A STUDY OF THE *IN VITRO* REPLICATION OF ADENOVIRUS TYPE 4 DNA

by

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SUMMARY

The work described in this thesis concerns the development and characterization of an *in vitro* system for the initiation of adenovirus type 4 DNA replication and the first steps towards the purification and characterization of the individual components of the system.

The study was suggested by the differences observed in the minimal DNA sequence requirements for adenovirus type 2 (Ad.2) and adenovirus type 4 (Ad.4) DNA replication *in vivo* (Hay,1985a,b). The minimal Ad.2 origin of replication consisted of the terminal 45b.p. of the genome containing two important sequence elements: The 10b.p. AT-rich sequence conserved in all the human adenoviruses (Stillman *et al*,1982) between nucleotides 9-18 and the consensus binding site for the cellular protein nuclear factor I (NFI) between nucleotides 19-45 (Nagata *et al*,1983). In contrast the Ad.4 origin of replication comprised only the terminal 18b.p. of the genome, which were identical to the terminal 18b.p. of Ad.2 (Hay,1985b). An *in vitro* study was seen as the best way to further define the mechanistic differences between Ad.2 and Ad.4 DNA replication.

Initially full-length Ad.4 DNA was isolated from virions by a number of treatments leaving it associated with either the terminal protein and core polypeptides (Ad.4 cores), the terminal protein alone (Ad.4 DNA-prot.), or completely protein-free (Ad.4 DNA) and it was shown that

these templates could support initiation of DNA replication in vitro. Initiation was defined as the formation of a covalent complex between 5'-dCMP and the 80,000-dalton precursor terminal protein (pTP-dCMP complex). In Ad.2 the formation of this complex is catalysed by the viral DNA polymerase which is tightly associated with the pTP. Use of (α^{32}P)dCTP enabled the detection of this product by SDS-polyacrylamide gel electrophoresis and autoradiography.

Subsequent experiments utilized a plasmid containing the terminal 140b.p. of Ad.4 as a template for reasons of stability and reproducibility of results. Formation of a pTP-dCMP complex was shown to be catalysed by either nuclear or cytoplasmic extract of Ad.4 infected cells alone. An inhibitory activity in nuclear extracts meant that cytoplasmic extracts were used exclusively to characterize the reaction. Various parameters of the reaction were studied including the formation of pTP-dCMP complex at different protein concentrations, the time course of pTP-dCMP complex formation and the nucleotide specificity of the reaction. The activity of cytoplasmic extract in forming pTP-dCMP complex was studied at varying concentrations of ATP and divalent cations and in varying conditions of ionic strength.

By including in the reaction dATP, dTTP and ddGTP it was shown that the pTP-dCMP complex could be elongated, presumably by the Ad.4 specific DNA polymerase, to the first dG residue in the sequence - the 30th nucleotide. This

resulted in the formation of an additional product with an apparent molecular weight of 90,000-daltons.

The template requirements for the formation of a pTP-dCMP complex were studied: Both the DNA sequence and template conformation requirements were investigated using both single- and double-stranded DNA. It was shown that the double-stranded DNA sequence requirements were the same as *in vivo*.

Finally the Ad.4 specific DNA polymerase was partially purified from Ad.4 infected HeLa cells and the response of this DNA polymerase to various inhibitors studied. A factor from uninfected HeLa cell nuclei that stimulated pTP-dCMP complex formation by cytoplasmic extract of Ad.4 infected cells was partially purified. This factor was shown to stimulate pTP-dCMP complex formation on a template containing only 18b.p. from the Ad.4 terminus, indicating that it might specifically recognize sequences within that region.

ABBREVIATIONS

A	adenine.
Å	angstrom.
Ad.	adenovirus.
ATP	adenosine-5'-triphosphate.
b.p.	base pairs(s).
BuDR	5-bromo-2'-deoxyuridine.
C	cytosine.
°C	degrees centigrade.
C_i	curies.
cm.	centimetres.
DBP	DNA-binding protein.
dAMP	2-deoxyadenosine-5'-monophosphate.
dATP	2-deoxyadenosine-5'-triphosphate.
dCMP	2-deoxycytidine-5'-monophosphate.
dCTP	2-deoxycytidine-5'-triphosphate.
dGMP	2-deoxyguanosine-5'-monophosphate.
dGTP	2-deoxyguanosine-5'-triphosphate.
dNMP	any 2-deoxyribonucleoside-5'-monophosphate (dAMP, dCMP, dGMP or dTMP).
dNTP	any 2-deoxyribonucleoside-5'-triphosphate (dATP, dCTP, dGTP or dTTP).
dTMP	2-deoxythymidine-5'-monophosphate.
dTTP	2-deoxythymidine-5'-triphosphate.
ddCTP	2,3-dideoxycytidine-5'-triphosphate.
ddGTP	2,3-dideoxyguanosine-5'-triphosphate.
ddTTP	2,3-dideoxythymidine-5'-triphosphate.
DMS	dimethylsulphate.
DNA	deoxyribonucleic acid.
DNA-prot.	DNA attached to terminal protein.
DNase	deoxyribonuclease.
DTT	dithiothreitol.
EDTA	ethylene-diamine tetra-acetic acid sodium salt.
fmole	femtomole(s).
g	gramme.
G	guanine.
GC	moles percent deoxyguanosine and deoxycytidine residues.

h.	hour(s).
h.p.i.	hours post infection.
ITR	inverted terminal repeat.
K	1000-dalton molecular weight.
kb.	kilobase(s).
l.	litre(s).
M	molar.
uC_i	microcurie(s).
mg	milligramme(s).
ug	microgramme(s).
min.	minute(s).
ml.	millilitre(s).
ul.	microlitre(s).
mm.	millimetre(s).
mM	millimolar.
uM	micromolar.
mmole	millimole.
mRNA	messenger ribonucleic acid.
m.u.	map units (for adenovirus 1m.u. = 359 nucleotides).
M.W.	molecular weight.
NFI	nuclear factor I.
NFII	nuclear factor II.
NFIII	nuclear factor III.
ng	nanogramme(s).
nm.	nanometre(s).
nM	nanomolar.
ORF	open reading frame.
oriC	Escherichia coli origin of DNA replication.
PAGE	polyacrylamide gel electrophoresis.
pfu	plaque forming units.
pmole	picomole(s).
PMSF	phenyl-methyl-sulphonyl-fluoride.
pTP	precursor terminal protein.
pTP-pol	precursor terminal protein - DNA polymerase complex.
rATP	adenosine-5'-triphosphate.
rCTP	cytidine-5'-triphosphate.
rGTP	guanosine-5'-triphosphate.

rNTP	any nucleoside-5'-triphosphate (rATP, rCTP, rGTP or rUTP).
rUTP	uracil-5'-triphosphate.
RNA	ribonucleic acid.
RNase	ribonuclease.
rpm	revolutions per minute.
SDS	sodium dodecyl sulphate.
sec.	second(s).
T-Ag.	Simian virus 40 large T antigen.
TP	terminal protein.
ts	temperature sensitive.
VP	viral protein.
v/v	volume to volume (ratio).
w/v	weight to volume (ratio).

INTRODUCTION

The aim of this introduction is to provide a background to the adenoviruses in general and adenovirus DNA replication in particular. Adenovirus DNA replication will be compared with other well characterized DNA replication systems, both procaryotic and eucaryotic. To this end I propose to cover 5 areas as follows:

1) To introduce the adenoviruses and describe the various components of the virions.

2) To describe the adenovirus transcriptional programme, relating it where possible to the life cycle of the virus by discussing the functions of the gene products.

3) To chart the developments in the understanding of adenovirus DNA replication and review the current state of knowledge regarding the proteins and DNA sequences involved. I shall also propose a model for the mechanism of adenovirus DNA replication based on the available facts.

4) To discuss 2 other *in vitro* viral DNA replication systems (SV40 and ϕ29) which are relevant to the adenovirus system and the work of Kornberg and colleagues in elucidating the mechanism of *Escherichia coli* DNA replication - pioneering work in the understanding of DNA replication in general.

5) To describe the properties of other eucaryotic DNA polymerases so far characterized, both viral and cellular, to compare them with the adenovirus DNA polymerases.

1:1 THE ADENOVIRIDAE

The Adenoviruses (family Adenoviridae) are a large group of viruses with DNA genomes. The viruses are divided into two genera depending on their natural hosts: The Mastadenovirus genus consists of 78 or more serotypes whose natural hosts are mammalian. These viruses infect a wide range of mammals including humans, cattle, horses, sheep, pigs, dogs, monkeys, mice and tree-shrews (Ishibashi and Yasue,1984). The Aviadenovirus genus consists of at least 15 serotypes that infect birds. These viruses infect chickens, turkeys, ducks and pheasants. In addition to these two genera an adenovirus-like agent has been isolated from the leopard frog (Clark et al,1973).

Human adenoviruses were discovered almost simultaneously by Rowe et al (1953) and Hilleman and Werner (1954). Since then 41 distinct serotypes of human adenoviruses have been identified. They produce a number of acute diseases in humans including ocular, respiratory, gastro-intestinal and urinary infections. The viruses are ubiquitous throughout the human population, affecting all ages and races, and both sexes equally.

Adenoviral infections are not very often associated with fatal diseases, and indeed the majority of infections are probably asymptomatic. Nevertheless they are considered important human pathogens. The initial motivation for studying the structure and function of adenoviruses was naturally the result of clinical requirements, but in the last 25 years their use as a tool in the study of eucaryotic molecular biology has been recognized and capitalized on.

1:2 THE STRUCTURE OF THE VIRION

Virion morphology is remarkably conserved throughout the adenoviruses. The virion consists of two major components- an icosahedral protein shell and an inner core containing the DNA. The dimensions of an icosahedron are derived mathematically from the edge. The edge of the adenovirus type 5 (Ad.5) capsid has been reported as 43nm (Nermut,1975) and 52nm (Devaux et al,1983), giving a diameter around the five-fold axis of symmetry of either 73nm or 88nm.

1:2:1 CAPSID STRUCTURE

The icosahedral protein shell consists of 252 capsomers- 240 hexons (designated viral protein II:VPII) and 12 pentons, each made up of a base and a fibre (VPIII and VPIV) (Valentine & Pereira,1965). Each of the penton bases, present at the vertices of the icosahedron, is surrounded by 5 'peripentonal' hexons. The remaining 180 hexons dissociate into 'groups of nine' (GON's) after various treatments:

heating at 56°C for 10min. (Russell et al,1967); or trypsin or deoxycholate treatment (Pereira & Wrigley,1974). The GON's appear in electron micrographs as left or right-handed depending on whether the inner or outer surface is visualized (Pereira & Wrigley,1974). Differences in the appearance of the GON's indicates a morphological difference between each end of the hexon, the outer face displays three identical subunits whilst the inner face has a large central hole. The intact hexon was shown (Jornvall et al,1981) to consist of three identical subunits of 108,000 molecular weight (108K).

The crystal structure of the hexon has recently been elucidated (Roberts et al,1986a): The complete hexon consists of two parts; a pseudo-hexagonal base 52A high with a triangular top 64A high consisting of three 'towers'. Each subunit consists of two domains P1 and P2 which each comprise an 8-stranded β-barrel with loop-out regions between the strands. The two domains P1 and P2 each have corresponding schematic structure, which is related to that found in the coat proteins of small spherical plant viruses, rhinoviruses and poliovirus. The towers are formed from loops l_1 and l_4 from domains P1 and P2 in adjacent subunits and loop l_2 from the third subunit. This interpenetration of the three subunits within the tower explains the hexon's resistance to denaturation and may also explain the requirement for the 100K protein in hexon assembly.

1:2:2 CORE STRUCTURE

The core consists of the viral DNA and three core proteins VPV, VPVII and mu. VPVII is an 18K protein shown to be closely associated with the DNA (Ginsberg,1979, Sato & Hosokawa,1981) whereas VPV, a less abundant 45K protein, is easily dissociated from the DNA-VPVII complex and has been shown to be situated adjacent to the hexon, penton and VPIIIa (Everitt,1975).

Electron microscopic analysis of cores in various stages of relaxation has shown a 'beads on a string' appearance or rod-like elements (Nermut,1979). In addition micrococcal nuclease treatment of cores produced DNA fragments from 150b.p. (Mirza & Weber,1982) to 200b.p. (Corden et al,1976). These data suggest two models for the organization of the nucleocapsid: Firstly the continuous helix model proposes a linear helical filament made up of VPVII, around which the DNA is wound. However that model is based on the assumption that only one protein is present in the core structure (Nermut,1980). The currently accepted model is the 'nucleosome' or discontinuous helix model (Corden et al,1976). This model proposes nucleosome beads consisting of 6 VPVII molecules with two turns of DNA per bead (164b.p.) and a spacer of 30b.p. between nucleosomes. It has been suggested (Vayda et al,1983) that the 4K protein mu has some role in maintaining the nucleosome structure. Mu is extremely basic, consisting of 54% arginine, and as such would neutralize the charge on the tightly packed DNA.

The hexons are associated with several other viral structural proteins: VPVI was shown to be closely associated with the hexon (Everitt et al,1975) although it was subsequently shown to be a DNA-binding protein (Russell & Precious,1982). VPVI cannot be labelled by iodination of intact virions, suggesting that it is located inside the hexon shell. VPVIII is associated with single hexons but not with GON's (Everitt & Philipson,1974) whereas VPIX is associated with the GON's (Boulanger,1979). VPIX is present at a ratio of 15 copies per GON and is thought to locate at the hexon contact points.

The 12 pentons each consist of a penton base and one (human adenoviruses) or two (avian adenoviruses) antennae-like projections called fibres. The base is a trimer, each polypeptide chain (VPIII) having a molecular weight of 85K (Devaux et al,1982). An additional protein VPIIIa (Devaux et al,1982) bridges the gap between the penton base and the 5 peripentonal hexons.

The fibre consists of a shaft 2nm in diameter varying in length between 9-31nm depending on the serotype (Norrby,1969), terminated by a knob 4nm in diameter. The fibre is thought to be a dimer of VPIV polypeptides, each having a molecular weight of 62K (Green et al,1983). The interaction between the fibre and the base is thought to be hydrophobic, as they can be dissociated by guanidine, pyridine or deoxycholate (Nermut,1984).

TABLE 1:1. ADENOVIRUS STRUCTURAL PROTEINS

No.	Molecular weight.	Number of copies/virion.	Name.	Location.	Remarks.
II	108K	720	Hexon.	Capsid.	Trimer.
III	85K	36	Penton base.	Capsid vertices.	Trimer.
IIIa	66K	60	-	Peripentonal region.	Phosphorylated.
IV	62K	24	Fibre.	Capsid vertices.	Glycosylated, carries antigenic determinants.
IVa_2	50K	-	-	Core.	DNA-binding.
-	55K	2	Terminal protein.	5' end of DNA.	-
V	48K	180	Core protein 1.	Core shell.	Moderately basic, weakly phosphorylated, DNA-binding.
VI	23.4K	420(?)	-	Hexon associated.	Phosphorylated, DNA-binding.
VII	18K	1070	Core protein 2.	Nucleocapsid,	Basic, DNA-binding.
VIII	13K	-	-	Hexon-associated.	-
IX	11.5K	300	-	Associated with GON's.	-
X	7K	50	-	Internal.	-
XI	4.5K	125	-	Internal.	mu- associated with core.
XII	3K	-	-	Internal.	Possibly cleavage product of VIII.

(Taken from Nermut,1984).

1:2:3 GENOME STRUCTURE

All adenovirus serotypes have a linear, double-stranded DNA genome of approximately 35-36,000 base pairs (35-36Kb.) in length, corresponding to a molecular weight of 19-24 $x10^6$ daltons (Green et al,1967), except for the avian adenovirus Chick Embryo Lethal Orphan virus (CELO) (Laver,1971), which has a much larger genome of 30 $x10^6$ daltons. A protein of apparent M.W. 55K is linked to the ends of the genome. This terminal protein (TP) was shown to be covalently linked to the DNA by virtue of the resistance of the DNA-protein complex to boiling and SDS (Robinson et al,1973, Rekosh et al,1977, Carusi,1977). DNA-protein complex and deproteinized DNA (which is linked to residual amino-acids) were both resistant to digestion with phosphatase and exonuclease VII and could not be labelled with polynucleotide kinase. However they were sensitive to digestion with exonuclease III and labelling with terminal transferase (Carusi,1977, Sharp et al,1976), thus indicating that the TP was bound to the 5' ends of the genome. The linkage was shown to be a phosphodiester bond between the hydroxyl group of a serine residue in the TP to the 5' phosphate group of the terminal deoxycytidine of the genome (Desiderio & Kelly,1981).

Green et al (1979) showed that the TP's of five different human serotypes were very similar by tryptic peptide analysis, suggesting a common cellular origin. However Rekosh (1981) showed that they had different

apparent molecular weights, suggesting that TP was a conserved viral protein. Stillman *et al* (1981) identified an mRNA originating from between coordinates 11-31.5 on the l-strand that was translated *in vitro* to give a protein with apparent M.W. 87K. This protein was identical to an 80K protein found attached to the 5' ends of viral DNA strands synthesized *in vitro* (Challberg,1980). The 80K/87K protein was structurally related to TP, suggesting that TP is synthesized as a precursor (pTP). Further evidence comes from studies with the temperature-sensitive (*ts*) mutant Ad2*ts*1. *ts*1 cannot cleave viral precursor proteins to the mature forms (Bhatti & Weber,1979) at the non-permissive temperature. As a result Ad2*ts*1 virions contain an 80K instead of a 55K TP (Stillman *et al*,1981).

It was noticed (Garon *et al*,1972, Wolfson & Dressler,1972) that when denatured adenoviral DNA was renatured at low concentrations and examined by electron microscopy single-stranded circles were seen. These circles had high thermal stability and were not seen after exonuclease III treatment. This suggested the presence of inverted terminal repeat sequences (ITR's). Early estimates of the size of these ITR's, based on data from exonuclease III experiments, put them between 350-1400b.p. (Garon *et al*,1972). Nucleotide sequence analysis later showed these to be overestimates: The length of the ITR's varies from 63b.p. for CELO virus (Alestrom,1982) to 162b.p. for Ad.12 (Shinigawa & Padmanabhan,1980). The first ITR to be sequenced, that of Ad.5 (Steenbergh *et al*,1977), is 103b.p.

long and shows features common to all adenoviruses. The distribution of GC and AT base pairs is asymmetrical, the first 50b.p. are 72% AT, whereas the second 50b.p. are 27% AT. All serotypes have a dCMP residue at the 5' termini except CELO virus, which has a dGMP (Alestrom,1982). The sequence 5'ATAATATACCTTAT from nucleotides 9-22 is conserved in all human adenoviruses (Tolun et al,1979) and has been shown to be important in the initiation of DNA replication. There are other blocks of high homology within the ITR's of various serotypes (Tolun et al,1979), some of which have been shown to bind cellular factors during DNA replication.

The entire nucleotide sequence for the Ad.2 genome has now been determined (Roberts et al,1986b) and comprises 35,937b.p. This data, in conjunction with data obtained from DNA-RNA hybridizations, in vitro transcription and translation, R-loop and S1 mapping (Weinmann et al,1984) has built up a detailed map of the organization of the Ad.2 genome. There are 10 transcription units which can loosely be classified as early or late, depending on whether they are transcribed before or after the onset of DNA replication. However the temporal regulation of adenoviral transcription is complex and mRNA species are now generally referred to as pre-early, early, intermediate or late. By convention the two strands of the viral genome are classified according to the direction of transcription: The genome consists of a GC-rich and an AT-rich half, designated left and right respectively. The strand transcribed to the right is termed the r-strand and the strand transcribed to

the left the l-strand (Bachenheimer et al,1977). 69% of the viral genes are transcribed from the r-strand, the remainder from the l-strand.

1:3 VIRAL TRANSCRIPTION

The adenovirus genes are transcribed from nine initiation sites by cellular RNA polymerase II (Price & Penman,1972, Wallace & Kates,1972), (and RNA polymerase III in the case of the VA RNA's) in the nucleus of the infected cell. mRNA species are capped and polyadenylated (Philipson et al,1971).

1:3:1 EARLY TRANSCRIPTION

The first mRNA species can be detected as early as 45min p.i. (Nevins et al,1979) and are transcribed from the pre-early Ela transcription unit. Transcription from Ela reaches a maximum at about 3h. p.i. and remains constant for at least a further 6h. Ela has been mapped to the r-strand between map units (m.u.) 1.3 and 4.6 (Berk & Sharp,1977) and transcribes 3 mRNA's of sedimentation coefficients 13S, 12S and 9S. These mRNA's share common 5' and 3' ends and only differ in the site of internal splicing. Early in infection (up to 6h.) the level of the 9S mRNA is only 5% of the 13S and 12S, but after 8-12 h.p.i. the 9S mRNA becomes the most abundant of the three (Spector et al,1978). This change can be explained in terms of increased message stability or changes in splicing activity. It has been shown (Svensson et al,1983) that the three mRNA's are generated by separate splicing events from a single precursor.

The 13S and 12S mRNA's are predicted to give rise to proteins of 289 and 243 amino-acids in length, with molecular weights 31.9 and 26.5K (Perricaudet et al,1979). However in practice the Ela proteins are heterogenous and have greater apparent M.W.'s than predicted (Lewis et al,1979), the latter due to the high percentage of proline residues in the proteins. The 9S mRNA gives rise to a protein 55 amino-acids long with a predicted M.W. 6.1K and observed M.W. 28K. All the proteins share a common N-terminus but the 9S product differs after the splice junction.

The Ela proteins are involved in both the regulation of transcription and the process of cellular transformation. An Ela product has been shown (Jones & Shenk,1979; Berk et al,1979) to increase transcription from other early viral promoters 50-fold, whilst only increasing it's own transcription 5-fold. Ela has also been shown (Nevins,1982; Wu et al,1986) to increase transcription from the cellular heat shock 70 protein promoter 8-fold, to repress transcription from the SV40 early promoter (Velcich & Ziff,1985) and to induce cellular DNA synthesis in quiescent, G_O-arrested cells (Kaczmarek et al,1986). It is now generally accepted that transcriptional control mediated by Ela is accomplished via interactions with various cellular proteins (Imperiale & Nevins,1986).

Ela is also important in cell transformation by adenoviruses. Ruley (1983) showed that Ela products could

induce primary cells to proliferate but could not induce complete morphological transformation alone. The latter requires the presence of E1b (van der Eb & Bernards,1984), or other transforming proteins such as ras or polyoma virus middle-T antigen (Ruley,1983).

The next mRNA's to be transcribed are from the E1b, E3 and E4 regions. These mRNA's are detected 1.5-2h.p.i. and reach a maximum level at 6-7h.p.i. for E1b, and 3-4h.p.i. for E3 and E4. Soon after the activation of these transcription units the E2 mRNA's are transcribed and reach a peak at 3-4h.p.i. (Nevins et al,1979).

The E1b transcription unit is located on the r-strand from m.u. 4.6-11.2 and was originally thought to produce 3 mRNA's, two early in infection, the 22S and 13S (Berk & Sharp,1978). Two additional early mRNA's, 14S and 14.5S, have recently been described (Virtanen & Pettersson,1985). All these mRNA's have common 5' and 3' ends and are produced by differential splicing of a precursor. The 14S and 14.5S mRNA's are identical to the 13S with the addition of a third exon. In the 22S mRNA two AUG's can be used for translation, giving rise to a 55-58K protein and, in a different but overlapping reading frame, a 19-21K protein. The latter is also coded for by the 13S, 14S and 14.5S mRNA's (Bos et al,1981; Virtanen & Pettersson,1985).

In addition all these mRNA's contain the coding regions for VPIX, a 14.3K structural protein associated with

the GON's (Boulanger,1979). However this protein is not translated from these mRNA's but from an additional mRNA synthesized at intermediate times in infection, between 8-12h.p.i. (Wilson et al,1979). This mRNA is unspliced (Alestrom et al,1980), making it unique amongst adenovirus mRNA's.

The precise functions of the E1b proteins is uncertain. Mutations affecting the 55-58K protein reduce virus yield 100-fold, by reducing late transcription and translation (Babiss & Ginsberg,1984). In transformed cells the 55-58K is found complexed to the cellular protein p53 (Sarnow et al,1982). p53 levels have been shown to increase in transformed cells and quiescent cells stimulated by serum (Reich & Levine,1984). p53 also complexes with the SV40 T-antigen and the formation of this complex increases the half life of p53 from 20min. to greater than 24h. (Oren et al,1981). This may represent an important mechanism in adenoviral transformation.

The E3 region maps on the r-strand from m.u. 76.8-85.9 (Berk & Sharp,1978). There are at least 9 mRNA's transcribed from this region, produced by differential splicing of two precursors. The mRNA's have a common 5' end and there are two major and one minor polyadenylation sites at m.u. 85.9, 82.9 and 85. The E3 region overlaps with L5, the late region that contains the gene for the fibre protein. The fibre mRNAs have three leaders x,y and z, the splice sites of which coincide with some of the E3 splice junctions (Chow & Broker,1978).

So far only three proteins have been assigned to the E3 region- a 19K glycoprotein (Persson et al,1980), an 11.6K protein (Wold et al,1984) and a 14K protein which has not been precisely mapped (Persson et al,1978). The 19K glycoprotein has been shown to complex with the heavy chain of the class I transplantation antigens (Signas et al,1982) and appears to result in the reduction of the class I antigens on the cell surface. The E3 region has been shown to be dispensable for lytic growth in tissue culture (Berkner & Sharp,1983).

Region E4 is transcribed from the 1-strand between m.u. 99.1-91.3 (Berk & Sharp,1978, Chow et al,1979). A primary transcript is spliced into 12 different mRNA's, all with identical 5' and 3' ends (Virtanen et al,1984). Sequence analysis (Roberts et al,1986b) predicts the existence of 7 open reading frames (ORF's) encoding proteins from 6-34K in size. Transcription of the E4 region is induced by a product of the E1a region (Berk et al,1979, Jones & Shenk,1979) and inhibited by a product of the E2 region (Nevins & Winkler,1980), later shown to be the 72K DNA binding protein (Handa et al,1983).

The functions of the E4 proteins are unknown. A 25K protein assigned to the E4 region was shown to be associated with the E1b 55-58K protein in productively infected cells (Sarnow et al,1984). Deletions in both these proteins (Halbert et al,1985; Babiss & Ginsberg,1984; Pilder et al,1986) have similar phenotypes: They exhibit low levels of

late mRNA and protein synthesis and inefficient shut-off of host-cell protein synthesis. In addition the E4-25K mutant exhibited low levels of viral DNA synthesis. These data suggest that the 25K-55K complex is involved in the regulation of late transcription, late translation and shut-off of host-cell macromolecular synthesis. This theory was strengthened by the work of Weinberg and Ketner (1986) using a deletion mutant dl808 which lacked most of the E4 region. In HeLa cells this mutant exhibited low levels of late mRNA, protein and viral DNA synthesis, but overproduced the 72K DBP. In a cell line expressing the E4 region (W162 cells) the mutant had a wildtype phenotype.

The E2 region is transcribed from the l-strand between m.u. 75.4 and 11.3 (Chow et al,1979). It is unique amongst adenovirus transcription units in that two promoter sites are used at different times in infection. E2 transcripts terminate at one of two possible poly-adenylation (poly-A) sites, on this basis they are termed E2a or E2b mRNA's. E2a mRNA's are transcribed early in infection from a promoter at m.u. 75.4 and from a second promoter at 72.2 late in infection, and terminate at a poly-A site at 62.4 (Baker et al,1979). E2b mRNA's bypass this poly-A site and terminate at 11.3 (Stillman et al,1981). In addition to the leader at 75.4/72.2, all E2 mRNA's have a common leader at 68.8. E2b mRNA's also have a leader at 39.

The only protein assigned to the E2a group is the 72K single-stranded DNA binding protein (DBP) (Lewis et al,1976). DBP is a multi-functional protein: It has a role in DNA replication, where it is thought to bind to displaced single-strands (van der Vliet & Sussenbach,1975, van der Vliet et al,1977) and increase the processivity of the viral DNA polymerase (Field et al,1984). It regulates it's own production (Carter & Ginsberg,1976; Nicholas et al,1982) and in addition regulates E1 and E4 transcription (Babich & Nevins,1981; Nevins & Winkler,1980). Whereas human adenoviruses normally undergo an abortive infection in monkey cells mutants in the N-terminus of the DBP are able to replicate normally in these cells (Klessig & Grodzicker,1979). This suggests a role for DBP in late mRNA splicing as the block to human adenovirus replication in monkey cells is thought to involve aberrant splicing of the fibre mRNA (Klessig & Chow,1980).

Three mRNA's are transcribed from the E2b region (Stillman et al,1981). The precursor terminal protein (pTP) (Stillman et al,1979) and the 140K DNA polymerase (Stillman, Tamanoi & Mathews,1982) have been assigned to two of these mRNA's. These proteins are present as a stoichometric complex in infected cells (Lichy et al,1982) and together with the DBP and 2 cellular factors (Nagata et al,1983b) are required for DNA replication (see Section 1:5). The third mRNA has an ORF coding for a 22.8K polypeptide, which has not been identified as yet.

1:3:2 INTERMEDIATE AND LATE TRANSCRIPTION

The E2b region completely overlaps with the mRNA for VPIVa2, a 50K protein involved in virion morphogenesis (Persson et al,1979). This mRNA is transcribed at intermediate times during infection from it's own promoter at m.u. 16.1 and is co-terminal with the E2b mRNA's (Lewis et al,1977). The class of intermediate mRNA's also includes the mRNA for VPIX. These two mRNA's can be detected prior to the onset of DNA replication (Wilson et al,1979) but have since been shown to require DNA replication for maximal transcription (Crossland & Raskas,1983).

Following the onset of DNA replication (6-8h.p.i.) (Green et al,1970), transcription from early sites is increased 3-10 fold (Shaw & Ziff,1980) presumably because of the increase in DNA template numbers. The onset of DNA replication also brings about a major change in the transcriptional programme. At late times in infection (from 8h. onwards) the majority of mRNA's are transcribed from the major late promoter (MLP) which is situated on the r-strand at m.u. 16.8 (Ziff & Evans,1978). The 5' end of all these mRNA's consists of a 201b.p. tripartite leader sequence present at m.u. 16.8, 19.8 and 26.9 (Berget et al,1977, Akusjarvi & Pettersson,1979). The tripartite leader is spliced to as many as 16 acceptor sites to generate a large number of unique mRNA species. These mRNA's are classified into five families L1 to L5, each with a common poly-A site (Evans et al,1977; Nevins & Darnell,1978). The primary transcript extends to m.u. 99 although the last poly-A signal (for L5) is at 91.3 (Fraser et al,1979).

The L1 region generates 3 major mRNA species with a poly-A signal at 39.3 and 5' cap sites at 29, 30.7 and 34. L1 is also transcribed early in infection (Shaw & Ziff,1980) but in this case the mRNA's have an additional leader from m.u. 21.5-23. This leader has an ORF coding for a hypothetical 15.9K polypeptide (Chow et al,1979). The L1 mRNA's encode two structurally related proteins of 52K and 55K (Miller et al,1980) whose function is unknown. The third mRNA codes for VPIIIa, a 66K protein associated with the hexons (Akusjarvi & Persson,1981).

The 3 major mRNA's from L2 have 5' cap sites at 39.4, 44.1 and 45.9 and a poly-A signal at 50 (Chow et al,1980; Alestrom et al,1984). They code for the structural proteins III, V and VII (Miller et al,1980; Akusjarvi & Pettersson,1981). L2 also contains a fourth ORF which codes for a hypothetical polypeptide of 85 amino-acids. This has been speculated to be the precursor for polypeptide mu, a component of the virus core.

L3 contains three mRNA's with 5' cap sites at 50.1, 52.3 and 60.2 and a poly-A signal at 62.4. These mRNA's code for VPVI-a protein associated with the hexon, the hexon itself (VPII) and a 23K endopeptidase, which is involved in the maturation of VP's VI,VII,VIII and the terminal protein (Bhatti & Weber,1979).

The L4 region contains 4 mRNA's with a poly-A signal at 78.5. Proteins have been assigned to 3 of these mRNA's

(Miller et al,1980). A 100K protein that is thought to function in hexon assembly (Gambke & Deppert,1984) which accumulates to high levels late in infection but is not present in virus particles. A 33K non-structural protein whose function is unknown and VPVIII, a structural protein associated with the hexon.

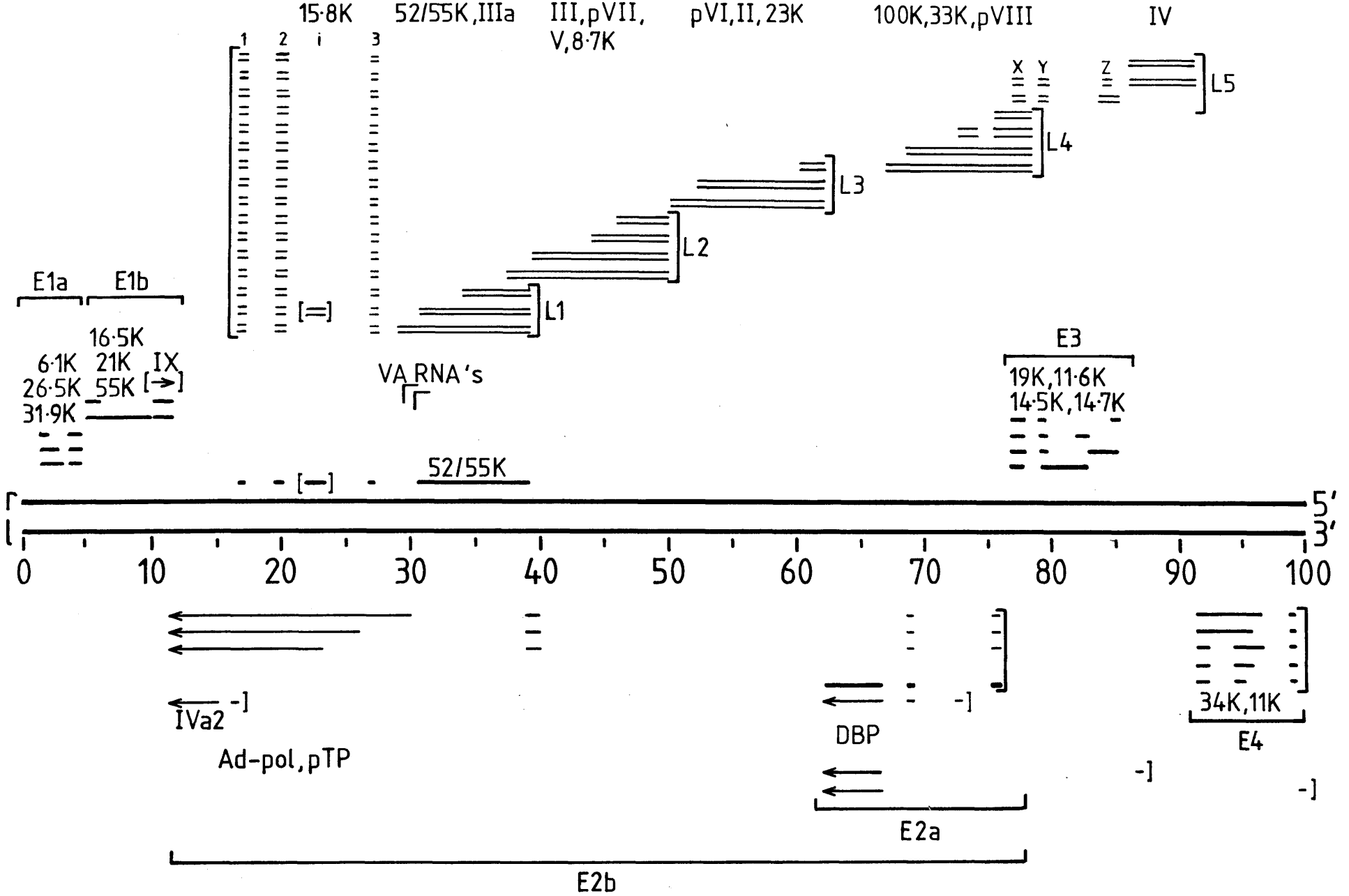
L5 differs from the other 4 late regions in that it only produces one mRNA, which codes for the fibre protein. 30% of fibre mRNA's also have various combinations of three ancillary leader sequences termed x,y and z (Uhlen et al,1982).

Two other mRNA's are transcribed from the adenovirus genome late in infection. These are the virus associated RNA's- VA RNA I and II. They are transcribed from the r-strand from two promoters at 29.5 and 30.2, within the non-coding region of L1 (Akusjarvi et al,1980) and accumulate to high levels (10^5-10^6/cell) late in infection. Unlike other adenoviral mRNA's they are transcribed by RNA polymerase III (Weinmann et al,1976). The VA RNA's are similar to each other, being about 160b.p. long, and are capable of forming similar secondary structures (Akusjarvi et al,1980). VA RNA II appears to be non-essential but VA RNA I has been shown to be required for late translation (Thimmapaya et al.1982).

VA RNA I acts to facilitate late viral translation by maintaining the activity of the eucaryotic initiation factor eIF2 (Schneider et al,1985; Reichel et al,1985). eIF2 is a

Figure 1:1 The adenovirus transcriptional programme.

The genome is divided into 100 map units (m.u.). Transcripts in the top half of the diagram are transcribed from the r-strand, in a left-to-right direction. Transcripts in the bottom half of the diagram are transcribed from the l-strand, in a right-to-left direction. Thick lines represent transcripts produced early in infection (prior to the onset of DNA replication). Thin, arrowed lines represent transcripts produced intermediate in infection (prior to and during DNA replication) and double lines represent transcripts produced late in infection. Gaps in lines represent intervening sequences which are spliced out. Polypeptides are designated by roman numerals in the case of structural proteins (see table 1:1), by their molecular weights (in kilodaltons - K) or by abbreviations in the case of the replication proteins. Adapted from Akusjarvi et al (1986).


15·8K
52/55K,IIIa
III,pVII,
V,8·7K
pVI,II,23K
100K,33K,pVIII
IV
1
2
i
3
X
Y
Z
L5
L4
L3
L2
L1
E1a
E1b
16·5K
6·1K
21K
IX
26·5K
55K
31·9K
VA RNA's
E3
19K,11·6K
14·5K,14·7K
52/55K
5'
3'
0
10
20
30
40
50
60
70
80
90
100
IVa2
Ad-pol,pTP
DBP
34K,11K
E4
E2a
E2b

trimeric protein required for the initiation of eucaryotic translation. It is inactivated by phosphorylation of it's ∝ subunit. This phosphorylation is mediated by a protein kinase that is induced by interferon and inhibited by high concentrations of double-stranded RNA. VA RNA I prevents the activation of this kinase and thus allows translation to continue. It is not known whether VA RNA I acts directly to inhibit the kinase or whether the effect is due to the increased levels of double-stranded RNA within the cell.

1:4 THE STUDY OF ADENOVIRUS DNA REPLICATION

1:4:1 EARLY IN VIVO STUDIES

A large number of workers throughout the 1970's used pulse-labelling and electron microscopic techniques to study the mechanics of adenovirus (Ad) DNA replication in infected cells.

Pulse-labelling techniques were first used to identify and characterize replicative intermediates. A proportion of the labelled DNA extracted from Ad-infected cells after a short pulse with (3H)-thymidine was shown to have a greater sedimentation rate in neutral sucrose gradients than mature viral DNA (Pearson & Hanaualt,1971; van der Eb,1973; Pearson,1975). Replicating DNA had a greater buoyant density in caesium chloride (CsCl) gradients than mature DNA (Pearson & Hanaualt,1971; van der Eb,1973; Pettersson,1973; Robin et al,1973; Pearson,1975) suggesting the presence of single-stranded regions. This was confirmed by showing that

the higher buoyant density was abolished by treatment with S1 nuclease (Pettersson,1973; Robins et al,1973; Pearson,1975) and that binding of replicating DNA to BND-cellulose was characteristic of DNA containing single-stranded regions (van der Eb,1973; Robins et al,1973).

Electron microscopic analysis of replicating DNA molecules revealed the presence of two classes of replicating molecules (van der Eb,1973; Ellens et al,1974; Lechner & Kelly,1977). Duplex molecules of genomic length with between one and four single-stranded branches were termed Type I replicative intermediates. Linear molecules with a single-stranded region extending from one end were termed Type II replicative intermediates. Ellens et al (1974) showed that when replication was synchronized after the release of a hydroxyurea block, branched (Type I) intermediates predominated early and unbranched (Type II) molecules at later times. Lechner & Kelly (1977) showed that terminal transferase would add dTTP residues to the terminus of the nascent strand to form poly(dT) tails. These were visible in electron micrographs at the growing points of Type I molecules and the double to single-strand transitions of Type II molecules. This indicated that both Type I and II synthesis proceeded in a 5' to 3' direction.

