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The analysis of JB3-B, a temperature-sensitive mutant of Chinese hamster ovary cells

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THE ANALYSIS OF JB3-B, A TEMPERATURE-SENSITIVE MUTANT OF CHINESE HAMSTER OVARY CELLS

by

BRIAN E. WOJCIK

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

1988

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

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Abstract

THE ANALYSIS OF JB3-B, A TEMPERATURE-SENSITIVE MUTANT OF CHINESE HAMSTER CELLS

by

BRIAN E. WOJCIK

Adviser: Dr. Harvey L. Ozer

A collection of over 200 Chinese hamster ovary cell mutants which are temperature-sensitive for growth, previously isolated by this laboratory, was analysed. Several of these mutants were identified as being primarily affected in DNA synthesis as contrasted to protein synthesis by using pulse labelling assays to determine the rates of macromolecular synthesis. The feasibilty of using adenovirus and polyomavirus to facilitate their preliminary characterization has been demonstrated. One of these putative ts DNA^- mutants, JB3-B, which fails to support the replication of either of these viruses at 39.5°C was further characterized through the use of adenovirus as simple model system for DNA synthesis. Evidence has been obtained which indicates that the elongation of Ad2 DNA is inhibited by the expression of the mutated function. Further data suggests that a decrease in the level of the nucleotide pools may be responsible. Additionally, genetic analysis of JB3-B indicates that it is a novel ts mutant and that the mutation is recessive to a human gene which corrects the ts

phenotype. An attempt to isolate this human gene has been initiated.

This work is dedicated to my father, Edward A. Wojcik. This endeavor and its successful completion is continually shared with him and represents the fulfillment of a mutual dream.

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And lastly my friends here in the City, for sharing the pain and joy of this doctorate with me, your love and support have kept me going. Being able to share this accomplishment with you gives it added significance and enjoyment.

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INTRODUCTION

DNA replication is a fundamental cell process required for the continuity of living cells and organisms. Although the general principle of DNA replication was suggested in 1953 when Watson and Crick (1953) presented the structure of DNA, work performed since then has revealed that the process is extremely elaborate. This problem has been attacked by many different approaches, using genetic, physiological, physical, biochemical, and molecular biological methods, by a number of laboratories. However, despite this effort, none of the mechanisms that regulate this basic phenomenon have been fully characterized.

Any discussion of DNA replication in a mammalian cell should start with an overview of cell division, since factors which are not directly involved in DNA replication may affect it or its experimental measurement (Sheinin and Humbert 1978, Pardee et al. 1978). In a strict sense, the cell cycle can be defined as the interval between a point in mitosis and the same point of the subsequent mitosis. However, since studies of the regulation of cell proliferation have become important in this area, this definition has been expanded to include all of the intrinsic and extrinsic biochemical aspects that regulate cell growth (i.e. growth factors, hormones, oncogenes, etc.)(Baserga 1981). In prokaryotes the cell cycle is essentially continuous. Cell division is largely regulated by the growth conditions (e.g. nutrient levels, presense of

growth inhibitors, etc.). Some strains of bacteria will undergo sporulation, a process which has been observed to occur in discrete stages, under poor growth conditions. Under favorable growth conditions DNA replication may occur continuously as evidenced by the observation of up to 3 to 4 copies of the genome per cell. This is due to the start of new rounds of replication prior to the completion of previous rounds. The events that occur during DNA replication in prokaryotes have been characterized in greater detail than those that occur in eukaryotes, although the mechanisms which govern them remain largely unknown. Since I will be focusing on eukaryotic DNA replication in this discussion, I will not mention any of this work except as it relates to the study of eukaryotic DNA replication (for reviews of prokaryotic DNA replication see Kornberg 1980, Marians 1985 and references therein).

In eukaryotes it has been possible to divide the cell cycle into discrete phases, even though it consists of a continuous array of processes. These stages are mitosis (M), gap1 (G1), DNA synthesis (S), and gap2 (G2). Mitosis is the stage during which the cell divides into two daughter cells. Mitosis is said to begin when the condensing chromosomes can be first visualized in the light microscope and ends when cytokinesis is complete. It is a well studied cellular process and has been shown to consist of a discreet series of events (for a review see Pardee et al. 1978). G1 is defined as the period between mitosis and the start of DNA synthesis.

Protein and RNA synthesis occur during this period. No nuclear DNA synthesis has been observed during G1, although replication of mitochondrial DNA occurs thoughout the cell cycle. The greatest growth in size of the cell usually occurs during this phase of the cell cycle. G1 exhibits a high variability in its length, while the lengths of the other phases are reasonably constant. The length of G1 is largely responsible for influencing the rate of cell proliferation. Thus, slowly growing populations of cells have longer G1 periods than more rapidly growing cells; some rapidly proliferating cell lines do not possess a G1 period (Prescott 1982). Progression of a cell through G1 is dramatically affected by environmental conditions (i.e. hormones, growth factors, etc.). Starvation conditions for cells in culture will result in those cells arresting in G1 and entering a quiescent state. Terminally differentiated mammalian cells also arrest in this state, which has come to be known as G0 (Marcus et al. 1985). The existance of G0 as a stage in the cell cycle is a controversial topic. Some investigators view the quiescent state as a lengthening of G1 rather than a separate stage (Rubin and Steiner 1975, Dell'orco et al. 1975). Others hold that quiescent cells leave the cell cycle and enter a distinct state - G0 (Prescott 1976). There is evidence to support both views. A third view, which reconciles the above two views, is the probabilistic cell cycle model (Smith and Martin 1973). This view proposes that cells which have just completed mitosis aren't directed

toward S phase; rather, they reach a point at which a decision is made to progress through G1 to the next stage of the cell cycle or to stop. Pardee et al. (Pardee 1974) have referred to this point as the restriction point. The probability of this transition is determined by cell type and by environmental conditions. Some of the conditions which have been determined to shift cells into G0 include high cell density, serum limitation, limitation of certain amino acids (e.g isoleucine) or other nutrients such as phosphate, glucose, a deficiency of lipids or biotin, and the addition of certain drugs (e.g. caffeine) (reviewed in Pardee et al. 1978). The presence of hormones or growth factors can shift cells into completing G1. Transformed cells are considered to have defective restriction point control or to have lost it altogether, thus they fail to enter G0 (Martin and Stein 1976). When quiescent cells enter the cell cycle, a set of metabolically unrelated biochemical reactions occur at varying times before the start of S phase. This phenomenon is known as the "pleiotypic response" (Hershko et al. 1971). However, it remains unknown whether these events are necessary or sufficient for entry into S phase; although it is known that RNA and protein synthesis are required (Sheinin and Humbert 1978).

The next stage chronologically is S phase. Since I will be elaborating on this stage in more detail, I will discuss G2 first. G2 is simply defined as the interval between the completion of DNA synthesis and the start of mitosis. It is

shorter than S or G1. Cells prepare for mitosis during this stage. Therefore this phase is marked by extensive chromosome condensation. Factors which disrupt any of these stages - M, G1, G2 - will indirectly affect DNA replication as cells which fail to reach S phase cannot undergo synthesis of new DNA. I would like to emphasize this distinction between DNA replication, which involves the totality of functions which are required for reproduction of a genome, and the biochemistry of DNA synthesis itself.

Initiation of DNA synthesis signals the beginning of S phase. Evidence is accumulating that DNA replication starts at specific origins of replication. In yeast, DNA sequences which are capable of autonomous replication in plasmids have been isolated (Struhl et al. 1979, Beach et al. 1980). These specific sequences, known as ARS (for autonomously replicating sequence), are found as individual sequences present 400 to 500 times in the yeast genome. This corresponds well with data from electron microscopic studies which suggest that there are approximately 400 replication origins per haploid yeast genome (Saffer and Miller 1986). ARS sequences, which have been localized to sequences as short as 57bp (Kearsey 1983), possess different primary sequences. However, a comparison of several ARS has demonstrated an 11 base pair consensus sequence (Broach et al. 1983).

In higher eukaryotes, researchers have been studying amplified regions of chromosomal DNA in order to isolate a

specific replication origin whose activation is believed to be responsible for the amplification of that region. Botchan and Dayton (1982) have reported that replication of the purified sea urchin ribosomal gene repeat starts in a specific region of the non-transcribed spacer, about 2kb from the 3' end of the 26S gene. Heintz et al. (1983) have performed *in vivo* labelling studies with a methotrexate resistant CHO cell line (CHOC 400) which contains a 500-fold amplification of a 135 Kb chromosomal DNA region. They report that DNA synthesis initiates in a 6 Kb Eco Rl restriction fragment within this amplified domain. Further studies have shown that a subclone of this 6 Kb fragment has ARS activity in yeast. Other investigators have found evidence of origin sequences in other systems and have developed new assays for the detecting the presence of replication origins (Grummt et al. 1987, James and Leffrak 1986, Spotila and Huberman 1985). The isolation of an eukaryotic origin of replication is considered an essential step in the development of *in vitro* DNA replication systems with which to elucidate the molecular mechanisms of eukaryotic DNA replication (i.e. oriC in E. coli replication, Kornberg et al. 1987).

Recently this work has been extended to the chromosomal level by Johnson and Jelinek (1986), who have shown that a human alu sequence is capable of initiating replication in monkey COS-7 cells. Alu sequences comprise a family of repetitive DNA sequences present at $>3 \times 10^5$ different positions in primate DNAs. A region of alu DNA bears strong

homology to the origin sequences of SV40, polyoma, and BK viral DNA and has been suspected of functioning as a chromosomal origin of replication. Monkey COS-7 cells synthesize T antigen and support replication of DNA bearing the SV40 origin sequence (Gluzman 1981). Ariga (1984) has reported that an alu sequence is capable of replication in an in vitro SV40 replication system.

The temporal sequence of DNA replication during S phase also strongly implies the existence of specific eukaryotic origins of replication. However, a number of other studies argue against the presense of specific DNA sequences solely determining the site and timing of initiation of DNA replication. The two copies of the mammalian female X chromosome are replicated at different times in S phase, despite their probable exact sequence homology (Willard and Latt 1976). Sequences used as origins in the early embryos of insects and amphibians cannot be used in the adult (Edenberg and Huberman 1975). When injected into Xenopus oocytes, plasmid or viral DNAs efficiently initiate DNA synthesis at multiple, apparently random sequences (Harland and Laskey 1980).

Initiation, whether requiring a specific origin or not, is believed to occur when a specific protein binds to a region of DNA (origin) and then interacts with other proteins to set up a replication fork. This simple model of initiation is based on viral models such as the SV40 T ag (which will be discussed later in the Introduction). Studies on prokaryotic

origin-recognizing proteins have shown that after binding occurs, a cascade of protein interactions (involving helicase, topoisomerase, primase, and polymerase activities) is initiated. Studies by deVilliers et al (deVillers et al. 1984) have shown that a transcriptional enhancer is required for Polyoma virus replication. This supports the growing belief that transcription is a component of replication, perhaps through the synthesis of the initial RNA primer.

The eukaryotic genome contains up to 10^{10} base pairs (bp) of DNA, all of which must be efficiently replicated during S phase. For the much smaller genomes of prokaryotes, Jacob, Brenner and Cuzin (1963) have proposed the replicon model for chromosome synthesis. This model states that the genetic element (chromosome or episome) replicates as a single unit, which they defined as the replicon. Since it is separated into different chromosomes, the eukaryotic genome cannot replicate as a single unit. It has even been observed that different portions of the same chromosome are replicated at different times during S phase (Taylor 1960). It was therefore proposed and later confirmed that there are multiple units of replication present in each chromosome (Huberman and Riggs 1968). This replication unit has been defined as a "stretch of chromosomal DNA replicated by two adjacent growing points which share a common origin and move in opposite directions from the origin" (Hand 1978). The rate of fork progression is 0.2 - 1.0 microns per minute. Greater than 10^4 such units have been observed in the genomes of

higher eukaryotes. DNA fiber autoradiography and electron microscopic studies have demonstrated that these units vary in size between 15 to 100 microns (50 - 330 Kb) with a low of 15 Kb in Chinese hamster ovary cell DNA to a high of 400 microns in DNA from human sources. The size of the replication unit has been observed to decrease in cell types with faster rates of DNA synthesis, in SV40 transformed Chinese hamster cells, and in Chinese hamster cells which were held at the G1/S border by a fluorodeoxyuridine block before release into S phase. Since the size of the replicating unit decreased directly upon increasing the duration of the block, it was proposed that the continued synthesis of initiation factors resulted in a greater number of initiation sites being used (reviewed in Edenberg and Huberman 1975, Hand 1978).

It has also been observed that these replication units are organized into clusters whose initiation is coordinated. In studies using fiber autoradiography Hand (1977) has demonstrated that adjacent units show similar grain track patterns at frequencies much higher than that predicted by random initiation. Second, studies employing the incorporation of bromodeoxyuridine have shown that DNA within localized regions of chromosomes is synthesized synchronously (Nakamura 1986). Third, the inhibition of DNA replication by UV or X-radiation inactivates replication of more than unit length DNA stretches (Porvik 1977). Fourth, Planck and Mueller (1977) have shown that simultaneously active

replication units are separated by at most one replication unit. Finally the temporal coordination of replication units has been observed in synchronized cells; the DNA sequences in a cell are not replicated at random times. In studying the increasing length of S phase as cells differentiate, Klevecz et al (1975), using four different assays for DNA synthesis, demonstrated that DNA synthesis occurs in two bursts (early and late) in S phase. They proposed this biphasic pattern of DNA synthesis as the reason for the observed increase in the length of S phase in differentiated cells. These results were recently confirmed and extended by Goldman et al. (1984) who have shown that the early replicating DNA is A-T rich and consists of active euchromatin which is thought to contain the essential "housekeeping genes", and others (Baserga 1976) who have shown that mutagens for certain loci are most effective during the early part of replication. The late replicating DNA was shown to be more G-C rich than the early replicating sequences and to consist primarily of heterochromatin (e.g. the late replicating X chromosome). Genes which are replicated at a particular time (early or late) during S phase tend to be replicated at the same time in subsequent S phases (Edenberg and Huberman 1975). However, Calza et al. (1984) have shown that many sequences are replicated differentially in cells depending on whether the function is highly expressed.

The DNA of eukaryotes also differs from the DNA of prokaryotes in the manner in which it is packaged. Because of

the small size of the nucleus, eukaryotic DNA is highly condensed and must replicate in this condensed form. Eukaryotic DNA is organized into chromatin which in turn is organized into 200 bp of DNA wrapped around a histone octamer. This structure, called a nucleosome, has been reviewed by Kornberg (1977) and Felsenfeld (1978). Studies have shown that histone synthesis correlates with DNA synthesis, although the mechanism of this coupling is not known (Heintz and Roeder 1984). Studies have also shown that the chromatin which is present during S phase can be distinguished from the chromatin which is present at other stages of the cell cycle (Felsenfeld 1978). These results have led to the suggestion that DNA synthesis proceeds as a component of chromatin replication. Hewish (1976) and Rosenberg (1976), noting the similarity in the length of DNA in the nucleosome and the eukaryotic Okazaki fragment (1968), have proposed that the exposed linker DNA (approximately 60 bp of the total 200 links the nucleosomes) acts as the origin for Okazaki fragment primer synthesis. Recent studies by Blow and Laskey (1986) and Blow and Watson (1987) have indicated that the structure of chromatin beyond the level of the nucleosome is involved with DNA synthesis. Their experiments on the control of DNA replication in cell-free Xenopus egg extracts showed that the assembly of a nucleus-like structure is a prerequisite for the initiation of DNA synthesis. Other investigators have produced evidence which indicates that the site of DNA replication is fixed at the nuclear matrix

(Jackson and Cook 1986). Accordingly, the origins of replication are DNA sequences which preferentially associate with the matrix prior to S phase. Once DNA synthesis is initiated, replicating DNA proceeds through these sites on the matrix.

The replication forks of eukaryotes are generally believed to progress in a manner similar to that of prokaryotes. For prokaryotes a multienzyme complex organized around a DNA polymerase has been shown to be the functional component of fork progression. Evidence is accumulating which indicate that this is true for eukaryotes as well. Recent improvements in purification techniques have resulted in the isolation of a complex composed of DNA polymerase alpha, primase, and various other polypeptide subunits from various eukaryotic cells. This complex has been called a "replicase" (Wong et al. 1986). These replicases contain subunits of 180-182 Kd, which possesses the DNA polymerase activity, 70-77 Kd, 55-60 Kd, and 47-50 Kd. The eukaryotic DNA polymerases polymerize nascent DNA only in the 5' to 3' direction (Weissbach 1977), thus suggesting that on one strand of DNA at the replication fork discontinuous DNA synthesis must occur. The 47-50 Kd and 55-60 Kd subunits are believed to possess the primase activity. The eukaryotic primase copies six to fifteen nucleotides from a DNA template into an oligonucleotide in a single burst of activity. In the absence of polymerase alpha subsequent bursts can extend this oligonucleotide. The primase can also incorporate

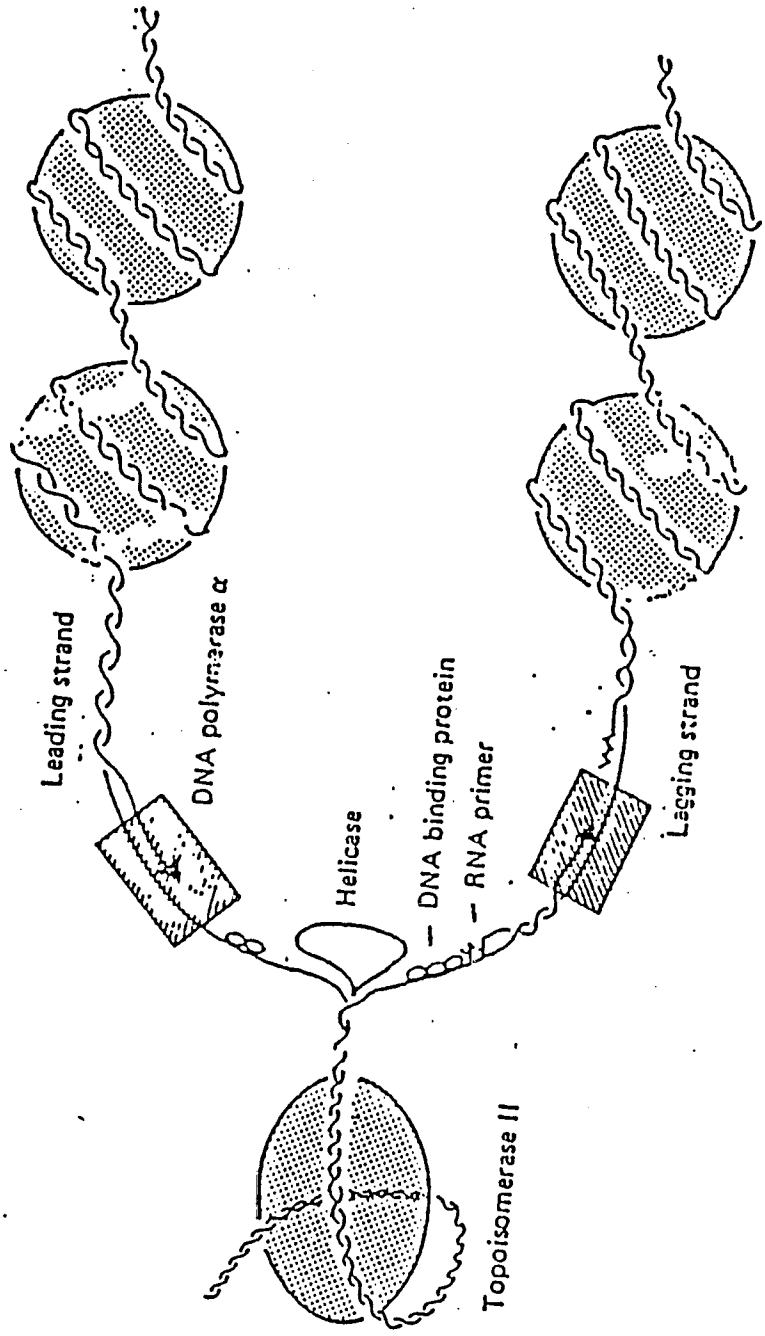
deoxynucleotide triphosphates (dNTP) into this oligonucleotide and may add the first dNTPs before the polymerase alpha takes over. Both of these activities are believed to be regulated by the presence of active polymerase alpha and the levels of available dNTP (Singh et al. 1986). Primase is responsible for priming the synthesis of new Okazaki fragments on the discontinuous strand at replication forks. It may also play a role in the initiation of DNA synthesis, as evidenced by detailed mapping of the sites of RNA primer synthesis and of the RNA to DNA transition at the SV40 origin of replication (Hay et al. 1984) and by the observation that aphidicolin (a strong inhibitor of DNA polymerase alpha but not primase) inhibits the progression of replication forks but not initiation of limited DNA synthesis (Decker et al. 1986).

Other investigators have reported finding additional subunits which possess other activities; such as DNA topoisomerase II (to prevent topological inhibition of the progressing replication fork), DNA helicase (to separate the base-paired strands), primer binding, diadenosine tetraphosphate binding (which may function in initiation), and exonuclease activities (Huberman 1987). Additionally, other activities involved in the replication of DNA have been isolated. Among these are factors C1 and C2 (which are required for the efficient function of DNA polymerase alpha) (Lamothe 1981), 3'-5' exonuclease (which probably maintains the fidelity of replication) (Lee et al. 1984), endonuclease

Figure 1. - Model of a replication fork. This model illustrates some of the proteins which act during the progression of the replication fork. See text for details. From Denhardt and Faust (1985).

Figure 1

Model of a replication fork.



(Wang and Furth 1977), ribonuclease H (Elgin and Weintraub 1975), DNA ligase (Soderhall and Lindahl 1976), single-strand DNA dependent ATPase (Otto 1977), DNA binding protein (Champoux 1978), and topoisomerases (Gellert et al. 1976). An overview of the putative eukaryotic replication fork and the possible roles of some of the activities discussed above is shown in Figure 1. The progression of the replication fork is thought to involve the coordinated separation of the base paired strands by a helicase and helix destabilizing proteins (single strand binding proteins) and through the action of a topoisomerase which permits the unwinding of the duplex. Other events required for the proper synthesis of nascent DNA strands include the excision of the RNA primers, the filling in of the resulting gaps, and the sealing of nicks, possibly by another eukaryotic polymerase. It is notable that although these activities have been isolated and biochemically studied only one has definitively been shown to function in <u>in vivo</u> DNA replication. Johnson et al (123) isolated the DNA polymerase alpha gene from yeast and demonstrated by gene disruption that it is essential for <u>in vivo</u> DNA replication. The complementation of biochemical analyses by genetic studies such as this will permit the <u>in vivo</u> roles of each gene product to be elucidated.

The biosynthesis of precursor dNTPs is closely coordinated with DNA replication. In prokaryotes it has been demonstrated that the dNTPs are channeled to replication sites by multienzyme complexes containing subunits which

function in the dNTP biosynthetic pathways (Mathews 1985). Various studies suggest a similar channeling mechanism in eukaryotes. Kuebbing and Warner (1975) have investigated the kinetics of thymidine incorporation, and Fridland (1973) the kinetics of thymidine and deoxyuridine incorporation. The results of these studies suggested that a specific pool (or compartment), located at or near the replication forks, is responsible for DNA synthesis. Various groups have demonstrated that the flux rates of the enzymes of dNTP synthesis closely reflect the rate of DNA synthesis in vivo (Nicander and Reichard 1985). Reddy and Pardee (1982) have generated data which suggest that nucleotides are reduced in situ by ribonucleotide reductase and then incorporated into DNA by using a labelled ribonucleotide diphosphate (rNDP) mixture to examine the rates of precusor incorporation in permeabilized Chinese hamster fibroblasts. Genetic analysis of the yeast cdc8 mutant has shown that the cdc8 gene codes for dTMP kinase. In vitro analysis of the effect of the molecular defect in this gene shows that it affects the polymerization step of DNA replication even in the presence of all four dNTPs which are exogenously provided (Sclafani and Fangman 1984). This strongly implies the existance of a multienzyme complex consisting of the enzymes responsible for dNTP biosynthesis directly coupled to the DNA synthetic apparatus (i.e. DNA polymerase alpha). Such a complex has been isolated by several independent laboratories (Baril et al. 1973, Reddy and Pardee 1980, Wickremasinghe et al. 1983,

Ayusawa et al. 1983). However the validity of this complex and the existance of channeling in eukaryotes remains a controversial issue as recent evidence has been reported which apparently contradicts the above evidence. Leeds et al. (1985), using a protocol which enables the nuclear and cytoplasmic pools to be measured independently, reported finding no ribonucleotide reductase activity in the nucleus at any time in the cell cycle. This has been confirmed by Engstrom et al. (1984), who localized the ribonucleotide reductase to the rough endoplasmic reticulum with antibodies against the M1 and M2 subunits. More recently, Leeds and Mathews (1987) have demonstrated temporal compartmentalization of dNTP pools. dNTPs are found in the cytoplasm during G1 and appear to move into the nucleus during S phase. Throughout all of these apparently contradictory data one fact stands clear - that the dNTP pools are in some manner compartmentalized. This is also true for the mitochondria which use a separate pool of dNTPs for their DNA replication than for nuclear DNA replication (Bestwick et al. 1982).

dNTP channeling was originally proposed to explain how replication systems could maintain the concentration gradients required to support the observed rates of DNA replication. This implies that the availability of any one of the dNTP pools could regulate the rate of DNA replication. The finding that the dCTP pool most closely follows the course of DNA synthesis has lead to suggestions that the dCTP

pool serves such a regulatory function, but no specific mechanism has been proposed (Bjursell and Reichard 1973). Alternatively, dGTP, the least abundant of the four dNTPs in all cell lines in which levels have been measured, may be responsible for the regulation of the rate of DNA synthesis. Leeds et al (1985) have calculated the average nuclear concentration of dGTP to be close to the reported K_m value of DNA polymerase alpha. It has also been shown that the rate of DNA replication in S49 cells are sensitive to guanine ribonucleotide depletion, leading to the proposal that a replication active pool of dGTP is sensitive to this depletion (Mathews and Slabaugh 1986).

Termination of replication in eukaryotes is not believed to be a result of sequence-specific signals in the DNA which halt the progression of replication forks. Rather, it appears that converging replication forks cancel each other out. However, two phenomena which are the result of the convergence of replication forks must be resolved by the cellular replication machinery; namely, the removal of the positive superhelical twists (which are the result of the unwinding of the parental duplex as well as the rewinding of the progeny DNA about the nucleosomes; Denhardt and Faust 1985), and the resolution of the topological links which exist between the parental strands as a result of replication. Either topoisomerase I or II is sufficient to resolve topologically linked strands during S phase in yeast (Viemura and Yanagida 1986), although type II, but not type

I, is essential for cell survival (Thrash et al. 1984).

It has long been recognized that replication of a linear DNA molecular could result in daughter molecules with protruding 3' ends. This would result in a shortening of the chromosomes over a period of replication cycles. Proposed models to account for this problem have come primarily from studies of viral DNA replication. Indeed most of the proposed mechanisms and observations of DNA replication have been based on viral model systems. Three classes of viruses have proven to be of particular merit in these studies. The parvoviruses and adenoviruses, with their respectively single and double stranded linear genomes, are believed to serve as models of DNA replication initiated at the telomeres of chromosomes. On the other hand, the double stranded circular genomes of the papovaviruses are considered to be models of a more complete (and more complex) system of DNA replication which is initiated at sites within the chromosome.

The parvoviruses are the smallest and least complex of the DNA animal viruses. They consist of two groups - the autonomous (helper-independent) group which contains the minute virus of mice and the defective (helper-dependent) group which contains the adeno-associated viruses. The genomes of both groups consist of linear, single stranded DNA molecules 4.5 - 5.2 Kb in size (Ward 1978). Because of their small genomes, it is generally believed that the parvoviruses are extensively, if not entirely, dependent on the host cell replication machinery. What makes this virus unique is the

presence of palindromic sequences 70 - 145bp long at the termini. These inverted repeats fold back to form hairpin structures which function as primers for continuous strand DNA synthesis. For more detailed explanations of the proposed mechanisms for parvovirus replication see Astell et al (1985, autonomous), Bohenzky et al. (1987, adeno-associated), and Challberg and Kelly (1982).

The Adenoviruses are a well characterized group of DNA tumor viruses. First discovered in 1953 (Rowe et al. 1953), human adenoviruses have been investigated as a model of eukaryotic gene expression and DNA replication since the early seventies. One way in which the adenoviruses differ from the other DNA tumor viruses is that they code for their own histone-like proteins. It was believed initially that this would serve as a model for the study of cellular chromatin. However, this has not been the case since it has proved difficult to study the viral nucleoprotein core. The life cycle of the adenoviruses can be divided into early (events occuring prior to the onset of viral DNA replication) and late (events which occur after the onset of DNA replication) periods. Four viral genes are expressed during the early period (designated E1A,E1B,E2A,E2B,E3,E4). The E1A gene is expressed first and functions in the transcriptional regulation of viral and cellular genes (Pettersson and Roberts 1986). The function of the E1B gene has not been specifically determined, although mutants in this gene display the phenotype of extensive DNA degradation (White et

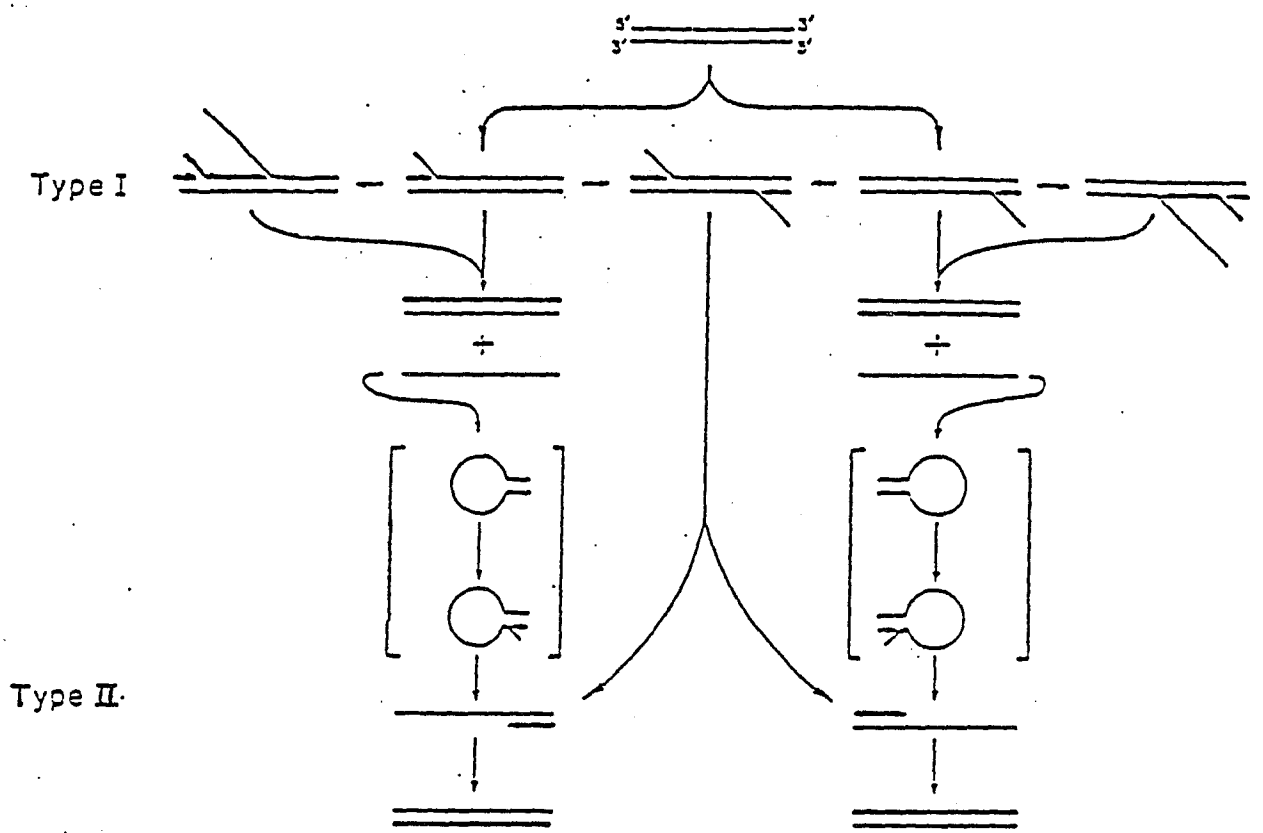
al. 1984). The E2 genes have been shown to code for the viral proteins which are required for Ad DNA synthesis *in vitro* (Stillman 1983). The E3 region can be deleted without any adverse effects on Ad DNA replication in cultured cells and is thought to function in the host range of the virus. Recently the E4 gene has been shown to encode functions which are essential for efficient DNA replication, late gene expression and inhibition of host cell synthesis (Halbert et al. 1985). The late genes predominantly encode the structural proteins of the virus.

The adenovirus genome consists of a linear, double-stranded DNA which is 36 Kb in length. Although it was first thought that these viruses would serve to produce a general model of eukaryotic DNA replication, several unique features of these viruses were later discovered which show that the adenoviruses are better suited as models of the mechanism of DNA synthesis which is initiated at the ends (telomeres) of chromosomes. Viral replicative intermediates were first indentified by pulse-labelling followed by analysis on neutral sucrose gradients (Pearson and Hanawalt 1971). Subsequent pulse-chase studies (Sussenbach et al. 1972) led to the proposal that Ad DNA replication proceeds in a continuous manner by strand displacement. The discovery of inverted terminal repeats (ITR) at the ends of the DNA (Garon et al. 1972) and electron microscopic studies of replicative intermediates (Lechner and Kelly 1977) led to the model which is shown in Figure 2. Briefly, this model states that Ad

Figure 2. - Model for adenovirus DNA replication. "Newly synthesized DNA strands are depicted by bold lines and parental DNA strands are depicted by thin lines. The circular "panhandle" forms enclosed in brackets are theoretical intermediates." From Lechner and Kelly (1977).

Figure 2 .

Model of adenovirus DNA replication.



replication initiates at either end of the genome, using the ITR as the origin, and proceeds by continuous strand displacement which has been designated type I replication. Initiation can occur at both ends simultaneously or at either end. If initiation occurs at only one end, the displaced single strand is replicated by the annealing of the ITR to form a proposed "panhandle" structure followed by continuous synthesis of the nascent strand. This has been designated type II replication. The initiation of Ad DNA synthesis requires a double stranded ITR to act as the origin, the first 18 bp of which are an absolute requirement (minimal origin) (Tamanoi and Stillman 1982). Another interesting feature of adenoviruses is the presence of a 55Kd protein which is bound to the 5' ends of the DNA (Desiderio and Kelly 1981). This terminal protein (TP) is covalently bound to dCMP through a serine residue. In infected cells the TP is synthesized as an 80Kd precursor (pTP) which is subsequently cleaved to the 55Kd form late in infection by a virally encoded endopeptidase. The pTP is associated with a 140Kd protein which has been shown to be the Ad DNA polymerase (Lichy et al. 1982).

Early attempts at obtaining mutants in the early genes which were believed responsible for coding functions involved in viral DNA replication were largely unsuccessful. One exception was the ts125 mutant of Ad5 isolated by Ensinger and Ginsberg (1972) which was subsequently shown to possess a lesion in the gene which codes for the 72Kd DNA binding

protein (DBP) (van der Vliet et al. 1975). The DBP binds cooperatively and tightly to single-stranded DNA. In vitro studies using endogenous templates in isolated nuclei (van der Vliet et al. 1977) as well as in vivo studies (van der Vliet and Sussenbach 1975) have indicated that the DBP is essential for strand elongation and may also function in viral initiation. The gene which encodes the DBP has been mapped to the E2A region of the viral genome (van der Vliet et al. 1977). Another group of mutants which were found to be defective in viral DNA replication comprise the N-group (Galos et al. 1979). These mutants map next to the TP gene in the E2B region of the genome (van der Vliet et al. 1977). Both of these genes as well as the gene for the DBP share the same promotor implying common regulation and involvement in similar viral processes. Later in vitro studies have suggested that the "N" gene encodes the viral DNA polymerase (Ostrove et al. 1983). Other interesting mutants involved in adenoviral DNA replication can now be isolated through the use of in vitro mutagenesis (White et al. 1984, Montell et al. 1982).

Work on the biochemistry and enzymology of Ad DNA replication has proceeded rapidly with the development of in vitro systems which utilize exogenous Ad DNA templates (Stillman 1983, Challberg and Kelly 1979, Guggenheimer et al. 1983). The basis of this system is the preparation of infected cell extracts (HeLa) which actively support the replication of exogenously added Ad DNA. The extracts are

prepared from hydroxyurea treated cells to prevent the accumulation of replicative intermediates. The Ad DNA must have a covalently linked TP for efficient replication as is the case for *in vivo* Ad replication. The *in vitro* reaction requires, in addition to the cell extract and Ad DNA, only the four dNTPs and magnesium ions. However, the addition of ATP, but not GTP,CTP, or UTP, results in a three fold stimulation of the reaction. This *in vitro* reaction has been shown to closely resemble type I *in vivo* viral DNA synthesis in that daugther strand synthesis initiates at either ITR and proceeds 5'-3', replication occurs by strand displacement, the rate of elongation (1200 nucleotides / minute) is comparable to the rate observed *in vivo*, and nascent strands are hydrogen bonded to the template (Challberg and Kelly 1979). Using this system, six proteins have been identified and characterized which are essential components for the complete replication of Ad DNA *in vitro*. Three of these are virally encoded: the DBP which binds single-stranded DNAs, the 80Kd TP precursor, and the Ad DNA polymerase which is associated with the TP precursor. In addition, fractionation of uninfected cell extracts has revealed that three cellular proteins are required for the complete replication of Ad DNA *in vitro*. The first, nuclear factor I (NF I), is a 47Kd protein which binds to a specific sequence on the ITR (nucleotides 19-39) and is required for initiation (Rosenfeld et al. 1987). Deletion of this region of the Ad origin results in a decrease to 10% of the levels of wild-type DNA.

Nuclear factor II (NF II) is not essential for initiation or partial elongation (up to 30%) of the nascent strand. It is, however, required for the completion of Ad DNA replication. A DNA topoisomerase activity co-purifies with NF II. Additionally, purified eukaryotic type I DNA topoisomerases can substitute for NF II in the in vitro assay (Nagata et al. 1983). This is consistant with NF II possessing in part or in total a topoisomerase I activity and demonstrates that a protein involved in higher order DNA structure is required for Ad DNA replication. The third host factor, NF III, is a 92 Kd protein which specifically recognizes and binds to nucleotides 36 to 54 of the Ad origin (O'Neill and Kelly 1987). Ad mutants in which this binding site is deleted replicate to only 30% of the levels of wild-type virus in vitro. Homology searches of the possible binding sequences of NF III and NF I indicate that both of these proteins can bind to several eukaryotic upstream regulatory sequences. This suggests that cellular proteins which which are required for the initiation of DNA synthesis may also function in the regulation of transcription.

All DNA polymerases commence replication from a primer; therefore, initiation of DNA synthesis is the manufacture of the primer. Elongation becomes simply the addition of nucleotides to the nascent strand. The protein-priming model for the initiation of adenovirus DNA replication, first proposed by Rekosh et al. (1977), has been confirmed by subsequent in vitro studies. Initiation starts when the pTP-

Ad polymerase complex together with NF I and other accessory proteins (see below) recognize specific sequences within the ITR and form a priming complex (Tamanoi and Stillman 1982). This reaction is stimulated by the Ad DBP (deVries et al. 1985). The first nucleotide in the nascent strand, dCMP, is covalently linked to a serine residue of the pTP by a phosphodiester bond. This reaction can be duplicated in vitro with NF I, pTP, Ad polymerase, $MgCl_2$, ATP, and dCTP; no other nucleotide will substitute. The dCMP is then used as the primer for subsequent strand elongation. The pTP that remains covalently bound to the 5' end of the replicated strand facilitates the initiation of the next round of DNA synthesis, but it is not essential (Challberg and Kelly 1979). The elongation of the nascent strand proceeds 5' to 3' from the bound dCMP and is absolutely dependent on the the Ad DBP (Ostrove et al. 1983). Ad elongation also requires topoisomerase I activity (NF II) for its completion.

The two papovaviruses, SV40 and Polyoma (Py), were first discovered several decades ago and were of interest initially due to their oncogenic properties. However, because of their relatively small genomes (5Kb) and ease of handling in the laboratory, they have come to be utilized as important models of eukaryotic systems including DNA replication (Gross 1983). Unlike the adenoviruses, these DNA tumor viruses possess covalently closed circular double-stranded genomes. The DNA is wound around cellular histone proteins to form a structure which is similar to the cellular nucleosome. Thus, the

replication of the viral genome can serve as a model for the replication of cellular chromatin. The genomes of these viruses can be divided into early and late regions. The late regions code for the structural proteins of the virus. The early region of the SV40 genome codes for two proteins, large and small T antigens (T ag and t ag, respectively), while the early region of the Py genome codes for three: large, middle, and small T antigens (LT, MT, and ST, respectively). The function of the small T antigens remains unclear. The T ag of SV40 possesses a multiplicity of functions including viral DNA replication and cell transformation (for review see DePamphilis and Wasserman 1982), whereas Py MT encodes the function for cell transformation and LT encodes the function for DNA replication. This was determined in part through the isolation of temperature-sensitive mutants in the genes which code for the large T antigens (Tegtmeyer 1972, Franke and Eckhart 1973). Several domains of the large T antigens are believed to play a role in DNA replication. T antigen specifically binds to sites (sites I and II) within the viral origin of replication. This binding is absolutely required for the initiation of viral DNA replication. In addition, an ATPase activity has also been shown to be a requirement for DNA replication (Clark et al. 1983). Recent evidence has suggested that a third function of SV40 T ag is required for viral DNA synthesis. This function is thought to involve a site for a specific protein-protein interaction (Stillman et al. 1985). Most recently it has been demonstrated that T ag

functions as a helicase (Dean et al. 1987). It is believed that the binding of T ag to the viral origin establishes the replication fork (opens a single-stranded region of DNA) at which the helicase activity continues to unwind the DNA. The ATPase activity of T ag may be used to drive this helicase.

In addition to T ag, both SV40 and Py require another viral factor - the cis-acting origin of replication (Hay et al. 1984). All of the other factors which are required for the replication of these viruses are of cellular origin; included are those that delineate permissive from nonpermissive cells (Murakami et al. 1986b). Tegtmeyer, DePamphilis and others have finely dissected the origins of both viruses (Tegtmeyer 1972, Hendrickson et al. 1987). A core origin (ori-core) has been defined which is highly homologous for both viruses. Each ori-core is flanked by a T ag binding site on its early gene side. These viral origins have also been found to differ in several pertinent aspects. First, the permissive host factors which are essential for viral DNA replication are species dependent. The recent development of <u>in vitro</u> DNA replication systems for both of these viruses (Li and Kelly 1984, Murakami et al. 1986c) has led to the demonstration that these factors are contained within the host replicase (polymerase-primase)(Murakami et al 1986b). Second, The other major T ag binding site (site II) for SV40 encompasses the ori-core, whereas both of the other Py LT binding sites are located farther into the early gene region. Third, the early mRNA TATA box and cap sites are

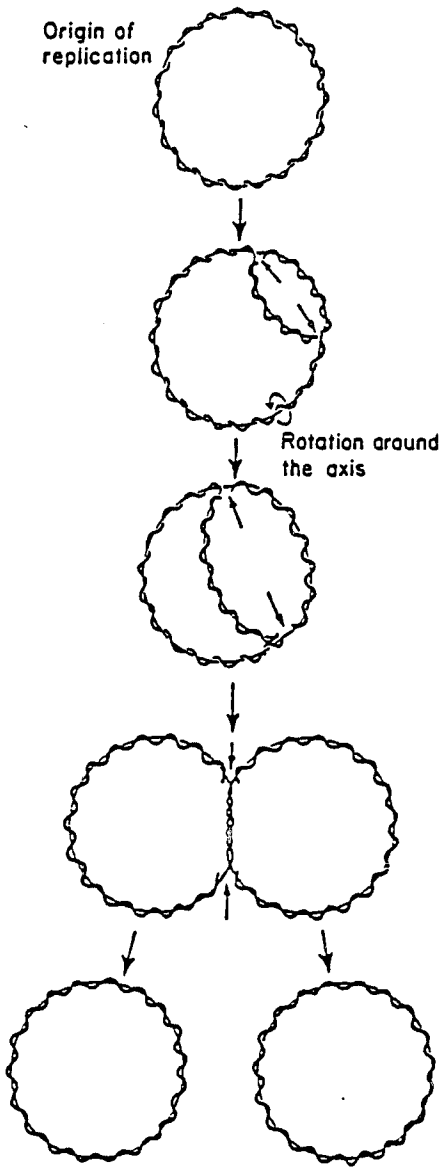
located within the SV40 ori-core, whereas they are located outside the Py ori-core. Fourth, the Py origin includes a transcriptional enhancer which is essential for replication (deVillers et al. 1984), whereas the SV40 origin does not. This may reflect the lack of a polyoma LT binding site in the ori-core. Finally, for SV40 the sites where continuous strand synthesis starts (defining the origin of bidirectional replication) are coincident with each other in a two base pair region between the ori-core and T ag binding site I. In Py, however, these sites lie 16 bp away from each other in a similar region between the ori-core and the LT binding site (Hendrickson et al. 1987). Furthermore these studies have suggested that the initiation of the first DNA strand at the origin may occur by the same mechanism used in the initiation of the Okazaki fragments. Others have speculated that, since T ag can be adenylated (Clertant et al. 1984) and the putative SV40 RNA primers start with an adenosine, the T ag may function in the priming of DNA synthesis.

Both intracellular and <u>in vitro</u> studies have indicated that the initiation of papovavirus DNA replication occurs in at least two stages (Hendrickson et al. 1987, Wobbe et al. 1986). The first, pre-elongation, is RNase A sensitive and occurs in the absence of dNTPs. It is believed that T ag binding to the origin induces a stretch of single stranded DNA with which the host factors (replicase) interact to form an initiation complex. The second stage, elongation, requires dNTPs and DNA polymerase alpha and can be separated from the

Figure 3. - Model for papovavirus DNA replication. Replication initiates at a unique origin sequence and proceeds bidirectionally. Topoisomerases relieve topological barriers as replication proceeds and resolve the resulting concatemers into daughter molecules. See text for details. From Tooze (1980).

Figure 3

Model for papovavirus DNA replication.



first stage *in vitro*. This is analogous to that which has been observed to occur during the *in vitro* DNA replication of E. coli oriC (van der Ende et al. 1985). After initiation occurs, a bidirectional replication fork is set up and elongation occurs in a continuous manner on one strand and in a discontinuous manner (via Okazaki intermediates) on the retrograde strand. In this manner a Cairns or theta intermediate is formed as shown in Figure 3. A minor population of molecules (1-2%) has been observed to replicate by a rolling circle mechanism. Before replication is completed the topological links which have been observed to exist between the parental strands must be resolved (Sundin and Varshavsky 1980). Yang et al. (1987) have shown that while either topoisomerase I or II is sufficient to provide the unlinking activity required during fork progression, only topoisomerase II provides the unlinking required for the separation of the daughter DNA molecules.

