

ANALYSIS OF THE CRYPTIC PROMOTER IN THE 5'-UTR
OF P27

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ABSTRACT

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Cyclin Dependent Kinase regulation is often manipulated by cancer cells to promote unlimited proliferation. P27 is an important regulator of Cyclin E/CDK 2, which has been found in low amounts in many types of malignant cancers. Lovastatin has been shown to cause cell cycle arrest in the G1 phase of the cell cycle by increasing the P27 protein. There has been some question, however, if lovastatin regulates P27 at the transcriptional or translational level. Although it has been claimed that P27 expression regulation is due to an IRES located in its 5'UTR, other studies suggested that P27 expression is regulated at the level of transcription. To further investigate the regulation mechanism of P27 expression, the 5'-UTR of P27 and its deletion mutants were examined using a luciferase reporter gene in HeLa cells following exposure to lovastatin. It was found that lovastatin stimulated a significant 1.4 fold increase in its promoter activity of the full length 5'UTR (575). Deletion of 35 nucleotides from the 5' end of the UTR eliminated the lovastatin-induced increase in promoter activity. Further mapping analyses of the first 35 bases showed that two regions, M1 (575-559) and M3 (543-527), were less sensitive to lovastatin than the other mutated constructs.

Since M1 and M3 still showed some activity, a construct was created with deletions in both the M1 and M3 regions. This showed no increase in luciferase activity when exposed to lovastatin. Looking at RNA levels, there was a 1.5 fold increase in RNA when the full length 5'UTR was inserted into HeLa cells and exposed to 81 μ M of lovastatin. In contrast, there was no increase in RNA when M1/M3 (575-559; 543-527)

was inserted into HeLa cells and exposed to 81 μ M of lovastatin. In addition, there was a 1.6 fold increase in endogenous P27 RNA levels after HeLa cells were exposed to 81 μ M of lovastatin. In all of these experiments, there seems to be two promoters that work cooperatively: M1 (575-559) and M3 (543-527).

Jian-Ting Zhang, Ph.D., Chairman

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CURRICULUM VITAE	

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CHAPTER 1. INTRODUCTION

1.1 Overview of Cancer: Initiation, Promotion, Progression

As shown in Figure 1, cancer can be divided into three stages: initiation, promotion, and progression. Initiation is the stage where an “initiated cell” or newly transformed cell is created. This irreversible process begins when chemical carcinogens interact with DNA to form stable adducts or mutations (such as transitions, transversions, or small deletions) which remain unless removed by repair. Cell division of transformed cells is required to “fix” cells in their mutated state. In addition, there is no threshold for initiators. It is conceivable that one dose of an initiating carcinogen is enough to form a tumor (Gamper, 1980).

Figure 1

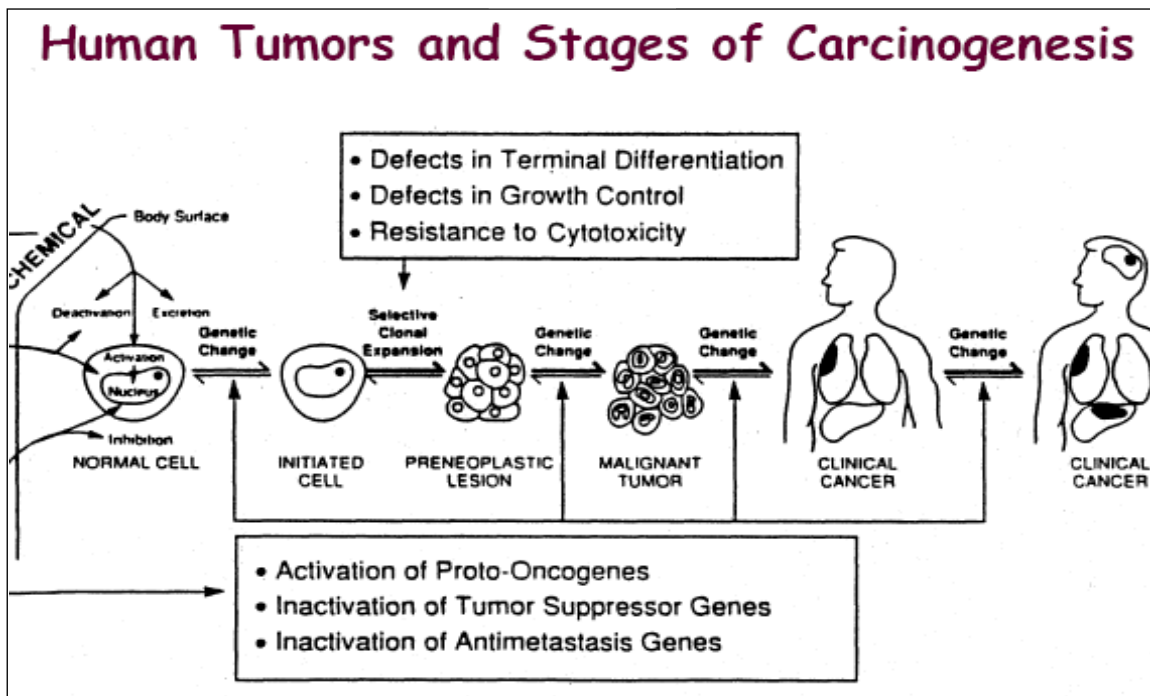


Figure 1: The stages of carcinogenesis. When an initiating carcinogen is added, there is a permanent genetic change that occurs if the DNA is not repaired. With the help of promoting agents, the initiated cell can then be replicated to form a preneoplastic lesion. Finally, progression involves multiple mutations in various genes and karyotypic instability. In the progression stage, we will observe benign and malignant tumors that are formed. (Adapted from www.unc.edu chemical carcinogenesis PowerPoint, 2007)

Promotion is a reversible stage that involves a reagent increasing the growth of an initiated cell. Instead of creating new types of cancer cells, promoters have a tendency to increase the growth of tumors through cellular proliferation and/or inhibition of apoptosis. Progression is the uncontrolled growth of a tumor. This stage is characterized by genetic changes which lead to different levels of activity for structural proteins, growth factors, and proteases (Hennings, 1993).

1.2 Cell Cycle in Relationship to Tumor Initiation, Promotion, and Progression

The cell cycle is divided into four distinct phases: G1-First gap phase to prepare cells for DNA synthesis, S-for DNA synthesis, G2-second gap phase and M-for cell division (See Figure 2). In addition to these four phases, cells can also be in a quiescent state or G0 phase (Caldon, 2006). In the G0 phase, cells are not dividing. If cells choose to undergo cell division, they will leave G0 and enter the G1 phase of the cell cycle.

Understanding how cells pause the cell cycle is essential to understanding the mechanism behind cellular proliferation. Stopping cell division allows for DNA repair after genetic integrity has been compromised by DNA damaging agents. Surveillance control mechanisms ensure proper completion of early events and cellular integrity before subsequent steps can occur (Shackelford, 1999). If damage is too severe to be adequately repaired, the cell may then undergo apoptosis.

Cyclin Dependent Kinases (CDKs) help to promote cell cycle progression. Mitogens and growth factors promote early events that commit cells to division. These events include the induction of D and E-type cyclins as well as phosphorylation of the retinoblastoma protein (pRB). The point at which the withdrawal of growth factors no longer halts cell cycle progression is called the G1 restriction point (Shackelford, 1990).

Kinases can be regulated at many levels to halt cell cycle progression and allow for DNA repair. Antiproliferative signals from proteins such as P27 and P16 help to prevent the formation of CDK complexes (Shackelford, 1990). When these mechanisms of CDK inhibition are blocked by carcinogens, transformed cells can then proliferate without DNA repair. Thus, understanding CDKs and their mechanism regulation is important for designing approaches to prevent the formation and spread of cancer cells.

Figure 2

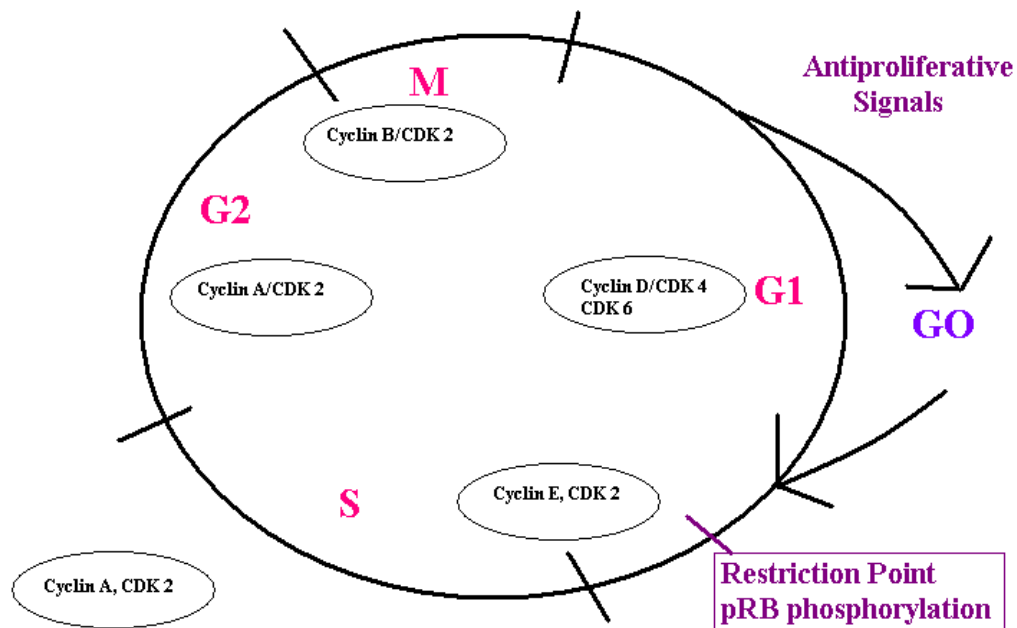


Figure 2: Diagram of Cyclin Dependent Kinases and where they act in the cell cycle. Helps to show how Cyclin Dependent Kinases are essential in cell cycle progression. (Adapted from a figure by Shakelford, 1999)

1.3 Cyclin Dependent Kinases

CDKs are dimeric complexes comprised of both a catalytic and a regulatory subunit. These serine/threonine kinases can be divided into two groups. Members of the first group include cyclin A dependent kinases 1 and 2 and cyclin B dependent kinase 1. These CDKs help to promote G₂-M phase transition. The other group of CDKs include cyclin D dependent kinases 4 and 6 as well as cyclin E dependent kinase 2. These cyclins, D and E, facilitate G₁-S transition (Shapiro 2006; Sherr 1999).