The right half of the Ad.2 genome is more easily denatured than the left half, due to the lower percentage of G-C base pairs (Doerfler & Kleinschmidt,1970). Thus after

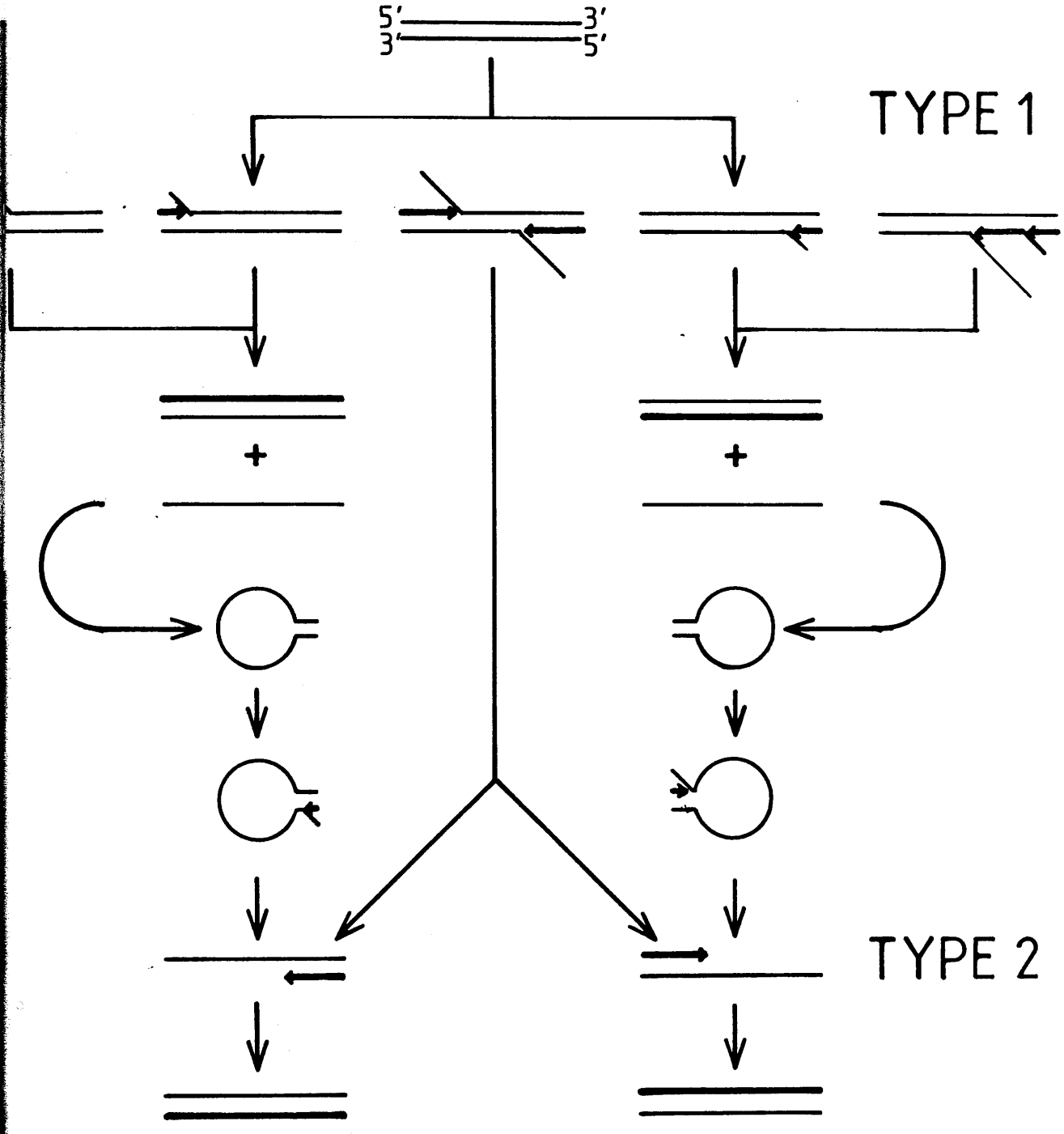
partial denaturation the polarity of Ad.2 DNA molecules is easily determined by electron microscopy. This method was used to determine at which end of the molecules initiation was occurring. Ellen et al (1974) indicated that initiation occurred almost exclusively at the right end but Lechner & Kelly (1977) and Revet & Benichou (1981) subsequently showed that in fact initiation occurred at each end with equal frequency.

On the basis of all this data Lechner & Kelly (1977) proposed a model of Ad. DNA replication. In this model Type I molecules are formed by initiation at one molecular end (or in a few cases at both ends) and elongation of the nascent strand occurred in a 5' to 3' direction with concomitant displacement of the non-template strand. Replication of the displaced strand then occurs by initiation at the 3' terminus and proceeds to the 5' end, thus generating a full-length duplex Ad.2 DNA molecule.

It has been proposed (Daniell,1976) that the ITR's of the displaced strand could associate to give a single-stranded circle with a double-stranded 'panhandle' structure. Incorporation of this proposal into the Lechner & Kelly model suggests that initiation events leading to the formation of both Type I and II molecules are identical. Evidence for panhandle formation comes from later experiments by Stow (1982) and Hay et al (1984). Stow (1982) showed that Ad.2 genomes with deletions within the left ITR would generate progeny virus with two intact ITR's after

Figure 1:2 A model for adenovirus DNA replication.

Taken from Lechner and Kelly (1977). Thin lines represent parental DNA, thick lines represent newly-synthesized DNA. Arrows show the direction of DNA synthesis.


5′
3′
3′
5′
TYPE 1
+
+
TYPE 2

transfection into permissive cells. This evidence was substantiated by the work of Hay et al (1984) using a linearized plasmid containing inverted repeats of a 94b.p. E.coli DNA fragment with a 570 b.p. Ad.2 terminal fragment at one end. After cotransfection of this plasmid into permissive cells with Ad.2 wildtype DNA, progeny molecules an extra 0.5Kb in length were generated. These studies indicate that during adenovirus infection the ITR's at each end do interact, possibly by means of a panhandle structure.

The sites of initiation and termination of replication were determined by studying the distribution of label in Ad.2 restriction fragments after pulse-labelling. After a pulse shorter than the time required for one round of replication, label in mature molecules would be preferentially accumulated in restriction fragments containing the termination sites (Tolun & Pettersson,1975; Schilling et al,1975; Sussenbach & Kruijk,1977). These studies showed that the restriction fragments from the termini of the genome also contained the sites for termination of DNA synthesis. These studies were extended by Ariga & Shimojo (1978) who used various combinations of degradative enzymes to reduce newly replicated DNA to oligonucleotides after pulse-labelling. Their data showed that initiation occurred within 20-30b.p. of the 5' ends and termination occurred at the extreme 3' end.

Using an Ad.5 temperature-sensitive mutant (ts125) defective for initiation of DNA replication Sussenbach &

Kuijk (1978) showed that label was present predominantly in terminal restriction fragments after pulse-labelling and shift down to the permissive temperature. This indicated that initiation occurred at, or near, the genomic termini.

By pulse-labelling it was also possible to determine the rate of chain elongation *in vivo*. Bodnar & Pearson (1980) estimated that one round of DNA replication took 20min. at a temperature of 37°C, corresponding to a rate of appproximately 1700 bases/min.

1:4:2 THE FIRST IN VITRO SYSTEMS: ELONGATION OF DNA CHAINS INITIATED IN VIVO

A number of groups have studied the *in vitro* elongation of nascent DNA chains that were initiated *in vivo*. These experiments were performed both in nuclei isolated from infected cells and in nuclear extracts.

Winnacker (1975) found that in nuclei isolated from infected cells Ad.2 DNA synthesis was discontinuous. After a 30sec. incubation newly synthesized viral DNA was found mainly in a slowly sedimenting (10S) band whereas after 60min. newly synthesized DNA comigrated with mature viral DNA (32S). He also found that in infected cells treated with hydroxyurea, short (10S) Ad.2 DNA fragments could be chased into full-length DNA after removal of the drug. Hydroxyurea reduces the pool of dNTP's in eucaryotic cells by inactivating the B2 subunit of ribonucleotide reductase, suggesting that formation of 10S fragments of Ad.2 DNA in isolated nuclei does not truly represent *in vivo* replication.

A number of methods were used to isolate Ad.2 replication complexes from infected nuclei. These included treatment of nuclei with the polyanion sodium heparin and exclusion chromatography of the products (Yamashita et al,1977), treatment with 0.15-0.3M ammonium sulphate (Brison et al,1977; Kaplan et al,1977; Horwitz et al,1978; Shaw et al,1979) and treatment with 0.27% Sarcosyl followed by collection of the complexes on a glass-fibre disc (Frenkel,1978).

These isolated complexes required Mg^{2+} and dNTP's to synthesize viral DNA (Yamashita et al,1977; Kaplan et al,1977) and were stimulated by ATP (Kaplan et al,1977; Frenkel,1978). Analysis of replicated DNA after incubation with BuDR revealed the presence of hybrid heavy/light (HL) DNA but no heavy/heavy (HH) DNA (Yamashita et al,1977; Brison et al,1977). This indicated that no initiation events were occurring in vitro, only elongation of DNA chains initiated in vivo. Large amounts of the viral 72K DBP were associated with these complexes (Arens et al,1977; Horwitz et al,1978; Shaw et al,1979). Horwitz et al (1978) also showed that the activity of extracts made from H5ts125 (a ts mutant in the 72K DBP) infected cells at the non-permissive temperature was enhanced by addition of purified wild-type DBP, implying a role for this protein in elongation.

Both DNA polymerase alpha (Arens et al,1977; Frenkel,1978) and gamma activity (Arens et al,1977; Brison et al,1977) were detectable in replication complexes, leading to the conclusion that these enzymes were involved in Ad. DNA synthesis.

However systems capable of elongation of DNA chains initiated *in vivo* were of limited use in analysing Ad.DNA replication. Their main drawbacks were that they did not support initiation of replication and contamination with cellular polymerases, obscured the presence of a viral polymerase later discovered (Enomoto *et al*,1981).

1:4:3 THE DEVELOPMENT OF SYSTEMS CAPABLE OF BOTH INITIATION AND ELONGATION IN VITRO

Probably the most important step in the understanding of adenovirus DNA replication was the development by Challberg and Kelly (1979a) of a cell-free extract capable of replicating exogenous templates. The vital component of this system was a salt extract of nuclei isolated from infected cells 22h.p.i. The cells had been treated with hydroxyurea from 2-22h.p.i. to inhibit DNA synthesis but at the same time allowing the synthesis of proteins to continue (Sussenbach & van der Vliet,1973). In the presence of Ad.DNA protein complex (Ad. DNA-prot) purified from virions and all four dNTP's the system catalysed the incorporation of (α32P)dTTP into acid-precipitatible radioactivity. *In vitro* replication proceeded via Type I (branched) intermediates to produce long adenovirus strands (40-60% of full length) hydrogen-bonded to the template strands. The fact that most of the nascent strands were hydrogen bonded to the template ruled out the involvement of a repair-like reaction.

Replication originated at each end of the genome (Challberg & Kelly,1979b; Kaplan *et al*,1979) and was

semi-conservative, as judged by the presence of HL DNA after replication in the presence of BUdR. By separating the two strands of terminal restriction fragments isolated from newly replicated DNA it was shown that the r-strand was preferentially labelled at the right end and the l-strand at the left end. This indicated that strand synthesis was occurring in a 5' to 3' direction.

The system was improved by the finding that *in vitro* replication was supported by a combination of cytosol from infected cells and uninfected nuclear extract (Ikeda *et al*,1980; Lichy *et al*,1981). Horwitz and Ariga (1981) showed that a 25-60% ammonium sulphate fraction of infected cytosol was active in this system. The modified two-component system was then used as a basis for the purification of the proteins involved in Ad.DNA replication. A number of assays were developed to analyse various aspects of the replication process in detail.

Rekosh *et al* (1977) had shown that a 55K protein (TP) was attached to the 5' ends of the genome and they proposed that it acted as a primer in DNA replication. Stillman *et al* (1981) showed that this protein was synthesized as an 80K precursor and was processed late in infection to the mature 55K form. Lichy *et al* (1981) showed that the initiation event involved the formation of a covalent complex between an 80K precursor terminal protein (pTP) and the first nucleotide of the sequence- 5'dCMP. They developed an assay to detect this complex in which uninfected nuclear extract,

infected cytosol and Ad.DNA-prot. were incubated with (α32P)dCTP. Complex was detected as a labelled 80K species on SDS-PAGE. The reaction was template dependent and required Mg^{2+} and ATP. They also showed that the complex could act as a primer for limited elongation at least as far as nucleotide 26, the first G in the sequence. Horwitz and Ariga (1981) developed an assay to look at more extensive elongation. They showed that when uninfected nuclear and infected cytoplasmic extracts were incubated with Xba-I cut Ad.DNA-prot., the terminal fragments of the Ad.DNA-prot were specifically labelled. These assays also showed that a second round of DNA replication could initiate at the termini, thus displacing the 5' strand synthesized during the first round.

These assays, in combination with the original assay of Challberg and Kelly (1979a) which measured the incorporation of labelled dNTP's in acid-precipitatible radioactivity, were used to follow the purification of the components of the adenovirus DNA replication system.

1:5 VIRAL PROTEINS REQUIRED FOR ADENOVIRUS DNA REPLICATION

1:5:1 THE ROLE OF THE TERMINAL PROTEIN AND THE TERMINAL PROTEIN PRECURSOR

As mentioned above the 5' end of newly-synthesized Ad. DNA is covalently linked to an 80K protein (pTP) (Challberg et al,1980) which is processed late in infection to a 55K form (Stillman et al,1981). The role of the pTP in replication was determined by purifying it from infected cells and studying it's replicative activity when combined with purified DBP and uninfected cell extracts.

Ikeda et al (1981) purified a fraction from Ad.2 infected HeLa cell cytosol through 3 columns: DEAE-cellulose, phosphocellulose and denatured-DNA cellulose. In the presence of uninfected nuclear extract and Ad.DBP the fraction catalysed the incorporation of dNMP's into acid-insoluble radioactivity using Ad.DNA-prot. as template. The products formed were covalently linked to an 80K protein at the 5' end and were approximately 25-50% of genomic length; full-length products were only observed in the presence of uninfected cytosol. The uninfected extract could be replaced with a mixture of purified polymerase alpha and beta.

This purification scheme was subsequently improved (Enomoto et al,1982) by the use of phosphate rather than Tris buffer in the purification and by the addition of a

native DNA-cellulose column and a glycerol gradient. The active fraction sedimented in the latter as a single peak of apparent M.W. 180K which separated on SDS-PAGE into two major equimolar bands of 80K and 140K. This fraction alone catalysed the formation of the pTP-dCMP complex showing that it contained pTP. The level of complex formation was stimulated 8-fold by the presence of uninfected nuclear extract (Enomoto et al,1982; Stillman et al,1982). The 80K protein was shown to be pTP by comparison of it's tryptic peptide map with that of TP purified from virions. A DNA polymerase activity which differed from the three cellular polymerases in both it's inhibition profile and chromatographic properties, copurified with the Ad-protein fraction. Lichy et al (1982) and Stillman et al (1982) separated the 80K and 140K proteins on glycerol gradients containing urea and showed that the Ad-specific polymerase activity resided in the 140K protein. Both pTP-dCMP formation and Ad-DNA replication in vitro required both proteins. Ikeda et al (1982) showed that the Ad-protein fraction (pTP-pol complex) would also form pTP-dCMP complex on single-stranded ØX174 DNA templates. pTP-dCMP formed on single strands could elongate to produce chains of about 200 nucleotides in length but further elongation required either E.coli DNA polymerase I or eucaryotic polymerase beta. In contrast to initiation on Ad-DNA-prot, ATP and uninfected nuclear extract were inhibitory, as was DBP.

Rijnders et al (1983a) showed that an antiserum raised against TP purified from virions would inhibit both pTP-dCMP

formation and specific labelling of Ad.DNA-prot. terminal fragments. Van Bergen et al (1983) showed that a cloned Ad.2 terminal fragment was specifically replicated by infected nuclear extract when the plasmids were linearized to expose the origin. This replication was also inhibited by anti-TP serum showing that inhibition was due to an effect on pTP, not the mature TP attached to the template. Rijnders et al (1983b) also showed that the anti-TP serum did not affect the polymerase activity of purified pTP-pol. These data indicate that functional pTP is required for initiation of Ad.DNA replication.

The binding of purified pTP-pol complex to duplex DNA was studied by Rijnders et al (1983b). The affinity of binding of adenoviral DNA was higher than that to calf thymus or pBR322 DNA. By analysing the binding to plasmids containing sequences from the Ad. DNA terminus, a sequence between nucleotides 9-22, which is perfectly conserved amongst all human adenoviruses (Stillman et al,1982), was implicated as a specific binding site for pTP-pol.

Van Bergen et al (1983), Tamanoi and Stillman (1982) and Guggenheimer et al (1984a) reported that protein-free DNA is less efficient as a template for initiation and elongation than Ad.DNA-prot. In addition, initiation did not always occur at the Ad. genomic terminus in protein-free templates- any G residue between 4-8 b.p. 3' of the conserved sequence could be used. Tamanoi and Stillman (1982) found that crude extracts would only catalyse

pTP-dCMP formation with Ad.DNA-prot. whereas partially purified extracts would utilize both Ad.DNA-prot. and protein-free templates. These data suggest several roles for the mature TP on viral DNA; TP could have a role in preventing exonuclease digestion of Ad. DNA during infection (Dunsworth-Browne et al,1980). Alternatively it could prevent aberrant internal starts. Robinson et al (1973) first demonstrated that Ad.DNA-prot. purified from virions by disruption with guanidinium-HCl would form duplex circles that were converted to linear molecules by protease treatment. This led to the suggestion that the TP's on mature virion DNA could interact. Such an interaction could occur between the TP on the genome and the incoming pTP, facilitating the initiation reaction. The greater efficiency of Ad.DNA-prot implies that TP has a role in the initiation of replication, it could possibly facilitate the unwinding of the helix (van Bergen et al,1983).

1:5:2 THE ADENOVIRUS-SPECIFIC DNA POLYMERASE

As mentioned in the previous section Enomoto et al (1981) purified a fraction from infected cytosol that was capable of catalysing the formation of a pTP-dCMP complex alone. The two major components of this fraction were an 80K protein (shown to be the pTP) and a 140K protein. The fraction also contained an unique DNA polymerase activity that could be distinguished from cellular DNA polymerases. Lichy et al (1982) separated this fraction on a glycerol gradient containing urea and showed that the 140K protein contained the DNA polymerase activity.

Stillman et al (1982) used a different approach to purify the DNA polymerase; They showed that nuclear extracts from cells infected with an Ad.5 ts mutant (ts149) which contained a mutation in the N-complementation group could not initiate DNA replication in vitro at the non-permissive temperature. They purified the DNA polymerase by it's ability to complement ts1[illegible] extracts for initiation and synthesis at the non-permissive temperature. They also showed that the DNA polymerase fraction contained two major proteins of 140K and 80K which co-sedimented through a glycerol gradient. They separated the two proteins on a glycerol-urea gradient and showed that the DNA polymerase activity coincided with the purified 140K protein, confirming the results obtained by Lichy et al (1982). pTP-dCMP formation and Ad. specific replication required both proteins but DNA polymerase activity of the 140K did not require pTP. No other DNA polymerases could function in the pTP-dCMP reaction; those tried included eucaryotic DNA polymerases alpha, beta, and gamma from various sources, bacteriophage T4 DNA polymerase, and E.coli DNA polymerases I, II and III (Lichy et al,1982,1983).

Both the pTP-dCMP synthesizing and DNA polymerase activities of the 140K protein were inactivated by 5mM N-ethylmaleimide or by heating at 55^{o}C for 15min. The Ad. DNA polymerase activity resembled eucaryotic DNA polymerase alpha both in it's use of nicked duplex (activated) DNA as template and in it's sensitivity to ara-CTP and NaCl. It differed from DNA polymerase alpha in it's resistance to

aphidicolin and it's sensitivity to ddTTP. However the replication of Ad.DNA both *in vivo* (Longiaru *et al*,1979) and *in vitro* (Nagata *et al*,1983) is inhibited by aphidicolin. The *in vitro* studies show that whereas in the presence of aphidicolin pTP-dCMP formation and elongation to the 26th nucleotide are unaffected (Lichy *et al*,1981), the products of more extensive elongation accumulate as DNA chains approximately 10Kb. in length (Nagata *et al*,1983). Thus the effect of aphidicolin on the Ad. DNA polymerase is only apparent after extensive elongation.

The Ad. DNA polymerase also resembled DNA polymerase alpha in it's use of homopolymer template:primers. Both enzymes were most active on activated DNA but would also use poly(dC):oligo(dG), poly(dC):oligo(rI), poly(dT):oligo(dA) and poly(dT):oligo(rA). Neither enzyme would use templates containing poly(dG), poly(dI) or poly(dGC). The relative efficiencies of utilization of these templates varied bwtween the two enzymes (Field *et al*,1984).

The purified Ad. DNA polymerase contained a 3'-5' exonuclease that was 10-fold more active on single-stranded than on duplex DNA (Field *et al*,1984). The exonuclease activity of the Ad. DNA polymerase is in common with other viral and procaryotic DNA polymerases including the vaccinia virus DNA polymerase, E.coli DNA polymerases I, II and III and T4 DNA polymerase. It is thought that this exonuclease activity increases the fidelity of replication by removing misincorporated nucleotides (Brown *et al*,1982). Reha-Krantz

and Bessman (1977) supported this model by showing that mutations which reduce the 3'-5' exonuclease activity of bacteriophage T4 DNA polymerase also increase the rate of spontaneous mutations and vice-versa.

The Ad. DNA polymerase exonuclease activity is inhibited up to 7-fold by the Ad. 72K DBP. This inhibition was shown to be due to a direct effect upon the DNA polymerase, rather than by binding to and protection of DNA (Lindenbaum et al,1986). The DBP also has several other profound effects on the DNA polymerase; DNA synthesis on poly(dT):oligo(dA) is stimulated 10-100 fold by the presence of DBP and can yield nascent chains of up to 30kb. in length, indicating that in the presence of DBP the Ad. DNA polymerase is a highly processive enzyme (Field et al,1984). This effect is template-specific as shown by the finding that synthesis on poly(dA):oligo(dT) was not affected by the presence of DBP. Lindenbaum et al (1986) studied the effect of DBP on the thermal stability of the Ad.polymerase. In the presence of an excess of DBP, the DNA polymerase activity was stable at 45°C for at least 2h., both in the presence and absence of DNA. DNA (in the form of poly(dT):oligo(dA)) was highly destabilizing in the absence of DBP; after 2h. at 45°C only 1% of the DNA polymerase activity was still present.

Despite the functional interactions between the two proteins it has not been possible to isolate DNA polymerase-DBP complexes (Lindenbaum et al,1986). The

presence of pTP reduced by two-thirds the amount of DNA synthesized by Ad. DNA polymerase and DBP using poly(dT):oligo(dA) as template. This suggests that the tight binding of pTP to the DNA polymerase inhibits elongation. The role of DBP *in vivo* may be to dissociate the complex and allow the DNA polymerase to move along the genome.

1:5:3 THE 72,000 DALTON MOLECULAR WEIGHT VIRAL DNA-BINDING PROTEIN

The first report of an adenovirus-infected cell specific DNA-binding protein was by van der Vliet and Levine (1973). They found two polypeptide species of apparent M.W.'s 72K and 48K which bound specifically to single-stranded DNA but not to duplex DNA. It was subsequently shown (Levinson & Levine,1977) that the 48K polypeptide was a breakdown product of the 72K.

Fowlkes *et al* (1979) and Schechter *et al* (1980) showed that in fact the 72K DNA-binding protein (DBP) bound to both duplex and single-stranded DNA. Melting studies using polynucleotide duplexes revealed that the protein stabilized the helix. Binding to duplex DNA was specific for the termini of a linear molecule, closed circular molecules were not bound to any great extent. However Kedinger *et al* (1978) showed by electron microscopic analysis of replicative intermediates isolated from infected cells that only the single-stranded regions were thickly covered with DBP, suggesting that *in vivo* the binding of DBP to DNA differs from that observed *in vitro*.

Van der Vliet *et al* (1977) and Kedinger *et al* (1978) first suggested that DBP was involved in elongation of nascent DNA chains when they observed that an anti-DBP antibody slowed down the rate of chain elongation. The role of DBP in DNA replication *in vitro* has been studied using the temperature-sensitive mutants of Ad.5: H5*ts*125 and H5*ts*107. Both these mutants carry base substitutions within the DBP gene and are defective for DNA replication at the non-permissive temperature. Nuclear extracts of H5*ts*125 infected cells were defective for full-length Ad.DNA synthesis *in vitro* on both endogenous (Horwitz,1978) and exogenous (Kaplan *et al*,1979) templates. In both cases this activity was restored by the addition of purified wildtype DBP. However DBP did not appear to be required for initiation *in vitro*: Lichy *et al* (1981) showed that addition of purified wildtype DBP had no effect on the formation of a pTPdCMP complex *in vitro* by crude infected cell extracts. Friefeld *et al* (1983) showed that extracts derived from H5*ts*125 infected cells could support wildtype levels of pTP-dCMP complex formation at the non-permissive temperature. Nagata *et al* (1983a) subsequently showed that pTP-dCMP formation by purified pTP-pol was inhibited by DBP, this inhibition was overcome by a host factor (see Section 4:5:1). Formation of the pTP.26mer limited elongation product by H5*ts*125 extracts at the non-permissive temperature was reduced and could be restored to 75% of wildtype levels by DBP. Friefeld *et al* (1983) also showed that purified *ts*125 DBP would complement wildtype pTP-pol and uninfected nuclear extract in assays to detect specific

labelling of Ad.DNA-prot. terminal restriction fragments at 30°C, but not at 38°C. These results confirmed that functional DBP was required for elongation.

Partial chymotryptic digestion of DBP produces a 44K C-terminal and a 26K N-terminal fragment (Klein et al,1979). The C-terminus is highly conserved- 60-80% at the amino-acid level- between adenoviruses of various subgroups (Kitchingman,1985). The N-terminus, which unlike the C-terminus is phosphorylated (Klein et al,1979), is less highly conserved- 30-40% at the amino-acid level.

The 44K C-terminal fragment was shown to complement ts125 extracts for Ad.DNA replication in vitro (Ariga et al,1980). This fragment has recently been crystallized (Tsernoglou et al,1984,1985) and the elucidation of it's three-dimensional structure will be extremely informative. Furthermore a 34K C-terminal fragment derived by more extensive chymotryptic digestion was also active (Friefeld et al,1983). Thus the unphosphorylated C-terminus of the DBP contains all the requirements for DNA replication. However the requirements for DNA-binding and DNA replication may not coincide: Recently Krevolin and Horwitz (1987) found that the intact ts107 DBP exhibited wildtype binding to DNA but was temperature sensitive for Ad.DNA replication. However the 34K fragment of the ts107 DBP was temperature sensitive both for DNA replication and DNA-binding, indicating that the amino terminal domain of the DBP, whilst not affecting the role of the DBP in DNA replication, is involved in binding of the protein to DNA.

1:6 CELLULAR FACTORS INVOLVED IN ADENOVIRUS DNA REPLICATION

1:6:1 NUCLEAR FACTOR I

Lichy et al (1981,1982) and Ikeda et al (1981) demonstrated that uninfected nuclear extract was required for optimal initiation and elongation in the presence of either infected cytosol or purified pTP-pol and DBP. Nagata et al (1982) purified an activity from uninfected HeLa cell nuclei that enhanced both initiation and elongation. The active fraction contained no detectable nuclease, topoisomerase, ATPase, RNA or DNA polymerase activities and contained a single major polypeptide of 47K, as revealed by SDS-PAGE (Nagata et al,1982). This protein was termed nuclear factor I (NFI).

The activity of NFI in stimulating initiation was affected by DBP. In the absence of DBP, NFI stimulated pTP-dCMP formation by purified pTP-pol 2-fold. The presence of DBP alone was inhibitory, but the further addition of NFI overcame this inhibition, giving a 6-fold increase in pTP-dCMP formation. ATP further enhanced the reaction 2-fold. NFI also enhanced the formation of the pTP-26mer limited elongation product (Nagata et al,1983a), and stimulated incorporation of labelled dTTP into acid-precipitatible radioactivity using Ad.DNA-prot. as template 5-fold (Lichy et al,1983). However these effects are likely to be an indirect effect of increased initiation efficiency.

Several groups looked at the specific binding of NFI to the adenovirus origin. DNase I protection experiments showed that NFI protected a region between nucleotides 17-48 (Nagata et al,1983b; Rawlins et al,1984; Leegwater et al,1985). Binding did not require ATP or Mg^{2+} and was resistant to high ionic strength. Again DBP had an effect on NFI activity; Nagata et al (1983b) showed that DBP would protect an entire 451b.p. terminal Ad.5 fragment against digestion with DNase I, but in the presence of both NFI and DBP a small amount of DNase I digestion was observed at either edge of the region protected by NFI alone. This suggested that NFI displaces DBP from it's binding site at the origin. This data also correlates with the finding that DBP inhibited initiation in the absence of NFI and that NFI could overcome this inhibition (Nagata et al,1982).

The region bound by NFI has two-fold rotational symmetry and therefore has the potential to form a hairpin structure. Methylation protection experiments (De Vries et al,1987) demonstrated that NFI protected symmetrically arranged G residues from methylation by DMS. DMS methylates G residues in the major groove, suggesting that NFI binds to that side of the helix. NFI thus appears to be analogous to the lambda repressor and cro proteins which also protect symmetrical G residues from methylation. Both repressor and cro bind to the same three 17b.p. repeats that also have the potential to form hairpin structures (Ptashne et al,1980). It has been suggested (Nagata et al,1983b) that this hairpin structure may be required for NFI binding. However Leegwater

et al (1985) subsequently showed that the 16b.p. region between nucleotides 25 to 40 was both necessary and sufficient for NFI binding. This excludes most of the possible hairpin structure, thus arguing against hairpin formation as a requirement for NFI binding. It seems likely however, given the comparison with the repressor/cro situation that NFI binds as a dimer. The radius of an NFI molecule, calculated from it's molecular weight (47K) is 27A (Nagata et al,1983b). The length of the 32b.p. protected sequence is 110A suggesting that two molecules interact with each binding site. Thus it is possible that a monomer of NFI could bind to half of the recognition sequence.

The role of NFI binding in initiation of Ad.DNA replication was demonstated using plasmids containing deletions within the Ad.2 terminal sequence. Tamanoi and Stillman (1982) showed that a plasmid containing an Ad.2 terminal fragment (the origin of replication), linearized such that the origin was placed at a molecular end, was active as a template for initiation of replication. Tamanoi and Stillman (1983) made sets of deletions extending both towards and away from the Ad. terminus. Their initial studies showed that the terminal 20b.p. of the genome were sufficient for efficient initiation, although in some cases the addition of sequences up to 35b.p. enhanced the efficiency slightly. These results were reiterated by Challberg and Rawlins (1984) who showed that the terminal 18b.p. constituted a functional origin of replication. However these studies were carried out using high

concentrations of template DNA which masked the effect of NFI. Lally et al (1984), using crude Ad.2 nuclear extracts, showed that a plasmid containing the terminal 38b.p. was three times more active than one containing the terminal 21b.p. It was only when purified NFI was used in these assays, and the concentration of template DNA was reduced, that the requirement for the NFI binding site on the template became more apparent.

Guggenheimer et al (1984a) showed that pTP-dCMP formation by purified pTP-pol and DBP on plasmids containing at least the terminal 48b.p. of the Ad. genome was stimulated 5-7 fold by NFI. These plasmids were also efficiently bound by NFI. Plasmids containing between 18 and 35b.p., although exhibiting a low level of activity, were not stimulated by, and did not bind to, NFI. Plasmids containing deletions extending either 7 or 15b.p. from the terminus were completely inactive in initiation although they bound efficiently to NFI. Rawlins et al (1984) showed that, whereas the terminal 18b.p. of the genome were sufficient for pTP-dCMP formation, addition of sequences up to 67b.p. greatly enhanced the efficiency of the reaction. In addition they showed that deletions that abolished enhancement also abolished binding of NFI.

NFI sites have also been found in a wide variety of eucaryotic DNA sources: Borgmeyer et al (1984) described a protein which bound to the sequence 5'TGGCA(N3)TGCCA upstream of the chicken lysozyme gene. Nowock et al (1985)

subsequently showed that this TGGCA-binding protein bound to sites in the MMTV LTR, the BK virus enhancer and the adenovirus origin of replication, tentatively identifying it as NFI. Leegwater et al (1986) showed that the TGGCA protein and NFI were one and the same by extensive functional comparisons. NFI sites were also shown to be present upstream of the IgM heavy chain gene (Hennighausen et al,1985) and within the promoter and first intron of the human cytomegalovirus major immediate-early gene (Hennighausen & Fleckenstein,1986), the expression of which is entirely dependent on host factors.

Gronostajski et al (1984,1985) isolated and analysed NFI sites from HeLa cell DNA. They estimated that one site was present per 100Kb. of DNA and derived a consensus sequence 5'TGG(N6-7)GCCAA for the binding site. Gronostajski (1986) then analysed the precise requirements for NFI binding using oligonucleotides. He showed that the spacer between the TGG and GCCAA motifs had to be 6 nucleotides. 4,5,7 or 8 nucleotide spacers were not bound by NFI. By synthesizing oligonucleotides with random sequences within the spacer and the 3b.p. either side of the conserved motifs he showed that the precise sequence within these regions was of minor importance for binding. De Vries et al (1985) showed that point mutations within the conserved motifs reduced binding to NFI. However reduced binding efficiency did not always result in a correspondingly reduced ability to enhance Ad. initiation. In addition a double point mutant at positions 22 and 23 on the Ad.2 ITR (outside the

conserved motifs) still bound NFI but was negative for enhancement of replication. These results indicated that binding of NFI and stimulation of Ad. initiation are not identical functions of the protein.

De Vries et al (1987) used various techniques such as methylation protection, methylation and ethylation interference and substitution with 5-BrdU to show that NFI interacted with pairs of symmetrically arranged G residues within the binding site. Using computer graphics they demonstrated that the contact points were located in two blocks showing dyad symmetry to each other. They also demonstrated that, as a consequence of this dyad symmetry, the NFI site enhanced Ad. initiation in either orientation (relative to the conserved sequence).

Adhya et al (1986) demonstrated that initiation in vitro was unaffected when the NFI site within the Ad. origin was replaced with a cellular NFI site. The HeLa NFI site was also active in either orientation but the distance from the conserved sequence was important. The centre to centre distance between the conserved sequence and the NFI site had to be less than 23b.p. The presence of two or more NFI sites did not further enhance the efficiency of initiation.

A number of physical studies have recently been performed on NFI. Rosenfeld and Kelly (1986) and Diffley and Stillman (1986) both purified NFI by affinity chromatography. Both groups constructed plasmids containing

many tandem repeats of the NFI binding site, linked them to cellulose and used the matrix as the final stage in NFI purification. Both groups however made different observations as to the size of NFI. Nagata et al (1982) had shown that NFI was a single polypeptide of M.W. 47K. Rosenfeld and Kelly observed several bands between 52-66K and a minor band at 32K on SDS-PAGE. These bands had similar polypeptide patterns after limited proteolysis. Diffley and Stillman however observed a major band at 160K on SDS-PAGE. They suggested that NFI consisted of two globular domains with a linker peptide that was highly sensitive to proteolysis. One domain (47-66K) was sufficient for DNA binding and stimulating Ad. replication. By analysing the stoichiometry of binding they predicted that 2 molecules of a 160K polypeptide bound to one binding site.

These studies show that there are two domains within the Ad.2 ITR that are required for initiation of replication. The region from nucleotides 1-18 is absolutely required, deletions into this region abolish initiation (Guggenheimer et al,1984a). Base changes within the highly conserved region from nucleotides 9-18, suggested as the binding site for the pTP-pol complex (Rijnders et al,1983a), also abolished initiation (Tamanoi & Stillman,1983; Challberg & Rawlins,1984). The presence of the region between nucleotides 19-48 greatly enhanced the efficiency of initiation, and this has been shown to be due to the binding of NFI. The requirements for these two regions have also been examined in in vivo replication systems (see Section 4:6).

1:6:2 NUCLEAR FACTOR II

In the presence of pTP-pol, DBP and NFI the products of DNA synthesis on Ad.DNA-prot. are only 25% of the size of full-length DNA (Nagata et al,1983; Lichy et al,1983). Full-length synthesis required the presence of a second factor from uninfected HeLa nuclei, termed nuclear factor II (NFII). NFII was purified through 4 columns by Nagata et al (1983). SDS-PAGE of purified NFII revealed the presence of 2 major bands of 14.5K and 15.5K, and a minor band at 30.5K. NFII preparations contained no detectable nuclease, ATPase, RNA or DNA polymerase activities but did contain Type I topoisomerase activity. Type I topoisomerases from eucaryotic, but not procaryotic, sources could substitute for NFII in Ad.DNA replication. Both eucaryotic topoisomerase and NFII were dependent on the presence of NFI for the formation of full-length Ad.DNA products.

1:6:3 NUCLEAR FACTOR III

Recently a third host cell factor affecting Ad.DNA replication has been identified. Nuclear factor III (NFIII) was purified from uninfected HeLa nuclei (Pruijn et al,1986) and was shown to stimulate the initiation efficiency 4-6 fold in the presence of NFI. Replication of a plasmid containing the terminal 73b.p. of the Ad.2 ITR was stimulated by both NFI and NFIII, whereas a plasmid containing only 40b.p., or one containing the complete ITR with a point mutation at position 46, were only stimulated by NFI. DNase I footprinting analysis revealed that NFIII bound to the ITR between nucleotides 36 and 54, overlapping

the NFI site. Despite this overlap both proteins could bind simultaneously. Methylation protection experiments showed that NFI protected a G-residue at position 36 and NFIII protected a G-residue at position 42, suggesting that the proteins bound to opposite sides of the helix. NFIII only stimulated initiation in the presence of NFI, but the binding of NFIII to the origin was unaffected by NFI.

The NFIII consensus sequence- 5'P_uP_yGNTAATGAP_uATNC has several homologies in eucaryotic DNA sequences. These include the histone H2B promoter, human and mouse immunoglobulin V_K and V_H promoters, upstream from the human interferon gene and upstream from the U1 and U2 snRNA genes in various species. It seems likely therefore that, as is the case for NFI, the virus is using a bona-fide cellular function to aid its replication. Whether NFIII has a role in Ad.DNA replication *in vivo* remains to be seen. Hay and McDougall (1986) (see Section 1:7) constructed viruses lacking parts of the left ITR and demonstrated that deletion of the NFIII site had no detectable effect on virus replication. In contrast deletion of the NFI site produced a non-infectious virus genome.

1:6:4 FACTOR pL: A HOST PROTEIN REQUIRED FOR REPLICATION OF TERMINAL PROTEIN-FREE TEMPLATES

Guggenheimer *et al* (1984b,c) purified a protein from uninfected HeLa nuclei that was specifically required for initiation on plasmid-borne origins of replication. Factor pL had no effect on the replication of Ad.DNA prot. but was

required for the replication of pronase treated Ad.DNA. Gel filtration studies indicated that factor pL had an apparent M.W. of 44K. The purified protein contained no RNA or DNA polymerase, double-stranded endonuclease or topoisomerase activities. Factor pL did not inhibit the binding of NFI to the origin of replication; initiation in the presence of pL was stimulated 8-10 fold by the presence of NFI.

Guggenheimer et al (1984c) suggested that pL displaces a DNA strand at the terminus, allowing the pTP-pol complex to initiate. Alternatively it is possible that pL digests the 5'- end of the genome, thus allowing the pTP-pol direct access to the 3'- end of the template strand. This would explain why pL has no effect on the replication of Ad.DNA-prot., as it is known that the TP protects the 5'-end of Ad.DNA-prot. from 5'-3' exonuclease activity. The fact that pL has no effect on the replication of Ad.DNA-prot. suggests that it does not play a role in replication in vivo.

1:7 STUDIES TO ELUCIDATE THE SEQUENCE REQUIREMENTS FOR ADENOVIRUS DNA REPLICATION IN VIVO.

