate infection by treatment of infected cultures with cycloheximide were unsuccessful (**Table III**). (O'Neill (1977) similarly failed to reactivate infection in suppressed cultres by this means.)

It is notable that the reactivation of infection in carrier cultures, whether occurring spontaneously (chapter 12) or following superinfection with ts mutants of HSV at the NPT (chapters 12 and 13), became apparent with the onset of c.p.e. whose manner of development (the focal and restrictive nature of lesions, the formation of syncytia of cells and their detachment from the substratum) closely resembled the characteristic behaviour of persistently HSV-infected Chinese hamster (MAL) cells described by Hampar and Copeland (1965), and of persistently HSV-1-infected rat C6 glial cell lines described by Dawson et al. (1983). (Here "persistence" refers to the continuous production of

detectable infectious virus.) These two systems belong to a class of long-term, HSV-infections established in non-permissive cells, requiring no supportive measures for their maintenance, and where infectious virus was persistently produced for extensive periods during cell culture (Hampar & Burroughs, 1969; Nii, 1969; Robey et al., 1976; Vahlne & Lykke, 1977; Doller et al., 1979; Rice et al., 1979; Levine et al., 1980; Dawson et al., 1983). However, in several cases cultures spontaneously ceased to produce detectable infectious virus for variable periods (i.e. showed periods of viral quiescence), c.p.e. becoming evident only during periods of high virus titres (Nii, 1966; Vahlne et al., 1977; Doller et al., 1979; Rice et al., 1979; Levine et al., 1980; Hammer et al., 1981). C.p.e. and virus synthesis sometimes were initiated and terminated in a cyclical manner, periods of cell destruction being followed by cell regrowth (Hampar and Burroughs, 1969; Valhne and Lycke, 1977; Rice et al., 1979). In particular, persistently HSV-1-infected C6-cell cultures have been reported to show not only cyclical production of infectious virus every 15 to 17 d with prolonged periods of viral quiescence (Rice et al., 1979), but also aperiodic fluctuations in virus titres, subsequently appearing to have been "cured" of the initial persistent infection (Dawson et al., 1983). These systems may therefore be viewed as constituting a broad range of long-term HSV-infections, whose establishment was dependent upon non-permissive conditions for viral replication (Nii, 1969; Levine et al., 1980; Dawson et al., 1983), and which display variable characteristics, including the dynamics of production of infectious virus (the demonstration of cycling or aperiodic behaviour) and the length of periods of persistence or quiescence, depending upon experimental conditions. And it is proposed that the carrier cultures described in these studies, and the antecedent cultures of Darai & Munk (1979, 1976), Darai et al. (1975) and Cameron (1982), all of which were derived similarly by abortive HSV-infection of otherwise permissive cells at hyperthermic temperatures, represent a class of HSV infections in which the production of infectious virus is undetectable for prolonged periods following establishment.

Virus was present in passaged carrier cultures below the level of detection by immunofluorescence using monoclonal antibodies to HSV-1 polypeptides (where roughly $4x10^4$ cells were processed per sample; chapter 14). Since the expression of viral antigens by persistently

HSV-infected cells is closely correlated with the production of infectious virus (Hampar, 1966a, b; Nii, 1969; Doller et al., 1979; Dawson et al., 1983), any persistent infection of these cultures would have been maintained at a low level. Virus was also undetectable by the less sensitive techniques - compared with immunofluorescence or titration (where $2x10^6$ cells in total were sonicated and assayed per sample) - of Southern blot analysis (at the level of about 1 viral genome per cell; chapter 15), or by transfection of cellular DNA (at the level of two genomes per cell; chapter 12). Other workers also were unable to detect viral DNA in cultures of human embryo (Darai et al., 1975) or rat (Cameron et al., 1985) cells surviving abortive infection with HSV-1 or HSV-2 at 42 ^{o}C, unless transformed rat cell lines were isolated which persistently produced infectious virus (Darai & Munk, 1976).

