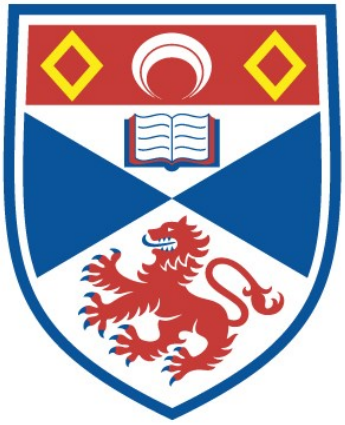
# A STUDY OF THE IN VITRO INITIATION OF ADENOVIRUS DNA REPLICATION

Simon M. Temperley

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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# A STUDY OF THE *IN VITRO* INITIATION OF ADENOVIRUS DNA REPLICATION.

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A Thesis presented for the degree of Doctor of Philosophy

at the University of St. Andrews, August 1991.

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**PUBLICATIONS RESULTING FROM THIS WORK.**

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## Abbreviations

| | |
|---|---|
| Ad | adenovirus |
| ATP | adenosine-5'-triphosphate |
| bp | base-pair(s) |
| $^{o}C$ | degrees celsius |
| Ci | curie(s) |
| cm | centimetre(s) |
| DBP | DNA binding protein |
| dATP | 2-deoxyadenosine-5'-triphosphate |
| dCMP | 2-deoxycytidine-5'-monophosphate |
| dCTP | 2-deoxycytidine-5'-triphosphate |
| dGTP | 2-deoxyguanosine-5'-triphosphate |
| dNTP | any 2-deoxyribonucleoside-5'-triphosphate (dATP, dCTP, dGTP or dTTP) |
| dTTP | 2-deoxythymidine-5'-triphosphate |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| DTT | dithiothreitol |
| EDTA | ethylene-diamine tetra-acetic acid sodium salt |
| fmol | femtomole(s) |
| g | gramme(s) |
| h | hour(s) |
| 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) |
| µl | microlitre(s) |
| mm | millimetre(s) |
| mM | millimolar |
| µM | micromolar |
| mmol | millimole(s) |
| µmol | micromole(s) |
| mRNA | messenger ribonucleic acid |
| m.u. | map units |
| M.W. | molecular weight |
| NFI | nuclear factor I |
| NFII | nuclear factor II |
| NFIII | nuclear factor III |
| NFIV | nuclear factor IV |
| ng | nanogramme(s) |
| nm | nanometre(s) |
| PAGE | polyacrylamide gel electrophoresis |
| pfu | plaque forming units |
| pmol | picomole(s) |
| PMSF | phenyl-methyl-sulphonyl-fluoride |
| pol | DNA polymerase |
| pTP | precursor terminal protein |
| pTP-pol | precursor terminal protein-DNA polymerase complex |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rpm | revolutions per minute |
| SDS | sodium dodecyl sulphate |
| sec | second(s) |
| T-Ag | Simian virus 40 large tumour antigen |
| TBE | Tris-borate EDTA buffer |
| TP | terminal protein |
| ts | temperature sensitive |

| | |
|---|---|
| VP | viral protein |
| v/v | volume to volume ratio |
| w/v | weight to volume ratio |

# CONTENTS

**Materials and Methods**

**Results**

## ABSTRACT

The development of systems in which adenovirus DNA can be replicated *in vitro* has led to the elucidation of the sequences essential for origin function and to the identification of the proteins required for viral DNA replication. Much of the information currently available has been derived from investigations carried out using adenovirus types 2 and 5 which in addition to the viral proteins, adenovirus DNA polymerase, precursor terminal protein and DNA binding protein, require cellular proteins nuclear factor I and nuclear factor III for efficient initiation of DNA replication. In contrast adenovirus type 4 replicates its DNA efficiently without these cellular proteins. Correspondingly its minimal origin of replication is remarkably simple in structure, consisting of only the terminal 18bp of the adenovirus genome ('the core sequence'). The effect of point mutations in the core region on adenovirus type 4 DNA replication *in vitro* was investigated and it was found that mutations within two discrete domains had a marked deleterious effect on initiation of DNA replication. The crude Ad4 infected cell extracts initially used for *in vitro* DNA replication were fractionated and it was found that only four detectable proteins, three of which were identified as viral DNA polymerase, precursor terminal protein and DNA binding protein gave efficient DNA replication *in vitro* and furthermore behaved similarly to unfractionated infected cell extracts in the presence of template which contained point mutations. To examine a possible role of the core region of the origin as containing sites for specific interactions with viral replication proteins, purified adenovirus type 5 precursor terminal protein and DNA polymerase were

assayed for their ability to recognise the terminal 1-18 sequence. It was found that both proteins independently and as a heterodimer bound specifically to a sequence corresponding to the core origin of replication, suggesting that sequences within this region are important for localisation of DNA replication proteins at the origin via a sequence specific DNA-protein interaction.

# INTRODUCTION

## 1. The Adenoviridae

Adenoviruses (family Adenoviridae) were discovered by Rowe *et al.*, (1953) whilst trying to identify and isolate the causative agents of acute viral respiratory infections. It was observed that cultured human adenoid and tonsil cells frequently underwent morphological changes, taking on a rounded grape-like appearance. The new virus identified as being the causative agent in these cytopathic changes was named after the tissue type in which it was first identified. At around the same time Hilleman and Werner (1954) isolated a virus from American army recruits suffering from acute respiratory infections which was subsequently shown to be identical to the virus identified by Rowe and co-workers. Since then 41 serologically distinct types of adenovirus have been found to infect humans causing acute respiratory, gastrointestinal, ocular and urinary diseases. In addition a wide range of other types have been identified which infect animal species.

Adenoviruses are divided into two genera according to their natural hosts: The Mastadenovirus genus, which in addition to the 41 types which infect humans, contains serotypes which infect a wide range of other mammals including monkeys, dogs, cattle, sheep, horses, rodents and tree-shrews; The Aviadenovirus genus, contains 15 or more serotypes that infect fowl, ducks and pheasants (Ishibashi and Yasue, 1984).

For the most part adenovirus infections in man are asymptomatic and seldom associated with life threatening disease, the exception being the enteric adenovirus types 40 and 41 which are important pathogens in acute diarrhoeal

disease of infants. Although initial studies focussed on the clinical aspects of adenovirus infection, many subsequent studies on various aspects of the viral life cycle have yielded results of much wider significance, shedding light on some of the underlying macromolecular biosynthetic mechanisms of the eukaryote cell.

## 2. Virion Architecture

Basic virion structure is highly conserved throughout the adenoviruses and consists of two major structural complexes: An icosahedral shell called the capsid which surrounds an internal core which is comprised of the viral DNA associated with at least two viral proteins. There are at least 11 species of polypeptides in the virion which range from 3kD to 110kD. The size of a virion is derived from its edge (the distance between two pentons) which, in adenovirus type 5 (Ad5) from neutron diffraction data, was calculated to be 52nm giving an icosahedral "diameter" (D) of about 88nm (Devaux *et al.*, 1983).

### 2.1. The Viral Capsid.

The adenovirus capsid is made up from three viral proteins which form 252 capsomeres. The major component of the capsid is hexon (viral protein II, VP II) which carries the major recognition sites for group and type specific neutralising antibodies (Haase and Pereira, 1972; Willcox and Mautner, 1976). The organisation of the 240 hexons dictates the overall icosahedral morphology of the capsid. At each of the 12 vertices of the capsid is a penton which consists of

a penton base (VP III) embedded in the capsid surface and an outward projecting fibre (VP IV) which provides the initial attachment site for the virion to the cell surface (Valentine and Periera, 1965).

Under various conditions such as treatment with SDS, acetone or heating to 56$^{o}$C the capsid dissociates into planar 'groups of nine' hexons (GON's) and peripentonal hexons which remain attached to the pentons (Russell *et al.*, 1967; Laver *et al.*, 1968). Viewed by electron microscopy GONs have a propeller like shape which is either left handed or right handed, depending from which side they are seen, with left handed GONs corresponding to an outside view of the capsid (Periera and Wrigley, 1974). Physical analysis showed that each hexon is a trimer consisting of three polypeptide chains of about 110kD (Grutter and Franklin, 1974; Akusjarvi *et al.*,1984). By determination of the 3-dimensional structure of hexon much information on the structure-function relationship has been obtained. X-ray crystallographic studies have revealed the 3-D structure of the hexon trimer as consisting of two main parts: A triangular top 6.4nm in height which contains three 'towers' and a pseudo hexagonal base 5.2 nm in height which has a central cavity (Burnett *et al.*, 1984). The three subunits of hexon make extensive contacts in both the base and the towers which confers a high degree of resistance to denaturation. Crystallographic studies conducted at higher resolution showed that the hexon trimer is formed from three copies each of two similar β-barrel domains, called P1 and P2, in the base. The three towers which comprise the top are each formed from three loops, two rising from P1

and P2 domains in adjacent subunits at their interface and a third which rises from P1 of the third, opposed subunit (Roberts *et al.*, 1986). A comparative study of the predicted amino acid sequences of the hexon of Ad2 and Ad40 revealed that most differences are localised in the loops which form the towers, consistent with the observation that the virion surface provides important type specific antigenic determinants (Toogood *et al.*, 1989).

The pentons located at each of the twelve vertices of the capsid are formed from a penton base and a thin projection called the fibre. The interaction between the fibre and the base is hydrophobic and the two can easily be separated using guanidine (Norrby, 1969). The penton base is comprised of five subunits of VPIII and the fibre is a trimer formed from three subunits of VP IV (Van Oostrum and Burnett, 1985). The fibre of Ad2 is about 2nm in diameter and 28nm in length terminated by a knob 4nm in diameter (Green *et al.*, 1983).

In addition to hexon and the penton complex VP IX has been found to be an essential component of the capsid. Colby and Shenk (1981) demonstrated that an Ad5 mutant containing a deletion in the gene encoding VP IX assembled virions which were more heat labile than wild type virus and which gave individual hexons rather than GONs when dissociated, suggesting that VP IX has a role in stabilisation of the capsid. This idea was supported by the observation that functional VP IX was essential for packaging of mature viral genomes during infection (Ghosh-Choudhury *et al.*, 1987). By EM analysis of GONs combined with crystallographic data on hexon structure Furcinitti *et al.* (1989) demonstrated that VP IX acts as a capsid 'cement' which is responsible

for holding hexons of dissociated virions in the highly stable GON configuration. Devaux *et al.* (1982) found that VP IIIa is associated with the vertices of the virion, suggesting it has a role in mediating an interaction between the five peripentonal hexons and the penton base.

### 2.2. The Viral Core

The viral core consists of viral DNA plus three viral proteins VP V, VP VII and Mu. It was shown that viral cores prepared by a variety of procedures such as disruption with pyridine, acetone or urea consistently yielded DNA in close association with VP VII (Ginsberg, 1979). VP VII has a molecular weight of 18kD and is highly basic, consisting of approximately 22% arginine, which is sufficient to neutralise the negative charge of DNA. In contrast, the less abundant 48kD VP V was found to dissociate from the DNA-VP VII complex under relatively mild conditions (Vayda *et al.*, 1983). It was proposed that VP V formed a shell on the surface of cores (Nermut, 1979). Electron microscopy of freeze fractured virions or cores prepared without the use of chemical treatment has provided evidence for the existence of a surface shell (Brown *et al.*, 1975).

The precise way in which viral proteins and DNA interact to package the genome has been the subject of some controversy. There were two models for the overall organisation of DNA and protein in the nucleocapsid: The continuous helix model where the DNA is wrapped around a single helical filament, and the discontinuous "nucleosome" model. The evidence provided by electron microscopy showed that cores prepared by various methods appeared to have a

"beads on a string" structure (Mirza and Weber, 1982) so the nucleosomal type arrangment of the nucleoprotein complex was the favoured model for core structure for a time. However more recently electron microscopy combined with Bal31 endonuclease activity assays on the DNA of subviral particles has revealed the presence of supercoiled domains within DNA packaged inside the virion (Wong and Hsu, 1989). On the basis of these observations a model has been proposed whereby adenovirus DNA is held in 8 supercoiled domains, each domain comprising 12% of the genome, by interaction with a central core protein. This model allows the prediction of the positions of the various parts of the genome within such a structure. Interestingly, the Ela and E4 promoters, being at the ends of the genome, lie just outside the putative supercoiled regions potentially making them accessible to transcriptional proteins prior to complete dissociation of the virion which suggests that a topological mechanism may exist for regulation of gene expression.

**2.3. The Viral Genome.**

Although there is considerable sequence divergence between the genomes of some adenovirus types all share many common structural features. Human adenovirus genomes range between 34300bp in Ad12 up to 36000bp for Ad2 and Ad5 (Sussenbach, 1984). The observation that denatured adenovirus DNA forms thermostable single stranded circles when reannealed at low concentrations led Garon *et al.* (1972) to propose that the genome contains inverted terminal repeats (ITR's). Prior treatment of DNA with exonuclease III

abolished the formation of such structures. Early estimates by exonuclease III digestion established that the size of the ITR varies from serotype to serotype but in general led to an over estimation of their size. Nucleotide sequence analysis has allowed an accurate comparison of the ITR sizes of several viruses ranging from 63bp in chicken embryo lethal orphan (CELO) virus (Alestrom *et al.*, 1982) to 165bp in human Ad18 (Garon *et al.*, 1982). Sequence analysis of the ITRs in several serotypes also revealed an assymetrical distribution of G/C and A/T base pairs within the ITR's. In Ad5 the first 50bp contains 72% A/T while the remainder has only 27% A/T (Steenbergh *et al.*, 1977). The ITR's of serotypes from the same subgroup appear to have a high degree of homology. Ad2 and Ad5 have identical ITR's of 103bp whilst both Ad3 and Ad7 have ITR's of 136bp which differ at only seven positions (Shinigawa and Padmanabhan, 1979). Variation within a serotype has been reported: the ITR's of two different isolates of Ad7 were found to vary at 5 positions (Shinigawa and Padmanabhan, 1980). The ITR's of all human serotypes examined have the sequence ATAATATACCTTAT from nucleotides 9-22 (Tolun *et al.*, 1979). The likely significance of this perfectly conserved sequence is addressed in later sections.

Analysis of several adenovirus genomes by thermal denaturation revealed that the distribution of A/T and G/C base pairs along the genome is assymmetrical which gave rise to the genome being divided into a right (AT rich) half and a left half (Doerfler and Kleinschmidt, 1970). A convention for defining the two complementary strands of the genome was adopted based on the direction of transcription from each strand: The strand transcribed towards the right hand AT

rich half of was designated the r-strand whilst the leftward transcribed strand was designated the l-strand. To describe position on the adenovirus genome it was divided into 100 map units (m.u.), reading from left to right. Although the units are arbitrary and the nucleotide equivalent of 1m.u. varies slightly depending on the position on the genome, owing to differences in nucleotide composition in different regions (Gingeras *et al.*, 1982), the system is still useful for giving a general description of the locations of transcription units on the adenovirus genome.

A distinguishing feature of the adenovirus genome is the presence of a 55kD terminal protein (TP) attached at either end of the genome. TP was first detected by Robinson *et al.* (1973) using a method to isolate viral DNA which avoided proteolytic digestion. The resistance of this DNA/protein complex to boiling and treatment with SDS suggested that the link is covalent in nature (Robinson *et al.*, 1973; Sharp *et al*., 1976; Rekosh *et al.*, 1977). De-proteinised DNA and DNA-protein complex were found to be inaccessible to kinase, phosphatase and exonuclease VII whilst they were vulnerable to exonuclease III digestion and could be labelled using terminal transferase (Sharp *et al.*,1976; Carusi, 1977). This was taken as evidence that TP is attached at the 5' end of either strand. The inaccessibility of apparently de-proteinised DNA was due to the continued presence of a short peptide on the 5' ends which could be removed by harsher treatment of DNA with alkali or piperidine. Treatment of DNA-protein complex with piperidine or S1 nuclease left a protein of apparent molecular weight 52kD or 55kD (Rekosh, 1981; Rijnders *et al.*, 1983). The linkage was shown to be

formed by a phosphodiester bond between the hydroxyl group of a serine residue on TP and the 5' phosphate group of the terminal dCMP (Desiderio and Kelly, 1981).

The origin of terminal protein was under debate for some time after its initial discovery. Analysis of the terminal proteins of five different human serotypes after treatment with trypsin showed them all to be very similar suggesting a common cellular origin (Green *et al.*, 1979). However, Rekosh (1981) observed that the TP's of different serotypes varied slightly in size which suggested they were of viral origin. The question was resolved when Stillman *et al.* (1981) demonstrated that an 87kD protein translated *in vitro* from mRNA's transcribed from the l-strand between map units 11 and 31.5 was very similar to an 80kD protein, structurally related to the TP, identified as being linked to the 5' ends of growing Ad2 strands synthesised *in vitro* (Challberg and Kelly, 1979; Challberg *et al.*, 1980). Furthermore this species was shown to be identical to a protein attached to the DNA in mature virions of a temperature sensitive Ad2 mutant, Ad2ts1 (Weber *et al.*, 1975). When grown at the non-permissive temperature Ad2ts1 is defective in the cleavage of virus coded precursor proteins to their mature states which suggested that the 80kD species represents a precursor form of the terminal protein (pTP). The central roles played by both pTP and TP in DNA replication will be dealt with in later sections.

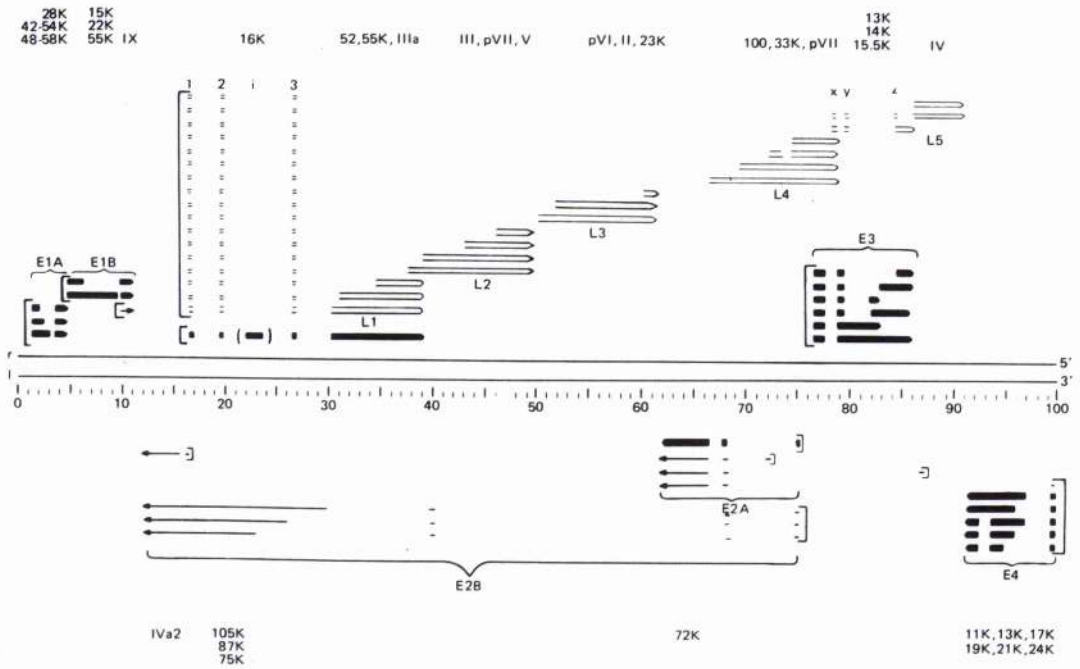
Early studies on the general organisation of the transcription units on the adenovirus genome made use of a variety of techniques including DNA-RNA hybridisation, genetic mapping and *in vitro* translation of selected mRNA's

(Kitchingman *et al.*, 1977; Berk and Sharp, 1978; Miller *et al.*, 1980). Since then nucleotide sequence analysis has allowed the precise location of transcription units on the genome to be described. The entire nucleotide sequence of the Ad2 genome has been elucidated and comprises 35,937bp (Roberts *et al.*, 1986). Although there is substantial sequence divergence between some serotypes all adenovirus genomes are similar with respect to the overall organisation of their transcriptional units. There are ten transcription units which are classified as early or late depending upon whether they are first transcribed before or after the onset of viral DNA replication. However given the complexity of the replicative cycle it is generally further subdivided into four stages: pre-early, early, intermediate and late. A schematic representation of the Ad2 genome divided into 100 map units is shown in Figure 1. The positions of the transcription units, their major mRNAs and the proteins they encode are indicated.

### 3. Adenovirus transcription

The replicative cycle of adenovirus commences when viral DNA enters the nucleus of the host cell about 30 minutes after adsorption. At about 45 minutes post infection (p.i.) transcription from viral promoters commences following a highly ordered sequence of events (Nevins *et al.*, 1979). Viral genes are transcribed in the host cell nucleus by cellular RNA pol II except for the virus associated (VA) RNA's which are transcribed by RNA pol III. All primary transcripts are capped with $^{7me}$G5'pppN and polyadenylated at the 3' end before entering the cytoplasm (Philipson *et al.*, 1971).

**Figure 1**- Schematic representation of the adenovirus type 2 genome, divided into 100 map units, showing the locations of the transcription units, the major transcripts including leader sequences, and the proteins. Early, intermediate and late transcripts are represented by solid, narrow and open arrows respectively. (From Ginsberg, H.S., 1984, p.47).


28K
42-54K
48-58K
15K
22K
55K IX
16K
52,55K, IIIa
III, pVII, V
pVI, II, 23K
100, 33K, pVII
13K
14K
15.5K
IV
1
2
3
x y
L5
L4
L3
L2
L1
E1A
E1B
E3
5'
3'
0
10
20
30
40
50
60
70
80
90
100
E2A
E2B
E4
IVa2
105K
87K
75K
72K
11K, 13K, 17K
19K, 21K, 24K

### 3.1. Early Transcription.

Transcription from pre-early region E1a commences in the absence of any other viral gene expression and can been detected as early as 45 minutes p.i. reaching a maximum during the early phase at about 3-4 hours p.i. (Nevins *et al.*,1979). E1a lies between map positions 1.3 and 4.6 at the extreme left of the r-strand. Three mRNA species are transcribed from E1a with sedimentation co-efficients 13S, 12S and 9S. All three species share common 5' and 3' termini and are products of differential splicing from a single primary transcript (Perricaudet *et al.*, 1979). Ela has two main functions: It is required for activation of transcription from other adenovirus promoters as well as transactivating transcription from a variety of promoters of both cellular and viral origin; and it also plays a central role in cellular transformation by adenoviruses. At early times during infection the predominant mRNAs transcribed from Ela are the 13S and 12S species which give rise to polypeptides of 289 and 243 amino acids respectively. They differ only in a 46 amino acid internal conserved region (CR3) which is unique to the 289R species (Moran and Mathews, 1987).

As mentioned above E1a plays a vital role in the regulation of transcription from the other viral promoters during the infectious cycle. Promoters of Elb, E2, E3, E4 and MLP are all activated by Ela (Berk, 1986). E1a is also a promiscuous transactivator, stimulating transcription from heterologous promoters of cellular origin such as globin promoters (Svensson *et al.*, 1984) and the rat preproinsulin promoter (Gaynor *et al.*, 1984) and viral promoters such as the HSVI glycoprotein D and thymidine kinase promoters (Everett and Dunlop., 1984) and

HTLVI and II promoters (Chen *et al.*, 1985). The 46R (CR3) region has been shown to be vital for transactivation by Ela, hence the 289R protein is largely responsible for this function (Moran and Mathews, 1987). In one study it was found that a synthetic peptide comprising the amino acid sequence of the 46R region plus 3 adjacent conserved amino acids alone was sufficient to stimulate transcription from several adenovirus promoters (Green *et al.*, 1988).

Promoters responsive to Ela transactivation share no common sequence element which could act as a recognition site for Ela protein. This led to the view that Ela modulates transcription by indirect means. Initially it was supposed that it did so by increasing the concentration of cellular transcription factors. However it has been observed that there is no significant increase in the concentration of several known cellular transcription factors such as ATF and MLTF, which are known to have a role in transactivation, during adenovirus infection (Flint and Shenk, 1989). It has also been proposed that Ela operates by activating existing transcription factors, an example being TFIIIC which is activated by phosphorylation in adenovirus infected cells. It was deduced that Ela was responsible for this since infection with an Ad mutant unable to express the 289R protein did not result in phosphorylation of TFIIIC (Hoeffler *et al.*, 1988). However such a mutant would have also been unable to synthesise other early proteins, so the lack of activation of TFIIIC may have represented an indirect consequence of the Ela defective phenotype. Recently evidence has come to light that Ela may stimulate transcription more directly as part of the transcriptional initiation complex at the promoter. It has been shown that many

cellular transcription activating proteins contain two functional domains, one a binding region which directs the protein to DNA, and the other an acidic activating region which directly interacts with a component of the transcriptional machinery to stimulate transcription (Ptashne, 1988). Lillie and Green (1989) reported that a hybrid protein comprising the putative transcription activating region of Ela protein fused to the DNA binding domain of the GAL4 protein transactivated transcription from E4 and Elb promoters with higher efficiency when heterologous GAL4 recognition sites were introduced into the promoters. This suggested that transcriptional activation was increased by more efficient binding of Ela, via the GAL4 DNA binding domain, at the promoter. However as Ela itself does not bind to any specific nucleotide sequence element in these promoters it would appear that the mechanism by which it is directed to the promoter region would be by recognition of a protein already bound.

The other major role played by Ela is in cellular transformation. Expression of Ela alone is sufficient to immortalise primary rodent cells but complete transformation requires expression of Elb as well (Van den Elsen *et al.*, 1983). Other oncogenes, including H-ras, and polyoma virus middle T-antigen can substitute for Elb in transformation. In addition Ela can be replaced by polyoma large T- antigen and the *myc* and p53 oncogenes (Ruley, 1983; Jenkins *et al.*, 1984). Mutational analysis of Ela has shown that the regions of the protein involved in transformation and transactivation are in separate domains. Two regions common to both the 289R and the 243R polypeptides are required for transformation but not for transactivation (Whyte *et al.*,1986). Immunological

studies have identified a number of cellular proteins with which Ela protein forms complexes. Prominent amongst these proteins are species of molecular weights 300kD and 107kD and a protein p105-RB, identified as being the product of the retinoblastoma susceptibilty gene (Harlow *et al.*, 1986; Whyte *et al.*, 1989). The RB protein has an important role in growth suppression, and correspondingly loss or inactivation of the RB gene has been found in a variety of malignant cell types (Weichselbaum *et al.*, 1988). Whyte *et al.* (1989) demonstrated that the regions of Ela involved in binding to p105-RB and the 107kD and 300kD proteins corresponded to those which have been identified as being essential for transformation. It therefore appears that an important part of Ela mediated transformation occurs by the direct interaction of Ela with proteins involved in regulation of cell growth and division.

Transcription from E1b, E2, E3, and E4 commences 1.5-2 hours p.i. The rates of transcription from E3 and E4 reach a maximum at 3-4 hours p.i. while E1b and E2 reach a maximum later at 6-7 hours p.i. (Nevins et al., 1979).

The E1b transcription unit is located between map positions 4.6 and 11.2 at the left end of the genome. Transcription from E1b is transactivated by Ela. The primary transcription product is spliced to give 22S, 14.5S, 14S and a 13S mRNA species which share common 5' and 3' termini (Virtanen and Peterson, 1985). The 14S and 14.5S mRNA's are identical to the 13S but have an additional exon. The 22S mRNA gives rise to two separate proteins of molecular weights 55kD and 19kD. The proteins are synthesised from separate but

overlapping reading frames depending upon which of two ATG triplets is utilised as the start codon (Bos *et al.*, 1981). The coding information for VPIX, a 14.3kD protein associated with the virus capsid is present on all the E1b mRNA's but is not translated at early times. It is translated from an additional mRNA transcribed at intermediate times during infection (Esche *et al.*, 1980). The mRNA synthesised is the only known adenovirus messenger which remains unspliced prior to translation (Alestrom *et al.*, 1980).

It has been demonstrated that the 55kD protein forms a functional complex with the 34kD protein encoded by E4. This complex has been shown to have a role in the transport of late mRNAs from the nucleus to the cytoplasm (Pilder *et al.*, 1986; Bridge and Ketner, 1990). The major function of Elb is its role in cellular transformation by adenovirus. Its expression, along with that of Ela, is essential for full transformation (Van der Eb and Bernards, 1984). Both the 19kD and the 55kD proteins are involved in transformation. The 55kD protein binds to p53, a cellular oncoprotein found at elevated levels in transformed cells (Sarnow *et al.*,1982). It was found that mutation of the region encoding the 19kD protein resulted in a range of mutant phenotypes which included degredation of all host cell and viral DNA, abnormal cytopathic effect and formation of large plaques (White *et al.*, 1988). By transfection of cells with an expression vector containing the gene for the 19kD protein it was observed that it specifically associated with and profoundly altered the organisation of intermediate filaments and the nuclear lamina (White and Cipriani, 1989). It has been proposed that this function plays an important role in cellular transformation by detaching

intermediate filaments from the plasma membrane, which could in turn affect cell-cell attachments and promote anchorage independent growth. Additionally, modification of the organisation of the nuclear lamina could be important in maintaining the integrity of cellular and viral DNA after infection, preventing the degradation of DNA observed during infection with virus defective for Elb production.

The E2 transcription unit is located between map positions 75.4 and 11.3 on the l-strand (Chow *et al.*, 1979). At early times in infection E2 mRNA's are transcribed from a promoter at map position 75 which is under the control of Ela but at late times transcription is initiated from a second, Ela independent promoter at map position 72. E2 transcripts are divided into two sets depending on the position at which they terminate. E2a transcripts terminate at a poly-A site at position 62.4 whereas E2b RNA is transcribed through to a poly-A site at 11.3 (Stillman *et al.*, 1981). All E2 mRNA's share a common leader sequence from position 68.8 as well as one derived from their promoter at position 75 or 72.

E2a mRNA's encode the viral single strand DNA binding protein (DBP), a multifunctional phosphoprotein which plays important roles in DNA replication, control of early and late gene expression and viral host range. The role of DBP in DNA replication will be addressed in later sections. Its role in early and late gene expression is probably a function of its ability to bind to RNA (Cleghon and Klessig, 1986). Studies involving limited proteolytic digestion have shown that DBP is preferentially degraded into a C-terminal domain and a highly

phosphorylated N-terminal portion (Schechter *et al.*,1980). The C-terminal domain binds to DNA and is functional in DNA replication (Tsernoglou *et al.*, 1985). In contrast, several mutations located in the N-terminal domain, whilst having no effect on DNA replication, have been shown to change the viral host range. In one study, viruses carrying a mutation in the coding sequence for the N-terminal portion of DBP were, unlike wild type, able to undergo a productive infection in monkey cells. This was found to be due to the aquisition of a functional fibre protein, whose mRNA is incorrectly spliced when monkey cells were infected with the wild type virus, implying that the N-terminal portion of DBP has a role in the processing of late mRNAs (Anderson and Klessig, 1984).

The E3 transcription unit is located between coordinates 76.6 and 86.0 on the r-strand. Six major species of mRNA's transcribed from this region have been identified and encode proteins ranging in size between 10.4 kD and 19kD (Sussenbach, 1984). The major functions which have been ascribed to the E3 proteins are in the evasion of the antiviral defences of the host cell and to down regulate the epidermal growth factor receptor (EGF-R). The 19kD E3 protein binds to class I MHC antigens and prevents their transport to the cell surface, thereby protecting adenovirus infected cells from recognition and cytolysis by cytotoxic T-cells (Andersson *et al.*, 1985; Burger *et al.*, 1987; Rawle *et al.*, 1989). The 14.7kD E3 protein on the other hand prevents cytolysis of infected cells by tumour necrosis factor (Gooding *et al.*, 1988). The 10.4kD E3 protein has been shown to be responsible for the down regulation of EGF-R, the target receptor for

EGF, which is a member of the tyrosine kinase family of receptors. On binding, EGF switches on the tyrosine kinase activity of EGF-R which in turn, by phosphorylation of a number of proteins, activates cellular metabolism and induces DNA synthesis and mitosis. It therefore appears that the E3 10.4kD protein has a role in down regulating cellular metabolism in infected cells (Carlin *et al.*, 1989; Tollefson *et al.*, 1990).

Early region E4 lies between coordinates 91.3 and 99 at the right end of the genome. DNA sequencing and analysis of E4 mRNAs have identified a number of open reading frames in the region indicating that E4 encodes at least seven distinct proteins (Fryer *et al.*, 1984; Virtanen *et al.*, 1984). Generation of mutants containing large deletions in E4 resulted in pleiotropic effects including defects in late protein synthesis, in late mRNA accumulation and of shutdown of host cell protein synthesis (Halbert *et al.*, 1985). Studies on mutations affecting individual ORFs within E4 has allowed functions to be ascribed to specific E4 proteins and explain some of the observed phenotypes. A 14kD and a 34kD E4 product are involved in the shutdown of host cell macromolecular synthesis and accumulation of late viral messengers (Huang and Hearing, 1989). Furthermore the 34kD has been found to be physically associated with a 55kD product of E1b in infected cells and the complex appears to have a role in transport of late mRNAs from the nucleus to the cytoplasm (Pilder *et al.*, 1986).

E4 has also been shown to transactivate transcription from the E2a promoter (Goding *et al.*, 1985). It appears that transactivation occurs via complex

formation with the cellular sequence specific DNA binding transcription factor E2F by a 19kD E4 protein. Complex formation leads to modification of E2F into an active state whereby its DNA binding activity is increased (Marton *et al.*, 1990).

### 3.2. Intermediate and late gene expression.

The two major products of the E2b region are the 140kD adenovirus specific DNA polymerase (Ad pol) and the 80kD precursor terminal protein (pTP) whose roles in DNA replication will be discussed in later sections. The main bodies of the messages encoded by this region start between map positions 23 and 30 and continue to position 11.2. A tripartite leader derived from the promoter at position 75, position 68 and position 41 is spliced onto the end of all transcripts. Both pTP and pol have the same three amino acids at the N terminal which are donated by an exon from map position 39 (Shu *et al.*, 1988).

After the onset of DNA replication transcription from the major late promoter (MLP) increases about a thousand fold. In addition transcription of early messages increases by 3-10 fold probably largely as a result of the increase in the number of templates available (Shaw and Ziff., 1980). The MLP lies at map position 16.5 on the r-strand. Transcription from the MLP can be detected as early as 1 hour after infection but at this time proceeds no further than position 39. After DNA replication has started transcription proceeds to position 99 (Fraser *et al.*, 1979). The 5' end of all mRNAs initiated from the MLP contain a tripartite leader of about 200 nucleotides which selectively enhances the

translation of mRNAs late in infection. It is comprised of 41 nucleotides from the promoter at map position 16.5, 72 nucleotides from map position 19.6 and 90 nucleotides from map position 26.5. The mRNA's transcribed from the MLP are classified into five families L1 to L5, each group sharing a common poly-A site (Nevins and Darnell, 1978).

The L1 family of transcripts are the only ones expressed at early times in infection. Three mRNA's are transcribed which share the poly-A site at map position 39 (Fraser *et al.*, 1982) and 5' cap acceptor sites at positions 29, 30.7 and 34. L1 transcripts have an additional leader fragment (i-leader) containing an open reading frame from position 21.5-23 (Chow *et al.*, 1979). The L1 mRNA's code for VP IIIa which is found associated with hexon in the mature virion (Akusjarvi and Persson, 1981), and two structurally related proteins of 52kD and 55kD whose function is unclear (Miller *et al.*, 1980).

The L2 family is transcribed from map position 39 and consists of 3 mRNA species which have 5' cap sites at 39.4, 44.1 and 45.9 with a polyadenylation site at position 50 (Chow *et al.*, 1980; Alestrom *et al.*, 1984). L2 mRNA's code for the 85kD VP III, a 20kD VP VII precursor later cleaved to an 18.5kD species and the 48.5 kD VP V (Miller *et al.*, 1980; Akusjarvi and Pettersson, 1981). All are structural components of the mature virion, VP III forming the penton base and VP VII and VP V constituting the two major proteins of the viral core.

The L3 family of transcripts consists of three mRNA's which are transcribed from map positions 49-62 with 5' cap sites at 50.1, 52.3 and 60.2. These encode a precursor of VP VI, hexon (VP II) and a 23kD protein (Akusjarvi and Pettersson, 1981). The 23kD protein is a viral endopeptidase which plays a critical role during virion maturation by specific cleavage of precursor forms of VP VI, VII, VIII, and the terminal protein. It is a cysteine protease exhibiting a high degree of substrate specificity which cleaves at an identified target amino acid sequence (Webster *et al.*, 1989).

Region L4 gives rise to 4 mRNA's with a poly-A site at position 78. Proteins encoded within this region are the 100kD protein, a 33kD protein and a 26kD precursor to VP VIII (Miller et al., 1980). The 100kD protein is apparently multifunctional. It is involved in the complex folding of hexon trimers (Gambke and Deppert, 1984) as well as forming ribonucleoprotein complexes with host and viral cytoplasmic RNAs whereby it appears to play a role in inhibition of translation of host messengers (Adam and Dreyfuss, 1987).