Several groups of workers have recently looked at the sequence requirements for replication in vivo. Hay et al (1984) demonstrated that plasmids containing two inverted adenoviral termini were replicated in vivo after co-transfection with wildtype viral DNA to provide replication functions. Efficient replication required the

ITR's to be present at the ends of a linear molecule, although a low level of replication was observed from internal origins. These replicons underwent multiple rounds of replication and reached high copy numbers (greater than 10,000 copies per cell). Newly replicated molecules were attached to protein, suggesting that they were replicated by the same mechanism as Ad.DNA.

Deletions were introduced into the ITR by Bal31 exonuclease and these molecules used to construct plasmids containing two inverted copies of partially-deleted ITR's (Hay,1985a). Analysis of the replication of these molecules demonstrated that the terminal 45b.p. were the minimum requirement for replication *in vivo*; molecules containing 36b.p. or less were not replicated to any detectable extent. Thus the NFI binding site, which enhances replication *in vitro* up to 10-fold, is absolutely required *in vivo*. This finding was repeated by Bernstein *et al* (1986) who also found that point mutations within the TGG and GCCAA motifs of the NFI binding site or within the 9-18 conserved sequence reduced replication at least 10-fold. Interestingly they found that point mutations at nucleotides 4 or 57 also reduced replication 5-10-fold. Nucleotide 57 may form part of the recognition site for NFIII, although it is not protected from DNase I digestion by NFIII (Pruijn *et al*,1986b).

Wang and Pearson (1985) modified this approach by constructing linear recombinants containing inverted repeats of pUC8 sequences with one copy of an Ad. terminal fragment at one end. After cotransfection with wildtype helper DNA these recombinants were replicated provided that they contained at least the terminal 30b.p. Molecules containing either 30 or 36b.p., lacking the complete NFI site, showed consistently less than 50% of wildtype replication levels. Wildtype levels were obtained by plasmids containing at least 67b.p. The products of replication were larger than the input molecules because they contained a copy of the Ad.DNA sequence at each end. This fact provides further evidence for the involvement of a panhandle structure in Ad.DNA replication as proposed by Daniell (1976) (see Section 1:4:1).

Although there are some minor discrepancies in the data presented by different laboratories these could be due to slight differences in the assays used. A general consensus seems to be that the NFI site is more important *in vivo* than *in vitro*. A possible reason for this is given by De Vries *et al* (1985). They demonstrated that the stimulation of replication by NFI was directly proportional to the concentration of DBP in the reaction, and that in the absence of NFI replication was inhibited by DBP. Thus in the presence of high concentrations of DBP, such as might be expected to occur in the nuclei of infected cells, NFI would appear to be absolutely required.

Hay and McDougall (1986) constructed Ad.2 genomes with deletions within the left ITR. They demonstrated that the terminal 45b.p. sequence, which was defined as the minimal Ad. origin *in vivo* by the plasmid replication system, also fulfilled all the requirements for replication of genomic Ad.DNA *in vivo*. Thus adenovirus genomes containing only 45b.p. of the left ITR showed wildtype growth kinetics, plaque morphology, viral DNA synthesis and Ela mRNA synthesis. This raises the question of the function of the remaining ITR sequences. These include several copies of the sequence GGGNGGAG which is conserved amongst the adenoviruses and is present in the SV40 origin of replication (Stillman *et al*,1982a). It could be that the copy of these sequences at the right end of the genome is sufficient for expression of their function.

1:8 A MODEL FOR THE MOLECULAR MECHANISM OF ADENOVIRAL DNA REPLICATION

Evidence from other *in vitro* DNA replication systems (eg. *E.coli oriC*, see section 1:11) has shown that initiation of replication involves the formation of a large nucleoprotein complex at the origin. Formation of this complex will require both protein-protein and protein-nucleic acid interactions. It seems likely that this situation also applies to adenovirus DNA replication.

Like these other systems initiation of adenoviral DNA replication requires a specific DNA sequence. In adenovirus

this sequence is located at the termini of the genome. Clearly therefore the first stage in initiation must involve interactions between cellular sequence-specific DNA binding proteins and the terminus of the adenovirus genome. Two cellular proteins have so far been shown to bind to specific sequences in the Ad.2 ITR; Nuclear Factor I (NFI; Nagata et al,1983b) and Nuclear Factor III (NFIII; Pruijn et al,1986). Both these proteins are thought to be involved in eucaryotic transcriptional activation (Jones et al,1987). A third sequence-specific DNA-binding protein which binds to the terminal 15b.p. of the Ad.2 ITR has recently been identified (Kelly, personal communication). However this protein did not appear to enhance pTP-dCMP formation under the conditions employed in vitro so may not have a role in initiation. The multiple protein:DNA interactions at the origin may also involve the Ad. 72K DNA-binding protein (DBP), which binds with high affinity to single-stranded DNA but also binds non-specifically to double-stranded DNA, and the terminal protein (TP) attached to the 5' ends of the genome. Evidence for the role of TP in forming the initiation complex comes from data showing that DNA attached to TP is more efficient than DNA lacking TP in acting as a template for initiation (Tamanoi & Stillman,1982; van Bergen et al,1983; Challberg & Rawlins,1984).

Secondly the ends of the genome are unwound with the concomitant hydrolysis of ATP. This process is likely to involve the site-specific DNA-binding proteins (unwinding must also occur during transcription so they may perform the

same function in two different processes) and may also involve the DBP. Binding of the pTP-pol complex to the single-stranded terminus may then occur. This interaction may involve either the non-specific binding of pTP-pol to single-stranded DNA, or recognition of a specific nucleoprotein structure at the origin. It may also involve the terminal protein, DBP and cellular sequence-specific DNA-binding proteins.

There is evidence to suggest that binding of pTP-pol to the termini occurs prior to unwinding (Rijnders et al,1983a). However both binding of pTP-pol and unwinding must occur before the final stage- transfer of dCMP on to pTP by the action of the Ad. DNA polymerase. The transfer reaction will not occur in the absence of a template suggesting that binding of pTP-pol to the terminal nucleo-protein complex enables the polymerase to catalyse the transfer of a dCMP residue to the pTP. The formation of a pTP-dCMP complex is stimulated by cellular factors including NFI and NFIII. This stimulation could occur at any of the stages of initiation, although the precise mechanism of stimulation is not known. There may be other factors involved in initiation: Kelly (personal communication) suggests that another fraction has to be added to NFI and NFIII to attain levels of pTP-dCMP synthesis comparable to those seen with crude extracts.

The pTP-dCMP complex then acts as a primer for DNA synthesis by the Ad. polymerase. Ad.DBP is known to increase

the processivity of the polymerase (Lindenbaum et al,1986) so it is likely that these two proteins act in concert to elongate the nascent strand. None of the other proteins known to be involved in Ad. DNA replication possess either helicase or DNA-dependent ATPase activities so the DBP and the polymerase must also act to unwind the helix. It is not known whether any of the sequence-specific DNA-binding proteins are involved in elongation. After synthesis of DNA chains approximately 10Kb in length further elongation requires the action of eucaryotic topoisomerase I. This activity is probably required to relax positive superhelical turns generated near the replication fork to allow it's translocation (Champoux,1978).

One round of DNA replication results in the displacement of a complete single-strand. This strand may form a panhandle structure, in which the ITR's associate to form a duplex region attached to a single-stranded loop. In this case initiation of replication on the displaced strand is mechanistically identical to initiation on the fully duplex genome. In the absence of panhandle formation initiation must occur on a single-stranded template. Presumably initiation on a single-strand would not involve sequence-specific DNA-binding proteins as there is no precident for the recognition of a specific single-stranded DNA sequence by a sequence-specific DNA-binding protein. Thus initiation would involve recognition of the terminus of a linear, single-stranded DNA molecule. The fact that initiation on single-stranded DNA would be relatively non-specific argues for the involvement of panhandle formation in adenovirus DNA replication.

1:9 PAPOVAVIRUS DNA REPLICATION IN VITRO

An *in vitro* system for the cell-free replication of exogenously added Simian Virus 40 (SV40) DNA was developed by Ariga and Sugano (1983). In the presence of uninfected HeLa nuclear extract and SV40-infected Cos-1 cell cytoplasm an exogenously added DNA template, either intact SV40 DNA or a plasmid containing the SV40 origin region (from map units 0.67-0.71), was replicated semiconservatively. Li and Kelly (1984) improved on this system by using a soluble extract of whole SV40-infected Cos-1 cells. Their system was more active than that of Ariga and Sugano (1983), synthesizing 320pmoles of DNA/ug of template as opposed to 9pmoles/ug of template. Both groups modified the system to include uninfected cell extract and purified SV40 large-T antigen (T-Ag).

The system was absolutely dependent on the presence of T-Ag and the origin region on the DNA template: In the absence of T-Ag, or after the addition of anti-T antiserum (Ariga & Sugano,1983), only a non-specific repair reaction was observed. Templates lacking the SV40 origin, or containing a 4b.p. deletion within the origin that abolished replication *in vivo* (Li & Kelly,1984), were not replicated. Further experiments (Ariga,1984) showed that the system would replicate plasmids containing the human *Alu* family repeat sequences. However Li and Kelly (1985) subsequently showed that their system would not use these *Alu* repeat sequences as origins of replication. It would use origins of

replication isolated from the human papovaviruses BKV and JCV at an efficiency of 10-20% of the SV40 origin. In addition the reaction required ATP and $MgCl_2$.

Replication was shown to be semiconservative and to proceed bidirectionally from the origin region (Ariga & Sugano,1983; Stillman & Gluzman,1985). Stillman and Gluzman (1985) also found that after replication in the presence of BUdR 12% of the products migrated as fully substituted heavy-heavy DNA on CsCl gradients, indicating that multiple rounds of replication had occurred. The products of the reaction in the absence of uninfected nuclear extract were relaxed, circular DNA molecules. In the presence of nuclear extract these molecules were negatively supercoiled.

SV40 DNA replication in vivo is semidiscontinuous; synthesis on the forward strand is continuous whereas synthesis on the lagging strand involves the formation of 4-5S Okazaki fragments from heterologous 9-10 nucleotide RNA primers (Hay et al,1984a). There is no concrete evidence as yet for the involvement of RNA primers in SV40 replication in vitro. Incubation of extracts with RNase A (Ariga & Sugano,1983; Wobbe et al,1985) reduced DNA synthesis by 60-70% but RNase H, which digests the RNA strand of DNA-RNA hybrids, had no effect. The effect of RNase A was studied in more detail by Wobbe et al (1986). Stillman and Gluzman (1985) had shown that when HeLa cytosol, T-Ag, SV40 origin DNA and dNTP's were incubated there was a lag phase of 10-20min. before elongation occurred. Wobbe et al (1986)

showed that this lag phase could be abolished by incubating the components without dNTP's for 10-30min. Addition of RNase A to the pre-elongation mixture inhibited subsequent elongation, but RNase A had no effect on the level of dNMP incorporation if added directly to the elongation reaction. However RNase A did prevent the occurrence of multiple rounds of replication.

1:9:1 THE ROLE OF LARGE-T ANTIGEN

Tegtmeyer (1972) first demonstrated that the product of SV40 gene A (the tumour or T-antigen) was required for DNA replication *in vivo*. T-antigen (T-Ag) has since been shown to be the only virally-encoded protein required for DNA replication both *in vivo* (Tooze,1980) and *in vitro* (Ariga & Sugano,1983; Li & Kelly,1984).

T-Ag is a multi-functional protein required for transcriptional control, initiation of DNA replication and cellular transformation. The protein binds non-specifically to DNA with higher affinity for single-stranded than duplex DNA. It also exhibits specific DNA-binding properties, binding to three sites located within or adjacent to the SV40 origin of replication (Tjian,1978). *In vivo* it appears that part of site I and all of site III are dispensable for initiation of replication (Subramanian & Shenk,1978). Site II is a perfect 27b.p. palindrome and together with part of site I (which consists of a 17b.p. palindrome and part of a 15b.p. palindrome) and an A-T rich region adjacent to site II constitutes a 65b.p. minimal origin *in vivo* (Subramanian

& Shenk,1978; Gutai & Nathans,1978; Myers & Tjian,1980; Bergsma *et al*,1982). In addition to these properties T-Ag has a DNA-dependent ATPase activity and a recently discovered DNA helicase activity (Stahl *et al*,1986).

Stillman *et al* (1985) demonstrated that the sequence requirements for replication *in vitro* were the same as those *in vivo*. Sequences to the early side of the 65b.p. minimal origin affected the efficiency of replication but were not absolutely required whereas sequences on the late side, including the three 21b.p. repeats, were not required. Stillman *et al* (1985) also looked at replication *in vitro* with purified mutant T-Ag's. None of the mutants tested had any replicative activity, even when they retained the wildtype ATPase and origin-binding activities. However they would all compete with purified wildtype T-Ag for replication. Clearly binding of T-Ag to the origin is not sufficient for DNA replication. In this respect T-Ag is similar to the Ad.2 72K DNA-binding protein (DBP) (see Section 1:5:3). Krevolin and Horwitz (1987) observed that a temperature-sensitive mutant in the DBP (*ts*107) bound to DNA like the wildtype protein but was *ts* for replication. Presumably the DNA helicase activity of T-Ag is important in DNA replication.

1:9:2 THE ROLE OF THE CELLULAR DNA POLYMERASE ALPHA:DNA PRIMASE COMPLEX

Studies of SV40 DNA replication *in vivo* with inhibitors implied that cellular polymerase alpha was the only polymerase involved (Krokan *et al*,1979). Several lines of evidence suggest that this is also the case *in vitro*: The initial experiments (Ariga & Sugano,1983; Li & Kelly,1984) showed that replication *in vitro* was inhibited by aphidicolin, an inhibitor of DNA polymerase alpha, and N-ethyl-maleimide, an inhibitor of polymerases alpha and beta. In contrast ddTTP, an inhibitor of polymerases beta and gamma, had no effect on replication. Wobbe *et al* (1985) demonstrated that treatment of extracts with anti-polymerase alpha antiserum abolished their replicative ability.

Murakami *et al* (1986a) purified polymerase alpha together with it's tightly associated DNA primase activity and showed that both activities were absolutely required for SV40 DNA replication *in vitro*: HeLa cell extracts that were depleted of polymerase alpha/primase complex by passage through an immuno-affinity column were inactive. This activity was restored by the addition of purified polymerase alpha/primase complex from either HeLa or Cos-1 cells. These cell types are permissive for SV40 replication *in vivo*. However the addition of polymerase/primase complex from cells that are non-permissive for SV40 replication (mouse or calf thymus) did not restore activity. Mouse cell extracts which did not support SV40 DNA replication *in vitro* were complemented by complex purified from HeLa cells.

These studies were extended (Murakami et al,1986b) to show that in vitro replication of polyoma virus (Py) DNA also required the presence of polymerase alpha/primase from permissive cells, in this case mouse cells. They also demonstrated that Py T-Ag could not function in SV40 DNA replication and vice-versa. The fact that both the T-Ag and the polymerase alpha/primase complex requirements are virus-specific suggests that there is a specific interaction between these proteins. These studies show that firstly the host-cell polymerase alpha/primase complex is of prime importance in papovavirus DNA replication and secondly that this enzyme complex is important in determining host-cell permissivity.

1:9:3 OTHER HOST-CELL REQUIREMENTS

Wobbe et al (1986) separated the HeLa cytosol required for SV40 DNA replication in vitro into two ammonium sulphate fractions- 0-40% (AS40) and 40-65% (AS65). AS40 had low activity that was stimulated 5-fold by AS65, which was by itself inactive. No DNA synthesis was detected when both fractions were treated with micrococcal nuclease. However if one fraction (either AS40 or AS65) was untreated the rate of synthesis was undiminished. This suggested that a nucleic acid component required for replication was present in both fractions.

Ariga (1986) fractionated an uninfected mouse cell extract into three components which, in conjunction with SV40-infected Cos-1 cell cytosol, would efficiently

replicate plasmid DNA containing the SV40 origin region. One fraction contained the polymerase alpha/primase complex, and the second contained a DNA topoisomerase activity. Presumably topoisomerase activity is required both for unwinding during chain elongation and for segregation of progeny molecules. The third fraction contained a DNA-binding activity (termed factor I) which bound specifically to the SV40 origin. Factor I alone enhanced the initiation of replication by SV40-infected Cos-1 cytosol as judged by the preponderance of 4-5S Okazaki fragments. Full-length synthesis required the addition of both the other fractions.

Stillman (personal communication) recently purified a 36K polypeptide that was required for efficient SV40 DNA replication *in vitro*. The protein sedimented as a dimer, had no DNA-binding properties and was highly hydrophobic and acidic in nature. He demonstrated that the protein was functionally homologous to three other previously identified proteins: Cyclin, a cell-cycle regulatory protein not present in quiescent cells, proliferating cell nuclear antigen and the polymerase delta auxiliary protein (see Section 1:12:2; Tan *et al*,1986) which increases the processivity of polymerase delta. This result suggests that polymerase delta may have a role to play in SV40 DNA replication.

Although the analysis of SV40 DNA replication *in vitro* has yielded valuable information as to the DNA and protein

requirements there is clearly much work to be done before our level of understanding of the system approaches that of the Adenovirus system. This knowledge will only be obtained by the purification and characterization of all the cellular proteins involved.

1:10 ϕ29 DNA REPLICATION IN VITRO

ϕ29 is a bacteriophage of Bacillus subtilis. It is discussed here because it's mode of replication is essentially the same as adenovirus. The genome of the phage is a linear, double-stranded DNA molecule of 18Kb. Like adenovirus it has a protein covalently linked via a phosphodiester bond from a serine residue to a 5'-dAMP, the terminal nucleotide of the genome. The protein is the product of gene 3 and has an apparent M.W. on SDS-PAGE of between 28-31K (Salas et al,1978). ϕ29 has an inverted repeat of 6b.p. at each terminus, the sequence of which is 5'-AAAGTA (Yoshikawa et al,1981).

The presence of terminal proteins and inverted terminal repeats (ITR's) is a common feature to several bacteriophage species including the B.subtilis phages ϕ15, Nf, M2Y and GA1, Streptococcus pneumoniae phages Cp-1, Cp-5 and Cp-7, and an E.coli phage PRD1 (Escarmis et al,1985). In addition the linear plasmid pSLA2 from Streptomyces rochai and the linear mitochondrial DNA's S1 and S2 from cytoplasmic male-sterile maize share these features. The lengths of the ITR's vary from 6b.p. in ϕ29 to 614b.p. in

pSLA2. The sequence AAAGTA is also well-conserved between the phages and the mitochondrial DNA termini (Escarmis et al,1985)

1:10:1 INITIATION OF Ø29 DNA REPLICATION

Initiation of Ø29 DNA replication involves a protein-priming mechanism analogous to that seen in the adenovirus system. Incubation of Ø29-infected B.subtilis cell extracts with (α^{32}p)dATP results in the transfer of dAMP to the terminal protein p3, this complex is then detected by SDS-PAGE and autoradiography (Penalva & Salas,1982). In the presence of dTTP, dGTP and ddCTP the p3-dAMP complex was elongated to the first C residue in the sequence:9b.p. from the left end or 12b.p. from the right end of the genome. The reaction required Mg^{2+}, ATP and the presence of Ø29 DNA-p3 complex, deproteinized DNA was not active (Penalva & Salas,1982). Garcia et al (1984) showed that purified terminal restriction fragments of the DNA-p3 complex would support initiation provided that they were at least 26b.p. in length. A fragment of 10b.p. was inactive, although activity could be restored by ligation of an unspecified sequence, suggesting a topological requirement for a longer DNA fragment rather than a specific sequence requirement. Crude extracts would not support the formation of complex on protein-free templates, however the subsequent use of purified proteins (see Section 1:10:2) (Gutierrez et al,1986) overcame this problem.

1:10:2 THE PROTEIN REQUIREMENTS FOR Ø29 DNA REPLICATION

The Ø29 genes 2, 3, 5, 6 and 17 have been implicated in viral DNA replication *in vivo* by genetic analysis (Salas,1983). However as yet only the products of genes 2, 3 and 6 have been shown to be involved in replication *in vitro*: As mentioned previously the product of gene 3 is the 28-31K terminal protein p3. Extracts from *B.subtilis* infected with a gene 3 mutant *sus*3 did not support p3-dAMP formation indicating the absolute requirement for p3 in replication.

The product of gene 2 is a 68K DNA polymerase (p2) which was shown to be absolutely required for initiation of replication *in vitro* (Blanco & Salas,1984; Watabe *et al*,1984). p2 and p3 copurify through a phosphocellulose column and on a glycerol gradient but appear to dissociate on a dnDNA cellulose column (Watabe *et al*,1984). This suggests that the terminal protein and the polymerase are associated but not so intimately as the corresponding adenovirus proteins. As in the adenovirus system the terminal protein (p3) and the polymerase (p2) are sufficient to catalyse initiation (Watabe *et al*,1984; Blanco & Salas,1984; Gutierrez *et al*,1986). This reaction was strongly stimulated by the presence of an extract from uninfected *B.subtilis* or *E.coli* cells (Blanco & Salas,1984), suggesting that, as for adenovirus, host factors are required for efficient initiation. Unlike initiation with crude extracts (Penalva & Salas,1982) ATP was not required

and in fact was inhibitory (Blanco & Salas,1985), Mg^{2+} was still required.

Unlike the adenovirus system p2 and p3 were sufficient to produce full-length ϕ29 DNA, no topoisomerase activity was required (Blanco & Salas,1985). However the rate of elongation was low, suggesting that host factors might be required for efficient elongation. Unusually ATP was not required for the elongation reaction.

Pastrana et al (1985) demonstrated that the purified product of gene 6 stimulated initiation in vitro up to 5-fold. Their preliminary results suggested that p6 was a single-stranded DNA binding protein. In vivo experiments with a ts mutant in gene 6 suggested that it was involved in elongation. If this is the case p6 would appear to be analogous to the adenovirus 72K DBP which stimulates both initiation and elongation.

The question of whether the mechanisms of ϕ29 and adenovirus DNA replication are completely analogous will only be answered when both systems have been completely reconstituted from purified proteins. Clearly both viruses require host cell factors for efficient initiation and elongation, It seems likely that these host factors will have similar functions given the striking similarities between the two replication systems observed so far. The existence of both procaryotic and eucaryotic viruses that utilize this unusual mode of DNA replication implies a form of divergent evolution.

1:11 INITIATION OF ESCHERICHIA COLI CHROMOSOMAL DNA REPLICATION

The replication of the Escherichia coli (E.coli) chromosome has been extensively studied over the last 20 years mainly by the group of Kornberg at Stanford University. Initiation of DNA replication takes place at a single site on the chromosome between map units 82 and 83 (Yasuda & Hirota,1977) termed oriC. OriC has been defined as a 245b.p. region (Oka et al,1980) consisting of two types of sequence elements (Asada et al,1982); repeated sequences constituting protein binding sites and spacer sequences serving to position the binding sites correctly. Tabata et al (1983) demonstrated that although oriC provides all the sequence requirements for initiation, DNA replication initiates at a site adjacent to oriC and proceeds bi-directionally.

Baker et al (1986) showed that initiation is a multi-step process that starts with the formation of a pre-priming complex. This complex consists of oriC DNA and the products of genes dnaA, dnaB and dnaC. The reaction requires ATP and Mg^{2+} and is stimulated by the presence of the protein HU- a double-stranded DNA-binding protein. The oriC sequence must be present on a supercoiled molecule (Funnell et al,1986), relaxed molecules are only active when treated with gyrase. DnaA was shown to be a site-specific DNA-binding protein which bound to a 9b.p. 'dnaA box', present throughout the genome with 4 copies located in oriC.

DNase I protection experiments (Fuller et al,1984) demonstrated that dnaA binding to oriC is highly co-operative; 20-30 protein molecules bind and protect 250b.p. from DNase I digestion. The dnaB and dnaC proteins interact to form a complex consisting of 6 dnaC molecules and one dnaB hexamer (Kobori & Kornberg,1982). This then interacts with the dnaA-oriC complex to form the complete pre-priming complex.

The next stage in initiation involves the unwinding of the DNA at oriC (Baker et al,1986). This requires the presence of the gyrase protein (a Type I topoisomerase) and SSB (the E.coli single-stranded DNA binding protein). In addition unwinding is dependent on a DNA helicase activity which is supplied by dnaB. Replication on the pre-primed template is initiated by the synthesis of a short RNA primer. Priming is mediated by either the primase, RNA polymerase or a combination of the two proteins (Ogawa et al,1985; van der Ende et al,1985). At low levels of the auxiliary proteins HU, RNase H and topoisomerase I (which act as specificity factors by inhibiting DNA synthesis that is not dependent on dnaA and oriC) primase alone could prime the template. However in the presence of high levels of these proteins primase alone is inactive and the addition of RNA polymerase is required for efficient priming. RNA polymerase would prime but only at very low efficiency. Priming is dependent on intact pre-priming complex; oriC DNA that has been pre-primed and then deproteinized does not support priming and subsequent elongation.

Elongation is performed by DNA polymerase III holoenzyme, a large and complex multi-subunit enzyme. Addition of this enzyme and dNTP's results in a burst of DNA synthesis, the rate of which slowly declines (Funnell et al,1986). In addition to the holoenzyme dnaB, dnaC and gyrase are required for elongation. Funnell et al (1986) also showed that the production of complete daughter molecules requires the action of DNA polymerase I and ligase. This process is stimulated by RNase H. The combined action of these three enzymes results in the excision of primers, filling in and ligation of the DNA strands to produce covalently closed circles, which are then supercoiled by the gyrase present throughout the reaction.

1:12 EUCARYOTIC DNA POLYMERASES

In this section I wish to briefly describe the various eucaryotic DNA polymerases that have so far been identified. I shall deal only with the DNA-dependent DNA polymerases, the RNA-dependent DNA polymerases (reverse transcriptases) encoded by the retroviruses will not be discussed.

1:12:1 OTHER VIRUS-CODED DNA POLYMERASES

Apart from adenoviruses there are several other DNA viruses with large genomes that encode, or induce in infected cells, an unique DNA polymerase. These include poxviruses, herpesviruses and baculoviruses. In addition the Dane particle of Hepatitis B virus and the Killham Rat parvovirus particle contain an endogenous DNA polymerase

activity that differs from the cellular DNA polymerases.

Poxvirus specific DNA polymerase activity was first demonstrated by Jungwirth and Joklik (1965) who detected an altered and enhanced DNA polymerase activity in HeLa cells after infection with vaccinia virus. The vaccinia virus specific enzyme was purified to homogeneity by Challberg and Englund (1979a) and consisted of a single polypeptide of apparent M.W. 110-115K. In common with other viral and procaryotic DNA polymerases it co-purified with a 3'-5' exonuclease activity which was most active on single-stranded DNA. The DNA polymerase activity required a divalent cation and was inhibited by aphidicolin, sodium chloride and N-ethylmaleimide. Maximal activity was observed on gapped duplex (activated) DNA, as is the case for the Ad. DNA polymerase. However the vaccinia enzyme would also elongate on a primed single-stranded DNA template, albeit inefficiently. Elongation was impeded by the presence of hairpin loops (Challberg & Englund,1979b).

Keir et al (1966) showed that there were alterations in cellular DNA polymerase activity after infection with herpes simplex virus (HSV). HSV DNA polymerase activity was separated from other viral proteins by Powell and Purifoy (1977). The major component of the purified DNA polymerase fraction was a polypeptide of apparent M.W. 150K. In addition there was a minor polypeptide of 54K. The DNA polymerase activity was inhibited by aphidicolin, phosphonoacetic acid and anti-serum to HSV-infected cells

but, unlike the other viral DNA polymerases discussed had optimal activity at high salt concentrations (75-150mM). Knopf (1979) showed that in common with the other viral DNA polymerases HSV DNA polymerase co-purified with a 3'-5' exonuclease activity. All members of the herpesvirus group have subsequently been shown to encode their own DNA polymerases (Weissbach,1979).

DNA polymerase activity is also stimulated in baculovirus infected cells although as yet this activity has not been shown to be virus coded (Kelly,1981). An infected cell specific DNA polymerase activity was purified (Wang & Kelly,1983) and shown to consist of a single polypeptide with apparent M.W. 126K. The activity was inhibited by aphidicolin and bromovinyl-deoxyuridine triphosphate (BVdU triphosphate) but not by BVdU. The authors did not look for exonuclease activity in the study.

In addition to these DNA viruses with large genomes the core of the Hepatitis B virus (HBV) Dane particle contains an associated DNA polymerase activity (Feitelson,1985). It is not known yet whether this DNA polymerase is virus or host-cell coded, however there is an open-reading frame on the 3.2Kb Dane particle DNA molecule which has the capacity to encode a 90K polypeptide, the approximate size of the DNA polymerase molecule. There is only one other example of the association of a specific DNA polymerase activity with a DNA virus possessing a small genome; Particles of the Killham Rat parvovirus were

reported to contain an endogenous DNA polymerase activity (Salzman,1971; Salzman et al,1978). However this finding has not been repeated and DNA polymerase activity has not been found in association with any other parvoviruses (Bates et al,1976) suggesting that this result was due to contamination with a cellular DNA polymerase.

It seems a reasonable assumption that viruses with small genomes will have to rely on host-cell functions to a much greater extent than those with larger genomes. Thus DNA viruses of small genome size are likely to use host-cell polymerases for replication of their genomes, as has been shown to be the case for papovaviruses both in vivo (Krokan et al,1979) and in vitro (Murakami et al,1986). If HBV is shown to encode it's own DNA polymerase it will be unique amongst small DNA viruses. Conversely it is highly probable that the baculovirus induced DNA polymerase will be shown to be virus-coded, given the large size of the baculovirus genome (130Kb.: Guarino & Summers,1986).

1:12:2 CELLULAR DNA POLYMERASES

It is now accepted that there are four different classes of cellular DNA polymerases: alpha, beta, gamma and delta (Kornberg,1982). They can be distinguished by means of four different criteria (Weissbach,1977); sensitivity to various inhibitors, selectivity in template usage, chromatographic properties, molecular weight and more recently by the use of monoclonal antibodies.

DNA POLYMERASE ALPHA: DNA polymerase alpha is the principal enzyme involved in eucaryotic chromosomal replication (Kornberg,1982; Hubscher,1984). It is ubiquitously present in the nuclei of actively growing cells in many species ranging from humans to yeast (Weissbach,1977) and comprises up to 90% of the intracellular DNA polymerase activity. As with it's procaryotic counterpart DNA polymerase III, DNA polymerase alpha exists as a multi-subunit holoenzyme (Hubscher,1984). The polymerizing subunit has an apparent M.W. of between 110-185K, depending on the species. In Drosophila melanogaster (the best characterized holoenzyme) the polymerizing subunit is 182K and is termed the alpha subunit. In addition the Drosophila holoenzyme has two other subunits- beta and gamma- of M.W.'s 60K and 50K.

Apart from the polymerizing subunit the holoenzyme contains a primase activity (Conaway & Lehman,1982) capable of synthesizing the short RNA fragments required to initiate DNA synthesis on the discontinuous (lagging) strand. In Drosophila the primase activity appears to be part of the beta and gamma subunits whereas in calf thymus both DNA polymerase and primase activities locate on a single subunit (Hubscher,1984).

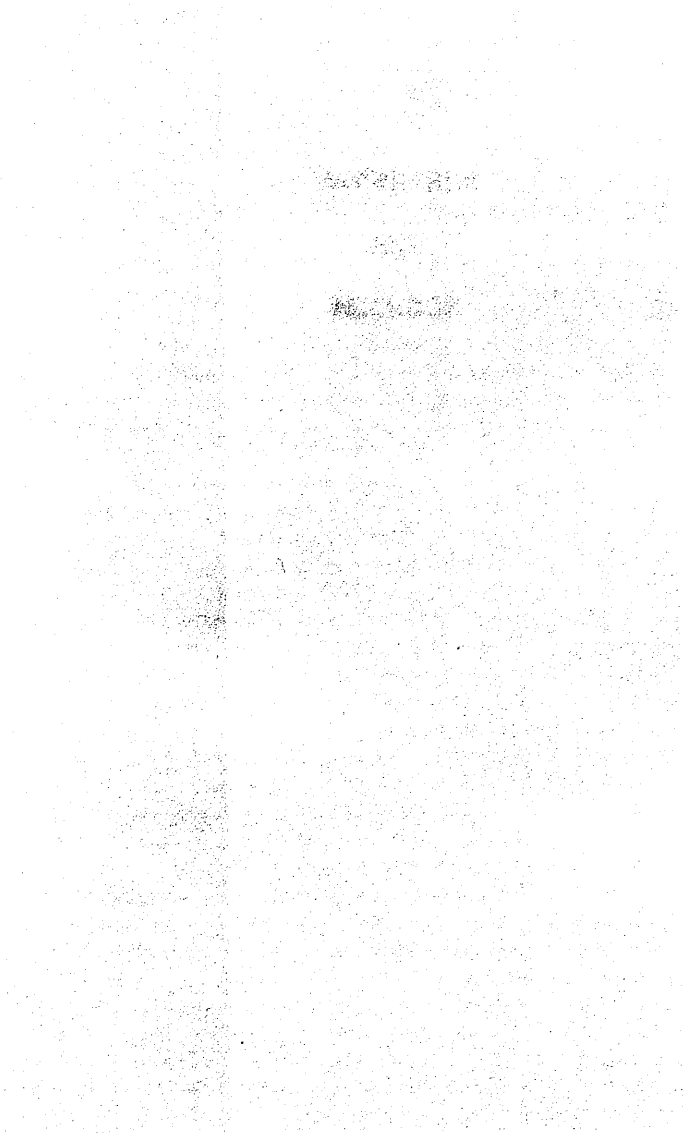
DNA POLYMERASE BETA: DNA polymerase beta is a low molecular weight DNA polymerase that is the predominant activity in quiescent or G_o-phase cells (Weissbach,1977), whose main function appears to be DNA repair (Hubscher,1984). The mammalian enzyme is a single polypeptide of 45K apparent

M.W. whereas the avian enzyme is present as a 27K form in the cytoplasm and a 50K dimer in the nucleus (Weissbach,1977). This dimerization may be involved in activation of the enzyme.

DNA POLYMERASE GAMMA: DNA polymerase gamma is associated with mitochondrial DNA replication (Hubscher,1984). Multiple forms of the enzyme exist, with reported M.W.'s from 100K-300K. Unlike the other cellular DNA polymerases gamma will copy synthetic ribohomopolymers such as poly(A). However it will not copy natural RNA and is not inhibited by antibodies to viral reverse transcriptases, suggesting that there is little if any structural or evolutionary relationship between these enzymes.

DNA POLYMERASE DELTA: DNA polymerase delta was the last cellular DNA polymerase to be discovered and is the only one to possess a 3'-5' exonuclease or 'proof-reading' activity (Byrne et al,1976), in common with most procaryotic and viral DNA polymerases. The core enzyme has an apparent M.W. of 173K and consists of two subunits of 125K and 48K (Lee et al,1984). Recently two high M.W. forms of DNA polymerase delta (240K and 290K) which also possess primase activity have been discovered (Crute et al,1986). In addition a DNA polymerase delta auxiliary protein has recently been characterized (Tan et al,1986). This protein is a dimer of 37K subunits which has no detectable enzymatic activity itself but increases the ability of DNA polymerase delta to copy primed single-stranded templates by 100-1000

fold. It has no effect on the ability of DNA polymerase delta to copy gapped duplex DNA. Tan et al (1986) showed that although the auxiliary protein does not bind to DNA it increases the affinity of DNA polymerase delta for poly(dA)/oligo(dT).



MATERIALS

AND

METHODS

2:1 VIRUS STOCKS

One Adenovirus serotype was used in this study. Adenovirus type 4 was originally obtained from W.C.Russell (St.Andrews) and consisted of a stock freeze-dried by M.R.Hilleman in 1957. The stock used in these experiments had been taken through 2 rounds of plaque purification before large scale production.

2:2 CELLS

The 293 cell line (Graham et al,1977) was used to prepare and titrate virus stocks. Nuclear and cytoplasmic extracts were made from the Hela WS cell line.

2:3 TISSUE CULTURE MEDIUM

Cells were cultured in Glasgow-modified Eagles medium supplemented with 100 units/ml of penicillin, 100ug/ml of streptomycin, 0.002% (w/v) phenol red and either 5% (Hela WS cells) or 10% (293 cells) foetal calf serum. All components were obtained from Gibco Ltd., Trident House, Renfrew Road, Paisley, PA3 4EF, Scotland.

2:4 BACTERIAL HOSTS FOR THE PROPAGATION OF PLASMIDS AND BACTERIOPHAGE M13mp18

All plasmids used in this study were propagated in the E.coli K12 strains DH1 (F^-, recA1, endA1, gyrA96, thi-1, hsdR17 [r_K-,m_K+], supE44, $\lambda-$; Maniatis et al,1982) and C600 (F^-, thi-1, thr-1, leuB6, tonA21, supE44, $\lambda-$; Maniatis et al,1982).

Bacteriophage M13mp18 was propagated in E.coli K12 strain JM101 (lacpro^{del}, supE thi, F'traD36, proAB, laclq, ZM15^{del}; Messing,1979).

2:5 BACTERIAL CULTURE MEDIUM

Bacteria were propagated in Luria Broth (LB) : 10g/l Difco bacto-peptone and 5g/l yeast extract in 177mM NaCl pH 7.5 prior to sterilization. Agar plate cultures contained LB supplemented with 1.5% agarose. Culture media were supplemented where appropriate with 100ug/ml ampicillin.

2:6 BUFFER SOLUTIONS

The following buffers were routinely used:

TE: 10mM Tris/HCl pH8, 1mM EDTA.

TEN: 10mM Tris/HCl pH8.3, 1mM EDTA, 100mM NaCl.

TBE: 89mM Tris/HCl pH8, 89mM Boric acid, 1mM EDTA.

Running Buffer: 6.32g/l Tris, 4g/l Glycine, 1g/l SDS.

Resolving Gel Buffer: 1.5M Tris, 0.4% SDS, pH 8.9.

Stacking Gel Buffer: 0.42M Tris, 0.4% SDS, pH6.7.

Gel Fix: 47:47:6 Methanol:Water:Glacial Acetic Acid.

Hypotonic Buffer: 20mM Hepes/KOH pH7.5, 5mM KCl, 0.5mM $MgCl_2$, 0.5mM DTT.

Nuclear Resuspension Buffer: 10mM Tris/HCl pH7.5, 10%(w/v) sucrose.

Tris/Saline: 140mM NaCl, 30mM KCl, 0.28mM Na_2HPO_4, 1mg/ml dextrose, 25mM Tris/HCl pH7.4, 0.0015% phenol red, supplemented by 100units/ml penicillin, 100ug/ml streptomycin.

Buffer A: 25mM Tris/HCl pH7.5, 1mM DTT, 1mM EDTA, 20% glycerol, 0.01% Nonidet P-40.

2:7 RESTRICTION ENDONUCLEASES

All restriction endonucleases were obtained either from Northumbria Biologicals Ltd. or Boehringer Mannheim, Ltd. and used as specified by the manufacturers.

2:8 REAGENTS

Reagents were obtained from the following sources:

Sigma Ltd., Fancy Road, Poole, Dorset, BH17 7NH, England:

Agarose, aphidicolin, N-2-Hydroxyethylpiperizine-N'-2-ethanesulphonic acid (Hepes), Gelatin, Lysozyme, Dithiothreitol (DTT), Dimethylsulphoxide (DMSO), ara-CTP, dideoxythymidine triphosphate (ddTTP), dideoxyguanidine triphosphate (ddGTP), all four deoxynucleoside triphosphates (dNTP's), Phenylmethylsulphonylfluoride (PMSF), Ethidium bromide, Hydroxyurea.

Koch-Light Ltd., Haverhill, Suffolk, England:

Acrylamide, Boric acid, Dimethylsulphoxide (DMSO), Glycerol, Trichloro-acetic acid (TCA), Tetra-sodium pyro-phosphate, Caesium chloride.

May & Baker Ltd., Dagenham, Essex, England:

Glacial acetic acid, chloroform, concentrated hydrochloric acid.

Boehringer Corporation Ltd., Bell Lane, Lewes, East Sussex, England:

Tris(hydroxymethyl)aminomethane (Tris).

Biorad, Richmond, California:

Ammonium persulphate (APS), Coomassie Brilliant Blue R250.

James Burroughs Ltd., 70 Eastways Industrial Park, Witham, Essex, England.

Absolute Ethanol.

All other reagents were of Analar grade and were obtained from BDH Chemicals Ltd., Broom Road, Poole, Dorset, BH12 4NN.

