EFFECTS OF DNA TUMOUR VIRUS INFECTION ON HOST NUCLEAR PROTEINS

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Abstract

Immunofluorescence techniques have been used to investigate the distributions of a number of host cell proteins upon infection with three different DNA tumour viruses: herpes simplex virus type 1 (HSV-1), adenovirus (types 2, 4 and 5) and simian virus 40 (SV40).

A number of host DNA replication proteins were shown to specifically redistribute to viral replication "compartments" labelled by antibody to the HSV-1 major DNA-binding protein (ICP8) during productive HSV-1 infection. These host proteins also colocalised to varying extents with ICP8 in the presence of a specific inhibitor of viral DNA replication which caused a marked alteration in the location of intranuclear ICP8. Two anti-oncogenic proteins, retinoblastoma and p53 were shown to relocate in the same manner as the cellular DNA replication proteins during both productive and abortive HSV-1 infections, raising the possibility that these proteins may be associated with DNA replication complexes in uninfected cells. Transfection experiments showed that the seven HSV-1 proteins shown to be essential for DNA replication can induce replication compartment formation in uninfected cells.

Host DNA replication proteins were also redistributed during productive (but not abortive) infection with adenovirus, although, of those tested, only proliferating cell nuclear antigen (PCNA) was detected in viral replication compartments. A specific population of PCNA was also detected at novel discrete nuclear foci.

SV40 infection resulted in the redistribution of single-stranded DNA-binding protein (SSB) to foci which enlarged as infection progressed and also contained SV40 large T antigen. SV40 infection of adenovirus-transformed 293 cells caused p53 to move from the cytoplasm to the nucleus. SV40 co-infections with adenovirus and HSV-1 were also studied.

The production and characterisation of monoclonal antibodies to the HSV-1 major DNA binding protein (ICP8) and polymerase accessory factor (UL42) are also described.
# Table of Contents

1. Introduction .........................................................................................................15

Compartmentalisation of DNA replication machinery ...........................................15

- Prokaryotes ...........................................................................................................15
- Eukaryotes ............................................................................................................15

Viruses of eukaryotes ...........................................................................................15

Eukaryotic organisms ............................................................................................15

Structural organisation of DNA replication sites ...................................................16

- The nuclear matrix .................................................................................................16

- Organisation of replication sites within the nucleus .............................................17

"Replication machines" ..........................................................................................18

- Prokaryotes ............................................................................................................18

- Viruses of prokaryotes .........................................................................................18

  - *Escherichia coli* (E. coli) ....................................................................................19

- Viruses of eukaryotes ............................................................................................21

  - Herpes simplex virus ..........................................................................................21

  - Major DNA binding protein / infected cell protein 8 (ICP8) .........................23

  - DNA polymerase ................................................................................................25

  - DNA polymerase accessory protein (UL42) .....................................................26

  - Origin-binding protein (OBP) .............................................................................26

  - Helicase-primase complex ..................................................................................27

- Adenovirus ............................................................................................................28

  - Terminal protein (TP) ..........................................................................................29

  - DNA polymerase (Ad Pol) ..................................................................................29

  - DNA-binding protein (DBP) .................................................................................30

  - Nuclear Factor I (NF-I) .......................................................................................32

  - Nuclear Factor II (NF-II) ....................................................................................33

  - Nuclear Factor III (NF-III) ..................................................................................33

  - pL ..........................................................................................................................33

- Simian virus 40 (SV40) .......................................................................................34

  - Large Tumour antigen (T Ag) .............................................................................36

  - DNA Polymerases ...............................................................................................40

  - Proliferating cell nuclear antigen (PCNA) .........................................................42

  - Single-stranded DNA binding protein (SSB) ......................................................42

  - Replication factor C (RF-C) .................................................................................43
Topoisomerases (topos) .............................................44
Other proteins ............................................................44
Eukaryotic organisms ......................................................45
Yeast ......................................................................................45
Xenopus laevis eggs .............................................................46
Mammalian cells ...............................................................47

2. Materials and methods ..........................................................48

1. Cells .........................................................................................48
   a) Cell types ...............................................................................48
   b) Cell culture ............................................................................48
   c) Cell staining ...........................................................................48
   d) Metabolic labelling of cells ...................................................50
   e) Preparation of cell extracts ................................................51

2. Viruses .....................................................................................51
   a) Virus stocks ...........................................................................51
   b) Viral infections ......................................................................52

3. Monoclonal antibody production .................................................53
   a) Antigens ...............................................................................53
   b) Injection regimes ...................................................................53
   c) Hybridoma fusions ..............................................................54
   d) Single-cell-cloning ...............................................................55
   e) Production of supernatant and ascites ................................ 55
   f) Purification of antibodies ....................................................55
   g) Labelling of antibodies .......................................................56

4. List of antibodies ......................................................................57

5. Immunochemical Techniques ..................................................59
   a) Immunoblotting ...................................................................59
   b) Immunoprecipitation .........................................................60
   c) Enzyme-linked immunoabsorption assays (ELISA’s) ...............61

6. DNA techniques .......................................................................61
   a) Plasmids ...............................................................................61
   b) Transformation into competent cells .....................................62
   c) Large-scale plasmid preparation .........................................62
   d) Calcium phosphate transfection .........................................62

3. Herpes simplex virus ..................................................................64
   Distribution of infected cell protein 8 (ICP8) in infected cells ..64
Distribution of SSB and PCNA in PAA-treated HSV-1-infected cells.................................64
Distribution of the tumour-suppressor proteins Rb and p53 in PAA-treated HSV-1-infected cells.................................70
Distribution of SSB and PCNA during productive HSV-1 infection........................................70
Distribution of Rb and p53 during productive HSV-1 infection........................................70
Controls .........................................................................................................................72
No physical association is detectable between ICP8 and colocalised proteins ..................72
Effects of HSV-1 infection on the distribution of other host nuclear proteins......................76
The distribution of PCNA changes when ongoing viral DNA replication is inhibited .......77
The redistribution of PCNA upon HSV-1 infection requires protein synthesis ..........77
Formation of replication compartments during productive infection of growth-arrested cells.........................................................81
A relationship between the growth state of the host cell and the number of viral prereplicative sites.....................................................82
Distribution of transiently-expressed ICP8 ...................................................................84
Distribution of transiently-expressed ICP8 in the presence of other HSV-1 DNA replication proteins...........................................84
Discussion .......................................................................................................................87
Cellular DNA replication proteins, Rb and p53 colocalise with ICP8 in HSV-1-infected cells.................................................................87
Do cellular DNA replication proteins participate in HSV-1 DNA replication?...............88
Rb and p53 are redistributed to the same sites as cellular DNA replication proteins......90
HSV-1 replication in growth-arrested cells......................................................................91
The redistribution of cellular DNA replication proteins cannot be mediated by ICP8 alone, but does occur in the presence of all seven HSV-1 DNA replication proteins.........................................................92
How are cellular DNA replication complexes redistributed?........................................93
4. Adenovirus

Adenovirus DNA-binding protein (DBP) is associated with replicating DNA in infected cells.

SSB and histones show similar distributions in adenovirus-infected cells.

Distinct populations of PCNA localise to different sites in adenovirus-infected cells.

Distribution of other proteins in adenovirus-infected cells.

Effect of 1-beta-D-arabinofuranosylcytosine (araC) on DBP distribution in adenovirus-infected cells.

SSB and histones are excluded from replication compartments in cells productively infected with Ad2 but are not redistributed in the presence of araC.

Comparison of PCNA and DBP distributions in cells infected with Ad2 in the presence or absence of araC.

Distributions of other proteins compared to DBP in cells infected with Ad2 in the presence or absence of araC.

The number of DBP foci in araC-treated Ad2-infected cells is dependent on the growth state of the host cell.

Discussion.

Host proteins are differentially included in adenovirus replication compartments.

SSB and histones are excluded from adenovirus replication compartments.

Two populations of PCNA are present in adenovirus infected cells.

Distributions of Rb and p53 in adenovirus infected cells.

Other proteins.

Effect of araC on the redistribution of host proteins by adenovirus.

Do the redistributed cellular DNA replication proteins function in adenovirus DNA replication?

Effects of the growth state of the host cell on the infection process.

5. Simian virus 40

SSB redistributes to intensely-staining foci during SV40 infection.
No other host proteins were shown to be colocalised with SSB in SV40-infected cells .....................................................124
SSB foci do not correspond to sites of replicating DNA.............130
SSB foci do not correspond to nucleoli........................................130
T Ag becomes colocalised with SSB as infection proceeds.............................................................130
Distributions of T Ag and host proteins in SV40-infected 293 cells ............................................................................................130
Interactions of SV40 with other viruses ........................................136
Discussion......................................................................................................139
The functional role of the SSB foci in SV40-infected cells ....................................................................................................139
Localisation of other host proteins in SV40-infected CV-1 cells.................................................................................................140
Localisation of host proteins in SV40-infected 293 cells .............141
Interactions of SV40 with HSV-1 ....................................................143
Interactions of SV40 with adenovirus .............................................144

6. Production and characterisation of monoclonal antibodies recognising HSV-1 DNA replication proteins ...........................................................145
ICP8 Fusion .............................................................. 145
Antibody production ....................................................... 145
Antibody characterisation...................................................146
UL42 Fusion ......................................................................................152
Antibody production ..............................................................152
Antibody characterisation ........................................................153
HSV Pol Fusion...................................................................................160
Antibody production ..............................................................160
Antibody characterisation ........................................................164
Discussion......................................................................................................165
Properties of the novel monoclonal antibodies ................................165
Monoclonal antibodies to ICP8.................................................166
Monoclonal antibodies to UL42....................................................167
Antibodies 1D5 and 5H7 ..................................................................168

7. General Discussion.................................................................................................170
A comparison of the major DNA-binding proteins encoded by HSV-1, adenovirus and SV40.....................................................170
Degree of reliance on the host cell.....................................................................................171
HSV-1 and adenovirus as helper viruses for parvovirus ........................................172
Further experiments ........................................................................173
List of tables
Table 1.1. T4 DNA replication proteins ................................................................. 19
Table 1.2. E. coli. DNA replication proteins ........................................................... 20
Table 1.3. HSV-1 DNA replication proteins .......................................................... 22
Table 1.4. Adenovirus DNA replication proteins ................................................... 29
Table 1.5. SV40 DNA replication proteins ............................................................ 35
Table 1.6. Additional SV40 DNA replication proteins ......................................... 45
Table 2.1. Antibodies to cellular proteins .............................................................. 58
Table 2.2. Antibodies to viral proteins ................................................................. 59
Table 3.1. Distribution of host proteins in CV-1 cells infected with
HSV-1 .............................................................................................................. 76
Table 4.1. Distribution of host proteins in CV-1 cells infected with Ad2 .......... 118
Table 5.1. Distribution of host proteins in CV-1 cells co-infected with
Ad5 and SV40 .................................................................................................. 137
Table 6.1 Properties of the newly-produced monoclonal antibodies .......... 165
Table 7.1. Functions of viral DNA binding proteins ........................................... 170

List of figures
Figure 1.1. The SV40 origin region ................................................................. 34
Figure 3.1. ICP8 staining in the presence or absence of viral DNA
replication ........................................................................................................ 65
Figure 3.2 Cell staining of mock-infected cells .................................................. 67
Figure 3.3. Double-staining of cells infected with HSV-1 in the
presence of phosphonoacetate ......................................................................... 69
Figure 3.4. Double-staining of HSV-1-infected cells ........................................ 71
Figure 3.5. Control Western blots ................................................................. 73
Figure 3.6. Effect of preincubation of anti-ICP8 serum with ICP8
protein ........................................................................................................... 73
Figure 3.7. Western blots of ICP8 immunoprecipitations from HSV-1-
infected cell extracts ..................................................................................... 75
Figure 3.8. Effects of HSV-1 infection on snRNP and c-myc
distributions .................................................................................................. 78
Figure 3.9. PCNA distribution after addition of PAA to cells in which
HSV-1 DNA synthesis is occurring ............................................................. 80
Figure 3.10. Effect of cycloheximide on PCNA redistribution ......................... 80
Figure 3.11. Cell staining of HSV-1-infected serum-starved cells ................. 81
Figure 3.12. Cell staining of HSV-1-infected and PAA-treated, serum-starved and serum-stimulated cells. .................................................................83
Figure 3.13. 3H-T/ICP8 double-labelling of HSV-1-infected, PAA-treated cells. ........................................................................................................83
Figure 3.14. ICP8 distribution in transiently-transfected cells. ..................85
Figure 3.15. SSB distribution in cells expressing HSV-1 DNA replication proteins. .................................................................................................86

Figure 4.1. Distributions of DBP and DNA replication sites in adenovirus-infected cells. .................................................................96
Figure 4.2. DBP distributions in cells infected with different adenovirus serotypes. .........................................................................................96
Figure 4.3. Distributions of SSB and histones in adenovirus-infected cells. .........................................................................................98
Figure 4.4. Distribution of PCNA in adenovirus-infected cells. .....................100
Figure 4.5. Distributions of p53 and heat-shock protein 70 (hsp70) in adenovirus-infected cells. .................................................................102
Figure 4.6. Distributions of Rb and E1a in adenovirus-infected cells. ..........104
Figure 4.7. Effect of araC on DBP distribution in adenovirus-infected cells. .................................................................................................106
Figure 4.8. Distributions of SSB and histones in cells infected with Ad2 in the presence or absence of araC. .................................................................108
Figure 4.9. Distribution of PCNA in cells infected with Ad2 in the presence or absence of araC. .................................................................110
Figure 4.10. Distributions of p53, heat-shock protein 70 (hsp70) and Ki67 in cells infected with Ad2 in the presence or absence of araC. ...............112
Figure 4.11. Distributions of Rb and E1a in cells infected with Ad2 in the presence or absence of araC. .................................................................114
Figure 4.12. Distribution of DBP in araC-treated Ad2-infected serum-starved and serum stimulated cells. .................................................................116

Figure 5.1. Distributions of SSB, PCNA and histones in SV40-infected CV-1 cells. .................................................................................................125
Figure 5.2. Simultaneous distributions of SSB and DNA replication sites in SV40-infected CV-1 cells (24hpi). .................................................................127
Figure 5.3. Simultaneous distributions of SSB and DNA replication sites in SV40-infected CV-1 cells (48hpi). .................................................................129
Figure 5.4. Comparison of SSB and nucleolar staining. ................................131
Figure 5.5. Distributions of T Ag, SSB and VP1 in SV40-infected CV-1 cells.

Figure 5.6. Distribution of host proteins in SV40-infected 293 cells.

Figure 5.7. Cell staining of cells doubly-infected with SV40 and HSV-1.

Figure 5.8. Distribution of host proteins in cells doubly-infected with SV40 and adenovirus.

Figure 6.1. Test bleeds from mice immunised with ICP8.

Figure 6.2. Purified anti-ICP8 monoclonal antibodies.

Figure 6.3. Cell staining using anti-ICP8 monoclonal antibodies.

Figure 6.4. Western blots using anti-ICP8 monoclonal antibodies.

Figure 6.5. 35S-labelled immunoprecipitations using anti-ICP8 monoclonal antibodies.

Figure 6.6. Blot and autoradiogram of a 35S-labelled immunoprecipitation using 11E2.

Figure 6.7. Western blots of EBV- and adenovirus-infected cell extracts using anti-ICP8 monoclonal antibodies.

Figure 6.8. Test bleeds from mice immunised with UL42.

Figure 6.9. Purified anti-UL42 monoclonal antibodies.

Figure 6.10. Cell staining using anti-UL42 monoclonal antibodies.

Figure 6.11. Western blots using anti-UL42 monoclonal antibodies.

Figure 6.12. 35S-labelled immunoprecipitations using anti-UL42 monoclonal antibodies.

Figure 6.13. Level of ICP8 and HSV Pol in UL42 immunoprecipitations.

Figure 6.14. Level of anti-UL42 monoclonal antibody binding to the peptide 278A as determined by ELISA.

Figure 6.15. Level of anti-UL42 monoclonal antibody binding to HSV Pol as determined by ELISA.

Figure 6.16. Test bleeds from mouse immunised with HSV Pol.

Figure 6.17. Cell staining using 1D5 and 5H7.

Figure 6.18. Western blots using 1D5 and 5H7.
**Abbreviations**

AAV - adeno-associated virus
Ad - adenovirus
ALP - alkaline phosphatase
araC - 1-β-D-arabinofuranosylcytosine
ARS - autonomously-replicating sequence
BHK cells - baby hamster kidney cells
bp - base pair
BSA - bovine serum albumin
°C - degrees Celsius
Ci - curies
cm - centimetres
CO₂ - carbon dioxide
DAB - 3, 3', 4, 4'-tetraminobiphenyl
DBP - adenovirus DNA-binding protein
dH₂O - distilled water
(ss or ds) DNA - (single- or double-stranded) deoxyribonucleic acid
EBV - Epstein Barr virus
_E. coli._ - _Escherichia coli_
ELISA - enzyme-linked immunoabsorption assay
E4 medium - Dulbecco's modified E4 medium
FCS - foetal calf serum
FITC - fluorescein
g - grams
HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRP - horse radish peroxidase
h - hours
hpi - hours postinfection
HSV-1 - herpes-simplex virus type 1
HSV-2 - herpes-simplex virus type 2
ICP8 - infected cell protein 8
ICRF - Imperial Cancer Research Fund
IP - intraperitoneal
IV - intravenous
k - kilodaltons
M - molar
mA - milliamperes
met - medium - E4 medium without methionine
min - minutes
ml - millilitres
mm - millimetres
mM - millimolar
MVM - minute virus of mice
μCi - microcuries
μg - micrograms
μl - microlitres
μM - micromolar
NF-I (or II, or III) - nuclear factor I (or II, or III)
NGS - normal goat serum
nm - nanometres
NMS - normal mouse serum
NP40 - nonidet-P40
(d)NMP, (d)NDP or (d)NTP (where N=A, C, G, T or U) - (deoxy)nucleotide
mono-, di-, or tri- phosphate (A=adenosine, C=cytidine, G=guanosine, 
T=thymidine, U=uridine)
OBP - origin binding protein
o/n - overnight
PBS - phosphate- buffered saline type A
p.f.u. - plaque-forming units
phage - bacteriophage
pol - polymerase
(p)TP - adenovirus (pre) terminal protein
PCNA - proliferating cell nuclear antigen
Rb - retinoblastoma
RF-C - replication factor C
(c)RNA - (complementary) ribonucleic acid
rpm - revolutions per minute
RT - room temperature
S. cerevisiae - Saccharomyces cerevisiae
S. pombe - Saccharomyces pombe
SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSB - single-stranded DNA binding protein (human unless otherwise 
defined)
SV40 - simian virus 40
T Ag - simian virus 40 large tumour antigen
topo - topoisomerase
Tris - Tris (hydroxymethyl) amino methane
TRITC - rhodamine
UL42 - 65k HSV-1 DNA binding protein
V - volts
v/v - volume / volume
xg - times gravitational force
2-D - two-dimensional
# - catalogue number
1. Introduction

The main subject of this thesis is the interaction of DNA tumour viruses with host cell DNA replication complexes. Besides providing important insights into viral mechanisms of replication, work in this field can yield information on cellular DNA synthesis and its control. Since the work to be presented focusses primarily on the ultrastructural aspects of replication, it seems appropriate to review what is already known about the compartmentalisation and structural organisation of replication sites, before going on to describe the actual DNA replication machinery of different organisms.

Compartmentalisation of DNA replication machinery

Prokaryotes

There is some evidence of compartmentalisation of the replication apparatus even in prokaryotes. *Escherichia coli* (*E. coli*) has no nucleus to separate the replicating chromosome from the numerous processes occurring around it. However, the origin of replication, oriC, is specifically sequestered from dam methyltransferase (Campbell and Kleckner, 1990), probably by binding to the cell membrane (Ogden, et al., 1988). The sequestration is transient, and may be one of many factors operating to ensure that initiation of DNA replication occurs only once per cell cycle (Boye and Lobner-Olesen, 1990) (see *E. coli* replication section below).

Eukaryotes

Viruses of eukaryotes

Viruses which infect eukaryotic cells often form specialised "compartments" in which replication of viral DNA occurs during lytic infection. Such sequestration of the viral replication machinery may be a way of escaping cellular replication controls. These compartments are discussed in more detail in the "replication of viruses" section below.

Eukaryotic organisms

Eukaryotic cells have nuclei, providing a simple way of sequestering and protecting the DNA replication apparatus. Such compartmentalisation creates a microenvironment where a finer degree of control can be exerted over reactions than could occur in the macroenvironment of the whole cell.
However, many other processes related to nucleic acid metabolism take place in the nucleus e.g. transcription, ribosomal RNA and transfer RNA synthesis, and DNA repair. Thus, some level of intranuclear compartmentalisation must be employed for tightly regulated DNA replication to occur.

A readily-visible nuclear compartment is the nucleolus. Also, immunological studies have identified specific areas for messenger RNA processing and transfer RNA synthesis (Carmo-Fonseca, et al., 1989; Nyman, et al., 1986), as well as domains of unknown function, such as "nuclear spots" (Chaly, et al., 1989) and "nuclear dots" (Ascoli and Maul, 1991). Three-dimensional mapping techniques indicate that chromosomes occupy spatially separated compact domains within the mammalian cell nucleus throughout interphase (reviewed by (Haaf and Schmid, 1991)). There is some preliminary evidence for consistent spatial relationships between interphase chromosomes ((Appels, 1989; van Dekken, et al., 1989) and references therein) but this awaits more general confirmation.

**Structural organisation of DNA replication sites**

**The nuclear matrix**

The degree of intranuclear organisation described above suggests a requirement for an underlying structural framework to support it. Evidence has been presented supporting the existence of such a framework, termed the nuclear matrix. The nuclear matrix has been defined as the biochemical entity that can be isolated after sequential extraction of cells with non-ionic detergents, nuclease and high-salt buffers (Shaper, et al., 1978). There is some confusion in the literature since investigators tend to use different extraction procedures which reveal a variety of sub-nuclear structures, given names such as ghosts, matrices, scaffolds and cages (Agutter and Richardson, 1980). Most extraction procedures leave a residual highly filamentous structure containing actin and vimentin (Capco, et al., 1982) and probably many other constituents e.g. small nuclear RNA, heterogeneous nuclear RNA and enzymes involved in DNA and RNA metabolism (reviewed by (Verheijen, et al., 1988)).

Perhaps the most conclusive evidence for a physiological nuclear substructure has been provided by the isotonic extraction procedure devised by Jackson and Cook (Jackson and Cook, 1986). After coating cells with agarose, lysing, and electroeluting digested chromatin, a "nucleoskeleton"
remains which has been isolated under isotonic conditions (as far as these can be estimated).

Nuclear matrices have been detected in various types of cells. However, no matrix is seen in the relatively inactive adult chicken erythrocyte nucleus (although the more active embryonic erythrocytes do have a matrix) (Lafond and Woodcock, 1983). Also, amphibian oocyte nuclei do not possess an extensive internal nuclear matrix (Benavente, et al., 1984), perhaps reflecting their relatively dormant state prior to fertilisation. Thus, the presence or absence of an extensive nuclear matrix appears to correlate with the level of metabolic activity within the nucleus.

The nuclear matrix should not be viewed as a static "scaffold", but rather as a dynamic network, which acts as a nucleation site for a large number of metabolic processes. Immunofluorescence using antibodies directed against HeLa cell nuclear matrix proteins shows different distributions of these proteins through the cell cycle (Bhorjee, et al., 1983). Other work has shown striking changes in matrix protein location during mitosis. During prophase many matrix proteins, including lamins and small nuclear ribonucleoproteins, show a diffuse distribution throughout the cell which excludes the condensed chromosomes. This movement is reversed during telophase. Other matrix proteins, such as topoisomerase II, remain associated with the condensed chromosomes as part of a putative chromosome scaffold structure (reviewed by (Verheijen, et al., 1988)). Thus, the matrix appears to be a relatively fluid structure, reflecting the changing demands of the nuclear environments surrounding it.

**Organisation of replication sites within the nucleus**

The association of enzymes such as DNA polymerase $\alpha$, DNA primase, 3'-5' exonuclease, RNaseH, and DNA methylase with the nuclear matrix (Jackson and Cook, 1986; Tubo and Berezney, 1987a) suggests that DNA replication may be occurring on this structure. Sedimentation analysis of extracts from regenerating rat liver cells suggests that 10 and 17S "prereplicative" complexes are assembled on the matrix before being recruited into 100 and 150 S "megacomplexes" during active DNA replication (Tubo and Berezney, 1987b). Two-dimensional electrophoresis of matrix-attached DNA from mammalian cells has shown that the matrix is markedly enriched for replication forks (Vaughn, et al., 1990b). Also, some regions of *S. cerevisiae*, *S. pombe* and *Drosophila melanogaster* DNA which remain associated with the nuclear matrix in histone-depleted nuclei
(scaffold attachment regions) can replicate autonomously on extrachromosomal plasmids (Amati and Gasser, 1990). Thus, the available evidence suggests that DNA replication is associated with the nuclear matrix.

The incorporation of detectable nucleotide analogues, such as bromodeoxyuridine and biotin-11-dUTP has enabled direct visualization of newly-replicated DNA within nuclei. Transient ring-like structures representing putative replicon domains (functional units of replication) were seen in mammalian cells after incubation with bromodeoxyuridine (Nakamura, et al., 1986), whereas discrete replication granules were seen after short incubations with biotin-11-dUTP (Nakayasu and Berezney, 1989). Moreover, the latter authors saw similar patterns of biotin labelling on matrices prepared in situ by DNase extraction. Confocal microscopy studies on synchronized 3T3 cells labelled with bromodeoxyuridine and stained with antibodies directed against bromodeoxyuridine and lamin suggest a controlled progression of replication site locations through a programmed sequence during S-phase. Replication begins at a small number of sites in the nuclear interior and extends to sites throughout the nucleus, with the condensed heterochromatic and nuclear peripheral regions containing the late-replicating sites (Fox, et al., 1991).

Replication foci have also been detected in the pseudonuclei formed in Xenopus egg extracts (Mills, et al., 1989). Replication in these extracts has been shown to require both the formation of a nuclear envelope (Blow and Sleeman, 1990) and the presence of lamin LIII (Meier, et al., 1991), implying an essential role for structural elements in DNA replication.

"Replication machines"

The multiprotein complexes employed by different organisms to carry out the complex process of DNA replication have been termed "replication machines" (Alberts, 1984). Current knowledge of the composition of these machines in selected organisms is shown below, with particular emphasis on the three eukaryotic viruses studied in the results section.

Prokaryotes

Viruses of prokaryotes

The diversity of the bacteriophages (phages) is illustrated by the large number of replication strategies they employ (for reviews see (Keppel, et al.,
Most of the large, virulent phages have evolved "replication machines", as exemplified by the well-characterised phage T4 replication complex. Phage T4 has a 166kb linear genome containing at least six replication origins (reviewed by (Mosig and Eiserling, 1988)). *In vitro* studies have defined the major T4 DNA replication proteins shown in table 1.1. below.

**Table 1.1. T4 DNA replication proteins**

<table>
<thead>
<tr>
<th>GENE</th>
<th>PROTEIN</th>
<th>MOL. WT.</th>
<th>ACTIVITIES</th>
<th>FUNCTIONAL HOMOLOGUE IN SV40 SYSTEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>DNA POLYMERASE</td>
<td>110K</td>
<td>DIMER SYNTHESISES LEADING AND LAGGING STRANDS; 3'-5' EXONUCLEASE</td>
<td>POLS α, δ (AND ε?)</td>
</tr>
<tr>
<td>44/62</td>
<td>POL ACCESSORY PROTEIN</td>
<td>34K/20K</td>
<td>DNA-DEPENDENT ATPase AND PRIMER RECOGNITION COMPLEX</td>
<td>RF-C</td>
</tr>
<tr>
<td>45</td>
<td>POL ACCESSORY PROTEIN</td>
<td>24.5K</td>
<td>STIMULATES GENE 44/62 ATPase; INCREASES RATE AND PROCESSIVITY OF POL</td>
<td>PCNA</td>
</tr>
<tr>
<td>41</td>
<td>DNA HELICASE</td>
<td>58K</td>
<td>GTP/ATP-DEPENDENT 5'-3' TEMPLATE UNWINDING</td>
<td>T ANTIGEN</td>
</tr>
<tr>
<td>61</td>
<td>RNA PRIMASE</td>
<td>40K</td>
<td>TEMPLATE-SPECIFIC RNA PRIMER SYNTHESIS</td>
<td>PRIMASE SUBUNITS OF POL α</td>
</tr>
<tr>
<td>32</td>
<td>HELIX-DESTABILISING PROTEIN</td>
<td>34.5K</td>
<td>ssDNA BINDING; STIMULATES POL ACTIVITY</td>
<td>SSB</td>
</tr>
<tr>
<td>dda</td>
<td>DNA HELICASE</td>
<td>47K</td>
<td>ALLOWS FORK PASSAGE THROUGH RNA POL BLOCKS (NON-ESSENTIAL IN VIVO)</td>
<td>T ANTIGEN?</td>
</tr>
</tbody>
</table>


T4 does not make extensive use of the host replication machinery, although transcription by the host RNA polymerase is required for the primary round of initiation (Luder and Mosig, 1982). The gene 32 protein product appears to play a key role in organising the replication complex, since it interacts with many T4 replication and recombination proteins (as well as some host proteins) (Alberts, 1984; Karam, *et al.*, 1983). The
similarity between the DNA replication machinery of phage T4 and human cells implies a possible common ancestor.

**Escherichia coli (E. coli)**

The *E. coli* genome consists of a 4000kb circular, dsDNA chromosome containing a single 245bp replication origin, *oriC*. Despite its relative complexity, the replication of *E. coli* is well-characterised, largely due to genetics and in vitro biochemical studies (Kornberg, 1980). Those proteins essential for *E. coli* DNA replication are shown in table 1.2 below. In addition to these essential proteins, various auxiliary replication proteins have been identified (detailed in (McMacken, et al., 1987)).

**Table 1.2. E. coli. DNA replication proteins**

<table>
<thead>
<tr>
<th>GENE</th>
<th>PROTEIN</th>
<th>MOL. WT.</th>
<th>ACTIVITIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>dnaA</td>
<td>DnaA</td>
<td>52.5K</td>
<td>ORIGIN RECOGNITION</td>
</tr>
<tr>
<td>dnaB</td>
<td>DnaB</td>
<td>52.3K</td>
<td>DNA HELICASE; PRIMING</td>
</tr>
<tr>
<td>dnaC</td>
<td>DnaC</td>
<td>29K</td>
<td>FACILITATES DnaB BINDING TO <em>oriC</em></td>
</tr>
<tr>
<td>ssb</td>
<td>SSB</td>
<td>18.8K</td>
<td>ssDNA BINDING</td>
</tr>
<tr>
<td>dnaG</td>
<td>primase</td>
<td>65.6K</td>
<td>SYNTHESIS OF RNA PRIMERS</td>
</tr>
<tr>
<td>polC (dnaE)</td>
<td>DNA pol III (α)</td>
<td>130K</td>
<td>REPLICATIVE DNA POLYMERASE</td>
</tr>
<tr>
<td>dnaQ (mutD)</td>
<td>DNA pol III (ε)</td>
<td>27.5K</td>
<td>3'-5' PROOFREADING EXONUCLEASE</td>
</tr>
<tr>
<td>N.D.</td>
<td>DNA pol III (θ)</td>
<td>10K</td>
<td>DNA POL ACCESSORY PROTEIN</td>
</tr>
<tr>
<td>dnaN</td>
<td>DNA pol III (β)</td>
<td>40.6K</td>
<td>DNA POL ACCESSORY PROTEIN</td>
</tr>
<tr>
<td>dnaZX</td>
<td>DNA pol III (τ)</td>
<td>71.1K</td>
<td>DNA POL ACCESSORY PROTEIN (DNA-DEPENDENT ATPase)</td>
</tr>
<tr>
<td>dnaZX</td>
<td>DNA pol III (γ)</td>
<td>52K</td>
<td>DNA POL ACCESSORY PROTEIN</td>
</tr>
<tr>
<td>dnaZX?</td>
<td>DNA pol III (δ)</td>
<td>32K</td>
<td>DNA POL ACCESSORY PROTEIN</td>
</tr>
<tr>
<td>gyrA</td>
<td>DNA gyrase (α)</td>
<td>97K</td>
<td>NICKING-CLOSING</td>
</tr>
<tr>
<td>gyrB</td>
<td>DNA gyrase (β)</td>
<td>89.8K</td>
<td>ATPase</td>
</tr>
<tr>
<td>polA</td>
<td>DNA pol I</td>
<td>102K</td>
<td>PRIMER REMOVAL; GAP-FILLING</td>
</tr>
<tr>
<td>lig</td>
<td>DNA ligase</td>
<td>74K</td>
<td>JOINING OF NICKED DNA</td>
</tr>
<tr>
<td>dnaT</td>
<td>DnaT</td>
<td>19.3K</td>
<td>TERMINATION OF DNA REPLICATION</td>
</tr>
<tr>
<td>rpoA, B, C, D</td>
<td>RNA pol</td>
<td>460K</td>
<td>TRANSCRIPTIONAL ACTIVATION OF <em>oriC</em></td>
</tr>
</tbody>
</table>

Data from (McMacken, et al., 1987). (Letters in brackets denote subunits).
E. coli DNA pol III (but not pol I) shares some homology with conserved region II of the eukaryotic DNA pol α. However, other prokaryotic polymerases, including T4 DNA pol are more closely related to pol α (Wong, et al., 1988). E. coli single-stranded DNA binding protein (SSB) will support unwinding of the simian virus 40 (SV40) origin of DNA replication, when substituted for human SSB in the in vitro SV40 DNA replication systems developed by various groups (see SV40 section below). However, E. coli SSB cannot substitute fully for human SSB, which has additional functions in SV40 DNA replication (Kenny, et al., 1989).