Cyclins are required for the activation of CDKs. When cells are stimulated by growth factors or mitogenic signals, they leave the G₀ phase to enter the cell cycle. The first cyclins to be synthesized during this period are the D type cyclins. These cyclins associate with CDK4 or CDK6 and function early in the G₁ phase. Cyclin E associates with CDK2 from the mid to late G₁ phase of the cell cycle (Lee, 2003; Sherr 1999). The Cyclin D1/CDK4 complex helps to mediate the phosphorylation of retinoblastoma protein (Rb). In a hypophosphorylated state, Rb interacts with E2F transcription family members. This family targets genes that are involved in G₁-S transition. By interacting with E2F, Rb prevents the transcription of genes that are required for S phase entry. In addition, Rb can also recruit histone deacetylases to the promoters of genes that are required for entering this phase of the cell cycle. These deacetylases help to lower transcription by removing acetyl groups from lysines on histone proteins. When Rb is phosphorylated, however, it no longer interacts with E2F and the transcription of genes that are required for entering the S phase occur uninterrupted (Shapiro, 2006; Sherr 1999).

1.4 CDK Regulation

CDKs have a number of positive regulators. C-Myc has been known to upregulate the transcription of CDK4 and cyclin D2 (Bouchard, 1999; Hermeking, 2000). Another positive regulator of CDK4 is p34 SEI 1 which activates its kinase activity at lower concentrations (Li, 2004). In addition, breast cancer cells have also been known to express a low molecular weight form of cyclin E which can increase the G1 to S phase transition when bound to CDK 2 (Lee, 2003).

CDKs also have a number of negative regulators. One of the main classes of these regulators are called CDK inhibitors or CDKIs. There are two main classes of CDKIs. The INK 4 family (p15, p16, p18) bind to and distort the catalytic cyclin binding site (Copfert, 2003). The Cip/Kip family (P21, P27, and P57) all share a highly conserved amino terminal domain that mediates cyclin and CDK binding (Zhang, 1997; Gopfert, 2003). Although the Cip/Kip family has a sequence that is highly conserved, much of their sequence is different which suggests that the proteins have distinct function and regulation (Benson, 2008). For example, P21 causes cell cycle arrest in the G1 and G2 phases in response to DNA damage whereas P57 regulates the cell cycle during embryonic development. However, P27 is upregulated in quiescent cells and is rapidly downregulated in other phases of the cell cycle (Benson, 2008). The protein P27 is being explored further because of its ability to induce cell cycle arrest by inhibiting CDK2 in the G1 phase of the cell cycle.

Figure 3

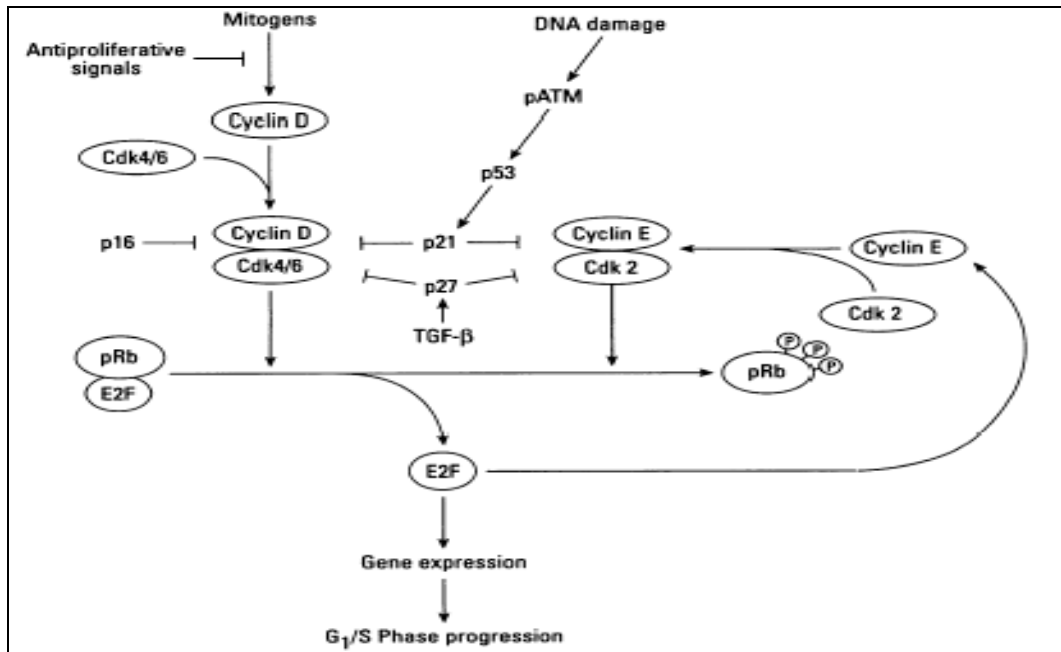


Figure 3: Schematic of how Cyclin Dependent Kinases are regulated. Regulation of Cyclin Dependent Kinases can occur at many levels. Antiproliferative signals help to prevent the induction of cyclin D; proteins such as P16, P21, and P27 help to inhibit complex formation and/or activation. Cancer cells try to manipulate this process by inhibiting one or more of these CDKIs (Shakelford, 1999).

1.5 P27: A Cip/Kip Inhibitor of CDK 2

P27 is a 27 kilodalton protein with its gene located on chromosome 12 (Lloyd, 1999). The protein is a prognostic indicator in many forms of cancer. Low levels of P27 protein in breast, prostate, and ovary cancers have all been shown to correlate with a poor medical prognosis (Benson, 2008). Transgenic mice with a P27 null (-/-) genotype have been known to display multiorgan hyperplasia. In addition, both P27 nullzygous (-/-) and heterozygous mice (-/+) were predisposed to tumorigenesis when exposed to gamma radiation (Paris, 2006; Gophert, 2003). In a study published in 2006, Troncione et al. analyzed P27 expression in 10 benign and 40 malignant breast lesions using

immunostaining and showed that P27 protein was significantly lower in carcinomas than in benign lesions (Troncone, 2004).

P27 has also been shown to induce cell cycle arrest in the G1 phase of the cell cycle. In 1997, Millard found that the expression of P27 in the G1 phase is much higher than in any other phases of the cell cycle (Millard, 1997). This observation suggests that high P27 expression results in G1 phase cell cycle arrest. Indeed, Rao et al. showed that lovastatin induces G1 phase cell cycle arrest by increasing P27 protein levels (Rao, 1998). P27 also causes cells to differentiate. In 2000, Quaroni showed that P27 expression in human intestinal epithelial cells led to the expression of differentiation markers at both the mRNA and protein levels (Quaroni, 2000). Troncone showed that poorly differentiated carcinomas had much less P27 immunostaining than carcinomas that were more differentiated (Troncone, 2004). Indeed, Millard showed high levels of P27 in HL60 cells treated with a known differentiation reagent called 12-*O*-tetradecanoylphorbol-13-acetate or TPA (Millard, 1997). P27 has also been implicated in apoptosis and has been shown to be a regulator of drug resistance in solid tumors (Lloyd, 1999).

Because P27 is known to cause cell cycle arrest as well as promote cellular differentiation, there is a great interest in the scientific community to discover how P27 is regulated. If the mechanism of P27 induction is fully understood, one could foresee future chemotherapies that might be used to treat cancer by activating or increasing P27 expression.

1.6 The Regulation of P27

The regulation of P27 has been under intensive debate. It has been shown by Carrano and Tsvetkov that P27 is phosphorylated at Thr 187 by CDK2/Cyclin E and this phosphorylation is then recognized by Skp 2 which targets it for proteasome degradation (Carano, 1999; Tsvetkov, 1999; Chen 2005). It was also found that lovastatin increased P27 protein levels without a significant increase in mRNA (Hengst, 1996). Thus, it was suggested that P27 expression may be regulated only by translation and/or protein stability. Indeed, Millard found a U rich region in the 5'UTR that seemed to be important for the translation of P27 (Millard, 2000). It was later suggested that the P27 5'UTR has an internal ribosome entry site (IRES) which allows for cap independent translation of its mRNA (Miskimins, 2001). This IRES activity appears to be increased by inducing differentiation of HL60 cells with TPA (Cho, 2005). However, the existence of an IRES in the 5'UTR of P27's mRNA has been challenged due to the inherited technical problems of the dicistronic vectors used in these studies. Using Northern blot analysis, Kulman et al. observed that the full length 5'-UTR of P27 (575 bases) potentially contains a cryptic promoter (Kullman, 2002). Later, Liu et al. (Liu, 2005) demonstrated that there is a cryptic promoter in the 5'-UTR of P27. This cryptic promoter may be present in a region between 549 and 511 nucleotides (Gizard, 2005). In addition, another promoter was identified in a region between 774 and 462 of the 5'UTR (Chen, 2005).

1.7 Aim and Significance of My Thesis Project

As mentioned above, it has been found that P27 expression is stimulated by lovastatin treatment (Hengst, 1996) and that this drug is known for its ability to cause cell cycle arrest in the G1 phase of the cell cycle by increasing P27 protein levels (Rao, 1998).

Lovastatin is a drug that helps to lower cholesterol levels in the body. It is in the class of medications called HMG-CoA reductase inhibitors. HMG-CoA reductase is required to convert 3-hydroxy-3methylglutaryl-coenzyme A to mevalonic acid. Biosynthesis of cholesterol requires mevalonic acid as a precursor. As a result, lovastatin is very effective in lowering low density lipoprotein levels (Lampercht, 1999). In addition to its role in forming cholesterol, mevalonic acid has been suggested to be involved in forming some non-sterol products that are important in cell cycle progression.