2:9 VIRUS PURIFICATION

293 cells in 80oz roller bottles were infected with 10 pfu/cell of virus in a volume of 5ml Tris/Saline supplemented with 2% calf serum per roller bottle. Virus was adsorbed for 90min. at 37°C and 100ml of medium with 2% calf serum was added per bottle. Cells were incubated at 37°C until complete CPE was observed, routinely about 48h. post-infection. Cells were shaken into the medium and pelleted by centrifugation at 2000rpm for 10min. in an MSE Coolspin centrifuge. The cell pellet was washed once in Tris/Saline ,repelleted and resuspended in 1ml Tris/Saline per roller bottle .

The cell suspension was frozen and thawed 3 times and virus was extracted by shaking with an equal volume of Arcton equilibrated with Tris/Saline. The aqueous phase was recovered, layered on top of a discontinuous

glycerol/caesium chloride gradient (2ml 40% glycerol in 10mM Tris/HCl pH7.9, 1mM EDTA, 3ml 0.32g/ml CsCl in 10mM Tris/HCl pH7.9, 2ml 0.418g/ml CsCl in 10mM Tris/HCl pH7.9) and centrifuged for 90min. at 25000rpm and 4°C in a Sorvall Tst-41 rotor. The lower opalescent virus band was recovered by bottom puncture, dialysed overnight against 4l TE, and an equal volume of sterile glycerol was added. Virus stocks were stored at -20°C.

2:10 PREPARATION OF VIRAL CORES

An aliquot (50 ul) of banded and dialysed virions was treated with 0.5% sodium deoxycholate (DOC) for 45sec. at 56°C and cooled on ice for 2-3 min. The DOC treated virions were layered on top of a 4ml 15% -60% glycerol gradient and centrifuged for 90min. at 30,000rpm at 4°C (Goding & Russell,1983). Glycerol gradients were prepared as step gradients using 15%, 30%, 45% and 60% glycerol solutions in 5mM Tris/HCl pH7.8 and allowed to stand at room temperature for 4h. prior to use. 0.5ml fractions were collected from the bottom of the tube and core containing fractions identified by measuring absorbance at 260nm and 280nm. Aliquots of each fraction were treated with Proteinase K (100 ug/ml) and 0.5% SDS for 2h/37°C and analysed by agarose gel electrophoresis for the presence of adenovirus genomic length DNA. Core containing fractions were stored at 4°C and were stable for up to 2 months.

2:11 PREPARATION OF VIRAL DNA-PROTEIN COMPLEX

An aliquot (250 ul) of banded and dialysed virions was incubated for 5min. on ice with an equal volume of 8M Guanidinium Hydrochloride (GuHCl) in the presence of 0.5mM PMSF. Treated virions were then loaded on to a 10ml column of Sepharose 4B equilibrated with TEN containing 4M GuHCl. The column was developed with TEN containing 4M GuHCl and 0.5ml fractions were collected. Fractions containing DNA-protein complex were identified by measuring absorbance at 260nm., pooled and dialysed against TE. An equal volume of glycerol was added and they were stored at -20°C.

2:12 PREPARATION OF VIRAL DNA

Banded virions dialysed against TE were treated with 100ug/ml Proteinase K in the presence of 0.25% SDS for 2h. at 55°C. The viral DNA was extracted once with an equal volume of a 1:1 mixture of phenol/chloroform, once with an equal volume of chloroform and concentrated by ethanol precipitation. For long-term storage viral DNA was kept as an ethanol precipitate at -20°C, otherwise it was resuspended in sterile TE and stored at 4°C.

2:13 PREPARATION OF SUPERCOILED PLASMID DNA

Bacteria transformed with the appropriate plasmid were grown to saturation in 350ml cultures of LB supplemented with 100ug/ml of ampicillin. The cells were pelleted by centrifugation at 6000rpm for 5min. in a Sorvall GS3 rotor. Cells were resuspended in 5ml of 25% (w/v) sucrose, 50mM Tris/HCl pH8 and 1ml of a freshly prepared 20mg/ml solution

of lysozyme in 0.25M Tris/HCl pH8 was added. The cells were incubated on ice for 5min., 1ml of 0.5M EDTA was added, followed by a further 5min. incubation on ice. 8ml of a solution containing 0.2% (v/v) Triton-X 100, 50mM Tris/HCl pH8, 62.5mM EDTA was rapidly added and the tube contents mixed by inversion. After incubation on ice for 15min. the mixture was centrifuged for 30min. at 20,000rpm., 0°C in a Sorvall SS34 rotor.

1g of caesium chloride (CsCl) per ml of supernatant and 0.8ml of a 10mg/ml solution of ethidium bromide (EtBr) was added and the solution was centrifuged at 45,000rpm. for 20h. at 20°C in a Dupont TV850 rotor. The band of supercoiled plasmid DNA was recovered using an 18G needle and recentrifuged at 40,000rpm. in a Dupont TV865 rotor. Supercoiled plasmid DNA was separated from unbound EtBr and CsCl by gel filtration through a column of Sepharose 4B CL. Bound EtBr was removed by ion exchange chromatography through a column of Dowex AG50W. The DNA was ethanol precipitated, resuspended in TE and treated with 50ug/ml RNase A for 30min. at 37°C, followed by treatment with 100ug/ml Proteinase K for 60min. at 55°C in the presence of 0.25% SDS. The DNA was extracted once with an equal volume of a 1:1 mixture of phenol:chloroform, once with an equal volume of chloroform and ethanol precipitated. Following resuspension in TE the DNA concentration was determined by spectroscopy and the DNA was stored at 4°C. For long term storage DNA was stored at -20°C as an ethanol precipitate.

2:14 PREPARATION OF NUCLEAR AND CYTOPLASMIC EXTRACTS OF INFECTED AND UNINFECTED HeLa CELLS

$6\text{-}8 \times 10^8$ Hela WS cells in 80oz roller bottles at approximately 90% confluence were infected with 30 plaque forming units/cell of virus in 5ml Tris/Saline per bottle. At 2h.p.i. medium containing 2% calf serum and 10mM hydroxyurea was added back and the cells were incubated at 37°C. At 22h.p.i. cells were dislodged by shaking with glass beads and collected in Tris/Saline. From this point all manipulations were carried out at 4°C.

The cells were pelleted by centrifugation at 2000rpm for 10min. in an MSE Coolspin, washed with 30ml hypotonic buffer containing 0.2M sucrose, repelleted and resuspended in 1ml. of hypotonic buffer per 10^8 cells. The cells were swollen on ice for 10min. and then lysed by 15 strokes with a tight fitting Dounce homogenizer. The nuclei were pelleted by centrifugation at 3000rpm for 5min. and the supernatant containing the cytoplasmic fraction was decanted.

The cytoplasm was clarified by centrifugation at 15000rpm for 30min., adjusted to 0.2M NaCl and applied to a 10ml column of DEAE-cellulose equilibrated in hypotonic buffer containing 0.2M NaCl. The column was washed with 20ml of hypotonic buffer containing 0.2M NaCl and the eluate collected.

The pelleted nuclei were washed twice in 5ml of 10mM Tris/HCl pH 7.5, 10% sucrose and resuspended in the same

buffer to give a total volume of 1ml per 10^8 cells. NaCl was added to give a final concentration of 0.3M and the nuclei were extracted by incubation for 60min. The nuclei were pelleted by centrifugation at 15000rpm for 30min., the supernatant was adjusted to 0.2M NaCl and applied to a 10ml column of DEAE-cellulose equilibrated in 10mM Tris/HCl pH7.5, 10% sucrose, 0.2M NaCl. The column was washed with 20ml of the same buffer and the eluate collected.

Solid ammonium sulphate was added to the column eluates to give 70% saturation and after 15min. incubation the precipitates were pelleted by centrifugation at 10000rpm for 10min. The precipitates were resuspended in 2ml of either hypotonic buffer (cytoplasmic extract) or 10mM Tris/HCl pH7.5, 10% sucrose (nuclear extract), dialysed overnight against 1l of the appropriate buffer, aliquoted and stored at -70 C. Extracts were stable for at least 6 months at this temperature.

2:15 IN VITRO ASSAY FOR DNA POLYMERASE ACTIVITY

Polymerase activity *in vitro* was determined by measuring the incorporation of radio-labelled deoxyribonucleotides into acid-precipitatible radio-activity using as a template salmon sperm DNA treated with pancreatic DNase I to introduce single-stranded nicks and gaps (activated DNA).

Enzyme fractions (2-5 ul) were incubated in a 50ul reaction mixture containing 50mM Tris/HCl pH 7.8, 7mM $MgCl_2$,

10mM DTT, 20uM dGTP, dTTP, dCTP, 4uM dATP, 0.5-2.0uC$_i$ (α^{32}P)dATP (specific activity 3000C$_i$/mmole) and 0.2ug/ul activated DNA for 60min. at 37^{o}C. Reactions were stopped by the addition of 5ml. of ice-cold 10% tri-chloro-acetic acid (TCA) containing 0.5% tetrasodium-pyrophosphate. After 15min. incubation on ice the mixtures were filtered under vacuum through glass-fibre discs (Whatman GF-C). The discs were washed twice with 5ml. 10% TCA containing 0.5% pyrophosphate, twice with 5% TCA and once with 5ml. 95% ethanol. After air drying the discs were placed in scintillation vials and 4ml. of Ecoscint (Nuclear Medical Electronic Services) was added. Sample radioactivity was determined by counting beta emissions in an Intertechnique SL4000 liquid scintillation counter.

2:16 IN VITRO ASSAY FOR THE FORMATION OF AN 80,000 MOLECULAR WEIGHT COMPLEX BETWEEN THE PRE-TERMINAL PROTEIN AND 5'dCMP

1-5 ul of the appropriate extracts were incubated in a 30ul reaction mixture containing 25mM Tris/HCl pH 8, 3mM $MgCl_2$, 2mM DTT, 3mM ATP, 3uC$_i$ (α^{32}P)dCTP (specific activity 3000C$_i$/mmole) and varying amounts of template DNA for 60min. at 37^{o}C. Reactions were stopped by heating at 70^{o}C for 5min. and treated with micrococcal nuclease (20U/ml) in the presence of 2mM $CaCl_2$ for a further 30min. at 37^{o}C. 12ul of boiling mix were added and the samples were heated at 100^{o}C for 2min. Reaction products were separated on a 10% SDS-polyacrylamide gel with a 5% spacer gel for 3-4h. at 45mA at 4^{o}C.

The gels were fixed in 47:47:6 methanol:water:glacial acetic acid for 30min., dried under vacuum and autoradiographed on Kodak XS film routinely for 15h. at -70°C using a Dupont Cronex intensifying screen. Bands corresponding to the 80K pTP-dCMP complex were cut out of the gel and radioactivity determined by scintillation counting.

2:17 IN VITRO ASSAY FOR THE FORMATION OF AN ELONGATED PRE-TERMINAL PROTEIN-30mer COMPLEX

The assay system was identical to that for the formation of the pTP-dCMP complex except that 50uM dATP, 50uM dTTP and 20uM ddGTP were added to the reaction mixtures. In addition, the reaction products were not treated with micrococcal nuclease.

2:18 QUANTITATION OF DNA

DNA concentrations were determined by measuring the absorbance of samples at 260nm. in a Cecil spectrophotometer. 1 A_{260} unit was taken to be equivalent to 50ug of double-stranded DNA or 20ug of single-stranded oligonucleotide. Concentrations were double-checked by separating DNA samples on agarose or polyacrylamide gels containing 0.5ug/ml. ethidium bromide and comparing the intensity of the fluorescence with that of known standards.

2:19 QUANTITATION OF PROTEIN

Protein concentrations were determined by the method of Bradford (1976). Protein samples in a total volume of

80ul were mixed with 1ml. of Bradford reagent (100mg Coomassie Brilliant Blue G, 30mg SDS, 50ml 95% (v/v) ethanol and 100ml 85% (v/v) phosphoric acid made up to 1l. with distilled water), incubated at room temperature for 15min. and the absorbance at 595nm. measured. Concentrations were calculated by comparison with a standard curve constructed using bovine serum albumin (Sigma).

2:20 AGAROSE GEL ELECTROPHORESIS

Gel slabs (265mm x 165mm) containing 0.8% (w/v) agarose, 1xTBE buffer and 0.5ug/ml ethidium bromide in a total volume of 200ml were run at up to 5V/cm at room temperature. One tenth volume of dye mix (50% (w/v) sucrose, 0.2% (w/v) bromophenol blue and 100mM EDTA) was added to samples prior to electrophoresis.

2:21 NON-DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

Gels were made up to the required acrylamide concentration (generally between 6% and 15%) from a 30% acrylamide stock (29% acrylamide, 1% N,N'-methylene-bis-acrylamide). Gels contained 1xTBE buffer, and were polymerized by t he addition of 1/250 volume 25% (w/v) ammonium persulphate and 1/2000 volume TEMED stock. Gels were poured in 1mM thick sandwiches sealed with Sylglas tape and run at up to 10V/cm. One tenth volume of dye mix (50% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol, 100mM EDTA) was added to the samples prior to electrophoresis. After electrophoresis gels were soaked in

1xTBE containing 0.5ug/ml ethidium bromide for 30min. to detect DNA by ultra-violet fluorescence.

2:22 DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

10% resolving gels were made up from a 30% acrylamide stock as above. Gels contained 0.375M Tris pH 8.9 and 0.1% SDS and were polymerized as described above. Gels were cast between glass plates separated by 1.5mm thick spacers and sealed with Teflon tape. Resolving gels were poured to leave room for a 1cm deep stacking gel and overlaid with approximately 1ml of butan-2-ol to ensure an even surface after polymerization.

Stacking gels contained 5% acrylamide in 0.105M Tris pH 6.7 and 0.1% SDS. Prior to electrophoresis samples were denatured by heating at 100°C for 2min. in a solution containing 8% (v/v) glycerol, 1.6% (w/v) SDS, 570mM 2-mercapto-ethanol, 33mM Tris pH 6.7 and 0.016% (w/v) bromophenol blue. Gels were run at 45mA in a buffer containing 52mM Tris, 53mM glycine and 0.1% SDS at 4°C until the dye front was 1cm from the bottom of the gel.

Gels were stained by soaking in 47:47:6 methanol:water:glacial acetic acid (fix) containing 0.2% (w/v) Coomassie Brilliant Blue R-250 for 60min. Excess stain was removed by soaking overnight in fix without the dye. For autoradiography gels were dried down under vacuum in a Biorad gel drier and exposed to Kodak XS film at -70°C.

2:23 PREPARATION OF DEAE-CELLULOSE COLUMNS

Preswollen DEAE-cellulose (Whatman DE52) was resuspended in 6ml/g of 0.2M Tris/HCl pH7.5 and stirred gently for 15min. The slurry was allowed to settle for 15min. and the supernatant containing any fines decanted. The ion exchanger was then resuspended in 6ml/g of 20mM Tris/HCl pH7.5 and allowed to settle for 1h. in a measuring cylinder. The supernatant was poured off until the final volume was the 'wet settled volume' of the ion exchanger plus 20%. Columns were poured with this slurry in disposable syringes blocked with either glass-wool in the nozzle, or a disc of glass-fibre filter paper (Whatman GF/C) at the bottom of the barrel. Columns were equilibrated with the appropriate buffer until the pH of the eluate was the same as that of the input buffer.

RESULTS

The background to this study into the *in vitro* replication of adenovirus type 4 (Ad.4) DNA was provided in experiments performed in this laboratory (Hay,1985b). The minimal *cis* acting DNA sequence requirements for *in vivo* origin of replication function had previously been identified for adenovirus type 2 (Ad.2) (Hay,1985a) by constructing plasmids containing two inverted copies of Ad.2 terminal sequences which, when cleaved with EcoR1, would release a linear DNA molecule with copies of these sequences at each end. These molecules were then co-transfected into susceptible cells with wildtype Ad.2 DNA to provide viral replication proteins. They were replicated to a high copy number provided that they contained a functional origin of replication. Using this technique it was shown that the terminal 45b.p. of Ad.2 was both necessary and sufficient for initiation of DNA replication *in vivo* (Hay,1985a).

This region had previously been defined in *in vitro* replication experiments as consisting of two domains (see Section 4:5:1). The domain from nucleotides 1-18 could support a limited amount of initiation *in vitro* and contained a 10b.p. sequence completely conserved amongst human adenoviruses (Stillman et al,1982). The second domain, from nucleotides 19-48, enhanced the efficiency of initiation *in vitro* and contained the binding site for the cellular protein nuclear factor I (NFI) (see Section 1:6:1). Thus the results obtained *in vitro* and *in vivo* did not entirely agree: *in vitro* it appeared that the minimal origin of replication for Ad.2 was the terminal 18b.p. and origin

function was enhanced by the presence of the 19-45b.p. domain. *In vivo* both domains were shown to be essential for origin function.

The *in vivo* assay was then used to examine the sequence requirements for Ad.4 origin function (Hay,1985b). It was demonstrated that *in vivo* Ad.4 only required the terminal 18b.p. of the genome and the addition of sequences outwith this region did not enhance replication. Ad.4 would replicate templates containing either Ad.2 or Ad.4 termini with equal efficiency whereas Ad.2 would only efficiently replicate homologous templates. Templates containing the Ad.4 ITR were replicated by Ad.2 at approximately 5% of the efficiency of Ad.2 templates, possibly due to the presence of a high affinity binding site for the cellular protein nuclear factor III (see Section 1:6:3). Sequence analysis revealed that the terminal 26b.p. of Ad.2 and Ad.4 were identical but that Ad.4 lacked the consensus sequence for the NFI binding site. It was concluded that, unlike Ad.2, Ad.4 did not require NFI for initiation of replication *in vivo*.

To characterize these differences at the molecular level it was decided to study the initiation of Ad.4 DNA replication *in vitro*. Two approaches were used: Firstly the initiation of Ad.4 replication *in vitro* by crude extracts of infected cells was studied with a view both to defining the DNA template and as an assay to purify and characterize the viral and cellular proteins involved. Secondly an attempt

was made to identify the Ad.4 pre-terminal protein-polymerase (pTP-pol) complex in infected cells and define the unique properties of those proteins. A cellular factor that enhanced Ad.4 initiation was also identified.

3:1 TEMPLATE DEPENDENT FORMATION OF A pTP-dCMP COMPLEX BY CYTOPLASMIC EXTRACT OF Ad.4 INFECTED CELLS

Initiation of adenovirus DNA replication involves the formation of a covalent complex between an 80K precursor terminal protein (pTP) and a dCMP residue, the first nucleotide at the 5'end of the genome. In adenovirus type 2 (Ad.2) this reaction is catalysed by the viral DNA polymerase which is found tightly associated with the pTP in infected cells. The pTP-dCMP complex then acts as a primer for DNA synthesis by the viral DNA polymerase. The formation of a pTP-dCMP complex *in vitro* can be assayed by incubating infected cell extracts and template DNA with (α32P)dCTP as the only nucleotide. The reaction products are separated by SDS-PAGE and an 80,000-dalton (80K) pTP-dCMP complex detected by auto-radiography (Lichy *et al*,1981). Formation of a pTP-dCMP complex can be quantitated by cutting out the appropriate area of the gel and counting in a liquid scintillation counter. Blank values are calculated by cutting out 2-3 equivalent areas of the gel not corresponding to pTP-dCMP complex and averaging the values obtained.

Four double-stranded DNA species were initially used as templates for the formation of an 80K pTP-dCMP complex by extracts of Ad.4 infected cells. These templates were:

1) Ad.4 viral cores consisting of nucleocapsids separated from the capsomeres by treatment with 0.5% sodium deoxycholate at 56°C and glycerol gradient centrifugation (Russell et al,1971; Goding & Russell,1983).

2) Ad.4 DNA-protein complex (DNA-prot.) consisting of naked viral DNA covalently attached to the 55K terminal protein at the 5'ends. Ad.4 DNA-prot. was made by treatment of intact virions with guanidinium hydrochloride followed by gel filtration (Goding & Russell,1983).

3) Ad.4 DNA with no attached protein made by treatment of virions with proteinase K in the presence of SDS, followed by extraction with phenol/chloroform and ethanol precipitation.

4) A plasmid DNA (p4A2; identical to p4A1;Hay, 1985b) containing the terminal 140b.p. of the Ad.4 genome linearized with EcoR1 such that the terminal Ad.4 sequences are located at the end of a linear molecule.

Initially nuclear extracts were made from Ad.4 infected HeLa cells as described for Ad.2 (Challberg & Kelly,1979a) but these extracts did not support detectable levels of pTP-dCMP complex formation. As described in

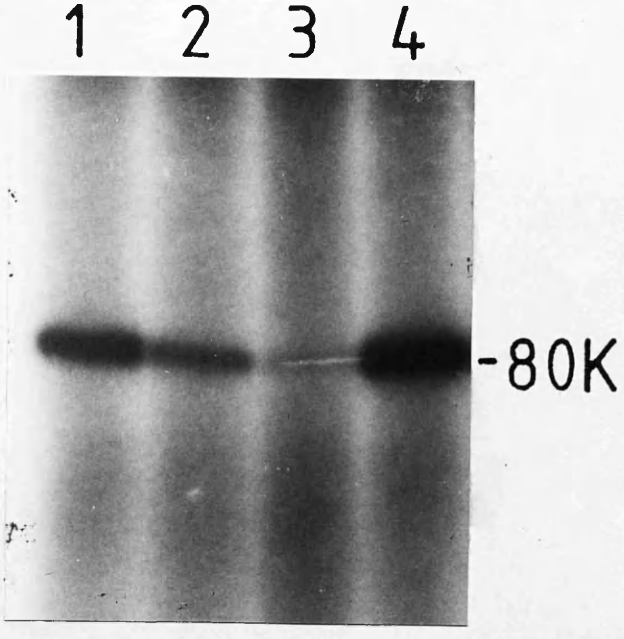
section 3:2:1 cytoplasmic extracts of Ad.4 infected cells were therefore used to characterize the system.

Figure 3:1 shows a comparison of the efficiency of Ad.4 cores, Ad.4 DNA-prot., Ad.4 DNA and a linearized plasmid containing the terminal 140b.p. of Ad.4 (p4A2; identical to p4A1, Hay,1985b) to support the formation of a pTP-dCMP complex. The reactions contained cytoplasmic extract of Ad.4 infected cells (4.2ug of protein) and 100ng of DNA, corresponding to 8.3fmoles of terminal sequences for cores, DNA-prot. or DNA (2 copies of the terminus per molecule) and 56fmoles of terminal sequences for p4A2 (1 copy per molecule). The results show that on a molar basis Ad.4 cores are the most active templates for complex formation, between 2-4 times more active than DNA-prot. and 8 times more active than the linearized plasmid.

It is possible that the nucleocapsid proteins closely associated with Ad.4 DNA within the core structure play a part in facilitating initiation. Alternatively the DNA-terminal protein complex might be in a better condition after treatment with deoxycholate than after the harsher treatment with guanidinium hydrochloride. The activity of the core structure must be highly resistant to denaturation since treatment with N-ethyl-maleimide or heating to 65^{o}C for 30min. had no effect on the ability of cores to support initiation (Figure 3:2). There is clearly a requirement for double-stranded DNA as exemplified by the fact that nuclease treatment (figure 2, tracks 5 and 6), abolished activity, and heating to 100^{o}C for

Figure 3:1 Comparison of the ability of various templates to support complex formation by cytoplasmic extract Ad.4 infected cells.

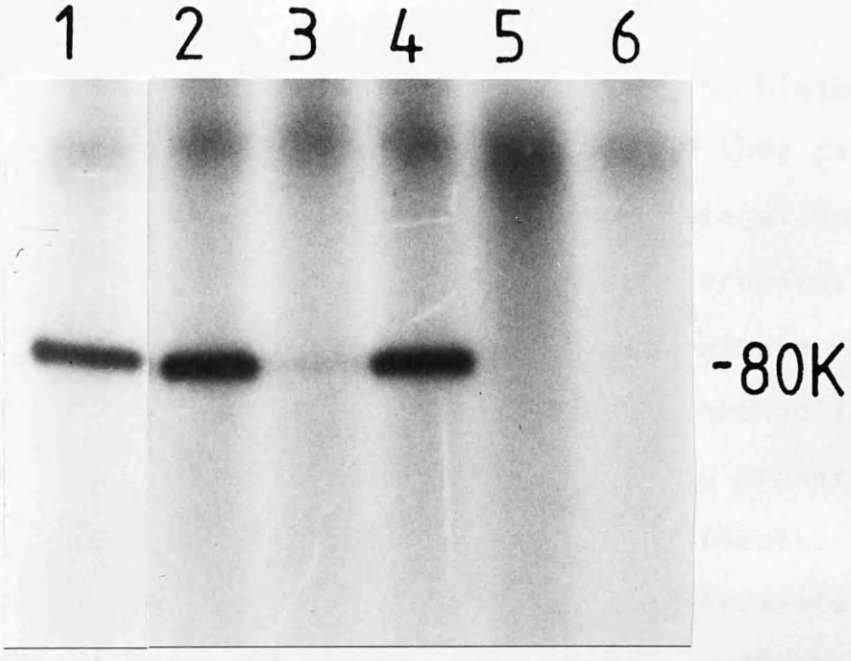
100ng of Ad.4 cores (track 1), Ad.4 DNA-prot. (track 2), deproteinized Ad.4 DNA (track 3) or plasmid p4A2 linearized with EcoRI (track 4) were incubated with 1ul of cytoplasmic extract (4.2ug protein) under the standard reaction conditions described in section 2:16: 25mM Tris/HCl pH8, 3mM $MgCl_2$, 3mM ATP, 2mM DTT and $3uC_i$ ($\alpha^{32}P$)dCTP (specific activity $3000C_i$/mmole) for 60min. at 37°C. The relative efficiency of complex formation is presented along with an autoradiograph of the part of the gel containing the pTP-dCMP complex. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.



TRACK	TEMPLATE	RELATIVE EFFICIENCY OF COMPLEX FORMATION
1	Ad.4 cores	100%
2	Ad.4 DNA-prot.	50%
3	Ad.4 DNA	25%
4	p4A2/EcoR1	12·5%

Figure 3:2 Effect of various treatments on the ability of Ad.4 cores to support complex formation.

Prior to assaying for their ability to support pTP-dCMP formation under standard conditions 100ng aliquots of Ad.4 cores were treated as indicated. N-ethyl-maleimide treatment was for 5min. at 4°C followed by the addition of DTT to 10mM. Micrococcal nuclease and pancreatic DNase treatments were for 60min. at 37°C followed by either the addition of EDTA to 20mM or heating to 65°C for 10min. An autoradiograph of the part of the gel containing the pTP-dCMP complex is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.



TRACK	TREATMENT
1	NONE
2	65°C / 30 min.
3	100°C / 5 min.
4	5 mM N-ethyl-maleimide
5	Micrococcal nuclease
6	Pancreatic DNase

5min. (figure 2, track 3) severely reduced template activity. In addition the ability to support pTP-dCMP complex formation is stimulated by the terminal protein.

Although cores were the most active and biologically relevant of the templates used in this study they proved to be unstable and rapidly lost activity on storage. Goding and Russell (1983) suggested that Ad.2 core preparations contained a nuclease activity which might explain the loss of activity. In addition the nature of the method for the preparation of cores meant that no two core preparations were the same, making data comparisons difficult, if not invalid. For these reasons the bulk of experiments were performed with plasmid templates as these proved to be more stable, easier to work with and provided reproducible results.

3:2 CHARACTERIZATION OF THE FORMATION OF A pTP-dCMP COMPLEX BY EXTRACTS OF Ad.4-INFECTED HELA CELLS

Unless specifically mentioned all the experiments in Section 3:2 were performed using plasmid p4A2 (identical to p4A1; Hay,1985b) linearized with EcoR1 as template DNA. A detailed analysis of the template requirements for complex formation is deferred until Section 3:3.

3:2:1 FORMATION OF A pTP-dCMP COMPLEX IS SUPPORTED BY BOTH CYTOPLASMIC AND NUCLEAR EXTRACTS OF Ad.4 INFECTED CELLS

Initially nuclear extracts of Ad.4 infected cells were prepared as described for Ad.2 (Challberg & Kelly,1979a). However these extracts did not support pTP-dCMP complex formation to any detectable levels. It was only when the extracts were depleted of nucleic acids by passage through a DEAE-cellulose column and concentrated by ammonium sulphate precipitation that template dependent pTP-dCMP complex formation was observed. Figures 3:3 to 3:5 show the results of experiments in which a constant amount of template plasmid DNA (0.5ug) was incubated with (α^{32}P)dCTP as the only nucleotide in the presence of increasing amounts of either cytoplasmic extract of Ad.4-infected cells (figures 3:3 and 3:4) or nuclear extract of Ad.4-infected cells (figure 3:5) for 60min. at 37^{o}C. Both cytoplasmic and nuclear extracts of Ad.4-infected cells supported pTP-dCMP complex formation. No pTP-dCMP complex formation was observed in reactions containing only uninfected extracts (data not shown).

At low protein concentrations (below 0.3mg/ml) the amount of pTP-dCMP complex formed by nuclear extract was significantly greater than by cytoplasmic extract. This could be due to differences in the relative amounts of replication proteins present in the cytoplasm and nucleus: If a protein required for initiation was more prevalent in the nucleus then pTP-dCMP complex formation would be

Figure 3:3 Formation of a pTP-dCMP complex by cytoplasmic extract of Ad.4 infected cells.

Cytoplasmic extract was prepared from Ad.4 infected cells as described in section 2:14. The protein concentration of the extract was 4.2mg/ml. Assays for the formation of a pTP-dCMP complex were performed in the presence of 0.5ug plasmid p4A2 linearized with EcoRI and increasing amounts of cytoplasmic extract under the standard reaction conditions described in section 2:16. Plasmid DNA was cleaved with EcoRI, extracted once with an equal volume of a 1:1 mixture of phenol:chloroform, once with an equal volume of chloroform, ethanol precipitated and resuspended in distilled water, prior to the reaction. An autoradiograph of the entire gel is presented. The low molecular weight bands present are likely to be partial proteolytic degradation products of the 80K pTP. The positions of the 80,000 molecular weight pTP-dCMP complex (80K) and the spacer gel (SP) are indicated.

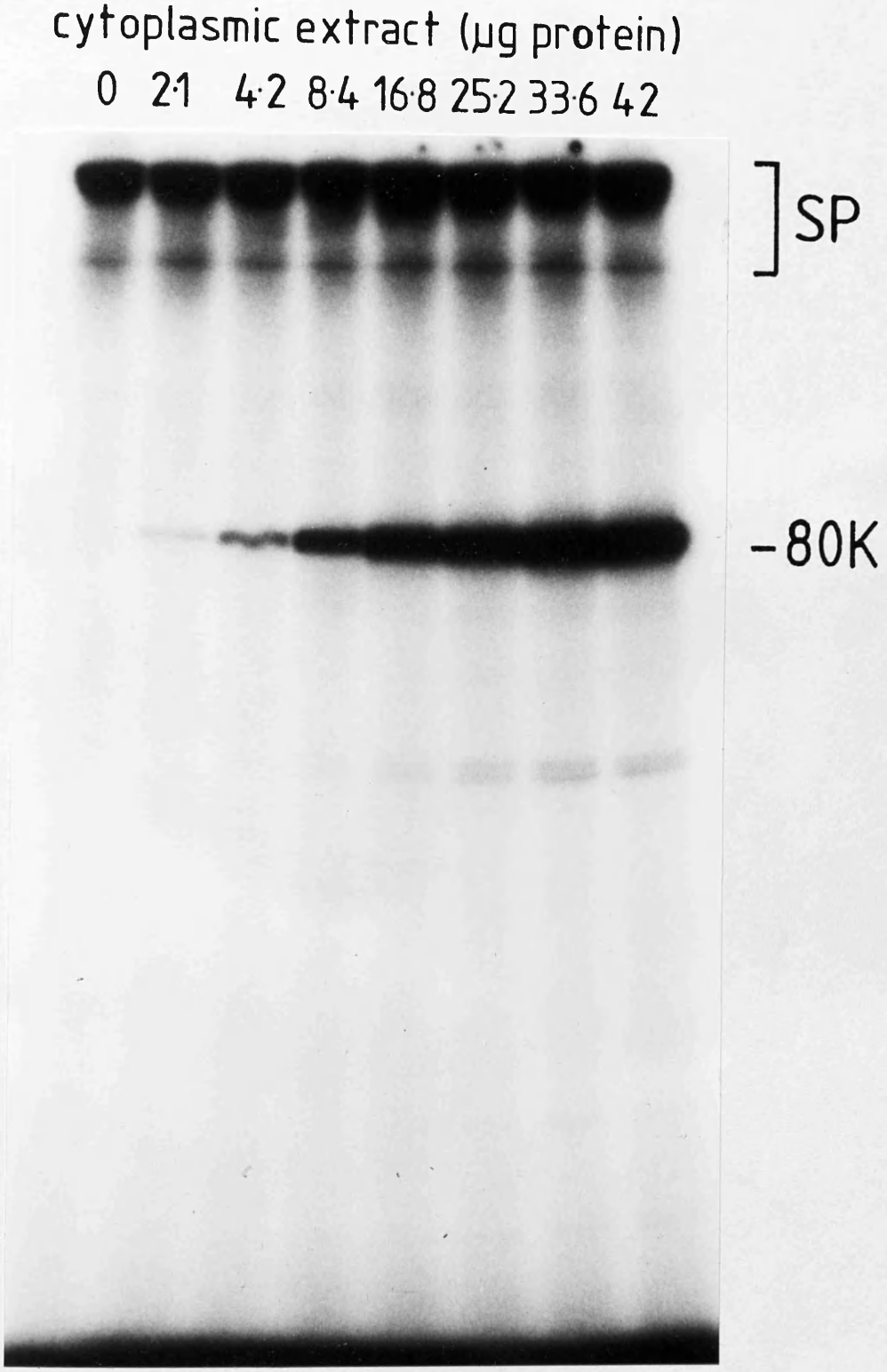

cytoplasmic extract (µg protein)
0 2·1 4·2 8·4 16·8 25·2 33·6 42
SP
-80K

Figure 3:4 Graphical representation of the formation of a pTP-dCMP complex by cytoplasmic extract of Ad.4 infected cells.

The amount of pTP-dCMP complex formation by varying amounts of cytoplasmic extract was quantitated by excising the area of the SDS-polyacrylamide gel corresponding to the 80K pTP-dCMP complex and counting in a liquid scintillation counter. The data were converted to femtomoles (fmoles) of pTP-dCMP complex formed and plotted against the amount of protein present in the reaction. A blank value was obtained from track 1 which contained no protein.

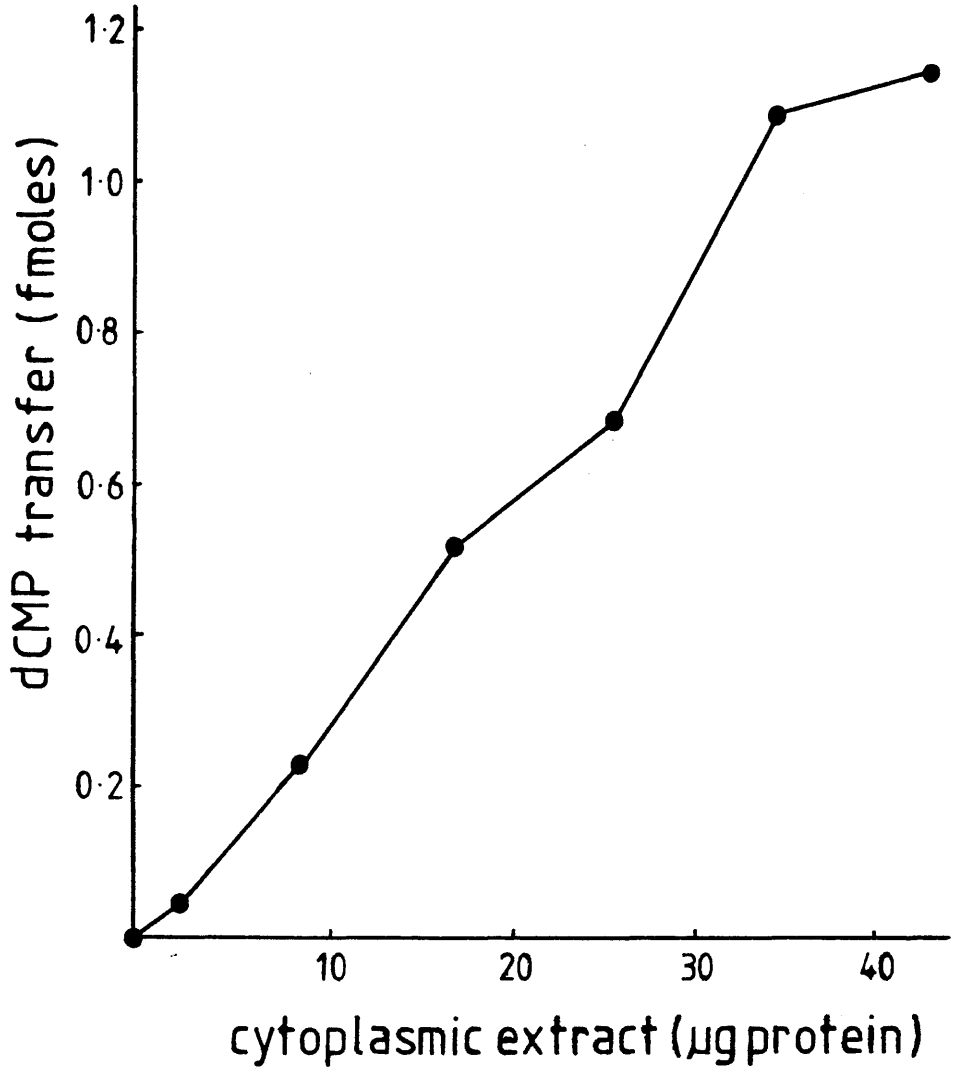
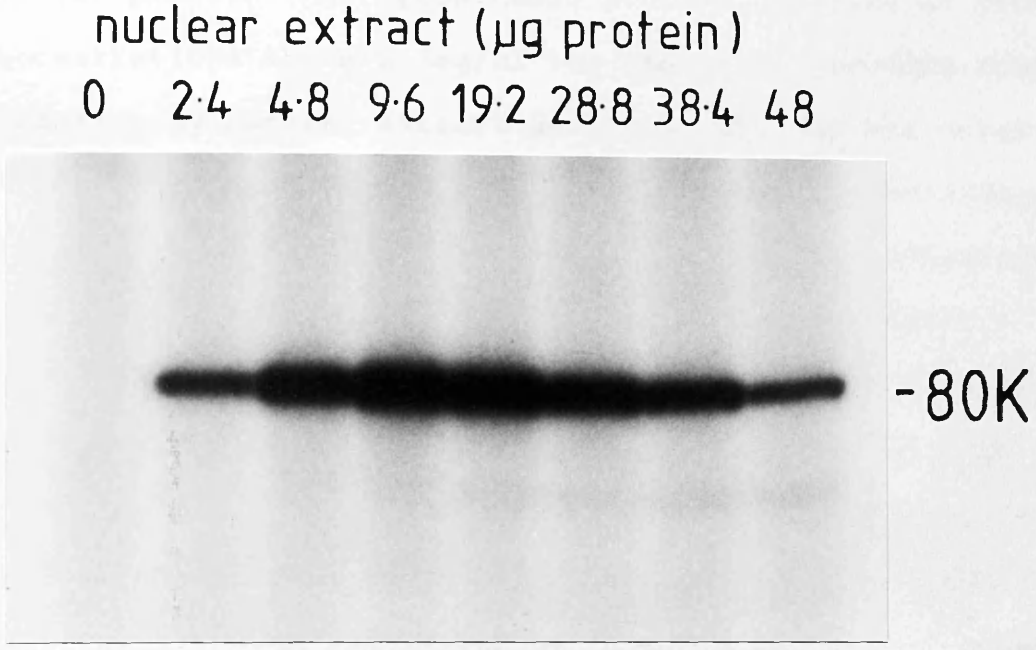
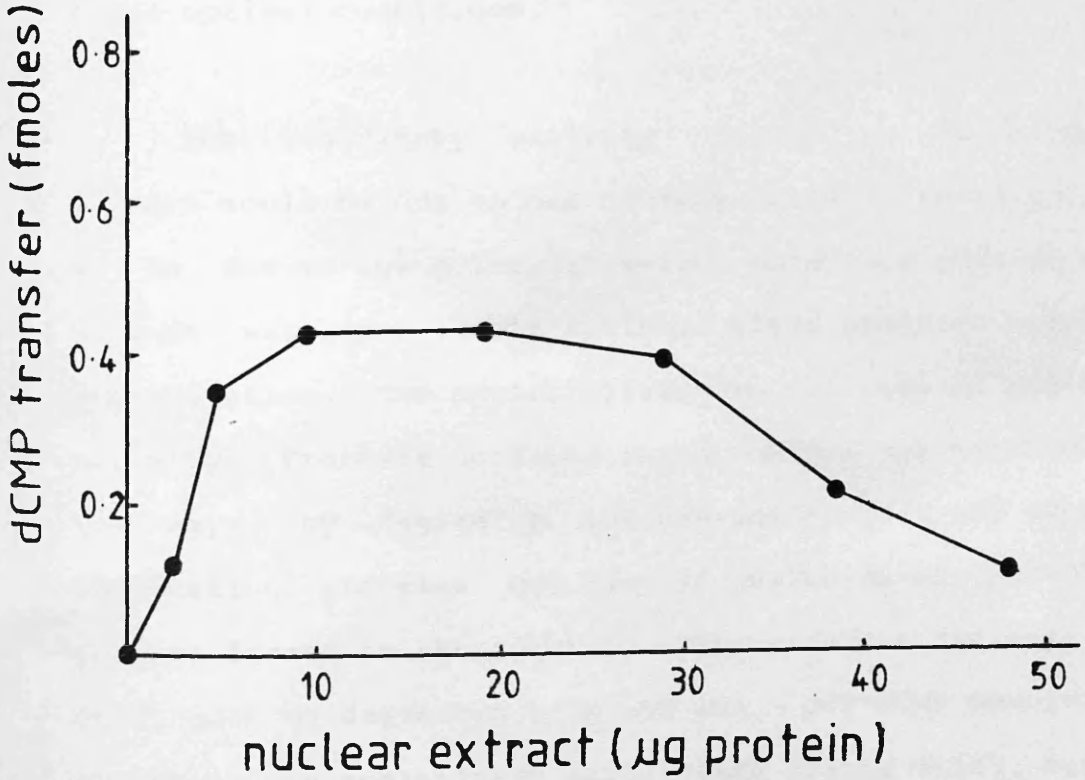

1·2
1·0
0·8
0·6
0·4
0·2
dCMP transfer (fmoles)
10
20
30
40
cytoplasmic extract (µg protein)

Figure 3:5 Formation of a pTP-dCMP complex by nuclear extract of Ad.4 infected cells.