The L5 family consists of two major species of mRNA's from between map positions 86 and 91.3 (Miller *et al.*, 1980). They differ from other late messengers in that they contain leader sequences derived from map positions 72.2, 78.6, and 84.7 in addition to the tripartite leader. These mRNAs encode the fibre protein (VP IV) (Uhlen *et al.*, 1982).

In addition to the transcripts produced under the control of the MLP, two low molecular weight RNA's designated virus associated RNA's I and II (VA I, 157-160 nucleotides and VA II, 158-163 nucleotides) are transcribed at high levels during late times in infection and are subsequently found at high levels in the cytoplasm of infected cells (Soderlund *et al.*, 1976). Located at map positions 28.8 and 29.5 on the viral genome these two RNA's are unique in that they are transcribed by RNA pol III. By introducing deletions into the promoters of both VA I and VA II Thimmapaya *et al.* (1982) demonstrated that VA RNA I is essential for efficient translation of late mRNAs. In addition it has been observed that it stimulates the expression of transfected genes (Kaufman and Murtha, 1987). VA RNA I functions by blocking the activation of the double stranded RNA dependent kinase, DAI which is in an inactive state in most cells (Akusjarvi *et al.*, 1987). However during viral infection DAI can become activated by symmetrical transcription of the viral genome (Maran and Mathews, 1988) in which case it can inhibit protein synthesis by phosphorylation of the translation initiation factor eIF-2 and trapping a second translation factor eIF-2B (O'Malley *et al.*, 1989). VA RNAs form complex secondary stem-loop structures by base pairing. Studies on mutant VA RNAs have established that two separate domains form structures which have independent functions; one which mediates binding to DAI and the other of which interferes directly with DAI activation (Mellits *et al.*, 1990).

**4. Adenovirus DNA replication.**

Replication of adenovirus DNA is first detected about 6-8 hours post infection reaching a maximum rate at about 19 hours. The onset of viral DNA replication is coincident with a virtual total shutdown of cellular DNA synthesis (Pina and Green, 1969). After 24 hours some $10^5$-$10^6$ new double stranded progeny molecules have been synthesised which nearly equal the total DNA content of the host cell (Green *et al.*, 1970). However only about 20% of the genomes are ultimately packaged into mature progeny virions.

The initial impetus for studying adenovirus DNA replication arose from the concept that analysis of the mechanism and control of replication of the relatively small and simple genomes of the DNA viruses which infect eukaryote cells would provide insights into the mechanisms by which the host cell replicates its own much larger and more complex genome. A lot of the important mechanistic features of adenovirus DNA replication were elucidated by studies on viral DNA replication *in vivo*. However the development of systems which were capable of replicating DNA *in vitro* represented a major breakthrough (Challberg and Kelly, 1979, Challberg *et al.*,1980). Studies on adenovirus DNA replication *in vitro* confirmed many of the features of the models for DNA replication arrived at on the basis of observations made *in vivo*. In addition *in vitro* studies have allowed the identification, purification and some characterisation of the proteins involved in viral DNA replication as well as elucidation of the sequences important for origin function. Through the use of cell-free adenovirus DNA replication systems reconstituted entirely from individual purified components, a much more detailed

view of the underlying molecular mechanisms is being arrived at.

### 4.1. The study of adenovirus DNA replication *in vivo*.

Initial studies utilised pulse labelling techniques to identify replicative intermediates. When adenovirus infected cells in culture were exposed to a short pulse of [$^3$H] thymidine and labelled DNA was examined by native sucrose gradient centrifugation it was observed that a significant proportion of the DNA sedimented more rapidly than mature viral DNA (Pearson and Hanawalt, 1971; Bellet and Younghusband, 1972). By pulse-chase studies it was established that this fraction corresponded to a precursor to mature viral DNA. Treatment of replicating DNA with S1 nuclease abolished this difference in density indicating that replicative intermediates consist of a large amount of single stranded DNA. Similar experiments carried out under denaturing conditions showed there was a broad range of nascent DNA strands of various sizes up to that of mature viral DNA (Pearson and Hanawalt, 1971).

A great deal was learned about the structure of replicative intermediates by direct examination of isolated replicating viral DNA by electron microscopy. Lechner and Kelly (1977) described two basic types of replicative intermediate based on EM observations: Type I intermediates which consisted of double stranded DNA of unit length with single stranded branches and type II molecules which consisted of unbranched molecules of unit length with both double and single stranded regions of variable length. It was observed that any single stranded branch on a type I intermediate was the same length as the double

stranded region up to the branch point. On the basis of these findings a model for adenovirus DNA replication was postulated whereby initiation of replication occurs at or near one end of the genome and synthesis of the nascent strand proceeds with the concomitant displacement of the non-template strand. In this model type II intermediates corresponded to second strand synthesis on the displaced strand.

Identification of the polarity of replicative intermediates by partial denaturation where only AT rich regions were melted revealed that DNA replication was initiated with equal frequency at either end of the genome. The polarity of DNA synthesis was determined by labelling of replicative intermediates with dTTP by terminal transferase. Poly-dT tails were observed to grow only at branch points of type I molecules and at double to single stranded transition points on type II molecules showing that the nascent strands contain only one free 3'-OH group located at their growing point. This indicated that all daughter strand synthesis occured continuously in a 5'-3' direction without lagging strand synthesis (Lechner and Kelly, 1977).

Models for DNA replication based on direct EM observations of replicative intermediates were backed up by other physical evidence. Earlier studies by Flint *et al.*, (1976) which analysed the single stranded DNA sequences present in a pool of replicating molecules were in agreement with this general model for the mechanism of DNA replication. Displaced single stranded sequences from replicative intermediates were quantified by hybridisation to each of the two complementary strands of the complete viral genome. It was found that single

stranded species corresponding to the r-strand were most abundant at the right hand end of the genome and that l-strand single strands were prevalent at the left end of the genome but that the total amount of l-strand and r-strand sequences present were equal. On the basis of this observation it was proposed that DNA replication results in the displacement of both the l-strand and the r-strand with equal frequency depending from which end of the genome replication originates.

The location of the termination points of DNA replication were predicted using a technique which results in preferential labelling of nascent DNA in regions close to the termini of DNA replication following exposure of infected cells to a short pulse of [$^3$H] thymidine. When progeny genomes were isolated and examined by restriction enzyme cleavage analysis it was observed that restriction fragments corresponding to the ends of the genome had incorporated the most radioactivity, indicating that the terminus of DNA replication lies at or close to the ends of the genome (Sussenbach and Kuijk, 1977). The location of the origins of DNA replication were determined by experiments using a temperature sensitive mutant defective in DNA replication, H5ts125. By maintaining infected cells at the non- permissive temperature for a time before switching to the permissive temperature the first round viral DNA synthesis was synchronised. At the start of synthesis nascent DNA was pulse labelled with [$^3$H] thymidine for a short time then isolated and cleaved with restriction enzymes. Fragments corresponding to the ends of the genome were labelled with the highest specificity with the r-strand being labelled with higher specific activity at

the right end of the genome and the l-strand being preferentially labelled at the left end of the genome. These observations suggested a model where replication of adenovirus DNA is initiated at the 5' end of each daughter strand at an origin located at or near the molecular ends of the parent molecule and terminates at a site at the opposite end of the genome with the concomitant displacement of the strand not being utilised as template (Sussenbach and Kuijk, 1978).

### 4.2. The study of adenovirus DNA replication *in vitro.*

Adenovirus was the first animal virus for which a system was developed whereby DNA replication could be faithfully initiated and elongated *in vitro* on exogenous templates at a rate and efficiency close to that obtained *in vivo*. The development of *in vitro* systems confirmed many of the earlier models concerning the mechanism of adenovirus DNA replication formulated on the basis of *in vivo* data and has allowed a precise breakdown of the major features of the replication pathway with identification and characterisation of the proteins involved.

The earliest *in vitro* studies of adenovirus DNA replication did not however use exogenous template but rather were carried out in nuclei isolated from infected cells. This technique relied on the permeability of the nuclear membrane to dNTP's and was useful for studies on replicative intermediates in which radioactive dNTPs and base analogues could be supplied for labelling of nascent strands. However attempts made to isolate replicating DNA and

associated replication proteins by disruption of isolated nuclei were largely unsuccessful because DNA so isolated became fragmented during isolation procedures (Frenkel, 1978). Chemical methods were more successfully used for isolation of whole replicating complexes. Kaplan *et al.* (1977) isolated a soluble extract containing replicating molecules by incubating nuclei with ammonium sulphate. The major drawback of such systems however was that elongation of nascent strands *in vitro* could only proceed on molecules which had been initiated *in vivo*. As such *in vitro* DNA synthesis occured only for a short time following extraction, making such systems unsuitable for further analysis by fractionation and reconstitution studies.

A major advancement was made by Challberg and Kelly, (1979) when they demonstrated that a soluble nuclear extract from Ad5 infected HeLa cells could support initiation of DNA replication and elongation utilising exogenous DNA protein-complex from adenovirus types 2 and 5 as template in the presence of ATP and $Mg^{2+}$. This represented the first adenovirus DNA replication system in which the whole process of DNA replication, including initiation ocurred entirely *in vitro*. As *in vivo*, synthesis of nascent strands was 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* producing type I replicative intermediates.

Although the development of *in vitro* adenovirus DNA replication systems has led to the identification and purification of the proteins involved in replicating adenovirus DNA and a clear picture of the overall mechanism has emerged a number of the molecular details concerning the interactions of the replication

proteins with each other and the template remain to be elucidated. In the following sections the current state of knowledge regarding the protein and the sequence requirements for adenovirus DNA replication as elucidated from studies mainly on *in vitro* systems will be reviewed with reference to a basic outline of the current model of the major stages in the replication of the adenovirus genome.

### 4.3. A model for the mechanism of adenovirus DNA replication.

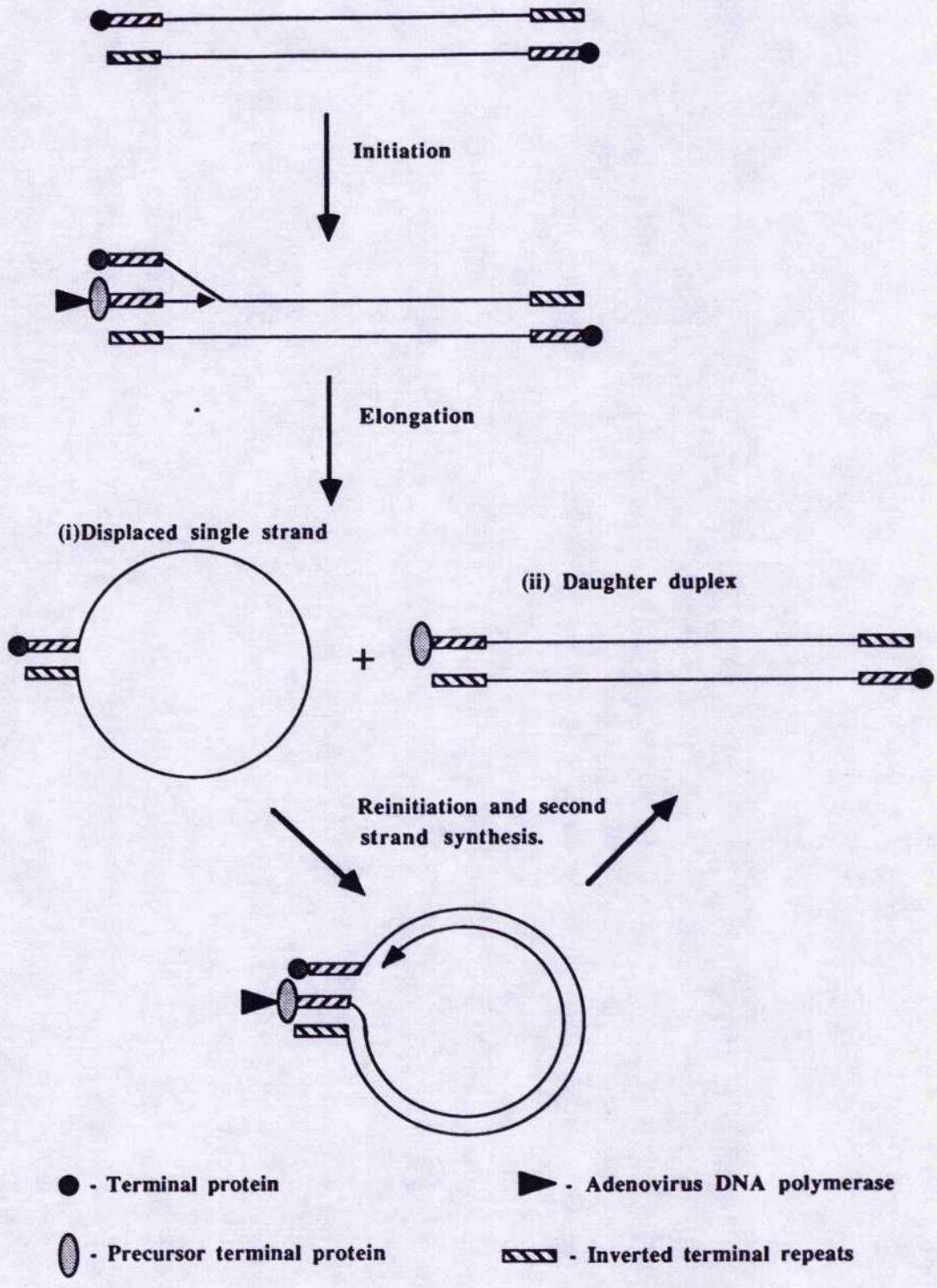
As already mentioned in an earlier section the genomes of human adenoviruses are linear double stranded DNA molecules consisting of around 36,000bp which have inverted terminal repeat sequences. Only one strand is utilised as template per initiation event and the nascent strand is synthesised continuously in a 5' to 3' direction by the adenovirus encoded DNA polymerase. Initiation occurs with equal frequency at either of the termini by a protein priming mechanism originally suggested by Rekosh *et al.* (1977). On the basis of the observation that the 5' ends of nascent DNA synthesised *in vitro* were attached to pTP by a phosphdiester bond between the β-OH of a serine residue in the protein and the 5'-$PO_4$ of the terminal dCMP residue Challberg *et al.* (1980) proposed that DNA replication is initiated by the template dependent formation of the phosphodiester bond and that the free 3' OH of the pTP-dCMP complex acts as a primer for further synthesis. Elongation proceeds catalysed by the adenovirus specific DNA polymerase with the concomitant displacement of the

non-template strand. There is strong evidence to suggest that the displaced strand forms a partially duplex panhandle structure (Stow, 1982a; Hay *et al.*, 1984) by intrastrand hybridisation of the ITR sequences recreating an origin of replication on which complementary strand synthesis is initiated. However, it is not clear whether panhandle formation is an absolute requirement for second strand synthesis since it has been demonstrated that initiation can take place on single stranded templates (Harris and Hay, 1988; Kenny and Hurwitz, 1988). Late during infection precursor terminal protein is cleaved by the adenovirus encoded endopeptidase to the mature 55kD terminal protein. A schematic outline of this model is shown in figure 2.

**5. The origin of DNA replication of adenovirus types 2 and 5.**

Various *in vitro* studies on adenovirus types 2 and 5, which possess nearly identical origin sequences, have shown that three domains within the terminal 51 bp of the viral genome are important for efficient DNA replication. The terminal 18bp form the core origin and are absolutely required for origin function but in isolation are only able to support very limited initiation (Tamanoi and Stillman, 1983, Challberg and Rawlins, 1984). The two additional sequences from nucleotides 19-39 and 40-51 contain the recognition sites for the cellular sequence specific DNA binding proteins NFI and NFIII respectively each of which as a result of binding to the origin independently act to increase the efficiency of initiation. The roles of NFI and NFIII in DNA replication will be discussed more thoroughly in a later section. Ad2 origin requirements have also

**Figure 2**- A model for adenovirus DNA replication with schematic representations of viral replication proteins shown. Lines with arrow heads represent newly synthesised DNA. The arrow indicates the direction of synthesis.


Initiation
Elongation
(i)Displaced single strand
(ii) Daughter duplex
Reinitiation and second strand synthesis.
- Terminal protein
- Precursor terminal protein
- Adenovirus DNA polymerase
- Inverted terminal repeats

been defined *in vivo* using a system in which transfected plasmids containing cloned origin sequences are amplified in the presence of a full complement of viral replication proteins provided by co-transfected helper virus (Hay *et al.*, 1984). This technique was initially developed for the isolation of other eukaryote origins such as that of SV40 (Myers and Tjian, 1980) and HSV (Stow, 1982), as well as being used for the identification of HSV genes required for DNA replication. It was observed that when a series of plasmids containing deletions in the Ad2 ITR were used in such an assay, with Ad2 as helper virus, that molecules containing ITR sequences deleted to within 45bp of the terminus were replicated as efficiently as those containing a complete ITR sequence. However plasmids containing ITR sequences deleted to within 36bp of the terminus were not replicated, demonstrating that all the sequences required for an origin of Ad2 DNA replication *in vivo* are contained within the terminal 45bp of the genome which contains the 1-18 domain and the NFI recognition site (Hay, 1985a). In a further study in which ITRs containing such deletions were reconstructed into viral genomes it was found that genomes containing deletions which removed the NFI site were non-infectious whereas genomes containing the terminal 45bp were fully infectious (Hay and McDougall, 1986).

## 6. Viral proteins required for adenovirus DNA replication.

Three viral proteins are required for adenovirus DNA replication. These are the DNA binding protein (DBP), the adenovirus specific DNA polymerase (Ad pol) and the precursor terminal protein (pTP). All three are products of the transcription unit E2 their transcripts being initiated from a single promoter at map position 75 on the adenovirus genome. pTP and pol are both translated from mRNAs which are products of differential splicing of the same E2b primary transcript expressed at early times. Since both proteins are required simultaneously during infection and cooperate with one another in a close physical interaction during DNA replication the presence of their genes in the same transcription unit probably represents a simple mechanism to ensure coordinate regulation of expression of pTP and pol. DBP is the product of the E2a transcript and despite sharing the same early promoter, levels of DBP mRNA are 10-fold higher than those of pTP and pol, probably as a result of a difference in the stability of the transcripts (Stillman *et al.*, 1981). In addition E2a comes under the control of a different promoter which allows expression of DBP at late times during infection when it has a role in the processing of late transcripts. In the following sections the roles of each of these proteins in adenovirus DNA replication will be discussed.

### 6.1. Adenovirus DNA binding protein (DBP).

DBP is the most abundant viral protein at early times in infection, reaching a maximum level of about $5x10^6$ molecules per cell. Its relatively high abundance

facilitated its identification and purification before cell free systems for replication of adenovirus DNA were developed, a step which was a prerequisite for the isolation of other proteins involved in adenovirus DNA replication. Van der Vliet and Levine, (1973) reported the isolation of two polypeptides of 72kD and 48kD from Ad5 infected cells, neither of which were present in uninfected cell extracts. Both of these species bound tightly to single stranded DNA-cellulose and could only be eluted with 1M NaCl. It was subsequently shown that the smaller of the two species was a proteolytic product of the larger, cleaved during isolation. Although DBP apparently has a molecular weight of 72kD this was subsequently shown to be due to aberrant electrophoretic mobility, its true molecular weight as predicted from its amino acid composition being around 59kD (Kruijer, 1981). The requirement for DBP in DNA replication was established by phenotypic studies of conditional mutants defective in DNA replication, in particular that of H5ts125 (van der Vliet *et al.*, 1975) which are incapable of elongating initiated molecules (Friefeld *et al.*, 1983). Infection of cells with this mutant at the non permissive temperature resulted in less than 5% of the wild type production of DBP. Furthermore DBP produced at the permissive temperature was found to be thermolabile and unable to bind to single stranded DNA. The DBP of Ad2 and Ad5 were both purified to homogeneity by virtue of their strong non-sequence specific binding to single stranded DNA (Van der Vliet *et al.*, 1975; Schechter *et al.*, 1980). In addition to binding to single stranded DNA, DBP also binds to double stranded DNA and to RNA (Cleghon and Klessig, 1986).

EM studies by Kedinger *et al.*, 1978 revealed that single stranded DNA in replicative intermediates isolated from infected nuclei was coated with DBP. DBP binds to single stranded DNA in repeating units forming a rigid structure which cover about 10 bases per protein molecule. The essential functional role played by DBP in adenovirus DNA replication was directly demonstrated in early *in vitro* systems where addition of anti-DBP antiserum was observed to inhibit elongation of nascent strands in isolated nuclei (Van der Vliet *et al.*, 1977). Conversely addition of DBP has been demonstrated to enhance the rate of polymerisation by Ad pol on poly(dT).oligo(dA) template-primer (Lindenbaum *et al.*, 1986). It was concluded that this effect was due to a cooperative interaction between DBP and Ad pol which results in an increase in the processivity of pol. Field *et al.*, 1984 observed that DBP could not be substituted for by single stranded DNA binding proteins from other sources such as E.coli, suggesting that the effect of DBP on pol is the result of a highly specific interaction. DBP probably fulfils more than one function in adenovirus DNA synthesis. It binds to the displaced single strand protecting it from nuclease attack and separating it from the replication complex, and in addition may act directly as a facilitator of chain elongation by interaction with Ad pol.

Although the effect of DBP on elongation has been well defined in several studies its role in initiation of DNA replication has been controversial. Crude nuclear extracts from cells infected with the DBP ts mutant H5ts125 grown at the non permissive temperature were found to support initiation at a normal level but were unable to support elongation from initiated products *in vivo* (Friefeld *et al.*,

1983; van Bergen and van der Vliet, 1983). This was interpreted to mean that although an absolute requirement for elongation, DBP was dispensable for initiation. Similarly studies where addition of purified DBP failed to stimulate initiation *in vitro* supported this view (Rosenfeld *et al.*, 1987). However others have observed a stimulatory effect of DBP on initiation (Kenny and Hurwitz, 1988; Cleat and Hay, 1989). It is most likely that DBP is necessary for initiation but that its effect is concentration dependent. At higher concentrations it probably inhibits initiation by saturation binding to the template and preventing the interactions of other replication proteins which are required for positioning themselves appropriately at the origin of replication prior to initiation. At lower concentrations a stimulatory effect may be attributable to a functional interaction with nuclear factor I (NFI), a cellular sequence specific DNA binding protein which stimulates initiation of Ad DNA replication on binding to the origin. The ability of NFI to stimulate DNA replication *in vitro* was observed to be influenced by the concentration of DBP present (De Vries *et al.*, 1985). It has been subsequently demonstrated that this is due to a cooperative interaction between the two proteins whereby the affinity of NFI for its binding site is increased in the presence of DBP in turn leading to an increase in initiation (Cleat and Hay, 1989; Stuiver and van der Vliet, 1990). The role of NFI in Ad DNA replication and its interaction with DBP is discussed in greater detail in a later section.

Limited proteolytic digestion of purified DBP yields a C-terminal fragment of around 40kD and a highly phosphorylated N-terminal fragment which migrates as a 27kD species. The aberrant migration of the whole protein is probably

largely due to the phosphorylation state of the N-terminal region. The C and N terminal portions of DBP are apparently functionally separate.

Dephosphorylation of DBP does not reduce DNA binding and it has been correspondingly demonstrated that the C-terminal domain alone is capable of binding to DNA and is functional in DNA replication (Ariga *et al.*, 1980; Friefeld *et al* ,1983). Nucleotide sequence analysis of the DBP genes of various adenovirus serotypes and examination of replication deficient DBP mutants has revealed the presence of highly conserved domains within the C terminal portion of the protein (Vos *et al.*, 1988) which appear to be essential for DBP function in DNA replication. Recently a series of mutants located in one of three highly conserved regions in the C-terminal portion of DBP were all shown to be altered in their ability to bind to single stranded DNA (Neale and Kitchingman, 1990). A domain which is rich in Hys and Cys residues may well represent the actual DNA binding domain since it bears similarity to the metal binding motif present in the known DNA binding domains of other DNA binding proteins. Whilst mutations in the C terminal of DBP often affect DNA replication it has been observed that a virus carrying mutations in the N terminal portion of DBP replicated their DNA normally but instead showed a change in host range due to altered processing of late transcripts, specifically that of the fibre protein (Anderson and Klessig, 1984). The nucleotide sequences for the DBP of several serotypes have been elucidated (Kitchingman, 1985; Vos *et al.*, 1988) and all appear to posses the characteristic two domain structure with the N-terminal region showing the most variation between serotypes whilst the C terminal contains several well

conserved regions.

### 6.2. Terminal protein and its precusor: Protein priming of DNA synthesis.

The presence of the 55kD terminal protein linked to either end of the mature viral DNA was first observed when viral DNA was isolated under mild conditions which left covalent bonds intact (Robinson *et al.*, 1973). TP was subsequently shown to be linked to the 5' dCMP residue of either strand of the virus genome by a phosphodiester bond. Challberg *et al.*, 1980 first identified precursor terminal protein as an 80kD species covalently linked to the 5' ends of nascent viral DNA *in vitro*. Furthermore it was observed that non-infectious progeny virions of cells infected at the non permissive temperature with the virus protease mutant H2ts1 had genomes with an 80kD attached instead of the 55kD TP (Stillman *et al.*, 1981). This evidence and the finding that both species for the most part had the same amino acid sequence suggested that the 80kD species represented a precursor form of the TP. The involvement of precusor terminal protein (pTP) in initiation of DNA replication was demonstrated by Lichy *et al.*, (1981) when it was shown that a covalent complex between pTP and [$^{32}$P] labelled dCMP, the 5' terminal nucleotide of the nascent strand, was formed in a cell free extract from infected cells in a reaction which required viral DNA-protein complex as template, $MgCl_2$, ATP and dCTP. pTP capable of forming initiation complex was purified from infected HeLa cell extracts by DNA affinity chromatography (Enomoto *et al.*, 1981). It was found to be in a tightly bound

complex with a 140kD protein from which it could only be dissociated by sucrose density centrifugation under strong denaturing conditions in the presence of 1.7M urea (Lichy *et al.* 1982). This protein was subsequently shown to be the adenovirus specific DNA polymerase.

Although the role of pTP in the protein priming of DNA synthesis has been elucidated the part played by the mature TP in DNA replication is unclear. Initially it was observed that the presence of TP considerably increased the infectivity of viral DNA (Sharp *et al.*, 1976). It was proposed that this was because the terminal protein simply protected transfected linear DNA from exonuclease attack. However a number of studies have established that template DNA containing TP can support levels of DNA replication in vitro up to 20 fold higher than template devoid of TP (Tamanoi and Stillman, 1982; van Bergen *et al.*, 1983; Challberg and Rawlins, 1984; Harris and Hay, 1988). One study even reported an absolute dependence on the presence of TP containing template for DNA replication *in vitro* (Ikeda *et al.*, 1981). It is likely that TP plays several roles in DNA replication. The observed interaction between TPs at the ends of mature viral genomes (Robinson *et al.*, 1973) suggests that it may be able to interact with pTP. Such an interaction could play a part in positioning of the replication proteins appropriately at the origin of replication. In support of this hypothesis is that aberrant utilisation of the fourth G on the template strand for initiation has been observed to take place on template lacking TP (Tamanoi and Stillman, 1982). There is indirect evidence that TP may also be involved in strand unwinding in the initial stages of DNA replication. Kenny *et al.* (1988)

demonstrated that when using TP free template for *in vitro* DNA replication it was required that the very end of the template strand be single stranded or that a cellular protein, pL, possessing a 5'-3' exonuclease activity be present to expose a single stranded region. However there was no such requirement for an exonuclease on template containing TP.

Recently evidence suggesting that TP mediates binding of adenovirus DNA to the nuclear matrix has been found. It was observed that the termini of the viral genome associated most tightly with the nuclear matrix and that a viral DNA with mutant TP attached was defective in nuclear matrix binding and additionally was not efficiently packaged into mature virions. Furthermore such mutants showed a much reduced efficiency in transcription of early genes (Schaack *et al.*, 1990). The nuclear localisation of Ad pol has been shown to be dependent on its interaction with pTP. In an assay where pTP and pol genes were cloned into expression vectors under the control of the RSV-LTR promoter and expressed in monkey cells it was observed that the cotransfection of the vector containing the pTP gene was necessary for direction of pol to the nucleus (Zhao and Padmanabhan, 1988). The formation of protein-protein complexes in the cytoplasm probably represents an important mechanism for location of proteins to their required destinations in the cell.

### 6.3. Adenovirus DNA polymerase.

During purification of precursor terminal protein a 140 kD species which was essential for *in vitro* DNA replication, and which had a DNA polymerase activity

distinguishable from that of cellular DNA polymerases α, β and γ was copurified and found to be in a tight complex with pTP (Enomoto *et al.*, 1981). The viral origin of this DNA polymerase was suggested from complementation experiments with a group of temperature sensitive E2 mutants of Ad5 (Group N mutants) defective in both intiation and elongation of DNA replication *in vivo* (Ostrove *et al.*, 1983; van Bergen and van der Vliet, 1983). Extracts prepared from cells infected with such mutant viruses were unable to support pTP-dCMP complex formation *in vitro*. Addition of the 140kD polymerase restored activity to these extracts. This complementation was utilised as an assay for purification of Ad pol (Stillman *et al.*, 1982, Ostrove *et al.*, 1983). When region E2b was cloned and expressed in cos cells an aphidicolin resistant DNA polymerase activity identical to that of the putative Ad pol was detected in extracts of transfected cells (Shu *et al.*, 1987). Both pTP and pol share an exon from map position 39 which donates 3 amino acids to the N terminal of both proteins which is absolutely essential for expression of the proteins in an active form (Shu *et al.*, 1988).

Ad pol can utilise a variety of homopolymer template.primer combinations such as poly(dC).oligo(dG) and poly(dT).oligo(rA) or d(A). When the latter is used as template it was observed that Ad pol activity is stimulated up to 100 fold by the presence of DBP and a further 3 to 10 fold by ATP (Field *et al.*, 1984). Consistent with a reported specific cooperative interaction between DBP and Ad pol (Lindenbaum et al., 1986) it was observed that E.coli SSB was unable to substitute for DBP and also that DBP did not stimulate the activity of cellular pol

α activity (Field *et al*., 1984). DBP acts by increasing the processivity of pol, probably by stabilising the interaction between pol and the template. There is evidence that the interaction may involve formation of a physical complex between the two proteins since the the thermostability of pol was observed to increase in the presence of DBP (Lindenbaum *et al.*, 1986). Such interactions between SSBs and DNA polymerases have been shown to occur in some bacteriophages such as T4 and T7 (Huberman and Kornberg, 1971; Reuben and Gefter, 1973). In addition to its polymerase function Ad pol also possesses an intrinsic 3' to 5' exonuclease activity, in common with several other eukaryote and prokaryote DNA polymerases. It is probable that this function has a role in proofreading of nascent DNA chains during elongation (Field *et al.*, 1984).

Recently it has been demonstrated that Ad pol interacts with nuclear factor I (NFI), a cellular protein which stimulates initiation of DNA replication. Bosher *et al.* (1990) observed binding of pTP-pol complex and pol alone but not pTP alone to a matrix containing NFI immobilised on Sepharose. Similarly it has been shown that NFI co-precipitates with pol by immunoprecipitation using antibody against Ad pol (Chen *et al.*, 1990). This interaction probably plays a role in the correct positioning and stabilisation of a multiprotein complex of replication proteins prior to initiation of DNA replication.

The Ad DNA polymerase contains several regions of homology with a range of other known DNA polymerases of eukaryote origin such as those of HSV, EBV, CMV, yeast pol I and cellular pol α and from prokaryote sources such as

bacteriophage T4 and ϕ29. However its primary amino acid structure does not have much in common with what are apparently a different class of DNA polymerases such as E.coli DNA polymerases I and II and DNA pol β.

Adenovirus DNA pol activity is similar to that of cellular DNA pol α when utilising activated calf thymus DNA as template in that it is sensitive to ara-CTP and high NaCl concentration but unlike pol α it is highly resistant to aphidicolin. However the replication of Ad DNA *in vivo* (Longiaru *et al.*, 1979) and *in vitro* (Nagata *et al.*, 1983) is inhibited by aphidicolin suggesting that another protein required for DNA replication is sensitive to its effect. *In vitro* studies have shown that pTP-dCMP complex formation and limited elongation is unaffected but that extended elongation is adversely affected by aphidicolin (Nagata *et al.*, 1983).

**7. Cellular proteins involved in adenovirus DNA replication.**

In the absence of other factors, very limited initiation of DNA replication occurs *in vitro* requiring only pTP, pol and DBP (Guggenheimer *et al.*, 1984; Rawlins *et al.*, 1984). The addition of a crude nuclear extract of uninfected HeLa cells was observed to restore efficient levels of initiation. This stimulatory effect was due mainly to the binding of two factors, NFI and NFIII to recognition sites in the origin of replication.

### 7.1. Nuclear Factor I (NFI).

NFI was initially purified as a 47kD protein from HeLa cell nuclear extract on the basis of its ability to stimulate pTP-dCMP complex formation (Nagata *et al.*, 1982). Subsequent DNAaseI footprint analysis revealed that it protected a region containing a sequence conserved in many adenovirus serotypes between nucleotides 17 and 48 of the ITR (Nagata *et al.*, 1983). Studies by several workers involving extensive mutagenesis and deletion analysis of the NFI binding site led to the determination of a core consensus recognition sequence for NFI of TGGC $(N)_5$ GCCAA (Gronostajski *et al.*, 1984,1987; De Vries *et al.*, 1985; Leegwater *et al.*,1985). The interaction of NFI with its binding site has been studied in detail by methylation interference and protection and ethylation interference studies. These reveal that NFI makes contacts with G and T residues in the major groove of the DNA helix as well as on the phosphate backbone. The observation that the contacts made are similar in each of the two blocks of symmetrical sequence and that inversion of the entire NFI binding site has no effect on the efficiency of Ad2 DNA replication *in vitro* or *in vivo* (Hay *et al.*, 1988) suggested that NFI binds as a dimer making base contacts on one side of the helix only (De Vries *et al.*, 1987).

As well as being located at the Ad ITR and involved in DNA replication NFI sites are also present as important components in the promoter regions of many eukaryote genes such as the human IgM gene, the myc oncogene, the chicken lsozyme gene and viral gene regulatory regions such as the Hepatitis B virus S-gene promoter and the CMV immediate early promoter. For the most part

deletion of NFI sites from upstream regulatory regions results in a reduction in the levels of transcription obtained. Correspondingly a five fold stimulation of transcription *in vitro* was observed when NFI sites were inserted upstream of the TATA box of the adenovirus MLP (Gronostajski *et al.*, 1988).

Although NFI was originally identified as a single 47kD species a wide range of proteins with NFI activity have been isolated from various sources. A family of NFI proteins ranging in size between 52 and 66kD have been identified in human cells (Rosenfeld and Kelly, 1986). In addition multiple mRNA species encoding NFI like proteins have been identified in the hamster, the rat and the pig (Gil *et al.*, 1988; Paonessa *et al.*, 1988; Meisterernst *et al.*, 1989). On the basis of immunological data, amino acid composition and proteolytic cleavage patterns it appears that NFI proteins are identical to CCAAT-binding transcription factor (CTF), a family of proteins involved in the expression of a variety of genes. Analysis of cDNAs from multiple human NFI/CTF mRNAs has shown that all are products of a single gene which gives rise to multiple transcripts by alternative splicing of a precursor (Santoro *et al.*, 1988). The proteins encoded by these mRNAs all contain a highly conserved N-terminal protease resistant region containing the DNA binding domain of the protein. This domain alone has been expressed using the vaccinia expression vector system and it has been demonstrated that the binding of this region of the protein in itself is sufficient to stimulate adenovirus DNA replication (Santoro *et al.*, 1988; Mermod *et al.*, 1989; Gounari *et al.*, 1990). Furthermore it appears that the transcriptional activation function of NFI is localised in the less highly conserved C-terminal portion of the

protein (Mermod *et al.*, 1989). Thus the binding of NFI to DNA is sufficient to stimulate DNA replication but transcriptional activation requires the additional presence of the C-terminal portion which is presumed to be important for interaction either directly with components of the transcriptional machinery or with proteins influencing transcription. It has been demonstrated that transcriptional activation of a collagen type I promoter by transforming growth factor (TGF-b) is mediated by an NFI site. This suggests that NFI protein may be modified by interaction with other proteins into an active state whereby it is able to activate transcription (Rossi *et al.*, 1988).

It is not known whether some members of the NFI/CTF family are exclusively involved with DNA replication and others with transcription or that all proteins are active in both functions. It has been demonstrated that a partially purified protein from chicken erythrocytes which binds a lysozyme promoter NFI site is able to substitute for human NFI in stimulating adenovirus DNA replication (Leegwater *et al.*, 1986). This suggests that there is a high degree of functional conservation amongst the NFI/CTF proteins and that their function in transcription and DNA replication is interchangeable.

