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Orlian, Martin S., Ph.D.

City University of New York, 1994

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CHARACTERIZATION OF ts2, A BALB/3T3 TEMPERATURE-SENSITIVE CELL CYCLE MUTANT DEFECTIVE IN DNA SYNTHESIS

by

Martin S. Orlian

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

1994

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

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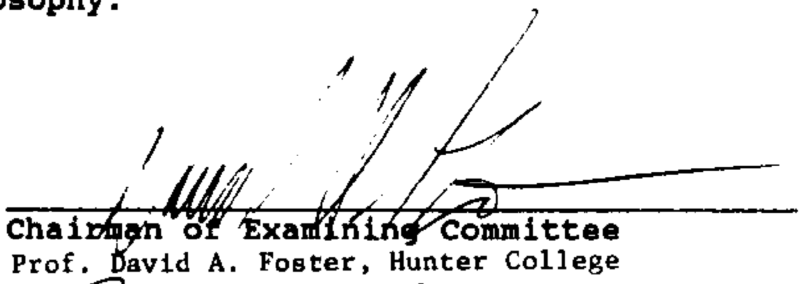

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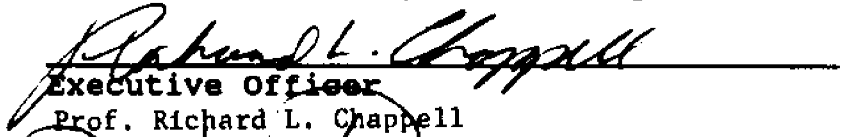

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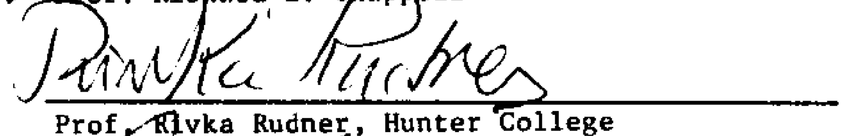

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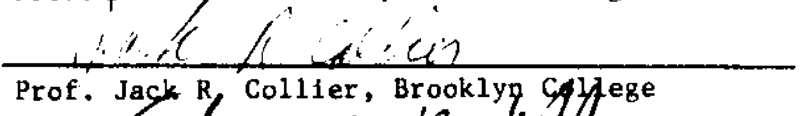

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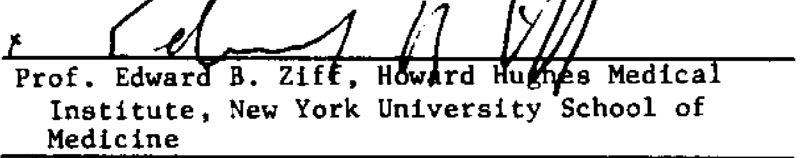

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ABSTRACT

CHARACTERIZATION OF ts2, A BALB/3T3 TEMPERATURE-SENSITIVE CELL CYCLE MUTANT DEFECTIVE IN DNA SYNTHESIS

by

Martin S. Orlian

Adviser: Professor David A. Foster

Ts2, a temperature-sensitive mutant cell line derived from Balb/3T3 cells, was isolated on the basis of the cell's inability to undergo DNA synthesis when incubated at 39°C. ^{3}H-TdR labeling shows that ts2 is defective in DNA synthesis following serum induction at 39°C. Flow cytometry shows that when ts2 cells are maintained at 39°C, if they manage to enter S phase, they arrest in G2+M. Infection with SV40 virions overcomes the block in DNA synthesis. Cells infected at 39°C accumulate in G2+M and undergo apoptosis. These data suggest that ts2 cells incubated at 39°C can arrest at two points in the cell cycle: the first prevents them from entering S phase and the second prevents them from undergoing normal cell division. SV40 infection bypasses or overrides the temperature-dependent block at G1/S, but not the block at G2+M.

Thymidine kinase (TK) mRNA levels were determined in ts2 cells following stimulation by serum addition or SV40 infection. Cells incubated at 39°C show significantly reduced TK mRNA expression following serum stimulation when compared to cells maintained at 33°C. SV40-infected cells show temperature-independent induction of the TK message. Several human TK plasmid constructs were stably transfected into ts2. Analysis of the ts2-derived cell lines show that the human TK gene, when linked to the human TK promoter, shows temperature-dependent cell cycle regulation following

serum stimulation. These data suggest that the temperature-dependent induction of human TK mRNA levels following serum stimulation is manifested at the level of the promoter.

A complex phenotype emerges when ts2 cells are incubated at 39°C. The transcription of a least one cell cycle-regulated gene whose expression is associated with the onset of DNA synthesis (TK) is defective. The observation that the genetic defect in ts2 blocks cell cycle progression at two places (G1/S and G2/M)- both major cyclin-dependent regulatory positions of the cell cycle- suggests that ts2 is not a DNA synthesis mutant *per se* but rather a cell cycle mutant.

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Table of Contents

page

Abstract.......... iv

Acknowledgements.......... vi

List of Tables.......... xi

List of Figures.......... xii

CHAPTER ONE: Introduction

The Mammalian Cell Cycle.......... 1

Cell Cycle Control Mechanisms.......... 3

The Use of *ts* Mutants to Study Cell Cycle Control Mechanisms.......... 6

Rationale for Studying Thymidine Kinase mRNA Expression in ts2.......... 7

Summary and Preview.......... 10

CHAPTER TWO: Materials and Methods

Cell Culture and Cell Manipulation

Mammalian Cell Lines and Culture Conditions.......... 12

Preparation of Growth-Arrested Cultures.......... 12

Serum Stimulation of Quiescent Cells.......... 13

Preparation of Cells for Flow Cytometry.......... 13

Transfection of ts2.......... 14

Selection of Transformants Using G418.......... 14

Cell Labeling Techniques

Pulse-labeling and TCA Precipitation.......... 15

Autoradiography of Labeled Nuclei.......... 16

Virological Techniques

SV40 Virus Preparation.......... 17

Determination of SV40 Virus Titer by Immunofluorescence.................. 18

SV40 Infection of Quiescent ts2 Cultures.. 18

Radiolabeled Probes

Random Primed DNA Probes.............................. 19

Riboprobes.. 19

Plasmids and Probes

Murine Thymidine Kinase Probes.. 20

Human Thymidine Kinase Probes.. 21

He7 Probe.. 23

Neomycin-Resistance Probe.. 23

Human Thymidine Kinase (TK) Constructs Used to Transfect ts2......... 24

DNA Isolation Protocols

Plasmid Preparation

Bacterial Culture Techniques.. 26

Competent *Escherichia coli* Cells.. 26

Transformation of *Escherichia coli* Cells.. 27

Plasmid Preparation by the Triton Lysis/CsCl Method.................... 27

Plasmid Isolation by Column Purification... 29

Plasmid Minipreparation by the Boiling Method.............................. 29

Preparation of High Molecular Weight (HMW) DNA from Mammalian Cells.. 30

DNA Analysis Protocols

Digestion of DNA with Restriction Enzymes... 31

Gel Electrophoresis of DNA.. 32

Recovery of DNA Fragments from Agarose Gels................................... 32

Southern Blot Analysis.. 33

RNA Isolation Protocols

Bentonite/SDS/Formaldehyde Method 34

Guanidinium Isothiocyanate (GIT/Sarkosyl) Method 35

Guanidinium/CsCl Method 36

Hot Phenol Method 37

RNA Analysis Protocols

Slot Blot Analysis of RNA 37

Preparation of RNA Samples for Gel Electrophoresis 38

RNA Gel Electrophoresis 38

Northern Blot Analysis 39

Prehybridization and Hybridization of RNA Filters 39

CHAPTER THREE: Results

Characterization of ts2

Kinetics of DNA synthesis in ts2 following growth arrest and stimulation 41

Determination of thymidine kinase (TK) mRNA levels following serum stimulation in both wild type A31N and ts2 cells 51

Analysis of TK mRNA levels in both serum-stimulated and SV40-infected ts2 cultures 56

Analysis of quiescent ts2 cells following serum addition or acute SV40 viral infection by flow cytometry 65

Characterization of ts2-derived cell lines 72

Establishment of ts2-derived cell lines 72

Kinetics of DNA synthesis in ts2-derived cell lines following serum addition 79

Determination of murine and human TK mRNA levels in ts2-derived

cell lines 82

CHAPTER FOUR: Discussion

Early Characterization of ts2: A Brief Review 102

The Cell Cycle Kinetics of ts2 Following Stimulation 103

The Expression of TK mRNA in ts2 Following Stimulation 105

Is Transcription Affected in ts2? 107

A Model to Account for the Defective Expression of TK mRNA in ts2 109

The Complex Regulation of TK Expression 111

The UBE1 Gene: A Candidate for the Gene Responsible for the ts2 Phenotype 113

Conclusion: ts2, a Cell Cycle Mutant Defective in DNA Synthesis 116

Bibliography 118

List of Tables

page

Table 1: Relative increase in ^{3}H-TdR incorporation following serum stimulation or acute SV40 infection.......... 49

Table 2: Autoradiography of ts2 nuclei following serum stimulation.......... 50

List of Figures

page

Figure 1: Analysis of ts2 during logarithmic growth and following rundown by flow cytometry........ 45

Figure 2: ^{3}H-TdR pulse-labeling of ts2 cells following serum stimulation or acute SV40 virus infection........ 47

Figure 3: Slot blot analysis of thymidine kinase (TK) and He7 mRNA levels in wild-type murine A31N cells following serum stimulation........ 54

Figure 4: Slot blot analysis of thymidine kinase (TK) and He7 mRNA levels in ts2 cells following serum stimulation........ 55

Figure 5: Northern blot analysis of thymidine kinase mRNA levels in ts2 cells following serum stimulation or acute SV40 infection.

Figure 5a: Thymidine kinase (TK) mRNA levels in ts2 cells following acute infection with SV40 virus........ 59

Figure 5b: Thymidine kinase (TK) mRNA levels in ts2 cells following serum stimulation........ 61

Figure 5c: He7 mRNA levels in ts2 following rundown and serum stimulation........ 62

Figure 6: SV40 mRNA expression in ts2 cells acutely infected by SV40 and incubated at the permissive temperature........ 63

Figure 7: Flow cytometric analysis of ts2 following rundown and stimulation by either serum addition or acute SV40 virus infection.

Figure 7a: Flow cytometric analysis of ts2 cells following serum addition........ 68

Figure 7b: Flow cytometric analysis of ts2 cells following SV40

infection........ 70

Figure 8: Southern blot analysis of ts2-derived cell lines.

Figure 8a: Verification of the presence of the G418-resistance gene in the ts2-derived cell lines........ 75

Figure 8b: Verification of the presence of human thymidine kinase (TK) sequences in the ts2-derived cell lines........ 77

Figure 9: ^{3}H-TdR pulse-labeling of ts2-derived cell lines following serum stimulation........ 80

Figure 10: Confirmation of the expression of the transfected human thymidine kinase (TK) genes in the ts2-derived cell lines by Northern blot analysis........ 87

Figure 11: Analysis of cell line P1-1 by Northern blot analysis.

Figure 11a: The expression of human thymidine kinase (TK) mRNA in the cell line P1-1 following serum stimulation........ 89

Figure 11b: The expression of murine thymidine kinase (TK) mRNA in the cell line P1-1 following serum stimulation........ 91

Figure 11c: The expression of He7 mRNA in the cell line P1-1 following serum stimulation........ 92

Figure 11d: The expression of the neomycin-resistance gene in the cell line P1-1 following serum stimulation........ 93

Figure 12: Analysis of cell line P2-1 by Northern blot analysis.

Figure 12a: The expression of human thymidine kinase (TK) mRNA in the cell line P2-1serum stimulation........ 95

Figure 12b: The expression of murine thymidine kinase (TK) mRNA in the cell line P2-1 following serum stimulation........ 97

Figure 12c: The expression of He7 mRNA in the cell line P2-1 following

serum stimulation.. 98

Figure 13: Analysis of the cell line P4-2 by Northern blot analysis.

Figure 13a: The expression of human thymidine kinase (TK) mRNA in the cell line P4-2 following serum stimulation... 99

Figure 13b: The expression of murine thymidine kinase (TK) mRNA in the cell line P4-2 following serum stimulation... 101

CHAPTER ONE: Introduction

The Mammalian Cell Cycle

The proliferation of mammalian cells is a stringently controlled process that is responsive to the presence of external growth factors (Gudas *et al.*, 1988). The cell cycle is composed of a series of steps which can be negatively and positively regulated (Levine *et al.*, 1991). Under optimal nutritional conditions, animal cell lines in tissue culture generally have cell cycle times of 10 to 30 hours (Pardee *et al.*, 1978). Most biologists divide the cell cycle into M phase (mitosis), G1 phase (the gap prior to DNA synthesis), S phase (period of DNA synthesis) and G2 (the gap between DNA synthesis and mitosis). The lengths of M, S, and G2 are remarkably similar in many different cells, while the length of G1 shows great variation among different cell lines. It is widely believed that during the G1 phase of the cell cycle, the major regulatory events leading to proliferation in animal cells occur.

In recent years, one of the most intensely studied areas of cell biology is how the cell cycle is controlled. Converging lines of evidence from many laboratories have led to the adoption of a "universal model", specifying that cell division follows essentially the same biochemical pathway in all eukaryotic organisms, from yeast to human beings (Murray and Kirschner, 1989). Central to this paradigm is the existence of two transition control points, at the G2/M boundary and during G1 phase (Lewin, 1990). The protein $p34^{cdc2}$, a serine-threonine kinase, acts as a master control unit, orchestrating the action of many other enzymes during the cell cycle. The *cdc2*$^{+}$ gene function is required for cells to successfully traverse the two major cell cycle transition points: G1 to S and G2 to M. The $p34^{cdc2}$ protein differs at its two execution points in terms of its protein kinase activity, its phosphorylation state, and its association with other proteins (especially cyclins) (Broeck *et al.*, 1991). These observations suggest that $p34^{cdc2}$ exists in at least two

states: one required for entry into S phase and the other for entry into mitosis (Broeck *et al.*, 1991).

Animal cells can exist in a non-growing quiescent state during which no DNA replication or cell division occurs. *In vivo*, most quiescent cells have a G1 DNA content (Campisi *et al.*, 1982). A normal animal cell upon reaching the G1 phase can either enter quiescence, often called G0, or undergo another round of DNA synthesis and cell division. In culture, normal cells become arrested in G0 under a variety of conditions that ultimately prevent or diminish proliferation: nutrient or serum deprivation, high cell density, and partial inhibition of protein synthesis (Campisi *et al.*, 1982). These G0 arrested cells have an unduplicated DNA content like cycling cells in G1, yet they show significant differences in many other fundamental properties. G0 cells decrease in size because their protein and RNA molecules are degraded and are not rapidly resynthesized since macromolecular syntheses in G0 cells are about one-third as rapid as in cycling cells. Enzymes and transmembrane transport activities are low in G0 cells, and ribosomes tend to exist as monosomes rather than as polysomes (Pardee, 1989). There is also considerable evidence that there are specific G0-induced RNAs and proteins (Wang, 1987, Schneider *et al.*, 1988, Ciccarelli *et al.*, 1990).

Pardee (1974) proposed that cells could enter the G0 state or make the commitment to continue proliferation at a single point in the G1 phase called the restriction (or R) point. According to this model, newly divided cells (in early G1) prepare for DNA synthesis and cell division while continually monitoring their environment. If conditions are favorable, the cells pass through the R point and become committed to complete the remainder of the cell cycle. Unfavorable conditions, on the other hand, cause them to cease proliferation, remaining with a G1 DNA content (Pardee, 1974, Campisi *et al.*, 1982). If a cell successfully traverses the R point, it proceeds through the rest of the cell cycle even after serum has been removed. The R point, as defined by protein synthesis inhibitors, occurs about two hours prior to the onset of DNA synthesis in 3T3 cells

(Rossow *et al.*, 1979, Campisi *et al.*, 1982). Rossow *et al.* (1979) proposed that a critical threshold concentration of a labile protein(s) (called the R proteins by Campisi *et al.*, 1982) must accumulate in order for cells to pass the R point and enter S phase. Lewin (1990) postulated that this labile protein could be a G1 cyclin.

Cell Cycle Control Mechanisms

Recent studies show that growth factors and oncogenes play a critical role in mammalian cell cycle control during the G1 phase until the restriction point, which is believed to occur about 2 hours prior to the onset of DNA synthesis. Following the restriction point, cells can progress into S phase in the absence of growth factors (Knight *et al.*, 1987).

The signals for induction and regulation of these enzymes could be critical event(s) leading to the onset of DNA synthesis since the onset of DNA synthesis in mammalian cells is accompanied by several biochemical events, including increases in activities of enzymes which synthesize precursors for DNA (Coppock and Pardee, 1985, Knight *et al.*, 1987). Much information has accumulated concerning the induction of genes in early G1 that are expressed when cells emerge from quiescence. However, very little is known about the regulatory events in late G1 that control the onset of DNA synthesis (Gudas *et al.*, 1988).

In order to gain insight into the processes which signal the induction of the enzymes functionally and temporally associated with DNA synthesis, it is important examine the mechanisms which ultimately lead to the functional expression of these cell cycle-dependent genes. In addition, the cloning of genes necessary for the initiation of DNA synthesis has facilitated the elucidation of many of the cellular regulatory events that occur at the G1/S boundary. Furthermore, since the mammalian cell cycle is regulated in the G1 phase by extracellular factors (Coppock and Pardee, 1987), the elaboration of mechanisms responsible for the conversion of extracellular signals into intracellular signals is of

critical importance to understanding the induction of the genetic program which eventually leads to DNA synthesis.

Polypeptide growth factors, regulatory peptides, and a variety of pharmacological agents acting alone or synergistically induce mitogenesis in cultured fibroblasts. Following the binding of a polypeptide growth factor to its target, a high-affinity receptor, the receptor undergoes changes which can include phosphorylation, redistribution in the membrane, and endocytosis (Rozengurt, 1986). The binding of a growth factor to its receptor promotes the generation of early signals in the membrane and cytosol, resulting in the rapid propagation of the mitogenic signal. These early events trigger a series of parallel sequences of molecular and cellular responses, all eventually converging into a common pathway leading to DNA synthesis and cell division. Since the initiation of DNA synthesis occurs 10-15 hours after mitogen stimulation, it is believed that the study of the early events which follow the addition of mitogens will provide insight into the cell cycle's primary regulatory mechanisms.

While it has become clear in recent years that the events of G1 occur sequentially over many hours, very few "biochemical landmarks" have been elucidated that permit positions in G1 to be fixed. The use of temperature-sensitive mutant cells that arrest at different positions in G1 provides a means to sequence metabolic events, thus allowing G1 to be subdivided into sequential subphases: competence, entry, progression and assembly (Pardee, 1989).

As knowledge of the biochemical events and the identification of genes that are involved in the response to growth factors is accumulated, it has become increasingly clear that genes whose expression is growth factor regulated are likely to be important components in the mechanism controlling cell proliferation and differentiation, and that a subset of these genes may play a critical role in cellular transformation (Almendral *et al.*, 1988). Since G1 is an important control point in cell proliferation where normal cells deprived of growth factors become quiescent while transformed cells do not, it has been

suggested that regulatory genes whose expression is uncontrolled in the neoplastic phenotype are expressed during this transition (Almendral *et al.*, 1988). For example, Greenberg and Ziff (1984) and Bravo *et al.* (1987) have shown that the expression of the proto-oncogenes c-*fos* and c-*myc* change immediately following stimulation with growth factors, thus indicating their importance in the G0 to G1 transition. Since both genes are induced in the presence of protein synthesis inhibitors, their induction is a direct result of growth factor-receptor interaction. The expression of c-*fos* is undetectable during the cell cycle, suggesting that its expression is essential during the G0 to G1 transition and not for logarithmically growing cells. In contrast, under the influence of growth factors, c-*myc* is expressed constitutively throughout the cell cycle (Thompson *et al.*, 1985). Recent evidence indicates that c-*myc* is essential for G1 progression and DNA synthesis rather than for the G0 to G1 transition. The c-*fos* and c-*myc* data suggest that growth factors induce the expression of genes required for both the transition from G0 to G1 and the progression from G1 to S phase.

The retinoblastoma susceptibility gene product (Rb) and the p53 protein are negative regulators of the cell cycle. Alteration or inactivation of these genes through mutation or through their interaction with oncogene products of some DNA tumor viruses can lead to cancer. Rb is considered to be an inhibitor of cell proliferation because it is absent or inactive in retinoblastoma and several other tumors. When the wild type gene (Rb1) is reintroduced into retinoblastoma or osteosarcoma cells, tumorogenicity is suppressed (Stein *et al.*, 1990). Rb, which is unphosphorylated in G0 and G1 phase cells, undergoes phosphorylation at the G1/S boundary, indicating that phosphorylation may be necessary for entry into S phase.

Similarly, Levine *et al.* (1991) propose two hypotheses to explain the role of p53 in the negative regulation of cells as they traverse the cell cycle. There is evidence that p53 could in some way act late in G1 to promote or prevent the assembly or function of a DNA replication-initiation complex (Levine *et al.*, 1991). However, the more likely hy-

pothesis is that p53 could act as a transcriptional transactivator, either promoting or repressing mRNA synthesis, perhaps of a set of genes that effect the passage of cells from late G1 to S phase (Levine *et al.*, 1991).

The Use of *ts* Mutants to Study Cell Cycle Control Mechanisms

In recent years, many different approaches have been used to analyze and characterize the different mechanisms which control cell proliferation. To date, however, none of the mechanisms which regulate this basic phenomenon has been fully characterized. By identifying specific components of the proliferation cycle, conditional lethal, temperature-sensitive (*ts*) mutants defective in DNA synthesis can provide considerable insight into the mechanisms controlling this process. Mutants with thermolabile proteins enable one to explore the role of a particular protein in its appropriate biological context. Since, in theory, any protein can be made thermolabile, the use of this genetic approach makes analysis of a wide range of cellular processes possible. However, unlike with many microbial systems, mammalian cell mutants are difficult to produce (owing to their diploidy) and are complex to study. Consequently, only limited studies have been described for mammalian cells in culture, and few mutants have been defined as defective in functions directly related to DNA synthesis (*ts*DNA), in contrast to those in cell cycle progression.

Our laboratory described the isolation of temperature-sensitive mutants in Balb/3T3 cells that are affected in cellular and polyoma DNA synthesis (Wittes and Ozer, 1973, Slater and Ozer, 1976, Jha *et al.*, 1980, Zeng *et al.*, 1984, Malkas, 1985). The mutants ts2 and ts20 mapped to the X-chromosome and complemented poorly or not at all to several other mouse *ts*DNA mutants (Jha *et al.*, 1980). The permissive and nonpermissive temperatures for cellular growth are 33°C and 39°C, respectively. These mutants were selected, after mutagenesis with ethylmethane sulfonate, by their inability to incorporate ^{3}H-thymidine (^{3}H-TdR) into DNA at 39°C. Cells capable of incorporating the

radiolabel at 39°C were killed by the deleterious effects of ^{3}H-TdR.

Several lines of evidence indicated that several of these mutants (ts2, ts20, ts22) were temperature sensitive (*ts*) for cellular DNA synthesis. Partial characterization of ts2 indicated that it was not defective in overall protein or RNA synthesis at the nonpermissive temperature (Slater and Ozer, 1976), and that the *ts* phenotype is independent of cell density. Ts2 cells only support DNA synthesis of the papovavirus, polyoma, after infection of these cells at the permissive temperature. With the exception of polyoma large-T antigen required for polyoma DNA synthesis, viral DNA replication depends solely upon the host cellular DNA replication machinery. Therefore, the inhibition in ts2 of both viral and cellular DNA synthesis to a similar degree at 39°C is indicative of the thermolability of the cellular DNA replication machinery. This inhibition of cellular DNA replication is at least partially reversible on shift of the cells back to 33°C. These data strongly suggest that ts2 is defective in some S phase function (Slater and Ozer, 1976).

Ts2 cells were chosen for this study because the majority of the data on this class of temperature-sensitive mutants is derived using this cell line. In addition, ts2 cells do not genetically complement ts20 or ts22, suggesting that these mutants contain lesions in the same genetic locus. This is important because considerable progress has been made towards the isolation of a human gene capable of correcting the *ts* defect in ts20. Therefore, it is likely that this human gene would also complement the *ts* defect in ts2 cells. In addition, it is possible that the information obtained concerning the defect in ts2 could provide insight into the thermolabile phenotypes of ts20 and ts22, as well.

Rationale for Studying Thymidine Kinase mRNA Expression in ts2

The use of ^{3}H-thymidine incorporation data to characterize the phenotype of ts2 cells is of limited value. Although labeling experiments show that ts2 cells are defective

in their ability to undergo DNA synthesis at the nonpermissive temperature, the ^{3}H-thymidine labeling criteria unites into a single category cells with defects in a variety of biochemical functions. It is likely that cell lines showing similar labeling kinetics would have widely divergent patterns of expression of growth regulated genes (Ide *et al.* 1986). Therefore, the study of the temporal expression of the protein and RNA levels of growth regulated genes should facilitate the further characterization of the nature of the temperature-sensitive defect in ts2 cells.

Earlier work in our laboratory (Malkas, 1985) showed that when density arrested ts2 cells are induced to resume cell growth by serum at the nonpermissive temperature, they are not only defective for DNA synthesis. They also fail to exhibit the expected increase in the activities of several enzymes closely associated with the onset of DNA synthesis, including thymidine kinase (TK). In order to further characterize the phenotype of ts2 cells at the restrictive temperature, the thymidine kinase (TK) gene was chosen for further study. We were interested in determining whether the failure to induce TK activity in serum-stimulated ts2 cells incubated at the nonpermissive temperature reflects a defect in TK mRNA accumulation in these cells.