Nuclear extract (protein concentration 4.8mg/ml) was prepared from Ad.4 infected HeLa cells as described in section 2:14. Assays for the formation of a pTP-dCMP complex were performed in the presence of 0.5ug of plasmid p4A2 linearized with EcoRI and increasing amounts of nuclear extract under standard reactions conditions (as described in section 2:16). An autoradiograph of the part of the gel containing the pTP-dCMP complex, together with a graphical representation of the data, is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.


nuclear extract (µg protein)
0 2·4 4·8 9·6 19·2 28·8 38·4 48
-80K


dCMP transfer (fmoles)
0·8
0·6
0·4
0·2
10
20
30
40
50
nuclear extract (µg protein)

expected to occur more readily at low concentrations of nuclear protein than cytoplasmic protein. However at protein concentrations above 0.3mg/ml the amount of pTP-dCMP complex formation by nuclear extract declines, whereas the activity of cytoplasmic extract continues to increase up to 1.4mg/ml, the highest concentration tested. Clearly an inhibitor which exhibits maximal activity at high protein concentrations is present in the nucleus. The apparent levelling off of pTP-dCMP complex formation at high concentrations of cytoplasmic protein could be due to saturation of the active template molecules in the reaction mixture: although 230 fmoles of template DNA was present in each reaction a maximum of 1.16 fmoles of complex was formed in 60min., a figure comparable to that obtained with Ad.2 under optimal conditions.

The inhibitory activity observed in the nuclear extract could be due to one of many factors. It is unlikely to be due to low molecular weight molecules because the extract was extensively dialysed after ammonium sulphate precipitation. Two possibilities are nuclease or protease activity. Protease activity would inhibit the reaction in two ways- by degrading the pTP-pol complex and other replication proteins and also by degrading any pTP-dCMP complex formed in the reaction. In contrast a nuclease would only act by degrading template DNA - pTP-dCMP complex is resistant to nucleolytic attack (see Figure 3:14). During the course of experiments designed to look at the template requirements for complex formation it was noticed that the

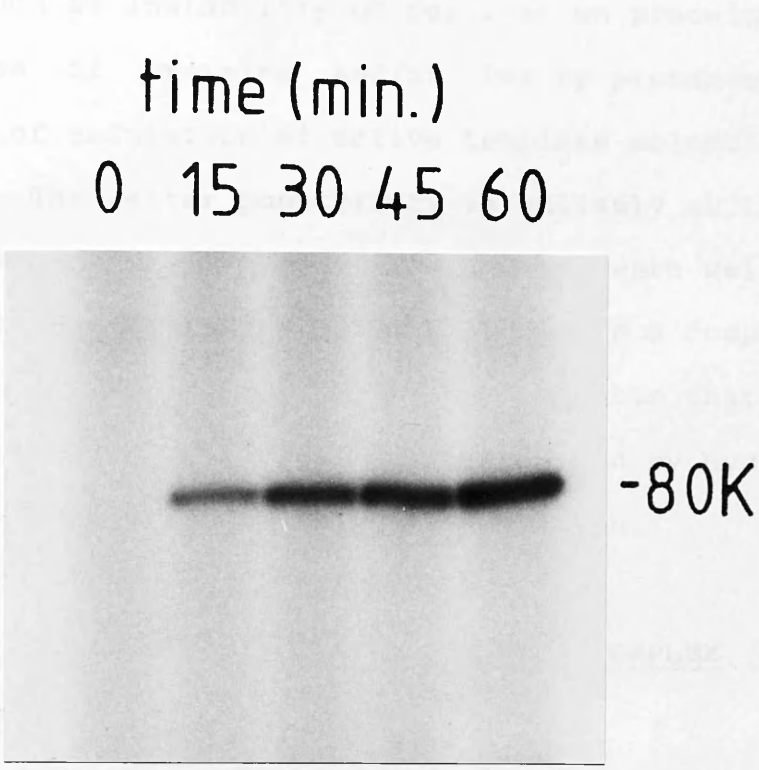
inhibitory effect of nuclear extract was more pronounced when a linearized plasmid instead of Ad.4 cores was used as template. Viral cores are resistant to 5'-3' exonuclease action by virtue of the TP attached to the 5' ends of the genome. This suggests that the inhibitory effect of nuclear extract is due, at least in part, to 5'-3' exonuclease activity. Alternatively cellular non-specific DNA binding proteins could be preventing the interaction between the template DNA and factors involved in initiation. In view of the presence of this inhibitor all further experiments were performed with Ad.4-infected cytoplasmic extract.

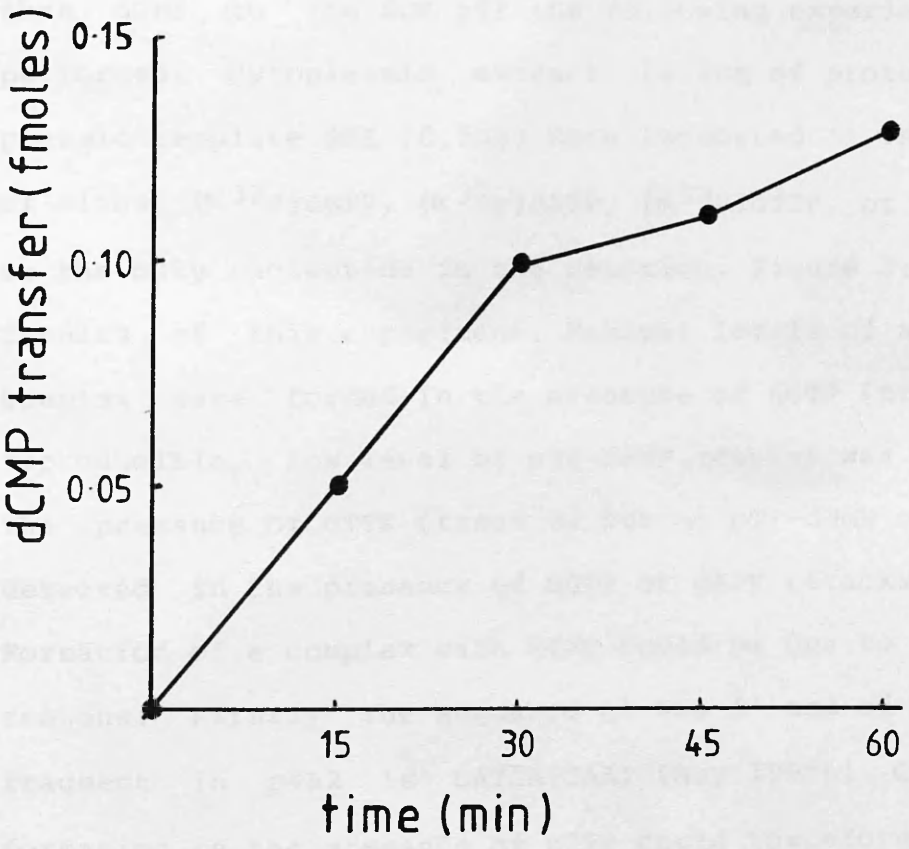
3:2:2 THE KINETICS OF pTP-dCMP COMPLEX FORMATION

To determine the kinetics of the formation of a pTP-dCMP complex reactions were performed in the presence of a constant amount of plasmid template DNA (0.5ug) and a non-saturating amount of cytoplasmic extract of Ad.4 infected cells (1ul=4.2ug). Reactions were terminated at various times after the addition of extract by heating at 70°C for 10min. The results are shown in figure 3:6. The rate of the reaction was constant for a period of approximately 30min. and then declined to 20% of it's initial value (Figure 3:6). One reason for this decline could be depletion in the number of un-complexed pTP molecules in the reaction. If most of the active pTP molecules were complexed with dCMP within the first 30min. of the reaction the concentration of uncomplexed molecules would decline and with it the rate of the reaction. However the decline in the rate of reaction could be due to other

Figure 3:6 Time course for the formation of a pTP-dCMP complex by cytoplasmic extract of Ad.4 infected cells.

Assays for the formation of a pTP-dCMP complex were performed in the presence of 0.5ug plasmid p4A2 linearized with EcoRI and lul (4.2ug protein) cytoplasmic extract under standard reaction conditions (as described in section 2:16). Assays were terminated as described at the specified times. An autoradiograph of the relevant portion of the gel, together with a graphical representation of the data, is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.


time (min.)
0 15 30 45 60
-80K


dCMP transfer (fmoles)
0·15
0·10
0·05
15
30
45
60
time (min)

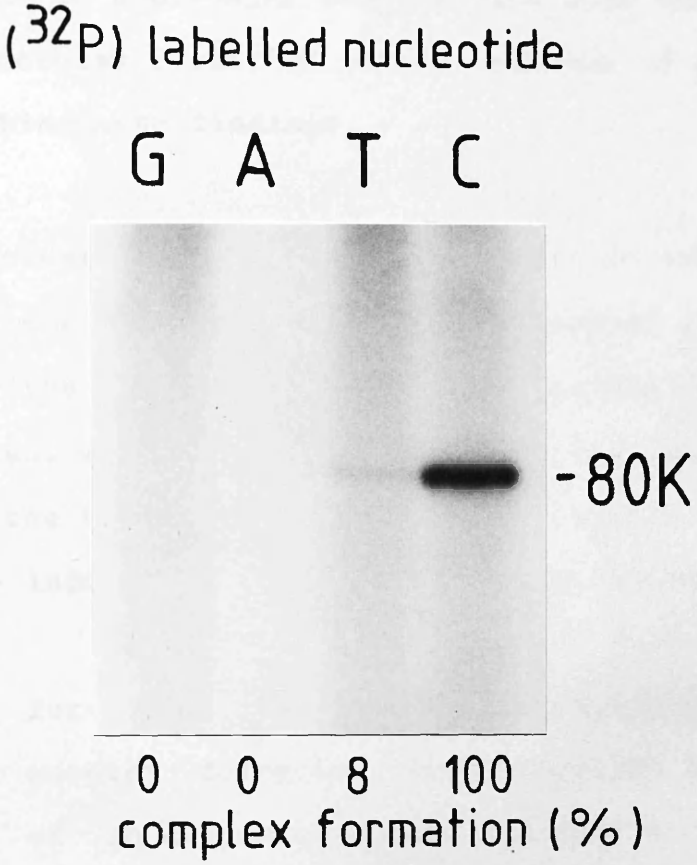
factors such as instability of replication proteins at 37°C, degradation of proteins and/or DNA by proteases and nucleases or saturation of active template molecules in the reaction. The latter possibility is unlikely as the levels of complex formation in this experiment were well below maximal levels such as those obtained in a comparable experiment (Figure 3:3). It is also possible that the dCTP concentration in the reaction was reduced by hydrolysis making it unavailable for complex formation.

3:2:3 NUCLEOTIDE SPECIFICITY OF pTP-dCMP COMPLEX FORMATION

To determine whether cytoplasmic extract of Ad.4 infected cells could catalyse the transfer of dNMP's other than dCMP to the 80K pTP the following experiment was performed. Cytoplasmic extract (4.2ug of protein) and plasmid template DNA (0.5ug) were incubated in the presence of either (α^{32}P)dGTP, (α^{32}P)dATP, (α^{32}P)dTTP, or (α^{32}P)dCTP as the only nucleotide in the reaction. Figure 3:7 shows the results of this experiment. Maximal levels of a pTP-dNMP complex were formed in the presence of dCTP (track 4). A reproducible, low level of pTP-dNMP complex was formed in the presence of dTTP (track 3) but no pTP-dNMP complex was detected in the presence of dGTP or dATP (tracks 1 and 2). Formation of a complex with dTMP could be due to one of two reasons: Firstly the sequence at the 5' end of the Ad.4 fragment in p4A2 is CATCATCAAT (Hay,1985b). Complex formation in the presence of dTTP could therefore be due to transfer of dTMP to a pTP-dCMP-dAMP complex. Low endogenous

Figure 3:7 Formation of a pTP-nucleotide complex by cytoplasmic extract of Ad.4 infected cells in the presence of different nucleotides.

0.5ug of plasmid p4A2 linearized with EcoRI and 1ul of cytoplasmic extract from Ad.4 infected cells (4.2ug protein) were incubated under standard reaction conditions (section 2:16) in the presence of either (α^{32}P)dGTP (track 1), (α^{32}P)dATP (track 2), (α^{32}P)dTTP (track 3) or (α^{32}P)dCTP (track 4) as the only nucleotide. The specific activity of all the (α^{32}P) labelled dNTP's was 3000C_i/mmole. An autoradiograph of the part of the gel containing the pTP-dCMP complex and the relative amounts of pTP-dCMP complex formation in each case is presented. 80K indicates the position of the 80,000 molecular weight pTP-dNMP complex.


(32P) labelled nucleotide
G A T C
-80K
0 0 8 100
complex formation (%)

levels of dNTP's in the extract could account for the formation of the latter complex. However if this is the case it would be reasonable to expect that dAMP would be transferred to a pTP-dCMP complex. The data show that there was no complex formation in the presence of dATP so this cannot explain the findings.

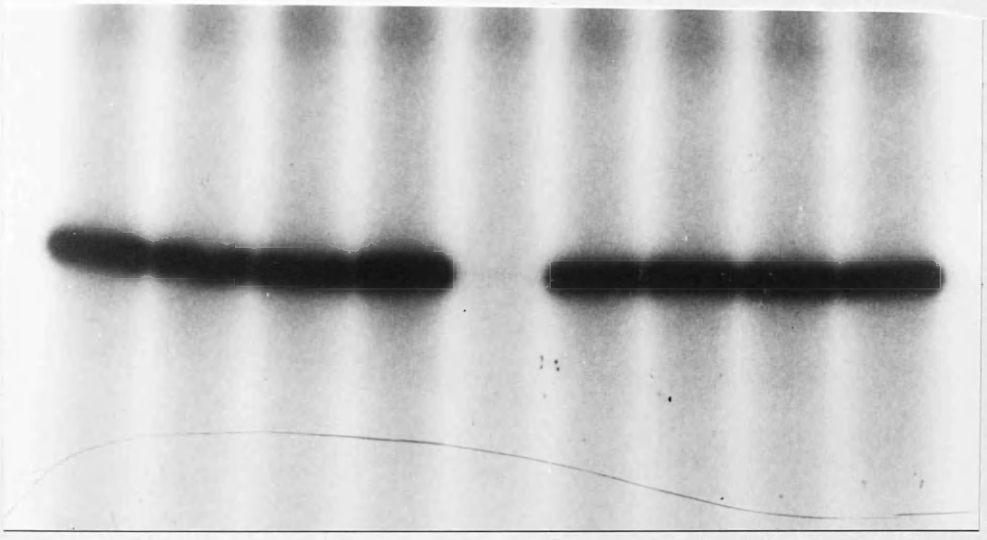
An alternative explanation is that in the presence of dTTP as the only nucleotide the degree of specificity regarding the site for initiation on the DNA sequence could be relaxed. In this case pTP-dNMP complex formation could occur at the third nucleotide in the sequence - the first dT residue - instead of at the terminal nucleotide.

To further analyse the nucleotide specificity of the reaction complex formation with labelled dCTP in the presence of an excess of unlabelled dNTP's or rNTP's was studied. Figure 3:8 shows the results of an experiment in which cytoplasmic extract (4.2ug of protein) and plasmid template DNA (0.5ug) were incubated with (α^{32}P)dCTP in the presence of a 300-fold excess of unlabelled dNTP's (tracks 2 to 5) or rNTP's (tracks 6 to 9). The data show that only unlabelled dCTP efficiently competed for complex formation. It might be expected from the data in figure 3:7 that, as dTTP forms a complex with pTP at a low efficiency, a large excess of unlabelled dTTP would compete with dCTP for complex formation. The data show that this is not the case. Given a choice of substrates for complex formation the pTP-pol shows a high specificity for dCTP. Complex formation

Figure 3:8 Competition by an excess of unlabelled nucleotides for pTP-dCMP formation by cytoplasmic extract of Ad.4 infected cells.

0.5ug of plasmid p4A2 linearized with EcoRI and 1ul of cytoplasmic extract (4.2ug protein) were incubated under standard reaction conditions (section 2:16) with 3uC$_i$ (α^{32}P)dCTP (specific activity 3000C$_i$/mmole) in the presence of 10uM (a 300-fold excess) of unlabelled deoxyribonucleotides (tracks 2-5) or ribonucleotides (tracks 6-9) as indicated. An autoradiograph of the part of the gel containing the pTP-dCMP complex is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.

competitor



with dTTP will only occur if that nucleotide is the only one present. As is the case for all DNA polymerases the Ad.4 DNA polymerase showed absolute specificity for dNTP's as a large excess of rCTP did not compete with (α^{32}P)dCTP for pTP-nucleotide complex formation.

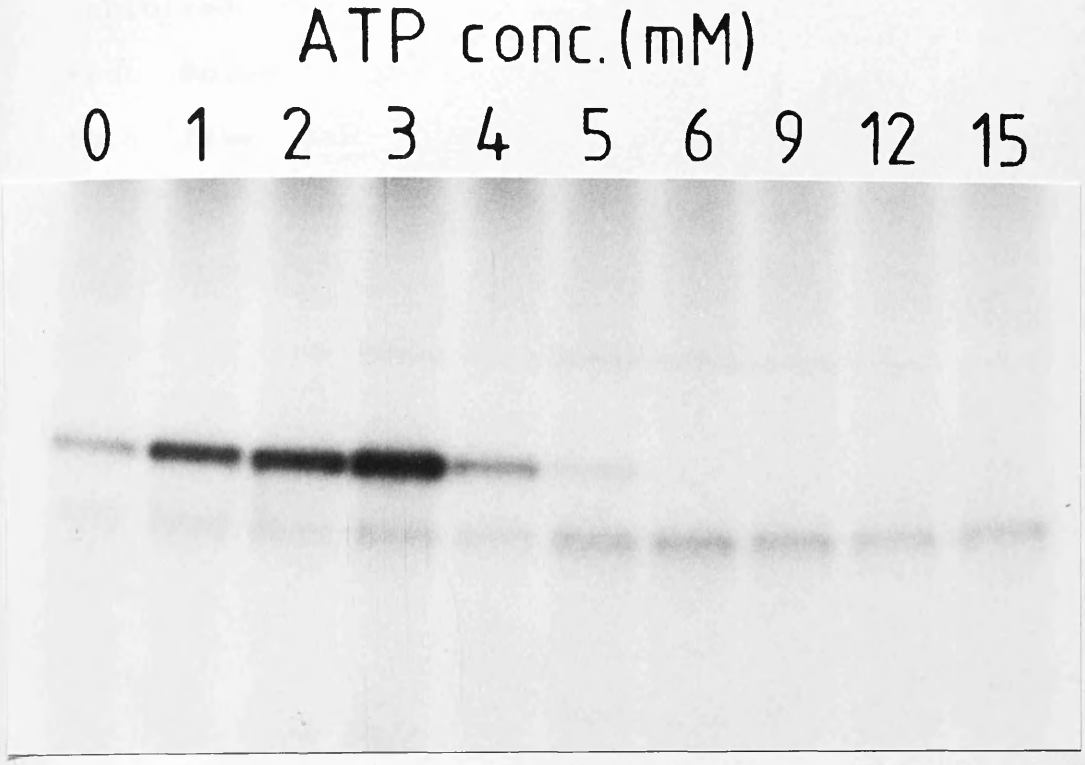
3:2:4 THE ROLE OF ATP AND DIVALENT CATIONS IN THE FORMATION OF A pTP-dCMP COMPLEX

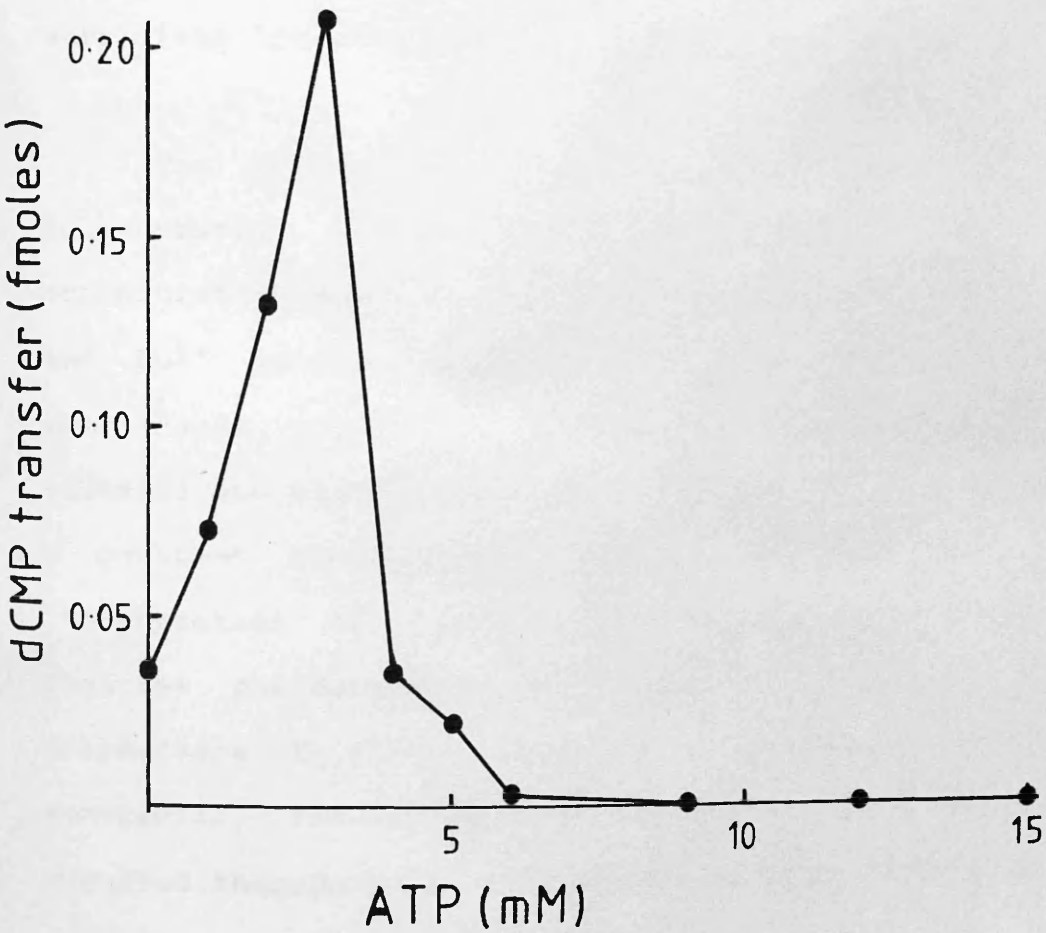
To determine the effect of ATP concentration on pTP-dCMP complex formation cytoplasmic extract of Ad.4 infected cells (4.2ug of protein) and plasmid template DNA (0.5ug) were incubated in the presence of a constant Mg^{2+} concentration of 3mM and a variable ATP concentration from 0-15mM. Figure 3:9 shows the results of this experiment. Although the results suggest that ATP is not absolutely required for complex formation it is possible that low endogenous levels of ATP were sufficient for limited complex formation (track 1). The amount of complex formed increased to a maximum at an ATP concentration of 3mM (track 4), a 6-fold increase over track 1. At higher ATP levels the amount of complex formation rapidly declined, becoming undetectable at 6mM. The level of stimulation by ATP varied between extracts and was clearly to some extent dependent on the endogenous ATP level.

ATP is known to chelate Mg^{2+} ions. In this context it was noticed that maximal pTP-dCMP complex formation was at a point where the ATP and Mg^{2+} concentrations were equal, and at higher ATP concentrations complex formation was sharply

Figure 3:9 Optimization of the ATP concentration for pTP-dCMP formation by cytoplasmic extract of Ad.4 infected cells.

1ul of cytoplasmic extract (4.2ug protein) was incubated with 0.5ug p4A2 linearized with EcoRI under standard reaction conditions (section 2:16) except that the concentration of ATP was varied from 0-15mM. The magnesium concentration (as $MgCl_2$) was maintained at the standard level of 3mM throughout the experiment. An autoradiograph of the part of the gel containing the pTP-dCMP complex, together with a graphical representation of the data, is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.


ATP conc.(mM)
0 1 2 3 4 5 6 9 12 15
-80K

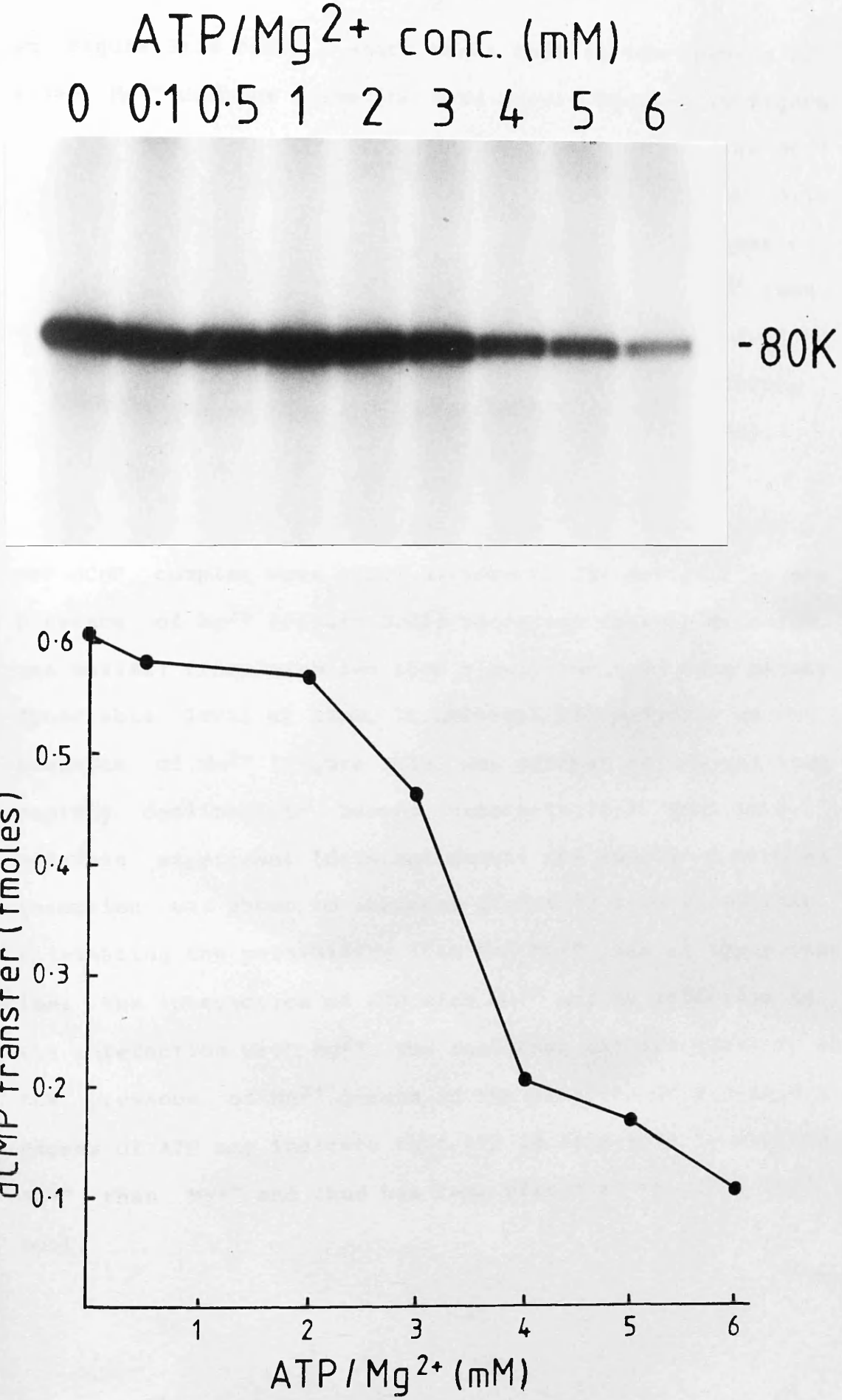

0·20
0·15
0·10
0·05
dCMP transfer (fmoles)
5
10
15
ATP (mM)

inhibited. These observations suggested either that free ATP (not bound to Mg^{2+}) was inhibitory to the reaction or that both free Mg^{2+} ions and Mg^{2+} ions chelated to ATP were required for the reaction. An excess of ATP over Mg^{2+} would reduce the pool of available free Mg^{2+} ions in the reaction. To test this hypothesis the experiment was repeated but in this case both ATP and Mg^{2+} concentrations were increased concurrently (Figure 3:10). In this experiment a greater amount of pTP-dCMP complex formation was observed in the presence of both ATP and Mg^{2+} at 6mM (figure 3:10, track 9) than in the presence of ATP at 6mM and Mg^{2+} at 3mM (figure 3:9, track 7). In figure 3:10 the highest level of pTP-dCMP complex formation was observed in the absence of added ATP and Mg^{2+}. Clearly the endogenous levels of both are sufficient for efficient complex formation.

The effect of varying the divalent cation concentration in the presence of a constant ATP concentration was then studied using both Mg^{2+} (Figure 3:11) and Mn^{2+} (Figure 3:12). Reactions were performed with cytoplasmic extract of Ad.4 infected cells (4.2ug of protein) and plasmid template DNA (0.5ug) in the presence of a constant concentration of ATP (3mM) and a Mg^{2+}/Mn^{2+} concentration varying between 0-15mM. Complex formation requires the action of the Ad.4 DNA polymerase and all DNA polymerases thus far studied, both procaryotic and eucaryotic, require a divalent cation for activity. As expected therefore, in the absence of added Mg^{2+} or Mn^{2+} no complex was detected. This appears to contradict the result

Figure 3:10 Optimization of the ATP/Mg^{2+} concentration for pTP-dCMP formation by cytoplasmic extract of Ad.4 infected cells.

1ul of cytoplasmic extract (4.2ug protein) was incubated with 0.5ug p4A2 linearized with EcoRI under standard reaction conditions (section 2:16) except that both the ATP and $MgCl_2$ concentrations were varied from 0-6mM. In each track both concentrations were equal. An autoradiograph of the part of the gel containing the pTP-dCMP complex, together with a graphical representation of the data, is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.

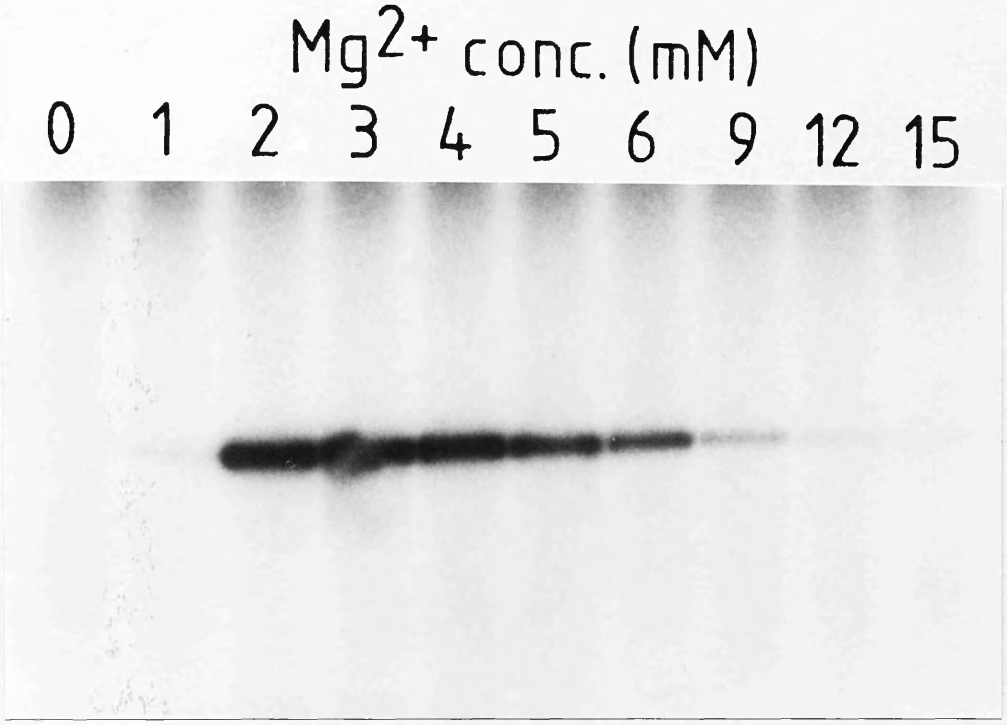

ATP/Mg2+ conc. (mM)
0 0·1 0·5 1 2 3 4 5 6
-80K
dCMP transfer (fmoles)
0·6
0·5
0·4
0·3
0·2
0·1
1 2 3 4 5 6
ATP / Mg2+ (mM)

in Figure 3:10 track 1 which shows that in the absence of added Mg^{2+} complex formation does occur. However in figure 3:10 track 1 no added ATP was present to chelate free Mg^{2+} and thereby inhibit the reaction. In figures 3:11 and 3:12 ATP was present at 3mM, a concentration well in excess of the possible endogenous Mg^{2+} level so free Mg^{2+}/Mn^{2+} ions would be at an extremely low level. The addition of EDTA to a reaction containing no added ATP or Mg^{2+} effectively eliminated pTP-dCMP complex formation (data not shown).

The effects of Mg^{2+} and Mn^{2+} on the formation of a pTP-dCMP complex were quite different. The activity in the presence of Mg^{2+} (Figure 3:11) increased sharply from 1mM, was maximal from 2-4mM and then slowly declined to a barely detectable level at 15mM. In contrast the activity in the presence of Mn^{2+} (Figure 3:12) was maximal at 1mM and then rapidly declined to become undetectable at 9mM. In a separate experiment (data not shown) the amount of complex formation was shown to increase gradually from 0-1mM thus eliminating the possibility that the Mn^{2+} peak is lower than 1mM. The interaction of ATP with Mn^{2+} may be different to the interaction with Mg^{2+}. The fact that maximal activity in the presence of Mn^{2+} occurs in the presence of a 3-fold excess of ATP may indicate that ATP is less able to chelate Mn^{2+} than Mg^{2+} and thus has less effect on the free Mn^{2+} pool.

Figure 3:11 Optimization of the Mg^{2+} concentration for pTP-dCMP formation by cytoplasmic extract of Ad.4 infected cells.

1ul of cytoplasmic extract (4.2ug protein) was incubated with 0.5ug p4A2 linearized with EcoRI under standard reaction conditions (section 2:16) except that the concentration of $MgCl_2$ was varied from 0-15mM. The ATP concentration was maintained at the standard level of 3mM throughout the experiment. An autoradiograph of the part of the gel containing the pTP-dCMP complex, together with a graphical representation of the data, is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.