The hypothesis being tested in this thesis is that cryptic promoters in the 5'-UTR of P27 are responsible for lovastatin stimulation of P27 expression. To this end, the full length 5'UTR sequence of P27 was inserted upstream of a luciferase reporter gene, transfected into HeLa cells, and subjected to treatment by 81 μ M of lovastatin for 24 hours. This treatment resulted in a two fold increase in the luciferase reporter protein as well as P27 mRNA. The response elements were mapped to the regions of 575-559 and 543-527 bases upstream of the start codon. These promoters are located in the same regions as those identified by Gizzard and Chen (Gizzard, 2005; Chen, 2005). In addition, the same two regions that are sensitive to lovastatin treatment were also responsive to serum starvation. Thus, it is possible that the 5'UTR of P27 has two cryptic promoters that may be responsive to cellular stress.

CHAPTER 2. MATERIALS AND METHODS

2.1 Reagents

Lovastatin (Mevolin) was purchased from Sigma Aldrich. Large scale preparation and plasmid purification kits were purchased from Qiagen. DMEM 1x for cell culture was a product of Cellgro while the Trypsin-Versene Mixture was purchased from BioWhitaker. Lipofectamine and Plus reagent were purchased from Invitrogen and iscript cDNA synthesis kit came from Biorad. Dnase and RNAsin were obtained from Promega.

2.2 Bacterial Strain and Media

E. coli DH5 alpha, the routinely used strain for molecular cloning and plasmid amplification, was grown in Luria's Broth, LB (for 1 liter: 10g Bacto tryptone, 5 g BAct yeast extract, 10 g sodium chloride and pH adjusted to 7.0 with 10 M NaOH). LB agar plates were made by adding 15 g Difco agar to 1 liter of LB. Ampicillin (100 µg/mL) was included in the LB plates for selection of ampicillin resistant transformants. *E. coli* BMH 71-18 mut S was used for molecular cloning in mutant plasmids. This *E. coli* was also grown in Luria's Broth that was made from the procedure described above. 50 ug/mL of tetracycline was included in the LB plates for selection of tetracycline resistant transformants.

2.3 Bacterial Cell Transformation - Large Scale Preparation

Cells were cultured in a LB agar plate supplemented with ampicillin and grown overnight in a 37° C incubator. One colony was chosen and then grown overnight in 250 mL of LB that contained .1% ampicillin. The ampicillin resistant clones were then harvested and the desired plasmid was isolated and purified using the Qiagen Midi Kit.

2.4 Buffers and Solutions

TSS solution was made for the creation of insertional mutations. TSS solution was made with 80% LB, 10% PEG, 5% DMSO and 50 mM of Magnesium Chloride. This solution was mixed together and then autoclaved to prevent bacterial contamination.

2.5 Bacterial Cell Transformation - Linker Scanning Mutagenesis

For BMH 71-18 mut S *E. coli* used for linker scanning mutagenesis, previously prepared BMH *E. coli* was streaked on an LB dish containing 1.5% agar and 50 µg/mL of tetracycline and incubated overnight in a 37° C humidified incubator. A single colony was then taken and placed in a tube with 5 mL of LB containing 50 µg/mL of tetracycline and shaken at 37° C overnight. After overnight incubation, 250 µL of this culture was then added to 25 mL of LB with no tetracycline and cultured at 37° C for about 2.5 hours or until the OD600 is about 0.5 on the spectrometer. The flask is then put on ice for 20 minutes. The *E. coli* is subsequently spun down at 4° C for five minutes, the LB is removed, and 2.5 mL of TSS solution is added. After resuspending the *E. coli* in TSS solution, the mixture is placed on ice for use in linker scanning mutagenesis transformation.

2.6 Primers for cDNA 16 Nucleotide Mutations

Mutation primers were made for the appropriate restriction enzymes (Bam H1 and Sal 1) as well as to create 16 nucleotide mutations from 575 to 496 of the P27 5'UTR. The mutated sections are underlined.

Restriction enzyme Mutations:

Bam H1: TGT GGT AAA ATC GAT AA GCTACG G TCG ACC GAT GCC CTT G

Sal 1: GT AAA ATC GAT AA G CTA CG G TA AAC C GAT GCC CTT GAG AGC CT

5'Untranslated Region Mutations:

M1: CTC TTA CGC GTG CTA GT ACTCTAGACTCTAGAC CTC GCC AGC CTC GGC GG

M2: TCC ACC TTA AGG CCG CG ACTCTAGACTCTAGAC GGG CGG CTC CCG CCG CC

M3: GCT CGC CAG CCT CGG CG ACTCTAGACTCTAGAC CGC AAC CAA TGG ATC TC

M4: GGG GCG GCT CCC GCC GC ACTCTAGACTCTAGAC CCT CCT CTG TTT AAA TA

M5: CCG CAA CCA ATG GAT CT ACTCTAGACTCTAGAC AGA CTC GCC GTC TCA AT

M1/M3 CTC TTA GCG GTC CTA GT ACTCTAGACTCTAGAC TCC ACC TTA AGG CCG CG ACTAGACTCTAGAC CGC AAC CAA
TGG ATC TC

2.7 Creation of cDNA Mutations—Linker Scanning

Primers, annealing buffer, and template were added to a microcentrifuge tube and boiled for ten minutes. Samples were then cooled in ice + water and 10 x synthesis buffer, T4 DNA polymerase, and T4 DNA ligase were subsequently added. After incubation at 37° C for two hours, the reaction was stopped by incubating at 70° C for seven minutes and cooled at room temperature for 30 minutes. Annealing buffer and the appropriate digestion enzyme (Sal 1 or Bam H1) were then added and incubated at 37° C for an additional two hours. After the appropriate mutant is added to 200 uL of TSS

solution, the plasmid is added to the *E. coli* mixture and cultured overnight in a 37 C shaker. The bacterial cells are then purified using the Qiaspin Prep Miniprep Kit. After the first transformation, plasmids are digested again with the appropriate digestion enzyme (Sal I or Bam HI). 30 or 100 uL of the plasmid are then plated on LB agar plates and incubated overnight. The plasmids are purified with the Qiaspin Prep Miniprep Kit.

2.8 Cell Culture and Transfection

Cells were plated in 35 mm dishes and cultured as described above. After three hours of transfection, the medium was changed to DMEM with serum and incubated for an additional 24 hours.

2.9 Lovastatin Treatment/Serum Starvation

Twenty four hours after transfection, HeLa cells were exposed to 81 μ M of lovastatin dissolved in Ethanol for an additional 24 hours. Control cells were given appropriate amounts of Ethanol only. Serum starvation cells were exposed to either “starvation conditions” (DMEM without serum) or control conditions (DMEM with 10% serum) for an additional 48 hours.

2.10 RNA Analysis

Harvesting Cells and Isolation of RNA: HeLa cells were washed and RNA was isolated and purified using Qiagen’s RNeasy Mini Kit.

Real-Time PCR Primers: Primers were developed from Invitrogen and had the following forward and reverse sequences:

Luciferous Forward: 5’ GCG AAG GTT GTG GAT CTG 3’

Luciferous Reverse: 5’ CAC ACA CAG TTC GCC TCT TTG 3’

B Galactosidase Forward: 5’ TGC TGC ACG CGG AAG AA 3’

B Galactosidase Reverse: 5' AGT CGT CGC CAC CAA TCC 3'

Endogenous P27 Forward: 5' CTC TGA GGA CAC GCA TTT GGT 3'

Endogenous P27 Reverse: 5' CGC ATT GCT CCG CTA ACC 3'

Endogenous GADPH Forward: 5' AAG GAC TCA TGA CCA CAG TCC 3'

Endogenous GADPH Reverse: CCA TCA CGC CAC AGT TTC 3'

Real-Time PCR Analysis: Reverse Transcriptase is added and RNA is subsequently converted to DNA using PCR. The samples are then given the appropriate primers, SYBR Green, and the resulting Ct value is measured with a thermocycler. Relative concentrations of DNA present during the exponential phase of the reaction are determined by plotting fluorescence against cycle number on a logarithmic scale. Amounts of DNA or RNA are then determined by comparing this value to a previously prepared standard curve. Samples are normalized with B-galactosidase.

2.11 Cell Cycle Analysis: Flow Cytometry

HeLa cells were washed with PBS and fixed with Ethanol. Fifty ug/mL of Propidium Iodide and 200 ug/mL of RNase are added and cell cycle is measured using FACS analysis.

2.12 Protein Analysis: Western Blot

Cells were rinsed with PBS and lysed with .1% SDS Page sample buffer. (3 mL TNN; 1 mM DDT; 2 mM of PMSF; .1% SDS). Equal amounts of protein were separated on SDS-polyacrylamide gels. The cells were then transferred to a PVDF membrane and were processed as previously described (Wei, 2002). Detection was carried out using a horseradish peroxidase conjugated secondary antibody and a chemiluminescent substrate.

2.13 Statistical Testing

Samples were analyzed with Microsoft Excel to determine the p value and t test.

CHAPTER 3. RESULTS

3.1 Lovastatin Causes G1 Phase Cell Cycle Arrest by Increasing the P27 Protein Level

It has been shown previously that lovastatin causes G1 phase cell cycle arrest in HeLa cells by increasing the level of P27 protein (Hengst, 1996). To confirm these results, HeLa cells were treated with 81 μ M of lovastatin (L+) or vehicle ethanol control (L-) for 24 hours. The cell cycle was subsequently analyzed by flow cytometry and P27 protein levels were examined by Western blot. Figure 4A shows that cells treated with 81 μ M of lovastatin had 70% of their cells in the G1 phase of the cell cycle. Twenty-five percent of the lovastatin-treated cells were in the S phase and 5% were in the G2 phase. In contrast, vehicle control treated cells had less than 50% of their cells in the G1 phase, 40% in the S phase and 10% in the G2 phase. Thus, lovastatin can arrest cells in the G1 phase of the cell cycle.

As shown in Figure 4B, HeLa cells treated with lovastatin had more than three times the amount of P27 protein compared with those treated by the vehicle control. Thus, it is likely that lovastatin increases P27 protein expression which in turn results in cell cycle arrest in the G1 phase. These results confirmed previous findings that lovastatin increases the levels of P27 protein (Hengst, 1996).