The TK gene has been used as model to study the regulation of expression of an important group of genes which could act as primary elements in the control of the cell cycle, because of its close association with the enzymes of DNA synthesis (Travali *et al.*, 1988). Thymidine kinase (TK) is a member of a group of enzymes involved in the synthesis of cellular DNA. The activity of TK, as well as that of other enzymes of the DNA-synthesizing machinery, is low in G0 and increases sharply at the G1/S boundary of the cell cycle and remains elevated throughout the S phase (Bradshaw and Deininger, 1984). Steady state levels of TK mRNA also increase sharply as cells stimulated to proliferate from quiescence enter S phase (Liu *et al.*, 1985b, Stuart *et al.*, 1985, Coppock and Pardee, 1987). It has been found, using DNA synthesis inhibitors, that increased

TK activity is not coupled to the onset DNA synthesis, despite its temporal correlation with entry into S phase (Johnson *et al.*, 1982, Malkas, 1985, Coppock and Pardee, 1987).

Studies measuring steady state mRNA levels for specific messages like TK is an accepted means to study gene expression. However, these types of studies by themselves fail to provide information as to whether the regulation in the accumulation of specific mRNA species is controlled at the transcriptional or posttranscriptional level. Control of an mRNA concentration in cells can conveniently be divided into three steps: control of the rate of transcription, control of the rate of nuclear turnover, and control of mature mRNA stability (Coppock and Pardee, 1987).

The current consensus is that the cell cycle regulation of TK mRNA expression in cells stimulated to proliferate from quiescence appears to be largely controlled at the level of the promoter (Gudas *et al.*, 1988, Kim *et al.*, 1988, Lieberman *et al.*, 1988, Travali *et al.*, 1988, Lipson and Baserga, 1989, Lipson *et al.*, 1989, Ito and Conrad, 1990, Roehl and Conrad, 1990, Fridovich-Keil *et al.*, 1991, Kim and Lee, 1991). The TK promoter requires multiple promoter elements for efficient transcription of the TK gene (Arcot *et al.*, 1989, Kreidberg and Kelly, 1986). Recent studies show several different binding activities to various elements within the promoter region of the TK genes. Some of these binding activities correlate closely with the G1/S phase induction of the gene, while others are constitutive (Knight *et al.*, 1987, Arcot *et al.*, 1989, Bradley *et al.*, 1990, Dou *et al.*, 1991, Dou *et al.*, 1992, Kim and Lee, 1992, Li *et al.*, 1993).

The fact that there is strong experimental evidence indicating that the transcriptional activation of the TK promoter is mediated by multiple binding activities to several promoter elements is not unexpected. In general, the transcriptional selectivity of eukaryotic genes is mediated by complex control regions composed of different combinations of promoter and enhancer elements arrayed in tandem that appear to allow multiple distinct regulatory factors to function coordinately to potentiate RNA synthesis (Lee *et al.*, 1987). Unique promoter sequences can be conferred by the particular composition and

spatial organization of multiple elements constituting the complete set used by any given gene (Lee *et al.*, 1987). Consequently, genetic and sequence analysis of regions 5' to the start of transcription should prove useful in understanding the transcriptional regulation of various genes.

Summary and Preview

Ts2 has thus far been described as a temperature-sensitive mutant defective in DNA synthesis (Wittes and Ozer, 1973, Slater and Ozer, 1976, Malkas, 1985). The goal of this research is to further characterize the nature of the thermolabile defect in ts2 cells. ^{3}H-thymidine incorporation and/or autoradiography was used to monitor the kinetics of the arrest and stimulation of ts2 cells at both the permissive and nonpermissive temperatures. In addition, ts2 cells were analyzed by flow cytometry in order to elucidate the kinetics of cell cycle progression following stimulation. In order to examine whether the temperature-sensitive defect in ts2 cells effects the steady state mRNA levels of genes associated with DNA synthesis, we used the thymidine kinase gene, a gene known to be cell cycle-regulated, as our "reporter gene". The levels of TK mRNA were determined by slot blot and Northern blot analysis at various times following stimulation. In an effort to ascertain whether the transcription of the TK gene is affected by the thermolabile defect in ts2 cells, cells lines were made by stably transfecting ts2 cells with a series of human TK plasmid constructs. Human TK mRNA expression was assessed by Northern blot analysis.

For these studies, cells were stimulated from quiescence either by the addition of fresh serum or by acute SV40 viral infection. Earlier studies have shown that unlike serum-stimulated cells, SV40-infected ts2 cells are capable of undergoing at least one round of DNA replication at the nonpermissive temperature (Malkas, 1985). The ability of acute SV40 infection to overcome the temperature-sensitive block to DNA synthesis

in late G1/S prompted us to ask the question whether SV40 infection could activate the same cell cycle regulated genes activated by serum- in this case, the TK gene.

The kinetic data indicate that ts2 cells can arrest at two important transition points within the cell cycle: one at the G1/S boundary and the other in G2/M. In addition, there appears to be a defect in the transcription of the TK gene when ts2 cells are incubated at the nonpermissive temperature. The results reported here indicate that ts2 may actually be a cell cycle mutant. The observed thermolabile defect in DNA synthesis appears to be one manifestation of a cellular phenotype defective in at least several biochemical and cell cycle-related functions. A potential model to account for the ts2 phenotype is discussed which involves a defect in the ubiquitin-mediated proteolytic pathway. The ubiquitin-conjugating system plays an important role in the regulation of multiple cellular processes (Trausch *et al.*, 1993), including the degradation of cyclins at two critical transition points in the cell cycle- late G1 and M (Glotzer *et al.*, 1991).

CHAPTER TWO: Materials and Methods

Cell Culture and Cell Manipulation

Mammalian Cell Lines and Culture Conditions

Ts2 is a temperature-sensitive (*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 the twice re-cloned subline ts2E2. The mouse cells were maintained in Dulbecco's modified Eagle's medium (DME, M.A. Bioproducts and KC Biologicals) or in a 1:1 mixture of DME and F-10 (DME/F10, M.A. Bioproducts and K.C. Biologicals) supplemented with 10% newborn calf serum (M.A. Bioproducts), penicillin (100 U/ml), and streptomycin (100 µg/ml). They were incubated, unless otherwise noted, in a 10% CO_2 atmosphere (when DME was used) or a 7.5% CO_2 atmosphere (when DME/F10 was used) at 33°C. These cell lines were expanded after passage without allowing cells to reach confluence, unless otherwise indicated. Earlier studies have shown that as ts2 cells are passaged, they lose their temperature-sensitive (*ts*) phenotype. In order to minimize this, experiments were performed with freshly plated cells no more than ten passages from frozen storage.

Cells were passaged by washing 1-2 times with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (0.15 M NaCl, 3 mM NaH_2PO_4, pH 7.4). When necessary, cell counts were obtained with a Royco Tissue Cell Counter after dilution in PBS. Cell lines were stored in NUNC cryotubes frozen in liquid nitrogen or in a -70°C freezer in complete medium containing 10% dimethyl sulfoxide (DMSO) (Fisher).

Preparation of Growth-Arrested Cultures

Low passage cells were grown to confluence in 100 mm tissue culture petri dishes

(Falcon). The cells were washed with PBS without Ca^{2+} and Mg^{2+} and trypsin and trypsinized with 1 ml of trypsin. The cells were removed from the dishes following the addition of 9 ml of DME or DME/F10 containing 10% NCS. Pools of cells were made and then 5 ml of cells were seeded into new 100 mm tissue culture dishes containing 5 ml of warm serum-supplemented medium, effectively diluting the cells 1:2. The cultures, with a final volume of medium and cells of 10 ml, were then incubated for 96 hours at 33°C in a 10% or 7.5% CO_2 atmosphere. These cells became arrested by a combination of density arrest due to contact inhibition and depletion of serum growth factors. Their growth arrest was monitored by measuring the amount of ^3H-thymidine incorporated into TCA precipitatable material at various times following plating.

Serum Stimulation of Quiescent Cells

Growth-arrested cultures were stimulated by aspirating the medium and washing the culture twice with 10ml of the appropriate medium, prewarmed and containing no serum. Ten ml of warm DME or DME/F10 containing 10% newborn calf serum was added. The cultures were incubated at 33°C or immediately shifted to 39°C.

Preparation of Cells for Flow Cytometry

Cells were trypsinized, resuspended in PBS, and counted. The cells were then pelleted by centrifugation and the cell pellets washed with PBS. The cells were resuspended in PBS to a concentration of 1-2 X 10^6 cells/ml. Aliquots of cells (1 ml) were added to 15 ml Falcon centrifuge tubes containing 9 ml of freshly prepared 70% ethanol. The tubes were mixed by inversion and stored at 4°C for later analysis at the UMDNJ Cellular Immunology Laboratory using a Becton Dickinson fluorescent flow sorter (following staining with propidium iodide).

Transfection of ts2

Transfections were carried out using the method of Graham and Van der Eb (1973) with modifications. Cells were seeded at a density of 5 x 10^6 cells per 100 mm tissue culture dish approximately 36 hours prior to transfection and were incubated in DME/F10 containing 10% NCS at 35°C and 7.5% CO_2. Just prior to transfection, calcium phosphate precipitates of DNA were prepared. Aliquots (1 ml) of freshly prepared 2X HEPES Buffered Saline (HBS; 280 mM NaCl, 50 mM HEPES (Calbiochem, Ultrol Grade), 1.5 mM $Na_2PO_4 \cdot 12H_2O$, adjusted to pH 7.10 with 1 M NaOH and filter sterilized) transfection buffer were added to sterile 15 ml tubes (Falcon). One ml double strength DNA-$CaCl_2$ solutions were prepared for each plasmid to be transfected that contained a total of 40 µg/ml DNA (15 µg TK plasmid DNA, 1 µg pWL*neo* (Stratagene), and 24 µg sonicated calf thymus DNA as a carrier) and 250 mM $CaCl_2$. Each DNA-$CaCl_2$ solution was pipetted gently and added dropwise (slowly) to the tube containing 1 ml 2X HBS. Mixing was accomplished by constantly bubbling air through the solution with a plugged pipet attached to a Pipet-aid. The tubes were allowed to stand at room temperature for 30 minutes to allow the precipitate to form and settle out. The precipitate was then gently resuspended in a pipet and for each human TK construct to be transfected, 1 ml of the precipitate was added to each of 2 100 mm tissue culture dishes in a dropwise manner over the entire surface of the dish. The dishes were then incubated overnight at 35°C and 7.5% CO_2. The following morning, the transfection medium was removed and the dishes refed with fresh DME/F10 and 10% NCS and incubated at 35°C and 7.5% CO_2 for an additional 48 hours.

Selection of Transformants Using G418

Three days after transfection (when the dishes were about 80% confluent), each

transfected dish was split into fresh 100 mm dishes such that 2 dishes of a 1:3, 1 dish of a 1:5, and 2 dishes of a 1:10 dilution were made. Cells were grown in selection medium, DME/F10 supplemented with 10% NCS, penicillin (100 U/ml), streptomycin (100 µg/ml), and 800 µg/ml G418 (Geneticin, Gibco), at 35°C and 7.5% CO_2. These dishes were fed twice weekly with selection medium until clones 2-4 mm in diameter could be observed. In dishes where there were more than several hundred clones, the cells were trypsinized and seeded into 5 100 mm dishes and grown as pooled cells lines in selection medium. Four dishes from each cell line were frozen down in NUNC vials for future use in DME/F10 supplemented with 10% NCS, penicillin (100 U/ml), streptomycin (100 ug/ml), plus 10% DMSO (DMSO; dimethylsulfoxide (Fisher)). Two vials from each cell line were stored at -70°C and two vials were stored in liquid nitrogen for future use. The remaining 100 mm dishes were expanded and the cells were used to prepare high molecular weight DNA for Southern blot analysis and RNA for Northern blot analysis. Since these cells were stably transformed, G418 selection was maintained for only several passages.

Cell Labeling Techniques

Pulse-labeling and TCA Precipitation

Cells were labeled by adding ^{3}H-thymidine (^{3}H-TdR) (NEN; NET 027Z, 82.4 Ci/mmole) to the dishes to a final concentration of 1 µCi/ml of medium (either DME or DME/F10) without removing the dishes from the incubator. The dishes were kept in a closed 33°C or 39°C incubator and pulse-labeled for 60 minutes. Following labeling, the cells were put on ice, washed 2 times with ice cold PBS and 1ml of 0.1% SDS in PBS was added to each 100 mm dish. Following cell lysis, the lysate was transferred to a 15

ml polystyrene Falcon tube with the aid of a Teflon-coated razor blade. All samples collected from a single experiment were frozen at -20°C and were processed together at a later time.

The frozen lysates (1 ml) were thawed on ice and 1 ml of 100% (w/v) trichloroacetic acid (TCA) was added to each tube. Following vortexing, each tube was incubated on ice for at least 20 minutes. The samples were vacuum filtered onto Whatman GF/A filters. The filters were then washed 3 times with 5% TCA, once with 70% ethanol, and air dried. Dried filters were placed into scintillation vials and 1 ml of Solvable (NEN) tissue solublizer was added to each vial. Each vial was vortexed and heated at 55-60°C for 20-30 minutes. The vials were then cooled to room temperature and 10 ml Aquassure scintillation fluid (NEN) and 50 µl of 2 N acetic acid were added. After vortexing, the vials were placed in the dark for at least 45 minutes. The samples were then counted in a Beckman scintillation counter and the amount of ^{3}H-TdR incorporation determined.

Autoradiography of Labeled Nuclei

Autoradiography of radiolabeled nuclei was performed as essentially described by Crowe *et al.* (1978) and Malkas (1985). Growth-arrested cultures of cells were trypsinized and seeded at a 1:10 dilution into tissue culture dishes containing sterile glass coverslips and 10 ml of medium (DME supplemented with 10% or 0.5% NCS, penicillin (100 U/ml), and streptomycin (100 µg/ml)). ^{3}H-TdR (5 µCi/ml of 20 Ci/mmole, NEN) was added at the time of seeding and the dishes were incubated at 33°C or 39°C. At various times after seeding, the coverslips were removed and washed twice with PBS. Following fixation in ice cold methanol for 15 minutes, the cells on the coverslips were extracted with ice cold 5% TCA for 30 minutes, rinsed twice with ddH_2O, mounted cell side up with a drop of Permount (Fisher), and allowed to air dry

overnight.

The slides were dipped into Kodak NTB-2 emulsion at 60°C which had been diluted 1:1 with ddH_2O. After drying, the slides were stored in a dark box containing a dessicant for one week. The coverslips were developed by soaking in Kodak D-19 developer for 3 minutes, stop bath for 1 minute, fixer for 5 minutes, followed by extensive washing with water. After drying, the slides were observed under low power and the number of cells containing dark staining nuclei, indicative of ^{3}H-TdR incorporation into DNA, was assessed.

Virological Techniques

SV40 Virus Preparation

SV40 virus was prepared by infection of confluent monolayers of CV-1P cells at low multiplicity of infection as previously described in this laboratory (Ozer, 1972). Cells were grown to confluence in T-175 flasks (Falcon) in DME with 10% fetal bovine serum (FBS). The growth medium was removed and replaced with DME containing 2% FBS. 1ml of SV40 virus stock (SV46-B, 12/84) was used to infect each flask. The virus was propagated at 37°C and 7.5% CO_2.

Infected cultures were harvested when the virus exerted its maximal cytopathic effect. Cells were repeatedly frozen and thawed in the original medium in order to disrupt the cells and release the intracellular virus. Cellular debris was removed by low speed centrifugation. The supernatant, designated as the virus stock, was aliquoted to minimize repeated cycles of freeze-thawing and stored at -20°C. The concentration of virus as plaque-forming units (pfu) was calculated by the proportion of cells infected by immunofluorescence assay for SV40 T-antigen using a standard curve.

Determination of SV40 Virus Titer by Immunofluorescence

Coverslip cultures of CV-1P cells were prepared by seeding cells in 60 mm tissue culture dishes containing sterile coverslips. These coverslips were then transferred to 6-well dishes and the cells were infected with 1ml of various dilutions of virus stock and incubated for 2 hours at 37°C and 10% CO_2. The coverslips were then washed with PBS and fresh medium (DME with 10% FBS) was added to each well. The infection was allowed to proceed by incubating under the same conditions overnight. The coverslips were then processed essentially as described by Solomon *et al.* (1979), by fixation in formaldehyde and permeabilization 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 (BMB) as routinely performed in this laboratory, Coverslips were examined with an Olympus fluorescence microscope under oil immersion. Approximately 200 cells were counted per coverslip and the percentage of cells with nuclear fluorescence calculated. This percentage was then used to extrapolate the virus titer from a standard curve.

SV40 Infection of Quiescent ts2 Cultures

Growth-arrested cultures of ts2 grown in DME with 10% NCS were infected using 1 ml of SV40 stock per 100 mm tissue culture dish or 0.5 ml virus stock per 60 mm dish (moi 5-10). The infection was carried out at 33°C for 2 hours. Following virus adsorption, the virus preparation was removed, the cultures washed three times with prewarmed DME and fed with DME containing 0.5% FBS. The cultures were incubated at 33°C or shifted immediately to 39°C. RNA was prepared from cells harvested at various times post-infection.

Radiolabeled Probes

Random Primed DNA Probes

Random primed DNA probes (Feinberg and Vogelstein, 1983, 1984) were synthesized using the Random Primed DNA labeling Kit from Boehringer Manheim (BMB). Heat denatured DNA (25-100 µg) was used in each reaction, according to the manufacturer's instructions. The DNA was radiolabeled by adding 50 µCi [α-^{32}P] dCTP (3000 Ci/mmole, NEG-013H) (NEN) to each reaction. Following labeling, the newly synthesized DNA was separated from unincorporated nucleotides by passing them over a pre-packed G-50 spun column (BMB). Probes made by this method routinely had a specific activity of 0.5-1.5 X 10^9 dpm/µg input DNA.

Riboprobes

Riboprobes were prepared according to the protocol kindly provided by M. Kauffman and T. Kelly (The Johns Hopkins University School of Medicine) with minor modifications. Riboprobes were made in 25 µl reactions containing 1X Transcription Buffer (Promega), 50 units RNasin (Promega), 600 µM each of rATP, rCTP, and rGTP (BRL), 1-2 µg linearized plasmid DNA, 10-20 units RNA polymerase (T3, T7, or SP6) (Promega or BRL), and 200 µCi [a^{32}P] UTP (800 Ci/mmole, 40.0 mCi/ml, NEG-007C) (NEN). Reactions were incubated for 2 hours at 37°C. 2 units RQ1 DNase (Promega) was added and the tube incubated for an additional 10 minutes at 37°C. The reaction was stopped by adding EDTA to a final concentration of 30 mM. The newly synthesized RNA was separated from the unincorporated nucleotides by passing it over a pre-packed Sephadex G-50 spun column for RNA probes (BMB). The specific activity of the probes prepared using this method was between 10^8-10^9 dpm/µg input DNA.

Plasmids and Probes

Murine Thymidine Kinase Probes

The plasmid containing the cloned murine thymidine kinase cDNA cloned into the expression vector pCD (Okayama and Berg, 1983), pMtk4, was obtained from H. Lieberman (Lin *et al.*, 1985). The DNA was used to transform competent DH-1 *E. coli* cells and preparative amounts of plasmid DNA was isolated from amplified cultures grown in the presence of 75 µg/ml ampicillin by the Triton X-100 lysis protocol. Preparative amounts of plasmid DNA was digested with BamH1 and electrophoresed on a preparative agarose gel. The gel region containing the 1.3 kb BamH1 fragment containing the murine TK cDNA insert was excised and the DNA isolated by electroelution, using an IBI electroeluter and following the manufacturer's instructions. The DNA was precipitated, washed, reconstituted, and quantitated. In early experiments, probes were prepared using this fragment as a template for the random primer labeling reaction.

The 1.3 kb BamH1 was recloned into the BamH1 site of pUC19 to make the plasmid pUCTK using conventional cloning techniques as described in Maniatis *et al.* (1982). The ligated DNA was used to transform competent XL1-Blue cells (Stratagene) and recombinants were selected using blue/white color selection by adding the recommended amounts of X-gal (5'-3') and isopropylthiogalactoside (IPTG; BMB) to the medium. Individual recombinants were screened by restriction digestion followed by gel electrophoresis of DNA prepared by the miniprep technique. Preparative amounts of plasmid DNA was prepared as described below.

In order to isolate a DNA fragment from the 1.3 kb fragment that was free of any SV40 sequences, a restriction map of the murine TK cDNA sequence as reported by Lin *et al.* (1985) was generated at the UMDNJ computer facility. A 647 bp RsaI fragment was determined to be most suitable. Preparative amounts of this DNA was prepared by first digesting pUCTK with BamH1 and recovering the 1.3 kb fragment from low melt-

ing agarose (Sea Kem) using an Elutip™-d column (S&S), following the instructions of the manufacturer. This fragment was then digested with RsaI and the 647 bp fragment of interest was isolated using the Elutip™-d protocol. Following precipitation, washing, resuspension, and quantitation, an aliquot of this DNA was used to verify the complete absence of any SV40 viral sequences by Southern blot analysis, using total SV40 DNA (BRL) as a probe. This 647 bp RsaI fragment was used as a template for a random primed probe to assess the presence of murine TK sequences in all subsequent experiments.

This 647 bp RsaI fragment was then ligated to phosphorylated BamH1 linkers (Stratagene), digested with BamH1, and cloned into the BamH1 site of pGEM ®-7Zf(+) (Stratagene), following digestion and treatment with calf intestinal phosphatase (CIP), to make the plasmid pRMTK. Additional random primer probe template DNA was made by digesting pRMTK, prepared using the Qiagen Maxiprep method, with BamH1 and recovering the 647 bp fragment. In addition, an antisense riboprobe was prepared by using EcoR1 linearized pRMTK DNA as a template for SP6 RNA polymerase (BRL) driven transcription.

Human Thymidine Kinase Probes

The plasmid pMGK23 was obtained from Mike Kauffman and Tom Kelly (The Johns Hopkin University School of Medicine) to be used to prepare a negative stand riboprobe. This plasmid contains the human TK cDNA linked to a -34 bp human TK promoter in pBSKS(+) (Stratagene). This plasmid was digested with SmaI, and purified cut plasmid DNA was used to synthesize a riboprobe in a standard T3 polymerase *in vitro* transcription reaction, as described above.

Since at times this probe seemed to show cross reactivity with murine TK in untransfected ts2 control RNA, even when stringent hybridization and wash conditions, it was

necessary to find an alternative probe and the appropriate reaction conditions in order to ensure that no cross reactivity with murine TK occurred. A comparison of the published sequences of between the human (Bradshaw and Deininger, 1984) and murine TK (Lin *et al.*, 1985) cDNA sequences revealed significant sequence divergence at the 3' end of the gene, especially within the 3' untranslated region. Oligonucleotides were synthesized that were complimentary to regions on opposite strands of DNA and bounded by a region totaling 350 bp, extending from the 3' end of the translated region into the untranslated region. These primers, synthesized by the Hunter College RCMI Sequencing and Synthesis Facility on an Applied Biosystems DNA Synthesizer, were the 25-mer O-5, 5'CCACTCCGTGTGTCGGCTCTGCTAC3' and the 26-mer O-6, 5'GCA-GACCAGTGGGTAGGAGAGGAGGG3', respectively. These oligonucleotide primers were purified and detritylized following deprotection by treatment in ammonium hydroxide at 55°C overnight, lyphilization, and reconstitution by passing them through NENSORB™ PREP cartridges (NEN) following the directions of the manufacturer. Further purification was carried out by passing them over NAPS-10 columns as directed by the manufacturer. The relevant fractions were pooled and the DNA quantitated.

These oligonucleotides were then used as primers in a polymerase chain reaction (PCR) with pMGK23 serving as the DNA template in order to produce preparative amounts of the 350 bp region of the human TK gene. The PCR reaction was set-up using a GeneAmp™ PCR Kit (U.S. Biochemical, Perkin-Elmer Cetus), according to the instructions of the manufacturer. Forty-five cycles of DNA amplification was carried out in a Perkin-Elmer Cetus GeneAmp™ thermocycler. During each cycle, the DNA was denatured at 94°C for 1 minute, annealed at 50°C for 2 minutes, and elongated at 72°C for 3 minutes.

Aliquots of amplified DNA were checked on 1.4% agarose minigels. Preparative amounts of DNA were recovered from low melting point agarose (Sea Kem) using

Elutip™-d columns (S&S) as described above. This DNA was then used in a random primer labeling reaction to synthesize a probe for the detection of human TK sequences in Northern blot analysis of the transfected ts2 cell lines. Under stringent hybridization and wash conditions, there was no evidence of hybridization between this probe and murine TK sequences.

He7 Probe

The plasmid pGEMHe7 containing a 300 bp fragment of the gene He7 cloned into the PstI site of pGEM1 was obtained from the laboratory of J. Nevins (Duke University). He7 has been reported by Kao and Nevins (1983) to be a gene whose RNA is expressed throughout the cell cycle in a cell cycle independent manner. Once it was determined that in ts2 the RNA level of this gene remained constant under all experimental conditions, this was used in subsequent experiments as an internal control to standardize and assess the amount and quality each RNA sample analyzed by Northern blot analysis.