Hampar (1966a) reported that cell proliferation parallelled the increased synthesis of infectious virus in persistently infected MAL-cell cultures; and Darai and Munk (1976) reported that in the producer cell lines obtained by abortive infection of rat embryo cells HSV-1 (ANG) at 42 ^{o}C, bursts of virus production were observed after renewal of the culture media or after trypsinization and passaging of the cells. However, in experiments here described, no correlation was observed between the trypsinization of cells and spontaneous reactivation in carrier cultures. Dawson et al. (1983) noted similarly that the fluctuations in virus titres in persistently infected C6 cultures could not be explained by effects of sub-culture.

Involvement HSV-Induced Transformation in Abortive Infections at Hyperthermic Temperatures.

It is postulated that hyperthermia was necessary to moderate the cytopathogenicity of HSV in the initial stages of infection and to provide conditions allowing the establishment of "carrier" cultures. It is generally accepted that the transformation of cells by HSV depends upon abortive infection, where the cytocidal effects of the virus are prevented (Rapp & Duff, 1973); and the incubation of HSV-infected cells at hyperthermic temperatures is one means by which the virus' transforming potential is revealed (Darai & Munk, 1973; Darai & Munk,

1975; Darai & Munk, 1976; Cameron, 1982). The studies of Darai and Munk (1976) may be crucial to an understanding of the nature of these infections: cultures of rat embryo cells surviving infection at the hyperthermic temperature of 42 ^{o}C were heterogeneous with respect to cellular morphology, being either epithelial-like or spindle-shaped, and derived cell lines showed a correlation between the production of infectious virus, oncogenic potential and resistance to superinfection: epithelial-like cells were non-virus-producing, and spindle-shaped cells persistently developed c.p.e. and produced infectious virus. The former cell lines were non-tumorigenic in rats and failed to form foci in soft agar, whereas the latter cell lines were tumorigenic and formed foci in soft agar, showing efficiencies of colony formation between 60 and 70%. In the experiments described in this thesis, the observed alterations in cellular morphology in abortively wt HSV-1-infected and wt HSV-2-infected HeLu cells, and the acquisition by abortively wt HSV-2-infected HeLu cells of the ability to form colonies in soft agar, indicate that the cells had acquired at least some of the properties associated with transformation. The absence of colony formation by wt HSV-1-infected cells in these experiments, compared with colony formation by abortively HSV-1 (ANG)-infected rodent cells in the study of Darai and Munk (1976), may reflect strain-dependent effects; Buultjens and Macnab (1981) reported that three rat embryo cell lines which had been transformed by HSV-1 17 syn $^{+}$ did not express many of the phenotypic properties associated with cellular transformation, whereas an HSV-2 (HG52)-transformed cell line expressed many.

Reactivation of Infection in Carrier Cultures by Superinfection with Ts Mutants of HSV at NPT.

On superinfection of carrier cultures with temperature-sensitive mutants of HSV at the NPT, infections by the original infecting viruses were reactivated (chapters 12 and 13). Although infection was reactivated by superinfection of carrier cultures with tsK, a mutant which is defective in Vmw IE 175 and induces synthesis of only the immediate-early class of viral polypeptides in infected cells (Preston, 1979a, b; Watson & Clements, 1980), this need not signify that IE viral polypeptides were sufficient to reactivate infection, since complementation by the resident virus might have permitted the involvement of later functions

of the superinfecting virus. Until the state of the viral genome in these carrier cultures is elucidated, mechanisms of reactivation must remain speculative. But it is anticipated that if virus were non-replicating, reactivation would occur due to functions of the superinfecting virus overcoming the block in viral replication; and if virus were persistently replicating at a low level, lytic functions of superinfecting viruses could overcome the resistance of a sufficient number of cells to exacerbate infection to detectable levels.

Genetic Variation of Reactivated Isolates of Wt HSV-2.

