

ABSTRACT

Title of Document: RNA INTERFERENCE MEDIATED SUPPRESSION OF TN-CASPASE-1 AS A MEANS OF INVESTIGATING APOPTOSIS AND IMPROVING RECOMBINANT PROTEIN PRODUCTION IN *TRICHOPLUSIA NI* CELLS

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The baculovirus expression system has proven to be a robust and versatile system for recombinant protein production in insect cells. A wide range of promoters is available for the facile expression of transgenes, and yields of up to 50% of total protein have been reported. However, in many cases production is decreased as a result of proteases and host cell apoptosis. To combat this problem, RNA interference (RNAi) has been used as a metabolic engineering tool to knockdown host genes responsible for decreasing the yield of recombinant protein. A novel caspase (Tn caspase-1) derived from *Trichoplusia ni* cells has been identified and characterized. Through modulation of caspase levels via either RNAi or through interaction with baculovirus protein p35, the overall level of apoptosis present in cell culture has been decreased. In addition, the use of *in vitro* RNAi targeted against Tn

caspase-1 has increased the production of recombinant green fluorescent protein. To further study the effect of suppressing Tn caspase-1, a stable cell line producing *in vivo* RNAi was developed, resulting in a nearly 90% decrease in caspase enzymatic activity. This suppression was able to improve culture viability under adverse conditions and increase recombinant protein production levels up to two-fold that of standard cells.

RNA INTERFERENCE MEDIATED SUPPRESSION OF TN-CASPASE-1 AS A
MEANS OF INVESTIGATING APOPTOSIS AND IMPROVING
RECOMBINANT PROTEIN PRODUCTION IN *TRICHOPLUSIA NI* CELLS

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Dedication

To my parents, for all the sacrifices they made and the love they gave.

Acknowledgements

There are so many people that have helped me along the way that I couldn't possibly name them all here, but I will do my best. Thanks to Dr. Bill Bentley, who gave me the opportunity to work in his lab with a great deal of intellectual freedom and support, and provided guidance when I needed it. Also to all the members of the Bentley lab, both past and present that I have encountered throughout my graduate career who taught me how to work in the lab and quickly became not only colleagues but friends. This includes other graduate students from the Center for Biosystems Research, Bioengineering, Chemical Engineering, and any other department I might be forgetting. Thanks also to the Bioengineering and CBR staff, including Julie Holbrook, Sandra Huskamp, Karen Lasher, and Brenda Brooks, who helped me with whatever I needed. To my friends on the cycling team, especially Eric Laing and Allan Wallace. Our shared time on the bike reminded me that there is a wonderful world outside if only you go looking for it. To my family, especially my brother, parents, and grandmother, for their support and companionship that reminds me what is truly important. And finally to my wife Julie, my best friend and most trusted companion, for being my partner on this beautiful journey called life. I am truly the luckiest.

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List of Abbreviations

Ac-DEVD-AMC - N-acetyl-Asp.Glu.Val.Asp-7-amino-4-methylcoumarin

AcMNPV - *Autographa californica* multiple nucleopolyhedrovirus

BEVS – Baculovirus expression vector system

bp – base pair

CAT – Chloramphenicol acetyl-transferase

cDNA – Complementary DNA

DNA – deoxyribonucleic acid

dsRNA – double stranded RNA

E. coli – Escherichia coli

GFP – Green Fluorescent Protein

His – Histidine

HRP – Horseradish peroxidase

kb – Kilobases

kDa – KiloDalton

MOI – Multiplicity of infection

mRNA – Messenger RNA

p35 – *AcMNPV* baculovirus protein p35

PBS – Phosphate buffered saline

PCR – polymerase chain reaction

RNA – ribonucleic acid

RNAi – RNA interference or Interfering RNA

RT-PCR – Reverse transcriptase polymerase chain reaction

SDS-PAGE – Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sf – *Spodoptera frugiperda*

Sf-9 – *Spodoptera frugiperda-9*

siRNA – short interfering RNA

T. ni or *Tn* – *Trichoplusia ni*

TUNEL - Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end
labeling

UV – Ultra violet

Chapter 1 : Introduction and Background

Project Summary

Since the introduction of Humulin in 1982, the market for recombinantly produced proteins and therapeutics has grown steadily and is predicted to reach over \$52 billion by the year 2010 (Pavlou and Reichert 2004). As demand increases, so too will the need to increase the efficiency of current production systems as well as develop new technologies for the production of recombinant proteins. Despite being an efficient and convenient method for the production of recombinant protein, one drawback associated with the baculovirus expression vector system (BEVS) is that host cells fight infection, through the production of proteases and the onset of apoptosis. Baculoviruses have evolved strategies to combat this, but protein production is still decreased by proteases and other host cell defenses, including apoptosis. One principal component of this apoptotic machinery is a group of proteases known as caspases, which respond to both external as well as internal apoptotic signals and carry out the proteolysis that leads to apoptotic cell death.

To combat this problem, we have identified a novel caspase present in cell lines derived from *Trichoplusia ni*, the cabbage looper, one of the main hosts for industrial protein production using the BEVS. Designated Tn-caspase-1, we have shown that it is responsible for the majority of effector caspase activity present in *T. ni* cells, and have demonstrated that its activity can be detected in the latter stages of baculovirus infection. Furthermore, through the suppression of Tn-caspase-1, apoptosis can be alleviated. Tn-caspase-1 was suppressed using interfering RNA

(RNAi), a decade old technology that has quickly become a powerful and widespread tool for the sequence specific knockdown of a gene of interest. Through the use of *in vitro* RNAi, Tn-caspase-1 transcript levels were decreased by 80%, resulting in recombinant protein levels more than 2 times that of untreated cells. Further, in order to take advantage of the simplicity and robustness of an *in vivo* approach, we have developed a construct for the expression of *in vivo* dsRNA and subsequently developed the first stable *T. ni.* derived cell line in which it is implemented. The stable cell line selected (designated dsTncasp-2) displays a 90% decrease in caspase enzymatic activity during chemically induced apoptosis. dsTncasp-2 also has increased resistance to nutrient starvation and high cell densities as is often seen during prolonged suspension culture and demonstrates protein production levels up to two-fold that of standard cells. Overall, this study supports the application of RNAi in metabolic engineering for the improvement of recombinant protein production and the development of cell lines with desirable production phenotypes.

Literature Review

Baculoviruses

Baculoviruses are a diverse family of rod-shaped, occluded double-stranded DNA viruses with a large, circular genome that ranges from 88 to 200 kilobase pairs (Ayres et al. 1994; King and Possee 1992). They are further divided into two genera: the Nucleopolyhedrovirus (NPVs) and the Granulovirus (GVs). In general, NPVs produce large occlusion bodies that contain multiple virions while GVVs produce smaller granular occlusion bodies containing only one virion (Willis et al. 2005). The most well characterized baculovirus is the *Autographa californica*

multiple nuclear polyhedrosis virus (AcMNPV), which is an NPV that exhibits a broad host range across several lepidopteron species, including the cabbage looper (*Trichoplusia ni*) and the fall armyworm (*Spodoptera frugiperda*) (Granados and Federici 1986; Willis et al. 2005).

The baculovirus replication cycle consists of two phases, which governs the transcription of viral genes. In the first phase, early and late genes are expressed, and infected cells produce many non-occluded or budded virus particles (BVs) into the medium. These BVs spread the infection within the insect and in insect cell culture. In the second stage of infection, the very late genes are expressed, and the virus particles are occluded by the polyhedrin protein to form polyhedra, which protect the virus particles from the environment and spread infection between insects. However, production of occluded virus particles is not required to spread infection in cell culture (Dalal 2001).

The two forms of AcMNPV virus particles are shown in Figure 2-1. The DNA is associated with p6.9, a highly basic, arginine-rich protein of 6.5 kilodaltons (kDa) and is packaged within a rod-shaped nucleocapsid composed of a 39-kDa capsid protein (vp39) and a 71-kDa protein (p80) (Miller 1997). The nucleocapsid is then packaged within the lipoprotein envelope to form the virion, which are occluded by the polyhedrin protein in the late stages of infection to form polyhedra ranging in size from 1-15 μm (King and Possee 1992).

Baculovirus Expression Vector System (BEVS)

In order to produce recombinant proteins using an insect cell/baculovirus system, the gene of interest is inserted in place of baculovirus genes not required for

replication. Usually, genes will be inserted under the control of either the polyhedrin or P10 promoter, which are transcribed during the very late time period of infection and are two of the strongest promoters known (King and Possee 1992). However, genes can also be inserted under earlier promoters, providing a convenient means of detecting infection (Dalal et al. 2005). Once the recombinant baculovirus particles have been constructed, they are introduced into the culture medium, subsequently infecting cells and producing the protein of interest. Genes placed under the control of the polyhedrin promoter can generally be detected at approximately 2 days post infection (dpi), while detection can be as early as 18 hours post infection (hpi) for genes under the control of early promoters. Cultures are usually harvested at 5-6 dpi, when the yield reaches a maximum (Caron et al. 1990). The insect cell/baculovirus expression system has been widely used as a means for producing recombinant protein, due to the high yield and correct functionality of the expressed protein (Luckow and Summers 1988; Smith et al. 1985). Yields approaching 50% of total cellular protein can be attained in infected cells (Wickham et al. 1992), and functionalities including secretion signal recognition (Davis et al. 1992a), assembly of oligomeric proteins (Kakker et al. 1999), glycosylation (James et al. 1995), phosphorylation (Hericourt et al. 2000), and disulfide bond formation (Hodder et al. 1996) have also been reported.

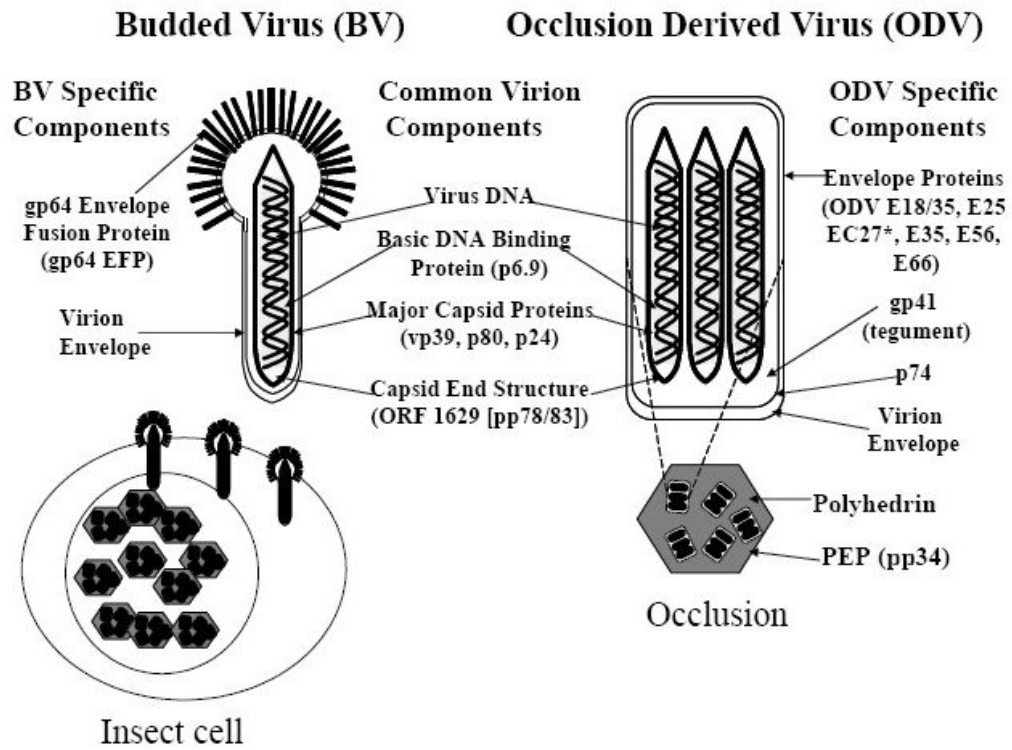


Figure 1-1. Two common forms of AcMNPV

The budded and occlusion derived virus forms of AcMNPV are represented. The genes that are responsible of each form and those that are common to both forms of the virus are indicated (adapted from (Miller 1997)).

In addition to a wide array of promoters and high yield the baculovirus system can also accept large amounts of foreign DNA, a task which can be difficult using bacterial or yeast systems (Shuler and Kargi 1997). Scale up is also relatively easy, though oxygen deprivation can still be a problem at high culture densities (Ikonomou et al. 2003). One way to circumvent this problem is to use the baculovirus in insect cell larvae to produce recombinant protein. Larvae can be fed an inexpensive nutrient source and scale linearly, but unfamiliarity with larval systems and easy access to cell culture facilities have made this practice relatively rare (Kost et al. 2005).

Baculovirus vectors can also be used to introduce DNA into mammalian cells.

Although the exact mechanism of baculovirus entry into mammalian cells has not yet been determined, these systems open up a host of new possibilities for the baculovirus system (Kost et al. 2005).

The baculovirus system has disadvantages as well. First of all, insect cell cultures infected with baculovirus are discontinuous, since the cells eventually die after infection. Also, despite their posttranslational ability, glycosylation in insect cells is not the same as it is in mammalian cells, making the production of proteins that require complex glycosylation less effective. However, a great deal of research is currently focused on engineering mammalian glycosylation pathways into the insect cell/baculovirus system (Betenbaugh et al. 2004; Jarvis 2003; Jarvis et al. 1998; Tomiya et al. 2003; Tomiya et al. 2004). Other disadvantages of the baculovirus system include decreased production of secreted or membrane proteins and product degradation due to proteases, both of which decrease product yield (Kost et al. 2005).

Improving Yield in the BEVS

In order to combat low protein yield in the baculovirus system due to improper folding, minimal secretion, or degradation by proteases, several strategies have been developed. One technique involves coexpression of chaperone proteins by the baculovirus to aid in the folding of recombinant proteins (Ailor and Betenbaugh 1999). A number of studies have documented an increase in functional protein as the result of coexpressing chaperones such as calnexin (Higgins et al. 2003), calreticulin (Fourneau et al. 2004; Zhang et al. 2003), or heat shock proteins (hsps) (Martinez-Torrecuadrada et al. 2005; Yokoyama et al. 2000). Examples in both Sf9 (Lenhard and Reilander 1997) and *T. ni* (Ailor and Betenbaugh 1998; Hsu and Betenbaugh 1997) cell culture have been reported. Another technique involves the addition of certain DNA enhancer elements to the baculovirus. Homology regions derived from baculovirus inserted upstream of the promoter for the gene of interest have been shown to increase luciferase production (Chen et al. 2004; Venkaiah et al. 2004). In addition, enhancer sequences can be constructed from heterologous elements. Incorporation of a 21 base pair element derived from a 5' untranslated leader sequence of lobster tropomyosin cDNA and an A-rich sequence found in the polyhedrin leader sequence enhanced the expression of tropomyosin and luciferase twenty and sevenfold, respectively (Sano et al. 2002). Protein degradation can also be combated by the addition of protease inhibitors to the media (Kato et al. 2003) or through the optimization of harvest time and conditions to minimize protease activity (Pham et al. 1999). Modification of the baculovirus itself can also be used to improve the yield of properly folded protein. Construction of a chitinase and v-

cathepsin (both of which help liquefy the host during the late stages of infection) negative bacmid improved the integrity of both intracellular and secreted recombinant protein (Kaba et al. 2004). Finally, a non-lytic baculovirus was constructed using a novel fluorescence resonance energy transfer (FRET) based assay, decreasing the percentage of lysed cells from 60% to 7% in Sf21 cells at 5 days post infection (Ho et al. 2004). The authors demonstrated this led to an increased amount of compactly folded luciferase with less degradation.

Apoptosis and Caspases

Apoptosis, or programmed cell death, was first described by Kerr et al. (1972) and has since been recognized as an important component of several biological processes including embryonic development, tissue homeostasis, tumorigenesis, and viral infection (Walker et al. 1988). Improper regulation of apoptosis has been associated with several diseases, including cancer, autoimmune disorders, and neurodegenerative diseases (Thompson 1995). Apoptosis can occur in a variety of cell types and can be triggered by either external or internal signals such as viral infection, UV light, or chemicals such as actinomycin D. During apoptosis, cells undergo a number of distinct morphological changes, including the blebbing of the plasma membrane, cellular shrinkage, and the formation of apoptotic bodies which are generally engulfed by neighboring cells (Kerr et al. 1972). Thus, the harmful contents of the cells are not released into the extracellular space but are instead absorbed by neighboring cells, preventing harmful inflammatory responses (Chang and Yang 2000). Apoptosis is morphologically distinct from necrosis, or passive cell death, which is generally typified by cell swelling and lysis, which results in the

release of cellular contents (Kim and Park 2003). Other characteristic biological changes associated with apoptosis include DNA fragmentation or laddering due to cleavage between nucleosomes, which can be visualized using conventional gel electrophoresis and the flipping of the phosphatidylserine from the inner side of the plasma membrane to the outside (Chang and Yang 2000).

The distinct nature of the apoptotic phenotype is in part due to its highly conserved nature as well as its tightly controlled machinery. The primary component in this machinery is a group of proteolytic enzymes known as caspases. First discovered in Cos-7 cells as the interleukin-1 beta converting enzyme (ICE) (Cerretti et al. 1992) and first linked to apoptosis with the cloning of the proapoptotic *ced-3* in *Caenorhabditis elegans* (Yuan et al. 1993), the term caspase means cysteine specific aspartase or aspartic protease. Thus, caspases use a cysteine group as the nucleophilic group to cleave the C-terminal peptide of an aspartic acid residue (Alnemri et al. 1996). Additional evidence for the involvement of caspases in the apoptotic process is provided by studies showing that certain synthetic or natural caspase inhibitors, such as the cowpox viral serpin CrmA (Tewari et al. 1995) and baculovirus protein p35 (Clem et al. 1991) can greatly reduce apoptosis induced by diverse stimuli. Further, animals lacking caspases show defects in apoptosis, and caspases are responsible for most of the proteolytic cleavage that takes place during apoptosis (Chang and Yang 2000).

Caspases are translated as latent pro-enzymes that require activation before they become active players in the apoptotic process. As a result, caspases generally act in cascades. Initiator caspases, such as human caspase-2, -8, and -10, have large

prodomains (Figure 1-2) and are generally autocatalytic or respond to an apoptotic stimuli. This signal can come from a cell surface receptor, such as the well studied CD95/APO-1/Fas receptor (Schmitt et al. 1999), or as through the release of a mitochondrial apoptotic factor such as apoptosis inducing factor (AIF) or cytochrome c (Susin et al. 1998). Effector caspases, such as human caspase-3 or -7, have relatively small prodomains and are directly activated by initiator caspases, resulting in the cleavage of cellular proteins and apoptotic cell death. Inflammatory caspases are a third type of caspase and are involved in cytokine activation.

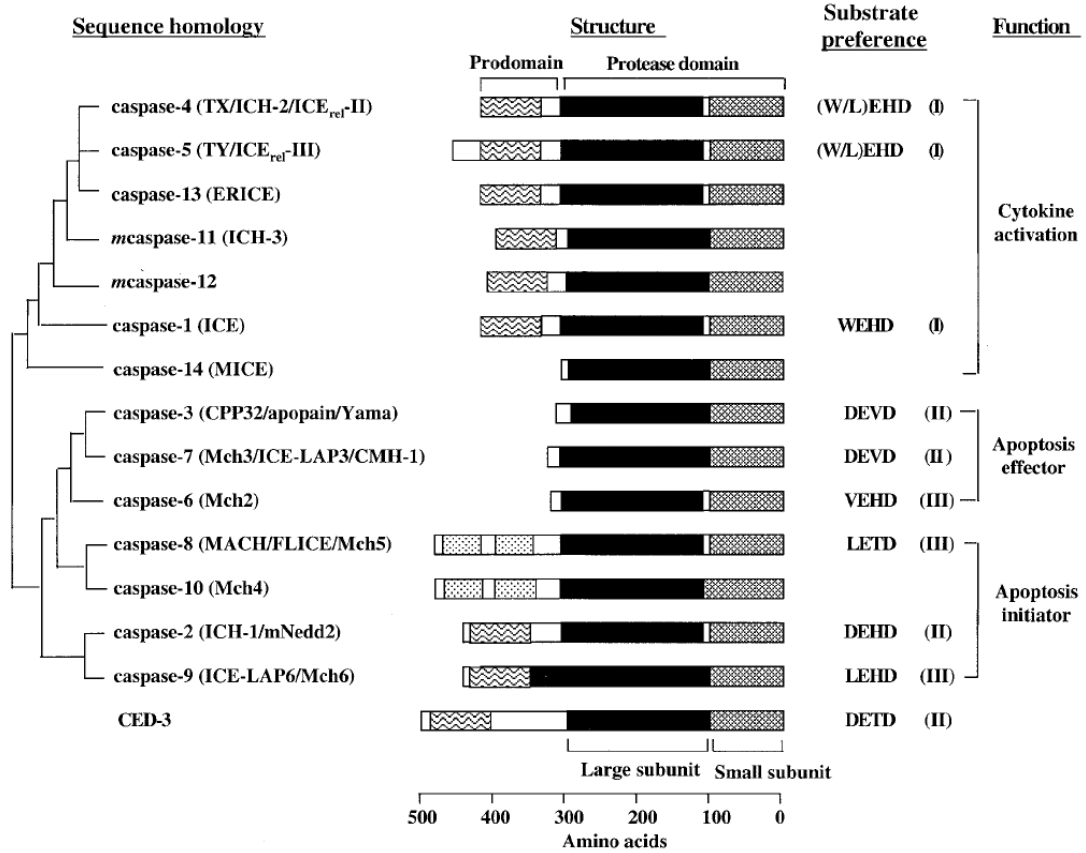


Figure 1-2. Mammalian caspases and *C. elegans* caspase CED-3.

All mammalian caspases are from human, except mcaspase-11 and mcaspase-12, for which human counterparts have not yet been discovered. Phylogeny is based on similarity between the protease domains. Adapted from (Chang and Yang 2000).

RNA Interference

RNA based gene silencing was first observed in plants and fungi, and was described as post-transcriptional gene silencing (PTGS) or quelling, respectively (Corgoni et al. 1996; Jorgensen 2003). In both cases, attempts to overexpress an endogenous gene by introducing transgenic copies backfired and blocked expression of the gene. The first case reported in animals came when Guo and Kemphues (Guo and Kemphues 1995) blocked the mRNA expression of the *par-1* gene using antisense RNA, but noticed that *par-1* mRNA itself repressed *par-1*. This seemingly contradictory observation, subsequently called RNAi (RNA mediated interference) by Rocheleau (Rocheleau et al. 1997), laid the foundation for later experiments that showed double-stranded RNA (dsRNA) was the trigger of gene silencing (Fire et al. 1998; Montgomery and Fire 1998).

Double-stranded RNA causes sequence specific degradation of mRNA to which it is homologous, resulting in decreased gene expression, while unrelated mRNA transcripts are not affected (Fire et al. 1998; Montgomery and Fire 1998; Sharp 1999; Tuschl et al. 1999). The RNA silencing pathway is triggered by small, 21-27 nucleotide long “small RNAs,” a term which can include small interfering RNAs (siRNAs), repeat-associated small interfering RNAs (rasiRNAs), and micro RNAs (miRNAs) (Tomari and Zamore 2005). siRNAs are produced *in vivo* by the ribonuclease Dicer, which cleaves long dsRNAs into siRNAs (Figure 1-3). Cellular miRNAs are processed by the endonuclease Drosha in the nucleus before being exported to the cytoplasm, where they are cut by Dicer to produce mature miRNA (Tomari and Zamore 2005). Although their processing is different, their functions are

the same. Once transcribed or introduced into the cell, these small RNAs affect gene expression through a common set of proteins known as RNA silencing effector complexes. These complexes, which include the RNA-induced silencing complex (RISC) (Hammond et al. 2000) or the RNA-induced initiation of transcriptional gene silencing (RITS) complex (Verdel et al. 2004), bind the small RNAs and direct gene silencing through a cascade of reactions. Once a complementary siRNA duplex has loaded onto RISC, Argonaute 2 (AGO2) cleaves the sense (passenger) strand, leaving the antisense (guide) strand still incorporated into the now active RISC complex. This complex can then recognize and cleave complementary mRNA through the catalytic domain of AGO2 (Matranga et al. 2005; Rand et al. 2005). In the case of miRNA, perfectly complementary RNA duplexes are processed in a manner similar to siRNA. Sequences that have imperfect homology use a bypass mechanism that unwinds and discards the sense strand, resulting in an active RISC complex that then directs translational repression and mRNA degradation (Bagga et al. 2005; Pillai et al. 2005). For a more rigorous discussion of the mechanisms of RNA gene silencing, see (Kim and Rossi 2007; Tomari and Zamore 2005).

