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ANALYSIS OF TS2, A TEMPERATURE-SENSITIVE MUTANT IN DNA SYNTHESIS

City University of New York PH.D. 1985

ANALYSIS OF TS2, A TEMPERATURE-SENSITIVE MUTANT IN DNA SYNTHESIS

by

LINDA HELEN MALKAS

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

1985

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

April 29, 1985
Date

Chairman of Examining Committee

April 29, 1985
Date

Executive Officer

Supervisory Committee

Abstract

ANALYSIS OF TS2, A TEMPERATURE-SENSITIVE MUTANT IN DNA SYNTHESIS

by

LINDA HELEN MALKAS

Adviser: Professor Harvey L. Ozer

A temperature-sensitive mutant (ts2) of the mouse cell line Balb/3T3 affected in DNA synthesis was further characterized. FMF analysis of actively growing ts2 cells shifted to the non-permissive temperature, 39°C, suggested an accumulation of these cells within the S phase with increasing incubation time at 39°C.

Serum stimulation of synchronized ts2 cells showed an induction of DNA synthesis and the enzymes thymidine kinase, thymidylate kinase, and DNA polymerase α at 33°C, while exhibiting little (or no) stimulation of these activities in cells incubated at 39°C. Similar experiments performed with the ts2 parent cell line and a revertant showed the stimulation of these activities at both 33°C and 39°C.

These synchronized ts2 cells did not show an increased requirement for serum at 39°C, and were fully capable of entering the cell cycle at 39°C; as indicated by an increase in cellular protein content, and the induction of both c-myc and proliferin mRNA's during the time course. The induction of enzyme activity

was not dependent on the stimulation of DNA synthesis, since TK was induced in the presence of hydroxyurea. These results suggest that the lack of enzyme induction in growth-arrested ts2 cells at 39°C after the addition of serum is associated with a broad cellular phenomena; the result of the ts lesion.

SV40 infection of quiescent ts2 cells stimulated the rate of ^{3}H-TdR incorporation and TK activity in these cells at both 33°C and 39°C. The induction of DNA synthesis was shown to be dependent on the presence of the SV40 large T-antigen. Those cells stimulated into the cell cycle by SV40 infection were able to complete DNA replication and progress through one round of cell division at 33°C and 39°C. SV40 transformants of ts2 were isolated to assess whether the continual presence of the T-antigen would complement the ts mutation. These transformants were found to be unable to support cell growth at 39°c.

A model postulated to explain the observed aberrant response of ts2 cells at 39°C considers a broadly based transcriptional block at the non-permissive temperature.

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This work is dedicated to Dr. Robert J. Hickey, my husband, friend, collaborator, and greatest cheerleader, and to the memory of my father, Anthony C. Malkas (1921 - 1984).

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CHAPTER ONE: INTRODUCTION

Animal cells duplicate their DNA during a discrete interval in interphase, permitting the cell cycle to be divided into four phases: G1, S (DNA synthetic period), G2, and M (mitosis). G1 is the period between mitosis and the initiation of DNA synthesis; G2 is the period between S and M. Most actively growing cell lines in tissue culture have cell cycle times of 10 to 30 hours (Pardee et al., 1978). Animal cells have also been observed to exist in a non-growing quiescent state during which no DNA replication or cell division occurs. Most normal cells, when in this state, contain the G1 content of DNA. Whether these cells have left the cell cycle to enter a distinct Go state or are arrested in a prolonged G1 is the subject of debate (Pardee et al., 1978).

A normal animal cell, upon reaching G1 phase, can enter a state of quiescence or proceed onto another round of DNA synthesis and cell division. This decision is a response to the cellular growth environment. Normal cells have been proposed to respond to suboptimal growth conditions by entering the Go (quiescent) state (Pardee, 1974). Support for this view is derived from observed biochemical and kinetic differences between Go and G1 cells. It has been shown that Go cells (upon induction) take a longer time to reach S phase then do cells in G1 progressing to S from mitosis (Epifanova et al., 1975, Martin and Stein, 1976). Also, WI-38 human fibroblasts which were kept in a non-growing state for prolonged periods enter a progressively deeper Go state characterized by reduced transcriptional activity. The cells were found to be

increasingly difficult to rescue from this deep arrested state (Rossini et al., 1976). Similarly, when Balb/3T3 cells were induced to enter Go by serum deprivation in sparse culture, it was observed that the longer the cultures were deprived of serum, the more time they required to enter S phase following addition of serum (Dooley and Ozer, 1979).

An alternate proposal suggests that quiescent cells slowly traverse the cell cycle with a greatly extended G1 period as opposed to entering a separate arrested state (Dell'Orco et al. 1975, Rubin and Steiner, 1975). It was observed that 96% of the nuclei in growth-arrested chick embryo cell cultures were labelled after 120 hours of continuous exposure to ^{3}H-thymidine (^{3}H-TdR) (Rubin and Steiner, 1975).

Diverse suboptimal growth conditions could result in the same quiscent state, and cells could enter the Go state or make the commitment to continue proliferation at a single point in G1 phase called the restriction point (Pardee, 1974). It has been proposed that some transformed cell lines are defective in their restriction point control (Martin and Stein, 1976, Pardee, 1974, Pardee and James, 1975, Schiaffonati and Baserga, 1977, Hatten et al., 1977), while other transformed lines retain the ability to enter a resting state under some conditions (Lindgren and Westermark, 1976, Lindgren and Westermark 1977, Holley et al., 1976, Lindgren et al., 1975). The biochemical nature of the restriction event is unknown.

Growth conditions that result in cells entering the proposed Go state include high cell density (Robinson and Smith, 1976, Temin, 1971, Canagaratna and Riley, 1975); serum limitations (Martin and

Stein, 1976, Rubin and Steiner, 1975, Lindgren and Westmark, 1976, Bartholomew et al., 1976); limitation of some amino acids (Martin and Stein, 1976, Holley and Kierman, 1974, Tobey, 1973) or of other nutrients, such as phosphate, glucose (Holley and Kierman, 1974, Kamely and Rudland, 1976) or lipids and biotin (Hatten et al., 1977); and the presence of certain drugs (Pardee and James, 1975, Fodge and Rubin, 1975, Fernandez-Pol et al., 1977, Mannino and Burger, 1975).

In order to perform biochemical studies on the cell cycle, it is generally necessary to obtain a population of cells that is synchronous with respect to the cell cycle. This can be done by selectively detaching mitotic cells from the growth surface and replating them, by blocking cells at a specific point in the cell cycle with a drug and then releasing them, by using serum or amino acid limitations or growth to confluence to shift cells into quiescence and then stimulating them to grow by the addition of complete medium, or by various combinations of these methods.

The studies employing synchronized cells described in this work used a combination of growth to confluence and serum deprivation to arrest the cultures. These arrested cells were stimulated into their cell cycle by the addition of complete medium. Serum, a complex mixture of substances, has been used routinely in cell culture medium to provide necessary cellular growth factors. The principal growth factor in serum for primate dermal fibroblasts, arterial smooth muscle cells, and glial cells is derived from platelets (Rutherford and Ross, 1976, Westermank and Wasteson, 1976), and called platelet-derived growth factor (PDGF). It is also

a growth factor for Balb/3T3 cells (Antoniades et al., 1975, Antoniades and Scher, 1977). PDGF has been purified to homogenity and shown to be a group of highly cationic heat-stable proteins (28,000-35,000 daltons); each member contains two polypeptide chains linked by disulfide bonds (Antoniades et al., 1979, Deuel et al., 1981, Heldin et al., 1979, Raines and Ross, 1982,). Like other polypeptide hormones, homogenous preparations of PDGF are active at 10^{-10}M. Many transformed derivatives of Balb/3T3 cells do not require PDGF for growth (Scher et al., 1978).

PDGF initiates the replication of density-arrested non-transformed Balb/3T3 cells by stimulating them to become "competent" to respond to a group of growth factors present in platelet poor plasma (Pledger et al., 1977). The number of cells rendered competent is a function of both the PDGF concentration and the duration of treatment. A high concentration of PDGF can induce the majority of Balb/3T3 cells within a culture to become competent in 30 minutes. Although PDGF binds to tissue culture plates (Smith et al., 1982), such binding is not responsible for competence. Smith and Stiles (1981) have shown that PDGF-treated cells remain competent after trypsinization and transfer to a fresh plate. The subsequent addition of the growth factors in platelet poor plasma allows these PDGF-treated cells to initiate DNA synthesis after a 12 hour lag. Treatment of cells with plasma before addition of PDGF does not stimulate replication (Pledger et al., 1978). Unlike PDGF, plasma must be present continuously to allow cellular replication.

The plasma growth factors have been identified (Scher et al., 1981, Stiles et al., 1979). One of these factors needed for optimal

replicative response is somatomedin (Van Wyk and Underwood, 1978), a group of proteins with sequence homology to insulin, whose plasma concentration is regulated by pituitary growth factor (EGF) (Carpenter and Cohen, 1979). PDGF, EGF or insulin alone induces a weak growth response in density-arrested Balb/3T3 cells. In combination, however, these agents function synergistically to stimulate the majority of cells to synthesize DNA (Scher et al., 1981).

When quiescent cells are stimulated to divide, an array of biochemical changes occur at various times before the initiation of DNA synthesis. This set of metabolically unrelated biochemical reactions that fluctuate coordinately with changes in cell growth has been termed the "pleiotypic response" (Hershko et al., 1971). It has been difficult to distinguish which of the many observed changes during Go to S or the M to S transition are either necessary or sufficient for entry into S phase. We do not know whether the animal cell cycle is a linear sequence of dependent events or whether it consists of several relatively independent biochemical pathways.

Approaches to the study of causal relationships in this process include the isolation of temperature-sensitive mutants that are blocked at a specific point in the cell cycle, and the study of the effects of drugs that inhibit biochemical processes. In addition, the study of differences between normal and transformed cells, as well as the study of the factors that stimulate quiescent cells to proliferate further suggest which biochemical parameters are important in growth regulation.

The actual biochemical mechanisms used by mammalian cells to initate and maintain their DNA synthesis are unknown. The simian virus 40 (SV40) origin of replication has been sequenced and shown to be an inverted repetitive sequence (palindrome) that can, in theory, generate one or two hairpin loops per strand due to intra-chain pairing (Jay et al., 1976). Additionally, the origins of other eukaryotic viruses have been shown to contain inverted repetitive sequences at their origins: Adenovirus 2 (Padmanabhan et al., 1976), adeno-associated virus (AAV) (Straus et al., 1976), and non-defective parvoviruses (Tattersall and Ward, 1976). A model has been proposed in which animal cells and their viruses may both employ inverted repetitive sequences as initiation sites for DNA synthesis (Tattersall and Ward, 1976). The putative hairpin loop contains two possible initiation regulatory features. First, it would provide a discontinuity in the DNA double helical structure, this, perhaps, being recognizable by initiation proteins. Second, due to a lack of base pairing at the turn, a few residues at the center of the palindrome are single stranded. These residues could serve a number of purposes, as a template of a RNA or DNA primer, a site for nuclease cleavage, or a site for unwinding proteins.

The actual event that triggers initiation at an origin of replication in eukaryotic cells is unknown. A proposed early event could be the generation of a nick, thereby, providing ends for the DNA or RNA polymerase. Along these lines endonucleases have been reported which interact with single and double stranded DNA (Wand et al., 1975, Urbanczyk and Studinski, 1974). One such enzyme was observed to generate 7S double stranded DNA fragments in calf thymus

which, upon denaturation, acted as both primer and template for DNA polymerase. These fragments were also found to contain hairpin loops (Wang et al., 1975).

Whatever the mechanism of initiation at a replicon, it must also explain the ordered synthesis of replicons within the S phase. The kinetics of DNA synthesis has been examined in synchronized Chinese hamster cells (Klevicz et al., 1975). They observed that the S phase can be subdivided on the basis of the amount of DNA synthesized to three time periods, namely, early, middle, and late S. Similar studies in Chinese hamster lung cells (Holmquist et al., 1982) have shown that certain bands of chromosomal DNA replicate early in S phase, and complete their replication, before a different set of bands, which replicate late in S. Additionally, it was shown in Balb/3T3 cells that satellite DNA replication did not begin until three hours into S phase , where upon its rate of synthesis increased very rapidly, reaching a maximum within the next two hours (Dooley and Ozer, 1977, Dooley and Ozer, 1979). Many studies (Hand, 1978) have shown that DNA replication is organized so that clusters of replicons replicate with a specific order and the different replicons in each cluster replicate simultaneously. Replication of DNA is controlled so that only a single replication occurs in every cell cycle (Hirschberg et al., 1980). This suggests that there are cellular regulatory functions which may regulate the early, middle, and late time periods of S phase.

After selection of a replicon for DNA synthesis, replication of the DNA occurs bidirectionally. As in prokaryotes, eukaryotic DNA polymerases have been observed to polymerize only in the 5' to 3'

direction (Weissbach, 1977), therefore, suggesting that on one strand of the double helix discontinuous DNA synthesis must occur. Thirty second pulses with radiolabelled thymidine in Chinese hamster ovary cells and in mouse cells resulted in the generation of 4S double stranded DNA fragments, which were chased to 20-60 S fragments within 2 to 8 minutes (Hildebrand and Walters, 1977 Gautschi and Clarkson, 1975). The label was found in high molecular weight DNA by 2 hours. These 4S fragments are presumed to correspond to Okazaki fragments (Okazaki and Okazaki, 1969), which are then ligated into replicon sized fragments, which, in turn, eventually form chromosome length fibers. Evidence has been obtained for the involvement of RNA primers in eukaryotic DNA synthesis. Radiolabelled uridine was found in the 4S Okazaki fragments after very brief pulses (Waqar and Huberman, 1975).

In prokaryotes, genetic analysis and the development of in vitro replication systems have resulted in detailed characterization of many of the components involved in DNA synthesis (Wickner, 1978). For eukaryotes, analagous systems have not been as finely developed; however, many enzymes and proteins which participate in replication have been identified. Among these are DNA polymerase α (Edenberg et al., 1978), factors C1 and C2 (required for the proper function of DNA polymerase α (Lamothe et al., 1981, Pritchard and DePamphilis, 1983, Pritchard et al., 1983)), DNA ligating enzyme (Soderhall and Lindahl, 1976), single strand DNA-dependent ATPase (Hachmann and Lezius, 1976, Otto, 1977a), DNA-binding protein (Champoux, 1978, Hock and McVey, 1977, Otto, 1977b), endodeoxyribonuclease (Wang and Furth, 1977), exodeoxyribonuclease

(Byrnes et al., 1976), ribonucleases (Elgin and Weintraub, 1975), RNA polymerase (Roeder, 1976), structural proteins of the chromatin (Elgi and Weintraub, 1975), and topoisomerases (Wang, 1971, Champoux and Dulbecco, 1972, Gellert et al., 1976).

Several lines of evidence have led to a proposal that DNA replication in eukaryotic cells proceeds via a multi-enzyme complex. There has been the additional suggestion that the assembly of this complex signals the initiation of the S phase. The various data supporting this proposal will be discussed in turn.

The rates of DNA synthesis are rapid (Collins, 1978), and are maintained although precursor pools appear to be small (Walter et al., 1978), when compared to the high levels of deoxyribonucleoside triphosphates (dNTPs) needed to sustain maximal DNA synthesis in vitro (Castellot et al., 1979). These results and other evidence dealing with the kinetics of incorporation of labelled precursors into cellular pools and DNA (Fridland, 1973, Kuebbing and Werner, 1975) have suggested the possibility of concentration gradients for deoxyribonucleosides, with the highest concentrations near DNA replication forks.

DNA replication in eukaryotic cells takes place at a limited number of sites on the nuclear membrane (Mizuno et al., 1971, Pearson and Hanawalt, 1971, O'Brien et al., 1972, LeBlanc and Singer, 1974). There is additional evidence which indicated that eukaryotic DNA synthesis begins at the nuclear membrane (Comings and Kakefuda, 1968). This apparent association of DNA and nuclear membrane is promoted by newly synthesized proteins 2-4 hours prior to the beginning of the S phase (Yamada and Hanaoka, 1973).

DNA-membrane aggregrates have been isolated from a variety of different sources and have been shown to be capable of synthesizing DNA from deoxyribonucleoside triphosphates (dNTPs) in vitro (Infante et al., 1973, Greene and Firshein, 1976, Infante et al., 1976). These aggregates have been extracted from bacteria (Firstein, 1973), from developing sea urchin nuclei (Infante et al., 1973), and from regenerating rat liver (Shearman and Kalf, 1977). These data suggest that during DNA replication the enzymes involved in DNA synthesis may be bound to both DNA and membrane.

Various macromolecular biosynthetic processes have been demonstrated to proceed via multienzyme complexes (Welch and Gaertner, 1975, Shoaf and Jones, 1973, Mathews et al., 1979). These complexes replace the concept of the cell as a bag of soluble enzymes catalyzing freely diffusing metabolites (reviewed in Gartner, 1978, Mosbach and Mathesson, 1978, Wombacher, 1983). Several lines of evidence from both prokaryotic and eukaryotic systems seem to indicate that DNA precursors are compartmentalized in replicating cells (Fridland, 1973, Kuebbing and Werner, 1975, Pato, 1979). Mathews and co-workers (Reddy et al., 1977, Reddy and Mathews, 1978) and Greenberg and co-workers (Tomich et al., 1974, Flenegan and Greenberg, 1977) have demonstrated that in bacteriophage T4-infected cells, compartmentation and metabolite channeling for DNA replication are facilitated by protein-protein interactions. Evidence for a possible similar functional compartmentation in eukaryotic cells has been found.

There was an observed association of at least four enzymes for DNA synthesis with the post-microsomal membrane fragment from

proliferating tissues (Baril et al., 1973). Three of these proteins function in the production of deoxyribonucleotide precursors. These enzymes are ribonucleotide reductase (2'deoxyribonucleoside-diphosphate:oxidized thioredoxin 2'-oxireductase, EC 1.17.4.1), thymidine kinase (ATP:thymidine 5'-phosphotransferase, EC 2.7.1.21), and thymidylate synthetase (5,10-methylene-tetrahydrofolate:dUMP c-methyltransferase, EC 2.1.1.45). DNA polymerase α is the fourth enzyme found in the isolated fragments. The activity of DNA polymerase, like the other three enzymes, is maximal in cells in S phase. These enzymes were found to remain associated and functional after treatment of the membrane fragments with a combined butanol and Triton X-100 solution or concentrated salt. These aggregates when examined by electron microscope have a uniform size of 8.5-12 nm. It seems unlikely, from these data, that the co-fractionation of these enzymes is fortuitous and the result of electrostatic absorption or aggregation. The membrane fragments do support the synthesis of TMP, TDP, and TTP from offered tritated labelled thymidine (^{3}H-TdR). In vitro, the labelled TTP is subsequently incorporated into a DNA template. Therefore, it is very likely that deoxyribonucleotide kinases are also present in these isolated membrane fragments.

Additional evidence for the existence of a multi-enzyme complex involved in DNA synthesis was presented by Reddy and Pardee (Reddy and Pardee, 1980, Noguchi et al., 1983). These authors proposed that in mammalian cells the pathway of DNA synthesis is catalyzed by enzymes that associated as a multi-enzyme complex which the authors

have named replitase. This readily sedimentable aggregate of enzymes was isolated from CHEF/18 (Chinese hamster embryo fibroblast) nuclei, but only if these cells were in S phase. The following enzymatic activities were found associated with the replitase: thymidine kinase, thymidylate synthetase, DNA polymerase α, dihydrofolate reductase (tetrahydrofolate dehydrogenase, EC 1.5.1.3), nucleoside-diphosphokinase, DNA methylase, and an ATP-dependent topoisomerase. It was also observed that these various activities were not complexed, but in a soluble form, when cells were in G1 phase, and in assembled form during S phase. These enzymes were found primarily in the cytoplasm of cells in the G1 phase, and in the nuclei of the cells in S phase. The authors suggest that the appearance of these activities in the nucleus of S phase cells may signal the assembly of these proteins into the replitase and, therefore, the initiation of DNA synthesis.

The approach of this laboratory to the unraveling of those cell cycle functions which are essential to the mechanism of DNA replication and the normal progress of cells through S phase has been the use of temperature- sensitive (ts) cell cycle mutants. Mutants with temperature-sensitive proteins provide a tool for the exploration of the role of a particular protein in metabolic pathways, biosynthetic reactions, cell proliferation, and regulation. In theory, virtually any protein can be altered to show thermolabile function. This makes accessible a wide range of processes for analysis using this genetic approach. It has been shown in procaryotic cell systems, that the use of thermolabile mutants in DNA synthesis has yielded detailed information on the

many different enzymes and co-factors coordinating the processes of initiation and chain elongation (Kornberg, 1980, Kornberg, 1982).

A variety of temperature sensitive mutants for DNA synthesis have been isolated from different cultured mammalian cell lines (reviewed in Hochstadt et al., 1981). Relatively few of these mutants have been characterized in sufficient detail to identify the defective protein or enzyme, an early exception being a group of mutants containing thermo-sensitive aminoacyl-tRNA synthetases (Thompson et al., 1973, Thompson et al., 1977, Thompson et al., 1978). In some of the other ts mutants the thermolabile function has been localized to a specific process resulting in an effect on DNA synthesis. Sheinin and co-workers (Sheinin, 1976, Colwill and Sheinin, 1983) reported that tsAIS9 cells, isolated from mouse L cells, showed defective maturation of the newly replicated DNA fragments into larger chromosomal DNA at the non-permissive temperature. These workers have evidence that the tsAIS9 locus may encode a novobiocin binding protein that is required for DNA topoisomerase II activity. TsC1, also derived from the mouse L cell line, is corrected by information carried on the human X-chromosome (Giles and Ruddle, 1976). The function of the tsC1 gene product has not yet been identified. It undergoes very rapid inactivation upon incubation at 38.5°C, and exhibits a half-life of approximately 3 hours (Sheinin et al., 1978). The tsC1 cells appear to be affected in a terminal event of chromosomal DNA synthesis. Additionally, polyoma virus is not replicated in these cells when incubated at 38.5°C (Sheinin et al., 1978). A ts DNA synthesis mutant, tsC8, was isolated from mutagenized Chinese hamster ovary cells

(McCracken, 1982). The tsC8 cells showed continued rates of ^{3}H-thymidine incorporation similar to the parental cell type for only 2 hours at the non-permissive temperatures after which DNA synthesis decreased while exhibiting little effect on the levels of RNA and protein synthesis. In tsBN2, a mutant of BHK-21, an inhibition in the incorporation of ^{3}H-thymidine into acid-precipitable material was observed at the non-permissive temperature(Eilen et al., 1980). By the use of the DNA-fiber autoradiography method to determine whether the rate of DNA chain elongation was affected at the non- permissive temperature, it was demonstrated that the block to ongoing replication in tsBN2 was at the level of initiation. Another mutant primarily characterized as containing a thermolabile function involved in the initiation of DNA synthesis by use of the DNA-fiber autoradiography procedure was ts131b, isolated from mouse FM3A cells (Hyodo and Suzuki, 1982). The mouse temperature sensitive mutant, ts85, isolated from mouse FM3A cells, was shown to be defective in DNA synthesis and chromosome condensation; both these events were thought to be ascribed to the decrease in H1 histone phosphorylation (Yasuda et al., 1981). A ts cell cycle mutant, E36 ts24, derived from the Chinese hamster lung cell line V79 also arrests in the S phase when incubated at its restrictive temperature (Fainsod et al., 1984). At the point of temperature arrest these cells were shown to continue to synthesize DNA at a high rate but practically all of the newly synthesized DNA is degraded. These various mutants and those not described should be useful for the study of the mechanism and regulation of DNA synthesis, since they are presumably defective at

different loci resulting in separate thermolabile gene products each having an effect on DNA synthesis.

Some mutants initially thought to be ts mutants in DNA synthesis, upon characterization were revealed to be defecive in a function not related to DNA synthesis itself. For example, tsF121, a ts mutant derived from rat 3Y1 cells contains a defect related to the efficiency of utilization of serum component(s) (Zaitsu and Kimura, 1984). A rapid decrease in the incorporation of ^{3}H-thymidine into DNA at a non-permissive temperature was observed in two temperature-sensitive mutants that were isolated from mouse FM3A cells. This change was not due to a decrease in the rate of DNA replication, but was closely associated with a decrease in thymidine kinase activity of these cells (Hyodo and Suzuki, 1981).

Other ts mutants have been reported which are more clearly affected in a G1 function. B54 is such a mutant, which was developed from a mouse cell line (Liskay, 1974). In this cell line, it was observed that cells in the G1 phase at the time of the shift to the non-permissive temperature failed to enter S phase. A ts mutant isolated from Chinese hamster fibroblasts, H3.5, is blocked in G1 phase at the non-permissive temperature apparently due to a block in transcription (Landy -Otsuka and Schaeffler, 1980). Membrane defects may account for the observed G1 block in the cold-sensitive mutants, cs4-D3(Crane and Thomas, 1976), and CR^{R}E5 (Ling, 1977).

A defect in a generally critical pathway can also preferentially arrest cells within a specific phase of the cell cycle as shown for

the mutant tsA58 (Burstin et al., 1974, Ingles, 1978, Rossini and Baserga, 1978, Rossini et al., 1980, Ingels and Shales, 1982). This mutant was observed to contain thermolabile RNA polymerase II molecules. Although this enzyme is utilized throughout the cell cycle, these mutant cells arrested in the G1 phase upon incubation at the non-permissive temperature.

This laboratory has isolated a series of ts mutants from Balb/3T3 cells (Wittes and Ozer, 1973, Slater and Ozer, 1976, Jha et al., 1980, Zeng et al., 1984). The permissive and non-permissive temperatures for cellular growth are 33°C and 39°C, respectively. These mutants were selected, after mutagensis with ethylmethane sulfonate, by their inability to incorporate ^{3}H-thymidine (^{3}H-TdR) into DNA at 39°C. Those cells capable of incorporating the radiolabel at 39°C were killed by its deleterious effects.

Several lines of evidence indicated one class of these mutants (ts2, ts20, ts22) was temperature-sensitive for cellular DNA synthesis. Ts2 was partially characterized and found not to be defective for overall protein or RNA synthesis at the non-permissive temperature (Slater and Ozer, 1976). The temperature-sensitive phenotype was found to be independent of cell density in contrast to another ts mutant derived from this cell line (Wittes and Ozer, 1973). Additionally, ts2 cells support DNA synthesis of the papovavirus, polyoma, after infection of these cells at 33°C, but not at 39°C. Viral DNA synthesis begun at 33°C is inhibited upon shift of the cells to an incubation of 39°C. With the exception of the A function (T-antigen) required for the initiation

of polyoma DNA synthesis (Francke and Eckhardt, 1973), viral DNA replication depends entirely upon the host cellular DNA replication machinery and some G1 functions of the host cells (Tooze, 1981). Initiation of papovavirus DNA synthesis is cell-cycle dependent; however, subsequent rounds of viral DNA replication are cell cycle independent (Pages et al., 1973). Therefore, the observed inability of ts2 cells to support polyoma DNA replication at 39°C is indicative of a thermo-sensitivity of the cell DNA synthesis machinery. The cellular and polyoma virus DNA synthesis are both inhibited to a similar degree at the non-permissive temperature. The inability of ts2 cells to replicate polyoma DNA at 39°C was not preferential for the viral DNA but indicative of a generalized mechanism affecting both the cellular and viral DNA synthesis. The inhibition of cellular DNA replication is at least partially reversible on shift of the cells back to 33°C. These data, taken together, suggest that ts2 is a S phase temperature-sensitive mutant, and that there appears to be a gradual decay of some function directly involved in DNA synthesis rather then in the progress toward its initation at 39°C.

Genetic complementation studies between ts2 cells and other temperature-sensitive mutants for DNA synthesis were performed (Jha et al., 1980). No complememtation for the growth phenotype was observed in the hybrids between ts2 and the other DNA⁻ts mutants (including ts20 and ts22). The ts phenotype of ts2 is, however, recessive in cell hybrids with non-ts mouse or human cells. The ts phenotype was also recessive (i.e. complemented) in a hybrid with a ts mutant containing a defect not related to DNA replication. The

genetic locus which corrects the ts phenotype in ts2 cells has been localized to a region on the human X-chromosome near the HPRT locus based on isozyme and karyotype analysis of cell hybrids (Jha et al., 1980).

My objective in this study was to extend the preliminary analysis of ts2. Ts2 cells were chosen for additional characterization because the majority of data obtained on this class of temperature-sensitive mutants had been derived using this cell line. Also, ts2 cells do not genetically complement ts20 or ts22 in cell hybrids suggesting that perhaps these mutants contain lesions in the same genetic locus. Therefore, it is possible that information obtained concerning the defect in ts2 would yield insight into the observed thermolability of DNA synthesis in ts20 and ts22 cells, as well.

CHAPTER TWO: Materials and Methods

2.1 Tissue culture and cell manipulation.

Mammalian Cell Lines and Culture Conditions

Ts2 is a ts cell mutant isolated from A31N (Slater and Ozer, 1976), a laboratory strain derived from the mouse embryo fibroblast line, Balb/3T3 clone A31 (Aaronson and Todaro, 1968). All studies were performed with a twice recloned subline ts2E2. Ts2R is a spontaneous revertant of ts2 isolated by David Neufeld (unpublished results). These mouse cells were maintained in Dulbecco's modified Eagle's medium (DME, M.A. Bioproducts and KC biologicals) supplemented with 10% calf or newborn calf serum (M.A. Bioproducts), penicillin, and streptomycin. They were incubated, unless otherwise noted, in a 10% CO_2 atmosphere at 33°C. These cell lines were expanded and stored after a passage without allowing cells to reach confluence, unless otherwise indicated. Experiments were performed with freshly plated cells no more than seven passages from frozen storage.

CHO-K1 is a Chinese hamster ovary cell line (Kao and Puck, 1968). MK42 was derived from the CHO-K1 cell line (Nunberg et al., 1978). Clone MK42 is approximately 50,000 times more resistant to methotrexate (MTX) then the parental CHO-K1 line (Urlaub and Chasin, 1980). The hamster cell lines were grown in a 1:1 mixture of DME and F-12 medium (M.A.Bioproducts) supplemented with 10%

newborn calf serum. Proline was added to the DME since CHO-K1 is a proline auxotroph. The cells were incubated in a 5% CO_2 atmosphere at 37°C, unless otherwise stated. All three sublines were kindly provided by Dr. L. Chasin.

Cells were passaged by brief treatment with 0.05% trypsin (Trypsin Versene, M.A.Bioproducts). Cell counts were obtained with a Royco Tissue Cell Counter after dilution in phosphate buffered saline (PBS) (0.15 M NaCl, 3 mM NaH_2PO_4, 7mM Na_2HPO_4, pH7.4). Cell lines were stored in Nunc cryotubes frozen in liquid nitrogen or in a -70°C freezer in complete medium containing 10% dimethyl sulfoxide (Fisher Scientific).

Chemicals

The following were purchased from Sigma: Trizma base (Tris), HEPES, adenosine triphosphate (ATP), cytidine triphosphate (CTP), cytidine diphosphate (CDP), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), thymidine (TdR), thymidine 5' monophosphate (TMP), thymidine 5'-diphosphate (TDP), thymidine 5'-triphosphate (TTP), deoxycytidine triphosphate (dCTP), deoxyguanosine 5'-triphosphate (dGTP), bovine serum albumin fraction V (BSA), isobutryic acid acid-washed charcoal, sodium dodecyl sulfate, ammonium formate, phenylmethylsulfonyl fluoride (PMSF).

Cleland's reagent (Dithiothreotol (DTT)), Calbiochem-Behring; Dextran T-2000, Pharmacia; formamide, MCB; ultrapure sucrose, Schwartz-Mann; agarose, FMC. Bio-Rad protein assay kit and PAGE

reagents were purchased from Bio-Rad. Aminoacetonitrile, Aldrich. Enzyme-grade Tris and DTT were purchased from GIBCO.

Preparation of growth-arrested cultures

Low passage cells were grown to confluence in 100 mm tissue culture petri dishes (Falcon). These cultures were trypsinized and the cells concentrated by centrifugation at 600 rpm for 5 minutes. The cells were seeded at a 1:2 dilution into 100 mm dishes containing DME supplemented with 10% calf serum (M.A. Bioproducts). The final volume of medium and cells in these dishes was 10 ml. The cultures were then incubated for 96 hours at 33°C in a 10% CO_2 atmosphere.

Preparation of conditioned medium

The medium was removed with a pipette from cultures seeded 96 hours earlier as described for growth-arrested cultures. This medium was stored at 4°C and used within a few hours.

Serum stimulation of quiescent cultures

Growth-arrested cultures were stimulated by aspirating the medium and washing the cultures twice with DME. Ten ml of warm DME containing 10% calf serum was added. The cultures were incubated at 33°C or immediately shifted to 39°C.

SV40 infection of quiescent cultures

Growth-arrested cultures were infected using 1 ml of SV40 stock virus (2 X 10^8 pfu/ml) per 100 mm petri dish. The infection was carried out for 2 hours at 33°C, unless otherwise specified. After virus adsorption, the virus preparation was removed and the cultures washed twice with prewarmed DME. The cultures were then fed with 10 ml of conditioned medium, unless otherwise indicated. These cultures were incubated at 33°C or shifted immediately to 39°C.

TCA precipitation of pulse-labelled cells to determine the amount of acid-insoluble material

Cultures of cells pulse-labelled for one hour with ^{3}H-TdR were washed once with PBS. The cells were then suspended in PBS containing 0.1% SDS, using 1 ml per 100mm petri dish. The suspension was TCA precipitated by adding 100% TCA, slowly with mixing, to a final concentration of 10%, followed by incubation for 30 minutes on ice. The sample was vacuum filtered onto Whatman GF/A filters. The filters were washed twice with 5% TCA, once with ethanol, and dried. The filters were then placed into scintillation vials containing 1 ml of the Amersham tissue solubilizer (NCS), heated at 60°C for 10 minutes, and then allowed to cool to room temperature prior to the addition of 10 ml of Liquifluor (NEN). The vials were vortexed and the radioactivity determined.

Autoradiography of ^{3}H-TdR labelled nuclei

Autoradiography of radiolabelled nuclei was performed essentially as described by Crowe et al. (1978). Growth-arrested cultures of cells were trypsinized, then seeded at a 1:10 dilution into tissue culture petri dishes containing glass coverslips and 10ml of medium. 5uCi per ml ^{3}H-TdR (20Ci per mmole in the methyl group, NEN) was added at the time of seeding. These cultures were incubated at 33°C or 39°C. At various times after seeding the coverslips were removed and washed twice with PBS. The cells on the coverslips were fixed in ice cold methanol for 15 minutes, then extracted with ice cold 5% TCA for 30 minutes. The coverslips were rinsed twice with distilled water, air dried, and mounted cell side up on slides with a drop of Permount. The mounted coverslips were allowed to harden overnight at room temperature.

The slides were dipped into Kodak NTB-2 emulsion at 60°C which had been diluted 1:1 with distilled water. The slides were allowed to dry, then stored in a dark box containing a dessicant. After 7 days, the coverslips were developed using Kodak D-19 developer (3 minutes), then washing the slide with 3% acetic acid. The slides were then placed in Kodak fixer for 3 minutes followed by extensive washing with water. The slides were drained and allowed to air dry, then observed under low power to count the number of cells containing dark nuclei which signifies ^{3}H-TdR incorporation into macromolecules.

Preparation of ^{35}S-methionine labelled cellular extracts

Cells were pulse-labelled with ^{35}S-methionine to determine pII (MEP) levels. The culture medium was removed and replaced with methionine-free DME containing 0.5% calf serum or platelet poor plasma. The cultures were incubated for 30 minutes at 33°C after which 50 uCi per ml ^{35}S-methionine was added. The cultures were again incubated at 33°C for 20 minutes. The cells were rinsed with DME, preparatory to cellular lysis.

Immune precipitation

The cells were scraped into 1 ml of immune precipitation buffer A (Gottesman and Cabral, 1981) (0.154 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% NP-40 (nonidet P-40, Shell), 0.05% SDS) with a rubber policeman, centrifuged for 5 minutes in a microfuge to remove cellular debris and the supernatants saved. The supernatants were spotted onto Whatman 3 MM paper and repeatedly washed with 5% TCA followed by ethanol to determine the acid-insoluble counts. Equal quantities of acid-insoluble material were diluted to 1 ml in buffer A and incubated overnight with rabbit antiserum to MEP, at antibody excess. 50ul of a 10% suspension of Staph aureus A (Pansorbin, Calbiochem-Behring) was added for one hour. The immune complex was pelleted in a microfuge and washed three times with immune precipitation buffer B (0.154 M NaCl, 0.05M Tris-HCl, pH 7.4, 2.5M KCl, 0.5% NP-40), once with buffer A, and once with distilled water. Appropriate buffers were added for one-

dimensional gel electrophoresis.

One-dimensional acrylamide gel electrophoresis (PAGE)

Samples in immune precipitation buffer A were adjusted to 62.5mM Tris-HCl (pH 6.8), 1% SDS, 0.0005% bromophenol blue, 10% 2-mercaptoethanol and 10% glycerol. Immune precipitates were eluted from the Staph A in the same buffer. The samples were then heated to 90°C for 2 minutes prior to application to 15% polyacrylamide slab gels (Laemmli, 1970). After electrophoresis, the gels were soaked in a fixation solution overnight (10% TCA, 10% acetic acid, 30% methanol) and processed for fluorography (Bonner and Laskey, 1974). The gels were overlaid with XAR-5 film (Kodak); the fluorograms were not fully exposed so that the density of the bands approximated the amount of ^{35}S-methionine incorporated.

Immunoblotting procedure (Western analysis)

Western analysis of proteins was performed essentially as described by Towbin et al. (1979). After the completion of PAGE, the gel was placed into electroblot buffer (24 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). The gel was then placed onto a nitrocellulose filter and transferred via a Hoeffer electroblot apparatus at 0.1 amps for 16 hours. The control lanes were cut from the transfer and the proteins visualized after staining with amido black. The filter was exposed to antibody to the protein of interest by first incubating the filter for one hour in blocking

buffer (3% gelatin in TBS, 20 mM Tris, 50 mM NaCl, pH 7.5), followed by incubation of the filter for one hour in a solution of the primary antibody suspended in 1% gelatin. The filter was washed 4 times with TBS and then incubated for one hour with horse radish peroxidase conjugated goat anti-mouse immunoglobulin (BioRad). The filter was washed 4 times in TBS and developed in a solution consisting of 0.05% 4-chloro-1-napthol, 0.01% H_2O in TBS. When the filter was fully developed (approximately 15 minutes) it was rinsed with water and photographed.

RNA isolation and Northern analysis

Total cellular RNA was isolated by phenol extraction at 60°C as described by Soeiro and Darnell (1969) and Hsuing et al. (1982). In brief, cells were harvested from 100mm tissue culture petri dishes by scraping with a Teflon tape-covered razor blade. The cells were washed twice in PBS, then suspended at 10^7 cells per ml in 0.1 M sodium acetate-1mM trisodium EDTA (pH 5.2). Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5% and immediately thereafter the mixture was extracted with buffer-saturated, redistilled phenol equilibrated at 60°C. RNA was recovered by ethanol precipitation at -60°C.

RNA samples were prepared for Northern analysis by drying the ethanol precipitated RNA and resuspending the sample in 1X electrophoresis buffer (Lehrach et al., 1977) containing 50% formamide and 1.85M formaldehyde. Samples were denatured by heating to 65°C for 10 minutes and fractionated through 1%

agarose gels (agarose prepared in 20 mM MOPS, adjusted to pH 7, 5mM sodium acetate, 1mM EDTA) containing 1M formaldehyde. Gels were blotted onto nitrocellulose filters by standard procedures (Thomas, 1980).

Hybridization probes were prepared by nick-translation as described by Rigby et al. (1977) with ^{32}P(TTP, dCTP, dATP) (3000 Ci per mmole, NEN) and non-radioactive dGTP. The specific activity of the nick-translated DNA was greater than 10^8 dpm per ug DNA. Hybridizations were performed for 18 hours at 65°C using 4X SSC, 5X Denhardt's solution (Denhardt, 1966), 0.1% SDS, 0.1% PPi, and 500 ug per ml heat-denatured salmon sperm DNA as described by Gunning et al. (1984). The nitrocellulose filters were washed and autoradiographed with Kodak XAR-5 film.

Recombinant plasmid DNA

Plasmid DNA's were obtained from other laboratories and used without further genetic manipulation. Purified 28H6 (proliferin) was provided by Linzer and Nathans (1983). pMc-myc 54 was provided by H. Lachman. All procedures involving recombinant DNA were performed in accordance with NIH guidelines.

Protein Determination

Protein concentration was determined by either the method described by Lowry et al. (1951) or the Bio-Rad Protein Assay (Bio-Rad).

Flow cytofluorographic analysis (FMF)

Cells were subjected to cytofluorographic analysis as described by Krishan (1975). In brief, the cells were washed twice with PBS, then once more with a fixation solution containing 0.1% sodium citrate and 50 ug per ml propidium iodide. 10 ml of the fixation solution was then added, and the cells were incubated at 4°C for 15 to 30 minutes. After the cells were swollen, they were collected by pipette and stored at 4°C for no more than two days. Analysis was performed with a Model 4800 A flow microfluorimeter (Biophysics Division System, Ortho Instruments) having a 488nM excitation wavelength.

2.2 Preparation of cell extracts and subcellular fractions.

Harvesting of cell cultures for the preparation of extracts

Cell cultures were harvested by washing the cultures twice with PBS and then scraping the cells into 1 ml PBS per culture. The cells were pelleted by centrifugation at 1000 rpm for 5 minutes at 4°C. The PBS was aspirated and the cell pellet was immediately extracted or frozen at -70°C until used.

