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Cell cycle regulation of thymidylate synthase in mouse fibroblasts

Nagarajan, Mahalakshmi, Ph.D.

The Ohio State University, 1989
Cell Cycle Regulation of Thymidylate Synthase in Mouse
Fibroblasts

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree of Philosophy in the Graduate
School of The Ohio State University

BY
Mahalakshmi Nagarajan M.Sc

The Ohio State University
1989

Dissertation Committee:
Dr. Lee F. Johnson
Dr. Thomas Byers
Dr. Roy Tassava
Dr. Caroline Breitenberger

Approved by:

Adviser
Molecular, Cellular and Developmental Biology Program.
To My Husband
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VITA

January 1, 1957 ......................... Born, New Delhi, India

1977 .......................... B.Sc.(Hons) Botany, Delhi University, India

1979 .......................... M.Sc. Botany, Department of Botany, Delhi University, India

1984-1985 ....................... Teaching Assistant, MCDB
The Ohio State University, Columbus, Ohio.

1986-1989 ....................... Research Assistant, MCDB, The Ohio State University, Columbus, Ohio.

PUBLICATION

Regulation of Thymidylate synthase gene expression in Mouse Fibroblasts Synchronized by Mitotic selection
Mahalakshmi Nagarajan and Lee F. Johnson
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INTRODUCTION

Importance of studying the regulation of cell cycle events

All cells except those that are terminally differentiated undergo growth, DNA replication and cell division. During each cycle, the cell responds to a series of precise signals that allow for an ordered progression through the cycle. Cell cycle can be defined as the interval between completion of mitosis in the parent cell and completion of the next division in the daughter cells (Baserga, 1985). Using autoradiography and following the status of DNA in synchronized cells, the cell cycle can be divided into four distinct phases (Fig.1). These are M (mitosis), G1 (interval between M and DNA replication), S (period where DNA replication occurs) and G2 (interval between S and M). There is yet another phase called G0 which refers to a quiescent phase where cells do not cycle. Todaro, Lazar and Green (Todaro et al. 1965) had shown that density inhibited 3T3 cells could remain quiescent for several days. However on addition of fresh medium with 10% serum these cells could re-enter the cell cycle. The G0 state is also referred to as resting state.

Studying the regulation of cell cycle is important for our understanding of the mechanisms that control normal cell proliferation. Since cell reproduction is
Figure 1. Cell Cycle. A single cell cycle can be divided into four main phases, M phase (Mitosis), G1, S(DNA Replication) and G2. G0 refers to a quiescent or resting phase.
FIGURE 1.
the final event of a cell cycle, I would briefly like to mention regulation of this event. Exhaustion of nutrients or unfavorable environmental conditions usually results in cell cycle arrest in both unicellular and multicellular organisms. However, in multicellular organisms, regulation of cell reproduction is not mainly achieved by regulating nutrients, instead it is achieved by specific processes brought about by hormones and cell-cell interactions (Prescott, 1976). Studies of these regulatory events are very important as they define the basis of normal versus abnormal cell growth.

In order to understand the mechanisms that control the sequential events of the cell cycle, it is important to identify and characterize genes that may be involved in this process. There are mainly three classes of genes that may play this role in the cell cycle. These are first, the protooncogenes, second, genes that can complement defects of temperature sensitive cell cycle mutants and lastly, genes that are growth regulated or regulated during the cell cycle (these classes overlap). Protooncogenes are believed to exert some regulatory role for the following reasons: i) transforming retroviruses have recombinant forms of cellular oncogenes that act as dominant growth stimulants e.g. c-myc protooncogene is the cellular counterpart of v-myc found in several avian retroviruses and in feline leukemia virus (Ratner et al. 1985); ii) these oncogenes are highly conserved suggesting an essential and fundamental role in normal cell growth e.g. c-ras has been identified in chickens, fruit flies, mollusks, plants, yeast and the striking property of evolutionary conservation is evident in the ability of c-ras to function in heterologous systems (Barbacid, 1987); iii) differential expression of
protooncogenes during embryonic development suggest their involvement in proliferation e.g. the cellular protein pp60^c-src is expressed at high levels during development of neural tissues in chick, especially during late prenatal and postnatal stages of development (Cotton and Brugge, 1983); iv) strong homologies between proteins encoded by viral oncogenes and growth factors/ receptors suggest an important role for these protooncogenes in cell proliferation e.g. v-erbB gene is strikingly homologous to epidermal growth factor receptor (Downward et al. 1984). Also, there is some evidence that some protooncogenes may control growth by interacting with specific regulatory DNA sequences responsible for controlling transcription and gene regulation e.g. the product of the protooncogene c-jun is thought to belong to the AP-1 family of transcription factors (Bohmann et al. 1987).

The second class of genes that may regulate the cell cycle are those that can complement temperature sensitive cell cycle mutants. Hartwell defined cell cycle mutations as those causing arrest in the cell cycle at a specific stage (Hartwell, 1974). This definition excludes some interesting mutants like G1-less (Liskay, 1977) and mutants that arrest at both G1 and G2 when grown at non permissive temperatures (Liskay et al. 1980). In the last 20 years many cell-cycle specific mutants have been isolated and partially characterized in both yeast and animal cells. These cells contain a mutation in a particular gene that affects directly or indirectly a function that is required only at a certain stage or stages in the cell cycle. These ts mutants, when placed at the restrictive temperature, are unable to proceed beyond the point of arrest in the cycle (Marcus et al. 1985).
Some of these mutants are described below. A mouse cell cycle mutant, ts85, arrests in S or early G2. This mutant is defective in the phosphorylation of histone and the addition of ubiquitin to histone H2A (Finley et al. 1984). In the mutant ts A1S9, a mouse cell mutant, the activity of DNA topoisomerase II is affected at restrictive temperature and this mutant is unable to proceed through the S phase (Colwill and Sheinin, 1983). tsAF8 is a Syrian hamster cell mutant, where RNA polymerase II is affected and at restrictive temperature cannot proceed beyond mid G1 (Rossini et al. 1980). Microinjection of RNA polymerase II corrected this ts mutation (Waechter et al. 1984). Several other S phase mutants seem to be defective in DNA polymerase activity.

Isolation and characterization of ts cell cycle defective mutants have been more successful in budding yeast and fission yeast than in mammalian cells. Most of these ts mutations have been identified to have some involvement with DNA replication machinery. One such gene CDC28 (Beach et al. 1982) from Saccharomyces cerevisiae has been shown to have protein kinase activity, and seems necessary for the cells to pass the G1-S phase commitment point (Reed et al. 1985). A gene analogous to CDC28, CDC2 has also been isolated from Schizosaccharomyces pombe (Hayles and Nurse, 1986). In S. pombe, the CDC2 gene encodes a function which is required at two control points, first, at G1 to S phase commitment point and second, at G2 to M phase transition. Recessive lethal cdc2 mutants are unable to proceed past G1 or G2 control points (Nurse et al. 1976).
In 1987, Lee and Nurse (Lee and Nurse, 1987) used complementation to clone the human CDC2 gene. The gene was cloned by expressing a human cDNA library in fission yeast and selecting for clones that can complement mutant cdc2. The gene has been sequenced and the predicted protein sequence indicates a protein of the same molecular weight as S. pombe cdc2 with 63% identity of amino acid residues. This experimental approach provides yet another way to isolate genes in mammals which are homologous to cell cycle specific genes in yeast.

The third class of genes that may be involved in regulating the cell cycle are genes that are regulated during the cell cycle or in response to growth factors. Genes that are regulated in response to mitogens can be isolated by the method utilized by Lau and Nathans. This was done by differential screening of cDNA libraries. Filter replicas of cDNA libraries were hybridized with radioactive cDNA probes prepared from poly(A)+ mRNA from cells before and after mitogenic stimulation. They isolated several serum inducible genes which were identified as c-fos, c-myc and p53. These genes were induced even in the presence of cycloheximide indicating that these were primary responders to growth stimulation (Lau and Nathans, 1985). Other examples of growth regulated genes that were identified include core histones, thymidine kinase, thymidylate synthase, dihydrofolate reductase, major excreted protein, ADP/ATP carrier and vimentin (Denhardt et al. 1986).

Some growth regulated genes are also regulated during the normal cell cycle. Most of these genes encode proteins that are synthesized at a specific time
during the cell cycle. The most well studied of these are the S phase genes. These
genes are expressed at low levels during G1 phase of the cell cycle and reach
maximal level of expression during S phase. The core histones H3, H2a, H2b and
H4 are all coordinately synthesized during the S phase of the mammalian cell
cycle (Heintz et al. 1983). Thymidine kinase (Littlefield, 1966) and dihydrofolate
reductase (Mariani et al. 1981a) are a few examples of enzymes involved in the
nucleotide biosynthetic pathway that are cell cycle regulated.

Cyclin is yet another gene that is cell cycle regulated and has increased
expression during the S phase (Bravo and Celis, 1980). Recently it has been
demonstrated that cyclin may be involved in inducing cells to divide. Kirschner
and Murray showed that addition of exogenous cyclin mRNA to extracts of frog
eggs (that can perform multiple cell cycles in vitro) is sufficient to produce
multiple cell cycles. They developed an in vitro cell-cycle extract that faithfully
mimics the key features of cell cycle in early Xenopus embryos. They destroyed
the endogenous mRNA in these extracts and showed that addition of exogenous
cyclin mRNA from either Xenopus or sea urchin could drive a number of cell
cycles. These results implied that cyclin synthesis can both activate MPF
(Maturation Promoting Factor) and entry into mitosis, as well as be able to bring
about its own degradation, inactivate MPF and progress into the next interphase
(Murray and Kirschner, 1989). In 1988 it was shown that the Xenopus cdc2
protein is a component of MPF (Dunphy et al. 1988; Gautier et al. 1988). Thus it
appears that at least some of the proteins that are involved in the regulation of
the cell cycle are being identified.
Genes involved in regulating the cell cycle form an interesting group of genes whose hierarchical function is still not clear. It is highly likely that activation of one gene may in turn activate several other genes. So far, what we know about cell cycle genes and their regulation is that the protooncogene c-fos is probably among the first genes to be turned on when cells are growth stimulated. This is followed by the activation of c-myc, followed by c-ras, actin, p53, proliferin etc., followed by the S phase genes (Chen and Wang, 1984). To be able to understand just how these genes interact and influence each other would give us some hint as to the regulation of the cell cycle. The important clue to our understanding of cell-cycle regulation is that these genes are expressed at precise times during the cycle and that regulation of expression of individual genes may help in our understanding of the cell cycle.

**Regulation of eukaryotic gene expression**

Most of our understanding of regulation of eukaryotic gene expression arises from work done on genes that are induced in a tissue specific manner or those that encode abundant proteins e.g. the lymphoid cell specific expression of immunoglobulin genes, ovalbumin, alpha amylase (Darnell Jr, 1982) etc. These proteins carry out specialized functions and therefore are not expressed in every cell type. Genes that are expressed at relatively low levels and perform basic cellular functions had been largely overlooked because regulation studies of these genes was difficult as their products were expressed at very levels. These genes
are called "housekeeping genes" were have been a recent focus of study.

Regulation of gene expression differs greatly in prokaryotes and eukaryotes. Prokaryotes lack compartmentalization and therefore transcription and translation are coupled. The mRNAs are true replicas of the gene. In contrast, eukaryotic cells are compartmentalized and transcription and translation are carried out in separate compartments. Transcription occurs in the nucleus and translation in the cytoplasm. Genes in eukaryotes can be divided into three categories depending on the polymerases used in their transcription. RNA polymerase II is the enzyme that transcribes genes that encode proteins (Corden et al. 1980). This enzyme synthesizes a primary transcript that is larger than the mature message and is localized to the nucleus. This RNA species is called the heterogeneous nuclear RNA or hnRNA. The hnRNA is then subjected to several processing steps to produce the final mature messenger RNA which is then exported to the cytoplasm to be translated. These processing steps include addition of the methylated guanylate to the 5' end of primary transcripts, the addition of a poly (A) tail to the 3' end of the primary transcript, and finally the removal of the introns to create the final mature mRNA.

Regulation of gene expression in prokaryotes occurs at the initiation of transcription and at termination. The most well known case of premature termination (also known as attenuation) occurs in the tryptophan operon in Escherichia coli. In E.coli in the presence of charged tRNA^{trp} attenuation / premature termination of transcription occurs resulting in the turning off of the trp operon. Therefore the cell decides whether or not to synthesize the amino
acid based on the intracellular concentration of tryptophan (Yanofsky, 1981). In eukaryotes, transcriptional arrest or pausing or attenuation may also regulate gene expression. In the case of c-myc, transcriptional arrest within the first exon seems to be a rapid control mechanism to control c-myc gene expression in response to DMSO. The steady state levels of c-myc RNA starts to decrease immediately after the addition of Dimethylsulphoxide (or DMSO, a potent inducer of granulocytic differentiation in HL60 cells) and this decrease is due to a block in transcriptional elongation (Eick and Bornkamm, 1986).

In the case of eukaryotes, regulation can occur at any one of the levels of mRNA biogenesis. In many cases, regulation of gene expression in eukaryotes occurs at the level of transcription. Regulation also occurs at the posttranscriptional level and at the translational level. Transcriptional control can occur in several ways. In differentiated cells, many proteins are produced due to hormonal induction e.g. in chicken oviduct, estrogen stimulates the production of egg white proteins like ovalbumin. This increased production of ovalbumin is due to increased rate of transcription of the gene in the presence of the hormone (Groudine et al. 1981). In the case of chick embryos the differential expression of α and β globin appears to be controlled at the level of transcription (McKnight and Palmiter, 1979). Lastly, the differential production of α-amylase in rat salivary gland and liver are attributed to transcriptional control by choice of different transcriptional initiation sites (Falck-Pederson et al. 1985). And as mentioned earlier, premature transcriptional termination or attenuation modulates the content of c-myc RNA in eukaryotic cells. Therefore attenuation can also be used
by cells as a means to regulate gene expression. Post transcriptional control can occur at any step of processing. It can occur at the level of polyadenylation, splicing, export of mature mRNA and at the level of mRNA stability. Finally, in the cytoplasm there is translation control. Soon after transcriptional initiation occurs, a methylated guanylate residue is added to the 5' terminus of the primary transcript (Darnell Jr, 1982). Processing of the 3' end and splicing of the introns are the next steps in the biogenesis of the mRNA. Use of alternative poly(A) addition sites, and/or splicing sites leads to the production of multiple RNAs in complex transcriptional units. Examples of some these complex transcriptional units are the rat calcitonin gene, immunoglobulin gene, rat fibrinogen and c-Ki-ras etc (Leff et al. 1986).

The switch from membrane bound IgM (immunoglobulin heavy chain) to secreted IgM results from alternative splicing that replaces a membrane anchoring exon for an exon that allows the protein to be secreted. Each exon has it's own poly(A) addition site, therefore it can be said that poly(A) site selection controls the formation of the two forms of IgM. This switch occurs in response to mitogen or antigen. The exact mechanism that brings about this control is not clear (Leff et al. 1986).

In the case of H4 histone mRNA in mouse mastocytoma cell cycle mutant (21-Tb), the conversion of the precursor RNA to mature mRNA seems to be dependent on a heat labile factor that is present in exponentially growing cells but is absent in growth arrested cells. This is a very interesting example of a cell cycle gene whose regulation is dependent on timely processing of the 3'end
(Luscher and Schumperli, 1987). Several genes use only alternative splicing to produce different mRNAs. In the case of c-Ki-ras protooncogene, exclusion of the 4th exon produces c-Ki-ras mRNA, whereas the inclusion of this exon produces a v-Ki-ras (Murine Sarcoma Virus) mRNA (Leff et al. 1986). Thus we see several genes utilize processing as a means to regulate gene expression. Late in adenovirus infection, although most cellular mRNA are transcribed and processed, they fail to be exported to the cytoplasm. This is a case of gene regulation at the level of mRNA export (Babich et al. 1983).

Regulation of mRNA stability is an important step at which eukaryotic gene expression can be controlled. Steady state level of mRNA reflects a balance between rates of transcription, processing and export, and rates of cytoplasmic mRNA degradation. In estrogen stimulated liver cells, vitellogenin mRNA accounts for at least 50% of the total mRNA. In the presence of estrogen the half life of the mRNA is 500 h, but in the absence of the hormone, the half life of the mRNA is only 16 h (Shapiro et al. 1987). In the case of histone mRNA synthesis, the half life of H3 mRNA during S phase is about 4-5 h whereas when DNA synthesis is blocked by cytosine arabinoside the half life is about 15 min. This lowered stability of the mRNA can be reversed in the presence of cycloheximide, a protein synthesis inhibitor. Thus it appears at least in the case of H3 mRNA stability, some factor/s (nucleases) are activated in the absence of DNA synthesis (DeLisle et al. 1983). In 1985 it was shown by Babiss et al. that the adenovirus E1B proteins are required for both accumulation of late viral mRNAs and for effectively reducing transport of cellular mRNA and their translation (Babiss et
The heat shock induced synthesis of HSP70 in chicken reticulocytes is controlled at the level of translation. It was shown that in non heat shocked cells, even though the HSP70 mRNA is associated with polysomes, it is translationally repressed. Heat shock appears to relieve this repression and allows normal translation of the HSP70 mRNA (Theodorakis et al. 1988). Thus it is clear that regulation of gene expression can be complex and may affect several levels of RNA processing.

**Eukaryotic Gene Structure**

Next, I would like to review the structure of a typical eukaryotic gene and compare it to the TS gene. Typically a RNA polymerase II transcriptional unit consists of a promoter, exons, introns and the 3' flanking regions. Each of these components of the transcriptional unit are characterized by DNA sequences. For the purpose of my work I shall briefly review only the sequences that are in the promoter which appear to play a role in regulation of transcriptional activity. The two types of DNA sequence elements that regulate transcription are enhancers and promoters. Enhancers are sequences that can exert their influence from great distances from the gene and in an orientation independent manner on cis-linked promoters. The general function of enhancers is to increase the rate of transcription. Promoters on the other hand are required for accurate and efficient transcription and are usually located immediately upstream of the transcriptional unit (Maniatis et al. 1987).
Structure of Inducible Promoters

Eukaryotic RNA polymerase II differs from the prokaryotic RNA polymerase holoenzyme in that it lacks the inherent ability to recognize promoters in an in vitro reaction. The factors present in the crude nuclear extracts seem to impart promoter specificity to the purified RNA polymerase II. These factors recognize DNA sequences that are either common to most promoters or restricted to promoters of few genes. Many RNA pol II promoters have an element called a TATA box in their promoter, located about 25-30bp upstream from the transcriptional start site. In addition to this there are several upstream elements that play an important role in regulating transcription. Some of these are the CCAAT and GGGCGG or the GC box (Breathnach and Chambon, 1981; Dynan and Tjian, 1985). Other upstream promoter elements are those found in inducible, tissue specific and developmentally regulated genes. Examples of inducible genes are those that respond to heavy metals or stress (heat shock) or growth factors or steroid hormones (Maniatis et al. 1987). To elaborate this point I would briefly like to review the promoter of the human HSP70 gene. This gene is expressed in response to a wide range of physiological stresses. Under non stress conditions, it is also serum regulated. Since this gene is induced by heat shock, heavy metals, cessation of anoxia, inhibitors of energy metabolism, amino acid analogues, infection by adenovirus or simian virus 40, serum and development, its promoter probably contains sequences that could interact with specific factors in response to these stimuli (Wu and Morimoto,
The HSP70 promoter can be divided into two distinct domains. The first domain lies between -107 and -68 nucleotides and the second lies between -68 and -28 nucleotides from the transcriptional start site. The distal domain contains regulatory elements that respond to heat shock and cadmium sulfate and the proximal domain contains the basic promoter elements and serum regulatory element. This gene has a TATA box around -28, a CAAT box at -68, a purine rich stretch GAAGGGAAAG between -58 and -47 that confers serum inducibility and at -100 the heat shock element represented by CTGGAATATTCCCG and the sequence CGCCCGG which is homologous to the core metal responsive element. All these regulatory elements lie within the 112 nucleotides of the transcriptional start site of the HSP70 gene (Wu et al. 1986).

**Structure of TS Promoter**

The complete mouse thymidylate synthase gene is about 12kb long. The 1kb coding region has 6 introns. Though this gene has overall features typical of a eukaryotic gene it also has certain unusual characteristics. The 3' end of the coding region of the TS cDNA is immediately followed by the poly(A) tail. This is the first example of a nuclear-encoded mRNA that lacks a 3' untranslated region.

