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Regulation of translation during adenovirus infection

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Regulation of Translation during Adenovirus Infection

by

Demetris C. Iacovides

DISSERTATION

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DOCTOR OF PHILOSOPHY

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of the

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To my beloved parents, who sacrificed everything to give me the world.

As you set out for Ithaca hope the journey may be long, full of adventure, full of discovery. May there be many a summer morning when with what pleasure, what joy, you enter harbors you're seeing for the first time. Keep Ithaca always in your mind. Arriving there is what you're destined for. But don't hurry the journey at all, Ithaca gave you the marvelous journey. Without her you wouldn't have set out.

Konstantinos P. Kavafis

Acknowledgements

As I am finishing the writing of this dissertation, I realize that it has proven to be a far more challenging task than I ever expected. Because of the five years of hard work that needed to be compiled into a book, because of all the scientific questions that still remain unanswered, even after these five years, and because of the numerous more questions that arose during this project. Still, nothing proved to be harder than writing this last piece, since it represents the most important of the experiences I had during Graduate School: the invaluable interactions and close relationships I developed with many of my mentors, my colleagues and my friends. Indeed, how would this dissertation even become possible, without your support? To all of you, I give my love and my gratitude, and I wish you all the best in every aspect of your lives!

First and foremost, I would like to thank my professor, Frank McCormick, for all the opportunities he offered me during the time I worked in his lab. Frank has been a great mentor a friend, and my role model, and the knowledge I gained from working closely with him is priceless. Dear Frank, from the bottom of my heart, I thank you! It has been an honor working with you!

To my dearest friend, colleague and mentor Clodagh O'Shea. Without you, your guidance and your support, none of these would have been possible. Thank you for believing in me, and for all the reagents, the advice and the knowledge that

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agåin soon, in or out of the lab, and live many more unforgettable moments together as a group!

My time in Graduate School has been an extraordinary journey, filled with unremarkable experiences. I met and interacted with hundreds of scientists, and developed strong relationships with many of them. And yet none of this work would have ever become possible without the love and the support from my beloved friend Maria Christophorou. She stood by me during all these years, and never failed to be there when I needed her; as a friend, as family and as a loving partner. My dearest Maria, I wish you all the best in your career, and a lifetime of great achievements and happiness! Βρεφος μου σ'ευχαριστώ!

Finally, I would like to dedicate this dissertation to my wonderful parents. Without their support, their love and their encouragement, I would have been nothing! My beloved parents, thank you for always being by my side, thank you for comforting me through all the tough times, and thank you for always believing in me, even when I stopped believing in my self…This dissertation is dedicated to you!

Regulation of Translation during Adenovirus Infection

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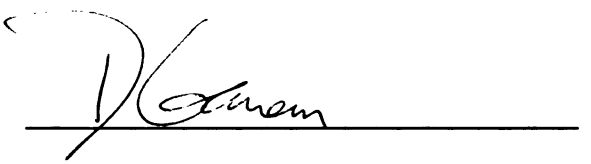

Donald E. Ganem, MD., Committee Chairman

Abstract

During the late stages of infection, adenoviruses inhibit cap-dependent host protein synthesis and allow the selective translation of viral mRNAs through ribosome shunting. This process is mediated by the non-structural L4100k protein, which also regulates hexon trimerization and assembly in the nucleus. However, it is not known how L4100k switches between these two functions. In this study, we show that the multifunctional properties of L4100k are regulated, at least in part, by arginine methylation of the RGG domain within the C-teminus of the protein, and that arginine methylation is a critical cellular function necessary for productive adenovirus infection.

To further understand the multiple functions of L4100k and the role this protein plays during adenovirus infection, we also identified novel cellular and viral proteins that interact with L4100k during the early and the late stages of infection. L4100k-interacting proteins included factors that have functions in transcription, translation, DNA damage response, RNA metabolism, and assembly of the virus capsid. These results suggest the exciting possibility that L4100k has a wider role during infection than previously recognized, and may function as a general modulator of many important processes during the late phase of infection. Finally, the identification in this study of novel L4100k-interacting proteins that have functions in translation regulation is an important first step towards our understanding of the mechanism by which adenoviruses modulate this process during infection, and should provide additional insights into the mechanisms of translational control in mammalian cells.

Declaration

This dissertation is the result of my own work. Some figures have been included which resulted from the work of other researchers and are published elsewhere and these have been appropriately attributed in the figure legends.

This work was carried out at the Comprehensive Cancer Center at the University of California, San Francisco from October 2001 to March 2006. This work was supported, in part, by UC Discovery Grant # bio-02-10242 and by ONYX Pharmaceuticals.

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CHAPTER 1

INTRODUCTION

1.1. *ADENOVIRIDAE* FAMILY

The adenoviruses constitute the Adenoviridae family of viruses, which is divided into two genera, Mastadenovirus and Aviadenovirus (Norrby et al., 1976). The Aviadenovirus genus is limited to viruses that only infect birds, whereas the Mastadenovirus genus includes serotypes with a wide host range which includes human, simian, murine, bovine, equine, porcine, ovine, canine and opossum viruses. There are forty nine different human adenovirus serotypes identified to date, classified into six different subgroups (A-F) based on their ability to agglutinate red blood cells (Rosen, 1960).

1.1.i *Pathogenesis and Pathology*

Adenoviruses were first isolated in 1953 from tonsils and adenoidal tissue surgically removed from children, when Rowe and colleagues recognized that a viral agent was causing degeneration of the epithelial-like cells (Rowe et al., 1953). Soon after similar viral agents were also isolated from febrile military personnel with a variety of respiratory illnesses (Hilleman and Werner, 1954; Huebner et al., 1954) and were subsequently called acute respiratory disease (ARD) agents. Epidemiologic studies confirmed that adenoviruses cause a wide spectrum of acute respiratory syndromes and also identified several serotypes as the causal agent of keratoconjunctivitis (EKC), acute hemorrhagic cystitis, hepatitis, gastroenteritis and myocarditis (De Jong et al., 1999; de Jong et al., 1983; Hierholzer et al., 1988) (Numazaki et al., 1973) (Schnurr and Dondero,

1993). However, the most common sites of infection are the respiratory tract, the eye and the gastrointestinal tract. Less frequently, adenoviruses can infect the urinary bladder and the liver ((Howley, 2001)). In most cases, these infections are subclinical and result in protective antibody production, which prevent re-infection with the same viral serotype. Even so, adenoviral infections of immunocompromised individuals can be more serious and may even result in fatalities.

Some adenovirus serotypes can induce malignant tumors in rodents (Trentin et al., 1962) and transform cells in culture. The transforming potential of adenovirus is attributed to the action of two early viral gene products, E1A and E1B, which are sufficient to induce transformation in cultured cells. However they have not been associated with any malignancies in humans (Green et al., 1979), which suggests a fundamental difference in the biology of rodent and human cells in terms of their response to adenovirus oncoproteins. Recent experiments have shown that activated telomerase can cooperate with SV40 T antigen plus an oncogenic H-ras protein to efficiently transform human fibroblasts to a tumorogenic phenotype (Hahn et al., 1999). T antigen plus H-ras fail to induce the same phenotype in the absence of activated telomerase. Therefore, the inability of adenovirus to induce tumors in humans may be at least in part due to the lack of telomerase expression in human somatic cells. Murine cells express telomerase and have longer telomeres, which may explain the transforming potential of E1A and E1B in these cells. Nevertheless, the inability of adenoviruses to induce tumors in humans may also be attributed to the fact that

they produce lytic, and not latent infections. Thus, they do not incorporate their genomes into the chromosome, and therefore do not express E1A and E1B for long enough periods to produce tumors. In contrast, infection of rodent cells is blocked at an early stage after virus entry, and viral replication and subsequent cell lysis is inhibited (Fognani et al., 1993; Lucher, 1995), which could result in persistent E1A/E1B expression and cell transformation.

1.1.ii *Virion Structure*

Adenoviruses are icosahedral particles consisting of a protein shell (capsid) surrounding a DNA-containing core. The protein component of the virus accounts for 87% of the total viral mass, whereas the DNA accounts for the rest 13%. The capsid is composed of 252 subunits (capsomeres) of which 240 are hexons and 12 are pentons, and each penton contains a base and a projecting fiber. The fiber part of the virion, the length of which varies among different serotypes, mediates the primary interaction with the cellular receptor on the cell surface and thus is the main determinant of viral tropism. Most adenovirus serotypes bind to the cell via a strong interaction of the fiber with the Coxsackie-Adenovirus Receptor (CAR) present in the tight junctions of epithelial cells. However members of at least one subgroup (subgroup B) enter the cell via a CAR-independent mechanism which involves binding to CD46 (Gaggar et al., 2003), and several other serotypes have been proposed to have secondary, CAR-independent mechanisms of entry (Hong et al., 1997). The mechanism of

attachment and internalization of human adenoviruses will be discussed in further detail in subsequent chapters of the introduction.