As mentioned in section 1.6 it was observed that stimulation of adenovirus DNA replication *in vitro* by NFI is increased in the presence of DBP (De Vries *et al.*, 1985). It has recently been demonstrated that this is due to a cooperative interaction between NFI and DBP whereby the affinity of NFI for its binding site and its rate of dissociation from DNA is decreased (Cleat and Hay, 1989; Stuiver and van der Vliet, 1990). It appears that the interaction is highly specific since

DBP cannot be substituted for by other single stranded DNA binding proteins like HeLa SSB or E.coli SSB. Two models accounting for this effect have been proposed. First, a direct protein-protein interaction between NFI and DBP could occur, bringing about a change in the structural configuration of NFI allowing it to bind to DNA more tightly. However since no evidence has been found for such a physical interaction it is thought more likely that the binding of DBP brings about localised structural changes which makes the NFI recognition site more accessible for NFI binding.

It has been observed that the position of the NFI site relative to the core origin sequence is critical since insertion of additional sequences between the 1-18 sequence and the NFI binding site abolishes NFI mediated stimulation of DNA replication *in vitro* (Adhya *et al.*, 1986; Wides *et al.*, 1987). This suggested that a strict constraint on the spatial arrangement between the 1-18 sequence and NFI site may be required to allow protein-protein contacts between NFI and other components of the replicative machinery. Recently a similar effect of altering the relative position of the NFI site was observed on adenovirus DNA replication *in vivo* (Bosher *et al.*, 1990). Futhermore it has been demonstrated that Ad pol and NFI make specific protein-protein contacts (Bosher *et al.*, 1990; Chen *et al.*, 1990). On the basis of the observed NFI-pol interaction it appears that one of the roles of NFI is to stabilise the multi-protein replication complex formed immediately prior to initiation of DNA replication.

### 7.2. Nuclear Factor III (NFIII).

Studies where crude nuclear extracts were screened for their ability to stimulate adenovirus DNA replication *in vitro* in the presence of excess NFI led to the identification of NFIII (Pruijn *et al.*, 1986). A protein with similar properties which was designated origin recognition protein C (ORP-C) was also identified at around the same time (Rosenfeld *et al.*, 1987). These two were subsequently found to be identical (O'Neill and Kelly,1988). NFIII has been purified as a 92-95kD species which on binding to its recognition site in the adenovirus origin of replication stimulates initiation of DNA replication between 3 and 7-fold. DNAaseI footprinting analysis showed that NFIII protected a region in the Ad2 origin of replication encompassing nucleotides 35 to 55. More detailed contact point analysis by methylation protection defined a core binding region TATGATAAT lying between nucleotides 39 and 47 (Pruijn *et al.*, 1988). By mutational analysis it was determined that every base in this core sequence is important, NFIII binding being reduced by between 2 and 10 fold depending on which nucleotide was changed. Unlike the NFI site, the NFIII site is not symmetrical and correspondingly NFIII binds to the sequence as a monomer. There is a direct relationship between NFIII binding and the rate of initiation of DNA replication. It has been demonstrated that adenovirus type 12, whose origin has a low affinity binding site for NFIII, can only be stimulated to increase initiation around 2-fold in the presence of NFIII (Pruijn *et al.*, 1988)

Similar to NFI, NFIII, in addition to its function in adenovirus DNA replication, plays a role in the regulation of transcription. NFIII is a member of the octamer

binding series of DNA binding proteins which recognise the consensus sequence TAATGARAT where R= A or G. This binding site has been identified in the regulatory regions of several cellular genes such as the immunoglobulin H and L chains, the histone H2B genes and the small nuclear RNAs U1, U2 and U6 genes. Additionally octamer binding sites are present in the SV40 enhancer and in the promoters of the IE genes of HSV. In the latter case a functional interaction between NFIII and a virion component,vmw65, leads to activation of transcription (O'Hare and Goding, 1988; Preston *et al.*, 1988). NFIII has been found to be functionally similar to another protein binding to cellular enhancers and promoters called octamer transcription factor (OTF-I). OTF-I is able to substitute for NFIII in stimulating adenovirus DNA replication. Another octamer binding protein OTF-2 has been identified in B-cells. All octamer binding proteins identified have in common a conserved region which is termed the POU domain.

The function of NFIII in intiation of adenovirus DNA replication is not as well defined as it is for NFI since efficient initiation is observed in its absence provided that NFI is present (Rosenfeld *et al.*, 1987). Furthermore Ad2 virus genomes with the NFIII site deleted were found to be as equally as infectious as those with an intact NFIII site (Hay and McDougall, 1986). However in a transfection assay it was observed that the presence of an NFIII site alone stimulated the basal level of Ad2 DNA replication obtained with the core origin only by between 10 and 20 fold (Hay *et al.*, 1988). More recently expression of truncated forms of the oct1 and oct2 genes in recombinant vaccinia vectors has

shown that, as with NFI, only the conserved DNA binding as distinct from the domain responsible for transcriptional activation, is required for stimulation of DNA replication (Mul *et al.*, 1990).

The location of the binding sites of NFI and NFIII in close proximity to one another suggested that the two proteins may function in a cooperative manner to stimulate DNA replication. However no evidence of any such interaction has been demonstrated and it appears that NFI and NFIII, by binding to their recognition sites in the origin function independently to stimulate initiation of DNA replication.

### 7.3. Nuclear Factor II (NFII).

NFII was identified as a factor required for full length elongation of nascent strands using adenovirus DNA-protein complex as template *in vitro* (Nagata *et al.*, 1983). Based upon its requirement for elongation it was purified and found to be a 30kD protein which had topoisomerase activity.

Unlike NFI and NFIII, NFII is not a sequence specific DNA binding protein and it is not required for initiation of DNA replication or elongation of shorter templates. It was observed that the function of NFII could be substituted for by the 100kD HeLa cell topoisomerase I. It may be that NFII represents a different species of cellular topoismerase with activity similar to that of topoisomerase I or it could be a core subunit of the enzyme. The requirement for NFII indicates that torsional stress results from the movement of the replication fork during adenovirus DNA replication *in vitro* indicating that the molecular ends are not free to rotate. That

this should occur on a linear molecule free of any nuclear matrix attachments implies that rotation must be restricted by proteins present on the template during DNA replication, possibly by an interaction between the TPs at either end of the DNA-protein complex.

### 7.4. Nuclear Factor IV (NFIV).

Using an exonuclease III protection assay for detection of cellular proteins binding to the origin of replication a protein consisting of a 72kD and an 84kD subunit was observed to bind with apparent sequence specificity to the termini of adenovirus DNA. However on a template devoid of other proteins it was found that NFIV bound to virus DNA via the molecular end and then migrated freely along double stranded DNA. Present in sufficiently high concentration NFIV was observed to saturate the DNA molecule forming a regular DNA-multimeric protein complex. The apparent specificity for binding to sequences at the termini initially observed appeared to be a product of blocking of translocation of NFIV by the presence of NFI and NFIII at their binding sites (De Vries *et al.*, 1989).

The role of NFIV, if any, in adenovirus DNA replication is unclear since it has not been observed to have any major effect on the level of DNA replication obtained *in vitro*. Moreover on TP free templates DNA replication was observed to be severely inhibited by the presence of high concentrations of NFIV presumeably by its perturbation of the interaction of other DNA replication proteins.

### 7.5. Factor pL

pL was first identified as a factor present in HeLa cell nuclear extracts and stimulated adenovirus DNA replication *in vitro* when DNA without TP was used as a template but not when DNA-protein complex was the template (Guggenheimer *et al.*, 1984). It was subsequently purified as a 44kD protein possessing a 5' to 3' exonuclease activity which stimulates DNA replication by rendering the 3' end of the template strand single stranded. Use of partially single stranded oligonucleotides as templates were shown to abolish the stimulatory effect of pL (Kenny *et al.*, 1988). It is unlikely that factor pL plays a role in adenovirus DNA replication *in vivo* since *in vitro* replication on TP containing template is not affected by pL. It is probable that partial unwinding at the end of the duplex is a role fulfilled *in vivo* by one or more of the known replication proteins. The fact that DNA- protein complex serves as a more efficient template for *in vitro* DNA replication suggests that TP may play a role in localised strand unwinding at the origin.

## 8. Adenovirus type 4 DNA replication.

The transfection experiments conducted by Hay *et al.*, 1984, described in section 5, which were used to determine the Ad2 origin of replication *in vivo* were extended to Ad4. It was observed that plasmids containing an Ad2 replication origin were replicated equally efficiently supported by either Ad2 or Ad4 helper virus. However plasmids containing Ad4 origin sequences were only efficiently replicated when co-transfected with Ad4 helper virus genome.

Co-transfection with Ad2 helper virus gave around a 20 fold reduction in replication by comparison (Hay, 1985b). Sequence analysis of the replication origins of Ad2 and Ad4 showed that whilst the 1-18 core domain of the origin is identical in both serotypes, Ad4 possesses an A/T rich domain in place of an NFI binding site. Furthermore it was demonstrated that Ad4, in contrast to Ad2, requires only the terminal 18bp of the viral genome for fully efficient DNA replication *in vivo*. Subsequent studies conducted using *in vitro* replication systems confirmed the *in vivo* observations. It was demonstrated using a crude Ad4 infected HeLa cell extract that linearised plasmid containing only the terminal 18bp of an adenovirus ITR could support initiation of DNA replication (pTP-dCMP complex formation) *in vitro* as effectively as a template containing a complete Ad4 ITR (Harris and Hay, 1988). The basis of the difference in origin sequence requirements between Ad2/5 and Ad4 is that Ad4 appears to be able to replicate its DNA without the need for auxilliary stimulatory functions provided by the binding of NFI and NFIII. Ad4 does not possess an NFI recognition site although it does have a strong consensus binding site for NFIII located at the same position as in the Ad2/5 origin of replication. However it has been unequivocally demonstrated that neither cellular factor stimulates Ad4 DNA replication even if their cognate binding site is present in a reconstructed origin of replication (Hay *et al.*, 1988). Since it appears that NFI and NFIII play auxilliary roles in Ad2/5 DNA replication, whereby the function of the viral replication proteins are enhanced or aided by their binding at the origin, the probability is that Ad4 is able to replicate its DNA without them because its own replication

proteins alone can perform the required functions. Ad4 therefore potentially provides a more simplified system in which to further study the molecular details of the mechanism of adenovirus DNA replication.

**9. Simian virus 40 DNA replication.**

Studies on the DNA replication of the Papovaviruses, polyomavirus and in particular Simian virus 40 (SV40) have led to a very detailed knowledge and characterisation of the molecular events and the proteins involved at each stage in the replicative process of these viruses. SV40 DNA replication is regarded as a good model system for providing information on the mechanisms underlying cellular DNA replication. DNA replication takes place in the nucleus of the host cell where the viral genome exists as a mini-chromosome complexed with cellular histones to form a nucleoprotein complex indistinguishable from that of chromatin. Only a single viral protein, T-antigen, an 82kD phosphoprotein is involved with viral DNA replication. As a result host cell replication proteins are extensively utilised for replication of the virus genome in a manner which probably differs little from that of cellular chromatin replication.

The SV40 genome is a duplex circular DNA molecule of 5243 bp containing a single origin of DNA replication. Initiation of DNA synthesis results in the establishment of two replication forks which move bi-directionally from the origin creating theta-form intermediates. At each fork one of the two nascent strands, the leading strand, grows continuously while the other lagging strand grows discontinuously by joining together of Okazaki fragments of around

200bp, each of which is independently initiated with RNA primers (reviewed by Hay and Russell, 1989; Stillman, 1989). Termination of replication occurs when the two forks moving in opposite directions meet.

As was the case in the study of adenovirus DNA replication, the development of a cell free system in which SV40 DNA replication could be initiated and elongated *in vitro* provided the opportunity for an in depth investigation of the molecular mechanisms of DNA replication and for purification and characterisation of the proteins involved. A major breakthrough came with the development of a method by Li and Kelly (1984) for making a soluble extract of SV40 infected cells that would support initiation and elongation of exogenous plasmid DNA containing an SV40 origin of replication. It was also shown that uninfected cell extract could support DNA synthesis if it was supplemented with T-antigen.

### 9.1. The SV40 origin of DNA replication.

By extensive mutational analysis the cis-acting DNA sequence elements contributing to the overall efficiency of SV40 DNA replication have been identified. A 64bp sequence forms the core origin and is both necessary and sufficient for DNA replication *in vitro* (Stillman *et al.*, 1985). *In vivo* however cis-acting elements adjacent to the core origin can have a profound effect on the efficiency of SV40 DNA replication. Two regions, aux-1 and aux-2 flank the origin core region. Aux-2 also forms part of a late transcriptional promoter and contains binding sites for sequence specific DNA binding transcription factors

Sp1 and Ap2 (Mitchell *et al.*, 1987). An additional region comprising the SV40 transcriptional enhancer, the 72bp repeats, lies close to aux-2. Deletion of aux-2 has been shown to reduce the efficiency of DNA replication 2 fold and the additional deletion of the 72bp repeats results in up to a 100 fold decrease in DNA replication (Hertz and Mertz, 1986). The observed stimulatory effect of these regions *in vivo* but not *in vitro* is thought to result from binding of transcriptional factors which perturb the chromatin structure in the region of the origin and facilitate initiation of DNA replication.

The SV40 core origin has been examined in detail and contains three domains essential for origin function. It is composed of a central element containing four repeats of the sequence 5'-GAGGC-3' arranged as a palindrome (Tegtmeyer *et al.*, 1983) which serves as the binding site for T-antigen. It has been demonstrated that T-antigen binds to this sequence in the absence of ATP forming a range of oligomeric structures from monomers up to tetramers (Mastrangelo *et al.*, 1985). The other two domains of the core origin are located on either side of the T-antigen binding site. One contains half of a palindromic sequence (the 'early palindrome'), which has been found to be perfectly conserved in other papovaviruses (Deb *et al.*, 1986) and corresponds to the initial site of T-antigen mediated duplex unwinding (Boroweic and Hurwitz, 1988a). The other, an A/T rich region 17bp in length brings about a stable bend in the DNA duplex in the region of the origin (Deb *et al.*, 1986).

## 9.2. Initiation of DNA replication and bidirectional DNA synthesis: The role of T-antigen.

T-antigen is expressed at early times in infection and plays multiple roles during the productive infection of cells by SV40. As well as its central role in the replication of SV40 DNA its other important function is its ability to induce cells to enter the cell cycle (Tjian *et al.*, 1978). Correspondingly it has been shown to interact with a number of cellular proteins implicated in cell growth control such as p53, transcription factor AP2, DNA polymerase $\alpha$ and, like Ad Ela, the retinoblastoma susceptibility gene product (Smale and Tjian, 1986; Mitchell *et al.*, 1987; de Caprio *et al.*, 1988; Dornreiter *et al.*, 1990).

T-antigen has two direct roles in SV40 DNA replication. Firstly it binds specifically to the core origin of replication bringing about important structural changes to the duplex DNA in the region of the origin which facilitate initiation of DNA synthesis (Boroweic *et al.*, 1990). Secondly it has a helicase activity which is required for bidirectional unwinding of the origin and extended unwinding during DNA synthesis. After initial binding of T-antigen, unwinding of the origin, initiation of DNA replication and elongation of the leading and lagging strands are carried out by host DNA replication proteins. Through detailed molecular studies of the DNA replication reaction *in vitro* most of these have been identified, purified and extensively characterised which has allowed the building of a detailed molecular model for the replication of SV40 DNA.

Following the binding of T-antigen at the origin, the origin region is more

extensively unwound in a bidirectional manner. This reaction, which involves the hydrolysis of ATP, requires the additional activity of a cellular topoisomerase to remove positive supercoils and single strand DNA binding protein (RF-A) to stabilise newly generated single strands (Wobbe *et al.*, 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988). This results in the formation of a nucleoprotein complex at the origin which is recognised by the cellular DNA replication machinery. DNA replication is initiated by the polymerase α/primase complex which binds to the single strands at the unwound origin via a protein/protein interaction with T-antigen and/or RF-A. The involvment of such an interaction was suggested by the observation that polymerase α co-precipitates with T-antigen (Smale and Tjian, 1986). In addition it has been observed that there is a requirement for the pol α/primase to originate from a cell type which is permissive for viral replication. Murakami *et al.* (1986) demonstrated that a cell extract depleted of pol α/primase which could not support SV40 DNA replication *in vitro* , had its activity restored by addition of purified pol α/primase derived only from primate cells and not with the analagous enzyme complex from murine or bovine sources.

In addition to polymerase α/primase a second cellular DNA polymerase, pol δ, is involved in the synthesis of SV40 DNA. Its role was first suggested when a 36kD factor essential for SV40 DNA replication *in vitro*, also found associated with cells undergoing rapid growth and division, proliferating cell nuclear

antigen (PCNA) was identified and purified (Mathews *et al.*, 1984; Prelich *et al.*, 1987a). PCNA was found to increase the processivity of DNA polymerase δ (Prelich *et al.*, 1987b) which implied that the polymerase had a role in SV40 DNA replication. The role of PCNA was investigated in a study which compared the structure of SV40 replication products formed *in vitro* in the presence and absence of PCNA (Prelich and Stillman, 1988). The results demonstrated that initiation of SV40 DNA replication and the formation of the first nascent strands at the replication origin did not require PCNA. However subsequent elongation was observed to be aberrant in the absence of PCNA: Leading strand synthesis did not occur and lagging strand products remained short and dissociated from the template. This implied that PCNA is required for co-ordinated leading and lagging strand synthesis and that DNA synthesised at the replication origin is not automatically elongated. A second factor called RF-C was also identified and found to be associated with elongation. Its omission from the SV40 *in vitro* DNA replication reaction was observed to have a similar effect on replication products to that of the absence of PCNA (Tsurimoto and Stillman, 1989a). Further investigations revealed that both PCNA and RF-C recognise and form a complex on primed single stranded DNA which blocks the primase of the pol α/primase complex from priming at the 3' end of Okazaki fragments (Tsurimoto and Stillman, 1990a). By extending studies of this type where SV40 DNA replication was reconstituted entirely from purified proteins and the effect of omitting components was examined, Tsurimoto and Stillman, (1990b) proposed a

'polymerase switching' model for initiation of bidirectional DNA replication and subsequent elongation of leading and lagging strands: The origin is unwound by the action of T-antigen and RF-A and the newly exposed single stranded region is recognised and bound by the polymerase α/primase complex mediated by an interaction with the T-antigen. The primase then synthesises short oligoribonucleotides that prime the first Okazaki fragments at the origin which are elongated by polymerase α. After synthesis of the first Okazaki fragment, PCNA interacts with RF-C at the end 3' end of the nascent strand and blocks further priming by the pol α/primase complex. The PCNA/RF-C complex is recognised by polymerase δ which replaces the pol α /primase complex and initiates leading strand DNA synthesis. The dissociated pol α/ primase then returns to the next priming site on the lagging strand by interaction with T-antigen and continues to copy lagging strand template discontinuously.

**10. Herpes simplex virus DNA replication.**

The most extensively studied and characterised members of the herpes virus group are human HSV1 and HSV2 which replicate lytically in epithelial cells of the infected host as well as in a range of mammalian cells in tissue culture. In addition HSV can maintain a latent infection, its genome being harboured in the neurons of sensory ganglia. HSV1 contains a large double stranded DNA genome of about 153 kb which has been completely sequenced and encodes at

least 70 polypeptides (McGeoch *et al.*, 1988) including all those required for the replication of its genome. The genome consists of a long ($U_L$) and a short ($U_S$) unique segment each of which is flanked by inverted repeat sequences. During the course of viral replication the two components invert relative to one another giving rise to an equal mix of four isomers which differ in relation to the orientation of the two segments (Jenkins and Roizman, 1986).

The overall mechanism of HSV DNA replication has been difficult to determine owing to the large size of the genome although analyses of replicative intermediates by physical means and electron microscopy has shown replicating HSV DNA to have two important features: [$^3$H] thymidine pulse labelled viral DNA replicating in infected cells has a higher sedimentation coefficient than unit length viral DNA (Jacob and Roizman, 1977); and is also fused at the molecular termini forming long concatemers (Jacob *et al.*, 1979). On the basis of these observations it was proposed that parental linear DNA is circularised after entry into the cell and subsequent replication takes place by a continuous rolling circle mechanism generating progeny genomes as linear concatemers of tandemly repeated virus genomes (Jacob *et al.*, 1979).

### 10.1. The HSV origins of DNA replication.

HSV has three origins of DNA replication located in separate regions of the genome; two are in the inverted repeats flanking the $U_S$ segment of the genome and are termed $ORI_S$ (Stow, 1982b) and the other is in the middle of the $U_L$

segment (Weller *et al.*, 1985). The functional necessity for the presence of three origins is not clear since HSV mutants lacking one copy of $ORI_S$ or $ORI_L$ are non-defective in growth (Longnecker and Roizman, 1986). Based on the observation that transfected plasmid containing viral origins of replication are amplified in HSV infected cells (Stow,1982b) the sequence requirements for origin function were defined. In initial studies a 90bp fragment which specified ORIs function was identified. It is comprised of a 45bp palindromic sequence of which the central 18bp are exclusively composed of A/T base pairs (Stow and McMonagle, 1983). In addition a region of around 40bp to the left of the palindrome is required for ORIs function. ORIL, though similar to ORIs in the region covering the ORIs palindrome, contains a more extensive palindromic region of 144bp (Weller *et al.*, 1985). Deletion of the A/T rich central component of the palindrome or replacement with G/C base pairs was shown to completely abolish origin function (Lockshon and Galloway, 1988). As with some other well characterised origins of replication the A/T rich region probably represents an initial site for unwinding of the duplex by a component of the replicative machinery. Both arms of the palindrome contain a binding site for the HSV origin binding protein, a product of the UL9 virus gene (Olivo *et al.*, 1988; Weir *et al.*, 1989). The probable importance of UL9 binding to origin function is discussed below. There is evidence that regions flanking the core origin sequence have a modest stimulatory effect on origin function (Stow, 1982b; Stow and McMonagle, 1983).Although both ORIs and ORIL are flanked by divergently transcribed genes Su and Knipe, (1987) reported that they found no evidence suggesting that

promoter or upstream regulatory elements of these transcription units had any direct influence on origin function.

### 1.10.2. HSV genes required for DNA replication.

Two approaches have been used to determine the HSV genes which are required for viral DNA replication. Firstly by genetic analysis; A large number of conditionally lethal mutants of HSV have been isolated, many of which are defective in DNA synthesis. Complementation studies have shown that mutants with defects directly affecting DNA replication fall into seven complementation groups (Challberg and Kelly, 1989). Secondly, by the use of an assay in which cloned segments of HSV genome were tested for their ability to support co-transfected plasmid containing an origin of replication (Challberg, 1986) seven genes have been identified which were found to be both necessary and sufficient for DNA replication (McGeoch *et al.*,1988; Wu *et al.*, 1988). Three of these genes encode proteins which have been reasonably well characterised; the major single stranded DNA binding protein, the viral DNA polymerase (Quinn and McGeoch, 1985) and a sequence independent DNA binding protein (Marsden *et al.*, 1987).

A novel DNA polymerase activity from extracts of HSV infected cells was identified on the basis of its having different sensitivity to a range of inhibitors from that of any of the cellular DNA polymerases (Weissbach *et al.*, 1973). The HSV DNA polymerase has since been purified as a 140kD monomer (O'Donnell *et al.*, 1987a) and is a product of the UL30 gene (Quinn and McGeoch, 1985).

Analysis of temperature sensitive and altered drug sensitivity polymerase mutants revealed that all such changed phenotypes arose from mutations in the C-terminal portion of the protein, which led to the suggestion that this region contains the nucleotide binding domain of the protein (Gibbs *et al.*, 1988). Biochemical studies on HSV DNA polymerase have shown it to possess an intrinsic 3' to 5' exonuclease activity which, in common with a number of other DNA polymerases, probably plays a role in proof reading during elongation (Powell and Purifoy, 1977; O'Donnell *et al.*, 1987a). It is highly processive, and apparently functions efficiently in the absence of accessory factors (O'Donnell *et al.*, 1987a). However it has been reported that a 55kD protein which binds strongly to double stranded DNA, subsequently shown to be a product of the UL42 gene, co-purified with the 140kD DNA polymerase (Vaughan *et al.*, 1984). UL42 is one of the seven genes shown to be absolutely required for HSV DNA synthesis, although the effect of its 55kD product on DNA polymerase is unclear.

Infected cell polypeptide 8 (ICP8) was first identified as a highly abundant protein in the HSV infected cell which bound tightly to single stranded DNA (Bayliss *et al.*, 1975). It is likely that the role of ICP8 in DNA replication is analagous to that of other single strand specific DNA binding proteins such as Ad DBP and E.coli SSB, whose primary function in DNA replication is to bind to single stranded DNA generated at the replication fork in order to facilitate elongation by DNA polymerase. However purified ICP8 has been observed to have only a twofold stimulatory effect on HSV DNA polymerase using activated calf thymus DNA as a template (Ruyechan and Weir, 1984). It was also observed

to inhibit HSV DNA pol on a single stranded template (O'Donnell *et al.*, 1987b). However it is thought that *in vivo* its function is modulated by interaction with other replication proteins. Some ICP8 temperature sensitive mutants have been observed to over express late genes at the non permissive temperature, suggesting that ICP8 may also play a direct role in the regulation of viral transcription (Godowski and Knipe, 1985). As discussed in an earlier section adenovirus DBP also appears to have an influence on mRNA synthesis. Such a function could be attributed to an intrinsic RNA binding activity, a property common to other DNA binding proteins such as adenovirus DBP.

By DNaseI footprinting analysis it was demonstrated that a sequence corresponding to that present in each arm of the palindromic region in ORIs was protected. It was subsequently shown that this was due to the binding of a virus induced polypeptide of molecular weight 83kD (Elias *et al.*, 1986; Elias and Lehman, 1988). Methylation interference experiments combined with mutagenesis showed that the protein recognised a consensus sequence YGYTCGCACT where Y can be a C or a T (Koff and Tegtmeyer, 1988; Deb and Deb, 1989). This sequence specific origin DNA-binding protein was found to be a product of the UL9 gene (Olivo *et al.*, 1988; Weir *et al.*, 1989). There have been conflicting data on the importance of the right arm of the palindrome for ORIs function. It was reported that a plasmid completely lacking the right arm replicated in a plasmid amplification assay as efficiently as a plasmid containing a complete origin (Deb and Doelberg, 1988). Interestingly studies on the related Varicella zoster virus (VZV) revealed that although its origin contains sequences

homologous to the left arm of the palindrome and the A/T rich region, VZV has no sequence equivalent to the right arm in the HSV origin (Stow and Davison, 1986). However it has more recently been demonstrated in a study where UL9 protein binding was disrupted by mutagenesis of the binding sites on either arm, that efficient origin function required that both sites be capable of binding UL9 (Weir and Stow, 1990). It has been observed that insertion of *n* additional A/T base pairs into the A/T rich domain at the centre of the palindromic sequence has a periodic effect on the efficiency of ORI function whereby DNA replication decreases sharply when $n = 1$ to 3, increases when $n = 5$ or 6 and then decreases again sharply when $n > 6$ (Lockshon and Galloway, 1988). This may reflect a strict spatial constraint on the positioning of the UL9 binding sites whereby UL9 proteins binding to the two arms of the palindrome have to be correctly orientated with respect to one another in order to facilitate a protein-protein interaction which may be critical for ORI function.

Although as discussed above some of the products of the HSV genes defined as being necessary for DNA synthesis have been reasonably well characterised a more comprehensive analysis of these proteins and their specific roles in viral DNA replication will probably require the development of a suitable system in which HSV DNA replication can be reconstituted *in vitro*.

# MATERIALS AND METHODS

**1. Reagents.**

Reagents were obtained from BDH Chemicals Ltd., Broome Road, Poole Dorset, England (AnalaR grade), Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, England and May and Baker Ltd., Dagenham, Essex, England. Radiochemicals were obtained from Amersham International plc.

**2. Restriction endonucleases and DNA modifying enzymes.**

Enzymes were obtained from Boehringer Mannheim Ltd., Lewes, East Sussex, England and New England Biolabs, Bishop's Stortford, Herts, England, and used according to manufacturer's directions.

**3. Bacterial culture medium.**

Bacteria were propogated in Luria Broth; 10g/l bacto-tryptone (Difco Labs., Detroit, USA) and 5g/l yeast extract (Difco) in 10mM NaCl, sterilised by autoclaving.

**4. Bacterial Strains.**

All plasmids were grown in *E. coli* K12 strain DH1 (Maniatis *etal*, 1982).

**5. Virus stocks.**

Adenovirus type 4 was obtained from R.T. Hay. Recombinant baculovirus vectors containing the genes for Ad5 pTP and pol were obtained from E-L. Winnaker.

**6. Plasmids.**

Plasmid pHR18 contains the terminal 18bp of the Ad2 ITR which is identical in adenovirus types 2 and 4. p4A2 contains the adenovirus type 4 ITR and is identical to p4A1 (Hay, 1985a). pHR1 contains the adenovirus type 2 ITR (Hay, 1985a). pUDI67 pm9 to 16 (pM9 to pM16) all contain the terminal 67bp of the Ad2/5 ITR carrying a single base transition in each base pair from position 9 to 16 from the terminus. pMDC10 pm17 and 18 (pM17 and pM18) contain the terminal 69bp of the Ad2/5 ITR and carry single base transitions at positions 17 and 18 (Rawlins *et al.*, 1984). Plasmids pM9 to pM18 were obtained from C. Burrow.

**7. Oligonucleotides.**

All synthetic oligonucleotides were synthesised using an Applied Biosystems model 381A DNA synthesiser.

**8. Antibodies.**

Monoclonal antibody α72k B6-10 raised against Ad2 DBP was obtained from A. Levine (Reich *et al.*, 1983). Guinea Pig antiserum raised against Ad2 DBP and rabbit antisera against Ad2 pTP and pol was obtained from R.T. Hay.

**9. Cells and tissue culture.**

HeLa S3 spinner cells were routinely used to prepare virus stock and as a source of virus infected cellular extract. Cells were grown in suspension in Earle's minimal essential medium (Gibco Europe Ltd., Paisley, Scotland) containing 50 units/ml of penicillin, 50μg/ml of streptomycin (Glaxo Labs. Ltd., Greenford, England) and 5% new-born calf serum (Gibco). A549 cells were used to titrate virus and assay for infectivity (Williams *et al.*, 1970). Cells were grown in monolayer in Glasgow modified minimal essential medium (Gibco) containing penicillin, streptomycin, sodium bicarbonate and 10% new-born calf serum (Gibco).

**10. Preparation of plasmid DNA.**

**10.1. Preparation of competent cells**

Competent *E.coli* cells were prepared as described by Hanahan (1983). Colonies were picked from a fresh streak of cells and resuspended in 6.5ml of SOB (2%bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$). The culture was maintained at 37°C in an orbital incubator until a cell density which gave an $OD_{550}$ = 0.4-0.5 was reached. Cells were kept on ice for 2 minutes, collected by centrifugation at 2500 r.p.m. for 10 min and resuspended in 2ml FSB (10mM KOAc, 100mM KCl, 45mM $MnCl_2.4H_2O$, 3mM $Co(NH_3)_6Cl_3$), kept on ice for 10 minutes then collected by centrifugation at 2500 r.p.m. for 10 minutes. The pellet was resuspended in 0.5

ml FSB. Freshly prepared DMSO was added to 0.5% v/v and the suspension kept on ice for 5 minutes. The DMSO step was repeated and competent cells were dispensed into 200ml aliquots, quick frozen in a solid $CO_2$/ethanol bath, and stored at -70$^0$C until required.

### 10.2. Transformation of bacteria

Competent cells were transformed by the method of Hanahan (1983), or the "one minute" method of Golub (1988): 1-10ng of plasmid DNA was added to 200µl of competent *E.Coli* cells and incubated on ice for 30 minutes prior to heat shock at 42$^0$C for 90 seconds. After 1-2 minutes, 800µl SOB containing 20mM glucose was added and the mixture incubated at 37$^0$C for 1 hour. Aliquots of 50-100µl of the culture were removed, plated onto Luria agar plates supplemented with 100µg/ml ampicillin and incubated at 37$^0$C for 12 hours to establish colonies or; 3µl of competent cells were mixed on ice with 1µl plasmid DNA (1-100ng DNA). The mixture was then heated at 44$^0$C for 1 minute and transferred immediately to 100µl of L-broth and the mixture plated out onto selective medium.

### 10.3. Large scale preparation of plasmid DNA

Transformed bacteria were grown to stationary phase in 500ml of L-broth containing 100µg/ml ampicillin and harvested by centrifugation at 4,000 r.p.m in

a Beckman JA14 rotor for 10 minutes. The pellet was resuspended in 5ml of buffer containing 50mM Tris-HCl pH8.0, 25% sucrose (w/v) to which 1ml of a freshly made solution of 0.25M Tris, 20 mg/ml lysozyme was added. After incubation at 20°C for 5 minutes 1ml of 0.5M EDTA (pH8.0) was added and the solution incubated for a further 5 minutes at 20°C. Cells were lysed by gentle agitation in a buffer containing 0.2% (v/v) Triton X-100, 50mM Tris (pH8.0) and 62.5mM EDTA and incubating at 20°C for 15 minutes, agitating gently. Cell debris were removed by centrifugation at 20,000 r.p.m for 30 minutes in a Beckman JA 20 rotor. The supernatant was collected and CsCl-EtBr was dissolved in the solution at 1.55mg/ml and 600mg/ml respectively. The solution was then transferred to 100x20mm Beckman polyallomer bell-top quick seal tubes and plasmid DNA was centrifuged to equilibrium at 50,000r.p.m for 16 hours. Plasmid DNA was removed by side puncture and ethidium bromide was removed by repeated extraction with caesium chloride saturated iso-butanol. The DNA was then desalted by ultrafiltration in a centricon-30.

**10.4. Quantitation of DNA.**

DNA concentrations in aquaeous solution were determined by scanning the absorbance between 220nm and 280nm on a Perkin-Elmer Lambda 5 UV/VIS Spectrophotometer. One optical density unit at 260nm was taken to correspond to 40µg/ml of double stranded DNA. In addition, concentrations were determined by separating DNA samples by electrophoresis in an agarose gel containing

0.5µg/ml EtBr and comparing the fluorescence under UV light with standards of known concentration.

**11. Radiolabelling of oligonucleotides.**

Oligonucleotides were 5' end labelled using the Klenow fragment of E.coli DNA polymerase. 1-50ng of DNA was incubated in a buffer containing 50mM Tris (pH 8.7), 5mM $MgCl_2$ and 0.1mM DTT with Klenow and 1mM dNTP's, including 20µCi of the appropriate [$\alpha^{32}$P] radiolabelled nucleotides. The reaction was incubated at 20°C for 15 minutes. Labelled DNA was then phenol/ chloroform extracted, ethanol precipitated and stored at 4°C.

**12. Virus purification.**

HeLa S3 cells in suspension were infected with 10pfu/cell of adenovirus stock in a tenth volume of serum free medium. Adenovirus was adsorbed for 90min at 37°C and medium containing 2% calf serum was added back to make up to the original volume. Cells were incubated at 37°C for 48 hours and collected by centrifugation at 2000rpm for 15min. The pellet was resuspended in Tris/saline, freeze/thawed 3 times and the virus extracted by shaking with an equal volume of Arcton for 20min. To further purify virus the Arcton extract was applied to a density gradient of 3ml of 3M CsCl overlayed with 2ml of 2M CsCl in a 14ml Beckman polycarbonate tube. The virus was then centrifuged to equilibrium at 30,000r.p.m for 90 minutes in a TST 40.1 swing bucket rotor and collected by

bottom puncture.

**13. Preparation of crude cytoplasmic extract from Ad4 infected HeLa cells.**

1litre of HeLa S3 cells were infected with 100 pfu/cell of Ad4 and after 90min medium containing 2% calf serum plus 10mM hydroxyurea was added. Cells were incubated for 22 hours and collected by centrifugation at 2000rpm for 15min and then washed in an isotonic buffer containing 20mM Hepes/NaOH (pH7.5), 5mM KCl, 0.5mM $MgCl_2$, 0.5mM DTT and 0.2M sucrose. Cells were collected by centrifugation again and resuspended in a hypotonic buffer containing 20mM Hepes (pH7.5), 5mM KCl, 0.5mM $MgCl_2$ and 0.5mM DTT, incubated at 4°C for 10min and lysed by 10 strokes of a Dounce homogenizer using a type B pestle. Nuclei were pelleted by centrifugation at 3000rpm for 5min, the supernatant containing the cytoplasmic fraction was removed and then clarified by further centrifugation at 15000rpm for 30min. The extract was depleted of nucleic acids by passage over DEAE-sepharose in 0.2M NaCl and then concentrated by precipitation with 70% saturation ammonium sulphate at 4°C. The precipitate was resuspended in 2ml of 20mM Hepes (pH7.5), 5mM KCl, 0.5mM $MgCl_2$ and 0.5mMDTT, dialysed against the same buffer and stored in small aliquots at -70°C.