The TS promoter also has certain interesting features. Transcription of the TS gene occurs at several sites within a 60 nucleotide region. The promoter lacks a TATA element and this may be responsible for the multiple start sites. Other
housekeeping genes like DHFR and HMG CoA reductase also lack a TATA element and have multiple transcriptional start sites (Reynolds et al. 1984; Sazer and Schimke, 1986). It also contains sequences that closely resemble the binding sites of several known transcriptional regulatory factors. The promoter contains potential binding sites for the transcription factors Sp1 (GGGCGG) and a USF (CACGTG) element which was identified in adenovirus and is important for the activity of the major late promoter. Both of these are found 35-50 nucleotides upstream of the most distal transcriptional initiation site. Site directed mutagenesis and deletion analysis of these sites indicated that both Sp1 and USF elements were not essential for promoter activity. Additional deletion analyzes revealed that the region critical for promoter activity lies very close to the first transcriptional start site. The sequence CCGGAAGTTTCCCCA lies in the critical region and in good agreement with the consensus binding site (CNNGAANNTTCNNG) for the heat shock transcription factor (Lindquist, 1986).

**Regulation of a cell cycle gene - Thymidylate Synthase**

Thymidylate synthase (TS) catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate to form thymidylic acid in the final reaction during the de novo synthesis of thymidylic acid (Fig 2). This gene is important because it is a target for many chemotherapeutic drugs. Also it is a housekeeping gene that is regulated in a cell cycle manner. Thymidylate synthase activity is
FIGURE 2.

dUMP  deoxyuridine monophosphate
TS   Thymidylate Synthase
dTMP  deoxythymidylic acid
FdUMP Fluorodeoxyuridine monophosphate
TdR   Thymidine
TK   Thymidine Kinase
DHFA Dihydrofolate
THFA Tetrahydrofolate
meTHFA Methylene tetrahydrofolate
MTX Methotrexate
elevated in rapidly dividing cells such as, regenerating liver (Maley and Maley, 1960), tumor tissues (Rode et al. 1980a) and in exponentially growing cells (Navalgund et al. 1980). Since this enzyme is essential in rapidly dividing cells it is a important target enzyme for chemotherapeutic drugs (Danenberg, 1977; Hartmann and Heidelberger, 1961). TS, along with ribonucleotide reductase, dihydrofolate reductase, DNA polymerase, cytidylate deaminase and thymidine kinase belong to a class of enzymes called S phase enzymes that are directly involved with DNA replication. These enzymes are generally present at much higher levels when cells are actively engaged in DNA replication than when cells are quiescent (Baserga, 1985). Our laboratory has been interested in studying the regulation of these S phase genes, mainly DHFR (Hendrickson et al. 1980), TS (Navalgund et al. 1980; Jenh et al. 1985b), TK (Johnson et al. 1982) and histones (DeLisle et al. 1983).

**Analysis of Cell Cycle Events**

When studying cell cycle regulated events, the method used to obtain synchronized cells is very important. Synchronization of cell populations can be achieved either by inducing or selecting for cells in a particular physiological state. The degree of synchrony is usually monitored by autoradiography. Ideally cells can be arrested at any phase during the cell cycle. However, most commonly used procedures arrest cells in M, G1, G1/S boundary or G0 phase.
Previous work on TS gene expression was conducted on cells that were serum arrested. The cells were grown on media which contained 0.5% serum and this caused them to enter a quiescent state or G0 arrest. During their arrest in G0, there is no net synthesis of protein or RNA and DNA synthesis occurs at a very low rate as compared to the rate in exponentially growing cells. This proved to be an ideal system to study the regulation of relatively stable proteins and RNA because the arrest in G0 allows the decay of stable proteins and RNA to low basal levels. The resting cells can be induced to reenter the cell cycle by the addition of 10% serum and the synthesis of the enzyme can be followed. This method to obtain synchrony is relatively free of artifacts as no drugs are used and there is no removal of essential nutrients. As with all methods, there are some limitations to this method. The cells undergo an unusually long G1 phase, as the cells take some time to recover from the G0 state. Due to this it may well be that regulatory methods governing cell cycle events in growth arrested cells are different from those in normally cycling cells. Some of the other methods to obtain synchronization are by amino acid deprivation (cells are synchronized at G1), by inhibiting DNA synthesis (cells accumulate at S phase) (Prescott, 1976) etc.

The second method to obtain synchronized cells is to select for cells that are in a particular phase. This can be achieved in a number of ways. The two most commonly used methods used are mitotic selection and centrifugal elutriation. The latter utilizes the fact that cells increase in volume as they progress from G1 to G2 and can be fractionated on the basis of size. The former
method is used in the case of cells that form monolayers. In culture, cells that undergo mitosis usually round up and are loosely attached to the surface. In any given population of exponentially growing cells about 5-8% of the cells are undergoing mitosis (Terasima and Tolmach, 1963). The degree of synchrony is high and the events that occur during G1 can be easily monitored. Cells selected in this way have a normal G1 phase. The three major drawbacks to this method are this can be applied only to cells that grow in monolayers, second, synchrony breaks down rapidly and lastly the yields are very low.

**Regulation of TS during Serum Induction**

Previous work from our laboratory has shown that when resting (G0) mouse fibroblasts are serum-stimulated to reenter the cell cycle, the amount of TS increased about 10-fold as the cells traverse S phase (Navalgund et al. 1980). Similar observations have been made with hamster (Conrad and Ruddle, 1972) and human (Ayusawa et al. 1986) cells. This increase was closely coordinated in time with DNA replication and was not blocked by the presence of inhibitors of DNA synthesis (Navalgund et al. 1980). Furthermore these studies indicated that the increase in the rate of synthesis of the enzyme is due to an increase in the transcription rate.

In order to overcome the low level of expression of this gene a drug resistant cell line was developed. A methotrexate resistant cell line M50L3 was exposed to increasing concentrations of the drug fluorodeoxyuridine, a substrate
analog for TS. An FdUrd-resistant cell line (LU3-7) was isolated that overproduced TS (Rossana et al. 1982). This cell line achieved resistance to the drug by amplification of the TS gene and therefore overproduction of the enzyme. The overproduced enzyme appeared the same as the normal enzyme and represented about 0.5% of the total soluble protein in these cells. TS activity was regulated in the same manner as in the parental cell line. This amplified cell line made it possible to clone the TS cDNA (Geyer and Johnson, 1984) and then the mouse thymidylate synthase gene (Deng et al. 1986).

The TS cDNA was used to determine the degree of gene amplification achieved by this drug resistant cell line. LU3-7 had achieved a 50 fold amplification of the TS gene as determined by Southern blots (Jenh et al. 1985c). Further it was shown that both TS mRNA and TS enzyme were overproduced by the same factor (Geyer and Johnson, 1984). Also TS gene expression appeared to be regulated in the same manner as in the parental cell line (Jenh et al. 1985a).

Further work on the regulation of transcription were done by Chung-Her Jenh of our lab. His studies showed that the increase in the mRNA content as the cells progressed from G1 to S phase was about 20-40 fold. Pulse labeling studies showed that 85% of the poly(A)+ TS mRNA was associated with polysomes at all times and the increase in TS poly(A)+ mRNA was the result of an eight fold increase in the rate of production of this species. He also showed that the mRNA was relatively stable and the half life of the message was similar in resting (9h) and growing (7h) cells. The rate of transcription of this gene went up by a factor of only 3-4 as the cells went from G1 to S phase. This increase in
the rate of transcription could not account for the 20-40 fold increase in the mRNA content during S phase therefore strongly suggesting that both transcriptional and posttranscriptional control mechanisms were regulating the expression of TS (Jenh et al. 1985b).

**Cell cycle versus Growth Induced Regulation**

Several investigators have compared changes in gene expression that are observed in growth stimulated cells with those occurring during the normal cell cycle, some examples include DHFR (Hendrickson et al. 1980; Farnham and Schimke, 1985b), histones (DeLisle et al. 1983), and TK (Thompson et al. 1985; Johnson et al. 1982). In these cases the regulation was similar. However it is also clear that some changes in gene expression that are observed in serum stimulated cells are the result of growth induction rather than normal cell cycle regulation. In the case of c-myc, a protooncogene, the mRNA content increased about 20 fold in response to growth stimulation (Kelly et al. 1983). However when the c-myc RNA was followed in cells that were synchronized by centrifugal elutriation, the content remained invariant throughout the cell cycle (Thompson et al. 1985). Recently it was shown that the expression of the small sub-unit p49 mRNA of primase, the enzyme that synthesizes oligoribonucleotides for initiation of DNA replication, increased in response to serum stimulation but is invariant during the cell cycle (Tseng et al. 1989). In addition Imam et al. reported that in mouse L1210 lymphoma cells, TS mRNA content is invariant in the cell cycle (Imam et
al. 1987). Thus at least in L1210 cell line, TS appears to be expressed constitutively. Our lab had shown that in the cell line LU3-7, during growth stimulation, TS enzyme and mRNA content increased 20-40 fold during G1/S phase transition. I wanted to know if the expression of TS was regulated in a cell-cycle manner in cells that were synchronized by mitotic selection. Therefore the first part of my dissertation deals with the regulation of TS in a normal cell cycle.

Effect of Protein Synthesis Inhibitors on Gene Expression

The next part of my dissertation deals with the mechanisms that may be involved in the accumulation of TS mRNA during the G1/S phase transition. As mentioned earlier, the rate of transcription during the serum induced transition from resting to growing state increases only about 3-4 fold. This increase does not concur with the 20-40 increase in the content of cytoplasmic TS mRNA during the same period. This disparity between the rate of transcription and the content of cytoplasmic TS mRNA can be due to the following reasons. First, during the G1/S phase there may be increased efficiency in the processing, including polyadenylation and splicing. Second, there may be an increased export of the mature mRNA from the nucleus and cytoplasm during the G1/S phase transition. Lastly stability of the message may be higher in the S phase than in G1, thus allowing substantial increase in the content of TS during S phase. The last possibility is not likely to account for the increase of TS mRNA in S phase because TS mRNA is more stable in resting cells (half life is 9.6 h) as compared
to growing cells (half life is 7.3 h).

To investigate which of these mechanisms may be responsible for the accumulation of TS mRNA during S phase I did the following. Since all post transcriptional events involve trans acting factors namely proteins, I wanted to see, if inhibiting protein synthesis during the cell cycle would affect the accumulation of TS mRNA content. The reason for studying the effect of protein synthesis inhibitors on the accumulation on TS mRNA during S phase stems from similar studies done the another S phase gene, TK. In 1982, Campisi et al. suggested that the major regulatory events that lead to cell proliferation seemed to occur during G1 phase of the cell cycle. They proposed that a single event controlled the commitment of cells to complete the cell-cycle. The event was termed as the "R" point (or the Restriction Point) and occurred 2-3 h prior to DNA synthesis. They showed that low doses of cycloheximide drastically reduced the rate at which normal 3T3 mouse fibroblasts in early G1 could enter S phase. They further characterized the biochemical nature of this R point and concluded that a labile protein or R protein that is sensitive to environmental conditions, needs to accumulate to a critical amount before the cells can proceed to S phase (Campisi et al. 1982). Further Coppock and Pardee showed that the appearance of TK mRNA during S phase was dependent on active protein synthesis preceding the S phase (Coppock and Pardee, 1987). These results were consistent with a requirement for a labile protein that is necessary for entry into S phase.

The question that I addressed was, is the R protein (or protein synthesis in general during G1) needed only for the appearance of TK during S phase or does
it affect other S phase genes like TS and DHFR too? If it does, then what steps in the biogenesis of TS mRNA are sensitive to protein synthesis inhibition?

**Attenuation and Gene Expression**

The other possible mechanism that may be responsible for the increase in the content of TS mRNA in S phase is attenuation. Attenuation or pausing has been reported in Adenovirus type 2, Simian virus-40 and in several protooncogenes (Maderious and Kiang-Chung, 1984; Coppola et al. 1983; Hay and Aloni, 1984). The protooncogenes where these elongation blocks have been identified are c-myc, c-fos and c-myb (Nepveu and Marcu, 1986; Bender et al. 1987; Fort et al. 1987). In all these studies premature termination or attenuation was identified on the basis of nuclear runoff assays. In analyzing the rate of transcription of the TS gene, Jenh et al. showed that there is a 3-4 fold increase in the rate as the cells traverse from G1 to S phase in serum-induced cells (Jenh et al. 1985b). The hypothesis that I wanted to test was based on the following. It was possible that initiation of transcription occurred at all times during the cell cycle but elongation occurred preferentially at G1/S phase boundary. This hypothesis could not have been tested by previous studies where the rates of transcription were being analyzed. In these studies labeled nuclear RNAs were hybridized to excess double stranded, denatured plasmid immobilized onto nitrocellulose membrane. The fact that a double stranded plasmid corresponding to the TS cDNA was used in the DNA excess studies indicates that these
experiments are limited in that they only measure overall rate of transcription of a gene. These studies will not indicate whether there is some sort of stalling or pausing of the polymerase.

To be able to distinguish between rate of transcriptional elongation over the whole gene and between different sections of the gene, I had used the following strategy. First I decided to carry out the nuclear run on assays in the presence of $^{32}$P UTP, so that I could both visualize and quantitate the amount of hybridizable counts. Second, by using single strand RNA corresponding to different sections of the gene, one can directly measure the rate of elongation over any one section of the gene. By this procedure I would be able to test my hypothesis and also be able to check for opposite strand transcription. Prior to my work, the question whether opposite strand transcription existed, had been addressed but the probes used were double stranded DNA probes that were from the 5' flanking region of the TS promoter. The main problem with using double strand DNA probes is decreased sensitivity and the inability to detect strand specific transcription. Thus I would like to present some of the work that I did which examined attenuation as a possible means of regulating TS gene expression.

Effect of Heat Shock on Gene Expression

Almost all organisms respond to elevated temperatures by synthesizing a small number of highly conserved proteins called the heat-shock proteins or hsp57.
This response is universal. The induction of hsps is very rapid but maximum induction temperature varies from one species to another depending on the normal range of environmental exposure. This highly conserved response - the activation of a small set of genes, and the repression of other normally active genes is in general a reflection of both selective transcription and translation of the heat shock gene (Lindquist, 1986). This phenomenon has been studied extensively in Drosophila melanogaster (Ashburner and Bonner, 1979). This response to heat shock can also be brought about by other physiological and chemically induced stress conditions. In the case of Drosophila the molting hormone ecdysone induces the expression of hsp26, hsp28 and hsp83 (Ireland and Berger, 1982). The human HSP70 gene is induced in response to heavy metals and serum (Wu and Morimoto, 1985). The exact role of these heat shock proteins is not known but it is a good model system to study eukaryotic gene regulation.

In higher eukaryotes, studies relating to heat shock response have been done extensively in human cells in culture. Most of our present knowledge about heat shock response comes from work done the human HSP70 gene. This gene codes for one of the most conserved of the heat shock proteins. The human protein is 73% identical to the Drosophila protein and 50% identical to the E.coli dnaK product (Lindquist, 1986). A common mechanism seems to be controlling all eukaryotic heat-shock genes. When Pelham analyzed deletion mutants of Drosophila hsp70 in COS cells (these are monkey cells containing a defective integrated SV40 genome and produce T antigen constitutively and thus support high-level replication of the input DNA), he found that the upstream regulatory
element of this gene is analogous to that of other heat shock genes (Pelham, 1982). This upstream regulatory element also known as heat shock element or HSE is also found in the human HSP70 gene. It is located 100 nucleotides upstream of the hsp70 gene and shares identity in 12/14 positions with the consensus sequence of the Drosophila heat shock element CTNGAATNTTTCNCG. Deletion of this sequence eliminates responsiveness to heat shock. In human cells a single HSE seems to enough to confer heat shock response as opposed to in Drosophila, where multiple HSEs are found closely linked upstream of heat shock genes (Wu et al. 1986). A heat shock transcription factor (HSTF) that binds to the HSE in vivo and in vitro has been isolated and characterized (Wu et al. 1987). In HeLa cells the HSF (heat shock factor) is present in control and heat-shocked cells and binds to the HSE to form a stable complex. Mobility shift assays revealed that the mobility of complexes from control and heat shocked cells were distinct and the dissociation rates of the complexes under the two conditions were different (Mosser et al. 1988).

When deletion analysis of the TS promoter was performed, the region between -105 and -85 appeared to be critical for the expression of this gene. This region appears to contain an essential promoter element. Sequence analysis of this region revealed a sequence CCGGAAGT TTCCCA which is in good agreement (7/8 match) with the consensus binding site of the heat shock transcription factor CNNGAANNTTCCNG (Deng et al. 1989b; Lindquist, 1986). The presence of a HSE in the critical region of the TS promoter was very puzzling. This led me to ask the following questions: Does the presence of a
single HSE confer heat inducibility on TS gene expression? How does the presence of a HSE affect TS gene expression?

To answer these questions I decided to study the effect of heat shock on the rate of transcription, the export of TS mRNA and the content of TS mRNA. Work is now in progress to identify the factors that interact with this region of the TS promoter as well as the role they play in the regulation of TS transcription.
MATERIALS AND METHODS

Cell Culture

LU3-7 cells are a FdUrd-resistant derivative of 3T6 fibroblasts, that were derived by serial passage in increasing concentrations of fluorodeoxyuridine (Rossana et al. 1982). Stock cultures were maintained on plastic petri dishes in the Dulbecco-Vogt modification of Eagle's medium (Gibco) supplemented with 10% NuSerum (Collaborative Research) or 10% calf serum (Colorado Serum Co.) 3 μM FdUrd, 1 mM uridine and 1 mM cytidine. The doubling time of exponentially growing LU3-7 cells is approximately 20 h (Rossana et al. 1982). M50L3 is a methotrexate resistant cell line of 3T6 that overproduces dihydrofolate reductase 300 fold. These were grown in medium containing 50 μM methotrexate (Wiedemann and Johnson, 1979). The 3T6 cell line was maintained in 10% calf serum.

Resting cells were prepared by seeding 7x10^4 cells/cm^2 in medium containing 0.5% calf serum. The medium was replaced on the second and fourth days, and the cells were used for the experiment on the seventh day following seeding (Abelson et al. 1974). Resting cells were stimulated to enter the cell cycle by feeding them with fresh medium containing 10% calf serum.
The procedure for the isolation of mitotic cells was similar to that described previously (Farnham and Schimke, 1985b). Approximately 2x10^6 cells were plated onto 150 cm^2 flasks in medium that lacked FdUrd. Twelve to fourteen hours after plating the flasks were preshaken to remove any dead or loosely adhering cells. 16 h later, the medium was removed and 10 ml of prewarmed medium was added to one flask which was tapped vigorously 10 times on each side. The medium was then transferred to the next flask and the process was repeated until the mitotic cells had been collected from all of the flasks. The cells were collected by centrifugation and counted. Yields of mitotic cells were typically about 2x10^5 mitotic cells per 150cm^2 flask that contained approximately 5x10^6 cells at the time of harvest.

To enrich for a population of cells that readily detach during mitosis a protocol similar to that described by Farnham and Schimke was used (Farnham and Schimke, 1985b). LU3-7 cells were grown in a 75 cm^2 flask to approximately 50% confluency. The medium was then removed, 10 ml of fresh prewarmed medium was added and the flask was tapped 6 times on each side. The dislodged cells were removed and plated on a 100 mm plastic dish. After 1 h, the medium was removed and fresh prewarmed medium was added. This ensured the removal of dead cells and cells that did not readily attach to the dish. The cultures were allowed to grow to confluency, split 1:10 and the selection process repeated. After several cycles of selection, the cells were plated at low density to permit the isolation of individual clones. Several of these were isolated using cloning cylinders and expanded for subsequent analyses.
The cells used in the heat shock experiments were heat shocked at $430^\circ C$ for the prescribed length of time depending on the experiment. The cells were grown in T-flasks and were sealed with Parafilm and placed in a $43^0 C$ water bath.

**Rate of DNA Synthesis**

To determine the rate of DNA synthesis, mitotically selected cells were plated on 35 mm dishes at a density of $2 \times 10^4$ cells/dish. At various times after plating they were exposed for 30 or 60 min to $5 \mu$Ci of $[^3 H]$ thymidine (ICN, 66Ci/mmol). The cells were then washed with ice cold phosphate buffered saline (0.14 M NaCl, 3 mM KCl, 8 mM Na$_2$HPO$_4$, pH 7.4) several times and dissolved in 0.1 M NaOH. The lysed cells were transferred from the plate with a pasteur pipette to a siliconized tube and neutralized with 0.1 ml of 1 M HCl. Nucleic acids and proteins were precipitated by adding 1 ml of ice cold 10% trichloroacetic acid and acid precipitable material was collected on glass fiber filters, which were dried and counted in a toluene-based scintillation fluid containing 10% Protosol (NEN).