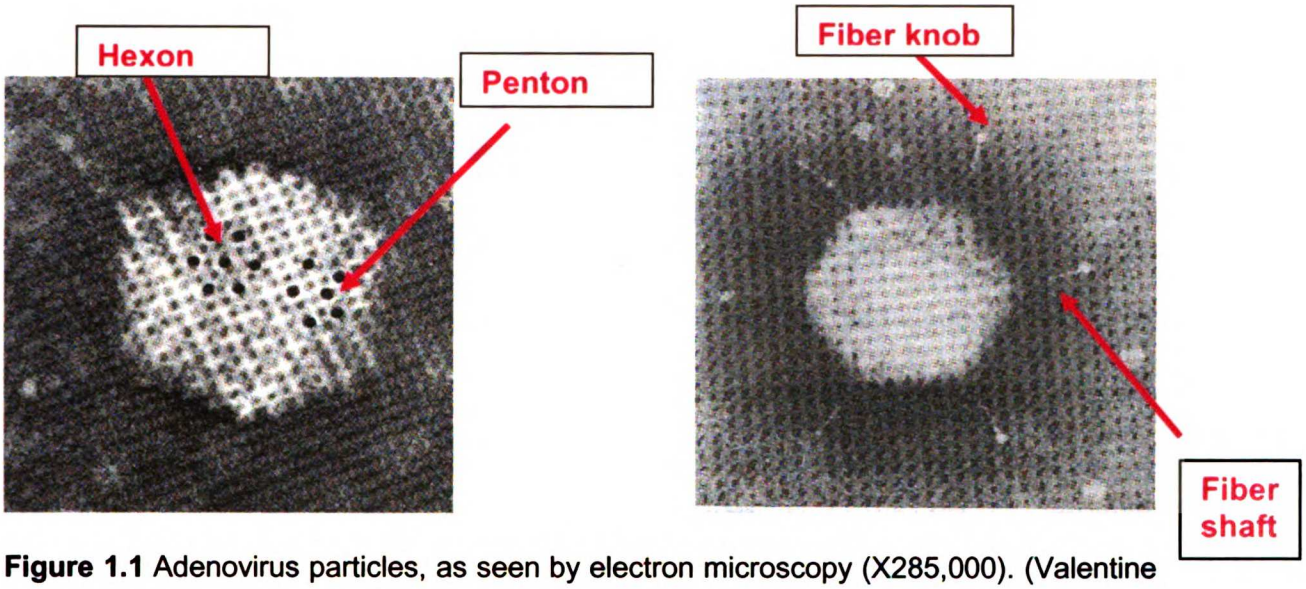


Figure 1.1 Adenovirus particles, as seen by electron microscopy (X285,000). (Valentine and Pereira, 1965). To the left, one of the 240 hexons and one of the 12 penton capsomeres are surrounded by six and five neighboring hexons respectively. To the right, 6 out of 12 fibers are shown projecting from penton capsomeres on the viral capsid. *Adapted from (Howley, 2001).*

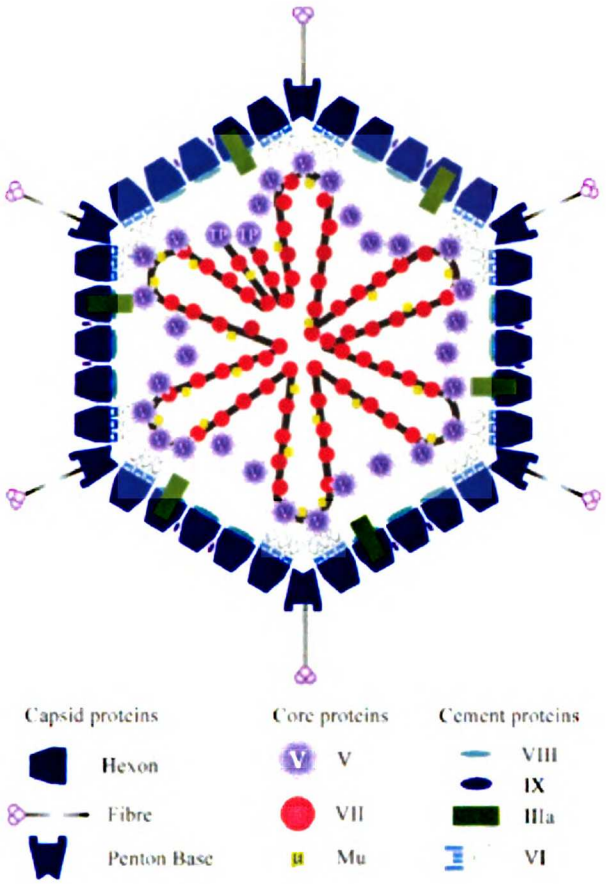


Figure 1.2 Cartoon of the structural components of adenovirus (Russell, 2000)

1.1.iii *Viral Genome*

The adenovirus genome is a linear, double stranded DNA molecule of 36kb, and all human adenoviruses examined to date present the same general functional organization. The genome has two identical origins for DNA replication, one at each of the two terminal repeats, and a cis-acting packaging sequence (Grable and Hearing, 1992) (Hammarskjold and Winberg, 1980), (Hearing and Shenk, 1983), (Hearing and Shenk, 1986) at the end of the viral chromosome which mediates the interaction of the DNA and the encapsidating proteins (Hearing et

al., 1987). The chromosome consists of eight transcription units- five early (E1A, E1B, E2, E3 and E4) two delayed early (IX and IVa2) and one late unit (major late) - based on the time that they are transcribed during infection (reviewed in (Pettersson, 1986). Even so, early genes continue to be expressed during the late stages of infection, and the major-late promoter is transcriptionally active at low levers soon after infection.

Both strands of the viral DNA are transcribed, and all transcription units are transcribed by RNA polymerase II. The genome also contains one or two (depending on the serotype) virus-associated genes transcribed by RNA polymerase III. Even though little is know about the evolutionary considerations that led to the current genome organization, it seems that the specific arrangement of the transcription units may serve a timing function and therefore their location on the chromosome might determine the order in which they are expressed relative to each other during the infection cycle (Howley, 2001).

Each of the viral transcripts give rise to multiple mRNAs, which are then differentiated by alternative splicing to give rise to different gene products (Berget et al., 1977), some of which are partially related in sequence, whereas others have no sequence homology. Many of the polypeptides encoded from the same transcription unit have related functions during infection and are involved in similar processes of the virus lifecycle. Thus, the E1A transcription unit encodes two proteins which activate transcription and push the cell into S-phase; E1B encodes two products that block apoptosis, whereas the E2 unit encodes genes involved directly in viral DNA replication. The E3 region encodes genes that

modulate the host immune response against the infection, and the major-late transcriptional unit encodes the structural proteins of the virus, along with two non-structural polypeptides which are involved in capsid formation and virion assembly (Cepko and Sharp, 1982) (Howley, 2001).

Transcription of the adenovirus genome

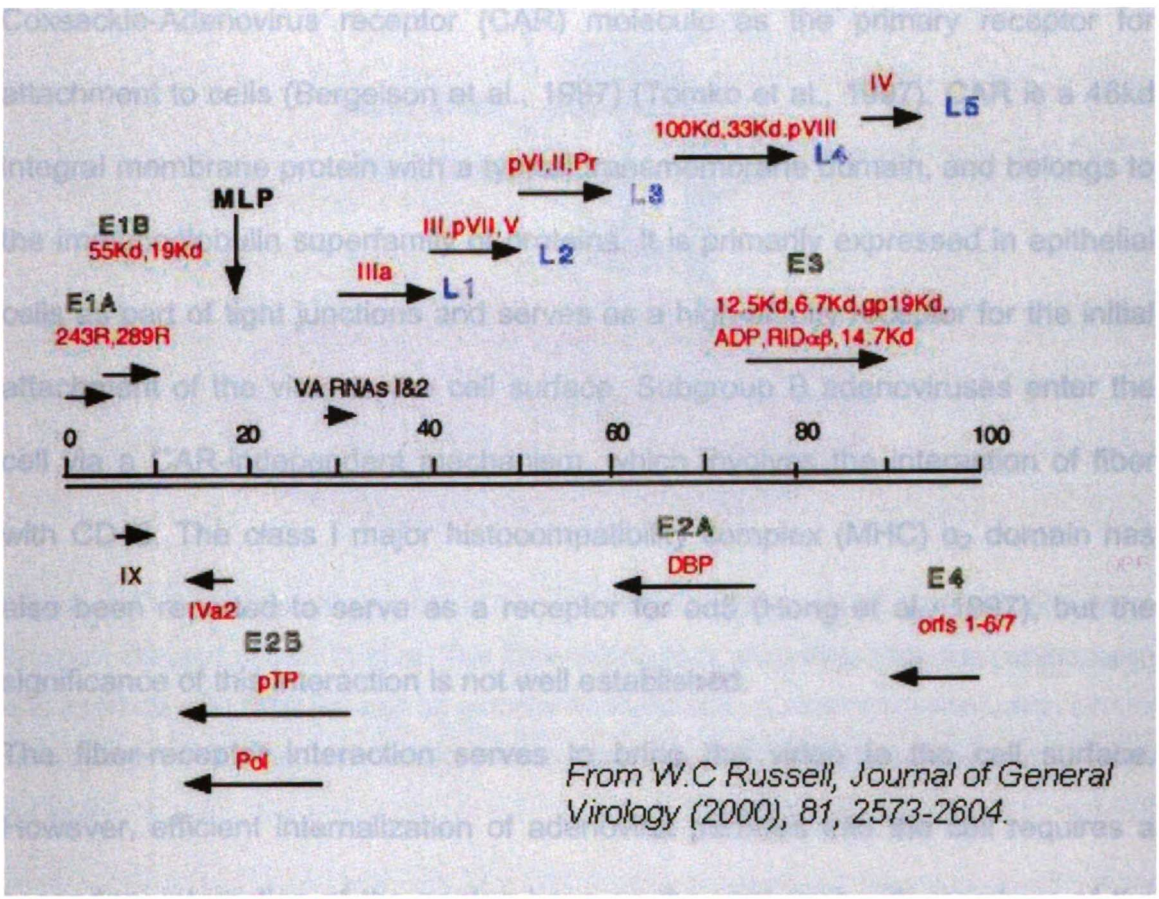


Figure 1.3 From (Russell, 2000). In green, the early gene transcripts and in blue the late. Polypeptide products are denoted in red. The direction of transcription is shown by the arrows. MLP: Major-late promoter.

