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**Polyomavirus DNA synthesis *in vitro*: Characterization of
wild-type and ts DNA⁻ mutant Chinese hamster ovary cell
extracts**

Lawlor, Kenneth Gerard, Ph.D.

City University of New York, 1991

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**POLYOMAVIRUS DNA SYNTHESIS IN VITRO: CHARACTERIZATION
OF WILD-TYPE AND ts DNA⁻ MUTANT CHINESE
HAMSTER OVARY CELL EXTRACTS**

by
Kenneth G. Lawlor

A dissertation submitted to the Graduate Faculty in
Biochemistry in partial fulfillment of the requirements of
the degree of Doctor of Philosophy. The City University of
New York.

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

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Abstract

POLYOMAVIRUS DNA SYNTHESIS IN VITRO: CHARACTERIZATION
OF WILD-TYPE AND ts DNA⁻ MUTANT CHINESE
HAMSTER OVARY CELL EXTRACTS

by

Kenneth G. Lawlor

Advisor: Dr. Harvey L. Ozer

Several ts DNA⁻ mutant CHO cell lines affected in cellular and polyomavirus DNA synthesis have been isolated in this laboratory. I have adapted a system for polyomavirus DNA synthesis in vitro and developed an in vitro complementation assay, using chromatographic fractions prepared from wild-type (wt) CHO cell extract, in an effort to identify cellular factors which stimulate polyoma in vitro DNA replication activity of defective CHO ts DNA⁻ mutant cell extracts.

The polyomavirus in vitro DNA replication activity is completely dependent upon the addition of polyomavirus large T antigen and a polyoma origin of replication. Analysis of in vitro DNA replication products synthesized by wild-type (wt) CHO cell extract, after deproteinization, included novel form I DNA monomeric products.

Detailed analysis of one CHO ts DNA⁻ mutant, JB3-O, has been performed. Cell extracts derived from JB3-O are defective for polyoma in vitro DNA replication, compared to wt CHO cell extract, at all temperatures assayed in vitro, suggesting that JB3-O extract exhibits a temperature-independent defect in vitro. JB3-O extract is not only

deficient in in vitro DNA replication activity but also synthesizes an aberrant replicative form. These replicative forms are molecules which are greater than 75% replicated. Poor cell growth is not the cause of the defective polyoma in vitro DNA replication activity.

We have attempted to complement the defective polyoma in vitro DNA replication activity of JB3-O cell extract by addition of chromatographic fractions prepared from wt CHO cell extract. One phosphocellulose fraction showed a low level of stimulation leading to an increase of incorporation into the aberrant replicative form typically synthesized by JB3-O cell extract. Addition of topoisomerase II led to stimulation and synthesis of more monomeric product but net synthesis was still significantly lower than that of wt CHO cell extract. These data suggest that JB3-O extracts are defective in more than one function required for efficient polyoma in vitro DNA replication.

This thesis is dedicated to my wife Bonnie, my daughter, Danielle, my son Matthew, my Father and my Grandmother and to my father-in-law Irv for their patience, understanding and most importantly their love.

ACKNOWLEDGEMENTS

It is with my deepest feelings that I acknowledge the following people for their support and assistance:

Dr. Harvey Ozer, my mentor, for his support, guidance, and encouragement. At times when he knew things were difficult, he offered words of encouragement. He helped guide me through a process of critical thinking and growth. I will always have kind thoughts of Harvey.

All the lab members, for their patience and time.

Krishna Jha, for being a thoughtful and helpful friend.

Dondaparti Chowdary, for being a good friend, and for help when it was needed so much.

Jim Dermody, for his enthusiastic help, critical comments and friendship.

Jose, Margaret, Martin, Gang Li, and many others, for their friendship and help, I thank all of you.

Linda Malkas and Bob Hickey, for their collaboration and friendship, I thank you both.

My thesis committee members, for their time, understanding and constructively critical comments, I thank all of you.

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DNA replication origin

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ABBREVIATIONS

CHO	Chinese hamster ovary cell
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
EtBr	ethidium bromide
EtOH	ethanol
MWCO	molecular weight cutoff
NP ₄₀	nonident 40 detergent
p.i.	post-infection
PyLT	polyomavirus large T antigen
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
SV LT	SV40 virus large T antigen
FMF	Flow microfluorimetry

CHAPTER 1 - INTRODUCTION

Understanding how cells perform and regulate functions required for cellular proliferation is of great interest. DNA replication must occur in order for cell proliferation to occur. Much of our basic knowledge of the mechanisms of DNA replication has been through biochemical and genetic studies involving the prokaryotic organism Escherichia coli and the bacteriophage which infect it. A current view of how E. coli replicates its single circular chromosome is presented below. This will not be a comprehensive review of the literature, but will hopefully be a salient lead-in to the following sections discussing the participants in and mechanisms of eukaryotic DNA replication.

Typically, prokaryotic replication proteins were identified using model in vitro DNA replication systems (1). There are two major approaches which have been particularly fruitful in identifying the proteins involved in prokaryotic DNA replication. The first approach has involved the chromatographic fractionation of cellular extracts into inactive fractions which, when combined, reconstitute in vitro DNA replication activity. Each fraction which is required for activity is further fractionated until all the proteins present in that fraction which are involved in replication are purified to homogeneity. A second approach has made use of temperature-sensitive mutants effected in proteins involved in DNA replication (ts DNA⁻ mutants) and

involves attempted complementation of defective mutant cell extracts with chromatographic fractions derived from wt cell extract. The chromatographic fractions prepared from wild-type (wt) cell extract serve as a source of factors to attempt to complement the defective function(s). A fraction which complements the defective extract may therefore contain the wild-type homologue of the defective protein. This fraction is then fractionated further until the protein which complements the defective extract is purified to homogeneity. These two approaches have been used toward the identification of a number of the polypeptides involved in prokaryotic DNA replication described below.

A model system which has been particularly fruitful in identifying proteins involved in prokaryotic DNA replication involves using the E. coli replication origin, oriC.

Initiation, the first required event, involves the interaction of several proteins with a 245-bp sequence which is necessary and sufficient to impart autonomous replication to plasmids lacking another functional origin. A number of prokaryotic duplex DNA replication origins resemble oriC in size and structure (see Figure 1). Therefore, a model which has been proposed for oriC may also apply to initiation at other origins (2).

One model, proposed by Bramhill and Kornberg (2), suggests that the following events lead to initiation at oriC. A protein involved in initiation of DNA replication termed dnaA protein performs three crucial steps. The

histone-like protein HU stimulates early initiation stages and is normally included in the in vitro reaction. First, dnaA binds to four 9-mer repeats (called dnaA boxes) and forms a so-called "initial complex" (see Figure 2). The exact structure of the "initial complex", as shown in Figure 2, and which contains 20-40 dnaA molecules, was deduced from electron microscopic and DNAase I digestion experiments. Secondly, it successively melts three AT-rich 13-mer repeats to form an "open complex". Starting with the nearest 13-mer, successive opening of the 13-mer repeats occurs (3). This reaction requires temperatures above 22°C and ATP (2). Finally, it guides dnaB-dnaC complex into the melted structure to form a so-called "pre-priming complex". At temperatures which were found to be too low to allow open or pre-priming complex, a dnaC-dependent association of dnaB with dnaA at oriC was observed (4), suggesting that dnaC-dnaA interactions are essential to bring in dnaB helicase. This "pre-priming complex" marks the point at which bidirectional DNA replication will initiate. Once dnaB has been localized to the melted duplex, it can then perform its two major functions. First, upon addition of single-strand-specific DNA binding protein (SSB) and ATP, dnaB acts as a helicase to unwind the template. Secondly, it acts to guide primase to lay down RNA primers for DNA synthesis by pol III holoenzyme (5).

After primers are laid down, the primers are then elongated by DNA polymerase III holoenzyme (5). DNA

polymerase III holoenzyme (approximately 900 kDa) is made up of the catalytic polymerase core of three subunits and seven auxiliary subunits which are responsible for processivity (reviewed in reference (6)). It has become apparent that the pol III holoenzyme exists as an asymmetric dimer, capable of both leading and lagging strand synthesis (7,8). Finally, primers are excised by DNA polymerase I (pol I) with the help of RNase H. The gaps are then filled in by pol I or pol III holoenzyme and the nicks are sealed by DNA ligase. Gyrase decatenates and supercoils the daughter molecules, generating two plasmids identical to the starting template (9).

Because of the inability to develop a bona fide eukaryotic in vitro DNA replication system utilizing cellular origins of DNA replication (10), early identification of eukaryotic cellular proteins involved in DNA synthesis relied on functional assays involving unnatural DNA templates such as activated DNA or single-stranded circular DNA containing a single annealed primer. Although these systems did not represent models of origin-dependent bidirectional replication, they allowed for the identification and characterization of a number of eukaryotic cellular factors involved in eukaryotic DNA replication.

DNA polymerase α accounts for the majority of total DNA polymerase activity in proliferating cells (1). It is generally accepted as a principal polymerase involved in cellular DNA replication. DNA polymerase α activity correlates with DNA synthesis during cellular proliferation

(11). DNA polymerase α inhibitors inhibit cell proliferation
(12). Additionally, monoclonal antibodies specific for DNA polymerase α inhibit DNA replication in permeabilized cells
(13). Determination of the subunit composition of DNA polymerase α has been hampered by proteolytic degradation during purification (14). The generally accepted subunit composition of DNA polymerase α -primase is a 182 kDa DNA polymerase catalytic core with three polypeptides ranging from 47-86 kDa that include a primase activity (15). Resolution of the primase from the polymerase α activity requires conditions which are typically used to disrupt hydrophobic interactions (16,17), suggesting that hydrophobic interaction is a major force contributing to the stability of the polymerase α -primase complex. The primase typically synthesizes a ribonucleotide primer of about 10 nucleotides in length, which is then used as a primer by DNA polymerase α , for initiating DNA synthesis (16,17). Single-stranded regions of approximately 1000 nucleotides are utilized efficiently by DNA polymerase α isolated from Drosophila melanogaster (18). But this enzyme is not capable of using single-stranded regions of approximately 5000 nucleotides in length because of dissociation from the template (18). Purified preparations of DNA polymerase α do not typically contain a tightly associated exonuclease with proofreading activity (15,18,19), although one report of isolation of DNA polymerase α with an associated proofreading activity has been published (20). Two factors which

stimulate primer recognition by DNA polymerase α have been identified (21,22,23). The primer recognition factors, C₁ and C₂, apparently enhance the ability of DNA polymerase α to initiate DNA synthesis by eliminating nonproductive binding of the enzyme to single-stranded DNA, allowing it to slide along the template until it recognizes primer (23). These proteins have been identified as part of a multienzyme complex which contains a number of DNA replication proteins (24), suggesting that they are functionally relevant for cellular DNA synthesis. The human gene encoding DNA polymerase α has been assigned to the X chromosome (25) and cDNA clones encoding the human DNA polymerase α catalytic polypeptide have been isolated (26) and used to demonstrate that serum-stimulated and transformed cells have elevated levels of DNA polymerase α mRNA compared to G1 arrested cells (26). Cells in G1, S, or G2 phases of the cell cycle do not have appreciably different levels of polymerase α mRNA (27) suggesting that DNA polymerase α activity is regulated by another mechanism(s). Indeed, phosphorylation effects the catalytic activity of DNA polymerase α (28,29).

Another DNA polymerase identified in crude cellular extracts has been termed DNA polymerase δ (30). An approximately 37 kDa protein and has been shown to stimulate the processivity of DNA polymerase δ (31) and has been subsequently identified as PCNA/cyclin (32,33). DNA polymerase δ differs from DNA polymerase α in chromatographic behavior (34), in inhibition by butylanilinodeoxyadenosine

triphosphate (BuAdATP), and in its immunological reactivity with antibodies prepared against DNA polymerase α (for review see reference 35). Additionally, DNA polymerase δ has a 3' to 5' exonuclease activity, a function which is absent from the vast majority of DNA polymerase α preparations (30).

Other enzymes, presumably having a function in eukaryotic cellular DNA replication, have been isolated because they possess a function(s) presumably required for cellular DNA replication. RNase H1 is an important enzyme aiding in the removal of RNA primers from Okazaki fragments (9) during DNA replication in E. coli. RNase H1 has been isolated from calf thymus and shown to consist of a 68 kDa polypeptide which can dimerize and remain active (36). Type II topoisomerase have been shown to have an essential function for cell division in S. pombe (37). Replicated circular DNA molecules accumulate as multiply intertwined, catenated dimers in the S. cerevisiae ts top2 mutant (38). Topoisomerase II has been shown to be a major component of mitotic chromosome scaffolds (39). Recently, a type II topoisomerase has been isolated from mouse FM3A cells (40). The topoisomerase II enzyme activity is modulated by phosphorylation/dephosphorylation, suggesting a possible mechanism of regulation of enzyme activity in vivo (40). A model single-stranded template has been used to identify five proteins which have been purified from mouse cells which efficiently convert the template into a covalently closed circle in a discontinuous manner (41). The above examples

show how artificial templates such as activated DNA templates or primed-single stranded DNA templates have been used to identify proteins which possess functions which would presumably function in eukaryotic DNA replication.

Because of the inability to develop a bona fide eukaryotic in vitro DNA replication system utilizing cellular origins of DNA replication (10), viral model systems have become very fruitful toward the identification of cellular proteins involved in eukaryotic DNA replication.

SV40 and polyomavirus are two small DNA viruses composed of circular double stranded genomes, each possessing a unique defined origin of replication (42). The maps of SV40 and polyomavirus are shown in Figures 3 and 4, respectively. SV40 was completely sequenced (43) and shown to contain 5,243 bp. The virus is separated into an early and a late regions which are separated by a noncoding viral regulatory region containing the origin of replication and transcriptional regulatory elements. During the infection of permissive primate or human cells, early genes encoding two viral tumor (T) antigens are transcribed. After a burst of DNA replication, the late region encoding the structural proteins of the virion are transcribed. The host cell eventually lyses and virus particles are released. Polyomavirus was completely sequenced and shown to contain 5297 bp (small variations exist between different strains) (44). In the infection of permissive mouse cells, early genes encoding the three polyoma viral tumor (T) antigens are

transcribed. After a burst of DNA replication, subsequent events are as noted above for SV40.

Both SV40 and polyoma represent excellent model systems to study eukaryotic DNA replication. Analysis of temperature-sensitive mutants of each virus has shown that only one virus-encoded protein is required for DNA replication after infection of a permissive host cell, the respective large T antigen (see reference 42 and references therein). The modes of replication of both SV40 and polyomavirus are very similar to that of the host eukaryotic cell (1, see reference 45). Polyomavirus and SV40 replicate in permissive cells as free minichromosomes (42). Therefore, analyses of the mechanisms of DNA replication of each of these viruses has led to new insights into the mechanisms of and proteins involved in eukaryotic cellular DNA replication. The Polyomavirus (PyLT) and SV40 (SV LT) large T antigens are required for the initiation of viral DNA replication. PyLT is a phosphoprotein with an apparent molecular weight of approximately 90-100 kDa (42). Phosphorylation has been identified in two clusters of phosphoserines and phosphothreonines located in the amino-terminal half of the protein (46,47). One cluster contains five phosphorylation sites and is located between amino acid residues 70 and 183. Three additional phosphorylation sites have been mapped between amino acid residues 270-280. This cluster is similar to a cluster found in the homologous region of the approximately 90 kDa SV LT phosphoprotein (48,49). SV LT

possesses ATPase and 3' to 5' helicase activities (50). Recently, PyLT has been shown to possess ATPase and 3' to 5' helicase activities (51,52). Both SV LT and PyLT are sequence specific DNA binding proteins and recognize the sequence 5' GAGGC 3'.

Although the viruses are similar in certain aspects, there are significant differences. SV40 and polyomavirus exhibit very different host ranges. SV40 is able to productively infect primate and human cells, whereas polyomavirus productively infects mouse cells. Additionally, SV40 encodes two tumor antigens. Polyoma encodes three tumor antigens. They interact with common and different cellular proteins. SV LT interacts with the cellular protein p53, whereas PyLT does not. Polyoma middle T antigen interacts with c-src. No comparable interaction between c-src and SV40 T antigens has been observed.

The development of SV40 in vitro DNA replication systems (53,54,55) were dramatic breakthroughs which have led to the identification of novel eukaryotic cellular factors involved in viral and presumably cellular DNA replication. In vitro DNA replication is dependent on the addition of a DNA molecule containing the origin of replication and addition of SV40 large T antigen (SV LT). Additionally, replication was shown to occur bidirectionally from the origin (53,54,55,56). Replication activities of origin mutants were determined both in vivo, by transfection of DNA containing each mutant

construct into cos-1 cells which constitutively produce SV40 LT (57), and in vitro (58).

Detailed genetic and biochemical analyses of the SV40 origin indicates that there are three domains which are critical for DNA replication (see Figure 5A): a central GAGGC element, a 10 bp region partially overlapping an imperfect inverted repeat (early palindrome (EP)), and a 17-bp region rich in A/T (59). The last two domains undergo structural transitions during the initial stages of origin unwinding (59). Although SP1 binding sites and enhancers had little or no effect in vitro, the presence of either element markedly increased replication in vivo, suggesting that these elements not only stimulate transcription but may have more general stimulatory effects on DNA replication as well (58). Additionally, SV LT binding site I was not required for DNA replication, but its presence increased replication efficiencies several-fold in vitro and in vivo (58). The presence of site I and site III, in addition to the core ori (site II), can effect core ori activity to various degrees depending on in vitro reaction conditions (57). A common feature of each site is the presence of 5' GA/GGGC 3' sequences to which SV LT has been shown to specifically bind. The number and spacing of these sequences is different in each of the three sites (see reference 59 and references therein for review).

An overview of the events occurring at the SV40 origin is presented in Figure 5B. SV LT molecules bind to the SV LT

binding sites in site II, and probably the early palindrome, which lead to structural changes in the A/T rich tract and early palindrome sequences. DNAase I and electron micrograph studies have revealed a "double-hexamer" of SV LT molecules which cover the entire core ori just described (59). As this structure forms on the ori core, the early palindrome is partially melted and the A/T rich tract is distorted. In the presence of ATP, SSB, and topoisomerase I, plasmid DNA is then unwound and ready to serve as a template for DNA synthesis. The replication activity of SV LT is modulated by phosphorylation. These phosphorylation/dephosphorylation events effect the ability of SV LT to initiate SV40 DNA replication in vitro (see reference 60 for review).

The SV LT/DNA complex exhibits similarities to and differences from the oriC initiation events which occur at the E. coli origin of replication. In both instances, a multimeric complex leads to distortions which ready the DNA replication origin for initiation. Each ori noticeably has a lengthy A/T rich tract. In the case of oriC, noticeable wrapping of the DNA around the protein into a nucleosome-like structure occurs. DNA is not wrapped around SV LT multimers. It should be noted that since SV LT is functioning as the origin recognition protein and helicase, it is probably not possible to identify proteins which normally perform these functions during cellular DNA replication by using an SV40 in vitro DNA replication assay.

The SV40 in vitro DNA replication system has been used by a number of laboratories using the fractionation/reconstitution approach to define cellular proteins involved in viral and cellular DNA replication. The paucity of mammalian ts DNA⁻ mutant cell lines has made using in vitro complementation systems, to identify mammalian cellular factors involved in DNA replication, less attractive. A current view of the proteins at a replication fork of SV40 is presented in Figure 6.

Numerous in vivo data suggest that DNA polymerase α is required for cell proliferation (as discussed earlier). Immunoaffinity depletion of DNA polymerase α -primase from HeLa or monkey cell extract resulted in the loss of SV40 in vitro DNA replication activity. Addition of the polymerase α and DNA primase back into the depleted extract restored SV40 in vitro DNA replication activity (61). It should be noted that addition of the human but not mouse polymerase α and DNA primase restored SV40 in vitro DNA replication activity. Additionally, human DNA polymerase α and DNA primase made mouse cell extracts competent for SV40 in vitro DNA replication (61). These data indicate that DNA polymerase α -primase is required for SV40 in vitro DNA replication and suggest that one determinant of host cell specificity for SV40 DNA replication involves productive interaction of polymerase α -primase derived from a permissive cell with SV LT leading to efficient SV40 DNA replication. Since SV40 replicates by a mechanism very similar to that of the host

cell, these data indicate that DNA polymerase α -primase is essential for viral as well as cellular DNA replication.

SV LT interacts with DNA polymerase α -primase complex (62,63) and may help initiate RNA primed-DNA synthesis by DNA polymerase α -primase complex. DNA polymerase α -primase, located on the lagging strand, synthesizes short RNA-primed DNA fragments in the presence of human single-stranded DNA binding protein (see Figure 6). A three subunit human single-stranded DNA binding protein (RP-A) has been identified to be essential for SV LT dependent in vitro DNA replication (64,65,66). Only the large 70 kDa subunit can bind single-stranded DNA (64). Monoclonal antibodies directed against either the human 70 kDa subunit or the 34 kDa subunit block the replication reaction (64), suggesting that both subunits are essential. The cell-cycle dependent phosphorylation (65) of the 34 kDa subunit suggests a possible mechanism of regulation, but the role of phosphorylation in the regulation of activity is not known. Joining of the "Okazaki fragments" to form a completed daughter DNA molecule on the lagging strand requires the actions of a 5' to 3' exonuclease and RNase H to remove primers, followed by filling in of the gaps by polymerase, and sealing the gaps by DNA ligase (described in reference 67).

One of the major findings utilizing the SV40 in vitro DNA replication assay involved implementing a second DNA polymerase in the synthesis of SV40 DNA and presumably

cellular DNA synthesis. A cellular protein which stimulates the processivity of DNA polymerase δ , PCNA (32,68), was found to be an essential component for SV40 DNA replication in vitro (33). In the absence of PCNA, only short products derived from lagging strand DNA synthesis occurred (69). These data suggest the possibility that two DNA polymerases are functioning at the replication fork and that short products were synthesized by a polymerase performing lagging-strand synthesis. These results suggest that DNA polymerase α may be the lagging strand polymerase (see Figure 6) and DNA polymerase δ is probably the leading strand DNA polymerase. The recent finding that DNA polymerase δ is essential for efficient SV40 in vitro DNA replication confirms that two polymerases are functioning during SV40 DNA replication in vitro and presumably in vivo (70,71,72,73).

DNA polymerase δ is shown carrying out elongation of the leading strand (see Figure 6). This elongation requires PCNA (69) and RF-C (74). Omission of RF-C (74) results in products being synthesized only from the lagging strand, virtually identical to the products synthesized in the absence of PCNA (32,68) once again suggesting that even though the two polymerases function asymmetrically, they may normally progress coordinately.

Although SV LT clearly acts as a helicase during the initial unwinding of the origin, the identity of the helicase acting during strand elongation is not known. Inhibition of elongation by various monoclonals directed against SV LT

suggest that SV LT may be functioning as a helicase throughout SV40 DNA replication in vitro (75,76). Finally, topoisomerase II can then decatenate the daughter molecules (77). DNA topoisomerase activity is limiting in human cell extracts prepared by typical hypotonic lysis (77). Additionally, immunoaffinity depletion experiments have shown that topoisomerase II alone can provide all the necessary topoisomerase functions required for SV40 in vitro DNA replication (77). Topoisomerase I is not capable of supplying all required topoisomerase functions because topoisomerase I is incapable of resolving catenated products into monomeric forms (77).

The model just presented is already being modified. Results from Stillman's laboratory (78,79,80) suggest that after initiation of SV40 DNA replication by DNA polymerase α , DNA polymerase δ replaces DNA polymerase α and synthesizes long stretches of DNA without dissociation. The model of a tightly associated asymmetric dimer model is refuted by the ability of several prokaryotic leading strand polymerase complexes to efficiently replace DNA polymerase as the leading strand DNA polymerase (78). This new hypothesis is presented in Figure 7.

Although these new data argue against a tightly associated asymmetric dimer coordinating synthesis simultaneously on the leading and lagging strand of DNA replication forks, multienzyme complexes involved in DNA replication are apparent (1). Additionally, a sedimentable

complex of enzymes for DNA synthesis has been isolated from HeLa cells. This complex is fully competent for SV40 in vitro DNA replication (81). The complex has a 640 kDa DNA polymerase α -primase complex (24), topoisomerase I, DNA-dependent ATPase, RNase H, DNA ligase, a simian virus 40 origin recognition protein, and PCNA. The characterization of a sedimentable complex involved in polyoma in vitro DNA replication has been initiated (see Appendix section I).

An SV40 in vitro DNA replication system has been used to identify a human cell nuclear factor, chromatin assembly factor (CAF-I), which promotes chromatin assembly on replicating SV40 DNA (82,83). CAF-I has been shown to target newly synthesized histones H3 and H4 to the replicating DNA. This reaction is dependent upon and coupled to DNA replication (84).

As described earlier, PyLT is a phosphoprotein which is required for the initiation of polyoma DNA replication. The protein has been shown to interact with a defined origin of replication which has been shown to consist of two genetic elements (see reference 85, and see Figure 8). The first element, termed the core origin, is a 85 bp region which encompasses a 32-bp palindrome consisting of G/C rich repeats to which PyLT can bind, a 15-bp A/T rich tract on the late side of the origin, and site A, a high affinity PyLT binding site located on the early side of the palindrome. The second element, located on the late side of the core origin, consists of two transcriptional enhancer elements,

termed A() and B(β), which are functionally redundant in their ability to activate DNA replication in vivo. At least one transcriptional element is required for significant amounts of DNA replication in vivo (see reference 86). In vitro DNA replication of polyoma virus exhibits no requirements for enhancers (87).

The development of a polyoma in vitro DNA replication assay provided another system which could be used to identify cellular proteins involved in eukaryotic DNA replication as well as define determinants of polyomavirus host cell specificity (88). Mouse cell extract which was depleted of DNA polymerase α -primase was inactive for polyoma in vitro DNA replication (88), showing that DNA polymerase α -primase is essential for polyoma in vitro DNA replication. The addition of purified DNA polymerase α -primase from mouse cells, which was inactive for polyoma in vitro DNA replication, to human cell extract made the human cell extract competent for polyoma in vitro DNA replication (88). These data indicate that DNA polymerase α -primase is required for polyoma in vitro DNA replication and suggest that one determinant of host cell specificity for polyomavirus DNA replication involves the productive interaction of DNA polymerase α -primase derived from a permissive cell with PyLT leading to efficient polyomavirus DNA synthesis.

The paucity of ts DNA⁻ mammalian mutant cell lines has made using in vitro complementation systems to identify mammalian cellular factors involved in DNA replication less

attractive. Although ts mutants have been isolated in diverse cell lines resulting in several large collections of ts mutants (89), few have been proven to be defective in DNA replication. Earlier genetic studies (90) of mouse ts DNA⁻ cell lines using independently isolated mutant cell lines mapped the ts mutations to the X chromosome (90). These mutant cell lines failed to complement each other, as determined by somatic cell hybridization (90), suggesting independent isolation of mutations in the same gene.

Chinese hamster ovary (CHO) was selected as the parental cell line from which to isolate a collection of ts DNA⁻ mutant cell lines for several reasons. It has favorable growth properties over a broad range of temperatures (33°C to 41°C) and has a plating efficiency which approaches 100%. One particularly important reason for selecting CHO as the parental cell line from which to isolate ts DNA⁻ mutants is the previous demonstration of regions of functional hemizygosity within the genome (91), suggesting that ts mutations in autosomal genes encoding DNA replication factors could be isolated. As noted earlier, the previously isolated mouse mutants in DNA replication map to the human X chromosome, indicating an inability to generate autosomal mutants. This is undoubtedly due to the presence of a second allele which masks any recessive mutations. CHO has been used to isolate a large number of mutant cell lines, by using selective and nonselective conditions, which are ts for growth (92). A set of nine mutants have been identified from

this collection which were designated ts DNA⁻ mutants, as upon shift of growing cells to the non-permissive temperature (NPT), they exhibited rapid inhibition of DNA synthesis but not protein synthesis (92).

Several CHO ts DNA⁻ mutants which restrict cellular and polyomavirus DNA replication in a temperature-dependent manner have been identified (92). Since the overall goal of the mutant isolation was to identify mutants temperature-sensitive in DNA synthesis itself, it was important to identify those mutants which involved macromolecules directly relevant to DNA synthesis (i.e., involved in initiation, elongation of DNA replication). In an effort to identify such mutants, the nine ts DNA⁻ CHO mutant cell lines were tested for their ability to support the DNA replication of two different classes of DNA viruses at the NPT: mouse polyomavirus and human adenovirus. The mode of DNA replication utilized by polyomavirus is very similar to that of the host cell, and since polyomavirus requires only one viral encoded protein for DNA replication within a permissive cell line, a number of the functions required for DNA replication are provided by host cell proteins. Adenovirus on the other hand, replicates by a very different mechanism and encodes a large number of the proteins which it uses for its own DNA replication. Of these nine ts DNA⁻ mutants tested, four have been shown to be defective for polyoma but not adenovirus DNA replication after shift to the NPT. One mutant, JB3-B, which restricts both polyomavirus and

adenovirus at the NPT, would be expected to be defective in a function required by both viruses. Indeed, JB3-B has been shown to possess a temperature-sensitive ribonucleotide reductase activity, an enzyme required for dNTP synthesis (93). Genetic studies of the CHO cell lines by cell hybridization have shown that four of these five CHO ts DNA⁻ mutants lie in different complementation groups (H.L. Ozer, unpublished data), suggesting the isolation of ts mutations in four different genes. In light of the inability of the mouse mutant cell hybrids to complement, and the subsequent mapping of the correcting gene to the human X chromosome, these data suggest that mutations which are autosomally isolated may have been isolated. Additionally, several of these mutants have been analyzed by FMF (flow microfluorimetry) to determine distribution of cells within the cell cycle at particular times after shift to the NPT; some have been shown to be blocked in particular stages of the cell cycle (H.L. Ozer, unpublished data).

These mutants represent a potential means to identify novel cellular factors involved in cellular DNA synthesis. Because of the inability to develop a highly reproducible, origin dependent in vitro DNA replication assay using putative eukaryotic cellular origins of replication, model virus in vitro DNA replication systems represented viable alternatives. The development of the SV40 in vitro DNA replication assay (53,54,55) was indeed a major accomplishment and has led to the identification of a number

of novel cellular proteins involved in viral and presumably cellular DNA replication, yet the system had several significant drawbacks if it were to be used to analyze CHO wt and ts DNA⁻ mutant cell extracts in vitro. In vitro DNA replication activity of wt CHO cell extract exhibited only a few percent of the activity of permissive cell extracts derived from human cells (56,94). Additionally, analysis of the efficiency of CHO to replicate polyoma or SV40 DNA intracellularly indicated that approximately 10-fold more polyomavirus DNA was synthesized per transfected cell (95). These data suggested that I try to develop an in vitro DNA replication system for polyomavirus. I successfully adapted a polyomavirus in vitro DNA replication assay and used the assay to analyze CHO wt and ts DNA⁻ mutant cell extracts (94). Analyses of these extracts is presented in this thesis.

CHAPTER 2 - MATERIALS AND METHODS

I. CELL CULTURE

A. GENERAL CELL GROWTH CONDITIONS

Wild-type and mutant CHO cells were cultured in an equal mixture of DME and Ham's F12 media (DF medium) supplemented with 10% donor calf serum (DCS) (Flow Laboratories) and penicillin/streptomycin (DF medium containing these supplements will be referred to as complete medium). These cell lines were incubated in a 7.5% CO₂ atmosphere at 33°C, unless otherwise indicated. CV-1, 293, and PyT54 cell lines were cultured in complete medium at 37°C. F4 hybridoma cells, which secrete F4 monoclonal antibody which binds to PyLT, were kindly provided by Carol Prives (96).

F4 hybridoma cells were grown in suspension in various media as described in Materials and Methods and Appendix section II. The insect cell line, Sf27, was kindly provided by Carol Prives and was cultured in spinner or monolayer culture in TC-100 medium (Gibco/BRL) supplemented with 1X tryptose phosphate broth, 10% fetal calf serum (FCS), 0.25 ug amphotericin B / ml and P/S. This cell line was incubated in sealed flasks at 28°C without CO₂.

Cell numbers were determined following trypsinization with a Royco cell counter (Royco 927TC cell counter) after dilution in Dulbecco's phosphate buffered saline without calcium and magnesium (PBS) (2.7 mM KCl, 1.1 mM KH_2PO_4 , 138 mM NaCl, 8.1 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.2). The plating efficiency (PE) was determined by seeding different numbers of cells in tissue culture dishes which were incubated at the appropriate temperature for approximately ten days to three weeks. The number of colonies were counted after staining culture dishes with crystal violet. Colonies were isolated using cloning cylinders. Cell lines were stored in Nunc cryotubes frozen in a -80°C freezer in complete medium containing 10% dimethyl sulfoxide.

B. ISOLATION OF REVERTANTS FROM JB3-O a CHO ts DNA⁻ MUTANT CELL LINE

Cell cultures of JB3-O growing at 33°C were trypsinized and used to seed 100 mm tissue culture dishes with approximately 5×10^5 cells. Dishes were placed directly into a 39.5°C incubator. After approximately two to three weeks, colonies were picked and expanded. After adequate numbers of cells of each revertant were obtained, cell freeze-downs were prepared. Two revertants isolated from JB3-O, JB3-O/R1 and JB3-O/R2 have been extensively characterized.

II. PROTEIN ANALYSIS

A. SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Cell extracts and chromatography fractions to be analyzed for protein content were separated in vertical polyacrylamide gels containing 12.5% acrylamide, 0.17% BIS-acrylamide and 0.1% SDS as described (97). Typically, gels were run at 150 V for 3 hours before subsequent processing.

B. WESTERN BLOTTING

The western immunoblot procedure was performed essentially as described (97). Protein preparations were separated by SDS-PAGE and transferred onto nitrocellulose filters. The filters were typically blocked using blocking buffer (5% w/v non-fat dry milk (NFDM) (Carnation), 0.1% NP₄₀, and 1X TBS (130mM NaCl, 20 mM TRIS-HCl, pH 7.5)) with gentle shaking at room temperature for 1 hour. The use of non-fat dry milk as a blocking agent led to significantly lower background than when using blocking buffers containing BSA or gelatin as the blocking agent.

Primary antibody buffer was prepared in 1X TBS containing one-third as much NFDM and NP₄₀ as the blocking buffer (antibody buffer). Typically, the F4 monoclonal

antibody used to detect PyLT was used at a 1:3 dilution of hybridoma tissue culture supernatants prepared in the antibody buffer just described. After incubation for 1 hour at room temperature the filter was washed twice for 5 minutes in 1X TBS for a total of two washes.

Alkaline phosphatase conjugated secondary antibodies were used to localize primary antibodies. The secondary antibody used to detect the mouse F4 monoclonal antibody was goat-anti-mouse IgG polyclonal antibody conjugated to alkaline phosphatase (Sigma). The secondary antibody was typically used at 1:1000 dilution. After incubating the filter with the secondary antibody solution for 1 hour at room temperature, the filter was washed twice for 5 minutes using 1X TBS for a total of two washes.

The alkaline phosphatase conjugated secondary antibodies were localized by using the alkaline phosphatase substrates NBT and BCIP which produce an insoluble purple dye. Developed westerns were typically stored in H₂O at 4°C.

C. SILVER STAIN PROCEDURE FOR SDS-PAGE

SDS-PAGE gels were silver stained as described (98). Gels were fixed for at least 3 hours (30% methanol, 10% acetic acid). Two 10 minute washes were performed in 10% ethanol. The gels were then washed in H₂O for a total of 30 minutes with two changes of H₂O. The gels were then placed in a 0.1% AgNO₃ solution for 30 minutes after which the gels

were rinsed in H₂O and developed using 2.5% Na₂CO₃ containing 0.02% formaldehyde for approximately 5 minutes. Development was stopped by briefly soaking the gels in 1% acetic acid. After several washes in H₂O, gels were either dried down or stored in H₂O at 4°C.

D. PROTEIN DETERMINATION

Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad) using BSA fraction V (Boehringer Mannheim Biochemicals (BMB)) as the protein standard.

III. DNA ANALYSIS

A. PLASMID PREPARATION

Plasmid DNAs were grown in dam⁺ E. coli (HB-101) and purified by the alkaline lysis method as described (100). Typically, plasmids were purified by two sequential CsCl-EtBr gradients to ensure isolation of highly purified plasmid of ϕ95% form I DNA. After removal of ethidium bromide from the form I plasmid DNA, the following procedures were performed. The plasmid was dialyzed extensively against TE, pH 8.0, extracted twice with 1 phenol : 1 isoamyl alcohol and once with 24 chloroform : 1 isoamyl alcohol, EtOH precipitated,

washed with 70% EtOH, dried in a speed vac (Savant), and resuspended in TE, pH 8.0. DNA was quantitated by O.D. at 260 nm and fluorometric analysis (Hoeffer).

O.D.260 / O.D.280 were approximately 1.9-2.0. Relative amounts of form I, II, and III DNA were judged by neutral agarose gel electrophoresis. Plasmid DNAs were judged to be $\geq$ 95% form I DNA. The plasmid pJLPYO, which contains the complete origin and enhancer region of the A-2 strain of polyomavirus (nucleotides 368-4973) cloned into the Bam HI site of the plasmid pKP54, was kindly provided by Thomas Kelly. The plasmid p53A6.6 contains a complete polyomavirus genome cloned into at the BamH I site of pAT153 and was kindly provided by Robert Kamen (112).

B. INTRACELLULAR POLYOMAVIRUS DNA SYNTHESIS

In order to access the ability of polyomavirus to replicate in CHO wt and ts DNA⁻ mutant cell lines under different growth conditions, transfections of a plasmid containing the entire polyoma genome were performed. CHO cell lines were seeded at approximately 4×10^5 cells per 100 mm tissue culture dish and incubated at 33°C. 18-24 hours later, dishes were transfected by the standard calcium phosphate precipitation technique without carrier DNA essentially as described (97). Dishes were transfected by adding 1 ml of CaPO₄ co-precipitate containing approximately 10 ug of the plasmid p53A6.6, which contains the entire

polyomavirus genome, to the culture medium. After 2 hours, chloroquine and amphotericin B boosts were performed by adding in amphotericin B to a final concentration of 5 ug/ml and chloroquine to a final concentration of 100 uM. After an additional 2 hours (total incubation of 4 hours), transfection medium was aspirated and the dishes were refed with complete medium. Dishes were then incubated in conditions described in Results. Low molecular weight viral DNA was extracted by the method of Hirt (101). DNA was phenol and chloroform extracted before precipitation. Purified viral DNA representing 1/10 of each transfected culture was digested with Sal I and Dpn I. After enzyme digestion, samples were run in a 1% agarose gel in 0.5 X TPE buffer. After Southern transfer, pre-hybridizations were carried out in Church buffer (1% BSA, 1 mM EDTA 0.5 M Na_2HPO_4 , and 7% SDS) containing 200 ug/ml denatured salmon sperm DNA at 68°C overnight. Hybridizations were performed under the same conditions as described for pre-hybridization except for the presence of the denatured random-primed linearized p53A6.6 probe. Unit-length replication products were visualized after autoradiography. In order to quantitate the level of replication, the areas of the nytran filters containing unit-length Dpn I resistant products were cut out, placed in scintillation vials containing 2 ml of H_2O and counted by Cerenkov counting.

IV. POLYOMAVIRUS DNA REPLICATION IN VITRO

A. PURIFICATION OF POLYOMA LARGE T ANTIGEN (PyLT)

1. EXPRESSION VECTORS USED TO PRODUCE PyLT

The helper-dependent adenovirus Ad-LTSVR587 (102) which expresses PyLT was kindly provided by Carol Prives and Terry Grodzicker. Expansions of virus stocks were typically accomplished by infecting cultures of CV-1 cells at approximately 80% confluence with Ad-LTSVR587 together with either 0, 3, 5, or 10 additional pfu of wild-type adenovirus. Typically, T-175 flasks or 150 mm dishes were infected with a volume of 3 ml of virus containing medium. Dishes were incubated at 37°C in a 7.5% CO₂ atmosphere and rocked at 10 minute intervals for 2 hours. After infection, the virus was aspirated and the cells were typically refed with 30 ml of complete medium. Infected cultures were incubated for approximately four to five days and showed extensive cytopathic effect. Virus pools containing dead cells were put through a total of three cycles of freeze-thawing. Cellular debris was pelleted by centrifuging virus stocks in an IEC clinical centrifuge at 1,500 RPM for 5 minutes. Supernatants containing virus were stored frozen at -80°C. Quality of AdLTSVR587 virus pools were determined by infecting 100 mm dishes containing CV-1 cells at approximately 80% confluence with 1 ml of each virus pool.

Infected cultures were separately extracted at 48 hours post-infection (p.i.). The level of PyLT within each extract was determined by western blotting; thereby reflecting the relative quality of each virus pool.

The recombinant baculovirus vector expressing PyLT, vEV55PyT (110) and Sf27 insect cells were kindly provided by Carol Prives. T-175 flasks containing Sf27 cells at approximately 80-90% confluent were infected with 3 ml of TC-100 medium (Gibco, BRL) without serum containing a 1:50 dilution of virus stock. Flasks were incubated at room temperature for 1 hour with rocking every 10 minutes. Flasks were then refed with 27 ml of complete TC-100 medium containing 10% FCS (Hyclone), tightly capped, and incubated at 28°C. Roughly five days p.i., medium and cells were removed and the cellular debris was removed by low-speed centrifugation in an IEC clinical centrifuge as has been described for recombinant adenovirus stock preparations. Virus stocks were stored at 4°C.

2. PREPARATION OF F4 IMMUNOAFFINITY MATRIX

i. PREPARATION OF INITIAL IMMUNOAFFINITY MATRICES

F4 is a mouse IgG monoclonal antibody originally prepared against polyomavirus small T antigen (96), but which also binds PyLT. F4 binds poorly to protein A. Two approaches were used to prepare F4 immunoaffinity columns to

be used in PyLT purification. One column was prepared by passing approximately 450 ml of centrifugally clarified F4 hybridoma tissue culture supernatant through a goat-anti-mouse-sepharose (Hyclone) chromatography matrix. This supernatant had been isolated from F4 cultures which had been grown and were allowed to die in 1 DME : 1 F-12 containing 20% FCS (M.A. Bioproducts). The matrix was then washed with PBS and subsequently cross-linked using dimethyl pimelimidate dihydrochloride (Pierce) according to the method of Schneider et al. (104). Briefly, the column matrix was washed batch-wise with more than five column volumes of 0.1 M borate buffer, pH 8.2. The column matrix was resuspended and washed with 0.2 M triethanolamine, pH 8.2. The matrix was pelleted by centrifuging at 500 g in an IEC clinical centrifuge for 5 minutes and resuspended in approximately twenty column volumes of 0.2 M triethanolamine, pH 8.2, containing 20 mM dimethyl pimelimidate dihydrochloride (Pierce). The matrix was carefully mixed by hand for 45 minutes at room temperature to allow crosslinking to occur. The reaction was terminated by the addition of 20 volumes of 0.2 M triethanolamine containing 20 mM ethanolamine, pH 8.2. The matrix was washed with TBS, pH 7.5, containing 0.02% NaN₃ and stored at 4°C. A second column was prepared by flowing Protein A MAPS II (Bio-Rad) purified F4 antibody through another aliquot of the same matrix and cross-linked by the same method. F4 cells had been grown and were allowed to die in HL-1 serum-free medium (Ventrex). Equivalent amounts of a

small-scale PyLT crude extract, prepared from Ad-LTSVR587 infected CV-1 cells, were flowed through each column. Each column was washed and PyLT antigen was eluted as described in the immunoaffinity purification section of Materials and Methods (section IV.A.3.). The levels of PyLT present in chromatogram fractions from each chromatogram were determined by western blotting. Analysis of the resultant F4 immunoaffinity matrices is presented in Appendix section II.

ii. PREPARATION OF SUBSEQUENT F4 IMMUNOAFFINITY MATRICES

Since a less degraded and more highly purified PyLT antigen preparation was obtained from the immunoaffinity column to which Protein A purified F4 had been crosslinked, protein A purified F4 monoclonal antibody was typically used as starting material for crosslinking.

In order to streamline the time-consuming process of protein A affinity purification of F4 antibody, centrifugally clarified HL-1 supernatants containing F4 monoclonal antibody were concentrated by batch binding to SP-Sephadex (Pharmacia). HL-1 serum-free medium containing F4 monoclonal antibody was adjusted to pH 4.0 by adding in 0.2 M sodium phosphate monobasic. The adjusted HL-1/F4 solution was batch bound to pre-equilibrated SP-Sephadex by rocking gently for 2 hours at room temperature. The matrix was loaded into a chromatography column and eluted by addition of 200 mM Tris-HCl, pH 8.0 containing 1 M NaCl. Fractions containing the

majority of the eluted protein were pooled and dialyzed against 1X TBS buffer. F4 antibody was subsequently isolated from this concentrated serum-free medium containing F4 monoclonal antibody using Protein A MAPS II antibody purification system obtained from Bio-Rad.