Preparation of crude cytoplasm extracts

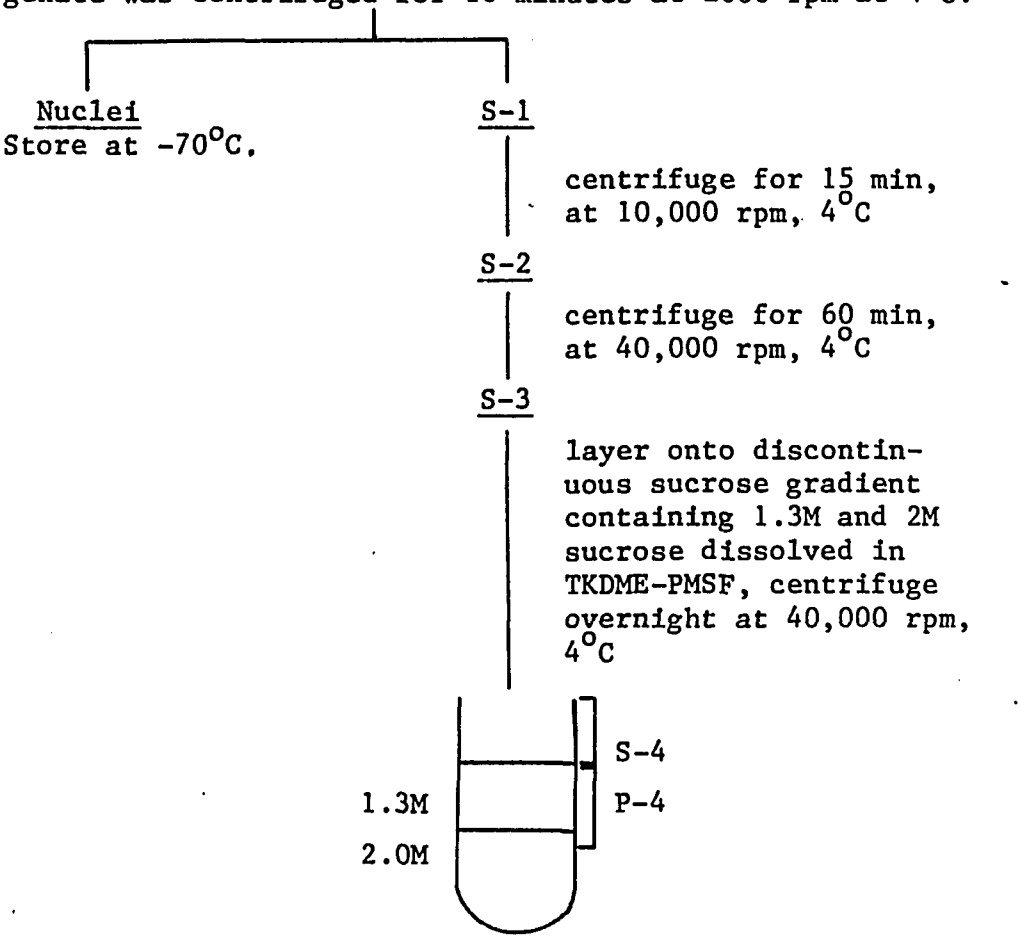
Cell pellets derived from duplicate cultures (fresh or previously frozen pellets thawed on ice) were resuspended in 1 ml of extraction buffer (50 mM Tris-HCl, pH 7.8, 10 uM DTT, 1mM EDTA), and homogenized by 20 strokes of a tight 7 ml Dounce homogenizer. The homogenate was centifuged at 12,000 rpm for 20 minutes in a Sorval SM24 rotor. The supernatant was used directly for enzymatic assay.

Preparation of fractionated cell extracts

Cell extracts were prepared and fractionated by the following diagrammed schemes to yield defined multi-enzyme complex fractions (which are described in the text). These schemes essentially diverge in the manner in which the "cytoplasmic" S-4 and P-4 are collected. They also vary as to the way the crude nuclei are extracted. Several different sample volumes were assessed to determine whether the enzymatic activities were within the linear range.

Fractionation Scheme One

The cell pellet was resuspended in 4 volumes of STKDME-PMSF. Cells were homogenized by 80 strokes with a Dounce homogenizer. The homogenate was centrifuged for 10 minutes at 2000 rpm at 4°C.



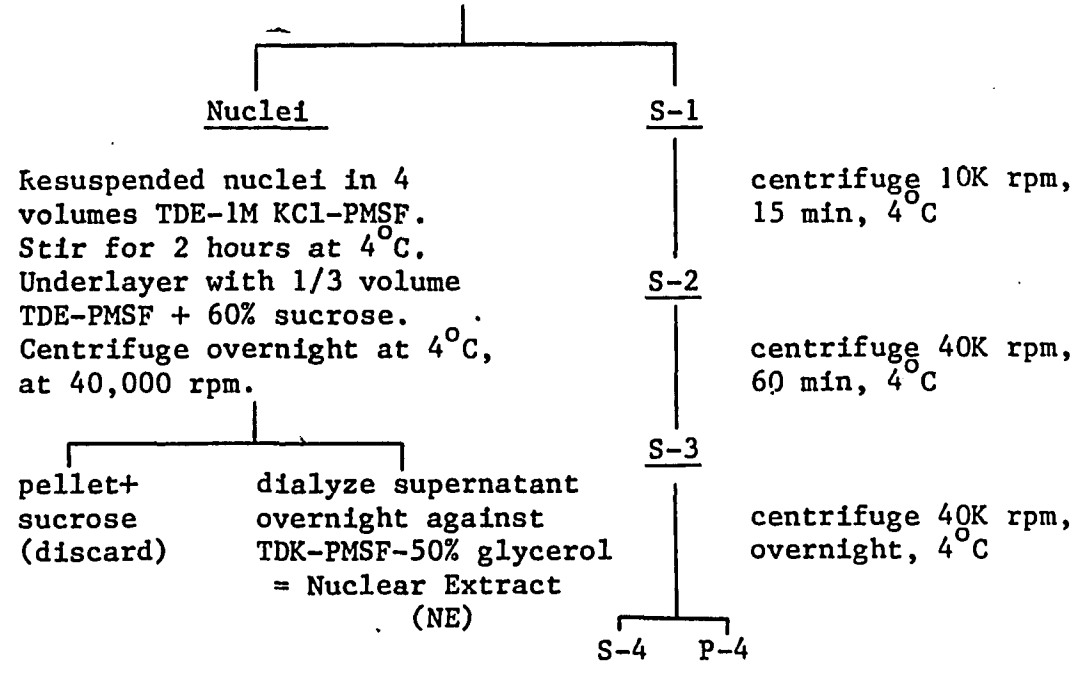
Buffer abbreviations:

STKDME-PMSF = .25M sucrose, 50mM Tris-Ac, pH 7.5, 25mM KCl, 1mM DTT, .1mM EDTA, .1mM PMSF.

TKDME-PMSF = the same as the above, without sucrose.

Fractionation Scheme Two

The cell pellet was resuspended in 4 volumes of STKDME-PMSF and homogenized by 80 strokes with a Dounce homogenizer. The homogenate was centrifuged for 10 minutes at 2000rpm, 4°C.



Buffer abbreviations:

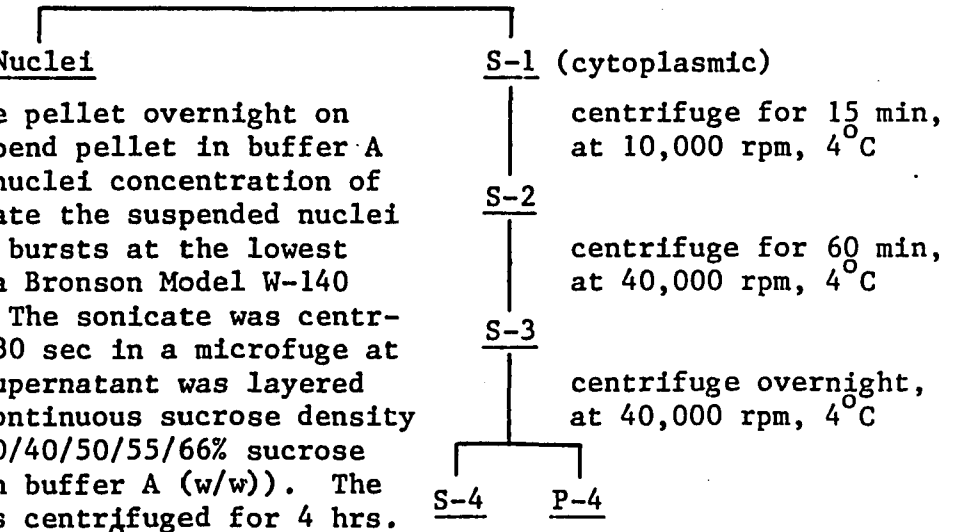
STKDME-PMSF = .25M sucrose, 50mM Tris-Ac, pH 7.5, 25mM KCl, 1mM DTT, 1mM EGTA, 0.1mM PMSF.

TDE-PMSF = 50mM Tris-Ac , ph 7.5, 1mM DTT, 25mM KCl, 1mM EGTA, .1mM PMSF.

TDE-1M KCl-PMSF = 50mM Tris-Ac, pH 7.5, 1mM DTT 1mM EGTA, .1 mM PMSF, 1M KCl.

Fractionation Scheme Three

The cell pellets were resuspended in 4 volumes of buffer A (20mM HEPES, pH 7.6, 1mM DTT, 1mM PMSF, and 5mM $MgCl_2$) and homogenized by 80 strokes in a tight 7 ml Dounce homogenizer. Nuclei were collected by centrifugation of cell homogenate for 10 minutes at 5000 rpm at 4°C in a Sorvall RC-5B centrifuge using a Sorvall SM-24.

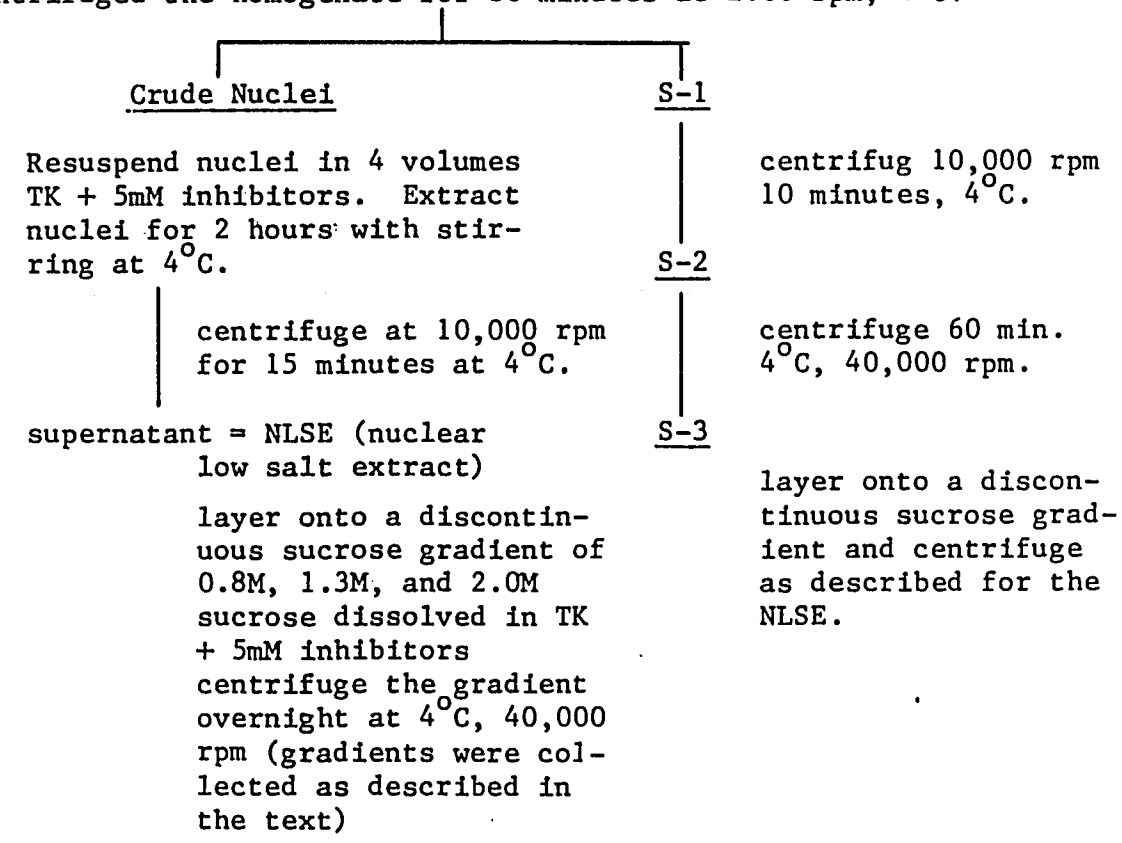


Incubate the pellet overnight on ice. Resuspend pellet in buffer A to a final nuclei concentration of 50%. Sonicate the suspended nuclei in 2-20 sec bursts at the lowest setting of a Bronson Model W-140 Sonicator. The sonicate was centrifuged for 30 sec in a microfuge at 4°C. The supernatant was layered onto a discontinuous sucrose density gradient (20/40/50/55/66% sucrose dissolved in buffer A (w/w)). The gradient was centrifuged for 4 hrs. at 23,000 rpm at 4°C in a Beckman L2-50 centrifuge using a Beckman SW27.1 rotor. The gradients were collected in 0.5 ml fractions. These fractions were assayed for various activities. That fraction which contained maximal activity was termed replitase[a] (Reddy and Pardee, 1980).

a) It was determined that overnight incubation of the nuclei on ice, the concentration of nuclei during sonicate, and the setting used to sonicate the nuclei all played a role in the distribution of replitase in the gradient.

Fractionation Scheme Four

Cells resuspended in 4 volumes of cold STK + 5mMinhibitors.
Cells homogenized by 80 strokes with a 7ml Dounce homogenizer.
Centrifuged the homogenate for 10 minutes at 2000 rpm, 4°C.



Buffer abbreviations:

STK = 0.25M sucrose, 50 mM Tris-acetate, pH7.5, 0.25M KCl

TK = 50 mM Tris-acetate, pH7.5, 0.15M KCl

inhibitors = PMSF (phenylmethylsulfonyl fluoride), AAN (amino-acetonitrile), EGTA, EDTA

2.3 Activity assays.

Thymidine kinase (TK)

The assay was performed as described by Postel and Levine (1976). 0-25ul samples were incubated with 25 ul of a TK-enzyme assay mix (TK-EAM, consisting of 110mM Tris-HCl, pH 7.8, 7mM $MgCl_2$, 4mM DTT, 12mM ATP, and 2.5uCi ^{3}H-TdR (20 Ci per mmole, New England Nuclear)) for 20 minutes at 37°C. The reaction was stopped by boiling the reaction mix for 2 minutes. The reaction mix was spotted onto Whatman DE-81 filter squares (2cm by 2cm), dried under an infra-red heat lamp, and washed 4 times in 1mM ammonium formate, followed by 2 washes in methanol. The filters were again dried and the levels of radioactivity determined by liquid sctillation spectrometry using 5 ml of a liquid scintillation cocktail (Liquifluor, New England Nuclear). Several different sample volumes were assessed to determine whether the enzymatic activities were within the linear range.

Thymidylate kinase (TMPK)

0-25 ul samples were incubated 20 minutes at 37°C with 25 ul of a thymidylate kinase enzyme assay mix (TMPK-EAM, containing 110mM Tris-HCl, pH 7.8, 7mM $MgCl_2$, 4mM DTT, 12mM ATP, and 2.5 uCi ^{3}H-TMP (40Ci per mmole, Amersham)). The reaction was stopped by boiling the samples for 2 minutes. 10ul of the reaction mix was spotted onto Whatman #1 paper chromotography strips (2.5cm by 60cm) along with carrier consisting of 60 mM of (TdR, TMP, TDP, TTP). The nucleotides were resolved for 16 hours in a system consisting

of a 66:1:33 ratio of isobutryic acid: ammonium hydroxide: water. The resolved nucleotides were visualized under long wave U. V. light and the spots corresponding to TDP and TTP were cut out and eluted overnight in 1 ml of 0.1N HCl. The radioactivity of each eluted sample was determined by liquid scintillation spectrometry in 10 ml of a liquid scintillation cocktail (Liquiscint, National Diagnostics).

DNA polymerase α (DNA pol α) (Weissbach, 1981)

Incubated 0-25 ul samples with 30 ul pol α-enzyme assay mix (α-EAM, containing 55 mM Tris-HCl, pH7.8, 3.5 mM $MgCl_2$, 2 mM DTT, 6 mM ATP, 1.25 uCi ^{3}H-TTP (70 Ci/mmole, Amersham), 50 ug heat-denatured bovine serum albumin fraction V, and 0.01M each of dATP, dGTP, and dCTP), and 5 ug of activated calf thymus DNA. This reaction mix was incubated for 30 minutes at 37°C.

The acid-insoluble material was isolated by first adding 50 ug BSA to act as carrier. The samples were precipitated by the addition of 20% TCA (to a final concentration of 10%) and then sodium pyrophosphate (NaPPi) to a final concentration of 0.1%. The samples were incubated for 15 minutes on ice, then diluted to 0.5 ml with 10% TCA, and centrifuged for 0.5 minutes at 4°C in a microfuge. The supernatants were decanted and discarded. The pellets were dissolved in 100 ul of 0.2M NaOH and incubated for 60 minutes at 37°C, neutralized in HCl, and then spotted onto Whatman DE-81 filter squares. Following drying, the filters were batch washed in 100 ml of 0.1M cold NaPPi, followed by 4 washes in 0.3M ammonium formate, and one wash in methanol. The filters were

dried and radioactivity determined by liquid scintillation spectrometry, using 5 ml of Liquifluor.

Nucleoside Diphospho Kinase (NDPK)

0-10 ul samples were incubated for 20 minutes at 37°C with 15 ul of a nucleoside diphosphokinase enzyme assay mix (NDPK-EAM, containing 83mM Tris-HCl, pH7.8, 0.016M Mg acetate, 83 uM DTT, 66mM NaF, 0.16mg/ml of heated BSA, 17mM ATP, 1.25 uCi ^{3}H-TDP (40 Ci/mmole, Amersham)). To stop the reaction, an equal volume (25ul) of 0.1M Na_3-EDTA, pH7.0 was added. 10ul of the reaction mix was spotted onto a pre-washed PEI plate (Brinkman). 1 ul of carrier containing 0.25 mM of dTTP, dTDP, dTMP, dTdR was also applied to the center of the sample spot. Ascending chromotography was performed in 1M LiCl until the solvent front was approximately 1 cm from the upper edge. The plate was dried at room temperature for 1-2 hours and viewed under U.V. light (mineral light), in order to mark the visible spots of carrier. The respective spots were scraped and placed into scintillation vials containing 1 ml of a solution consisting of 0.1N HCl and 0.2M KCl. The vials were incubated at 50°C for 60 minutes. 10 ml of Liquiscint was added and the radioactivity measured in a liquid scintillation counter.

Dihydrofolate reductase-methotrexate binding assay (DHFR-^{3}H-MTX)

The assay was used essentially as described by Johnson et al., (1978) and Kamen et al., (1976). Sample was incubated for 10 minutes at room temperature with 50 ul of a DHFR-^{3}H-MTX binding assay mix (DHFR-^{3}H-MTX-BAM consists of 0.01M phosphate buffer,

pH 6.0, 0.15M KCl, 1 mg/ml BSA, 3 x 10^{-4}M NADPH, and 4 x 10^{-8}M ^{3}H-MTX (18Ci/mmole, Amersham)). After incubating the samples, they were washed 2X in 300 ul of a charcoal-dextran suspension (1g acid-washed charcoal and 0.01g Dextran T-2000, 0.25g BSA, 30 ml H_2O, pH adjusted to 6.2 with phosphate buffer). The samples were then centrifuged for 5 minutes at 2000 rpm. The supernatant was poured into scintillation vials containing 10 ml Liquiscint and radioactivity determined.

Channeling of ^{3}H-thymidine to ^{3}H-thymidine-triphosphate

Conversion of ^{3}H-TdR by subcellular fractions is defined in the test. 25ul samples were incubated with 25 ul TK-EAM as for the TK assay for 60 minutes at 37°C. The reactions were stopped by boiling for 2 minutes and 10 ul aliquots of each sample were spotted onto 60 cm long Whatman #1 chromatography paper strips along with 2 ul of a carrier (60 mM each of TdR, TMP, TDP, and TTP). Descending chromatography was performed with development solvent of isobutyric acid : NH_4OH : H_2O in a ratio of 66:1:33 for 16 hours. All the carrier spots were visualized with U.V. light, cut from the paper, and eluted overnight in 1 ml of 0.1N HCl. The radioactivity for each was determined by liquid scintillation spectrometry, using 10 ml of Liquiscint. Data are generally presented for ^{3}H-TTP only.

In Vitro incorporation of ^{3}H-thymidine into DNA

25 ul samples were incubated with 25 ul TK-EAM for 60 minutes at 37°C as described for the TK assay. 50 ug of BSA was then

added along with 5 ug of heat denatured calf thymus DNA, and 50 nM dATP, dGTP, dCTP, and dTTP. The samples were reincubated at 37°C for 60 minutes. Acid-insoluble material was isolated as described for the DNA polymerase ∝ assay.

2.4 Preparation of virus stocks and the purification of virions.

Virus preparation

SV40 virus was prepared by infection of confluent monolayer of permissive CV-1V cells (obtained from P. Berg) at a low multiplicity of infection (less than 0.01% pfu/cell) as previously described by this laboratory (Ozer, 1972). Wild type virus (SV-5) was propagated at 37°C and tsA58 at 33°C. Infected cultures were harvested at maximal cytopathic effect. Cells were repeatedly frozen and thawed in the original medium to disrupt the cells and release intracellular virus. Cell debris was removed by low speed centrifugation and the supernatant designated as stock virus. Virus was stored in aliquots at -20°C and used without further repeated freeze-thawing. The concentration of virus as plaque-forming units (pfu) was determined by plaque assay on CV-1P monolayers (obtained from P. Berg) or by determining the proportion of cells infected by immunofluorescence assay for SV40 T antigen. In the latter case, the equivalent number of pfu was calculated by use of a standard curve.

Purification of SV40 virions from an infected cellular lysate

Virus was concentrated from a crude virus preparation (stock

virus) by PEG precipitation and purified by CsCl equilibrium centrifugation as previously described by this laboratory (Kidwell, et al., 1972). In brief, sodium chloride was added to a virus suspension to a final concentration of 0.5M, followed by PEG 6000 to 10%, with stirring at room temperature. The mixture was incubated at 4°C overnight. The precipitate was collected by low speed centrifugation (10,000 rpm for 10 minutes). The pellet was washed twice with TE buffer (10mM Tris-HCl, pH7.5, 1 mM EDTA), and redissolved in TE buffer with stirring. 0.51g CsCl per ml buffer was added to the viral suspension to result in a density of 1.34. The suspension was centrifuged for 20 hours at 40,000 rpm in a Beckman SW50.1 rotor. The virion bands were visualized in the gradients by blue light. The lower band was collected and dialyzed extensively against 10 mM Tris-HCl, pH7.4 and 0.1M NaCl at 4°C.

Determination of SV40 T-antigen by immunofluorescence (IF)

Coverslip cultures were prepared and infected as described in the text. At appropriate times post infection (pi) coverslips were processed essentially as described by Solomon et al., (1979) in formaldehyde and permeabilized with acetone at -20°C. T-antigen was detected using a monoclonal antibody pAb101 (Gurney et al., 1980) and a fluorescein conjugated goat anti-mouse IgG (Yeda-Miles) as routinely performed in this laboratory. Rhodamine conjugated BSA was used as a counterstain. Coverslips were examined with a Leitz U.V. microscope with epi-illumination. 200 cells were counted per coverslip under oil immersion and the percentage of cells with nuclear fluorescence calculated.

2.5 Calculations.

One unit of activity is defined as the following for each of the following activities:

Thymidine Kinase (TK)

One fentomole of ^{3}H-nucleotide formed from ^{3}H-TdR per 20 minutes at 37°C.

Thymidylate Kinase (TMPK)

One femtomole ^{3}H-TdR and TTP formed from ^{3}H-TMP per 20 minutes at 37°C.

Nucleotide-Diphosphokinase (NDPK)

One femtomole ^{3}H-TTP formed from ^{3}H-TDP per 30 minutes at 37°C.

Dihydrofolate Reductase (DHFR)

One femtomole ^{3}H-MTX bound per 15 minutes at room temperature.

DNA polymerase α (DNA pol α)

One femtomole ^{3}H-TTP incorporated per 30 minutes at 37°C.

^{3}H-TdR Channeling to ^{3}H-TTP

One femtomole ^{3}H-TTP formed per 60 minutes at 37°C.

^{3}H-TdR Incorporation Into DNA In Vitro

One femtomole ^{3}H-TdR incorporated per 60 minutes at 37°C.

2.6 Abbreviations.

AAN	aminoacetonitrile
BSA	bovine serum albumin
CHO	Chinese hamster ovary fibroblasts
CS	calf serum
DHFR	dihydrofolate reductase
DME	Dulbecco modified Eagle's medium
DNA pol α	DNA polymerase α
EAM	enzyme assay mix
FMF	flow (micro) cytofluorographic analysis
^{3}H-MTX	tritiated methotrexate
HPRT	hypoxanthine-guanine phosphoribosyl transferase
^{3}H-TdR	tritiated thymidine
MEC	multi-enzyme complex
MEP	major excreted protein
NDPK	nucleoside diphosphokinase
NE	nuclear extract
NEN	New England Nuclear
NLSE	nuclear low salt extract
NP-40	non-idet P-40
PAGE	polyacrylamide gel electrophoresis
pfu	plaque forming unit
PLF	proliferin
PMSF	phenylmethylsulfonyl fluoride
PPi	sodium pyrophosphate

PPP	platelet poor plasma
RR	ribonucleotide reductase
SDS	sodium dodecylsulfate
Staph A	Staph aureus A
STKDME-PMSF	sucrose, tris, KCl, DTT, $MgCl_2$, EDTA, PMSF
SV40	simian virus 40
TCA	trichloroacetic acid
TDP	thymidine 5'-diphosphate
TMP	thymidine 5'-monophosphate
TMPK	thymidylate kinase
TK	thymidine kinase
TKDME-PMSF	tris, KCl, DTT, $MgCl_2$, EDTA, PMSF
ts	temperature-sensitive
TS	thymidylate synthetase
TTP	thymidine 5'-triphosphate

RESULTS

CHAPTER THREE: Properties of ts2 at the permissive and restrictive temperature

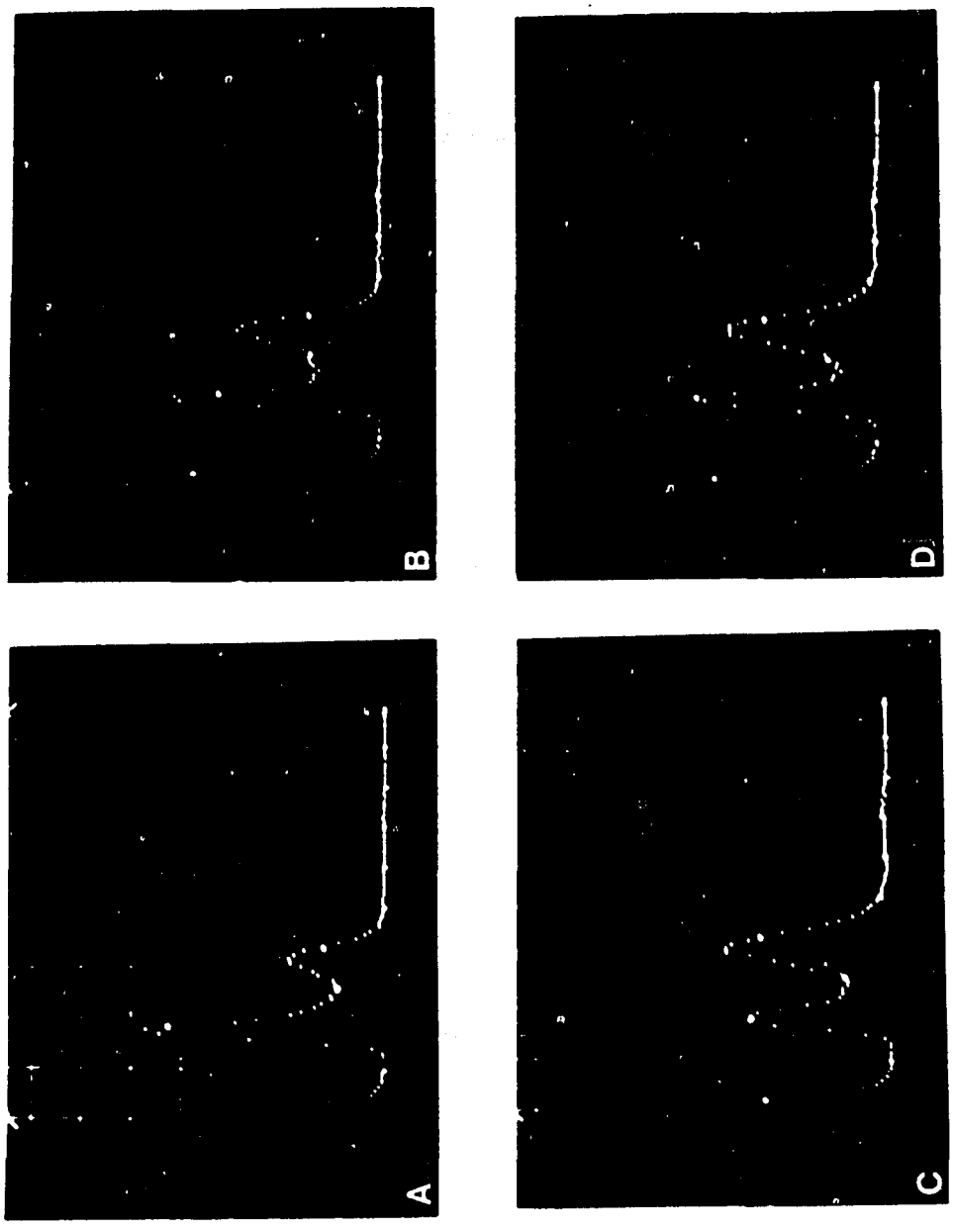
3.1 Cell cycle analysis of ts2.

Several lines of evidence suggest that ts2 is a temperature-sensitive (ts) mutant affected in DNA synthesis (Slater and Ozer, 1976), as described earlier in the text. Further support for the genetic defect in ts2 as contributing to the observed thermolability of its DNA synthesis was obtained by cell cycle analysis using flow-cytofluorographic (FMF) analysis (Sugarbaker et al., 1979). By measuring the DNA content of each cell in a given population, it is possible to correlate individual cells with phases in the cell cycle (ie., phases G1, S G2, and M). The proportion of cells with increased DNA content can thus be used as a parameter for proliferative activity.

Cultures of actively growing ts2 cells were shifted to their non-permissive temperature for growth of 39°C for 9,12, or 16 hours, then stained with propidium iodide (Krishan, 1975), a fluorescent dye for DNA. The FMF profiles obtained from such an experiment are shown in figure 1 (panels A-D). Figure 1A shows a profile of actively growing (log phase) ts2 cells which were not subjected to incubation at 39°C. The first peak on the left side of each panel represents that portion of the tested cell population

Figure 1. Flow-cytofluorographic (FMF) analysis of actively growing ts2 cells at 33°C upon shift to 39°C. Cultures of ts2 cells were incubated at 39°C for (A) 0 hours, (B) 9 hours, (C) 12 hours, (D) 16 hours. At each time point the cultures were prepared for FMF analysis and stained with propidium iodide as described in Material and Methods.

FIGURE 1



with a unit complement of DNA, ie., G1 phase. The second peak indicates those cells containing two complements of DNA; these are cells in either the G2 or M phases. The valley between the two peaks is representative of the cells in S phase.

If ts2 were defective in a G1 function or in the initiation of DNA synthesis we could expect to observe an FMF profile in which the cells accumulate within the G1 peak. Those cells which had passed the defective block in G1 or initiated DNA replication prior to the shift of the cultures to 39°C would complete their cell cycle, and then collect in the G1 phase. The observed profiles (figure 1B-D) indicate that ts2 cells incubated at 39°C accumulate within S and G2 (late S?) phases of the cell cycle. A spontaneous revertant of ts2, designated ts2R, when incubated at 39°C does not show the accumulation of cells within S phase (data not shown). The pattern observed with ts2R cells was indistinguishable from that of log phase cells at all times tested. These data lend further support for ts2 being defective in some function involved in S phase.

3.2 Enzymatic activity and DNA synthesis in actively growing ts2 cells incubated at 39°C.

The kinetics of the decay in DNA synthesis had been investigated in ts2 cells incubated at 39°C (Slater and Ozer, 1976). It was observed that there was a decline in DNA replication continuing for over a generation time and no substantial immediate loss of viability. Those data and that obtained from FMF analysis indicated that the thermolabile function required in S phase

decayed over time at 39°C, thus preventing ts2 cells from progressing in their cell cycle. Studies were undertaken to examine what effect, if any, this labile function would have on other S phase activities in ts2.

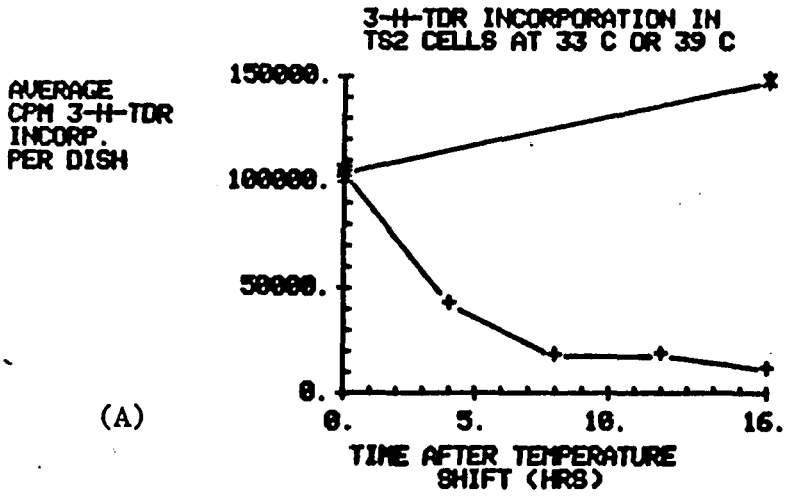
It had been shown (Prescott, 1976, Johnson et al., 1978) that many enzymes and proteins involved in nucleotide metabolism and DNA replication have maximal activities only during the S phase of the cell cycle. Since ts2 was shown to have temperature-sensitive DNA synthesis, we questioned what effect temperature would have on such enzymes. Two enzymes examined were thymidine kinase (TK) and thymidylate kinase (TMPK). Thymidine kinase usually exhibits relatively high activities in proliferating mammalian cells, while activities are low in cells during proliferative quiescence (Kit, 1976, Munch-Peterson and Tyrsted, 1977). TK was also described as undergoing marked changes during the cell cycle. Maximal levels of TK activity occur in cells during S and G2 phases, with lower levels observed (10-50% of observed maximum) in G1 phase (Gelbard et al., 1971, Brent, 1971, Bello, 1974, Piper et al., 1980). Thymidylate kinase also appears to be a member of the class of enzymes and proteins that is required for DNA replication and synthesized primarily, if not exclusively, during the S phase (Prescott, 1976).

The activities of TK and TMPK were examined in ts2. Cultures of actively growing ts2 incubated at 33°C were either shifted to 39°C or kept at 33°C for 0-16 hours. At four hour intervals cultures were harvested, crude extracts prepared, and these extracts were assayed for their TK and TMPK activities, see

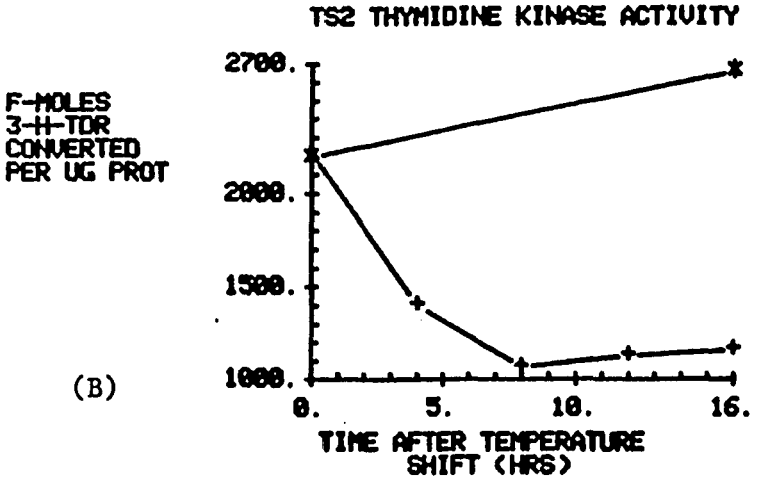
Materials and Methods. Parallel cultures of ts2 were examined for their rate of DNA synthesis by their ability to incorporate ^{3}H-TdR into TCA precipitable material during a one hour labelling time. The results of such an experiment are presented in figure 2. During the time course, there is a large decrease in the rate of DNA synthesis at 39°C when compared to that observed at 33°C. There also appears to be a corresponding, although less severe, decline in TK activity. TMPK activity is not greatly affected, if at all, during this period of incubation at 39°C. There is no observable change in TK and TMPK activities or the rate of DNA synthesis at 33°C during the time course.

The decrease in TK activity observed, as ts2 are incubated at 39°C, could explain the decline in the rate of ^{3}H-TdR incorporation, although the level of enzyme is still well-above minimal levels. A control to examine this possible connection between TK activity and the rate of ^{3}H-TdR incorporation is the use of the ts2 revertant, ts2R. Ts2R is capable of normal proliferation at 39°C. TK activity was assayed in crude extracts prepared from ts2R incubated for 0-8 hours at 39°C. Parallel cultures at 33°C were also assayed. The rate of DNA synthesis was assessed by pulse-labelling cultures for one hour with ^{3}H-TdR. Figure 3 indicates that the rate of ^{3}H-TdR incorporation is higher at 39°C when compared to that observed at 33°C during the 8 hour time course. This would be expected if the kinetics of DNA synthesis is occuring at a higher rate at increased temperature. While there is no decrease in DNA replication noted at 39°C in ts2R, there is a lowered TK

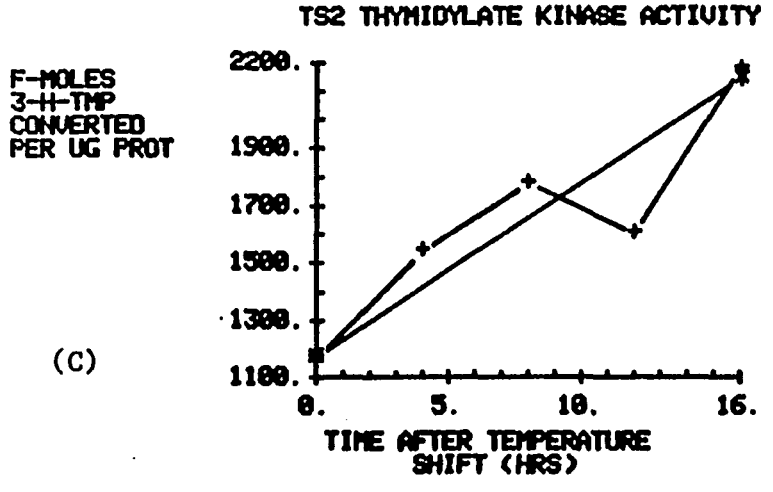
Figure 2. Effect of incubating actively growing ts2 at 33°C and 39°C on its (A) rate of ^{3}H-TdR incorporation, (B) TK activity, (C) and TMPK activity. Ts2 cells were incubated at 33°C of shifted to 39°C for various lengths of time. The cultures were harvested, and crude extracts prepared, and assayed for enzymatic activities in duplicate, as described in Materials and Methods. Protein concentration was determined by the method of Lowry et al. (1951). Sister cultures were examined for the rate of ^{3}H-TdR incorporation into acid-insoluble material by pulse-labelling the cultures for one hour with 1 uCi ^{3}H-TdR (20 Ci/mmole, NEN) per ml of medium. These labelled cultures were than processed to determine the amount of isotope incorporated into acid-insoluble material as described in the Materials and Methods.


3-H-TDR INCORPORATION IN
TS2 CELLS AT 33 C OR 39 C
AVERAGE
CPM 3-H-TDR
INCORP.
PER DISH
150000.
100000.
50000.
0.
0.
5.
10.
16.
TIME AFTER TEMPERATURE
SHIFT (HRS)

(A)


TS2 THYMIDINE KINASE ACTIVITY
F-MOLES
3-H-TDR
CONVERTED
PER UG PROT
2700.
2000.
1500.
1000.
0.
5.
10.
16.
TIME AFTER TEMPERATURE
SHIFT (HRS)

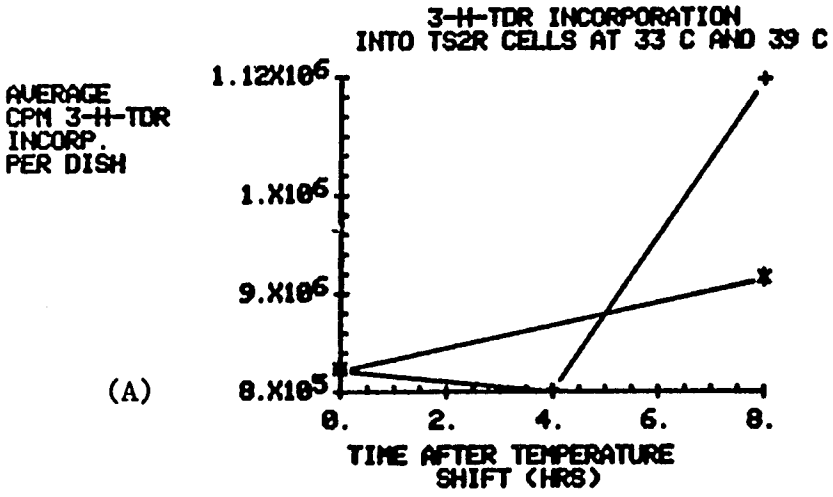
(B)


TS2 THYMIDYLATE KINASE ACTIVITY
F-MOLES
3-H-TMP
CONVERTED
PER UG PROT
2200.
1900.
1700.
1500.
1300.
1100.
0.
5.
10.
16.
TIME AFTER TEMPERATURE
SHIFT (HRS)
-*- 33 C
-+- 39 C

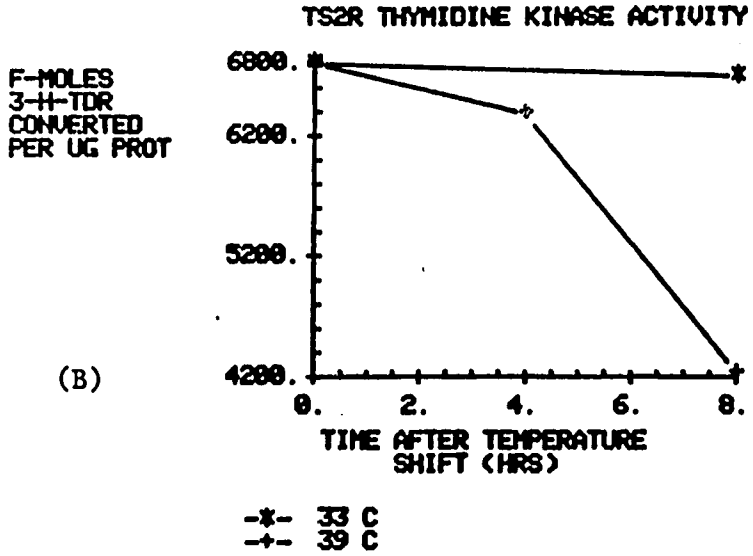
(C)

FIGURE 2.