In summary it can be stated that, analogous to the manner that phages were utilized as simpler model systems for the elucidation of the biochemistry of DNA replication in prokaryotes, DNA viruses are being utilized as models with which to elucidate the mechanisms of eukaryotic DNA replication. The development of *in vitro* DNA replication systems for adenovirus and the papovaviruses has accelerated this work. Differences between these viruses can be observed primarily in the mechanics of initiation, although differences in elongation (Ad - only continuous strand

synthesis) and termination (Ad - only requires topoisomerase I, and not topoisomerase II) are also observed.

Biochemical analyses of polypeptides which are believed to play a role in DNA replication cannot by themselves establish that these polypeptides function in the intracellular replication of DNA. So too studies of viral DNA replication can only serve as an indication of polypeptides which may also function in cellular DNA replication. These studies need to be complemented by genetic approaches, as they have been in the continuing elucidation of prokaryotic DNA replication. Fortunately, genetic studies of eukaryotic DNA replication have been initiated, although they have not as yet been as fruitful as those with prokaryotes.

One approach is the isolation of cellular mutants which are resistant to drugs which are potent inhibitors of DNA synthesis. Aphidicolin-resistant mutants have been used to demonstrate that DNA polymerase alpha is involved in DNA synthesis and DNA repair (Liu et al. 1983, Sugino and Nakayama 1980). Among the aphidicolin-resistant mutants which have been partially characterized are those which overproduce DNA polymerase alpha and those which display alterations in the kinetics of dCTP binding by polymerase alpha (reduced K_m). Further analysis of these mutants may lead to an elucidation of the functional role(s) of polymerase alpha at the molecular level. For example, some of these mutants display a mutator phenotype. Mutator DNA polymerases have been isolated in prokaryotes. These mutant DNA polymerases

have been characterized as possessing a defective 3'-5' exonuclease activity. Most of the mammalian DNA polymerases which have been isolated thus far lack this associated exonuclease activity. Thus, a detailed analysis of eukaryotic mutator mutants may reveal the mechanism by which higher eukaryotes maintain fidelity during DNA replication. A camptothecin(CPT)-resistant mutant has recently been isolated in a human lymphoblastic leukemia cell line (Andoh et al.1987). CPT is a potent inhibitor of RNA and DNA synthesis. Purification of the topoisomerase I from these cells established that this enzyme is the cellular target of CPT.

Another approach has been the study of human cells isolated from individuals suffering genetic diseases which display a high incidence of chromosome rearrangements, breakage and sister chromatid exchange. This is indicative of a defect in DNA replication or repair with the free ends of DNA initiating the exchanges. Most of these cell lines exhibit evidence of a defect in DNA repair. However, two of these cell lines, from patients with Bloom's Syndrome and megaloblastic anemia, have been characterized as being defective for DNA synthesis; specifically, the elongation of replicative intermediates (Gianelli et al. 1977, Hoffbrand et al. 1976). Willis et al. (1987) have recently demonstrated a defect in DNA ligase I which they propose is responsible for Bloom's Syndrome. The further characterization of these cell lines may yield insights into the mechanism of chain elongation.

Since the incidence of spontaneous mutants in eukaryotic DNA replication which are not lethal to the cell is extremely low, procedures for the selection and screening of temperature-sensitive (ts) and non ts conditional mutants have been developed. These methods are reviewed in Hochstadt et al. (1981). The majority of the ts conditionally lethal mutants affecting DNA replication which have been isolated to date result in the arrest of the cells in a phase of the cell cycle other than S phase (for a review see Marcus et al. 1985). Additionally, many non ts conditional mutants have been isolated which affect nucleotide metabolism (for a review see Hochstadt et al. 1981). The earliest isolation of temperature-sensitive (ts) mutants which are affected in DNA synthesis was by Thompson et al. (Thompson et al. 1971, Thompson et al. 1970) in mouse L cells. One of these ts DNA$^-$ isolates, tsA1S9, has been more extensively characterized (Sheinin 1976, Colwill and Sheinin 1983). Upon shift to the nonpermissive temperature (NPT) tsA1S9 cells continue to synthesize DNA in an apparently normal fashion for 6-8 hours but falls rapidly thereafter. Studies at these later times have shown that these cells are still capable of synthesizing Okazaki fragment sized DNAs and converting them to larger forms. Viral analysis has shown that the replication of Polyoma DNA is supported at both the permissive temperature (PT) and the non-permissive temperature (NPT) in infected cells. Other studies have indicated that modifications in the supercoiling of the DNA and in chromatin structure occur at

the NPT. These data and the hypersensitivity of tsA1S9 cells to novobiocin have suggested that DNA topoisomerase II activity may be affected by the ts mutation. Affinity chromatography of novobiocin binding proteins from tsA1S9 and wild-type mouse L cells have indicated that the gene product of the mutated locus in tsA1S9 is required for the proper function of DNA topoisomerase II (Colwill and Sheinin 1983).

Another ts DNA$^-$ mutant from this collection which has been partially characterized is tsC1 (Thompson et al. 1971, Guttman and Sheinin 1979). There is an immediate and rapid decline in the incorporation of tritiated thymidine (^{3}H-TdR) for the first 24 hours after shift to the NPT, although the incorporation rises then falls again during the subsequent 24 hours. Further analysis of this cell line has shown that mitochondrial DNA replication is not affected by the ts lesion. Studies with synchronized cells have shown that tsC1 cells can enter, but cannot complete, S phase. Genetic analysis of this ts mutant has revealed that the defect can be corrected by sequences on the human X chromosome (Giles and Ruddle 1979). However, the function affected by the genetic lesion has not yet been identified.

A collection of ts mutants were isolated from mutagenized BALB/3T3 mouse cells by this laboratory (Slater and Ozer 1976). Three of these ts mutants, ts2, ts20, ts22, displayed kinetics of DNA inhibition which were similar to each other - a progressive decline after an initial 3 hour

rise. The mutants do not complement each other, although each is recessive to wild-type (Jha et al. 1980). Flow microfluorimetry data have indicated that ts2 and ts20 arrest in S phase. Both ts2 and ts20 fail to support Polyoma DNA replication at the NPT (Zeng et al. 1985, Zeng et al. 1984). In vitro complementation of the defect in ts20 with extracts from wild-type cells has been used to obtain a fraction of the wild-type extract which corrects the cellular defect at the NPT. This fraction contains at least 10 polypeptides including topoisomerase I activity, thus suggesting that the genetic lesion in ts20 affects DNA topoisomerase I. Also the analysis of chain elongation rates by fiber autoradiography reveals a reduction in the rate at the NPT, thus providing support for this conjecture. An additional defect in the stability of the purified DNA polymerase alpha complex has recently been observed (Dermody et al. in press). Studies with synchronized ts2 cells have shown an induction of thymidine kinase, thymidylate kinase, and DNA polymerase alpha activities which is independent of a simultaneous induction of DNA when density arrested (G0?) cells at the PT are stimulated by serum. This serum stimulated induction does not occur at the NPT,although the cells are capable of entering the cell cycle. Infection of ts2 cells with SV40 does, however, induce thymidine kinase and DNA synthesis at both temperatures. These data have lead to the proposal that a broad based transcriptional block is responsible for the ts defect (Malkas 1985). Genetic analysis of ts2 has shown that

the ts defect can be corrected by sequences on the human X chromosome (Jha et al. 1980).

Ts DNA$^-$ mutants have also been isolated from mouse FM3A cells. Ts131b is ts DNA$^-$ as shown by the incorporation of ^{3}H-TdR and FMF analysis (Hyodo and Suzuki 1982). The rate of DNA chain elongation is diminished 6 hours after shift to the NPT by DNA fiber autoradiography. Since this decrease is insufficient to account for the decrease in ^{3}H-TdR, it was proposed that a simultaneous decrease in the rate of initiation must also occur. In the case of ts85, the majority of cells arrest in G2 with many cells distributed in S phase as well (Yasuda et al. 1981). Further analysis of this mutant has revealed a gradual decrease in histone H1 phosphorylation at the NPT. This decrease is thought responsible for the failure to complete DNA replication and the defect in chromosome condensation which has been observed at the NPT. The best characterized ts DNA$^-$ mutant of FM3A cells is tsFT20 (Murakami et al. 1986). DNA synthesis, as measured by ^{3}H-TdR incorporation, decreases immediately upon shift to the NPT. Cell cycle analysis has shown that tsFT20 cells at the NPT arrest in S phase in a manner similar to that observed when wild-type cells are treated with aphidicolin. The DNA polymerase alpha activity has subsequently been shown to decrease at the NPT, whereas this activity is restored in ts$^+$ revertants of tsFT20. These data are consistant with tsFT20 possessing a genetic lesion in the gene for DNA polymerase alpha or a factor which is essential for its normal function

Hamster cells have also been utilized as parents of ts DNA$^-$ mutants. ts BN-2, derived from BHK cells, is defective in DNA synthesis at the NPT due to a defect in initiation on individual replicons (Narkhammer and Hand 1985). These cells also display premature chromosome condensation at the NPT. The protein responsible for this defect has not yet been identified. Another ts DNA$^-$ mutant which is derived from Chinese hamster ovary (CHO) cells is tsC8 (McCracken 1982). DNA synthesis is inhibited immediately upon shift to the NPT. These cells fail to complete S phase at this temperature. Complementation by cell hybridization was accomplished with tsA1S9, tsC1, ts2, and tsBN2, indicating that the genetic lesion was in a gene which is unaffected in other ts DNA$^-$ mutants but the function encoded by this gene has not yet been identified. Two other CHO derived mutants are ts13A and ts15C (Srinivasan et al. 1980). Although the kinetics of the inhibition of DNA synthesis at the NPT differs for these mutants, they fail to complement each other, suggesting that they are defective in the same gene function. These cell lines display increased sensitivity to alkalating agents, but not to ultraviolet light. The exact nature of the defect in these mutants is unknown. Ts DNA$^-$ mutants have also been isolated from V79 hamster lung cells (Marcus et al. 1985). One, ts24, arrests two hours into S phase. Further analysis has shown that most of the DNA which is synthesized at the NPT is rapidly degraded. The gene which encodes this function has been recently cloned (Fainsod et al. 1985) which should

greatly facilitate the further analysis of its role in DNA replication. The occurance of multiple rounds of replication within a single S phase has been observed in a second V79 mutant, ts41, indicating that it is defective in a function which regulates the amount of DNA replication which occurs during a single S phase.

From this brief survey of the available mutants in DNA replication it can be seen that a relative paucity of mutant functions required for replication has been obtained in mammalian cells. It should also be noted that mutants in other biochemical pathways can mimic a ts DNA$^-$ state. For example, Wong and Scheffler (1982) has described a ts mutant in which a rapid inhibition of DNA synthesis is secondary to a less dramatic effect in a mRNA population. Presumably the effect on DNA synthesis is mediated through a short-lived protein encoded by an mRNA which turns over rapidly. Similarly, a defect in the synthesis of one or more ribonucleotides would also be expected to result in a deficiency in deoxyribonucleotide triphosphate synthesis, although such a ts mutant has not been reported thus far. Furthermore, most of these mutants in DNA replication map to the human X chromosome, indicating an inability to generate mutants in autosomally encoded functions. This is undoubtedly due to the presence of a second allele which masks any recessive mutations. Furthermore, difficulties in analysing these mutants have resulted from the complexity of the eukaryotic genome.

In an effort to remedy these deficiencies in the genetic analysis of eukaryotic DNA replication, Dr. James Dermody of this laboratory isolated a collection of over 200 mutants of Chinese hamster ovary (CHO) cells which are temperature-sensitive for growth by utilizing a variety of selective and nonselective strategies (Dermody et al. 1986). Although CHO cells have often been used for the isolation of mutants, few mutants directly affecting DNA synthesis have been described (see previous section) and no studies have been reported on the extensive isolation of ts mutants in CHO cells. Several considerations indicated that this cell line would be a good choice to serve as the parent of a mutant collection. First, it has favorable growth properties over a broad range of temperatures (33°C to 41°C) and its efficiency of colony formation (EOC) approaches 100%. Esko and Raetz (1978) have also demonstrated the efficiency of direct screening by replica plating for identification of mutants. Second, autosomal recessive mutants dispersed throughout the genome have been isolated in CHO cells at unusually high frequency (Gupta 1980, Siminovitch 1976). If genes important to DNA synthesis reside in regions of functionally hemizygous chromosomes, mutants at these loci should be easier to obtain. Finally, human adenovirus (Type C) (Radna et al. 1987) and the papovaviruses SV40 and polyoma (LaBella and Ozer 1985) replicate in CHO cells.

Four Chinese hamster cell lines were used in the isolation of the ts mutant cell collection to be studied in

this thesis. Three were sublines of Chinese hamster ovary fibroblasts (CHO-K1 (Denver), CHO-S (Toronto), and CHO thy^{-}49 (Meuth et al. 1982), a mutant with disturbed nucleotide pools resulting in a mutator phenotype) and V79 Chinese hamster lung cells (clone 380-6). Exponentially growing cells were mutagenized with ethyl methanesulfonate (25 micrograms/ml medium) for 16 hours at 33°C, followed by 3 days incubation to allow phenotypic expression and recovery from the mutagen. The mutagenesis was performed in separate dishes to maximize deriving mutants of independent origin. Following mutagenesis each culture was split and and the sister subcultures were subjected to different selection protocols. Ts mutants have been isolated using a variety of selective agents (see Bascilico 1977 for reviews). The commonly utilized selection regimens depend on slowing (or arrest) of DNA synthesis of the rare ts mutants after shift of the mass population to an elevated (restrictive) temperature. The culture is then exposed to an agent which will kill the wild-type cells that vigorously incorporate it into their DNA. Upon removal of the agent and return of the culture to the lower (permissive) temperature, the surviving population becomes enriched for mutants. High specific activity ^{3}H-TdR, arabinofuranosylcytosine (ara C), and fluordeoxyuridine (FUdR) have been successfully used for recovery of ts mutants (Thompson et al. 1970, Nishimoto and Basilico 1978). Bromodeoxyuridine (BUdR) in conjunction with Hoechst 33258 and visible light has also been shown to be highly toxic to

rapidly growing cells (Killary et al. 1984). When this laboratory compared these agents for their kinetics of cell killing, it was found that the BUdR regimen was the most efficient (Dermody, unpublished data).

Three methods of selection were employed in the isolation of this ts mutant collection. The first involved a single selection with prolonged exposure to the selective conditions. Mutagenized cultures were shifted to 39°C, 16 hours later 2 x 10^{-5}M BUdR was added. 48 hours later the cultures exposed to Hoechst 33258 (1 microgram/ ml) for 2 hours followed by long-wave UV light for 60 seconds. However, as previously discussed in the Introduction, there are limitations to this method of ts mutant isolation; most importantly, the possible failure to isolate mutants directly involved in DNA synthesis which display a rapid inhibition of DNA synthesis. In an effort to overcome this difficulty, a second selection system, involving multiple selections with brief exposures to selective conditions was employed. Mutagenized cultures were grown at 39°C for 2 to 4 hours, BUdR was added for an additional 2 to 4 hours followed by Hoechst 33258 and UV light. Cultures were then refed and incubated at 33°C for 16 hours and this procedure repeated two more times for a total of three cycles of selection. Since exposure to selective conditions, however brief, may result in the loss of interesting mutants, a third method of isolation, involving nonselective replica plating, was also employed. Mutagenized cells were diluted into 96-well

microtiter plates at 4 cells per well. After growth of colonies (40-70% of the wells), cells were typsinized and one half volume distributed into each of two 96-well plates, one incubated at 33°C and the other at 39°C. After four days incubation, wells at 39°C were examined under the microscope. Wells with no or aberrantly growing cells were compared to the replica at 33°C.

Following selection, cultures were incubated for 1-2 weeks at 33°C to allow surviving cells to form colonies. Colonies were picked and grown into mini-cultures in multiwell dishes. Alternatively, putative mutants identified by replica plating were recovered from the 33°C wells. In both cases, arbitrary aliquots of cells were inoculated into dishes and incubated at 33°C and 39°C. Those exhibiting ts growth behavior (growth at 33°C, poor or no growth at 39°C) were expanded into mass culture and subjected to further analysis. Mutants temperature-sensitive for growth had either of two phenotypes: cells stopped growing at 39°C, most often followed by loss of attachment 1 to 3 days later (these were designated RD for rapid death), or cells continued growing very slowly at 39°C and remained attached, forming microcolonies (these were designated SG for slow growth). A collection of over 200 ts mutants were obtained by these selection regimens.

OBJECTIVES

This mutant collection provides the basis of my thesis research. It entails several goals which may be summarized as follows. First, to screen this collection of ts CHO mutants for those which are of value to a study of eukaryotic DNA replication (i.e. primary defect in DNA synthesis, unique gene affected); Second, to test the feasibility of using DNA viruses as simple model systems in which to study the cellular defect, an approach which, although successfully applied to prokaryotic DNA replication, has yet to be fully exploited in the elucidation of the mechanisms of eukaryotic DNA replication in cell mutants; Third, to perform a preliminary analysis of the defect in one of these mutants (JB3-B) utilizing this approach. I expect to obtain data which will suggest that the defect affects one of the following areas: a macromolecule which is directly involved in DNA synthesis, intracellular dNTP pools, a phase of the cell cycle other than S phase, chromatin, or another as yet unknown area which affects DNA synthesis; Fourth, to initiate the genetic analysis of the human gene which corrects for the defect in one of these mutants (JB3-B).

MATERIALS AND METHODS

Cells and Culture Methods. The temperature-sensitive Chinese hamster cell lines used in this study were isolated in this laboratory by mutagenic treatment with ethyl methanesulfonate followed by several selective or non-selective regimens (Dermody et al. 1986). These mutant cell lines were derived from the following cell lines: CHO-K1 (Denver) Chinese hamster ovary fibroblasts (Kao and Puck 1967) which were obtained from L. Chasin; CHO-S, a subclone of CHO-K1, isolated in Toronto (Stanley and Siminovitch 1976) was obtained from P. Stanley (this cell line will be used as a wild-type control in experiments involved in this study.); V79 Chinese hamster lung cells (Chu et al. 1965) (clone 380-6) were obtained from U. Francke. 3B·THO, a ouabain (OUA^r) and thioguanine (TG^r) resistant cell line, was derived from JB3-B at 33°C by sequential isolation of resistance to ouabain and thioguanine. Two days after mutagenesis by exposure to 125 ug of ethyl methanesulfonate (Eastman Kodak), JB3-B cells were plated in medium containing 3 X 10^{-3}M ouabain; individual colonies (3B OUA^R) were picked, and tested for colony formation at 33°C and 39.5°C. A clone which exhibited the most rapid loss of viability at 39.5°C was chosen for further study. It was plated without further mutagenesis in medium supplemented with 5 X 10^{-5}M thioguanine at 33°C and colonies isolated as above. A suitably ts colony was designated 3B·THO. Its further characterization is described in the Results. The human diploid fetal bone marrow

TABLE 1

ts mutants not isolated as a part of this collection

CELL LINE	SOURCE	LABORATORY	REF.	COMMENTS
tsH1	CHO-S	L. Thompson	1	ts leucyl tRNA synthetase
tsC8	CHO-K1(AdeC$^-$)	A.A. McCraken	2	mutated function unknown[a]
ts13A	CHO-S	L. Siminovitch	3	mutated function unknown
ts15C	CHO-S	L. Siminovitch	3	mutated function unknown
ts2	BALB/3T3	This laboratory	4	mouse; mutated function unknown
ts20	BALB/3T3	This laboratory	5	mouse; mutated function unknown[b]
tsA	L cells	L. Thompson	6	mouse; subclone of tsA1S9
tsA1S9	L cells	R. Sheinin	7	mouse; possible DNA topoisomerase II[b]
tsC1	L cells	R. Sheinin	8	mouse; mutated function unknown[b]

a - complements with ts2

b - does not complement with ts2

1 - Thompson et al. 1973

2 - McCracken, A.A. 1982

3 - Srinivasan et al. 1980

4 - Slater and Ozer 1976

5 - Zeng et al. 1984

6 - Thompson et al. 1970

7 - Colwill and Sheinin 1983

8 - Guttman and Sheinin 1979

fibroblast cell line HS74BM was obtained from H. Smith (Smith et al. 1976). The immortalized human cell line SV/HF-5/39 was isolated in this laboratory (Neufeld et al. 1987). Monkey COS-7 cells were obtained from Y. Gluzman (1981). Other cell lines used in these studies and the sources from which they were obtained are listed in Table 1.

Cells were cultured in an equal mixture of Dulbecco's modified Eagles medium and F12 Ham's medium (DF, Hazelton/KC) with 10% newborn calf serum (NBS, M.A. Bioproducts) supplemented with proline (50 ug/ml final concentration, Sigma), penicillin "G" phosphate (0.05 gm/l, Gibco) and streptomycin sulfate (0.05 gm/l, Gibco). The human cell lines were cultured in Dulbecco's modified Eagle medium and Ham's F10 in a 1:1 mixture supplemented with 10% fetal bovine serum (M.A. Bioproducts) as previously described (Neufeld et al. 1987). Cells were removed from culture dishes in trypsin-EDTA (M.A. Bioproducts), and cell numbers were determined with a Royco 927TC cell counter. Cell lines were stored in Nunc Cryotubes frozen in liquid nitrogen or in a -70°C freezer in complete medium containing 10% dimethyl sulfoxide (DMSO).

Assays for assessment of cell growth and viability. The efficiency of colony formation (EOC) was obtained by seeding either 100 or 250 cells per 60mm dish (Falcon). When colonies of observable size appeared, the dishes were stained with Giemsa and the colonies counted. The growth rates of the cell lines were determined by seeding 5 $x10^4$ cells per 60mm dish in triplicate and counting the cells on a dish following

trypsinization at various times post-attachment. Survival curves were performed by seeding at 33°C as for an EOC; these cultures were then shifted to 39.5°C for designated periods of time; at the end of these times, triplicate dishes were returned to 33°C and incubated until colonies appeared (14 days). Colonies were stained and counted as above. The incidences of revertants were calculated by seeding 10^4 to 10^6 cells in a 100mm dish and growing parallel cultures at both 33°C and 39°C. The dishes were scored after 13 days and any surviving colonies at 39°C were picked, grown out and frozen down as described above for later use as controls.

Cell fusion. Preliminary complementation analysis was performed as follows: 2 x 10^5 cells of each of two cell lines which are to be fused were seeded into a 60mm dish. On the following day, when the cells have attached, the dish was washed with DF (no serum); then 50% polyethylene glycol (PEG, Koch-Light Labs, M.W.= 1000)/DF was added dropwise to a total volume of two ml. (Jha et al. 1980). After two minutes, the PEG was aspirated and the cells washed twice with DF; two ml DF was added and the cells incubated at 33°C for five minutes followed by five ml complete medium for 30 minutes; the cells were then refed with complete media and incubated at 33°C. The next day the cells were trypsinized and replated onto a 100mm dish at a range of cell densities. After allowing a day for the cells to reattach, the dish was shifted to 39°C. Colonies were scored after two weeks. Complementation analysis between 3B-THO and other ts mutants or human cell

lines was performed as above except for the following: 5 x 10^5 cells of each cell line was seeded initially. On the day following fusion the cells were split into five 100mm dishes; after allowing the cells to attach, the dishes were refed with complete medium containing hypoxanthine (6.47 x 10^{-5}M, Sigma), aminopterin (5 x 10^{-6}M, Sigma), thymidine (1.15 x 10^{-5}M, Sigma)(designated HAT media) and ouabain (3 x 10^{-3}M, Sigma). In the case of hybrids with human cells 10^{-5}M ouabain was used instead. Chromosomes were prepared for karyotypic analysis as previously described (Bignone et al. 1983). The chromosomes were not banded.

Flow Microfluorimetry. At various times of interest exponentially growing cells were harvested by trypsinization, washed with phosphate buffered saline (PBS), and fixed in a 50:50 mixture of 80% ethanol/acetone at -20°C. 10^6 cells were stained with propidium iodide in triton X-100, treated with RNase, and analysed with a System 50H flow cytometer (Ortho Diagnostic) interfaced to an Ortho 2150 data analysis system by Dr. F. Traganos (Sloan-Kettering Institute) as described elsewhere (Klein et al. 1988).

Determination of Rates of DNA and Protein Synthesis. 5 X 10^5 cells in 3 mls were seeded into flat based culture "ambi-tubes" (NUNC 1409) in the prone position and incubated for 24 hours at 33°C. 2 ml medium was added and tubes were shifted to an upright position to mimic later labelling conditions (some mutants had altered morphology and might be less adherent) for an additional 16 hours. Subsequently, one set

of tubes was shifted to a 39°C water bath, the rest remaining at 33°C. At appropiate intervals thereafter, duplicate cultures were pulse labeled at 33°C and 39°C: the growth medium was aspirated and replaced with methionine-free DME (which lacks thymidine as well) supplemented with 1 microCurie/ml ^{35}S-methionine (800 Ci/mmole, NEN) and ^{3}H-thymidine (74 Ci/mmole, NEN). After 2 hours incubation, labeling medium was removed, the monolayer washed with PBS, and the cells lysed by addition of 1 ml lysis solution (0.1% SDS, 10mM Tris pH 7.5, 1mM EDTA). Samples were precipitated with trichloroacetic acid, collected onto GF/A filters (Whatman), and dissolved with NCS tissue solubilizer (Amersham). Radioactivity was determined in Liquifluor (NEN) by liquid scintillation spectroscopy after double label correction as previously described (Wittes and Ozer 1973).

Virus and Viral DNA. Human adenovirus 2 (Ad2) virus stocks were prepared from infected HeLa cells grown in suspension. Infected cells were pelleted from the medium at 48 hours post infection (p.i.) and resuspended in HEPES buffered (50 mM, pH 7.6) DME. After 3 cycles of freeze-thaw to lyse the cells, the released virions were separated from the cellular debris by centrifugation at 2,000 RPM for 15 minutes at 4°C in a Sorvall RC5 centrifuge. Two milliliter aliquots of the resulting supernatent were stored at -70°C. For infection, the required amount of virus was diluted in DME containing 1% fetal bovine serum (FBS, M.A. Bioproducts). Virus stocks were titered for infectious units in JB3-B cells

(cultured at the permissive temperature) by indirect immunofluorescence for viral DBP as previously described (Radna et al. 1987).

Recombinant DNAs containing viral sequences were propagated and harvested from DH-1 (dam^+) bacteria using standard methodology (Maniatis et al. 1982). pLA1 contains the 0 - 9.4 Bgl II fragment of the Ad 5 genome cloned into Eco R1, Bam H1 digested pAT (Tamanoi and Stillman 1982). It was kindly supplied by B. Stillman. p53A6.6 DNA contains a complete polyoma genome cloned at the Bam H1 site in pAT153 and was kindly provided by R. Kamen (Triesman et al. 1981). All recombinant DNAs were used in accordance with NIH guidelines.

Determination of Adenovirus and Polyoma virus DNA Replication. Cell lines were seeded at 3 X 10^5 cells per 60mm dish and infected the following day with Ad2 virus at a multiplicity of infection (MOI) between 400-700 for CHO. Cultures, in duplicate, were incubated at 33°C or 39.5°C. At various times p.i. viral DNA was selectively extracted by a modified Hirt procedure which included pronase in the lysis buffer (Kimelman et al. 1985). Hirt supernatents were extracted with organic solvents and DNA was precipitated in 2.5 volumes cold ethanol. Dried pellets were dissolved in 50 microliters of 10mM Tris, 1 mM EDTA (TE) per culture. Dot blot analysis was performed as described by Kafatos and Efstradiatis (1979). Briefly, 15 microliters of each DNA sample was denatured in 0.3M NaOH, serially diluted, and

spotted onto a nitrocellulose(S&S) filter using the S&S minifold apparatus. The plasmid pLA1, nick-translated with ^{32}P-dATP and ^{32}P-dCTP (NEN) according to Rigby et al.(1977), was used for hybridization as previously described (LaBella and Ozer 1985). Known amounts of purified pLA1 DNA (Tamanoi and Stillman 1982) were used as a quantitation standard. The replication of polyoma virus was performed as previously described (Dermody et al. 1986).

Analysis of Ad2 DNA synthesis by alkaline sucrose gradients. Pulse and pulse-chase labelling and alkaline sucrose gradient centrifugation were performed as described by Longiaru and Horwitz (Longiaru and Horwitz 1981). Briefly, at various times of interest p.i., cells were pulse-labelled for one hour with ^{3}H-TdR (5uCi/ml, NEN), followed by the addition of non-radioactive TdR for 30 minutes so as to chase intermediates to full genomic length, washed with PBS, scraped and lysed directly on a 5 - 20% alkaline sucrose gradient with desoxycholate. A cesium chloride cushion was used to collect cellular DNA. Centrifugation was performed in a SW27 rotor for 16 hours at 22,000 RPM at 4^{o}C. 0.5 ml fractions were collected and TCA precipitation performed as described earlier. Analysis of Ad2 DNA elongation was performed by a modification of this protocol. Prior to the pulse with ^{3}H-TdR, the infected cultures were incubated for 2.5 hours with ^{14}C-TdR (0.1 uCi/ml, 57 mCi/mmole). The duration of the pulse was shortened to 4 minutes (40 uCi/ml) and the duration of the chase was varied (see Results,

Chapter 3). The remainder of the protocol was as described above. Aphidicolin was obtained from Sigma.

High Performance Liquid Chromatography. Deoxyribonucleotide triphosphates (dNTP) were isolated from JB3-B cells for HPLC analysis by the method of Tanaka et al. (Tanaka et al.1984) with modifications. Briefly, nucleotide(NTP)-containing extracts were prepared from 1 X 10^8 JB3-B cells by TCA precipitation followed by freon/tri-n-octylamine extraction. The aqueous upper layer containing the NTP was either used immediately or was frozen at -70°C for up to 1 month. rNTP were destroyed by oxidation with periodate. Immediately thereafter the samples were passed through a G-10 column (Bio-Rad) to remove as many interfering substances (rNTP breakdown products and cellular contaminants) from the dNTP as possible prior to injection. HPLC analyses were performed on a Beckman model 334 HPLC system. Chromatography was performed on a Whatman Partisil-10 SAX column eluting with a gradient of 10% acetonitrile/0.4 M $NH_4H_2PO_4$ pH 2.90 at time = 0 to 25% acetonitrile/0.4 M $NH_4H_2PO_4$ pH 2.90 at time = 10 minutes. Each elution was of 32 minutes duration.

Identification of human sequences which correct the ts defect in JB3-B. Cell fusions were performed as decribed earlier except that the exposure to 50% PEG was shortened to one minute for fusions involving human cells. DNA-mediated gene transfer was performed using the calcium phosphate coprecipitation technique (Graham and van der Eb 1973) under the conditions specified in the text. pCV103-gpt (human

genomic cosmid library) was obtained from Y.W. Kan (Lau and Kan 1983). Plasmid DNAs pBLUR8 and pSV2 gpt were obtained from W. Jelinek (Jelinek et al. 1980) and P. Berg (Mulligan and Berg 1981) respectively. These DNAs were propagated in appropiate strains of E. coli and harvested by standard methodology (Maniatis et al. 1982). High molecular weight cellular DNA was isolated as previously described (Small et al. 1984).

Southern analysis for exogenously introduced sequences was performed by conventional methodology (Maniatis et al. 1982). High molecular weight DNA (and plasmid DNA controls) was digested with different restriction endonucleases at 2 U/ug of DNA overnight under conditions specified by the supplier. Fragments of digested DNA were separated by electrophoresis in 0.8% agarose gels. Hybridizations were performed with either the gel purified 270 bp Alu fragment from pBLUR8 or the gel purified 1.8 Kb gpt specific (Bgl II to Bam HI) fragment of pSV2 gpt. These probes were ^{32}P-labelled by the random primer method (Feinberg and Vogelstein 1984). DNA containing filters were dehybridized in 0.3 M NaOH for 20 minutes followed by neutralization in 0.5 M Tris-HCl, pH 7.0.

RESULTS

Chapter 1 - The isolation and initial characterization of the temperature-sensitive CHO mutant cell collection.

A collection of over 200 CHO mutants which are temperature-sensitive (ts) for cell growth was isolated in this laboratory as described in the Introduction. As a first step in identifying mutants which were defective in DNA synthesis, I undertook to screen the collection using pulse-labelling assays to determine the rates of macromolecular synthesis. Since the primary goal was to identify mutants with rapid onset of the ts defect, we determined the rate of DNA synthesis (incorporation of ^{3}H-TdR) only within the first 24 hours after shift of growing cultures from 33°C to 39°C. As a control, cells were simultaneously monitored for protein synthesis (incorporation of ^{35}S-methionine). Cells were seeded at a density which would grow logarithmically. Two days later some of the cultures were shifted to the nonpermissive temperature (NPT), the rest left at the permissive temperature (PT). Cultures were labelled for two hour pulses at 1, 7, and 17 or 25 hours post shift. Cells were then processed as described in the Methods and Materials. This protocol permitted us to distinguish at the outset mutants which were secondarily affected in DNA synthesis. The results of this preliminary screen were used to sort mutants into four categories. The first catagory

included those mutants which were phenotypically indistinguishable from wild-type CHO-S cells (i.e. no obvious defect in protein or DNA synthesis). These were designated wild type-like and would include mutants with a delayed onset of phenotype requiring more than one cell generation at the restrictive temperature as well as mutants in cellular processes that do not rapidly affect protein or DNA synthesis. Not surprisingly the majority of the mutants fell into this catagory. Thirty one of the mutants fell into the second catagory and were designated rapid ts PRO^-. Phenotypically these mutants show a rapid inhibition of protein synthesis - within two hours - and are thus secondarily affected in DNA synthesis. The 26 mutants which fell into the third catagory were those which demonstrated a concomitant decline in both DNA and protein synthesis at 7 hours or later. These mutants were designated slow PRO^-. This group of mutants was given a low priority (in terms of further analysis) since the inhibition of DNA synthesis is most likely secondary to the inhibition of protein synthesis observed in these mutants. Twenty mutants were placed into the fourth catagory. These mutants exhibited a decline or plateau in DNA synthesis with very little or no concomitant decline in protein synthesis. These mutants were designated ts DNA^- (primary defect in DNA synthesis). These mutants represented 6% (5/84) of the ts mutants isolated by multiple short selections, 9% (4/43) of the ts mutants isolated by a single long selection, and 20% (12/60) of the ts mutants

Table 2

Macromolecular synthesis in preliminary ts DNA$^-$ mutants

		Incorporation of ^{3}H-TdR and ^{35}S-met (CPM x 10^{-3})[a] hours after temperature shift				
Cell line	Temp.	0	5	25	Viability at 39.5°C	Remarks
CHO-S	39°C	134/30[b]	182/28[f]	200/83		Wild-type parent
	33°C	61/17	74/15[f]	150/18		
JB1-C	39°C	40/11	16/7	17/7	RD[c]	DNA synthesis decreases
	33°C	24/9	31/8	42/7		to 15 hours, then rises
JB1-R	39°C	26/8	21/7	19/8	RD	Progressive decrease in
	33°C	14/5	19/4	31/4		DNA synthesis
JB3-B	39°C	37/12	19/12	02/11	RD	Rapid and progressive
	33°C	18/12	29/10	45/10		decrease in DNA synthesis
JB3-C	39°C	29/8	30/8	25/11	SG[d]	Not ts
	33°C	22/6	13/5	22/11		
JB3-O	39°C	36/12	42/12	16/11	SG	DNA synthesis decreases
	33°C	21/8	19/6	31/12		after 10 hours
JB4-E	39°C	12/ND[e]	15/ND	31/ND	SG	Not ts
	33°C	07/ND	21/ND	27/ND		
JB5-G	39°C	23/5	20/4	58/14	RD	DNA synthesis decreases
	33°C	12/5	08/2	22/8		to 10 hrs., then rises
JB5-J	39°C	30/11	22/6	64/20	RD	Not ts
	33°C	17/7	23/5	43/8		
JB6-N	39°C	22/10	23/9	08/7	SG	Progressive decrease
	33°C	25/9	14/6	22/10		after five hours
JB7-K	39°C	21/6	12/4	10/8	SG	Progressive decrease
	33°C	13/5	09/3	18/11		in DNA synthesis
JB8-D	39°C	19/7	15/6	09/3	RD	Progressive decrease
	33°C	06/2	07/2	22/4		in DNA synthesis
JB8-G	39°	13/ND	18/ND	42/ND	SG	Not ts
	33°C	06/ND	18/ND	17/ND		
JB8-K	39°C	23/ND	18/ND	37/ND	RD	Not ts
	33°C	14/ND	20/ND	34/ND		
JB9-I	39°C	16/8	23/9	27/12	SG	Not ts
	33°C	05/3	08/3	17/4		

continued on the next page

Table 2 continued

Incorporation of ^{3}H-TdR and ^{35}S-met (CPM x 10^{-3})[a]

Cell line	Temp.	hours after temperature shift 0	5	25	Viability at 39.5°C	Remarks
JB10-F	39°C	04/ND	06/ND	33/ND	RD	Not ts
	33°C	04/ND	05/ND	16/ND		
JB10-M	39°C	08/ND	13/ND	17/ND	SG	Not ts
	33°C	06/ND	05/ND	11/ND		
JB10-O	39°C	18/7	15/5	10/6	SG	Progressive decrease
	33°C	07/3	09/3	11/4		in DNA synthesis
JB11-J	39°C	45/ND	32/ND	12/ND	RD	Rapid and progressive
	33°C	21/ND	19/ND	52/ND		decrease in DNA synthesis
JB12-C	39°C	40/15	31/9	53/27	SG	Not ts
	33°C	20/10	26/5	42/9		
JB12-D	39°C	28/ND	33/ND	31/ND	SG	Not ts
	33°C	18/ND	19/ND	38/ND		

a - cells were incubated for one hour intervals with radioactive precursors and assayed for TCA precipitable radioactivity as described in the Methods

b - incorporation of ^{3}H-TdR / incorporation of ^{35}S-met.

c - rapid death

d - slow growth

e - not determined

f - 10 hours after shift

isolated by a nonselective regimen. Additionally 9 mutants were not classified.

These preliminary labelling experiments were then repeated with the 20 mutants which were suspected of being ts DNA$^-$ in order to confirm their phenotype. Cultures at both temperatures were pulse labelled for 1 hour with ^{3}H-TdR and ^{35}S-methionine at 0, 5, 10, 15, 25, and 30 hours after shift to 39°C and the equivalent 0, 5, and 25 hours for those cultures left at 33°C. The results are summarized in Table 2. Ten of these mutants were re-catagorized as not tsDNA$^-$. This was not surprising as these mutants were borderline tsDNA$^-$ in the initial screen. Of the other 10 mutants which retested as good tsDNA$^-$ mutants (i.e. warranting further study) a variety of phenotypes can be observed. Some, such as JB3-B and JB7-K, demonstrate a progressive decline in DNA synthesis from time 0 (immediately after shift up to 39°C). For other mutants, such as JB3-O and JB6-N, there are brief lag periods prior to the decline in DNA synthesis. The mutants JB1-C and JB5-G show an initial decline in DNA synthesis followed by a rise which starts 15 and 10 hours after shift to the NPT, respectively. This variety of phenotypes suggests that these tsDNA$^-$ mutants may represent a collection of mutations affecting different genes. Previous isolations of ts mutants in DNA synthesis have resulted in the preferential isolation of mutants which fail to complement each other. All of those which have been mapped have been localized to the X chromosome. Therefore a preliminary complementation analysis

Table 3

Preliminary complementation analysis with ts DNA$^-$ mutants[a]

Cell Line[b]	JB1-C	JB1-R	JB3-B	JB3-O	JB5-G	JB6-N	JB7-K	JB8-D	JB11-J
JB1-C	-[c]	++	++	++	++	++	++	ND	++
JB1-R	++	-	+	+	+	+	+	+	++
JB3-B	++	+	-	+	-	+	+	+	++
JB3-O	++	+	+	-	+	-	++	++	ND
JB5-G	++	+	-	+	-	+	+	+	++
JB6-N	++	+	+	-	+	-	+	++	ND
JB7-K	++	+	+	++	+	+	-	-	ND
JB8-D	ND	+	+	++	+	++	-	-	ND
JB11-J	++	++	++	ND	++	ND	ND	ND	-

a - Cells were fused with PEG and plated directly for assessment of colony formation at 39.5°C as described in the text.

b - mutant cell lines as in Table 1. All other mutants were classified as containing revertants; i.e. colonies appeared when these cell lines were hybridized to themselves.

c - - = no colonies at 39.5°C
+ = few colonies at 39.5°C
++ = many colonies at 39.5°C
ND = not determined

was performed to determine if this collection of tsDNA$^-$ mutants represented one or more than one complementation group and if so, approximately how many groups are present. The cells were fused to each other with PEG as described in the Methods. One caveat in interpreting the data obtained from these experiments is that there is difficulty in confirming that the colonies which grow out are from hybrid cells or from revertants of one of the cell lines. However, an approximation of the number of revertants can be obtained by hybridizing each cell line to itself. Colony growth observed in such dishes would be due to revertants since a mutant cell line would not be expected to complement itself. The results of these experiments are presented in Table 3. These data indicate that there is more than one complementation group represented among these mutants. Thus the approach taken in the isolation of these mutants was successful in achieving a collection of ts DNA$^-$ mutants affected in more than one function (gene). However, a high incidence of apparent revertants was observed in some cases (data not shown). This could be due to a high genetic reversion frequency which one might expect in a functionally hemizygous region of the chromosome. For example, if the region is functionally hemizygous due to methylation of one set of alleles, then demethylation of those alleles may correct the defective allele. Alternatively, each mutant may represent a heterogeneous population of cells. In order to isolate the homogeneous populations which are required in the

further analyses of these tsDNA$^-$ mutants, ten mutants (JB1-C, JB1-R, JB3-B, JB3-O, JB5-G, JB6-N, JB7-K, JB8-D, JB10-O, and JB11-J) were seeded at low density at 33°C for recloning. Cultures were also seeded at 39°C to obtain an estimation of the incidence of reversion for each cell line prior to being recloned (i.e. to identify cell lines exhibiting high rates of reversion). The results can be seen in Table 4. Six clones which were isolated at 33°C were picked and grown up. Two cultures of each subline (designated /#, e.g. JB3-B/2) were seeded; one was shifted to 39°C and the other left at 33°C. The subline exhibiting the most rapid death at the NPT was the one used for all subsequent studies (the symbol /# is not used hereafter for convenience). Of the ten, the recloning had to be repeated for JB6-N and JB7-K due to poor growth at the initial low densities when seeded at 33°C or failure of the picked colonies to grow out or exhibit the ts phenotype (clones were isolated in a second attempt by seeding at higher cell densities and picking more colonies). JB1-C failed to die in mass cultures at 39°C, but formed colonies with significantly lower efficiency than at 33°C. JB5-G and JB10-O still demonstrated growth at 39.5°C indicative of high reversion frequencies or a leakiness of the defect. JB7-K grew well at 39°C at high cell density, suggesting that its growth at this temperature is density dependent (subsequent analysis of this mutant by another graduate student in this lab has confirmed this phenomenon). The pulse-label analysis was repeated on the recloned mutants to confirm their ts DNA$^-$

Table 4

Colony formation of ts DNA⁻ mutants prior to recloning[a]

Cell Line	33°C Cell number inoculated		39.5°C Cell number inoculated		
	20	200	2×10^3	1×10^4	2×10^5
JB1-C	6	141	0	10	TNTC[b]
JB1-R	6	57	0	0	ND[c]
JB3-B	6	120	0	0	0
JB3-O	1	21	0	0	0
JB5-G	6	53	1	113	TNTC
JB6-N	0	0	2	116	TNTC
JB7-K	0	15	0	0	+[d]
JB9-D	5	47	0	0	0
JB10-O	6	40	3	179	confluent
JB11-J	5	157	0	0	0

a - Cells were inoculated at 33°C and 39.5°C, and observed for colony formation over a four week period. Discrete colonies were picked as a source of recloned mutants for later study

b - not determined

c - too numerous to count

d - dish was contaminated with yeast but growing cells were evident

phenotype. The times of retesting at 39°C were 0, 2, 5, 10, 15, and 25 or 30 hours post-shift. Figure 4 depicts the the data obtained on macromolecular synthesis. All of the mutants tested showed a rapid inhibition of ^{3}H-TdR incorporation with delayed or no inhibition of protein synthesis upon shift to 39°C. Seven mutants exhibited progressive decreases from the initial time; two (JB11-J and JB8-D) maintained high incorporation of ^{3}-TdR for 5 to 10 hours followed by a progressive inhibition. The results were generally consistant with those in the mutants prior to recloning.

The recloned mutants were also tested for their growth properties - efficiency of cloning (EOC), reversion frequency, and growth curve - as described in the Methods. The results are presented in Table 5. All of the mutants display normal frequencies of colony formation at 33°C (58-100%). Some, however, exhibit moderate colony formation at 39°C (1 in 10^3 to 1 in 10^4 cells plated). This occurs particularly in the mutants which were derived from the long selection. For these mutants an effort was made to determine whether the surviving cells at 39°C (after 16 days) were true revertants or a phenotypic property of the cell line. Survivors were picked and grown out at 33°C. These cells were reseeded for EOC determination as before. For the cell lines JB5-G and JB10-O, cells which were seeded at 10^4 or higher grew to confluence after shift to 39°C. The ratio of EOC (33°C/39°C) for JB5-G survivors was 2.1 X 10^3 (the ratio for JB10-O was not determined) as compared to 1.2 X10^4 in the

FIGURE 4. - Macromolecular synthesis in temperature-sensitive mutants. Cultures of each cell line were seeded and incubated at 33°C for 40 hours; half were shifted to 39.5°C at time 0. Cultures were pulse-labelled with ^{3}H-TdR (●—) or ^{35}S-methionine (o--) at appropiate intervals and analyzed for radioactivity as described in the Methods. WT, wild-type.

FIGURE 4

Macromolecular synthesis in temperature-sensitive mutants.