Subsequent F4 immunoaffinity columns were prepared by cross-linking protein A purified F4 antibody to Affi-Gel HZ immunoaffinity matrix (Bio-Rad) in order to increase quantity of antibody bound per ml of matrix and to optimize antibody orientation (105). Briefly, typical IgG molecules are glycoproteins which contain approximately 3% carbohydrate localized on the Fc region (heavy chain) of the antibody. Periodate oxidation of sugar hydroxyl groups of the sugars form aldehydes which react with the Affi-Gel HZ matrix and form stable covalent hydrazone bonds. This coupling through the carbohydrate eliminates the loss of antibody activity experienced by coupling at or near the antigen binding site thus allowing correct orientation of antibody for antigen binding.

3. PURIFICATION OF PyLT FROM INFECTED CELL EXTRACTS

i. PREPARATION OF INFECTED CELL EXTRACTS

PyLT preparations prepared from the helper-dependent adenovirus vector system involved extraction of sixty to one-hundred 150mm dishes of infected CV-1 or 293 cells.

Typically, 3 ml of AdLTSVR587 virus pools were used to infect 150 mm dishes containing CV-1 or 293 cells grown at approximately 80% confluence. Infections were carried out as described for recombinant adenovirus pool expansions in section IV.A.1. of Materials and Methods. Infected CV-1 cells were harvested at approximately 48 hours as described by Murakami et al. (88) with minor modifications. Briefly, 150 mm dishes of infected cells were washed two times with 3 ml of pH 9.0 buffer (20 mM Tris-HCl, pH 9.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.2 mg/ml PMSF (BMB)). Infected cells were scraped loose using a teflon tape coated razor blade. Infected cells were gently pelleted and lysed by resuspension in ten cell volumes of pH 9.0 buffer containing 1% NP₄₀. The lysate was then incubated on ice for 30 minutes and subsequently clarified by centrifuging at 20,000g for 20 minutes at 4°C. Infected 293 cell extracts were prepared by the same method as described for infected CV-1 cells except that since the cells were not firmly attached, cells were collected after tapping dishes before being washing in pH 9.0 buffer and subsequently processed. Lysates were used immediately for immunoaffinity purification of PyLT.

PyLT preparations isolated from the baculovirus vector system typically involved preparing extracts from thirty 150mm dishes of infected Sf27 cells. T-175 flasks containing Sf27 cells at approximately 80-90% confluence were infected with 3 ml of TC-100 medium without serum containing

a 1:50 dilution of vEV55PyT virus stock. Infections were carried out as described for baculovirus pool expansions in section IV.A.1. of Materials and Methods. Infected cells were harvested at approximately 48 hours p.i.. T-175 flasks containing infected cells were washed twice with 10 ml of ice-cold PBS pH 6.2. Cells were lysed by adding 1 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP₄₀, and 0.2 mg/ml PMSF) to each flask. The lysate was then incubated on ice for 30 minutes and subsequently clarified by centrifuging at 20,000g for 20 minutes. Clarified lysates were either frozen at -80°C or used immediately for immunoaffinity purification of PyLT.

ii. IMMUNOAFFINITY PURIFICATION OF PyLT

Immunoaffinity purification of PyLT was performed as described by Murakami et al. (88) with minor modifications. Infected cell extracts prepared from CV-1 or 293 cells were diluted with an equal volume of pH 6.8 sample dilution buffer (100 mM Tris-HCl, pH 6.8, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% NP₄₀, and 0.2 mg/ml PMSF). Typically, a 1.5 ml immunoaffinity column was equilibrated with pH 8.0 buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% NP₄₀, and 10% glycerol). Flow rates were approximately 1 drop/7-10 seconds. The immunoaffinity column was then sequentially washed with approximately 3-4 column volumes of pH 8.0

buffer, then with 3-4 column volumes of buffer B (20 mM Tris-HCl, pH 8.5, 1 mM EDTA, 1 mM DTT, 500 mM NaCl, and 10% glycerol). PyLT was eluted using buffer B + 50-55% ethylene glycol (Aldrich). Typically, 2 ml fractions were collected. Fractions were separately dialyzed overnight on ice in Spectrapor 2 dialysis membrane (Spectrum, MWCO 12,000-14,000) against a total of approximately 100 volumes of dialysis buffer (10 mM HEPES-NaOH, pH 7.5, 5 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, containing 50% glycerol. After dialysis, each fraction was assayed for polyoma in vitro DNA replication activity and analyzed by western blot to determine the level of PyLT present. Fractions were either frozen separately or fractions possessing the highest levels of in vitro DNA replication activities were pooled. Analysis of these fractions by silver staining showed approximately 80% of the silver-stained protein migrated to the apparent molecular weight of PyLT. Initially, purified PyLT was stored in a liquid state at -20°C. Subsequently, purified PyLT was stored frozen at -80°C because less in vitro replication activity was lost during storage at this temperature (Heather Lorimer, personal communication).

B. POLYOMAVIRUS DNA REPLICATION IN VITRO

1. PREPARATION OF AN IN VITRO REACTION

25 ul polyomavirus in vitro DNA replication assays were assembled on ice unless otherwise stated. The reactions were assembled by adding the following components into 650 ul microfuge tubes (Marsh):

(A) 7 ul of a reaction mix containing:

- (i) 2.5 ul of Polyoma system 10X mix which contained
800 mM TRIS-HCl (Sigma), pH 7.8, 70 mM MgCl₂ (Sigma),
40 mM ATP (disodium salt, Pharmacia), 2 mM CTP
(disodium salt, Pharmacia), 2 mM UTP (trisodium
salt, Pharmacia), 2 mM GTP (quatrassodium salt,
Pharmacia), 1 mM dATP, dGTP, dTTP (100 mM trisodium
salt, Pharmacia) 0.5 mM dCTP (100 mM trisodium salt,
Pharmacia).
- (ii) 0.5 ul of 25 mM DTT (BMB).
- (iii) 0.5 ul of 300 ng/ul pJLPYO DNA in TE, pH 8.0.
- (iv) 1 ul of 1 M phosphocreatine (disodium salt, Sigma)
80 mM TRIS-HCl, pH 7.8.
- (v) 1 ul of 80 mM TRIS-HCl, pH 7.8, 50% glycerol (Sigma),
containing 2.5 mg. creatine phosphokinase/ml.
(Sigma, rabbit muscle type I).

- (vi) 1 ul d-H₂O.
- (vii) 0.5 ul of 3000 Ci/mmol, 10 Ci/ml, ³²P-dCTP (NEN)
- (B) A total of 14 ul of sample prepared in HB buffer
(20 mM HEPES-NaOH, pH 7.5, 5 mM KCl 1.5 mM MgCl₂, 1 mM DTT) was added. The tube was mixed.
- (C) Finally, 4 ul of Polyoma large T antigen dialysis buffer
(10 mM HEPES-NaOH pH 7.5 (Sigma, MB grade), 5 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF (Sigma), 50% glycerol) either containing Polyoma large T antigen or not was added and the tube was mixed. The tubes were very briefly microfuged. Assays were initiated by placing the tubes at the appropriate temperature. The total Na⁺ concentration was 90 mM.

2. TERMINATION AND PROCESSING OF AN IN VITRO REACTION

Typically, reactions were terminated by the addition of 75 ul of a stop mixture containing 0.27% SDS, 267 ug/ml proteinase K, 16.7 mM EDTA, pH 8.0. The final concentrations were 0.2% SDS, 200 ug/ml proteinase K, 12.5 mM EDTA, pH 8.0. Microfuge tubes were then incubated at 37°C for 1 hour. After incubation, 50 ul of 7.5 M NH₄OAc containing 300 ug/ml tRNA (Sigma), which had been purified by repeated 1 phenol: 1 chloroform and 24 chloroform: 1 isoamyl alcohol extractions was added, followed by the addition of 150 ul of isopropanol. Tubes were then either incubated overnight at

-20°C or at -80°C for 30 minutes. Tubes were then microfuged for 15 minutes at 4°C. Supernatants were discarded and precipitates were resuspended in 200 ul of 0.3 M NaOAc, pH 7, containing 300 ug/ml tRNA. Samples were then extracted once with 1 phenol : 1 (24 chloroform : 1 isoamyl alcohol) and once with 24 chloroform : 1 isoamyl alcohol. Samples were then precipitated by adding 500 ul of absolute EtOH to each microfuge tube and incubated either overnight at -20°C or at -80°C for 30 minutes. Microfuge tubes were then microfuged for 15 minutes at 4°C. Supernatants were discarded and precipitates were washed with 500 ul of 70% EtOH. Pellets were then dried in a speed vac (Savant) and resuspended in an appropriate volume of TE. Samples which were not digested were electrophoresed in agarose gels without further processing.

The plasmid containing the polyoma DNA replication origin, pJLPYO, was grown in the *dam*⁺ E coli strain HB-101. Dpn I digests DNA when its restriction site is fully methylated. Therefore, Dpn I digests only input DNA which has not been replicated. Bona fide polyoma in vitro DNA replication products contain Dpn I restriction sites which are not fully methylated and are therefore not digested by Dpn I (i.e. they are Dpn I resistant). Samples to be digested were digested in buffer H (BMB) with Sal I and/or Dpn I (BMB).

3. NEUTRAL AND ALKALINE AGAROSE GEL ELECTROPHORETIC ANALYSIS OF IN VITRO REPLICATION PRODUCTS

Typically, samples to be analyzed in neutral agarose gels were electrophoresed in 1.4% agarose gels in 0.5 X TPE buffer. Some gels were photographed after being stained with ethidium bromide (EtBr) before further processing. Gels were then slowly shaken for approximately 1 hour in EtOH to help remove unincorporated nucleotides and expedite drying down of the gels at 60°C onto Whatman paper using a gel dryer (Hoeffer). After drying, the resultant dried down gels were exposed to x-ray film at -80°C. Bands requiring quantitation were cut from the dried down gels, after careful localization, and quantitated by Cerenkov counting. Alkaline agarose gel electrophoretic analysis was carried out essentially as described (97). Typically 1.4% agarose gels were electrophoresed using alkaline running buffer (50 mM NaOH, 1 mM EDTA). Dried down sample pellets after EtOH washes were resuspended in the desired volume of alkaline running buffer. Marker lanes included the polyoma origin containing plasmid (pJLPYO) and a 1 kb ladder of linear denatured DNA molecules (Gibco/BRL). Typically, alkaline gels were run for 18-20 hours at approximately 30-40 volts. After running, the gels were typically fixed in 7% TCA for 30 minutes. Some gels were stained with EtBr before being dried down. Gels were dried down without heating and were exposed to x-ray film at -80°C.

V. CHARACTERIZATION OF CHO WILD-TYPE (WT) AND
ts DNA⁻ MUTANT CELL EXTRACTS

A. ANALYSIS OF CELL EXTRACTS PREPARED FROM
DENSE CELL CULTURES

1. CELL GROWTH

Early in these studies, relatively dense cell cultures of CHO wt and ts DNA⁻ mutant cell lines were extracted. Typically, dense cell cultures growing at 33°C and containing approximately $2.5-4 \times 10^7$ cells per 150mm cell culture dish were split approximately 1:6 or 1:8 and extracted by the HB extraction protocol described below 2-3 days later. At the time of extract preparation, cell densities typically ranged between $2.5-4 \times 10^7$ cells per 150mm dish.

2. PREPARATION OF HYPOTONIC EXTRACTS (HB EXTRACTS)

Hypotonic extracts were prepared essentially as described by Li and Kelly (53) with minor modifications. Typically, 150mm cell culture dishes containing cells to be extracted were placed onto stainless-steel trays which were placed on ice. The trays contained a small quantity of ice cold water. The dishes were tilted every few minutes. After

15 minutes on 'ice', the medium was aspirated and 10 ml of ice cold hypotonic buffer (HB buffer) (20 mM HEPES-NaOH, pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, 1 mM DTT) was added to each plate. Each dish was swirled to ensure adequate washing by HB buffer before aspirating the buffer. Another 10 ml of HB buffer was added to each plate. Dishes were tilted every 5 minutes and left on 'ice' for a total of 15 minutes. HB buffer was then aspirated as extensively as possible from each dish. A teflon tape-coated razor blade was then used to scrape the swollen cells off the bottom of the dish. The crude suspension was then transferred to a small type B dounce homogenizer and dounced with approximately 5 strokes of the pestle. A small aliquot was viewed under the microscope to ensure that complete (95%) lysis had occurred. Crude cell lysates were kept on ice with gentle mixing every 10 minutes for a total of 1 hour. Cell lysates were then microfuged for 15 minutes at 4°C. The supernatant (HB extract) was frozen and stored at -80°C.

3. EFFECTS OF EXTRACT PRE-INCUBATION ON SUBSEQUENT POLYOMA IN VITRO DNA REPLICATION ACTIVITY

Before pre-incubation, cell extracts were diluted to the desired final concentration of protein per 14 ul volume. Pre-incubation of HB cell extract was performed by placing the indicated amount of HB cell extract protein in a water bath of the appropriate temperature. Pre-incubated extracts

were removed from the appropriate water bath to room temperature and placed into assay tubes containing the typical in vitro reaction mixture as described for extracts which were not pre-incubated except reactions were assembled at room temperature (see section IV.B.1. of Materials and Methods). The tubes were then mixed. The appropriate amounts of PyLT were added and mixed. The reaction mixtures were then assayed for Polyoma in vitro DNA replication activity and processed as described in Results and section IVB. of Materials and Methods.

B. ANALYSIS OF CHO WT AND ts DNA⁻ MUTANT CELL EXTRACTS PREPARED FROM EXPONENTIALLY GROWING CELL CULTURES

1. PREPARATION OF EXPONENTIALLY GROWING CELL CULTURES

It became particularly important to know the growth state of the cells from which extracts were being prepared. Therefore, a protocol was developed to ensure that actively growing cultures were being extracted (see Figure 18). Subconfluent monolayers were used to seed 150mm dishes at approximately 2×10^6 cells per dish. Cell counts were performed on a daily basis. When the cell densities reached approximately 8×10^6 cells per dish, these dishes ("seeder cultures") were used to seed a second set of 150 mm dishes at approximately 2×10^6 cells per dish ("extraction cultures"). Cell counts were subsequently performed on sister "seeder

culture" dishes to ensure that actively growing cells had been used to seed the "extraction culture" dishes. Extracts were prepared from the "extraction culture" dishes when cell densities typically were between 6×10^6 and 1.5×10^7 cells per 150 mm dish. Cell counts were subsequently performed on remaining sister "extraction culture" dishes to ensure actively growing cultures were actually extracted.

2. PREPARATION OF HB CELL EXTRACTS

HB extracts were prepared from exponentially growing cultures exactly as described for preparation of HB extracts from dense cultures of CHO wt and ts DNA⁻ mutant cell lines described in the section above.

3. PREPARATION OF 0.2 M NaCl/HB CELL EXTRACTS

HB and 0.2 M NaCl/HB cell extracts were prepared as follows. HB lysates were prepared as described above. After lysis by dounce homogenization in HB buffer, one-half of each extract was then adjusted to 0.2 M NaCl by addition of 5 M NaCl; an equal volume of dH₂O was added to the other half of the lysate. Lysates were incubated on ice and gently mixed at 10 minute intervals. After a total of 60 minutes incubation, the lysates were microfuged for 15 minutes at 4°C. Supernatants were removed and were immediately placed in Spectrapor 2 dialysis membranes (Spectrum, 12,000-14,000

MWCO) and dialyzed against 100 volumes of HB buffer with stirring at 2-4°C for 2 hours. This dialysis procedure was repeated. After a total of 4 hours of dialysis, the dialyzed extracts were removed, aliquoted and frozen at -80°C. When desired, the set of HB and 0.2 M NaCl/HB cell extracts was thawed, microfuged and concentrated as described below for HB cell extracts before being analyzed.

4. CELL CYCLE ANALYSIS BY FLOW MICROFLOURIMETRY (FMF)

In order to determine cell cycle distribution of cell cultures at the time of cell extract preparation, sister dishes were used to prepare samples for FMF analysis at the same time HB extracts were being prepared. Typically, two sister dishes were separately trypsinized and resuspended in a total volume of 10 ml using PBS. The cells from each plate were processed as follows. The cells were pelleted at low speed and resuspended in 10 ml of PBS. The suspension was counted and the cells were pelleted as before. The cell pellet was resuspended such that there were $2-3 \times 10^6$ cells per ml of PBS. 1 ml of this cell suspension was carefully pipetted into 9 ml of 70% EtOH and this suspension of fixed cells was gently mixed by inversion. The fixed cells were stored at 4°C until further processing could be performed. The fixed cells were then sedimented, washed once with PBS (at 10^6 cells per ml), stained with propidium iodide (50 ug/ml) in the presence of Triton X-100 (1% W/V) and RNase

treated for 20 minutes at room temperature. Cells were then analyzed for DNA content using a system 50H flow cytometer (Ortho Diagnostics Institute, Westwood, MA) interfaced to an Ortho 2150 data analysis system. FMF analysis of fixed cells was kindly performed by Thomas Denny.

5. PREPARATION OF CONCENTRATED CELL EXTRACTS

Cell extracts were thawed on ice using warmth from the hand to facilitate thawing. Cell extracts were then microfuged for 15 minutes at 4°C. The resulting supernatant was used for the following concentration step. RCF-ConFilt/Hollow Fiber Bundles were obtained from Bio-Molecular Dynamics, Beaverton, Oregon (see Figure 9). Before concentration of an extract each concentration unit was placed into a 50 ml centrifuge tube, loaded with 5 ml of HB buffer and centrifuged at 1000 g in an IEC clinical centrifuge at 4°C for 1.5 minutes without the concentrate cup attached. The HB buffer inside the centrifuge tube was discarded. The concentration cup was then seated and the unit immediately loaded with up to 5 ml of an extract to be concentrated. The 50 ml centrifuge tube containing the concentration unit set-up in the concentration mode was then centrifuged for 5-15 minutes until the desired final volume, as indicated on the unit, was obtained. The concentrate cup was then unscrewed the specified number of turns to permit collection of the concentrate and the 50 ml centrifuge tube

containing the concentration unit was centrifuged at 1000 g for 1.5 minutes. The concentrate was removed from the concentrate cup and the filtrate was collected from the 50 ml centrifuge tube the concentration unit was centrifuged within. The concentrate was then microfuged at 4°C for 15 minutes and the supernatant was removed and represented the concentrated HB cell extract. Protein assays were performed on the concentrate and the filtrate. Typically, in vitro assays were performed without another freeze/thaw cycle.

7. DETERMINATION OF DNA POLYMERASE ACTIVITY

DNA polymerase activity was measured using activated DNA as a template (Sigma). 25ul DNA polymerase assays were performed in the polyoma in vitro DNA replication reaction mix except that PyLT dialysis buffer was always used (containing no purified PyLT), and instead of using the plasmid pJLPYO, which contains the polyomavirus origin of replication, the equivalent amount of TE containing ug of activated calf thymus DNA (Sigma) was added into the reaction mix. The reactions were terminated by adding 100 ul of 0.1 M sodium pyrophosphate and 100 ul of 1 mg / ml heat denatured salmon sperm DNA followed by the addition of 1 ml of 5% TCA. The microfuge tubes were then mixed by vortexing briefly and incubated on ice for 30 minutes. The contents of the microfuge tube was passed over a GF/A filter (Whatman) with suction; the microfuge tube was washed with 5% TCA. The

filter was then washed with 95% EtOH and placed into a scintillation vial containing 2 ml of H₂O. Incorporation into acid-insoluble material was determined by Cerenkov counting.

7. DETERMINATION OF TOPOISOMERASE I OR TOPOISOMERSE II ACTIVITY

Topoisomerase I activity was measured using form I DNA of the plasmid pJLPYO in topoI buffer (50 mM TRIS-HCl, pH 7.5, 80 mM KCl, 1 mM DTT, 30 ug/ml BSA, 5 mM EDTA). Reactions were typically run at 30°C for incubations of 0.5 to 2 hours in length. Reactions involving purified topoisomerase I (Gibco/BRL) were not typically deproteinized before gel electrophoresis. Reactions involving analysis of crude cell extracts were typically terminated by the standard polyoma in vitro DNA replication assay termination method and the nucleic acids were purified as described for processing of in vitro replication reactions (section IV.B.2. of Materials and Methods). Products were typically separated by running the purified products in 1-1.4% neutral agarose gels. Topoisomers were visualized and photographed after staining with EtBr.

Topoisomerase II activity was measured using purified kinetoplast DNA isolated from Crithidia fasciculata which was kindly provided by Robert Hickey. Topoisomerase II assays were performed in either polyomavirus in vitro DNA

replication reaction mixes or in topoII buffer (50 mM TRIS-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 0.5 mM DTT, 0.4 mM EDTA, 30 ug/ml BSA, 1 mM ATP). Products produced after incubation with purified topoisomerase II (kindly provided by Bruce Stillman) or after incubation with crude cell extracts were processed in a fashion analogous to that described for products of topoisomerase I assays. Neutral agarose gel electrophoresis was typically carried out at approximately 50 volts for about 18-20 hours. Topoisomers were visualized and photographed after staining with ethidium bromide.

8. DETERMINATION OF THE LEVEL OF REPLICATION PROTEIN A (RP-A) IN CELL EXTRACTS

The levels of RP-A in CHO wt and ts DNA⁻ mutant cell extracts were determined. Rabbit polyclonal serum prepared against human RP-A was kindly provided by Lorne Erdile and Thomas Kelly. This polyclonal serum was used at 1:1000 dilution in conditions identical to those used during the incubation of goat-anti-mouse IgG conjugated to AP for detection of PyLT in western blotting. Rabbit antibodies were detected using an alkaline phosphatase conjugated goat-anti-rabbit IgG antibody (Sigma). The secondary, alkaline phosphatase conjugated, antibody was localized as described in section II.B. of Materials and Methods.

VI. PHOSPHOCELLULOSE FRACTIONATION OF
WT CHO CELLULAR EXTRACTS

Exponentially growing wt CHO cells were extracted using HB buffer as previously described. Extracts were prepared from 40-60 150 mm dishes (containing approximately 2×10^7 cells per dish) and were frozen at -80°C . The extracts were thawed on ice and an S100 was prepared by centrifugation in a 50.2 Ti rotor at 30,000 RPM at 4°C for 1 hour. The soluble supernatant was dialyzed 2 times against 2 l of TDE (50 mM TRIS-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT) + 5 mM KCl, for 2 hours each.

Phosphocellulose (Whatman P-11) was pre-cycled according to the manufacturer. The matrix was equilibrated with TDE buffer containing 5 mM KCl at 4°C by washing the matrix with approximately twenty column volumes of ice cold buffer. Equilibration was checked by performing pH and/or conductivity measurements. The matrix was then washed with several column volumes of dialysis buffer in which the wt CHO S100 cell extract had been dialyzed.

The phosphocellulose fractionation was performed using 1 ml of equilibrated phosphocellulose matrix per 6 mg of CHO wt cell extract. A typical fractionation involved 24 mg of wt CHO cell extract. Therefore, a matrix volume of 4 ml was loaded into an approximately 1 X 10 cm disposable column (Bio-Rad) and the dialyzed wt CHO cell extract was flowed

through at a rate of approximately 1 drop per 5-7 seconds. The flow-through was collected in 3.5 ml fractions. The matrix was washed with approximately 6 column volumes of TDE + 5 mM KCl. 3.5 ml fractions were collected. The column was eluted with approximately 6 column volumes of TDE + 1 M KCl. 3.5 ml fractions were collected. Each flow-through, wash and TDE + 1 M KCl eluate was assayed for protein concentration using the Bio-Rad protein assay. The combined flow-through/wash fractions and the combined TDE+1 M KCl elution fractions were each separately dialyzed simultaneously in the same vessel against 4 l of HB buffer. The high salt fractions were dialyzed using Spectrapor 2 dialysis tubing (12,000-14,000 MWCO, 10 mm diameter). The larger volume low salt flow-through/wash was dialyzed using Spectrapor 2 dialysis tubing (12,000-14,000 MWCO, 25 mm diameter). Dialysis was performed for 2-3 hours. After a change of dialysis buffer, dialysis was continued for another 2-3 hours. The combined dialyzed flow-through and wash was termed CF I (cellular fraction 1) and the combined dialyzed 1 M KCl eluate was termed CF II (cellular fraction 2). The dialyzed fractions were frozen at -80°C. When desired, the fractions were thawed and concentrated using RCF-ConFilt units (as described for the concentration of CHO cell extracts prepared from exponentially growing cell cultures, section IV.B.5. of Materials and Methods).

CHAPTER 3 - RESULTS

I. CHARACTERIZATION OF WILD-TYPE AND MUTANT CHO CELL EXTRACTS PREPARED FROM DENSE CELL CULTURES

A. FACTORS AFFECTING POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF WILD-TYPE (WT) CHO CELL EXTRACT

1. EFFECTS OF ASSAY TEMPERATURE ON POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF WT CHO CELL EXTRACT

Several CHO ts DNA⁻ mutant cell lines have been isolated and shown to restrict cellular and polyomavirus DNA in a temperature-dependent fashion (92). The identification of mutant cell lines which restrict polyoma but not adenovirus DNA replication suggest that these mutants are defective in cellular proteins involved in DNA replication. Analysis of extracts prepared from these CHO ts DNA⁻ mutant cell lines may lead to the identification of novel cellular factors involved in polyomavirus and presumably cellular DNA replication.

The most straightforward approach to the analysis of the CHO ts DNA⁻ mutant cell lines involved the preparation of extracts from wt and mutant cell lines grown at the permissive temperature followed by subsequent analyses of the polyoma in vitro DNA replication activities of these

extracts at various temperatures in vitro. The first experiment that was performed, with this approach in mind, involved determining the effects of assay temperature on the polyoma in vitro DNA replication activity of wt CHO cell extract. Initially, extracts were prepared from relatively dense cell cultures containing approximately 4×10^7 cells per 150 mm dish. High cell density cultures of monolayer cells allowed for the preparation of HB extract with the highest protein concentration. Polyoma in vitro DNA replication activity was completely dependent upon addition of PyLT and a plasmid containing the polyomavirus origin of replication. In vitro reactions containing 100 ug of extract protein prepared by HB extraction of dense cultures of wt CHO cells were assayed at various temperatures for 1 or 2 hours. Unit-length Dpn I resistant products were quantitated after agarose gel electrophoresis as described in Materials and Methods. The results are presented in Figure 10. The relative amount of polyoma in vitro DNA replication activity of wt CHO cell extract assayed at temperatures greater than 30°C compared to assays performed at 30°C is presented in Table 1. A very significant decrease in the polyoma in vitro DNA replication activity of wt CHO cell extract, greater than 60%, was evident when assays were performed for 1 hour at 36°C instead of 30°C. The dramatic effects of assay temperature on the polyoma in vitro DNA replication activity of wt CHO cell extract was more exaggerated when in vitro reactions were incubated for 2 hours. As is seen in Table 1,

there was more than a 45% decrease in the level of polyoma in vitro DNA replication activity of wt CHO cell extracts when assays were performed at 34°C instead of 30°C. Extract prepared from wt CHO cell cultures clearly exhibited significantly less polyoma in vitro DNA replication activity when assayed at temperatures as low as 34°C. Since wt CHO cell extracts exhibited significant amounts of temperature-sensitivity when assayed for polyoma in vitro DNA replication, analysis of the polyoma in vitro DNA replication activities of CHO ts DNA⁻ mutants cell extracts at elevated temperatures was significantly hampered.

2. FACTORS INFLUENCING THE EFFECTS OF ASSAY TEMPERATURE ON POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF WT CHO CELL EXTRACT

Analysis of polyoma in vitro DNA replication activity of CHO ts DNA⁻ mutant cell extract at elevated temperature would be virtually impossible due to the dramatic temperature-sensitive phenotype of the polyoma in vitro DNA replication activity of wt CHO cell extract. I therefore wanted to assess which factors could affect the deleterious effects of elevated assay temperature on the polyoma in vitro DNA replication activity of wt CHO cell extract in order to determine whether I would be able to circumvent this problem.

The amount of wt CHO extract protein assayed did not significantly affect the temperature-sensitive phenotype of

polyoma in vitro DNA replication activity of wt CHO cell extract. Assays containing either 61 ug or 142 ug of wt cell extract protein did not show any significant differences with respect to the effects of assay temperature on wt extract polyoma in vitro DNA replication activity (see Figure 11). The relative polyoma in vitro DNA replication activity of wt CHO cell extract assayed at 37°C compared to assays performed at 30°C is presented in Table 2. The relative polyoma in vitro DNA replication activity of wt CHO cell extract assayed at 37°C for 1 hour at either 142 or 61 ug per assay is lower than 30% of the activity when assayed at 30°C, regardless of the amount of protein assayed. The effects of elevated assay temperature on in vitro replication activity of wt CHO cell extracts is even more pronounced after 2 hour incubations at the respective temperatures. The relative polyoma in vitro DNA replication activity of wt extract assayed at 37°C for 2 hours at either 142 or 61 ug of extract protein per assay is lower than 10% of the activities of the duplicates assayed at 30°C for the same period.

I also wanted to determine how the amount of PyLT present in a polyoma in vitro DNA replication reaction effects the temperature-sensitive phenotype of wt CHO cell extract. As shown in Figure 12, panel A, 62 ug of wt CHO cell extract prepared from dense cell cultures was assayed at 30°C, 33°C, 34°C, 36°C, and 37°C, for 1 or 2 hours (data previously shown in Figure 1). Duplicate samples assayed at 30°C, 36°C, and 37°C, which contained more PyLT in the same

final volume of PyLT dialysis buffer, were assayed simultaneously (Figure 12, panel B). Incorporation into unit-length Dpn I resistant products was determined after agarose gel electrophoresis as described in Materials and Methods. The additional PyLT stimulated polyoma in vitro DNA replication activity by wt CHO cell extract assayed at 30°C for 2 hours by approximately 80 fold, incorporating more than 3 pmol of dCTP into full-length products during the incubation. More pertinent to this discussion, is the fact that although the additional PyLT had a dramatic effect on the level of synthesis, it did not completely overcome the temperature-sensitive phenotype of polyoma in vitro DNA replication activity of wt CHO cell extract.

The effect of PEG-8000 on increasing certain macromolecular interactions (107) as well as results from DePamphilis' laboratory, which showed that PEG-8000 stimulated origin dependent DNA replication in an SV40 in vitro DNA replication assay (108), suggested that PEG-8000 may offer a mechanism to diminish the deleterious effects of assay temperature on the polyoma in vitro DNA replication activity of wt CHO cell extract. The effects of various concentrations of PEG-8000 on polyoma in vitro DNA replication activity of wt CHO cell extract assayed at 30°C or 37°C was determined. (See Figure 13) 61 ug of wt CHO cell extract was assayed at 30°C or 37°C in the presence of 0%, 2%, 5%, or 8% PEG-8000 for 2 hours. Incorporation into unit-length Dpn I resistant products was determined after agarose

gel electrophoresis as described in Materials and Methods. PEG-8000 stimulated polyoma in vitro DNA replication activity of wt CHO cell extracts at 30°C and 37°C. The fold stimulation over assays performed without PEG-8000 is presented in Table 3. In the presence of 5% PEG-8000 polyoma in vitro DNA replication activity was stimulated 3.2-fold at 30°C and 15.7-fold at 37°C compared to assays performed in the absence of PEG-8000. Because of the lower polyoma in vitro DNA replication activity of wt CHO extracts in the presence of 8% PEG-8000 compared to reactions containing 5% PEG-8000, and such as not to bias the effects of PEG-8000 in favor of higher relative polyoma in vitro DNA replication activity of wt extract assayed at 37°C compared to assays performed at 30°C, subsequent analyses of mutant cell extracts at elevated temperatures in the presence of PEG-8000 were performed in the presence of 5% PEG-8000.

3. EFFECTS OF EXTRACT PRE-INCUBATION ON SUBSEQUENT POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF WT CHO CELL EXTRACT

In order to attempt to circumvent the deleterious effects of elevated assay temperature on the polyoma in vitro DNA replication activity of wt CHO cell extract, attempts to pre-incubate wt extracts at elevated temperatures were made. Extract was pre-incubated under defined conditions and subsequently assayed at 30°C for 1 hour to determine the

effects of the pre-incubation on polyoma in vitro DNA replication activity. 100 ug of wt CHO cell extract was independently pre-incubated, as described in Materials and Methods, at either 30°C, 35°C, or 37°C for times ranging from 0.5 to 2 hours. As can be seen in Figure 14, pre-incubation at all temperatures led to loss of activity of wt extracts, compared to extracts pre-incubated on ice. Yet the degree of inactivation after 2 hour pre-incubations did not dramatically differ. This suggests that factors other than the presence of a labile factor(s) in wt CHO cell extract contributes significantly to the dramatically decreased polyoma in vitro DNA replication activity of wt CHO cell extract when assayed at 37°C (see Figures 10, 11, and 12). The potentially bi-phasic nature of the inactivation curve shown in Figure 14, suggests that one or more components (or functions) which stimulates polyoma in vitro DNA replication is comparatively labile, while other factors which are involved in polyoma in vitro DNA replication loose stimulatory activity at slower rates. In order to determine the feasibility of using higher pre-incubation temperatures to heat-treat CHO ts DNA⁻ mutant cell extracts, the effects of higher pre-incubation temperatures on the subsequent polyoma in vitro DNA replication activity of wt CHO cell extract activity was determined. 15 minute pre-incubations at temperatures ranging from 30°C to 41°C were performed on 143 ug of wt CHO cell extract protein before subsequent analysis of the remaining polyoma in vitro DNA replication

activity by assaying at 30°C for 1 hour. The results are shown in Figure 15. A 15 minute pre-incubation at 30°C does not lead to very significant inactivation of wt CHO cell extract. A loss of approximately 35% of the replication activity of wt CHO cell extract occurred when extract was pre-incubated at 37°C for 15 minutes. Virtually all of the subsequent polyoma in vitro DNA replication activity of wt CHO cell extract was lost when extract was pre-incubated for 15 minutes at temperatures greater than 39°C. Since wt CHO cell extract exhibited very significant amounts of inactivation during pre-incubation experiments, analysis of the polyoma in vitro DNA replication activities of CHO ts DNA⁻ mutant cell extracts after pre-incubations at elevated temperatures would be significantly hampered.

**B. ANALYSIS OF POLYOMA IN VITRO DNA REPLICATION ACTIVITIES
OF CHO WT AND ts DNA⁻ MUTANT CELL EXTRACTS
ASSAYED AT VARIOUS TEMPERATURES IN VITRO**

Analysis of polyoma in vitro DNA replication activities of CHO ts DNA⁻ mutants cell extracts at elevated temperatures in vitro was significantly hampered because wt CHO cell extract exhibited temperature-sensitive behavior when assayed for polyoma DNA replication in vitro. The deleterious effects of elevated assay temperature on the polyoma in vitro DNA replication activity of wt CHO cell extract can be somewhat alleviated by including 5% PEG-8000 to in vitro

reaction mixes and by adding additional PyLT. We therefore decided to assay CHO ts DNA⁻ mutant cell extracts at 30°C and 37°C, using the most concentrated PyLT available, in the presence of 5% PEG-8000.

Cell extracts were prepared from dense cell cultures of CHO wt, and several CHO ts DNA⁻ mutant cell lines, JB3-O, JB7-K and JB11-J, grown at 33°C. 55 ug of extract protein was assayed in the presence of 5% PEG-8000 for 1 or 2 hours at 30°C or 37°C. Incorporation in unit-length Dpn I resistant products was determined after agarose gel electrophoresis as described in Materials and Methods. The results are presented in Figure 16. The relative polyoma in vitro DNA replication activity at 37°C compared to the replication activity at 30°C after 1 or 2 hours of incubation is presented in Table 4. The extracts prepared from the CHO ts DNA⁻ mutants did not show any dramatic temperature-dependent defects for polyoma in vitro DNA replication, compared to wt CHO cell extract. However, decreased polyoma in vitro DNA replication activities of cell extracts prepared from the CHO ts DNA⁻ mutant cell lines JB3-O and JB7-K is clearly evident when the relative polyoma in vitro DNA replication activities of wt and mutant CHO cell extracts assayed at 30°C for 1 or 2 hours is analyzed (see Figure 17). Extracts prepared from the two CHO ts DNA⁻ mutant cell lines JB3-O and JB7-K consistently showed significantly lower polyoma in vitro DNA replication activities than extract prepared from wt CHO or from another CHO ts DNA⁻ mutant cell

line, JB11-J. This led us to hypothesize that JB3-O and JB7-K cell extracts were exhibiting temperature-independent defects in vitro.

II. CHARACTERIZATION OF WT AND CHO ts DNA⁻ MUTANT CELL EXTRACTS PREPARED FROM EXPONENTIALLY GROWING CULTURES

A. PROTOCOLS DEVISED TO PREPARE EXTRACT FROM EXPONENTIALLY GROWING CELL CULTURES FOR ANALYSIS OF POLYOMA IN VITRO DNA REPLICATION ACTIVITY

HB cell extracts prepared from dense cultures of CHO ts DNA⁻ mutant cell lines JB3-O and JB7-K showed consistently significantly lower polyoma in vitro DNA replication activities at all temperatures assayed in vitro than HB cell extracts prepared from wt CHO or another CHO ts DNA⁻ mutant cell line, JB11-J. These data led us to hypothesize that extracts derived from the CHO ts DNA⁻ mutant cell lines JB3-O and JB7-K were exhibiting temperature-independent defects for polyoma in vitro DNA replication activities. Since we were hypothesizing that extracts prepared from the CHO ts DNA⁻ mutant cell lines were exhibiting temperature-independent defects in vitro, it became essential to ensure that deficient in vitro DNA replication activities were not the result of poor cell growth in these very dense cell cultures.

A protocol was devised that ensured the extraction of

cells which had been growing rapidly for a number of days. The protocol is shown in Figure 18 and is described in Materials and Methods. Since exponentially growing cultures were used to seed dishes which were to be extracted, and because sister dishes of cultures which were extracted were counted at time points after extraction to ensure cells which were extracted were indeed growing logarithmically, I was certain that extracts were prepared from cells which had been growing exponentially for several days.

One drawback of this extraction protocol is that the HB cell extract prepared from tissue culture dishes containing less dense cell cultures have lower extract protein concentrations. I therefore decided to attempt to concentrate the CHO HB cell extract prepared from exponentially growing wt CHO cell cultures.

A RCF-ConFilt hollow fiber concentration unit was obtained from Bio-Molecular Dynamics and used to concentrate wt CHO HB cell extract. Concentration factors of greater than 20-fold were accomplished in less than 15 minutes. Both the concentrate and the filtrate were recovered. There are several reasons why I believe that extract concentration did not produce any artifacts. (1) Approximately 98% of the recovered protein was recovered in the concentrate, although this fraction generally represented only about 10% of the final total volume of concentrate and filtrate. Typically, approximately 90% of the total input protein was recovered.

(2) When assayed for polyoma in vitro DNA replication activity or for DNA polymerase activity, concentrated cell extract diluted down to the same protein concentration as unconcentrated HB cell extract, using the filtrate as a diluent, results in virtually indistinguishable results.

(3) Concentrated HB cell extracts from each CHO cell line, analyzed for polyoma in vitro DNA replication activities, gave reproducible maximal levels of polyoma in vitro DNA replication activities characteristic of that cell line, when increasing amounts of HB cell extract protein were assayed (for example see Figure 21). For these reasons, I believe that concentration of HB cell extract prepared from exponentially growing cell cultures did not produce any artifacts.

I wanted to determine the effects of cell extract protein concentration assayed on polyoma in vitro DNA replication activity. The results of an experiment assaying various amounts of concentrated wt CHO HB cell extract for polyoma in vitro DNA replication activity at 30°C for 2 hours are shown in Figure 19. Purified products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. Note that there is clearly a non-linear response to the amount of cell extract protein assayed when reactions contained less than approximately 60 ug of cell extract protein. The non-

linear response to protein in this concentration range has been routinely observed (see later experiments).

**B. CONCENTRATED HB CELL EXTRACTS PREPARED FROM THE *ts* DNA⁻
CHO MUTANT JB3-0 ARE DEFECTIVE FOR POLYOMA
IN VITRO DNA REPLICATION**

Since we hypothesized that JB3-0 HB cell extract prepared from dense cell cultures exhibited temperature-independent defects in vitro, I decided to concentrate and assay both wt CHO and JB3-0 HB cell extracts prepared from exponentially growing cell cultures for analysis of polyoma in vitro DNA replication activities of these extracts. Increasing amounts of concentrated cell extracts prepared from exponentially growing cultures of both cell lines were assayed at 30°C for 2 hours. Purified products were digested with Dpn I and linearized with Sal I, fractionated by agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. The results are shown in Figure 20. Virtually all of the polyoma in vitro DNA replication activity was dependent upon addition of PyLT and a plasmid containing a polyomavirus origin of DNA replication. Concentrated HB cell extracts prepared from JB3-0 exhibit significantly lower polyoma in vitro DNA replication activity than extracts derived from wt CHO cells at all protein concentrations assayed. In two experiments, described in Figure 21, increasing amounts of concentrated HB cell

extracts prepared from CHO wt and the ts DNA⁻ mutant JB3-O cell lines were assayed at 30°C for 2 hours. Products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. The maximal polyoma in vitro DNA replication activity of concentrated JB3-O HB cell extracts was only approximately 10-20% of that of concentrated CHO wt HB cell extract. The polyoma in vitro DNA replication activity of concentrated JB3-O HB cell extracts was dramatically lower than that of concentrated CHO wt HB cell extract at all protein concentrations assayed. There is no suggestion that if polyoma in vitro DNA replication reactions contained additional concentrated JB3-O cell extract, that this conclusion would be significantly affected. These data support the hypothesis that HB cell extracts derived from JB3-O exhibit temperature-independent defect(s) for polyoma in vitro DNA replication.

In order to determine if product formation occurred at a linear rate during two hour incubations and to see if any aberrant replicative forms were synthesized by concentrated HB cell extract prepared from JB3-O, a time-course of polyoma in vitro DNA replication activity of concentrated HB cell extracts prepared from CHO wt and the ts DNA⁻ mutant cell line, JB3-O, was performed. Additionally, concentrated HB cell extract prepared from JB7-K was also analyzed in order

to determine whether JB7-K exhibited any temperature-independent defects in vitro, as had been hypothesized earlier when extracts prepared from dense cell cultures were assayed in vitro.

I wanted to use a concentration of extract protein that would produce maximal polyoma in vitro DNA replication activity levels when extracts prepared from CHO wt, JB3-O and JB7-K extracts were assayed. As can be seen in Figure 21, the maximal level of polyoma in vitro DNA replication activity is apparent when extracts derived from these cell lines are assayed using approximately 175 ug of cell extract protein per assay. Therefore, 175 ug of concentrated HB cell extract derived from CHO wt, JB3-O and JB7-K were assayed at 30°C for 0.5, 1.0, 1.5 or 2.5 hours. Purified products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. The results are presented in Figure 22. Concentrated HB cell extracts prepared from JB7-K and JB3-O synthesized low levels of full-length Dpn I resistant products when compared to products synthesized by concentrated HB cell extract prepared from wt CHO cell cultures and did not show significant additional incorporation into full-length Dpn I resistant products when reactions were incubated for periods longer than 1 hour.

Neutral agarose gel electrophoretic analysis of the products synthesized during this time-course experiment by

extracts derived from CHO wt and ts DNA⁻ mutant cell lines JB3-O and JB7-K, without prior enzyme treatment, are shown in Figure 23. Products were purified, fractionated by agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. Several general observations were made. (1)The total amount of incorporation of radiolabel into product by extracts derived from JB3-O and JB7-K cells was significantly lower than that incorporated into products by wt CHO cell extracts. (2)Polyoma in vitro DNA replication reactions containing extracts derived for JB7-K or JB3-O accumulated replicative forms which were greater than replicon size and which did not accumulate in polyoma in vitro DNA replication reactions containing CHO wt cell extract. (3)Dramatically lower levels of plasmid monomeric products were synthesized by polyoma in vitro DNA replication reactions containing extracts derived from JB3-O and JB7-K, (4)As described above, virtually no additional full-length product is synthesized during the incubation period of 1 to 2.5 hours, as determined by analysis of DpnI/SalI digested samples. (5)Finally, monomeric products synthesized by wt CHO migrate predominately as form I DNA after deproteinization. Assays performed using lower concentrations of CHO wt extract protein synthesized monomeric products which predominantly migrated between form I and form II DNA when electrophoresed in a neutral agarose gel (see later experiments).

C. POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF OTHER ts DNA⁻ MUTANT CELL LINES

I wanted to determine how extracts prepared from other ts DNA⁻ mutant cell lines behaved in vitro in order to see whether the defective polyoma in vitro DNA replication activities of JB3-O and JB7-K were unique to extracts prepared from these mutant cell lines. Two other CHO ts DNA⁻ mutant cell lines, JB11-J and JB3-B, and one mutant, JB17-D, isolated from the Chinese hamster lung cell line, V79, were analyzed in an analogous fashion.

Reaction mixes containing increasing amounts of concentrated cell extract prepared from CHO wt and several CHO ts DNA⁻ mutant cell lines were assayed for 2 hours at 30°C. Purified products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. As shown in Figure 24, JB11-J and JB3-B were also defective for polyoma in vitro DNA replication. Extracts prepared from JB3-B and JB11-J exhibited significantly lower polyoma in vitro DNA replication activities compared to wt CHO cell extract polyoma in vitro DNA replication activity. Analysis of the products synthesized by JB3-B and JB11-J cell extracts by neutral agarose gel electrophoresis showed that the products synthesized were virtually identical to those synthesized by

concentrated CHO wt HB cell extract (indistinguishable from those seen in Figure 31, short exposure).