Figure 3. Effect of incubating actively growing ts2R at 33°C and 39°C on the (A) rate of ^{3}H-TdR incorporation, (B) TK activity. Ts2R cells were incubated at 33°C or shifted to 39°C for varying lengths of time. The cultures were harvested, crude extracts prepared, and assayed for enzyme activity in duplicate, as described in the Materials and Methods. Protein concentration was determined by the method of Lowry et al. (1951). Sister cultures were examined for the rate of ^{3}H-TdR incorporation into acid-insoluble material by pulse-labelling the cultures for one hour with 1 uCi ^{3}H-TdR (20 Ci/mmole, NEN) per ml of medium. The amount of isotope incorporated into acid-insoluble material was determined as described in the Material and Methods.


3-H-TDR INCORPORATION
INTO TS2R CELLS AT 33 C AND 39 C
AVERAGE
CPM 3-H-TDR
INCORP.
PER DISH
1.12X10^6
1.X10^6
9.X10^6
8.X10^5
0.
2.
4.
6.
8.
TIME AFTER TEMPERATURE
SHIFT (HRS)

(A)


TS2R THYMIDINE KINASE ACTIVITY
F-MOLES
3-H-TDR
CONVERTED
PER UG PROT
6800.
6200.
5200.
4200.
0.
2.
4.
6.
8.
TIME AFTER TEMPERATURE
SHIFT (HRS)
-*- 33 C
-+- 39 C

(B)

FIGURE 3.

activity. The heat sensitive property of the TK enzyme in ts2R does not, apparently affect the rate of ^{3}H-TdR incorporation at 39°C. This initial result implies that the observed thermolability of TK in ts2 is not the basis for the genetic defect in DNA synthesis at 39°C.

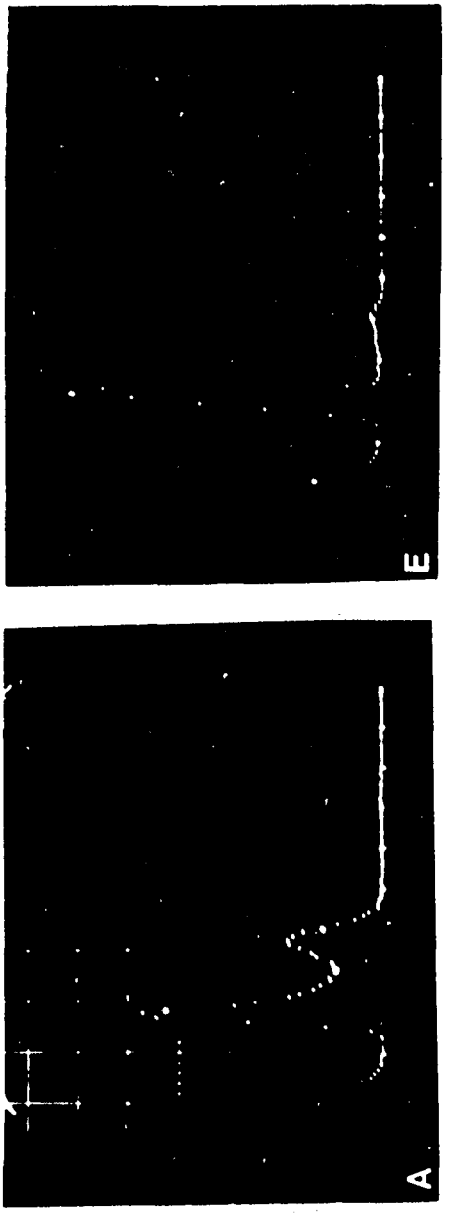
3.3 DNA synthesis and enzyme induction in ts2 by serum stimulation of growth-arrested cells.

To better understand the kinetics of DNA synthesis and enzyme activity during S phase in ts2, relating to its temperature-sensitive growth, experiments employing synchronized cells were performed. These synchronized populations of cells facilitate the observation of cell cycle specific events. Since the majority of cells are in the same phase of the cell cycle, this allows examination of cellular activities involved only with that phase.

A convenient way to synchronize 3T3 cells, including ts2, is by density-dependent inhibition growth. Confluent cultures of ts2 at 33°C were trypsinized and seeded at high cell density (equivalent to half the cell number at confluence) in complete medium as described in Materials and Methods. After 4 days of subsequent incubation of these cultures at 33°C, FMF analysis shows the cells to be arrested in the G1 phase of the cell cycle (figure 4, panel B). Figure 4A, as described earlier, shows a profile of actively growing cells. The profile in figure 4B indicates that there is little or no cell population undergoing DNA synthesis and

Figure 4. Flow-cytofluorographic (FMF) analysis of growth-arrested ts2 cells at 33°C. Cultures of ts2 cells were seeded at high cell density, incubated for 96 hours at 33°C than prepared for FMF analysis and stained with propidium iodide as described in Materials and Methods. (A) is a profile of actively growing ts2 cells at 33°C. (E) is a profile of growth-arrested ts2 cells at 33°C.

FIGURE 4.

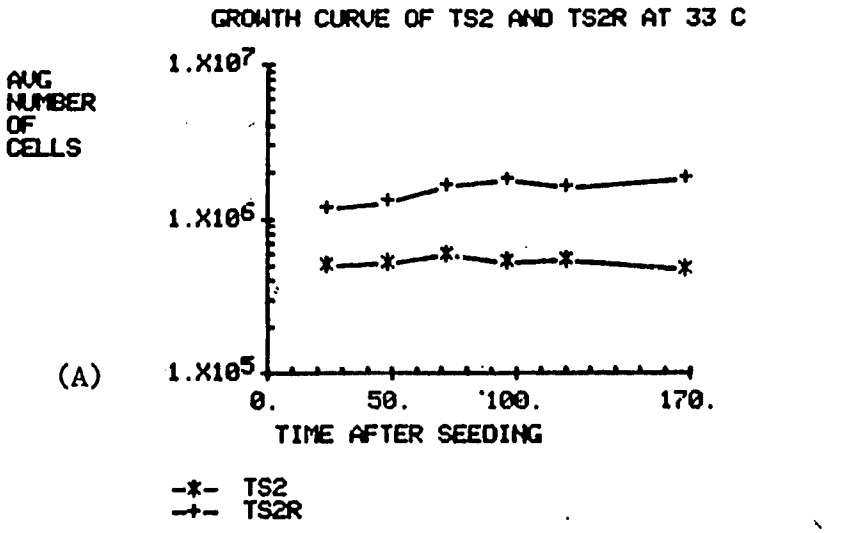


mitosis. Integration of the area under the G1 peak in figure 4B indicates that greater than 95% of the cells have a G1 complement of DNA. ^{3}H-TdR incorporation into ts2 DNA assayed by a one hour pulse label of cultures each day over a seven day time course (figure 5A) indicates that the overall rate of DNA synthesis in the culture is at a basal level by 96 hours after the seeding of the cells. Furthermore, the cell number in the cultures is no longer increasing by that time (figure 5B). Using these criterion, we defined ts2 cells as growth-arrested at 96 hours after seeding the cultures.

Synchronized cultures of ts2 were stimulated into their growth cycle by removing the "spent" medium and rinsing the cultures twice with DME (without added serum). Fresh medium was added and these cultures were then re-incubated at 33°C or shifted immediately to 39°C. Triplicate cultures were harvested for assay of TK and TMPK activity (as described in Materials and Methods) at four hour intervals and compared to controls in which the medium had not been changed. The time course for these experiments included 0-28 hours after the addition of fresh serum. Enzymatic activity was determined in crude extracts (see Materials and Methods) and compared after correction for protein content (Lowry et al., 1951). As expected, ts2 shows an increase in total cellular protein content during such a time course experiment at both 33°C and 39°C since ts2 is not temperature-sensitive in protein synthesis (figure 6).

DNA synthesis (as measured by ^{3}H-TdR incorporation) is induced at 33°C after a lag period of 16 hours. The rate of

Figure 5. Cell number and the rate of ^{3}H-TdR incorporation into acid insoluble material into ts2 seeded at high cell density and incubated at 33°C for 7 days. Confluent 100 mm petri dish cultures of ts2 and ts2R cells were trypsinized, as described in the Materials and Methods. The cells were seeded at a 1:6 dilution of each 100 mm culture into six 35 mm tissue culture plates containing 2 ml of DME (with 10% calf serum). Approximately 5 x 10^{5} ts2 cells were seeded per 35 mm tissue culture plate, and 1 x 10^{6} ts2R. The cultures were incubated at 33°C throughout the time course. (A) is the average number of cells per culture, determined in triplicate cultures as described in the Materials and Methods. (B) represents the average cpm ^{3}H-TdR incorporated into acid-insoluble material after a one hour pulse with 1 uCi ^{3}H-TdR (20 Ci/mmole, NEN) per ml of medium determined in triplicate cultures.


GROWTH CURVE OF TS2 AND TS2R AT 33 C
AVG NUMBER OF CELLS
1.X10^7
1.X10^6
1.X10^5
(A)
0.
50.
100.
170.
TIME AFTER SEEDING
-*- TS2
-+- TS2R

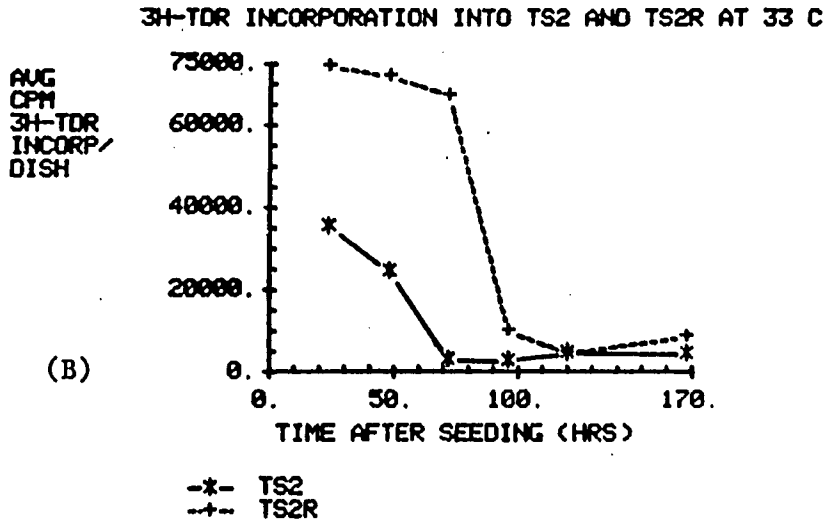

3H-TDR INCORPORATION INTO TS2 AND TS2R AT 33 C
AVG CPM 3H-TDR INCORP/ DISH
75000.
60000.
40000.
20000.
0.
(B)
0.
50.
100.
170.
TIME AFTER SEEDING (HRS)
-*- TS2
-+- TS2R

FIGURE 5.

Figure 6. Protein concentration in crude extracts of serum stimulated ts2 cells incubated at 33°C or 39°C. The experiment was performed as described in figure 7 and the text. Triplicate cultures at each time point were harvested and extracts prepared as described in the text. The concentration of soluble protein in the extracts was determined by the method of Lowry et al. (1951).

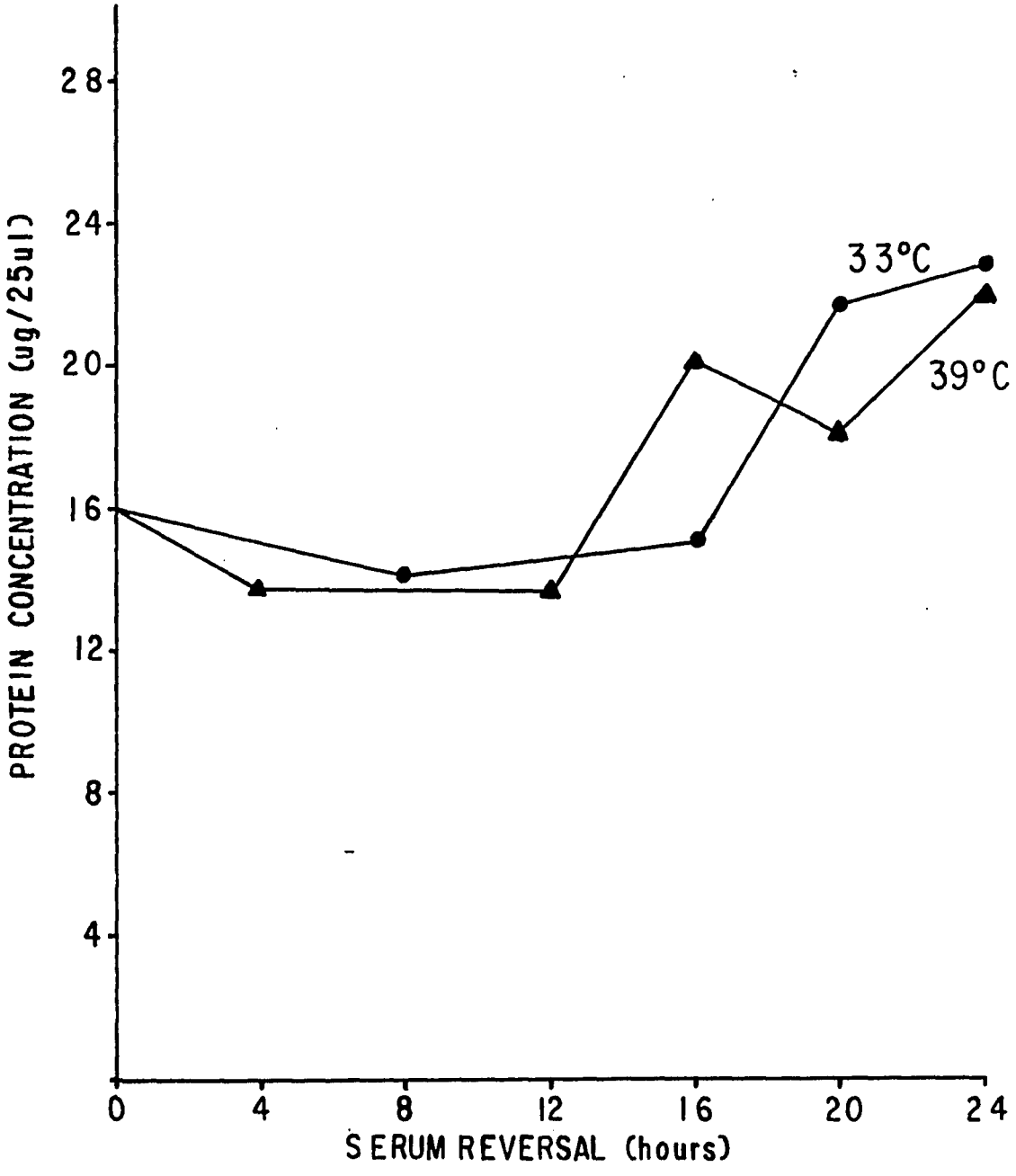
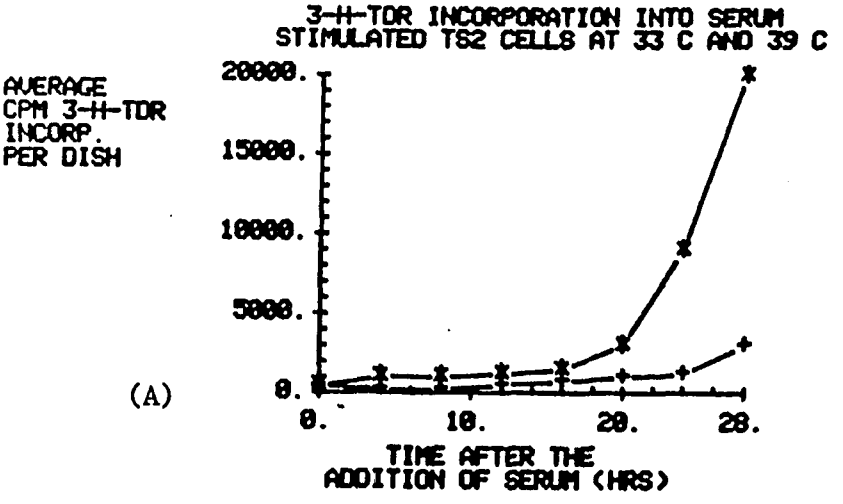

28
24
20
16
12
8
4
PROTEIN CONCENTRATION (ug/25ul)
33°C
39°C
0
4
8
12
16
20
24
SERUM REVERSAL (hours)

FIGURE 6.

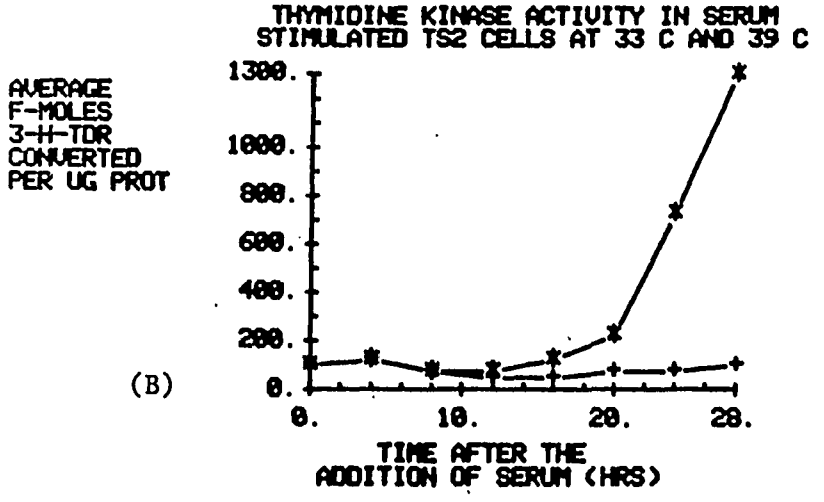
^{3}H-TdR incorporation is about 80 fold higher at 24-28 hours after the addition of serum when compared to that observed at the beginning of the time course (figure 7A). Fifty to 60% of the cells in each culture participated in the induction of DNA synthesis, as determined by autoradiography of nuclei labelled with ^{3}H-TdR, see Materials and Methods. TK activity follows a similar induction profile as seen for DNA synthesis at 33°C (figure 7B). Figure 7C shows that TMPK activity is also induced at approximately the same times after serum stimulation. However, at 39°C there is no (or a much reduced) induction of ^{3}H-TdR incorporation (figure 7A) in ts2 at any time after the addition of fresh medium. There is little or no stimulation of TK activity observed during the time course at 39°C (figure 7B). Likewise, there is no induction of TMPK activity at 39°C (figure 7C). Figure 7C also indicates that TMPK activity continues to fall after serum stimulation and immediate incubation at 39°C, suggesting that the TMPK activity is not at its cellular basal level at the beginning of the time course. Other cellular proteins have been shown to require longer periods of time to reach their basal levels (Johnson et al., 1978). However, for the purpose of this time course the TMPK activity is at a low enough level to observe its induction in serum stimulated ts2 at 33°C, and its lack of induction at 39°C.

For comparison with the results obtained with serum stimulated ts2, this type of experiment was repeated using the ts2 revertant, ts2R, to verify that the effects were associated with the genetic defect. Growth-arrested ts2R (figure 5) were stimulated into their

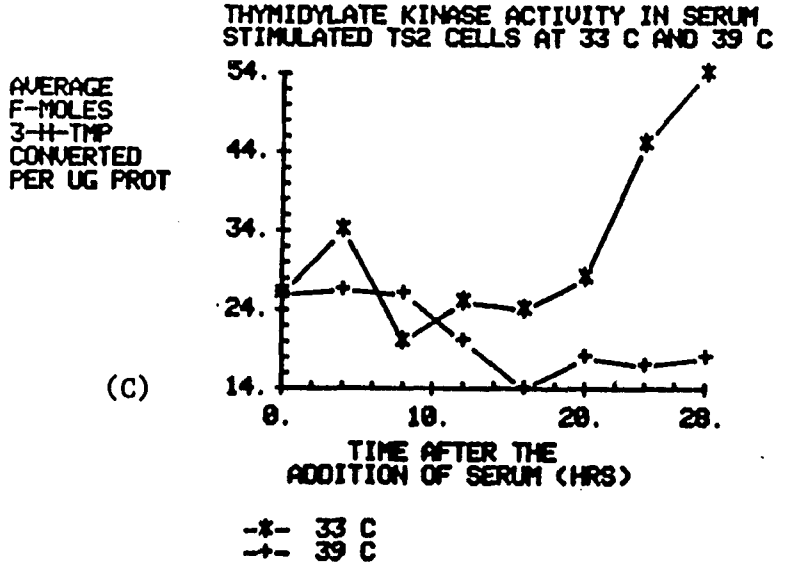
Figure 7. Effect of incubation temperature on quiescent cultures of ts2 after the addition of fresh medium. Quiescent cultures of ts2 were prepared as described in Materials and Methods. These cultures were stimulated into their growth cycle by the addition of fresh medium containing 10% calf serum. The cultures were again incubated at 33°C or immediately shifted to 39°C. At four hour intervals after the medium change the rate of ^{3}H-TdR incorporation into acid-insoluble material was determined in triplicate cultures at each temperature. (A) is the average rate of ^{3}H-TdR incorporation during a one hour pulse with 1 uCi ^{3}H-TdR (20 Ci/mmole, NEN) per ml of medium. Triplicate sister cultures were harvested at these four hour intervals, crude extracts prepared, and assays for enzymatic activity were performed in duplicate, as described in the Materials and Methods. (B) and (C) represent the average TK and TMPK activities, respectively, determined in these extracts. Protein concentration was determined by the method of Lowry et al. (1951).


3-H-TDR INCORPORATION INTO SERUM
STIMULATED TS2 CELLS AT 33 C AND 39 C
AVERAGE
CPM 3-H-TDR
INCORP.
PER DISH
20000.
15000.
10000.
5000.
0.
0.
10.
20.
28.
TIME AFTER THE
ADDITION OF SERUM (HRS)

(A)


THYMIDINE KINASE ACTIVITY IN SERUM
STIMULATED TS2 CELLS AT 33 C AND 39 C
AVERAGE
F-MOLES
3-H-TDR
CONVERTED
PER UG PROT
1200.
1000.
800.
600.
400.
200.
0.
0.
10.
20.
28.
TIME AFTER THE
ADDITION OF SERUM (HRS)

(B)


THYMIDYLATE KINASE ACTIVITY IN SERUM
STIMULATED TS2 CELLS AT 33 C AND 39 C
AVERAGE
F-MOLES
3-H-TMP
CONVERTED
PER UG PROT
54.
44.
34.
24.
14.
0.
10.
20.
28.
TIME AFTER THE
ADDITION OF SERUM (HRS)
-*- 33 C
-+- 39 C

(C)

FIGURE 7.

cell cycle by the addition of fresh medium containing serum, as had been done with ts2. These cultures of ts2R were incubated at 33°C or immediately shifted to 39°C. At 33°C it was observed that TK and TMPK activities and DNA synthesis are induced after an initial lag period of 12-16 hours (see figure 8A-C). Cultures incubated at 39°C show a stimulation in ^{3}H-TdR incorporation along with TK and TMPK activity after a period of 8-12 hours following addition of serum. These data suggest that when the defect which contributes to the observed temperature-sensitivity of DNA synthesis in ts2 was corrected in ts2R, the thermolabile induction of the activities for TK and TMPK was correspondingly rectified. Similarly, the observed thermolability of TK in this subline noted in log phase cultures shifted to 39°C could not be a major factor in the failure to observe TK induction in these experiments.

A31, the parent cell line of ts2, served as a further control for the serum stimulation experiments performed with ts2 and ts2R. Growth-arrested A31 cultures were subjected to the experimental conditions described for the stimulation of ts2 and ts2R. The results from this type of experiment with A31 are shown in figure 9. TK and TMPK activities along with DNA synthesis are induced at 33°C and 39°C, The reversion of the ts mutation in ts2 restores the complete parental phenotype, defined by the ability of the cells to respond to serum at 33°C and 39°C. These data strongly imply that the mutation leading to temperature-sensitive DNA synthesis in ts2 is not independent of the observed failure to induce TK and TMPK activities at the non-permissive temperatures.

Figure 8. Effect of incubation temperature on quiescent cultures of ts2R after the addition of fresh medium. (A) is the average rate of ^{3}H-TdR incorporation into acid-insoluble material during a one hour pulse with 1 uCi ^{3}H-TdR (20 Ci/mmole, NEN) per ml medium. (B) and (C) represent the average TK and TMPK activities, respectively, determined in the prepared crude cellular extracts. The protein concentration was determined by the method of Lowry et al. (1951).

FIGURE 8

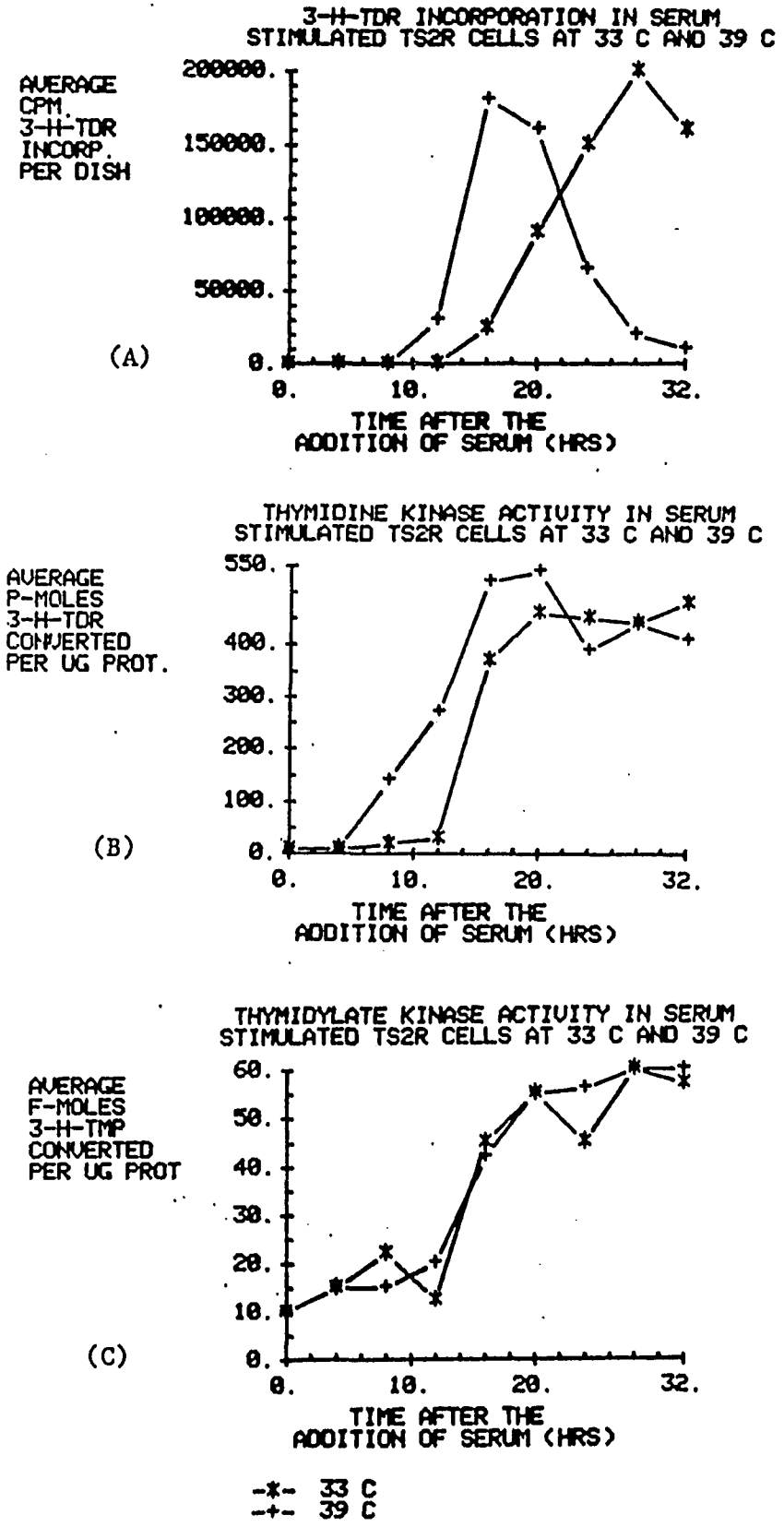
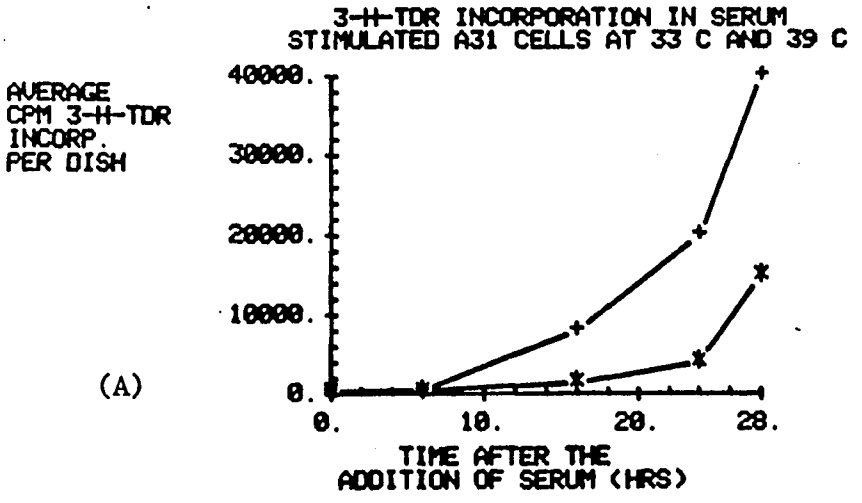
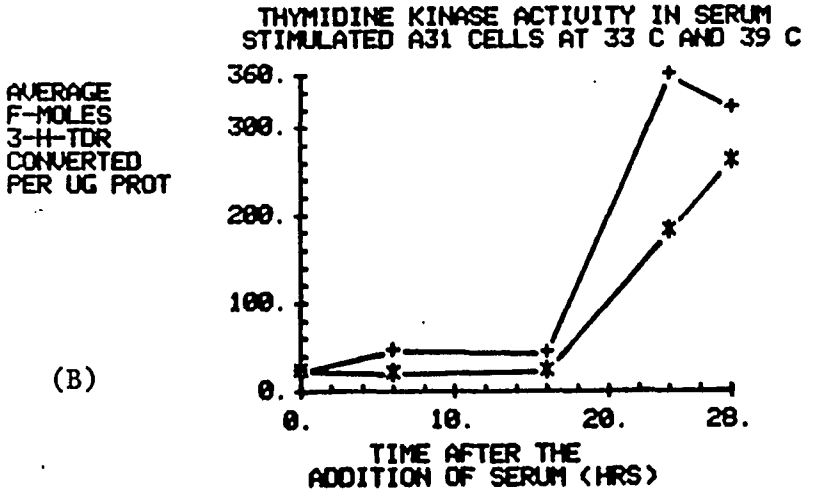

3-H-TDR INCORPORATION IN SERUM
STIMULATED TS2R CELLS AT 33 C AND 39 C
AVERAGE
CPM
3-H-TDR
INCORP.
PER DISH
200000.
150000.
100000.
50000.
0.
(A)
0.
10.
20.
32.
TIME AFTER THE
ADDITION OF SERUM (HRS)
THYMIDINE KINASE ACTIVITY IN SERUM
STIMULATED TS2R CELLS AT 33 C AND 39 C
AVERAGE
P-MOLES
3-H-TDR
CONVERTED
PER UG PROT.
550.
400.
300.
200.
100.
0.
(B)
0.
10.
20.
32.
TIME AFTER THE
ADDITION OF SERUM (HRS)
THYMIDYLATE KINASE ACTIVITY IN SERUM
STIMULATED TS2R CELLS AT 33 C AND 39 C
AVERAGE
F-MOLES
3-H-TMP
CONVERTED
PER UG PROT
60.
50.
40.
30.
20.
10.
0.
(C)
0.
10.
20.
32.
TIME AFTER THE
ADDITION OF SERUM (HRS)
-*- 33 C
-+- 39 C

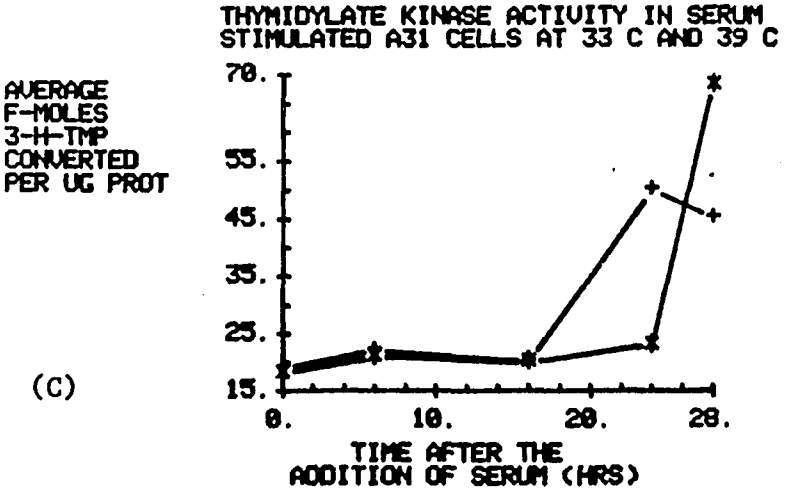
Figure 9. Effect of incubation temperature on quiescent A31 cells after the addition of fresh medium. The experiment was performed as described in figure 7 and the text. (A) is the average rate of ^{3}H-TdR incorporation into acid-insoluble material during a one hour pulse with 1 uCi ^{3}H-TdR (20 Ci/mmole, NEN) per ml medium. (B) and (C) represent the average TK and TMPK activities, respectively, determined in the prepared crude cellular extracts. The protein concentration was determined by the method of Lowry et al. (1951).


3-H-TDR INCORPORATION IN SERUM
STIMULATED A31 CELLS AT 33 C AND 39 C
AVERAGE
CPM 3-H-TDR
INCORP.
PER DISH
40000.
30000.
20000.
10000.
0.
0.
10.
20.
28.
TIME AFTER THE
ADDITION OF SERUM (HRS)

(A)


THYMIDINE KINASE ACTIVITY IN SERUM
STIMULATED A31 CELLS AT 33 C AND 39 C
AVERAGE
F-MOLES
3-H-TDR
CONVERTED
PER UG PROT
360.
300.
200.
100.
0.
0.
10.
20.
28.
TIME AFTER THE
ADDITION OF SERUM (HRS)

(B)


THYMIDYLATE KINASE ACTIVITY IN SERUM
STIMULATED A31 CELLS AT 33 C AND 39 C
AVERAGE
F-MOLES
3-H-TMP
CONVERTED
PER UG PROT
70.
55.
45.
35.
25.
15.
0.
10.
20.
28.
TIME AFTER THE
ADDITION OF SERUM (HRS)
-*- 33 C
-+- 39 C

(C)

FIGURE 9.

These two different S phase activites in ts2, ie., enzyme induction and DNA synthesis, appear to be connected to the same genetic mutation which leads to the observed ts phenotype.

3.4 Studies undertaken to clarify the failure to observe enzyme induction in ts2 cells at 39°C.

Several possibilities were considered in attempting to clarify the failure to observe TK and TMPK induction after the addition of serum in growth-arrested cultures of ts2 cells incubated at 39°C. Among these were that the ts2 TK and TMPK enzymes were perhaps more labile at 39°C when compared with those of the parent or revertant cell lines; that possible stimulatory factors for TK and TMPK activities were present in extracts of ts2 cells, incubated at 33°C and absent in those prepared from cells at 39°C; that the induction of TK and TMPK activities is dependent on DNA synthesis, and the failure to stimulate DNA synthesis at 39°C results in the observed lack of induction of enzyme activity; a possible increased requirement of ts2 cells stimulated with serum and incubated at 39°C for the growth factors present in serum; that growth-arrested ts2 cells stimulated with serum and incubated at 30°C are incapable of progressing from the quiescent state, therefore, leading to observed lack of S phase enzyme activites. Each of these proposed explanations were investigated and will be discussed in turn.

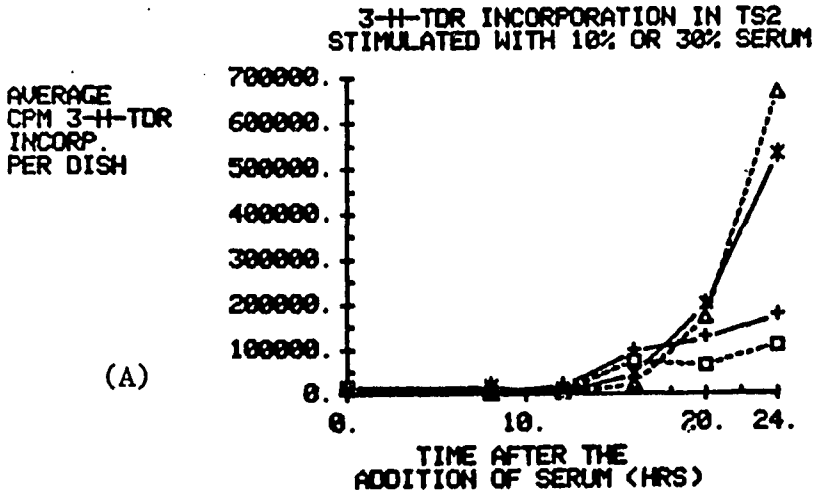
To determine whether growth-arrested cultures of ts2 require a higher serum level at 39°C to permit progression through the

early portions of the cell cycle, synchronized ts2 was stimulated with either 10 or 30 % calf serum. These cells were incubated at 33°C or shifted to 39°C. Extracts were prepared at different times after the addition of serum and assayed for TK activity or their rate of ^{3}H-TdR incorporation. Figure 10 shows that varying the amount of serum used in the stimulation of arrested ts2 from 10 to 30 % had no significant effect on the overall experimental results. There is still the failure to observe the stimulation of DNA synthesis or enzyme activity in cultures incubated at 39°C. It was concluded from these data that ts2 does not have a higher serum requirement for the progression of growth-arrested cells into their cell cycle.

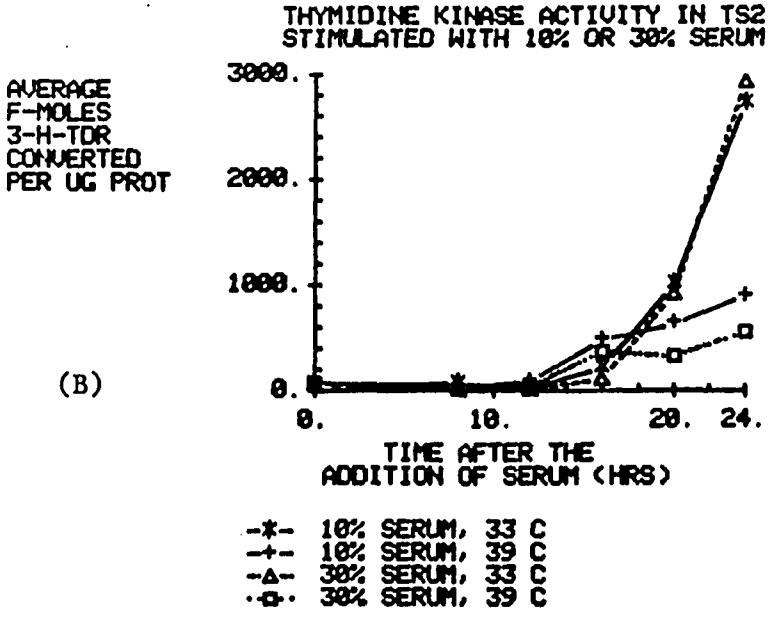
The failure to induce TK and TMPK activites in growth-arrested ts2 cells after the addition of serum at 39°C could result from the enzymes themselves, if these activites were readily inactivated at 39°C. To test this possibility, the effect of temperature on the *in vitro* activity of TK and TMPK was examined in crude extracts of ts2. Extracts were prepared from actively growing ts2 incubated at 33°C. These extracts were then incubated for one hour at the following temperatures: 33°C, 35°C, 37°C, or 39°C. They were then assayed for TK and TMPK activites at 37°C; the results are shown in table 1. These data indicate that the *in vitro* TK and TMPK activites in ts2 are somewhat heat labile.

