Umidahan DJAKBAROVA

M.S. Thesis In Genetics and Bioengineering

February-2012

INVESTIGATING THE ROLE OF CYCLIN DEPENDENT KINASE 2 IN TRIGGERING LATE G1 EXPRESSION OF STEM LOOP BINDING PROTEIN AS A MAJOR MECHANISM TO REGULATE HISTONE PRODUCTION

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Umidahan DJAKBAROVA

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in

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

_____
Prof. Dr. Halil Rıdvan ÖZ
Head of Department

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

_____
Assist. Prof M. Murat KÖSEOĞLU
Supervisor

Examining Committee Members

Assist. Prof. M. Murat KÖSEOĞLU

Assoc. Prof. Barık SALİH

Assist. Prof. Gönül SCHARA

It is approved that this thesis has been written in compliance with the formatting rules laid down by the Graduate Institute of Sciences and Engineering.

_____
Assoc. Prof. Nurullah ARSLAN
Director

February, 2012

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Supervisor: Assist. Prof. Mehmet Murat KÖSEOĞLU

ABSTRACT

Metazoan replication dependent histone mRNAs are the only eukaryotic cellular mRNAs that are not polyadenylated. Synthesis of mature histone mRNA requires only a single processing reaction: an endonucleolytic cleavage between a conserved stem-loop and a purine-rich downstream element to form the 3' end. The stem-loop binding protein (SLBP) is required for histone mRNA processing and involved in transport of the mRNA to the cytoplasm, where SLBP participates in translation of histone mRNA and regulation of histone mRNA stability. SLBP expression is limited to S phase and cell cycle regulation of SLBP is one of the major mechanisms that restrict histone mRNA metabolism and thus histone production to S phase.

The level of SLBP is low during early G1 and dramatically increase as cells enter to S phase. It has been shown that low level of SLBP is due to low translational efficiency of SLBP mRNA and translation of SLBP increases as cells approach to S phase. High level of SLBP is maintained throughout the S phase and degraded at S/G2 border depending on phosphorylation of N-terminal SFTTP sequence. Another regulated degradation of SLBP was shown to be present at mid G1 phase, which keeps SLBP level low until the subsequent S phase. The molecular details of G1 regulation of SLBP needs to be elucidated further.

In this dissertation, I have worked on investigating the G1 phase regulation of SLBP. In order to facilitate studies concerning the G1 regulation of SLBP, I have generated stable cells expressing wild type and 'S/G2 degradation mutant' SLBP. The mutant form of SLBP will contribute for several factors including; determination of whether the degradation at late G1 phase is mediated via the same or distinct motif that is responsible for S/G2 degradation, providing detectable amount of SLBP enabling the

studies of SLBP regulation in G1 phase and elimination of the interference of S/G2 degradation with regulated degradation in G1/S transition.

In the second part of my thesis, I have analyzed the role of Cdk2, the major player of G1/S transition, on SLBP expression. Asynchronous Hela cells treated with chemical inhibitor (Roscovitine) of Cdk1 and Cdk2 have decreased SLBP level. For specific inhibition of Cdk2, I have transfected Hela cells with Cdk2 dominant negative and have shown that SLBP expression has dropped off in these cells. Further, in order to particularly examine the role of Cdk2 on rapid increment of SLBP at G1/S border, I have synchronized cells by different methods and treated them with Roscovitine at late G1 phase. Inhibition of Cdk2 activity at late G1 phase prevented SLBP increase. Thus, we have concluded that Cdk2 is required for SLBP expression and it triggers rapid increment of SLBP as cells approach to S phase.

Keywords: cell cycle, histone mRNA, SLBP, G1 phase, Cdk 2.

HİSTON ÜRETİMİNİN REGÜLASYONUNDA ÖNEMLİ OLAN STEM LOOP BINDING PROTEİN'İN GEÇ G1 FAZINDA CDK2 TARAFINDAN TETİKLENMESİNİN ARAŞTIRILMASI

Umidahan DJAKBAROVA

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ÖZ

Replikasyona bağlı histon mRNA'ları polyadenin kuyrugu olmayan tek ökaryotik hücre mRNA' larıdır. Histon premRNA'larının olgun hale gelmesi 3' ucunda bulunan korunmuş stem loop ikincil yapısından hemen sonraki endonükleatik kesimle mümkün olmaktadir. mRNA üzerindeki bu ikincil yapıya bağlanan SLBP (Stem Loop Binding Protein) histon mRNA larının işlenme süreci için gereklidir. Bununla birlikte SLBP olgun histon mRNA sının sitoplazmaya taşınmasında ve translasyonunda görevlidir. SLBP sadece S fazında ifade edilir. SLBP ifadesinin bu şekilde hücre döngüsüne bağlı olarak düzenlenmesi, olgun histon mRNA ve dolayısıyla histon üretiminin S faza sınırlandırılmasını sağlayan ana mekanizmalardandır.

SLBP seviyesi erken G1 fazında düşüktür ve hücre S fazına yaklaştığında ise önemli ölçüde artış meydana gelir. G1 fazının başlarında SLBP seviyesinin düşük olmasının SLBP mRNA translasyonun veriminin düşük olmasına bağlı olduğu ve bu verimin G1 fazının ortalarına doğru yükseldiği gösterilmiştir. Yüksek miktardaki SLBP ifadesi S fazı boyunca devam eder ve S fazının sonunda N uçta bulunan threonine 60 ve 61'in fosforilasyonuna bağlı olarak yıkılır. G1 fazda SLBP mRNA translasyonunun verimi yükselmesine rağmen protein seviyesi S faza kadar düşük seviyede tutulmasının sebebinin kontrollü bir yıkım mekanizmasi olduğu düşünülmektedir. Bununla beraber, SLBP nin G1 fazındaki regülasyonu hala bütünüyle açıklığa kavuşturulmamıştır.

Bu tez çalışmasının ilk bölümünde, G1 faz SLBP çalışmalarında kullanılmak üzere Thr 61 ➔ Ala 61 mutant SLBP üretilmiş ve bu mutant SLBP'yi stabil olarak

ifade eden hücre hattı oluşturulmuştur. Bu hücre hattı ile SLBP nin G1 fazındaki yıkımının S/G2 fazındaki yıkımı kontrol eden motiften bağımsız olup olmadığını göstermek mümkün olacaktır.

Tezin ikinci bölümü olarak, hücre döngüsünde G1 fazından S faza geçişte ana faktör olan Cyclin/Cdk2 nin SLBP ifadesindeki rolü araştırılmıştır. Bu amaçla, senkronize olmayan Hela hücreleri Cdk lerin kimyasal inhibitörü olan Roscovitine ile muamele edilmiş ve SLBP seviyesinin düştüğü gözlemlenmiştir. Hususi olarak Cdk 2 nin rolünü incelemek için Hela hücrelerde Cdk 2 nin dominant negatif mutant formu ifade edilmiş ve SLBP ifadesinin önceki deneyde olduğu gibi düştüğü gösterilmiştir. Bununla birlikte Cdk 2 nin özellikle G1 fazından S fazına geçişteki SLBP artışındaki rolünü incelemek için bu fazda senkronize edilmiş hücreler Roscovitine ile muamele edilmiş ve G1 fazından S fazına geçerken SLBP artışının engellendiği gösterilmiştir. Bu deneyler ışığında, Cdk 2 nin SLBP ekspresyonu için gerekli ve G1/S teki SLBP artışından sorumlu olduğu gösterilmiştir.

Anahtar kelimeler: hücre döngüsü, histone mRNA, SLBP, G1 fazı, Cdk2.

To my most beloved one…

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SYMBOLS/ABBREVATIONS

Ala	Alanide
APS	Ammonium PerSulphate
BrdU	Bromodeoxyuridine
$CaCl_2$	Calcium Chloride
Cdk 1	Cyclin Dependent Kinase 1
Cdk 2	Cyclin Dependent Kinase 2
Cdk 4	Cyclin Dependent Kinase 4
CFU	Colony Forming Unit
CKI	Cyclin Dependent Kinase Inhibitor
DMSO	Dimethyl Sulphoxide
dn	Dominant Negative
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemiluminescence
ELISA	Enzyme Linked İmmunosorbant Assay
FACS	Flourescence Activated Cell Sorter
FBS	Fetal Bovine Serum
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
HDE	Histone Downstream Element
HiNF-P	Histone H4 transcription factor
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
KCl	Potassium Chloride
KH_2PO	Potassium Pyrophosphate
LB Broth/Agar	Luria Betani Broth/Agar

LDH	Lactate Dehydrogenase
$MnCl_2$	Manganase Chloride
MOPS	3-(N-morpholino)propanesulfonic Acid
mRNA	Messenger Ribonucleic Acid
Na_2HPO_4	Sodium pyrophosphate
NaCl	Sodium Chloride
NaF	Sodium Floride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NPAT	Nuclear protein of the ataxia telangiectasia
OD	Optical Density
ON	Over Night
PBS	Phosphate Buffer Saline
Pen/strep	Pennicilin/streptomycin
PI	Propodium İodide
PVDF	Polyvinilidine Fluoride
$RbCl_2$	Rubidium Chloride
ROSC	Roscovitine
RT	Room Temperature
SDS PAGE	Sodium Dedocyl Sulfate Polyacrilamide Gel Electrophoresis
SDS	Sodium Dedocyl Sulfate
Ser	Serine
SL	Stem-Loop
SLBP	Stem Loop Binding Protein
snRNP	Small Nuclear Ribonucleoproteins
TEMED	Tetramethylethylenediamine
Thr	Threonine
WST-1	Proliferation marker
WT	Wild type

CHAPTER 1

1.1 INTRODUCTION

Progression through the eukaryotic cell cycle is defined by a timed succession of distinct events. A cell's regulatory mechanisms must guarantee the completion of DNA replication in S phase before chromosomes are segregated into two daughter cells in M phase. The periodic movement through cell cycle is driven by programmed oscillations in the CDKs, Ser/Thr kinases activities. The activity of CDK is dependent on binding of a cyclin regulatory subunit; inactivation regulated by association with a Cdk inhibitor (CKI). Entrance into S phase and progression through it requires cyclin E/Cdk2 and Cyclin A/Cdk2 respectively, ultimately triggering initiation of DNA replication and proper progression through S phase. After completion of S phase, the progression through Mitosis is sustained by cyclin B/Cdk1. In coordination with DNA replication, sufficient amount of histone proteins need to be synthesized in order to package newly synthesized genomic material. Histone mRNAs are unique among eukaryotes that they lack polyA tail but instead end up with conserved stem loop structure. An only event required for mature histone mRNA formation is the endonucleatic cleavage in between conserved stem loop and purine rich downstream element. Histone mRNA is tightly cell cycle regulated mostly via posttranscriptional mechanisms mediated by both *cis* acting and trans regulating elements. Stem Loop Binding Protein binds to histone mRNA stem loop at 3' end and is required/involved in all aspects of histone mRNA regulations including mRNA processing, translation and stability of histone mRNA.

SLBP expression is also tightly cell cycle regulated together with histone production, however SLBP mRNA level does not change significantly during cell cycle (Whietfield et al., 2000). SLBP protein level increases dramatically in G1/S border and kept high throughout the S phase followed by phosphorylation dependent degradation at the end of S phase. Degradation of SLBP at S/G2 border shuts down the histone biosynthesis in order to prevent excess expression of histones other than in S phase. The mechanism for rapid decrease in SLBP level at the end of S phase was elucidated by Dr. Koseoglu, where SLBP is phosphorylated at Thr 61 and Thr 60 by cyclin A/Cdk1 and CK II respectively and marks for proteasome mediated degradation (Koseoglu et al., 2008).

The previous studies have elucidated some parts of regulations of SLBP during G1 phase. It was shown that translation efficiency of SLBP is low during early G1 in Hela cells and somewhere in between early and mid G1, SLBP translation efficiency recovers back to S phase level (Koseoglu, 2008). However it is known that SLBP expression level does not recover until G1/S phase, which occurs around 3-4 hours later. To examine the reason for this regulation, SLBP stability was checked during this period and showed that the stability of SLBP is low after mid-G1 till the beginning of S phase. This reduced stability was proposed to be due to regulated proteasome mediated degradation of newly synthesized SLBP (Koseoglu, 2008). Despite of regulated degradation, the level of SLBP increases 10-20 fold at G1/S border. The mechanism that triggers rapid increase of SLBP in G1/S transition remains to be unknown.

In this dissertation, I aimed to investigate G1 regulation of SLBP. I have studied with two separate but related topics as my Masters project. Firstly, I have generated stable cell lines expressing wild type and mutant SLBP, which will be used as a tool for G1 phase SLBP regulation studies of. Secondly, I have investigated the role of Cdk2, the major regulator of G1/S transition, on rapid increment of SLBP at G1/S border.

In the first part of my thesis, I have generated stable cells expressing both wild type and ‘S/G2 degradation’ mutant SLBP. Due to its low expression at G1 phase, it is very hard to study regulatory mechanisms of SLBP. By preventing SLBP degradation at S/G2 border, we would be able to keep this level high as long as the half-life of SLBP in G1 phase allows. This can be achieved by mutating Thr 60 or Thr61 of SLBP into

Alanine. It has been shown that both Thr 60➔ Ala 60 and Thr 61➔ Ala 61 are stable at S/G2 (Koseoglu et al., 2008).

The mutated construct of SLBP will enlighten the responsible motif for regulated degradation in G1 phase. The similar regulation of mutant SLBP together with wild type, would indicate that the regulation in G1 phase is independent from S/G2 regulation.

Lastly, it has been proposed that the coding sequence of SLBP is sufficient and required for translation efficiency recovery of SLBP at mid-G1 phase (Koseoglu, 2008). Different assays targeting just the exogenous SLBP, which lacks the UTR regions and contains just the coding sequence, would further support the notion of the posttranscriptional regulations of SLBP.

To sum up, as the first part of my thesis, I have aimed to obtain Hela cells stably expressing 'S/G2 degradation' mutant of SLBP constructed from His-tagged wild type SLBP. Namely, I have mutated Thr61 into Ala61 with site directed mutagenesis and transfected Hela cells with both wild type and mutant SLBP constructs. Furthermore, in order to have stable expression of exogenous SLBP, I have treated transfected cells with selective antibiotics until the construct has been incorporated into the genome of the cells. The expression of exogenous SLBP in both transient transfected and stable Hela cells was confirmed by Western Blot analysis.

The aim of the second part of my thesis was to investigate the role of Cdk2 on SLBP expression at G1/S transition. Cyclin E /Cdk2 is upstream of both DNA synthesis and NPAT. NPAT is involved in both histone transcription and mRNA processing. The regulation of histone production is sustained majorly via posttranscriptional mechanisms and histone synthesis is tightly coupled to progression of DNA replication. SLBP is known to be essential for all steps of histone mRNA biosynthesis and it restricts histone mRNA expression to the S phase. All these evidences arise a question if Cyclin/ Cdk2 could be an upstream regulator of major player of histone mRNA production, namely SLBP synthesis just before entry into S phase. Although Cyclin/Cdk2 is predicted to be upstream regulator of SLBP, there is still ambiguity concerning this regulation which has to be investigated.

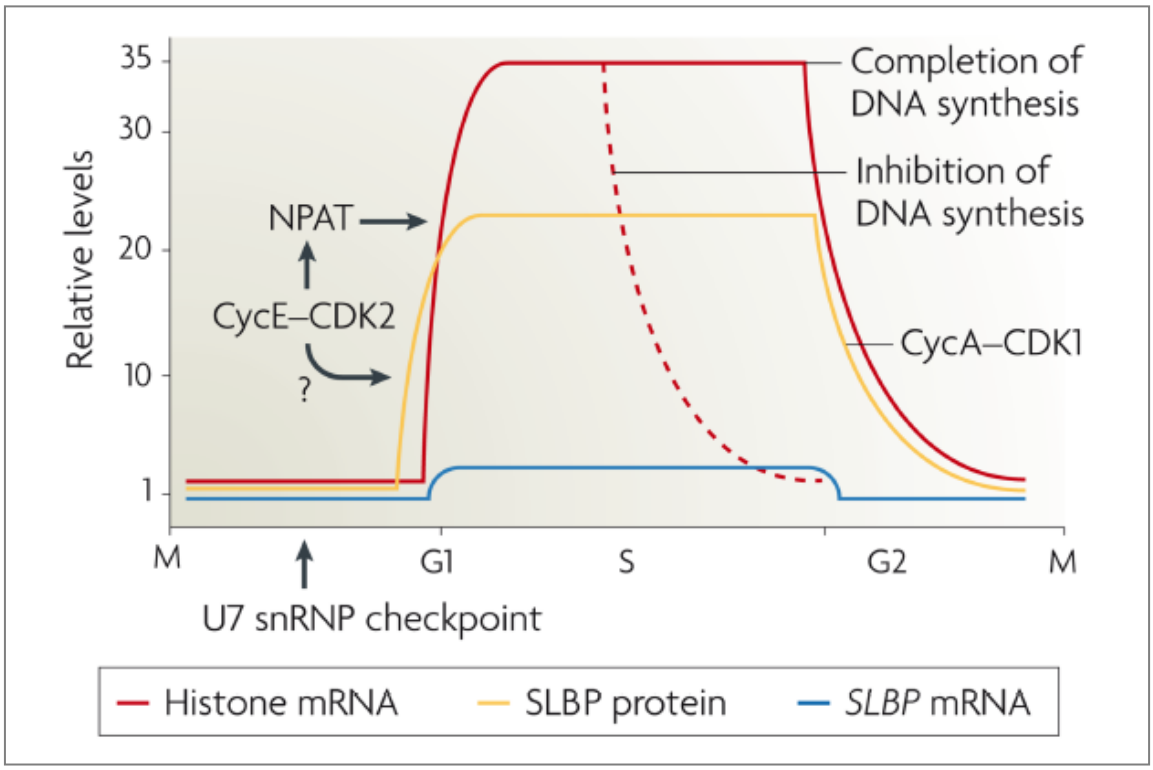

Figure 1.1 Cell cycle regulations of canonical Histone mRNA and SLBP (Marzluff, 2008).

Here, I have analyzed the role of Cdk2 in SLBP expression of both synchronized and asynchronous cells. In order to examine this, I have used a selective inhibitor of Cdks, Roscovitine and overexpressed Cdk2 dominant negative and analyzed their effect on SLBP expression by Western Blotting. Roscovitine is a cell permeable purine analog which competes for the binding site of ATP in the catalytic cleft of Cdk1 and Cdk2, thus selectively inhibits them (Meijer, 1987). For more selective inhibition of just Cdk2, I have used Cdk2 dominant negative, which is kinase dead mutant of Cdk2. In case of overexpression, it will compete and predominate the effect of Cdk2 activity (Hu et al., 2001, Osumu et al., 2003). These inhibitors were first tested on asynchronized cells and upon their treatment SLBP level has declined. Thus, we have concluded that Cdk2 activity is required for SLBP expression.

In order to examine the specific role of Cdk2 on rapid increase of SLBP at G1/S border, I have synchronized cells with different methods and released for one more cycle. Treatment of cells with Roscovitine at late G1 phase has prevented SLBP

increase at G1/S transition. Thus, this suggests that Cdk2 triggers rapid increment of SLBP as cells enter to S phase.

1.2 LITERATURE REVIEW

1.2.1 Cell Cycle and Its Control

Precise control of cell proliferation is essential for development and survival of all multicellular organisms. The deregulation of cell proliferation is a fundamental hallmark of all cancer cells (Hanahan and Weinberg, 2000). At the center of cellular proliferation is the cell division which is conducted by highly regulated series of events, also known as **cell cycle (**Baserga, 1985; Murray and Hunt, 1993; Morgan, 2007**)**. The most basic function of cell cycle is to duplicate accurately the vast amount of genome and segregate copies equally into two genetically identical daughter cells (Harris, 2000). So these processes define two major phases of cell cycle, S phase (S stands for DNA synthesis) and M phase (stands for Mitosis) separated by two *gap* phases, the G1 and G2 (Murray and Hunt, 1993; Enders, 2002). Chromosome duplication occurs during S phase, after the S phase: chromosomes are segregated and cell is divided into two daughter cells during the M phase followed by cytokinesis (Dean and Hinshelwood, 1965). Cell division comprises two events: nuclear division, which is also known as mitosis and cellular division, known as cytokinesis. Most cells require much more time to grow and double their mass organelles than they require to duplicate their chromosomes and divide. This time is sustained by the two extra gaps between two phases. Thus, the eukaryotic cell cycle is traditionally divided into four sequential phases: G1, S, G2, and M. The two gap phases are more than just simple time delays allow for cell growth, they also provide time for the cell to monitor the internal and external environment to ensure that conditions are suitable and preparations are complete before the cell commits itself to the major upheavals of S phase and mitosis.

A key concept of cell cycle is that it has to be progressed unidirectional; S phase must always follow M phase and M phase must not start until S phase has been completed successfully (Murray and Hunt, 1993; Garrett, 2001). In other words, subsequent DNA replication must not commence until mitosis and cytokinesis is

completed and mitosis must not begin until the previous round of DNA replication has been ended, thus, the integrity of genome is maintained.

Programmed progression through the cell cycle phases is driven by periodic activation and inactivation of family of Ser/Thr kinases called Cyclin Dependent Kinases (CDKs). Cyclin–dependent protein kinases (Cdks) are the engines that conduct the events of eukaryotic cell cycle and the clock that times them. In complex cell cycle, they function as information processors that combine and interpret extracellular and intracellular cellular signals to ensure proper coordination of cell cycle events taking into account the environmental change or mechanical failure (Morgan, 2001; Morgan, 1997).

Cdk catalytic subunits do not act alone; their activity depends on binding of Cyclin subunits, whose oscillating concentration underlie the stage-specific timing of Cdk activity and together trigger the cell cycle events (Tyson et al., 2003; Morgan, 2001). Cyclin/Cdks promote cell cycle progression by phosphorylating critical downstream substrates to alter their activity, localization and stability (Suryadinata et al., 2010). The cell cycle oscillation of cyclin concentration is achieved via two key sites of cyclin regulations: gene transcription and protein degradation. Although cyclin binding is primary determinant of Cdk functioning, many other additional regulatory subunits and protein kinases also modulate Cdk activity, substrate recognition and its subcellular localization. This finely tuned regulatory network ensures the precise timing and coordination of the events that leads to duplication of genome and division of the cell.

Based primarily on studies of CDKs involved in the cell cycle control, at least four mechanisms appear to govern Cdk activity. The primary mechanism of CDK activation is binding of a Cyclin subunit as mentioned above. Complete activation of most Cdks also requires further posttranslational modifications. Lastly association of Cdk with group of inhibitory proteins which are collectively called CKI, shut down the activity of Cdks (Ferrel, 1996; Morgan, 2001). One class of CKIs, comprised of p21, p27 and p57 bind and inhibit Cyclin/Cdk complexes, whereas the other class of CKIs are Ink4, p15, p16 and p18 bind to Cdk4/Cdk6 and prevent their activation by Cyclin D (Morgan, 1995; Pavletich, 1999).

Different Cyclin/Cdks have been shown to be involved in regulation of different phases of the cell cycle. According to the classical view, Cyclin E-CDK2 is required to initiate S phase, CDK2 and CDK1 together with Cyclin A, are then responsible for the continuation of S phase and for entry into mitosis respectively; and the CDK1, together with B type Cyclins catalyze entry into mitosis.

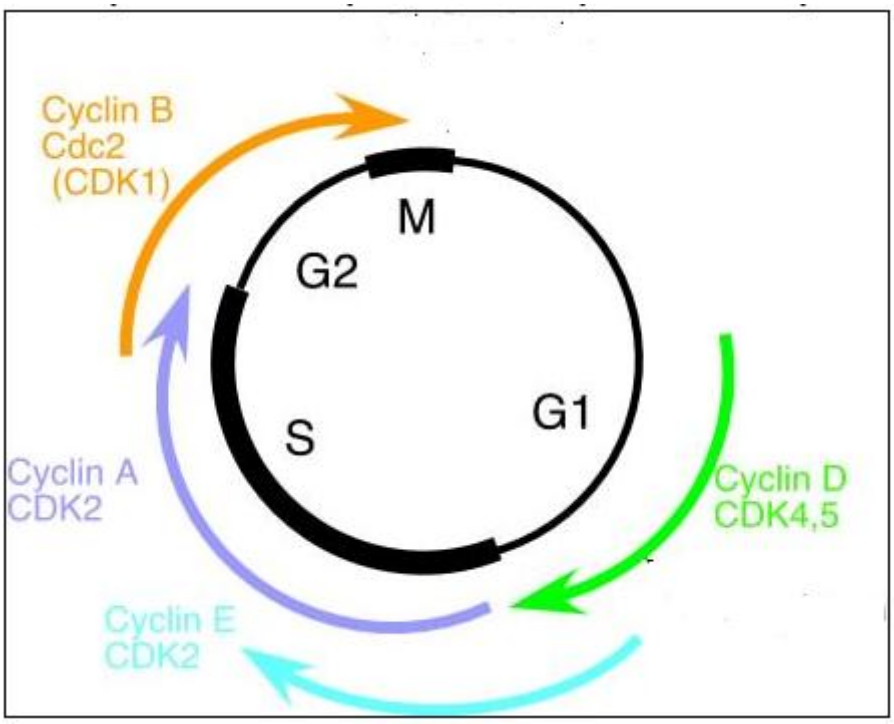

Figure 1.2 The Cyclin-CDK complexes involved in each phase of the cell cycle (Forsburg's online webpage, 2007).