1.2 ADENOVIRUS LIFECYCLE

1.2.i *Adsorption and Internalization*

Binding of all adenoviruses to the cell surface is mediated via the interaction of the fiber to the cellular receptor (Lonberg-Holm and Philipson, 1969). All human adenoviruses, with the exception of subgroup B (Gaggar et al., 2003), utilize the Coxsackie-Adenovirus receptor (CAR) molecule as the primary receptor for attachment to cells (Bergelson et al., 1997) (Tomko et al., 1997). CAR is a 46kd integral membrane protein with a typical transmembrane domain, and belongs to the immunoglobulin superfamily of proteins. It is primarily expressed in epithelial cells as part of tight junctions and serves as a high-affinity receptor for the initial attachment of the virus to the cell surface. Subgroup B adenoviruses enter the cell via a CAR-independent mechanism, which involves the interaction of fiber with CD46. The class I major histocompatibility complex (MHC) α_2 domain has also been reported to serve as a receptor for ad5 (Hong et al., 1997), but the significance of this interaction is not well established.

The fiber-receptor interaction serves to bring the virion to the cell surface. However, efficient internalization of adenoviral particles into the cell requires a secondary interaction of the penton base on the viral coat with members of the integrin family of heterodimeric cell surface receptors (Wickham et al., 1993). The penton base contains arg-gly-asp (RGD) sequences through which interaction with integrins, specifically the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, occurs.

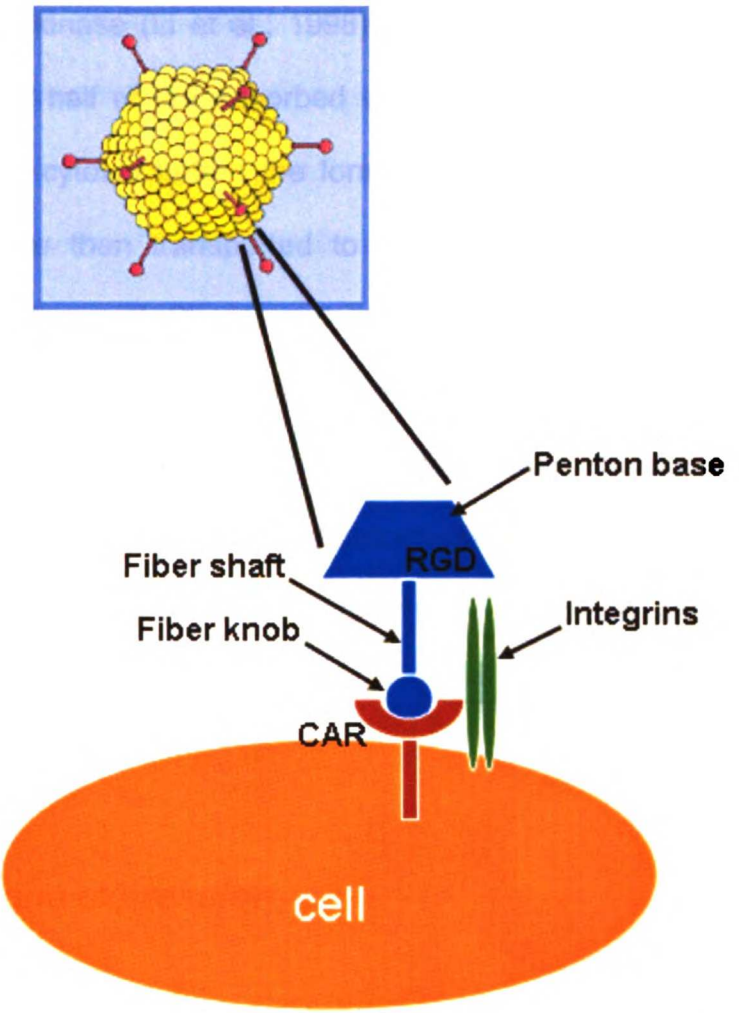


Figure 1.4 Adenovirus attachment to the cell surface. One of the 12 penton capsomeres is blown out and shown in blue. The fiber molecule is projecting from the penton base and interacts with CAR through its globular knob domain. A secondary interaction occurs between the RGD domain on the penton base and $a_v\beta_3$ or $\alpha_v\beta_5$ integrins on the cell surface.

After attachment, adenovirus-receptor complexes diffuse into coated pits, and they are internalized by receptor-mediated endocytosis (Chardonnet and Dales, 1970a; Chardonnet and Dales, 1970b) (FitzGerald et al., 1983) (Varga et al., 1991), a process triggered by the penton-integrin interaction and requires

activation of PI3 kinase (Li et al., 1998), which induces reorganization of actin filaments. Almost half of the adsorbed virus moves to endosomes and rapidly escapes into the cytosol before the formation of a lysosome (Mellman, 1992). Viral particles are then transported to the nucleus, a process that involves microtubules (Dales and Chardonnet, 1973) (Suomalainen et al., 1999) and subsequently the viral particles, which are sequentially disassembled during the internalization process, release their DNA which enters the nucleus through the nuclear pore (Greber et al., 1997) and associates with the nuclear matrix through its terminal protein (TP) (Bodnar et al., 1989) (Fredman and Engler, 1993), (Schaack et al., 1990).

1.2.ii Early gene expression

S-phase induction and inhibition of apoptosis

An important aspect of adenovirus infection is the induction of quiescent cells to enter the S phase of the cell cycle. This is the optimal period for viral replication, because of the abundance of building blocks and cellular factors required for DNA synthesis. Modulation of the cell cycle is mainly a function of the E1A proteins, which are the first viral proteins to be expressed after internalization of the viral particles. E1A proteins bind to several cellular polypeptides required for activation of transcription and entry of the cell into the S-phase (Nevins, 1990). One of the most important E1A-binding proteins is the retinoblastoma tumor suppressor protein, pRb (Whyte et al., 1988), which regulates the ability of the

E2F cellular transcription factor to activate transcription (Nevins, 1992). Downstream targets of E2F include a variety of genes that regulate entry into S-phase and cell growild-typeh. In quiescent cells, pRb is bound to E2F and sequesters it away from its transcriptional targets, thus keeping the cells in G_0. E1A binds to pRb and liberates E2F, which then enters the nucleus and initiates cell cycle progression (references). In addition to this mechanism, E1A facilitates cell cycle progression through binding to p300 and CBP proteins, which serve as coactivators for many transcription factors including CREB, STATs and nuclear receptors (Howe et al., 1990) (Lillie et al., 1987) (Zerler et al., 1987). p300/CBP complexes also serve as a coactivator for the p53 tumor suppressor, the gene most commonly mutated in human cancer. p53 induces cell cycle arrest and apoptosis in cells with deregulated cell cycle, and adenovirus-infected cells are no exception. Thus, indirect inhibition of p53 through inhibition of its coactivators by E1A relieves the block to cell cycle progression and allows cells to enter S-phase. However, p53 can also induce apoptosis in infected cells, and this function does not require its ability to stimulate transcription (references). Therefore, adenoviruses have evolved additional mechanisms to inhibit p53 function. Inhibition of p53 is primarily mediated by the genes encoded by the E1B transcription unit. Specifically, E1B encodes two polypeptides, E1B55k and E1B19k, both of which are involved in inhibiting apoptosis. E1B19k is the viral homologue of bcl-2, a cellular protein which acts as a survival factor and counteracts the pro-apoptotic function of bax and the subsequent activation of caspases (Rao et al., 1992). E1B55k mediates its anti-apoptotic effects by

binding to p53 (Sarnow et al., 1982) (Kao et al., 1990) in a complex which also involves another viral protein, E4orf6, and targets it for degradation, therefore preventing it from exerting its pro-apoptotic function. Based on these observations, it has been proposed that a mutant adenovirus deleted for E1B55k could be a conditionally-replicating cancer selective agent against tumor cells with deleted or mutant p53 (Bischoff et al., 1996). In normal cells where the p53 function is intact, replication of this virus should be restricted. The use of this and other conditionally replicating adenoviruses in cancer therapy will be discussed in the next chapter.