An analogous experiment was performed using concentrated HB cell extracts prepared from CHO wt, JB11-J and JB17-D. The results are shown in Figure 25. Extracts prepared from JB17-D exhibited different in vitro DNA replication activity versus protein concentration profiles, reaching a level of polyoma in vitro DNA replication activity approaching that of wt CHO.

Neutral agarose gel electrophoretic analysis of undigested replication products demonstrated that only extracts prepared from JB3-O and JB7-K synthesized aberrant replicative forms which were typically not seen when wt CHO cell extract or extract prepared from two other ts DNA⁻ CHO mutant cell lines, JB11-J and JB3-B, were assayed for polyoma in vitro DNA replication.

III. CHARACTERIZATION AND ATTEMPTED COMPLEMENTATION OF THE DEFECTIVE POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF JB3-O HB CELL EXTRACT

A. THE DEFECTIVE POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF JB3-O AND JB7-K ARE NOT DUE TO POOR CELL GROWTH, ANOMOLOUS CELL CYCLE DISTRIBUTION, OR ANOMOLOUS CELL EXTRACTION BEHAVIOR

We were concerned that the decreased polyoma in vitro DNA replication activities of JB3-O and JB7-K concentrated HB cell extracts prepared from dense cell cultures could be the result of poor cell growth. Since these extracts were prepared from exponentially growing cell cultures, the deficiencies in polyoma in vitro DNA replication activities exhibited by concentrated JB3-O and JB7-K HB cell extracts are not due to the result of poor cell growth.

Roberts and D'Urso (109) have shown that extracts prepared from S phase human cells are approximately 10-fold more efficient in initiating SV40 in vitro DNA replication than extracts prepared from G1 phase human cells. Therefore, in order to rule out the potential effect of anomalous distribution of cells within the cell cycle could have on Polyoma in vitro DNA replication activities of CHO cell extracts, samples were prepared for FMF (flow microfluorometric) analyses to determine the distribution of cells within the cell cycle at the time of extract preparation. Samples for FMF analysis were prepared from sister dishes of cell cultures which were being extracted. Variation between sister dishes typically varied by only 2-3% for each of the stages of the cell cycle assessed. The results are presented in Table 5. FMF analyses show that there are no significant differences in the distribution of cells when HB extracts were prepared from CHO wt and the ts DNA⁻ mutant cell lines JB3-O, JB7-K and JB11-J. I wanted to rule out gross extraction abnormalities as the reason for the

deficient polyoma in vitro DNA replication activities of JB3-O and JB7-K. I therefore decided to determine whether the extracts prepared from JB7-K and JB3-O were grossly anomalous.

The first analysis involved determining the level of replication protein A (RP-A) within the concentrated HB cell extracts. A rabbit polyclonal prepared against human RP-A was used to determine the level of RP-A within each of these cell extracts. Samples containing 75 ug, 150 ug and 300 ug of concentrated HB cell extract from each of these cell lines were separated by SDS-PAGE and subsequently transferred to a nitrocellulose filter and the level of RP-A was determined by western blotting as described in Materials and Methods. The results are presented in Figure 26. Although densitometric analysis was not performed on this western blot, there were obviously very similar levels of RP-A in each of these concentrated HB cell extract. Additionally, preliminary experiments show extracts prepared from JB11-J and JB3-B also have levels of RP-A 34 kDa subunit similar to that of wt CHO HB cell extract. These data strongly argue against any gross extraction abnormalities being exhibited by any of these cell lines.

I next decided to use a functional assay to assess the relative quality of the HB cell extracts prepared from CHO wt and the ts DNA⁻ mutant cell lines. DNA polymerase assays were performed using 25 ug of each concentrated HB cell extract. Using concentrated wt HB cell extract, this

quantity of concentrated HB cell extract was shown to exhibit a near-linear rate of incorporation for at least 2 hours. Assays were incubated at 30°C for either 0.5, 1.0 or 1.5 hours. The DNA polymerase assays were performed and processed as described in Materials and Methods. The results are shown in Figure 27. These extracts exhibit virtually indistinguishable levels of DNA polymerase activity, as measured using activated DNA as a template. These data strongly suggest that the extracts prepared from the ts DNA⁻ mutants JB3-O and JB7-K are generally qualitatively similar to the concentrated extracts prepared from wt CHO. Additionally, these data are in sharp contrast to the defective polyoma in vitro DNA replication activities exhibited by these same extracts.

In summary, the virtually indistinguishable levels of RP-A 34 kDa subunit and DNA polymerase activities of extracts derived from JB3-O and JB7-K suggest that these extracts are not grossly anomalous, compared to wt CHO.

B. DEFECTIVE POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF JB3-O AND JB7-K HB CELL EXTRACTS ARE NOT DUE TO THE PRESENCE OF POTENT INHIBITORS OF DNA REPLICATION

We next wanted to determine whether the decreased polyoma in vitro DNA replication activities of JB3-O and JB7-K concentrated HB cell extracts were due to the presence of a potent inhibitor(s) of DNA replication in the extracts.

A mixing experiment involving concentrated HB cell extracts prepared from CHO wt, JB3-O, and JB7-K was performed.

Polyoma in vitro DNA replication assays were incubated for 2 hours at 30°C. Purified products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. The results are presented in Figure 28. Polyoma in vitro DNA replication assays containing 125 ug each of CHO wt and JB7-K cell extracts and assay tubes containing 125 ug each of CHO wt and JB3-O exhibited significantly more polyoma in vitro DNA replication activity than did assays containing only 125 ug of concentrated CHO wt HB cell extract. These data suggest that there is no potent polyoma in vitro DNA replication inhibitor(s) present in JB3-O and JB7-K concentrated HB cell extracts.

**C. MIXING EXPERIMENTS INVOLVING EXTRACTS PREPARED FROM
CHO WT, JB3-O AND JB7-K SHOW NO OBVIOUS
COMPLEMENTATION IN VITRO**

In vitro complementation between two mutant cell extracts, which are defective in vitro, suggests that when mixed together they possess all the replication factors required for efficient in vitro DNA replication. If only one DNA replication factor is defective in each of the extracts and the factor which is defective is unique to that extract,

then mixing of the two extracts should produce an extract mixture which exhibits near wild-type in vitro DNA replication activity level.

While simultaneously assembling the mixing experiment described above (results shown in Figure 28), additional reaction mixes were assembled to see whether there was any obvious in vitro complementation between extracts derived from CHO wt, JB3-O and JB7-K. Data have been compiled and are presented in Figure 29. The polyoma in vitro DNA replication activity of reaction mixes containing either wt and JB7-K extracts or wt and JB3-O extracts (125 ug of each extract) exhibited significantly lower polyoma in vitro DNA replication activity than when 250 ug of wt CHO cell extract was assayed alone. These data show that there is no obvious complementation when concentrated wt HB cell extract is mixed with concentrated HB cell extract derived from JB3-O or JB7-K. Several possible explanations could account for these observations. One possibility is that the defective function in the mutant is present in limiting quantities in wt HB cell extract. Another possibility is that concentrated HB cell extracts prepared from JB7-K and JB3-O are deficient in a number of functions, one of which is present in limiting quantities in wt CHO cell extract. Therefore, mixing of these extracts does not result in complementation.

Mixing of concentrated HB cell extracts prepared from the CHO ts DNA⁻ mutant cell lines JB3-O and JB7-K does not show any obvious complementation (Figure 29). Polyoma in

vitro DNA replication activity of these mixed extracts is dramatically lower than the polyoma in vitro DNA replication activity of equivalent total amounts of wt CHO HB cell extract and is approximately equivalent to the polyoma in vitro DNA replication activity of equivalent amounts of each extract assayed separately. Agarose gel electrophoretic analysis of the replication products synthesized in another mixing experiment is presented in Figure 30. The products synthesized in polyoma in vitro DNA replication reactions containing extracts derived from JB3-O and JB7-K are virtually indistinguishable from the products synthesized when an equivalent amount of each cell extract is assayed separately. These data, taken together with the fact that mixing of concentrated HB cell extracts derived from JB3-O and JB7-K does not lead to any obvious complementation, suggest that extracts derived from JB3-O and JB7-K may have one or more defective replication functions in common.

**D. POTENTIAL LINKAGE OF THE DEFECTIVE IN VITRO DNA
REPLICATION ACTIVITY OF JB3-O AND THE *ts* CELL GROWTH
PHENOTYPE**

Several data suggested that we re-evaluate the hypothesis that JB3-O and JB7-K HB cell extracts were exhibiting temperature-independent defects for polyoma in vitro DNA replication activity which are linked to the *ts* growth defect. Extracts prepared from a number of the *ts*

DNA⁻ CHO mutant cell lines exhibited defective polyoma in vitro DNA replication activities, suggesting a possible common defect in a number of the mutant cell extracts. I believe that it is unlikely all of the mutant cell extracts exhibit temperature-independent defects in vitro. Additionally, mixing experiments involving extracts prepared from CHO wt, JB3-O and JB7-K did not show any obvious complementation in vitro suggesting at least one common factor is rate-limiting.

The ultimate goal of this project, at its inception, was to identify extracts derived from CHO ts DNA⁻ mutant cell extracts which exhibited temperature-dependent or temperature-independent defects in vitro, with the hopes of developing an in vitro complementation system which could lead to the identification of the wild-type homologous protein which could correct the in vitro defect. Sequencing of the purified protein could lead to the subsequent ability to clone the wild-type gene. This approach could ultimately be used to show that the mutated gene product is required for cellular and polyomavirus DNA replication and that it is required for cell viability. With these long term goals in mind, and in light of the data discussed above, it became important to determine whether the defective polyoma in vitro DNA replication activity of concentrated JB3-O HB cell extract could be linked to the ts growth phenotype.

Revertants were isolated from JB3-O as described in Materials and Methods. Since I felt it was important to maintain cell populations at 33°C for a number of passages before extract preparation to ensure cells are well adapted to growth at 33°C before extract preparation, I decided to determine if the revertants I had isolated from JB3-O were stable after being maintained at 33°C for extended periods. Two revertants isolated from JB3-O, JB3-O/R1 and JB3-O/R2 were chosen for characterization. These two cell lines were passaged at 33°C for approximately 1 month before being used to determine the relative plating efficiency (PE) of each of these two cell lines incubated at 39.5°C compared to the plating efficiency observed for cells incubated at 33°C. The results are presented in Table 6. When JB3-O/R1 was seeded to determine relative PE at 39.5°C compared to the PE observed at 33°C, there were 80% as many colonies observed in the dishes which had been incubated at 39.5°C as had been observed in dishes which were incubated at 33°C. When JB3-O/R2 was seeded to determine the relative PE at 39.5°C compared to the PE observed at 33°C, there were 46% as many colonies in the dishes which were incubated at 39.5°C as had been observed in dishes which were incubated at 33°C. These data suggest that the revertant phenotypes of JB3-O/R1 and JB3-O/R2 are relatively stable and extraction of cells grown at 33°C could be performed under conditions in which the revertants had not lost there revertant phenotype in vivo.

Concentrated HB cell extracts were prepared from CHO wt, JB3-O, JB3-O/R1 and JB3-O/R2. Increasing concentrations of these HB cell extracts were assayed at 30°C for 2 hours. Products were purified, fractionated by agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. Locations of form I, II, and III DNA of the input plasmid are marked. The results of polyoma in vitro DNA replication reactions involving extracts prepared from CHO wt and JB3-O/R1 are presented in Figure 31. Extract prepared from the revertant JB3-O/R1 exhibited the same low level of polyoma in vitro DNA replication activity exhibited by extract prepared from JB3-O. Additionally, apparently the same aberrant replication product(s), as was described for extracts derived from JB3-O, is the prominent replicative form(s) synthesized by concentrated JB3-O/R1 HB cell extract. Extract prepared from JB3-O/R2 gave virtually indistinguishable results. In summary, the levels of polyoma in vitro DNA replication activity and the replicative forms synthesized by JB3-O, JB3-O/R1 and JB3-O/R2 extracts are indistinguishable. We were therefore unable to correlate the correction of the growth defect with the correction of the defective polyoma in vitro DNA replication activity exhibited by JB3-O HB cell extract.

Since we were not able to correlate the ability of the revertants to grow at the non-permissive temperature (NPT) with an increase in the polyoma in vitro DNA replication activities of extracts derived from the revertants. I

decided that it was important to determine how temperature affected the intracellular replication of polyomavirus DNA in CHO wt, JB3-O, JB3-O/R1 and JB3-O/R2 cell lines. Cell cultures were transfected with the plasmid p53A6.6, which contains the entire polyomavirus genome as described in Materials and Methods. After transfection, cultures were either (1) incubated at 33°C for 48 hrs or (2) incubated at 33°C for 24 hrs and shifted to 39.5°C and incubated for 24 additional hrs or (3) incubated at 33°C for 72 hrs. After incubation of cell cultures, the viral DNA was extracted, purified, and digested with Sal I and Dpn I. Linearized replication products were visualized after autoradiography of the Southern blots as described in Materials and Methods. The results are presented in Figure 32. Only CHO wt cells exhibit enhanced polyoma in vivo DNA replication activity when shifted to 39.5°C. The CHO ts DNA⁻ mutant JB3-O and the revertant JB3-O/R2 restrict in vivo polyoma DNA replication at 39.5°C, compared to CHO wt. The revertant JB3-O/R1 shows no dramatic effects of incubation temperature on the amount of polyoma DNA replication in vivo. It should be noted that the effects of incubation temperature on in vivo polyoma DNA replication activity of JB3-O/R1 is not similar to that of CHO wt. Quantitation of the unit-length products as described in Materials and Methods and as described in Figure 33 were performed. The absolute (Figure 33) and relative values (Figure 34) of the levels of polyoma DNA replication in vivo in each cell line support the above interpretation of

the autoradiograms. Observation of cell cultures at various points after the transfection were performed. The only cells that looked unhealthy after being shifted to 39.5°C were JB3-O cells.

In summary, although the revertants JB3-O/R1 and JB3-O/R2 are stable revertants and are able to form colonies when seeded at 39.5°C, polyomavirus DNA replication in vivo at 39.5°C is only partially restored, at best. Therefore, we were unable to conclusively show linkage of the in vitro defect to the ts growth defect.

E. CHARACTERIZATION OF ABERRANT POLYOMA IN VITRO

DNA REPLICATION PRODUCTS SYNTHESIZED BY JB3-O

HB CELL EXTRACT

In spite of the uncertainty of whether the defective polyoma in vitro DNA replication activity is linked to the ts growth defect, we wanted to attempt to characterize the replication products synthesized by concentrated JB3-O HB cell extracts in an attempt to gain insight into the defective function(s) in extracts derived from this CHO ts DNA⁻ mutant cell line. Analysis of the structure of the aberrant replication products, along with experiments to determine the rate of accumulation of specific replicative forms during time-course experiments, could help determine whether initiation, elongation, or termination are effected,

and thereby potentially narrow down the scope of potentially defective functions.

The first characterization involved determining the size of the products synthesized by concentrated wt and JB3-0 HB cell extracts. The first relevant piece of information was obtained from a time-course experiment. Some of these data were presented earlier in Results section II.B. (Figure 22). In vitro reactions containing 175ug of concentrated JB3-0 HB cell extract were incubated at 30°C for 0.5, 1, 1.5 or 2.5 hours. Polyoma in vitro DNA replication products were purified and one-half of each assay was digested with Sal I and DpnI. Undigested and Dpn I/Sal I digested products were fractionated by agarose gel electrophoresis and visualized by autoradiography of the dried down agarose gel. The results are presented in Figure 35. As can be seen in panel A, after one hour of incubation at 30°C, the majority of the incorporated radiolabel is present in replicative forms whose mobilities are slower than that of the monomeric topoisomers. Digestion of the other half of this sample with Sal I and Dpn I show a band of significant intensity (both autoradiograms were exposed simultaneously for the same length of time) which migrated as form III DNA, suggesting fully replicated molecules (or molecules greater than approximately 95% replicated) are present.

Analysis of SalI/DpnI digested and undigested samples after longer incubations are also informative. Note that, as shown in Figure 22, there is virtually no additional

incorporation into full-length DpnI products after 1 hour of incubation. Yet analysis of the undigested samples show that during the period between 1 and 2.5 hours of incubation there is an increase in the amount of monomer seen. If one carefully examines the total amount of slower migrating replicative forms, its total level appears to decrease during the same time interval that additional monomer appears. Collectively, these data suggest that at least some of the less mobile replicative form can be resolved into monomeric products.

In summary, the results of neutral agarose gel electrophoretic analysis of replicative forms synthesized during a time-course experiment suggest that after 1 hour of incubation at 30°C the majority of the polyoma in vitro DNA replication products have not been resolved into monomers and that they are near unit-length. Additionally, since there is a decrease in the amount of the slower migrating replication product and an increase in the amount of monomeric product seen, without a net increase in the amount of full-length Dpn I resistant product (as was presented in Figure 22), at least some of the slower migrating replicative forms are able to ultimately be resolved into monomeric products.

The size of the DNA fragments synthesized by concentrated JB3-0 HB cell extract was characterized by alkaline gel electrophoretic analysis of the polyoma in vitro DNA replication products synthesized by JB3-0 extract. In vitro replication reactions containing 175 ug of concentrated

HB cell extract derived from JB3-O or CHO wt were assayed for 10, 20, 30, 60, or 120 minutes. Products were purified, fractionated in a 1.4% alkaline agarose gel and visualized by autoradiography of the dried down agarose gel. The results are presented in Figure 36. Analysis of the products synthesized by wt CHO cell extract during the time-course experiment show that after 60 minutes of incubation at 30°C a prominent product co-migrates with an input plasmid marker. A significant proportion of products seen in the neutral agarose gel reinforce the argument that the major band seen in the alkaline gel analysis represents monomeric products. Analysis of the polyoma in vitro DNA replicative forms synthesized during the time-course experiment (see Figure 36) shows that, even after an incubation period of 1 hour, the majority of the replicative forms synthesized by concentrated JB3-O HB cell extract are generally greater than 2 kb in length but do not co-migrate with the monomeric marker. The input plasmid is approximately 2.6 kb, suggesting that the product molecules seen on a neutral agarose gel are more than 75% replicated. The neutral agarose gel analysis of the products synthesized by JB3-O cell extracts after a 1 hour incubation at 30°C are predominantly not monomeric products (see Figure 23). Only during the time period between 1 hour and 2 hours of incubation does a significant amount of monomeric product appear, as determined by observation of both neutral and alkaline gels.

These results suggest that concentrated JB3-O HB cell extract synthesized products which were predominantly greater than 75% replicated but not completed and resolved into monomeric products. These data support the interpretation of the neutral agarose gel analysis of undigested and SalI/DpnI digest products synthesized during a time-course experiment by JB3-O cell extract, as discussed above.

Neutral and alkaline gel electrophoretic analysis of polyoma in vitro DNA replicative forms synthesized by concentrated JB3-O HB cell extract consisted predominantly of molecules which were greater than 75% replicated. I therefore decided to see whether the products were simply catenated dimers or some other replicative forms which were topologically related to monomeric products. I attempted to resolve the polyoma in vitro DNA replication products synthesized by concentrated HB cell extracts derived from CHO wt, JB3-O and JB7-K into monomeric products by incubation with purified topoisomerase I or topoisomerase II. The results of these attempts are presented in Figure 37. Incubation of polyoma in vitro DNA replication products with topoisomerase I or topoisomerase II did not lead to significant changes in the predominant aberrant products synthesized by extracts derived from JB3-O and JB7-K. These data suggest that these aberrant products are not catenated dimers nor are they topologically related to monomeric products.

**F. ANALYSIS OF CHO WILD-TYPE AND JB3-O CELL EXTRACTS
PREPARED UNDER DIFFERENT EXTRACTION CONDITIONS**

I wanted to see if changing extraction conditions could significantly increase the polyoma in vitro DNA replication activity of JB3-O cell extract to a level approaching that of wt CHO cell extract. It has been observed that certain proteins require high salt in order to be extracted from the nucleus. A protein, CAF-I (chromatin assembly factor I) (84), which is involved in distributing histones to the parental and newly replicated DNA molecules during SV40 DNA replication in vitro, requires greater than 0.2 M NaCl in order for significant amounts of the protein to be extracted from the nucleus. Additionally, extraction of CHO hypotonic lysates with 0.2 M NaCl yields extracts which contain several-fold higher levels of topoisomerase activity than if no NaCl is added. Therefore, HB and 0.2 M NaCl/HB cell extracts were prepared from wt and JB3-O cell cultures as described in Materials and Methods in order to address this issue.

I first wanted to determine whether concentrated JB3-O 0.2 M NaCl/HB cell extracts exhibited increased polyoma in vitro DNA replication activity compared to simultaneously produced concentrated JB3-O HB cell extract. A similar analysis of 0.2 M NaCl/HB and HB extracts prepared from wt CHO cells was simultaneously performed.

Increasing amounts of concentrated cell extract were assayed at 30°C for 2 hours. Purified products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. The results of this experiment are shown in Figure 38. Assays containing greater than approximately 75 ug of 0.2 M NaCl/HB cell extract isolated from wt CHO cells exhibited significant amounts of inhibition of polyoma in vitro DNA replication activity. These effects were not as dramatic when JB3-O 0.2 M NaCl/HB cell extract was assayed. Although JB3-O 0.2 M NaCl/HB cell extracts were significantly more active than the simultaneously prepared JB3-O HB cell extract, the 0.2 M NaCl/HB JB3-O cell extract still had only approximately 25% of the maximal polyoma in vitro DNA replication activity of analogous extracts prepared from wt CHO cells.

Undigested purified polyoma in vitro DNA replicative forms synthesized by 175 ug of HB or 150 ug of 0.2 M NaCl/HB cell extracts prepared from JB3-O and wt CHO during a time-course experiment were analyzed by neutral and alkaline agarose gel electrophoresis. Purified polyoma in vitro DNA replication products were fractionated by neutral agarose gel electrophoresis and visualized by autoradiography of the dried down gel as described in Material and Methods. The results are presented in Figure 39. The 0.2 M NaCl extracts prepared from CHO wt and JB3-O synthesize products which have

different topological distributions than the analogous simultaneously prepared HB cell extract, as has been described previously. During the time-course, form I monomeric products synthesized by wt CHO HB cell extract not predominantly seen until time points greater than 1 hour , whereas form I monomeric products are clearly the predominant monomeric form seen after 1 hour when the 0.2 M NaCl/HB cell extract prepared from wt CHO cells is assayed. Observation of the products synthesized by 0.2 M NaCl/HB cell extracts prepared from JB3-O cells shows that significantly more monomeric products are synthesized than after a comparable incubation of JB3-O HB cell extracts.

Alkaline agarose gel electrophoretic analysis of the replicative products synthesized during this time-course experiment is presented in Figure 40. The most significant observation pertaining to the results of the products synthesized by wt CHO cell extracts is that the 0.2 M NaCl/HB cell extracts assayed at this protein concentration have significantly less polyoma in vitro DNA replication activity than does the HB cell extract, as has been shown for the synthesis of full-length products in Figure 38. There are two related observations that I have made. HB cell extract prepared from JB3-O synthesized significant amounts of disperse products typically greater than 2 kb, these products are not as prominent in the time-course involving 0.2 M NaCl/HB JB3-O cell extracts. Indeed, the 0.2 M NaCl/HB JB3-O cell extracts synthesize significantly more monomeric

products, as is evidenced by the increase in the intensity of the band on the autoradiogram at the position to which monomeric products migrate. Neutral and alkaline agarose gel electrophoretic analysis of the products synthesized during polyoma in vitro DNA replication activities of 0.2 M NaCl/HB and HB cell extract derived from JB3-O have been informative. High resolution neutral agarose gel electrophoretic analysis of the polyoma in vitro DNA replication products synthesized by these extracts is presented in Figure 41. The products synthesized during 2 hour incubations at 30°C by 75 ug, 150 ug, 200 ug, or 250 ug of HB or 0.2 M NaCl/HB cell extract protein derived from CHO wt of JB3-O were purified, fractionated by agarose gel electrophoresis and visualized by autoradiography of the dried down gel as described in Material and Methods.

HB cell extracts prepared from wt and JB3-O cell cultures synthesized different distributions of monomeric topoisomers. At virtually all protein concentrations assayed, HB cell extracts prepared from JB3-O synthesized monomeric products which, after deproteinization, migrate predominantly as form I DNA. HB cell extracts prepared from wt CHO synthesized a different spectrum of monomeric topoisomers which was dependent upon the amount of extract protein assayed. In vitro reactions containing 75 ug of extract protein synthesized products which, after purification, migrate predominantly as topoisomers ranging in migration on the gel from form I to form II DNA. When

greater levels of wt HB cell extract were assayed more of the purified products migrated closer to form I plasmid DNA.

0.2 M NaCl/HB cell extracts prepared from wt and JB3-O cell cultures synthesized different distributions of monomeric topoisomers. At virtually all protein concentrations assayed, the predominant monomeric products synthesized by wt CHO cell extract, after purification, migrated predominantly as form I DNA. 0.2 M NaCl/HB cell extract prepared from JB3-O synthesized monomeric products which migrated predominantly as relaxed DNA or topoisomers migrating between form I and form II. With an increase in the protein concentration assayed, relatively more products, after purification, tended to run shifted toward topoisomers near form I DNA. Monomeric products synthesized by HB and 0.2 M NaCl/HB cell extracts prepared from CHO wt and JB3-O differ significantly.

Different replicative forms migrating between the origin and relaxed (form II) plasmid DNA were observed. The replication intermediate which was a predominant product synthesized by JB3-O HB cell extract is evident and appears to still be synthesized by 0.2 M NaCl/HB JB3-O cell extract. Interestingly, a new atypical replicative form is seen when 0.2 M NaCl/HB cell extract prepared from wt CHO is assayed. The replicative forms migrate to a position similar to that of predominant replicative forms synthesized by HB cell extracts prepared from JB3-O; some subtle differences are noted.

In summary, although the 0.2 M NaCl/HB extract prepared from JB3-O is significantly more active than the simultaneously prepared JB3-O HB cell extract, a change in the extraction procedure does not overcome the dramatically defective polyoma in vitro DNA replication character of extracts prepared from JB3-O (see Figure 38).

**G. ATTEMPTED COMPLEMENTATION OF THE DEFECTIVE POLYOMA
IN VITRO DNA REPLICATION ACTIVITY OF HB CELL EXTRACT
PREPARED FROM JB3-O BY ADDITION OF PHOSPHOCELLULOSE
CHROMATOGRAPHIC FRACTIONS PREPARED FROM
WT CHO CELL EXTRACT**

Although we were unable to link the defective polyoma in vitro DNA replication activity of concentrated JB3-O HB cell extracts to the ts growth phenotype. We decided to try to complement the deficient in vitro replication activity using phosphocellulose chromatographic fractions prepared from wt CHO cell extract.

Phosphocellulose chromatographic fractionation was performed as described in Materials and Methods. Protein assays were performed to determine the protein concentration of phosphocellulose flow-through and wash fractions (CF I) and 1 M KCl eluate fractions (CF II). The results of these protein assays is presented in Figure 42. Typically, CF I contained a total of approximately 75% of the total protein in CF I and approximately 25% of the total in the CF II

fraction. After dialysis and concentration of CF I and CF II, as described in the Material and Methods, the concentrated fractions were assayed for DNA polymerase activity. The most desirable characteristics of fractions to use in attempts to complement mutant cell extracts would be that they would be separately inactive and when mixed together would reconstitute polyoma in vitro DNA replication activity. A successful phosphocellulose fractionation yielded the DNA polymerase activity results presented in Figure 43. Fractionations which yielded these ideal fractions typically had over 80% of the DNA polymerase activity present in CF II. Several phosphocellulose fractionations yielded concentrated CFI fractions which independently had polyoma in vitro DNA replication activity and which could be stimulated by the simultaneously prepared inactivate concentrated CF II. DNA polymerase assays of these fractions showed a significantly greater percentage of total DNA polymerase in the CF I fraction (30% or more). Reconstituting of polyoma in vitro DNA replication activity typically yielded roughly 5-20% of the activity of a crude wt CHO extract when similar levels of protein were assayed. These results are similar to those obtained during fractionation of human cell extracts focused on identification of cell factors required for SV40 in vitro DNA replication (David Weinberg, personal communication) and are presented in Figure 44. The reconstituted polyoma in vitro DNA replication activity is dependent upon the addition of

PyLT and a plasmid containing the polyomavirus origin of replication.

Since I had prepared concentrated CF I and CF II fractions which were separately inactive yet could reconstitute polyoma in vitro DNA replication activity, I decided to use these phosphocellulose fractions to try to complement the defective polyoma in vitro DNA replication activity exhibited by JB3-O HB cell extract using these fractions. Attempts to complement the defective polyoma in vitro DNA replication activity of JB3-O cell extract using these phosphocellulose fractions was performed in the presence of 100 ug of concentrated JB3-O HB cell extract. In vitro reactions containing 100 ug of concentrated wt CHO extract were also assayed under the identical conditions. In vitro reactions were incubated for 2 hours at 30°C. Purified products were split into aliquots. One aliquot was digested Dpn I and Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. An undigested aliquot was fractionated by neutral agarose gel electrophoresis, and visualized by autoradiography of the dried down gel as described in Material and Methods.

The effects of the addition of CF I or CF II on the polyoma in vitro DNA replication products synthesized by wt CHO cell extract is shown in Figure 45. Increasing concentrations of CF I inhibit the polyoma in vitro DNA replication activity of wt CHO cell extract. Additionally,

upon addition of CF I an additional band was seen in the same location of the gel as form III plasmid DNA; no other topological changes were observed. Although CF II exhibits some inhibitory effects on the polyoma in vitro DNA replication activity of wt CHO cell extract, a noticeable change in the distribution of monomeric topoisomers was observed when 26 ug of CF II was added. This is clearly not just a general effect of the addition of more protein because other lanes containing added protein did not show a similar response (e.g. see CF I results). The same product, which showed migration similar to that of form III DNA upon addition of CF I, was also evident when CF II was added. The quantitation of unit-length Dpn I resistant products synthesized when CF I or CF II was added is presented in Figures 47 and 48. Both CF I and CF II contain factor(s) which inhibit polyoma in vitro DNA replication by wt CHO cell extract. Note that the filtrates prepared during the concentration of CF I and CF II are not inhibitory (see Figures 47 and 48), suggesting that protein(s) found in CF I and CF II are inhibitory.

The effects of addition of CF I and CF II on the polyoma in vitro DNA replication activity and replicative forms synthesized by concentrated JB3-O HB cell extract were determined simultaneously under identical conditions. The effects of the addition of CF I and CF II on the polyoma in vitro DNA replication products synthesized by 100 ug of concentrated HB JB3-O cell extract is shown in Figure 46.

Increasing concentrations of CF I clearly inhibit the polyoma in vitro DNA replication activity of JB3-O HB cell extract. A decrease in the amount of purified replication product co-migrated with form I DNA was observed and product migrating similar to form III DNA is observed, as has been described when CF I or CF II were added to concentrated wt HB cell extract. Addition of CF II to JB3-O HB cell extract stimulates the level of incorporation of radiolabel into certain monomeric products as well as into the aberrant in vitro DNA replication product. No significant change in the distribution of monomeric topoisomers other than that already noted, was observed when 26 ug of CF II was added. The amount of unit-length Dpn I resistant products synthesized when CF I or CF II is added is presented in figures 34 and 35. As discussed with the addition of CF I to wt HB cell extracts, addition of CF I to JB3-O HB cell extract inhibits polyoma in vitro DNA replication. Note that the filtrates prepared during the concentration of CF I and CF II are not inhibitory (see Figures 47 and 48). The addition of CF II stimulates polyoma in vitro DNA replication by JB3-O HB cell extract. This observation is very different than that observed when CF II was added to the polyoma in vitro replication activity of CHO wt HB cell extract. One or more factors in CF II stimulates polyoma in vitro DNA replication by JB3-O HB cell extracts. The addition of only 10 ug of CF II stimulates polyoma in vitro DNA replication activity by approximately 1.7-fold (see Figure 48). Since there are

obviously inhibitory factor(s) present, as demonstrated by the inhibitory effect on in vitro activity of CHO wt HB cell extract, this stimulation may actually be under-estimated. This stimulation, although significant, does not stimulate polyoma in vitro DNA replication activity of JB3-O HB cell extracts to levels approaching that of wt CHO HB cell extracts, as is clearly seen in Figure 47. The presence of inhibitors and the fact that the fractions reconstituted only approximately 15% of the original polyoma in vitro DNA replication activity, compared to wt CHO HB cell extract, could partly explain why more stimulation is not seen.

**G. ATTEMPTED COMPLEMENTATION OF THE DEFECTIVE POLYOMA
 IN VITRO DNA REPLICATION ACTIVITY OF JB3-O HB
 CELL EXTRACT BY THE ADDITION OF PURIFIED TOPOISOMERASE I
 OR TOPOISOMERASE II**

I felt that a significant amount of data had accumulated to warrant attempts to complement concentrated JB3-O cell extracts with purified topoisomerase. Concentrated HB cell extracts prepared from JB3-O synthesized aberrant slowly migrating replicative forms, some of these products appear to have been able to be resolved, in time, into monomeric products. Additionally, analysis of the polyoma in vitro DNA replication products synthesized by JB3-O HB cell extracts were determined to be greater than 75% replicated. Analysis of human cell extract, prepared by HB extraction, showed SV40

in vitro DNA replication activity was limited by the levels of topoisomerase activities in the extract (77).

Additionally, topoisomerase II alone could perform all the functions need for monomeric product synthesis, whereas topoisomerase I could not decatenate the final products.

These data suggest that there may be a topoisomerase functional deficiency in these extracts, and in particular a topoisomerase II functional deficiency. I therefore decided to try to complement concentrated JB3-O HB cell extracts with purified topoisomerase II (topo II), which was kindly provided by Bruce Stillman, and with purified topoisomerase I which had been obtained from Gibco/BRL. In vitro reaction mixes containing 175 ug of concentrated HB cell extract prepared from CHO wt or JB3-O cell cultures was assayed for 2 hours at 30°C in the presence or absence of purified topoisomerase I or topoisomerase II. An aliquot of the purified in vitro DNA replication products was digested with DpnI and SalI followed by subsequent autoradiography and quantitation of the unit-length DpnI resistant products. The results of these analyses are shown in Figures 49 and 50. There is significant stimulation of the polyoma in vitro DNA replication activity of JB3-O HB cell extracts seen in all assay tubes containing added topoisomerase II. This effect is not seen in tubes containing additional HB cell extract protein nor in the buffer only controls. Topoisomerase II also stimulates the polyoma in vitro DNA replication activity of concentrated wt CHO HB cell. The stimulation of polyoma

in vitro DNA replication activity of JB3-O was approximately 2-fold, while the stimulation of wt CHO was only approximately 1.2-fold. Although the addition of topoisomerase II significantly stimulates JB3-O HB cell extract polyoma in vitro DNA replication activity, the absolute polyoma in vitro DNA replication activity is still only approximately 35% of that of wt CHO cell extract assayed under the same conditions (see Figure 50). Even though a topoisomerase II titration was not performed, due to limited supply, enough topoisomerase II had been added to completely decatenate 500 ng of kinetoplast DNA in 1 hour. Another aliquot of the polyoma in vitro DNA replication products was fractionated by agarose gel electrophoresis and visualized by autoradiography of the dried down gel as described in Material and Methods. These results are presented in Figure 51. When the products synthesized by concentrated JB3-O HB cell extracts are fractionated by agarose gel electrophoresis more monomeric products are observed exclusively in lanes to which topo II had been added to the in vitro reactions. An analogous increase is seen in the in vitro reactions that contained wt CHO HB cell extract, although the increase is not significant.

JB3-O HB cell extract may exhibit multiple deficiencies which lead to the defective phenotype for polyoma in vitro DNA replication activity exhibited by JB3-O HB cell extract. Addition of the phosphocellulose fraction CF II stimulated incorporation into the aberrant replicative form typically

synthesized by JB3-O concentrated HB cell extract, without an apparent increase in the amount of monomeric product synthesized. The stimulation afforded by the introduction of exogenously added topo II with the concomitant increase in the amount of monomer synthesized suggests that this stimulation may be by a different mechanism. Taken together, these observations suggest that the defective polyoma in vitro DNA replication activity exhibited by JB3-O HB cell extracts may be caused by a number of defective replication functions which are required for efficient polyoma in vitro DNA replication.

CHAPTER 4 - DISCUSSION

The development of SV40 (53,54,55) and polyomavirus (88,94) in vitro DNA replication assays has allowed unprecedented progress in the isolation of novel cellular factors involved in viral and presumably cellular DNA replication. The SV40 in vitro DNA replication system has been used by a number of laboratories using the fractionation/ reconstitution approach to define cellular proteins involved in viral and cellular DNA replication (53,54,55). The paucity of mammalian ts DNA⁻ mutant cell lines has made using in vitro complementation systems to identify mammalian cellular factors involved in DNA replication less attractive.

I have adapted a system for polyomavirus DNA synthesis in vitro using CHO cell extracts and developed an in vitro complementation assay, using chromatographic fractions prepared from wt CHO cell extract, in an effort to identify cellular factors which stimulate polyoma in vitro DNA replication activity of defective CHO ts DNA⁻ mutant cell extracts. The polyomavirus in vitro DNA replication system requires a DNA molecule containing the polyomavirus origin of replication, permissive cell extracts, rNTPs, dNTPs, an ATP regenerating system, salts and buffer, and one virus encoded protein, polyomavirus large T (PyLT) antigen. There is virtually no incorporation of radiolabel in the absence of

either PyLT or a DNA molecule containing a polyomavirus origin of replication.

Analysis of the polyoma in vitro DNA replication products synthesized by wt CHO HB cell extract exhibited novel monomeric polyoma in vitro DNA replication products. Murakami, et al. (88) reported that products synthesized by a 0.2 M NaCl extract of FM3A cells, were predominantly form II monomeric products. We have observed large amounts of form I DNA after neutral agarose gel electrophoretic analysis of undigested replication products, which have been deproteinized by phenol/chloroform extractions, suggesting that these molecules exist as chromatin-like structures within the extract. Plasmid DNA introduced into HB cell extract is relaxed within the extract and migrates as relaxed DNA, presumably having been relaxed by endogenous topoisomerase activities. The presence of form I, after deproteinization, suggests that plasmid DNA was present in nucleosome-like structures in the extracts before deproteinization. Deproteinization introduces superhelical turns which cause the plasmid to assume a form I DNA configuration. Since a large amount of incorporation into form I is seen under defined conditions, and the majority of the input DNA is relaxed, this nucleoprotein complex (e.g.- nucleosomal complex) may be dependent upon polyoma in vitro DNA replication. These data are similar to those described for chromatin assembly during SV40 DNA replication in vitro (82,83,84). The amount of form I product observed depended

upon the concentration of wt CHO cell extract protein assayed as well as the incubation period. Similar results were observed when HB cell extracts prepared from FM3A spinner cell cultures were analyzed in an analogous fashion.

The kinetics of form I DNA product synthesis was different when extract was prepared by 0.2 M NaCl extraction of HB lysates of wt CHO cells; clarified lysate was subsequently dialyzed against HB buffer before being assayed for polyoma in vitro DNA replication (HB/0.2 M NaCl wt CHO cell extract). Whereas most of the form I DNA products are observed when high concentrations of HB cell extract protein were incubated at 30°C for periods greater than one hour, the predominant monomeric products first observed after approximately 1 hour, when dialyzed HB/0.2M NaCl cell extract was assayed, were form I DNA. These results parallel the expected behavior of a chromatin assembly factor (CAF-I)-like activity isolated from human cells (83,84). CAF-I activity was not efficiently extracted unless the salt concentration was greater than 150 mM NaCl (83). Detailed pulse-chase experiments, analysis of ori⁺ and ori⁻ plasmid behavior and utilization of relaxed ori⁺ DNA as a template would be particularly informative. Micrococcal nuclease digestion to determine nucleoprotein phasing and electron microscopic analysis of nucleoprotein structure can be used to help characterize the nucleoprotein structure present. Further analysis will be required to determine whether these

observations are indeed the result of a CAF-I-like activity present in wt CHO cell extracts.

Several ts DNA⁻ mutant CHO cell lines affected in cellular and polyomavirus DNA synthesis have been identified (89). The most straightforward approach to the analysis of the CHO ts DNA⁻ mutant cell lines involved the preparation of extracts from wt and mutant cell lines grown at the permissive temperature followed by subsequent analyses of the polyoma in vitro DNA replication activities of these extracts at various temperatures in vitro. The first experiment that was performed, with this approach in mind, involved determining the effects of various assay temperatures on the polyoma in vitro DNA replication activity of wt CHO cell extract prepared from dense cell cultures. HB cell extracts prepared from dense cell cultures of wt cells have dramatically decreased polyoma in vitro DNA replication activity at elevated temperatures in vitro.

Several factors did have significant effects on the temperature-sensitive phenotype of wt CHO polyoma in vitro DNA replication activity. Although including additional PyLT had an effect on the level of synthesis, it did not dramatically effect the temperature-sensitive phenotype. The addition of 5% PEG-8000 had a qualitatively similar effect on the temperature-sensitive phenotype as adding additional PyLT, although the level of synthesis was not stimulated as dramatically.

Pre-incubation of wt CHO cell extract for periods of up to two hours showed significant, but not dramatic, loss of subsequent polyoma in vitro DNA replication activity, suggesting that factors other than the presence of labile factor(s) in the wt CHO cell extract significantly contribute to the dramatically lower polyoma in vitro DNA replication activity of wt CHO cell extract assayed at elevated temperatures in vitro.

Elevated temperature presumably disrupts molecular interactions between PyLT and cellular factors which are required for efficient polyoma in vitro DNA replication. Addition of 5% PEG-8000 or addition of additional PyLT rendered the polyoma in vitro DNA replication activity of wt CHO cell extract somewhat less temperature-sensitive, suggesting that increased interaction of PyLT with other cellular factors (and possibly with other PyLT molecules) enhances the ability of wt CHO cell extract to perform efficient polyoma in vitro DNA replication at elevated temperatures in vitro. Wang and Prives (103) have shown that PyLT is differentially dephosphorylated at 37°C and 33°C, suggesting additional possible mechanisms which lead to the deleterious effects of assay temperature on in vitro DNA replication activity of wt CHO cell extract.

Utilizing the highest levels of polyoma large T antigen available, in the presence of 5% PEG-8000, extracts prepared from CHO wt and the ts DNA⁻ mutant cell lines JB3-O, JB7-K and JB11-J were assayed at elevated temperatures in vitro and

revealed no dramatic temperature-dependent defects in vitro. HB cell extracts prepared from JB3-O and JB7-K extracts exhibited consistently significantly lower polyoma in vitro DNA replication activities than wt and JB11-J HB cell extracts at all temperatures assayed in vitro. These observations led to the hypothesis that HB cell extracts prepared from dense cell cultures of JB3-O and JB7-K were exhibiting temperature-independent defects in vitro.

There are numerous examples of ts mutant phenotypes showing aberrant behavior at permissive temperature. An example of an in vitro temperature-independent functional defect has been described for the E. coli DNA replication protein, Pol III (ts mutant allele E2) (1) which is non-functional at all temperatures assayed in vitro. Another example of aberrant behavior of a temperature-sensitive mutant at permissive temperature involves the the aberrant behavior of yeast actin mutants. A number of alleles show aberrant actin cable deposition within cells even at the permissive temperature (111).

We decided to determine whether the defective polyoma in vitro DNA replication activities of extracts derived from JB3-O and JB7-K were the results of these extracts exhibiting temperature-independent defects in vitro.

Extracts originally showing deficient polyoma in vitro DNA replication activities prepared from JB3-O and JB7-K were prepared from cells growing at high cell densities (greater than 2×10^7 cells/150 mm dish) and the growth state of the

cells from which extracts were prepared was not determined.

Therefore, extracts were prepared from CHO wt and mutant cell lines while the cells were verified to be growing exponentially, by using the seeding/extraction protocol described earlier. These extracts were more dilute because there were less cells per tissue culture dish at the time of extraction. Dilute HB cell extracts were concentrated by a centrifugal ultrafiltration device. In vitro analyses of increasing protein concentrations of concentrated HB cell extracts prepared from JB3-O and JB-7K gave consistently significantly lower polyoma in vitro DNA replication activities than analogously prepared CHO wt HB cell extracts at all levels of cell extract protein assayed. Independently prepared extracts from wt, or JB3-O, or JB7-K cell lines exhibit characteristic, cell line specific, in vitro polyoma DNA replication saturation levels. Maximal levels of polyoma in vitro DNA replication activities of concentrated HB cell extracts prepared from JB7-K and JB3-O were only approximately 10-20% of the maximal level of polyoma in vitro DNA replication activity exhibited by HB cell extract prepared from wt CHO cell cultures. These results support the hypothesis that concentrated HB cell extracts prepared from JB3-O and JB7-K exhibit temperature-independent defects for polyoma in vitro DNA replication. Additionally, since these cultures were verified to be growing logarithmically, poor cell growth does not explain the defective polyoma in

vitro DNA replication activities exhibited by extracts prepared from the CHO ts DNA⁻ cell lines JB7-K and JB3-O.