**$^3H$ FdUMP Binding Assay**

Cells were grown in medium lacking FdUrd (to avoid inactivation of the enzyme). Cells were synchronized by mitotic selection and seeded at a density of $10^5$ cells/60 mm dish. Cells were harvested by washing the monolayer several times with ice cold 1X PBS. All subsequent steps were performed at 0-4$^0$ C. Cultures were harvested at various times in 600 $\mu$l of 100 mM Tris-HCl (pH 7.3)
containing 0.5% NP-40. The cell suspension was vortexed for 30 sec to complete the lysis and nuclei and cell debris were removed by centrifugation and TS enzyme level in the supernatant was determined using the sensitive $^3$H FdUMP binding assay, which measures the formation of the ternary complex between TS, FdUMP and 5,10-methylene-tetrahydrofolate (Rossana et al. 1982; Lockshin et al. 1979).

The complex was formed using a modification of the procedure of Lockshin et al. (Lockshin et al. 1979). $N^5, N^{10}$-methylene tetrahydrofolate was prepared by dissolving 4 mmoles of tetrahydrofolate (Sigma) in 0.5 ml of 0.26 M formaldehyde, 90 mM dithiothreitol and 50 mM Tris-HCl pH 7.3, and incubating the mixture at 37°C for 5 min. The complex was formed by incubating the cell extract with 235 μM $^3$H-FdUMP, 706 μM $N^5, N^{10}$-methylene tetrahydrofolate, 0.5 mg/ml bovine serum albumin, 50 mM Tris-HCl pH 7.3 in a final volume of 255 μl at 37°C for 20 min. A small sample was removed and assayed for TCA precipitable counts.

**Percentage of Labeled Nuclei**

The percentage of labeled nuclei was determined by exposing cultures growing on 35mm dishes to 2 μCi of $^3$H-thymidine (ICN 77.2Ci/m mole) for 1 h in the warm room. After labeling, the plates were rinsed five times with ice cold 1X PBS and drained well. The plates were then gently exposed for about 1 min to a few ml of ice cold 1X PBS containing 0.5% NP-40 to remove the cytoplasmic compartment and make the nuclei visible. The cells were fixed by adding a few
ml of ice cold, freshly prepared 10% formalin in 1X PBS. After 30 min at room temperature, the plates were rinsed under a gentle stream of tap water or were allowed to soak in a beaker of water for 30 min. The plates were the dried and coated with 40% Kodak NTB-2 emulsion : 60% water.

After 5 days of exposure, the plates were developed (16 g of Kodak D-19 dissolved in 100 ml of distilled water) for 2 min. They were rinsed with distilled water, drained and fixed for 2 min with the Kodak fixer. They were rinsed well with water, drained and stained with 1 ml of hematoxylin (10 ml hematoxylin stain + 0.4 ml of glacial acetic acid, filtered through nitrocellulose twice before use). for 15-30 min. Then the plates were rinsed with water and dried. To determine the percent labeled nuclei, at least 400 nuclei were counted at random with a Zeiss microscope at 400X with an oil immersion lens.

**Large Scale Isolation of Plasmid DNA**

Plasmid preparation was essentially carried out as per Maniatis et al. (Maniatis et al. 1982) with a few minor modifications. All plasmids that derived from pUC vectors or pIBI vectors were not amplified with chloramphenicol but were grown in TB medium overnight (16-18hrs). The plasmid preparations were purified twice on a CsCl gradients.
**High Molecular Weight DNA Isolation**

High molecular weight DNA was prepared as described previously (Jenh et al. 1985b). All steps were performed at 0-4°C. Cells were isolated from exponentially growing cultures in 100 mm dishes. The cells were lysed in RSB + 0.5% NP-40 and nuclei recovered by centrifugation. The nuclei were then resuspended in STE containing 0.1 mg/ml heat treated RNase A. SDS was added to a final concentration of 0.2%. At this point the solution becomes very viscous and care was taken to avoid shearing the DNA. This was incubated at 37°C for 1-2 h. SDS and self digested Pronase (Pronase incubated at 37°C) were added to a final concentration of 0.5% and 500 µg/ml respectively. The DNA solution was incubated overnight at 37°C. Then, to ensure complete removal of proteins, phenol-chloroform extraction was performed. The aqueous phase was then dialyzed against four changes of STE (0.25 M NaCl, 10 mM Tris-HCl pH7.4, and 1 mM EDTA).

**Subcloning of TS cDNA**

The pIBI vectors are derived from pEMBL plasmids and can be used to obtain either single or double stranded DNA (Dente, 1983). In addition they also have T7 and T3 RNA promoters on either side of the multiple cloning sites. Different sections of the cDNA pMTS-3 were subcloned into pIBI30 vectors. pIBI30(687) and pIBI31(687) contained the middle 687bp PstI fragment. pIBI30(270) contained the 270 bp PstI-PstI fragment from the 3' end of the
cDNA. pIBI30(EB) contained exon 1 and exon 2 of the TS gene. These plasmids were constructed for transcriptional elongation studies. pIBI30 and pIBI31 were obtained from IBI (International Biotechnologies Inc.) Ligation conditions were standard and the ligated DNA was transformed into JM103 or HB101 competent cells (Norgard et al. 1978).

Random Primer Labeling

Random primer labeling of DNA was done according to Feinberg & Vogelstein (Feinberg and Vogelstein, 1983). First, 100 ng of linearized DNA was taken in an eppendorf tube and the volume made up to 20 μl with water. This was denatured at 100°C for 15 min and quickly cooled on ice. The reaction mixture contained the following: 10 μl of 5X OLB [ Solution O: 1.25 M Tris-HCl, 0.125 M MgCl₂ pH 8.0 ; Solution A: 1ml Solution O + 18 μl of 2-mercaptoethanol + 5 μl of dATP, 5 μl dTTP and 5 μl dGTP, each triphosphate previously dissolved in TE (3 mM Tris-HCl, 0.2 mM EDTA) at pH 7 at a concentration of 0.1 M, stored at -20°C. Solution B: 2 M HEPES pH 6.6 stored at 4°C. Solution C: hexadeoxyribonucleotides (Calf Thymus, Pharmacia 9000 units/ml) evenly resuspended in TE, stored at -20°C. Solution A:B:C: were mixed in a ratio 100:250:150 to make 5X OLB, Stored at -20°C, 5-10 μl (50-100 μCi) of α-³²P-dCTP (3000-4000Ci/mmole), 2 μl BSA (10mg/ml), 2 units of Klenow fragment of E.coli DNA Polymerase I (USB) and water to make up the volume to 50 μl. This was incubated at 25-30°C for 2.5-3.0 h. The reaction was then stopped by the addition of the Stop Solution (20 mM NaCl, 20 mM Tris-Cl
pH 7.5, 2 mM EDTA 0.25% SDS, 1 μm dCTP.) Free unincorporated radionucleotides were removed by using a G-50 spin column. The specific activity of the probe was about $10^9$ cpm / μg DNA.

**Uniformly Labeled RNA Probe**

This was done as per the protocol given by Promega (Melton et al. 1984). The plasmid used was linearized downstream of the appropriate promoter and subjected to phenol-chloroform treatment. To generate a high specific activity probe, the following were added to a final volume of 20 μl. 4 μl of 5X transcription buffer, 2 μl of 100 mM DTT, 0.8 μl of RNasin (25 U/μl), 4 μl of 2.5 mM each of ATP, GTP and CTP, 2.4 μl of 100 μm CTP, 1.0 μl of linearized plasmid (0.2-1.0 μg), 5 μl of α-$^{32}$P CTP and 0.5-1.0 μl of T7 or T3 RNA polymerase (5-15U). This mixture was incubated at $37^0$ C for 60 mins. Following RNA synthesis, RQ1 DNase was added to the concentration of 1U/μg. This was then subjected to phenol chloroform extractions and RNA was recovered by two precipitations using 1/10 vol of 3 M NaOAc and 2.5 vol of ethanol. The RNA was analyzed on a denaturing polyacrylamide gel system to determine the size and quality of the probe.

**Southern Blot Analysis**

This was essentially done using the standard Southern transfer procedure (Southern, 1975). Agarose gels were run, stained and photographed. DNA standards were marked with pin pricks containing India Ink. The gel was then
soaked in 1.5 M NaCl and 0.5 M NaOH to denature the DNA. After 30 min the gel was rinsed several times with distilled water and placed in 2 M NaCl and 1 M Tris-Cl pH 5.5 for another 30 min. Then, the neutralized gel was inverted onto the transfer apparatus. The transfer buffer was 10X SSC. (1X SSC:- 0.15 M NaCl, 0.015 M Na Citrate, pH 7.3). All air bubbles were removed to allow for proper transfer. The membrane used for transfer was either nitrocellulose or Nytran (Schleicher and Schuell). Care was taken to mark both the orientation of the gel and the standards. After transferring for 2-16 h, the membrane was air dried for 15-20 min, then baked in a vacuum oven at 80°C for 2 h.

**Hybridization**

The filter was used for hybridization either immediately or after storage at -20°C. It was hybridized for 16 h at 65°C in a deaerated solution of 2X SSC, 1% SDS, 5X Denhardt's solution (Denhardt, 1966) (1X Denhardt's solution: 0.02% BSA, 0.02% Ficoll type 400, and 0.02% polyvinylpyrollidone ), 300 µg/ml of denatured, sonicated salmon sperm DNA and 500 µg/ml of poly (A). The prehybridization solution was replaced by the hybridization solution, which is essentially the same except that it contained 10% Dextran Sulphate (Wahl et al. 1979) and 10 x10⁶ cpm of denatured ³²P-probe. Hybridization was carried out at the same temperature for 16-24 h. The filter was then washed in two changes of 2X SSC at 65°C and two changes of 1X SSC. The extent of washing depended upon the level of background radioactivity detected by the Geiger Counter. After the washes the filter was wrapped in Saran Wrap and exposed to X-ray film with
an intensifying screen at \(-70^0\text{C}\).

**Isolation of RNA**

All solutions were made with double distilled water that was treated with 0.1% DEPC (diethyl pyrocarbonate) and autoclaved and all glassware was baked prior to use. Monolayers were rinsed three times with 1X PBS. All subsequent steps were performed at 4\(^0\text{C}\). 2 ml of 1X PBS was added and the cells were scraped with a rubber policeman and the cells transferred to a 15 ml plastic conical tube (Sarstedt) and centrifuged at 2000 rpm for 2 min. This step allows the separation of the cytoplasmic and nuclear compartments. To isolate cytoplasmic RNA the following is done. The supernatant was removed and 2 ml of RSB (0.01 M Tris-Cl, pH 8, 0.01 M NaCl, 0.003 M MgCl\(_2\) containing 0.5% NP40 (Sigma) was added to the cell pellet. The cells were incubated on ice for 5 min, vortexed for 30 sec and then spun at 2000 rpm for 2 min. 2 ml of the supernatant was transferred to another plastic conical tube and 0.5 ml of 5X buffer (0.5 M NaCl, 0.05 M EDTA pH 8, 2.5% SDS) was added. Also in some cases 20 mM of vanadyl ribonucleosides (BRL) was added. The suspension was subjected to proteinase K digestion for 0.5-2 h at 37\(^0\text{C}\), then extracted three times with phenol-chloroform and twice with chloroform. The aqueous phase was transferred to a fresh Corex tube and two volumes of ethanol were added to it and the sample was stored at \(-20^0\text{C}\) for at least 2 h. The RNA pellet was then resuspended in the buffer of choice depending on what it was used for.
Nuclear RNA was isolated in the one of the following ways. After the cells were pelleted and lysed in the presence of RSB + 0.5% NP40 the nuclear pellet was resuspended in RSB and 0.3 ml of Tween DOC was added. This was then vortexed and spun down. The nuclei were processed as indicated below.

**Isolation of total RNA or nuclear RNA by the Guanidinium/Cesium Chloride Centrifugation Method.** (Maniatis et al. 1982)

The cells were scraped in 1X PBS and pelleted. Then 2 ml of Guanidinium Lysis Solution was added to the cell pellet (4 M guanidinium isothiocynate containing 0.1 M Tris-Cl pH 7.5, and 1% 2-mercaptoethanol). To disperse the cells and to ensure thorough shearing of the nuclear DNA the solution was passed several times through a syringe. Then 0.4 g of CsCl was added for every ml of the homogenate. The homogenate was layered onto a 1.1 ml cushion of 5.7 M CsCl in 0.1 M EDTA (pH 7.5) in a Beckman SW60.1 polyallomer thick walled tube. This was centrifuged at 41,000 rpm for 12 h at 20°C. The supernatant solution was carefully aspirated and the walls of the tube dried. The pellet was resuspended in SET buffer (10 mM Tris-Cl, pH 7.4, 5 mM EDTA, 0.1% SDS). The RNA was precipitated by the addition of 0.1 volume of 3M sodium acetate and 2.5 volumes of ethanol.

**Isolation of total RNA and nuclear RNA by Guanidinium Isothiocyanate-Phenol-Chloroform Method.** (Chomczynski and Sacchi, 1987)
This is a rapid procedure that combines guanidinium isothiocyanate and phenol-chloroform extraction method. This method provides a pure preparation of RNA and is relatively quick. 1 ml of solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol) was added to the cell pellet. Sequentially the following were added and after each addition the solution was thoroughly mixed by inversion; 0.1 ml of 2 M sodium acetate (pH 4), 1 ml of phenol (water saturated) and 0.2 ml of chloroform-isoamyl alcohol 49:1. The final solution was mixed vigorously for 10 sec and cooled on ice for 10 min. The samples were then centrifuged at 12000 rpm at 4°C for 20 min. RNA is present in the aqueous phase whereas DNA and proteins are present in the interphase and the phenol phase. The aqueous phase was then transferred to a fresh tube and mixed with equal volume of isopropyl alcohol and placed at -20°C for 1 h. RNA was then pelleted by centrifuging at 12000 rpm for 20 min in the Sorvall centifuge. The pellet was then resuspended in 0.3 ml of solution D and an equal volume of isopropyl alcohol in an Eppendorf tube and placed at -20°C for 1 h. After centrifugation at 12000 rpm for 20 min the pellet was dried and then resuspended in the appropriate buffer.

Oligo(dT) Cellulose Chromatography

Poly (A)+ RNA was isolated by passing the total RNA over an oligo (dT) cellulose column (Aviv and Leder, 1972). An Econo column was packed with about 0.2 ml of oligo (dT) cellulose in elution buffer (0.01 M Tris-Cl pH 7.4, 0.05% SDS). The cellulose was rinsed several times with about 10 ml of Elution
Buffer at 65°C and then with 5 ml of Binding Buffer (0.4 M NaCl, 0.01 M Tris-Cl pH 7.4, 0.5% SDS) at room temperature. The RNA sample was dissolved in 1 ml Binding Buffer and passed through the column. This was repeated at least twice to ensure complete binding of all the poly(A)+ RNA. The column was then washed with 5-10 volumes of Binding Buffer (0.01 M Tris-Cl at pH 7.4, 0.5 M NaCl, 1 mM EDTA, 0.5% SDS) and 5 volumes of the Wash Buffer (0.01 M Tris-Cl at pH 7.4, 0.1 M NaCl, 1 mM EDTA). The bound RNA was eluted with 2-3 ml of Elution Buffer at 65°C. The eluted RNA was precipitated with 2.5 vol of ethanol in the presence of 1/10 volume of 3 M sodium acetate and 200 μg of yeast tRNA.

**3H Poly (U) Binding Assay**

This was done according to Bishop et al. (Bishop et al. 1974). 20 μl of RNA was added to a tube containing 1 ml of 2X SSC containing 20,000 cpm of 3H- poly (U) (Amersham, 20-72 Ci/mmol). This was incubated at 45°C for 10 min. The mixture was cooled for 10 min. Then 50 μl of pancreatic RNase (2mg/ml) was added and the mixture was incubated for 20 min at 0°C. 20 μl of BSA (1mg/ml) was added and mixed well. To this, 1 ml of 10% TCA was added and the tube was incubated at 4°C for 1 h. The precipitates were collected on glass fiber filters, dried and counted in a toluene based scintillation fluid.
Northern Blot Analysis

Northern blot analysis was conducted as per Lehrach et al. & Thomas, (Lehrach et al. 1977; Thomas, 1980) as modified by Schleicher & Schuell, Inc. 1-10 μg of RNA was pelleted in an Eppendorf tube and dried. The pellet was resuspended in 20 μl of the following, 50% formamide (freshly deionized or special grade, BRL), 2.2 M formaldehyde, 20 mM MOPS (morpholino-propanesulfonic acid, pH 7), 5 mM sodium acetate and 0.5 mM EDTA and denatured for 15 min at 55°C. To this, 5 μl of loading buffer was added (50% glycerol, 1 mM EDTA (pH 8), 0.4% bromophenol blue and 0.4% xylene cyanol) and the samples were electrophoresed for 3-3.5 hrs at 100 V. The gel system used was 1.5% agarose (BRL) in 20 mM MOPS, 2.2 M formaldehyde, 10 mM sodium acetate and 1 mM EDTA. For markers either SP6/T7 generated antisense RNA from the cDNA or standard markers from BRL were used.

After the run the gel was placed over the transfer apparatus and transfer was done essentially as per the instructions of Schleicher and Schuell Inc. Membrane used was Nytran or nitrocellulose. Transfer was complete in 12-16 h and the filters were baked at 80°C for either 1 h or 2 h depending on whether the membrane was either Nytran or nitrocellulose. Prehybridization and hybridization was done essentially as per Thomas, (1980). The filters were then wrapped up in Saran Wrap and exposed to film with an intensifying screen and kept at -70°C.
Transcription in Isolated Nuclei

Isolation of Nuclei:

Monolayers were washed several times with ice cold 1X PBS. The cells were scraped into a 15 ml conical plastic tube and spun for 3 min at 2000 rpm. The cell pellet was resuspended in RSB + 0.5% NP40 and incubated on ice for 5 min. The suspension was vortexed for 30 sec to ensure complete lysis. The nuclei were then suspended very gently in 1 ml of cold nuclear storage buffer( 40% glycerol, 50 mM Tris-Cl, pH7.5, 5 mM MgCl₂, 0.1 mM EDTA) and spun for 2 min at 2000 rpm. The pellet was resuspended in 50 μl of nuclear storage buffer and either used immediately or frozen in liquid nitrogen and stored at -70°C. Intactness of nuclei was monitored by visualizing each preparation under the microscope.

Nuclear Run On Transcription: (Groudine and Casimir, 1984)

To 40 μl of the nuclei in the storage buffer the following were added: 20 μl of 5X nuclear run on transcription buffer (0.5 M Tris-Cl, pH7.8, 0.25 M NaCl, 1.75 M ammonium sulfate, 10 mM EDTA ), 20 mM Mn₂Cl₂·H₂O, 5 μl Heparin (20mg/ml), 5 μl mixed rNTPs, (20 mM each), 100-200 μCi (10-20 μl) of α³²P UTP 800 Ci/mmol (NEN, Dupont), and DEPC treated water to make up the volume to 100 μl. The transcription mix was then incubated at 32°C for 45 min. After the incubation, 10 μg of yeast tRNA and 350 μl of 20 mM Tris-Cl pH 7.5, 10 mM CaCl₂ were added. To remove the nuclear DNA, 25 μl of RNase free
DNasel was added and incubated at least 30 min at $37^\circ C$ with occasional gentle agitation. To terminate the reaction, 50 $\mu l$ each of 0.2 M EDTA and 10% SDS were added and phenol-chloroform extraction was performed. The aqueous phase was then subjected to two ammonium acetate/ethanol precipitations to ensure complete removal of all free unincorporated nucleotides. The pellet was resuspended in 100 $\mu l$ of DEPC water, 1 $\mu l$ was counted and $10^7$ cpm /ml was used in the hybridizations which were performed as per Thomas et al. (Thomas, 1980).

**Filter Preparation:**

The plasmid DNA was linearized with an appropriate restriction enzyme. 0.1 vol of 1 M NaOH was added to this and the DNA was heated to $100^\circ C$ for 2 min. 5 M NaCl was added to a final concentration was 2 M NaCl. 5-10 $\mu g$ of DNA was blotted onto Nytran using a Schleicher & Schuell Dot Blot Apparatus. The filters were air dried for 2 h and baked at $80^\circ C$ for 1 h. RNA filters were made by heating the RNA to $65^\circ C$ for 15 min and then adding 3 volumes 2XSSPE, and then blotting to Nytran as above.