Knowledge of the regulation of E. coli DNA replication initiation is still incomplete. The DnaA protein seems to play a key role in control of initiation (Lobner-Olesen, et al., 1989; Skarstad, et al., 1989; Skarstad, et al., 1988) and a 33k protein, IciA, has recently been identified which blocks DnaA binding to oriC, preventing DNA synthesis in vitro, although null mutants of the iciA gene show wild type growth rates in vivo. Cells with elevated IciA levels grow at a normal rate, but show a pronounced lag in growth on transfer into fresh medium (Hwang and Kornberg, 1990; Thony, et al., 1991). Other regulatory mechanisms may include: activation by transcription in the proximity of oriC, membrane attachment of DNA and inactivation of oriC by hemi-methylation (Baker and Kornberg, 1988; Boye and Lobner-Olesen, 1990; Ogden, et al., 1988; Skarstad, et al., 1990). It is likely that further replication control proteins have yet to be identified.

Viruses of eukaryotes

Three viruses have been studied in this thesis, all having quite different replication mechanisms. An outline of each is given below, in the same order as presented in the results section (see contents page).

Herpes simplex virus

Herpes simplex is one member of a family of at least 80 herpesviruses, all of which have a double-stranded (ds) DNA genome within an icosadeltahedral capsid containing 162 capsomeres (reviewed by (Roizman and Sears, 1990)). Herpes simplex exists in two forms; type-1 (HSV-1) causes oral lesions in humans, whereas type-2 (HSV-2) causes genital lesions. These viruses have a wide host range and are closely related, so that much of the following description of HSV-1 also applies to HSV-2.
The 150kb genome of HSV-1 contains three replication origins; two copies of ori₅ (in the short unique sequence) and one of ori₇ (in the long unique sequence). These were suggested by analysis of the structure of defective viral genomes and defined by the replication of plasmids containing them on transfection into HSV-infected cells (Spaete and Frenkel, 1982; Stow and McMonagle, 1983; Vlasny and Frenkel, 1982). The sequences of ori₅ and ori₇ are very similar, and one copy of either can be deleted with no adverse effect on virus growth (Longnecker and Roizman, 1986; Polvino-Bodnar, et al., 1987), implying some functional equivalence.

Analysis of HSV-1 replication products suggests that the virus employs a rolling circle method of replication (Jacob, et al., 1979). Recent studies have defined seven HSV-1 replication proteins which are necessary and sufficient for viral replication. These were identified by co-transfecting different HSV-1 restriction fragments and assaying for the replication of an accompanying ori-containing plasmid. The results obtained agreed with and extended earlier genetic and biochemical studies. The seven genes identified are shown in table 1.3. below.

Table 1.3. HSV-1 DNA replication proteins

<table>
<thead>
<tr>
<th>HSV-1 GENE</th>
<th>REPPLICATION PROTEIN</th>
<th>PREDICTED MOL. WT.</th>
<th>OBSERVED MOL. WT.</th>
<th>IN REPLICATION COMPARTMENTS?</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL5</td>
<td>COMPONENT OF HELICASE-PRIMASE COMPLEX. (DNA HELICASE?)</td>
<td>99K</td>
<td>95K</td>
<td>NO</td>
</tr>
<tr>
<td>UL8</td>
<td>COMPONENT OF HELICASE-PRIMASE COMPLEX</td>
<td>80K</td>
<td>75K</td>
<td>NO</td>
</tr>
<tr>
<td>UL9</td>
<td>ORIGIN-BINDING PROTEIN</td>
<td>94K</td>
<td>82K</td>
<td>YES</td>
</tr>
<tr>
<td>UL29</td>
<td>MAJOR DNA-BINDING PROTEIN (INFECTED CELL PROTEIN 8)</td>
<td>130K</td>
<td>130K</td>
<td>YES</td>
</tr>
<tr>
<td>UL30</td>
<td>DNA POLYMERASE</td>
<td>136K</td>
<td>140K</td>
<td>YES</td>
</tr>
<tr>
<td>UL42</td>
<td>DNA POLYMERASE ACCESSORY PROTEIN</td>
<td>51K</td>
<td>62K</td>
<td>YES</td>
</tr>
<tr>
<td>UL52</td>
<td>COMPONENT OF HELICASE-PRIMASE COMPLEX</td>
<td>114K</td>
<td>115K</td>
<td>NO</td>
</tr>
</tbody>
</table>

(Data from (Olivo, et al., 1989)).
Attempts have been made to reconstitute viral replication in vitro, using highly purified forms of these seven proteins. However, as yet, this has not been accomplished, suggesting that either the correct reaction conditions have not yet been defined, or that cellular proteins may also be needed for HSV replication. HSV replication in resting cells may also require additional viral proteins. The viral thymidine kinase and ribonucleotide reductase are essential for normal viral DNA replication and growth in resting cells, whilst thymidine kinase at least is dispensable in proliferating cells (Goldstein and Weller, 1988; Jamieson, et al., 1974; Tenser, 1991). The viral alkaline nuclease may also be required for viral replication (Moss, 1986), though a physiological role specifically in DNA synthesis has not yet been demonstrated (Knipe, 1990).

The seven proteins above have been studied in detail since their replicative role was discovered. An outline of the properties of each is given below.

**Major DNA binding protein / infected cell protein 8 (ICP8)**

ICP8 can bind to both single-stranded (ss) DNA and to dsDNA in vitro in a sequence-independent manner. ICP8 binds cooperatively to ssDNA, and has a higher affinity for ssDNA than for dsDNA (Bayliss, et al., 1975; Purifoy and Powell, 1976; Ruyechan, 1983; Ruyechan and Weir, 1984). The use of mutants, in vitro translation products and renatured protein fragments (Gao and Knipe, 1989; Leinbach and Heath, 1989; Wang and Hall, 1990) has identified several regions in the protein which influence DNA binding; amino acid regions 332-564 (includes a zinc finger motif) and 571-1160 are thought to contain DNA binding sites, although interpretation of results is complicated by an inhibitory effect of the N-terminus.

Two conformational forms of ICP8 have been detected; a slower migrating form 'a', which is thought to be oxidised to produce a disulphide-bonded folded form 'b' (Knipe, et al., 1982). In the absence of DNA, helical filaments of ICP8 have been observed in the presence of magnesium in conditions of low ionic strength (O'Donnell, et al., 1987b). The physiological significance of these structures is not known.

ICP8 appears to be a multifunctional protein. Putative functions are detailed below.
(i) Viral DNA replication

The abundance of ICP8 and its DNA binding properties suggest a function analogous to that of *E. coli* SSB (Kornberg, 1980) i.e. to bind to ssDNA at replication forks, creating an activated template for replication by the DNA polymerase. However, the data regarding the effect of ICP8 on the viral DNA polymerase varies according to the type of DNA template used. ICP8 has been reported to stimulate HSV-1 DNA polymerase activity on heat-denatured salmon sperm DNA by 1.7-fold but to inhibit its activity on singly-primed ssDNA. HSV-1 DNA polymerase activity on a circular duplex DNA template appears to be enhanced in the presence of ICP8 and infected cell proteins (O’Donnell, *et al.*, 1987b; Ruyechan and Weir, 1984). It has been suggested that ICP8 increases the processivity of the enzyme by melting out secondary structure in the template (Hernandez and Lehman, 1990).

(ii) Organisation of viral replication complexes within the nucleus

The suggestion of a structural role for ICP8 arises mainly from immunofluorescence studies which have identified two different locations for ICP8 within the host cell nucleus; "prereplicative sites" (small, discrete foci containing ICP8 attached to a peripheral component of the nuclear matrix) and "replication compartments" (large, globular regions containing ICP8 bound to viral DNA). Prereplicative sites are seen in cells where viral DNA replication has been inhibited (either by chemicals, or by the use of viral mutants) and also in productively-infected cells during early stages of infection (presumably stages independent of HSV polymerase function). Replication compartments are formed in infected cells within which viral replication is occurring (de Bruyn Kops and Knipe, 1988; Quinlan, *et al.*, 1984).

Bromodeoxyuridine labelling of cells infected HSV-1 in the presence of a specific inhibitor of viral DNA replication revealed that newly-replicated cellular DNA was relocated to viral prereplicative sites. This redistribution of replicating cellular DNA was not seen when viral mutants defective for ICP8 were used to infect cells, indicating that ICP8 is required for the movement of cellular replication complexes (de Bruyn Kops and Knipe, 1988). This result, along with the ability of ICP8 to bind both to the nuclear matrix and to viral DNA gave rise to the hypothesis that ICP8 plays a role in organising cellular and viral elements into HSV replication structures. This theory is supported by recent work showing that functional ICP8 is required for the HSV-1 DNA
polymerase to localise correctly to prereplicative sites although nuclear localisation per se is independent of ICP8 (Bush, et al., 1991).

(iii) Stimulation of late gene expression

This function of ICP8 was suggested by the isolation of a trans-dominant mutant of ICP8 (d105, deleted between amino acids 1082-1169) which inhibits viral replication by around 80%, but exhibits a level of late gene expression comparable to that of cells in which replication is completely blocked (Gao and Knipe, 1991). Thus, ICP8 may be involved in determining when the switch from replication to late gene expression occurs.

(iv) Other functions

That ICP8 has other, as yet undefined, functions is implied by the existence of a mutant form of the protein (d101) which can localise to the cell nucleus and bind to DNA in vitro, but cannot support viral DNA replication (Gao and Knipe, 1989). This mutant is deleted within the amino-terminal region of ICP8, a region which has some homology to proliferating cell nuclear antigen (PCNA) (Matsumato, et al., 1987). This homology is provoking, since both ICP8 and PCNA demonstrate intranuclear movement in association with DNA replication and are proposed to interact with their cognate polymerases. However, the homology is relatively weak at the amino acid level, with many of the nucleotide matches positioned upstream of the PCNA transcriptional start site.

DNA polymerase

The HSV-1 polymerase (HSV Pol) activity is readily distinguished from that of host cell polymerases by its unique sensitivity to the compound phosphonoacetate. This drug does not appear to act by binding to the DNA template or by mimicking of polymerase substrates. Instead, it appears to act directly and specifically on the HSV-1 polymerase enzyme, blocking the elongation phase of DNA synthesis (Mao and Robishaw, 1975). The possibility of targeting anti-viral drugs to the HSV Pol has prompted extensive mutational analysis and structure-function studies of the protein, defining both drug resistance and functional domains (Haffey, et al., 1990; Marcy, et al., 1990b).

The HSV-1 polymerase gene has six regions which are conserved within a family of replicative DNA polymerases (both viral and cellular), which includes DNA polymerase α (Gibbs, et al., 1985; Quinn and McGeoch, 1985; Wong, et al., 1988). However, recent studies suggest that HSV Pol is more closely related to DNA polymerase δ (Boulet, et al., 1989). The
conserved DNA regions code for functional activities such as dNTP binding, pyrophosphate hydrolysis and substrate recognition. Purified HSV Pol contains a 3'-5' proofreading exonuclease activity (O'Donnell, et al., 1987a) and a 5'-3' exonuclease activity which is capable of removing RNA primers from a DNA template and is also active on duplex DNA (Crute and Lehman, 1989).

**DNA polymerase accessory protein (UL42)**

The UL42 gene encodes a 65k double-stranded DNA binding protein, which binds non-cooperatively to DNA in a sequence-independent manner (Gallo, et al., 1988). The UL42 polypeptide can be purified either singly, or complexed to the HSV Pol catalytic subunit (Gallo, et al., 1988; Gottlieb, et al., 1990). UL42 binds to the carboxy-terminal region of HSV Pol and increases its processivity, probably by raising its affinity for primer termini (Digard and Coen, 1990; Gallo, et al., 1989; Gottlieb, et al., 1990). In this role, it seems analogous to PCNA, which increases the processivity of polymerase δ, an enzyme closely related to HSV Pol. However, no sequence homology has been identified between UL42 and PCNA, and purified PCNA does not stimulate HSV Pol activity (Gottlieb, et al., 1990).

UL42 is present in molar excess relative to HSV Pol and characterisation of null UL42 mutants in UL42-transformed cells indicates that only small amounts of the protein are necessary for viral replication and the production of infectious virus (Johnson, et al., 1991). Also, despite being localised in replication compartments during normal infection, the UL42 (along with some HSV Pol) is not exclusively restricted to prereplicative sites in PAA-treated cells (Goodrich, et al., 1990). Thus, UL42 may have another function(s) apart from its role in viral DNA replication. UL42 is a phosphoprotein (Marsden, et al., 1987), suggesting that its function(s) may be regulated during the viral life cycle.

**Origin-binding protein (OBP)**

The UL9 gene encodes a protein (OBP) which binds specifically to multiple sites within HSV ori₅ in a cooperative manner (Elias, et al., 1990; Elias, et al., 1986; Olivo, et al., 1988). The DNA binding domain of OBP has been localised to the C-terminal portion of the protein, between amino acids 564 and 832 (Deb and Deb, 1991; Weir, et al., 1989). This region contains a helix-turn-helix motif and a pseudo-leucine zipper, both of which appear to be required for DNA binding (Deb and Deb, 1991). Efficient binding of OBP
to oriS is required for viral DNA replication (Hernandez, et al., 1991). OBP loops and distorts the AT-rich region between its two binding sites (Koff, et al., 1991), possibly opening up the origin to allow the entry of other proteins.

An apparently natural truncated form of OBP (approx 45k) forms a labile, replication-dependent complex with oriS DNA, whereas a more stable, replication-independent complex is formed between full-length OBP and oriS. Band-shift experiments indicate that a cellular factor(s) is able to bind to oriS and that this factor(s) may facilitate OBP-oriS interactions (Dabrowski and Schaffer, 1991).

OBP appears to form a dimer in solution and possesses both DNA-dependent NTPase and DNA helicase activities (Bruckner, et al., 1991; Challberg and Kelly, 1989). These properties suggest that OBP may play a role in replication initiation by unwinding the origin to allow entry of a replication complex in a manner analogous to SV40 large T antigen. However, it should be noted that unwinding of oriS by OBP has not yet been detected.

HSV-1 infection induces amplification of the viral DNA integrated into the genome of SV40-transformed human and hamster cells (Matz, et al., 1984; Schlehofer, et al., 1983). This amplification requires SV40 large T antigen, the SV40 origin of DNA replication and all the HSV replication genes except UL9. Thus, it has been proposed that large T antigen may substitute for OBP and direct the HSV-1 replication complex to SV40 replication origins (Danovich and Frenkel, 1988; Heilbronn, et al., 1990; Heilbronn and zur Hausen, 1989). The production of double-minute chromosomes in HSV-1-infected cells suggests that the virus may also induce amplification of some host genes (Chenet-Monte, et al., 1986). However, this has not yet been demonstrated directly, since the lytic mode of HSV-1 infection does not permit selection for the amplified phenotype. Transfection experiments should resolve this issue. A model similar to that put forward for HSV-1-induced SV40 amplification has been suggested for host gene amplification whereby a cellular protein(s) substitutes for OBP and directs the HSV-1 replication machinery to cellular replication origins (Heilbronn and zur Hausen, 1989). Thus, OBP may impart specificity to the HSV-1 replication complex.

Helicase-primase complex

The three polypeptides encoded by the UL5, UL8 and UL52 genes form a heterotrimeric complex containing DNA-dependent ATPase and
GTPase activities, as well as helicase and primase activities (Crute and Lehman, 1991; Crute, et al., 1988; Crute, et al., 1989). The 5'-3' helicase activity of the complex probably resides within the UL5 subunit (deduced from DNA sequence analysis (Hodgman, 1988)) and this activity is dependent on the presence of ICP8 (Crute and Lehman, 1991). A complex of only UL5 and UL52 has all the enzyme activities described above, though neither subunit demonstrates such activities independently (Calder and Stow, 1990; Dodson and Lehman, 1991). The helicase-primase subunits, along with OBP, are present in low abundance in infected cells relative to the other HSV replication proteins. Immunofluorescence using antisera raised against UL5, UL8 and UL52 fusion proteins showed faint diffuse nuclear staining of infected cells (Olivo, et al., 1989). The functional significance of this distribution is not known.

Complexed helicase and primase activities are not unique to HSV; bacteriophages T4 (gene 41 and gene 61) and T7 (gene 4) also utilise such complexes, allowing close coordination of DNA unwinding at the replication fork and priming of lagging strand synthesis.

Adenovirus

Adenoviruses are non-enveloped, regular icosahedral viruses, with dsDNA genomes of about 35kb. They generally produce respiratory, ocular and enteric disease in humans, and some serotypes are tumorigenic in animals (Horwitz, 1990). Adenovirus was the first of the eukaryotic viral DNA replication systems to be established in vitro (Challberg and Kelly, 1979). Adenovirus DNA replication can begin at either end of the genome; one strand is protein-primed and copied, displacing a single strand which circularises, forming a "panhandle" structure which can then also be primed and replicated. Thus, all replication occurs in a 5'-3' direction (Kelly, et al., 1988; Stillman, 1983).

Those proteins which are required for efficient adenovirus DNA replication in vitro are shown in table 1.4 on the following page.
### Table 1.4. Adenovirus DNA replication proteins

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>MOL. WEIGHT</th>
<th>ORIGIN</th>
<th>IN COMPARTMENTS?</th>
<th>KNOWN FUNCTION(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(PRE)TERMINAL PROTEIN</td>
<td>(80K) 55K</td>
<td>VIRAL</td>
<td>YES</td>
<td>INITIATION</td>
</tr>
<tr>
<td>DNA POLYMERASE</td>
<td>140K</td>
<td>VIRAL</td>
<td>YES</td>
<td>INITIATION AND ELONGATION</td>
</tr>
<tr>
<td>DNA-BINDING PROTEIN</td>
<td>59K (72K IN SDS-PAGE)</td>
<td>VIRAL</td>
<td>YES</td>
<td>MULTIFUNCTIONAL (SEE TEXT)</td>
</tr>
<tr>
<td>NF-I (=CTF)</td>
<td>52-66K</td>
<td>CELLULAR</td>
<td>YES</td>
<td>INITIATION</td>
</tr>
<tr>
<td>NF-II</td>
<td>30K</td>
<td>CELLULAR</td>
<td>ND</td>
<td>ELONGATION (TOPOISOMERASE)</td>
</tr>
<tr>
<td>NF-III (=OTF-1)</td>
<td>92K</td>
<td>CELLULAR</td>
<td>ND</td>
<td>INITIATION</td>
</tr>
<tr>
<td>pL</td>
<td>44K</td>
<td>CELLULAR</td>
<td>ND</td>
<td>ALLOWS INITIATION ON TP-MINUS TEMPLATES</td>
</tr>
</tbody>
</table>


**Terminal protein (TP)**

The 5' end of each strand of the adenovirus genome is covalently attached to the mature TP protein (Rekosh, et al., 1977). This protein is produced during packaging of the viral genome, by the proteolytic cleavage of a precursor (pTP), which is active in the initiation of DNA synthesis (Challberg, et al., 1980; Challberg and Kelly, 1981; Smart and Stillman, 1982). After (or during) localised unwinding of the origin (possibly mediated by TP), the pTP protein binds the adenovirus DNA template within an 18bp minimal origin sequence, in a 1:1 complex with the adenovirus polymerase (Chen, et al., 1990; Lichy, et al., 1982). A dCMP molecule (the first residue of the new strand) is then covalently linked to a serine group in the pTP, and elongation occurs from the 3' hydroxyl group of this dCMP (Rekosh, et al., 1977).

**DNA polymerase (Ad Pol)**

DNA sequence analysis reveals that Ad Pol is a member of the same family of replicative polymerases as HSV Pol and DNA pol α (Wong, et al., 1988). However, it has several properties which distinguish it from pol α (Field, et al., 1984). Ad Pol can utilise a variety of homopolymeric DNA
primers, but is much less efficient at using RNA primers. It possesses a 3'-5'
proofreading exonuclease activity, which is specific for single-stranded DNA.
Aphidicolin inhibits the production of short DNA chains by pol α, whereas Ad
Pol is inhibited only after extensive DNA synthesis.

As mentioned above, Ad Pol forms a tight complex with pTP, which is
thought to bind to the origin via a zinc-finger motif in Ad Pol (Chen, et al.,
1990). This interaction facilitates the transport of Ad Pol into the nucleus
(Zhao and Padmanabhan, 1988). Ad Pol also interacts with NF-I, resulting in
the stabilisation and correct positioning of pTP-Ad Pol binding to the origin

Ad Pol activity can be stimulated up to 100-fold by the adenovirus
DNA-binding protein (DBP) using a poly(dT) template and an oligo(dA)
primer. Ad Pol becomes highly processive under these conditions. E. coli
SSB cannot substitute for DBP in this reaction. Also, DBP cannot stimulate
other polymerases such as pol α, arguing for a specific interaction between
DBP and Ad Pol (Field, et al., 1984; Lindenbaum, et al., 1986). However,
such a protein-protein complex has not yet been directly detected.

DNA-binding protein (DBP)
The early region 2A of the human adenovirus genome encodes the
DBP phosphoprotein (Klein, et al., 1979; Linne, et al., 1977), which is
synthesised at both early and late times during infection (Axelrod, 1978).
The protein consists of two functional domains which can be separated by
proteolytic cleavage; a 26k amino-terminal fragment involved in regulating
late gene expression, and a 44k carboxy-terminal fragment responsible for
the other known functions of the protein (Klein, et al., 1979; Klessig, et al.,
1986).

Immunofluorescence and in situ hybridisation studies indicate that,
like ICP8, DBP is associated with viral DNA in globular replication
compartments within infected cell nuclei (Sugawara, et al., 1977). In cells
where viral replication is inhibited (by hydroxyurea or use of an Ad Pol
mutant virus) DBP staining is diffuse. Salt-extraction experiments suggest
that the diffuse form of DBP is not matrix-associated, unlike the
compartmentalised form (Klessig, et al., 1986; Voelkerding and Klessig,
1986)(note that this is the reverse of the ICP8 situation (Quinlan, et al.,
1984)). During infection in the presence of 1-β-D-arabinofuranosylcytosine,
(arac, a compound which is phosphorylated within cells to form a
deoxycytosine triphosphate analogue, inhibiting the transition from early to
late stages of infection (Cohen, 1977)) DBP is localised to discrete foci similar to the prereplicative sites seen in PAA-treated HSV-1-infected cells (Sugawara, et al., 1977).

DBP is a multifunctional protein. Its known roles (mainly deduced from the behaviour of DBP mutants) are detailed below.

(i) Initiation of DNA replication

DBP is not essential for the initiation of viral DNA synthesis (Kenny and Hurwitz, 1988; Rosenfeld, et al., 1987) but does stimulate this process by increasing the affinity, stability and rate of association of NFI binding to the origin (Cleat and Hay, 1989; Stuiver and van der Vliet, 1990). Thus, initiation of adenovirus DNA synthesis involves multiple protein-protein and protein-DNA interactions.

(ii) Elongation of nascent DNA molecules

DBP binds cooperatively and preferentially to ssDNA in a sequence-independent manner, presumably activating the DNA template for polymerase action (van der Vliet, et al., 1978). However, studies of DBP mutants suggest that ssDNA binding alone is not sufficient to account for the role of DBP in adenovirus DNA replication (Meyers, et al., 1990). As discussed above, DBP may also interact specifically with the Ad Pol. It has been suggested that DBP, in concert with Ad Pol, may mediate unwinding of the DNA duplex in an ATP-independent manner, obviating the need for a separate helicase for fork-passage (Challberg and Kelly, 1989).

(iii) Regulation of gene expression

The role of DBP in early gene expression appears to be limited (Klessig, et al., 1986). However, the protein does seem to activate late gene expression. This is suggested by host-range mutants with altered DBP genes, which can overcome a restrictive block to late gene expression in non-permissive cells (Johnston, et al., 1985; Klessig and Grodzicker, 1979). Analysis of the effects of DBP on expression levels of a reporter gene under the control of the late promoter confirmed the role of DBP in late gene activation (Chang and Shenk, 1990). DBP may also enhance its own expression; a process requiring phosphorylation of the DBP protein (Klessig, et al., 1986; Morin, et al., 1989).

(iv) Virion assembly

A ts revertant of a replication-defective DBP mutant has been isolated and found to produce normal amounts of late virion proteins but not to assemble virions in 293 cells at the non-permissive temperature (Nicolas, et al., 1983).
(v) Transformation

Several ts DBP mutants have been shown to transform rat cells at higher frequencies than wild-type virus (Ginsberg, et al., 1975; Logan, et al., 1981). However, DBP-negative deletion mutants have no effect on transformation efficiency, indicating that loss of DBP per se is not the cause of the elevated transformation frequency. The ts transforming mutants are dominant over wild-type virus, indicating that they encode a form of DBP with altered functional properties (Klessig, et al., 1986).

(vi) RNA-binding

Some of the above functions may be mediated by the RNA-binding activity of DBP. This binding has been demonstrated both in vitro and in vivo, using filter-binding and cross-linking studies. Inhibition of transcription by actinomycin D causes partial breakdown of DBP replication compartments at late times in infection (Klessig, et al., 1986). The blocks overcome by DBP mutants which allow virus production in non-permissive cells suggest a role for DBP in RNA transcription and processing (Klessig and Grodzicker, 1979).

Nuclear Factor I (NF-I)

Sequence analysis of human cDNA clones of NF-I suggests that the family of NF-I polypeptides is generated, at least partly, by differential mRNA splicing (Santoro, et al., 1988).

NF-I binds to a conserved motif within domain B (nucleotides 19-39) of the adenovirus origin of replication (Leegwater, et al., 1985). NF-I binding is not essential for in vivo adenovirus replication in all serotypes (Ad4 does not have an NF-I binding site) (Harris and Hay, 1988; Hay, 1985). However, in serotypes such as Ad 2, NF-I binding increases the efficiency of replication initiation in vitro (Guggenheimer, et al., 1984b; Nagata, et al., 1982; Wides, et al., 1987) and in vivo (Bernstein, et al., 1986; Hay, 1985; Wang and Pearson, 1985). This increased initiation is probably due to the interactions of NF-I with Ad Pol and DBP (Chen, et al., 1990; Cleat and Hay, 1989). NF-I colocalises with DBP in replication compartments during Ad 2; but not Ad 4; infection suggesting a specific targeting of NF-I to Ad 2 replication sites (Bosher and Hay, 1991).

NF-I is identical to CTF, a cellular transcription factor recognising a GCCAAT motif present in several cellular promoters (Jones, et al., 1987).
Nuclear Factor II (NF-II)

NF-II is required for the synthesis of full-length adenovirus DNA molecules. In the absence of NF-II, only 25% of the complete chain length is replicated. HeLa NF-II copurifies with DNA topoisomerase I activity and can be functionally replaced in vitro by human, or calf-thymus (but not E. coli) DNA topoisomerase I (Nagata, et al., 1983).

Nuclear Factor III (NF-III)

NF-III (also called ORP-C) binds to domain C (nucleotides 40-51) of the adenovirus origin. Like NF-I, NF-III binding is not essential for adenovirus replication, but increases replication efficiency in vitro (O'Neill and Kelly, 1988; Pruijn, et al., 1986; Rosenfeld, et al., 1987; van der Vliet, et al., 1988). This stimulatory effect has not yet been observed in vivo. The enhancing effects of NF-I and NF-III are independent and non-synergistic, suggesting that the two proteins do not interact (Mul, et al., 1990).

NF-III is also a cellular transcription factor, being indistinguishable from the octamer transcription factor (OTF-I) (O'Neill, et al., 1988). The transcriptional activation domains of NF-I and NF-III are probably not required for adenovirus replication, since the DNA binding domain of NF-III has been shown to be sufficient for stimulation of replication (Verrijzer, et al., 1990). NF-I binding to a engineered site adjacent to a core SV40 origin significantly increases replication efficiency if the DNA is assembled into a minichromosome, but has no effect on naked DNA. This suggests a model whereby transcription factor-binding increases the accessibility of the origin by perturbing the local distribution of histones (Cheng and Kelly, 1989). This model may not be applicable to adenovirus, whose DNA is packaged by viral core proteins rather than by histones (Vayda, et al., 1983).

pL

This host nuclear factor permits replication on adenovirus DNA templates devoid of TP (Guggenheimer, et al., 1984a). The protein has 5'-3' exonuclease activity, which appears to degrade the 5' end of the strand to be displaced, facilitating binding of the initiation complex to the exposed 3' end of the template strand (Guggenheimer, et al., 1984a; Kenny, et al., 1988). This suggests that, on normal TP-bound templates, the TP plays a role in unwinding the origin to allow entry of the initiation complex.
Simian virus 40 (SV40)

SV40 has a circular dsDNA genome of around 5kb, which contains a single 64bp core replication origin. The viral genome becomes complexed with host histones to form a minichromosome with the same structure as cellular chromatin. SV40 encodes a single replication protein, large tumour antigen (T Ag), and therefore makes extensive use of the host DNA replication machinery. Thus, SV40 provides a good model for studying the mechanism of cellular DNA replication (for reviews see (Challberg and Kelly, 1989; Hurwitz, et al., 1990; Kelly, 1988; Stillman, 1989)).

SV40 DNA replication is bidirectional, with both DNA strands being synthesised simultaneously. One strand is synthesised continuously (the leading strand), whilst the other is produced by the joining of Okazaki fragments synthesised discontinuously (the lagging strand).

A 64bp region of the genome constitutes the core origin of DNA replication. This region has at least three functional domains; four pentameric motifs which form an inverted repeat, flanked by a 17bp AT-tract on one side and a 15bp imperfect (early) palindrome on the other (Deb, et al., 1986) (see figure 1.1). Sequences outside the core origin significantly increase the efficiency of initiation in vivo, but not in vitro. Such sequences include a T Ag binding site, transcriptional enhancers, and Sp-I binding sites (DeLucia, et al., 1986; Li, et al., 1986; Stillman, et al., 1985). Transcription factor binding may increase the accessibility of the origin by perturbing nucleosome distribution, as discussed above (see adenovirus NF-III section).

Figure 1.1. The SV40 origin region

Schematic representation of the SV40 origin region. Small arrows indicate the position and direction of GAGGC pentameric motifs. T Ag binding sites I and II, the early palindrome (EP) and AT-tract (A/T) are shown, along with the three 21bp GC-rich repeats containing Sp1 binding sites and the two 72bp transcriptional enhancers. The general region protected by p53 is also shown. Figure based on diagrams in (Bargonetti, et al., 1991; Borowiec, et al., 1990; Kelly, et al., 1988).
Unlike HSV and adenovirus, SV40 does not form novel replication compartments. Instead, in situ hybridisation experiments indicate that replicating SV40 DNA is localised primarily to the nucleoli of host cells (Geuskens and May, 1974).

The development of in vitro SV40 DNA replication systems using crude fractions (Li and Kelly, 1984; Li and Kelly, 1985; Stillman and Gluzman, 1985; Wobbe, et al., 1985) and highly purified proteins (Tsurimoto, et al., 1990; Weinberg, et al., 1990; Wobbe, et al., 1987) has facilitated detailed study of the mechanism of DNA replication. Those proteins sufficient for SV40 DNA replication in vitro are shown in table 1.5.

Table 1.5. SV40 DNA replication proteins

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>MOL. WT.</th>
<th>ORIGIN</th>
<th>PUTATIVE FUNCTION(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T ANTIGEN</td>
<td>82K (95K IN SDS-PAGE)</td>
<td>VIRAL</td>
<td>ORIGIN BINDING, ORIGIN UNWINDING (DNA HELICASE)</td>
</tr>
<tr>
<td>POL-α-PRIMASE</td>
<td>180K, 70K, 59K, 49K *</td>
<td>CELLULAR</td>
<td>LAGGING STRAND SYNTHESIS</td>
</tr>
<tr>
<td>POL δ</td>
<td>130K, 47K *</td>
<td>CELLULAR</td>
<td>LEADING STRAND SYNTHESIS</td>
</tr>
<tr>
<td>POL ε</td>
<td>215K, 55K *</td>
<td>CELLULAR</td>
<td>POSSIBLE ROLE IN LAGGING STRAND SYNTHESIS</td>
</tr>
<tr>
<td>PCNA</td>
<td>36K</td>
<td>CELLULAR</td>
<td>INCREASES POL- δ AND POL-ε PROCESSIVITY</td>
</tr>
<tr>
<td>SSB (RF-A/RP-A)</td>
<td>70K, 34K AND 11K</td>
<td>CELLULAR</td>
<td>ORIGIN UNWINDING AND STIMULATION OF POLYMERASES</td>
</tr>
<tr>
<td>RF-C (A1)</td>
<td>140K, 41K AND 37K</td>
<td>CELLULAR</td>
<td>ATPase. STIMULATES POL-α AND POL-δ</td>
</tr>
<tr>
<td>TOPO-ISOmerASE I</td>
<td>100K</td>
<td>CELLULAR</td>
<td>REMOVAL OF TORSIONAL STRAIN IN DNA TEMPLATE</td>
</tr>
<tr>
<td>TOPO-ISOmerASE II</td>
<td>172K</td>
<td>CELLULAR</td>
<td>REMOVAL OF TORSIONAL STRAIN; SEGREGATION OF DAUGHTER STRANDS</td>
</tr>
</tbody>
</table>

* The reported molecular weights of the DNA polymerases differ according to the source of purified material and the purification method used. Molecular weights shown are for polymerases purified from HeLa cells (Syvaoja, et al., 1990).

It should be noted that the rate of replication fork movement is only a tenth of that found in vivo and abnormally large Okazaki fragments are
synthesised (Alberts, 1990; Tsurimoto, et al., 1990). Thus, additional proteins may be utilised for efficient SV40 DNA synthesis in vivo and reaction conditions in the in vitro system may not be optimal.

Large Tumour antigen (T Ag)

T Ag is a multifunctional phosphoprotein, active in many aspects of the infectious process. These functions may be carried out by different populations of T Ag variously associated with the plasma membrane, cytoplasm, nucleoplasm, chromatin, and nuclear matrix (Butel and Jarvis, 1986; Michel and Schwyzer, 1982; Schirmbeck and Deppert, 1989).