The He7 DNA was used to transform competent RJ7B *E.coli* cells (received from K. Jha, this laboratory). Plasmid DNA was isolated from unamplified mass cultures in the presence of ampicillin using the Triton X-100 method. The 300 bp PstI fragment was recovered from agarose using the GENECLEAN™ Kit (Bio 101) or by Elutip™-d (S&S), following the directions of the respective manufacturer. This fragment was then used in the random primed labeling reaction in order to make a radiolabeled probe.

Neomycin-Resistance Probe

E. coli containing the plasmid pSV2*neo* (Southern and Berg, 1982) was received from R. Athwal (UMDNJ). Preparative amounts of this DNA was prepared by the Triton X-100 method. The plasmid was digested sequentially with HindIII followed by NaeI.

The 1 kb HindIII-NaeI fragment containing *neo* coding sequences along with some 5' sequences from Tn5 was recovered from low melting point agarose by the Elutip™-d (S&S) method and ligated to pGEM®-7Zf(+) digested sequentially with Hind III and SmaI. The ligation reaction was used to transform competent DH-5a *E. coli* cells. Recombinant clones were picked on the basis of blue/white selection in the presence of X-gal and IPTG. Several clones were analyzed by restriction enzyme digestion and a single clone was used to prepare preparative amounts of the plasmid pMO*neo* by the Qiagen MaxiPrep method. The plasmid was linearized with HindIII so that an antisense riboprobe would be synthesized in a standard *in vitro* transcription reaction containing T7 RNA polymerase (BRL).

Human Thymidine Kinase (TK) Constructs Used to Transfect ts2

A number of constructs (pTK8, pTK21, pMGK85, pMGK86) were obtained from Mike Kauffman and Tom Kelly (The Johns Hopkins University School of Medicine) for this analysis. Of these, several were cotransfected into ts2 along with the *neo* containing plasmid pWL*neo* (Stratagene). 20μg of each plasmid DNA in TE was obtained and aliqouts of these DNAs were used directly transfect ts2 cells as described above.

The plasmid pTK8 (Kreidberg and Kelly, 1986, and M. Kauffman, personal communication) was constructed by subcloning the EcoRI-SacI fragment of the phage λTK46 (Bradshaw, 1983) into pUC8. This construct consists of approximately 13.3 kb of human TK genomic sequences, and is complete except for 200 bp of 3' untranslated region including the poly (A) addition site. It contains about 430 bp of sequence 5' to the transcription start site, but is missing 200bp of the extreme 5' genomic sequences, shown to play no role in promoter function (M. Kauffman, personal communication).

pTK21 (Kreidberg and Kelly, 1986), a plasmid containing the human TK cDNA under the control of the human TK promoter, was constructed making use of the unique

XmaI site at the 3' end of the first exon. The plasmid pTK11 (Bradshaw and Deininger, 1984), a full-length TK cDNA clone obtained by screening the Okayama-Berg expression library, served as a starting point for this construction. pTK11 contains the TK cDNA positioned between the SV40 early promoter and an SV40 poly (A) site. Following linearization of pTK11 with SalI, the recessed 3' ends were blunted with Klenow fragment, and after the addition of SacI phosphorylated linkers, the DNA was digested with SacI and XmaI. The larger fragment that contains the TK cDNA sequences 3' to the XmaI site, the SV40 poly (A) addition site, and pBR322 vector sequences was ligated to the SacI-to-XmaI fragment derived from pTK8 containing the genomic sequence upstream of the start site and mRNA coding sequences 5' to the XmaI site. Therefore, pTK11 consists of the TK promoter derived from pTK8 driving the complete TK cDNA, including its endogenous poly (A) addition signal, and an SV40 poly (A) region from pTK11 (Kreidberg and Kelly, 1986, and M. Kauffman, personal communication).

The plasmid pMGK85 consists of the Herpes Simplex Virus (HSV) TK promoter driving the human TK capsite and genomic coding region in pUC. This plasmid deletes 8bp from the human 5' untranslated region, and, like pTK8, is missing the distal 200 bp of the 3' untranslated region of the human gene. The plasmid pMGK85 also lacks poly (A) addition signals (M. Kauffman, personal communication).

Plamid pMGK86, used only as a negative control in several experiments, consists of the human TK promoter and mRNA cap sites driving the Herpes viral TK coding region (M. Kauffman, personal communication).

DNA Isolation Protocols

Plasmid Preparation

Bacterial Culture Techniques

Overnight cultures of *Escherichia coli (E. coli)* were grown from single colonies in 10 ml LB (LB; 1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl) medium containing 75 µg/ml ampicillin (Sigma) or carbenicillin (Sigma) or 15 µg/ml tetracycline (Sigma) in 50 ml Falcon tubes at 37°C with vigorous shaking. Large-scale cultures were inoculated with 0.1 ml of overnight culture. Minipreps were prepared directly from the overnight cultures.

Competent *Escherichia coli* Cells

Competent XL1-Blue cells were obtained from Stratagene and competent DH-5 and DH-5a cells were obtained from BRL. The were aliquoted and stored at -70°C until ready for use. In addition, competent cells from these cell lines as well as from RJ7B were prepared by K. Jha and J.Dermody (this laboratory) using the method of Hanahan (1983). Competent HB101 or DH-1 cells were prepared by growng cells picked from a single colony in 5 ml of LB broth for 2 hours at 37°C with vigorous shaking. This culture was transferred to 100 ml of fresh LB broth and shaken vigorously at 37°C for 2.5 hours, until the cells reached a density of 25-30 Klett units. The cells were pelleted by centrifugation at 2500 rpm for 5 minutes at 4°C. The supernatant was drained and the pellet was resuspended in 30ml of Transformation Buffer I (30 mM potassium acetate, 50 mM $MgCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15% (w/v) glycerol, pH 5.8 with 0.2 M acetic acid) and incubated on ice for 100 minutes for HB101 cells or 10 minutes for DH-1 cells. The cells were pelleted by centrifugation at 2000 rpm for 5 minutes at 4°C. After the supernatant was drained, the cells were gently resuspended in

4ml of Transformation Buffer II (10 mM Na-MOPS, pH 7.0, 75 mM $CaCl_2$, 10 mM KCl, 15% (w/v) glycerol) and stored in 100 µl aliquots in Nunc freezedown vials at -70°C.

Transformation of *Escherichia coli* Cells

Competent *E. coli* cells were transformed with plasmid DNA from various sources by adding no more than 100 µg of DNA to thawed aliquots of cells and incubating on ice for 30 minutes. The cells were then heat shocked for 2 minutes at 37°C. 800 µl of LB was added and the cells were incubated at 37°C for 60 minutes at 37°C with vigorous shaking. Aliquots of cells were then spread on antibiotic-containing plates and incubated overnight at 37°C.

Competent HB101, XL1-Blue, DH-5, or DH-5a cells were transformed following thawing on ice. Aliquots of cells (100 µl) were placed in 15 ml polypropylene (Falcon 2059) tubes. Freshly diluted β-mercaptoethanol was added to a final concentration of 25 mM. The cells were incubated on ice for 10 minutes with gentle swirling every 2 minutes. Approximately 10 ng of DNA was added and the cells were incubated in ice for an additional 30 minutes. The cells were heat pulsed in a 42°C water bath for 45 seconds and then incubated in ice for 2 minutes. S.O.C. medium (0.9 ml) (S.O.C.; 2% Bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added and the cells were incubated with vigorous shaking at 37°C for 1 hour. Varying amounts of cells were then plated on LB plates containing the appropriate antibiotic. In the case of DH-5a and XL1-Blue cells, when recombinant plasmids were being selected for, these plates also contained X-gal and IPTG.

Plasmid Preparation by theTriton lysis/CsCl Method

Bacterial cultures were grown overnight at 37°C in LB (LB; 1% Bacto-tryptone,

0.5% yeast extract, 1% NaCl) medium containing 75 μg/ml of ampicillin (Sigma) or carbenicillin (Sigma) in either the presence or absence of 34 μg/ml chloramphenicol (Sigma). Cells were pelleted by centrifugation in a Sorvall GSA rotor at 6,000 rpm for six minutes at 4°C. Following washing and recentrifugation, the pellet from each liter of bacterial culture was resuspended in 8 ml 25% sucrose, 50 mM Tris-HCl, pH 8.0, and transferred to a prechilled 30 ml Oakridge tube. Lysozyme(2.5 ml of 10 mg/ml stock) in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) and incubated on ice for 5 minutes. EDTA (2.5 ml of 0.5M EDTA, pH 8.0) was then added and the tubes were incubated on ice for an additional 5 minutes. Triton lysis mix (12.5 ml) (0.1% TritonX-100 (Sigma), 60 mM EDTA, pH 8.0, 50 mM Tris-HCl, pH 8.0) was then added and following mixing by gentle inversion, the tubes were incubated on ice for 15 minutes. The lysate was cleared by centrifugation at 4°C in a Beckman 70Ti rotor for 30 minutes at 32,000 rpm. The supernatant was then treated with RNase A (10 μg/ml) (Sigma) at 37°C for 30 minutes.

To each 25 ml of lysate, 25 grams CsCl (BMB, molecular biology grade) and 2.5 ml ethidium bromide (10 mg/ml) was added and the lysate was transferred to Seton Easy Seal tubes for either the 70Ti on v50Ti rotor. The plasmid DNA was banded by centrifugation at 20°C in a 70Ti rotor at 38,000 rpm for 48 hours or in a v50Ti rotor at 45,000 for 16-20 hours. The plasmid band was removed by puncturing the side of the tube with an 18-gauge needle connected to a 10 ml syringe. If further purification was desired, this material was banded a second time using the same conditions. The DNA was then extracted several times with water saturated *n*-butanol in order to remove the ethidium bromide, followed by dialysis overnight at 4°C against TE to remove the CsCl. The solution was then extracted two times with phenol/chloroform/isoamyl alcohol (25:24:1 v/v) and once with chloroform/isoamyl (24:1 v/v). The DNA was then precipitated by adding 1/10th volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of absolute ethanol and storage at -70°C for 1 hour. The DNA was recovered by centrifu-

gation at 4°C for 15 minutes at 11,000 rpm in a HB-4 rotor. After washing the pellet with cold 70% ethanol, it was dried in a Savant Speed-vac. The DNA was dissolved in sterile TE and quantitated spectrophotometrically. The quality of the plasmid DNA was determined by electrophoresis of aliquots of the DNA both with and without the use of the appropriate restriction enzymes. The DNA was stored in sterile microfuge tube at -20°C.

Plasmid Isolation by Column Purification

Plasmid DNA was also isolated using Qiagen plasmid isolation kits (Qiagen). For large-scale plasmid preparation Qiagen-tip 500 (Maxi prep kit) columns were used to isolate plasmid DNA from 250 ml of unamplified bacterial cultures, while Qiagen-tip 20 (Mini prep kit) columns were used to isolate plasmid DNA from 1.5-3 ml of overnight culture. The isolation procedure followed the manufacturer's protocol and used the solutions supplied with each kit. Essentially, after the alkaline lysis of the bacterial cells, the cleared lysate was applied to a specially prepared anion exchange column under the appropriate ionic conditions. After extensive washing, the plasmid DNA was eluted off the column by altering the ionic strength and the pH of the buffer. The DNA was then precipitated with isopropanol. Following reconstitution of the DNA in sterile TE, the DNA was quantitated spectrophotometrically and assessed for purity on the basis of the OD_{260}/OD_{280} ratio. The integrity of the DNA was determined by gel electrophoresis of small aliquots of both undigested DNA and DNA digested with diagnostic restriction enzymes. In order to assess the biological activity of the DNA, the ability of the DNA to transform bacterial and/or ts2 cells was examined.

Plasmid Minipreps by the Boiling Method

Rapid, small-scale isolation of plasmid DNA by the boiling method was carried out as described in Maniatis *et al.* (1982) with some modifications. Ten ml cultures contain-

ing the appropriate antibiotic were grown overnight at 37°C with vigorous agitation. Cells from 3 ml of culture were pelleted by consecutively centrifuging 1.5 ml of culture in a microfuge tube. The pellet was resuspended in 350µl of lysis buffer (8% sucrose, 50 mM Triton X-100, 50 mM EDTA, pH 8.0, and 100 mM Tris-HCl, pH 8.0), followed by the addition of 25 µl of lysozyme solution (10 mg/ml) in 10 mM Tris-HCl, pH 8.0. After vortexing for 3 seconds, the tube was placed in a boiling water bath for 40 seconds and then centrifuged at room temperature in a microfuge. Following removal of the pellet with a toothpick, the supernatant was extracted two times with phenol/chloroform/isoamyl alcohol (25:24:1 v/v) and once with chloroform/isoamyl alcohol (24:1 v/v). After adjusting the volume to about 400 µl, 40 µl of 2.5 M sodium acetate, pH 5.2, and 420 µl of isopropanol was added and the tube was vortexed for several seconds. Plasmid DNA was precipitated by storing the tube in a dry ice/ethanol bath for 15 minutes. The DNA was precipitated by centrifuging for 15 minutes at 4°C. The pellet was then washed with 70% ethanol and Speed-Vac dried. The pellet was resuspended in 50-100 µl TE, pH 8.0, containing 50 µg/ml DNase-free RNase (BMB). The quality of the DNA was assessed by analyzing 5-10 µl aliquots of the DNA by gel electrophoresis with and without digestion with selected restriction enzymes.

Preparation of High Molecular (HMW) Cellular DNA from Mammalian Cells

Cells were pelleted by low speed centrifugation and either frozen at -20°C or used immediately to prepare HMW DNA. Cell pellets were suspended in approximately 1 ml of lysis buffer per 1-2 x 10^7 cells (lysis buffer= 50 mM Tris-HCl, pH 7.5, 50 mM Na_2EDTA, 100 mM NaCl). The cells were lysed with the addition of 10% SDS to a final concentration of 0.1%. After mixing by gentle inversion, the tubes were placed in a 60°C water bath for 10-15 in order in inactivate endogenous DNase activity. Following cooling to room temperature, Proteinase K (BMB, prepared as a 20 mg/ml stock) was

added to a final concentration of 100 μg/ml and the tubes were incubated at 37°C or 50°C for 24 hours.

The lysate was extracted gently 2 times with phenol/chloroform/isoamyl alcohol (25:24:1 v/v) and once with chloroform/isoamyl alcohol (24:1 v/v). Cold absolute ethanol (2.5 volumes) was added to the viscous lysate in order to precipitate the DNA. The DNA was picked up on the sealed end of a hooked Pasteur pipette and was rinsed by dunking into 5 ml of 70% ethanol. The DNA was then allowed to air dry in the hood and then was transferred to a Falcon tube containing sterile TE. The DNA was allowed to dissolve by rocking the tube at 4°C for 1-2 days or by incubating at 37°C or 50°C for several hours. The HMW DNA was then subjected to RNase A treatment, by first adjusting the salt concentration of the TE to 150 mM NaCl by adding the appropriate amount of 5 M NaCl, followed by the addition of DNase-free RNase (BMB prepared stock) to a final concentration of 100 μg/ml and incubation at 37°C for 4 hours. The HMW was either first treated with Proteinase K or immediately extracted as described above. The DNA was once again ethanol precipitated, and reconstituted in sterile TE as described above. The DNA was quantitated spectrophotometrically (OD_{260}) (1OD=50μg/ml) and it purity assessed by determining the OD_{260}/OD_{280} ratio (≥1.8), while DNA quality was assessed by gel electrophoresis of small aliquots of both undigested and restriction enzyme digested DNA.

DNA Analysis Protocols

Digestion of DNA with Restriction Enzymes

Restriction enzymes from several commercial sources (BMB, IBI, BRL, and Promega) were used to digest both plasmid and high molecular weight cellular DNA. Digestions were carried out in the appropriate buffers as supplied by the manufacturer at the recommmended temperatures. Generally, at least 5 units of enzyme per μg of DNA

was used and the time of incubation varied from 1 hour to overnight, depending upon the purpose of the digestion. HMW genomic DNA used for Southern blot analysis was subjected to overnight digestion. The glycerol concentration of individual digestions never exceeded 5%.

Gel Electrophoresis of DNA

For analysis of DNA by gel electrophoresis, either digested or undigested, 1-1.5% agarose (IBI- Molecular Biology Grade) gels were used. Either 0.5X TPE (1X TPE; 0.08 M Tris-acetate, 0.002 M EDTA) or 1X TAE (1X TAE; 0.04 M Tris-phosphate, 0.002 M EDTA), prepared according to Maniatis *et al.* (1982), was used in the gels as well as for running buffer. Prior to loading, the appropriate amount of 6X gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll 400 in H_2O) was added to each sample. For rapid analysis, minigels were run at 100 volts for one hour. Gels to be used for Southern blot analysis were run in an IBI MPH gel electrophoresis apparatus at 5-7 volts/cm until the leading edge of the dye front moved down about 75% the length of the gel. Ethidium bromide (0.5 µg/ml final concentration) was either included in the gel and running buffer during electrophoresis or the gel was stained with 0.5 µg/ml ethidium bromide following electrophoresis.

Recovery of DNA Fragments from Agarose Gels

Several different methods were used to recover DNA fragments from agarose gels after electrophoretic separation, following very brief staining with ethidium bromide and excision of the appropriate band. The DNA was electroeluted from the agarose using the IBI electroeluter or the GENELUTER™ apparatus (Invitrogen) as directed by their respective manufacturers, or by using the dialysis bag technique as described by Maniatis *et al.* (1982). In other instances, the DNA was recovered by using the GENECLEAN™

Kit (BIO 100) or from low melting point agarose using Elutip™-d columns (NEN) as directed by the respective manufacturer.

Southern Blot Analysis

Southern blot analysis of DNA was carried out using the method of Southern (1975) with modifications. Following electrophoresis, gels were rinsed in ddH_2O. The DNA was depurinated by soaking the gel in 0.25 N HCl for 10 minutes and denatured by soaking in 1 N NaOH for 40 minutes. After rinsing with water, the gels were neutralized by soaking for 1 hour in 0.5 M Tris-HCl, pH 7.0, and 1.5 M NaCl and kept in 6X SSC until they were set-up for blotting.

The DNA was blotted overnight onto NYTRAN membranes (0.45 µm) (S&S) pre-moistened by soaking in ddH_2O and 6X SSC by capillary transfer against 20X SSC using a transfer set-up as described in Maniatis *et al.* (1982). Following transfer, the filters were soaked in 6X SSC for 5 minutes, baked at 80°C under vacuum for 2 hours, put in Seal-A-Meal bags and stored at 4°C up to several days until prehybridization.

Prehybridization and hybridization were carried out essentially following the protocol kindly provided by Mike Kauffman. Prehybidization and hybridization solutions were identical except for the absence or presence of probe. The solution consisted of 1X PE (5X PE; 250 mM Tris-HCl, pH 7.5, 0.5% (w/v) sodium pyrophosphate, 5% SDS, 1% polyvinylpyrolidone (40,000 MW), 1% Ficoll 400, 25 mM EDTA, 5% BSA) 3X SSC, 50% deionized formamide, and 150 µg/ml sonicated salmon sperm DNA. Filters were prehybridized at 51°C for 2 hours, and hybridized overnight in fresh solution with 1.5 X 10^6 cpm/ml of the appropriate riboprobe at 51°C. Blots were then soaked for 10 minutes in ddH_2O at room temperature, and then at 61°C for 1 hour in 2X SSC and 0.1% SDS and then twice for one hour each in 0.1X SSC and 0.1% SDS. Blots were then blotted dry and exposed for various times to Kodak XAR-5 film at -70°C with a

pair of Dupont Cronex intensifying screens.

RNA Isolation Protocols

Bentonite/SDS/Formaldehyde Method

Total cellular RNA was isolated using the procedure of Hatch and Bonner (1987) with some modifications. This method was particularly useful because of its relative simplicity. It greatly facilitated the preparation and handling of RNA from multiple samples, while minimizing the number cells needed for each condition analyzed. Although the RNA prepared by this method appears undegraded, it is impure and the amount of RNA cannot be quantitated spectrophotometrically. When electrophoresed through agarose gels, the mobilities of samples prepared at different times were variable. However, Northern blots of gels using RNA prepared by the bentonite/SDS/formaldehyde method were of excellent quality. In addition, since this technique is limited to single cell culture dishes, this method is not useful when preparative amounts of RNA is needed. In most cases, these limitations are easily overcome by using multiple dishes for each condition and by including the appropriate internal controls when analyzing these samples.

Following aspiration of cell culture medium, each 100 mm cell culture dish was washed two times with 10 ml PBS at room temperature. The cells were then washed once with 2 ml of trypsin and then subjected to trypsinization using 1 ml of trypsin at 37°C for three minutes. The cell were then knocked loose and scraped off the dish with a Teflon tape-covered single edge blade and pipetted into a chilled sterile microfuge tube. Cells were pelleted by spinning at room temperature for two minutes and the supernatant was removed by aspiration. The cell pellet was then resuspended by ticturation in 90 μl of cold sterile PBS containing 2% bentonite (Sigma). 270 μl of formaldehyde-SDS (FSDS= 46% deionized formamide, 6.3% formaldehyde, 1X gel buffer, and 1.7%

SDS) buffer preheated to 65°C was then added to each tube. After gentle mixing, the tubes were incubated at 65°C for 10 minutes and then microfuged for 10 minutes. Following removal of the pelleted cellular debris with a sterile micropipet tip, the samples were stored at -70°C until aliquots were electrophoresed on an RNA gel.

Guanidinium Isothiocyanate (GIT/Sarkosyl) Method

When large amounts of total RNA was needed, the method of Chomczyski and Sacchi (1987) was used. This simplicity of this technique facilitated the preparation of relatively pure and undegraded RNA from multiple samples of cells.

Cells were rinsed two times with PBS at room temperature. Lysis mixture (4M guanidinium thiocyanate (Sigma), 0.5% Sarkosyl (Sigma), and 0.7% β-mercaptoethanol (Sigma)) preheated to 65°C was added to each dish (2 ml/100 mm dish). Following cell lysis, the dishes were scraped with a Teflon tape-coated blade and the lysate decanted into a polypropylene centrifuge tube. One-tenth volume of 2 M sodium acetate, pH 4.5, was added, followed by an equal volume of water equilibrated phenol and 1/5th volume of chloroform/isoamyl alcohol (24:1). The tube was vortexed for 10 seconds and spun in a tabletop centrifuge for 10 minutes at 2,500 rpm. The aqueous layer was carefully pipetted into a autoclaved tube and an equal volume of cold (-20°C) isopropanol was added. The contents were mixed well and stored overnight at -20°C or at -70°C for one hour to precipitate the RNA. Following precipitation, the RNA was pelleted by centrifugation at 11,000 rpm in an Sorvall HB-4 rotor for 20 minutes at 4°C. The supernatant was discarded and the pellet was carefully washed with 70% ethanol and allowed to air dry. The pellet was then resuspended in DEPC-treated autoclaved ddH_2O and quantitated spectrophotometrically by measuring OD_{260} ($1OD_{260}$=40 µg/ml). The RNA was then aliquoted into microfuge tubes and 1/10 volume sodium acetate, pH 4.5, and 2.5 volumes absolute ethanol were added. Following mixing, the tubes were stored

at -70°C. The samples were prepared for elecrophoresis by spinning in a microfuge for 15 minutes, removing the supernatant, washing the pellet with 70% ethanol, drying the pellet and resuspending it in the appropriate amount of sample buffer (50% deionized formamide, 6.6% formaldehyde, 1X gel running buffer).

Guanidinium/CsCl Method

In early slot blot experiments, total cellular RNA was isolated from ts2 using the guanidinium/cesium chloride method as described by Maniatis *et al.* (1982) with several modifications. Since this method is relatively unwieldy, the preparation of multiple samples was relatively difficult. Therefore, this method was abandoned in favor of either the guanidinium isothiocyanate method (Chomczyski and Sacchi, 1987) or the bentonite/SDS/formaldehyde method (Hatch and Bonner, 1987).

In brief, cells were collected by scraping them into cold sterile PBS and the pelleted by low speed centrifugation in a tabletop centrifuge at 4°C. The cells were lysed upon the addition of 6 ml of lysis buffer (4 M guanidinium isothiocyanate, 5 mM sodium citrate, pH 7.0, 0.1 M β-mercaptoethanol, 0.5% Sarkosyl) and vigorous vortexing for 30 seconds. The cell lysates were immediately frozen at -70°C for later processing. Upon thawing, 2.8 g CsCl was added to the lysate. The homogenate was layered onto a 3 ml cushion of filtered 5.7 M CsCl in 0.1 M EDTA, pH 7.5, in a sterile Beckman SW41 pollyallomer tube and centrifuged at 32,000 rpm for 16 hours at 20°C. Following centrifugation, the supernatant was carefully removed from the tube and the walls of the tube thoroughly dried with a Kimwipe. The RNA pellet was dissolved in 10 mM Tris-HCl, pH 7.4, 5 mM EDTA , and 1% SDS, extracted with phenol/chloroform/isoamyl alcohol (25:24:1 v/v) and reextracted with chloroform/isoamyl alcohol (24:1 v/v). The RNA was then precipitated by adding 0.1 volume 3 M sodium acetate, pH 5.2, and 2.2 volumes of ethanol. After storage at -20°C for at least 2 hours, the RNA was recovered

by centrifugation. The RNA was then dissolved in H_2O, quantitated spectrophotometrically, aliquoted, reprecipitated in ethanol, and stored at -70°C in 70% ethanol.