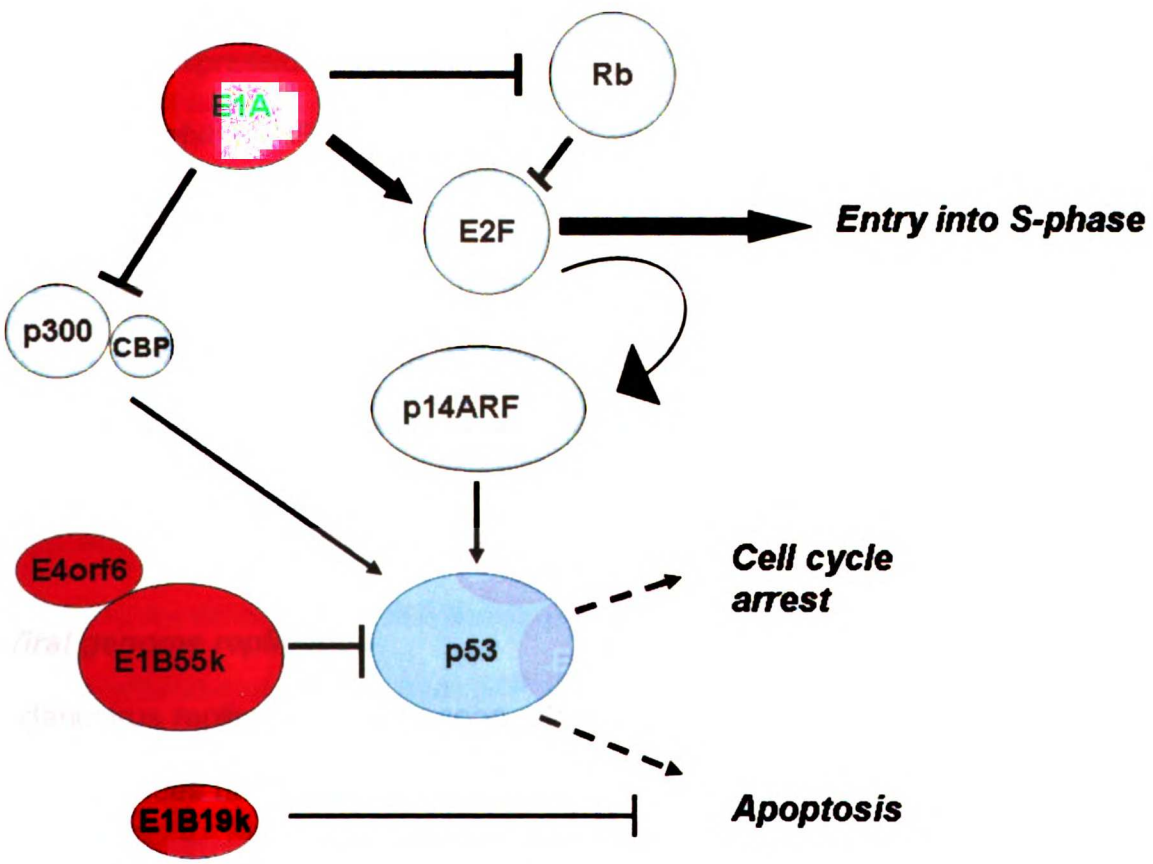


Figure 1.5 Adenovirus-mediated induction of S-phase and inhibition of apoptosis. Cellular proteins are shown in blue and viral proteins in red. Induction of S-phase by E1A

signals to p53 through p14ARF, which would lead to cell cycle arrest and apoptosis. E1A represses p53 transcription by inhibiting its coactivators p300/CBP. p53 transcription-independent function is inhibited by the E1B55k/E4orf6 complex, which targets it for degradation. E1B19k inhibits p53-independent apoptosis.

Since adenoviral infections naturally occur in the context of a whole organism, the immune response of the host against viral infections must be considered. Thus, adenoviruses encode additional proteins from the E3 transcription which modulate the immune system and suppress the immune defense against the virus. One of these proteins, the E3 gp19k, localizes to the ER and binds to the MHC class I heavy chain, preventing its transport to the cell surface and thus preventing viral antigen presentation and recognition by CTLs. This protein also delays the expression of MHC I (Bennett et al., 1999). The E3 proteins RIDα&β and 14.7k inhibit TNF apoptosis by internalizing the TNF receptor, and degrade fas ligand (Wold, 2000) (Tollefson et al., 1998). Finally the E3 11.6kd protein, also termed adenovirus death protein (ADP) induces apoptosis when it accumulates during the late stages of infection, and renders the cell more susceptible to lysis, thus promoting virus release (Tollefson et al., 1996a; Tollefson et al., 1996b).

Viral genome replication

Adenovirus replication begins soon after the accumulation of E2 gene products, and continues until the host cell is lysed. Replication is initiated at the cis-acting sequences composing the replication origins at the two ends of the viral chromosome (first and last 51 bp), and requires binding of two viral proteins: the

preterminal protein (pTP) and the DNA polymerase (Chen et al., 1990), (Mul and Van der Vliet, 1992), (Temperley and Hay, 1992), which are both encoded by the E2 region. *In vitro* studies showed that pTP and the polymerase form a complex together (Enomoto et al., 1981) (Lichy et al., 1982), (Stillman et al., 1982), (Temperley and Hay, 1992), and therefore are thought to act at the origin as a unit. After DNA replication initiation, the pTP protein is cleaved by proteolysis during virion assembly to generate a 55-kd fragment that remains covalently attached to the genome (Challberg and Kelly, 1979). The viral DNA polymerase contains both 5'-to-3' polymerase activity and 3'-to-5' exonuclease activity (Field et al., 1984) and along with pTP binds to the first 18bp of the DNA, which is the minimal origin of replication. Even though bp 19-51 of the origin are dispensable for DNA replication, they substantially enhance the efficiency of the initiation reaction, since a number of cellular factors are recruited and bind to these elements in order to stabilize the viral DNA replication complex (Bosher et al., 1990), (Chen et al., 1990), (Mul et al., 1990). These factors include nuclear factors I (NFI) and NFIII, the binding of which is stabilized by another viral protein, the E2-coded single stranded DNA-binding protein (Mul et al., 1990), (Stuiver and van der Vliet, 1990).

After the synthesis of the first three or four nucleotides of the new DNA strand, the polymerase separates from pTP (King et al., 1997) and it becomes a more efficient elongation enzyme. Besides the DNA polymerase, elongation of the newly synthesized strand also requires the single-stranded binding protein and the cellular nuclear factor II (NFII). The single-stranded DNA binding protein

forms multimeric complexes on the single strand portion of the newly synthesized strand (Tucker et al., 1994), and these protein chain formation drives strand separation, which negates the requirement for a DNA helicase to unwind the double-stranded template. Thus, in the presence of the single-stranded DNA binding protein, the polymerase can travel the entire length of the viral chromosome. The function of NFII in the elongation process appears to be the recruitment of a cellular DNA topoisomerase, since it copurifies with topoisomerase activity from adenovirus-infected cells (Nagata et al., 1983). In support of this finding, cellular topoisomerase I can substitute for NFII in an in vitro replication reaction (Nagata et al., 1983) of the viral chromosome.

Functions of the E4 products

The adenovirus E4 cassette encodes genes with diverse functions during both the early and the late phases of infection. Most of the E4 products have overlapping functions and therefore are partially dispensable for virus replication, even though a complete deletion of E4 results in a grossly defective phenotype (Halbert et al., 1985). The most well characterized E4 products are the E4 orf3 and E4 orf6 proteins. E4 orf4 increases late viral protein production by enhancing the cytoplasmic accumulation of viral late mRNAs, functioning in a complex with E1B55k to increase the rate of export of these transcripts from the nucleus (Flint and Gonzalez, 2003). In addition, this protein as well as E4 orf3 enhance the stability of unprocessed late RNA in the nucleus, which increases the amount of viral RNA available for maturation and transport (Goodrum and Ornelles, 1999),

(Ohman et al., 1993) (Nordqvist et al., 1994). Both orf3 and orf6 also play a role in viral DNA replication, apparently with overlapping functions, since orf3 or orf6 Ad mutants show only modest reduction in the overall viral genome synthesis, whereas the double mutants are substantially defective (Bridge and Ketner, 1989); (Huang and Hearing, 1989). These viruses produce concatemeric Ad DNA (Weiden and Ginsberg, 1994), and since expression of late products, including the structural components of the virion, is severely defective, it seems that the viral DNA remains unprotected and is ligated and recombined by the cellular DNA repair machinery, leading to diminished viral replication. Viral concatemerization is mediated by the cellular DNA repair complex Mre11-Rad50-NBS1, which is important for DNA double stranded break repair, and E4orf3/orf6, along with E1B55k were shown to inactivate this complex, and prevent Ad genome concatemerization (Stracker et al., 2002). As mentioned earlier, E4 orf6 also functions in a complex with E1B55k and blocks p53-dependent apoptosis, by targeting p53 for degradation (Dobner et al., 1996; Querido et al., 2001a; Querido et al., 2001b). Moreover, E4orf3 affects the distribution of a group of essential transcription and replication factors in the nucleus, which collectively function in organized nuclear structures known as PML-organizing domains (PODs) (Carvalho et al., 1995; Doucas et al., 1996), but the reason for this redistribution is still unclear.

E4orf1 and E4orf4 were recently shown by our lab to activate the mTOR pathway, and activation of this pathway overrides cellular checkpoints for protein translation in infected cells (O'Shea et al., 2005a). mTOR is a pivotal regulator of

protein translation and is activated in response to nutrient and growild-typeh factor signals (Inoki et al., 2003a; Inoki et al., 2003b; McManus and Alessi, 2002). After its activation, mTOR induces the phosphorylation of 4EBP1 and p70S6K kinase, which leads to stimulation of translation in quiescent cells (reviewed in (Richardson et al., 2004)). E4orf1 activates PI3-kinase, which in turn activates the mTOR signaling pathway (O'Shea et al., 2005a). E4orf4 also stimulates mTOR activity, but it does so in a PI3-kinase independent manner, via a mechanism that is likely to involve PP2A (O'Shea et al., 2005a). Thus, E4orf1 and E4orf4 functions seem to have evolved to ensure efficient viral replication, even in a limiting cellular environment, devoid of nutrient and growild-typeh factor signals (O'Shea et al., 2005b).

1.2.iii *Late gene expression*

Activation of late genes-translation of viral mRNAs

After the onset of viral DNA replication adenovirus late genes begin to be expressed efficiently. All of the late adenovirus polypeptides are encoded into a single large transcription unit whose primary transcript is about 29KB (Evans et al., 1977), (Nevins and Darnell, 1978). This transcript is processed by differential poly(A) site utilization and splicing to generate multiple and distinct mRNAs. These mRNAs are classified into five families, (L1 to L5) based on the use of

common poly(A) addition sites (Chow et al., 1977), (Nevins and Darnell, 1978), (Ziff and Fraser, 1978). Expression of late mRNAs is controlled by the major late promoter (MLP). MLP is activated by E1A, but the fact that its expression is delayed suggests that there is another regulatory component, since E1A is expressed immediately after infection. There appear to be at least two distinct components that control the delayed activation of the major late promoter; an unknown cis-acting component, and a transacting factor, the virally-coded IVa2 protein.