**In Vitro Run Off Transcription:**

This was done essentially as per the protocol given by Promega (Melton et al. 1984). The plasmids were pIBI vectors that contained T3 and T7 promoters. The plasmid was linearized with a suitable restriction enzyme and then subjected
to phenol-chloroform extraction before being used in the transcription reaction. Briefly the following were added to an Eppendorf tube in order at room temperature. 20 μl of 5X transcription buffer (200 mM Tris-Cl, pH 7.5, 30 mM MgCl₂, 10 mM Spermidine, 50 mM NaCl), 10 μl of 100 mM DTT, 4 μl of RNasin (Ribonuclease inhibitor 25 U/μl), 20 μl rNTPs (2.5 mM each of ATP, CTP, GTP, and UTP), 2.0 μl of linearized template in water, (2-5 μg), 10-50 units of T3 RNA polymerase or T7 RNA polymerase and DEPC water to make up the volume to 100 μl. The reaction mix was incubated at 37°C for 60-120 min. Following the RNA synthesis the template DNA was removed by the addition of RQ1 DNase to the concentration of 1 U/μg of template DNA. After a 15 min incubation at 37°C the mix was subjected to phenol-chloroform extraction and the RNA in the aqueous phase was recovered by sodium acetate-ethanol precipitations. Usually 5-10 μg of RNA /μg of template was obtained. To make sure the transcript was a full length representative of the template, the RNA was run on a denaturing acrylamide mini-gel, stained with ethidium bromide and photographed.

Prehybridization and hybridization were done at 42°C in the presence of 50% formamide. The nuclear RNA was denatured by the addition of 0.25 M of NaOH, kept on ice for 10 min and then neutralized by the addition of 0.5 M of HEPES prior to addition to the hybridization solution (Nevins, 1987).
DNA Excess Filter Hybridization

5 μg of plasmid DNA was denatured in alkali and immobilized on a 13 mm nitrocellulose filter (Schleicher & Schuell, BA85) as described (Hendrickson et al. 1980). Blank filters without any DNA were used as controls. Prehybridization of filters containing different plasmids were done separately at 65°C for at least 6 h. Prehybridization solution contained 2X SSC, 1% SDS, 5X Denhardt's solution and 300 μg/ml of denatured salmon sperm DNA. Labeled RNA was dissolved in 400 μl of 2X SSC containing 0.5 mg/ml poly (A). 395 μl was used for hybridization and 5 μl was used to determine the total counts used for hybridization. The nitrocellulose filters were placed in a one dram siliconized vial, the hybridization fluid was added to this and this in turn was overlayed with 400 μl of mineral oil to prevent evaporation. The vials were capped and submerged in a 65°C water bath for 18-24 h. After hybridization the washes were done in 2X SSC several times at room temperature to remove the oil. The filters were then treated with 20 μg/ml RNase A in 2X SSC at room temperature for 4-6 h. Lastly the filters were rinsed in 2X SSC overnight at room temperature, dried and counted in toluene omniflour in a scintillation counter.

5'End Labeling

The probe was prepared by digesting 20 μg of DNA with the suitable restriction enzyme that gives a 5' overhang and deproteinized with phenol-chloroform. The DNA was dephosphorylated in 0.1 M Tris-Cl pH 7.9 containing
calf intestinal phosphatase (Boeringer) at a concentration of 1 U/μg of DNA. The reaction was terminated by the addition of NaOAc to a final concentration of 500mM and then deproteinized with phenol-chloroform and precipitated with 3 volumes of ethanol. The pellet was resuspended in 20 μl of 2X denaturation buffer (1 M Tris-Cl, pH 7.9, 10 mM spermidine, and 1 mM EDTA) and heated at 65°C for 15 min. (This allows the ends to become single stranded). After quenching on ice, 7 μl of 10X Kinase Buffer (500 mM Tris-Cl, pH 9.5, 100 mM MgCl₂, 50 mM DTT, 50% Glycerol), 500 μCi of gamma ³²P-ATP, and 2 μl of T₄ polynucleotide kinase are added and the mix was incubated at 37°C for 30-40 min. The addition of 1.2 M of NH₄OAc terminated the reaction and the mixture was deproteinized with phenol-chloroform. The aqueous phase was then precipitated with ethanol and the pellet was dried and resuspended in 20 μl of water. The labeled DNA was digested with a suitable restriction enzyme and the fragments were resolved on a 6% acrylamide-25% glycerol gel system. The probe was then band isolated and electroeluted using D-Gel and concentrated using an Amicon microconcentrator.

**3' End Labeling**

One μl (0.5-1.0 μg) of restricted DNA having the appropriate 3' overhang was used in a 20 μl reaction volume. To this was added 2 μl of T₄ DNA polymerase buffer, 12 μl of H₂O, 1 μl of 2 mM solution of three dNTPs (barring the one that was radioactive) and 3 μl of α-³²P dCTP. To this 1 μl (2.5 U) of T₄ DNA polymerase was added and the entire reaction mixture was incubated at
37°C for 15 min. Then, 1 μl of unlabeled dCTP was added and the reaction was further incubated for 10 min. The reaction was terminated by a phenol chloroform extraction and two ethanol precipitations in the presence of 2.5 M NH₄OAc. The probe was then subjected to digestion by the second enzyme.

**S1 Analysis**

This was done essentially as per Favaloro et al. (Favaloro et al. 1980). Briefly, 100 ng-1 μg of poly(A)+ RNA was coprecipitated with 100 ng (100,000-200,000 cpm) of the labeled DNA probe. The pellet was resuspended in 10 μl of Hybridization Buffer (40 mM PIPES pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% deionized formamide). The pellet was incubated at 85°C for 15 min and then rapidly transferred to a water bath of desired temperature. Hybridization was carried out for 10-14 h. 250 μl of ice cold S1 digestion buffer (280 mM NaCl, 30 mM NaOAc pH 6.4, 45 mM Zn(OAc)₂) was added to each tube while it was submerged at the desired temperature. The sample was vortexed and 150 units of S1 nuclease (Pharmacia) was added and incubated at 37°C for 30 min. The reaction was terminated by the addition of 75 μl of Termination Buffer, (2.5 M NH₄OAc, 50 mM EDTA) 2 μl of yeast tRNA (10mg/ml) and 400 μl of isopropyl alcohol. The pellet was washed with 70% ethanol. The pellet was resuspended in 5 μl of Sequencing Loading Dye. The protected fragments were analyzed on a 6% polyacrylamide/urea sequencing gel.
RESULTS

SECTION I

The primary goal of my research was to analyze the regulation of TS gene expression in a normal unaltered cell cycle. To obtain synchronized cells, serum or amino acid starvation has been used by several investigators to study stage specific events. Previously regulation of TS gene expression was studied during a serum-induced transition from resting to growing state in both 3T6 and LU3-7 cell line. These studies showed that the enzyme activity remained low during G0-G1 phase but increased sharply as the cells entered the S phase. Further it was shown that the increase in enzyme activity was due to the de novo synthesis of the enzyme and that the increase required gene transcription (Navalgund et al.1980; Jenh et al.1985a). The main question to be answered was, are regulatory mechanisms utilized during growth stimulation similar to those used in a normal cell cycle?

In order to distinguish between events that are growth regulated from those that are regulated in a cell cycle manner, I wanted to study the regulation of TS in mouse fibroblasts that were synchronized by mitotic selection. Mitotic selection is an excellent method for achieving a high degree of synchrony without perturbing the normal physiological parameters of the cell. This method does not use starvation or inhibitors to arrest cells in any phase of the cycle but simply selects for cells that are undergoing mitosis. Therefore synchrony in this
case is not only phase specific but also point specific since the duration of mitosis is usually about 30-60 minutes. There are, however, two drawbacks to this method. The first is, it can only be applied to cells that can grow in monolayers and second is, a relatively small number of mitotic cells are obtained by this selection procedure. This limitation becomes especially important when studying the expression of genes whose products are present at low concentrations, such as those of a typical housekeeping gene. To reduce this problem, I used the overproducing cell LU3-7. It has already been shown by Dr. Chung-Her Jenh that TS gene expression appears to be regulated in the same manner in the LU3-7 cells as in the parental 3T6 cells in cells undergoing a serum-induced transition from resting to growing state (Jenh et al. 1985b).

**Synchronization by Mitotic Section**

Before I started studying the regulation of TS gene expression in mitotic cells I wanted to standardize the method used to select for these cells. First, I isolated mitotic cells from a mass culture of LU3-7 and followed the degree of synchrony attained in this population. Mitotic cells were isolated as described in the Methods and Materials. The degree of synchrony was analyzed by measuring the rate of incorporation of labeled thymidine into DNA as a function of time following mitotic selection. The results of several such analyses are shown in Figure 3. The rate of DNA synthesis was relatively low during the first 4 h, increased to a maximum at about 16 h and then decreased at later times.
Unfortunately, the difference in the rate of DNA synthesis between cells in G1 phase (2-4 h after mitotic selection) and those in mid S phase (16 h after selection) was only about 4-fold, indicating that the cells were not well synchronized. It was possible that the relatively low degree of synchrony observed in Figure 3 was the result of heterogeneity in the population of cells or it is inherent to this cell line.

There are several ways to improve both the synchrony and yield of mitotic cells. The most popular one is to treat cells with Colcemid for 2 hr. This arrests cells in metaphase in a reversible manner. At least in Chinese hamster cells Stubblefield and Klevecz (reviewed in Ashihara and Baserga, 1979) found that treatment with Colcemid had no adverse effect on the biochemical events of these cells. However, Ashihara and Baserga observed that cells treated with Colcemid had different kinetics of entry into S phase (Ashihara and Baserga, 1979). Another way to increase the yield of cells is to do repeated harvesting. Here the monolayers are shaken at 10 min interval and each harvest is cooled and stored at 4°C (Ashihara and Baserga, 1979). However repeated shaking, may decrease the purity of the population i.e. the percent of mitotic cells may be low in this population.
Figure 3. Rate of DNA synthesis in mitotically selected cells selected from a mass culture of LU3-7 cells. Mitotic cells were selected from LU3-7 cells and were plated at a density of 2x10^6 cells/35 mm dish. At the indicated times, duplicate cultures (closed circles vs open circles) were labeled for 30 min with 5 μCi of [3H] thymidine (ICN, 77 Ci/mmole) and the amount of incorporation into acid-precipitable material determined. The data points are plotted at the end of the labeling interval.
Therefore to improve the degree of synchrony as well as the yield of mitotic cells without using drugs, I used the procedure of Farnham and Schimke (Farnham and Schimke, 1985b). In this method I selected for individual clones that would readily detach from the culture dish during mitosis. Mitotic cells from a mass culture of LU3-7 were subjected to several rounds of serial selection of mitotic cells. Individual clones were isolated and expanded and the degree of synchrony achieved in each was analyzed. The clone designated L2 showed the highest degree of synchrony in preliminary experiments and was used in all subsequent analyses. Figure 4A shows that the degree of synchrony was much better with the cloned cell line than with the mass culture. The rate of incorporation of labeled thymidine into DNA increased 10-20-fold between 2.5 and 16 h after mitotic selection. From Figure 4A the length of G1 appears to be approximately 6-7 h and mid S-phase appears to be at 16 h after mitotic selection. As seen in Figure 5A in serum stimulated LU3-7 cells the length of G1 was about 12 h and the peak of S-phase occurred at about 20 h after the cells reentered the cell cycle. The increase in the incorporation of labeled thymidine in these cells appears to be approximately 200 fold between 2 h and 20 h after the cells enter the cell cycle (Jenh's dissertation 1985). Thus it is clear that when cells undergo serum induced transition from resting to growing state they spend an unusually long time in G0/G1 phase. However this is not the case in cells undergoing a normal cell-cycle.

To ensure that the degree of synchrony was high, similar analyses were included in each subsequent experiment. There was little variation from
Figure 4. DNA Synthesis and TS enzyme level in mitotically selected clone from LU3-7 cells.
Mitotic cells were selected from a culture derived from a clone of LU3-7 cells that detached readily during mitosis called L2.

Panel A. Rate of DNA synthesis in L2. Results from three independent experiments (different symbols) are shown.

Panel B. TS enzyme levels in mitotically selected cells. Mitotic cells were selected from a culture derived from a clone of LU3-7 cells that detached readily during mitosis.
Cells were seeded at a density of $2 \times 10^5/100$ mm dish. At the indicated times, duplicate cultures were harvested and TS enzyme level was measured by the $[^3H]F$dUMP binding assay. The results of 4 independent experiments (different symbols) are shown. The values for the different experiments were normalized to the average value at 15 hr so that they could be plotted on the same ordinate scale.
Figure 5. DNA Synthesis and TS enzyme level in serum stimulated LU3-7 cells. Cultures of resting cell were serum stimulated at time=0 and assayed for:

Panel A. Incorporation of $^3$H thymidine into DNA during a 30 min labeling period (closed circles) and the percentage of nuclei labeled with $^3$H thymidine during a 60 min labeling period (open circles) as described previously (Navalgund et al., 1980). The data plotted at the end of the labeling period.

Panel B. TS enzyme activity per culture as measured by $^3$H water assay (closed circles) or $^3$H FdUMP binding assay (open circles). TS activity expressed in arbitrary units (cpm X 10$^{-4}$) for both assay procedures in this figure.

This figure has been taken from Dr. Jenh's dissertation to allow for comparisons between the two methods utilized to obtain synchrony in mouse fibroblasts.
FIGURE 5.

THYMIDINE INCORPORATION

TS activity (•)

(cpm x 10⁻³)

TS activity (○)

Labeled nuclei (%)

Time (hr)
### TABLE I

<table>
<thead>
<tr>
<th>Time after mitotic selection</th>
<th>*Percent labeled nuclei</th>
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<tbody>
<tr>
<td>3 h</td>
<td>4.9</td>
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<tr>
<td></td>
<td>8.6</td>
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<tr>
<td>5 h</td>
<td>16.3</td>
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<td></td>
<td>9.6</td>
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<td>10 h</td>
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<td>12 h</td>
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<td>15 h</td>
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<td>17 h</td>
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<td>19 h</td>
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<td>15.3</td>
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*Percent Labeled Nuclei: Average of duplicates from two independent experiments.*
experiment to experiment in the fold stimulation of the rate of DNA synthesis. This shows that the method employed to obtain mitotic cells was standardized and the clone L2 was indeed a good model system to study the regulation of TS gene expression.

To check the degree of synchrony, autoradiographic analysis were conducted to calculate the percent labeled nuclei in cells as they traverse the cell cycle. Table 1. shows that 50-70% of the nuclei were labeled at 17 h following stimulation. In the case of serum stimulation the percent labeled nuclei at 24 h is about 60%-80%. Thus in both these methods a high degree of synchrony is obtained.

**TS Enzyme Content in Mitotically Synchronized Cells**

Next, the level of the TS enzyme per cell was measured at different times after mitotic selection using a sensitive $^3$H FdUMP binding assay. Since previous studies showed that in growth-stimulated cells, the increase in TS enzyme and mRNA levels occur primarily during the transition between G1 and S phase, measurements were focused primarily on these phases of the cell cycle. Figure 4B shows that the TS enzyme level per cell increased only by a factor of 2 between 2.5 and 15 h following mitotic selection. Since total cellular protein also increased during this interval, the specific activity of TS increased less than 2-fold as cells progressed from G1 through S phase. The fact that there was little increase in TS enzyme level does not necessarily mean that the gene is not regulated during the
normal cell cycle. TS is a rather stable enzyme, with a half-life of roughly 36 h in exponentially growing LU3-7 cells (Navalgund et al. 1980). The content of such a stable component is not expected to fluctuate by much more than a factor of 2 during a 20 h cell cycle. However, in serum stimulated cells there was almost a 20 fold increase in TS activity during the G0-S phase transition (Fig 5B). This was due to the fact that in serum stimulated cells the cells rest in the G0 state for 7 days prior to addition of mitogens thereby allowing the relatively stable enzyme to decay to low basal levels. In a normal cell cycle the basal levels of the enzyme are going to be high since there has been no time for the enzyme to decay. Therefore, it is possible that the rate of synthesis of the enzyme and the content and metabolism of TS mRNA may be regulated during the cell cycle. Although it is difficult to judge conclusively (due to the small extent of the increase and the experimental scatter), the data in Figure 4B is consistent with the possibility that the enzyme is synthesized primarily during S phase.

**TS mRNA Content in Mitotically Synchronized Cells**

In the case of serum stimulated cells, the TS mRNA content increases by a factor of 20-40 as the cells progress from G1 to S phase. From these studies it was clear that the increase in the rate of synthesis of TS was due to an increase in the mRNA content. These studies also looked at the stability of the TS message in G1 and S phase. Jenh et al. found that the TS mRNA is relatively stable in both G0 (half life 9.6 h) and S (half life 7.3 h) phase (Jenh et al. 1985b).
Figure 6. TS mRNA content. Mitotic cells were seeded at a density of $5 \times 10^5$ cells/100 mm dish. At indicated times cells were harvested and cytoplasmic poly(A)+ RNA was isolated. Equal amounts (5μg) of RNA were denatured, electrophoresed on a 1.5% agarose/formaldehyde gel, transferred to nitrocellulose and probed with labeled DNA corresponding to TS mRNA or L30 ribosomal protein mRNA.

Panel A. Cytoplasmic mRNA from cells harvested at 5, 7.5, 10, 12.5 and 15 hrs after mitotic selection (Lanes 1-5 respectively).

Panel B. Cytoplasmic mRNA from cells harvested at 2.5, 5, 7.5, 10, 12.5 and 15 hrs after mitotic selection (Lanes 1-6 respectively).

Panel C. The filter in panel B was also probed with a cDNA for ribosomal L30 protein.
FIGURE 6.
In serum stimulated cells the overall mRNA content goes up by a factor of 3 between 0 and 20 h after stimulation (Johnson et al. 1974) and TS mRNA content goes up by 40 fold. I wanted to look at the content of TS mRNA relative to total mRNA in a normal cell cycle. Therefore TS content was measured by northern blot analysis at different times following mitotic selection. The results of two such experiments (Figure 6A), shows that the content of TS mRNA increased to a much greater extent than TS enzyme level. Densitometric analysis revealed that the amount of TS mRNA increased 5-8 fold between 5 and 15 h following mitotic selection. Since the amount of TS mRNA was even lower at 2.5 h the overall increase in TS mRNA content was even greater than this. The filters were also probed with cDNA corresponding to the mouse L30 ribosomal protein mRNA (Meyuhas and Perry, 1980). Figure 6C shows that the amount of this mRNA remained relatively constant during the cell cycle. At most, the content of L30 mRNA goes up by a factor of 2.

The difference in the fold increase in the content of TS mRNA in serum stimulated (20-40 fold) vs mitotically selected cells (8-10 fold) is due to the fact that, in the case of serum starvation, resting the cells for 7 days allows the relatively stable message to decay to low basal levels whereas this is not the case in the mitotically synchronized cells.
Figure 7. SI nuclease protection analysis of TS mRNA.

Probe used in the S1 nuclease protection analysis was a 1.2kb BamHI-HindIII fragment from pTSMG2 5’ end-labeled at the BamHI site. The marker used in this gel was 5’ end labeled pBR322 cut with Msp I. Mitotically selected (MS) cells: 400 ng of poly(A)+ mRNA was isolated from cells harvested at
Lane 1. 4 hr after mitotic selection (G1 phase) and
Lane 2. 14 hr after mitotic selection (S phase). Serum-stimulated (SS) cells: 15 μg of total cytoplasmic RNA was isolated from cells that were serum-stimulated for
Lane 3. 0 hr (resting cells) and
Lane 4. 12 hr (early S phase).
SI protection assays were performed as described in Materials and Methods.
FIGURE 7.
**Promoter Utilization During the Cell Cycle**

Transcription of the TS gene is initiated at multiple sites. This has been observed in other genes that lack a TATAA element in the promoter e.g. DHFR, HMG CoA Reductase, etc. (Reynolds et al. 1984; Sazer and Schimke, 1986). Since TS had multiple transcriptional start sites I wanted to know if there was a preferential use of different start sites during the cell cycle. These start sites are located between 24 and 90 nucleotides upstream of the AUG initiation codon (Deng et al. 1986). It was possible that different transcriptional initiation sites were used during different stages of the cell cycle. To examine this possibility, S1 nuclease protection analyses were performed on RNA isolated from G1 and S-phase cells, 4 and 14 hr after mitotic selection, respectively. For comparison, similar analyses were performed on mRNA isolated from serum-arrested (GO) and 12 hr serum-stimulated (early S phase) cells. The probe used was a 1200bp BamHI/ HindIII fragment of the TS minigene2 (DeWille et al. 1989). This probe contains approximately 1 kb of 5'flanking sequence and 300 bases of the cDNA. The probe was 5' end labeled with T4 polynucleotide kinase at the BamHI site. Routinely the probes had a specific activity of $7 \times 10^6$ cpm/µg DNA. S1 analyses were performed as mentioned in Methods and Materials. Figure 7 shows that the same patterns of 5' termini were observed for all RNA preparations, indicating that the increase in TS mRNA in cells synchronized by either method is not due to the utilization of different promoters at different stages of the cell cycle.
Densitometric analysis revealed that the intensities of the signals increased about 4-fold for the mitotically selected cells and about 10-fold for the serum-stimulated cells. This is consistent with the results of the RNA blot analyses shown in Figure 6A. The 5-10 fold difference in TS mRNA content that is observed between S-phase and G1 phase cells is in line with that which would be expected, given that the half-life of TS mRNA is about 7.5 hr (Jenh et al. 1985b) and the doubling time of LU3-7 cells is about 20 hr. If we assume that the synthesis of the message terminates during mid S phase, the content of TS mRNA relative to total mRNA should decrease roughly 8-fold (two half lives of the mRNA and a doubling of total mRNA content) between S phase and G1 phase. The increase is much greater in serum-stimulated cells due to the fact that the relatively stable mRNA is allowed to decay for many days prior to serum-stimulation (see above). The above results clearly show that in LU3-7 cells, TS mRNA content is regulated during the normal cell cycle as well as during the transition from the resting to growing state.