Hot Phenol Method

Some of the RNA samples analyzed by slot blot analysis were kindly provided by L. Malkas and were prepared by the hot phenol extraction method. As described by Malkas (1985), total cellular RNA was isolated by phenol extraction at 60°C as originally described by Soeiro and Darnell (1969) and Hsuing *et al.* (1982). Cells were harvested from 100 mm tissue culture petri dished by scraping with a Teflon tape-covered razor blade. The cell pellet was washed twice in PBS, then suspended at 10^7 cells per ml in 0.1 M sodium acetate-1 mM trisodium EDTA, pH 5.2. Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5% and the mixture was immediately extracted with buffer-saturated, redistilled phenol equilibrated at 60°C. RNA was recovered by ethanol precipitation at -60°C. The RNA was dissolved in autoclaved DEPC-treated ddH_2O, quantitated spectrophotometrically, aliquoted, reprecipitated in ethanol, and stored in ethanol at -60°C or -20°C.

RNA Analysis Protocols

Slot Blot Analysis of RNA

RNA samples were bound to nitrocellulose according to the protocol of Han *et al.* (1986). Aliquots of RNA stored in ethanol at -20°C were centrifuged in a microfuge for 15 minutes at 4°C. The RNA pellets were washed with 70% ethanol, dried in a Savant Speed-Vac, and dissolved in 10 µl of denaturing solution (2.2 M formaldehyde, 50% (v/v) deionized formamide, 10 mM sodium phosphate buffer, pH 7.0) and heated for 10 minutes at 60°C. After quenching on ice, 90 µl of 20X standard saline citrate (SSC;

1X SSC = 0.1 M NaCl, 15 mM sodium citrate) was added to each sample. The samples were then passed through a nitrocellulose filter that has been moistened with water and equilibrated with 20X SSC in a HYBRI-SLOT™ (BRL) 24-slot manifold with the aid of water-generated vacuum. Each slot was then rinsed twice with 100 µl 20X SSC and the filters were baked at 80°C under vacuum for 2 hours. Individual filters were stored dry in Seal-A-Meal (Daisy) bags at 4°C until prehybridization solution was added.

Preparation of RNA Samples for Gel Electrophoresis

Samples prepared by the bentonite/SDS/formaldehyde method were thawed on ice, heated for 10 minutes at 68°C, and quenched on ice. 2 µl of 50% glycerol containing bromophenol blue were added to 18 µl aliquots of each sample. The samples were then loaded onto agarose gels for electrophoresis.

Aliquots of RNA samples prepared by other methods and stored in ethanol were centrifuged in a microfuge for 15 minutes at 4°C, the pellets washed with 70% ethanol and dried in a Speed-vac. The RNA pellet was resuspended in 18 µl of 1X Sample Buffer (50% deionized formamide, 1X MOPS, and 2.2 M formaldehyde). If the samples were run on gels containing no bentonite/SDS/ formaldehyde samples, the sample buffer also contained 40 µg/ml of ethidium bromide. The samples were heated to 68°C for 10 minutes and quenched on ice. Before loading onto a gel, 2 µl of 50% glycerol containing bromophenol blue were added to each sample.

RNA Gel Electrophoresis

Once loaded, the samples were electrophoresed in an IBI MPH horizontal gel electrophoresis apparatus on a 1.2% agarose (BRL) gel containing 1X MOPS running buffer (10X MOPS; 200 mM MOPS (Sigma), 50 mM sodium acetate, and 10 mM EDTA, the pH adjusted to 7.0 with glacial acetic acid), and 0.66 M formaldehyde at 100 volts for

approximately 2 hours. The 1X MOPS running buffer was circulated every 30 minutes with a 60 ml syringe.

Following electrophoresis, gels whose samples contained ethidium bromide were immediately visualized under UV light and photographed and soaked in ddH_2O until set-up for blotting. Gels run without ethidium bromide in the samples, were stained in ddH_2O containing 1µg/ml ethidium bromide for 30-60 minutes, followed by destaining in ddH_2O for several hours. They were then photographed and set-up for blotting.

Northern Blot Analysis

The gels were set-up for blotting as was described in the Southern blot procedure. The RNA was transferred overnight by capillary transfer using 10X SSC onto NYTRAN membranes. The filters were then soaked in 6X SSC for 5 minutes and the RNA immobilized on the filters by baking at 80°C under vacuum for 2 hours or by baking followed by UV-crosslinking in a Stratolinker. The filters were placed in Seal-A-Meal bags and prehybridized immediately or stored at 4°C up to several days.

Prehybidization and Hybridization of RNA Filters

Filters containing RNA either from slot blotting or Northern blotting were handled in the same manner. Prehybidization mix (50% formamide, 5% dextran sulfate, 2X SSC, 0.1% SDS, 1X Denhardt's (Maniatis, 1982), 50 mM sodium phosphate buffer, pH 7.5, and 100 µg/ml sonicated, boiled and quenched salmon sperm DNA) was added to each bag. Filters were prehybridized for 3-4 hours. The prehybridization mix was removed and hybridization mix (50% formamide, 5% dextran sulfate, 2X SSC, 0.1% SDS, 5 mM EDTA, 50 mM sodium phosphate, pH 7.5, 100 µg/ml sonicated, boiled, and quenched salmon sperm DNA, along with 1.5 X 10^6 cpm/ml of random primed radiolabeled DNA probe) was added to the bags and hybridization was carried out

overnight. All prehybridizations and hybridizations, with the exception of those involving the transfected cell lines and TK probes which required higher stringency, were carried out at 45°C. Incubations involving the transfected cell lines analyzed with TK probes were carried out at 50°C. The filters were washed for 30 minutes at room temperature in 2X SSC and 0.1% SDS, followed by two washings in 0.2X SSC and 0.1% SDS and two washings in 0.1X SSC and 0.1% SDS. All blots, with the exception of those involving samples from the transfected cell lines probed for TK, were washed at 50°C. Blots with samples from the transfected cell lines analyzed for TK were washed at 62°C. Following washing, the filters were blotted semi-dry, wrapped in plastic, and exposed to Kodak XAR-5 film with two Dupont Cronex enhancing screens at -70°C for varying lengths of time.

CHAPTER THREE: Results

Characterization of ts2

Kinetics of DNA synthesis in ts2 following growth arrest and stimulation. The kinetics of the decay in DNA synthesis has been investigated in logarithmically growing ts2 cells shifted to 39°C (Slater and Ozer, 1976). It was observed that the level of DNA synthesis declined, continuing for over a cell generation time without any substantial loss of viability. To better understand the kinetics of DNA synthesis during S phase as it relates to the temperature-sensitive growth of ts2, experiments employing synchronized cells were performed. Since the vast majority of cells are in the same phase in synchronized cultures, the analysis of cellular activities in particular phases of the cell cycle is greatly facilitated. 3T3 cells, including ts2, can easily be synchronized by density-dependent inhibition of growth. As described in *Materials and Methods*, cells were plated at high density in complete medium and incubated at 33°C for 4 days. By measuring ^{3}H-TdR incorporation into ts2 cells plated under these conditions by a one hour pulse label of cultures each day over a seven day period, Malkas (1985) showed that the overall rate of DNA synthesis is at a basal level 96 hours after seeding with no further increase in cell number. Therefore, 96 hours after seeding ts2 cells at high density, ts2 cells become quiescent by a combination of contact inhibition and depletion of serum growth factors. In all experiments, quiescence was confirmed by pulse-labeling ts2 cultures just prior to stimulation (at time zero).

These G0-arrested cultures were then stimulated to reenter their growth cycle by either the addition of fresh medium containing 10% NCS, or by acute infection with SV40 virions. Flow cytometric analysis of the arrested cultures show that at the start of these experiments 94% of the cells were in G0/G1 (Figure 1, panel B) as compared to

only 67% of cells in G1 in logarithmically growing cultures (Figure 1, panel A). These cultures were then incubated further at 33°C or immediately shifted to 39°C. At various times following stimulation, the level of DNA synthesis was assayed in duplicate dishes as measured by ^{3}H-TdR incorporation following one hour pulse label of cultures. In addition, at the time of serum stimulation several dishes of confluent cells were trypsinized, pooled and plated onto coverslips in complete DME containing 5 μCi/ml ^{3}H-TdR and incubated at either 33°C or 39°C. The coverslips were fixed and mounted onto slides and the proportion of positive nuclei (indicative of DNA synthesis) was determined by autoradiography at various times following the addition of fresh serum.

Pulse-labeling experiments using ^{3}H-TdR, as summarized in Figure 2 and Table 1, show that at 20 h post-serum stimulation, the level of increase of TCA precipitatable counts at 33°C is approximately two-fold over that observed at 39°C. At 28 h following the addition of serum, this difference increases to about 3.5-fold. The level of incorporation remains high at 33°C while falling considerably at 39°C. By 46 h, these cells have completed one round of DNA replication and as expected, the level of ^{3}H-TdR incorporation into newly synthesized DNA decreases significantly at both temperatures. However, the level of incorporation of the radiolabel at the permissive temperature is still two-fold greater than at the nonpermissive temperature. These data indicate that following serum stimulation of quiescent ts2 cultures, the ability of ts2 cells to reenter the cell cycle and synthesize new DNA is significantly impaired at 39°C.

In marked contrast to these data, when acute SV40 infection is used to stimulate quiescent ts2 cultures, the peak level of incorporation of the radiolabel is substantially greater than what is observed in serum-stimulated cells. In addition, at the 20 h point, the the level of incorporation at 39°C is about three times that seen at 33°C. At 33°C, the level of induction of DNA synthesis is approximately equal to that observed at 20h at 33°C in serum-stimulated cells. At 39°C, however, there is a 45-fold induction of

DNA synthesis over basal levels in serum induced cells as compared to a 225-fold induction in virally infected cells. By 28 h at 33°C, the level of induction shows a further moderate increase, while decreasing significantly at 39°C. At 46 h, the levels of ^{3}H-TdR incorporation decreases significantly at both temperatures. From these data, it appears that the block in DNA synthesis observed at the nonpermissive temperature is overcome when SV40 infection is used to stimulate cells out of quiescence. The fact that the peak levels of incorporation are greater than those observed in serum-stimulated cells could at least in part be accounted for by the induction of viral DNA synthesis along with cellular DNA synthesis. Furthermore, the increased kinetics of the reactions involved in DNA synthesis at the higher temperature could at least in part explain the three-fold difference observed at 39°C when compared to 33°C at 20 h post-infection.

In order to determine the percentage of ts2 cells within a given culture that are actually synthesizing DNA, coverslips were plated at the time zero point and incubated at either 33°C or 39°C in fresh DME containing either 0.5% or 10% NCS along with 5 µCi/ml ^{3}H-TdR. At various times following plating, these coverslips containing cells that were subjected to steady-state ^{3}H-TdR labeling were fixed and prepared for autoradiography as described in *Materials and Methods*. In each case, the percentage of labeled nuclei, indicating ^{3}H-TdR incorporation and thus DNA synthesis, was determined and the results presented in Table 2. As expected, relatively few cells replated in 0.5% NCS (5-10%) were able to reenter the cell cycle at both temperatures, even at 26 h after plating. However, while there are some cells synthesizing DNA by 16 h after seeding in 10% NCS (10-15%), it is not until 26 h that we see that between 80-90% of cells incubated at 33°C have labeled nuclei as compared with only 30-40% of cells incubated at 39°C. These autoradiographic data support the pulse-labeling data, suggesting that ts2 has a thermolabile mutation that effects the ability of ts2 cells to enter S phase when growth arrested cells are stimulated with fresh serum and then incubated at

the nonpermissive temperature. The fact that 30-40% of the nuclei incubated at the nonpermissive temperature was positive is likely attributable to the inherent "leakiness" of the *ts* defect in these cells. In addition, it is likely that some of the effects observed under these steady-state labeling conditions are artifactual and some positive nuclei could conceivably represent DNA repair synthesis and not true DNA synthesis.

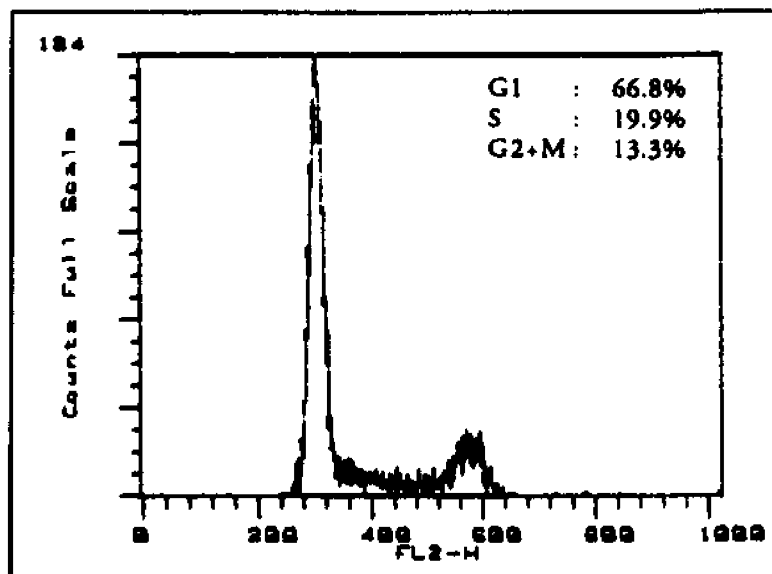
Figure 1: Analysis of ts2 during logarithmic growth and following rundown by flow cytometry.

Ts2 cells growing at 33°C were prepared for flow cytometry (as described in *Materials and Methods*) 24 hours after plating (for logarithmically growing cells) and 96 hours after plating (for quiescent cells). Cells were analyzed by the UMDNJ Cellular Immunology Laboratory using a Becton Dickinson cell sorter equipped with CellFIT Cell-Cycle Analysis Version 1.2 software.

Histograms for exponentially growing ts2 cells, panel A, and quiescent cells, panel B.

Panel A:

Log Phase Cells
Growing at 33°C



Panel B:

Quiescent Cells
Grown at 33°C
(time zero)

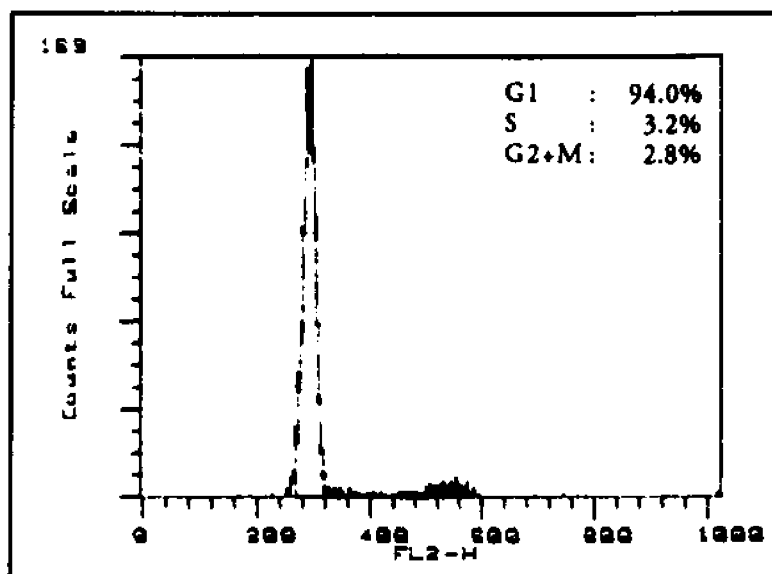


Figure 2: ^{3}H-TdR pulse-labeling of ts2 cells following serum stimulation or acute SV40 virus infection.

Following serum arrest of ts2 cells grown at 33°C, 100 mm cell culture dishes were either harvested (time zero) or washed 2X with DME and refed with DME + 10% NCS (for serum reversal), or with DME + 0.5% NCS (as control for serum reversal), or acutely infected with SV40 virus or treated with uninfected cellular lysate (as control for virus infection). Following incubation at either 33°C or 39°C for 20, 28, or 46 hours, duplicate dishes from each experimental condition were pulse-labeled for 60 minutes and processed as described in *Materials and Methods*. ^{3}H-TdR incorporation was measured by determining the number of counts per minute (cpm) in the TCA precipitatable material from a single dish using a Beckman scintillation counter on an open channel.

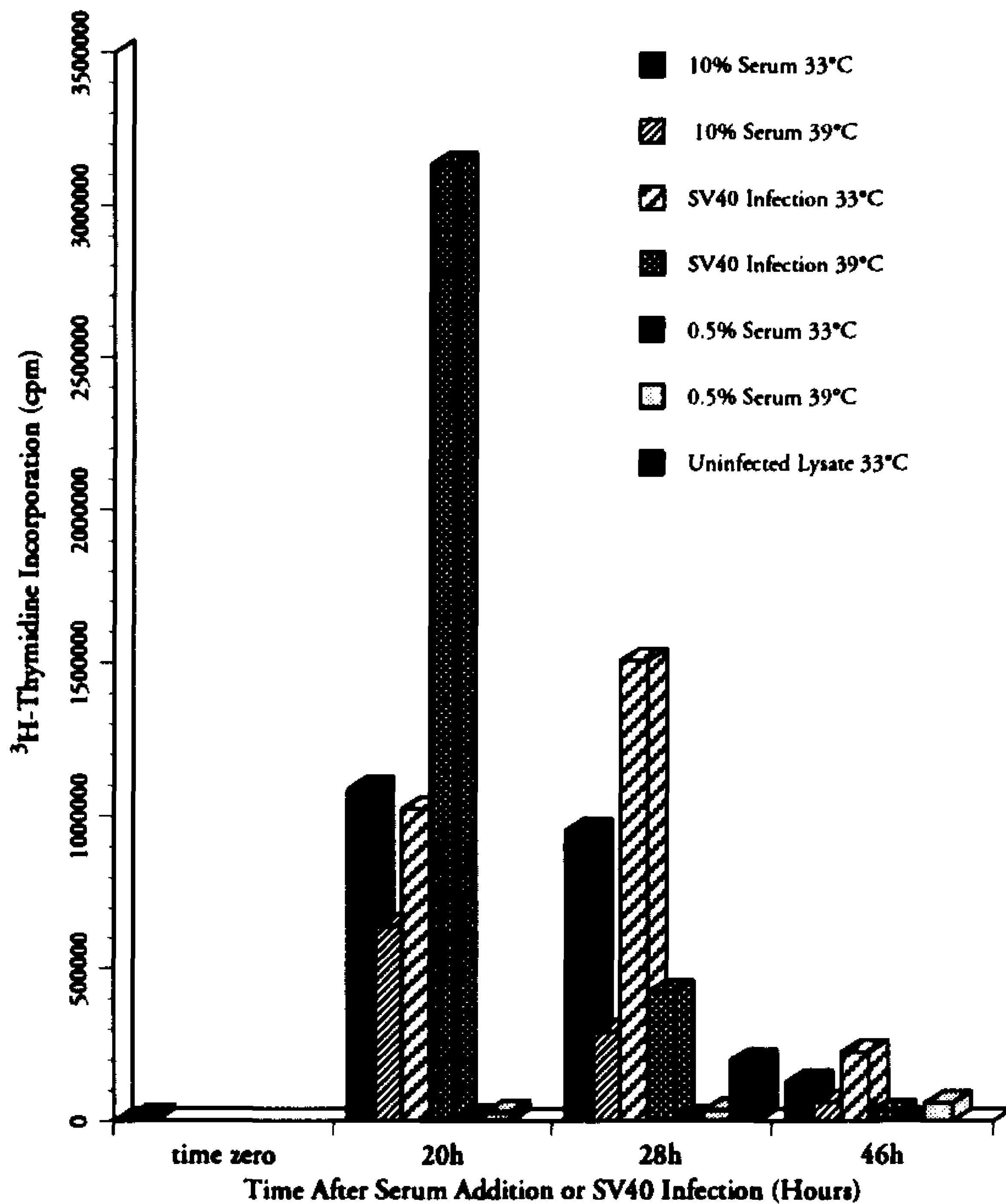

10% Serum 33°C
10% Serum 39°C
SV40 Infection 33°C
SV40 Infection 39°C
0.5% Serum 33°C
0.5% Serum 39°C
Uninfected Lysate 33°C
3H-Thymidine Incorporation (cpm)
0
500000
1000000
1500000
2000000
2500000
3000000
3500000
time zero
20h
28h
46h
Time After Serum Addition or SV40 Infection (Hours)

Table 1: Relative increase in ^{3}H-TdR incorporation following serum stimulation or acute SV40 infection.

Experimental condition	Fold increase over basal level (time zero)			
	10% NCS	SV40	0.5% NCS	Uninfected lysate
20h at 33°C	79X	77X	9.2X	nd
20h at 39°C	46X	225X	20.4X	nd
28h at 33°C	69X	109X	8.2X	14.2X
28h at 39°C	21X	31X	2.5X	nd
46h at 33°C	9.2X	16X	0.3X	nd
46h at 39°C	4.3X	22X	4.3X	nd

nd = not determined

Table 2: Autoradiography of ts2 nuclei following serum stimulation.

Experimental condition	% Positive nuclei	
	0.5% Serum	10% Serum
5 hours at 33°C	5%	5%
5 hours at 39°C	5%	5%
16 hours at 33°C	5%	15%
16 hours at 39°C	5%	10%
26 hours at 33°C	5-10%	80-90%
26 hours at 39°C	10%	30-40%

Determination of thymidine kinase (TK) mRNA levels following serum stimulation in both wild-type A31N and ts2 cells. In an effort to further characterize the *ts* defect in ts2 cells, we examined the kinetics of induction of thymidine kinase (TK) activity following stimulation of quiescent ts2 cultures. TK was chosen for these studies because although TK activity increases dramatically as cells enter S phase, the induction of TK enzyme activity been shown to be uncoupled to DNA synthesis (Johnson *et al.*, 1982, Malkas, 1985, Coppock and Pardee, 1987). Work previously reported by this laboratory showed that in addition to being defective in DNA synthesis, as determined by ^{3}H-TdR labeling studies, ts2 cells fail to show an induction of (TK) enzyme activity when incubated at the nonpermissive temperature following serum stimulation (Malkas, 1985). At the permissive temperature, however, ts2 cells showed a marked induction of TK activity that correlates with the entrance of these cells into S phase, as expected. In contrast, wild-type A31N cells not only fail to show a defect in DNA synthesis at 39°C, they also show temperature-independent induction of TK activity. In this report, we determined whether the failure to induce TK activity at the nonpermissive temperature could at least in part be accounted for by a temperature-dependent defect in the induction of TK mRNA expression in serum-stimulated cells. The kinetics of TK mRNA induction were assessed at 33°C and 39°C in both ts2 and A31N cells following serum stimulation.

Quiescent wild-type A31N and ts2 cultures were stimulated by the addition of fresh serum as described in *Materials and Methods*. Total cellular RNA was isolated from these cells at both 33°C and 39°C at various times following the addition of serum by the hot phenol method (A31N) or by the guanidinium/CsCl method (ts2) (see *Materials and Methods*). Aliquots of these RNA samples were slot blotted onto nitrocellulose membranes. Duplicate filters from each cell line were hybridized to ^{32}P-dCTP-labeled random primed DNA probes for either murine TK mRNA or He7

mRNA. Since the level of He7 has been shown to be essentially invariant throughout the cell cycle (Kao and Nevins, 1983), the levels of mRNA for this gene at each time point was used as a standard to which the level of the murine TK message could be normalized to. It should be noted that while the basal levels (at 0 hours) for these messages varied from experiment to experiment for each cell line, the overall kinetics of the response from different experiments were highly reproducible. In the case of A31N (Figure 3), the analysis of RNA samples isolated from cells both 4 and 8 hours following the addition of serum and incubated at either 33°C or 39°C shows that the level of TK mRNA is unchanged from what is observed in quiescent cells (0 h). By 16 h, the TK message level increases significantly at 39°C, while showing no increase over basal levels (0 h) at 33°C. At 26 h following serum stimulation, the level of TK message remains high at 39°C (at about the same level as 16 h, 39°C), but now there is a similar increase in the TK mRNA level in cells incubated at 33°C. In contrast, in ts2 cells incubated at either 33°C or 39°C (Figure 4), the level of TK mRNA observed 5 and 16 h after the addition of serum remains at the basal level (0 h). At 26 h, a significant increase in the level of TK mRNA is observed in cells incubated at 33°C, whereas cells incubated at 39°C fail to show any significant induction of TK mRNA expression.

The data indicate that, following the addition of fresh serum, wild-type A31N cells show a temperature-independent induction of TK mRNA expression. In contrast, the thermolabile defect affecting DNA synthesis in ts2 cells also affects the induction of TK mRNA expression in cells incubated at the nonpermissive temperature. As expected, the timing of the induction of TK mRNA expression in both A31N (at both 33°C and 39°C) and ts2 (at 33°C) correlates with the onset of DNA synthesis in the serum-stimulated cultures.

The data show that the induction of TK mRNA following serum stimulation of ts2 cells is significantly impaired at the nonpermissive temperature. The manifestations of

the temperature-sensitive defect in ts2 observed at the mRNA level for a cell cycle-regulated gene (TK) could be studied by determining the pattern of TK mRNA expression. The regulation of TK mRNA levels, therefore, served the function of a "reporter gene" in subsequent experiments. We were interested in determining whether acute infection of quiescent ts2 by SV40 virion, which overcomes the temperature-dependent block in DNA synthesis, can overcome the block in TK mRNA expression, as well. In addition, other experiments were carried out in order to ascertain whether the failure to induce TK mRNA expression in ts2 cells incubated at the nonpermissive results from a block in the transcription of the TK gene or from a defect in some posttranscriptional process.

Figure 3: Slot blot analysis of thymidine kinase (TK) and He7 mRNA levels in wild-type murine A31N cells following serum stimulation.