Mg2+ conc. (mM)
0 1 2 3 4 5 6 9 12 15
-80K

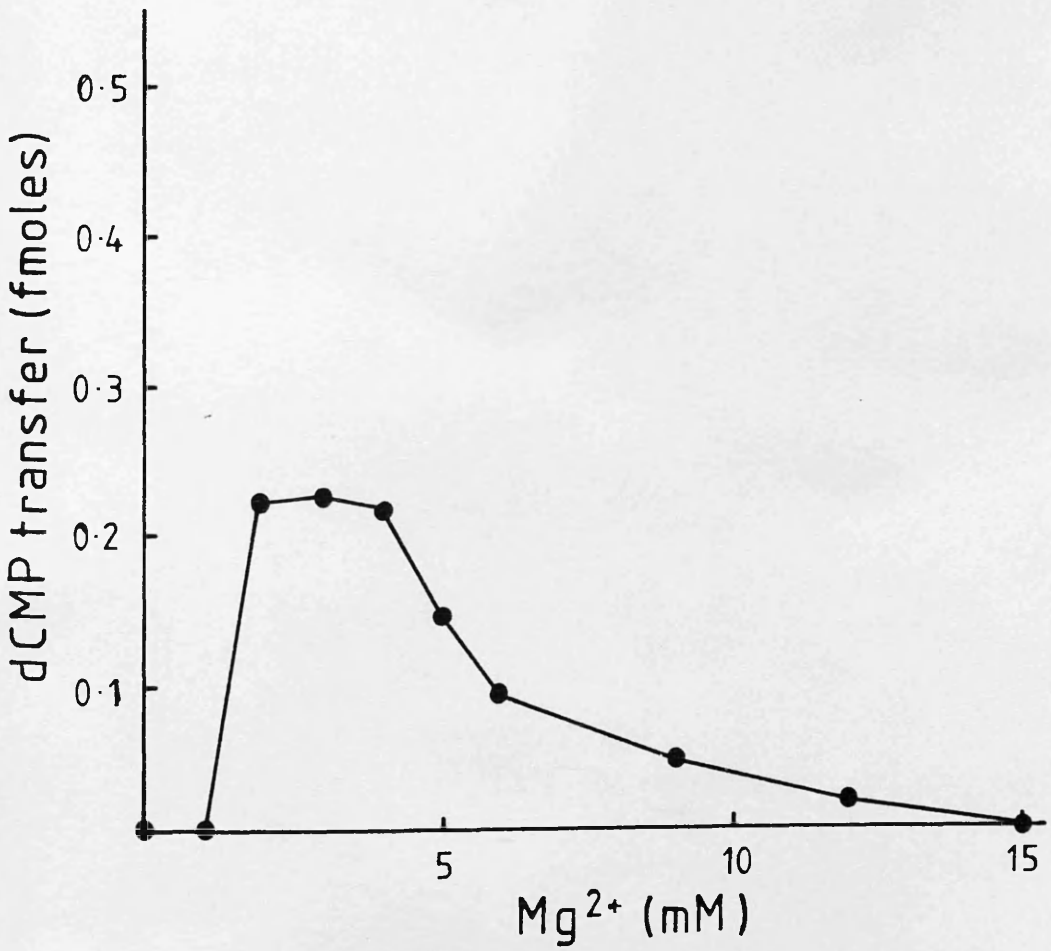
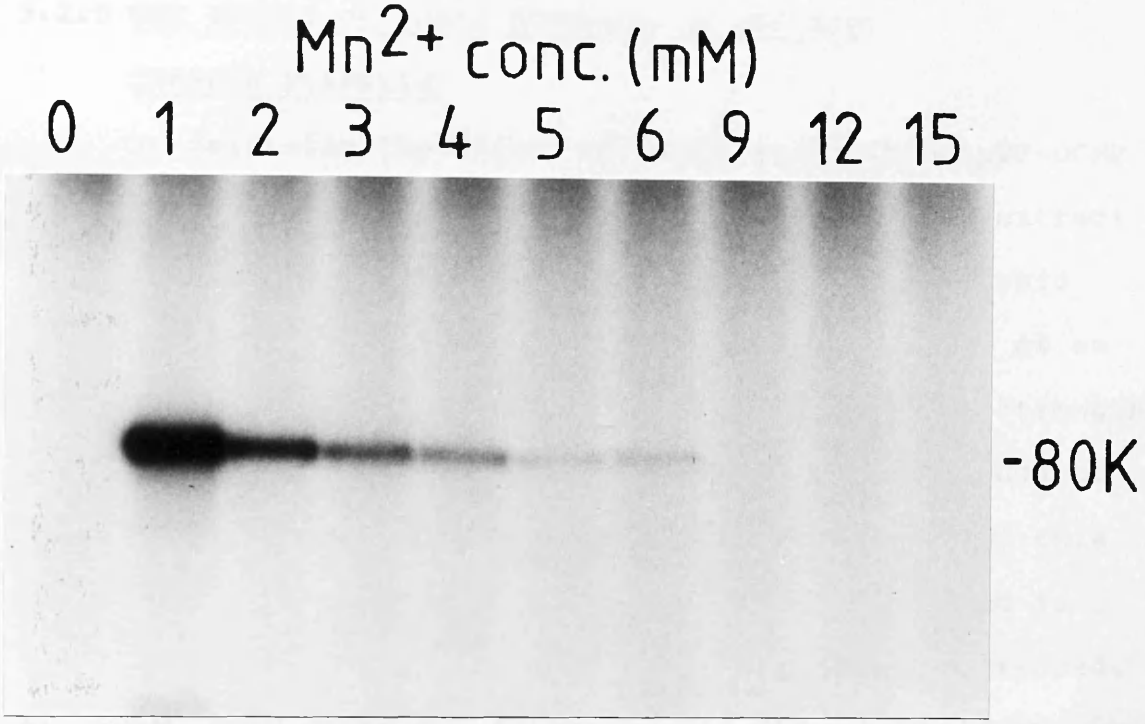
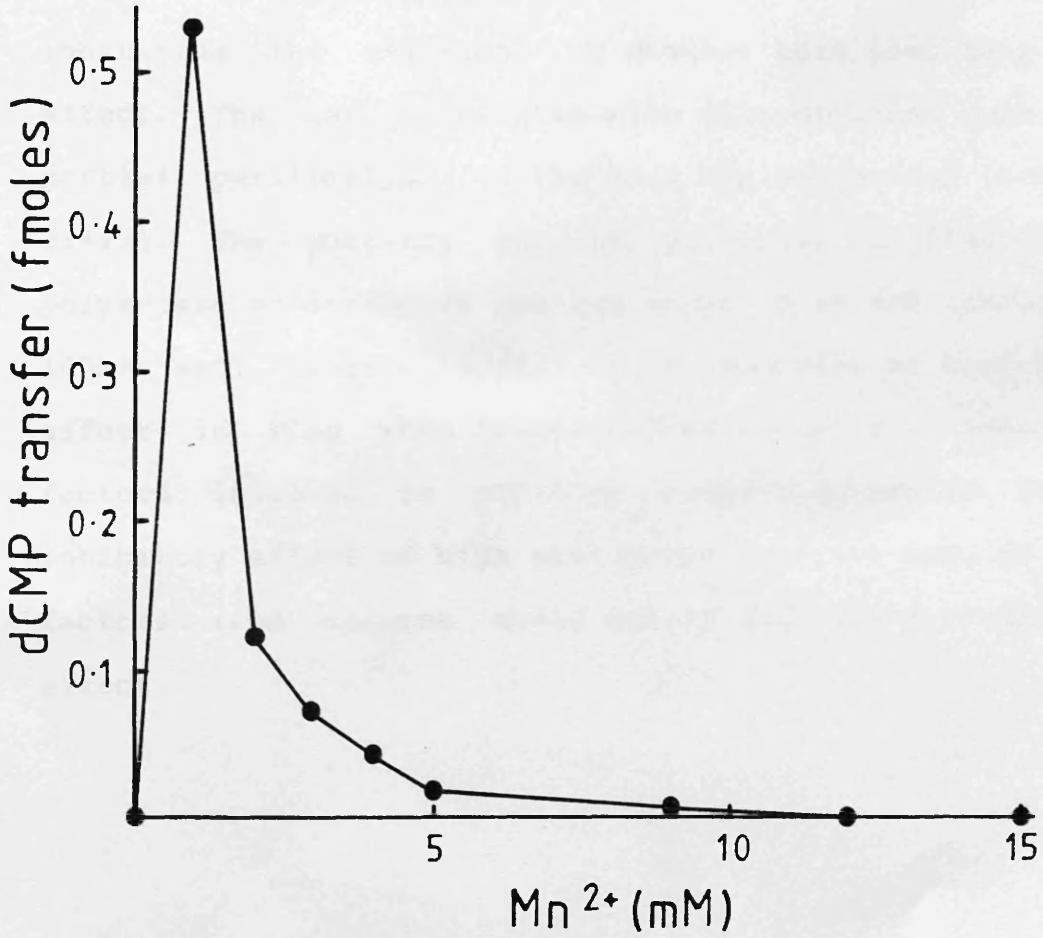

dCMP transfer (fmoles)
0·5
0·4
0·3
0·2
0·1
5
10
15
Mg2+ (mM)

Figure 3:12 Optimization of the Mn^{2+} concentration for pTP-dCMP formation by cytoplasmic extract of Ad.4 infected cells.

1ul of cytoplasmic extract (4.2ug protein) was incubated with 0.5ug p4A2 linearized with EcoRI under standard reaction conditions (section 2:16) except that $MnCl_2$ was substituted for $MgCl_2$ and the concentration of $MnCl_2$ was varied between 0-15mM. The ATP concentration was maintained at the standard level of 3mM throughout the experiment. An autoradiograph of the part of the gel containing the pTP-dCMP complex, together with a graphical representation of the data, is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.


Mn2+ conc. (mM)
0 1 2 3 4 5 6 9 12 15
-80K

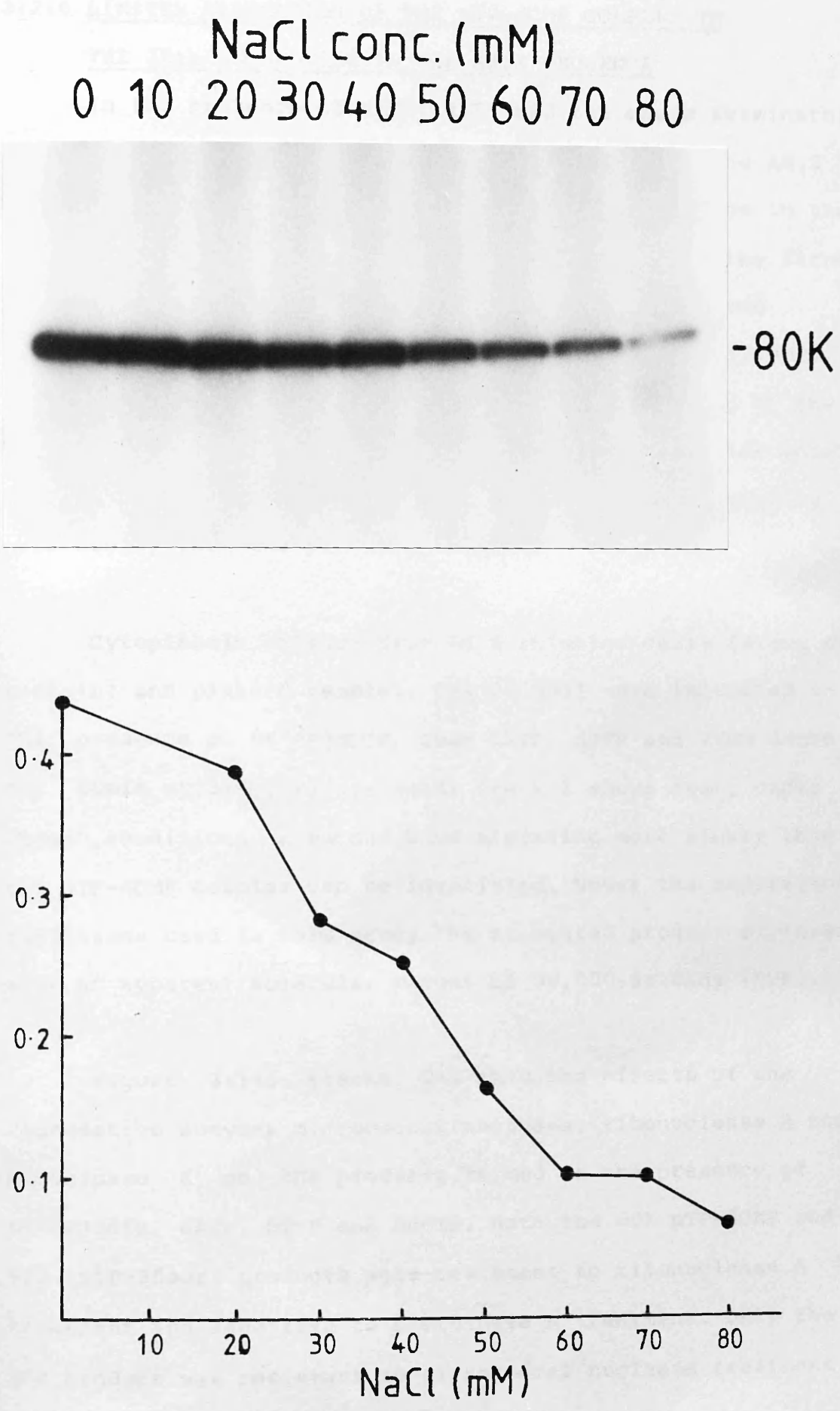

dCMP transfer (fmoles)
0·5
0·4
0·3
0·2
0·1
5
10
15
Mn 2+ (mM)

3:2:5 THE EFFECT OF IONIC STRENGTH ON pTP-dCMP COMPLEX FORMATION

To determine the effect of ionic strength on pTP-dCMP complex formation reactions containing cytoplasmic extract of Ad.4 infected cells (4.2ug of protein) and plasmid template DNA (0.5ug) were performed in the presence of an increasing concentration of NaCl. The initial ionic strength of the reaction mixture was 28mM, due to the presence of Tris/HCl and $MgCl_2$. Figure 3:13 shows the results of this experiment. The amount of complex formation declined in a linear fashion as the concentration of salt was increased. At 80mM NaCl complex formation was 85% inhibited in comparison to the level obtained in the absence of NaCl. It is not clear whether the anion or cation are responsible for inhibiting the reaction, or whether both ions have an effect. The data correlates with that obtained from the partial purification of the Ad.4 DNA polymerase (Section 3:4:1). The activity of the partially purified DNA polymerase on activated DNA was shown to be 90% inhibited at 100mM NaCl (Figure 3:26). It is important to bear this effect in mind when fractionating extracts to identify factors involved in pTP-dCMP complex formation. The inhibitory effect of high salt concentrations used to elute factors from columns could easily mask any stimulatory effect.

Figure 3:13 Optimization of the NaCl concentration for pTP-dCMP formation by cytoplasmic extract of Ad.4 infected cells.

1ul of cytoplasmic extract (4.2ug protein) was incubated with 0.5ug p4A2 linearized with EcoRI under standard reaction conditions (section 2:16) the ionic strength of which was varied by increasing the concentration of NaCl from 0-80mM. An autoradiograph of the part of the gel containing the pTP-dCMP complex, together with a graphical representation of the data, is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.


NaCl conc. (mM)
0 10 20 30 40 50 60 70 80
-80K
dCMP transfer (fmoles)
0·4
0·3
0·2
0·1
10
20
30
40
50
60
70
80
NaCl (mM)

3:2:6 LIMITED ELONGATION OF THE pTP-dCMP COMPLEX TO THE 30th NUCLEOTIDE IN THE Ad.4 SEQUENCE

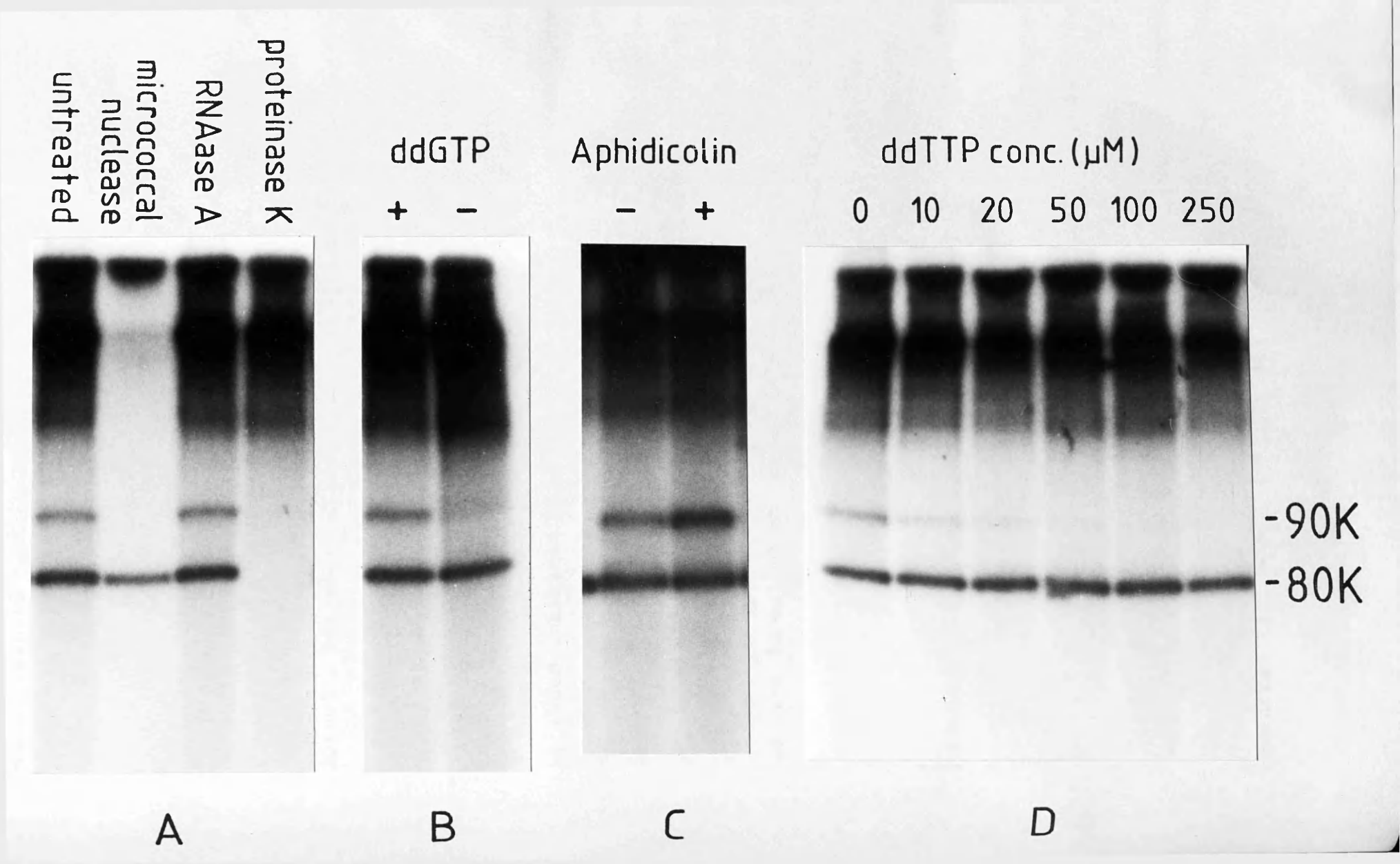
In the presence of dTTP, dATP and the chain terminator ddGTP, Ad.2 pTP-dCMP complex can be elongated by the Ad.2 polymerase (Lichy et al,1982) to the first dG residue in the sequence, the 26th nucleotide. In the case of Ad.4 the first dG residue is the 30th nucleotide from the 5' end (Hay,1985b; Tokunaga et al,1982,1986; Stillman et al,1982). The product of this reaction is a complex consisting of the 80K pTP covalently bound to a oligodeoxyribonucleotide which can be detected as a distinct band on SDS-PAGE, migrating more slowly than the pTP-dCMP complex.

Cytoplasmic extract from Ad.4 infected cells (4.2ug of protein) and plasmid template DNA (0.5ug) were incubated in the presence of (α^{32}P)dCTP, 50uM dATP, dTTP and 20uM ddGTP for 60min at 37^{o}C. Figure 3:14a track 1 shows that, under these conditions, a second band migrating more slowly than the pTP-dCMP complex can be identified. Under the separation conditions used in this study the elongated product migrated with an apparent molecular weight of 90,000-daltons (90K).

Figure 3:14a tracks 2-4 show the effects of the degradative enzymes micrococcal nuclease, ribonuclease A and proteinase K on the products formed in the presence of (α^{32}P)dCTP, dATP, dTTP and ddGTP. Both the 80K pTP-dCMP and 90K pTP-30mer products were resistant to ribonuclease A treatment and sensitive to proteinase K treatment. Only the 80K product was resistant to micrococcal nuclease treatment.

Figure 3:14 Limited elongation of the pTP-dCMP complex to the 30th nucleotide in the Ad.4 sequence.

1ul of cytoplasmic extract from Ad.4 infected cells (4.2ug protein) was incubated with 0.5ug p4A2 linearized with EcoRI in the presence of $3uC_i$ ($\alpha^{32}P$)dCTP (specific activity $3000C_i$/mmole), 50uM dATP, dTTP and 20uM ddGTP as described in section 2:17. Following termination of the reaction the products were processed directly for electrophoresis without micrococcal nuclease treatment (apart from 3:13a, track 2). Reactions contained additional components (ddTTP and aphidicolin) as indicated. The reaction products in 3:13a tracks 3 and 4 were treated with 50ug/ml RNase A or 100ug/ml proteinase K respectively for 30min. at 37°C before processing for electrophoresis. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex and 90K indicates the position of the 90,000 molecular weight pTP-30mer complex.


untreated
micrococcal nuclease
RNAase A
proteinase K
ddGTP
+
−
Aphidicolin
−
+
ddTTP conc. (μM)
0
10
20
50
100
250
-90K
-80K
A
B
C
D

These results confirm the predictions that the 90K product consists of an oligodeoxyribonucleotide chain linked to an 80K protein molecule and that the 80K product is a protein covalently linked to a single deoxyribonucleotide.

Figure 3:14b demonstrates that in the absence of the chain terminator ddGTP very little pTP-30mer product was formed but more radio-activity accumulated at the top of the gel. This was presumably due to further elongation using trace amounts of endogenous dGTP in the extract.

As demonstrated in Figures 3:25 and 3:26 aphidicolin at 100uM inhibited Ad.4 DNA polymerase activity on activated DNA by 50%. Figure 3:14c shows that in contrast aphidicolin had no effect on the limited elongation of pTP-dCMP complex to the 30th nucleotide. Figure 3:14d shows the effect of increasing concentrations of a second chain terminator ddTTP on the formation of the pTP-dCMP and pTP-30mer complexes. Whereas the formation of the pTP-dCMP complex was unaffected by the presence of a second chain terminator, pTP-30mer production was severely inhibited. Elongation of pTP-dCMP to the first dG residue by the Ad.4 DNA polymerase is thus more sensitive to ddTTP inhibition, but less sensitive to aphidicolin, than Ad.4 DNA polymerase activity on activated DNA (figure 3:26). These results can be partly explained by the specificity of the assay: The Ad.4 DNA polymerase is likely to be able to re-initiate at another single-stranded gap on activated DNA after chain termination whereas if this occurred it would not be detected by the assay for

elongation of the pTP-dCMP complex. The absence of detectable intermediate products (with apparent M.W.'s between 80K and 90K) suggests that chain termination by ddTTP occurs at an early stage in elongation, possibly at the first dT residue, the third nucleotide in the sequence. The effect of DBP and other replication proteins not present in the partially purified Ad.4 DNA polymerase fraction may explain the differences in aphidicolin sensitivity.

In all the experiments in Figure 3:14 only a proportion of the pTP-dCMP complex formed supported elongation to the 30th nucleotide. The reason for this is unclear. One possible explanation is that a proportion of pTP-dCMP complex dissociates from the template and thus is unable to prime elongation. Alternatively exonuclease activity within the extract could degrade elongated strands.

3:3 THE DNA TEMPLATE REQUIREMENTS FOR THE FORMATION OF A pTP-dCMP COMPLEX IN VITRO

3:3:1 CHARACTERIZATION OF THE ABILITY OF PLASMID TEMPLATES TO SUPPORT pTP-dCMP COMPLEX FORMATION

To determine whether complex formation could occur on templates in which the Ad.4 origin fragment was not at the molecular end assays for the formation of a pTP-dCMP complex were performed with 0.5ug of either circular p4A2 or p4A2 cleaved with various restriction enzymes: EcoR1 cleaves once in p4A2 at a site adjacent to the Ad.4 terminus, producing a linear molecule which at one end is identical to the

terminal 140b.p. of Ad.4 apart from a 4b.p. 5' overhang. BamH1 cleaves once at the other end of the insert and Nde1 cleaves once within the pUC8 backbone 230b.p. from the EcoR1 site (see Figure 3:15). Figure 3:16 tracks 1-4 demonstrate that the Ad.4 terminal fragment was only active for pTP-dCMP complex formation when located in the correct orientation at the terminus of a linear molecule (p4A2 cleaved with EcoR1). Tracks 5-8 demonstrate that the sequences required for complex formation are contained within the Ad.4 fragment; no complex formation was detected in the presence of either circular or EcoR1, BamH1 or Nde1 cleaved pUC8. These negative controls rule out the possibility that either pUC8 sequences or the 5' overhang produced by EcoR1 cleavage supported complex formation.

To determine the effect of template concentration on the level of pTP-dCMP complex formation reactions were performed in the presence of a constant amount of cytoplasmic extract of Ad.4 infected cells (4.2ug of protein) and a variable amount of plasmid template DNA (0-200ng). The results are shown in figures 3:17 and 3:18. At very low template concentration pTP-dCMP complex formation was minimal. Above 12.5ng per assay the amount of pTP-dCMP complex formed increased linearly to 50ng per assay and then reached a plateau which was maintained up to 200ng per assay. This plateau is presumably maintained as far as 500ng per assay, the level used for most assays, as the average amount of pTP-dCMP complex formed in other experiments containing 4.2ug of cytoplasmic protein and 0.5ug plasmid

Figure 3:15 The structure of plasmid p4A2.

Plasmid p4A2 is identical to p4A1 - a plasmid constructed and described previously (Hay,1985b). The plasmid consists of an 140b.p. terminal SalI fragment of Ad.4 blunt end ligated into EcoRI cleaved, T4 DNA polymerase treated pUC9. This figure shows the single cleavage sites of the three restriction endonucleases used in this study. EcoRI cleaves at the Ad.4 terminus, BamHI cleaves at the other end of the Ad.4 fragment and NdeI cleaves in the backbone 231b.p. from the EcoRI site.

Structure of p4A2.
(not drawn to scale)

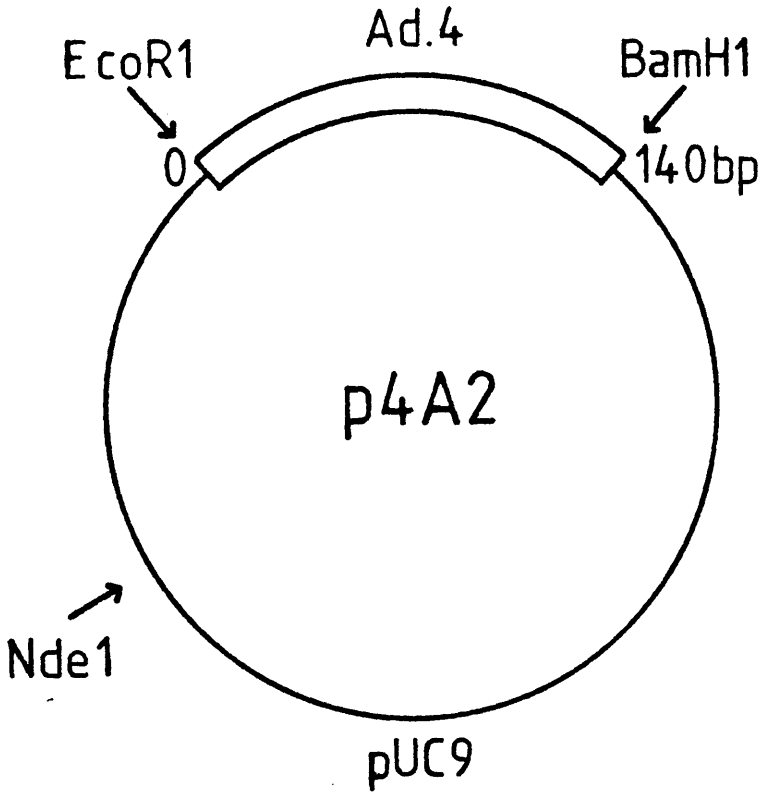
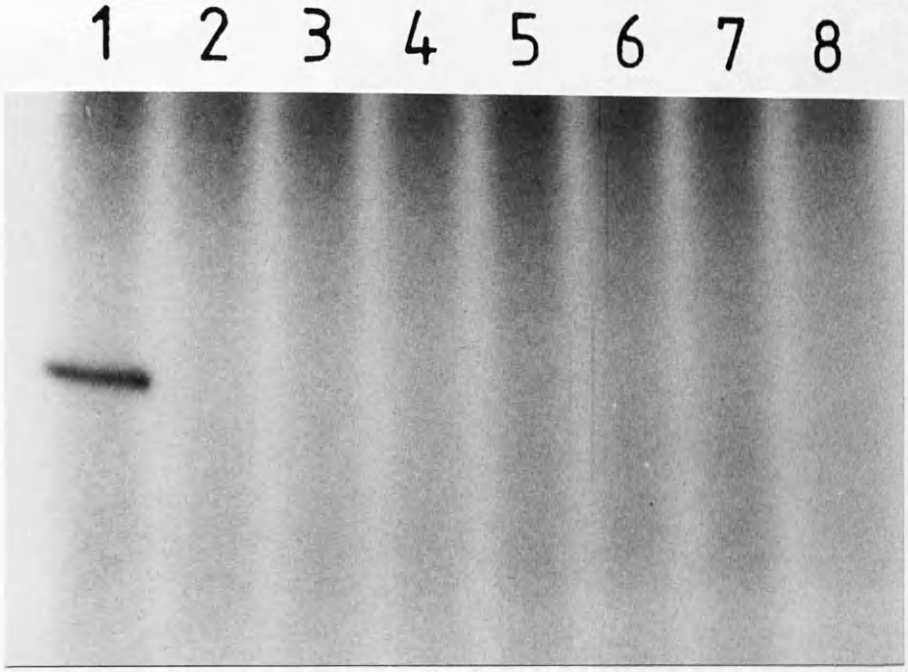


Figure 3:16 Effect of template configuration on the ability of plasmid templates to support pTP-dCMP complex formation.

Plasmid DNA (either p4A2 or pUC8) treated with restriction endonucleases as indicated or uncut were extracted once with an equal volume of a 1:1 mixture of phenol:chloroform, once with an equal volume of chloroform, ethanol precipitated and resuspended in distilled water. 100ng aliquots of each sample were incubated with 1ul of cytoplasmic extract (4.2ug protein) under standard reaction conditions (section 2:16). 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.



TRACK	TEMPLATE
1	p4A2/EcoR1
2	p4A2/BamH1
3	p4A2/Nde1
4	p4A2/uncut
5	pUC8/EcoR1
6	pUC8/BamH1
7	pUC8/Nde1
8	pUC8/uncut

Figure 3:17 Effect of increasing the plasmid template concentration on pTP-dCMP complex formation.

1ul of cytoplasmic extract (4.2ug protein) was incubated under standard reaction conditions (section 2:16) in the presence of increasing amounts (0-200ng) of EcoRI linearized plasmid DNA (either p4A2 or pUC8). 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.

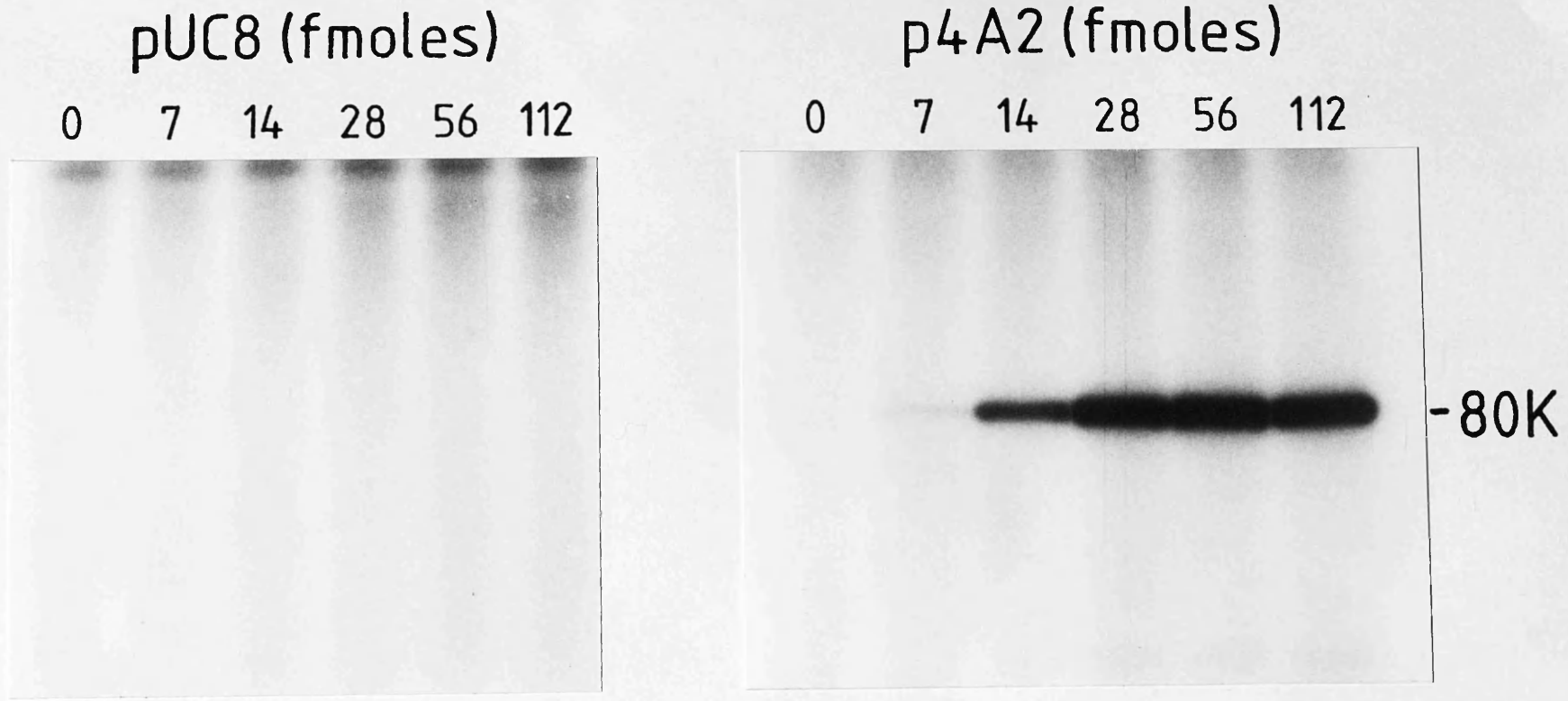
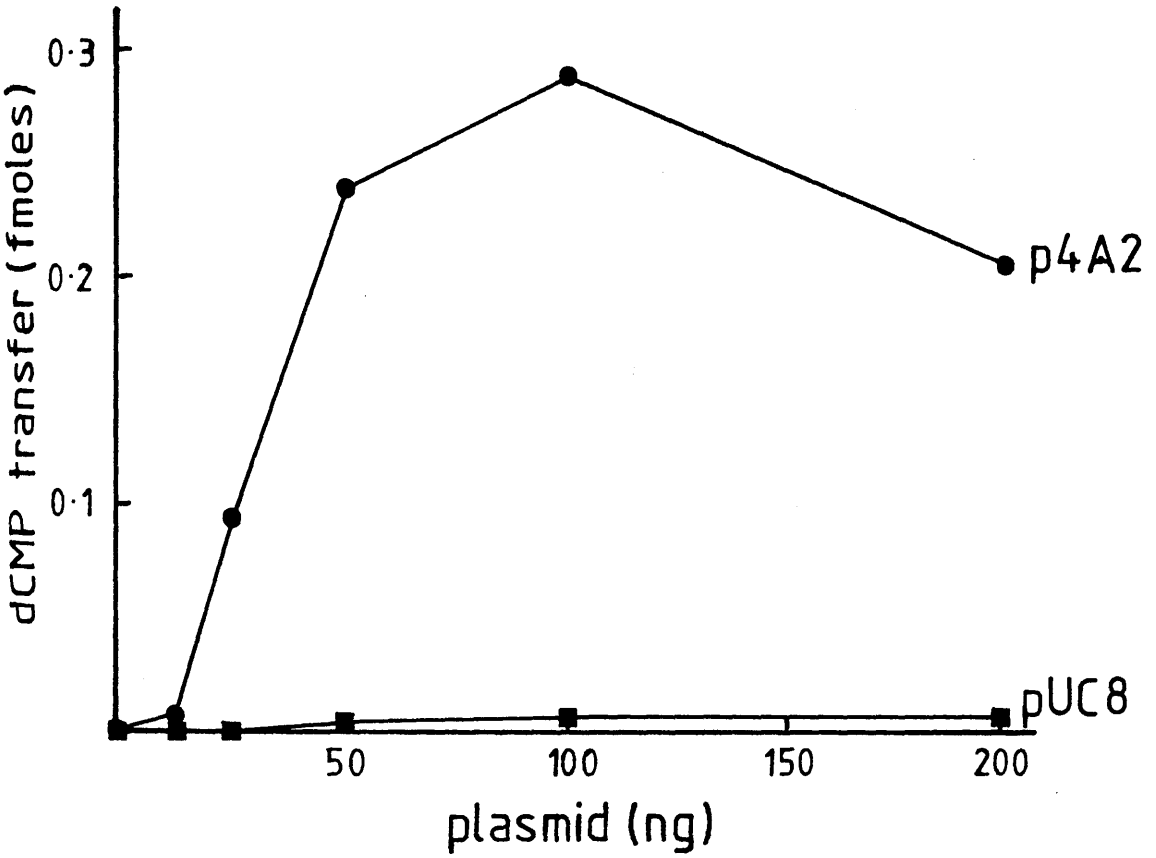

pUC8 (fmoles)
0
7
14
28
56
112
p4A2 (fmoles)
0
7
14
28
56
112
-80K

Figure 3:18 Graphical representation of the effect of increasing the plasmid template concentration on pTP-dCMP complex formation.

The data in figure 3:17 is presented as a graph of amount of complex formed plotted against the amount of template present in the reaction.


0·3
0·2
0·1
dCMP transfer (fmoles)
p4A2
pUC8
50
100
150
200
plasmid (ng)

DNA was 0.26fmoles. The apparent decline in pTP-dCMP complex formation between 100 and 200ng per assay is thus most likely due to experimental variation.

Plasmid pUC8 linearized with EcoR1 was included as a negative control in these experiments. No pTP-dCMP complex formation was detected in the presence of this plasmid under any conditions, again emphasizing that the DNA sequence requirements for pTP-dCMP complex formation are located within the 140b.p. Ad.4 terminal fragment.

3:3:2 THE DNA SEQUENCE REQUIREMENTS FOR THE FORMATION OF A pTP-dCMP COMPLEX

To determine the precise DNA sequence requirements for the formation of a pTP-dCMP complex *in vitro* plasmids containing deletions extending into the Ad.4 or Ad.2 ITR's were examined for their ability to support pTP-dCMP formation. The p4AnΔ plasmids contain a single copy of sequences from the Ad.4 ITR where n represents the number of bases of the ITR remaining (Hay,1985b). The pHRnΔ plasmids were constructed and described previously (Hay,1985a,b) and contain one copy of sequences derived from the ITR, where n is the number of nucleotides from the terminus present. pHR1 is the parental plasmid of this series and contains the complete Ad.2 ITR (102b.p.). Figure 3:19 shows the results of an experiment in which reactions were performed with cytoplasmic extract of Ad.4 infected cells (4.2ug of protein) and 0.5ug of each of the p4AnΔ plasmids cleaved with EcoR1. Figure 3:20 shows the results of a similar experiment involving the pHRnΔ plasmids.

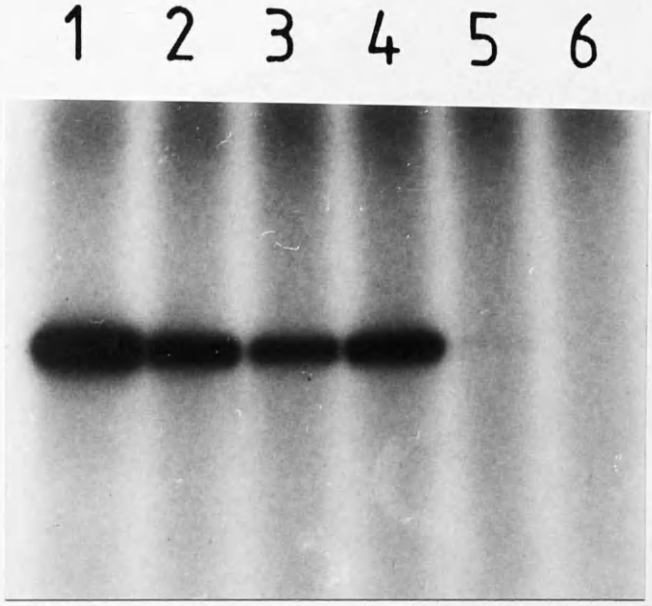
The terminal 18b.p. of the Ad.2 and Ad.4 ITR's are identical.

The results show clearly that the terminal 18b.p. of either the Ad.4 or Ad.2 ITR are both necessary and sufficient as a template for the formation of a pTP-dCMP complex by cytoplasmic extract of Ad.4 infected cells. A plasmid containing less than 18b.p. from the Ad.2 ITR (pHR7Δ), or a control plasmid containing no adenovirus sequences (pUC8) did not support any pTP-dCMP complex formation. Small variations in the levels of complex formation can be attributed to small differences in plasmid concentrations. These results are consistent with those defining the minimal sequence requirements for Ad.4 origin function in vivo (Hay,1985b).

The stimulatory fraction from uninfected nuclei described in section 3:4:2 also exerted it's effect in the presence of a plasmid template containing only 18b.p. from the Ad.4 origin (data not shown). It seems likely that host cell factor(s) are interacting with the terminal 18b.p., either in a sequence specific manner or non-specifically, to enhance pTP-dCMP complex formation. Purified nuclear factor III, a protein that enhances Ad.2 pTP-dCMP complex formation (Pruijn et al,1986b) and which has a high affinity binding site on the Ad.4 ITR (Hay, unpublished observations), did not enhance Ad.4 pTP-dCMP complex formation either in the presence or absence of it's binding site.

Figure 3:19 The DNA sequence requirements for the formation of a pTP-dCMP complex by cytoplasmic extract of Ad.4 infected cells. I. Use of Ad.4 ITR sequences as a template.

The p4AΔn series of plasmids were constructed and described previously (Hay,1985b). The plasmids consist of fragments of the Ad.4 ITR generated by Bal31 digestion ligated into EcoRI/BamHI cleaved pUC9. 0.5ug of each plasmid was cleaved with EcoRI to expose the Ad.2 terminus and, after phenol extraction and ethanol precipitation, incubated under standard reaction conditions (section 2:16) with 1ul of cytoplasmic extract of Ad.4 infected cells (4.2ug of protein). An autoradiograph of the part of the gel containing the pTP-dCMP complex, together with the structure of the deletions, is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex and I indicates the minimal Ad.4 origin in vivo. NFIII indicates the binding site for nuclear factor III a sequence that is not required for Ad.2 replication in vivo but enhances Ad.2 initiation in vitro, and is also present on the Ad.4 ITR.