Rate of Transcription

Although I was interested in studying the rate of transcription in mitotically isolated cells I was faced with a major problem. This problem was technical and inherent to the method used to obtain synchronized cells. Nuclear run on assays requires at least $10^7$ nuclei per assay. Since obtaining large amounts of synchronized cells ($10^7 - 10^8$) would require me to grow large quantities of cells, these experiments were not carried out.
SECTION II A

TS, TK and DHFR genes all encode enzymes that are involved in the nucleotide biosynthetic pathway. The expression of these genes is not tightly coupled to DNA replication and the regulation of these genes appears to be quite similar (Navalgund et al. 1980; Johnson et al. 1982; Wu and Johnson, 1982). All three genes show an increase in both the rate of transcription and content of the mRNA during G1/S phase boundary during a serum induced transition from the resting to growing state. Therefore the regulation of expression of these genes is thought to be controlled at both transcriptional and posttranscriptional levels.

Several laboratories have been involved in studying the expression of this class of S phase genes. Currently our knowledge about the events that regulate the cell cycle is very limited. Transition from a quiescent state into an 'active' cell cycle is generally accompanied by temporal patterns of activation of specific genes. Based on their timely appearance and their maximal transcript abundance they can be placed in different classes. With the information that is available at present, it appears that several pathways or cascades, of gene expression will be discovered where the activation of one gene will determine in turn the activation of another gene (Denhardt et al. 1986).
Effect of Cycloheximide on the accumulation of TS mRNA

To study the events that may affect the activation of TS gene I decided to analyze the effect of cycloheximide on the transcription of this gene. The cell line that I used to do these studies was the TS overproducing cell line LU3-7. The rationale behind doing these experiments was the following. If transcription of the TS gene was dependent on events occurring in G1, then, inhibiting protein synthesis in G1 would affect the content of the TS mRNA in S phase. These events could include the synthesis of a TS gene specific or S phase specific transcription factors, general transcription factors, processing factors, factors involved in the export of this message etc. If this were the case it would support the theory that regulation of cell cycle events is controlled by a cascade of events. If addition of cycloheximide during G1 had no effect of the content of TS mRNA, then it means that the factors needed for the gene expression are synthesized very early in the serum induced transition from G1 to S phase (e.g. like c-fos). To further analyze these possibilities the following experiments were done.

Initial studies were done with the overproducing cell line LU3-7. Here the cells were rested for 7 days in 0.5% calf serum. The cells were fed on days 2 and 4 after plating, stimulated on day 6 and harvested on day 7. Cells that were treated with the protein synthesis inhibitor were handled in the following manner. 10% calf serum was added to these cells at time 0. At various times after the addition of serum, cycloheximide (1μg/ml) was added to these plates. These cells
were then harvested at 24 hrs after the addition of serum. As a control I used cells that were serum stimulated for different periods of times. Cytoplasmic RNA was isolated as indicated in the Materials and Methods.

Cytoplasmic total RNA or poly(A)+ RNA were then subjected to S1 protection analysis. The probe that I used was a 1.2 kb BamHI-XbaI fragment from the TS minigene2 (DeWille et al. 1989) that was 5' end-labeled at the BamHI site using T4 polynucleotide kinase (Materials and Methods). This probe had been previously used to map the 5' end of the TS mRNA (Deng et al. 1989a). The results are shown in Figure 8. In this figure, S1 analyses were performed on poly(A)+ RNA isolated from 6 and 24 hr serum stimulated cells and from cycloheximide treated cells. (Cycloheximide was added at times 0,6,8,9,10,12 hrs after serum stimulation and the cells were harvested 24 hrs after serum stimulation).

As seen in Figure 8, lanes 1 and 2 contain 1 μg of poly(A)+ RNA from cells that were serum stimulated for 6 and 24 hrs. Lanes 3,4,5,6,7 and 8 contain 1 μg of poly(A)+ RNA from cells that were serum stimulated for 24 hrs, with the addition of cycloheximide at 0,6,8,9,10, and 12 hrs after serum stimulation. Therefore all cycloheximide treated cells were harvested 24 hrs after serum stimulation. When analyzing the data the mRNA content of the cycloheximide treated cells is compared to that of the 24 hr serum stimulated cells because all these cells were serum stimulated for 24 hrs. When comparing the intensities between lanes 1 and 2 there is a greater than 20 fold increase in the TS mRNA content between 6 and 24 hrs serum stimulated cells. This is consistent with
**Figure 8. Effect of Cycloheximide of the TS Poly(A)+ mRNA content in Serum Stimulated LU3-7 cells.**

**5' S1 Mapping.**

Control Cells were stimulated for 6 and 24 hrs with 10% Calf Serum. Cells that were treated with cycloheximide were serum stimulated at time 0. Cycloheximide (1μg/ml) was added at 0, 6, 8, 9, 10, 12 hrs after serum stimulation and cytoplasmic RNA was isolated 24 hrs after the addition of serum at time 0. 1 μg of Poly(A)+ RNA from each time point was subjected to S1 nuclease protection analysis. The probe used was a 1.2kb BamHI-HindIII fragment from pTSMG2, 5'end-labeled at the BamHI site. Marker used was 5' end labeled pBR322 restricted with MspI.

Lane1. 6h Serum Stimulated;
Lane2. 24h Serum Stimulated;
Lane3. Serum Stimulated 24 hr, cycloheximide added at 0h;
Lane4. Serum Stimulated 24 hr, cycloheximide added at 6h;
Lane5. Serum Stimulated 24 hr, cycloheximide added at 8h;
Lane6. Serum Stimulated 24 hr, cycloheximide added at 9h;
Lane7. Serum Stimulated 24 hr, cycloheximide added at 10h;
Lane8. Serum Stimulated 24 hr, cycloheximide added at 12h;
previous studies from our lab where a 20-40 increase was seen in the TS mRNA content during this same transition (Jenh et al. 1985b). When the content of TS mRNA from cycloheximide treated cells is compared to TS mRNA content from the 24 hr serum stimulated cells, it appears that the content increases gradually. This increase seems to depend on the how late the cycloheximide is added after serum stimulation. As seen in Figure 9, similar results were obtained when S1 mapping of the 3' end of the TS mRNA were performed. Here 1 μg of poly(A)+ RNA from 6 and 20 hr serum stimulated and 1 μg of poly(A)+ RNA from 20 hr serum stimulated cells (in the presence of cycloheximide, added at 6 and 12 hr after serum stimulation) were hybridized to a 644 bp PstI-EcoRI fragment 3' end labeled at the PstI site. These hybrids were then subjected to S1 digestion and the protected fragments were analyzed on a denaturing polyacrylamide gel system. As seen in the Figure 9, (lane 3) when cycloheximide is added 6 hr after serum stimulation there is a decrease in the content of TS mRNA. However when cycloheximide is added (lane 4) 12 hr after serum stimulation then the content of TS mRNA decreases slightly. Whereas in the control lanes 1 and 2 there is a 20 fold increase seen in the TS mRNA content when cells traverse the cell cycle. This suggests that the overall content of the TS mRNA is affected when cycloheximide is added during the first 10 hr after serum stimulation.

If cycloheximide had no effect on the accumulation of TS mRNA during S phase, then there should be no change in the content of mRNA between the cycloheximide treated cells and the 20 hr serum stimulated cells. However, this does not seem to be the case. It is very clear the addition of cycloheximide during
Figure 9. Effect of Cycloheximide on the TS Poly(A)+ mRNA content in Serum Stimulated LU3-7 cells.

3' S1 Mapping.

1 µg of poly(A)+ RNA was used in these 3' S1 protection analysis. The probe used for this assay was a 644 bp PstI-EcoRI fragment from the pTSMG2, 3' end-labeled at the PstI site. The protected fragments are F1, F2 and F3 (DeWille, J.W., 1989).

F1 represents protection of probe to the termination codon;

F2 represents protection of probe to the U-rich region beginning 32 nt downstream of the termination codon;

F3 represents protection to the a minor polyadenylation site;
Marker used was 5' end-labeled puc18 restricted with HinfI.

Lane 1. Serum stimulated for 6h;
Lane 2. Serum stimulated for 20h;
Lane 3. Serum stimulated 20 hr + cycloheximide added at 6h;
Lane 4. Serum stimulated 20 hr + cycloheximide added at 12h.
the first 10 hrs after serum stimulation affects the accumulation of TS mRNA in S phase. However when the content of TS mRNA was analyzed in cells where cycloheximide was added 10 hrs or later after serum stimulation, the level is comparable to the level of TS mRNA in the 20 hr serum stimulated cells. This strongly suggests that blocking protein synthesis during G1 (0-10 hrs) after serum stimulation drastically affects the content TS mRNA in S phase. Therefore it appears that for the accumulation of both TK and TS mRNA during S phase, active protein synthesis in G1 phase is necessary.

To see if inhibiting protein synthesis would affect another S phase gene in a similar manner, I performed similar experiments using a DHFR overproducing cell line M50L3 in which the dhfr gene is amplified 300 fold. Here I measured the content of DHFR mRNA in cells that were serum stimulated in the presence and absence of cycloheximide. Cytoplasmic RNA was hybridized to a 5' end labeled 1.4 kb Bgl II/ Eco RI fragment of the cDNA pDHFR21 and subjected to S1 analysis. The fragment protected by this probe is 625 nt long. Fig 10 shows that inhibiting protein synthesis during G1 had the same effect on the content of DHFR mRNA. These results indicate that addition of cycloheximide during G1 affected the accumulation of TS mRNA to a greater extent than DHFR mRNA.

These results strongly suggest that inhibiting protein synthesis during G1 affects the synthesis or stability of factor/s that are involved in transcription or processing or export of these messages. In order to determine which of these processes may be affected I looked at the rate of transcription, processing kinetics and distribution of TS mRNA in the nuclear and cytoplasmic compartments in
Figure 10. Effect of Cycloheximide on dhfr mRNA content.

Control M50L3 cells were serum stimulated for 6 and 20 hrs. Cells treated with cycloheximide were serum stimulated for 20 hrs and cycloheximide (1 μg/ml) was added at 6 and 12 hrs after the addition of serum at time 0. Cytoplasmic RNA was isolated and 20 μg of total RNA from each time point was subjected to S1 protection analysis. S1 resistant hybrids were analyzed on a 8% polyacrylamide 8.3 M Urea gel system. The probe used was a 1.5 kb BglII-EcoRI fragment from pDHFR21 5’end-labeled at the BglII site.

Lane 1. Serum Stimulated for 6h;
Lane 2. Serum Stimulated for 20h;
Lane 3. Serum Stimulated 20 hr + cycloheximide added at 6h;
Lane 4. Serum Stimulated 20 hr + cycloheximide added at 12h;

Marker used was 5’ end-labeled pBR322 restricted with MspI.
cells that were serum stimulated in the presence and absence of cycloheximide.

**Rate of transcription in the presence of cycloheximide**

To analyze transcription rate, I used run on transcription assays as stated in the *Materials and Methods*, with nuclei isolated from serum stimulated cells that were treated with cycloheximide at 6 and 12 hrs and harvested at 20 hrs. Here the nuclei were incubated in a suitable buffer and transcribed in the presence of $\alpha^{32}$P UTP. This labeled RNA was hybridized to plasmid DNA bound to Nytran. Since reinitiation of RNA polymerase II is prevented in the presence of heparin, this assay really measures the density of active polymerases and their elongation activity. Run on assays were also conducted in the control nuclei, that were harvested 6 and 20 hrs after serum stimulation. As shown in Figure 11 the fold increase in the rate of transcription between 6 hr and 20 hr serum stimulated nuclei was comparable to the 3 fold increase seen earlier by Chung-Her Jenh (Jenh et al. 1985b). The rate of transcription in cells treated with cycloheximide at 6 and 12 hrs after serum stimulation but harvested at 20 hrs shows a slight decrease. The fold decrease ranged between 1.5 to 2 fold as compared to the 20 hr serum stimulated control. The signal to noise ratio is rather high but the experiment was repeated many times with the same conclusion that transcription is not abolished though it maybe reduced.
Figure 11. Rate of Transcription in the presence and absence of cycloheximide in serum stimulated LU3-7 cells. $10^8$ nuclei isolated from cells serum stimulated for 6 and 20 hrs and from cells serum stimulated in the presence of cycloheximide (added at 6 and 12 h) were transcribed in vitro in the presence of $\alpha^{32}$P UTP. Labeled nuclear RNA was isolated and $10^7$ cpm/ml were used in hybridization assays. 10 $\mu$g of linearized plasmid DNA corresponding to the following cDNA was immobilized onto nitrocellulose filter and hybridized.

Panel A. 6h serum stimulated;

Panel B. 20h serum stimulated;

Panel C. Serum stimulated 20 hr + cycloheximide added at 6h;

Panel D. Serum stimulated 20 hr + cycloheximide added at 12h;

Order of Plasmids

1. pUC18
2. pMTS-3
3. pDHFR21
4. pActin
FIGURE 11.
Cytoplasmic mRNA Production

These experiments were done in the presence and absence of cycloheximide in cells that were serum stimulated. Control cells were serum stimulated for 6 and 20 hrs. Cells were labeled for 90 min with 100 μCi/ml of $^3$H Uridine (44 Ci/mmol). The 6 hr serum stimulated cells were labeled from 4.5 hr to 6.0 hr and the 20 hr cells were labeled from 18.5 hr to 20.0 hr. In the case of cycloheximide treated cells, the inhibitor was added at 6 and 12 hr after serum stimulation and cells were labeled from 18.5 hr to 20.0 hr. Cytoplasmic RNA was isolated and used in the DNA excess filter hybridization assay as detailed in the Methods and Materials. The labeled RNA was hybridized to the following plasmids that were immobilized to nitrocellulose pBR322, pMTS-3, and pActin. Table 2 shows the results from this experiment. There is a twenty fold increase in the appearance of cytoplasmic TS mRNA in the 20 hr serum stimulated cells as compared to the 6 hr serum stimulated cells. If inhibiting protein synthesis did not have any effect on the processing of TS mRNA then I should see the same ratio of TS/total RNA as the control 20 hr sample. However in cells treated with cycloheximide at 6 hrs after serum stimulation there is a 2 fold decrease and at 12 hrs a 7 fold decrease as compared to the 20 hr serum stimulated cells. These results thus indicate that inhibiting protein synthesis during G1 does indeed affect the appearance of cytoplasmic TS mRNA. Previously from our lab Muralidhar and Johnson had shown that cycloheximide does delay processing and this delay
TABLE 2

<table>
<thead>
<tr>
<th></th>
<th>INPUT COUNTS CPM</th>
<th>COUNTS HYBRIDIZED CPM</th>
<th>TS/TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 6h SS</td>
<td>18 X 10^6</td>
<td>150</td>
<td>8.3 X 10^-6</td>
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<tr>
<td></td>
<td>16 X 10^6</td>
<td>140</td>
<td>8.7 X 10^-6</td>
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<tr>
<td>2. 20h SS</td>
<td>6 X 10^6</td>
<td>1001</td>
<td>166 X 10^-6</td>
</tr>
<tr>
<td></td>
<td>5 X 10^6</td>
<td>800</td>
<td>160 X 10^-6</td>
</tr>
<tr>
<td>3. CH6</td>
<td>6 X 10^6</td>
<td>372</td>
<td>62 X 10^-6</td>
</tr>
<tr>
<td></td>
<td>5 X 10^6</td>
<td>225</td>
<td>45 X 10^-6</td>
</tr>
<tr>
<td>4. CH12</td>
<td>12 X 10^6</td>
<td>265</td>
<td>22 X 10^-6</td>
</tr>
<tr>
<td></td>
<td>10 X 10^6</td>
<td>220</td>
<td>22 X 10^-6</td>
</tr>
</tbody>
</table>

Note: Results of two independent experiments.

SS = Serum Stimulated
CH = serum stimulated for 20 hrs but cycloheximide added at 6 and 12 hrs after serum stimulation.
TS/Total = Counts corresponding to TS mRNA wrt the Total RNA
affects different genes in a similar manner and therefore may be a general processing delay (Muralidhar and Johnson, 1988). Studies of this nature only give an overall picture of processing but do not indicate exactly at which step mRNA production is affected. Here only relative amounts of RNA were observed, however all messages (or many messages) may increase or decrease in the presence of cycloheximide, without having any change in the ratios.

So the next step was to see at which level production of TS mRNA is affected. As already mentioned regulation at the level of processing can be at the level of polyadenylation, splicing, export or stability of the message. Since studies on the 3' processing of TS mRNA were being done by Chris Harendza, a student in our lab, I looked at the distribution of TS mRNA in the two compartments, nuclear and cytoplasmic during the G1-S phase transition.

**Distribution of TS mRNA in Nuclear and Cytoplasmic Compartments**

The hypothesis that I wanted to test was the following. It has already been shown that the transcription of this gene goes up only 3-4 fold during G1/S phase transition and the content goes up by 20-40 fold (Jenh et al. 1985b). Therefore it is possible that even though the gene is transcribed during G1 the transcript is not processed or exported until the G1/S phase boundary. This has been shown to be the case with the TK gene (Gudas et al. 1988). If this is indeed the case, then, unprocessed mRNA might accumulate in the nucleus during G1. This lack of processing in G1 could then be attributed to the fact that some
processing factor/s synthesized during G1 may be involved in the efficient splicing of the unprocessed message at the G1/ S phase boundary.

If this were the case then, the distribution of RNA in the nucleus and cytoplasm should vary during the cell cycle. First, during serum-induced transition from resting to growing state I should see an accumulation of unprocessed or processed RNA in the nucleus during G1, prior to the appearance of the mature message in the cytoplasm during S phase. Second, if this factor/s is important for the processing of the hnRNA is synthesized in G1, then, inhibiting protein synthesis during G1, should be reflected in the build up of unprocessed hnRNA in S phase.

To test if there is indeed an accumulation of TS RNA in the nucleus in G1 during serum-induced transition from resting to growing state, I isolated nuclear and cytoplasmic RNA from cells that were serum stimulated for 4,7,14 and 20 hrs. The cells were harvested at the specific times after serum stimulation and separated in nuclear and cytoplasmic fractions. The cytoplasmic RNA was isolated as per the Materials and Methods and 30 μg of total RNA was used in northern blot analyses. To isolate the nuclear RNA and to minimize cytoplasmic RNA contamination, the nuclear pellet was treated with another detergent Tween-deoxycholate (before the isolation of nuclear RNA by the guanidinium-isothiocyanate-phenol-chloroform method). 60 μg of total nuclear RNA was analyzed on northern blots. In an eukaryotic cell, 10% of total cellular RNA is nuclear RNA, the remaining 90% is cytoplasmic. 1-5% of total cellular RNA is polyadenylated. In the overproducing cell line TS mRNA represents about 0.1%
of this poly(A) fraction. These numbers become important when determining the
distribution of the TS mRNA in the nuclear and cytoplasmic compartments.

**Figure 12** shows the results from one such experiment. The probe used to
hybridize this northern blot was a uniformly labeled cDNA probe, pMTS-3. The
markers were a RNA ladder from BRL ranging from 9.5 to 0.24 kb. As seen in
this figure, at times 4 and 7 hrs after serum stimulation there appears to be an
accumulation of TS mRNA in the nuclear fraction (lanes 1 and 2) as compared
to the cytoplasmic fraction (lanes 4 and 5). In this experiment, to be able to pick
up the nuclear RNA species, I had loaded twice the amount of nuclear RNA as
cytoplasmic RNA. Given the distribution of total RNA in the two compartments
of any cell (10% in the nucleus and 90% in the cytoplasmic compartment), one
can conclude that under these conditions the content of TS mRNA in the nucleus
is much lower. These analyses were conducted with total RNA and hence the
smearing of the RNA could be due to TS poly(A) RNA. In a second experiment
I analyzed the same amount of nuclear and cytoplasmic RNA by northern blot
analysis. When this analysis was done it was very clear that there was no
appreciable accumulation of the message in the nucleus during G1. I repeated
this experiment several times with the same conclusion that there is no significant
accumulation of the TS mRNA during G1 during the serum-induced transition
from G1 to S phase.

I had earlier proposed that if regulation of TS gene expression was at the
level of mRNA processing, then one should see a build up of unprocessed
Figure 12. Distribution of Cytoplasmic and Nuclear TS RNA Content in Serum-Stimulated LU3-7 cells.