## 14. Purification of Ad4 DNA replication proteins

### 14.1. Preparation of single stranded calf thymus DNA.

Type 1 calf thymus DNA (Sigma No.D-1501) was dissolved in $H_2O$ at 10mg/ml by stirring for 12 hours and sonicating using an MSE Soniprep 150 until the viscosity of the solution was lost. The solution was then denatured by boiling for 15 minutes and cooled rapidly to 4°C.

### 14.2. Preparation of single stranded calf thymus DNA-sepharose.

100ml settled volume of sepharose (Whatman CL-4B) was washed twice with 1litre of ultra-pure $H_20$, resuspended in 200ml of $H_20$ and stirred gently for 30minutes. 1.5g of CNBr was added dropwise to the sepharose over 1 minute followed by the addition of 9ml of 10M NaOH dropwise over 10 minutes. The reaction was stopped by addition of 1 litre of ice-cold $H_20$. The slurry was then transferred to a Buchner funnel and filtered with 4 litres of ice-cold $H_2O$ followed by 2 litres of 10mM potassium phosphate buffer(pH8.0). 16mg of single stranded calf thymus DNA was added and shaken gently with the CNBr activated sepharose for 12 hours at 20°C. The DNA-sepharose was then washed with 100ml of 1M ethanolamine and gently shaken for 4 hours at 20°C followed by 200ml washes with 50mM sodium phosphate buffer(pH8.0), 1M NaCl and finally with buffer containing 10mM TRIS (pH8.0), 1mM EDTA and 0.3 M NaCl.

### 14.3. DNA affinity purification.

Crude cytoplasmic extract was prepared from Ad4 infected Hela S3 spinner cells as described, excluding the last two stages, and applied to a DEAE-sephacel column equilibrated in buffer containing 20mM HEPES (pH8.0), 5mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT and 50mM NaCl. The column was washed with two volumes of buffer containing 50mM NaCl and then with two volumes of buffer containing 0.2M NaCl. Fractions were collected, assayed for *in vitro* DNA replication activity and their protein concentration determined. Activity was recovered in the 0.2M eluate. This was applied to a single strand calf thymus DNA-sepharose column equilibrated in buffer containing 0.2M NaCl. The column was washed with two volumes of buffer containing 0.2M NaCl and 5 volumes of a concentration gradient from 0.2M to 2M NaCl, collecting 2ml fractions. The protein concentration of each fraction was determined and peak fractions were dialysed against 20mM HEPES (pH8.0), 5mM KCl, 0.5mM $MgCl_2$, 5mM DTT and 1mM PMSF and stored at -70°C.

### 14.4. Immunoaffinity purification.

Mouse ascitic fluid containing 5mg of monoclonal antibody α72k B6-10 raised against Ad2 DBP was collected and bound to 1ml of protein G-sepharose (Sigma) by direct coupling in 3M NaCl (Simanis and Lane,1985) and used to prepare a column. Crude extract from Ad4 infected HeLa cells was prepared as described above and dialysed against 10mM sodium phosphate (pH 7.5). The

extract was applied to the antibody column and unbound protein was removed by extensive washing with 10mM sodium phosphate, 0.05% NP40. Bound protein was eluted by increasing stepwise washes of 10, 20, 30, 40, 50 and 100mM $MgCl_2$ and fractions were analysed by SDS-PAGE. DBP was eluted in a near homogeneous state at 50-100mM $MgCl_2$.

**15. Preparation of crude extract and purification of Ad5 pTP and pol from Spodoptera frugiperda cells infected with recombinant baculovirus.**

For expression of proteins using the recombinant baculovirus vectors 500ml of Spodoptera frugiperda insect cells were grown in liquid culture in TC-100 medium supplemented with 15% foetal calf serum at 28°C and infected with recombinant Autographa californica polyhedrosis virus expression vectors containing the genes for Ad5 pTP and pol at 10 p.f.u. per cell. Infected cells were incubated for a further 72 hours at 28°C in TC-100 medium supplemented with 5% foetal calf serum and then harvested by centrifugation at 2500 r.p.m. for 15 minutes and resuspended in 25mM Hepes (pH8.0), 5mM KCl, 0.5mM $MgCl_2$ and 0.5mM DTT, incubated at 4°C for 10 minutes, then disrupted by 10 strokes in a Dounce homogeniser using a type-B pestle. NaCl was added to bring the final concentration to 0.2M, and the extract was incubated at 4°C for 30 minutes prior to removal of insolubilised protein by centrifugation at 15000 r.p.m. for 5 minutes followed by further centrifugation at 50000 r.p.m. for 15 minutes.

The method for purification of recombinant Ad5 pTP and pol from crude insect cell extract was identical for both proteins. Crude clarified extract at 0.2M NaCl was applied to denatured calf thymus DNA-Sepharose equilibrated with 0.2M NaCl. After extensive washing with 0.2M NaCl bound proteins were eluted with 0.6M NaCl. Fractions were collected and their protein concentration determined. Peak fractions were pooled and dialysed against 2 changes of 0.4M KCl, 5mM $KPO_4$ (pH7.0), 1mM DTT and 10% glycerol for 8 hours and then applied to hydroxylapatite equilibrated in the same buffer. After extensive washing with 5mM $KPO_4$ bound proteins were eluted with 100mM KCl. Proteins were aliquoted and stored in small volumes at -70$^{o}$C.

**16. Separation of proteins by glycerol density gradient centrifugation.**

Two types of gradient were used: (1) A native gradient was created by layering 0.8ml each of a 50%, 35%, 30%, 25%, 20% and a15% glycerol solution in 20mM HEPES pH7.5, 5mM KCl and 0.1% NaCl into a 5ml tube and; (2) A denaturing gradient by layering 0.8ml each of a 50%, 30%, 27.5%, 22.5%, 17.5% and a12.5% glycerol solution in 20mM HEPES pH7.5, 5mM KCl, 0.1M NaCl and 1.7M urea into a 5ml tube. Gradients were allowed to form by incubating the tubes at 4$^{o}$C for 3 hours. A 100-200µl protein sample was loaded onto the gradient and proteins were separated by centrifugation at 49000rpm for 14 hours at 4$^{o}$C in a Beckman SW40Ti rotor. Fractions were collected by bottom

puncture and then analysed.

**17. Quantitation of protein.**

Protein concentrations were determined by the method of Bradford (1976). Protein samples in a total volume of 10µl, were mixed with 1ml of Bradford's reagent (100mg coomassie blue G250, 100ml orthophosphoric acid and 50ml of ethanol made up to 1 litre with distilled $H_20$), incubated at room temperature for 5 minutes and the absorbance at 595nm measured on an LKB Biochrom Ultrospec II spectrophotometer. Concentrations were calculated by comparison with a standard curve constructed using BSA.

**18. Agarose gel electrophoresis**

Gel slabs (100mm x 100mm) containing either 1% or 2% (w/v) agarose, 1x TBE and 0.5µg/ml EtBr in a total volume of 50ml were run at 5V/cm. One fifth volume of sample buffer (50% (v/v) glycerol, 100mM EDTA, 0.2%(w/v) bromophenol blue and 0.2% (w/v) xylene cyanol) was added to samples prior to electrophoresis.

**19. Native polyacrylamide gel electrophoresis.**

Gels were made up to the required acrylamide concentration from a 40% acrylamide stock solution (38% acrylamide, 2%N,N'-methylene-bis- acrylamide). Gels contained 1xTBE buffer and were polymerised by the addition of 1/100

volume of 10% (w/v) ammonium persulphate and 1/2000 volume of TEMED. Gels were cast 1.5mm thick and run at up to 10V/cm. 1/10 volume of sample buffer (50% glycerol (v/v), 0.2%(w/v) bromophenol blue, 0.2%(w/v) xylene cyanol, 100mM EDTA) was added to samples prior to electrophoresis.

**20. SDS-polyacrylamide gel electrophoresis.**

10% separation gels were made up from a 30% acrylamide stock (29% acrylamide, 1% N,N'-methylene-bis-acrylamide). Gels contained 0.375M Tris.HCl (pH8.9) and 0.1% SDS and were polymerised as described above. Gels were cast 1.5mm thick and poured to leave sufficient space for a 1.5cm deep stacking gel. An overlayer of $H_2O$ was used to ensure an even surface after polymerisation. Stacking gels contained 5% acrylamide in 0.105M Tris.HCl (pH6.7) and 0.1% SDS. Prior to electrophoresis protein samples were denatured by heating at 100$^{o}$C for 2min in a buffer containing 20% glycerol (v/v), 5%SDS (w/v), 570mM 2-mercaptoethanol, 33mM Tris pH6.7 and 0.2% (w/v) bromophenol blue. Gels were run at 45mA for 4 hours.

If necessary polypeptide species were stained by soaking gel in 47:47:6 methanol: water: acetic acid containing 0.2% Coomassie Brilliant blue R-250 for 60 minutes. Excess stain was removed by soaking in methanol: water: acetic acid mix. Alternatively proteins were visualised by silver staining using a Bio Rad silver staining kit according to the manufacturers directions. If required, gels were dried in a Biorad 583 vacuum gel dryer.

## 21. Western Blot analysis

All blots were carried out using Ancos semi-dry Western blot equipment according to the manufacturers directions. Proteins were transferred from polacrylamide gel to nitrocellulose by applying a current of 8mA per $cm^2$ of gel. Filters were blocked by incubation with 10% w/v powdered non-fat milk in PBS and, after washing in PBS, were probed by incubating with 200µl of immune serum in 5ml of PBS, at 37°C for 1 hour. Antibody-antigen complex was detected by incubation of the filter with [$I^{125}$] protein A at 2µCi/ml and after extensive washing with PBS, 0.05% NP40, labelled species were detected by exposure to X-ray film at -70°C.

## 22. Analysis of proteins by Gel filtration.

250µg samples of Ad5 pTP and pol and pTP-pol complex were loaded onto a Zorbax G-250 hydrophilic gel filtration column installed on a Waters HPLC system. The column was calibrated by running protein standards of known molecular weight. Their $K_{av}$ was derived ($K_{av}= V_e-V_o/V_t-V_o$, where $V_o$= void volume of column, $V_t$= total column volume and $V_e$= elution volume) and plotted against the log of their molecular weight. The buffer used throughout contained 100mM $KPO_4$ (pH7.0), 400mM KCl and 1mM DTT.

**23. Assay for the transfer of dCMP to pTP.**

1-12µl of Ad4 infected HeLa cell cytoplasmic extract or purified replication proteins were incubated in a 30µl reaction containing 25mM Tris/HCl (pH8), 3mM $MgCl_2$, 2mM DTT, 3mM ATP, and 3µCi [$\alpha^{32}$P] dCTP (specific activity 3000Ci/mmol) and varying amounts of template DNA for 90min at 37°C. Reactions were stopped by heating at 70°C for 5min and treated with micrococcal nuclease at 1 unit/reaction in the precence of 2mM $CaCl_2$ for 30min at 37°C. 12µl of gel loading buffer containing 20% glycerol (v/v), 5%SDS (w/v), 570mM 2-mercaptoethanol, 33mM Tris pH6.7 and 0.2% bromophenol blue (w/v) was added and the samples denatured by heating at 100°C for 2 min. Reaction products were resolved and visualised in a 10% SDS-polyacrylamide gel at 35mA for 4 hours and which was dried and then subjected to autoradiography at -70°C in the presence of an intensifying screen.

**24. Assay for elongation of DNA initiated *in vitro*.**

Restriction enzyme cleaved template DNA was pre-incubated in a total volume of 7µl for 30 minutes at 30°C with 1-3µl of Ad4 infected HeLa cell cytoplasmic extract or purified viral replication proteins in a buffer containing 25mM Hepes-KOH (pH 7.5), 4mM $MgCl_2$, 1mM DTT, 0.1 mg/ml BSA and 0.2 mM aphidicolin. Elongation proceeds by addition of 7µl of a buffer containing 80µM

each of dTTP, dATP, and dGTP, 5μM dCTP, 0.3μCi [$\alpha^{32}$P] dCTP (specific activity 3000Ci/mmol), 4mM ATP, 10mM creatine phosphate, 10mg/ml creatine phosphokinase, 0.1mg/ml BSA, 25mMHepes-KOH, 4mM Mg $Cl_2$ and 1mM DTT to give a total reaction volume of 14μl. The total reaction was incubated at 30°C for 90 minutes. Reactions were stopped by addition of 6μl of buffer containing 5% SDS, 50% glycerol, 100mM EDTA and bromophenol blue and heated at 70°C for 5 minutes. Reaction products were resolved on a 2% agarose gel containing 0.1% SDS at 35mA for 4 hours which was fixed in 10% acetic acid, dried and subjected to autoradiography at -70°C in the presence of an intensifying screen.

Quantitation of *in vitro* initiation and elongation data was carried out by excising bands from the gel and measuring the amount of radioactivity incorporated by scintillation counting using an Intertechnique SL 30 liquid scintillation spectrometer or, alternatively for elongation, precipitating nascent DNA incorporating $^{32}$P by addition of 5ml/ reaction of 10% TCA, filtering under vacuum through a Whatman GF-C filter and measuring the acid precipitable radioactivity by scintillation counting.

**25. Assay for DNA polymerase activity**

2-10μg of protein sample were incubated with 10μg of activated calf thymus DNA, 100mM dTTP, dGTP, dCTP, 20mM dATP, 1mCi [$\alpha^{32}$P] dATP (specific

activity 3000Ci/mmol), 5mM Tris pH8.0, 7mM $MgCl_2$, 10mM DTT and 100mM aphidicolin in a total volume of 50µl for 1 hour at 37°C. Reactions were terminated by addition of 10% TCA, 0.5% sodium pyrophosphate and insoluble radioactivity was captured on a Whatman GF-C disc by filtration under vacuum. The disc was washed once with 10% TCA, O.5% sodium pyrophosphate, twice with 5% TCA, once with 95% ethanol, then air dryed and the radioactivity measured by scintillation counting.

**26. Gel mobility shift assay.**

0.5-1.0 µg of purified protein(s) were incubated with $^{32}P$ end labelled double stranded oligonucleotide for 30 minutes at 21°C either in the presence or absence of unlabelled competitor DNA. DNA-protein complexes were resolved from free probe by electrophoresis in an 8% acrylamide gel at 10 volts per cm for 60 minutes. Reaction products were visualised by exposure of the gel to X-ray film at -70°C in the presence of an intensifying screen.

# RESULTS

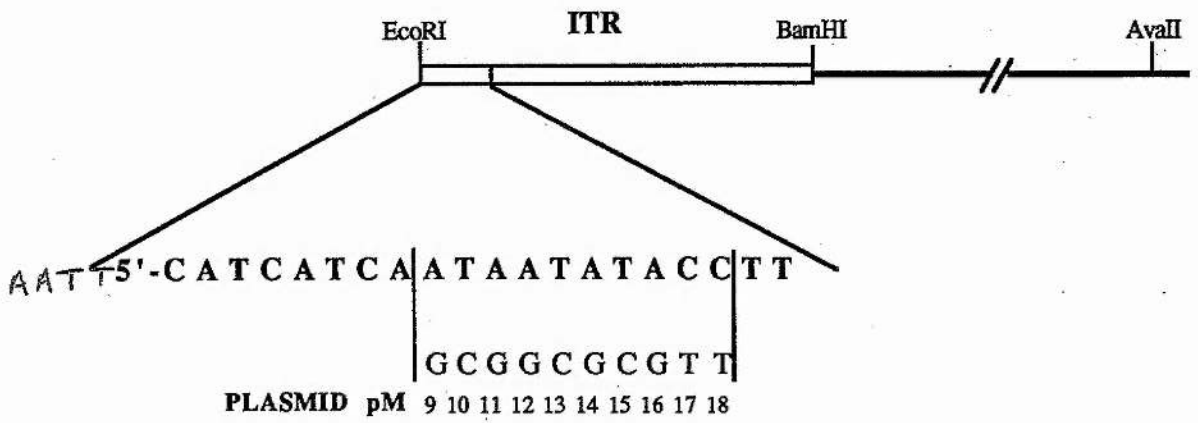
## Chapter 1. Analysis of the sequence requirements for Ad4 DNA replication.

Previous studies have demonstrated that the terminal 18bp of the ITR which are identical in adenovirus types 2/5 and 4 are sufficient to serve as an origin for Ad4 DNA replication (Hay, 1985b; Harris and Hay, 1988; Hay *et al.*, 1988). Within this region bp's 9 to 18 from the terminus are perfectly conserved in all human serotypes so far examined (Alestrom *et al.*, 1982; Ishino *et al.*, 1987) suggesting that the specific sequence in this region plays an important functional role in DNA replication.

### 1.1. The effect of point mutations in the adenovirus origin of replication on initiation of adenovirus type 4 DNA replication *in vitro*.

To examine the sequence requirement for initiation of DNA replication in Ad4 the consequence of single base changes from positions 9 to 18 on initiation of DNA replication *in vitro* was determined. The relative efficiency of initiation was assayed quantitatively by measuring the transfer of [$^{32}$P] dCMP to pTP, this being the first synthetic step of DNA replication. A series of ten plasmids, pM9 to pM18, each of which contains an Ad5 ITR with a single base change in positions 9 to 18 respectively were cleaved with EcoRI to expose the ITR sequence at a molecular end (Fig 1.1). Incubation of the template with an Ad4 infected HeLa cell cytoplasmic extract, [a$^{32}$P] dCTP and optimal levels of $MgCl_2$ and ATP results in the transfer of dCMP to pTP. Reaction products

**Figure 1.1-** Structure of plasmids containing an adenovirus ITR. A single copy of the ITR is inserted between the EcoRI and BamHI sites. Cleavage with EcoRI linearises the plasmid exposing the origin sequence at one of the molecular ends. The single point mutations at positions 9 to 18 from the terminus and the plasmid in which they are contained is indicated. The non-template strand is shown.


EcoRI
ITR
BamHI
AvaII
AATT 5'-CATCATCA ATAATATACC TT
GCGGCGCGTT
PLASMID pM 9 10 11 12 13 14 15 16 17 18

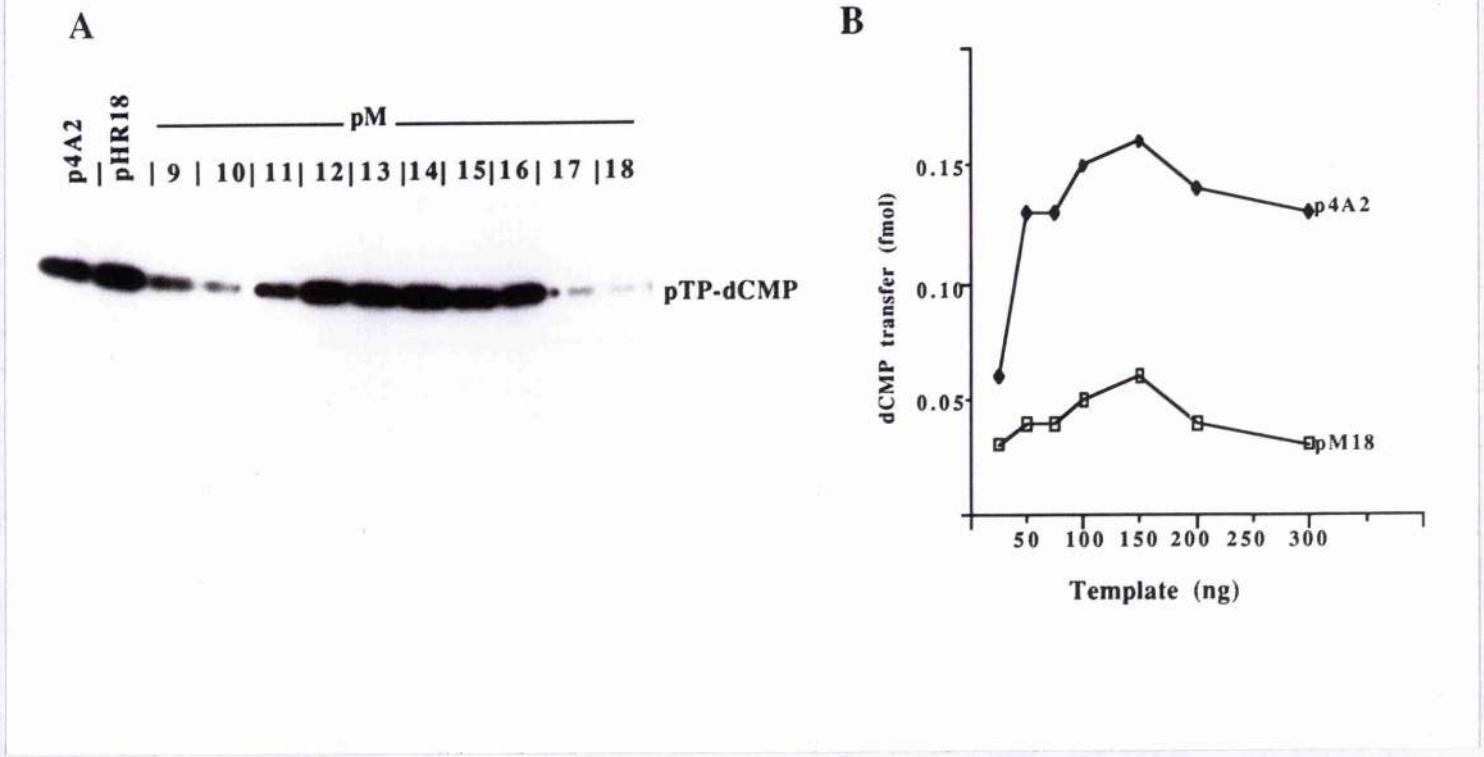
were denatured and the polypeptides resolved by electrophoresis on a polyacrylamide gel containing SDS. Labelled species were detected by autoradiography, excised from the gel and the radioactivity determined by liquid scintillation counting.

Plasmid p4A2 which contains one complete copy of an Ad4 ITR and pHR18 which contains the terminal 18bp of an Ad2/5 ITR, both supported initiation with approximately equal efficiency consistent with previous reports establishing that Ad4 requires only the terminal 18bp for efficient initiation (Fig 1.2A, lanes 1 and 2). Similarly, single base substitutions from positions 12 to 16 from the terminus had no deleterious effect on the efficiency of initiation (Fig 1.2A, lanes 6 to 10). However, alterations at positions 9, 10, 11, 17 and 18 all resulted in a reduction in efficiency of initiation by between 50% and 90% (Fig 1.2A, lanes 3,4,5,11 and 12).

**1.2. The effect of template concentration on initiation of DNA replication *in vitro*.**

To test the effect of varying template concentration on initiation of DNA replication *in vitro* increasing concentrations of EcoRI cleaved p4A2 and pM18 were used as template in a series of standard initiation reactions and the amount of dCMP transferred to pTP was quantitated by excising the bands and measuring the radioactivity in a scintillation counter. The efficiency of initiation on both p4A2 and pM18 increased with concentration to a maximum at 150ng/reaction followed by a marked decrease (Fig 1.2B). Increasing the

**Figure 1.2- A.** Initiation using mutated templates. Plasmids (50ng) containing the adenovirus type 4 ITR (p4A2), the terminal 18bp of the adenovirus type 2 ITR (pHR18) and the adenovirus type 5 ITR with point mutations in each position from 9-18 (pM9 to pM18 respectively) were cleaved with EcoRI and incubated with cytoplasmic extract (3μg) from adenovirus type 4 infected HeLa cells in a standard initiation reaction (see materials and methods) containing 1μCi [$\alpha^{32}$P] dCTP (3000Ci/mM). After incubation at 32$^{o}$C for 90 minutes and micrococcal nuclease digestion polypeptides were resolved by SDS polyacrylamide gel electrophoresis and labelled species were detected by autoradiography. **B.** Titration of template. EcoRI cleaved p4A2 and pM18 were supplied to standard initiation reactions containing 3μg of Ad4 infected cell extract and [$\alpha^{32}$P] dCTP in the quantities shown. Samples were processed for autoradiography, and the region of the gel containing the pTP-dCMP complex was excised and the radioactivity determined by scintillation counting. The information was then used to quantify dCMP incorporated.


A
p4A2
pHR18
pM
9
10
11
12
13
14
15
16
17
18
pTP-dCMP
B
dCMP transfer (fmol)
0.15
0.10
0.05
p4A2
pM18
50
100
150
200
250
300
Template (ng)

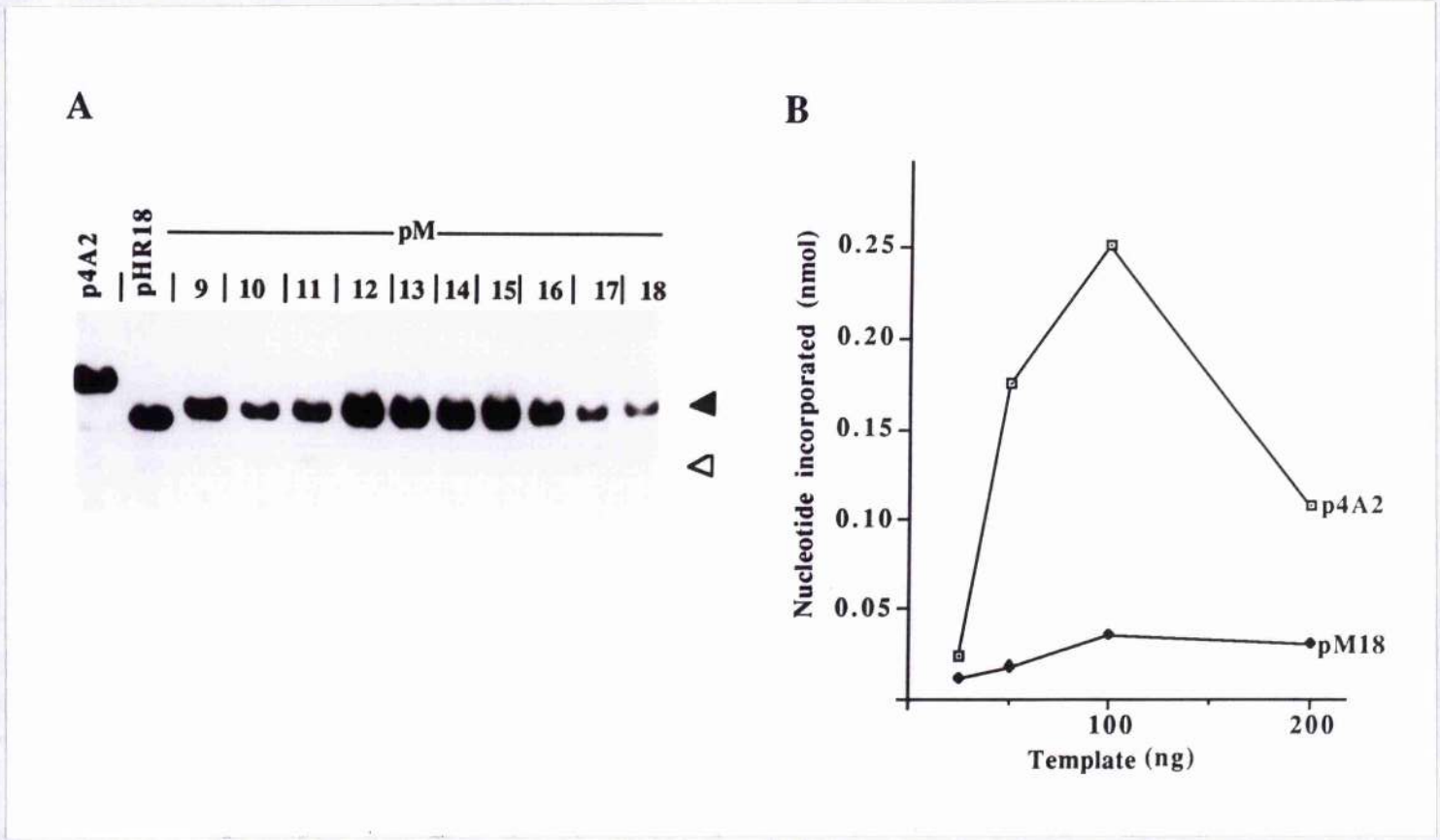
concentration of template pM18 could not compensate for the deleterious effect of the mutation.

### 1.3. Elongation of initiated molecules *in vitro*.

To further examine the effect of the single point mutations the relative efficiency with which wild type 1 to 18 sequences and plasmids pM9 to pM18 could support elongation from initiated products *in vitro* was tested. Templates were cleaved with EcoRI and AvaII to give a 1.6kb fragment with the origin sequence at the EcoRI terminus (Fig 1.1) and an 0.9kb fragment containing only plasmid sequences, and incubated with Ad4 infected HeLa cell extract, dNTP's (including [$\alpha^{32}$P] dCTP), optimal $MgCl_2$ and an ATP regeneration system. Reaction products were fractionated by electrophoresis in an agarose gel containing SDS and replicated templates which had incorporated [$^{32}$P] dCMP were detectable by autoradiography. Correctly initiated products have pTP linked at the 5' end resulting in a lower electrophoretic mobility (Fig 1.3A, black arrow) than input template molecules which incorporated $^{32}$P at a low level as a result of repair synthesis (Fig 1.3A, open arrow). p4A2 and pHR18 both supported elongation with equal efficiency, as did pM12 to pM16 (Fig 1.3A, lanes 1,2 and 6 to 10). pM9, 10, 11, 17 and 18 however all replicated at a reduced efficiency corresponding to the observed reduction for initiation. (Fig 1.3A, lanes 3,4,5,11 and 12).

**Figure 1.3- A.** Elongation of initiated template. Plasmid templates (100ng) p4A2, pHR18 and pM9 to pM18 were cleaved with EcoRI/AvaII and incubated with 3μg of cytoplasmic extract from adenovirus type 4 infected HeLa cells in a standard elongation reaction containing [$\alpha^{32}$P] dCTP (see materials and methods). After incubation at 30°C for 90 minutes reaction products were resolved by electrophoresis on a 2% agarose gel containing 0.1% SDS. Labelled species were detected by autoradiography. Nascent DNA incorporates [$^{32}$P] dCMP and has pTP attached (black arrow head) so is resolved from uninitiated input template (white arrow head) which is labelled at a low level due to repair synthesis. The 0.9 kb fragment containing only plasmid sequences, under these conditions, ran out of the gel.

**B.** Titration of template. EcoRI/AvaII cleaved plamids p4A2 and pM18 were supplied to standard elongation reactions containing 3μg of Ad4 infected cell extract in the amounts shown. Reactions were stopped by addition of 10% TCA and then filtered under vacuum through a glass fibre disc. The acid precipitable radioactivity was then determined by scintillation counting and the information used to calculate total nucleotide incorporation.

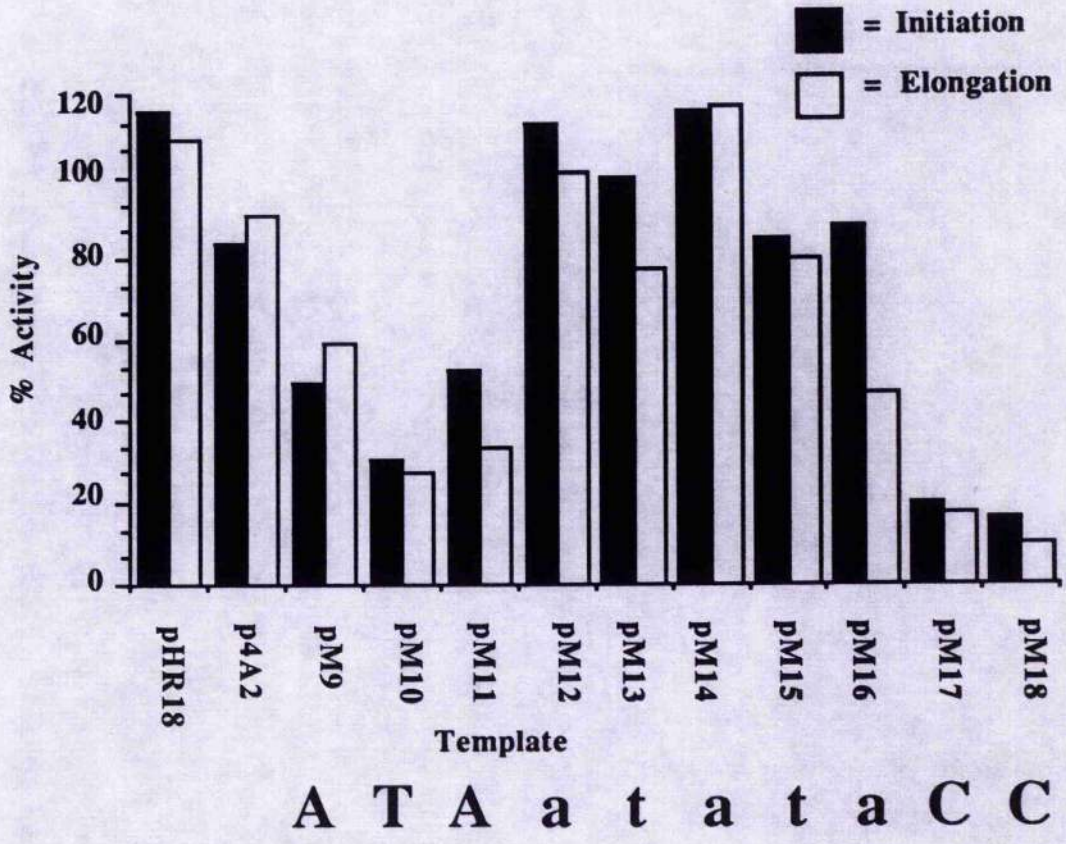

A
p4A2
pHR18
pM
9
10
11
12
13
14
15
16
17
18
B
Nucleotide incorporated (nmol)
0.25
0.20
0.15
0.10
0.05
p4A2
pM18
100
200
Template (ng)

### 1.4. The effect of template concentration on elongation *in vitro*.

To determine the effect of template concentration on elongation increasing concentrations of EcoRI/AvaII cleaved p4A2 and pM18 were incubated under standard elongation conditions. Both templates gave an increase in elongation levels in response to increased template concentration to a maximum of 100ng. Above this level a marked decrease in the total amount of elongated product was observed particularly with p4A2 (Fig 1.3B). As was the case for initiation the reduced level of elongated product obtained using the mutant template could not be compensated for by increasing the concentration of template added to the reaction.

Mutants which were defective in initiation were found to be similarly defective in elongation (fig 1.4). These data indicate that as predicted, the conserved sequence from bp's 9 to 18 in the adenovirus ITR plays a vital functional role in viral DNA replication even though certain limited sequence changes appear to be tolerable for *in vitro* DNA replication. The corresponding decrease in the efficiency of elongation which is observed for mutants exhibiting impaired initiation suggests that it is only initiation of DNA replication that is being affected by changes in the origin sequence and that elongation can proceed unimpaired from molecules which have been initiated.

**Figure1.4-** The relative activities of template shown as a percentage of wild-type activity by initiation assay (black bars) and elongation assay (white bars). The data was obtained by excision of the part of the gel containing the pTP-dCMP complex and measuring the radioactivity by scintillation counting. The mean of the activities of pHR18 and p4A2 was taken as 100%. The wild type sequence of the non-template strand between bases 9 and 18 is shown beneath the graph. The bases which are critical for initiation of DNA replication are shown in upper case and those which can be altered without an effect on DNA replication *in vitro* are shown in lower case.