(i) Initiation of viral DNA synthesis

T Ag binds to the four -GAGGC- pentanucleotide motifs at the centre of the core origin (DeLucia, et al., 1983; Tjian, 1978) (figure 1.1). In the presence of ATP, T Ag binds to these sites with increased affinity (Borowiec and Hurwitz, 1988a; Deb and Tegtmeyer, 1987), forming a double hexamer, which covers the core origin and extends 12bp beyond it in both directions (Mastrangelo, et al., 1989). This complex is visible in electron micrographs as a bilobed structure (Dean, et al., 1987b).

T Ag binding causes structural distortions of the origin DNA as detected by hypersensitivity to both methylation and potassium permanganate oxidation (reviewed by (Borowiec, et al., 1990)). The 15bp early palindrome is inherently unstable due to stretches of purines and pyrimidines on opposite strands (Wells, 1988) and is melted on T Ag binding. The bent 17bp AT-tract undergoes a conformational change and the origin region is untwisted by up to two helical turns (Dean and Hurwitz, 1991). These structural changes occur independently of ATP hydrolysis (Borowiec and Hurwitz, 1988b).

T Ag is also a DNA helicase, i.e. it can separate the two strands of a DNA duplex through an enzymatic unwinding reaction (see (Matson and Kaiser-Rogers, 1990) for a review of DNA helicases). The DNA helicase activity of T Ag is ATP-dependent and acts in a 3'-5' direction (Stahl, et al., 1986), to locally unwinds the SV40 origin in a reaction dependent on the presence of SSB and topoisomerase (Dean, et al., 1987a; Dodson, et al., 1987; Matsumoto, et al., 1990; Wold, et al., 1987)). This unwinding allows the pol-α-primase complex to bind to the exposed origin region.

Also, T Ag interacts with the catalytic subunit of pol α (Dornreiter, et al., 1990; Gannon and Lane, 1987; Smale and Tjian, 1986). At low DNA template and polymerase concentrations (≤100ng and ≤0.012U per 25μl
reaction), T Ag markedly stimulates the synthesis of RNA primers by the pol-α-primase complex, as well as increasing the average length of the DNA product on both primed and unprimed ssDNA templates. Thus, T Ag may facilitate pol-α-primase association with the DNA template (Collins and Kelly, 1991). The T-pol-α-primase interaction may also be important in determining the host-range specificity of SV40, since addition of human, or monkey, pol-α-primase allows SV40 replication in normally non-permissive mouse cells (Murakami, et al., 1986).

(ii) Elongation

The helicase activity of T Ag is capable of extensive unwinding of the DNA duplex and monoclonal antibodies which inhibit this helicase activity also inhibit chain elongation in vitro (Dodson, et al., 1987; Goetz, et al., 1988; Wiekowski, et al., 1987; Wiekowski, et al., 1988). Thus, at least in vitro, T Ag is likely to unwind the DNA template ahead of the moving replication fork. The directionality of the helicase activity suggests that T Ag propagates along the leading DNA strand (Goetz, et al., 1988; Wiekowski, et al., 1988). Inhibition of DNA replication results in the formation of overwound replicative intermediates, implying that DNA synthesis and unwinding are normally linked (Droge, et al., 1985).

(iii) Regulation of DNA replication

T Ag has two clusters of phosphorylation sites, in amino acid regions 106-124 and 639-701 (Prives, 1990; Scheidtmann, et al., 1982). Treatment of T Ag with calf intestinal alkaline phosphatase to remove serine phosphates results in increased initiation of DNA synthesis in vitro (Grasser, et al., 1987; Mohr, et al., 1989). Later studies revealed a stimulation of T Ag origin-unwinding activity by protein phosphatase 2A, which removes one or two serine phosphates from T Ag. This dephosphorylation is inhibited by SV40 small-t antigen, which is associated with the regulatory A subunit of protein phosphatase 2A (Pallas, et al., 1990; Scheidtmann, et al., 1991; Walter, et al., 1990). The catalytic subunit of protein phosphatase 2A was shown to be identical to RP-C, a protein which stimulates initiation of SV40 replication in vitro (Virshup, et al., 1989)(see below).

cdc 2 kinase-mediated phosphorylation of threonine-124 on the otherwise unmodified T Ag from E. coli results in an increase in origin binding efficiency and initiation (McVey, et al., 1989). Thus, the net state of phosphorylation of T Ag determines its level of activity, allowing a fine degree of control over DNA replication (reviewed by (Prives, 1990)).
(iv) Stimulation of quiescent cells

T Ag plays an important role in SV40 infection of quiescent cells by inducing proliferation, possibly through its interaction with the tumour-suppressor protein retinoblastoma (Rb) (see below) (Mueller, et al., 1978; Tjian, et al., 1978).

(v) Transformation

The transformation effects of T Ag are presumably mediated through its interactions with host cell proteins. T Ag has been reported to interact with a number of cellular proteins, including p53, retinoblastoma (Rb), pol α, 73k heat shock protein, tubulin and the AP2 transcription factor (Schreier and Gruber, 1990). The interactions of T Ag with the tumour-suppressor proteins p53 and Rb are the best characterised and these are described briefly below, since results concerning these two proteins will be presented later.

p53

p53 forms complexes with adenovirus E1b and oncogenic human papilloma virus E6 proteins as well as with SV40 T Ag (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow, et al., 1982; Werness, et al., 1990). The p53 gene is altered in many types of human cancer; generally one allele has a mis-sense mutation and the other is lost (reviewed in Hollstein, et al., 1991)). Mutant forms of p53 can immortalise primary cells in culture and, in some cases, can cooperate with oncogenes to transform such cells (Lane and Benchimol, 1990). Wild-type p53 can do neither of the above, but instead suppresses such changes (Finlay, et al., 1989; Michalovitz, et al., 1990). Mutant p53 generally has a much longer half-life than the wild-type protein (Finlay, et al., 1988; Ginsberg, et al., 1991) and has an altered structural conformation, often binding heat shock protein and a particular subset of monoclonal antibodies (Gannon, et al., 1990; Sturzbecher, et al., 1987).

Recently, it has been shown that wild-type, but not mutant p53 binds specifically to cellular DNA sequences associated with replication origins and probably also to SV40 DNA sequences adjacent to the SV40 origin of replication (see figure 1.1)(Bargonetti, et al., 1991; Kern, et al., 1991). p53 has also been shown to contain a strong transactivation domain, so that DNA-binding of p53 may be mediating the activation of gene expression (Fields and Jang, 1990). T Ag can inhibit specific DNA binding by p53, thus preventing p53 from binding near the SV40 replication origin and possibly from binding to cellular DNA (Bargonetti, et al., 1991).
Retinoblastoma (Rb)

Rb, similarly to p53, associates with the protein products of at least three DNA tumour viruses; SV40 T Ag, adenovirus E1a and human papillomavirus E7 protein (DeCaprio, et al., 1988; Dyson, et al., 1989; Whyte, et al., 1988). The Rb-binding regions of these viral proteins overlap their transformation domains, implying that these proteins transform cells by complexing and inactivating Rb.

The Rb gene was first identified through its involvement in retinoblastoma, a rare inherited disease causing childhood eye-cancers. The disease is passed on in a dominant manner with incomplete penetrance. Deletion of 13q14 was shown to be associated with the disease, and the Rb gene was mapped within this region and shown to be expressed normally in most tissues, but to be absent in retinoblastomas (Francke, 1978; Friend, et al., 1986; Lee, et al., 1987). Thus, retinoblastomas arise from loss-of-function mutations in the Rb gene, indicating that Rb is a tumour-suppressor gene.

The Rb protein is phosphorylated in a cell-cycle-dependent manner with maximum levels of phosphorylation at the G1-S transition (Buchkovich, et al., 1989). Some, or all, of this phosphorylation is mediated by the p34cdc2 protein kinase (Lin, et al., 1991). T Ag binds exclusively to the underphosphorylated form of Rb, presumably inactivating its role in regulating entry into S-phase (Ludlow, et al., 1989; Ludlow, et al., 1990).

Rb appears to regulate S-phase entry by complexing with a number of cellular proteins through a common "pocket" region (Kaelin Jr., et al., 1991; Wagner and Green, 1991). One such protein is E2F, a transcription factor with binding sites in the promoters of several proliferation-related genes (Chellappan, et al., 1991; Chittenden, et al., 1991; Mudryj, et al., 1990). The E2F-Rb complex is present in G1 cells (S-phase cells contain E2F complexed to cyclin A) and can be dissociated by adenovirus E1a protein (Chellappan, et al., 1991; Mudryj, et al., 1991). Rb and cyclin A co-complexes have been reported with a differentiation-regulated transcription factor (DRTF) in undifferentiated F9 embryonic carcinoma cells. DRTF is a multi-protein complex and may be related to E2F (Bandara and La Thangue, 1991). Another Rb-binding protein, at least in vitro is the c-myc proto-oncogene product (Rustgi, et al., 1991). Other Rb-binding proteins have yet to be characterised, but they too are likely to be involved in cell proliferation. Thus, T Ag binding to the Rb "pocket" presumably releases sequestered cell proliferation proteins, allowing S-phase to begin.
Regulation of gene expression

T Ag negatively regulates early gene expression and stimulates late gene expression (Keller and Alwine, 1984; Reed, et al., 1976).

RNA metabolism

T Ag has a 3'-5' RNA helicase activity which utilises UTP, GTP or CTP as a cofactor (Scheffner, et al., 1989). The association of T Ag with cellular heterogeneous nuclear RNA and with cytoplasmic messenger ribonucleoproteins has been reported (Darlix, et al., 1984; Michel and Schwyzer, 1982).

DNA Polymerases

Five distinct DNA polymerase activities have been identified in mammalian cells: polymerases α, β, γ, δ and ε (Fry and Loeb, 1986; Lee (M. Y. W. T.), et al., 1991; Syvaoja, et al., 1990; Wang, 1991). Of these, polymerases α, δ and possibly ε are thought to be involved in SV40 DNA replication. These three enzymes share common properties with the yeast DNA polymerases I, III, and II respectively (Syvaoja, et al., 1990). In accordance with this discovery, the yeast polymerases were recently renamed; polymerases I, II and III are now termed yeast polymerases α, ε and δ (Burgers, et al., 1990).

Pol-α-primase

The 180k catalytic subunit of polymerase α (pol α) has been cloned and shown to be a member of a large family of prokaryotic and eukaryotic polymerases (Wong, et al., 1988). No exonuclease activity has been detected in the human four-subunit enzyme, unlike the Drosophila pol α, which has a 3'-5' cryptic exonuclease in its catalytic subunit (Cotterill, et al., 1987). However, exonuclease and other enzymatic activities have been detected within higher molecular weight complexes containing DNA polymerase α activity (Skarnes, et al., 1986). Pol α gene expression and enzymatic activity are not cell-cycle-regulated, but are greater in proliferating cells than in quiescent cells (Cripps-Wolfman, et al., 1989; Wahl, et al., 1988).

The pol-α-primase complex is thought to be positioned at the SV40 origin through its species-specific interaction with T Ag (see above). This interaction can be blocked by the protein product of the cellular tumour-suppressor gene, p53, which competes with pol α for binding to T Ag (Gannon and Lane, 1987). This result raises the possibility that p53 may
control cell proliferation through interaction with a cellular homologue of TAg bound pol α.

Once in position at the origin, pol-α-primase complex initiates DNA synthesis from both DNA strands. A polymerase switch then occurs on the leading strand, with pol δ taking over leading strand synthesis (Tsurimoto, et al., 1990). It is currently unclear whether pol α plays a role in elongation of the lagging strand, once initiation is complete (see pol ε section below).

Polymerase δ

Pol δ differs from pol α in its sensitivity to various chemical inhibitors, its immunological properties, its possession of a 3'-5' exonuclease activity, its lack of associated primase activity, and its dependence on proliferating cell nuclear antigen (PCNA) (Bauer and Burgers, 1988; Byrnes, et al., 1976; Syvaoja, et al., 1990; Tan (C.-K.), et al., 1986; Wong, et al., 1989). Pol δ is also stimulated synergistically by RF-C and RF-A (Tsurimoto and Stillman, 1989a). RF-C and PCNA, aided by RF-A, form a complex on the 3' end of the first nascent leading strand. This primer recognition complex blocks pol α binding and simulates pol δ binding to the primer. Pol δ, RF-C and PCNA then move along the leading strand as a complex (Tsurimoto and Stillman, 1991a; Tsurimoto and Stillman, 1991b). This complex can be replaced by a number of DNA polymerases including the bacteriophage T4 holoenzyme (Tsurimoto, et al., 1990) and the E. coli DNA pol III holoenzyme (Matsumoto, et al., 1990), suggesting that non-specific leading-strand synthesis can occur.

Polymerase ε

Pol ε is distinguishable from pol δ mainly by its lack of stimulation by PCNA at low salt concentrations (PCNA stimulation of pol ε processivity is reportedly observed at high salt concentrations (Matsumoto, et al., 1990)) (Focher, et al., 1988; Lee (M. Y. W. T.), et al., 1991; Syvaoja and Linn, 1989; Syvaoja, et al., 1990). Preliminary evidence suggests a role for pol ε in DNA replication. Mutations of yeast POL ε are lethal and have a replication-defective phenotype, suggesting the existence of three essential replicative polymerases (Morrison, et al., 1990). Selective inhibition experiments indicate that another DNA polymerase (δ or ε) takes over lagging strand synthesis after the initial production of an intermediate "DNA primer" by the pol-α-primase (Nethanel and Kaufmann, 1990). This is further supported by the observation that lagging strand synthesis is inhibited by antibodies which neutralise PCNA (Bullock, et al., 1991). The relative abundance and fidelity of pol ε, and lack of processivity of pol-α-primase are compatible with
pol ε functioning as a lagging strand polymerase (Kelly, 1988; Syvaoja, et al., 1990).

**Proliferating cell nuclear antigen (PCNA)**

A 36k protein essential for SV40 replication was found to be identical to proliferating cell nuclear antigen (PCNA), a protein recognised by sera from systemic lupus erythematosus patients (Miyachi, et al., 1978; Prelich, et al., 1987a). PCNA is synthesised during S-phase of the cell cycle, and is associated with sites of cellular DNA replication (Bravo and Celis, 1980; Bravo and Macdonald-Bravo, 1985; Bravo and Macdonald-Bravo, 1987; Mathews, et al., 1984). Also, the identity of PCNA with a previously characterised accessory protein for pol δ provided the first real evidence for pol δ's role in DNA replication (Bravo, et al., 1987; Prelich, et al., 1987b; Tan (C.-K.), et al., 1986). A PCNA homologue has been purified from *S. cerevisiae* and shown to stimulate both yeast and mammalian pol δ (Bauer and Burgers, 1988). A homologue has also been cloned from *S. pombe* (Waseem and Lane, manuscript in preparation).

In the absence of PCNA, initiation of SV40 nascent strand synthesis occurs normally *in vitro*. However, elongation of the leading strand does not occur and lagging strand products consist of displaced Okazaki fragments, suggesting roles for PCNA in leading strand elongation and in coordinating leading and lagging strand synthesis (Prelich and Stillman, 1988).

PCNA may have other roles in the cell besides DNA replication, since it is present in excess within the nucleus. Two populations of PCNA can be distinguished using different fixation techniques; an S-phase specific class associated with sites of DNA replication and detected by methanol fixation, and a class in the nucleoplasm of both quiescent and growing cells detected by formaldehyde fixation (Bravo and Macdonald-Bravo, 1987). Also a monoclonal antibody has been produced which recognises an epitope on PCNA found only in the nucleoli of cells, raising the possibility of a role for PCNA in this compartment (Waseem and Lane, 1990).

**Single-stranded DNA binding protein (SSB)**

SSB (also known as human SSB, RF-A, or RP-A) is a trimeric protein complex required for the initiation of SV40 DNA replication *in vitro* (Fairman, et al., 1988; Wobbe, et al., 1987; Wold and Kelly, 1988). Preferential binding of SSB to ssDNA is mediated by the 70k subunit alone (Kenny, et al., 1990; Wold, et al., 1989). SSBs from various prokaryotes and viruses will support
origin-unwinding but will not substitute fully for human SSB in the SV40 replication system, suggesting additional roles for SSB in replication (Kenny, *et al*., 1989).

Further roles for SSB in SV40 DNA replication have been defined by experiments using purified replication proteins on model templates and by studies using monoclonal antibodies raised against SSB. Human SSB specifically and independently stimulates pol α activity, possibly by increasing its processivity. Pol δ is also stimulated by human and other SSBs which act cooperatively with PCNA and RF-C to increase the association of pol δ with primer ends (Kenny, *et al*., 1989; Tsurimoto and Stillman, 1989a; Tsurimoto and Stillman, 1991a; Tsurimoto and Stillman, 1991b). In addition, there are reports of SSB stimulation of pol ε (Coverley, *et al*., 1991). SSB also inhibits non-specific binding to primer ends by pols α and δ (Collins and Kelly, 1991; Tsurimoto and Stillman, 1991a; Tsurimoto and Stillman, 1991b).

A homologue of SSB has been purified from *S. cerevisiae* (Brill and Stillman, 1989) and the gene encoding its 70k subunit (the RPA1 gene) cloned and sequenced. The product of this gene stimulates the activity of a yeast strand exchange protein, SEP1, implying a role for SSB in recombination. RPA1 is essential in yeast and is continuously required for mitotic growth. Mutants of RPA1 have a replication-defective phenotype (Heyer, *et al*., 1990).

Antibodies to SSB inhibit human excision repair in vitro. This inhibition can be overcome by the addition of purified SSB, implying a role for SSB in DNA repair (Coverley, *et al*., 1991).

SSB may be a key protein for the regulation of DNA replication, since the middle subunits of both the human and yeast SSBs are phosphorylated in a cell-cycle-dependent manner. SSB is phosphorylated at the G1-S transition and is dephosphorylated at mitosis (Din, *et al*., 1990).

**Replication factor C (RF-C)**

RF-C has been purified from human 293 cells on the basis of its essential role in the elongation phase of SV40 DNA replication (Tsurimoto and Stillman, 1989b). RF-C is probably the same as "activator 1" (A1), a multiprotein complex purified from HeLa cells which, together with PCNA and pol δ, binds to primer ends, overcoming the inhibitory effects of poly(ADP-ribose) polymerase on elongation of DNA strands in the
monopolymerase (pol α only) system (Eki and Hurwitz, 1991; Lee (S.-H.), et al., 1991).

Replication in the absence of RF-C produces products similar to those found when PCNA is omitted from the SV40 in vitro system (see above), suggesting a role in coordination of strand synthesis (Tsurimoto and Stillman, 1989b). Like RF-A, RF-C stimulates pol α activity independently and pol δ activity cooperatively. RF-C increases both the processivity and the frequency of primer recognition of pols α and δ (Tsurimoto and Stillman, 1989a). Purified RF-C binds specifically to primed DNA templates and forms part of the primer-recognition complex for pol δ (Tsurimoto and Stillman, 1990; Tsurimoto and Stillman, 1991a; Tsurimoto and Stillman, 1991b). A DNA-dependent ATPase activity copurifies with RF-C. This ATPase is stimulated by PCNA and is probably required for processive DNA synthesis by pol δ (Tsurimoto and Stillman, 1990).

**Topoisomerases (topos)**

Specific topoisomerase inhibitors and monoclonal antibodies have been used to define the roles of topos I and II in vitro. Either topo I or topo II can act to relieve torsional strain resulting from unwinding at the replication fork (Yang, et al., 1987). Genetic studies in yeast support this conclusion, although the kinetics of replication in S. cerevisiae mutants suggest that topo I is probably the major enzyme performing this function (Kim and Wang, 1989; Uemura and Yanagida, 1984). Topo I sites have been mapped in vivo to clusters within the early palindrome and AT-rich regions of the SV40 origin. These sites are predominantly on the lagging strand. Thus, topo I may be specifically associated with the replication complex in a unique functional orientation (Porter and Champoux, 1989; Tsui, et al., 1989).

Topo II has been shown to be uniquely required for the separation of multiply intertwined daughter molecules and parental strands both in the SV40 system and in yeast (DiNardo, et al., 1984; Sundin and Varshavsky, 1980; Yang, et al., 1987). In vivo, SV40 infection causes an increase in topo II, but not topo I protein levels (Rainwater and Mann, 1990).

**Other proteins**

A number of additional cellular proteins have been identified as being involved in the efficient replication of SV40 DNA to produce mature products. These are shown in table 1.6 on the following page.
Table 1.6. Additional SV40 DNA replication proteins

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>MOL. WT.</th>
<th>FUNCTION</th>
<th>REFS.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP-C (PP2Ac)</td>
<td>34K, 32K</td>
<td>STIMULATION OF ORIGIN UNWINDING BY T ANTIGEN</td>
<td>(Virshup, et al., 1989; Virshup and Kelly, 1989)</td>
</tr>
<tr>
<td>DNA LIGASE I</td>
<td>125K</td>
<td>JOINING OF OKAZAKI FRAGMENTS</td>
<td>(Soderhall and Lindahl, 1975; Tomkinson, et al., 1990)</td>
</tr>
<tr>
<td>CAF-I</td>
<td>150K, 62K, 60K, 58K, 50K</td>
<td>CHROMATIN ASSEMBLY (COUPLED TO DNA REPLICATION)</td>
<td>(Smith and Stillman, 1989)</td>
</tr>
<tr>
<td>POLY(ADP-RIBOSE) POLYMERASE</td>
<td>120K</td>
<td>INHIBITS ELONGATION BY POL α</td>
<td>(Eki and Hurwitz, 1991; Lee, et al., 1989)</td>
</tr>
<tr>
<td>RNaseH</td>
<td>49K, 39K</td>
<td>PRIMER REMOVAL</td>
<td>(Hurwitz, et al., 1990)</td>
</tr>
<tr>
<td>5'-3' EXONUCLEASE</td>
<td>44K</td>
<td>PRIMER REMOVAL</td>
<td>(Hurwitz, et al., 1990)</td>
</tr>
</tbody>
</table>

Eukaryotic organisms

Yeast

Autonomously replicating sequences (ARS elements) have been defined in *S. cerevisiae* and in other eukaryotes by their ability to replicate in artificially-constructed extrachromosomal plasmids. Those ARS elements which have been identified have a high A+T content and contain an 11bp consensus sequence (reviewed by (Newlon, 1988)). Two-dimensional mapping techniques have shown that some ARS elements function as origins of replication both in plasmids and in genomic DNA (Brewer and Fangman, 1987; Huberman, et al., 1987). ARS-containing plasmids replicate once per cell cycle and may therefore provide a good model for studying the control of eukaryotic DNA replication. A yeast *in vitro* DNA replication system has not yet been successfully established. However, several ARS-binding proteins have already been identified; ABF-I (probably a transcriptional activator/repressor) (Buchman and Kornberg, 1990; Diffley and Stillman, 1988a), ABF-II (induces bending specifically in ARS1) (Diffley and Stillman, 1988b), OBFI (Eisenberg, et al., 1988), and ACBP (Hofmann and Gasser, 1991).

The powerful genetics of the yeast system has enabled the identification and study of several *S. cerevisiae* homologues of mammalian cell replication proteins, including DNA pols α, δ, and ε, DNA primase,
PCNA and SSB (Bauer and Burgers, 1988; Burgers and Bauer, 1988; Heyer, et al., 1990; Newlon, 1988). Thus, the combination of yeast genetics and mammalian in vitro biochemical studies should provide a powerful approach to understanding DNA replication mechanisms and control.

**Xenopus laevis eggs**

Insight into the regulation of eukaryotic DNA replication has been gained from an in vitro system derived from activated *Xenopus laevis* eggs. These eggs contain large stockpiles of replication proteins and, unlike the less mature *Xenopus oocytes*, carry out multiple rounds of rapid DNA replication in vivo. Naked DNA or nuclei microinjected into such eggs are replicated semiconservatively, in synchrony with the ongoing cell cycle. All DNA sequences tested were replicated, reflecting either a simple origin sequence requirement, no origin sequence requirement, or perhaps a relaxed sequence stringency during early developmental stages (Harland and Laskey, 1980; Mechali and Kearsey, 1984).

Cell-free extracts from such eggs can replicate nuclei or naked DNA in a similar fashion. The replication of naked DNA is preceded by its decondensation and assembly into a "pseudonucleus", a process essential for the initiation of DNA synthesis (Blow and Laskey, 1986; Blow and Sleeman, 1990; Newport, 1987). Each nucleus within the extract initiates DNA synthesis independently and rapidly completes DNA replication from multiple origins. Thus, initiation is coordinated within, but not between, nuclei (Blow and Watson, 1987).

In the absence of cycloheximide, re-replication of DNA is observed in the egg extract. Re-replication is also seen in cycloheximide-containing extracts after addition of maturation promoting factor, a protein which induces nuclear envelope breakdown. Also, re-replication is observed in some nuclei after their artificial permeabilisation. These experiments led to the proposal of a "licensing factor" which can promote one round of initiation and can only enter the nucleus at mitosis when the nuclear envelope breaks down (Blow and Laskey, 1988).

*Xenopus* homologues of some mammalian replication proteins have been identified, and PCNA and pol α have been shown to colocalise within nuclei replicating in *Xenopus* egg extracts (Hutchison and Kill, 1989). Also, p34cdc2, or a closely related protein, has been implicated in the initiation of DNA synthesis in such extracts (Blow and Nurse, 1990). This system should yield much information on the regulatory mechanisms operating to ensure
that chromosomal DNA replication within nuclei occurs once and only once per cell cycle.

**Mammalian cells**

The study of DNA replication in mammalian cells is hindered by their complexity. However, techniques have recently been developed to map origins of DNA replication within such cells, providing the beginnings of an understanding of mammalian origin structure. Research has focussed on the well-characterised amplified DNA region surrounding the Chinese hamster ovary dihydrofolate reductase (DHFR) gene. Determination of strand polarity using nucleosome association has identified two sites downstream of the gene at which leading and lagging strand synthesis switch (Handeli, *et al.*, 1989). "Run-on" experiments in synchronised cells confirmed the existence of two replication origins within this region (Burhans, *et al.*, 1986; Heintz and Hamlin, 1982), one of which has been identified as an origin of bidirectional replication and precisely mapped to a 0.45kb sequence (Burhans, *et al.*, 1990). However, two-dimensional (2-D) gel techniques suggest a broad initiation zone extending over 28kb (Vaughn, *et al.*, 1990a). This discrepancy has been reconciled in a model where clusters of replication origins form microbubbles (not detected on 2-D gels), which fuse to form minibubbles (detected on 2-D gels) and then macrobubbles (Linskens and Huberman, 1990). However, since 2-D gels are notoriously difficult to interpret, and discrete origins have been identified using two independent techniques, the existence of defined replication origins is more likely than an extensive initiation zone.

The SV40 system has provided much information on the proteins involved in mammalian DNA replication. However, a cellular helicase which will unwind DNA ahead of the replication fork in a manner equivalent to T Ag has not yet been identified. This remains a major stumbling block in studies of eukaryotic DNA replication. However, it is likely that much more will be discovered in the next few years using the model systems described above.
2. Materials and methods

1. Cells

a) Cell types

The different cell types used are detailed below:

- BHK; Baby hamster kidney cells (from ICRF cell production).
- MDA-MB 468; a breast epithelial cell line which overexpresses p53 (and is Rb-negative) (Bartek, et al., 1990).
- BT20; a breast epithelial cell line which has high levels of p53 and Rb (Bartek, et al., 1990).
- 293; human embryonic kidney cells transformed with adenovirus type 5 DNA (Graham, et al., 1977) (provided by Dr. P. Gallimore, University of Birmingham).
- Sp2/0-Ag14; myeloma cells used as fusion partners for hybridoma production (Harlow and Lane, 1988; Kohler and Milstein, 1976; Shulman, et al., 1978).

b) Cell culture

Cells were routinely propagated in Dulbecco's modified Eagle medium (E4 medium) without antibiotics and supplemented with foetal calf serum (FCS; Gibco) to 10%. Trypsin diluted in versene (supplied by ICRF media service) was used to detach cells from plates before dilution (generally 3 to 5-fold) and re-seeding. All cells were grown in a humid atmosphere containing 5-10% CO\textsubscript{2} at 37°C. For storage, cells were frozen by resuspension in a small volume of 8% dimethylsulphoxide in FCS and cooled slowly to -70°C overnight (o/n), before transfer to liquid nitrogen.

c) Cell staining

For optimal staining, cells were generally seeded the previous day to produce subconfluent cell monolayers. For immunofluorescence, cells were grown on glass coverslips (for improved optics) whereas peroxidase staining was performed on plastic dishes. Cell monolayers were washed in phosphate buffered saline type A (PBS) and then fixed either:

(A) for 3-5 minutes (min) at room temperature (RT) in acetone/methanol (50/50 v/v) or
(B) for 10 min at RT in 5% formaldehyde/2% sucrose/PBS, followed by PBS washes and permeabilisation in 1% Triton/5% sucrose/PBS, for 5 min.

The monolayers were then either allowed to dry (for A), or washed several times in PBS (for B), before application of antibody. First antibody(ies) was diluted in 10% FCS/PBS if necessary. Where no dilution is given, hybridoma supernatant (i.e. culture medium from which hybridoma cells have been removed by centrifugation) was used. In some instances, polyclonal antibodies were precleared by incubating with cell extracts produced by NP40 lysis (section 1e) for 1 hour (h) and centrifuging at 100,000 x g for 30 min at 4°C to remove spurious antibody-antigen complexes. First antibody(ies) was applied as a single large spot (on coverslips), or as several discrete spots (on plates) and samples were left in a humid atmosphere either for 2 h at RT, or o/n at 4°C. Cells were then washed 3-4 times in PBS and the next antibody layer was applied.

Detection by fluorescence

To reduce non-specific staining, normal goat serum (NGS; Vector laboratories) was applied to coverslips and left for 1 h at RT. After rinsing in PBS, second antibody conjugates were applied. Several conjugates were available; those most commonly used are shown below, together with their typical dilutions for optimum staining. Conjugates were diluted in PBS containing 10% FCS and 1% NGS. For double-labelling, two conjugates were generally applied simultaneously.

FITC-goat anti-mouse polyvalent immunoglobulins (Sigma; #F-1010) 1/20
FITC-goat anti-mouse immunoglobulins (Cappel; #1611-0081) 1/30
TRITC-goat anti-rabbit immunoglobulins (Sigma; #T-5268) 1/100
FITC-goat anti-rabbit immunoglobulins (Cappel; #1612-0081) 1/20
Texas-Red-Streptavidin (Amersham; #RPN 1233) 1/100

After 1-2 h in a humid atmosphere at RT, cells were washed 3-4 times in PBS. Generally Hoescht dye (diluted 1/1000 in PBS) was applied for 5-10 min before final rinses with PBS and then distilled water (dH2O). Samples were allowed to partially dry before being mounted in Gelvatol (Monsanto Chemicals; prepared as described in (Harlow and Lane, 1988)).

Detection by peroxidase

Two layers of horse-radish-peroxidase (HRP)-conjugated antibodies were generally used, giving improved sensitivity of staining:

HRP-rabbit anti-mouse immunoglobulin (Dako) 1/200
HRP-swine anti-rabbit immunoglobulin (Dako) 1/200
The conjugates were diluted in PBS containing 10% FCS, and each was applied sequentially for either 2h at RT, or o/n at 4°C.

Diaminobenzidine (3, 3', 4, 4'-tetraaminobiphenyl, Sigma) was used as a substrate, producing a brown insoluble product upon reaction with HRP. Approximately 0.1g of solid substrate was dissolved in 10ml of PBS and the solution filtered through a Whatman No. 1 paper. 3% nickel sulphate was added to 1/100th volume to deepen the colour of the reaction product. 5-10µl of 30% hydrogen peroxide was added as a catalyst, and the solution was poured onto the dish and left for 5-10min, or until sufficient colour had developed. After several washes in PBS, cells were rinsed in dH2O and viewed either wet, or mounted in Gelvatol.

**Photography**

Conventional photographs were taken using a Zeiss epifluorescence microscope. Fluorescence images were photographed on Kodak "TMax" P3200 film exposed at 1600 ASA. Peroxidase-stained cells and phase-contrast/bright-field images were photographed on Kodak "technical pan" film exposed at 25 ASA.

Confocal pictures were produced using a BioRad confocal microscope set up to detect dual-labelled samples (models 500 and 600 were both used). Images were photographed from the screen at 100 ASA, using Ilford XP1, or XP2 film.

**d) Metabolic labelling of cells**

**35S methionine**

For radioactive labelling of proteins, cells were rinsed in E4 medium without methionine (met - medium), before addition of 35S-methionine (Amersham, specific activity 1000Ci/mmol; #SJ1515) in a small volume of met - medium containing 10% FCS. Typically, isotope was added at 100µCi per 10cm plate in a volume of 2ml. During long labelling periods, plates were intermittently rocked, and the medium was topped up if necessary. After incubation at 37°C in a humid atmosphere containing 5-10% CO2, the cells were harvested as described in *t.e.*

**3H-thymidine**

For detection of newly-replicated DNA, tritiated thymidine (3H-T) (Amersham, specific activity 5Ci/ml; #TRA.61) was added to cells at 1µCi/ml of culture medium. Cells were incubated at 37°C for the desired labelling period (generally between 30min and 2h), then fixed in acetone/methanol (50/50) at RT for 3-5min. At this stage, fluorescent cell
* Lysis buffer was supplemented with 1mM phenyl methyl sulphonyl fluoride to prevent protein degradation.
staining was performed if required (see 1.c). Cell monolayers were then dried thoroughly before addition of photographic emulsion. Emulsion was prepared by mixing the supplied gel (K2, Ilford Scientific) with an equal volume of dH2O at 40°C in the dark, until dissolved. The prepared emulsion was then poured onto the cell plates, and quickly poured off, leaving a thin, even layer coating the plate. Plates were stored, inverted, in the dark, for 3-6 days, before fixing with "Superamp" (Champion photochemistry; diluted 1/5 in dH2O and precooled to 15°C) for 10 min. After removal of the fixative, D-19 developer (Kodak) was added to the plates and left for 10 min at RT. Cells were then viewed by bright-field microscopy. (The above method was provided by Dr. J. Southgate, University of Leeds).

e) Preparation of cell extracts

For immunoblotting, immunoprecipitation, and ELISA assays, cell extracts were made by rinsing cell monolayers twice in cold PBS, before adding lysis buffer (1% nonidet P40 (NP40) in NET buffer {150mM sodium chloride; 5mM EDTA; 50mM Tris, pH8}). For general purposes lysis was performed directly on cell monolayers (2ml lysis buffer per 15cm plate), although for more concentrated extracts e.g. for use in ELISA's, cells were scraped off the plate into cold PBS and centrifuged at low speed (1000 rpm in an MSE Centaur 2 benchtop centrifuge) before addition of approximately two volumes of lysis buffer to the cell pellet and vortexing. Plates were incubated for 30min at 4°C with occasional rocking, before collection of the resulting extract. Extracts were then spun at maximum speed in an Eppendorf centrifuge for 30min at 4°C to remove cell debris. If not used immediately, extracts were stored at -70°C.

