ASSEMBLY OF CELLULAR AND VIRAL PROTEINS INTO NUCLEOPROTEIN COMPLEXES IN ADENOVIRUS TYPE 2 INFECTED CELLS

Julia Bosher

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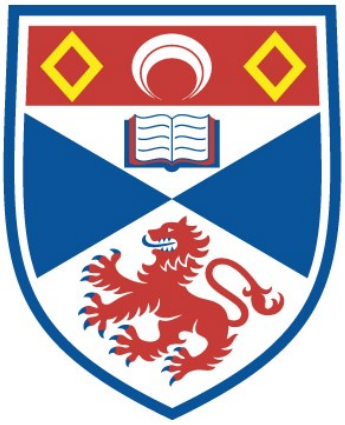

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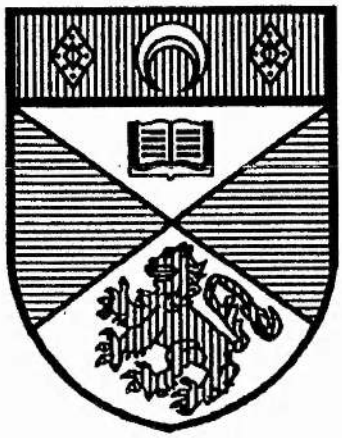

ASSEMBLY OF CELLULAR AND VIRAL PROTEINS INTO NUCLEOPROTEIN COMPLEXES IN ADENOVIRUS TYPE 2 INFECTED CELLS

A THESIS PRESENTED BY JULIA BOSHER FOR THE DEGREE OF DOCTOR OF PHILOSOPHY AT THE UNIVERSITY OF ST. ANDREWS

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DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY

SUPERVISED BY DR. R. T. HAY

DECLARATION:

I, Julia Margaret Bosher hereby certify that this thesis has been composed by myself, that it is a record of my work and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed ..... Date 15/3/93.....

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 on the 1st October 1989 and as a candidate for the degree of Ph.D on the 1st October 1989.

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PUBLICATIONS RESULTING FROM THIS WORK

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CONTENTS

ABBREVIATIONS

aa	amino acid(s)
Ad	adenovirus
ATP	adenosine-5'-triphosphate
bp	base-pair(s)
BSA	bovine serum albumin
BrdU	bromodeoxyuridine
Br-UTP	5-bromouridine-5'-triphosphate
Br-DNA	BrdU labelled DNA
Br-RNA	BrdU labelled RNA
Biotin-DNA	Biotin-16-dUTP labelled DNA
^{o}C	degrees celsius
Ci	curie(s)
CTP	cytidine-5'-triphosphate
Cys	cysteine
DBP	adenovirus DNA binding protein
dATP	2-deoxyadenosine-5'-triphosphate
dCMP	2-deoxycytidine-5'-monophosphate
dCTP	2-deoxycytidine-5'-triphosphate
dGTP	2-deoxyguanosine-5'-triphosphate
dTTP	2-deoxythymidine-5'-triphosphate
dUTP	2-deoxyuridine- 5'-triphosphate
DMSO	dimethlysulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double stranded
DTT	dithiothreitol
EDTA	ethylene-diamine tetra-acetic acid sodium salt
FITC	fluorescein isothiocyanate
g	gramme(s)
G-MEM	Glasgow modified Earle's minimal essential medium
GTP	guanosine-5'-triphosphate
h	hours(s)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
His	histidine
HSV	Herpes simplex virus

ITR	inverted terminal repeat
kD	1000-dalton molecular weight
kb	kilobase(s)
l	litre(s)
M	molar
µCi	microcurie(s)
mg	milligramme(s)
µg	microgramme(s)
min	minute(s)
ml	millilitre(s)
MLP	major late promoter
MTase	methyltransferase
µl	microlitre(s)
mM	millimolar
µM	micromolar
mRNA	messenger ribonucleic acid
m.u.	map units
M. W.	molecular weight
NFI	nuclear factor I
NFI_{FL}	full length nuclear factor I
NFI_{DBD}	DNA binding domain of nuclear factor I
NFII	nuclear factor II
NFIII	nuclear factor III
NHS	N-hydroxysuccinimide
ng	nanogramme(s)
nm	nanometre(s)
NP40	nonidet-P40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline type A
p.f.u.	plaque forming units
p.i.	post infection
PMSF	phenyl-methly-sulphonyl-fluoride
pol	adenovirus DNA polymerase
pTP	precursor terminal protein
pTP-pol	precursor terminal protein-DNA polymerase heterodimer
Rb	retinoblastoma gene product
RNA	ribonucleic acid

RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
sec	second(s)
SMBS	sodium metabisulphate
ss	single stranded
TBE	Tris-borate EDTA buffer
TEMED	N,N,N',N'tetramethylethylenediamine
TP	terminal protein
ts	temperature sensitive
TTP	thymidine-5'-triphosphate
UTP	uridine-5'-triphosphate
v/v	volume to volume ratio
w/v	weight to volume ratio

ABSTRACT

The adenovirus type 2 origin of DNA replication is located within the terminal 51 bp of the viral genome and contains three recognisable domains: the minimal origin or 'core' and binding sites for the cellular transcription factors Nuclear Factor I and Nuclear Factor III. Initiation of Ad2 DNA replication is preceded by the assembly of a nucleoprotein complex at the viral origin of DNA replication. Recombinant baculoviruses were previously constructed which express full-length Nuclear Factor I (NFI_{FL}) or its DNA binding domain (NFI_{DBD}) for use in experiments. DNase I footprinting experiments were carried out to examine the cooperative interaction between NFI and DBP. DBP can increase the binding affinity of both NFI_{FL} and NFI_{DBD} for their recognition site in the Ad2 origin of DNA replication. Mini-columns containing NFI_{DBD} covalently linked to CNBr-activated Sepharose were used to demonstrate that the adenovirus DNA polymerase (pol) interacts with NFI on its own and as part of the precursor terminal protein (pTP)-pol heterodimer.

Immunofluorescence experiments have shown that NFI is specifically targeted to sites of ongoing viral DNA replication in Ad2 infected cells but not in Ad4 infected cells. This localisation of NFI to discrete subnuclear sites reflects the requirement for NFI for efficient initiation of Ad2 DNA replication. The viral transcriptional activator E1a is also localised to discrete subnuclear sites which are very different from those of DBP and are cell cycle dependant. Antibodies to RNA polymerase II show that although its cellular distribution is also cell cycle dependant it is different from both DBP and E1a. When the sites of active transcription were labelled in Ad2 infected cells they were shown to be distinct from both the E1a and RNA polymerase II nuclear distribution.

INTRODUCTION

The main subject of this thesis is to investigate the formation and cellular location of a nucleoprotein complex at the Adenovirus Type 2 (Ad2) origin of DNA replication. The focal point is the role that the cellular transcription factor Nuclear Factor I (NFI) plays in the formation of this nucleoprotein complex which is involved in the initiation of Ad2 DNA replication.

ADENOVIRUSES

Adenoviruses were discovered during attempts to establish tissue culture lines from tonsils and adenoidal tissue from children by Rowe *et al.* (1953) when they realised that a new virus was causing the degeneration of the cells. Hilleman and Werner (1954) isolated a similar virus from cultured human tracheal cells whilst investigating respiratory illnesses in army recruits. Since then 47 distinct serotypes infecting humans have been recognised (Heirholzer *et al.*, 1991). Adenoviruses belong to the family Adenoviridae which is divided into two genera according to their natural hosts. The Mastadenovirus genus includes human, simian, bovine, equine, porcine, ovine, canine and opossum viruses whereas the Aviadenovirus genus includes fowl and turkey viruses.

Originally human adenoviruses were sub-group classified into four groups (I-IV) on the basis of their ability to attach to the erythrocyte receptor in rat or rhesus monkeys producing four hemagglutination patterns (Table 1). An alternative classification scheme (sub-groups A-F) was devised when it was discovered that Ad12 could induce tumours in hamsters (Trentin *et al.*, 1962) and was based on the oncogenicity in rodents of the various serotypes (Table 1).

Subgroup	Hemagglutination groups	Serotypes	Oncogenic potential: Tumors in animals	Oncogenic potential: Transformation in tissue culture	Percentage of G+C in DNA
A	IV (little or no agglutination)	12,18,31	High	+	48–49
B	I (complete agglutination of monkey erythrocytes)	3,7,11,14,16,21,34, 35	Moderate	+	50–52
C	III (partial agglutination of rat erythrocytes)	1,2,5,6	Low or none	+	57–59
D	II (complete agglutination of rat erythrocytes)	8,9,19,37,10,13,15, 17,19,20,22–30, 32,33,36,37,38, 39,42	Low or none	+	57–61
E	III	4	Low or none	+	57–59
F	III	40,41	Unknown		

TABLE 1. Classification schemes for human adenoviruses (Horwitz, 1990a).

Adenoviruses can infect and replicate at various sites of the respiratory tract as well as in the eye, gastrointestinal tract and urinary bladder. Most of the human disease is associated with only a third of the 47 recognised serotypes and many infections are asymptomatic resulting in protective antibody formation. Table 2 describes the more common illnesses associated with Ad infections.

Disease	Individuals most at risk	Principal serotypes
Acute febrile pharyngitis	Infants, young children	1, 2, 3, 5, 6, 7
Pharyngoconjunctival fever	School-age children	3, 7, 14
Acute respiratory disease	Military recruits	3, 4, 7, 14, 21
Pneumonia	Infants, young children	1, 2, 3, 7
Pneumonia	Military recruits	4, 7
Epidemic keratoconjunctivitis	Any age group	8, 11, 19, 37
Pertussis-like syndrome	Infants, young children	5
Acute hemorrhagic cystitis	Infants, young children	11, 21
Gastroenteritis	Infants, young children	40, 41
Hepatitis	Infants and children with liver transplants	1, 2, 5
Persistence of virus in urinary tract	Acquired immunodeficiency syndrome (AIDS), other immunosuppression, and bone marrow transplant recipients	34, 35

TABLE 2. Illnesses associated with adenovirus infections (Horwitz, 1990b).

VIRION ARCHITECTURE

Adenoviruses are nonenveloped, regular icosahedrons (20 triangular surfaces and 12 vertices) that are 65-80nm in diameter and can be divided into two major structural complexes called the capsid and the core (Valentine and Pereira, 1965; Brown *et al.*, 1975). The capsid consists of three viral proteins which make up the icosahedral shell and the core contains the linear double stranded (ds) DNA genome (approximately 35kbp) plus associated proteins (fig 1). The number of proteins contained within the virion has been variously estimated at between 11 and 15 (Maizel *et al.*, 1968; Weber *et al.*, 1977).

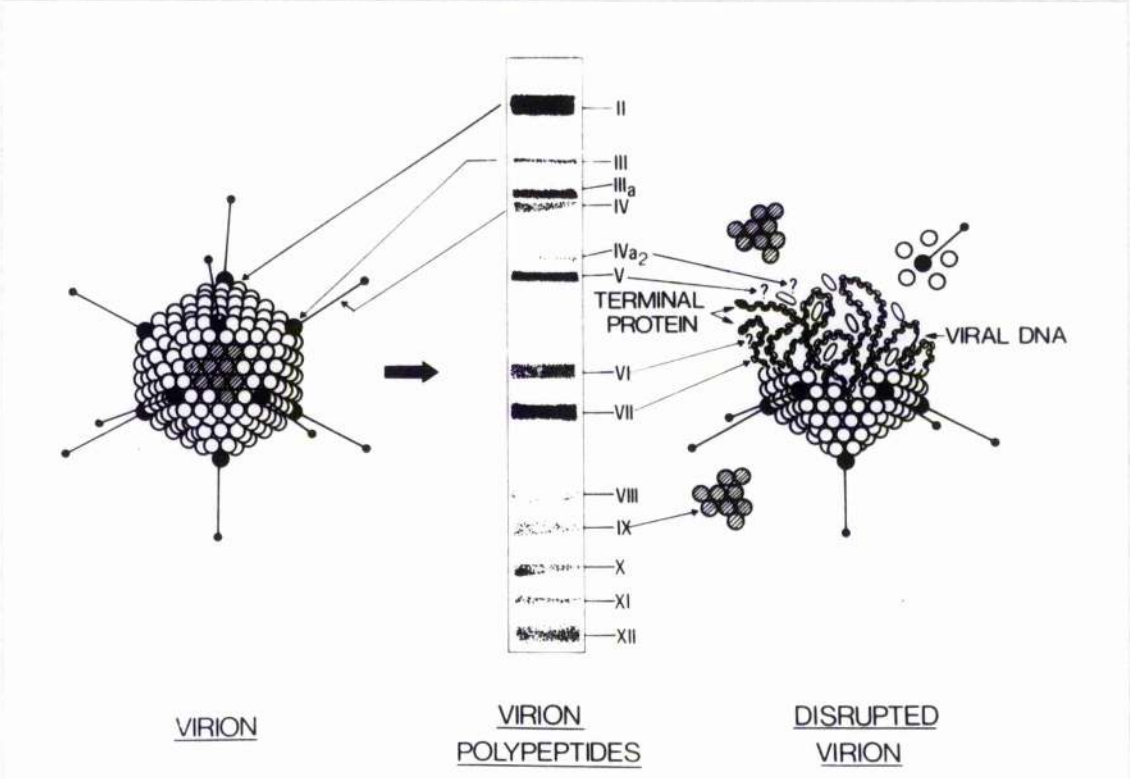


FIGURE 1. Schematic model illustrating the architecture of the adenovirus particle. The SDS-PAGE pattern of the virion polypeptides is shown along with their proposed locations (Russell and Precious, 1982).

THE VIRAL CAPSID

The capsid is composed of 252 subunits (capsomers) of which 240 are hexon protein and 12 are pentons which are located at the vertices of the icosahedron. The hexon capsomere is actually made up of three indentical subunits (Gruther and Franklin, 1974) which are held tightly together by noncovalent interactions.

The sequence of the Ad2 hexon protein reveals a chain length of 967 residues and a molecular mass of 109,058 (Akusjarvi *et al.*, 1984). Hexon was the first animal viral protein to be crystallised (Pereira *et al.*,1968) with the crystals produced having a tetrahedral shape. Subsequent X-ray crystallographic studies of hexon have revealed that it has a total height of 11.6nm (Burnett *et al.*, 1984) of which 5.2nm is a pseudo-hexagonal base which contains a central cavity and 6.4nm is a triangular top which comprises of three towers. Higher resolution X-ray crstallography (Roberts *et al.*, 1986) showed that the hexon trimer is formed from three copies each of two very similar pedestal domains called P1 and P2. Each pedestal domain consists of an eight stranded flattened ß barrel and has four loop structures projecting from it. Loops L1, L2 (from P1) and L4 (from P2) form one of the towers of the hexon trimer whereas L3 (from P2) does not protrude beyond the upper part of the base where it stabilises the interaction of the P domains within one subunit (fig 2). It is the three towers of the hexon trimer that contain the major antibody recognition sites which are important type specific antigenic determinants (Toogood *et al.*, 1992).

The penton capsomere consists of a wide penton base and a long protruding fibre with a distal knob (Laver *et al.*, 1969). The penton base is a pentamer of protein III (85kD) and has a diameter of approximately 9.5nm at its widest point and a height of approximately 11nm (Ruigrok

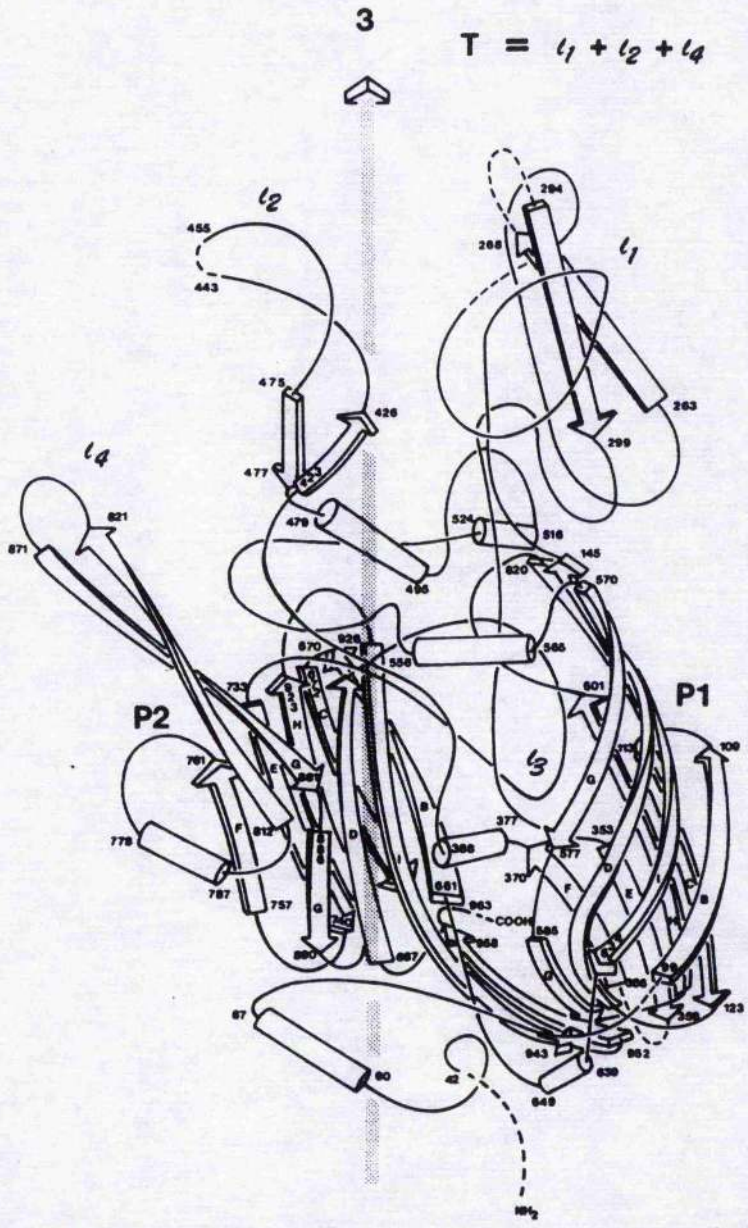


FIGURE 2. A sketch of the hexon subunit viewed from within the central cavity of the trimeric molecule. The bottom of the molecule lies toward the adenovirus capsid interior and the top forms its external surface. The secondary structure has been overemphasised by drastically reducing the lengths of the connecting loops with the gaps being indicated by dashed lines. The two domains P1 and P2 overlap through a very long β–strand forming hydrogen bonds with both domains. In each β–barrel, an α–helix precedes the first strand and an α–helix is followed by a tower loop between the fifth and sixth strands. These loops are L2 rising from P1 and L4 from P2 and both contain two antiparallel β–strands, those from L2 forming the β–constriction about the molecular axis. The tower domain T is formed from three loops (L1, L2, L4), each from a different subunit. The bulk of the tower is provided by L1, an excursion in P1 between the third and forth β–strands. Its counterpart in P2 is L3; although not as extensive , it is still the second longest connecting bridge of this barrel. Loop L3 is restricted to the upper part of the base, where it stabilises the interaction of the P domains within one subunit (Roberts *et al.*, 1986).

et al., 1990). The fibre is a trimer of the 62kD protein IV (van Ostrum and Burnett, 1985; Ruigrok *et al.*, 1990) the N-terminus of which non-covalently interacts with the penton base (Devaux *et al.*, 1987; Boudin and Boulanger, 1982). Green *et al.* (1983) showed that the N-terminal region of the Ad2 fibre consists of 43 residues followed by a shaft region of about 357 residues and then a C-terminal knob of 181 residues. The shaft region contains a 15 amino acid repeat of which there are 22 in Ad2 but only 6 in Ad3 each of which adds 0.132nm to the shaft length (Ruigrok *et al.*, 1990). The C-terminal knob like region contains the viral receptor-binding region and has a diameter of 4nm. Proteins IIIa (66kD), VI (24kD), VIII (13kD) and IX (12kD) are also known to be associated with the capsid.

THE CORE

The core consists of the linear double-stranded (ds) DNA genome (35937bp in Ad2) plus associated proteins (V, VII, μ and TP). The 55kD terminal protein (TP) is covalently linked to the dCMP residue at the 5' end of each strand of the DNA genome. The core proteins V (48.5kD) and VII (18.5kD) are basic proteins rich in arginine. VII accounts for approximately 14% of the viral protein and contains 21-23% arginine residues (Vayda *et al.*, 1983) which is sufficient to cover about 60% of the phosphate charges of the viral DNA. There are 1070 copies of VII and 180 copies of V in each core and they are referred to as the major and minor core protein, respectively (Russell *et al.*, 1968). The 4kD μ protein is also rich in arginine (54%) and histidine (13%).

Several models have been proposed to explain the detailed arrangement of the DNA and proteins within the cores. Cores can be isolated preparatively by extracting whole virions with several different agents including 0.5% deoxycholate, acetone, Sarkosyl or 10% pyridine.

Cores prepared by Sarkosyl extraction contain the viral DNA plus protein VII whereas those isolated by other methods contain protein V as well.

Cores prepared by Sarkosyl extraction and examined by electron microscopy (Brown *et al.*, 1975) led to the suggestion that they consist of twelve 21.6nm spheres which are found at the vertices of the capsid icosahedron. Newcomb *et al.* (1984) used ion etching to erode the virion capsid so that the core could be examined by electron microscopy. They also found that the virion core consists of 12 spheres that they called adenosomes which are positioned towards the vertices of the icosahedron capsid.

The Nucleosome Model suggests that the DNA is packaged in structures similar to cellular nucleosomes where well defined lengths of DNA (200bp) are complexed with an octamer of histones. Corden *et al.* (1976) used micrococcal nuclease (which cleaves between nucleosomes) to investigate the structure of the Ad core in pentonless virions. They found that the Ad genome was cut into 180 units of about 200bp and calculated that each DNA unit would be complexed with six copies of VII and one copy of V similar to cellular nucleosomes. Later experiments by Mirza and Weber (1982) involved electron microscopy on micrococcal nuclease digested pyridine cores. They proposed that 180 units are spaced at irregular intervals along the Ad genome each consisting of approximately 150bp of DNA wrapped around three dimers of VII and about 30bp of linker DNA interacting with one copy of V.

More recent experiments (Wong and Hsu, 1989) showed that the adenovirus virion DNA is organised in torsionally stressed, supercoiled loops. They digested pentonless cores with the nuclease Bal31 which has supercoiling-dependant endonuclease activity as well as extracting

Ad DNA from virus particles that had been crosslinked with psoralen. As a result they have proposed a loop-domain model where the DNA is organised into eight supercoiled loops which are anchored to the center of the virus core by proteins. The ends of the genome containing the two origins of replication and the promoters of E1a and E4 are not thought to be contained within supercoiled domains because of their insensitivity to Bal31 digestion (fig. 3).

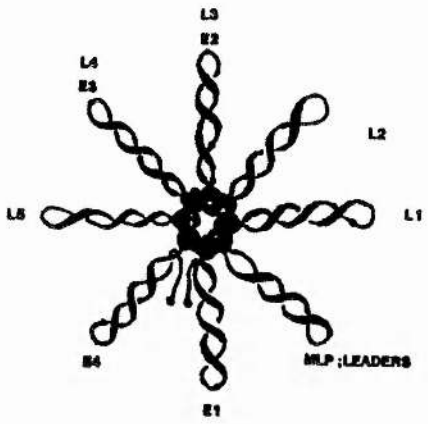


FIGURE 3. The Wong and Hsu (1989) loop domain model for the organisation of Ad5 DNA in the virus particles. E1-E4 represent Ad5 early genes, L1-L5 represent late genes and MLP is the major late promoter. DNA at the two ends of the Ad5 genome containing promoters of E1a and E4 as well as replication origins are shown outside of supercoiled domains because of their insensitivity to Bal31 digestion.

Futhermore Wong and Hsu (1990) have shown that these supercoiled loops are found in intracellular Ad DNA by the observation of compensatory supercoiling of portions of Ad DNA when the DNA double helix was unwound through the intercalation of ethidium bromide. Their observation that the packaging of Ad DNA into virus particles was inhibited by topoisomerase II inhibitors suggests that topoisomerase II maybe involved in core assembly.

LYTIC INFECTION

Adenovirus infection has a great effect on the host cells with cell DNA synthesis and protein synthesis being inhibited in adenovirus infected cells (Hodge and Scharff, 1969; Pina; Green, 1969). Lindberg *et al.* (1972) suggested that the inhibition of cell protein synthesis could in part be due to the depletion of cell mRNA from late cytoplasmic polyribosomes. However primary transcription of host cell sequences is still active in the nucleus of adenovirus infected cells and in fact this RNA makes up 80-90% of the nuclear transcripts (McGuire *et al.*, 1972; Price and Penman, 1972). It has been shown that suppression of cellular translation requires progression into the late phase of infection but does not involve alterations in cellular mRNA stability or translatability *in vitro* (Babich *et al.*, 1983). Adenovirus infection blocks the transport of cellular RNAs from the nucleus to the cytoplasm at late times after infection (Beltz and Flint, 1979) however this does not significantly reduce the level of most cell mRNAs although their translation is inhibited (Babich *et al.*, 1983). It is thought that the extremely effective inhibition of protein synthesis involves a translational control mechanism that discriminates between late viral and cellular mRNAs. Adenovirus late mRNAs all possess a common 200 nucleotide 5' noncoding region called the tripartite leader which enhances translation of most mRNAs (Logan and Shenk, 1984; Mansour *et al.*, 1986). The presence of the tripartite leader on mRNAs allows them to be translated without the critical initiation factor CBP (cap-binding protein) complex (eIF-4F) (Dolph *et al.*, 1988; 1990). Factor 4F functions as a cap-dependant RNA helicase required for translation of most capped RNAs so that independance from 4F activity would provide a means by which Ad late mRNAs can be distinguished from cellular and Ad early mRNAs. The CBP complex (eIF-4F) is composed of eIF-4A, eIF-4E

(CBP) and p220. Huang and Schneider (1991) demonstrated that the inhibition of the translation of cellular mRNAs and possibly adenovirus early mRNAs is a result of a decrease in the phosphorylation of CBP and the subsequent inactivation of the CBP complex.

Viral DNA synthesis begins at 6-9 hours after infection whereupon cellular DNA synthesis is gradually inhibited. One suggestion is that inhibition could be a side effect of cellular protein synthesis shut off. Alternatively Hodge and Scharff (1969) found that when Ad DNA synthesis was timed to start during G1 there was no cellular DNA synthesis in the following S phase whereas when Ad DNA synthesis was timed to start during S phase then cellular DNA synthesis was normal for many hours. This suggests that Ad infection inhibits the initiation of cellular DNA synthesis but can not interupt it. Certainly the cellular chromatin is visibly displaced within adenovirus infected nuclei to the margins of the nucleus, excluded from viral replication sites (Puvion-Dutilleul and Puvion, 1991).

CELL INVASION

Adenoviruses are internalised via receptor mediated endocytosis where the C-terminal head portion of the Ad fiber is thought to attach to specific receptors on the cell membrane (Londberg-Holm and Philipson, 1969). The virus migrates within the plasma membrane to clathrin coated pits which form endocytic vessicles or receptosomes. Receptosomes have an internal pH of 5.0 which causes a conformational change in the hexon protein so that hydrophobic regions are exposed (Everitt *et al.*, 1988) resulting in the rupture of the receptosome membrane on contact with the virions. The virus is then thought to be transported across the cytoplasm via an interaction between hexon and the microtubules (Dales and Chardonnet, 1973; Luftig and Weitung,

1975). At the nucleur pores uncoating of the virus is completed and the DNA plus closely associated proteins enter the nucleus whereupon adenovirus transcription can begin.

ADENOVIRUS TRANSCRIPTION

Human adenovirus genomes consist of linear ds DNA which range in size from 34300bp in Ad12 to 35937bp in Ad2 and although there is substantial sequence diversity between the serotypes the basic genetic organisation is the same. Figure 4 shows the positions of the major transcripts on the Ad2 genome which has been divided up into 100 map units (mu) to make describing their locations easier.

The infectious cycle can be divided into early and late phases with the dividing line being the onset of viral DNA replication which occurs at 6-9 hours post infection. The exact controls of this are not fully understood although Thomas and Mathews (1980) found that the switch mechanism is a cis-acting function of the viral DNA itself. The late and early phases can be further divided into immediate early (E1a); delayed early (E1b, E2a, E2b, E3, E4); intermediate (IX, IVa2) and late (L1-L5) phases. These are misleading descriptions of adenovirus transcription which really only indicate when the peak amount of a transcript is produced. Experiments using the protein synthesis inhibitor anisomycin (Lewis *et al.*, 1980; Shaw and Ziff, 1982) indicated the presence of two mRNA species that are independant of E1a function. These are a mRNA for a 13.5kD protein encoded between 17.0 and 21.5 map units and the L1 mRNA for the late 52-55kD protein encoded between 29 and 34 map units. Although L1 is considered a late gene in reality it is the earliest gene expressed and expressed at about the same level as other early genes although at late times post infection its expression is greatly increased.