= Initiation
= Elongation
% Activity
120
100
80
60
40
20
0
pHR18
p4A2
pM9
pM10
pM11
pM12
pM13
pM14
pM15
pM16
pM17
pM18
Template
A T A a t a t a C C

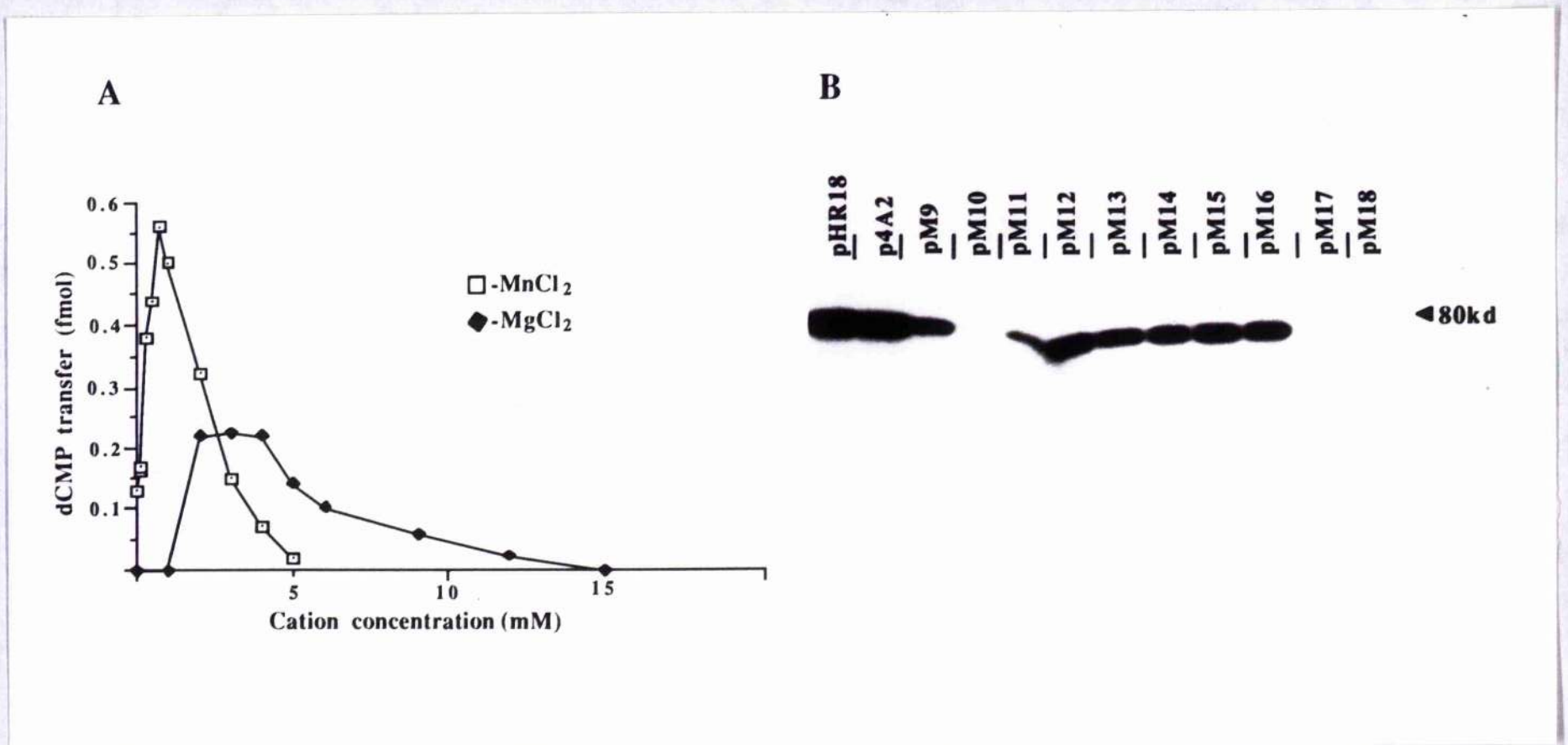
### 1.5. Initiation in the presence of $MnCl_2$.

It has been previously observed that initiation of replication can proceed in the absence of $MgCl_2$ if manganese chloride is provided (R.T. Hay, unpublished) and furthermore that a marked stimulation of initiation is obtained in the presence of $MnCl_2$. A stimulatory effect of $MnCl_2$ has also been observed for certain DNA modifying enzymes although this is often accompanied by a decrease in substrate specificity. To accurately determine the optimal $MnCl_2$ concentration for Ad4 DNA replication *in vitro* a $MgCl_2$ free cytoplasmic extract dialysed against $MgCl_2$ free extract buffer from Ad4 infected He La cells was incubated with EcoRI and AvaII cleaved p4A2 and assayed for initiation at various $MnCl_2$ concentrations, holding the ATP concentration constant. A similar titration with $MgCl_2$ was carried out for comparison (Fig 1.5A). $MgCl_2$ had to be present at 2mM before any detectable level of initiation was obtained. When the concentration of $MgCl_2$ was increased to 3mM an optimal level of initiation was attained above which a gradual decrease in initiation was seen. In contrast the maximum level of initiation in the presence of $MnCl_2$ was attained at 0.75mM and was nearly three fold higher than at the optimal $MgCl_2$ concentration. When the concentration of $MnCl_2$ was increased above 0.75mM a rapid decrease in the level of initiation was observed.

To test whether the requirement for sequence specificity of the template was

**Figure 1.5-A.** Initiation in the presence of $MnCl_2$. 50ng of EcoRI cleaved p4A2 was incubated under standard initiation conditions with 3μg of adenovirus type 4 infected cell extract in $MgCl_2$ free buffer in the presence of [$^{32}$P] dCTP and a series of increasing $MnCl_2$ concentrations. A separate set of reactions containing a series of increasing $MgCl_2$ concentrations was also carried out for comparison. In all reactions the ATP concentration was held constant at 3mM. Reactions were processed for autoradiography and the region of the gel containing the pTP-dCMP complex was excised from the gel and the radioactivity determined by scintillation counting. This information was then used to quantify the transfer of dCMP to pTP.

**B.** Initiation using mutant templates in the presence of $MnCl_2$. 50ng each of EcoRI cleaved pHR18, p4A2 and pM9 to pM18 were used as template for initiation under $MgCl_2$ free conditions in the presence of 0.75mM $MnCl_2$. All other conditions were standard.


A
B
dCMP transfer (fmol)
0.6
0.5
0.4
0.3
0.2
0.1
5
10
15
Cation concentration (mM)
□-MnCl2
◆-MgCl2
pHR18
p4A2
pM9
pM10
pM11
pM12
pM13
pM14
pM15
pM16
pM17
pM18
◀80kd

maintained in the presence of $MnCl_2$ 50ng of EcoRI cleaved p4A2, pHR18 and pM9 to pM18 was incubated under standard initiation conditions with 0.75mM $MnCl_2$. p4A2 and pHR18 both supported initiation efficiently as did pM12 to pM16 (Fig 1.5B, compare lanes 1,2 and 6-10). pM9,10, 11, 17 and 18 all gave reduced levels of initiation which corresponded to the reduction in efficiency obtained in the presence of optimal $MgCl_2$ (Fig 1.5B, lanes 3, 4, 5, 11 and 12). These data demonstrate that initiation of DNA replication *in vitro* in the presence of $MnCl_2$ was equally as sensitive to changes in the template sequence as in the presence of $MgCl_2$.

## Chapter 2. Purification and characterisation of the proteins required for Ad4 DNA replication *in vitro*.

Previous studies *in vivo* and *in vitro* have established that Ad4 has a unique and very simple origin of DNA replication compared to the more intensively studied Ad2/Ad5. Accordingly, the protein requirements differ markedly, in that Ad4 is able to replicate its DNA without the need for cellular factors NFI/CTF and NFIII/Oct-1. Although a crude extract of Ad4 infected cells was used for the initial experiments presented in this study it was conceivable that contaminating activities present in the extract may have complicated interpretation of the data. For example it has been shown that partially single stranded templates generated as a result of exonuclease action behave differently to double stranded templates (Kenny and Hurwitz, 1988). Additionally the presence of cellular DNA polymerases in a crude extract could potentially interfere with the levels of DNA replication observed.

### 2.1. Purification of a fraction from Ad4 infected HeLa cells capable of DNA replication *in vitro*.

To examine the proteins involved in Ad4 DNA replication and to exclude the possibility that contaminatory activities may have affected any data already obtained, Ad4 infected HeLa extract which was capable of *in vitro* DNA replication was fractionated. Crude Ad4 infected extract was prepared from 10 litres of HeLa suspension cells as described in materials and methods. Soluble extract was adjusted to 50mM NaCl and applied to DEAE-Sephacel

**Figure 2.1-** Purification of a fraction capable of replication of adenovirus DNA *in vitro*. **A.** Proteins bound to denatured calf thymus DNA-Sepharose were eluted with a 0.2M to 2M NaCl gradient. The protein concentration of each fraction was determined and the two major peaks designated P1 and P2(white squares). 2µl of each fraction was incubated in a standard DNA polymerase reaction (see methods) and the activity determined (black diamonds). **B.** Plasmid p4A2 (50ng) containing a copy of the Ad4 ITR was cleaved with EcoRI and incubated with 8µl of each fraction (dialysed against 0.1M NaCl buffer) from DNA-Sepharose in a standard assay for initiation of DNA replication (see methods) containing [$\alpha^{32}$P] dCTP (3000Ci/mM) and 100µM aphidicolin. After incubation at 32$^{o}$C for 90 minutes and micrococcal nuclease digestion polypeptides were fractionated by SDS polyacrylamide gel electrophoresis and labelled species were detected by autoradiography.

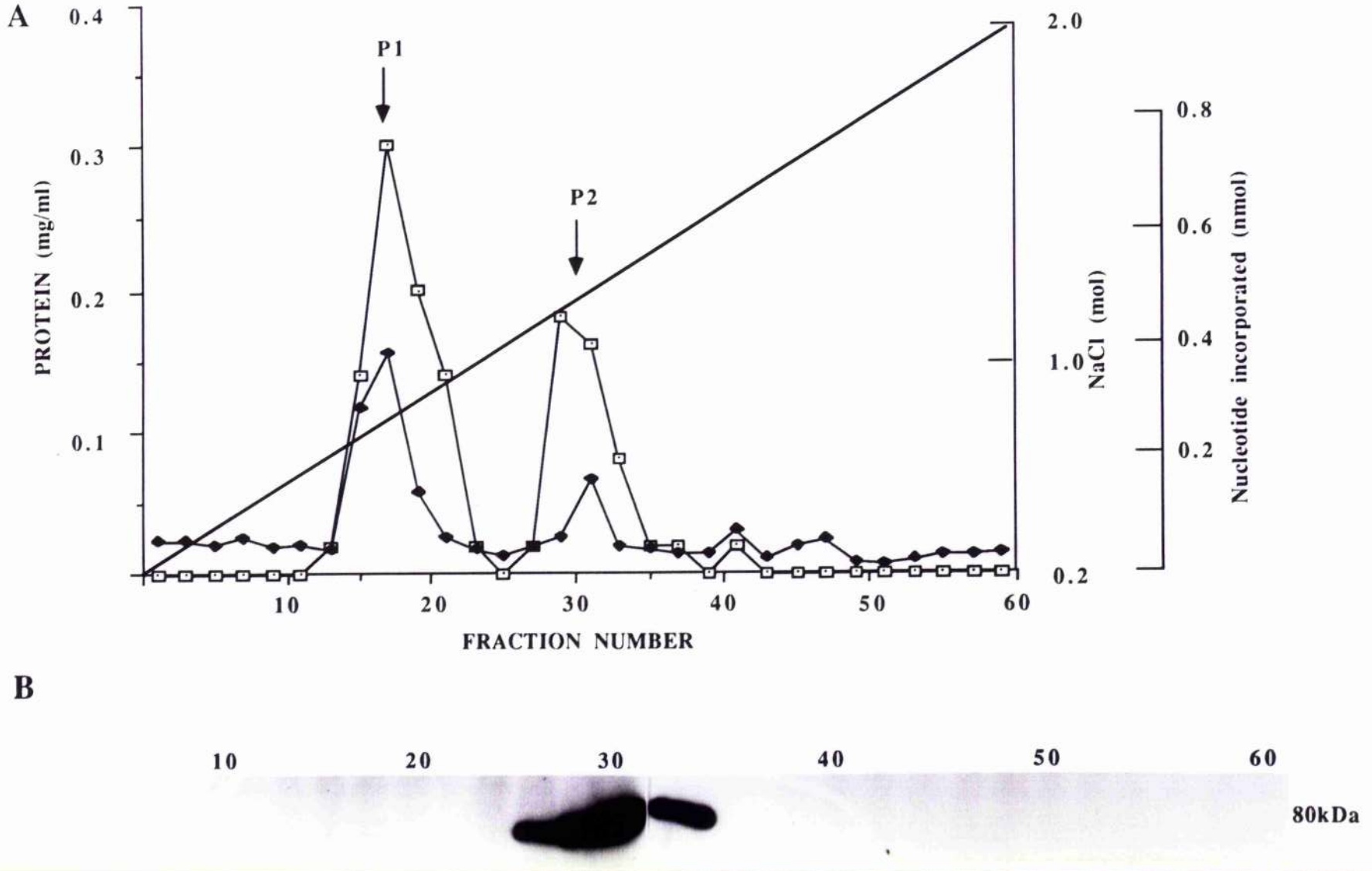

A
0.4
0.3
0.2
0.1
PROTEIN (mg/ml)
P1
P2
2.0
1.0
0.2
NaCl (mol)
0.8
0.6
0.4
0.2
Nucleotide incorporated (nmol)
10
20
30
40
50
60
FRACTION NUMBER
B
10
20
30
40
50
60
80kDa

**Table 2.1**- Purification of the Ad4 DNA replication proteins. 1μg of crude infected extract, DEAE-Sephacel eluate and denatured DNA-Sepharose eluate was incubated with EcoRI cleaved p4A2 as template in standard initiation reactions containing [$\alpha^{32}$P] dCTP. Polypeptides were fractionated by electrophoresis in a polyacrylamide gel containing SDS and labelled species were detected by autoradiography. The region of the gel corresponding to pTP-dCMP complex was excised, the radioactivity quantitated by scintillation counting and the information was used to calculate the data shown.

* 1 unit corresponds to the amount of protein required to transfer 1fmol of dCMP to pTP.

| Fraction | Total Protein (mg) | *Total Units | % Yield | Specific Acivity (units/mg) | Fold Purification |
|---|---|---|---|---|---|
| Crude extract | 149.2 | 1798 | 100 | 12 | 1 |
| DEAE-Sephacel | 29.5 | 983 | 55 | 33 | 2.75 |
| Denatured DNA-Sepharose | 1.1 | 220 | 12 | 200 | 16.6 |

equilibrated with 50mM NaCl, followed by extensive washing with 50mM NaCl. Bound proteins were eluted with 0.2M NaCl and fractions were collected. The protein concentration of each fraction was determined and the peak fractions were assayed for their ability to catalyse pTP-dCMP complex formation using EcoRI cleaved p4A2 as template. This step gave an overall 5-fold increase in specific activity with 50% yield and freed the extract of nucleic acids (Table 2.1). The peak fractions were pooled and applied to denatured calf thymus DNA-Sepharose equilibrated with 0.2M NaCl. After extensive washing with 0.2M NaCl bound proteins were eluted with a linear gradient of 0.2-2M NaCl. Fractions were collected and the protein concentration determined. Two peaks were eluted between 0.5-0.8M NaCl (P1) and 1.0-1.2M NaCl (P2) respectively (Fig 2.1A, white squares). Individual fractions were tested for their ability to initiate DNA replication *in vitro* by assaying the efficiency with which they were able to catalyse pTP-dCMP complex formation using EcoRI cleaved p4A2 as template. Only fractions in P2 were capable of initiation (Fig 2.1B). The purification procedure resulted in a 16 fold increase in the specific activity with a 12% overall yield (Table 2.1).

## 2.2. Determination of DNA polymerase activity in denatured DNA-sepharose eluate.

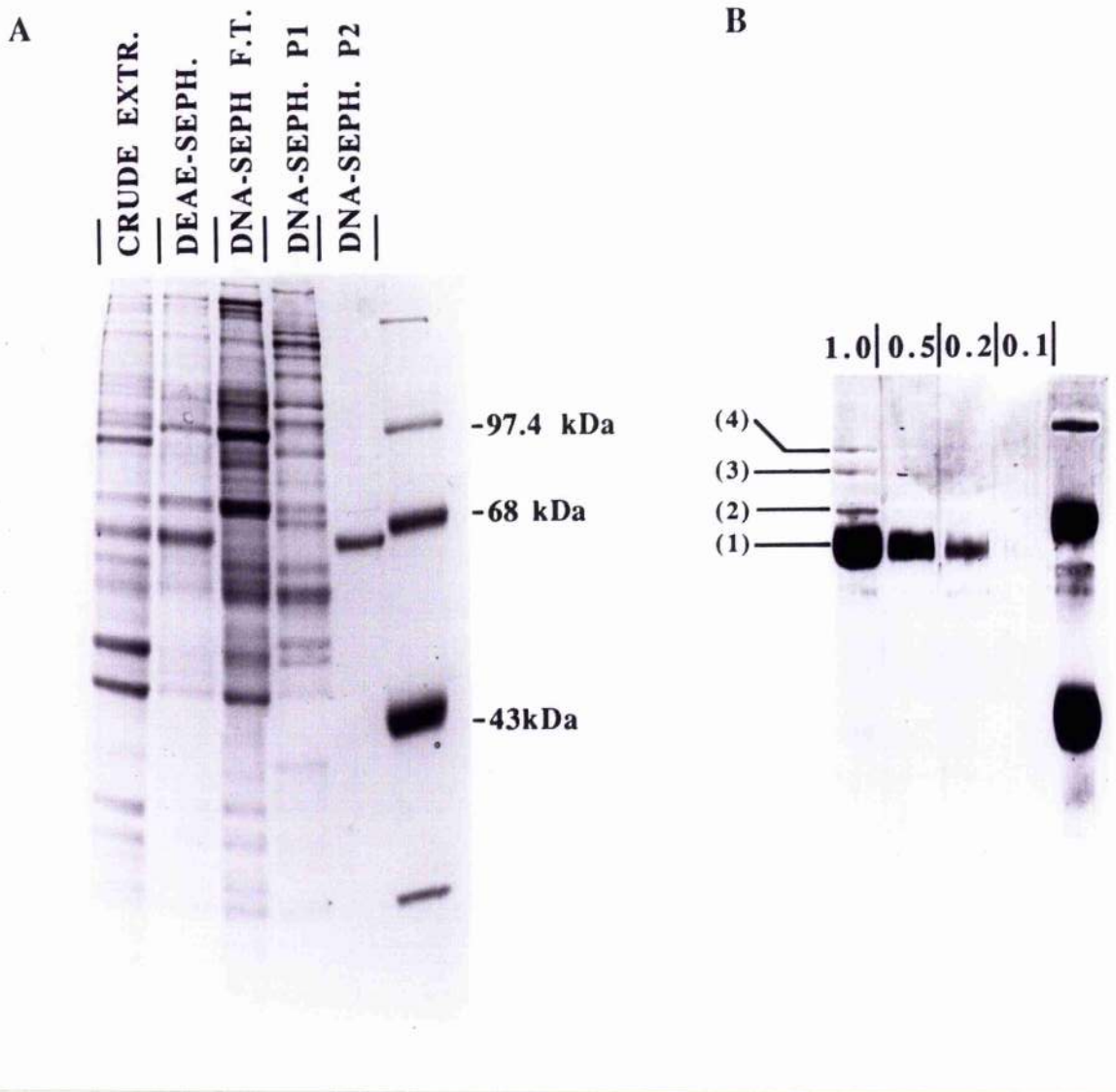
To assay for DNA polymerase activity 3µl of each fraction eluted from DNA-Sepharose was incubated with dATP, dTTP, dGTP and dCTP including

[$\alpha^{32}$P] dATP in the presence of DNAaseI nicked calf thymus DNA and aphidicolin. After TCA precipitation radioactive products were quantitated by scintillation counting and the overall nucleotide incorporation was calculated. An aphidicolin resistant DNA polymerase activity co-eluted from the denatured DNA sepharose column with the template dependent pTP-dCMP transfer activity (Fig 2.1A, black diamonds). In addition a considerable level of DNA polymerase activity was present in P1. As P1 was unable to support initiation of adenovirus DNA replication the polymerase activity present was probably that of cellular polymerase $\gamma$.

**2.3. Analysis of proteins in fraction P2.**

To examine the proteins present at each stage of the purification samples of crude Ad4 infected extract, 0.2M eluate from the DEAE-Sephacel, flowthrough from the denatured DNA-Sepharose and eluates P1 and P2 from the denatured DNA-Sepharose were analysed by SDS polyacrylamide gel electrophoresis. Proteins were visualised by staining with Coomassie Brilliant Blue. All fractions analysed, with the exception of P2, contained multiple species. P2 apparently contained only one species which from its relatively high abundance, molecular weight and elution characteristics was deduced to be viral DBP (Fig 2.2A). Since this fraction was capable of initiation of DNA replication *in vitro* it was clear there must be other replication proteins present. To further analyse fraction P2 various quantities of P2 were

**Figure 2.2**- Protein analysis of peak fractions. **A.** 5µg each of crude extract, eluate from DEAE-Sephacel, flowthrough from DNA-Sepharose, and peak fractions P1 and P2 eluted from DNA-Sepharose were denatured by heating at 100°C in SDS and mercaptoethanol and the polypeptides present fractionated by electrophoresis. Species were visualised by staining with Coomassie Blue. **B.** Samples of 0.1µg, 0.2µg, 0.5µg and 1µg of fraction P2 eluate from DNA-Sepharose were denatured in SDS and 2-mercaptoethanol and the polypeptides present fractionated by electrophoresis. Species were visualised by silver staining.


A
CRUDE EXTR.
DEAE-SEPH.
DNA-SEPH F.T.
DNA-SEPH. P1
DNA-SEPH. P2
-97.4 kDa
-68 kDa
-43kDa
B
1.0
0.5
0.2
0.1
(4)
(3)
(2)
(1)

separated by SDS polyacrylamide gel elctrophoresis and silver staining was used to detect the polypeptides present. This revealed the presence of three additional species of apparent molecular weights approximately 70kD, 85kD and 95kD (Fig 2.2B, lane 1; 2, 3 and 4).

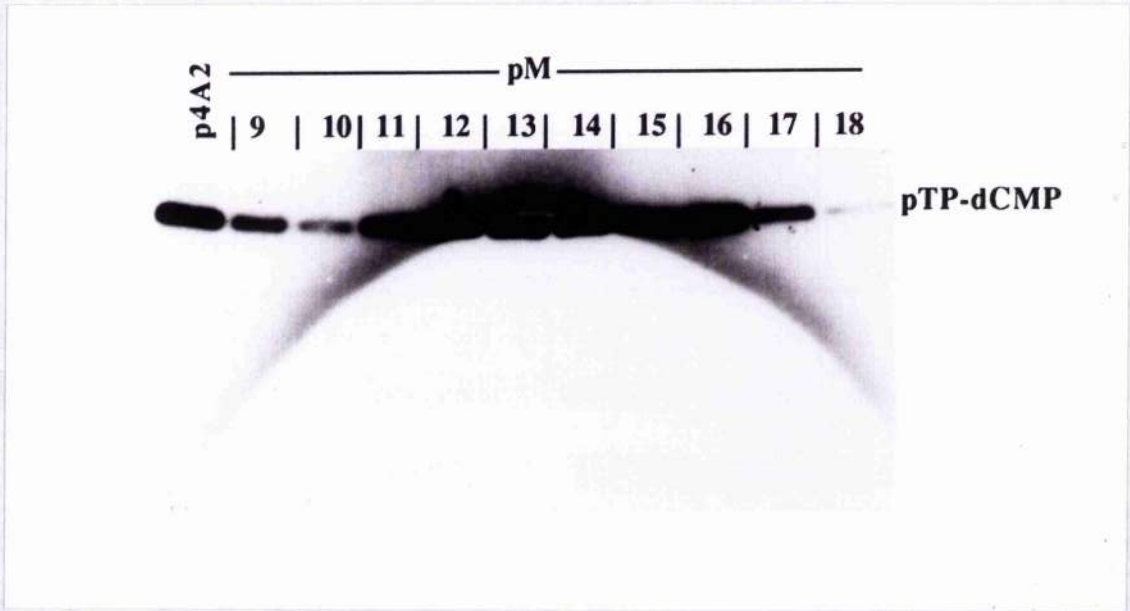
**2.4. Utilisation of mutant templates pM9 to pM18 by purified fraction P2.**

To check that the data obtained using crude extract on templates pM9 to pM18 were not affected by contamining activities present in the extract, standard initiation reactions were carried out in which crude Ad4 infected extract was replaced with purified fraction P2, using p4A2 and pM9 to pM18 as templates. Initiation was supported by p4A2 and pM12 to pM16 with equal efficiency (Fig 2.3, lanes 1,5,6,7,8 and 9 ). pM9,10,11,17 and 18 all gave very much reduced levels of initiation (Fig 2.3, lanes 2,3,4,10 and11) which corresponded to those obtained when using a crude extract for initiation. It was therefore concluded that any contaminatory enzymic activity in the crude Ad4 infected cell extract had little or no effect on DNA replication *in vitro*.

**2.5. Elongation of initiated molecules *in vitro* by P2.**

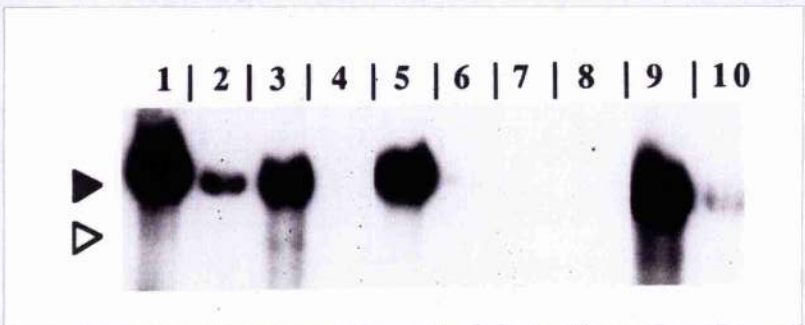
Although the fraction containing the four polypeptide species was capable of initiation of DNA replication it was not clear if the fraction could also carry out elongation of initiated molecules. To compare the activities of crude and fractionated extracts two different templates were used; p4A2 which as

**Figure 2.3-** Utilisation of mutant templates by fraction P2. 50ng of EcoRI cleaved templates pHR18, p4A2 and pM9 to pM18 were incubated under standard initiation conditions with 3µg of protein and [$\alpha^{32}$P] dCTP. The templates used for each reaction are indicated. Reaction products were analysed by electrophoresis on a polyacrylamide gel containing SDS followed by autoradiography.


p4A2
pM
9
10
11
12
13
14
15
16
17
18
pTP-dCMP

described previously contains one copy of an Ad4 ITR, and pM18 which contains an Ad5 ITR carrying a C to T base change at position 18 which severely reduces template efficiency *in vitro*. Templates were cleaved with EcoRI and AvaII to give a 1.6kb fragment with the origin sequence at the EcoRI terminus and a 0.9 kb fragment containing only plamid sequences, and incubated with various protein samples, dNTP's (including [$\alpha^{32}$P] dCTP), optimal $MgCl_2$ and an ATP regeneration system. Reaction products were fractionated by electrophoresis in an agarose gel containing SDS and replicated templates which had incorporated [$^{32}$P] dCMP were detectable by autoradiography. Correctly initiated products have pTP linked at the 5' end which results in a lower electrophoretic mobility (Fig 2.4, black arrow) than input template molecules which incorporate [$^{32}$P] at a low level (Fig 2.4, open arrow). Crude Ad4 infected extract gave efficient elongation on p4A2 but greatly reduced elongation on pM18, consistent with pM18's previously observed compromised ability to support *in vitro* DNA replication (Fig 2.4, lanes 1 and 2). The DEAE-Sephacel eluate and DNA-Sepharose eluate fraction P2 both gave efficient elongation on p4A2 but utilised the pM18 template inefficiently (Fig 2.4, lanes 3 to 6). DNA-Sepharose eluate P1 was unable to elongate either template consistent with its inability to initiate DNA replication *in vitro* (Fig 2.4, lanes 7 and 8). Although fraction P1 itself was unable to replicate adenovirus DNA *in vitro* the presence of factors involved in DNA replication could not be ruled out. To determine if fraction P1

**Figure 2.4**- Elongation of initiated templates by fraction P2. Plasmid templates p4A2 and pM18 (100ng) were cleaved with EcoRI/AvaII and incubated with 3μg of crude Ad4 infected extract (lanes 1 and 2), 3μg of DEAE-Sephacel eluate (lanes 3 and 4), 2μg of DNA-Sepharose eluate P2 (lanes 5 and 6), 2μg of DNA-Sepharose eluate P1 (lanes 7 and 8) and a mixture of 2μg each of P1 and P2 (lanes 9 and 10) in a standard elongation reaction containing [$\alpha^{32}$P] dCTP (see methods). After incubation at 30°C for 90 minutes reaction products were resolved by electrophoresis in a 2% agarose gel containing 0.1% SDS. Labelled species were detected by autoradiography. Correctly initiated molecules have pTP attached (black arrow head) and so have a lower electrophoretic mobility than input template (white arrow head) which is labelled at a low level due to repair synthesis. A second fragment containing only plamid sequences is run out of the gel.


1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10

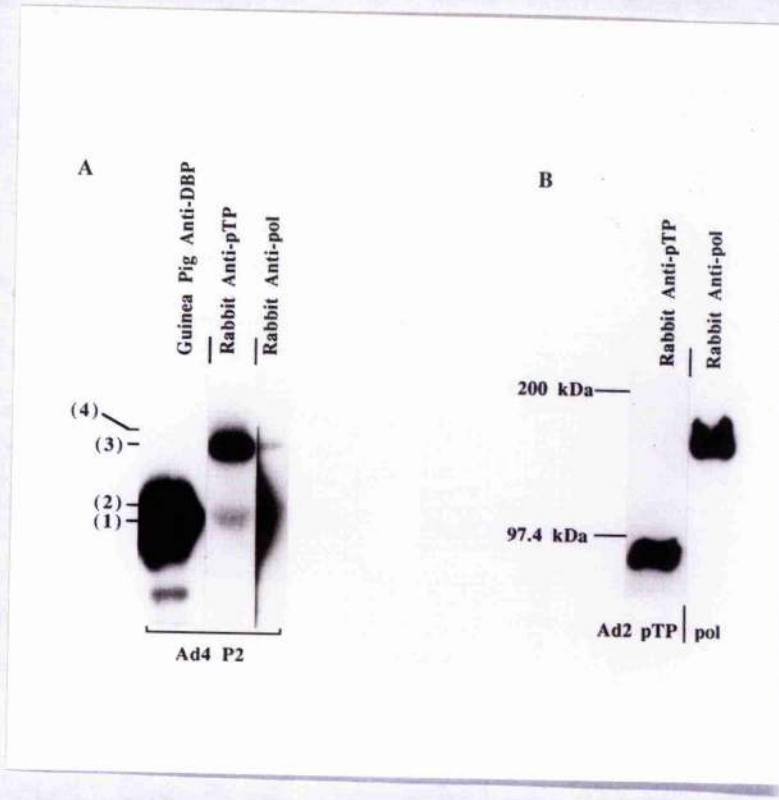
contained any factors important for DNA replication P1 and P2 were incubated together with templates p4A2 and pM18 in a standard elongation reaction (Fig 2.4, lanes 9 and 10). The combined fractions did not support DNA replication at a significantly higher level than fraction P2 alone.

These data demonstrated that all the proteins necessary for Ad4 DNA replication *in vitro* were present in fraction P2 from the DNA-Sepharose column. The presence of viral DNA polymerase and pTP was inferred by the ability of P2 to initiate on and elongate from input template.

**2.6. Western blot analysis of P2.**

To identify the polypeptide species present in P2 samples were fractionated by SDS-PAGE, transferred to nitrocellulose by Western blot and incubated with antisera raised against Ad2 DNA replication proteins. Antibody-antigen complexes were detected by incubation of nitrocellulose with [$I^{125}$] protein-A followed by autoradiography. A polyclonal guinea-pig antiserum raised against Ad2 DBP strongly reacted with the 65kD species 1 (Fig 2.2B and Fig 2.5A, lane1) confirming the identity of this species as DBP. Antisera raised in rabbits against purified Ad2 pTP and pol respectively were also used to probe blots of P2. The antiserum against Ad2 pTP recognised only the second highest molecular weight species 3 (Fig 2.2B and Fig 2.5A, lane 2). The antiserum against Ad2 polymerase however failed to recognise any of the species present (Fig 2.2B and Fig 2.5A, lane 3). The presence of Ad pol in the fraction however was implied by the data from both DNA

**Figure 2.5-** Western blot analysis of P2. Samples containing 10μg each of the second peak eluted from the DNA-sepharose (P2), Ad2 pTP and Ad2 pol were denatured in SDS and mercaptoethanol and fractionated by electrophoresis in a polyacrylamide gel containing SDS. Proteins were then transferred to nitrocellulose. **A.** P2 was probed with antisera against Ad2 DBP, pTP and pol and, **B.** Ad2 pTP and pol were probed with antisera against Ad2 pTP and pol respectively. The positions indicated of species 1,2,3 and 4 in P2 were determined by staining of Western blot with napthalene black (not shown). Antibody-antigen complexes were detected with $I^{125}$ protein-A followed by autoradiography.


A
Guinea Pig Anti-DBP
Rabbit Anti-pTP
Rabbit Anti-pol
(4)
(3)
(2)
(1)
Ad4 P2
B
Rabbit Anti-pTP
Rabbit Anti-pol
200 kDa
97.4 kDa
Ad2 pTP
pol

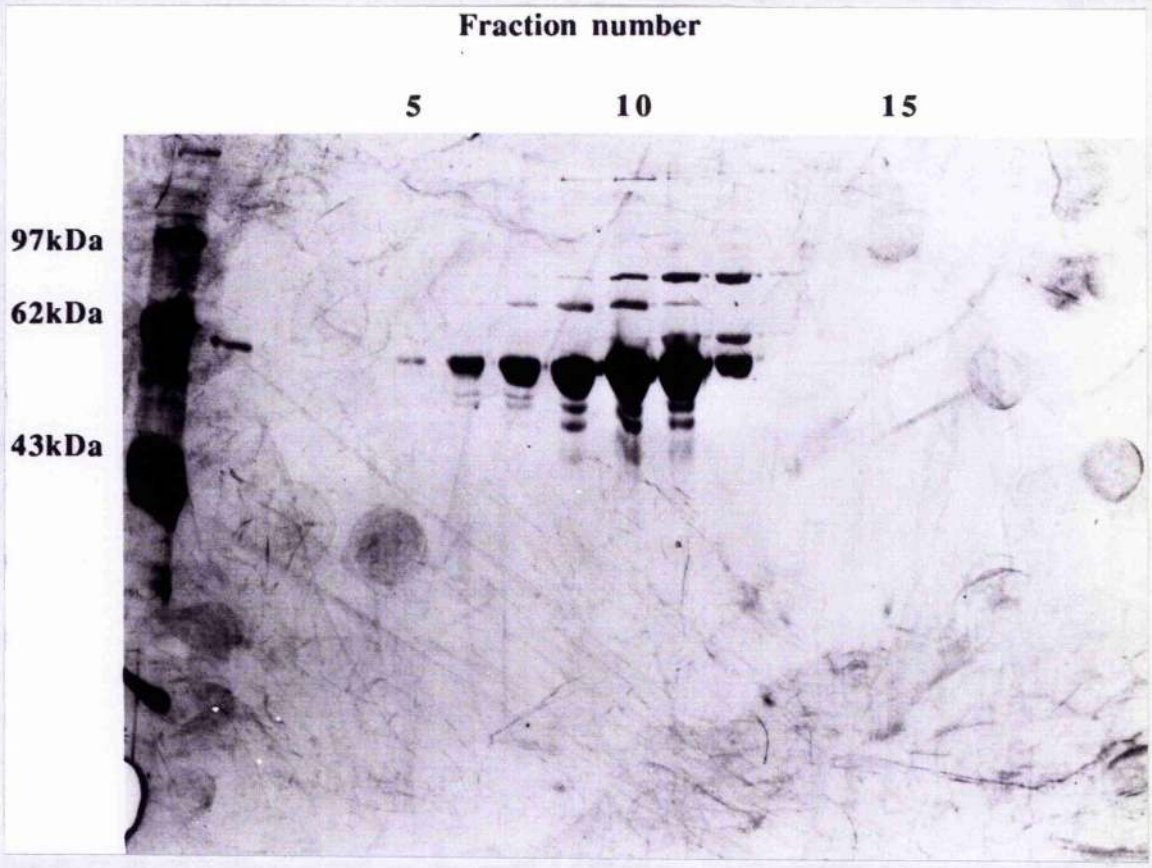
polymerase and elongation assays. Since the antiserum detects Ad2 pol (Fig 2.5B, lane 2) this result suggests that the differences in the DNA polymerases of the two serotypes prevented cross reaction with Ad4 pol. These data confirmed the identity of two of the species present in P2. As predicted, the most abundant protein corresponded to DBP and the presence of pTP, as predicted from pTP-dCMP transfer assay was confirmed.