Analysis of restriction profiles of DNA from reactivated HSV-2 isolates (chapter 13) revealed a large degree of variation in the electrophoretic mobility of only fragment BamHI g (and the related fragment, BamHI g′). This differs from the invariability of BamHI g, and from the tendency towards heterogenity in other fragments, that is observed for cloned isolates of HSV-2 propagated under permissive conditions (chapter 13; Davison & Wilkie, 1981), and may have reflected selective factors operating during the establishment or maintenance of carrier cultures, or during the reactivation of infection. The variation observed in BamHI g between reactivated HSV-2 isolates was likely to represent reduplications and deletions of a sequences at the joint region of the viral genome, since this type of heterogeneity is known to occur in strain HG52 of HSV-2 (Davison & Wilkie, 1981). Reduplications of the a sequence are considered by Davison and Wilkie (1981) to occur as a result of misaligned genetic recombination or by direct ligation of the termini of the L and S components during the replication and maturation of viral DNA. Variations corresponding to those in BamHI g were absent from the S (and L) terminal fragment, BamHI u (and BamHI v), of reactivated HSV-2 isolates; and this may be related to the fact that multiple a sequences are not observed at the S terminus of HSV-2 (HG52) (Davison & Wilkie, 1981).

Variation in the electrophoretic mobility of BamHI g similar in magnitude to that observed for reactivated HSV-2 isolates has been reported to occur between clinical isolates of HSV-2, in a strain-independent manner (Chaney et al., 1980). And in an

epidemiological study conducted by Smith et al. (1981), BamHI g was the fragment showing the highest frequency of variation between paired isolates from patients suffering primary HSV-2-infections. For non-primary infections, most paired isolates demonstrated no heterogeneity in their restriction profiles. To account for these results, it was proposed by Smith et al. (1981) that the viral genome is altered during adaptation to the host, several cycles of viral replication being necessary before variation is observable, and that the genome of adapted virus is invariant thereafter. The data of Smith et al. (1981) and the restriction profiles for reactivated isolates of HSV-2 obtained in these experiments agree with the notion that heterogeneity in otherwise variable restriction fragments is reduced during prolonged infections by HSV, and it is concluded that studies of the genetic variation of HSV in carrier cultures established by abortive infection at hyperthermic temperatures may have relevance to infections in vivo.

Detection of Cellular Sequences Hybridizable to the DNA of HSV-1 or of Plasmids Bearing HSV-1-sequences.

Results of Southern blot hybridizations (chapter 15) indicated that HSV-1-virion DNA and plasmid DNA containing HSV-1-specific sequences are homologous with sequences in the DNA of uninfected human cells, and also raise the possibility that fragments of DNA from different regions of the genome hybridize to cellular sequences, with varying efficiencies. The stringency of the hybridization and washing conditions indicates that these cellular sequences are (G+C) rich, and either highly homologous or partially homologous and highly reiterated. Cellular sequences homologous to HSV-1 or HSV-2 DNA, in particular to the repetitions (TR_S) in the S component, have been detected in the human genome (Peden et al., 1982; Puga et al., 1982; Park, 1983) and are transcribed in normal tissues (Maitland et al., 1981). Such sequences have been detected also in the genome of the mouse (Puga et al., 1982) and the rat (Park, 1983), and regions homologous to the short repeats of the viral genome are transcribed in control and HSV-transformed rat-cell lines (Cameron, 1982).

Puga et al. (1982) investigated the nature of the homology

between sequences from mouse-cell or human-cell DNA and the terminal repetition of the TR_S component of the genome of HSV-1. From a library of cloned DNA, prepared from the trigeminal ganglia of mice latently infected with HSV-1, were obtained three recombinant clones which cross-hybridized to each other and to the TR_S component of the viral genome. The region of homology was mapped to a 40-350 bp sequence contained within a 1.1 kb SmaI fragment which is situated approximately 400 bp from the terminus of TR_S (that is, within BamHI k, the HSV-1-specific sequence carried by plasmid pBam k, used in these experiments). The insertion sequences in these clones, as well as the SmaI fragment of viral DNA containing the region of homology, hybridized to DNA from the brains of uninfected mice with a pattern indicating the presence of repetitive sequences, and also hybridized to discrete EcoRI fragments of human DNA. It was concluded from patterns of hybridization that the insertion sequences represented intermediate repetitive sequences of mouse-cell DNA, that they contained a region highly homologous with a short sequence of the viral TR_S, and that such sequences are evolutionarily conserved in mammalian DNA. (The possible correspondence between the data of Puga et al. (1982) and those data presented here was noted in chapter 15.)