Figure 4A

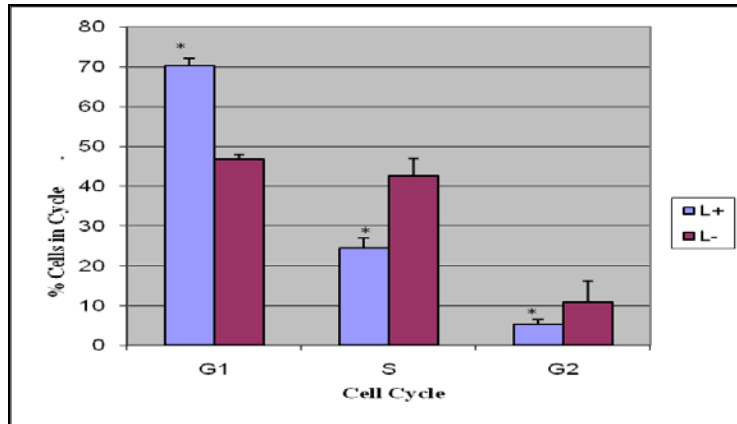
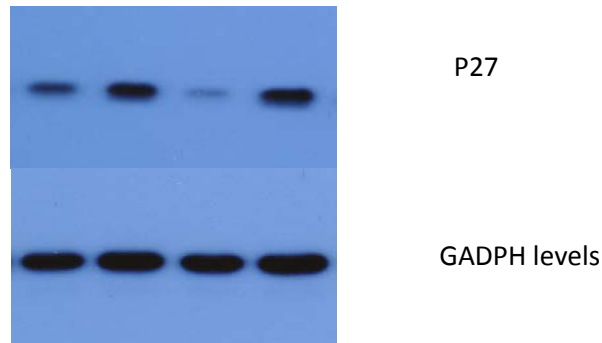


Figure 4B



Lanes	1	2	3	4
Lovostatin	yes	no	yes	no

Figure 4: Cell cycle analysis and western blot of HeLa cells exposed to 81 μM of Lovastatin. 1.2×10^6 HeLa cells were plated on 6 well plates, incubated for 48 hours, and then subject to 81 μM of lovastatin (L+) or ethanol (L-) for an additional 24 hours. HeLa cells were then analyzed by flow cytometry for cell cycle distribution (A) and by western blot for P27 expression (B). Statistically significant results in the G1 phase of the cell cycle ($p < .05$) are shown with the * symbol. GADPH was used as a loading control.

3.2 Lovastatin and its Effect on P27 5'UTR Deletion Mutations

Previously, it has been found that P27 was regulated by translation via an IRES site in its 5'UTR (Miskimins, 2001). However, this finding has been disputed by other studies which showed that the presumed IRES in the 5'-UTR may be a cryptic promoter instead (Kullman, 2002; Liu, 2005; Gizard, 2005). In order to understand if P27 is regulated by a cryptic promoter rather than an IRES and if this promoter is responsible for lovastatin stimulation, various monocistronic DNA constructs were created (Figure 5A). These constructs were then transfected into HeLa cells followed by treatment with or without lovastatin and the promoter activity was measured by determining the reporter level. As shown in Figure 5B, the full length P27 5'UTR (575 bases) showed a 1.4 fold increase in luciferase activity. However, the deletion constructs with 540, 496, and 150 bases of the 5'-UTR had very little increase in luciferase expression following the exposure to lovastatin. In addition, the total level of luciferase activity decreases substantially when a 5'-UTR with 540 nucleotides or less is used. These results suggest that a lovastatin response element may be located between 540 and 575 nucleotides.

Figure 5A

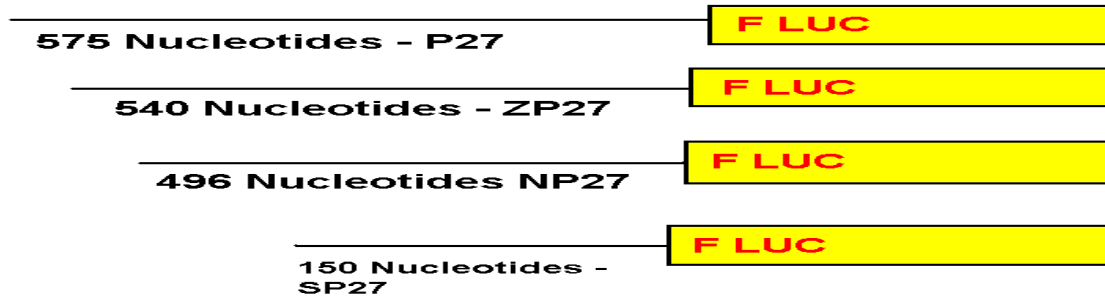


Figure 5B

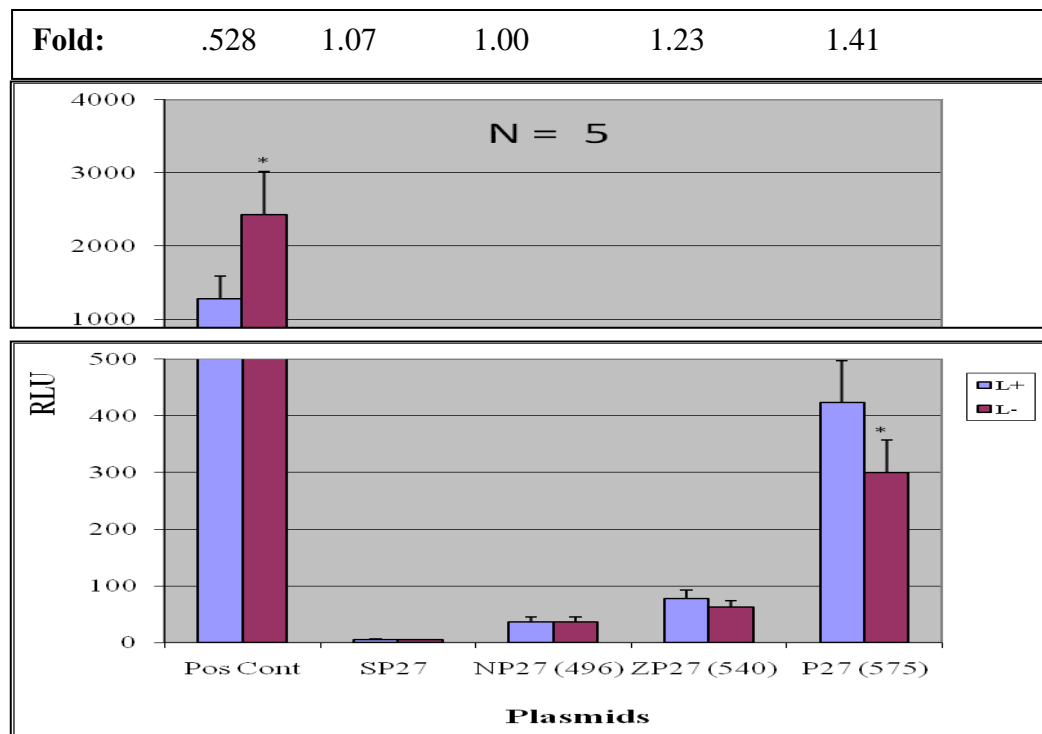


Figure 5: Luciferase Analysis of P27 deletion mutations. **5A:** Deletion mutation constructs inserted upstream of the luciferase gene. F Luc. Stands for Firefly Luciferase. **5B:** Relative luciferase activity for HeLa cells transfected with deletion mutations and exposed to 81 μ M of lovastatin for 24 hours. Cells not exposed to lovastatin, L-, were given equal amounts of ethanol. Cells were then harvested and analyzed for luciferase analysis. All samples are measured in RLU (Relative Light Units). Samples were normalized with B-galactosidase. All samples are expressed relative to the PGL3 vector control. (N=number of experiments. * = $p < .05$; ** = $p < .01$; *** = $p < .001$)

3.3 Mapping the Lovastatin Response Element in the Region From 575-540 of P27's 5'UTR by Linker Scanning Mutagenesis

To define the putative lovastatin response element between 540 and 575 nucleotides of the P27 5'UTR, linker scanning mutagenesis was used. Figure 6A shows the resulting constructs. These constructs were transfected into HeLa cells to determine their response to lovastatin. As shown in Figure 6B, the total luciferase activity was decreased by mutations M1 and M3 in the regions of 575-559 and 543-527. This indicates that promoter elements may exist in the regions of M1 or M3. These promoter elements also appear to be sensitive to lovastatin. At 81 μ M of lovastatin, both M1 and M3 showed a decrease in lovastatin-stimulated luciferase expression compared with the wild type P27 5'-UTR. Taken together, it is possible that there may be two lovastatin response elements; one in the M1 region (575-559) and the other in the M3 region (543-527). In addition, it is possible that these two response elements may cooperatively work together and mutating any one of them may not be enough to completely eliminate the lovastatin response.