Samples of total RNA (2.5 µg) from A31N cells were isolated at various times following arrest and serum stimulation at both 33°C and 39°C by the hot phenol method. Samples were denatured and immobilized onto nitrocellulose filters and hybridized to either a ^{32}P-labeled murine TK (1.3 kb BamHI fragment from pMtk4) or He7 (300 bp PstI fragment from pGEMHe7) DNA probe prepared by the random primer method. Duplicate filters were used.

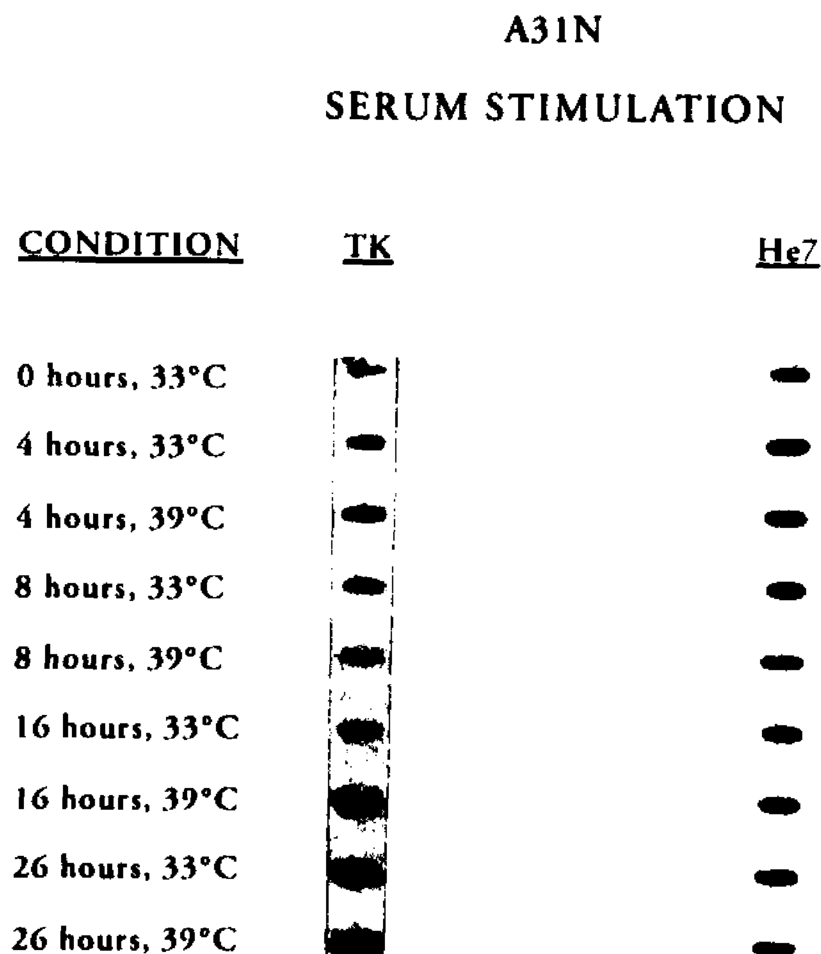
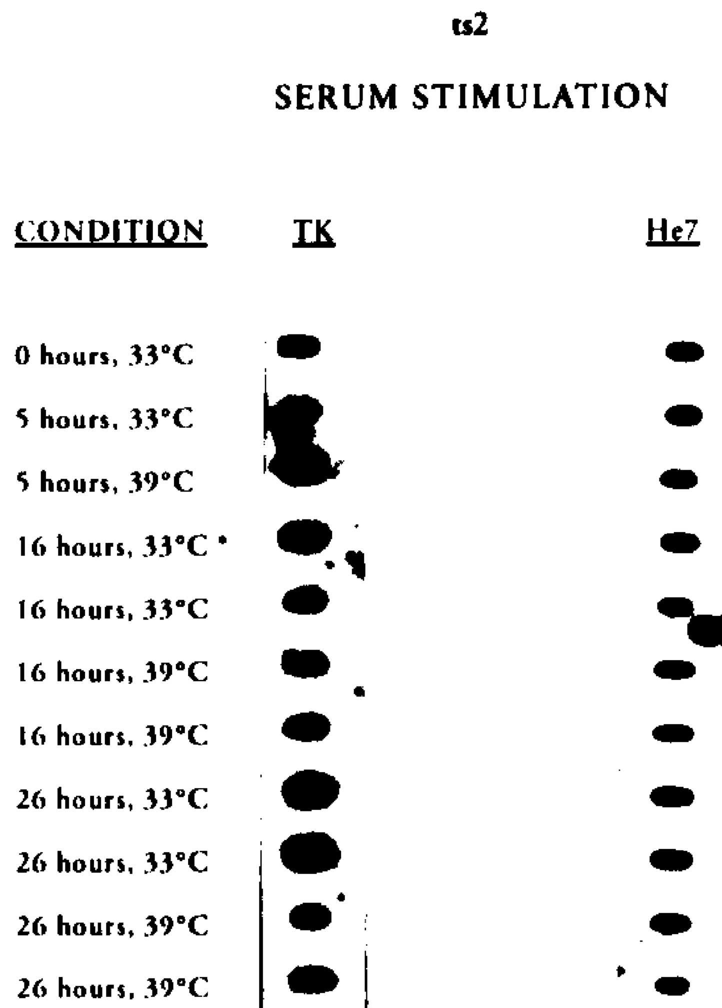


Figure 4: Slot blot analysis of thymidine kinase (TK) and He7 mRNA levels in ts2 cells following serum stimulation.

Total RNA isolated from ts2 cells at various times following arrest and serum stimulation at both 33°C and 39°C using the guanidinium/CsCl method. Samples were denatured and immobilized onto nitrocellulose filters and hybridized to either a ^{32}P-labeled murine TK (1.3 kb BamHI fragment from pMtk4) or He7 (300 bp PstI fragment from pGEMHe7) DNA probe prepared by the random primer method. All samples with the exception of the one designated by an asterisk (*) contained 2.5 µg of total RNA. The sample denoted by * contained 5 µg of RNA. Duplicate filters were used.



<u>**Analysis of TK mRNA levels in serum-stimulated and SV40-infected ts2 cultures.**</u> Ts2 cells were made quiescent as described in *Materials and Methods*. Quiescent cells were stimulated to reenter the cell cycle either by the addition of fresh serum or by a 2 h infection with SV40 virus (see *Material and Methods*). The cells were then incubated at 33°C or immediately shifted to 39°C. At various times following stimulation, several dishes from each condition were processed and total cellular RNA was isolated. Each sample represents the total RNA isolated from a single 100 mm tissue culture dish. Aliquots of each sample were electrophoresed through agarose gels, transferred to NYTRAN membranes and hybridized to either a murine TK probe or to an He7 probe (Figure 5). Figure 5a shows that by 20 h post-infection there is a significant induction of TK message at both temperatures (lanes g-h, 33°C, and lanes i-j, 39°C). These increased levels of TK mRNA persisted at 28 h at both temperatures (lanes k-m and b', 33°C, and lanes o-p, 39°C) and at 46 h at 33°C (lanes e'-f'). However, by 46 h post-infection, the TK mRNA level at 39°C fell to undetectable levels (lanes g'-h'). The precipitous decline of TK message is probably due in large part to the observation that at about 48 h post-infection at 39°C, there appears to be significant cell death (the death of these cells will be addressed later). The 0.5% serum controls show no induction of TK message at either temperature (lanes i'-p').

Figure 5b verifies the slot blot data presented in Figure 4 that show that the induction of TK mRNA expression is defective in ts2 cells incubated at 39°C following serum stimulation. Figure 5b shows that TK mRNA is undetectable in quiescent cells (time zero, lanes a-b). Twenty hours after the addition of fresh serum, there is a significant induction of TK message levels at both temperatures (lanes c-d, 33°C, and lanes e-f, 39°C). However, at 20 h, the level of TK message at 33°C is significantly more than what is observed at 39°C. The observed temperature-dependent disparity in TK mRNA accumulation is much more striking at the 28 h time point (lanes g-h, 33°C,

and lanes i-j, 39°C). While the level of TK mRNA increased significantly between 20 and 28 h following serum addition in cells incubated at 33°C (compare lanes c-d, 20 h, and lanes g-h, 28 h), the level of TK message declines in cells incubated at 39°C (compare lanes e-f, 20 h, and lanes i-j, 28 h). There is a significant decline in message levels by 43 h post-stimulation at both temperatures, as expected (lanes m-n, 33°C, and lanes o-p, 39°C). In addition, at 28 h post-stimulation the level of TK mRNA induction SV40-infected cells incubated at 33°C is significantly less than what is observed in serum-stimulated cells (compare lanes g-h, serum, and lanes k-l, SV40).

Figure 5c is a duplicate of the blot shown in Figure 5b that was probed with a He7 probe. This blot shows that that this gene is expressed at about the same level during all phases of the cell cycle and under the various experimental conditions. These data verify two things: (1) the amount of RNA loaded in each sample is relatively constant throughout the cell cycle and under the various experimental conditions used, and (2) the temperature-sensitive expression of TK message observed following serum stimulation does not represent a generalized defect in mRNA accumulation in cells incubated at the nonpermissive temperature.

When the RNA samples isolated from ts2 cells acutely infected with SV40 virus are probed with the entire SV40 genome (BRL), a heterogeneous population of RNA species is observed. Figure 6 shows that RNA samples isolated at 33°C have a significant level of SV40-specific message by 16 h post-infection (lanes c-d), this level increases at 24 h (lanes e-f) and 43 h (lanes g-h), while leveling off at 65 h (lanes i-j) post-infection. As the time course progresses, the high molecular weight species increase dramatically, while the smaller ones show a moderate decline. These data verify the fact that SV40 has successfully entered the cells and is capable of expressing its genome in ts2.

Northern blot analysis supports the slot blot data that show that following serum stimulation of quiescent ts2 cells, the induction of TK mRNA expression is impaired in

cells incubated at the nonpermissive temperature. In contrast, acute SV40 infection induces the expression of TK message in a temperature-independent manner, although it appears that the maximal level of TK mRNA that is induced by SV40 infection is significantly less than what is observed in serum-stimulated cells. These data suggest that acute SV40 infection bypasses or overrides the temperature-sensitive defects in both DNA synthesis and TK expression observed when ts2 cells are incubated at the nonpermissive temperature. These data also suggest that the temperature-dependent defect in TK enzyme activity in ts2 cells reported by Malkas (1985) could at least in part be accounted for by their failure to induce TK message when incubalted at the nonpermissive temperature. Similarly, the ability of SV40 infection to induce TK mRNA expression could also explain the SV40 large T-dependent induction of TK enzyme activity in virally infected cells (Malkas, 1985).

Figure 5: Northern blot analysis of thymidine kinase mRNA levels in ts2 cells following serum stimulation or acute SV40 infection.

Aliquots of total RNA (18 μl) isolated by the bentonite/SDS/formaldehyde method (Hatch and Bonner, 1987) from a single 100mm tissue culture dish were electrophoresed at 100 V through a 1.2% agarose gel containing 1X MOPS buffer and 0.66 M formaldehyde for 2 hours. Following visualization by ethidium bromide staining, the RNA was transferred to NYTRAN membranes and immobilized by UV-crosslinking and baking. The filters were then hybridized to a ^{32}P-labeled murine TK (647 bp RsaI fragment from pMtk4) or He7 (300 bp PstI fragment from pGEMHe7) probe prepared using the random primer method. Mobility differences result from artifact introduced by bentonite/SDS./formaldehyde method.

Figure 5a: Thymidine kinase (TK) mRNA levels in ts2 cells following acute infection with SV40 virus.

Top panel:

Lanes a-b, uninfected quiescent ts2 cells grown at 33°C (time zero).

4 hours post-SV40 infection incubated at 33°C, lanes c-d, and at 39°C, lanes e-f.

20 hours post-SV40 infection incubated at 33°C, lanes g-h, and at 39°C, lanes i-j.

28 hours post-SV40 infection incubated at 33°C, lanes k-n, and at 39°C, lanes o-p.

Bottom panel:

Lane a', uninfected quiescent ts2 cells grown at 33°C (time zero). Same as lane a on top panel.

Lane b', 28 hours post-SV40 infection incubated at 33°C. Same as lane k on top

panel.

Uninfected lysate control 28 hours after treatment incubated at 33°C, lanes c'-d'.

46 hours post-SV40 infection incubated at 33°C, lanes e'-f', and at 39°C, lanes g'-h'.

DME + 0.5% NCS control: 16 hours at 33°C, lanes i'-j', and at 39°C, lanes k'-l'.

24 hours at 33°C, lanes m'-n', and at 39°C, lanes o'-p'.

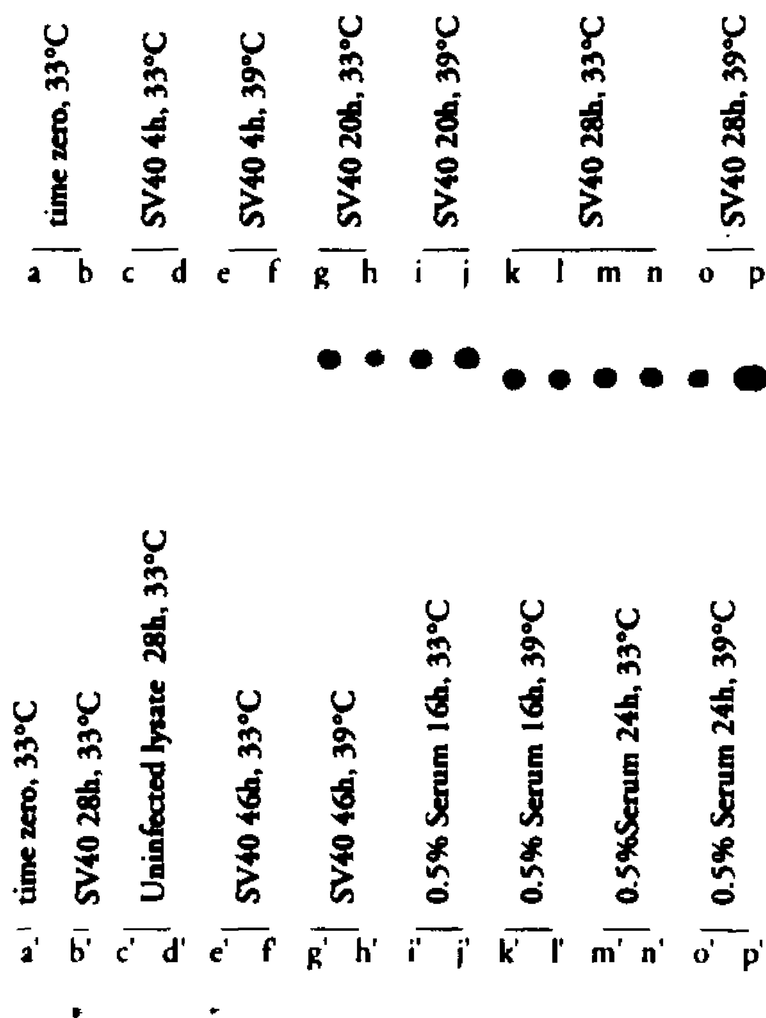


Figure 5b: Thymidine kinase (TK) mRNA levels in ts2 cells following serum stimulation.

Samples were treated as in Figure 5a and the filter was hybridized to the same murine TK probe under the same experimental conditions.

Lanes a-b, uninfected quiescent ts2 cells grown at 33°C. Same samples as lanes a-b in Figure 4a.

20 hours after serum addition incubated at 33°C, lanes c-d, and at 39°C, lanes e-f.

28 hours after serum addition incubated at 33°C, lanes g-h, and at 39°C, lanes i-j.

28 hours post-SV40 infection incubated at 33°C, lanes k-l. Same samples as lanes m-n in Figure 5a.

46 hours after serum addition incubated at 33°C, lanes m-n, and at 39°C, lanes o-p.

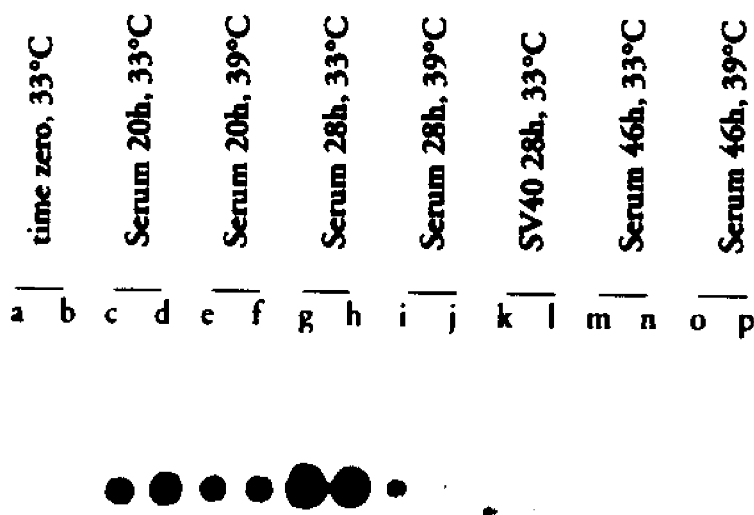


Figure 5c: He7 mRNA levels in ts2 following rundown and serum stimulation.

Duplicate filter of one shown in Figure 5b. Filter was hybridized to a ^{32}P-labeled He7 probe prepared by the random primer method.

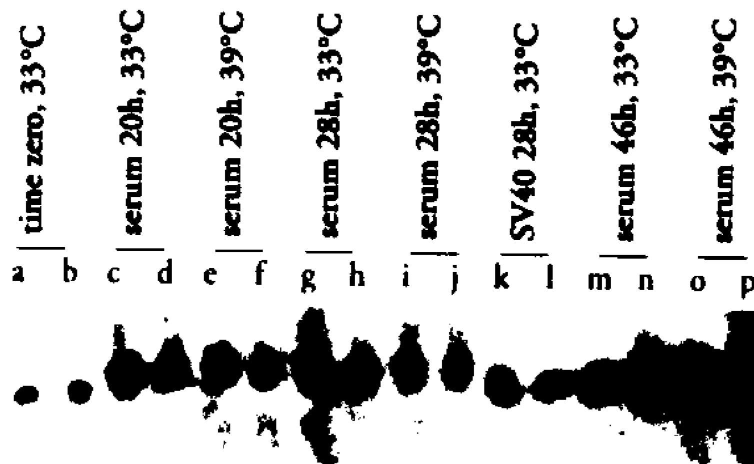


Figure 6: SV40 mRNA expression in ts2 cells acutely infected by SV40 and incubated at the permissive temperature.

Total RNA (18 μl aliquouts) isolated by the bentonite/SDS/formaldehyde method from a single 100 mm tissue culture dish at various times following SV40 infection and incubation at 33°C were electrophoresed at 100 V through a 1.2% agarose gel containing 1X MOPS buffer and 0.66 M formaldehyde for 2 hours. Following visualization by ethidium bromide staining, the RNAs were transferred to a NYTRAN membrane and immobilized by UV-crosslinking and baking. The filter was then hybridized to a ^{32}P-labeled probe made from pure SV40 virion DNA by the random primer method.

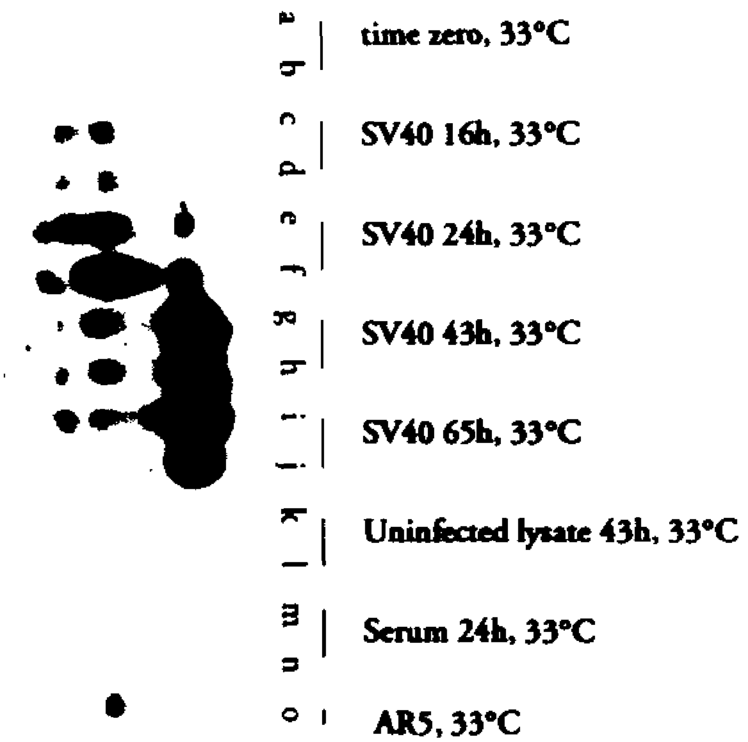
Lanes a-b, uninfected quiescent ts2 cells.

16, 24, 43, and 65 hours post-SV40 infection, lanes c-d, e-f, g-h, and i-j, respectivly. The following controls were included in this experiment:

43 hours after treatment with uninfected lysate, lanes k-l.

24 hours following serum addition incubated at 33°C, lanes m-n.

AR5, SV40-transformed human fibroblast cell line, logarithmically growing at 33°C, lane o.


a b c d e f g h i j k l m n o
time zero, 33°C
SV40 16h, 33°C
SV40 24h, 33°C
SV40 43h, 33°C
SV40 65h, 33°C
Uninfected lysate 43h, 33°C
Serum 24h, 33°C
AR5, 33°C

<u>Analysis of quiescent ts2 cells following serum addition or acute SV40 viral infection by flow cytometry.</u> The ^{3}H-TdR pulse-labeling and Northern blot data show that when fresh serum is added to quiescent ts2 cells and these cultures are incubated at the nonpermissive temperature (39°C), temperature-dependent defects in both DNA synthesis and TK mRNA expression are observed. In contrast, neither defect is manifested when quiescent ts2 cells are incubated at 39°C following acute infection by SV40 virions, suggesting that SV40 infection plays a role in overriding both defects. However, as noted earlier, it is consistently observed that by 48 h post-infection these cultures show massive cell death, with >50% of cells floating in the culture medium (data not shown). Therefore, taken together, these data suggest that although these virally infected cells can reenter the cell cycle and undergo substantial DNA synthesis at the nonpermissive temperature, they cannot successfully traverse the cell cycle. In order to validate this hypothesis and further chraracterize these cells, flow cytometric analysis of quiescent ts2 cells at various times following serum addition or acute SV40 infection at both 33°C and 39°C was undertaken.

Histograms of ts2 cells following serum stimulation at the permissive temperature (Figure 7a, panel B) show a significantly different pattern of cell cycle kinetics than what is observed at the nonpermissive temperature (Figure 7a, panel C). At 33°C, ts2 cells start to move out of G1 and into S phase by 24 h (Figure 7a, panel B). The percentage of cells in S phase reaches 24% at 30 h (Figure 7a, panel B). In contrast, at 39°C, about 10% of the cells enter S phase by 16 h after serum addition (Figure 7a, panel C). By 24 h, these cells have moved from S into G2+M, where they remain at 30 h (Figure 7a, panel C). At 39°C, it appears that only a small percentage of cells are capable of reentering the cell cycle. These cells, however, cannot successfully traverse the cell cycle and are blocked in G2+M (Figure 7a, panel C).

The histograms presented in Figure 7b (panel C) show that by 24 h post-infection

by SV40 virus, cultures incubated at 39°C show a significant accumulation (12%, up from 3% at 16 h) of cells in the G2+M portion of the cell cycle. This accumulation increases substantially (to 25%) at 30 h post-infection (Figure 7b, panel C). In contrast, the histograms of cultures incubated at 33°C following SV40 infection show no significant changes throughout the time course of this experiment (Figure 7b, panel B). It is conceivable that synchronous populations of cells, with cell cycle kinetics like ts2, could complete DNA replication and mitosis between time points, thereby generating histograms that shows no apparent change over the course of the experiment.

Earlier flow cytometric studies conducted in our laboratory (Malkas, 1985) show that when ts2 cells logarithmically growing at 33°C are shifted to 39°C, there is a progressive accumulation of cells in G2+M. These data indicate that in growing populations of cells, the temperature-sensitive defect observed in ts2 cells arrests these cells in late S phase or in G2+M following shift-up to the nonpermissive temperature. These data appear to contradict both the ^{3}H-TdR labeling data and TK mRNA data obtained when quiescent cells are stimulated to reenter the cell cycle following the addition of fresh serum at the nonpermissive temperature (39°C), which indicate that the temperature-sensitive defect in ts2 is manifested in late G1 or early S phase.

The finding that approximately 10% of cells in serum-stimulated ts2 cultures incubated at the nonpermissive temperature (39°C) (Figure 7a, panel C) are capable of reentering the cell cycle only to become blocked in G2+M could be accounted for if at the outset of these experiments (at time zero), not all the cells are truly quiescent (Figure 7a, panel A). These "cycling cells" could undergo DNA synthesis but then become blocked in G2+M. This is similar to what is observed in exponentially growing cultures of ts2 following shift-up to 39°C. On the basis of the data, it appears that the temperature-sensitive defect in ts2 cells is manifested at two points in the cell cycle: at late G1/S and at late G1/G2+M. Cells capable of undergoing DNA synthesis follow-

ing shift-up to 39°C become blocked again later in the cell cycle.