Production of two cells from one requires duplication of all molecules and organelles that comprise each cell. DNA does not duplicate throughout the cycle but only several hours in S phase. DNA replication requires orderly assembly of many proteins on chromosomes and once the replication is fired it has to be completed. Therefore extracellular signals like growth factors must not and do not control S-phase progression (Reed et al., 1994). After successful completion of DNA synthesis, cells enter G2 phase in preparation for mitosis. Protein kinases activated during G2 phase prevent rereplication of the DNA. Hence, control by growth factors is also unnecessary in the G2 phase. Whereas, as cells exit mitosis, the cell cycle is reset, allowing establishment of a new, competent replication state in G1 phase. Therefore as logic

dictates, G1 is only part of cell cycle that can and must be growth factor dependent (Blagosklonny, 2001).

1.2.2 Restriction Point and G1 Regulation

The major regulatory events leading to proliferation occurs during G1 phase of the cell cycle. Most of the cell population in culture and *in vivo* has G1 phase genomic content (Reed et al., 1994). Growth factors are important to initiate and maintain proper transition from G1 to S phase. In normal cells, lack of growth factors retains cells from entering to S phase and they will instead enter G0 (also known as quiescence) state. The point at which commitment occurs and the cell is no longer dependent on growth factors for completion of cell cycle is called as restriction (R) point. This is the point at which the cell ascertains whether it has received the necessary growth signals, so that it can pass out of G1 to S phase, replicate its DNA and complete one round of cell division (Planas-Silva, 1997). The R point is estimated to be mapped 2-3 hours before the onset of DNA replication.

Mitogen dependent progression through G1 is mediated by induction of cyclin D family. Growth factors regulate cyclin D by four simultaneous mechanisms. Firstly, Cyclin D transcription is induced followed by stabilization of Cyclin D protein. Next, it is translocated to nucleus and assembled with their partners Cdk 4 and Cdk6. After cyclin D/Cdk4,6 is assembled in nucleus, it phosphorylates the retinoblastoma (Rb) protein preventing its binding to E2F, thus activating E2F1 mediated transcription. E2F transcription factors activate genes whose production are involved in S phase entry and progression and repress genes which inhibits S phase entry. Cyclin E is one of the transcriptional targets of E2F1 and together with Cdk2, they positively induce Rb phosphorylation until cell becomes independent of mitogenic signals. This positive loop ensures irreversibility of commitment. Further expression of Cyclin E activates more Cdk2 by direct binding, they together result in phosphorylation of proteins involved in the initiation of DNA replication and progression of cell cycle from G1 to S phase (van den Heuvel, 2005).

R point is considered as prototype of cell cycle checkpoints. The places where cell monitors the completion of previous phase and onset the subsequent phases are

called checkpoints (Collins and Garrett, 2005). They are defined first by Hartner and Weinert as the sensor mechanisms within the cell that surveys the cellular environment and determine whether appropriate conditions have been fulfilled before it may progress through a cell division cycle. Each checkpoint is composed of three components. The first is sensor mechanism that detects abnormal or incomplete cell cycle events such as DNA damage. This triggers signal transduction pathway, which carries the signal from the sensor to the third components effector that invoke a cell cycle arrest until the problem has been resolved (Lydall and Weinert, 1995; Mal A et al., 1996).

The restriction point is related to mitogens deprivation whereas checkpoints are mostly related to DNA damage and mitotic progression. DNA damage leads to arrest in G1 via p53 dependent transactivation of genes, primarily $p21^{CIP1}$, which binds to and inhibits CDKs required for S phase entry and progression (Vousden and Prives, 2009). Damaged DNA might propagate during S-phase and mitosis. Therefore arrest should occur in G1 (before S) and in G2 (before mitosis) to prevent propogation of damaged DNA. Arrest in G1 prevents aberrant replication of damaged DNA and arrest in G2 prevents segregation of defective chromosomes (Blagasklonny, 2001). In addition to this, proper segregation of chromosomes is ensured by spindle checkpoint, which inhibits cell cycle progression in response to signals generated by mitotic spindle damage or chromosomes that are not attached to microtubules (Clarke and Gimenez-Abian, 2000). If any of these checkpoints are not in place then inappropriate cell proliferation can occur, this is one of the hallmarks of cancer (Hanahan, 2000).

1.2.3 DNA Replication and Histone Biosynthesis

1.2.3.1 Replication of Chromosomes

To transit genetic information stably over generations, chromosomal DNA must be duplicated faithfully before segregation. To achieve this goal, eukaryotic cell has the regulatory mechanism to limit chromosomal DNA replication to exactly once per cell cycle (Sclafani and Holzen, 2007). Eukaryotic cells must replicate large amount of genomic DNA distributed on multiple chromosomes. To accomplish this feat in a reasonable period of time, replication initiates throughout S phase at multiple origins along each chromosome (Waga and Stillman, 1998). Initiation from these origins must be coordinated so that no region of the genome is left unreplicated and no region is replicated more than once. DNA replication must also coordinate with chromosome segregation to ensure each daughter cell receives a complete and unaltered complement of genetic information (Bell and Dutta, 2002).

The assembly of the pre-RC is central to the regulation of DNA replication initiation (Sclafani and Holzen, 2007). In vertebrates, Cyclin E/CDK2 is required for DNA replication initiation by contributing to formation of pre-RC complex (Zhang, 2007). CDKs active in other than G1 phase inhibit the activity of all known pre-RC components, either by leading to delocalization from nucleus to cytoplasm or by triggering their degradation (Jackson et al., 1995; Diffley, 2004). During the S phase, cyclin A/Cdk2 is essential for initiation and ongoing DNA replication (Bell and Dutta, 2002; Nguyen et al., 2001).

On besides of replication of DNA, each passage through S phase requires the synthesis of histones, which are assembled with newly synthesized DNA into chromatin fibers (Dorigo et al., 2004; Hayes and Hansen, 2001). Disturbance in chromosome assembly results in misregulation of gene expression (Lam et al., 2005), cell cycle arrest (Straight, 1997) and chromosomal instability (Carr et al., 1994) which eventually results in developmental failure (Khorasanizadeh, 2004; Meek-Wagner and Hartwell, 1986). Cells deposit newly synthesized histones behind the replication fork almost as soon as DNA has emerged from replication machinery to allow formation of nucleosomes

(Annunziato and Seale, 1983; Kelly and Brown, 2000). So, sufficient amount of histone has to be synthesized rapidly as cells enter to the S phase.

1.2.3.2 DNA and Histone Synthesis are Coordinated S Phase Events

The majority of histone synthesis is restricted to S phase, in a way that strictly coupled to rate of DNA replication; therefore they are called as replication dependent histones. Histone synthesis is coordinated with DNA synthesis not only under normal growth conditions but also under conditions where DNA damage may occur (Zhao, 2004). DNA synthesis inhibition causes rapid histone mRNA destabilization that leads to histone biosynthesis shutdown in mammalian cells (Heintz et al., 1983). Similarly, inhibition of histone synthesis triggers concerted arrest of DNA replication (Nelson et al., 2002). The fact that DNA and histone synthesis are tightly coupled during S phase indicates that impairment of the coordination of these two events have deleterious consequences. Indeed, overexpression of canonical histones or expression of histones outside of the S phase, disrupt proper incorporation of noncanonical histone proteins into chromosomes in all cell types, therewithal causes loss of chromosomes in yeast (Carr et al., 1994) and developmental arrest in Drosophila (Sullivan et al., 2001).

The coordination mentioned above is sustained partially by Cyclin E/Cdk2. NPAT a downstream target of Cyclin E/Cdk2 accumulates at G1/S transition and is essential for coordination of histone gene transcription with S phase entry (Zhao, 2004). In addition to this, two major S phase events, DNA replication and centrosome duplication, also have been shown to require the activity of Cyclin E–Cdk2 (Fang and Newport, 1991; Lange and Gull, 1996; Hinchcliffe et al., 1999; Lacey et al., 1999).

1.2.3.3 Replication Dependent Histones and Their Cell Cycle Regulation

As it is mentioned above, histones are primary protein components of chromatins. They are involved in both DNA packaging and gene regulation. In metazoans majority of histones are canonical histone proteins and they are encoded by a family of replication dependent histone genes. These genes encode only four core histones: H2A, H2B, H3 and H4 all together make up nucleosome and H1 linker, which

links nucleosomes (Figure 1.3). Synthesis of replication dependent histones is cell cycle regulated. In addition to canonical histones, there are several variant histones whose synthesis is not cell cycle regulated and they are different in mRNA structure from canonical ones. Replication-independent histone genes are constitutively expressed at low levels throughout the cell cycle. In mammalian cells, there are four distinct regulatory mechanisms that contribute to the proper rate of canonical histone protein accumulation during the cell cycle: transcription of histone genes, efficiency of pre-mRNA processing, change in half-life of histone mRNA and degradation of excess histone proteins. (Heintz 1991; Osley 1991; Stein et al., 1992; Marzluff and Duronio, 2002). Major contribution to cell cycle regulation of replication dependent histones biosynthesis occurs at mRNA level in all eukaryotes (Marzluff and Duronio, 2002).

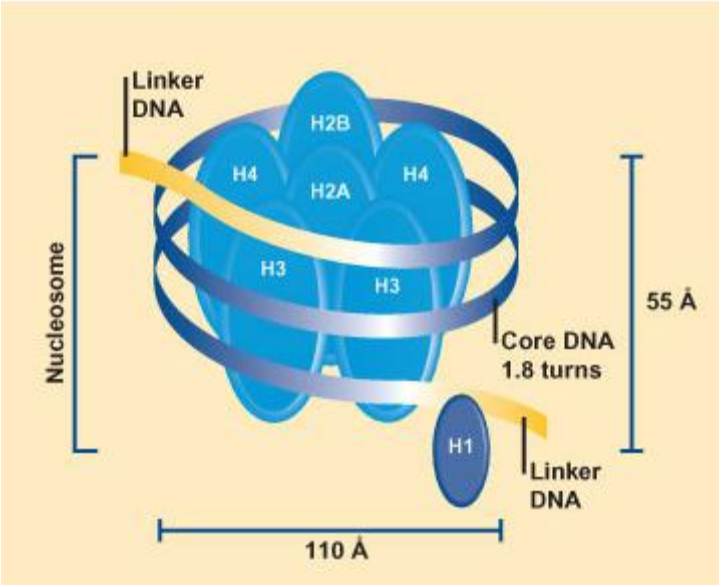

Figure 1.3 The schematic representation of nucleosome. (Carpenter P, webpage of Abcam).

1.2.3.3.1 Structure of Replication Dependent Histone mRNAs

Like other mRNAs, replication dependent histone mRNAs have a 7-methyl-guanosine cap at 5' end. However they end up with conserved stem-loop structure, instead of a poly-A tail. The stem-loop consists of 6 base stem and 4 nucleotide loop

(Figure 1.4). This unique structure requires a distinct set of factors for many aspects of their expression and regulation (Marzluff et al., 2008).

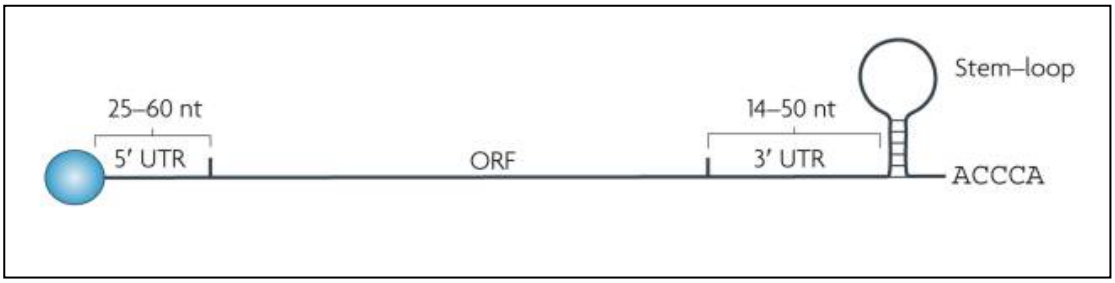

Figure 1.4 Structure of canonical histone mRNA (Marzluff et al., 2008).

1.2.3.3.2 Transcription of Histone Genes

Another important feature of histone genes is that they are physically linked in a large cluster, enabling easier rapid transcription. Human replication-dependent histone genes are clustered on chromosomes 1 and 6. Six histone genes have been identified so far in the histone gene cluster at 1q21, while ~50 histone genes have been found in the cluster at 6p21 (Albig and Doenecke, 1997). Genes encoding histones are constitutively transcribed by RNA polymerase II and their rate increases as cells approach S phase (DeLisle et al., 1983, Zhong 1983). Transcriptional regulation plays an important role in replication-dependent histone gene expression at the G1/S boundary. As cells enter S phase, histone mRNA transcription increases three- to 5-fold compared to the basal level in G1 phase (Heintz, 1991). This increase at the beginning of S phase is sustained by Cyclin E/Cdk2 phosphorylation of NPAT in Cajal bodies (Ma et al., 2000; Wei et al., 2003). Depletion of NPAT results in substantial decrease in replication dependent histone RNA transcripts (Ye et al., 2003). Besides this, overexpression of NPAT promotes S phase entry, and coexpression of Cyclin E–Cdk2 enhances the effects of NPAT on cell cycle progression (Wang et al., 2004).

1.2.3.3.3 Mature Histone mRNA Formation

Replication dependent histones lack introns and endonucleotic cleavage that releases the nascent pre-mRNA from DNA template is the only processing event

necessary to form mature histone mRNA. For histone pre-mRNAs, cleavage occurs between the stem-loop and the histone downstream element (HDE), a purine rich sequence located about 15 nucleotides after the cleavage site and this process requires U7snRNP (Mowry and Steitz, 1987; Dominski and Marzluff, 2007). U7snRNP is composed of 60 nucleotide U7 snRNA and Sm ring. It is recruited to histone pre-mRNA primarily through formation of double stranded RNA between the 5' end of U7 snRNA and histone downstream element (HDE). Stem Loop Binding Protein (SLBP), which is also known as hairpin binding factor (HBF), binds to stem loop of histone mRNA, interacts with one of Sm ring proteins and stabilize the U7snRNP on the HDE, which is required for cleavage catalyzed by CPSF-73 (Dominski et al., 2005b) as shown in Figure 1.5 (Dominski et al., 2003; Marzluff and Duronio, 2002; Yang et al., 2009; Marzluff et al., 2008). In vitro processing of histone mRNA is absolutely dependent on SLBP if Histone Downstream Element (HDE) is mutated in a way that prevents strongly binding of U7 snRNA (Dominski et al., 1999). Mutations in the stem loop at 3' end that prevents binding of SLBP results in no expression of processed histone mRNA in vivo (Pandey et al., 1994). The loss of normal histone mRNA processing can result in the production of polyadenylated mRNAs from the replication dependent histone genes (Narita et al., 2007; Wagner et al., 2007). The level of polyadenylated canonical histone mRNAs are very low in proliferating cells (Narita et al., 2007, Pirngruber et al., 2009) but increase during tumorigenesis (Zhao et al., 2004). This is one of the few evidences where regulated switch mode of pre-mRNA 3'end processing serves as an altered important physiological role during tumorigenesis.

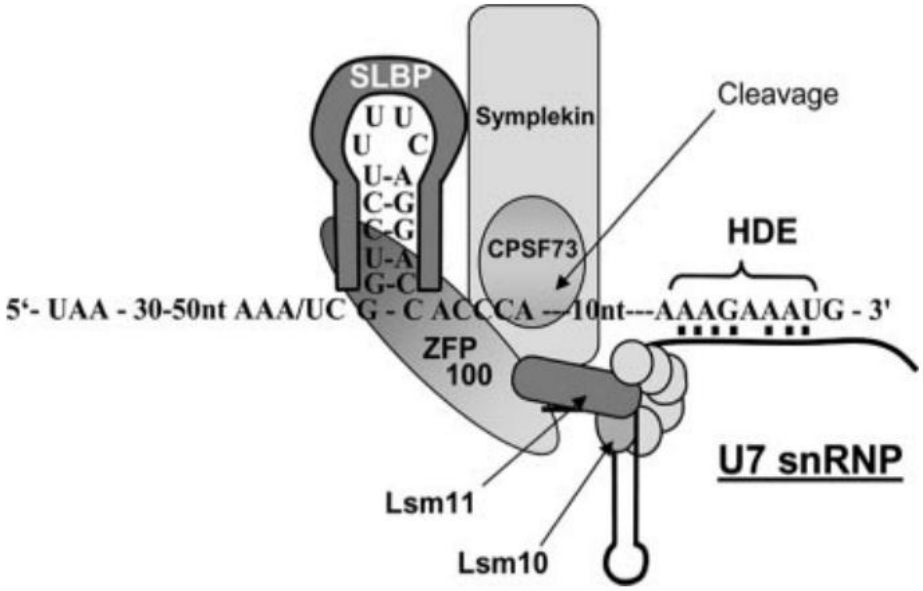

Figure 1.5 Hypothetical model depicting possible interactions in the stable processing complex assembled on histone mRNA before cleavage (Wagner and Marzluff, 2006).

1.2.3.3.4 Export and Translation of Histone mRNA

Like other mRNA, replication dependent histones mRNA are exported from nucleus to cytoplasm by antigen peptide transporter and once exported they are efficiently translated. Histone mRNA is exported in TAP dependent manner in a very short time (Schochetman and Perry, 1972; Erkmann et al., 2005a; Sullivan et al., 2009). Mammalian cells with knockdown SLBP fails to export processed histone mRNA to cytoplasm (Sullivan et al., 2009).

3' end of histone mRNA is essential for translation *in vivo (*Gallie, 1996). SLBP is bound to histone mRNA throughout the transport and is required for its translation (Whietfield, 2004) and it is present in the cytoplasm only when histone mRNA is present (Erkmann et al., 2005b). SLBP in coordination with other proteins help to circularize histone mRNA and mediate the efficiency of translation of histone mRNA by a mechanism similar to poly-A tailed mRNAs (Sanchez and Marzluff, 2002). Some of the mechanisms that mediate translation by SLBP have been recently revealed: SLBP interacts with SLIP1 that binds to E1F4G and plays role in translation of histone mRNA (Cakmakci et al., 2008).

1.2.3.3.5 Regulated Degradation of Histone mRNAs

The stem-loop at the 3' end of replication dependent histone mRNA is the *cis* element that mediates mRNA degradation. Rapid decay of histone mRNA requires SLBP, which is involved in recruiting the proteins necessary to add short oligo (U) tail to histone mRNA that is being translated (Mullen et al., 2008). Degradation of histone mRNAs requires Upf1 which binds to SLBP is a key regulator of the nonsense-mediated decay pathway, and ATR, a key regulator of the DNA damage checkpoint pathway activated during replication stress (Kaygun and Marzluff, 2005).

1.2.3.4 SLBP is Required for All Steps of Histone mRNA Metabolism

These all evidences mentioned above show that both the stem loop structure (SL) at 3' end of replication dependent histone mRNA and SLBP are crucial for all three steps in histone mRNA metabolism: processing, translation and stability of histone mRNA (Figure 1.6). Reducing the SLBP level decreases the efficiency of histone mRNA metabolism, thus the histone protein level and reduces the cell growth by accumulating cells in S phase. This is mostly likely due to the defects in replication fork where sufficient amount of replication dependent histones to assemble the chromatin structure could not be provided and eventually may lead to S phase checkpoint activation. The processed histone mRNA is not rapidly degraded when DNA synthesis is halted in these cells. (Sullivan et al., 2009). Expression of RNAi resistant SLBP restores proper S phase progression (Wagner et al., 2005). Loss of SLBP in Drosophila causes genomic instability and disrupted cellular proliferation (Wagner et al., 2005). Overexpression of SLBP increase the level of histone mRNA but does not increase number of cells in S phase in mammalian cells (Wagner and Marzluff, 2006). The degradation of SLBP is temporally associated with the disappearance of histone mRNA from the cell (Wagner, 2005; Sulvian et al., 2009). On the other hand, stabilizing SLBP does not prevent histone mRNA degradation at the end of S phase.

SLBP functions as a versatile regulator that ensures high levels of histone mRNA and proteins only during S phase concomitants with DNA replication and prevents harmful accumulation of free histone proteins in cells outside of S phase.

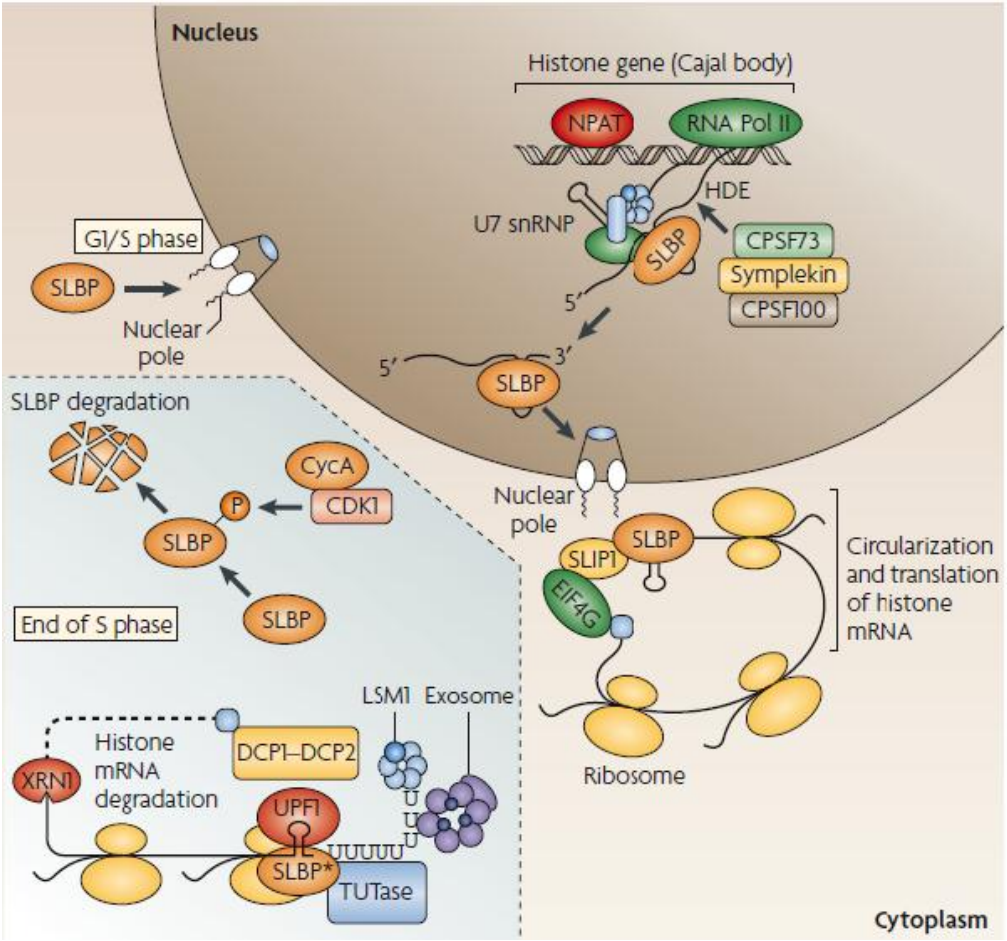

Figure 1.6 SLBP is essential for all steps of histone mRNA's life. (Marzluff and Duronio, 2008).

1.2.3.5 Cell Cycle Regulation of SLBP

All five classes of canonical histone proteins are cell cycle regulated. Accumulation of histone protein is majorly due to posttranscriptional regulations of histone mRNA. Although both gene transcription and the half-life of histone mRNA are regulated during cell cycle, major regulatory step is processing of 3' end of histone mRNA. Histone mRNA transcription increases 3-5 fold, but there is almost 10 fold increase in processing efficiency of histone mRNA (the percentage of histone transcripts that reach to cytoplasm (Marzluff et al., 2008)), making up around overall 35 fold increase in histone mRNA level just before entry into S phase. At the end of S phase, histone mRNA are rapidly degraded and processing of the mRNA is inactivated (Whietfield et al., 2000). There is histone gene transcription in other phases other than S

phase, but increased processing efficiency is limited to S phase and this determines the expression of histone mRNA throughout the cell cycle (Dominski and Marzluff, 1999; Wang, 1996; William,1995) .