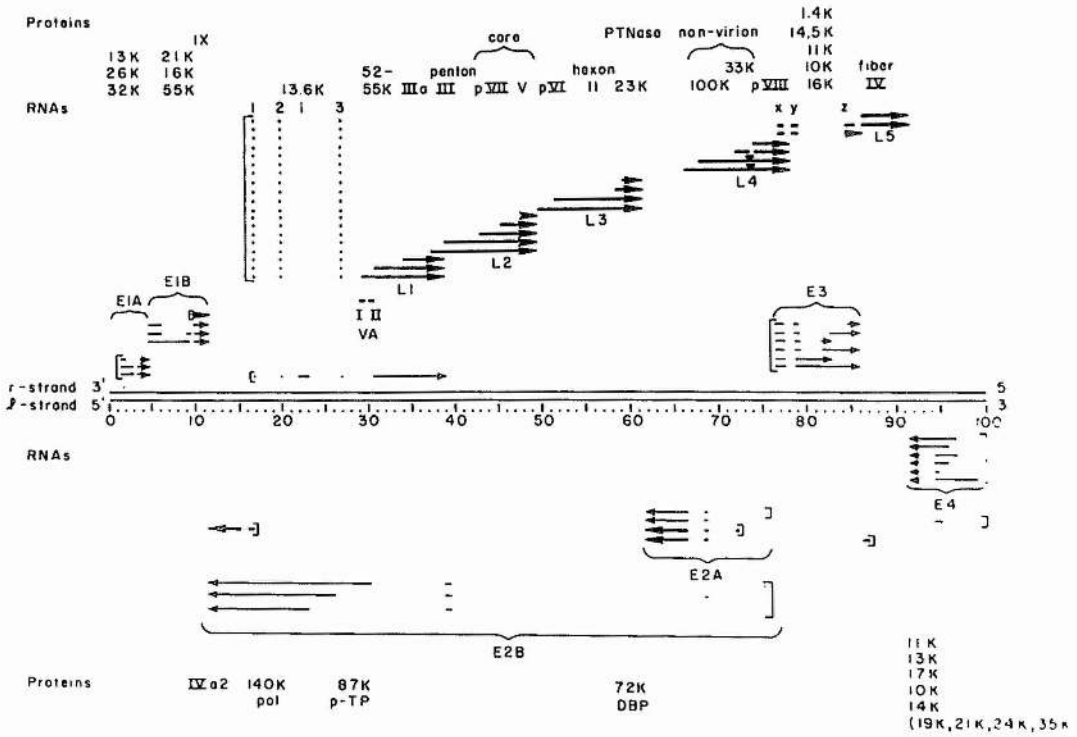


FIGURE 4. A transcription and translation map of the Ad2 genome. By convention the r-strand is transcribed from left to right and the l-strand is transcribed from right to left. The virion structural proteins are designated by roman numerals whereas the nonstructural proteins are designated in kiloDaltons (Horwitz, 1990a).

All the mRNA transcripts that code for polypeptides are synthesised by cellular RNA polymerase II and are capped and polyadenylated before being transported to the cytoplasm (Philipson *et al.*, 1971). However there are two small RNAs which have considerable secondary structure which are synthesised from the r strand at map units 28.8 and 29.5 by cellular RNA polymerase III. These are called virus associated (VA) RNAI and VA RNAII and are 155 (Ohe and Weissman, 1970) and 158-163 nucleotides long respectively. VA RNAI is required for efficient initiation of translation with protein synthesis being decreased 10 fold in cells infected with Ad mutants that do not have VA RNAI (Thimmappaya *et al.*, 1982). Mutants which do not have VA

RNAII show no such decrease but mutants which have neither VA RNA are 5-6 times more affected than those lacking VA RNAI alone (Bhat and Thimmappaya, 1982). This suggests that in the absence of VA RNAI VA RNAII is able to compensate in some way. VA RNAI is involved in blocking an interferon-induced mechanism of inhibiting protein synthesis after infection (Kitajewski *et al.*, 1986; O'Malley *et al.*, 1986) . Interferon induces higher levels of the cellular protein kinase DAI (for double stranded RNA-activated inhibitor) which phosphorylates the α-subunit of the initiating factor eIF2 thereby inhibiting protein synthesis. VA RNAI has been shown to bind directly to DAI thereby inhibiting its activation (Katze *et al.*, 1987; Galabru *et al.*, 1989).

Region E1

The E1 region is located on the r strand at 1.3-11.2 map units and can be subdivided into E1a at 1.3-4.5 map units and E1b at 4.6-11.2 map units. The E1 region codes for the transformation function of the adenoviruses so that in Ad2 E1a is required for the immortalisation of cells in tissue culture but E1b is required for full transformation (Ruley, 1983; Kelekar and Cole, 1987).

Five mRNAs are transcribed (commencing at about 45min post infection and reaching a maximum at 3-4 hours post infection) from the E1a region with sedimentation coefficients of 13S, 12S, 11S, 10S, and 9S (Nevins, 1987; Stevens and Harlow, 1987; Ulfendahl *et al.*, 1987). They are all (except the 9S transcript) translated in the same reading frame and have the same 5' and 3' ends but they are diffentially spliced (Perricaudet *et al.*, 1979; Boulanger and Blair, 1991). 13S and 12S are produced early and late in infection whereas 9S is only produced late in infection (Spector *et al.*, 1978). The primary translation products of 13S

and 12S are 289 and 243 amino acids long respectively and give rise to more than six polypeptide species (38-51kD) as a result of postranslational modifications (Richter *et al.*, 1988). The only difference between the 289 and 243 amino acid polypeptides is a 46 amino acid internal conserved region between amino acids 140-185 in the 289 amino acid species (Perricaudet, 1979). The 13S product contains three highly conserved domains and a nuclear localisation sequence at the C-terminus with only domain 3 being absent in the 12S product (Moran and Mathews, 1987). Mutations in domain 2 greatly reduce the ability of the E1a products to induce mitosis or proliferation of primary cells (Zerler *et al.*, 1987). The transformation function of E1a is located within domain 2 and although, for example, Ad12 and Ad5 can transform cells in tissue culture only Ad12 is oncogenic in animals. Bernards *et al.* (1983) and Schrier *et al.* (1983) used transfection with E1a segments from Ad5 or Ad12 in the presence of an active E1b gene to show that only the Ad12 E1a turns off the synthesis of class I major histocompatibility (MHC) polypeptides allowing the transformed cells to survive in the presence of cytotoxic T lymphocytes due to the down-regulation of MHC. Domain 1 seems to be involved in inducing DNA synthesis. Domain 3 which is unique to the 13S product is the primary transactivator of early viral genes (Moran *et al.*, 1986). E1a products are unable to bind directly to DNA (Ferguson *et al.*, 1985) and so must modify the activity of cellular transcription factors. One example of a cellular transcription factor involved in the E1a regulation of gene transcription is E2F. E2F undergoes a dramatic change in activity during adenovirus infection (Kovesdi *et al.*, 1986) which is mediated by E1a. The 19kD E4 gene product interacts with E2F enabling it to bind to the E2 promoter so that transcription can proceed (Huang and Hearing, 1989; Bagchi *et al.*, 1990; Raychaudhuri *et al.*,

1990). However the retinoblastoma gene product inhibits E2F DNA binding by interacting with it (Bagchi *et al.*, 1991) and thereby its transcriptional activity by interacting with it . E1a disrupts this interaction thereby freeing E2F to bind to the E2 promoter along with the 19kD E4 gene product resulting in a marked stimulation of transcription.

Transcription from E1b is transactivated from E1a at 1.5-2 hours post infection reaching a maximum at 6-7 hours post infection. A primary transcript is produced which is differentially spliced to give products 22S, 14.5S, 14S and 13S which all have identical 5' and 3' termini (Virtanen and Peterson, 1985). Three polypeptides are coded for with sizes 19kD, 20kD and 53-58kD. Both the 19kD and the 53-58kD proteins are involved in transformation along with E1a. The 19kD protein was found (White and Cipriani, 1989) to specifically disrupt both intermediate filaments and the nuclear lamina which could affect cell-cell attachment and growth regulation thereby promoting anchorage independant growth and tumorigenicity. The 19kD protein also has transactivating functions for E1a, E1b, E2, E3 and E4 as well as for the cellular heat shock protein promoter (hsp 70). When the 19kD protein is defective the host DNA is degraded and the Ad cytopathology is increased (White and Stillman, 1987). The 53-58kD protein binds to the cellular tumour suppressor protein p53 (Sarnow *et al.*, 1982) a mutated form of which is found in large amounts in transformed cells. The 53-58kD protein also interacts with the E4 34kD protein both of which are reqired for the efficient production of viral DNA, the expression of late viral genes and host cell shut-off (Halbert *et al.*, 1985).

Region E2

The E2 region is located on the l strand at 11.3-75.4 map units and can be divided into E2a at 61.5-75.4 map units and E2b at 11.3-75.4 map units from both of which transcription starts at 1.5-2 hours post infection and peaks at 6-7 hours post infection.

E2a codes for the 72kD DNA binding protein (DBP) which is a multifuctional phosphoprotein which binds specifically to ssDNA as well as to dsDNA although with a lower affinity. It is required for Ad DNA replication both *in vivo* and *in vitro* which will be discussed later. The N-terminus of DBP is not required for DNA replication but has been shown to have a role in the regulation of transcription (Klessig and Quinlan, 1982).

E2b codes for the 80kD precursor to the terminal protein (pTP) and the 140kD DNA polymerase (pol) both of which are required for DNA replication and will be discussed later.

Region E3

The E3 region is located on the r strand at 76.6-86 map units from which transcription commences ar 1.5-2 hours post infection and reaches a maximum at 3-4 hours post infection. At least six mRNA species have been identified (Ross *et al.*, 1980) coding for a number of proteins which are nonessential in tissue culture but are involved in modulating the host response to adenovirus infection. For example a 19kD protein binds to the MHC polypeptide heavy chain in the endoplasmic reticulum and prevents their transport to the cell surface thereby decreasing the target for recognition of infected cell antigens by cytotoxic T lymphocytes (Burgert *et al.*, 1987). Another example of how Ad by-passes the host's antiviral mechanisms is carried out by the 14.7kD protein which inhibits the lysis of Ad infected cells by tumour necrosis factor (Gooding *et al.*,

1988) also the 10.4kD protein binds to and thereby down regulates the epidermal growth factor receptor (Carlin *et al.*, 1989).

Region E4

The E4 region is located on the l strand at 91.3-99 map units and codes for a number of polypeptides, transcription commencing at 1.5-2 hours post infection and peaking at 3-4 hours post infection. As mentioned earlier the 34kD protein binds to the 53-58kD protein of E1b (Halbert *et al.*, 1985). The 19kD protein transactivates E2 transcription by binding to the cellular factor E2F thereby increasing its affinity for the E2a promoter (Neill *et al.*, 1990). The 11kD protein was found to bind to the nuclear matrix and may have a role in DNA replication, transcription or RNA processing (Sarnow *et al.*, 1982).

Intermediate Transcription

The IX and IVa2 genes are located at the ends of the E1b region and the E2b region respectively. Control of the intermediate transcripts is very complex since they are synthesised in large amounts at 6-8 hours post infection but are also synthesised in small amounts early in infection and late in infection (Horwitz, 1990).

Major Late Promoter (MLP)

The MLP is located on the r strand at 16.5 map units. It is active early in infection but transcription proceeds no further than map unit 39 which is at the end of the L1 region. At late times in infection transcription proceeds to map unit 99 through additional regions L2, L3, L4 and L5. All of the mRNAs initiated from the MLP possess a common 200 nucleotide 5' noncoding region called the tripartite leader which enhances their translation (see under Lytic Infection).

Regions L1-L5

The L1 region is located on the r strand at 29-39 map units. Three mRNA species are produced starting at map units 29, 30.7 and 34 and all ending at map unit 39. These code for three proteins; pIIIa which is a precursor of the structural component of the viral capsid protein IIIa and two phosphoproteins of 53kD and 55kD which are required for virion assembly.

The L2 region is located on the r strand at 39-50 map units. Three mRNA species are produced starting at map units 39.4, 44.1 and 45.9 and all ending at map unit 50. These code for a number of proteins including III (penton base), pVII which is the precursor of the core protein VII, V (core protein) and an 11kD protein which is the precursor of the core protein μ.

The L3 region is located on the r strand at 50-62 map units. Three mRNA products have been identified which code for the proteins pVI which is the precursor protein of VI, II (hexon) and the 23kD cysteine protease which cleaves the precursor proteins pVI, pVII, pVIII and pTP during maturation of the virion (Webster *et al.*, 1989).

The L4 region is located on the r strand at 66.5-77.3 map units. Four mRNA products have been identified which code for the proteins 100kD which is involved in the folding of hexon trimers (Gambke and Deppert, 1984), 33kD and pVIII which is the precursor of the hexon associated protein VIII.

The L5 region is located on the r strand at 86-91.3 map units. Two mRNA species are produced which code for protein IV (fiber).

ADENOVIRUS DNA REPLICATION

Adenovirus DNA replication is a highly structured process which takes place within nuclear globular 'replication compartments' and begins at between 6 and 9 hours post infection coinciding with a reduction in host DNA synthesis of more than 90% (Horwitz, 1990). As previously mentioned the human adenovirus DNA genome consists of linear dsDNA which range in size between 34300bp in Ad12 to 35937bp in Ad2. At each end of the genome there is an inverted terminal repeat (ITR, Garon *et al.*., 1972) which ranges in size from 63bp in chicken embryo lethal orphan virus (Alestrom *et al.*, 1982) to 165bp in human Ad 18 (Garon *et al.*, 1982). The group C viruses Ad2 and Ad5 have identical ITRs of 103bp and are indistinguishable from each other with respect to their DNA replication. At each end of the genome the 55kD terminal protein (TP) is covalently attached to the 5' end of each DNA strand (Robinson *et al.*, 1973; Challberg *et al.*, 1980) via a phosphodiester bond between the ß-OH of a serine residue in TP and the 5' phosphate group of the terminal dCMP (Desiderio and Kelly, 1981).

A MODEL FOR DNA REPLICATION

Some early studies investigating the mechanism of adenovirus DNA replication involved pulse-labelling the termini of replicating DNA with ^{3}H-thymidine and analysing the distribution of radioactivity in completed DNA molecules. Weingartner *et al.* (1976) determined that replication initiates at the 3' end of the parental template strand with each progeny strand being synthesised unidirectionally along its entire length possibly displacing the complementary parental strand. Wolfson and Dressler (1972) demonstrated that the Ad2 genome contains inverted terminal repeats (ITR) which allow the DNA to form a panhandle structure held together by hydrogen bonds between the two

ITRs when it is rendered single stranded which could have a role to play in DNA replication. Electron microscopy of adenovirus DNA pulse-labelled with ^{3}H-thymidine revealed two types of putative replicating DNA molecules (Lechner and Kelly, 1977). Type I consists of a duplex DNA molecule with one or more single stranded branches and type II consists of a duplex DNA molecule with a single stranded region extending from one end. They proposed that replication initiated at or near the ends of the adenovirus genome with progeny strand synthesis proceeding in the 5' to 3' direction displacing the complementary parental strand. Type I replicating molecules can be formed when one or more replication events are initiated at either or both ends of the DNA. Type II replication molecules can be formed in two ways one of which is when two oppositely moving replication forks meet resulting in separation of the parental strands. Alternatively type II replication molecules can be formed when replication is initiated at one end of the adenovirus genome and is completed before replication is initiated from the other end resulting in the non-template stand being displaced so that its replication is initiated from its 3' end.

When viral DNA was isolated using extraction procedures that did not contain proteolytic enzymes it was found to be circular or oligomeric. These complexes were disrupted with proteases or SDS to give the usual linear duplex formation leading to the conclusion that a protein is attached to each end of the viral genome (Robinson *et al.*, 1973). Rekosh *et al.* (1977) demonstrated that this protein (Terminal Protein, TP) is 55kD in size and is covalently attached to the 5' ends of each DNA strand. They proposed a novel protein priming model for DNA replication where a newly synthesised molecule of TP binds to a region near the 3' end of the parental strand. Then , either before or after binding, it becomes covalently attached to the 5' terminal deoxycytidine

of the progeny strand and acts as a primer for DNA replication. This model was modified when it was discovered that an 80kD protein is covalently attached to the 5' ends of the progeny DNA rather than the 55kD TP (Challberg *et al.*, 1980). They suggested that this 80kD protein is a precursor of TP (pTP) which is cleaved during virion maturation. The primary initition event is the formation of a covalent linkage between the α-phosphoryl group of the 5' terminal residue dCTP and the β-OH group of a serine residue in pTP. The 3' OH group in the pTP-dCMP complex then acts as a primer for synthesis of the progeny strand by pol which proceeds displacing the parental strand.
The displaced strand can either form a panhandle structure via its ITRs thereby recreating the adenovirus replication origin from which replication can be initiated (Stow, 1982; Hay *et al.*, 1984) or alternatively there is evidence that initiation can take place on a single stranded template (Harris and Hay, 1988; Kenny and Hurwitz, 1988) without the necessity of the panhandle structure formation.

REPLICATION *IN VITRO*

In order to study the component requirements of initiation of adenovirus DNA replication the development of an *in vitro* initiation of DNA replication system was highly desirable. The first such *in vitro* system was developed by Challberg and Kelly (1979) and consisted of Ad DNA covalently linked to TP as a template plus a soluble extract from the nuclei of Hela cells infected with Ad5. Synthesis of progeny strands were initiated at the termini of the input template and elongation proceeded in a 5' to 3' direction at a rate comparable to that *in vivo* resulting in the formation of type I replicative intermediates. To further define the requirements for DNA replication active components from the system had to be purified and characterised. Initially it was recognised

that the nuclear extract of Ad5 infected Hela cells could be replaced by cytoplasmic extracts of Ad infected cells plus nuclear extracts from uninfected cells (Ikeda *et al.*, 1980). This indicated that cellular components were required for Ad5 DNA replication as well as viral ones. It is now known that three virally encoded proteins (DBP, pTP and pol) and two cellular proteins are required for efficient Ad2 and Ad5 DNA replication (reviews Hay and Russell, 1989; Salas, 1991).

ORIGIN OF DNA REPLICATION

In vivo experiments to define the minimum requirement for the origin of DNA replication were carried out (Hay *et al.*, 1984; Hay, 1985) whereby linear plasmid molecules containing copies of the Ad2 ITR were introduced into cells along with helper virus. By deletion analysis it was shown that the terminal 45bp of the Ad2 genome were required for viral DNA replication. This was confirmed when a series of virus genomes were constructed with deletions in the left ITR (Hay and McDougall, 1986) and it was found that those viruses containing the terminal 45bp were fully infectious whereas those containing the terminal 36bp or less were not.

In Ad2 *in vitro* experiments allowed the origin of DNA replication to be divided into three domains, contained within the terminal 51bp of the viral genome, which each contribute to replication efficiency (fig. 5). Each of these domains contains the binding sites of viral and cellular proteins which assemble into a nucleoprotein complex at the origin of replication. The terminal 18bp is referred to as the 'core' of the origin of DNA replication and contains a 10bp sequence (9-ATAATATACC-18) which is perfectly conserved in all human adenoviruses sequenced to date (Tamanoi and Stillman, 1983; Challberg and Rawlins, 1984). In Ad2 this core region is absolutely required for

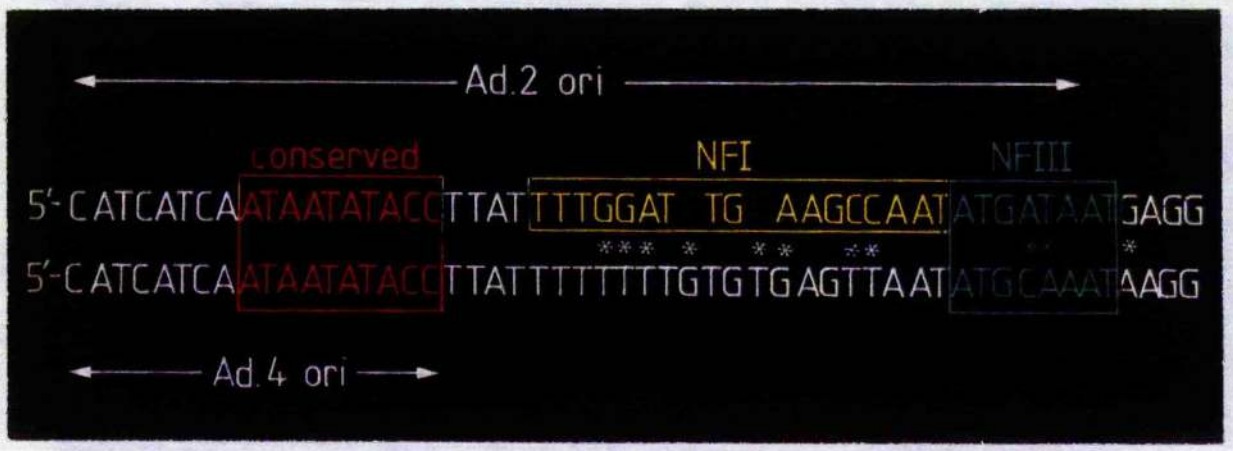


Figure 5. The origin of DNA replication of Ad2 and Ad4 showing the conserved core region and the NFI and NFIII recognition sites.

DNA replication but in isolation can only support a limited level of replication. Temperley and Hay, (1992) showed that the conserved region of the core is the binding site of the pTP-pol heterodimer. The second domain lies between bp 19 and 39 and contains the recognition sequence of the cellular protein Nuclear Factor I (NFI). *In vitro* experiments have shown that initiation of Ad2 DNA replication is stimulated 10 fold in the presence of NFI (Nagata *et al.*, 1982). *In vivo* experiments already mentioned demonstrate that if deletions are made into the NFI binding site then Ad2 DNA replication is abolished. Transfection experiments where a series of plasmids were constructed where 0.5, 1. 1.5. and 2 turns of the DNA helix were inserted between the NFI binding site and the core region of Ad2 were carried out (Bosher *et al.*, 1990). Plasmids containing any of the inserted DNA sequence replicated only as well as plasmids containing the core region only demonstrating that the position of NFI in relation to the core is very important for efficient Ad2 DNA replication. The third domain lies between bps 40 and 51 and contains the recognition sequence of another cellular protein Nuclear Factor III (NFIII). The requirement for NFIII in

Ad2 and Ad5 DNA replication is at present ambiguous since although *in vitro* experiments have shown that the addition of purified NFIII stimulates DNA replication 4-6 fold (Pruijn *et al.*, 1986) *in vivo* experiments have demonstrated that only the terminal 45bp are required for Ad2 DNA replication (Hay, 1985; Hay and McDougall, 1986). More recently Coenjaerts *et al.* (1991) created mutant origins and demonstrated that the insertion of two or more base pairs between the NFI and NFIII binding site abolished stimulation of replication by NFIII indicating that NFIIIs role in Ad2 replication is position dependant. Since the stimulation of Ad2 DNA replication by NFIII is independant of NFI (Mul *et al.*, 1990) it suggests that the distance between it and the core region is critcal. The roles of NFI and NFIII will be discussed in more detail in a later section.

Unlike Ad2 Ad4 only requires the core 18bp for efficient initiation of DNA replication (Hay, 1985). It does not have an NFI recognition site within its ITR and addition of purified NFI to an *in vitro* replication assay does not stimulate Ad4 initiation of DNA replication. Although Ad4 does have an NFIII recognition site within its ITR it is not required for efficient initiation of DNA replication either *in vivo* (Hay, 1985) or *in vitro* (Harris and Hay, 1988).

VIRAL REPLICATION PROTEINS

Three viral proteins are required for efficient initiation of adenovirus DNA replication. These are the DNA binding protein (DBP), the DNA polymerase (pol) and the preterminal protein (pTP).

DNA Binding Protein

DBP is a multifunctional phosphoprotein (Russell and Blair, 1977) that is coded for by the E2a region. It is produced in very large

amounts (up to $5x10^6$ molecules per cell) early in infection and was first isolated by van der Vliet and Levine (1973) when they fractionated Ad infected and uninfected cell extracts by DNA-cellulose affinity chromatography. They originally purified two forms of DBP of sizes 44kD and 72kD the smaller of which was later found to be a proteolytic product of the larger. DBP is absolutely required for elongation of Ad DNA (Friefeld *et al.* 1983) and has been shown using electron microscopy to be bound to ssDNA actively engaged in Ad DNA replication (Kedinger *et al.* 1978). One of the roles DBP plays in adenovirus DNA replication is to protect the non-replicated displaced strand from nuclease attack for which it can be replaced by other ssDNA binding proteins such as *E. coli* SSB (Lindenbaum *et al.*, 1986). Using the H5ts107 DBP mutant Krevolin and Horwitz (1987) showed that DNA binding function was not sufficient to ensure that DNA replication would occur indicating that DBP may interact with another replication component. DBP stimulates poly(dA) synthesis by pol I 10-100 fold resulting in the synthesis of poly(dA) chains greater than 30kb suggesting that in the presence of DBP pol can synthesise the complete adenovirus genome (Field *et al.*, 1984). Using poly(dT).oligo(dA) templates Lindenbaum *et al.* (1986) showed that DBPs role in increasing the processivity of pol was independant of its ability to bind to ssDNA and can not be substituted by other ssDNA binding proteins. They suggested that a DBP-pol cooperative complex is capable of synthesising the entire adenovirus genome in a single binding event resulting in the displacement of the non-replicated strand. As yet no direct interaction between DBP and pol has been demonstrated.

DBP was originally classified as a single strand specific DNA binding protein but it is now known to bind to double stranded DNA as well. DBP has only a slightly higher affinity for ssDNA than it does for

dsDNA but its dissociation from dsDNA is rapid whereas the complex with ssDNA is much more stable (Stuiver *et al.*, 1992) which indicates the possibility that two different binding methods are involved. Circular dichroism and optical density measurements have been used to study DBP binding to ssDNA and RNA. Various templates have been used and the size of the DNA-binding site has been found to range from 10 nucleotides for M13 DNA to 10-11 nucleotides for poly(dA) to 13 nucleotides for poly(rA) to 15 nucleotides for poly (dT) (van Amerongen *et al.*, 1987; Kuil *et al.*, 1989). The secondary structure of the DBP-ssDNA complex indicates that there is an increase in the length of the polynucleotides (as a result of a decrease in the rotation per base and/or the bases move closer to the helix axis) and a strong tilt of the bases as a result of DBP binding (van Amerongen *et al.*, 1987). The structure of the DBP-dsDNA complex was also studied using circular dichroism (Stuiver *et al.*, 1992) which revealed distortions in base-to-base positions of the DNA possibly reminiscent of those found in the DBP-ssDNA complex. Hydroxyl radical degradation confirmed that DBP distorts the DNA at the level of base-pair positions by the altered susceptibility of sugar residues to hydroxyl radical degradation (Stuiver *et al.*, 1992). These DBP induced distortions of the DNA may explain how DBP increases the processivity of pol since the helical distortions may reduce the energy required to separate the DNA strands at the replication fork.