The accumulation of a significant percentage of ts2 cells in the G2+M phase of the cell cycle between 16 h and 30 h after SV40 infection at 39°C (Figure 7b, panel C) further supports the hypothesis that there are two arrest points for these cells. Although acute SV40 infection of ts2 cells overcomes the temperature-dependent block affecting their ability to enter S phase, these cells become blocked again at some later stage in the cell cycle (in late S, G2 or M). While these data do not pinpoint where this second block occurs, the resulting unbalanced growth state of these cells due to their inability to successfully complete DNA replication and/or cell division provides a possible explanation for the observation that these cells undergo massive cell death by 48 h post-infection at 39°C.

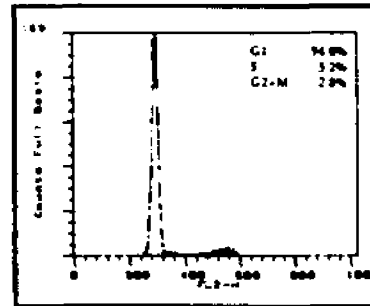
Figure 7: Flow cytometric analysis of ts2 following rundown and stimulation by either serum addition or acute SV40 virus infection.

Ts2 cells were prepared for flow cytometric analysis as described in *Materials and Methods* following rundown and at 16, 24, or 30 hours after the addition of fresh serum or acute infection by SV40 virus and incubation at both 33°C and 39°C. Cells were analyzed by the UMDNJ Cellular Immunology Laboratory using a Becton Dickinson cell sorter equipped with CellFIT Cell-Cycle Analysis Version 1.2 software.

Figure 7a: Flow cytometric analysis of ts2 cells following serum stimulation.

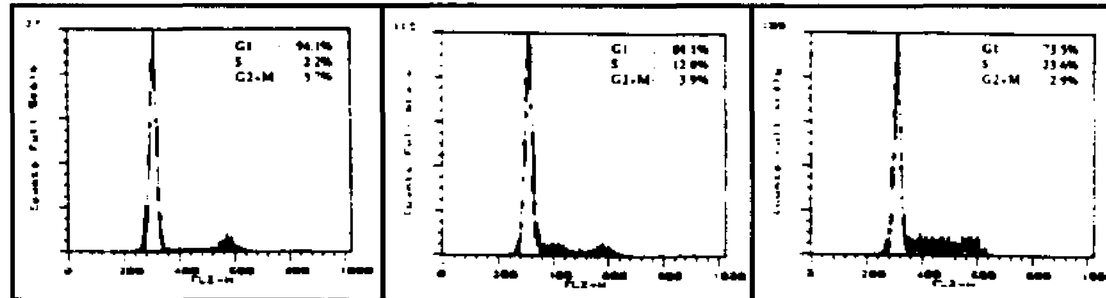
Panel A, histogram of quiescent ts2 cells (time zero).
Histograms of ts2 cells 16, 24, and 30 hours after the addition of fresh serum and incubated at 33°C, panel B, and at 39°C, panel C.

Panel A:
time zero, 33°C



Time After Serum Addition → **16 hours** **24 hours** **30 hours**

Panel B:
10% Newborn Calf Serum, 33°C



Panel C:
10% Newborn Calf Serum, 39°C

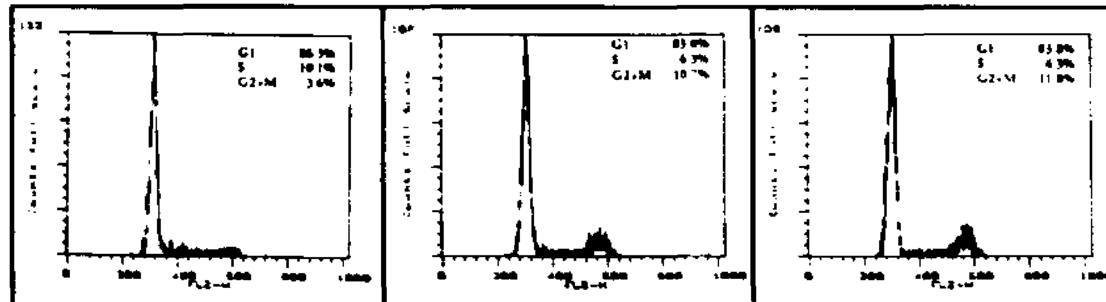
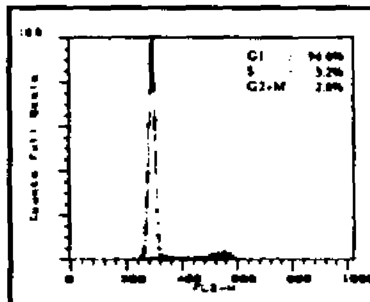


Figure 7b: Flow cytometric analysis of ts2 cells following SV40 infection.

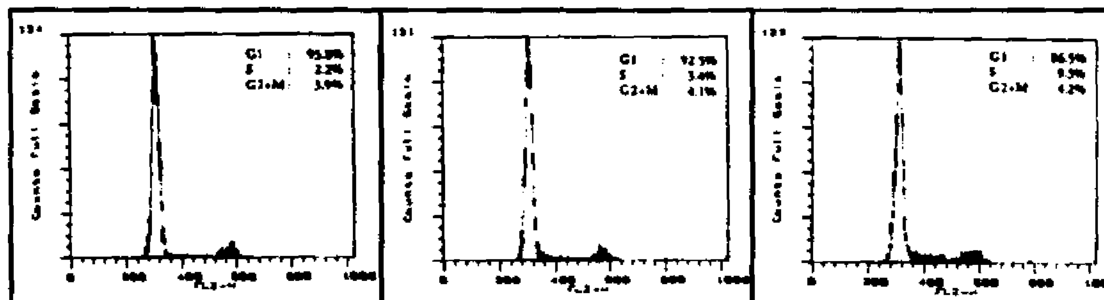
Panel A, histogram of quiescent ts2 cells prior to SV40 infection (time zero). Histograms of ts2 cells 16, 24, and 30 hours after SV40 infection and incubated at 33°C, panel B, and at 39°C, panel C.

Panel A:
time zero, 33°C

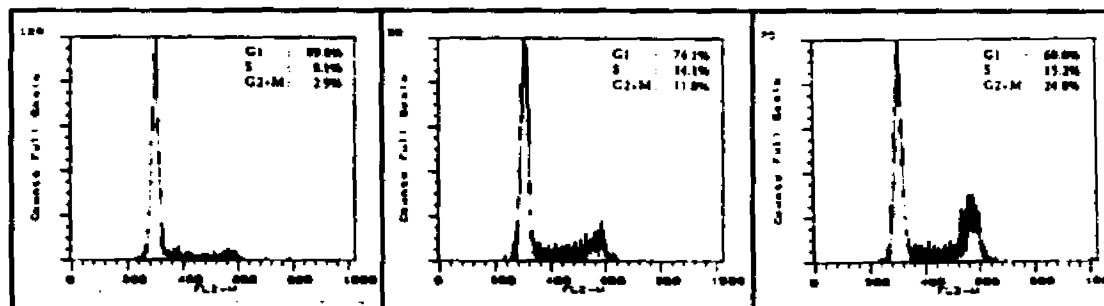


Time After Infection → **16 hours** **24 hours** **30 hours**

Panel B:
SV40 Infection at 33°C



Panel C:
SV40 Infection at 39°C



<u>Characterization of ts2-derived cell lines</u>

The slot blot andNorthern blot data presented thus far show that TKmRNA expression is defective in serum-stimulated ts2 cells incubated at the nonpermissive temperature. However, studies measuring the steady state levels of TK message do not provide information about the biochemical basis of this defect. In order to determine whether the reduced levels of TK mRNA accumulation results from a defect in the transcription of the TK gene or from a defect in its posttranscriptional regulation, we studied the patterns of mRNA expression of several human TK gene constructs introduced into ts2 cells by stable transfection (see *Material and Methods*).

<u>Establishment of ts2-derived cell lines.</u> Logarithmically growing cultures of early passage ts2 cells were transfected with several human TK constructs on plasmids obtained from M. Kauffman and T. Kelly (The Johns Hopkins University School of Medicine) along with the plasmid pWL*neo* which carries our selectable marker, the G418-resistance gene, as described in *Materials and Methods*. In order to control for differences in gene expression that can result from the integration of the transfected plasmids into specific sites in the mouse genomic DNA, cells from several hundred G418-resistant colonies derived from the same transfection were trypsinized and pooled. By using cell lines derived from pools of transformants, we are in effect normalizing for both integration sites (if, in fact, integration is a relatively random process) and copy number of the inserted gene. These pools of cells were then expanded into mass culture and were designated P1-1, P2-1, P3-1, and P4-2. P1-1, cotransfected with the plasmid pTK8, contains the human TK promoter linked to the human TK genomic coding sequence, with the deletion of 200 bp from the 3' untranslated region and lacking a poly(A)-addition site. P4-2, cotransfected with the plasmid pMGK85,

contains the same human coding sequence as does P1-1, but is linked to an HSV-TK promoter. P2-1, cotransfected with the plasmid pTK21, contains the same promoter as does P1-1, but is linked to the human TK cDNA which includes a poly(A)-addition site. P3-1, cotransfected with the plasmid pMGK86, contains the human TK promoter linked to the Herpes Simplex Virus (HSV) TK coding sequence.

We confirmed the presence of both *neo*^{r} gene and human TK gene sequences in these cells before analyzing these transfected cell lines for their ability to express RNA from the transfected genes. High molecular weight DNA was prepared from each of these cell lines. To confirm the presence of the *neo*^{r} gene, samples of each DNA along with the plasmid pWL*neo* were sequentially digested with HindIII followed by XhoI. Following electrophoresis and transfer to a NYTRAN membrane, the filter was hybridized to a radiolabeled probe for the *neo*^{r} gene. Figure 8a shows that all transfected cell lines (lanes b-e) as well as pWL*neo* (lane h) have the expected 1.1 kb internal HindIII-XhoI fragment, confirming the presence of the G418-resistance gene in these cell lines.

The presence of human TK sequences in these cell lines was confirmed by digesting high molecular weight DNA prepared from these transfected cell lines along with cellular DNA from two human cell lines, HS74 and HAL/Py*neo*, as well as pTK8 and pTK21 plasmid DNA with HindIII followed by XhoI. Following electrophoresis and transfer, the filter was hybidized to a human TK probe. Figure 8b shows that both the P1-1 (lane b) and P4-2 (lane d) cell lines probed with a human TK probe contain the two diagnostic HindIII-XhoI bands of 6.5 and 4.4 kb seen in human TK genomic sequences (lanes f, g, and i). P2-1 (lane c) shows the same prominant 1.6 kb band as does pTK21(lane h), its original input plasmid, when hybridized to a human TK probe. These data confirm that the cell lines under study do, in fact, contain the appropriate human TK sequences. In addition, on the basis of signal intensity, it appears that the

signals seen in the transfected cell lines is a result of the integration of multiple copies of the human TK sequences (compare HS74 (lane f), a normal diploid human fibroblast cell line, with any of the transfected cell lines (lanes b-e)). This is not unexpected since the ratio of the human TK plasmid DNA to pWL*neo* DNA used in all transfections was approximately 15:1(w/w).

Figure 8: Southern blot analysis of ts2-derived cell lines.

Figure 8a: Verification of the presence of the G418-resistance gene in the ts2-derived cell lines.

High molecular weight DNA from ts2 cells and from cell lines derived from the transfection of ts2 that were selected on the basis of their resistance to 800 μg/ml G418. Each DNA sample (5 μg) was sequentially digested by HindIII followed by XhoI. Following electrophoresis through a 1.2% agarose gel at 100 V for 4 hours, the DNAs were immobilized by baking on a NYTRAN filter. The filter was hybridized to a ^{32}P-labeled riboprobe prepared from the plasmid pMO*neo* linearized by digestion with HindIII. The presence of a 1.1 kb HindIII-XhoI fragment indicates the presence of the G418-resistance gene from the plasmid pWL*neo*.

ts2, untransfected murine control, lane a.

P1-1, ts2 transfected with pTK8 and pWL*neo*, lane b.

P2-1, ts2 transfected with pTK21 and pWL*neo*, lane c.

P3-1, ts2 transfected with pMGK86 and pWL*neo*, lane d.

P4-2, ts2 transfected with pMGK85 and pWL*neo*, lane e.

HS74, human diploid fibroblast negative control, lane f.

HAL/Py*neo*, immortalized human fibroblast containing G418-resistance gene, lane g.

Lane h, 20 pg HindIII and XhoI digested pWL*neo* DNA.

a tu2
b P1-1
c P2-1
d P3-1
e P4-2
f HS74
g HAL/Py*neo*
h pWL*neo*, 20pg

Figure 8b: Verification of the presence of human thymidine kinase (TK) sequences in the ts2-derived cell lines.

High molecular DNA was prepared as in Figure 8a. Each DNA sample (15 μg) was digested with HindIII and electrophoresed and transferred to NYTRAN as in Figure 8a. The filter was hybridized to a ^{32}P-labeled riboprobe prepared from the plasmid pMGK23 linearized by digestion with SmaI. The presence of a 1.6 kb HindIII fragment is diagnostic for the presence of human TK cDNA sequences, whereas the presence of 6.5 and 4.0 kb HindIII fragments indicate the presence of human genomic sequences. All transfected cell lines were cotransfected with pWL*neo*. See *Materials and Methods* for descriptions of the cell lines and plasmids.

ts2, untransfected murine control, lane a.

P1-1, ts2 transfected with pTK8, lane b.

P2-1, ts2 transfected with pTK21, lane c.

P4-2, ts2 transfected with pMGK85, lane d.

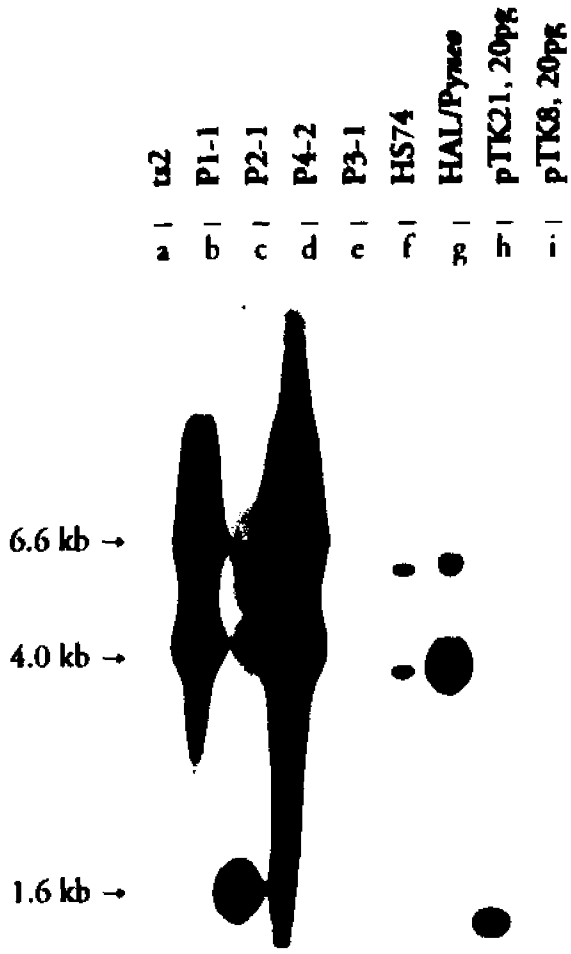
P3-1, ts2 transfected with pMGK86, lane e.

HS74, human diploid fibroblasts negative control, lane f.

HAL/Py*neo*, immortalized human fibroblasts containing G418-resistance gene, lane g.

Lane h, pTK21, 20 pg digested with HindIII. Control for human TK cDNA sequences.

Lane i, pTK8, 20 pg digested with HindIII. Control for human genomic sequences.

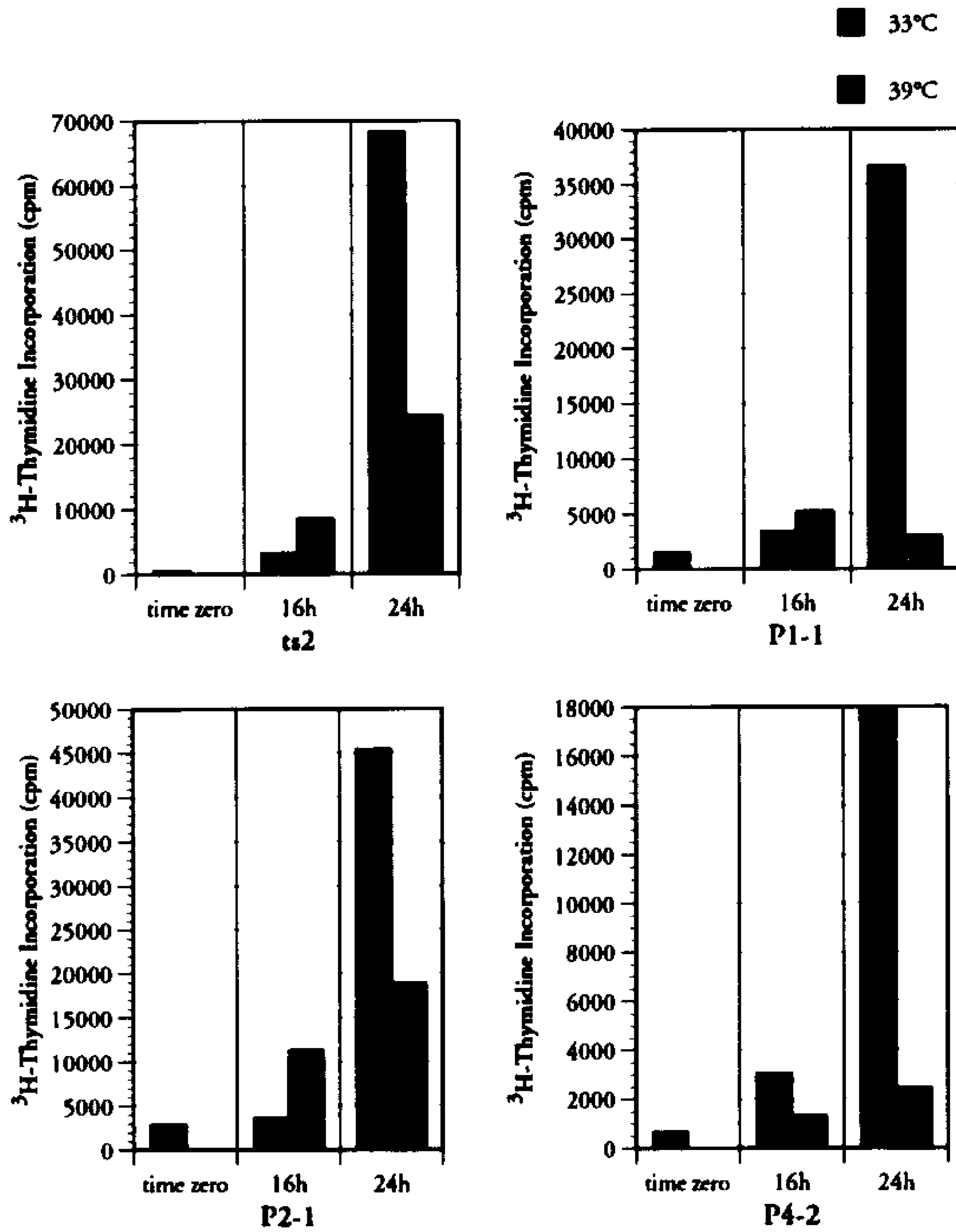


a b c d e f g h i
ts2
P1-1
P2-1
P4-2
P3-1
HS74
HAL/Pyneo
pTK21, 20pg
pTK8, 20pg
6.6 kb →
4.0 kb →
1.6 kb →

Kinetics of DNA synthesis in ts2-derived cell lines following serum addition. The cell lines P1-1, P2-1, and P4-2 were chosen for further study by Northern blot analysis. Before this analysis was undertaken, it was necessary to establish that these cell lines reach quiescence and then become restimulated following the addition of fresh serum in a temperature-sensitive manner like the ts2 parental cell line. Quiescent cultures incubated at 33°C as well as serum-stimulated cultures incubated at both 33°C and 39°C were pulse-labeled with ^{3}H-TdR for 60 minutes at 16 and 24 hours following the addition serum as described in *Materials and Methods*. Figure 9 shows that all three cell lines, P1-1, P2-1, and P4-2, reach quiescence 96 hours after plating, as expected. At twenty-four hours following serum addition, all three cell lines show a reduced incorporation of the radiolabel at the nonpermissive temperature (Figure 9). Therefore, these pulse-labeling data indicate that these cell lines all show serum arrest/stimulation kinetics similar to parental ts2 cells.

Figure 9: ^{3}H-TdR pulse-labeling of ts2-derived cell lines following serum stimulation.

Following serum arrest of ts2 and ts2-derived cell lines, P1-1, P2-1, and P4-2, cells grown at 33°C, 100 mm cell culture dishes were either harvested (time zero) or washed 2X with DME/F10 and refed with DME/F10 + 10% NCS. Following incubation at either 33°C or 39°C for 16 or 24 hours, duplicate dishes from each experimental condition were pulse-labeled for 60 minutes and processed as described in *Materials and Methods*. ^{3}H-TdR incorporation was measured by determining the number of cpm in the TCA precipitatable material from a single dish using a Beckman scintillation counter counting on an open channel.


33°C
39°C
^{3}H-Thymidine Incorporation (cpm)
70000
60000
50000
40000
30000
20000
10000
0
time zero
16h
24h
ts2
40000
35000
30000
25000
20000
15000
10000
5000
0
time zero
16h
24h
P1-1
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
time zero
16h
24h
P2-1
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
time zero
16h
24h
P4-2

Determination of murine and human TK mRNA levels in ts2-derived cell lines. Southern blot analysis verified the presence of human TK gene sequences in the ts2-derived cell lines (Figure 8b). In addition, ^{3}H-TdR labeling experiments show that the cell lines P1-1, P2-1, and P4-2 have similar arrest and serum stimulation kinetics as does the parental ts2 cell line (Figure 9). Since the cell lines P1-1, P2-1, and P4-2 are defective in their ability to undergo DNA synthesis at the nonpermissive temperature (Figure 9), we measured the levels of human TK message by Northern blot analysis in these three cell lines following serum stimulation in order to determine whether any of these transfected human TK genes show temperature-dependent and/or cell cycle-regulated patterns of expression.

Preliminary experiments verified the expression of the human TK genes in logarithmically growing cultures. Figure 10 shows that in the cell lines containing the human genomic TK coding region and no poly(A)-addition site, P1-1(lanes d), P1-2 (lanes e-f), and P4-2 (lanes m-n), there are two prominent bands when hybridized to the human TK probe, suggesting the presence of some intermediate form of incompletely processed RNA. On the other hand, P2-1, the cell line containing the human TK cDNA coding sequence along with a poly(A) addition site shows a single discrete band when hybridized to the same probe. The cell line, P3-2, which contained the HSV-TK coding region showed no evidence of hybridization to the human-specific TK probe, as expected (lanes i-j). In all cases, however, when these RNA samples were hybridized to a murine-specific TK probe, a single band was always seen (data not shown).

Further experimentation showed that in order to get suitable signals from RNA isolated from cell lines transfected with the human TK genomic constructs (P1-1 and P4-2) during the course of a serum arrest/stimulation experiment, it was necessary to load 25-30 μg samples on the gels. The GIT/Sarkosyl method(Chomczyski and Sacchi, 1987) was used to further analyze these cell lines because the large volumes generated

by the bentonite/SDS/formaldehyde method (Hatch and Bonner, 1987) could not be loaded onto gels(see *Materials and Methods*). When this method was used, each RNA sample was made by pooling the cells from 4 100 mm tissue culture dishes. It was shown experimentally that the bentonite/SDS/formaldehyde method was suitable for studying the cell cycle regulation of the human TK message transcribed from the TK cDNA in P2-1. The amount of RNA loaded for each sample using the bentonite/ SDS/formaldehyde was determined by ethidium bromide staining to be approximately 5 μg (data not shown).

Figure 11a shows that when 30 μg samples of RNA were hybridized to a human TK specific probe, there is significant heterogeneity in message size. This is not unexpected because this message is transcribed from a genomic sequence and various processing intermediates are likely to be formed. The smears emanating from the two prominent RNA bands are probably due to some message instability because these RNAs lack poly(A) tails. Figure 11a shows that the levels of these human TK transcripts are in fact regulated in a cell cycle dependent manner. However, this regulation is not nearly as stringent as what is observed when these samples are probed with a murine TK specific probe (Figure 11b). Figure 11a shows that there is a significant diminution in signal level at time zero (lanes e-f) when compared to what is observed in RNA isolated one day after seeding (lanes c-d), presumably representing a logarithmically growing culture. By 16 h after serum addition, there appears to be an increase in the level of message at both 33°C (lanes g-h) and 39°C (lanes i-j). At this time point, however, the results of several independent experiments show that any temperature-dependent effect is tenuous at best. By 24 h after the addition of serum, the level of induction at 33°C (lanes k-l) is is clearly several-fold greater than what is observed at 39°C (lanes m-n).

In contrast to these data, when the levels of the endogenous murine TK message in 10 μg samples were analyzed, there is much tighter cell cycle regulation (Figure 11b).

Basal levels (lanes e-f) are almost undetectable and there is significant temperature-dependent induction by 16 h post-serum addition (lanes g-h, 33°C, and lanes i-j, 39°C). This level of induction increases significantly by the 24 h point at 33°C(lanes k-l), while decreasing at 39°C (lanes m-n).