**2.7. Separation of P2 proteins by glycerol gradient centrifugation.**

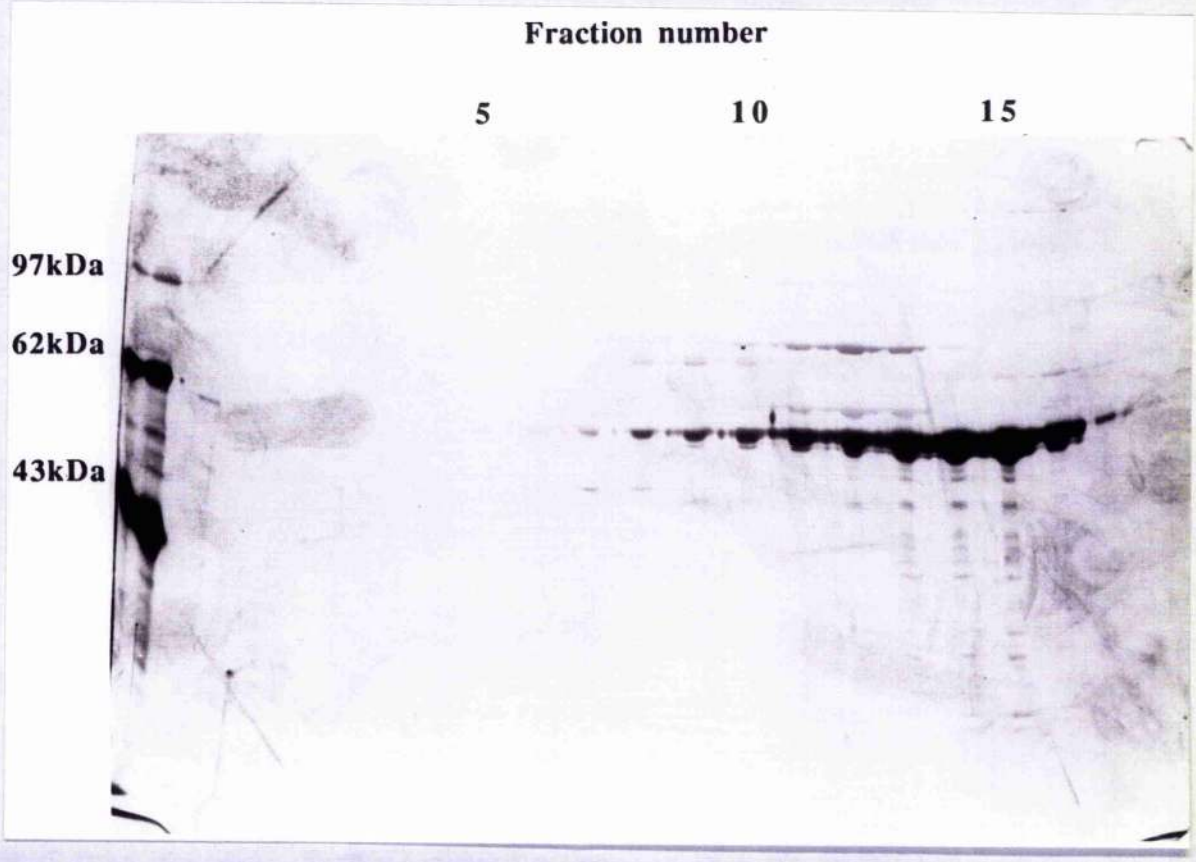
In an attempt to further fractionate the components present in fraction P2, 150mg of P2 was applied to each of two 15%-50% glycerol gradients. One was run under native conditions and the other under denaturing conditions containing 1.7M urea (Stillman *et al.*, 1983). The gradients were centrifuged at 49000 rpm for 18 hours and 24 hours respectively and fractions were collected by bottom puncture. The proteins present in each fraction were analysed by SDS polyacrylamide gel electrophoresis followed by silver staining. Limited fractionation of the components of P2 was achieved under both native and denaturing conditions, although none of the species was isolated in a homogeneous state. DBP was distributed throughout several fractions in both the native and denaturing gradients although the highest concentration of DBP was present in a different fraction from the three higher molecular weight species 2, 3 (pTP) and 4 with a greater separation being achieved under denaturing conditions (Fig 2.6A, compare lanes 7-12 with B, lanes 8-16). Under both native and denaturing conditions species 2 and 4

**Figure 2.6-** Glycerol gradient centrifugation of P2.150µg of fraction P2 was loaded onto **A.** a native 15%-50% glycerol gradient and **B.** a denaturing 15%-50% glycerol gradient containing 1.7M urea. Both were centrifuged at 49000rpm, for 18 and 24 hours respectively. Fractions were collected and samples were analysed by electrophoresis on a polyacrylamide gel containing SDS followed by silver staining.

A


Fraction number
5
10
15
97kDa
62kDa
43kDa

B


Fraction number
5
10
15
97kDa
62kDa
43kDa

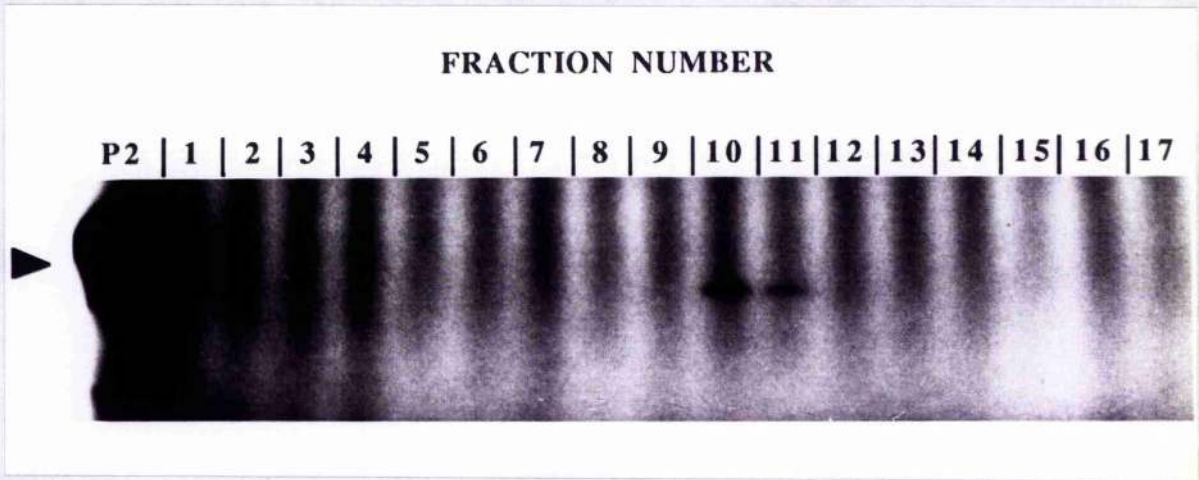
appeared to co-sediment (Fig 2.6A, lanes 10-12 and B, lanes 11-13) implying that they may be present in a tightly bound complex.

**2.8. Initiation of DNA replication by fractions from glycerol gradient.**

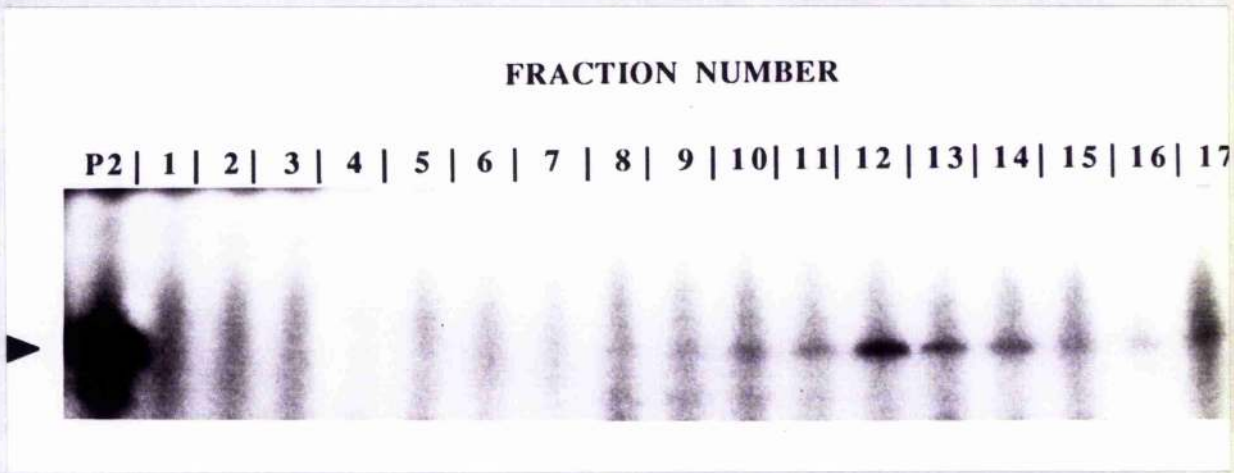
To test the ability of fractions from the glycerol gradients to initiate DNA replication *in vitro*, a 5µl sample of each fraction dialysed against buffer containing 25mM HEPES, 5mM KCl, 0.5mM $MgCl_2$ was incubated with 50ng of EcoRI cleaved p4A2 as template in a standard pTP-dCMP complex formation assay. Of the fractions obtained from the native gradient number 10, which contained the highest concentration of DBP, supported the most efficient initiation (Fig 2.7A, lane 10). However, of the fractions obtained from the denaturing gradient maximum initiation was obtained from the fraction containing the highest concentration of species 4 and 2 (Fig 2.7B, lane 12). Although undetectable by silver staining, pTP (species 3) in fraction 12 (Fig 2.6B, lane 12), was present in sufficient quantity to give some initiation. However, the maximum efficiency of initiation of DNA replication by any of the glycerol gradient fractionated P2 samples was around 100-fold lower than that obtained with the equivalent amount of unfractionated P2 (Fig 2.7, A and B, lane 1). The efficiency of initiation probably requires the presence of all four proteins but is in addition influenced in a complex way by changes in their relative concentrations. However further analysis where the efficiency with

**Figure 2.7-** Initiation of DNA replication by glycerol gradient fractionated P2. 5µl samples of fractions dialysed against buffer containing 25mM Hepes (pH7.5), 5mM KCl, 0.5mM $MgCl_2$ from **A.** the native glycerol gradient and **B.** the denaturing glycerol gradient were incubated with 50ng of EcoRI cleaved p4A2 template DNA in a standard pTP-dCMP complex formation assay. In addition 1µl of unfractionated P2 was incubated with template under similar conditions. Reaction products were resolved by electrophoresis in a polyacrylamide gel containing SDS and pTP-dCMP complex was detected by autoradiography.

A


FRACTION NUMBER
P2 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17

B


FRACTION NUMBER
P2 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17

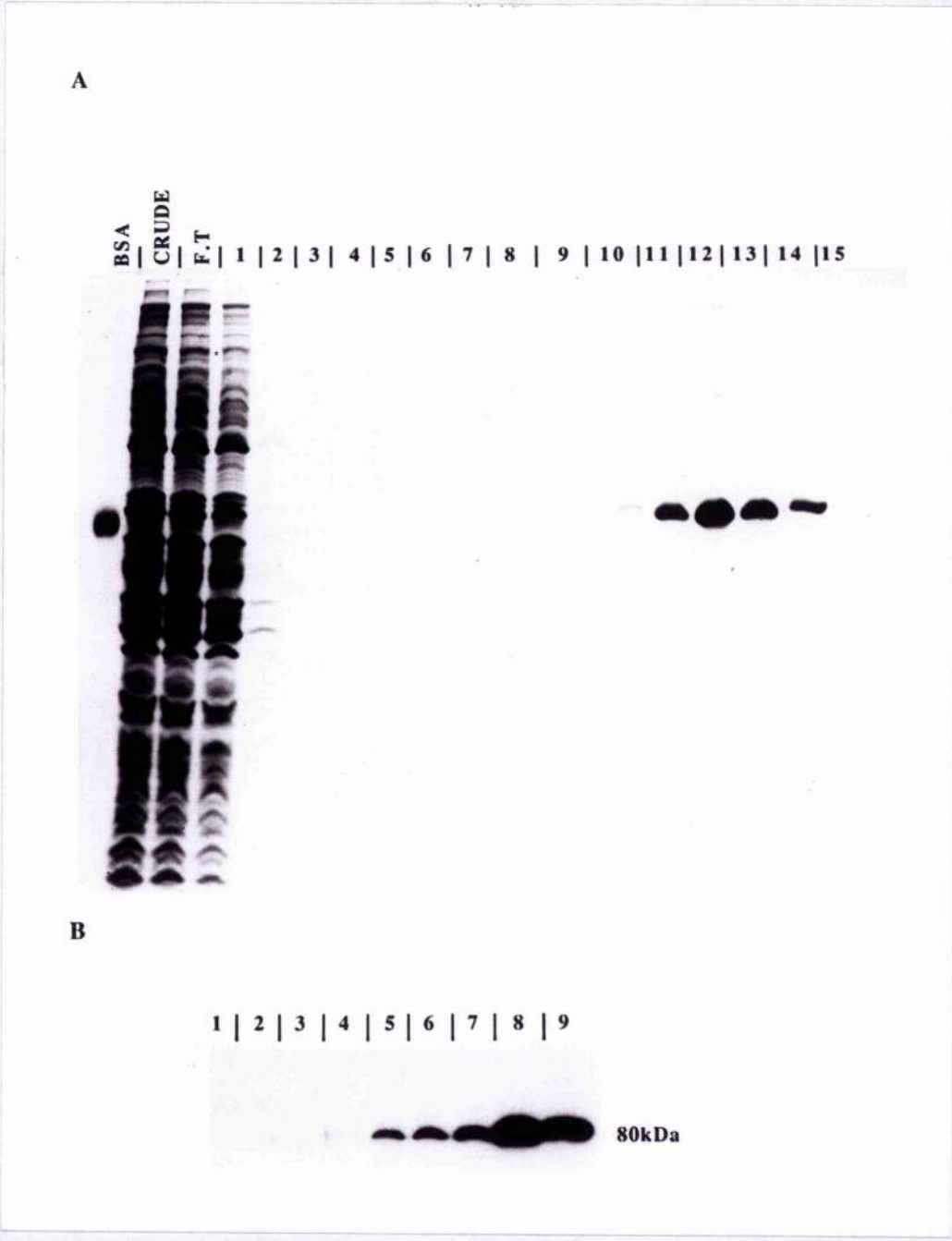
which combinations of fractions were assayed for their ability to support pTP-dCMP complex formation proved to be unsuccessful due to a loss of activity of one or more of the fractionated P2 species.

**2.9. Immunoaffinity purification of Ad4 DBP.**

Owing to the limited success achieved at separating the components of fraction P2 by glycerol gradient centrifugation a different approach was adopted whereby DBP was fractionated from the other components present in P2. A column was prepared using protein G-sepharose linked to α72K B6-10 mouse monoclonal antibody directed against an epitope present in Ad2 DBP (Reich *et al.*, 1983). Crude extract from Ad4 infected HeLa cells was prepared as described in materials and methods and the soluble extract freed from nucleic acids by passage over DEAE-Sepharose in 0.2M NaCl. The buffer was changed by dialysis against 10mM $NaPO_4$ (pH7.2) and applied to the antibody column equilibrated in 10mM $NaPO_4$ (pH7.2). After extensive washing with 10mM $NaPO_4$ and 0.05% NP40 bound proteins were eluted by washing the column with increasing stepwise concentrations of 10mM, 20mM, 30mM, 50mM and 100mM $MgCl_2$. Fractions were collected and samples were analysed by SDS polyacrylamide gel electrophoresis followed by staining with coomassie blue. The majority of the protein remained unbound and was washed from the column with 0.05% NP40 (Fig 2.8A, lane 2). DBP was eluted from the column at 50mM to 100mM $MgCl_2$ (Fig 2.8A, fractions 11 to 15) at

**Figure 2.8-** Immunoaffinity purification of Ad4 DBP. **A.** A crude extract from Ad4 infected HeLa cells was applied to a column containining monoclonal antibody raised against Ad2 DBP linked to protein-G Sepharose. Bound protein was eluted by application of increasing concentrations of $MgCl_2$. Proteins present in each fraction were analysed by electrophoresis in an acrylamide gel containing SDS followed by staining with Coomassie blue.

**B.** DBP peak fraction (no.12) was incubated at 1μg, 2μg and 4μg alone (lanes 1-3) in a standard initiation assay with 50ng of EcoRI cleaved p4A2 as template. In addition, 2μg each of immunoaffinity column flowthrough and crude extract were incubated alone (lanes 4 and 7) and with 0.5μg and 1μg respectively of fraction12 (lanes 5 & 6 and 8 & 9) under standard initiation conditions.


A
BSA
CRUDE
F.T
1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15
B
1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9
80kDa

about 98% purity. Approximately half of the DBP was not bound to the matrix. Because of the reported heterogeneity of DBP it was thought that the unbound portion may represent modified states of the protein which the monoclonal antibody was unable to recognise. To test this possibility the flowthrough was re-applied to the antibody matrix and a series of increasing stepwise elutions of $MgCl_2$ were used to elute bound protein as described. The DBP remaining in the flowthrough after the first round of purification bound to the column and was eluted at 50-100mM $MgCl_2$. This showed that the monoclonal antibody recognised all the DBP present in the crude extract. The binding capacity of the antibody-Sepharose matrix was reached during the first application of the crude extract leading to the observed presence of DBP in the flowthrough.

## 2.10. The effect of purified DBP on initiation of DNA replication *in vitro*.

To test the effect of purified DBP on initiation of DNA replication, DBP in the peak fraction (Fig 2.8A, fraction 12) was added to standard pTP-dCMP complex formation assays containing [$\alpha^{32}$P] dCTP and 50ng of EcoRI cleaved p4A2 as template. The fraction containing DBP alone (fraction 12) was incapable of *in vitro* initiation of DNA replication (Fig 2.8B, lanes 1 to 3). The flowthrough from the second round of immunoaffinity purification which was depleted of DBP was capable of *in vitro* DNA replication at a very low level (Fig 2.8B, lane 4). However addition of DBP (fraction 12) resulted in an

approximately 20 fold stimulation of initiation activity (Fig 2.8B, lanes 5 and 6). Initiation in crude Ad4 infected extract was stimulated by addition of DBP (fraction 12) at 0.5 µg but was inhibited at 1µg (Figure 2.8B, lanes 8 and 9).

These data show that by immunoaffinity purification active and near homogeneous DBP could be isolated from crude Ad4 infected cell extract in a single step, separating it from viral DNA polymerase and pTP. The data obtained from initiation assays suggest, contrary to some previous reports, that DBP plays an important role in initiation of DNA replication.

## Chapter 3. Recognition of the adenovirus minimal origin of replication by pTP and pol.

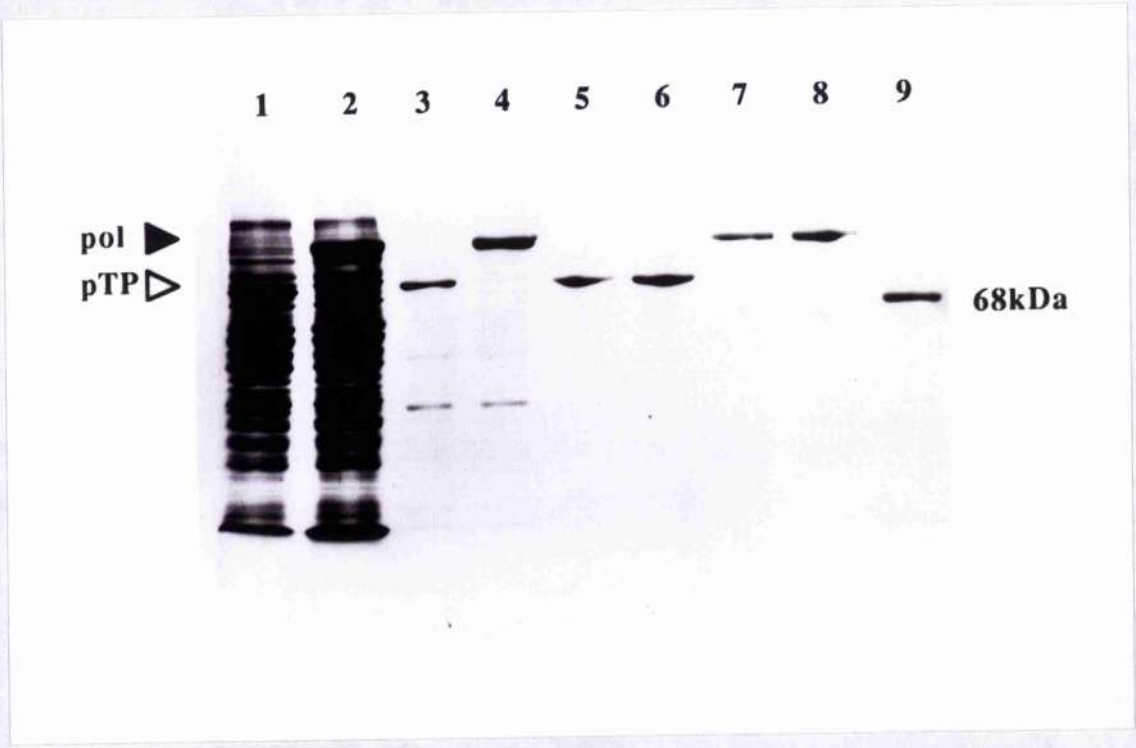
The results presented in chapter 1 demonstrated that only single base changes at some positions in the region between base pairs 9 and 18 of the origin of replication have a deleterious effect on initiation of Ad4 DNA replication *in vitro*. The perfect conservation of this sequence in all human serotypes so far examined suggests that it is essential for DNA replication. Point mutants which decreased initiation most profoundly were located at either end of the 9-18 sequence, set approximately one turn of the helix apart, implying that the bases at these positions may be important for a DNA-protein interaction. Recognition of a specific sequence in the origin of DNA replication directly by one or more of the viral replication proteins would provide one of the most straightfoward means of positioning the replicative machinery in proximity to the origin of DNA replication prior to initiation of DNA synthesis. There is indirect evidence implying that this occurs: It has been observed that pTP-pol complex binds with greater affinity to DNA of sequence corresponding to the adenovirus termini than to DNA of non specific sequence (Rijnders *et al.*, 1983) but direct evidence of a sequence specific interaction has been difficult to demonstrate. In addition, protein free template containing only the terminal 18bp supports initiation of DNA replication by adenovirus replication proteins indicating that replication proteins must, by some route, specifically recognise the 1-18 sequence. Previous studies may have been hampered by the lack of sufficient quantities of purified active pTP and pol.

Cloning of pTP and pol in baculovirus expression vectors where they are highly over-expressed has allowed purification of relatively large quantities of both proteins.

### 3.1 Purification of Ad5 pTP and pol from recombinant baculovirus infected Spodoptera frugiperda cells.

Crude cellular extracts were prepared as described in materials and methods from two 500ml batches of Spodoptera frugiperda cells infected with recombinant baculovirus containing the genes for Ad5 pTP and pol respectively. The procedure was identical for both proteins and the purifications were carried out in parallel. Crude extract was adjusted to 0.2M NaCl and applied to denatured calf thymus DNA-Sepharose. Bound protein was eluted from the column with 0.6M NaCl and then dialysed against 5mM $KPO_4$ (pH7.0), 0.4M KCl and 1mM DTT and applied to hydroxylapatite. Bound protein was eluted with buffer containing 100mM $KPO_4$ (pH7.0). Samples of crude extract, DNA-sepharose eluate, and hydroxylapatite eluate were analysed by electrophoresis in a polyacrylamide gel containing SDS followed by staining with Coomassie brilliant blue. Two distinct species corresponding to over-expressed pTP and Ad pol were observed in the crude extract (Fig 3.1, lanes 1 and 2). 80-90% of the total protein eluted from the DNA-Sepharose corresponded to pTP and pol (Fig 3.1, lanes 3 and 4 respectively). The eluate from hydroxylapatite consisted of near homogeneous pTP and pol (Fig 3.1,

**Figure 3.1-** Purification of pTP and pol from recombinant baculovirus infected Spodoptera cell extracts. Spodoptera frugiperda cells were infected with recombinant baculovirus vectors containing the genes for Ad5 pTP and pol. A crude extract was fractionated by denatured DNA affinity chromatography followed by passage over hydroxylapatite. 15μg of crude extract (lanes 1 and 2), 3μg of 0.6M NaCl eluate from DNA-Sepharose (lanes 3 and 4) and 3μg and 5μg respectively of eluate from hydroxylapatite (lanes 5 to 8) were denatured in mercaptoethanol and SDS and analysed by SDS polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue.

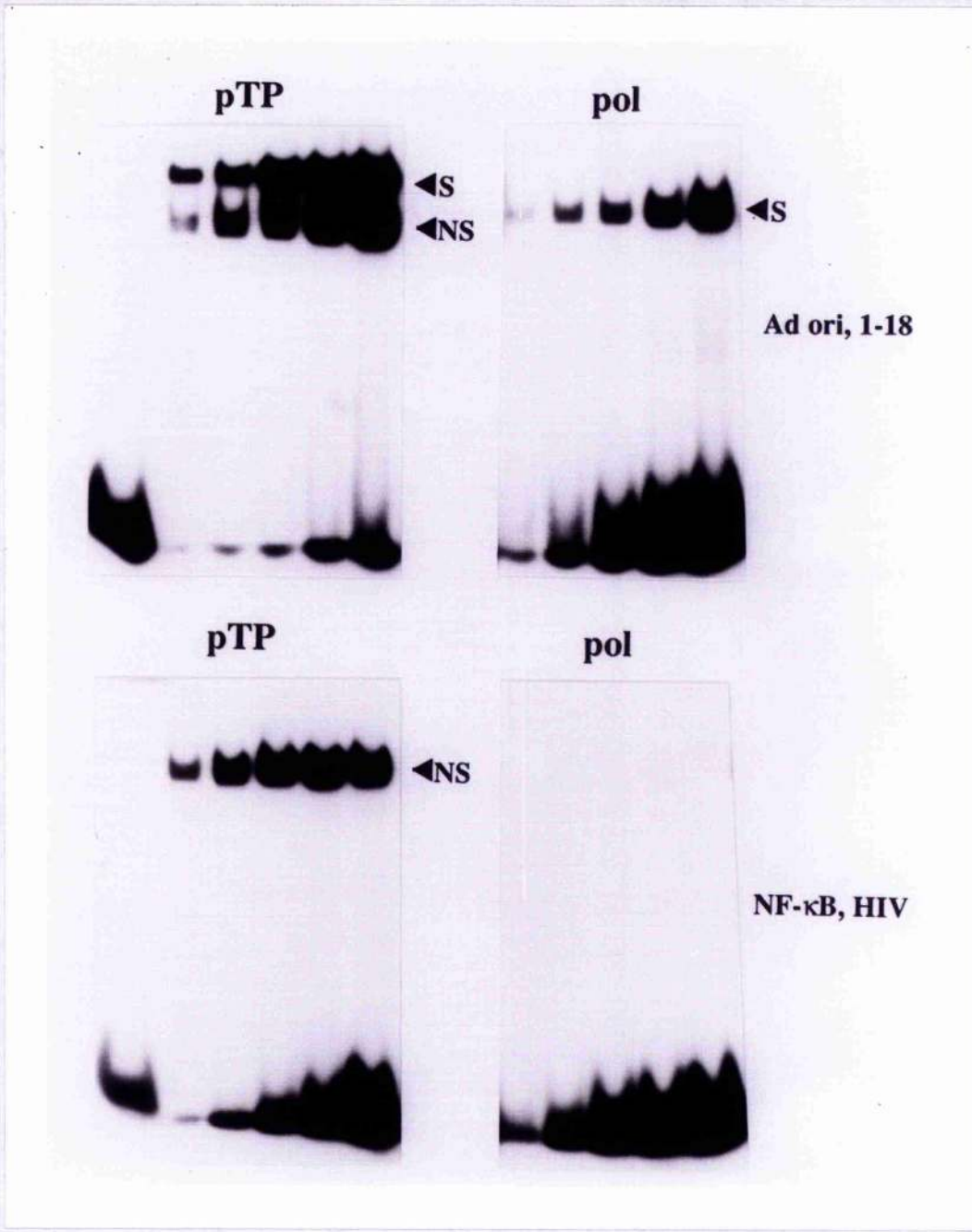

1
2
3
4
5
6
7
8
9
pol
pTP
68kDa

lanes 5 & 6 and 7 & 8).

### 3.2. Specific DNA-protein complex formation of pTP and pol with the terminal 18bp sequence of the adenovirus origin of DNA replication.

To examine the DNA binding characteristics of pTP and pol produced from the baculovirus system a gel mobility shift assay was used. 0.5μg of purified pTP and pol were incubated separately with increasing amounts of $^{32}P$ end labelled double stranded 1-18 oligonucleotide and an oligonucleotide of equal size but unrelated sequence derived from the HIV enhancer (NFk-B binding site) and the reaction products were resolved by electrophoresis in a native 8% polyacrylamide gel. Two species of altered mobility corresponding to complexes formed between pTP and the 1-18 probe were detected (Fig 3.2, upper left panel, N and NS). A single species of retarded mobility corresponding to the lower of the two species was detected using the NFκ-B oligonucleotide (Fig 3.2, lower left panel, NS). A single species formed between pol and the 1-18 sequence was detected (Fig 3.2, upper right panel, S). No complex formation was obtained between pol and the NF-κB probe (Fig 3.2, lower right panel). These data show that both pTP and pol form sequence specific protein-DNA complexes with a sequence corresponding to the 1-18 domain of the adenovirus origin of DNA replication. This implies that viral DNA replication proteins specifically recognise the core (1-18) sequence

**Figure 3.2-** Sequence specific complex formation of pTP and pol with the Ad origin 1-18 sequence. 0.5μg of purified Ad pol and pTP were incubated with 20, 40, 80, 100 and 200pg of $^{32}P$ end labelled double stranded oligonucleotides containing the terminal 18bp sequence of the adenovirus origin and an unrelated HIV enhancer sequence (NF-κB binding site). DNA protein complexes (black arrow heads) were resolved from free probe by electrophoresis in an 8% native polyacrylamide gel.


pTP
pol
S
NS
S
Ad ori, 1-18
pTP
pol
NS
NF-κB, HIV

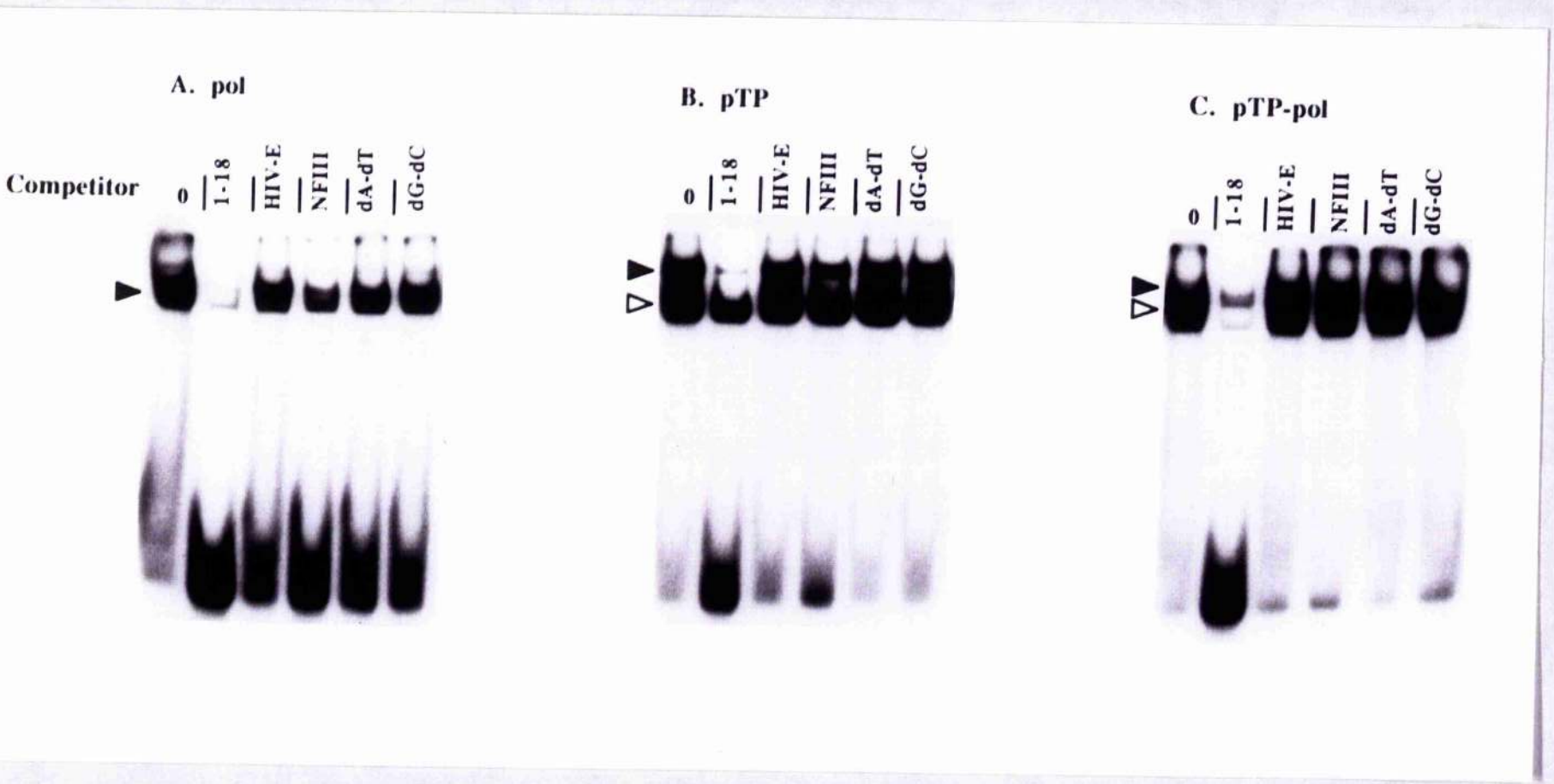
in the origin of DNA replication.

### 3.3. Challenge of DNA-protein complex formation with unlabelled oligonucleotides.

To further examine the specificity with which pTP and pol binds to the 1-18 sequence 0.5μg of purified pTP and pol was incubated with $^{32}P$ end labelled 1-18 oligonucleotide as described and the formation of DNA/protein complex was challenged by addition of a 100-fold molar excess of various unlabelled competitor oligonucleotides. Virtually all labelled 1-18 oligonucleotide was complexed when incubated alone with pol (Fig 3.3A, lane 1, black arrow head). The addition of excess unlabelled homologous competitor to this reaction abolished nearly all complex formation (Fig 3.3A, lane 2). When reactions were challenged with an excess of unlabelled oligonucleotide to the HIV-E (NFκ-B) binding site, oligo dG-dC and oligo dA-dT complex formation remained unaffected (Fig 3.3A lanes 3, 5 and 6). Interestingly, addition of oligonucleotide containing the recognition site for NFIII as competitor resulted in a reduction in complex formation of about 50% (Fig 3.3A, lane 4). The binding of pTP and pTP-pol complex to the origin of replication was also examined. 0.5 μg of pTP was incubated with $^{32}P$ end labelled 1-18 oligonucleotide producing the double retarded complex previously observed (Fig 3.3B lane 1, arrow heads). Challenge with a one hundred fold molar excess of homologous unlabelled oligonucleotide virtually abolished

**Figure 3.3-** Challenge of DNA binding of Ad pol, pTP and pTP-pol complex to the 1-18 sequence using excess unlabelled oligonucleotide.

**A.** 0.5μg of purified pol was incubated with 100pg of $^{32}P$ end labelled double stranded oligonucleotide containing the sequence of the terminal 1-18bp of the origin of DNA replication and challenged with 10ng of the various oligonucleotides indicated. **B.** 0.5μg of pTP was incubated with 100pg of 1-18 oligonucleotide and challenged with 10ng each of the various unlabelled oligonucleotides indicated. **C.** 20μg of pol was pre-incubated with 10μg of pTP for 30 minutes at 21$^{o}$C to allow formation of the pTP-pol heterodimer. 1μg of pTP-pol complex was incubated with 100pg of 1-18 oligonucleotide and challenged with 10ng of the various unlabelled oligonucleotides indicated. DNA-protein complexes were separated from free probe by electrophoresis in a native 8% polyacrylamide gel.


A. pol
B. pTP
C. pTP-pol
Competitor
0
1-18
HIV-E
NFIII
dA-dT
dG-dC

formation of the upper complex (black arrow head) but had only a minor effect on the lower complex (white arrow head) suggesting that the upper band represented formation of a specific complex whereas the lower one was due to a non-specific interaction. Challenge with a molar excess of unlabelled oligonucleotide competitors NFIII, HIV-E, oligo dA-dT and oligo dG-dC did not lead to a reduction in the formation of either complex (Fig 3.3B lanes 3, 4, 5 and 6).

To examine the binding of pTP-pol complex to the 1-18 origin sequence an equimolar mix of pTP and pol was preincubated at 21$^{o}$C for 20 minutes to allow formation of pTP-pol complex. 1μg of pTP-pol was incubated with $^{32}$P end labelled 1-18 oligonucleotide in various reactions with competitor DNAs. Two retarded complexes resulted (Fig 3.3C lane 1, arrow heads). The faster migrating complex (white arrow head) was deduced to be caused by the binding of pol alone since it had similar mobility to the complex obtained between pol and the 1-18 olignucleotide. The slower migrating complex (black arrow head) fell between the complex formed by pol alone and the specific complex formed by pTP alone. Formation of both complexes was virtually abolished by challenge with excess unlabelled 1-18 oligonucleotide (Fig 3.3C, lane 2) but not by non homologous oligonucleotides HIV-E, NFIII, dA-dT and dG-dC (Fig 3.3C, lanes 3,4,5 and 6).

These data confirm that Ad pol, pTP and pTP-pol complex bind with a high degree of specificity to sequences within the 1-18 region of the adenovirus

origin of DNA replication.