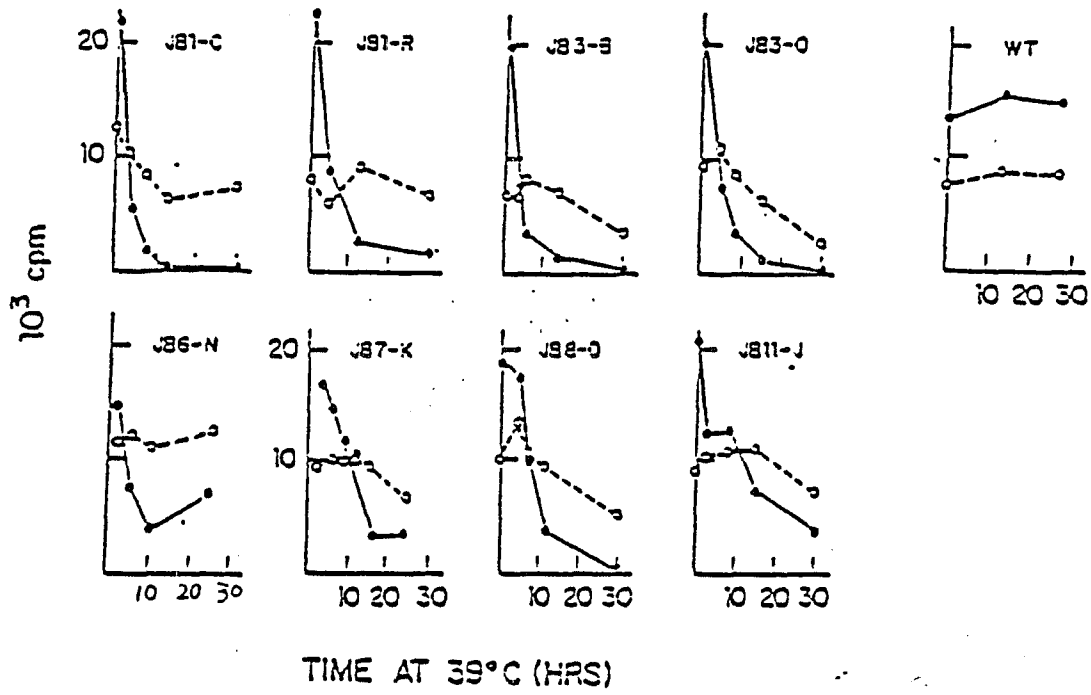


Table 5

Growth properties of ts DNA$^-$ mutants of CHO cells

CELL LINE	PARENT STRAIN	SELECTION	EOC (%) 33°C	EOC (%) 39°C	RATIO 33°C / 39°C	COLONY MORPHOLOGY AT 39°C
JB1-C	CHO-K1	I[b]	86	0.01	$1X10^4$	smaller size than 33°C
JB1-R	CHO-S	I	48	0.005	$\geq 1X10^4$	normal appearance
JB3-B	CHO-S	II[c]	58	0.0002	$3X10^5$	revertants only
JB3-O	CHO-S	I	60	<0.005	$\geq 6X10^4$	microcolonies only no revertants
JB5-G	CHO-K1	I	50	0.004	$1X10^4$	vigorously growing revertants only
JB6-N	CHO-S	I	48	<0.005	$\geq 1X10^4$	microcolonies only no revertants
JB7-K	CHO-S	I	60	<0.005	$\geq 1X10^4$	microcolonies only no revertants
JB8-D	CHO-K1	II	100	<0.005	$\geq 2X10^4$	microcolonies only no revertants
JB10-O	CHO-S	I	30	0.02	$2X10^3$	revertants only
JB11-J	CHO-S	III[d]	87	0.0001	$1X10^6$	revertants only

a - Cells were seeded at various densities to determine EOC at 33°C and 39°C.

b - Single long selection

c - multiple short selection

d - non-selective replica plating.

Table 6

Growth properties of mutants maintained at 39.5°C[a]

Cell line	Efficiency of colony formation Ratio 33°C / 39.5°C: Cells maintained at 33°C[b]	Cells maintained at 39.5°C for two weeks
JB1-C	1×10^4	1.6×10^4
JB1-R	$\geq 1 \times 10^4$	4.8×10^5
JB3-O	$\geq 6 \times 10^4$	7.5×10^3
JB8-D	$\geq 2 \times 10^4$	1.8×10^4

a - Mutant cells were plated at 39.5°C at 1×10^4 cells per 60mm dish and incubated for two weeks until the appearance of microcolonies. The cultures were then trypsinized and reseeded at 33°C and 39.5°C for assessment of colony formation. Colonies at 39.5°C were typically microcolonies.

b - data obtained from Table 4.

original determination (see Table 5). This could be indicative of an enrichment for revertants, although it is not as great a change as one would have expected, or of a leakiness in the defect which allows the limited growth of cells at the NPT while impairing DNA synthesis. Growth curve data for JB5-G and JB10-0 also supports these explanations in that only these cell lines are capable of growth at 39°C in mass culture. The labelling data indicate that for JB10-O the inhibition of DNA synthesis is gradual (maintaining almost a constant level, data not shown), for JB5-G there is a delayed inhibition followed by a rise. Taken together these data indicate that both JB5-G and JB10-O possess a phenotypic leakiness in the defective function at the NPT. This degree of leakiness does not make these mutants good candidates for further analysis and thus both mutants were frozen down and are no longer used. Survivors at 39°C of the cell lines JB1-R, JB1-C, JB3-O, and JB8-D were also tested. The results indicated that some growth at 39°C (formation of microcolonies) was a phenotypic property of these cell lines. Table 6 briefly summarizes these results. As can be seen, the ratios (33°C / 39°C) of the survivors are similar to those obtained for the original cultures for these four mutants. Additionally, colonies large enough to be classified as true revertants appeared at the frequencies of approximately 1 per 10^6 cells plated for two of these mutants (JB1-R and JB3-O). The growth curve data for the mutants is summarized in Table 7. For the cell line JB1-C the slight rise in cell number

observed after 50 hours is most likely due to the growth of the microcolonies which this cell line exhibits as a phenotypic property at 39°C (see tables 5 and 6). None of the other cell lines exhibited any growth at 39°C (except JB5-G and JB10-O as mentioned above). A variety of growth rates (as evidenced by estimated cell doubling times) can be observed for these cell lines, all of which are slower than the wild-type. This could be indicative of the mutant DNA$^-$ phenotype being expressed to a certain degree at 33°C. Alternatively it could be due to an additional effect of the mutagenesis (i.e. another function(s) mutated in such a manner as to slow growth in a temperature-independent manner).

In an effort to gather more information about the effect of the mutated function in these ts DNA$^-$ mutants, two more properties of these mutants were assayed - EOC at various intermediate temperatures (33°C to 39.5°C) and loss of viability after increasing lengths of exposure to the nonpermissive temperature for cell growth (survival curve). The data obtained by determining the EOC at a series of temperatures gives a picture of the growth range of each mutant which in turn is an indication of the lability of the affected function. Additionally, this phenotypic property of the mutant could be exploited in future analyses (e.g to enhance the growth of cells which are under stress due to experimental conditions; see Chapter 3 on cloning). The results of this assay are shown in Table 8. The cell lines JB1-R, JB1-C, and JB8-D display a sharp decrease in viability

Table 7

Growth rates of ts DNA$^-$ mutants[a]

Cell line	Culture doubling time (hours) 33°C	39.5°C	Appearance at 39.5°C
CHO-S	16	11	Normal
JB1-C	23	>48	Some increase in cell number after 50 hours
JB1-R	27	>60	Loss of cells after 24 hours
JB3-B	28	>60	No growth. Floating cells after 12 hours; loss of cells after 24 hours
JB3-O	21	>60	No growth
JB5-G	17	37	Doubling time at 39.5°C decreases with longer incubation at 39.5°C
JB6-N	21	ND[b]	
JB7-K	24	ND	
JB8-D	21	>60	No growth
JB10-O	ND	ND	Grows more rapidly at 39.5°C
JB11-J	20	>60	No growth

a - Cells were plated at 2.5 x 10^4 cells per 60mm dish and cell number determined at 4 - 12 hour intervals as described in the Methods.

b - not determined

Table 8

Efficiency of colony formation at different temperatures[a]

Cell line	Temperature (degrees Centigrade) 33	34.5	37	38.5	39.5	41
JB1-C	69	80	82	0	2[c]	0
JB1-R	82	35	68[b]	0	0	0
JB3-B	58	58	13	5	0	-
JB3-O	81	75	5	0	0	0
JB8-D	102	108[b]	85	-	0	0
JB11-J	87	84	28	-	0	-

a - One hundred cells were plated at each temperature and observed for 16 days for colonies to appear. Numbers are the average of triplicate dishes

b - colonies larger than at 33°C

c - microcolonies

between 37°C and 38°C. The cell lines JB3-B, JB3-O, and JB11-J display a more gradual decrease in viability above 34.5°C with significant loss of viability observed even at 37°C.

Survival curves were determined by incubating cells at low density at 39.5°C for periods of a few hours to several days before returning them to 33°C for colonies to appear. The cultures were stained 14 days later. The data obtained by this assay provide information on another phenotypic property of these mutants; that is, the ability of the mutated function to support cell viability for some time at the nonpermissive temperature. It is a complex phenomonology most likely reflecting the interaction of the mutated phenotype with other cellular processes. The results of the survival curves which I performed on these ts DNA$^-$ mutants are shown in Table 9. Four of the mutants display a gradual loss of viability until a point is reached at which less than one percent of the cells are viable. One mutant, JB1-C, exhibits a similar gradual loss of viability, although it reaches a point at which 25% to 30% of the cells remain viable up to four four days at the NPT. This result is in accordance with the observation that JB1-C forms small colonies at the nonpermissive temperature and is suggestive of a mutation which results in a G0-like arrest at the NPT. One mutant, JB3-B, exhibits a more rapid loss of viability than the others.

Table 9

Survival of colony formation after incubation at 39.5°C[a]

Cell line	Hours at 39.5°C										
	0	5-9	23-27	39-43	48	51-53	60-69	84-95	120	139	160
JB1-C	69[b]	64	36	28	-	30	26	24	-	-	-
JB1-R	82	59	57	19	-	15	4	2	-	-	-
JB3-B	58	49	4	-	0	0	0	-	0	-	-
JB3-O	81	-	-	13	-	-	6	0.7	0	0	0
JB8-D	102	-	-	67	-	-	20	0.7	0	0	0
JB11-J	87	62	26	-	4	2	0.4	-	0	-	0

a - One hundred cells were plated at 33°C for 16 hours and shifted to 39.5°C for the indicated periods of time. They were then returned to 33°C for an additional 16 days for colonies to appear. Numbers are the average of triplicate dishes

b - number of colonies per 100 cells plated.

In an effort to facilitate the further characterization of these mutants, I examined the effect of the mutation on the ability of the cells to support viral DNA synthesis at the permissive and restrictive temperatures. This approach offers several potential advantages. First and foremost, it identifies mutants which are amenable to intensive biochemical investigations (i.e. in vitro analysis - see Introduction). However, the approach also permits an initial characterization of the primary defect as well, limiting greatly the number of possible biochemical bases for the ts DNA^- phenotype (Tooze 1980). A differential effect on two classes of viruses could be particularly informative.

Ad2 was chosen for initial studies because of the ability of CHO cells to support appreciable levels of viral replication (Longiaru and Horwitz 1981, Radna et al. 1987), its amenablity to biochemical analysis both in vivo and in vitro (Longiaru and Horwitz 1981, Challberg and Kelly 1979), and the multifold dependence of Ad2 on host cell functions for viral replication. In order to look at Ad2 DNA replication in these ts DNA^- mutants I have employed a simple methodology which determines the accumulation of nascent viral DNA and enabled me to screen a large number of mutants easily. In the initial experiments, mutant cell lines were infected at a multiplicity of infection (MOI) of 40 with Ad2 virions at 33°C for 2 hours and either shifted to 39°C or reincubated at 33°C. Viral DNA was quantitated by "dot blotting" as described in the Methods. Representative

Figure 5. - Dot blot analysis of Ad2 infected CHO-S. Cells were infected at an MOI of 40 with Ad2 virions. Viral DNA was extracted at different times p.i. and quantitated by dot blot hybridization with nick-translated pLA1 DNA as described in the Methods. All DNA was diluted serially tenfold. Infected cells were incubated at 39.5°C (lanes 1 - 10) or 33°C (lanes 11 - 20). DNA was extracted p.i. at 4 hours (lanes 1,2,11,12), 15 hours (lanes 3,4,13,14), 25 hours (lanes 5,6,15,16), 35 hours (lanes 7,8,17,18), and 42 hours (lanes 9,10,19,20).

Figure 5

Dot blot analysis of Ad2 infected CHO-S.

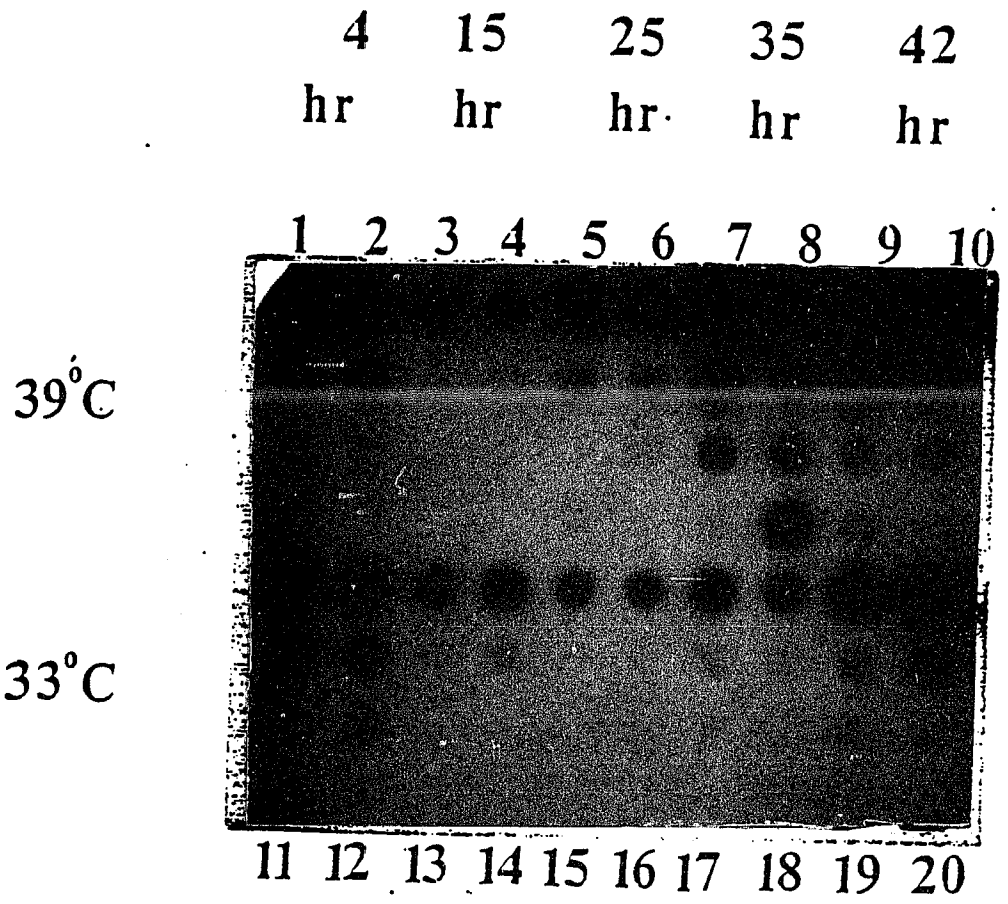


Figure 6. - Dot blot analysis of Ad2 infected JB3-B. Procedures were as described in Figure 5. Infected cells were incubated at 33°C (lanes 1 - 8) and 39.5°C (lanes 9 - 13,15,16). DNA was extracted p.i. at 24 hours (lanes 1,2,9,10), 48 hours (lanes 3,4,11,12), 72 hours (lanes 5,6,13), and 96 hours (lanes 7,8,15,16). Lane 14 contains DNA from uninfected cells.

Figure 6 .

Dot blot analysis of Ad2 infected JB3-B.

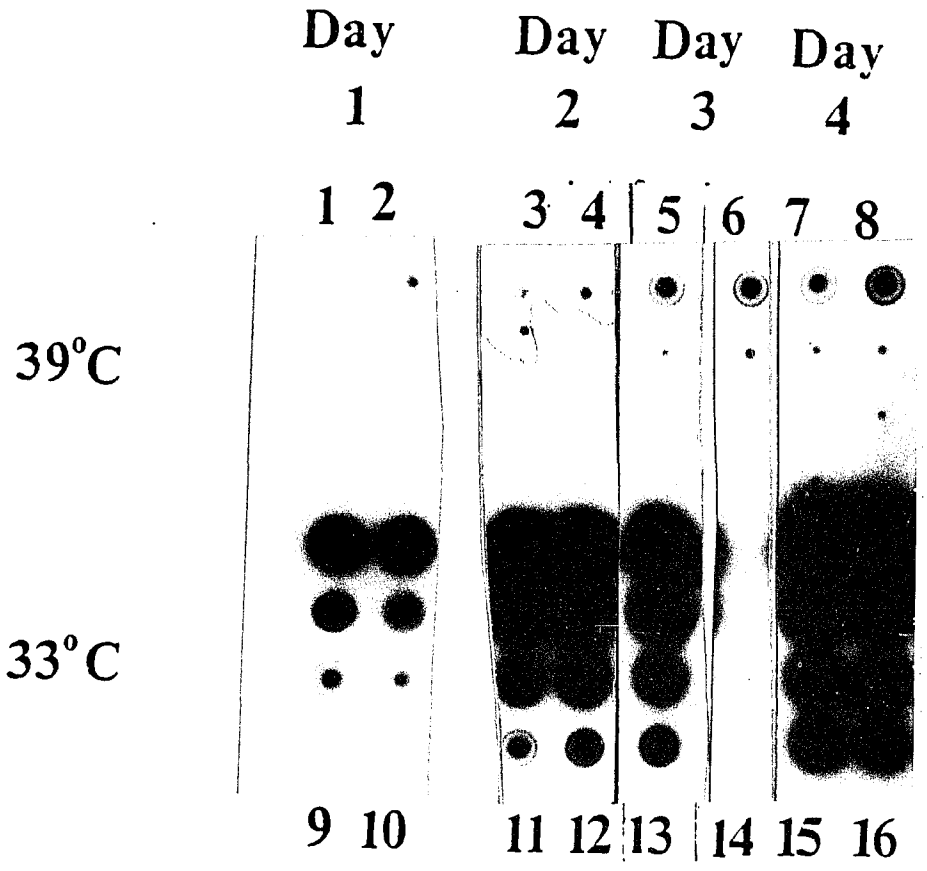
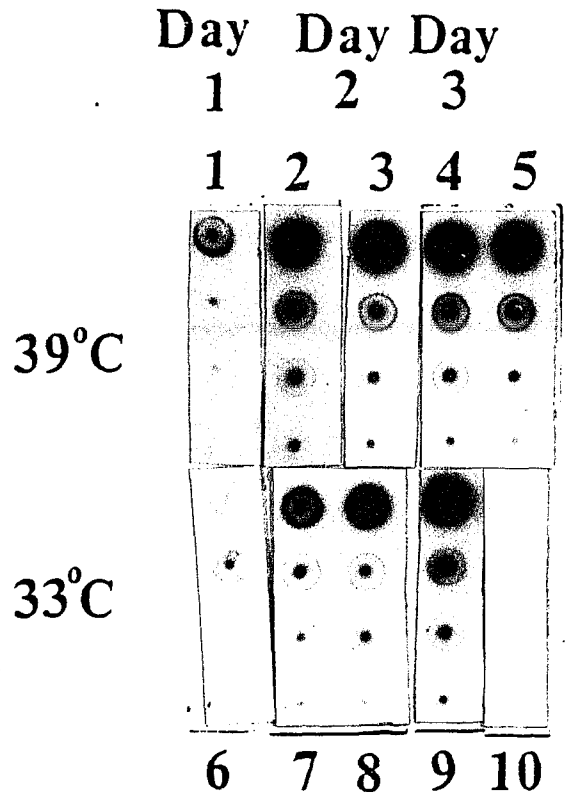


Figure 7. - Dot blot analysis of Ad2 infected JB1-R. Procedures were as described in Figure 5. Lane 5 contains tenfold dilutions of pLA1 from 1.0 ug. to 1.0 ng.. Infected cells were incubated at 33°C (lanes 6 - 10) and 39.5°C (lanes 1 - 4). DNA was extracted p.i. at 24 hours (lanes 1 and 6), 48 hours (lanes 2,3,7,8), and 72 hours (lanes 4 and 9). Lane 10 contains DNA from uninfected cells.

Figure 7

Dot blot analysis of Ad2 infected JB1-R.



results of these initial experiments are shown in Figures 5 through 7. Figure 5 describes the time course of Ad2 infection in wild-type CHO-S cells at both 33°C and 39.5°C using a series of 10-fold dilutions. As can readily be seen in this blot, the time course of Ad2 replication is accelerated at 39.5°C as compared to 33°C. The input Ad2 DNA can be observed at 4 hours p.i. at both 33°C and 39.5°C. By 15 hours p.i., the replication of Ad2 DNA can be detected as increased, consistant with the previously published data of Longiaru and Horowitz (1981). At 25 hours p.i. tenfold greater nascent Ad2 DNA has accumulated at 39.5°C than at 33°C. By 35 and 42 hours p.i. this difference appears to increase somewhat. Thus if any of the mutants display equal or greater accumulations of nascent Ad2 DNA at 39.5°C than at 33°C, it can be classified as wild-type like for Ad2 synthesis; that is, the mutated cellular function is not a function which is required for Ad2 DNA synthesis.

Figure 6 describes the time course of Ad2 replication in the ts DNA$^-$ mutant JB3-B using a series of 10-fold dilutions. As can be seen, this mutant supports the replication of Ad2 DNA at the permissive temperature, with the accumulation of nascent Ad2 DNA rapidly increasing to 48 hours (detectable in a 1000-fold dilution) and accumulating at a slower rate thereafter. However, at the restrictive temperature, very little accumulation of nascent Ad2 DNA can be observed (barely detectable with 9/10 of the sample on the filter), indicating that little or no Ad2 DNA replication has

occurred. Thus the mutated cellular function in JB3-B is one which is required for Ad2 DNA replication as well.

Figure 7 describes the time course of Ad2 DNA replication in JB1-R. As can be seen, replication of Ad2 DNA occurs equally well at both temperatures up to 72 hours p.i. (detectable in a 100-fold dilution at 48 and 72 hrs. p.i.). Thus it can be concluded that the mutated gene product is one which is required for cellular DNA replication but is not required for Ad2 DNA replication. An analysis of the mutant JB11-J yielded similar results.

Several conclusions can be made can be made from these results. First, of the mutants screened by these initial experiments only JB3-B demonstrates a dramatic temperature dependent restriction on Ad2 DNA replication. Second, the mutants JB1-R and JB11-J support the replication of Ad2 DNA at both the permissive and restrictive temperatures, as does wild-type CHO-S, and thus have been classified as wild-type like. Third, the analyses of the mutants JB1-C, JB3-O, and JB8-D yielded inconclusive data due to the insufficient accumulation of nascent viral DNA at either temperature. Infecting these cell lines at higher MOI should allow their analysis to proceed. Thus all subsequent analyses of these and other mutants was performed by infecting at MOIs of approximately 400. Fourth, no essential data can be obtained by harvesting viral DNAs at times greater than 50 hours p.i.. Thus viral DNA will be harvested at 24 and 48 hours p.i. for each temperature. The data could also be improved by

decreasing the incremenent of dilution from tenfold to threefold while increasing the number of dilutions made. Additionally, another modification of the protocol was made. The reduced level of viral DNA observed for the mutant JB3-B could be explained by multiple mechanisms, some of which would be expected to be secondary to the arrest of cell growth (i.e. cell death or cell loss at the restrictive temperature). To distinguish further between such and a primary defect, cultures were infected at 33°C, incubated at 33°C for 24 hours, and shifted to 39°C for an additional 24 hours.

All of the mutants in this collection as well as two others were analysed by this protocol. They could be classified into three groups based on this analysis. Group 1 includes those that support Ad2 DNA replication at both temperatures, designated wild-type like. Group 2 are those that exhibit some degree of temperature dependence when the data obtained at 33°C is compared to that obtained at 39.5°C; but analysis of the shift up data indicate that the defect is not the primary cause of viral inhibition. Group 3 are those mutants for which all data demonstrate an inhibition at 39.5°C under all conditions, indicative of a primary defect in Ad2 DNA replication.

Figure 8 presents the data obtained by this protocol for the mutant JB11-J. At 24 hours p.i. there is a 30-fold increase in nascent viral DNA at 39.5°C as compared to 33°C. Similar amounts of viral DNA can be observed at 48 hours p.i.

Figure 8. - Ad 2 DNA replication in JB11-J. Dot blot analysis was performed as described in the Methods. Lane 1 contains tenfold concentrations of pLA1 DNA from 0.1 pg. to 1.0 ug.. All other DNA was diluted serially threefold. The DNA in lane 2 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 3 (33°C) and 4 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 5 (33°C) and 6 (39.5°C) were extracted at 48 hours p.i.. The DNA in lanes 7 and 8 are from duplicate infected cultures which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 8

Ad 2 DNA replication in JB11-J.

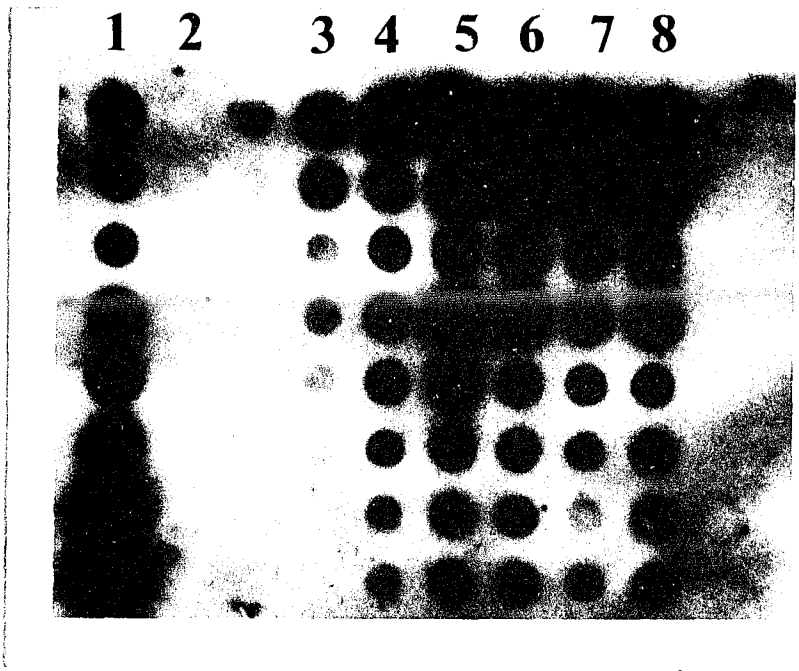


Figure 9. - Ad 2 DNA replication in JB1-R. Dot blot analysis was performed as described in the Methods. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. The DNA in lane 2 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 3 (33°C) and 4 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 5 (33°C) and 6 (39.5°C) were extracted at 48 hours p.i.. The DNA in lanes 7 and 8 are from duplicate infected cultures which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 9

Ad 2 DNA replication in JB1-R.

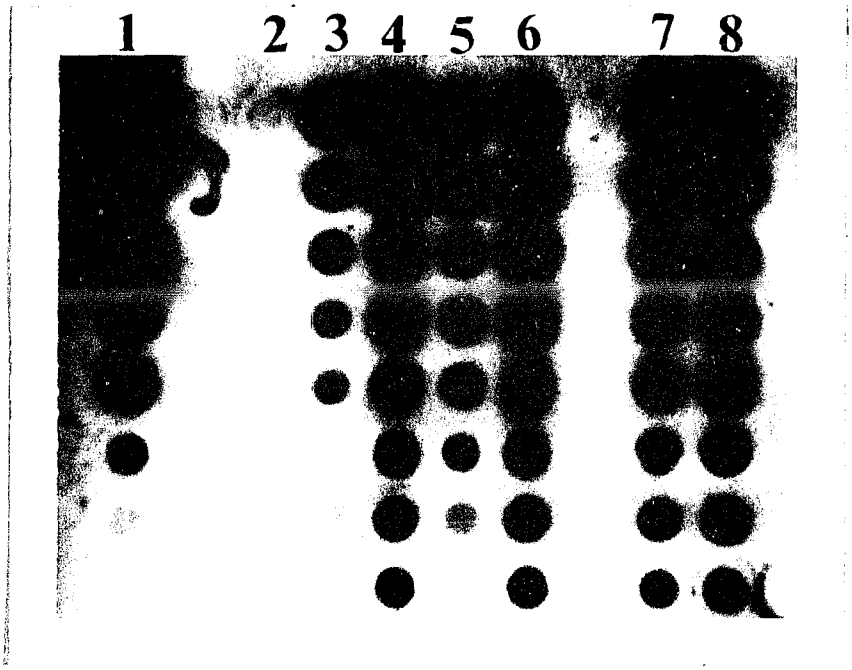


Figure 10. - Ad 2 DNA replication in JB10-O. Dot blot analysis was performed as described in the Methods. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. The DNA in lane 2 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 3 (33°C) and 4 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 5 (33°C) and 6 (39.5°C) were extracted at 48 hours p.i.. The DNA in lane 7 is from infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 10

Ad 2 DNA replication in JB10-O.

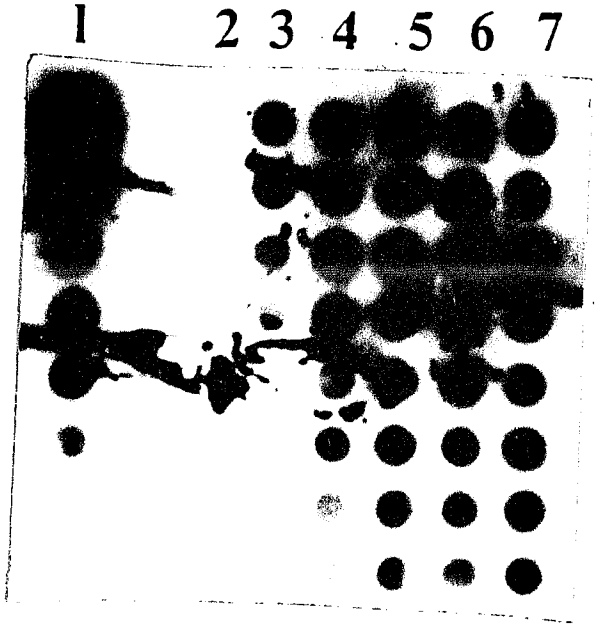


Figure 11. - Ad 2 DNA replication in tsC8. Dot blot analysis was performed as described in the Methods. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. The DNA in lane 4 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 2 (33°C) and 3 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 5 (33°C) and 6 (39.5°C) were extracted at 48 hours p.i.. The DNA in lane 7 is from infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 11

Ad 2 DNA replication in tsC8.

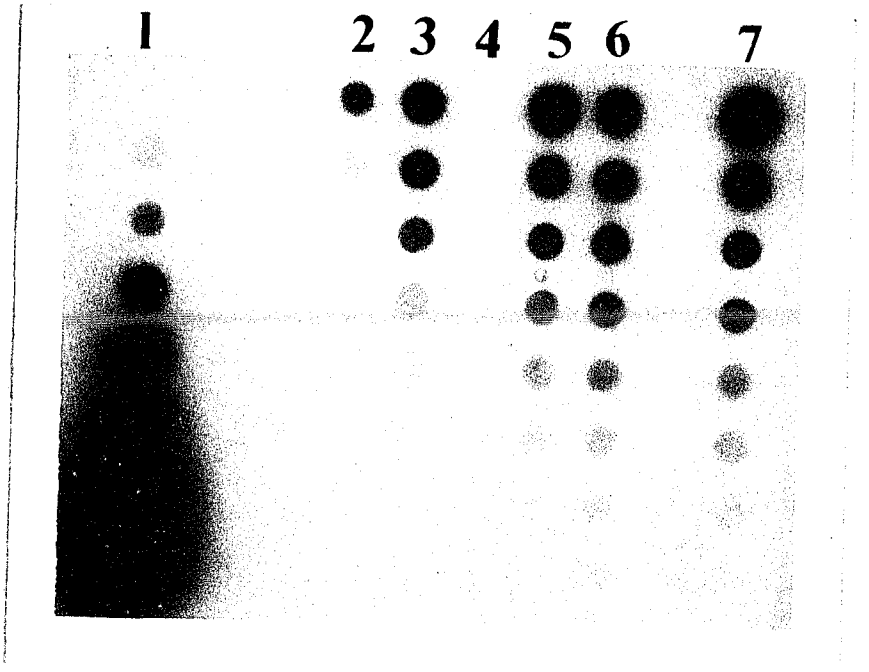
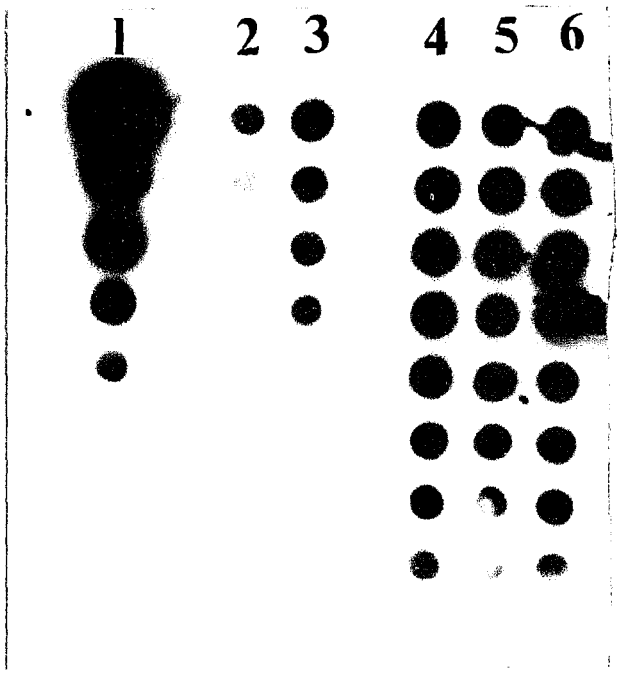


Figure 12. - Ad 2 DNA replication in ts13A. Dot blot analysis was performed as described in the Methods. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 2 (33°C) and 3 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 4 (33°C) and 5 (39.5°C) were extracted at 48 hours p.i.. The DNA in lane 6 is from infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 12

Ad 2 DNA replication in ts13A.



for both temperatures. A comparison of the amount of viral DNA accumulated in cultures incubated for 24 hours at 33°C followed by 24 hours at 39.5°C (the 24 hr./24 hr. shift up lanes) to the amount accumulated after 24 hours at 33°C indicates that a large amount of DNA was replicated after shift to 39.5°C. Therefore, it can be concluded that the preliminary analysis was correct. JB11-J supports the replication of Ad2 DNA at both temperatures and thus falls into the first group - wild-type like. Clearly better (more quantitative) data are obtained due to the dilution scheme employed. Also there were no adverse effects of the increased MOI observed.

The data obtained for the mutant JB1-R can be seen in Figure 9. From the data presented it can be seen that viral DNA replicates at least 80-fold greater at 39.5°C than at 33°C by 24 hours p.i.. As was the case for JB11-J, these data support the conclusion derived from the initial screen that JB1-R is wild-type like. The mutant JB10-O was also subjected to this analysis as a example of a mutant which exhibits some growth at 39.5°C. The results are shown in Figure 10. The data similarly demonstrate that this mutant is wild-type like.

In addition to the mutants in this collection two other mutants were screened; tsC8, a temperature-sensitive CHO cell mutant, obtained from A. A. McCraken (1982), and ts13A, a temperature-sensitive CHO cell mutant obtained from L. Siminovitch (Srinivasan et al. 1980). The results are shown

in Figures 11 and 12, respectively. Viral replication results in an accumulation of greater amounts of nascent DNA at 39.5°C than at 33°C up to 48 hours p.i. for both of these mutant cell lines. Therefore both of these ts mutants have been classified as wild-type like.

Figure 13 presents the results of Ad2 DNA replication in the mutant JB1-C. The first conclusion which can be made from these data is that the higher MOI increased the signals to measureable levels. A comparison of the viral DNA levels at 24 hours p.i. shows that the viral DNA is equally capable of being replicated at both temperatures. By 48 hours p.i. the viral DNA has been replicated to a greater extent at 33°C than at 39.5°C, suggesting that Ad2 DNA replication is restricted at 39.5°C in this mutant. However, a comparison of of the amount of viral DNA accumulated in the 24 hour period at 39.5°C following a 24 hour period at 33°C to the amount accumulated after only 24 hours at 33°C demonstrates that a significant amount of viral DNA replication has occured at 39.5°C. These data thus indicate that the decrease in Ad2 DNA replication observed at 39.5°C at 48 hours p.i. is most likely due to a secondary effect of the cellular defect on viral replication. Based on this analysis, JB1-C has been placed into group 2. Four other mutants behave in a similar fashion: JB8-D, JB3-O, JB7-K, and JB6-N. The data for these mutants are shown in Figures 14,15,16, and 17, respectively.

The only mutant to demonstrate a restriction of Ad2 DNA replication at 39.5°C in the initial screen was JB3-B. The

Figure 13. - Ad 2 DNA replication in JB1-C. Dot blot analysis was performed as described in the Methods. Lane 7 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. The DNA in lane 8 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 1 (33°C) and 2 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 3 (33°C) and 4 (39.5°C) were extracted at 48 hours p.i.. The DNA in lanes 5 and 6 are from duplicate infected cultures which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 13

Ad 2 DNA replication in JB1-C.

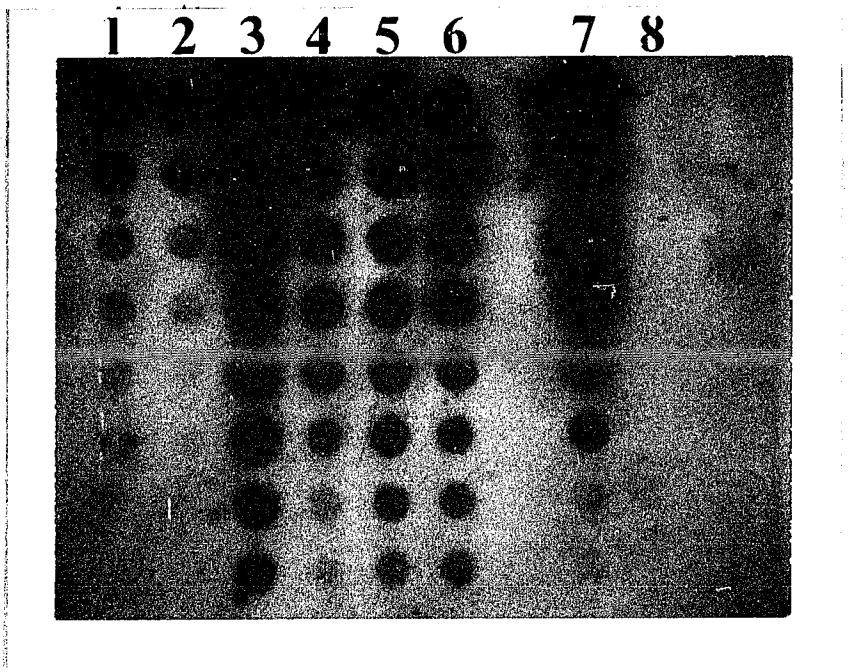


Figure 14. - Ad 2 DNA replication in JB8-D. Dot blot analysis was performed as described in the Methods. Lane 7 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. The DNA in lane 1 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 2 (33°C) and 3 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 4 (33°C) and 5 (39.5°C) were extracted at 48 hours p.i.. The DNA in lane 6 is from infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 14

Ad 2 DNA replication in JB8-D.

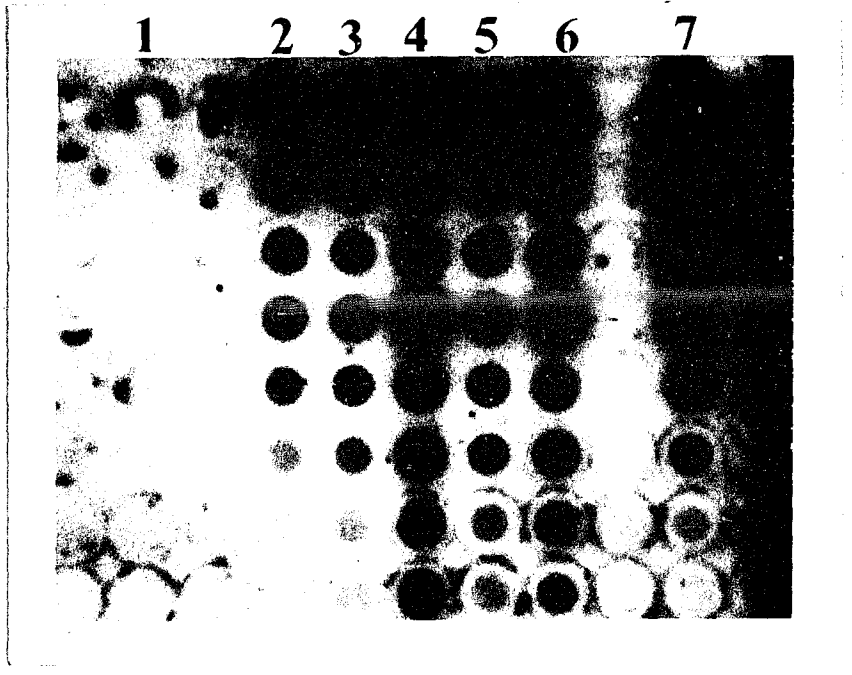


Figure 15. - Ad 2 DNA replication in JB3-O. Dot blot analysis was performed as described in the Methods. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. The DNA in lane 2 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 3 (33°C) and 4 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 5 (33°C) and 6 (39.5°C) were extracted at 48 hours p.i.. The DNA in lane 7 is from infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 15

Ad 2 DNA replication in JB3-O.

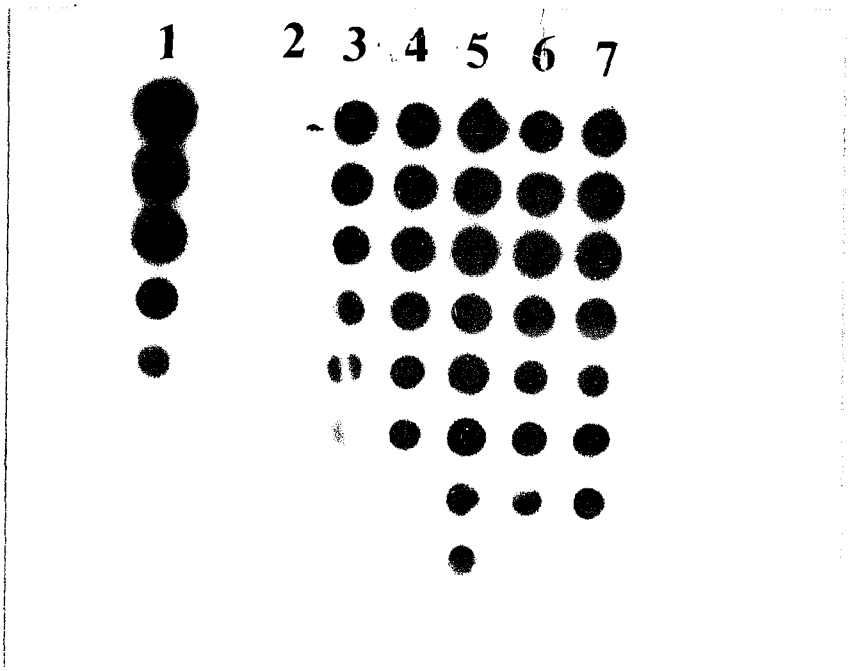


Figure 16. - Ad 2 DNA replication in JB7-K. Dot blot analysis was performed as described in the Methods. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. The DNA in lane 2 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 3 (33°C) and 4 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 5 (33°C) and 6 (39.5°C) were extracted at 48 hours p.i.. The DNA in lane 7 is from infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 16

Ad 2 DNA replication in JB7-K.

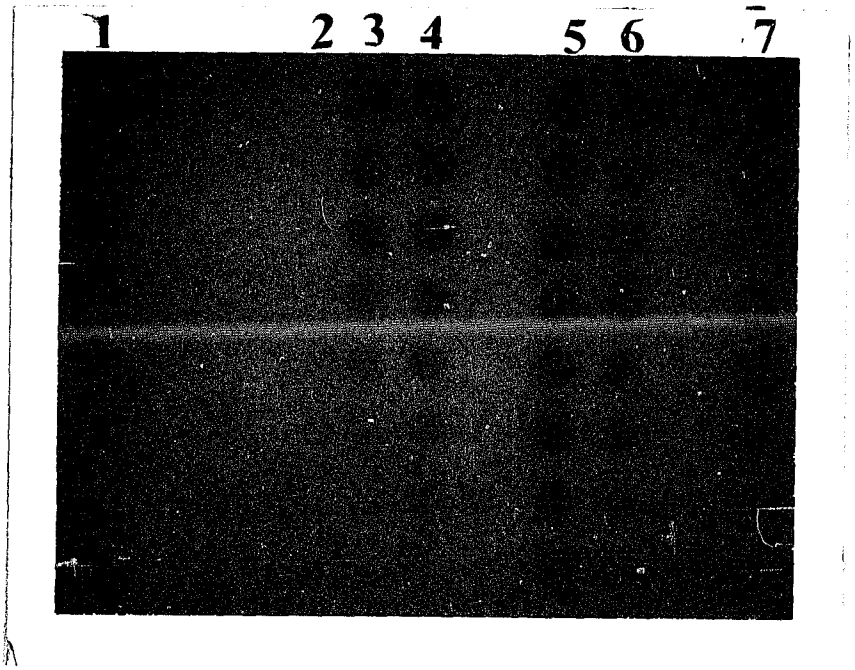


Figure 17. - Ad 2 DNA replication in JB6-N. Dot blot analysis was performed as described in the Methods. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 2 (33°C) and 3 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 4 (33°C) and 5 (39.5°C) were extracted at 48 hours p.i.. The DNA in lane 6 is from infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 17

Ad 2 DNA replication in JB6-N.

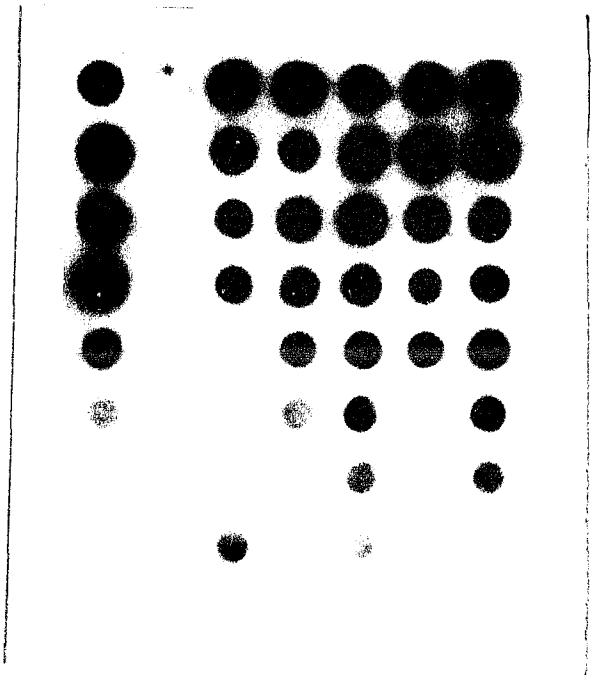
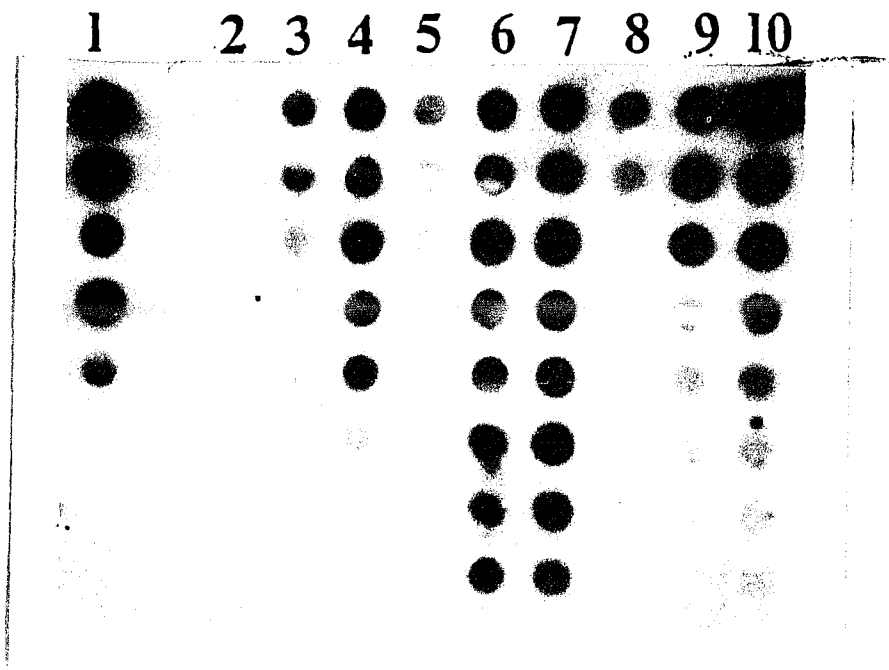


Figure 18. - Ad 2 DNA replication in JB3-B. Dot blot analysis was performed as described in the Methods. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. The DNA in lane 2 was extracted from uninfected cells. Infected cells were incubated at 33°C, 37°C, and 39.5°C. DNA in lanes 3 (33°C), 4 (37°C), and 5 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 6 (33°C), 7 (37°C), and 8 (39.5°C) were extracted at 48 hours p.i.. The DNA in lanes 9 and 10 are from duplicate infected cultures which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 18

Ad 2 DNA replication in JB3-B.



results of the second screening are presented in Figure 18. This confirms that there is a marked inhibition of viral DNA replication in cultures which are maintained at 39.5°C throughout infection. Moreover the cultures which were incubated initially at 33°C before shift to 39.5°C showed at least a tenfold reduction in the accumulation of nascent viral DNA as compared to those incubated continuously at 33°C. This result is indicative of a rapid primary defect in Ad2 DNA replication. JB3-B therefore is the sole member of group 3, consistant with a primary effect of the cellular mutation on viral replication. Viral DNA synthesis does occur at the intermediate temperature of 37°C.