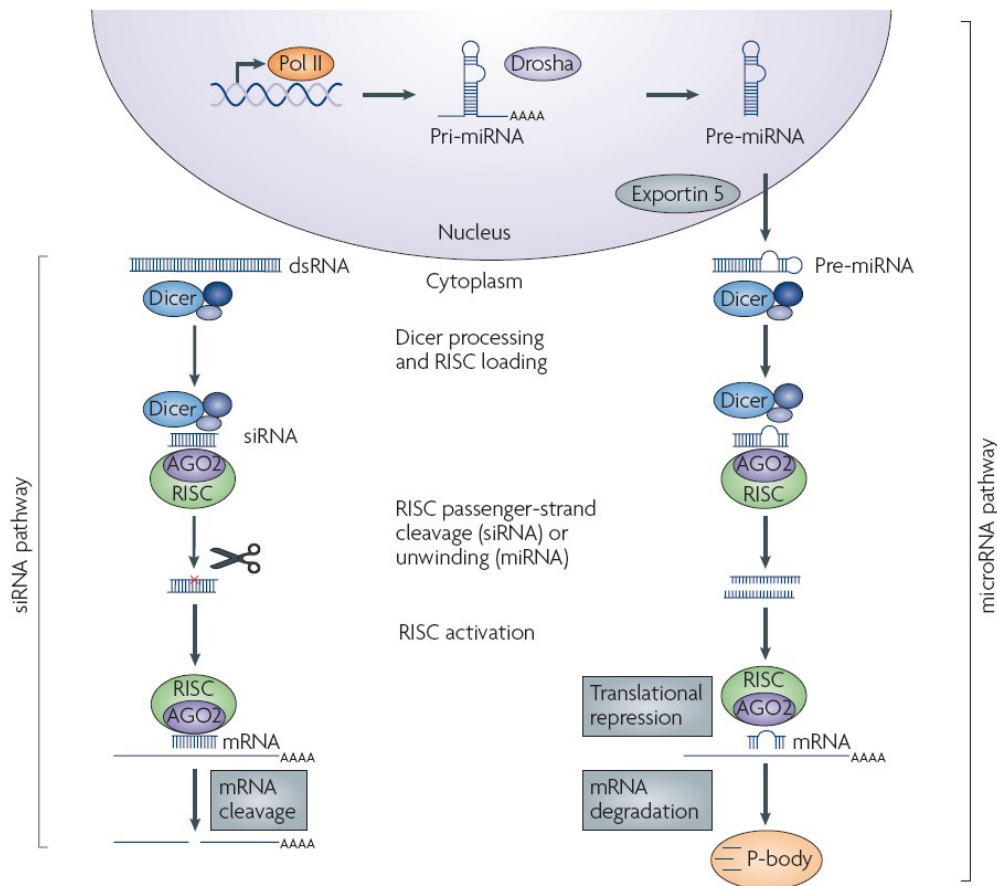


Figure 1-3. Mechanism of RNAi in mammalian cells

The RNA interference pathway is directed by small RNAs such as small interfering RNAs (siRNAs) and microRNAs (miRNAs). In the siRNA pathway, long double stranded RNA (dsRNA) is cleaved into siRNAs by the enzyme Dicer. These siRNA duplexes then associate with Argonaute 2 (AGO2) and the RNA-induced silencing complex (RISC). When these duplexes have complementary sequences, AGO2 cleaves the sense strand, leaving the antisense strand still associated with the now active RISC complex. The active RISC complex then binds and cleaves (through the catalytic domain of AGO2) complementary mRNA. miRNAs are first processed in the nucleus by Drosha before exportation to the cytoplasm. While perfectly complementary miRNAs are processed similarly to siRNAs, imperfectly complementary strands are processed differently. Instead of cleavage as with the siRNA duplex, the miRNA duplex is unwound and the sense strand is discarded, leaving the antisense complex that now functions to either repress or degrade the complementary mRNA target. Figure from (de Fougères et al. 2007).

Applications of RNAi

Since its initial discovery in 1998 (Fire et al. 1998), RNA interference (RNAi) has quickly moved from a tool for single gene loss-of-function studies to a means of implementing genetic changes in a specific manner over a wide range of organisms and cell types. In addition to unraveling the mechanisms, origins, and purpose of RNAi (Scherer and Rossi 2003; Tomari and Zamore 2005), research has focused on improving and creating new delivery methods for both cells and animals (Dann 2007; Kumar and Clarke 2007; Mittal 2004), as well as genome wide RNAi screens for functional analysis in both mammalian (Aza-Blanc et al. 2003; Cullen and Arndt 2005; Silva et al. 2004; Sturzl et al. 2008) and drosophila cells (Armknecht et al. 2005; Boutros et al. 2004). A recent review examines many of the issues involved in genome wide RNAi screens, including cell line selection, screening techniques, and infrastructure requirements (Echeverri 2006). While these screens are a relatively new technique for elucidating gene function and interaction, they are rapidly becoming powerful and widespread.

Since the discovery that RNAi was mediated by short-interfering RNA (siRNA) in mammalian cells (Elbashir et al. 2001) and the subsequent demonstration of its efficacy in an animal disease model (Song et al. 2003), RNAi has also been touted as the next great therapeutic tool for combating a wide range of diseases, including cancer (Pai et al. 2006; Pento 2007), neurodegenerative disease (Raoul et al. 2006), viral infection (Leonard and Schaffer 2006), and ocular disorders (Campochiaro 2006). Great potential exists, but difficulties remain, including effectively delivering the RNAi of interest into the target cell and precisely

quantifying specific and non-specific side effects. These issues have been addressed in greater detail in numerous recent reviews on RNAi as a therapeutic tool (Aigner 2006; Bumcrot et al. 2006; Corey 2007; de Fougères et al. 2007; Dorsett and Tuschl 2004; Dykxhoorn and Lieberman 2005; Dykxhoorn et al. 2006; Gewirtz 2007; Grimm and Kay 2007; Novobrantseva et al. 2008; Shankar et al. 2005). Additional applications include antisense applications in bacteria, RNAi in plant biotechnology, and RNAi in metabolic engineering (Figure 1-4) (Hebert et al. 2008a).

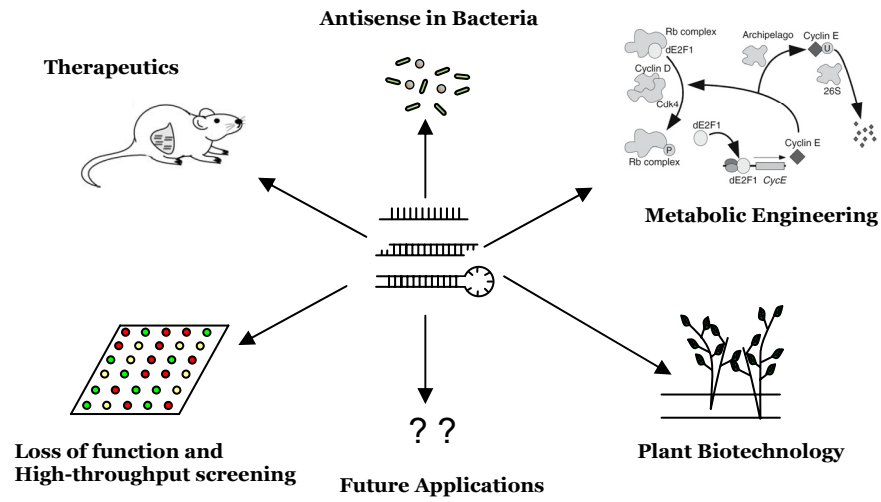


Figure 1-4. RNA interference as a means of altering phenotype.

RNA constructs are used in research (screening and loss of function), therapeutics (Dykxhoorn and Lieberman 2005), metabolic engineering (March and Bentley 2006), plant biotechnology, and, in single-stranded form, bacteria.

RNAi and Metabolic Engineering

Metabolic engineering typically involves the rational alteration of a host cell's genotype or regulatory network in order to achieve a desired phenotype (Bailey 1991; Stephanopoulos and Vallino 1991). In addition to efficient production of the molecule of interest, desirable phenotypes include the ability to survive well in the adverse conditions normally experienced by cells overproducing protein, such as decreased oxygen and nutrients or increased cellular and oxidative stress (diStefano et al. 1995; Rao and Bredesen 2004; Scott et al. 1992). In general, this involves altering the central metabolism, seeking to increase the flux through specific pathways while decreasing others. Traditionally, this has been accomplished either through the overexpression of specific targets directly involved in the pathway (Chen et al. 2001; Irani et al. 1999) or alteration of the overall protein machinery, such as the expression of chaperone proteins (Borth et al. 2005; Davis et al. 2000; Higgins et al. 2003; Kitchin and Flickinger 1995; Martinez-Torrecuadrada et al. 2005; Yokoyama et al. 2000) or transcriptional factors involved in the secretory pathway (Tigges and Fussenegger 2006). A similar approach can be taken using RNAi and other antisense technologies; instead of overexpressing desirable genes, potentially deleterious genes are silenced.

RNAi initially found limited use in metabolic engineering, however, perhaps due to its transient nature, relatively inefficient delivery methods, and the more typical metabolic engineering objective functions that are based on permanent changes in genotype. As RNAi technology improved, several noteworthy examples

based on antisense RNA have appeared, targeting apoptosis, the cell growth cycle, and glycosylation.

Since apoptosis (programmed cell death) can account for up to 80% of cell death in bioreactors and lead to decreased product yield and quality (Goswami et al. 1999; Mastrangelo and Betenbaugh 1998; Mercille and Massie 1994), anti-apoptosis engineering has become an important area of metabolic engineering. This strategy has also been implemented using RNAi. Examples include RNAi against proteases in CHO (Lai et al. 2004; Wong et al. 2006b) and Sf9 cells (Lin et al. 2007) and produced by recombinant baculovirus (Kim et al. 2007). In each of these examples, the yield of recombinant protein was enhanced as a result of the downregulation of a target protease.

A more global approach involves altering the cell growth cycle using RNAi. An optimal production cycle is generally biphasic. The cells first grow quickly to a high density before transitioning to a production phase characterized by low growth and high productivity (Fussenegger 2001; Gammell et al. 2007). Achieving this regime is relatively straightforward in bacterial systems, but can be more problematic in eukaryotic cell culture since their state is influenced by the cell cycle. Studies have investigated the relationship between the cell cycle and heterologous protein expression and some attempts have been made to optimize product synthesis by using a cycle-specific promoter (Banik et al. 1997; Banik et al. 1996), or controlling the cell cycle itself (Ibarra et al. 2003; Kim et al. 2000; Simpson et al. 1999; Watanabe et al. 2002). RNAi has again proven a viable alternative, as it has been used to increase cell growth and protein production in *Drosophila* S2 cells through the downregulation

of cell growth controllers (March and Bentley 2006; March and Bentley 2007) and in HEK 293 cell through the downregulation of transcriptional regulators (Hacker et al. 2004).

A final area of metabolic engineering in which RNAi has played a role is glycosylation. Engineering specific glycosylation patterns can increase the potency and stability of therapeutic proteins while at the same time decreasing their immunogenic effects (Butler 2006; Jenkins 2007; Kuystermans et al. 2007), and has been the subject of study in several systems including mammalian (Butler 2006), insect (Tomoya et al. 2004), and yeast (Wildt and Gerngross 2005). Several specific examples of using RNAi to fine tune glycosylation patterns include increasing the antibody-dependent cellular cytotoxicity of antibodies produced in CHO cells through the reduction of a 1,6 fucosyltransferase (FUT8) (Mori et al. 2004), GDP mannanose 4,6 dehydrogenase (GMP) (Kanda et al. 2007), or both FUT8 and GMP (Imai-Nishiya et al. 2007), as well as improving product quality by reducing sialidase activity in CHO cells (Ngantung et al. 2006).

As the amount of genomic and metabolomic data increases, additional RNAi-based metabolic engineering targets will surely be identified. Some such screens are already underway (Gammell et al. 2007; Lee et al. 2007; Wong et al. 2006a), and as more targets are identified, RNAi will continue to expand its influence in the field of metabolic engineering.

RNAi and Baculovirus

Several instances of using RNAi in conjunction with baculovirus have been reported. One of the first studies used antisense RNA made by the baculovirus under the control of the p10 promoter to decrease juvenile hormone esterase in the tobacco budworm *Heliothis virescens* and affect its development (Hajos et al. 1999). Several more recent studies have used dsRNA against essential viral genes made either in vitro (Valdes et al. 2003) or in vivo (Isobe et al. 2004) to confer baculovirus resistance to both cells and larvae, or to study the mechanics of baculovirus infection (Flores-Jasso et al. 2004; Means et al. 2003). RNAi against genes encoded by the baculovirus has proven effective (Agrawal et al. 2004; Kramer and Bentley 2003), but the silencing of host genes by RNAi during baculovirus has not been examined thoroughly. Some preliminary work has shown that RNAi can be used to increase protein production in the baculovirus system (Kim et al. 2007; Kramer 2002), laying the groundwork for further improvements.

Project Objectives

Previous studies in by both the Bentley lab and others have demonstrated the efficacy of RNAi as a means of improving recombinant protein production. The objectives of this project focus around the application of RNAi to improve protein production in the baculovirus expression system and are given below:

Objective 1: Identify and characterize novel gene targets, such as cell death genes and proteases, which could be responsible for decreasing yield in the baculovirus expression system.

Objective 2: Evaluate potential targets using *in vitro* synthesized dsRNA. Both the level of suppression and the effect on recombinant protein production will be measured.

Objective 3: Select the most promising targets for implementation with *in vivo* dsRNA by developing a cell line that expresses a dsRNA hairpin construct. Evaluate cell lines for their ability to survive well in culture and produce recombinant protein.

Chapter 2 : Characterization and Analysis of *Trichoplusia ni* Caspase-1 Through Overexpression and RNAi Mediated Silencing

Abstract

In both mammals and invertebrates, caspases play a critical role in apoptosis. Although Lepidopteron caspases have been widely studied in *Spodoptera frugiperda* cells, this is not the case for *Trichoplusia ni* cells, despite their widespread use for the production of recombinant protein and differences in baculovirus infectivity between the two species. We have cloned, expressed, purified and characterized Tn-caspase-1 in several situations: in its overexpression, in silencing via RNA interference (RNAi), during baculovirus infection, and in interactions with baculovirus protein p35. Overexpression can transiently increase caspase activity in *T. ni* (High Five™) cells, while silencing results in a greater than 6-fold decrease. The reduction in caspase activity resulted in a reduction in the level of apoptosis, demonstrating the ability to affect apoptosis by modulating Tn-caspase-1. During baculovirus infection, caspase activity remains low until approximately 5 days post infection, at which point it increases dramatically, though not in those cells treated with dsRNA. Our results demonstrate that Tn-caspase-1 is the principal effector caspase present in High Five cells, and that it is inhibited by baculovirus protein p35. Finally, our results indicate differences between RNAi and p35 as effector molecules for modulating caspase activity and apoptosis during cell growth and baculovirus infection.

Introduction

Although apoptosis was first described over thirty years ago (Kerr et al. 1972), it remains an active area of research in many fields as a result of its role in development, homeostasis, and host defense (Steller 1995; White 1996), and because a multitude of questions remain to be answered. Deficiency in apoptosis has been linked to human diseases including cancer, certain autoimmune disorders, and neurodegenerative diseases (Peter et al. 1997; Thompson 1995). Well conserved throughout higher organisms, a central component of the machinery of apoptosis is a family of aspartate-specific cysteine proteases known as caspases (Chang and Yang 2000; Earnshaw et al. 1999; Salvesen and Dixit 1997).

First described in mammalian systems (Cerretti et al. 1992; Thornberry et al. 1992) and shortly thereafter in *Caenorhabditis elegans* (Lazebnik et al. 1994; Yuan et al. 1993), caspases are generally divided into three groups. Inflammatory caspases, which are involved in cytokine activation, comprise the first group and include human caspases-1, -4, -5, -11, -12, -13, and -14 (Chang and Yang 2000). The second group, initiator caspases, includes caspase-2, -8 and -9, and the third group, effector caspases, includes caspase-3 and -7 (Chang and Yang 2000). Although the exact mechanism is not fully understood, initiator caspases respond to a variety of signals that result in the cleavage of their long N-terminal prodomain, which in turn results in the cleavage and subsequent activation of effector caspases, which have relatively short prodomains. These activated effector caspases cleave cellular proteins, resulting in apoptotic cell death (Chang and Yang 2000; Earnshaw et al. 1999).

In invertebrates, caspases also play an important role in apoptosis (Bergmann et al. 1998; Clem 2005; Clem et al. 1996). *Drosophila melanogaster* has been the most studied insect system of apoptosis, with seven caspases identified based on sequence similarity and biochemical activity (Vernooy et al. 2000). *Drosophila* Dronc, Dcp-2/Dredd, and Strica have long prodomains, similar to initiator caspases, while Drice, Dcp-1, Damm and Decay have short prodomains, similar to effector caspases (Muro et al. 2004). Another well studied insect system includes the Sf9 and Sf21 cell lines, both of which are derived from the lepidopteron species *Spodoptera frugiperda*. These lines are particularly suited for studies in apoptosis due to their susceptibility to a wide array of apoptotic stimuli, including UV radiation, baculovirus infection, chemical agents such as actinomycin D, and overexpression of *Drosophila* death genes such as *reaper* or *hid* (Clem et al. 1991; Manji and Friesen 2001; Vucic et al. 1997b).

Sf-caspase-1 is the principal effector caspase in Sf cell lines (Ahmad et al. 1997; LaCount et al. 2000). Its activation is believed to follow a two-step process, in which an unidentified initiator caspase (*Sf*-caspase X) cleaves pro-*Sf*-caspase-1 D¹⁹⁵, producing the small subunit p12, and an intermediate fragment, p25. The second step cleaves the small prodomain from the p25 fragment at D²⁸, resulting in the formation of P6 and the large subunit p19 (Manji and Friesen 2001). A recent study has identified an alternative activation pathway, in which pro-*Sf*-caspase-1 is first cleaved at D²⁸ and then D¹⁹⁵, resulting in active *Sf*-caspase-1 (Liu and Chejanovsky 2006). As a result of evidence suggesting that apoptosis is used by the cells as an antiviral response (Clem 2005), antiapoptotic proteins from several baculoviruses have been

characterized with respect to Sf-caspase-1 activation. Inhibitors of apoptosis (IAPs), including Op-IAP from the baculovirus *Orgyia pseudotsugata* nucleopolyhedrovirus (OpNPV), block apoptosis upstream of Sf-caspase-1, perhaps by preventing the activation of Sf-caspase-X or other pro-apoptotic proteins (Birnbaum et al. 1994; Vucic et al. 1997a; Zoog et al. 2002). P35, a caspase inhibitor from the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), blocks the cleavage of the prodomain (Ahmad et al. 1997; Bertin et al. 1996; LaCount et al. 2000; Manji et al. 1997), while P49 from *Spodoptera littoralis* nucleopolyhedrovirus (SINPV) blocks cleavage into the large and small subunits (Zoog et al. 2002). Because of their ability to inhibit apoptosis, IAP and p35 containing vectors have been successfully employed in recombinant protein expression systems to extend production times and increase product yield (Jabbour and Hawkins 2004; Lin et al. 2001; Sauerwald et al. 2003; Vives et al. 2003).

While Sf-derived host cells are widely used in the Baculovirus Expression Vector System (BEVS) for the production of recombinant protein (Cha et al. 1999c; Cruz et al. 1999); *Trichoplusia ni* cells (Ailor et al. 2000; Davis and Wood 1995; Jarvis 2003) and larvae (Cha et al. 1999a; Cha et al. 1997; Kramer and Bentley 2003; Pham et al. 1999) have been reported to have superior productivity (Davis et al. 1992b; Wickham et al. 1992; Wickham and Nemerow 1993) and yet no reports have appeared on caspases in *T. ni*. Indeed, only two reports have appeared concerning caspases in lepidopteron cell lines other than Sf (Liu et al. 2005; Pei et al. 2002). Additionally, differences in infectivity seen between different species of lepidopteron larvae with respect to p35 mutant baculoviruses (Clem 2005; Clem and Miller 1993;

Haas-Stapleton et al. 2003; Hershberger et al. 1992) and the effects of protease activity on recombinant protein yield (Cruz et al. 1999; Naggie and Bentley 1998; Pham et al. 1999) on have stimulated our interest in *T. ni* caspases.

In this study, we have identified, cloned, expressed and purified Tn-caspase-1 based upon the existing Genbank sequence for Tn-caspase (**AAO17788**). Subsequent experimental objectives included 1.) Modulation of the caspase activity in *T. ni* cells through overexpression and knockdown of Tn-caspase-1 via double-stranded RNA (dsRNA), 2.) Determination of caspase activity during baculovirus infection, 3.) Evaluation of baculovirus protein p35 as an inhibitor of caspase activity, and 4.) Characterization of apoptosis with respect to caspase activity. Our results demonstrate that overexpression of Tn-caspase-1 can transiently increase caspase activity, while silencing of Tn-caspase-1 results in a greater than 6-fold decrease in caspase activity, which also translates into a reduction in overall apoptosis. dsRNA can also reduce the high levels of caspase activity seen during the latter stages of baculovirus infection. Finally, heterologously expressed baculovirus protein p35 can also inhibit caspase activity and apoptosis, although not as effectively as RNAi under the conditions studied.

Materials and Methods

Cell culture, transfection, and infection

Trichoplusia ni BTI-TN-5B1-4 (High Five™, Invitrogen) cells were cultivated in EX-CELL™-405 media (SAFC Biosciences) at 27°C. Unless otherwise noted, cells were initially plated on 24 well plates at a density of 3×10^5 cells/mL and transfected the following day using Cellfectin® (Invitrogen) according to the manufacturer's procedure. Briefly, culture media was aspirated from the wells and the transfection mixture was added for a 4 hour incubation period. Subsequently, the transfection mixture was removed; cells were washed with media, and then returned to the incubator in fresh media. In the case of infection with baculovirus, cells were infected 24 hours post transfection as previously described with a Multiplicity of Infection (MOI) of 2 (Eun Jeong Kim 2007).

Baculovirus

A recombinant *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) that expresses GFPuv under the control of the polyhedron promoter was created previously (Cha et al. 1999a; Cha et al. 1999b; Cha et al. 1999c). The baculovirus was propagated in High Five™ cells (Invitrogen), and *Spodoptera frugiperda* (Sf-9) cells (Invitrogen) following the general protocols outlined in O'Reilly et al. (O'Reilly et al. 1992). The titer of baculovirus was determined by the endpoint dilution method (O'Reilly et al. 1992).

RNA Isolation, cDNA cloning, and In vitro dsRNA synthesis

Total RNA was extracted using RNAqueous (Ambion) according to the manufacturer's instructions. Cells were sloughed from the well plate, spun at 500 g for 5

minutes, and lysed using the provided lysis/binding buffer. Following the RNA isolation, samples were subjected to a DNase digest for 30 minutes per the manufacturer's instructions in order to remove any contaminating DNA. First strand templates of each target gene were synthesized from 500 ng of total mRNA using oligo-dT primers and Superscript RT III (Invitrogen). 700-900 base-pair regions of first strand DNA templates were PCR amplified by AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen) using gene-specific primers for *T. ni*-caspase (5'-CGA TCA AAA TGC TGG ACG GTG-3'; 5'-AAG TCT GCA TGC ACA GGA AT 3'). T7 sequences (TAA TAC GAC TCA CTA TAG GGA) were added in a subsequent PCR step, resulting in T7 templates. To make the dsRNA, sense and anti-sense RNA was transcribed from T7 templates using the Megascript™ kit (Ambion) following the manufacturer's instructions. The single-stranded RNA (ssRNA) synthesized was extracted using phenol/chloroform, resuspended in nuclease free water, and then incubated at 65° C for 10 min before cooling to room temperature, annealing the two strands to form dsRNA. dsRNA was checked for size and integrity using agarose gel electrophoresis and then diluted to a concentration of 3 µg/µL. dsRNA was transfected into cells at a concentration of 15ug/mL.