### 3.4. Analysis of pol/pTP interactions by gel filtration.

To analyse the interactions between either protein alone, or after the two had been allowed to interact, 250µg samples of pTP and pol separately and as a pTP-pol complex were loaded onto a Zorbax G-250 hydrophilic gel filtration column installed on a Waters HPLC system. Protein concentration was continuously monitored by absorption at 280nm and samples of fractions corresponding to the peaks of protein were incubated with $^{32}P$ end labelled 1-18 oligonucleotide and DNA-protein complexes formed were detected by gel mobility shift assay. The column was calibrated using proteins of known molecular weight and a standard curve of elution time against the log of the molecular weight was plotted and used to calculate the molecular weights of the species corresponding to the major peaks of protein obtained in each run. pTP yielded a single peak after 9 minutes corresponding to a molecular weight of about 100kD (Fig 3.4, 1st trace and table 3.1). It gave retarded species of two different mobilities when incubated with the 1-18 oligonucleotide (Fig 3.4, inset, 1st trace). The two complexes corresponded to the specific and non specific complexes observed previously. Ad pol gave two major peaks after 7.73 and 8.39 minutes which corresponded to molecular weights of approximately 270kD and 150kD respectively (Fig 3.4, 2nd trace and table 3.1). As the previously published molecular weight of Ad pol is 140kD, this suggests that the peaks corresponded to dimeric and monomeric

**Figure 3.4-** Gel filtration analysis of Ad pol, pTP and pTP-pol complex.

100μg each of pTP, pol and a mixture of 100μg of pol and 50μg of pTP preincubated at 21°C for 30 minutes were applied to a Zorbax GF250 gel filtration column installed on a Waters HPLC system. Protein concentration was determined by absorbance at 280nm and fractions were collected. 10μl of fractions corresponding to the peaks of protein for each run were incubated with 100pg of $^{32}P$ end labelled 1-18 oligonucleotide and DNA-protein complexes were separated from free probe by electrophoresis in an 8% native polyacrylamide gel.

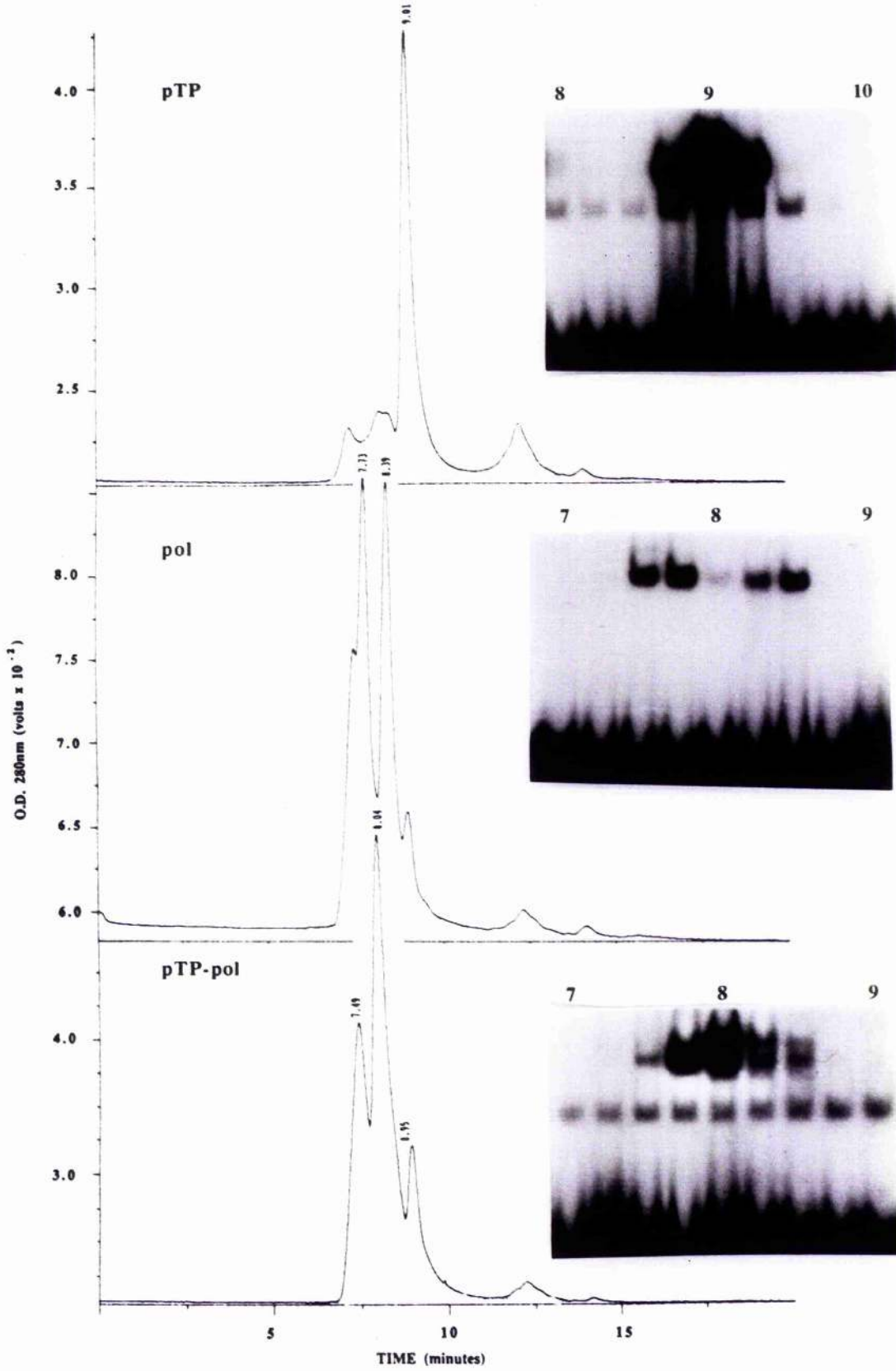

pTP
8
9
10
4.0
3.5
3.0
2.5
pol
7
8
9
8.0
7.5
7.0
6.5
6.0
pTP-pol
7
8
9
4.0
3.0
5
10
15
TIME (minutes)
O.D. 280nm (volts x 10⁻²)

**Table 3.1.**- Analysis of Ad5 pTP and pol and pTP-pol heterodimer by gel filtration. 250μg samples of pTP, pol and pTP-pol complex were loaded onto a Zorbax G-250 hydrophilic gel filtration column installed on a Waters HPLC system. The elution times of the major peaks of protein from each run are shown along with the corresponding molecular weight as determined from a calibration curve derived from running molecular weight standards under similar conditions.

| Protein | Elution time of major peaks(min) | Molecular weight (kD) |
|---|---|---|
| pTP | 9.01 | 100 |
| Ad pol | 7.73<br>8.39 | 270<br>150 |
| pTP-pol | 8.04 | 210 |

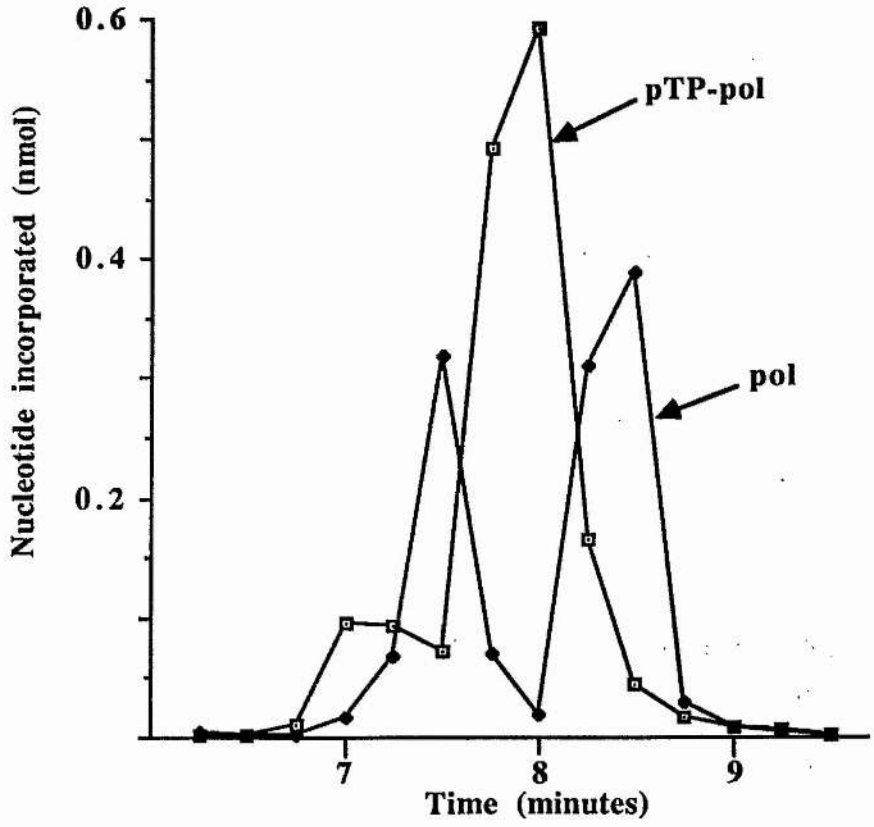
forms of pol, each present in roughly equal proportions. Both peaks formed a complex of the same mobility with the 1-18 oligonucleotide which was similar to the specific complex initially observed (Fig 3.4, inset, 2nd trace). pTP-pol yielded one major peak after 8.04 minutes corresponding to a molecular weight of around 210kD (Fig 3.4. 3rd trace and table 3.1 ). It was concluded that this species corresponded to a pTP-pol heterodimer which, from existing data, would have an approximate molecular weight of 220kD. The lesser peak of higher molecular weight obtained at 7.49 minutes may possibly represent formation of a higher order multimer of pol since it corresponds to a shoulder obtained when pol alone was analysed (compare 2nd and 3rd traces). Fractions corresponding to the major peak of protein formed protein-DNA complexes of two different mobilities when incubated with 1-18 oligonucleotide (Fig 3.4, 3rd trace, inset). These species corresponded to those initially observed and described in the above section.

The observed correspondence of the peaks of protein obtained by HPLC purification of pTP, pol and pTP-pol complex with sequence specific DNA binding activity confirms that DNA-protein complexes initially observed do form as a result of binding of pTP and pol to the 1-18 sequence domain of the Ad origin.

### 3.5 Analysis of DNA polymerase activity in HPLC fractions.

The activity of many DNA polymerases, including Ad pol, is increased by cooperative interaction with other proteins. The processivity of Ad pol has

**Figure 3.5-** DNA polymerase activity of HPLC fractions. 10µl each of fractions corresponding to the peaks of protein obtained from HPLC runs with Ad pol (black diamonds) and pTP-pol complex (white squares) were incubated in standard DNA polymerase assays with DNAaseI nicked calf thymus DNA and [$^{32}$P] dATP. Reactions were terminated by addition of 10% TCA and acid precipitable counts were quantitated in a scintillation counter. This information was used to calculate the specific activity of pol in the peak fractions of pol alone and as part of the pTP-pol complex.


0.6
0.4
0.2
Nucleotide incorporated (nmol)
pTP-pol
pol
7
8
9
Time (minutes)

been shown to increase in the presence of DBP (Lindenbaum *et al.*, 1986). To compare the DNA polymerase activity of Ad pol alone and in complex with pTP fractions surrounding and corresponding to the peaks of protein obtained from HPLC runs performed with pol and pTP-pol complex were assayed for their ability to synthesise nascent DNA using nicked calf thymus DNA as template. 10µl samples of fractions were incubated with DNAase I nicked calf thymus DNA in a standard polymerase assay containing all four nucleotides including [$\alpha^{32}$P] dATP. The radioactivity incorporated into nascent DNA was precipitated in 10% TCA, collected by filtration and quantitated by scintillation counting. DNA polymerase activity corresponding to the peaks of protein were obtained in both cases (Fig 3.5). The specific activity of pol was calculated for the two peak fractions corresponding to pol alone (peak 7.73= 4.4 nmol of nucleotide incorporated/pmol of pol; peak 8.39= 5.14 nmol of nucleotide incorporated/pmol of pol) and the peak fraction corresponding to pTP/pol complex (peak 8.04= 22.7 nmol of nucleotide incorporated/pmol of pol. Thus the specific activity of Ad pol was found to increase more than four fold in complex with pTP.

### 3.6. Initiation of DNA replication by purified pTP and Ad pol.

To test the ability of pTP and pol purified from baculovirus infected insect cell extracts to support initiation of DNA replication *in vitro* 2µg of pTP-pol complex were incubated with 1µg of adenovirus type 2 DNA binding protein

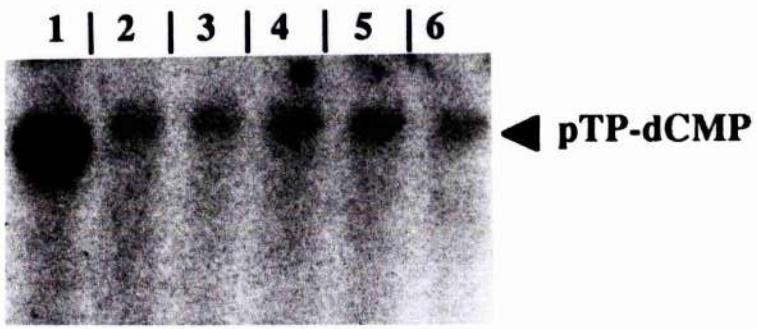

1 | 2 | 3 | 4 | 5 | 6
pTP-dCMP

**Figure 3.6-** Initiation of DNA replication by purified pTP and pol. 2μg of pTP-pol and 1μg of Ad2 DBP were incubated with 100ng of EcoRI cleaved plasmid pHR1 in a standard initiation reaction. Ad4 crude cell extract was also incubated with template as a positive control (lane 1). NFI was added at 0.1, 0.5, 0.75 and 1μg to reactions (lanes 3-6). Polypeptides were denatured in SDS and mercaptoethanol and fractionated in an 8% polyacrylamide gel containing SDS. Labelled species were detected by autoradiography.

and increasing amounts of NFI with 100ng of EcoRI cleaved pHR1, which contains the Ad2 origin of DNA replication, in a standard pTP-dCMP transfer assay. A positive control using 1μg of crude adenovirus type 4 infected HeLa cell extract gave efficient initiation (Fig 3.6, lane 1). Baculovirus pTP and pol supported initiation of DNA replication, the efficiency of pTP-dCMP complex formation slightly increased as more NFI was supplied to the reaction up to a maximum of 0.75μg after which initiation was inhibited (Fig 3.6, lanes 2 to 6). The difference in electrophoretic mobility of Ad4 pTP-dCMP complex compared to that of Ad5 probably reflects variation in amino acid composition between the precusor terminal proteins of the two serotypes.

# DISCUSSION

**1. The adenovirus origin of DNA replication: Constraints on the sequence suggest that the core region contains points of contact for a DNA-protein interaction.**

Much of the information which has led to the current understanding of the mechanism of adenovirus DNA replication has been derived from work carried out using adenovirus types 2 and 5. Ad2/5 relies on the interaction of host factors NFI/CTF and NFIII/Oct-1 with their respective binding sites at the origin of replication for efficient DNA replication. Ad4 has apparently dispensed with the need to employ these host DNA binding proteins in the replication of its DNA and as a result has a remarkably simple origin construction which potentially makes it an ideal system for studying the mechanism of adenovirus DNA replication. The data presented in Chapter 1 identify DNA sequences within the conserved domain of the minimal origin of replication between bp's 9-18 that are essential for Ad4 DNA replication. Two critical regions separated by a region in which single base changes could be tolerated were defined. The latter spans bases 12 to 16 and any single base transition in this region had no effect on DNA replication and could support a level of initiation comparable to the wild type sequence. Any base change in the two critical flanking regions had a serious deleterious effect on DNA replication *in vitro*. The sequence between bp's 9 and 18 may well play an important part in recognition by and positioning of the replication proteins at the origin prior to initiation of DNA replication. Employing an indirect binding assay it has been suggested that pTP can recognise the 1-18 region (Rijnders

*et al*, 1983), but confirmation using direct binding studies has been inconclusive (Nagata *et al.*, 1983; Adhya *et al.*, 1986). Clearly, a route by which pTP and/or pol are able to specifically recognise a specific sequence or sequences in the origin would be the simplest way of positioning the replication apparatus on the template. Recent evidence demonstrating a direct interaction between pol and NFI suggests that Ad2/5 utilises the binding of NFI/CTF adjacent to the origin to stabilise a pre-initiation complex between the viral replication proteins and the template prior to initiation (Bosher *et al.*, 1990; Chen *et al.*, 1990; Mul *et al.*, 1990). The importance of precise spacing between these functionally important sequences has been demonstrated by the observation that mutations at either positions 19 or 20 do not effect levels of Ad5 DNA replication *in vitro* but an insertion of more than 2bp at this position which moves the NFI site relative to the 1-18 sequence reduces the efficiency of initiation *in vitro* (Adhya *et al.*, 1986; Wides *et al.*, 1987) and *in vivo* (Bosher *et al.*, 1990). The affinity of NFI for its binding site is in turn further increased by the presence of viral DBP although it has not been demonstrated that there is any direct protein-protein interaction involved in this functional cooperation (Cleat and Hay, 1989; Stuiver and van der Vliet, 1990). It has been demonstrated that mutations in positions 17 and 18, whilst being adjacent to the NFI/CTF binding site, do not affect the binding of NFI (Rosenfeld *et al.*, 1984). Because mutations in either of these positions result in a substantial reduction in the level of DNA replication *in vitro* in both Ad2/5 (Rawlins *et al.*, 1984) and Ad4, which has no requirement for NFI/CTF, it is

apparent that a decrease in NFI/CTF binding is not responsible for the observed effect on DNA replication in Ad2/5. However, the three domain structure of the 9-18 region suggests it may be part of a protein recognition site since the boundaries of the two domains in which mutations have the profoundest effect on DNA replication lie approximately one turn of the helix apart and thus could be contact points in a DNA/protein interaction on one side of the DNA helix. The results presented in chapter 3 show that Ad2/5 pTP-pol heterodimer recognises and binds to a sequence in the terminal 18bp. Each protein in the heterodimer may contact distinct points on the template such that a mutation in one site would reduce the stability of the complex formed between the template and the heterodimer thereby reducing the efficiency of initiation. The results obtained are consistent with such a model since a single mutation in one of the regions which results in a reduction in the efficiency of initiation fails to abolish initiation completely suggesting that whilst one protein is able to bind to the template a pre-initiation complex still forms by virtue of the protein-protein interaction, albeit with a reduced stability. Clearly analysis using templates with mutations in both sites will be required to test this hypothesis.

Although single point mutations in positions 12 to16 do not affect Ad4 DNA replication *in vitro* it is likely that this region plays an essential role in maintaining the conformation of the DNA. It has been shown that a template carrying a double mutation at positions 13 and 14 gave a marked reduction in initiation of Ad5 DNA replication (Tamanoi and Stillman, 1983). The double

mutation, unlike a single base change, may prevent effective interaction of replication proteins with the origin or alternatively may alter the conformation of the origin template such that the concerted structural changes that are essential for origin function do not take place. The length of this region may also be critical if it serves to separate two regions where interactions with proteins occur. A functional analysis using mutants with insertions at positions 12 to 16 will be required to comprehensively characterise this region.

The template titration data shown in figure 1.2B and 1.3B demonstrated that the dramatic reduction in the efficiency of initiation seen using mutant template pM18 could not be compensated for by increasing the template concentration, suggesting that the mutation may be exerting its effect by way of severely destabilising an interaction between replication proteins and the origin of replication. The data clearly shows that mutations at positions 9,10,11,17 and 18, all of which reduce the efficiency of DNA replication *in vitro*, affect only initiation. If this inhibition is due to an effect on a DNA/protein interaction which is in turn critical for initiation of replication, then clearly a way of directly assaying for binding of these proteins to the mutant templates will be necessary. Some evidence does exsist from comparison of various mutant oligonucleotides that formation of a complex on an ITR sequence roughly correlates to the ability of the same oligonucleotides to support initiation *in vitro* (Kenny and Hurwitz, 1988).

## 2.1. The protein requirements for Ad4 DNA replication.

Two different approaches, described in chapter 2 were used to isolate and characterise the proteins required for Ad4 DNA replication. In the first a fraction containing only four detectable polypeptides able to both initiate DNA replication and elongate initiated molecules *in vitro* was purified. In the second, near homogeneous DBP was isolated from crude Ad4 infected cell extract. The data obtained indicates that there are important differences in the viral replication proteins between Ad4 and the more extensively characterised Ad2.

Initially a combination of ion exchange and single strand DNA affinity chromatography was employed with the intention of purifying DBP as described in the method of Schechter *et al.* (1980). Surprisingly, a fraction whose major component was DBP, but which contained three detectable higher molecular weight species was isolated. Furthermore this fraction was capable of initiation of DNA replication and elongation *in vitro* implying the presence of pTP and viral DNA polymerase in the fraction. Two of the species, which were visualised by SDS PAGE, were identified as DBP and pTP by probing of a Western blot with anti Ad2 DBP antiserum and anti Ad2 pTP antiserum respectively. However an antiserum against Ad2 pol, which recognised Ad2 pol in Western blots, failed to recognise any species present in the fraction. Sequencing data reveals that there is considerable variation in the region near the amino termini of the DNA polymerases of Ad2, Ad7 and Ad12 (Shu *et al.*, 1986, 1987). Although no sequence data for Ad4 pol exists the variation that has been observed in the DNA polymerases from the subgroups thus far analysed suggests that perhaps

there is insufficient homology between the DNA polymerases of Ad2 and Ad4 for serum raised against Ad2 pol to recognise that of Ad4. In agreement with this data it has been observed that the same antiserum is unable to recognise native Ad4 pol in immuno-fluoresence of Ad4 infected HeLa cells (J.Bosher, personal communication).

It was demonstrated that Ad4 pTP and pol co-elute with DBP from denatured DNA-Sepharose with 1M NaCl. This differs markedly from Ad2 where denatured DNA-Sepharose chromatography separates pTP/pol and DBP: pTP/pol is eluted with 0.36M NaCl whilst 1.0M NaCl is required to elute DBP (Enomoto *et al.*, 1981; Stillman *et al.*, 1982). Thus the requirement for 1.0M NaCl to elute the Ad4 pTP/pol complex may reflect a higher affinity of the Ad4 proteins for single stranded DNA or alternatively may be a result of a direct interaction between the Ad4 pTP/pol complex and DBP. However, under the conditions used for immunoaffinity purification DBP was separated from pTP/pol in low ionic strength buffer suggesting that the formation of such a complex may be DNA dependent. Alternatively the possible site of interaction on DBP with pTP and pol may have been partially or wholly obscured by antibody binding. Another possibility is that bound DBP alters the structure of the DNA template resulting in higher affinity binding by Ad4 pTP/pol. This latter possibility is reminiscent of the effect of the Ad2 DBP on the DNA binding properties of NFI: DBP increases the affinity of NFI for its recognition site in the Ad2 origin of replication although no direct interaction between the two proteins could be detected (Cleat and Hay, 1989; Stuiver and van der Vliet, 1990). Although Ad2 pTP/pol and DBP do not co-elute

from DNA-Sepharose, the thermostability and processivity of pol is increased in the presence of DBP (Lindenbaum *et al.*, 1986), suggesting the existence of functionally significant interactions between the proteins. Specific interactions between single stranded DNA binding proteins and DNA polymerases have previously been reported in prokaryotic phage systems such as T4 and T7 (Huberman and Kornberg, 1971; Reuben and Gefter, 1973). Such an interaction may play a critical functional role in light of the independence of Ad4 DNA replication from transcription cellular factors. The recent observation that NFI and Ad2 pol interact (Bosher *et al.*, 1990; Chen *et al.*, 1990) and the implied role of NFI in the formation of a preinitiation complex suggests that such a function in Ad4 may be fulfilled by the viral replication proteins themselves. Thus Ad4 pTP and/or pol may themselves be required to recognise and bind to the origin of replication with higher affinity than their Ad2 counterparts.

**2.2. The role of DBP in initiation of adenovirus DNA replication.**

A marked stimulation of initiation of DNA replication was observed in the presence of the immunoaffinity purified fraction of DBP indicating the important role of this protein in initiation of Ad4 DNA replication (see results section 2.10). While there is ample biochemical and genetic evidence for the role of Ad2 DBP in elongation (reviewed by Hay and Russell, 1989), its role in initiation has been subject to debate. In earlier studies it was found that crude nuclear extracts from cells infected with a temperature sensitive mutant of DBP grown at the non-permissive temperature would support initiation but were unable to extend

initiated molecules (Friefeld *et al.*, 1983; Van Bergen and van der Vliet, 1983). In another study it was observed that purified DBP did not stimulate initiation *in vitro* (Rosenfeld *et al.*, 1987). The evidence at this time therefore strongly suggested that DBP, whilst being required for elongation, was not needed for initiation of replication. In contrast the data presented in chapter 2 of this study and other more recent investigations have clearly shown that there is a marked stimulation in initiation as the concentration of DBP is increased (Kenny and Hurwitz, 1988 Cleat and Hay, 1989; Stuiver and van der Vliet, 1990). However all these studies found that initiation is inhibited if the DBP concentration is increased beyond an optimal point. It is therfore most likely that DBP is required for initiation but that if the concentration is too high its non-specific binding to the template could prevent the access of other replication proteins to the origin, thereby decreasing the efficiency of initiation. In earlier studies contamination of apparently purified pTP-pol with low concentrations of DBP may have obscured the stimulatory effect reported here and in other more recent investigations. The observations made during studies with conditional DBP mutants could be explained by the fact that DBP is a multi-functional protein in which separate domains often fulfill different roles; A mutation disrupting the elongation function of DBP may therefore have no effect on its ability to increase the frequency of initiation.

## 2.3. The role of mature terminal protein in initiation of adenovirus DNA replication.

It should be noted that the data presented in this study was obtained using plasmids as templates for *in vitro* DNA replication. The importance of terminal protein in the adenovirus life cycle was initially reported when it was observed that the infectivity of viral DNA was much enhanced if intact terminal protein remained attached to the genome (Sharp *et al.*, 1976). In subsequent studies it has been observed that viral DNA with terminal protein attached (DNA-protein complex) serves as a more efficient template than plasmid for adenovirus DNA replication *in vitro* (Tamanoi and Stillman, 1982; van Bergen *et al.*, 1983; Challberg and Rawlins, 1984; Harris and Hay, 1988). A possible explanation for this is that terminal protein plays an important part in the positioning of the replication apparatus in relation to the template prior to initiation. The early observation that TPs at opposite ends of the genome interact (Robinson et al., 1973) suggests the possibility that TP may interact with incoming pTP prior to initiation. It has also been demonstrated in Ad2/5 that the presence of a host protein, pL, which has 5'-3' exonuclease activity is required to expose a single stranded region at the 3' end of the template strand as a prerequisite for DNA replication on a template which does not contain terminal protein (Guggenheimer *et al.*, 1984; Kenny *et al.*, 1988). This strongly suggests that TP may have an important role in unwinding of the duplex at the origin to allow access of the replicative machinery to single stranded template so that initiation can take place. However the *in vitro* DNA replication assays conducted using

Ad4 replication proteins in this study contained no detectable 5' to 3' exonuclease activity present since it was observed that the specific activity of $^{32}P$ 5'-end labelled origin DNA did not decrease when incubated under conditions similar to those used for *in vitro* DNA replication. Clearly, under the conditions used in this study for Ad4 DNA replication *in vitro*, the replication proteins supplied to the naked DNA template are able to unwind origin DNA effectively in their own right.

**3. Sequence specific DNA-protein interactions mediate recognition of the origin of replication by pTP-pol.**

The data presented in chapter 3 of this study shows that pTP and pol of adenovirus type 5 individually and as a pTP-pol heterodimer bind with high specificity to the terminal 18bp of the adenovirus genome, forming nucleoprotein complexes which are readily detectable by gel retardation assay. This suggests that the mechanism by which pTP and pol, which exist *in vivo* as a tightly associated pTP-pol complex (Enomoto *et al.*, 1981), locate themselves specifically at the origin prior to initiation of replication by recognition and binding to a sequence or sequences within the 1-18 domain. Functionally important domains of Ad pol have been identified through introduction of linker insertion mutations into the cloned Ad pol gene (Chen and Horwitz, 1989). Functional analyses on a range of such mutants showed that for the most part mutant proteins which were unable to form a heterodimer with pTP also failed to recognise origin DNA. However one of the mutants studied was found to be able

to bind to pTP but failed to bind to the Ad origin of replication. The mutated region was shown to contain a putative zinc binding domain (Chen *et al.*, 1990). Although there is no direct evidence that Ad pol is a zinc binding protein clearly the presence of a potential zinc finger motif in a region found to be important for DNA binding argues strongly that this region forms an essential point of contact with origin DNA. The perfect conservation of the sequence from base pairs 9 to 18 throughout all human adenovirus serotypes probably reflects a crucial functional constraint on this domain, which makes it a very likely candidate for containing the sequences which are critical for recognition by pTP-pol. When specific formation of a DNA-protein complex between pol and the 1-18 sequence was challenged with unlabelled oligonucleotide containing the recognition site for NFIII a reduction of about 50% in the amount of specific complex formed was observed. Comparison of the 1-18 and NFIII binding site sequences show that the sequence TAAT, which constitutes bp's 10 to 13 of the conserved domain of the adenovirus origin is common to both sites and could therefore be an important element for the recognition of the origin by Ad pol. The effect of introducing mutations specifically in the TAAT sequence on Ad pol binding would be valuable in directly assessing the importance of this motif for recognition by pol. Functional analysis of a series of point mutations from base pairs 9 to 18 of the origin of DNA replication showed that the *in vitro* initiation of adenovirus type 4 DNA replication is seriously reduced but not entirely abolished by base substitutions at either end of the 9-18 conserved region. It may be that mutations at these critical positions perturb a DNA protein

interaction which is crucial for initiation of DNA replication. An analysis comparing pTP-pol complex formation on mutant templates with the ability of the same templates to support DNA replication *in vitro* would show if there was a correlation between the level of pTP-pol binding and the efficiency of DNA replication *in vitro*.

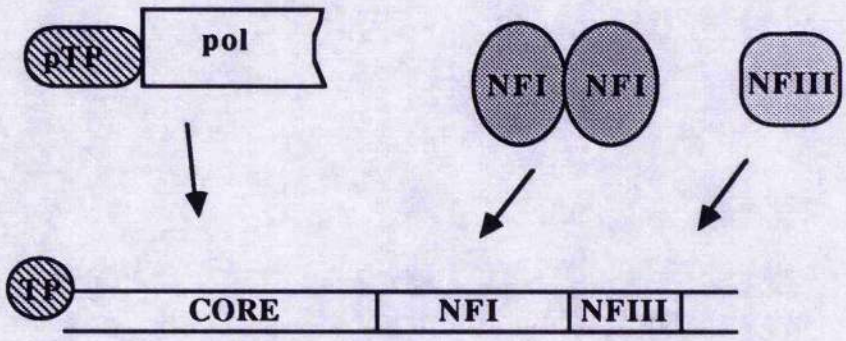
The results of the analysis of Ad pol, pTP and pTP-pol heterodimer by gel filtration (presented in chapter 3) showed that the major peaks of protein obtained formed complexes with the core region of the Ad origin which were similar to those initially observed when less purified preparations of pol and pTP were used. Furthermore determination of the molecular weights of the protein peaks confirmed that these corresponed with previously determined molecular weights of pol and pTP. Analysis of pol purified by gel filtration showed there to be two distinct species of protein whose molecular weights suggested that pol was present both as a monomer and as a dimer. The observation that both peaks formed complexes with the 1-18 sequence of similar electrophoretic mobility and which furthermore corresponded to the complex observed in the initial data confirmed the identity of each of the species. Clearly pol binds to the origin sequence in only one of these two forms since the mobility of the nucleoprotein complex formed by each is identical. Futher analysis by cross-linking of the DNA-protein complex could yield more information. Challenge of complex formation between pTP and the 1-18 sequence showed that pTP alone forms both a specific and a non-specific complex with the origin core sequence. However such non-specific binding is probably prevented *in*

*vivo* by the tight association of pTP with pol since it was observed that pTP and pol which had been pre-incubated to allow formation of heterodimer formed only specific complexes with the 1-18 sequence.

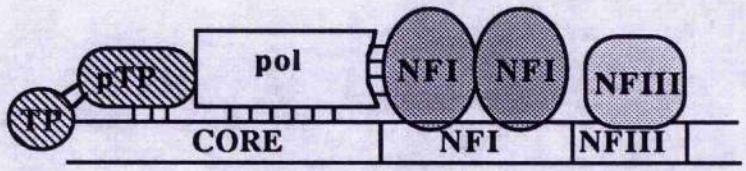
**4. A model for initiation of adenovirus DNA replication.**

The molecular events leading up to the initiation of DNA replication in adenovirus have yet to be precisely established although the data presented in this study combined with that which has been obtained in other recent studies which have examined the functional interactions of viral and cellular proteins with each other and with the origin of DNA replication, allows predictions to be made concerning pre-initiation events. The data presented in chapter 3 of this study demonstrate that both precursor terminal protein and adenovirus DNA polymerase of Ad5 specifically recognise and bind to the adenovirus core origin of replication. In addition it has been shown that Ad pol interacts with NFI (Bosher *et al.*, 1990; Chen *et al.*, 1990) which binds to the Ad2 and Ad 5 origins as a dimer stimulating initiation of DNA replication (Nagata *et al.*, 1983; Mul *et al.*, 1990). As already discussed there is a strict spatial constraint on the positioning of the NFI site relative to the core origin (Wides *et al.*, 1987). This is probably important for allowing specific contacts to take place between Ad pol and NFI (Bosher *et al.*, 1990; Mul *et al.*, 1990). On the basis of these findings it is possible to propose a model for the sequence of events which lead to the formation of a pre-initiation complex. The important features of this model are summarised in the figure overpage. Initially pTP and pol, which exist as a tightly

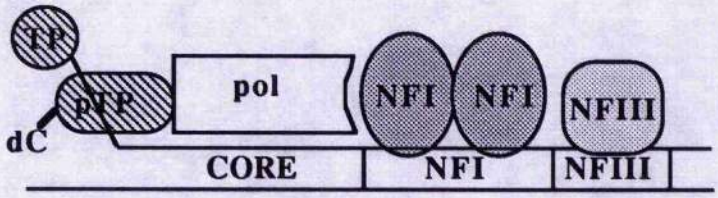
Formation of a pre-initiation complex at the adenovirus origin of DNA replication. Representations of known and potential DNA-protein and protein-protein interactions are included.




Sequence specific recognition of the origin by pTP-pol, NFI and NFIII. Active pre-initiation complex established and stabilised by protein-protein interactions between pol-NFI and pTP-TP.




Component of complex, perhaps TP, starts to unwind duplex at the terminus exposing template strand and allowing initiation of DNA replication.



bound pTP-pol heterodimer, bind specifically to the origin by recognition of sequences in the core domain, probably between positions 9-18, and in the case of Ad2/5, NFI binds to its recognition site. The pre-initiation complex is then further stabilised by protein-protein interactions between pol and NFI and possibly between pTP and TP. An alternative sequence of events could be envisaged whereby pTP-pol is loaded onto the origin primarily by specific interactions between pol and NFI already bound at the origin. However it has been demonstrated that template containing only the core region of the origin does specifically supports initiation of Ad2 DNA replication in the absence of NFI, albeit at a very low level (Rawlins *et al.*, 1984; Wides *et al.*, 1987). This suggests that pTP-pol is initially located at the origin via a site specific DNA-protein interaction which is followed and stabilised by protein-protein contacts. NFIII on the other hand apparently acts independently to stimulate initiation of DNA replication. There is no evidence of a cooperative interaction with NFI, as might be suggested by the close proximity of the binding sites for the two proteins and it does not interact with pTP-pol. It could be envisaged therefore that it acts by binding at the origin and changing the local structure of the DNA in such a way that initiation of replication is favoured. In the case of Ad4, which does not require cellular factors for efficient replication of its DNA, the loading of Ad4 pTP-pol onto the origin may rely predominantly on a more stable site specific interaction between pTP-pol and the core 1-18 sequence.

After the replication proteins have stably engaged the origin the next step which is required is for one or more of the components of the nucleoprotein

complex to begin unwinding of the origin region in order to make single stranded template DNA available. Although TP has not been shown to be directly involved in initiation, it has, as discussed above been well established that templates containing intact TP are replicated much more efficiently than protein-free templates *in vitro*. The role of TP in the initial unwinding of the duplex was implied when it was observed that efficient *in vitro* DNA replication with protein-free template required the presence of an exonuclease to expose the template strand (Kenny *et al.*, 1988). No such requirement was observed for template containing TP, suggesting that TP has a direct role in unwinding of the origin.

Although the model for the sequence of events leading to initiation of DNA replication described above refers mainly to Ad2 and Ad5 which require host factors NFI and NFIII for DNA replication it could be that the relatively simple protein requirements for Ad4 DNA synthesis may prove to be advantageous to the further study of the underlying mechanisms of DNA replication. Specifically, studies on Ad4 may be valuable in giving a clearer insight into the means of recognition of the origin by viral DNA replication proteins and to how they then go on to fulfill their roles in initiation and elongation.