In continuing mammalian cells, the expression of SLBP is also cell cycle regulated. SLBP is synthesized as cells enter S phase, increases 10-20 fold and degraded at the end of S phase, leading to shut down of histone mRNA processing, thus histone protein production. The degradation of SLBP ensures the cessation of histone mRNA biosynthesis; preventing accumulation of histone mRNA until SLBP is synthesized just before entry into the next S phase (Koseoglu et al., 2008). The proteasome mediated degradation of SLBP is triggered by phosphorylation of Thr 61 and 60 sequentially and respectively by Cyclin A/Cdk1 and CK II (Zheng et al., 2003; Koseoglu et al., 2008). The level of SLBP mRNA is almost constant during cell cycle in both CHO and Hela cells (Whietfield et al., 2000). SLBP has half-life of about 2 hours in either asynchronous or S phase cells) and stability of SLBP decreases dramatically at S/G2 border (Whietfield et al., 2000). This leads to anticipation of being regulated via translational or posttranslational mechanism.

Low level of SLBP in G1 is due to synthesis and rapid degradation of SLBP during G1 and low efficiency of SLBP mRNA translation. It was shown that the translation efficiency of SLBP is low at early G1 phase but it is restored to S phase level somewhere in mid-G1 (Koseoglu, 2008). However, the level of SLBP is still kept low for 3-4 more hours until G1/S transition. To examine the reason of this regulation, SLBP stability was checked during this period by adding Cyclohexamide to synchronized Hela cells. Cyclohexamide blocks the translation elongation, thus will give an idea about the stability of the protein. After treatment of mid-G1 cells with Cyclohexamide, the level of SLBP was decreased indicating that the stability of SLBP is low after mid-G1 till the beginning of S phase. In order to examine if proteasome mediated degradation is the reason for reduced stability of SLBP, cells were treated with MG132. MG132 inhibits the proteasome activity and MG132 treatment prevents SLBP degradation. By this data, it was proposed that regulated degradation of SLBP may be a new mechanism to keep SLBP level low until S phase. This is the case in G1 for several other proteins like Cyclin A, cdc6 and skp2 in mammalian cells (Petersen et al., 2000; Bashir et al., 2004; Wei et al., 2004a).

Although both histone mRNA and SLBP stability are regulated in parallel during the cell cycle, the molecular signals regulating these two factors are different (Koseoglu et al., 2010). The expression level of replication dependent histone mRNA and DNA replication are tightly coupled during S phase. Thus, inhibition of DNA replication due to treatment of cells with chemicals or DNA damage inducing agents causes rapid histone mRNA destabilization that results in histone synthesis shutdown (DeLisle et al., 1983; Chuan et al., 2004), but has no effect on SLBP level (Whitfield et al., 2004). Similarly inhibition of histone synthesis triggers a concerted suspension of DNA synthesis (Nelson DM et al., 2002) but depletion in SLBP does not cause cell cycle arrest, thus no influence on DNA synthesis in mammalian cells, but results in S phase accumulation in Drosophila (Wagner et al., 2005).

In the beginning of S phase, histone gene transcription is achieved by recruitment of Cyclin E/Cdk2 phosphorylated p220 ^{NPAT} by transcriptional activator HiNF-P to histone promoter (Meile et al., 2005). Cyclin E/Cdk2 is on the top both histone transcription via NPAT phosphorylation and DNA replication initiation. NPAT is also shown to be involved in histone mRNA processing (Pingruber and Johnson, 2010). SLBP, a major factor required for histone mRNA processing which is essential for all steps of histone mRNA metabolism and restricts histone mRNA expression to S phase. SLBP level dramatically increases as cells enter S phase and Cyclin E/Cdk2 is the major regulator of S phase entry. Thus, this raises a question if Cyclin/Cdk2 could be an upstream regulator of SLBP at G1/S border. Thus, in this dissertation I have investigated the G1 regulation of SLBP. I have generated a tool for studies concerning G1 regulation of SLBP, namely I have established stable cells expressing wild type and 'S/G2 degradation' mutant of SLBP. Furthermore, I have particularly focused on analyzing the role of predicted upstream regulator, Cyclin/Cdk2 on rapid increment of SLBP at G1/S transition.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 List of Equipments

Table 2.1 List of Equipments.

Orbital Shaker	Hangzhou allsheng instruments	OS-100
Magnetic stirrer and heater	Daihan	Wisestir
Sensitive Balance	PRECISA	
Highly sensitive balance	Sartorius	
Centrifuge 15ml TC	NÜVE	NF800
Centrifuge 1.5 ml	Thermo Scientific	
Western blot apparatus	Hoeffer	
Laminar Flow	ESCO	Type A2
CO2 incubator	Thermo Scientific	
Invert Microscope	Leica	
Ph meter	Thermo Scientific	
-80 C freezer	Daihan	
Refrigerator	BOSCH	
Ice machine	Scotsmann	
Incubator shaker	Thermo Scientific	
Pipette set	Denville	X3000i
Pipette gun	Drummond	
KODAK film cassette	KODAK	
Dry block		
Refrigerated centrifuge	Thermo Scientific	
ELISA reader	BIOTEK	
QUBIT	Invitrogen	
Spectrophotometer	Shimadzu	UV-1700
Distilled water machine	Millipore	
Autoclave	ALP	
Vortex	Biosan	
Benchtop mini centrifuge	Wisd	
Flow Cytometry	FACS Calibur	

2.1.2 List of Chemicals

Table 2.2 List of Western Blot Chemicals.

Acrylamide/Bis solution (30%)	Bio-Rad	161-0156
Tris	Wisent INC	600-127-LG
Glycine	Merck	5.00190.1000
TEMED	BIOMATIK	A4008-100ml
Ammonium per sulfate	Sigma Aldrich	31117-1kg
SDS loading buffer pack	Fermentas	R0891
Protein ladder	Pierce	26619
SDS	Merck	
NP-40	Applichem	A1694,0250
Tween-20	Sigma Aldrich	P1376-100ml
Phenylmethanesulfonyl fluoride (PMSF)	sigma	P7626-1G
P-coumaric acid	Sigma	C9008-10G
Luminol	Sigma	A8511-5G
Ponseu-S	Sigma-Aldrich	P3504-10G
Copmassie Brilliant Blue R-250	Bio-Rad	161-04000
QUBIT protein assay	Invitrogen	Q33211
Sodium Hydroxide pellets	Merck	1.06482.1000
Sodium Chloride	Merck	1.06404.1000
KODAK X ray film		
ROCHE PVDF membrane		03010040001
Complete mini EDTA-free protease inhibitor cocktail	Roche	04693159001
Isopropanol	EMBOY	
Bio-Rad Protein Assay	Bio-Rad	500-0006
Gel Blot Papers	Whatman	GB003
Fixer	KONIX	
Developer	KONIX	
Hydrogen peroxide	Sigma	H1009
Acetic Acid	Merck	1,00063,2511
Methanol, HPLC grade	Merck	1.06009.2500
Lumi-Light plus western blotting substrate	Roche	12 015 196 001
Lumi light western blotting substrate	Roche	12 015 200 001
NaF	Sigma Aldrich	SK.S.30105-250G
KCl	Sigma Aldrich	SK.R.12636-1 KG
NaCl	Sigma Aldrich	SK.R.13423-1 KG
Na_2HPO_4	Sigma Aldrich	S3264
KH_2PO	Sigma	P9791
Nitrocellulose membrane	Amersham	RPN303D
Non Fat Dry Milk	Bought from grocery	

Table 2.3 List of Tissue Culture Chemicals.

PBS	Hyclone	SH30256.01
Lipofectamine 2000 reagent	Invitrogen	11668-027
Turbofect	Fermentas	R0539
DMEM/high Glucose	Hyclone	SH30243.01
Trypsin	LONZA	CC-5012
Penicillin/Streptomycin	HyClone	SV30010
FBS	LONZA	DE14-802F
Trypan Blue	Sigma	T 815
WST1	Roche	11 644 807 00
LDH	Roche	11 644 793 001
BrDU	Roche	11 647 229 00
Dimethyl Sulphoxide	Sigma	D2650
Bio-Rad Transfection reagent	Bio-Rad	Sample
Thymidine	Sigma	T1895-5G
Roscovitine	sigma	R7772-1MG
Nocodazole	Sigma	M1404-10MG
RNAase	Sigma	R6513
PI stain	Abcam	ab14083
Geneticin, liquid	Invitrogen	10130 035
Geneticin, powder	Invitrogen	11811 031

Table 2.4 List of Chemicals for Bacterial Culture and Cloning.

LB agar	Merck	1.10283.0500
LB broth	Merck	1.10285.0500
Site directed mutagenesis	Stratagene	#20052
Rubidium Chloride	Sigma	83-979-25 G
Genopure Maxi Prep	Roche	03143422001
Gene-jet miniprep	Fermentas	K0503
Ampicillin	Sigma	A0166-25G
Potassium Acetate	Sigma	SK.S.25059-1KG
$CaCl_2$	Sigma	SK.R.12022-1 KG
$MnCl_2$	sigma	M3634
Glycerol	Sigma	15524-1L
MOPS	Sigma	M1254
NaOH	Sigma	SK.S.38215-1EA
HCl	Sigma	SK.R.30721-2,5 L

Table 2.5 List of constructs.

His SLBP in pcDNA3.1(-)(SFTTP)	MMK LAB
His SLBP in pcDNA3.1(-) (SFTAP)	constructed
pcDNA3	Nesrin Özerin's Lab
pCMV myc	Nevzat Kazgan' Lab
Cdk2 dn in pCMV	Enders Lab
Cdk4 dn in pCMV	Enders Lab
Cdk2 WT in pCMV	Enders Lab

Table 2.6 List of antibodies.

Cyclin A	(H-432):sc-751 (Santa Cruz)
SLBP	Serum (Marzluff Lab)
Cdk 2	(m2):sc-163 (Santa Cruz)
Cdk 4	(c-22):sc-260 (Santa Cruz)
Goat anti Rabbit IgG HRP	AP132P (Millipore)

2.2 METHODS

2.2.1 Cell Culture

2.2.1.1 Subculturing

Hela cells (Provided from Dr. Seval Korkmaz) were grown in DMEM/High glucose media containing 10% FBS and 1% pen/strep. The old media of cells were removed followed by PBS wash. Cells were detached adding enough volume of trypsin and subsequently incubating at 37 ºC for 3-4 minutes. FBS was added to inhibit the activity of trypsin and cells were seeded in a new plate in particular ratio. Hela cells were subcultured in 3-4 days when they reached the confluence. The number of cell passage did not exceed 40.

2.2.1.2 Freezing Hela Cells

Hela cells were detached by trypsinization and collected by spinning them at 200g for 5 minutes. The pellet was washed with PBS and spinned again. The pellet was dissolved in 1 ml of DMEM media with 5% DMSO, 20% FBS in specific cryovial tubes. They were frozen by gradual temperature decrease and stored at -80 ºC.

2.2.1.3 Thawing Hela Cells

The Hela cells at -80 ºC were thawed at 37 ºC water bath and immediately transferred to plate containing 10ml of DMEM/High Glucose with 20% FBS and 1% pen/strep. The media was refreshed after cells were attached to the surface of the plate. Hela cells have been passaged for 5-6 times and considered as ready for experiments. Namely, they have been given enough time for recovery from freezing.

2.2.1.4 Cell Collection

Hela cells were detached from surface of the plate as mentioned above and pelleted at 200g for 5 minutes. The pellet was washed with cold PBS and spinned again. All the supernatant was removed carefully. The pellets are immediately used or stored at -80 ºC until usage.

2.2.1.5 Cell Lysis

Hela cells were collected as mentioned above. The cell pellet was dissolved in cold Lysis Buffer. The Lysis Buffer solution contains 150mM NaCl, 50mM Tris, Ph: 7.5 and 1% NP-40. And protease inhibitor cocktail (1X) and PMSF (1mM) were added freshly. The pellet was lysed at cold for 30 minutes with agitation. In order to get rid of insoluble material, the lysate was centrifuged at maximum for 10 minutes at 4 ºC. The supernatant was collected and stored at -80 ºC for further use.

2.2.2 Transfection

2.2.2.1 Optimization of DNA concentration for Transfection with Lipofectamine 2000

Cells have to be at confluence of around 80-90 % at the day of transfection in 6 well plate. 1 µg, 2 µg, 3ug and 4 µg of HA tagged SLBP in pcDNA3 (308 ng/µl) were diluted in 250 µl of OPTIMEM individually. –DNA (No DNA added to transfection reagent) wad used as a negative control. 4x 10ul of Lipofectamine 2000 was diluted in 4x 250 µl of OPTIMEM. They were incubated at RT for 7 minutes. After incubation 250 µl from Lipo 2000 solution was added to each tube containing different DNA concentrations. The mixtures are incubated at RT for 25-30 minutes and during this incubation the media is changed with –antibiotics DMEM/FBS (10%). The mixture was

added to the plates carefully drop by drop and mixed well. The media of the plates were refreshed after 6 hours with normal media. The cells were collected after 48 hours of transfection. 2 µg of DNA was chosen to be most optimum, thus all further transfections were done with 2 µg of DNA.

2.2.2.2 Optimization of proper cell confluence for Lipo 2000 transfection

Cells were seeded at different confluence percentage. The Hela cells were transfected with 2 µg of HA-SLBP in pcDNA3.1 (-) in a same manner mentioned in section 2.4.1 the number of viable and dead cells was analyzed under invert microscopy.

2.2.2.3 Efficiency Comparison of different transfection reagents

3 different transfection reagents (Lipo 2000 (Invitrogen), Turbofect (Fermentas), Transfectin Lipid Reagent (Bio-Rad)) were tested with 2 µg of pCMV, Cdk 4 dn in pCMV and Cdk2 dn in pCMV were transfected with transfection reagents according to the manufacturer's protocols. They were grown in –antibiotics media for 6 hours and refreshed with normal media. Cells were splitted to 96 well plate for cell cycle and performed proliferation analysis.

2.2.2.4 Generation of Stable Cell Lines

Hela cells were transfected with 2 µg of his SLBP in pcDNA3.1(-) with SFTTP ➔ SFTAP mutation constructs and wild type as described in section 2.4.1. After 48 hours of incubation, 1/3rd of cells were collected for lysis and SLBP expression and 2/3rd was seeded again for selection and incubated at 37 ºC . After a day, 400 µg/µl of selective antibiotics, geneticin (G-418) was added to both negative control and transfected cells. The media was refreshed in 3-4 days and cells were grown (splitted if needed) until all the cells in negative control were died. The stable cells were tested for expression of SLBP and frozen for further use at -80 ºC.

2.2.2.5 Cytotoxicity Assays

Hela cells were seeded in 200 µl media to assess cytotoxicity and proliferation rates. In order to examine effect of cytotoxicity of transfected constructs on Hela Cells, 100 µl of suspension media was taken as a replica to another 96 well-plate.100 µl LDH/well were added and incubated for 2 hours. The absorbance was measured at 420-480 nm.

2.2.2.6 Proliferation Assay (WST-1)

Cells were grown in 96 well micro plates (TC grade, flat bottom) in a volume of 200 µl/well culture in a humidified atmosphere (37 C, 5% CO2). As described above, 100 µl was taken for cytotoxicity kit (section 2.4.5) .Cell proliferation reagent WST-1 is added in 10 µl/well volume and incubated for 4 hours in the incubator. The samples absorbance was measured at 420-480 nm with ELISA reader.

2.2.2.7 BrdU incorporation assay

The ROCHE BrdU incorporation assay kit was used for assessment of cell proliferation. Cells were seeded in 96 well plate. The media was removed and cells were treated with FixDenat solution. Fix solution was tapped off and anti-BrdU was added and cells were incubated for 2 hours at RT. The 96 well plate was washed off for three times. As a last step the substrate was added and the presence and quantity of BrdU was assessed by measuring at 540 nm.

2.2.3 Bacterial Culture and Cloning

2.2.3.1 Culture and storage of DH5alpha cells

The DH5alpha cells were grown in LB broth at 37 ºC with agitation. They were frozen in 1 ml of autoclaved 15% glycerol and quick frozen with liquid nitrogen. For new culture, only a small piece of frozen culture is scraped and seeded in liquid LB media.

2.2.3.2 Generation of competent cells

All the chemicals and equipments used for this protocol are kept cold. Some piece from the DH5α cells were scraped and grown in 3 ml LB broth ON at 37 C. 1 ml from this culture is subcultured into 100 ml of LB broth and grown until OD reaches 0.4-0.6. Cells are pelleted at 4 ºC centrifuge at max for 10 minutes and TFbI buffer is added. Solution is spinned and TFbII buffer is added. It is aliquited and quick frozen with liquid nitrogen and stored at -80 °C. The recipe for TFbI and TFbII buffers is shown in Table 2.7 and 2.8.

Table 2.7 Chemical composition of TFb I buffer.

30 mM Potassium Acetate	1.465 g
100 mM $RbCl_2$	6.045 g
10 mM $CaCl_2$	0.735 g
50 mM $MnCl_2$	4.954 g
15% Glycerol	75 ml

400 ml of dH_2O was added. The pH was adjusted to 5.8 with acetic acid. The volume was brought to 500 ml. Sterilized with 0.2 µm filter.

Table 2.8 Chemical composition of TFb II buffer.

10 mM MOPS	0.210 g
10 mM $RbCl_2$	0.121 g
75 mM $CaCl_2$	1.100 g
15% Glycerol	75 ml

75ml of dH_2O was added and pH was adjusted to 6.5. The solution was sterilized with 0.2 µm filter.

2.2.3.3 Estimating efficiency of competent cells and transformation

DH5α competent cells were transformed with three different concentration of HA-SLBP in pcDNA3 (1 ng, 10 ng and 100 ng). Appropriate volume of DNA was added to 50 µl of competent cells and incubated on ice for 30 minutes. The mixture was heat shocked at 42 ºC for 45 seconds and subsequently incubated on ice for 2 more minutes. 950 µl of LB broth was added to competent cells and incubated at 37 ºC with 225 rpm agitation. 50 µl of culture was spreaded on 100 µg/ml of Ampicillin containing plates. After 16 hours, colonies were counted and efficiency of competent cells was calculated in terms of CFU/µg.

2.2.3.4 MiniPrep

A single colony was chosen from the antibiotics containing plate and grown in LB broth with antibiotics for no more than 16 hours. The cells were pelleted and 250 µl of resuspension buffer was added followed by gently mixing. 250 µl of lysis buffer was added to lyse cells completely and 350 µl of neutralization buffer was added to recover just the plasmid DNAs. The mixture was spinned at max for 5 minutes and supernatant was transferred to columns where DNA binds. In order to remove cell debrid remaining in the column was washed twice with 500ul Wash Buffer. 40-50 µl of elution was added

to elute DNA from the column. The concentration of DNA was measured by using 2 µl with Nanodrop.

2.2.3.5 Maxiprep

A single colony after transformation was grown in 3 ml of LB broth and 1ml of this culture was subcultured into 100 ml of LB Broth with antibiotics. Cells were pelleted in cold and resuspended, lysed and neutralized as mentioned in section 2.5.4. All the steps were carried out on ice. Isopropanol was used as an eluting agent and DNA was washed with cold 70% ethanol. After air dry, the pellet of DNA was dissolved in 200-300 µl ddH_2O. The concentration of DNA was measured by Nanodrop.

2.2.4 Generation of SFTAP from SFTTP by Using Site Directed Mutagenesis Kit

In order to generate a point mutation, Site directed Mutagenesis (Stratagene kit) was used. The summary of procedure is described as shown in the Figure 2.2. Primers were designed for Thr 62➔ Ala 62 construct from his SLBP in pcDNA3.1- as shown in Figure 2.1.

Table 2.9 Primers for SFTAP construct generation.

	Tm	**Primer length**	**Sequence**
Forward Primer	67.3 °C	28 bp	CCGAGAGCTTTACCGCTCCTGAAGGCCC
Reverse Primer	67.3 °C	28 bp	GGGCCTTCAGGAGCGGTAAAGCTCTCGG

The primers were diluted and aliquited. The PCR reaction was set according to the Table 2.10. The PCR conditions are also shown in Table 2.11.

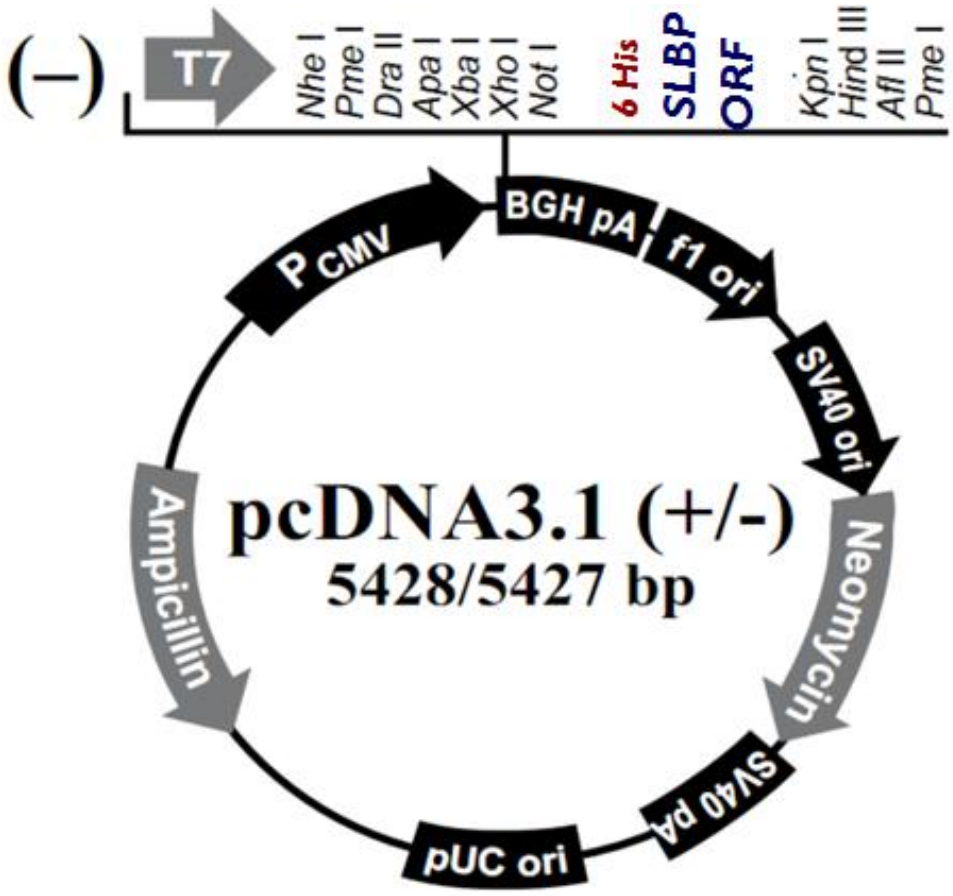

Figure 2.1 The map of SLBP in His-tagged pcDNA3.1 (-).

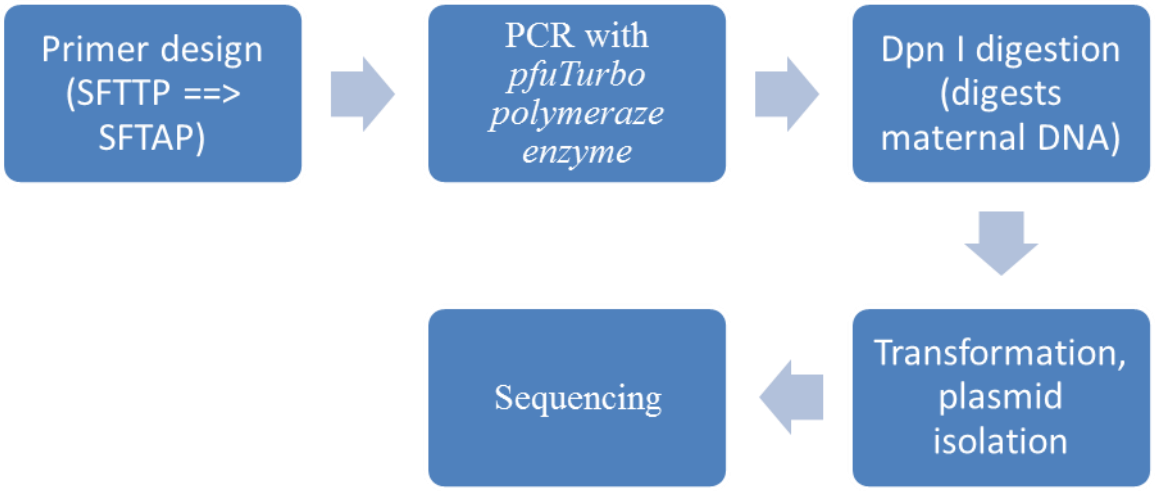

Figure 2.2 The schematic illustration of generation of site directed mutagenesis reaction.