After initiation of DNA replication and synthesis of all the late mRNAs, accumulation of cellular mRNAs in the cytoplasm is blocked (Beltz and Flint, 1979). The block to cellular mRNA accumulation is mediated by the E1B55K (Babiss and Ginsberg, 1984), (Pilder et al., 1986) and by the E4orf6 polypeptide (Halbert et al., 1985), (Weinberg and Ketner, 1986)), which function as a complex. The same proteins are also required for efficient cytoplasmic accumulation of viral mRNAs during the late phase of infection (Babiss et al., 1985), (Halbert et al., 1985), (Pilder et al., 1986), (Weinberg and Ketner, 1986). The mechanism by which nuclear export of cytoplasmic mRNAs is blocked while the viral mRNAs are efficiently exported to the cytoplasm is complex, but it seems to involve recruitment of a cellular factor, necessary for cellular mRNA export, from the many sites of host transcription and processing to the viral centers. Besides facilitating export of their own mRNAs and blocking cellular messages from reaching the cytoplasm, adenoviruses have evolved additional mechanisms to ensure efficient translation of late viral transcripts. Viral mRNAs

are preferentially translated when they reach the cytoplasm during the late phases of infection (Zhang and Schneider, 1994). At that point, viral mRNAs constitute about 20% of the total RNA in the cytoplasm (Tal et al., 1975), but they are translated at the exclusion of the host messages (Tal et al., 1975), (Yoder et al., 1983). The mechanism of preferential translation of viral mRNAs involves several components. The first involves activation of the cellular protein kinase R (PKR) (Maran and Mathews, 1988), (O'Malley et al., 1986), which in turn phosphorylates and inactivates the eukaryotic initiation factor-2a (eIF-2a) and blocks translation. Second, inactivation of eIF-4F has been suggested to contribute to the selective translation of viral mRNAs during infection (Huang and Schneider, 1990). This translation initiation factor, when phosporylated by mnk1 kinase, binds to the cap structure at the 5' end of cellular mRNAs and facilitates scanning of the 40s ribosome from the cap to the AUG start codon through its helicase activity. Late viral messages posses a unique 5'-noncoding region (termed the *tripartite leader*) which allows adenovirus late mRNAs to be translated independent of eiF-4F, because the 40s ribosome can scan from cap to AUG by a process called *ribosome shunting* (Yueh and Schneider, 1996), without the need for eiF-4F helicase activity. In addition to these mechanisms, selective activation of late viral protein synthesis is induced by another viral protein, the L4100K. L4100K has been show to bind to the translation initiation complex and displace mnk1 kinase, therefore preventing phosphorylation of eiF-4F and consequently inhibiting host protein synthesis. However, several studies have questioned the significance of eiF-4F phosphorylation in translation

initiation (reviewed in (Scheper and Proud, 2002), and it seems that, at least in mammals, this modification is not necessary for normal development and growild-typeh (Waskiewicz et al., 1997).

The mechanism of adenovirus-specific translation, within the context of L4100K, is the subject of the work presented here, and will be discussed in greater detail in subsequent introductory chapters and throughout this dissertation.

Virus Assembly and release of viral progeny

Initiation of virus assembly begins right after replication of the viral DNA and the production of large quantities of the adenovirus structural proteins. Monomeric hexon polypeptides, which constitute the major part of the viral coat, are rapidly assembled into mature trimers immediately after their synthesis in the cytoplasm (Horwitz et al., 1969). Hexon trimerization and assembly requires the L4100K protein, the same protein that mediates host protein synthesis shutoff and preferential translation of late viral mRNAs. The exact process is unclear, but L4100K may function as a scaffold to bring the monomeric hexons together and catalyze the formation of the trimeric capsomeres. The penton base and fiber assemble independently in the cytoplasm (Horwitz et al., 1969) and then join together to form the complete penton capsomere. Hexon and penton capsomeres accumulate in the nucleus and assembly of the virion occurs, with an empty capsid forming first (Sundquist et al., 1973). Packaging of the viral DNA into the empty capsid is mediated by the packaging sequence on the left end of the chromosome (Grable and Hearing, 1992) (Hammarskjold and Winberg, 1980), (Hearing et al., 1987), (Tibbetts, 1977) which appears to recruit several

cellular factors that are presumably important for this process, even though their exact function is still unclear (Schmid and Hearing, 1998).

After capsid assembly and packaging of the viral chromosome, the newly synthesized viral particles are ready to escape the cell. This process (termed *lysis*) is facilitated by three different mechanisms. Firstly, the intermediate filament network of the cell (which normally helps to maintain the structural integrity of the cell) is disrupted. Disruption of the intermediate filaments is initiated early during infection through the rapid cleavage of vimentin by a reaction triggered during adsorption (Belin and Boulanger, 1987) and is completed during the late stages of infection by the virus-coded L3 proteinase, which cleaves the cellular cytokeratin K18 (reference). It has also been proposed that a major function of host protein synthesis shutoff, induced during the late stages of infection, is to inhibit the production of key cytoskeletal elements and consequently compromise the integrity of the cell structure (Zhang and Schneider, 1994).

A second mechanism facilitating cell lysis and release of the viral progeny involves the E3 11.6-kd protein, which is also referred to as the adenovirus death protein. After it accumulates, during the late stages of infection, this protein induces cell death by a mechanism which is still unclear (Tollefson et al., 1996a).

Lastly, at least during infection of the animal host, excess production of fiber seems to be important for the efficient release of the viral progeny and the infection of neighboring cells within the epithelium monolayer. A recent report suggested that excess fiber, which is not used during the assembly of the virion

capsomeres, is secreted from the infected cell and binds to the CAR receptor, which is part of the tight junctions that mediate cell-cell interactions within the epithelium. This results in the disruption of the epithelium, which in turn facilitates infection of neighboring cells by the released infectious progeny after cell lysis (Walters et al., 2002).

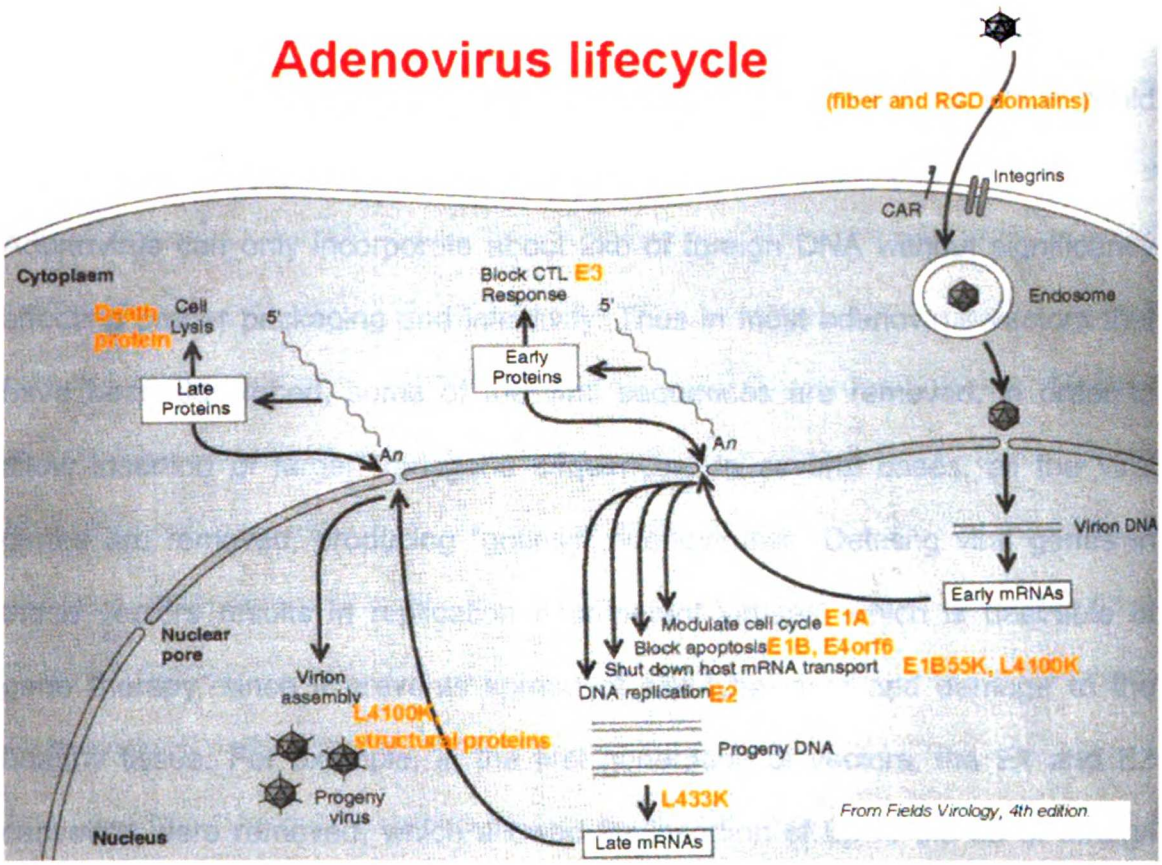


Figure 1.6 The adenovirus lifecycle. Adapted from *Fields, Virology,* 4th edition.

1.3 Adenovirus vectors and applications in human therapy

Adenoviruses can infect a wide spectrum of tissues and cell types, a characteristic that made these viruses attractive for use as vectors in the clinic. Adenoviral vectors have been constructed for use in cancer therapy, either as replication competent viruses engineered to only replicate in tumor cells, or as replication incompetent vectors engineered to deliver genes that will lead to tumor suppression and elimination. Adenoviruses have also been designed for use in gene therapy, as vectors to deliver genes, expression of which could augment defective genes, or alt disease progression.