1 2 3 4 5 6
-80K

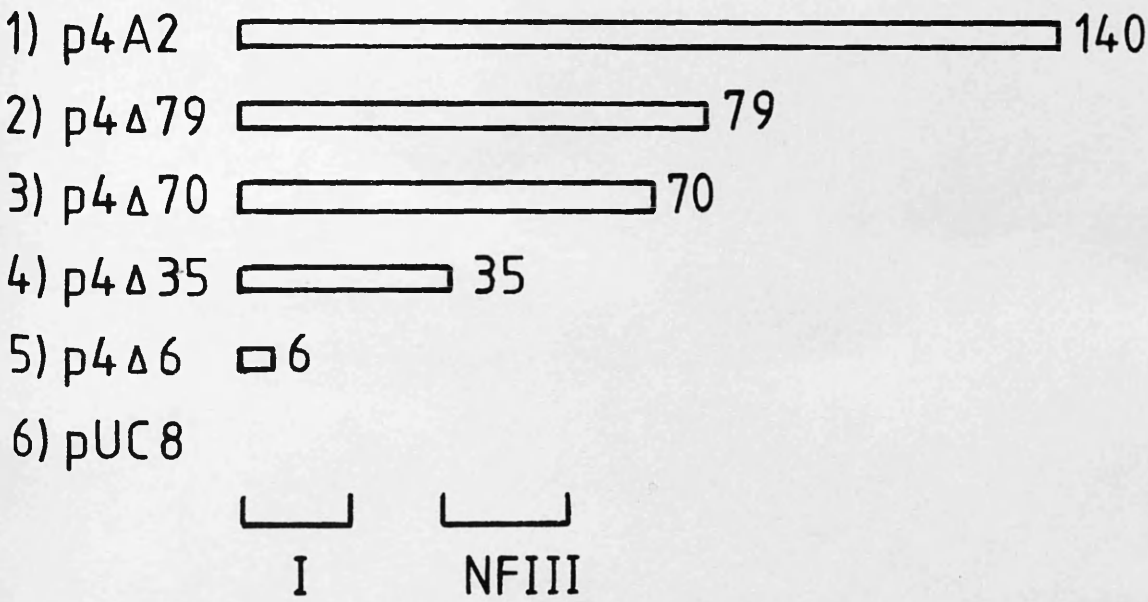
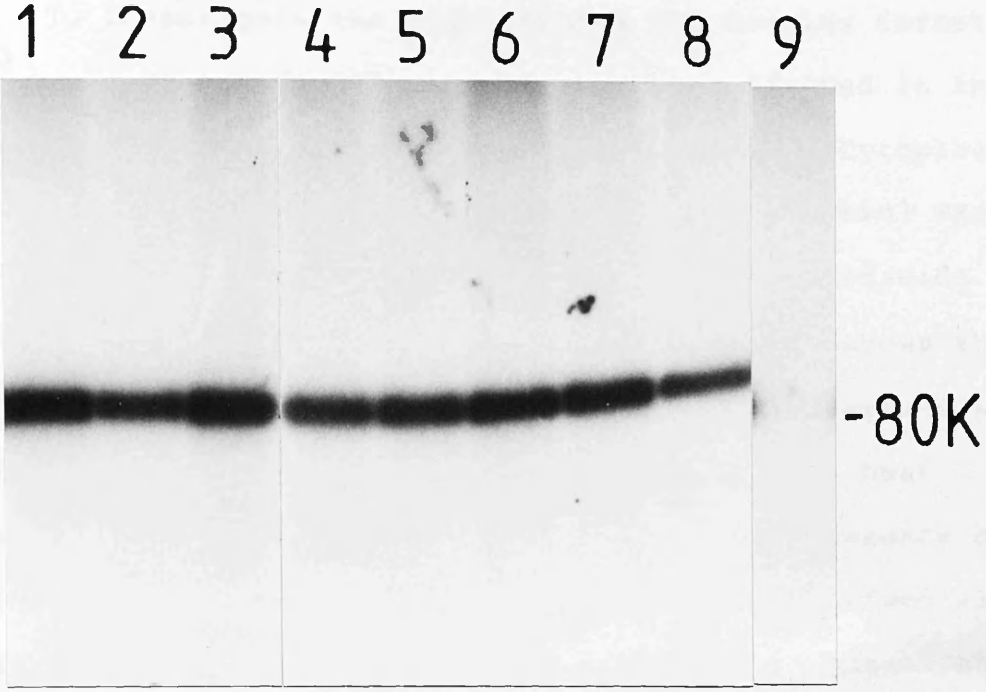
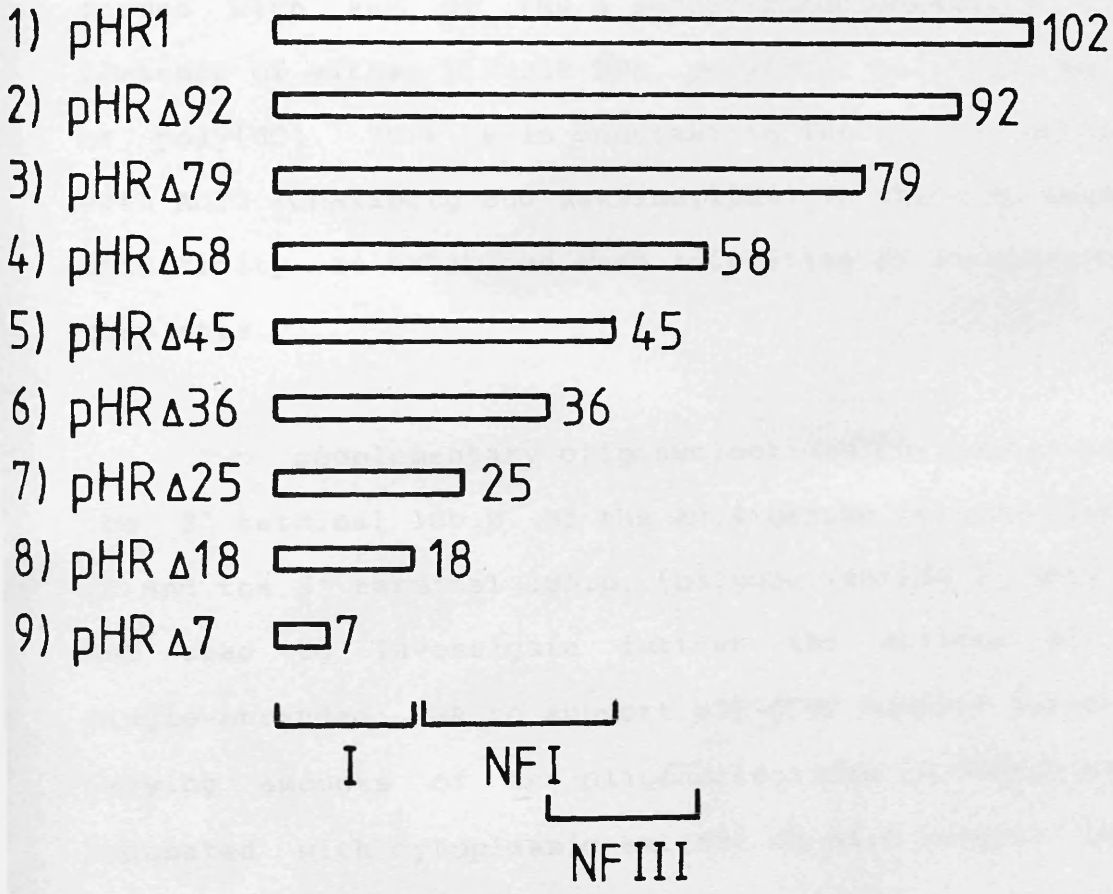

1) p4A2
140
2) p4Δ79
79
3) p4Δ70
70
4) p4Δ35
35
5) p4Δ6
6
6) pUC8
I
NFIII

Figure 3:20 The DNA sequence requirements for the formation of a pTP-dCMP complex by cytoplasmic extract of Ad.4 infected cells. II. Use of Ad.2 ITR sequences as a template.

The pHRΔn series of plasmids was constructed and described previously (Hay,1985a,b). The plasmids consist of fragments of the Ad.2 ITR generated by Bal31 digestion ligated into EcoRI/BamHI cleaved pUC9. 0.5ug of each plasmid was cleaved with EcoRI to expose the Ad.2 terminus and, after phenol extraction and ethanol precipitation, incubated under standard reaction conditions (section 2:16) with 1ul of cytoplasmic extract of Ad.4 infected cells (4.2ug of protein). An autoradiograph of the part of the gel containing the pTP-dCMP complex, together with the structure of the deletions, is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex and I indicates the minimal Ad.4 origin in vivo, NFI indicates the binding site for nuclear factor I a sequence required for Ad.2 replication in vivo and which enhances Ad.2 replication in vitro but is not present on Ad.4 ITR. NFIII indicates the binding site for nuclear factor III a sequence that is not required for Ad.2 replication in vivo but enhances Ad.2 initiation in vitro, and is also present on the Ad.4 ITR.


1 2 3 4 5 6 7 8 9
-80K


1) pHR1
102
2) pHRΔ92
92
3) pHRΔ79
79
4) pHRΔ58
58
5) pHRΔ45
45
6) pHRΔ36
36
7) pHRΔ25
25
8) pHRΔ18
18
9) pHRΔ7
7
I
NFI
NFIII

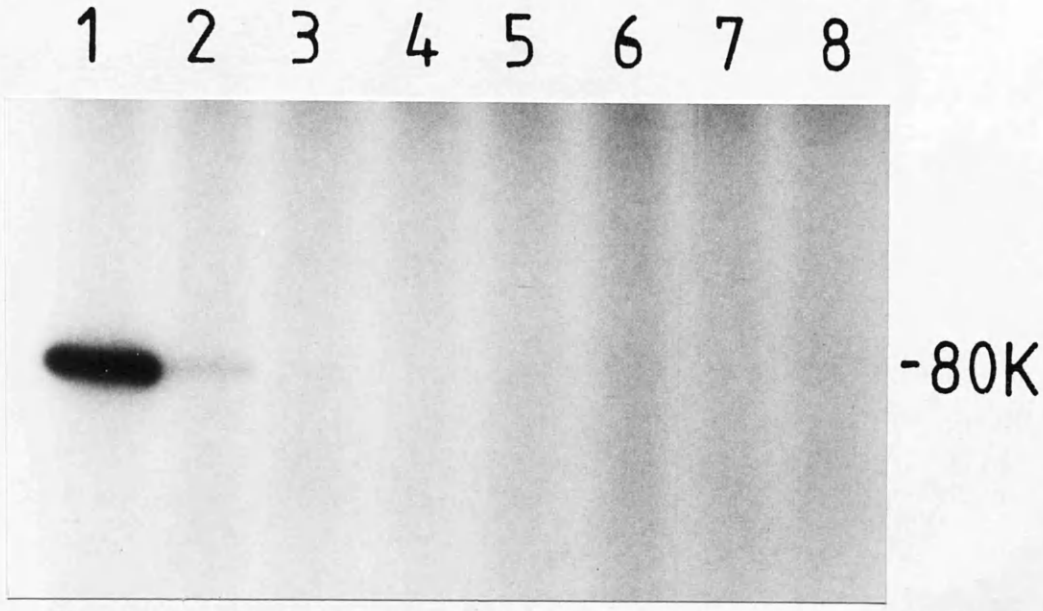
3:3:3 THE USE OF SINGLE-STRANDED DNA AS A TEMPLATE FOR pTP-dCMP COMPLEX FORMATION

To investigate the requirements for complex formation on single-stranded DNA, reactions were performed in the presence of native and heat denatured plasmids. Cytoplasmic extract of Ad.4 infected cells (4.2ug of protein) was incubated in the presence of 0.5ug of native plasmids or plasmids heated at 100°C for 5min. Figure 3:21 shows that the efficiency of complex formation on p4A2 linearized with EcoR1 was reduced approximately 24-fold by heat denaturation. No complex was formed in the presence of either native or denatured forms of p4A2 linearized with BamH1 or Nde1. This indicated that, even on single-stranded templates, complex formation required the Ad.4 sequences to be located at the end of a linear molecule. No complex was formed with any of the 4 deoxyribonucleotides in the presence of either M13mp18 DNA, poly(dG), poly(dA), poly(dT) or poly(dC). This is in contrast to the results obtained with Ad.2 (Challberg and Rawlins,1984) in which no sequence specificity was exhibited when initiating on single-stranded templates.

Two complementary oligonucleotides corresponding to the 3' terminal 18b.p. of the Ad.4 genome (oligonucleotide 1) and the 5' terminal 18b.p. (oligonucleotide 2) were made and used to investigate further the ability of single-stranded DNA to support pTP-dCMP complex formation. Varying amounts of the oligonucleotides (0-500ng) were incubated with cytoplasmic extract of Ad.4 infected cells

Figure 3:21 Ability of native and denatured plasmids to support pTP-dCMP complex formation.

Linearized plasmid DNA was denatured by heating to 100°C for 2min. and placed on ice. 0.5ug aliquots of either native or denatured plasmid DNA as indicated were incubated under standard reaction conditions (section 2:16) with 1ul of cytoplasmic extract of Ad.4 infected cells (4.2ug of protein). An autoradiograph of the part of the gel containing the pTP-dCMP complex is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.



TRACK	TEMPLATE
1	native p4A2/EcoR1
2	denatured p4A2/EcoR1
3	native p4A2/BamH1
4	denatured p4A2/BamH1
5	native p4A2/Nde1
6	denatured p4A2/Nde1
7	native pUC8/EcoR1
8	denatured pUC8/EcoR1

Figure 3:22 Ability of single-stranded oligonucleotides to support pTP-dCMP formation.

Two complementary oligonucleotides corresponding to the 3' and 5' terminal 18b.p. of the Ad.4 genome were synthesized on a Biosearch 8600 DNA synthesizer. For future use in constructing a plasmid containing multiple copies of this oligonucleotide for use in an affinity purification scheme for factors binding to the terminal 18b.p. of the Ad.4 genome, these oligonucleotides contained 3' and 5' sequences which would enable the double stranded oligonucleotide to be ligated in a head to tail configuration. Increasing amounts of single stranded oligonucleotides were incubated under standard reaction conditions (section 2:16) with 1ul of cytoplasmic extract of Ad.4 infected cells (4.2ug of protein). The sequence of the oligonucleotides used and an autoradiograph of the part of the gel containing the pTP-dCMP complex are presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.

1) 3'-AGTAGTAGTTATTATATGGCCTAG

2) 5'-GATCTCATCATCAATAATATACCG

Ad.4 sequence

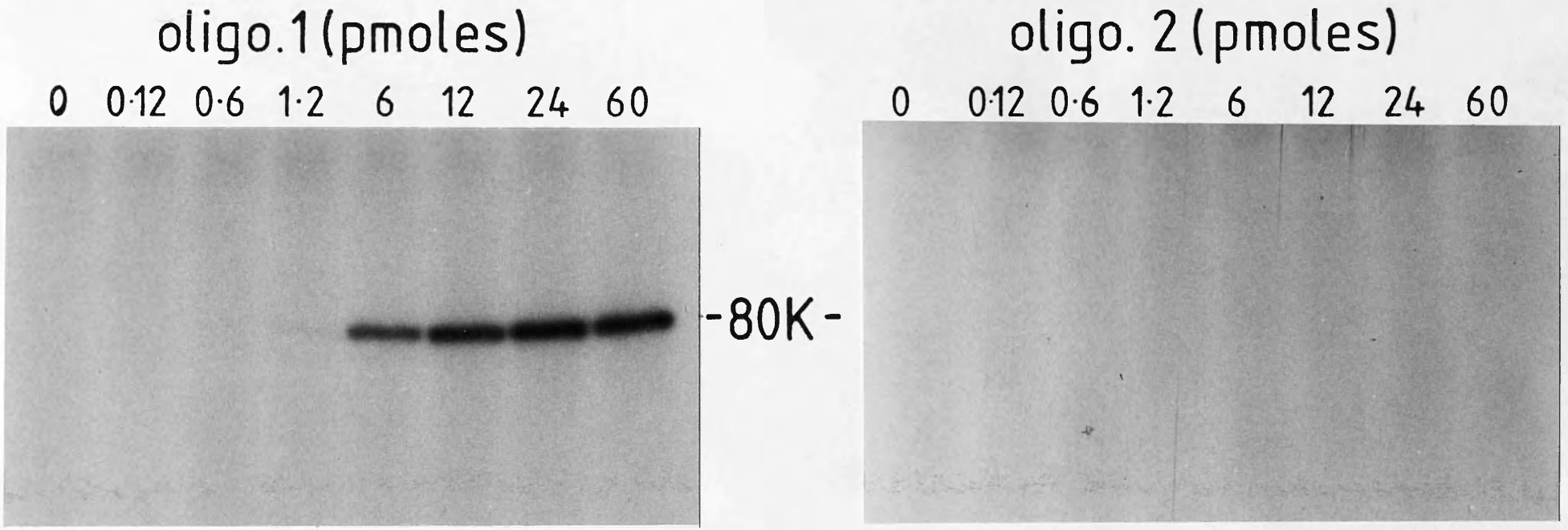
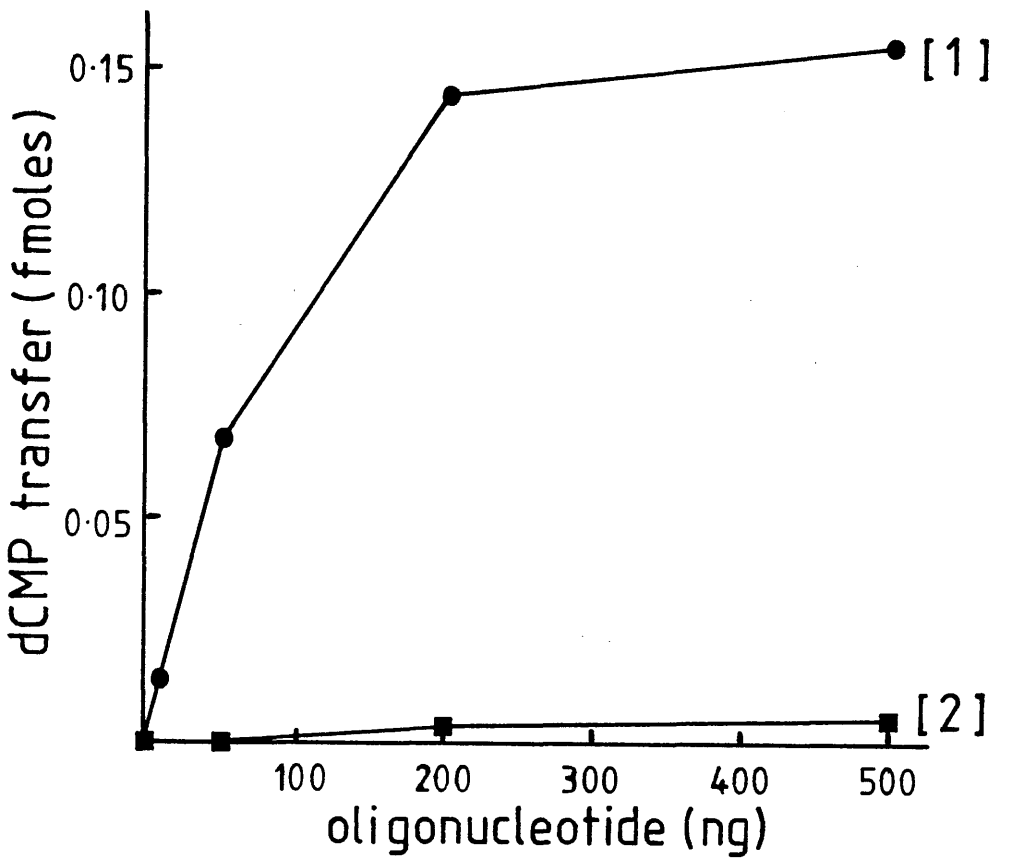


Figure 3:23 Graphical representation of the formation of a pTP-dCMP complex by single stranded oligonucleotides.

The data in figure 3:22 are presented as a graph of the amount of complex formed plotted against the amount of oligonucleotide present in the reaction.


0·15
0·10
0·05
dCMP transfer (fmoles)
[1]
[2]
100
200
300
400
500
oligonucleotide (ng)

(4.2ug of protein) (figures 3:22 and 3:23). Formation of a pTP-dCMP complex was detected in the presence of an oligonucleotide corresponding to the 18b.p. at the 3' terminus of Ad.4, whereas an oligonucleotide corresponding to the complementary strand did not support pTP-dCMP complex formation at detectable levels. Figure 3:22 shows that the efficiency of pTP-dCMP complex formation was extremely low, in the order of 600-fold less than native, EcoR1 linearized p4A2. The fact that, in comparison to plasmids, the molarity of the reaction with regard to template sequences could be extremely high when using oligonucleotides meant that this level of pTP-dCMP complex formation could be readily detected.

The finding that one single-stranded oligonucleotide was able to support pTP-dCMP complex formation and yet the complementary oligonucleotide did not was surprising. This result is unlikely to be explained by specific recognition of a single-stranded DNA sequence by DNA-binding proteins. The nucleotides in a single-strand are able to rotate in relation to each other making it very difficult for a protein to recognize a specific sequence. There are several more probable explanations to explain the findings:

Firstly it cannot be ruled out that the active oligonucleotide can adopt a secondary structure that could support pTP-dCMP complex formation, whereas the complementary oligonucleotide was unable to do so. Secondary structure formation could also involve the interaction of

the oligonucleotide with non-specific DNA binding proteins. The Ad.4 DBP may also have an, as yet unknown, role to play in the process.

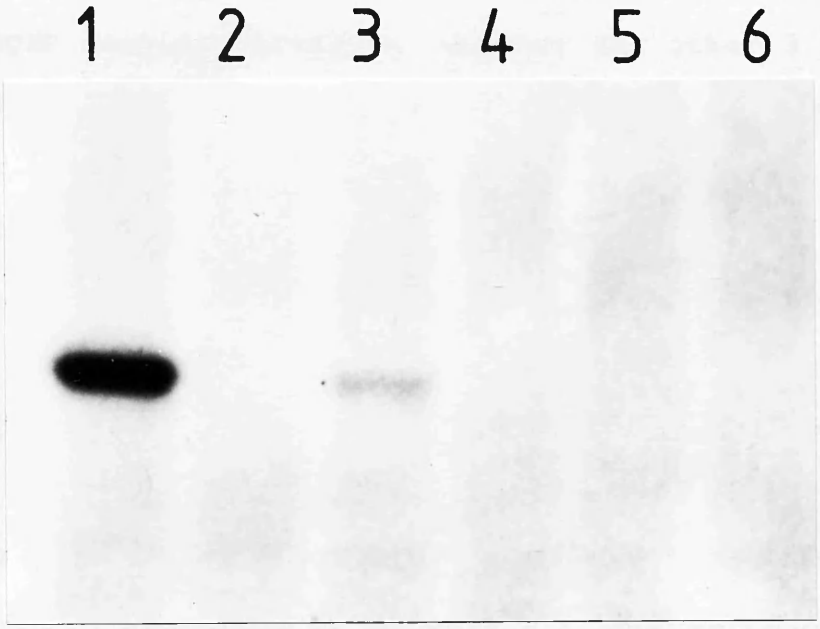
Secondly, examination of the sequences of the two oligonucleotides reveals that whereas the active oligonucleotide contains 5 internal dG residues at which pTP-dCMP complex formation could occur, the inactive oligonucleotide has none. It is possible that the pTP-pol complex and other replication proteins bind non-specifically to a single-stranded DNA molecule and then dCMP is transferred to the pTP at a dG residue near the binding site. The terminal dG residues of oligonucleotide 2 may not fulfill these requirements. This explanation is borne out to some extent by the data presented in figure 3:24.

Figure 3:24 shows the results of an experiment to determine the sequence elements on oligonucleotide 1 that were responsible for supporting pTP-dCMP complex formation. Four oligonucleotides (numbers 3-6) corresponding to the 3' terminal 18b.p. of the Ad.4 genome were constructed with G to A base changes at the positions indicated in figure 3:24 and 500ng of each oligonucleotide was incubated with cytoplasmic extract of Ad.4 infected cells (4.2ug of protein).

Substitution of the dinucleotide GG 18 bases from the 3' end with the dinucleotide AA (figure 3:24, track 3) clearly had a profound effect on the ability of the

Figure 3:24 Ability of mutated, single-stranded oligonucleotides to support pTP-dCMP complex formation.

Oligonucleotides corresponding to the 3' terminal 18b.p. of th Ad.4 genome were constructed with various G to A base changes as indicated. 500ng of each oligonucleotide was incubated under standard reaction conditions (section 2:16) with lul of cytoplasmic extract of Ad.4 infected cells (4.2ug of protein). An autoradiograph of the part of the gel containing the pTP-dCMP complex, together with the sequences of the oligonucleotides, is presented. 80K indicates the position of the 80,000 molecular weight pTP-dCMP complex.



1) 3′-AGTAGTAGTTATTATATGGCCTAG

2) 5′-GATCTCATCATCAATAATATACCG

3) 3′-AGTAGTAGTTATTATATAACCTAG

4) 3′-AATAATAATTATTATATAACCTAG

5) 3′-AATAATAATTATTATATGGCCTAG

6) 3′-AGTAATAATTATTATATGGCCTAG

(altered nucleotides underlined)

oligonucleotide to support pTP-dCMP complex formation, reducing it at least 10-fold. However substitution of the dG residues 5 and 8 bases from the 3' end completely abolished pTP-dCMP complex formation, whether the other 3 internal dG residues were present or not (figure 3:24, tracks 4-6). These data indicate that the choice of dG residues for pTP-dCMP complex formation on single-stranded templates is not entirely random. It is possible that secondary structure and other proteins associated with initiation may be involved in the positioning of the initiation event.

3:4 PARTIAL PURIFICATION OF FACTORS INVOLVED IN THE FORMATION OF AN AD.4 pTP-dCMP COMPLEX

3:4:1 PARTIAL PURIFICATION AND CHARACTERIZATION OF THE Ad.4 SPECIFIC DNA POLYMERASE FROM INFECTED CELLS

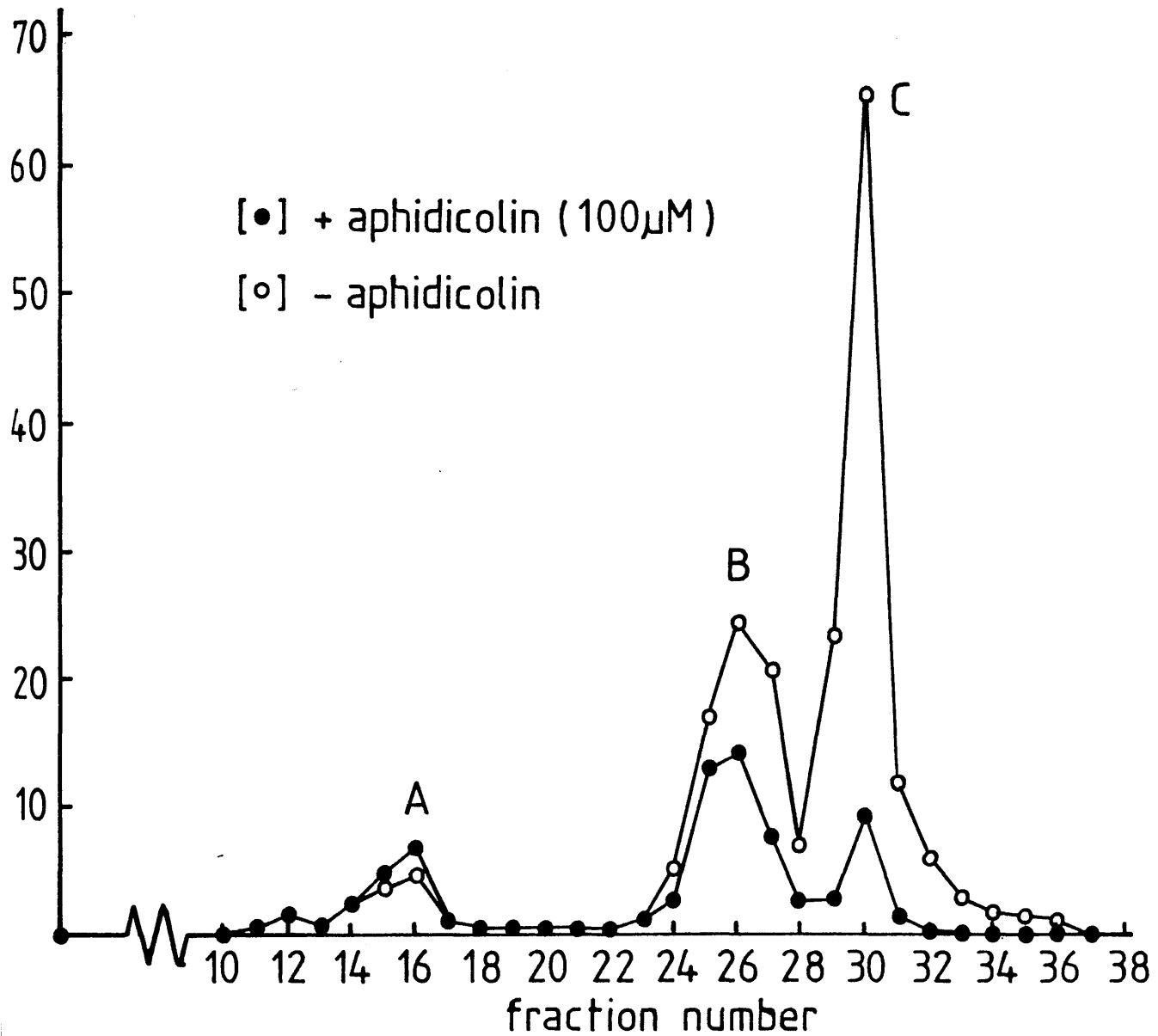
8l of HeLa spinner cells at a density of $4x10^5$ cells/ml. were infected with 50pfu/cell Ad.4 virus. The cells were treated with 5mM hydroxyurea from 2-22h.p.i. to inhibit viral DNA synthesis and extracts were made as described in Materials and Methods (Section 2:14). The clarified cytosol was adjusted to 50mM NaCl, 1mM DTT and 1mM EDTA and applied to a 50ml column of DEAE-sepharose equilibrated in buffer A containing 50mM NaCl. The column was washed with 100ml of buffer A containing 50mM NaCl and protein was eluted with buffer A containing 0.2M NaCl. 7ml fractions were taken, the absorbance at 280nm measured and peak protein-containing fractions pooled. Protein was precipitated by adding ammonium sulphate to 70% saturation,

resuspended in buffer A and dialysed. 5ml samples were then loaded on to a Pharmacia FPLC system fitted with a Mono-Q anion exchange column. The column was washed with 5ml buffer A and developed with a 20ml linear gradient from 0-0.35M NaCl followed by a 4ml linear gradient from 0.35-1M NaCl, both in buffer A. The column was finally washed with 4ml buffer A containing 1M NaCl. 1ml fractions were collected throughout the fractionation.

Fractions were assayed for DNA polymerase activity using an activated DNA template as described in Materials and Methods both in the presence and absence of aphidicolin (0.1mM) (Figure 3:25). Three peaks of DNA polymerase activity were obtained which differed greatly in their sensitivity to aphidicolin. To identify the virus-specific DNA polymerase the effect of various inhibitors on the activity of each of the peaks was determined. 5 inhibitors were used in this study: dideoxythymidine triphosphate (ddTTP) and 1-β-D-arabinofuranosylcytosine 5'-triphosphate (ara-CTP) are nucleotide analogues which cause chain termination. Aphidicolin is a tetracyclic diterpenoid known to inhibit purified DNA polymerase alpha and the growth of herpes simplex and vaccinia viruses (Longiaru et al,1979) as a result of inhibition of the viral DNA polymerases. N-ethylmaleimide (NEM) binds to sulphydryl groups and is known to inhibit both DNA polymerases alpha and gamma (Weissbach,1977). 100mM NaCl inhibits only DNA polymerase alpha but not DNA polymerases beta or gamma (Weissbach,1977). The results are shown in Figure 3:26.

Figure 3:25 FPLC fractionation of DNA polymerase activity from the cytoplasm of Ad.4 infected cells.

Cytoplasmic extract from Ad.4 infected cells was fractionated as described in the text and 2ul aliquots of each fraction were assayed for DNA polymerase activity on gapped duplex DNA both in the presence and absence of aphidicolin at 100uM. Fractions 16 (peak A), 26 (peak B) and 30 (peak C) were taken for further characterization (figure 3:26).


70
60
50
40
30
20
10
[•] + aphidicolin (100µM)
[○] - aphidicolin
A
B
C
10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
fraction number

DNA polymerase beta has been shown not to bind to DEAE-cellulose either in phosphate buffer at pH 7.5 (Edenberg et al,1978) or Tris buffer at pH 7.7 (Longiaru et al,1979). It was probable therefore that the material loaded on to the Mono-Q column did not contain any DNA polymerase beta and that the three peaks of DNA polymerase activity obtained represented alpha, gamma and an Ad.4 specific activity. This was borne out by the data obtained from the inhibitor study (Figure 3:26). DNA polymerase beta is resistant to N-ethyl-maleimide whereas none of the DNA polymerase peaks exhibited resistance to this compound, indicating that they do not correspond to DNA polymerase beta. Although peak A was sensitive to both ddTTP and ara-CTP it was more sensitive to ddTTP, as is the case for DNA polymerase gamma. Clearly peak C corresponds to DNA polymerase alpha as it is the only peak to show resistance to ddTTP and is sensitive to all other inhibitors tested. Peak B however bears no ressemblance to any of the cellular DNA polymerases in that is was sensitive to all inhibitors tested except aphidicolin, to which it was partially resistant. Peak B clearly represents an Ad.4 specific DNA polymerase activity. This activity is very similar to the Ad.2 DNA polymerase activity studied by Lichy et al (1982) in it's sensitivity to ddTTP, ara-CTP, NaCl and NEM. It differs only in that it is 50% resistant to aphidicolin at 0.1mM, as opposed to 85% for Ad.2. Given the variability encountered in this assay system the difference may not be significant.

Several attempts were made to detect Ad.4-specific initiation activity in the Ad.4 polymerase fraction. Neither fractions containing peak B alone, or in combination with uninfected nuclear extract, uninfected cytoplasmic extract or both uninfected nuclear and cytoplasmic extracts showed any pTP-dCMP complex formation. In addition pTP-dCMP complex formation was not observed when other fractions from the Mono-Q column were added back to peak B. Although it is likely that this would have replaced factors required for pTP-dCMP formation they may have been too dilute to have any effect.

3:4:2 PARTIAL PURIFICATION OF A CELLULAR FACTOR THAT STIMULATES THE FORMATION OF AN Ad.4 pTP-dCMP COMPLEX IN VITRO

A fraction from uninfected HeLa cell nuclei that stimulated pTP-dCMP complex formation by cytoplasmic extract of Ad.4 infected cells was partially purified as follows: Nuclei were prepared from uninfected HeLa cells as described (Challberg and Kelly, 1979a) and washed three times in 25mM Tris/HCl pH7.5, 5mM KCl, 0.5mM $MgCl_2$, 0.5mM DTT, 0.2M sucrose. Nuclei were resuspended in the same buffer containing 0.35M NaCl, stirred on ice for 30min. and particulate material removed by centrifugation at 100,000 x g for 60min. The supernatant was adjusted to 0.3M NaCl and applied to a DEAE-sepharose column equilibrated in buffer A containing 0.3M NaCl. The flow-through was dialysed against buffer A containing 30mM NaCl and particulate material removed by centrifugation at 100,000 x g for 30min. The

Figure 3:26 Characterization of the inhibitor profiles of the DNA polymerase peaks isolated from the cytoplasm of Ad.4 infected cells.

2ul aliquots of each of the peak fractions described in figure 3:1 were assayed for DNA polymerase activity on gapped duplex DNA in the presence of various inhibitors as follws: Aphidicolin at 100uM, ddTTP and ara-CTP at ddTTP:dTTP and ara-CTP:dCTP ratios between 0.1-50:1, NaCl at 100mM and N-ethyl-maleimide at 5mM. All the inhibitors were added tothe reaction mixture except N-ethyl-maleimide which was added to the enzyme fraction for 5min. at 4°C and then neutralized with 10mM DTT. As described in the text the inhibitor profile of peak B identifies it as the most likely candidate for an Ad.4 infected cell specific DNA polymerase activity.

To facilitate a comparison between the inhibitor profiles of the three DNA polymerase peaks isolated from the cytoplasm of Ad.4 infected cells, the cellular DNA polymerases and the Ad.2 specific DNA polymerase the latter are set out. Data for the Ad.2 specific DNA polymerase were taken from Lichy et al (1982) and data for the cellular DNA polymerases were taken from Krokan et al (1979) and Weissbach (1977).

PEAK	N-ethyl-maleimide	Aphidicolin	ddTTP (50:1)	ara-CTP (50:1)	100mM NaCl
A	I	R	S (90%)	S (80%)	R
B	S	I	S (70%)	S (70%)	S
C	S	S	R	I	S

YMERASE	N-ethyl-maleimide	Aphidicolin	ddTTP	ara-CTP	100mM NaCl
Alpha	S	S	R	S	S
Beta	R	R	S	R	R
Gamma	S	R	S	R	R
Ad.2	S	R	S	S	S

S - sensitive (%) REDUCTION

R - resistant

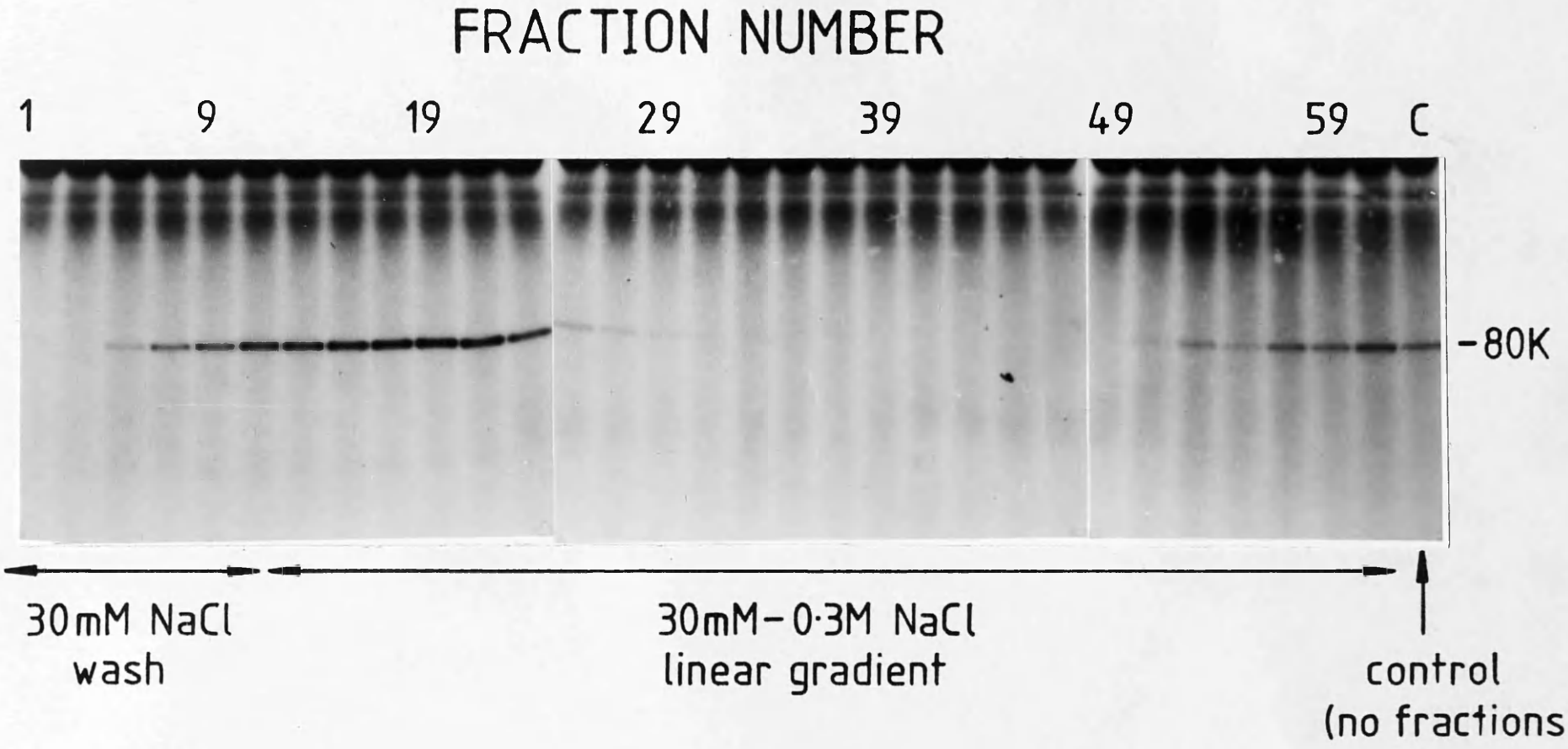
I - intermediate (50% inhibition)

supernatant was applied to a DEAE-sepharose column equilibrated with buffer A containing 30mM NaCl. The column was washed with 2 column volumes of buffer A containing 30mM NaCl and proteins were eluted with a linear gradient from 30mM to 0.3M NaCl in buffer A. Fractions were taken and 5ul aliquots were assayed for stimulation of Ad.4 pTP-dCMP complex formation activity.