Two separate controls were used to verify these results. Samples of these RNAs (10 μg) were hybridized to a He7 probe. The level of the He7 message stays approximately the same throughout the course of this experiment (Figure 11c). Figure 11d shows that when 20 μg of these RNA samples are hybridized to a probe for the cotransfected *neo*^{r} gene, the level of this message throughout the entire course is also invariant. The constitutive expression of the *neo*^{r} gene is expected because it is driven by the HSV-TK promoter which shows cell cycle-independent regulation. However, the pattern of expression of the *neo*^{r} gene confirms the fact that both the cell cycle regulation and temperature-sensitive effect observed in the analysis of the expression of exogenous human TK gene is not a generalized phenomenon observed for all expression vectors transfected into ts2.

Analysis of RNA samples prepared from P2-1 and hybridized to a human TK probe (Figure 12a) show that the transcription of the exogenous gene containing the human TK cDNA coding region as well as a poly(A)-addition site exhibits a much tighter cell cycle and temperature-sensitive regulation than does P1-1, even though both coding regions are under the control of the same human TK promoter. In addition, Figure 12a shows that in P2-1, since no splicing occurs, only a single species of RNA corresponding to the human TK gene is observed. The level of its expression is much higher than the RNA levels detected in P1-1 because it readily detected in 5 μg samples. Figure 12b shows that when these samples are hybridized to a murine TK probe, the kinetics of the temperature-sensitive induction of the endogenous TK gene mirrors

that observed for the exogenous gene. This is especially evident at the 24 h time point where there is a high level of induction of both genes at 33°C (Figures 12a and 12b, lanes k-l) with minimal induction at 39°C (Figures 12a and 12b, lanes m-n). The He7 blot of P2-1 RNA samples (Figure 12c) shows that the level of expression of this gene is relatively invariant throughout the cell cycle, thus serving as a valid control for the TK RNA blots.

The analysis of the cell line P4-1, transfected with the human TK genomic coding region, the same coding region as in P1-1, linked to the HSV-TK promoter. Samples of RNA (25 μg) isolated from these cell lines at both 33°C and 39°C at various times following serum induction of quiescent cells showed an no evidence of either cell cycle or temperature-sensitive regulation of the exogenous human TK gene when hybridized to a human TK probe. The overall expression of this gene remained low throughout. In addition, the heterogeneity of the messages detected in these samples (Figure 13a) is what was observed when P1-1 (Figure 11a) was hybridized to the same probe. Figure 13b shows unequivocally that the RNA levels for the endogenous murine TK gene is regulated in both a cell cycle and temperature-sensitive manner, as expected.

These results show that despite some very striking structural differences between the murine and human TK promoters (Seiser *et al.*, 1989 and Weichselbaum *et al.*, 1990), both the exogenous human (linked to the human TK promoter) and endogenous murine TK genes are regulated in a cell cycle dependent manner in ts2, with their expression being induced at the permissive temperature. At the nonpermissive temperature, however, both genes manifest the *ts* defect similarly. On the other hand, the human TK gene under the control of the HSV-TK promoter shows temperature-independent constitutive expression. In both cases (P1-1 and P4-2), when the genomic sequence lacking the polyadenylation signal is used, Northern blot analysis shows the presence of a heterogeneously sized population of human TK RNA species, whereas

only a single band was observed for the cell line containing the human TK cDNA with its polyadenylation signal(compare Figures 11a (P1-1) and 13a (P4-2) with Figure 12a (2-1)). In addition, when under the control of the human TK promoter, the cell line containing the cDNA sequence had a much higher level of TK expression and showed much tighter cell cycle regulation than the cell line containing the same promoter linked to the genomic coding sequence. It also appears that neither the polyadenylation signal nor the 3' untranslated region is required for the cell cycle regulation of this gene. However, it is possible that the lack of these sequences accounts at least in part for the relatively low levels of expression of these genes as well as for the heterogeneity in message size observed in these cell lines.

These data strongly suggest that both the cell cycle regulation and the *ts* defect observed in TK expression at the nonpermissive temperature occur at the level of the promoter. Therefore, the defect in TK mRNA expression at 39°C is likely to involve a transcriptional block. Since it is likely that at least a subset of the genes whose transcription are induced at the G1/S boundary (like TK) are linked to the onset of DNA synthesis in ts2 cells, these genes can serve as markers for the *ts* phenotype, whose prominent characteristic is the inability of these cells to replicate their DNA at the nonpermissive temperature.

Figure 10: Confirmation of the expression of the transfected human thymidine kinase (TK) genes in the ts2-derived cell lines by Northern blot analysis.

Total RNA (25µl aliquots) isolated by the bentonite/SDS/formaldehyde method (Hatch and Bonner, 1987) from a single 100 mm tissue culture dish of logarithmically growing cells were electrophoresed at 100V through a 1.2% agarose gel containing 1X MOPS buffer and 0.66 M formaldehyde for 5 hours. Following visualization by ethidium bromide staining, the RNA was transferred to a NYTRAN filter and immobilized by UV-crosslinking and baking. The filter was then hybridized to a ^{32}P-labeled probe prepared by random priming a 350 bp PCR synthesized human TK cDNA fragment.

ts2, untransfected control, lane a.

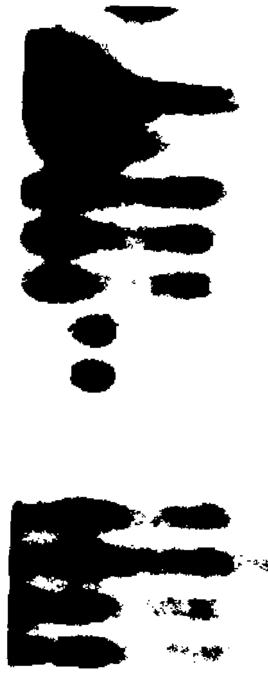
AR5, immortalized human fibroblasts, and HS74, diploid human fibroblasts, lanes b and c, respectively. Human positive controls.

P1-1 and P1-2, transfected with pTK8, human TK promoter linked to human TK genomic sequence, lanes d and e-f, respectively.

P2-1, transfected with pTK21, human TK cDNA linked to human TK promoter, lanes g-h.

P3-2, transfected with pMGK86, HSV-TK coding sequence linked to the human TK promoter, lanes i-j.

P4-1 and P4-2, transfected with pMGK85, HSV-TK promoter linked to the human TK genomic sequence, lanes k-l and m-n, respectively.



a ts2
b AR5
c HS74
d P1-1
e f P1-2
g h P2-1
i j P3-2
k l P4-1
m n P4-2

Figure 11: Analysis of cell line P1-1 by Northern blot analysis.

The P1-1 cell line was established by pooling several hundred G418-resistant colonies following transfection of ts2 cells by both pWL*neo* and pTK8. The plasmid pTK8 contains the human TK promoter and complete genomic coding region with the exception of 200 bp at the 3' untranslated region.

Figure 11a: The expression of human TK RNA in the cell line P1-1 following serum stimulation.

Total RNA (30 μg) isolated by the GIT/Sarkosyl (Chomczyski and Sacchi, 1987) method at various times following plating were electrophoresed through a 1.2% agarose gel containing 1X MOPS running buffer and 0.66 M formaldehyde at 100 V for 3 hours. Following visualization by ethidium bromide staining, the RNA was transferred to a NYTRAN membrane and immobilized by UV-crosslinking and baking. The filter was then hybridized to a ^{32}P-labeled probe prepared by random priming a 350 bp PCR synthesized human TK cDNA fragment.

HAL/Py*neo*, human control, 18 μl RNA sample prepared by the bentonite/SDS/ formaldehyde method from logarithmically growing cells, lanes a.

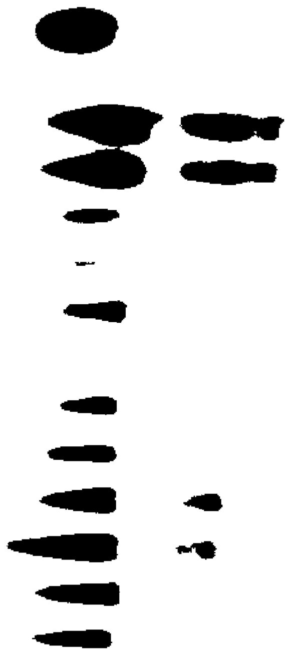
untransfected logarithmically growing ts2 cells, murine control, 30 μg, lane b.

Lanes c-d, P1-1 logarithmically growing cells at 33°C.

Lanes e-f, P1-1 quiescent cells (time zero) grown at 33°C.

P1-1 cells 16 hours after serum addition incubated at 33°C, lanes g-h, and at 39°C, lanes i-j.

P1-1 cells 24 hours after serum addition incubated at 33°C, lanes k-l, and at 39°C, lanes m-n.



a | HAL /Py *neo*
b | ts2, log, 33°C
c, d | log, 33°C
e, f | time zero, 33°C
g, h | serum 16h, 33°C
i, j | serum 16h, 39°C
k, l | serum 24h, 33°C
m, n | serum 24h, 39°C

P1-1 Samples

Figure 11b: The expression of murine thymidine kinase mRNA in the cell line P1-1 following serum stimulation.

Samples (10 μg) of total RNA were isolated by the GIT/Sarkosyl method at various times following plating were electrophoresed and immobilized onto a NYTRAN membrane as described in Figure 11a. The filters were hybridized to a ^{32}P-labeled probe prepared from the 647 bp RsaI fragment of murine TK cDNA by the random primer method.

Lanes a-m, as described in Figure 11a. All samples contained 10 μg of total cellular RNA with the exception of lane a which contained 18 μl of RNA isolated by the bentonite/SDS/formaldehyde method.

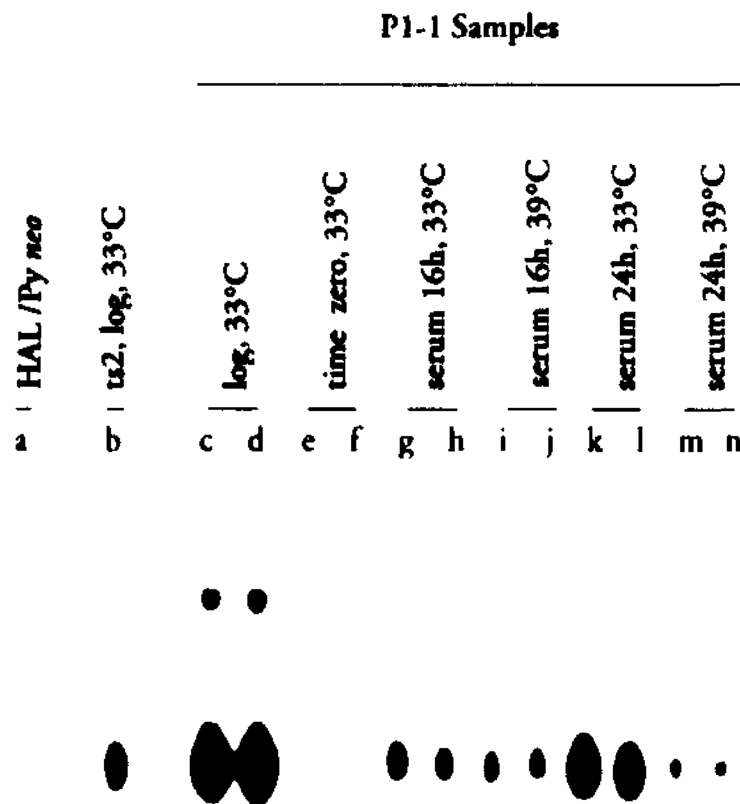


Figure 11c: The expression of He7 mRNA in the cell line P1-1 following serum stimulation.

Samples of RNA (10 µg) isolated by the GIT/Sarkosyl method at various times following plating were electrophoresed for 2 hours at 100 V and immobilized onto a NYTRAN filter as described in Figure 11a. The filter was hybridized to a ^{32}P-labeled probe prepared from the 300 bp PstI He7 fragment isolated from pGEMHe7 by the random primer method. Duplicate of filter shown in Figure 11b.

Lanes a-b, logarithmically growing P1-1 cells at 33°C.

Lanes c-d, quiescent P1-1 cells grown at 33°C.

16 hours following serum addition incubated at 33°C, lanes e-f, and at 39°C, lanes g-h.

24 hours following serum addition incubated at 33°C, lanes i-j, and at 39°C, lanes k-l.

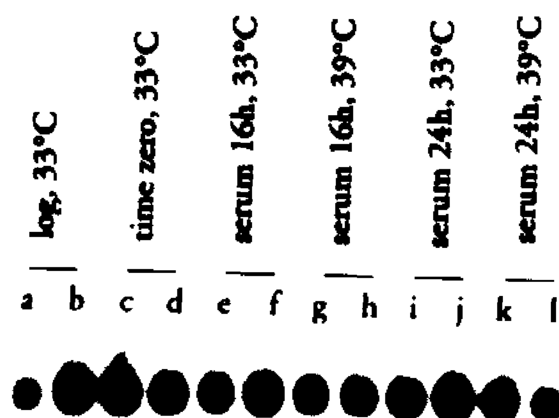


Figure 11d: The expression of the neomycin-resistance gene in the cell line P1-1 following serum stimulation.

Samples of RNA (20 μg) isolated by the GIT/Sarkosyl method at various times following plating were electrophoresed at 110 V for 2 hours and transferred to a NY-TRAN membrane as described in Figure 11a. The filter was then hybridized to a ^{32}P-labeled *neo^r^* probe prepared from a 1 kb NaeI-HindIII fragment isolated from pSV2*neo* by the random primer method.

Lane a, HAL/Py*neo*, human cell line containing the *neo*^{r} gene used as a positive control, 18μl sample isolated by the bentonite/SDS/formaldehyde method.

Lane b, ts2 cells, 20 μg of total RNA isolated from logarithmically growing ts2 cells at 33°C, used as a negative control.

Lanes c-d, logarithmically growing P1-1 cells at 33°C.

Lanes e-f, quiescent P1-1 cells grown at 33°C (time zero).

16 hours following serum addition incubated at 33°C, lanes g-h, and at 39°C, lanes i-j.

24 hours following serum addition incubated at 33°C, lanes k-l, and at 39°C, lanes m-n.

a Hal /*Pyneo*
b ts2, log, 33°C
c d log, 33°C
e f time zero, 33°C
g h serum 16h, 33°C
i j serum 16h, 39°C
k l serum 24h, 33°C
m n serum 24h, 39°C

Figure 12: Analysis of cell line P2-1 by Northern blot analysis.

The P2-1 cell line was established by pooling several hundred G418-resistant colonies following transfection of ts2 cells with both pWL*neo* and pTK21. The plasmid pTK21 contains the complete human TK cDNA under the control of the human TK promoter.

Figure 12a: The expression of human thymidine kinase (TK) mRNA in the cell line P2-1 following serum stimulation.

Samples of RNA (18 μl) isolated by the bentonite/SDS/formaldehyde method (Hatch and Bonner, 1987) at various time following plating were electrophoresed through a 1.2% agarose gel containing 1X MOPS buffer and 0.66 M formaldehyde at 100V for 2 hours. Following visualization by ethidium bromide staining, the RNA was transferred to a NYTRAN filter and immobilized by UV-crosslinking and baking. The filter was then hybridized to a ^{32}P-labeled probe prepared by random priming a 350 bp PCR synthesized human TK cDNA fragment.

Lane a, HAL/Py*neo*, logarithmically growing as human control.

Lane b, ts2, logarithmically growing at 33°C as murine control.

Lanes c-d, logarithmically growing P2-1 cells at 33°C.

Lanes e-f, quiescent P2-1 cells (time zero) grown at 33°C.

16 hours following serum addition incubated at 33°C, lanes g-h, and at 39°C, lanes i-j.

24 hours following serum addition incubated at 33°C, lanes k-l, and at 39°C, lanes m-n.

a HAL /Py *neo*

b ts2, log, 33°C

c d log, 33°C

e f time zero, 33°C

g h serum 16h, 33°C

i j serum 16h, 39°C

k l serum 24h, 33°C

m n serum 24h, 39°C

P2-1 Samples

Figure 12b: The expression of murine thymidine kinase (TK) mRNA in the cell line P2-1 following serum stimulation.

Filter is a duplicate of the filter used in Figure 12a but is hybridized to a random primer synthesized ^{32}P-labeled probe using the 647 bp RsaI fragment of pMtk4 as a template.

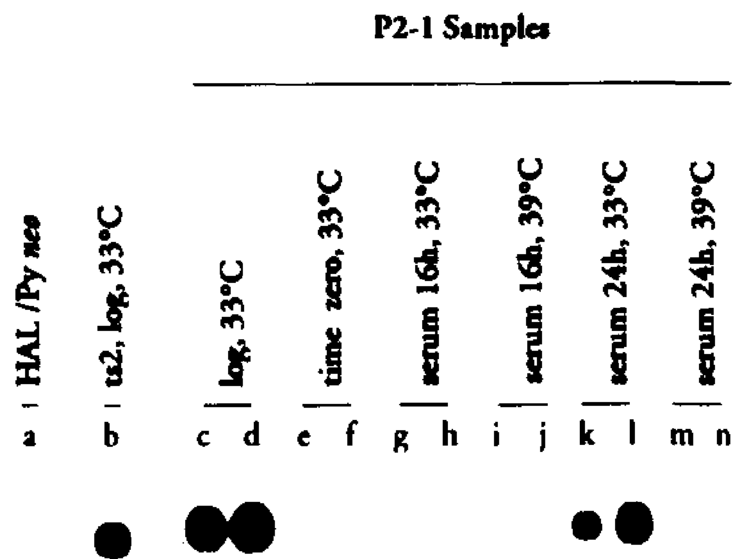


Figure 12c: The expression of He7 mRNA in the cell line P2-1 following serum stimulation.

The same RNA samples (18 µl) used in Figures 12a and 12b from the cell line P2-1 were electrophoresed and transferred to a NYTRAN membrane that was hybridized to a probe made from the 300 bp PstI He7 fragment of pGEMHe7 ^{32}P-labeled using the random primer method.

Lanes a-b, logarithmically growing P2-1 cell at 33°C.

Lanes c-d, quiescent (time zero) P2-1 cells grown at 33°C.

16 hours after serum addition incubated at 33°C, lanes e-f, and at 39°C, lanes g-h.

24 hours after serum addition incubated at 33°C, lanes i-j, and at 39°C, lanes k-l.

log, 33°C | time zero, 33°C | serum 16h, 33°C | serum 16h, 39°C | serum 24h, 33°C | serum 24h, 39°C

a b c d e f g h i j k l

Figure 13: Analysis of the cell line P4-2 by Northern blot analysis.

The P4-2 cell line was established by pooling several hundred G418-resistant colonies following transfection of ts2 cells by both pWL*neo* and pMGK85. The plasmid pMGK85 contains the HSV-TK promoter driving the human TK capsite and genomic coding region with the exception of 200 bp at the 3' untranslated end.

Figure 13a: The expression of human thymidine kinase (TK) mRNA in the cell line P4-2 following serum stimulation.

Samples of RNA (25 μg) isolated by the GIT/Sarkosyl method (Chomczyski and Sacchi, 1987) at various times following plating were electrophoresed through a 1.2% agarose gel containing 1X MOPS buffer and 0.66 M formaldehyde at 100 V for 4 hours. Following visualization by ethidium bromide staining, the RNA was transferred to a NYTRAN membrane and immobilized by UV-crosslinking followed by baking. The filter was then hybridized to a ^{32}P-labeled probe prepared by random priming a 350 bp PCR synthesized human TK cDNA fragment.

Lane a, logarithmically growing ts2 at 33°C, 18 μl sample prepared by bentonite/SDS/formaldehyde method used as a murine control.

Lane b, HAL/Py*neo*, 18 μl sample prepared by bentonite/SDS/formaldehyde method used as a human control.

Lanes c-d, logarithmically growing P4-2 cells at 33°C.

Lanes e-f, quiescent (time zero) P4-2 cells grown at 33°C.

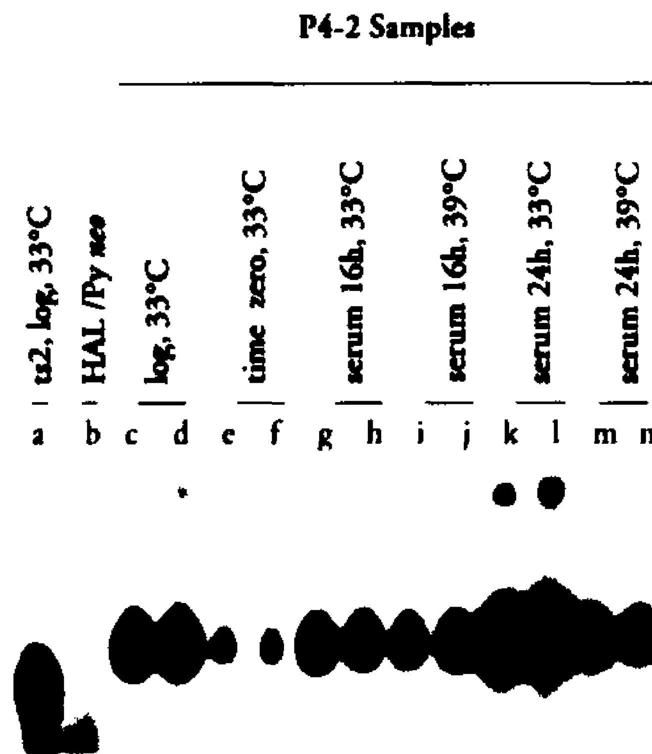
16 hours after serum addition incubated at 33°C, lanes g-h, and at 39°C, lanes i-j. (Lane i contained less RNA, as determined by ethidium bromide staining.)

24 hours after serum addition incubated at 33°C, lanes k-l, and at 39°C, lanes m-n.

a b c d e f g h i j k l m n
ts2, log, 33°C
HAL /Py *neo*
log, 33°C
time zero, 33°C
serum 16h, 33°C
serum 16h, 39°C
serum 24h, 33°C
serum 24h, 39°C
P4-2 Samples

Figure 13b: The expression of murine thymidine kinase (TK) mRNA in the cell line P4-2 following serum stimulation.

Samples of RNA (10 μg) isolated from P4-2 cells were processed as described in Figure 13a. The filter was hybridized to a ^{32}P-labeled probe prepared from the 647 bp RsaI fragment of pMtk4 prepared by the random primer method.



CHAPTER FOUR: Discussion

Early Characterization of ts2: A Brief Review

In contrast to other biological systems, relatively few mutants involved in DNA synthesis in mammalian cells have been isolated. The cell line ts2, derived from Balb/3T3 cells, has been shown to be temperature-sensitive (*ts*) for both cellular and polyoma DNA synthesis (Slater and Ozer, 1976). Jha *et al.* (1980) showed via genetic analysis of ts2 in cell hybrids that the defect is corrected by the human X-chromosome and is recessive in hybrids with other mouse cells with a *ts* lesion in a non-S phase function. Ts2 cells were not complemented in hybrids with several other cell lines *ts* for DNA synthesis, suggesting that these *ts* mutants have lesions in the same genetic locus (Jha *et al.*, 1980). In addition, the levels of DNA synthesis and different enzymatic activities associated with cells in S phase were studied in ts2 cells incubated at the nonpermissive temperature for various lengths of time (Malkas, 1985). As measured by ^{3}H-TdR incorporation, the rate of DNA synthesis declined when ts2 cells are shifted to the restrictive temperature. The activity of several enzymes (i.e. TK and DNA polymerase α) associated with DNA synthesis exhibited a corresponding decrease in cells incubated at 39°C (Malkas, 1985). When quiescent cells were stimulated by the addition of serum, there was a clear correlation between temperature and the ability of ts2 cells to progress through the cell cycle. Serum-stimulated ts2 cells showed a significant induction of DNA synthesis along with the activities of several cell cycle-regulated enzymes, including TK and TMPK (thymidylate kinase), only when incubated at the permissive temperature (Malkas, 1985). Malkas (1985) hypothesized that the *ts* defect observed in ts2 cells results from a block in the transcription of at least several of the genes required for DNA synthesis (i.e. TK, TMPK, and DNA polymerase α). However, Malkas (1985) maintained that the predominant phenotype manifested by ts2 cells is the defect in their ability to undergo

DNA synthesis at the nonpermissive temperature, thus supporting the view that ts2 is a DNA synthesis mutant and not a cell cycle mutant (Wittes and Ozer, 1973, Slater and Ozer, 1976).

The Cell Cycle Kinetics of ts2 Following Stimulation

In this text, additional characterization of ts2 was presented. Initial experiments were conducted to verify that ts2 is affected in cellular DNA synthesis following serum induction of quiescent cells at 39°C by measuring ^{3}H-TdR incorporation by TCA precipitation and by autoradiography. Data obtained using either technique show that when fresh serum is added to quiescent ts2 cells grown at 33°C, cultures shifted to the nonpermissive temperature (39°C) are defective in their ability to incorporate the radiolabel when compared to cells maintained at the permissive temperature (33°C). Although the thermolabile effect is not absolute, multiple experiments using serum-induced cells show an approximately 2-3 fold reduction in labeling at the nonpermissive temperature.