Figure 6A

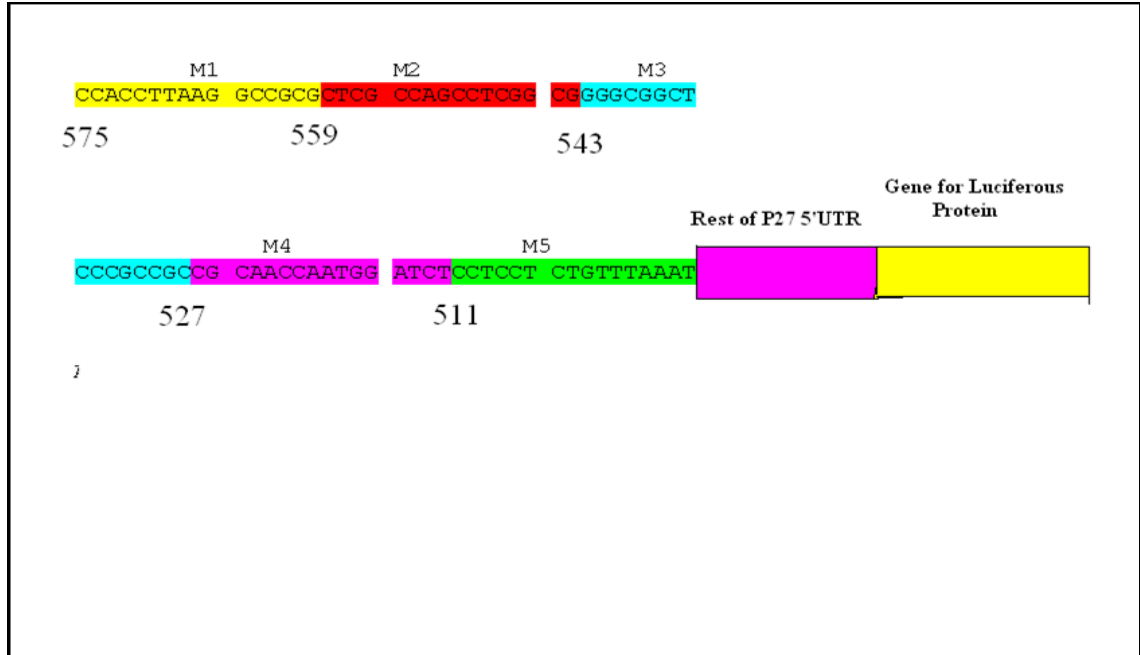
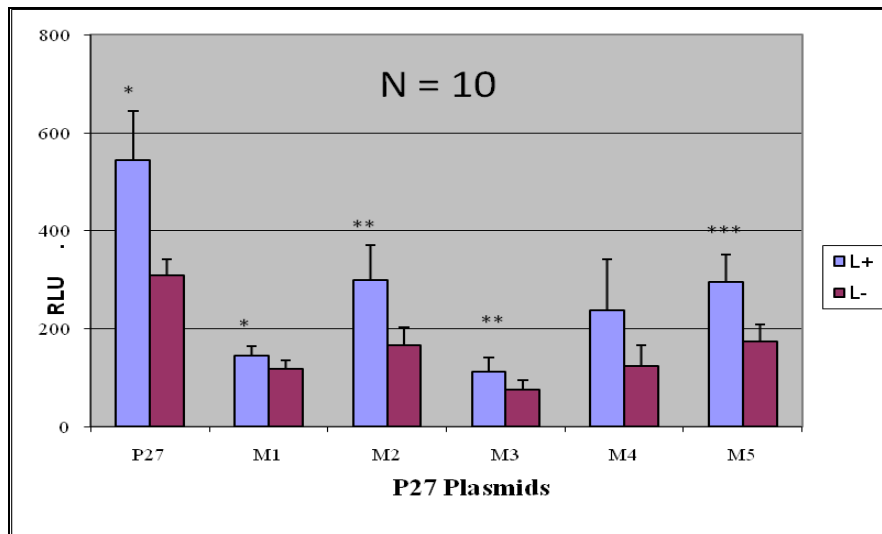


Figure 6B

Fold:	1.44	1.22	1.81	1.51	1.97	1.71
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KEY:
M1 = 575-559
M2 = 559-543
M3 = 543-527
M4 = 527-511
M5 = 511-495

Figure 6: Luciferase analysis of 16 nucleotide insertional mutations transfected into HeLa cells and exposed to Lovastatin. **6A:** Mutations made by linker scanning mutagenesis. **6B:** Luciferous activity of Mutants 1-5 after transfecting them into HeLa cells and incubating them with 81 μ M of lovasatin for 24 hours. (N=number of experiments. RLU = Reflective Light Units. * = $p < .05$; ** = $p < .01$; *** = $p < .001$)

3.4 Effect of the M1/M3 Double Mutant (575-559, 543-527) on Lovastatin Response

To test if M1 and M3 do indeed work cooperatively, a new plasmid was created (M1/M3) that has mutations in both the M1 and M3 of P27's 5'UTR. (575-559; 543-527) (Figure 7A). This newly created plasmid was then transfected into HeLa cells and subject to 81 μ M of lovastatin as described above (See 3.4). Figure 7B indicates that the total luciferase activity with the M1/M3 construct is significantly lower than either the M1 or M3 constructs and all are much lower compared with the wild type 5'UTR of P27. In addition, HeLa cells transfected with M1/M3 show a statistically insignificant 1.18 fold increase with cells exposed to 81 μ M of lovastatin. This provides further evidence that the lovastatin response elements are likely present in both the M1 and M3 regions.

Figure 7A

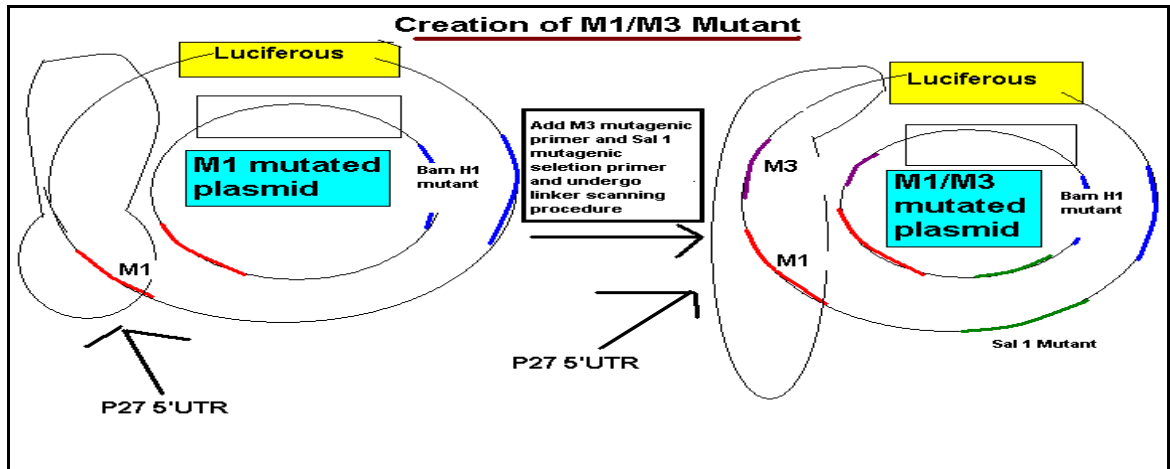


Figure 7B

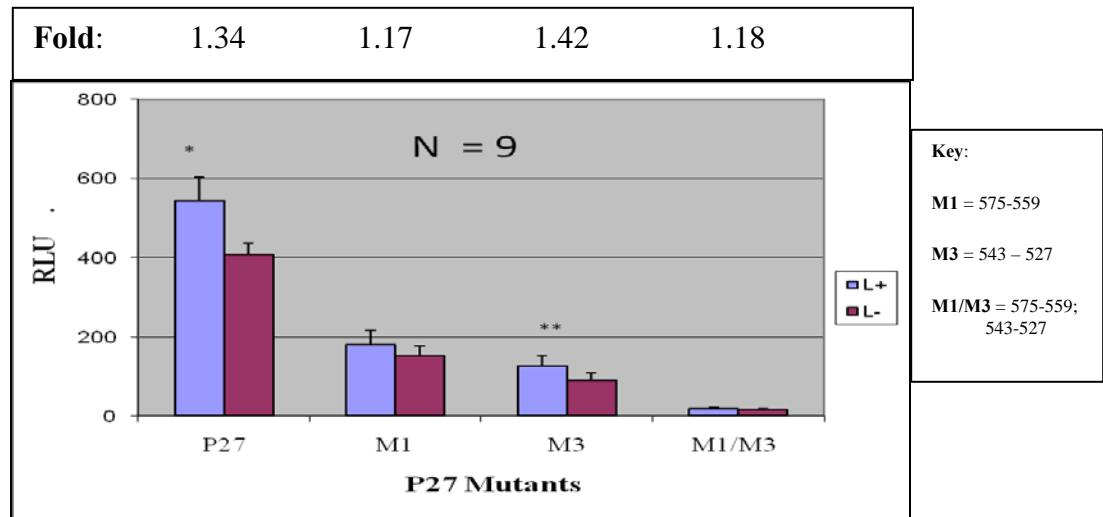


Figure 7: Luciferase analysis of newly transfected M1/M3 mutant (575-559; 543-527) transfected into HeLa cells and exposed to Lovastatin. **7A:** Creation of the M1/M3 mutant using the same linker scanning procedure described previously. **7B:** P27 5'UTR; M1, M3, and M1/M3 were transfected into HeLa cells and treated with 81 μ M lovastatin for 24 hours. Luciferase activities were then measured on a luminometer. (N=number of experiments run. * = $p < .05$; ** = $p < .01$; *** = $p < .001$)

3.5 RNA Analysis of P27 5'UTR Mutants Exposed to 81 μ M of Lovastatin

Since there seems to be lovastatin response elements in both the M1 (575-559) and M3 (543-527) regions of P27's 5'UTR, the RNA level of luciferase was then measured to confirm the existence of these elements in our newly created plasmids. HeLa cells were transfected with either P27 (575), M2 (559-543), M1/M3 (575-559; 543-527), or SP27 (150) (a very minimal P27 promoter) and exposed to 81 μ M of lovastatin for 24 hours. RNA levels were then measured by real-time PCR (RT-PCR). Figure 8 indicates that both the wild type P27 and the mutant M2 control had an increase in RNA levels following lovastatin treatment. (Figure 8) As expected, the double mutant M1/M3 shows no change in RNA levels when subject to these conditions (Figure 8). Thus, there may be two lovastatin sensitive promoters (M1: 575-559 and M3: 543-527) that function cooperatively to increase the transcription of P27.