Adenovirus can only incorporate about 2kb of foreign DNA without significantly affecting proper packaging and infectivity. Thus in most adenovirus vectors that have been developed, some of the viral sequences are removed, in order to allow insertion of larger transgene sequences. In several cases, all the viral genes are removed, producing "gutless" adenoviruses. Deleting viral genes in these vectors results in replication-incompetent viruses, which is desirable in gene therapy, since it prevents spread of wild-type virus and damage to the healthy tissue. For example, in the first generation of vectors, the E1 and E3 cassettes were removed, which allowed for insertion of up to 6.5 kb of foreign DNA. Deleting the E1 genes has the apparent advantage of impairing transcription of the E2 genes, which are necessary for replication of the viral genome. However, deleting E1 also results in a much less optimal cellular environment for the virus, and p53-dependent apoptosis is unobstructed. Moreover, even though replication of these vectors is attenuated, cellular E2F

can partially complement the function of deleted E1 genes resulting in the production of replicating-competent adenovirus (RCA). Even though RCA is produced in very low amounts, it is enough to spread to healthy tissue and produce an infection. In addition, deletion of the immunoregulatory genes coded in the E3 region results in host-mediated antiviral response (Poller et al., 1996) which eliminates cells that are infected with these vectors, thus preventing the expression of the desired transgene in the target cells.

Second generation vectors were constructed by deleting some the E2A and pTP genes encoded by the E2 region of the viral genome (Lusky et al., 1998) (Moorhead et al., 1999). Even though there was no unwanted production of replication-competent adenovirus, the immune response against these vectors was still a major problem, since several studies have shown that the infecting recombinant virus itself, without viral DNA replication, was sufficient to induce the host immune response. This occurs because of the high antigenicity of the capsid components (Liu et al., 2003), and the activation of signaling cascades from the induction of the E1 products that lead to a potent immune response (Bennett et al., 1996; Muruve, 2004).

The "gutless" adenoviruses introduced earlier constitute the third generation of adenovirus-based vectors (Hardy et al., 1997), (Kumar-Singh and Chamberlain, 1996), (Steinwaerder et al., 1999), (Morsy et al., 1998) (Steinwaerder et al., 1999), and retain only the ITR and the packaging sequence. These vectors eliminate the potential problems associated with the induction of the immune response or the generation of RCA particles (Xiong et al., 2006). However, they

cannot be propagated and purified as single agents, and require "helper" virus and appropriate complementing cell lines in order to be produced. This makes the purification and isolation process of these vectors extremely hard, and in many cases the purified "gutless" vector also contains contaminating "helper" virus.

Finally, in the case of cancer therapy, adenovirus-based vectors have been designed based on several strategies in order to suppress tumor progression, lyse the tumor cells or serve as vaccines to develop a host-mediated elimination of tumor cells by components of the immune system. For example, a number of vectors incorporating wild-type p53 have been constructed (Blagosklonny et al., 1998) (Cirielli et al., 1999), (Li et al., 1999b) (Putzer et al., 1998), in order to reintroduce wild-type p53 in tumor cells with p53 mutations or deletions, since loss of p53 function drives the development of a wide variety of human tumors. Some of these vectors showed promise in cell culture and in animal models (Ganjavi et al., 2005; Horowitz, 1999), and clinical studies are under way (Lang et al., 2003; Wen et al., 2003). In some cases, administration of these vectors in combination with a cytotoxic drug, such as adriamycin (Blagosklonny *et al.*, 1998) proved more effective.

Another generation of adenovirus vectors is designed to replicate selectively in cancer cells and cause lysis of the tumor. These vectors, termed oncolytic adenoviruses, explore molecular signaling pathways that differ between tumor and normal cells, and they are conditionally replicating viruses engineered to replicate only in tumor cells. The prototype of oncolytic viruses is an E1B55k-

deleted mutant adenovirus, which was originally developed to replicate in p53-deficient tumors, or in cancer cells with a defective p53 pathway (Bischoff et al., 1996). In normal cells, where the p53 pathway is intact, this virus should be unable to replicate, since E1B55k is necessary to target p53 for degradation and eliminate p53-dependent apoptosis. This concept led to the commercial development of this mutant adenovirus (ONYX-015), and after showing promise in cell culture and in animal models, it entered clinical trials for the treatment of head and neck cancer. Even though a number of publications have suggested that the original premises of this concept for p53 selectivity may have not held true (Harada and Berk, 1999), (Rothmann et al., 1998), (Edwards et al., 2002; Goodrum and Ornelles, 1998; O'Shea et al., 2004) the virus has proven efficacious in treatment of several tumors (Chiocca et al., 2004; Heise et al., 1999; Khuri et al., 2000; Nemunaitis et al., 2001) and combination with standard chemotherapy also looks promising (Heise et al., 1997) (Hecht et al., 2003; Heise et al., 2000; Khuri et al., 2000; Nemunaitis et al., 2001). The tumor selectivity of ONYX-015 is therefore well demonstrated in the clinic, even though it does not appear to be dependent of p53. Rather, recent work in our lab demonstrated that the tumor selective replication of ONYX-015 relies on the p53-independent functions of E1B55k (O'Shea et al., 2004). Expression of late viral messages during infection of normal cells and non-permissive tumor cells is severely impaired, and this was shown to be due to a block in late viral RNA export and the inability of the virus to induce host protein synthesis shutoff in these cells (O'Shea et al., 2004). E1B55k regulates the export of L4100k mRNA, which upon

translation in the cytoplasm, mediates inhibition of cellular protein synthesis and enhances the expression of late viral proteins. Complementing this function of E1B55k by ectopic expression of L4100k rescues ONYX-015 replication in refractory tumor cells. Permissive tumor cells somehow complement the RNA export function of E1B55k and L4100k is expressed. In these cells, host protein synthesis is inhibited, and late proteins are efficiently expressed (O'Shea et al., 2004).

The use of adenovirus vectors in the clinic, as means of delivering a gene in deficient cells, or as oncolytic agents, shows many promises for the future treatment of such diseases. However, several problems associated with this approach still remain to be solved. Targeting of these vectors to the desired tissue is still a major hindrance. Even though CAR receptor is expressed in all epithelial cells in the body, the lymphoid and mesenchymal tissues can not be targeted. Moreover, CAR is downregulated in a number of primary tumors (Douglas et al., 2001; Li et al., 1999a), and in tumors that undergo epithelial-mesenchymal transition (EMT). For example, immunostaining experiments with metastatic colon cancer cells isolated from the liver of patients with colon cancer showed that in about 50% of those cells, CAR expression was strongly downregulated, and this correlated with loss of epithelial morphology, a characteristic feature of EMT (Korn, 2002). A number of retargeting strategies are developed in order to bypass this problem, including changes in the fiber knob of the capsid to direct infection through different receptors, and the use of adenovirus serotypes that use CAR-independent mechanisms for attachment to

cells (such as subgroup B viruses). For example, immunologic retargeting approaches have been developed that employ the use of bispecific antibodies which contain a ligand for a cellular receptor expressed on the targeted cell (like a growild-typeh factor), tethered to a monoclonal antibody specific for a viral surface protein (Li et al., 2000). However, there are certain limitations to this approach. Firstly, large scale production of bispecific antibody conjugates of consistent configuration is very difficult. In addition, the stability of the virion-antibody complex remains of concern, especially following intravenous delivery. More importantly, clearance of these complexes and activation of the complement system may prove a major obstacle in using this approach *in vivo* (Bell et al., 2002). Finally, even though immunologic retargeting may prove useful for gene delivery when the vector must target the cell and express the transgene of interest, it will not be sufficient to improve the efficacy of oncolytic viruses to eliminate malignant tumors. In this case, the progeny produced by the initial viral dose will not be retargeted to the neighboring tumor cells, or to cancer cells that metastasized in different sites.

Another approach to retarget adenovirus vectors employs genetic modifications of the fiber knob. For example several groups incorporated small ligands at the carboxy-terminus of the fiber, in an attempt to retarget the virus to cells expressing the receptors specific for the ligands. However, fibers must trimerize to enable attachment to the penton base for proper capsid formation (Novelli and Boulanger, 1991), and usually modifications to the knob perturb trimerization of the fiber. In several cases, this is because of steric hindrance

effects due to the presence of the ligand, or more importantly because of the introduced changes in the sequence which alter the ability of fiber monomers to interact with each other (Michael et al., 1995), (Curiel, 1999).

A second problem in the use of adenoviruses in therapy is the immune response induced upon viral infection. Prolonged persistence of a transgene-expressing adenovirus in a tissue results in many cases in a strong CTL response, which eliminates the infected cells (Harrod et al., 1998). In the case of oncolytic viral therapy, the immune response against the viral infection might be beneficial, since the virus only replicates in cancer cells, and elimination of infected cells is desirable. However, when adenovirus vectors are used as means to deliver a therapeutic gene in deficient cells, elimination of the infected cells by CTLs prevents prolonged expression of the transgene. Finally, the majority of the human population has naturally developed antibodies against most adenovirus serotypes, especially against adenoviruses type 5 and type 2, which are the most commonly used serotypes in gene therapy. Thus, systemic delivery of these vectors is severely hindered by the humoral response of the host.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell lines and culturing conditions.

U20S and 293 cells were obtained from American Type Culture Collection and grown at 37°C/5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with high glucose,10% fetal bovine serum (FBS), 100 µg/ml of nonessential amino acids, 4mM L-glutamine,10 U/ml of penicillin, and 10 µg/ml of streptomycin.

2.2 Plasmids and antibodies.

The plasmid pMYC-100k was generated by PCR amplification of the L4100k sequence and cloning into pCAN-myc. Plasmids pMYC-100kΔC and pMYC-100k^{RGG3} were generated by PCR mutagenesis of the pMYC-100k. All constructs were confirmed by sequencing.