2. Viruses

a) Virus stocks

Herpes simplex virus

A master stock of herpes-simplex virus type 1 (HSV-1) strain 17 syn+ (Brown, et al., 1973) was obtained from Dr. N. Stow (MRC, Glasgow). All HSV stocks used were derived from this preparation. Stocks were prepared according to the method described by Killington and Powell (Killington and Powell, 1986). Subconfluent monolayers of BHK cells were rinsed in medium without serum, before adsorption of virus for 1h at a multiplicity of infection (m.o.i.) of 0.001-0.01 plaque-forming units (p.f.u.) per cell, in a small
volume of medium (typically 0.5ml/10cm plate), with occasional rocking. Plates were then filled with E4 medium containing 10% FCS, and incubated for 2-3 days at 37°C. The virus produced a cytopathic effect which generally resulted in the detachment of cells from the plate. Cells were harvested by centrifugation and resuspended in cold E4 medium. Virus was released from the cells by sonication for 9min in an ice-cold water bath (Langford Electronics model H75E). Cell debris was removed by low speed centrifugation, and the resulting stock was stored in small aliquots at -70°C.

Plaque assays were performed according to the suspension method of Killington and Powell (Killington and Powell, 1986). Each viral dilution tested was added to 10^6-10^7 BHK cells in 2ml E4 medium and incubated with gentle agitation for 30min at 37°C. 8ml of 2% carboxymethylcellulose (medium viscosity, Sigma) in E4 medium containing 5% FCS was added to each suspension and the resulting viscous mixture divided between two 6cm dishes. After 2-3 days in a CO_2 incubator at 37°C, the cells were fixed in acetone/methanol (50/50) and stained with 1% methylene blue (Sigma). Plaques were counted with the aid of a microscope. Titres were typically between 10^7-10^9 p.f.u.'s per ml.

**Adenovirus**

Adenovirus type 5 was obtained from Dr. K. Lee (ICRF). Adenovirus types 2 and 4 were provided by Dr. R. Hay (University of St. Andrews). No viral sub-stocks were made.

**Simian virus 40 (SV40)**

SV40 (E728) stocks were obtained from Mr. J. Gannon (ICRF). No viral sub-stocks were made.

**b) Viral infections**

All infections were performed as described by Killington and Powell (Killington and Powell, 1986). Cells were seeded the previous day to produce a monolayer of 60-90% confluence for infection (lower confluencies were used for cell staining than for the preparation of cell extracts). Virus was added at approximately 10-20 p.f.u.'s per cell in a small volume of E4 medium (typically 50μl per 19mm coverslip, or 0.5ml per 10cm plate). The virus was then allowed to adsorb to the cells for 45min-1h at 37°C with occasional rocking, before addition of E4 medium containing 10% FCS and incubation at 37°C for the remainder of the infection period.

Mock infections were performed in exactly the same manner, except that virus stock was omitted from the adsorption medium (note that cell
staining results using mock-infected cell extract as adsorption medium were no different from those obtained using E4 medium alone).

3. Monoclonal antibody production

a) Antigens

Purified antigens were kindly provided by Drs. M.D. Challberg and J. Gottlieb (National Institutes of Health, Bethesda, MD). All antigens were stored at -70°C and were diluted in PBS to obtain a reasonable volume for injection.

ICP8

ICP8 was purified from U-35-VERO cells. The U-35 cell line contains multiple copies of the ICP8 gene (Orberg and Schaffer, 1987) thus facilitating the purification of large quantities of ICP8. ICP8 was purified as described previously (Gottlieb, et al., 1990). Virtually homogeneous fraction IV protein was supplied at 660 μg/ml.

POL/UL42

POL/UL42 complex was purified through five steps from HSV-infected HeLa cells (Gottlieb, et al., 1990). It was supplied at 190 μg/ml and was approximately 90% pure.

b) Injection regimes

Two groups of five 3-6 month-old, female Balb/c mice were given antigen injections (performed by the ICRF animal unit). For first injections, antigen was mixed with complete Freund's adjuvant. Subsequent intraperitoneal (IP) injections used incomplete Freund's adjuvant. Adjuvant was not used for intravenous (IV) injections. The injection regimes of the mice used for fusions are shown below:

<table>
<thead>
<tr>
<th>Date</th>
<th>Antigen</th>
<th>Quantity/mouse</th>
<th>Route</th>
</tr>
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<tbody>
<tr>
<td>20/2/90</td>
<td>ICP8</td>
<td>2 μg</td>
<td>IP</td>
</tr>
<tr>
<td>2/4/90</td>
<td>ICP8</td>
<td>2.5 μg</td>
<td>IP</td>
</tr>
<tr>
<td>17/5/90</td>
<td>ICP8</td>
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<td>IP</td>
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<tr>
<td>22/8/90</td>
<td>ICP8</td>
<td>3.3 μg</td>
<td>IP</td>
</tr>
<tr>
<td>5/10/90</td>
<td>ICP8</td>
<td>5 μg</td>
<td>IV</td>
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UL42 fusion

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<th>Date</th>
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POL fusion

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</table>

C) Hybridoma fusions

Fusions were performed essentially as described in (Harlow and Lane, 1988). Sp² myeloma cells were thawed 1 week prior to the fusion and subcultured every day to obtain a population of rapidly-dividing cells. Approximately 2 x 10⁸ Sp² cells were used per spleen. The mouse was sacrificed by the ICRF animal unit 4 days after a final injection intravenously. The spleen was aseptically removed and freshly supplied in E4 medium. The spleen was teased apart for several minutes and larger clumps of tissue were omitted from the final cell suspension. The stirring method rather than the spinning method of fusion was used. Fusion by stirring was performed exactly as described in (Harlow and Lane, 1988), except for the inclusion of 0.8ng/ml interleukin-6 (British Biotechnology Ltd.) in the plating medium in place of OPI (oxaloacetate, pyruvate and insulin). The cells were left to recover in an atmosphere of 5-10% CO₂ for 30min-1h before plating out.

Fusions were plated over approximately fifteen 96-well tissue-culture plates (Falcon) at 100µl/well. 100µl of fresh plating medium was added to each well 5-6 days after the fusion to feed the hybridoma cells.
Fusions were screened by cell staining when each clone had, on average, grown to cover about half of the well surface (generally 10 days-2 weeks after the fusion date). Hybridoma culture medium from each well was spotted onto plates of HSV-1-infected cells, which had been fixed in acetone/methanol 5h.p.i. and peroxidase staining was performed (see 1.c).

Clones from positive wells were transferred up to 48, 24, and then 6-well plates by dilution in E4 medium containing 10% FCS. After transfer to 6cm. and then 10cm plates, hybridomas were frozen in 8% dimethylsulphoxide/FCS (see 1.b).

d) Single-cell-cloning

To obtain hybridoma lines derived from a single cell, cloning was carried out using a dilution method. Hybridomas from positive wells (generally at the 24- or 6-well plating stage) were diluted in E4 medium containing 20% prescreened FCS and 8ng/ml interleukin-6, so as to obtain approximately 50 clones per 96-well plate. Plates were then incubated at 37°C and screened by cell staining as described above (3.a). Cloning was repeated until all hybridoma-containing wells in a dilution plate were positive in the screening process.

e) Production of supernatant and ascites

Large scale production of hybridoma supernatant was carried out by the ICRF hybridoma development unit, using spinner cultures. Sub-typing was also performed by this unit, using ELISA assays.

For ascites production, hybridoma cells were resuspended in 0.5ml E4 medium containing 0.01% FCS and injected intraperitoneally into mice (ICRF animal unit) which had been intraperitoneally injected with 0.5ml pristane (2, 6, 10, 14-tetramethyldecanoic acid) 11 days earlier. 7 mice were used per hybridoma, with approximately 10^6 cells injected per mouse. This yielded around 20ml ascites per hybridoma.

Supernatants were stored at 4°C, in the presence of 0.02% sodium azide. Ascites were stored at 4°C in the presence of 0.02% sodium azide, or at -20°C for longer-term storage.

f) Purification of antibodies

Various techniques were employed according to the subclass of antibody being purified (see (Harlow and Lane, 1988) for a general description of the techniques and the rationale for deciding which
purification strategy to use for a particular antibody. Purified antibodies were quantified by absorption at 280nm and kept at 4°C in the presence of 0.02% sodium azide, or at -20°C for longer-term storage.

**Ammonium sulphate precipitation**

Ammonium sulphate precipitation was employed for concentration of large volumes of antibody-containing solution. Ultrapure ammonium sulphate (Sigma) was added slowly, with stirring, to cold antibody supernatant or column fractions to 277g/litre, creating a 45% saturated solution. The solution was stirred for 2h-o/n at 4°C and then spun at 8000xg for 15min at 4°C to pellet the precipitated antibody fraction. The pellet was resuspended in PBS to one tenth of the starting volume and dialysed versus 3 changes of PBS.

**Caprylic acid purification**

This was employed to obtain ascites/sera of sufficient purity to use in ELISA techniques. Purification was performed exactly as described in (Harlow and Lane, 1988), using 0.4ml caprylic acid (Sigma) per ml of ascites/serum.

**Protein A columns (high salt)**

This method was used for antibodies of the IgG1 subclass. The sodium chloride concentration of the tissue culture supernatant was adjusted to 3.3M by adding 184g sodium chloride (BDH) to 1litre of supernatant. 0.1 volumes of 0.1M sodium borate (0.1M borax plus 0.1M boric acid to pH8.9) were added, and the resulting solution was recirculated through a 5ml protein A-sepharose column (Pharmacia), o/n at 4°C. The column was washed with 10 volumes of 3M sodium chloride, 50mM borate (pH8.9), then with 10 volumes of 3M sodium chloride, 10mM borate (pH8.9). Antibody was eluted with 1ml steps of 100mM glycine (pH 3.0), directly into tubes containing 100μl of 1M Tris (pH8.0). Immunoglobulin-containing fractions were identified by absorbance at 280nm.

The column was regenerated by washing sequentially with 2M urea, 1M lithium chloride and 100mM glycine.

**Hydroxylapatite columns**

Hydroxylapatite chromatography was used in the purification of antibodies of the IgM subclass. Biogel HTP (BioRad) was de-fined twice by stirring in 10mM sodium phosphate (pH6.8), allowing to settle and decanting to remove small particles. The gel was resuspended in an equal volume of 10mM sodium phosphate (pH6.8) prior to column packing. Antibody was purified as described in (Harlow and Lane, 1988), eluting with increasing
concentrations of sodium phosphate pH6.8 (50mM increments of 10ml each from 50mM to 500mM).

g) Labelling of antibodies
Biotinylation of purified antibodies was carried out as described in (Harlow and Lane, 1988), using succinimide ester (Clontech). Antibodies were spun through Centricon 30 units (Amicon) to remove azide and allow dilution into the correct reaction buffer (sodium borate pH8.9). The biotinylated reaction product was dialysed extensively against PBS, before storage at 4°C, in the presence of 0.02% azide.

4. List of antibodies
The tables on the following pages list all the antibodies used in the results section, apart from the novel antibodies described in Chapter 6.
<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Type</th>
<th>Antigen</th>
<th>Source</th>
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(Individuals shown without an address were members of the Lane laboratory when the antibody was donated.)
### Table 2.2. Antibodies to viral proteins

<table>
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<th>Name</th>
<th>Species</th>
<th>Type</th>
<th>Antigen</th>
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<tr>
<td>anti-HSV Pol</td>
<td>rabbit</td>
<td>polyclonal</td>
<td>N-terminal-deleted HSV Pol fusion protein</td>
<td>D.I. Dorsky (Harvard Medical School)</td>
<td>(Dorsky and Crumpacker, 1988)</td>
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<td>Z1F11</td>
<td>mouse</td>
<td>monoclonal</td>
<td>HSV-1 UL42 protein</td>
<td>H.S. Marsden (MRC, Glasgow)</td>
<td>(Murphy, et al., 1989)</td>
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<tr>
<td>anti-DBP</td>
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<td>polyclonal</td>
<td>DNA binding protein of adenovirus type-2</td>
<td>R. Hay (University of St. Andrews)</td>
<td></td>
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<tr>
<td>anti-E1a</td>
<td>rabbit</td>
<td>polyclonal</td>
<td>adenovirus early protein 1a</td>
<td>E. Harlow (Massachusetts General Hospital)</td>
<td></td>
</tr>
<tr>
<td>anti-T Ag</td>
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<td>polyclonal</td>
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<tr>
<td>PAb419</td>
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<td>SV40 VP1 capsid protein</td>
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</table>

#### 5. Immunochemical Techniques

**a) Immunoblotting**

Denaturing gel electrophoresis was performed according to the method of Laemmli (Laemmli and Favre, 1973), using polyacrylamide gels cast in a BioRad (or Hoeffer) minigel unit. Samples were diluted 1:1 in 2x Laemmli sample buffer (1x = 62.5mM Tris pH6.8, 2% sodium dodecyl sulphate, 10% glycerol, 0.001% Bromophenol Blue). 2-mercaptoethanol was added to 5% and samples were placed in a boiling water bath for 5min.
Proteins were run at 50 volts through the stacking gel (5% acrylamide) and at 150 volts through the running gel (10% acrylamide).

Transfer on to nitrocellulose (Schleicher & Schuell BA85) was performed by the method of Towbin (Towbin, et al., 1979) using a BioRad mini-gel blotting apparatus. Transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol, 0.1% sodium dodecyl sulphate) was freshly prepared. Proteins were transferred for 2h at 200mA or o/n at 100mA.

After blotting, the nitrocellulose was air-dried, rinsed in dH2O and stained briefly in Ponceau S (0.2% Ponceau S in 3% trichloroacetic acid). The blot was then rinsed in dH2O and photocopied as a record of the protein staining pattern. After rinsing in PBS to remove the Ponceau stain, blots were blocked in 5% dried milk (Marvel) in PBS, for 1h, with rocking. Blots were rinsed briefly in PBS before incubation, on a rocker, in first antibody (diluted in 10% FCS/PBS where necessary) for 2h (RT) or o/n (4°C). First antibody was removed by two 15min washes in 0.2% NP40 in PBS. Alkaline-phosphatase(ALP)-conjugated second antibody (DAKO) was then applied, diluted 1/1000 in 10% FCS/PBS, with incubation and washing as for the first antibody. The substrate was developed using nitro blue tetrazolium and bromochloroindolyl phosphate in ALP buffer (100mM Tris, 100mM sodium chloride, 5mM magnesium chloride, pH 9.5), (Harlow and Lane, 1988). Colour development (purple-black) was stopped by incubation in 1% acetic acid. Blots were then rinsed in water before drying and mounting.

b) Immunoprecipitation

Cell extracts were prepared as described in 1.e. The number of cells used varied with the experiment, but was generally around 2 x 10^6 per immunoprecipitation. Extracts were spun for 30min in an Eppendorf centrifuge at maximum speed, before preclearing by incubating for 30min with protein G sepharose (Pharmacia) diluted 1:1 with sepharose 4B beads (Pharmacia). All incubations were done on a rotating wheel at 4°C. Samples were spun briefly in an Eppendorf centrifuge, and the resulting supernatant was immunoprecipitated by incubation with antibody o/n. Approximately 20μl protein G sepharose/sepharose 4B beads (1:1) were incubated with the mixture for 30min-1h, before being pelleted by brief centrifugation. The supernatant was removed and the beads were washed 3 times in cold lysis buffer (see 1.e),
before resuspension in Laemmli sample buffer for electrophoresis as
described in 5a.

Gel electrophoresis was performed as described in 5.a. Unlabelled
immunoprecipitations were immunoblotted (see 5.a). If $^{35}$S-labelled extracts
were used, the gel was stained with 0.25% Coomassie Brilliant Blue R in
50% methanol/10% acetic acid, destained in 10% methanol/14% acetic acid
for 30 min and soaked in "Amplify" (Amersham) for 15-30min, before drying
onto 3MM paper under vacuum and exposing to pre-flashed
autoradiographic film (Kodak X-omat or Fuji X-ray).

c) Enzyme-linked immunoabsorption assays (ELISA's)

Two-site sandwich assays were performed essentially as described in
(Harlow and Lane, 1988). Flat-bottomed 96-well plates were coated with
antibody diluted in PBS for 2h at RT, washed twice in PBS and blocked in
3% BSA o/n at 4°C. Cell extracts were prepared as described in 1.e,
quantitated by the method of Bradford (Bradford, 1976), using BioRad
protein quantitation reagent, and applied across blocked, PBS-washed 96-
well plates in serial 1/2 dilutions (in 1% BSA/0.1% NP40/PBS). After 2h at
4°C, plates were washed 3 times in NP40 and once in PBS, before addition
of second antibody diluted in 1%BSA/0.1% NP40/PBS for 2h at 4°C. Plates were then

HRP was detected using 10mg/ml 3', 3', 5', 5',-tetramethylbenzidine
(TMB) diluted 1/100 in 0.1M citric phosphate (0.1M disodium-hydrogen-
orthophosphate plus citric acid to pH6.0) with hydrogen peroxide at 1/5000
v/v (50µl developer/well). Colour development (blue) was stopped after 10
min with 1M sulphuric acid (50µl/well), turning the reaction product yellow.
Absorbance readings were taken at 450nm.

6. DNA techniques

a) Plasmids

Plasmids expressing individual HSV-1 DNA replication proteins
(Heilbronn and zur Hausen, 1989) were obtained from R. Heilbronn
(Heidelberg). In these plasmids, the replication genes were placed under
the control of the human cytomegalovirus immediate early promoter. The
plasmids are detailed on the following page:
<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Replication gene</th>
<th>HSV-1 DNA replication protein</th>
</tr>
</thead>
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<tr>
<td>pH5</td>
<td>UL29</td>
<td>Infected cell protein 8 (ICP8)</td>
</tr>
<tr>
<td>pH1 (C57) pCM-pol</td>
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<td>DNA polymerase</td>
</tr>
<tr>
<td>pCM-UL42</td>
<td>UL42</td>
<td>65k DNA binding protein</td>
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<tr>
<td>pCM-UL8</td>
<td>UL8</td>
<td>Component of helicase-primase</td>
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</tbody>
</table>

**b) Transformation into competent cells**

DH5α library-efficient competent cells (BRL) were thawed on ice and transformed using the "one-minute" transformation protocol described by (Golub, 1988). Transformed cells were plated onto L-agar containing 100μg/ml ampicillin and incubated at 37°C o/n.

c) Large-scale plasmid preparation

500ml cultures were grown up overnight at 37°C, in L-broth containing 100μg/ml ampicillin. A Qiagen 500 gravity flow column (Diagen) was used for each culture. DNA was prepared according to the manufacturer's instructions. The DNA obtained was extracted once in phenol (pH8.0) and once in phenol/chloroform (1:1), before ethanol precipitation at -20°C, resuspension of the pellet in TE (10mM Tris, 1mM EDTA, pH8.0), and storage at -20°C.

DNA was size-checked by electrophoresis in 1% agarose gels, run in TAE buffer (40mM Tris, 5mM sodium acetate, 10mM EDTA, pH7.8) at 100V, and stained with ethidium bromide.

d) Calcium phosphate transfection

CV-1 cells were generally seeded onto 6cm tissue culture plates 12-20h prior to transfection, such that their confluency at the time of transfection was 60-70%. The tissue culture medium was changed 5 min-2h before transfection. For each 6cm plate, 7-25μg DNA (prepared as described in 5.c. was mixed with 167μl filter-sterilised TSB (6mM glucose, 20mM Hepes, 137mM sodium chloride, 5mM potassium chloride, 0.7mM disodium-hydrogen-orthophosphate, pH 6.98) and 17μl 2.5M calcium chloride (filter-sterilised) in a polystyrene tube. After 15min at RT, a fine, bluish precipitate was visible. This mixture was then added dropwise to the cell medium, with gentle swirling. After 4-5h at 37°C, numerous fine, black particles were
visible on the surface of the cells. The medium was removed at this stage, and the cells were "glycerol-shocked" by incubation with 2ml of prewarmed 15% glycerol/TSB at 37°C for 4min. The cells were then washed twice in E4 medium, and incubated in E4 medium containing 10% FCS at 37°C, in an atmosphere of 5-10% CO₂, for 24-48h, before fixation and cell staining (see 1.c). The above method was provided by Dr. Lu Xin (CRC, Dundee) and was based on that described in (Land, et al., 1983).
3. Herpes simplex virus

This chapter describes the effects of HSV-1 infection on the intranuclear location of various host nuclear proteins. Photographs are presented of immunofluorescently-labelled cells infected in the presence or absence of a specific inhibitor of viral replication. It is shown that the number of prereplicative sites formed by the virus is determined by the growth state of the host cell. Finally, transfection experiments are described which examine the roles of individual HSV-1 proteins in the formation of viral DNA replication structures.

Distribution of infected cell protein 8 (ICP8) in infected cells

The distribution of ICP8 in CV-1 and Vero cells infected with HSV-1 strains KOS1.1 and mP respectively has been described previously (Quinlan, et al., 1984; de Bruyn Kops and Knipe, 1988). Figure 3.1 confirms that ICP8 located in a similar manner in CV-1 cells infected with HSV-1 strain 17 syn+ i.e. it was present in large "globular" replication compartments in cells where viral DNA replication was occurring, but was localised to small discrete prereplicative sites when infection was carried out in the presence of a specific inhibitor of the HSV DNA polymerase, phosphonoacetate (PAA). Thus, CV-1 cells and HSV-1 strain 17 syn+ were used in the following experiments. The large nuclei of CV-1 cells made them particularly suitable for cell staining and cells infected with HSV-1 strain 17 syn+ formed syncitia, providing a simple assay for infection levels.

Distribution of SSB and PCNA in PAA-treated HSV-1-infected cells

The discovery that newly-replicated cellular DNA relocates to viral prereplicative sites in PAA-treated HSV-1-infected cells suggested that host DNA replication proteins may also be associated with viral prereplicative structures (de Bruyn Kops and Knipe, 1988). To examine this question, immunofluorescence was carried out on PAA-treated HSV-1-infected cells using a panel of antibodies raised against cellular proteins known to have a role in DNA replication. The distributions of two such proteins, SSB and PCNA are shown in figures 3.3a and 3.3b together with the pattern of ICP8 staining revealed by simultaneous double-labelling. Comparison with mock-infected cells stained in the same manner (figure 3.2) clearly shows that both proteins were redistributed upon infection. (Note that PAA had no effect on
Figure 3.1. ICP8 staining in the presence or absence of viral DNA replication.
CV-1 cells were infected with HSV-1 as described in Materials and Methods section 2b. Cells were fixed 5hpi in acetone/methanol and immunofluorescence was carried out using polyclonal anti-ICP8 serum diluted 1/500 (see Materials and Methods section 1c) and TRITC-conjugated goat anti-rabbit immunoglobulin (diluted 1/100).

A. HSV-1-infected cells.
B. Cells infected with HSV-1 in the presence of 400μg/ml phosphonoacetate.
Figure 3.2 Cell staining of mock-infected cells.
CV-1 (a-c) or MDA-MB-468 cells (d) were mock-infected as described in Materials and Methods section 2b. Cells were fixed 5hpi in acetone/methanol and immunofluorescence was carried out using: a, anti-SSB (70C); b, anti-PCNA (PC4); c, anti-Rb (1F/8); d, anti-p53 (PAb421). FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30) was used as to visualise the first antibodies. Cells were viewed under a x40 objective.
Figure 3.3. Double-staining of cells infected with HSV-1 in the presence of phosphonoacetate (PAA).
Cells and methods are as described in fig.3.4., except that PAA was included in the cell medium at 400μg/ml throughout the infection. Right-hand panels show ICP8 (polyclonal anti-ICP8, 1/500). Left-hand panels show: a) SSB (70C); b) PCNA (PC2); c) Rb (IF/8); d) p53 (PAb421); e) p68 (biotinylated PAb204, diluted 1/10). Scale bar, 25μm (a, b) or 10μm (c, d, e).
the distribution of these proteins in mock-infected cells; data not shown). SSB was always colocalised exactly with ICP8, whereas PCNA was also present in areas other than prereplicative sites. The non-replicative host nuclear protein p68 (Iggo and Lane, 1989) did not redistribute upon infection (figure 3.3e).

**Distribution of the tumour-suppressor proteins Rb and p53 in PAA-treated HSV-1-infected cells**

Since antibodies to Rb and p53 were readily available, it was of interest to examine the effect of HSV-1 infection on their nuclear location. Both Rb and p53 were seen to relocate upon infection (compare figures 3.2 and 3.3). Rb was found exclusively at a subset of prereplicative sites (figure 3.3c), whereas p53 was present at all prereplicative sites as well as other regions of the nucleus (figure 3.3d). Figures 3.3c and 3.3d show particularly clear-cut examples of colocalisation of these proteins with ICP8.

**Distribution of SSB and PCNA during productive HSV-1 infection**

Bromodeoxyuridine labelling has been detected in viral replication compartments during productive infection (de Bruyn Kops and Knipe, 1988). However, this labelling is likely to represent largely viral DNA replication, since cellular DNA replication is inhibited during productive HSV-1 infection. Using antibodies to DNA replication proteins, the question of cellular DNA replication site redistribution upon productive infection can be better addressed.

Figures 3.4a and 3.4b show that both SSB and PCNA were present in viral replication compartments during HSV-1 infection. In each case, all of the protein appeared to be colocalised with ICP8. The non-replicative protein p68 behaved in the same way as in PAA-treated cells, i.e. it did not redistribute upon productive infection (figure 3.4e).

**Distribution of Rb and p53 during productive HSV-1 infection**

Rb and p53 were also seen in replication compartments during productive HSV-1 infection (figures 3.4c and 3.4d). Incomplete colocalisation was observed, as was the case for PAA-treated cells. Rb was only present in a subset of areas within replication compartments, whereas p53 was present both within and outside the compartments defined by ICP8.
Figure 3.4. Double-staining of HSV-1-infected cells.
Confocal microscope images of fluorescein and rhodamine fluorescence in CV-1 (a, b, c, e) or MDA-MB-468 (d) cells, 5hpi. Fixation and staining methods as for fig. 3.1. Right-hand panels show ICP8 staining (polyclonal anti-ICP8 serum diluted 1/500). Left-hand panels show: a) SSB (70C); b) PCNA (PC4); c) Rb (IF/8); d) p53 (PAb421); e) p68 (biotinylated PAb204 {pure antibody diluted 1/10}). Conjugates used were: TRITC-conjugated goat anti-rabbit immunoglobulin (1/100) plus FITC-conjugated goat anti-mouse immunoglobulin (Sigma, 1/20) for a-d, and FITC-conjugated goat anti-rabbit immunoglobulin (1/20) plus Texas-Red-streptavidin for e. Scale bar, 25μm (a, b, c) or 10μm (d, e).
Controls

The antibodies used for immunofluorescence did not cross-react with HSV-1 proteins on Western blots of total infected-cell extracts (figure 3.5). In addition, the anti-ICP8 antibody did not react with any of the cellular proteins studied (figure 3.5.b).

It is interesting to note that the polyclonal anti-ICP8 antibody cross-reacted with a band of a similar molecular weight in mock-infected-cell extracts (figure 3.5b). However, detection of this band was not blocked by preincubation of the ICP8 antiserum with pure ICP8 protein (obtained from M.D. Challberg, see Materials and Methods section 3a), a procedure which inhibited detection of ICP8 in infected-cell extracts (figure 3.6). Thus, detection of the cellular band was probably the result of a spurious cross-reaction.

Control cell staining experiments were performed in which one of the first antibodies was omitted from the incubation, but both fluorescent conjugates were added in the normal way. These experiments confirmed that no cross-reaction was occurring between the conjugates themselves, or between conjugates and opposite first antibodies. Also, no significant bleed-through into opposing fluorescence channels was seen in these experiments, a fact confirmed by the incomplete colocalisation seen in some samples. Staining of mock-infected cells with anti-ICP8 revealed no significant signal beyond the general background often seen with polyclonal sera.

The results shown in figures 3.3. and 3.4. were repeatable with other monoclonal antibodies recognising different epitopes on SSB, PCNA, Rb and p53 (antibodies are listed in table 3.1). Redistribution to replication compartments was also observed in other cell types, including HeLa (human) Ptk-2 (kangaroo) and BT20 (human) cells.

No physical association is detectable between ICP8 and colocalised proteins

ICP8 immunoprecipitations from HSV-1-infected-cell extracts were run on denaturing gels, transferred to nitrocellulose and probed with antibodies to SSB, PCNA, Rb and p53. None of these proteins were co-precipitated by anti-ICP8 antibody in this assay (figure 3.7). Thus, any interaction between the colocalised proteins and ICP8 must be either weak or indirect.
Figure 3.5. Control Western blots.
Cell extracts prepared as described in Materials and Methods section 1e were run on denaturing gels and transferred to nitrocellulose. The nitrocellulose was cut into strips which were blotted with the antibodies shown (see Materials and Methods section 5a). Note that some degradation of ICP8 has occurred in A and C.
A. CV-1 cell extracts prepared 5hpi with HSV-1 and blotted with: polyclonal anti-ICP8 serum diluted 1/1000; anti-PCNA (PC2); anti-p53 (PAb421); polyclonal anti-ligase diluted 1/50; anti-Rb (IF/8); purified anti-SSB (34A) diluted 1/1000.
B. Mock (m) or 5h-HSV-1-infected (+) CV-1 cell extracts were prepared and blotted as in A using: polyclonal anti-ICP8 serum (1/1000); anti-PCNA (PC9); anti-PCNA (PC2); polyclonal anti-ligase serum (1/50); purified anti-SSB (34A, 1/500).
C. Mock (m) or 5h-HSV-1-infected (+) MDA-MD-468 cell extracts prepared and blotted as in A using: anti-p53 (PAb421) and polyclonal anti-ICP8 serum (1/1000).

Figure 3.6. Effect of preincubation of anti-ICP8 serum with ICP8 protein.
1μl of polyclonal anti-ICP8 serum was incubated with 5μg pure ICP8 for 1h, on ice. 1ml of 10%FCS/PBS was added, and this mixture was divided equally for incubation with nitrocellulose strips 2 and 4. Lanes 1 and 3 were incubated with anti-ICP8 alone, diluted in the same way. Lanes 1 and 2 contain mock-infected extracts. Lanes 3 and 4 contain 5h-HSV-1-infected extracts. Methods as in figure 3.5.
Figure 3.7. Western blots of ICP8 immunoprecipitations from HSV-1-infected cell extracts.
Cell extracts were prepared 5hpi as described in Materials and Methods section 1e. Immunoprecipitation from these extracts was performed as described in Materials and Methods section 5b. Bead eluates were run on a denaturing polyacrylamide gel, transferred to nitrocellulose and Western-blotted (see Materials and Methods section 5a).
A. Immunoprecipitations from CV-1 cell extracts were carried out using polyclonal anti-ICP8 (1/1000). Mock (m) and infected (+) extract immunoprecipitates were blotted with polyclonal anti-ICP8 (1/1000), anti-Rb (IF/8), or anti-SSB (pure 34A antibody diluted 1/500). Blotting of immunoprecipitation supernatants revealed that Rb and SSB were present in the extracts (not shown).
B. Immunoprecipitations from CV-1 cell extracts were carried out using polyclonal anti-ICP8 (1/1000) (lanes 1-4) or anti-PCNA (PC10) antibodies (lanes 5-8). Immunoprecipitates were blotted with polyclonal anti-ICP8 (1/1000) (lanes 1, 2, 5 and 6) or anti-PCNA (PC2) antibodies (lanes 3, 4, 7 and 8).
C. Immunoprecipitations from BT20 cell extracts were carried out using 11E2, a monoclonal antibody directed against ICP8 (see chapter 6), or BG2, a monoclonal antibody to β-galactosidase. Immunoprecipitates and immunoprecipitation supernatants were blotted with 10A3, a monoclonal antibody to ICP8 (see chapter 6) and then with PAb421 anti-p53 antibody.
Effects of HSV-1 infection on the distribution of other host nuclear proteins

Table 3.1 summarises the results obtained from staining HSV-1-infected CV-1 cells with monoclonal antibodies raised against a variety of host nuclear proteins. All immunofluorescence was performed on cells infected in the presence or absence of PAA and fixed 5hpi.