We sought to clarify whether this observed *in vitro* thermolability of the enzymes was related to the genetic defect in ts2. Two cell lines were chosen for comparison, ts2R, the ts2 revertant, and the ts2 parental cell line A31. It has already been

Figure 10. Effect of incubation temperature on quiescent ts2 cells after the addition of fresh medium containing 10% or 30% calf serum. Cultures of quiescent ts2 were prepared as described in the Materials and Methods. These cultures were stimulated into their growth cycle by the addition of fresh medium containing either 10% or 30% calf serum. The cultures were again incubated at 33°C or shifted immediately to 39°C. At various time intervals after the medium change the rate of ^{3}H-TdR incorporation into acid-insoluble material was determined in duplicate cultures at each temperature. (A) is the average rate of ^{3}H-TdR incorporation during a one hour pulse with 1 uCi ^{3}H-TdR (20 Ci/mmole, NEN) per ml medium. Duplicate sister cultures were harvested at these time intervals, crude extracts prepared, and assayed for TK activity. (B) represents the average TK activity determined in these extracts. The protein concentration was determined by the method of Lowry et al. (1951).


3-H-TDR INCORPORATION IN TS2
STIMULATED WITH 10% OR 30% SERUM
AVERAGE
CPM 3-H-TDR
INCORP.
PER DISH
700000.
600000.
500000.
400000.
300000.
200000.
100000.
0.
0.
10.
20.
24.
TIME AFTER THE
ADDITION OF SERUM (HRS)

(A)


THYMIDINE KINASE ACTIVITY IN TS2
STIMULATED WITH 10% OR 30% SERUM
AVERAGE
F-MOLES
3-H-TDR
CONVERTED
PER UG PROT
3000.
2000.
1000.
0.
0.
10.
20.
24.
TIME AFTER THE
ADDITION OF SERUM (HRS)
-*- 10% SERUM, 33 C
-+- 10% SERUM, 39 C
-Δ- 30% SERUM, 33 C
·□· 30% SERUM, 39 C

(B)

FIGURE 10.

Table 1. Effect of Temperature on TK and TMPK Activities in Crude Extracts Prepared From Ts2 Cells.

extract incubation[b] temperature	% TK activity[c]	% TMPK activity[c]
33°C	100	100
35°C	97.6	93.1
37°C	95.2	86.9
39°C	91.4	85.2

a) Ts2, actively growing at 33°C, were harvested and a crude extract prepared as described in the Materials and Methods.

b) Aliquots of the extracts were incubated for one hour at the above described temperatures. The aliquots were then assayed at 37°C in duplicate for TK and TMPK activities as described in Materials and Methods.

c)Enzyme activity was calculated as femtomoles of substrate converted in 20 minutes at 37°C per ug protein. The activites determined for the aliquot incubated for one hour at 33°C were used to represent 100% activity.

shown that ts2R and A31 are capable of a full response to serum stimulation at 39°C, ie., the induction of DNA synthesis and TK and TMPK activites. If the *in vitro* thermolability of TK and TMPK in ts2 contributes to the failure to induce these enzyme activites at 39°C in growth-arrested cells, then we may expect to observe that temperature has little or no effect on the *in vitro* enzymatic activity of ts2R and A31.

Cell extracts were prepared from actively growing cultures of ts2R and A31 incubated at 33°C. These extracts were incubated at 33°C, 35°C, 37°C, 39°C for one hour, they then were assayed for TK and TMPK enzymatic activities. The results are presented in table 2. Both the TK and TMPK enzymes show heat sensitivity in extracts prepared from ts2R and A31. Ts2R shows a degree of sensitivity for both TK and TMPK activities greater than that observed in ts2. The heat lability of TK and TMPK *in vitro* appears to be intrinsic to the cell line, Balb/3T3, and is not in itself an explanation for the absence of their induction at 39°C in ts2 after the addition of serum. These results also serve to confirm the apparent heat lability of TK *in vivo* in ts2R (and ts2) (see figures 2 and 3).

The absence of possible stimulatory factors for the TK and TMPK enzymes in cell extracts prepared from ts2 incubated at 39°C was examined. Both the TK and TMPK activities may be induced and the polypeptides capable of function in ts2 at 39°C, but the absence of these factors may prevent readily assaying their activities. Conversely, an inhibitory substance may accumulate in extracts of 39°C.

Table 2. Effect of Temperature on TK and TMPK Activities in Crude Extracts Prepared from Ts2R and A31 Cells.[a]

	Ts2R		A31	
extract incubation temperature[b]	% TK activity[c]	% TMPK activity[c]	% TK activity[c]	% TMPK activity[c]
33°	100	100	100	100
35°C	69.1	100.6	91.6	90.4
37°C	61.3	82.9	87.0	81.8
39°C	58.3	69.0	76.7	80.9

a) Actively growing ts2R and A31, growing at 33°C, were harvested and crude extracts prepared as described in the Materials and Methods.

b) Aliquots of each extract were incubated for one hour at the above described temperatures. The aliquots were then assayed at 37°C in duplicate for TK and TMPK activities as described in the Materials and Methods.

c) Enzyme activity was calculated as fentomoles of substrate converted in 20 minutes at 37°C per ug protein. The activities determined for the aliquots incubated for one hour at 33°C were used to represent 100% activity.

An experiment was performed to determine if these proposed stimulatory or inhibitory factors were present in crude extracts prepared from cultures of ts2 which were serum stimulated and then incubated at 33°C for 24 hours. Extracts from cultures incubated at 33°C were mixed with extracts prepared from ts2 incubated at 39°C for 24 hours after serum stimulation. This mixture, as well as each of the extracts alone, were assayed for TK activity. We would expect a significant enhancement of the enzyme activity in the extracts from ts2 incubated at 39°C when mixed with extract from cells incubated at 33°C, if stimulatory factors were indeed present. The results of such an assay indicate the TK activity in the extract mixture is an average of the two individual enzyme activities (data not shown). These data suggest that extract prepared from ts2 incubated at 33°C do not contain readily extractable stimulatory factors which could activate a dormant TK enzyme, if it were present in extracts of ts2 at 39°C. The data similarly do not suggest the presence of inhibitory substances in the extract under the assay conditions used. We did not choose to explore a variety of experimental conditions after this initial result.

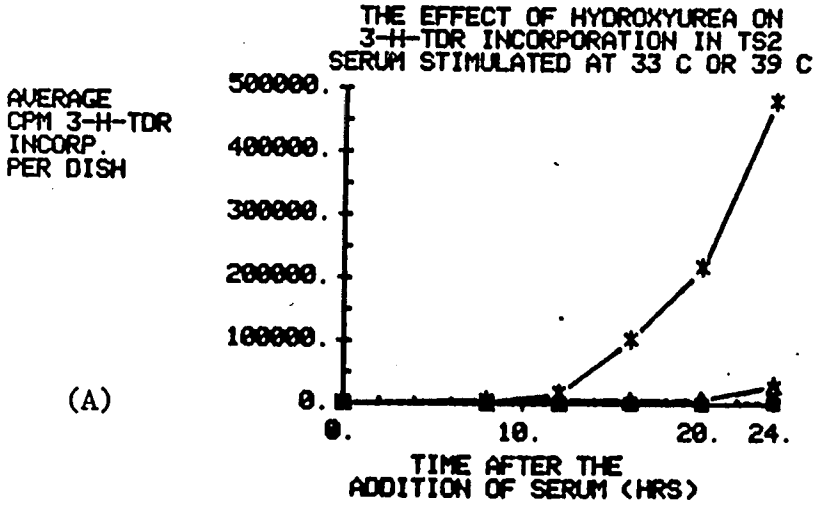
TK activity and DNA synthesis had been thought to be intimately associated (Kit et al., 1968). The serum stimulation experiments with ts2 suggested DNA synthesis may be needed in order to observe an increase in TK activity as well as TMPK activity. To examine this possibility, the serum stimulation experiment with ts2 was repeated except that 1mM hydroxyurea (HU) was included at the time of the addition of fresh medium. This drug allows cellular

progression into the S phase of the cell cycle, but prevents bulk DNA synthesis (Walters et al., 1973, Walters et al., 1976). If DNA synthesis were required for enzyme induction, then at 33°C there would be a lack of stimulation of TK activity. The results of this experiment, shown in figure 11, indicate that TK activity was induced at 33°C without the corresponding induction of DNA synthesis. This observation demonstrates that the stimulation of cellular DNA synthesis is not a strict requirement for the induction of TK activity. Therefore, the absence of an observable TK activity induction in ts2 at 39°C is in an unspecified way linked to the ts phenotpye, but is not, in itself, the cause of the absence of enzyme activity.

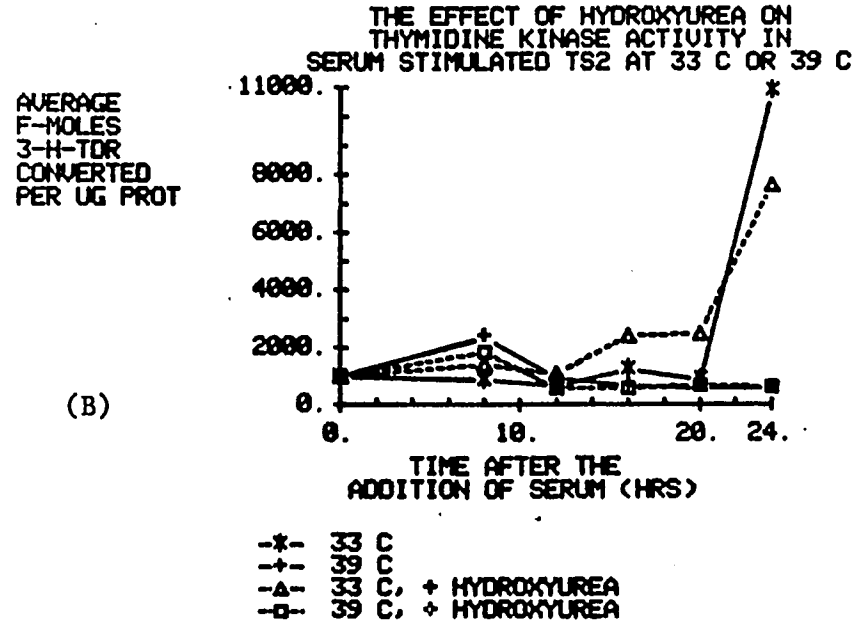
Another possibility considered for the lack of stimulation of TK and TMPK activities at 39°C could be that ts2 never progresses from its arrested state at the non-permissive temperature. Perhaps ts2, although containing a ts defect in DNA synthesis, may also harbor a mutation which prohibits the entry of cells from the quiescent (Go) state into the G1 phase of the cell cycle at 39°C. This particular mutation would not manifest itself in actively growing cultures shifted to 39°C, since the cells would never pass through Go phase. Experiments were performed to determine whether ts2 progressed into the G1 phase from its growth-arrested state.

It has been shown that either whole serum (Thomas et al., 1981) or PDGF (Pledger et al., 1981) rapidly stimulates quiescent Balb/3T3 cells to preferentially synthesize certain proteins. Such protein synthesis may be required for the PDGF-modulated competence

Figure 11. Effect of hydroxyurea on quiescent ts2 cells after the addition of fresh medium containing 10% calf serum and subsequent incubation at 33°C. Quiescent cultures of ts2 were prepared as described in Materials and Methods. Fresh DME (with or without 1mM hydroxyurea) containing 10% calf serum was added to the cultures. The cultures were again incubated at 33°C. At various time intervals after the medium change the rate of ^{3}H-TdR incorporation into acid-insoluble material was determined in duplicate cultures. (A) is the average rate of ^{3}H-TdR incorporation during a one hour pulse with 1 uCi ^{3}H-TdR (20 Ci/mmole, NEN) per ml of medium. Duplicate sister cultures were harvested at these time points, crude extracts prepared, and assayed for TK activity in duplicate. (B) represents the average TK activity determined in these extracts. The protein concentration was determined by the method of Lowry et al. (1951).


THE EFFECT OF HYDROXYUREA ON
3-H-TDR INCORPORATION IN TS2
SERUM STIMULATED AT 33 C OR 39 C
AVERAGE
CPM 3-H-TDR
INCORP.
PER DISH
500000.
400000.
300000.
200000.
100000.
0.
0.
10.
20.
24.
TIME AFTER THE
ADDITION OF SERUM (HRS)

(A)


THE EFFECT OF HYDROXYUREA ON
THYMIDINE KINASE ACTIVITY IN
SERUM STIMULATED TS2 AT 33 C OR 39 C
AVERAGE
F-MOLES
3-H-TDR
CONVERTED
PER UG PROT
11000.
8000.
6000.
4000.
2000.
0.
0.
10.
20.
24.
TIME AFTER THE
ADDITION OF SERUM (HRS)
-*- 33 C
-+- 39 C
-Δ- 33 C, + HYDROXYUREA
-□- 39 C, + HYDROXYUREA

(B)

FIGURE 11.

because plasma does not stimulate the synthesis of these proteins (Pledger et al., 1981). Both the synthesis of these proteins and the acquisition of competence is blocked by inhibitors of RNA synthesis (Pledger et al., 1981, Smith and Stiles, 1981). A group of these cell proteins (pII, 35Kd) have been studied in detail because their synthesis dramatically increases in response to PDGF (Pledger et al., 1981, Scher et al., 1983). These proteins also share antigenic determinants with proteins (major excreted proteins, MEP) secreted by retro-virus transformed NIH or Balb 3T3 cells (Gottesman, 1978). It was found that the addition of PDGF to growth-arrested cultures of Balb/3T3 stimulated increased synthesis of pII (MEP) proteins within 40 minutes after the addition (Scher et al., 1983). The amount synthesized continued to increase until seven hours after the PDGF addition. From seven through 24 hours after PDGF addition, the amount of pII (MEP) synthesized was approximately 9-fold greater than at time zero.

Since increased synthesis of pII (MEP) proteins is noted rapidly after the addition of PDGF, these proteins could perhaps serve as a marker for the transition of cells from the quiescent (Go) state to the growing phase of the cell cycle (Pledger et al., 1982). To assess whether these pII (MEP) proteins would be a convenient marker for the progression of arrested ts2 into the cell cycle, it was first necessary to determine if these proteins reached a level of synthesis low enough to readily allow the observation of their stimulation after the addition of serum. The levels of pII (MEP) synthesis were evaluated as ts2 cells entered quiescence.

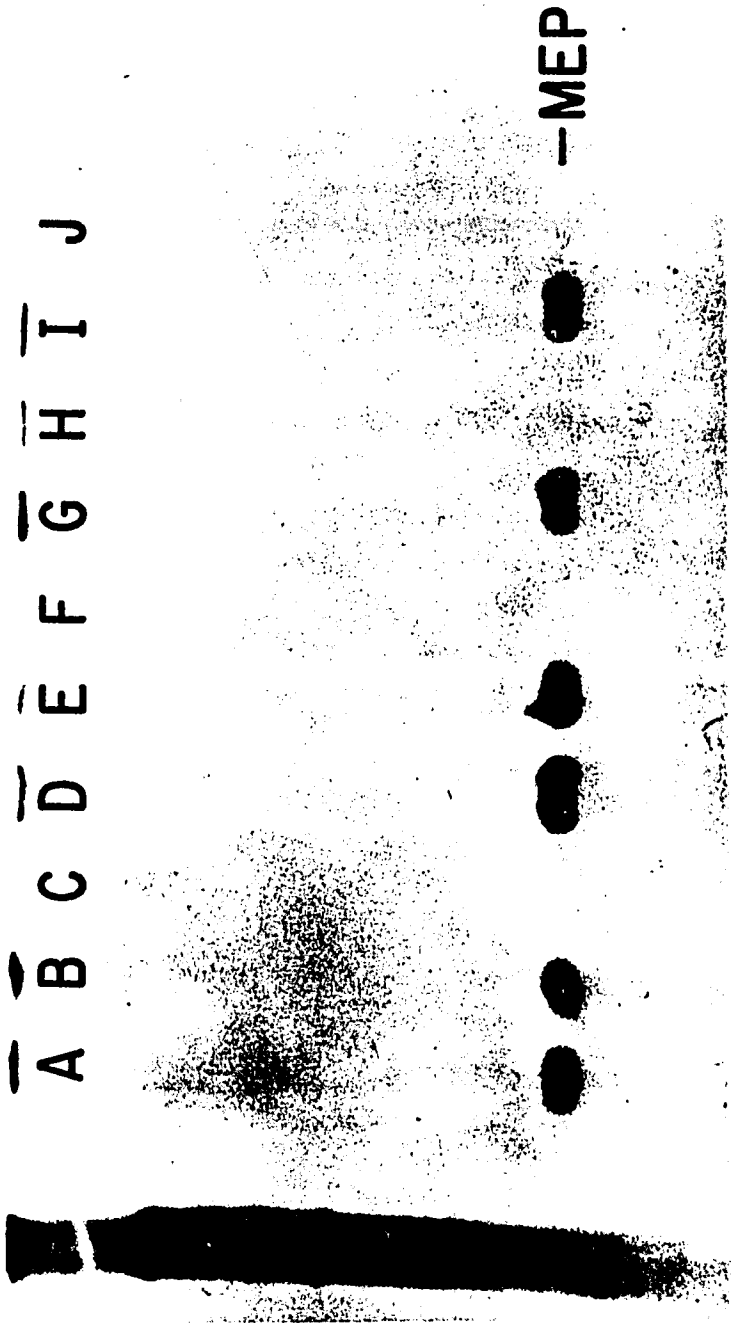
Confluent cultures of ts2 were trypsinized and seeded at a 2-fold dilution into 100 mm tissue culture dishes containing 10 ml of medium (DME containing 10% calf serum). These cultures were than incubated at 33°C. On the second through the sixth days after seeding, two of these cultures were rinsed twice with methionine-free DME. One and a half milliliters of methionine-free DME containing 0.5% calf serum were added to each culture. The cultures were incubated at 33°C for 30 minutes and then pulse-labelled for 20 minutes with 50 uCi/ml ^{35}S-methionine at 33°C. After this labelling period, the cultures were rinsed twice with DME (containing methionine), and twice with cold PBS. The cells were scraped into cold PBS and pelleted by centrifugation at 1000 rpm for 5 minutes. Cell extracts were prepared for immunoprecipitation as described in Materials and Methods.

Aliquots of the cell extracts containing equivalent TCA precipitable radioactivity ($5x10^5$ cpm) were immunoprecipitated with rabbit antiserum to pII (provided by M. Gottesman) and Staph aureus A. The bound radioactivity was eluted and analysed by PAGE and fluorography. The molecular weight of newly synthesized proteins was estimated from the position of ^{35}S-labelled human adenovirus infected Hela extract proteins used as a marker (kindly provided by R. Radna of this laboratory).

The fluorogram of such an experiment with ts2 is shown in figure 12. The rate of pII (MEP) protein synthesis does not decrease as ts2 approaches and enters quiescence. Even at 6 days after seeding a strong signal is observed. Cytofluorometric analysis and the rate of ^{3}H-TdR incorporation both indicate that

Figure 12. Level of pII (MEP) proteins as ts2 cells approach and enter quiescence at 33°C. Confluent 100 mm culture dishes containing ts2 were trypsinized as described in the Materials and Methods. The cells were seeded at a 1:3 dilution of each 100 mm culture into three 60 mm tissue culture plates at approximately 1 x 10^6 cells per plate. These cultures were incubated at 33°C throughout the time course. Duplicate cultures were processed at 2 days (A, B, C), 3 days (D), 4 days (E, F), 5 days (G, H), or 6 days (I, J) post-seeding. (A), (D), (E), (G), and (I) were immuno-precipitated with 1 ul of antibody solution, (B) was immuno-precipitated with 3 ul of antibody solution. (C), (F), (H), and (J) were incubated without antibody then treated with Staph aureus A as described in the Materials and Methods.

FIGURE 12.



ts2 arrested in their growth by 96 hours after seeding (see figures 3 and 4).

We questioned whether the 0.5% calf serum present in the medium used to pulse-label the cells was sufficient to stimulate pII (MEP) synthesis. The experiment was ,therefore, repeated using 0.5% platelet poor plasma (PPP) in the labeling medium instead of calf serum. There was no decline in pII (MEP) synthesis noted during a time course spanning seven days after seeding (data not shown).

These data suggest that the pII (MEP) proteins are synthesized constitutively in ts2. The function of pII (MEP) is not known, but appears to be related to PDGF action (Scher et al., 1983). Agents which behave like PDGF, in stimulating cellular competence, also stimulate pII (MEP) synthesis. If the pII (MEP) proteins are markers for the progression of cells from the quiescent (Go) state, then we could possibly conclude that ts2 arrests in the Go phase, but not to the same extent as that observed for other Balb/3T3 cell lines (Scher et al., 1983). Alternately, ts2 may not arrest in the Go state as it has been defined but within early G1. Since the pII (MEP) proteins do not serve as markers for the progression of ts2 from the resting phase, another approach was employed.

Various mRNAs have been shown to be differentially transcribed in resting verses growing cells (Wu and Johnson, 1982, Kelly et al., 1983, LaBella et al., 1983, Santiago et al., 1984, Campisi et al., 1984). Kelly et al. (1983) presented data indicating that the cellular gene that encodes the proto-oncogene, c-myc, is an inducible gene regulated by specific growth signals in a cell cycle dependent manner. Agents which initiate cellular competence

(Pledger et al., 1977) in lymphocytes (lipopolysaccharide or Concanavalin A) and fibroblasts (PDGF) induce c-myc mRNA. It was noted that c-myc mRNA levels increased 10- to 40-fold, within one to three hours after the addition of these specific mitogens to the appropriate cells. Similar results were obtained by Campisi et al. (1984). These authors observed that growth stimulation of quiescent cells by serum elevates the expression of the myc proto-oncogene in Balb/3T3 cells.

These results demonstrate that the expression of the c-myc gene is dependent upon the cellular growth state, and that growth control exhibits growth-factor dependent, cell cycle timed c-myc expression. We therefore chose its mRNA transcription as a possible indicator for the transit of ts2 from the Go phase into the cell cycle. Experiments were performed to examine whether serum growth factors activated the proto-oncogene, c-myc, in quiescent ts2 at 33°C and 39°C. Ts2 cells were growth-arrested as defined earlier, (figure 5). These cultures were stimulated by first washing the cultures twice, followed by the addition of fresh DME containing 10% calf serum. The cells were incubated at 33°C or 39°C. At 0, 4, 8, 16, and 28 hours after the addition of serum, cultures were harvested and the total cellular RNA was extracted (Materials and Methods). Equal amounts of total cellular RNA were analyzed for c-myc mRNA levels by Northern transfer and hybridization to ^{32}P nick translated c-myc specific sequences, see figure 13.

The level of c-myc mRNA was estimated by densitometry to be 4.3 fold higher after 4 hours at 33°C, and 2.3-fold higher after 4

Figure 13. Demonstration of c-myc expression in ts2 cells at 33°C and 39°C after the addition of fresh medium containing 10% calf serum. Quiescent cultures of ts2 were prepared as described in the Materials and Methods. The cultures were stimulated by a change to fresh medium combining 10% calf serum. These cultures were than incubated at 33°C or shifted immediately to 39°C. At each of the following time intervals after the addition of fresh medium, 0, 4, 8, 16, and 28 hours at 33°C, and 4, 8, 16, and 28 hours at 39°C, twenty 100 mm petri dish cultures were harvested and the total RNA extracted as described in the Materials and Methods. The RNA was fractionated on a 1% agarose gel as described in the text. 40 ug of total RNA was loaded per lane. The RNA was transferred to nitrocellulose by standard procedures. The RNA was hybridized to a ^{32}P-labelled nick-translated c-myc DNA as described in the Materials and Methods.

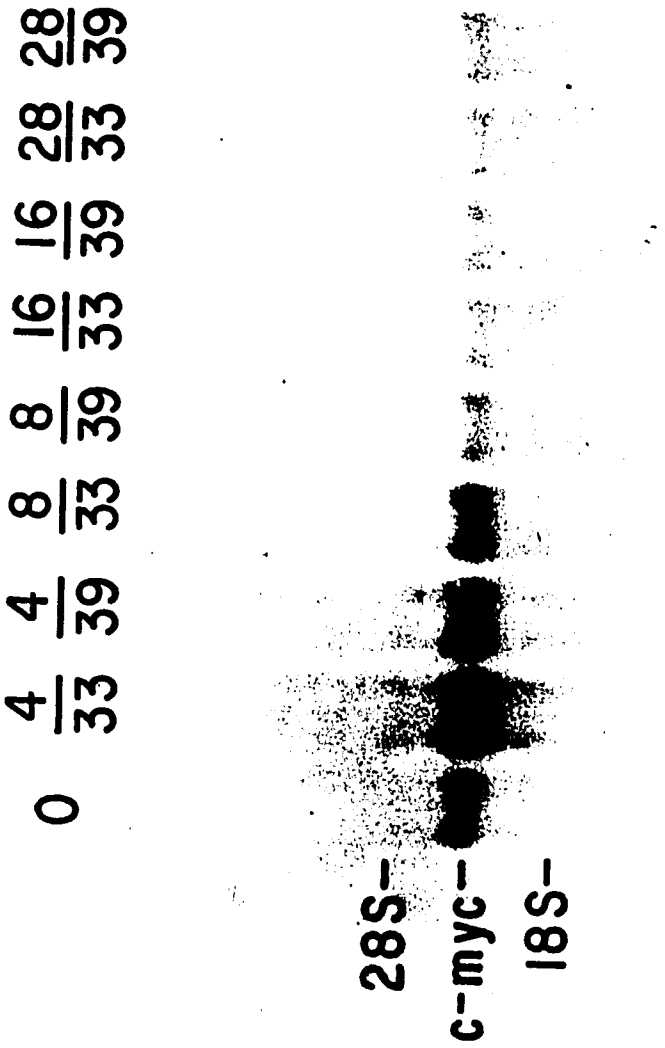


FIGURE 13

hours at 39°C when compared to zero hour. Densitometry on autoradiograms was performed on a Scanner Model Joyce Automatic Recording Microdensitometer (Model MK 111C Joyce, Loebl, and Co.). Although the levels of c-myc mRNA observed upon serum stimulation of ts2 are lower then those described by others for the Balb/3T3 cell line, we may possibly account for this by one or some combination of the following possibilities. First, the number of cells in the ts2 cultures responding to the serum stimuli is lower (approximately 50-60%) than that in the reported Balb/3T3 cell experiments (75-95%) (Kelley et al., 1983). Secondly, the highest levels of c-myc were noted within one to three hours after the addition of PDGF (Kelley et al., 1983) in Balb/3T3 cells; our first time point is four hours after the addition of serum. Therefore, we may be observing the downside of the c-myc expression after stimulation. Also, if this were true, the lower 39°C level of c-myc mRNA when compared to that observed at 33°C could be explained, ie., the rate of c-myc mRNA degradation occurs at a higher rate at 39°C as opposed to that at 33°C. Thirdly, the observed difference in the level of c-myc expression in ts2 when compared to the Balb/3T3 cell line could also be due to a cell subline variation.

These results obtained on c-myc expression after serum stimulation of ts2 suggest that the proto-oncogene is induced in ts2 after the addition of serum, whether the cells are incubated at 33°C or 39°C. This indicates that growth-arrested ts2 cells stimulated at 39°C can progress from the quiescent state. Therefore, ts2 is not additionally defective in the Go phase.

Further evidence for the ability of quiescent ts2 to progress into the G1 portion of the cell cycle was obtained by examining another mRNA which shows a cell cycle timed, growth-factor dependence. This mRNA, named proliferin, was described by Linzer and Nathans (1983). The authors constructed a c-DNA plasmid library from the poly(A)$^+$ RNA present in Balb/3T3 cells after serum stimulation. Thirty five hundred clones were screened, and an estimated 0.5% of the clones contained inserts related to mRNAs occurring in higher levels in serum stimulated Balb/3T3 cells when compared to quiescent cultures. One clone hybridized to a 1000 bp mRNA, present at minimal levels in resting cells, which increases 15 to 20-fold after serum stimulation. The maximal level for this mRNA is coincident with the onset of DNA synthesis. Although the function of the proliferin protein is unknown, it has been determined that it contains prolactin-like sequences (Nathans, personal communication). The inducibility of the proliferin (PLF) mRNA was examined in growth-arrested ts2.

The same Northern transfer of the total cellular RNAs extracted from ts2 and hybridized to c-myc specific sequences (figure 13) was used. The Northern blot was re-hybridized to nick-translated plasmid DNA containing PLF sequences (Materials and Methods). The PLF mRNA is induced in ts2 after 8 hours at both 33^{o}C and 39^{o}C (figure 14). This induction represents an approximate 20-fold higher level in the PLF mRNA when compared to that observed at zero hour.

From these data and those obtained concerning c-myc expression in ts2, we may conclude that growth-arrested ts2 can readily

Figure 14. Proliferin mRNA expression in serum stimulated ts2 cells incubated at 33°C or 39°C. Northern analysis of total RNA extracted from serum stimulate ts2 cells. ^{32}P-labelled nick-translated proliferin (PLF) DNA was hybridized to a blot previously hybridized to a c-myc probe (figure 13).

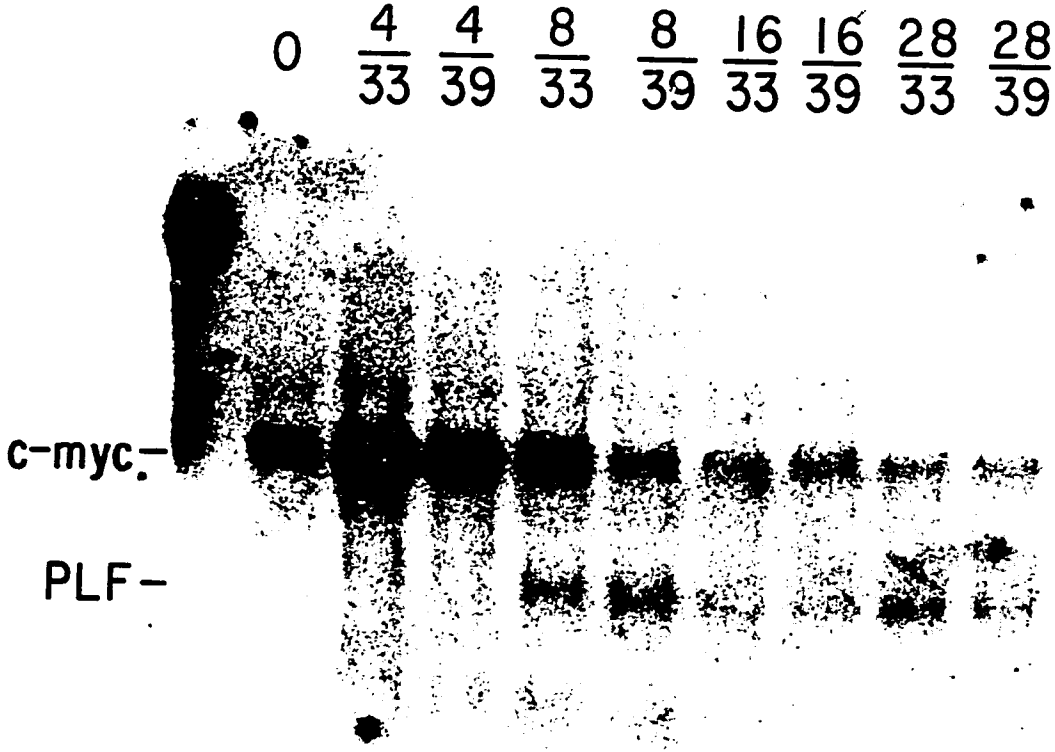


FIGURE 14

transit from its resting phase into the G1 phase at 39°C after the addition of serum.

Summary:

Additional characterization of ts2 by cytofluorographic analysis reveals that ts2, after incubation at 39°C, accumulates in the S and G2 (late S) phases of the cell cycle. This result adds further support to prior evidence that ts2 is a temperature-sensitive mutant affected in DNA synthesis.

The investigations presented with growth-arrested ts2 reveal a correlation between the stimulation of DNA synthesis and the induction of the enzymes of the thymidine metabolizing pathway at the permissive and non-permissive temperatures for growth. Upon serum stimulation of growth-arrested ts2, there is induction at 33°C of DNA synthesis (as measured by ^{3}H-TdR incorporation) and the enzyme activities for TK and TMPK. Maximal enzyme activity is coincident with maximal ^{3}H-TdR incorporation. At 39°C, there is no (or a much reduced) induction of DNA synthesis and TK and TMPK activities.

The observed failure to stimulate enzyme activity after the addition of serum to growth-arrested ts2 incubated at the non-permissive temperature was not dependent wholly on the absence of DNA synthesis itself. Serum stimulation of ts2 cells in the presence of hydroxyurea at the permissive temperature revealed the induction of TK activity without the stimulation of DNA synthesis.

The *in vitro* acitivity of the TK and TMPK enzymes in ts2 appeared to be somewhat heat labile. However, the thermolability of the enzymes does not account for the *in vivo* experimental results obtained with ts2. The *in vitro* enzymatic activity of TK and TMPK was examined in ts2R and the ts2 parental cell, A31. These enzymatic activities were found to be also heat labile. Ts2R was shown to have an even more heat sensitive *in vitro* activity for TK and TMPK then that observed with ts2. Both A31 and ts2R were determined to be proficient in the stimulation of DNA synthesis and induction of TK and TMPK activities at 39°C. Therefore, the observed *in vitro* temperature-sensitivity of the TK and TMPK enzymes in A31 and ts2R does not interfere with enzymatic induction and stimulation of DNA synthesis at 39°C.

Investigation of mRNA transcription as growth-arrested ts2 cells are serum stimulated and incubated at 33°C and 39°C shows that the cells at 39°C are able to respond to the growth factors present in serum. This response was determined by the induction of two mRNAs, c-myc and PLF, in quiescent ts2 after the addition of serum. Both these mRNAs have been shown to be growth-factor dependent and their induction cell cycle timed (Kelly et al., 1983, Campisi, 1984, Linzer and Natnans, 1983). The expression of these mRNAs in ts2 incubated, after adding serum, at 33°C and 39°C indicates that quiescent ts2 can progress from their resting state into the growing phase of the cell cycle at 39°C. Therefore, the failure to induce TK and TMPK activities at 39°C in growth-arrested ts2 is selective.

Two conclusions from these different observations are, first, the temperature-sensitive decrease in DNA synthesis in ts2 is not caused by the thermolability of its TK activity ,per se, and second, the failure to induce TK and TMPK activities at the non-permissive temperature in quiescent ts2 is attributed to the same genetic defect which results in the observed thermolabile phenotype.

Experiments performed in collaboration with this laboratory by Dr. Earl Baril, suggested that ts2 could contain a thermolabile multi-enzyme complex (MEC) which is involved in DNA synthesis. Previous evidence from Novikoff tumor (a rat liver tumor) reveals there is a readily isolatable subcellular fraction which contains many of the DNA synthetic and nucleotide metabolism enzymes(Baril et al., 1973). Among the different components of this fraction are the following enzymatic activities: TK, TMPK, thymidylate synthetase (TS), ribonucleotide reductase (RR), and DNA polymerase α (DNA pol α). Baril defines this fraction, termed P-4, as a MEC involved in eukaryotic DNA replication. DNA precursors, namely ^{3}H-TdR, can channel through the complex and subsequently be incorporated into an offered DNA template.

The data, produced in collaboration with Dr. Baril, indicating that ts2 may contain a heat sensitive MEC (defined as P-4 fraction) are presented in table 3. S-4 and P-4 are designations given different subcellular fractions which will be described in more detail later in the text. For the present discussion, the key facts are that P-4 represents the fraction in which the various enzymes are associated in the putative complex form, and S-4

Table 3. Assay of subcellular fractions isolated from actively growing ts2 cells grown at 33°C or 39°C for TK, TMPK, NDPK, TS, and RR activities.

cell line	incubation temp. °C	subcellular fraction	ENZYME ACTIVITY[b] TK	TMPK	NDPK	TS	RR
ts2	33	S-4	18.5	21.6	2.7	2.3	1.1
		P-4	11.6	2.4	3.1	8.4	10.7
	39	S-4	8.5	25.4	10.3	15.7	17.2
		P-4	2.0	0.9	0.6	0.9	0.8
A31	33	S-4	0.8	4.3	1.4	3.1	1.7
		P-4	10.0	38.6	5.1	11.4	4.1
	39	S-4	1.8	6.1	2.1	6.2	3.2
		P-4	18.9	45.0	13.0	25.7	16.5

a) Actively growing cultures of ts2 and A31 were incubated at 33°C or shifted to 39°C for 24 hours. the cells were harvested as described in Materials and Methods. The cell pellets were frozen at -70°C and shipped in dry ice. The cell pellets were fractionated as described in fractionation scheme two in the Material and Methods. the isolated subcellular fractions were assayed for the described activities. All assays were performed at 35°C for comparative purposes.

b) One unit of enzyme activity defined as one nanomole of substrate converted per 30 minutes at 35°C.

TK = thymidine kinase
TMPK = thymidylate kinase
NDPK = nucleoside diphosphokinase
TS = thymidylate synthetase
RR = ribonucleotide reductase

describes the fraction in which the enzymes are not in complex form and soluble. A31 cells were analyzed for the association of the various enzymes as a comparison for ts2. Ts2 and A31 cells were incubated at 33°C or 39°C. The cells were fractionated and the S-4 and P-4 fractions were assayed for their respective enzyme activities. It is readily observable that for A31 cells the majority of activity for each of the enzymes fractionates with the P-4 fraction and, therefore, they are in an associated form at both these temperatures. The data from ts2 indicate that at 33°C, a large percentage of each of the different activities, is present in the P4 fraction. At 39°C, however, the majority of each enzymatic activity in ts2 is found in the S-4 fraction. These results intimate that the proposed MEC is partially disrupted at 33°C in ts2 and completely dissociated at the non-permissive temperature.

The existence of a thermolabile MEC in ts2 could account for the observed temperature-sensitive phenotype. The MEC in actively growing ts2 would disrupt after shifting the cells to 39°C leading to the observed decrease in DNA synthesis. Serum stimulation of quiescent ts2 and subsequent incubation at 39°C would not be expected to induce DNA synthesis since the MEC could not aggregate into a functioning unit at the non-permissive temperature. Other mammalian ts mutants in DNA synthesis could conceivably be thermolabile in different components of the putative MEC, thus all leading to dissociated MEC at their non-permissive temperature. Therefore, hybrids produced between ts2 and other ts mutants in DNA synthesis would not be able to complement each other

at the non-permissive temperature, as observed (Jha et al., 1980), although the defect would be recessive to wild-type.

The following studies in this text extrapolate on these initial data concerning a thermolabile MEC in ts2, as isolated from cells at their permissive and non-permissive temperatures. Further experimental data as to the existence of such a putative MEC are also presented later in this text.

CHAPTER FOUR: Examination of the MEC in ts2

4.1 Isolation of a MEC involved in DNA synthesis from HeLa cells.

Initial attempts to isolate the putative multi-enzyme complex (MEC) involved in eukaryotic cellular DNA synthesis were performed with the HeLa cell line. The definition of the multi-enzyme complex we employed in these studies was that used by Baril et al. (1973). The HeLa cells used in the isolation had been stored at -70°C as a cell pellet. The MEC fraction was isolated using subcellular fractionation scheme one diagrammed in Materials and Methods. In brief, the cells were disrupted by homogenization in hypotonic buffer. Nuclei and other subcellular material were removed by differential centrifugation, yielding a post-microsomal supernatant, designated S-3. Prolonged high speed centrifugation of S-3 layered onto a discontinuous sucrose gradient, consisting of 1.3M and 2.0M sucrose, resulted in the separation of two fractions: P-4, composed of the pellet (the interphase between the 1.3M and 2.0M sucrose layers) and overlapping 1.3M sucrose layer, and S-4, the layer above the 1.3M sucrose layer. The S-4 and P-4 fractions were assayed for different enzymatic activities(Table 4). Eighty-nine percent of the assayed TK activity partitioned to the P-4 along with 69% of the TMPK activity, and 66% of the DHFR-^{3}H-methotrexate (^{3}H-MTX) binding activity.

Baril et al. (1973) had found in Novikoff tumor that 98% of the

Table 4. Assay of subcellular fractions isolated from HeLa cells for TK, TMPK, and DHFR activities.

subcellular fraction[a]	TOTAL ACTIVITIES[b,c] x 10^{-4}		
	TK	TMPK	DHFR
S-4	2.9	5.3	290
P-4	20.0	12.0	570

a) A Hela cell pellet, stored at -70°C, was thawed on ice and the cells were subjected to fractionation as described in the text and fractionation scheme one in the Materials and Methods.

b) One unit of activity is defined as described in the Material and Methods per 25 ul of assay volume.

c) Activities (b) were corrected for the total fraction volume ((b) x total fraction volume).

TK activity was associated with the P-4 fraction along with 99.8% of the DNA polymerase, 99% RR, and 88% of the TS activities. Although he had not assayed Novikoff tumor for the additional enzymes, TMPK and DHFR, I felt confident in our ability to isolate the proposed MEC fraction based on the similar TK data obtained for HeLa cells and Novikoff tumor.

4.2 Isolation of the MEC fraction from mouse cells.

The partitioning of nucleotide metabolism and DNA replication enzymes was examined in a mouse cell line. The Balb/3T3 cell line, A31, was used. Subcellular fractionation was performed on frozen cell pellets as described for HeLa cells. Enzymatic activities were assessed in the S-4 and P-4 fractions, the results of which are presented in Table 5. TK (99.8%), DHFR (99.1%), DNA pol (85%), and nucleotide-diphosphokinase (NDPK) (99%) activities partitioned to the P-4 fraction from A31 cells. Again the TK results agree with those obtained by Baril et al. (1973) in Novikoff tumor. Also, the DNA pol activity demonstrates a similar association with the P-4 fraction as observed by Baril and co-workers. NDPK, another nucleotide metabolizing enzyme, also appears to associate with the defined MEC fraction. The data in table 5 additionally suggest that a greater association of DHFR-^{3}H-MTX binding activity exists with the P-4 fraction of A31 cells, then that observed with HeLa cells. This higher affinity of DHFR-^{3}H-MTX binding activity for the MEC fraction of A31 could be the result of possible cell line differences or variability in the

Table 5. Assay of subcellular fractions isolated from A31 cells for TK, NDPK, DHFR, DNA pol α activities.