The functional domains of DBP were mapped by chymotryptic and tryptic partial digests of the purified protein that had been extensively labeled *in vivo* with [^{32}P]orthophosphate. Friefeld *et al.* (1983) found that a 34kD chymotryptic peptide from the carboxy end was completely functional for Ad DNA elongation and unlike the N-terminal region it is not phosphorylated suggesting that phosphorylation

is not involved in DNA replication. Krevolin and Horwitz (1987) showed that the 34kD C-terminal domain of DBP is able to bind to ssDNA and that it is functional in *in vitro* DNA replication assays. The C-terminal domain of a number of adenovirus serotypes contain four conserved regions at positions 178-186 (CR1), 322-330 (CR2) and 464-475 (CR3) in the Ad5 sequence and position 273-286 in the Ad2 sequence which may be involved in DNA binding (Vos *et al.*, 1988; Neale and Kitchingman 1990). The conserved region between amino acids 273 and 286 consists of a symmetrical arrangement of two Cysteine (Cys) and two Histidine (His) residues. This $HXCX_8CXH$ motif shows some similarity to several proposed metal-binding sites and since it contains two out of the three conserved His. residues and two ts mutants are located between the two Cys-His pairs it seems likely that it is (Vos *et al.*, 1988). This was confirmed when DBP was shown to bind specifically to Zn^{2+} even in the presence of other divalent ions such as Ca^{2+}, Mg^{2+}, Cd^{2+}, Co^{2+} and Mn^{2+} (Eagle and Klessig, 1992). Mutants were constructed which had the region 273-286 deleted or a point mutation of Cys. to Ser at position 284. The deletion mutant was unable to bind zinc and ssDNA-cellulose and the point mutant was only able to bind zinc and ssDNA-cellulose with decreased ability (Eagle and Klessig, 1992). Cleghon and Klessig (1992) used limited proteolysis followed by ssDNA-cellulose chromatography and amino acid sequence analysis to isolate the ssDNA fragment of DBP. They obtained a fragment af 35kD which consisted of amino acids 153 to approximately 470. Previously Tsernoglou *et al.* (1985) isolated a chymotrytic fragment of amino acids 174 to 525 which is able to substitute for native DBP in *in vitro* DNA replication assays. Combining this information suggests that the DNA binding domain of DBP is localised to amino acids 174 to approximately 470 which contains all four of the conserved

regions. DBP was photochemically cross-linked to the oligonucleotide $p(dT)_{14}$ so that the contact points between DBP and ssDNA could be identified (Cleghon and Klessig, 1992). These were found to be methionine 299 and phenylalanine 418. Methionine 299 is only 13 amino acids away from the zinc-binding region which could be involved in maintaining the proper local conformation to allow mehionine 299 to make contact with the DNA (Eagle and Klessig, 1992). Phenylalanine 418 contact with ssDNA supports the idea that aromatic residues are involved in binding to ssDNA by the stacking of aromatic residues between the nucleic acid bases (Cleghon and Klessig, 1992). It is also positioned between the conserved regions CR2 and CR3 which have both been shown to be involved in DNA binding (Neale and Kitchingman, 1990).

In the past there was some controversy over whether or not DBP has a stimulatory affect on initiation of DNA replication. Early work involving the DBP ts mutant H5ts125 found that nuclear extracts of infected cells grown at the non-permissive temperature were blocked in elongation but allowed the formation of the pTP-dCMP intiation complex suggesting that DBP is not required for initiation. Some workers have failed to demonstrate that addition of DBP to *in vitro* initiation assays results in stimulation (Rosenfeld *et al.*, 1987) whereas others have demonstrated just such a stimulation (Kenny and Horwitz, 1988). The confusion seems to be centred on the amount of NFI used in the assays. Cleat and Hay (1989) and Stuiver and van der Vliet (1990) showed that DBP can stimulate the frequency of initiation of Ad2 DNA replication when NFI is present in non-saturating amounts. DBP increases the affinity NFI has for its recognition site (Cleat and Hay, 1989) probably by altering the DNA structure so that base-specific contacts by NFI are brought into better alignment (Stuiver and van der

Vliet, 1990; Stuiver *et al.*, 1992). An added complication involves Ad4 which does not require NFI for efficient initiation of DNA replication suggesting that DBP would have no effect on initiation. However Ad4 DBP has been found to stimulate initiation of Ad4 DNA replication in *in vitro* assays (Temperley and Hay, 1991) indicating that DBP stimulation is not confined to increasing NFI binding.

Adenovirus DNA Polymerase

Initially polymerase activity, that was distinguished from those of HeLa cell DNA polymerases α, β and γ, was copurified with preparations of pTP from adenovirus infected HeLas (Enomoto *et al.*, 1981). It resembled DNA polymerase α in its sensitivity to N-ethlymaleimide, NaCl and cytosine β-D-arabinofuranoside-5'-triphosphate but unlike polymerase α it proved to be insensitive to aphidicolin all of which suggested that adenoviruses code for their own DNA polymerase. It was ascertained that the 80kD pTP forms a very tight heterodimer with a protein of 140kD which requires glycerol gradient sedimentation in the presence of 1.7M urea to be broken (Lichy *et al.*, 1982). This 140kD protein is the adenovirus DNA polymerase which is coded for by the E2b region which is expressed early in infection reaching a maximum at 6-7 hours post infection just as Ad DNA replication commences. Shu *et al.* (1987) cloned and expressed full length active pol and found that the HindIII-J fragment that encodes the exon at map position 39 (shared with pTP) is required together with the DNA fragment from 24.7 to 9.2 map units which contains an open reading frame for a protein of 120kD.

A unique function of pol is its ability to use pTP as a primer for DNA replication when it catalyses the transfer of dCMP directly to the pTP (Lichy *et al.*, 1982). Another distinctive property of pol is its DBP

dependant elongation reaction on poly(dT).oligo(dA) template primers (Field *et al.*, 1984; Lindenbaum *et al.*, 1986). DBP stimulates the poly(dA) chain synthesis 10-100 fold and ATP further stimulates it 3-10 fold. Under optimal conditions pol is a highly processive enzyme which can synthesise poly(dA) chains greater than 30kbp in length, that is, it is capable of synthesising DNA the size of the Ad genome. Added to this it also has a 3'-5' exonuclease activity (Field *et al.*, 1984) which could be associated with the high fidelity of DNA synthesis by removing nucleotides which are misincorporated.

Chen and Horwitz (1989) constructed various 16 linker-insertion Ad pol mutants to investigate whether the various pol activities are segregated into functional domains within the whole protein. They found that this is not the case and therefore propose that some of the separate regions on the linear Ad pol molecule interact to produce active sites that act as functional domains for the properties of pol. The Ad2 pol sequence contains two potential zinc finger motifs (Cys-His-rich sequences) which may be involved in DNA binding (Chen *et al.*, 1990). The N-terminal (amino acids 228-256) proposed motif consists of COYC$(aa)_{20}$HINSH and the C-terminal (amino acids 1056-1080) proposed motif consists of CGAC$(aa)_{22}$HCPSC. Several point and linker insertion mutants in these regions were expressed and analysed (Joung and Engler, 1992) *in vitro* for any effects on pol function. Mutants in the C-terminal region had a great effect on DNA synthesis, DNA binding and *in vitro* initiation. Mutants in the N-terminal region had a moderate effect on DNA synthesis and elongation but failed to make dCMP-pTP initiation complexes or to bind to DNA. They suggest that pol has two DNA binding domains: the C-terminal domain is responsible for binding to DNA primer-template complexes whereas the

N-terminal domain is involved in the recognition of adenovirus origin sequences.

Terminal Protein and Preterminal Protein

The 80kD pTP is coded for by the E2b region of the Ad genome early in infection, reaching a maximum at 6-7 hours post infection just as Ad DNA replication commences. pTP is specifically cleaved to the 55kD TP via a 62kD intermediate by the Ad 26kD cysteine protease during maturation of the virion (Webster *et al.*, 1989). As already mentioned TP is covalently linked to the 5' end of each DNA strand of the Ad genome via a phosphodiester linkage between serine residue 580 and the first nucleotide dCMP (Robinson *et al.*, 1973; Smart and Stillman, 1982). The exact role of TP in adenovirus DNA replication is still unclear. Originally it was demonstrated that the attachment of TP to the Ad DNA greatly increases its infectivity (Sharp *et al.*, 1976) possibly by preventing nucleolytic degradation. In addition Ad DNA has been found to bind to the nuclear matrix of the host via TP (Schaack *et al.*, 1990; Bodnar *et al.*, 1989). In addition Schaak *et al.* (1990) found that tight binding of TP to the DNA is correlated with efficient transcription probably as a result of its attaching the Ad genome to the nuclear matrix. Template DNA attached to TP can support levels of DNA replication *in vitro* up to 20 fold higher than template in isolation (Tamonoi and Stillman 1982; van Bergen *et al.* 1983; Challberg and Rawlins 1984; Harris and Hay 1988). It has been suggested that this may in part be due to an interaction with pTP thereby positioning the pTP-pol heterodimer at the origin of replication and indeed band-shift assays have shown that TP-DNA binds pTP-pol more efficiently than TP free DNA (Pronk and van der Vliet, in preparation; cited in Pronk *et al.*, 1992). Also TP may be involved in initial strand unwinding (Kenny *et al.* 1988) since in TP

free templates there has to be a single stranded region at the end or the 5'-3' exonuclease activity or Factor pL is required for *in vitro* replication. Lichy *et al.* (1981) demonstrated that the pTP of the pTP-pol heterodimer is the primer for Ad DNA replication when a covalent complex is formed between the ß-OH of a serine residue in pTP and the phosphate of dCMP.

CELLULAR REPLICATION PROTEINS

Two cellular proteins are required for efficient initiation of Ad2 and Ad5 DNA replication. These are the cellular transcription factors Nuclear Factor I (NFI) and Nuclear Factor III (NFIII). In addition Nuclear Factor II (NFII) is required for complete elongation and Factor pL is required in some *in vitro* experiments..

Nuclear Factor I

Ikeda *et al.* (1980) demonstrated that the nuclear extract of Ad5 infected HeLa cells required by Challberg and Rawlins (1979) in their Ad5 DNA replication assay could be replaced by cytoplasmic extracts of Ad5 infected cells plus nuclear extracts from uninfected cells. This indicated that Ad5 requires at least one cellular component for efficient DNA replication. Nuclear Factor I (NFI) was isolated, characterised and named by Nagata *et al.* (1982) from extracts of uninfected nuclei. This apparent 47kD protein stimulates pTP-dCMP formation in Ad DNA replication assays and this NFI mediated stimulation is itself stimulated in the presence of DBP and/or ATP.

NFI was (Nagata *et al.*, 1983a) found to bind selectively to base pairs 17-48 near the terminus of the Ad5 DNA. Leegwater *et al.* (1985) further clarified the NFI recognition site as being between base pairs 24 and 39/40 and showed that the stimulation of Ad DNA replication is

dependant on NFI binding to its recognition site and that binding is affected by the DBP concentration. DBP stimulates Ad2/5 DNA replication via its affect on NFI binding by increasing the affinity NFI has for its recognition site. It also increases the association rate and decreases the dissociation rate of NFI for its recognition site with the overall effect being that when NFI is in non-saturating amounts its recognition site occupancy is optimised at any given moment in the presence of DBP (Cleat and Hay, 1989). The NFI recognition site is adjacent to the core of the origin of DNA replication (bp 1-18) and for optimal activity *in vitro* the relative postions of the core region and the NFI recognition site is critical (Adhya *et al.*, 1986). NFI maximally stimulates pTP-dCMP formation when the DNA template contains the intact Ad2 or Ad5 origin. A reduced level of stimulation is still obtained when up to 2bp are removed or 3bp added to the origin between the core and NFI site but if there are any more deletions or additions NFI stimulation is abolished. Adhya *et al.* (1986) also found that the orientation of the NFI site had no effect on stimulation of DNA replication suggesting that NFI binds DNA in a symmetrical manner, for example, as a dimer. In contrast to Ad2 and Ad5 Ad4 does not have an NFI recognition site adjacent to the core region in its origin of DNA replication. Ad4 replication proteins are able to replicate Ad DNA efficiently and so do not require stimulation from NFI (Hay, 1985).

Jones *et al.* (1987) made the discovery that purified NFI and purified CTF (CCAAT-binding transcription factor), which are actually both families of proteins of sizes 52-66kD, are indistinguishable from each other in DNA binding properties, immunological cross-reactivity and *in vitro* stimulation of DNA replication and transcription initiation. They concluded that the terms NFI and CTF refer to the same family of proteins which stimulate RNA polymerase II transcription and initiation

of Ad2/5 DNA replication via their binding to DNA control elements that contain the sequence motif GCCAAT. In reality the presence of a CAAT motif does not automatically result in strong NFI binding which seems to require the presence of a TGG(C/A) motif and a GCCAA motif separated by 6bp (Jones *et al.*, 1987; Gronostajski, 1987). Site directed mutagenesis of the consensus sequence reduces ar abolishes NFI binding and mutagenesis of sequences just outside it modulates binding affinity (de Vries *et al.*, 1985; Leegwater *et al.*, 1985). Detailed contactpoint analysis of HeLa NFI (de Vries *et al.*, 1987) revealed that all but one of the contacts are accessible from one side of the helix and are made within two successive turns of the major groove with the phosphate backbone and G and T residues.

The family of proteins (52-66kD) which makes up NFI/CTF are thought to originate from a single gene in human cells which gives rise to at least three distinct mRNA species as a result of alternative splicing (Santoro *et al.*, 1988; Meisterernst *et al.*, 1989). Various clones of NFI (Santoro *et al.*, 1988; Meisterernst *et al.*, 1989) have been expressed and found to have virtually indistinguishable DNA binding activities and to stimulate initiation of Ad2/5 DNA replication *in vitro* ten fold much like HeLa NFI does. Mermod *et al.* (1989) mutagenised the cDNA encoding for the largest NFI protein (CTF-1) and identified the domains that are responsible for DNA binding, dimerisation and stimulation of Ad DNA replication. The 185 N-terminal amino acids of related NFI factors are highly conserved whereas the amino acids of the C-terminal half show significant divergence (Mermod *et al.*, 1989; Meisterernst *et al.*, 1988; Paonessa *et al.*, 1988). The N-terminal region is sufficient for site-specific DNA recognition, protein dimerisation and adenovirus replication whereas transcriptional activation requires the additional proline rich C-terminal region (Mermod *et al.*, 1989). Possibly the

proline residues are involved in specific interactions with other factors that play a role in the initiation of transcription but are not required for DNA binding or Ad DNA replication.

Nuclear Factor III

Pruijn *et al.* (1986) purified a cellular protein (NFIII) that stimulates initiation of Ad2 DNA replication 4-6 fold *in vitro* and has a recognition site in the Ad2 origin of DNA replication between nucleotides 36 and 54. Since NFI's recognition site is between nucleotides 24 and 39/40 (Leegwater *et al.*, 1985) it appears that NFI and NFIII can partially overlap each other on the Ad2 origin when they bind simultaneously (Pruijn *et al.*, 1986). Both proteins have contacts with nucleotide 39 for example (Pruijn *et al.*,1988) with NFI contacting the T base in the major groove and NFIII contacting the complementary A base in the minor groove as well as the phosphate backbone. *In vitro* experiments indicate that like NFI the 92kD NFIII protein exerts its stimulatory effect on the level of initiation of Ad2 DNA replication (Pruijn *et al.*, 1986 and O'Neill and Kelly, 1988) with stimulation being dependant on NFIII binding to its recognition site in the replication origin (O'Neill and Kelly, 1988). Deletion of this site results in a decrease in the efficiency of initiation of Ad2 DNA replication *in vitro* to about 30% of the normal level. However unlike NFI the role of NFIII in stimulating Ad2 DNA replication *in vivo* is unclear since plasmids containing sequences 1-45 or 1-67 are fully active in replication *in vivo* (Hay and McDougall, 1986). The NFIII recognition site contains an octanucleotide sequence which is partially conserved in human adenoviruses at similar positions within the origin. Ad4 has the optimal binding consensus sequence of 5'-TATGCAAAT-3' between nucleotides 41 and 49 whereas the Ad2 sequence (5'-TATGATAAT-3') between

nucleotides 39 and 47 has a relative binding affinity 2.5 times lower than Ad4 (Pruijn *et al.*,1987). NFIII also binds to its consensus sequence present in a number of transcriptional regulatory elements, for example, the V_H promoter and the H2B promoter resulting in transcriptional activation (Pruijn *et al.*, 1987; O'Neill and Kelly, 1988).

Pruijn *et al.* (1989) demonstrated that NFIII and OTF-I (octamer transcription factor I) are the same protein when they showed that they have indistinguishable mobilities on SDS-polyacrylamide gels, both stimulate Ad2/5 DNA replication and antisera raised against OTF-I recognises NFIII as well as inhibiting stimulation of Ad5 DNA replication by NFIII. NFIII contains a central conserved DNA binding domain (POU domain) which is sufficient for stimulation of Ad2 initiation of DNA replication although transcription requires additional regions as well (Verijzer *et al.*, 1990). The POU domain can be subdivided into an N-terminal 74 amino acid POU-specific domain and a C-terminal POU homeo domain. The POU homeo domain binds to DNA thereby inhibiting DNA replication which suggests that the POU-specific domain is responsible for stimulation of Ad 2/5 DNA replication and possibly contributes to DNA binding. The position of the NFIII recognition site in the origin of DNA replication is critical (Coenjaerts *et al.*, 1991) with the insertion of two or more base pairs between the NFI and NFIII sites abolishing NFIII stimulation. This sort of strict spatial arrangement suggests an NFI-NFIII protein interaction however both proteins can stimulate initiation independantly and without steric hindrance (Mul *et al.*, 1990). Verrijzer *et al.* (1991) have shown that NFIII is able to bend DNA via the POU-specific domain which could result in an altered origin structure that promotes pTP-pol binding to the core.

Nuclear Factor II

A third cellular factor, Nuclear Factor II (NFII) is required for full elongation of the Ad DNA but is not required for efficient initiation of Ad DNA replication. NFII is a 30kD complex of two proteins of 15kD and 17kD which has topoisomerase activity. *In vitro* assays demonstrate that on addition of NFII the DNA products are increased in size from 25-35% to the full length genome (Nagata *et al.*, 1983b). Purified eukaryotic topoisomerase I (100kD) can substitute for NFII and may represent either an active proteolytic fragment or a new type of eukaryotic topoisomerase I activity.

Factor pL

Factor pL is a 44kD protein that has 5'-3' exonuclease activity on both single stranded and duplex DNA (Kenny *et al.*,1988). It is not thought to be involved in Ad DNA replication *in vivo* where TP is probably involved in initial strand unwinding but it is required *in vitro* if TP free templates are used. Then pL degrades the 5' terminus of the displaced strand to create a single stranded region at the 3' end of the template strand.

REPLICATION COMPARTMENTS

NUCLEAR ARCHITECTURE

During the S phase of the eukaryotic cell cycle DNA replication takes place at discrete sites in the nucleus. For many years there was controversy over whether these replication sites are fixed to a nuclear sub-structure with the replicating DNA moving through them or if the replicating complex is unattached to the nucleoskeleton and progresses along the replicating DNA. The problem was that evidence for fixed

replication sites came from experiments using cells that had been extracted in hypertonic salt concentrations that could result in the formation of artefacts that bear no resemblance to structures *in vivo*. There are many terms used to describe various nuclear structures which correlate with the extraction procedure used to obtain them, the most commonly used being defined as follows. The term nuclear matrix was first used to describe the residual framework obtained from rat liver nuclei by extraction with 0.2mM $MgCl_2$, 2M NaCl, detergent and then nucleases. This matrix consisted of 98.4% protein , 0.1% DNA, 1.2% RNA and 0.5% phospholipid (Berezney and Coffey, 1974). Today nuclei extracted simply with hypertonic salt buffers are called nuclear matrices (Jackson, 1991). Whatever the exact method used they all share common structural characteristics such as residual elements of the nuclear envelope, residual nucleoli and a granular and fibrous internal matrix structure that extends throughout the nucleus (Verheijen *et al.*, 1988). Nucleoids are prepared by gently extracting the protein from nuclei with non-ionic detergents and hypertonic salt solutions. Because nucleases are not used they contain the DNA and nearly all of the RNA of the nucleus but are depleted of most of their nuclear proteins(Cook *et al.*, 1976). Due to concerns that structural information derived from nuclei that had been extracted with high salt buffers could be the result of artefacts led to more gentle procedures being developed. Nuclear scaffolds are prepared in low salt buffers that contain spermidine and lithium diiodosalicylate (LIS) which removes most of the histones and many other proteins but maintains the DNA in a folded conformation (Mirkovitch *et al.*, 1984). Finally the nucleoskeleton was developed (Jackson *et al.*, 1988) where the cells were first encapsulated in agarose microbeads which allows ionic detergents to extract nearly all the protein and RNA but leave the naked DNA. The cells are lysed and

washed in a 'physiological' buffer whose composition is approximately cytoplasmic. Since nucleoskeletons are prepared using mild conditions whilst protected by agarose with the preservation of nuclear function a priority they should be the least plagued by artefacts.

The initial experiments examining how chromatin is organised within the nucleus involved the analysis of nuclear matrices (Verheijen *et al.*, 1988) and nucleoids (Jackson *et al.*, 1984). Chromatin appears to be organised into domains or loops which are held together by a nuclear framework. However results from experiments using nuclear matrices and nucleoids were doubted because it was argued that DNA loop formation could have been an artefact created by hypertonic extraction.

Preparations of nuclear scaffolds were developed which do not involve hypertonic extraction to counter part of the artefact argument (Mirkovitch *et al.*, 1984). These preparations are histone depleted which allows complete restriction enzyme digestion of the DNA. The scaffold is composed of a number of nonhistone proteins which form a peripheral lamina with a complex internal network. The highly organised chromatin loops are attached to the nuclear scaffold at specific attachment sites that occur in nontranscribed spacer regions of the DNA.

The encapsulation of cells in agarose microbeads has allowed permeabilisation and subsequent manipulations to be carried out in a 'physiological buffer' (Jackson *et al.*, 1988) which minimises the possibility of artefacts since significant ionic changes have been eliminated. This approach has revealed that chromatin loops have an average size of 85kb which is constant throughout the cell cycle and is larger than those seen in nuclear matrices (48.4kb) and nuclear scaffolds (15kb) and smaller than those seen in nucleoids (123kb) (Jackson *et al.*, 1990). Analysis of these procedures demonstrated that treatment with 2M NaCl used in the isolation of nuclear matrices and nucleoids resulted

in the formation of larger loops probably by destroying some attachment sites whereas treatment with hypotonic buffers also used in the isolation of nuclear matrices significantly reduced loop size. The very small loops found in nuclear scaffolds were a result of 'thermal stabilisation' which was responsible for 5 out of 6 loops. Jackson *et al.* (1990) suggest that their preparations of nuclear skeletons reflect most the *in vivo* situation with the maintenance of isotonic coditions being critical. Their loops are free of nicks, gross nuclear morphology has been retained and they have nearly all the replicational and transcriptional activity of the cell (Jackson *et al.*, 1988) indicating that nuclear function has been preserved. The organisation of chromatin loops attached at specific sites to a nuclear protein framework suggests that nuclei are highly organised, integrated structures where nuclear architecture and function could be inextricably linked. (Jackson, 1991).

EUKARYOTIC REPLICATION COMPARTMENTS

DNA synthesis was shown to occur at several hundred discrete sites within the nuclei of eukaryotic cells in S-phase by pulse labelling replicating DNA with 5-bromodeoxyuridine *in vivo* (Nakamura *et al.*, 1986; Fox *et al.*, 1991) or biotinylated dUTP in nuclei of permeabilised cells (Nakayasu and Berezney, 1989; Mills *et al.*, 1989). Each discrete centre of DNA synthesis was found to contain approximately 20 replicating complexes. Time course experiments showed that DNA synthesis was initiated in the replication centres at the beginning of S-phase and continued for about an hour when a second wave of DNA synthesis was initiated at different sites (Nakamura *et al.*, 1986)..Jackson and Cook (1986) demonstrated that progeny DNA and active DNA polymerase did not electroelute from cells with most of the chromatin after they had been encapsulated in agarose microbeads, lysed and

treated with endonucleases. They interpret this to mean that the replication complex is attached to the nucleoskeleton and that replication proceeds by the movement of DNA through the fixed replication complex. In support of this *in vitro* experiments by Nakayasu and Berezney (1989) demonstrated that even after the removal of histones and more than 90% of the chromatin from cells the replication centres were unmoved having been successfully labelled by incorporation of biotinylated-dUTP both before and after extraction. Mills *et al.* (1989) were able to use *Xenopus laevis* eggextracts and sperm DNA for the *in vitro* reconstitution of nuclei. Biotin-dUTP incorporation revealed that they also contained discrete centres of replication numbering 100-300 per nucleus. The nucleus replicates in about an hour with each replication centre containing 300-1000 replication forks. Since pulse-labelling at various times throughout the replication phase revealed a similar patern without a raised level of background they also suggested that replication complexes are immobilised at fixed sites and that DNA is passed through them. Leonhardt *et al.* (1992) demonstrated that the DNA methyltransferase (MTase) that methylates deoxycytidine is localised to sites of ongoing DNA synthesis in S-phase nuclei whereas in G_1 and G_2 cells it has a diffuse nuclear distribution. They determined that MTase has a replication focus targeting seqence between amino acids 207 and 455 as well as a nuclear localisation signal between amino acids 72 and 92.

VIRAL REPLICATION COMPARTMENTS

The replication proteins of various eukaryotic viruses are also known to localise to discrete sites within the nucleus. Early immunofluorescence experiments demonstrated that adenovirus early proteins or P antigens, which includes DBP, are not evenly distributed

throughout the nucleus but are localised to discrete subnuclear sites (Hayashi and Russell, 1968). Later immunofluorescence experiments have shown that DBP (Sugawara *et al.*, 1977; Reich *et al.*, 1983) and pol (Sasaguri *et al.*, 1987) are localised to discrete sites within the nucleus of Ad infected cells. A small amount of DBP remains diffusely distributed throughout the nucleus and can be extracted with 1% NP40, 150mM NaCl but the majority is contained within globular foci and requires further treatment with 2m NaCl to be extracted (Voelkerding and Klessig, 1986). However the formation of these globular foci are dependant upon ongoing viral DNA replication with treatment with the DNA replication inhibitor hydroxyurea abolishing their formation. *In situ* DNA hybridisation with biotinylated Ad5 probes demonstrates that DBP (Voelkerding and Klessig, 1986) and pol (Sasaguri *et al.*, 1987) colocalises with Ad DNA. Murti *et al.* (1990) used immunogold electron microscopy to investigate the distribution of DBP, pol and pTP in Ad5 infected cells. They hypothesise that the replication complex is translocated from compact initiation foci which contain large amounts of pol and pTP and little DBP and ssDNA to larger elongation foci which contain large amounts of DBP and ssDNA and small amounts of pol and pTP. The morphology of Ad5 infected cells has been studied (Puvion-Dutilleul and Puvion, 1991) using *in situ* hybridisation with a biotinylated Ad5 DNA probe followed by immunogold labelling and electron microscopy to investigate the intranuclear distribution of replicating viral DNA. They report the existance of very small foci containing replicating viral DNA which develop into larger sites of accumulated viral single stranded DNA.

The existance of replication compartments in Herpes simplex virus (HSV-1) comes from immunofluorescence studies of the localisation of the viral DNA binding protein ICP8 which is required for

viral DNA replication. In cells where viral DNA replication has been inhibited by sodium phosphoacetate ICP8 is localised to small, discrete foci evenly distributed throughout the nucleus but in cells where viral DNA replication is proceeding ICP8 is localised to large, globular foci distributed throughout the nucleus (Quinlan *et al.*, 1984). These two types of foci have been named 'prereplicative sites' and 'replication compartments' respectively. Randall and Dinwoodie (1986) showed that ICP8, ICP4 (a transcriptional cofactor) and the DNA polymerase all colocalise at sites of active viral DNA replication. Several cellular DNA replication proteins such as PCNA, SSB and pol α as well as the retinoblastoma protein and p53 have also been shown to colocalise with ICP8 in HSV-1 infected cells (Wilcock and Lane, 1991).

TRANSCRIPTION COMPARTMENTS

The traditional view of transcription is that a mobile RNA polymerase moves along the DNA (Miller and Beatty, 1969; McKnight and Miller, 1979) but more recently (Jackson *et al.*, 1981) studies using nucleoids indicated that transcription occurs as DNA passes through a transcription complex fixed to a sub-nuclear structure. As mentioned previously results from experiments involving nuclear derivatives such as nucleoids have to be cautiously assessed because of the possibility of artefacts. The use of preparations of nucleoskeletons circumvents the problems of artefacts since the cells are protected within agarose microbeads before lysing them in a physiological buffer (Jackson *et al.*, 1988). When the sites of RNA synthesis were labeled using Br-UTP several hundred discrete sites were seen distributed throughout the nucleus which in late S-phase were distinct from replication foci (Jackson *et al.*, manuscript submitted for publication). These sites do not elute with most of the chromatin indicating that they are attached to

a nuclear sub-structure. Puvion-Dutilleul and Puvion (1991) labelled sites of active transcription by short pulse tritiated uridine incorporation and found that active replication sites and single stranded DNA accumulation sites were also transcription sites although only 10-15% of nascent viral RNA was contained in the single stranded accumulation sites.

METHODS AND MATERIALS

1. Cells and tissue culture media

A549 cells (human lung carcinoma cell line) and HeLa cells (human cervical carcinoma cell line) were grown as monolayers at 37°C in Glasgow modified Eagle's Medium containing 50units/ml of penicillin and 50μg/ml of streptomycin (G-MEM, GIBCO) supplemented with 10% newborn calf serum (Sera Lab Ltd) and maintained post infection in G-MEM supplemented with 1% newborn calf serum.

Spodoptera frugiperda cells were maintained either as monolayers or suspension cultures at 28°C in TC100 medium (GIBCO) supplemented with 10% foetal calf serum (Sera Lab Ltd).