Table 3.1. Distribution of host proteins in CV-1 cells infected with HSV-1

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>ANTI-BODIES</th>
<th>COLOCALISATION WITH ICP8 (NO PAA)</th>
<th>COLOCALISATION WITH ICP8 (PAA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TOTAL</td>
<td>PART</td>
</tr>
<tr>
<td>SSB</td>
<td>70C; 34A</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>PCNA</td>
<td>PC2; PC4; PC1</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>NUCLEOLAR PCNA</td>
<td>PC9</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Rb</td>
<td>IF/8; 245</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>PAb421; PAb1801</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>DNA LIGASE I</td>
<td>POLyclonal Serum</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>POL α</td>
<td>SJK-132; 4E9 †</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>LP4N</td>
<td>LP4N</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>HISTONE</td>
<td>J2B2</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Sm</td>
<td>Y12</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>C-MYC</td>
<td>9E10</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>p68</td>
<td>PAb204</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>LAMIN B</td>
<td>LN43</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>B23</td>
<td>ANTI-B23</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Ki67</td>
<td>Ki67</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

† Note that a cross-reaction between these antibodies and HSV Pol cannot be ruled out.
(For details of antibodies see Materials and Methods section 4a).
Thus, during productive HSV-1 infection, all DNA replication proteins tested were redistributed to replication compartments. Rb, p53 and the uncharacterised nuclear antigen recognised by LP4N were also present in replication compartments. c-myc and the small nuclear ribonucleoprotein Sm were found in clusters associated with the peripheral regions of viral replication compartments, in contrast to their distributions throughout the nucleus of mock-infected cells (see figure 3.8). p68, lamin B, B23 and Ki67 protein distributions were not altered by a 5h infection with HSV-1.

The distribution of PCNA changes when ongoing viral DNA replication is inhibited

ICP8 has been shown to move from replication compartments to prereplicative sites when active viral DNA replication is inhibited either by temperature shifting a HSV Pol temperature-sensitive mutant or by adding PAA (Quinlan, et al., 1984). Similarly, addition of PAA during productive infection produced PCNA staining of prereplicative sites, rather than replication compartments (figure 3.9). In some cells, a mixture of compartment and prereplicative site staining patterns was visible, suggesting that PCNA moves between sites according to the status of viral replication. PCNA was colocalised with ICP8, even in these intermediate cells, implying coordinate movement of both proteins. Thus, active viral DNA synthesis is required for the maintenance of PCNA in HSV-1 replication compartments.

The redistribution of PCNA upon HSV-1 infection requires protein synthesis

The immediate early (α) genes of HSV-1; ICP4, ICP0, ICP27, ICP22 and ICP47 are transactivated by a component of the infecting virion, Vmw65 (also known as αTIF or VP16) (Campbell, et al., 1984). This tegument protein acts in concert with the cellular Oct-1 transcription factor and possibly other cellular proteins to activate transcription of the HSV-1 immediate early genes (Katan, et al., 1990; Stern, et al., 1989). It was of interest to determine whether this transactivation alone was sufficient for the redistribution of cellular proteins.

Figure 3.10 shows the effects of cycloheximide treatment on the redistribution of PCNA by HSV-1. It can be seen that, when protein synthesis was inhibited by cycloheximide, PCNA did not move to replication compartments upon HSV-1 infection, but remained at mock-infected cell
Figure 3.8. Effects of HSV-1 infection on snRNP and c-myc distributions.

A. Sm distribution in productively-infected cells. CV-1 cells were infected with HSV-1, fixed with acetone/methanol 5hpi and stained with anti-Sm antibody (purified Y12 antibody, 1/200) and polyclonal anti-ICP8 (1/500) (see Materials and Methods section 1c). FITC-conjugated goat anti-mouse immunoglobulin (Sigma, 1/20) plus TRITC-conjugated goat anti-rabbit immunoglobulin (1/100) were used as to visualise the first antibodies.

B. c-myc distribution in productively-infected cells. HSV-1-infected cells were formaldehyde-fixed 5hpi and stained with anti-myc antibody (9E10) (affinity-purified, diluted 1/2) and polyclonal anti-ICP8 serum (1/500).

C. Sm distribution in PAA-treated HSV-1-infected cells. CV-1 cells were infected in the presence of PAA, fixed and stained 5hpi as described in A.
Figure 3.9. PCNA distribution after addition of PAA to cells in which HSV-1 DNA synthesis is occurring. Infection was allowed to proceed for 3h, before addition of PAA to 400μg/ml. After a further 2h, cells were fixed in acetone/methanol and stained with polyclonal anti-ICP8 (1/500) and PC2, as described in the legend to figure 3.8a.

Figure 3.10. Effect of cycloheximide on PCNA redistribution. A. CV-1 cells grown and infected with HSV-1 in the absence of cycloheximide. B. CV-1 cells pretreated with 10μg/ml cycloheximide for 90min prior to HSV-1 infection in the presence of 10μg/ml cycloheximide. Cells were fixed in acetone/methanol 5hpi and stained with polyclonal anti-ICP8 serum (1/500) and monoclonal anti-PCNA (PC2) antibody. Fluorescent conjugates were used as described in the legend to figure 3.8a.
sites (compare with figure 3.2b). Similar results were observed for SSB and Rb (data not shown). Thus, the redistribution of these proteins requires active protein synthesis and cannot be mediated solely by the transactivation of immediate early genes by Vmw65.

Preliminary results with the temperature sensitive tsK mutant, which is mutated in the ICP4 gene and synthesises only the five immediate early proteins (Preston, 1979) suggest that early (β) gene transcription is required for the redistribution of PCNA by HSV-1 (data not shown).

**Formation of replication compartments during productive infection of growth-arrested cells**

![Image](image.png)

**Figure 3.11. Cell staining of HSV-1-infected serum-starved cells.** CV-1 cells were allowed to attach to coverslips in E4 medium containing 10% FCS. Cells were then rinsed twice in E4 medium alone before addition of E4 medium containing 0.1% FCS. After 5 days, infection was performed in the normal manner (Materials and Methods section 2b). 5hpi, cells were fixed in acetone/methanol, and stained with anti-PCNA (PC2) and polyclonal anti-ICP8 (1/500) antibodies followed by FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/20) and TRITC-conjugated goat anti-rabbit immunoglobulin (1/100) (Materials and Methods section 1c).

Redistribution to replication compartments was also observed during infection of serum-starved cells (figure 3.11). SSB, PCNA, Rb and p53 were all present within compartments in such cells. The compartment areas appeared more clearly defined under these conditions than in S-phase cells (serum-starved cells stimulated 16h prior to infection), where the ICP8 tended to fill the whole cell apart from the nucleoli. This may simply reflect faster kinetics of infection in S-phase cells than in growth-arrested cells,
since ICP8 occupies the whole nucleus at late times of infection (Randall and Dinwoodie, 1986).

Thus, redistribution of host nuclear proteins to replication compartments may occur during natural HSV-1 infections of resting epithelioid cells.

A relationship between the growth state of the host cell and the number of viral prereplicative sites

During infections of mixed populations of PAA-treated cells, variable numbers of prereplicative sites were seen in individual nuclei (figure 3.3). It has been shown previously that the number of prereplicative sites formed in Vero cells infected at the non-permissive temperature with the HSV Pol mutant tsD9 is independent of the multiplicity of infection. This is not the case for replication compartments, whose initial number is dependent on the multiplicity of infection with wild-type HFEM/STH2 virus (Randall and Dinwoodie, 1986). Thus, some other factor must be influencing prereplicative site number. Therefore, it was of interest to determine whether the host cell growth state at the time of infection had any effect on prereplicative site number.

Figure 3.12 shows immunofluorescence photographs of serum-starved, or serum-stimulated cells infected with HSV-1 in the presence of PAA. Ki67 antibody specifically stains the nucleoli of proliferating cells (G1, S, G2 and M), but not of quiescent cells (G0) (Gerdes, et al., 1984). Thus, this antibody was used to check the proliferative state of the two groups of cells. Over 95% of the serum-starved cells were negative for Ki67, whereas over 95% of the serum-stimulated cells were Ki67-positive (3.12 a and b). Parallel groups of cells were infected for 5h in the presence of PAA and stained with 10A3, a monoclonal anti-ICP8 antibody (3.12 c-f). Serum-starved cells were found to have a few discrete foci superimposed on a weak, diffuse pattern of ICP8 staining. Conversely, proliferating cells generally contained numerous ICP8 foci, which varied from cell to cell in their degree of coarseness.

The incorporation of 3H-T was used to confirm the above results by simultaneously detecting cellular DNA replication sites and ICP8-labelled viral prereplicative sites. Cells with few ICP8 foci were unlabelled, whereas cells with many ICP8 foci were heavily labelled with 3H-T (figure 3.13).
Figure 3.12. Cell staining of HSV-1-infected and PAA-treated, serum-starved and serum-stimulated cells.
CV-1 cells were serum-starved as described in the legend to figure 3.11. for 7 days. One group of cells (shown in b, d, and f) was then stimulated by addition of E4 medium containing 10% FCS, whilst the other group (shown in a, c, and e) was kept in the same medium. After 17h, the cells were infected with HSV-1 in the presence of 400μg/ml PAA (Materials and Methods section 2b). 5hpi, cells were fixed in acetone/methanol, and stained with Ki67 (a, b) or anti-ICP8 (10A3) antibodies (see Chapter 6) (c-f) followed by FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30). a-d show low-power images. e and f show higher-power images.

Figure 3.13. 3H-T/ICP8 double-labelling of HSV-1-Infected, PAA-treated cells.
CV-1 cells grown in E4 medium containing 10% FCS, were infected with HSV-1 as described in Materials and Methods section 2b, except that 3H-T was included in the medium added to the cells after the adsorption stage of infection (Materials and Methods section 1d). Thus, cells were labelled with 3H-T from 45min to 5hpi. Cells were fixed and stained with anti-ICP8 (10A3) antibody as described in the legend to figure 3.13.
* Note that none of these plasmids contained a HSV origin of DNA replication (Heilbronn and zur Hausen, 1989).
Distribution of transiently-expressed ICP8

Figure 3.14a shows the pattern of ICP8 staining seen in cells which were transiently-transfected with pH5, a plasmid with the ICP8 gene under the control of the human cytomegalovirus immediate early promoter. The cells shown were fixed 24h after glycerol shock treatment, since expression was optimal at this time. 0.5-1% of cells in the monolayer were positive for ICP8 by immunofluorescence. In such cells ICP8 filled the whole nucleus, except the nucleoli. The distribution of ICP8 was generally uniform, although a few cells showed minor irregularities in fluorescence staining intensity within the nucleus. Thus, ICP8 alone does not localise to prereplicative site structures when transiently expressed in host cells. The distribution of ICP8 in transfected cells is the same as that of SSB and PCNA (see figure 3.15a), which exhibit staining patterns similar to those seen in untransfected cells.

Distribution of transiently-expressed ICP8 in the presence of other HSV-1 DNA replication proteins

Seven HSV-1 proteins have been defined as being necessary and sufficient for viral DNA replication (see introduction). These proteins were co-expressed in cells by transient transfection of seven plasmids, each containing an individual HSV-1 gene under the control of the human cytomegalovirus immediate early promoter (see Materials and Methods section 6a for details of the plasmids used).

When all seven plasmids were transfected, the percentage of cells in the monolayer which were positive for ICP8 was approximately one seventh that of cells transfected with pH5 alone, presumably because of the lower amounts of ICP8 plasmid present. The majority of ICP8-positive cells exhibited a uniform staining pattern as described for cells transfected with pH5 alone. However, approximately 10-20% of ICP8-positive cells had a markedly different ICP8 distribution (figure 3.14b-d). A variety of ICP8 staining patterns were seen, ranging from many small foci through intermediate-sized larger foci (the most common pattern) to large clumps in the nucleus. These patterns strongly resembled ICP8 distributions seen in PAA-treated and productively-infected HSV-1 infected cells. Non-uniform ICP8 distributions were seen in a number of independent transfection experiments and were never seen in parallel transfections of the ICP8 plasmid alone. The amount of DNA added per plate was the same in the parallel transfections, suggesting that the altered patterns are not simply non-specific changes caused by an excess of DNA in the transfected cell.
Figure 3.14. ICP8 distribution in transiently-transfected cells. CV-1 cells were transfected using the calcium phosphate technique as described in Materials and Methods section 6d. 24h after glycerol shock treatment, cells were fixed in acetone/methanol and stained as described in Materials and Methods section 1c.

A to C. Cells transfected with a plasmid expressing ICP8. 7μg pH5 (see Materials and Methods section 6a) was added to each 6cm plate. ICP8 was detected using anti-ICP8 monoclonal antibody (10A3) followed by FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30).

D to L. Cells transfected with plasmids expressing all seven HSV-1 DNA replication proteins. 7μg of a mixture containing equal masses (1μg each) of the seven plasmids listed in Materials and Methods section 6a was added to each 6cm plate. Immunofluorescence was performed as described above.
Figure 3.15. SSB distribution in cells expressing HSV-1 DNA replication proteins.
CV-1 cells were transfected as in 3.14, except that 25μg DNA was added per 6cm plate. Double-staining was performed with anti-SSB (34A) and polyclonal anti-ICP8 antibodies using FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30) and TRITC-conjugated goat anti-rabbit immunoglobulin (1/100). Scale bar is 10μm.
A. Cells transfected with pH5, a plasmid expressing ICP8.
B. Cells transfected with plasmids expressing all seven HSV-1 DNA replication proteins.
In transfected cells with non-uniform ICP8 distributions, SSB was colocalised with ICP8 (see figure 3.15b). Thus, the transfection of all seven HSV-1 replication proteins can mimic HSV-1 infection by redistributing SSB to restricted areas within the nucleus which also contain ICP8.

**Discussion**

Cellular DNA replication proteins, Rb and p53 colocalise with ICP8 in HSV-1-infected cells

The results presented in table 3.1. show that the proteins studied fall into two general groups; DNA replication and tumour suppressor proteins, which redistribute to ICP8-containing sites on HSV-1 infection, and non-replicative proteins, which do not. There are some exceptions to this general rule. Some, but not all histone protein is found in replication compartments. Typical nucleosomal chromatin has been reported to be present, adjacent to regions of non-nucleosomal DNA, in capsid-associated HSV DNA molecules, including the areas around replication branch points (Muller, et al., 1980). Therefore, it is likely that some histone protein is used to coat progeny viral DNA. Also, the small ribonucleoprotein, Sm, an RNA splicing factor, appears to be present at a subset of viral prereplicative sites in PAA-treated infected cells (figure 3.8c). Any role for these Sm foci in HSV-1 RNA processing is likely to be limited, since the viral genome contains few introns and the HSV-1 early (α) gene products are diffusely distributed in PAA-treated cells (McGeoch, et al., 1988; Knipe, et al., 1987). Sequestration of snRNPs by HSV-1 may contribute to the inhibition of host RNA processing observed in infected cells (Martin, et al., 1987). It appears that snRNPs associate with a subset of prereplicative sites and migrate to the periphery as replication proceeds and these sites coalesce to form replication compartments. The nucleolar form of PCNA does not appear to colocalise exactly with ICP8, since, although it is released from the nucleoli of some cells 5hpi, not all of it enters replication compartments or prereplicative sites at this time. The antigen recognised by LP4N, which is normally present throughout the nucleus, is found in HSV-1 replication compartments, suggesting that it might be associated with DNA replication complexes in uninfected cells.

Sm and c-myc are found in discrete clusters at the periphery of replication compartments in cells productively-infected with HSV-1 (figures 3.8a and b). This is in agreement with a previous report of Sm redistribution...
in human epidermoid carcinoma (HEp-2) cells infected with HSV-1 (HFEM and KOS strains), where clusters of snRNP antigens, including S_m, were shown to condense throughout the nucleus, before migrating to its periphery. The clusters described were not detectably associated with ICP8 (Martin, et al., 1987). S_m and c-myc have similar distributions in HSV-1-infected cells, in agreement with their reported colocalisation in uninfected cells (Spector, et al., 1987).

Lamin B, p68, B23 and Ki67 exhibit mock-infected patterns of staining in HSV-1-infected cells 5hpi. B23 has previously been reported to redistribute in herpesvirus-infected HeLa cells (Walton, et al., 1989). However, no data was supplied as to the strain of herpesvirus used, the type of cell infected, or the duration of the infection.

The differential inclusion of host nuclear proteins in replication compartments and prereplicative sites argues against the observed redistributions being simply a case of non-specific aggregation of nuclear proteins upon infection. Hoescht staining shows that there is no gross movement of cellular DNA into replication compartments, but rather an exclusion of the dye from these areas. Also, only a subset of HSV-1 proteins are present in replication compartments (Knipe, et al., 1987; Olivo, et al., 1989), suggesting that these structures are functional assemblages of defined viral and cellular proteins.

**Do cellular DNA replication proteins participate in HSV-1 DNA replication?**

The results presented here show that cellular DNA replication proteins exhibit significantly altered distributions in HSV-1-infected cells and are present in the same novel structures as the viral replication protein ICP8. Furthermore, this colocalisation with ICP8 is seen in two quite different structures, according to the infection conditions used. This raises the question of whether host DNA replication proteins have any functional role in HSV-1 DNA replication.

Seven HSV-1 genes have been identified as being essential for viral DNA replication. These genes encode a DNA polymerase and associated processivity factor, ICP8, an origin-binding protein and a three-subunit helicase-primase complex. A description of these proteins and their known functional properties is presented in the introductory chapter. The presence of pol-α, PCNA, and SSB in the same structures as HSV Pol, UL42 and ICP8 implies an element of functional redundancy at these sites. The evolution of comparable proteins by HSV-1 would suggest that these host
proteins are not used to replicate the viral DNA and may be redistributed simply as a consequence of their binding to replicating cellular DNA. However, functional roles for some, or all, of these proteins in viral replication cannot be ruled out.

There is evidence for the involvement of some host cell proteins in HSV-1 DNA replication. Band-shift experiments suggest that a cellular factor may mediate OBP-oriS interactions (Dabrowski and Schaffer, 1991). The identity of this factor is not yet known, but it may well be involved in cellular DNA replication. Also, host topoisomerase II has been shown to act on progeny, but not parental, viral DNA (Ebert, et al., 1990) and a topoisomerase I activity, which may be cellular or viral, is associated with the HSV-1 virion (Muller, et al., 1985).

The redistribution of DNA ligase I to viral replication compartments along with host cell replication proteins (see table 3.1) provides further evidence that DNA ligase I functions in cellular DNA replication (Soderhall and Lindahl, 1975). Ligation of HSV-1 replication products may be performed by the host cell DNA ligase I in replication compartments. HSV-1 is thought to replicate via a rolling circle type mechanism whereby one strand is replicated continuously prior to discontinuous synthesis of the remaining parent strand (Jacob, et al., 1979; Kornberg, 1980; Rabkin and Hanlon, 1990). Thus, it is probable that HSV requires a ligase activity to join the fragments produced during discontinuous DNA synthesis. A HSV-1 gene encoding a DNA ligase enzyme has not yet been identified, despite knowledge of the entire genome sequence (McGeoch, et al., 1988). However, a change in the chromatographic properties of a DNA ligase activity in BHK-21 cells upon HSV-1 infection has been reported (Evans and Keir, 1975). This may represent a change in the properties of the host cell DNA ligase I upon its redistribution to replication compartments. Vaccinia virus may also make use of a host DNA ligase activity, since although it encodes a ligase enzyme, this is non-essential for viral replication in cultured cells (Colinas, et al., 1990).

An in vitro system has been developed which supports ATP-dependent rolling circle HSV-1 DNA synthesis from a preformed replication fork (Rabkin and Hanlon, 1990). This system uses HSV Pol-containing fractions purified from infected Vero cells and is at an early stage of characterisation. Specific inhibitors of HSV Pol inhibit DNA replication in this system to the same degree as they inhibit HSV Pol activity. This would seem to preclude a functional role for cellular DNA polymerases in viral DNA
synthesis from pre-primed templates. The incorporation of $^{3}$H-T into replication compartments within aphidicolin-treated cells infected with an aphidicolin-
also suggests that the aphidicolin-sensitive cellular DNA polymerases are not required for HSV DNA replication (Randall and Dinwoodie, 1986). However, DNA synthesis in the in vitro system occurs only after a 45min lag and takes place at a much slower rate than in vivo. This suggests non-optimal conditions and/or a requirement for additional factors.

Rb and p53 are redistributed to the same sites as cellular DNA replication proteins

As discussed in the introductory chapter, Rb and p53 are the products of tumour-suppressor genes and are thought to function in the control of cell growth. Rb and p53 interact with proteins from a number of DNA viruses. It may be that such interactions eliminate the growth-suppressive functions of Rb and p53, creating an environment for viral DNA replication free of cellular controls.

The mechanism of inactivation of Rb by viral proteins such as adenovirus E1a has recently been elucidated (Bandara and La Thangue, 1991; Chellappan, et al., 1991; Wagner and Green, 1991) (see introductory chapter). E1a binds to a "pocket" region of Rb which normally interacts with a range of cellular transcription factors. E1a competes with these transcription factors for binding to the Rb pocket region, freeing them to promote transcription of proliferation-related genes.

The experiments presented previously (figure 3.7a and c) did not detect an interaction between ICP8 and Rb or p53. Also, immunoprecipitations from $^{35}$S-labelled HSV-1-infected cell extracts using anti-Rb or anti-p53 antibodies did not reveal obvious HSV-1 proteins interacting with Rb and p53, though the existence of such proteins cannot be ruled out. Alternatively, Rb and p53 may be redistributed by an indirect mechanism as a consequence of an association with cellular DNA replication sites. Such an association has not yet been demonstrated for Rb. However, preliminary evidence for an indirect association of p53 with cellular replication complexes is provided by the demonstration of specific binding of p53 to a DNA sequence present in the vicinity of a number of putative cellular replication origins (Kern, et al., 1991). p53 also binds to DNA sequences adjacent to the SV40 origin of DNA replication (Bargonetti, et al., 1991). It would be interesting to determine whether p53 also binds to sequences associated with HSV-1 replication origins, since the region
around oriS contains a number of GGGCGG repeats and a single copy of the TGCCT motif, both of which have been implicated as target sequences for p53 binding.

Thus, the redistribution of Rb and p53 upon HSV-1 infection may alter the functional properties of these two proteins, allowing a deregulation of cellular growth controls. This may allow HSV-1 to overcome the barriers to DNA replication present in quiescent cells and to replicate its DNA continuously in a cellular environment permitting permanent S-phase.

**HSV-1 replication in growth-arrested cells**

The results presented previously (figure 3.11) show that the redistribution of host replication proteins to replication compartments occurs in growth-arrested HSV-1-infected cells, suggesting that this phenomenon may occur in natural infections of quiescent tissues. Viral prereplicative sites are also present in quiescent PAA-treated HSV-1-infected cells, but are very much reduced in number (figure 3.12). This may be a consequence of limiting amounts of constituent DNA replication proteins in resting cells. Another possibility is that such cells have a shortage of suitable structural elements for prereplicative site assembly.

Previous work suggests that viral prereplicative structures are formed by the incorporation of preformed cellular DNA replication complexes, rather than by de novo assembly of individual cellular and viral proteins (de Bruyn Kops and Knipe, 1988). This conclusion is based on bromodeoxyuridine incorporation into synchronised PAA-treated HSV-1-infected cells; 75% cells infected in S-phase had incorporated bromodeoxyuridine 5hpi, compared with less than 12% of cells infected during G1. Thus, most cells infected during G1 do not appear to synthesise cellular DNA at prereplicative sites, although the authors point out that components of the cell replication machinery insufficient to support DNA replication may still be assembled into prereplicative sites under these conditions. The results presented here suggest that this is indeed the case, although the number of prereplicative sites is reduced.

The same authors report that ICP8 is distributed at prereplicative sites in cells infected both during G1 and S-phase. However, no mention is made of prereplicative site number in such cells. A detailed study of PAA-treated synchronised cells may reveal that the variable prereplicative site patterns observed in mixed populations of cells are due to differences in the cell-cycle stages of the cells at the time of infection.
The redistribution of cellular DNA replication proteins cannot be mediated by ICP8 alone, but does occur in the presence of all seven HSV-1 DNA replication proteins.

Transiently-expressed ICP8 has a distribution typical of many cellular DNA replication proteins i.e. it has a fine granular staining pattern which excludes the nucleoli (figure 3.14a). Thus, ICP8 may possess an intrinsic ability to localise to host cell DNA replication sites. However, a change in the distribution of these sites is not observed when ICP8 is the only viral protein present, suggesting that other viral functions are required for this process. These functions appear to be provided by some, or all, of the six other essential HSV-1 DNA replication proteins, since their co-expression with ICP8 can result in a dramatic change in both SSB and ICP8 distributions (figure 3.15). A relatively high frequency of transfected cells had redistributed proteins (10-20% of ICP8-positive cells).

The range of staining patterns observed may be due to variations in transfection kinetics between individual cells, or it may be that different combinations of proteins produce redistribution to different types of structures within the nucleus. Perhaps a lack of HSV Pol results in the formation of prereplicative site-type structures, as those seen in figure 3.14c. Further transfection experiments are needed to define the roles of individual HSV-1 proteins in replication compartment formation.

Co-transfection of the same seven HSV-1 expression constructs into SV40-transformed hamster cells induces the amplification of SV40 DNA (Heilbronn and zur Hausen, 1989). This amplification will occur in the absence of viral DNA replication and requires all of the HSV-1 DNA replication proteins except OBP (Heilbronn, et al., 1990) (see OBP section in introductory chapter). As discussed in the introduction, indirect evidence for the amplification of host cell genes also exists (Chenet-Monte, et al., 1986). It has been proposed that a cellular protein can replace OBP causing the redirection of the HSV-1 DNA replication machinery to cellular replication origins and the subsequent amplification of host genes. The results presented here suggest that such amplification may occur within replication compartment-type structures. Thus, it appears that the HSV-1 DNA replication machinery can assemble within replication compartments in the absence of viral DNA, perhaps by utilising cellular DNA replication origins.
The results obtained by transiently expressing ICP8 in cells are at odds with a previous report of ICP8 localisation to "prereplicative site type" structures in Vero cells transfected with a different ICP8 expression construct (Quinlan, et al., 1984). Although it is debatable whether the previously-reported ICP8 distribution resembles that of prereplicative sites, it is certainly more irregular than that described here. This may be due to the use of different expression constructs. The pH5 ICP8 construct used here is derived from pUC19, with the HSV-1 DNA from map units 0.38-0.42 under the control of the cytomegalovirus immediate early promoter (Challberg, 1986; Heilbronn and zur Hausen, 1989; Wu, et al., 1988). The pSG18 construct used in the previous report was derived from pBR325 and contains HSV-1 DNA from map units 0.31-0.42 (Goldin, et al., 1981; McGeoch, et al., 1988). Thus, whilst the pH5 construct contains only the open reading frame for ICP8 (UL29), the pSG18 construct also contains reading frames UL24-28 which encode two proteins of unknown function, a virion protein, a capsid protein and virion glycoprotein B (McGeoch, et al., 1988). Thus, these additional proteins may be responsible for the irregular ICP8 distribution seen in cells transfected with pSG18. It is interesting to note that the transfection of a construct containing the gene for HSV Pol into cells produces a uniform HSV Pol staining pattern very similar to that observed for ICP8 in pH5-transfected cells (Bush, et al., 1991).

How are cellular DNA replication complexes redistributed?

ICP8 appears to be involved functionally in the redistribution of cellular DNA replication complexes, since newly-replicated DNA is not redistributed during infection with mutant HSV-1 strains expressing defective ICP8 (de Bruyn Kops and Knipe, 1988). Also, the coordinate movement of PCNA and ICP8 from replication compartments to prereplicative sites (figure 3.9) is compatible with a role for ICP8 in the redistribution process. Redistribution requires active protein synthesis (figure 3.10), consistent with a requirement for ICP8.

There are two general ways in which ICP8 can be envisaged to cause redistribution of cellular DNA replication complexes. Either ICP8 can localise to novel sites in the nucleus and attract cellular proteins to these sites, or ICP8 can localise to established DNA replication sites and modify them. The distribution of transiently-expressed ICP8 (figure 3.14a) would tend to favour the latter theory, with another viral protein(s) necessary for the modification step.
The change in distribution of a number of DNA replication proteins together with replicating cellular DNA suggests that HSV-1 targets a key component necessary for the correct assembly of DNA replication complexes. This key component or "nucleation element" may be a protein such as SSB, by analogy with the T4 gene 32 SSB which interacts with a large number of other T4 replication proteins (see introduction). It could also be the DNA replication origin itself, since the replication machinery can assemble in compartments in the absence of viral DNA (figure 3.14b) and may amplify cellular genes. Alternatively, HSV-1 may target some element or configuration of the nuclear matrix which is specific for DNA replication sites, reflecting a structural requirement of HSV-1 DNA replication. This "nucleation element" may be limiting or relatively inaccessible in quiescent cells (figure 3.12). Using this targeting strategy, the virus would be able to assemble viral DNA replication structures around this "nucleation element" incorporating both viral and cellular replication proteins. However, this model is highly speculative and has no direct experimental evidence. Experiments which simplify the infection process by expressing defined HSV-1 proteins in cells may provide more information on the mechanism of host DNA replication complex redistribution and the assembly of viral DNA replication complexes.
4. Adenovirus

This chapter extends the localisation studies presented in Chapter 3 to adenovirus infections. The effects of infection with three different adenovirus serotypes on the distributions of host nuclear proteins are described. Immunofluorescence photographs are presented of cells infected in the presence or absence of an inhibitor of adenovirus DNA replication. A relationship between the pattern of DBP foci in replication-blocked cells and the growth state of the host cell is demonstrated.

Adenovirus DNA-binding protein (DBP) is associated with replicating DNA in infected cells

Figure 4.1 shows the simultaneous distributions of DBP and replicating DNA in Ad5-infected CV-1 cells, 24hpi. These cells are semi-permissive for adenovirus infection, but the block to virus production occurs at a late stage in infection and viral DNA replication occurs at the same time and rate as seen in permissive cells (Klessig and Grodzicker, 1979). Adenovirus, like HSV-1, forms replication compartments during the infection process. These generally take the form of small circular regions within infected cells which appear to enlarge and coalesce as infection proceeds (Sugawara, et al., 1977; Voelkerding and Klessig, 1986). Figure 4.1 confirms previous results showing that DBP is present within such compartments and that DNA synthesis is occurring within these structures in CV-1-infected cells. Thus, DBP can be used as a marker for sites of adenovirus DNA synthesis.

Ad2 and Ad5 are both group C adenoviruses, whereas Ad4 is classified as a group E adenovirus. All three serotypes transform cells in culture, but show low or zero levels of tumorigenicity in animals (Horwitz, 1990). Figure 4.2 shows patterns of DBP staining in cells infected with Ad2, Ad4, and Ad5, 24hpi. The compartments formed by Ad2 had often coalesced at this stage, whereas those formed by Ad5 were usually circular. Ad4 compartments were more numerous and diffuse than seen with the other two serotypes. This probably reflects differences in the kinetics of infection of CV-1 cells by these viruses, since Ad4 infection is generally faster than that of Ad2 or Ad5.
Figure 4.1. Distributions of DBP and DNA replication sites in adenovirus-infected cells.
CV-1 cells seeded onto 6cm plates were infected with adenovirus type 5 as described in Materials and Methods section 2b. Cells were labelled with $^3$H-T from 23hpi-24hpi, as detailed in Materials and Methods section 1d. After fixation in acetone/methanol 24hpi, cell staining was carried out using rabbit polyclonal anti-DBP serum (1/100, precleared against CV-1 cell extract) and goat anti-rabbit TRITC (1/100). Photographic emulsion was then applied to the plates which were then left for three days before developing (Materials and Methods section 1d).

Figure 4.2. DBP distributions in cells infected with different adenovirus serotypes.
CV-1 cells grown on coverslips were infected with adenovirus type 2, 4, or 5 as described in Materials and Methods section 2b. Cells were fixed in formaldehyde 24hpi and permeabilised with 1% Triton (see Materials and Methods section 1c), prior to incubation with polyclonal anti-DBP serum (1/50, precleared against CV-1 cell extract). TRITC-conjugated goat anti-rabbit immunoglobulin was used as second antibody.
A. Mock-infected cells. (Increased sensitivity of detection and longer film exposure used than for B, C, and D). Scale bar is 10µm.
B. Ad2 infection. Scale bar is 25µm.
C. Ad4 infection. Scale bar is 10µm.
D. Ad5 infection. Scale bar is 25µm.
Figure 4.3. Distributions of SSB and histones in adenovirus-infected cells.

CV-1 cells grown on coverslips were infected with adenovirus type 2, 4, or 5 and fixed 24hpi as described in the legend to figure 4.2. Cell staining was performed using anti-SSB (34A) (a, c, e, g) or anti-histone (J2B2) (b, d, f, h) antibodies and FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30). Photographs show: mock-infected cells (a, b); Ad2-infected cells (c, d); Ad4-infected cells (e, f); Ad5-infected cells (g, h). Scale bars are 10μm (a, d, f, h) and 25μm (b, c, e, g).
SSB and histones show similar distributions in adenovirus-infected cells

Figure 4.3 shows that SSB and histone proteins exhibit major changes in distribution during adenovirus infection. Both proteins were present throughout the nucleus in mock-infected cells (excluding the nucleoli). However, these proteins were excluded from many areas of infected cell nuclei producing a very irregular staining pattern.

Distinct populations of PCNA localise to different sites in adenovirus-infected cells

Figure 4.4 shows the cell staining patterns produced by two different antibodies directed against PCNA. PC4 recognised PCNA present in DSB-like compartments (seen more clearly for Ad2 in figure 4.9), whereas PC10 recognised a small number of intensely-staining foci.

The PC10 foci were smaller and more numerous in Ad4- than in Ad2- or Ad5- infected cells at this time-point. Similar foci were observed for PC2, but not for PC3, PC5, PC8, PC9 and PC11. In Ad5-infected cells at least, the PCNA foci were not present at 8.5hpi, were present as shown in some cells at 24hpi, were slightly larger and present in almost every cell at 30hpi, but had disappeared by 45hpi.