Figure 3:27 shows the results of the experiment. Five ul of every second fraction from the column was incubated with cytoplasmic extract of Ad.4 infected cells (4.2ug of protein) and 50ng of plasmid template DNA. Sub-optimal quantities of extract and template DNA were used so that any stimulatory effect was not obscured by saturating the assay system. The data show that a peak of stimulatory activity eluted from the column in fractions 15-21 (50-80mM NaCl). This stimulatory activity was also active on a template containing only 18b.p. from the Ad.4 terminus (data not shown). Thus if the stimulatory activity is a sequence-specific DNA-binding protein it must therefore recognize a sequence within the terminal 18b.p. Alternatively it could be interacting with the viral proteins involved in the initiation reaction so as to enhance the specific interaction of the pTP-pol and other factors with the Ad.4 origin. The fraction did not alter the specificity of the reaction as no pTP-dCMP complex was formed in the presence of pUC8 linearized with EcoR1 either with or without the stimulatory factor. No pTP-dCMP complex formation was observed in the presence of fractions 25-53. These fractions presumably contained the inhibitory activity previously observed in unfractionated nuclear extracts.

Figure 3:27 Fractionation of uninfected cell extract on DEAE-sepharose: Identification of a fraction that stimulates Ad.4 pTP-dCMP complex formation.

Uninfected nuclear extract was fractionated as described in the text and 5ul of each fraction were incubated in a standard reaction mixture (section 2:16) with 1ul of cytoplasmic extract from Ad.4 infected cells and 50ng plasmid DNA template. Throughout the assay the salt concentration of the reaction mixture was kept constant.


FRACTION NUMBER
1
9
19
29
39
49
59
C
-80K
30mM NaCl
wash
30mM-0·3M NaCl
linear gradient
control
(no fractions)

DISCUSSION

The objective of this study was to develop an *in vitro* system capable of authentically reproducing the *in vivo* initiation of adenovirus type 4 DNA replication. This system would then be utilized to investigate the mechanistic differences between the initiation of adenovirus type 2 (Ad.2) and adenovirus type 4 (Ad.4) DNA replication. The data presented in this thesis concerns two aspects of this system. Firstly the characterization of the initiation of Ad.4 DNA replication *in vitro* by extracts derived from infected cells. Secondly the partial purification and characterization of an Ad.4 specific DNA polymerase activity present in infected cells and the identification of a fraction from uninfected cell nuclei that stimulates Ad.4 initiation *in vitro*. In chapter 4 I shall compare the data obtained with that published by other workers concerning Ad.2 DNA replication *in vitro* and make some suggestions as to the precise nature of the differences between the two viruses at the level of initiation of DNA replication.

4:1 THE USE OF FULL-LENGTH VIRAL DNA AS A TEMPLATE FOR THE INITIATION OF ADENOVIRAL DNA SYNTHESIS IN VITRO.

The use of adenoviral cores as templates for *in vitro* replication was first described by Goding and Russell (1983). They showed that Ad.2 cores produced by deoxycholate treatment of virions were as efficient as DNA-protein complex (DNA-prot.) produced by guanidinium hydrochloride treatment in supporting initiation and limited elongation. Ad.4 cores proved to be reproducibly more efficient than

template for *in vivo* DNA synthesis. This result may be due to the fact that deoxycholate treatment of virions is certainly less severe than guanidinium hydrochloride and may leave the DNA-terminal protein in a more active state for replication. The effect of the major core protein on Ad.2 pTP-dCMP complex formation has not been documented. Presumably the termini of the genome in adenovirus cores must be accessible to initiation factors both in Ad.2 (Goding & Russell,1983) and Ad.4 (this thesis).

Unlike Ad.2 (Lichy *et al*,1981) deproteinized Ad.4 DNA reproducibly supported pTP-dCMP complex formation at about 25% of the efficiency of Ad.4 cores. Tamanoi and Stillman (1982) demonstrated that the ability of deproteinized Ad.2 DNA to support pTP-dCMP complex formation was restored by piperidine treatment. This treatment removed residual amino acids not digested by Pronase which were presumably interfering with pTP-dCMP complex formation. There are two reasons why deproteinized Ad.4 DNA could support pTP-dCMP complex formation: Firstly the residual amino acids left after treatment with proteinase K may not interfere with Ad.4 complex formation in the same way as they interfere with Ad.2 complex formation. This would imply differences in the protein-DNA interactions at the Ad.4 origin compared to the Ad.2 origin. Secondly the phosphodiester bond between the terminal protein and the 5' terminal dC may be very sensitive to alkaline hydrolysis in Ad.4. Even at pH 8 - the conditions used in this study - the bond between residual amino acids and the DNA may be unstable.

DNA-prot. in supporting initiation *in vitro* although this ability varied greatly between core preparations.

In vivo adenovirus infection results in the introduction of cores into the nucleus at 1h.p.i. (Chardonnet & Dales,1972). However soon after this viral genomes in the nucleus have been reported to be almost protein free (Lonberg-Holm & Philipson,1969) suggesting that the cores are not used as templates for *in vivo* DNA replication. In addition Tate and Philipson (1979) showed that between 1 and 6h.p.i. viral DNA in the nucleus became increasingly present in nucleosome-like structures, larger than those found in isolated cores (Corden *et al*,1976). Tate and Philipson (1979) suggested that viral DNA was stripped of core proteins and possibly became associated with cellular histones prior to DNA replication. Later in infection (20h.p.i.) adenovirus DNA is found associated with core proteins (Weber & Philipson,1984).

However the major core protein pVII has been shown to inhibit Ad.2 DNA synthesis *in vitro* by binding non-specifically to DNA and presumably preventing the interaction of the DNA with replication proteins (Korn & Horwitz,1986). If this inhibition is also the case *in vivo* then core proteins must be removed from the DNA prior to replication. It is somewhat surprising therefore that cores are the most efficient template for pTP-dCMP complex formation *in vitro*, given that they appear not to be the

4:2 COMPARISON OF THE Ad.2 AND Ad.4 IN VITRO INITIATION REACTIONS

Formation of an Ad.4 pTP-dCMP complex *in vitro* was catalysed by either cytoplasmic or nuclear extracts of Ad.4 infected HeLa cells (figures 3:3 and 3:4). Ad.4 initiation *in vitro* thus differs from Ad.2 initiation *in vitro*. Lichy *et al* (1981) demonstrated that formation of an Ad.2 pTP-dCMP complex was only catalysed by cytoplasmic extract of Ad.2 infected cells in the presence of uninfected nuclear extract. In the absence of the latter no pTP-dCMP complex was detected. The activity of cytoplasmic extract of Ad.4 infected cells in forming pTP-dCMP complex alone was comparable to that of cytoplasmic extract of Ad.2 infected cells in the presence of uninfected nuclear extract: In the system of Lichy *et al* (1981) 0.93 fmoles of complex were formed in 60min. by a reaction mixture containing 70ug of cytoplasmic protein and 15ug of nuclear protein. In the Ad.4 system described here 1.16 fmoles of complex were formed in 60min. by a reaction mixture containing 33ug of cytoplasmic protein. Thus Ad.4 initiation is occurring at a similar (if not higher) efficiency to Ad.2 in the absence of the specific nuclear factors required by Ad.2.

Ad.2 initiation is strongly inhibited by the viral DNA-binding protein (DBP) in the absence of purified nuclear factor I (Nagata *et al*,1982; De Vries *et al*,1985). DNase I footprinting studies demonstrated (Nagata *et al*,1983b) that DBP protects the entire ITR from DNase I digestion. In the

presence of both DBP and NFI small amounts of DNase I digestion were observed at the boundaries of the NFI site suggesting that NFI displaces DBP from the NFI binding site, enabling initiation to occur. However additional experiments are required to prove this point. It is possible that DBP binds only to the phosphate backbone of the DNA as is the case for the bacteriophage T4 gene 32 protein (Kowalczykowski et al,1981). DBP and the gene 32 protein are similar in that they both bind to single- and double-stranded DNA. They also both stimulate the in vitro activity of the homologous DNA polymerases but not other DNA polymerases. Inhibition of pTP-dCMP complex formation by DBP seems unlikely in the case of Ad.4 as the Ad.4 DBP is known to be present in the cytoplasm of infected cells (Hay, unpublished observations). Cellular non-sequence specific DNA-binding proteins are also likely to be present in the cytoplasm. In spite of this initiation occurs with high efficiency (in comparison with Ad.2) in the absence of nuclear extract.

The specificity of complex formation with different nucleotides varies between Ad.2 and Ad.4: Both viruses exhibit the most efficient complex formation with dCMP. Ad.2 DNA polymerase will also transfer dAMP to pTP (Challberg et al,1982; Tamanoi & Stillman,1982) at a level that is at least 20-fold lower than complex formation with dCMP. This is presumably due to the formation of a pTP-dCMP-dAMP complex, dA being the second residue in the sequence at the 5' end of Ad.2 DNA. A trace amount of complex was formed

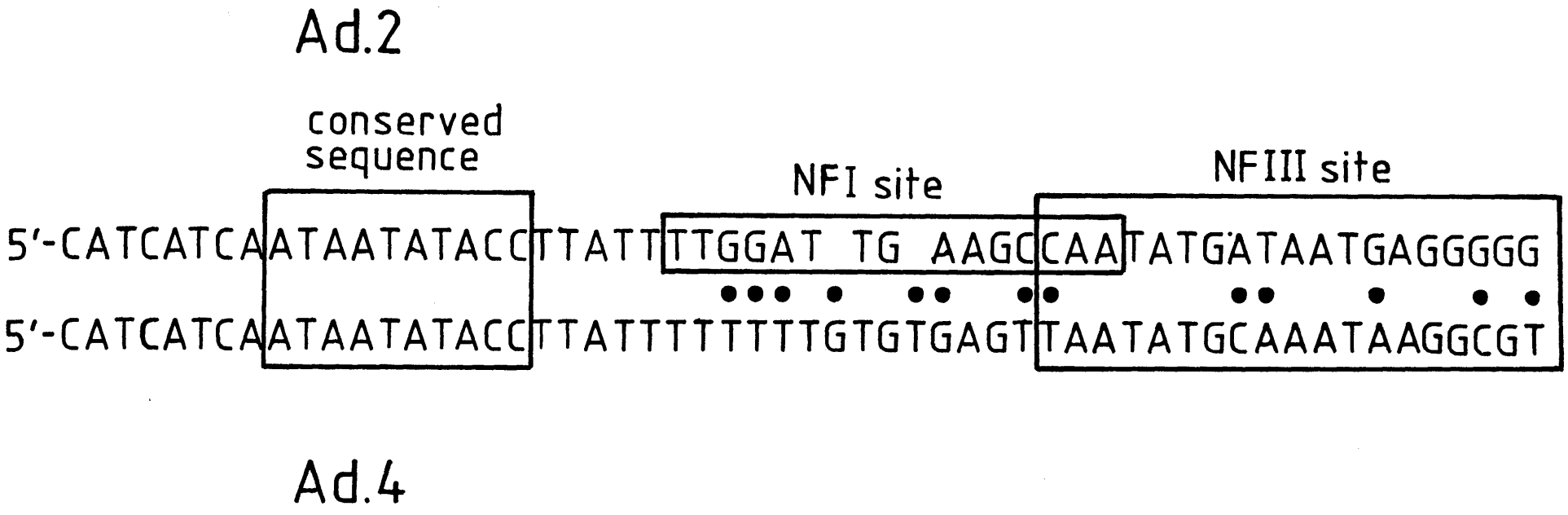
with dTMP (Tamanoi & Stillman,1982), presumably due to the formation of a pTP-dCMP-dAMP-dTMP complex. This is also consistent with the sequence of the 5'end of Ad.2. No complex was formed with dGMP (Lichy et al,1981; Tamanoi & Stillman,1982).

In contrast cytoplasmic extract of Ad.4 infected cells reproducibly catalysed the formation of a low level of a pTP-dTMP complex in the presence of dTTP and either Ad.4 cores or plasmid p4A2 linearized with EcoRI as template. By analogy with Ad.2, if the mechanism of initiation is the same, the Ad.4 DNA polymerase should transfer dAMP to a pTP-dCMP complex given that the sequence of the terminal 18b.p. of Ad.2 and Ad.4 are identical (see figure 4:1). Figure 3:7 shows that this is not the case: no pTP-dNMP complex was formed in the presence of either dATP or dGTP. The formation of a low level of a pTP-dNMP complex in the presence of dTTP cannot therefore be explained by the transfer of dTMP to a pTP-dCMP-dAMP complex.

It is possible that the Ad.4 DNA polymerase is transferring dTMP on to pTP at the terminal 3' nucleotide in the Ad.4 sequence, i.e. the terminal dG residue. This is unlikely because it would not be possible for the primer to base pair with the template. In general terms efficient priming of DNA synthesis must involve hydrogen bonding between the primer and the template. Data obtained for Ad.2 (Challberg & Rawlins,1984) suggests that in the presence of a template containing no dG residues the Ad.2 DNA polymerase

Figure 4:1 Comparison of the DNA sequence of the 5' termini of Ad.2 and Ad.4.

Adapted from Hay (1985b). The box labelled conserved sequence is the 9-18b.p. AT-rich sequence perfectly conserved in all human adenoviruses. The NFI box is the minimal sequence requirements for NFI binding (Leegwater et al,1985) whereas the NFIII box is the sequence protected from DNase I digestion by NFIII (Pruijn et al,1986b). The variant nucleotides are marked by black dots.


Ad.2
conserved sequence
NFI site
NFIII site
5'-CATCATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGG
5'-CATCATCAATAATATACCTTATTTTTTTTGTGTGAGTTAATATGCAAATAAGGCGT
Ad.4

will only transfer to the pTP a nucleotide that can base pair with the template, i.e. in the presence of poly(dA) as template only a pTP-dTMP complex was formed.

What seems more likely is that pTP-dTMP complex formation is occurring at one of the dA residues at the 3'end of the Ad.4 sequence - nucleotides 3, 6 or 10. In this case hydrogen bonding would be possible and a stable primer-template complex would be generated. Clearly this would result in an aberrant initiation event and, if followed by DNA synthesis, would produce a genome lacking at least the terminal 2 nucleotides. There must be an evolutionary pressure to prevent this occurrence in vivo but it should be recognized that initiation in vitro in the presence of a single nucleotide species is a highly artificial environment. It is unlikely that the Ad.4 DNA polymerase would ever be in the situation of being unable to choose between nucleotides in vivo. This is borne out by the data in figure 3:8 which demonstrate that, given a choice between dCTP and a vast excess of dTTP, the Ad.4 DNA polymerase transfers only dCMP to the pTP.

The use of aberrant start sites has been documented for Ad.2 initiation in vitro (Tamanoi & Stillman,1982; van Bergen et al,1983). In both these studies initiation on Ad.2 DNA-prot. was highly specific for the terminal nucleotide. When plasmid DNA containing an Ad.2 terminal fragment was used as a template initiation occurred at both the 3' terminal dG and at the second dG residue 4 nucleotides in

from the 3' terminus. It was suggested that one function of the Ad.2 terminal protein may be to prevent these internal starts. This relaxed specificity for start sites in the absence of the TP may be mimicked in Ad.4 by coercing the DNA polymerase to transfer dTMP to the pTP. The fact that pTP-dTMP complex formation occurred in the presence of either Ad.4 cores or linearized plasmid DNA suggests that the Ad.4 TP is not influencing the initiation site as strongly as the Ad.2 TP.

4:3 OPTIMIZATION OF THE CONDITIONS FOR Ad.4 pTP-dCMP COMPLEX FORMATION

To characterize the Ad.4 *in vitro* initiation system fully experiments were performed to elucidate the optimal concentrations of divalent cations and ATP, and the optimal ionic strength (figures 3:9 to 3:13).

The rate of the reaction proved to be highly sensitive to the level of divalent cations. Substitution of Mn^{2+} for Mg^{2+} gave a higher level of activity which was attained at a lower concentration. In this respect the reaction is similar to that catalysed by eucaryotic DNA polymerase beta (Kornberg,1980). DNA polymerase beta shows a 100-fold increased affinity for primer-templates in the presence of Mn^{2+} instead of Mg^{2+}.

The ATP concentration also had a profound effect upon the formation of a pTP-dCMP complex and as expected this

effect was influenced by the divalent cation concentration (figures 3:9 to 3:10). In the presence of a constant Mg^{2+} concentration (3mM) the level of pTP-dCMP complex formation was maximal at an ATP concentration of 3mM and then declined rapidly. When the ATP and Mg^{2+} concentrations were increased concurrently pTP-dCMP complex formation was most efficient in the absence of exogeously added ATP and Mg^{2+} and thereafter declined gradually. The fact that the highest level of pTP-dCMP complex formation was observed in the absence of added ATP and Mg^{2+} implies that the endogenous levels of both are sufficient for efficient pTP-dCMP complex formation. This was further shown by the fact that in the absence of exogenously added ATP and Mg^{2+} the addition of EDTA completely inhibited the reaction (data not shown).

ATP and Mg^{2+} form an equimolar chelate so clearly when the concentrations of both are equal neither is present as a free entity. The formation of a pTP-dCMP complex may require both ATP:Mg^{2+} and free Mg^{2+} ions. In this case any excess of ATP over Mg^{2+} will result in a reduction of the pool of free Mg^{2+} ions, thus inhibiting the reaction. This prediction is borne out by the results in figures 3:9 and 3:10. Cytoplasmic extracts were prepared using a buffer containing 0.5mM $MgCl_2$ and were present at a 1 in 30 dilution in these assays, giving a minimal Mg^{2+} concentration (excluding that bound by proteins and other factors in the extract) of 17uM. The endogenous levels of ATP in Ad.5 infected HeLa cells has been reported as 0.16uM (De Jong et al,1983) in a suspension of 5x107 nuclei/ml. Clearly there is an excess of Mg^{2+} ions

unchelated to ATP in the endogenous population. It appears that the requirement for ATP in the formation of an Ad.4 pTP-dCMP complex is negligible - the energy requirements may come from the hydrolysis of phosphates on the dCTP substrate.

However exogenously added ATP has been shown to stimulate Ad.2 pTP-dCMP complex formation (Lichy et al,1981) and Ad.5 pTP-dCMP complex formation (De Jong et al,1983). In both these systems the absence of added ATP resulted in the labelling of additional bands of higher and lower molecular weights than the pTP-dCMP complex. This phenomenon was also observed for Ad.4 (data not shown). In Ad.4 it may be related to the presence of added $MgCl_2$ in the absence of ATP because in the absence of both these other bands are not seen (figure 3:10 track 1). The explanation for this is as yet unknown.

4:4 LIMITED ELONGATION OF THE pTP-dCMP COMPLEX TO THE FIRST dG RESIDUE IN THE ADENOVIRAL SEQUENCE

Figure 3:13 shows that, in the presence of dATP, dTTP and ddGTP, the Ad.4 pTP-dCMP complex is capable of limited elongation to the first dG in the sequence of the 5'end. This results in the formation of a complex between the pTP and a 30 base oligonucleotide. As is the case for Ad.2 (Lichy et al,1981) this limited elongation reaction is insensitive to the action of aphidicolin. Elongation in the Ad.4 system is highly sensitive to premature chain

termination by ddTTP, much more so than the Ad.2 system: Ad.4 elongation is virtually undetectable at a ddTTP:dTTP ratio of 1:1 whereas Ad.2 elongation is still apparent at a ratio of 6:1 and is only undetectable at a ratio of 20:1.

Although Ad.4 contains more dT residues at the 5'end than Ad.2 most of these are clustered between base pairs 18-30 (Hay,1985b) (see figure 4:1). Presumably premature termination by ddTTP in this region would result in the formation of a complex migrating between pTP-dCMP and pTP-30mer on an SDS-polyacrylamide gel and would thus be easily detectable. No such products were observed. Incorporation of ddTTP is most likely to be occurring at an early stage in elongation, close to the 5'end, such that the products would be indistinguishable from the pTP-dCMP complex. At first sight it seems unusual that the Ad.4 DNA polymerase should be so strongly inhibited by a low ratio of ddTTP:dTTP, one might expect a 50% inhibition at a 1:1 ratio. However activity of both DNA polymerases beta and gamma and Ad.2 DNA synthesis in isolated nuclei are 50% inhibited by ddTTP:dTTP ratios of between 0.02-0.2 (Krokan et al,1979). Clearly ddTTP is not only inhibiting chain elongation by acting as a chain terminator but it must also be having a direct effect on the DNA polymerase to inhibit polymerization.

In contrast, when assayed on gapped duplex DNA, purified Ad.2 DNA polymerase activity is more sensitive to ddTTP inhibition (Lichy et al,1982) than the partially purified Ad.4 DNA polymerase studied here: At a ddTTP:dTTP

ratio of 2.5:1 the Ad.2 DNA polymerase is 97% inhibited whereas the Ad.4 DNA polymerase is only 80% inhibited at a ratio of 50:1 (figure 3:26). Both enzymes are more sensitive to ddTTP when assayed for elongation of a pTP-dCMP complex than for DNA polymerase activity on gapped duplex DNA. This effect can be explained by the differences in the two assay systems. The gapped duplex DNA polymerase assay will detect reinitiation by the DNA polymerase following chain termination by ddTTP but this will not be detected by the specific assay for formation of the pTP-30mer complex.

Again it must be emphasized that the limited elongation of the Ad.4 pTP-dCMP complex to the first dG residue in the sequence was catalysed by cytoplasmic extract of Ad.4 infected cells alone, whereas Ad.2 elongation also required the presence of uninfected nuclear extract.

4:5 DNA SEQUENCE REQUIREMENTS FOR INITIATION OF ADENOVIRUS DNA SYNTHESIS IN VITRO

As is the case for Ad.2 (Tamanoi & Stillman,1982; Challberg & Rawlins,1984) a plasmid-borne Ad.4 origin of replication had to be located at the end of a linear DNA molecule, such that the end of the DNA molecule was analogous to the end of intact viral DNA (Figure 4:2). Embedded origins were not active on either a linear molecule or a closed-circular molecule, but activity of the origin was not inhibited by the presence of a 4b.p. 5' overhang (the result of EcoRI cleavage). As shown in figure 4:2 the

Figure 4:2 Comparison of the structure of the ends of Ad.4 DNA-protein complex and plasmid p4A2 linearized with EcoRI.

In both cases the 5' end of the Ad.4 sequence is protected, either by the terminal protein (TP) or by a 4b.p. 5' overhang, whereas the 3' end is at the very end of the molecule. Sequence from Hay (1985b).

Ad.4 DNA-protein complex

p4A2 linearized with EcoR I

ends of both Ad.4 cores/DNA-prot. and EcoRI cleaved p4A2 are similar in that the 3' end of the viral sequence is exposed. This may represent a requirement for the binding of either the pTP-pol or another initiation factor.

There were important differences between Ad.2 and Ad.4 regarding the sequence requirements for pTP-dCMP complex formation *in vitro*. In the Ad.2 system the terminal 18b.p. alone could support a low level of pTP-dCMP complex formation (Tamanoi & Stillman,1983; Lally *et al*,1984; Challberg & Rawlins,1984). This level was enhanced by the presence of sequences up to nucleotide 48 and the protein nuclear factor I (NFI), which binds to a specific sequence within the 19-48b.p. region (Guggenheimer *et al*,1984a; Rawlins *et al*,1984). Recently another cellular protein which enhances adenovirus DNA replication *in vitro* has been purified from uninfected HeLa nuclei (Pruijn *et al*,1986b). This factor, termed nuclear factor III (NFIII), enhanced Ad.2 replication 4-6 fold in the presence of NFI, exerting it's effect at the level of pTP-dCMP formation. NFIII bound to a specific DNA sequence between bases 34 and 54 in the Ad.2 ITR, overlapping the NFI site. Whereas Ad.4 lacks the consensus sequence for binding of NFI (Hay,1985b), it does contain the sequences required for binding of NFIII (Pruijn *et al*,1986b) and NFIII has been shown to protect analogous regions on both the Ad.2 ITR (Pruijn *et al*,1986b) and the Ad.4 ITR (Hay, unpublished observations) from digestion by DNase I. NFIII showed a higher affinity for the binding site on the Ad.4 ITR than on the Ad.2 ITR (Hay, unpublished

observations). Kelly (personal communication) has also purified a factor, termed ORP-C, with similar properties to NFIII.

There is, as yet, no evidence indicating a role for NFIII/ORP-C in either Ad.2 or Ad.4 DNA replication *in vivo*. The minimal Ad.2 origin of replication *in vivo* has been localized to the terminal 45b.p. of the ITR (Hay,1985a), including the 9-18b.p. AT-rich conserved sequence and the binding site for NFI. The addition of sequences spanning the NFIII site (up to base 56) did not enhance replication. The minimal Ad.4 origin *in vivo* was defined as the terminal 18b.p. of the genome (Hay,1985b) which are identical to the terminal 18b.p. of Ad.2. Addition of sequences spanning the NFI and NFIII sites did not enhance replication.

The results obtained in this study confirm those obtained for Ad.4 *in vivo* (Hay,1985b). Figures 3:19 and 3:20 demonstrate that the terminal 18b.p. of either the Ad.4 or the Ad.2 ITR are both necessary and sufficient for pTP-dCMP complex formation by cytoplasmic extract from Ad.4 infected cells. Although neither NFI or NFIII appear to enhance Ad.4 initiation *in vitro* (this thesis) or *in vivo* (Hay,1985b) it cannot be ruled out that another cellular factor is interacting with the terminal 18b.p. of the genome to enhance initiation. A candidate for this cellular factor may be the stimulatory factor described in figure 3:27 which was active on a template containing only the terminal 18b.p of the Ad.4 ITR. Kelly (personal communication) has recently

identified a cellular factor which binds to the terminal 15b.p. of the Ad.2 genome and protects that region against DNase I digestion. Although this factor did not enhance Ad.2 complex formation *in vitro* it is possible that it has a role in Ad.4 initiation. The terminal 15b.p. of Ad.2 is identical to that of the Ad.4 strain used in this study but not to other Ad.4 strains sequenced (Tokunaga *et al*,1982,1986; Stillman *et al*,1982). If this factor is involved in Ad.4 initiation *in vivo* then clearly it would have to be able to tolerate some degeneracy within it's (as yet undefined) recognition sequence.

It is likely that there are other, as yet unidentified, factors involved in Ad. DNA replication. Kelly (personal communication) suggested that, in addition to NFI and ORP-C (NFIII), other fraction(s) are required in order to obtain levels of DNA replication comparable with those obtained from crude extracts. Pruijn *et al* (1986a) demonstrated that sera from patients with various autoimmune diseases inhibited Ad.2 DNA replication *in vitro*. They showed that this inhibitory effect was due to an antibody to a nuclear component. The inhibitor was only effective in the presence of crude nuclear extract, assays performed in the presence of purified NFI were not inhibited. The antibody only inhibited elongation on terminal restriction fragments of Ad.2 DNA-prot., it did not inhibit pTP-dCMP formation or elongation to the first dG (Pruijn *et al*,1986a). Thus the factor(s) inhibited by auto-immune sera cannot include NFIII/ORP-C as the latter enhanced pTP-dCMP formation.

4:6 THE USE OF SINGLE-STRANDED DNA AS A TEMPLATE FOR THE INITIATION OF ADENOVIRUS DNA SYNTHESIS IN VITRO

The ability of single-stranded DNA to act as a template for the formation of an Ad.2 pTP-dCMP complex depends, at least in part, on the DNA concentration. Tamanoi and Stillman (1982) observed that at a DNA concentration of 4ug/ml initiation on single-stranded DNA required a specific sequence (an Ad.2 terminal fragment) which had to be present at the end of a linear molecule. Ikeda et al (1982) showed that initiation took place at two specific sites on circular single-stranded ØX174 DNA. Both of these sites contained the sequence 5'TATTTTG, present between nucleotides 19-25 of Ad.2. This reaction also occurred at low DNA concentration- 2ug/ml. In contrast Challberg and Rawlins (1984) found no sequence specificity for pTP-dCMP complex formation at a DNA concentration of 20ug/ml. All the single-stranded DNA species that they tested, including circular M13 DNA with and without an Ad.2 insert, and the synthetic oligonucleotide $(dG\text{-}dT)_5$, were effective.

The results obtained in this study show that the situation is quite different for Ad.4. The specificity of Ad.4 pTP-dCMP complex formation on single-stranded templates was independent of DNA concentration and required the presence of sequences from the Ad.4 terminus at the end of a linear molecule. Cytoplasmic extract from Ad.4 infected cells was unable to catalyse complex formation with any $(\alpha^{32}p)$dNTP in the presence of single-stranded M13.mp18 DNA,

an Ad.4-M13 recombinant, poly(dG), poly(dA), poly(dT) or poly(dC). The only active single-stranded templates were heat-denatured, EcoRI linearized p4A2 and a synthetic oligonucleotide corresponding to the 3'terminal 18b.p. of Ad.4. It appears that, unlike Ad.2, at high DNA concentrations Ad.4 retains a requirement for the presence of a defined DNA sequence when initiating on either single- or double-stranded DNA.

As mentioned previously it is highly unlikely that the sequence specificity for Ad.4 pTP-dCMP complex formation on single-stranded templates is mediated by sequence-specific DNA-binding proteins. To date no such proteins recognizing a specific single-stranded DNA sequence have been identified in either procaryotic or eucaryotic systems. Protein binding to single-stranded DNA is non-specific, binding occurs mainly to the phosphate backbone eg. T4 gene 32 protein (Kowalczykowski et al,1981).

Secondary structure may be involved in the formation of an active configuration for pTP-dCMP complex formation. The active oligonucleotide (figure 3:22, number 1) may have the potential to form some type of secondary structure which the inactive oligonucleotide (figure 3:22, number 2) cannot. For example the active oligonucleotide contains the sequence element 3'-GGCC-5' which could conceivably base pair with the same element on a second molecule, especially at the high DNA concentration used in the assay. Formation of a stable secondary structure could be assisted by the action of non-specific DNA binding proteins.

However it is more likely that the reason for the ability of oligonucleotide 1 to support pTP-dCMP complex formation is due to the presence of internal dG residues. Both oligonucleotides 1 and 2 contain an AT rich sequence corresponding to the 9-18b.p. conserved sequence in Ad.4. The active oligonucleotide has dG residues both 3' and 5' relative to this sequence whereas the inactive oligonucleotide only has terminal dG residues (figure 3:22). One hypothesis is that the pTP-DNA polymerase complex and other replication proteins may bind non-specifically to a single-stranded AT-rich sequence and the DNA polymerase then transfers dCMP to the pTP at a dG residue adjacent to the binding site. The strongest evidence for this comes from the data in figure 3:24 which demonstrates that substitution of the dG residues 1 and 4 base 3' of the AT-rich sequence completely abolished template activity. Substitution of the dG residues 5' to the AT-rich sequence severely reduced template activity but did not abolish it completely. Interestingly the dG residue 7 bases 3' to the AT-rich sequence did not appear to be utilized as a site for pTP-dCMP formation (compare tracks 5 and 6, figure 3:24).

Only limited studies have been carried out into the formation of an Ad.2 pTP-dCMP complex on single-stranded oligonucleotides corresponding to the viral termini. Stillman and Tamanoi (1983) demonstrated that an oligonucleotide corresponding to the 3'terminal 15b.p. of the Ad.2 genome supported complex formation whereas an oligonucleotide corresponding to bases 16-30, or a random

15mer oligonucleotide, were inactive. Tamanoi and Stillman (1983) showed that an oligonucleotide corresponding to the 3'terminal 20b.p. of Ad.2 was active. However they also found that a mutated 20mer, in which bases 13 and 14 in the middle of the conserved sequence were changed from TA to GG, was also active. This mutated oligonucleotide, unlike the wildtype, was inactive as part of a duplex, linear plasmid template. The complementary 5'strand of the mutated oligonucleotide was also inactive. They did not mention whether the complementary wild type oligonucleotide was active or not. All their experiments were done at low DNA concentrations and are therefore consistent with the their earlier data (Tamanoi & Stillman,1982) showing that at low DNA concentrations initiation on single-stranded DNA required a specific sequence. These results are also explicable in the context of internal dG residues available for pTP-dCMP complex formation.

It is not clear how relevant pTP-dCMP complex formation on single-stranded templates is to initiation *in vivo*. It has been postulated that initiation *in vivo* involves unwinding of the helix and that pTP-dCMP complex formation takes place on the single-stranded terminus of the genome (Nagata *et al*,1983b). Formation of a pTP-dCMP complex *in vitro* on single-stranded DNA templates may involve only those events that occur after helix unwinding. The inefficiency of pTP-dCMP complex formation on single-stranded DNA could be due to the fact that sequence-specific interactions between cellular or viral

DNA-binding proteins and the origin cannot occur on single-stranded DNA. This would preclude the formation of a specific nucleoprotein complex which may be required for replication. Assembly of this complex may also require a longer DNA molecule than the 24b.p. oligonucleotides used in this study.

4:7 PARTIAL PURIFICATION OF THE COMPONENTS OF THE ADENOVIRUS TYPE 4 INITIATION REACTION

4:7:1 COMPARISON OF THE CHARACTERISTICS OF THE Ad.4 SPECIFIC AND THE Ad.2 SPECIFIC DNA POLYMERASES

The Ad.4 specific DNA polymerase appears to be very similar in inhibitor profile to the Ad.2 specific polymerase described by Lichy et al (1982). When assayed for polymerase activity on gapped duplex DNA both enzymes are sensitive to N-ethyl-maleimide, ddTTP, ara-CTP and 0.1M NaCl. The Ad.2 polymerase is more resistant to 0.1mM aphidicolin; 85% as opposed to 50%. This difference may not be significant and may conceivably represent differences in experimental conditions. The levels of dNTP's in the reaction may be responsible, at least in part, for this difference. Aphidicolin is known to inhibit purified DNA polymerase alpha in a manner that is competitive for dCTP, whereas it inhibits Ad.2 DNA synthesis in vitro in a manner that is competitive with dTTP (Pincus et al,1981). The purified Ad.2 DNA polymerase was assayed at a dNTP concentration of 40uM (Lichy et al,1982) whereas the DNA polymerase assays in this study were performed at a dNTP concentration of 20uM. The ratio of aphidicolin to dNTP's was thus higher in the Ad.4

assays, a fact which may well explain the difference in aphidicolin sensitivity.

Although the purified Ad.2 polymerase is resistant to aphidicolin when assayed on gapped duplex DNA, extensive synthesis of adenoviral DNA both in vivo (Longiaru et al,1979; Pincus et al,1981) and in vitro (Longiaru et al,1979; Ikeda et al,1981; Lichy et al,1982; Nagata et al,1983a) is inhibited by the drug. These data suggest that the site of aphidicolin action in Ad.2 DNA replication is not the virus specific polymerase itself but some other factor involved in Ad. DNA synthesis. Nagata et al (1983a) demonstrated that DNA synthesis on Ad.2 DNA-prot. with five purified proteins (pTP, pol., DNA-binding protein, NFI and NFII) was inhibited by aphidicolin. However the topoisomerase activity of NFII, as assayed by it's ability to change the linking number of a plasmid DNA species, was unaffected by aphidicolin. It appears therefore that aphidicolin does not affect the activity of individual proteins when assayed separately but may affect the organization of these proteins into large nucleo-protein complexes involved in DNA replication. Alternatively the drug could inhibit an as yet unidentified replication factor contaminating one of the purified fractions.

Aphidicolin does not inhibit pTP-dCMP formation or partial elongation to the first dG residue in either Ad.2 (Pincus et al,1981; Lichy et al,1981) or Ad.4 (this thesis) so the effect must occur during more extensive elongation.

In this respect, the effect of aphidicolin is similar to the inhibitory effect of sera from patients with various autoimmune diseases observed by Pruijn et al (1986a) (see section 4:5).

There are several reasons why no pTP-dCMP complex forming activity was observed in the Ad.4 specific polymerase fraction: Firstly the Ad.4 pTP-polymerase complex might have dissociated during the FPLC fractionation. Secondly the polymerase could have been separated from one or more cellular factors required for Ad.4 initiation. However no activity was detected in combination with crude extracts of uninfected cells so this seems unlikely. Finally a virus-specific factor other than the pre-terminal protein could have been separated from the DNA polymerase. One possible candidate for a virus-specific factor is the viral DNA-binding protein. In the presence of purified nuclear factor I (NFI) Ad.2 initiation is enhanced at least 3-fold by the addition of the purified Ad.2 DNA-binding protein (Nagata et al,1983), in Ad.4 which does not require NFI this effect may be greater. However, in the absence of NFI Ad.2 initiation is strongly inhibited by the DNA-binding protein (see Section 4:2) (Nagata et al,1983; De Vries et al,1985). Experiments were also performed in which other fractions from the Mono-Q column were mixed with the Ad.4 specific DNA polymerase fraction. It is possible that the lack of pTP-dCMP complex formation in these assays was due to the low concentrations of other components required.

4:7:2 PARTIAL PURIFICATION OF A CELLULAR FACTOR THAT STIMULATES THE FORMATION OF A pTP-dCMP COMPLEX IN VITRO

Figure 3:27 shows that a factor that stimulates the formation of a pTP-dCMP complex by cytoplasmic extract of Ad.4 infected cells is present in the nuclei of uninfected HeLa cells. This factor was identified during the course of an experiment to determine the role of NFIII in Ad.4 pTP-dCMP complex formation. Whereas the peak of stimulatory activity eluted from the DEAE-sepharose column between 50-80mM NaCl the peak of NFIII activity, as detected by DNase I footprinting to an Ad.4 ITR fragment (Hay, personal communication), eluted from the column in the 30mM wash fractions. The stimulatory factor is clearly not NFIII and the latter factor does not appear to enhance the formation of an Ad.4 pTP-dCMP complex.

NFIII appears not to be required for DNA replication *in vivo* by either Ad.2 or Ad.4 (Hay,1985a,b). It seems surprising therefore that NFIII, which binds more strongly to the Ad.4 ITR than to the Ad.2 ITR, enhances Ad.2 pTP-dCMP complex formation *in vitro* but has no effect on Ad.4 pTP-dCMP complex formation *in vitro*. There are several possible explanations for this: Firstly the stimulation of Ad.2 pTP-dCMP complex formation appears to be dependent on the presence of NFI (Pruijn *et al*,1986b). It may be that another factor is required for the stimulation of Ad.4 pTP-dCMP complex formation by NFIII. Secondly an inhibitory factor for Ad.4 pTP-dCMP complex formation may have

co-eluted from the column with NFIII, thus masking any stimulation. Lastly the NFIII activity may be labile under the conditions used or simply too dilute to have any effect.

The Ad.4 pTP-dCMP complex formation stimulatory factor was active on plasmid templates containing either the complete ITR or the terminal 18b.p. as well as on Ad.4 cores (data not shown). The fact that it stimulated the formation of a pTP-dCMP complex in the presence of Ad.4 cores suggests that, unlike factor pL which stimulates Ad.2 pTP-dCMP complex formation on protein-free templates (Nagata et al,1984c), it is not a 5'-3' exonuclease. If the stimulatory factor is a sequence-specific DNA-binding protein it must recognize a sequence within the terminal 18b.p. As mentioned earlier Kelly (personal communication) has purified a factor that binds to the terminal 15b.p. of the Ad.2 ITR but did not enhance pTP-dCMP complex formation. This factor may have a role in Ad.4 initiation and may correspond to the factor purified in this study. Attempts to purify the stimulatory activity further were unsuccessful. No stimulatory activity was recovered from either phosphocellulose or denatured DNA-cellulose columns indicating that the factor may be unstable or possibly may be a complex of proteins.

In conclusion an in vitro system that faithfully initiates adenovirus type 4 DNA replication has been developed and characterized. The DNA template requirements of the system have been defined and progress has been made

towards the purification and identification of the components of the system. Future work should concentrate on this latter aspect. Detailed information about Ad.4 DNA replication, and the ways in which it differs from Ad.2, will only be obtained by reconstituting the system from purified components. The system described here can be used as an assay system for the purification of cellular factors involved in the initiation of Ad.4 DNA replication. However the first step should be to purify, in an active form, the viral proteins involved - the preterminal protein/ DNA polymerase complex and the DNA-binding protein. The role of the DNA-binding protein in particular should be studied in detail as it clearly differs from it's Ad.2 counterpart in it's effect on initiation in vitro.

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