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# Replication of adenovirus type 4 DNA by a purified fraction from infected cells

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## ABSTRACT

**An extract from Adenovirus type 4 infected HeLa cells was fractionated by ion-exchange and DNA affinity chromatography. One fraction, which bound tightly to single stranded DNA, contained predominantly a protein of apparent molecular weight 65,000 and three less abundant proteins. Immunological cross-reactivity with adenovirus type 2 proteins confirmed the presence of preterminal protein and indicated that the abundant species was the virus coded DNA binding protein. This fraction contained an aphidicolin resistant DNA polymerase activity and in the presence of a linearised plasmid containing the adenovirus type 4 origin of DNA replication efficient transfer of dCMP onto preterminal protein, indicative of initiation, was observed. Furthermore, addition of all four deoxyribonucleotide triphosphates and an ATP regenerating system resulted in the elongation of initiated molecules to generate plasmid molecules covalently attached to preterminal protein. Adenovirus type 4 DNA binding protein was extensively purified from crude adenovirus-4 infected HeLa extract by immunoaffinity chromatography using a monoclonal antibody raised against adenovirus type 2 DNA binding protein. A low level of initiation of DNA replication was detected in the fraction depleted of DNA binding protein but activity was restored by addition of purified DNA binding protein. DNA binding protein therefore plays an important role in the initiation of Ad4 DNA replication.**

## INTRODUCTION

Human adenoviruses contain linear duplex DNA genomes of approximately 35kb with inverted terminal repeats (ITR's) (1) and a 55kDa terminal protein (TP) covalently bound to each 5′ end (2, 3). Initiation of DNA replication occurs at the termini by a protein priming mechanism in which dCMP, the terminal nucleotide is covalently attached to an 80kDa precursor of the terminal protein (pTP) in a template dependent reaction. The nascent strand is elongated by viral DNA polymerase using the 3′-OH of the pTP-dCMP complex as a primer resulting in the displacement of the non-template strand which can then act as a template for a second round of DNA synthesis (4). There is evidence that the second round of synthesis occurs on a partially duplex 'panhandle' template formed by base pairing of the ITR's of the displaced strand (5, 6, 7).

The development of cell free systems, initially by Challberg and Kelly (8), in which adenovirus DNA can be synthesised from template molecules of viral or plasmid origin *in vitro* has played a key role in the identification of the sequences important for origin function and the isolation and purification of the proteins involved in DNA replication. The replication of Ad2 and Ad5 DNA has been the most extensively studied and has been shown to require three virus encoded proteins, the 80kDa pTP, the 140kDa Ad DNA polymerase (Ad pol) and the 72kDa DNA binding protein (DBP) as well as two host proteins, nuclear factor I (NFI) (9, 10, 11, 12, 13) and nuclear factor III (NFIII) (14, 15, 16, 17) which on binding to their cognate sites in the origin of replication can stimulate initiation of DNA replication up to 100-fold. *In vitro* studies have shown that the origins of Ad2 and Ad5 consist of a core domain comprising the terminal 18bp of the genome which alone is only capable of supporting a low level of initiation (18, 19), and an auxiliary region encompassing nucleotides 19−50 which contains the recognition sequences for NFI and NFIII. The sequence requirements of the Ad2 origin have also been defined *in vivo* where deletion analysis has demonstrated that the terminal 45bp are required for efficient replication of adenovirus mini chromosomes co-transfected with helper Ad2 DNA (20), and similarly that virus genomes with deletions extending into the terminal 45bp where rendered non-infectious (21). Transfection assays carried out using Ad4 as helper virus showed that unlike Ad2 only the terminal 18bp of the genome, which in Ad2 constitute the core origin, were required for efficient DNA replication *in vivo* (22). This has been borne out *in vitro* where it was demonstrated that linearised plasmid containing only the terminal 18bp of an adenovirus ITR could support initiation of DNA replication as effectively as a template containing a complete Ad4 ITR (23, Temperley *et al.*, manuscript in preparation). Correspondingly the protein requirements for Ad2/5 differs from Ad4 in that Ad4 shows no dependence on NFI or NFIII for efficient DNA replication. The

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Ad4 inverted terminal repeat does not contain a NFI recognition site and whilst it does have a binding site for NFIII neither site is required for DNA replication *in vivo* and the purified proteins are incapable of stimulating DNA replication *in vitro* even when the templates contain the cognate binding site (24). The mechanisms by which host factors increase the efficiency of DNA replication has yet to be properly defined but it has been demonstrated that the DNA binding domains of NFI and NFIII alone are sufficient to stimulate adenovirus Ad2 DNA replication (25, 26, 27). Recent evidence of a direct interaction between NFI and Ad2 polymerase has led to the suggestion that NFI increases the rate of initiation by stabilising a pre-initiation complex with pTP and pol via its interaction with pol (28, 29, 30).

The likelihood is that host proteins play auxilliary roles which enhance the functions of the viral replicative machinery and it is possible that Ad4 is able to efficiently replicate its DNA in the absence of host proteins because viral DNA replication proteins can adequately fulfill such roles themselves. To study the proteins required for Ad4 DNA replication we fractionated crude Ad4 infected HeLa cell extract which was capable of *in vitro* DNA replication by a combination of ion exchange and DNA affinity chromatography. A fraction eluted from DNA-sepharose with 1M NaCl containing 4 polypeptide species was sufficient for initiation and elongation of Ad4 DNA replication *in vitro*. Immunological and biochemical experiments confirmed the presence of Ad4 DBP, pol and pTP in these fractions. Using a single step immunoaffinity purification, we isolated near homogeneous DBP from crude Ad4 infected HeLa extract and demonstrated the important role of this protein in the initiation reaction.

## MATERIALS AND METHODS

### Cells and virus

HeLa S3 spinner cells were used to prepare virus stock and, after infection with Ad4, as a source of virus infected cell extract. Cells were grown in Spinner medium containing 50 units/ml of penicillin, 50μg/ml of streptomycin and 5% new-born calf serum. A549 cells were used to titrate virus by the method of Williams (31). Cells were grown in monolayer in Glasgow modified minimal essential medium containing penicillin, streptomycin, sodium bicarbonate and 10% new-born calf serum.

### Templates

Plasmid p4A2 contains the adenovirus type 4 ITR and is identical to plasmid p4A1 (20). pMDC10 pm18 (pM18) contains the terminal 69bp of the Ad5 ITR carrying a single base transition in the 18th base pair from the terminus (10) . Plasmid DNA was prepared by two rounds of CsCl/ethidium bromide centrifugation. Ethidium bromide was removed by repeated extraction with caesium chloride saturated iso-butanol and the DNA was desalted by ultrafiltration in a centricon-30.

### Preparation of cell extracts

1 litre of HeLa S3 cells were infected with 100 plaque forming units/cell of adenovirus type 4. After 90min medium containing 2% calf serum plus 10mM hydroxyurea was added. Cells were incubated for 22 hours at 37°C and extract was prepared as described (8). Extract was depleted of nucleic acids by passage over DEAE-sepharose in 0.2M NaCl and concentrated by precipitation with ammonium sulphate as described (32). The precipitate was resuspended in 2ml of 20mM Hepes (pH7.5), 5mM KCl, 0.5mM $MgCl_2$ and 0.5mM DTT per $3\times10^8$ cells, dialysed against the same buffer and stored in small aliquots at −70°C.

### Purification of Ad4 DNA replication proteins

*DNA affinity chromatography.* Crude cytoplasmic extract was prepared from Ad4 infected Hela S3 spinner cells as described. NaCl was adjusted to 50mM and the extract was applied to DEAE-sephacel equilibrated in 50mM NaCl. The column was washed with two volumes of buffer containing 50mM NaCl, and then with two volumes of buffer containing 0.2 M NaCl. Fractions were collected, assayed for *in vitro* DNA replication activity by pTP-dCMP complex formation assay and their protein concentration determined by the method of Bradford (33). Activity recovered in the 0.2M eluate was applied to a denatured calf thymus DNA-sepharose column equilibrated in buffer containing 0.2M NaCl. The column was washed with two volumes of buffer containing 0.2M NaCl and 5 volumes of a concentration gradient from 0.2M to 2M NaCl. Fractions were collected, assayed for their ability to initiate adenovirus DNA replication *in vitro* and their protein concentration was determined. Active fractions were dialysed against 20mM HEPES (pH8.0), 5mM KCl, 0.5mM $MgCl_2$, 5mM DTT and 1mM PMSF and stored at −70°C in small volumes.

*Immunoaffinity Purification.* Mouse ascitic fluid containing 5mg of monoclonal antibody α72k B6−10 raised against Ad2 DBP (34) was collected and bound to 1ml of protein G-sepharose (Sigma) by direct coupling in 3M NaCl as described (35) and used to prepare a column. Crude extract from Ad4 infected HeLa cells was prepared as described and dialysed against 10mM sodium phosphate. The extract was applied to the antibody column and unbound protein was removed by extensive washing with 10mM sodium phosphate, 0.05% NP40. Bound protein was eluted by increasing stepwise washes of 10, 20, 30, 40, 50 and 100mM $MgCl_2$ and fractions were analysed by SDS-PAGE. DBP was eluted in a near homogeneous state at 50−100mM $MgCl_2$.

### Assay for the transfer of dCMP to pTP

1−12μg of Ad4 infected HeLa cell cytoplasmic extract or purified replication proteins were incubated in a 30μl reaction containing 25mM Tris/HCl (pH8), 3mM $MgCl_2$, 2mM DTT, 3mM ATP, 3μCi [$\alpha^{32}$P] dCTP (specific activity 3000Ci/mole) and varying

**Table 1.** Purification of the Ad4 DNA replication proteins. 1μg of crude infected extract, DEAE-Sephacel eluate and denatured DNA-Sepharose eluate was incubated with EcoRI cleaved p4A2 as template in standard initiation reactions containing [$\alpha^{32}$p] dCTP. Polypeptides were fractionated by electrophoresis in a polyacrylamide gel containing SDS and labelled species were detected by autoradiography. The region of the gel corresponding to pTP-dCMP complex was excised, the radioactivity quantitated by scintillation counting and the information was used to calculate the data shown.

| Fraction | Total Protein (mg) | *Total Units | % Yield | Specific Activity (units/mg) | Fold Purification |
|---|---|---|---|---|---|
| Crude extract | 149.2 | 1798 | 100 | 12 | 1 |
| DEAE-Sephacel | 29.5 | 983 | 55 | 33 | 2.75 |
| Denatured DNA-Sepharose | 1.1 | 220 | 12 | 200 | 16.6 |

* 1 unit corresponds to the amount of protein required to transfer 1fmol of dCMP to pTP.

amounts of restriction enzyme cleaved template DNA for 90min at 37°C. Reactions were stopped by heating at 70°C for 5min and treated with micrococcal nuclease at 33 units/ml in the precence of 2mM $CaCl_2$ for 30min at 37°C. 12$\mu$l of gel loading buffer containing 20% glycerol (v/v), 5%SDS (w/v), 570mM 2-mercaptoethanol, 33mM Tris pH6.7 and 0.2% bromophenol blue (w/v) was added and the samples denatured by heating to 100°C for 2min. Reaction products were resolved on a 10% SDS-polyacrylamide gel at 35mA for 4 hours, fixed in 10% acetic acid, dried and then subjected to fluorography at −70°C in the presence of an intensifying screen.

### Assay for elongation of DNA initiated *in vitro*

Restriction enzyme cleaved template DNA was pre-incubated in a total volume of 7$\mu$l for 30 minutes at 30°C with 1−3$\mu$l of Ad4 infected HeLa cell cytoplasmic extract or purified viral replication proteins in a buffer containing 25mM Hepes-KOH (pH 7.5), 4mM $MgCl_2$, 1mM DTT, 0.1 mg/ml BSA and 0.2 mM aphidicolin. The reaction was started by addition of 7$\mu$l of a buffer containing 80$\mu$M each of dTTP, dATP, and dGTP, 5$\mu$M dCTP, 0.3$\mu$Ci [$\alpha^{32}$P] dCTP (specific activity 3000Ci/mM), 4mM ATP, 10mM creatine phosphate, 10$\mu$g/ml creatine phosphokinase, 0.1mg/ml BSA, 25mMHepes-KOH pH7.5, 4mM Mg $Cl_2$ and 1mMDTT to give a total reaction volume of 14$\mu$l. The total reaction was incubated at 30°C for 90 minutes. Reactions were stopped by addition of 6$\mu$l of buffer containing 5% SDS, 50% glycerol, 100mM EDTA and bromophenol blue and heated at 70°C for 5 minutes. Reaction products were resolved on a 2% agarose gel containing 0.1% SDS at 35mA for 4 hours which was fixed in 10% acetic acid, dried and subjected to fluorography at −70°C in the presence of an intensifying screen.

### Assay for DNA polymerase activity

2−10$\mu$g of protein sample were incubated with 10$\mu$g of activated calf thymus DNA, 100$\mu$M dTTP, dGTP, dCTP, 20$\mu$M dATP, 1$\mu$Ci [$\alpha^{32}$P] dATP (specific activity 3000Ci/mole), 5mM Tris pH8.0, 7mM $MgCl_2$, 10mM DTT and 100$\mu$M aphidicolin in a total volume of 50$\mu$l for 1 hour at 37°C. Reactions were terminated by addition of 10% TCA, 0.5% sodium pyrophosphate and insoluble radioactivity measured by scintillation counting.

### Western Blot analysis

All blots were carried out using semi-dry Western blot equipment according to the manufacturers directions. Filters were blocked by incubation with 10% w/v powdered non-fat milk in PBS and, after washing in PBS, were probed by incubating with 200$\mu$l of immune serum in 5ml of PBS, at 37°C for 1 hour. Antibody-antigen complex was detected by incubation of the filter with [$I^{125}$] protein A at 2$\mu$Ci/ml and after extensive washing with PBS, 0.05% NP40, labelled species were detected by exposure to X-ray film at −70°C.

## RESULTS

### Purification of a fraction from Ad4 infected HeLa cells capable of DNA replication *in vitro*

Previous studies *in vivo* and *in vitro* have established that Ad4 has a unique and very simple origin of DNA replication compared to the more intensively studied Ad2/Ad5. Accordingly, the protein requirements differ markedly, in that Ad4 is able to replicate its DNA without the need for cellular factors NFI/C-TF and NFIII/Oct-1.

To examine the proteins involved in Ad4 DNA replication we fractionated Ad4 infected HeLa extract which was capable of *in vitro* DNA replication. Crude Ad4 infected extract was prepared from 10 litres of HeLa suspension cells as described (see methods). Soluble extract was adjusted to 50mM NaCl and applied to DEAE-Sepharose equilibrated with 50mM NaCl, followed by extensive washing with 50mM NaCl. Bound proteins

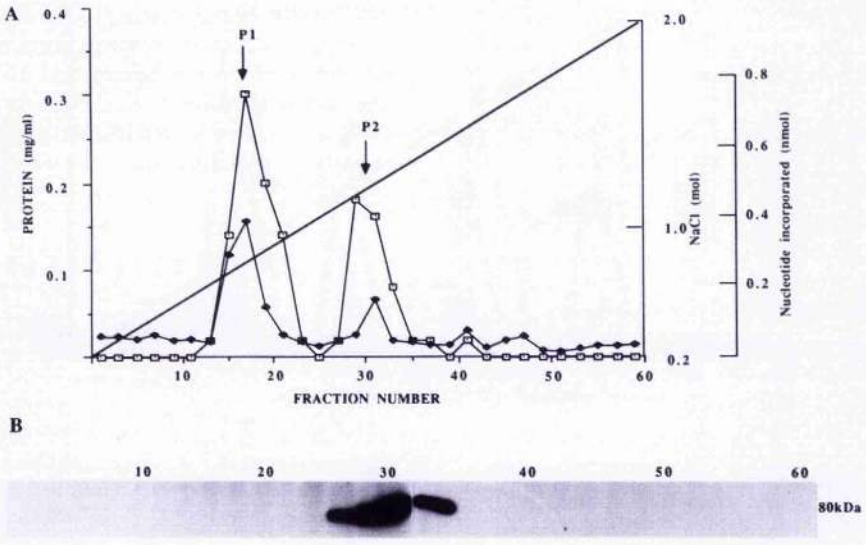


**Figure 1.** Purification of a fraction capable of replication of adenovirus DNA *in vitro*. **A.** Proteins bound to denatured calf thymus DNA-Sepharose were eluted with a 0.2M to 2M NaCl gradient. The protein concentration of each fraction was determined and the two major peaks designated P1 and P2(white squares). 2$\mu$l of each fraction was incubated in a standard DNA polymerase reaction (see methods) and the activity determined (black diamonds). **B.** Plasmid p4A2 (50ng) containing a copy of the Ad4 ITR was cleaved with EcoRI and incubated with 8$\mu$l of each fraction (dialysed against 0.1M NaCl buffer) from DNA-Sepharose in a standard assay for initiation of DNA replication (see methods) containing [$\alpha$32P] dCTP (3000Ci/mole) and 100$\mu$M aphidicolin. After incubation at 32°C for 90 minutes and micrococcal nuclease digestion polypeptides were fractionated by SDS polyacrylamide gel electrophoresis and labelled species were detected by autoradiography.

were eluted with 0.2M NaCl and fractions were collected. The protein concentration of each fraction was determined and the peak fractions were assayed for their ability to catalyse pTP-dCMP complex formation using EcoRI cleaved p4A2 as template. This step gave an overall 5-fold increase in specific activity with 50% yield and freed the extract of nucleic acids (Table 1). Peak fractions were pooled and applied to denatured calf thymus DNA-Sepharose equilibrated with 0.2M NaCl. After extensive washing with 0.2M NaCl bound proteins were eluted with a linear gradient of 0.2−2M NaCl. Fractions were collected and the protein concentration determined. One peak of protein eluted between 0.5−0.8M NaCl (P1) followed by a second peak eluting at 1.0−1.2M NaCl (P2) (Figure 1A). P1 corresponded to a single peak of protein obtained when extract of uninfected cells was fractionated under similar conditions (data not shown). Individual fractions were tested for their ability to initiate DNA replication *in vitro* by assaying the efficiency with which they were able to catalyse the transfer of dCMP onto pTP using EcoRI cleaved p4A2 as template. Only fractions in P2 were capable of initiation (Figure 1B). The purification procedure resulted in a 16 fold increase in the specific activity with a 12% overall yield (Table1).

An aphidicolin resistant DNA polymerase co-eluted from the denatured DNA-Sepharose column with the template dependent pTP-dCMP transfer activity (Figure 1A).

### Analysis of proteins involved in Ad4 DNA replication

To examine the proteins present at each stage of the purification samples of crude Ad4 infected extract, 0.2M eluate from the DEAE-Sephacel, flowthrough from the denatured DNA-Sepharose and eluates P1 and P2 from the denatured DNA-Sepharose were analysed by SDS polyacrylamide gel electrophoresis. Proteins were visualised by staining with Coomassie brilliant blue. All fractions analysed, with the exception of P2, contained multiple species. P2 apparently contained only one species of apparent molecular weight 65kDa which from its relatively high abundance and elution characteristics was likely to be viral DBP (Figure 2A). Since this fraction was capable of initiation of DNA replication *in vitro* it was clear there must be other replication proteins present. To further analyse fraction P2 we examined various quantities of P2 by SDS PAGE and used silver staining to detect the fractionated polypeptides. This revealed the presence of three additional species of apparent molecular weights approximately 70kDa, 85kDa and 95kDa (Figure 2B, lane 1; 2, 3 and 4).

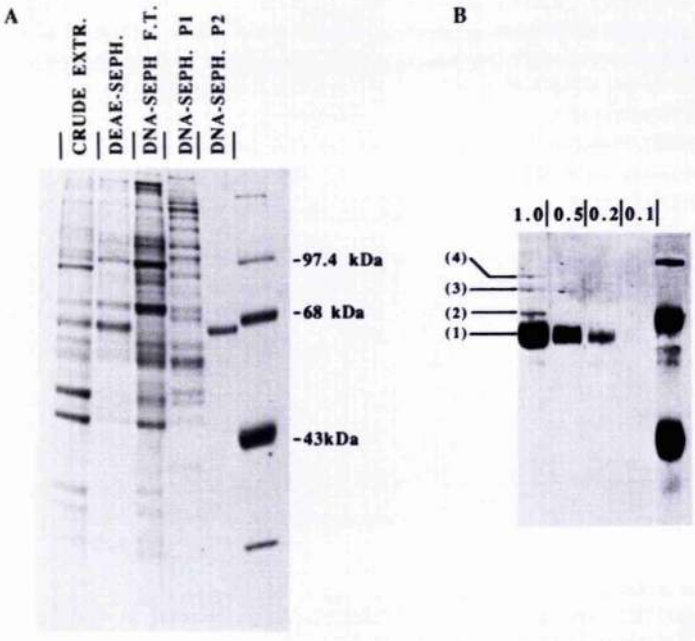


**Figure 2.** Protein analysis of peak fractions. **A.** 5μg each of crude extract, eluate from DEAE-Sephacel, flowthrough from DNA-Sepharose, and peak fractions P1 and P2 eluted from DNA-Sepharose were denatured by heating at 100°C in SDS and mercaptoethanol and the polypeptides present fractionated by electrophoresis. Species were visualised by staining with Coomassie blue. **B.** Samples of 0.1μg, 0.2μg, 0.5μg and 1μg of fraction P2 eluate from DNA-Sepharose were denatured in SDS and mercaptoethanol and the polypeptides present fractionated by electrophoresis. Species were visualised by silver staining.

### Elongation of initiated molecules *in vitro* by P2

Although it had been demonstrated that the fraction containing the four polypeptide species was capable of initiation of replication it was not clear if the fraction could also carry out elongation of initiated molecules. To compare the activity of crude and fractionated extracts two different templates were used; p4A2 which as described (22) contains one copy of an Ad4 ITR and pM18 containing an Ad5 ITR carrying a C to T base change at position 18 which has been shown to severely reduce template efficiency *in vitro* (Temperley *et al.*, manuscript in preparation). Templates were cleaved with EcoRI and AvaII to give a 1.6kb fragment with the origin sequence at the EcoRI terminus and a 0.9 kb fragment containing only plamid sequences and incubated with various protein samples, dNTP's (including [$\alpha^{32}$P] dCTP), optimal $MgCl_2$ and an ATP regeneration system. Reaction products were fractionated by electrophoresis in an agarose gel containing SDS and replicated templates which had incorporated [$^{32}$P] dCMP were detectable by autoradiography. Correctly initiated products have pTP linked at the 5′ end which results in a lower electrophoretic mobility (Fig 3, black arrow) than input template molecules which incorporate [$^{32}$P] at a low level (Fig 3, open arrow). Crude extract gave efficient elongation on p4A2 but greatly reduced elongation on pM18, consistent with its previously observed compromised ability to support *in vitro* DNA replication (Figure 3, lanes 1 and 2). The DEAE-Sephacel eluate and DNA-Sepharose eluate fraction P2 both gave efficient elongation on p4A2 but utilised the pM18 template inefficiently (Figure 3, lanes 3 to 6). DNA-Sepharose eluate P1 was unable

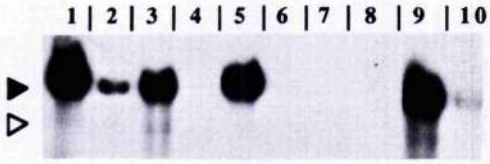


**Figure 3.** Elongation of initiated templates by fraction P2. Plasmid templates p4A2 and pM18 (100ng) were cleaved with EcoRI/AvaII and incubated with 3μg of crude Ad4 infected extract (lanes 1 and 2), 3μg of DEAE-Sephacel eluate (lanes 3 and 4), 2μg of DNA-Sepharose eluate P2 (lanes 5 and 6), 2μg of DNA-Sepharose eluate P1 (lanes 7 and 8) and a mixture of 2μg each of P1 and P2 (lanes 9 and 10) in a standard elongation reaction containing [$\alpha^{32}$P] dCTP (see methods). After incubation at 30°C for 90 minutes reaction products were resolved by electrophoresis in a 2% agarose gel containing 0.1% SDS. Labelled species were detected by autoradiography. Correctly initiated molecules have pTP attached (black arrow head) and so have a lower electrophoretic mobility than input template (white arrow head) which is labelled at a low level due to repair synthesis. A second fragment containing only plamid sequences is run out of the gel.

to elongate either template consistent with its inability to initiate DNA replication *in vitro* (Figure 3, lanes 7 and 8). Although fraction P1 itself was unable to replicate adenovirus DNA *in vitro* the presence of factors involved in DNA replication could not be ruled out. To determine if fraction P1 contained factors important for DNA replication P1 and P2 were incubated together with templates p4A2 and pM18 in a standard elongation reaction (Figure 3, lanes 9 and 10). The combined fractions did not support DNA replication at a significantly higher level than fraction P2 alone. Various combinations of flowthrough, P1 and P2 were examined under initiation conditions but no combination gave initiation levels in excess of that obtained with P2 alone.

These data demonstrate that all the proteins necessary for Ad4 DNA replication *in vitro* were present in fraction P2 from the DNA-Sepharose column. The presence of viral polymerase and pTP was inferred by the ability of P2 to initiate on and elongate from input template.

### Western blot analysis of P2

To identify the polypeptide species present in P2 samples were fractionated by SDS-PAGE, transferred to nitrocellulose and incubated with antisera raised against Ad2 replication proteins. Antibody-antigen complexes were detected with [$I^{125}$] protein-A followed by autoradiography. A polyclonal guinea-pig antiserum raised against Ad2 DBP strongly reacted with the 65kDa species (Figure 2B 1. and Figure 4A; lane1) confirming the identity of this species as DBP. Antisera raised in rabbits against purified Ad2 pTP and pol respectively were also used to probe blots of P2. The antiserum against Ad2 pTP recognised only the second highest molecular weight species (Figure 2B 3. and Figure 4A, lane 2). The antiserum against Ad2 polymerase however failed to recognise any of the species present (Figure 4A, lane 3) . The presence of Ad4 pol in the fraction however was implied by the data from both DNA polymerase and elongation assays. Since the antiserum has been shown to detect Ad2 pol (Figure 4B, lane 2) this result suggests that differences in the DNA polymerases of the two serotypes prevented cross reaction with Ad4 pol.

These data confirmed the identity of two of the species present in P2. As predicted, the most abundant protein corresponded to DBP and the presence of pTP, as predicted from pTP-dCMP transfer assay was confirmed.

### Immunoaffinity purification of Ad4 DBP

To fractionate DBP from the other components present in P2 a different approach was adopted. A column was prepared using protein G-sepharose linked to mouse monoclonal antibody directed against an epitope present in Ad2 DBP (34) as described (35). Crude extract from Ad4 infected HeLa cells was prepared as described (8) and the soluble extract freed from nucleic acids by passage over DEAE-Sepharose in 0.2M NaCl. The buffer was changed by dialysis against 10mM $NaPO_4$ pH7.2 and applied to the antibody column equilibrated in 10mM $NaPO_4$ pH7.2. After extensive washing with 10mM $NaPO_4$ pH7.2 and 0.05% NP40 bound proteins were eluted with increasing stepwise concentrations of 10mM, 20mM, 30mM, 50mM and 100mM $MgCl_2$. Fractions were collected, samples analysed in an SDS polyacrylamide gel and proteins visualised by Coomassie staining.

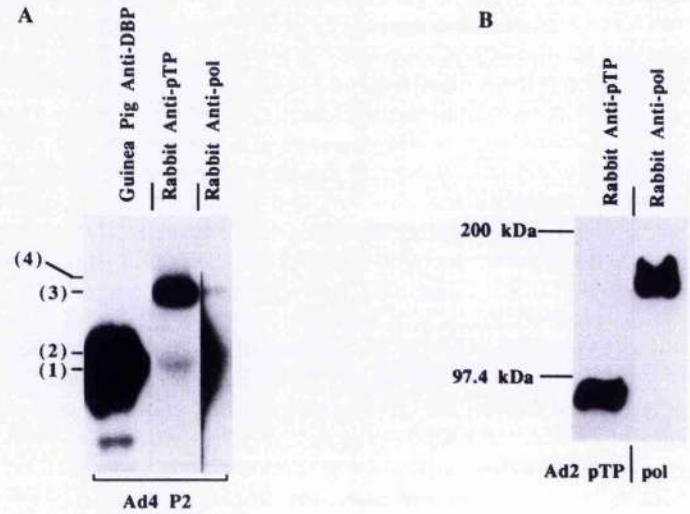


**Figure 4.** Western blot analysis of P2. Samples containing 10$\mu$g each of the second peak eluted from the DNA-sepharose (P2), Ad2 pTP and Ad2 pol were denatured in SDS and mercaptoethanol and fractionated by electrophoresis in a polyacrylamide gel containing SDS. Proteins were then transferred to nitrocellulose. **A**. P2 was probed with antisera against Ad2 DBP, pTP and pol and, **B**. Ad2 pTP and pol were probed with antisera against Ad2 pTP and pol respectively. The positions indicated of species 1,2,3 and 4 in P2 were determined by staining of Western blot with napthalene black (not shown). Antibody-antigen complexes were detected with $I^{125}$ protein-A followed by autoradiography.

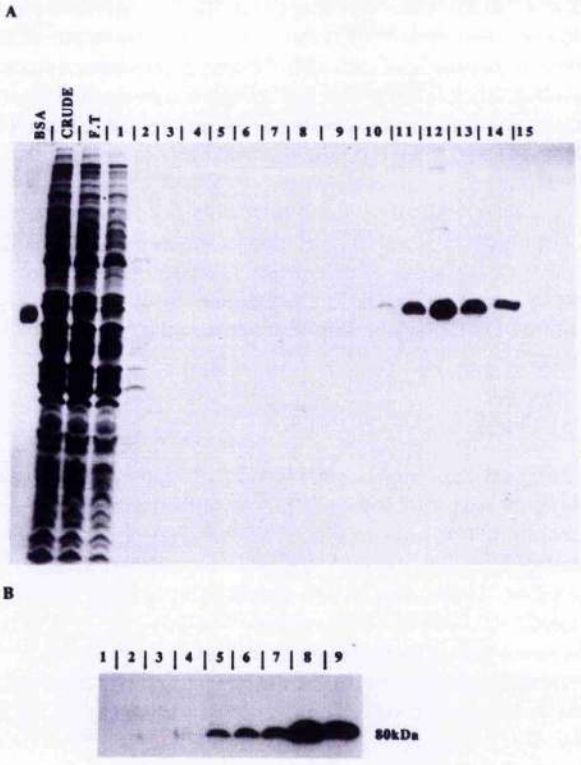


**Figure 5.** Immunoaffinity purification of Ad4 DBP. **A.** A crude extract from Ad4 infected HeLa cells was applied to a column containing $\alpha$72K monoclonal antibody against Ad2 DBP linked to protein-G Sepharose. Bound protein was eluted by application of increasing concentrations of $MgCl_2$. Proteins present in each fraction were analysed by electrophoresis in an acrylamide gel containing SDS followed by staining with Coomassie blue. **B.** DBP peak fraction (no.14) was incubated at 1$\mu$g, 2$\mu$g and 4$\mu$g alone (lanes 1–3) in a standard initiation assay with 50ng of EcoRI cleaved p4A2 as template. In addition, 2$\mu$g each of immunoaffinity column flowthrough and crude extract were incubated alone (lanes 4 and 7) and with 0.5$\mu$g and 1$\mu$g respectively of fraction14 (lanes 5 & 6 and 8 & 9) under standard initiation conditions.

The majority of the protein remained unbound and was washed from the column with 0.05% NP40 (Figure 5A lane 2). DBP was eluted from the column at 50mM to 100mM $MgCl_2$ (Figure 5A lanes 14 to 18) at about 98% purity. Approximately half of the DBP was not bound to the matrix. Because of the reported heterogeneity of DBP it was thought that the unbound portion may represent modified states of the protein which the monoclonal antibody was unable to recognise. To test this possibility the flowthrough was re-applied to the antibody matrix and a series of increasing stepwise elutions of $MgCl_2$ were used to elute bound protein as described. The DBP remaining in the flowthrough after the first round of purification bound to the column and was eluted at 50−100mM $MgCl_2$ (data not shown). These data demonstrated that the monoclonal antibody recognised all DBP present in the crude extract. The binding capacity of the antibody-Sepharose matrix was reached during the application of the crude extract leading to the observed presence of DBP in the flowthrough.

To test the effect of purified DBP on DNA replication, DBP in the peak fraction (Figure 5A, lane 14) was added to standard pTP-dCMP complex formation assays containing [$\alpha^{32}$P] dCTP and 50ng of EcoRI cleaved p4A2 as template. The fraction containing DBP (14) alone was incapable of *in vitro* initiation of DNA replication (Figure 5B, lanes 1 to 3). The flowthrough from the second round of immunoaffinity purification which was depleted of DBP was capable of initiation of DNA replication but only at a very low level (figure 5B, lane 4), however addition of DBP (fraction 14) resulted in an approximately 20-fold stimulation of initiation activity (Figure 5B, lanes 5 and 6). Initiation in crude extracts was stimulated by addition of DBP (fraction 14) at 0.5 $\mu$g but was inhibited at 1$\mu$g (Figure 5, lanes 8 and 9).

These data show that by immunoaffinity purification active and near homogeneous Ad4 DBP could be isolated from crude infected extract in a single step, separating it from viral polymerase and pTP. The data obtained from initiation assays suggest that DBP plays an important role in the initiation of DNA replication.

## DISCUSSION

Two different approaches were used to isolate and characterise the proteins required for Ad4 DNA replication. In the first a fraction containing only four detectable polypeptides able to both initiate DNA replication and elongate initiated molecules *in vitro* was purified. In the second, near homogeneous DBP was isolated from crude Ad4 infected cell extract. The data obtained indicates that there are important differences in the viral replication proteins between Ad4 and the more extensively characterised Ad2.

Initially a combination of ion exchange and single strand DNA affinity chromatography was employed with the intention of purifying DBP as described in the method of Schechter *et al.* (36). Surprisingly, a fraction whose major component was DBP, but which contained three detectable higher molecular weight species was isolated. Furthermore this fraction was capable of initiation of DNA replication and elongation *in vitro* implying the presence of pTP and viral DNA polymerase in the fraction. Two of the species, which were visualised by SDS PAGE, were identified as DBP and pTP by probing of a Western blot with anti Ad2 pTP antiserum and anti Ad2 DBP antiserum respectively. However an antiserum against Ad2 pol, which recognised Ad2 pol in Western blots, failed to recognise any species present in the fraction. Sequencing data reveals that there is considerable variation in the region near the amino termini of the DNA polymerases of Ad2, Ad7 and Ad12 (37,38). Although no sequence data for Ad4 pol exists the variation that has been observed in the DNA polymerases from the subgroups thus far analysed suggests that perhaps there is insufficient homology between the DNA polymerases of Ad2 and Ad4 for serum raised against Ad2 pol to recognise that of Ad4. In agreement with this data it has been observed that the same antiserum is unable to recognise native Ad4 pol in immunofluoresence of Ad4 infected HeLa cells (J.Bosher, unpublished observation).

As stated previously pTP and pol co-elute with DBP from denatured DNA-Sepharose with 1M NaCl. This differs markedly from Ad2 where denatured DNA-Sepharose chromatography separates pTP/pol and DBP: pTP/pol is eluted with 0.36M NaCl whilst 1.0M NaCl is required to elute DBP (39,40). Thus the requirement for 1.0M NaCl to elute the Ad4 pTP/pol complex may reflect a higher affinity of the Ad4 proteins for single stranded DNA or alternatively may be a result of a direct interaction between the Ad4 pTP/pol complex and DBP. However, under the conditions used for immunoaffinity purification DBP was separated from pTP/pol in low ionic strength buffer suggesting that the formation of such a complex forms, it may be DNA dependent. Another possibility is that bound DBP alters the structure of the DNA template resulting in higher affinity binding by Ad4 pTP/pol. This latter possibility is reminiscent of the effect of the Ad2 DBP on the DNA binding properties of NFI: DBP increases the affinity of NFI for its recognition site in the Ad2 origin of replication although no direct interaction between the two proteins could be detected (41, 42). Although Ad2 pTP/pol and DBP do not co-elute from DNA-Sepharose, the thermostability and processivity of pol is increased in the presence of DBP (43), suggesting the existence of functionally significant interactions between the proteins. Specific interactions between single stranded DNA binding proteins and DNA polymerases have previously been reported in prokaryotic phage systems such as T4 and T7 (44, 45). Such an interaction may play a critical functional role in light of the independence of Ad4 DNA replication from transcription cellular factors. The recent observation that NFI and Ad2 pol interact (28, 29, 30) and the implied role of NFI in the formation of a preinitiation complex suggests that such a function in Ad4 may be fulfilled by the viral replication proteins themselves. Thus Ad4 pTP and/or pol may themselves recognise and bind to the origin of replication with higher affinity than their Ad2 counterparts.

A marked stimulation of initiation of DNA replication was observed in the presence of the immunoaffinity purified fraction of DBP indicating the important role of this protein in initiation of Ad4 DNA replication. While there is ample biochemical and genetic evidence for the role of Ad2 DBP in elongation (46), its role in initiation is less clear cut, although under certain conditions it too can stimulate the initiation of Ad2 DNA replication (41, 42).

The relatively simple protein requirements for Ad4 DNA synthesis may well reflect the evolutionary development of a more autonomous system of viral self replication, where the reliance on host factors is reduced. This may well prove to be advantageous to the investigation of the underlying mechanisms of DNA replication. Further fractionation of the proteins will be needed to facilitate a more detailed functional analysis of each component.

## ACKNOWLEDGEMENTS

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