Distribution of other proteins in adenovirus-infected cells

p53 appeared uniform in Ad2- and Ad5- infected cells, but was present in compartment-like structures in some Ad4-infected cells (figure 4.5). Antibodies to the heat-shock protein hsp70 recognised compartment-like structures in Ad2-, Ad4- and Ad5- infected cells, although the pattern and intensity of staining varied from cell to cell (figure 4.5). Rb was weak in most infected cells, though a few cells showed more intense staining. Rb-staining was generally quite uniform throughout the nucleus (figure 4.6). E1a staining was also variable in intensity in Ad2- and Ad5- infected cells, but E1a was not detected in the nuclei of Ad4-infected cells (figure 4.6). E1a showed a variety of distributions, from diffuse to speckled to peripheral (figure 4.6) as noted previously (White, et al., 1988). Peripheral or negative staining was the predominant pattern seen in Ad4-infected cells consistent with the late stage of Ad4 infection at this time postinfection.
Figure 4.4. Distribution of PCNA in adenovirus-infected cells.
CV-1 cells grown on coverslips were infected with adenovirus type 2, 4, or 5 and fixed 24hpi as described in the legend to figure 4.2. Cell staining was performed using PC4 (a, c, e, g) or PC10 (b, d, f, h) anti-PCNA monoclonal antibodies and FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30). Photographs show: mock-infected cells (a, b); Ad2-infected cells (c, d); Ad4-infected cells (e, f); Ad5-infected cells (g, h). Scale bars are 10μm (a) and 25μm (b-h).
Figure 4.5. Distributions of p53 and heat-shock protein 70 (hsp70) in adenovirus-infected cells. CV-1 cells grown on coverslips were infected with adenovirus type 2, 4, or 5 and fixed 24hpi as described in the legend to figure 4.2. Cell staining was performed using anti-p53 (PAb421) (a, c, e, g) or anti-hsp70 (hsp72/73) (b, d, f, h) antibodies and FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30). Photographs show: mock-infected cells (a, b); Ad2-infected cells (c, d); Ad4-infected cells (e, f); Ad5-infected cells (g, h). Scale bars are 10μm (a, e, f) and 25μm (b, c, d, g, h).
Figure 4.6. Distributions of Rb and E1a in adenovirus-infected cells.
CV-1 cells grown on coverslips were infected with adenovirus type 2, 4, or 5 and fixed 24hpi as described in the legend to figure 4.2. Cell staining was performed using anti-Rb (IF/8) (a, c, e, g) or anti-E1a (b, d, f, h) antibodies and FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30) (a, c, e, g) or TRITC-conjugated goat anti-rabbit immunoglobulin (1/100) (b, d, f, h). Photographs show: mock-infected cells (a, b); Ad2-infected cells (c, d); Ad4-infected cells (e, f); Ad5-infected cells (g, h). Scale bars are 10μm (a, h) and 25μm (b, c, d, e, f, g).
Effect of 1-β-D-arabinofuranosylcytosine (araC) on DBP distribution in adenovirus-infected cells

AraC is taken up by cells and phosphorylated to form araCTP, which competes with dCTP for incorporation into newly-synthesised DNA, thus inhibiting both viral and host DNA synthesis (Cohen, 1977). Adenovirus infection in the presence of araC inhibits the transition from early to late stages of infection and results in the formation of foci of DBP (Sugawara, et al., 1977) and figure 4.7. These DBP foci resemble those seen early in infection. Note that these intensely-staining DBP foci were not visible in araC-treated Ad4-infected cells 24hpi (figure 4.7b).

SSB and histones are excluded from replication compartments in cells productively infected with Ad2 but are not redistributed in the presence of araC.

Figure 4.8 extends the results of figure 4.3 by using double-labelling techniques. In productively-infected cells, SSB and histones were seen to redistribute to areas where DBP was not present, i.e. they were excluded from replication compartments (figures 4.8b and d). At later stages of infection, the replication compartments were larger and thus the redistribution more extensive than at early times. In araC-treated cells, SSB and histones were distributed throughout the nucleus, as seen in mock-infected cells (compare figures 4.8c and e with 4.3a and b).

Comparison of PCNA and DBP distributions in cells infected with Ad2 in the presence or absence of araC

Figure 4.9a confirms that PC4 is present in replication compartments within Ad2-infected cells. This redistribution did not occur in araC-treated infected cells (figure 4.9b). The foci recognised by PC10 were present only in cells with many DBP compartments, suggesting that they were formed relatively late in infection (figure 4.9c). These foci were not detected in araC-treated infected cells (figure 4.9d). Staining of cells fixed with acetone/methanol (not shown) suggested that PC10-positive foci were present within DBP compartments.

Distributions of other proteins compared to DBP in cells infected with Ad2 in the presence or absence of araC

Hsp70 was present in a variety of compartment-like structures in Ad2-infected cells, whereas p53 was generally diffusely distributed (figures 4.10a and c). Ki67 redistributed to sites within compartments in cells that were in later stages of infection (figure 4.10e). p53 and Ki67 were not redistributed in
Figure 4.7. Effect of araC on DBP distribution in adenovirus-infected cells.
CV-1 cells grown on coverslips were infected with adenovirus type 2, 4, or 5 in the continuous presence of 25μg/ml araC (Sigma). Cells were fixed and stained 24hpi as described in the legend to figure 4.2.
A. Ad2 infection. Scale bar is 10μm.
B. Ad4 infection. Scale bar is 10μm.
C. Ad5 infection. Scale bar is 25μm.
Figure 4.8. Distributions of SSB and histones in cells infected with Ad2 in the presence or absence of araC.
CV-1 cells grown on coverslips were infected with adenovirus type 2 in the presence (c, e) or absence (a, b, d) of 25μg/ml araC. The cells were fixed in formaldehyde 24hpi and permeabilised with 1% Triton (see Materials and Methods section 1c), prior to incubation with polyclonal anti-DBP serum (1/50, pre cleared against CV-1 cell extract) and either anti-SSB (34A) or anti-histone (J2B2) antibodies. TRITC-conjugated goat anti-rabbit immunoglobulin (1/100) and FITC-conjugated goat anti-mouse immunoglobulin (Sigma, 1/20 (b, c), 1/30 (a, d, e)) conjugates were used. Left-hand panels show DBP staining; right-hand panels show background (a), SSB (b, c) or histone (d, e) staining. Scale bars are 25μm.
Figure 4.9. Distribution of PCNA in cells infected with Ad2 in the presence or absence of araC.
CV-1 cells were infected in the presence (b, d) or absence (a, c) of araC and stained as described in the legend to figure 4.8, but using the PC4 (a, b) and PC10 (c, d) antibodies (right-hand panels) to compare with the anti-DBP staining pattern (left-hand panels). FITC-conjugated goat anti-mouse immunoglobulin was diluted 1/20 for a and b and 1/30 for c and d. TRITC-conjugated goat anti-rabbit immunoglobulin was used at 1/100. Scale bars are 25μm.
Figure 4.10. Distributions of p53, heat-shock protein 70 (hsp70) and Ki67 in cells infected with Ad2 in the presence or absence of araC.
CV-1 cells were infected in the presence (b, d, f) or absence (a, c, e) of araC and stained as described in the legend to figure 4.8 using PAb421 (a, b), hsp72/73 (c, d) or Ki67 (e, f) antibodies (right-hand panels) in combination with anti-DBP serum (left-hand panels). FITC-conjugated goat anti-mouse immunoglobulin was used at a dilution of 1/30. TRITC-conjugated goat anti-rabbit immunoglobulin was used at 1/100. Scale bars are 25µm except for d which is 10µm.
Figure 4.11. Distributions of Rb and E1a in cells infected with Ad2 in the presence or absence of araC.
CV-1 cells were infected in the presence (b, d) or absence (a, c) of araC and stained as described in the legend to figure 4.8 using anti-Rb (IF/8) antibody in combination with either polyclonal anti-DBP (a, b) or polyclonal anti-E1a sera (c, d). FITC-conjugated goat anti-mouse immunoglobulin was used at a dilution of 1/30 (right-hand panels). TRITC-conjugated goat anti-rabbit immunoglobulin was used at 1/100 (left-hand panels). Scale bars are 25μm except for c which is 10μm.
CV-1 cells were allowed to attach to coverslips in E4 medium containing 10% FCS. Cells were then rinsed twice in E4 medium alone before addition of E4 medium containing 0.1% FCS. After 5 days, one group of cells (shown in c, and d) was stimulated by addition of E4 medium containing 10% FCS, whilst the other group (shown in a, b, e and f) was kept in the same medium. 15h later, Ad2 infection was performed in the normal manner (Materials and Methods section 2b) except that the infected cells were incubated in E4 medium containing 0.1% FCS. Cells were fixed in formaldehyde 24hpi, permeabilised with 1% Triton (see Materials and Methods section 1c) and stained with Ki67 (1/60) (a, c, e) and anti-DBP (1/50) (b, d, f) antibodies followed by FITC-conjugated goat anti-mouse immunoglobulin (Sigma, 1/20) and TRITC-conjugated goat anti-rabbit immunoglobulin (1/100). Cells were photographed using x40 (a-d) or x63 (e, f) objectives.
cells infected in the presence of araC (figures 4.10b and f). Hsp70 was present at a small number of intensely-staining foci in some araC-treated Ad2-infected cells, which appeared to correspond to a subset of the DBP foci observed in such cells (figure 4.10d).

Rb did not appear to colocalise with DBP or E1a compartments in Ad2-infected cells (figures 4.11a and c) (the foci of Rb staining in figure 4.11a were probably due to bleedthrough or cross-reactions, since they were not observed in cells stained with Rb alone, see figure 4.6). Levels of Rb and E1a were generally greater in araC-treated cells, but did not appear to correlate with one another (figure 4.11d). Both Rb and E1a were diffusely distributed in araC-treated cells (figures 4.11b and d).

The number of DBP foci in araC-treated Ad2-infected cells is dependent on the growth state of the host cell

Figure 4.12 shows the simultaneous distributions of DBP and Ki67 in cells infected with Ad2 in the presence of araC. The number of DBP foci in S-phase cells was generally greater than in quiescent cells (compare figures 4.12d and b). In this particular experiment, complete growth arrest was not observed in the serum-starved culture, with some cells still undergoing proliferation (Ki67-positive). In these proliferating cells DBP generally accumulated in relatively large structures, whereas in Ki67-negative cells DBP was either diffusely distributed, or was present at smaller foci (figures 4.12b (low power) and f (high power)). The cells containing larger DBP foci were probably not in S phase at the time of infection, since this type of DBP distribution was not observed in serum-stimulated cells, which tended to have more numerous, smaller foci (compare figures 4.12b and f with figure 4.12d). Thus, these larger DBP foci may be formed in cells infected during some other stage of the proliferating cell cycle (G1, G2 or M).

Discussion

Host proteins are differentially included in adenovirus replication compartments

A summary of the effects of Ad2 infection on a number of host proteins is presented in table 4.1, which was compiled using data from this study and from previous reports.
Table 4.1. Distribution of host proteins in CV-1 cells infected with Ad2

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>ANTIBODIES</th>
<th>STAINING PATTERN IN Ad2-INFECTED CELLS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSB</td>
<td>34A, J2B2</td>
<td>EXCLUDED FROM REPLICATION COMPARTMENTS</td>
<td>FIG 4.8</td>
</tr>
<tr>
<td>HISTONE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCNA</td>
<td>PC2; PC10</td>
<td>INTENSELY-STAINING FOCI</td>
<td>FIG. 4.9</td>
</tr>
<tr>
<td>PCNA</td>
<td>PC3; PC5;</td>
<td>IN REPLICATION COMPARTMENTS</td>
<td>FIG. 4.9.</td>
</tr>
<tr>
<td>LP4N</td>
<td>PC8; PC9;</td>
<td></td>
<td>NOT SHOWN</td>
</tr>
<tr>
<td></td>
<td>PC11</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LP4N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb</td>
<td>IF/8, PAb421</td>
<td>DIFFUSE</td>
<td>FIG. 4.11</td>
</tr>
<tr>
<td>p53</td>
<td>4E9</td>
<td></td>
<td>FIG. 4.10</td>
</tr>
<tr>
<td>POL α</td>
<td></td>
<td></td>
<td>NOT SHOWN</td>
</tr>
<tr>
<td>HSP70</td>
<td>HSP72/73</td>
<td>IN E1a COMPARTMENTS</td>
<td>FIG. 4.10; (White, et al., 1988)</td>
</tr>
<tr>
<td>Ki67</td>
<td>Ki67</td>
<td>PRESENT AT A SUBSET OF DBP SITES AND IN NUCLEOLI</td>
<td>FIG. 4.10</td>
</tr>
<tr>
<td>p68</td>
<td>PAb204, Y12</td>
<td>AT MOCK-INFECTED SITES AND IN REPLICATION COMPARTMENTS</td>
<td>NOT SHOWN (Chaly, et al., 1987; Walton, et al., 1989)</td>
</tr>
<tr>
<td>Sm</td>
<td>AF/CDC3, P11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U1</td>
<td>MATRIX Ag</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U2</td>
<td>ANTI-U2</td>
<td>AT MOCK-INFECTED SITES AND NOVEL FOCI (NOT REPLICATION COMPARTMENTS)</td>
<td>(Walton, et al., 1989)</td>
</tr>
<tr>
<td>B23</td>
<td>ANTI-B23</td>
<td>IN REPLICATION COMPARTMENTS AND NUCLEOLI</td>
<td>(Walton, et al., 1989)</td>
</tr>
<tr>
<td>NOR90</td>
<td>ANTI-NOR90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA POL I</td>
<td>ANTI-RNA POLI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NUCLEOLAR Ag</td>
<td>AF/CDC6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th</td>
<td>ANTI-Th</td>
<td>NUCLEOLAR AND DIFFUSE</td>
<td>(Walton, et al., 1989)</td>
</tr>
<tr>
<td>TOPOISO-</td>
<td>AF/CDC9</td>
<td>DIFFUSE AND IN REPLICATION COMPARTMENTS</td>
<td>(Walton, et al., 1989)</td>
</tr>
<tr>
<td>MERASE I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U3</td>
<td>ANTI-U3</td>
<td>UNCHANGED</td>
<td>(Chaly, et al., 1987; Walton, et al., 1989)</td>
</tr>
<tr>
<td>L5/5S</td>
<td>Fi ANA, Ku ANA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dsDBP</td>
<td>AF/CDC8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CENTRO-</td>
<td>P1, P12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MERIC Ag</td>
<td>ANTI-LAMIN B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMIN B</td>
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</tbody>
</table>

("Matrix" refers to the nuclear matrix. "Ag" means antigen. Sm, U1, U2, U3, Th, L5/5S are small nuclear ribonucleoprotein antigens. The present work was performed using monkey CV-1 cells which are semi-permissive for adenovirus replication since they block viral growth at a late stage of infection (Klessig and Grodzicker, 1979), whereas the work of Walton et al used HeLa cells which are fully-permissive for adenovirus replication (Walton, et al., 1989)).
Thus, PCNA (PC4-positive), LP4N, Ki67, topoisomerase I and various nucleolar antigens are redistributed to replication compartment-like structures in Ad2-infected cells, whereas SSB, histones, PCNA (PC10-positive), hsp70, U2 and Th antigens are redistributed in such cells, but are not compartmentalised in the same way. The distribution of other tested nuclear proteins is not significantly altered by Ad2 infection. Thus, the inclusion of host proteins into viral replication structures appears to be a specific process, rather than a general aggregation of nuclear proteins.

SSB and histones are excluded from adenovirus replication compartments

The exclusion of SSB from viral replication compartments (figure 4.8) suggests that adenovirus viral DNA replication in vivo utilises solely viral DBP, with no requirement for host SSB. The exclusion of histones from sites containing replicated adenovirus DNA supports previous results which suggested that adenovirus core proteins and not cellular histones are used to package progeny adenovirus DNA (Vayda, et al., 1983). The distribution of histones in Ad2-infected cells also suggests that the bulk of the cellular chromatin is excluded from these compartments (figure 4.8). The lack of an inhibitor specific for adenovirus DNA replication is an obstacle to determining whether replicating host DNA is redistributed to adenovirus replication compartments as has been shown to occur during HSV-1 infections (de Bruyn Kops and Knipe, 1988).

Two populations of PCNA are present in adenovirus infected cells

Different populations of PCNA are selectively redistributed in Ad2-infected cells (figures 4.4 and 4.9). The selective recognition of intensely-staining foci by PC2 and PC10 does not correlate exactly with previously defined epitope mapping studies placing these antibodies in the same group as PC3, PC8 and PC11, antibodies which recognise PCNA in Ad2 replication compartments (Waseem and Lane, 1990). PC2 and PC10 could form a further subset within this group, by selectively recognising an epitope on a different conformational form of PCNA present in these foci during adenovirus infection. The differential distribution of two forms of PCNA provides further evidence that the redistribution of cellular proteins during adenovirus infection is a specific process.
Distributions of Rb and p53 in adenovirus infected cells

Rb and p53 have been shown to bind to adenovirus E1a and E1b proteins respectively (Sarnow, et al., 1982; Whyte, et al., 1988). Thus, it might be expected that Rb and p53 would colocalise with these associated proteins in adenovirus-infected cells. The distributions of E1a and E1b have been described previously (Ornelles and Shenk, 1991; White, et al., 1988). The location of E1a is dependent on the time postinfection and on the cell cycle stage of the host cell at the time of infection. At least five patterns of E1a distribution can be distinguished: diffuse, reticular, nucleolar, punctate and peripheral. Rb generally showed a diffuse staining pattern (figure 4.11c), although more irregular staining was occasionally observed (figures 4.6e and g). Thus, the two proteins may be complexed in cells displaying diffuse E1a staining, an E1a pattern characteristic of cells infected in late G1 and S phase (White, et al., 1988). Although the distributions of Rb and E1a appear different in cells where E1a is compartmentalised, there are still areas of overlap and thus complexing between the two proteins in these cells is still a possibility. A more detailed, systematic analysis of cells infected at different stages in the cell cycle and for different lengths of time is needed to define the relationship between the distributions of Rb and E1a. Such studies may contribute to models of E1a transformation through interaction with Rb, as discussed in the introductory chapter.

E1b also has a complex distribution in infected cells with five distinct localisation patterns exhibited: diffuse, filamentous, clustered (at the periphery of replication compartments), cytoplasmic and in discrete cytoplasmic bodies (Ornelles and Shenk, 1991). p53 staining was generally diffuse, although more irregular staining was seen in Ad4-infected cells (figure 4.5e). Thus, as for Rb and E1a, p53 and E1b may be complexed in diffusely-stained cells and possibly in cells with other E1b distributions. p53 is transactivated during adenovirus infection of non-permissive or semi-permissive cells, but increased levels of protein are not visible until 40hpi (Braithwaite, et al., 1990) and were thus not observed in the present experiments with Ad2 and Ad5, but were seen using the more rapidly-infecting Ad4 (figure 4.5). E1a and E1b are multi-functional proteins and thus it is unlikely that all of the E1a or E1b in a cell is complexed to Rb or p53 throughout the infection process.

Both Rb and p53 were seen to have more irregular distributions in Ad4-infected cells than in Ad2-, or Ad5- infected cells 24hpi. This may be a feature of Ad4 infection or may simply be due to a difference in the kinetics of
infection by this virus. Cell staining at different times postinfection with each of the three serotypes should resolve this issue.

Other proteins

E1a and DBP are diffusely distributed in infected cells at first, and then localise to different regions of viral inclusions (Chaly, et al., 1987; White, et al., 1988). Hsp70 has previously been shown to be induced by adenovirus infection and to colocalise with compartmentalised E1a in Ad2-infected HeLa cells (Nevins, 1982; White, et al., 1988). The hsp70 distributions shown in figures 4.5 and 4.10 are consistent with these results, although an E1a/hsp70 double label was not performed.

Polymerase α did not appear to be compartmentalised with DBP during Ad2 infection, despite preliminary reports of a pol-α-primase-NF-I interaction in vitro. The LP4N antigen was present in viral replication compartments as was the case during HSV-1 infections (see chapter 3). Various nucleolar proteins were also found in compartments containing DBP. These included B23, Sm, p68, PC9-positive PCNA and Ki67. Ki67 was present only at a subset of DBP sites (figure 4.10). Also, clusters of Sm and p68 were sometimes visible at the periphery of compartments as occurred during HSV-1-infection (see chapter 3, figure 3.8).

Effect of araC on the redistribution of host proteins by adenovirus

The compound araC inhibits viral DNA replication by a different mechanism to that used by PAA (chapter 3) in that it forms a competitor substrate for the viral polymerase rather than directly inhibiting the polymerase enzyme (Cohen, 1977; Mao and Robishaw, 1975). The cytosine analogue formed by araC enters newly synthesised DNA and forms links with other nucleotides in the chain. Thus, a small amount of DNA synthesis does occur in the presence of araC. Hence, the DBP foci formed during adenovirus infection should perhaps be referred to as "early-replicating sites" rather than the prereplicative sites produced during HSV-1 infection of PAA-treated cells. The much larger number of DBP sites formed during adenovirus infection in the presence of hydroxyurea may be more equivalent in function to HSV-1 prereplicative sites (Bosher and Hay, pers. comm., 1991). DBP foci were observed in Ad2- and Ad5-, but not Ad4-infected araC-treated cells (figure 4.7). This may reflect differences in the Ad Pol and/or DBP proteins and replication origins of the different serotypes (Hay, 1985; Kitchingman, 1985). The distribution of DBP foci in Ad2-infected
araC-treated cells appeared to related to the growth state and cell-cycle stage of the infected cell (figure 4.12).

The redistribution of SSB, histones, PCNA and Ki67 and Sm does not occur in araC-treated cells (figures 4.8, 4.9 and 4.10), suggesting that this relocalisation occurs after the initiation of viral DNA synthesis during normal infections. Hsp70 is induced in some cells, but is restricted to only a few foci which are often associated with the nucleoli and appear to represent a subset of DBP sites (figure 4.10).

Do the redistributed cellular DNA replication proteins function in adenovirus DNA replication?

Certain cellular proteins are known to function in adenovirus DNA replication in vitro (see introductory chapter) and at least one of these, NF-I, is present in replication compartments in vivo (Bosher and Hay, 1991). NF-II may also be present in these compartments, since it copurifies with DNA topoisomerase I activity and antibodies to DNA topoisomerase I recognise adenovirus replication compartments (Nagata, et al., 1983; Walton, et al., 1989). Topoisomerase II activity is also required for adenovirus DNA replication in vivo (Schaak, et al., 1990; Wong and Hsu, 1990).

The presence of PCNA in replication compartments raises the possibility that this protein may be required for efficient adenovirus DNA replication in vivo. PCNA transcription and protein levels are elevated during adenovirus infection of quiescent non-permissive or semi-permissive cells (Liu, et al., 1985; Morris and Mathews, 1990; Zerler, et al., 1987). Transactivation of PCNA and a number of other cell cycle-regulated genes is mediated by adenovirus E1a protein (Braithwaite, et al., 1990; Zerler, et al., 1987). Thus, although the transactivation of PCNA may reflect a specific functional requirement for this protein, it may equally well be a general consequence of the mechanism of PCNA gene regulation in uninfected cells. A functional role could be envisaged for PCNA as an accessory factor for Ad Pol or for a cellular DNA polymerase involved in adenovirus DNA replication in vivo. The presence of a population of PCNA at discrete foci in adenovirus-infected cells raises the possibility that PCNA may have multiple roles in adenovirus infection.

Electron microscopic studies of Ad5-infected cells have further divided the nuclear compartments formed during infection into subcompartments where different viral functions are thought to take place. For example, DNA initiation and chain elongation are postulated to occur in
different types of inclusions (Murti, et al., 1990; Puvion-Dutilleul and Puvion, 1990). Thus, higher-resolution studies of the host proteins associated with replication compartments may yield more precise information on the colocalisation of specific proteins, thereby suggesting more precise putative functions for host proteins in adenovirus nucleic acid metabolism.

Effects of the growth state of the host cell on the infection process

DBP was diffusely distributed or present at a few "pinpoint" foci in quiescent CV-1 cells infected in the presence of araC. In contrast, some proliferating cells infected under the same conditions had larger DBP foci, whilst those in S phase had more numerous foci (figure 4.12). This suggests a difference in the organisation of early-replicating sites formed by adenovirus in quiescent and proliferating cells. These findings resemble those seen in cells infected with HSV-1 in the presence of PAA (chapter 3). Thus, these results may also be a consequence of limiting amounts of DNA replication proteins or structural elements in quiescent cells.

Adenovirus infection has been shown to induce cellular DNA synthesis in non-permissive and semi-permissive quiescent cells (Braithwaite, et al., 1981). This stimulation of cellular DNA synthesis can be induced by the microinjection of purified E1a proteins into cells, suggesting that it is caused by the transactivation of cellular genes by E1a (Kaczmarek, et al., 1986). Ad2 infection activates transcription of a number of cellular genes expressed in G1, including PCNA, p34cdc2, c-myc and thymidine kinase (Draetta, et al., 1988; Hiebert, et al., 1989; Liu, et al., 1985). Thus some, or all, of these factors may be required for the assembly of fully-functional adenovirus DNA replication complexes. The altered DBP distribution in quiescent CV-1 cells may reflect the presence of limiting amounts of these proteins and the inhibitory controls on DNA replication which exist in these cells. Therefore, it could be argued that DBP is present at prereplicative sites in quiescent araC-treated cells where initiation of DNA synthesis is inhibited and at early-replicating sites in proliferating araC-treated cells where initiation of DNA synthesis can occur.

Ad2 has been shown to induce the amplification of integrated SV40 DNA in permissive cells (Schlehofer and zur Hausen, 1990). The mechanism of this amplification is not known. It is possible that the adenovirus replication machinery acts in the same way as HSV-1, by amplifying cellular origins of DNA replication within replication compartments (see chapter 3).
5. Simian virus 40

The results presented in this chapter concern the effects of SV40 infection on the host cell. SV40 DNA replication has been studied extensively in vitro (see introductory chapter), but much less is known about its replication in vivo where the full complement of viral and cellular proteins is present. Photographs showing the distribution of host proteins in SV40-infected CV-1 and 293 cells are presented. Particular attention is paid to the distribution of SSB, which is markedly altered during SV40 infection. The interactions of SV40 with HSV-1 and adenovirus are also studied.

SSB redistributes to intensely-staining foci during SV40 infection.

Figure 5.1a shows the distribution of SSB in mock-infected and SV40-infected CV-1 cells 24hpi. SSB was localised to a number (typically between 10 and 30) of discrete foci within SV40-infected cells, in marked contrast to its granular distribution in mock-infected cells. The intensity of staining suggested that SSB protein levels may have been elevated in SV40-infected cells.

No other host proteins were shown to be colocalised with SSB in SV40-infected cells.

The distribution of (PC4-positive) PCNA in mock-infected and SV40-infected CV-1 cells is shown in figure 5.1b. PCNA staining was more irregular in SV40-infected cells than in mock-infected cells, resembling the background (non-focal) pattern of SSB staining in figure 5.1a. However, unlike SSB, PCNA was not localised to discrete foci in SV40-infected cells. PC2 and PC10 gave the same staining pattern as PC4, indicating that the differential localisation of PCNA observed during adenovirus infection did not occur during SV40 infection (see chapter 4). PC9 stained the nucleoli of most cells 24hpi.

Rb and p53 showed distributions similar to that of PCNA in SV40-infected CV-1 cells 24hpi. The distributions of proteins such as nucleophosmin (B23), Ki67 and $S_m$ were unchanged at this stage of infection. Histones were redistributed during SV40 infection, becoming more irregularly distributed throughout the nucleus with especially high concentrations in the nucleoli of host cells (figure 5.1c).
Figure 5.1. Distributions of SSB, PCNA and histones in SV40-infected CV-1 cells.
CV-1 cells grown on coverslips were mock-infected (M) or infected with SV40 (+) (see Materials and Methods section 2b) and fixed in acetone/methanol 24hpi. Cells were stained as described in Materials and Methods section 1c using:
A. anti-SSB (34A) (mock and infected cells)
B. anti-PCNA (PC4) (mock and infected cells)
C. rabbit anti-T Ag (1/500) and mouse anti-histone (J2B2) antibodies (infected cells only).
After blocking in NGS, FITC-conjugated goat anti-mouse immunoglobulin (Sigma, 1/20) (A, B, and C) and TRITC-conjugated goat anti-rabbit immunoglobulin (C) were applied. Cells were photographed using a x40 (A(+), C) or a x63 objective (A (M); B).
Figure 5.2. Simultaneous distributions of SSB and DNA replication sites in SV40-infected CV-1 cells (24hpi).

CV-1 cells grown on 6cm plates were infected with SV40 as described in Materials and Methods section 2b. The cells were labelled with $^3$H-T for 70min (see Materials and Methods section 1d) immediately before fixation 24hpi with acetone/methanol. Cell staining was then performed as described in the legend to figure 5.1, using anti-SSB (34A) antibody and FITC-conjugated goat anti-mouse immunoglobulin (Sigma 1/20). Emulsion was then applied and the plates were left for 3 days before developing (Materials and Methods section 1d). Fluorescence pictures are shown on the left (i) (SSB) and bright field images of the same cells are shown on the right (ii) ($^3$H-T). Cells were photographed using a x40 (B(i)) or x63 (A and B(ii)) objective. Arrowheads indicate paired SSB foci.

A. Mock-infected cells.
B. SV40-infected cells.
Figure 5.3. Simultaneous distributions of SSB and DNA replication sites in SV40-infected CV-1 cells (48hpi). Methods were exactly as for figure 5.3 except that cells were fixed 48h after infection with SV40. A number of different fields are shown. Cells were photographed using a x63 objective. Arrowheads indicate paired SSB foci. V symbols indicate colocalised SSB and $^3$H-T sites.
SSB foci do not correspond to sites of replicating DNA

Figures 5.2 and 5.3 compare the distributions of SSB and newly-replicated DNA as revealed by a 70min pulse of $^3$H-T. SV40 infection was seen to inhibit cellular DNA replication late in infection (compare figures 5.2a and 5.3). The majority of SSB foci present in SV40-infected cells did not appear to colocalise with sites of ongoing DNA synthesis. However, a small number of foci appeared associated with sites of $^3$H-T incorporation (shown by v symbols on figure 5.3a and c), though the limited resolution of $^3$H-T labelling did not allow defining observations of colocalization.

The foci of SSB increased in size from 24hpi to 48hpi (compare figures 5.2 and 5.3). At both timepoints, paired foci were observed in some cells (shown by small arrowheads in figures 5.2 and 5.3).

SSB foci do not correspond to nucleoli

T Ag was present throughout the nuclei of SV40-infected cells 24hpi, but was excluded from the nucleoli (compare v symbols in figure 5.4a). A comparison of SSB and T Ag staining patterns revealed that the SSB foci in SV40-infected cells 24hpi were not associated with the host cell nucleoli, although some of the more diffusely-distributed SSB was observed in nucleolar regions (see arrows in figure 5.4b).

T Ag becomes colocalised with SSB as infection proceeds

As mentioned previously, the foci of SSB observed in SV40-infected cells were observed to increase in size as infection proceeded. This is evident in the time-course of infection shown in figure 5.5a. An increasing proportion of T Ag became colocalised with SSB as infection proceeded. T Ag was present at SSB foci in some, but not all cells 24hpi (figures 5.5a and b). However, at later stages of infection, all cells had sites containing colocalised SSB and T Ag. Late in infection (>72hpi), inclusion structures were visible which contained SSB, but often not T Ag (see arrows in figure 5.5a). The distribution of SSB did not correspond to that of the SV40 capsid protein VP1 (figure 5.5c).

Distributions of T Ag and host proteins in SV40-infected 293 cells

The 293 cell line was derived by transforming human embryonic kidney cells with Ad5 DNA. The resulting 293 cells were found to constitutively express the adenovirus E1a and E1b genes (Graham, et al., 1977). Untransformed human cells were shown to be semi-permissive for SV40 replication, but 293 cells supported the replication of transfected...
Figure 5.4. Comparison of SSB and nucleolar staining.
CV-1 cells grown on coverslips were infected with SV40 and fixed 24hpi with acetone/methanol as described in figure 5.1. Double staining was performed (Materials and Methods section 1c) using FITC-conjugated goat anti-mouse immunoglobulin (Sigma, 1/30) and TRITC-conjugated goat anti-rabbit immunoglobulin (1/100). Cells were photographed using a x63 objective. V and arrow symbols highlight particular nucleoli for comparisons.
A. Cells stained with precleared rabbit anti-T Ag (1/500) (left) and Ki67 (1/60) (right).
B. Cells stained with precleared rabbit anti-T Ag (1/500) (left) and mouse anti-SSB (34A) (right).
Figure 5.5. Distributions of T Ag, SSB and VP1 in SV40-infected CV-1 cells.

CV-1 cells grown on coverslips were infected with SV40 (see Materials and Methods section 2b) and fixed in acetone/methanol. Immunofluorescence was performed as described in Materials and Methods section 1c. In A and B, rhodamine labelling (T Ag) is shown on the left and fluorescein labelling (SSB) is shown on the right.

A. T Ag/SSB staining during a timecourse of infection. Confocal microscope pictures of mock (a) or SV40-infected (b-d) cells fixed at: a, 24hpi; b, 24hpi; c, 48hpi; d, 72hpi. Cells were stained with anti-SSB (34A) and rabbit anti-T Ag (1/500) using FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/20) and TRITC-conjugated goat anti-rabbit immunoglobulin (1/100) conjugates. Scale bar is 25μm (a-c) or 10μm (d). Arrows highlight particular inclusions for comparison.

B. T Ag/SSB staining (higher power). Conventional microscope photograph of SV40-infected cells fixed at 24hpi and stained with anti-SSB (34A) and precleared rabbit anti-T Ag (1/500) using FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/20) and TRITC-conjugated goat anti-rabbit immunoglobulin (1/100) conjugates. Cells were photographed using a x63 objective.

C. VP1 staining. Conventional microscope photograph of SV40-infected cells fixed at 72hpi and stained with anti-VP1 hybridoma supernatant and FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30). Cells were photographed using a x63 objective.
plasmids containing the SV40 genome (Lebkowski, et al., 1985). However, the levels of T Ag in these transfected 293 cells were found to be very low and it was proposed that SV40 replication antagonised T Ag expression in these cells (Lebkowski, et al., 1985; Lewis and Manley, 1985).