Roberts and D'Urso (108) have shown, using centrifugally elutriated human cell cultures, that extracts from S phase cells are approximately ten-fold more efficient at initiating SV40 large T antigen dependent DNA replication in vitro . Therefore, extraction of cell cultures anomalously distributed within the cell cycle could potentially contribute to the defective polyoma in vitro DNA replication activities of extracts derived from the CHO ts DNA⁻ cell lines JB3-O and JB7-K. CHO wt and ts DNA⁻ mutant cell lines have virtually identical distribution of cells within the cell cycle, as determined by flow microfluorometric analysis, at the time of extract preparation. Therefore, abnormal distribution of cells within the cell cycle at the time of HB extract preparation is not the cause of the defective polyoma in vitro DNA replication activities of concentrated HB cell extracts prepared from JB3-O and JB7-K.

Anomalous cell extraction behavior could account for the defective polyoma in vitro DNA replication activities of HB cell extracts prepared from JB3-O and JB7-K. Preparation of abnormal extracts deficient in a number of replication factors could account for the defective polyoma in vitro DNA replication activities of JB3-O and JB7-K. In order to address this possibility, the level of replication protein A (RP-A) was determined by using a rabbit polyclonal antibody prepared against human RP-A to determine the level of RP-A

within the concentrated HB cell extracts prepared from CHO wt and the ts DNA⁻ CHO cell lines JB3-O and JB7-K. The level of the RP-A 34 kDa subunit in each of these extracts is comparable. Additionally, DNA polymerase activities, using activated DNA as the template, was determined for CHO wt, JB3-O and JB7-K concentrated HB cell extracts. These cell extracts exhibited very similar levels of DNA polymerase activity. Results from the DNA polymerase assays are in sharp contrast to the defective polyoma in vitro DNA replication activities of extracts prepared from JB3-O and JB7-K. These data argue against the possibility of gross extraction abnormalities being the cause of the defective in vitro DNA replication activities of JB3-O and JB7-K.

DNA replication inhibitors present in JB3-O and JB7-K concentrated HB cell extracts could cause the defective polyoma in vitro DNA replication activities of these extracts. Mixing experiments involving extracts derived from wt, JB3-O, and JB7-K cells indicate extracts derived from JB3-O and JB7-K do not contain potent inhibitors of DNA replication. Therefore, the presence of potent inhibitors of DNA replication are not the cause of the defective polyoma in vitro DNA replication activities of these extracts derived from JB3-O and JB7-K.

Since concentrated HB cell extracts prepared from JB3-O and JB7-K exhibited defective polyoma in vitro DNA replication activities, analysis of the replicative forms synthesized could be informative and help gain insight into

the defective function(s) within these mutant cell extracts. Aberrant topological forms are synthesized by extracts derived from JB3-O and JB7-K. Virtually all radiolabel incorporation was dependent upon input plasmid containing a functional polyomavirus origin of replication and the addition of PyLT. A time-course experiment utilizing extracts derived from the CHO ts DNA⁻ mutant cell lines JB7-K and JB3-O produce aberrant replication forms with little synthesis of monomeric full-length replication products during a two hour time-course. Neutral and alkaline gel electrophoretic analysis of the replicative forms synthesized by concentrated HB cell extracts prepared from JB3-O indicate replicative forms predominantly forms greater than 75% replicated and which can potentially lead to monomeric products if the in vitro reactions are incubated for periods longer than 1.5 hours. It is important to note that very little additional full-length Dpn I resistant product is synthesized, yet monomeric products start to accumulate. These results suggest a possible problem in the final stages of elongation and/or subsequent resolution of the replicative forms into monomeric products. These aberrant topological forms may represent dead-end products/substrates which do not lead to significant full length product formation and which may also "tie-up" cellular factors required for subsequent DNA synthesis thereby contributing to the deficient replication activities of these mutant cell extracts. Only concentrated HB cell extracts prepared from JB3-O and JB7-K

synthesize aberrant forms, all other CHO ts DNA⁻ mutants analyzed synthesize a distribution of products typically synthesized by concentrated wt CHO HB cell extract (which will be discussed shortly).

Two other ts DNA⁻ mutants isolated from CHO and one mutant, JB17-D, isolated from the Chinese hamster lung cell line V79, were analyzed in an analogous fashion. JB3-B and JB11-J concentrated HB cell extracts showed significantly lower replication activities when compared to wt CHO cell extract at all protein concentrations assayed. JB17-D showed a different polyoma in vitro DNA replication activity versus protein concentration profile, reaching a level which approaches the maximal level of concentrated wt CHO HB cell extracts. The deficient polyoma in vitro DNA replication activity of concentrated HB cell extract prepared from JB3-B was not expected since measurement of dNTP pools in this CHO mutant cell line JB3-B as a function of time after shift to the NPT (non-permissive temperature) revealed that all four dNTP pools declined at similar rates (93). In concordance with this observation, JB3-B possesses a thermolabile ribonucleotide reductase activity (93). We, therefore, did not expect extracts prepared from JB3-B to exhibit any defects for polyoma in vitro DNA replication activity because dNTPs are supplied in the polyoma in vitro DNA replication reaction mix. It should be noted, however, that human DNA sequences which correct the ts defect in JB3-B do not encode ribonucleotide reductase, leaving open the possibility that

other replication factors are also defective. However, none of the concentrated HB cell extracts prepared from the CHO ts DNA⁻ mutants exhibited polyoma in vitro DNA replication activity levels which attained that of concentrated HB cell extracts prepared from wt CHO cell cultures. It seemed unlikely that all of the CHO ts DNA⁻ mutant cell extracts were exhibiting defective polyoma in vitro DNA replication activities linked to the respective ts mutation.

Therefore, two stable revertants isolated by the ability to form colonies at the non-permissive temperature, JB3-O/R1 and JB3-O/R2, were isolated from JB3-O and characterized in detail. Concentrated HB cell extracts were prepared from the two JB3-O revertants and analyzed in vitro. Polyoma in vitro DNA replication assays which contained increasing concentrations of concentrated cell extracts derived from JB3-O, JB3-O/R1, and JB3-O/R2 gave virtually indistinguishable levels of full-length Dpn I resistant products. Neutral gel electrophoretic analysis of undigested products showed that in vitro reactions containing extracts prepared from JB3-O/R1 and JB3-O/R2 synthesized aberrant replicative forms which were indistinguishable from those synthesized by concentrated HB cell extract derived from JB3-O cell cultures. The defective polyoma in vitro DNA replication activities of the JB3-O revertants JB3-O/R1 and JB3-O/R2 suggested that the in vitro defect did not correlate to ts growth defect.

Although the revertants, JB3-O/R1 and JB3-O/R2 were able to form colonies at the non-permissive temperature we did not know if these cell lines could support polyomavirus DNA replication at the non-permissive temperature. We therefore determined the effect of incubation temperature on the ability of JB3-O/R1 and JB3-O/R2 to support polyomavirus DNA replication in vivo. Cell cultures of CHO wt, JB3-O, JB3-O/R1 and JB3-O/R2 were transfected with the plasmid p53A6.6, which contains the entire polyoma genome. Sister transfected cell cultures were incubated at different temperatures in order to access the effects of temperature on the level of polyomavirus DNA replication within each cell line. Results showed that JB3-O/R1 and JB3-O/R2 exhibited significant temperature-restriction of polyomavirus DNA replication in vivo. Therefore, although the revertants could form colonies at the non-permissive temperature, these cell lines' abilities to replicate polyomavirus DNA in vivo at the non-permissive temperature (NPT) was not significantly restored. The restoration of growth capacity without the restoration of the ability to support polyoma DNA replication in vivo can be explained by a number of mechanisms. Although cellular and polyomavirus DNA synthesis occur by very similar mechanisms, there are differences. Therefore a second-site suppressor mutation which allows cellular chromatin to be replicated and not polyomavirus chromatin can be envisioned. An alternative possibility is that the ts growth mutation and the ts

mutation which restricts polyomavirus DNA replication are not linked.

These transfection experiments were designed to determine whether JB3-O/R1 and JB3-O/R2 restricted polyomavirus DNA synthesis at the NPT in order to clarify the defective polyoma in vitro DNA replication activities of these revertants. Data show that these two cell lines restrict polyomavirus DNA synthesis at the NPT. Since these cell lines restrict polyomavirus DNA synthesis in vivo one would not be surprised that they are defective for polyoma in vitro DNA replication. In summary, we have not been able to demonstrate a correlation between the defective polyoma in vitro DNA replication activity of JB3-O cell extract with the ts growth phenotype.

Despite these uncertainties, we attempted to complement the defective polyoma in vitro DNA replication activity of JB3-O concentrated HB cell extract by adding phosphocellulose chromatographic fractions prepared from wt CHO HB cell extract. These fractions were each separately inactive and when reconstituted had approximately 15% of the polyoma in vitro DNA replication activity of similar levels of concentrated CHO wt HB cell extract. This level of incorporation after reconstitution of crude phosphocellulose chromatographic fractions is comparable to that described for the SV40 in vitro DNA replication system (David Weinberg, personal communication). This relatively low level of polyoma in vitro DNA replication activity of the

reconstituted fractions should be noted because factors could have been inactivated or otherwise have lost significant amounts of activity and therefore their ability to complement defective extracts may have been compromised.

Phosphocellulose fractions were also added into polyoma in vitro DNA replication reactions containing equivalent amounts of concentrated wt HB cell extract to serve as controls. The flow-through and wash fractions, termed CF I, and the high salt eluate (1 M KCl), termed CF II, were used. Addition of CF I to polyoma in vitro DNA replication reactions containing either JB3-O or wt CHO concentrated HB cell extract resulted in significant inhibition of 50% or more of the respective polyoma in vitro DNA replication activity. Addition of CF I to reaction mixes containing concentrated JB3-O HB cell extract resulted in the significant loss of form I product with the appearance of a slower migrating replicative product migrating close to form III plasmid marker. This replicative form was also seen when CF I was added to concentrated wt HB cell extract. In summary, CF I inhibited polyoma in vitro DNA replication activities of concentrated HB cell extracts prepared from JB3-O and wt cell cultures.

Addition of CF II to polyoma in vitro DNA replication reactions containing concentrated CHO wt HB cell extract inhibited the polyoma in vitro DNA replication activity of the wt cell extract. Appearance of a DNA replicative form migrating close to plasmid form III marker was observed as has been described for reactions containing added CF I.

Additionally, more monomeric products migrating as form I DNA were present. Addition of equivalent amounts of either wt HB cell extract or CF I did not produce the same distribution of replicative forms. Data suggest that this change in monomer topoisomer distribution may be the result of a chromatin assembly factor I -like (CAF-I -like) activity as has been described for SV40 DNA replication in vitro (82,83,84). Preliminary analysis, as discussed earlier in this Discussion, suggest that CAF-I -like activity may be present in CHO cell extract and, as can be seen in these data, possibly in phosphocellulose fraction CF II.

Addition of CF II to polyoma in vitro DNA replication reactions containing concentrated JB3-O HB cell extract exhibit significant stimulation of polyoma in vitro DNA replication activity (approximately 1.7-fold). There are no dramatic changes in the distribution of products, although the appearance of a DNA replicative form migrating close to form III plasmid marker was evident, as has been described earlier. The most obvious increase in the level of incorporation into replicative forms was seen in the slowly migrating replicative form, which has been described earlier. No dramatic increases in the amount of monomeric products were observed, suggesting that 'maturation' of the slowly migrating replicative forms into monomeric products was not significantly expedited by factors present in CF II.

Although the polyoma in vitro DNA replication activity of concentrated JB3-O HB cell extract was stimulated

approximately 1.7-fold, the absolute level of in vitro DNA replication activity was still only approximately 20% of that of concentrated wt HB cell extract. Collectively, these data suggest that factors other than those present in CF II are required in order to stimulate the polyoma in vitro DNA replication activity of concentrated JB3-O cell extracts to polyoma in vitro DNA replication activity levels approaching that obtained when assaying concentrated CHO wt HB cell extract.

The replicative forms synthesized by JB3-O concentrated HB cell extracts were near unit-length and appeared after prolonged incubation to be able to ultimately be converted into monomeric products. These data suggested that there may be topoisomerase functional deficiency in JB3-O extracts. Additionally, Yang et al. (77) have shown that topoisomerase activities are the limiting functions in human cell extract, determining the absolute amount of in vitro DNA replication in SV40 in vitro DNA replication reactions using extracts prepared in HB buffer. These data led us to attempt to complement the defective concentrated JB3-O HB cell extract with purified topoisomerase I or topoisomerase II. Since replicative forms synthesized by JB3-O concentrated HB cell extract were greater than 75% replicated, and since it has been demonstrated that only topoisomerase II can resolve SV40 in vitro DNA replicative forms which are near completion (77), topoisomerase II complementation seemed to be the most likely to succeed. Topoisomerase II stimulated the polyoma

in vitro DNA replication activity of concentrated CHO wt HB cell extract by approximately 1.2-fold. The addition of purified topoisomerase II to concentrated JB3-O HB cell extract stimulated polyoma in vitro DNA replication activity approximately 2-fold. The addition of topoisomerase II also led to the synthesis of significantly higher levels of monomeric product. It was difficult to assess whether there was less accumulation of the slower migrating replicative form in the presence of added topoisomerase II. The addition of purified topoisomerase I did not significantly effect the in vitro DNA replication activities of wt and JB3-O concentrated HB cell extracts. Additionally, topoisomerase I did not complement the defective polyoma in vitro DNA replication activity of concentrated JB3-O HB cell extract.

Topoisomerase II stimulated the polyoma in vitro DNA replication activity of concentrated JB3-O HB cell extract by approximately 2-fold. Although topoisomerase titrations were not performed, enough topoisomerase II was added to completely decatenate 500 ng of kinetoplast DNA in only 1 hour (data not shown). Even though the in vitro DNA replication activity was stimulated significantly, the absolute level of polyoma in vitro DNA replication activity of concentrated JB3-O HB cell extract in the presence of added topoisomerase II is still only approximately 35% of the maximal polyoma in vitro DNA replication activity of concentrated wt HB cell extract assayed under the identical conditions.

These data suggest that factors other than topoisomerase II are required in order to stimulate the polyoma in vitro DNA replication activity of concentrated JB3-O cell extracts to polyoma in vitro DNA replication activity levels approaching those obtained when assaying concentrated CHO wt HB cell extract.

Data from complementation attempts using phosphocellulose fractions prepared from wt CHO HB cell extract and complementation attempts using purified topoisomerase I or topoisomerase II suggest that concentrated JB3-O HB cell extract is defective in more than one function required for efficient polyoma in vitro DNA replication. Addition of the phosphocellulose fraction CF II into concentrated JB3-O HB cell extract predominantly stimulated the incorporation into the slowly migrating replicative forms typically synthesized by JB3-O extract. Addition of purified topoisomerase II stimulated the polyoma in vitro DNA replication activity leading to the production of significantly more monomeric products. Therefore, the observed stimulation by CF II and topoisomerase II apparently occurred by different mechanisms, suggesting that the defective polyoma in vitro DNA replication activity of concentrated JB3-O HB cell extract is caused by the deficient function of more than one protein involved in polyoma in vitro DNA replication. The rate limiting function in concentrated JB3-O HB cell extracts is not known. One function which occurs late in the synthesis of monomeric replicative forms is the resolution of daughter

molecules into two separate molecules. During SV40 in vitro DNA replication this process has been shown to require topoisomerase II (77). Since net incorporation was increased without a significant increase in monomeric replication forms being synthesized, it seems likely that the CF II phosphocellulose fraction stimulated a function(s) needed earlier on in the DNA replication pathway, before topoisomerase II function is most required. It is not known whether the initiation and/or elongation stages of polyoma in vitro DNA replication are enhanced by addition of CF II into concentrated JB3-O HB cell extract.

The next question which must be addressed is: Why are the concentrated HB extracts prepared from the CHO ts DNA⁻ mutant cell lines defective for polyoma in vitro DNA replication? A number of possibilities exist. Although we tried to rule out gross extraction abnormalities of each cell line, we are not able to determine the levels of all replication factors and therefore have not been able to rule out specific extraction abnormalities. One other possibility is related to the fact that each of the mutants grows slower than wt CHO (see table 7). Recent literature citing post-translational modification of DNA replication factors such as DNA polymerase α (28,29), RP-A (65), and other proteins which function during DNA replication, substantiates the possibility that a number of replication factors present in the mutants may not be modified for full function, whereas in wt CHO cells the replication factors may be modified for

maximal activity. Additionally, the functional integrity of replication competent multienzyme complexes, which have been described for both SV40 (81) and Polyomavirus (appendix) involved in in vitro DNA replication, present in extracts prepared from each of the CHO ts DNA⁻ mutants described is unknown. The functional integrity could be effected both by a ts defective replication protein or because they have been isolated from a cell with a slow growth rate and the replication proteins are not fully activated, therefore leading to a less functionally active multi-enzyme complex.

ORIGIN			SEQUENCE	NUMBER OF MATCHES
	← AT-RICH →	← TIGHTLY BOUND →		
		dnaA protein		
<i>E. coli</i> ori C	13 13 13 A	A A A A	5' G A T C T n T T T n T T T T	12, 12, 11
		dnaA protein		
<i>B. subtilis</i> ori C	16 16 16 A	A A A A	G A T A C C T T A n T T T T T C	14, 14, 15
pSC101	A 13 13		G A T C T n T T T n T T T T	13, 11
P1	A A 7 7 7 7		A G A T C C $\frac{A}{T}$	7, 6, 7, 7, 7
F	A A 8 8 8 8		T T T T T A $\frac{T}{G}$ A	8, 8, 8, 8
R1	9 9 9		T T T A A A n G A	9, 9, 9
R6K ori γ	10 10 10		T A T T n A T T T T	9, 8, 10
λ	11 11 11		T T n T C T T T T G T	9, 11, 10
ϕ 82	11 11		T T G T C T T T T G T	11, 11
ϕ 80	6 6 6		T C T T G T	5, 6, 6
RK2	9 9 9 9		G G T T $\frac{T}{A}$ A A A A	9, 8, 9, 8
P4	9 9 9		C A C T T A A A G	9, 8, 9
		α protein		

Figure 1. **COMPARISON OF AT RICH REPEATS IN PROKARYOTIC ORIGINS.** The arrows indicate the locations of the AT-rich repeats in each origin, with the length (in bp) marked above each. Stippling identifies the sequences that bind tightly to the origin-specific initiator protein. The stippled boxes marked "A" match the 9-mer box to which the *E. coli* dnaA protein binds. (Taken from reference 2 and see references therein).

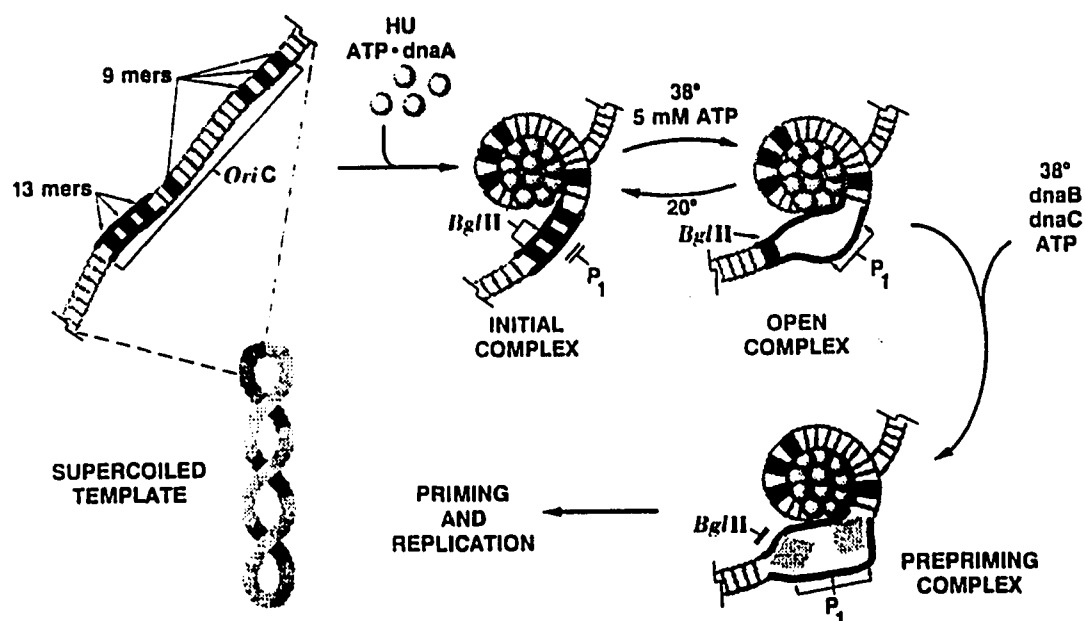


Figure 2. **A MODEL FOR INITIATION AT ORIC OF THE *E. COLI* CHROMOSOME.** *dnaA* protein binds four 9-mers, organizing *oriC* around a protein core to form an "initial complex". The three 13-mers are then melted serially by *dnaA* protein creating an "open complex". The *dnaB*-*dnaC* complex can now be directed to the 13-mer region to extend the duplex opening and generate a "prepriming complex". The "prepriming complex" unwinds the template for subsequent priming and replication. Taken from reference 2.

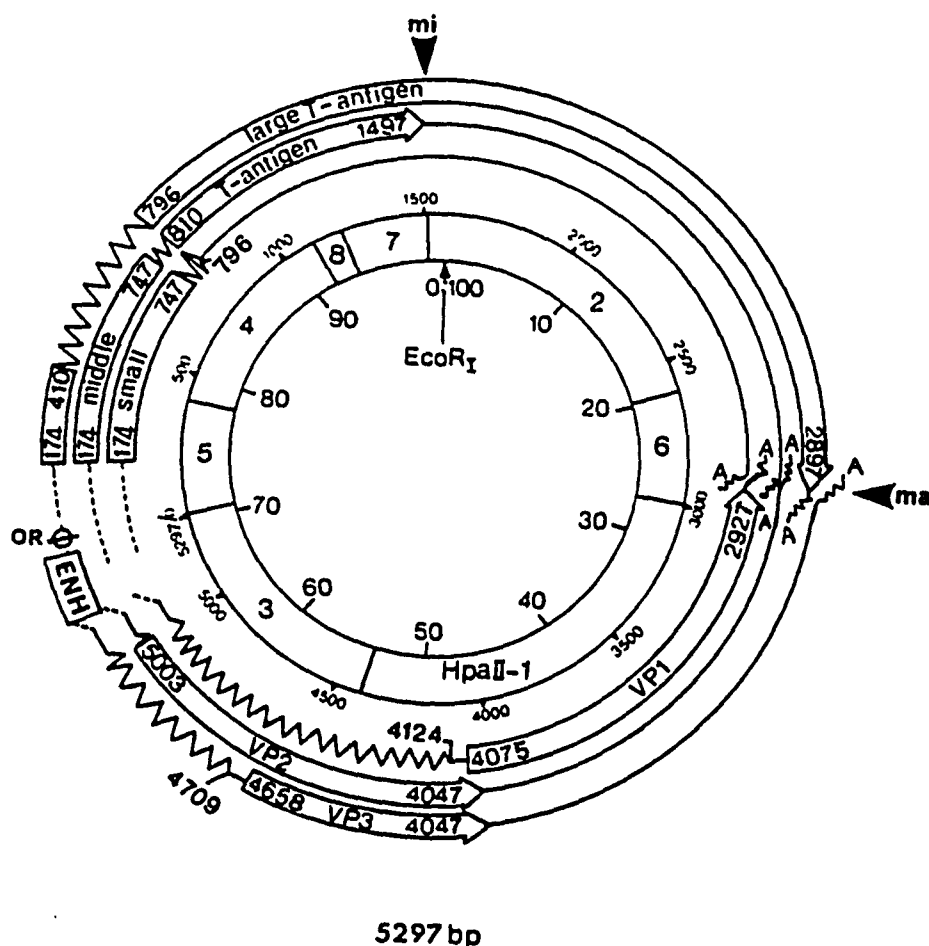


Figure 4. **A SCHEMATIC REPRESENTATION OF THE POLYOMAVIRUS A-2 STRAIN GENOME.** Some important features are shown. The early and late mRNAs (thin lines) and their coding regions (boxed regions) are shown. The nucleotide numbers at the starts and ends of the coding regions and at the splice donors and acceptors are shown. Introns are indicated by jagged lines. The positions of the origin of DNA replication (OR), the enhancer region (ENH), and the major (ma) and minor (mi) early region polyadenylation sites are shown. Taken from reference 42.

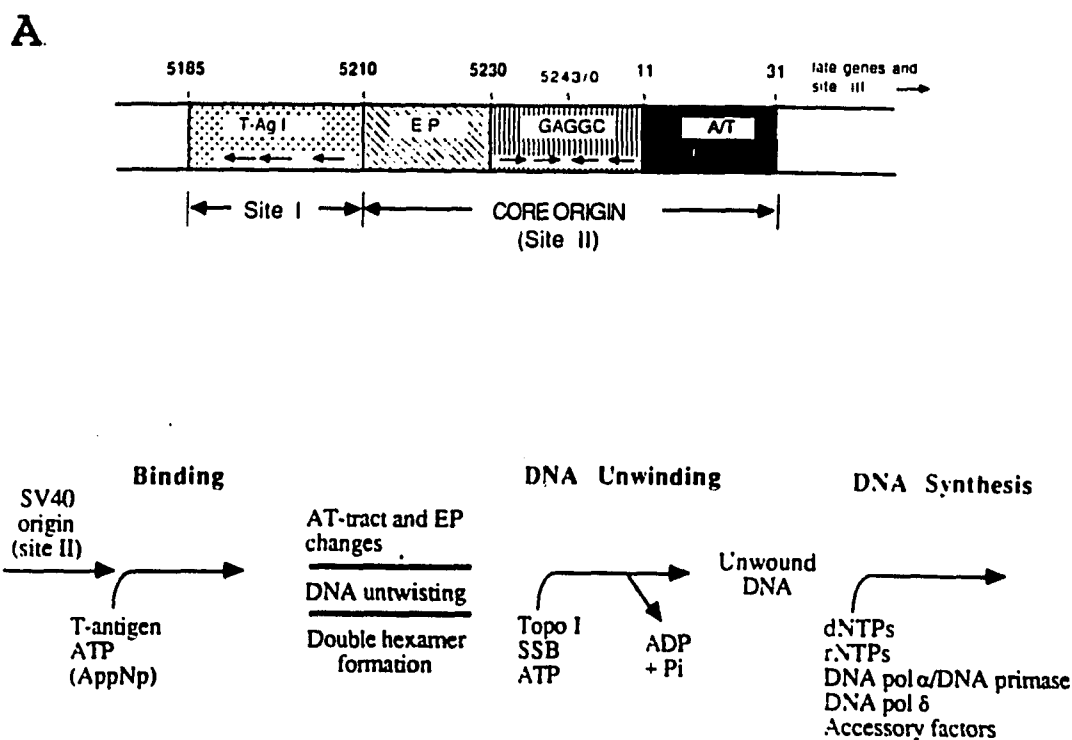


Figure 5. A. The SV LT binding site (T-AG I), early palindrome element (EP), central core containing the GAGGC repeats, and A/T rich tract is shown. The GAGGC binding sites are represented by short arrows. B. Schematic model for T antigen dependent reactions occurring during the initial stages of SV40 DNA replication. Taken from reference 59.

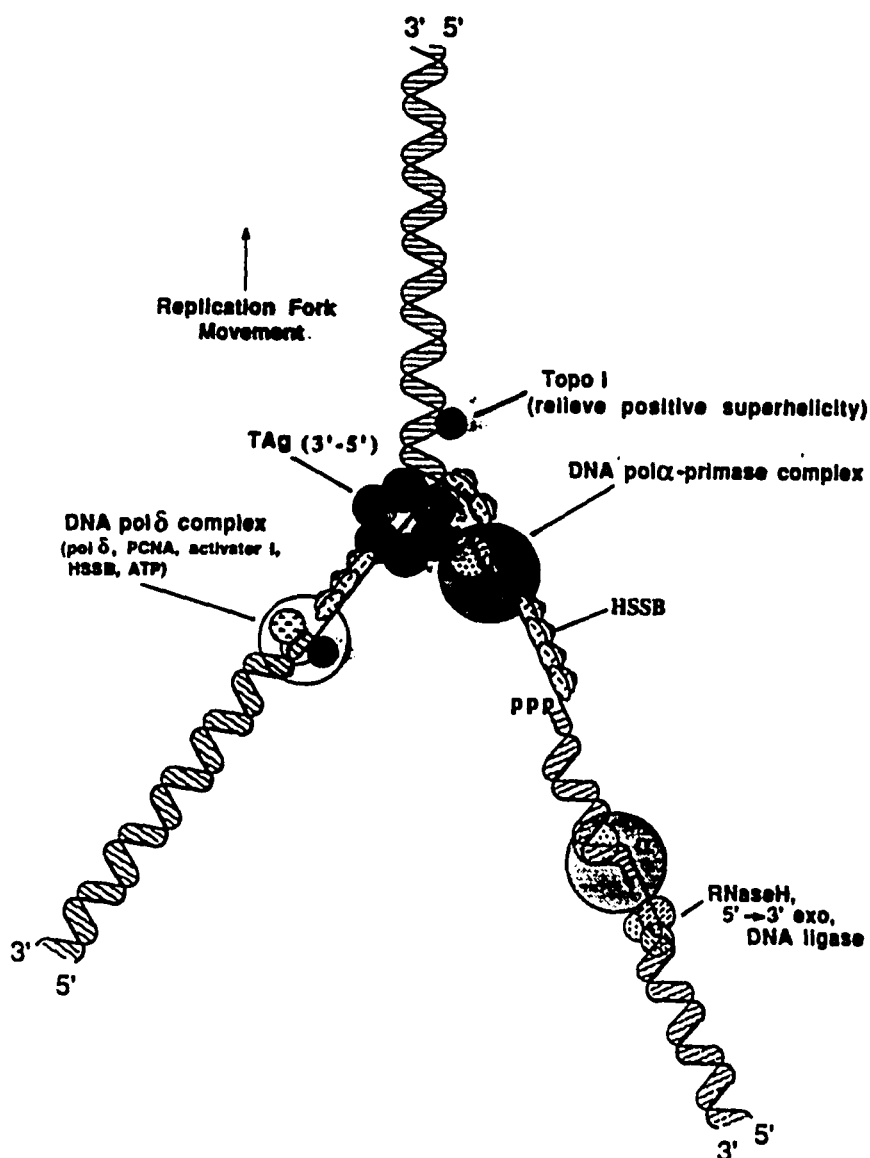


Figure 6. PROPOSED MODEL OF SV40 DNA REPLICATION FORK AND THE POSITIONING OF VARIOUS PROTEINS. See text in the Introduction section for description. Taken from ref 106.

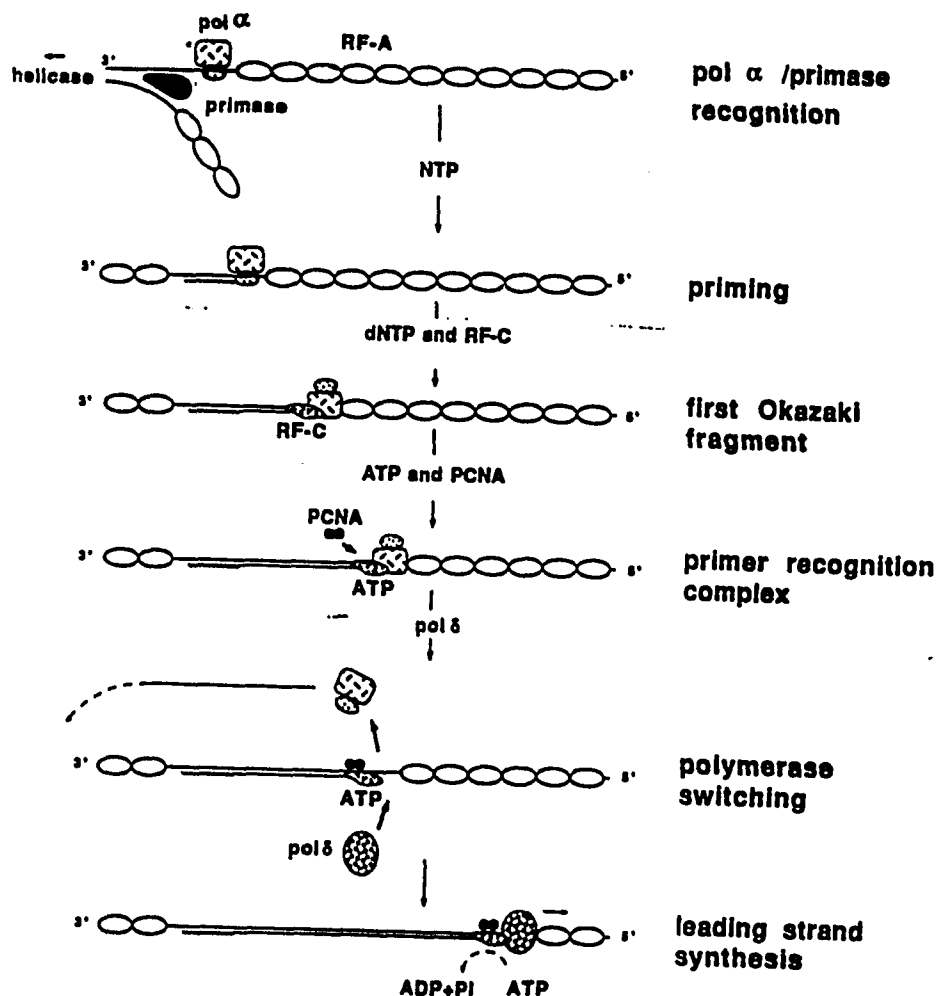


Figure 7. PROPOSED DNA POLYMERASE SWITCHING FOR INITIATION OF LEADING STRAND SYNTHESIS AT THE SV40 REPLICATION ORIGIN. In the presence of RF-A and SV LT, the SV40 origin is locally unwound yielding RF-A coated single strands. DNA polymerase α primase then synthesizes a primer followed by synthesis of the first Okazaki fragment in cooperation with RF-C. PCNA then recognizes the RF-C bound to the 3' end, thus displacing the DNA polymerase α -primase. Polymerase δ recognizes the active primer recognition complex, resulting in the initiation of leading strand synthesis. Taken from reference 80.

RCF-ConFilt CENTRIFUGAL-CONCENTRATOR/FILTRATOR

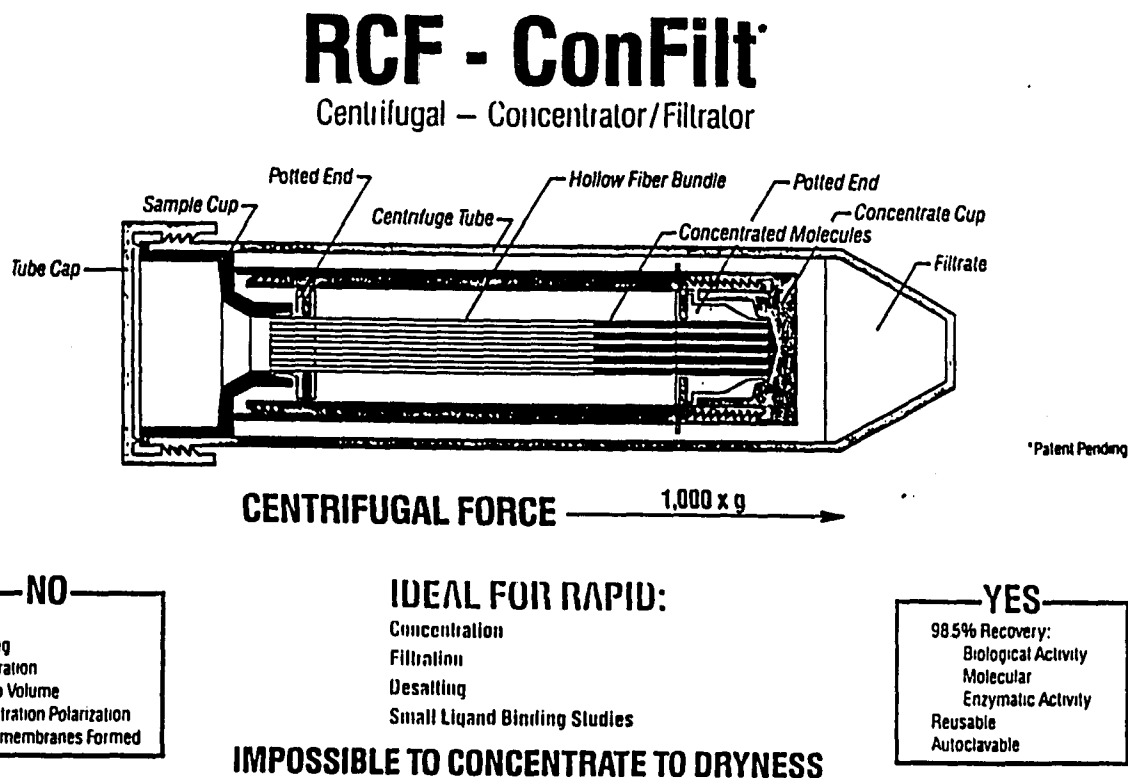


Figure 9. RCF-ConFilt CENTRIFUGAL-CONCENTRATOR/FILTRATOR. This unit was obtained from Bio-Molecular Dynamics (Beaverton, Oregon) and was used to concentrate CHO cell extracts and phosphocellulose chromatography fractions. Concentration was performed as described in Materials and Methods.

EFFECTS OF ASSAY TEMPERATURE ON POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF WT CHO CELL EXTRACT

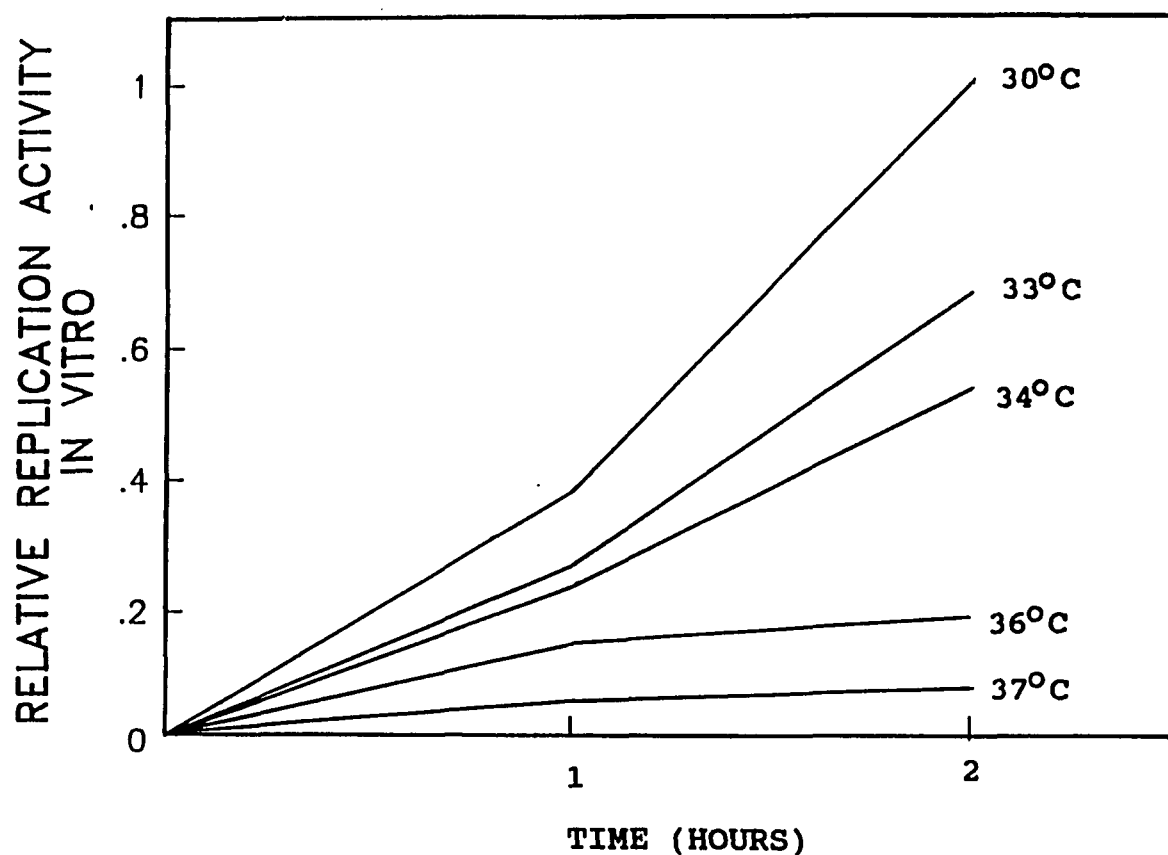


Figure 10. EFFECTS OF ASSAY TEMPERATURE ON POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF WT CHO CELL EXTRACTS PREPARED FROM DENSE CELL CULTURES. Replication mixes containing 100 ug of wt CHO cell extract protein prepared from dense cell cultures were assayed at the indicated temperatures for 1 or 2 hours. Incorporation into unit-length Dpn I resistant products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity is standardized to the replication activity of wt CHO cell extract assayed for 2 hours at 30°C and is equivalent to the incorporation of approximately 40 fmol of dCTP.

**EFFECTS OF EXTRACT CONCENTRATION ON THE
TEMPERATURE-SENSITIVE PHENOTYPE OF WT CHO CELL
EXTRACT POLYOMA IN VITRO DNA REPLICATION
ACTIVITY**

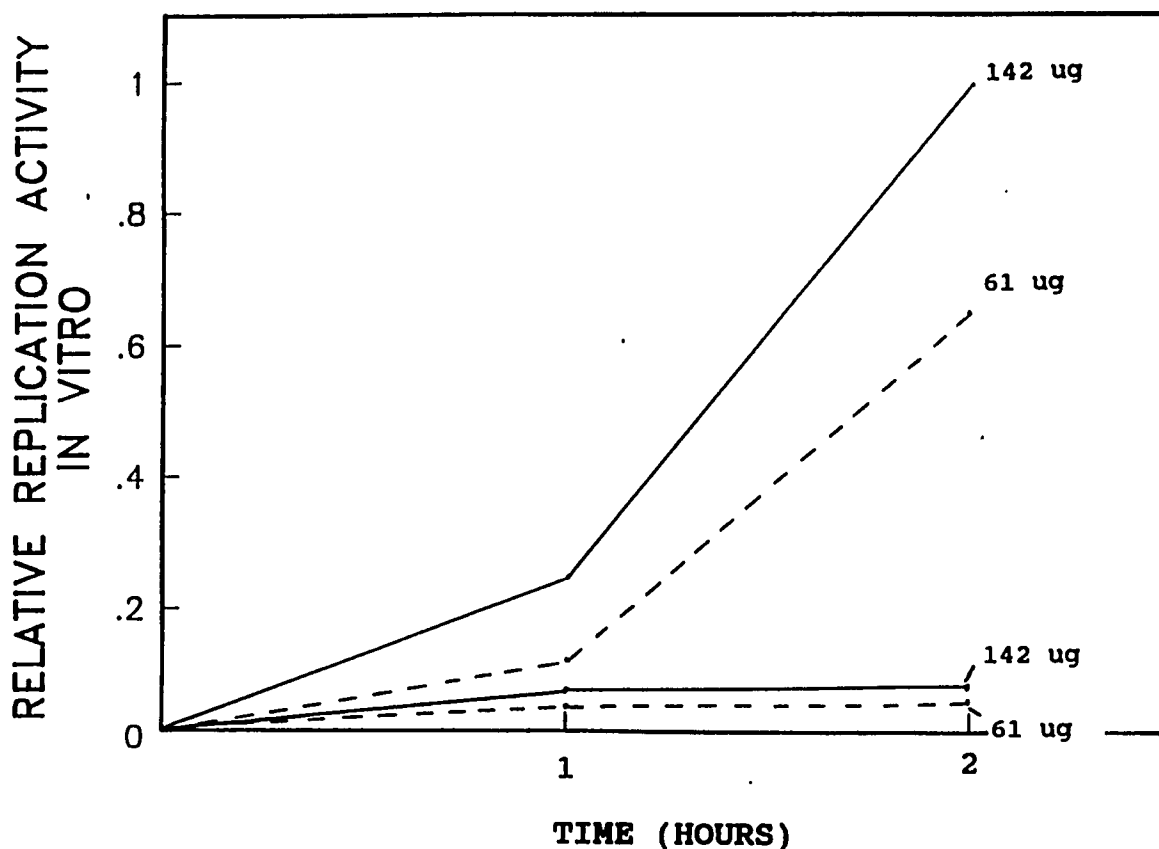


Figure 11. EFFECTS OF EXTRACT PROTEIN CONCENTRATION ON THE TEMPERATURE-SENSITIVE PHENOTYPE OF POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF WT CHO CELL EXTRACT. In vitro reaction mixtures containing either 142 or 61 ug of CHO wt cell extract prepared from dense cell cultures was assayed at 30°C or 37°C for 1 or 2 hours. Incorporation into unit-length Dpn I resistant products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity is standardized to the replication activity of 142 ug of wt CHO cell extract assayed for 2 hours at 30°C and is equivalent to the incorporation of approximately 540 fmol of dCTP.