Figure 8

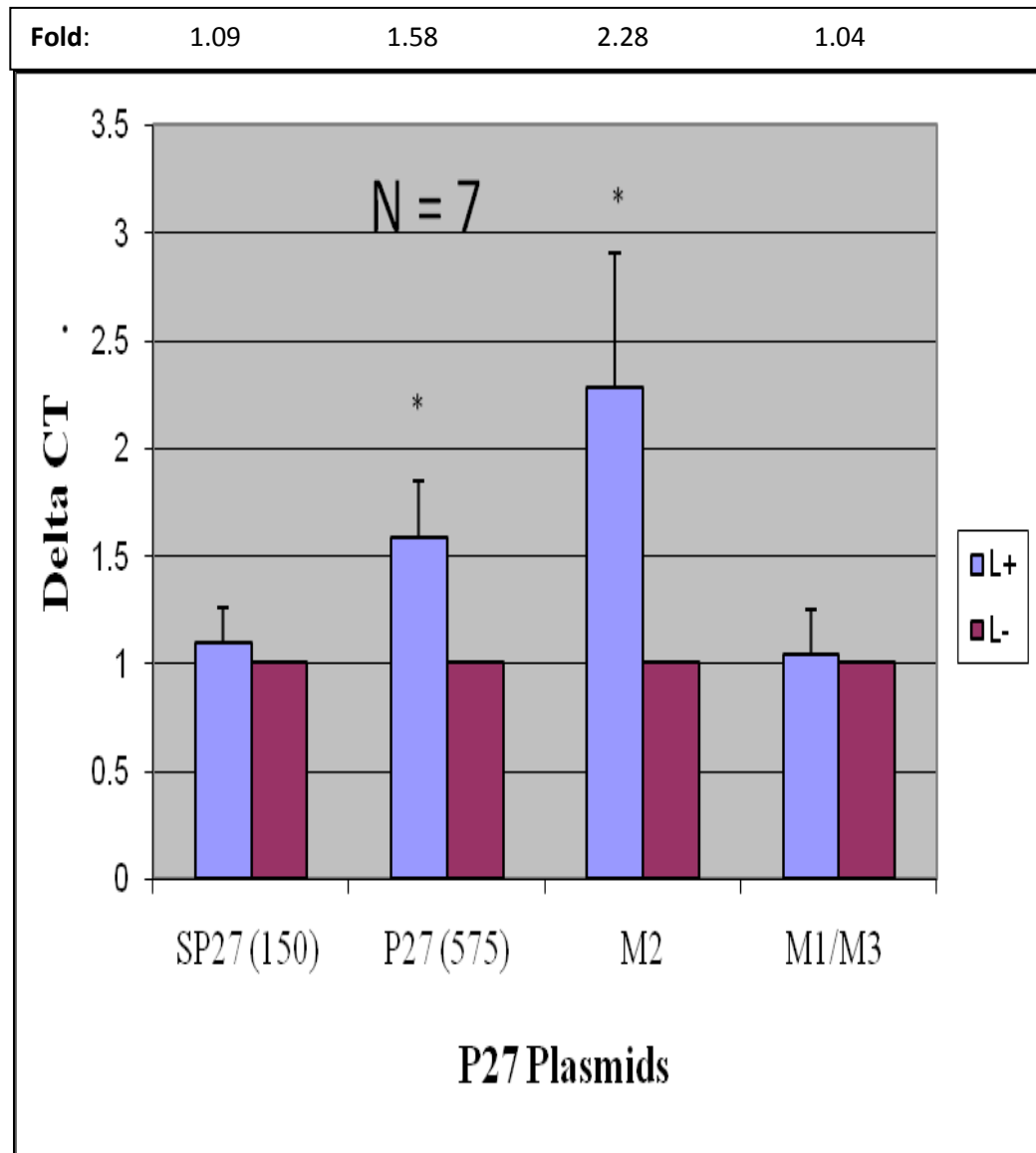


Figure 8: RNA analysis of P27 mutated plasmids exposed to 81 μ M of lovastatin. SP27 deletion mutation (150), P27 (575), Mutant 2 – (559-543), and M1/M3 [(575-559); (543-527)] were transfected into HeLa cells, incubated for 24 hours, and then subject to 81 μ M of lovastatin in procedures described previously. RNA was then harvested and analyzed by real-time PCR. (N = number of experiments. * = $p < .05$)

3.6 Endogenous RNA Analysis of P27 Exposed to 81 μ M of Lovastatin

Previous results indicate that two lovastatin sensitive elements are located in the 5'UTR of P27. To further determine if endogenous P27 RNA is also increased following lovastatin treatment, RT-PCR was performed with endogenous P27. As shown in Figure 9, a 1.6 fold increase in endogenous P27 RNA was observed with HeLa cells exposed to 81 μ M of lovastatin for 24 hours. These results are comparable to the 1.5 fold increase observed with the P27 5'UTR luciferase construct in HeLa cells treated with the same conditions. Thus, it is possible that lovastatin increases the endogenous P27 expression via two response elements in its 5'UTR: M1 (575-559) and M3 (543-527).

Figure 9

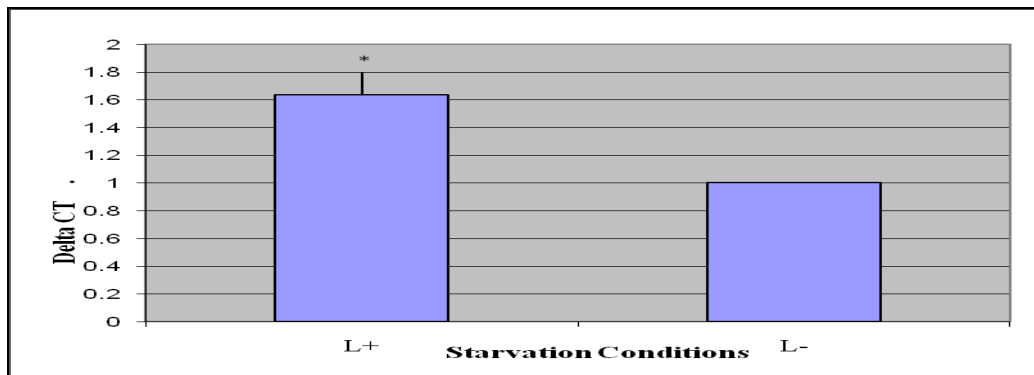


Figure 9: Endogenous RNA analysis of P27 plasmids exposed to 81 μ M of lovastatin. Cells were plated, incubated for 48 hours, and exposed to 81 μ M of lovastatin for an additional 24 hours. HeLa cells were then harvested and RNA was subsequently isolated. Endogenous P27 was measured using Real Time PCR. Endogenous P27 RNA samples were normalized with the housekeeping gene GADPH. (N = number of experiments. * = $p < .05$)

3.7 Cell Cycle Analysis of HeLa Cells Exposed to Starvation Conditions

In addition to lovastatin, cell synchronization in the G1 phase of the cell cycle can be achieved by serum starvation. Chou published a paper in 1999 showing that a Chinese hamster ovary cell line (CHO K1) could be effectively arrested in the G1 phase of the cell cycle through serum starvation (Chou, 1999). In a more recent study on dermal fibroblasts, a statistically significant increase of cells in the G1 phase was observed upon exposure to serum starvation (Khammanit, 2008). Moreover, Shin observed decreases in CDK2 and CDK4 activity in ovarian cancer cells that were exposed to serum starvation (Shin, 2008).

To see if HeLa cells could also be synchronized in the G1 phase, cells were plated onto six well plates and incubated for 48 hours. Cells were then subjected to starvation conditions for 48, 96, or 144 hours respectively. Cells that were incubated for 48 hours under normal conditions showed 40% in G1, 55% in S, and 5% in the G2 phase of the cell cycle. In contrast, cells incubated without serum showed 53% in G1, 30% in S, and 12% in the G2 phase of the cell cycle. As expected, cells incubated without serum at 96 and 144 hours showed similar results to those incubated for only 48 hours (Figure 10). This shows either serum starvation or lovastatin incubation can arrest cells in the G1 phase of the cell cycle. Indeed, starved HeLa cells exposed to 81 μ M of lovastatin had 83% of their cells arrested in the G1 phase.

Figure 10

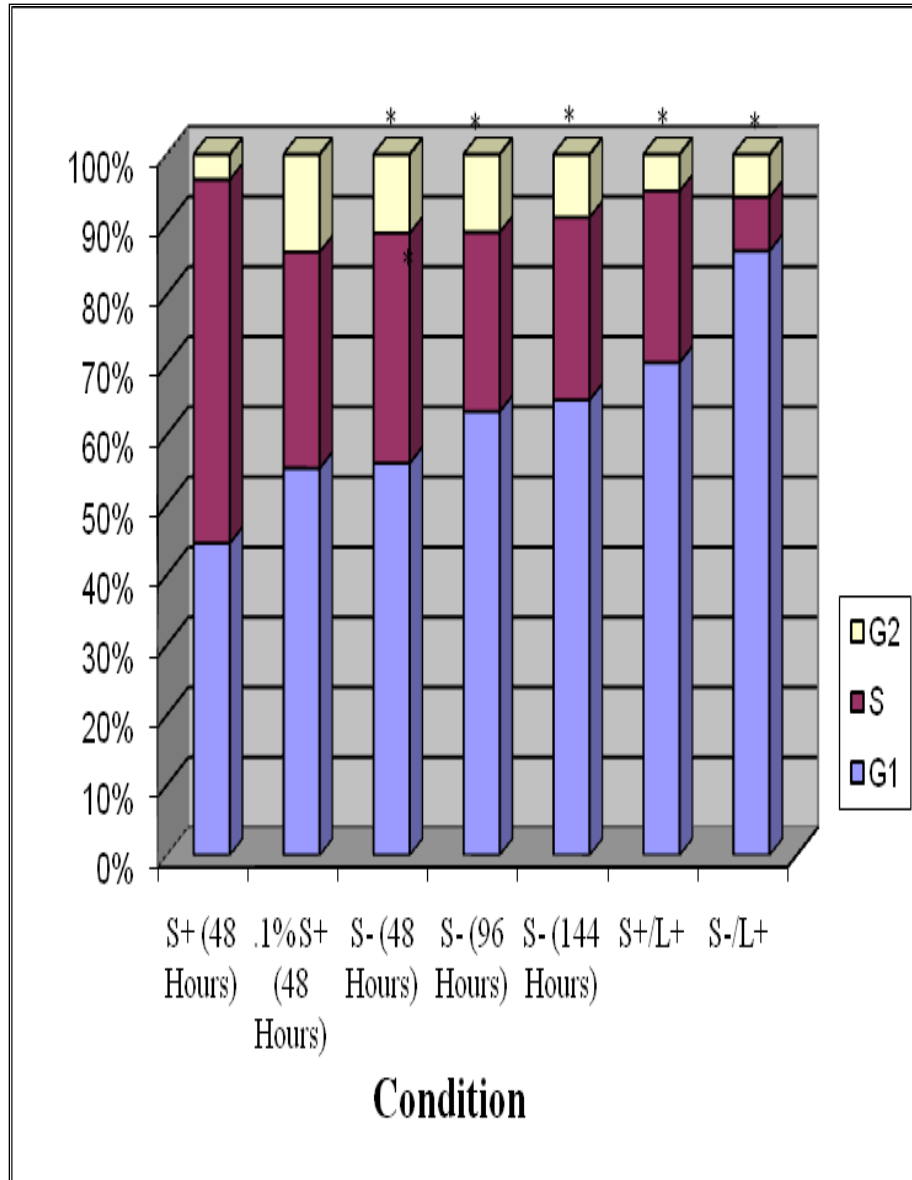


Figure 10: Cell cycle analysis of HeLa cells exposed to starvation conditions. 1.2×10^5 cells (S+, S+/L+, S- 48 hours, S- 96 hours, S- 144 hours) were plated on a 6 well plate, incubated for 48 hours, and then subject to starvation conditions for 48, 96, or 144 hours. Cells were then analyzed by FACS analysis (* = $p < .05$).