2. Virus stocks

Adenovirus types 2 and 4 were obtained from R. T. Hay. A549 cells were used to titrate virus by the method of Williams (1970). Recombinant baculovirus *(Autographa californica)* expressing full length Nuclear Factor I (NFI_{FL}) and the DNA binding domain of NFI (NFI_{DBD}) were also obtained from R. T. Hay (Bosher *et al.*, 1991). Recombinant baculovirus expressing the adenovirus type 5 preterminal protein (pTP) and DNA polymerase (pol) were obtained from E-L. Winnacker. Titers of virus stocks were determined by plaque assay (Summers and Smith, 1987).

3. Antibodies

Monoclonal antibody α72k B6-10 directed against the Ad2 DNA binding protein (DBP) was obtained from A. Levine (Reich *et al.*, 1983). Monoclonal antibodies M2, M37 and M73 directed against E1a and

rabbit polyclonal serum containing antibodies against E1a were a gift from N. Jones.

Rabbit polyclonal serum containing antibodies against bovine RNA polymerase II were a gift from M. Dahmus.

Polyclonal antisera were raised in rabbits against purified NFI_{FL}, NFI_{DBD}, DBP (obtained from I. Leith), pTP and pol (both obtained from S. Temperley). 100µg of purified protein in 1ml PBS was mixed with 1ml of Freund's complete adjuvant (SIGMA) and sonicated until a white, viscous emulsion had formed. Two Dutch rabbits were each subcutaneously injected with half of this emulsion. The rabbits were given booster injections every two weeks as before except that Freund's incomplete adjuvant (SIGMA) was used. 5ml of blood was removed from the rabbit's ears before the initial immunisation (pre-immune sera) and one week after subsequent immunisations (test sera). The blood collected was allowed to clot at 4°C overnight and tthe sera separated by centrifugation at 1000rpm for 10 min and stored in 0.5ml aliquots at -70°C.

5. Preabsorption of polyclonal antisera, raised against Ad2 replication proteins

A confluent monolayer of HeLa or A549 cells were fixed with formaldehyde and permeabilised with NP40 as described in the Immunofluorescence method. The polyclonal antisera raised against DBP, pTP or pol was diluted 1 in 3 with 1% newborn calf serum, 0.1% sodium azide in PBS and poured onto the monolayer of cells. This was placed on a rocker and left for two hours at room temperature (RT) or overnight at 4°C. The antisera was then removed and filter sterilised and stored at -20°C.

6. Affinity purification of polyclonal antisera raised against cellular proteins

1ml HiTrap NHS-activated columns (Pharmacia) were used to prepare affinity chromatography columns for the purification of polyclonal antisera raised against cellular proteins. The purified protein was coupled to the NHS-activated Sepharose according to the manufacturers instructions before purification was carried out.

The affinity column was pre-equilibriated with 10mM Tris-HCl, pH7.5. 100µl of polyclonal antisera was microfuged for 3 min to clear any debris and then diluted 1 in 10 with 10mM Tris-HCl, pH7.5. This was loaded onto the column and left for 60 min to maximise antibody-antigen binding. The column was washed with 10ml of 10mM Tris-HCl, pH7.5 followed by 10ml of 10mM Tris-HCl, 500mM NaCl, pH7.5. The bound antibodies were eluted using ten 1ml aliquots of 100mM triethylamine, pH11.5 and were collected in 100µl of Tris-HCl, pH8.0. The presence of specific antibodies in the eluates was tested for by Western Blot analysis.

7. SDS polyacrylamide gel electrophoresis

Bio-Rad Mini Protein System Gel Rigs were assembled and used as directed by the manufacturers instructions. 10% running gels were made up using a 30% acrylamide stock (29.25% acrylamide, 0.75% N,N'-methylene-bis-acrylamide) and contained 0.375M Tris-HCl, pH8.9 and 0.1% SDS. Polymerisation was carried out by the addition of 1/250 volume of 25% (w/v) ammonium persulphate (APS) and 1/2500 volume of TEMED (Kodak). Running gels were overlayed with isopropanol to ensure an even surface and was rinsed off with H_2O after the gel had fully polymerised. The stacking gel contained 5% acrylamide in 0.105M Tris-HCl, pH6.7 and 0.1% SDS. Samples to be loaded onto the

gel were first denatured by heating at 100°C for 3 min in buffer containing 30% (v/v) glycerol, 15% (v/v) b-mercaptoethanol, 6% (w/v) SDS, 0.128M Tris-HCl, pH6.7 and 0.05% bromophenol blue. Electrophoresis was carried out at 100 volts in an electrophoresis buffer consisting of 0.19M glycine, 0.025M Tris-HCl and 0.1% SDS.

8. Preparation of cytoplasmic and nuclear extracts

Cells (either A549 or *S. frugiperda*) were grown as monolayers in 90mm diameter dishes to 80% confluence. A549 cells were infected at 10pfu/cell with Ad2 and were harvested at various times post infection (0, 12, 24, 36 or 48 hours). *S. frugiperda* cells were infected at 10pfu/cell with the appropriate protein (pol or pTP) expressing recombinant baculovirus and were harvested at 72 hours post infection. To harvest cells were scraped into the media and collected by centrifugation. The cells were washed twice with PBS and then lysed on ice in 50µl of 25mM Hepes, 5mM KCl, 1mM dithiothreitol (DTT), 0.5mM $MgCl_2$, 1mM sodium metabisulphite (SMBS), 1mM phenylmethylsulphonylfluoride (PMSF), 0.5% NP40, 1mM benzamidine and 10µg/ml of antipain, pepstatin and leupeptin for 30 min. The nuclei were collected by centrifugation in a microfuge for 3min and the cytoplasmic extract removed. Nuclei were lysed on ice in 50µl of 0.4M NaCl, 25mM Hepes, 5mM KCl, 1mM DTT, 0.5mM $MgCl_2$, 1mM SMBS, 1mM PMSF, 0.5% NP40, 1mM benzamidine and 10µg/ml of antipain, pepstatin and leupeptin for 30 min and then the cell debris removed by centrifugation in a microfuge for 3 min and the nuclear extract removed. The protein content of the cytoplasmic and nuclear extracts were quantified by the method of Bradford (1976).

9. Radiolabelling of a double stranded oligonucleotide for use in gel electrophoresis DNA binding assays

A double stranded oligonucleotide (obtained from R.T. Hay) that contained the NFI DNA binding site present in the Ad2 ITR (positions 18-41) was ^{32}P end labelled using the Klenow fragment of *E.coli* DNA polymerase (Amersham). 500ng of the oligonucleotide was incubated with 20μCi[α-^{32}P]dCTP, 20μCi[α-^{32}P]dATP and unlabelled dTTP and dGTP to final concentrations of 100μM in a buffer containing 50mM Tris-HCl, pH7.8, 5mM $MgCl_2$, 1mM DTT and 100μg/ml (w/v) BSA with 10 units of Klenow for 15 min at 20°C. Then unlabelled dCTP and dATP were added to final concentrations of 100μM and incubation continued for a further 15 min at 20°C. The reaction was stopped by addition of 1/5 final volume of 50% glycerol, 100mM EDTA and bromophenol blue. Unincorporated dNTPs were separated from the labelled oligonucleotide by gel electrophoresis in an 8% polyacrylamide gel (29:1, acrylamide:N-N'-methylene-bis-acrylamide) and 100mM Tris borate, 1mM EDTA, pH8.3 and was polymerised by addition of 0.6% (v/v) of 25% APS and 0.06% (v/v) TEMED (Kodak). Gels were run at 200 volts for 45-60 min in 1X TBE buffer. The band containing the labelled oligonucleotide was identified by autoradiography, excised from the gel and soaked in 500μl of 10mM Tris-HCl, 0.1mM EDTA, 100mM NaCl, pH8.0 buffer overnight at 37°C to elute the DNA. Labelled double stranded oligonucleotide was stored at 4°C.

10. Gel electrophoresis DNA binding assays

Either 10-100ng of purified NFI or nuclear extracts containing 4μg (as determined by Bradford, 1976) of total protein were incubated with 20μg BSA and 1μg of poly(dA.dT)poly(dG.dC) in 20μl of 150mM NaCl, 25mM Hepes-NaOH pH8, 5mM $MgCl_2$, 20mM DTT and 0.01%

NP40. After 15 min at 20°C ^{32}P end labelled double stranded oligonucleotide (1-2ng) containing the NFI DNA binding site was added and incubated at 20°C for a further 15 min. For competition experiments the assay mixtures were incubated with an excess of unlabelled double stranded oligonucleotides containing the NFI, NFIII or NF-κB DNA binding sites for 15min at 20°C prior to the addition of 32P labelled double stranded oligonucleotides. 3µl of dye (50% glycerol, 150mM NaCl, 25mM Hepes, 5mM $MgCl_2$, 20mM DTT, 0.01% NP40 and 0.05% bromophenol blue) was added to each assay mixture prior to loading onto a 6% polyacrylamide gel (55:1, acrylamide:N-N'-methylene-bis-acrylamide) and 50mM Tris borate, 0.5mM EDTA, pH8.3 which was polymerised by addition of 0.6% (v/v) of 25% APS and 0.06% (v/v) of TEMED (Kodak). Gels were run at 200 volts for 45-60 min in 50mM Tris borate, 0.5mM EDTA, pH8.3, fixed in 10% acetic acid for 10 min, dried and exposed to X-ray film at -70°C with an intensifying screen. Alternatively each NFI-DNA complex was cut out of the dried gel, submerged in 15ml of scintillation solution (Ecoscint A, National Diagnostics) and its radioactivity determined by a SL 30 Liquid Scintillation Spectrometer (Intertechniques).

11. Purification of NFI from *S. frugiperda* cells infected with recombinant baculovirus

One litre of *S.frugiperda* cells were infected at 2pfu/cell with recombinant baculovirus containing either NFI_{FL} or NFI_{DBD} cDNA (Bosher *et al.*, 1991). Infected cells were incubated for 72 hours at 28°C and were then collected by centrifugation. The cells were washed once and resuspended in 5ml of a lysis buffer containing 25mM Hepes-NaOH (pH8.0), 1mM ethylene-diamine tetra-acetic acid sodium salt (EDTA), 2mM DTT, 0.4M NaCl, 1mM benzamidine, 1mM PMSF, 1mM SMBS,

0.5% NP40 and 10µg/ml antipain, pepstatin and leupeptin. This was left on ice for 10 min and the cells were further disrupted with 10 strokes in a Dounce homogeniser using a type B pestle. After a further 20 min on ice it was centrifuged at 45000g for 30 min at 4°C. The supernatent was diluted 1 in 2 with column buffer (25mM Hepes-NaOH (pH8.0), 1mM DTT, 10% (v/v) glycerol, 2mM SMBS, 1mM PMSF and 10µg/ml antipain, pepstatin and leupeptin) and clarified by centrifugation at 45000g for 15 min at 4°C. To purify the NFI the clarified extract was applied directly to a column of Bio-Rex (Bio-Rad) equilibrated in column buffer containing 0.2M NaCl. Unbound proteins were removed by washing the column with two bed volumes of column buffer containing 0.2M NaCl. Bound proteins were eluted using 1ml aliquots of column buffer containing an increasing concentration of NaCl from 0.25M to 1.0M (increasing in 0.05M steps). The fractions were collected and their protein concentration determined by the method of Bradford (1976). The NFI DNA binding activity of these fractions was ascertained by gel electrophoresis DNA binding assays and scintillation counting of the NFI-DNA complex formed.

The fractions found to contain active NFI were pooled and diluted with column buffer to bring the NaCl concentration down to 0.25M. 6µg of poly(dI-dC) was added per mg of protein and then it was applied to a pre-equilibrated column containing the immobilised NFI-binding site linked via a 5'-amino link (Applied Biosystems) to CNBr-activated Sepharose (Pharmacia) as described (Clark *et al.*, 1990). Unbound proteins were removed by washing the column extensively with column volumes of column buffer. The bound NFI was eluted from the column in the same way as for the Bio-Rex column and the fractions tested for the presence of NFI as before.

12. Radiolabelling of DNA fragments

A plasmid pHRI, obtained from R. T. Hay, (Hay, 1985) that contains the 103bp Ad2 ITR was cut with EcoRI and Pst I to excise the Ad2 ITR which was then ^{32}P end labelled using the Klenow fragment of *E.coli* DNA polymerase (Amersham). 1μg of the cut plasmid was incubated with 20μCi[α-^{32}P]dATP and unlabelled dGTP, dCTP and dTTP to final concentrations of 100μM in a buffer containing 50mM Tris (pH7.8), 5mM $MgCl_2$, 1mM DTT and 100μg/ml (w/v) bovine serum albumin (BSA) with 10 units of Klenow for 15 min at 20°C. Then unlabelled dATP was added to a final concentration of 100μM for a further 15 min at 20°C. The reaction was stopped by addition of 1/5 final volume of 50% glycerol, 100mM EDTA and 0.05% bromophenol blue. Unincorporated dNTPS were separated from the labelled fragment by gel electrophoresis in an 8% polyacrylamide gel (29:1, acrylamide:N-N'-methylene-bis-acrylamide) containing 100mM Tris borate, 1mM EDTA, p H8.3 and was polmerised by addition of 0.6% (v/v) of 25% APS and 0.06% (v/v) of TEMED (Kodak). Gels were run at 200 volts for 45-60 min in 100mM Tris borate, 1mM EDTA, pH8.3. The band containing the labelled DNA fragment was located by autoradiography, excised from the gel and the DNA electroeluted.

13. DNase I footprinting

Labelled DNA (1.5ng) was incubated with varying amounts of NFI_{FL}, NFI_{DBD} and DBP in a final volume of 50μl containing 25mM Hepes-NaOH pH8.0, 5mM $MgCl_2$, 20mM DTT, 150mM NaCl, 0.01% NP40, 0.5μg BSA and 100ng poly(dA.dT) and poly(dG.dC) for 60 min at 20°C. 0.25 units of DNase I (Amersham) was added and the incubation allowed to proceed for 60 seconds at 20°C and the reaction stopped by the addition of 200μl of 0.3M NaOAc, 20mM EDTA and

100µg/ml tRNA. DNA was isolated by organic extraction followed by ethanol precipitation and resuspended in deionised formamide containing 2mM EDTA, 20mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanole. The samples were run on an 8% polyacrylamide gel (20:1, acrylamide:N-N'-methylene-bis-acrylamide) and containing 100mM Tris borate,1mM EDTA, pH8.3, 46% urea and polymerised by addition of 1/200 volume of 25% APS and TEMED. Gels were run in 100mM Tris borate, 1mM EDTA, pH8.3 at 2000 volts at 50°C until the leading dye front was approximately 1 inch from the bottom. The gels were dried and exposed to X-ray film at -70°C in the presence of an intesifying screen.

14. Expression and radiolabelling of replication proteins in *S.frugiperda* cells infected with recombinant baculovirus.

S.frugiperda cells were grown as monolayers in 90mm diameter dishes and infected with 10pfu/cell with the appropriate recombinant baculovirus. After 48 hours at 28°C the cells were collected by centrifugation , washed once in PBS and resuspended in 1ml of methionine-free TC100 (GIBCO) containing 500µCi of [^{35}S] methionine (NEG-072 EXPRES^{35}S^{35}S, Du Pont). Incubation was continued at 28°C for a further 3 hours. Cells were again collected by centrifugation, washed once with PBS, resuspended in 100µl of 0.4M NaCl, 25mM Hepes-NaOH pH8.0, 1mM DTT, 0.5mM $MgCl_2$, 2mM SMBS, 0.5% NP40 and 10% glycerol and incubated on ice for 30 min. The cell debris was collected by centrifugation at 12000g for 1min and the supernatent was diluted to a final NaCl concentration of 50mM and clarified by centrifugation at 100,000g for 30 min at 4°C.

15. Affinity chromatography

Six 50µl columns containing either BSA or NFI_{DBD} coupled to CNBr-activated Sepharose 4B were prepared and equilibrated with 50mM NaCl, 25mM Hepes-NaOH pH8.0, 1mM DTT, 0.5mM $MgCl_2$, 2mM SMBS, 0.05% NP40 and 10% glycerol. The columns were saturated with an unlabelled extract of *S.frugiperda* cells infected with wild type baculovirus and unbound proteins were washed away with the above buffer. Labelled extracts from *S.frugiperda* cells infected with recombinant baculovirus expressing either pol, pTP or a mixture of both viruses were loaded onto NFI_{DBD} and BSA columns and unbound proteins were washed away with the above buffer. Bound proteins were eluted with 50µl aliquots of 200mM NaCl, 25mM Hepes-NaOH pH8.0, 1mM DTT, 0.5mM $MgCl_2$, 2mM SMBS, 0.05% NP40 and 10% glycerol and then analysed by SDS-polyacylamide gel electrophoresis and fluorography.

16. Analysis of SDS-PAGE gels using fluorography

The SDS-PAGE gels were fixed in 10% acetic acid, 20% methanol for 10 min and then carefully placed in a sealed box and washed twice in DMSO for 30 min each time on a gentle rocker. The DMSO was replaced with 22% PPO (w/v) dissolved in DMSO and rocked gently for 2 hours. The PPO/DMSO solution was removed and tap water was run onto the gel for 30 min. The gel was then dried at 60°C and exposed to X-ray film at -70°C.

17. Western blot analysis

Proteins were transferred from SDS-PAGE gels to nitrocellulose (soaked in 20% methanol, 0.02M Tris) by applying a current of 60mA for 60 min. The nitrocellulose was blocked by incubating with 10%

(w/v) non-fat powdered milk (Marvel) in PBS for 60 min at 20°C. The nitrocellulose was briefly washed in 1% marvel/PBS and then incubated with antibody diluted in 1% marvel/PBS for 60 min at 20°C. The nitrocellulose was washed thoroughly in several changes of PBS and then in 1% Marvel/PBS. The antibody-antigen complex was visualised using ^{125}I-Protein A by incubating the nitrocellulose in ^{125}I-Protein A diluted in 5ml of 1% Marvel/PBS for 60 min at 20°C. The nitrocellulose was washed in frequent changes of PBS until the background count was lowered to 2-5cps and then exposed to X-ray film at -70°C.

18. Immunoprecipitations

Cell extracts from cells labelled with [^{35}S]-methionine (as described previously) were also used in immunoprecipitations. 10µl of the appropriate antisera was added to 200µl of extract and incubated at 4°C for 60 min with constant agitation. A 10% suspension of Protein A-Sepharose beads (150µl, Sigma) was added and incubated at 4°C for 60 min with constant agitation. The Protein A-Sepharose beads were collected by centrifugation and washed three times in 1ml of 25mM Hepes-NaOH pH8.0, 5mM KCl, 1mM DTT, 0.5mM MgCl, 0.5% NP40 before being resuspended in 30µl of 30% (v/v) glycerol, 15% (v/v) ß-mercaptoethanol, 6%(w/v) SDS, 0.128M Tris-HCl (pH6.7) and 0.05% bromophenol blue. After being heated at 80°C for 10 min the beads were removed by centrifugation and the proteins were fractionated on a 10% SDS-PAGE gel which was analysed by fluoragraphy.

19. Immunofluorescence

A549 cells were grown on circular glass coverslips to subconfluence and then infected with Ad2 or Ad4 at varying

multiplicities of infection and for varying times post infection. Cells were carefully washed twice in PBS and then fixed in one of two ways: i) for 10 min at RT in 5% (v/v) formaldehyde, 2% (w/v) sucrose in PBS followed by two washes in 1% normal donkey serum in PBS. The cells were permeabilised for 5 min at 20°C in 0.5% NP40, 10% (w/v) sucrose, 1% normal donkey serum in PBS followed by two washes in 1% normal donkey serum. When the cells were not used immediately they were stored in 1% normal donkey serum, 0.1% NaN_3 in PBS at 4°C after fixation but before permeabilisation.
ii) for 10 min at -20°C in absolute methanol (-20°C) followed by two washes with 1% normal donkey serum in PBS. When the cells were not used immediately they were stored in the absolute methanol at -20°C.

The primary antibodies (either polyclonal or monoclonal) were appropriately diluted in 1% normal donkey serum in PBS and applied to the cells in a 10µl drop. They were either incubated for 60 min at 20°C or for 30 min at 37°C. The cells were then washed for 1 min in 1% normal donkey serum in PBS; for 2 min in 1% normal donkey serum, 0.5% NP40 in PBS; for 10 min in 1% normal donkey serum in PBS and for 20 min in 1% normal donkey serum.

Sites of proteins indirectly labelled with rabbit polyclonal antisera were visualised using FITC-conjugated donkey anti-rabbit IgG (Jackson Immuno Research Laboratories Inc.) diluted 1/100 with 1% normal donkey serum in PBS. Sites of proteins indirectly labelled with mouse monoclonal antibodies were visualised using Texas Red-conjugated donkey anti-mouse IgG (Jackson Immuno Research Laboratories Inc.) diluted 1/100 with 1% normal donkey serum in PBS. These antibodies were applied to the cells in a 10µl drop and and incubated for 60 min at 20°C or 30 min at 37°C. The cells were then washed for 1 min in 1% normal donkey serum in PBS; for 2 min in 1% normal donkey serum,

0.5% NP40 in PBS; for 10 min in 1% normal donkey serum in PBS; for 20 min in normal donkey serum in PBS and finally two 10min washes in PBS.

The cells were mounted in a glycerol/PBS solution (Citifluor Ltd) and sealed using dental wax or nail varnish before they were viewed under a Nikon Microphot conventional immunofluorescence microscope or a laser scanning confocal microscope (Bio-Rad 500).

20. Bromodeoxyuridine labelling

After 24 hours of Ad2 infection A549 cells grown on coverslips were labelled with 5-bromodeoxyuridine (BrdU) for 15 min at 37°C by replacing their media with prewarmed media that contained 40μM BrdU, 5μM uridine, 0.4μM 5-fluorodeoxyuridine and 20mM Hepes-NaOH ph8.0. Then the cells were washed in cold PBS and fixed with absolute methanol as described previously. The cells were removed from the methanol, washed twice in PBS and were either stained with the appropriate antibodies or were pretreated, prior to staining, for 30 min with 22.5 units of *Eco* RI in 150μl of buffer (React 3, BRL) followed by 45 units of exonuclease III in 150μl of buffer (50mMTris-HCl, pH8.0, 5mM $MgCl_2$, 1mM DTT) for 30 min.

Sites containing BrdU labelled DNA (Br-DNA) were indirectly labelled using 10μl of undiluted monoclonal antibodies specific for bromodeoxyuridine (Becton Dickinson Immunocytometry Systems) for 60 min at 20°C or 30min at 37°C. Then the cells were treated as for standard immunofluorescence .

21. Synchronisation of cells at the G1/S boundary of the cell cycle

A549 cells were grown to 80% confluence in 90mm dishes under normal growth conditions and then the media was replaced with G-MEM supplemented with 10% newborn calf serum containing 1μg/ml of aphidicolin (Sigma). The aphidicolin block on DNA synthesis was removed after 18 hours at 37°C by washing the cells four times with normal growth media.

The effectiveness of the aphidicolin block and the cell's passage through the cell cycle was monitored using the CycleTEST (Becton Dickinson) and a FACScan flow cytometer.

21. CycleTEST and FACScan Flow Cytometry

At various times (0, 2, 4, 6, 8, 10 and 12 hours) after the aphidicolin block was removed the cells were collected by centrifugation for 15 min at 300g at RT and washed twice with citrate buffer (Becton Dickinson). Then they were collected in a final volume of 500μl of citrate buffer. If the cells were not to be stained immediately then they were snap frozen in a mixture of dry ice and 99% ethanol and stored at -70°C.

Before staining frozen samples were thawed rapidly in a 37°C waterbath without the sample itself reaching that temperature. Then 0.9ml of trypsin buffer (Solution A, Becton Dickinson) was added to a 200μl aliquot of the cell suspension and was gently inverted continuously for 10 min at 20°C. 0.75ml of trypsin inhibitor and RNase buffer (Solution B, Becton Dickinson) was added and gently inverted continuously for a further 10 min at 20°C. 0.75ml of ice cold propidium iodide stain (Solution C, Becton Dickinson) was added for 10 min at 20°C and kept protected from the light. This was transferred to Falcon

tubes and kept on ice and protected from light until flow cytometric analysis (no longer than three hours after addition of Solution C).

22. Cell encapsulation and lysis

An aliquot ($2x10^6$ cells) of uninfected and Ad2 infected (for 12 hours) A549 cells were encapsulated in agarose beads as follows. The cells were collected by centrifugation, washed once in PBS and then resuspended in 1ml of PBS. Each aliquot was transferred to a round bottomed flask in a 33°C waterbath and 250µl of prewarmed 2.5% agarose was added and gently mixed. 10ml of parafin was added and the flasks sealed and put on a flask shaker for 10-15 sec and then immediately plunged into ice. 35ml of ice cold PBS was added and then the mixtures were transferred to 50ml tubes and centrifuged at 2K for 3 min to collect the beads. The interface was disturbed and then they were centrifuged again. The parafin was aspirated down to 20ml remaining volume and 10ml was transferred to a 10ml sterilin tube and centrifuged as before. The supernatent was discarded and the remaining beads were added and centrifuged. The beads were washed with PBS and resuspended in 10ml of G-MEM supplemented with 1% newborn calf serum and incubated at 33°C for 30 min.

The beads were collected by centrifugation and washed with PBS three times. 10ml of Streptolysin O (40 units, Wellcome) was added for 30 min on ice. The beads were washed once in ice cold PBS and then resuspended in prewarmed 'physiological' buffer (PB) for 3 min at 33°C. PB (pH7.4) contains 22mM Na^+, 130mM K^+, 1mM Mg^{2+}, <0.3µM free Ca^{2+}, 31mM Cl^-, 100mM acetate, 11mM phosphate, 1mM ATP, 1mM DTT and 0.2mM PMSF. The beads were chilled on ice and then washed once with PB buffer and pelleted. The beads containing uninfected and

Ad2 infected cells were each aliquoted into four lots of 250µl and kept on ice.

24. Labelling sites of transcription

The encapsulated beads were pre-incubated at 33°C for 2 min and prewarmed transcription mix (10X concentration) was added to give final concentrations of 2mM ATP, 2mM $MgCl_2$, 100units/ml RNase inhibitor (Pharmacia), 50µM CTP, GTP (Pharmacia) and Br-UTP (Sigma). Reactions were incubated at 33°C for 15 min and then beads were washed twice in ice cold PB containing 20units/ml RNase inhibitor.

25. Labelling sites of replication

The encapsulated beads were pre-incubated at 33°C for 2 min and prewarmed replication mix (10X concentration) was added to give final concentrations of 2mM ATP, 2mM $MgCl_2$, 100µM dATP, dCTP, dGTP (Pharmacia) and Biotin-16-dUTP (Boehringer) and 50µM CTP, GTP and UTP (Pharmacia). Reactions were incubated at 33°C for 15 min and then the beads were washed twice in ice cold PB.

26. Labelling sites of transcription and replication

The encapsulated beads were pre-incubated at 33°C for 2 min and prewarmed replication and transcription mix (10X concentration) was added to give final concentrations of 2mM ATP, 2mM $MgCl_2$, 100units/ml RNase inhibitor, 100µM dATP, dCTP, dGTP and Biotin-16-dUTP and 50µM CTP, GTP and Br-UTP. Reactions were incubated at 33°C for 15 min and then the beads were washed twice in ice cold PB containing 20units/ml RNase inhibitor.

27. Visualisation of transcription and replication sites

The pelleted beads were immediately permeabilised by incubation in 0.2% Triton X-100 in PB (20units/ml Rnase inhibitor was in all buffers and washes used for the labelled transcription sites) for 15 min on ice. The beads were fixed for 15 min on ice in 0.3% paraformaldehyde in PB and then washed three times with 0.05% Tween in PBS.

Sites containing Br-UTP labelled RNA (Br-RNA) were indirectly labelled using 100µl of a 1/20 dilution of monoclonal antibodies specific for bromodeoxyuridine (Boehringer) for 4 hours on ice. The beads were washed twice with 0.05% Tween in PBS followed by a 15 min wash with 0.05% tween, 0.05% BSA in PBS. Then they were labelled using 100µl of a 1/500 dilution of Texas Red-conjugated sheep anti-mouse Ig (Amersham) overnight at 4°C.

Sites containing Biotin-16-dUTP labelled DNA (Biotin-DNA) were directly labelled using 100µl of a 1/20,000 dilution of Streptavidin-FITC (Sigma) overnight at 4°C.