Differential display

For determining relative transcript levels, RNA was extracted from High Five cells using an RNAqueous kit (Ambion) as described above. RNA concentration was determined by measuring the absorbance of a diluted sample at the 260 nanometer wavelength in a UV spectrophotometer (Beckman). 500 ng of total RNA was subject to reverse transcription using oligodT primers to obtain the first-strand cDNA template. The cDNA template was subject to PCR with sequence-specific primers: *T. ni-caspsae* (5'-TTC ATT CGA TCC CTG GAT AGC-3'; 5' TAG TAT CCA GGC ACG GTG GAG 3'). PCR products were run on a 1% agarose gel stained with ethidium bromide to compare band intensities under fluorescent

light. Primers against a 300 bp section of actin (5'-GAT ATG GAG AAG ATC TGG CA 3'; 5'-GCG TAG CCC TCG TAG ATG-3') were used to PCR amplify a loading control from the reverse transcript. Photographs of agarose gels under UV light were taken with an AlphaImager[®] HP (Alpha Innotech) and analyzed for band intensity using AlphaEaseFC[™] (Alpha Innotech).

Plasmid Construction

pIB-TnCasp was constructed by PCR amplification of *T. ni* cDNA using the following gene specific primers for *tn-caspase-1* (5'-TAA GCC ACC ATG CTG GAC GGT GAA TCC CAG-3; 5' GCG CTT TTT ACC AAA TAC AAG TAG ACG 3'). The forward primer included a Kozak consensus sequence (Kozak 1987; Kozak 1990; Kozak 1991), and the reverse primer included the native stop codon. This PCR fragment was then TOPO[®] cloned into pIB/V5-His-TOPO[®] (Invitrogen). This plasmid uses the OpIE2 promoter (Theilmann and Stewart 1992) for constitutive expression of the gene of interest in insect cells. pIB-TnCasp-HIS was constructed using the same forward primer, but a reverse primer that omitted the native stop codon (5' GCG CTT TTT ACC AAA TAC AAG TAG ACG), allowing for the translation of the poly-histidine tag included in the pIB/V5-His-TOPO[®] backbone to aid in purification. pIB-p35 was constructed through the PCR amplification of baculovirus infected *T. ni* cDNA using the following gene specific primers for *Autographa californica multiple nucleopolyhedrovirus p35* (gi 9627742:116492-117391) (5'-TAA GCC ACC ATG TGT GTA ATT TTT CCG GTA -3'; 5' GGG GCT GCT TAT TTA ATT GTG TTT AAT AT 3'). This PCR fragment was then TOPO cloned into pIB/V5-His-TOPO[®] to produce pIB-p35. DNA sequencing was performed at the DNA core facility of the Center of Biosystems Research (University of Maryland Biotechnology Institute). All constructs made by PCR were sequenced to verify their integrity.

Protein Purification

In order to isolate Tn-caspase-1-HIS, crude cell lysates were purified using TALON[®] Metal Affinity Resin (ClonTech) per the manufacturer's instructions. Briefly, 250uL of lysate was bound to 60uL resin and washed several times with 1mL Equilibration/Wash buffer (50 mM Sodium Phosphate, 300 mM NaCl, pH 7.5). Purified protein was then eluted in two steps using 100 uL Elution buffer (50 mM Sodium Phosphate, 300 mM NaCl, 150 mM Imidazole, pH 7.0) in each step and stored for later analysis.

Immunoblot Analysis

Cell lysates or purified protein fractions were subjected to SDS polyacrylamide gel electrophoresis at 160V for ~1 hour using the BioRad Mini Protean 3 system and then transferred to a nitrocellulose membrane using a BioRad Trans-Blot semi-dry transfer cell. Tn-caspase-1 was detected using a 1:1000 dilution of α -SfCasp-1 (LaCount et al. 2000), kindly provided by P.D. Friesen (University of Wisconsin-Madison), and a 1:2000 dilution of goat anti-rabbit HRP (GE Healthcare), which was then developed using the SuperSignal[®] West Pico chemi-luminescence kit (Pierce) and visualized using a Bio-Rad Chemidoc XRS. Protein size was approximated using the Magic Mark[™] XP Western Protein Standard (Invitrogen).

Assay of Caspase activity

After various experimental treatments, including actinomycin D (see Results and Discussion), cells were collected and washed with PBS (pH 7.5). The cells were then suspended in a lysis buffer (from caspase 3 assay kit, BD Bioscience) and

incubated on ice for 30 min. Cell debris was removed by centrifugation and supernatants were stored for further use or assayed directly. The tetrapeptide N-acetyl-Asp.Glu.Val.Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was used as the substrate. Cell lysates were added to the reaction buffer (from caspase 3 assay kit, BD Bioscience) with Ac-DEVD-AMC and incubated for several hours at 37°C. The release of AMC was measured using a Spectramax M5 Plate Reader (Molecular Devices) (excitation $\lambda_{380\text{nm}}$, emission $\lambda_{440\text{nm}}$). Caspase activity is defined as the slope of the plot of Fluorescence vs. Incubation time.

TUNEL Staining

Apoptosis was visualized using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Sciences) according to the manufacturer's instructions. After various treatments, including actinomycin D, cells were washed with PBS prior to fixation with a 4% paraformaldehyde solution for 1 hour at room temperature. The cells were then washed once with PBS and permeabilized using a solution of 0.1% Triton-X in 0.1% Sodium Citrate for 5 minutes on ice. Subsequently, cells were washed twice with PBS before incubation with the TUNEL reaction mixture for 1 hour at 37 °C. Cells were again washed twice with PBS before visualization under a fluorescent microscope or analysis via flow cytometry. Fluorescent images were taken using a Zeiss Axiovert 40 CFL. Flow cytometry was conducted on a FACSCalibur (BD Bioscience, NJ, USA). Fluorescence intensity was determined for 10,000 events per sample at an excitation of 488nm and emission at 530nm.

DNA Laddering Assay

Cells were seeded in 24 well plates at a density of 300,000 cells/mL (150,000 cells/well). The following day, actinomycin D was added at various concentrations (see supplemental Figure 2a) for a period of 24 hours. Cells were then sloughed from well plates and spun at 500 x g for 5 minutes. Cell pellets were then resuspended in 400 uL lysis buffer (Ambion, RNAqueous kit) and extracted using an equal volume of phenol/chloroform. An equal volume of isopropanol was added to the extracted aqueous fraction which was then stored overnight at -80 °C. The following day, samples were spun for 10 minutes at 14,000 x g (4 °C) to pellet the DNA/RNA. The pellets were then washed twice with 100% ethanol before resuspension in 100 uL nuclease free water. The samples were then incubated with 1 uL RNase H (Invitrogen) for 45 minutes at 37 °C to digest any remaining RNA. Samples were quantified using UV spectrometry and 2 ug of each sample was run on a 1.5% agarose gel. Bands were visualized using ethidium bromide and UV light.

Viable cell count

Cells were seeded in 60 mm² plates at a concentration of 250,000 cells/mL and exposed to 2 ug/mL actinomycin D 24 hours post seeding. Counts were made using Trypan Blue exclusion at various timepoints (see supplemental Figure 2b).

Labeling using Vybrant[®] Apoptosis Assay Kit #4

Cells were seeded in 24 well plates at a density of 300,000 cells/mL (150,000 cells/well). The following day, actinomycin D was added at a concentration of 2 ug/mL for a period of 24 hours. Cells were then labeled using the Vybrant Apoptosis

Assay Kit #4 (Invitrogen, CAT #V13243) according to the manufacturer's instructions. The labeling solution was then prepared using 1 uL of each labeling component (YO-PRO[®]-1 and Propidium Iodide) per 1 mL of PBS buffer (pH 7.4). Following incubation with actinomycin D, media was aspirated from each well and cells were washed once with 500 uL PBS buffer before addition of 500 uL labeling solution to each well. Cells were incubated with the labeling solution on ice for 30 minutes before visualization with fluorescence microscopy.

Results

cDNA cloning and sequence analysis

In order to isolate *tn-caspase-1*, total RNA was extracted from High Five cells and a cDNA library was constructed using RT-PCR. Using the existing Genbank sequence for *T. ni* caspase ([AAO17788](#)) as a guide, gene specific primers that omitted the native stop codon were used to amplify *tn-caspase-1*, which was subsequently cloned into pIB/V5-His-TOPO[®] (Invitrogen) to produce pIB-TnCasp-HIS. Upon the sequencing of positive transformants, it was apparent that discrepancies existed between the Genbank (gb) sequence and all the clones sequenced for this study. A reduced ClustalW sequence alignment of the protein sequence from this study, subsequently denoted Tn-caspase-1, (done so because it is the first caspase described in *T. ni* and not because it is necessarily the *T. ni* analog to Sf caspase-1), gb|[AAO17788](#), and closely related caspases is shown in Figure 2-1. Black boxes indicate identical amino acids, gray boxes indicate related amino acids, white boxes indicate a difference in amino acids, and dashes indicate a blank position. Tn-caspase-1 is most closely related to the three Lepidopteron caspases, Sl-caspase 1 (gb [AAO16241](#), 88% sequence identity), Sf-caspase 1 (gb [AAC47442](#), 85%) and Bm-Caspase (gb [NP_001037050](#), 79%). Tn-caspase-1 also shares similarity with Caspase-7 from both mouse (gb [NP_031637](#), 41%) and human (gb [AAH15799](#), 41%).

The cleavage domains of Sf-caspase 1 (LaCount et al. 2000) are indicated by boxes above the sequence alignment, with the line in the middle of the box

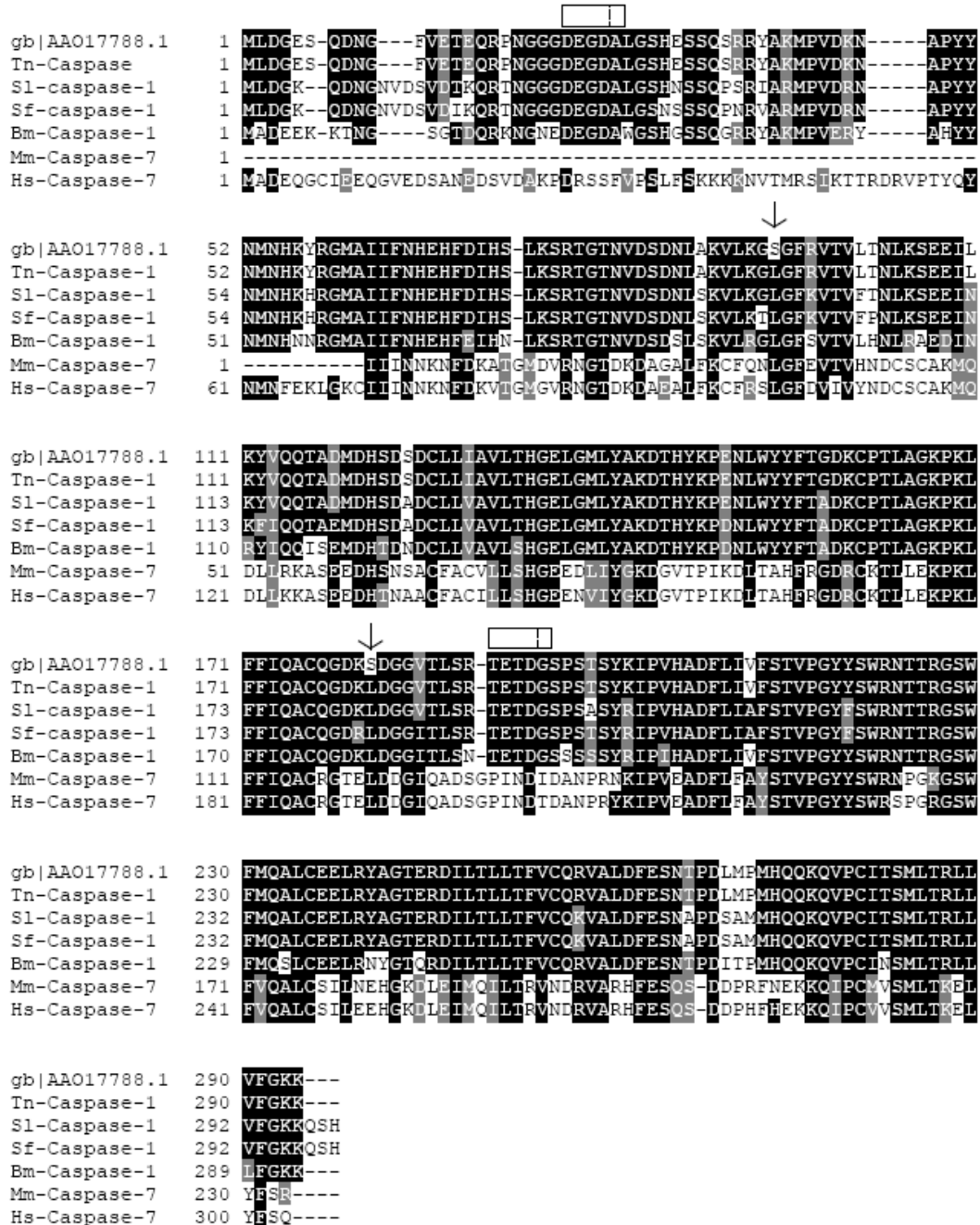


Figure 2-1. ClustalW multiple sequence alignment of Tn-caspase-1 and related caspases
 Default alignment values were used (ClustalW 1.82) and shading was performed using BOXSHADE. Black boxes indicate identical amino acids, gray boxes indicate related amino acids, white boxes indicate a difference in amino acids, and dashes indicate a blank position. The Genbank™ accession numbers of the protein sequences used for the alignment are: **AAO16241** (Sl-Caspase-1), **AAC47442** (Sf-Caspase-1), **NP_001037050** (Bm-Caspase-1), **NP_031637** (Mm-Caspase-7), and **AAH15799** (Hs-Caspase-7). Arrows indicate differences in the protein sequence of Tn-caspase-1 between **AAO17788** and the results of this study, while the boxes above the sequence represent the binding domains of Sf-caspase-1 (LaCount et al. 2000).

representing the cleavage site. Tn-caspase-1 has identical amino acids at each of these cleavage sites, indicating that they might also serve as cleavage sites in Tn-caspase-1. The sequence from this study differs from gb|[AAO17788](#) at two amino acid positions (indicated by arrows), 94 and 181. In gb|[AAO17788](#), both amino acids 94 and 181 are serine; however, in Tn-caspase-1, as well as all the other caspases analyzed, these amino acids are leucine. The conserved nature of these amino acids throughout all caspases suggests that the published Genbank sequence is incorrect. Since these caspases are effector caspases with relatively short prodomains (Chang and Yang 2000), it seems likely that Tn-caspase-1 is as well.

Protein expression, purification and detection

In order to isolate Tn-caspase-1, cells were transfected with pIB-TnCasp-HIS, which produces a modified Tn-caspase-1 with a polyhistidine tag for purification using a metal affinity resin. Two sets of cells were transfected: one was exposed to actinomycin-D, a chemical apoptosis inducer, at 2 days post-transfection (24 hours at 2 μ g/mL), while the other remained untreated. Both crude lysates and purified protein fractions from untreated and actinomycin D exposed cells were subjected to SDS-PAGE and Western Blot analysis. Tn-caspase-1 was visualized using α -SfCasp-1 as the primary antibody and Anti-Rabbit HRP as the secondary antibody. Results are presented in Figure 2-2. Crude lysates from untreated cells show a strong band at ~35 kDa which presumably represents uncleaved Tn-caspase-1, and a faint band at ~23 kDa which represents a cleavage product. Crude lysates from cells exposed to actinomycin D have a much stronger band at ~23, and a band at ~ 19 kDa, which represents either a subsequent cleavage step or a different cleavage product. This

shows that chemically induced apoptosis results in the cleavage of Tn-caspase-1. The purified fractions, while fainter, show similar bands in both untreated and actinomycin-D exposed cells.

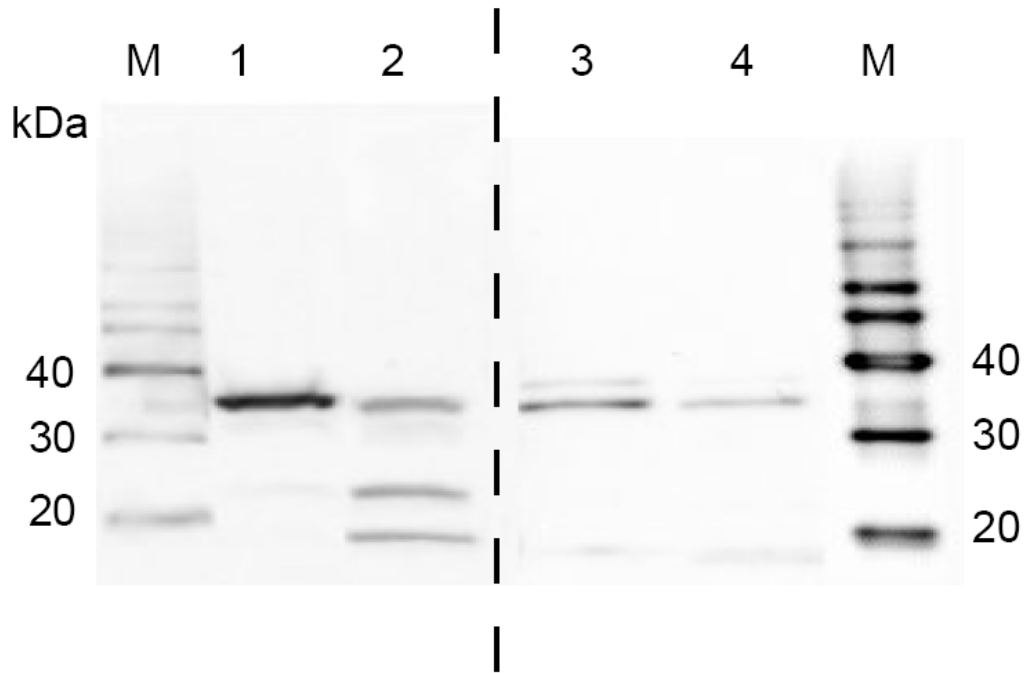


Figure 2-2. Tn-Caspase-1 Western Blot.

Western blot of native and HIS-purified Tn-caspase-1, in the presence and absence of actinomycin-D. Lanes are as follows: M. Protein Standard, 1. Crude lysate from untreated High FiveTM Cells, 2., Crude lysate from cells treated with Actinomycin-D (24 hours at 2 $\mu\text{g}/\text{mL}$), 3. HIS-purified protein fraction from untreated cells, 4. HIS-purified protein fraction from cells treated with Actinomycin-D (24 hours at 2 $\mu\text{g}/\text{mL}$). The dashed line represents the combination of two different western blots.

Tn-caspase-1 overexpression and RNAi silencing – *In vivo* protein activity

In order to determine the nature of Tn-caspase-1 *in vivo*, overexpression and RNAi silencing experiments were conducted by transfecting High Five cells with pIB-TnCasp (2 ug/mL) and dsRNA synthesized against *tn-caspase-1* (15 ug/mL), respectively. Control cells were transfected with pIB-CAT (2ug/mL, Invitrogen). Cells were seeded in 24 well plates and harvested 3 days post transfection. Initial experiments showed no increase in caspase activity as a result of overexpression (data not shown), suggesting that an apoptotic stimuli was required to fully activate Tn-caspase-1. This behavior is consistent with previous studies involving overexpression of *Sf*-Caspase-1 (Ahmad et al. 1997; LaCount et al. 2000). In subsequent experiments, a subset of cells was treated with actinomycin D, a chemical inducer of apoptosis. Although it was previously reported (Clem and Miller 1994) that a different *T. ni* derived cell line (TN-368) was resistant to actinomycin D induced apoptosis, this was not the case with High Five™ cells, as we observed both DNA laddering and cell death upon exposure to actinomycin D (see appendix, Figures 5-1 and 5-2). Additional evidence for the induction of apoptosis upon exposure to actinomycin D is given in appendix Figure 5-3, which shows labeling results using the Invitrogen's Vybrant® Apoptosis Assay Kit #4 (CAT V13243), which labels necrotic cells red using Propidium Iodide and apoptotic cells green using YO-PRO®-1. While dead cells are seen in both groups (with a greater number of dead cells in the actinomycin D treated cells), a much greater number of green (apoptotic) cells are present in cells treated with actinomycin D. Cells were exposed to actinomycin D

for 9 or 24 hours at a concentration of 2 $\mu\text{g}/\text{mL}$. Actinomycin D was added in a manner that allowed the cells to be harvested at 72 hours post transfection. Thus, in the case of 24 hours exposure, actinomycin D was added at 48 hours post transfection, while in the case of 9 hours exposure, actinomycin D was added 63 hours post transfection. Results are presented in Figure 2-3. As determined by RT-PCR and visualized using differential display, knockdown of *tn-caspase-1* was quite effective in both the presence and absence of actinomycin D (Figure 2-3). However, no significant difference in transcript levels was seen between cells transfected with pIB-CAT and pIB-TnCasp.

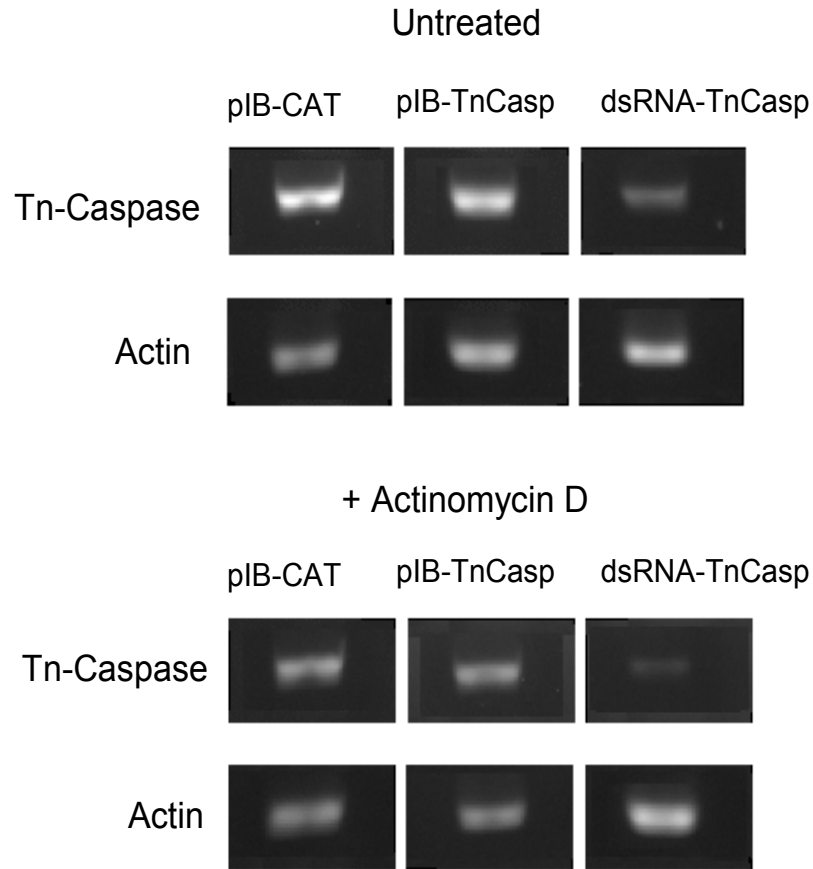
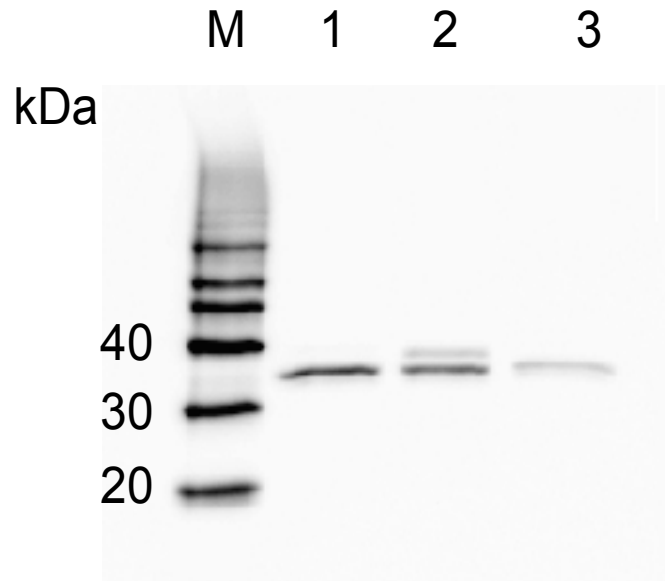


Figure 2-3. RT-PCR results for overexpression and RNAi silencing of Tn-caspase-1. Cells were transfected with either pIB-CAT (control), pIB-TnCasp (caspase overexpression), or dsRNA against tn-caspase-1 (silencing). Transcript levels of both Tn-caspase-1 and actin measured at 3 days post transfection, in the presence or absence of actinomycin D (9 hours at 2µg/mL). Bands were visualized on a 1% agarose gel using ethidium bromide and UV light.