Table 2.10 PCR reaction components.

	SFTTP➔ SFTAP	- control
10X reaction buffer	5 µl	5 µl
ds DNA template	1 µl	1 µl
125 ng of forward primer	1 µl	-
125 ng of reverse primer	1 µl	-
dNTP	1 µl	1 µl
ddH2O	40 µl	42 µl
PfuTurbo DNA polymerase (2.5 U/ µl)	1 µl	1 µl

Table 2.11 PCR conditions.

Cycles	Temperature	Time
1	95 ºC	30 sec
22	95 ºC	30 sec
	52 ºC	1 min
	68 ºC	10 min
1	68 ºC	10 min
1	4 ºC	infinite

In order to digest parental DNA in amplification reaction, 1 µl of Dpn I enzyme was added and incubated for 1 hour at 37 ºC. 10 µl of each reaction was run in a 1% agarose gel at 80 V. The bands were visualized under UV.

2.2.4.1 Transformation of SFTAP constructs

4 µl of amplification reaction was used to transform 50ul of homemade competent cells as mentioned in 2.5.2 section and the cells were pelleted and spreaded on antibiotics containing plates. Several colonies were chosen and plasmids were isolation.

2.2.4.2 Sending Constructs to Sequencing

All the sequencing services were carried out via IONTEK Company in Turkey. 100 ng of 25 µl samples were sent and all constructs were sequenced both from 5’ and 3’ by using T7 and SP6 universal primers.

Table 2.12 List of primers for sequencing constructs in the pcDNA 3.1 (-).

	Length	**Sequence**
T7 (forward)	18	ATT-TAG-GTG-ACA-CTA-TAG
SP6 (reverse)	20	TAA-TAC-GAC-TCA-CTA-TAG-GG

2.2.5 Western Blotting

2.2.5.1 Measurement of Protein Concentration by Bradford Assay

The BIORAD protein assay solution was 4 times diluted with dH_2O. Standards were prepared according to the manufacturer's protocol. 2 µl of standards or samples were diluted in 198 µl of Bio-Rad solution and incubated at RT for 5 minutes. The concentrations of proteins were measured by ELISA reader at 595 nm. For some proteins, 950 µl of Bio-Rad solution and 50 µl of standards and samples were mixed and measured by spectrophotometer at 595 nm.

2.2.5.2 Measurement of Protein Concentration by QUBIT

The reaction was set according to manufacturers' protocol (Invitrogen). The concentration was measured after 15 minute incubation at RT by QUBIT.

2.2.5.3 Preparation of 12% SDS Gel

The two glasses were washed very carefully with tapped warm water and rinsed with distilled water followed by 70% Ethanol. According to the size and width of the glasses, appropriate amount of 12% gel was prepared based on Table 2.13.

Table 2.13 Solutions for preparing resolving gels for 12% Tris-glycine SDS-polyacrylamide gel electrophoresis.

12% SDS PAGE gel	8 ml (ml)	10ml (ml)	15ml (ml)
Distilled H_2O	2.7	4	5
Acrylamide/Bis (30%)	3.2	3,3	6
1 M Tris, pH: 8.8	2	2,5	3,8
10% APS	0.08	0,1	0,15
10% SDS	0.08	0,1	0,15
TEMED	0.0035	0,004	0,006

After Gel has been polymerized, the Isopropanol was removed and washed for several times. The proper volume of stacking gel was prepared and combs were placed.

Table 2.14 Solutions for Stacking gel.

	2 ml (ml)	3 ml (ml)	8 ml (ml)
Distilled H_2O	1,4	2,1	5,5
Acrylamide/Bis (30%)	0,33	0,5	1,3
1.5 M Tris, pH: 6.8	0,25	0,38	1,0
10% APS	0,02	0,03	0,08
10% SDS	0,02	0,03	0,08
TEMED	0,002	0,003	0,008

2.2.5.4 Preparation of Samples for Loading

Samples were aliquated after calculation of concentrations. Appropriate volume of 5X Loading Buffer was added and both together boiled for 5 minutes at 100 ºC. In order to prevent formation of aggregates, the mixture was short spinned and brought to RT.

2.2.5.5 Running Conditions for SDS PAGE

After samples were loaded on the determined wells, the gel casting system is assembled. The running buffer was added to both upper and lower chambers and proteins were let to move at constant Amper. (20 mA per 1 mm gel). The composition of Running Buffer is listed in the Table 2.15.

Table 2.15 Recipe for Running Buffer

	1X	10X
Glycine	28.8 g	288 g
Tris	6.04 g	60.04 g
SDS	2 g	20 g
dH_2O	Fill up to 2 L	Fill up to 2 L

pH should be around 8.3

2.2.5.6 Semidry Transfer

The system is assembled is seen in the Figure 2.3. Membrane (just for PVDF) has to be activated by soaking in HPLC grade Methanol and soaked in the transfer buffer. Transfer buffer contain same ingredients as running buffer except SDS and it has 20% methanol. The system is run at surface area*0.8 mA for at least 1 hour for 0.75 mm gel.

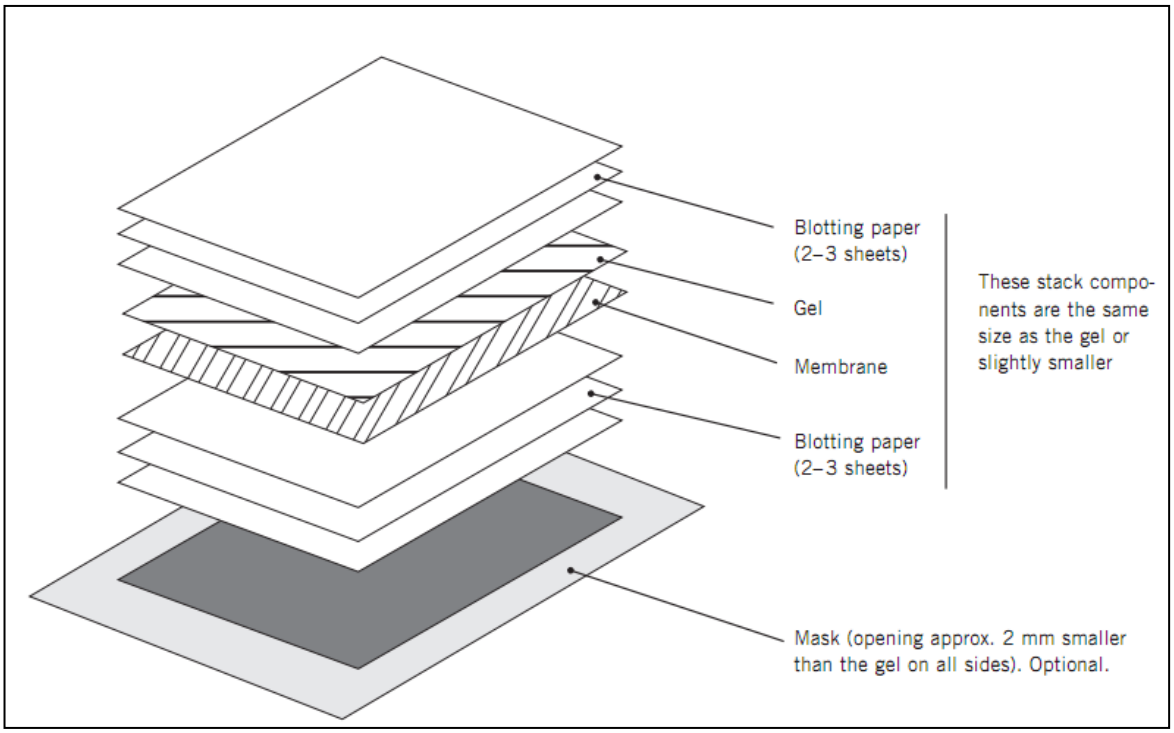

Figure 2.3 Semidry transfer system assembly. (Hoefer TE70X Semi-dry Blotters instruction manual).

2.2.5.7 Wet Transfer

The wet transfer is carried out by again soaking all towels and membranes in transfer buffer for at least 5 minutes. The wet transfer system is filled up with transfer buffer and outer chamber is filled up with chilled deionized water.

2.2.5.8 Staining the Membrane with Ponseu S

Immediately after transfer has been completed the membrane has to be put into Ponseu S and if not needed directly put into the blocking solution. The membrane is stained at least for 5 minutes with agitation and destained with distilled water.

2.2.5.9 Staining the Gel with Comassie Brilliant Blue and Destaining

In order to check complete transfer, the gel is stained with CBB (Comissie Brilliant Blue Solution) for 30 minutes with shaking and destained with destaining solution. Destaining solution is made up of 10% acetic acid and 20% methanol.

2.2.5.10 Blocking and Antibody Incubation of Membrane

The membrane is blocked with 5% milk in PBS-t solution for 2 hrs. at RT or O/N at 4 ºC. The PBS was prepared as described in elsewhere. 0.1% Tween-20 is added to PBS and this solution is called as PBS-t solution.

Primary antibody incubation is also carried out in antibody diluted in blocking solution. It can either be ON at 4 ºC or 2 hrs. at RT. After primary incubation, the membrane is washed with PBS-t solution for 10 minutes under agitation. This step is repeated for three times. Secondary antibody is diluted in blocking solution and incubated with membrane for 45 minutes-1 hour at RT. The membrane is washed for three times as described above.

2.2.5.11 ECL and Film Development

2.2.2.5.11.1 Homemade ECL

Homemade ECL (Solution A: 250 mM Luminol, 90 mM p-coumaric acid, 100 mM Tris pH: 8.6, Solution B: 30% H_2O_2 and 100 mM Tris ph: 8.6) was prepared freshly. The membrane was incubated with 1:1 mixture of solution A and solution B of ECL for 1 minute and immediately used for film development.

2.2.5.11.2 The Commercial ECL

The membrane was incubated with commercial for at least 1 minute and exposed to film for short period of time.

In order to develop the film, Developer and Fixer solutions were prepared freshly and prewarmed to 30 ºC before use. The film is incubated 3-4 minutes in Developer solution followed by rinsing in water for 1 minute. Then it is fixed for 3-4 minutes in Fixer solution and again rinsed with water.

2.2.6 Synchronization

2.2.6.1 Double Thymidine Synchronization

Hela cells at confluency of 20% were treated with 2 mM Thymidine for 19 hours. They are released by refreshing the media for 9 hours and blocked for second time again with 2 mM thymidine for 18 hours. Cells are refreshed and released. The samples are collected on my interest.

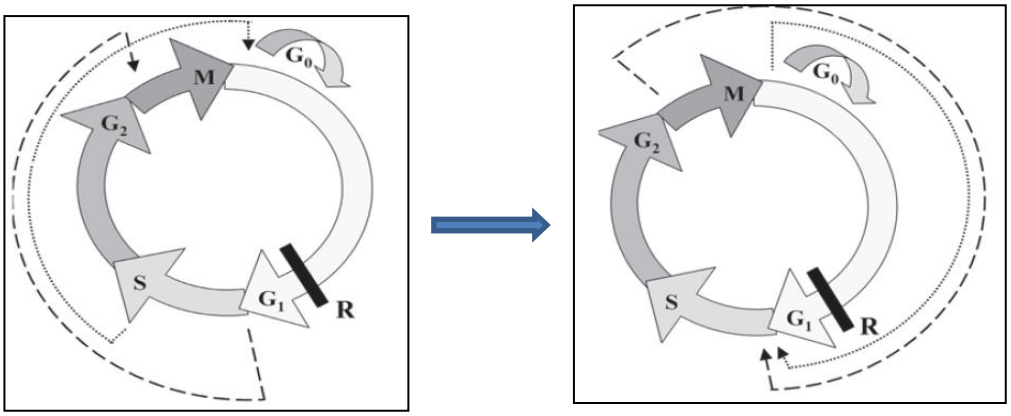

Figure 2.4 Representation of cell cycle synchronization in G1/S by double thymidine (Adapted from Harper J, 2005).

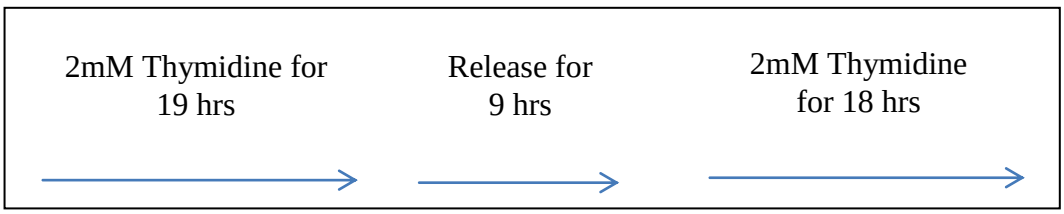

Figure 2.5 Schematic illustrations of G1/S synchronization methods.

2.2.6.2 Double Thymidine-Nocodazole Synchronization

Hela cells at confluency of 20% were treated with 2 mM Thymidine for 19 hours. They were released by refreshing the media for 9 hours and blocked for second time again with 2 mM Thymidine for 18 hours. After 1.5 hours of release, cells were treated with 100 ng/ml Nocodazole for 16 hours and collected by hitting on the edge. The collected cells are seeded again and collected at time of interest.

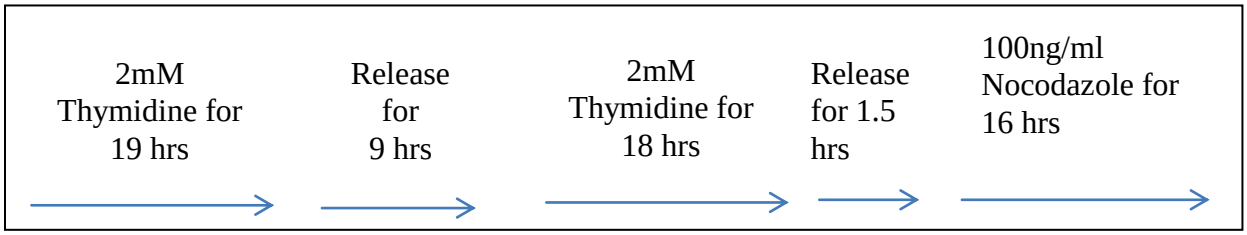

Figure 2.6 Schematic illustration of G2/M synchronization method.

2.2.6.3 Nocodazole Synchronization

Hela cells at 60% confluency are treated with 100 ng/ml Nocodazole for 22 hours and collected with trypsinization. Reseeded cells were collected at time of interest.

2.2.6.4 Transfection of Synchronized Cells

2.2.6.4.1 Transient Transfection in Double Thymidine-Nocodazole Synchronization

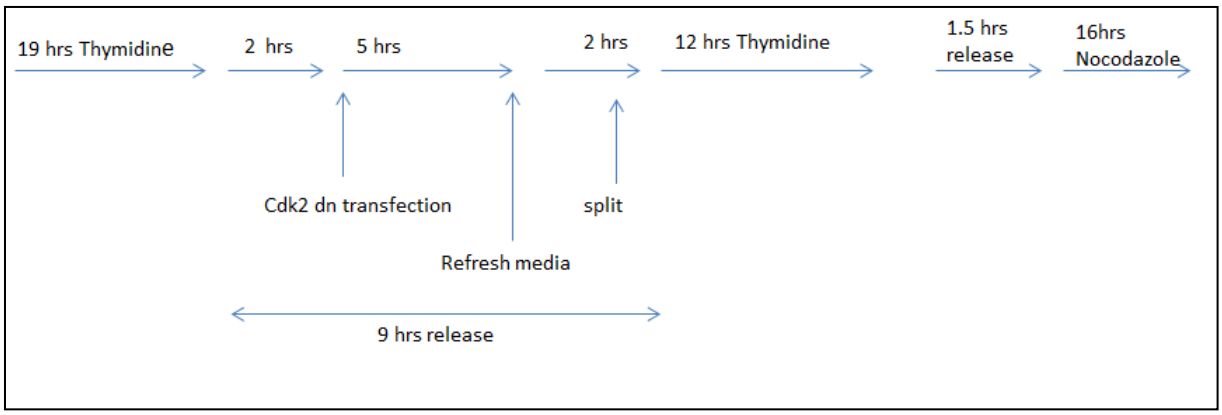

Figure 2.7 Schematic illustration of transient transfection in double thymidine Nocodazole synchronization.

2.2.6.4.2 Transient Transfection in Thymidine-Nocodazole Synchronization.

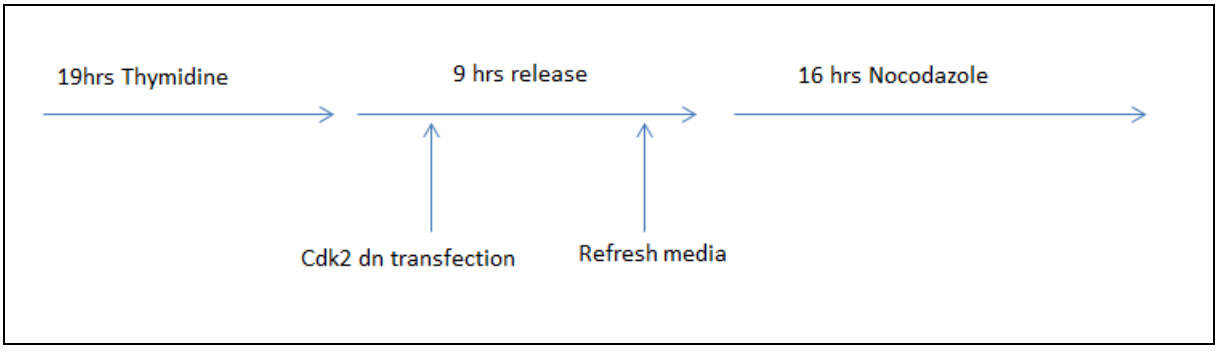

Figure 2.8 Schematic illustration of transient transfection in Thymidine-Nocodazole synchronization.

2.2.7 Flow Cytometry

2.2.7.1 Fixation of Cells with Ethanol

After cells have been collected they were mixed very gently in order to prevent clusters. They were fixed in 4.5 ml of cold 70% Ethanol and stored at -20 ºC.

2.2.7.2 Staining the Cells

Cells were pelleted and washed with PBS. Cells were incubated at 37 ºC for 30 minutes with staining solution, PI/RNAase (25 µg/ml PI and 200 µg/ml RNAase A).

2.2.7.3 Analysis by FACS Machine

FACS analysis was carried out by FACS CALIBUR from BOUN AKIL LAB. The data was evaluated by CellQuest software program.

CHAPTER 3

RESULTS

3.1 PRODUCTION OF THR61 ➔ALA61 CONSTRUCT OF SLBP FOR G1 REGULATION STUDIES

As a first part of my thesis, I have generated a tool for G1 regulation studies of SLBP. I have established stable cell lines expressing wild type and mutant SLBP constructs.

3.1.1 Generation Thr61➔ Ala61 mutant construct of His-tagged SLBP in pcDNA3.1(-)

In order to mutate Thr 61 to Ala 61, I have designed forward and reverse primers and mutated hisSLBP in pcDNA3.1(–) (SFTTP motif) into his SLBP in pcDNA3.1(-) (SFTAP motif) with single nucleotide change (Figure 2.1). The steps for generation of point mutation were described in Methods part (Figure 2.2). I have mutated Adanine at position 84 of coding sequence into Guanine in order to obtain Thr 61 ➔ Ala 61.

Subsequently, to confirm if I got successful PCR result, I run my PCR samples with 1% agarose, but could not detect any band. The protocol suggests to continue even if no band is detected, so I transformed PCR samples and obtain around 20 colonies. There were 2 colonies in negative control PCR sample (no primers) which indicates that the DpnI digestion may not have been sufficient. I have selected 5 colonies and send them to sequencing and all of the five constructs had successful point mutation at desired position.

I have aligned 3 of the sequence results (Figure 3.2). In protein alignment of Figure 3.4, it can be clearly seen that Thr61 has been successfully mutated into Ala 61 with proper His tag (not shown here) and stop codon. After confirmation with sequencing , I have maxiprepped one of these constucts (UD126_2) and aliqoited for further usage. Figure 3.3 shows the sequence chromatogram of the mutated construct.