Peden et al. (1982) also detected strong homology between human-cell DNA and several specific regions of the genomes of HSV-1 and HSV-2. The cellular sequences were estimated to be repeated from 10^3 to 10^5 times per human-cell genome, and the viral sequences were located predominantly within the L and S inverted repeat regions and near the centre of the L unique region, correlating with regions of inter- and intra-strain size heterogeneity in the genomes of HSV-1 and HSV-2. The authors speculated that this heterogeneity might reflect interactions between viral and cellular DNA, in which either the inverted repeats or the entire S segment act as insertion sequences or translocatable elements (Skare & Summers, 1977); and the finding of homology to repetitive cellular DNA sequences was considered consistent with the notion that imprecise integration or excision of viral sequences, or homologous recombination with viral sequences, might occur at such sites in the cellular genome. (These hypotheses therefore involve different mechanisms for the generation of size heterogeneity to that proposed by Davison and Wilkie (1981), which involves recombination between sequences of replicating, viral DNA.) None of the cloned

fragments of viral DNA tested in the study of Peden et al. (1982) consisted of HSV-1-specific sequences corresponding with those carried by pBam k and pBam f, the plasmids used in these experiments. However, minor sites of homology in the HSV-1 (MP) genome were reported to include BamHI q and Eco RI k, which share sequences homologous with fragment BamHI k.

Apart from herpesviruses, nucleic acid sequence homology with regions of the host genome has been found for adenovirus type 2 (Jones et al., 1979), SV40 (McCutchan & Singer, 1981; Jelinek et al., 1980) and retroviruses (Stehelin et al., 1976; Frenkel et al., 1979; Oskarsson et al., 1980). Furthermore, such homologous sequences may be expressed in normal human tissues (Jones et al. 1979) as reported for HSV-related sequences (Maitland et al., 1981). Cellular sequences homologous with the origin of replication of SV40 have been identified as the Alu-family of closely-related, repetitive sequences (Jelinek et al., 1980; McCutchan & Singer, 1981), which represent 5% of the genome, being repeated about 300,000 times or every few thousand bp throughout the genomes of the Chinese hamster, monkey and man (Jelinek et al., 1980; McCutchan & Singer, 1981); the homology with SV40 consists of 14 of the 30 bp of the most highly conserved segment. This homology prompted the suggestion that the Alu-family of interspersed repetitive sequences might function as origins of replication in mamalian cells (Jelinek et al., 1980). The homologous components are grouped in viral DNA but disordered in monkey-cell DNA (McCutchan and Singer, 1981). However, Peden et al. (1982) have shown that the homology between cellular DNA and HSV is more extensive and abundant by several orders of magnitude than the homology between cellular DNA and SV40.

Amplification of Hybridizable Cellular Sequences following Abortive Infection of HeLu Cells with Wt HSV-1 at Hyperthermic Temperatures.

HSV-1 virion DNA, and DNA from plasmids bearing HSV-1 sequences, showed increased hybridization to DNA from cultures abortively infected with wt HSV-1 at 41.5-42 ^{o}C compared with uninfected cultures, which may be explained by the amplification or rearrangement of repetitive cellular sequences (chapter 15). Infection with HSV-1 or HSV-2 has been shown to

cause amplification of SV40 sequences integrated into the DNA of Chinese hamster ovary cells (Schlehofer et al., 1983), as well as numerous other effects upon host DNA, including: chromosomal aberrations (Hampar & Ellison, 1961); stimulation of host DNA synthesis (Maroon & Kucera, 1976); mutagenic activity for host cell DNA (Schlehofer & Zur Hausen, 1982); induction of host DNA repair (Lorentz et al., 1977; Nishiyama & Rapp, 1981); and activation of endogenous retroviral genes (Hampar et al., 1976).