The results of the second Ad2 infections are summarized in Table 10. Of the 12 temperature-sensitive mutants screened, six display wild-type like phenotypes, five fell into the second group - exhibiting a delayed restriction of viral replication which is probably secondary to the effect on the cellular defect, and one, JB3-B, inhibited viral DNA replication in a direct manner. No mutant proved to be unanalysable by this protocol. For the mutants in groups 1 and 2 this analysis indicates that their mutated function is not required for the replication of Ad2. This aids in their further characterization by eliminating a number of the biochemical bases for their ts DNA$^-$ phenotypes. However this also means that Ad2 cannot be used as a probe for the further elucidation of the affected function. Alternately, for JB3-B the mutated function has been proven to be one which is

Table 10

SUMMARY OF VIRUS REPLICATION STUDIES[a]

CELL LINE	ADENOVIRUS 2	POLYOMA
JB1-C	+	+
JB1-R	+	+
JB3-B	-	-
JB3-O	+	-
JB6-N	+	+
JB7-K	+	-
JB8-D	+	-
JB11-J	+	-
tsC8	+	+
ts13A	+	+

a - + indicates that viral replication is supported at the temperature restrictive for cellular growth (39°C)
- indicates that the cell line fails to support viral replication at 39°C. All cell lines support viral replication at 33°C.

directly required for Ad2 replication. This function is now amenable to more intensive biochemical investigations utilizing Ad2 as a probe of this system in much the same manner as phages and plasmids have been used to probe bacterial replication. These results indicate that divergent approaches are required for the further characterization of this mutant collection. I decided to pursue the further characterization of JB3-B utilizing Ad2 rather than continuing to screen the collection.

Subsequently the collection of cell mutants was also screened for their ability to support Polyoma (Py) replication by others in the laboratory (Dermody et al. 1986)). Since CHO cells are resistant to infection by papovavirus virions, a method was utilized which introduced viral DNA into the cells and measure replicated molecules (LaBella and Ozer 1985). Briefly, a recombinant DNA, containing the Py genome methylated by amplification in dam^+ bacteria, was introduced into cultures of the ts DNA^- mutants by DNA-mediated gene transfer. Replicated recombinant DNA was harvested by Hirt lysis and identified after digestion with DpnI, which digests methylated (imput) but not unmethylated (replicated) DNA, by Southern analysis. All mutants support Py replication at the permissive temperature for cell growth. A summary of the results at the restrictive temperature, with the corresponding Ad2 results is presented in Table 10. It can be seen that four of the mutants which supported Ad2 replication at the restrictive temperature also support Py

replication. For these mutants, Py, like Ad2, could not be used as a probe of cellular function and will most likely have to be characterized by other means. Four of the other mutants which support Ad2 replication at the restrictive temperature fail to support Py replication at the same temperature. This limits the possible bases of their biochemical defect to cellular functions which are required for Py but not for Ad2 replication (e.g. initiation factors) and eliminates those functions which are required for both (e.g. nucleotide pools).

The mutant JB3-B restricts the replication of both viral DNAs at 39.5°C. This limits the possible biochemical basis of the mutated function to those functions which are required for the replication of both viruses while eliminating those required for one but not the other. For example, it is not probable that the defect is in the priming mechanism as Ad2 and Py use different mechanisms (see Introduction). Likewise, the defect would not involve chromatin proteins as only Py DNA is present in this form. However the nuclear matrix could be involved with the defective function, as evidence is accumulating that it plays a role in the initiation of both viruses. Since the replication of both these viral DNAs are cell cycle independent under the conditions used for these experiments, there is a low probability of the defect involving a function required solely for cell cycle progression. It is generally believed that both of these viruses employ some aspects of the host elongation apparatus

in the replication of their genomes, although Ad2 doesn't undergo discontinuous strand synthesis. Therefore it is probable that a defect in continuous strand synthesis is responsible for the ts DNA$^-$ phenotype. Especially likely is a defect in the nucleotide pools, which is a common requirement of all DNA viruses.

CHAPTER 2 - Analysis of JB3-B

Based on the data previously generated, JB3-B is a mutant highly worthy of further characterization. It was isolated by a short selection protocol not employed outside of this collection. (Within this collection, the only other ts DNA$^-$ mutant isolated by this protocol is JB8-D.) The kinetics of DNA synthesis at the restrictive temperature shows a rapid inhibition expected of a ts mutant with a primary defect in DNA synthesis. A preliminary complementation analysis suggests that the gene which is mutated in JB3-B is unique from the rest of the collection. Furthermore, viral analysis demonstrates that JB3-B fails to support the replication of either Ad2 or Py at the temperature which is restrictive for cell growth but supports their replication at the permissive temperature. These data also indicated that the inhibition of DNA synthesis was a direct result of the mutation. The cell cycle independence of both Ad2 and Py under the conditions employed in those experiments have suggested that the inhibition of DNA synthesis observed at 39.5^{o}C was not a result of a growth arrest at a stage of the cell cycle other than S phase. In an effort to assess this possibility directly, an analysis of the cell cycle at 33^{o}C and 39.5^{o}C using flow microfluorimetry (FMF) was performed in collaboration with Dr. Frank Traganos at the Memorial Sloan Kettering Cancer Center. Cells were prepared as described in the Methods. For each FMF analysis,

growth data was also obtained to insure that log phase cells were being sampled at 33°C (see Table 11). The profiles obtained at the permissive temperature are presented in Figure 19. A comparison shows that they are equally typical of cells in log phase growth, except for the profile taken at T=0 (Figure 19B) which displays fewer cells in G2/M than the others. All of the cultures were refed at the end of day -1. This refeeding appears to have driven many of the G2/M cells forward into G1. This effect is temporary, as can be seen from the later time points; the typical pattern of cells in log phase of growth seen at Day -1 is restored within 10 hours.

The profiles which were obtained at various times after shift up to 39.5°C are presented in Figure 20. An examination of these profiles reveals that no change has occured in cell cycle distribution from T=0 to T=10. The equilibrium observed prior to refeeding, which is restored after 10 hours incubation at 33°C, is not restored at 39.5°C. This indicates that cells are not progressing through the cell cycle to replenish G2/M. Also noted is that cells are not accumulating in any one phase of the cell cycle up to 10 hours after shift to 39.5°C. However, after 25 hours incubation at this temperature an accumulation of cells in S phase can be observed (Figure 20F). This result is confirmed by a second 25 hour time point. Since cells can be observed floating after 12 hours of incubation at 39.5°C, two harvestings were done, one in which only cells which remained attached to the

Table 11

Cell cycle distribution of JB3-B[a].

Day[b]	Time (hours)	Temp.	Total cells ($\times 10^6$)	Percent cells in each phase G1	S	G2/M
-1	-20	33°C	1.0	35.7	37.6	26.7
0	0	33°C	2.1	53.6	32.2	14.2
0	2.5	39.5°C	1.8	55.3	32.6	12.1
0	5.0	39.5°C	1.8	50.7	32.6	16.7
0	7.0	39.5°C	2.1	56.3	30.6	13.1
0	10.0	39.5°C	1.9	60.4	27.0	12.6
0	10.0	33°C	1.7	44.1	29.6	26.3
+1	25.0	33°C	4.1	52.3	29.6	18.1
+1	25.0	39.5°C	1.5	41.4	42.3	16.3
+1	25.0[c]	39.5°C	2.0	38.2	41.3	20.5

a - Replicate cultures were trypsinized for cell count and fixed for FMF as described in the Methods.

b - Day 0 = day of temperature shift of replicate cultures.

c - Floating cells added to attached cells prior to analysis.

Figure 19. - Cell cycle analysis of JB3-B at 33°C. At the times indicated replicate cultures were trypsinized for cell count and fixed for FMF as described in the Methods. A, Time = -20 hours; B, Time = 0 hours (time of temperature shift); C, Time = +10 hours; D, Time = +25 hours. Y-axis - cell number; X-axis - Fluorescence.

Figure 19

Cell cycle analysis of JB3-B at 33°C.

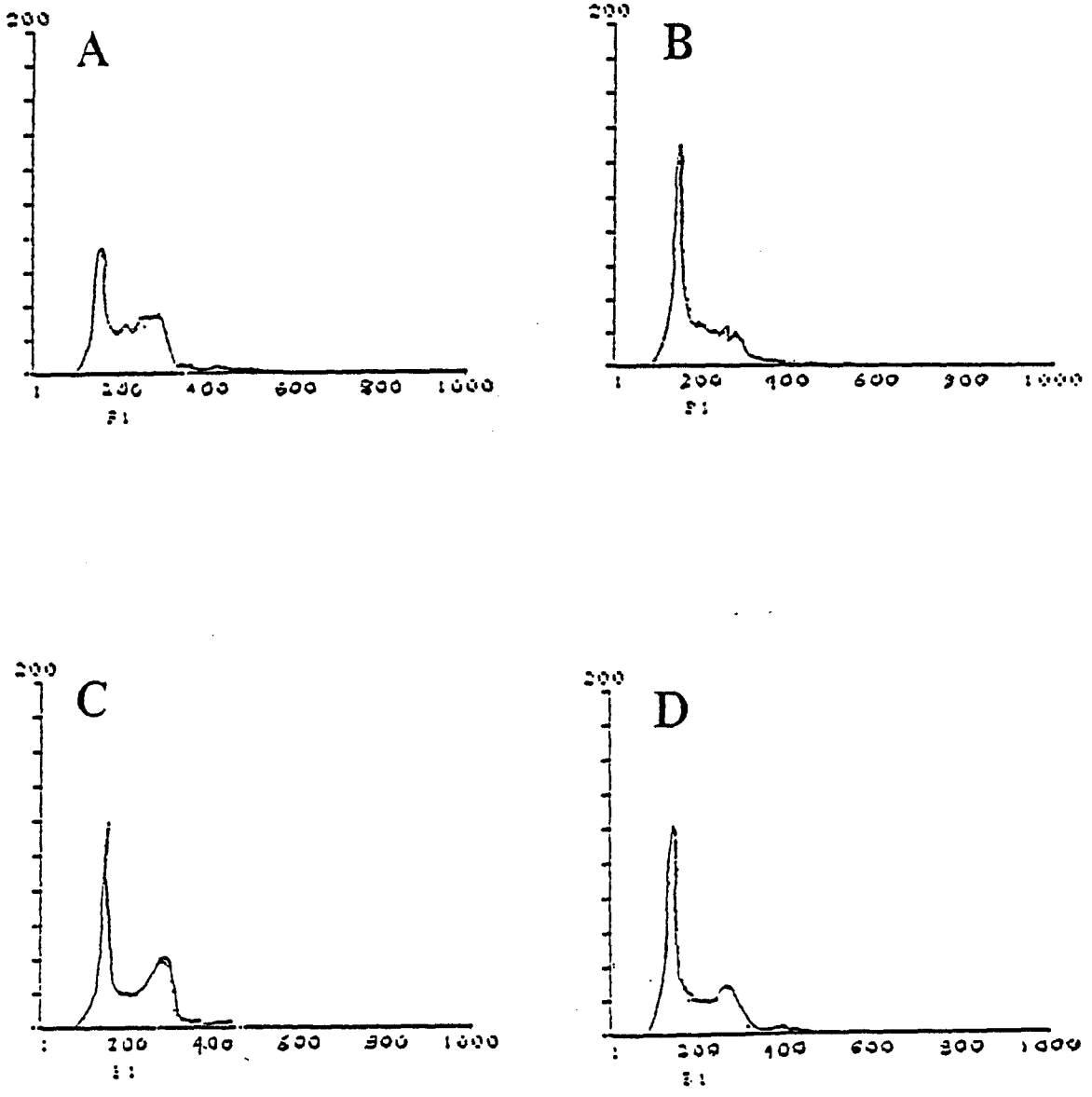
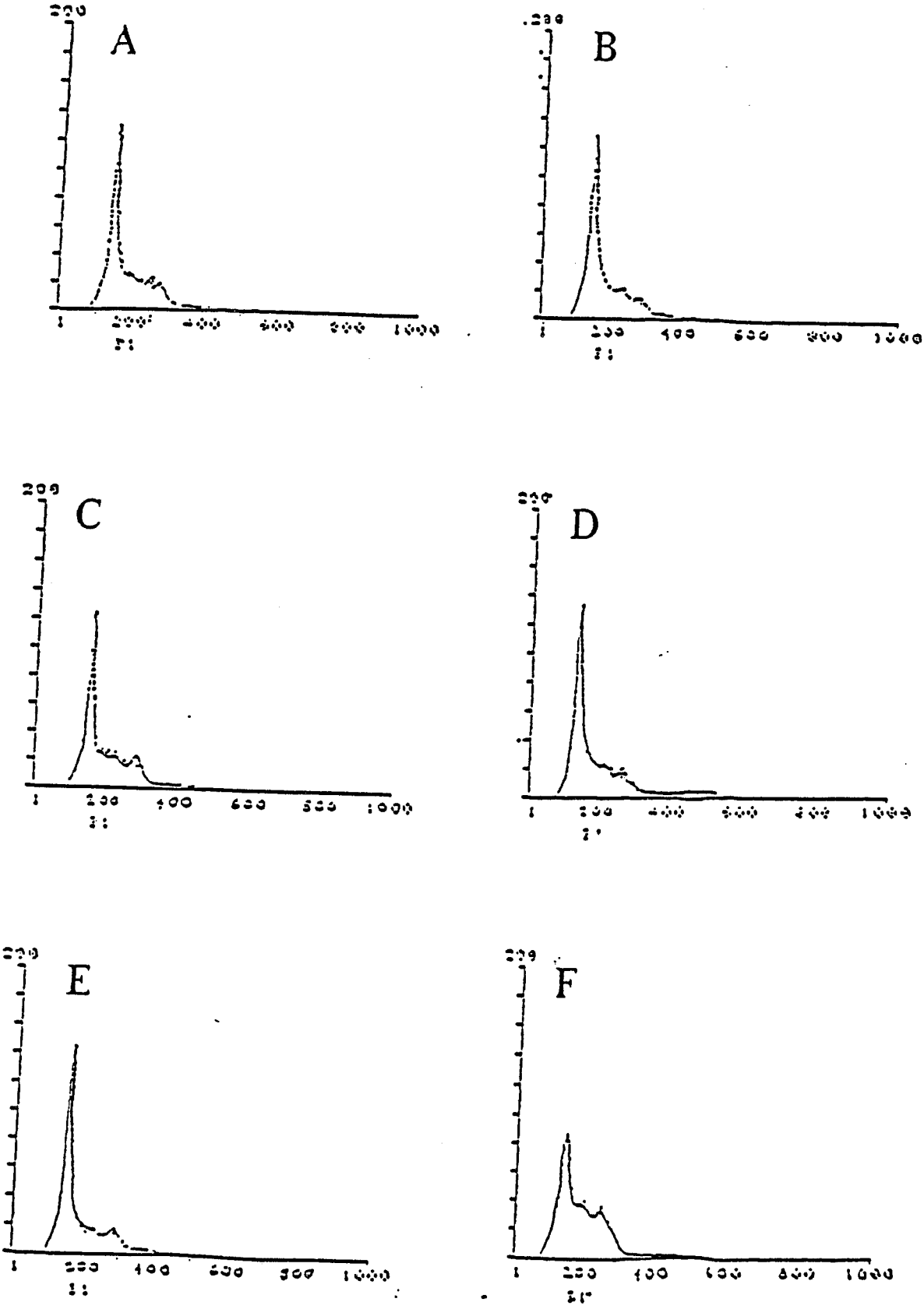


Figure 20. - Cell cycle analysis of JB3-B at 39.5°C. At the times indicated replicate cultures were trypsinized for cell count and fixed for FMF as described in the Methods. A, Time = 0 hours (time of temperature shift); B, Time = 2.5 hours; C, Time = 5.0 hours; D, Time = 7.0 hours; E, Time = 10 hours; F, Time = 25 hours. Y-axis - cell number; X-axis - Fluorescence.

Figure 20

Cell cycle analysis of JB3-B at 39.5°C.



dish were harvested and another in which the floating cells were also included. This was done to eliminate any bias which might be produced by their exclusion. The floating cells are most likely to be those which were affected the earliest. Therefore their loss could produce artifactual results (i.e. a preferential exclusion of cells in a specific stage of the cell cycle from experimental analysis). A comparison of these two populations reveals only a slight increase in the number of cells which are in G2/M (see Table 11). Thus it can be concluded that the exclusion of the floating cells doesn't introduce any significant bias into this data.

A comparison of these profiles (T=25 at 39.5°C) to the profile obtained at T=25 at 33°C (Figure 19D) reveals an increase in the number of cells in S phase for the 39.5°C profiles. A numerical calculation of these data (Table 11) shows this increase to be rather marked (30%). Either of two possibilities could account for this increase. The first is that the expression of the mutant phenotype of JB3-B cells results in a failure of cells to complete S phase and that measurable accumulations do not occur prior to 10 hours incubation. Alternatively, the cultures could be undergoing a period of increased cell division. In order to differentiate between these possibilities the number of cells present at each time of cell harvest was determined. The growth data presented in Table 11 demonstrate that during the interval from T=0 to T=25 cultures incubated at 33°C doubled in cell number (2.1 to 4.1 X 10^6 cells/dish, respectively). However,

no change in cell number was observed in cultures incubated at 39.5°C during the same interval (2.1 to 2.0 X 10^6 cells/dish). Thus the latter possibility can be excluded.

Therefore it can be concluded that the mutant cell line JB3-B does not arrest at a stage of the cell cycle other than S phase upon incubation at the restrictive temperature. This supports the previous interpretation of the data obtained from the viral studies. Thus JB3-B is not a cell cycle mutant which is affected at stage other than S phase. Furthermore the accumulation of cells in S phase observed after 25 hours incubation at the restrictive temperature indicates that the mutation in JB3-B affects a function which is directly involved in DNA synthesis. This results in an accumulation of cells which are not able to leave S phase because they have failed to complete DNA synthesis.

In an effort to avoid the duplication of studies on a previously isolated mutant which might be defective for the same function as JB3-B, data were obtained to confirm the unique nature of the defective gene in JB3-B. To accomplish this, another, more conclusive complementation analysis than the one described in Chapter 1 was performed. As a first step toward this goal, a thioguanine resistant (TG^R), ouabain resistant (OUA^R) subclone of JB3-B was isolated. This subclone would permit the selection of hybrid cells at the permissive temperature in HAT/ouabain media prior to asking for correction of the defect at the restrictive temperature. In this manner, a possible false complementation caused by

self-fusion (dosage effect) or a revertant can be minimized. An initial attempt was made to isolate spontaneously occurring OUA^R cells by plating 3 X 10^7 cells under conditions for logarithmic growth followed by selection in media containing 3mM ouabain. After the failure of two such attempts to isolate a OUA^R subclone of JB3-B, a different approach was employed. Exponentially growing cells were exposed overnight to 125 micrograms of ethyl methanesulfonate per ml. of growth media. Survivors were subcultured for 3 days, and mutants selected in 3mM ouabain. Four OUA^R colonies were isolated at 33°C. All remained ts for growth. (A OUA^R which was a revertant at the ts locus was isolated when one of these clones (3B·OUA^R-3) was tested at the restrictive temperature.) 3 X 10^7 cells of 3B·OUA^R-3 were then plated under conditions for logarithmic growth followed by selection in 5 X 10^{-5}M 6-thioguanine without further mutagenesis. One spontaneously occuring TG^R OUA^R clone, designated 3B·THO, was isolated. Figure 21 summarizes the relationship of these cell lines.

3B·THO was assayed for the proper maintainance of the mutant phenotype by labelling and Ad2 dot blot analysis prior to its use in any complementation analysis. The data thus obtained, shown in Figures 22 and 23, demonstrate that 3B·THO displays a mutant phenotype similar to that of the parental JB3-B. These results, as well as those obtained for the intermediate cell lines (e.g. 3B·OUA^R) and the revertants

Figure 21

The relationship of JB3-B, its sublines and revertants.

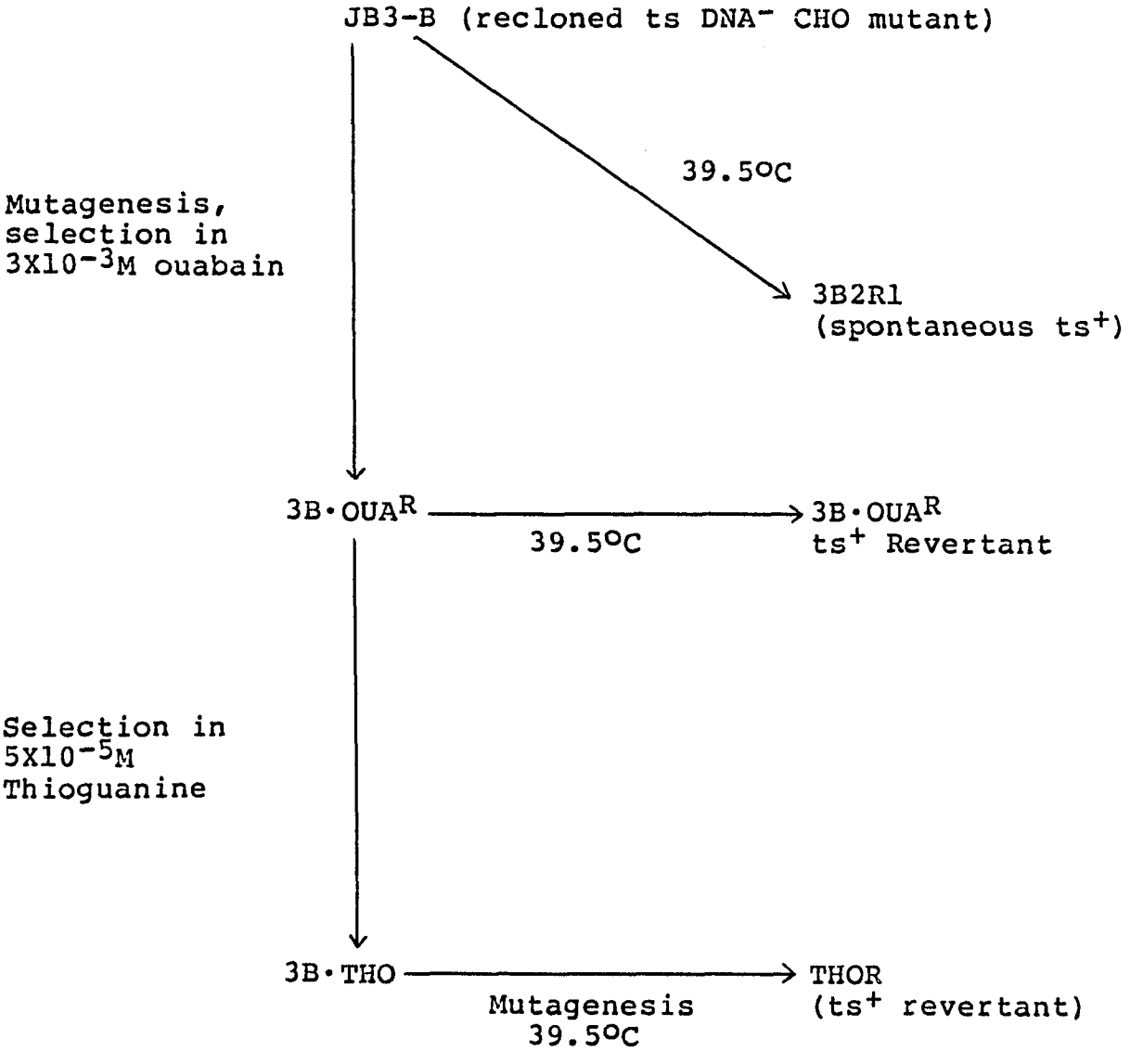


Figure 22. - Macromolecular synthesis in JB3-B, and its ts^- and ts^+ sublines. Cultures of each cell line were seeded and incubated at 33°C for 40 hours; half were shifted to 39.5°C at time 0. Cultures were pulse-labelled with ^{3}H-TdR (o—) or ^{35}S-methionine (▲--) at appropriate intervals and analysed for radioactivity as described in the Methods. A, JB3-B; B, 3B·OUA^R; C, 3B·THO; D, 3B2R1; E, 3B·OUA^R ts^+ revertant; F, THOR.

Figure 22

Macromolecular synthesis in JB3-B, and its ts$^-$ and ts$^+$ sublines.

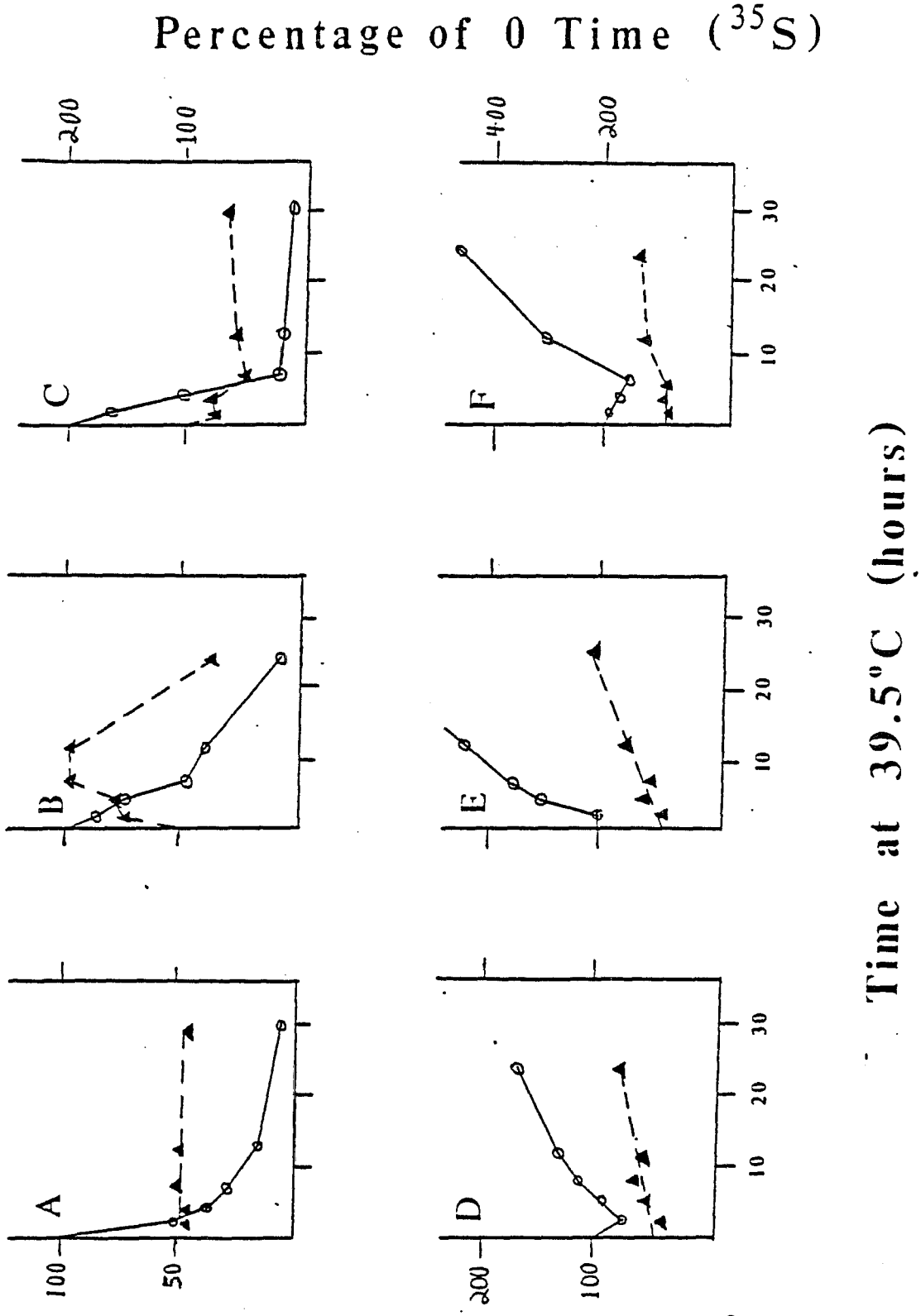


Figure 23. - Ad 2 DNA replication in 3B·THO. Dot blot analysis was performed as described in the Methods. Lane 7 contains tenfold concentrations of pLA1 DNA from 0.1 pg. to 1.0 ug.. All other DNA was diluted serially threefold. The DNA in lane 6 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 1 (33°C) and 2 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 3 (33°C) and 4 (39.5°C) were extracted at 48 hours p.i.. The DNA in lane 5 is from infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 23

Ad2 DNA replication in 3B·THO.

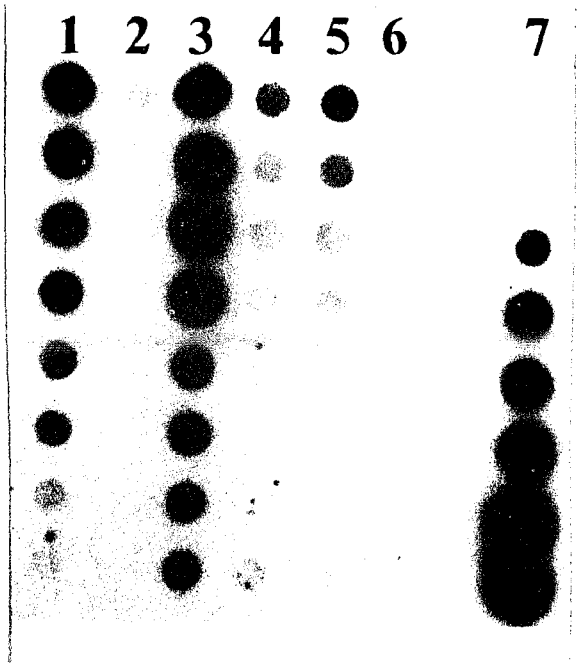


Table 12

Complementation analysis of JB3-3.

	Cell line	Complementation[a]	Remarks
I[b].	JB1-C	++++	Complements
	JB1-R	++	Complements
	JB3-B	-	Does not complement
	JB3-O	++++	Complements
	JB6-N	+++	Complements
	JB7-K	+++	Complements
	JB8-D	+++	Complements
	JB11-J	+++	Complements
II[c].	ts2	++	Complements
	ts20	++	Complements
	tsA1S9	++	Complements
	tsC1	++	Complements
	tsC8	+++	Complements
	ts13A	+++	Complements
	ts15C	+++	Complements
III.	tsH1	++++	Control - leucyl tRNA transferase, will complement DNA$^-$ mutants.

a - Based on average number of clones per 100mm dish seeded with 10^5 cells per dish. - = no colonies, ++ = 10 - 100 colonies, +++ = 100 - 500 colonies, ++++ = >500 colonies. All self-self fusions failed to grow in non-selective media for each of the cell lines included in this table.

b - ts DNA$^-$ CHO mutants from this collection.

c - ts DNA$^-$ mutants which are not a part of this collection.

which have been isolated will be discussed below.

For the complementation analysis, 3B·THO cells were seeded together with the cells with which they were to be tested at 33°C on day -2. On the following day (-1), these cells were fused with PEG and allowed to recover overnight. On day 0 these cultures were split into 100mm dishes at 10^5 and 10^4 cells per dish and incubated at 33°C overnight in complete medium to allow the cells to attach. The next day these dishes were refed with complete medium containing HAT/ouabain and incubated at 33°C. After the appearance of colonies (a few days later) the dishes were shifted to 39.5°C. Each cell line was also fused to itself and grown at 39.5°C without HAT/ouabain as a control. The results are presented in Table 12. A 3B·THO X JB3-B fusion was used as a negative control; colonies, which were seen growing at 33°C in HAT/ouabain media, rapidly died upon being shifted to 39.5°C. This also indicates that the functional levels of the affected gene product are sufficiently low that the combined levels of two cells cannot overcome the defect. As a positive control a 3B·THO X tsH1 fusion was performed. Since tsH1 is defective in a function (ts leucyl t-RNA synthetase) which is not directly required for DNA synthesis, hybrid cells produced by a fusion between it and any ts DNA^- mutant would be expected to grow at 39.5°C. My results indicate that such a cell fusion is an excellent control as dishes which were seeded with 10^5 cells were nearly confluent after shift to 39.5°C.

A total of 14 suspected ts DNA$^-$ cell lines were used for this analysis. These mutants can be placed into either of two groups: the ts DNA$^-$ CHO mutants which were isolated and screened as a single collection as described in Chapter 1, or suspected ts DNA$^-$ mutants which are not a part of this ts CHO collection. This last group includes two mouse cell mutants which were previously isolated by this laboratory. As can be seen from Table 12, the results of this complementation assay confirm those of the original assay (see Chapter 1). More than 100 colonies grew out at the restrictive temperature for each of the cell lines in this collection except forJB1-R (40-60% of JB1-R cells are floaters; these cells probably were not fused, thus accounting for the lower number of hybrids observed.). None of the self fusions grew at the restrictive temperature, except for JB8-D which formed microcolonies - a phenotypic property of this cell line. Thus JB3-B is defective in a different function from that of the other mutants in this collection. Varying numbers of colonies were obtained at 39.5^oC when 3B·THO was fused with the other hamster and mouse ts DNA$^-$ cell lines (see Table 12). This result indicates that JB3-B is mutated in a separate function than the ones which are defective in these cell lines. Since the mutated function in each of these cell lines has not been identified, no function can be conclusively eliminated as being the one affected in JB3-B.

When 3B·THO and 3B·OUA^R were assayed for the mutant phenotype by radio-labelling and Ad2 dot blot analyses, the

ts^+ revertants were also analysed to determine if they possessed a true revertant phenotype and thus could be used as controls in future experiments. These revertants would be potentially more informative as controls than the wild-type as they are genetically closer to the mutant (same history except for correction of the mutant phenotype). Figure 22 presents the results of the labelling experiments conducted on the 3B·OUA^R and 3B·OUA^R ts^+ revertant cell lines. Although the slope of the curve is not as steep as that for JB3-B, 3B OUA^R displays a progressive inhibition of DNA synthesis (50% after 8 hours) upon incubation at the temperature which restricts its growth. Protein synthesis continues to increase for 12 hours and then decreases as a probable result of cell loss or cell death. Floating cells can be observed after 12 hours incubation at 39.5°C - as can also be observed for JB3-B. Conversely, the 3B·OUA^R ts^+ revertant displays an increase in the incorporation of ^{3}H-TdR upon incubation at 39.5°C, similar to that observed for the wild-type. Protein synthesis also increases. In order to assess whether these phenotypes also hold true for Ad2 DNA synthesis dot blot analysis was performed with these cell lines. Figure 24 presents the results for 3B·OUA^R. Very little viral DNA synthesis occurs at 39.5°C at both 24 and 48 hours p.i., whereas nascent viral DNA continues to be synthesized at 33°C. A 24 hr./ 24 hr. shift up was not done. Figure 25 presents the results of a dot blot analysis which was performed with the 3B·OUA^R ts^+ revertant. At 33°C progeny Ad2 DNA is synthesized to the same

Figure 24. - Ad 2 DNA replication in 3B OUA^R. Dot blot analysis was performed as described in the Methods. Lane 7 contains tenfold concentrations of pLA1 DNA from 0.1 pg. to 1.0 ug.. All other DNA was diluted serially threefold. The DNA in lane 6 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 1 (33°C) and 2 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 3 (33°C), 4 (39.5°C) and 5 (39.5°C) were extracted at 48 hours p.i..

Figure 24

Ad 2 DNA replication in 3B·OUA^R.

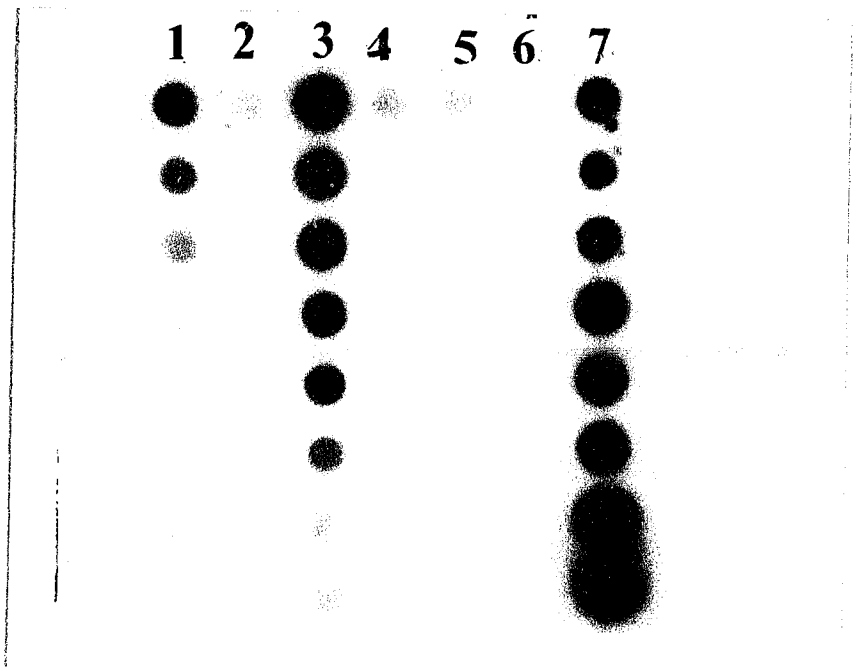


Figure 25. - Ad 2 DNA replication in 3B·OUA^R ts$^+$ revertant. Dot blot analysis was performed as described in the Methods. Lane 7 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. The DNA in lane 6 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 1 (39.5°C) and 3 (33°C) were extracted at 24 hours p.i.. DNA in lanes 2 (39.5°C) and 4 (33°C) were extracted at 48 hours p.i.. The DNA in lane 5 is from infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 25

Ad 2 DNA replication in 3B·OUA^{R} ts^{+} revertant.

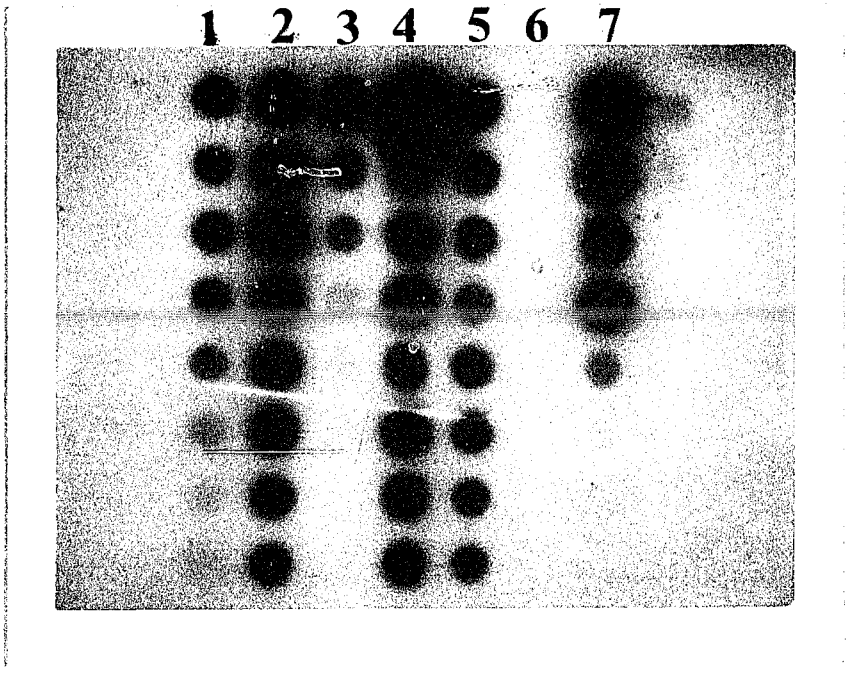
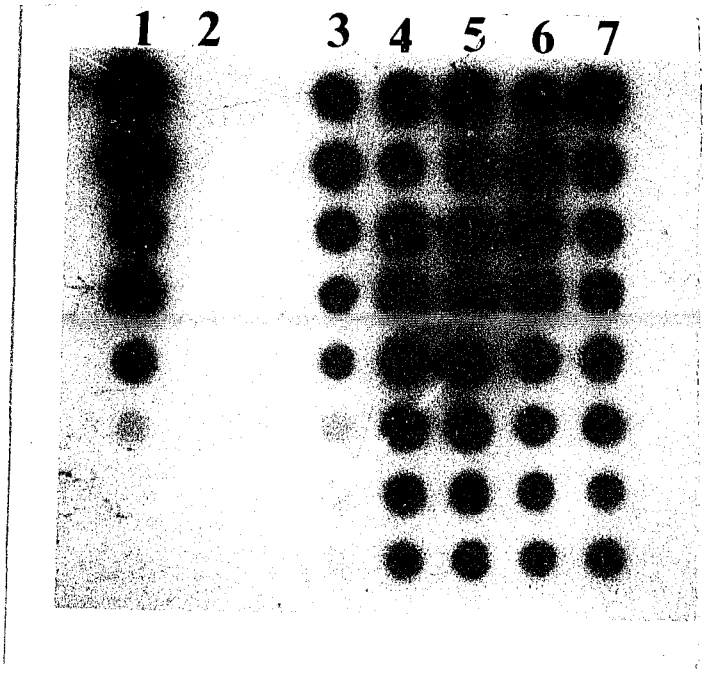


Figure 26. - Ad 2 DNA replication in 3B2R1. Dot blot analysis was performed as described in the Methods. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. The DNA in lane 2 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 3 (33°C) and 4 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 5 (33°C) and 6 (39.5°C) were extracted at 48 hours p.i.. The DNA in lane 7 is from infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 26

Ad 2 DNA replication in 3B2R1.



extent in this cell line as it is in the 3B·OUA^R cell line. However more progeny Ad2 DNA is synthesized at 39.5°C, both 24 and 48 hours p.i., than at 33°C for the revertant. Another ts$^+$ revertant, 3B2R1, was also analysed. This revertant was isolated from the original JB3-B (see Figure 21). From the labelling data presented in Figure 22, the rate of DNA synthesis increases at 39.5°C for 3B2R1. After 24 hours at 39.5°C the rate of DNA synthesis doubled, whereas for the 3B·OUA^R revertant the rate of DNA synthesis tripled. This could be an indication of separate mechanisms of correction of the ts DNA$^-$ phenotype for each revertant. The results of a dot blot analysis of Ad2 DNA synthesis in 3B2R1 is presented in Figure 26. At 24 hours p.i. more progeny Ad2 DNA has been synthesized in cultures incubated at 39.5°C than identical cultures incubated at 33°C. By 48 hours p.i. the amounts of progeny Ad2 are so great that a distinction cannot be made. Thus both revertants possess greater rates of DNA synthesis at 39.5°C than 33°C.

Through the use radiolabelling of macromolecular synthesis (Figure 22) and dot blotting (Figure 23) it has been shown that the cell line 3B·THO displays a mutant phenotype similar to the parental JB3-B. There is, however, one difference between these cell lines. When growing cultures of 3B·THO are shifted to 39.5°C a failure to isolate revertants is observed. Over a period of time a total of 3 X 10^8 3B·THO cells have been shifted to the restrictive temperature in a number of separate attempts to isolate a ts$^+$

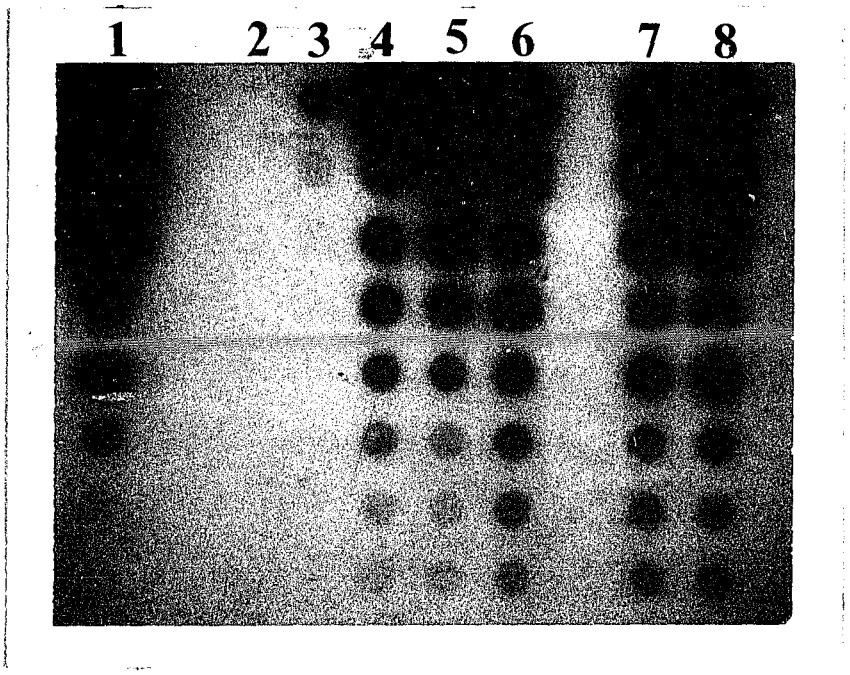
revertant of this cell line. These experiments were tried with cells at different passages and seeded at various densities. A revertant has been isolated from the 3B·OUA^R cell line at a similar incidence as observed for JB3-B (3 X 10^{-5}). This raises the possibility that the presence of the 6-thioguanine resistance marker is related to the failure to isolate revertants in 3B·THO.

In an effort to isolate a revertant of THO, the following experiment was performed. Exponentially growing cultures were exposed overnight to 125 micrograms of ethyl methanesulfonate per ml. of growth media. The cultures were washed with growth media and the surviving cells were shifted to 39.5°C on the following day. Five revertants were isolated by this procedure at a frequency similar to that of JB3-B. All of these revertants exhibit slow rates of growth. The one with the most rapid growth rate, designated THOR-3A, was grown up and used for future analysis. Figure 22 presents the labelling data obtained for this cell line. There is an increase in the rate of DNA synthesis over the 26 hour time course in this experiment observed when cultures are incubated at 39.5°C. Thus THOR-3A exhibits characteristic revertant behavior similar to the other previously isolated revertants (3B2R1 and the 3B OUA^R revertant). As presented in Figure 27, the results of a dot blot analysis reveal that this revertant is capable of supporting Ad2 DNA synthesis at both 33°C and 39.5°C and at a greater rate at 39.5°C than at 33°C.