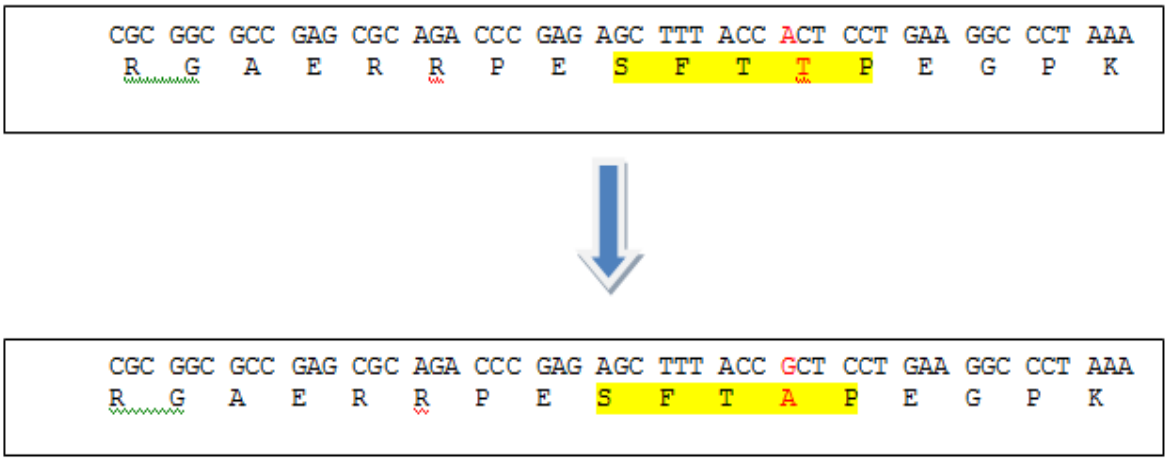

Figure 3.1 The nucleotide and protein sequence of SLBP region used for primer design. The Adenine nucleotide at position 84 was mutated into Guanine in order to obtain Ala 61 instead of Thr 61.

```
                          51                                                  100
      SLBP ORF    (51)  TGACGCCAGCCCGCCGTCCCCCGCGCGATGGAGCCTGGGACGGAAGCGCA
UD 126_1 SFTAP    (51)  TGACGCCAGCCCGCCGTCCCCCGCGCGATGGAGCCTGGGACGGAAGCGCA
UD 126_2 SFTAP    (51)  TGACGCCAGCCCGCCGTCCCCCGCGCGATGGAGCCTGGGACGGAAGCGCA
 ud126_3 SFTAP    (51)  TGACGCCAGCCCGCCGTCCCCCGCGCGATGGAGCCTGGGACGGAAGCGCA
     Consensus    (51)  TGACGCCAGCCCGCCGTCCCCCGCGCGATGGAGCCTGGGACGGAAGCGCA
                         101                                                 150
      SLBP ORF   (101)  GAGCCGACGGCAGGCGCTGGAGGCCCGAAGACGCCGAGGAGGCAGAGCAC
UD 126_1 SFTAP   (101)  GAGCCGACGGCAGGCGCTGGAGGCCCGAAGACGCCGAGGAGGCAGAGCAC
UD 126_2 SFTAP   (101)  GAGCCGACGGCAGGCGCTGGAGGCCCGAAGACGCCGAGGAGGCAGAGCAC
 ud126_3 SFTAP   (101)  GAGCCGACGGCAGGCGCTGGAGGCCCGAAGACGCCGAGGAGGCAGAGCAC
     Consensus   (101)  GAGCCGACGGCAGGCGCTGGAGGCCCGAAGACGCCGAGGAGGCAGAGCAC
                         151                                                 200
      SLBP ORF   (151)  CGCGGCGCCGAGCGCAGACCCGAGAGCTTTACCACTCCTGAAGGCCCTAA
UD 126_1 SFTAP   (151)  CGCGGCGCCGAGCGCAGACCCGAGAGCTTTACCGCTCCTGAAGGCCCTAA
UD 126_2 SFTAP   (151)  CGCGGCGCCGAGCGCAGACCCGAGAGCTTTACCGCTCCTGAAGGCCCTAA
 ud126_3 SFTAP   (151)  CGCGGCGCCGAGCGCAGACCCGAGAGCTTTACCGCTCCTGAAGGCCCTAA
     Consensus   (151)  CGCGGCGCCGAGCGCAGACCCGAGAGCTTTACCGCTCCTGAAGGCCCTAA
                         201                                                 250
      SLBP ORF   (201)  ACCCCGTTCCAGATGCTCTGACTGGGCAAGTGCAGTTGAAGAAGATGAAA
UD 126_1 SFTAP   (201)  ACCCCGTTCCAGATGCTCTGACTGGGCAAGTGCAGTTGAAGAAGATGAAA
UD 126_2 SFTAP   (201)  ACCCCGTTCCAGATGCTCTGACTGGGCAAGTGCAGTTGAAGAAGATGAAA
 ud126_3 SFTAP   (201)  ACCCCGTTCCAGATGCTCTGACTGGGCAAGTGCAGTTGAAGAAGATGAAA
     Consensus   (201)  ACCCCGTTCCAGATGCTCTGACTGGGCAAGTGCAGTTGAAGAAGATGAAA
                         251                                                 300
      SLBP ORF   (251)  TGAGGACCAGAGTTAACAAAGAAATGGCAAGATATAAAAGGAAACTCCTC
UD 126_1 SFTAP   (251)  TGAGGACCAGAGTTAACAAAGAAATGGCAAGATATAAAAGGAAACTCCTC
UD 126_2 SFTAP   (251)  TGAGGACCAGAGTTAACAAAGAAATGGCAAGATATAAAAGGAAACTCCTC
 ud126_3 SFTAP   (251)  TGAGGACCAGAGTTAACAAAGAAATGGCAAGATATAAAAGGAAACTCCTC
     Consensus   (251)  TGAGGACCAGAGTTAACAAAGAAATGGCAAGATATAAAAGGAAACTCCTC
                         301                                                 350
      SLBP ORF   (301)  ATCAATGACTTTGGAAGAGAGAGAAAATCATCATCAGGAAGTTCTGATTC
UD 126_1 SFTAP   (301)  ATCAATGACTTTGGAAGAGAGAGAAAATCATCATCAGGAAGTTCTGATTC
UD 126_2 SFTAP   (301)  ATCAATGACTTTGGAAGAGAGAGAAAATCATCATCAGGAAGTTCTGATTC
 ud126_3 SFTAP   (301)  ATCAATGACTTTGGAAGAGAGAGAAAATCATCATCAGGAAGTTCTGATTC
     Consensus   (301)  ATCAATGACTTTGGAAGAGAGAGAAAATCATCATCAGGAAGTTCTGATTC
                         351                                                 400
      SLBP ORF   (351)  AAAGGAGTCTATGTCTACTGTGCCGGCTGACTTTGAGACAGATGAAAGTG
UD 126_1 SFTAP   (351)  AAAGGAGTCTATGTCTACTGTGCCGGCTGACTTTGAGACAGATGAAAGTG
UD 126_2 SFTAP   (351)  AAAGGAGTCTATGTCTACTGTGCCGGCTGACTTTGAGACAGATGAAAGTG
 ud126_3 SFTAP   (351)  AAAGGAGTCTATGTCTACTGTGCCGGCTGACTTTGAGACAGATGAAAGTG
     Consensus   (351)  AAAGGAGTCTATGTCTACTGTGCCGGCTGACTTTGAGACAGATGAAAGTG
                         401                                                 450
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UD 126_1 SFTAP   (401)  TCCTAATGAGGAGACAGAAGCAGATCAACTATGGGAAGAACACAATTGCC
UD 126_2 SFTAP   (401)  TCCTAATGAGGAGACAGAAGCAGATCAACTATGGGAAGAACACAATTGCC
 ud126_3 SFTAP   (401)  TCCTAATGAGGAGACAGAAGCAGATCAACTATGGGAAGAACACAATTGCC
     Consensus   (401)  TCCTAATGAGGAGACAGAAGCAGATCAACTATGGGAAGAACACAATTGCC
                         451                                                 500
      SLBP ORF   (451)  TACGATCGTTATATTAAAGAAGTCCCAAGACACCTTCGACAACCTGGCAT
UD 126_1 SFTAP   (451)  TACGATCGTTATATTAAAGAAGTCCCAAGACACCTTCGACAACCTGGCAT
UD 126_2 SFTAP   (451)  TACGATCGTTATATTAAAGAAGTCCCAAGACACCTTCGACAACCTGGCAT
 ud126_3 SFTAP   (451)  TACGATCGTTATATTAAAGAAGTCCCAAGACACCTTCGACAACCTGGCAT
     Consensus   (451)  TACGATCGTTATATTAAAGAAGTCCCAAGACACCTTCGACAACCTGGCAT
                         501                                                 550
      SLBP ORF   (501)  TCATCCCAAGACCCCTAATAAATTTAAGAAGTATAGTCGACGTTCATGGG
UD 126_1 SFTAP   (501)  TCATCCCAAGACCCCTAATAAATTTAAGAAGTATAGTCGACGTTCATGGG
UD 126_2 SFTAP   (501)  TCATCCCAAGACCCCTAATAAATTTAAGAAGTATAGTCGACGTTCATGGG
 ud126_3 SFTAP   (501)  TCATCCCAAGACCCCTAATAAATTTAAGAAGTATAGTCGACGTTCATGGG
     Consensus   (501)  TCATCCCAAGACCCCTAATAAATTTAAGAAGTATAGTCGACGTTCATGGG
                         551                                                 600
      SLBP ORF   (551)  ACCAGCAAATCAAACTCTGGAAGGTGGCTCTGCATTTTTGGGATCCTCCA
UD 126_1 SFTAP   (551)  ACCAGCAAATCAAACTCTGGAAGGTGGCTCTGCATTTTTGGGATCCTCCA
UD 126_2 SFTAP   (551)  ACCAGCAAATCAAACTCTGGAAGGTGGCTCTGCATTTTTGGGATCCTCCA
 ud126_3 SFTAP   (551)  ACCAGCAAATCAAACTCTGGAAGGTGGCTCTGCATTTTTGGGATCCTCCA
     Consensus   (551)  ACCAGCAAATCAAACTCTGGAAGGTGGCTCTGCATTTTTGGGATCCTCCA
                         601                                                 650
      SLBP ORF   (601)  GCGGAAGAAGGATGTGATTTGCAAGAAATACACCCTGTAGACCTTGAATC
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UD 126_1 SFTAP    (601) GCGGAAGAAGGATGTGATTTGCAAGAAATACACCCTGTAGACCTTGAATC
UD 126_2 SFTAP    (601) GCGGAAGAAGGATGTGATTTGCAAGAAATACACCCTGTAGACCTTGAATC
 ud126_3 SFTAP    (601) GCGGAAGAAGGATGTGATTTGCAAGAAATACACCCTGTAGACCTTGAATC
     Consensus    (601) GCGGAAGAAGGATGTGATTTGCAAGAAATACACCCTGTAGACCTTGAATC
                        651                                             700
      SLBP ORF    (651) TGCAGAAAGCAGCTCCGAGCCCCAGACCAGCTCTCAGGATGACTTTGATG
UD 126_1 SFTAP    (651) TGCAGAAAGCAGCTCCGAGCCCCAGACCAGCTCTCAGGATGACTTTGATG
UD 126_2 SFTAP    (651) TGCAGAAAGCAGCTCCGAGCCCCAGACCAGCTCTCAGGATGACTTTGATG
 ud126_3 SFTAP    (651) TGCAGAAAGCAGCTCCGAGCCCCAGACCAGCTCTCAGGATGACTTTGATG
     Consensus    (651) TGCAGAAAGCAGCTCCGAGCCCCAGACCAGCTCTCAGGATGACTTTGATG
                        701                                             750
      SLBP ORF    (701) TGTACTCTGGCACACCCACCAAGGTGAGACACATGGACAGTCAAGTGGAG
UD 126_1 SFTAP    (701) TGTACTCTGGCACACCCACCAAGGTGAGACACATGGACAGTCAAGTGGAG
UD 126_2 SFTAP    (701) TGTACTCTGGCACACCCACCAAGGTGAGACACATGGACAGTCAAGTGGAG
 ud126_3 SFTAP    (701) TGTACTCTGGCACACCCACCAAGGTGAGACACATGGACAGTCAAGTGGAG
     Consensus    (701) TGTACTCTGGCACACCCACCAAGGTGAGACACATGGACAGTCAAGTGGAG
                        751                                             800
      SLBP ORF    (751) GATGAGTTTGATTTGGAAGCTTGTTTAACTGAACCCTTGAGAGACTTCTC
UD 126_1 SFTAP    (751) GATGAGTTTGATTTGGAAGCTTGTTTAACTGAACCCTTGAGAGACTTCTC
UD 126_2 SFTAP    (751) GATGAGTTTGATTTGGAAGCTTGTTTAACTGAACCCTTGAGAGACTTCTC
 ud126_3 SFTAP    (751) GATGAGTTTGATTTGGAAGCTTGTTTAACTGAACCCTTGAGAGACTTCTC
     Consensus    (751) GATGAGTTTGATTTGGAAGCTTGTTTAACTGAACCCTTGAGAGACTTCTC
                        801                                             850
      SLBP ORF    (801) AGCCATGAGC----------------------------------------
UD 126_1 SFTAP    (801) AGCCATGAGCTAACCGCTCGAGCATGCATCTAGAGGGCCCTATTCTATAG
UD 126_2 SFTAP    (801) AGCCATGAGCTAACCGCTCGAGCATGCATCTAGAGGGCCCTATTCTATAG
 ud126_3 SFTAP    (801) AGCCATGAGCTAACCGCTCGAGCATGCATCTAGAGGGCCCTATTCTATAG
     Consensus    (801) AGCCATGAGCTAACCGCTCGAGCATGCATCTAGAGGGCCCTATTCTATAG
```

Figure 3.2 Nucleotide alignments of SFTAP construct and wildtype SLBP. UD 126 samples (1, 2 and 3) represent DNA samples from three different colonies.

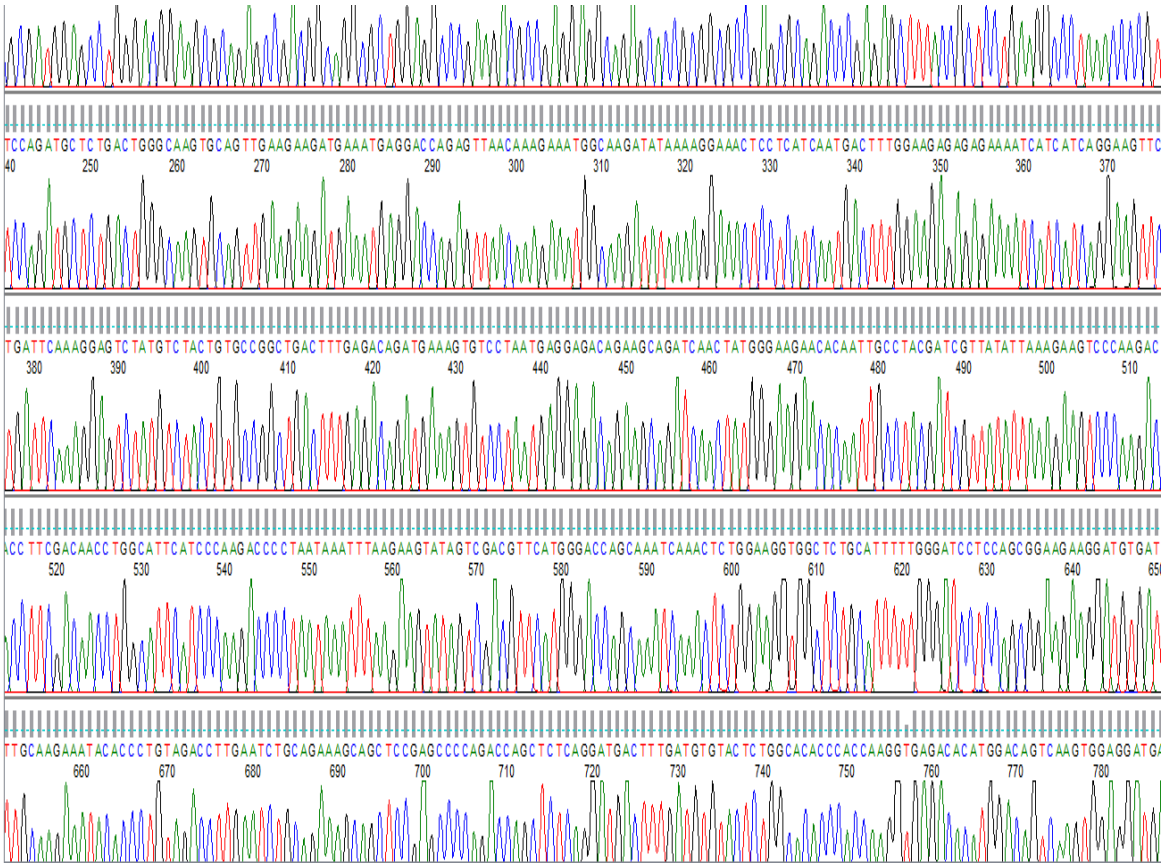

Figure 3.3 Chromatogram of SFTAP SLBP (UD 126_2) construct sequence analysis.

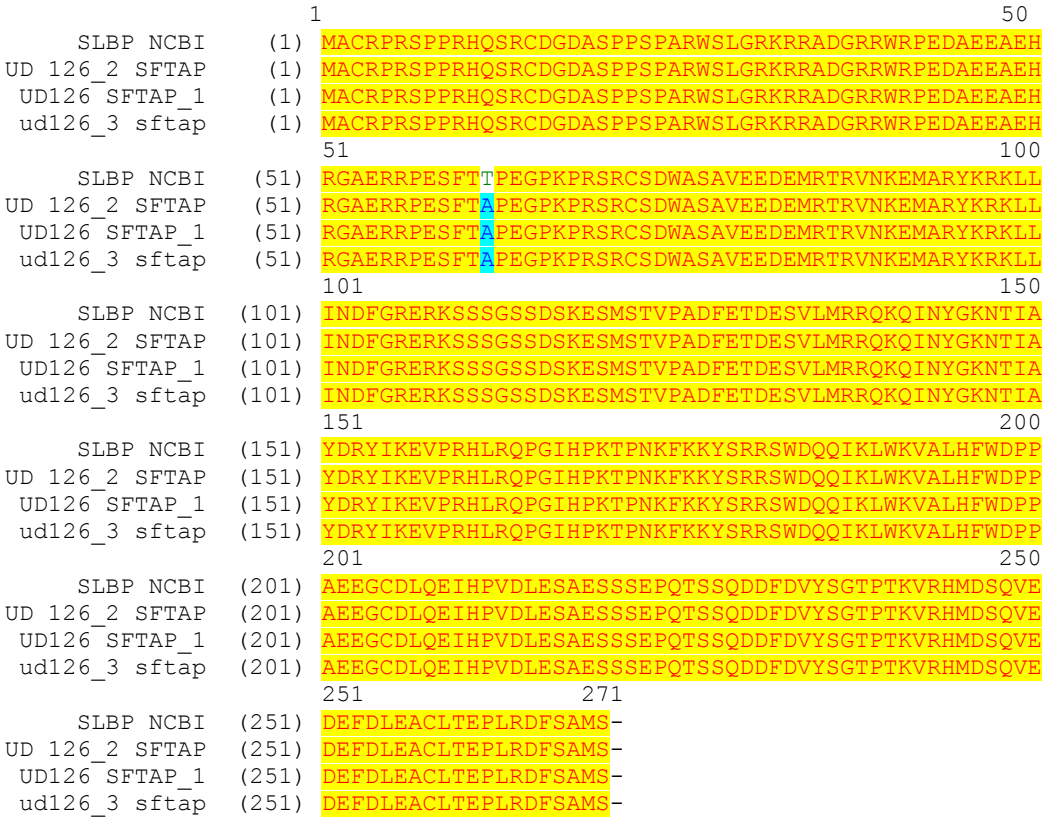

Figure 3.4 Protein alignment of mutated and wild type SLBP. UD 126 samples (1, 2 and 3) represents DNA samples from three different colonies.

3.1.2 Optimization of Transfecting Hela Cells with Wild Type and Mutant SLBP Constructs

During transfection of Hela cells, I have encountered several problems which led to either cell death or low expression efficiency. So, in order to have proper stable Hela cells with wild type and mutant SLBP expression, I have optimized the transfection protocol.

3.1.2.1 Optimizing the DNA Concentration

High amount of SLBP may be toxic for the most of the cells, so it was important to choose most optimum DNA concentration to be transfected. For this reason I have used HA-SLBP with different concentrations (1µg, 2 µg, 3 µg and 4 µg). The expression of HA tagged and His tagged SLBP does not show difference thoughout cell

cycle, so I have tested HA tagged SLBP for concentration optimization. As shown in Figure 3.5, 2-3 μg for 6 well plates seems to be most optimum. 4 μg has high cytotoxicity to Hela cells, whereas 1 μg may not be enough to have enough exogenous expression. Another important finding about the plasmid DNA used for transfection is that, when constructs are miniprepped by using Fermentas Gene-Jet Miniprep kit, the rate of cell death was higher, most probably due to either high volume of elution buffer or presence of endotoxins in plasmid DNA. To test if elusion buffer is toxic, I have transfected Hela cells just with Elusion Buffer and observed that it does not cause any cell death. (Figure 3.6). So I suspected about the presence of endotoxins in my miniprepped plasmids and in order to eliminate this effect, I have maxiprepped my constructs and used less volume for transfection. Further I had figured out that the death of Hela cells after the transfection was due to some problem in Hela cell attachment to 6 well plate (Greiner). When I splitted cells into 24 well plate I did not observed these problem (not shown here).

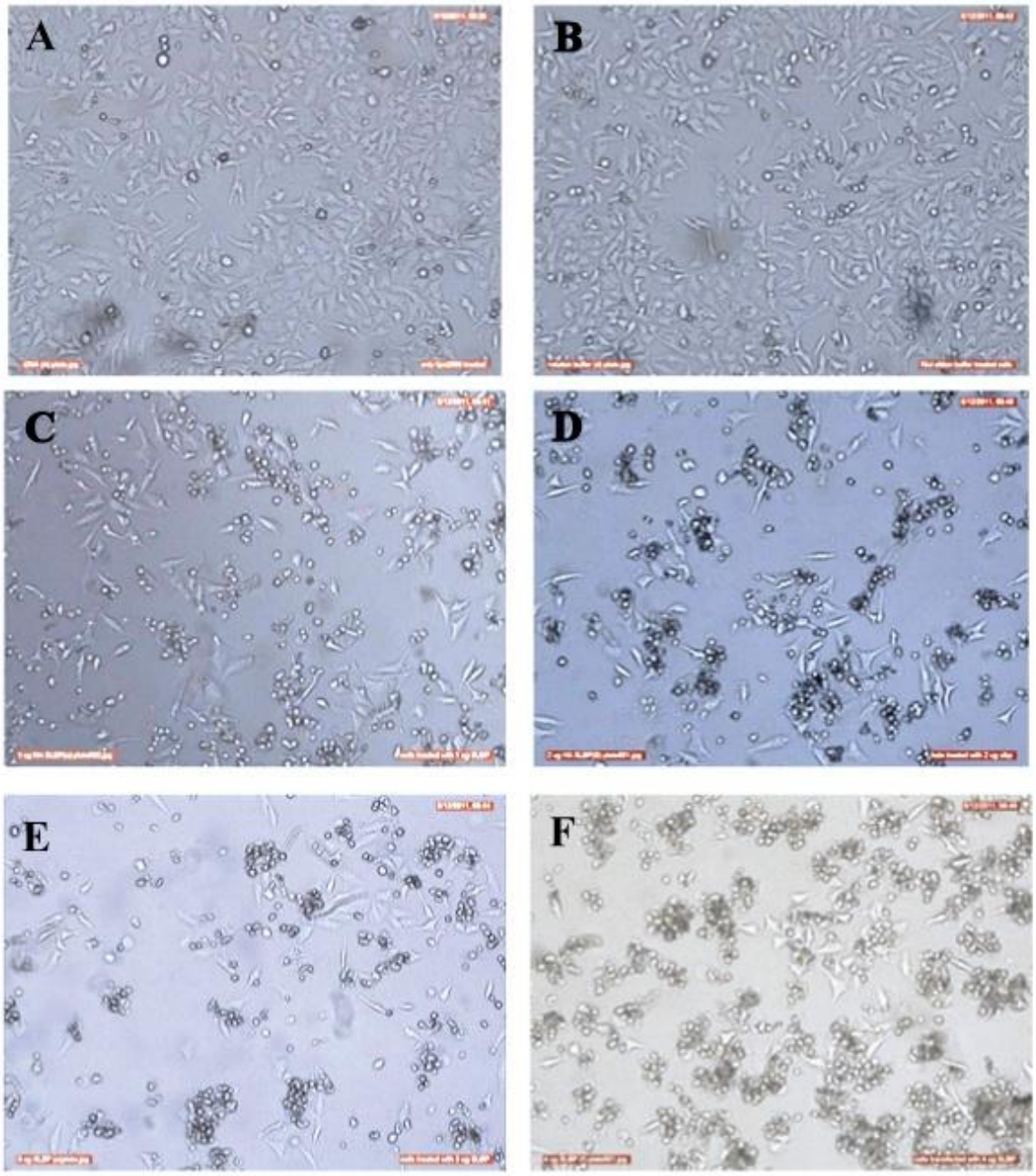

Figure 3.5 The effect of DNA concentration on cell viability. Hela cells were transfected with **(1A)** Lipo 2000 reagent without any DNA, **(1B)** Lipo 2000 reagent and Elusion Buffer alone, and (**1C)** 1µg of HA-SLBP; (**1D)** 2 µg of HA-SLBP; (**1E**) 3 µg of HA-SLBP and (**1F**)4 µg of HA-SLBP.

3.1.2.2 Optimizing Proper Cell Population for Transfection

The confluency rate of cells is one of the factors that affect death during transfection and it changes depending on cell type and construct used for transfection. So I tried to optimize the proper confluency of Hela cells, which causes less death and high expression. The most effective cell percentage was found to be 90%-95% (Table 3.1), where I observed relatively less death compared to other populations. Interestingly, as mentioned above 24 well plates gave better result compared to 6 well plates even with less confluent cell population, so I used 2 wells of 24 well plate in order to have sufficient samples for further studies.

Table 3.1 Effect of cell population percentage during transfection with Lipo 2000.

% cells before transfection	% cells after transfection
60	10-15
70	40-50
90	80

3.1.2.3 Optimizing Transfection Reagent

In order to find out most proper transfection reagent, namely most efficient and less cytotoxic one, I have tried out several types. We have requested sample tests for different transfection reagent. To test the efficiency, the best way is to do a Western Blotting of transfected protein but at that time we had not totally optimized the Western Blotting protocol, so I tested the efficiency of transfection reagents by different approach. I have transfected Hela cells with 2 µg of pCMV, Cdk2 dn and Cdk4 dn with 3 types of transfection reagents: Lipo 2000, Turbofect, Biorad transfection reagents. have been tested. The Cdk dominant negative proteins are kinase dead Cdks, which reduces the Cdks activity. After 48 hours, cells were splitted into 96 well plate and incubated for 24 hours (Figure 3.6). The efficiency of transfection reagents were tested

with BrdU incorporation assay, as Cdk 2 dn and Cdk4 dn are expected to arrest cells in G1 phase of cell cycle, namely would lead to decrease in BrdU incorporation. BrdU is a thymidine analog and incorporates into newly synthsized DNA strands of actively proliferating cells. Thus, in order to estimate the percentage of cells in S phase, where DNA is replicated, I have normalized BrdU values by dividing into WST-1 values for each well. The normalized values were converted into percentage by comparing with pCMV (no insert) sample values. As a result, I found that, Lipo 2000 is the most efficient and relatively less toxic transfection reagent compared to others. Samples transfected with Turbofect have very high proliferation rate, namely almost all cells have survived after transfection but they were not arrested in G1, they had high BrdU incorporation values. The normalized values of BrdU and WST-1 values are shown in Figure 3.7. Biorad Tranfection reagent was were toxic to Hela cells, as they have killed around 70% of total population, but as it can be seen from the Figure 3.7 it has very high expression efficiency.

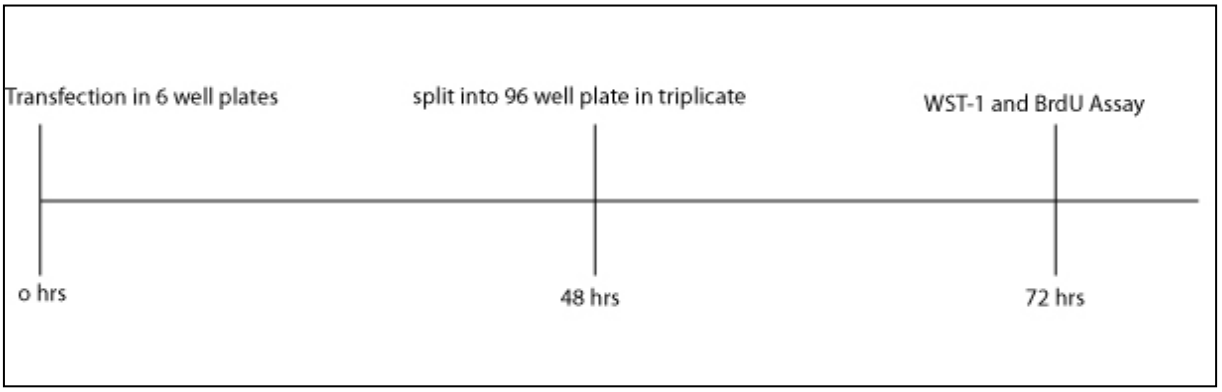

Figure 3.6 Schematic illustration of the experiment carried out to measure transfection efficiency of reagents.