	TOTAL ACTIVITIES[b] x 10^{-2}			
subcellular fraction[a]	TK	DNA POL α	DHFR	NDPK
S-4	3.8	1.3	NIL	170
P-4	2000.	7.2	28,000	1700

a) Actively growing A31 cells were harvested from 30-100mm cultures as described in the Materials and Methods. The cell pellet was stored at -70°C until used. The cell pellet was thawed on ice and the cells fractionated as described in scheme one in the Materials and Methods.

b) Activities were calculated as described in table 4.

fractionation procedure.

4.3 Isolation of the MEC fraction from ts2.

Actively growing cultures of ts2 incubated at 33°C were harvested, and the cells fractionated to yield the S-3, S-4, and P-4 fractions. TK, TMPK, and DHFR assays were performed and high levels of these different enzyme activities were observed in the P-4 fraction (Table 6). 99.8% of the TK, 98.5% of the TMPK, and 100% of the DHFR activities partitioned to the P-4. These results are similar to those obtained, previously, with the HeLa and A31 cell lines, and in Novikoff tumor (Baril et al., 1973). From these data it was concluded that a MEC fraction, defined as the P-4, is isolatable from ts2 cells growing at their permissive temperature.

4.4 Enzymatic activities in the subcellular fractions isolated from ts2 grown at its permissive and non-permissive temperatures.

To determine whether ts2 contained a structurally thermolabile MEC at the non-permissive temperature, growing cultures of ts2 were incubated for 20 hours at 39°C or maintained at 33°C. The cells were harvested and fractionated. The resulting S-3, S-4, and P-4 fractions were assayed for their respective TK and DHFR activities (table 7). The S-3 fraction isolated from ts2 incubated at 39°C exhibits a slight decrease in TK activity when compared to that obtained from ts2 growing at 33°C, and a pronounced decrease in the ability to bind ^{3}H-MTX. A large loss in

Table 6. Activities of subcellular fractions isolated from ts2 cells growing at its permissive temperature.

subcellular fractions[a]	TOTAL ACTIVITIES[b] x 10^{-2} TK	TMPK	DHFR
S-3	2900	2000	10000
S-4	4	2	NIL
P-4	1600	1300	5000

a) Actively growing ts2 cells were harvested from 30 -100 mm cultures as described in the Materials and Methods. the cell pellet was stored at -70°C until used. The cell pellet was thawed on ice and the cells fractionated as described in scheme one in the Materials and Methods.

b) Activities were calculated as described in table 4.

Table 7. Activities of subcellular fractions isolated from ts2 cells growing at 33°C or incubated at 39°C.

cell incubation temp °C	subcellular fraction[a]	TOTAL ACTIVITIES[b] x 10^{-3} TK	DHFR
33	S-3	1000	1100
	S-4	3.1	150
	P-4	150	320
39	S-3	840	360
	S-4	1.2	90
	P-4	63	130

a) Actively growing ts2 cells at 33°C or incubated at 39°C, were harvested from 30-100 mm cultures as described in the Materials and Methods. The cell pellets were thawed on ice and the cells fractionated as described in scheme one in the Material and Methods.

b) Activities were calculated as described in table 4.

assayable activities is also observed in fractionation of the S-3 to the resulting S-4 and P-4 at both temperatures. This loss in activity on fractionation had been observed previously and will be addressed later in the text. Nonetheless, an initial conclusion as to the effect of temperature on the partitioning of the enzymes into the P-4 fraction from ts2 cells can be reached. The majority of the TK activity is located in the P-4 isolated from ts2 at both 33°C and 39°C. 72.2% and 60.1% of the DHFR-^{3}H-MTX binding activity is found in the P-4 from ts2 at 33°C and 39°C, respectively. These results contradict the preliminary data (table 3) suggesting the enzymatic activities found in the P-4 from ts2 growing at 33°C partitioned in the soluble (S-4) fraction upon incubation of the cells at 39°C. However, since the observed TK and DHFR activities in the P-4 fraction at 39°C are only approximately 30% of those found at 33°C, an alternate interpretation could be that, although the MEC in ts2 may not be thermolabile structurally at 39°C, the complex, could be functionally temperature- sensitive instead.

Several experimental approaches were evaluated in an attempt to clarify the basis for the discrepancy observed concerning the heat-sensitivity of the MEC in ts2 cells at its non-permissive temperature. Basically two points were addressed. First, did the manner in which the S-3 was fractionated to yield the S-4 and P-4 account for the observed difference in the results? Second, was the proposed thermolability of the MEC in ts2 masked by the low recovery of enzyme activity in the S-4 and P-4 fractions when compared to that observed in the S-3? These alternative concepts

will be described in turn.

The fractionation scheme used by our collaborators, arriving at their preliminary data indicating that ts2 cells contained a heat labile MEC was somewhat different then that used in this laboratory to generate the MEC fraction. The difference in the two schemes lies in the way the S-3 is fractionated yielding the S-4 and P-4. In their case, the S-3 is subjected to prolonged centrifugation without first layering it onto a discontinuous sucrose gradient (scheme two in Materials and Methods). The upper 80-90% of the high-speed supernatant is designated the S-4, the remaining portion constitutes the P-4. I, therefore, used this method of fractionation of the S-3 in a new experiment with cultures of ts2 incubated for 20 hours at 39°C or remaining at 33°C to determine whether this scheme was capable of detecting a thermolabile MEC. The resulting S-3, S-4, and P-4 fractions were assayed for TK, TMPK, and DHFR activities (table 8). The distribution of the enzymes within these fractions follows the pattern previously observed. The majority of the activities are located in the P-4 whether the ts2 cells were incubated at 33°C or 39°C. Again a large loss in activity is noted as the S-3 is fractionated to the S-4 and P-4. These data concerning the partitioning of the assayed activities do not contradict our previous results indicating that ts2 cells incubated at 39°C do not appear to contain a structurally labile MEC.

The second point considered as to account for the discrepancy in the results concerning the temperature-sensitivity of the MEC in ts2 was whether the large losses of enzyme activity as S-3 was

Table 8. Enzymatic activities of subcellular fractions isolated from ts2 cells at 33°C or incubated at 39°C using a different fractionation scheme.

cell incubation temp °C	subcellular fraction[a]	TOTAL ACTIVITIES[b] x 10^{-3} TK	TMPK	DHFR
33	S-3	1200	270	220
	S-4	2.5	11	50
	P-4	130	27	70
39	S-3	950	39	70
	S-4	1.5	.002	12
	P-4	19	.017	44

a) Actively growing ts2 cells at 33°C or incubated at 39°C were harvested from 30-100 mm cultures as described in the Materials and Methods. The cell pellets were thawed on ice and the cells fractionated as described in scheme 2 in the Materials and Methods.

b)Activities were calculated as described in table 4.

fractionated to S-4 and P-4 masked an observable lability of the complex. Our concern was founded on whether the question of the thermolability of the MEC in ts2 could be accurately assessed with only a 10% recoverability of activity from the S-3. Within the lost 90% of enzyme activity, perhaps an observable labile MEC would be uncovered.

Several possibilities were considered to account for the observed loss in enzyme activity: the presence of a possible inhibitory substance in the S-4; the effect of concentrating the S-4; possible aggregation of P-4 elements leading to an underestimation of enzyme activity; the effect of different methods of storage of the S-3; the effect of sterile reagents on the isolation of the P-4. Each of these alternatives will be examined in turn.

A possible source for the observed loss in enzyme activity as S-3 is fractionated to S-4 and P-4 could be a substance inhibitory to TK and TMPK activities fractionates with the S-4. Therefore, an underestimation of these activities in this fraction would be made. Ts2 cells growing at 33°C were harvested and fractionated (scheme two). TK and TMPK assay of the resulting S-4 and P-4 showed essentially no enzyme activity in the S-4; all apparent activity resided in the P-4. To assess the presence of the proposed inhibitory substance, the S-4 and P-4 were added together and the resulting mixtures assayed for TK and TMPK activity (table 9). Were an inhibitory substance present in the S-4, we could expect to observe a decrease in TK and TMPK activities associated with the P-4. No significant change in activity was observed.

Table 9. Assaying for inhibitory substances in S-4 isolated from ts2.

VOLUMES (ul)			ACTIVITIES OF MIXTURES[c]	
P-4[a]	S-4[a]	buffer[b]	TK	TMPK
			(x 10^{-2})	
1	----	24	0.5	2.5
5	----	20	3.6	6.7
15	----	10	14.0	20.0
1	24	----	0.5	1.3
5	20	----	4.8	6.2
15	10	----	16.0	19.0

a) Aliquots of previously isolated S-4 and P-4 from ts2 growing at 33°C (see table 7) were thawed and mixtures of the two subcellular fractions or the P-4 and buffer were assayed for their resulting TK and TMPK activities as described in the text and the Materials and Methods.

b) Buffer used in mixture with P-4 was STKDME-PMSF, as described in fractionation scheme 1 in the Material and Methods.

c) Activities were calculated as described in the Materials and Methods/assay volume.

These data failed to demonstrate that any apparent inhibitory substances for TK and TMPK activities are present in the S-4.

The concentration of protein is lower in the S-4 compared to that found in the P-4. To determine whether a dilute protein concentration in S-4 masks the lost activity of the S-3, ts2 cells were incubated at 33°C and 39°C, harvested, and fractionated to S-4 and P-4 fractions. TK assay of the S-4 revealed little activity present. The S-4 from both temperatures was concentrated against solid sucrose approximately 8-fold. The concentrated S-4 showed no additional TK activity.

It was proposed that aggregation of the elements in the P-4 may prevent substrate from reacting with all available enzyme, therefore, masking additional enzyme activity in the P-4. To examine this putative aggregation of components in the P-4 leading to an underestimate of enzyme activity, three methods were employed to gently disrupt the proposed aggregate: homogenization; 0.5% NP-40, a non-ionic detergent; homogenization together with non-ionic detergent. A P-4 isolated from ts2 growing at 33°C with known activity was treated with the above mentioned methods. The homogenization consisted of 10 strokes with a 1 ml tissue grinder. Table 10 describes the results of this experiment. The data for TMPK indicate that the non-ionic detergent, NP-40, greatly decreases the activity of this enzyme. Approximately a 4-fold loss in activity is observed after homogenization. The data concerning TK activity after treatment of the P-4 shows the activity decreases slightly after the additon of NP-40. Homogenization decreases TK activity by 3.4-fold, although the addition of NP-40 following

Table 10. Attempts to disrupt proposed aggregates in P-4.

Treatment of P-4[a]	TOTAL ACTIVITIES[b] x 10^{-2}	
	TK	TMPK
untreated (diluted P-4)	120	31
Homogenization	36	7.3
0.5% NP-40	89	0.3
Homogenate + 0.5% NP-40	49	0.14

a) An aliquot of a previously isolated P-4 from ts2 growing at 33°C (see table 7) was thawed. The P-4 was suspended 5-fold in STKDME-PMSF (described in fractionation scheme 1 in the Materials and Methods) prior to treatment. The diluted P-4 was treated as described in the text. The treated P-4 were assayed for their resulting TK and TMPK activities.

b) Activities were calculated as described in table 4.

homogenization of the P-4 stimulates the observed TK activity slightly. These results do not permit an unambiguous determination of whether aggregation of the elements in the P-4 prevents the actual estimation of the total activity for TK and TMPK, although they do not support such an interpretation. Furthermore, these results do suggest the apparent fragility of the putative MEC or at least the assayed enzymes within the complex.

The question as to whether the reagents used to isolate the MEC fraction played a role in the low recoverability of activities was addressed. Although all solutions were prepared just prior to the cellular extractions, there could be some element present in the reagents (ie., bacterial growth or some type of unspecified inhibitory substance), which could interfere with either enzymatic recovery or activity. To test this assumption in a most elementary way, we questioned whether sterile solutions prepared in sterile plasticware (tissue culture quality) would increase the yield of enzyme activity. Just prior to MEC isolation, all solutions used in the subcellular fractionation were prepared in deionized water as usual, then passed through sterile 0.45 um filters into autoclaved glassware. Cultures of ts2 growing at 33°C were harvested and fractionated (scheme two) using the sterile reagents. The resulting S-3, S-4, and P-4 fractions were assayed for TK, TMPK, and DHFR activities (table 11). The data indicate the recovery was greatly improved. The recovery of activity in S-4 and P-4 is 40-70% of that found in the S-3. Prior fractionations resulted in S-4 and P-4 together containing only 10% of the original activity of the S-3. Ts2 cells incubated at 39°C and

Table 11. Activities of subcellular fractions isolated from ts2 growing at 33°C using sterile solutions in the fractionation.

subcellular fractions[a]	TOTAL ACTIVITIES[b,c] x 10^{-5}		
	TK	TMPK	DHFR
S-3	41	20	17
S-4	NIL	NIL	1.7
P-4	16	14	9.5

a) 30-100 mm cultures of ts2 cells growing at 33°C were harvested and fractionated as described in the text and fractionation scheme in the Material and Methods.

b) Activities were calculated as described in table 4.

c) Protein concentration (determined by method of Lowry et al., (1951)) did not show an increased yield in the isolated fractions when compared to previous fractionations (data not shown).

fractionated using sterile solutions also showed a similar increase in enzyme activity recovery as observed with ts2 cells grown at 33°C. However, the additional activity did not uncover a change in the partitioning of enzymes into the S-4 and P-4. The majority of the different activities was found associated with the P-4 from ts2 cells incubated at 33°C or 39°C.

Explanation for the remaining loss of enzyme activity was provided when the aliquot of the S-3, normally saved for assay of its activity, at -70°C was stored at 4°C. An aliquot of S-3 incubated overnight at 4°C would mimic more accurately the conditions of isolation of S-4 and P-4. The effect of this different method of storage of the S-3 aliquot was examined using ts2 cells growing at 33°C. These cells were harvested and fractionated as described in scheme two using sterile solutions. Two aliquots of S-3 were removed prior to overnight high-speed centrifugation resulting in S-4 and P-4 fractions. One aliquot was promptly stored at -70°C, the other at 4°C. The S-4, P-4, and two aliquots of S-3 were assayed for TK and TMPK activities(table 12). These results show a large loss in enzyme activity for TK and TMPK in the S-3 aliquot stored at 4°C when compared with that stored at -70°C. Using the activities for TK and TMPK observed in the S-3 stored at 4°C as the baseline against which the S-4 and P-4 activities are measured, it is found that the recoveries for TK and TMPK are quite good. The large decrease in TK and TMPK activities noted in the S-3 stored overnight at 4°C as opposed to storage at -70°C could perhaps be attributed to MEC fragility or a cellular protease(s) which is liberated as the cells are

Table 12. Activities of subcellular fractions isolated from ts2 growing at 33°C. Test of S-3 storage conditions.

subcellular fractions[a]	TOTAL ACTIVITIES[b] x 10^{-5}	
	TK	TMPK
S-3[c]	3.2	9.7
S-3[d]	2.2	5.4
S-4	NIL	0.02
P-4	2.4	3.3

a) 20-100 mm cultures of ts2 growing at 33°C were harvested and fractionated as described in the text and the Materials and Methods.

b) Activities were calculated as described in table 4.

c) S-3 stored overnight at -70°C prior to assay.

d) S-3 stored overnight at 4°C prior to assay.

fractionated (Baril, personal communication).

Evaluation of the discrepancy of whether ts2 contained a thermolabile MEC involved in DNA synthesis lead to the following observations. The method used to fractionate S-3 to S-4 and P-4 does not contribute to the difference in data. A series of experiments performed in attempts to discover the cause for the observed decrease in enzyme activities as S-3 is fractionated to S-4 and P-4 disclosed that the preparation of sterile solutions for the isolation of MEC is most important to high recoverability of enzyme activity. Improving the recovery of enzyme activity in S-4 and P-4 did not reveal a thermolability in the structure of the MEC for ts2 as the complex has been defined.

4.5 Examination of MEC from nuclear extracts.

The MEC found in the P-4 is presumably leakage of nuclear contents into the cytosol during cellular extraction (Baril, personal communication). Nuclear extracts of ts2 grown at 33°C or incubated for 20 hours at 39°C were examined for enzyme activity and MEC levels to assess whether the cells would display a ts effect in either the distribution of the enzyme activities in the nuclear extract (N.E.) versus P-4 or the partitioning of enzymes of the MEC isolated from the N.E..

Ts2 cells incubated at either 33°C or 39° were harvested and fractionated to yield N.E. and P-4 as described in scheme two. In brief, the N.E. was isolated by resuspending the cells in hypotonic buffer and the cells homogenized. The homogenate was

subjected to low speed centrifugation, the supernatant was processed to P-4 as described earlier. The crude nuclei (pellet) was resuspended in isotonic buffer and stirred for 2 hours at 4°C. This crude extract was underlayed with a 1/3 volume 60% sucrose solution dissolved in isotonic buffer and subjected to prolonged high-speed centrifugation. The resulting pellet and sucrose layer are discarded and the supernatant is dialyzed overnight against hypotonic buffer containing 50% glycerol. This dialyzate is designated the nuclear extract. The S-1, S-3, S-4, P-4 and N.E. from ts2 were assayed for TK and TMPK activities (table 13). The majority of TK and TMPK activities at either temperature are associated with the P-4 fraction when compared with the N.E. Approximately 20-fold or more TK and TMPK activity is found in the P-4. Optimization of the isolation conditions, as previously described, resulted in the increased recovery of activity on fractionation of S-3. The data indicate that there is no difference in the distribution of enzyme activities between the N.E. or P-4 in ts2 at either temperature.

The isolated N.E. prepared from ts2 cells incubated at 33°C and 39°C were subjected to prolonged high-speed centrifugation and fractionated to a soluble (N.E.S.) and MEC fraction (N.E.P.) (analogous to S-3 fractionation to S-4 and P-4) to determine whether the activity in the N.E. was associated in MEC form. TK activity was determined in the isolated soluble and MEC fractions derived from the N.E. (table 13). The results demonstrate that essentially all of the activity for TK found in N.E. prepared from cells at 33°C and 39°C is associated with the MEC fraction.

Table 13. Enzymatic activities of subcellular fractions isolated from ts2 cells incubated at 33°C or 39°C.

cell incubation temp °C	subcellular fraction[a]	TOTAL ACTIVITY[b] x 10^{-3} TK	TMPK
33	S-1[c]	520	230
	S-3[d]	200	110
	S-4	NIL	64
	P-4	170	75
	NE	6.6	2.8
	NES[e]	NIL	ND
	NEP[e]	5.5	ND
39	S-1[c]	350	150
	S-3[d]	150	49
	S-4	NIL	0.2
	P-4	120	41
	NE	5.1	2.1
	NES[e]	NIL	ND
	NEP[e]	3.5	ND

a) 20-100 mm cultures of ts2 cells at 33°C amd 39°C were harvested and fractionated as described in fractionation scheme two in the Materials and Methods and the text.

b) Activities were calculated as described in table 4.

c) S-1 stored at -70°C until assayed.

d) S-3 stored overnight at 4°C, then assayed at the same time as S-4, P-4, and NE.

e) NES and NEP were assayed immediately after sedimentation.

Therefore, the enzymes associated with the MEC isolated from N.E. do not show a thermolabile partitioning in ts2.

A MEC involved in DNA synthesis was isolated from the nuclear fraction of CHEF cells by Reddy and Pardee (1980, 1983). This complex, which the authors termed "replitase" sedimented rapidly in a discontinuous sucrose density gradient. This replitase contained TK, TMPK, DHFR, DNA pol α, RR, and TS activities. In addition, it was noted that newly replicated DNA co-sedimented with these enzymatic activities.

The possibility of the temperature-sensitive lesion in ts2 revealing itself in this replitase fraction was explored. The migration of newly replicated DNA in a discontinuous sucrose density gradient from ts2 cells incubated for 20 hours at 39°C or growing at 33°C was determined. For this analysis, cultures of ts2 were incubated with ^{3}H-TdR for 15 minutes before harvesting. The cells were processed for the isolation of replitase as outlined in scheme three in Materials and Methods. In brief, the cells were resuspended in hypotonic buffer and Dounce homogenized, followed by low speed centrifugation to collect the nuclei. The supernatant was fractionated to S-4 and P-4, as described earlier. The crude nuclei were incubated overnight on ice. The nuclei were resuspended in hypotonic buffer and sonicated in 2-20 second bursts at a low setting. The sonicate was clarified by brief centrifugation in a microfuge; the resulting supernatant was layered onto a discontinuous sucrose density gradient consisting of 20, 40, 50, 55, 60 % sucrose (w/w) dissolved in hypotonic buffer. The gradient was centrifuged for four hours at 23,000 rpm in an

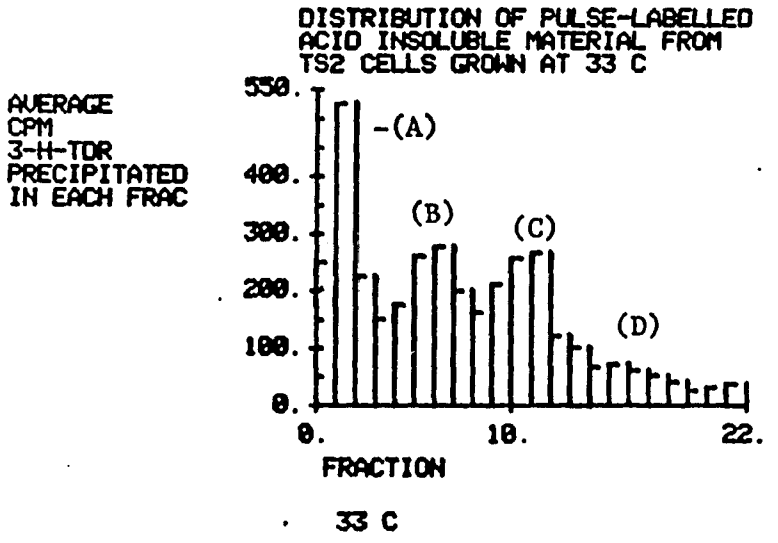
Beckman SW27 rotor, collected in 0.3 ml fractions, and assayed for acid-insoluble material. Reddy and Pardee (1983) have defined the 40/50% sucrose border as the replitase fraction. The activity of the replitase can move from this border to other locations in the gradient depending on isolation conditions, as described in Materials and Methods. Figure 15 shows the distribution of DNA from ts2 cells labelled at 33°C and 39°C, and isolated in the above manner. These data suggest that there is no significant difference in patterns of TCA-precipitable radioactivity (ie., putative newly replicated DNA) in ts2 at the permissive or non-permissive temperatures. Although no significant differences in the level of incorporation for ts2 at 33°C and 39°C were noted, controls verified ts2 expressed the ts phenotype under conventional conditions (one-hour pulse label of cultures of ts2 at 33°C and 39°C with ^{3}H-TdR, followed by TCA-precipitation of acid-insoluble material); ie., ^{3}H-TdR incorporation into high molecular weight DNA is decreased in TS2 at 39°C when compared with the cells at 33°C.

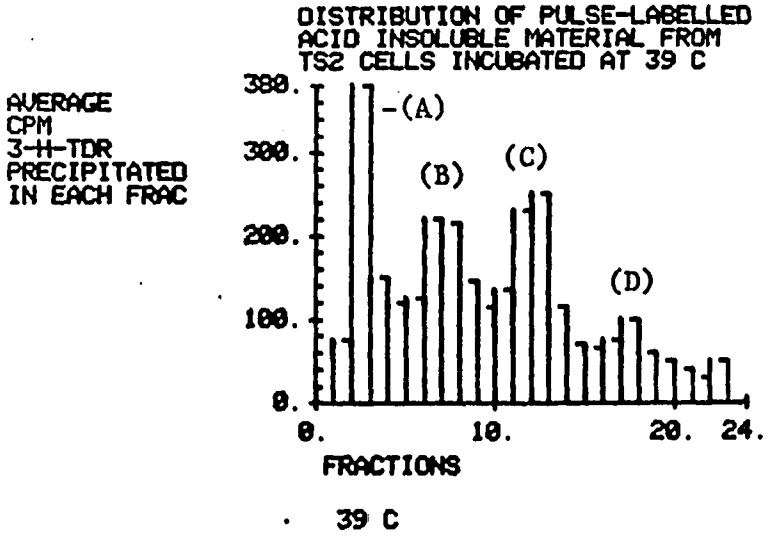
TK and DNA pol α activities were compared in the replitase fractions, from analogous discontinuous sucrose density gradients as described above, isolated from ts2 cells at 33°C and 39°C (table 14). These assayed activities do not reveal any dramatic difference between 33°C and 39°C for TK, although the activity of DNA pol α at 39°C is about 50% of that observed at 33°C.

As replitase was extracted from the nuclear fraction of ts2 cells at 33°C and 39°C, the remaining cytoplasmic fraction was processed to yield the S-4 and P-4, as described earlier for scheme

Figure 15. Distribution of radiolabelled acid-insoluble material from ts2 cells growing at 33°C or incubated at 39°C in a discontinuous sucrose gradient. Ts2 cells grown at 33°C or incubated at 39°C for 16 hours were pulse-labelled for 15 minutes with 1 uCi/ml of ^{3}H-TdR (20 Ci/mmole, NEN). The cultures were harvested and the cells fractionated as described in the text. The quantity of radiolabel incorporated into acid-insoluble material in each fraction was determined by TCA precipitation of the cellular macromolecules as described in the Materials and Methods. (A) represents the 55/66%, (B) 50/55%, (C) 40/50%, and (D) 20/40% sucrose borders.

FIGURE 15


DISTRIBUTION OF PULSE-LABELLED
ACID INSOLUBLE MATERIAL FROM
TS2 CELLS GROWN AT 33 C
AVERAGE
CPM
3-H-TDR
PRECIPITATED
IN EACH FRAC
500.
400.
300.
200.
100.
0.
-(A)
(B)
(C)
(D)
0.
10.
22.
FRACTION
33 C


DISTRIBUTION OF PULSE-LABELLED
ACID INSOLUBLE MATERIAL FROM
TS2 CELLS INCUBATED AT 39 C
AVERAGE
CPM
3-H-TDR
PRECIPITATED
IN EACH FRAC
380.
300.
200.
100.
0.
-(A)
(B)
(C)
(D)
0.
10.
20.
24.
FRACTIONS
39 C

two. The S-4 and P-4 were assayed for TK and DNA polα activities (table 14). Better then 99% of the TK activity was associated with the P-4 from ts2 cells at 33°C and 39°C. The majority of the DNA pol α activity also partitioned to the P-4 isolated from ts2 at both temperatures.

It is readily apparent from the data in table 14 that P-4 contains considerably more units of TK activity from cells at both temperatures than the replitase fraction. There is also 2-fold more DNA pol α activity in P-4 compared with the replitase fraction from ts2 at 33°C, and approximately 4-fold more from ts2 at 39°C. When an isolated P-4 is layered onto an analogus discontinuous sucrose density gradient as in the replitase isolation and centrifuged for four hours at 23,000 rpm the maximum TK and DNA pol α activities are found in the 20% sucrose layer indicating that the MEC associated with P-4 is smaller than that in the replitase fraction (data not shown). This suggests that the replitase is perhaps a higher order complex then that in the P-4. Cellular extraction may disrupt the replitase into smaller units which then leak into the cytoplasm. This lower order complex then fractionates, because of its size, to the P-4 or to the 20% sucrose layer in the discontinuous sucrose density gradient. Since cellular extraction methods are rather crude when compared to the fine architecture of the cell, the replitase, most likely, is readily disrupted to smaller units. It is much more likely, therefore, that the concentration of this proposed lower order complex would be high in relation to the large replitase. The data represented in table 14 would suggest that this proposal may be

Table 14. Activities of subcellular fractions isolated from ts2 cells growing at 33°C or incubated at 39°C.

cell incubation temp. °C	subcellular fraction[a]	TOTAL ACTIVITIES[b] x 10^{-4} TK	DNA pol α
33	S-4	0.019	33
	P-4	68	170
	replitase	0.56	85
39	S-4	1.2	26
	P-4	130	160
	replitase	0.49	42

a) 30-100 mm cultures of ts2 cells at 33°C and 39°C were harvested and fractionated as described in fractionation scheme 3 in the Material and Methods.

b) Activities were calculated as described in table 4.

correct, since there is more enzyme activity associated with the P-4 than with the replitase.

The yields of enzyme activity recovered for P-4 from ts2 at 33°C and 39°C are much higher than observed earlier in this chapter. Repeated isolation and optimization of the fractionation conditions resulted in this improved activity. As indicated earlier, sterile reagents improved the overall recovery. Also, the quality of reagents (e.g. freshly prepared DTT and enzyme-grade Tris (GIBCO)) used in the isolation was paramount to increased yields. Still these later data and those obtained from earlier experiments in which complex was isolated contradict the preliminary results which indicated the enzymatic activities associated with the MEC remained in the soluble (S-4) fraction at 39°C.

4.6 ^{3}H-thymidine channeling to ^{3}H-TTP by complex fractions from ts2 grown at 33°C and 39°C.

No significant differences were observed in the fractionation of enzymatic activities from ts2 cells incubated at 33°C and 39°C. Perhaps the defect in ts2 would manifest itself by a lack of coordinate function of the MEC rather than with the individual enzymes themselves. To assess this aspect of P-4 and replitase ability, the reactions which result in ^{3}H-TTP formation from the substrate ^{3}H-TdR were examined. The sequential reactions of the thymidine pathway are shown in figure 16. Channeling of ^{3}H-TdR to ^{3}H-TTP and DNA is defined as described by Baril et al (1973).

Figure 16. Sequential reactions utilizing thymidine.

FIGURE 16

It is the ability of the MEC fraction to demonstrate the sequential reactions that produce ^{3}H-TTP from ^{3}H-TdR and subsequently incorporate the ^{3}H-TTP into DNA. Ts2 cells incubated at 33°C or for 20 hours at 39°C were harvested and fractionated to yield S-4, P-4, and replitase fractions, their enzyme activites are shown in table 14. Aliquots of these fractions were incubated for 60 minutes at 37°C with ^{3}H-TdR. The quantities of the resultant ^{3}H-TMP, ^{3}H-TDP, ^{3}H-TTP were determined by paper chromatography (table 15). The total yields for these nucleotides were normalized for the concentration of protein in each fraction. The data indicate the P-4 and replitase fractions are each capable of channeling ^{3}H-TdR through the complex to ^{3}H-TTP, although, the P-4 is more efficient; this is perhaps related to its higher associated TK activity. The apparent ability of P-4 and replitase from ts2 to readily channel substrate at 39°C suggests that this is not the basis of its observed ts defect.

4.7 Incorporation of channeled ^{3}H-TdR into DNA <u>in vitro</u> by complex fractions.

Examination of the ts2 P-4 and replitase partitioning patterns, levels of enzyme activities, and the ability to channel ^{3}H-TdR did not reveal any deficiency in cells incubated at 39°C when compared with those growing at 33°C. Another biological activity examined was the ability of the isolated complex fractions to incorporate ^{3}H-TTP, accumulated by the MEC from ^{3}H-TdR, into an offered DNA template. S-4, P-4, and replitase fractions, isolated

Table 15. Channeling of ^{3}H-TdR to ^{3}H-TTP and DNA by subcellular fractions isolated from ts2 cells incubated at 33°C or 39° C.

cell incubation temp. °C	subcellular fraction[a]	TOTAL FEMTOMOLES OF ^{3}H-TdR CONVERTED[b] to ^{3}H-TTP (x 10^{-3})	into DNA
33	S-4	0.57	NIL
	P-4	400	5.1
	replitase	8	NIL
39	S-4	1.5	NIL
	P-4	430	17
	replitase	10	NIL

a) ts2 cells were fractionated as described in table 14. Channeling is defined as in text.

b) Activity = unit activity per ug protein (corrected for the volume of the fraction).

from ts2 cells at 33°c and 39°C (table 14), were incubated for 60 minutes at 37°C with ^{3}H-TdR to accumulate ^{3}H-TTP pools. A DNA template was then added and the fractions re-incubated for 60 minutes at 37°C. The samples were then assayed for acid-insoluble material. Data were normalized for the concentration of protein in each fraction (table 15). The P-4 from ts2 cells incubated at 39°C is as capable, if not better, of incorporating ^{3}H-TdR into DNA when compared to P-4 from ts2 growing at 33°C. Non-radiolabelled TTP does not inhibit this incorporation (table 16), as expected if the radioactive TTP pool was not equilibrated with the total reaction volume. The S-4 is unable to channel ^{3}H-TdR to DNA, consistent with the observed low levels of ^{3}H-TTP generated. The replitase fraction from ts2 cells at 33°c and 39°c is incapable of channeling ^{3}H-TdR to DNA, as we have measured it. This failure to observe ^{3}H-TdR incorporation into DNA by replitase may be attributed to the low level of TK and DNA pol activities present in this fraction (table 14).

4.8 The MEC in serum stimulated ts2R.

Data presented in the preceeding chapter correlated the stimulation of DNA synthesis and the induction of nucleotide metabolism enzymes at the permissive and non-permissive temperatures after the addition of serum in ts2. Growth-arrested ts2 cells immediately incubated at 39°C after the addition of serum showed no (or a much reduced) induction of TK and TMPK activities along with little or no stimulation of ^{3}H-TdR

Table 16. Demonstration of the channeling of ^{3}H-TdR by MEC to an offered DNA template.

subcellular fraction[a]	TOTAL FENTOMOLES ^{3}H-TdR INCORPORATED[b] NUCLEOTIDE-TRIPHOSPHATES ADDED[c]				
	A	B	C	D	E
S-4	75	1040	100	90	140
P-4	9360	9220	380	7770	7720
replitase	170	240	70	130	200

a) Subcellular fractions were isolated, from CHO-K1 cells growing at 37°C, by fractionation scheme 3 in Materials and Methods. Aliquots of the subcellular fractions were incubated in ^{3}H-TdR for 60 minutes at 37°C to generate ^{3}H-TTP pools (as described in the text). Then an activated calf thymus DNA template was presented to the aliquots (as described in the Materials and Methods) along with various non-radiolabelled nucleotide triphosphate combinations (A-E)[b]. The aliquots were incubated for an additional 60 minutes at 37°C, then processed to determine the level of radiolabel incorporated into acid-insoluble material (Materials and Methods).

b) Activities were calculated as described in table 4.

c) A = standard assay (50 nanomoles TTP, dGTP, dCTP, dATP)
B = 1000X standard assay
C = no nucleotide triphosphates added
D = (standard assay - TTP) + 50 uM rCTP
E = standard assay - TTP

incorporation. It was now evaluated whether a population of ts2 cells with basal levels of MEC, as in quiescent cultures, would upon serum stimulation be capable of assembling new complex at its non-permissive temperature. Additionally, would the MEC assemble in such a way that sedimentation of the enzyme activities would vary with time in a discontinuous sucrose density gradient, and would the temperature of cellular incubation play a role in this assembly. To examine these questions experiments were initially performed with ts2R, a spontaneous revertant of ts2, to characterize a non-ts response of cells to serum stimulation at 33°C and 39°C.

A different fractionation scheme was employed to isolate the P-4 and NE fractions, see scheme 4 in Materials and Methods. Briefly, the cells are suspended in hypotonic buffer and Dounce homogenized. The homogenate is subjected to low speed centrifugation to obtain crude nuclei and a cytoplasmic fraction (S-1). The S-1 is subjected to differential centrifugation to yield an S-3 as described previously. The S-3 is layered onto a discontinuous sucrose gradient consisting of 0.8M, 1.3M, and 2.0M sucrose dissolved in hypotonic buffer. This gradient is centrifuged overnight at 40,000 rpm in a Beckman SW50.1 rotor. Each layer of the gradient is drawn off using a bent pasteur pipette and designated A through D. D=2.0M sucrose, C=1.3M sucrose, B=0.8M sucrose, A is equivalent to the previously defined S-4. Nuclear extract is prepared from the crude nuclei pellet by stirring for 2 hours in four volumes of hypotonic buffer at 4°C followed by centrifugation at 10,000 rpm for 15 minutes in a

Sorvall SM24 rotor. The supernatant is then layered onto a discontinuous sucrose gradient and processed as described for the S-3. The extraction buffers in this scheme contain the additional chelating agent EGTA and the protease inhibitor AAN since a strong calcium ion dependent protease apparently is released on extraction of cells (Baril, personal communication).

Quiescent ts2R cells were serum stimulated, then incubated at 33°C or immediately shifted to 39°C. Ten cultures were harvested at each of the following time points after the addition of serum: 0, 12, and 24 hours at 33°C, and 10 and 16 hours at 39°C. The cells were fractionated for both crude nuclear and cytoplasmic fractions as outlined above, scheme 4. The discontinuous sucrose gradient fractions were collected as described and designated A - D, each prefixed with c (cytoplasmic) or n (nuclear). TK, DNA polα, and TMPK activities were evaluated in these fractions, as were the ability to channel ^{3}H-TTP and its subsequent incorporation into an offered DNA templated (table 17 and 18). A graphic representation of the data is presented in figure 17.

These data indicate that TK, DNA polα, and TMPK activities are induced after serum stimulation of ts2R at both33°C and 39°C. Maximal DNA synthesis, as measured by ^{3}H-TdR incorporation <u>in vivo</u>, occurs 24 hours after the addition of serum in cells incubated at 33°C and after 16 hours for cultures at 39°C. At the time of maximal stimulation the TK and TMPK activities distributed primarily through gradient fractions A, B, and C as isolated from both cytoplasmic and nuclear fractions. The

Table 17. Assay of activities in different gradient fractions for serum stimulates ts2R (cytoplasmic fractions).

	ENZYME ACTIVITIES[b] (x 10^{-2})			FMOLES OF TdR CONVERTED[b] (x 10^{-2})	
fraction	TK	TMPK	DNA POL ∝	to TTP	to DNA
CA 0 hr	10	1	NIL	2.5	1.1
12/33	85	0.8	0.08	63	1.4
24/33	1000	480	0.16	1200	4.4
10/39	210	NIL	0.13	17	1.7
16/39	1400	580	0.35	970	0.13
CB 0 hr	38	NIL	NIL	12	1.1
12/33	81	NIL	7.4	24	1.8
24/33	760	1200	15.9	1300	1.3
10/39	60	NIL	0.56	36	1.4
16/39	800	1200	11.0	1500	140.0
CC 0 hr	34	16	5.2	13	1.7
12/33	34	73	3.9	NIL	1.3
24/33	830	690	1.2	370	15.0
10/39	37	NIL	5.4	3.3	1.2
16/39	590	780	9.5	61	18.0
CD 0 hr	5.6	2.2	0.78	NIL	1.4
12/33	14	2.4	0.66	NIL	0.3
24/33	460	120	0.73	30	2.6
10/39	8.5	2.3	1.5	NIL	1.5
16/39	76	170	1.1	16	0.05

a) Quiescent ts2R cells were serum stimulated, harvested, and fractionated as described in the text. These fractions were immediately assayed for the described activities.

b) Activities calculated as described in table 4.

Table 18. Assay of activities in different gradient fractions from serum stimulated ts2R (nuclear fractionation).

Fraction[a]	ENZYME ACTIVITIES[b] (x 10^{-2}) TK	TMPK	DNA POL ∝	FEMTOMOLES OF ^{3}H-TdR CONVERTED (x 10^{-2}) to ^{3}H-TTP	into DNA
NA 0 hr	20	NIL	1.6	0.4	0.43
12/33	2	NIL	0.77	NIL	0.17
24/33	220	220	3.8	NIL	0.55
10/39	9.3	13	1.1	12	1.3
16/39	210	430	0.64	3.5	2.3
NB 0 hr	35	1	3.1	NIL	0.89
12/33	10	NIL	7.3	8.6	1.3
24/33	530	550	43	NIL	0.89
10/39	13	8.2	1.7	24	0.68
16/39	530	560	24	NIL	1.1
NC 0 hr	25	30	36	NIL	0.91
12/33	8.1	NIL	5.2	2.5	0.86
24/33	500	540	69	NIL	1.5
10/39	23	NIL	1.8	36	0.46
16/39	520	620	29	23	1.4
ND 0 hr	5.3	NIL	0.46	NIL	0.55
12/33	3.7	7	0.43	1.5	NIL
24/33	75	48	14	NIL	0.44
10/39	6	NIL	0.36	8	0.33
16/39	74	120	3.7	NIL	0.19

a) Fractionation of cells described in text and table 17.

b) Activites were calculated as described in table 4.

Figure 17. Distribution of activities from serum stimulated ts2R cells in a discontinuous sucrose gradient. Quiescent ts2R cells were prepared as described in the Materials and Methods. These cultures were stimulated into their growth cycle by the addition of fresh medium containing 10% calf serum. The cultures were incubated at 33°C or shifted immediately to 39°C. At different times after stimulation cultures were harvested and fractionated as described in the text. These fractions were assayed for TK, TMPK, DNA pol α-activities as well as the ability to channel ^{3}H-TdR to ^{3}H-TTP and DNA as described in the Materials and Methods. One unit of activity is defined as described in the Materials and Methods per 25 ul of assay volume. The activities were corrected for the total fraction volume. Only the activities derived from the "cytoplasmic" fraction (S-1) are shown since those observed from the nuclear extract were not shown to differ greatly.