3.8 Luciferase Analysis of P27 Transfected HeLa Cells Exposed to Starvation Conditions

Since HeLa cells could be synchronized in the G1 phase of the cell cycle, P27 5'UTR mutant plasmids were transfected into HeLa cells to determine how luciferase activity was affected. After cells were transfected with P27 plasmids, they were incubated for 24 hours and then subject to either starvation (DMEM without serum) or control (DMEM with serum) conditions for an additional 48 hours followed by analysis of luciferase activity. As shown in Figure 11, the full length P27 5'UTR had a 1.4 fold increase in luciferase activity. (Figure 11) This is identical to the results seen in HeLa cells exposed to 81 μ M of lovastatin (Figures 5 and 7). In contrast, the 496 deletion construct (NP27) showed no increase in luciferase activity in response to starvation conditions which is also consistent with our previous results. Furthermore, the plasmids M1 and M3 showed a statistically insignificant increase in luciferase activity when exposed to starvation conditions for 48 hours. When the double mutant M1/M3 was analyzed, there was no increase (Fold = .81) in luciferase activity in response to serum starvation (Figure 11B). These results suggest that the two lovastatin response elements, M1: 575-559 and M3: 559-527, may also respond to serum starvation.

Figure 11A

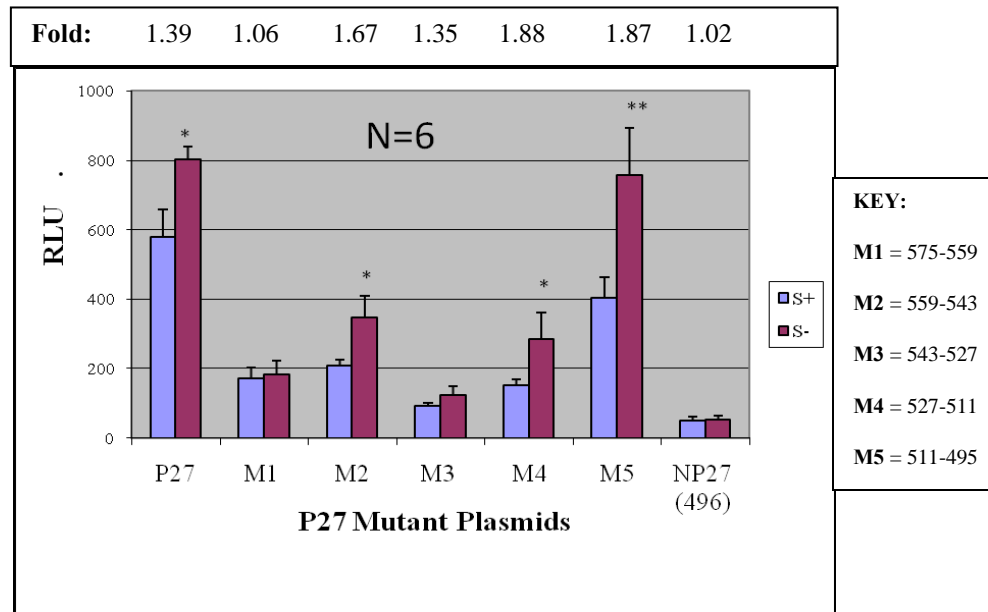


Figure 11B

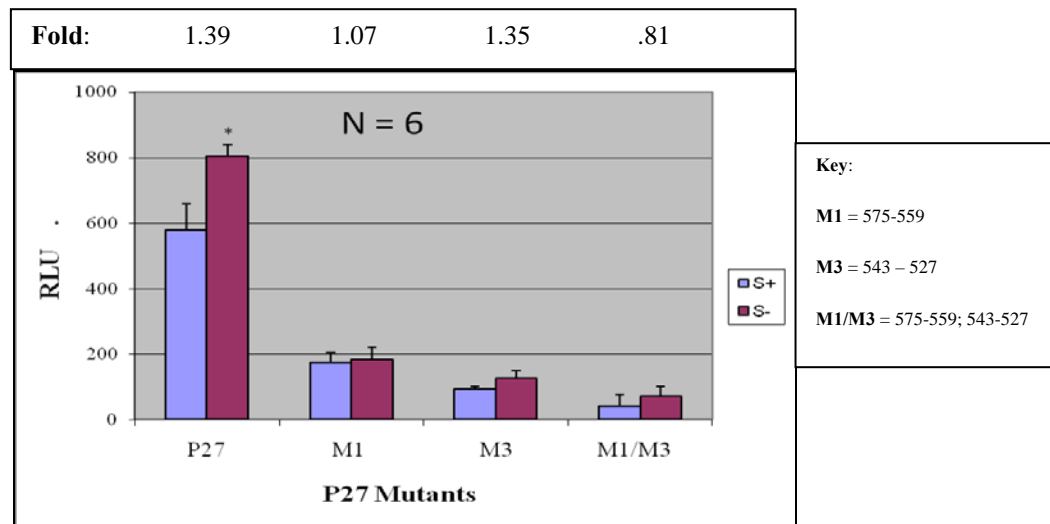


Figure 11: Luciferase Analysis of 16 nucleotide deletion mutations transfected into HeLa cells and exposed to starvation conditions. **11A & B:** P27 mutants were transfected into HeLa cells, incubated for 24 hours and subject to serum starvation conditions for 48 hours. Cells were then harvested, lysed, and activity was analyzed on a luminometer. (N = number of experiments. * = p<.05; ** = p<.01; *** = p<.001)

3.9 RNA Results of HeLa Cells Exposed to Starvation Conditions

We next determined RNA levels to confirm that serum starvation causes an increase in P27 transcription. HeLa cells were exposed to starvation conditions for 48 hours with the resulting total RNA being measured. Figure 12A showed a 1.5 fold increase in P27 RNA which is comparable to the 1.5 fold increase in RNA when HeLa cells were exposed to lovastatin. When cells were exposed to starvation conditions and M2 RNA was measured, there was a 2.5 fold increase in RNA compared to the serum control. In contrast, there was no RNA increase in SP27 (150) or M1/M3 (575-559, 543-527) (Figure 12A). To see if this only affects manufactured plasmids, endogenous P27 levels were measured from HeLa cells exposed to starvation conditions for 48 hours. Figure 12B indicates a statistically significant 1.3 fold increase in P27 levels. This statistically significant increase provides further evidence that there are two starvation sensitive promoters: M1 (575-559) and M3 (543-527). Since these results are similar to those when HeLa cells were exposed to lovastatin, it suggests that cellular stress increases P27 levels through the process of transcription. This hypothesis, however, requires a Western Blot analysis to confirm if serum starvation does in fact cause an increase in endogenous P27 protein.

Figure 12A

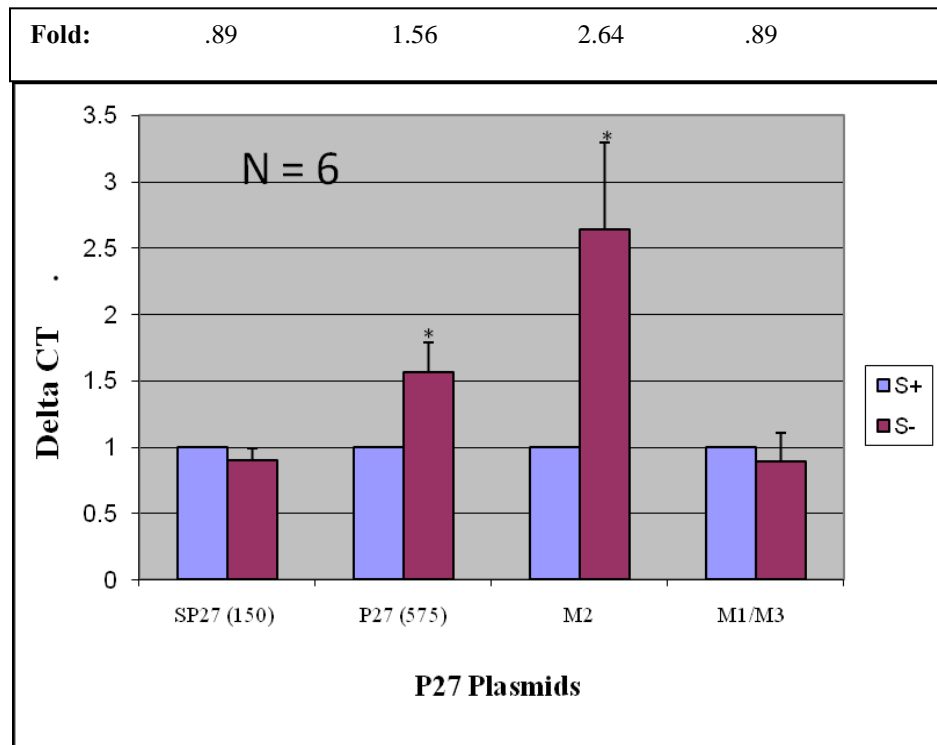


Figure 12B

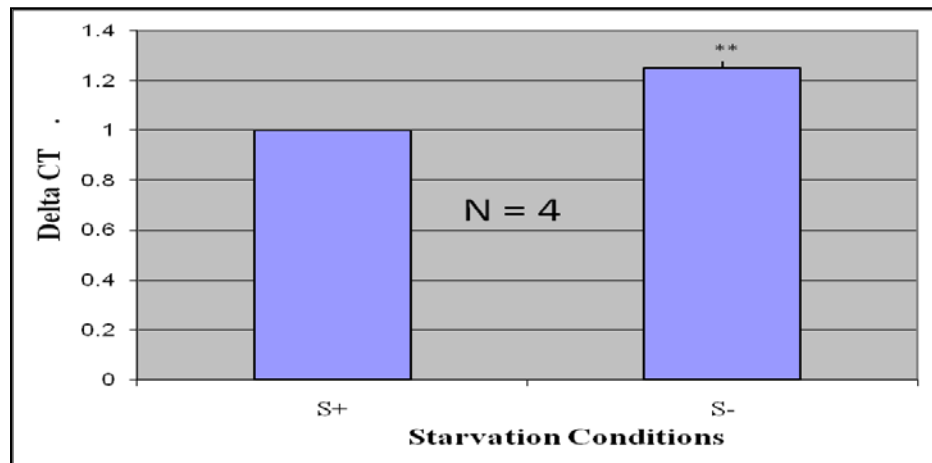


Figure 12: RNA analysis of HeLa cells exposed to starvation conditions. **12A:** SP27 deletion mutation (150), P27 (575), Mutant 2 (559-543), and M1/M3 (575-559; 543-527) were transfected into HeLa cells, incubated for 24 hours, and subject to starvation conditions for 48 hours. RNA was then isolated and analyzed through Real Time PCR. **12B:** HeLa cells were plated, incubated for 48 hours, and subject to starvation conditions for 48 hours. HeLa cells were then harvested and RNA was subsequently isolated. Endogenous P27 RNA levels were then measured using Real Time PCR. Endogenous P27 samples were normalized with the housekeeping gene GADPH. (N=number of experiments. * = p<.05; ** = p <.01; *** = p<.001)