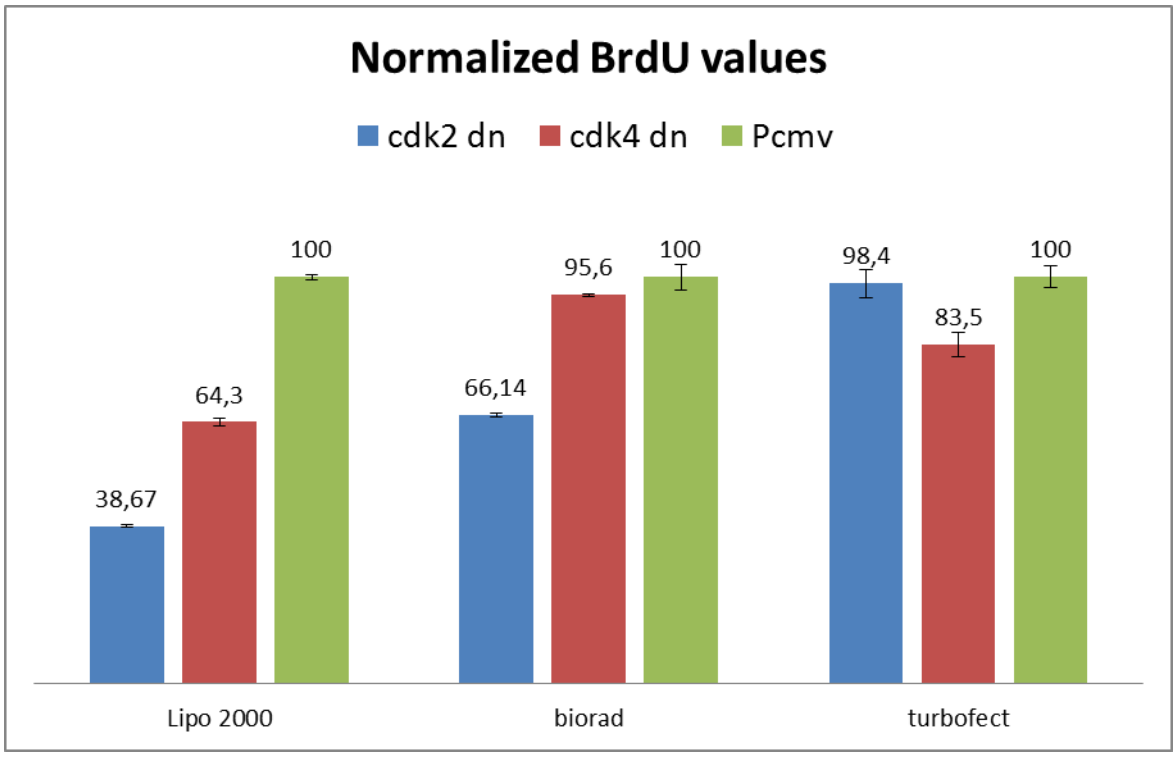

Figure 3.7 Normalized BrdU values of Cdk2 dn and Cdk4 dn transfected Hela cells with different transfection reagents.

3.1.3 Generation of Stable Hela Cell Lines with His-Tagged Wild Type and Mutant SLBP Constructs in pcDNA 3.1(-)

Hela cells were transfected with wild type His-tagged SLBP (SFTTP) and mutated SLBP (SFTAP). pcDNA3.1 (-) was used as a negative control. After 48 hours of transfection 2/3rd of cells were taken for Western blotting in order to confirm the protein expression and 1/3rd was seeded back for stable cell generation. To achieve long term expression of SLBP constructs, I have selected transfected cells by treating them with Geniticin G-418 (neomycin, Figure 2.1). After several cycles, the plasmid will be integrated into the genome of the host cell. The selection was performed until all the negative control cells (No DNA) have died. The cells in control plate have completely diminished in 4 weeks.

I have successfully established stable cell lines with both SFTTP and SFTAP SLBP constructs. The expression of SLBP in both transiently transfected and stable

Hela cells are shown in Figure 3.8. These samples were collected and lysed as discussed in methods part and run in a 12% gel. There is no exogeneous SLBP in –insert (pcDNA3) lane, indicating that band over the 45 kDa is His-tagged SLBP. SLBP is itself has 269 amino acids and molecular weight is around 31 kDa. It has an unusual electrophoretic mobility and migrated at around 45 kDa.

The transient expression of exogenous SLBP constructs are very high, there is almost 5-6 fold higher expression than the endogenous one (Figure 3.8 A). After the constructs have been incorporated into the genome, the expression level of both endogenous and exogenous SLBP have been equalized (Figure 3.8 B).

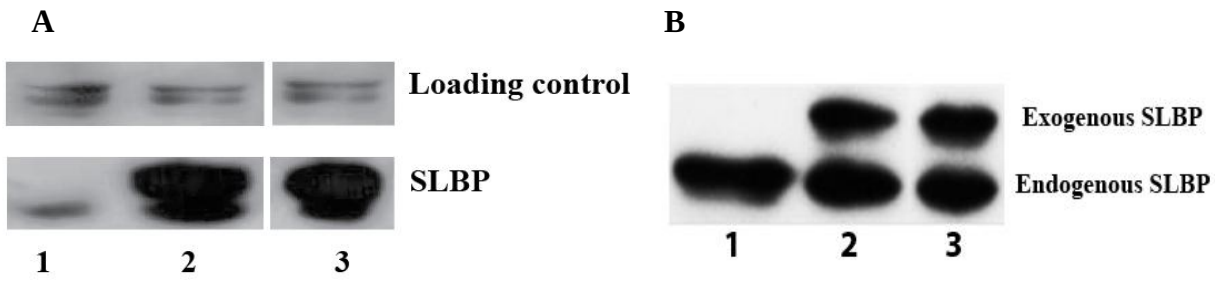

Figure 3.8 Western blot analysis of Hela cells samples of transient and stable expression of exogenous SLBP. **A:** Transient expression of exogenous SLBP, **B**: Stable expression of SLBP in Hela Cells. For both Panels, lane 1 is a negative control, has only pcDNA3.1(-) vector, lane 2 has his tagged wild type SLBP in pcDNA3.1(-) (SFTTP) and lane 3 has S/G2 degradation mutant SLBP in pcDNA3.1(-) (SFTAP).

3.2 INVESTIGATING THE ROLE OF CDK2 ON G1 REGULATION OF SLBP

3.2.1. Establishment of Western Blotting Technique for Different Proteins

Before going through the main experiments, several steps of Western Blotting for different proteins were optimized. Firstly, equal loading of samples was very crucial for my experiments. Even a slight difference in protein concentration of samples might lead to incorrect conclusions. I have spent a lot of time on this problem and tried out several different approaches.

Another major step in Western Blotting that had to optimized was transfer of proteins from gel to PVDF membrane. I have played with concentration of Transfer Buffer contents. Higher concentration of SDS enables better transfer of bigger proteins (> 100kDa) but has reverse effect on small ones. Methanol enhances transfer efficiency of all proteins but it tends to remove SDS from proteins. So the concentration of SDS and methanol percentage has to be optimized for proteins based on their size. Proteins that I am interested in are in between 25 kDa to 70 kDa, so I have used very low amount of SDS (0.04%) and 20% HPLC grade Methanol.

The dilution and incubation period of antibodies had to be optimized for different proteins. I aimed to find out the lowest amount with highest effect. In Figure 3.8 it is shown that western blotting analysis of SLBP, Cdk2, Cdk4 and Cyclin A has been optimized successfully. The sharpest bands were obtained with 1:1000 primary antibody dilutions for SLBP serum, 1:1250 for Cdk4, 1:500 for Cyclin A and 1:1000 for Cdk2. Cdk4 and the nonspecific band of SLBP serum was used as a loading control in most of the Western Blots, as their expression does not change throughout the cell cycle.

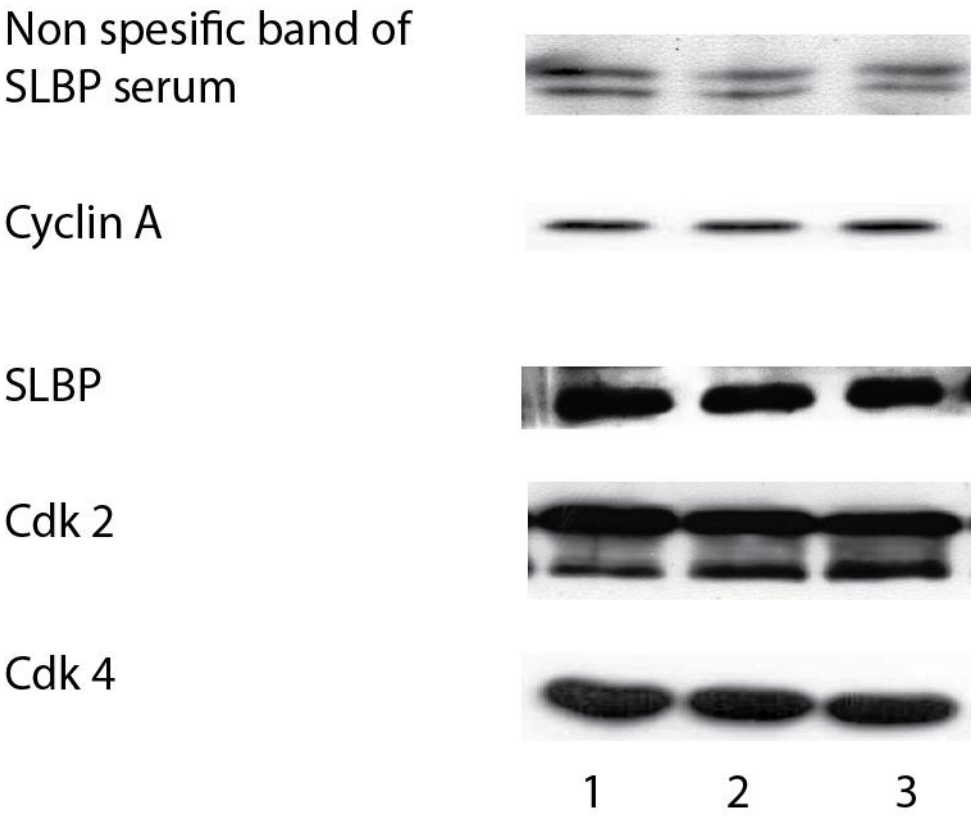

Figure 3.9 Western Blot analyses of three different Hela cell samples for different proteins.

3.2.2 The Effect of Cdk Inhibition on Asynchronized Cells

3.2.2.1 Treatment of Asynchronous Cells with Roscovitine

Roscovitine selectively diminishes activity of Cdk1 and Cdk2. In order to analyze effect of Cdks on SLBP expression, I have treated Hela cells at 60% confluency with 2mM Roscovitine or corresponding volume of DMSO (vehicle) as a control.

15 hours treatment of cells with Roscovitine was toxic for Hela cells, as 50 % of cells were dead compared to control one. In order to optimize duration of Roscovitine treatment, I have treated 6 plates (for 0hr, 2 hrs, 4 hrs, 6 hrs, 8 hrs and 10 hrs) with Roscovitine and analyzed their viability by Trypan Blue staining. The percentage of dead cells was calculated by dividing the number of dead cells over the total cell number. I have observed that, Roscovitine starts to kill cells after 6 hours of treatment (Table 3.2).

Cdk inhibition seems to decrease SLBP expression in asynchronized cells. The level of Cyclin A increases as cells enter to S phase in Cdk2 dependent manner, thus I have checked effect of Roscovitine on Cyclin A expression along with SLBP (Figure 3.10). The cell cycle distribution of Hela cells after Roscovitine treatment did not change significantly and the cell cycle was assessed by FACS analysis (Table 3.3). The G2 population has increased slightly in Roscovitine treated cells compared to the control.

Table 3.2 The effect of Roscovitine treatment on cell viability.

Duration of treatment	**Percentage of dead cells**
0 hr	2 %
2 hrs	3 %
4 hrs	2 %
6 hrs	2.7 %
8 hrs	18 %
10 hrs	47 %

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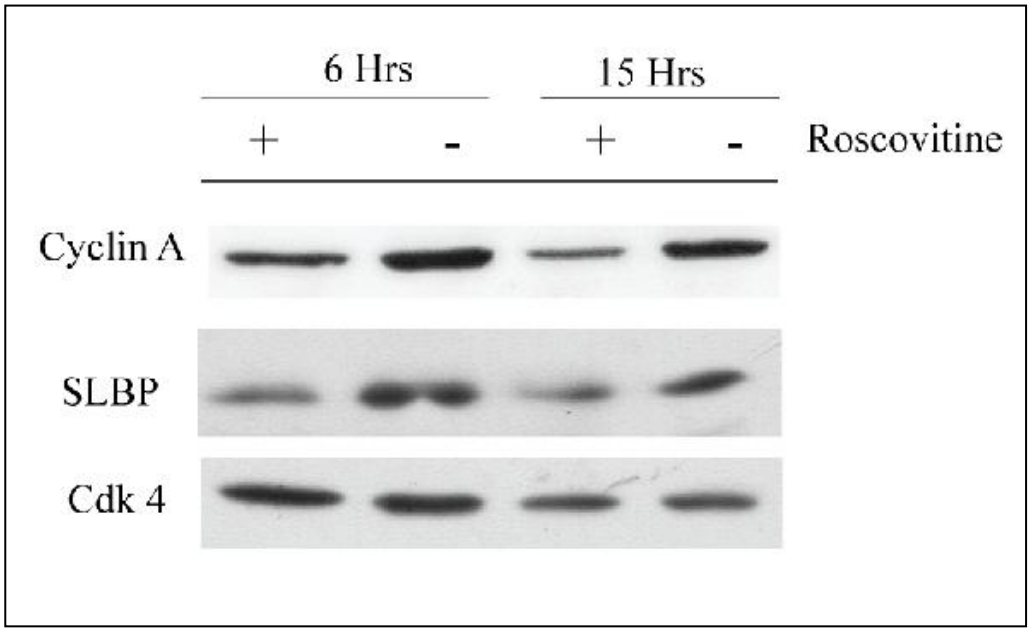

Figure 3.10 The effect of Roscovitine on cyclin A and SLBP expression of asynchronized Hela cells. 1st and 3rd lanes were treated with Roscovitine for 6 hrs and 15 hrs respectively. 2nd and 4th lanes were treated with DMSO (vehicle) and used as a control. The level of SLBP, Cdk 4 and Cyclin A was assessed by Western Blotting.

Table 3.3 The effect of Roscovitine on cell cycle distribution of Hela cells. The cell cycle distribution was assessed by FACS analysis. Samples treated with Roscovitine (for 6 hrs and 15 hrs) or DMSO was analyzed with PI staining in FACS machine.

	DMSO	**Roscovitine (6hrs)**	**Roscovitine (15hrs)**
G1 phase	66%	57 %	60 %
G2 phase	22%	26 %	26 %

3.2.2.2 Transfection of Asynchronized Hela Cells with Cdk2 Dominant Negative

In order to confirm whether the decline in SLBP level is Cdk 2 dependent, I have transfected cycling Hela cells with Cdk2 dn construct, which encodes kinase dead Cdk 2 protein. As I had expected based on the previous data, Cdk2 dn transfected Hela cells, had decrease in SLBP expression compared to pCMV control (Figure 3.11). In Figure 3.11, the loading of the samples were not equal but change in SLBP is greater than the change in loading control expression. Cdk2 was overexpressed successfully which can be observed clearly despite the difference in loading.

The cell cycle distribution does not change significantly with Cdk2 dn transfection. But slight increase in G1 population can be observed which indicates that Cdk2 inhibition might have arrested very few cells in G1 phase, which has no effect on the current result (Table 3.4).

In conclusion, the data above suggest that SLBP expression decreases depending on Cdk 2 inhibition in unsynchronized cells.

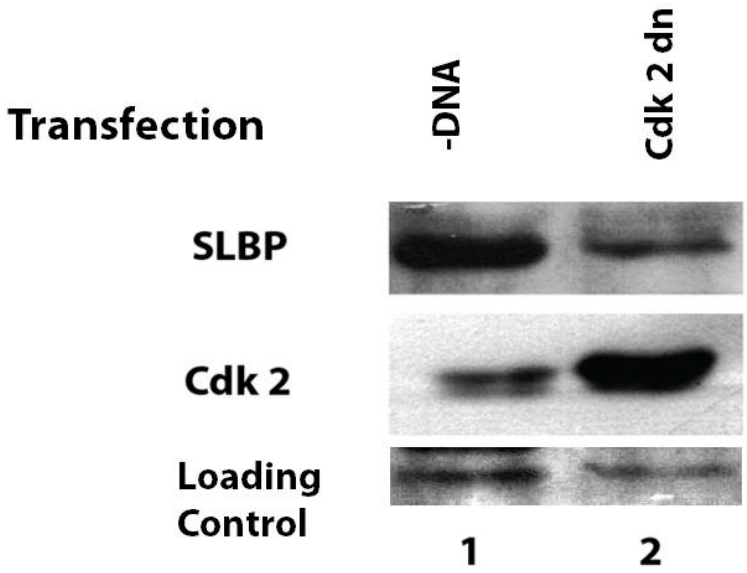

Figure 3.11 The effect of Cdk2 dn transfection on SLBP level of asynchronous cells. Hela cells were transfected with Cdk 2 dn construct and cells were collected 48 hrs after transfection. SLBP, Cdk2 and L.Control were assessed by Western Blotting.

Table 3.4 The effect of Cdk2 inhibition on cell cycle distribution. Transfection of Hela cells with Cdk2 dn transfection does not change cell cycle distribution much.

	- **DNA**	**Cdk 2 dn**
G1 phase	66%	73%
G2 phase	22%	20%

3.2.3 The Effect of Cdk2 Inhibition on G1/ S Expression of SLBP

In order to check the role of Cdk2 in SLBP increment at G1/S transition, I have synchronized Hela cells with different synchronization techniques. By inhibiting Cdk2 activity in late G1 phase, I have analyzed the effect on SLBP expression by Western Blotting. For inhibition of Cdk2, I have treated synchronized cells with Roscovitine or transfected them with Cdk2 dn.

3.2.3.1 The Effect of Roscovitine on SLBP Expression at G1/S Transition

Hela cell synchronization has been well optimized, but it may change depending on cell passage number or other properties of Hela cells in different Laboratories. I have carried out two different synchronization methods in order to better analyze SLBP regulation in G1 phase. Synchronization of cells in G1/S by double Thymidine block is widely used methods but it takes around 20 hours to complete the cycle. So, as cells approach to S phase, there might be impairment in cell synchrony. Using an additional approach would also support the notion of G1 regulation of SLBP.

Firstly, I have synchronized Hela cells at G1/S border with double thymidine block and released them for one more cycle. I have collected cells every two hours after release and divided samples for FACS and Western Blot analysis. In order to inhibit Cdk2 activity, I have treated cells at estimated G1 phase with Roscovitine for determined time and collected as they approached to S phase.

Further, I have synchronized Hela cells with double thymidine followed by Nocodazole treatment in order to arrest cells at G2/M phase released till subsequent S phase. In literature there are some evidences where G2/M synchronization was done by just treating with Nocodazole for longer time. The longer the exposure the higher the toxicity of Nocodazole on cells, thus I have shortened the duration of Nocodazole treatment by firstly blocking cells at G1/S border with double thymidine treatment. I have added Roscovitine in assumed mid-G1 phase and collected as cells approached to S phase.

3.2.3.1.1 The Effect of Roscovitine on Late G1 Expression of SLBP in Double Thymidine Synchronized Cells

I have synchronized Hela cells with double Thymidine at G1/S border and check the effect of Roscovitine on SLBP expression in next G1 phase . Figure 3.11 shows the illustration of the experiment flow; the arrows show the time of sample collection. Roscovitine and its control was added separately at indicated time points and collected later as shown again in the Figure 3.12.

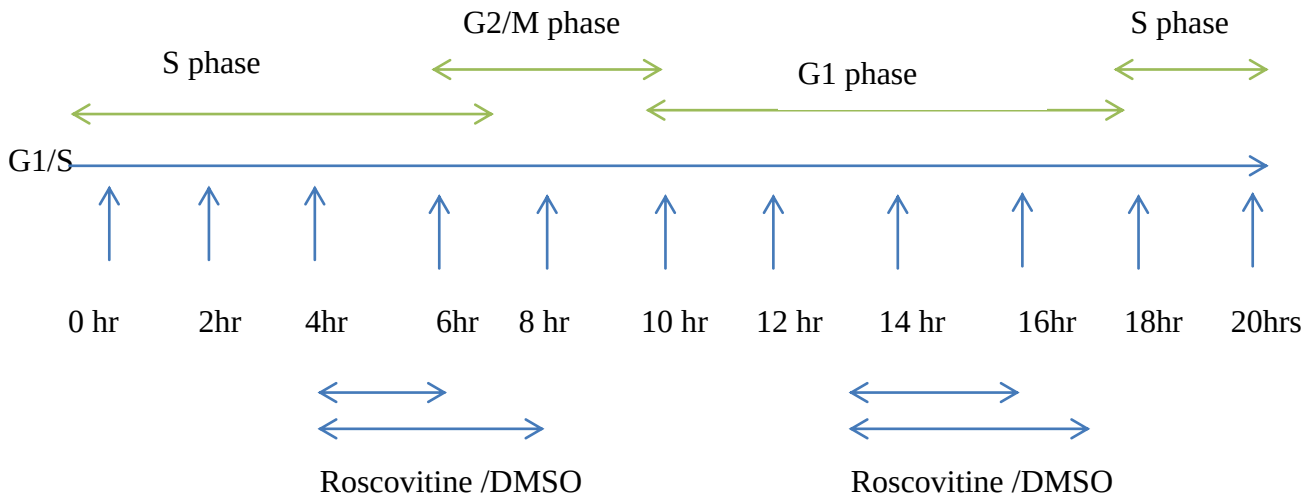

Figure 3.12 Schematic representation of double Thymidine synchronized of cells at G1/S border and time points for Roscovitine treatment.

The cell cycle distribution of synchronized cells was assessed by FACS analysis (Figure 3.13). At 0 hr., almost all of the cells seem to be at G1 phase and they start to enter G2 phase after 6 hours and S phase after 18 hours. This data indicates that, synchronized Hela cells successfully have entered next cycle in synchrony.

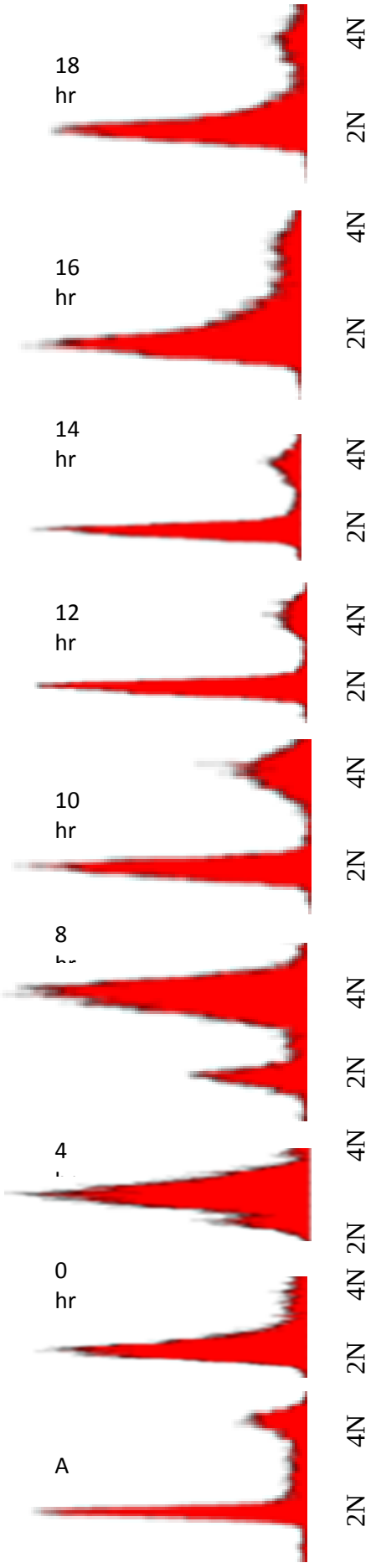

Figure 3.13 FACS analysis of double Thymidine synchronized Hela cells. Cells were synchronized at G1/S border and released for one more cycle. A indicates asynchronous cells. At O hr, almost all of cells seem to be at G1 phase and they start to enter G2 phase at 8 hrs after release. Cells started to enter to next S phase 16 hrs after release.