The beads were washed twice with 0.05% Tween in PBS followed by two washes in PBS. The beads were mounted under coverslips in Mowiol 4-88 (Hoechst) containing 0.1µg/ml DAPI (Boehringer) and 2.5% DABCO (1,4-diazobicyclo-[2.2.2]-octane; Sigma). Photographs were taken using a Zeiss Axiophot microscope (100X oil-immersion objective).

RESULTS

CHAPTER ONE

Direct and indirect interactions of NFI with Ad2 replication proteins

Expression of replication proteins in insect cells

One difficulty in detecting specific protein-protein interactions in adenovirus infected cells is that many of the proteins may already exist in specific protein complexes thereby making it hard to determine their individual properties. The use of recombinant baculoviruses that express the various replication proteins addresses this problem and also provides larger quantities of protein for purification and characterisation.

Spodoptera frugiperda cells were infected with 2 p.f.u./cell of the appropriate recombinant baculoviruses and cell extracts were prepared after 72 hours, aliquots of which were analysed by SDS-PAGE followed by staining with Coomassie blue. Proteins corresponding to the 140kD pol, 80kD pTP and the 35kD NFI_{DBD} are detected in substantial levels (fig. 6).

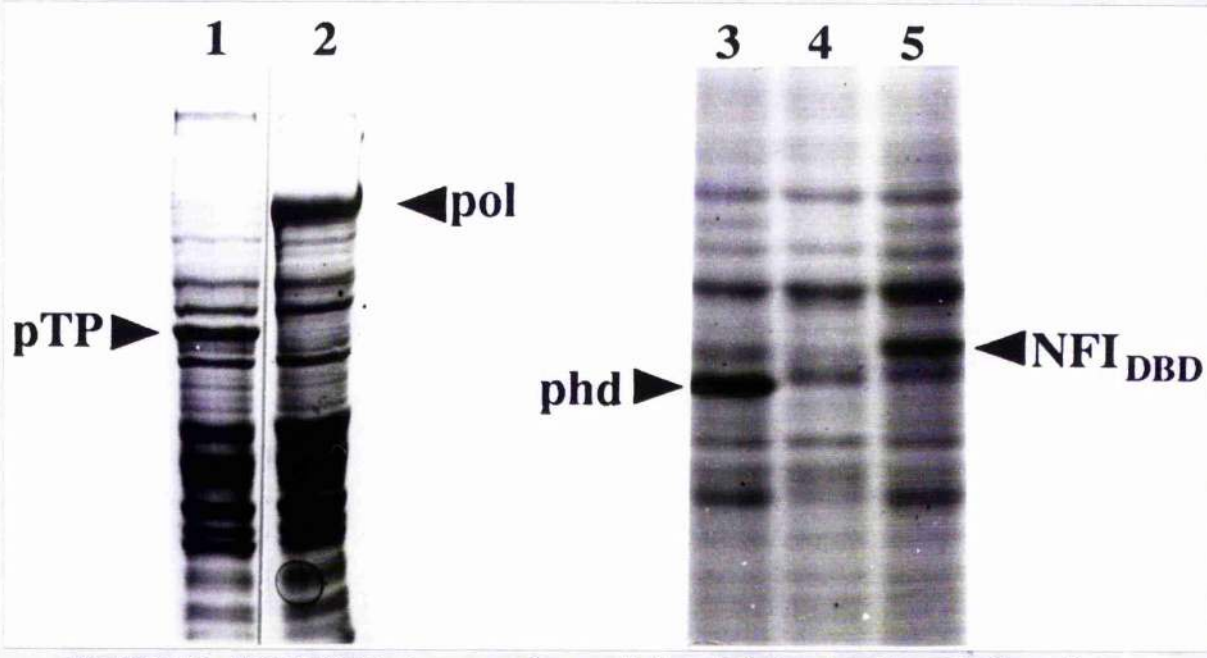


FIGURE 6. SDS-PAGE of extracts from uninfected *S. frugiperda* cells (lane 4) or cells infected with wild-type baculovirus expressing polyhedrin (phd, lane 3), or recombinant baculoviruses expressing pTP (lane1), pol (lane 2) or NFI_{DBD} (lane 5).

Purification of NFI_{FL} and NFI_{DBD}

The proteins of the NFI family have a conserved N-terminal domain which is required for DNA binding plus additional sequences which are involved in transcriptional regulation (Mermod *et al.*, 1989). To determine the role of the different NFI domains in Ad2 DNA replication the CTF1 cDNA (full length protein) and the DNA-binding domain of CTF1 were inserted into recombinant baculoviruses (Bosher *et al.*, 1991). Both NFI_{FL} and NFI_{DBD} were individually purified from cell extracts of *S. frugiperda* cells that had been infected with 2p.f.u./cell of baculoviruses containing either NFI_{FL} or NFI_{DBD} cDNA for 72 hours. They were purified by a combination of ion-exchange chromatography and DNA affinity chromatography.

Ion-exchange chromatography involves the separation of proteins on the basis of their net charge. Positively charged proteins bind to the Bio-Rex ion-exchange column and are then eluted with sodium chloride. Sodium ions compete with positively charged groups on the protein for binding to the column. Proteins with a lower density of net positive charge will be eluted first followed by those having a higher charge density. The CTF1 DNA binding domain has a net positive charge due to a number of lysine and arginine residues which cluster on one side of the a-helical protein structure to form a novel a-helical sequence motif which may contact backbone phosphates of the DNA (Meisterernst *et al.*, 1989).

To determine the optimum NaCl concentration required to elute NFI_{FL} and NFI_{DBD} from the Bio-Rex column 0.05M stepwise increases in the NaCl concentration of the column buffer (25mM Hepes-NaOH pH8.0, 1mM DTT, 10% (v/v) glycerol, 2mM SMBS, 1mM PMSF and 10μg/ml antipain, pepstatin and leupeptin) were initially used. The eluted fractions that contained NFI were identified using gel electrophoresis

DNA-binding assays with the ^{32}P-DNA-NFI complex being measured by scintillation counting (fig. 7).

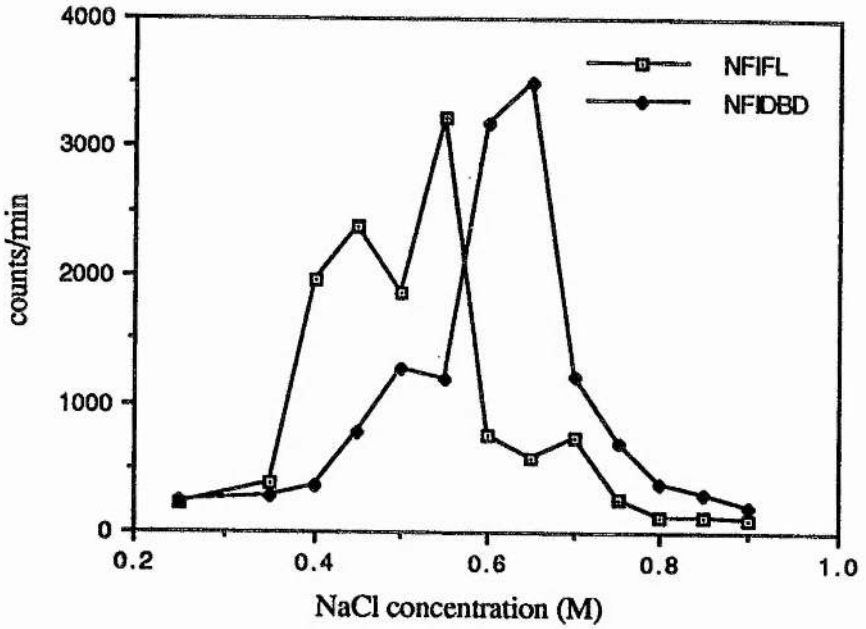


FIGURE 7. NFI DNA-binding activities of fractions eluted from the Bio-Rex column were determined by gel electrophoresis DNA-binding assays where the NFI-DNA complex was measured using scintillation counting.

As expected the peak of NFI_{DBD} eluting from the column occured at a higher NaCl concentration than that of NFI_{FL} because NFI_{DBD} has a higher net positive charge due to the cluster of basic residues which make up the 'lysine helix'. Figure 7 shows that NFI_{FL} should be eluted with 0.6M NaCl whereas NFI_{DBD} should be eluted with 0.7M NaCl.

DNA affinity chromatography makes use of the specific DNA binding property of NFI by using a column containing the NFI-binding site linked via a 5'-amino link to CNBr-activated Sepharose. To determine the optimum NaCl concentration required to elute NFI_{FL} and NFI_{DBD} from the affinity column 0.05M stepwise increases in the NaCl concentration of the column buffer were initially used. The eluted fractions that contained NFI were identified using gel electrophoresis

DNA-binding assays with the ^{32}P-DNA-NFI complex being measured by scintillation counting (fig. 8).

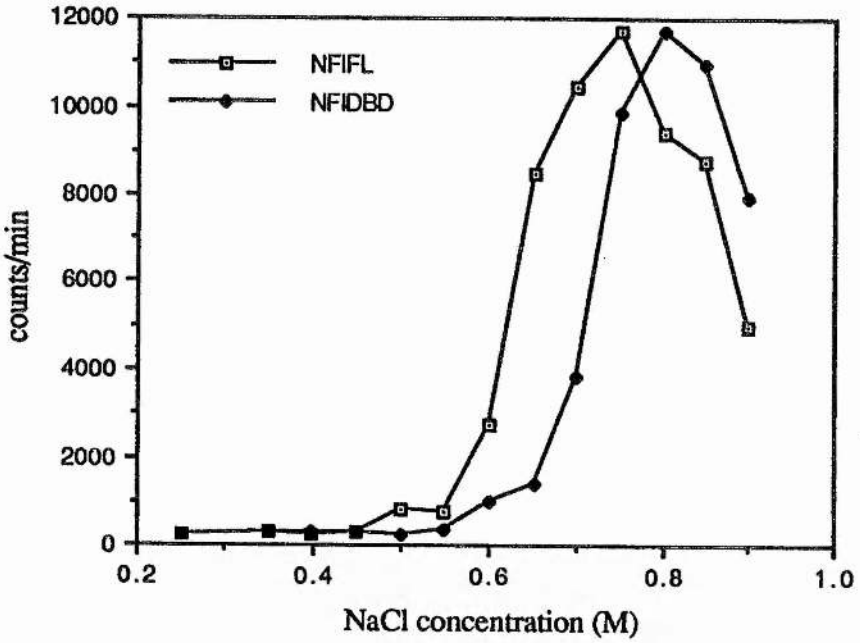


FIGURE 8. NFI DNA-binding activities of fractions eluted from the DNA affinity column which contains a double stranded synthetic oligonucleotide representing positions 18 to 41 from the Ad2 origin of DNA replication.

The peak of NFI_{FL} elution occured at 0.75M NaCl whereas the peak elution of NFI_{DBD} occured at 0.8M NaCl which again could be a reflection of the more basic nature of the DNA binding domain and the negatively charged DNA. In future purifications both species were eluted with 1.0M NaCl, having been loaded on at 0.25M NaCl, with washes being carried out with 0.4M NaCl in column buffer.

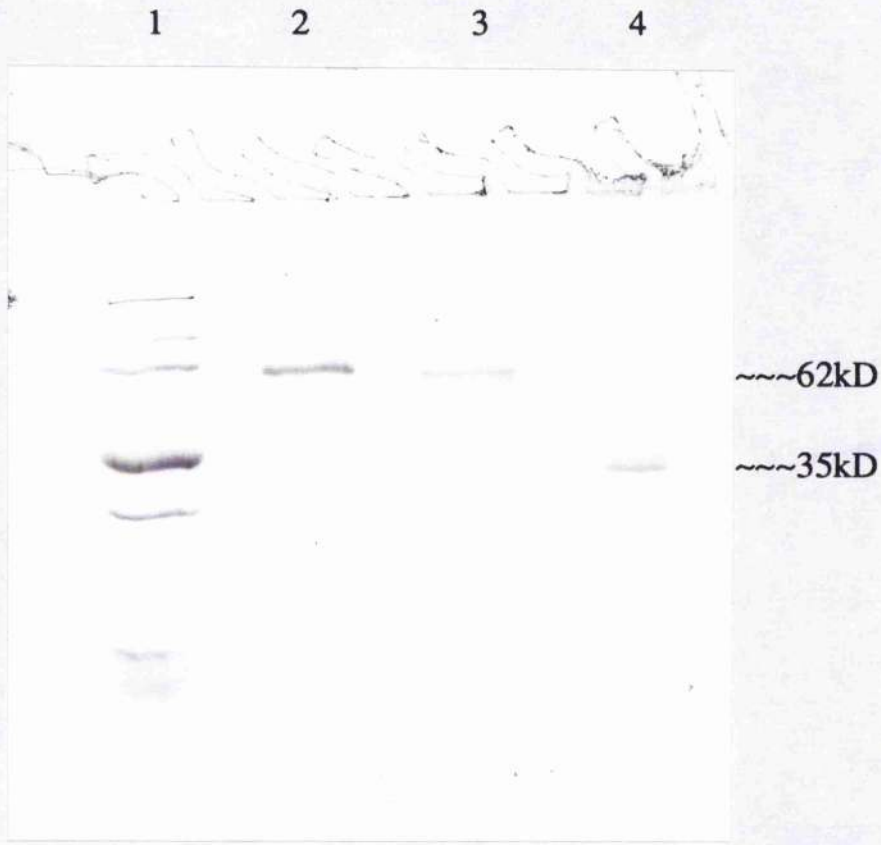


FIGURE 9. SDS-PAGE followed by Coomassie blue staining on NFI_{FL} and NFI_{DBD} samples purified by ion exchange chromatography and DNA affinity chromatography. Lane 1 contains molecular weight markers (200, 97.4, 68, 43, 29, 18.4 and 14.3kD, GIBCO BRL), lane 2 DBP, lane 3 NFI_{FL} and lane 4 NFI_{DBD}.

NFI_{FL} is purified predominanty as a single species of 62kD whereas NFI_{DBD} elutes from the column as two species, one of which appears to be generated from the other by proteolysis. It was usual to purify 1-1.5mg of NFI from 1 litre of infected *S. frugiperda* cells.

Stimulation of binding of NFI to DNA by Ad2 DBP

Ad2 DBP can increase the affinity of NFI for its recognition site in the Ad2 origin of DNA replication (Cleat and Hay, 1989; Stuiver and van der Vliet, 1990). To determine whether the full-length protein or only the DNA-binding domain of NFI is required for this, DNase I footprinting analysis was carried out. DNase I footprinting involves incubating a DNA fragment (radioactively labelled at one end) with a possible DNA binding protein and then digesting the DNA with a limited amount of DNase I so that on average there is one cut per DNA molecule. In the presence of a sequence specific DNA binding protein such as NFI the DNA bound by the protein is protected from DNase I digestion thereby resulting in a footprint (protected region) in the DNA cleavage pattern.

A 3'-labelled DNA fragment containing the NFI binding site from the Ad2 replication origin was incubated with increasing amounts of purified NFI_{FL} or NFI_{DBD} in the absence of DBP for 60 min at 20°C and then subjected to digestion with DNase I. The full length protein and the DNA binding domain appeared to bind to the Ad2 origin of DNA replication with similar affinity and indistinguishable patterns of DNase I protection (fig 10A). Following on from this NFI_{FL} and NFI_{DBD} were used in further experiments at concentrations giving only partial protection of the binding site from cleavage with DNase I. Addition of increasing concentrations of DBP to NFI_{FL} and NFI_{DBD} resulted in an increase in NFI site occupancy in each case (fig 10B) indicating that DBP only required the DNA binding domain of NFI to increase its affinity for its recognition site.

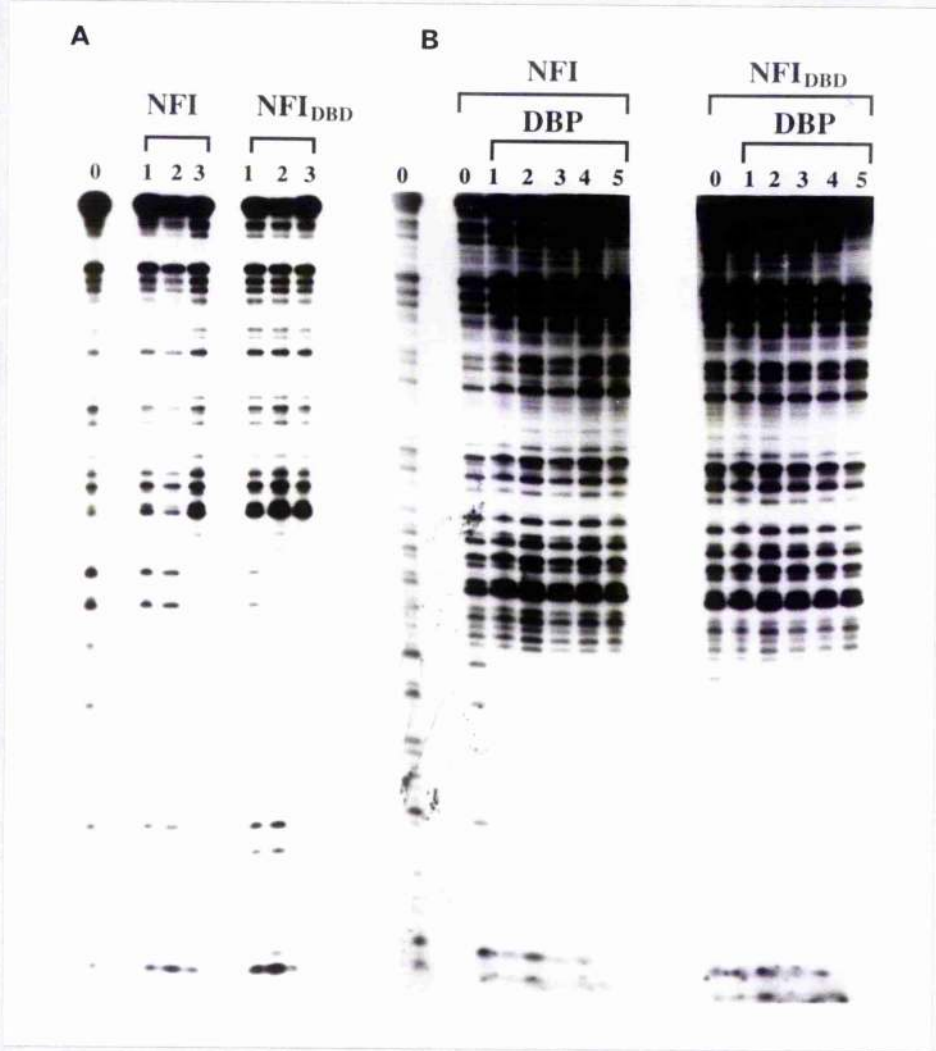


FIGURE 10. The *Eco* RI-*Pst* I fragment from pHRI containing the Ad2 origin of DNA replication was 3'end-labelled at the *Eco*RI site and incubated with various concentrations of NFI and DBP prior to DNase I digestion.
A. DNase I cleavage pattern produced in the absence of NFI and DBP (lane 0), and increasing concentrations of NFI_{FL} (lane 1,1.6ng; lane 2, 4.8ng; lane 3, 16ng) or NFI_{DBD} (lane1, 1.5ng; lane 2, 5ng; lane 3, 15ng).
B. Labelled DNA was incubated alone (lanes 0) or in the presence of NFI_{FL} (0.8ng) or NFI_{DBD} (0.6ng) and increasing concentrations of DBP (lanes 1, 10ng; lanes 2, 20ng; lanes 3, 100ng; lanes 4, 200ng; lanes 5, 250ng) prior to DNase I digestion.

Interaction of the DNA polymerase-preterminal protein heterodimer with NFI_{DBD}

To identify proteins that interact with NFI_{DBD} six 50µl columns containing either NFI_{DBD} or BSA coupled to CNBr-activated Sepharose 4B were prepared and equilibriated with 50mM NaCl, 25mM Hepes-NaOH pH8.0, 1mM DTT, 0.5mM $MgCl_2$, 2mM SMBS, 0.05% NP40 and 10% glycerol. Because of the charged nature of NFI_{DBD} proteins will bind to it non-specifically so the columns were saturated with unlabelled extracts of *S. frugiperda* cells infected with wild type baculovirus to block these non-specific binding sites. Insect cells were infected with recombinant baculoviruses expressing either pol or pTP or a mixture of both. After 72 hours the proteins were labeled *in vivo* with [^{35}S] methionine and then whole cell extracts were prepared. Extracts were loaded onto the columns in a buffer containing 50mM NaCl and unbound protein removed by extensive washing with 25mM Hepes-NaOH pH7.5, 1mM DTT, 0.5mM $MgCl_2$, 50mM NaCl, 2mM SMBS, 0.05% NP40 and 10% glycerol. Bound proteins were eluted by increasing the NaCl concentration to 200mM and analysed by SDS-PAGE and fluorography. Even in the presence of saturating amounts of unlabelled extract from wild-type baculovirus infected cells the bulk of the Ad2 DNA polymerase was bound to the NFI_{DBD} column and eluted by 200mM NaCl (fig. 11B and 11D). In contrast no specific binding of pTP to NFI_{DBD} was detected from extracts that did not contain pol (fig. 11C). However pTP was eluted from NFI_{DBD} when pol was also present in the extracts (fig. 11B). Neither pol or pTP bound to the BSA (fig. 11A). Since pTP and pol exists as a heterodimer in adenovirus infected cells this demonstrates that pTP alone can not bind to NFI but as part of the heterodimer can do so via pol which does interact with NFI.

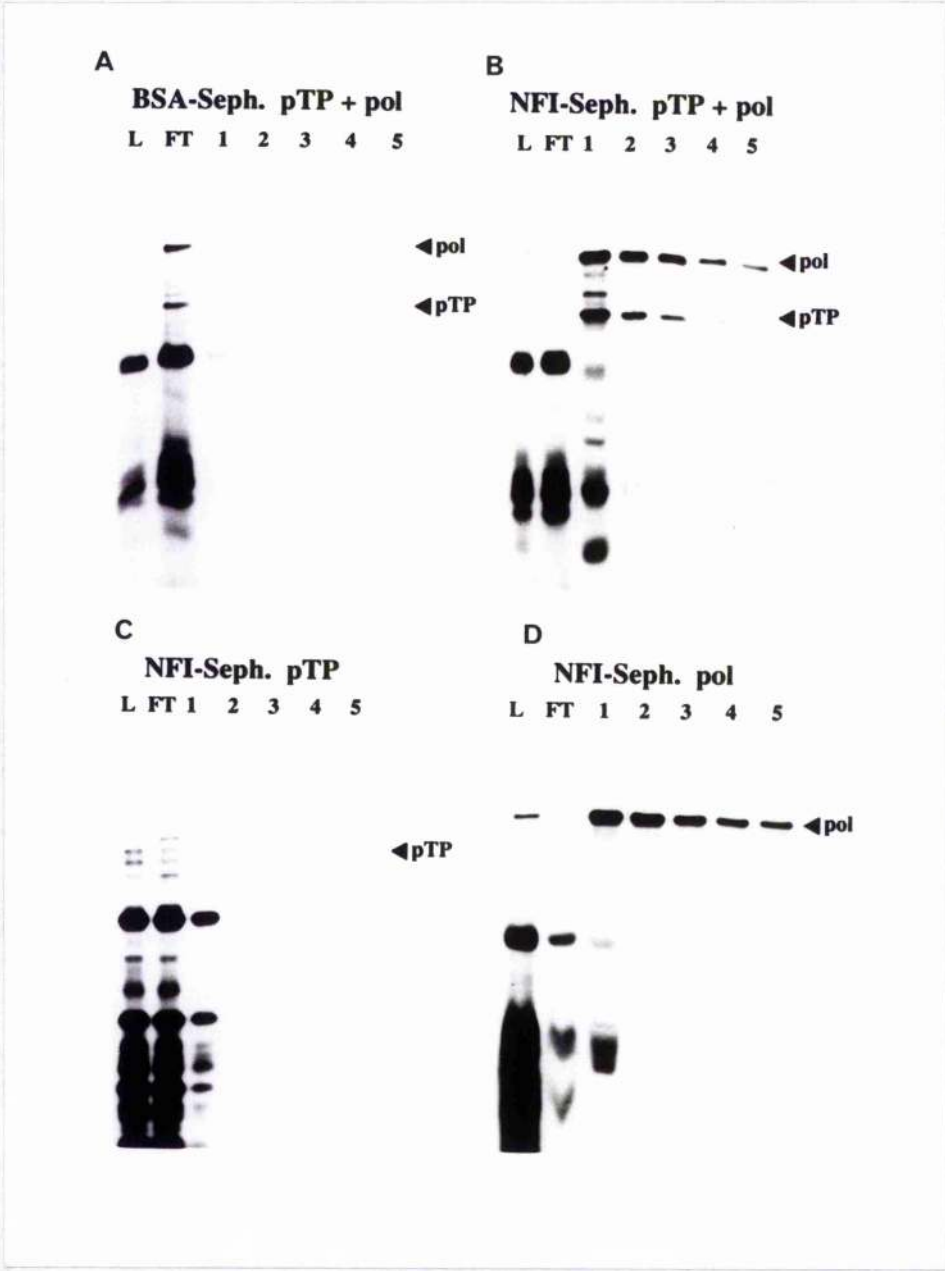


FIGURE 11. *S. frugiperda* cells infected with baculovirus expressing either pTP, pol or both were labelled with [^{35}S] methionine and cell extracts were loaded onto small (50µl) columns of NFI_{DBD}-Sepharose or BSA-Sepharose that had been previously saturated with an extract from *S. frugiperda* cells infected with wild-type baculovirus. Columns were washed and eluted and the protein fractions were analysed by SDS-PAGE and fluorography.

A. Neither pol or pTP were eluted from the BSA columns.
B. Both pol and pTP were eluted from the NFI_{DBD} column.
C. pTP was not eluted from the NFI_{DBD} column when pol was absent
D. pol was eluted from the NFI_{DBD} column when pTP was absent.

SUPPLEMENT

Purification of NFI_{DBD} and NFI_{FL}

The object of purifying both NFI_{FL} and NFI_{DBD} was to obtain enough protein, as quickly as possible, to allow the production of polyclonal antisera in rabbits as well as to carry out some experiments. The protocol settled upon routinely resulted in 1-1.5mg of purified NFI per litre of *Spodoptera frugiperda* cells infected with the appropriate recombinant baculovirus.

Figure 7 demonstrates that NFI_{FL} is eluted from the Bio-Rex column with 0.6M NaCl whereas NFI_{DBD} requires 0.7M NaCl. As a result these were the NaCl concentrations used in all subsequent purifications. Figure 8 shows a similar elution profile from the DNA affinity column where both NFI_{FL} and NFI_{DBD} both require a higher NaCl. Although the NaCl concentration does not go high enough to elute all of the protein it was felt that there was enough information available to ascertain that both NFI_{FL} and NFI_{DBD} could be eluted with 1M NaCl. In subsequent purifications the affinity column was washed with 0.4M NaCl after NFI was bound to it and then it was eluted with 1M NaCl.

Figure 9 illustrates the good level of purity obtained using the finalised purification protocol. The doublet seen in purified NFI_{DBD} seems to be the result of proteolysis. Supporting evidence of this comes from the knowledge that the DNA binding activity of NFI is present in a highly protease resistant domain (Cleat and Hay, 1989b) and that the slower migrating band can be converted into the faster one by limited proteolysis *in vitro*. A possible alternative explanation for the doublet was that it could represent two phosphorylation states of NFI. However this seems unlikely since radioactive labelling of the potential

phosphorylation sites of NFI_{FL} and NFI_{DBD} indicated that they exist outside of the DNA binding domain.

Stimulation of binding of NFI to DNA by Ad2 DBP

The DNase I footprinting in figure 10 demonstrates that Ad2 DBP stimulates the binding of NFI to its recognition site in the Ad2 origin of DNA replication. This experiment could have been greatly improved had the relative amounts of the various proteins been adjusted more. In both footprints the concentrations of NFI_{FL}, NFI_{DBD} and DBP should have ranged significantly lower to obtain the gradual emergence of the protection pattern rather than the all or nothing result obtained.

Interaction of the DNA polymerase-preterminal protein heterodimer with NFI_{DBD}

Figure 11 clearly demonstrates that the N-terminal 35kD of NFI is able to interact with the pol-pTP heterodimer via pol. BSA was used in the control columns because it is cheap and readily available. Any doubt over whether this provides the experiment with an acceptable control is unnecessary because the behaviour of pTP presents itself as an inbuilt control. Pol binds to the NFI_{DBD}-Sepharose column with or without pTP whereas pTP requires the presence of pol before it is able to bind. This indicates that when the pol-pTP heterodimer interacts with the DNA binding domain of NFI it does so via pol. This could also have been shown by binding pol to an NFI_{DBD}-Sepharose column and then adding pTP to see if it then bound.