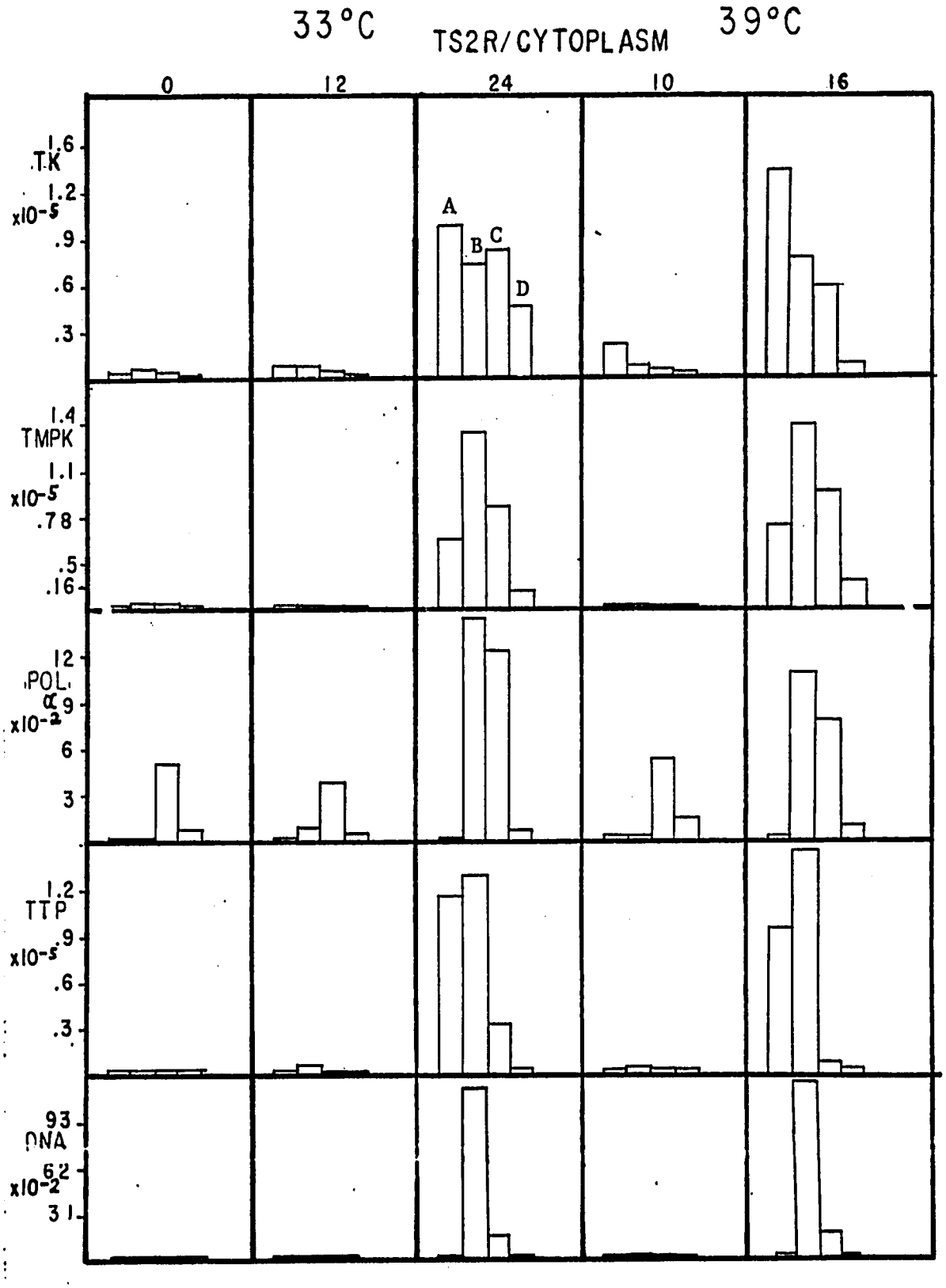

33°C
TS2R/CYTOPLASM
39°C
0
12
24
10
16
TK
x10-5
1.6
1.2
.9
.6
.3
A
B
C
D
TMPK
x10-5
1.4
1.1
.78
.5
.16
POL α
x10-2
12
9
6
3
TTP
x10-5
1.2
.9
.6
.3
DNA
x10-2
93
62
31

FIGURE 17.

distribution of TK and TMPK activities through the gradient fraction (in most instances) takes the appearance of an average distribution, suggesting that there is an average size for the MEC as reflected in the partial breakdown of fragile complex. DNA polα activity partitions differently in that it is only found in fractions B and C in both cytoplasmic and nuclear fractions. Perhaps the DNA pol α is anchored in some unspecified way to larger components of the MEC, therefore, it sediments into the gradient in higher proportions than observed with TK and TMPK activities. For the cytoplasmic fraction in which the levels of TK, TMPK, and DNA pol α activities are high, as at 24 hours at 33°C and 16 hours at 39°C, channeling of ^{3}H-TTP is observed, with high concentrations of ^{3}H-TTP generated. The high level of ^{3}H-TTP is then incorporated into an exogenous DNA template.

The fractions isolated from the extraction of crude nuclei did not seem capable of channeling ^{3}H-TdR to ^{3}H-TTP efficiently, therefore, there is no <u>in vitro</u> DNA synthesis observed by these fractions. This deficiency may be related to the lower enzyme activities of the nuclear fractions. Also, the method used to extract the crude nuclei, the stirring of nuclei for 2 hours at 4°C in buffer, could damage the coordinate function of the enzymes but not their individual activities.

We had postulated that as new complex assembled, the sedimentation of the enzymatic activities in the discontinuous sucrose gradient would change, the initially soluble components of the MEC would assemble into higher orders of complex in a time dependent fashion. The enzymes at zero hour would primarily be

associated with the soluble (A) fraction of the gradient, as the complex matured in the later time points, the activities would be associated with gradient fractions requiring particles to have a higher sedimentation value. Review of the different assay results derived from serum stimulated ts2R (tables 17, 18, and figure 17) do not reveal a cell cycle dependent distribution of the different enzyme activities in the gradient. The data indicate the biologically active MEC (defined by its ability to channel ^{3}H-TdR to DNA in vitro) is found primarily in fraction B of the gradient (cytoplasmic results). Fraction A can channel ^{3}H-TdR to ^{3}H-TTP, but cannot incorporate the nucleotide into DNA. The B fraction contains the maximal activity for both these fractions. Fraction C can channel ^{3}H-TdR to DNA, but not as efficiently as fraction B. These data do not rule out the possibility of the assembly of complex resulting in particles with increasing sedimentation values as the MEC matures. The MEC appears to be a fragile cell component, and the methods of isolation are still crude, such that the different components of the complex could readily dissociate, resulting in an overall average distribution of activities.

4.9 The MEC in serum stimulated ts2.

To determine whether ts2 cells were capable of assembling complex at its non-permissive temperature, quiescent ts2 were serum stimulated and incubated at 33°C and 39°C. Ten cultures were harvested at each of the following time points: 0, 12, and 24 hours

at 33°C, and at 10 and 16 hours at 39°C. The cells were fractionated using the scheme described for the serum stimulated ts2R, scheme 4 in Materials and Methods. The nuclear and cytoplasmic fractions were sedimented into the previously described discontinuous sucrose density gradients. The gradients were collected in fractions designated A, B, C, and D, and prefixed with c or n to specify their origin as cytoplasmic or nuclear, respectively.

TK, TMPK, and DNA pol α activities were assessed in the gradient fractions (tables 19 and 20). Figure 18 is a graphic representation of these data. The results indicate an induction in the assayed activities in cells incubated at 33°C for 24 hours after the addition of serum. Cells incubated at 39°C after serum stimulation show no (or a much reduced) induction in the enzyme activities. The partitioning of the enzymes from ts2 cells cytoplasmic fraction is somewhat different than that observed with serum stimulated ts2R. TK and TMPK activities are found primarily in the B and C fractions isolated from cells incubated at 33°C for 24 hours after the addition of serum. The DNA pol α activity is observed in fractions A and B, unlike the results obtained with ts2R in which DNA pol α was found primarily in the B and C fractions.

TK activity isolated from this nuclear fraction of ts2 cells 24 hours after the addition of serum and incubated at 33°C is associated with the B and C fractions as observed with the cytoplasmic fraction. DNA pol α and TMPK activities are very low in the nuclear fraction. Presumably, the low activities contribute

Table 19. Assay of activities in different gradient fractions from serum stimulated ts2 (cytoplasmic fractionation).

Fraction	ENZYME ACTIVITIES[b] (x 10^{-2}) TK	TMPK	DNA POL	FMOLES OF TdR CONVERTED[b] (x 10^{-2}) ^{3}H-TdR channeling to TTP	^{3}H-TdR incorp. in vitro
CA 0 hr	22	3.7	0.3	2.2	NIL
12/33	14	6.2	11	3.2	NIL
24/33	7.1	37	21	7.4	NIL
10/39	42	4.5	1.8	5.2	NIL
16/39	56	15	0.7	5.4	NIL
CB 0 hr	75	2.7	2.5	12	1.3
12/33	86	4.9	14	29	1.4
24/33	210	120	18	500	130
10/39	81	4.6	0.2	15	NIL
16/39	92	15	0.4	60	1.4
CC 0 hr	40	30	12	5.9	2.4
12/33	57	3.8	3.7	8.2	1.7
24/33	210	68	7.8	320	19.
10/39	29	3.5	3.4	29	0.3
16/39	23	12	3.9	8.2	0.7
CD 0 hr	9	NIL	1.2	NIL	NIL
12/33	12	1.6	0.4	NIL	NIL
24/33	8.8	6.5	1.6	8.2	0.2
10/39	6	NIL	3	NIL	NIL
16/39	6.7	2.6	0.2	NIL	NIL

a) Quiescent ts2 cells were serum stimulated, harvested, and and fractionated as described in the text. These isolated fractions were immediately assayed for the described activities.

b) Activities were calculated as described in table 4.

Table 20. Assay of activities in different gradient fractions from serum stimulated ts2 (nuclear fractionation).

Fraction[a]	ENZYME ACTIVITIES[b] (x 10^{-2}) TK	TMPK	DNA POL	FMOLES OF TdR CONVERTED[b] (x 10^{-2}) femtomoles of ^{3}H-TdR converted to ^{3}H-TTP	into DNA
	(ENZYME ACTIVITY[b])$_2$ (x 10^{-2})				
NA 0 hr	13	0.4	1.1	NIL	NIL
12/33	NIL	3.1	2.6	0.5	NIL
24/33	30	5.8	3	NIL	NIL
10/39	NIL	3	1.8	NIL	0.2
16/39	5.8	2.7	NIL	0.4	NIL
NB 0 hr	2.2	5	0.6	NIL	NIL
12/33	40	2.6	2.6	0.5	1.1
24/33	130	3.6	2.9	NIL	NIL
10/39	3.3	6.3	1.2	0.7	NIL
16/39	87	2.5	0.3	NIL	0.3
NC 0 hr	38	5.5	1.1	2.5	NIL
12/33	29	3.1	0.7	0.5	NIL
24/33	250	NIL	1.1	0.4	NIL
10/39	61	4.9	0.7	NIL	NIL
16/39	63	4.4	0.3	NIL	0.4
ND 0 hr	10	5	NIL	NIL	NIL
12/33	NIL	6.5	NIL	0.2	NIL
24/33	83	8.1	NIL	0.2	0.2
10/39	13	NIL	NIL	NIL	NIL
16/39	4.3	NIL	3.2	NIL	NIL

a) Fractionation of cells described in the text and table 19.

b) Activities were calculated as described in table 4.

Figure 18. Quiescent ts2 cells were serum stimulated, harvested, fractionated, and assayed as described in figure 17. One unit of activity is defined as described in the Materials and Methods. The activities were corrected for the total fraction volume. Only the activities derived from the "cytoplasmic" fraction are shown.

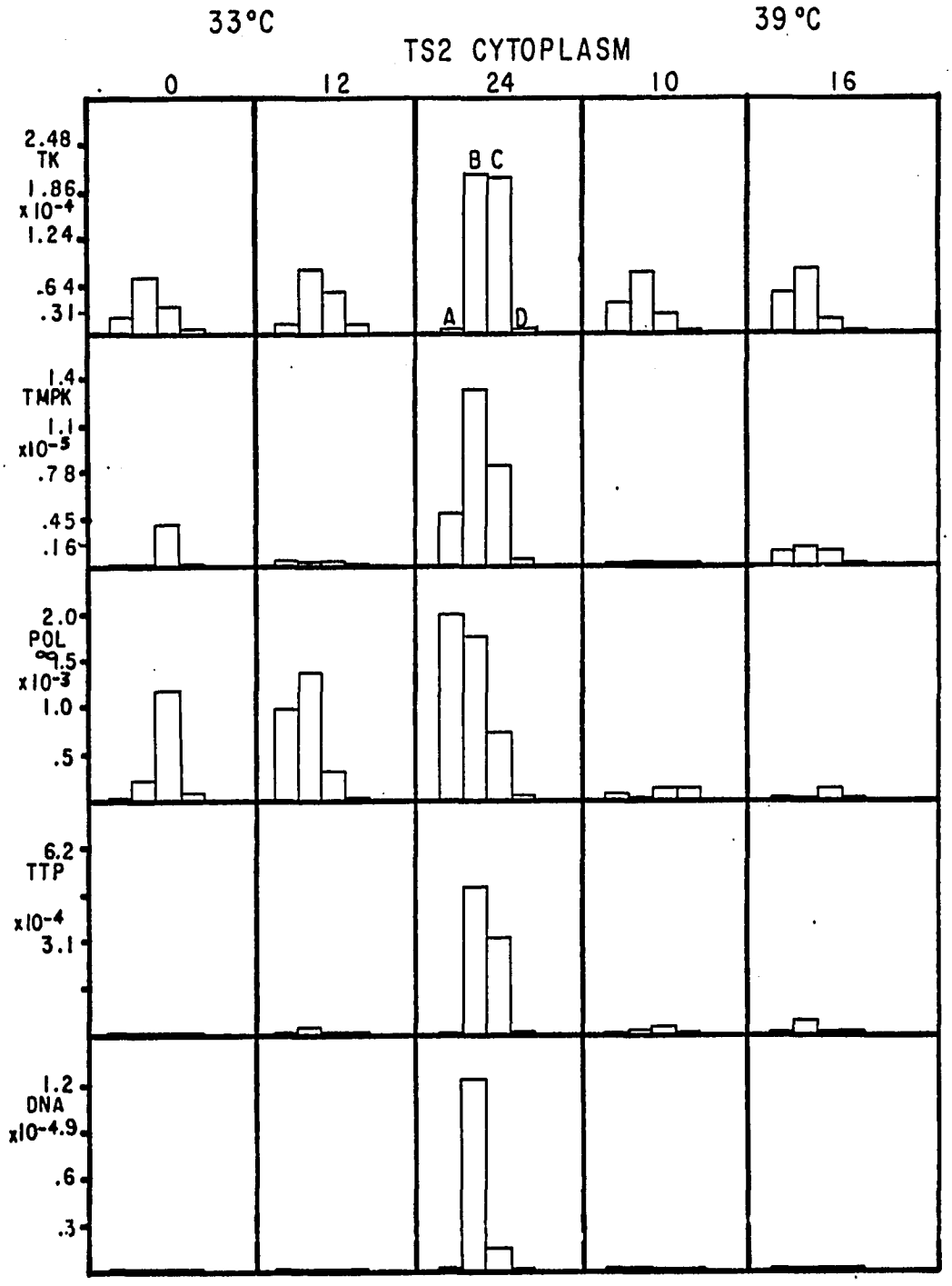

33°C
39°C
TS2 CYTOPLASM
0
12
24
10
16
2.48
TK
1.86
x10-4
1.24
.64
.31
A
B C
D
1.4
TMPK
1.1
x10-5
.78
.45
.16
2.0
POL
1.5
x10-3
1.0
.5
6.2
TTP
x10-4
3.1
1.2
DNA
x10-4.9
.6
.3

FIGURE 18.

to the inability of this fraction to channel ^{3}H-TdR to ^{3}H-TTP and DNA. In addition, as discussed previously, concerning the absence of substrate channeling by the nuclear fraction from ts2R, the method used to extract the nuclei may result in the loss of the ability of the MEC to function coordinately.

The highest levels of ^{3}H-TdR channeling to ^{3}H-TTP and its subsequent incorporation into DNA in vitro is exhibited with the cytoplasmic fraction. Fractions B and C, where the maximal levels of TK, TMPK, and DNA pol α activities are measured, are the most efficient fractions for channeling as was observed with ts2R cells.

These results with ts2 cells when compared with those obtained with ts2R cells indicate the levels of the different activities extracted from ts2R were higher than those observed with ts2, yet the ts2 fractions were as capable of channeling ^{3}H-TdRinto acid-insoluble material. Perhaps on fractionation of the ts2R cells the MEC was damaged resulting in lowered channeling ability or maybe this discrepency is the result of cell line variation yielding a very efficient MEC in ts2 cells. Most likely, the assay for the ability of MEC to channel substrate, was closer to optimal, leading to the observed difference. It should be noted that the assay is performed at a single concentration. It is simply used as an indicator for each separate isolation of MEC, of those fractions capable of conducting DNA synthesis in vitro, and should not be construed as a measure of absolute rates.

The observance of DNA pol α activity in the soluble (A) fraction from the cytoplasm of serum stimulated ts2 incubated at 33^{o}C may represent a deficiency on the part of ts2 MEC assembly.

This tendency may perhaps only be observed with ts2 cells upon serum stimulation since it was readily observed that the majority of DNA pol α distributed into the complex fraction (P-4) in actively growing cells. This possibility was not further pursued since the main objective of these experiments was to disclose whether ts2 cells are capable of assembling complex at 39°C since there is an overall lack of induction of the assay enzyme activities. The ts DNA synthesis exhibited in ts2 cells does not correlate with a structurally thermolabile MEC in actively growing cells or with a readily apparent failure to assemble the complex at the non-permissive temperature after stimulation of quiescent cells. Rather, the ts lesion in ts2 cells may be related to the observed lack of expression of the proteins involved in nucleotide metabolism and DNA synthesis at 39°C and not the activity of the proteins or their coordinate function.

CHAPTER FIVE: The MEC in Mutants of CHO

The putative MEC involved in nucleotide metabolism and DNA synthesis was shown not to be structurally thermolabile in ts2 cells at the non-permissive temperature in our preparations. Other approaches were attempted to further define the MEC contained in the P-4 and replitase fractions. The CHO (Chinese hamster ovary fibroblast) cell line was used because of its convenience and the availability of two sublines which might be affected in a component of the MEC. Two cell mutants were examined, MK42 (Nunberg et al., 1978), and DUK22 (Urlaub and Chasin, 1980). MK42, due to gene amplification, contains a 200-fold increase in the level of DHFR activity. DHFR appears to be a component of the MEC isolated both from the cytoplasm and nucleus, as shown in this text and the literature (Reddy and Pardee, 1980, Noguchi et al., 1983). MK42 was examined to determine whether the enzymes were non-specifically absorbed to the MEC. As DHFR is amplified in a cell, it is assumed that the other members of the complex are not. Therefore, the level of DHFR associated with the isolated MEC fractions should be in ratio to the level of the other complex components. Hence, the level of DHFR activity in the P-4 and replitase fractions isolated from MK42 and its parent cell line, CHO-K1, should be approximately equivalent.

The other CHO mutant examined, DUK22, has only 2% of the wild-type DHFR activity. This residual activity has been shown to

be heat labile *in vitro* at 43°C (Chasin, unpublished data). Since DHFR is a member of the MEC, the possibility of whether a known ts component of the complex would destabilize the MEC on incubation of the cells at the protein's labile temperature was examined.

5.1 The isolation of the P-4 and N.E. fractions from MK42 and its parent, CHO-K1.

The levels of three enzymes, TK, TMPK, and DHFR, identified as components of the putative MEC in this text, and in the literature (Baril et al., 1973, Reddy and Pardee, 1980, Noguchi et al., 1983), were compared in isolated P-4 and N.E. (nuclear extract) fractions isolated from MK42 and CHO-K1 cells. The cells were incubated at 37°C (in the absence of methotrexate), then harvested and fractionated into the N.E. and P-4 as described in scheme 2 in Materials and Methods. In brief, the cells were suspended in hypotonic buffer and Dounce homogenized. The homogenate was centrifuged at low speed and the supernatant removed. The supernatant was subjected to differential centrifugation to yield a post-microsomal fraction, designated S-3. The S-3 was centrifuged overnight at high-speed. The top 80-90% of the centrifugate was removed by bent pasteur pipette and designated S-4; the remaining liquid was designated the P-4. The crude nuclear pellet resulting from the low-speed centrifugation of the cellular homogenate was resuspended in isotonic buffer and extracted by stirring for 2 hours at 4°C. The nuclear fraction was underlayered with 1/3

volume of 60% sucrose dissolved in isotonic buffer and subjected to prolonged high-speed centrifugation. The resultant pellet and sucrose were discarded while the supernatant was dialyzed overnight against hypotonic buffer and 50% glycerol, this fraction was termed the N.E. The S-3, S-4, P-4, and N.E. isolated from MK42 and CHO-K1 cells were assayed for their enzymatic activities (table 21). 98.4% of the TK activity isolated from the crude cytoplasmic fraction from both MK42 and CHO-K1 partitions in the P-4. 86.7% and 91.2% of the TMPK activity also distributes into this fraction isolated from CHO-K1 and MK42, respectively. Similar levels of TK activity were found in the N.E. from MK42 and CHO-K1. These data suggest that components of the MEC, other than DHFR, were not amplified in the MTX selection of MK42. This implies that the two cell lines probably contain similar levels of the MEC.

26.7% of the DHFR-^{3}H-MTX binding activity is found in the P-4 isolated from CHO-K1 and 41.6% in MK42. The actual levels of DHFR in the two P-4 fractions reveal a 40-fold increase in ^{3}H-MTX binding activity in the fractions isolated from MK42 compared to that of CHO-K1. This suggests that a large portion of the DHFR activity in the P-4 from MK42 may be associated with the fraction as non-specifically bound material and not representative of the actual level of the DHFR component in the complex fraction. An abundance of DHFR activity is also noted in the N.E. from MK42 cells when compared to CHO-K1 cells. Additionally, this supports the possibility of excessive non-specific adsorption of DHFR protein to the MEC itself or some other component within this fraction.

Table 21. The Activities in Subcellular Fractions Isolated From CHO-K1 and MK42 Cells.

cell line	subcellular fraction[a]	TOTAL ACTIVITIES[b] TK	TMPK (x 10^{-4})	DHFR
CHO-K1	S-3[d]	470	310	840
	S-4	3.3	21	460
	P-4	190	140	170
	NE	46	ND[c]	190
MK42	S-3[d]	450	220	20000
	S-4	3.4	12	9600
	P-4	210	120	6800
	NE	78	ND	4100

a) 30-100 mm cultures of CHO-K1 and MK42 were grown at 37°C then harvested as described in the text.

b) Activities were calculated as described in table 4.

c) ND = not determined

d) S-3 fraction was stored overnight at 4°C until assayed with the S-4, P-4, and NE.

Several mechanisms were postulated in attempting to account for the excessive level of DHFR activity associated with the MEC fraction in MK42 cells: the abundant DHFR protein is perhaps "sticking" to the complex in some unspecified interation which is promoted by the isolation conditions; the overproduced protein may form large aggregates which co-sediment with the MEC into the P-4; the DHFR protein is binding non-specifically to some other component, besides the MEC, which partitions to the P-4. Various experimental approaches were attempted to decipher which of the above proposed mechanisms were responsible for the high level of DHFR activity in the P-4 of MK42 cells, and to possibly separate the MEC from the excess DHFR to determine more accurately the quantitative level of this protein in the complex. Among the methods employed in efforts to partition the abundant DHFR protein from the MEC fractions were gentle homogenization of the P-4; repeated sedimentation of the MEC fraction through sucrose cushions; incubation of the complex fraction in the presence of chelator, non-ionic detergent, or salt; sedimentation of MEC fraction in sucrose gradients. The result of the use of each of these methods on the distribution of the DHFR protein and the MEC fraction will be discussed in turn.

5.2 Efforts to partition excessive levels of DHFR protein from the MEC fraction..

An initial attempt to isolate the MEC fraction away from the overly abundant DHFR protein present in the P-4 extracted from MK42

cells consisted of the gentle homogenization of the fraction followed by sedimentation into a sucrose cushion in an effort to disrupt proposed large DHFR protein aggregates. The homogenization consisted of rolling the pestle of a 1 ml tissue grinder slowly through the P-4, since it was previously observed in this text, that conventional homogenization of the fraction resulted in decreased enzyme activity. A previously isolated P-4 fraction from MK42 (stored at -70°C) containing known levels of TK and DHFR activities was diluted 5-fold in STKDME-PMSF containing 10mM ATP and 1 mg/ml heat-denatured BSA. The addition of ATP and BSA in the dilution of P-4 had been found to stabilize TK activity in the MEC on subsequent sedimentation of the P-4 (data not shown). The diluted P-4 was divided into two aliquots, one of which was homogenized as described above. Each aliquot was layered onto a 15% sucrose cushion dissolved in TKDME-PMSF, 10 mM ATP, 1 mg/ml BSA and subjected to prolonged centrifugation at 35,000rpm in a Bechman SW50.1 rotor. The top 80% of the centrifuged sample (initial sample + 15% sucrose cushion) was collected by bent pasteur pipette from each cushion and designated S-5. The remaining liquid of each cushion was designated P-5. These resultant fractions were assayed for their TK and DHFR activities (table 22). The data indicate that the TK activity of the P-4 is stable to this homogenization as shown by its ready partitioning to the P-5 on sedimentation into the sucrose cushion. 33 % of the ^{3}H-MTX binding activity is recovered in the P-5 resulting from the homogenized P-4, and 38% from the unhomogenized P-4. Gentle homogenization of the MK42 P-4 prior to sedimentation into a

Table 22. An Attempt To Remove Excess DHFR From the MEC Fraction.

Subcellular[a] fractions	TOTAL ACTIVITIES[b] TK (x 10^{-3})	DHFR
Diluted P-4	170	2200
S-5 recentrifugation	0.4	1600
P-5	160	1000
Homogenization and recentrifugation		
S-5	1.4	1900
P-5	180	900

a) A previously isolated P-4 from MK42 (table 21), stored at -70°C, was treated as described in the text.

b) Activities calculated as described in table 4.

sucrose cushion does not in itself appear to disrupt the unspecific association of DHFR in this fraction. Sedimentation, alone, into a sucrose cushion apparently separates a large level of the DHFR protein.

The re-sedimentation of the P-4 isolated from MK42 cells into a 15% sucrose cushion resulted in a high level of DHFR activity partitioning into the soluble (S-5) fraction. Still, the level of DHFR activity which co-sedimented with the MEC (P-5), as measured by TK activity, was higher than that observed in a P-4 extracted from CHO-K1. We questioned whether a portion of this non-specific DHFR activity, which co-sedimented with the TK activity, would be separable from the MEC after sedimentation in a sucrose gradient during a short high-speed centrifugation time, particularly since the P-4 had been previously frozen. To examine this possibility, two aliquots of a MK42 P-4 (which had been stored at -70°C) containing known DHFR and TK activities were gently homogenized as described above. The aliquots were layered onto 15% sucrose cushions as previously described. One tube was centrifuged for one hour at 45,000 rpm. The upper 80% of the cushion was removed by pasteur pipette and designated S-5A, the remaining 20% was designated P-5A. The second cushion was subjected to prolonged centrifugation at 35,000 rpm. S-5 and P-5 fractions were isolated as previously described. The S-5A, P-5A, S-5, and P-5 were assayed for TK and DHFR activities (table 23). The results suggest that a rapidly sedimenting component of the P-4 from MK42 partitions readily after centrifugation of the fraction for a short time into a sucrose cushion. This component could represent an exceptionally

Table 23. Effect of a Short Sedimentation Time on P-4.

Subcellular[a] fraction	TOTAL ACTIVITIES[b] X 10^{-4} TK	DHFR
P-4	17	220
S-5A	6.6	140
P-5A	3.9	80
S-5	NIL	170
P-5	14	70

a) Origin of these subcellular fractions are described in the text and table 22.

b) Activity = unit activity per assay volume (corrected for the fraction volume).

high order of the MEC, though, more likely, it is composed of non-specific DHFR protein bound either to aggregates of itself or some other unspecified element of the P-4, possibly induced by the freezing or storage at -70°C.

We then attempted to exploit this observation by determing whether repeated sedimentation of the MEC fraction from MK42 cells protein would successfully remove the non-specifically associated DHFR from this fraction. A P-4 fraction from MK42 cells, which had been stored at -70°C was thawed and an aliquot of this fraction containing known TK and DHFR activities was diluted 5-fold in STKDME-PMSF-ATP-BSA, then gently homogenized. The sample was layered onto a 15% sucrose cushion, as described earlier in the text, and centrifuged for 60 minutes at 45,000 rpm. The S-5A and P-5A fractions were collected as described previously. The S-5 was diluted 1.5-fold in STKDME-PMSF-ATP-BSA and layered onto another 15% sucrose cushion. Again the cushion was centrifuged for 60 minutes at 45,000 rpm and collected, generating S-5AA and P-5AA. The S-5AA fraction was centrifuged for 16 hours at 35,000 rpm. S-5 and P-5 were collected. All fractions were assayed for TK and DHFR activities (table 24). The assay results indicate that during each short sedimentation, rapidly sedimenting material partitioned from the MEC fraction. When the S-5AA was subjected to prolonged centrifugation, virtually all the DHFR activity in this fraction co-sedimented with the TK activity.

In view of the ready partitioning of high levels of DHFR activity from the MEC fraction extracted from MK42 cells after repeated sedimentation, we questioned the assumption that the level

Table 24. The Activities of Subcellular Fractions Isolated From MK42.

subcellular[a] fractions	TOTAL ACTIVITIES[b] X 10^{-3} TK	DHFR
S-5A	26	540
S-5AA	14	270
P-5AA	12	180
S-5	0.4	23
P-5	8.1	170

a) Origin of these fractions are described in the text and table 21.

b) As described in table 4.

of DHFR activity associated with P-4 isolated from CHO-K1 cells represented specifically bound activity in the MEC. To assess whether the DHFR activity found in P-4 from CHO-K1 cells represents activity which is specifically associated with the MEC, a P-4 was isolated from actively growing CHO-K1 cells. The fraction (without prior freezing) was diluted 5-fold in STKDME-PMSF- ATP- BSA, gently homogenized, then layered onto a 15% sucrose cushion as previously described, centrifuged for 60 minutes and 45,000 rpm. S-5A and P-5A fractions were collected. The S-5A was layered onto another 15% sucrose cushion and centrifuged overnight at 35,000 rpm, resulting in S-5 and P-5. The fractions were assayed for their TK and DHFR activities (table 25). Upon sedimentation of the S-5A, virtually all the associated TK activity partitions to the P-5, whereas, 44% of the ^{3}H-MTX binding activity distributes in this fraction. Two possibilities were suggested by these data. Some portions of DHFR activity in the P-4 from CHO-K1 cells, though required <u>in vivo</u> by the MEC, are more loosely attached to the complex; thus, they are readily dissociated upon repeated sedimentation. Alternatively, the level of DHFR activity partitioning with the P-4 from CHO-K1 cells is not indicative of the actual level of activity specifically associated with the MEC. These data should also be applicable to MK42. If there is a loosely bound component of the MEC containing substantial levels of DHFR activity, then repeated sedimentation of the MEC fraction of MK42 cells can remove two kinds of activity: a specific activity associated with complex, and a very high non-specific activity. If not all the DHFR activity partitioning into the P-4 of CHO-K1 cells

Table 25. Activities of Subcellular Fractions Isolated From CHO-K1.

subcellular[a] fraction	TOTAL ACTIVITIES[b] X 10^{-4} TK	DHFR
S-5A	77	36
P-5A	9.2	5
S-5	NIL	23
P-5	85	18

a) The origin of these subcellular fractions are described in the text.

b) Activities were calculated as described in table 4.

represents the level required by the MEC, then this suggests that the DHFR protein has a high affinity for the MEC. The very high level of DHFR activity associated with the P-4 of MK42 cells would be largely non-specific. Such high level non-specific binding of the proteins to the complex, of course, posses a problem in determining the actual level of activity associated with the complex. If this latter supposition were true, the use of different reagents, such as salt, chelator, or NP-40 might dissociate the non-specific DHFR activity from the MEC.

To determine whether any of these reagents would effect the partitioning of the proposed non-specifically associated DHFR activity from the MEC fraction, a previously isolated P-4 fraction from MK42 cells was diluted 5-fold in STKDME-PMSF-ATP-BSA and gently homogenized. The fraction was layered onto a 15% sucrose cushion and centrifuged for 60 minutes at 45,000 rpm. The resultant S-5A was divided into 6 aliquots and subjected to different incubation conditions in an attempt to dislodge non-specific DHFR activity. The S-5A aliquots were incubated for 30 minutes at 4°C after one of the following treatments: dilution of the S-5A aliquot 5-fold in buffer containing no salts (STDE-PMSF-ATP-BSA); adjustment of the KCl concentration to 0.5 M KCl (high salt); adjustment of the EDTA concentration to 5 mM; addition of NP-40 to a final concentration of 0.5%; incubation of S-5A in complete buffer (STDME-PMSF-ATP-BSA). One untreated aliquot of S-5A was layered onto a 15% sucrose cushion dissolved in buffer containing no ATP or BSA. Each of the other aliquots were layered onto 15% sucrose cushions dissolved in TKDME-PMSF-ATP-BSA.

All cushions were centrifuged for 16 hours at 35,000 rpm followed by the collection of S-5 and P-5 from each cushion, as described earlier. The fractions were assayed for their DHFR and TK activities (table 26). In each case, the data show that the majority of TK activity partitions to the P-5, whereas, approximately 20-30% fo the ^{3}H-MTX binding activity is found associated with this fraction, except in the case of the S-5A incubated in buffer containing low salts in which it is found that 60% of the DHFR activity distributes to the P-5. Apparently, incubation of S-5A in the presence of a low salt concentration promotes an association of DHFR activity with the MEC fraction. The untreated S-5A aliquot which was sedimented into the sucrose cushion containing no ATP and BSA demonstrates a decrease in the level of TK activity found in P-5, while evidencing no effect on the ^{3}H-MTX binding ability or partitioning of the DHFR. This agrees with data indicating the presence of ATP and BSA as important reagents for the stabilization of TK activity on sedimentation of the MEC fraction. The demonstrated lack of effect of these reagents on DHFR activity or partitioning demonstrates that the observed association of non-specific activity with the complex fraction is not promoted by the presence of ATP and BSA.

Repeated sedimentation of the MEC fraction into 15% sucrose for short lengths of time resulted in the partitioning of high levels of rapidly sedimenting DHFR activity from the MEC fraction from both MK42 and CHO-K1 cells. This suggested that a high percentage of the DHFR activity associated with the P-4 was non-specifically associated with this fraction or a component of a more loosely

Table 26. Effect of Salt, EDTA, and NP-40 On the Partitioning Of the MEC Fraction.

conditions of treatment[a]	subcellular fraction[b]	TOTAL ACTIVITIES[c] X 10^{-3} TK	DHFR
low salt	S-5	0.33	930
	P-5	130	1400
0.5M KCl	S-5	7.8	1500
	P-5	140	670
5mM EDTA	S-5	5.0	1800
	P-5	130	480
0.5% NP-40	S-5	1.3	1800
	P-5	130	810
No treatment	S-5	5.0	1800
	P-5	110	600
without ATP and BSA	S-5	0.3	1800
	P-5	88	830
	S-5A	210	2000
	P-5A	26	280

a) The P-4 treatment conditions are described in the text.

b) Origins of the isolated fractions are described in the text and table 21.

c) Activities calculated as described in table 4.

attached unit of the MEC (possibly in the case of CHO-K1 cells). The above series of experiments suffered from a potential difficulty due to their reliance on a sucrose cushion or short step gradient. Since, only two fractions were obtained it was possible that separation of DHFR aggregates from MEC was being obscured. These data suggested a possibility, that on sedimentation of an S-3 into a sucrose gradient, the bulk of the non-specific DHFR activity or the loosely attached component of the MEC would successfully partition from the MEC activities (measured by TK activity). This would, perhaps, result in a level of DHFR activity which co-sedimented with the MEC reflecting that level of activity required by the complex. Since a low salt concentration was shown to effect the partitioning of DHFR activity (table 26), the sucrose gradients were prepared with high (0.5 M KCl) or low (25mM KCl) salt concentrations to assess the effect of salt on the sedimentation of the different enzyme activities.

CHO-K1 and MK42 cells were harvested to yield an S-3 (scheme 2). The S-3 were each divided into two aliquots. One aliquot from each cell line was made 0.5M KCl, and then all the aliquots were incubated at 4°C for 30 minutes. The S-3 incubated in 0.5 M KCl were each layered onto 3.8 ml sucrose gradients (5-20% sucrose dissolved in TDME-PMSF-ATP-0.5 M KCl) containing 0.5 ml 70% sucrose cushions. The untreated S-3 were layered onto similar gradients prepared with 25mM KCl. The gradients were centrifuged for 18 hours at 45,000 rpm in a Bechman SW50.1 rotor, then collected in 0.3 ml fractions, which were assayed for TK and DHFR activities (figure 19 and 20).

The data on the sucrose gradient analysis demonstrate that the bulk of the DHFR activity does not co-sediment with the TK activity in cytoplasmic fractions isolated from both MK42 and CHO-K1 cells. The S-3 incubated in 0.5 M KCl and sedimented into sucrose gradients containing 0.5 M KCl retards the sedimentation of TK activity by a small degree, and the ^{3}H-MTX binding activity to a larger extent in fractions prepared from both cell lines. These results do not show the presence of aggregates of DHFR protein which are as large or larger than the MEC. Shorter centrifugation times confirmed this result (data not shown). This DHFR activity could represent aggregates of DHFR protein which are small when compared to the size of the MEC or activity associated with some smaller component of the MEC which is easily detached from the complex. The data do not resolve which of these possible phenomenon is occurring. Since the bulk of the DHFR activity does enter the gradient, it explains the observation of high levels of this acitvity in the P-4. Supporting the possibility that this sedimentable DHFR activity is non-specific is the fact that the level of TK and TMPK activities in P-4 is essentially the same for MK42 and CHO-K1 cells. Only the DHFR activities differ in the two cell lines, and in both cell lines this activity partitions from the MEC.

Various methods were employed in efforts to determine whether the use of the MK42 cell line could yield information on the level of DHFR activity present in the MEC, therefore, defining a property of the complex. Although the P-4 isolated from CHO-K1 and MK42 cells contained similar levels of TK and TMPK, a high degree of

Figure 19. Activities of fractions from sucrose gradient analysis of the S-3 fraction from CHO-K1 cells. S-3 fraction was prepared from CHO-K1 cells and subjected to sedimentation in a continuous sucrose gradient as described in the text. Panels A and C represent TK activity, B and D, DHFR-^{3}H-MTX binding activity. The activities of a S-3 sedimentated into a neutral sucrose gradient (5-20% sucrose) are shown in panels A and B. The activities of a S-3 incubated in 0.5M KCl and sedimentation into a 5-20% sucrose gradient containing 0.5M KCl are shown in panels C and D. TK activity was calculated as the average picomoles of ^{3}H-TdR converted per 25 ul assay volume, corrected for the volume of the fraction. DHFR activity was calculated as the average number of picomoles ^{3}H-MTX bound per 25 ul assay volume corrected for the volume of the fraction.

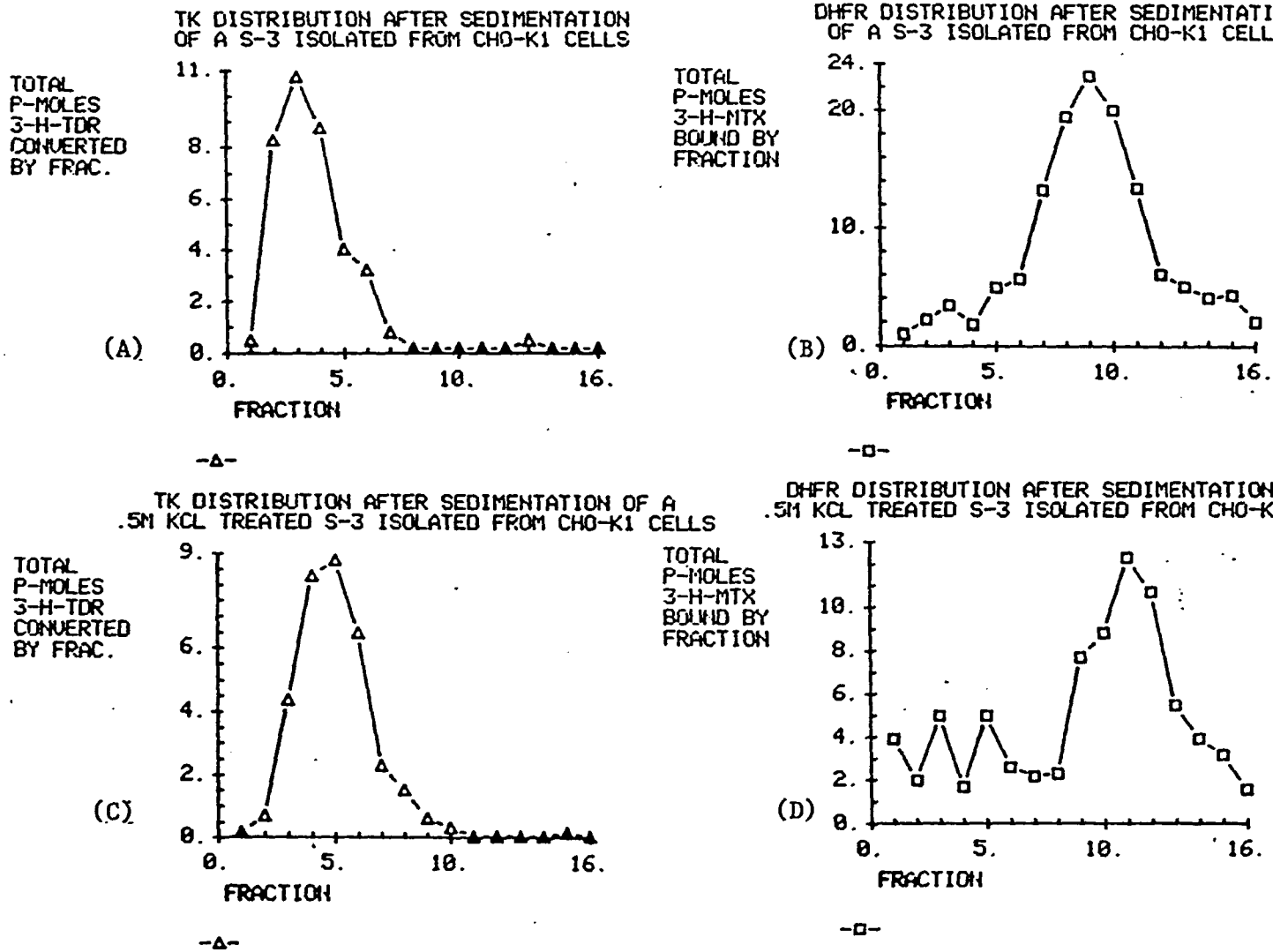

TK DISTRIBUTION AFTER SEDIMENTATION OF A S-3 ISOLATED FROM CHO-K1 CELLS
TOTAL P-MOLES 3-H-TDR CONVERTED BY FRAC.
11.
8.
6.
4.
2.
0.
(A)
0.
5.
10.
16.
FRACTION
-Δ-
DHFR DISTRIBUTION AFTER SEDIMENTATION OF A S-3 ISOLATED FROM CHO-K1 CELLS
TOTAL P-MOLES 3-H-MTX BOUND BY FRACTION
24.
20.
10.
(B) 0.
0.
5.
10.
16.
FRACTION
-□-
TK DISTRIBUTION AFTER SEDIMENTATION OF A .5M KCL TREATED S-3 ISOLATED FROM CHO-K1 CELLS
TOTAL P-MOLES 3-H-TDR CONVERTED BY FRAC.
9.
6.
4.
2.
0.
(C)
0.
5.
10.
16.
FRACTION
-Δ-
DHFR DISTRIBUTION AFTER SEDIMENTATION OF A .5M KCL TREATED S-3 ISOLATED FROM CHO-K1 CELLS
TOTAL P-MOLES 3-H-MTX BOUND BY FRACTION
13.
10.
8.
6.
4.
2.
0.
(D)
0.
5.
10.
16.
FRACTION
-□-

FIGURE 19

Figure 20. Activities of sucrose gradient fractions after sedimentation of S-3 isolated from MK42 cells. The S-3 was prepared from MK42 cells and subjected to sedimentation in a discontinuous sucrose gradient as described in the text. The activities of a S-3 sedimented into a neutral sucrose gradient (5-20% sucrose) are shown in panels A and B. Activities of a S-3 incubated in 0.5M KCl and sedimented in a sucrose gradient containing 0.5M KCl are shown in panels B and D. Panels A and C represent TK activity, B and D, DHFR activity. TK activity was calculated as the average picomoles of ^{3}H-TdR converted per 25 ul assay volume. DHFR activity was calculated as the average picomoles of ^{3}H-MTX bound per 25 ul assay volume. The activities were corrected for the volume of the fraction.