Serum-induced cultures of ts2 cells were analyzed by flow cytometry in order to gain additional insight into the nature of the temperature-sensitive defect as well as to ascertain where in the cell cycle they arrest. Prior to the addition of fresh serum (at time zero), 94% of the cells grown at 33°C are in G1 (or G0). From 16 to 30 hours following the addition of fresh serum, there is a steady increase in the number of cells that reenter the cell cycle when these cultures are incubated at the permissive temperature (33°C). In contrast, in ts2 cultures shifted to the nonpermissive temperature (39°C) following the addition of stimulation, only approximately 10% of cells reenter the cell cycle. Furthermore, the histograms show that although these cells enter S phase by 16 hours after serum addition, they become blocked in G2+M at later time points. These results support the ^{3}H-TdR labeling data because they show that there is a significant defect in the ability of serum-stimulated ts2 cells to undergo DNA synthesis (or enter S phase) at

the nonpermissive temperature. However, the data suggest that at the beginning of the time course (at time zero), not all cells are truly quiescent. The "incomplete arrest" of ts2 cultures could at least in part account for the apparent "leakiness" of the *ts* phenotype observed in the ^{3}H-TdR labeling experiments. In addition, it appears that the thermolabile defect in ts2 is manifested at two points in the cell cycle. The first block involves the inability of G0-arrested ts2 cells of reentering the cell cycle and undergoing DNA replication following serum stimulation at the nonpermissive temperature. Cells that are not truly quiescent and cells with a "leaky" *ts* phenotype can pass the execution point of the first block, enter S phase, and become blocked in late S or G2+M. This hypothesis is supported by the finding that actively growing cultures of ts2 cells accumulate in late S or G2+M following shift-up to the nonpermissive temperature (Malkas, 1985).

Malkas (1985) showed that if acute infection of ts2 cultures with SV40 virions is used to stimulate growth-arrested cells instead of serum, both DNA synthesis (as measured by ^{3}H-TdR incorporation) and TK enzyme activity are induced at both the permissive and nonpermissive temperatures. Ts2 cells stimulated into the cell cycle by SV40 infection were found to be able to complete one round of cellular DNA replication even at the nonpermissive temperature. On the basis of experiments carried out using the SV40 virus mutant tsA58, which contains a thermolabile mutation affecting T-antigen function, it was determined that both the induction of DNA synthesis and TK activity require the presence of functional T-antigen (Malkas, 1985).

The ^{3}H-TdR labeling data reported here confirm that acute infection of quiescent ts2 cells by SV40 virions bypasses the block in DNA synthesis at the nonpermissive temperature. Flow cytometric analysis of SV40-infected cells indicates that as the cells reenter the cell cycle at the nonpermissive temperature, they accumulate in G2+M. Taken together, the data strongly suggest that although SV40 infection bypasses the thermolabile defect that prevents serum-induced ts2 cells incubated at 39°C from initiating DNA

synthesis, there seems to be a second temperature-dependent block that occurs in late S or G2+M which is not overcome by SV40 infection.

In addition, it was observed that after SV40-infected ts2 cells are incubated at the nonpermissive temperature for more than 48 hours, the cells rapidly die and become detached from the cell culture dish. The arrest of these cells in late S or G2+M likely causes them to enter a state of "unbalanced growth" which leads to their rapid death. Rodent cells whose DNA replication is arrested in the absence of protein synthesis inhibitors continue to grow and reach abnormal size (Murray and Hunt, 1993). Once the replication block is lifted, these cells die. Murray and Hunt (1993) postulate that as these cells grow to abnormal size, the feedback controls that hold them at various checkpoints in the cell cycle decline. This would result in aberrant mitoses accompanied by massive genetic damage. Instead, rodent cells released from a replication block respond by inducing their own death without ever undergoing mitosis. This programmed cell death, called apoptosis, serves an adaptive purpose in multicellular organisms because although individual cells die, the organism avoids producing abnormal cells that could give rise to cancer (Murray and Hunt, 1993).

The Expression of TK mRNA in ts2 Following Stimulation

Since the defect that affects the ability of ts2 to undergo DNA synthesis at the nonpermissive temperature also affects the level of activity of at least several enzymes commonly associated with DNA synthesis, we were interested in determining whether the observed effects on the induction of these enzymes could at least in part be accounted for by a decrease in the steady state levels of the mRNAs associated with these activities. Measuring the steady state mRNA levels for specific messages is a useful approach to study gene expression because it allows one to study the control of the expression of a mRNA from a single gene using molecular techniques. However, these types of analyses by themselves are of limited value because they fail to provide information as to whether

the regulation in the accumulation of specific mRNA species is controlled at the transcriptional or posttranscriptional level.

The thymidine kinase (TK) gene was used as a "reporter gene" for these studies because its expression is closely associated with DNA synthesis, although it can be shown to be uncoupled to DNA synthesis using DNA synthesis inhibitors (Johnson *et al.*, 1982, Coppock and Pardee, 1987, Malkas, 1985). In addition, the TK gene has been used as a model to study the regulation of an important group of genes which can act as primary elements in the control of the cell cycle (Travali *et al.*, 1988). TK activity has been shown to be low in G0 and increases sharply at the G1/S boundary, remaining high throughout S phase (Bradshaw and Deininger, 1984). Steady state levels of TK mRNA also increase sharply as cells stimulated to proliferate from quiescence enter S phase (Coppock and Pardee, 1987, Liu *et al.*, 1985b, Stuart *et al.*, 1985). Malkas (1985) showed that the expression of TK enzyme activity in serum-stimulated ts2 cells is both cell cycle-regulated and temperature-dependent (Malkas, 1985). We measured the steady state levels of TK mRNA in ts2 cells following serum addition and determined that the temperature-sensitive induction of TK activity observed in ts2 cells as they enter S phase could at least in part be accounted for by their pattern of TK mRNA expression.

Slot blot experiments measured the level of TK mRNA at various times following serum induction at both 33°C and 39°C in both ts2 and its parental cell line, A31N. In ts2 cells, the induction in TK message is significantly reduced when serum-stimulated cells are shifted to the nonpermissive temperature. No temperature-dependent effect was observed in the parental cell line. The kinetics of TK mRNA induction in ts2 cells closely parallels what is observed when ^{3}H-TdR labeling is used as a criterion for assessing the cell's ability to undergo DNA synthesis. These results were confirmed using Northern blot analysis of serum reversed ts2 cells, which shows that by 24 hours after the addition of fresh serum, the level of TK message induced in ts2 cells incubated at 39°C

is significantly less than what is observed in cells incubated at 33°C.

Is Transcription Affected in ts2?

Transcriptional regulation of eukaryotic genes is mediated by a complex array of promoter and enhancer elements at the 5' end of the gene that allows multiple regulatory factors to coordinately function to potentiate or inhibit RNA synthesis. Therefore, the identification of the structural and functional properties of the regulatory elements affecting the transcription of the TK gene is important in order to elucidate the mechanisms by which this gene is regulated. Several recent studies have shown that in fibroblasts the sequences responsible for the regulation of TK mRNA expression lies in the promoter region of the TK gene (Travali, *et al.*, 1988, Lieberman *et al.*, 1988, Kim *et al.*, 1988, Lipson *et al.*, 1989, Ito and Conrad, 1990, Roehl and Conrad, 1990, Fridovich-Keil *et al.*, 1991, Kim and Lee, 1991, Kim and Lee, 1992). In addition, several different binding activities to various elements within the promoter region of the TK gene have been elucidated (Knight *et al.*, 1987, Arcot *et al.*, 1989, Bradley *et al.*, 1990, Dou *et al.*, 1991, Dou *et al.*, 1992, Kim and Lee, 1992, Li *et al.*, 1993), with at least one of these activities correlating closely with the G1/S phase induction of this gene. We determined that both the cell cycle and temperature-dependent expression of human TK genes transfected into ts2 cells is at least in part regulated at the level of the TK promoter.

Several human TK constructs were stably transfected into ts2 cells in order to determine whether a *ts* defect in TK gene transcription could account for the reduced levels of TK message observed in cells incubated at 39°C following serum-stimulation. Since the transfected cell lines contained both a murine TK gene as well as a transfected human TK gene, it was possible to simultaneously monitor both the levels of the endogenous murine and exogenous human transcripts. Three different human TK constructs were used for this analysis. One contained a human promoter linked to its genomic coding region with approximately 200 bp deleted from the 3' noncoding region and contained

no polyadenylation sequence, another the same human coding region linked to the HSV-TK promoter, and another contained the human promoter linked to a human TK cDNA with the appropriate poly(A) addition signal.

The sequence of the the mouse TK promoter shows little resemblance to other TK promoters (Weichselbaum *et al.*, 1990, Seiser *et al.*, 1989). The upstream regulatory elements of the murine TK gene differ markedly from those of the human gene, lacking a TATA box but containing two putative Sp1 binding sites (GGGCGG motifs) and sequences resembling CCAAT boxes (Seiser *et al.*, 1989). However, the results presented reveal that despite some very striking structural differences both the exogenous (linked to the human TK promoter) and endogenous TK genes are regulated in a cell cycle dependent manner, with their expression being induced at the permissive temperature. At the nonpermissive temperature, however, both genes manifest the *ts* defect and show diminished levels of their respective transcripts. On the other hand, the human TK gene under the control of the HSV-TK promoter, shows temperature-independent constitutive expression.

When the genomic sequence lacking the polyadenylation signal was used, Northern blot analysis showed the presence of a heterogeneously sized population of human TK RNA species, regardless of which promoter it was linked to (human TK or HSV-TK). By contrast, only a single band was observed for the cell line containing the human TK cDNA linked to the appropriate polyadenylation signal. In addition, of the two cell lines studied under the control of the human TK promoter, the cell line containing the cDNA sequence had a much higher level of TK expression and showed much tighter cell cycle regulation than the construct containing the genomic coding sequence. It also appears that neither the polyadenylation signal nor the 3' untranslated region is required for the cell cycle regulation of this gene.

To reiterate, these data strongly suggest that both the cell cycle regulation and the *ts* defect observed in TK mRNA expression at the nonpermissive temperature occur at least

in part at the level of the promoter and is likely to involve a transcriptional block. It is conceivable that genes whose expression at the mRNA level are induced at the G1/S boundary are in some way linked to the onset of DNA synthesis, and in at least the case of ts2, serve as markers for the *ts* phenotype, whose selected for characteristic is the inability of this cell line to undergo DNA replication at the nonpermissive temperature. Furthermore, these results indicate that the transcription or modification of some "factor(s)" required for the initiation and/or the progression of DNA synthesis is defective in ts2. Alternatively, it could be argued that the removal of a repressor molecule is required for cells to synthesize DNA under permissive conditions. A defect in the process by which this derepression occurs could also result in a *ts* phenotype similar to what is observed in ts2 cells at 39°C.

A Model to Account for the Defective Expression of TK mRNA in ts2

Liu *et al.* (1985a) and Pardee (1989) point out that the oncogene products of DNA tumor viruses, such as SV40 large T antigen and adenovirus E1A protein, appear to activate or short-circuit the various steps in G1 that are normally initiated by growth factors, thus making growth less restricted. Roehl *et al.* (1993) analyzed human TK promoter deletion mutants and localized a region sufficient to confer transcriptional regulation on SV40-infected cells. These elements are distinct from those required for serum induction, suggesting that SV40 activates novel cellular pathways that are not activated by serum stimulation of quiescent cells (Roehl *et al.*, 1993). When acute SV40 viral infection is used to stimulate quiescent ts2 cells instead of serum, the level of TK mRNA is induced in a temperature-independant manner. This finding supports both the flow cytometric and ^{3}H-TdR labeling data, indicating that acute infection by SV40 virions either overrides or bypasses the temperature-sensitive block in DNA synthesis manifested by these cells when they are stimulated from quiescence by the addition of fresh serum.

Ogris *et al.* (1993) showed that the murine TK gene promoter contains a binding site for transcription factor E2F. Using cell lines (3T3-LT) capable of expressing polyomavirus large T antigen from a hormone-responsive promoter, they demonstrated that this E2F site is the target for transactivation by the viral protein. Induction of transcription from the TK promoter in quiescent cells by polyomavirus large T antigen requires an intact binding site for the retinoblastoma protein in the viral protein. This same promoter region plays a critical role in the growth regulation of this gene (Ogris *et al.*, 1993). Furthermore, the promoters of several other genes coding for enzymes involved in DNA synthesis also contain E2F binding sites, suggesting that these genes are regulated by a common mechanism.

Since SV40 large T antigen binds the retinoblastoma protein (Rb) in a similar manner, it is possible that the temperature-independent induction of both TK transcription and DNA synthesis observed in SV40-infected ts2 cells results at least in part from the transactivation of E2F binding sites that is mediated by the interaction between SV40 large T antigen and Rb. Rb acts as a brake on cell cycle progress by binding to and inhibiting transcription factor E2F, which plays a role in the transcription of both early and delayed response genes (Murray and Hunt, 1993). In uninfected cells, Cdk-G1 cyclin complexes phosphorylate and inactivate Rb in late G1, at a time roughly corresponding to the restriction (R) point. The phosphorylation of Rb blocks its ability to bind to E2F, allowing E2F to stimulate a cascade of events that leads to the initiation of DNA replication.

On the basis of these findings, it is possible to formulate an hypothesis that at least in part accounts for the fact that quiescent ts2 cells acutely infected by SV40 virions do not exhibit defects in either TK mRNA induction or DNA synthesis when they are incubated at the nonpermissive temperature, while serum-stimulated cells are blocked in both functions. According to this model, the binding of Rb to SV40 large T antigen would inactivate Rb in a manner similar to what occurs in uninfected cells when Rb is phospho-

rylated by Cdk-G1 cyclin complexes in late G1, thus freeing E2F from inhibition and allowing it to bind to DNA. The resulting activation of transcription of the TK gene along with other growth regulated genes that contain E2F binding sites would culminate in the initiation of DNA replication in these cells. Furthermore, the report by Dou *et al.* (1992) demonstrating that the induction of transcription of the murine TK promoter at G1/S involves the phosphorylation of Rb (p107) associated in complexes believed to contain E2F by an H1 kinase related to cdc2 supports suggests that the failure to activate TK gene transcription in serum-induced cells at the nonpermissive may involve the failure to activate E2F by first inactivating Rb. By postulating that the actual defect in these cells likely involves a gene associated with a modification step, this model would help explain at least some of the pleiotropic effects of the *ts* phenotype exhibited when ts2 cells are incubated at the nonpermissive temperature.

The Complex Regulation of TK Expression

The demonstration that both the cell cycle regulation and the *ts* defect in TK mRNA accumulation occur at the level of the TK promoter strongly suggests that the reduced level of TK activity observed in serum-stimulated cells incubated at the nonpermissive temperature (Malkas, 1985) results from a defect in the transcription of the TK gene. However, on the basis of these studies, additional defects affecting posttranscriptional and/or posttranslational mechanisms cannot be ruled out. Hofbauer and Denhardt (1991) point out that when studying a gene whose expression varies during the cell cycle, the question arises as to whether that gene regulates the cell cycle, whether the cell cycle regulates that gene, or whether the correlation results from the fact that both the cell cycle and that gene respond to the same signal(s). Furthermore, they contend that since gene expression is regulated at multiple levels, the relative importance of regulation at these different levels depends upon the operational meaning one ascribes to the cell cycle (Hofbauer and Denhardt, 1991). For example, exponentially growing cells proceed di-

rectly from mitosis into G1. They are regulated primarily by posttranscriptional and posttranslational mechanisms, with transcriptional control playing a secondary role. In contrast, cells stimulated to exit quiescence, G0, require new gene transcription as well as changes in the posttranscriptional role of gene expression. Therefore, the relative importance of the different cellular mechanisms involved in the regulation of the cell cycle expression of the TK gene is significantly influenced by the physiologic state of the cells analyzed.

Sherley and Kelly (1988) studied TK gene expression in cycling HeLa cells synchronized by centrifugal elutriation. They found that despite a dramatic increase in TK activity as these cycling cells move from G1 into S, the steady state levels of TK mRNA show relatively small changes during the cell cycle. Their data show that two different posttranscriptional mechanisms largely account for the cell cycle regulation of TK expression in cycling cells: one involving an increase in the efficiency of translation of TK mRNA in S phase and the other involving a dramatic decrease in TK protein stability upon cell division (Sherley and Kelly, 1988). Several studies have shown that both transcriptional and posttranscriptional mechanisms are responsible for the dramatic increase in TK expression that occurs at the onset of S phase in G0-arrested cells stimulated to reenter the cell cycle by the addition of fresh serum (Coppock and Pardee, 1987, and Gudas *et al.*, 1988). However, Lipson and Baserga (1989) used the highly sensitive technique of reverse transcription coupled to polymerase chain reaction to show that in truly quiescent human diploid fibroblasts there is no transcription of the TK gene. Following serum stimulation, the transcriptional activity of the TK gene reached maximal levels in early S phase (Lipson and Baserga, 1989). Since our studies used G0-reversed ts2 cells, an increase in the rate of transcription of the TK gene could play a significant role in the accumulation of TK message as cells enter S phase at 33°C. Similarly, a thermolabile defect affecting the transcription of the TK gene could account for the reduced levels of TK message seen at the restrictive temperature.

The UBE1 Gene: A Candidate for the Gene Responsible for the ts2 Phenotype

The data reported here provide additional information about the phenotype of the *ts* defect manifested by ts2 cells when they are stimulated from quiescence at the nonpermissive temperature. On the basis of these data, we proposed that the defect in TK mRNA expression (which appears to involve a block in TK gene transcription) and the inability of these cells to undergo DNA replication (which results in the arrest of these cells in G1) when incubated at 39°C are both manifestations of a defect in a modification step. This likely results from a lesion in a gene that controls multiple cell cycle-related functions. However, as described, our hypothesis fails to account for the observation that ts2 cells capable of entering S phase at the nonpermissive temperature due to the "leakiness" of the *ts* phenotype, the incomplete arrest of ts2 cultures, or acute SV40 infection become blocked in G2+M. The results of ongoing experiments directed towards the identification of the genetic lesion responsible for the *ts* phenotype in ts20, another temperature-sensitive Balb/3T3 mutant defective in DNA synthesis, may provide the conceptual basis for a unified model which could account for the temperature-dependent blocks in cell cycle progression that cause ts2 cells to arrest at either the G1/S boundary or in G2+M. Furthermore, this model suggests that ts2 is not a DNA synthesis mutant *per se* but rather a cell cycle mutant.

In order to gain further insight into the nature of the genetic defect responsible for the temperature-sensitive phenotype in ts2 cells, the most useful strategy to pursue would be the cloning and identification of a gene capable of correcting the *ts* defect in these cells. Considerable progress has been made in this regard. A thioguanine (TG)-resistant derivative of ts20 has been corrected by high molecular weight human DNA using DNA-mediated gene transfer and co-selection with a plasmid containing ECOgpt. Primary transfectants (*ts*$^{+}$ gpt^{+}) containing human sequences which codes for the human ubiquitin-activating enzyme E1, UBE1, gene have been isolated. Back selection in TG results in the both the restoration of the *ts* phenotype and the loss of the UBE1 gene se-

quences. Ts2 and ts20 are independent 3T3 mutants that do not complement each other in cell hybrids. In addition, they are both corrected by a gene on the human X-chromosome (Jha *et al.*, 1980). The UBE1 gene has been localized to both the mouse (Disteche *et al.*, 1992) and human (Takahashi *et al.*, 1992) X-chromosome. It is likely, therefore, that the human UBE1 gene would also correct the *ts* defect in ts2 cells.

In recent years, several other temperature-sensitive mammalian cell cycle mutants have been complemented by a functional copy of the human UBE1 gene (Kudo *et al.*, 1991, Ayusawa *et al.*, 1992, Disteche *et al.*, 1992, and Nishitani *et al.*, 1992). Each of these mutant cell lines has a distinct *ts* phenotype and shows a characteristic pattern of cell cycle arrest in S and/or G2 when incubated at the restrictive temperature, suggesting pleiotropic functions for this enzyme. The finding that lesions in the UBE1 gene are responsible for the phenotypes of a number of well-studied *ts* mammalian cell cycle mutants relates to the fact that the UBE1 gene product is essential for the activation of an important intracellular regulatory pathway.

Intracellular levels of proteins are determined by the balance between the rates of protein synthesis and protein degradation. One prominent degradation pathway in eukaryotic cells involves the conjugation of ubiquitin, a 76-amino acid protein, to abnormal and short-lived proteins, thus marking them for ATP-dependent proteolysis (Trausch *et al.*, 1993). Ubiquitin-activating enzyme E1 (UBE1) catalyzes the first step in the ubiquitin-mediated proteolytic cascade. Trausch *et al.* (1993) examined the subcellular distribution of UBE1 in several eukaryotic cell lines. UBE1 was found in both the nucleus and cytoplasm in all cell lines analyzed. However, not only did the relative abundance of the enzyme in each cellular compartment differ markedly between cell lines, the nuclear distribution even within a single cell line was variable (Trausch *et al.*, 1993). These findings suggest pleiotropic functions for this enzyme and the ubiquitin-conjugating system.

In addition to the growing number of cell cycle mutants that can be corrected by a functional UBE1 gene, there is considerable evidence that protein degradation triggered

by ubiquination is crucial to the sequential regulation of cellular events as cells traverse the cell cycle. For example, in frog embryos, cyclin degradation by the ubiquitin-conjugating system is crucial for the transition from metaphase to anaphase. Mutants containing cyclins that cannot be degraded by this pathway become arrested in mitosis (Glotzer *et al.*, 1991, and Murray and Hunt, 1993). In addition, there is evidence that the ubiquitin-mediated degradation of cyclin proteins plays an important role in the G1 to S transition (Glotzer *et al.*, 1991).

Nuclear oncoproteins are among the most rapidly degraded intracellular proteins (Ciechanover *et al.*, 1991). Ciechanover *et al.* (1991) found that *in vitro* the nuclear oncoproteins encoded for by the N-*myc*, c-*myc*, c-*fos*, p53, and E1A genes are degraded by the ubiquitin-mediated proteolytic cascade, suggesting that these proteins possess specific signals that target them for rapid turnover by this pathway. The failure to degrade any of these proteins at the appropriate time could affect the ability of cells to successfully traverse the cell cycle and continue normal growth.

The foregoing discussion postulates that the genetic lesion responsible for the *ts* phenotype observed in ts2 cells is in the UBE1 gene. Based on what is known about both ts2 and the UBE1 gene, one can easily see how a cell line with a mutation affecting an enzyme responsible for the activation of an important intracellular protein degradation pathway that acts throughout the cell cycle could have multiple defects. Specifically, we could propose that the temperature-dependent arrest of ts2 cells at G1/S and G2+M results from the failure of the ubiquitin-conjugating system to degrade cyclins at these two important transition points in the cell cycle. Since cDNA clones for both the human and murine UBE1 genes are currently available (Disteche *et al.*, 1992), the next logical step would be to transfect ts2 cells with a functional UBE1 gene and see whether or not the *ts* phenotype is complemented.

Conclusion: ts2, a Cell Cycle Mutant Defective in DNA Synthesis

When the cell line ts2 was isolated over twenty years ago, the criterion for its selection was the inability of cells to incorporate lethal amounts of ^{3}H-TdR when incubated at 39°C (Wittes and Ozer, 1973). Initial studies of ts2 examined macromolecular synthesis by measuring the incorporation of radioactive precursors into TCA-precipitable macromolecules (i.e. proteins, DNA, and RNA) and found that DNA synthesis is preferentially affected in this cell line (Slater and Ozer, 1976). In addition, cell cycle analysis consisting of kinetic studies which measured ^{3}H-TdR incorporation following synchronization of cells with hydroxyurea showed that the these cells were defective in DNA synthesis (Slater and Ozer, 1976). On the basis of these studies, it was concluded that since the predominant phenotype of these cells was their inability to undergo DNA synthesis when incubated at the restrictive temperature, ts2 must be a DNA synthesis mutant. Although these methodologies were "state of the art" when these studies were reported, they are relatively crude and provide only limited information regarding the complexity of the *ts* phenotype manifested by ts2 cells. Although the studies reported by Malkas (1985) correlated the ^{3}H-TdR labeling data with the activity of several enzymes associated with DNA synthesis, it was argued that the temperature-dependent expression of these enzymes was a manifestation of a block to DNA synthesis.

Advances in molecular biology have facilitated the elucidation of a variety cellular regulatory processes, including mechanisms involved in the regulation of DNA synthesis and cell cycle progression. By studying the expression of specific genes or sets of genes under different experimental conditions, it becomes obvious that the expression of a given gene is regulated at multiple levels. For example, in addition to a pattern of transcriptional regulation that involves at least several factors, posttranscriptional, translational, and posttranslational mechanisms are involved in the control of TK expression. Furthermore, not only is the study of gene expression difficult because many of these

mechanisms operate simultaneously, the relative importance of a particular regulatory mechanism varies with the physiological state of the cells analyzed. As discussed earlier, transcriptional control mechanisms are likely to play a greater role in TK gene expression in serum-induced cells than in exponentially growing cells.

The analysis of TK mRNA expression of both the endogenous murine TK gene and the exogenous human TK gene in ts2 suggests that there is a *ts* defect in the of transcription the TK promoter in serum-stimulated cells. A model has been proposed to account for this which involves the failure to activate transcription factor E2F. However, on the basis of the studies, we cannot rule out the possibility that other mechanisms affecting TK expression could be defective in these cells, as well. Therefore, the studies involving TK mRNA expression provided additional characterization of the *ts* phenotype without addressing the nature of the underlying defect in ts2. The isolation and identification of the gene responsible for the *ts* phenotype should enable us to understand how a lesion is a single genetic locus can affect a variety of cellular processes.

The observation made by flow cytometric analysis of ts2 cells incubated at the restrictive temperature that shows that they can arrest in both G1 and G2+M argues that a more appropriate description of ts2 is that it is a temperature-sensitive cell cycle mutant. The thermolabile defect in DNA synthesis in ts2 cells appears to be only one manifestation of a *ts* phenotype that affects the cell cycle progression of these cells. A model which involves the failure of the ubiquitin-mediated proteolysis of cyclins at critical cell cycle transition points has been proposed to account for the temperature-dependent arrest of thse cells in G1 and G2+M.

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