Repetitive sequences are a characteristic feature of the organization of animal-cell DNA (Davidson and Britten, 1973) and may be involved in regulating the expression of structural genes (Davidson et al., 1977). Roberts et al. (1983) have proposed a model for the amplification of endogenous cellular genes that involves repetitive genomic sequences; according to this model, amplification is a rapid event, occurring during one cell-cycle, and generates a tandem array of irregularly repeated sequences via replication at a single locus and homologous recombination.

There is increasing evidence that transformed and tumours cells display rearranged or amplified genes, including oncogenes (Rowley, 1980; Balaban-Malenbaum & Gilbert, 1977; Bishop, 1983; Kuff, 1983). Morphological transformation is a consequence of infection with HSV at hyperthermic temperatures (Darai & Munk, 1973; Darai & Munk, 1976; Cameron, 1982), and these abortively HSV-infected cultures showed morphological alterations soon after their establishment (chapter 12). These results may have reflected DNA rearrangements or amplification associated with the process of HSV-induced cellular transformation.

SUGGESTIONS FOR FUTURE WORK.

An important implication of the results of this study is that induction of the cellular stress response may alter the susceptibility of cells to productive infection by HSV. This, in turn, suggests that the cytopathogenicity of HSV could be influenced by cellular factors manifest by high levels of constitutive synthesis of stress proteins, and may have some bearing on the development of latent or transforming infections. To substantiate these hypotheses would require interactions between the cellular stress response and infection by HSV to be investigated in a wider range of cell types (for example, in cells of nerve tissue, the site of herpesvirus latency.) It would be of particular interest to confirm that primary cells are more sensitive to stress-inducing agents; and that cells which are more susceptible to activation of the stress response are, in general, less able to support productive infection by HSV.

Concerning induction of the stress response by infection with HSV, it would be useful to determine if any particular IE viral function(s) is involved; this might be studied by use of mutants defective in IE viral polyeptides other than Vmw IE 175, as they become available. The loss of response of infected cells to treatment with disulfiram several hours p.i. is a finding worth investigating further, as it implies a loss of cellular control processes during infection.

Procedures were described for the derivation of carrier cultures following abortive infection with HSV at hyperthermic temperatures: the nature of the transition to an inapparent infection, the role of serum in causing this transition, and the possible involvement of HSV-induced transformation in the maintenance of the carrier state, should be examined. It is important to ascertain the nature of infection in carrier cultures so derived, whether they contain virus in a non-replicating or a persistently replicating state. (Accurate reconstruction experiments to test the sensitivity of the titration assay would set a lower limit to the number of infectious particles which might be carried.) The cloning of cell lines from carrier cultures would provide homogenous cell populations, which would facilitate the analysis of any viral genetic information they contained. Once the state of the viral genome in such these carrier cultures is elucidated,

mechanisms of reactivation will become more amenable to study.

Restriction enzyme mapping or DNA sequencing could be used to determine the cause of the change in electrophoretic mobility of BamHI g in reactivated isolates of HSV-2. The study of isolates reactivated from carrier cultures may provide information concerning the mechanisms of genetic variation of HSV during prolonged infections in vivo.

Cloned viral DNA must be separated from vector DNA for use in the analysis of cellular sequences which are hybridizable to viral sequences, and of hybridizable sequences which are amplified following the abortive infection of cells at hyperthermic temperatures.

Other results which should be extended are:

- the electrophoretic separation of forms of Vmw IE 175, leading to the characterization of the functional and defective forms of that polypeptide in the nuclear fractions of wt HSV-1-infected and tsK-infected cells at a NPT.

- the observation of aberrant processing of Vmw IE 110 in tsK-infected cells at a NPT, which may be significant in view of the proposed function of this polypeptide in the transcription of early viral genes (Everett, 1984).

- the possible interaction of IE viral polypeptides, Vmw IE 68 in particular, with components of the cytoskeleton.

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