Figure 27. - Ad 2 DNA replication in THOR. Dot blot analysis was performed as described in the Methods. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. All other DNA was diluted serially threefold. The DNA in lane 2 was extracted from uninfected cells. Infected cells were incubated at 33°C and 39.5°C. DNA in lanes 3 (33°C) and 4 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 5 (33°C) and 6 (39.5°C) were extracted at 48 hours p.i.. The DNA in lanes 7 and 8 are from infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 27

Ad 2 DNA replication in THOR.



A karyotypic analysis of these cell lines as well as the CHO-S wild-type and several of the other ts DNA$^-$ CHO mutants was performed to determine if any gross cytogenetic changes had occured. Specifically, it was performed to assess whether a dosage effect is responsible for the phenotype of any of the revertants and to check that the mutants had maintained a parental karyotype. A preliminary analysis was performed by myself as described in the Methods (the chromosomes were not banded and no attempt was made to identify each chromosome). The results of this analysis revealed that all of the mutants analysed possessed a pseudodiploid chromosome number similar to the wild-type (22 chromosomes). These mutants were JB3-B, 3B·OUA^R, JB8-D, JB1-C, JB3-O, and 3B·THO. Additionally the 3B·OUA^R ts$^+$ revertant was also shown to possess a similar pseudodiploid chromosome number. However, the five revertants which were isolated directly from JB3-B, 3B2R1 to R5, possessed a chromosome number between 30 and 36. Since these revertants were isolated from a single original culture of JB3-B, these revertants may be subclones of a single revertant. Furthermore, these data also suggest that the ts DNA$^-$ phenotype of the mutant JB3-B can be corrected by a gene dosage effect. In an attempt to explore this result further a more detailed analysis is being performed in collaboration with an experienced cytogeneticist at this institution.

Growth data for 3B·THO and all three of the ts$^+$ revertants are shown in Table 13.

Table 13

Growth rates of 3B·THO and the ts$^+$ revertants[a]

Cell line	Culture doubling time (hours) 33°C	39.5°C	Appearance at 39.5°C
3B·THO	42	>120	No Growth
3B2R1	29	26	Normal
3B·OUA^R ts$^+$ revertant	29	22	Normal
THOR	42	34	Normal

a - Cells were plated at 2.5 x 10^4 cells per 60mm dish and cell number determined at 4 - 12 hour intervals as described in the Methods.

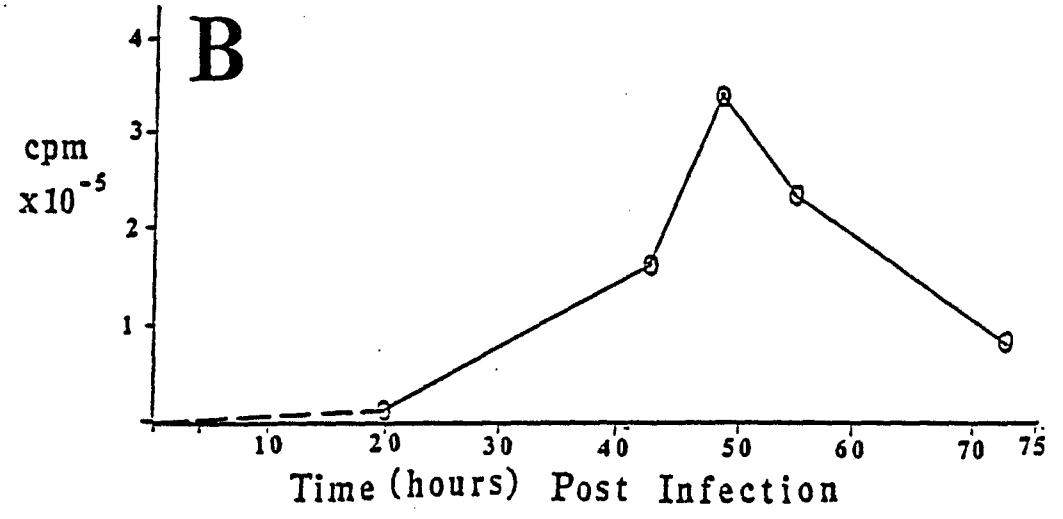
The preceeding data obtained from the viral and FMF analyses of JB3-B indicated that the inhibition of DNA synthesis was a direct result of the mutation. (The cell cycle independence of both Ad2 and Py under the conditions employed in those experiments have suggested that the inhibition of DNA synthesis observed at 39.5°C was not a result of a growth arrest at a stage of the cell cycle other than S phase. This interpretation was further supported by the analysis of cell cycle kinetics utilizing flow cytometry.) I therefore sought to exploit the effect of the cellular mutation on adenoviral DNA synthesis toward further identification of the ts defect. Ad2 DNA replication in JB3-B had been analysed initially by dot blotting (see Chapter 1). This technique, however, allows only the measurement of the accumulation of progeny DNA molecules. Therefore studies were initiated on the rate and steps of Ad2 DNA synthesis using pulse-chase labelling experiments. The synthesis of viral intermediates was analysed on alkaline sucrose gradients. Such studies will permit a direct examination of the rates of viral DNA synthesis. A discrete peak of unit length viral DNA can be obtained free from cellular DNA which pellets at the bottom of the gradient. These studies also permit a direct examination of viral intermediates which are separate from the template strand of DNA. The procedure was as described in the Methods.

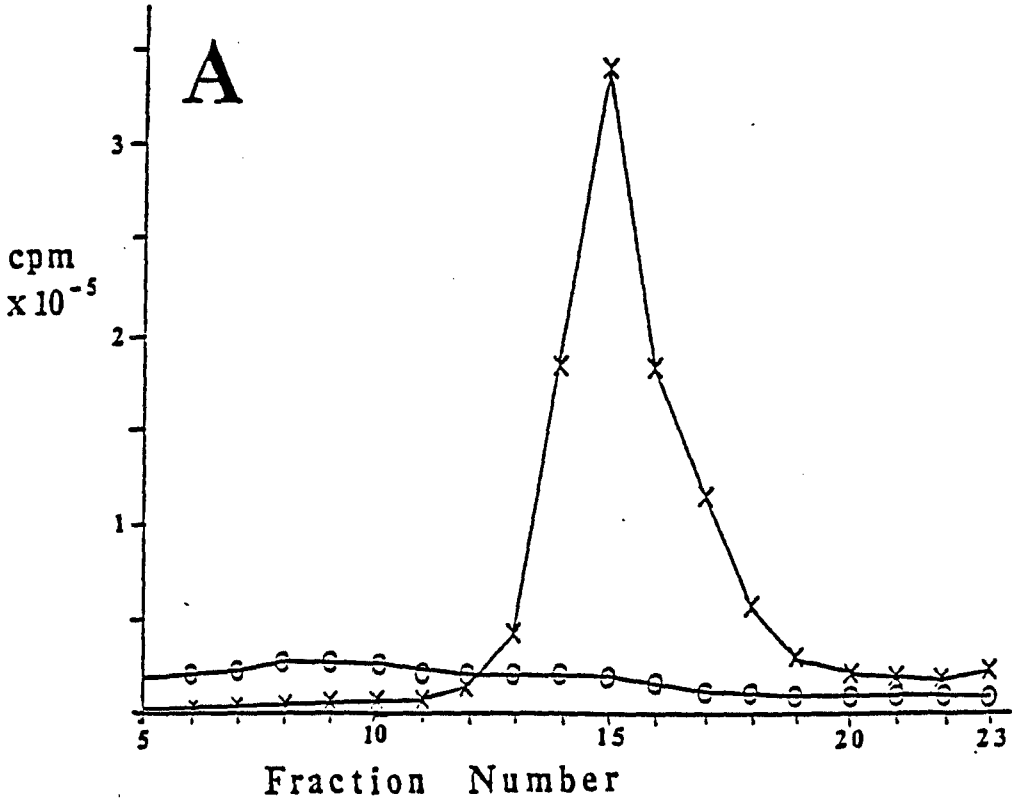
Initially, a time course was performed at 33°C to determine when the maximum rate of Ad2 DNA synthesis occurs.

Figure 28. - Time course of the rate of Ad2 DNA synthesis in infected JB3-B cells. At different times p.i., infected cells were pulse-labelled for 1 hour with ^{3}H-TdR, followed by addition of nonradioactive TdR for 30 minutes and analysed for virus DNA on an alkaline sucrose gradient as described in the Methods. A) TCA-precipitable CPM in each fraction of a gradient. (O), uninfected; (X), infected 49 hours p.i.. B) ^{3}H-TdR incorporated into full genome Ad2 fractions over the course of infection.

Figure 28

Time course of the rate of Ad2 DNA synthesis in infected JB3-B cells.





A typical gradient profile of TCA-precipitable ^{3}H-TdR counts from infected cells is shown in Figure 28A. A discrete peak is observed in fractions 14 - 17. The only incorporation observed for the uninfected cells is into cellular DNA which pellets onto the cesium chloride cushion at the bottom of the gradient. Only background levels of incorporation are seen in the gradient itself. Figure 28B shows a time course for the incorporation observed at 25 hour, 40 hour, 49 hour, 55 hour, and 72 hour p.i.. Longiaru and Horwitz (1981) reported that maximum synthesis occurs between 36 and 42 hours p.i. in infected wild-type CHO cells at 37^{o}C. Because the growth properties of JB3-B at the permissive temperature (33^{o}C) would be expected to differ from that of the wild-type CHO-S, this experiment was repeated for JB3-B. Infected cells were pulse labelled for 1 hour at 33^{o}C and incorporation analysed on an alkaline sucrose gradient. The data show that the maximum rate of Ad2 DNA synthesis occurs at approximately 49 hours p.i.. The rate rapidly increases after 20 hours p.i. at which time no Ad2 DNA synthesis can be observed in comparison to an uninfected cell. After reaching its peak at 49 hours p.i. the rate decreases to 73 hours p.i. almost as rapidly as it increased. The incorporation of ^{3}H-TdR into cellular DNA at all times p.i. was significantly less than the incorporation into cellular DNA in the uninfected control (data not shown). This was expected, as previous studies (Longiaru and Horwitz 1981) have shown that host DNA synthesis is shut off approximately 12 hours p.i. in wild-

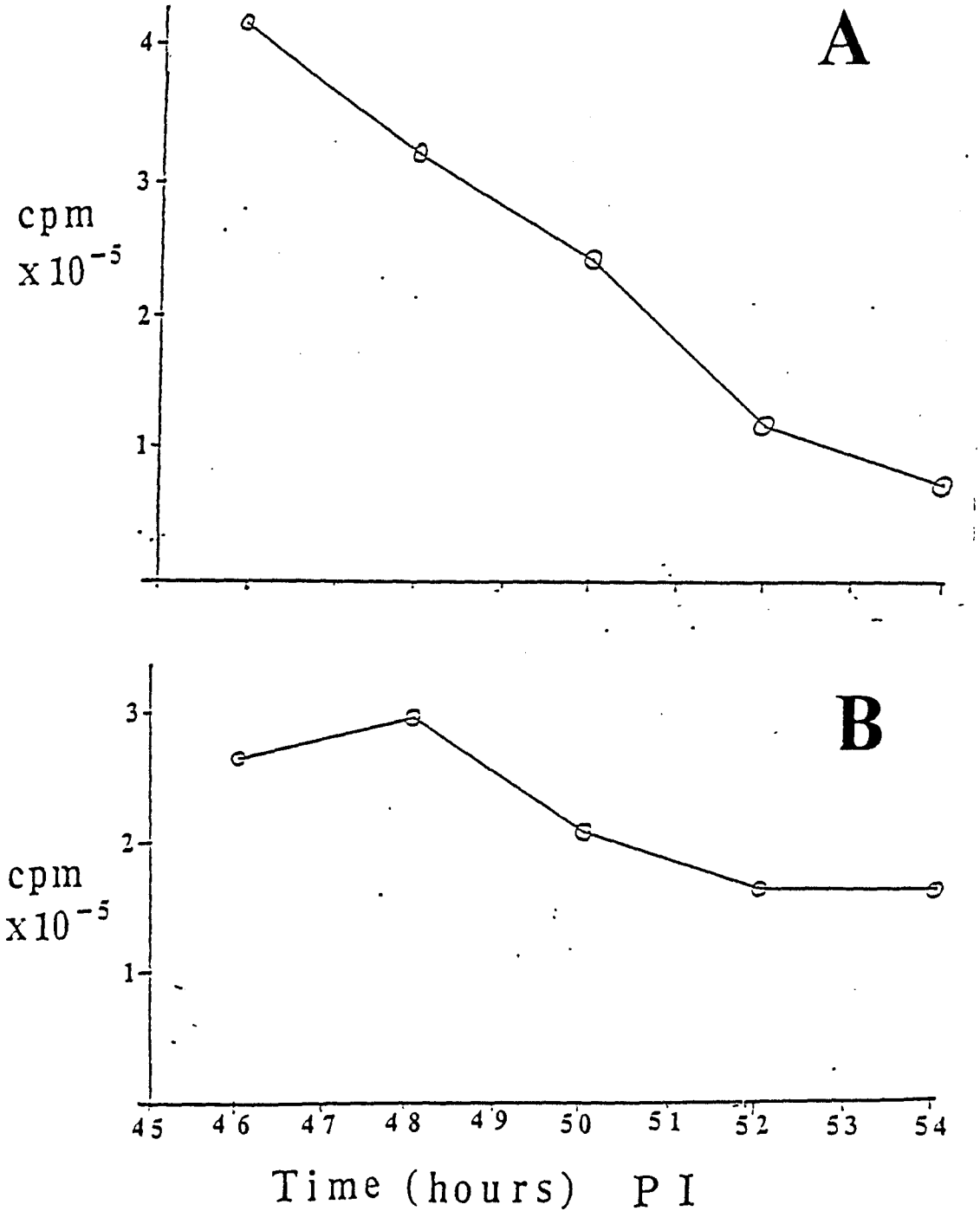
type CHO cells. Thus it can be concluded that the course of viral infection at 33°C in JB3-B is similar to that observed in the wild-type although it may be delayed somewhat

Knowledge of the time p.i. when the maximal rate of viral DNA synthesis occurs is a necessary prerequisite for the analysis of Ad2 DNA synthesis at the restrictive temperature. Cultures can be shifted up to the nonpermissive temperature prior to the occurrance of maximum DNA synthesis to facilitate the observation of the effects of incubation at this temperature. (If cultures were shifted up at later times, interpretation of the data becomes difficult as the only way to distinguish between a true decrease in rate caused by the expression of the mutated function and that of the normal decrease observed late in a virus infection would be the difference in the slope of the respective curves, if any can be observed.). Since the observed maximum in JB3-B cells at 33°C was 49 hours p.i., a preliminary experiment in which cultures of JB3-B were shifted up to the nonpermissive temperature at 46 hours p.i. was performed. A parallel experiment in which cultures were left at the permissive temperature was also performed both as a control and to more precisely determine the maximal rate of Ad2 DNA synthesis. The results of these experiments are presented in Figure 29. The data from the 33°C time course indicates that the peak rate of Ad2 DNA synthesis occurs at 48 hours p.i. and the rate decreases rapidly thereafter, declining to approximately two thirds of the 48 hour level by 50 hours p.i.. When the

Figure 29. - Effect of temperature shift 46 hours p.i. on the rate of Ad2 DNA synthesis in infected JB3-B cells. Cultures of JB3-B cells were infected for 46 hours at 33°C. Cultures were then divided into two sets: one was shifted to 39.5°C (A) and the other maintained at 33°C (B). Parallel sets were pulse-labelled for 1 hour every 2 hours and analysed for synthesis of viral DNA as described in the Methods.

Figure 29

Effect of temperature shift 46 hours p.i. on the rate of Ad2 DNA synthesis in infected JB3-B cells.



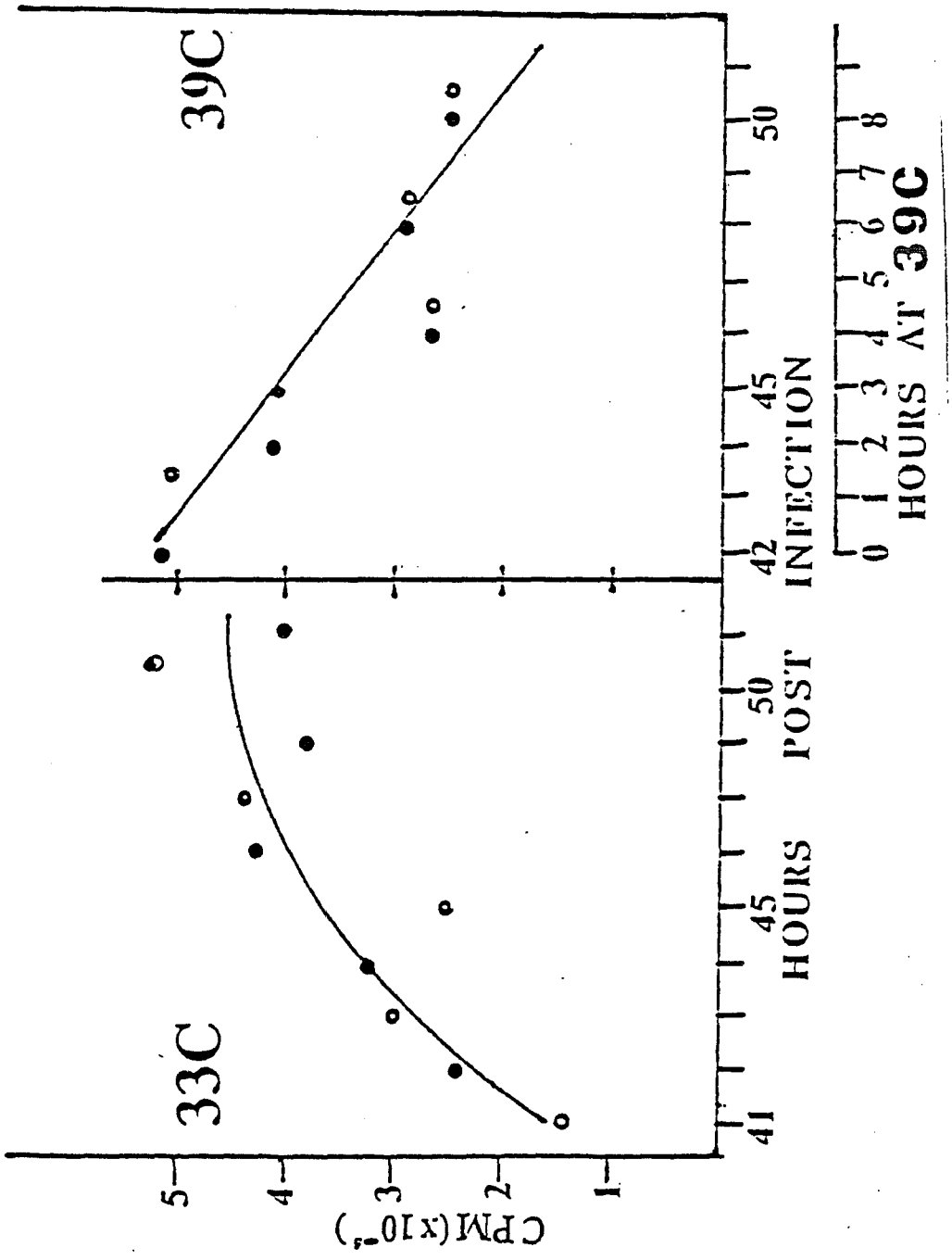
cultures are shifted up to 39.5°C at 46 hours p.i. a marked decrease in the rate of viral DNA synthesis is observed. This is in contrast to the initial rise followed by a less steep decrease seen after 48 hours p.i. at 33°C. This result supports the conclusion that the expression of the mutated function affects Ad2 DNA synthesis. Furthermore this effect is immediate (compare difference from 46 to 48 hours p.i. at each temperature). However, the time chosen for temperature shift is too late in infection since the rate is falling for the last six hours at both temperatures.

In an effort to improve the observation of the differential effects of infection at the two temperatures, two other pairs (33°C and 39.5°C) of experiments were performed with temperature shift times of 42 and 43 hours p.i.. The data from these experiments are presented in Figure 30. When virus infection is allowed to continue at 33°C, an increase in the rate of viral DNA synthesis is observed. This increase continues in a linear fashion to 48 hours p.i. at which time the rate begins to plateau. The peak rate of viral DNA synthesis appears to be 49 hours, which confirms the results of the previous experiment. When the cultures were shifted to 39.5°C at 42 hours p.i., however, a rapid decrease in the rate of viral DNA synthesis is observed. This decrease begins almost immediately after shift up as evidenced by the observed drop of 20% in the rate of viral DNA synthesis just two hours after temperature shift. Eight hours after shift up the rate of viral DNA synthesis has decreased to 40% of what

Figure 30. - Effect of temperature shift 42 and 43 hours p.i. on the rate of Ad2 DNA synthesis in infected JB3-B cells. Cultures of JB3-B cells were infected and analysed as in Figure 29 except that cultures were shifted at 42 or 43 hours p.i..

Figure 30

Effect of temperature shift 42 and 43 hours p.i. on the rate of Ad2 DNA synthesis in infected JB3-B cells.



it was at the time of shift up. This is comparable to what has been observed to occur for cellular DNA synthesis (see Figure 4). (Although it is possible to measure the amount of ^{3}H-TdR incorporated into cellular DNA in infected cells since it is trapped by the CsCl cushion, such data do not accurately measure the effects of incubation at the restrictive temperature on cellular DNA synthesis since Ad2 shuts off host cell DNA synthesis.) From these data it can be stated that Ad2 DNA synthesis in JB3-B is dependent on a host cell function which is labile at 39.5°C. Based on the similarity of the kinetics of inhibition of DNA synthesis, this is probably the same function which affects cellular DNA synthesis at this temperature. Alternatively, the data can also be explained by a preferential loss of infected cells at the nonpermissive temperature, or by the degradation of newly synthesized DNA.

Analyses of the kinetics of Ad2 DNA synthesis upon temperature shift in two other cell lines were performed to substantiate this conclusion: namely, wild-type CHO-S and the temperature independent revertant of JB3-B, 3B2R1. For example, the shift to 39.5°C after ~ 42 hours of incubation at 33°C may result in an acceleration of the normal sequence of events which occur during the course of infection; specifically, the earlier appearance of the decline in the rate which is observed commencing 49 hours p.i. at 33°C. In an effort to determine whether this is true or not, the experiment was repeated using wild-type CHO-S cells. The

Figure 31. - Effect of temperature shift 42 hours p.i. on the rate of Ad2 DNA synthesis in infected CHO-S cells. Cultures of CHO-S cells were infected and analysed as in Figure 30.

Figure 31

Effect of temperature shift 42 hours p.i. on the rate of Ad2 DNA synthesis in infected CHO-S cells

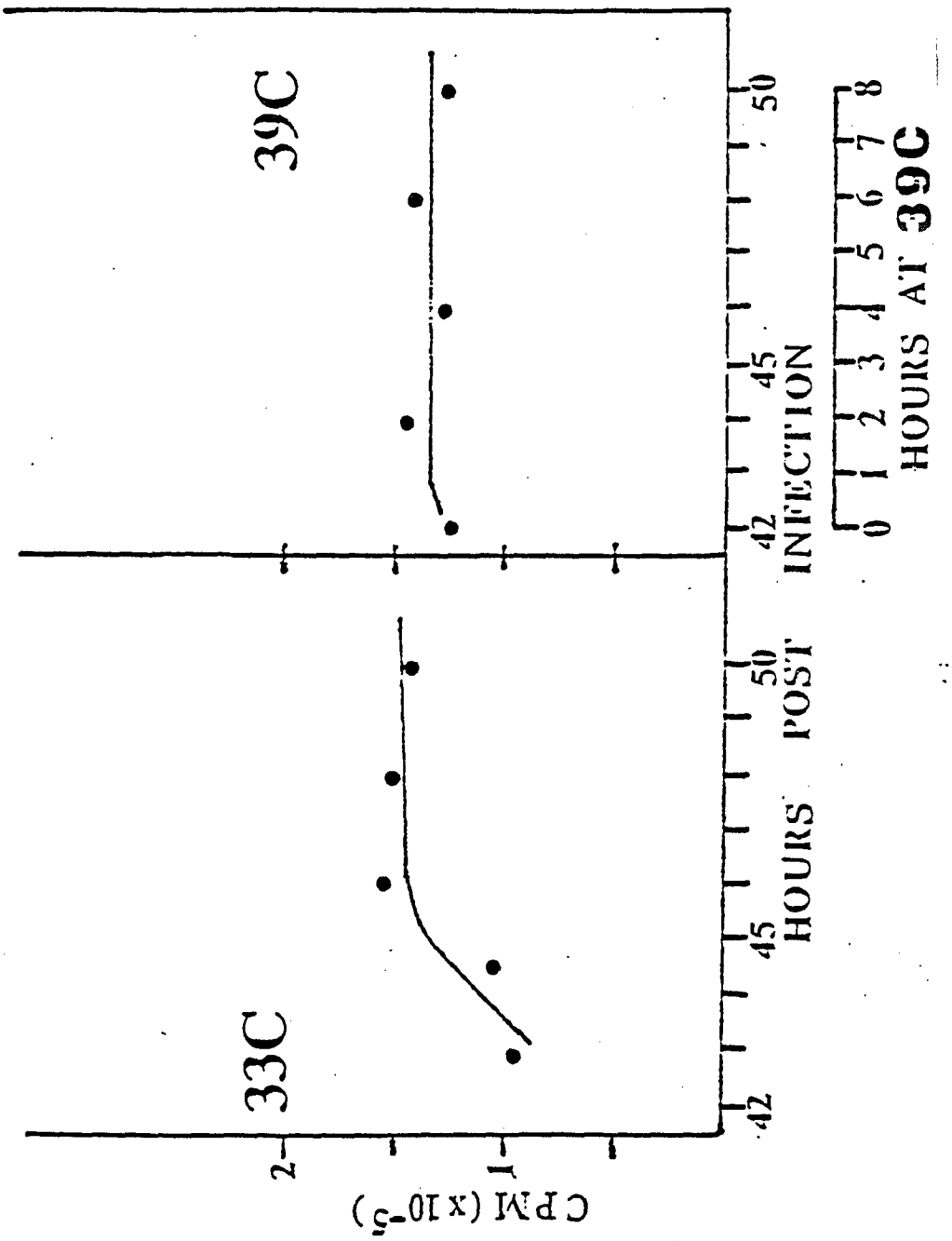
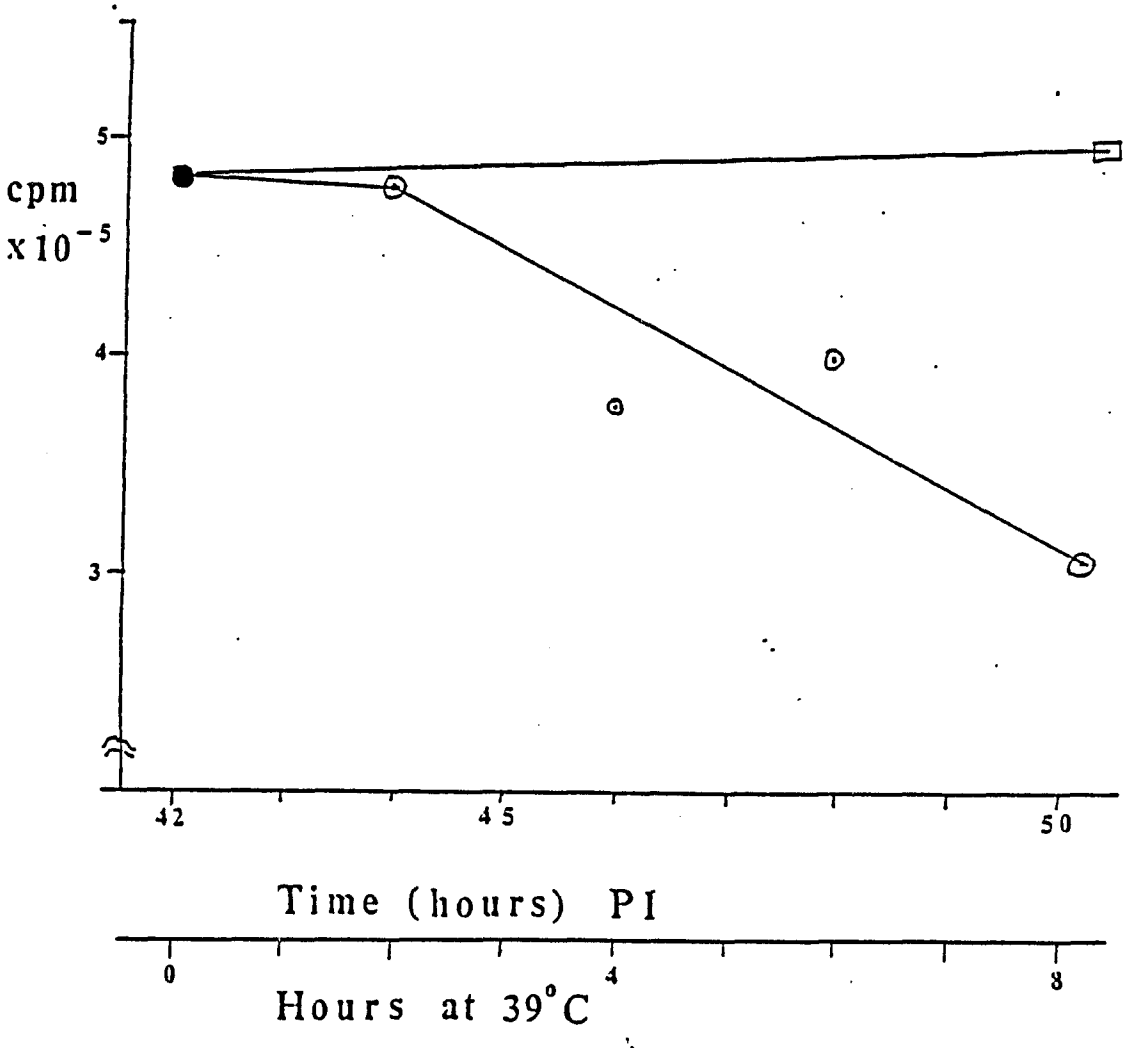


Figure 32. - Effect of temperature shift 42 hours p.i. on the rate of Ad2 DNA synthesis in infected 3B2R1 cells. Cultures of 3B2R1 cells were infected and analysed for virus DNA as in Figure 30 and shifted to 39.5°C at 42 hours p.i. (○). Cultures maintained at 33°C were labelled at 42 hours p.i. (●) and 50 hours p.i. (□)

Figure 32

Effect of temperature shift 42 hours p.i. on the rate of Ad2 DNA synthesis in infected 3B2R1 cells



results are presented in Figure 31. At 33°C the rate increases slightly between 42 and 45 hours p.i., and is stable or falls slightly up to 50 hours p.i.. Similar results are obtained when infected cultures of CHO-S were shifted up to 39.5°C at 42 hours p.i.. The difference between the time of the maximum rate of viral DNA synthesis reported here (46 hours p.i.) and that reported by Longiaru and Horwitz (36 hours p.i.) is most likely due to the difference in the temperatures at which the infections occurred, 33°C and 37°C respectively.

A more accurate control than wild-type CHO-S would be a spontaneous revertant of JB3-B. This is due to the fact that both JB3-B and its revertant share the same history (i.e. same mutagenesis protocol) except for the event which occured in the revertant to correct the ts defect. The revertant 3B2R1 has been described earlier. The results from pulse-chase studies which were analysed on alkaline sucrose gradients are presented in Figure 32. An increase in the rate of viral DNA synthesis can be observed to occur from 42 hrs p.i. to 48 hrs p.i. at which point the rate levels off. This is identical to the curve observed for JB3-B under the same conditions. The cell line 3B2R1 has been observed to grow and replicate its DNA at the temperature which restricts the growth and replication of JB3-B. Dot blot analysis demonstrates that this cell line is capable of supporting Ad2 DNA replication at this temperature as well (see Figure 26). Therefore it was expected that the difference in the rate of

Ad2 DNA synthesis at both temperatures will not be greater than that observed for the wild-type. The data in Figure 32B, however, shows that there is a decrease in the rate of viral DNA synthesis starting about 2 hours after shift up to 39.5°C. Except for the delay in the onset of the decrease, this result is identical to that which is observed for JB3-B. The best possible explanation for this result is that I am still too near the point at which the rate of viral DNA synthesis decreases as a natural consequence of the course of infection. That is, the shift to the higher temperature accelerates the sequence of infection and the normal decrease in rate occurs earlier. This implies that I am still looking too late in infection and that I need to shift the cultures to the high temperature earlier than 42 hours p.i.. In the wild-type a slight acceleration of the time course at 39.5°C was observed. The more dramatic acceleration seen in 3B2R1 could be an unanticipated effect of the mutagenesis - either of the function which is affecting DNA synthesis or another cellular function which participates in viral infection.

In an effort to rule out that a similar phenomenon was responsible for the results with JB3-B, alkaline sucrose gradient analysis experiments were repeated with JB3-B as before, but with the following modifications: 1) cultures were also shifted from 33°C to 39.5°C at 36 hours p.i. 2) two cultures were left at the permissive temperature and harvested for analysis at the same time as the final 39.5°C data point (8 hours after temperature shift; 44 hours p.i.

for the 36 hour temperature shift and 50 hours p.i. for the 42 hour temperature shift). By combining these 33°C data points from both experiments (36 and 42 hour temperature shifts), four 33°C data points (including time of shift) were obtained which describes the events that occur simultaneously at 33°C. Thus three sets of data are generated: 1) the effect of incubation at 33°C from 36 to 50 hours p.i. 2) the effect of incubation at 39.5°C when temperature shift occurs 36 hours pi. and 3) the effect of incubation at 39.5°C when temperature shift occurs 42 hours p.i.. The data which were obtained for the mutant JB3-B are presented in Figure 33. The rate of viral DNA replication in cells incubated at 33°C accelerates rapidly from 36 to 42 hours p.i. and less so thereafter until 50 hours p.i., confirming previous observations. When cultures of JB3-B cells are shifted to the nonpermissive temperature at 42 hours p.i. there is a marked decrease in the rate of Ad2 DNA synthesis which starts immediately after temperature shift. This result confirms the data presented in Figure 30. When cultures are shifted to the nonpermissive temperature at 36 hours p.i., the rate of Ad2 DNA synthesis remains constant to 44 hours p.i.. Clearly Ad2 DNA synthesis is affected by the shift to the nonpermissive temperature as the rate of Ad2 DNA synthesis should be rapidly increasing during this interval of time p.i., as shown in the cultures maintained at 33°C.

A similar analysis of the revertant 3B2R1 was performed with a temperature shift occuring at 36 hours p.i. in an

Figure 33. - Comparative effects of time of temperature shifts on the rate of Ad2 DNA synthesis in infected JB3-B cells. Cultures of JB3-B cells were infected and analysed for virus DNA as in Figure 30. Cultures were maintained at 33°C throughout the experiment (o—), shifted to 39.5°C at 36 hours p.i. (Δ--), or shifted to 39.5°C at 42 hours p.i.(□--).

Figure 33

Comparative effects of time of temperature shifts on the rates of Ad2 DNA synthesis in infected JB3-B cells

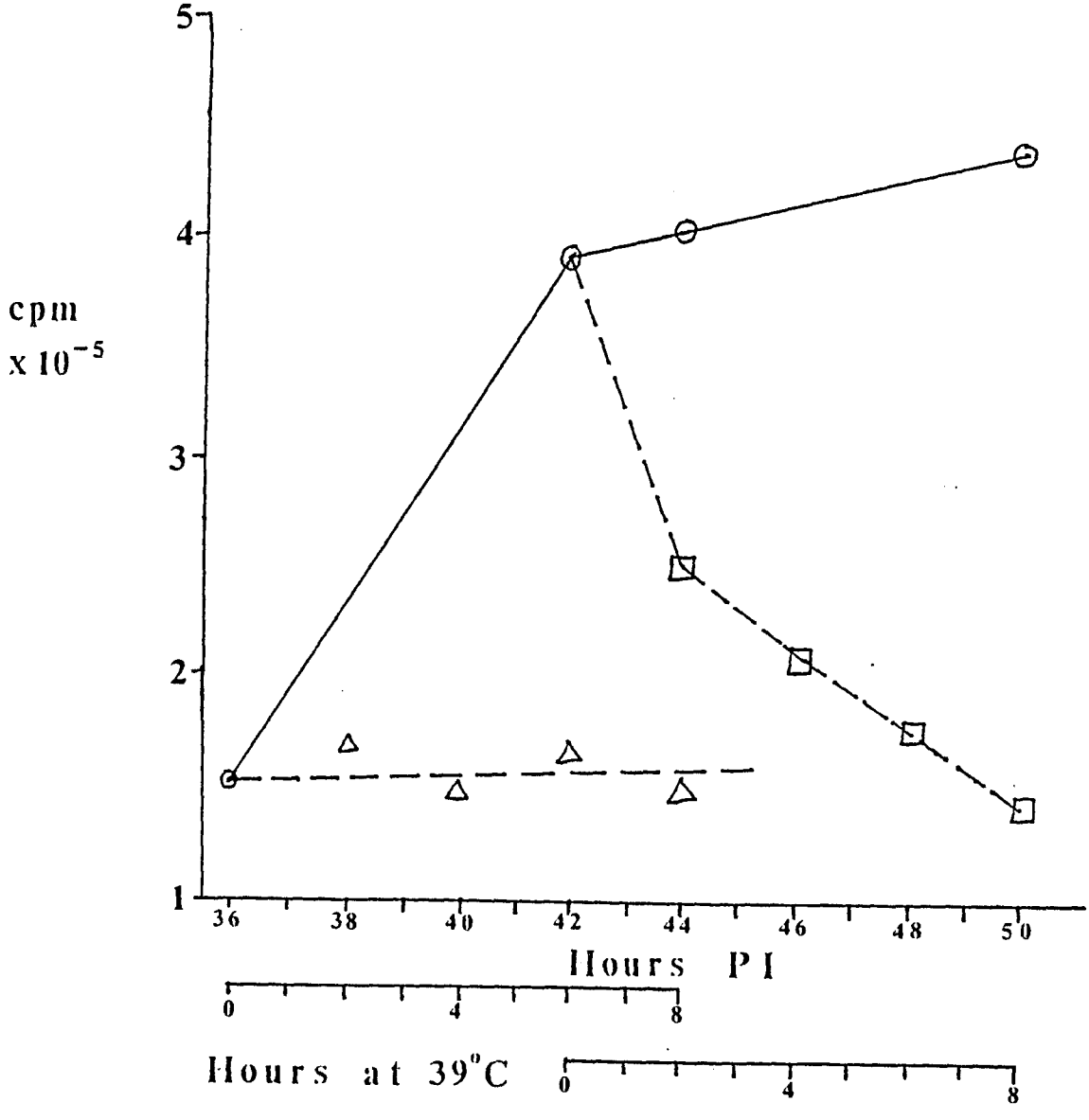
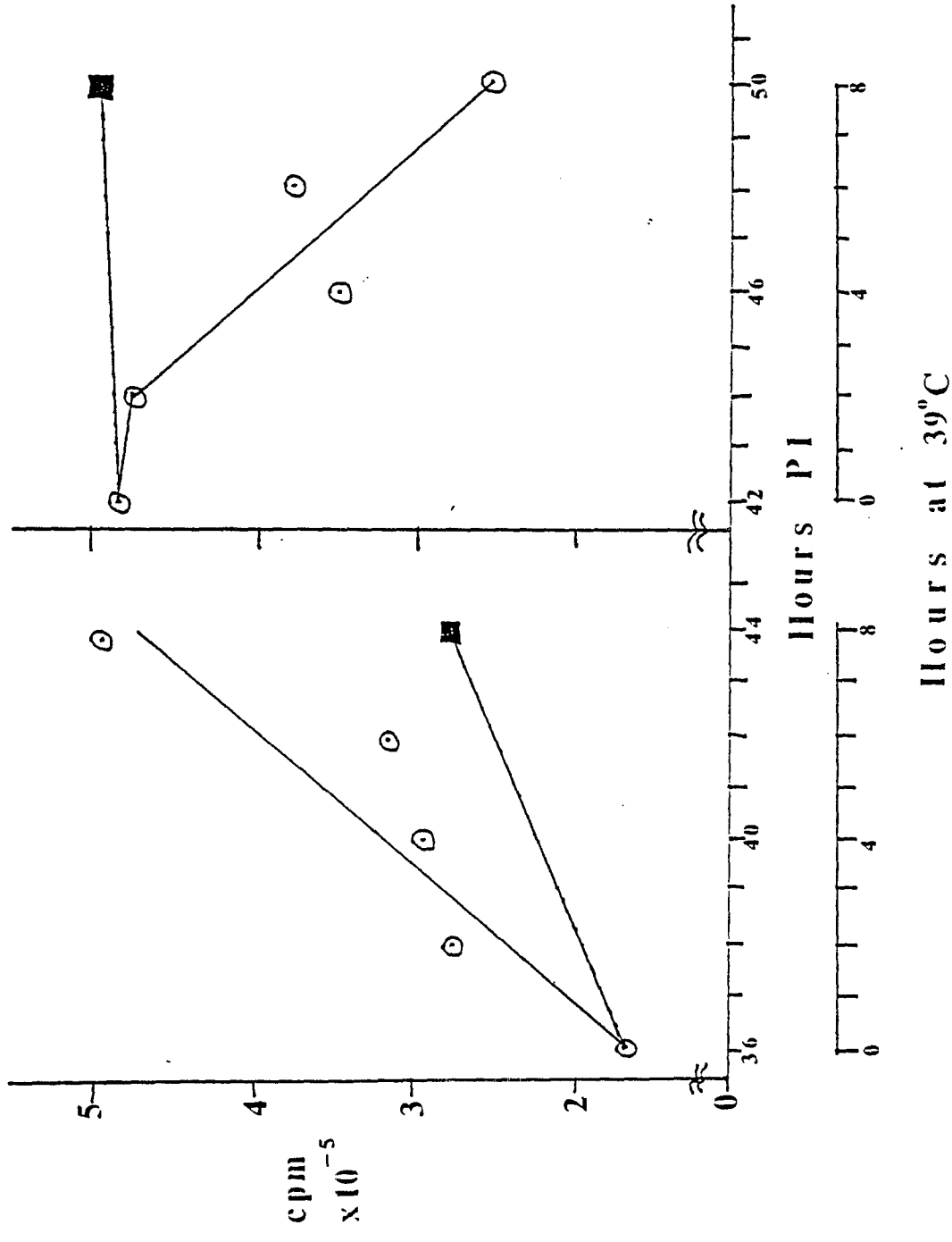


Figure 34. - Comparative effects of time of temperature shifts on the rate of Ad2 DNA synthesis in infected 3B2R1 cells. Cultures of 3B2R1 cells were infected and analysed for virus DNA as in Figure 30. Cultures were shifted at 36 hours p.i. (A) or 42 hours p.i. (B) to 39.5°C and labelled at 2 hour intervals (O). Cultures maintained at 33°C were labelled 8 hours after replicates were shifted (■).

Figure 34

Comparative effects of time of temperature shifts on the rates of Ad2 DNA synthesis in infected 3B2R1 cells



effort to determine whether there is a differential effect of 39.5°C upon the mutant JB3-B as opposed to its revertant at this earlier time p.i.. The protocol followed was exactly the same as that followed for JB3-B above. Figure 34 A and B presents these data as well as the data obtained by the 42 hour p.i. temperature shift previously shown in Figure 32 for comparison. At 33°C the rate of Ad2 DNA synthesis increases from 36 hours p.i. to approximately 42 hours p.i. then plateaus (negligible increase from 42 to 50 hours p.i.). The data obtained when infected cultures are shifted to 39.5°C at 42 hours p.i. demonstrate that, after a two hour lag, a decline in the rate of viral DNA synthesis similar to that observed in JB3-B under the same conditions can be seen. However, when infected cultures of 3B2R1 are temperature shifted to 39.5°C at 36 hours p.i., the rate of viral DNA synthesis is accelerated as compared to the rate at 33°C. This was an expected result of the effect of increased temperature on the rate of viral DNA synthesis. Thus these data indicate that 3B2R1 behaves as a true revertant at this point during viral infection. Cultures which are shifted to the 39.5°C later in infection (e.g. 42 hours p.i.) are accelerated to a later point in the time course of Ad2 infection (i.e. the decline in the rate of viral DNA synthesis). These data thus support the previous conclusion that the rate of Ad2 DNA synthesis is negatively affected by a shift to the restrictive temperature during the course of infection in JB3-B cells.

More data to support this conclusion was obtained by a similar analysis of another subclone of JB3-B. The subclone used here was also used for other studies contained in this thesis (i.e. complementation analysis, gene cloning) and therefore it was also important to conduct this analysis to confirm its phenotype. Thus, the rate of Ad2 DNA synthesis was also followed during infection of 3B·THO cells (the ouabain and thioguanine resistant subclone of JB3-B). Figure 35 presents the data obtained for this cell line when assayed under the same conditions as JB3-B in the preceding experiment. The data which describe the rate of viral DNA replication in infected 3B·THO cells continuously incubated at 33°C clearly differs from the analogous data obtained with JB3-B. The rapid acceleration in the rate of viral DNA synthesis which is seen between 36 and 42 hours p.i. for JB3-B is lacking in 3B·THO. Instead, there is a gradual increase in the rate of viral DNA synthesis up to 50 hours p.i.. When the cultures of Ad2 infected 3B·THO are shifted to 39.5°C 42 hours p.i., a result similar to that observed for JB3-B under the same conditions is obtained; namely, a rapid and progressive decrease in the rate of viral DNA synthesis. When cultures of Ad2 infected 3B·THO are shifted to 39.5°C at 36 hours p.i., a parallel rapid decrease in the rate of viral DNA synthesis is observed. This result is different from that which is seen for JB3-B under the same conditions. The data obtained at 33°C suggest that the time course of Ad2 infection in 3B·THO may be accelerated as compared to JB3-B

Figure 35. - Comparative effect of time of temperature shifts on the rate of Ad2 DNA synthesis in infected 3B·THO cells. Cultures of 3B·THO were infected and analysed for virus DNA as in Figure 30. Cultures were maintained at 33°C throughout the experiment (O—), shifted to 39.5°C at 36 hours p.i. (Δ--), or shifted to 39.5°C at 42 hours p.i. (□--).

Figure 35

Comparative effect of time of temperature shifts on the rate of Ad2 DNA synthesis in infected 3B·THO cells.