To investigate the effects of knockdown and overexpression at the protein level, Western Blot analysis was performed on crude lysates from cells transfected with pIB-CAT, pIB-TnCasp, and dsRNA targeting Tn-caspase-1 (in the absence of actinomycin D). Results are shown in Figure 2-4a. While the Tn-caspase-1 level is similar in cells transfected with pIB-CAT and pIB-TnCasp, there is a marked decrease in those cells transfected with dsRNA. The caspase activity of cell lysates was measured using Ac-DEVD-AMC, which acts as a substrate for Human Caspase 3/7 and emits fluorescence upon cleavage. In the absence of actinomycin D, there was no significant difference between the caspase activity of cells transfected with pIB-CAT versus pIB-TnCasp (Figure 2-4b). However, cells transfected with dsRNA against Tn-caspase-1 showed a significant ($p < 0.05$) reduction in caspase activity. Upon exposure to actinomycin D for 9h, cells transfected with pIB-CAT had a nearly 7-fold increase in caspase activity, while cells transfected with pIB-TnCasp had an even larger increase at greater than 12-fold compared to pIB-CAT in the absence of actinomycin D. The caspase activity of cells transfected with dsRNA remained low even in the presence of actinomycin D, indicating that the silencing of Tn-caspase-1 was effective at the protein level. Cells exposed to actinomycin D for 24 hours showed no significant difference between those transfected with pIB-CAT and pIB-TnCasp, which had caspase activities of roughly 14 and 13 times that of the unexposed control, respectively. The caspase activity of cells treated with dsRNA and exposed to actinomycin D for 22h did increase as compared to the 9h exposure, but remained far lower than either pIB-CAT or pIB-TnCasp transfected cells, at just over 2 times the activity of unexposed control cells. The reduction in caspase activity

A.



B.

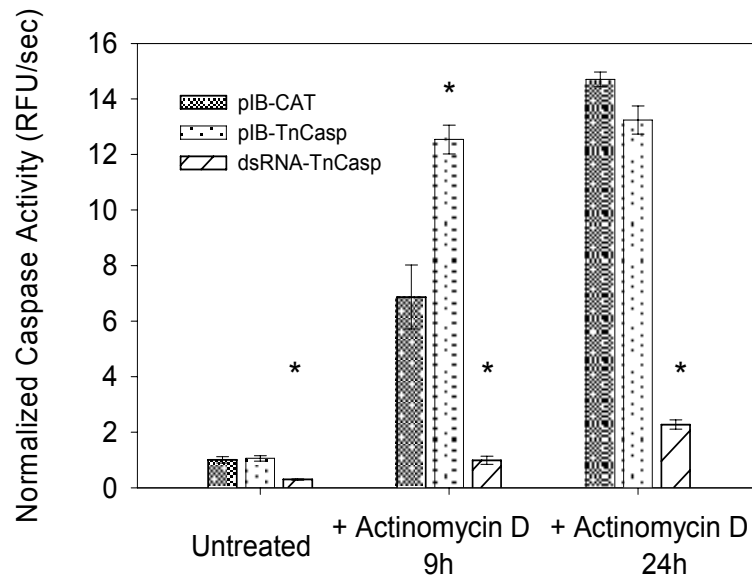


Figure 2-4. Western Blot, and Caspase activity results for overexpression and RNAi silencing of Tn-caspase-1.

A, Western blot of Tn-caspase-1, all lysates in the absence of Actinomycin D. Lanes are as follows: M. Protein Standard, 1. Crude lysate from cells transfected with pIB-CAT, 2., Crude lysate from cells transfected with pIB-TnCasp, 3. Crude lysate from cells transfected with dsRNA against tn-caspase-1. *B*, caspase activity of cell lysates measured at 3 days post transfection, in the presence or absence of actinomycin D (9 or 24 hours at 2mg/mL). * - indicates a statistically significant ($p < 0.05$) difference between the treated and control values at that condition (+ or -actinomycin D).

as a result of dsRNA treatment at all conditions, including cells not exposed to actinomycin D, indicates that *tn-caspase-1* is responsible for the majority of the caspase 3/7 activity in High Five cells, and is likely the primary effector caspase in this cell line.

Tn-caspase-1 overexpression and RNAi silencing – Effects on apoptosis

Photomicrographs were taken of both control and treated cells in the presence and absence of actinomycin D in order to examine cellular morphology. In the absence of actinomycin D (Figure 2-5), all treatment groups (CAT, TnCasp, dsRNA) display normal morphology. However, upon exposure to actinomycin D (24 hours), both pIB-CAT and pIB-TnCasp transfected cells present apoptotic symptoms, including degradation of the cell membrane and the formation of apoptotic bodies, while cells treated with dsRNA against Tn-caspase-1 present a far more normal morphology with only minor apoptotic characteristics. Fluorescein based TUNEL staining was also used to determine the effect of overexpression and knockdown of *tn-caspase-1* on the overall level of apoptosis in transfected cells. Terminal transferase catalyzes the addition of fluorescein-dUTP to single and double-stranded DNA breaks that occur as cells undergo apoptosis. As a result, apoptotic cells appear green when visualized with fluorescent microscopy or analyzed via flow cytometry. Labeling results are presented in Figure 2-6. Under all three conditions, untreated cells showed minimal fluorescence. However, upon exposure to

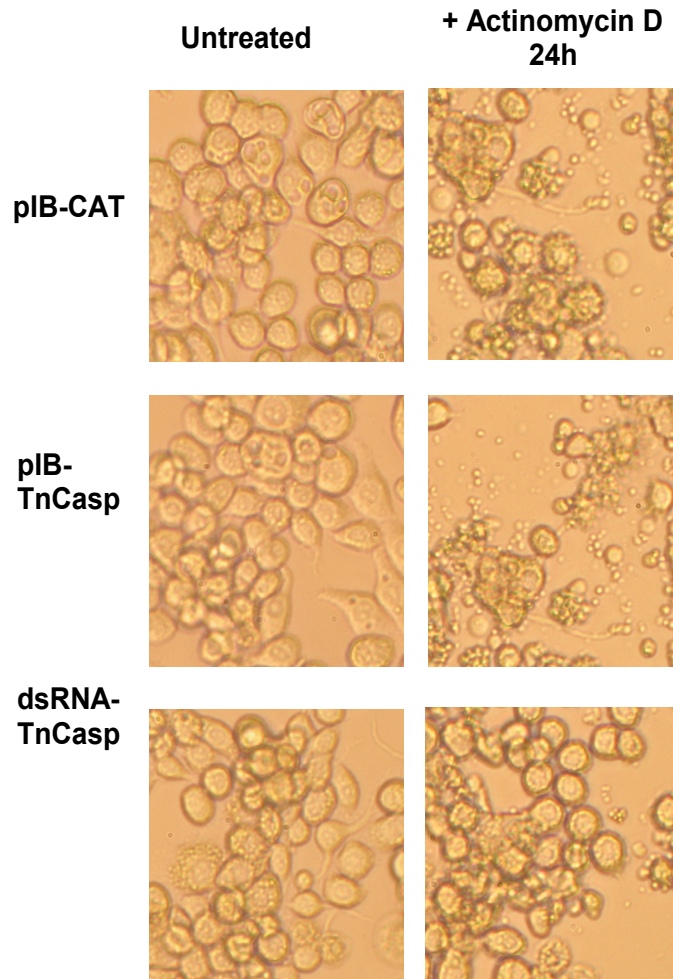


Figure 2-5. Photomicrographs for overexpression and RNAi silencing of Tn-caspase-1. Cells were transfected with either pIB-CAT (control), pIB-TnCasp (caspase overexpression), or dsRNA against *Tn-caspase-1* (knockdown). A, photomicrographs (100x magnification) of either untreated cells or cells exposed to actinomycin D (24 hours at 2ug/mL) taken 72 hours post transfection for each condition.

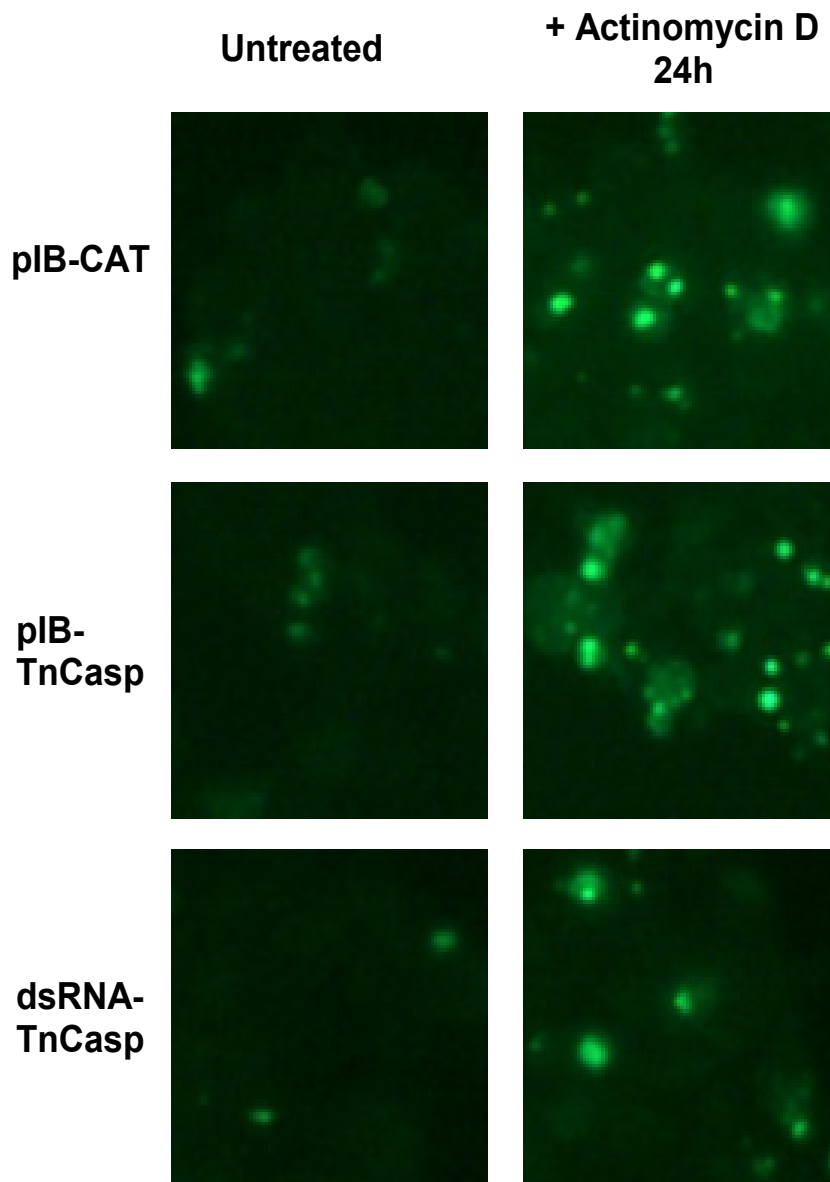


Figure 2-6. Fluorescent TUNEL staining for overexpression and RNAi silencing of Tn-caspase-1.

Fluorescent micrographs (100x magnification) of either untreated cells or cells exposed to actinomycin D at each condition visualized using Fluorsecin based TUNEL staining. Green fluorescence indicates cells are positive for apoptosis.

actinomycin D for 24h, both pIB-CAT and pIB-TnCasp transfected cells showed strong fluorescence, while dsRNA transfected cells exhibited noticeably less fluorescence, though still greater than untreated cells. This fluorescence was quantified using flow cytometry, the results of which are presented in Figure 2-7. Populations were first gated based on forward and side scatter to ensure only cells were counted. Then the cells were gated on fluorescence such that 5% of untreated control cells were considered positive for apoptosis. Representative histograms, labeled with the percentage of cells considered to be positive for apoptosis, are shown in Figure 3c. Consistent with the caspase activity data, both pIB-CAT and pIB-TnCasp treated with actinomycin D showed high levels of apoptosis, with 57 and 48 percent apoptosis positive cells, respectively. Although pIB-CAT had a higher percentage of apoptotic cells, pIB-TnCasp had a higher mean fluorescence, at 92, versus only 77 for pIB-CAT. Cells transfected with dsRNA had both a lower percentage of apoptotic cells and mean fluorescence than controls in both the presence and absence of actinomycin D (19% and 50, respectively), indicating that knockdown of Tn-caspase-1 was effective in reducing the amount of chemically induced apoptosis present in the cells.

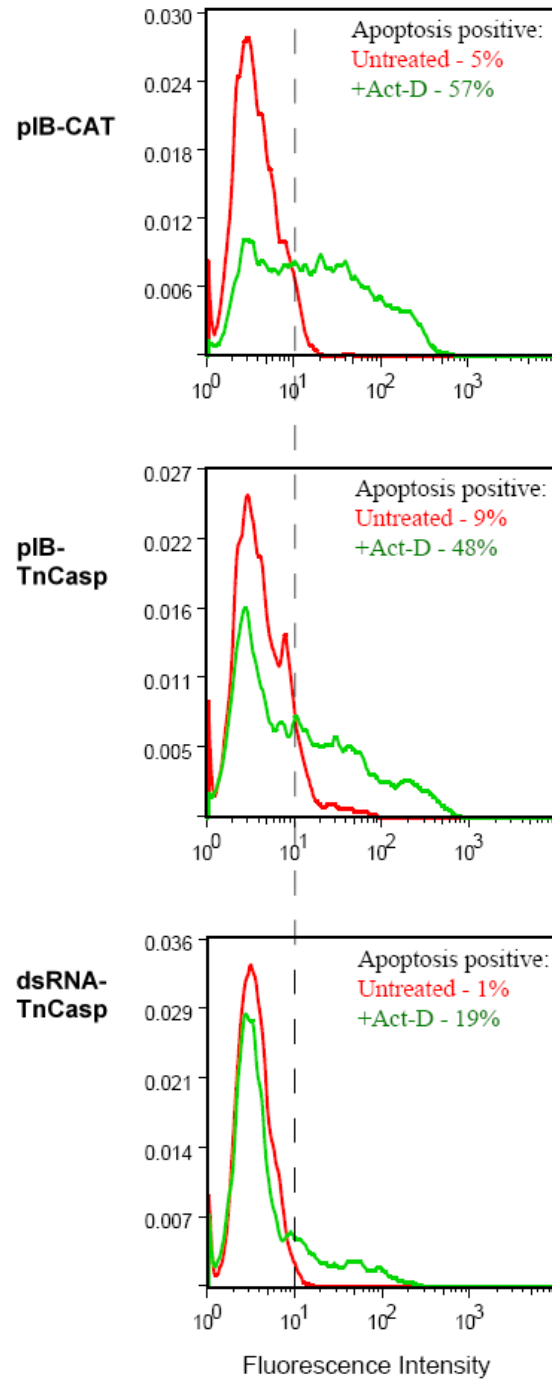


Figure 2-7. Fluorescein based TUNEL Staining for overexpression and RNAi silencing of Tn-caspase-1.

Flow cytometry histograms comparing untreated and actinomycin D-exposed cells for each condition. Numbers on the graph represent the percentage of cells considered to be positive for apoptosis at that condition and the dashed line represents the fluorescence intensity above which cells are considered apoptosis positive.

Tn-caspase-1 activity during baculovirus infection

Several previous studies have focused on the activity of Sf-caspase-1 during baculovirus infection (Ahmad et al. 1997; Clem 2005; LaCount et al. 2000). However, no study has examined *in vivo* caspase activity at time greater than 3 days post infection which is often of interest in protein expression systems. As such, experiments were conducted to determine the activity of Tn-caspase-1 throughout the entire course of baculovirus infection. In addition to cells infected with baculovirus (AcMNPV-GFPuv), a group of cells were also transfected 24 hours prior to infection with dsRNA against Tn-caspase-1. Tn-caspase-1 transcript levels and caspase activity were analyzed from 1-5 days post infection. Transcript levels of *tn-caspase-1* remained relatively constant in infected and control (uninfected) cells, but remained negligible throughout the entire course of infection for those cells transfected with dsRNA (Figure 2-8a and b). Caspase activity levels, shown in Figure 2-9, of both infected and dsRNA cells remained below that of the control for the first 4 days post infection. However, at 5 days post infection, the caspase activity of infected cells jumps greatly up to nearly 19 times that of control cells at day 1. The caspase activity of cells transfected with dsRNA also increased, but only to 2.5 times that of the control cells at day 1, which was in fact less than the control cells at day 5. Thus, knockdown of Tn-caspase-1 was effective at the protein level during baculovirus infection, even after the large increase in caspase activity at day 5 post infection.

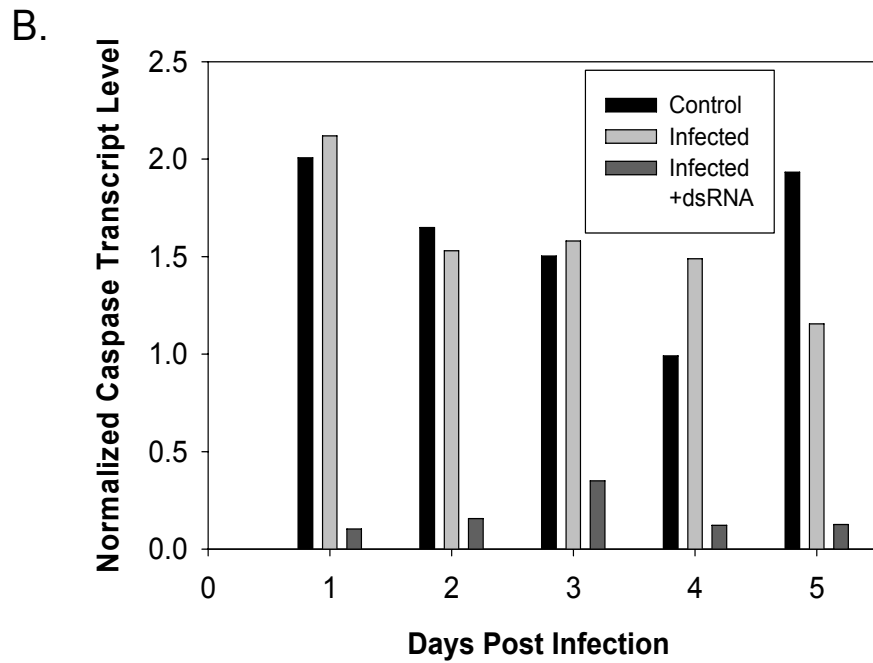
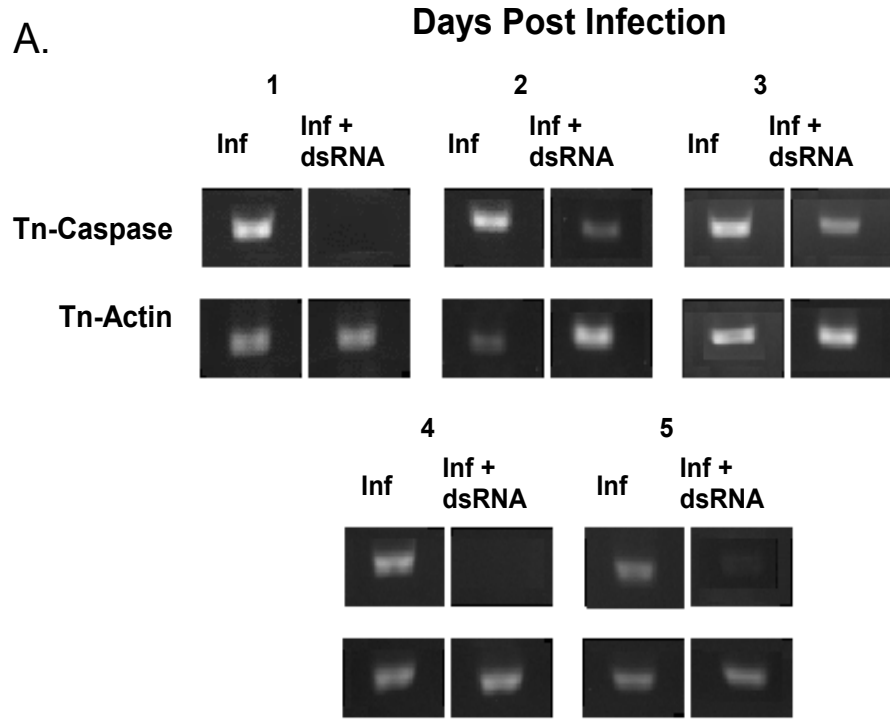


Figure 2-8. RT-PCR activity results for cells infected with recombinant baculovirus AcMNPV-GFPuv.

Cells were either uninfected (control), infected, or transfected with dsRNA against *Tn-caspase-1* 24h prior to infection. *A*, transcript levels of both *Tn-caspase-1* and *actin* for infected and infected+dsRNA cells from 1-5 days post infection. *B*, normalized *Tn-caspase-1* transcript levels for control, infected, and dsRNA treated cells from 1-5 days post infection. Normalized transcript level is defined as the *Tn-caspase-1* transcript level divided by the *tn-actin* transcript level at each time point.

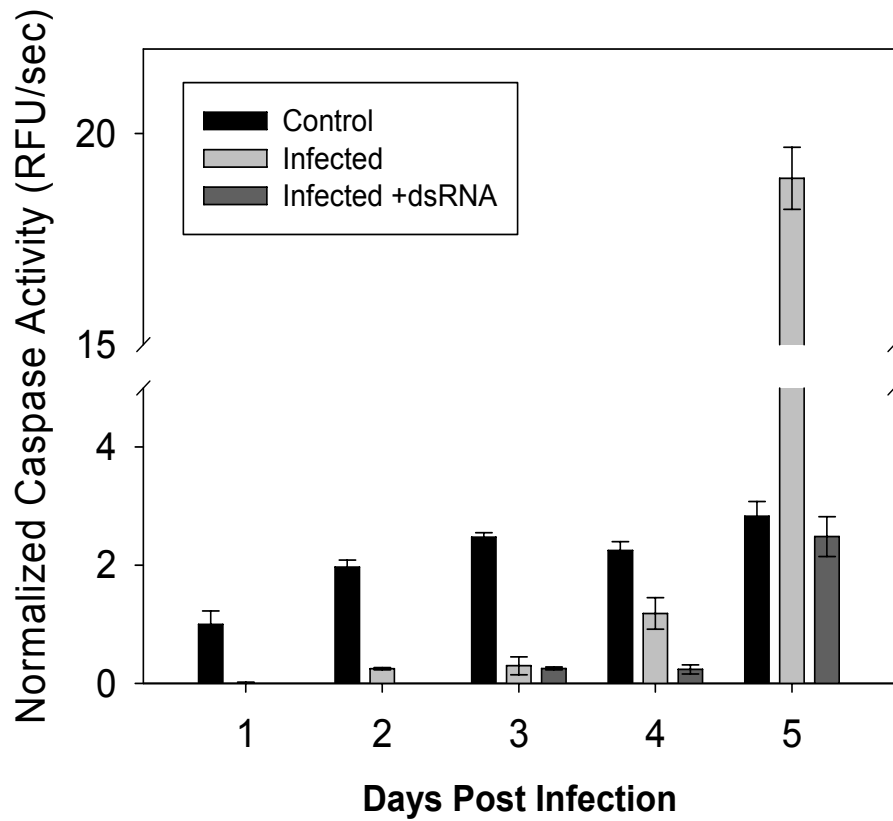


Figure 2-9. Caspase activity results for cells infected with recombinant baculovirus AcMNPV-GFPuv

Caspase activity of cell lysates measured from 1-5 days post infection. Activity values are normalized to the level of caspase activity in control (uninfected) cells on day 1 post infection.

Interaction between Tn-caspase-1 and baculovirus protein p35

During analysis of the caspase activity during baculovirus infection, it was determined that the caspase activity curve (Fluorescence Intensity vs. Incubation Time, see Mat. and Meth.) of infected cells became non-linear after about 2 hours of incubation (data not shown). It was unlikely this was due to exhaustion of substrate, however, because a similar trend was seen in the caspase activity curves of cells transfected with dsRNA, which had a much lower caspase activity. Since the caspase activity curves of cells treated with actinomycin D remained linear, it was likely that a baculovirus protein (or proteins) was responsible for this behavior. A likely choice was baculovirus protein p35, which has been shown to be a potent inhibitor of Sf-Caspase 1 (Ahmad et al. 1997; LaCount et al. 2000). Furthermore, stable Sf9 cell lines producing p35 demonstrated increased resistance to actinomycin D induced apoptosis (Lin et al. 2001).