EFFECTS OF PyLT LEVELS ON THE TEMPERATURE-SENSITIVE PHENOTYPE OF WT CHO CELL EXTRACT POLYOMA IN VITRO DNA REPLICATION ACTIVITY

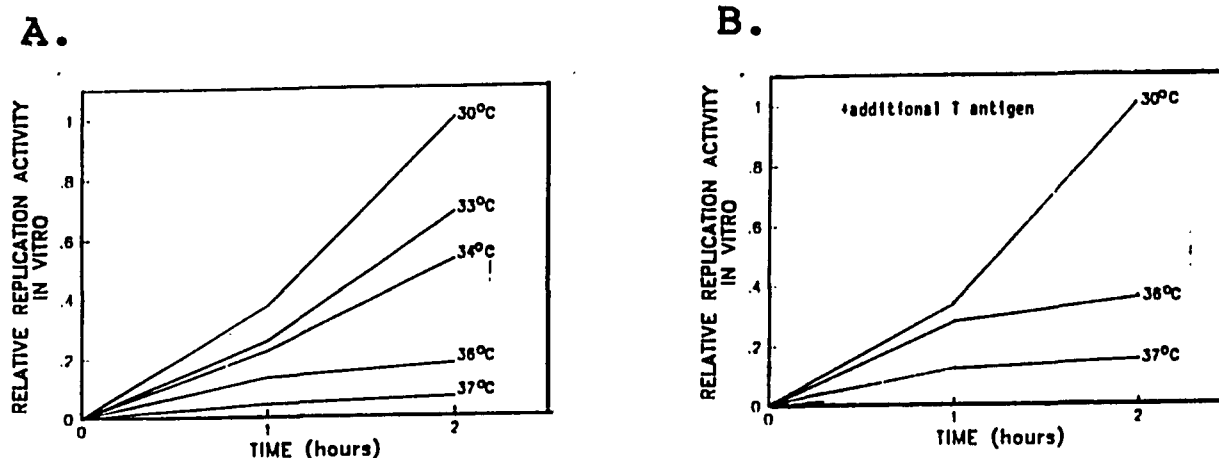


Figure 12. EFFECTS OF INCREASED LEVELS OF ACTIVE PyLT ON IN VITRO REPLICATION ACTIVITY OF WT CHO CELL EXTRACTS. 62 ug of wt CHO cell extract prepared from dense cultures was assayed at the indicated temperature for 2 hours. Assays were performed (A) without and (B) with additional PyLT added. The total volume of PyLT dialysis buffer was equivalent. In (A) 1 unit of relative replication activity in vitro is equivalent to the incorporation of approximately 40 fmol of dCTP and in (B) 1 unit of relative replication activity in vitro is equivalent to the incorporation of approximately 3.2 pmol of dCTP. Incorporation into unit-length Dpn I resistant products was determined after agarose gel electrophoresis as described in Materials and Methods.

**EFFECTS OF PEG-8000 ON POLYOMA IN VITRO DNA
REPLICATION ACTIVITY OF WT CHO CELL EXTRACTS
ASSAYED AT 30°C OR 37°C**

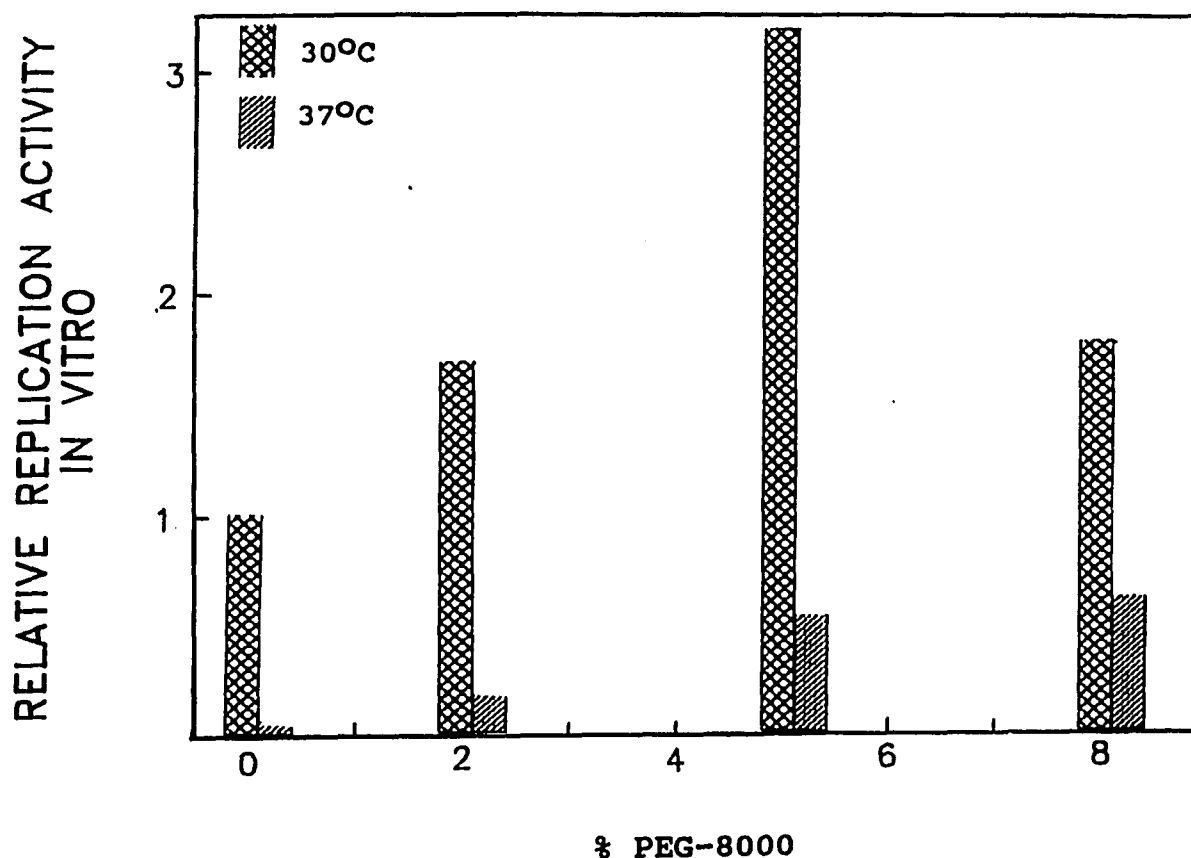


Figure 13. EFFECTS OF PEG-8000 ON POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF WT CHO EXTRACT ASSAYED AT 30°C OR 37°C FOR 2 HOURS IN VITRO . 61 ug of wt CHO cell extract prepared from dense cell cultures was assayed at 30°C or 37°C for 2 hours in the presence of 0%, 2%, 5%, or 8% PEG-8000. Incorporation into unit-length Dpn I resistant products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity in vitro is standardized to the replication activity of wt CHO extract assayed for 2 hours at 30°C and is equivalent to the incorporation of approximately 220 fmol dCTP.

EFFECTS OF EXTRACT PRE-INCUBATION ON SUBSEQUENT
POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF WT
CHO CELL EXTRACTS

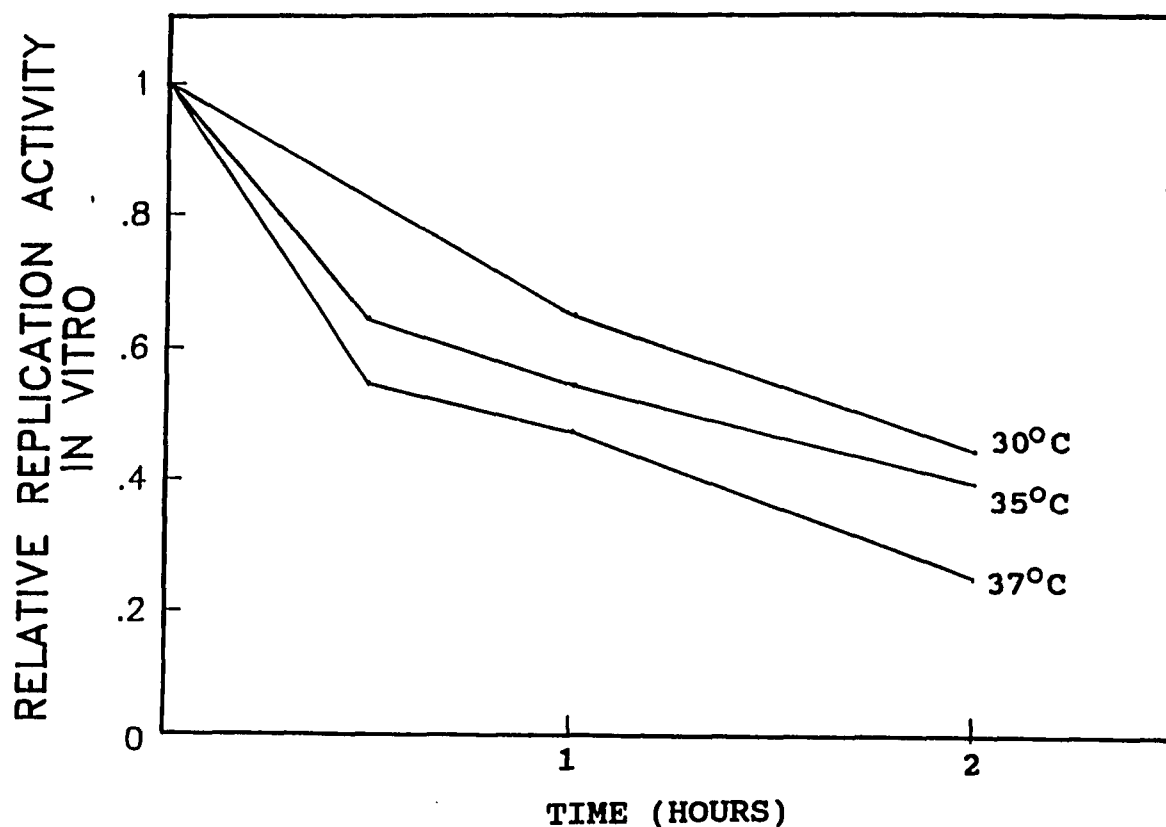


Figure 14. **EFFECTS OF EXTRACT PRE-INCUBATION ON SUBSEQUENT POLYOMA IN VITRO REPLICATION ACTIVITY OF WT CHO CELL EXTRACTS PREPARED FROM DENSE CULTURES.** 100 ug of extract derived from dense cultures of wt CHO cells were either kept on ice or incubated at the temperatures shown for either 0.5, 1.0, 1.5 or 2.0 hours before being assayed at 30°C for 1 hour. Incorporation into unit-length Dpn I resistant products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity in vitro represents the replication activity of wt CHO cell extract which had been pre-incubated on ice and represents the incorporation of approximately 20 fmol dCTP.

**EFFECTS OF PRE-INCUBATION TEMPERATURE ON
SUBSEQUENT POLYOMA IN VITRO DNA REPLICATION
ACTIVITY OF WT CHO CELL EXTRACT**

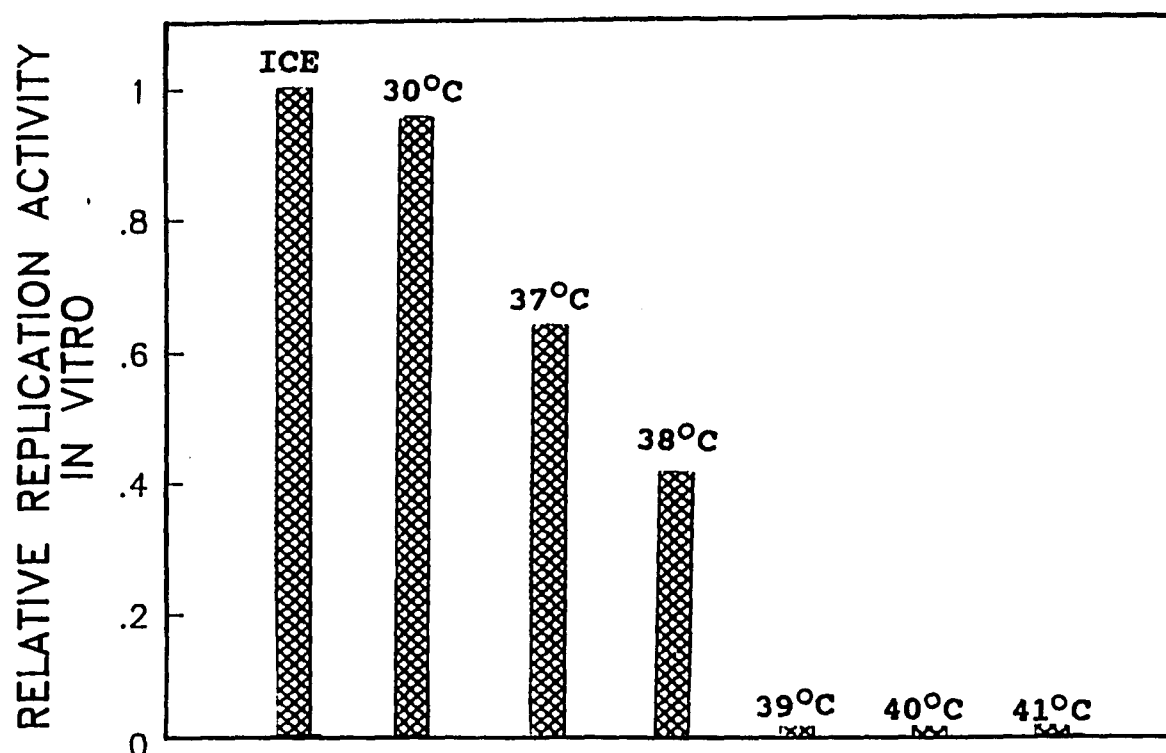


Figure 15. EFFECTS OF 15 MINUTE PRE-INCUBATION AT VARIOUS TEMPERATURES ON SUBSEQUENT POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF WT CHO CELL EXTRACTS. 71 ug of wt CHO cell extract protein prepared from dense cell cultures was incubated at the indicated temperatures for 15 minutes and subsequently assayed in vitro for 1 hour at 30°C. Incorporation into unit-length Dpn I resistant products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity in vitro represents the replication activity of CHO wt extracts pre-incubated on ice and represents the incorporation of approximately 24 fmol of dCTP.

EFFECTS OF ASSAY TEMPERATURE OF POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF CHO WT AND ts DNA⁻ MUTANT CELL EXTRACTS

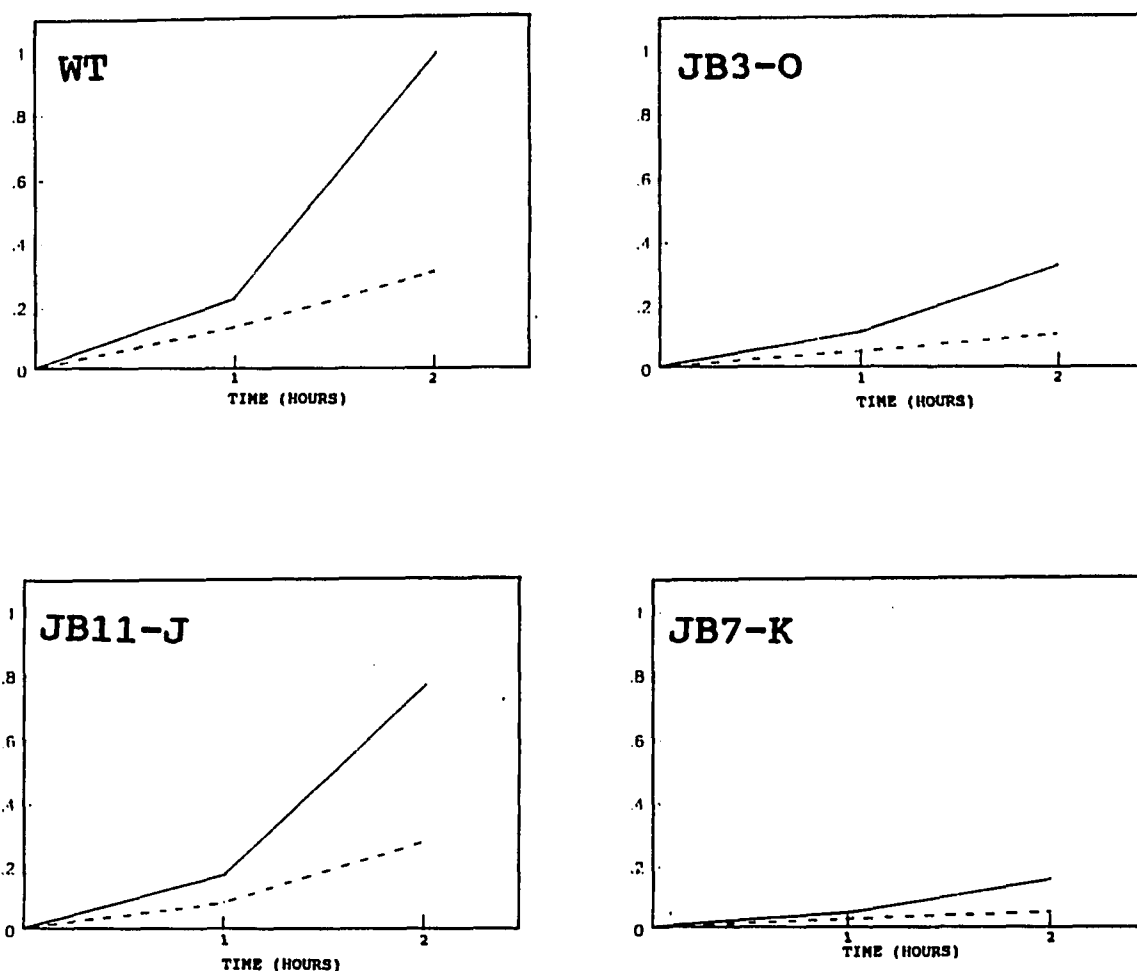


Figure 16. POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF WT AND ts DNA⁻ MUTANT CELL EXTRACTS PREPARED FROM DENSE CELL CULTURES. Extracts prepared from dense cultures of wt and ts DNA⁻ mutant cells were assayed at 30°C or 37°C for 1 or 2 hours. 55 ug of extract protein was assayed in the presence of 5% PEG-8000. Incorporation into unit-length Dpn I resistant products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity in vitro is standardized to the replication activity of wt extract assayed for 2 hours at 30°C and is equivalent to the incorporation of approximately 58 fmol of dCTP.

**EFFECTS OF ASSAY TEMPERATURE OF POLYOMA IN VITRO
DNA REPLICATION ACTIVITIES OF CHO WT AND
ts DNA⁻ MUTANT CELL EXTRACTS**

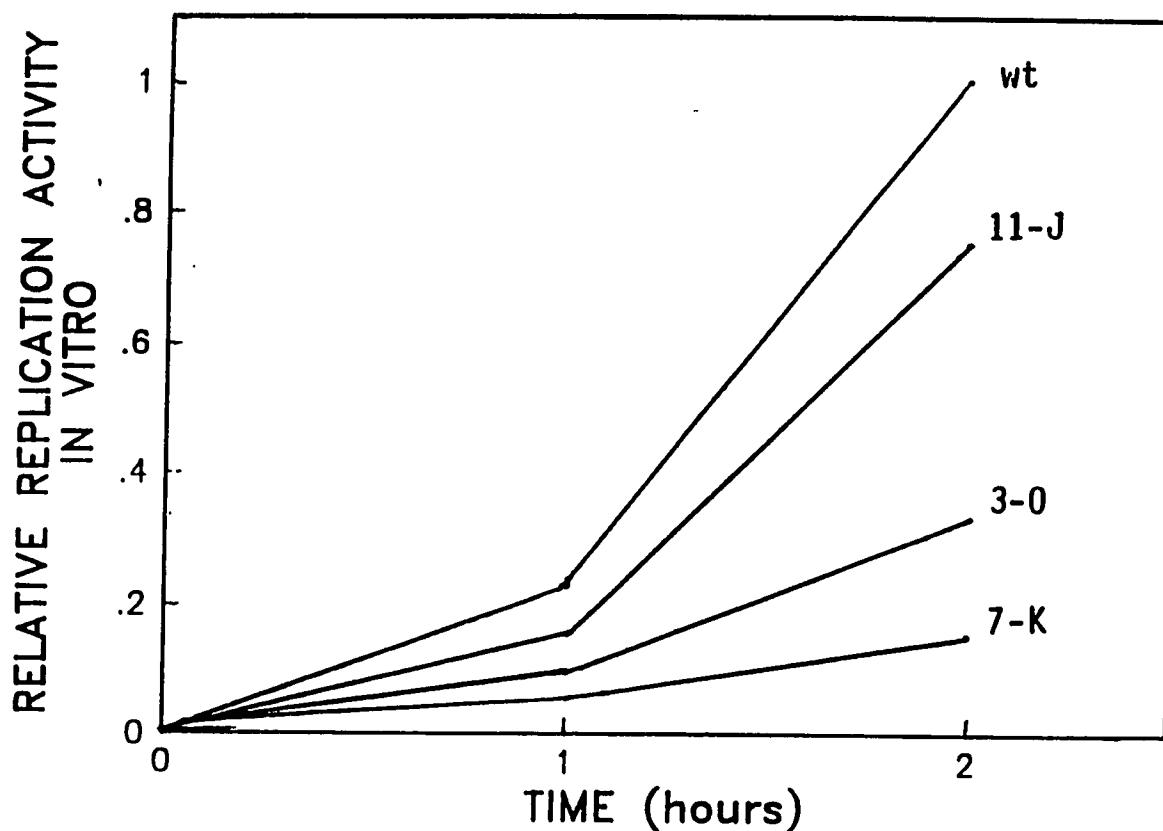


Figure 17. POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF WT AND ts DNA⁻ MUTANT CELL EXTRACTS PREPARED FROM DENSE CELL CULTURES. Extracts prepared from dense cultures of wt and ts DNA⁻mutant cells were assayed at 30°C for 1 or 2 hours. 55 ug of extract protein was assayed in the presence of 5% PEG-8000. Incorporation into unit-length DpnI resistant products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity in vitro represents the replication activity of wt CHO cell extract assayed at 30°C for 2 hours and is equivalent to the incorporation of 58 fmol of dCTP.

PROTOCOL USED TO ENSURE THE EXTRACTION OF
EXPONENTIALLY GROWING CELL CULTURES

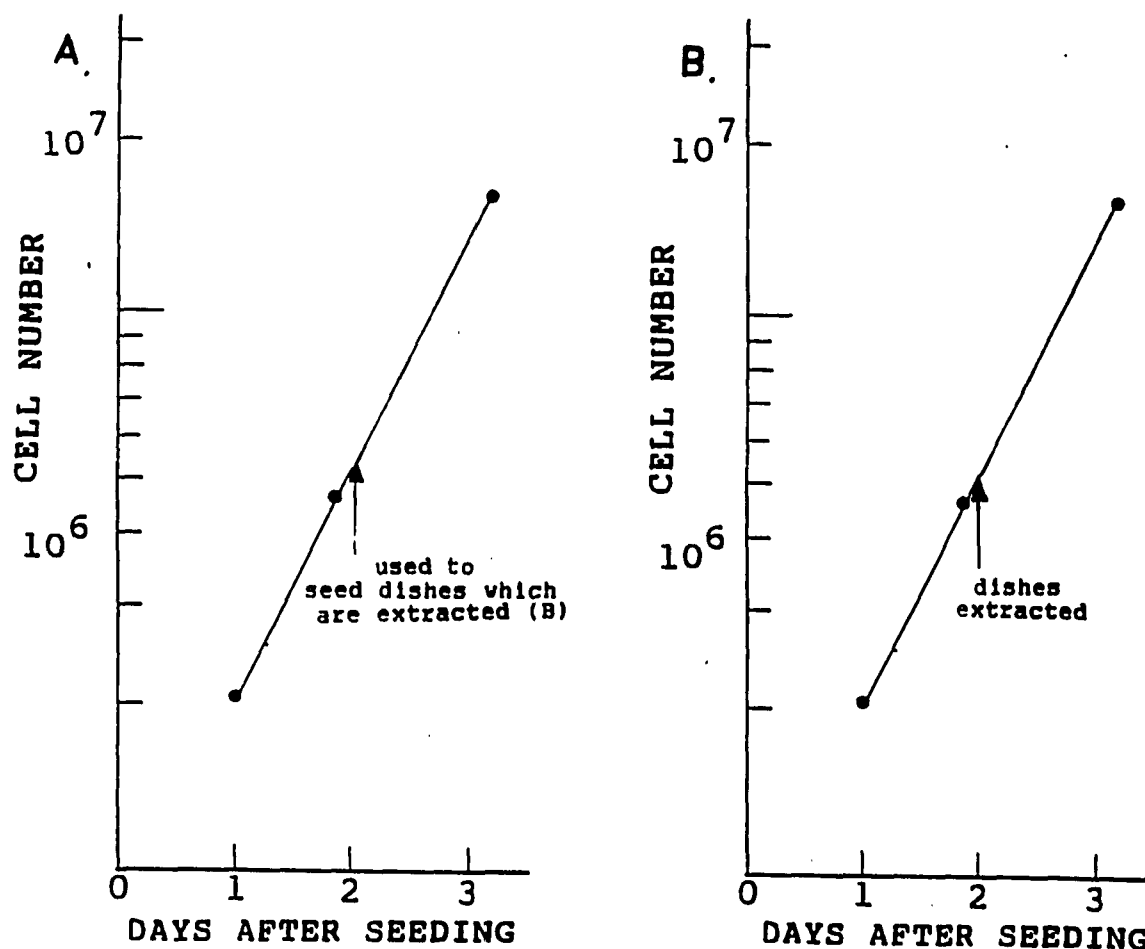


Figure 18. **PROTOCOL UTILIZED TO ENSURE EXPONENTIALLY GROWING CELLS WERE EXTRACTED.** Exponentially growing cells (A) were used to seed dishes which were extracted (B). Dishes in (B) are extracted at the indicated point ($\uparrow$).

**EFFECTS OF EXTRACT PROTEIN CONCENTRATION ON
POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF WT
CHO CELL EXTRACT**

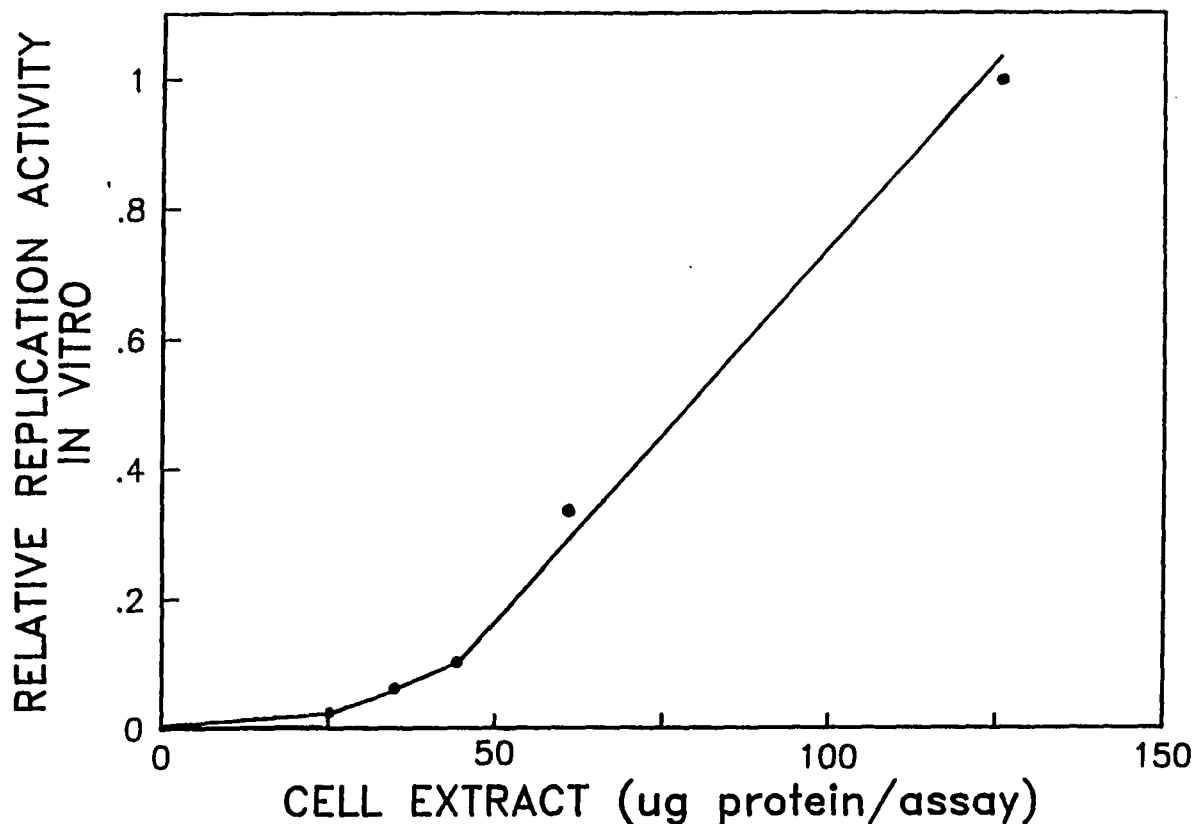


Figure 19. EFFECTS OF PROTEIN CONCENTRATION ON POLYOMA IN VITRO REPLICATION ACTIVITY OF WT CHO CELL EXTRACTS PREPARED FROM EXPONENTIALLY GROWING CELL CULTURES. Extract was prepared from exponentially growing cultures of wt CHO cells. Extract was concentrated as described in Materials and Methods. In vitro reactions containing various amounts of concentrated cell extract were assayed for 2 hours at 30°C. Incorporation into unit-length Dpn I resistant products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity in vitro is equivalent to the incorporation of 0.9 pmol dCTP.

**POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF
CHO WT AND THE ts DNA⁻ MUTANT JB3-O CELL
EXTRACTS**

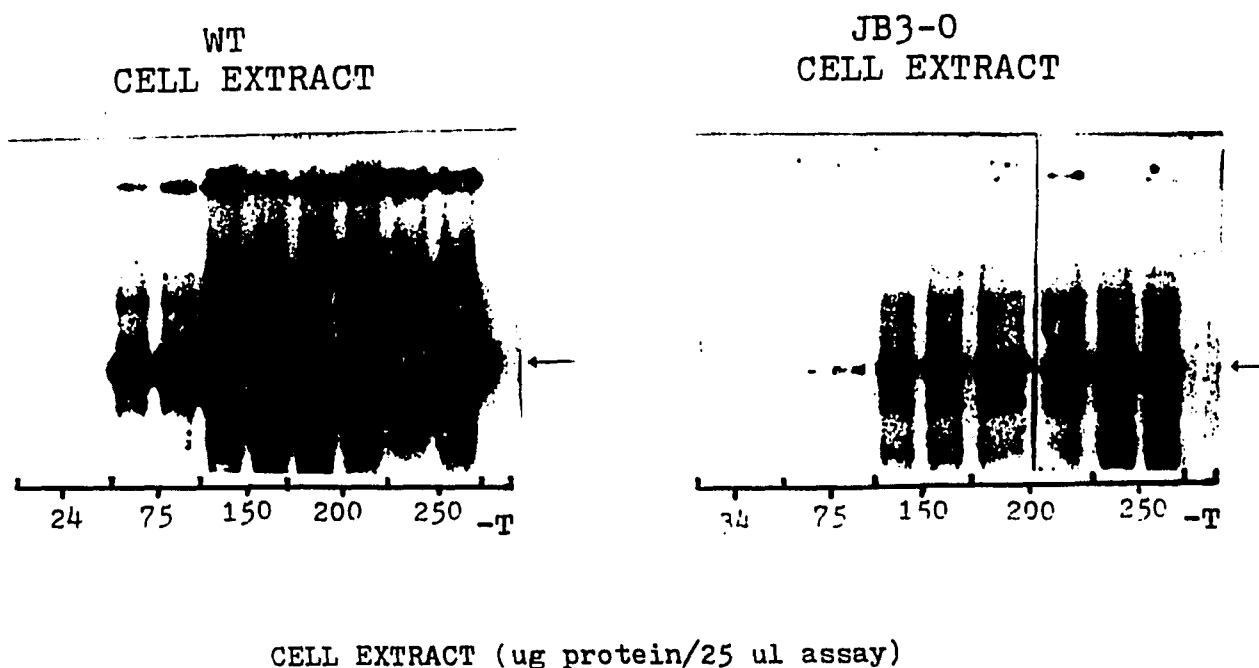


Figure 20. POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF WT AND THE ts DNA⁻ MUTANT JB3-O CELL EXTRACTS. Increasing amounts of concentrated cell extracts prepared from exponentially growing cultures of CHO wt or the ts DNA⁻ mutant JB3-O were assayed at 30°C for 2 hours. Purified products were digested with Dpn I and linearized with Sal I, fractionated by agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. Unit-length replication products are indicated (←). Reactions assayed with PyLT dialysis buffer alone (no PyLT protein) are shown (-T).

**POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF
CHO WT AND THE ts DNA⁻ MUTANT JB3-O CELL
EXTRACTS**

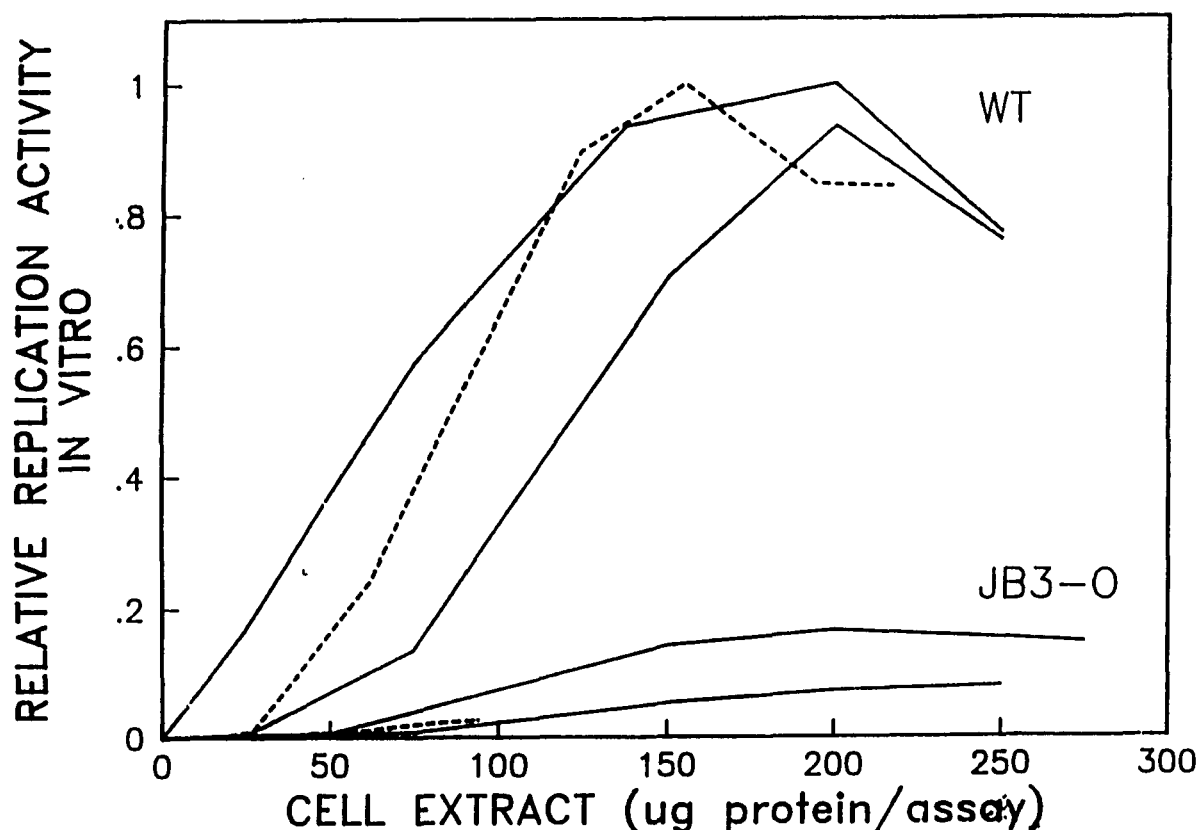


Figure 21. **POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF WT AND THE ts DNA⁻ MUTANT JB3-O CELL EXTRACTS.** Increasing amounts of concentrated cell extracts prepared from exponentially growing cultures were assayed at 30°C for 2 hours. The results of two independent experiments are presented (experiment 1 (---) and experiment 2 (—)). Two independently prepared extracts from both wt and JB3-O cells were assayed in experiment 2. Products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity in vitro represented the maximal level of in vitro DNA replication by wt cell extract in each experiment. 1 unit of relative replication activity in vitro in experiment 1 was equivalent to the incorporation of 1.25 pmol dCTP. 1 unit of relative replication activity in vitro in experiment 2 was equivalent to the incorporation of 5.4 pmol dCTP.

**TIME-COURSE OF UNIT-LENGTH POLYOMA IN VITRO DNA
REPLICATION PRODUCT FORMATION BY EXTRACTS
PREPARED FROM CHO WT AND THE *ts* DNA⁻ MUTANT CELL
LINES JB3-O AND JB7-K**

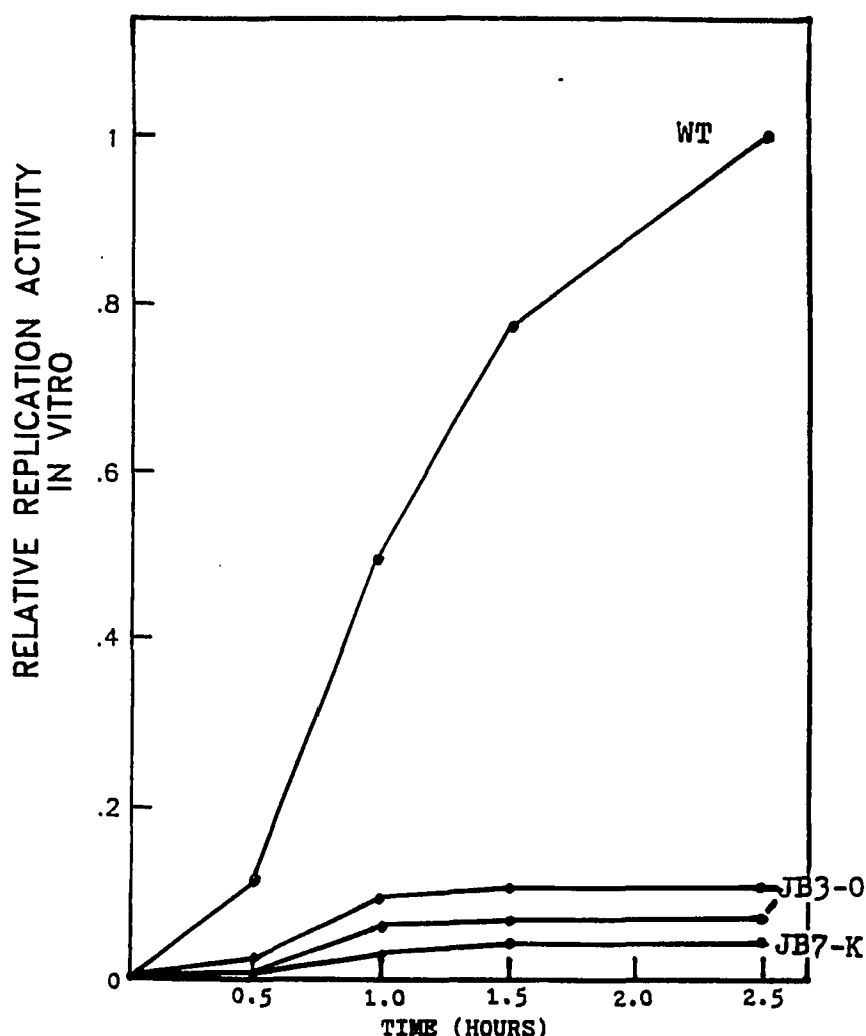


Figure 22. TIME-COURSE OF SYNTHESIS OF FULL LENGTH DPN I RESISTANT PRODUCTS BY EXTRACTS PREPARED FROM CHO WT AND THE *ts* DNA⁻ MUTANT CELL LINES JB3-O AND JB7-K. 175 ug of concentrated cell extract protein were assayed at 30°C for 0.5, 1.0, 1.5, or 2.5 hours. Purified products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity in vitro represents the in vitro replication activity of wt CHO cell extract assayed for 2.5 hours at 30°C and is equivalent to the incorporation of 6.8 pmol dCTP.

**TIME-COURSE EXPERIMENT INVOLVING EXTRACTS DERIVED
FROM CHO WT AND THE ts DNA⁻ MUTANT
CELL LINES JB3-O AND JB7-K**

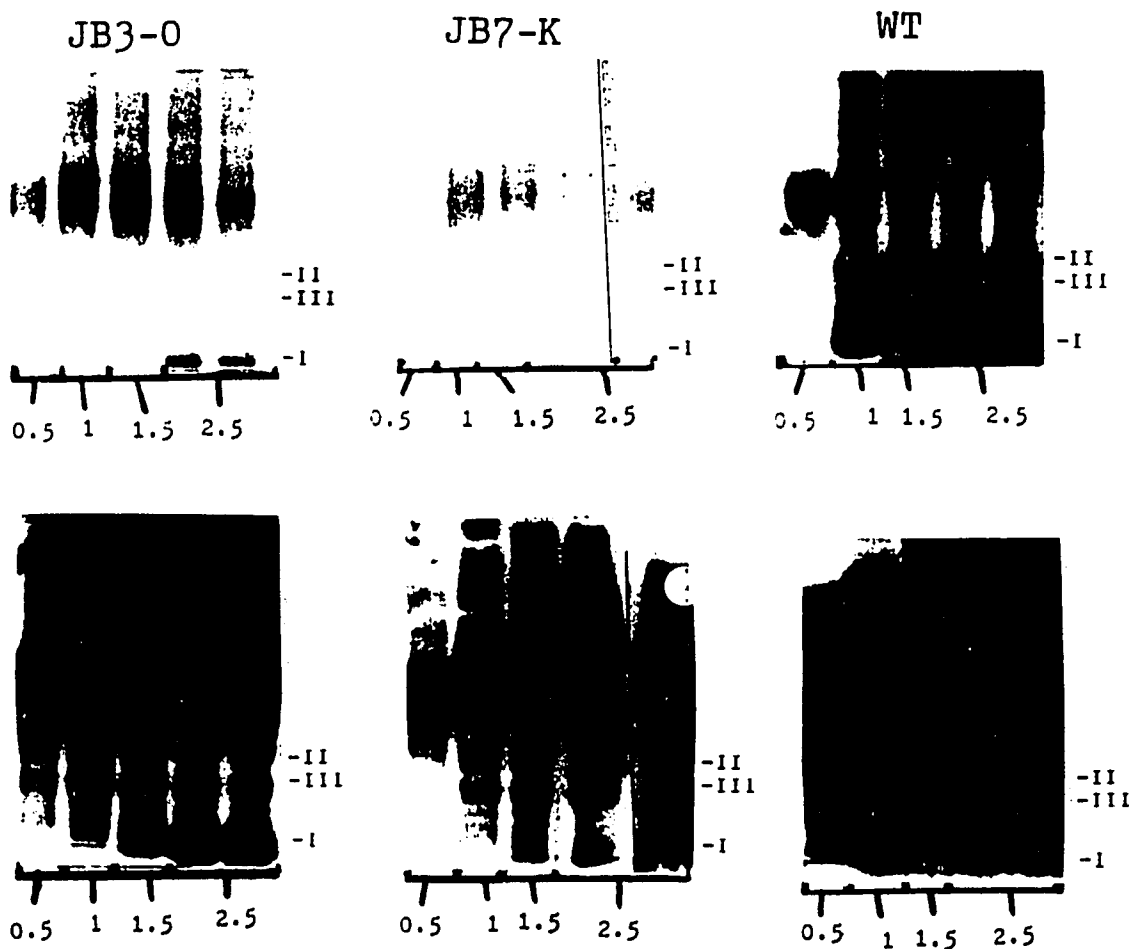


Figure 23. TIME COURSE OF POLYOMA IN VITRO DNA REPLICATION PRODUCTS SYNTHESIZED BY EXTRACTS DERIVED FROM CHO WT AND THE ts DNA⁻ MUTANT CELL LINES JB3-O AND JB7-K. 175 ug of concentrated cell extract protein was assayed at 30°C for 0.5, 1.0, 1.5, or 2.5 hours. Products were purified, fractionated by agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. Location of form I, II, and III DNA of the input plasmid DNA is marked. Top panel, short exposure. Bottom panel, long exposure. Data corresponds to aliquots of the same samples as was analyzed in Figure 13.

POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF CHO WT AND ts DNA⁻ MUTANT CELL EXTRACTS

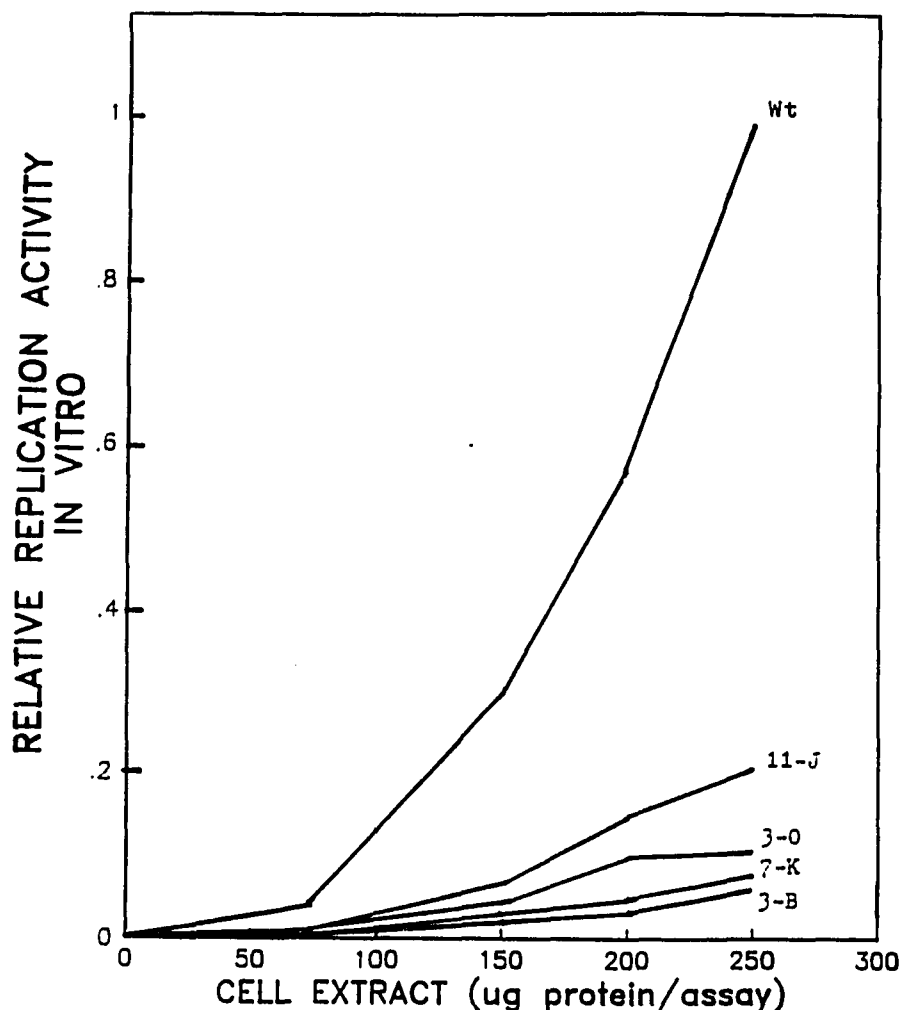


Figure 24. **POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF CHO WT AND ts DNA⁻ MUTANT CELL EXTRACTS.** Increasing amounts of concentrated cell extracts prepared from exponentially growing cultures of the cell lines shown above were assayed at 30°C for 2 hours. Products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity in vitro is standardized to the in vitro replication activity of wt CHO cell extract assayed at 30°C for 2 hours and is equivalent to the incorporation of 8.3 pmol of dCTP.

POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF CHO WT, JB11-J, AND JB17-D CELL EXTRACTS

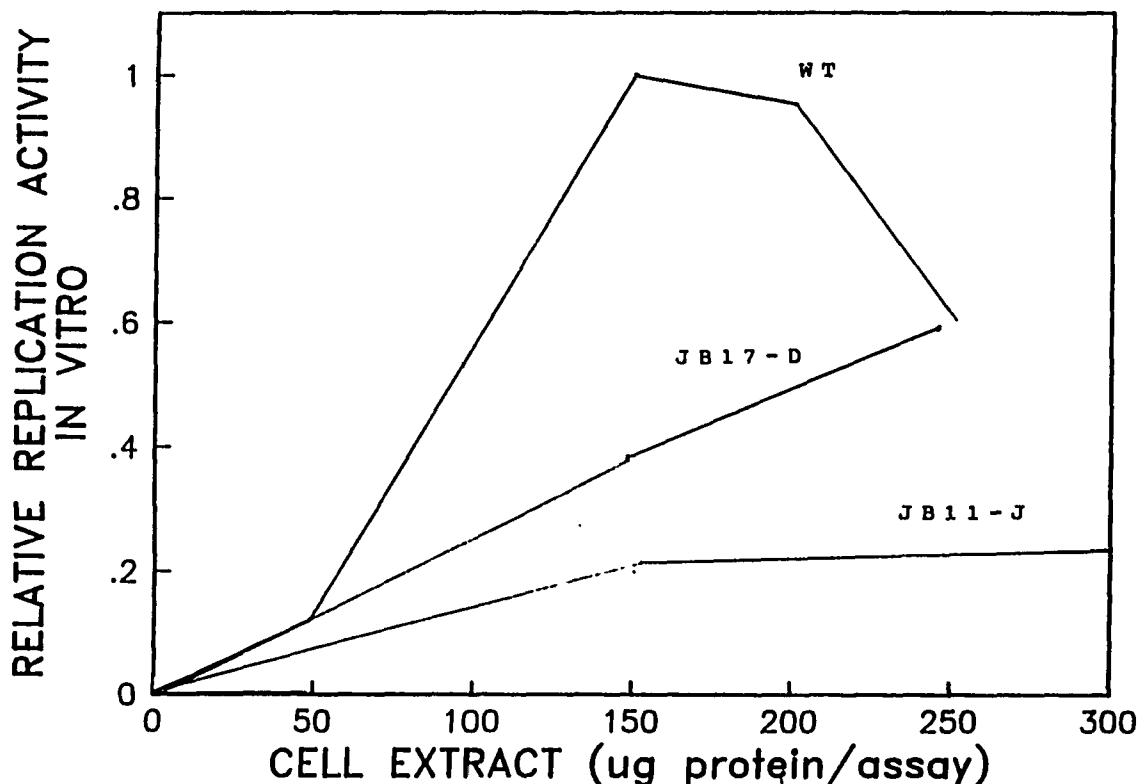


Figure 25. **POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF CHO WT, JB11-J and JB17-D ts DNA⁻ MUTANT CELL EXTRACTS.**

Increasing amounts of concentrated cell extracts prepared from exponentially growing cultures of the cell lines shown above were assayed at 30°C for 2 hours. Products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity in vitro is standardized to the in vitro replication activity of 150 ug of wt CHO cell extract assayed at 30°C for 2 hours and is equivalent to the incorporation of approximately 6 pmol of dCTP.

**DETERMINATION OF THE LEVELS OF REPLICATION
PROTEIN A (RP-A) IN CONCENTRATED HB
CELL EXTRACTS PREPARED FROM
CHO WT, JB3-O AND JB7-K**

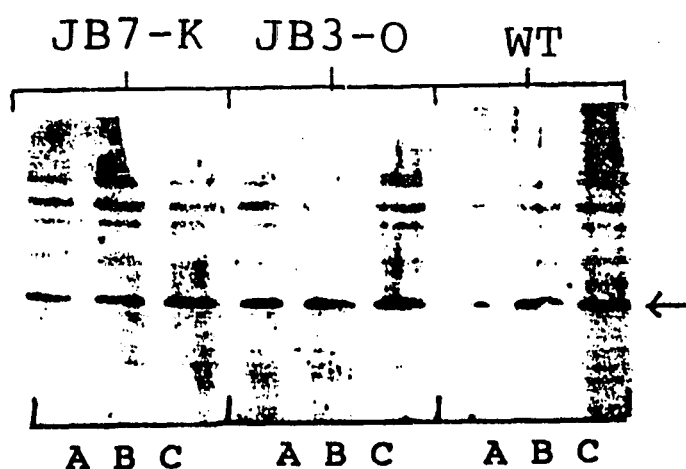


Figure 26. DETERMINATION OF THE LEVELS OF REPLICATION PROTEIN A (RP-A) IN CONCENTRATED HB CELL EXTRACTS PREPARED FROM CHO WT, JB3-O AND JB7-K. 75 (A) , 150 (B) , and 300 (C) ug of each cell extract was separated by SDS-PAGE and a rabbit polyclonal serum prepared against human RP-A was used to detect the levels of RP-A in these extracts by western blotting as described in Materials and Methods. The prominent band migrating at approximately 32 kDa represents 1 of 3 subunits of RP-A and is indicated above (←).

DNA POLYMERASE ACTIVITY OF WT AND ts DNA⁻ MUTANT CELL EXTRACTS

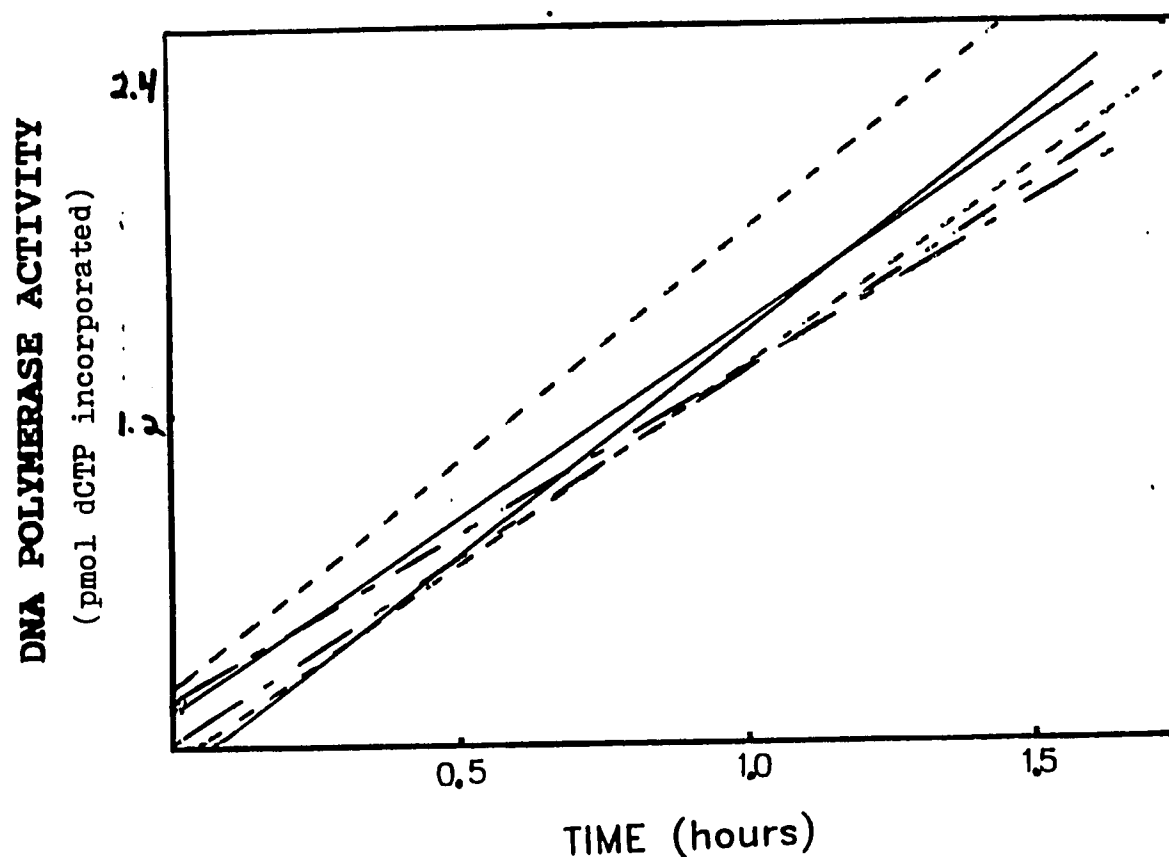


Figure 27. **DNA POLYMERASE ACTIVITIES OF CONCENTRATED CHO WT AND TS DNA⁻MUTANT CELL CONCENTRATED HB EXTRACTS.** 25 ug of concentrated HB cell extract was assayed at 30°C for 0.5, 1.0 or 1.5 hours. DNA polymerase assays using activated DNA as a template were performed as described in the material and methods section. Samples were TCA precipitated and processed before being counted by Cerenkov counting to determine incorporation into acid-insoluble product. DNA polymerase activity for each cell line was assessed using two independently prepared concentrated HB cell extracts. The DNA polymerase activities of extracts prepared from CHO wt (---), JB3-O (——) and JB7-K (—.—) are shown above.

**UNIT-LENGTH DPN I RESISTANT PRODUCTS SYNTHESIZED
DURING MIXING EXPERIMENT INVOLVING EXTRACTS
DERIVED FROM CHO WT, JB3-O AND JB7-K**

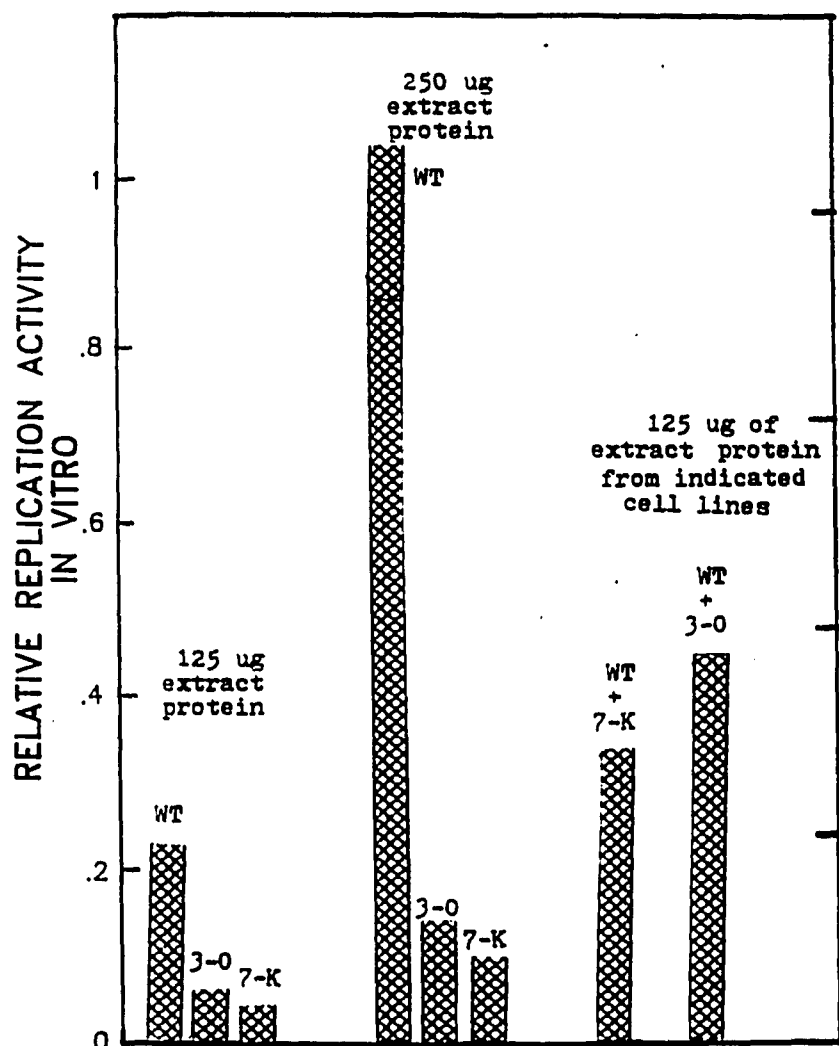


Figure 28. ANALYSIS OF MUTANT CELL EXTRACTS FOR THE PRESENCE OF DNA REPLICATION INHIBITORS. The indicated amounts of HB cell extracts prepared from CHO wt, JB3-O and JB7-K were assayed at 30°C for 2 hours. Purified products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity in vitro is standardized to the in vitro replication activity of 250 ug of wt CHO cell extract and is equivalent to the incorporation of approximately 8.2 pmol dCTP.

**UNIT-LENGTH DPN I RESISTANT PRODUCTS SYNTHESIZED
DURING MIXING EXPERIMENT INVOLVING EXTRACTS
DERIVED FROM CHO WT, JB3-O AND JB7-K**

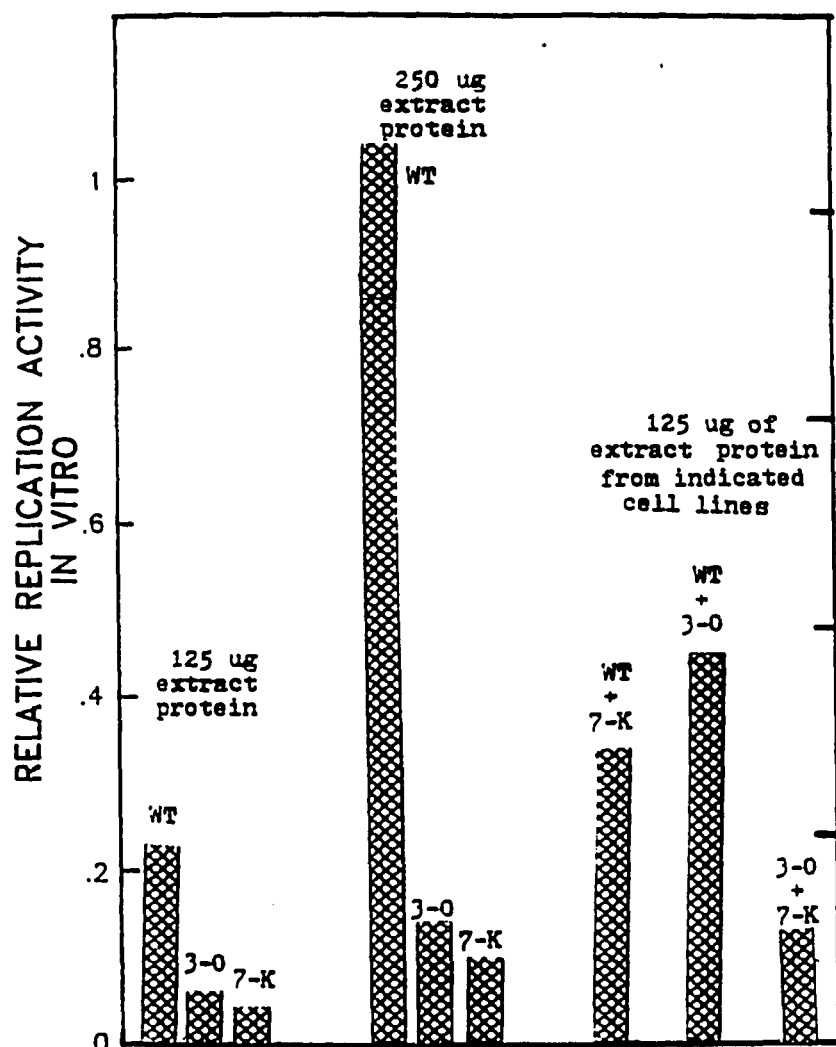


Figure 29. MIXING EXPERIMENTS INVOLVING EXTRACTS DERIVED FROM CHO WT, JB3-O AND JB7-K. The indicated amounts of HB cell extracts prepared from CHO wt, JB3-O and JB7-K were assayed at 30°C for 2 hours. Purified products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in Materials and Methods. 1 unit of relative replication activity in vitro is standardized to the in vitro replication activity of 250 ug of wt CHO cell extract and is equivalent to the incorporation of approximately 8.2 pmol dCTP. Most of these data were presented previously in Figure 28.

**ANALYSIS OF POLYOMA IN VITRO DNA REPLICATION
PRODUCTS SYNTHESIZED IN MIXING EXPERIMENT
INVOLVING EXTRACTS DERIVED FROM CHO WT, JB3-O,
AND JB7-K CELL LINES**

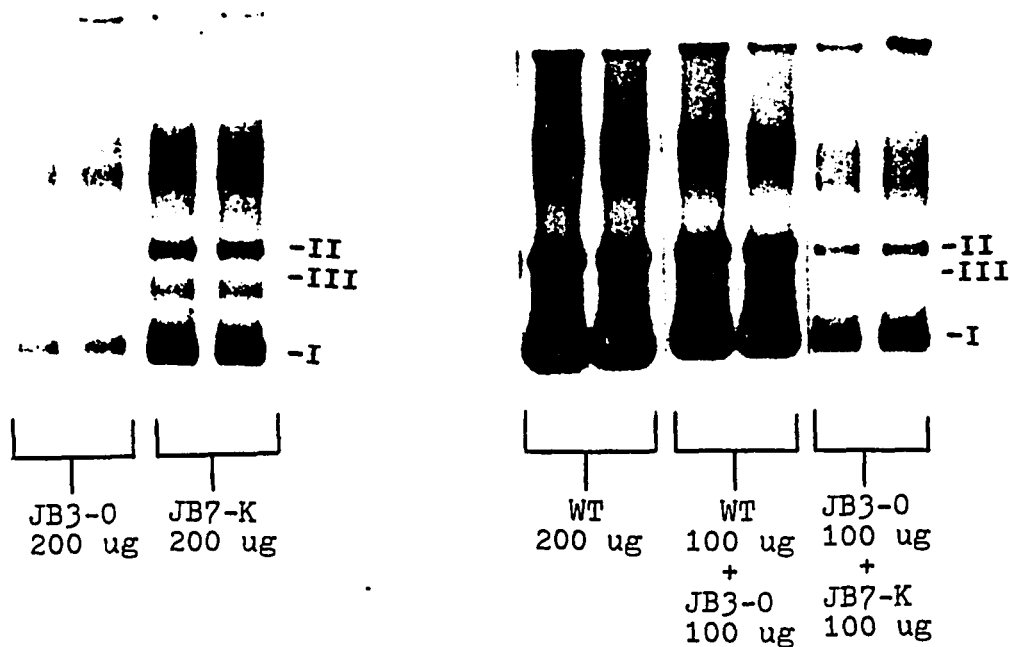


Figure 30. ANALYSIS OF REPLICATION PRODUCTS SYNTHESIZED IN MIXING EXPERIMENTS INVOLVING EXTRACTS DERIVED FROM CHO WT AND JB3-O CELL LINES. *In vitro* reactions containing the indicated amounts of concentrated cell extracts derived from CHO wt and the ts DNA⁻ mutant JB3-O were assayed at 30°C for 2 hours. Products were purified, fractionated by agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. Locations of form I, II, and III DNA of the input plasmid are marked.

POLYOMA IN VITRO DNA REPLICATION ACTIVITY OF CHO
WT AND JB3-O/R1, A ts^+ REVERTANT
ISOLATED FROM JB3-O

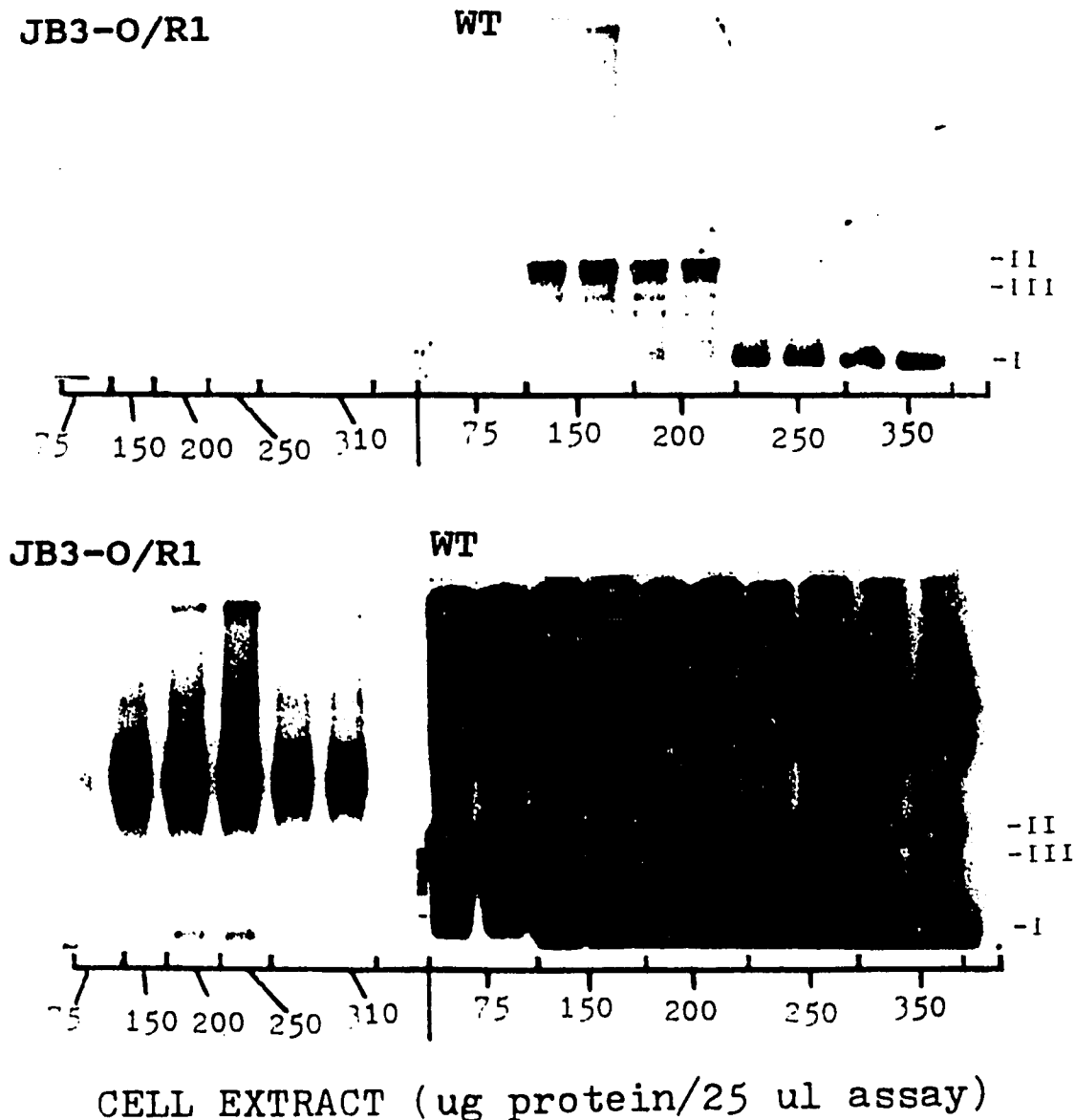


Figure 31. **POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF CHO WT AND JB3-O/R1, A ts^+ REVERTANT ISOLATED FROM JB3-O.** In vitro reaction mixes containing the indicated amounts of cell extracts derived from CHO wt or JB3-O/R1 were incubated at 30°C for 2 hours. Products were purified, fractionated by agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. Locations of form I, II, and III DNA of the input plasmid are marked.

**POLYOMAVIRUS DNA REPLICATION IN CHO WT, JB3-O AND
JB3-O REVERTANT CELL LINES JB3-O/R1 AND JB3-O/R2**

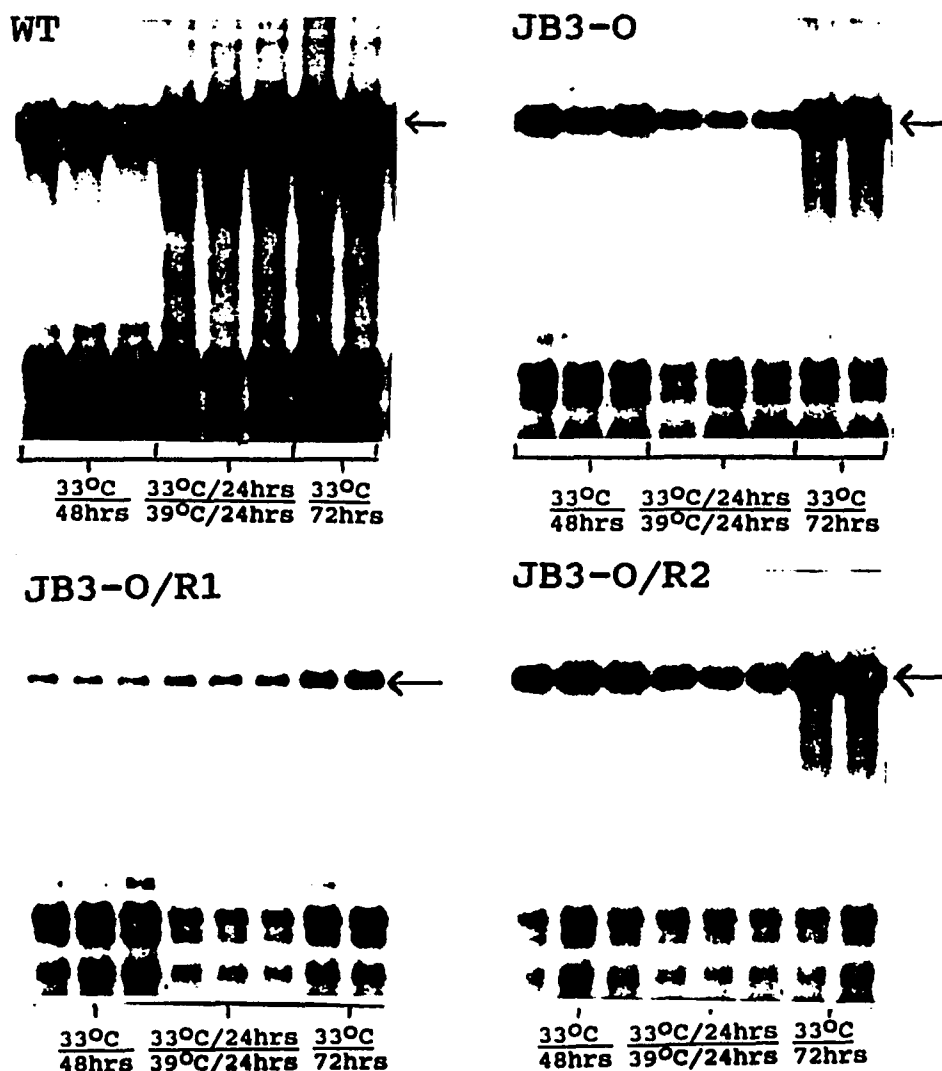


Figure 32. POLYOMAVIRUS DNA REPLICATION IN CHO WT, JB3-O AND JB3-O REVERTANT CELL LINES JB3-O/R1 AND JB3-O/R2. Cell cultures were transfected with the plasmid p53A6.6, which contains the entire polyoma genome, as described in Materials and Methods. Transfected cultures were incubated in the conditions described above. Viral DNA was extracted and purified as described. Purified DNA representing 1/10 of each transfected culture was digested with Sal I and Dpn I. After enzyme digestion, samples were run in a 1% agarose gel in 0.5 X TPE buffer. Unit-length replication products were visualized after Southern blotting and subsequent probing using random primed p53A6.6 linearized DNA as described in the materials and methods section. The migration of form III p53A6.6 DNA is indicated (←). The lower molecular weight Dpn I digested products are also visible.

**ABSOLUTE VALUES OF POLYOMAVIRUS DNA REPLICATION
IN CHO WT, JB3-O AND JB3-O REVERTANT
CELL LINES JB3-O/R1 AND JB3-O/R2**

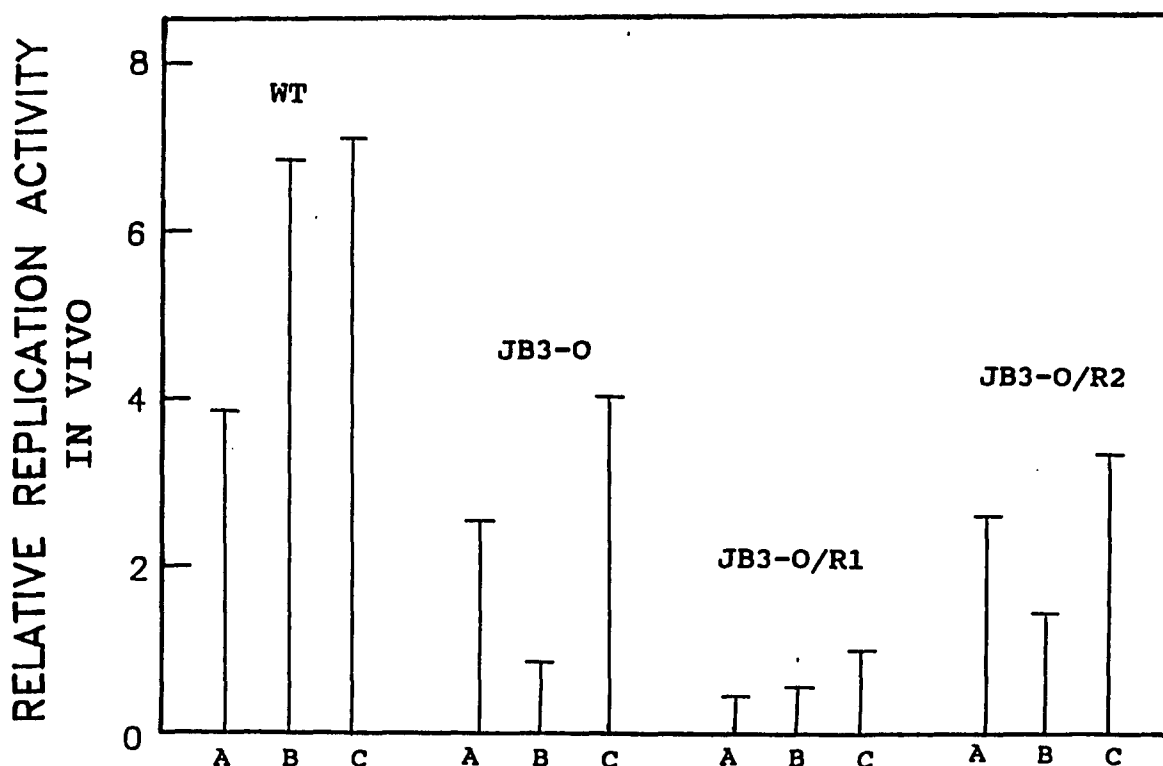


Figure 33. ABSOLUTE AMOUNTS OF POLYOMAVIRUS DNA REPLICATION IN CHO WT, JB3-O AND JB3-O REVERTANT CELL LINES JB3-O/R1 AND JB3-O/R2. Transfections and sample processing were described in Materials and Methods and in Figure 22. Transfected cultures were incubated as follows: (A) 33°C / 48 hrs., (B) 33°C / 24 hrs followed by 39.5°C / 24 hrs and (C) 33°C / 72 hrs.. Unit-length Dpn I resistant products were localized and the areas of the nytran filters producing the signals were cut out and placed in scintillation vials containing 2 ml of dH₂O and counted by Cerenkov counting. Absolute number of counts is shown above.

RELATIVE VALUES OF POLYOMAVIRUS DNA REPLICATION
IN CHO WT, JB3-O AND JB3-O REVERTANT
CELL LINES JB3-O/R1 AND JB3-O/R2

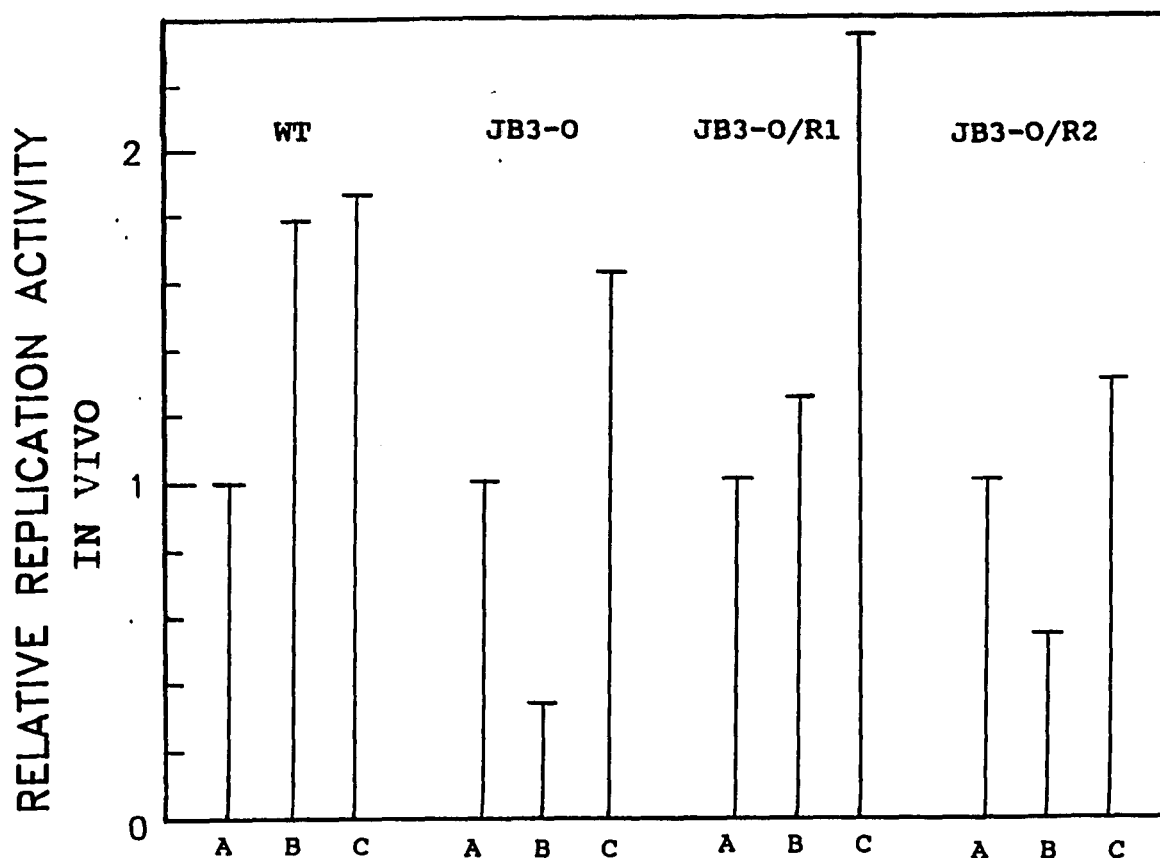


Figure 34. **RELATIVE AMOUNTS OF POLYOMAVIRUS DNA REPLICATION IN CHO WT, JB3-O AND JB3-O REVERTANT CELL LINES JB3-O/R1 AND JB3-O/R2.** Transfections and sample processing were described in the materials and methods section and in figures 22 and 23. Transfected cultures were incubated as follows: (A) 33°C / 48 hrs., (B) 33°C / 24 hrs followed by 39.5°C / 24 hrs and (C) 33°C / 72 hrs.. 1 unit of relative replication activity in vivo is assigned to the amount of unit-length Dpn I resistant replication products synthesized by each cell line after incubation at 33°C for 48 hours.

**EFFECTS OF EXTRACT CONCENTRATION ON THE
TEMPERATURE-SENSITIVE PHENOTYPE OF WT CHO CELL
EXTRACT POLYOMA IN VITRO DNA REPLICATION ACTIVITY**

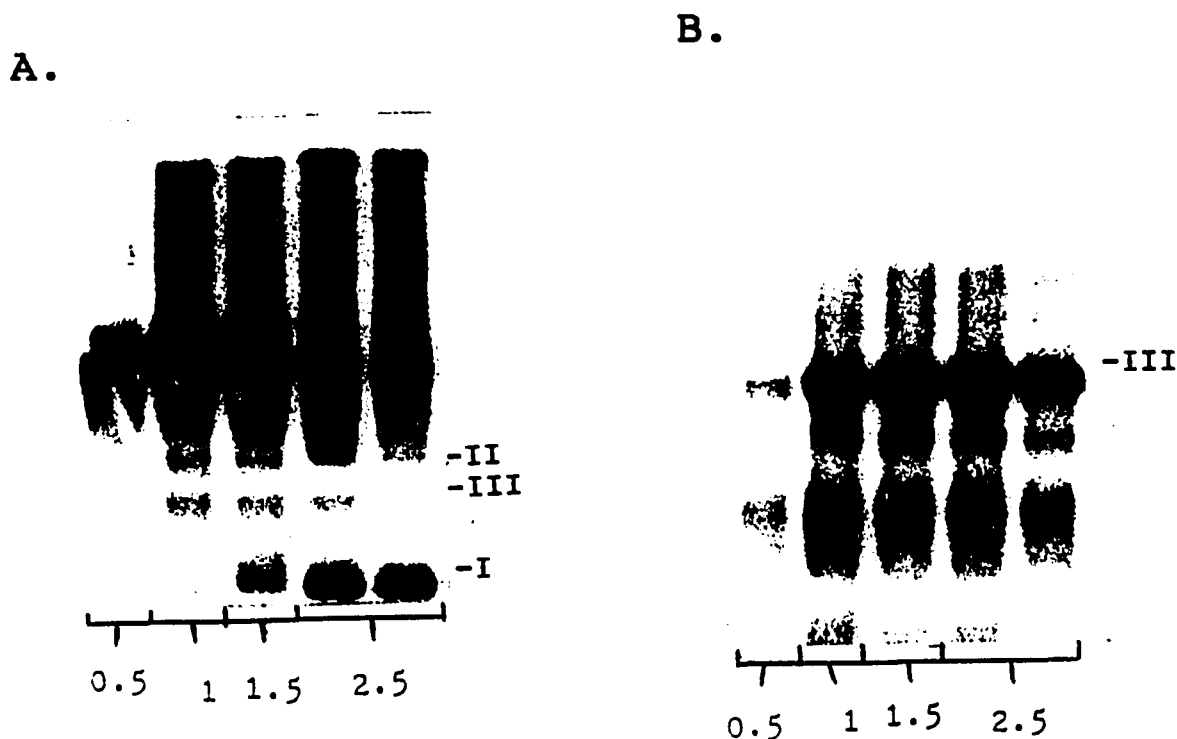


Figure 35. TIME-COURSE OF POLYOMA IN VITRO DNA REPLICATION PRODUCTS SYNTHESIZED BY EXTRACTS DERIVED FROM THE *ts* DNA⁻ MUTANT CELL LINE JB3-O. Reaction mixes containing 175 ug of HB cell extract prepared from JB3-O were incubated at 30°C for the times indicated above. Purified products were (A) undigested or (B) cut with Sal I and Dpn I before being fractionated by agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. The migration of form I, II, and III DNA of the input plasmid are marked. Some of these data were presented in Figure 23.

NEUTRAL GEL

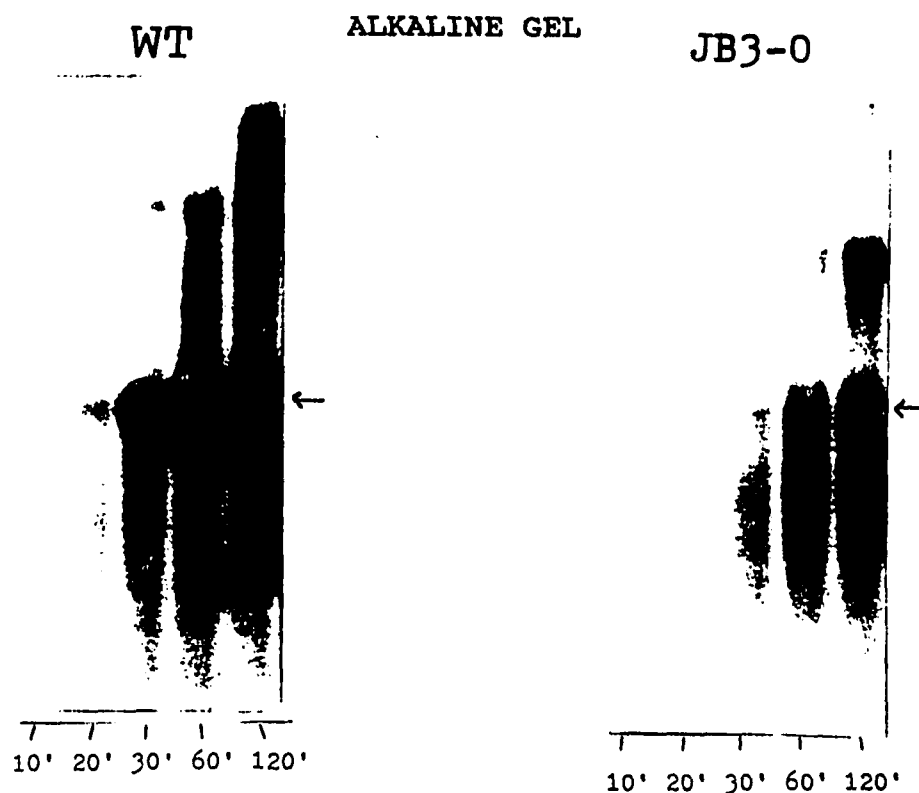
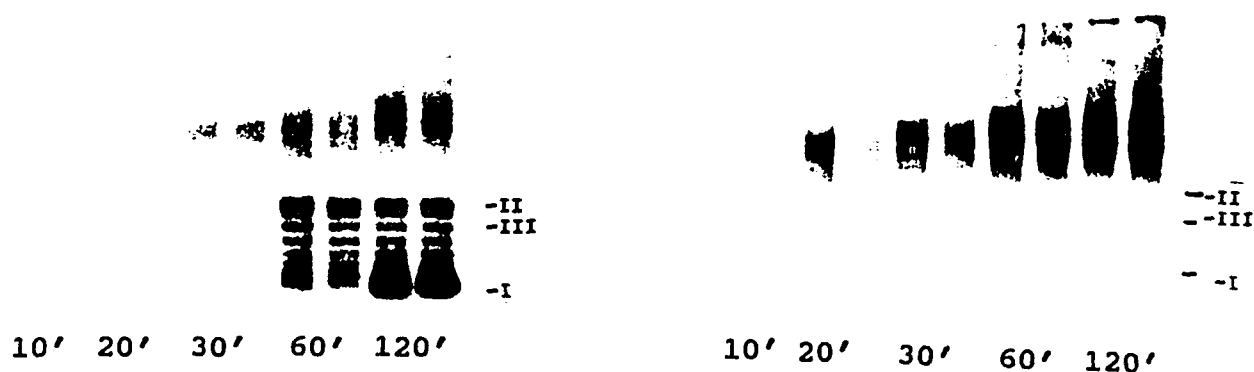


Figure 36. ALKALINE GEL ELECTROPHORETIC ANALYSIS OF POLYOMA IN VITRO DNA REPLICATION PRODUCTS SYNTHESIZED DURING A TIME COURSE BY CELL EXTRACTS PREPARED FROM CHO WT AND JB3-0 CELL LINES. *In vitro* reaction mixes containing 175 ug of HB cell extract protein from each cell line was assayed at 30°C for the times shown above. Products were purified, fractionated by alkaline agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. The migration of linearized denatured size-markers are shown as well as the migration of input supercoiled plasmid pJLPYO DNA (←). (1)10 minutes, (2)20 minutes, (3)30 minutes (4) 60 minutes (5)120 minutes.