CHAPTER 4. DISCUSSION

Our results indicate that P27 contains two cooperatively functioning lovastatin response elements in both the M1 (575-559) and M3 (559-543) regions of the 5'UTR. This was shown through both luciferase reporter analysis and real-time PCR. In addition to being responsive to lovastatin, these two regions are also sensitive to serum starvation. This suggests that P27 expression may be regulated by other aspects of cellular stress in addition to lovastatin at the transcriptional level.

Lovastatin has been shown to arrest HeLa cells in the G1 phase of the cell cycle. (Hengst, 1996). Indeed, Figure 4 indicates that over 70% of the cells were arrested in the G1 phase after being exposed to 81 μ M of lovastatin for 24 hours. Furthermore, there was also a two fold increase in P27 protein expression in cells exposed to lovastatin. HeLa cells that were subject to starvation for 96 hours had 60% of the cells arrested in the G1 phase (Figure 10). This suggests that both lovastatin and serum starvation arrest cells in the G1 phase of the cell cycle by increasing the expression of P27. Although there is no previous study indicating the effects of serum starvation on P27 levels, Khammanit showed a statistically significant increase of cells in the G1 phase upon exposure to starvation conditions for 48 hours (Khammanit, 2008).

Furthermore, Shin observed decreases in CDK2 and CDK4 activity in ovarian cancer cells that were exposed to serum starvation (Shin, 2008). P27 is a known inhibitor of CDK2. This suggests that P27 may cause cell cycle arrest in starved HeLa cells in addition to cells exposed to 81 μ M of lovastatin. To confirm this, however, a Western blot needs to be performed to determine if P27 protein levels increase under starvation conditions.

When HeLa cells transfected with the P27 5'UTR-Luciferase construct were exposed to 81 μ M of Lovastatin for 24 hours, there was a statistically significant 1.4 fold increase in luciferase activity (Figure 5). In turn, there was also a 1.4 fold increase in luciferase activity in cells exposed to starvation conditions (Figure 11). In contrast, there was no change in luciferase activity in 540, 496, or 150 5'UTR deletion mutations. Since these were monocistronic constructs, this data contradicts the concept that Minikimis proposed where P27 regulation is controlled by an IRES site (Minikimis, 2001). If P27 5'UTR did indeed contain an IRES that regulated translation, there should be no luciferase activity present when the cell lysate was analyzed on a luminometer. Instead, however, there was an increase in luciferase activity when transfected HeLa cells were subject to 81 μ M of lovastatin or starvation conditions. This suggests that lovastatin or serum starvation increases the P27 protein through transcription by activating a promoter in the 5'UTR.

When deletion mutations were made from nucleotides 496-575, all five mutations had some response to lovastatin. Two mutations, however, had a lower total luciferase activity: M1 (575-559) and M3 (543-527). Since these two mutations had significantly lower activity than P27, M2, M4, or M5, a new M1/M3 mutant was made with deletions in both regions of the 5'UTR (575-559; 543-527). This newly created mutant showed no lovastatin-stimulated increase in luciferase activity. Since there was an increase in luciferase activity with both M1 and M3 but not M1/M3, there seems to be two cooperatively functioning luciferase response elements: M1 (575-559) and M3 (543-527). When Real Time RT-PCR was run, there was no increase in RNA levels with either serum starvation (Figure 12A) or 81 μ M of lovastatin (Figure 8). These results are further validated by Gizard which showed a region between 549 and 511 nucleotides that

caused an increase of P27 mRNA with the addition of progesterone (Gizard, 2005). Taken together, these results suggest that cellular stress causes G1 phase cell cycle arrest by increasing the transcription of P27 via activating both the M1 and M3 response elements.

In 1996, Hengst indicated that endogenous P27 protein increased upon lovastatin incubation. This paper also showed no change in P27 RNA when measured on a Northern blot. A Northern blot, however, is not as sensitive as real-time PCR. Because of this and our previous results, HeLa cells were exposed to 81 μ M of lovastatin or starvation conditions after which endogenous P27 RNA was measured via real-time PCR. With 81 μ M of lovastatin, there was a 1.6 fold increase in endogenous P27 mRNA. There was also a statistically significant 1.3 fold increase in P27 mRNA when HeLa cells were exposed to starvation conditions. These results contradict Hengst conclusion that the increase in P27 protein is entirely due to translational regulation. Furthermore, this data confirms that P27 RNA does increase via transcription when HeLa cells are subject to cellular stress.

Uncontrolled cellular proliferation is one of the major causes of tumorigenesis. If cells continue to proliferate indefinitely, repair is unable to occur which allows for transitions, transversions, double stranded breaks and other errors to permanently incorporate themselves into DNA. In addition, uncontrolled cellular proliferation causes already formed initiated cells to develop into full blown tumors. This is why it is important to understand the regulation of cyclin dependent kinases. These kinases, which promote the growth of the cell cycle, are regulated by various proteins such as P27, P21, and P57.

P27 causes cells to be arrested in the G1 phase of the cell cycle by inhibiting Cyclin E/CDK2. Low amounts of P27 have lead to a poor prognosis in many types of human cancers including breast, prostate, and ovary. This is partly because low P27 amounts prevent cells from being arrested in the G1 phase of the cell cycle. It is also because P27 causes cellular differentiation which prevents the formation of metastatic cancers. Trocone helped to verify this by publishing a paper showing higher amounts of P27 protein in benign lesions in contrast to those in malignant breast lesions (Troncone, 2004).

Although our P27 promoter only gives a 1.5 fold increase in response to lovastatin or serum starvation, it does suggest that P27 is regulated at the level of transcription. This regulation has also been shown by Kulman who found two bands on a northern blot when the full length 5'UTR (575 nucleotides-located on a bicistronic construct) was analyzed (Kulman, 2002). P27's 5'UTR has also been shown to be regulated by transcription in other cell lines such as T467D and prostate cancer cells. In all of these experiments, the promoter has been shown to be in the region of 575 to 540 nucleotides of the P27 5'UTR (Gizard 2005; Roy 2007). Since results are similar when HeLa cells are exposed to either lovastain or starvation conditions, it is possible that P27 is regulated by transcription via cellular stress. Indeed, Gizzard showed that Silibinin increases P27 RNA levels and Roy indicated that progesterone also increased P27 through transcription (Gizard 2005; Roy 2007). Transcriptional regulation does not explain the total increase in P27 protein, since there was only a 1.6 fold increase in endogenous P27 RNA while there was over a three-fold increase in P27 protein when 81 μ M of lovastatin was incubated for 24 hours (Figure 4 and 9). Furthermore, other studies from Carrano and Hengst suggest that P27 is

regulated during translation and at the level of proteosomal degradation (Carano, 1999; Hengst, 1996). This suggests that P27 is regulated at multiple levels in response to cellular stress. Hopefully, scientists will be able to manipulate one or more of these aspects of regulation in the future. This could lead to treatments that increase P27 to prevent the formation and progression of cancer.

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CURRICULUM VITAE

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Advisor: Dr. Jian-Ting Zhang

- Focuses on looking at promoter/reporter assays to see how lovastatin increases the P27 protein.

Bachelor of Arts in Chemistry, July 2000.

Ball State University, USA.

Project: Analyzed cell structure using Nuclear Magnetic Resonance technology.

Research Interests

- Utilized Real Time PCR to measure RNA levels
- Utilized NMR to analyze cell structure

Experience

Microbiologist, A-Tek, Washington D.C.

Jan 2010 – Present

- Uses real time PCR to measure bacterial DNA from the outside environment.
- Involved in Quality Control analysis of various DNA primers and probes.
- Proficient in chain of custody procedures.

Chemical Technician, Indiana University at Riley Children's Hospital

Sep 2000 – Jan 2010

- Use tissue homogenization to understand protein formation/turnover in children with active Chron's Disease who are given the anti-tumor necrosis factor drug Inflixmab.
- Develop methods to analyze tissue biopsies from tissues with inactive Chron's disease to see if enteral nutrition actually suppresses proteolysis and increases protein formation.

- Isolate, purify, and derivitize amino acids to develop a greater understanding of proteolysis in extremely low birth weight infants intravenously given labeled amino acids.
- Perform ion exchange chromatography to analyze how insulin and/or recombinant human IGF-1 infusion can affect ovine fetal phenylalanine kinetics and protein synthesis.
- Troubleshoot various problems with amino acid analysis.

Tutor/Teacher's Assistant, Ball State University

Aug 1998 – Jul 2000

- Presented complicated mathematics to diverse classrooms of 20+ students.
- Created lesson plans.
- Organized and held test preparation sessions. Perform ion exchange chromatography to analyze how insulin and/or recombinant human IGF-1 infusion can affect ovine fetal phenylalanine kinetics and protein synthesis.