On the basis of the above reports, SV40-infected 293 cells were predicted to produce low levels of T Ag having a largely replicative function. Hence, this system was used for further studies on T Ag and host replication protein localisation. Figure 5.6a shows the distribution of T Ag observed in SV40-infected 293 cells 48hpi. T Ag was readily visible in a small proportion of cells, though at much lower levels than those observed in CV-1-infected cells at the same time-point of infection. Generally, a small number (<10) of intensely-staining T Ag foci were superimposed on a general granular T Ag staining pattern which extended throughout the nucleus, but excluded the nucleoli (figure 5.6a). However, some cells contained only T Ag foci, whereas others contained only diffusely-staining T Ag (figure 5.6b).

Figures 5.6c-e show the distributions of various host proteins relative to T Ag in SV40-infected 293 cells. A biotinylated mouse monoclonal antibody directed against T Ag was used in preference to the rabbit polyclonal anti-T Ag serum which had cross-reacted with mock-infected cells in some previous experiments (possibly a reaction with co-precipitated Rb in the immunogen preparation used for inoculation). SSB was present at T Ag foci in some, but not all, SV40-infected 293 cells (figure 5.6c), and was uniformly distributed in mock-infected 293 cells (not shown). Foci were observed in infected cells labelled singly with anti-T Ag (figures 5.6a and b) or anti-SSB (not shown) antibodies and no significant bleedthrough from the TRITC to the FITC channel was observed (figure 5.6b), suggesting that genuine colocalisation was occurring. PCNA (PC10-positive) was not concentrated in these foci, but showed a granular distribution throughout the nucleus with some areas of exclusion and occasional slightly larger, more intense, foci (see arrows on figure 5.6d). hsp70 was localised to the cytoplasm of these cells, whereas histones were often marginalised to the nuclear periphery (data not shown). Rb staining was undetectable under these conditions.

Discrete cytoplasmic bodies containing p53 have been observed close to the nucleus in Ad5-transformed cells (Zantema, et al., 1985a). During SV40 infection of 293 cells, these cytoplasmic p53 bodies showed a decreased staining intensity or disappeared completely, and nuclear
Figure 5.6. Distribution of host proteins in SV40-infected 293 cells.

293 cells grown on coverslips were infected with SV40 (Materials and Methods section 2b) and fixed 48hpi with acetone/methanol. First antibody was applied for 2h, followed by FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30). Cells were then post-fixed in acetone/methanol for 2min and incubated with 10% normal mouse serum (NMS) for 90min to block remaining binding sites on the anti-mouse conjugate. Biotinylated PAb419, anti-T Ag mouse monoclonal antibody (provided by J. Gannon, ICRF) was then applied at a dilution of 1/100 in 10%FCS/5%NMS/PBS and left o/n at 4°C. Cells were finally incubated with Texas-Red conjugated streptavidin for 2h at RT. Texas-Red fluorescence images are shown on the left and fluorescein images on the right.

A. T Ag. Method as described above but first antibody was omitted. Scale bar is 10µm.

B. T Ag (photographed through both fluorescence channels). Method as described above but first antibody was omitted. Scale bar is 10µm.

C. T Ag/SSB. Method as described above using anti-SSB (34A) as first antibody. Scale bar is 10µm.

D. T Ag/PCNA. Method as described above using anti-PCNA (PC10) as first antibody. Scale bar is 10µm.

E. T Ag/p53. Method as described above using anti-p53 (PAb421) as first antibody. Scale bar is 25µm.
inclusions of p53 at sites corresponding to T Ag foci were visible (figure 5.6e).

**Interactions of SV40 with other viruses**

When SV40-infected CV-1 cells were super-infected with HSV-1 18hpi, compartments containing both T Ag and ICP8 were formed (figure 5.7). These compartments were very similar in size and shape to those formed during normal HSV-1 infections. Thus SV40 infection did not block subsequent HSV-1 infection and morphologically normal HSV-1 replication compartments were formed, which sequestered T Ag.

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**Figure 5.7. Cell staining of cells doubly-infected with SV40 and HSV-1.**

CV-1 cells were infected with SV40 as described in Materials and Methods section 2b. The medium was removed 18hpi, and the cells were re-infected in the same way, but with HSV-1. Cells were fixed in acetone/methanol 4.5h after HSV-1 infection and cell staining was performed using rabbit polyclonal anti-ICP8 serum and mouse monoclonal antibody to T Ag (PAb419). FITC-conjugated goat anti-mouse immunoglobulin (Sigma, 1/20) and TRITC-conjugated goat anti-rabbit immunoglobulin (1/100) conjugates were used. Cells were photographed using a x63 objective.

A. Rhodamine label (ICP8).
B. Fluorescein label (T Ag).
The distributions of a number of host proteins in CV-1 cells co-infected with SV40 and Ad5 are summarised in table 5.1 below.

**Table 5.1. Distribution of host proteins in CV-1 cells co-infected with Ad5 and SV40**

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>ANTIBODY</th>
<th>DISTRIBUTION IN CO-INFECTED CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Ag</td>
<td>PAb419</td>
<td>GENERAL DIFFUSE STAIN; SOME RING-SHAPED STAINING</td>
</tr>
<tr>
<td>DBP</td>
<td>ANTI-DBP</td>
<td>IN COMPARTMENTS</td>
</tr>
<tr>
<td>E1a</td>
<td>ANTI-E1a</td>
<td>DIFFUSE</td>
</tr>
<tr>
<td>SSB</td>
<td>70C</td>
<td>IN COMPARTMENTS (FOCI TOO IN SOME CELLS)</td>
</tr>
<tr>
<td>PCNA</td>
<td>PC4</td>
<td>IN COMPARTMENTS</td>
</tr>
<tr>
<td>Ki67</td>
<td>Ki67</td>
<td>IN SOME AREAS OF COMPARTMENTS</td>
</tr>
<tr>
<td>NUCLEOPHOSMIN</td>
<td>B23</td>
<td>IN NUCLEOLI AND COMPARTMENTS</td>
</tr>
<tr>
<td>p68</td>
<td>PAb204</td>
<td>IN COMPARTMENTS</td>
</tr>
<tr>
<td>Sm</td>
<td>Y12</td>
<td>IN CLUSTERS AT PERIPHERY OF COMPARTMENTS</td>
</tr>
<tr>
<td>HISTONES</td>
<td>J2B2</td>
<td>EXCLUDED FROM COMPARTMENTS</td>
</tr>
</tbody>
</table>

Thus, for the majority of proteins, a distribution similar to that seen in adenovirus-infected cells was observed. T Ag was present throughout the nucleus and was also observed in rings around the periphery of replication compartments in some cells (indicated by arrows in figure 5.8a). Co-infected cells appeared to contain a combination of SSB-staining patterns, since both compartments and foci containing SSB were observed (figure 5.8b). PCNA was present in compartments, which often appeared more circular than those observed in adenovirus-infected cells fixed and stained in the same manner (figure 5.8c). The intensely-staining foci detected by PC2 and PC10 in adenovirus-infected cells did not appear to be present in co-infected cells. A variety of patterns was present in co-infected cells, representing single- and double- infected cells at different stages of infection. Thus, a precise interpretation of these staining patterns was not possible.
Figure 5.8. Distribution of host proteins in cells doubly-infected with SV40 and adenovirus.

CV-1 cells grown on coverslips were co-infected with Ad5 and SV40 (see Materials and Methods section 2b) and fixed in acetone/methanol 24hpi. Cells were stained with: A, precleared polyclonal anti-DBP serum (1/100) and monoclonal anti-T Ag antibody (PAb419); B, anti-SSB (70C); C, anti-PCNA (PC2) using FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30) and (for A) TRITC-conjugated goat anti-rabbit immunoglobulin (1/100) conjugates (see Materials and Methods section 1c).
Discussion

The functional role of the SSB foci in SV40-infected cells

SSB was redistributed to discrete foci in permissive CV-1 cells during lytic infection with SV40. Other host proteins studied were not localised to such foci, but were more diffusely distributed in the host cell nucleus. A population of T Ag molecules was associated with SSB in these foci (figure 5.5a). The majority of SSB foci did not appear to be associated with sites of active DNA replication (figures 5.2 and 5.3), nor were they equivalent to nucleoli (figure 5.4).

Although not associated with actively replicating DNA, SSB may be colocalised with fully-replicated progeny SV40 DNA. Perhaps the small number of $^3$H-T sites associated with SSB foci in figure 5.3 represented those SV40 genomes which completed their replication within the 70min pulse time, thus being fully-replicated at the end of the pulse but also containing $^3$H-T. SSB may function to coat progeny viral DNA and thus protect it from the action of nucleases prior to encapsidation. Similar roles have been suggested for the major DNA binding proteins of pseudorabies virus (a herpesvirus which encodes a 136k major DNA binding protein antigenically related to ICP8) and adenovirus (Ben-Porat, et al., 1983; Ginsberg, et al., 1975). Perhaps SV40 uses host SSB for this protective role, reflecting its increased dependence on the host cell compared with other larger viruses.

Previous studies found that SV40 DNA detected by in situ hybridisation to a tritiated SV40 cDNA probe was preferentially associated with host cell nucleoli during infection of CV-1 cells (Geuskens and May, 1974). However, cross-hybridisation of the probe with cellular DNA and/or viral RNA could not be ruled out. The number and distribution of extranucleolar SV40 DNA sites detected by hybridisation resemble those of SSB, with a decrease in the overall number of sites as infection proceeds and the presence of "paired foci" in some cells. Neither the SSB foci, nor the SV40 DNA were specifically associated with areas of the nucleus containing viral capsid proteins 44-48hpi (figure 5.5c, (Kasamatsu and Nehorayan, 1979)) and areas containing viral particles were not labelled by in situ hybridisation with SV40 cRNA at this stage of infection (Geuskens and May, 1974). Simultaneous in situ hybridisation and SSB localisation studies should determine whether the detected SSB foci correspond to sites of SV40 DNA
accumulation, thus supporting or disproving the above theory for a protective function of SSB.

It is of interest to note that another host single-stranded DNA binding protein, HMG1, has been proposed to be required for SV40 replication in vivo (Bonne-Andrea, et al., 1986). This protein functions in the assembly of nucleosomes in vitro and may act to stabilise ssDNA at the replication fork whilst maintaining chromatin structure (see discussion on histones below).

Previous experiments using immunoelectron microscopy and autoradiography showed that sites of active SV40 DNA replication were not nucleolar, but were preferentially localised to discrete areas of the nucleoplasm. These areas also contained large amounts of T Ag and were thus named "viral DNA/T Ag loci" (Harper, et al., 1985). On the basis of this report the colocalisation of T Ag with foci of SSB shown in figure 5.5a implies that SSB is present at viral DNA replication sites. However, this is not generally the case in figures 5.2 and 5.3. This discrepancy may be due to the longer labelling times used in this study (70min compared with 5min in the previous report), and/or the different fixation methods employed (acetone/methanol versus embedded sections). Interpretation is also complicated by the multi-functional nature of T Ag, which exists as a number of sub-populations involved in different aspects of the viral life-cycle (see introductory chapter).

Localisation of other host proteins in SV40-infected CV-1 cells

The distributions of PCNA, Rb and p53 were similar in SV40-infected CV-1 cells, with antibodies to all three proteins displaying slightly irregular staining which extended throughout most of the nucleus (figure 5.1b). These staining patterns resembled the distributions of those populations of T Ag and SSB which were not present in discrete foci. Thus, SSB, PCNA, and T Ag, which have been shown to participate in SV40 DNA replication in vitro, and p53, which binds regions close to SV40 origins of DNA replication (see introductory chapter), are colocalised throughout most areas of the nucleus.

Histones were observed to accumulate to high levels in the nucleoli of SV40-infected CV-1 cells (figure 5.1c), suggesting that the nucleolus may be the site of assembly of SV40 DNA into chromatin. The synthesis of all five classes of histone is stimulated by SV40 infection (Kay and Singer, 1977), consistent with the presence of all five types of histone protein in SV40 chromatin (Keller, et al., 1978). As mentioned above, HMG1, which interacts with core histones and acts as a nucleosome assembly factor in vitro, may
be involved in SV40 replication. This protein is present in the nucleus of proliferating or SV40-infected quiescent cells, but is solely cytoplasmic in uninfected non-proliferating cells. The sites of HMG1 accumulation in infected cells correspond to T Ag-rich sites of SV40 DNA replication. It has thus been suggested that HMG1 may act to stabilise ssDNA at the replication fork, whilst maintaining basic nucleosomal structure in a form which allows polymerase access to nucleotides (Bonne-Andrea, et al., 1986). The distribution of histones in SV40-infected cells is similar to that of HMG1, consistent with this hypothesis.

Localisation of host proteins in SV40-infected 293 cells

The large quantities of multi-functional T Ag produced in SV40-infected CV-1 cells often made colocalisation studies difficult to interpret. Therefore, SV40-infected 293 cells were studied, since SV40 infection of these cells produces only small amounts of T Ag (Lebkowski, et al., 1985; Lewis and Manley, 1985). Furthermore, the transactivation functions of E1a are postulated to substitute for those of T Ag in these cells, so that the T Ag produced is thought to function largely in viral DNA replication.

Previous studies reported undetectable levels of T Ag in 293 cells transfected with plasmids containing the SV40 genome. T Ag was only detected in such cells when SV40 DNA replication was inhibited, either by chemicals or by the use of a plasmid containing a mutated SV40 origin region (Lebkowski, et al., 1985; Lewis and Manley, 1985). Under these conditions of replication inhibition, both diffuse and focal T Ag nuclear staining was apparently observed, although the resolution of the published pictures is too low for detailed interpretation (Lebkowski, et al., 1985). However, in the present experiments, 5-10% of 293 cells productively infected with SV40 were positive for T Ag. This could represent more sensitive immunofluorescence procedures (unlikely, since two laboratories using different staining techniques reported negative T Ag fluorescence) or may be a consequence of the use of infection rather than transfection for the introduction of virus into cells. Another possibility is that SV40 replication was somehow inhibited in the T Ag positive 293 cells observed here.

SSB was concentrated at T Ag foci in some SV40-infected 293 cells at both 24 and 48hpi, but not all cells with T Ag foci had corresponding SSB foci. Perhaps SSB accumulates at sites containing only fully-replicated progeny DNA as postulated above for SV40-infected CV-1 cells.
PCNA (PC10-positive) was not concentrated at T Ag foci, but showed a granular distribution throughout the nucleus. A few PCNA foci were more intensely-stained, but these were much smaller and fewer in number than those observed in adenovirus-infected cells (compare figures 5.6d and 4.4). Immunofluorescence of SV40-infected 293 cells using other anti-PCNA antibodies was not performed. Hsp70, which is induced in 293 cells (Nevins, 1982), was present in the cytoplasm during SV40 infection (data not shown) and thus its distribution was unchanged from that displayed in uninfected 293 cells (White, et al., 1988).

Cytoplasmic bodies containing p53 protein have been observed previously in cells transformed with early region 1 of the non-oncogenic Ad5 virus, but not in cells transformed with the same region of the oncogenic Ad12 virus (Zantema, et al., 1985b). These cytoplasmic bodies also contain the majority of adenovirus E1b protein produced by such cells (Zantema, et al., 1985a). Similar cytoplasmic bodies (one to five per cell) containing E1b have also been detected in HeLa cells lytically-infected with Ad5, although E1b is also present at a number of other nuclear and cytoplasmic sites in such cells (Ornelles and Shenk, 1991). These authors did not perform p53 staining of these lytically-infected cells but other work suggests that p53 is unlikely to be colocalised with E1b in such cells ((Braithwaite and Jenkins, 1989) and chapter 4).

In the experiments presented here, p53 was detected in a single cytoplasmic body near the nuclear periphery of 293 cells as was previously reported for Ad5-transformed cells (Zantema, et al., 1985). However, when 293 cells were infected with SV40, p53 staining of these cytoplasmic bodies decreased and, in many cases, was not detected (figure 5.6e). Furthermore, nuclear p53 staining, which was weakly positive in uninfected 293 cells (Zantema, et al., 1985), was markedly altered upon SV40 infection, with the appearance of intense foci of p53 corresponding to sites of T Ag accumulation (figure 5.6e). These changes in p53 distribution suggest two possibilities; either p53 dissociates from E1b and migrates to the nucleus where it associates with T Ag, or p53 in cytoplasmic bodies is degraded and newly-synthesised p53 associates with T Ag in the nucleus. Distinct regions of p53 have been shown to bind E1b and T Ag, so these two viral proteins are unlikely to compete for the same binding site on p53 (Braithwaite, et al., 1991; Jenkins, et al., 1988; Kao, et al., 1990; Tan (T.-H.), et al., 1986). SV40 infection produces a T Ag-mediated change in the phosphorylation state of p53 (Scheidtmann and Haber, 1990) which has been suggested to interfere
with binding to E1b (Kao, et al., 1990). This theory is supported by results showing that the ability of p53 and E1b to form a complex is determined by some property (probably a protein modification) of p53 (Braithwaite and Jenkins, 1989). Thus, SV40 infection of 293 cells may produce sufficient T Ag to activate a protein kinase(s) which phosphorylates p53, resulting in a decreased affinity of p53 binding to E1b and, consequently, the formation of p53-T Ag complexes.

The formation of a p53-E1b complex may not be a prerequisite for viral transformation, since Ad12 transforms cultured cells and increases the stability of p53, but does not form an E1b-p53 complex (Zantema, et al., 1985). Also, the formation of a p53-T Ag complex has recently been suggested to be involved in viral propagation rather than in viral transformation (Bargonetti, et al., 1991). Hence, the precise functions of the interactions of p53 with E1b and T Ag are currently unclear.

Thus, three main differences were observed between the process of SV40 replication in 293 and CV-1 cells. Firstly, the level of T Ag was lower in 293 cells and its distribution was rather more even than in CV-1 cells at the same time post-infection. Secondly, SSB was less frequently associated with T Ag foci in 293 cells. Lastly, p53 was colocalised with T Ag in discrete foci in 293 cells, but was diffusely distributed in CV-1 cells. These results suggest that the process of SV40 replication in 293 cells is different to that in CV-1 cells. This difference could be due to the species origin of the two cell types or, more likely, to the presence of adenovirus early region 1 proteins in 293 cells.

**Interactions of SV40 with HSV-1**

T Ag was found to colocalise with the HSV-1 ICP8 protein in structures resembling replication compartments in CV-1 cells co-infected with SV40 and HSV-1 (figure 5.7). Thus, the compartmentalisation machinery of HSV-1 appears unaffected by SV40 infection and sequesters T Ag and presumably the cellular DNA replication machinery associated with this protein, into compartments. As mentioned in chapter 3, HSV-1 can induce the amplification of SV40 sequences in SV40-transformed cells. This amplification is mediated by six of the seven essential HSV-1 DNA replication proteins (Heilbronn and zur Hausen, 1989) and requires SV40 T Ag and an SV40 origin of DNA replication (Danovich and Frenkel, 1988). The presence of T Ag in compartments containing ICP8 lends support to the theory that T Ag binds to the HSV-1 DNA replication machinery and directs it
to SV40 origins of DNA replication (Heilbronn and zur Hausen, 1989). It is possible that this process is mediated through an interaction between T Ag and HSV Pol, analogous to that between T Ag and the cellular pol α. It would be of interest to determine whether SV40 DNA is in fact replicated within the replication compartments observed.

**Interactions of SV40 with adenovirus**

Co-infection of CV-1 cells with SV40 and Ad2 produced a variety of host protein distributions. The majority of cells displayed staining patterns similar to those seen during single adenovirus infections (figure 5.8 and table 5.1). Thus, Ad2 was still able to form compartments in SV40-infected cells. T Ag was present throughout the nucleus and appeared especially concentrated around replication compartment boundaries.

SV40 has been shown to enhance Ad2 replication in CV-1 cells (Rabson, et al., 1964). The SV40 helper function is provided by the carboxy-terminal region of T Ag, which overcomes the block to adenovirus late gene expression in CV-1 cells, possibly by functioning in the processing of late adenovirus mRNAs (Klessig and Grodzicker, 1979; Tjian, et al., 1978). The distribution of T Ag in co-infected cells is consistent with this role since it resembles that of the population of E1b protein which is thought to function in mRNA metabolism i.e. it is located around the periphery of DBP compartments (Ornelles and Shenk, 1991).

Ad2 inhibits SV40 replication in CV-1 cells through the action of an early gene product(s) (Friedman, et al., 1970; van Roy and Fiers, 1978). The mechanism of this inhibition is not known, but may involve a decrease in T Ag binding to Rb and/or p53 due to the presence of E1a and/or E1b.
6. Production and characterisation of monoclonal antibodies recognising HSV-1 DNA replication proteins

This chapter is divided into three sections, each one describing the antibodies produced from an individual fusion (details of immunisation schedules, fusions and methods of antibody production are presented in Materials and Methods section 3). Monoclonal antibodies recognising the HSV-1 proteins ICP8 and UL42 are described. Details of two other antibodies are also presented; one of these recognises a cellular protein(s) present in replication compartments in HSV-1-infected cells, whilst the other recognises an antigen present within the nuclei of HSV-1 infected, but not mock-infected, cells.

ICP8 Fusion

Antibody production

Five mice were immunised at regular intervals with purified ICP8 (see Materials and Methods section 3a for ICP8 purification details). Figure 6.1 shows Western blots of HSV-1-infected cell extracts using test bleeds taken from the mice after four injections (only three mice were remaining at this point). It can be seen that bleeds from mice 2 and 4 reacted strongly with ICP8. The bleed from mouse 4 showed more intense peroxidase staining of replication compartments than that from mouse 2 when tested on HSV-1-infected CV-1 cells. Therefore, mouse 4 was selected for the subsequent fusion.

Fusion of spleen cells from the selected mouse with Sp2 myeloma cells was performed as described in Materials and Methods section 3c using approximately $1.2 \times 10^8$ myeloma cells. Hybridomas were fed after 10 days and screened after 14 days. Thirty clones were selected on the basis of peroxidase staining of HSV-1 infected CV-1 cells and transferred to 24-well plates. These clones were further characterised by testing media from their wells in Western blotting and $^{35}$S-labelled-immunoprecipitation assays and by peroxidase staining of both mock-infected and HSV-1-infected CV-1 cells. Six clones were found to detect a single band of the same molecular weight as ICP8 on Western blots. Antibody derived from only one clone strongly immunoprecipitated a band of the same molecular weight as ICP8. Culture media from fourteen clones were found to stain HSV-1-infected cells strongly, though not all of these stained replication compartments. Cell staining of mock-infected cells was not observed using culture media from
these fourteen clones. Four clones were stable through several rounds of single-cell-cloning (see Materials and Methods section 3d) and these were named 2F4, 7D7, 10A3 and 11E2, according to the wells from which they were isolated. 7D7 was not stable in spinner culture and thus large-scale preparations were made from just three antibodies: 2F4, 10A3 and 11E2.

Figure 6.1. Test bleeds from mice immunised with ICP8. HSV-1-infected CV-1 cell extracts were made 20hpi by sonicating cells which had been pelleted, washed in PBS and resuspended in dH2O (Killington and Powell, 1986). Extracts were run on a denaturing polyacrylamide gel and transferred to nitrocellulose (see Materials and Methods 5a). Strips were then blotted with test bleeds taken on 6/9/90 (see Materials and Methods section 3a for immunisation schedule). Lane 1, polyclonal anti-ICP8 (diluted 1/1000); lane 2, mouse 2 test bleed (1/50); lane 3, mouse 2 pre-bleed (1/50); lane 4, mouse 4 test bleed (1/50); lane 5, mouse 4 pre-bleed (1/50); lane 6, mouse 5 test bleed (1/50); lane 7, mouse 5 pre-bleed (1/50).

Antibody characterisation
2F4, 10A3 and 11E2 were all sub-typed and found to be of the IgG1 subclass. Thus, they were purified using protein A columns in conditions of high salt concentration (see Materials and Methods section 3f). A denaturing polyacrylamide gel of the resulting purified antibodies is shown in figure 6.2. The rather fuzzy bands may be accounted for by the presence of contaminating bovine immunoglobulin from the FCS used in the hybridoma growth medium.
Figure 6.2. Purified anti-ICP8 monoclonal antibodies. 5μg of each purified anti-ICP8 monoclonal antibody was run on a denaturing polyacrylamide gel and Coomassie-stained (Materials and Methods sections 5a and 5b). See text for purification details.

The patterns of staining exhibited by the three antibodies are shown in figure 6.3. Each of the antibodies stained replication compartments in productively-infected cells and prereplicative sites in PAA-treated cells, whilst showing little reaction with mock-infected cells. In double-labelling experiments, the areas of staining observed using the monoclonal antibodies were found to colocalise with those recognised by polyclonal anti-ICP8 serum.

All of the characterised antibodies were positive in Western blots, recognising a band specific to HSV-1-infected cell extracts that was also detected by polyclonal anti-ICP8 serum (figure 6.4.). 10A3 was the strongest-reacting antibody followed by 2F4 and 7D7, with 11E2 as the weakest. However, this should not be taken as a quantitative measure of affinity since hybridoma supernatants may contain variable amounts of antibody. The lower bands detected by 10A3 were not consistently reproducible, and may simply be degradation products detected by this strongly-reacting antibody. In some blots ICP8 appeared to run as a doublet. No preferential specificity of the antibodies for a particular band in the doublet was reproducibly observed.
Figure 6.3. Cell staining using anti-ICP8 monoclonal antibodies. CV-1 cells were mock-infected (M) or infected with HSV-1 for 5h in the presence (PAA) or absence (+) of phosphonoacetate. Cells were then fixed in acetone/methanol and stained as described in Materials and Methods section 1c using single-cell-cloned anti-ICP8 hybridoma supernatants 2F4, 10A3 or 11E2 followed by FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30).
Figure 6.4. Western blots using anti-ICP8 monoclonal antibodies.
CV-1 cell extracts were made 20h after mock- (m) or HSV-1- (+) infection, electrophoresed and transferred to nitrocellulose as described in the legend to figure 6.1. Nitrocellulose strips were blotted with polyclonal anti-ICP8 (1/1000) or with hybridoma supernatant as indicated. All hybridomas were single-cell-cloned, except for 7D7, which had been through one round of cloning.

Figure 6.5. 35S-labelled immunoprecipitations using anti-ICP8 monoclonal antibodies.
A. HSV-1-infected CV-1 cell extracts were prepared 6.5hpi as described in Materials and Methods section 1e, after labelling from 3-6.5hpi with 200μCi 35S-met per 15cm plate (see Materials and Methods section 1d). Immunoprecipitations were performed as described in Materials and Methods section 5b, using 5μg pure monoclonal antibody or 1μl of polyclonal anti-ICP8. The bead eluates were electrophoresed and the gel was exposed to Kodak X-omat film for 6 days, producing the autoradiogram shown.
B. As for A, but extracts were made 5hpi after labelling from 2.5-5hpi with 150μCi 35S-met per 10cm plate. Immunoprecipitations were performed with 5μg 2F4 or 11E2, or with 100μl 10A3 hybridoma supernatant (single-cell-cloned). The gel was exposed to Fuji X-ray film for 7 days, producing the autoradiogram shown.

Figure 6.6. Blot and autoradiogram of a 35S-labelled immunoprecipitation using 11E2.
A sample of the 11E2 immunoprecipitation described in figure 6.5b was run on a denaturing polyacrylamide gel and transferred to nitrocellulose (Materials and Methods section 5a). The nitrocellulose was then blotted with polyclonal anti-ICP8 (1/1000) (lanes 1 and 2). The finished blot was sprayed three times with "Enhance" (Du Pont) and exposed to Kodak X-omat film overnight. The resulting autoradiogram is shown (lanes 3 and 4).
Figure 6.5a shows the proteins immunoprecipitated from $^{35}$S-labelled mock-infected and HSV-1-infected cell extracts by the anti-ICP8 monoclonal antibodies. It can be seen that only 11E2 immunoprecipitated significant amounts of ICP8. A number of other bands were co-precipitated by this antibody and also by polyclonal anti-ICP8 serum (see arrows). These are more clearly visible in figure 6.5b, which shows a longer exposure of a gel from a similar experiment. The approximate molecular weights of the co-precipitated bands in figure 6.5b were 125k, 90k, 85k, 68-69k and 55-60k. At least some of these bands were not ICP8 degradation products, since they were not recognised by polyclonal anti-ICP8 serum on a Western blot (figure 6.6). This blot also provides formal proof that the protein immunoprecipitated by 11E2 was ICP8.

![Figure 6.5a](image)

**Figure 6.5a.** Western blot showing the proteins immunoprecipitated from mock-infected and HSV-1-infected cell extracts using anti-ICP8 monoclonal antibodies. The bands at 116, 98, 68, 45, and 29 kDa are indicated.

![Figure 6.5b](image)

**Figure 6.5b.** Enlarged view of the gel from figure 6.5a, showing the co-precipitated bands at 116, 98, 68, 45, and 29 kDa.

**Figure 6.7. Western blots of EBV- and adenovirus-infected cell extracts using anti-ICP8 monoclonal antibodies.**

Cell extracts were prepared and transferred to nitrocellulose as described in Materials and Methods sections 1e and 5a.

**A. EBV.** B-cell lymphoblast cells transformed with EBV (PH3RI cells) were treated with tumour promoting agent for 72h to induce lytic infection (this procedure was performed by Dr. M. Ginsberg, ICRF). Extracts were made by NP40-lysis of both induced (+) and uninduced (m) cells and blotted with hybridoma supernatant or polyclonal anti-ICP8 (1/1000) as shown.

**B. Adenovirus.** CV-1 cells were infected with adenovirus type 5 at an m.o.i. of >10 pfu's/cell and harvested 24hpi. Extracts were prepared by NP40-lysis of mock-infected (m) or infected (+) cells. Nitrocellulose strips were blotted with polyclonal anti-DBP (1/500), polyclonal anti-ICP8 (1/1000) or hybridoma supernatant as shown.
Figure 6.7 shows Western blots of Epstein Barr virus (EBV)- and adenovirus- infected cell extracts using the anti-ICP8 monoclonal and polyclonal antibodies. No virus-specific band was detected in these extracts by any of the anti-ICP8 antibodies used.

**UL42 Fusion**

**Antibody production**

Five mice were immunised at intervals with baculovirus-produced HSV Pol or purified HSV Pol/UL42 complex (see Materials and Methods section 3a for purification details). It was originally intended to produce only anti-HSV Pol antibodies from these particular mice, but injection of the HSV Pol/UL42 complex (used because of a limited supply of HSV Pol immunogen) produced a strong immune response to UL42 (figure 6.8a) making the mice suitable for production of anti-UL42 antibodies. Mouse 1 was selected for fusion on the basis of Western blotting and peroxidase staining of HSV-1-infected CV-1 cells. This mouse responded strongly to an intravenous injection of UL42 performed 4 days prior to fusion (figure 6.8b).

![Figure 6.8. Test bleeds from mice immunised with UL42.](image)

A. Test bleeds. Extracts were prepared and blotted as described in figure 6.1. See Materials and Methods section 3a for the immunisation schedule. Lanes 1-5 show nitrocellulose strips blotted with test bleeds from mice 1-5 respectively. Test bleeds were diluted 1/50.

B. Serum from boosted mouse. Methods as in A but using bleed-out from mouse 1 taken just before fusion.
Fusion of spleen cells from the selected mouse with Sp2 myeloma cells was performed as described in Materials and Methods section 3c. Hybridomas were fed after 6 days and screened by cell staining after 11 days (2 positives) and 14 days (many positives). Positive clones were further characterised by testing media from their wells in Western blotting and 35S-labelled-immunoprecipitation assays. On this basis, a number of hybridomas were selected for single-cell-cloning, of which five were subsequently single-cell-cloned and grown in large quantities. These were named 2H4, 6F1, 7C5, 13C9 and 13D11 according to the wells from which they were isolated.

**Antibody characterisation**

The five anti-UL42 monoclonal antibodies were all of the IgG1 subclass and were thus purified in the same way as the anti-ICP8 monoclonal antibodies (see above). A denaturing polyacrylamide gel of the purified anti-UL42 antibodies is shown in figure 6.9. As with the anti-ICP8 antibodies, there was some contamination with bovine immunoglobulin from the FCS in the growth medium. The purified antibodies were still functional in cell staining and Western blot assays.

![Figure 6.9. Purified anti-UL42 monoclonal antibodies.](image)

5μg of each purified anti-UL42 monoclonal antibody was run on a denaturing polyacrylamide gel and Coomassie-stained (Materials and Methods section 5a and 5b). See text for purification details.
Figure 6.10. Cell staining using anti-UL42 monoclonal antibodies.
CV-1 cells were mock-infected (M) or infected with HSV-1 for 5h in the presence (PAA) or absence (+) of phosphonoacetate. Cells were then fixed in acetone/methanol and stained as described in Materials and Methods section 1c using single-cell-cloned anti-UL42 hybridoma supernatants or Z1F11 ascites (1/1000) and FITC-conjugated goat anti-mouse-immunoglobulin (Cappel, 1/30).
Figure 6.10.(continued) Cell staining using anti-UL42 monoclonal antibodies.
CV-1 cells were mock-infected (M) or infected with HSV-1 for 5h in the presence (PAA) or absence (+) of phosphonoacetate. Cells were then fixed in acetone/methanol and stained as described in Materials and Methods section 1c using single-cell-cloned anti-UL42 hybridoma supernatants or Z1F11 ascites (1/1000) and FITC-conjugated goat anti-mouse-immunoglobulin (Cappel, 1/30).
The patterns of cell staining exhibited by the five antibodies are shown in figure 6.10 along with the staining pattern produced by Z1F11, a previously characterised mouse monoclonal antibody to UL42 (Murphy, et al., 1989) (kindly provided by Dr. H. S. Marsden, MRC, Glasgow). Each of the new antibodies stained replication compartments in productively-infected cells, but stained the whole nucleus of PAA-treated cells. Thus, their staining patterns were the same as those displayed by Z1F11. There was little reaction with the nuclei of mock-infected cells (the mock-infected cell photographs were exposed for much longer than the infected-cell photographs, amplifying their relative staining intensity).