**ATTEMPTED RESOLUTION OF POLYOMA IN VITRO DNA
REPLICATION PRODUCTS SYNTHESIZED BY EXTRACTS
DERIVED FROM CHO WT, JB3-O AND JB7-K BY ADDITION
OF PURIFIED TOPOISOMERASE I OR TOPOISOMERASE II**

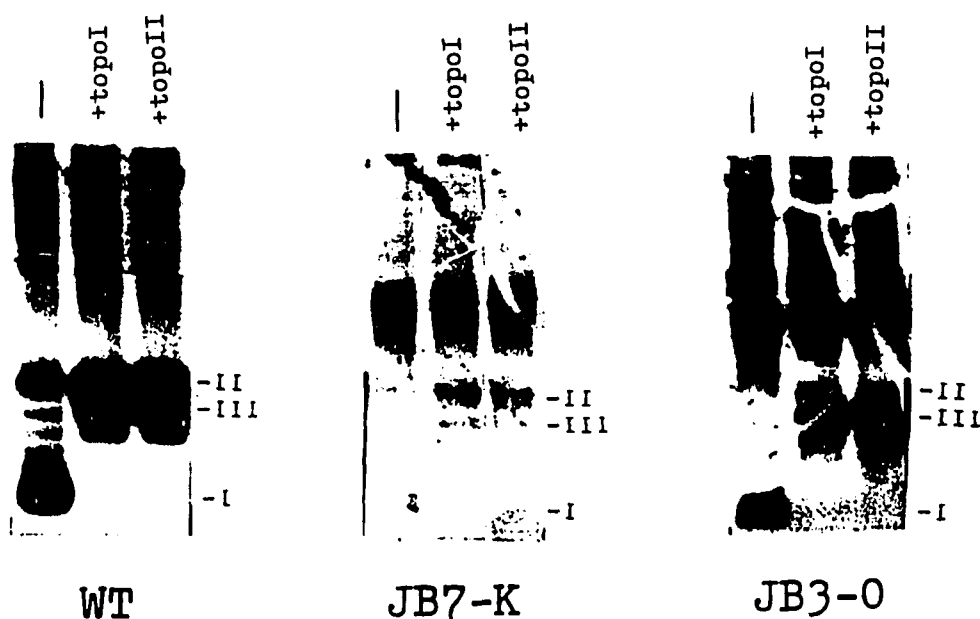


**Figure 37. ATTEMPTED RESOLUTION OF POLYOMA IN VITRO DNA
REPLICATION PRODUCTS SYNTHESIZED BY EXTRACTS DERIVED FROM CHO
WT, JB3-O AND JB7-K BY ADDITION OF PURIFIED TOPOISOMERASE I
OR TOPOISOMERASE II.** In vitro reactions containing 200 ug of
cell extract protein from CHO wt, JB3-O and JB7-K cell lines
were incubated at 30°C for 2 hours. Identical aliquots of
purified replication products were incubated in the absence
(-) or in the presence of topoisomerase I (+topoI) or
topoisomerase II (+topoII). Only one-half of the products
synthesized by wt CHO cell extract were used such that lanes
containing these replication products would not be grossly
overexposed. The migration of form I, II, and III DNA of the
input plasmid are marked.

POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF HB AND 0.2 M NaCl/HB CELL EXTRACTS PREPARED FROM CHO WT AND THE ts DNA⁻ MUTANT JB3-O CELL LINES

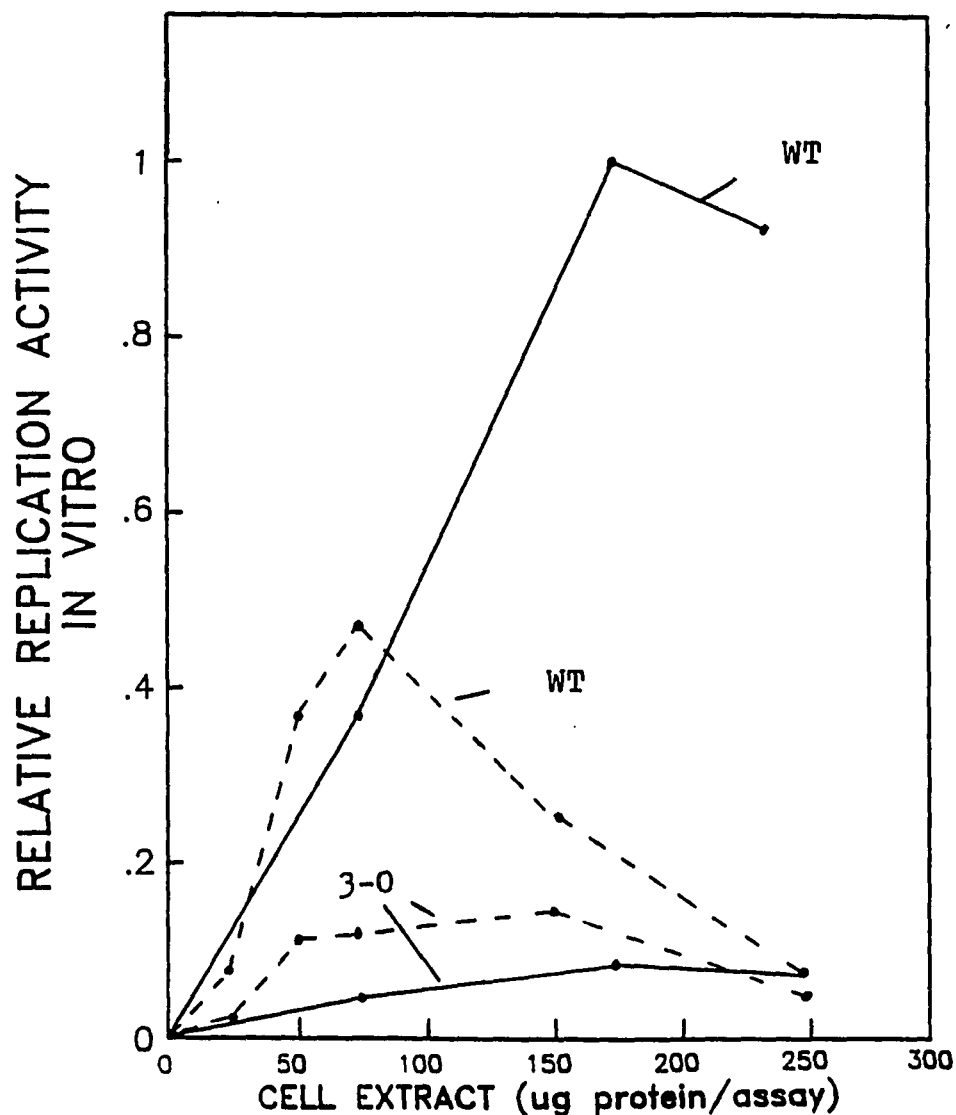


Figure 38. POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF HB AND 0.2 M NaCl/HB CELL EXTRACTS PREPARED FROM CHO WT AND THE ts DNA⁻ MUTANT JB3-O CELL LINES. Increasing amounts of concentrated cell extracts were assayed at 30°C for 2 hours. Polyoma in vitro DNA replication activities of HB cell extracts (—) and of 0.2 M NaCl/HB cell extracts (---). Purified products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in the materials and methods section. 1 unit of relative in vitro replication activity is standardized to the replication activity of 175 ug of CHO wt HB cell extract and is equivalent to the incorporation of 4.2 pmol dCTP.

**POLYOMA IN VITRO DNA REPLICATION PRODUCTS SYNTHESIZED
BY HB AND 0.2 M NaCl/HB CELL EXTRACTS PREPARED FROM
WT CHO AND THE ts DNA⁻ MUTANT JB3-O CELL LINES**

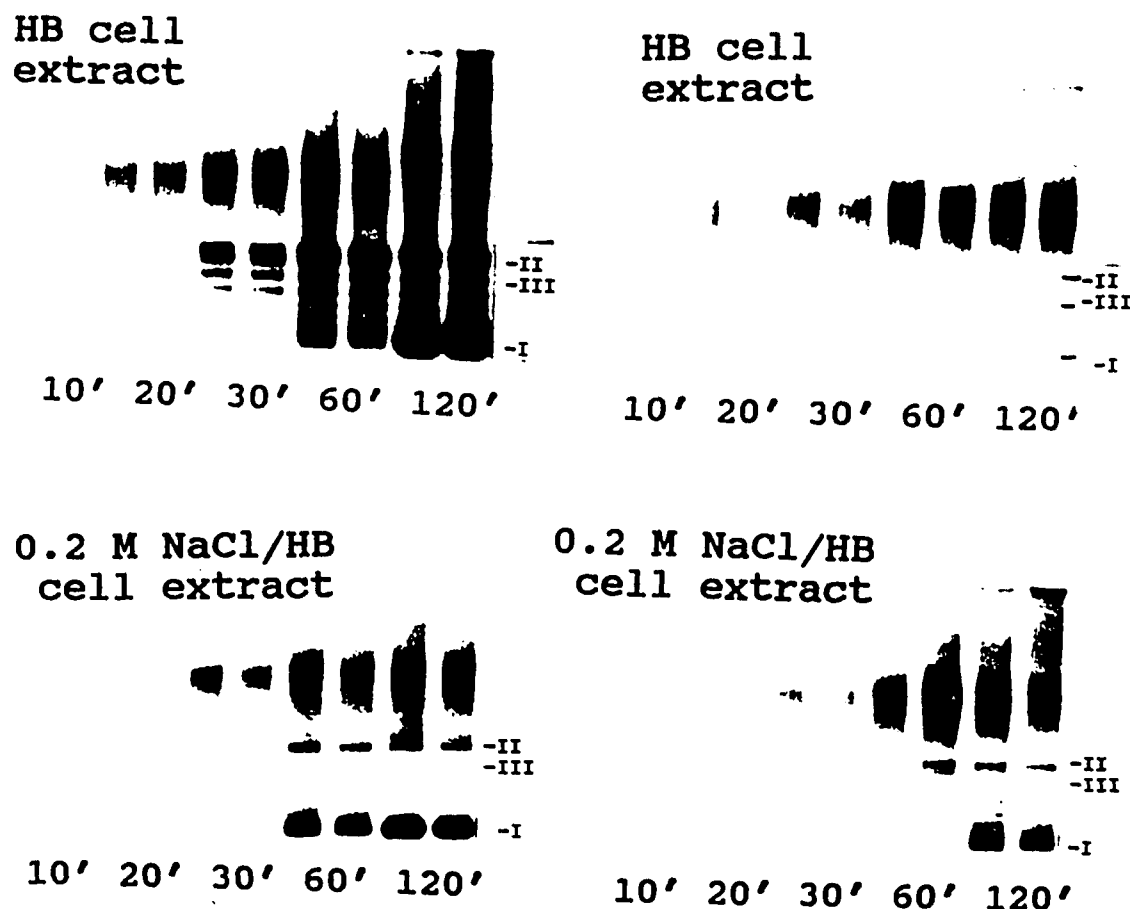


Figure 39. TIME-COURSE OF POLYOMA IN VITRO DNA REPLICATION PRODUCTS SYNTHESIZED BY HB AND 0.2 M NaCl/HB CELL EXTRACTS PREPARED FROM CHO WT AND THE ts DNA⁻ MUTANT JB3-O. *In vitro* reaction mixes containing 150 ug of 0.2 M NaCl/HB cell extract or 175 ug of HB cell extract prepared from CHO wt or JB3-O cell lines were assayed at 30°C for times ranging from 10 minutes to 2 hours. Products were purified, fractionated by agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. Locations of form I, II, and III DNA of the input plasmid are marked.

**ALKALINE GEL ELECTROPHORETIC ANALYSIS OF POLYOMA
IN VITRO DNA REPLICATION PRODUCTS SYNTHESIZED
BY HB AND 0.2 M NaCl/HB CELL EXTRACTS
PREPARED FROM CHO WT AND JB3-O CELL LINES**

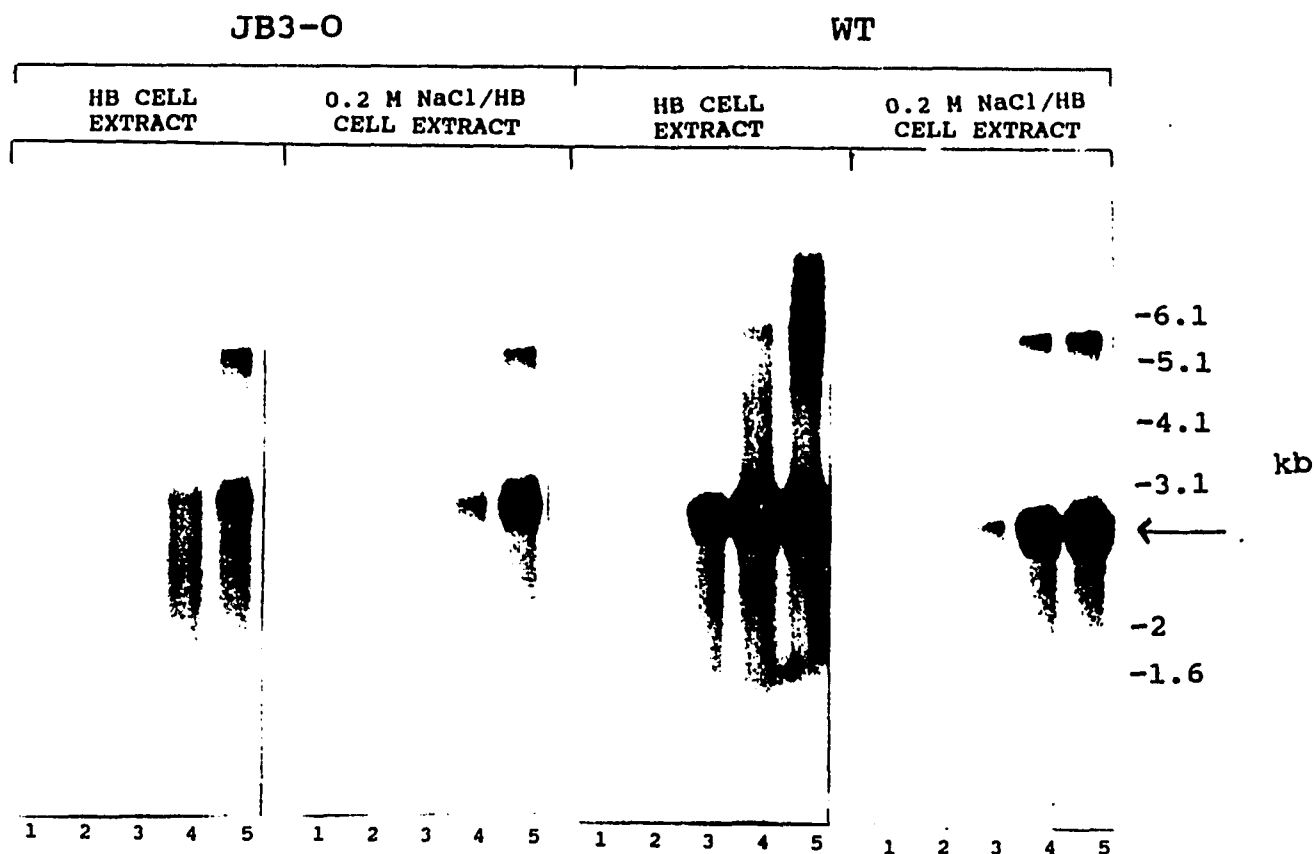


Figure 40. ALKALINE GEL ELECTROPHORETIC ANALYSIS OF POLYOMA IN VITRO DNA REPLICATION PRODUCTS SYNTHESIZED BY HB AND 0.2 M NaCl CELL EXTRACTS PREPARED FROM CHO WT AND JB3-O CELL LINES. In vitro reaction mixes containing either 175 ug of HB cell extract protein or 150 ug of 0.2 M NaCl/HB cell extract protein from CHO wt or JB3-O were assayed at 30°C for the times shown above. Products were purified, fractionated by alkaline agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. The migration of linearized denatured size-markers are shown as well as the migration of input supercoiled plasmid pJLPYO DNA (←). (1)10 minutes, (2)20 minutes, (3)30 minutes (4) 60 minutes (5)120 minutes.

**POLYOMA IN VITRO DNA REPLICATION PRODUCTS
SYNTHESIZED BY HB AND 0.2 M NaCl/HB CELL EXTRACTS
PREPARED FROM CHO WT AND THE ts DNA⁻
MUTANT JB3-O CELL LINES**

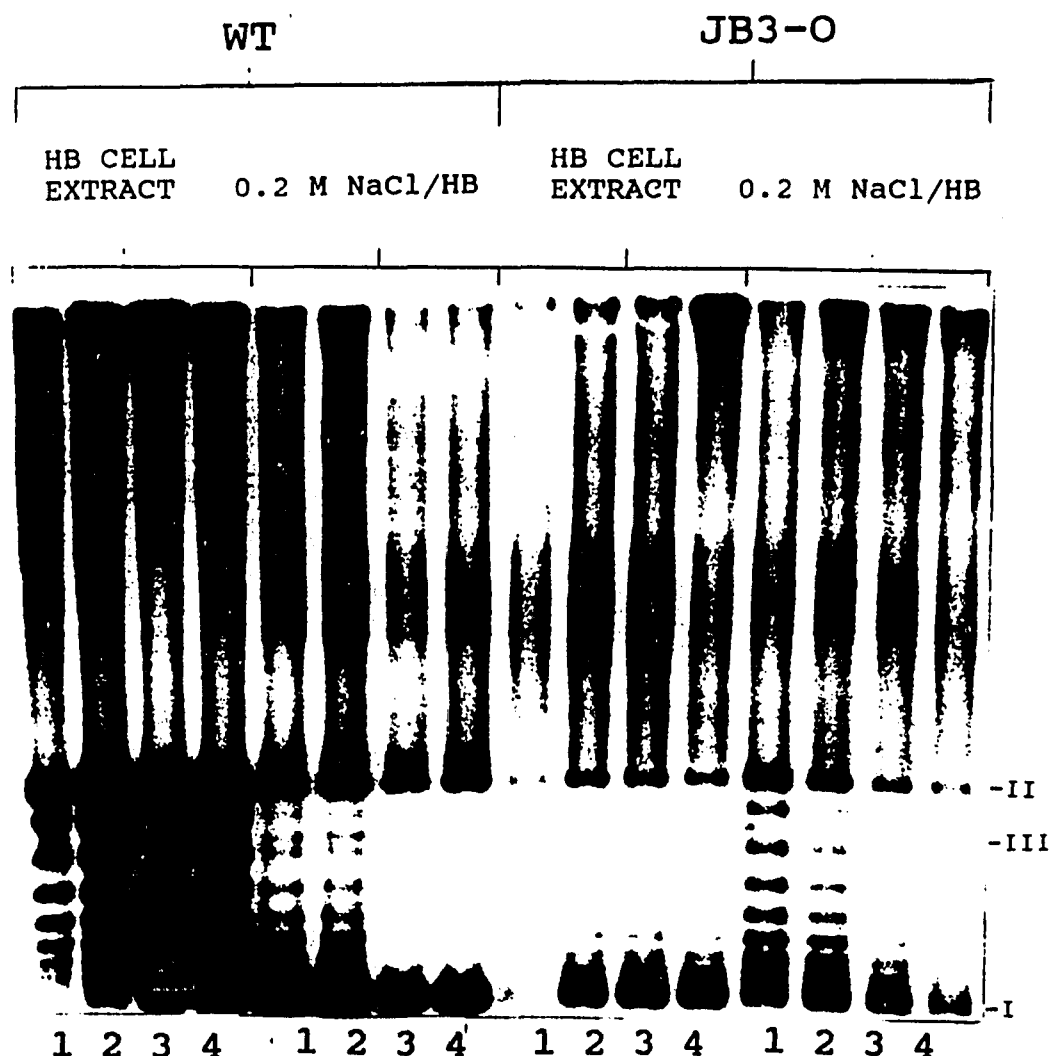


Figure 41. POLYOMA IN VITRO DNA REPLICATION PRODUCTS SYNTHESIZED BY HB AND 0.2 M NaCl/HB CELL EXTRACTS PREPARED FROM CHO WT AND THE ts DNA⁻ MUTANT JB3-O. *In vitro* reaction mixtures containing either 75 (1), 150 (2), 200 (3), or 250 (4) ug of concentrated cell extract protein were assayed at 30°C for 2 hours. Products were purified, fractionated by agarose gel electrophoresis by running in a 1.4% agarose gel for approximately 16 hours at 40 volts, and visualized by autoradiography of the dried down agarose gel. Locations of form I, II, and III DNA of the input plasmid are marked.

PHOSPHOCELLULOSE CHROMATOGRAPHIC FRACTIONATION OF WT CHO CELL EXTRACT

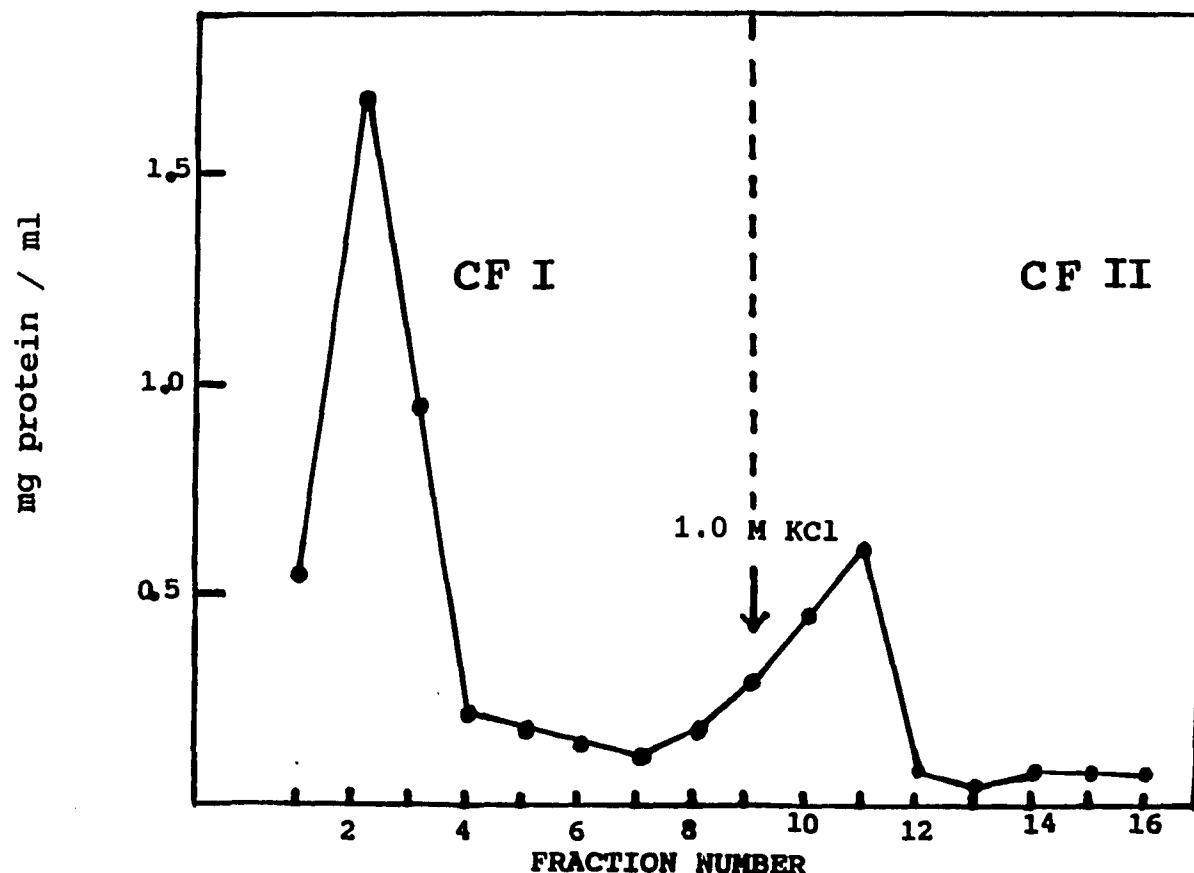


Figure 42. **PHOSPHOCELLULOSE CHROMATOGRAPHIC FRACTIONATION OF WT CHO CELL EXTRACT** Phosphocellulose chromatographic fractionation was performed as described in Materials and Methods. Protein assays were performed to determine the protein concentration of phosphocellulose flow-through and 1 M KCl eluate fractions. The results of the protein assays are presented above. (↓) indicates addition of buffer containing 1 M KCl.

DNA POLYMERASE ACTIVITIES OF CONCENTRATED PHOSPHOCCELLULOSE FRACTIONS CF I AND CF II

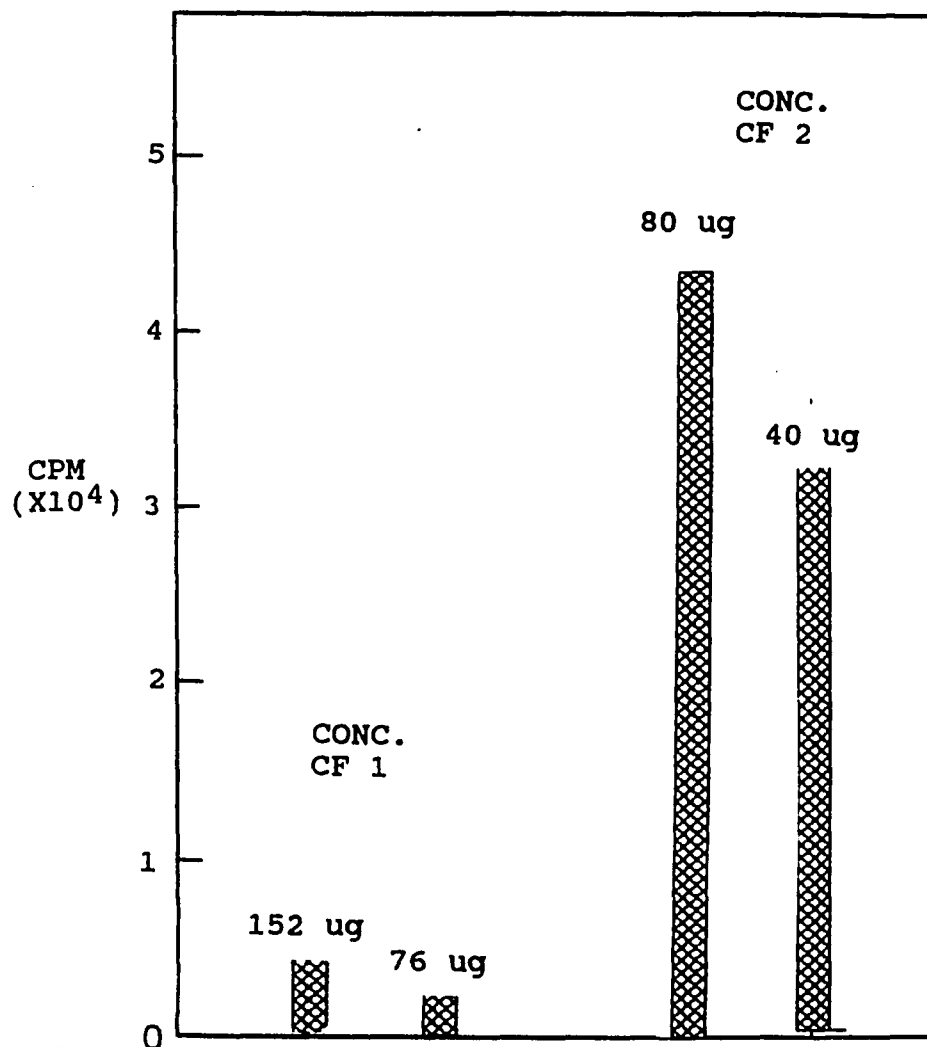


Figure 43. **DNA POLYMERASE ACTIVITY OF PHOSPHOCCELLULOSE FRACTIONS CF I AND CF II.** The indicated amounts of concentrated phosphocellulose fractions CF I and CF II, prepared as described in Materials and Methods from phosphocellulose fractionation of wt CHO HB cell extracts, were assayed for DNA polymerase activity using activated DNA as a template. DNA polymerase assays were incubated at 30°C for 1 hour before processing, TCA precipitation, and determination of levels of incorporation by Cerenkov counting as described in Materials and Methods.

RECONSTITUTION OF POLYOMA IN VITRO DNA REPLICATION ACTIVITY USING PHOSPHOCCELLULOSE COLUMN FRACTIONS

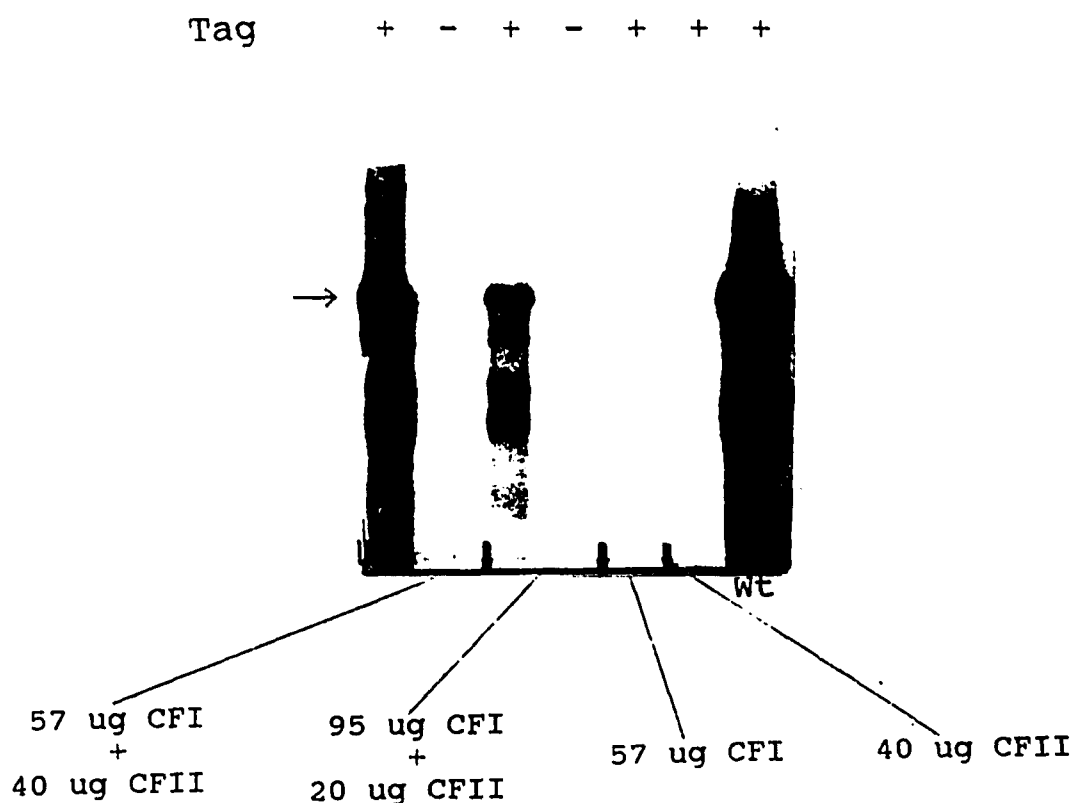


Figure 44. RECONSTITUTION OF POLYOMA IN VITRO DNA REPLICATION ACTIVITY USING PHOSPHOCCELLULOSE COLUMN FRACTIONS. In vitro reactions containing the indicated amount of each concentrated phosphocellulose fraction was assayed for Polyoma in vitro DNA replication activity by incubation for 2 hours at 30°C. PyLT dialysis buffer containing PyLT was added (+) or PyLT dialysis buffer alone was added (-). Products were purified, digested with Sal I and Dpn I, fractionated by agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. Migration of unit-length Dpn I resistant products is shown (→).

EFFECTS OF ADDITIONAL CHO WT HB CELL EXTRACT OR
PHOSPHOCCELLULOSE FRACTIONS CF 1 OR CF 2 ON THE
POLYOMA IN VITRO DNA REPLICATION PRODUCTS
SYNTHESIZED BY CHO WT HB CELL EXTRACTS

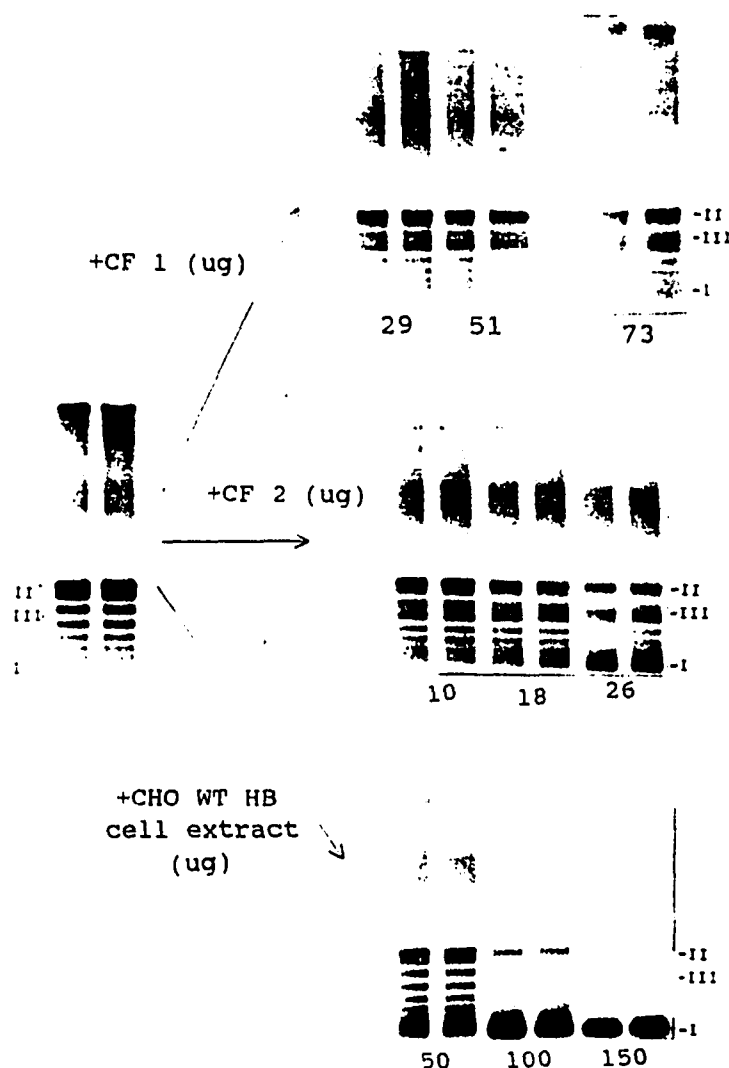


Figure 45. EFFECTS OF INCLUSION OF ADDITIONAL WT CHO HB CELL EXTRACT OR PHOSPHOCCELLULOSE FRACTIONS CF 1 OR CF 2 ON THE POLYOMA IN VITRO DNA REPLICATION PRODUCTS SYNTHESIZED BY WT CHO HB CELL EXTRACTS. In vitro reaction mixes containing 100 ug of concentrated HB cell extract prepared from wt CHO were assayed at 30°C for 2 hours. In vitro reactions were supplemented with either additional HB cell extract derived from wt CHO or phosphocellulose fractions CF I or CF II. Products were purified, fractionated by agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. Locations of form I, II, and III DNA of the input plasmid are marked.

EFFECTS OF INCLUSION OF ADDITIONAL JB3-O HB CELL EXTRACT OR PHOSPHOCCELLULOSE FRACTIONS CF 1 OR CF 2 ON THE POLYOMA IN VITRO DNA REPLICATION PRODUCTS SYNTHESIZED BY JB3-O HB CELL EXTRACTS

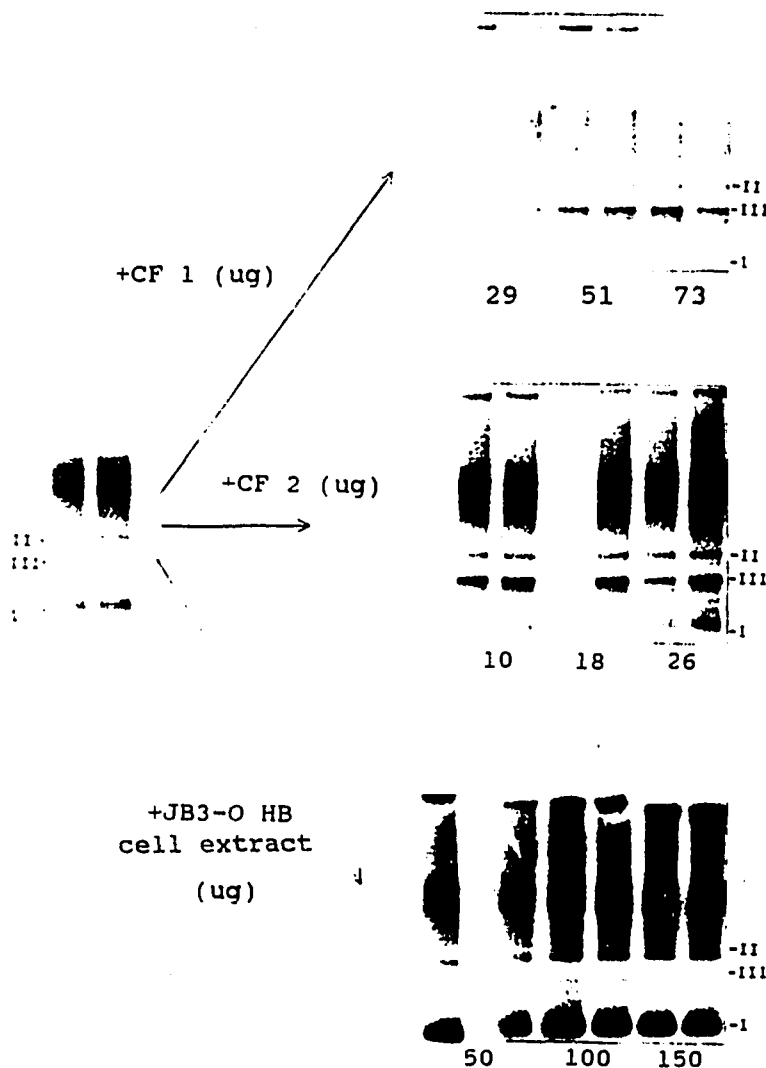


Figure 46. EFFECTS OF INCLUSION OF ADDITIONAL JB3-O HB CELL EXTRACT OR PHOSPHOCCELLULOSE FRACTIONS CF 1 OR CF 2 ON THE POLYOMA IN VITRO DNA REPLICATION PRODUCTS SYNTHESIZED BY JB3-O HB CELL EXTRACTS. In vitro reaction mixes containing 100 ug of concentrated HB cell extract prepared from JB3-O were assayed at 30°C for 2 hours. In vitro reactions were supplemented with either additional HB cell extract derived from JB3-O or phosphocellulose fractions CF I or CF II. Products were purified, fractionated by agarose gel electrophoresis, and visualized by autoradiography of the dried down agarose gel. Locations of form I, II, and III DNA of the input plasmid are marked.

EFFECTS OF ADDITIONAL HB CELL EXTRACT OR
PHOSPHOCELLULOSE FRACTIONS CF I OR CF II ON THE
POLYOMA IN VITRO DNA REPLICATION ACTIVITIES
OF JB3-O AND WT CHO HB CELL EXTRACTS

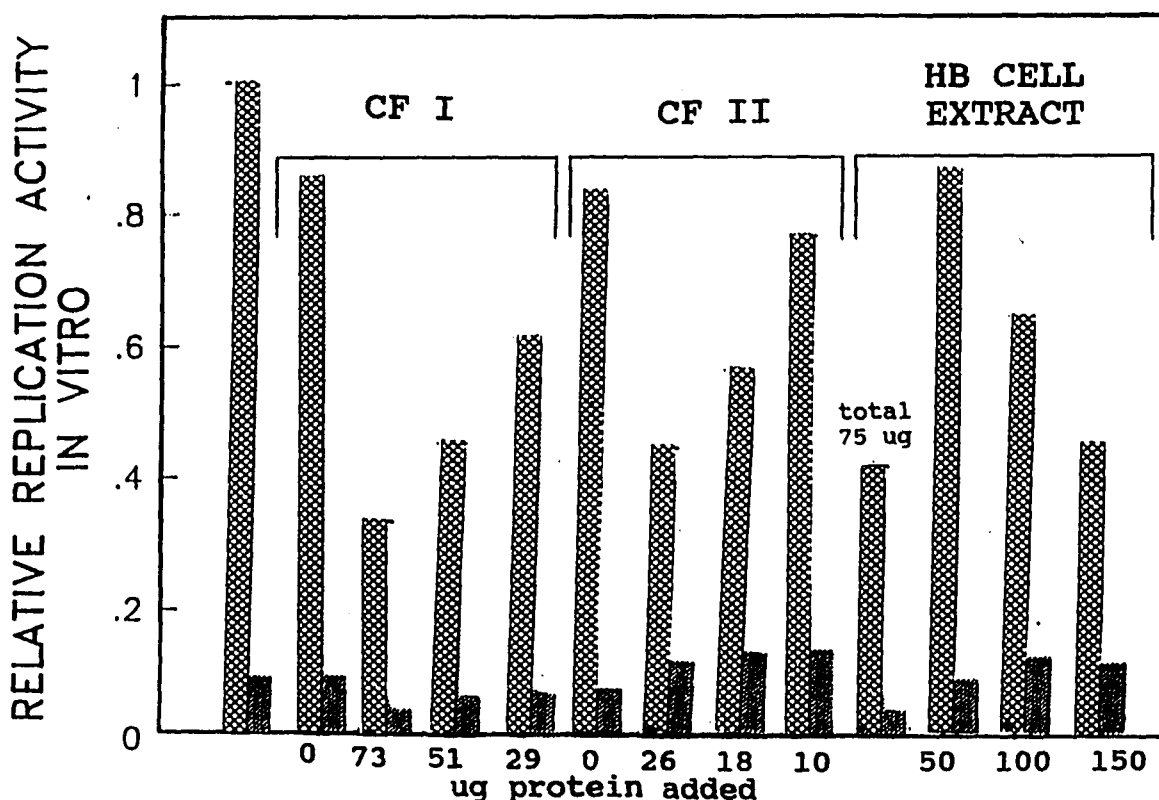


Figure 47. **ABSOLUTE EFFECTS OF INCLUSION OF ADDITIONAL HB CELL EXTRACT OR PHOSPHOCELLULOSE FRACTIONS CF 1 OR CF 2 ON THE POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF JB3-O AND WT CHO HB CELL EXTRACTS.** In vitro reaction mixes containing 100 ug of concentrated HB cell extract prepared from JB3-O or wt CHO were assayed at 30°C for 2 hours. In vitro reactions were supplemented with either additional HB cell extract derived from that respective cell line or phosphocellulose fractions CF 1 or CF 2 as shown above. Polyoma in vitro DNA replication activities of wt CHO (■) or JB3-O (▨) under the conditions shown above. Purified products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in the materials and methods section. 1 unit of relative replication activity in vitro was standardized to represent the replication activity of 100 ug of HB cell extract protein derived from wt CHO cells and is equivalent to the incorporation of 7.6 pmol of dCTP.