To check out the role of Cdk2 on expression of SLBP at G1/S transition phase, I have treated Hela cells at late G1 phase with Roscovitine. The change in SLBP level was detected by Western Blot analysis of collected samples. The Roscovitine was added 13.5 hrs after release, where cells still are in G1 phase (Figure 3.12) and treated for 2.5 or 3.5 hrs. In Figure 3.14, it can be seen that the expression level of SLBP has decreased when Cdk2 was inhibited with Roscovitine. And the level of SLBP has increased 17 hrs after released compared to 1st lane (16hrs after release). The Western analysis shows that SLBP has decreased about 40% upon treatment with Roscovitine at late G1 phase (Figure 3.14). Treatment of cells with Roscovitine did not change cell cycle distribution, majority of cells are still in G1 phase at 16hrs and 17hrs after release (Table 3.5).

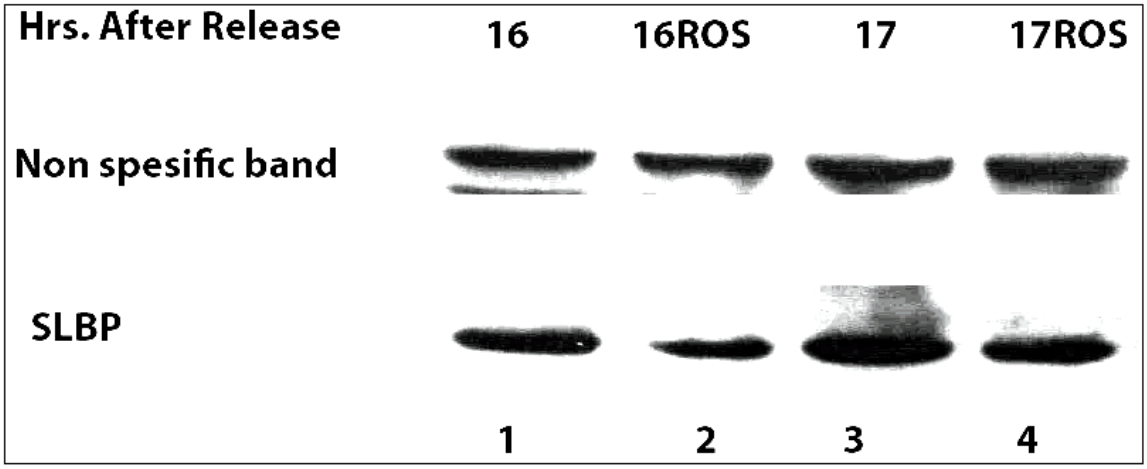

Figure 3.14 The effect of Roscovitine on late G1 SLBP expression of double thymidine synchronized cells. Hela cells were synchronized with double thymidine block at G1/S border and released for one more cycle. Cells were treated with Roscovitine or DMSO at late G1 phase and collected prior to S phase. The SLBP expression was assessed by Western Blot analysis.

Table 3.5 Cell cycle distribution of double thymidine synchronized cells upon Roscovitine treatment. The cell samples 16 and 17 hrs. after release still are in G1 phase in both DMSO and ROSC treated cells.

	Asynchronized	16 HR DMSO	16 HR ROS	17 HR DMSO	17 HR ROS
G1	66%	87 %	87 %	88 %	86%
G2	22%	5%	8%	4%	6%

In order to check the efficiency of Roscovitine in our hands, I have analyzed the effect of Cdk inhibition on SLBP at S/G2 border. At S/G2, SLBP is degraded upon phosphorylation by Cyclin A/Cdk1, so inhibiting Cdk1 at this point should recover SLBP level back to S phase level. Previously, it has been shown that treatment of synchronized cells with Roscovitine at S phase, prevented degradation of SLBP in particular amount (Koseoglu, 2008).

Cdk inhibition in S/G2 cell with Roscovitine could not totally recover the SLBP level back to S phase level (Figure 3.15). There is a problem with equal loading, the Roscovitine treated sample were loaded with less protein concentration, but anyway the level of recovered SLBP is not good as expected. After correction of loading of the samples, we again can see Rosvovitine does not fully inhibit the Cdks (Figure 3.16). Thus, we can conclude that Roscovitine could prevent around 60% of SLBP degradation at S/G2 phase.

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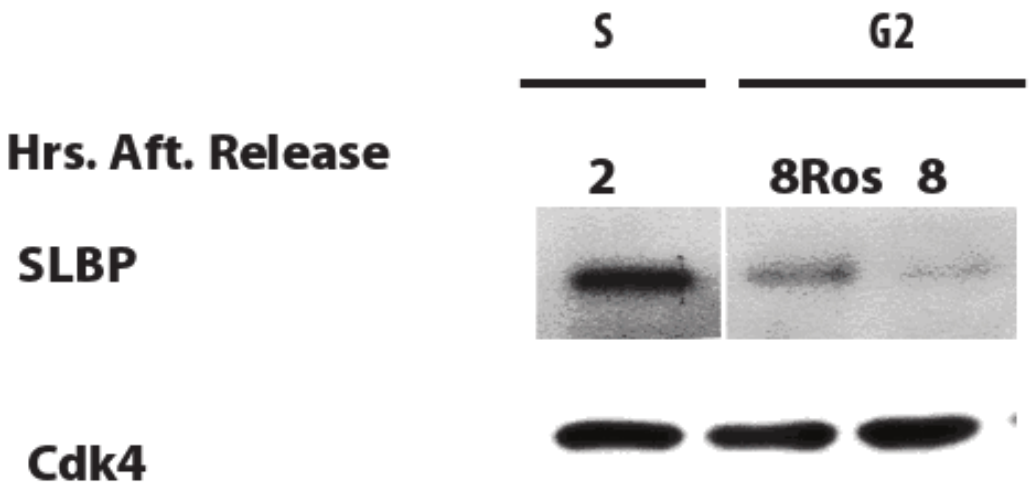

Figure 3.15 The effect of Roscovitine on S/G2 level of SLBP. Hela cells synchronized at G1/S border by double thymidine block were released for one more cycle. They were treated with Roscovitine for 4 hours at 4 hrs after release and collected at 8 hrs after release. The level of SLBP and Cdk4 are assessed by Western Blotting analysis.

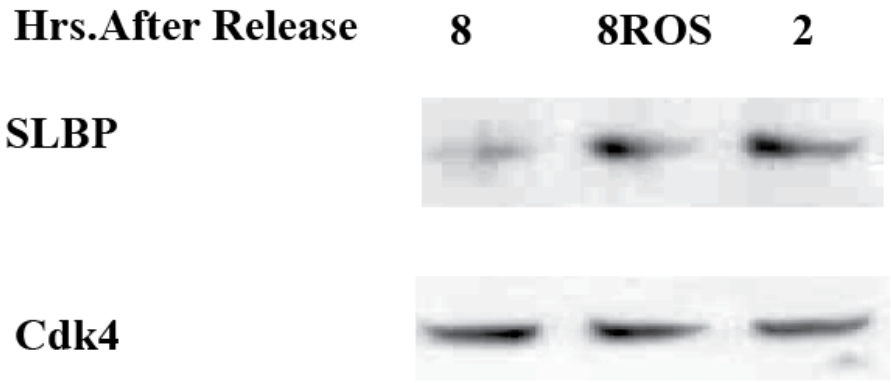

Figure 3.16 The Western Blot analysis of SLBP in Roscovitine treated samples at S/G2 phase (repeated). Hela cells synchronized at G1/S border by double thymidine block were released for one more cycle. They were treated with Roscovitine for 4 hours at 4 hrs after release and collected at 8 hrs after release. The level of SLBP and Cdk4 are assessed by Western Blotting analysis.

3.2.3.1.2 The Effect of Roscovitine on G1/S Expression of SLBP in Thymidine/Nocodazole Synchronized Cells

In order to arrest cells at G2/M phase, Hela cells were blocked in G1/S phase with double thymidine method and further treated with Nocodazole that blocks the cells in G2/M phase. This synchronization technique takes longer time but the success of synchronization can easily be detected just by visualizing the cells. The cells at M phase are round in shape and can be collected by gently dislodging. Thus, only cells which are definitely in M phase will be collected and released for one more cycle.

G2/M synchronized cells were released and collected at indicated time points (Figure 3.17). Roscovitine was added at 7 hrs, 8 hrs, 9 hrs and 10 hrs after release and collected after 2 or 4 hrs. The cell cycle distribution after release is shown in Figure 3.19. The cell cycle expression of SLBP was assessed by Western Blot (Figure 3.18) and there is almost no expression of SLBP at G2 phase. The SLBP starts to increase in between 8hr and 10 hrs. In order to analyze the effect of Cdk2 inhibition on SLBP, synchronized cells were treated with Roscovitine but any change in SLBP level cannot be detected upon addition of Roscovitine (Figures 3.20).

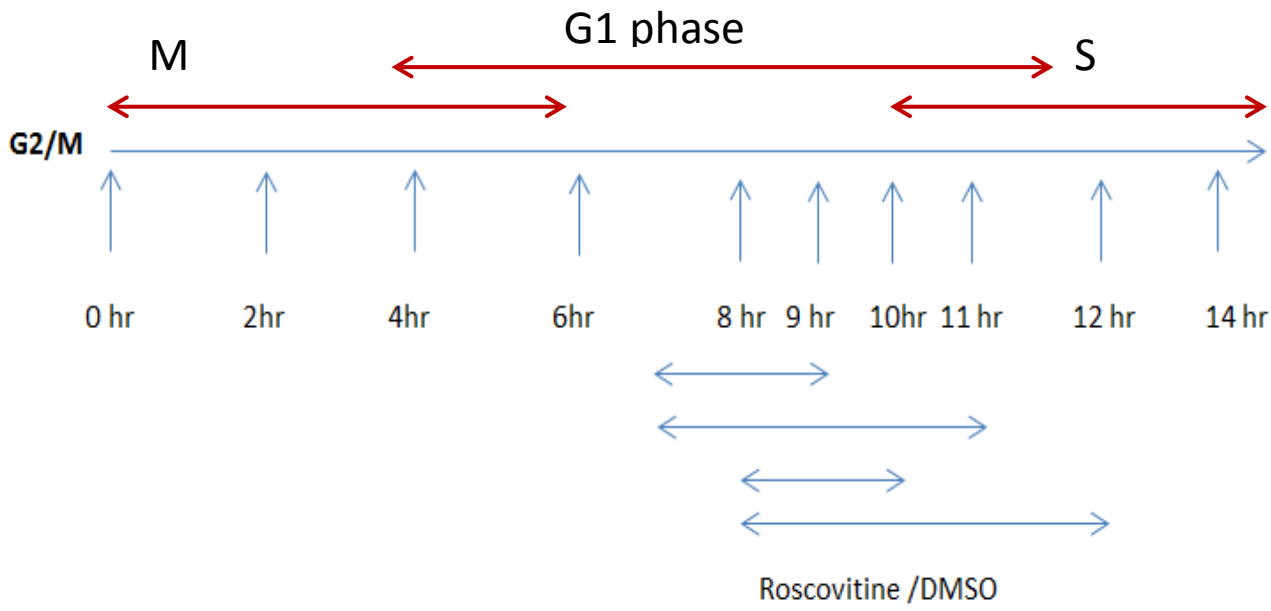

Figure 3.17 Schematic representation of G2/M synchronization by double Thymidine/Nocodazole synchronization and time points for Roscovitine treatment.

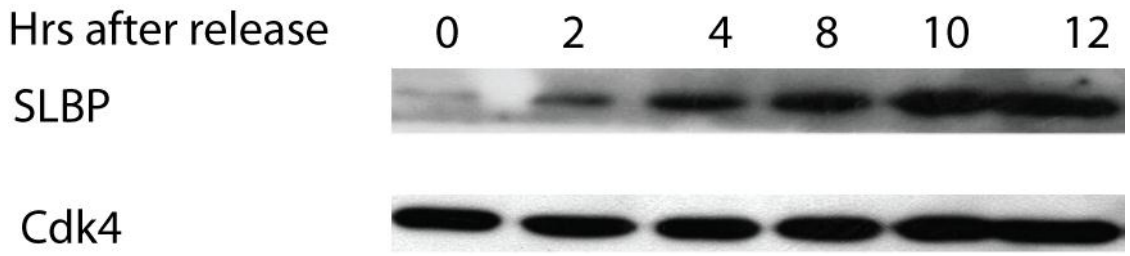

Figure 3.18 Cell cycle regulation of SLBP after G2/M arrest by double Thymidine/Nocodazole treatment. The Hela cells were synchronized in Promethaphase with Double Thymidine followed by Nocodazole synchronization. The expression of SLBP increases as cells approach to S phase.

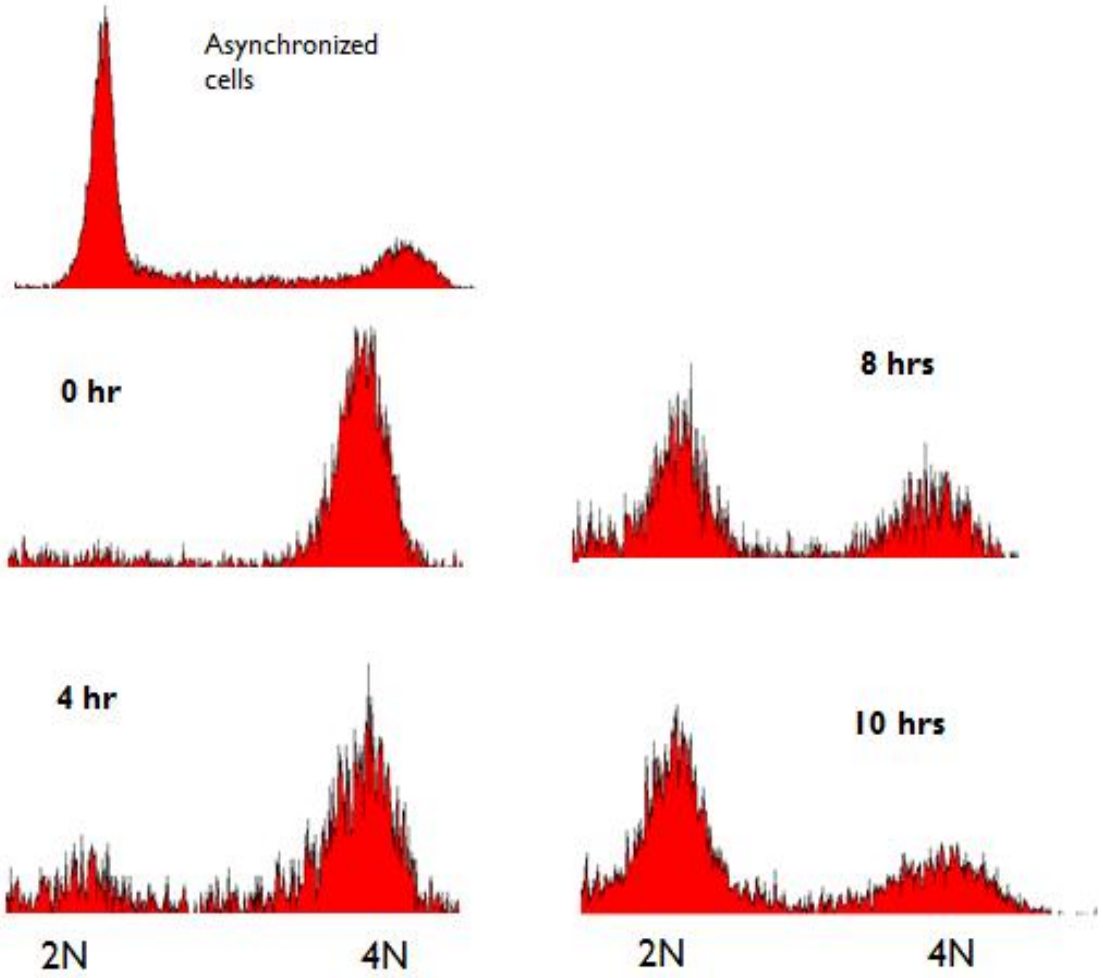

Figure 3.19 FACS analysis of cell cycle distribution of Thymidine/Nocodazole synchronized cells. All of the cells were at G2 phase after release (0 hr). After 4 hrs, some of cells were still in G2 phase and they remained to be there event after 8 and 10 hrs.

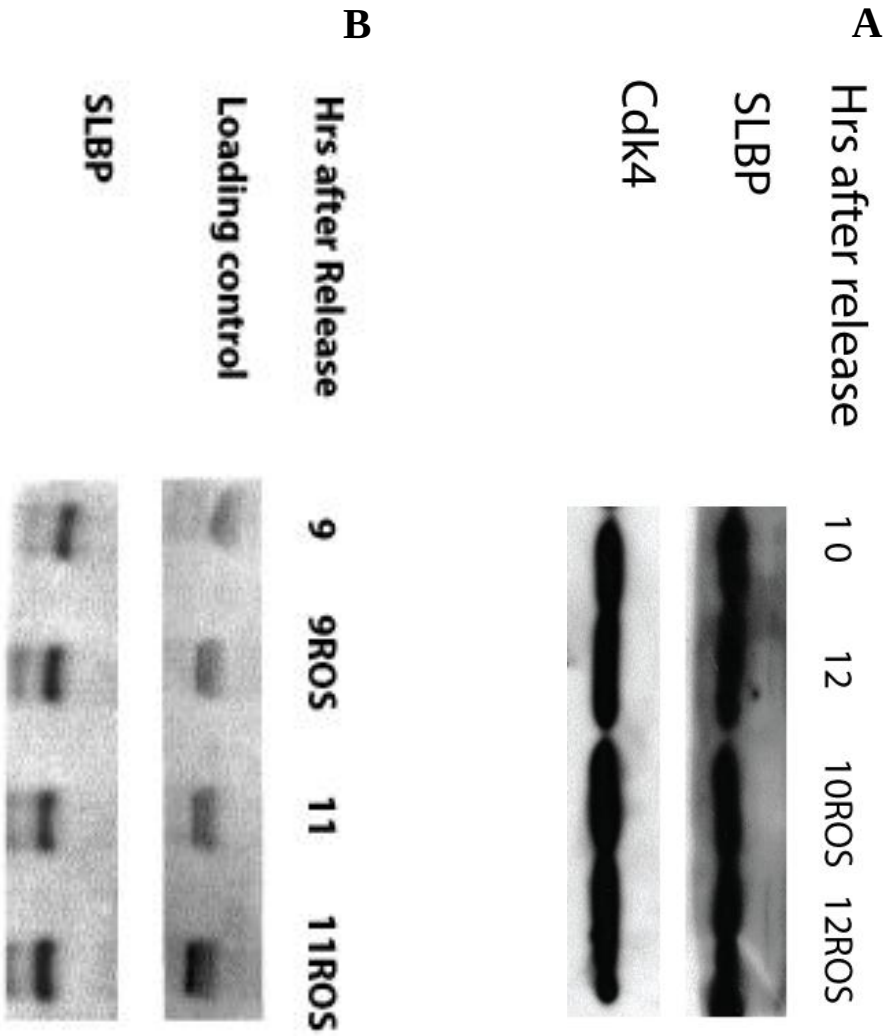

Figure 3.20 The effect of Cdk inhibition on SLBP in late G1 phase in G2/M synchronized cells. Hela cells were synchronized with double Thymidine-Nocodazole synchronization at G2/M phase and released for one more cycle. Cells were treated with Roscovitine at late G1 phase and collected at S/G2 transition. The level of SLBP was assessed by Western Blotting. Panel **A**, shows Western Blot analysis of SLBP of ROSC/DMSO treated cells at 10-12 hrs. Panel **B** shows Western Blot analysis of SLBP of ROSC/DMSO treated cells at 9-11 hrs.

3.2.3.2 Optimizing Cdk2 dn Transfection of Thymidine-Nocodazole Synchronized Cells

Roscovitine diminishes the effect of both Cdk1 and Cdk2 and it cannot completely inhibit their activity. Roscovitine may target any other kinases at G1 phase. Transfection of Hela cells with Cdk2 dominant negative is specific for Cdk2 inhibition. Transfection of synchronized cells with Cdk2 dn constructs would give more precise conclusion about the role of Cdk2 on SLBP increment at G1/S border. Thus, I have optimized transfection of G2/M synchronized cells with Cdk2 dominant negative and analyzed the effect of Cdk2 inhibition in G1/S SLBP level.

The inhibition of Cdk2 is very critical for cell cycle distribution, thus it had to be well optimized. I have tried two different approaches in order to achieve Cdk2 inhibition just in G1, not any other phases of cell cycle, namely I did not wanted to arrest cells before they reach to G1/S border. Firstly, I have transfected Hela cells within synchronization period with double Thymidine block followed by Nocodazole treatment for G2/M arrest (Figure 3.21). But majority of cells at the end of synchronization seems to be arrested at G1 or S phase. The shape of Hela cells were not round, indicating that they could not reach the M phase (Figure 3.22).

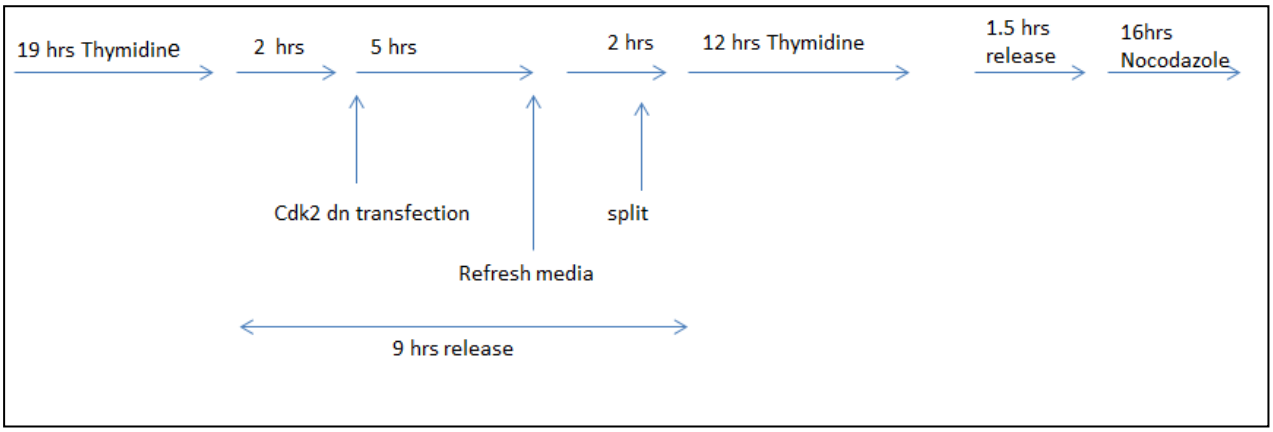

Figure 3.21 Schematic illustration of Cdk2 dn transfection within G2/M synchronization by double Thymidine-Nocodazole treatment.

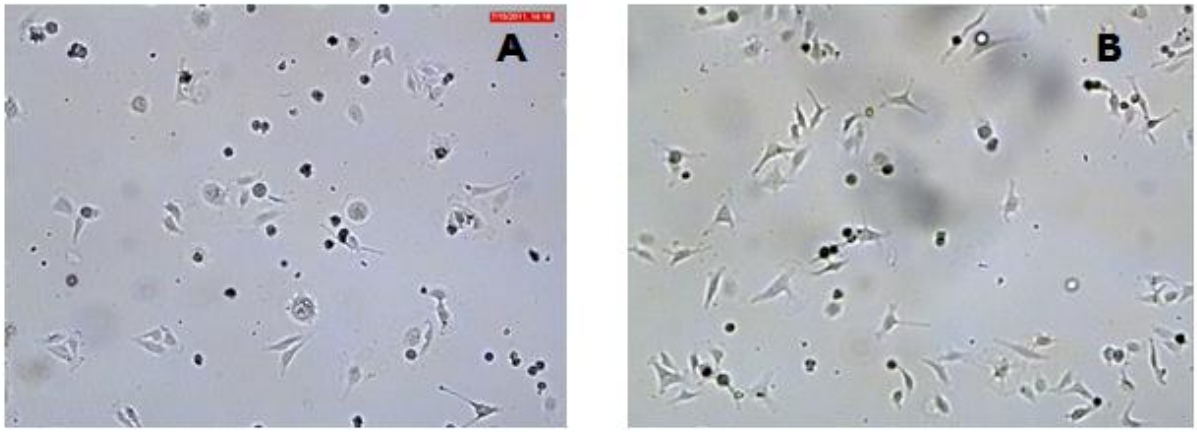

Figure 3.22 The morphology of Cdk2 dn transfected double Thymidine/Nocodazole synchronized cells. **A** panel shows cells with pCMV (negative control) transfection and in **B** panel, cells were transfected with Cdk2 dn.