In order to determine the effect of baculovirus protein p35 on the activity of Tn-caspase-1, High five cells were co-transfected with either pIB-CAT and pIB-p35 or pIB-TnCasp and pIB-p35 (all plasmid concentrations 2ug/mL). Caspase activity of cell lysates was determined as described previously and is presented in Figure 2-10a. In both the presence and absence of actinomycin D, cotransfecting with pIB-p35 greatly reduced the caspase activity of cells transfected with both pIB-CAT and pIB-TnCasp. In fact, at 9 hours of exposure, caspase activity of co-transfected cells is well below that of untreated control cells. At 24 hours of exposure, the caspase activity of pIB-TnCasp/pIB-p35 cells is slightly higher than that of pIB-CAT/pIB-p35

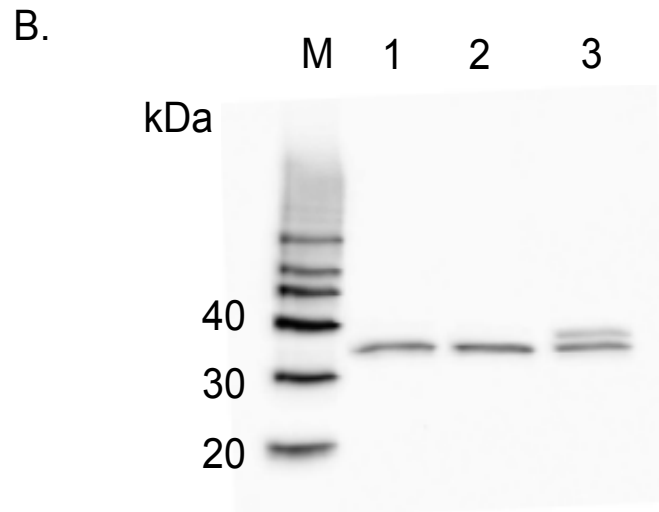
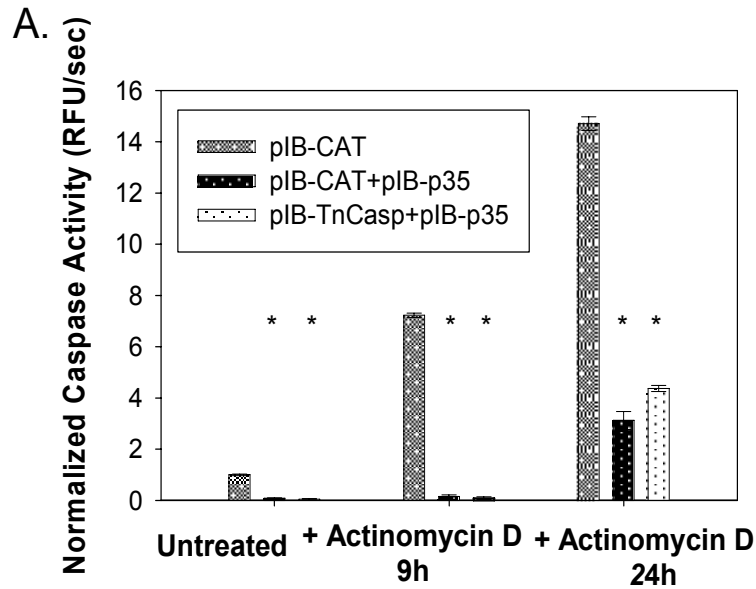


Figure 2-10. Caspase activity and Western Blot results for cells transfected with pIB-CAT, pIB-CAT+pIB-p35, or pIB-TnCasp+pIB-p35

Cells were transfected with pIB-CAT or cotransfected with pIB-CAT+pIB-p35, or pIB-TnCasp+pIB-p35. Caspase activity was measured from cell lysates harvested 3 days post transfection, in the presence or absence of actinomycin D (9 or 24 hours at 2 μ g/mL). * - indicates a statistically significant ($p < 0.05$) difference between the treated and control values at that condition (+ or -actinomycin D). *B*, Western blot of Tn-caspase-1, all lysates in the absence of Actinomycin D. Lanes are as follows: M. Protein Standard, 1. Crude lysate from cells transfected with pIB-CAT and pIB-p35, 2., Crude lysate from cells transfected with pIB-TnCasp and pIB-p35.

co-transfected cells, but remains lower than that of cells transfected with only pIB-CAT or pIB-TnCasp. It is also important to note that the caspase activity of co-transfected cells was higher than that of cells transfected with dsRNA.

Western Blot analysis was also performed on lysates from co-transfected cells (Figure 2-10b), with both groups having similar caspase levels to cells transfected with only pIB-CAT.

Tn-caspase-1 and p35 – TUNEL Staining

Photomicrographs were also taken of co-transfected cells, both in the presence and absence of actinomycin D. As with the other treatments, in the absence of actinomycin D (Figure 2-11), both groups display normal morphology. However, upon exposure to actinomycin D (24 hours), co-transfected cells both display a reduction in apoptotic morphology as compared with cells transfected with only pIB-CAT or pIB-TnCasp, similar to the effect of dsRNA. Co-transfected cells were analyzed using TUNEL staining to determine if the overall apoptotic level of the cells was affected by p35 as it was with dsRNA. Fluorescent images are shown in Figure 2-12. Untreated cells, as before, show little fluorescence, while those cells exposed to actinomycin D for 24 hours show strong fluorescence. Flow cytometry was used to quantify the fluorescence, which is shown in Figure 2-13. Cells co-transfected with pIB-CAT and pIB-p35 had 30% apoptosis positive cells with a mean fluorescence of 50, while cells co-transfected with pIB-TnCasp and pIB-p35 had 34% apoptosis positive cells with a mean fluorescence of 42. Consistent with the caspase activity

data, co-transfection had a lower percentage of apoptosis than cells transfected with only pIB-CAT and pIB-Tncasp, but higher than that of cells transfected with dsRNA.

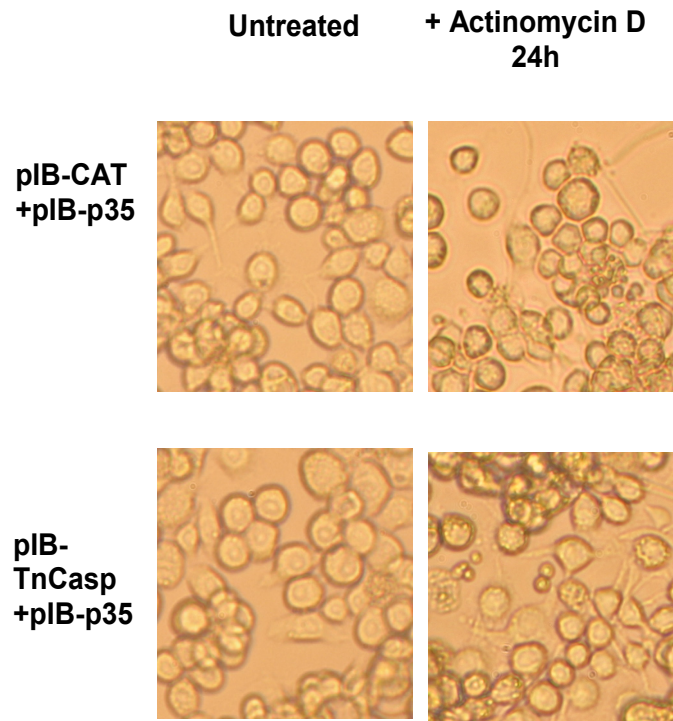


Figure 2-11. Photomicrographs for cells transfected with pIB-CAT+pIB-p35 and pIB-TnCasp+pIB-p35

Cells were transfected with pIB-CAT or cotransfected with pIB-CAT+pIB-p35, or pIB-TnCasp+pIB-p35. *A*, photomicrographs (100x magnification) of either untreated cells or cells exposed to actinomycin D (24 hours at 2ug/mL) taken 72 hours post transfection for each condition.

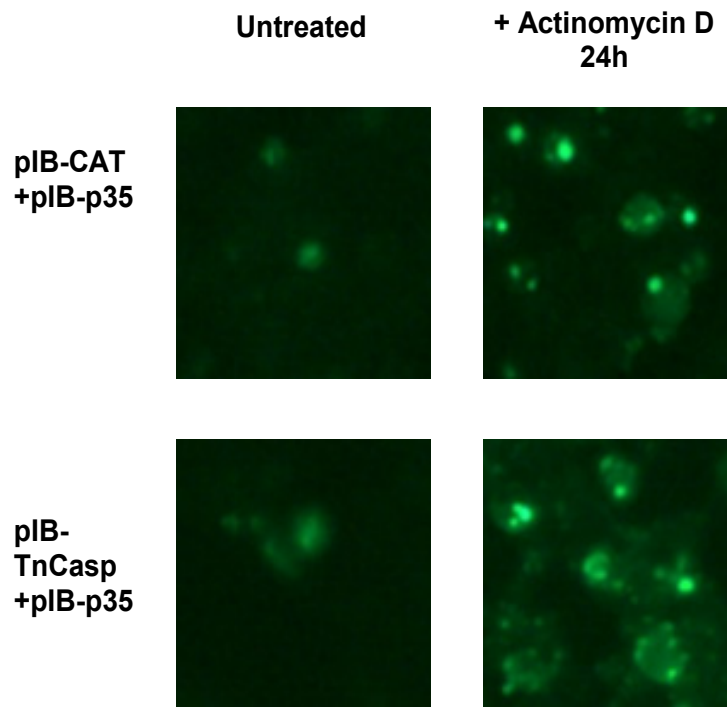


Figure 2-12. Fluorescein based TUNEL Staining for cells transfected with pIB-CAT+pIB-p35 and pIB-TnCasp+pIB-p35
 Fluorescent micrographs (100x magnification) of either untreated cells or cells exposed to actinomycin D for 24 hours (2 μ g/mL) at each condition. Green fluorescence indicates cells are positive for apoptosis.

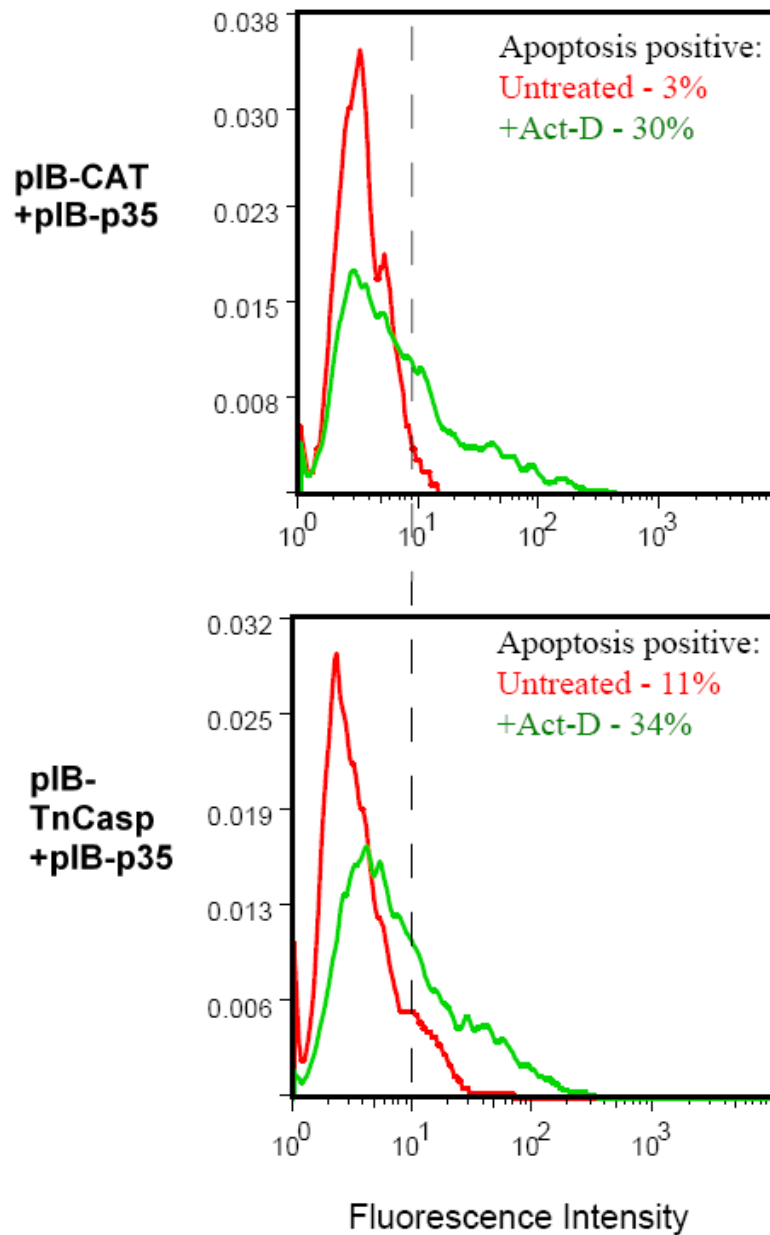


Figure 2-13. Fluorescein based TUNEL Staining for cells transfected with pIB-CAT+pIB-p35 and pIB-TnCasp+pIB-p35

Flow cytometry histograms comparing untreated and actinomycin D-exposed cells for each condition. Numbers on the graph represent the percentage of cells considered to be positive for apoptosis at that condition and the dashed line represents the fluorescence intensity above which cells are considered apoptosis positive.

Discussion

Tn-caspase-1 is the main effector caspase in *T. ni* cells

Based on the high sequence similarity of Tn-caspase-1 to other lepidopteron and mammalian caspases, including the well studied Sf-Caspase-1, it is likely that Tn-caspase-1 behaves in a similar fashion. Tn-caspase-1, as well as other lepidopteron caspases, has the same amino acid sequences (Figure 2-1) as Sf-Caspase-1 at the cleavage sites between the prodomain and large subunit (DEGD²⁸↓A), and between the large and small subunits (TETD¹⁹⁵↓G), and similar sequences at the linker cleavage site (DXXD↓G) (LaCount et al. 2000). Tn-caspase-1 also has a short prodomain that is indicative of group II effector caspases. Furthermore, Tn-caspase-1 can be detected on a Western blot using an antibody developed for Sf-Caspase-1 (Figure 2-2), and is cleaved into two subunits upon exposure to actinomycin D. Purified Tn-caspase-1-HIS can also be detected on a western both in lysates with and without actinomycin D.

Assuming Tn-caspase-1 is cleaved into two subunits in a manner similar to Sf-caspase-1, the expected size of the large subunit is 21 kDa with the small prodomain and 19 kDa without, while the size of the small subunit would be 12 kDa. Thus, it seems likely that the 21 kDa protein seen on the western blot in Figure 1b is the large subunit plus the prodomain, while the 19 kDa protein is the large subunit without the prodomain. Also, since the small subunit of Sf-caspase is not detected by the α -SfCasp1 antibody (LaCount et al. 2000), it is likely that it does not recognize the small subunit of Tn-caspase-1 either.

Further evidence supporting a role similar to Sf-caspase 1 is provided by experimental data showing that Tn-caspase-1 must be activated by an apoptotic stimuli, in this case actinomycin D, before any upregulation in caspase activity is seen (Figure 2-4). Although overexpression of Tn-caspase-1 can provide a transient increase in caspase activity, at 24h exposure to actinomycin D, the caspase activity of cells transfected with pIB-TnCasp is no different than that of cells transfected with pIB-CAT, suggesting that either the upregulation of native *tn-caspase-1* transcription as a result of exposure to actinomycin D is greater than the plasmid driven expression of *tn-caspase-1*, or that post-transcriptional regulation of *tn-caspase-1* is responsible for the increase in caspase activity, or both. Recent studies have suggested that much of the upregulation of Sf-Caspase-1 also occurs post-transcriptionally (Liu and Chejanovsky 2006).

Since knockdown of *tn-caspase-1* via dsRNA eliminates nearly all caspase 3/7 activity as measured by cleavage of Ac-DEVD-AMC, it is likely that Tn-caspase-1 is not only an effector caspase, but is also responsible for the majority of the effector caspase activity in High Five cells, as Sf-Caspase-1 is in Sf cells (Ahmad et al. 1997; LaCount et al. 2000). Silencing via dsRNA even reduces the caspase activity of cells not exposed to actinomycin D, indicating there is some basal level of caspase activity that is reduced in the absence of *tn-caspase-1* transcript.

Tn-caspase-1 activity during baculovirus infection

During baculovirus infection, the caspase activity of both infected and infected+dsRNA cells remained below that of the control for the first 4 days post infection (Figure 2-9). This is likely due to a baculovirus protein such as p35, which

have been shown to inhibit apoptosis in Sf cells (Ahmad et al. 1997; LaCount et al. 2000; Manji and Friesen 2001). Further support is given by the fact that cotransfecting cells with pIB-p35 greatly reduces caspase activity, both in the presence and absence of actinomycin D (Figure 2-10). However, at 5 days post infection and beyond (data not shown), caspase activity greatly increases in infected cells, though not in cells transfected with dsRNA against Tn-caspase (Figure 2-9). To our knowledge, no other published study shows caspase activity levels throughout the entire course of baculovirus infection. This data suggests that at a certain point, the baculovirus proteins can no longer fully inhibit caspase activity, though the reason for this is for the moment unclear. Perhaps at a certain level, p35 and other baculovirus proteins can no longer compete with the upregulation of Tn-caspase-1, resulting in a subsequent increase in caspase activity level. Although differences have been seen in the replication of p35 negative baculoviruses with respect to *S. frugiperda* and *T.ni* derived cell lines (Clem and Miller 1993; Clem et al. 1994; Hershberger et al. 1992) , it seems clear that p35 inhibits the principal effector caspase activity in both.

Modulating Caspase Activity – dsRNA vs p35

Although both dsRNA and p35 reduce the caspase activity in cells exposed to actinomycin D, it is important to contrast their method of action and how this affects the reduction in caspase activity. A model for this modulation is presented in Figure 2-14. Transfecting with dsRNA lowers caspase activity through degradation of *tn-caspase-1* mRNA, while p35 presumably acts post-transcriptionally by preventing Tn-caspase-1 cleavage and activation, similar to its action on Sf-Caspase-1 (Ahmad et al.

1997; LaCount et al. 2000). Further evidence for the post-translational action of p35 is provided by the fact that Tn-caspase-1 protein levels (as visualized by Western Blot, Figure 2-10) are similar in cells with and without p35. In untreated cells, as well as those exposed to actinomycin D for only 9 hours, p35 is more effective at reducing caspase activity than dsRNA. This is likely due to the fact that levels of active Tn-caspase-1 are relatively low, allowing p35 to effectively inhibit nearly all the Tn-caspase-1 present in the cell. Alternatively, while dsRNA can substantially lower transcript levels, it cannot fully eliminate translation, resulting in a small but detectable amount of active caspase. As apoptosis progresses, the situation is reversed. This is demonstrated in the cells exposed to actinomycin D for 24 hours, in which dsRNA is more effective at lowering caspase activity than p35. This trend is also reflected in the flow cytometry data, as the percentage of apoptotic cells in dsRNA treated cells is lower than that of cells expressing p35 (Table 2-1). At this point, levels of Tn-caspase-1 may have increased to a level where p35 can no longer completely inhibit its cleavage and activation. However, by lowering transcript levels, dsRNA has decreased the amount of caspase available for activation, thereby limiting the pool of *tn-caspase-1* available for translation. The difference between these situations suggests that the principal form of regulation for Tn-caspase-1 is post-transcriptional, which recent studies have suggested is the case for Sf-Caspase-1 (Liu and Chejanovsky 2006). It is important to note however, that the performance of p35 and dsRNA might change when presented with alternative apoptotic stimuli. Since actinomycin D is a transcriptional inhibitor, it may affect p35 in a manner different than it does dsRNA. Thus, further investigation is warranted to determine

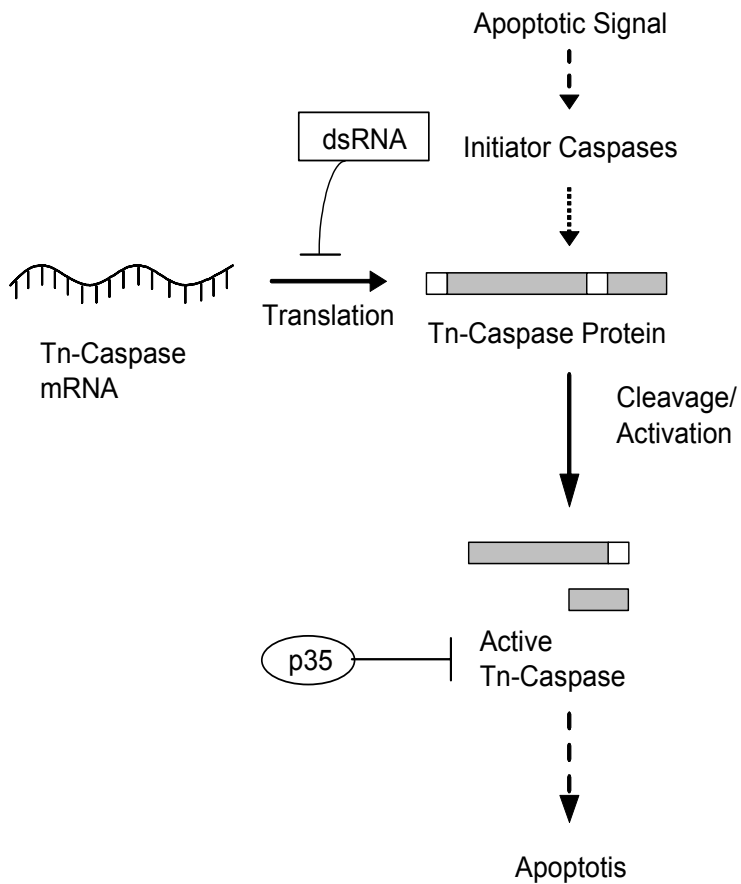


Figure 2-14. Theoretical Model of Caspase Activation and Modulation.

Tn-caspase-1 can be activated by an apoptotic signal, such as actinomycin-D or baculovirus infection. Once in its active form, Tn-caspase-1 triggers apoptosis. Both dsRNA and baculovirus protein p35 can inhibit caspase activity, but at different steps. dsRNA directed against Tn-caspase-1 works post-transcriptionally, blocking the translation of Tn-caspase-1 mRNA into protein, while p35 acts post-translationally, presumably by targeting active Tn-caspase-1.

Table 2-1. Modulating caspase activity with overexpression or RNAi silencing of Tn-caspase, or through cotransfection with baculovirus protein p35.

Cells were transfected with either pIB-CAT (control), pIB-TnCasp (overexpression), dsRNA synthesized against TnCasp (silencing), pIB-p35, or combinations of the above.

Actinomycin-D was added three days post transfection, and cells were harvested after the listed exposure time. Caspase activity was normalized to the untreated pIB-Cat lysates.

	Normalized Caspase Activity (RFU/sec)		
	Untreated	9h Act-D	24h Act-D
pIB-CAT	1.000 ± 0.117	6.863 ± 1.150	14.702 ± 0.265
pIB-TnCasp	1.054 ± 0.099	12.540 ± 0.516	13.240 ± 0.511
dsRNA-TnCasp	0.295 ± 0.021	0.985 ± 0.1466	2.276 ± 0.169
pIB-CAT+pIB-p35	0.086 ± 0.008	0.155 ± 0.059	3.122 ± 0.351
pIB-TnCasp+pIB-p35	0.047 ± 0.022	0.089 ± 0.0483	4.367 ± 0.118

the exact nature and relative effectiveness of p35 and dsRNA under a wide range of apoptotic conditions.

Modulating Caspase Activity and Apoptosis – Implications for Metabolic Engineering

In addition to modulating caspase activity, both dsRNA and p35 also affect the overall level of apoptosis present in cells treated with actinomycin D, as reflected in the TUNEL staining and flow cytometry data. This presents several interesting applications for metabolic engineering. Several studies (Ailor and Betenbaugh 1998; Ailor et al. 1997; Arden and Betenbaugh 2004; Figueroa et al. 2005; Figueroa et al. 2002; Yung et al. 2006a) have used the overproduction of anti-apoptotic proteins in order to prolong production time and increase recombinant protein production. Relatively few studies have used RNAi to similar effect (Desai and Papoutsakis 1999; March and Bentley 2006; March and Bentley 2007; Srivastava et al. 2000; Sung et al. 2005). This study has not only demonstrated the potential of using RNAi to decrease caspase activity and apoptosis, but has also contrasted some of the differences between post-transcriptional (RNAi) versus post-translational (p35) regulation. In the case of Tn-caspase-1, RNAi has proven to be more effective at both decreasing caspase activity levels and apoptosis, bolstering the potential of RNAi as a metabolic engineering tool.