POLYOMAVIRUS DNA REPLICATION IN CHO WT, JB3-O
AND JB3-O REVERTANT CELL LINES
JB3-O/R1 AND JB3-O/R2

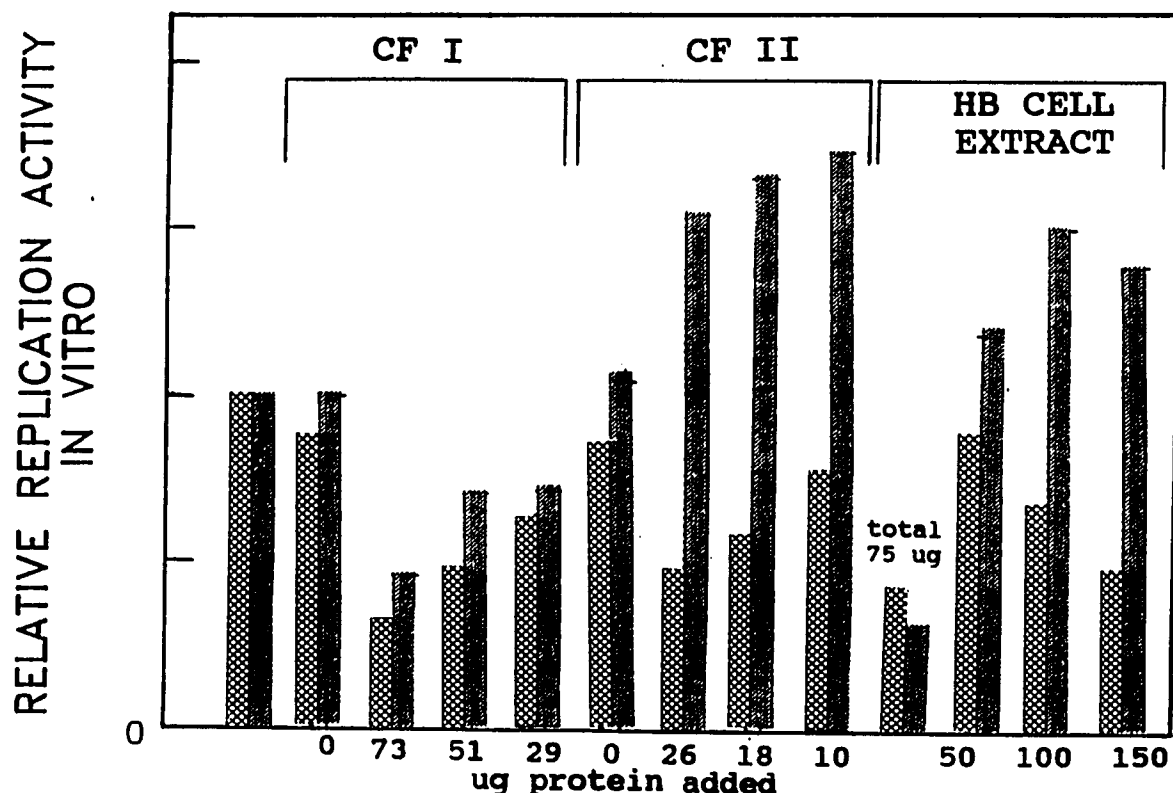


Figure 48. **RELATIVE EFFECTS OF INCLUSION OF ADDITIONAL HB CELL EXTRACT OR PHOSPHOCELLULOSE FRACTIONS CF 1 OR CF 2 ON THE POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF JB3-O AND WT CHO HB CELL EXTRACTS.** In vitro reaction mixes containing 100 ug of concentrated HB cell extract prepared from JB3-O or wt CHO were assayed at 30°C for 2 hours. In vitro reactions were supplemented with either additional HB cell extract derived from that respective cell line or phosphocellulose fractions CF 1 or CF 2 as shown above. Purified products were digested with Dpn I and linearized with Sal I. Incorporation into unit-length products was determined after agarose gel electrophoresis as described in the materials and methods section. 1 unit of relative replication activity in vitro was standardized to represent the replication activity of 100 ug of HB cell extract protein of each cell line. Polyoma in vitro DNA replication activities of wt CHO (▨) or JB3-O (■)

EFFECTS OF ADDITION OF PURIFIED TOPOISOMERASE I
OR TOPOISOMERASE II ON THE POLYOMA IN VITRO DNA
REPLICATION ACTIVITIES OF HB CELL EXTRACTS
PREPARED FROM CHO WT AND THE TS DNA⁻
MUTANT JB3-O CELL LINES

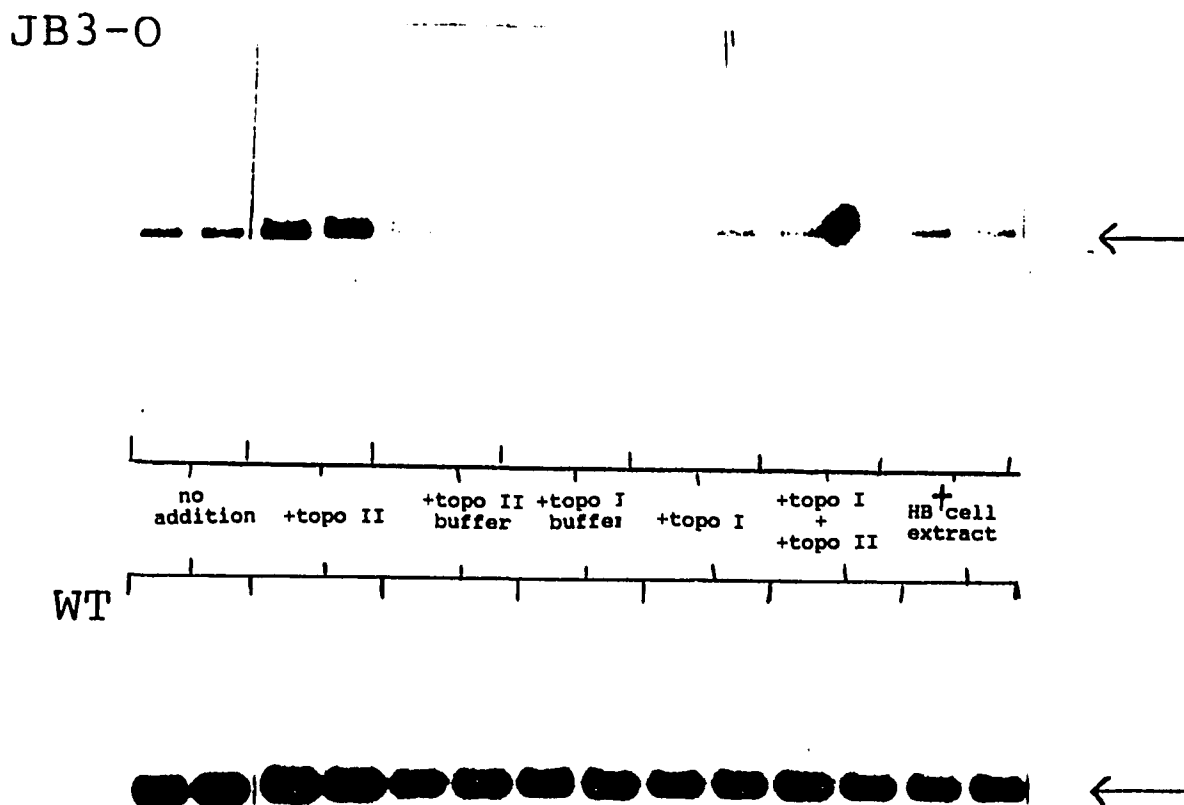


Figure 49. EFFECTS OF ADDITION OF PURIFIED TOPOISOMERASE I OR TOPOISOMERASE II ON THE POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF HB CELL EXTRACTS PREPARED FROM CHO WT AND THE TS DNA⁻ MUTANT JB3-O CELL LINES. 175 ug of concentrated HB cell extract prepared from CHO wt or JB3-O cell lines was assayed at 30°C for 2 hours. Additions are shown above. Purified polyoma in vitro DNA replication products were digested with Sal I and Dpn I and were fractionated by agarose gel electrophoresis and visualized by autoradiography of the dried down gel as described in Materials and Methods. Migration of form III DNA is shown (←).

**EFFECTS OF ADDITION OF PURIFIED TOPOISOMERASE I
OR TOPOISOMERASE II ON THE POLYOMA IN VITRO DNA
REPLICATION ACTIVITIES OF HB CELL EXTRACTS
PREPARED FROM CHO WT AND THE ts
DNA⁻ MUTANT JB3-O CELL LINES**

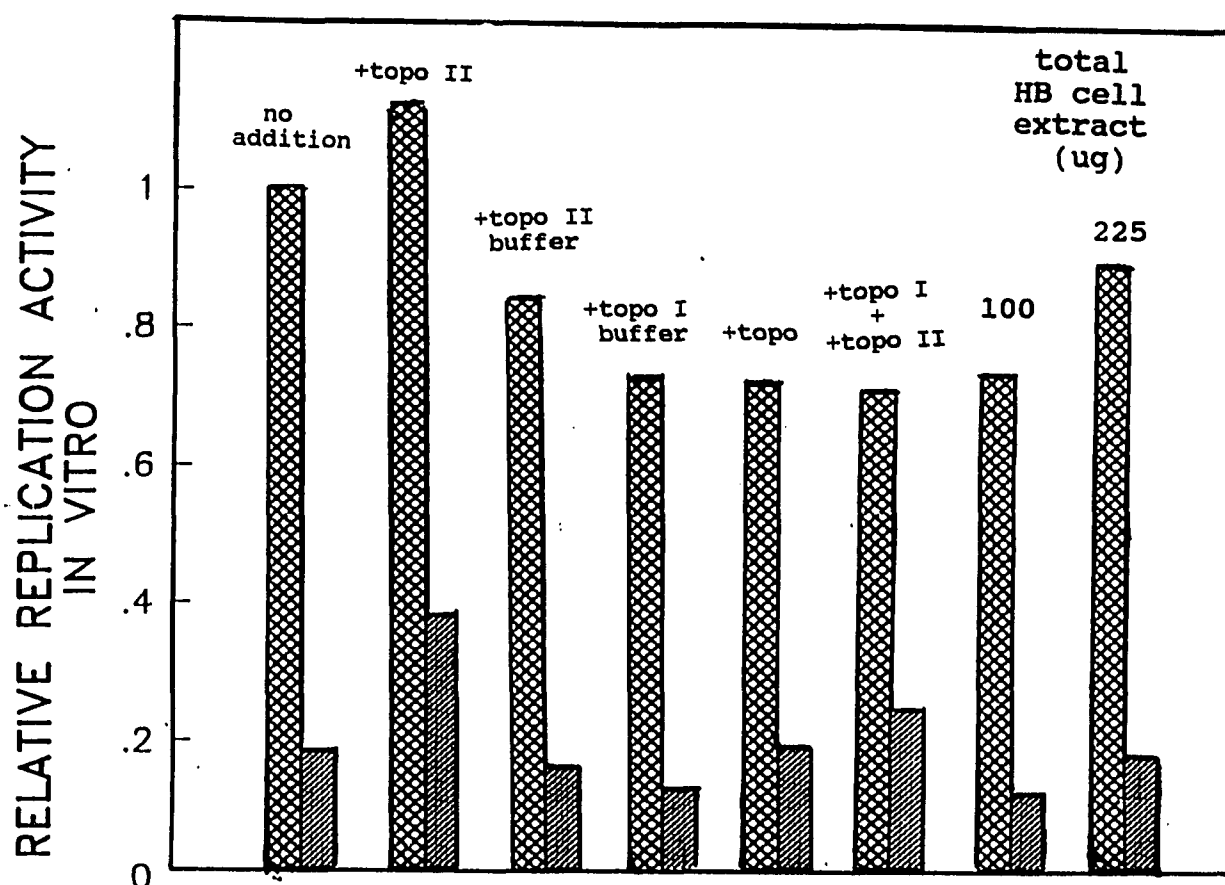


Figure 50. EFFECTS OF ADDITION OF PURIFIED TOPOISOMERASE I OR TOPOISOMERASE II ON THE POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF HB CELL EXTRACTS PREPARED FROM CHO WT AND THE TS DNA⁻ MUTANT JB3-O CELL LINES. 175 ug of concentrated HB cell extract prepared from CHO wt or JB3-O cell lines was assayed at 30°C for 2 hours. Additions are shown above. Purified products were digested with Dpn I and linearized with Sal I. Incorporation into unit length products was determined after agarose gel electrophoresis as described in Materials and Methods. Relative replication activity in vitro was standardized to the replication activity of wt CHO cell extract assayed without any additions. Relative replication activity in vitro of wt CHO (▨) and JB3-O (▧).

**EFFECTS OF ADDITION OF PURIFIED TOPOISOMERASE I
OR TOPOISOMERASE II ON THE POLYOMA IN VITRO DNA
REPLICATION ACTIVITIES OF HB CELL EXTRACTS
PREPARED FROM CHO WT AND THE TS DNA⁻
MUTANT JB3-O CELL LINES**

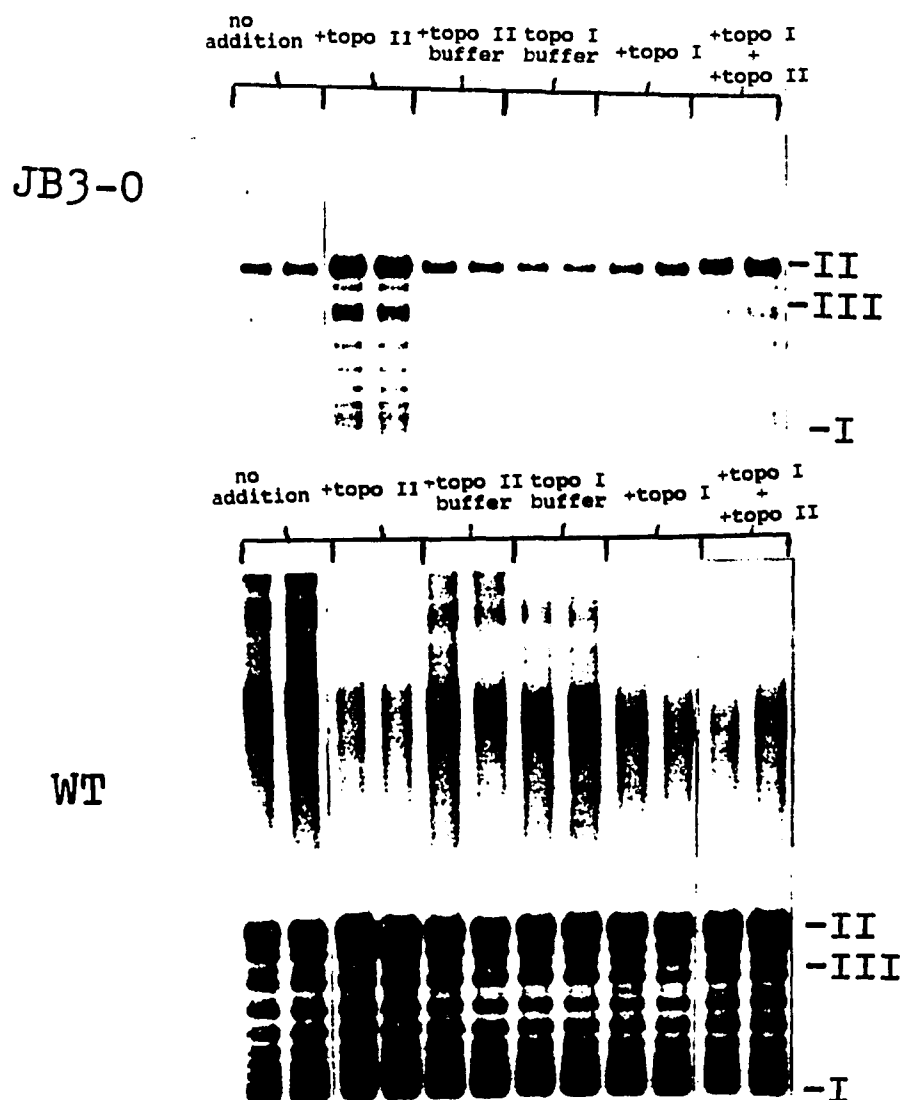


Figure 51. EFFECTS OF ADDITION OF PURIFIED TOPOISOMERASE I OR TOPOISOMERASE II ON THE POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF HB CELL EXTRACTS PREPARED FROM CHO WT AND THE TS DNA⁻ MUTANT JB3-O CELL LINES. 175 ug of concentrated HB cell extract prepared from CHO wt or JB3-O cell lines was assayed at 30°C for 2 hours. Additions are shown above. Purified polyoma in vitro DNA replication products were fractionated by agarose gel electrophoresis and visualized by autoradiography of the dried down gel as described in Material and Methods. Migration of plasmid form I, II and III DNAs is shown.

**TABLE 1 - EFFECTS OF ASSAY TEMPERATURE ON THE POLYOMA
IN VITRO DNA REPLICATION ACTIVITY OF WT CHO CELL EXTRACTS**

ASSAY TEMPERATURE (°C)	PERCENT RELATIVE IN VITRO REPLICATION ACTIVITY AT X °C ^b	
	INCUBATION PERIOD	
	1 HOUR	2 HOURS
30	100%	100%
33	72%	69%
34	61%	53%
36	37%	19%
37	13%	8%

$$\frac{\text{replication activity at X } ^\circ\text{C}}{\text{replication activity at } 30^\circ\text{C}} \times 100 = \text{PERCENT RELATIVE IN VITRO REPLICATION ACTIVITY AT X } ^\circ\text{C}$$

a- In vitro reaction mixtures containing 100 ug of CHO wt cell extract prepared from dense cell cultures was assayed at the temperatures shown above for 1 or 2 hours. Incorporation into unit length Dpn I resistant products was determined after agarose gel electrophoresis as described in Materials and Methods.

TABLE 2 - EFFECTS OF CELL EXTRACT PROTEIN CONCENTRATION
ASSAYED ON THE TEMPERATURE-SENSITIVE PHENOTYPE OF POLYOMA IN
VITRO DNA REPLICATION ACTIVITY OF WT CHO HB CELL EXTRACT

EXTRACT PROTEIN CONCENTRATION (ug / assay)	PERCENT RELATIVE <u>IN VITRO</u> REPLICATION ACTIVITY AT 37°C ^b	
	INCUBATION PERIOD	
	1 HOUR	2 HOURS
142	28%	7.4%
61	28%	7.0%

$$\frac{\text{replication activity at } 37^{\circ}\text{C}}{\text{replication activity at } 30^{\circ}\text{C}} \times 100 = \text{PERCENT RELATIVE } \underline{\text{IN VITRO}} \text{ REPLICATION ACTIVITY AT } 37^{\circ}\text{C}$$

a- In vitro reaction mixtures containing either 142 or 61 ug of CHO wt cell extract prepared from dense cell cultures was assayed at 30°C or 37°C for 1 or 2 hours. Incorporation into unit length Dpn I resistant products was determined after agarose gel electrophoresis as described in the materials and methods section

TABLE 3 - EFFECTS OF PEG-8000 ON POLYOMA IN VITRO
DNA REPLICATION ACTIVITY OF WT CHO CELL EXTRACTS
ASSAYED AT 30°C OR 37°C FOR 2 HOURS^a

% PEG-8000 (W/V)	FOLD STIMULATION OVER ASSAY PERFORMED WITHOUT PEG-8000	
	<u>IN VITRO</u> REPLICATION ACTIVITY AT 30°C	<u>IN VITRO</u> REPLICATION ACTIVITY AT 37°C
2	1.7	5.6
5	3.2	15.7
8	1.8	18.6

a- 61 ug of wt CHO cell extract prepared from dense cell cultures was assayed at 30°C or 37°C for 2 hours in the presence of 0%, 2%, 5%, or 8% PEG-8000. Incorporation into unit length Dpn I resistant products was determined after agarose gel electrophoresis as described in the materials and methods section.

TABLE 4 - EFFECTS OF ASSAY TEMPERATURE ON POLYOMA IN VITRO
DNA REPLICATION ACTIVITIES OF CHO WT AND
ts DNA⁻ MUTANT CELL EXTRACTS^a

CELL EXTRACT	PERCENT RELATIVE <u>IN VITRO</u> DNA REPLICATION ACTIVITY AT 37°C AFTER INCUBATION OF ^b	
	1 HOUR	2 HOURS
WT	60%	31%
JB7-K	64%	27%
JB3-O	50%	29%
JB11-J	50%	38%

$$\frac{\text{replication activity at } 37^{\circ}\text{C}}{\text{replication activity at } 30^{\circ}\text{C}} \times 100 = \frac{\text{bPERCENT RELATIVE } \underline{\text{IN VITRO}} \text{ REPLICATION ACTIVITY AT } 37^{\circ}\text{C}}$$

^a- Extracts prepared from dense cultures of wt and ts DNA⁻ mutant cells were assayed at 30°C or 37°C for 1 or 2 hours. 55 ug of extract protein was assayed in the presence of 5% PEG-8000. Incorporation into unit length DpnI resistant products was determined after agarose gel electrophoresis as described in the materials and methods section

TABLE 5 - CELL CYCLE DISTRIBUTION OF WT AND
MUTANT CHO CELLS AT TIME OF EXTRACTION ^a

CELL LINE	G1	S	G2 + M
WT	50	34	16
JB3-O	52	34	14
JB7-K	51	36	13
JB11-J	51	37	11

^a- percentage of cells in each phase of the cell cycle

TABLE 6 - PLATING EFFICIENCY OF JB3-O REVERTANT CELL
LINES JB3-O/R1 AND JB3-O/R2 AT 39.5 C

CELL LINE	TEMPERATURE OF MAINTENANCE	PERCENT PLATING EFFICIENCY AT 39.5 C ^a
JB3-O/R1	33°C	80
	39.5°C	145
JB3-O/R2	33°C	46
	39.5°C	78

^a-
$$\frac{\text{plating efficiency at } 39.5^\circ\text{C}}{\text{plating efficiency at } 33^\circ\text{C}} \times 100 = \frac{\text{PERCENT RELATIVE PLATING EFFICIENCY AT } 39.5^\circ\text{C}}{\text{PERCENT RELATIVE PLATING EFFICIENCY AT } 33^\circ\text{C}}$$

TABLE 7 - GENERATION TIMES OF CHO WT AND ts DNA⁻
MUTANT CELL LINES

CELL LINE	APPROXIMATE GENERATION TIME (HOURS)
WT	17
JB3-O	25
JB11-J	26
JB7-K	26
JB3-B	32

APPENDIX

I. PARTIAL CHARACTERIZATION OF A MULTIENTZYME COMPLEX REQUIRED FOR POLYOMA DNA SYNTHESIS IN VITRO

In collaboration with Linda Malkas, Robert Hickey, and Yan Wu we have started to characterize a sedimentable complex required for polyomavirus DNA replication in vitro. Our collaborators have performed the cellular fractionation procedures and biochemical characterizations of various fractions as described below. Our laboratory has been performing analyses of the polyoma in vitro DNA replication activities of various fractions.

In order to determine whether mouse replication proteins associate into a readily sedimentable multienzyme complex, as had been previously isolated from human cells (24,81), FM3A cells were subjected to the fractionation scheme shown and described in Figure 1A.

1. MOUSE CELL DNA POLYMERASE AND DNA PRIMASE CO-FRACTIONATE IN A READILY SEDIMENTABLE FORM

S-4 and P-4 fractions were prepared by our collaborators as described in Figure 1A and assayed for DNA polymerase and DNA primase activities. The results obtained by our collaborators are presented in Table 1A. Greater than 75% of the total DNA polymerase activity and greater than 90% of the

DNA primase activity were found in the P-4 fraction. Therefore, the majority of the DNA polymerase and DNA primase activities contained within the NE/S-3 were observed to partition with the sedimentable P-4 fraction. This pattern of partitioning for DNA polymerase and DNA primase activities has been previously observed during the isolation of a 21S Hail cell DNA replication complex which is competent for SV40 DNA replication in vitro (24,81), suggesting that an analogous DNA replication complex may also be present in mouse cells.

2. THE SEDIMENTATION BEHAVIOR OF FM3A MOUSE CELL DNA POLYMERASE AND DNA PRIMASE IS INDEPENDENT OF ASSOCIATIONS WITH OTHER CELLULAR COMPONENTS

In order to determine whether the co-sedimentation of mouse cell DNA polymerase and DNA primase was the result of non-specific aggregation with a cellular component(s) such as membrane, RNA or DNA, our collaborators made attempts to disrupt such aggregates by treating the NE/S-3 with a number of reagents before attempting to prepare P-4 fractions. The results are presented in Table 2A. Treatment of the NE/S-3 with 2M KCl, RNase, DNase, 0.5% NP₄₀, or 2.5% n-butanol/containing 5% triton X-100 did not significantly effect the sedimentation profiles of DNA polymerase and DNA primase to the P-4 fraction. These results suggest that the co-sedimentation of FM3A DNA polymerase and DNA primase is

independent of non-specific aggregation with other cellular components.

**3. DNA POLYMERASE ACTIVITY PRESENT IN THE P-4 AND S-4
EXHIBIT DIFFERENT SEDIMENTATION BEHAVIOR ON SUCROSE
GRADIENTS**

S-4 and P-4 fractions were subjected to zonal sedimentation analysis in order to determine the sedimentation behavior of DNA polymerase activity that was present in each fraction. S-4 and P-4 fractions were subjected to sucrose gradient analysis as described in Figure 2A. Fractions were collected from each tube and assayed for DNA polymerase activity. The results are shown in Figure 2A. The DNA polymerase activity in the S-4 fraction sediments predominantly with a sedimentation value of 7S, whereas the majority of the DNA polymerase activity present in the P-4 fraction sediments with a sedimentation value of 17S. These data suggest that the DNA polymerase activity present in the P-4 fraction is organized into a larger multi-protein complex than the DNA polymerase present in the S-4.

**4. ONLY THE SEDIMENTABLE P-4 FRACTION POSSESSES POLYOMA IN
VITRO DNA REPLICATION ACTIVITY**

Since the majority of mouse cell DNA polymerase and DNA primase co-sediment with the P-4 fraction, it became of

interest to determine whether other enzymatic activities, which are required for DNA replication, also co-sediment to the P-4 fraction. Polyomavirus requires only one viral-encoded protein, PyLT, for replication in a permissive host cell. All other factors required for polyomavirus DNA replication are supplied by the host cell. Therefore, an in vitro polyomavirus DNA replication assay affords one the capability of looking for a number of host cell factors required for polyomavirus DNA replication in vitro. When S-4 and P-4 fractions are assayed for polyoma in vitro DNA replication activity, the ability to support PyLT dependent polyoma in vitro DNA replication was found exclusively with the P-4 fraction (see Figure 3A). Data suggest that not only do DNA polymerase and DNA primase co-sediment to the P-4 fraction, but all of the functions required for polyoma in vitro DNA synthesis are also associated with the sedimentable P-4 fraction. Data suggest that the enzymes required for polyoma in vitro DNA replication exist in a sedimentable complex.

II. PURIFICATION OF POLYOMA LARGE T ANTIGEN (PyLT)

1. VECTOR SYSTEMS TO PRODUCE ADEQUATE AMOUNTS OF PyLT

One of the major problems in adapting a polyomavirus in vitro DNA replication assay had been obtaining an appropriate vector system to produce adequate amounts of PyLT for

purification. At the time of the initiation of this project, the only vector system that was available was a helper-dependent adenovirus vector Ad-LTSVR587 (102). Selection to maintain the recombinant which over-expresses PyLT is due to the presence of sequences coding for SV40 LT. SV40 LT provides a helper-function activity that allows human adenoviruses to grow in otherwise nonpermissive monkey cells. As described in Materials and Methods, 3 ml of virus preparation was used to infect a tissue culture vessel which finally yielded approximately 30 ml of virus preparation (ten fold more virus). This ten fold increase in pool size during a round of scale-up is dramatically lower than scale up which can be obtained using other helper-independent adenovirus systems. A helper-independent adenovirus which overexpresses PyLT has been described (111), although no reports of its use have appeared in the literature to the best of my knowledge. Recently, recombinant baculoviruses which overexpress PyLT have been described (102,103). These baculovirus expression vectors have made the preparation of cell extracts containing significant amounts of PyLT for immunoaffinity purification less tedious.

2. IMMUNOAFFINITY PURIFICATION OF PyLT

Immunoaffinity columns are easily prepared when antibodies which have a high affinity for protein A are to be immobilized. Typically, antibodies are purified by protein

A-sepharose chromatography. The purified antibodies are then once again bound to protein A and then crosslinked to the protein A-sepharose in situ (104). The mouse monoclonal antibody, F4 (96), which we and others were using to detect PyLT in western blots was chosen to prepare an immunoaffinity matrix. This antibody binds to protein A under appropriate buffer conditions (MAPS II binding buffer, Bio-Rad) but is not able to be cross-linked in situ because the antibody dissociates from protein A in cross-linking conditions. The development of the polyoma in vitro DNA replication assay in Hurwitz's laboratory by Prives, Murakami, and co-workers (88) using PyLT purified from an immunoaffinity column prepared by cross-linking crude hybridoma supernatants, from cells grown in the presence of serum, to a goat-anti-mouse immunoaffinity column by standard cross-linking methodologies prompted me to perform the experiment described in Figure 4A and section IV.A.2 of Materials and Methods.

The western blot results of the eluted column fractions containing the peak of PyLT from each column is shown in Figure 4A. The immunoaffinity column prepared by flowing through crude hybridoma supernatant yielded significantly more degraded PyLT than the column prepared by cross-linking purified F4 antibody to the goat-anti-mouse IgG Sepharose matrix. Because of these results we decided to grow the hybridoma cells producing F4 in HL-1 serum-free medium (Ventrex) and purify F4 before preparing an F4 immunoaffinity column for large-scale PyLT purifications. Approximately 0.5

mg of protein A purified F4 was cross-linked per ml of goat-anti-mouse matrix. The amount of F4 cross-linked by flowing the crude serum containing supernatant through the goat-anti-mouse matrix was not determined.

Subsequently, protein A purified F4 monoclonal antibody was cross-linked to Affi-Gel HZ immunoaffinity matrix (Bio-Rad) as described in Materials and Methods section IV.A.2.. Cross-linking efficiencies averaged approximately 60% and yielded matrices which contained an average of 2.5 mg of purified F4 per ml of matrix. This method yielded approximately 5-fold more F4 antibody cross-linked per ml of matrix than did cross-linking purified F4 to a goat-anti-mouse matrix (approximately 0.5 mg of purified F4 / ml of matrix). The net yields of purified PyLT from either extracts prepared from roughly 75 150mm dishes of 293 cells infected with the helper-dependent adenovirus vector or extracts prepared from 30 150mm dishes of Sf27 cells infected with the recombinant baculovirus vector were comparable. Protein concentrations of purified PyLT were approximately 0.1 mg/ml and were typically used at 4 ul per 25 ul in vitro reaction.

SUBCELLULAR FRACTIONATION SCHEME FOR THE ISOLATION OF MOUSE P-4 FRACTION

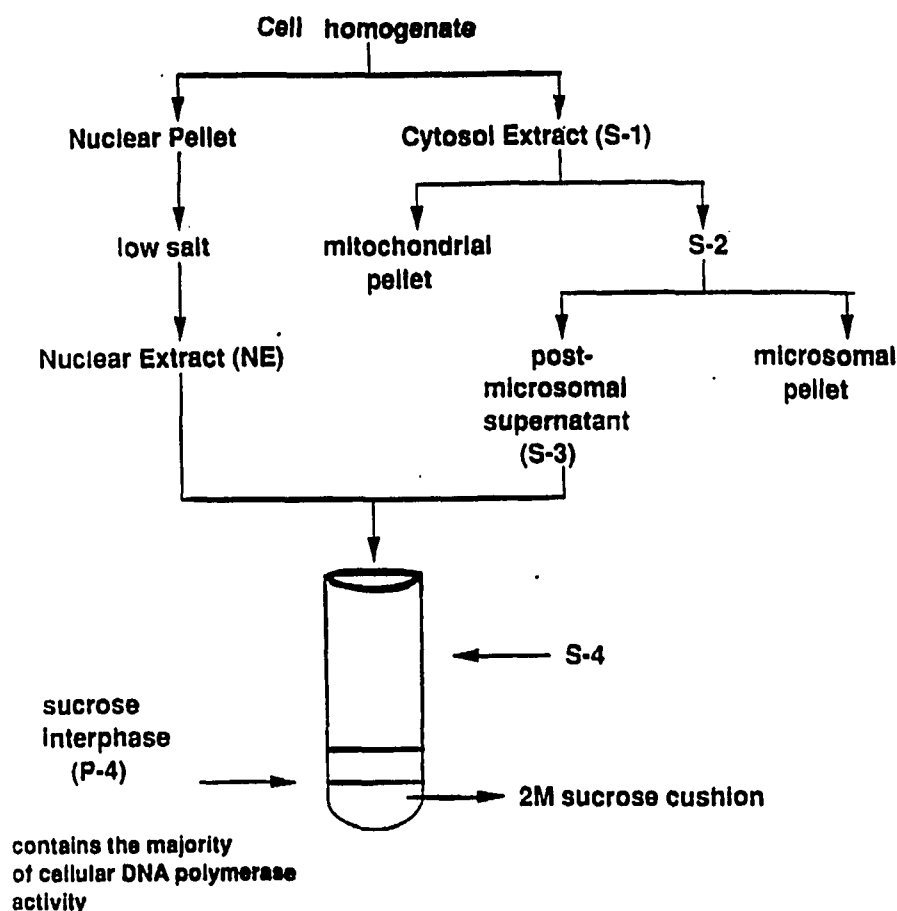


Figure 1A. SUBCELLULAR FRACTIONATION SCHEME FOR THE ISOLATION OF MOUSE P-4 FRACTION. Mouse FM3A cells were grown in suspension in Joklik's modified Eagle's medium supplemented with 5% irradiated calf serum and 5% fetal calf serum. Exponentially growing suspension cultures were harvested by low speed centrifugation and the cell pellets were frozen at -80°C . Our collaborators prepared S-4 and P-4 fractions as follows. Cell pellets were thawed and resuspended in 3 volumes of buffer containing 50 mM TRIS-HCl, pH 7.5, 0.25 M sucrose, 5 mM MgCl_2 , 0.1 mM phenylmethyl

sulfonyl fluoride (PMSF) from a stock prepared in isopropanol, 0.1 mM aminoacetonitrile hemisulfate (AAN), pH 7.5, and 1 mM DTT. After resuspension of the cell pellet, the cells were homogenized by 30 strokes of a loose-fitting dounce homogenizer. The homogenate was then centrifuged at 2000 RPM for 10 minutes at 4°C. The crude cytosolic supernatant (S-1) and crude nuclear pellet were collected separately. The S-1 was centrifuged at 15,000 RPM for 15 minutes at 4°C to remove the majority of the mitochondria and the resultant supernatant was designated the S-2. The S-2 was then centrifuged at 100,000g for 1 hour to remove microsomes. The post-microsomal supernatant was designated the S-3 fraction. The crude nuclear pellet, obtained from the 2,000 RPM centrifugation step immediately after dounce homogenization, was resuspended in 2 volumes of buffer containing 50mM TRIS-HCl, pH 7.5, 150mM KCl, 5 mM EGTA, 5 mM EDTA, 0.1 mM each PMSF and AAN, and 1 mM DTT. After gently stirring for 2 hours at 4°C, the suspension was centrifuged at 15,000 RPM for 15 minutes. The supernatant was collected and was designated the nuclear extract (NE). The S-3 and NE fractions were pooled, layered onto a 2M sucrose cushion and subjected to centrifugation at 100,000g for 16-18 hours. The material above the sucrose interphase was collected and was designated the S-4 fraction. The sucrose interphase fraction was collected and was designated the P-4 fraction. Both the S-4 and P-4 fractions were dialyzed extensively against HB buffer before being assayed for polyoma in vitro DNA replication activity.

ZONAL SEDIMENTATION ANALYSIS OF DNA POLYMERASE ACTIVITIES OF S-4 AND P-4 FRACTIONS

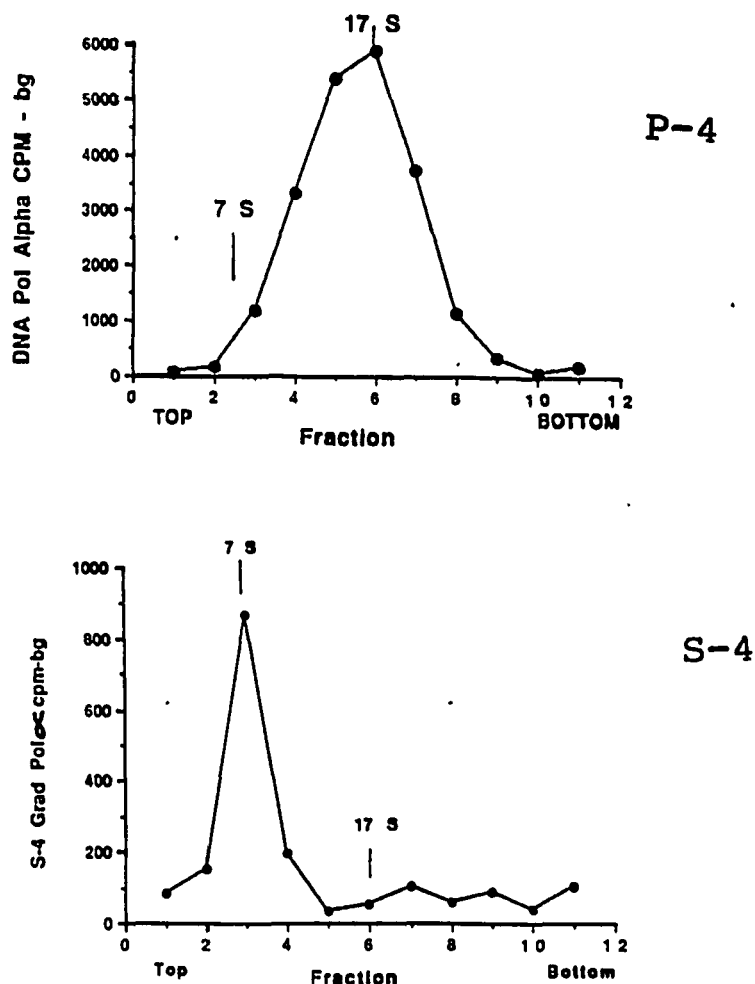


FIGURE 2A. ZONAL SEDIMENTATION ANALYSIS OF DNA POLYMERASE ACTIVITIES IN S-4 AND P-4 FRACTIONS. 1 ml fractions of P-4 or S-4 were loaded onto 9 ml 10-35% sucrose gradients in 50 mM TRIS-HCl pH 7.5, 0.5 M KCl, 1 mM EDTA, which were formed over a 1.0 ml cushion of 2 M sucrose in polyallomer tubes. Tubes were centrifuged at 100,000g for 16 hours at 4°C. After centrifugation, the gradients were collected by puncturing the bottom of the centrifuge tube and collecting the gradient dripping from the tube. Sedimentation of marked proteins (horse spleen apoferritin, 17S; and yeast alcohol dehydrogenase, 7S) were run on parallel gradients and were used to verify that the 16 hour gradients were isokinetic. Fractions were assayed for DNA polymerase activity as described in Table 1A. Absolute values of DNA polymerase activity are presented.

**POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF
NE/S-3, S-4 AND P-4 FRACTION ISOLATED
FROM FM3A CELLS**

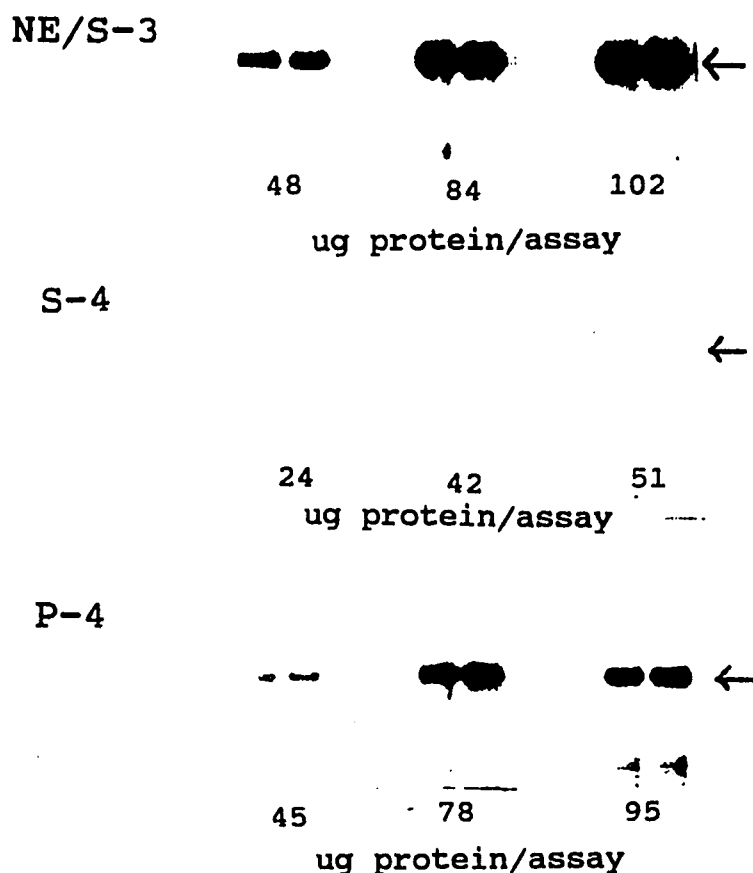


FIGURE 3A. POLYOMA IN VITRO DNA REPLICATION ACTIVITIES OF NE/S-3, S-4 AND P-4 FRACTIONS ISOLATED FROM FM3A CELLS. The indicated amounts of NE/S-3, S-4 or P-4 were incubated at 30°C for 2 hours. Purified polyoma in vitro DNA replication products were digested with Sal I and Dpn I, fractionated by agarose gel electrophoresis and visualized by autoradiography of the dried down gel as described in Materials and Methods. Maximal polyoma in vitro DNA replication activity of NE/S-3 led to the incorporation of approximately 3 pmol of dCTP into unit-length Dpn I resistant products. Maximal polyoma in vitro DNA replication activity of P-4 fraction was approximately 1.5 pmol of dCTP into unit-length Dpn I resistant product. The migration of form III DNA is shown (←).

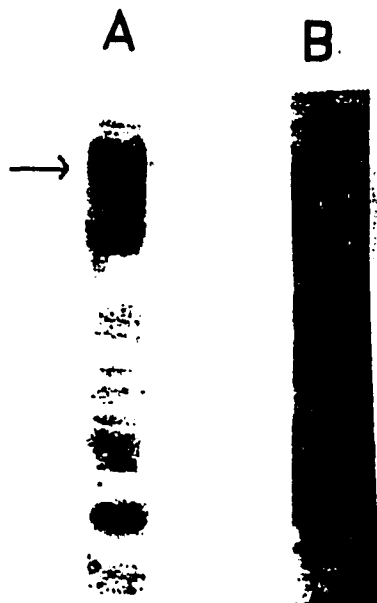


Figure 4A. PyLT PURIFICATION ON F4 IMMUNOAFFINITY COLUMNS PREPARED BY VARIOUS PROTOCOLS. Equivalent aliquots of crude cell extract containing PyLT prepared from CV-1 cells infected with the helper-dependent adenovirus Ad-LTSVR587 was passed over two separately prepared immunoaffinity columns. As described in section IV.A.2. of Materials and Methods, one immunoaffinity matrix was prepared by cross-linking protein A affinity purified F4 monoclonal antibody. The second immunoaffinity column was prepared by cross-linking crude supernatant from F4 hybridoma cells grown in the presence of 20% FCS. After washing and elution of PyLT as described in Material and Methods, the elutions containing PyLT were analyzed by SDS-PAGE followed by western blotting for detection of PyLT. The PyLT eluted from the column prepared by cross-linking protein A affinity purified F4 is shown in panel A. The PyLT eluted from the column prepared by cross-linking crude hybridoma supernatant from F4 hybridoma cells grown in the presence of 20% FCS is shown in panel B.

TABLE 1A - FRACTIONATION OF MOUSE FM3A DNA
POLYMERASE AND DNA PRIMASE ACTIVITIES

FRACTION	TOTAL UNITS ^a	
	DNA POLYMERASE ACTIVITY	DNA PRIMASE ACTIVITY
NE/S-3	52	64
S-4	12	4
P-4	42	56

^a- DNA polymerase activity was measured using activated DNA as a template and was assayed according to published procedures (78). DNA polymerase assays were incubated at 35°C for 1 hour. One unit of DNA polymerase activity equals 1 nmol of total dNMP incorporated into DNA during the one hour incubation. DNA primase activity was measured by determining the incorporation of H-ATP (800-100 cpm/pmol) into acid insoluble material using poly(dT) as template. Primase assays were incubated at 30°C for 1 hour. One unit of DNA primase activity equals 1 nmol of NMP incorporated into acid insoluble material during the one hour incubation.

TABLE 2A -PROPORTION OF DNA POLYMERASE AND DNA PRIMASE^a
IN S-4 AND P-4 FOLLOWING TREATMENT OF
NE/S-3 WITH VARIOUS REAGENTS

REAGENT	% DNA POLYMERASE		% DNA PRIMASE	
	S-4	P-4	S-4	P-4
2 M KCl	16	84	4	96
RNase	20	80	8	92
DNase	18	82	9	91
0.5% NP ₄₀	23	77	3	97
2.5% n-butanol/ 5% triton	20	80	10	90
untreated	16	84	6	95

^a- DNA polymerase and DNA primase assays were performed as described in Table 1A

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