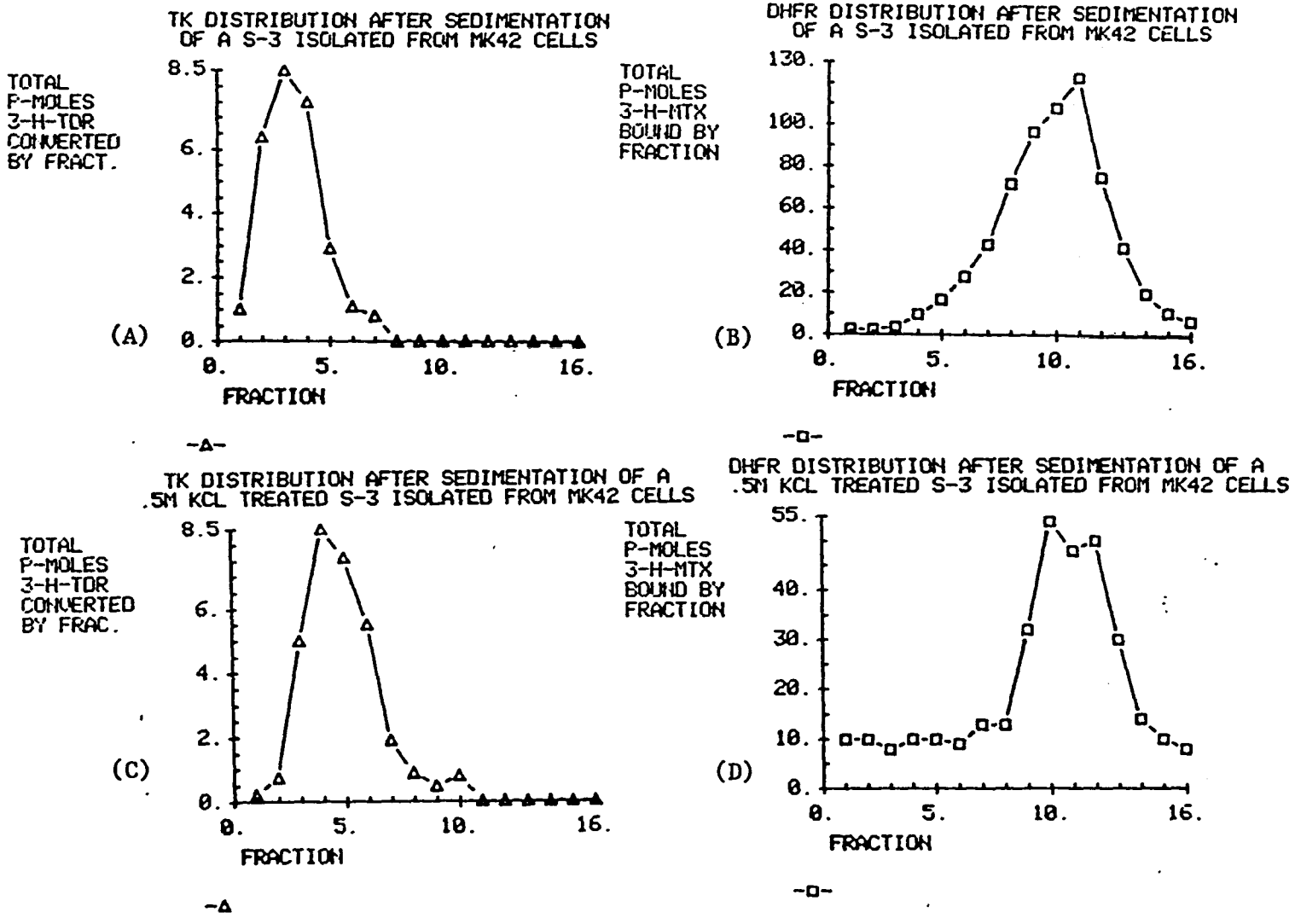

TK DISTRIBUTION AFTER SEDIMENTATION OF A S-3 ISOLATED FROM MK42 CELLS
TOTAL P-MOLES 3-H-TDR CONVERTED BY FRACT.
8.5
6.
4.
2.
0.
0.
5.
10.
16.
FRACTION
(A)
-Δ-
DHFR DISTRIBUTION AFTER SEDIMENTATION OF A S-3 ISOLATED FROM MK42 CELLS
TOTAL P-MOLES 3-H-MTX BOUND BY FRACTION
130.
100.
80.
60.
40.
20.
0.
0.
5.
10.
16.
FRACTION
(B)
-□-
TK DISTRIBUTION AFTER SEDIMENTATION OF A .5M KCL TREATED S-3 ISOLATED FROM MK42 CELLS
TOTAL P-MOLES 3-H-TDR CONVERTED BY FRAC.
8.5
6.
4.
2.
0.
0.
5.
10.
16.
FRACTION
(C)
-Δ
DHFR DISTRIBUTION AFTER SEDIMENTATION OF A .5M KCL TREATED S-3 ISOLATED FROM MK42 CELLS
TOTAL P-MOLES 3-H-MTX BOUND BY FRACTION
55.
40.
30.
20.
10.
0.
0.
5.
10.
16.
FRACTION
(D)
-□-

FIGURE 20

DHFR activity was associated with the P-4 of MK42 cells. Repeated attempts failed to successfully parition this activity from the MEC fraction. Eventually, it was determined that not all the DHFR activity associated with the MEC fraction of CHO-K1 cells would co-sediment with the MEC on repeated sedimentation in sucrose cushions. Although the different experimental approaches for the separation of non-specific DHFR activity from the P-4 have not demonstrated a level of activity representing that which may be required by the complex, it has been shown that the MEC (based on TK activity) is stable to a number of different biochemical criteria. The MEC is stable to gentle homogenization, repeated sedimentation into a sucrose cushion, and sucrose gradients containing high salt concentrations. Additionally, the MEC was shown to be unaffected by non-ionic detergent, concentrated salt solution, and chelation. That the complex remains associated after these manipulations and treatments adds further support that the enzymes of the complex are not a fortuitous aggregation resulting from a fractionation artifact or electrostatic adsorption.

The use of the MK42 cell line to define properties of the P-4 was discontinued based on the repeated failure to clearly determine the level of DHFR activity associated with this fraction.

5.3 Comparison of replitase fractions from CHO-K1 and MK42 cells.

Noguchi et al. (1983) also attempted to determine whether the enzymes of their MEC fraction ,termed replitase, were the result of non-specific adsorption. A test was conducted by these authors

using CHEF/18 cells which overproduced DHFR protein. Even with vast overproduction of the enzyme in the mutant cells, only about 2- to 3-fold more DHFR activity was recovered in the replitase fraction when compared with normal cells, with the surplus activity remaining in the soluble fraction. The distribution of TK activity was also higher in the replitase fraction made from the mutant cells. An effort was made to possibly repeat this observation concerning replitase with the MK42 cells in an attempt to determine the level of DHFR activity required by the MEC.

CHO-K1 and MK42 cells were harvested and replitase extracted from the crude nuclear pellets (scheme 3). Multiple experiments were performed to maximize recovery of the fraction containing "replitase" including concentration of extract, time and intensity of sonication, etc. (data not shown). In brief, cell pellets were resuspended in hypotonic buffer and homogenized. The homogenates were centrifuged at low speed, after which the crude nuclear pellets were collected, and incubated on ice overnight. The pellets were again resuspended in hypotonic buffer, and sonicated at a low setting. The sonicates were centrifuged for 30 seconds in a microfuge, and supernatants were layered onto a discontinuous sucrose density gradient (20-60% dissolved in hypotonic buffer) and centrifuged for 4 hours at 23,000 rpm. The gradients were collected in 0.5 ml fractions and assayed for TK and DHFR activities (table 27). The TK activity is not significantly different between the two replitase fractions indicating the replitase fraction is perhaps similar in the two cell lines. Negligible ^{3}H-MTX binding activity was associated with the

Table 27. Activities of Isolated Replitase Activities From CHO-K1 and MK42.

cell line	TOTAL ACTIVITY[a] X 10^{-5} TK	DHFR
CHO	1.9	NIL
MK42	1.6	NIL

a) Replitase fraction was isolated from CHO-K1 and MK42 cells as described in the Materials and Methods and the text. The replitase fraction was assayed for TK and DHFR activities as described in the Materials and Methods. Activity was calculated as described in table 4.

replitase fraction from either CHO-K1 or MK42 cells. The majority of DHFR activity was observed at the top of the gradients, above the 20% sucrose layer, from both cell lines. The protocol used to isolate the replitase fraction does rid the fraction of most of the over abundant DHFR activity. This is not surprising in that after sedimentation of the S-3 from these cell lines, the majority of DHFR activity did not co-migrate with the TK activity (figures 19 and 20), revealing negligible activity for DHFR within the MEC. These data do not necessarily contradict those of Noguchi et al. (1983). The observed discrepancy may be due to a possible decreased sensitivity of the ^{3}H-MTX binding assay when compared with the DHFR enzymatic assay.

5.4 Effect of various manipulations on the coordinate function of the P-4.

The definition of a MEC we have employed throughout this text is that it is a readily sedimentable fraction containing assayable activities involved in DNA synthesis and which is also capable of channeling ^{3}H-TdR to ^{3}H-TTP and DNA *in vitro*. Evidence to prove that it is, indeed, the MEC channeling substrate to DNA cannot be conveniently obtained. One possible approach to the problem is in determining conditions which disrupt the coordinate function of the MEC, but not the individual enzyme activities of the complex, thereby, obtaining support that it is by the enzymes functioning together as an organized unit in the P-4 that ^{3}H-TdR is incorporated into DNA *in vitro*.

Various conditions to disrupt the coordinate function of the MEC were examined using a freshly prepared P-4 isolated from CHO-K1 cells (scheme 3). Five aliquots of the P-4 were subjected to one of the following manipulations: 1:1 dilution in buffer; dialysis; incubation overnight in an ice bath; freezing; no treatment. These aliquots were then assayed for the ability to channel ^{3}H-TdR to ^{3}H-TDP and ^{3}H-TTP, the subsequent incorporation of the ^{3}H-TTP into DNA, and TK activity (table 28).

These data indicate that the manipulations themselves do not greatly affect TK activity, but decrease the coordinate function of the complex. TMPK activity was determined for both the untreated and diluted P-4 aliquots and observed to be, like TK, not affected by dilution (data not shown). Although TK and TMPK activities did not decrease on dilution of the P-4, a low level of ^{3}H-TDP is produced when ^{3}H-TdR was offered, suggesting that these enzymes could no longer function as a unit. The results of this experiment and those indicating that non-radiolabelled TTP did not dilute the pool of ^{3}H-TTP generated by the MEC are taken as evidence that the complex is a functioning entity.

5.5 DUK22, a CHO mutant containing a temperature-sensitive DHFR protein is also ts for growth.

Another cell system examined in efforts to define properties of the putative MEC was DUK22. As described earlier in the text, DUK22 cells contains low and temperature-sensitive DHFR activity. We postulated that a ts protein in the MEC could lead to a

Table 28. Effect of Different Manipulations On the Coordinate Function Of P-4 From CHO-K1.

P-4 treatment[c]	TOTAL FEMTOMOLES OF ^{3}H-TdR CONVERTED TO[b]			
	^{3}H-TMP	^{3}H-TDP	^{3}H-TTP	DNA
		(X 10^{-3})		
untreated	350	320	280	21
1:2 dilution	200	5.5	4	1.8
dialysis	320	30	24	3.8
4°C for 16 hours	310	ND[a]	ND	6.3
-70°C	340	10	7.1	3.0

a) Not-determined.

b) Activities calculated as described in table 4.

c) Aliquots of a freshly prepared P-4 (scheme 3 in Materials and Methods) were subjected to a variety of conditions. One aliquot was diluted 1:2 in buffer A (scheme 3). Another aliquot was dialyzed overnight at 4°C against buffer B (2.5mM Tris-HCl, pH 7.6, 2.5mM EGTA, 1mM PMSF). In addition, two other aliquots were subjected to overnight incubation at 4°C or freezing overnight at -70°C.

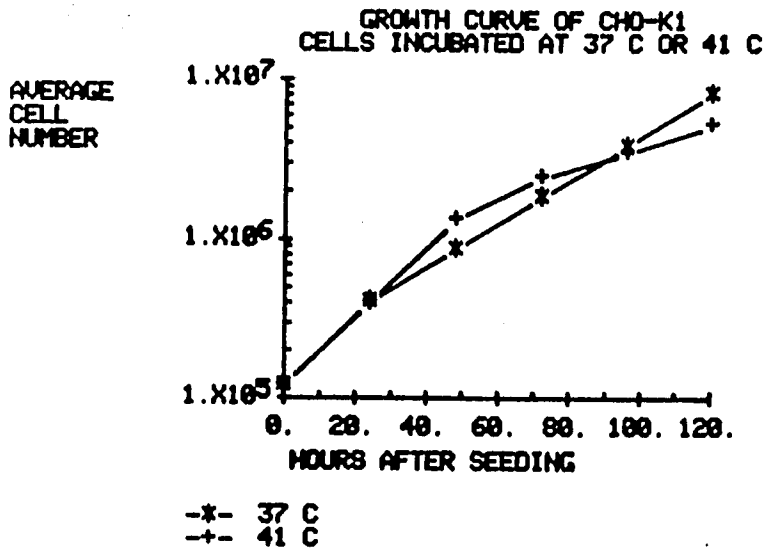
structurally labile complex when the cells are incubated at 41°C.

An initial test of the hypothesis was conducted on the premise that if the ts DHFR protein in DUK22 cells labilized the MEC at 41°C, then we would predict that cells would not be viable at this temperature. DUK22 cells were seeded at low cell density in medium containing glycine, hypoxanthine, and thymidine. These cultures were incubated at either 37°C or 41°C in the presence of these additives which should have by-passed the DHFR deficiency at both temperatures. On each of the next five days, the cells of duplicate cultures at each temperature were counted. CHO-K1 cells, the parental cell line, were treated similarly for comparison. The resultant growth-curves for DUK22 and CHO-K1 cells at 37°C and 41°C are shown in figure 21 (panels A and B). CHO-K1 cells grow well at both 37°C and 41°C, while, DUK 22 cells shown a cessation of growth within 24 hours at 41°C, consistent with the hypothesis.

5.6 Enzymatic activities of isolated P-4 and replitase fractions from DUK22 cells at 37°C and 41°C.

DUK22 cells were incubated at 37°C or for 20 hours at 41°C, then harvested, and the P-4 and replitase fractions were isolated as described in scheme 3, in an attempt to assess whether the ts DHFR protein led to an observable labile MEC. CHO-K1 cells were incubated for 20 hours at 41°C, and the MEC fractions extracted to serve as a control for the DUK22 cells. These fractions were assayed for TK and DNA pol α. The majority of the activities is found in the P-4 isolated from DUK22 at both 37°C and 41°C.

Figure 21. Growth curves for CHO-K1 and DUK22 cells at 37°C or 41°C. Cultures were seeded at approximately 5 x 10^5 cells per 100 mm tissue culture petri dish and incubated at either 37°C or 41°C. Each day after the initial seeding the cells in duplicate cultures at each temperature were counted as described in the Materials and Methods.


GROWTH CURVE OF CHO-K1
CELLS INCUBATED AT 37 C OR 41 C
AVERAGE
CELL
NUMBER
1.X10^7
1.X10^6
1.X10^5
0. 20. 40. 60. 80. 100. 120.
HOURS AFTER SEEDING
-*- 37 C
-+- 41 C

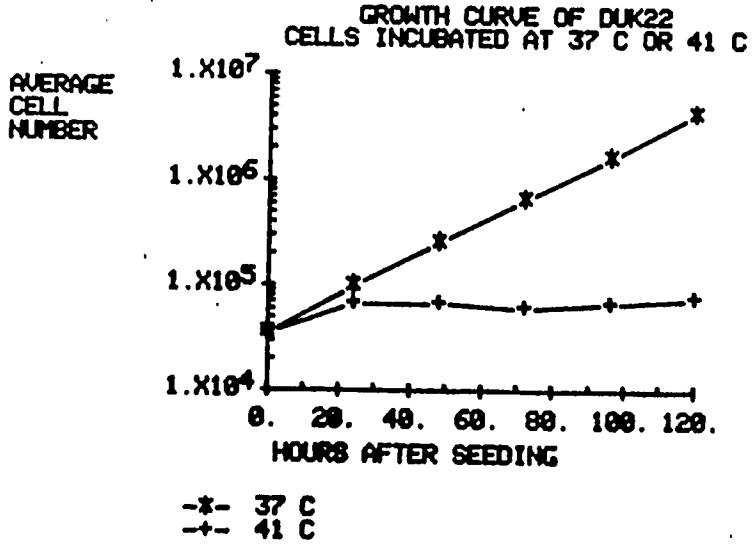

GROWTH CURVE OF DUK22
CELLS INCUBATED AT 37 C OR 41 C
AVERAGE
CELL
NUMBER
1.X10^7
1.X10^6
1.X10^5
1.X10^4
0. 20. 40. 60. 80. 100. 120.
HOURS AFTER SEEDING
-*- 37 C
-+- 41 C

FIGURE 21

The level of the enzymatic activities observed at 41°C in the P-4 from DUK22 cells are lower when compared to those at 37°C (table 29). These data may suggest that the cytoplasmic MEC is functionally thermolabile.

The replitase fractions extracted from DUK22 cells, does not appear to be affected by the temperature of incubation. Perhaps, the cytoplasmic P-4, if a functional component of the nuclear replitase, represents a more thermolabile component of replitase in DUK22 cells.

Also examined was the coordinate function of the P-4 isolated from DUK22 cells incubated at 37°C and 41°C. Table 30 shows the results of the abilities of the S-4 and P-4 isolated from DUK22 cells to channel ^{3}H-TdR to ^{3}H-TTP, then incorporate this ^{3}H-TTP into an offered DNA template. P-4 isolated from DUK22 cells at either temperature can channel ^{3}H-TdR to ^{3}H-TTP and DNA, although, the level of the activity is decreased in the P-4 from cells incubated at 41°C. The MEC fraction from cells at 41°C channels ^{3}H-TdR to ^{3}H-TTP, approximately, at a sixth of the level observed by P-4 isolated from DUK22 at 37°C. There is a 20-fold decrease in the ability to incorporate ^{3}H-TdR into DNA by the P-4 from cells at 41°C. The decreased ability to channel ^{3}H-TdR to ^{3}H-TTP and DNA by the P-4 extracted from DUK22 at 41°C may be reflective of the 7-fold decreased polymerase α activities (table 29), and not a decline in the ability of the MEC to function coordinately. It should be noted that both aspects of thymidine metabolism are sensitive to variations in concentration as observed by the disproportionate effect in channeling activity

Table 29. The Activity in Isolated MEC Fractions from DUK22 and CHO-K1 Incubated at 37°C and 41°C.

subcellular[a] fractions	TOTAL ACTIVITIES[b] X 10^{-3} TK	DNA POL ∝
CHO-K1, 41°C		
S-4	170	5.2
P-4	230	7.9
replitase	1.2	0.1
DUK22, 37°C		
S-4	0.4	NIL
P-4	71	4.8
replitase	2.6	0.5
DUK, 41°C		
S-4	0.4	NIL
P-4	12	0.7
replitase	2.8	0.41

a) 30-100 mm cultures of CHO-K1 growing at 37°C, DUK22 cultures growing at 37°C or incubated at 41°C were harvested, and fractionated (scheme 3) as described in the Materials and Methods and the text.

b) Activity was calculated as described in table 4.

Table 30. Capability of MEC Fraction from DUK-22, Incubated at 37°C and 41°C, to Channel ^{3}H-TdR to ^{3}H-TTP and an Offered DNA Template.

subcellular[b] fractions	TOTAL FEMTOMOLES OF ^{3}H-TdR CONVERTED[c] to ^{3}H-TTP (X 10^{-3})	incorporated into DNA
CHO-K1, 41°C		
S-4	14	0.72
P-4	270	5.1
DUK22, 37°C		
S-4	0.7	0.17
P-4	120	23
DUK22, 41°C		
S-4	ND[a]	NIL
P-4	21	1.1

a) Not determined

b) Subcellular fractions prepared as described in the text.

c) Activities calculated as described in table 4.

as compared to individual enzyme activities as shown in table 28. A 1-fold dilution of P-4 isolated from CHO-K1 shows a decrease in TK and TMPK proportional to the dilution whereas ^{3}H-TdR channeling to ^{3}H-TTP and its subsequent incorporation into DNA is greatly effected.

These results indicate that although DUK22 cells contain a ts DHFR protein which apparently contributes to the observed lack of cell viability at 41°C, it does not demonstrate a structurally thermolabile MEC, as it has been defined and measured. The MEC appears affected in DUK22 at 41°C in its individual enzyme activities, perhaps leading to the observed decrease in coordinate function of the MEC. From these data it was decided that the DUK22 cell line could not be readily exploited to determine the physical properties of the MEC.

CHAPTER SIX: The Effect of SV40 on Growth-Arrested Ts2

The addition of serum to quiescent ts2 cells was shown to induce cellular DNA synthesis along with the TK and TMPK activities only in those cultures maintained at 33°C. A much reduced induction, if any, for these activities was observed at 39°C. In experiments to examine whether a putative MEC is assembled at 39°C in ts2 cells, it was further noted that DNA polα activity was maximally stimulated only in cells incubated at 33°C after the addition of serum.

TK is one of a collection of cellular enzymes induced by infection of cells with either polyoma virus or Simian virus 40 (SV40) (Kit, 1968). This induction is dependent on the A-gene of SV40 as shown by the temperature- sensitive induction of TK activity in infections by a variety of tsA mutants (Postel and Levine, 1976). Such induction is generally thought to occur at the level of transcription; in the case of dihydrofolate reductase induction by polyoma virus, this has been directly confirmed (Kellems et al., 1979).

There are also data indicative that the A-gene product of SV40 is involved in the induction of cellular DNA synthesis. Microinjection of the D2 protein, an adenovirus-SV40 hybrid protein (Patch et al., 1979), stimulates cellular DNA synthesis (Tijan et al., 1978). In labelling experiments to measure cell DNA synthesis, tsA mutants can be either somewhat (Chou and Martin,

1975) or largely (Pockl and Winterberger, 1980) deficient at the non-permissive temperature. Mutants that have a normal A gene, but lacking the small T, induce cellular DNA synthesis (Setlow et al., 1980, Hiscott and Defendi 1979, 1981, Rundell and Cox, 1979).

Since SV40 induces cellular TK and other enzymatic activities along with the stimulation of host DNA synthesis, and since serum has been shown to stimulate these activities in ts2 cells differentially depending on the temperature of incubation, the effect of this virus by infection of quiescent ts2 cells incubated at 33°C or 39°C was examined.

6.1 SV40 infection of growth-arrested ts2 cells.

Quiescent cultures of ts2 cells were prepared as described previously in this text. The medium was removed from these cultures and saved as "conditioned medium." The cultures were rinsed twice with DME and infected by SV40 using 1 ml per dish of a crude viral preparation. The infection was performed for 1.5 hours at 33°C. The virus was removed after this absorption time and 10 ml of conditioned medium was added to each culture. A mock infection of some of the quiescent cultures was performed as a control for the viral infection. The conditioned medium was removed and the cultures were rinsed twice with DME. Ten ml of conditioned medium was added back to each culture. The viral and mock-infected cultures was incubated at 33°C or immediately shifted to 38.5°C. Triplicate cultures were harvested and crude cell extracts prepared to assess the level of TK activity at the

following time points after infection: 0, 8, 12, 16, 20, and 24 hours at 33°C and 39°C. At these time points, the rate of cellular DNA synthesis was also measured by incubating cultures for one hour with 1 uCi/ml ^{3}H-TdR (20 Ci/mmol, NEN), after which the cells were processed to determine the level of ^{3}H-TdR incorporated into acid-insoluble material.

The results of this type of experiment with ts2 cells are represented in figure 22 (panels A and B). Unlike the experiments, described previously, in which serum was used to stimulate growth-arrested ts2 cells, SV40 infection of this quiescent cells induces TK activity at the non-permissive temperature (figure 22A). A partial stimulation of ^{3}H-TdR incorporation is observed at 39°C (figure 22B). Although the level of TK activity appears higher than that observed at 33°C at 24 hours after infection, this may be attributed to the effect of temperature on the rate at which cells cycle. As presented earlier in this text, a revertant of ts2, ts2R, shows the induction of TK and DNA synthesis occuring earlier at 39°C, when compared to 33°C, during a time course followed after the addition of serum (figure 8). Therefore, at 39°C, cells apparently transit the cell cycle faster than those at 33°C. That this may be the case with the TK induction observed in ts2 cells after infection and incubated at 39°C is shown at the 20 hour time point (figure 22A). The stimulation of TK activity is started by this time in cells at 39°C, whereas, for cells at 33°C stimulation does not occur until 24 hours after infection. The data in figure 22A only represents the onset of the induction of TK activity and are not a reflection of the maximal

Figure 22. SV40 infection of quiescent ts2 cells incubated at 33°C or 39°C. Quiescent cultures of ts2 cells were prepared as described in the Materials and Methods. The cultures were infected with SV40 stock virus for 2 hours at 33°C. After infection 10 ml of conditioned medium were added to the cultures and the cells incubated again at 33°C or immediately shifted to 39°C. At various times after infection duplicate cultures were harvested, crude extracts prepared, and assayed for TK activity in duplicate, as described in the Materials and Methods. Protein concentration was determined by the method of Lowry et al. (1951). Sister cultures were assessed for their rate of ^{3}H-TdR incorporation into acid-insoluble material by pulse-labelling these cultures with 1 uCi of ^{3}H-TdR/ml of medium for one hour. The samples were TCA precipitated and the acid-insoluble radioactivity was determined as described in the Material and Methods. (A) average TK activity, (B) the average rate of ^{3}H-TdR incorporation determined in duplicate cultures.

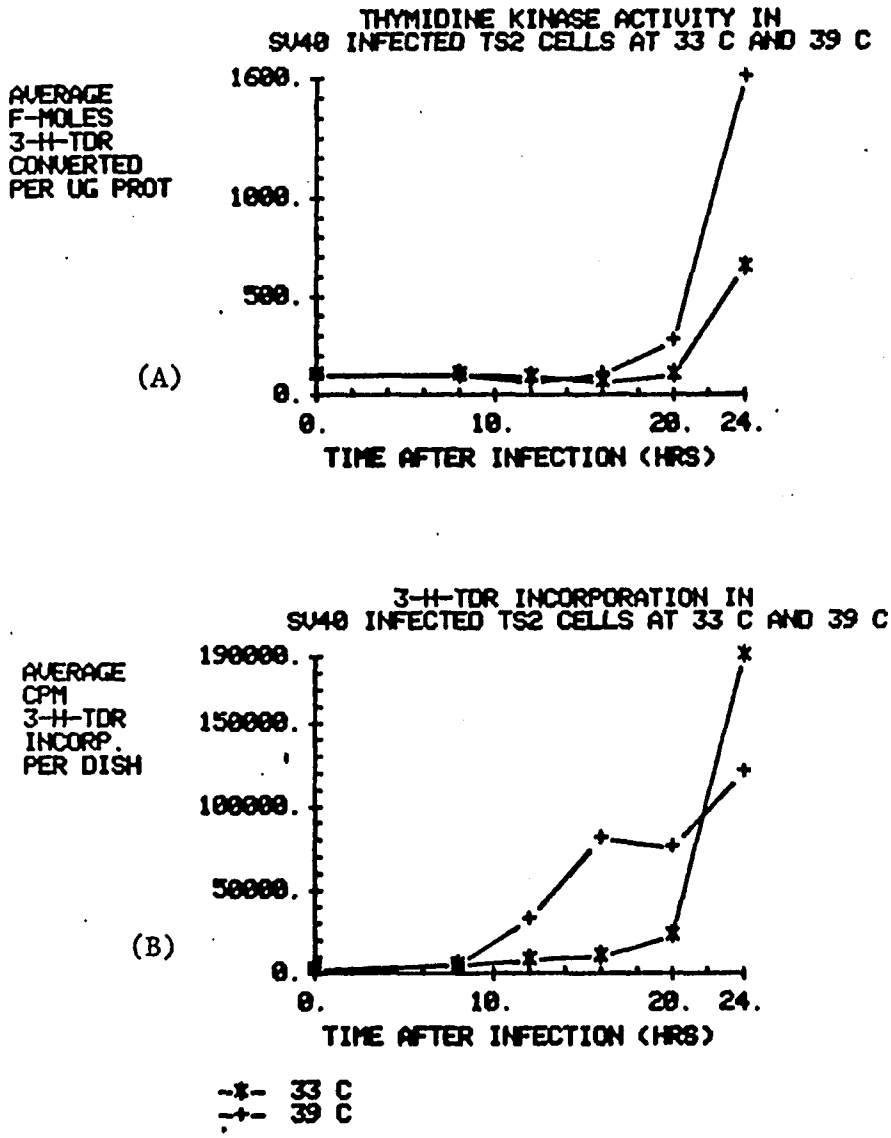

THYMIDINE KINASE ACTIVITY IN
SV40 INFECTED TS2 CELLS AT 33 C AND 39 C
AVERAGE
F-MOLES
3-H-TDR
CONVERTED
PER UG PROT
1600.
1000.
500.
0.
(A)
0.
10.
20.
24.
TIME AFTER INFECTION (HRS)
3-H-TDR INCORPORATION IN
SV40 INFECTED TS2 CELLS AT 33 C AND 39 C
AVERAGE
CPM
3-H-TDR
INCORP.
PER DISH
190000.
150000.
100000.
50000.
0.
(B)
0.
10.
20.
24.
TIME AFTER INFECTION (HRS)
-*- 33 C
-+- 39 C

FIGURE 22

levels of stimulation.

A partial stimulation of DNA synthesis is observed after the infection of ts2 cells which were then incubated at 39°C (figure 22B), when compared to that at 33°C. Although induction of TK activity is observed at 39°C, infection with SV40 as described above only compensates for the ts defect on DNA synthesis to a limited degree.

Two arguments could be raised against SV40 being solely responsible for the induction of TK activity and the partial stimulation of DNA synthesis observed in ts2 cells. First, the cell lysate (SV40 stock virus) used in the SV40 infection could contain cellular factors which are responsible for inducing TK activity and DNA synthesis at the non- permissive temperature. Another possible explanation for the observed results could be that incubating the ts2 cells in the presence of the SV40 stock solution for 2 hours at 33°C permits these cells to pass a thermolabile portion of their cell cycle as they more toward S phase. This last point was essentially ruled out by two pieces of evidence: quiescent ts2 cells, when serum stimulated and incubated at 39°C, were shown to readily induce the expression of c-myc and proliferin mRNA indicating suggesting ts2 is capable of leaving the growth-arrested state at 39°C (Chapter Three). Additionally, Slater and Ozer (1976) reported that ts2 showed a direct correlation between time at 33°C and subsequent DNA synthesis at 39°C. Two hours at 33°C had no significant effect in those experiments, although the conditions of growth-arrest were different.

To determine whether cellular factors from the SV40 stock preparation used to infect ts2 were involved in stimulating DNA synthesis in ts2 at 39°C, SV40 virions were purified from a SV40 stock preparation by banding in CsCl (Materials and Methods). Growth-arrested cultures of ts2 were infected with either a SV40 stock preparation or the purified SV40 virions for 2 hours at 33°C. After infection, conditioned medium was added back to the cultures. These cultures were then incubated at 39°C for 24 hours at which point their rate of DNA synthesis was measured by the incorporation of ^{3}H-TdR into acid-insoluble material during a one hour labelling time. Mock-infected cultures were prepared as described earlier for comparison. Table 31 contains the results of this experiment.

The mock-infected cultures show no stimulation of DNA synthesis. Those cells infected with the viral infected lysate and then incubated for 24 hours at 39°C show an induction in DNA synthesis. Ts2 cultures infected with the purified SV40 virions also show the stimulation in ^{3}H-TdR incorporation into acid-insoluble material. The rate of synthesis observed after infection with the virions is 2-fold higher than that occuring with the SV40 stock prepartion. Also, cells infected with SV40 stock preparation at 39°C for 2 hours, instead of 33°C, reveal no significant difference in the ability to stimulate ts2 DNA synthesis. These observations indicate that the effect is due to SV40 virus, itself. In addition, the incubation of ts2 in the presence of the infected cell lysate for 2 hours at 33°C do not permit the cells to bypass a possible ts lesion in the transition

Table 31. SV40 Infection of Ts2: Comparison of Crude Virus and Purified SV40 Virions.

Virus[a] Source	Incubation Temperatures during infect. °C	post-infect. °C	Time (hrs) post-infect.	cpm ^{3}H-TdR incorporated
SV40 stock preparation	33	33	0	3521 2660
	33	38.5	24	55553 56903
	38.5	38.5	24	59038 47211
purified virions	33	38.5	24	111741 120390
mock	33	38.5	24	1855 1561

a)Quiescent cultures of ts2 cells were infected with either SV40 stock solution or purified virions (10 ug per culture) as described in the text and the Materials and Methods.

from the quiescent state resulting in an ability to partially synthesize DNA.

6.2 The stimulation of DNA synthesis in growth-arrested ts2 by SV40 requires the viral T-antigen.

As discussed earlier in the text, the A-gene product of SV40 has been shown to be involved in the induction of cellular DNA synthesis. An experiment was performed to determine whether the stimulation of DNA synthesis observed in ts2 at both 33°C and 39°C was dependent on the presence of the T-antigen.

Growth-arrested cultures of ts2 cells were infected by a ts mutant of SV40, tsA58 (Teghmeyer, 1970). TsA58 contains a lesion which renders the SV40 T-antigen thermolabile. Sister cultures were infected with a SV40 stock preparation. The infections were performed for 2 hours at 33°C after which conditioned medium was added to the cultures. Other cultures of ts2 cells were stimulated by the addition of DME containing 10% calf serum. All these cultures were incubated at 33°C or shifted immediately to 39°C. The rate of DNA synthesis was determined by pulse-labelling cultures for one hour with ^{3}H-TdR at 12 and 24 hours after infection or the addition of serum.

The data presented in table 32 indicates that the SV40 T-antigen is required for the stimulation of DNA synthesis in ts2 cells at the non-permissive temperature. Wild-type virus induces DNA synthesis at both temperatures with a higher rate of ^{3}H-TdR

Table 32. Tsa58 Infection of Quiescent ts2[a]

Virus infection	Hours (pi)	Incorporation of ^{3}H-TdR (cpm) 33°C	38.5°C
SV40 (SV-S)[b]	0	708	ND
	12	519	1113
	24	15,158	24,105
TsA58	12	387	1902
	24	33,270	5328
uninfected[c]	24	57,677	270

a) Quiescent ts2 cultures were prepared and infected with either SV40 virus or tsA58 virus as described in the text and the Materials and Methods.

b) SV40 stock diluted 1:10 in conditioned medium to mimic titer of tsA58 virus.

c) Fresh medium containing 10% calf serum was added to these uninfected cultures.

incorporation at 39°C than at 33°C at the times selected. The results upon infection with tsA58 are markedly different. Good induction is observed at 33°C, even better than with wild-type. However, induction at 39°C is much reduced. Although incorporation of ^{3}H-TdR is significantly greater than the "negative controls" (i.e., the culture at the time of infection at 33°C or the serum stimulated culture at 38°C), the level at 24 hours post-infection only approximates 15% of the same virus at 33°C. The level of synthesis resulting from infection of ts2 cells with tsA58 and incubation at 33°C is approximately 2-fold higher than that with infection by wild-type SV40. This is most likely due to the titer of the two virus stocks. The wild-type viral stock was diluted to mimic the titer of tsA58. The rate of DNA synthesis observed in ts2 cells after the addition of serum is higher than that of either viral infection. It has been shown that SV40 induces the activity of TK in ts2 cells incubated at either temperature and serum stimulates its activity only at 33°C. The stimulation of DNA synthesis also follows this pattern in ts2, though the stimulation observed after viral infection is only a partial response at either temperature, not on the same order as noted with serum induction. It is in these experiments that the correlation between TK induction and DNA synthesis breaks down. SV40 is capable of generating a full response on the part of TK activity but fails to yield an optimum stimulation of DNA synthesis at either temperature. Whether this partial stimulation of DNA synthesis by virus is due to the inability of the virus to induce a full response when compared to serum or in some unspecified way

related to the conditions used to stimulate the cells was examined.

6.3 Optimization of conditions for stimulation of ts2 cells with serum or SV40.

Variations in the level of DNA snythesis after stimulation of ts2 cells with either serum or SV40 were observed repeatedly in experiments. Some experiments yielded results in which the induction of DNA synthesis was very poor whether SV40 or serum were used. Others showed SV40 having a higher stimulatory effect on quiescent ts2 at 33°C than that observed with serum. Such data suggested that the conditions used to stimulate the growth-arrested ts2 cells by SV40 and serum were not optimized. A series of studies were undertaken to account for the observed variations in the stimulatory response.

Initial experiments were performed to examine the effects of DME and serum on the stimulation of DNA synthesis in ts2 cells. The levels of ^{3}H-TdR incorporation observed previously in ts2 cells after infection by SV40 were markedly lower than those resulting from stimulation with serum. An obvious difference in the conditions between these two types of stimulation was that after SV40 infection of the cells, conditioned medium was added to the cultures, whereas fresh DME was added to cultures used to examine the effect of serum. The results of experiments to examine the effect of fresh serum and DME on both infected and uninfected quiescent ts2 cells are shown in table 33.

Growth-arrested cultures of ts2 cells were rinsed once with DME. The following various medium combinations were added to the cultures: conditioned medium; conditioned medium containing fresh 10% calf serum; 1/2 conditioned medium plus 1/2 fresh DME; 1/2 conditioned medium plus 1/2 fresh DME containing 10% calf serum; fresh DME containing either 0.5% or 10% calf serum. Other quiescent cultures of ts2 cells were infected for hours at 33°C by a SV40 stock preparationAfter infection the cultures were fed with one of the following medium: conditioned medium; 1/2 conditioned medium plus 1/2 fresh DME; fresh DME containing 0.5% calf serum. All cultures were incubated for 28 hours at 33°C after stimulation and their rate of DNA synthesis was measured by their ability to incorporate ^{3}H-TdR into cellular DNA (table 33).

The data demonstrate that the type of medium used to refed the ts2 cells has an influence on the level of ^{3}H-TdR incorporation. The use of conditioned medium to feed cultures appears to inhibit (or greatly reduce) the induction of DNA synthesis in ts2 cells, even after SV40 infection. Conditioned medium containing fresh 10% calf serum does not result in the stimulation of DNA synthesis in quiescent ts2, nor does fresh DME containing 50% conditioned medium. A combination of the two media results in an improved but partial response; the stimulation is 20-fold, but less than 10% of that observed with cultures fed with fresh DME containing 10% calf serum (CS). Cultures fed with fresh DME containing 0.5% CS respond with a low level of ^{3}H-TdR incorporation, less than 5% of that resulting from fresh DME containing 10% calf serum. SV40-infected

Table 33. Effect of medium on the rate of ^{3}H-TdR incorporation by SV40 infected quiescent ts2 cells at 33°C.[a]

medium conditions	SV40 infected ts2 (cpm)	uninfected ts2 (cpm)
conditioned medium[b]	791 572	317 209
cond. med. + 10% C.S.[c]	ND[d]	208 247
1/2 cond. med. + 1/2 DME	4037 4495	465 480
1/2 cond. med. + 1/2 DME + 10% C.S.	ND	9917 9236
DME + 0.5% C.S.	65716 50917	3771 2827
DME + 10% C.S.	ND	117136 111300

a) Cultures were growth-arrested, then infected with SV40 stock virus as described in the Materials and Methods. The rate of ^{3}H-TdR incorporation was determined by pulsing the cultures for one hour at 33°C (28 hours after infection) with 1 uCi/ml of ^{3}H-TdR. These cultures were then washed 2 times with cold PBS and processes to determine the amount of radiolabel incorporated into acid-insoluble material (see Materials and Methods).

b) Conditioned medium.

c) Calf serum.

d) Not determined.

ts2 cultures fed with fresh DME containing 0.5% calf serum after infection show a strong level of ^{3}H-TdR incorporation, approximately 50% of that observed in uninfected cultures fed with fresh DME containing 10% calf serum. A full stimulatory response in DNA synthesis by uninfected ts2 cells was only observed in cultures fed with fresh DME containing 10% calf serum.