CHAPTER 2

NFI is specifically targeted to discrete subnuclear sites in Ad2 infected cells.

NFI has a crucial role to play in Ad2 DNA synthesis and is incorporated into nucleoprotein complexes containing the Ad2 origin of DNA replication, the pTP-pol heterodimer and DBP.

Fate of NFI after Adenovirus type 2 infection

Since NFI is required for efficient Ad2 DNA replication its fate in cells after Ad2 infection was examined. NFI levels were determined using a polyclonal antisera raised against NFI_{DBD} purified from insect cells infected with the NFI_{DBD} expressing recombinant baculovirus. The specificity of this antibody for NFI_{FL} was tested using immunoprecipitation and western blot analysis. Insect cells infected with wild type baculovirus or the recombinant virus expressing NFI_{FL} were labelled with [^{35}S] methionine and extracts immunoprecipitated with the NFI_{DBD} antisera. The antibody efficiently immunoprecipitated the 62,000 molecular weight NFI_{FL} protein from extracts of insect cells infected with the recombinant vector, but does not recognise proteins from the cells infected with a baculovirus containing the Ad2 pol gene (fig. 12A).

A549 cells were infected (10p.f.u./cell) with Ad2 and harvested at various times after infection for Western blot analysis. In infected cells the NFI family of proteins is apparent as a multiple series of immunoreactive species ranging in size from 55-62,000 molecular weight. Infection of these cells with Ad2 appears to have little effect on the overall levels of NFI protein (figure 12B). To ensure that cells had

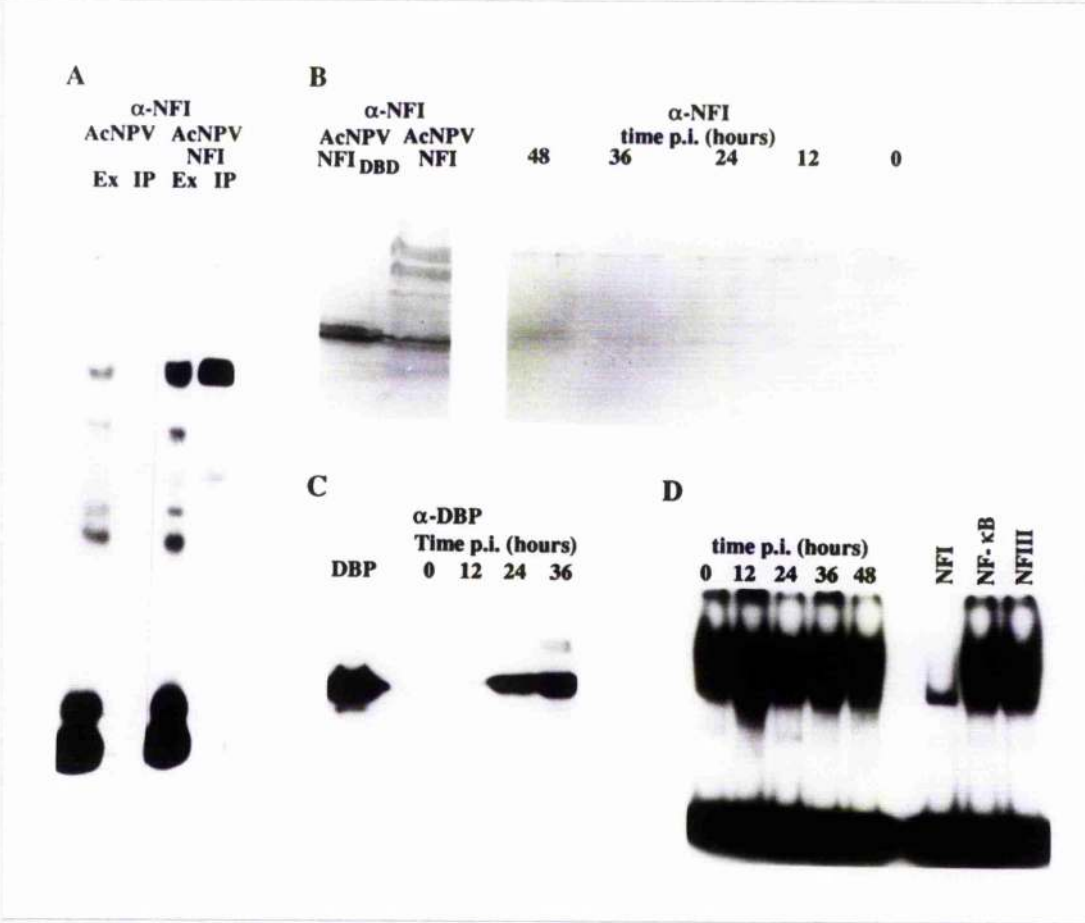


FIGURE 12. Fate of NFI after Ad2 infection of A549 cells.
A. Extracts from *S. frugiperda* cells infected with recombinant baculovirus expressing NFI (ACNPV NFI) or Ad2 DNA polymerase (AcNPV) were immunoprecipitated with polyclonal antibodies raised againstNFI_{DBD} (α-NFI). Whole-cell extracts (Ex) or immunoprecipitated proteins (IP) were resolved by electrophoresis in an 8% polyacrylamide gel and labelled proteins were detected by fluorography.
Extracts were prepared from *S. frugiperda* cells infected with a recombinant baculovirus expressing NFI_{DBD} or the NFI_{FL} (NFI) and from A549 cells infected for the indicated times with Ad2 (p.i., postinfection).
B. Extracts were fractionated by SDS-PAGE and NFI proteins were detected with anti-NFI (α-NFI) primary antibodies and alkaline phosphatase-conjugated secondary antibodies by using BCIP-NBT colour development.
C. Extracts were fractionated by SDS-PAGE and DBP was detected with anti-DBP (α-DBP) primary antibodies followed by ^{125}I-protein A. Purified DBP was analysed in parallel.
D. Extracts were used in gel electrophoresis DNA binding assays to investigate the NFI DNA-binding activities present at various times post infection. Unlabelled oligonucleotide competitors contained binding sites for NFI, NFIII or NF-κB and were added in 100-fold molar excess.

been successfully infected samples of the same extracts were tested for the presence of the adenovirus coded DBP. Western blot analysis with a DBP specific antiserum indicated that, as expected, DBP levels increase throughout the course of the infection (figure 12C).

While it is possible that the level of NFI proteins does not change during the course of Ad2 infection it is conceivable that the activity of the protein is modified. Gel electrophoresis DNA binding assays were carried out on samples of the same extracts used previously to investigate whether the DNA binding activity of NFI varied as Ad2 infection proceeds. In uninfected and Ad2 infected cells a typically heterogeneous series of DNA-protein complexes was formed between NFI proteins and their DNA recognition site with no apparent variation in NFIs ability to bind DNA. Competition experiments demonstrated the specificity of the interaction with addition of unlabelled oligonucleotides containing the NFI recognition site in the binding reaction precludeding formation of the DNA-protein complex, whereas addition of oligonucleotides containing unrelated binding sites had no effect on formation of the DNA-protein complexes (figure 12D). Thus the ability of NFI proteins to bind to a double stranded oligonucleotide containing the NFI recognition site present in the Ad2 origin of DNA replication did not change after Ad2 infection (figure 12D).

The nuclear distribution of NFI is altered after adenovirus type 2 infection.

Although the level of NFI proteins or NFI DNA binding activity did not appear to change after adenovirus infection, it is entirely possible that other properties of the protein are altered. It has long been known that adenovirus early proteins or P-antigens are not evenly distributed throughout the nucleus but are localised in discrete nuclear sites

(Hayashi and Russell, 1968). Viral DNA and replication proteins are contained in these foci, which are thought to be the sites of viral DNA synthesis. In view of the involvement of NFI in Ad2 DNA replication the subcellular location of NFI, before and after infection with Ad2, was determined by immunofluorescence using the polyclonal antiserum raised against NFI_{DBD} that had been antigen affinity purified. A polyclonal antiserum specific for the virus coded DBP was used in parallel to identify the presumed foci of viral DNA replication.

Uninfected cells or cells infected at less than 1 p.f.u. per cell with Ad2 were fixed in formaldehyde, permeabilised with detergent and incubated with either the NFI or DBP specific rabbit antiserum. An FITC conjugated second antibody was used to permit visualisation of the antibody locations by fluorescence microscopy. Uninfected cells displayed no cross reaction with the DBP antibody (figure 13A), but in infected cells the DBP antibody revealed a strikingly punctate localisation that is characteristic of the protein (figure 13C). In uninfected cells NFI was localised to the nucleus with a slightly granular distribution (figure 13B), but its distribution in infected cells was dramatically different, taking on a punctate appearance (figure 13D) that was reminiscent of that observed with DBP (figure 13C).

Colocalisation of NFI and the adenovirus DBP.

To determine if NFI and DBP are localised to identical sites in Ad2 infected cells, antibodies to DBP and NFI were applied to the same cells and then visualised by double fluorescence. A mouse monoclonal antibody directed against an epitope present on the Ad2 DBP (Reich *et al.*, 1983) was used in conjunction with a Texas Red conjugated goat anti-mouse immunoglobulin whereas an FITC conjugated goat anti-

rabbit immunoglobulin was used to localise the rabbit antibodies bound to NFI.

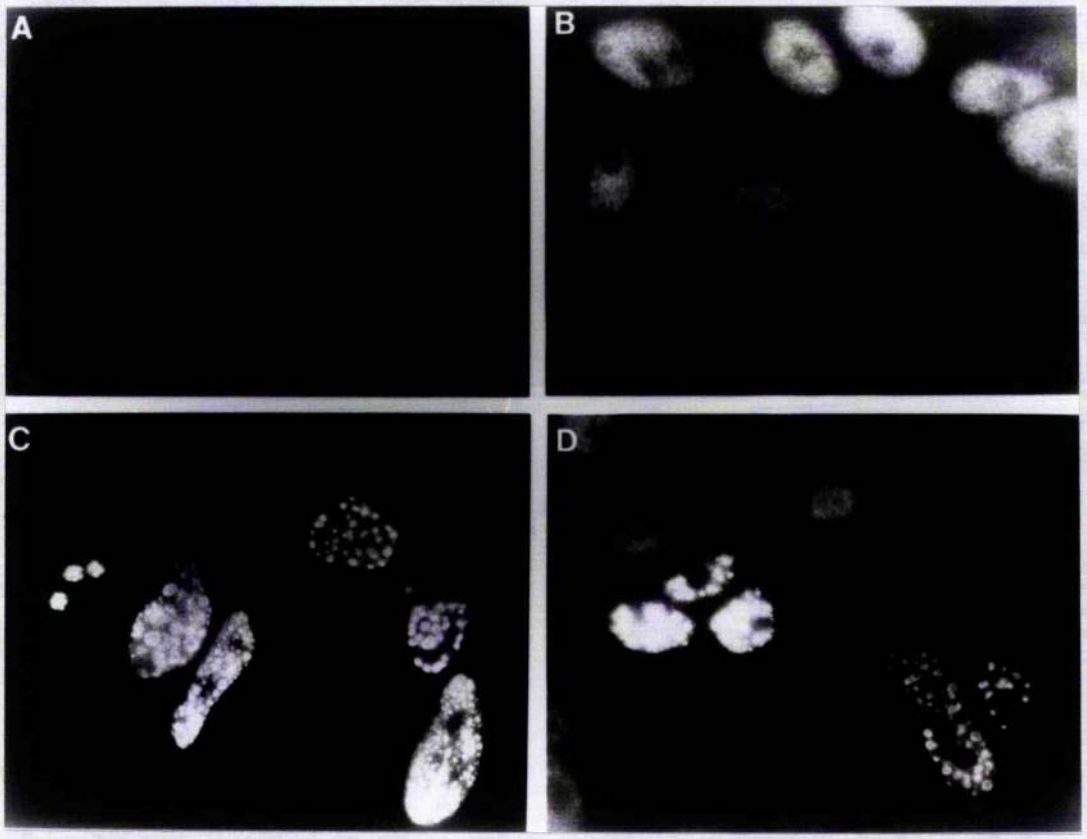


FIGURE 13. Cellular distribution of NFI (B and D) and DBP (A and C) before (A and B) and after (C and D) infection for 24 hours with Ad2.

Figure 14 clearly shows that NFI and DBP are colocalised to the same sites within Ad2 infected cells.

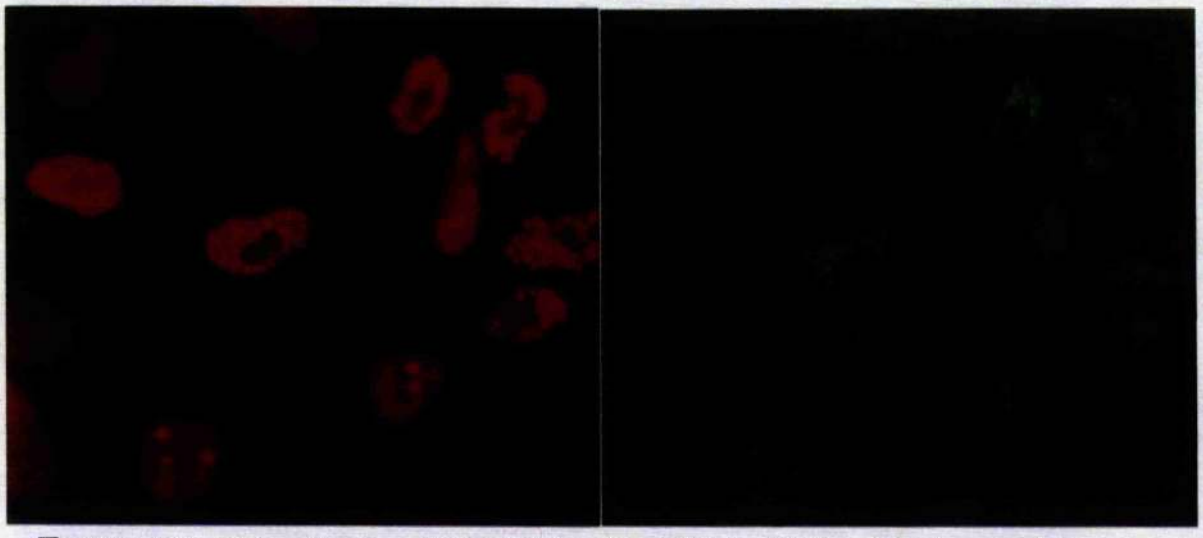


FIGURE 14. Colocalisation of DBP (A) and NFI (B) in A549 cells infected with Ad2 and examined by conventional fluorescent microscopy.

To more precisely demonstrate the identity of the sites of NFI and DBP localisation Ad2 infected cells were examined by laser scanning confocal microscopy. Shown in figure 15 is the fluorescence emmision from a 0.5mm thick optical section taken through Ad2 infected A549 cells treated with NFI and DBP antibodies as described previously. It is evident from this optical section that NFI (fig. 15H) and DBP (fig. 15G) are indeed present at the same subcellular locations in infected cells thus confirming the results of the conventional microscopy. Confocal microscopy allows the two separate NFI and DBP images to be merged to demonstrate absolutely that they are colocalised (fig 15M). Control experiments where one of the primary antibodies had been omitted indicated that, under the conditions employed in these experiments, crossover in the FITC and Texas Red channels was negligible.

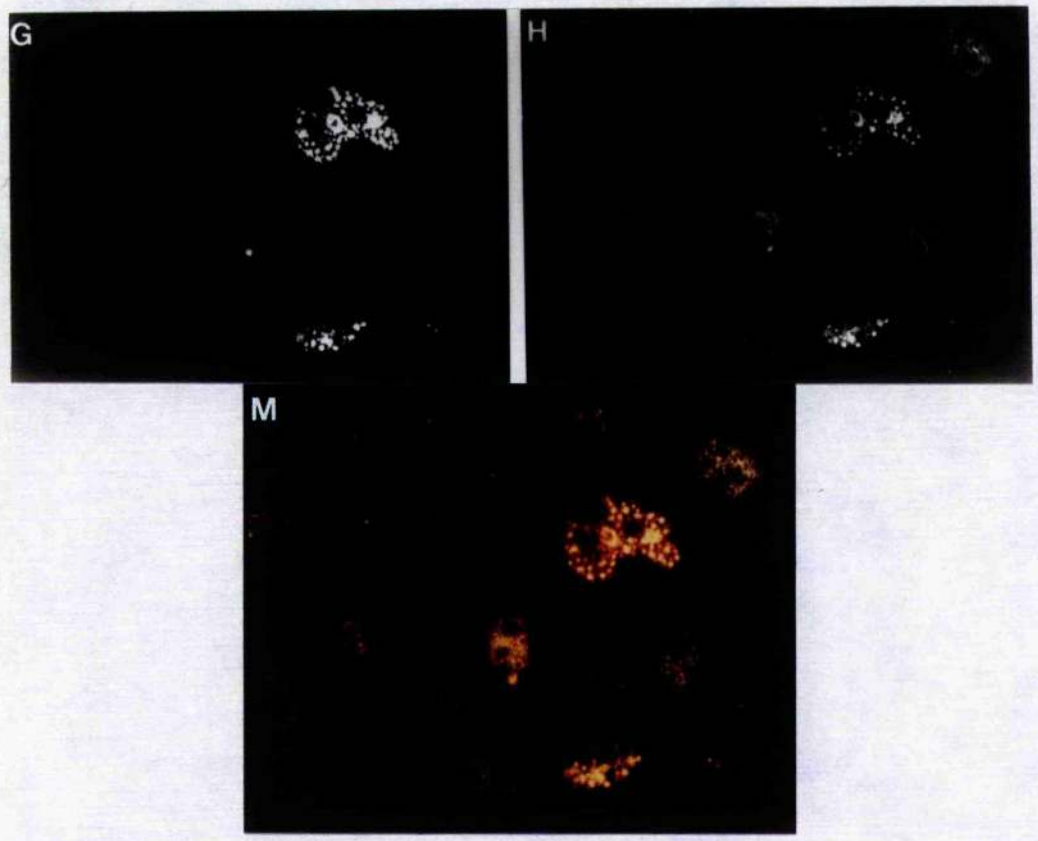


FIGURE 15. Colocalisation of DBP (G) and NFI (H) in A549 cells infected with Ad2 and examined by laser scanning confocal microscopy. M. The image from G and H has been coloured and merged to demonstrate how precisely they colocalise.

Effect of various times post infection.

To investigate the point in the infectious cycle when DBP and NFI distribution is altered, cells were fixed at various times after infection and processed for microscopy as described previously. At a low multiplicity of infection (1p.f.u./cell), 18 hours after infection DBP localisation takes one of two forms, being either punctate or evenly distributed (fig. 16A). In the cells where DBP is localised to discrete sites it is apparent that NFI is also concentrated in these sites (fig. 16B). As the infection proceeds the proportion of DBP in discrete sites

increases, being initially present in a small number of large foci (fig. 16C) and at later stages in a larger number of smaller foci (fig. 16E). This behaviour is also observed with NFI with the protein apparently being localised to the same discrete sites as DBP (fig.. 16D and 16F).

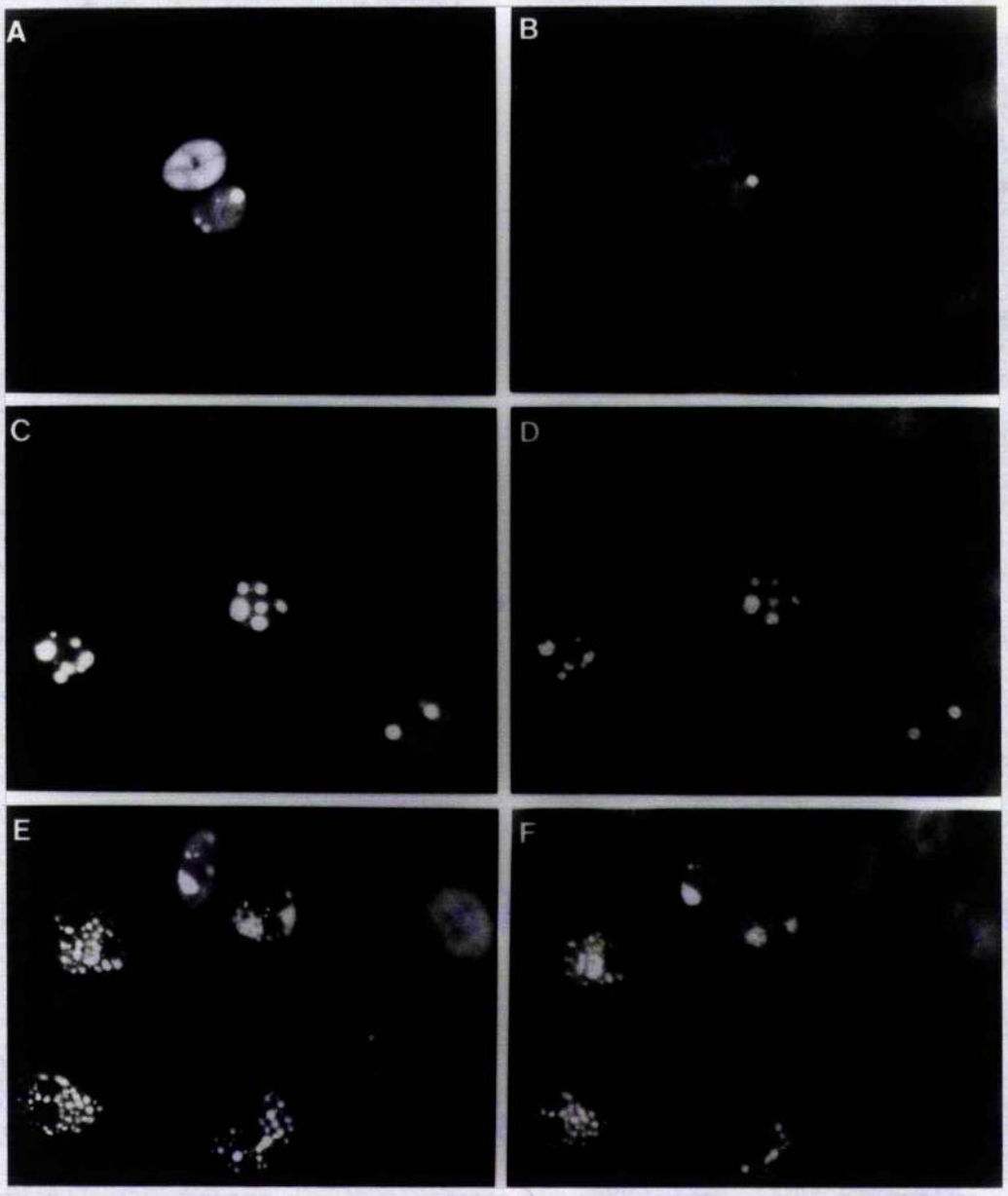


FIGURE 16. Effect of various times post infection on the distribution of DBP and NFI. A549 cells were infected at a low multiplicity of infection (1p.f.u./cell) for 18 hours (A and B), 24 hours (C and D) and 48 hours (E and F).

Ad2 DBP is localised to sites of viral DNA replication.

To determine if the regions to which NFI and DBP are colocalised are sites of viral DNA replication Ad2 infected A549 cells were pulse labelled with the thymidine analogue 5-bromodeoxyuridine (BrdU). Newly synthesised DNA which incorporated BrdU can then be immunolocalised with a monoclonal antibody specific for BrdU, provided that the DNA has been rendered single stranded. In the procedures developed for the detection of newly synthesised cellular DNA (Fox *et al.*, 1991) single stranded DNA is generated by the combined action of *Eco* RI and exonuclease III. However, since adenovirus replicates by a strand displacement mechanism which generates single stranded DNA nuclease treatment of the cells is unnecessary. This difference therefore provides a convenient means of distinguishing between newly replicated cellular and viral DNA. Ad2 DNA, but not cellular DNA, will be detected in the absence of nuclease treatment whereas both Ad2 and cellular DNA will be detected after nuclease pretreatment. A549 cells infected with Ad2 for 24 hours were pulse labelled for 15 minutes with BrdU and either pretreated with nuclease or processed directly. Ad2 DBP was detected with a rabbit polyclonal antibody (fig. 17A and 17C) whereas BrdU was detected with a mouse monoclonal antibody (fig. 17B and 17D). It is clear that the previously identified sites of DBP accumulation are also sites of BrdU incorporation (fig. 17; compare panels A with B and C with D). As these sites of BrdU incorporation are detected either with (fig. 17D) or without (fig. 17B) nuclease pretreatment this indicates that the areas of DBP accumulation are also sites of viral DNA replication. It is worth noting that the pattern of DBP fluorescence in nuclease treated cells is less well defined than in untreated cells (fig. 17; compare panels A and

C) and this may be a consequence of the nuclease treatment releasing genome bound DBP.

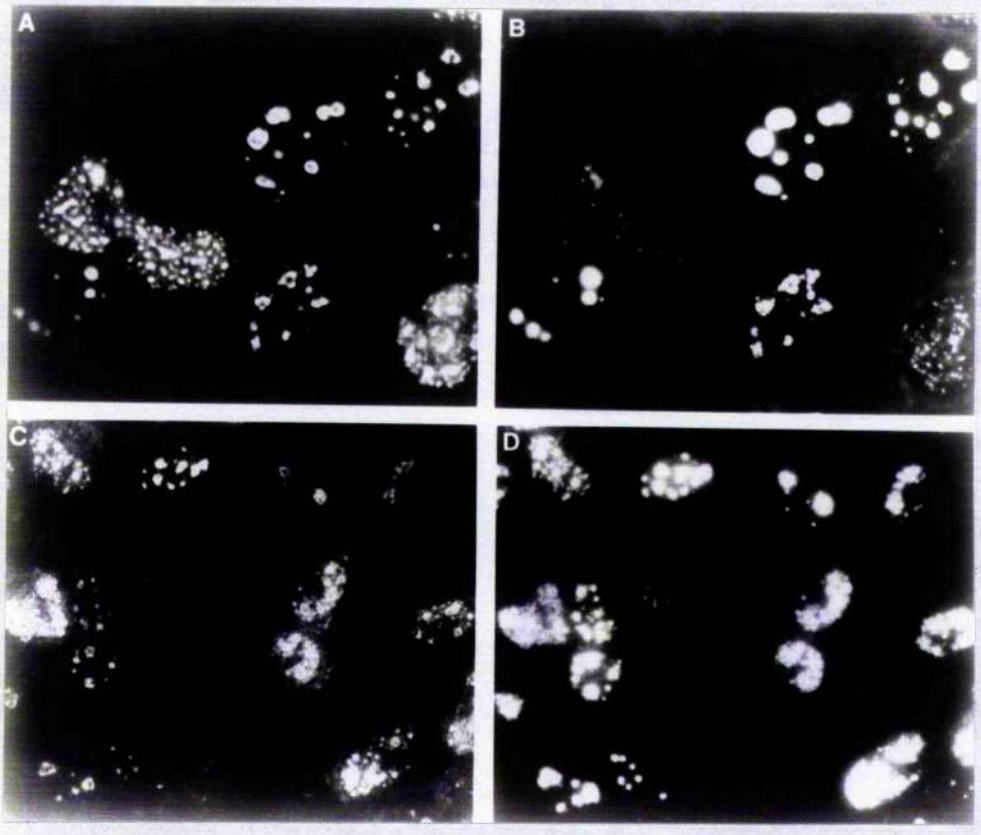


FIGURE 17. Colocalisation of DBP (A and C) and BrdU-labelled DNA (B and D) in A549 cells infected with Ad2. Panels A and B are immunofluorescence pictures of cells that were not treated with *Eco* RI-exonuclease III, whereas panels C and D are of cells that were treated with *Eco* RI-exonuclease III.

NFI does not colocalise with DBP in adenovirus type 4 infected cells

While it is evident from figures 14 and 15 that Ad2 DBP and NFI are present in the same subnuclear sites it is not clear whether this is a specific targeting event or whether it is a non-specific consequence of structural rearrangements that take place in the nucleus after infection. To address this question we made use of adenovirus type 4 (Ad4) which replicates efficiently in A549 cells but which does not appear to require the involvement of NFI. The Ad4 origin of DNA replication lacks an NFI binding site and addition of purified NFI fails to stimulate Ad4

DNA replication *in vitro* (Hay, 1985; Harris and Hay, 1988). A549 cells were infected with Ad4 and the location of NFI and DBP determined by double immunofluorescence using rabbit antibodies specific for NFI and mouse antibodies specific for DBP. The mouse monoclonal antibody that recognises an epitope present on Ad2 DBP (Reich *et al.*, 1983) also recognises a similar epitope present on Ad4 DBP (Temperley *et al.*, 1991). As in Ad2 infected cells, Ad4 DBP is localised to discrete sites within the nucleus (fig. 18A and 18C) but in contrast the distribution of NFI remains diffuse and clearly does not colocalise with Ad4 DBP (fig. 18B and 18D). Thus the redistribution of NFI in Ad2 infected cells is likely to be the result of a targeting event specific to this particular virus that reflects the requirement for NFI in Ad2 DNA replication.