Nuclear and Cytoplasmic RNA was isolated from LU3-7 cells serum stimulated for 4, 7, 14 and 20 h. Total RNA was subjected to Northern blot analysis as per Materials and Methods. Probe used was $^{32}$P labeled pMTS-3, labeled by random primer labeling method.

Lanes (1-3) contain 60 $\mu$g of total nuclear RNA from 4, 7 and 20 h serum stimulated LU3-7 cells; lanes (4-7) contain 30 $\mu$g of total cytoplasmic RNA from 4, 7, 14 and 20 h serum stimulated LU3-7 cells.

Marker used was a BRL RNA ladder.
RNA during G1. This would be consistent with the observations made by Gudas et al. where, the authors show a build up unprocessed TK mRNA in the nucleus of G1 cells (Gudas et al. 1988). But the results from the above experiment also indicated that there is no build up of high molecular weight or unprocessed TS RNA in the G1 nuclear fraction. As seen in the Figure 13 the TS mRNA that accumulates in the nucleus seems to be of the same size as the mature cytoplasmic TS mRNA. This could mean that, during G1 there could be a build up of hnRNA but it may be turning over very rapidly and therefore I could not detect it under these conditions.

To further check the molecular weight of the RNA in the nuclear fraction I isolated RNA from nuclear and cytoplasmic compartments from exponentially growing cells as well as from whole cells. I subjected 20 µg of RNA from each fraction to northern blot analysis. The probe that I used in this case was a uniformly labeled antisense RNA probe that spanned an internal 402bp PstI-Bam HI fragment of the cDNA pMTS-3 (spans exons 4 and 5). As shown in Figure 13 there was no high molecular weight precursor in the nuclear fraction. Rather the RNA in the nuclear fraction was the same size as the mature cytoplasmic message.

To see if the distribution of TS RNA in the nuclear and cytoplasmic compartments in serum stimulated cells changed in the presence of cycloheximide, cytoplasmic and nuclear RNA was isolated from serum stimulated cells in the presence and absence of cycloheximide and were subjected to northern blot and S1 analyses. I was not very successful and was
Figure 13. RNA Content in Exponentially growing LU3-7 cells. Total RNA, cytoplasmic and nuclear RNA were isolated from exponentially growing cells and subjected to northern blot analysis.

Lane 1. 20 µg of total nuclear RNA from exponentially growing LU3-7 cells;
Lane 2. 20 µg of total cytoplasmic RNA from exponentially growing LU3-7 cells;
Lane 3. 20 µg of total whole cell RNA from exponentially growing LU3-7 cells.

Probe used was a uniformly labeled RNA antisense probe corresponding to the exons 5 and 6 of the cDNA, pMTS-3.

Marker used was the BRL RNA ladder.
FIGURE 13.
constantly faced with degradation of the nuclear RNA fractions as well as problems of low signal to noise ratio.

Summarizing the results and observations from these experiments leads to the following conclusions. First, it is evident that for the S phase accumulation of the TS mRNA, active protein synthesis must occur during G1. Measuring the cytoplasmic content of TS RNA in serum-induced cells in the presence of cycloheximide, seem to suggest the possibility that, a factor/s synthesized during G1 affects the accumulation of TS mRNA during S phase. When the rate of transcription was analyzed in cells that were serum-induced in the presence of cycloheximide, it appears that there is a slight decrease. Here the rate of transcription of the 20 hr serum stimulated nuclei (control) was compared to that of the 20 hr serum stimulated nuclei in the presence of cycloheximide (cycloheximide was added at 6 and 12 hrs after serum stimulation). Since the rate of transcription of TS gene in the control nuclei (6 and 20 hr serum stimulated) showed a 3 fold increase, as already shown by Jenh et al. (Jenh et al. 1985b), this method should have been able to detect any significant change. The fact that the decrease was so subtle, makes it almost impossible to determine what the effect of inhibiting protein synthesis has on the rate of transcription, it is certainly not a major effect or specific for TS gene alone. Third, when the production of TS mRNA was analyzed in serum-induced cells in the presence of the inhibitor, there appears to be a two fold decrease in the production of TS mRNA in the S phase. This indicates that protein synthesis does affect the biogenesis of TS mRNA to a much greater extent as compared to the DHFR mRNA. Lastly, in the serum-
induced transition from resting to growing state, I could not detect any significant accumulation of unprocessed or processed TS RNA in the nucleus during G1. When the same analyses were done in the serum induced cells in the presence of cycloheximide, I was not able to detect any build up of the unprocessed TS RNA in the nucleus during S phase. Due to technical problems I was not successful in detecting accumulation of unprocessed mRNA in the nucleus of S phase serum-induced (+cycloheximide) cells. The fact that I did not pick up hnRNA in the nucleus of serum-induced G1 cells could mean that during G1, there may be a rapid turnover of the TS hnRNA and/or the rate of export of TS mRNA may be more efficient during S phase.
SECTION IIB

Attenuation or premature termination of transcription occurs more frequently in prokaryotes than in eukaryotes. However there are several eukaryotic genes that use this mechanism to modulate the levels of RNA. As already mentioned in the introduction, c-myc is one of the genes that regulates the content of its RNA by attenuation. Levels of c-myc RNA decrease upon growth arrest and differentiation. Changes in the steady state level of c-myc RNA have been attributed to regulation of both rate of transcription and stability of myc RNA (Lachman and Skoultchi, 1984; Einat et al. 1985). However when measurement of the rate of transcription across different regions of the myc gene was carried out, it appeared that the level of myc RNA was modulated by a "block" near the 3' end of the first exon that prevented transcription elongation (Eick and Bornkamm, 1986; Nepveu and Marcu, 1986). Wright and Bishop in 1989 showed that a 180 nt fragment from the 3' end of exon 1 acts in an orientation-dependent manner to partially block transcription elongation by polymerase II through the human α-globin gene (Wright and Bishop, 1989). This data corroborated the results of Bentley and Groudine who showed that a 95 nt sequence at the 3' end of exon 1 of human MYC gene mediates termination of transcription (Bentley and Groudine, 1988). I wanted to see if attenuation is
involved in the modulation of TS RNA during the G1/S transition.

When the rate of transcription was studied in LU3-7 cells, Jenh et al. reported a 3-4 fold increase as cells traversed the G1/S phase boundary (Jenh et al. 1985b). These studies were done by hybridizing labeled nuclear run on transcripts to double stranded cDNA corresponding to the entire TS coding region. By using double stranded DNA it would not be possible to detect bidirectional transcription. Furthermore, since full length cDNA probe was used, it would have been very hard to differentiate between rate of elongation over the whole gene versus over different sections of the gene. Therefore it is possible that in previous experiments where double stranded cDNA was used, the occurrence of attenuation could have been overlooked. In order to see if attenuation modulates TS transcription I modified the above procedure.

### Analysis of Premature Termination

Analyzing premature termination requires that different parts of the gene be separated and subcloned. Since I wanted to measure the relative densities of active RNA polymerases in the 5' and 3' ends of the gene, I did the following. As shown in Figure 14A I subcloned a 256 bp fragment from the 5'coding region of a deletion clone of pTSMG2,(generated by Tiliang Deng in our lab) an Eco RI-Bam HI fragment into pIBI30 (IBI). This fragment contain exons 1 and 2. I also subcloned a 270 bp PstI-PstI fragment from the 3' end of the cDNA pMTS-3 into a pIBI30 vector. This fragment contain the last two exons. The reason that I
subcloned these fragments into pIBI30 was the following. This vector has T3 and T7 RNA polymerase promoters on either side of the multiple cloning sites, that would enable me to synthesize strand specific transcripts. I then transcribed both sense and antisense transcripts from these plasmids and immobilized them to Nytran filters. I probed these with α-32 P labeled nuclear RNA from nuclear run on transcriptions from cells that were serum stimulated for 6 and 20 hrs. As a control I linearized the vector downstream of the T3 RNA polymerase promoter and transcribed the multiple cloning site (175 nt). The reason I chose to immobilize sense and antisense transcripts on the filter was the following. Studies where transcriptional elongation are studied generally use single stranded DNA immobilized to the filter. Another approach used by Stewart et al. to study rate of transcription of TK in isolated nuclei was to immobilize sense and antisense RNA onto the filter (Stewart et al. 1987). This procedure is far more sensitive and more accurate, as RNA-RNA duplexes form very stable hybrids. It was also important to choose similar sized fragments spanning the two ends to eliminate any preferential hybridization to different sized fragments. The strategy used to study attenuation was the following. First, sense and antisense transcripts corresponding to the 5' and 3' ends of the cDNA were transcribed and immobilized to the Nytran membrane. These were probed with labeled nuclear run on assays that were performed on nuclei isolated from cells that were serum stimulated for 4 and 18 hrs. If attenuation did regulate the TS mRNA content in S phase, I should see the same amount of RNA corresponding to the 5' end of the TS cDNA in both G1 and S phase serum stimulated nuclei but should see
more RNA corresponding to the 3' end of the TS cDNA in S phase. This would then imply that initiation of transcription occurs at all times during the cell cycle but elongation occurs only in S phase.

The results from one such assay are shown in Figure 14B. From this experiment it appears that there is more nuclear RNA corresponding to the 3' end of the TS cDNA in S phase as compared to the 3' end of the TS cDNA in G1. However the amount of RNA corresponding to the 5' end of the TS cDNA also seems to increase in S phase. To further quantitate this individual dots were counted. Table 3 shows the following, the ratio between the amount of RNA corresponding to the 5' end of the TS cDNA from 4 and 18 hrs is 3.6 and the ratio between the 3' end of the TS cDNA from 4 and 18 hrs is 4.2. This ratio indicates that within experimental error there is same increase in the amount of RNA corresponding to the two ends of the cDNA in G1 and S phase. These results also indicate that the rate of transcription during serum-induced transition from G1 to S phase goes up 3-4 fold. This agrees with previous studies carried out by Jenh et al. (Jenh et al. 1985b) where he showed that the rate of transcription of the TS gene goes up 3-4 fold as cells traverse from G1 to S phase during serum stimulation.

Next, I looked at the amounts of labeled RNA corresponding to the 5' and 3' ends during G1 and S phase. The rationale was that if there is more RNA corresponding to the 5' or 3' end at any given time then it will indicate a higher density of active polymerases at that end of the gene. When comparing the amount of RNA corresponding to the 5' end to the amount of RNA
Figure 14 A. Subcloning strategy to generate sense and antisense transcripts corresponding to the 5' and the 3' coding regions of the TS mRNA.

BTD(-13) is a deletion clone of pMG2, that contains 13 nt of the 5'flanking sequence upstream of the AUG codon and the rest of the pTSMG2 (gift from Tiliang Deng).
Thick lines = region corresponding to the cDNA.
Thin lines = corresponds to the 5' and 3'flanking sequences in Btd-13, and to the vector sequences in the cDNA.

Panel a pIBI30(EB) contains exons 1 and 2, in addition to the 13 nt upstream of the AUG cloned into pIBI30 as a 256 bp EcoRI-BamHI fragment.
pIBI30(270) is 270 bp PstI-PstI fragment corresponding to exons 6 and 7 in the cDNA, pMTS-3, cloned into pIBI30 into the PstI site such that the 5' end lies near the T7 RNA polymerase promoter.

Figure 14 B. Nuclear Run On Transcription.

$10^8$ nuclei were isolated from 4 and 18 h serum stimulated LU3-7 cells. Nuclear run on transcription was performed using these nuclei in the presence of $\alpha^{-32P}$ UTP. $10^8$ cpm/ml was used in each hybridization assay.

Panel A. Hybridization of 4h SS and Panel B. 18h SS nuclear run ons to the following transcripts immobilized to a nytran membrane.

Lane 1. Control, 10 $\mu$g of transcript corresponding to the pIBI30 multiple cloning sites, 155 nt.
Lane 2. 10 $\mu$g of antisense transcript corresponding to the 5’coding region of the TS cDNA (exons 1 and 2).
Lane 3. 10 $\mu$g of sense transcript corresponding to 5’coding of the TS cDNA (exons 1 and 2).
Lane 4. 10 $\mu$g of antisense transcript corresponding to the 3’coding region of the TS cDNA (exons 6 and 7).
Lane 5. 10 $\mu$g of sense transcript corresponding to the 3’coding region of the TS cDNA (exons 6 and 7).
FIGURE 14.

**A**

Btd-13 [deletion clone of pMG2] cDNA

X E P S BB Bg P C Sa

T7 T3

**B**

A B

1 2 3 4 5

**C**

T7 T3
### TABLE 3

<table>
<thead>
<tr>
<th>Transcript Immobilized on the filter</th>
<th>Nuclei from Serum Stimulated Cells</th>
<th>4h</th>
<th>18h</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'antisense</td>
<td></td>
<td>655</td>
<td>2378</td>
</tr>
<tr>
<td>5' sense</td>
<td></td>
<td>270</td>
<td>1219</td>
</tr>
<tr>
<td>3'antisense</td>
<td></td>
<td>287</td>
<td>1212</td>
</tr>
<tr>
<td>3' sense</td>
<td></td>
<td>239</td>
<td>867</td>
</tr>
</tbody>
</table>

| RATIOS                               |                                    |     |     |
|--------------------------------------|                                    |     |     |
| 5' 18h AS/ 5' 4h AS                  |                                    | 3.6 |     |
| 3' 18h AS/ 3' 4h AS                  |                                    | 4.2 |     |
| 5' 4h AS / 3' 4h AS                  |                                    | 2.28|     |
| 5, 18h AS/ 3'18H AS                  |                                    | 1.98|     |
corresponding to the 3' of the TS cDNA in G1, there appeared to be 2X more RNA corresponding to the 5' end. When similar comparisons were done at S phase, again there was 2X more RNA corresponding to the 5' end as compared to RNA corresponding to the 3' end of the TS cDNA. The fact that at any given point there is more message corresponding to the 5' end of the cDNA indicates that elongation does not occur at a uniform rate across the gene. Another reason why I may have detected more 5' end corresponding to the TS gene as compared to the 3' end could be due unequal number of uridines present in the two ends of the TS gene. This is a very important factor because the nuclear run on assays were done in the presence of $\alpha^{32}$-P UTP. If there were more uridines in the 5' end of the gene than the 3' end of the gene then, the number of hybridizable counts to the 5' end will appear greater than those to the 3' end. This however may not be a problem because, there are roughly the same number of uridines at the two ends of the coding region of the TS gene. This means that attenuation probably occurs but is not regulated by the cell cycle.

In the above studies, I had used both the sense and antisense RNA spanning the first two exons and the last two exons in the run on assays. In all these experiments I always detected appreciable hybridization to the sense transcripts corresponding to the 5'end and (to a lesser extent) to the 3' end of the cDNA. The intensity of the signal varied slightly from one experiment to another but was consistent. This led to be ask the question is this an artifact or is there bidirectional transcription of this gene?
Before I explain the experimental strategy, I would like to point out that these experiments were all conducted in the amplified cell line. Since it is not clear how the amplified genes are arranged, that is, whether in a head to head or in a head to tail manner, it was possible that what I was detecting as opposite strand transcription may have been just an artifact inherent to the system. With this in mind, I analyzed the rate of transcription in serum stimulated parental cell line 3T6 mouse fibroblasts. Since there is only one copy of the TS gene I should be able to eliminate any artifact introduced in the amplified cell line. These experiments were difficult to do as I was constantly faced with problems of high signal to noise ratio as well as the large number of nuclei that had to be harvested for each assay. The couple of times the assay worked reasonably I saw the same pattern of hybridization (data not shown). Therefore I decided to see if I can detect any RNA that would hybridize to the sense probe.

To do this I isolated whole cell, cytoplasmic and nuclear RNA from exponentially growing LU3-7 cells. 10 μg of total RNA was subjected to Northern blot analysis. The blot was probed with a uniformly labeled RNA probe that corresponded to the first two exons of the cDNA. As a control I probed the same preparation of RNA with a uniformly labeled antisense RNA. If TS was transcribed bidirectionally, then I should detect a normal sized TS message with the antisense probe and different sized message/s with the sense probe. If there was no opposite strand transcription then I should not detect any RNA with the sense probe.
Figure 15 shows the results from this experiment. Panel A shows the results when a northern blot is probed with a uniformly labeled antisense RNA probe. The size of the message is about 1.4 kb and corresponds to the size of the TS mRNA. Panel B shows the results of a northern blot that was probed with a uniformly labeled sense RNA probe. As you can see, the bands detected by this probe are not the same size as the TS message. This probe detects a series of bands in the nuclear fraction that range in size from 4.4 kb to about 1 kb. The fact the this RNA species is not present in the same amount as the TS message further supports the observation that TS may be transcribed bidirectionally.

These observation suggesting that TS gene may be transcribed bidirectionally was one of the last experiments that I did. The reason why I believe that this probably occurs is the following. From the northern blot analyses, it is clear that the RNA species that hybridizes to the sense probe appears to be present in both nuclear and cytoplasmic compartments. It is highly unlikely that it is due to non specific hybridization because this filter was subjected both to RNAse treatment, as well as very stringent washes.
Figure 15. Analysis of opposite strand transcription in the TS gene.

Total whole cell, cytoplasmic and nuclear RNA was isolated from exponentially growing LU3-7 cells. RNA was subjected to northern blot analysis and probed with uniformly labeled sense and antisense RNA probes.

Lane 1. 10 µg of total whole cell RNA from exponentially growing LU3-7 cells.

Lane 2. 20 µg of total cytoplasmic RNA from exponentially growing LU3-7 cells.

Lane 3. 20 µg of total nuclear RNA from exponentially growing LU3-7 cells.

Panel A. Northern blot probed with uniformly labeled antisense RNA probe corresponding to exons 5 and 6 in the cDNA, pMTS-3.

Panel B. Northern blot probed with uniformly labeled sense RNA probe corresponding to exons 1 and 2 in the cDNA, pMTS-3.
SECTION III

Effect of Heat Shock on Mouse Thymidylate synthase Gene Expression

When deletion and site directed mutagenesis analyses were done on the TS promoter to define the essential promoter elements, it was found that a region -105 to -85 was critical for the expression of this gene. Deletion of this region completely abolished transcription of the gene. When sequences in this region were closely examined, there was a sequence CCGGAAGTTTCCCA that had a 7/8 match to the consensus sequence of the Heat Shock Element (HSE) CNNGAANNTTCNNG (Figure 16) (Deng et al. 1989b; Lindquist, 1986). The presence of an HSE element in this region led to the following series of experiments.

Since there was an element that had a 7/8 bp match to the consensus sequence of HSE and is present in the critical region of the TS promoter, the first question was what role does it play in the expression of TS gene? Does it confer heat inducibility to this gene? To answer these questions I decided to see what effect does heat shock have on TS gene expression. The first experiment that I did was to see what effect heat shock would have on the rate of transcription of TS. Next, I studied the export of TS mRNA under normal and under heat shock conditions and lastly I looked at the content of the TS mRNA
FIGURE 16.
in both normal and heat shock conditions.

**Effect of Heat Shock on the transcription of TS gene**

Here I used the TS overproducing cell line LU3-7 mouse fibroblasts. These cells were grown in T-150 flasks to about 75% confluency. The cells were then exposed to elevated temperature 43°C for 15 mins and nuclei were isolated as indicated in the Materials and Methods. As a control, nuclei from exponentially growing cells were isolated. The same number of nuclei were used to carry out in vitro run on transcription in the presence of α-32P UTP. The nuclear RNA was isolated and hybridized to plasmids pUC18, pIBI30, pMTS-3, pH2.3 and p50. (pH2.3 was the plasmid containing the hsp70 cDNA kindly given to us by Dr. R.Morimoto) that were immobilized to Nytran membrane. Each filter was hybridized with the same number of cpm of labeled RNA. As seen in Figure 17, signal intensities reveal that the rate of transcription of TS remains unchanged after a 15 min heat shock. As expected the rate of transcription of hsp70 is low in the control cells but goes up in cells that were heat shocked. Rate of transcription of hsp70 gene goes up 20 fold (as measured by densitometric analysis) upon a short exposure to elevated temperature. As compared to the control p50 (cDNA of ribosomal protein L3) there is definitely no change in the transcription of TS during heat shock. Therefore, these results indicate that exposing LU3-7 cells to elevated temperatures does not affect the transcription of the TS gene. In most cases heat shock blocks transcription of genes that are not
Figure 17. Effect of Heat Shock on the Rate of Transcription of the TS gene.

Panel A. Control, hybridization performed using nuclear run on $^{32}$P transcripts from nuclei isolated from exponentially growing LU3-7 cells.

Panel B. Hybridization performed using nuclear run on $^{32}$P transcripts from nuclei isolated from exponentially growing cells that were heat shocked at 42°C for 15 min. 10$^7$ cpm/ml of counts were added to each hybridization bag and the filters were exposed for 72 hrs.