In order to reduce the time for Cdk2 dn expression in synchronized cells, so that I would not arrest cells before they reach to G1 phase, I have synchronized Hela cells with Thymidine followed by Nocodazole treatment (Figure 3.23).

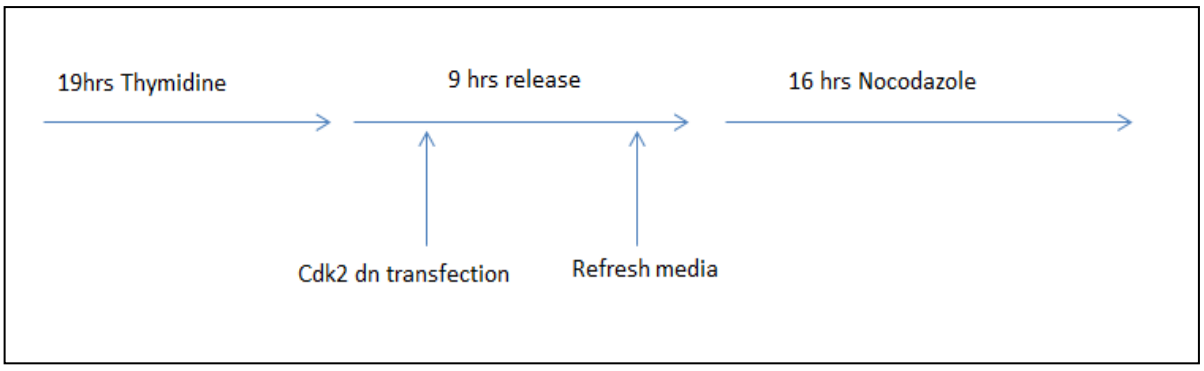

Figure 3.23 Schematic illustration of the Cdk2 dn transfection of G/M synchronization by Thymidine-Nocodazole treatment.

The majority of cells were assessed to be at G2/M phase by microscopic observations, namely most of them were at round shape. The cells at G2/M phase are easy to dislodge, thus I have collected cells by gently rocking, by this way I have collected only the cells which are less adherent and released further for next G1 phase. The cells were observed 10 hrs after release and they seem to be attached well.

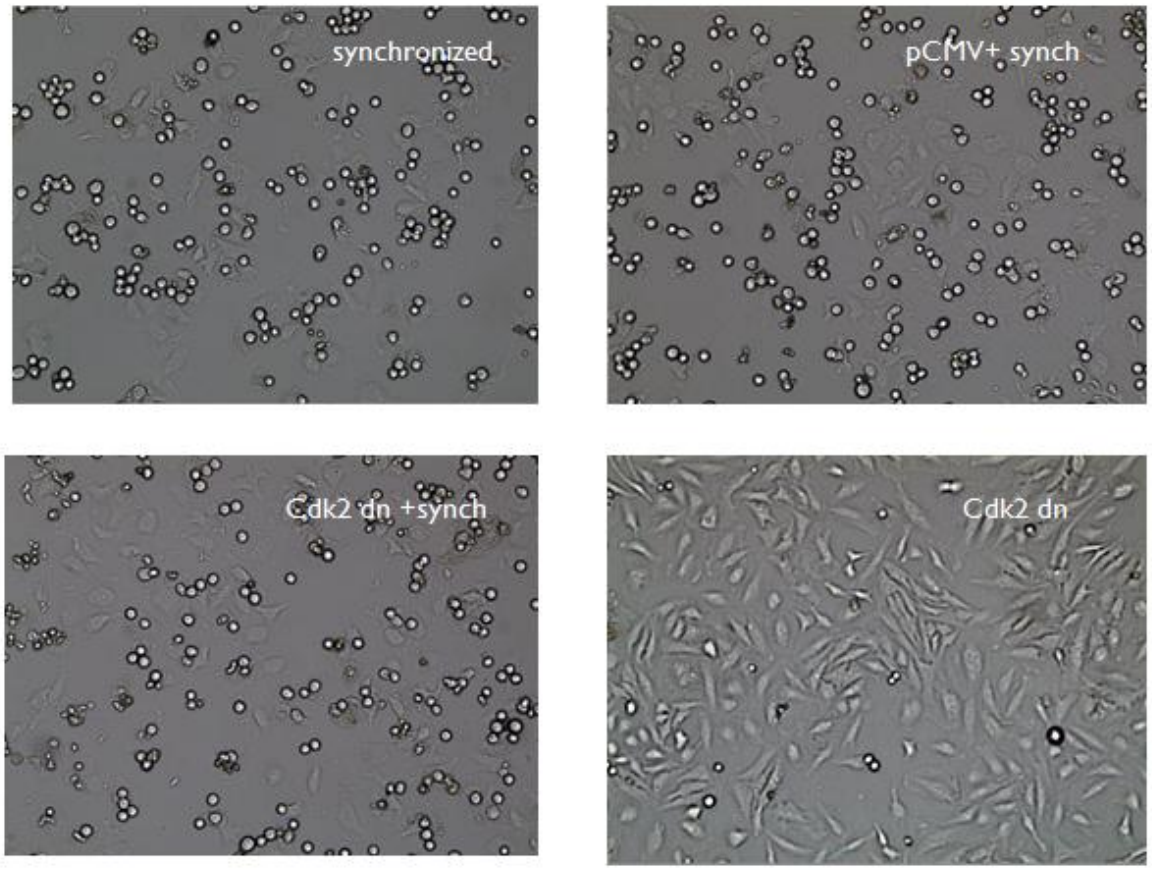

Figure 3.24 Shape of G2/M phase synchronized cells with Cdk2 dn transfection. In first panel, almost half of Hela cells were at round shape indicating they are at M phase. The majority of transfected synchronized cells were also at M phase. The Cdk2 dn transfected cells without synchronization was used a control.

CHAPTER 4

DISCUSSION

4.1 PRODUCTION OF THR61 ➔ALA61 CONSTRUCT OF SLBP FOR G1 REGULATION STUDIES

As a sole player in histone mRNA metabolism, SLBP is known to be tightly cell cycle regulated. It has been shown that cyclin A/CDK 1 triggers degradation of SLBP by phosphorylating Th61 and leading to subsequent phosphorylation of Thr60 by CKII. At the end of S phase, SLBP is degraded by proteasomes depending on these phosphorylations. So, mutating Thr 61, will inhibit phosphorylation of Thr 60 and thus inhibits S/G2 degradation of SLBP. As a result, SLBP will be stable until G1 phase and this mutant construct can be used for studying the G1 regulation of SLBP. Here I have mutated Thr 61 into Ala 61 by site directed mutagenesis and successfully generated stable cell lines with these constructs (both wild type and mutant His-tagged SLBP).

The transfection only impacts the cells that directly receive the transfected DNA. The transfected DNA is not passed from generation to generation during cell division and therefore the genetic alteration is not permanent. In a very low number of cases, the transfected DNA will integrate into a chromosome. This allows the transfected DNA to be carried stably from generation to generation. To achieve this, cells must be treated with selective antibiotic which kills non-gene transformed cells. In our case, SLBP is carried in pcDNA 3.1 (-) which has a geneticin resistance coding gene, thus geneticin is used to select cells with plasmid incorporated into their genome. This step is very

critical and time consuming, because in some cases only a small portion of plasmid may incorporate and ending up with no exogenous expression even after selection in surviving cells. Thus, the expression of exogenous proteins has to be assessed after completion of stable cell generation.

The expression of both wild type and mutant SLBP constructs were assessed by Western Blotting in transient transfected cells and in cells which have incorporated the exogenous genomic material into the genome. In both cases, we can see wild type and his-tagged SLBP, indicating Hela cells were successfully transfected by SLBP constructs and the plasmids with these constructs have been incorporated into their genome properly. These cells can be further used for G1 regulation studies of SLBP

4.2 INVESTIGATING THE ROLE OF CDK2 ON G1 REGULATION OF SLBP

One of the major events of cell cycle is replication of chromosomes. During S phase, proper chromosome replication requires both synthesis of DNA and sufficient amount of histone to properly package the nascent DNA. Cells limit the bulk of histone production to the S phase and these histones are encoded by so called replication-dependent histone genes. Histone synthesis is majorly regulated via histone mRNAs level. Expression of histone mRNAs are cell cycle regulated and in metazoans a major regulatory mechanism is S phase restriction of Stem-loop binding protein (SLBP) expression which is a required for histone mRNA processing. At the end of S phase SLBP degradation is triggered by Cyclin A/Cdk1 and its expression is kept low at G1 until next S phase where it is needed again for histone synthesis. In this study, I have focused on investigating signaling pathways inducing the SLBP expression at G1/S border which is an important mechanism to coordinate cell cycle and histone synthesis. Cyclin E/Cdk2, as the major player that triggers S phase entry; DNA replication and histone transcription, is proposed to be best candidate for rapid accumulation of SLBP as cells approach to S phase.

The best way of analyzing the effect of a protein on its targets is to either overexpression or down regulating its expression in cells. To detect the role of Cdk2 on SLBP expression, I have inhibited Cdk2 activity with chemical inhibitor and by transfecting cells with Cdk2 dominant negative construct. Overexpression of Cdk2 would enhance S phase entry, which might compromise an effect that interferes with SLBP expression. Thus, I have examined the effect of Cdk2 on SLBP in both asynchronous cells and in G1/S phase by inhibiting its activity.

Roscovitine is a reversible selective inhibitor of Cdks, which is a purine analog that competes for binding site ATP in the catalytic site. Treatment of asynchronous Hela cells with Roscovitine no more than 6 hours have decreases SLBP level expression despite the increase in S/G2 SLBP level due to Cdk1 inhibition. In Figure 3.10, it can be clearly seen that, Roscovitine treatment have decreased the level of SLBP as well as Cyclin A. This is an interesting finding in terms of SLBP regulation by both Cdk1 and Cdk2. Roscovitine also targets Cdk1, which is responsible for S/G2 degradation of

SLBP. So, by inhibiting the Cdk1, SLBP degradation should not take place at S/G2 border, leading to increase in SLBP expression. On the other hand, a candidate kinase, Cdk2 is also inhibited by the drug treatment and we propose that it triggers SLBP accumulation at G1/S border. So Cdk2 inhibition is expected to decrease the level of SLBP. In Figure 3.10, the level of SLBP has decreased almost 2 fold. This may be overall effect of both Cdk1 and Cdk2 inhibition. Namely, despite the increase due to Cdk1, we can clearly see the decrease in SLBP level in unsynchronized cells. Thus, this suggests that SLBP expression has decreased by Cdk2 inhibition.

In addition to this, most of cell population is known to be in G1 phase, so these results majorly might be reflecting G1 level of SLBP after Cdk inhibition. Long term Cdk inhibition disrupts the cell cycle distribution. In our case, Roscovitine did not cause significant cell cycle arrest. Thus, it can be proposed that this decrease is not due to cell cycle arrest, it is most likely due to the direct effect of Cdk2 inhibition.

Roscovitine targets majorly Cdk1 and Cdk2, but it also has unintended effect on other kinases or Cdk family members like Cdk 5. Thus, for specifically inhibiting Cdk2, I have transfected Hela cells with Cdk2 dominant negative gene, which expresses kinase dead Cdk2 and ultimately decrease the Cdk2 activity. This would end up with more clear inhibition of Cdk 2 compared to Roscovitine. Again as in the previous result, the Cdk2 inhibition led to decrease in SLBP level. The FACS analysis showed that Cdk2 dn transfection did not have significant effect on cell cycle distribution. So, it can be suggested that the decline in SLBP expression is not due to cell cycle arrest at any phase but due to Cdk2 inhibition.

The Cdk inhibition by Roscovitine treatment in double Thymidine synchronized cells has partially prevented SLBP increase. Partial prevention of SLBP by Roscovitine treatment may be due to either the efficiency of the drug was not good enough, namely Cdk2 was not inhibited completely or there may be another mechanism present together with Cdk2 which triggers increase in SLBP expression in late G1. Anyway, this data suggests that Cdk2 is required for accumulation of SLBP at G1/S border.

In order to examine the efficiency of the drug on Cdk inhibition, cells at S/G2 phase were treated with Roscovitine. Cdk 1 is responsible for S/G2 degradation of SLBP and inhibition of Cdk1 should prevent SLBP degradation. In our case, we could just prevent around 60% of SLBP degradation at S/G2 phase, which indicates that the current concentration and the duration of Roscovitine treatment does not fully inhibit Cdks activity. Taking these factors into consideration, we can support the notion of Cdk2 requirement for SLBP expression at G1/S border.

As an additional method for investigating the role of Cdk2 in G1 regulation of SLBP, Hela cells were arrested at G2/M phase by Thymidine-Nocodazole treatment and released further. As expected the level of SLBP at G2/M is very low and continues to be nearly low during G1 (4 hours after release) and near G1/S border (around 9hrs after release) SLBP expression has increased significantly. Next, we have introduced Roscovitine at two different points in order to intercept with the SLBP increment and check the expression of SLBP at G1/S border. We could not see any detectible effect on SLBP expression, which was unexpected based on previous results of asycnronized and G1/S synchronized cells.

In order to check the proper progression through synchronization, the cell cycle distribution of cells released from G2/M phase were analyzed by Flow Cytometry. Although cells had to enter to G1 phase based on data from the literature, a good portion of cells coming from G2/M synchronization seems to be arrested at G2 phase. It was the case for 8 and 10 hrs. after release. If we interpret Western Blot data together with FACS data, we can conclude that, good portion of cell population at 10 hrs. and 12 hrs. seem to be in G2 phase. Thus, SLBP level at these time points does not reflect G1 phase expression. This synchronization method has to be optimized and the experiment can be repeated again in order to confirm the effect of Cdk2 on SLBP expression.

Lastly, for specific inhibition of Cdk2 in synchronized cells, I have developed a protocol. I have optimized the transfection of synchronized Hela cells with Cdk2 dominant negative, which diminishes Cdk2 activity more specific than Roscovitine treatment. The duration of Cdk2 inhibition might affect the cell cycle, so I have designed an experiment in way that inhibits Cdk2 only at G1/S border after release.

Here I have observed that, transfection of Hela cells within double Thymidine synchronization cannot completely synchronize cells into G2/M phase. This is most probably due to overexpression of Cdk2 dn arrests cells before they reach to G2/M phase. Thus I have repeated the same approach with lesser time for Cdk2 dn expression. I have synchronized cells with single thymidine block followed by Nocodazole treatment. Here I got more than half of cells population at G2/M phase (based on their rounded shapes). I have collected cells and released till subsequent G1 phase. Cells were collected after 10 hrs but there were very few cells. Thus, here it can be concluded that, Cdk2 dn transfection can be done by synchronizing Hela cells with single thymidine block followed by Nocodazole treatment. This method can be used with higher number of transfected plates for further investigations.

CHAPTER 5

CONCLUSION

Histone production is limited to S phase and majority of regulation posttranscriptional. Histones are unique in that they lack poly-A tail but end with conserved stem loop and have no introns. All the steps of mRNA regulation require or involve both *cis* acting and trans regulatory elements; 3' end stem loop and Stem Loop Binding Protein. SLBP expression is limited to S phase, where it is needed to trigger histone synthesis. Like histone biosynthesis, SLBP expression is also tightly cell cycle regulated. It has been previously shown that, SLBP is degraded by double phosphorylation at Th60 and Thr 61 and caused rapid decline of histone synthesis. A major regulator of histone mRNA metabolism, the SLBP dramatically increases at G1/S border where Cyclin/Cdk2 is the major player. Most probable upstream regulator of SLBP is predicted to be Cyclin/Cdk2, which is required for DNA replication and histone transcription and processing in some point and eventually triggers S phase entry. Thus there is an ambiguity in literature concerning upstream regulator of SLBP at G1/S border.

In this dissertation, I have concentrated on finding out upstream signaling pathways of SLBP in G1 phase. Particularly, I have generated mutant SLBP construct that will not be degraded at S/G2 border in order to study G1 regulation of SLBP. Besides this, I have

investigated the role of Cdk2 on late G1 expression of SLBP and determined that, Cdk2 regulates SLBP expression in G1 phase.

5.1 Generation Thr 61➔ Ala61 SLBP

SLBP is rapidly degraded at the end of S phase, so the level of SLBP is almost undetectable in early G1 phase due to low translation efficiency. This makes studies regarding the G1 regulation of SLBP very difficult. The stability of SLBP can be extended by impeding the S/G2 depredation, so there will be detectible amount of SLBP as long as its half-life allows. Mutating Thr 61➔ Ala61 prevents subsequent phosphorylation of Thr 60 and eventually precludes proteasome mediated degradation of SLBP at the end of S phase. Therefore, the 'S/G2 degradation' mutant can facilitate G1 studies of SLBP by providing expression of detectable amount of SLBP. The level of mutant SLBP will not drop at S/G2 border and they will be present at G1 phase as long as their stability allows until next regulated degradation after mid G1 phase is stimulated. Thus, Hela cells stably expressing mutant SLBP may facilitate and enlighten G1 studies of SLBP regulation.

Firstly, the wild type SLBP would enlighten the sufficiency of coding sequence for posttranscriptional regulations. Namely, if exogenous wild type SLBP behaves like the endogenous SLBP throughout the cells cycle, particularly in G1 phase, than one can conclude that coding sequence of SLBP is sufficient for posttranscriptional regulations.

Secondly, recent studies revealed that there is another regulated depredation at G1 phase other than an S/G2 degradation of SLBP. Thus, the mutant construct of SLBP would enlighten whether these depredation regulations are mediated via the same motif or not.

Lastly, Thr61➔ Ala61 mutant of SLBP would facilitate the SLBP studies in asynchronized cells with chemical Cdk inhibitors. Cdks are very similar in sequence and structure, thus the drugs targeting the Cdks cannot distinguish them from each other. For instance, Roscovitine, targets Cdk1, Cdk2 and Cdk5. In our case, SLBP is regulated by Cdk1 and supposed to be regulated by Cdk2. In order to specifically inhibit Cdk2 with

Roscovitine, the cell cycle synchronization needs to be carried out, that takes more than 3 days and lots of endeavor. The specific inhibition by transfection has some toxicity to cells and it also requires long time. Thus, treating Hela cells stably expressing Thr61➔ Ala61 mutant of SLBP with Roscovitine will eliminate the interference of S/G2 and G1/S regulation. The Roscovitine treatment of stable cells with mutant SLBP most likely would reflect the G1/S regulation of SLBP.

5.2 Effect of Cdk2 Inhibition on SLBP Expression

In this dissertation I have shown that Cdk2 activity is required for SLBP expression. The depletion of Cdk2 activity in late G1 phase prevents rapid accumulation of SLBP at G1/S phase to a certain amount in Hela cells. The partial decrease in SLBP expression at G1/S upon Roscovitine treatment was shown and proposed to be due to inefficient inhibition of Cdks by Roscovitine. Another reason for partial decrease in SLBP level in Roscovitine treated G1 phase cells may be due to presence of additional mechanism together with Cdk2. Inhibition of both mechanisms may lead to complete inhibition of SLBP at G1/S phase. For better and precise inhibition of Cdk2 in G1 phase I have optimized transfection of Cdk2 dn in synchronized cells. These cells would further be used for investigating the role of Cdk2 on rapid increase of SLBP just before S phase. In order to support the notion of this regulation, Cdk2 can be further silenced with siRNA and its effect on SLBP at G1/S phase could be observed by Western Blot analysis.

Cdk2 is proposed to be required for SLBP increment at G1/S border and Cdk2 might be targeting the SLBP directly or via another proteins. If it is not direct regulation, then the timing of Cdk2 inhibition in synchronized cells would be very crucial for accurate conclusions. With slight difference in time of Roscovitine treatment, one can miss the point where the intermediate protein triggers SLBP expression. The intermediate protein may be regulating SLBP either ‘catalytically’ or stoichiometrically. If it just triggers firing of SLBP catalytically, then it would be important to enclose that time of regulation. If it is regulating stoichiometrically, one should see an effect of Cdk2 inhibition throughout the late G1 phase.

In addition to this, in further studies, it would be interesting to find the partner of Cdk2 responsible in G1 increment of SLBP. Cdk2 is known to function together either with Cyclin E or Cyclin A. Cyclin A expression increases at mid-S phase, but it has some regulatory functions in late G1 phase too. The effect of candidate cyclins can be detected by downregulation of their expression at G1 phase and observe the effect on SLBP expression. Other than this, co-Immunoprecipitation (coIP) analysis of SLBP can be done. If Cdk2 can be detected with CoIP analysis, then this would indicate that the Cdk2 directly regulates SLBP. With further investigations of IP we can easily detect the Cdk2 partner for this regulation. If this regulation is mediated directly by Cdk2, than it would be exciting to find out the regions for this regulation. Cdk2 is a Ser/Thr kinase and phosphorylates together with Cyclins at consensus motif of [S/T]-P-X-[K/R]. There are at least five likely Cyclin/Cdk consensus sites on SLBP. Further, the effect of Cdk2 can be examined on SLBP with mutant consensus target sites. As a result of this investigation, we would be able to identify the target region for Cdk2.

Furthermore, it would be interesting to investigate how Cylin/Cdk2 regulates the SLBP level. It is well known that the level of SLBP mRNA does not change significantly throughout the cell cycle. So, the rapid accumulation of SLBP just before the S phase is due to majorly translational or posttranslational regulations. Previously it has been shown that the efficiency of SLBP translation is kept low at early G1 and recovers back to S phase level at around mid-G1 phase. But the level of SLBP is still kept low until G1/S border. This indicates that, there is a posttranslational mechanism that regulates rapid accumulation of SLBP just before S phase, just where it is needed. Namely, there is a regulatory mechanism that removes newly synthesized SLBP for several times and it is inhibited as cells approach to S phase. It has proposed previously that the stability of SLBP increases as cells approach to S phase by preventing proteasome mediated degradation. If we combine these two evidences we may suggest that Cdk2 may trigger rapid accumulation of SLBP by increasing the protein stability. This is the case in G1 for several other proteins including cdc6, skp2 and Cyclin A. So, finding out players in proteasome mediated degradation would bring majority of G1 regulation of SLBP to the light.

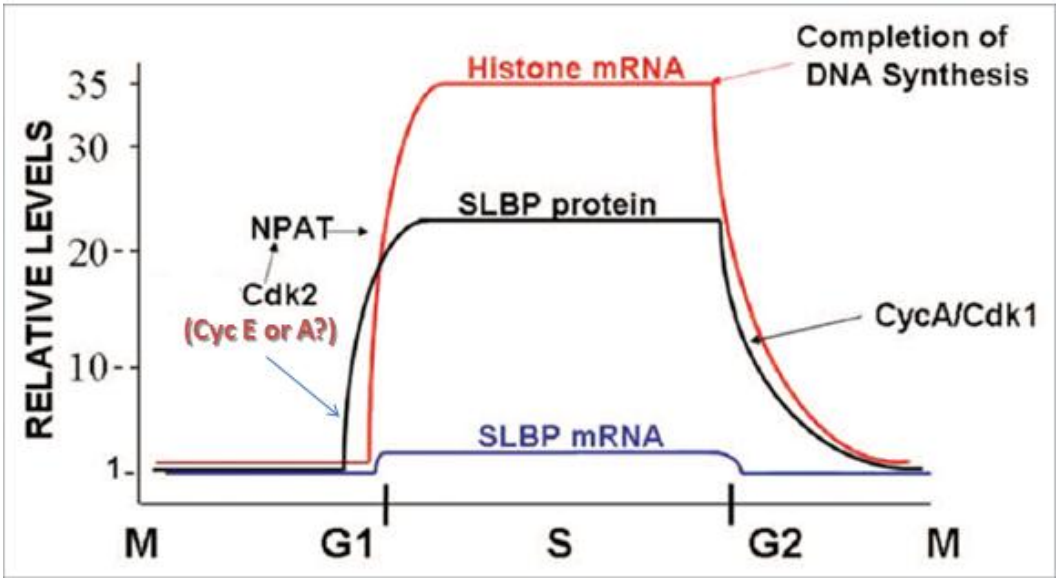

Figure 5.1 Cell cycle regulation of SLBP and histone mRNA. Cdk2 is required for SLBP increment at G1/S border. But it is not yet known with which Cyclin does Cdk2 partners with for this regulation (Adapted from Koseoglu et al., 2010).

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