Chapter 3 : *In vitro* and *in vivo* suppression of Tn caspase-1 for improved recombinant protein production in High Five cell culture with the baculovirus expression vector system

Abstract

While traditional metabolic engineering generally relies on the augmentation of specific genes and pathways in order to increase the yield of target proteins, the advent of RNA interference (RNAi) as a biological tool has given metabolic engineers another tool capable of rationally altering the host cell's biological landscape in order to achieve a specific goal. Given its broad applicability and potent specificity, RNAi has the ability to suppress genes whose function is contrary to desired phenotype. In this study, RNAi has been used to increase recombinant protein production in a *Trichoplusia ni* derived cell line (High Five™) using the Baculovirus Expression Vector System. The specific target investigated is Tn-caspase-1, a protease involved in apoptosis that is likely the principle effector caspase present in *T. ni.* cells. Experiments were first conducted using *in vitro* synthesized dsRNA to verify silencing of Tn-caspase-1 and increased protein production as a result. Subsequent experiments were conducted using a cell line stably expressing *in vivo* RNAi in the form of an inverted repeat that results in a hairpin upon transcription. Using this construct, Tn-caspase-1 transcript levels were decreased by 50% and caspase enzymatic activity was decreased by 90%. This cell line, designated dsTncasp-2, demonstrates superior viability under low nutrient culture conditions and resulted in as much as 2 times the protein yield when compared to standard High Five cells.

Introduction

In general, metabolic engineering involves altering a gene or regulatory network to bring about a desired phenotype (Bailey 1991; Stephanopoulos and Vallino 1991). Traditionally, this has been accomplished either through the overexpression of specific targets directly involved in the pathway (Chen et al. 2001; Irani et al. 1999) or alteration of the overall protein machinery, such as the expression of chaperone proteins (Borth et al. 2005; Davis et al. 2000; Higgins et al. 2003; Kitchin and Flickinger 1995; Yokoyama et al. 2000) or transcriptional factors (Tigges and Fussenegger 2006) involved in the secretory pathway. However, as illustrated in Figure 3-1a, a similar strategy can be implemented using RNA Interference (RNAi) (Hebert et al. 2008a). First described in 1998 (Fire et al.), RNAi involves a broad class of RNA molecules that can be used to suppress or “silence” homologous RNA through a pathway that is thought to be conserved throughout eukaryotes (Dykxhoorn and Lieberman 2005; Fagard et al. 2000). The fundamental goal of metabolic engineering with RNAi remains the same, though the method of execution is different. Instead of introducing new genes or pathways, potentially deleterious genes and pathways are suppressed in an effort to optimize the host’s native production machinery.

With respect to the production of recombinant protein, specific desirable phenotypes include the ability to produce a specific product at a high yield and quality, as well as the ability to maintain viability in the harsh environments generally seen during the production process, including decreased oxygen and nutrients or increased cellular and oxidative stress (diStefano et al. 1995; Rao and Bredesen 2004;

Scott et al. 1992). Efforts have been made using RNAi to increase protein production in *Drosophila S2* cells through manipulation of the cell growth cycle (March and Bentley 2006; March and Bentley 2007) and in HEK cells through the downregulation of transcriptional regulators (Hacker et al. 2004). Protein quality has also been improved using RNAi, both through increased antibody-dependent cellular cytotoxicity for anticancer therapeutics (Imai-Nishiya et al. 2007; Kanda et al. 2007; Mori et al. 2004), as well as higher sialic acid content in recombinant glycoproteins (Ngantung et al. 2006). Specific past examples of anti-apoptosis engineering involving RNAi include enhancing viability and protein production in CHO cells through the downregulation of caspase-3 (Lai et al. 2004; Sung et al. 2005) or both caspase-3 and caspase-7 (Sung et al. 2007) as well as the suppression of the anti-apoptotic genes *Alg-2* and *Requiem* (Wong et al. 2006b). The latter study was the second paper in a series that used transcriptional profiling to determine potential RNAi targets (Wong et al. 2006a). A similar screen is underway in HEK cells (Lee et al. 2007).

RNAi for increasing protein production in the Baculovirus Expression Vector System (BEVS) has been less common, despite its high productivity (Caron et al. 1990; Luckow and Summers 1988; Smith et al. 1985). Initial reports in *Trichoplusia ni* larvae and *Spodoptera frugiperda* derived cell lines demonstrated that genes produced on a baculovirus could be silenced with RNAi, and subsequent applications of this technique aimed to reduce baculovirus infectivity through the downregulation of several key genes (Hajos et al. 1999; Isobe et al. 2004; Valdes et al. 2003). Relatively few studies have examined the potential of RNAi to increase protein

production in the baculovirus expression vector system, though several examples have appeared in both cells and larvae demonstrating the effectiveness of the approach (Kramer and Bentley 2003; Lin et al. 2007).

In a previous work (Kim et al. 2007), we demonstrated that protein production in the baculovirus expression vector system could be enhanced using *in vitro* dsRNA against baculovirus genes in Sf9 cells. Here, we demonstrate that the same principle holds true for High Five cells, which have been shown to have superior productivity (Davis et al. 1993; Wickham et al. 1992; Wickham and Nemerow 1993). The metabolic engineering target was Tn-caspase-1, which has been shown to be the primary effector caspase in High Five cells and an accurate indication of the overall level of apoptosis present in the cells (Hebert et al. 2008b). In this study, we first show that protein production can be enhanced by silencing Tn-caspase-1 with chemically synthesized *in vitro* dsRNA (Figure 3-1b). Further, in order to take advantage of the simplicity and robustness of an *in vivo* approach, we have developed a construct for the expression of *in vivo* dsRNA and subsequently developed the first stable *T ni.* derived cell line in which it is implemented (Figure 3-1c). The stable cell line selected (designated dsTncasp-2) displays a 50% decrease in caspase transcript level and a 90% decrease in caspase enzymatic activity during chemically induced apoptosis. dsTncasp-2 also has increased resistance to nutrient starvation and high cell densities as is often seen during prolonged suspension culture and demonstrates protein production levels up to two-fold that of standard cells. In order to illustrate the generality of this approach, recombinant baculoviruses producing either of the two model proteins Green Fluorescent Protein (GFP) and Chloramphenicol

acetyltransferase (CAT), were used to compare the production levels of dsTncasp-2 and control cells at several multiplicities of infection (MOI).

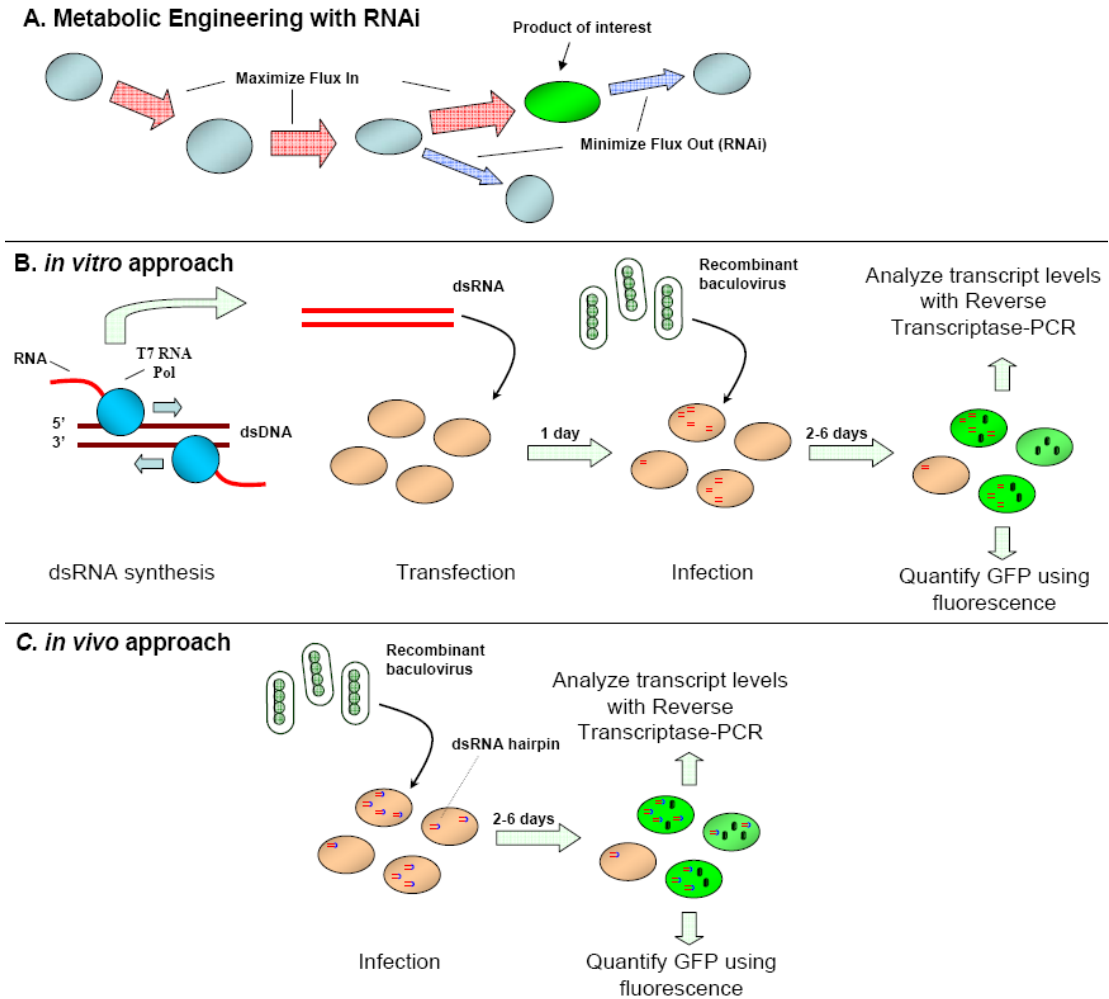


Figure 3-1. RNAi in Metabolic Engineering and Experimental Design

A. The goal of RNAi as a metabolic engineering tool is to suppress genes that would divert flux away from the product of interest. Potential target genes include enzymes involved in alternative pathways or that degrade the product of interest. *B.* Experimental design – *in vitro* dsRNA and baculovirus infection. dsRNA is first synthesized *in vitro* from a dsDNA template and subsequently transfected into High Five™ cells at a concentration of 1µg/mL. The following day, cells are infected with a recombinant baculovirus. Throughout infection, RNA and proteins levels are monitored using RT-PCR and fluorescence measurements. *C.* Experimental design – *in vivo* dsRNA and baculovirus infection. Since the dsRNA construct is stably integrated, cells can be seeded and infected without a separate transfection step.

Materials and Methods

Cell culture, transfection, and infection

Trichoplusia ni BTI-TN-5B1-4 (High Five™, Invitrogen) cells were cultivated in EX-CELL™-405 media (SAFC Biosciences) at 27°C. Unless otherwise noted, cells were initially plated on 24 well plates at 2×10^5 cells/mL and transfected the following day using FuGene HD® (Roche) according to the manufacturer's procedure. Briefly, DNA:FuGene complexes at a ratio of 2:7 (ug:uL) were formed in distilled and deionized water and then added to the appropriate wells. For studies involving *in vitro* synthesized dsRNA, 0.5ug was added to each well (concentration of 1ug/mL). In the case of infection with baculovirus, cells were infected 24 hours post transfection as previously described with a Multiplicity of Infection (MOI) of 5, unless otherwise noted (Kim et al. 2007).

Plasmid Construction and Selection of Stable Cell Line

The hairpin construct in pIB-dsTnCasp was created in two steps. First, the sense strand plus a 100bp loop were PCR amplified from pIB-TnCasp (Hebert et al. 2008b) using the following primers (5' - CGA TCA AAA TGC TGG ACG GTG - 3', 5' - GAA CCA CGA GTT GTG TTC CTC CAA - 3'). This PCR fragment was then TOPO® cloned into pIB/V5-His-TOPO® (Invitrogen), which allows for the insertion of gene products under the control of the constitutive Opie-2 promoter and allows for the selection of stable cells using blasticidin, creating pIB-TnCasp-Sense. In a separate cloning step, the antisense fragment was first cloned into pCR®-Blunt II-Topo® (Invitrogen) using the following primers (5' - GCA CCG GTC GAT CAA AAT GCT GGA CGG TG - 3', 5' - GCC TCG AGA AGT CTG CAT GCA CAG

GAA T - 3'), creating pCR-TnCasp-Antisense. Next, pIB-dsTncasp-Sense and pCR-dsTnCasp-Antisense were digested using the restriction enzymes AgeI and XhoI, and then ligated using T4 DNA Ligase (NEB) to create pIB-dsTnCasp which retains the blasticidin resistance gene and contains an insert that will form a RNA hairpin loop upon transcription. To create a stable cell line containing this construct, High Five cells were seeded in a 6 well plate at a density of 5×10^5 cells/mL and transfected with 2 μ g of pIB-dsTnCasp. 2 days post transfection, cells were split at a ratio of 1:5 and subject to blasticidin selection at a concentration of 100 μ g/mL based on the manufacturer's recommendation. Several populations that showed resistance to blasticidin were selected and analyzed for Tn caspase-1 transcript and protein levels.

Baculovirus

A recombinant *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) that expresses GFPuv or Chloramphenicol acetyltransferase (CAT) under the control of the polyhedron promoter was created previously (Cha et al. 1999a; Cha et al. 1999b; Cha et al. 1999c). The baculovirus was propagated in High FiveTM cells (Invitrogen), and *Spodoptera frugiperda* (Sf-9) cells (Invitrogen) following the general protocols outlined in O'Reilly et al. The titer of baculovirus was determined by the endpoint dilution method (O'Reilly et al. 1992).

In vitro dsRNA synthesis

Total RNA was extracted using RNAqueous (Ambion) according to the manufacturer's instructions. Cells were sloughed from the well plate, spun at 500 g

for 5 minutes, and lysed using the provided lysis/binding buffer. Following RNA isolation, samples were subjected to a DNase digest as per the manufacturer's instructions in order to remove any contaminating DNA. First strand templates of each target gene were synthesized from 500 ng of total mRNA using oligo-dT primers and Superscript RT III (Invitrogen). 700-900 base-pair regions of first strand DNA templates were PCR amplified by AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen) using gene-specific primers for Tn-caspase-1 (5'-CGA TCA AAA TGC TGG ACG GTG-3'; 5'-AAG TCT GCA TGC ACA GGA AT 3') or CAT (5'-ACT GGA TAT ACC ACC GTT GAT-3'; 5'-GTG CTG GAT ATC TGC AGA ATT-3'). T7 sequences (TAA TAC GAC TCA CTA TAG GGA) were added in a subsequent PCR step, resulting in T7 templates. To make the dsRNA, sense and anti-sense RNA was transcribed from T7 templates using the Megascript™ kit (Ambion) following the manufacturer's instructions. The single-stranded RNA (ssRNA) synthesized was extracted using phenol/chloroform, resuspended in nuclease free water, and then incubated at 65° C for 10 min before cooling to room temperature, annealing the two strands to form dsRNA. dsRNA was checked for size and integrity using agarose gel electrophoresis and then diluted to a concentration of 3 µg/µL.

Differential display

For determining relative transcript levels, RNA was extracted from High Five cells using an RNAqueous kit (Ambion) as described above. RNA concentration was determined by measuring the absorbance of a diluted sample at the 260 nanometer wavelength in a UV spectrophotometer (Beckman). 500 ng of total RNA was subject

to reverse transcription using oligodT primers to obtain the first-strand cDNA template. The cDNA template was subject to PCR with sequence-specific primers: *Tn-caspase-1* (5'-TTC ATT CGA TCC CTG GAT AGC-3'; 5' TAG TAT CCA GGC ACG GTG GAG 3'). PCR products were run on a 1% agarose gel stained with ethidium bromide to compare band intensities under fluorescent light. Primers against a 300 bp section of actin (5'-GAT ATG GAG AAG ATC TGG CA 3'; 5'-GCG TAG CCC TCG TAG ATG-3') were used to PCR amplify a loading control from the reverse transcript. Photographs of agarose gels under UV light were taken with an AlphaImager[®] HP and quantitatively analyzed for band intensity using the “Spot Denso” function of the AlphaEaseFC[™] software package (Alpha Innotech). Exposure times were metered to ensure brightness values were not saturated.

Assay of caspase activity

After various experimental treatments, cells were collected and washed with PBS (pH 7.5). The cells were then suspended in a lysis buffer (from caspase 3 assay kit, BD Bioscience) and incubated on ice for 30 min. Cell debris was removed by centrifugation and supernatants were stored for further use or assayed directly. The tetrapeptide N-acetyl-Asp.Glu.Val.Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was used as the substrate. Cell lysates were added to the reaction buffer (from caspase 3 assay kit, BD Bioscience) with Ac-DEVD-AMC and incubated for several hours at 37°C. The release of AMC was measured using a Spectramax M5 Plate Reader (Molecular Devices) (excitation $\lambda_{380\text{nm}}$, emission $\lambda_{440\text{nm}}$). Caspase activity is defined as the slope of the plot of Fluorescence vs. Incubation time.

Analysis of GFP Expression

GFP fluorescence was measured using a SpectraMax M5 plate reader using an excitation wavelength of 395 nm and an emission wavelength of 509 nm.

Fluorescence was measured *in vivo* using the well scan method of the SoftMax Pro software, which determines well fluorescence by averaging the fluorescence of 21 distinct points throughout the well. Thus, each well could be measured non-invasively and could be used for subsequent time points.

Analysis of CAT Activity

For each time point, cells were harvested by sloughing and spun for 5 minutes at 500g. The media was then removed and saved for later analysis, and the cell pellet was resuspended in 100uL Cytobuster (Novagen) for 5 minutes. Cell lysates were then spun at 10,000 g for 5 minutes to pellet the insoluble lysates. Samples were either analyzed directly or frozen at -20 °C for later analysis. CAT was measured in both the extracellular media and soluble cell lysates using a colorimetric assay. A reaction mix was prepared consisting of 100uM Acetyl CoA (Sigma) and 1mM 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.1 M Tris-HCl buffer (pH 7.8). For each sample, 200 uL of the reaction mix was added to a well in a 96-well plate and allowed to reach 37 °C. Then, an appropriate amount of sample (extracellular media or lysates) was added to the well. Depending upon the concentration of CAT, samples were diluted 5-20 fold in order to fall within the linear range of the assay. Once the sample had been added and allowed to reach 37 °C, 25uL of 5mM chloramphenicol was

added to each well and the absorbance (412nm) was measured over a period of several minutes. CAT activity was determined from the initial slope of the plot of OD vs. time, and normalized by the amount of crude, undiluted sample. The reported CAT activity is a sum of the CAT present in the media and in the cell lysate, scaled appropriately by the relative volume of each.

Results and Discussion

Silencing Tn caspase-1 via in vitro RNAi – Effects on Recombinant Protein Production

In a previous study (Hebert et al. 2008b), we investigated the behavior of Tn-caspase-1 both under chemically induced apoptosis and during baculovirus infection. In addition to showing that Tn-caspase-1 is presumably the main effector caspase present in High Five cells, we demonstrated that the enzymatic activity of Tn-caspase-1 could be linked to the overall level of apoptosis present in High Five cells, and that caspase activity was present during baculovirus infection, especially at 5 days post infection and longer. Thus, an initial goal of this study was to see if *in vitro* suppression of Tn caspase-1 increased recombinant protein production in the baculovirus expression vector system. An overall experimental scheme is shown in Figure 3-1b. dsRNA is first synthesized in vitro from dsDNA before it is transfected at a concentration of 1 $\mu\text{g}/\text{mL}$ into High Five cells that have been seeded in 24 well plates the previous day at a density of 1×10^5 cells/well (2×10^5 cells/mL). In addition to dsRNA against the target gene, two additional conditions were used; no dsRNA and non-specific dsRNA (dsRNA synthesized against CAT). The day following transfection, cells were infected with a recombinant baculovirus. RNA and protein levels were measured during infection using RT-PCR (RNA) and fluorescence (GFP) measurements, respectively.

In order to determine the effectiveness of the dsRNA against Tn caspase-1, total RNA was harvested from each group at 72 hours post infection and analyzed using RT-PCR and differential display. Results are presented in Figure 3-2. Infected

and uninfected cells have nearly identical caspase transcript levels, while there is a slight decrease (~30%) in cells transfected with non-specific dsRNA against CAT. Cells transfected with dsRNA against Tn caspase-1 show a much larger decrease in transcript level, at just under 80% when compared to non-transfected cells, demonstrating that *tn caspase-1* is successfully silenced at the transcriptional level.

Once suppression at the transcript level was confirmed, a recombinant baculovirus producing GFP was used to determine if protein production levels were affected by the silencing. Controls of non-specific dsRNA (dsCAT) and no dsRNA (Infected) were included, along with an additional control of only transfection reagent (Fugene Only). As shown in Figure 3-3, all transfection conditions demonstrated higher protein levels than non-transfected cells from days 3 to 6. However, the highest protein level at those time points was in cells transfected with dsRNA against Tn caspase-1, which was just over 2.5 times higher than infected cells at days 5 and 6.

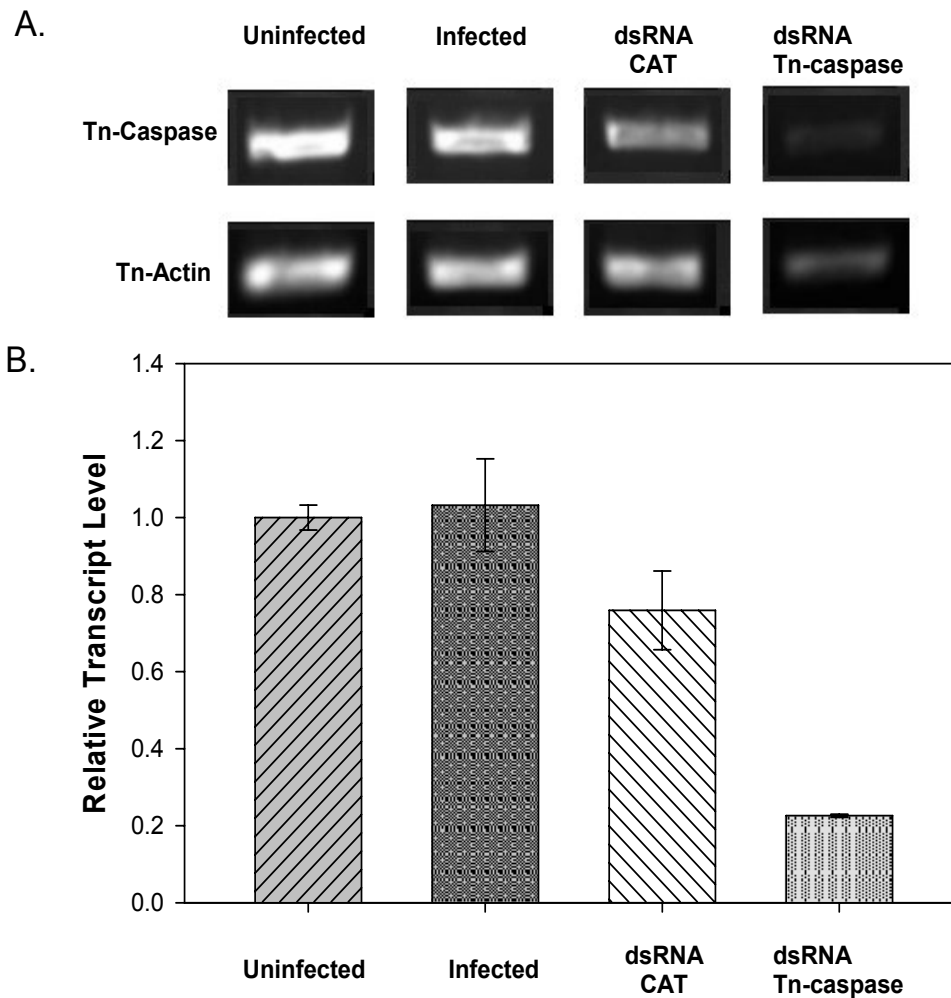


Figure 3-2. RT-PCR results from suppression of Tn-caspase-1 via *in vitro* RNAi during baculovirus infection

Treatment groups included uninfected, infected (no dsRNA), infected + dsRNA against CAT, and infected + dsRNA against Tn-caspase. A. RT-PCR showing transcript levels of both Tn caspase-1 and Actin. Bands were visualized using ethidium bromide under exposure to UV light. B. Graphical representation of the relative transcript levels of Tn caspase-1 under all conditions, normalized to the level of Actin transcript at each condition.