The results of this experiment demonstrate that in infected ts2 cells it is the combination of serum and fresh DME that stimulates the induction of DNA synthesis observed. The data in table 33 are suggestive of a possible inhibitor of the induction of DNA synthesis being present in conditioned medium, although it does not inhibit the expression of TK activity, as observed earlier in this chapter. Data presented by Holley, et al., (1978) indicate that density-dependent regulation of growth in BSC-1 cells, a monkey epithelial cell line, results from the combined effects of inhibitors formed by the cells, decreased availability of receptor sites for serum growth factors as the cells become crowded, and limiting concentrations for low molecular weight nutrients in the medium. Additionally, the data of Downes et al., (1983) indicate that cellular uptake of nucleosides is inhibited as the cells in culture condition their growth medium. The inhibition of uptake acts at the level of nucleoside phosphorylation. Schor and Rozengurt (1973) presented results suggesting that the suppression of nucleoside uptake by conditioned medium in mouse 3T3 cells may directly maintain cellular quiescence.

Additional evidence for the presence of a proposed inhibitor(s) for DNA synthesis in the conditioned medium of ts2 cells was

obtained after observing that manipulation of the ts2 cultures during the rinsing of the cells prior to re-feeding had an effect on the level of stimulation of DNA synthesis. If the cultures are rocked repeatedly in fresh DME after infection of the cells with SV40 or before the addition of fresh medium to study the effect of serum, it is observed that the level of ^{3}H-TdR incorporation into the cells is further increased as shown in table 34. As in previous experiments, both virus and medium change with 10% serum result in significant (greater than 15-fold) enhancement over basal level or medium change in low serum alone ("standard conditions"). However, this incorporation can be markedly enhanced further by more vigorous washing. Stimulation in 0.5% serum is increased slightly; approximately 2- to 3-fold but remains at a low level (less than 10^{4} cpm). The stimulation with 10% serum is enhanced 7-fold to 40-fold the value in 0.5% serum. Virus infection (in 0.5% serum) results in the highest observed level, approximately 100-fold that in 0.5% serum and 1000-fold the basal level.

Thus, these data demonstrate that both the type of medium employed in studying the effects of SV40 and serum stimulation of ts2 cells as well as the manipulation of these cultures by just washing the cells play a role in the level of ^{3}H-TdR incorporation observed. These two conditions, together, would seem to explain the seeming variation in DNA synthesis induction noted in different experiments. If an inhibitor(s) were present in conditioned medium of growth regulated cells, the simple removal of the medium may not entirely rid the culture of this agent. This possibiltiy is supported by the data of Harel, et al., (1978)

Table 34. Effect of culture washing conditions on the rate of ^{3}H-TdR incorporation[a].

wash conditions[c]	medium	infection[b]	incorporation[d] (10^{-4})
Standard	0.5% serum	-	0.3
Vigorous	0.5% serum	-	0.8
Standard	0.5% serum	+	5.9
Vigorous	0.5% serum	+	100.
Standard	10% serum	-	4.8
Vigorous	10% serum	-	31.9

a) Quiescent cultures of ts2 were prepared as in previous tables.

b) The medium was removed, and those cultures indicated were infected with a SV40 stock preparation as described in the Materials and Methods.

c) Infected and uninfected cultures were washed twice with DME as previously described or vigorously with agitation.

d) ^{3}H-TdR incorporation into acid insoluble material was measured by pulsing cultures for one hour at 33°C (28 hours after stimulation) with 1 uCi/ml of ^{3}H-TdR (20 Ci/mmole, NEN), then processing the cultures as described in the Materials and Methods. The zero time point = 862 cpm.

indicating that the incorporation of phosphate into cells and the phosphorylation of small organic compounds were increased by shaking dense cultures of Swiss 3T3 mouse fibroblasts. These authors suggest that an inhibitor(s) is released by dense cultures of 3T3 cells; therefore, it appears that shaking dense cultures would not increase phosphate metabolism and DNA synthesis by changing the cellular uptake of serum components, but instead, by increasing the release from cells (or the close proximity of cells) of an inhibitory factor into the medium.

After optimization of the conditions to maximize the response of quiescent ts2 cells to serum and SV40 stimulation, the effect, if any, that these conditions would have on the cells at their non-permissive temperature was examined. Quiescent ts2 cultures were infected with a SV40 stock preparation for 2 hours at 33°C, washed repeatedly, with agitation with fresh DME. Fresh DME containing 0.5% calf serum was added to the cultures and the cells incubated at 33°C or 39°C. At various times after infection the rate of DNA synthesis was measured by pulsing the cultures for one hour with ^{3}H-TdR(figure 23). Sister cultures to those which were infected by SV40 were handled similarly and refed with fresh DME containing 10% calf serum for incubation at 33°C or 39°C. At 16 and 28 hours after serum stimulation the rate of DNA synthesis was measured. The optimized stimulation conditions do not alter the overall effects of SV40 and serum on quiescent ts2 at 33°C and 39°C. DME containing 10% calf serum is capable of stimulating a response in DNA synthesis only in uninfected ts2 cells incubated at 33°C and not in those cells at 39°C at any

Figure 23. The effect of SV40 infection on quiescent ts2 cells incubated at 33°C and 39°C. Quiescent cultures of ts2 cells were prepared, and infected with SV40 stock virus solution as described in Materials and Methods. The rate of ^{3}H-TdR incorporation into acid-insoluble material was measured by pulse-labelling duplicate cultures for one hour at each time point with 1 uCi of ^{3}H-TdR/ml of medium. The cultures were then processed to measure the level of radiolabel incorporated into acid-insoluble material, as described in the Materials and Methods.

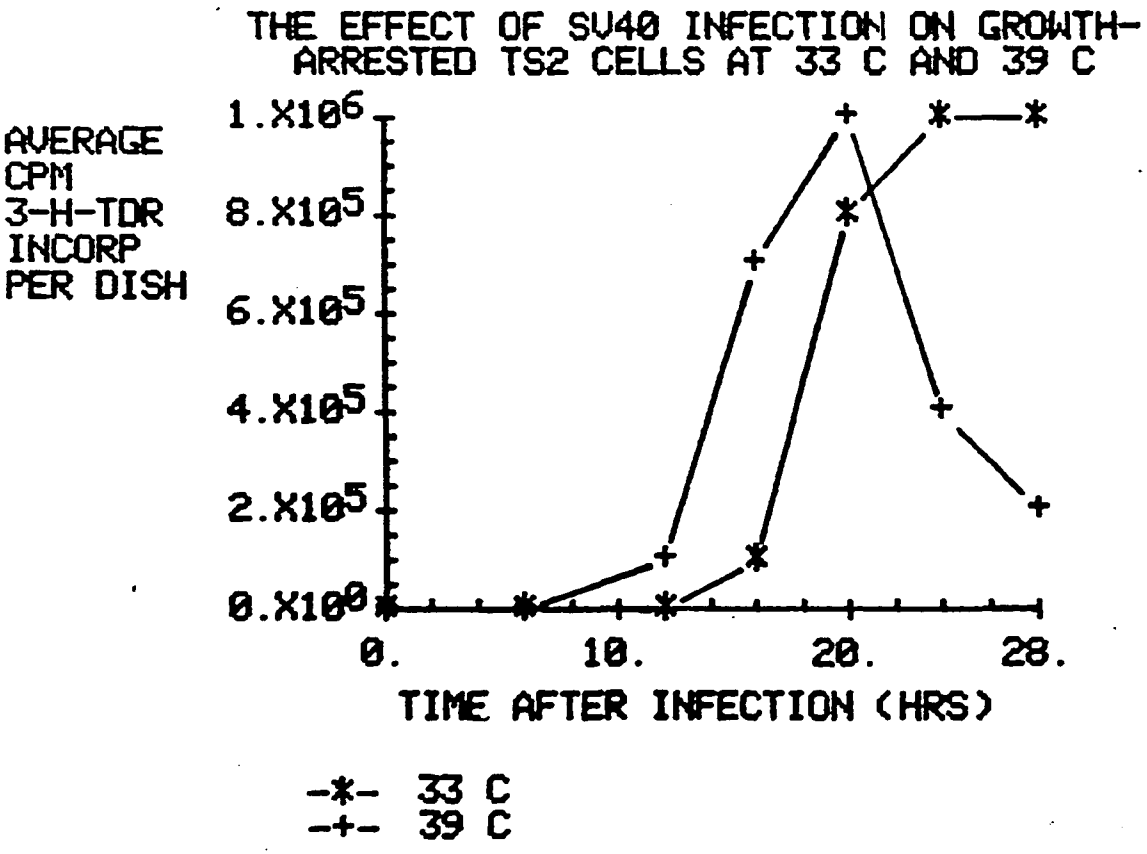

THE EFFECT OF SV40 INFECTION ON GROWTH-ARRESTED TS2 CELLS AT 33 C AND 39 C
AVERAGE CPM 3-H-TDR INCORP PER DISH
1.X10^6
8.X10^5
6.X10^5
4.X10^5
2.X10^5
0.X10^0
0.
10.
20.
28.
TIME AFTER INFECTION (HRS)
-*- 33 C
-+- 39 C

FIGURE 23

time after the addition of serum: SV40 infection of quiescent ts2 cells, on the other hand, successfully induces DNA synthesis at both temperatures (figure 23). DNA synthesis at 39°C shows a sharp peak at 20 hours post-infection, whereas, a comparable level at 33°C is reached at 24-28 hours post infection. Negligible incorporation is observed with the medium change in low serum at either temperature at any time, as expected. The rate of DNA synthesis in cells infected by SV40 and incubated at 33°C is approximately 2-fold higher at 28 hours than observed in the ts2 cells stimulated with DME containing 10% CS.

The number of nuclei in the ts2 cultures participating in the induction of DNA synthesis by serum and SV40 were determined by autoradiography of ^{3}H-TdR labelled nuclei (table 35). Two hundred nuclei were counted in each of three cultures at each time point, each number is an average of the determined values. Also represented in table 35 are the number of nuclei which stained positive for the SV40 large T-antigen by indirect immunofluorescence. These values also represent the average of determinations made in triplicate cultures. On serum stimulation of growth-arrested ts2 cells with DME containing 10% calf serum, approximately 50% of the culture participates in the induction at 33°C, whereas, only 12% of the nuclei are labelled at 39°C. Ts2 cells infected by SV40 show 60-80% of the culture population at both 33°C and 39°C taking part in the induction. The SV40 T-antigen staining of infected ts2 nuclei show a similar level of positive nuclei, approximately 70-80% of the cells stain for the T-antigen. These data support the results obtained on the rate of

Table 35. The Nunber of Quiescent Ts2 Cells Participating in the Stimulation by Serum or SV40 infection.[a]

Conditions	Temperature °C	Hours after serum or infect.	DNA synthesis (percent positive nuclei)	T-antigen (percent positive nuclei)
uninfected (0.5% C.S.)	33	4	4	0
		28	8.5	0
	39	4	0.5	0
		28	7	0
uninfected (10% CS)	33	4	0	ND[b]
		28	45	ND
	39	4	2	ND
		28	12	ND
infected	33	4	0	1
		28	77	81.5
	39	4	0	0
		28	61.5	68.5

a) 200 nuclei were counted in each culture. Values reported represent an average of the determinations. Triplicate cultures were analyzed for each point for both SV40 large T-antigen positive nuclei and ^{3}H-TdR labelled nuclei, as described in Materials and Methods.

b) Not determined.

DNA synthesis indicating that infection of ts2 cells by SV40 was higher at 33°C than that occuring after stimulation of the cells with DME containing 10% calf serum. There appears to be a greater participation of the culture population after infection by SV40 than after serum stimulation.

6.4 The effect of SV40 on the growth of ts2 cells at 39°C.

Since SV40 infection of growth-arrested ts2 cells induced TK activity and cellular DNA synthesis at both 33°C and 39°C in a high proportion of the population, the effect of the virus on the abiltiy of the cells to complete the cell cycle was examined. An experiment was performed to determine whether the stimulated cells at 33°C and 39°C would complete S phase and go on to divide.

Quiescent cultures of ts2 were stimulated by either SV40 infection or the addition of serum. The cells were incubated at 33°C or 39°C. Daily for 5 days, the cells in duplicate cultures for each stimulation condition were counted. A parallel series of cultures were subcultured onto coverslips in the appropriate medium for determining population participating in DNA synthesis (cells were labelled for 24 hour periods). The number of SV40 T-antigen positive nuclei were also determined in the latter cultures infected by virus. The results of this experiment are shown in table 36.

Cells stimulated by the addition of serum show a 34% increase in cell number at 33°C after 48 hours, while 42% of the nuclei in the population after 24 hours were labelled with ^{3}H-TdR. The

Table 36. The Effect of Serum on SV40 Stimulation on the Cell Number in Quiescent ts2 cultures.[a]

Hours after stimulation		CONDITIONS: uninfected (10% CS) 33°C	uninfected (10% CS) 39°C	infected (0.5%) 33°C	infected (0.5%) 39°C
0	cell number[b] (10^5)	6.0	----	----	----
	% DNA synth.[c]	----	----	----	----
	% virus infect[d]	----	----	----	----
24	cell number (10^5)	6.2	6.2	6.4	6.4
	% DNA synth.	42	8.5	84.5	78
	% virus infect.	----	----	76.5	71
48	cell number (10^5)	8.1	5.5	9.4	9.1
	% DNA synth.	31	3.5	72.5	56
	% virus infect.	----	----	12	8.5
72	cell number (10^5)	9.9	5.1	9.5	9.4
	% DNA synth.	7	1.5	16	8.5
	% virus infect.	----	----	13.5	10.5
96	cell number (10^5)	9.6	4.8	9.5	9.1
	% DNA synth.	4.5	0	12	12
	% virus infect.	----	----	15	13.5
120	cell number (10^5)	9.6	4.2	9.5	8.2
	% DNA synth.	2	1	7	4
	% virus infect.	----	----	9.5	2.5

a) Quiescent ts2 cultures were prepared and treated as described in the text.

b) Average of duplicate cultures.

c) Determined by autoradiography of ^{3}H-TdR labelled cultures (Materials and Methods). Cultures were labelled for 24 hours prior to fixing. 200 nuclei counted in triplicate cultures. Values represent an average.

d) Positive nuclei determined by indirect immuno-fluorscence (Materials and Methods). Values represent an average of triplicate determinations.

serum stimulated cultures at 39°C do not demonstrate any increase in cell number at anytime during the 5 day time course.

The SV40-infected ts2 cultures show an increase in cell number by 48 hours at both 33°C and 39°C of 56% and 51%, respectively. Twenty four hours after infection 85% of the nuclei are labelled in cells at 33°C, while at 39°C, 78% of the nuclei have been stimulated to replicate DNA. It is to be noted that SV40 infection of the quiescent ts2 cells causes only one round of cell division at either temperature since no increase in cell population occurs after 48 hours. This presumably is due to a decrease in the expression or presence of SV40 sequences as demonstrated by the decline in the number of T-antigen positive nuclei after 48 hours. Serum stimulation of ts2 cells subsequently incubated at 33°C shows an increase in cell number also after 72 hours unlike SV40-infected cells at either temperature. Whether this represents cells undergoing a second round of cell division or a population of cells with a long transit through a single cell cycle was not explored.

The stimulation of ts2 cells by SV40 infection induces TK activity and DNA synthesis at both 33°C and 39°C. This induction is a full cellular response to the stimuli in that it results in cell division at both temperatures for at least one cell cycle after acute infection. This ability on the part of SV40 suggests that virus may stimulate a cellular pathway toward DNA synthesis and cell division, by passing the ts2 ts defect. Serum stimulation, on the other hand, is unable to do this, thereby, suggesting the possible existence of different stimulation pathways

resulting in DNA replication and ultimately cell division.

6.5 SV40 transformants of ts2.

The observations that the large T-antigen of SV40 was capable of stimulating the induction of DNA synthesis in quiescent ts2 at 39°C (table 32), and the correlation between the presence of the T-antigen and persistent synthesis at either 33°C or 39°C (table 36), suggested that a SV40 transformant of ts2 could be capable of growing at the non-permissive temperature.

SV40 transformants of ts2 were obtained in the following manner. Subconfluent cultures of ts2 were infected for 2 hours at 33°C with a SV40 stock preparation. After infection the cultures were washed thrice with DME. Ten ml of fresh DME containing 10% calf serum was added to the dishes and the cultures incubated at 33°C for five days. Cells were then subcultured and incubated at 33°C for several weeks. Seven foci of phenotypically transformed cells were picked using a wooden dowel. These foci were grown up in DME containing 10% calf serum at 33°C. The cells from all 7 foci stained positive for the presence of the SV40 T-antigen by indirect immunofluorescence.

To determine whether these transformants were capable of growth at 39°C, each of the seven cell strains were seeded at 10^4 cells per 60 mm tissue culture dish. These cultures were incubated at either 33°C or 39°C. Those cells incubated at 39°C did not grow over a 7 day period, while the cells at 33°C grew to subconfluence. Thus, although the SV40 T-antigen is

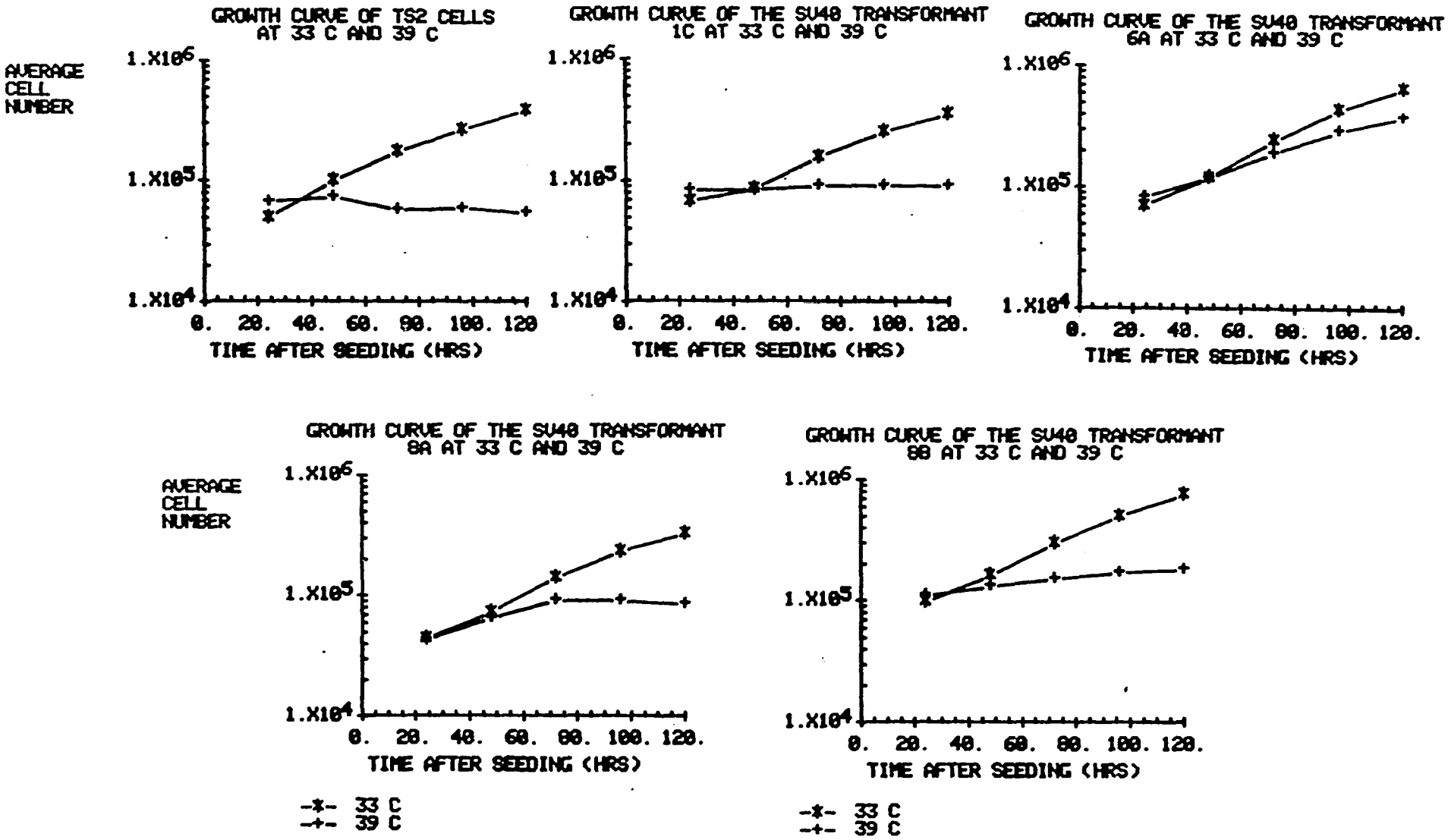
present in these transformants it does not render ts2 capable of long-term growth at 39°C.

Whether the SV40 transformants of ts2 were able to grow for a short time at 39°C was next examined. Ts2 cells along with four of the SV40 transformant cell strains (designated 1C, 6A, 8A, 8B) were seeded at low cell density into 60 mm tissue culture dishes. These cultures were incubated at 33°C and 39°C and cell number determined daily. The growth curves for each cell line at both 33°C and 39°C are diagramed in figure 24. Three of the transformants stop growing early in the 5 day period at 39°C. 6A appears to grow slowly at 39°C when compared to cultures at 33°C, but nonetheless, does grow over the 5 day time course.

We examined the possibility that the behavior of 6A may be associated with an increased expression of the T-antigen in these cells. Extracts were prepared from ts2 cells and the transformants 1C, 6A, and 8A using the procedure for the preparation of extracts for immunoprecipitation and examined by Western analysis (see Material and Methods). Similar minimal levels of T-antigen were detected in all the transformants.

In this chapter data were presented indicating that the small DNA tumor virus, SV40, is capable of stimulating TK activity and DNA synthesis in quiescent ts2 cells. Unlike serum this induction occured at both 33°C and 39°C. Infection of ts2 cells with the ts mutant of SV40, tsA58, demonstrate that the stimulation of cellular DNA synthesis at 39°C was dependent on the SV40 T-antigen. Additionally, it was found that the cells stimulated by SV40 infection were capable of cell division at either 33°C or

Figure 24. Growth curves for SV40 transformed ts2 cells at 33°C and 39°C. Cultures were seeded at 1 x 10^5 cells per 60 mm tissue culture petri dish. The cultures were incubated at 33°C or 39°C. Each day after seeding the cells in duplicate cultures at each temperature were counted as described in the Materials and Methods. The ts2 curve presented was obtained using low passaged cells. Later passaged ts2 show essentially no difference (data not shown).



-210-
Figure: 24

39°C. There apparently, is only a single round of division by the infected cells, perhaps due to dilution of viral sequences. SV40 transformants of ts2 were shown to be unable to grow for extended periods at 39°C, even though T-antigen is present in these cells. This inability may be related to a difference in the levels of T-antigen present in cells after acute infection versus that required for cell transformation. One transformant, 6A, was shown to grow slowly at 39°C when compared to cells at 33°C. The increased ability of 6A to grow for an extended short time at 39°C does not appear, on Western analysis, to be attributed to a higher expression of the T-antigen protein when compared to the other transformants. This ability to slowly grow at 39°C could be related to some unspecified genetic change in the cell strain rendering a "leaky" phenotype for growth a 39°C.

Medium conditions and culture manipulation of growth-arrested ts2 cells were demonstrated as important for the levels of ^{3}H-TdR incorporation observed. Data concerning the use of conditioned medium to stimulate quiescent ts2 cells suggest that an inhibitor(s) may be present in conditioned medium which prohibits a full DNA synthesis response. When conditions for the stimulation of growth-arrested ts2 were optimized, it was observed that SV40 was fully capable of inducing a high level of DNA synthesis at both 33°C and 39°C. This high level of induction was attributed to the increased number of in the virally stimulated population which participated in the induction of DNA synthesis.

Chapter Seven: DISCUSSION

Few mutants involved in mammalian cell DNA synthesis have been isolated. One cell line, ts2, derived from Balb/3T3 cells is temperature-sensitive for DNA synthesis (Slater and Ozer, 1976). The mutation in ts2 cells was also shown to affect the replication of polyoma DNA at the non-permissive temperature. Genetic analysis of ts2 in cell hybrids revealed that the defect is corrected by the human X-chromosome and recessive in hybrids with other mouse cells containing a ts lesion in a non-S phase function (Jha et al., 1980). Ts2 cells were not complemented in hybrids with other ts mutants of DNA synthesis. The replication of the retrovirus, murine leukemia virus (MuLV), was also recently examined in this mutant (Richter et al., 1984). The mutation was found to inhibit MuLV production when the cells were shifted up to the non-permissive temperature early in the infection cycle. The level of unintegrated linear MuLV DNA was not notably affected when ts2 were incubated at 39°C, but the level of supercoiled viral DNA was significantly lowered at this temperature. The MuLV linear DNA at the non-permissive temperature was shown by restriction endonuclease analysis not to contain any structural modifications, but these molecules exhibited poor infectivity in transfection assays.

In this text, additional characterization of ts2 cells was

presented. FMF analysis of actively growing ts2 cells shifted to 39°C suggested an accumulation of these cells within the S phase with increasing incubation time at the non-permissive temperature, lending further support that ts2 cells are ts for DNA synthesis. The levels of DNA synthesis and different enzyme activities associated with S phase cells were also examined in ts2 cells incubated for various lengths of time at the non-permissive temperature. The rate of DNA synthesis declined in these cells (measured by ^{3}H-TdR incorporation) during the time course. TK activity also exhibited a corresponding decrease, while, TMPK activity was shown not to be significantly affected by incubation at 39°C.

Several lines of evidence argue against the inability of ts2 cells to incorporate ^{3}H-TdR at 39°C as being the result of a thermolabile TK activity. First, the TK locus is not located on the human X-chromosome which complements the ts mutation (Jha et al., 1980). Second, when actively growing ts2 revertant cells were shifted to 39°C, a decrease in TK activity was also observed, as in ts2 cells, but, no affect on cellular DNA synthesis was noted. This suggests that the decline in TK activity may be intrinsic to the cell line since the rate of spontaneous reversion of the ts2 cell line has a frequency of 10^{-7} (David Neufeld, unpublished results). Therefore, it is most likely that a single locus mutated in ts2 to generate the ts2R subline, this locus apparently affects DNA synthesis, but not TK activity. Additionally, the TK activity of ts2, ts2R, and A31 was shown to be unstable <u>in vitro</u> in extracts (even more so for ts2R then that observed with ts2), again

supporting the observed lability of the activity in actively growing ts2R *in vivo* at 39°C. We conclude from these results that the decrease in TK activity in actively growing ts2 cells incubated at 39°C is not the basis for the observed ts phenotype in DNA synthesis in these cells.

Synchronized ts2 cells were examined at both 33°C and 39°C after the addition of serum to correlate the effect of temperature on their ability to progress through the cell cycle and on the induction of DNA synthesis and enzyme activities specific for the S phase. Serum stimulation of these cells showed an induction of DNA synthesis and the TK and TMPK at 33°C, while exhibiting little (or no) stimulation of these activities in cells incubated at 39°C. Similar experiments performed with the ts2 revertant, ts2R, and its parent, A31, showed the stimulation of these activities at both 33°C and 39°C.

These synchronized ts2 cells did not show an increased requirement for serum at the non-permissive temperature. They were also found fully capable of entering the cell cycle at 39°C as indicated by an increase in cellular protein content during the time course, and the induction of both c-myc and proliferin mRNAs.

Further attempts to clarify the failure to observe TK induction in synchronized ts2 cells at 39°C after serum stimulation revealed that postulated stimulators of TK activity were not present in extracts of these cells at 33°C or possible inhibitors of this activity in cells at 39°C. Also, the induction of TK activity in ts2 cells was not dependent on the stimulation of DNA

synthesis, since it was observed that these cells were capable of enzyme induction in the presence of hydroxyurea. These results suggest that the lack of TK induction in growth-arrested ts2 cells at 39°C after the addition of serum is associated with a broad cellular phenomena, the result of the ts lesion. Additional evidence for this proposal is the observed failure to induce TMPK activity in ts2 cells after serum stimulation and incubation at the non-permissive temperature. Likewise, the data derived from the isolation of the multi-enzyme complex from serum stimulated ts2 cells at 39°C showed a much reduced DNA pol α activity in this fraction, indicating that there is also a lack of induction of this activity in cells at 39°C after the addition of serum.

SV40 infection of quiescent ts2 cells stimulated ^{3}H-TdR incorporation and TK activity in these cells at both 33°C and 39°C. The induction of DNA synthesis was shown to be dependent on the presence of the SV40 large T-antigen. Those cells stimulated into the cell cycle by SV40 infection were found to be able to complete cellular DNA replication and progress through one round of cell division at both the permissive and non-permissive temperatures for ts2. SV40 transformants of ts2 were isolated to assess whether the continual presence of the T-antigen would complement the ts mutation. These transformants were found to be unable to support cell growth at 39°C. This inability was interpreted as perhaps related to a deficient level of T-antigen in the transformants as opposed to that of an acute infection of ts2 cells.

Our initial model proposed to account for these various data with ts2 cells was that the ts defect in these cells resulted in a thermolabile MEC. Repeated isolation of the MEC fraction from ts2 cells, as obtained from both nuclear and cytoplasmic preparations, did not reveal an unstable complex, as it has been defined (Baril et al., 1973, Reddy and Pardee, 1980, Nogushi et al., 1983).

A model postulated to explain the observed aberrant response of ts2 cells considers a broadly based transcriptional block at the non-permissive temperature. I propose that the ts defect in ts2 cells results in an unstable protein (or factor) at 39°C which is needed to transcribe a particular class of mRNAs required for DNA synthesis, among these would be the mRNA for TK, TMPK, and DNA pol α. This factor or protein could act on transcription in either of two ways. The gene product may be required by RNA polymerase II for proper transcription of this class of genes or it may be a type of gene activator which binds to particular DNA sequences and renders these sequences more assessible for transcription. This transcriptional regulator would be made in the G1 phase, but is required continually in S phase. The class of mRNAs required for DNA replication would no longer be transcribed when the ts2 cells are incubated at the non-permissive temperature. The decline in these mRNAs would result in a gradual decay in DNA synthesis, as observed in actively growing ts2 shifted to 39°C (Slater and Ozer, 1976, this text), as DNA replication proteins with various half-lives are degraded. The cells would accumulate within S phase as observed in FMF profiles. Quiescent cultures of ts2 cells stimulated by the addition of serum, then

incubated at 39°C, would progress into the cell cycle to the S phase, but be unable to replicate DNA due to the lack of transcription of the various proteins required for DNA synthesis. The observed inhibition of polyoma DNA synthesis in ts2 cells incubated at 39°C (Slater and Ozer, 1976) is consistent with this proposed model since polyoma viral DNA replication requires essentially all host proteins (Tooze, 1981). As DNA synthesis is gradually arrested at 39°C, the packaging or conformation of the replicated DNA becomes defective. These illegimate DNA conformations may not present a large problem in the cellular DNA when the cells are shifted back to the permissive temperature, since it was shown these cells gradually resume DNA synthesis (Slater and Ozer, 1976), but, may pose a serious diffuculty in a small DNA molecule, such as a virus (Slater and Ozer, 1976, Richter et al., 1984).

The activity of a variety of enzymes involved in the biosynthesis of pyrimidine deoxyribonucleotides increases on infection of cells by SV40 along with DNA ligase and DNA polymerase α activities (Tooze, 1981). SV40 infection of monkey cells, CV1, showed a 2 to 3 fold greater RNA polymerase II activity then uninfected cells (Bagshaw and Rightland, 1974). The rate of transcription in isolated nuclei of primary mouse kidney cells increases by a factor of approximately 1.5 early after infection with SV40 (Pockl and Winterberger, 1980). Strong evidence for an involvement of the large T-antigen in this observed increase in RNA transcription was derived from experiments employing tsA mutants of SV40. Furthermore, it was shown that the increase in transcription

rate is a prerequisite for the induction of DNA replication in SV40 infected mouse kidney cells (Pockl and Winterberger, 1980).

The results obtained on SV40 infection of ts2 cells can be explained by our proposed model due to the evidence that SV40 T-antigen causes an enhanced transcription rate in infected cells. In this case, SV40 acts as a much better mitogen in ts2 cells then serum. If the ts transcription regulator in ts2 has a low activity in cells incubated at 39°C, then on SV40 infection of these cells the level of this defective gene product would be higher due to the increased transcription. The level of regulator, although "weak" functionally, may be high enough to drive the system through the observed single round of replication.

Another explanation for the observed effect of SV40 on growth-arrested ts2 cells incubated at 39°C could be that SV40 infection employs an alternate pathway (as opposed to serum stimulation) leading to the induction of cellular DNA synthesis. There are data from other laboratories to support this explanation. Both cellular and viral DNA replication were induced in adenovirus type 5 infected cells in the presence of dibutryl cyclic AMP at concentrations which inhibited induction by serum, which suggested that some of the controls of DNA synthesis in serum-treated and virus-infected cells are different (Braithwaite et al., 1981). Additionally, it has been shown that butyrate does not inhibit SV40-induced DNA synthesis (Kawaraki et al., 1981, Daniell et al., 1982). This proposed pathway could bypass the ts defect in these cells resulting in a successful round of DNA replication.

An initial attempt to obtain evidence for our proposed model for the observed ts defect in ts2 DNA replication entailed an effort to determine whether the mRNA for TK was induced in growth-arrested cells at 39°C after the addition of serum. Northern analysis of RNA extracted from these cells failed to disclose whether the TK message was induced at either temperature due to limitations with the available TK sequences (Lewis et al., 1983). To test our model, other cellular genes, the products of which are known to be required for DNA replication, need to be examined in ts2 cells to determine whether these sequences are expressed at the non-permissive temperature.

The P-4 (Baril et al., 1973) isolated from ts2 cells has been shown in different extractions not to contain a thermolabile MEC; contradicting previous data indicating that the ts phenotype was related to a structurally thermo-sensitive MEC. Conditions for the optimum recovery of enzyme activity in the P-4 were found to be the use of sterile, high quantity reagents, and storage of these solutions in plasticware. A proper baseline was found against which to measure the recoveries of enzyme activities in S-4 and P-4. Assay of nuclear extracts (N.E.) prepared from ts2 cells incubated at either 33°C of 39°C indicated that the enzymes of the MEC distribute themselves, upon extraction, in equivalent proportion at either temperature between the crude nuclear and cytoplasmic fractions, with most of the enzymatic activity found in the "cytoplasmic" P-4. The replitase fraction (Reddy and Pardee, 1980) was isolated from ts2 cells incubated at 33°C and 39°C and shown to have no significant differences in assayed activities

from cells at either temperature. Newly labelled acid-insoluble material was found to migrate in discontinuous sucrose density gradients in similar patterns whether the cells were incubated at 33°C or 39°C. The level of replitase in crude nuclei is low compared to the sedimentable material fractionated after high-speed centrifugation of N.E. P-4 contains the most activity of the three defined complex fractions (P-4, N.E., replitase). The low enzyme levels noted in the nuclear fractions could result from a possible leakage of MEC into the cytoplasm as the cells were extracted. The P-4 and replitase fractions isolated from ts2 cells at 33°C and 39°C were each able to successfully channel ^{3}H-TdR to ^{3}H-TTP, although P-4 was shown to be more efficient then replitase. Only P-4 was capable of channeling accumulated ^{3}H-TTP into DNA in vitro. This activity was present in P-4 isolated from ts2 at either temperature and shown to be independent of non-radiolabelled TTP, added after ^{3}H-TTP was generated by the complex fraction. The inability of replitase to incorporate ^{3}H-TdR into DNA in vitro was attributed to the low level of TK present in this fraction resulting in a small ^{3}H-TTP pool. In addition, the level of DNA pol observed is low in the replitase fraction when compared to that in P-4. Based on these different observations, it is concluded that actively growing ts2 cells incubated for a period of 20 hours at 39°C do not contain a thermolabile MEC, as it has been defined in this text.

Isolation of MEC from growth-arrested ts2 cells stimulated by the addition of serum and then incubated at 33°C or 39°C did not reveal whether ts2 cells are capable of assembling complex at

39 C, since there is an overall failure to induce the assayed enzyme activities. The ts DNA synthesis exhibited in ts2 cells does not correlate with a structurally thermolabile MEC in actively growing cells or with a readily apparent failure to assemble the complex at the non-permissive temperature after stimulation of quiescent cells.

It could be argued that the MEC is thermolabile in ts2 cells at 39°C, but, those unstable members of the complex were not among the assayed activities. Two lines of evidence suggest that the monitoring of TK activity was a good choice to assess whether ts2 cells contained a heat-sensitive MEC. One, the preliminary data indicating a thermolabile complex in ts2 (table 3) showed that TK was one of the enzymes exhibiting a dramatic change in partitioning on incubation of the cells at 39°C. Two, on serum stimulation of quiescent ts2 cells, TK activity was shown not to be induced at 39°C, therefore, suggesting that this enzyme was indeed affected by the ts defect and a good candidate for determining whether these cells contained a labile complex. These two points can also be made for the choice of TMPK activity to monitor the stability of the complex.

Although a structurally temperature-sensitive complex was not uncovered in ts2 *in vivo*, the isolated MEC fraction was not examined *in vitro* for the effect of temperature on the integrity of the complex from cells incubated at 39°C. Perhaps, *in vitro*, the MEC isolated from ts2 cells at 39°C would exhibit an increased lability related to temperature. This experiment could not be conveniently performed since the activities of both TK and TMPK in

the Balb/3T3 cell line were shown to be affected by temperature *in vitro*.

In an effort to obtain additional evidence for a fractionable MEC the cell line MK42 was used. MK42, a CHO mutant (Nunberg et al., 1978), has a 200 fold increase in the level of DHFR due to gene amplification. When the parental cell line, CHO-K1, and MK42 were compared, it was found that similar percentages of the total TK and DHFR activities partitioned in the S-4 and P-4 in each case; therefore, a high level of DHFR was associated with the P-4 in MK42. This result contradicts our expectations that the MEC is a specific structure with stoichiometric units of enzyme. Various procedures were attempted to remove the excessive level of DHFR from the P-4 in MK42, as compared to TK or parental cell line extracts. The replitase fraction was compared in CHO-K1 and MK42. The procedure used to isolate replitase successfully fractionates the DHFR activity from the MK42 fraction. The resultant level of DHFR activity was too low to accurately assess whether CHO-K1 and MK42 contained similar ratios of DHFR to TK activity, although the measured levels of TK activity were similar in the two cell lines. Another CHO mutant, DUK22, was examined to assess whether a ts DHFR protein would labilize the MEC on incubation at the defective temperature. The cell line replicated well at 37°C, but not at 41°C in medium containing glycine, hypoxanthine, and thymidine (compounds which overcome DHFR deficiency induced by methotrexate). Cell growth was shown to cease within 24 hours at 41°C, lending support that the ts DHFR protein labilized a functional MEC. The complex was shown not to be labile at 41°C.

Although we did not uncover a cell system which would facilitate a better defining of the properties of the MEC, our experiments do provide additional evidence to support the existence of the putative complex. First, those fractions defined as the MEC (P-4, N.E. replitase) could be readily generated from all the cell lines examined. Additionally, in the case of the overproducing DHFR cell, MK42, and its parent, CHO-K1, the levels of TK activity in the MEC fraction were observed to be approximately equivalent; thus suggesting a stoichiometry for this activity in the complex. Data from another laboratory suggested that the conformation of at least one enzyme of the complex was important for it to be a member of the MEC. It was shown that the human thymidylate synthetase introduced by DNA-mediated gene transfer into mouse cells did not co-sediment with DNA pol α and TK in sucrose gradients, suggesting that the human enzyme was unable to incorporate into the MEC for DNA replication (Ayusaiva et al., 1983). That the conformation of the enzyme played a role in the observed inability of the human thymidylate synthetase to join the mouse MEC was indicated by the absence of hybrid dimer enzyme between the human and mouse enzymes, since each consist of two identical subunits. The P-4 was stable by various biochemical criterion; repeat sedimentation, exposure to the non-ionic detergent, NP-40, and sedimentation through sucrose gradients containing 0.5 M KCl. The levels of various enzyme activities associated with the MEC fraction increased as cells progressed to the S phase as was also observed by Baril et al., 1973, Reddy and Pardee, 1980. The strongest evidence for the MEC is its ability to channel ^{3}H-TdR to ^{3}H-TTP, and subsequently

incorporate this ^{3}H-TTP into an offered DNA template (Baril et al., 1973). We presented data indicating that the preferred route of entry of nucleotide into DNA is via the MEC. This was shown by the inability of non-radiolabelled TTP to dilute the nucleotide pool generated by the complex. In addition, the coordinate function of the MEC was disrupted by several means, dilution, freezing, incubation overnight on ice, and dialysis, while exhibiting little effect on the enzymatic activities of the complex. This suggested that although the individual enzymes were unaffected by these manipulations, the enzymes could no longer function together as an organized unit.

CONCLUDING REMARKS

The studies in this text show the importance of ts mutants in unraveling the mechanisms of DNA replication. Although the actual ts defect in ts2 was not determined it was shown that a single mutation can effect a broad range of activities indicating the complex regulation of DNA synthesis in mammalian cells. In addition, the differential response on the part of quiescent ts2 cells to serum and SV40 may lead to insights as to the role that this virus plays in causing aberrant cell growth. The eventual full characterization of ts2, as well as other ts mutants will disclose the methods by which a cell decides to initiate a round of DNA replication, execute this synthesis, and regulate the DNA

synthetic machinery such that only a single round of replication occurs per cell cycle. With this basic understanding of eukaryotic cell biology we can explore ways in which to regulate the unchecked cellular proliferation that generate aberrant growth.

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