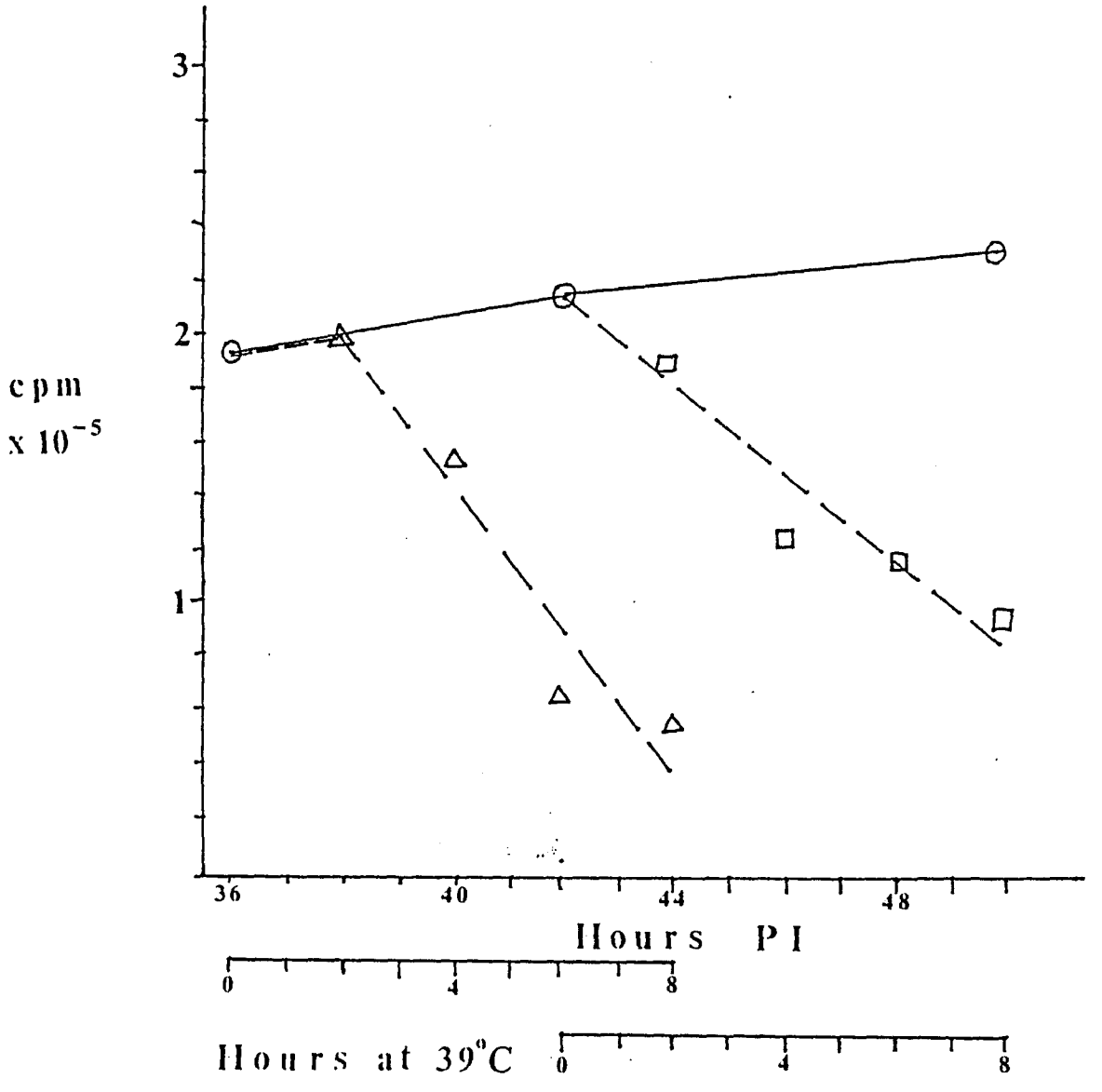
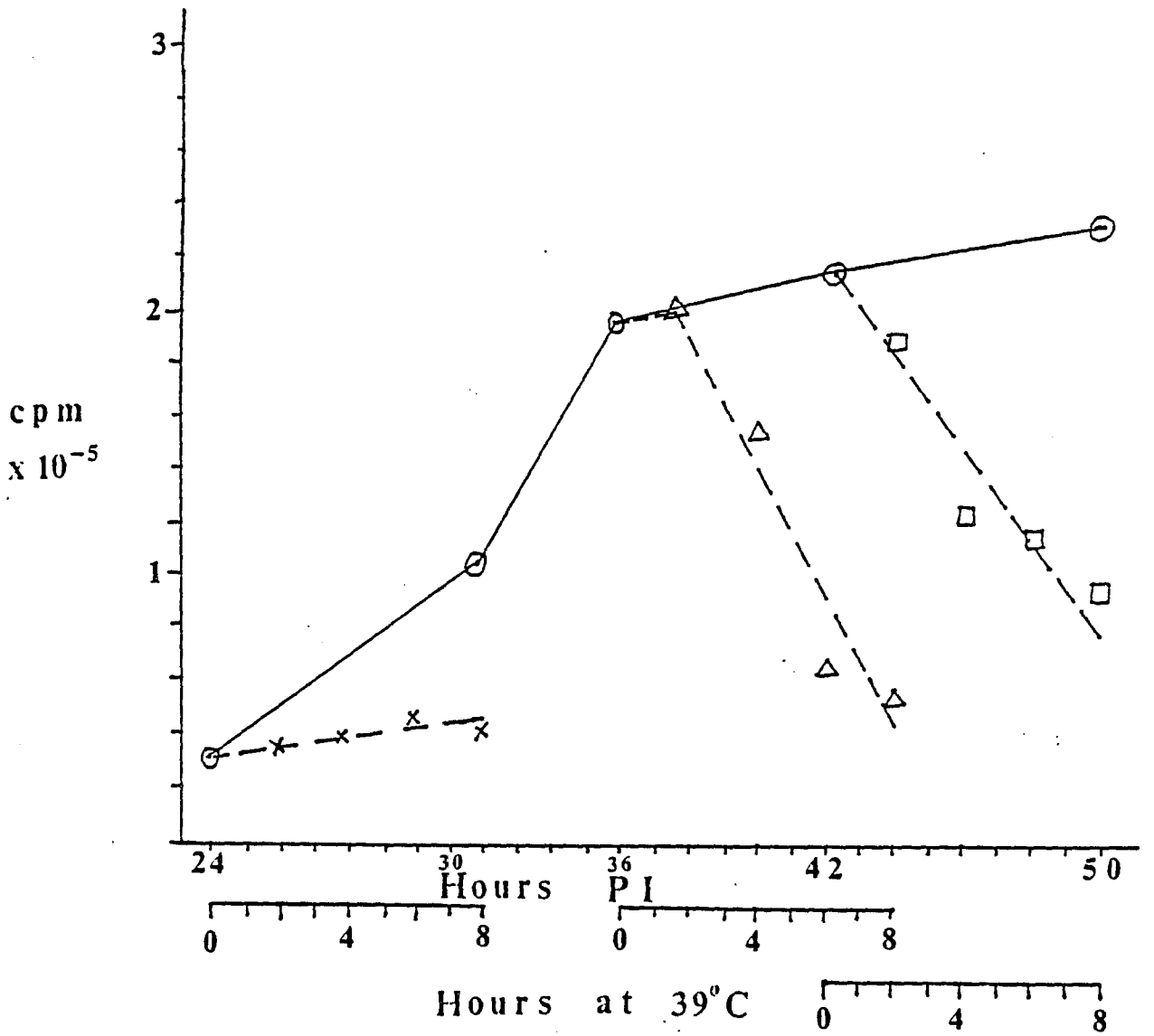


Figure 36. - Effect of temperature shift at 24 hours p.i. on the rate of Ad2 DNA synthesis in infected 3B·THO cells. Cultures of 3B·THO were infected and analysed for virus DNA as in Figure 30. Cultures infected for 24 hours at 33°C were shifted to 39.5°C and pulse labelled every two hours (X--) or maintained at 33°C and pulse labelled 8 hours later (32 hours p.i.). All samples at 36 hours p.i. and thereafter were redrawn from Figure 35 for comparative purposes.

Figure 36

Effect of temperature shift at 24 hours p.i.
on the rate of Ad2 DNA synthesis in infected 3B·THO cells.



itself. Therefore the rate of Ad2 DNA synthesis was analysed from 24 hours p.i. to 32 hours p.i. at 33°C and at 39.5°C when temperature shift occurs at 24 hours p.i.. The data obtained is presented in Figure 36 together with the data which was already shown in Figure 35. The data obtained by continuous incubation at 33°C shows that there is a rapid acceleration in the rate of viral DNA synthesis between 24 and 36 hours p.i.. This result is similar to that obtained with JB3-B between 36 and 42 hours p.i.. When cultures of infected 3B·THO are shifted to 39.5°C at 24 hours p.i., the rate of viral DNA synthesis remains approximately constant. This result is similar to that obtained with infected JB3-B cells temperature shifted at 36 hours p.i.. When these data are taken together (Figures 33 and 36), it can be seen that a rapid decline in viral DNA synthesis in temperature shifted cultures is best seen when the curve at 33°C is plateauing (i.e. the rate of viral DNA synthesis is not rapidly accelerating). When infected cultures are shifted to 39.5°C at times when the rate at 33°C is accelerating, however, a constant rate is observed. This constant rate is probably a result of a decreasing number of new templates available to start new rounds of DNA synthesis.

After the initial experiments demonstrated a fall in recoverable Ad2 DNA in JB3-B in the preceding experiments, it was considered that possible artifacts could be responsible; for example a degradation of viral DNA or a loss of infected cells. Thus, the constant rate seen after temperature shift

early in infection can be produced by an equilibrium between new synthesis of DNA and its degradation. At later times during infection when the rate of viral DNA synthesis is not increasing, the decline in the rate of viral DNA synthesis directly reflects the degradation of DNA as not enough new DNA is being produced to balance this decline. An alternate explanation might be that Ad2 infected cells become loosely attached to the dish when shifted to the restrictive temperature. Cells which are infected to a greater degree may preferentially become detached (possibly due to being in the early stages of cell death) and become lost during the experimental manipulations, especially during the PBS washes prior to cell lysis on the gradient. These cells may even be expected to contain a disproportionate amount of incorporated radiolabel than the cells which remain. Their loss may thus result in skewed data.

To assess these possibilities the experimental protocol employed in the previous experiments was modified as follows. Prior to Ad2 infection, cellular DNA was labelled with ^{14}C-TdR. After 36 or 42 hours p.i., cells were incubated with ^{3}H-TdR for one hour followed by a 30 minute chase with non-radioactive TdR to label the viral DNA pool. The medium from each of the dishes was collected, and any cells which may have been present were pelleted and frozen for later TCA precipitation. At the times of cell harvest for gradient analysis, the media and all of the PBS washes were likewise saved for later TCA analysis. The harvested cells were

resuspended in a final volume of 0.5 mls - 0.1 ml in duplicate for TCA analysis, 0.1 ml for cell counts, 0.2 ml loaded on the gradient. The cell counts were performed with trypan blue staining for viability. The results are presented in tables 14 and 15. There is no significant degradation of the viral (^{3}H-TdR) or the cellular (^{14}C-TdR) DNA observed. In cultures labelled at both 36 and 42 hours p.i. and subsequently chased, there is a consistant pattern of high recovery of unit length viral DNA and no systematic fall in total TCA-precipitable counts. The apparent low recovery of viral DNA in the 36 hour samples most likely reflects the presence of cellular DNA synthesis. The counts which are lost in the washes are negligible under all conditions (less than 12% of the total counts). The ^{14}C counts in the washes tend to increase during the course of infection from 36 to 44 hours p.i., but do not increase progressively from 42 to 50 hours p.i. at which time viral DNA synthesis is severely impaired. The ^{3}H counts also indicate that Ad2 infected cells are not preferentially lost when cultures are shifted to the nonpermissive temperature since there is no differential accumulation of ^{3}H-TdR labelled DNA in the washes.

In conclusion, it can be stated that there is a defect in the rate of Ad2 DNA synthesis when infected cultures of the ts DNA^{-} CHO mutant JB3-B are shifted to 39.5^{o}C. This defect is clearly related to the ts cell phenotype. An attempt was therefore made to determine the stage at which viral DNA synthesis was affected. The initiation of Ad2 DNA

Table 14

Stability of cell and virus DNA at 42 hours post infection[1]

Time after shift	Virus DNA (^{3}H-TdR) CPM X 10^{-6} (A) cell associated cpm[2]	(B) virus DNA cpm	(C) percent recovery of virus DNA (B/A X100)[3]	(D) percent ^{3}H recovered as unattached cells
0	6.06	7.72	127.0	2.0
2	7.99	6.51	81.5	2.5
4	7.42	8.28[4]	111.6	2.6
6	5.99	8.28[4]	138.0	3.3
8	6.51	7.40	113.7	4.0
8(33)	5.79	7.22	124.7	6.0

Cellular DNA (^{14}C-TdR) CPM X 10^{-4}

Time after shift	(E) cpm recovered as cell DNA	(F) percent recovered in unattached cells[5]	(G) percent viable cells[6]
0	2.26	9.3	65.7
2	1.51	15.7	73.3
4	1.95[4]	12.4	55.5
6	1.95[4]	12.5	60.9
8	2.00	11.9	67.7
8(33)	2.62	11.9	80.0

1 - Cells were labelled with ^{14}C-TdR, infected for 42 hours at 33°C, and pulse labelled with ^{3}H-TdR before being shifted to 39.5°C as described in the text. Viral DNA (^{3}H-TdR) was calculated from peak and cell DNA (^{14}C-TdR) from the CsCl cushion after alkaline sucrose gradient fractionation.

2 - A = cell associated ^{3}H-TdR TCA-precipitable cpm applied to an alkaline sucrose gradient.

3 - [^{3}H-TdR TCA-precipitable cpm recovered in the viral DNA peak (B) divided by cpm applied to the gradient (A)] X 100.

4 - samples from the gradient were pooled and values halfed.

5 - [^{14}C-TdR TCA-precipitable cpm recovered in unattached cells divided by cpm recovered in CsCl cushion of alkaline sucrose gradient] X 100

6 - Trypan blue exclusion test.

Table 15

Stability of cell and virus DNA at 36 hours post infection[1]

Time after shift	(A) cell associated cpm[2]	(B) virus DNA cpm	(C) percent recovery of virus DNA (B/A X100)[3]	(D) percent ^{3}H recovered as unattached cells
	Virus DNA (^{3}H-TdR) CPM X 10^{-6}			
0	13.22	2.34	17.7	4.0
2	4.00	1.86	46.5	6.7
4	9.30	2.63	28.2	9.0
6	10.74	2.72	25.3	11.1
8	11.51	5.44	47.2	6.7
8(33)	8.61	6.89	79.9	7.6

Time after shift	(E) cpm recovered as cell DNA	(F) percent recovered in unattached cells[5]	(G) percent viable cells[6]
	Cellular DNA (^{14}C-TdR) CPM X 10^{-4}		
0	2.85	4.7	94.3
2	2.18	8.0	72.7
4	2.57	7.3	63.6
6	0.42	48.2	57.7
8	2.10	10.4	62.5
8(33)	2.17	14.4	81.6

1 - Cells were labelled with ^{14}C-TdR, infected for 42 hours at 33°C, and pulse labelled with ^{3}H-TdR before being shifted to 39.5°C as described in the text. Viral DNA (^{3}H-TdR) was calculated from peak and cell DNA (^{14}C-TdR) from the CsCl cushion after alkaline sucrose gradient fractionation.

2 - A = cell associated ^{3}H-TdR TCA-precipitable cpm applied to an alkaline sucrose gradient.

3 - [^{3}H-TdR TCA-precipitable cpm recovered in the viral DNA peak (B) divided by cpm applied to the gradient (A)] X 100.

4 - [^{14}C-TdR TCA-precipitable cpm recovered in unattached cells divided by cpm recovered in CsCl cushion of alkaline sucrose gradient] X 100

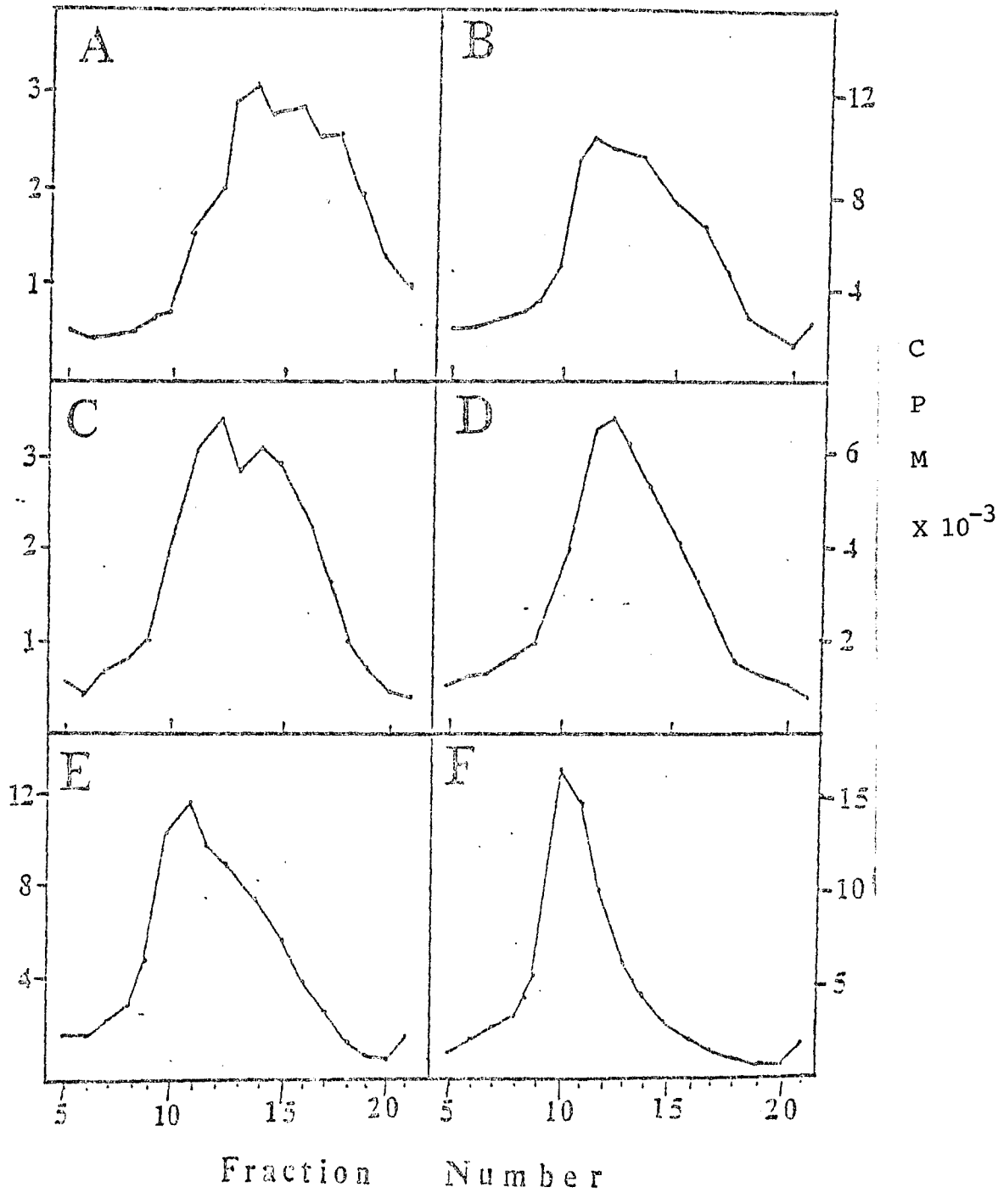
5 - Trypan blue exclusion test.

replication involves the use of a terminal protein which functions as a primer for DNA synthesis. It is generally believed that the DNA synthetic machinery of eukaryotic cells does not employ such a mechanism for initiation of cellular DNA synthesis. Therefore it seemed more likely that a defect in strand elongation rather than initiation could be responsible for the ts DNA$^-$ phenotype of JB3-B cells. Ad2 can be employed as a probe for strand elongation in JB3-B cells by modifying the conditions of the pulse-chase labelling in the alkaline sucrose gradient experiments; specifically, shortening the pulse to (4 minutes) and performing a series of chases of increasing duration. Although it has been reported that the time for replicating Ad2 DNA to elongate to full genomic length is approximately 22 minutes in wild-type CHO cells (grown in suspension at 37^{o}C), it was thought that the differences in the growth properties between these cells and JB3-B might also result in differing rates of viral elongation. Therefore, a preliminary experiment was performed at 33^{o}C to determine the optimum duration of the chases with which to best observe elongation in this cell system. Cultures of JB3-B were infected with Ad2 virions at the same MOI as the previous experiments. After 49 hours at 33^{o}C the cultures were pulsed with ^{3}H-TdR for 4 minutes, chased for 0, 3, 6, 9, 12, or 30 minutes with excess TdR (1 X 10^{-6}M), and analysed on alkaline sucrose gradients. The results, which are presented in Figure 37, indicate that the replicating viral DNA elongates to full genomic length between 12 and 30

Figure 37. - The elongation of Ad2 DNA in infected JB3-B cells at 33°C. Cultures of JB3-B cells were infected for 49 hours at 33°C, pulse labelled for 4 minutes, and chased for 0 - 30 minutes with 1 X 10^{-6}M TdR. Virus DNA was analysed on alkaline sucrose gradients as described in the Methods and 30. Each frame corresponds to an individual gradient: A, 0 chase; B, 3 minute chase; C, 6 minute chase; D, 9 minute chase; E, 12 minute chase; and F, 30 minute chase.

Figure 37

The elongation of Ad2 DNA in infected JB3-B cells at 33°C.



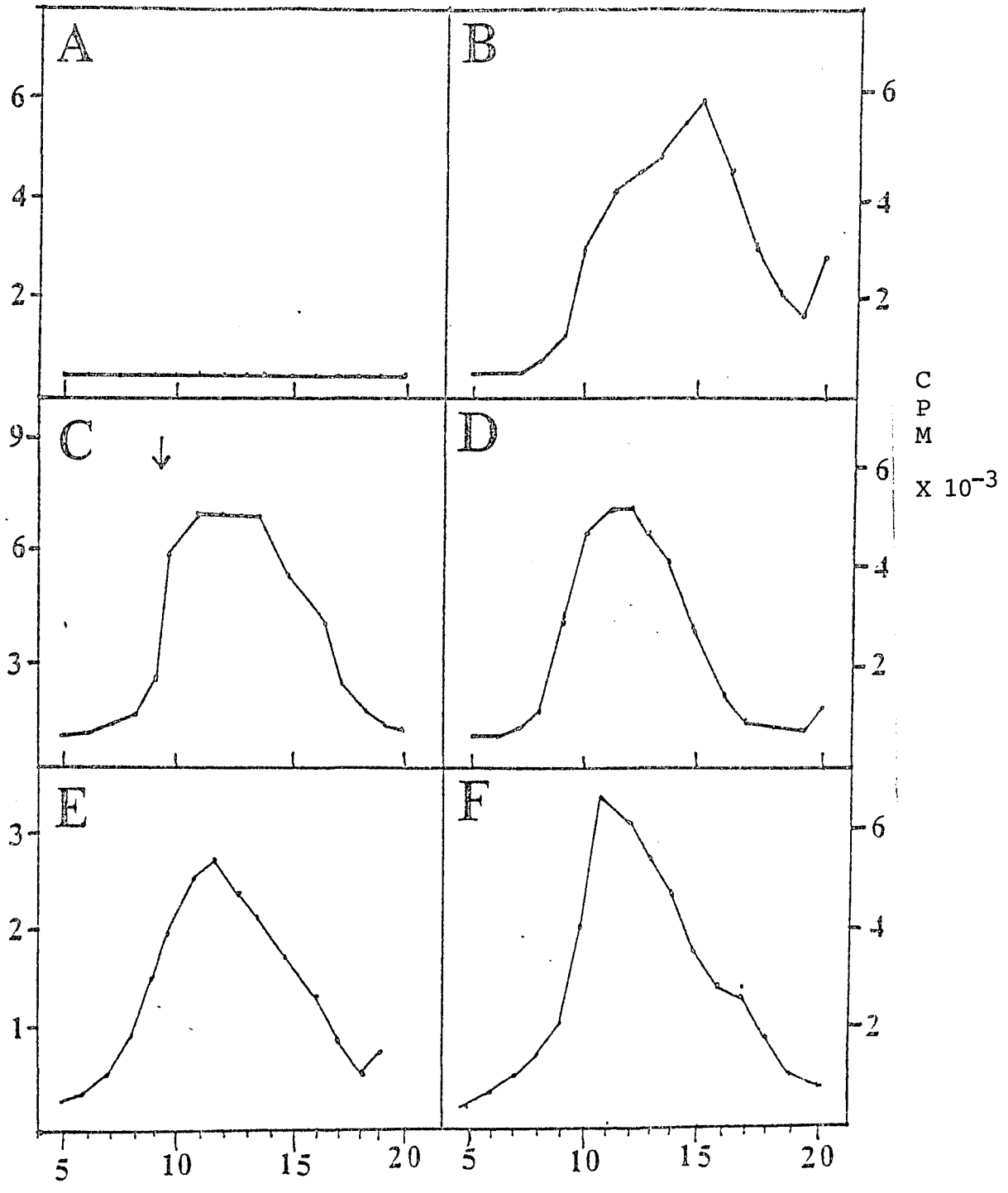
minutes. This is consistant with the rate which has been observed in wild-type CHO cells.

A second preliminary experiment was performed at 39.5°C. JB3-B cells, infected with Ad2 at 33°C, were shifted to 39.5°C at 42 hours p.i.. Eight hours later the cultures were pulsed with ^{3}H-TdR for 4 minutes and chased for 0, 8, 12, and 30 minutes. The cultures were left at 39.5°C for eight hours to attain the maximal effect of the nonpermissive temperature on Ad2 DNA synthesis. Uninfected cells were also analysed as a control to insure that the ^{3}H-TdR present in the gradient is incorporated into viral and not cellular DNA. Additionally, an infected culture was prelabelled with ^{14}C-TdR at 33°C prior to temperature shift to serve as a marker of full-length DNA in the gradient. The results are presented in Figure 38. A comparison of the uninfected cultures with the infected cultures demonstrates that the incorporated ^{3}H-TdR present in the gradient does not represent cellular DNA. The feasibility of prelabelling genomic length Ad2 DNA by this protocol is demonstrated by a comparison of the 8 minute chase gradients. The data which are presented in Figure 38, frame C show that the presence of the ^{14}C labelled marker DNA does not significantly interfere with the determination of the subsequent incorporation of ^{3}H-TdR into viral DNA. Although, on the average, 30% more ^{3}H-TdR is incorporated into viral DNA in the presence of the marker, the overall distribution of incorporated ^{3}H-TdR is not altered to a significant degree. (In subsequent experiments duplicate

Figure 38. - The elongation of Ad2 DNA in infected JB3-B cells at 33°C and 39°C. Cultures of JB3-B cells were infected for 42 hours at 33°C and shifted to 39.5°C for 8 hours prior to pulse labelling for 4 minutes and chasing for 0 - 30 minutes (B-F) as in Figure 40. Figure C and D are duplicates except that infected cultures were also labelled with ^{14}C-TdR at 33°C (40 - 42 hours p.i.). Appropriate double label corrections were performed. The arrow corresponds to the peak of ^{14}C-TdR corresponding to full length viral DNA. Frame A, uninfected cells; frame B, 0 chase; C, 8 minute chase; D, 8 minute chase; E, 12 minute chase; and F, 30 minute chase.

Figure 38 .

The elongation of Ad2 DNA in infected JB3-B cells at 33°C and 39°C.



gradients did not differ to a significant degree.) A time of 8 minutes is insufficient to chase the viral DNA to genomic length. This is clearly demonstrated by a comparison of all of the gradient profiles. However, it is difficult to establish the degree to which elongation has been completed at each time of chase. The use of ^{14}C-TdR to prelabel full length genomic DNA clearly permits a more accurate measurement of this parameter. This can be demonstrated by a comparison of both 8 minute chase gradients, with and without prelabelled marker. A visual comparison of the data from these experiments and the ones performed at 33°C suggests that elongation of viral DNA is retarded at 39.5°C. Full length viral genomic DNA is typically found in gradient fraction 10 or 11. An apparent preferred intermediate length DNA fragment can be observed in fractions 15 and 16. A quantitative comparison of fractions 10 + 11 (A) to fractions 15 + 16 (B) from the gradients is shown in Table 16. At 33°C the ratio of full genomic length DNA to intermediate length viral DNA increases from approximately one third, when no chase is applied, to five after a thirty minute chase. At 39.5°C this ratio also increases as the duration of the chase is increased. The magnitude of this increase (seven tenths), however, is not as great as that seen at 33°C (approximately two). A graphic representation of these data, shown in Figure 40, clearly shows the magnitude of this difference.

A more rigorous experimental comparison of the effect of the nonpermissive temperature upon Ad2 elongation can be

Table 16

Calculated rate of Ad2 DNA strand elongation in JB3-B[a]

Chase Conditions	Fraction A[a]	Fraction B[a]	Ratio[b]
33°C			
0	2047	5257	0.39
3	14376	13648	1.05
6	4837	5096	0.95
9	10062	7187	1.40
12	21636	9386	2.30
30	28855	5427	5.32
39.5°C			
0	7411	10428	0.71
8	9711	4482	2.17
8[c]	12752	8974	1.42
12	4396	3155	1.39
30	10486	6135	1.71

a - Data determined from fractions in Figures 37 (33°C) and 38 (39.5°C). Fraction A corresponds to TCA-precipitable cpm (^{3}H-TdR) in fractions 10 and 11. Fraction B corresponds to fractions 15 and 16.

b - Fraction A divided by Fraction B

c - contains ^{14}C-TdR labelled viral marker.

Figure 40. - Calculated rate of Ad2 DNA strand elongation in JB3-B. Illustration of the ratios of full genomic length Ad2 DNA to a particular intermediate fragment as calculated in Table 16. 33°C (o—); 39.5°C (▲--).

Figure 40

Calculated rate of Ad2 DNA strand elongation in JB3-B.

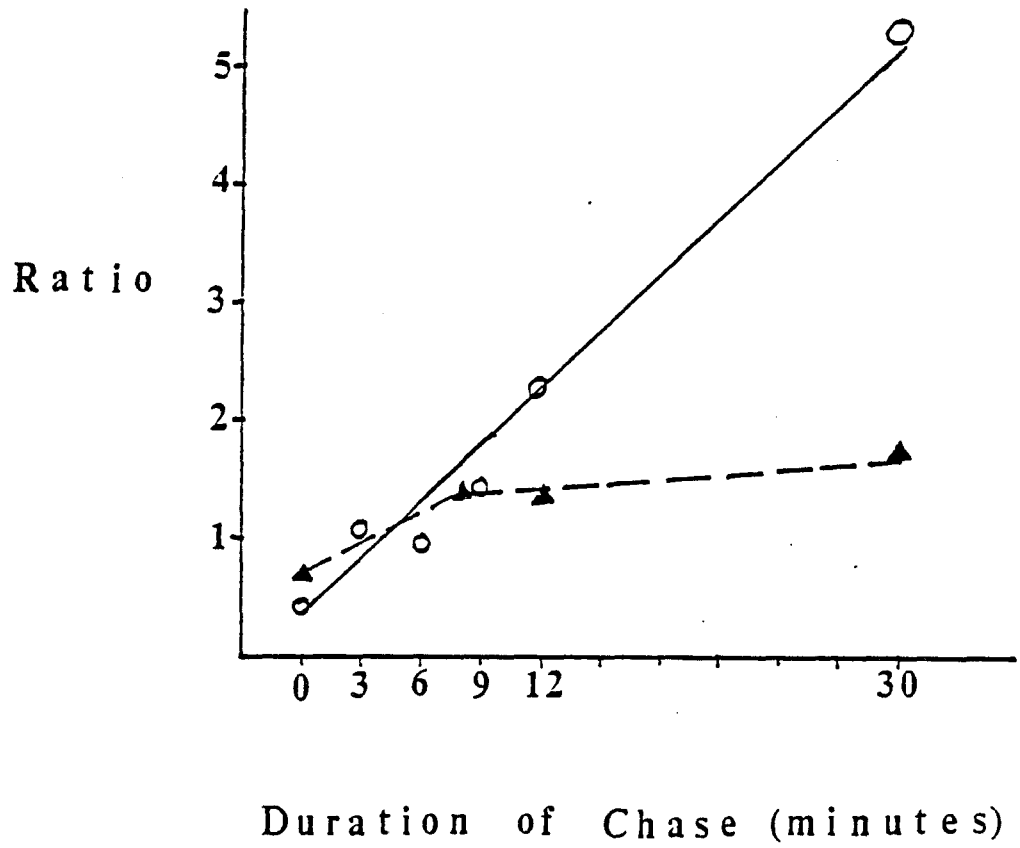


Figure 41. - Effects of shift to 39.5°C on the elongation of Ad2 DNA in infected JB3-B cells. Cultures of infected JB3-B cells were incubated for 42 hours at 33°C and either shifted to 39.5°C for 8 hours or maintained at 33°C. Equivalent cultures were pulse labelled and chased in parallel at the two temperatures as described in Figure 37.

Figure 41

Effects of shift to 39.5°C on the elongation of Ad2 DNA in infected JB3-B cells.

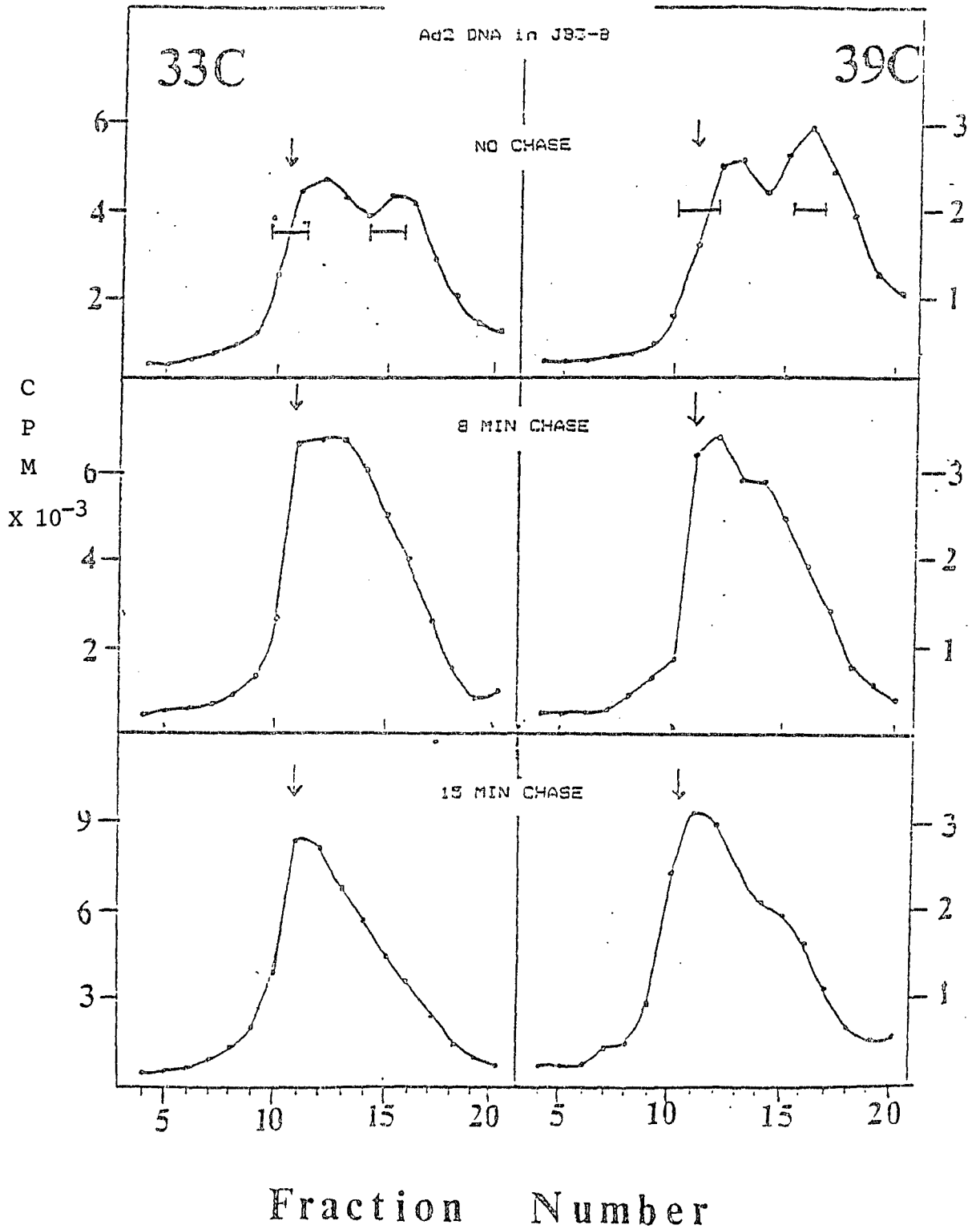


Table 17

Differential rate of Ad2 DNA strand elongation in JB3-B cells at 33°C and 39.5°C[a]

Chase Conditions	Fraction A[a]	Fraction B[a]	Ratio[b]
33°C			
0	6925	8375	0.83
8	6634	4054	1.64
15	8478	3595	2.36
39.5°C			
0	1674	2957	0.56
8	2859	1926	1.48
15	2456	1987	1.24

a - Data determined from fractions in Figures 41 (33°C) and 38 (39.5°C). Fraction A corresponds to TCA-precipitable cpm (^{3}H-TdR) in a fraction which corresponds to the ^{14}C-TdR full length viral marker. Fraction B corresponds to a fraction containing an intermediate sized viral DNA.

b - Fraction A divided by Fraction B

Figure 42. - Differential rate of Ad2 DNA strand elongation in JB3-B cells at 33°C and 39.5°C. Ratio of the data obtained in Figure 41 and Table 17 were plotted for 33°C (o—) and 39.5°C (▲--).

Figure 42

Differential rate of Ad2 DNA strand elongation in JB3-B cells at 33°C and 39.5°C

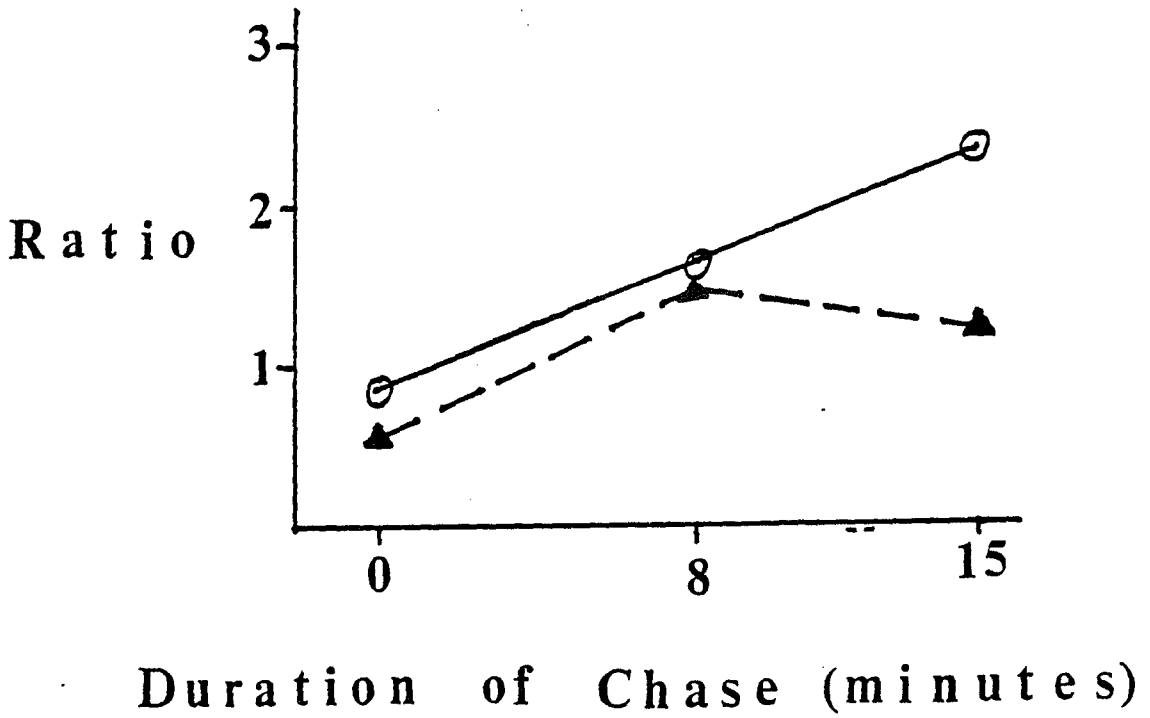


Table 18

Percent incorporation into full length Ad2 DNA in JB3-B cells at 33°C and 39.5°C[a]

Chase Conditions	A cpm incorporated in full viral DNA	B Total cpm in gradient	Percent (A/B)[b]
33°C			
0	3468	42763	8.1
8	9055	50190	18.0
15	12183	54968	22.2
39.5°C			
0	2166	25093	8.6
8	3650	22442	16.2
15	3335	25328	13.2

a - The data was determined from fractions in Figure 41. A is the total cpm (^{3}H-TdR) present in the fraction which corresponds to the leading edge of the full length viral DNA (as determined by the ^{14}C-TdR labelled viral DNA). B is the total cpm (^{3}H-TdR) present in the gradient. All numbers are corrected for spillover of ^{14}C-TdR cpm.

b - The cpm due to ^{3}H-TdR present in the leading edge of the ^{14}C-TdR containing fractions divided by the total ^{3}H-TdR cpm in the gradient.

obtained from a further experiment in which a prelabelled marker is used to precisely indicate the gradient fractions to which full genomic length viral DNA migrates. Cultures were shifted to the NPT at 42 hours p.i. and pulse-chased 8 hours later. The durations of the chase are 0, 8, and 15 minutes at each temperature. Furthermore, all samples were processed together. The data from each of the gradients is presented in Figure 41. When no chase is applied, two peaks of less than unit length are observed. Table 17 provides the data in the unit length and intermediate regions. The full genomic DNA migrates to fraction 10 or 11, as indicated in these gradients by the prelabelled marker. This form can be seen to accumulate with increasing duration of chase. A visual comparison of the graphs suggests that elongation occurs at a slower rate at 39.5°C. This is especially evident when the 15 minute chases are compared (i.e. the shoulder observed at 39.5°C, but not at 33°C). These data correspond well to the data from the previous experiments. A graph of the ratio of the tritium labelled DNA which migrates to the same gradient fraction as the genomic sized marker to the intermediate fragments contained in this peak is shown in Figure 42. The difference between these data, 33°C and 39.5°C, is not as great as it was in Figure 39. However these data (Table 17 and Figure 42) show that the ratios, which when taken together describe the rate of elongation at 39.5°C are consistently lower than the ratios at 33°C. It has been shown that replication occurs at a greater rate at higher

temperatures within the host cell temperature range. Therefore it seems logical to expect the rate of viral elongation to be greater at 39.5°C than at 33°C. Thus these data suggest that a defect in the elongation of replicating viral DNA exists at 39.5°C. This interpretation is supported by an alternate method of calculating the data; namely, the percent incorporation into full genome length viral DNA. The leading edge of the viral incorporated ^{3}H-TdR in the gradient was employed as a measure of the full genome length DNA present in the gradient. Only the leading edge was used to minimize as much as possible any interference attributable to the spillover of less than full length DNAs. The incorporated tritium present in the leading edge is shown as a percent of the total incorporation, corrected for the percent ^{14}C incorporated into the same gradient fractions, in Table 18. These data demonstrate that with increasing durations of chase the percent incorporation into full genomic length DNA increases in a more marked fashion at 33°C than it does at 39.5°C. These data also demonstrate the consistant incorporation of both ^{3}H-TdR and ^{14}C-TdR into viral DNA found in each gradient.

This interpretation is substantiated by performing similar experiments with Ad2 infected wild-type CHO-S cells and the revertant 3B2R1. The data obtained from the wild-type have been processed and plotted in the same manner as the data obtained from the mutant and are shown in Table 19 and Figure 43. As can be seen, the ratio of genome length DNA to

Table 19

Differential rate of Ad2 DNA strand elongation in CHO-S and 3B2R1 cells at 33°C and 39.5°C

Chase Conditions	Ratio[a] CHO-S	3B2R1	JB3-B[b]
33°C			
0	1.04	1.06	0.83
8	ND[c]	2.01	1.64
15	3.24	1.53	2.36
39.5°C			
0	1.01	0.83	0.56
8	1.64	2.25	1.48
15	4.49	2.01	1.24

a - Ratio = TCA-precipitable cpm (^{3}H-TdR) in a fraction which corresponds to the ^{14}C-TdR full length viral marker divided by TCA-precipitable cpm (^{3}H-TdR) in a fraction containing an intermediate sized viral DNA.

b - Ratios previously shown in Table 17 are presented here for comparison.

c - not determined.

Figure 43. - Differential rate of Ad2 DNA strand elongation in 3B2R1 and CHO-S cells at 33°C and 39.5°C. Ratios shown in Table 19 were plotted for 33°C (o—) and 39.5°C (▲--). Part A corresponds to CHO-S and Part B corresponds to 3B2R1. Ratios were calculated as described in Table 17.

Figure 43

Differential rate of Ad2 DNA strand elongation in 3B2R1 and CHO-S cells at 33°C and 39.5°C

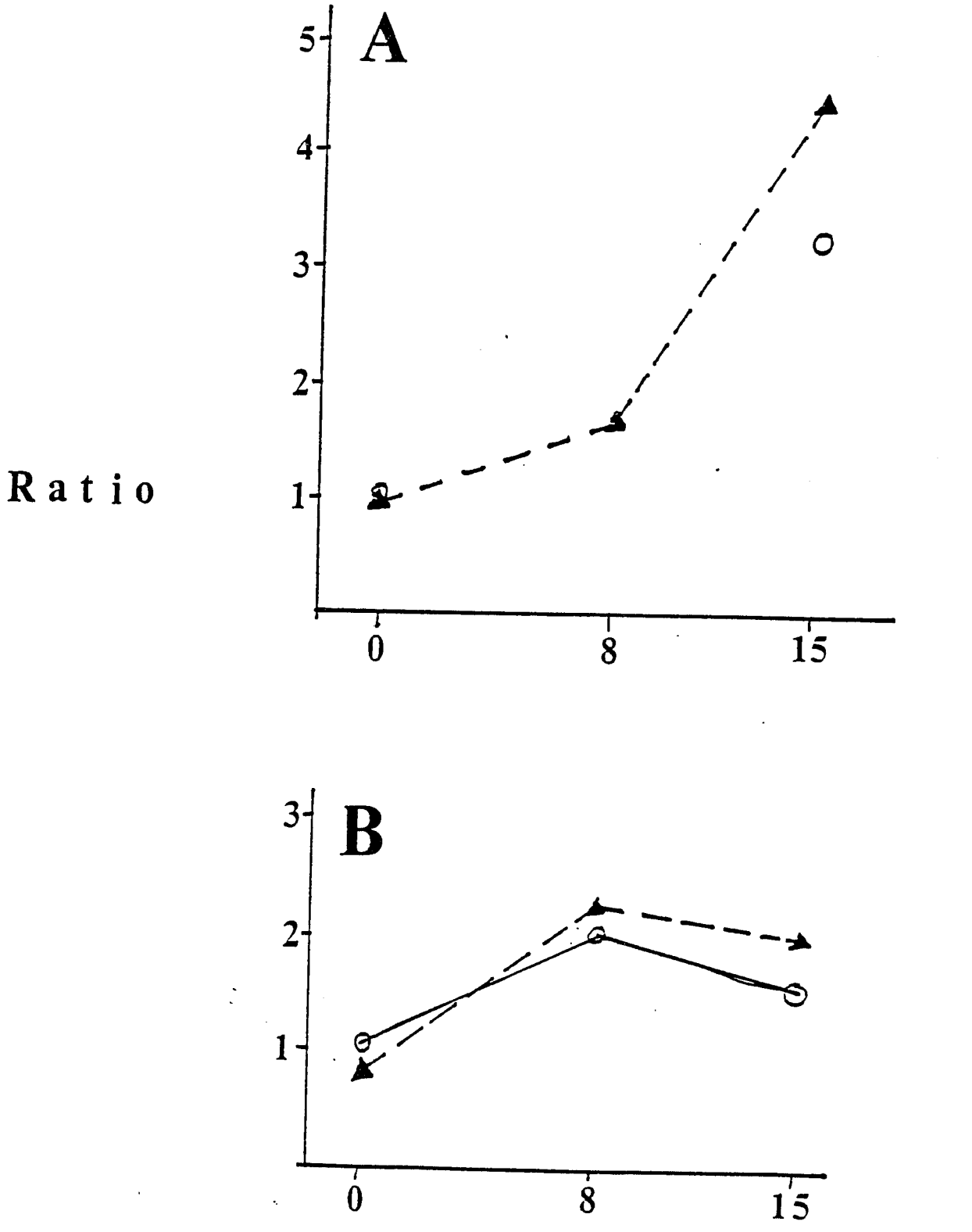


Table 20

Percent incorporation into full length Ad2 DNA in 3B2R1 and CHO-S cells at 33°C and 39.5°C[a]

Chase Conditions	Percent incorporation into genomic DNA[a]		
	CHO-S	3B2R1	JB3-B[b]
33°C			
0	9.9	13.1	8.1
8	ND[c]	13.5	18.0
15	16.0	13.1	22.2
39.5°C			
0	12.4	13.2	8.6
8	16.3	22.2	16.2
15	19.9	16.4	13.2

a - The data was calculated as described in Table 18. cpm (^{3}H-TdR) present in the fraction which corresponds to the leading edge of the full length viral DNA (as determined by the ^{14}C-TdR labelled viral DNA). B is the total cpm (^{3}H-TdR) present in the gradient divided by the total ^{3}H-TdR cpm in the gradient. All numbers are corrected for spillover of ^{14}C-TdR cpm.

b - Percentages previously shown in Table 18 are presented here for comparison.

c - not determined.

an intermediate size DNA increases with increasing duration of chase at both temperatures. However the ratio for each duration of chase is consistantly greater (or equal) at 39.5°C than at 33°C. This is in contrast to what has been observed for the mutant JB3-B (see table 16 and Figure 44). Thus the defect in strand elongation in JB3-B does not exist in wild-type CHO-S cells under the same conditions.