All of the anti-UL42 antibodies were strongly positive in Western blot assays, recognising a band specific for infected cells that was also detected by Z1F11 (figure 6.11). The new antibodies did not recognise the additional cellular proteins detected by Z1F11 ascites. These additional bands were probably due to contaminating mouse antibodies present in the Z1F11 ascites fluid.

Figure 6.12 shows the proteins immunoprecipitated from 35S-labelled mock-infected and HSV-1-infected cell extracts by the anti-UL42 monoclonal antibodies. The new anti-UL42 antibodies immunoprecipitated UL42 only very weakly when compared with Z1F11. Z1F11 co-precipitated a higher molecular weight protein (see arrow in figure 6.12) which was weakly present in the other anti-UL42 lanes and may be HSV Pol (see discussion). Western blots of similar immunoprecipitations from unlabelled HSV-1-infected cells are shown in figure 6.13. The levels of UL42 and ICP8 in these immunoprecipitations were visualised by blotting simultaneously with anti-UL42 and anti-ICP8 monoclonal antibodies (figure 6.13a). The ICP8 signal was slightly stronger in those lanes where a UL42 band was present (compare 2H4, 6F1 and 7C5 with 13C9 and 13D11), but did not increase in proportion to the quantity of UL42 immunoprecipitated (compare Z1F11 with 2H4, 6F1 and 7C5). Similar results were observed for HSV Pol (figure 6.13b). It should be noted that the peptide anti-HSV Pol serum cross-reacted with UL42. The 13C9 antibody preparation was not recognised as mouse immunoglobulin on Western blots (figure 6.13a) although a band was visualised by Ponceau staining. Thus, although functional in cell staining and Western blot assays, the purified 13C9 preparation may contain diminished amounts of 13C9, with an increased amount of contaminating bovine immunoglobulin contributing to the quantitative OD280 reading.
Figure 6.11. Western blots using anti-UL42 monoclonal antibodies.
CV-1 cell extracts were prepared and transferred to nitrocellulose as described in the legend to figure 6.4. Nitrocellulose strips were blotted with supernatant from single-cell-cloned hybridomas or with Z1F11 ascites (diluted 1/1000).

Figure 6.12. 35S-labelled immunoprecipitations using anti-UL42 monoclonal antibodies.
CV-1 cell extracts were prepared as described in the legend to figure 6.5a and immunoprecipitated using 5µg of purified monoclonal antibody or 1µl of Z1F11. The bead eluates were electrophoresed and the gel was exposed to Kodak X-omat film for 6 days, producing the autoradiogram shown.

Figure 6.13. Level of ICP8 and HSV Pol in UL42 immunoprecipitations.
Extracts were produced from six 10cm plates of HSV-1-infected CV-1 cells 5hpi. Immunoprecipitation was performed with 5µg pure monoclonal antibody or 2µl Z1F11 ascites. After electrophoresis and transfer to nitrocellulose, immunoprecipitates were blotted with:
A. anti-UL42 (6F1) and anti-ICP8 (10A3) hybridoma supernatants
B. peptide II anti-HSV Pol serum (1/100) (see Materials and Methods sections 1e, 5a and 5b).
Previous peptide mapping studies located the Z1F11 epitope to an apparently immunodominant region of UL42 between amino acids 360 and 366. Thus, it was of interest to determine whether the new anti-UL42 antibodies also mapped to this region. This was tested in an ELISA assay, using a synthetic peptide (278A) which encompassed this region (kindly provided by Dr. H.S. Marsden, MRC, Glasgow). As expected, Z1F11 reacted strongly with the peptide at dilutions of up to 1/100,000 (figure 6.14a). Antibodies 2H4, 6F1 and 7C5 also reacted strongly with this peptide, whereas 13D11 reacted more weakly and 13C9 reacted weakly or not at all, showing signals only slightly above background levels (figure 6.14b).

6.14A

![Graph showing the OD 450 values for different dilutions of Z1F11 antibody. The graph has a linear scale on the x-axis labeled 'Z1F11 dilution (x1000)' and a logarithmic scale on the y-axis labeled 'OD 450'. The graph shows a curve that rises as the dilution decreases, indicating increased reactivity. There is a legend indicating the data points for Z1F11 antibody.](image-url)
Figure 6.14. Level of anti-UL42 monoclonal antibody binding to the peptide 278A as determined by ELISA.

Two flat-bottomed 96-well plates were coated overnight with 278A peptide at a concentration of 1μg/well. After blocking, pure monoclonal antibodies or Z1F11 were applied in serial two-fold dilutions across the plates. The plates were washed before application of HRP-conjugated rabbit anti-mouse immunoglobulin (1/1000) as a second antibody layer. Bound HRP was detected with TMB (see Materials and Methods section 5c). Optical density measurements were taken at 450nm and the averages of the readings from the two plates were used to plot graphs.

A. Z1F11. Z1F11 diluted 1/1000 in PBS was further diluted across the 96-well plate in 1% BSA/0.1% NP40/PBS. These further dilutions are plotted on the horizontal axis.

B. UL42 monoclonal antibodies. Purified antibodies were applied in serial two-fold dilutions across the plates from a starting concentration of 12.5μg/well.

The ability of the antibodies to bind to HSV Pol was measured by a two-site sandwich ELISA assay (figure 6.15). A schematic representation of this assay is shown in the legend to figure 6.15. 2H4, 6F1 and 7C5 clearly bound to HSV Pol, whilst 13D11 may have bound weakly and 13C9 not at all.

HSV Pol Fusion

Antibody production

As mentioned previously, injection of the HSV Pol/UL42 complex produced a very strong response to UL42. This rather masked the response to HSV Pol, which had previously been quite weak. One mouse did not respond strongly to UL42, and this was given further injections of HSV Pol
Figure 6.15. Level of anti-UL42 monoclonal antibody binding to HSV Pol as determined by ELISA. A schematic diagram of the assay used is shown below.

Flat-bottomed 96-well plates were coated with purified antibodies at 1μg/well. A two-site sandwich assay was then performed as described in Materials and Methods section 5c using extracts prepared 5h after mock-infection (m) or HSV-1-infection (+) of CV-1 cells. Peptide II anti-HSV Pol serum was applied as the second antibody layer at 1/1000, followed by HRP-conjugated swine anti-rabbit immunoglobulin at 1/1000. The results for all antibodies tested are shown graphically in A, with results for individual antibodies shown in B-F for clarity.
alone in the hope of producing a response to HSV Pol. A weak response to HSV Pol was generated (figure 6.16a). Thus, the mouse was boosted with HSV Pol and used for fusion. The HSV Pol response was weaker in the boosted mouse than expected (figure 6.16b); however the fusion was continued.

Figure 6.16. Test bleeds from mouse immunised with HSV Pol. Cell extracts were prepared, electrophoresed and transferred to nitrocellulose as described in figure 6.1. See Materials and Methods section 3a for immunisation schedule.

A. Test bleed. nitrocellulose strips were blotted with: lane 1, peptide II anti-HSV Pol serum (1/100); lane 2, test bleed taken on 1/3/91 (1/50).

B. Serum from boosted mouse. nitrocellulose strips were blotted with: lane 1, peptide II anti-HSV Pol serum (1/100); lane 2, bleed-out from mouse taken just before fusion.

Approximately 70% of the fusion wells contained hybridoma clones after 14 days, but many of these showed cytoplasmic staining when tested on HSV-1-infected cells. However, two clones were selected and cloned on
Figure 6.17. Cell staining using 1D5 and 5H7.
CV-1 cells were mock-infected (M) or infected with HSV-1 for 5h in the presence (PAA) or absence (+) of phosphonoacetate. Cells were then fixed in acetone/methanol and stained as described in Materials and Methods section 1c using single-cell-cloned 1D5 and 5H7 hybridoma supernatants and FITC-conjugated goat anti-mouse immunoglobulin (Cappel, 1/30).
the basis of their cell staining patterns. One of these (5H7) stained HSV-1 replication compartments, whilst the other (1D5) displayed a punctate nuclear staining pattern in HSV-1-infected cells.

Antibody characterisation

Both antibodies belonged to the IgM subclass and were characterised using hybridoma supernatant.

The results of immunofluorescence staining using these antibodies are shown in figure 6.17. 1D5 did not stain mock-infected cells, but did stain the nuclei of HSV-1-infected cells displaying a granular staining pattern which appeared to exclude the nucleoli. A small number of intense foci were visible in about 20% of cells. These foci were also present in cells infected in the presence of PAA and were often localised to the nuclear periphery. 5H7 stained the entire nucleus of mock-infected CV-1 cells, and did not exclude the nucleoli. The staining pattern was very granular. The antibody recognised an antigen within replication compartment type structures in HSV-1-infected cells, but in PAA-treated HSV-1-infected cells showed a staining pattern very similar to that seen in mock-infected cells.

Figure 6.18. Western blots using 1D5 and 5H7.
Cell extracts were prepared from mock-infected (m) or HSV-1-infected (+) CV-1 cells 5hpi (see Materials and Methods section 1e). After electrophoresis and transfer to nitrocellulose, extracts were blotted with 1D5 or 5H7 hybridoma supernatants (Materials and Methods section 5a).
The two antibodies were also tested in Western blots (figure 6.18). 1D5 did not blot cell extracts from mock- or HSV-1-infected CV-1 cells. 5H7 recognised a number of bands in both uninfected and infected cell extracts. Ponceau staining of the blot shown in figure 6.18 revealed that there was more protein in the infected than in the mock-infected lane and thus the detected proteins were probably present at approximately equal levels in infected and uninfected cells (this was confirmed in other Western blots). Four major bands were recognised by 5H7 on this and other blots. The molecular weights of these bands were approximately 65k, 90k, 125k and 140k (a doublet).

1D5 and 5H7 did not detectably immunoprecipitate any proteins from 35S-labelled cell extracts, even though rabbit anti-mouse immunoglobulin was used as a coupling agent between these IgM antibodies and protein G.

**Discussion**

Properties of the novel monoclonal antibodies

A summary of the properties of the new antibodies is shown in table 6.1.

Table 6.1 Properties of the newly-produced monoclonal antibodies

<table>
<thead>
<tr>
<th>NAME</th>
<th>ANTIGEN</th>
<th>CELL STAINING</th>
<th>WESTERN BLOTS</th>
<th>IMMUNOPRECIPITATION</th>
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<td></td>
<td></td>
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<td>infected</td>
<td></td>
</tr>
<tr>
<td>2F4</td>
<td>ICP8</td>
<td>-</td>
<td>++</td>
<td>++</td>
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<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>13D11</td>
<td>UL42</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>1D5</td>
<td>UNKNOWN (VIRAL?)</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>5H7</td>
<td>UNKNOWN (CELLULAR)</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

+ indicates a weak reaction
++ indicates a strong reaction
Monoclonal antibodies to ICP8

The three antibodies studied were not epitope-mapped, but the ability of 11E2 to immunoprecipitate ICP8 suggests that it recognises an epitope distinct from those of 2F4 and 10A3. The antibodies often detected a doublet of ICP8, probably by recognising the two conformational forms of ICP8 described previously as being present in HSV-1-infected cells 3-6hpi (Knipe, et al., 1982) (see introductory chapter). Analysis of a number of blots showed that the antibodies were able to recognise both species of ICP8 and did not appear to be specific for a particular conformational form.

Five bands with approximate molecular weights of 125k, 90k, 85k, 68-69k and 55-60k were immunoprecipitated by 11E2 (see figure 6.5b). Immunoprecipitations using 39S, a previously-produced monoclonal antibody to ICP8, showed that an 85k viral polypeptide and cellular polypeptides of 125k, 68-70k and 58-61k co-precipitated with ICP8 (Quinlan, et al., 1984). Thus, four of the proteins co-precipitated by 11E2 also seem to be recognised by 39S, whilst 11E2 also precipitated a 90k protein. The 85k viral protein recognised by 11E2 and 39S is probably the HSV-1 alkaline exonuclease, since antibodies to the HSV-2 major DNA-binding protein (which is closely related to ICP8) co-precipitate the 85k HSV-2 alkaline exonuclease (Thomas, et al., 1988). The identity of the other co-precipitated proteins is unknown, though those recognised by 39S are not specific to replication compartments or prereplicative sites and may be indirectly associated with ICP8 through binding to DNA (Quinlan, et al., 1984). The common immunoprecipitation of these four proteins by two independent monoclonal antibodies to ICP8 provides evidence that these interactions are not due to spurious antibody cross-reactions, but represent an association of these proteins with ICP8.

The anti-ICP8 monoclonal antibodies did not cross-react with any EBV or adenovirus type 5 proteins in Western blotting assays. ICP8 is homologous to the protein encoded by the BALF2 sequence of EBV (Quinn and McGeoch, 1985). Regions of similarity exist throughout the two proteins, but the amino acid identity after optimal alignment is only 25% (Albrecht and Fleckenstein, 1990). Thus the chances of one of the anti-ICP8 monoclonal antibodies cross-reacting with the 135k BALF2 were low. The polyclonal anti-ICP8 serum also did not appear to react with BALF2. The lack of cross-reaction with adenovirus proteins was unsurprising since, although adenovirus has a major double-stranded DNA binding protein, protein
sequence analysis showed no significant homology between this protein and ICP8.

Monoclonal antibodies to UL42

The anti-UL42 monoclonal antibodies all stained replication compartments in HSV-1 infected cells, but showed a diffuse staining pattern in the nuclei of cells infected in the presence of PAA (figure 6.10). This confirms previous results using MAb 6898 monoclonal anti-UL42 antibody on HSV-1 KOS-infected Vero cells (Goodrich, et al., 1990).

At least three of the antibodies, 2H4, 6F1 and 7C5 mapped to the same epitope as Z1F11, a previously characterised anti-UL42 monoclonal antibody (figure 6.14). All seven of the anti-UL42 monoclonal antibodies which have been produced previously map to epitopes between amino acids 360 and 369 of UL42. However, since six of these antibodies were derived from a single fusion, it was possible that these antibodies arose from the same clone within the mouse (Murphy, et al., 1989). The mapping of three new anti-UL42 monoclonal antibodies to the same epitope as recognised by the previous seven confirms that this region is immunodominant in BALB/c mice.

Despite mapping to the same epitope as Z1F11, 2H4, 6F1 and 7C5 detected lower amounts of UL42 in Western blot and immunoprecipitation assays (figures 6.11 and 6.12). This may be due to lower affinities of these antibodies for UL42, but may also be accounted for by the use of greater quantities of Z1F11 antibody which was added as ascites (note the higher levels of immunoglobulin chains in the Z1F11 lane of figure 6.11).

No conclusive association was detected between UL42 and ICP8 as assayed by immunoprecipitation (figure 6.13a). Physical ICP8-HSV Pol interactions demonstrated by this method have not been reported elsewhere.

Z1F11 immunoprecipitated a band of around 140k which may be HSV Pol (figure 6.12). Previous immunoprecipitations using Z1F11 hybridoma supernatant did not detect any co-precipitating bands, although a UL42-HSV Pol interaction was deduced from the specific retention of HSV Pol by a MAb 6898 affinity column (Gallo, et al., 1988; Murphy, et al., 1989). In the present experiments Z1F11 ascites was used, raising the possibility that a contaminating mouse antibody was responsible for the co-precipitated band. However, the molecular weight of the band was very similar to that of HSV Pol and thus it may be that the use of large quantities of Z1F11 can co-
precipitate HSV Pol. Blotting of UL42 immunoprecipitations with peptide II antiserum revealed a slight increase in HSV Pol levels in lanes containing UL42, although the increase in levels was not very significant and does not provide conclusive evidence for co-precipitation of HSV Pol by UL42 (figure 6.13b). However, evidence for a UL42-HSV Pol interaction was provided by ELISA assays (figure 6.15) which detected binding of a UL42-HSV Pol complex to 2H4, 6F1 and 7C5. The ability of these antibodies to bind UL42 complexed to HSV Pol provides further evidence that amino acids 360-366 of UL42 are not directly involved in binding to HSV Pol (Murphy, et al., 1989).

13C9 and 13D11, whilst recognising UL42 in cell staining and Western blot assays, showed different behaviour to the other anti-UL42 antibodies in immunoprecipitation and ELISA assays. 13D11 bound UL42-HSV Pol complexes more weakly than 2H4, 6F1 and 7C5, perhaps reflecting a lower affinity for native UL42. The lower affinity of 13D11 for the peptide 278A may be intrinsic to the antibody, or may be due to incomplete representation of the 13D11 epitope by the 278A peptide sequence. 13C9 did not bind to UL42-HSV complexes or to the 278A peptide, suggesting either that the preparation contained diminished amounts of purified antibody (see results concerning figure 6.13a) or that the 13C9 epitope is distinct from that of the other anti-UL42 antibodies and is involved in UL42 binding to HSV Pol. Repetition of the ELISA experiments using another preparation of purified 13C9 is necessary to clarify this point.

Antibodies 1D5 and 5H7

The strong response elicited by UL42 as compared to that induced by HSV Pol in BALB/c mice may be a general obstacle to the production of anti-HSV Pol antibodies as evidenced by the lack of mouse monoclonal anti-HSV Pol antibodies reported in the literature. Thus, any future attempts to produce such antibodies would be best performed using solely baculovirus-produced HSV Pol as an immunogen which would be completely free of UL42. Although no anti-HSV Pol antibodies were isolated, two interesting monoclonal antibodies were produced from the HSV Pol fusion, 1D5 and 5H7.

The antigen recognised by 1D5 is probably viral, since it did not stain mock-infected cells (figure 6.17). However, the possibility that 1D5 recognises a cellular protein induced by HSV-1 infection cannot be ruled out. Since 1D5 did not detectably Western blot or immunoprecipitate any

168
proteins, characterisation of the 1D5 antigen was not possible. However, its association with the nuclear membrane of infected cells suggests that it might be a HSV-1 glycoprotein.

5H7 recognised a number of cellular proteins on Western blots. The 5H7 antigen was present in HSV-1 replication compartments but was diffusely distributed in PAA-treated HSV-1-infected cells (figure 6.17). Similar cell staining patterns were observed using anti-histone antibodies (table 3.1). However, the molecular weights of the proteins recognised by 5H7 on Western blots were greater than those predicted for histone proteins (figure 6.18). Perhaps the 5H7 antigen, like histones, is associated with cellular DNA and is recruited to sites containing progeny HSV-1 DNA once viral DNA replication is completed.

The isolation of the 5H7 antibody reveals the power of the cell staining screening technique for detecting antibodies directed against replication compartment-associated proteins. Therefore, this strategy has the potential for isolating antibodies against possible novel cellular DNA replication proteins recruited to replication compartments by HSV-1, provided a suitable immunogen could be produced i.e. one consisting of native DNA replication complexes.

The antibodies described above should prove useful in further studies of the HSV-1 infection process.
7. General Discussion

A comparison of the major DNA-binding proteins encoded by HSV-1, adenovirus and SV40

Table 7.1 compares the known functions of the major DNA-binding proteins encoded by the viruses studied in this thesis. It should be emphasised that only functions which have been demonstrated are described and further undetected roles for these proteins are possible. A more detailed account of the functions of the three proteins is given in the introductory chapter.

Table 7.1. Functions of viral DNA binding proteins

<table>
<thead>
<tr>
<th>FUNCTION</th>
<th>ICP8 (130K)</th>
<th>DBP (59K)</th>
<th>T Ag (82K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA BINDING</td>
<td>SEQUENCE - INDEPENDENT, COOPERATIVE ds&gt;ss</td>
<td>SEQUENCE - INDEPENDENT, COOPERATIVE ss&gt;ds</td>
<td>SEQUENCE - INDEPENDENT ss&gt;ds ALSO ORIGIN - SPECIFIC</td>
</tr>
<tr>
<td>DNA REPLICATION</td>
<td>YES (ELONGATION)</td>
<td>YES (INITIATION AND ELONGATION)</td>
<td>YES (INITIATION AND ELONGATION)</td>
</tr>
<tr>
<td>DNA HELICASE</td>
<td>NO</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>INTERACTION WITH DNA</td>
<td>SUGGESTED</td>
<td>SUGGESTED</td>
<td>YES</td>
</tr>
<tr>
<td>HELICASE POLYMERASE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STIMULATION OF LATE VIRAL</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>GENE EXPRESSION</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRANSFORMATION</td>
<td>NO</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>ROLE IN RNA MEtabolism</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
</tr>
</tbody>
</table>

The molecular weight given refers to that predicted from the DNA sequence of the viral genome. DBP and T Ag behave abnormally in SDS-PAGE, running at positions corresponding to molecular weights of 72k and 95k respectively.

Thus, each of the studied viruses has evolved a key multi-functional protein, which binds to DNA as well as participating in a number of processes in the viral life-cycle. The number of functions of these key proteins appears to be related to the size of the virus with SV40 (5kb
T Ag having more functions than Ad (35kb genome) DBP, which in turn has more functions than HSV-1 (150kb genome) ICP8. This is to be expected since larger viruses have the coding capacity to divide these functions between different proteins; for example, HSV-1 encodes a DNA helicase protein as well as the major DNA-binding protein ICP8, whereas SV40 T Ag has both DNA helicase and DNA-binding activities.

Degree of reliance on the host cell

All three of the viruses studied replicate in the host cell nucleus, suggesting a requirement for some component(s) of this organelle. The degree of dependence on host cell proteins correlates with the size of the viral genome. The number of host proteins shown to be essential for viral DNA replication in vitro is: eight for SV40, three for adenovirus and none for HSV-1 (see introductory chapter). However, further host proteins are likely to be required for efficient viral DNA replication in vivo. The results presented here support this view since, for HSV-1 and adenovirus, differences were observed between infections of quiescent and proliferating cells and various host replication proteins colocalised with replicating viral DNA. Structural elements of the nucleus may also be required by these viruses.

An additional, possibly non-replicative, role for SSB in the SV40 life-cycle is suggested by its distribution in infected cells. The use of host SSB by SV40 may reflect a higher affinity of SSB than T Ag for sequence-independent binding to ssDNA, since it has been suggested that T Ag is displaced from DNA cellulose columns by ssDNA binding proteins present in crude nuclear extracts prepared from human cells (Spillman, et al., 1979).

The replication compartments of the three viruses vary in their constitution and structure. HSV-1 forms novel compartments, which extend throughout much of the nucleus and contain a number of host DNA replication proteins. Adenovirus also forms novel compartments, which appear to contain a population of PCNA as well as a number of nucleolar components, prompting the suggestion that "pseudonucleoli" are formed around input adenovirus DNA (Walton, et al., 1989). SV40 does not appear to form clearly delimited replication compartments, although it may utilise the pre-existing nucleolar compartment (Geuskens and May, 1974) and distinct compartments containing SSB are observed which may or may not be involved in viral DNA replication. It may be that since SV40 relies heavily on the host DNA replication machinery, it utilises pre-existing cellular DNA
replication sites and does not change their location and organisation within the nucleus.

**HSV-1 and adenovirus as helper viruses for parvovirus**

Parvoviruses are divided into two main groups: autonomous parvoviruses such as minute virus of mice (MVM) and adeno-associated viruses (AAVs). AAV's normally require co-infection with adenovirus or herpesvirus (HSV-1, HSV-2, cytomegalovirus or pseudorabies virus) helper viruses for productive infection of cultured cells (Berns, 1990). Parvoviruses will only replicate in proliferating cells and autonomous parvoviruses have been shown to require a host protein expressed late in S phase, which may bind to the genome terminus (Metcalfe, *et al.*, 1990; Wolter, *et al.*, 1980).

It is possible that the recompartmentalisation of host proteins by adenovirus and HSV-1 forms at least a part of their parvovirus helper function in some cases. MVM was shown to replicate weakly in the nucleoli of HeLa cells (a non-permissive host) but Ad2 co-infection markedly increased MVM replication which was recompartmentalised to Ad2 replication sites. However, efficient MVM replication was also observed in the nucleoli of 293 cells in the absence of co-infected adenovirus, suggesting that recompartmentalisation of the MVM replication machinery itself is not required for efficient DNA replication in these cells (Fox, *et al.*, 1990). Thus, E1a and E1b are sufficient to induce efficient MVM DNA replication and may act by transactivating host genes and/or by sequestering growth-suppressive host proteins such as Rb and p53.

HSV-1 infection also sequesters Rb and p53 (chapter 3) which may thus act similarly to create a "replication-competent" host cell. Cotransfection studies have identified four HSV-1 genes which are sufficient for the induction of AAV DNA synthesis (Weindler and Heilbronn, 1991). These four genes, UL5, UL8, UL52 and UL29, code for the helicase-primase complex and major DNA-binding protein (ICP8) of HSV-1 (see introductory chapter). It has been suggested that ICP8 interacts with a cellular DNA polymerase to facilitate replication of AAV DNA, since a cellular DNA polymerase(s) is thought to be involved in parvovirus DNA replication (Kolleck, *et al.*, 1982; Weindler and Heilbronn, 1991). AAV replication is thought to be self-primed using a hairpin structure at the origin, and the AAV rep68 protein has a DNA helicase activity (Im and Muzychka, 1990; Samulski, *et al.*, 1982). Hence, additional roles for the HSV-1 helicase-primase complex have been proposed to be involved in AAV replication.
Weindler and Heilbronn (1991). It is possible that the four required HSV-1 replication proteins stimulate AAV DNA replication by redistributing host and AAV replication proteins to replication compartments, since compartments can be formed in the absence of HSV-1 DNA (see chapter 3). It would be of interest to determine whether these four proteins are sufficient for replication compartment formation when transiently expressed in uninfected cells.

Adenovirus activation of AAV DNA replication is mediated by the E1a, E1b, E2a and E4 genes, and is thought to operate via transcriptional and post-transcriptional mechanisms (Berns, 1990). However, it is possible that recompartmentalisation of host and viral proteins may also be involved.

**Further experiments**

Suggestions for future experiments have been put forward in the discussion sections at the end of each chapter. The main suggestions are summarised below.

Further transfection studies defining the roles of individual HSV-1 DNA replication proteins in replication compartment formation should provide information on the mechanism of host protein redistribution. The monoclonal antibodies described in chapter 6 should be useful in defining the interactions of HSV-1 proteins at the replication fork. Experiments could be performed to determine whether the host cell DNA replication proteins present in HSV-1 and adenovirus replication compartments are physically associated with the viral replication machinery. These host proteins could also be tested for functions in viral DNA replication. Also, simultaneous immunofluorescence and *in situ* hybridisation studies of SV40-infected cells could reveal any relationship between the SSB foci observed in these cells and sites of SV40 DNA accumulation.
References


185


192


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Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells

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Repl i ca tion of DNA occurs at discrete sites in eukaryotic cell nuclei, where replication proteins are clustered into large complexes, or 'replicases'.1-3. Similarly, viral DNA replication is a highly structured process, notably in herpes simplex virus type-1 (HSV-1; reviewed in ref. 4) in which large globular replication compartments containing the viral replication machinery exist. Replicating cellular DNA redistributes to these compartments upon HSV-1 infection. We have now used antibodies raised against several cellular proteins to detect changes in their subnuclear localization on HSV-1 infection. We found that various proteins involved in cellular DNA replication move to sites of viral DNA synthesis, whereas a selection of non-replication proteins do not. The retinoblastoma protein and p53 (the products of two putative anti-oncogenes6,7) relocate to the same sites as known DNA replication proteins, suggesting that they may be associated with DNA replication complexes in normal, uninfected cells.

Infected cell protein 8 (ICP8), the HSV-1 major DNA binding protein, is associated with viral DNA in replication compartments2, thus antiserum to ICP8 was used as a convenient marker for viral replication sites. Immunofluorescent double-labelling, using both confocal and conventional microscopy, showed that the cellular single-stranded DNA binding protein SSB (also known as R-f-A, or RPA) proliferating cell nuclear antigen (PCNA, an accessory factor to polymerase δ)3,11, the retinoblastoma protein (Rb) and p53 all colocalize with ICP8 in HSV-1-infected cells (Fig. 2a-d), in contrast to their granular distribution in the nuclei of uninfected cells (Fig. 1). The colocalization of SSB is complete, whereas p53 is found exclusively at certain sites and some Rb fails to enter the compartments.

The results are clearer when cells are infected in the presence of phosphonoacetate (PAA), a specific inhibitor of the HSV-1 DNA polymerase.12 ICP8 is now present in smaller, more numerous regions, called 'prereplicative sites'8. On PAA treatment, SSB is always found at these sites (Fig. 3a), whereas PCNA, Rb and p53 generally show only partial colocalization with ICP8 (Fig. 3b-d). This indicates that SSB may redistribute by a mechanism different from that used for PCNA, Rb and p53 relocation, or perhaps binding sites for the latter proteins become saturated. The extent of colocalization of these latter proteins varies from cell to cell (Fig. 3c-d shows particularly clear-cut examples of colocalization). Thus the accumulation of host proteins at these sites may occur in distinct stages.

Infection of serum-starved cells (5 days in 0.1% bovine serum) produced similar results, suggesting that redistribution of host proteins may occur during natural infections of resting cells. Control staining experiments showed no cross-reaction between the fluorescent conjugates used. Immunoblotting of total infected cell extracts showed that none of the antibodies used cross-reacted with HSV-1 proteins. In addition, the anti-ICP8 antibody did not cross-react with HSV-1 proteins. Small nuclear ribonucleoprotein particles (snRNPs) were visualized with antibody Y12 (from J. Steitz, Yale)38, and c-Myc was visualized with antibody 9E10. Antibody LN43 (from B. Lane) was used to detect lamin B, a structural nuclear protein. The nucleolar protein B23 was detected with a monoclonal antibody (from P. K. Chan, Texas Medical Center)42. Ki67 is also a nucleolar protein, present in proliferating cells43 (antibody from Immunotech, France).

The table shows particularly clear-cut examples of colocalization. Thus the accumulation of host proteins at these sites may occur in distinct stages.

| TABLE 1 Status of host proteins in cells infected with HSV-1 (no PAA treatment) |
|-----------------------------------------------|-----------------------------------------------|
| Colocalization with ICP8                      | No colocalization with ICP8                      |
| SSB                                           | snRNP                                         |
| PCNA                                         | c-Myc                                         |
| Rb                                            | lamin B                                       |
| p53                                           | p68                                           |
| DNA ligase 1                                  | nucleophosmin (B23)                           |
| p53 α                                        | KI67                                          |

Fig. 1 Cell staining of uninfected cells. Immunofluorescence pictures of monkey CV-1 cells (a, b, c) or a p53-overproducing breast cancer cell line, MDA-MB-468 (d). Cells stained with a, 70C, anti-SSB; b, PC4, anti-PCNA; c, IF/8, anti-Rb; d, PAN421, anti-p53.
Table 1 groups the proteins studied in terms of their distribution in infected cells. It can be seen that, at least on this basis, Rb and p53 behave as proteins associated with replication complexes. Although Rb, p53 and ICP8 colocalize, no physical association between the proteins was detected by western blotting, following immunoprecipitation, suggesting that any interaction must be either weak or indirect.

The redistribution of replicating cellular DNA on HSV-1 infection suggested that replication proteins might also be moved by the virus. Functional ICP8 is required for the redistribution of replicating cellular DNA, implying that this protein may be involved in moving replication complexes.

Whether some, or all, of the redistributed proteins play a role in HSV-1 replication is unclear. Seven viral genes essential for DNA replication have been identified by transient transfection studies and analysis of HSV mutants. These genes encode a DNA polymerase and accessory factor, ICP8, a three-subunit helicase-primase complex, and an origin-binding protein. Thus there seems to be an element of functional redundancy between the cellular and viral proteins in replication compartments. Perhaps some component of the replicase is targeted for transport by the virus (perhaps through ICP8) and as a result the whole complex redistributes to a replication compartment, where certain replication proteins may be used by the virus, while others remain only passively.

Recompartamentalization of nuclear proteins is also a feature of adenovirus replication, where certain host nucleolar proteins are moved to viral 'replication factories'. Both herpesvirus and adenovirus can act as helper viruses for minute virus of mice, which can normally replicate only in S-phase cells. The helper-virus function may be to make the host cell competent for replication by recompartamentalizing nuclear proteins.

Other proteins from DNA viruses interact with p53 and Rb; simian virus 40 (SV40) large T antigen, adenovirus Elb and papillomavirus E6 proteins all bind to p53, whereas large T, adenovirus E1a and papillomavirus E7 bind to Rb. It may be that the function of p53 and Rb needs to be altered for these viruses to overcome quiescence in the host cell, thus creating an environment in which viral replication can occur. This model relates to the proposed roles of p53 and Rb as tumour suppressors: elimination of p53 and Rb function may lead to a cell which permits permanent S phase, thus facilitating viral replication and tumour development. Dissection of the HSV-1 infection system may shed light on the functions and cellular targets of these two anti-oncogenes.

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FIG. 2 Double-labelling of HSV-1-infected cells. Confocal microscope pictures showing fluorescence in CV-1 cells (a, c, e) or in MDA-MB-468 cells (d), 5 h. after infection. Right-hand panels show ICP8 staining. Left-hand panels show: a, SSB; b, PCNA; c, Rb; d, p53; e, p68. a-d antibodies as for Fig. 1, e, biotinylated 204 antibody. Cells were infected with HSV-1 strain 17 syn + ref. 32, fixed in acetone-methanol, incubated overnight with polyclonal rabbit anti-ICP8, and the mouse antibody of interest, blocked with normal goat serum and incubated with appropriate rhodamine and fluorescein antibody conjugates. Scale bar, 25 μm (a, b, c) or 10 μm (d, e).
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Fig. 3 Double-labelling of cells infected in the presence of 400 μg ml⁻¹ PAA, 5 h after infection. Left panels: a, SSB; b, PCNA (PC2); c, Rb; d, p53; e, p68. Right panels show ICP8. Antibodies, cells and methods as described in Fig. 2. Scale bar, 25 μm (a, b) or 10 μm (c, d, e).