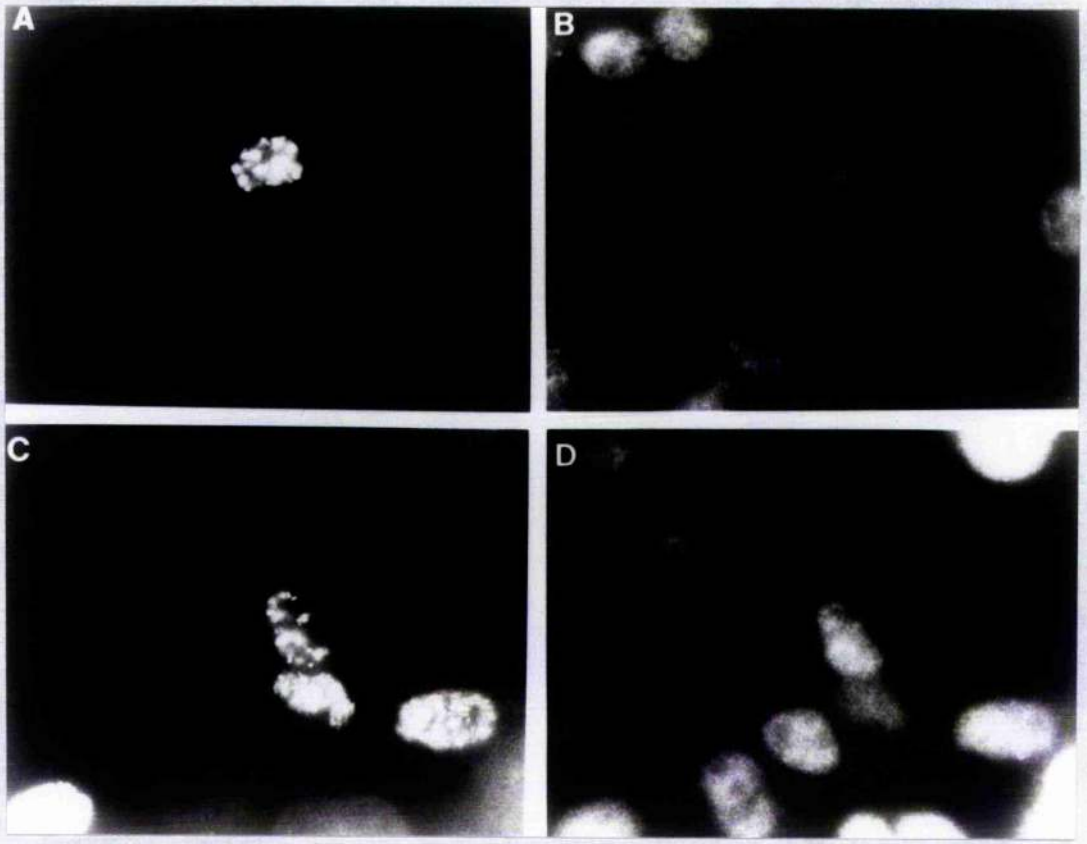


FIGURE 18. NFI does not colocalise with DBP in Ad4 infected cells. DBP (A and C) and NFI (B and D) distribution in A549 cells infected for 12 hours (A and B) or 18 hours (C and D) with Ad4.

Nuclear distribution of NFI and Ad2 DBP in the absence of viral DNA replication

Although the results obtained above indicate that the nuclear redistribution of NFI that takes place after infection is specific to Ad2, it is not clear if this event precedes, or is a consequence of viral DNA replication. To provide an answer to this question A549 cells were infected with Ad2 at a high multiplicity of infection in the presence of 10mM hydroxyurea which inhibits viral and cellular DNA replication by depleting the intracellular pool of nucleotides. Double immunofluorescence analysis of untreated cells infected at the same multiplicity of infection revealed the expected colocalisation of NFI and DBP at a limited number of nuclear sites (fig. 19C and 19D). However analysis of infected cells in which DNA replication was blocked with hydroxyurea indicated that although the two proteins still appeared to colocalise the distribution of sites within the nucleus was different from that observed in untreated infected cells (fig. 19A and 19B). In hydroxyurea treated cells NFI and DBP were present in a large number of 'prereplicative sites', that although punctate in appearance were considerably smaller than the areas of fluorescence observed in untreated Ad2 infected cells (fig. 19; compare A and B with C and D). NFI and DBP therefore appear to colocalise in Ad2 infected cells even in the absence of viral DNA replication.

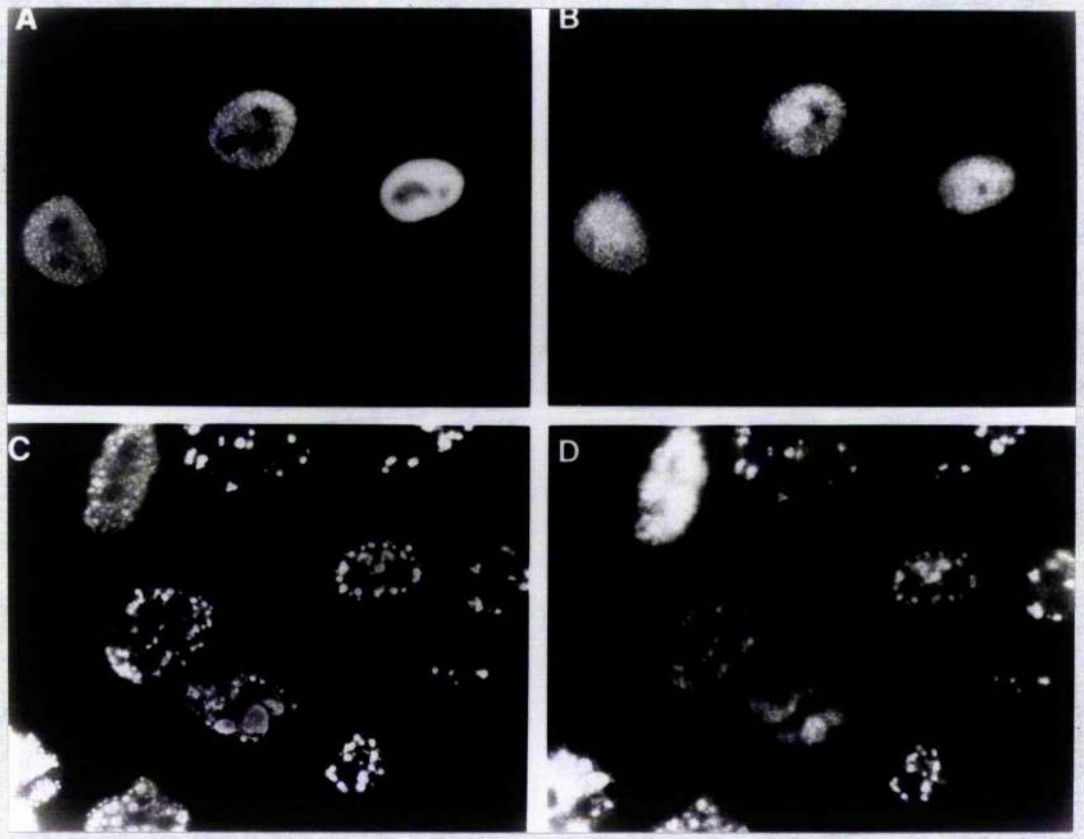


FIGURE 19. A549 cells were infected at 10p.f.u./cell with Ad2 for 18 hours in the presence (A and B) or absence (C and D) of 10mM hydroyurea to investigate whether the distribution of DBP (A and C) and NFI (B and D) changed when DNA replication was inhibited.

SUPPLEMENT

Fate of NFI after Ad2 infection of A549 cells

Figure 12 is a composite figure showing that the overall amount and DNA binding activity of NFI does not change throughout the course of infection.

A. demonstrates that the polyclonal antisera raised against NFI_{DBD} (α-NFI) immunoprecipitates (IP) NFI from an extract (Ex) of *Spodoptera frugiperda* cells infected with NFI expressing baculovirus (AcNPV NFI). This immunoprecipitation should have been duplicated using antisera obtained from the rabbit before it was immunised with NFI to show that the precipitating antibodies were the result of an immune response against NFI.

B. The first panel is a Western Blot which demonstrates that α-NFI antibody recognises denatured, full length and the DNA binding domain of NFI from extracts of *Spodoptera frugiperda* cells infected with recombinant baculovirus. Unfortunately the second track is hard to interpret because NFI_{FL} is prone to proteoytic attack resulting in only the DNA binding remaining intact. To clarify this it is advisable to leave empty wells between samples so that the charge of overspill can be properly refuted. This figure should also have included a sample of uninfected cells or cells infected with an alternative recombinant baculovirus to demonstrate more clearly the specificity of the antisera.

D. is a gel retention assay demonstrating that the DNA binding activity of NFI does not vary as infection proceeds. The last three lanes are competition assays where 100-fold molar excess of competitor oligonucleotides containing the DNA binding sites of either NFI, NF-κB or NFIII were also added. These demonstrate that the major protein-DNA complex formed is specific to NFI binding to its recognition site. This assay could also have been used to test the antisera by

preincubating the extracts with it before addition of the labelled oligonucleotide to see if the antibodies abolished the DNA binding activity of NFI or supershifted the protein-DNA complex.

CHAPTER THREE

Discrete subnuclear sites of transcription in Ad2 infected cells.

Since DBP and NFI colocalise at discrete sites of viral DNA replication perhaps other nuclear processes also occur at discrete subnuclear sites.

E1a does not colocalise with DBP in Ad2 infected cells

To investigate the relative distribution of DBP and the viral transcriptional activator E1a in Ad2 infected cells antibodies to DBP and E1a were applied to the same cells and visualised by double fluorescence and laser-scanning confocal microscopy. Monoclonal antibodies directed against epitopes on E1a were used in conjunction with a Texas Red conjugated donkey anti-mouse immunoglobulin whereas an FITC conjugated donkey anti-rabbit immunoglobulin was used to localise the rabbit antibodies bound to DBP. Figure 20A shows the usual punctate distribution of DBP in A549 cells infected at a high multiplicity of infection (50 p.f.u./cell) for 18 hours as viewed by laser scanning confocal microscopy. Clearly although E1a (fig. 20B) is distributed to discrete sites within the nuclei they are very distinct from those of DBP a fact that is highlighted when the two separate DBP and E1a images are coloured and merged (fig. 20C).

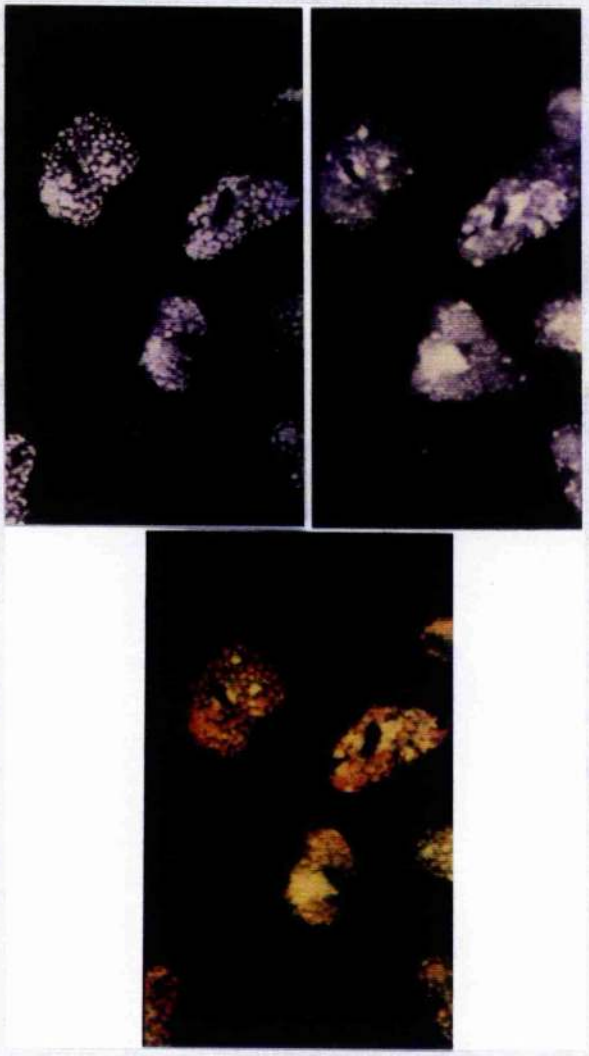


FIGURE 20. E1a (B) does not colocalise with DBP (A) in A549 cells infected with Ad2 and examined by laser scanning confocal microscopy.
C. The image from A and B has been coloured and merged to demonstrate how they do not colocalise.

Synchronisation of cells at the G1/S boundary of the cell cycle

A549 cells were grown to 80% confluence and then 1μg/ml aphidicolin was used to block DNA synthesis thereby synchronising the cells at the G1/S boundary of the cell cycle. The aphidicolin block was removed after 18 hours and its effectiveness and the passage of the cells through the cell cycle was monitored using FACScan Flow Cytometry.

Figure 21 illustrates the effectiveness of aphidicolin-mediated synchronisation of uninfected cells: two G1 peaks occur 12 hours apart and S-phase occurs between 1 and 7 hours after the removal of aphidicolin peaking at 4 hours with 78% of the cells.

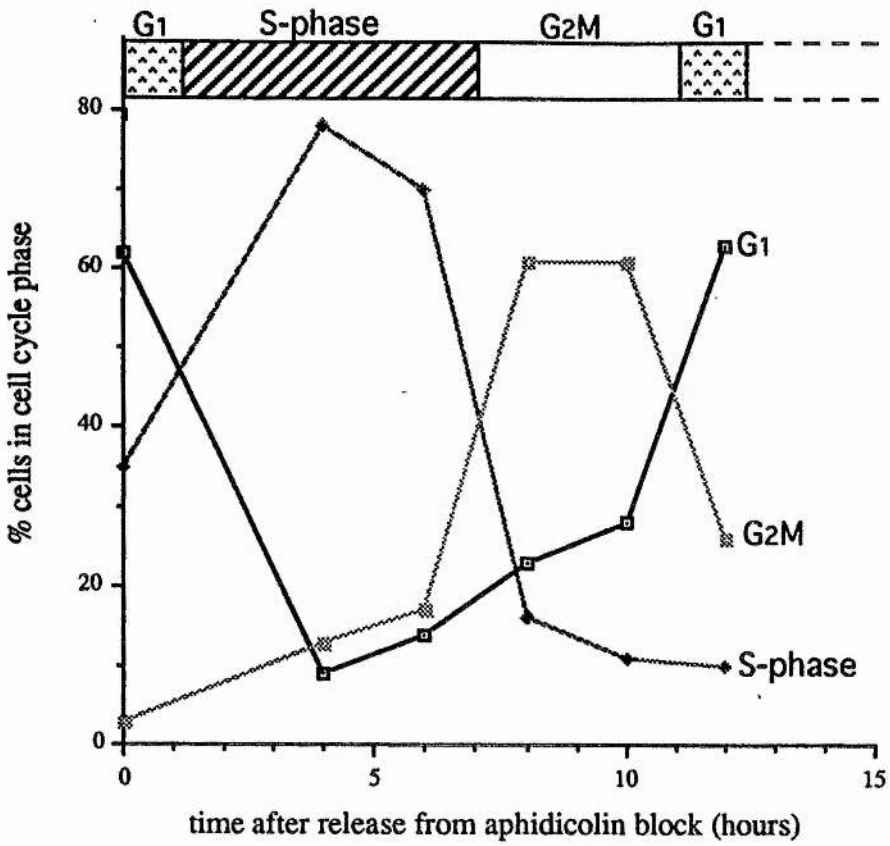


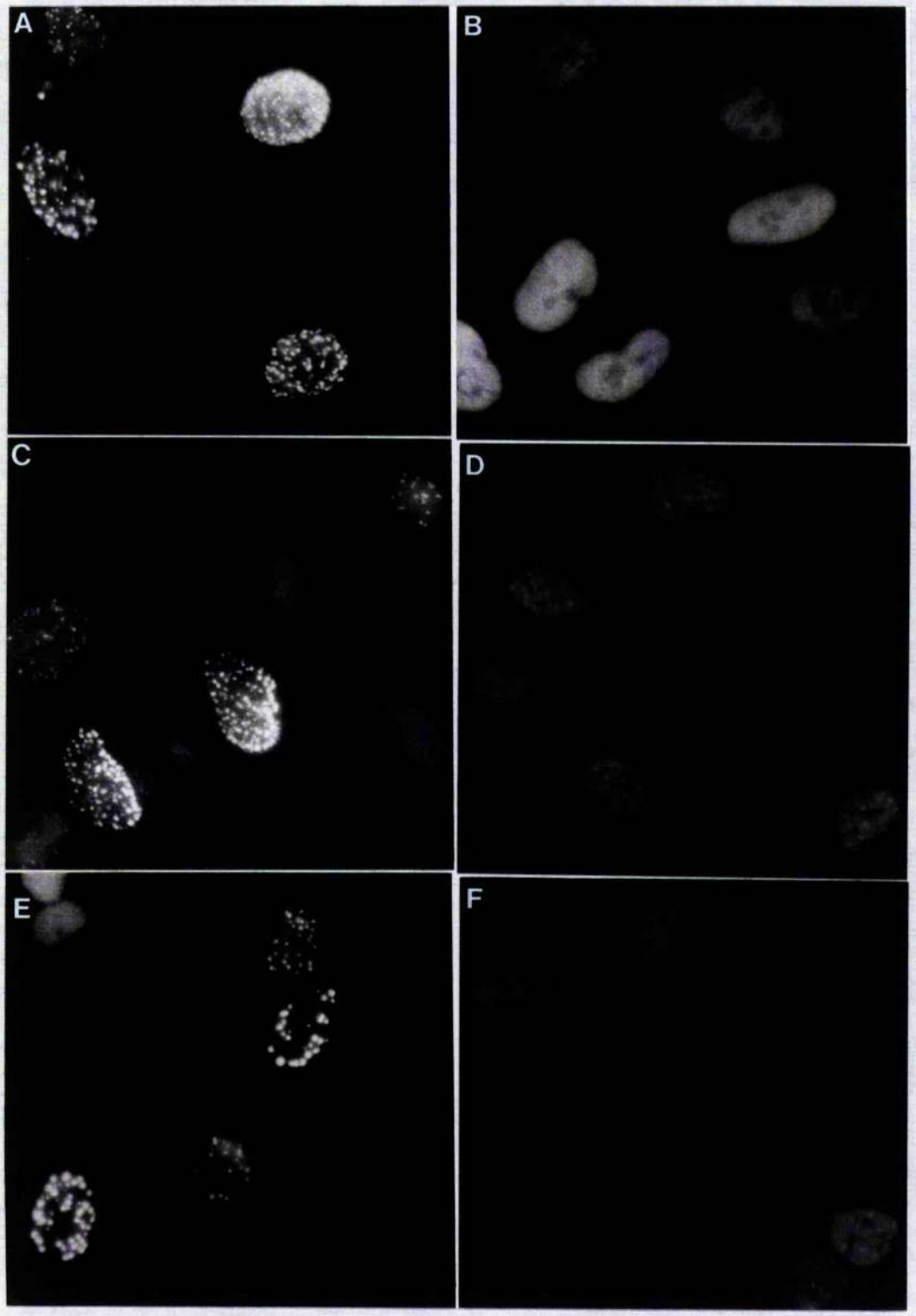
FIGURE 21. The passage of uninfected cells through the cell cycle after release from their aphidicolin-mediated synchronisation at the G_1/s-phase boundary.

Subsequent experiments involved studying the nuclear localisation of a number of proteins in A549 cells that were in G_1, S-phase or G_2M at the point of Ad2 infection. The cells were infected at specific times after the aphidicolin block on DNA synthesis was removed: zero hours for G_1, five hours for S-phase and eight hours for G_2M.

Effect of host cell cycle on DBP and E1a localisation

A549 cells were infected in G_1, S-phase and G_2M at a high multiplicity of infection (50 p.f.u./cell) for 12 hours. The distribution of DBP and E1a was investigated by single fluorescence and conventional fluorescence microscopy. Monoclonal antibodies directed against DBP or E1a were used in conjunction with a Texas Red conjugated donkey anti-mouse immunonglobulin. Figures 22A, C, E show the pattern of DBP distribution in cells that were infected in G_1, S-phase and G_2M respectively. There are no striking differences in DBP distribution between cells infected in G_1 (fig. 22A), S-phase (fig. 22C) and G_2M (fig. 22E). Conversely figures 22B, D, F show that E1a distribution is dependant on the cell cycle phase of the cells at the point of infection. Those infected in G_1 show a diffuse distribution of E1a throughout the nuclei (fig. 22B) whereas E1a in cells infected in S-phase (fig. 22D) and G_2M (fig. 22F) is localised to discrete subnuclear sites that are not at all reminiscent of DBP distribution.

FIGURE 22 (next page). A549 cells were infected at 50p.f.u./cell for 12 hours in G_1 (A and B), S-phase (C and D) and G_2M (E and F) and the distributions of DBP (A, C and E) and E1a (B, D and F) were examined using conventional fluorescence microscopy.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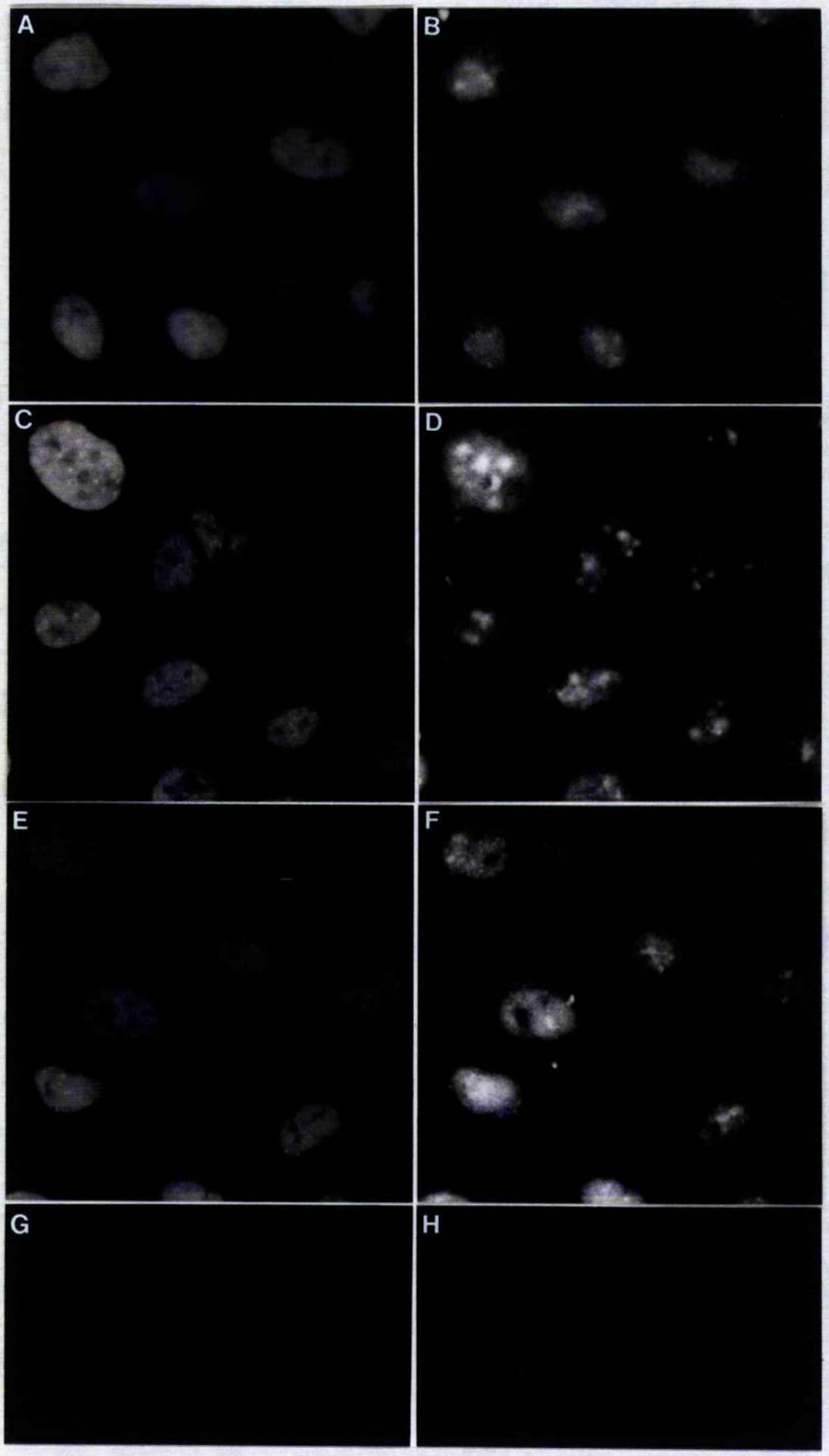
F

E1a does not colocalise with RNA polymerase II in Ad2 infected cells

Since the primary role of the E1a proteins is to stimulate viral gene transcription it seemed reasonable that the discrete subnuclear sites of E1a distribution in Ad2 infected cells could be sites of viral transcription. All mRNA transcripts that code for proteins are synthesised by cellular RNA polymerase II so its distribution in Ad2 infected cells was compared with that of E1a to see if they colocalised. Monoclonal antibodies directed against E1a were used in conjunction with a Texas Red conjugated donkey anti-mouse immunoglobulin whereas an FITC conjugated donkey anti-rabbit immunoglobulin was used to localise the rabbit antibodies bound to RNA polymerase II. The distribution of these proteins was examined in cells whose cell cycle had been blocked at the G_1/S boundary with aphidicolin and then released so that the cells were in G_1 (fig. 23A and B), S-phase (fig. 23C and D) and G_2M (fig. 23E and F). Once again it can be seen that the distribution of E1a is diffuse in G_1 (fig. 23A) but finely punctate in S-phase (fig. 23C) and G_2M (fig. 23E). RNA polymerase II is also found at discrete sites within the nuclei of cells infected with Ad2 during S-phase (fig. 23D) but these sites do not correspond with E1a localisation, in fact RNA pol II appears to be localised to the nucleoli (fig. 23D). In cells infected in G_1 or G_2M there are some feint indications that RNA polymerase II is distributed to the nucleoli but on the whole it seems diffusely distributed throughout the nucleus. Control experiments in which one of the primary antibodies were omitted indicated that there was no crossover in the FITC (fig. 23G) and Texas Red (fig. 23H) channels.