Mouse monoclonal antibodies to L4100k were a kind gift from Prof W.C. Russell (University of St. Andrews, Fife, UK) and were previously described (Russell et al., 1981). Mouse monoclonal α-myc 9E10 antibody was a generous gift from Dr. G. Evan. Other antibodies were from commercial sources: rabbit polyclonal α-myc (Cell Signaling Technology), mouse monoclonal α-PRMT-1 (Abcam cat.# ab7027), mouse monoclonal α-hexon (Abcam cat# ab7428), mouse monoclonal α-fiber (Abcam cat# ab3233), mouse monoclonal α-E1A (Calbiochem cat# DP11) and mouse monoclonal asymmetric α-dimethyl- arginine (Upstate cat#07414).

2.3 Transfections

Transfections were done using Lipofectamine 2000 (Invitrogen). Cells were plated in 6- well plates at 5 x 10^5 cells/well, or in 10cm dishes at 5 x 10^5 cells/well the day before transfection. Next day 2-5ug of DNA was diluted in 250ul of DMEM (no serum, no antibiotics), and in a separate tube 7-15ul of Lipofectamine 2000 reagent was diluted in 250ul of DMEM (no serum, no antibiotics). Tubes were mixed within 5min and the 500ul mix was incubated at RT for 20min. The medium on cells was then changed to 2ml of serum-free, antibiotics-free DMEM and the transfection mix added in each well. 4-6hrs later, medium was changed back to DMEM + 10%FBS + drugs as needed.

2.4 Bacterial Transformation and plasmid isolation

To transform bacteria, a 10ml FALCON tube was put on ice for 5min, and 2-4ul of DNA was added into the tube, mixed with 40ul of transformation competent bacteria. The volume of the DNA should never exceed 1/10 of the volume of bacteria. For standard transformations, DH5a cells work great, but for transformations expected to give very few colonies, commercially available, high-efficiency competent cells should be used)in several occasions I used XL1-blue for INVITROGEN. The mixture was then let sit on ice for 30-40 min, during which time a water bath was prepared at 42C and LB was pre-warmed at 37C. At the end of incubation on ice, the tubes were heatshocked at 42C for 30-40 seconds. The duration of heatshock is critical for the efficiency of transformation. Cells

were then put back on ice for 2min, and then 500ul of pre-warmed LB medium (no antibiotic selection) was added. The mixture was then incubated at a 37C shaker for 1hr, and cells were subsequently plated on agar plates with the appropriate antibiotic selection. For transformations with high efficiency, the cells were first diluted 1:500 before plated, to ensure single colony formation.

Next day, desired colonies were picked incubated in 5ml of LB with selection. At the end of the day the 5ml inoculum was added in 100-200ml of LB medium + selection and grown at 37C shaker O/N. Next day, the bacterial pellet was collected by centrifugation at 5,000 x g and either frozen at -20C for use later, or directly used for plasmid purification. Plasmid isolation was performed using Plasmid-Prep kits from QIAGEN (Maxi, or Midi kits) according to the manufacturer's protocol.

2.5 Construction of stable cell lines using retrovirus constructs

To make retroviral constructs, Phoenix cells were seeded at 4 x 10^6 cells/10cm dish (70% confluency) in high glucose DMEM + 10% FBS. Next day, 6ug of the retroviral plasmid was mixed with 500ul DMEM (serum-free, antibiotics-free). In a separate tube 24ul of Lipofectamine 2000 were mixed with 500ul DMEM (serum-free, antibiotics-free). The two tubes were mixed together and incubated at RT for 20min. During that time, cells were washed once with DMEM (serum-free, antibiotics-free), and left with 4ml of just DMEM. At the end of the 20min incubation, the transfection mixture was added in onto the cells and incubated at 37C for 3hrs. Next, medium was removed and 10ml of fresh medium was added

(DMEM + 10% FBS). Next day at the end of the day, medium was aspirated and 5ml of fresh medium was added. The next day (48hrs post transfection), the 5ml medium was collected with a 10ml syringe and filtered through a 0.45um filter into a 15ml FALCON tube. This is the first batch of virus. Another 5ml of medium was subsequently added on the cells and left O/N. In the medium collected, containing the retrovirus, polybrene was added to 5ug/ml (hexadimethrine bromide, SIGMA H-9268, 5mg/ml stock in water), and the virus was then aliquoted into 1.5ml tubes and either used immediately to infect target cells, or frozen down in dry ice/ethanol and stored at -80C. The same procedure was repeated with the second batch of virus (next day, 72hrs post transfection). For infection of target cells, an aliquot of 1.5ml was added to the cells and incubated for 4hrs-O/N. After incubation of cells with the virus, medium was removed and fresh medium was added. Next day, cells were split 1:2 and plated back, this time with medium containing the appropriate resistance for selection of stable cell lines that incorporated the retroviral vector in the chromosomes.

2.6 Immunofluorescence microscopy.

U2OS cells were transfected with plasmids expressing myc-100k, myc-100kΔC or myc-100k^{RGG3} proteins and grown on coverslips. Cells were fixed for 15min at room temperature with 4% paraformaldehyde freshly made in phosphate-buffered saline (PBS), permeabilized for 5min with 0.1% Triton X-100, blocked with 5% normal goat serum (NGS) for 20 minutes followed by incubation with antibodies to myc-tag epitope or to L4-100k in blocking buffer for 1hr at 37 C.

Cells were then washed 3 times for 20min. each time with PBS, reacted with Alexα-488 anti-mouse fluorescently labeled antibody (Molecular Probes) for 30min at 37 C, washed three times for 20 min each time with PBS, stained with Hoechst 33258 and mounted with DAKO fluorescent mounting medium. Cells were visualized and photographed using a Zeiss-LSM confocal microscope.

2.7 Immunoprecipitation and western immunoblot analysis.

U2OS cells were lysed in NP-40 lysis buffer (1% NP-40, 150mM NaCl, 10mM HEPES [pH 7.4], 2mM EDTA, 2mM sodium orthovanadate, 0.1% β-mercaptoethanol and protease inhibitor cocktail tablet (Roche) and clarified by centrifugation for 20min at 14,000 X g. Lysates were precleared for 20min at 4 C with protein G agarose and incubated with 9E10 agarose beads for 2hrs at 4 C. Lysates were then washed five times with lysis buffer, immunoprecipitated proteins were eluded off the agarose beads with SDS sample buffer. Samples were run on 4-20% or 6% tris-glycine gels (Invitrogen) in 1X SDS-running buffer (INVITROGEN) at 80-150V and transferred at 300mAmps for 90min onto Immobilon PVDF membranes using a semi-dry transfer apparatus and 1X SDS buffer + 20% methanol. Membranes were washed with 1XTNT buffer (50ml 1M Tris pH 8.0, 30ml 5M NaCl, 1ml Tween 20, dH2O to 1000ml final volume) and blocked with 5% milk in TNT for 45min-1hr at RT on a shaker. All primary antibodies were incubated with the membranes at 4 C O/N on a shaker, unless otherwise suggested by the manufacturer.

2.8 S^{35} labeling

Cells in 6-well plates were washed twice with PBS and supplemented with cysteine and methionine-free DMEM with 2% dialyzed FBS. Cells were starved for 2hrs and then 100uCi of S^{35} was added in each well and cells were incubated at 37C for two hrs to label. At the end of incubation the medium was removed and cells were washed twice with cold PBS before harvested in the appropriate lysis buffer for subsequent analysis.

2.9 Nucleocytoplasmic fractionation.

Fractionation was performed using a SIGMA CelLytic NuCLEAR EXTRACTION KIT (SIGMA product code N-XTRACT) according to the manufacturer's protocol. Cells were trypsinized with 0.25mM trypsin-EDTA solution (Gibco), washed with PBS once and pellets were incubated in hypotonic lysis buffer for 15min at RT. Samples were centrifuged at 11,000 x g for 30sec at RT and the supernatant was collected (cytoplasmic fraction). Nuclei were then incubated with extraction buffer and samples were agitated for 30min at RT. Samples were then centrifuged at 21,000 x g for 5 min and the supernatant collected (nuclear fraction).

2.10 Ligations

DNA fragments generated by PCR were purified using a Queen PCR purification kit, according to the manufacturer's protocol. The fragments and the vectors were then digested with the appropriate restriction end nuclease, and ran on a thick 1% low-melting gel (Lag) for 1hr. Bands were visualized under UV light, and

desired fragments were cut out of the gel with a scalpel. To minimize the chance of T-T dimers, the minimum intensity of UV light was used. Cut bands were then put in an eppendorf tube and melted at 65 C in a waterbath for 10min. Samples were vortexed, and the following reaction prepared, while the melted bands were kept at 65 C (to prevent solidification): 14µl ddH2O, 2µl 10X ligation buffer (INVITROGEN), 1µl vector DNA, 3µl insert DNA and 1µl T4 DNA ligase (INVITROGEN). When possible, the ratio of vector:insert was always kept at 1:3. The reaction was left O/N at RT to ligate, and the next day transformation-competent bacteria were transformed with 3µl of the reaction mix, as described elsewhere in this section.

2.11 Tag-100K fusion constructs

L4100k was cloned by PCR into the pENTR vector (Life Techonologies). Genes were transferred into CMV-promoter driven expression plasmids with N-terminal Myc-, Flag- or GST-tags using recombination-mediated Gateway technology (Life Technologies). Mutations were generated using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene). For TAP purification, an N-terminus TAP cassette (kind gift from Justin Cross) was cloned into the FBneo retroviral vector (Stratagene) and made Gateway-compatible by cloning of the Gateway cassette following manufacturer's protocols.