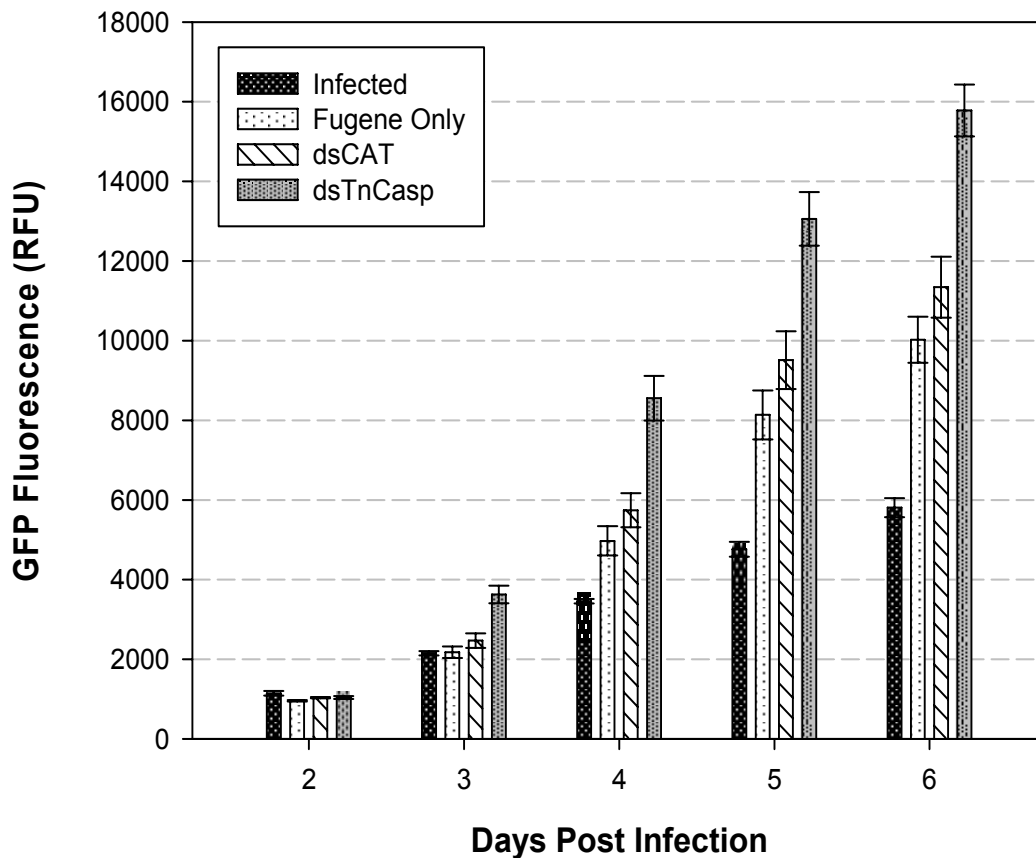


Figure 3-3. GFP production during baculovirus infection of cells treated with *in vitro* RNAi

GFP production levels from days 2-6 post infection during suppression of Tn caspase-1 with *in vitro* dsRNA. Treatment groups include uninfected, infected (no dsRNA), infected + Fugene (transfection reagent), infected + dsRNA against CAT, and infected + dsRNA against Tn-caspase-1.

Protein levels in Tn caspase-1 suppressed cells were also significantly higher than those receiving only transfection reagent or non-specific dsRNA, indicating that the increase was indeed due to a sequence specific effect. This demonstrates that *in vitro* RNAi against Tn caspase-1 is an effective means of increasing recombinant protein production in the baculovirus system.

Development of Tn caspase-1 suppressed cell line

Once the beneficial effects of Tn caspase-1 suppression were verified using *in vitro* dsRNA, steps were taken in order to create a cell line capable of expressing *in vivo* dsRNA against Tn caspase-1 in order to reduce any non-specific effects caused during the transfection process. Using the pIB/V5-His-TOPO[®] plasmid as a backbone, a ~600bp region of Tn caspase-1 plus a 100 bp spacer was PCR amplified from pIB-TnCasp (Hebert et al. 2008b) and cloned into the vector in sense orientation to create pIB-Tncasp-Sense. The same region was PCR amplified using a different set of primers to create pCR-dsTncasp-Antisense, containing the 600bp region of Tn caspase-1 in the opposite orientation. The antisense fragment was then digested using the restriction enzymes AgeI and XhoI and subsequently ligated into pIB-dsTncasp-Sense, forming pIB-dsTncasp (Figure 3-4). Cells were then transfected with pIB-dsTnCasp and transformants were selected using the antibiotic Blasticidin.

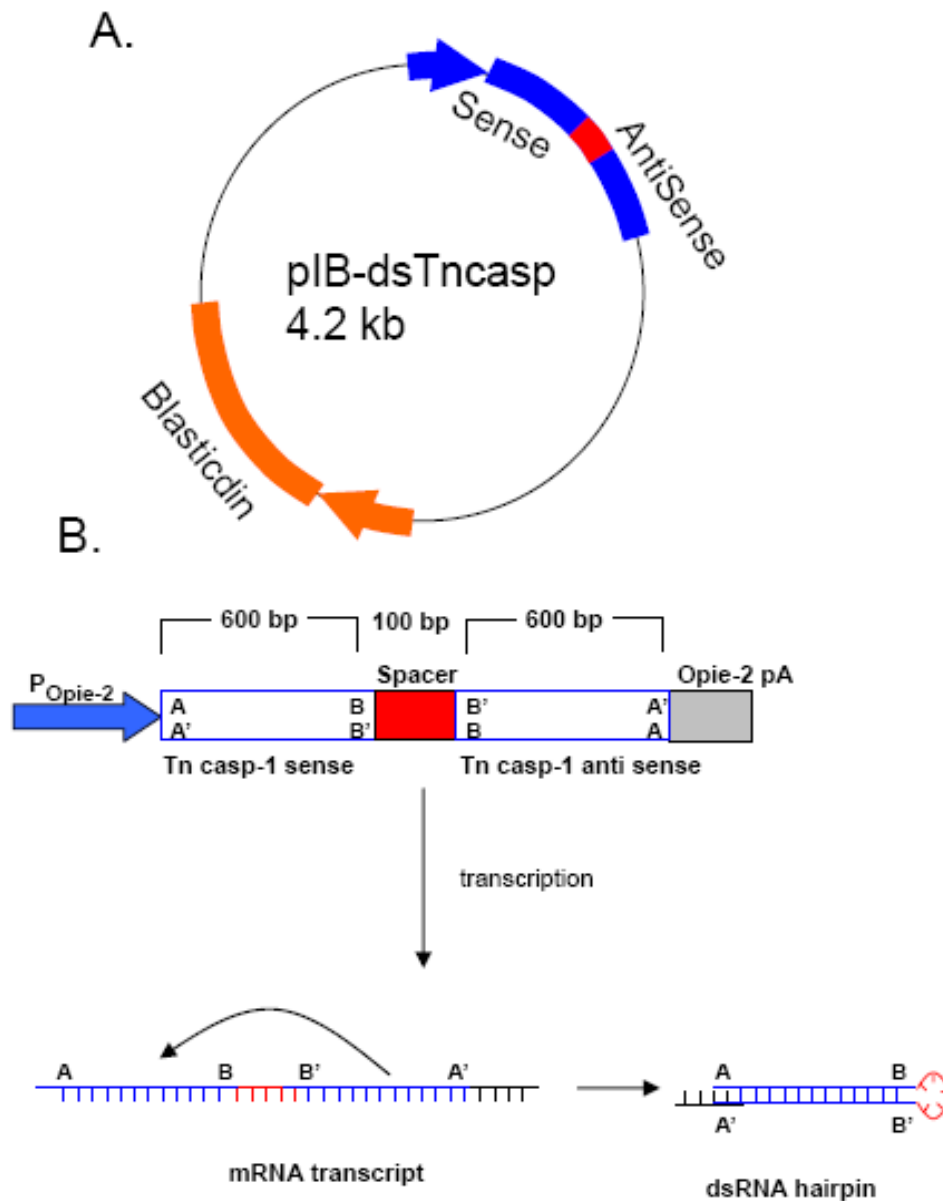


Figure 3-4. Plasmid Design

Plasmid map and detail of the in vivo dsRNA plasmid for the suppression of Tn caspase-1. A. Plasmid map of pIB-dsTncasp, which contains a 600bp portion of Tn caspase-1 in the sense and antisense orientation separated by a 100bp spacer, all under the control of the constitutive Opie-2 promoter. pIB-dsTncasp also contains a Blastidicin resistance gene under the control of the Opie-1 promoter. B. Detail of the dsRNA construct. Upon transcription, the mRNA transcript folds upon itself, forming a dsRNA hairpin, which is recognized by dicer and subsequently processed into siRNA.

Several populations of Blasticidin resistant cells (designated dsTncasp-1 and dsTncasp-2) were then tested for their level of Tn caspase-1 transcription and enzymatic activity. Results are shown in Figure 3-5. Both dsTncasp-1 and dsTncasp-2 show a reduction in Tn caspase-1 transcription levels as shown via RT-PCR (Figure 3-5A). However, dsTncasp-2 has a greater decrease, at 50% versus 40% in dsTncasp-1 (Figure 3-5B). This difference is more apparent when examining the level of caspase enzymatic activity present in dsTncasp-1 and dsTncasp-2 versus control cells (Figure 3-6). As demonstrated previously, Tn caspase-1 and other caspases have minimal activity unless cells are exposed to an apoptotic stimulus, such as the chemical apoptosis inducer actinomycin D (Ahmad et al. 1997; Hebert et al. 2008b; LaCount et al. 2000). Thus, the caspase activity of both dsTncasp-1 and dsTncasp-2 was compared to control cells under normal conditions and after exposure to actinomycin D (24 hours at 2 µg/mL). As shown in Figure 3-6, the caspase activity levels of untreated cells are similar for each cell line. However, when treated with actinomycin D, the caspase activity of control cells jumps to nearly 70 times control levels, while both dsTncasp-1 and dsTncasp-2 show a much smaller increase; 25 and 7 times control levels, respectively. In other words, dsTncasp-1 has a 60% decrease in caspase activity levels when treated with actinomycin D, while dsTncasp-2 shows a nearly 90% decrease when compared to control cells. So while the level of suppression at the transcript level is relatively modest when compared to *in vitro* dsRNA, suppression at the protein level, which is arguably more important, remains quite effective. This large increase in level of suppression when moving from transcript to protein could be explained by the self-activating nature of caspases.

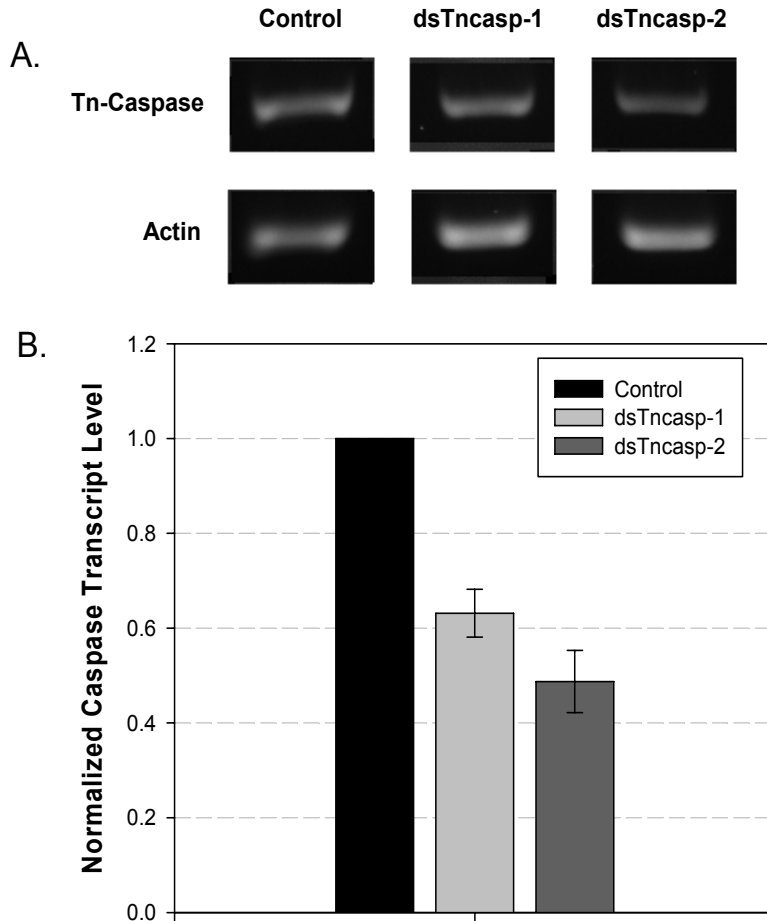


Figure 3-5. In vivo suppression of Tn caspase-1 - RNA Levels

A. RT-PCR of Tn caspase-1 and Actin transcript levels in control High Five cells versus two stable cell populations, dsTnCasp-1 and dsTncasp-2. Bands were visualized using ethidium bromide under exposure to UV light. B. Graphical representation of the relative transcript levels of Tn caspase-1 in standard High Five cells versus stable cell populations, normalized to the level of Actin transcript at each condition.

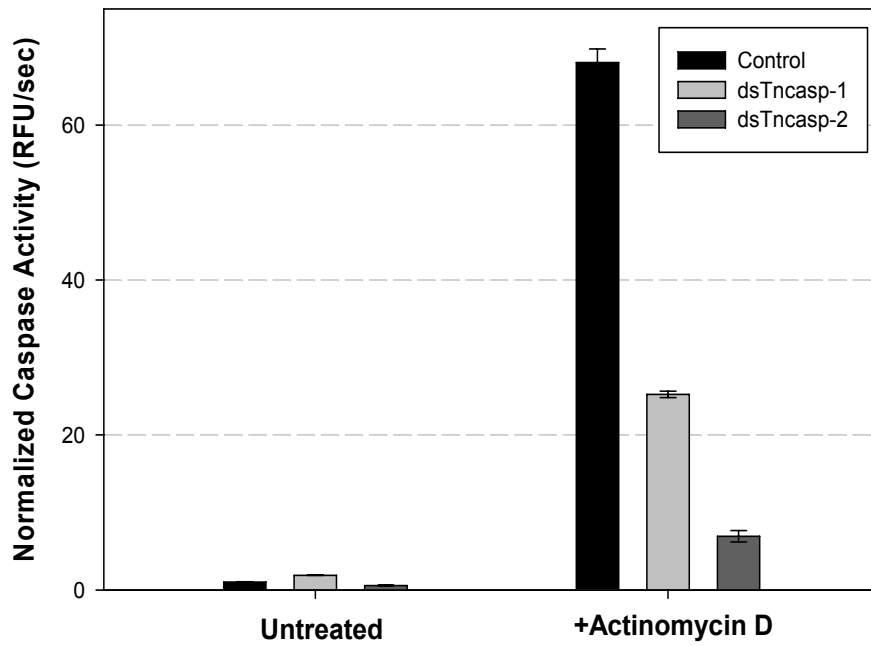


Figure 3-6. In vivo suppression of Tn caspase-1 - Caspase activity levels

Caspase enzymatic activity of standard High Five cells versus the two stable cell populations. Caspase activity was measured from cell lysates in the presence or absence of actinomycin D (24 hours at 2 μ g/mL) and is normalized for each condition to untreated control cells.

Because Tn-caspase-1 presumably plays the role of both effecting apoptosis as well as activating itself, a given reduction in the available amount of inactive Tn-caspase-1 would lead to a proportionally greater decrease in the amount of active caspase. Since dsTncasp-2 showed a greater level of suppression than dsTncasp-1, it was selected for subsequent experiments examining growth, viability, and protein production.

Growth and Viability of Stable cell line

Once the suppression of Tn caspase-1 had been verified, experiments were conducted in order to test the growth characteristics of dsTncasp-2 against control cells. Both lines were seeded at a concentration of 5×10^5 cells/mL in 60mm^2 culture dishes. A total of four culture conditions were used. For both control and dsTncasp-2 cells, one group received 500uL of media each day before counting, while the other group received 500uL of PBS (ph 7.4), in order to maintain a consistent culture volume. Cell count and viability was determined each day for 7 days post seeding. Results are shown in Figure 3-7. From days 0-3, all four groups of cells show similar growth and viability, but difference between the conditions appear on day 4 and beyond. The viable cell count for control cells, both with and without supplemental media, drops sharply from days 4-7. Cells that did not receive media fare the worst, dropping from a count of $\sim 2.6 \times 10^6$ cells at day 3 to $\sim 1.4 \times 10^6$ cells at day 4 and then finally to $\sim 6 \times 10^5$ cells at day 7, only just above the number of cells at seeding. Those cells that did receive media fare better than those without, but still drop steadily from $\sim 2.9 \times 10^6$ cells/mL at day 3 to $\sim 1.3 \times 10^6$ cells/mL at day 7. dsTncasp-2 cells with and

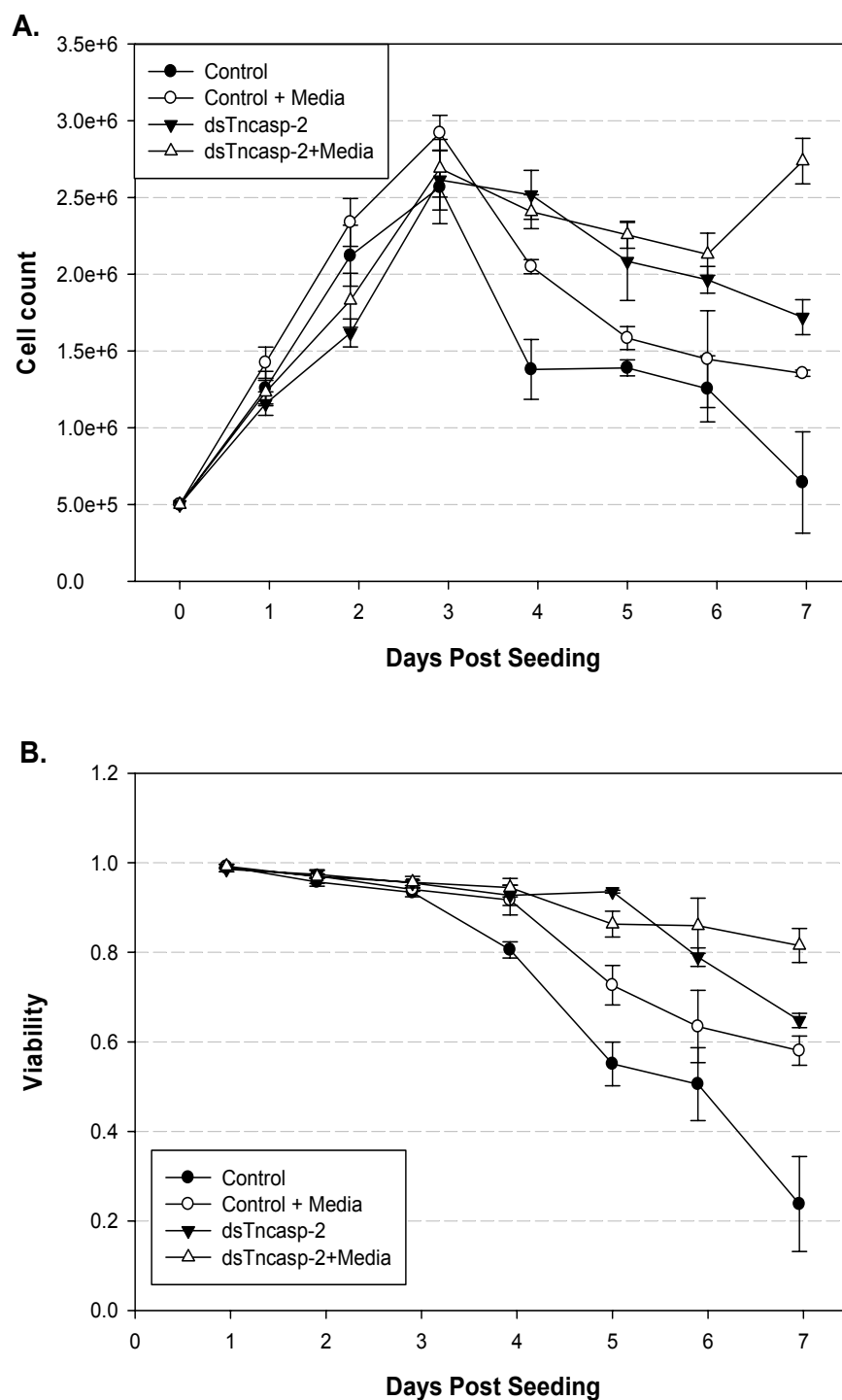


Figure 3-7. Cell growth and viability in suspension culture

Treatment groups included control, control+media, dsTncasp, and dsTncasp+media. In ‘+media’ groups, 500uL of fresh media was added each day before counting. In other groups, 500uL of PBS buffer was added each day before counting to maintain a consistent volume. *A.* Viable cell count of each treatment group, determined using a hemocytometer. *B.* Viability of each treatment group, determined by trypan blue exclusion.

without additional media show greater resilience to the adverse culture conditions. dsTncasp-2 cells that only received PBS did decrease in cell count from days 3-7, but still maintained a higher cell count than both groups of control cells. dsTncasp-2 cells that did receive additional media have similar cell counts from days 4-6, only pulling ahead of the PBS cells at day 7. Viability results show a similar trend. From days 1-3 post seeding, all four groups of cells maintain viability at approximately 95%. However, from days 4-7, both groups of control cells show a rapid decline in viability, dropping to 24% (PBS) and 58 % (media) on day 7. In contrast, dsTncasp-2 cells, especially those that received additional media, maintain higher viability, remaining at 65% (PBS) and 82% (media) on day 7. Since low nutrient concentrations can result in apoptosis (Goswami et al. 1999; Mastrangelo and Betenbaugh 1998), it is likely that the decreased caspase activity present in the dsTncasp-2 cells helps prevent this effect, allowing the cells to maintain higher cell count and viability. The suppression of Tn-caspase-1 seems to me a more effective means of preventing cell death than the addition of media. It is interesting to note that while the addition of media clearly benefits both the cell count and viability of the control group of cells, the effect was less dramatic in the dsTncasp-2 cells, which only showed a significant enhancement of cell count and viability at day 7. It is possible that since dsTncasp-2 cells have a higher tolerance for poor culture conditions, it takes a longer time for the lack of media to have a significant impact, thus delaying the onset of beneficial effects due to additional media. Overall, these results demonstrate that the silencing of Tn caspase-1 is an effective means of maintaining cell count and viability in adverse culture conditions.