Lanes 1-5: Plasmids immobilized to the Nytran Membrane.

Lane 1. 10 μg of linearized control plasmid, pUC18;
Lane 2. 10 μg of linearized control plasmid, pIBI30;
Lane 3. 10 μg of linearized pMTS-3, TS cDNA;
Lane 4. 10 μg of linearized pH2.3, Hsp70 cDNA;
Lane 5. 10 μg of linearized p50, ribosomal protein L30 cDNA.
heat shock genes (Ashburner and Bonner, 1979). The fact that heat shock did not alter the transcription of TS suggests that the presence of the HSE in the critical region prevents the suppression of TS gene expression during heat shock.

**Effect of Heat Shock on the Export of TS mRNA**

Since the transcription of TS seems to be unaffected by heat shock, I decide to analyze the effect of heat shock on the export of TS mRNA. Control cells were incubated in the presence of 100 μCi/ml of $^3$H uridine for 90 min at $37^\circ$C while experimental cells were incubated at $43^\circ$C. Cytoplasmic RNA was isolated and hybridized to linearized plasmids pUC18, pdhfr21, pH2.3, pMTS-3 and pActin immobilized to nitrocellulose. The filters were then washed, dried and sprayed with three coats of EN$^3$ HANCE (NEN) and exposed to an X-Ray film. Figure 18, panel A shows that under normal temperature TS mRNA is transported to the cytoplasm but not HSP 70 mRNA is not. However when the temperature is raised to $43^\circ$C, there is no signal corresponding to the TS mRNA but there is at least a 20 fold increase in the signal corresponding to the HSP70 mRNA. This experiment was repeated a couple of times with the same results. This method to follow the transport of *in vivo* labeled RNA seems to be relatively simple and rather informative. Therefore at least in this respect, the TS gene seems to behave like any other cellular gene.
Figure 18. Effect of heat shock on the export of TS mRNA.
Exponentially growing LJ3-7 cells were labeled in vivo with 100 μCi/ml of \(^3\)H uridine for 90 min at 37°C (Panel A) and (Panel B) at 42°C. Cytoplasmic RNA was isolated from each and used in hybridization assays to linearized plasmid immobilized to nytran membrane. The filters were then dried and sprayed with EN\(^3\)HANCE and exposed for 5 to 7 days.

Lane 1. 10 μg of linearized pUC18;
Lane 2. 10 μg of linearized pDHFR21;
Lane 3. 10 μg of linearized pH2.3;
Lane 4. 10 μg of linearized pMTS-3;
Lane 5. 10 μg of linearized pActin.
Finally I decided to look at the effect of heat shock on the content of cytoplasmic TS mRNA. During heat shock generally there is a preferential translation of heat shock mRNA. The translation of normal cellular genes ceases within minutes of temperature shift while the translation of heat shock mRNA increases very rapidly. I wanted to know if raising the temperature would affect the content of TS mRNA. Here the cells were exposed to elevated temperature for 2 hrs and cytoplasmic RNA was isolated. The RNA was then subjected to S1 analysis. The probe used was the same Bam HI-Hind III fragment that was used to map the 5' ends of the TS mRNA. As seen in Figure 19 the content of cytoplasmic TS mRNA remains unchanged in the heat shocked cells as compared to that in the control cells.

The results presented above can be summarized as follows. The TS gene behaves like any other normal cellular gene under elevated temperatures, with the exception that heat shock has no effect on the rate of transcription. This may be attributed to the presence of a nearly perfect HSE in the critical region of the TS promoter. The fact that heat shock did not alter the rate of transcription of TS, may mean that a factor other than (but perhaps related to) the heat shock transcription factor may recognize this sequence and bind here. Further studies are being conducted by Keith Joliff, in our lab to identify the protein/s that bind to this region.
Figure 19. Effect of Heat Shock on the TS mRNA Content.

Cytoplasmic mRNA was isolated from cells growing at 37°C and 42°C. 20 μg of total RNA from each was hybridized to a 1.2 kb BamHI-HindIII fragment 5' end labeled at the BamHI site and then subjected to S1 protection analysis. The S1 protected fragments were analyzed on a 6% polyacrylamide-8.3 M urea sequencing gel. Marker used was 5’ end labeled pBR322 restricted with MspI.

Lane 1. RNA from exponentially growing cells.
Lane 2. RNA from cells heat shocked at 42°C for 120 min.
FIGURE 19.
DISCUSSION

The main focus of my research was to study the regulation of TS gene expression in a normal cell cycle. All previous work in our lab were done on cells that were synchronized by serum starvation. Under these conditions TS appears to be regulated in a cell cycle specific manner with increased expression at the onset of S phase. However, since this increased expression could be attributed to a response to growth induction, it was very important to study the regulation of TS in a normal cell cycle. The first part of my work deals with the regulation of TS in mitotically selected mouse fibroblasts. My subsequent studies tested several different hypotheses that concern the control of regulation of TS gene expression in growth stimulated cells. To identify the regulatory events that control the content of TS mRNA during S phase, I determined the effect of cycloheximide on TS gene expression. Next, could the premature termination in G1 account for low levels of TS mRNA in this phase of the cell cycle? Finally, I analyzed the effect of heat shock on the expression of TS. In this section I shall be discussing each of these projects separately.
Regulation of TS in mitotically selected cells

When studying stage specific events in cultured cells, importance should be given to the method used to obtain synchronized population of cells. There are generally two ways to obtain synchrony, first by selection and second by induction. Selection synchrony is when cells in the same stage are physically separated from an asynchronous population. In the second method, induced synchrony, the progress of the cell cycle is blocked so that all cells are brought to the same position in the cycle (Prescott, 1976). Each of these methods has its advantages and disadvantages. Ideally synchronized populations in culture should meet the following criteria:-(1) they should be perfectly synchronized at a specific point of the cell-cycle; (2) the procedure used to obtain synchronized cells should have very little effect on the metabolic processes of the cell; and (3) the procedure should allow for harvesting large quantities of cell.

No known procedure satisfies all three conditions (Ashihara and Baserga, 1979). The methods in which cells are selected and not induced are model systems to study events in an unperturbed cell cycle. The two commonly used methods in which cell are selected are i) mitotic selection and ii) centrifugal elutriation. The latter method uses a special rotor to size select cells as they traverse the cell cycle. The disadvantage of this method is that first one must have access to a rotor and second there is some variability between cell size and cell cycle position. Therefore synchrony may not be tight. In the case of mitotic
selection the cells undergoing mitosis are selected and followed into the next cycle. This is probably the best way to analyze cell cycle regulated events without perturbing the cell cycle. There are a few disadvantages to this method. First, yield of synchronized cells is low because in any exponentially growing population only 5% of cells are undergoing mitosis. Second, as is the case with most methods, synchrony breaks down very rapidly. Since I was interested in events occurring during G1-S phase transition this was not a major problem.

In the case of induced synchrony, the cells in culture are brought to a single point in the cell cycle by manipulating the culture conditions. For example, animal cells in culture arrest in G0 phase when they are confluent and/or when plated in low serum concentrations. In order for cells to reenter the cycle one can either subculture them into fresh medium or feed them with 10% serum. This method has several advantages. For example, synchronization by serum starvation allows one to harvest large quantities of cells. Second, since the G0-G1 phase is prolonged in a serum induced transition from resting to growing state, it is a convenient model system to study G1-S phase events. In many instances this method has no adverse effect on the metabolic processes of the cell cycle because, drugs are not used to induce synchrony. Other methods use amino acid deprivation to arrest cells in G1, inhibition of DNA synthesis etc (Prescott, 1976).

There have been cases where regulation in response to mitogens have been misinterpreted as cell cycle regulation. One such example is that of c-myc. Initial experiments classified c-myc as "an inducible gene that is regulated by specific growth signals in a cell-cycle-dependent manner" (Kelly et al. 1983).
There was a 20 fold increase in the content of c-myc RNA shortly after the cells were stimulated to reenter the cycle. Thus it was inferred that expression of this gene may be specific to the G1 portion of the cell cycle (Kelly et al. 1983; Campisi, 1984). However when Thompson et al. looked at the levels of c-myc mRNA in cells that were growing exponentially and thus under constant stimulation, they found that levels of c-myc remained invariant throughout the cell cycle (Thompson et al. 1985). Therefore in order to distinguish events that occur during the activation of quiescent cells to proliferate from those events that are regulated during the cell cycle, I chose to study the regulation of TS gene expression in mitotically selected cells. Since large quantities of cells are usually not obtained by this method, I chose to use the overproducing cell line LU3-7 to study regulation of TS gene expression in mitotically selected cells. This cell line has been shown to regulate TS gene expression in a normal fashion as compared to the parental cell line (Jenh et al. 1985a; Navalgund et al. 1980). The generation time of this cell line is about 20 hrs. This becomes a very important issue, especially when studying levels of relatively stable enzymes and RNA species.

As indicated in the results, I used a method that was employed by Farnham and Schimke to obtain fairly large yields of cells that were reasonably well synchronized. This selection method allows for the isolation of a population of cells that are less adhesive and that would readily detach during the selection procedure. One can argue, that one may be introducing an artifact while doing such a selection. But one has to keep in mind that these studies were conducted
as is the case with most studies, in cells growing in culture, which itself is an artificial situation. The mitotic selection employed by Farnham and Schimke, does not really alter the normal physiology of the cells. When viewed under the microscope these cells are able to attach to the petri dishes within an hour or so after they have been plated. Therefore this method, significantly improved the yields of mitotic cells without having to use drugs. The degree of synchrony obtained from mitotic cells isolated from a heterogeneous population of LU3-7 cells showed only a 4 fold increase in the rate of DNA synthesis during the G1-S phase transition. This was in sharp contrast to the synchrony obtained when the clone L2 was analyzed. Here there was a 20 fold increase in the rate of incorporation of \(^3\)H thymidine between 2.5 and 16 hrs after mitotic selection. Autoradiographic analysis showed that 70 \% of the nuclei at 17 hrs were actively engaged in DNA synthesis. Therefore, this method of synchronization does indeed give a high degree of synchrony and was a good system to study cell cycle regulated events. The degree of synchrony that I obtained with this cell line closely agreed with that which Farnham and Schimke obtained with the MTX-resistant mouse 3T6 fibroblast. In their case the difference in incorporation between 2 h and 14 h was approximately 45 fold (Farnham and Schimke, 1985b).

Next, to see if the TS enzyme content fluctuated in a normal cell cycle, I measured the level of the enzyme/cell at different times after mitotic selection. As the results indicate the level of the enzyme went up only by a factor of 2. The fact that overall protein content also goes up by a factor of 2 as cell traverse from G1 to S phase, implies that the specific activity of TS increased less than 2 fold.
As mentioned earlier TS is a relatively stable enzyme with a half life of 36 hrs (Navalgund et al. 1980), therefore is not expected to fluctuate by more than a factor of 2. This is supported by the observation made by Conrad et al. who observed only a 2 fold increase in the enzyme activity during G1-S phase transition in colcemid synchronized hamster cells (Conrad, 1971). Even though this is the case, it does not mean that TS is not a S phase enzyme. Histones, are another example of proteins that are very stable with extremely long half lives, that show only a 2 fold increase as cell traverse the cell cycle. But the amount of histone mRNA increases 50 fold during S phase, strongly suggests that histones are indeed S phase proteins (DeLisle et al. 1983). Further the presence of translatable histone mRNA on the polysomes of S phase and not of G1 phase cells, suggests that this is a S phase specific protein (Heintz et al. 1983). Therefore the fact that there is an increase in the TS mRNA content in S phase supports the view that TS enzyme is synthesized primarily in the S phase (Nagarajan and Johnson, 1989).

Similar results were obtained by Mariani et al. in the case of dihydrofolate reductase. They looked at the cell cycle regulation of DHFR in methotrexate-resistant Chinese hamster ovary cells that were synchronized by mitotic selection. They found that the content goes up by a factor of 2.5 while the content of the total soluble protein goes up by 50% (Mariani et al. 1981b). Since DHFR and TS are both involved directly with DNA replication it is not surprising that they are regulated in a similar manner. These enzymes are primarily needed during the S phase therefore it is logical their synthesis occurs
during the G1-S phase transition.

Navalgund et al. had shown that in 3T6, the increase in thymidylate synthase activity was due to an increased rate of synthesis of this enzyme and that the synthesis of this enzyme required transcription of the gene. This, therefore suggested that the mRNA for this enzyme also accumulated at the same time as the enzyme (Navalgund et al. 1980). Later it was shown in the overproducing cell line LU3-7, that in a serum induced transition from resting to growing state, the content of TS mRNA went up 20-40 fold in the S phase. Like the enzyme, the TS mRNA is relatively stable. The half life of the mRNA in resting cells is 9 h and in growing cells it is 7 h (Jenh et al. 1985b). When I analyzed the levels of TS mRNA in cells that were mitotically selected, I observed that there was an 8 fold increase in the mRNA between 5 and 15 hrs after selection. When I looked at the level of the mRNA at 2.5 hrs the difference in the fold increase between 2.5 and 15 hrs after selection was even greater. The 5-10 fold increase in the mRNA accumulation during S phase is in line with the fact the TS mRNA has a half life of 7 hrs and the doubling time of the LU3-7 cells is about 20 hr. If synthesis of the message ceases during mid S phase, then the content of the TS mRNA relative to the total mRNA could be expected to decrease 8-fold (Two half lives of the mRNA and a doubling of total mRNA content) between S phase and G1 phase.

As mentioned before there are several cases where growth induced transcriptional increases have been observed. Primase p49, is one such gene that shows changes in the mRNA content under growth induced conditions but
remains invariant during the normal cell cycle. This enzyme synthesizes oligoribonucleotides for the initiation of DNA replication. Tseng et. al. analyzed the fluctuation of the Primase p49 mRNA in 3T6 cells undergoing a serum-induced transition from resting to growing state. Upon serum stimulation the primase mRNA increased about 10 fold around 10 hr after reentering the cycle. However when the level of mRNA was studied in continuously growing L1210 mouse lymphoma cells synchronized by centrifugal elutriation, Primase p49 mRNA remained invariant during the cell cycle. 10 fractions were obtained by centrifugal elutriation and the cell cycle stage of each fraction was based on the cellular DNA content. p49 mRNA showed little change between early G1 and mid-S-phase whereas histone H3 mRNA levels increased about 4 fold (Tseng et al. 1989). There are several problems in these analyses, first, they compared regulation of a stage specific event in two different cell lines. 3T6 mouse fibroblasts were used in their serum stimulation experiments and L1210 a mouse lymphoma cell line, was used in the continuously growing cells experiments. Not only are the two cells different but their generation times are quite different. The generation time of the 3T6 cells is 20 hr (Navalgund et al. 1980) and the generation time of L1210 is 12 hrs (Rode et al. 1980b). Therefore to arrive at a conclusion that p49 is growth regulated and not regulated in a cell cycle manner may not be accurate. To be able to differentiate between growth regulated and cell cycle regulated event they should have followed the transcription of p49 gene in the same cell line but synchronized by two independent methods.
Similar observations were made regarding the expression of thymidylate synthase gene in L1210 cells by Imam et al. (Imam et al. 1987). In this paper the authors looked at the changes in the level of TS mRNA in mouse lymphoma cells in continuously growing cells. Again centrifugal elutriation was used to separate cells in different stages of the cell cycle and mRNA levels were analyzed on northern blots. They came to the conclusion that TS mRNA remained invariant in a normal cell cycle. They also implied in their discussion, accumulation of TS mRNA in serum induced transition from resting to growing state need not imply that the mRNA accumulates in a normal cell cycle (Imam et al. 1987). However when making their observations they did not take into consideration that TS mRNA is relatively stable and that one does not expect to see changes of such a stable message in a cell line with a generation time of 12 hrs. Again here, the authors were comparing accumulation of TS mRNA in two different cell lines which have vastly different generation times 12 hrs (L1210) versus 20 hrs (LU3-7). However in my case TS mRNA levels was analyzed in the same cell line using two independent methods to obtain synchronization. Therefore, it is very evident that in studies presented here, TS gene expression is regulated in a cell cycle manner in both serum induced transition as well as in a normal cell cycle in the overproducing cell line LU3-7 mouse fibroblasts.

These studies also bring out the following: i) comparisons between growth induced events and cell cycle regulated events should be conducted in the same cell line; ii) when studying fluctuations of stable mRNA, in a normal cell cycle, the generation time of the cell line should be taken into consideration; iii) the
method used to obtain synchrony is very important when studying stage specific events. When analyzing changes in the level of a stable message like TS, a slight contamination of G1/S phase cells can give rise to erroneous conclusions. In such a case it will appear as if (within experimental error) there is little or no change in the levels of the mRNA as the cells progress from G1 to S phase. This can occur in a synchronization procedure such as centrifugal elutriation. Obtaining stage specific homogeneous populations of cells based on cell size may not be the best method to obtain synchronization.

**Events in G1 Influence the content of TS mRNA in S phase**

Regulation of the cell cycle appears to occur due to interactions between different gene products and genes. So far very little is known about the genes involved and the roles they play. To identify and analyze these genes would give us more insight as to the hierarchial order of events in a cell cycle. Most of the work done so far has been with respect to the protooncogenes. When serum arrested cells are stimulated to reenter the cell cycle, several genes are activated. When activation of genes occurs even in the presence of protein synthesis inhibitors it implies that these are probably the primary events in the cycle. For example, when inhibitors of protein synthesis were used to see which genes (during growth stimulation) were dependent on new protein synthesis, Greenberg et al. came to the following conclusion (Greenberg et al. 1986). They found that in 3T3 mouse cells, c-fos and c-myc cytoplasmic mRNA expression occurred not
only in cycloheximide pretreated serum stimulated cells but also in cells treated with another protein synthesis inhibitor, anisomycin. They used two different protein synthesis inhibitors to further confirm their results. However when the same experiments were done in another cell line PC12 cells (a pheochromocytoma cell line) induction of c-myc is completely blocked in the presence of cycloheximide. They concluded that the activation of c-myc may be mediated by the same factor in both cell lines, but in PC12, the factor may be present in limiting amounts. These results from PC12 also suggest that c-myc activation requires active protein synthesis but c-fos accumulation (which precedes c-myc), does not (Greenberg et al. 1986). Thus it is very tempting to speculate that regulation of the cell cycle may involve a cascade of events.

If the activation of c-fos is a primary event, then what are the events that affect the activation of genes that turned on later in the cell cycle? The hypothesis that I wanted to test was the following. If regulation of the cell cycle was a consequence of a series of events, then activation of the TS gene must also be under the control of events that occur during G1. First of all I wanted to know if protein synthesis was necessary for the activation of TS gene expression. Second, if it was necessary, was it also necessary for the activation of other S phase genes. Third, what steps in the biogenesis of TS mRNA needed active protein synthesis during G1.

First I will briefly review the regulation of two housekeeping genes thymidine kinase and dihydrofolate reductase and then later try to compare the regulation of these S phase genes. Thymidine kinase catalyses the phosphorylation
of thymidine to form thymidylic acid in the salvage pathway during the biosynthesis of thymidylate. TK enzyme content goes up 10-20 fold as the cells progress from G1 to S phase (Denhardt, 1966; Littlefield, 1966). The increase of the enzyme in S phase has been attributed to an increase in the transcription of this gene (Stuart et al. 1985). When the rate of transcription was analyzed in serum induced cells it was found the rate of transcription of TK went up only by a factor of 3-4 as cells went from resting to growing state (Stewart et al. 1987). However when rate of transcription was analyzed at close intervals around the G1/S phase boundary, there was a sharp burst of transcription at 9h after serum stimulation, but the level dropped by 12 hrs. These results showed that this increase in transcription was about 6 fold. As is the case in TS, the increase in TK mRNA content far exceeds that of the rate of transcription of this gene. This strongly suggested that the expression of this gene was controlled at both the transcription and post-transcriptional levels.

According to Stewart et al. the post-transcriptional regulation was independent of the promoter. That is, the sequences within the TK cDNA are sufficient to confer cell-cycle regulated expression of the gene (Stewart et al. 1987). However, in 1988, Travali et al. reported that the 5' flanking sequences of the human TK promoter played an important role in the cell-cycle regulation of TK mRNA (Travali et al. 1988). Both of these groups used chimeric constructs of the TK gene. The reasons for the conflicting reports from these two labs seems to be in the choice of the vectors which have different SV40 polyadenylation signals. Thus, when analyzing regulatory elements of a gene, great caution should be
exercised as far as interpretation of data and the vectors used.

Coppock and Pardee had suggested that regulation of TK mRNA content was governed by a labile protein. This labile protein seems to complete its function 2-3 hr before the S phase and the appearance of TK mRNA. The role played by this protein is not clear, but a high rate of protein synthesis seems critical during the first 10 hours after serum stimulation for the accumulation of TK mRNA (Coppock and Pardee, 1987). Further Gudas et al. presented evidence to suggest that quiescent cells lacked some factor required for processing of TK hnRNA. They suggested that this function is gained at the onset of S phase. Therefore in this case the authors suggest that post-transcriptional control of TK mRNA abundance is regulated by a nuclear processing event(s) (Gudas et al. 1988).