An analysis of the rates of Ad2 strand elongation in the JB3-B revertant, 3B2R1, was also performed, as shown in Table 19 and Figure 43. As predicted, the ratio at 39.5°C is greater than the corresponding ratio at 33°C for all durations of chase employed. Again this interpretation is supported by a calculation of the percent incorporation into genome length DNA. The data were calculated as previously noted and the resulting percentages for 3B2R1, CHO-S, and JB3-B are shown in Table 20. As can be seen, the percent incorporation into genomic size DNA are greater for 3B2R1 and CHO-S at 39.5°C than at 33°C at all durations of chase. JB3-B, however, is the opposite; the percent incorporation into genomic size DNA is greater at 33°C than 39.5°C for all durations of chase. These results are similar to those obtained from the ratio of full genome to intermediate size DNAs. Thus two separate criteria for interpreting these data yield the same conclusion.

Similar experiments have been performed with cultures of JB3-B and its revertant 3B2R1 shifted to the NPT after 36 hours infection instead of 42 hours (i.e. at a period during

infection when the rate of Ad2 DNA synthesis was increasing at 33°C for both cell lines). For these experiments the labelling conditions were also modified, increasing the concentration of TdR and the addition of deoxycytidine (CdR) to the chase medium. The increased concentration of TdR in the chase media could result in sufficiently increased levels of dTTP to inhibit the conversion of CDP into dCDP by ribonucleotide reductase (Mathews 1985) which in turn could result in a slowing of DNA synthesis by depleting the available pool of dCTP. Since high levels of TdR are required in the chase due to the inability to remove all of the ^{3}H-TdR from the cells by washing after the pulse, addition of CdR to the medium allows the cells to replenish the pool of dCTP. The addition of $1X10^{-5}M$ CdR permitted the concentration of deoxythymidine (TdR) in the chase to be increased 200 fold (from $1X10^{-6}M$ to $2X10^{-4}M$) in order to make the chase more efficient. The chase conditions were otherwise not changed. The data obtained from these experiments was processed and plotted as before. The resulting ratios for JB3-B and 3B2R1 are shown in Table 21 and Figure 44. At 33°C the data show good agreement for each cell line; the ratios for each cell line increase from 0.84 when no chase is applied to approximately 3.0 after a chase of 15 minutes, although the difference at 8 minutes of chase creates somewhat of a difference in the shape of the curves. When the ratios obtained eight hours after temperature shift to 39.5°C are considered, however, a striking difference in the pattern of

Table 21

Differential rate of Ad2 DNA strand elongation in JB3-B and 3B2R1 cells at 33°C and 39.5°C (modified chase)

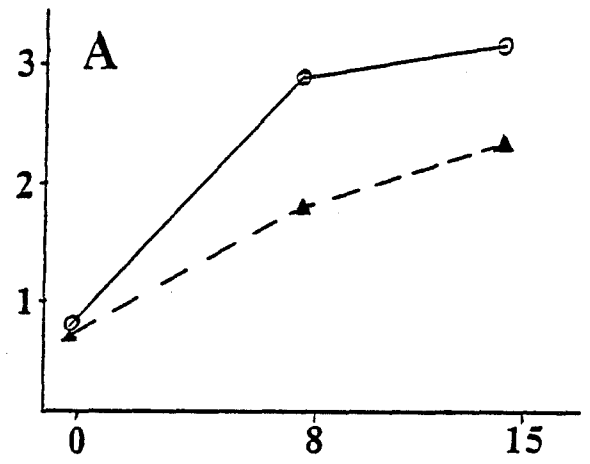
Chase Conditions	Ratio[a] 3B2R1	JB3-B
33°C		
0	0.84	0.84
8	1.80	2.90
15	2.86	3.05
39.5°C		
0	0.99	0.75
8	3.02	1.72
15	3.91	2.18

a - Ratio = TCA-precipitable cpm (^{3}H-TdR) in a fraction which corresponds to the ^{14}C-TdR full length viral marker divided by TCA-precipitable cpm (^{3}H-TdR) in a fraction containing an intermediate sized viral DNA. Data were corrected for spillover of ^{14}C.

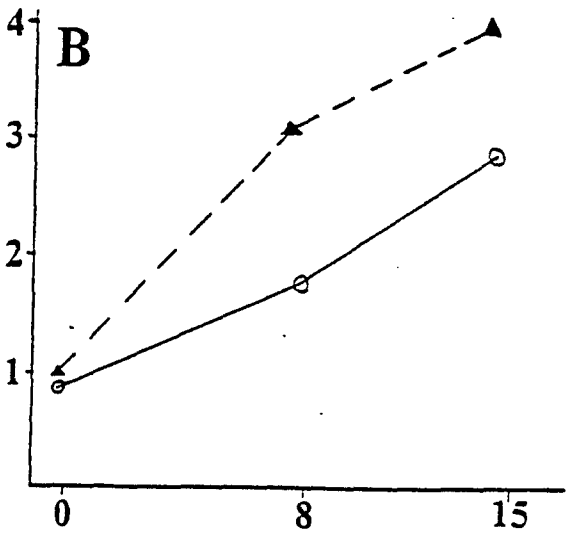
Figure 44. - Differential rate of Ad2 DNA strand elongation in 3B2R1 and JB3-B cells at 33°C and 39.5°C (modified chase). Ratios shown in Table 21 were plotted for 33°C (o—) and 39.5°C (▲--). Part A corresponds to JB3-B and Part B corresponds to 3B2R1. Ratios were calculated as described in Table 21.

Figure 44

Differential rate of Ad2 DNA strand elongation in 3B2R1 and JB3-B cells at 33°C and 39.5°C (modified chase)



Ratio



Duration of Chase (minutes)

the ratios can be observed. For the revertant, 3B2R1, the ratios are consistantly greater than those obtained at 33°C. Also the overall increase is from 1.0 when no chase is applied to approximately 4.0 after a chase of 15 minutes. For the mutant, JB3-B, the ratios obtained at 39.5°C are consistantly lower than those obtained at 33°C. The overall increase at 39.5°C is from 0.75 when no chase is applied to only 2.2 after a chase of 15 minutes. These data, obtained with more rigorous chase conditions, strongly support the interpretation of the previous data; namely, that JB3-B cells, when shifted to the nonpermissive temperature, are defective for a function which is required for the efficient elongation of replicating Ad2 DNA.

The wild-type and the revertant function as controls in these studies. Data generated from repeating this analysis with a cell line in which the rate of Ad2 strand elongation is inhibited or in which conditions can be manipulated to create such an inhibition (i.e. addition of a drug which inhibits a cellular elongation factor) would serve as a further control in these studies. Aphidicolin is an antibiotic drug which inhibits the function of the cellular enzyme DNA polymerase alpha. Several studies have demonstrated that this drug inhibits adenovirus chain elongation <u>in vivo</u> and <u>in vitro</u> (Longiaru et al. 1979). Thus, a positive control can be created by observing the effects of this drug on Ad2 chain elongation in JB3-B cells in the assay system employed in this thesis. Briefly, the conditions of

infection remain the same. Aphidicolin at the appropiate concentration was added to the media 4 hours prior to labelling. The conditions of labelling were as before except that the appropiate concentration of aphidicolin was included in both the pulse and chase media. The gradient analysis was performed as descibed in the preceding section. Previously published studies have shown that at a concentration of 50 micromolar, in vivo Ad2 strand elongation is severely inhibited (10%, as compared to the uninhibited activity level). These studies have also shown that at a concentration of 5 micromolar, viral strand elongation is only moderately inhibited (approximately 50% of uninhibited activity level). Thus both of these concentrations were employed in my studies. This latter concentration is particularly interesting since DNA synthesis is inhibited to 40% of the uninhibited levels after 8 hours at the restrictive temperature in JB3-B.

The data obtained from these experiments is presented in Figure 45. When no chase is applied, a large number of intermediate size DNA fragments are observed. In the presense of 5 micromolar aphidicolin, some of these intermediates are chased into larger sized intermediates and genomic length DNA as longer chases are applied. In the presence of 50 micromolar aphidicolin, however, these intermediate size DNAs fail to elongate to even larger intermediates. This data has been processed and plotted as before and is presented in Table 22 and Figure 46. In the presence of 50 micromolar

Figure 45. - Elongation of Ad2 DNA in infected JB3-B cells in the presence of Aphidicolin. Cultures of infected JB3-B cells were incubated at 33°C for 36 hours followed by addition of 5 uM or 50 uM aphidicolin for 4 additional hours. Cultures were pulse labelled, chased, and analysed as in Figure 44.

Figure 45

Elongation of Ad2 DNA in infected JB3-B cells in the presence of Aphidicolin

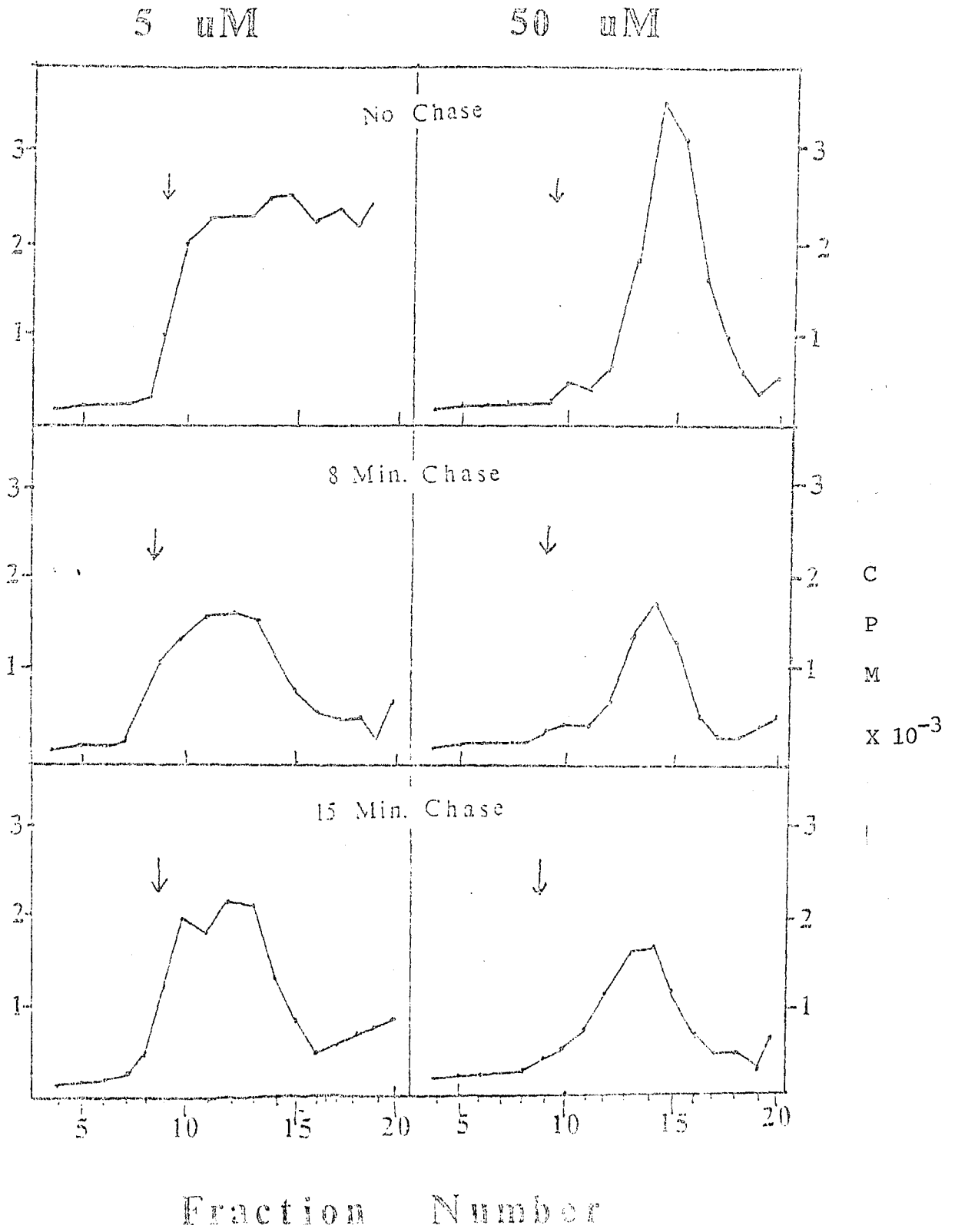


Table 22

Rate of elongation of Ad2 DNA in infected JB3-B cells in the presence of Aphidicolin

Chase Conditions	Ratio[a]			
	3B2R1[b]	JB3-B[b]	JB3-B + 5 uM aphidicolin	JB3-B + 50 uM aphidicolin
33°C				
0	0.84	0.84	0.60	0.09
8	1.80	2.90	0.87	0.10
15	2.86	3.05	1.30	0.12
39.5°C				
0	0.99	0.75		
8	3.02	1.72		
15	3.91	2.18		

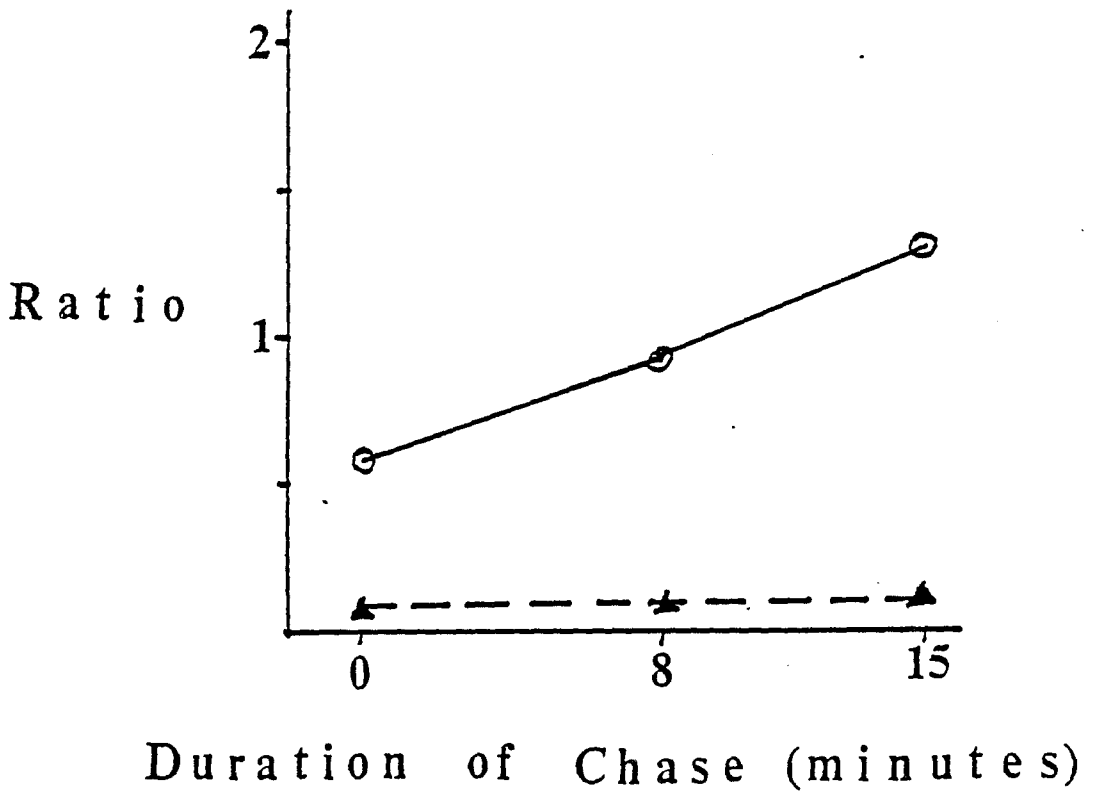
a - Ratio = TCA-precipitable cpm (^{3}H-TdR) in a fraction which corresponds to the ^{14}C-TdR full length viral marker divided by TCA-precipitable cpm (^{3}H-TdR) in a fraction containing an intermediate sized viral DNA. Data were corrected for spillover of ^{14}C.

b - Ratios previously shown in Table 21 are presented here for comparison.

Figure 46. - Rate of Ad2 DNA strand elongation in JB3-B cells at 33°C in the presence of aphidicolin. Ratios shown in Table 22 were plotted for 5 uM aphidicolin (o—) and 50 uM aphidicolin (▲--). Ratios were calculated as described in Table 22.

Figure 46

Rate of Ad2 DNA strand elongation in JB3-B cells at 33°C in the presence of aphidicolin.



aphidicolin a severe inhibition of Ad2 DNA strand elongation can be seen. The ratios are extremely low (0.09 as opposed to 0.84 in the absense of the drug when no chase is applied) and they fail to increase when increasing durations of chase are applied (0.09, when no chase is applied, to 0.12, after a chase of 15 minutes). In the presence of 5 micromolar aphidicolin, a less severe inhibition is observed. These ratios, while lower than those obtained in the absense of the drug, are much larger than those obtained in the presense of 50 micromolar aphidicolin (compare the ratios obtained when no chase is applied for JB3-B in the absense of the drug and at both concentrations of aphidicolin in Table 22). There is also a greater increase in the ratios as increasing durations of chase are applied at this concentration of the drug than in the presence of a greater concentration of the drug (from 0.6 when no chase is applied to 1.3 after a chase of 15 minutes). A more moderate degree of inhibition is obtained by the effect of incubation at the nonpermissive temperature in the absense of aphidicolin. Taken together, the data presented in Table 22 indicate that there is an inhibition of the elongation of replicating Ad2 DNA in JB3-B at 39.5°C. This inhibition, however, is not as severe as that observed in presence of even moderate concentrations of the drug aphidicolin (5.0 micromolar) in JB3-B at 33°C. These data also show that the cellular defect responsible for this inhibition has been corrected in the revertant, 3B2R1.

Studies on Ad2 DNA replication indicate that JB3-B is

defective for a function which is required for the elongation of relatively long stretches of DNA. Since the synthesis of RNA primers and Okazaki fragments is not required for Ad DNA synthesis to be completed, it can be stated that continuous strand synthesis is affected rather than discontinuous strand synthesis. There are two likely cellular mechanisms responsible: 1) a defect in a protein which is an integral component of the macromolecular mechanism for cellular DNA replication; 2) an imbalance in the deoxynucleotide triphosphate (dNTP) pools caused by either a defect in the synthetic pathway or lack of proper compartmentalization (i.e. failure of the available pools to be efficiently utilized). For all of the Ad in vitro DNA synthesis systems currently available the dNTP must be added to achieve efficient levels of replication. This results in a masking of the effects of the dNTP pools on Ad DNA synthesis. Therefore, before initiating in vitro analyses of Ad DNA synthesis in JB3-B, an attempt was made to assess the levels of the dNTP pools in JB3-B at both temperatures by high performance liquid chromatography (HPLC). Extracts of JB3-B cells incubated at 33°C or at 39.5°C for 8 to 10 hours were prepared according to the method of Tanaka et al. (1984) as described in the Materials and Methods. The data obtained was not sufficiently reproducible to be conclusive. However, the data did suggest that the levels of dTTP and dATP display moderate differences between 33°C and 39.5°C (30% decrease at 39.5°C). The levels of dGTP were not sufficiently detectable

in all analyses to be accurately measured. The data obtained from analyses in which the levels were detectable, however, suggest that there is a similarly moderate difference in the levels of dGTP in cells which had been incubated at the restrictive temperature for 10 hours. The levels of dCTP varied by more than 50% from one analysis to another due in part to interference from earlier eluting material and hence could not be accurately measured, although a review of all of the data suggests that the levels of dCTP are lower in cells which were incubated at the restrictive temperature (70% decrease at 39.5°C). Examples of the data obtained are presented in Figure 47 (33°C) and Figure 48 (39.5°C). Since the lack of reproducibility obtained by HPLC analysis led to inconclusive results, a collaboration was set up with Dr. Christopher Mathews at Oregon State University to measure the dNTP pool levels by an *in vitro* assay using DNA polymerase.

Figure 47. - HPLC analysis of dNTP pools in JB3-B cells incubated at 33°C. Procedures are as described in the Methods. Peak A corresponds to deoxyguanosine; Peak B to dCTP; Peak C to dTTP; Peak D to dATP; and Peak E to dGTP.

Figure 47

HPLC analysis of dNTP pools in JB3-B cells incubated at 33°C

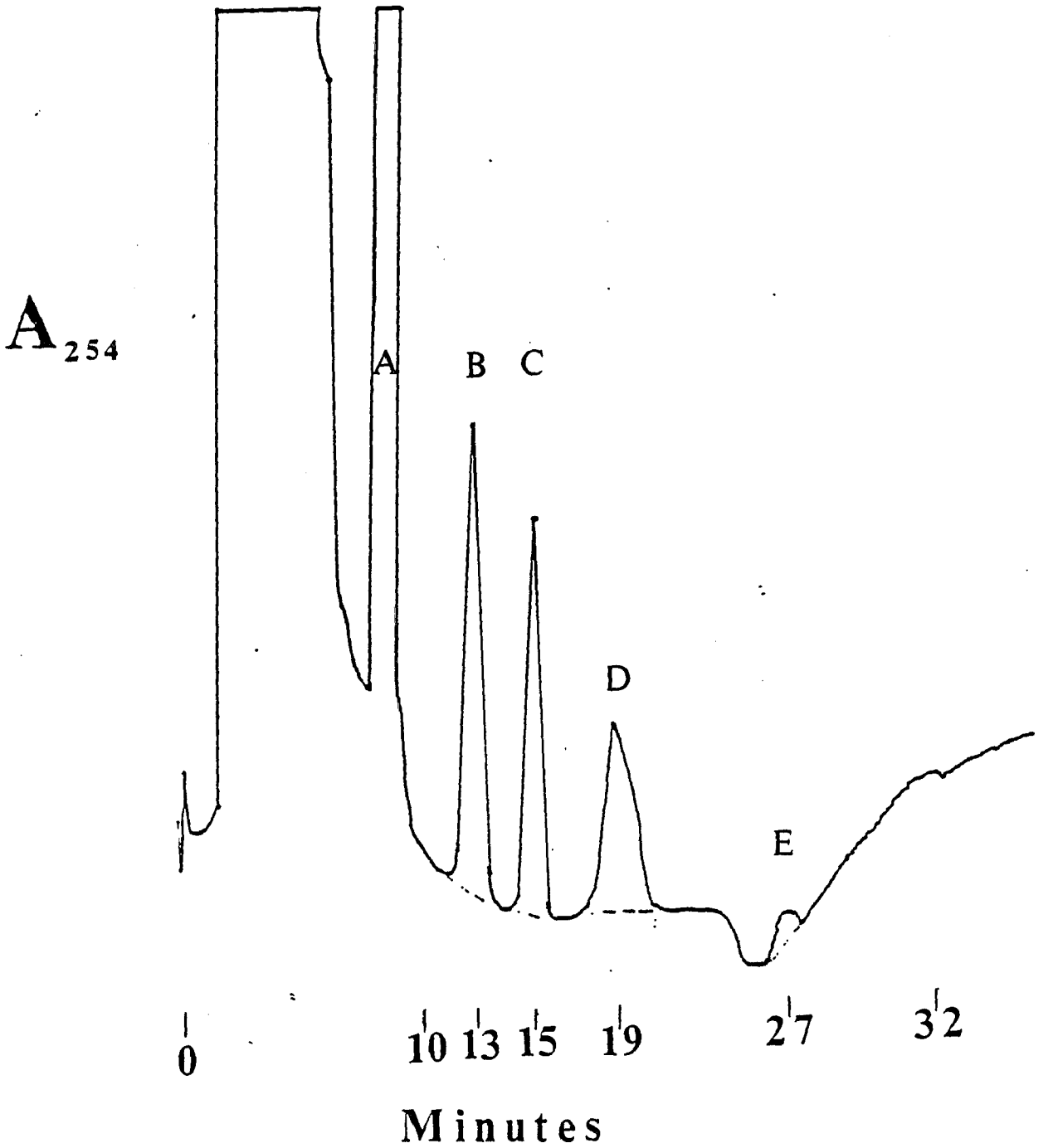
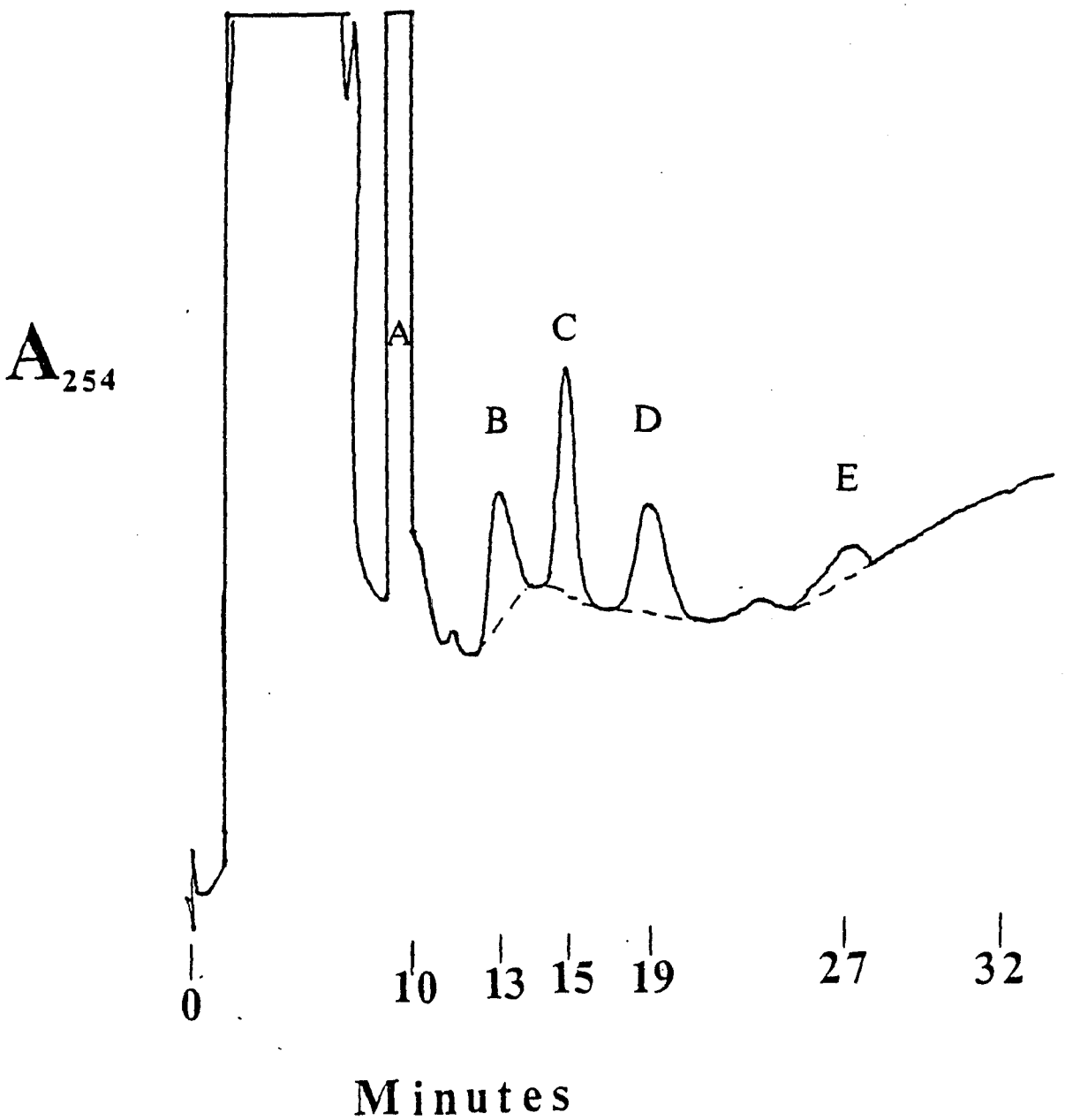


Figure 48. - HPLC analysis of dNTP pools in JB3-B cells incubated at 39.5°C. Procedures are as described in the Methods. Peak A corresponds to deoxyguanosine; Peak B to dCTP; Peak C to dTTP; Peak D to dATP; and Peak E to dGTP.

Figure 48

HPLC analysis of dNTP pools in JB3-B cells incubated at 39.5°C



Chapter 3 - The identification of human DNA sequences which correct the ts defect in JB3-B.

Previous studies have shown that there is a long lag time between the isolation of random replication mutants in eukaryotes and the identification of the gene products affected. One alternative is the use of well characterized host dependent viruses as probes with which to analyse the cellular defect. This is the primary approach which has been utilized in this thesis. Another approach is to clone a gene which corrects the defect in such a mutant. A general strategy to be employed in such an approach is to introduce DNA from another closely related organism into the replication defective cell line to obtain a primary transformant in which the defect has been corrected. Introduced sequences which contain the correcting gene can be enriched for by creating secondary and tertiary transformants. Probes made from appropiate linked sequences can be used to identify the introduced DNA sequences. The correcting gene can be then be isolated by screening a library made from the tertiary with these probes.

We have endeavored to utilize a variation of this strategy in an attempt to isolate a human DNA sequence which corrects the defect in JB3-B cells. Prior to initiating the actual molecular cloning of a human gene which corrects the ts DNA^- defect in JB3-B, confirmation of the ability of human DNA to correct the defect was obtained through the use of

somatic cell genetics. Cell hybrids were created by PEG-mediated cell fusion of human HS74 fibroblasts with the thioguanine, ouabain resistant ts DNA$^-$ mutant subclone, 3B·THO, followed by selection in HAT/ouabain medium at the permissive temperature (33^{o}C). Successful hybrids were then shifted to the restrictive temperature (39.5^{o}C) to determine whether the ts DNA$^-$ defect had been corrected. Selection in HAT/ouabain medium at the permissive temperature prior to the temperature selection should minimize the occurance of false positives caused by spontaneous revertants or self-self fusions which correct the defect. An initial series of fusions with equal numbers of cells (1:1 ratio) was performed. A number (46) of successful hybrids were obtained. When these hybrids were shifted to the restrictive temperature, however, none survived, indicating that the defect was not corrected (Table 23, section I). Two further series of fusions were done in an effort to obtain corrected hybrids; one, in which the ratio of HS74 cells to 3B·THO cells was increased by varying amounts (see Table 23, section II), the other identical to the first except that SV/HF-5/39, an HS74 cell line immortalized by the transfection of origin defective SV40 DNA (Neufeld et al. 1987), was used instead of the HS74. The ratios were increased in an effort to increase the efficiency of cell fusion. Since the HS74 cells are particularly sensitive to the effects of PEG, it was thought that the increased numbers of cells would increase the likelihood of their survival to form hybrids. Karyotypic

Table 23

Correction of the ts defect in 3B·THO by fusion with human cell lines[a]

Number of fusions	Total # of cells/fusion Human	3B·THO	ratio	hybrids obtained	ts corrected hybrids
I. HS74 X 3B·THO					
16	5.0×10^5	5.0×10^5	1:1	46	0
II. HS74 X 3B·THO					
1	5.0×10^5	5.0×10^5	1:1	0	0
2	1.0×10^6	5.0×10^5	2:1	0	0
1	5.0×10^5	2.5×10^5	2:1	0	0
1	1.5×10^6	5.0×10^5	3:1	0	0
2	2.5×10^6	5.0×10^5	5:1	0	0
3	1.3×10^6	2.5×10^5	5:1	0	0
1	2.5×10^6	2.5×10^5	10:1	0	0
III. SV/HF-5/39 X 3B·THO					
1	5.0×10^5	5.0×10^5	1:1	0	0
1	1.0×10^6	5.0×10^5	2:1	1	0
1	1.3×10^6	2.5×10^5	5:1	2	1
1	1.5×10^6	5.0×10^5	3:1	0	0
IV. HS74 X 3B·THO[b]					
1	1.0×10^6	5.0×10^5	2:1	0	0
1	1.5×10^6	5.0×10^5	3:1	0	0
1	2.5×10^6	5.0×10^5	5:1	1	1
1	1.3×10^6	2.5×10^5	5:1	1	1
V. JB3-B X 3B·THO					
1	5.0×10^5	5.0×10^5	1:1	61	0

a - Mixed cell populations were fused with PEG and selected in HAT/ouabain medium at 33°C as described in the Methods. Colonies growing in HAT/ouabain at 33°C were then shifted to 39.5°C to test for correction of the ts defect.

b - coselected in HAT/ouabain medium at 39.5°C.

analysis of SV/HF-5/39 reveals a greater than normal chromosome number, many of these extra chromosomes are rearranged and/or fragmented. It was thought that fusion with SV/HF-5/39 would increase the chances of a given hybrid retaining a chromosome or fragment thereof which corrects the ts defect in 3B·THO cells. Additionally it was thought that the gene(s) responsible for the senescence of HS74 cells may cause the premature death of successful hybrids. No successful hybrids were isolated when the ratio of HS74 to 3B·THO cells was increased. Three hybrids from two fusion regimens were isolated when SV/HF-5/39 was fused with 3B·THO (Table 23, section III). Upon shift to the restrictive temperature one of these hybrids survived, suggesting that a sequence of human DNA can correct the defect in the JB3-B subclone 3B·THO. Since HAT medium selects for the retention of the human X chromosome, it is possible that if the gene which corrects the ts defect is located on a human autosome it will be lost from the hybrid prior to its selection by temperature shift. In an effort to circumvent this possibility, another series of fusions with the ratio of HS74 to 3B·THO cells increased by varying amounts were performed. For this series the experimental protocol was altered by the simultaneous application of selection for both true hybrids (HAT/ouabain medium) and temperature correction (shift to the restrictive temperature) (Table 23, section IV). Two temperature corrected hybrids were isolated from two independent fusions in which there was a ratio of five HS74

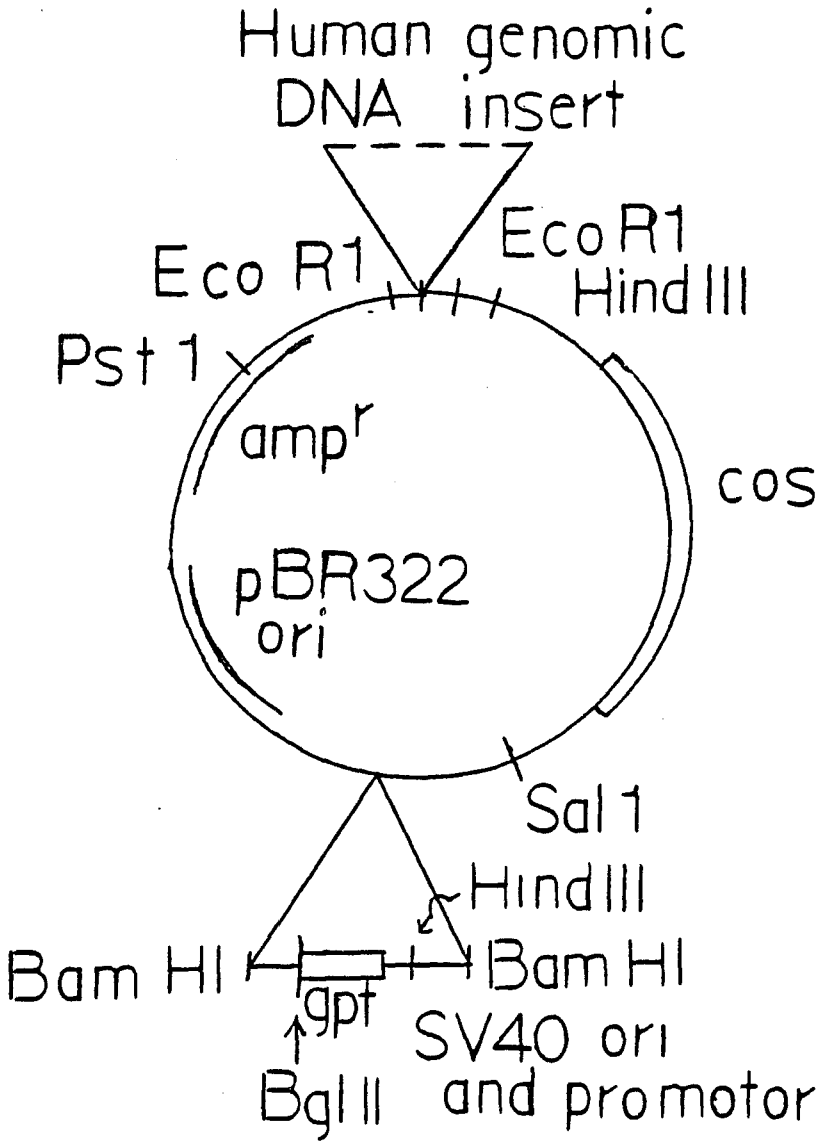
cells to one 3B·THO. Cell hybrids from 3B·THO X JB3-B fail to grow at 39.5°C indicating that a self-self fusion does not correct the ts defect (Table 23, section V) as previously noted. The isolation of three independent temperature corrected hybrids indicates that a human gene can correct the ts defect in JB3-B cells. A collaboration with an experienced cytogeneticist has been initiated in an attempt to identify the human chromosomes which have been retained in these hybrids.

The first step in the isolation of the human gene which corrects the ts defect in JB3-B is to introduce human DNA into JB3-B (or 3B·THO) cells. Rather than transfecting high molecular weight DNA from human cells into JB3-B, a human genomic DNA cosmid library, pCV103-gpt (shown in Figure 51) isolated by Lau and Kan (1983), was utilized as the source of human DNA. The use of this cosmid library confers several advantages over the use of high molecular weight DNA. Cosmids are bacterial plasmids to which the cohesive ends (cos sites) of the bacteriophage lambda have been attached to allow packaging with extracts of lysogenic bacteria (Collins and Hohn 1978). Up to 45 Kb of DNA can be inserted into cosmid vectors. Cloning such large DNA fragments increases the likelyhood of isolating an intact gene(s) from the genomic DNA and thus of preserving any functional domain present in the genes and their flanking sequences. If successful, it also defines an upper size limit of the (human) gene. The bacterial origin of replication present in the vector can be

Figure 49. - Structural map of pCV103-gpt. The Bam HI fragment containing the bacterial gpt gene and the SV40 origin from pSV2-gpt was inserted into the Bal I site of pJB8 which had been converted into a Bgl II site. The total size of the cosmid vector is 10.8 Kb. Genomic human leukocyte DNA was partially digested with Mbo I and DNA in the 30 - 45 Kb range was inserted into the Bam HI site on the cosmid vector.

Figure 49

Structural map of pCV103-gpt



utilized to rapidly and efficiently obtain large quantities of high purity DNA. This DNA can then be introduced into JB3-B by either transfection (co-precipitation with $CaPO_4$) or, if the mutant cells are not good recipients for this method, protoplast fusion (Litzkas, Jha, and Ozer 1984). Additionally the vector used in the manufacture of the cosmid library contains the bacterial gene guanine phosphoribosyltransferase (gpt) as a selectable marker in mammalian cells. This marker serves two very useful functions. First, it can be used as a probe for the successful introduction of the library. This would be especially useful if there are no repetitive sequences flanking the correcting gene(s). Secondly, successful transformants can be selected prior to testing for temperature correction, thus eliminating the possibility of false positives as a result of spontaneous reversion. Also, if 3B·THO is used as the recipient, correction of the defect by the successful introduction of human DNA can be confirmed by back-selection of ts thioguanine resistant cells. Another useful feature of the pCV103 cosmid library is that a number of different methods can be employed to isolate the correcting gene from the secondary or tertiary transformant (i.e. cosmid rescue, COS cell fusion). Thus this approach may eliminate the tedious manufacture and screening of a DNA library from the DNA of the secondary or tertiary transformant.

The ts DNA$^-$ subclone 3B·THO was utilized as the recipient cell line for these studies for several reasons.

Cells in which the human DNA containing cosmid vector has successfully integrated can be selected on the basis of the expression of the bacterial gpt gene by their ability to grow in HAT medium. This eliminates the requirement for mycophenolic acid and xanthine as components of the selection medium. Mycophenolic acid is highly toxic to cells and its concentration must be carefully adjusted for each cell line with which it is used. By eliminating its use, one can eliminate the necessity for performing a series of experiments to determine the conditions of optimal selection. Correction of the ts defect by the integrated human DNA in ts^+ transformants can be confirmed by back-selection of thioguanine resistant clones (loss of gpt) which should simultaneously reacquire their temperature sensitivity (loss of the linked ts correcting gene). This loss of both genes can be confirmed by Southern analysis. Other advantages of using 3B·THO cells as recipients are their low incidence of spontaneous reversion (see Chapter 2) and the fact that it is a good recipient for transfection using the calcium phosphate co-precipitation technique. In preliminary experiments, a frequency of 2.5×10^{-5} colonies per cell has been obtained using pRSV-gpt (Gorman et al. 1983) as the donor DNA.

An initial transfection was performed to determine the optimal conditions for transfection of the pCV103-gpt DNA. 100mm dishes containing 5×10^6 3B·THO cells each were transfected by the calcium phosphate co-precipitation technique as described in the Methods and Materials except

Table 24

Comparative efficiency of introducing pCV103-gpt DNA into 3B·THO

ug of DNA	CQ[a]	Number of Cells transfected	gpt+ colonies	Transfection Frequency
25	-	1×10^8	592	6.0×10^{-6}
25	+	2×10^7	69	3.5×10^{-6}
50	-	1×10^7	18	1.8×10^{-6}
100	-	1×10^7	22	2.2×10^{-6}

a - +, 1×10^{-4}M chloroquine added to each dish during transfection.
-, no chloroquine added.

that the amount of DNA used per dish was varied (25 ug, 50 ug, and 100 ug) and that for one set of dishes (25 ug DNA) 0.1 mM chloroquine was added in an effort to boost the transfection frequency (Luthman and Magnusson 1983). The results, which are presented in Table 24, show that the frequency of obtaining gpt^+ clones did not vary greatly (no more than 3-fold) as the amount of pCV103 DNA per 5 X 10^6 cells was doubled (50 ug) or quadrupled (100 ug). Likewise chloroquine treatment did not significantly increase the frequency of gpt^+ clones obtained (only 2-fold). None of the 701 gpt^+ clones survived after being shifted to 39.5°C for temperature correction.

Prior to the completion of this experiment a second transfection experiment was initiated. Twenty 100mm dishes were seeded with 5 X 10^6 cells each. Twenty five ug of pCV103 DNA was used to transfect each dish. One day post-transfection each dish was split into five 150mm dishes. For this experiment, however, the concentration of the components in the HAT media was halfed (3.24 X 10^{-5}M hypoxanthine, 2.5 X 10^{-6}M aminopterin, 5.75 X 10^{-6}M thymidine). This was done in an attempt to increase the survival of gpt^+ clones in which the gpt gene may be expressed at low levels (possibly due to the mode of integration). Five thousand gpt^+ clones, representing 2,500 independent integration events, were growing four weeks after the initial application of selection. At this time these colonies, continuing to be refed with 1/2 HAT media, were shifted to 39.5°C for

determination of temperature correction. Three temperature corrected clones, designated B23, B39, and B51, were observed two weeks after temperature shift. All three primary transformants exhibit very slow grow rates at both temperatures. However, since B51 grew more rapidly than the others at 39.5°C, it was used for further studies. Primary transformants B23 and B39 were frozen in liquid nitrogen for possible later analysis.

The authenticity of the primary transformant B51 was confirmed by back-selection; specifically, the co-loss of the gpt gene and the linked human sequence which corrects the ts defect in 3B·THO. Cultures of B51 were shifted to 33°C. After being passaged eight times (1/10 split each passage) in the absence of HAT medium, the cultures were refed with medium containing 5 X 10^{-5}M 6-thioguanine (TG). In this manner seven TG^R clones were obtained. These were seeded into duplicate cultures, one was grown at 33°C and the other was shifted to 39.5°C. The cultures at 33°C continued growing. However, the cultures which had been shifted to 39.5°C stopped growing and died, indicating that both the gpt gene and the gene which corrects the ts defect were lost simultaneously. This has been confirmed by Southern analysis using either gpt (Figure 50) or human alu DNA from pBLUR8 (Figure 51) as a probe. The Southern analysis also reveals that abundant amounts of human DNA sequences were retained in B51. All of these human sequences were lost in the back-selected clones.

Most of the human-specific sequences found in the

Figure 50. - Detection of gpt sequences in B51, BBS-7, 206, and BS206 DNA. Southern analysis was performed as described in the Methods. Lanes 1 through 12 contain ten micrograms of high molecular weight DNA per lane. DNA in lanes 1,4,7, and 10 were cut with Eco RI. DNA in lanes 2,5,8, and 11 were cut with Hind III. DNA in lanes 3,6,9, and 12 were digested with Pst I. Lane 13 contains 100 pg of pJB8 DNA linearized with Hind III. Lane 14 contains 80 pg of pSV2-gpt DNA linearized with Eco RI. Lanes 15 through 17 contain pBLUR8 DNA in the following amounts: 5 pg, 10 pg, and 100 pg, respectively. All pBLUR8 DNA was linearized with Eco RI. The gel purified Bgl II to Bam HI fragment of pSV2-gpt was labelled with ^{32}P using the random primer method and used to probe the blot.