FIGURE 23 (next page). E1a (A, C and E) does not colocalise with RNA polymerase II (B, D and F) in cells that were infected in G_1 (A and B), S-phase (C and D) or G_2M (E and F).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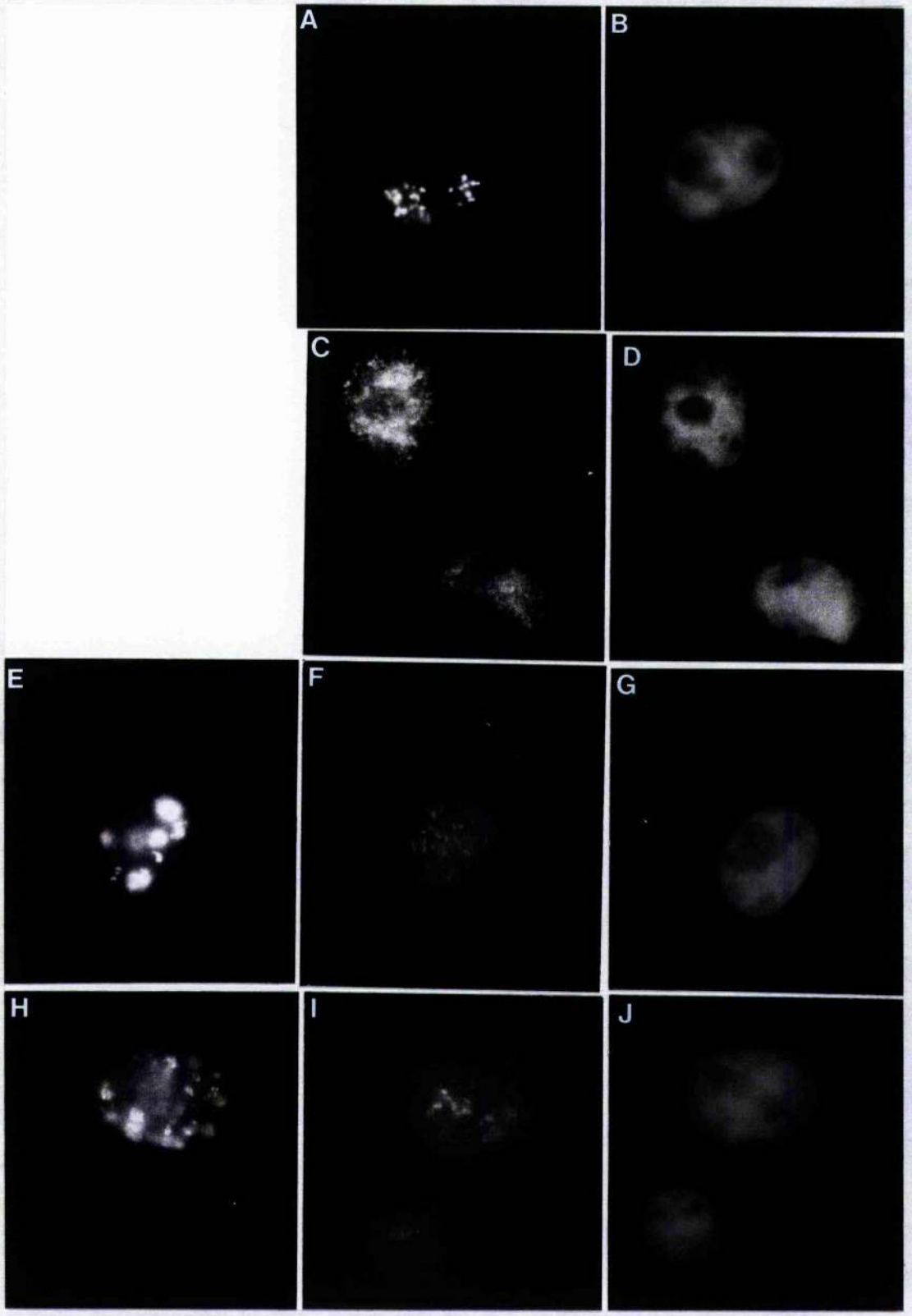
Visualisation of sites of transcription and replication in Ad2 infected cells

Ad2 infected cells (50 p.f.u./cell for 12 hours) and uninfected cells were encapsulated, lysed with streptolysin O and incubated for 15min with Br-UTP and/or Biotin-16-dUTP. After permeabilising the nuclear membrane with Triton X-100 and fixation with paraformaldehyde sites of Br-UTP incorporation were indirectly labelled using an antibody that reacts with Br-RNA followed by a second antibody conjugated with Texas Red. Sites of Biotin-16-dUTP incorporation were directly labelled using Streptavidin-FITC.

In uninfected cells most of the cells exhibit RNA polymerase II activity with the nuclei containing several hundred fluorescent foci spread throughout the extranucleolar regions (fig.24C). The nucleoli appear as dark 'holes' in DAPI stained cells (fig. 24B and D). In a minority of cells RNA polymerase I labelling is apparent with a number of fluorescent foci confined to the nucleoli (fig 24A).

In Ad2 infected cells there is a general reduction in Br-UTP incorporation (fig. 24F and I). DAPI staining of these cells (fig. 24G and J) illustrates the general reorganising of the cellular DNA which is excluded from the sites of viral DNA replication and the breakdown of the nucleoli. Labelled transcription sites mainly seem to be concentrated outside the sites of viral DNA replication (fig. 24E and H) although further experiments are required to optimise the double labelling of replication and transcription sites.

FIGURE 24 (next page). Sites of transcription were visualised in uninfected (A and C) and Ad2 infected (F and I) A549 cells by conventional fluorescence microscopy. Uninfected (B and D) and Ad2 infected (G and J) cells were also stained with DAPI and sites of DNA replication were visualised in Ad2 infected cells (E and H).


A
B
C
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SUPPLEMENT

Discrete subnuclear sites of transcription in Ad2 infected cells

Although E1a is the primary transactivator of early viral gene expression (Moran *et al.*, 1986) it is unable to directly bind to DNA (Ferguson *et al.*, 1985) and so operates by modifying the activity of cellular transcription factors. Therefore it would not be unreasonable for E1a not to localise to sites of transcription. A more appropriate candidate for studying the location of transcription sites is the transcription factor E2F which undergoes a dramatic change in activity during adenovirus infection (Kovesdi *et al.*, 1986) which is mediated by E1a. The 19kD gene product interacts with E2F enabling it to bind to the E2 promoter so that transcription can proceed (Huang and Hearing, 1989; Bagchi *et al.*, 1990; Raychaudhuri *et al.*, 1990). E1a's role in this is to interact with Rb thereby preventing it from interacting with E2F which would inhibit its ability to bind to the E2 promoter (Bagchi *et al.*, 1991). Since E2F actually binds to the E2 promoter it is much more likely to colocalise with sites of transcription than E1a. It would be interesting for further experiments to investigate whether E2F actually does localise to sites of transcription labelled with Br-UTP. Other future experiments could involve the use of inhibitors of transcription such as α-amanitin (which inhibits RNA pol II) to characterise the types of transcription being labelled using Br-UTP.

DISCUSSION

Adenovirus types 2 and 5 require the viral proteins pTP, pol and DBP plus two cellular proteins NFI and NFIII for efficient initiation of DNA replication. These proteins form a nucleoprotein preinitiation complex at the origin of DNA replication which involves a number of direct protein-protein and protein-DNA interactions as well as some indirect cooperative interactions. The data presented in Chapter 1 concerns the role of NFI in the formation of this nucleoprotein complex.

Ad2 initiation of DNA replication is affected by the relative concentrations of NFI and DBP. The initiation reaction is inhibited by DBP in the absence of NFI and stimulated in the presence of NFI (Nagata *et al.*, 1982). The stimulation of replication by NFI is strongly dependant on the concentration of DBP (de Vries *et al.*, 1985). DBP increases the affinity NFI has for its recognition site in the adenovirus origin of DNA replication (Cleat and Hay, 1989a; Stuiver and van der Vliet, 1990) in a specific reaction that cannot be substituted for by other single stranded DNA binding proteins such as *E. coli* SSB, HeLa SSB or T4 gene 32 protein (Cleat and Hay, 1989a). DBP increases the rate of NFI association with and decreases the rate of dissociation from its DNA recognition site (Cleat and Hay, 1989b) thereby resulting in increased binding of NFI. No direct interaction has been found between NFI and DBP so it seems most likely that DBP distorts the structure of the DNA so as to allow optimal base contacts between NFI and its recognition site thereby increasing its binding affinity (Stuiver *et al.*, 1992).

Native NFI consists of a family of related proteins which range in size from 52 to 66kD (Jones *et al.*, 1987) and are generated as a result of differential RNA splicing (Santoro *et al.*, 1988). Functional domains were identified (Mermod *et al.*, 1989; Meisterernst *et al.*, 1989; Gounari

et al., 1990) and found to consist of the N-terminal region which is required for DNA binding, dimerisation and stimulation of adenovirus DNA replication *in vitro* and the proline rich C-terminal region which is additionally required for transcriptional activation.

As expected the DNase I footprinting experiments in Chapter 1 showed that NFI_{FL} and NFI_{DBD} bind to the Ad2 origin of DNA replication with similar affinity and indistinguishable pattrerns of DNase I protection. Futhermore they demonstrate that DBP can cooperate with the DNA-binding domain of NFI to increase its affinity for its recognition site which is reasonable since NFI_{DBD} is sufficient to stimulate Ad2 initiation of DNA replication. Although it was known that initiation of adenovirus DNA replication is stimulated by the N-terminal region of NFI and that this stimulation is dependant on NFI binding to its recognition site (Leegwater *et al.*, 1985) its role in adenovirus DNA replication was still not clear.

The NFI recognition site lies between base pairs 24 and 39/40 (Leegwater *et al.*, 1985) in the Ad2 and Ad5 origins of DNA replication. The terminal 18bp is referred to as the 'core' of the replication origin and is present in Ad4 as well as Ad2 and Ad5. Ad4 only needs this core region for efficient initiation of DNA replication (Hay, 1985) but in Ad2 and Ad5 it can only support a limited level of DNA replication (Tamonoi and Stillman, 1983; Challberg and Rawlins, 1984). The spacer region between the core and the NFI recognition site was found to be essential with any insertions of DNA sequences reducing the level of initiation of DNA replication to that of the core only (Bosher *et al.*, 1990). This strict spatial arrangement of the NFI binding site with respect to the core region suggested the possibility that NFI mediated stimulation of DNA replication involves a direct or indirect interaction with a replication component at the core. If the role of NFI in

adenovirus replication involves a direct protein-protein interaction then if must be accomplished by the DNA binding domain since it carries out all the Adenovirus DNA replication functions of NFI. The most likely candidate for a protein-protein interaction with NFI at the origin core was the DNA polymerase. Initiation of adenovirus DNA replication takes place when a covalent linkage between the α-phosphoryl group of the terminal nucleotide (dCMP) and the β-OH group of a serine residue in pTP is formed. Pol and pTP form a very tight heterodimer which requires glycerol gradient sedimentationin the presence of 1.7M urea to be broken (Lichy *et al.*, 1982). It seemed likely that the pol-pTP heterodimer would be positioned at the core region of the origin of replication and one or both proteins could interact with NFI. This was investigated using very small columns containing 50µl of NFI_{DBD} coupled to CNBr activated sepharose. Extracts of *S. frugiperda* cells that had been infected with recombinant baculoviruses expressing pTP, pol or a mixture of both were applied to the columns. It was shown that pol interacts with NFI_{DBD} in isolation and as part of the pTP-pol heterodimer. So the proposed role for NFI in Ad2 DNA replication is to load the pol-pTP heterodimer onto the origin of DNA replication so that replication can be initiated. NFI binds to the DNA as a dimer making contacts with its symmetrical recognition site in two major grooves (de Vries *et al.*, 1987) which means that each dimer of NFI could potentially bind two molecules of the pTP-pol heterodimer. Unfortunately only one of these would be correctly positioned for initiation (fig. 25) which suggests that other interactions may exist to stabilise the pTP-pol heterodimer position at the terminus of the adenovirus genome. One possibility is that the pTP-pol heterodimer could specifically recognise DNA sequences in the core region thereby stabilising the preinitiation complex. Previously Kenny and Hurwitz (1988) demonstrated that

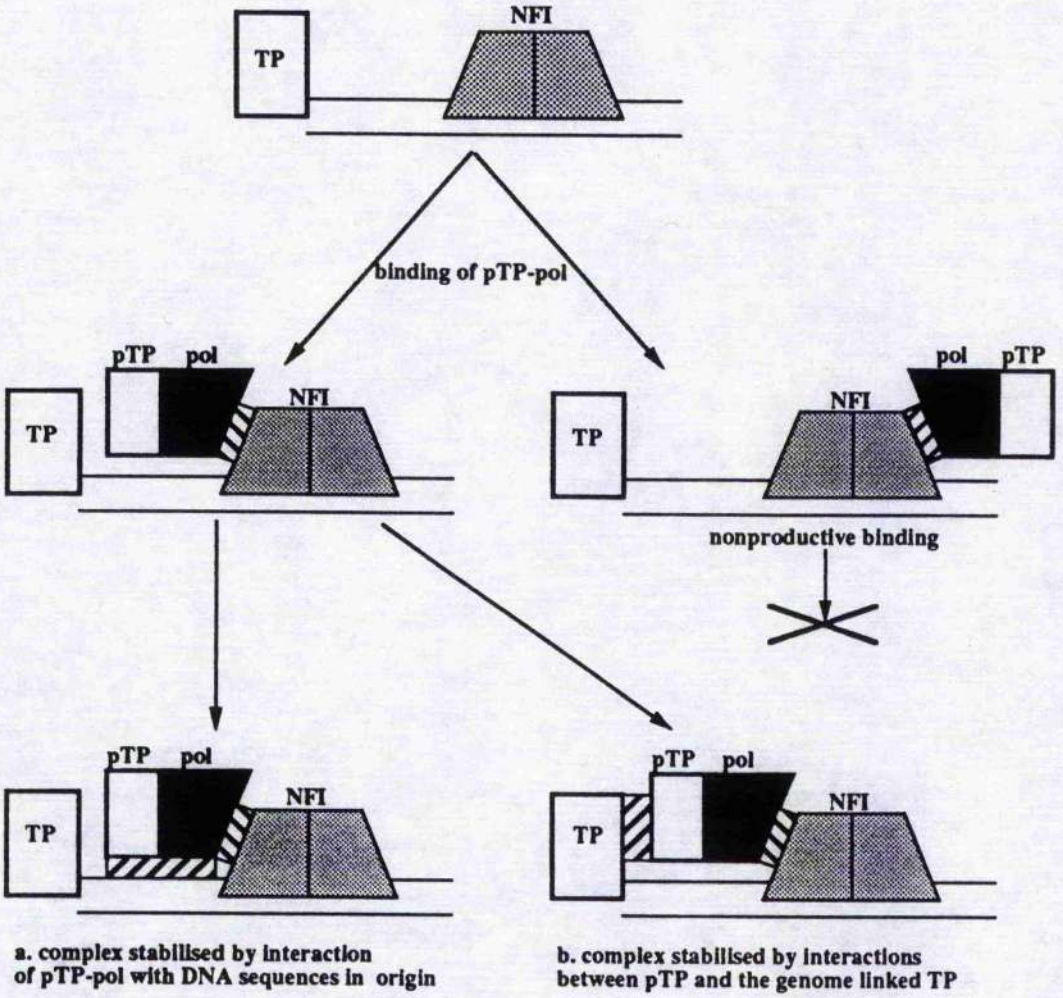

FIGURE 25. A model for the interaction of viral and cellular proteins at the adenovirus type 2 origin of DNA replication.

nucleotides between positions 15 and 22 on the displaced strand are crucial for pTP-pol complex formation and that they increase the affinity of pTP-pol for the DNA. However this was suggesting that the origin DNA had to be unwound before the pTP-pol heterodimer could bind to it. It has since been demonstrated using gel electrophoresis DNA binding assays and DNase I footprinting that purified pol and pTP can

specifically bind to the core region protecting base pairs 8 to 17 from DNase I cleavage (Temperley and Hay, 1992). This pTP-pol heterodimer binding site corresponds to the sequence in the core that is perfectly conserved in all human adenoviruses sequenced to date (Tamonoi and Stillman, 1983; Challberg and Rawlins, 1984). Another possibility is that pTP makes direct contact with the genome linked TP and it has been found that template DNA attached to TP can support levels of DNA replication *in vitro* up to 20 fold higher than template in isolation (Tamonoi and Stillman, 1982; van Bergen *et al.*, 1983; Challberg and Rawlins, 1984; Harris and Hay, 1988). Indeed it has been reported that TP-DNA is able to bind pTP-pol more efficiently than protein free DNA with there being no effect on NFI binding (Pronk and van der Vliet, manuscript in preparation; cited in Pronk *et al.*, 1992). A third possibility is that the binding of NFIII to its recognition site simply blocks the interaction of the pTP-pol heterodimer to that side of the NFI dimer.

It is therefore possible to propose a sequence of events that lead to the formation of a preinitiation complex at the origin of DNA replication in Ad2 and Ad5 (fig. 26). DBP which is produced in large amounts, interacts nonspecifically with the adenovirus genome thereby facilitating the binding of NFI (Cleat and Hay, 1989; Stuiver and van der Vliet, 1990). NFIII binds to its recognition site adjacent to NFI. The pol-pTP heterodimer interacts with NFI (Bosher *et al.*, 1990; Chen *et al.*, 1990; Mul *et al.*, 1990) via pol and is loaded onto the core region. This preinitiation complex is stabilised by additional interactions such as between genome TP and pTP and/or between pTP-pol and the core region DNA (Temperley and Hay, 1992). This nucleoprotein complex must then isomerise to unwind the origin region and expose the single stranded DNA that is the template for the initiation reaction. Ad4 does

not require cellular proteins for efficient initiation of DNA replication. The loading of Ad4 pTP-pol heterodimer onto the origin may involve a more stable site specific interaction between pTP-pol and the core of the replication origin resulting in no need for cellular factors.

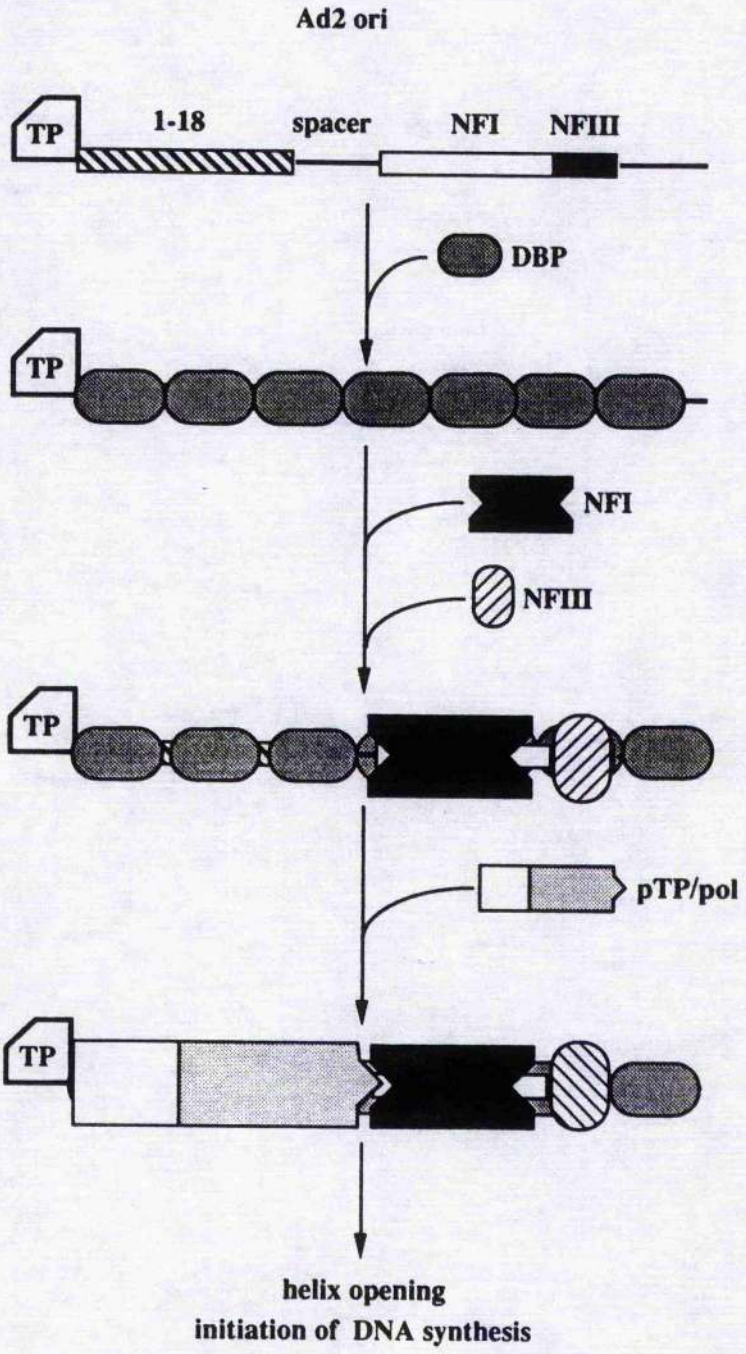

FIGURE 26. A model for the formation of the preinitiation complex at the adenovirus type 2 origin of DNA replication.

The location and function of nucleoprotein complexes in adenovirus type 2 infected cells

Nuclei contain numerous distinct compartments which are not membrane bound where processes such as DNA replication and transcription take place in uninfected cells and cells infected by a variety of viruses.. As discussed in the introduction immunofluorescence microscopy and electron microscopy have demonstrated that DBP, pol and pTP colocalise at presumed sites of adenovirus DNA replication (Sugawara *et al.*, 1977; Reich *et al.*, 1983; Sasaguri *et al.*, 1987; Voelkerding and Klessig, 1986; Murti *et al.*, 1990).

Immunofluorescence microscopy has revealed that NFI is distributed evenly throughout the nuclei of human cells. However upon infection with Ad2 this distribution is dramatically altered with NFI being localised to discrete subnuclear sites which correspond to the location of DBP. The sites of viral DNA synthesis were labelled by incorporation of bromodeoxyuridine into synthesising DNA within Ad2 infected A549 cells. It was found that NFI and DBP colocalise to these sites of active viral DNA synthesis. A convenient way of differentiating between labelled sites of viral and cellular DNA synthesis is provided for by the mechanism of adenovirus DNA replication. The monoclonal antibody used to visualise the incorporated BrdU requires that the DNA be rendered single stranded which normally involves treating the cells with *Eco* RI and exonuclease III. However since adenovirus replicates by a strand displacement mechanism this is unnecessary and allows for the visualisation of viral replication sites only.

This redistribution of NFI to sites of viral DNA replication in Ad2 infected cells is thought to be a specific targeting event that reflects the requirement for NFI in Ad2 DNA replication, rather than a non-specific aggregation of nuclear proteins brought about by infection. The

most striking evidence to support this proposal is that infection of A549 cells with Ad4 does not result in the relocalisation of NFI even though the Ad4 DBP is localised to discrete sites that are indistinguishable from those present in Ad2 infected cells. Ad4 does not contain an NFI binding site in its origin of DNA replication and the presence of an NFI binding site *in vivo* (Hay, 1985; van Bergen *et al.*, 1983) or the addition of NFI to an *in vitro* replication assay, even when the template contains the cognate binding site (Harris and Hay, 1988; Temperley and Hay, 1991) does not stimulate viral DNA replication.

How NFI is directed to these subnuclear sites is unknown but a number of possibilities exist. It may simply be a matter of NFI binding to its DNA recognition site which is stimulated and stabilised by a number of protein-protein and protein-DNA interactions (Cleat and Hay, 1989a; Stuiver and van der Vliet, 1990; Bosher *et al.*, 1990; Chen *et al.*, 1990; Mul *et al.*, 1990) to form the nucleoprotein complex. On the other hand it could be more complicated. NFI contains a recognisable nuclear localisation signal within its DNA binding domain (Gounari *et al.*,1990) which is presumably required to direct the protein into the nucleus however it seems unlikely that nuclear localisation sequences (Silver, 1991; Dingwall, 1991) are also used for intranuclear targeting. NFI may have an intranuclear targeting sequence such as the one found in DNA methyltransferase (Leonhardt *et al.*, 1992) that is exploited by Ad2 during DNA replication. DNA MTase has a diffuse nuclear distribution throughout G_1 and G_2 but during S-phase it is localised to the sites of DNA synthesis. The sequence required for this localisation has been defined as being towards the N-terminus of the protein between amino acids 207 to 455 which is separate from the nuclear localisation sequence at amino acids 72 to 92.

A number of DNA replicative proteins have been found to localise to sites of DNA synthesis during S-phase some of which have also been shown to interact with the nuclear matrix at these sites. Proliferating cell nuclear antigen (PCNA), a component of the DNA polymerase δ haloenzyme, has several nuclear patterns during DNA synthesis which allow S-phase to be subdivided and is therefore often used as a marker to assess S-phase synchrony (Bravo and McDonald, 1987; Celis and Celis, 1985; Celis *et al.*, 1987). Polymerase α has also been shown to localise to sites of DNA synthesis in S-phase cells (Nakamura *et al.*, 1984) and has been found to bind tightly to granular structures in the nuclear matrix (Yamamoto *et al.*, 1984). Various experiments have provided evidence that the replication complex is attached to the nucleoskeleton via one or more of its components suggesting that replicating DNA is moved through it (Jackson and Cook, 1986; Nakayasu and Berezney, 1989; Mills *et al.*, 1989).

Herpes simplex virus (HSV) DNA replication also occurs in discrete subnuclear sites. The HSV DNA-binding protein, ICP8, is localised to very small, evenly distributed discrete foci prior to DNA replication (prereplicative sites) and to larger, globular foci during DNA replication (replication compartments, Quinlan *et al.*, 1984). ICP8 in prereplicative sites appears to be associated with the nuclear matrix since it is not extracted along with 99% of the cellular DNA. ICP8 in replication compartments on the other hand is extracted along with most of the cellular and HSV DNA which suggests that ICP8 dissociates from the nucleoskeleton for DNA replication to proceed (Quinlan *et al.*, 1984). In cells infected with HSV in the presence of phosphonoacetate (PAA, a specific inhibitor of HSV DNA polymerase) cellular DNA replication sites are found to localise to HSV prereplicative sites leading to the suggestion that cellular DNA replication complexes formed prior

to infection are incorporated into the viral prereplicative sites (de Bruyn Kops and Knipe, 1988). The HSV DNA polymerase is normally found in preplicative sites and replication compartments except when cells are infected with mutant viruses encoding defective ICP8 when the polymerase is diffusely distributed throughout the nucleus. In cells infected with mutant viruses encoding defective polymerase ICP8 is still localised to prereplicative sites indicating that ICP8 is involved in the assembly of prereplicative sites (Bush *et al.*, 1991). DNA synthesis inhibited in adenovirus infected cells by hydroxyurea results in a redistribution of both NFI and DBP to a very large number of very small discrete sites evenly distributed throughout the nucleus reminiscent of HSV prereplicative sites.

Although there is a body of evidence to support the proposal that cellular DNA replication occurs at fixed replication centres the same cannot be said for adenovirus and HSV DNA replication. However in SV40 Wessel *et al.* (1992) found that when bidirectional DNA unwinding is initiated at the viral origin of replication two T-antigen helicase hexamer complexes form a dodecamer complex at the replication fork through which the DNA is threaded producing single stranded loops rather than each hexamer complex moving along the DNA in opposite directions. This supports the model of SV40 DNA replication where DNA is threaded through a fixed replication center.

E1a, a viral transcriptional activator, and DBP were found to be distributed to different discrete sites within the nuclei of Ad2 infected cells. Futhermore the distribution of DBP is not dependant on the stage of the cell cycle that the cells are in when they are infected whereas E1a distribution is. Those infected in G_1 have a diffuse E1a distribution whereas those infected in S-phase and G_2M have a fine, punctate E1a distribution. White *et al.* (1988) distinguished five E1a patterns which

correlate with the time post infection. At 6-12 hours E1a staining was absent or diffuse; at 12-24 hours it was reticular, nucleolar or punctate and at 24-48 hours it was peripheral ar absent. For this thesis the cells were infected with 50p.f.u./cell for 12 hours because that gave the brightest E1a fluorescence. E1a is known to bind to the retinoblastoma gene product (Rb, Whyte *et al.*, 1988) which is diffusely distributed in Ad2 infected cells (Wilcock, 1991). Possibly E1a is bound to Rb in cells showing diffuse E1a distribution such as those in G_1. Since one of the goals of viral early protein expression is to push the cells into S-phase perhaps E1a binding to Rb helps achieve this by blocking the normal function of Rb of constraining growth.

In an effort to assign the E1a punctate pattern a functional signficance its distribution was compared to that of RNA polymerase II which transcribes all protein encoding mRNAs. However these proteins were shown not to colocalise in cells infected with 50p.f.u./cell for 12 hours. RNA pol II appears to be targeted to the nucleoli of the cells in S-phase whereas its distribution in G_1 and G_2M seems much more diffuse. In order to ascertain whether either or none of these patterns correspond to adenovirus transcription attempts were made to label sites of ongoing transcription. This was achieved by incubating encapsulated and permeabilised A549 cells with Br-UTP in a 'physiological buffer' followed by indirect labelling of nascent Br-RNA. RNA polymerases I and II are responsible for most nucleolar and extra-nucleolar cellular transcription respectively. Labelling sites of transcription in uninfected cells showed that RNA pol I transcription occurs at a number of discrete sites within the nucleoli and that RNA pol II transcription occurs at several hundred discrete subnuclear sites which are reminiscent of the pattern of replication sites in early S-phase cells. However later on in S-phase the two distribution patterns are very different (P.R. Cook,

personal communication). In the Ad2 infected cells the discrete sites of transcription seem to be clustered outside of the labelled sites of viral replication although further experiments are required to better define this. Adenovirus infection has a great affect on cellular processes resulting in the nucleoli being degraded (Phillips and Raskas, 1972) and rRNA synthesis being inhibited (Castiglia and Flint, 1983). Also host chromatin is excluded from sites of viral DNA replication to the margins of the nucleus (Puvion-Dutilleul and Puvion, 1991). From the results here it appears that transcription in adenovirus infected cells is occuring at different sites to Ad2 DNA replication.

Further experiments would involve optimising the simultaneous labelling conditions of transcription and replication sites as well as the use of inhibitors such as α-amanitin (which inhibits RNA pol II) to characterise the types of transcription being labelled. Also the localisation of transcription factors such as E2F could be investigated since E2F is known to bind to the E2 promotor along with the 19kD E4 gene product to stimulate transcription.

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