Recombinant Protein production with dsTncasp-2 cell line

In order to test whether the suppression of Tn-caspase 1 via *in vivo* dsRNA would lead to increased recombinant protein production, both control and dsTncasp-2 cells were infected with a recombinant baculovirus producing GFP, this time at three different MOIs: 0.1, 1, and 10. Expression levels were measured from days 2-6 and are presented in Figure 3-8a. From days 3-6, GFP levels at all MOIs are significantly ($p < .05$) higher for dsTncasp-2 cells versus control cells, averaging about 1.5 times greater at MOI 0.1 and 1 and 2 times greater at MOI 10. In order to further investigate the performance of dsTncasp-2 cells, additional experiments were conducted using a recombinant baculovirus that produces Chloramphenicol acetyltransferase (CAT). Both control and dsTncasp-2 cells were again infected at MOIs 0.1, 1, and 10. Results are shown in Figure 3-8b. Although the difference is not as dramatic as with GFP, dsTncasp-2 cells again show higher levels of recombinant protein, especially at 6 days post infection, when both MOI 1 and 10 show a significant increase in CAT production (2.4x and 1.6x respectively). Overall, these results demonstrate that suppression of Tn caspase via *in vitro* dsRNA is an effective means of increasing protein production in the baculovirus expression vector system. As such, we successfully created several cell lines expressing a dsRNA hairpin that resulted in the constitutive suppression of Tn-caspase 1 at both the transcript and protein levels. Based on its high level of suppression, dsTncasp-2 was chosen for studies examining growth, viability and protein

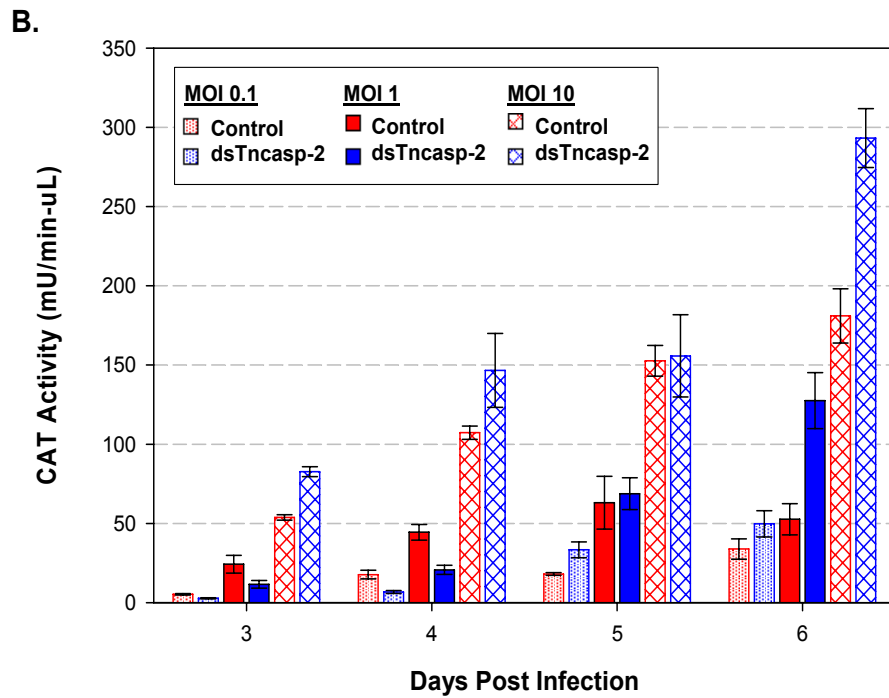
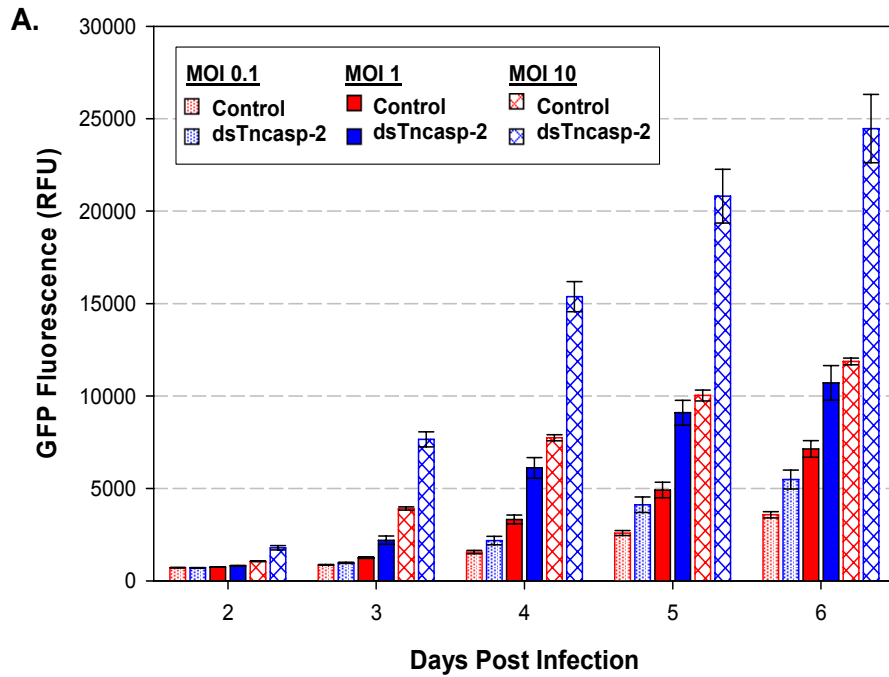


Figure 3-8. Protein production of dsTnCas2 versus control cells with varying MOI and a recombinant baculovirus producing either GFP or CAT
 A. GFP production at MOI 0.1, 1.0, and 10 from 2-6 days post infection. B. CAT production at MOI 0.1, 1.0, and 10 from 3-6 days post infection. GFP was measured *in vivo* using a fluorescence plate reader, while CAT activity was determined using the enzymatic assay described in materials and methods.

production. dsTncasp-2 cells demonstrated an ability to maintain high cell densities and viability under low nutrient conditions, as well as superior protein productivity upon infection with recombinant baculoviruses producing both GFP and CAT. Finally, this work continues to support the application of RNAi in metabolic engineering for the improvement of recombinant protein production and the development of cell lines with desirable production phenotypes.

Chapter 4 Conclusions and Future Directions

Conclusions

Investigation of Tn-caspase-1 in High Five Cells

In chapter 2, the novel lepidopteron effector caspase Tn-caspase-1 was identified based upon an existing genbank sequence. While Sf-caspase-1, the primary effector caspase in Sf cells, had been well characterized, the same was not true for *T. ni* derived cell lines. This, coupled with the differences in the infectivity of p35 negative baculoviruses that have been reported between Sf derived cells and *T. ni* derived cells, warranted an investigation into the nature of caspases in *T. ni* cells. Therefore, in order to further characterize this protein and its function, a vector was constructed to overexpress Tn-caspase-1 and chemically synthesized dsRNA was used to suppress Tn-caspase-1. While overexpressed Tn-caspase-1 only temporarily increased caspase enzymatic levels during actinomycin D chemically induced apoptosis, dsRNA mediated suppression of Tn-caspase-1 resulted in decreased caspase enzymatic levels both in the presence and absence of actinomycin D (30 and 15 percent of control, respectively). This reduction in caspase activity was accompanied by a decrease in the overall level of apoptosis present in the cells, as measured by several indicators, including cellular morphology, DNA fragmentation, and fluorescent TUNEL staining. As a result, it became evident that by manipulating the level of Tn-caspase-1, one could affect the overall level of apoptosis present in High Five cells.

Since Sf-caspase-1 is known to be inhibited by the baculovirus protein p35 (Ahmad et al. 1997; LaCount et al. 2000; Manji and Friesen 2001), another important area of consideration was the behavior of Tn-caspase-1 during baculovirus infection. It was discovered that during baculovirus infection, levels of Tn-caspase-1 remained below uninfected cells for the first 4 days post infection. Beyond 4 days, the level of Tn-caspase-1 enzymatic activity in baculovirus infected cells increased to nearly 20 fold the initial level in uninfected cells, though this increase was far less (only 2.5 fold) in infected cells that had also been treated with dsRNA targeting Tn-caspase-1. This data suggests that at a certain point, the baculovirus proteins can no longer fully inhibit caspase activity, though the reason for this is at the moment unclear. Perhaps at a certain level, p35 and other baculovirus proteins can no longer compete with the upregulation of Tn-caspase-1, resulting in a subsequent increase in caspase activity level.

In order to more fully investigate the behavior between Tn-caspase-1 and p35, a vector was constructed to express p35 in the absence of baculovirus infection. In both the presence and absence of actinomycin D induced apoptosis, expressing p35 reduced both the caspase activity of cells as well as the overall level of apoptosis. In untreated cells and during the early stages of apoptosis, p35 was more effective than dsRNA at decreasing caspase activity levels. This is likely due to the fact that levels of active Tn-caspase-1 are relatively low, allowing p35 to effectively inhibit nearly all the active Tn-caspase-1 present in the cell. Alternatively, while dsRNA can substantially lower transcript levels, it cannot fully eliminate translation, resulting in a small but detectable amount of active caspase. As apoptosis progresses, the situation

is reversed. At this point, levels of Tn-caspase-1 may have increased to a level where p35 can no longer completely inhibit its cleavage and activation. However, by lowering transcript levels, dsRNA has decreased the amount of caspase available for activation, thereby limiting the pool of *tn-caspase-1* available for translation. It is important to note however, that the performance of p35 and dsRNA might change when presented with alternative apoptotic stimuli. Since actinomycin D is a transcriptional inhibitor, it may affect p35 in a manner different than it does dsRNA. Thus, further investigation is warranted to determine the exact nature and relative effectiveness of p35 and dsRNA under a wide range of apoptotic conditions.

RNAi based suppression of Tn-caspase 1 for increased recombinant protein production

In chapter 3, RNAi based suppression of Tn-caspase-1 is used to enhance recombinant protein production in the baculovirus expression system. Despite its high productivity, the baculovirus expression system can be limited by its induction of host cell apoptosis and high protease activity. As demonstrated in chapter 2, RNAi based suppression of Tn-caspase-1 leads to not only a decrease in the caspase activity level, but also a reduction in apoptosis. Thus, Tn-caspase-1 became an ideal target for increasing recombinant protein production as well. Initial studies using chemically synthesized *in vitro* dsRNA demonstrated that Tn-caspase-1 transcript levels could be reduced by 80 percent, resulting in GFP levels greater than 2 times that of untreated cells. However, some non-specific effects relating to the transfection process were observed. In order to help address this problem as well as take advantage of the robustness and relative simplicity of an *in vivo* approach, a

plasmid was developed containing a construct designed to integrate a dsRNA hairpin that could be used to produce *in vivo* RNAi. Several populations of cells were created using this plasmid and tested for their level of Tn-caspase-1 suppression. One particular cell line, dsTncasp-2, achieved a 50 percent decrease in Tn-caspase-1 transcript levels, which resulted in a nearly 90 percent decrease in caspase enzyme activity during actinomycin D induced apoptosis. dsTncasp-2 was also tested for its ability to remain viable under low nutrient growth conditions and demonstrated superior viability when compared to standard High Five cells. Finally, dsTncasp-2 demonstrated increased recombinant protein production using two different model proteins, GFP and CAT, resulting in as much as twice the production of control cells.

RNAi as a metabolic engineering tool

One of the main goals of this project was to further the Bentley lab's application of RNAi as a tool for metabolic engineering, specifically through the application of *in vivo* RNAi. While traditional metabolic engineering generally adds new proteins or pathways, RNAi can present a more nuanced approach capable of optimizing the host natural protein production system by taking advantage of pre-existing regulatory mechanisms or pathways. Although research in RNAi has certainly exploded in the medical arena as a next generation therapeutic, it has seen relatively little use in metabolic engineering, resulting in opportunities for further research, some of which are discussed below. As RNAi technology continues to develop, its application in the field of metabolic engineering will continue to expand.

Future Directions

Bacterial expression of Tn-caspase-1

In order to conduct *in vitro* studies with Tn-caspase-1, it must be effectively produced and purified. Although Tn-caspase-1-HIS was produced in High Five cells, it proved difficult to produce large amounts of pure activated Tn-caspase-1-HIS due to the background of native Tn-caspase-1. Thus, expression in bacterial systems was conducted using the pTrcHis vector system in the TOP10 strain of *E. coli*. While an approximate yield of 0.5 mg/mL was achieved as measured by UV absorbance and the Bradford assay, no measurable caspase activity was detected. However, literature results and personal communications indicate that the expression of active caspases, including Sf-caspase-1, in *E. coli* is possible using the BL21 production strain and the pET200 vector system. Thus, if future studies require active *in vitro* Tn-caspase-1, production should be attempted using those conditions.

Tn-caspase-1 and baculovirus proteins p35 and FP-25

The major difference between Sf versus *T. ni* derived cell lines is their behavior when infected with p35 negative baculoviruses. In Sf derived cell lines, infection with a p35 negative baculovirus prevents both protein production and virus replication, while viruses replicate normally in *T. ni* derived cells (Clem and Miller 1993). Recent studies have shown that baculoviruses lacking both p35 and another protein, FP-25, have resulted in apoptotic characteristics in TN-368 cells, suggesting it also interacts with apoptotic proteins in lepidopteron cells. It would be interesting to examine the interactions of Tn-caspase-1 with FP-25 *in vivo*, as well as its

interactions with both p35 and FP-25 *in vitro*, and the kinetics and stoichiometry of Tn-caspase-1 – baculovirus protein interactions.

Additional RNAi targets and host systems

While Tn-caspase-1 proved to be an effective metabolic engineering target, an additional goal would be to develop a cell line in which several genes were suppressed simultaneously. This could potentially further increase a cell line's ability to cope with the stresses associated with recombinant protein production in general and baculovirus infection in particular. However, one of the difficulties in working with lepidopteron cell lines is the relative lack of genomics data to widely screen and identify novel targets. Thus, such a technique could be attempted in either *Drosophila* S2 cells, or HEK293 (Human Embryonic Kidney) cells. HEK293 cells have emerged as a promising cell line for recombinant protein production (Han et al. 2007; Sun et al. 2006), including adeno-associated virus particles (Hildinger et al. 2007). This, coupled with the vast amount of human sequence data and readily available microarrays would make HEK293 cells an excellent choice for such a study. *Drosophila* S2 cells have been used in the past by the Bentley lab and others for the production of recombinant protein (March and Bentley 2006; March and Bentley 2007; McCarroll and King 1997), are genetically well characterized, and also have readily available microarrays. By taking advantage of these additional genetic tools, additional targets could be identified and implemented

Target identification and implementation strategies

One method for identifying new targets would be to expose cells to a number of focused stresses designed to simulate the conditions faced during recombinant protein production, coupled with corresponding microarray analysis to determine which genes are up- and down-regulated during this process. Through the exposure of cells to focused stresses including hypoxia, ER stress, and apoptosis and then subjecting them to a time dependent microarray analysis, genes associated with the presence of abnormal proteins and their processing could be identified. In order to tie transcriptional data to the desired phenotype (increased recombinant protein production); several “stressing” strategies will be linked to protein expression using reporter cell lines created in the Bentley lab that express red fluorescent markers (DsRed or HcRed) and a secreted product, human interleukin-2 (IL-2) (Yung et al. 2006b). Cells could be sorted based on their fluorescence and used to determine the transcriptional landscape present during the high production of recombinant protein. Genes that are differentially expressed could then be selected as metabolic engineering targets for either RNAi or overexpression.

Since the Bentley lab has previously demonstrated the importance of tuning the level of RNAi based suppression (March and Bentley 2007) and increasing cell growth rate through RNAi (March and Bentley 2006), one could envision a production scheme for first improving growth rate of cells through the suppression of a specific set of genes, such as cell growth and metabolic regulators, and then transitioning cells into a production mode in which a different set of genes is targeted, such as proteases or cell death regulators (Figure 4-1). Through the use of directed

stresses and subsequent microarray analysis, these key regulators could be identified and implemented in a dynamic and focused manner to increase recombinant protein production.

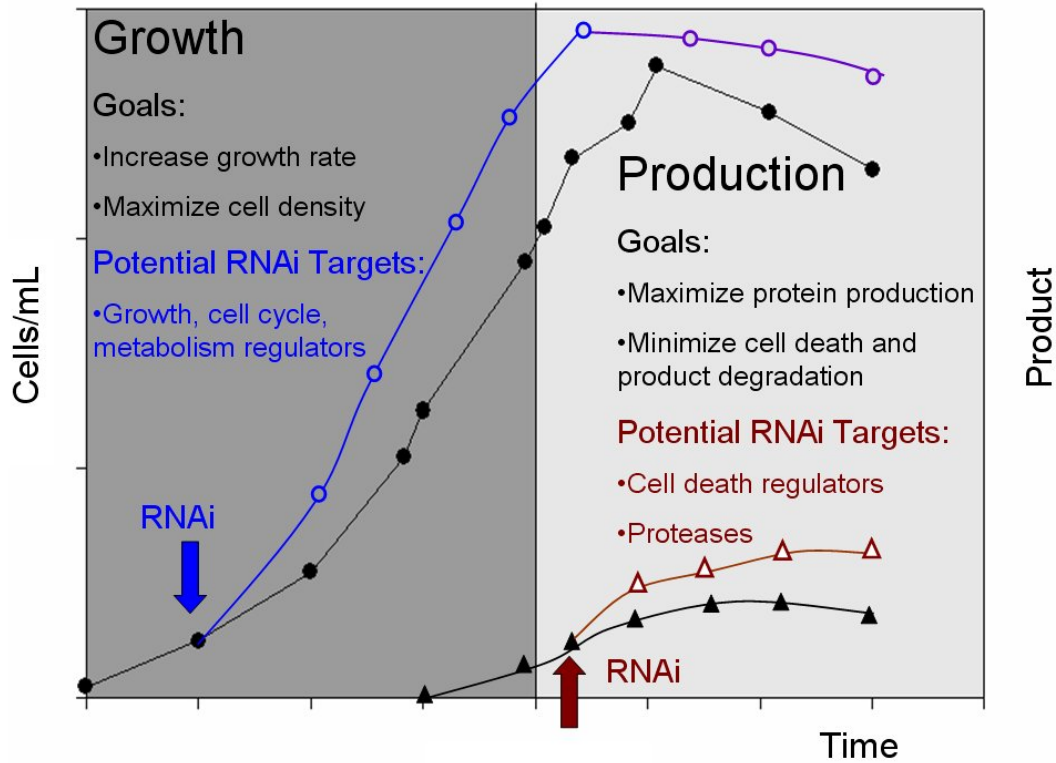


Figure 4-1. Regulating multiple RNAi targets throughout the production cycle.
In order to maximize the potential of RNAi as metabolic engineering tool, targets will be identified for regulation during both the growth and production phases.

Chapter 5 Appendix

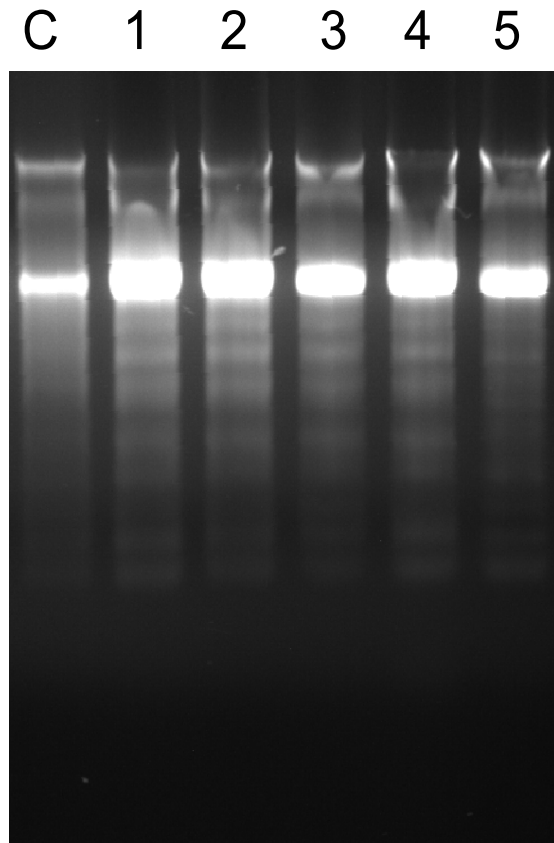


Figure 5-1. DNA laddering following exposure to actinomycin D.

2 ug of DNA was run on a 1.5% agarose gel and visualized using UV light. The lanes are as follows: C - control/no actinomycin D, 1 - actinomycin D at a concentration of 1 ug/mL, 2 - 0.5 ug/mL, 3 - 0.25 ug/mL, 4 - 0.1 ug/ mL, 5 - 0.05 ug/mL. Actinomycin D was added for a period of 24 hours in all samples (except control).

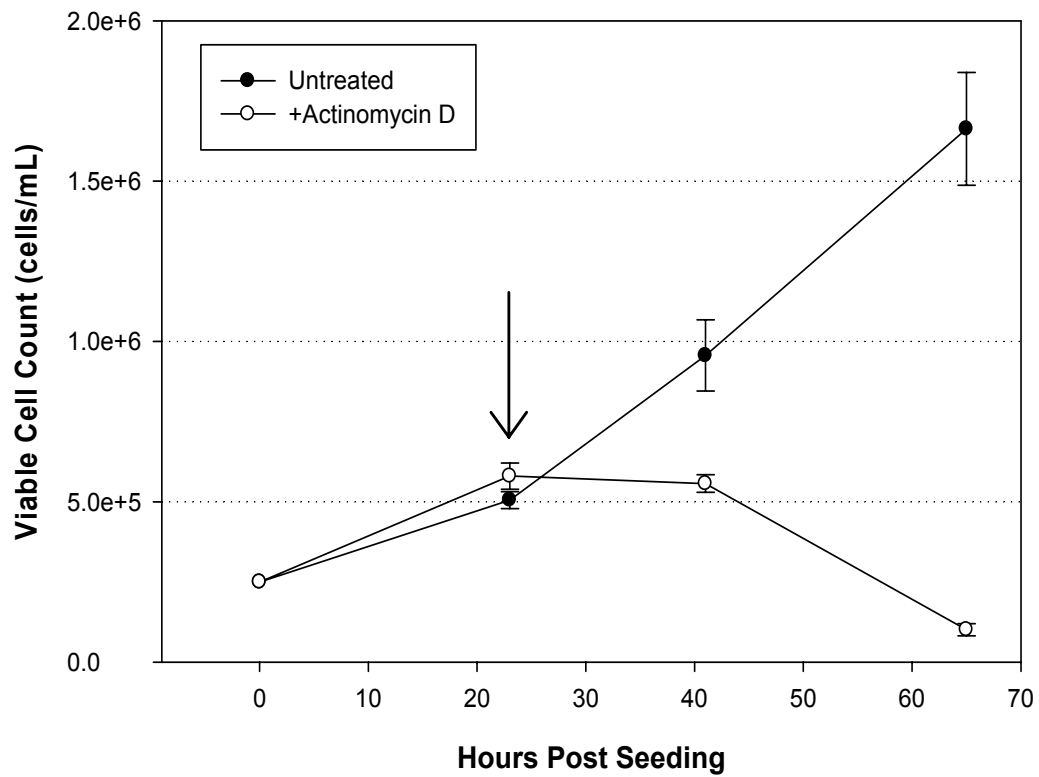
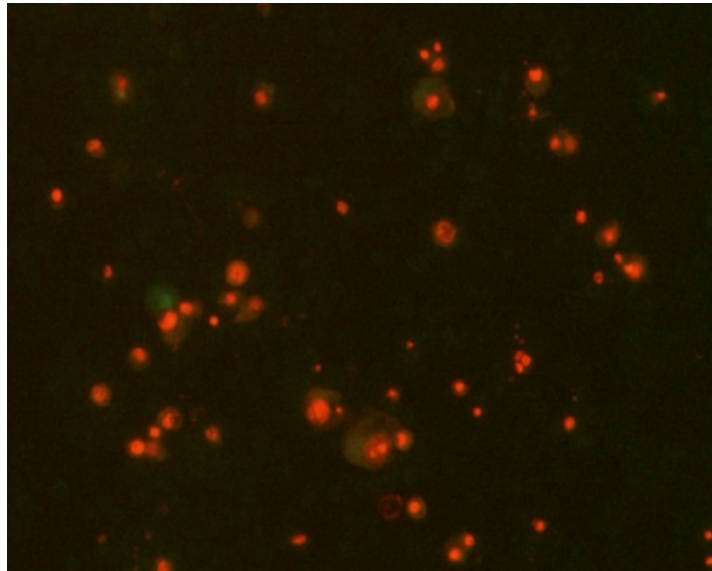


Figure 5-2. Viable cell count following exposure to actinomycin D.

Cells were seeded at a density of 250,000 cells/mL and grown for 24 hours before the addition of 2 μ g/mL actinomycin D (indicated by the arrow). Viable cell count was determined using a hemocytometer and trypan blue exclusion.

Untreated



**+ Actinomycin D
24h**

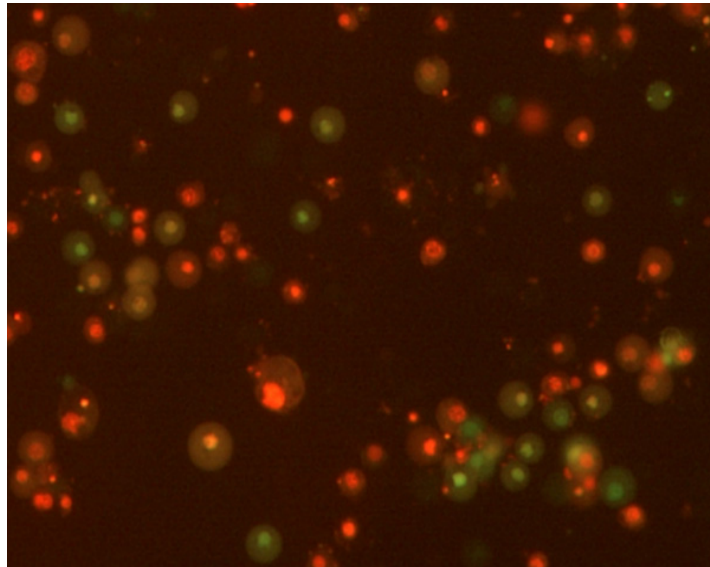


Figure 5-3. Cell labeling

Cells were exposed to actinomycin D for 24 hours and then labeled using the Vybrant[®] Apoptosis Assay Kit #4 (Invitrogen), which labels necrotic cells red using Propidium Iodide and apoptotic cells green using YO-PRO[®]-1. Pictures were taken at 100x magnification and identical exposure lengths. Cells were either untreated or exposed to actinomycin D (24 hours at 2 ug/mL).

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