Thus at least in the case of TK, regulation of gene expression appears to be complex and occurs in a number of different ways. To date no clear picture seems to be emerging as to how the cell-cycle regulation of TK actually occurs.

DHFR is required for the reduction of dihydrofolic acid to tetrahydrofolic acid. The latter is an important co-factor in many single carbon transfer reactions as well as in the de novo synthesis of thymidylic acid and purines. Regulation of DHFR gene expression again seems to be both transcriptional and post-transcriptional. Previous work from our lab (Wu and Johnson, 1982; Santiago et al. 1984) showed that regulation of DHFR gene expression is at the transcriptional level during serum-induced transition from resting to growing state. Farnham and Schimke reported that in cells synchronized by mitotic selection
regulation was controlled at the level of transcription (Farnham and Schimke, 1985b).

However under growth-stimulated conditions Leys and Kellems reported that the appearance of cytoplasmic DHFR mRNA depended on the relative stability on the hnRNA and not on the rate of transcription. When metabolism of dhfr mRNA was studied in cells synchronized by amino acid-starvation, processing of the hnRNA appeared less efficient in starved vs growing cells. Also the content of the DHFR RNA in the nuclei was three times greater in starved cells as compared to content in the exponentially growing cells (Leys et al. 1984; Leys and Kellems, 1981). Later it was shown that under these conditions, delayed processing and/or export could cause the reduced level of cytoplasmic mRNA in starved cells (Leys et al. 1984).

Thus it appears that under different conditions used for obtaining synchrony, the regulation of gene expression appears to be different. The fact that during a normal cell cycle the regulation appears to be at the transcriptional level seems to suggest at least in the case of DHFR this may be a major regulatory mechanism. However, control at other levels cannot be ruled out.

The results presented here indicate that accumulation of TS mRNA is dependent on active protein synthesis during G1. It appears, some factor/s made in G1 during the first ten hours after serum stimulation may be responsible for the increase in the content of the TS mRNA during S phase. These results strongly suggest that the same R protein as defined by Coppock and Pardee may be involved in the appearance of TK and TS during the cell cycle. The fact that
the accumulation of three S phase mRNAs appears to be dependent on active protein synthesis during G1, strongly supports the hypothesis that, a G1 protein/s may be playing a key role in the activation of these S phase genes.

Next I wanted to identify the step/s in the biogenesis of TS mRNA that were sensitive to protein synthesis inhibitors. Inhibiting protein synthesis during the first ten hours after serum stimulation does not seem to affect the rate of transcription significantly. This rules out the possibility that a specific or a general transcriptional factor with a fairly short half life is being synthesized during the first ten hours after serum stimulation. Further when the production of TS mRNA was studied, there was a two fold decrease in the presence of cycloheximide. This suggests that the addition of cycloheximide in G1 affected the production of TS mRNA. Since mRNA processing occurs at several steps, I first wanted to see if it affected the distribution of the TS mRNA in the two cellular compartments. That is, if addition of cycloheximide in G1 affected splicing of the TS hnRNA in S phase then, there should be a build up of unprocessed RNA in S phase nuclei. Before I addressed that question, I first studied the distribution of the TS RNA in cells undergoing a serum-induced G1 to S phase transition.

The first step in analyzing the distribution of TS mRNA in the nuclear and cytoplasmic compartments was to isolate nuclear RNA free of any cytoplasmic contamination. Given the fact that only 10% of total cellular RNA is nuclear, even a slight contamination of cytoplasmic RNA fraction would give erroneous results. To minimize this, the nuclear pellet was subjected to a second detergent, so that the outer nuclear membrane and the attached endoplasmic reticulum were
removed (Penman, 1969). Northern blot analyses, indicated that there is no build up of unprocessed message during G1. To further confirm these observations, nuclear and cytoplasmic RNA from exponentially growing cells were analyzed by northern blot analyses. Using a uniformly labeled probe I was still unable to detect unprocessed RNA in the nuclear compartment. One can argue that the RNA in the nucleus could be due contamination by the cytoplasmic fraction. This may be the possibility even though I had tried to minimize this from occurring by treating the nuclear pellet with a double detergent (NP-40 and Tween-DOC). Besides, since I was only looking at the content (steady state levels) in the two compartments in G1 and S phase, it is highly probable that TS hnRNA may be rapidly processed or destroyed in the nuclei of G1 and exponentially growing cells and therefore could not be detected in these analyses. However it was puzzling that RNA corresponding in size to the mature TS mRNA, appeared in the nuclear fraction during G1 prior to its appearance in the cytoplasm. This could mean that mature TS RNA accumulated in the nucleus in G1, and during S phase there was an increase in the efficiency of transport of TS message. These results suggest that the regulation of TS gene expression could be at the level of nuclear RNA processing or export.

If regulation did in fact occur at the level of processing, was this event sensitive to protein synthesis inhibition during G1? If this processing event was indeed sensitive to protein synthesis inhibitors, then, in the presence of cycloheximide the turnover of the hnRNA and/or the export of the mature mRNA in S phase would be inhibited. This would result in the accumulation of
unprocessed hnRNA and/or mature RNA in the nucleus. Based on this assumption, I analyzed the distribution of TS RNA in the nucleus and the cytoplasm in cells that were treated with cycloheximide during G1. From my analyses, I was unable to resolve this question. I detected only mature RNA in the nucleus but the distribution of TS RNA between the two compartments did not suggest a build up in the nucleus.

Summarizing the results in this section, I can conclude that first, TS gene expression is dependent on the events occurring in the G1 phase and second the cycloheximide sensitive event appears to be regulating the production of TS mRNA. The accumulation of TS mRNA in S phase, may be dependent on a factor synthesized during G1 that is involved in 3' processing. If this were the case, addition of cycloheximide in G1 would result in the increase of unprocessed RNA in the nucleus. The fact I could not detect any unprocessed RNA in the nucleus could be attributed to the fact that this species of RNA may be highly unstable making it virtually impossible to detect. Also, if the TS RNA in the nucleus was not polyadenylated, the size of the hnRNA would be very heterogeneous and would spread over the whole lane making it harder to detect.

Luscher and Schumperli suggest that, the regulation of histone H4 RNA levels in the cell cycle may be controlled at the level of 3' processing. They studied the processing of histone H4 in vitro, using extracts from arrested and exponentially growing 21-Tb cells. Their studies strongly indicated that a factor needed for the 3' processing becomes limiting in the G1 arrested cells (Luscher and Schumperli, 1987). Extending this hypothesis, one can propose that TS
mRNA level may also controlled by regulating polyadenylation during the cell cycle. If this were the case, then, inhibiting protein synthesis during G1 would further limit the amount of this factor, resulting in a delay in the appearance of mature message in the S phase. This may well be the reason why inhibiting protein synthesis during the first 10 hours after serum stimulation showed a decrease in the S phase accumulation of TS mRNA.

**Attenuation and TS gene expression**

The increase in the rate of TS gene transcription in S phase does not account for the increase in the content of the TS mRNA. Recently, several laboratories have suggested that attenuation or RNA polymerase pausing may be involved in the regulation of transcription in a number of eukaryotic genes (Nepveu and Marcu, 1986; Bender et al. 1987; Fort et al. 1987). Therefore there was a possibility that attenuation may regulate the content of TS mRNA during the G1-S phase transition. The results of my experiments indicated that attenuation is not involved in the regulation of TS gene expression. Even though attenuation may not be regulated, the fact that there is twice the amount of RNA corresponding to the 5' end of the TS cDNA as compared to the 3' end suggests that there maybe some sort of pausing of the RNA polymerase as it transcribes the gene. If premature termination or pausing occurs during transcription, the next question would be where does it occur. To answer this, first the sequence of the gene (introns and exons) has to analyzed to see if there is a potential for the
formation of secondary structures. If this is the case, then one can hypothesize that, these secondary structures may stall or slow the movement of the polymerase resulting in a higher density of active polymerases in the 5' end of the gene.

While doing run on analysis, I had detected hybridization to sense TS RNA transcripts that were immobilized to the filter. This prompted me to investigate the occurrence of bidirectional transcription from the TS gene. This had not been a focus of our earlier studies. To check if there was indeed bidirectional transcription from this gene, I analyzed RNA isolated from whole cell, cytoplasmic and nuclear compartments from exponentially growing LU3-7 cells. As mentioned in the results, with the antisense RNA probe, a 1.3 kb RNA transcript (corresponding to the TS mRNA) was detected. However when the same RNA preparations were probed with the sense RNA probe, I detected several different sized RNA species. The sizes of these transcripts ranged from 4.4 kb to about 1 kb. These multiple transcripts have yet to characterized. Since all the above studies were done using total RNA isolated from LU3-7 cells, one has to analyze whether this occurs also in the parental 3T6 cells. Next, are these opposite strand transcripts polyadenylated or not. If they are not, are they confined only to the nuclear compartment? If they are polyadenylated, then, what do they encode? The next step would be to map these transcripts. Finally, the most important question to be answered would be, does the transcription of this gene affects TS gene expression. These investigations are yet to be carried out.
Occurrence of promoters within the coding region of eukaryotic genes is an unusual feature. In the case of DHFR, there appears to opposite strand transcription but not from within the coding region of the gene (Farnham et al. 1985a). If these transcripts do indeed originate from a promoter located within the TS gene, then this could be a very exciting observation. It is very tempting to speculate that the promoter may be located in the introns. So far, the sequence of introns 5 and 6 of the TS gene are known (Deng et al. 1989a). There are TATAA boxes, octamer binding sites, Z DNA etc in these introns. The significance of these sequence remains to be determined.

These studies indicate that the use of RNA-RNA hybridization in run on assays is a good, reproducible and highly sensitive method to study rate of transcription. Also it emphasizes the importance of using single stranded RNA probes when analyzing northern blots. These probes are very specific and label very efficiently. The northern blots probed with these probes can be treated with RNase and can be washed under very stringent conditions. Not only does this reduce the background but it also indicates that any bands that are detected are genuine and not due to non specific hybridization.

**Effect of Heat Shock on TS gene Expression**

In the third section of my dissertation I analyzed the effect of heat shock on the transcription of TS. The reason for doing these analyses has been mentioned earlier. Briefly there is a sequence present in the critical region of the
TS promoter that has a 7/8 bp match to the consensus sequence of the heat shock element (Lindquist, 1986). When the rate of TS genetranscription was analyzed in nuclei of heat shocked LU3-7 cells, there was no change in the rate as compared to the rate in the control nuclei. This result was rather puzzling as transcription of most cellular genes is turned off during heat shock except those of the heat shock genes (Ashburner and Bonner, 1979). This could mean either the presence of the heat shock element in the promoter facilitates the binding of the HSTF or the binding of some other factor that recognizes the same motif. Studies are currently being conducted in our lab to identify this factor/s. When the export and content of TS mRNA was analyzed in heat shocked cells, it was evident that TS behaved as any other cellular gene. It is not yet clear exactly how processing is affected by heat shock. According to Yost and Lindquist, heat shock is thought to have a global effect on RNA splicing. They suggest that the rapid transport of the heat shock genes during heat shock could be attributed to the fact that almost all heat shock genes lack introns. Of the 70 or so heat shock genes that have been cloned and analyzed, only two have been identified as having introns, the hsp 83 gene of *Drosophila* and one of the small hsp genes of nematodes (Lindquist, 1986). Thus it is thought that at higher temperatures, there is general block in intron processing. This could explain why during heat shock there is preferential transport of the heat shock mRNA to the cytoplasm where it is translated. Therefore these genes are able to circumvent the global block on splicing whereas most cellular genes cannot (Lindquist, 1981). Also during heat shock most cellular RNAs are not degraded during heat shock, there is just a
preferential translation of heat shock RNA (Storti et al. 1980; McGarry and Lindquist, 1985). The fact that the cytoplasmic content of the TS mRNA content remained unchanged during heat shock further confirmed our finding that TS gene expression is not heat inducible. It has been known that normal cellular messages are not degraded or inactivated, since they can be isolated and translated in vitro. Apparently high temperature just establishes a change in translational specificity (Storti et al. 1980). Therefore it was concluded by McGarry and Lindquist that, the preferential translation of heat shock messages depends of certain sequences that are present in the 5' untranslated leader regions of the heat shock genes (McGarry and Lindquist, 1985).

These results strongly suggest that TS gene expression responds to elevated temperatures like most other cellular non heat shock genes but differs from them in one aspect i.e. heat shock does not affect the rate of transcription of this gene. Preliminary studies from our lab have indicated that some protein does indeed bind to the critical region of the TS promoter but the identity and the physiological significance of this interaction is not known. As mentioned earlier in the Introduction, a single HSE is enough to confer heat inducibility in mammalian cells. The human HSP70 gene has a single HSE element (Wu et al. 1986). Therefore it is possible that some transcription factor related to the HSTF may bind to the TS gene promoter and regulate transcription of this gene.

Summarizing the work presented here, it is evident that TS is regulated in a cell cycle manner under serum-induced transition from resting to growing as well as in a normal cell cycle. The increase in the content of TS mRNA is
dependent on active protein synthesis during G1. Regulation of TS mRNA content during the cell cycle seems to occur at the level of processing, most likely at the level of polyadenylation. Attenuation does not seem to regulate the level of TS mRNA during the cell cycle. This gene appears to be transcribed bidirectionally and it appears that opposite strand transcription occurs within the coding region of the gene. In spite of the fact that there is a HSE element in the promoter of the TS gene, this gene is not heat inducible. At the same time, heat shock does not turn off the transcription of this gene.
Another project that I was involved in was the characterization of a cell line that was developed in our lab by Dr. Chung-Her Jenh. He exposed the TS overproducing mouse fibroblast cell line LU3-7 to increasing concentrations of methotrexate in the presence of $3 \mu M$ flurodeoxyuridine. He then isolated populations of cells that were able to grow in the presence of both these drugs. Therefore these cells MF1 were able to grow in the presence of $3 \mu M$ FdUMP and $50 \mu M$ Methotrexate. The rationale for developing this cell line was the following. If resistance to these two drugs was achieved by gene amplification, i.e. amplification of the mouse TS gene (which in this case is the starting cell line with 50 fold amplification of the TS gene) as well as amplification of the mouse DHFR gene, then, this system would be a good model system to study regulation of both these genes in the same environment.

I characterized the degree of gene amplification by performing Southern blot analyses on the genomic DNA from MF1 and compared it to the TS overproducing cell line LU3-7 and the DHFR overproducing cell line M50L3. I further looked at the content of these two RNA species, TS and DHFR in the double drug resistant cell line to see if there was a corresponding increase in the RNA levels when compared to the overproducing cell lines.
Degree of Amplification

Here high molecular weight genomic DNA was isolated as described in the Materials and Methods from MF1, LU3-7 and M50L3 cell lines. 10 μg of genomic DNA from each cell line was subjected to digestion with the restriction enzyme PstI. The digestion products were then subjected to gel electrophoresis. The gel system was used in these experiments was 1% Agarose dissolved in TAE buffer. Before transferring the gel, it was stained and photographed to ensure that the DNA was completely digested and there was equal amount of present in each lane. The DNA was transferred onto a Nytran membrane as described earlier. The filter was hybridized with either a uniformly labeled pMTS-3 or a pDHFR21 (pMTS-3 is the cDNA of TS and pDHFR is the cDNA of DHFR gene). The results are seen in Figure 20, in Panel A the southern blot was hybridized with pMTS-3 and Panel B with pDHFR. Lane 1 contains 10 μg of genomic DNA from LU3-7, lane 2 has 10 μg of DNA from MF1 and lane 3 has 10 μg of DNA from M50L3 cell lines. The marker used in these gels was pBR322 restricted with HindIII. This figure clearly shows that in the case of MF1 resistance to DHFR was achieved by amplification of the DHFR gene. The degree of amplification appears to be the same as the overproducing cell line M50L3, (Panel B lanes 2 and 3) that is, 300 fold (Weidemann and Johnson,1979). At the same time this cell line maintains the 50 fold amplification of the TS gene as seen in Panel A, lanes 1 and 2 (Jenh et al. 1985a). Therefore this cell line has both TS and DHFR genes co-amplified in the presence of FdUMP and methotrexate.
Figure 20. Southern Blot Analyses

High molecular weight genomic DNA was isolated from LU3-7, MF1 and M50L3 cell lines and 10 μg of this DNA from each was restricted with the restriction enzyme PstI. The DNA was then subjected to Southern blot analyses as described in the Materials and Methods.

Panel A. This filter was probed with a uniformly labeled plasmid corresponding to the TS cDNA, pMTS-3.

Panel B. This filter was probed with a uniformly labeled plasmid corresponding to the DHFR cDNA, pDHFR21.

Lane 1. 10 μg of genomic DNA from LU3-7 cell line.
Lane 2. 10 μg of genomic DNA from MF1 cell line.
Lane 3. 10 μg of genomic DNA from M50L3 cell line.

The marker used was pBR322 restricted with HindIII.
**Content of TS and DHFR mRNAs in MF1**

Next, I analyzed the content of TS and DHFR mRNA in this co-amplified cell line MF1. Here cytoplasmic RNA was isolated from the three cell lines and subjected to poly(A) selection. 5 µg of poly(A)+ RNA from LU3-7, MF1 and M50L3 cell lines were subjected to northern blot analyses. Figure 21 shows the results of one such northern blot where the filter was hybridized with a uniformly labeled probe corresponding to the TS cDNA, pMTS-3. Lanes 1 and 2 contain RNA from LU3-7, where lane 1 has 10 µg and lane 2 has 5 µg of poly(A)+ RNA. Lanes 3 and 4 contain RNA from the co-amplified cell line MF1, lane 3 has 10 µg and lane 4 has 5 µg of poly(A)+ RNA. As one can see, there appears to be 4 fold decrease in the amount of TS message in the MF1 cell line. This could be due to unequal amounts of RNA analyzed. Further experiments confirmed that this was the case. Therefore there appears to a corresponding increase in the TS mRNA as compared to the overproducing cell line LU3-7.

Similar northern blot analyses was carried out to determine the content of DHFR mRNA in this cell line. Here 10 µg of poly(A)+ RNA was subjected to northern blot analyses and hybridized with uniformly labeled pDHFR21, a DHFR cDNA. Figure 22, shows the results from one such experiment. Here lanes 1,2 and 3 contain 10 µg of poly(A)+ RNA from LU3-7, MF1 and M50L3 cell lines respectively. As one can see unlike the TS mRNA, there are several species of DHFR mRNA (Setzer et al. 1982). The MF1 cell line appears to have the same
Figure 21. Northern Blot Analyses

Here poly(A)+ RNA from LU3-7 and MF1 was subjected to Northern blot analyses. The probe used here was uniformly labeled pMTS-3, the TS cDNA.

Lane 1. 10 µg of poly(A)+ RNA from LU3-7 cell line.
Lane 2. 5 µg of poly(A)+ RNA from LU3-7 cell line.
Lane 3. 10 µg of poly(A)+ RNA from MF1 cell line.
Lane 4. 5 µg of poly(A)+ RNA from MF1 cell line.

Markers used corresponded to the 28S and 18S ribosomal RNA species.
Figure 22. Northern Blot Analyses

Here poly(A)+ RNA isolated from LU3-7, MF1 and M50L3 cell lines were subjected to Northern blot analyses as described in the Materials and Methods. The probe used here was uniformly labeled DHFR cDNA, pDHFR21.

Lane 1. 10 \( \mu \text{g} \) of poly(A)+ RNA from LU3-7 cell line.
Lane 2. 10 \( \mu \text{g} \) of poly(A)+ RNA from MF1 cell line.
Lane 3. 10 \( \mu \text{g} \) of poly(A)+ RNA from M50L3 cell line.

The marker used corresponded to the 28S and 18S ribosomal RNA species.
FIGURE 22.
amount of DHFR mRNA as the overproducing cell line M50L3. This further supported the earlier Southern blot analyses, that this cell line achieved resistance to methotrexate by amplification of the DHFR gene.

Thus, this cell line MF1 has achieved resistance to methotrexate by amplification of the target gene, DHFR. The degree of amplification of this gene is 300 fold, similar to the overproducing cell line M50L3. This cell line also shows a corresponding increase in the DHFR mRNA content. This cell line would form a good model system where regulation of TS and DHFR can be studied in the same environment. I did use this cell line in some experiments especially where I wanted to look at the effect of cycloheximide on the content of TS and DHFR mRNAs in the same cell. The two genes showed the same response as seen in LU3-7 and M50L3 cell lines. They are able to rest well in 0.5% calf serum and appear to regulate both these genes in a cell cycle manner.
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