Figure 50

Detection of gpt sequences in B51, BBS-7, 206, and BS206 DNA

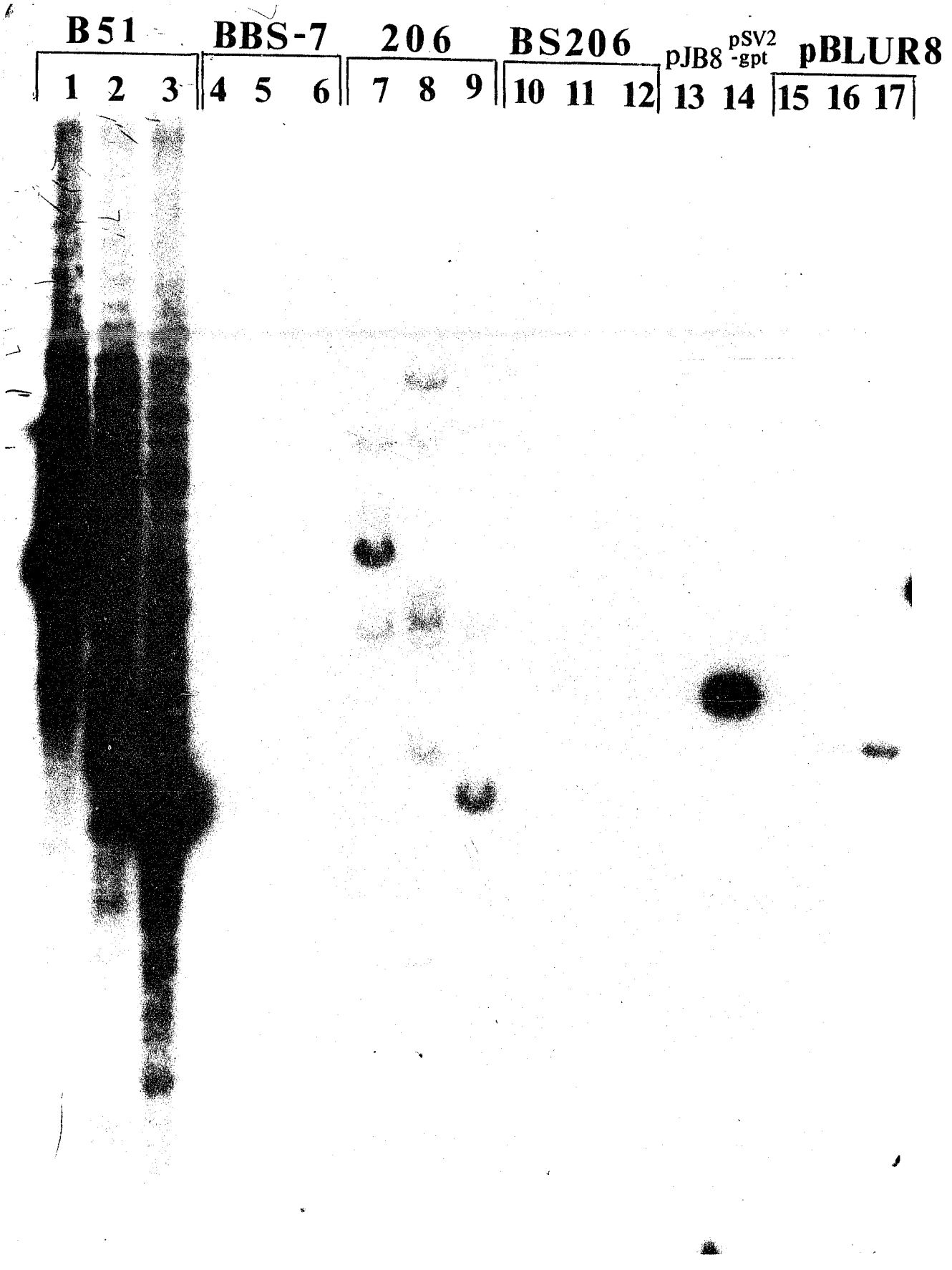
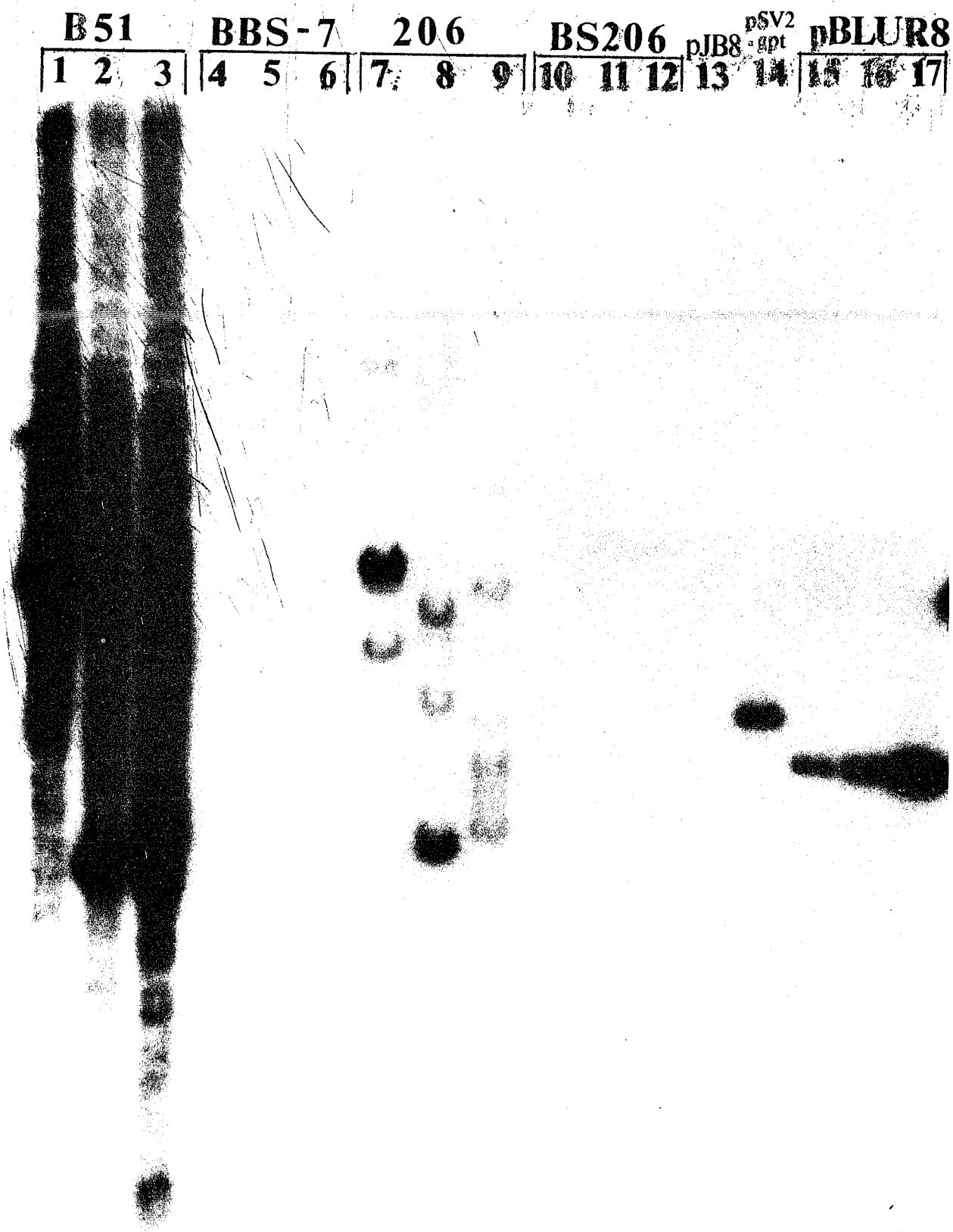


Figure 51. - Detection of human alu sequences in DNA from B51, BBS-7, 206, and BS206. The filter shown in the previous Figure (50) was dehybridized as described in the Methods and rehybridized with the gel purified human alu sequence from pBLUR8.

Figure 51

Detection of human alu sequences in DNA from B51, BBS-7, 206, and BS206



primary transformant, B51, may arise by co-transfer, and thus not be responsible for the temperature correction in this clone. These human sequences will likely be lost during a secondary transfection. Thus to enrich for the human DNA sequence(s) which correct the ts defect in 3B·THO a secondary transformant was made. High molecular weight DNA from the primary transformant, B51, was made as described in the Methods. This DNA was sheared by passing it through a 18g needle 3 to 5 times to increase the transfection efficiency. The sheared DNA was introduced into 3B·THO cells by the calcium phosphate co-precipitation technique as described in the Methods. Four weeks after transfection of 10^8 3B·THO cells 822 gpt^+ clones, one of which was growing rapidly and shedding many satellite colonies, were obtained. Two growing clones were isolated three weeks after the gpt^+ clones were shifted to 39.5°C. Both of these temperature corrected clones, designated 206 and 2010, came from the same dish which contained the large colony which was shedding satellites. Therefore it was thought that these clones were subclones of the same secondary transformant. Southern analysis revealed that 206 and 2010 are indeed indistinguishable (data not shown). A more detailed southern analysis of the secondary transformant 206 is shown in Figure 50 (gpt as a probe) and Figure 51 (human alu sequence as a probe). For this analysis high molecular weight DNA was obtained from 206 (as well as the other cell lines) as described in the Methods. The DNA was cut with each of three

restriction enzymes: Eco RI, which cuts the human DNA insert away from the the cosmid vector. This enzyme does not cut anywhere else in the vector but may cut one or more times within the human DNA sequence (see Figure 49 for the map of the vector); HindIII, which cuts twice in the vector (once between the insert and the cos sequence and once in the SV40 origin and promotor sequence) and at any number of possible sites within the human DNA insert; PstI, which cuts once within the ampicillin resistance gene and at any number of possible sites within the human DNA insert. The primary transformant B51 shows a large number of integrated DNAs whether probed with gpt or alu. The secondary transformant possesses fewer integrated DNAs indicating that an enrichment for the human sequence which corrects the ts defect has occured. When this DNA is cut with Eco RI and hybridized with gpt, 4 bands, each greater than 5.2 Kb, can be observed. One of these bands is of an intensity which indicates that it represents a tandem repeat. When the secondary DNA is cut with HindIII or PstI and probed with gpt, 7 and 2 bands respectively are observed. Since the restriction pattern of these enzymes within the vector are known and the gpt probe will only hybridize to the gpt present in the vector, these data indicate that from 4 to 7 different integrated vector DNAs are present in the secondary transformant. This blot was then dehybridized as described in the Methods and rehybridized with the human alu sequence from the plasmid, pBLUR8. The results are shown in Figure 51. When the Eco RI

cut DNA is hybridized with alu, 7 bands, all greater than 5.2 Kb, are observed. When the DNA from the secondary transformant is cut with HindIII or PstI, 8 and 7 bands, respectively, can be observed. These data confirm that human DNA sequences are present in the secondary transformant.

The authenticity of the secondary transformant was also confirmed by back-selection. Cultures of 206 were shifted to 33°C. After being passaged eight times the cultures were refed with medium containing 5 X 10^{-5}M TG. One TG^R clone at 33°C which failed to grow at 39.5°C was isolated. Southern analysis revealed that both the gpt gene (Figure 50) and the human alu sequences (Figure 51) are no longer present in the back-selected clone.

Ad2 infection followed by dot blot analysis was performed to determine whether the inhibition of Ad2 DNA synthesis at the nonpermissive temperature observed in 3B·THO was also corrected in the primary and secondary transformants. The methods employed are the same as those employed in Chapter 1. The blots obtained for the primary B51 and secondary subclones 206 are shown in Figures 52 and 53, respectively. These data indicate that the defect in Ad2 DNA synthesis present in 3B·THO (see Figure 22, Chapter 2) has been corrected in the primary and secondary transformants. Dot blot analysis was also performed with two of the back-selected primary clones. An example of which, shown in Figure 54, indicates that there is an inhibition of Ad2 DNA synthesis at 39.5°C. Taken together these data indicate that

Figure 52. - Ad 2 DNA replication in B51. Dot blot analysis was performed as described in Figure 18. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg.. The DNA in lane 5 was extracted from uninfected cells. DNA in lanes 2 - 4 were extracted at 24 hours p.i.. DNA in lanes 6 - 9 were extracted at 48 hours p.i.. DNA in lanes 2,3, and 6 are from infected cells which had been incubated at 33°C. DNA in lanes 4 and 7 are from infected cells which had been incubated at 39.5°C. The DNA in lanes 8 and 9 are from infected cells which were incubated for 24 hours at 33°C, then shifted to 39.5°C and incubated for an additional 24 hours.

Figure 52

Ad 2 DNA replication in B51

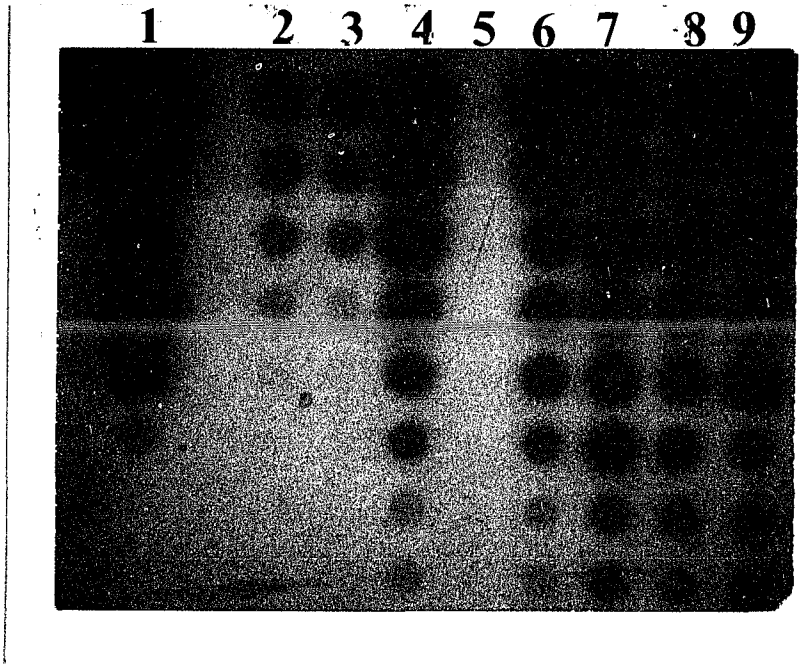


Figure 53. - Ad 2 DNA replication in 206. Dot blot analysis was performed as described in Figure 18. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg..The DNA in lane 2 was extracted from uninfected cells. DNA in lanes 3 (33°C) and 4 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 5 (33°C) and 6 (39.5°C) were extracted at 48 hours p.i.. The DNA in lane 7 is from infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 53

Ad 2 DNA replication in 206

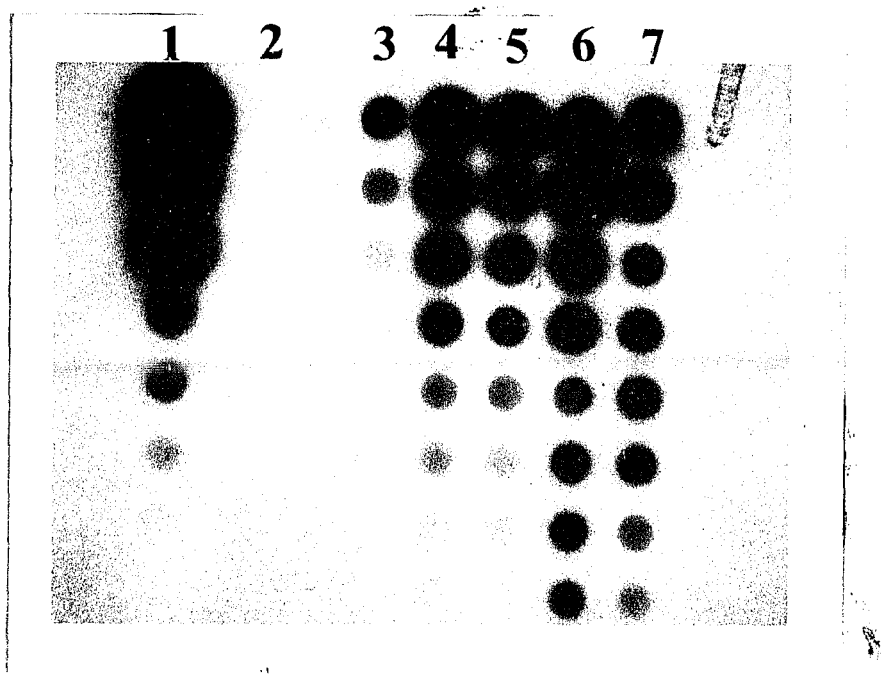
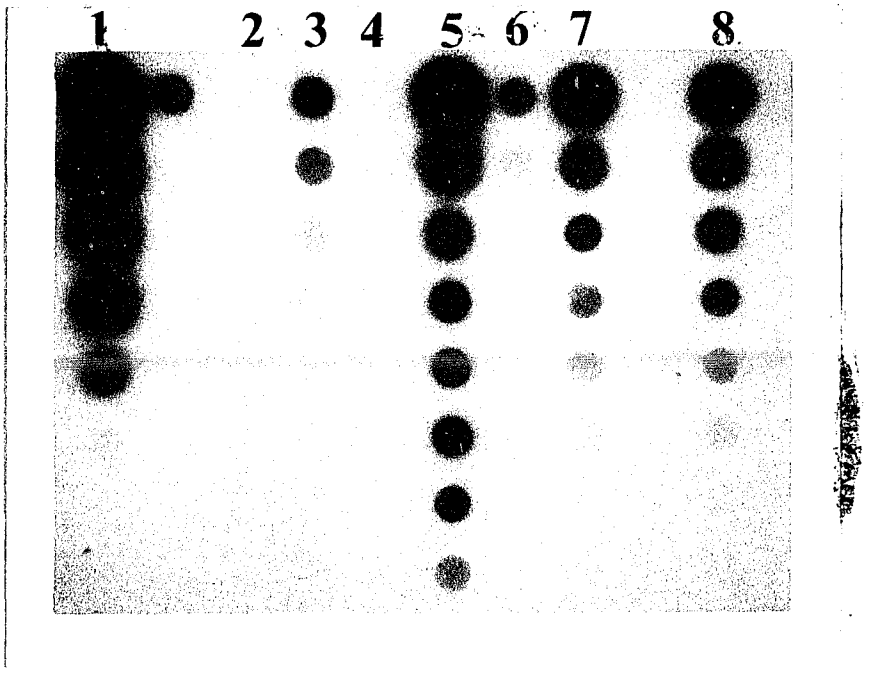


Figure 54. - Ad 2 DNA replication in BBS-7. Dot blot analysis was performed as described in Figure 18. Lane 1 contains tenfold dilutions of pLA1 DNA from 1.0 ug. to 0.1 pg..The DNA in lane 2 was extracted from uninfected cells. DNA in lanes 3 (33°C) and 4 (39.5°C) were extracted at 24 hours p.i.. DNA in lanes 5 (33°C) and 6 (39.5°C) were extracted at 48 hours p.i.. The DNA in lanes 7 and 8 are from replicate infected cells which had been incubated at 33°C for 24 hours, shifted to 39.5°C and incubated for an additional 24 hours.

Figure 54

Ad 2 DNA replication in BBS-7



the human DNA sequence(s) which correct the cellular defect in DNA replication also correct the defect in Ad2 DNA synthesis. This supports the interpretation that the same defect is responsible for the inhibition of both cellular and viral DNA synthesis and that the use of Ad2 to determine the nature of the cellular defect is a valid one.

Initially the cosmid which contains the correcting human DNA sequence was to be recovered by the in vitro packaging of DNA from the secondary which had been partially digested with MboI according to the method of Lau and Kan (1983). However other researchers in this lab have been experiencing difficulties in obtaining sufficient yields of phage due to a problem in obtaining sufficient quantities of DNA in the proper size range for efficient packaging. Therefore a different approach was taken to recover the correcting human DNA sequence. Previous studies by Conrad et al. (1982) and Tsui et al. (1982) have demonstrated the ability to recover sequences containing the SV40 origin of replication from cells into which they had been transfected by somatic cell fusion with COS cells. COS cells constitutively express SV40 T antigen (T ag). When these cells are fused to cells which contain an SV40 origin the T ag stimulates the in situ ("onion skin") replication of the SV40 origin and some flanking DNA which then excises. This DNA can then be isolated by Hirt extraction of the hybrid cells. Exogenous DNA which has integrated as a tandem repeat can be isolated intact; exogenous DNA which has integrated as a single copy

insert has been recovered in a variety of different sizes.

COS-7 cells and 2010 cells which had been seeded in ratios of 5:1, 1:1, and 1:5 were fused by PEG-mediated cell fusion. Hirt lysates were made from the hybrids at 24, 48 and 72 hours post-fusion. Aliquots of these lysates were electrophoresed on agarose gels, transfered to Nytran filters and probed with gpt as described in the Methods. The results indicate that a wide range of different size DNAs are obtained (data not shown). However, there are also present 4 bands which represent DNAs of a specific size due to excision at preferential sites (possibly within the tandemly integrated sequence observed in the secondary transformant by Southern analysis). Since the cosmid also contains a bacterial origin of replication and the gene which encodes ampicillin resistance, competant DH-5 (Stratagene) was transformed with the DNAs in the Hirt supernatants and successfully transformed colonies were isolated on agar plates containing ampicillin. Sixty colonies were isolated in this manner. Southern analysis was performed with uncut DNA obtained from minipreps of 34 of these colonies (data not shown). It shows that the majority of the plasmids are of two sizes which correspond to two of the prominent DNAs seen in the Hirt lysate (the four bands are thought to represent the closed circular and nicked circle forms of two different sized plasmids).

Since none of these plasmids are thought to contain the complete sequence of DNA which corrects the ts phenotype, the

next step in this strategy is to isolate a unique human sequence from one of these plasmids which can be used to probe a human library for the complete gene(s). Since these plasmids represent multiple integrated cosmids it would be a laborious task to use all of them to screen a library and determine the correct gene(s) which corrects the ts phenotype. Therefore an effort is being made to obtain a tertiary transformant which is further enriched for the correcting human DNA sequence.

DISCUSSION

The isolation of many ts mutants and the use of simple (viral) model systems to characterize them - the primary approach which led to the fruitful study of prokaryotic DNA replication - has been applied in this study of eukaryotic DNA replication. Mutant isolations in diverse cell lines have resulted in several large collections of ts mutants (Marcus et al. 1985). However, few of these mutants have thus far proved to be defective in DNA synthesis. One possible reason for this phenomenom is suggested by genetic studies done with ts DNA$^-$ mutants. In mouse cells, independent mutants such as ts2 (Jha et al. 1980), ts20 (Donegan and Ozer, unpublished data), and tsC1 (Giles and Ruddle 1979) are clustered on the X chromosome and fail to complement each other by somatic cell hybridization; thus suggesting repeated isolation of mutations in the same gene or related genes. Nishimoto and collegues (Nishimoto and Basilico 1978) observed a similar phenomenom in Syrian hamster cells. CHO cell lines were selected as parents of this ts mutant collection because of the previous demonstration of regions of functional hemizygosity (Siminovitch 1976). It was anticipated that these regions could be exploited in order to derive increased numbers of mutations in autosomal genes required for DNA synthesis.

Another possible explanation for the paucity of mutants in DNA synthesis is the long exposure of the cell population

to the restrictive temperature during the selection process. Such a protocol would tend to select against any mutants which exhibit a rapid inhibition of DNA synthesis. Indeed, mutants previously reported in this laboratory and elsewhere often lose viability when exposed to the restrictive temperature for periods exceeding one cell cycle. In an effort to ameliorate this condition in this study several selective (including multiple short exposures) and nonselective protocols were used in the isolation of the ts mutant collection. Although no enrichment for defects in DNA synthesis might be expected, it was found that from 6% (multiple short selections) to 20% (nonselection) of the mutants ts for growth were also DNA$^-$ mutants (as determined by incorporation of ^{3}H-TdR). Thus reducing or eliminating selection involving exposure to the restrictive temperature may facilitate isolation of ts DNA$^-$ mutants. Viability studies with several of the ts DNA$^-$ mutants isolated in this study confirm the expectation that there is cell lethality associated with prolonged exposures to the restrictive temperature (see Table 8).

Eight of the ts DNA$^-$ mutants isolated in this study, including representatives from all three isolation regimens, were chosen for further analysis based on the relatively rapid and progressive inhibition of DNA synthesis observed (see Figure 4 and Table 2). The kinetics of DNA synthesis at the restrictive temperature differed for some of the mutants (e.g. a delay in the onset of inhibition). No obvious

correlation exists between these kinetics of DNA synthesis in an individual mutant and the regimen used to isolate it.

The overall goal was, however, the isolation of mutants ts in DNA synthesis itself, which would be expected to be a subset among these ts DNA$^-$ mutants. Therefore, it would be most important to identify those mutants which involve macromolecules which are most relevant to cellular DNA synthesis in S phase, i.e. replicon initiation and elongation. It would also be advantageous to distinguish those mutants which affect the metabolism of the dNTP pools as well as those which are unable to complete other stages of the cell cycle, precluding replication of DNA. To facilitate their further characterization, each of the ts DNA$^-$ mutants was screened for the ability to support viral DNA synthesis at the temperature restrictive for cellular DNA synthesis. Ad2 and polyoma were chosen because of the ability of CHO cells to support appreciable levels of viral replication for both (Radna et al. 1987; Longiaru and Horwitz; LaBella and Ozer 1985) and the multifold dependence of both viruses on host functions for viral replication. In addition, both viruses are well characterized. In the case of Ad2 a fully defined <u>in vitro</u> system is available, thus allowing the further characterization and biochemical analysis of the cellular lesion. An <u>in vitro</u> system has also recently been developed for polyoma. Both systems have been shown to be applicable to extracts prepared from CHO cells (Longiaru and Horwitz 1981; Dermody et al., in press; Ozer and Kelly,

unpublished data) A summary chart of the analysis of the mutants for intracellular viral DNA synthesis is shown in Table 9. The mutants can be categorized into one of three groups. The first includes those mutants which support the replication of both Ad2 and polyoma at the restrictive temperature. Three of the mutants fall into this category. The lesion in these mutants may be in a gene not directly involved with DNA synthesis (e.g. cell cycle mutants) or may be in a gene coding for a function required for cellular DNA synthesis but either supplied by the virus itself or not required for viral DNA replication.

The second group includes the mutants JB3-O, JB7-K, JB8-D and JB11-J. These mutants support Ad2 DNA synthesis at 39°C but fail to support polyoma DNA synthesis at wild type levels at the same temperature. With this group the similarities and the differences between the mode of replication of these two viruses can be exploited to narrow the number of possible functions in which the defect may have occured. Since both viruses depend on cellular nucleotide pools for their synthesis, it would be unlikely that a defect in generating deoxyribonucleotide pools adequate for DNA synthesis is responsible. The defect is also unlikely to solely involve arrest of the cell mutants in a stage of the cell cycle other than S phase. Although papovavirus DNA synthesis depends on the infected cell proceeding through G1 into S, subsequent synthesis of viral replicons is not cell cycle dependent (Slater and Ozer 1976, Pages et al. 1973). Polyomavirus DNA

synthesis was inhibited even when infected cultures were shifted to 39.5°C after its onset. In vivo and in vitro systems for polyoma replication in CHO cells are currently being developed to facilitate more definitive characterization of this class of mutants. One might have expected to have found mutants which restricted polyoma DNA synthesis only when the entire course of infection was at 39°C. It can only be speculated that the failure to observe such putative cell cycle mutants is related to the kinetics of the manifestation of the cell ts phenotype and the onset of viral DNA synthesis in an asynchronous cell population.

The third group of mutants has a single member, JB3-B, which fails to support either Ad2 or polyoma DNA replication at the restrictive temperature. This mutant is therefore defective in a function required for the replication of both viruses. As in the discussion of the mutants of the second group, it is unlikely that a defect in cell cycle progression is involved. Since both viruses are dependent on several cell functions, it was not possible to define the exact step at this time; however, it should be noted that a defect in the priming mechanism is unlikely as Ad2 and Py utilize different mechanisms. No firm evidence exists that discontinuous synthesis occurs during the replication of Ad DNA. Therefore a function uniquely required in the synthesis and ligation of Okazaki fragments is an equally unlikely possibility. Since both viruses elongate at least one of the nascent DNA strands by continuous strand synthesis, it is probable that a defect

in this process is responsible for the ts DNA⁻ phenotype. Especially likely is a defect in the nucleotide pools, which are a common requirement of all DNA viruses. Both DNA topoisomerase activities (I and II) are involved in the termination of papovavirus DNA replication. DNA topoisomerase I activity, which has been isolated as nuclear factor II *in vitro*, is thus far the only host cell factor shown to function in the completion of Ad DNA replication. Therefore the gene which codes for DNA topoisomerase I activity is a possible site for the genetic lesion. Although the papovaviruses rely on the host chromatin proteins to package their DNA, the adenoviruses code for their own and therefore a defect which affects these proteins is an unlikely possibility. However, both of these viruses have been observed to associate with the nuclear matrix during replication. Thus a defect which alters this association cannot be excluded.

JB3-B was chosen as the best candidate for further analysis. It was isolated by a short selection protocol not employed in the isolation of any mutant outside of this collection. Complementation analysis indicates that JB3-B is complemented by several previously or simultaneously isolated ts DNA⁻ mutants. The kinetics of DNA synthesis at the restrictive temperature shows a rapid inhibition. Additionally, both Ad2 and Py DNA replication are directly affected by the ts defect.

Data obtained from the initial viral analysis (Ad2 and

Py) indicated that the inhibition of DNA synthesis exhibited as a phenotypic property of JB3-B at 39.5°C was not a result of growth arrest at a stage of the cell cycle other than S phase. This indication was confirmed by a direct analysis of cell cycle kinetics through the use of flow microfluorimetry. An observation of the accumulation of cells in S phase after 24 hours at the restrictive temperature by this technique strongly suggests that JB3-B cells are defective in a function required for the completion of S phase.

The novel nature of the gene affected was confirmed by complementation of the defect through cell hybridization. To accomplish this, a ouabain and thioguanine resistant subclone of JB3-B was isolated (designated 3B·THO). Although this cell line grew more slowly at the permissive temperature, it possesses the same ts DNA$^-$ qualities as the parental JB3-B (i.e. rapid inhibition of DNA synthesis at the restrictive temperature; failure to support Ad2 DNA synthesis at the restrictive temperature due to a primary effect of the mutated function). When fused to other (wild-type or mutant) cells which do not contain ouabain resistance and thioguanine resistance, 3B·THO permits the selection of hybrids in HAT/ouabain medium prior to selecting for those hybrids in which the ts defect has been complemented (growth of the hybrids at the restrictive temperature). A total of 21 ts DNA$^-$ cell lines isolated within and outside of this laboratory complemented 3B·THO. This result indicates that JB3-B may possess a genetic lesion in a genetic function for

which a ts DNA$^-$ mutant has not previously been isolated. Since the mutated function in each of these cell lines has not been identified, no function can be conclusively eliminated as being one affected in JB3-B.

In addition to 3B·THO several additional cell lines derived from JB3-B were isolated analysed by radiolabelling and Ad2 dot blotting. 3B2R1, a putative spontaneous ts$^+$ revertant, was isolated as a colony of JB3-B which grew at the restrictive temperature. When assayed for the incorporation of ^{3}H-TdR into DNA, its kinetics of DNA synthesis indicate that the inhibition of DNA synthesis displayed by JB3-B has been corrected (see Figure 22) in this cell line. Further indications of the correction of the ts defect in 3B2R1 come from data on its ability to form colonies at the restrictive temperature (data not shown) Additionally, Ad2 is capable of replicating its DNA at the nonpermissive temperature (39.5^{o}C) at levels equal to or greater than those at the permissive temperature as shown by dot blotting (see Figure 26). As a result of this correction and its common lineage with JB3-B, 3B2R1 is an excellent choice for use as a cell which replicates its DNA at the restrictive temperature in subsequent analyses. It should be noted, however, that JB3-B and all of its sublines (defective and corrected) display a pseudodiploid karyotype which is characteristic of the parent CHO cell line. Only 3B2R1 possesses a karyotype (30 - 36 chromosomes) which is different from this number (22 chromosomes). A collaboration

with an experienced cytogeneticist (Sally Ripley of Dr. Ann Henderson's laboratory) has been started to investigate the significance and relationship of its phenotype to this increased chromosome number in 3B2R1.

3B·OUA^R ts$^+$ revertant and THOR (a ts$^+$ revertant of 3B·THO) are two other cell lines originally derived from JB3-B. 3B OUA^R ts$^+$ revertant was isolated as a colony of a ouabain resistant subclone of JB3-B, 3B OUA^R, which grew at the restrictive temperature. THOR was isolated as a colony of a mutagenized 3B·THO culture which grew at the restrictive temperature. The kinetics of DNA synthesis and the accumulation of nascent Ad2 DNA at 39.5^o indicate that the ts DNA$^-$ defect is corrected in these cell lines. However, since these cell lines are not directly obtained from JB3-B (ouabain resistance was introduced through a mutagenesis of JB3-B), they are not as good choices as 3B2R1 as an example of a corrected cell line in subsequent analyses. The lineage of all of the cell lines derived from JB3-B has been presented in Figure 21. Taken together, data from these revertants support the premise that the mutation responsible for the ts growth phenotype is also responsible for the defect in both cell and adenovirus DNA synthesis. Further, it suggests that a mutation in a single gene is responsible.

Dot blots were employed in the initial assays of Ad2 DNA replication to facilitate the preliminary characterization of all of the ts DNA$^-$ mutants in this collection. The limitation of this approach is in that only the accumulation of nascent

viral DNA is measured. No direct assessment of the kinetics of Ad2 DNA replication is obtained. Pulse-chase labelling experiments of infected JB3-B cells analysed on alkaline sucrose gradients were employed in an effort to exploit the effect of the cellular mutation on Ad2 DNA replication in the further characterization of the ts defect. This method permits the direct measurement of the rate of Ad2 DNA synthesis in infected JB3-B cells at both the permissive and nonpermissive temperatures. Additionally, it permits the direct examination of viral replicative intermediates which are seperate from the template strand of DNA.

The initial experiments were performed to determine the optimal time p.i. for the the analysis of the effect of the cellular defect on the kinetics of Ad2 DNA replication in infected JB3-B cells; specifically, the time p.i. of maximal rate of Ad2 DNA synthesis at the permissive temperature and at what point prior to this infected cultures should be shifted to the nonpermissive temperature for optimal observation of the effect of the mutated cell function on the rate of Ad2 DNA synthesis. When infected cultures of JB3-B are shifted to the nonpermissive temperature 42 hours p.i. a decrease in the rate of Ad2 DNA synthesis can be observed. This decrease is in contrast to the gradual increase in the rate which is observed at the permissive temperature. Thus it can be stated that there is a defect in the rate of Ad2 DNA synthesis when infected cultures of this mutant are shifted to 39.5^{o}C. This defect is clearly related to the ts cell

phenotype as can be demonstrated by a comparison of the data obtained during continuous incubation at 33°C to that obtained after temperature shift. This conclusion has been supported by data obtained for the wild-type CHO-S and a spontaneously occuring temperature independent revertant of JB3-B, 3B2R1. An important parameter in the consideration of these data are the conditions of infection. This is especially evident for the ts subclone 3B·THO and the revertant 3B2R1. When infected cultures of 3B2R1 are shifted to 39.5°C 42 hours p.i. an acceleration of the time course at 33°C is observed. This acceleration results in a progressive decrease in the rate of viral DNA synthesis which is similar to that seen for JB3-B under the same conditions. When infected cultures of 3B2R1 are shifted to 39.5°C at 36 hours p.i., however, an increased rate of viral DNA synthesis is observed. This increase contrasts with the constant rate of viral DNA synthesis for JB3-B under the same conditions. When infected cultures of 3B·THO are shifted to 39.5°C at 36 or 42 hours p.i., a rapid and progressive decrease in the rate of viral DNA synthesis is observed. This is in contrast to the data obtained with JB3-B for which a constant rate of viral DNA synthesis is observed following temperature shift at 36 hours p.i.. An explanation is provided by the data obtained for continuous incubation at 33°C. These data show that the time course of infection is accelerated by approximately 12 hours in 3B·THO as compared to JB3-B. The similarity of the data obtained for temperature shift of 3B·THO at 24 hours

p.i. to that obtained for temperature shift of JB3-B at 36 hours p.i. supports this interpretation. Further experiments have shown that these data are not the result of experimental artifacts such as cell loss due to experimental handling of infected cells or due to the degradation of newly synthesized viral DNA.

I then sought to determine whether the cellular defect preferentially affects the initiation or the elongation (and termination) of nascent Ad2 DNA. The in vivo elongation of Ad2 DNA can be measured by a modification of the pulse-chase regimen. Two criteria were used to facilitate the interpretation of the data obtained from these experiments. The first was a comparison of the ratio of the ^{3}H-TdR incorporated into the whole genomic sized DNA to the ^{3}H-TdR incorporated into a viral intermediate which is moved into genomic sized DNA by a series of chases of increasing duration. A convenient intermediate is readily identified when no chase is applied. This intermediate is similar in size to a DNA fragment which is observed when aphidicolin is added to the cells prior to the time of pulse-chase (Nagata et al. 1983). It is approximately 20S in the gradient and represents a nascent strand which is 30-35% of the full sized viral DNA. Deletion of NF II from an in vitro reaction will also yield a similarly sized DNA. The second criterion was based on the ^{3}H-TdR present in the leading edge of the full sized viral DNA as a percentage of the total ^{3}H-TdR incorporated into DNA. Although not all of the full sized DNA

is included, the spillover of ^{3}H-TdR incorporated into large intermediates is minimized so that only full sized viral DNA is counted. Since both of these criteria depend on the identification of the precise location of genomic DNA in the gradients, viral DNA was prelabelled with ^{14}C-TdR as described in the Methods. Inclusion of this marker had no significant effect on the distribution of viral DNA in the gradients. The ts^{+} revertant, 3B2R1, and aphidicolin treated JB3-B cells were employed as controls in these studies. 3B2R1 serves as an example of cell line in which the ts defect has been corrected and thus should display no significant difference in the rate of Ad2 nascent strand elongation at both temperatures. Aphidicolin inhibits the elongation of Ad2 DNA (Longiaru et al 1979). Thus aphidicolin treated JB3-B cells should serve as an indication of an inhibition of Ad2 elongation.

The data obtained from these studies indicate that the elongation of Ad2 DNA is inhibited at the nonpermissive temperature. This statement is supported by interpretations of the data based on both of the criteria described above. For infected JB3-B cells at 33^{o}C the ratio of full length genomic DNA to the intermediate increases as the duration of the chase is increased as does the amount of ^{3}H-TdR incorporated into the leading edge of the full sized viral DNA. This is also true of infected 3B2R1 cells. When infected cultures of both of these cell lines are shifted to 39.5^{o}C a marked difference can be observed. In the case of 3B2R1, the

ratios obtained for each duration of chase at 39.5°C are greater than the corresponding ratio at 33°C. Additionally, the data suggest that the rate of elongation may be somewhat increased at 39.5°C. The opposite is true of JB3-B. The ratios obtained for each duration of chase at 39.5°C are less than those obtained at 33°C. Treatment of infected JB3-B cells with two different concentrations of aphidicolin for four hours prior to analysis showed a concentration dependent decrease in the ratios. This was interpreted as an indication that when the ratios of full length to intermediate length DNA decrease an inhibition of DNA elongation has occured. An examination of the ^{3}H-TdR incorporated into the fractions corresponding to the leading edge of the viral DNA confirms this interpretation of the data.

Taken together these data indicate that JB3-B is defective for a function required for the elongation of replicating Ad2 DNA. Since no strong evidence exists for the occurrence of discontinuous DNA strand synthesis in Ad2 replication, it can be stated that continuous strand synthesis is affected. Thus the number of possible functions affected by the genetic lesion can be further narrowed to one of two possiblities. First, that there exists an imbalance in the deoxynucleotide triphosphate (dNTP) pools caused by either a defect in the synthetic pathway or lack of proper compartmentalization (i.e. failure of the available pools to be efficiently utilized). Alternatively, the defect could be in a protein which is an integral component of the

macromolecular mechanism for cellular DNA replication. The use of the in vitro Ad DNA replication system developed by Challberg and Kelly (1979) should facilitate the identification of such a protein. However, for this and all other Ad in vitro DNA synthesis systems currently available the dNTP must be added to achieve efficient levels of replication. This results in a masking of the effects of the dNTP pools on Ad DNA synthesis.

Therefore, prior to initiating in vitro analyses of Ad DNA synthesis in JB3-B, an attempt was made to assess the levels of the dNTP pools in JB3-B at both temperatures by high performance liquid chromatography (HPLC). The data obtained from the HPLC analysis of the dNTP pool levels suggested that the levels of the pools were decreased at 39.5°C. Each pool was affected differently by the temperature shift with dCTP levels decreasing by more than 50%; a greater decrease than that seen with any of the other pools. Reproducible data could not be obtained however, due to methodological problems. Therefore, in an effort to corraborate these data, a collaboration was formed with Dr. Christopher Mathews. Dr. Mathews laboratory utilizes an enzymatic assay (Leeds and Mathews 1987) to measure the level of the pools. The data obtained by Dr. Mathews are shown in Table 25. These data indicate that the levels of all four of the dNTP pools decrease in cultures of JB3-B shifted to 39.5°C. This is true of both the nuclear and whole cell

Table 25

dNTP pool levels in JB3-B at 33°C and 39.5°C[a]

dNTP	Time (hours) at 39.5°C	pmoles/10^6 cells (percent of 0 Time) Whole Cell	Nuclear
dATP	0	83 (100)	12.0 (100)
	3	61 (73)	5.0 (42)
	6	40 (48)	3.0 (25)
	12	47 (57)	2.2 (18)
	30	19 (23)	2.5 (21)
dTTP	0	151 (100)	18.0 (100)
	3	128 (85)	15.0 (83)
	6	68 (45)	9.5 (53)
	12	44 (29)	9.5 (53)
	30	44 (29)	4.7 (26)
dCTP	0	86 (100)	15.0 (100)
	3	51 (59)	7.0 (47)
	6	45 (52)	3.2 (21)
	12	25 (29)	2.5 (17)
	30	27 (31)	1.1 (7)
dGTP	0	40 (100)	8.0 (100)
	3	27 (68)	3.7 (46)
	6	19 (48)	1.7 (21)
	12	8 (20)	1.7 (21)
	30	10 (25)	1.8 (23)

a - All procedures are as described in Leeds and Mathews 1987.

pools, although the levels of the nuclear dTTP pool do not decrease as rapidly as the nuclear pools of the other dNTPs. The kinetics of this decrease in the sizes of the pools correlates extremely well with the kinetics of the inhibition of DNA synthesis which is observed at 39.5°C for this mutant. Therefore the data presented here provides evidence for the coupling of DNA synthesis and the size of the dNTP pools.

The first question which this data presents is: where is the primary defect? Is in the pools or is it elsewhere and the decrease in the levels of the pools is a secondary effect of the inhibition of DNA synthesis? Studies by P. Reichard (Nicander and Reichard 1985) have shown that when the incorporation of dNTPs into DNA is inhibited by aphidicolin in mouse 3T6 cells the levels of dATP and dTTP increase while the levels of dCTP exhibit a moderate decrease. The levels of dGTP remain approximately the same. When compared to the data obtained for JB3-B this suggests that a secondary effect of the cellular mutation on the dNTP pools is unlikely; although the effect of an inhibition of DNA synthesis could be cell specific. Also the inhibition of DNA synthesis by aphidicolin may not have the same effect on the size of the dNTP pools as does the inhibition caused by the cellular mutation in JB3-B (i.e. the inhibition of DNA synthesis in JB3-B may accelerate the turnover of the dNTP).

If the primary defect is in the pools themselves then the possible functions affected can be narrowed considerably. Since the all of the pools are decreased in size at 39.5°C

the occurance of the mutation in a protein which functions in the biosynthesis of all of the dNTPs is a strong possibility. Ribonucleotide reductase catalyses the reduction of all four common ribonucleotide diphosphates (rNDP) to the corresponding deoxyribonucleotide diphosphates (dNDP). Thus all four dNTPs are processed by this enzyme. Measurement of the activity of this enzyme in cultures of JB3-B incubated at 33 and 39.5°C should demonstrate the existance of a temperature labile reductase. Alternatively, DNA from the secondary transformant of 3B•THO can be probed with cloned DNA sequences from the coding regions for the M1 and M2 subunits. The ribonucleotide reductase genes are highly conserved among organisms. Bacterial, viral, and mammalian genes possess regions of strong sequence homology and exhibit cross hybridization. Experiments are in progress using the cloned mouse M1 and M2 subunits as probes for Southern analysis with DNA prepared from JB3-B containing human sequences which correct the ts defect (i.e. primary and secondary transformants) described in Chapter 4.

Alternatively the mutation could be in a gene which regulates the turnover of the dNTP pools. Expression of the mutated protein at 39.5°c would then be expected to result in an increased rate of degradation of the dNTP. DNA synthesis is then inhibited as a result of the depletion of the available pools. Nicander and Reichard (1985) have developed a methodology to assay the rate of the turnover of the dNTP. This methodology will be used, possibly in collaboration with

another laboratory, to study the effect of the genetic lesion on the kinetics of dNTP turnover in JB3-B at the permissive and restrictive temperatures.

However, the simplest hypothesis is that the dNTP pool defect reflects a defect in an earlier step in nucleotide biosynthesis. I therefore examined the incorporation of ^{3}H-uridine into TCA precipatates as an assessment of one of the ribonucleotide pathways. The results of a preliminary experiment indicate that the incorporation of ^{3}H-uridine is inhibited at 39.5^{o}C but not at 33^{o}C (data not shown). The kinetics of this inhibition are similar to the kinetics observed for the incorporation of ^{3}H-TdR in this mutant. This suggests that the DNA^{-} phenotype is the result of a defect in the ribonucleotide pools. Two possible models of the effect of this defect on DNA synthesis in JB3-B have been proposed which are based on the data presented in this thesis. For the first model I propose that the defect in the ribonucleotide pools results in a decrease in the size of the deoxyribonucleotide pools available for DNA synthesis. This in turn directly inhibits DNA synthesis. Alternatively, the defect in the ribonucleotide pools results in a failure to make all RNAs. This leads to a rapid depletion of mRNAs with rapid turnover rates. One such mRNA may code for a short lived protein which is essentially required for DNA synthesis. Thus DNA synthesis is inhibited by the decreased synthesis of this protein. The rapid decrease in the deoxyribonucleotide pools is not a direct result of the

defect in the ribonucleotide pools but is a secondary effect of the inhibition of DNA synthesis. Further experiments to confirm the dNTP pool and uridine data are in progress using both the mutant JB3-B and its revertant 3B2R1.

Complementation of the defect in hybrids of 3B·THO and human HS74 cells has demonstrated that the mutated gene is recessive to a human gene which corrects the defect at the restrictive temperature. This indicates that this human gene can be isolated by standard cloning methodologies. Therefore an attempt has been initiated to clone this gene from a human genomic cosmid library. Thus far a secondary transformant enriched for this sequence has been isolated. However, it contains several copies of transfected human DNA. The subsequent strategy is to isolate a tertiary transformant which is further enriched for this sequence and then to isolate it by one of several methodologies.

In conclusion, several ts DNA$^-$ mutants were isolated from CHO cells. The feasibilty of using adenovirus and polyomavirus to facilitate their preliminary characterization has been demonstrated. One of these ts DNA$^-$ mutants, JB3-B, which fails to support the replication of either of these viruses at 39.5^{o}C was further characterized through the use of adenovirus as simple model system - an approach which has been successfully exploited in the study of prokaryotic DNA replication. Evidence has been obtained which indicates that the elongation of Ad2 DNA is inhibited by the expression of the mutated function. Analyses of the deoxynucleotide pools

has suggested that a decrease in the level of the pools may be responsible for this inhibition. Further studies are in progress to confirm this interpretation. Additionally, genetic analysis of JB3-B indicates that it is a novel ts DNA$^-$ mutant and that the mutation is recessive to a human gene which corrects the ts phenotype. An attempt to isolate this human gene has been initiated.

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