2.12 Virus amplification and purification

Viral supernatant containing L4100k ts mutant viruses were obtain from Dr. Hamish Young. In order to amplify these viruses, 293/E4 cells were used, which contain the whole E4 adenovirus cassette under the control of a dex-inducible promoter. These cells are used since expression of E4 products is known to increase the overall viral yield (unpublished observations). 50ul of viral sup was used to infect 1 T150 flask of 293/E4 cells in DMEM + 2% FBS at 32C (permissive temperature), and allowed to proceed to full CPE (~48-60hrs). The whole medium was then collected, centrifuged at 5,000 x g to pellet the cell debris, and virus-containing medium was then used to infect 30 T150 flasks of 293/E4 cells (1ml sup/flask). Cells were treated with Dex 24hrs before infection, in order to induce E4 expression. Infected cells were incubated at 32C till the first signs of CPE appeared. At that time, cells started rounding up, but where still attached to the flask surface. Cells were then forced to detach by agitation, and centrifuged at 5,000 x g for 10min. The medium was then discarded, and the virus-containing cell pellet was collected in a 50ml FALCON tube. To release the virus, the cell pellet was resuspended in 4ml of DMEM and freeze-thawed three times to burst the cells. The sample was then centrifuged at 6,000 x g to get rid of the cell debris, and the supernatant was collected and loaded on a CsCl column for purification. The viral band was separated by ultracentrifugation at 50,000 x g O/N at 4C and collected using an 18-gauge syringe, by puncturing the side of the tube.

2.13 Virus quantification.

Viruses were quantified on 293/E4/pIX cells using an ELISA assay. For the ELISA, 293/E4/pIX cells were seeded at $2x10^7$cells per T150 on day 1.On day 2, the cells (80%–90% confluent) were trypsinized, counted, diluted to $5x10^5$cells/ml in infection medium (standard growild-typeh medium supplemented with 2% heat-inactivated FBS), seeded at $5x10^4$cells (100 µl)/well, and then incubated for 1 hr at 37°C/5% CO2. During the incubation time, the viral standard and unknown samples were diluted. All viral dilutions were initiated in tubes and then serially diluted in a round bottom 96-well plate. One flat bottom plate of cells and one round bottom plate for viral dilutions were required per 3 "unknown" viral samples as serial dilutions for both the standard and unknowns were carried out in triplicate on each plate. For the "unknown" viral samples, dilutions were prepared in infection medium based upon a pre-defined target dilution protocol. Samples expected to have a titer between $1x10^9$ to $1x10^{10}$pfu/ml were diluted to an initial dilution of $1x10^{-5}$in tubes. For every log drop in expected titer, the initial dilution was similarly decreased by 10X (e.g., expected titer of $1x10^8$to $1x10^9$pfu/ml would be diluted initially at $1x10^{-4}$). Samples with an expected titer below $1x10^4$pfu/ml fell below the detection limits of this assay and were quantified using a standard plaque assay. Initial dilutions were seeded at 250 µl/well in row A of columns 4-12 of a 96-well round bottom master dilution plate. The standard was developed using either ONYX-015 (for 2-day assays) or ONYX-410 (for 3-day assays). Standards were diluted to an initial dilution of $5x10^5$pfu/ml in tubes and then seeded at 250 µl/well in row A of columns 1-3 of

each 96-well round bottom, master dilution plate. 125 µl of each initial dilution in row A was then serially diluted 6X's to rows B-G at 1:2 (for a 2-day assay) and 1:3 for a 3-day assay) into 125 µl of infection medium. Serial dilutions were performed using a multichannel pipetman and each serial dilution was mixed thoroughly by pipetting up and down 10X. Using a multichannel pipette, 100 µl from each row of the master dilution plate was transferred to the corresponding row of the 96-well plate that had been seeded with293/E4/pIX cells.100 µl of infection medium was added to the cells in row H and served as mock infection control. Plates were incubated for 40 to 48 hr at 37°C/5% CO2 for 2-day assays or for ~72 hr for 3-day. At this point, cells should exhibit increased CPE as the dilution decreased, whereas the cell monolayer should remain intact in the mock controls. Plates were aspirated gently using a gel-loading, fine aspirate pipette tip. Cells were gently washed 1X with 200 µl/well of PBS and aspirated as before. Virus/cells were then fixed by adding 200µl/well of cold (-20°C) EtOH/5% glacial acetic acid and incubating the plates at –20°C for at least 15 min. Cells/virus were then washed 1X with 200 µl/well of PBS and blocked for at least 1hr at RT (or up to 1 wk at 4°C) in 200 µl/well of SuperBlock (Pierce). SuperBlock was decanted and plates incubated with100 µl/well of primary rabbit polyclonal anti-Ad5 antibody (Access BioMedical Diagnostic Research Laboratories, Inc.). diluted at 1:500 or 1:1,000 (lot-specific) in TBS (BupH Tris-buffered saline pack: 25 mM Tris·Cl (pH 7.2), 150 mM NaCl, Pierce) plus 1.35% normal goat serum (NGS, Pierce) or 3%BSA (Sigma) for at least 1 hr at RT on a shaker. Plates were washed 3X in TBST (TBS + 0.1% Tween-20) using a Skanwasher 300 and then

incubated for 1 hr at RT in 100 µl/well of secondary goat anti-rabbit alkaline phosphatase conjugated antibody (Pierce) diluted at 1:1,000 in TBS plus 1.35% NGS or3% BSA. The substrate solution was prepared by dissolving 1tablet of p-nitrophenyl phosphate (PNPP, Pierce) in 8 ml of distilled water for 1 hr during the secondary antibody reaction step;2 ml of 5X diethanolamine (Pierce) was then added after the tablet had completely dissolved. Plates were washed 3X in TBST using a Skanwasher 300 and then incubated on a plate shaker with 100 µl/well of the PNPP substrate solution for ~10min or until a strong yellow color developed. Reactions were stopped by adding 100 µl/well of 2N NaOH in the same order as substrate addition and incubating the plate on a shaker for 5min.Plates were read at 405 nm using a SpectraMax250 plate reader (Molecular Devices) and analyzed using SOFTmaxPRO. Assay criteria were: R2value ≥0.96 for the standard curve, only those values that fell within the linear portion of the curve were used, OD readings had to decrease with decreasing amounts of virus, and the mean value was used from sample dilutions exhibited a %CV ≤30. When possible, the final titer was determined as an average of the mean titer from 2 to 4 different dilutions.

2.14 Tandem Affinity Purification (TAP)

Buffers

- IPP150: 10mM Tris-Cl pH 8.0, 150mM NaCl, 0.1% NP40, H2O to 100ml final volume.

- IPP150 Calmodulin binding buffer: 100ml of IPP150 buffer, 10mM b-mercaptoethanol,
1mM Mg-acetate, 1mM imidazole, 2mM CaCl2.
- IPP150 Calmodulin elution buffer: 100ml of IPP150 buffer, 10mM b-mercaptoethanol, 1mM Tris-Cl pH 8.0, 1mM Mg-acetate, 1mM imidazole, 2mM EGTA.

For isolation of novel cellular and viral 100K binding proteins using TAP, a U2OS cell line stably expressing TAP-100K was generated by retroviral infection as described above. TAP tag consists of a protein A binding portion (which binds to IgG agarose beads) and a calmodulin-binding domain (which binds to beads covalently attached to calmodulin molecules). Between these two domains, TAP tag contains a TEV protease cleavage site. For this experiment, 25 15cm dishes of the TAP-100K expressing cells were infected with wild-type adenovirus and harvested at 18hrs and 36hrs post infection in 1% Np40 lysis buffer. Also included in the experiment were non-infected TAP-100k U2OS cells and 100k non-expressing U2OS cells as a negative control.
At the appropriate time point, cells were washed twice with PBS and lysed in 30ml of lysis buffer. Lysates were centrifuged at 20,000 x g for 30min and the supernatant was incubated with 300ul packed volume of IgG beads for 2hrs at 4 C on a rocker. At the end of incubation, samples were transferred to a plastic column to facilitate washes, and were washed four times with 10ml of IPP150 buffer. After washing, the beads were incubated with 15ul of TEV protease in 1ml

of IPP150 buffer and left O/N at 4C on a shaker. Next day, samples were eluded from the beads by gravity flow into a 15ml FALCON tube and 5µl of 1M CaCl2 was added along with 2.5ml of Calmodulin binding buffer. The mixture was put onto a new column with 200µl packed volume of Calmodulin beads, pre-washed with 5ml of calmodulin binding buffer and the column was incubated at 4C on a shaker O/N. Next day, samples were eluted form the column by gravity flow, and the eluates were put through another round of calmodulin binding (eluate A). Calmodulin beads were washed on the column three times with 10ml of calmodulin binding buffer, and eluted with 1.4ml of calmodulin elution buffer in 7 steps x 200ul of buffer in 1.5ml eppendorf tubes (eluate B). Eluates A and B were subjected to TCA precipitation as follows: In each tube, DOC was added to 0.015% final concentration and after vortexing, TCA was added to 10% final concentration and vortexed again. Samples were incubated on ice for 30min and centrifuged for 20min at 20,000 x g. The protein pellets were washed with 500ul of acetone, centrifuged again for 5min at 20,000 x g and resuspended in 20ul of SDS loading buffer. Samples A and B were then pooled together and ran on a 4-20% Tris-Glysine gel (INVITROGEN). The gel was stained with SimplyBlue SafeStain Coomasie stain (INVITROGEN) for 2hrs at RT shaking. After 2hrs, the stain was removed and the gel was washed with 150ml of ddH2O for 1hr at RT shaking. After 1hr, 30ml of a 20% (w/v) NaCl solution was added to the gel and kept on shaker at RT O/N. Next day, bands on the gel were cut, and sent for Mass-Spec Analysis.

Tandem Affinity Purification (TAP) strategy

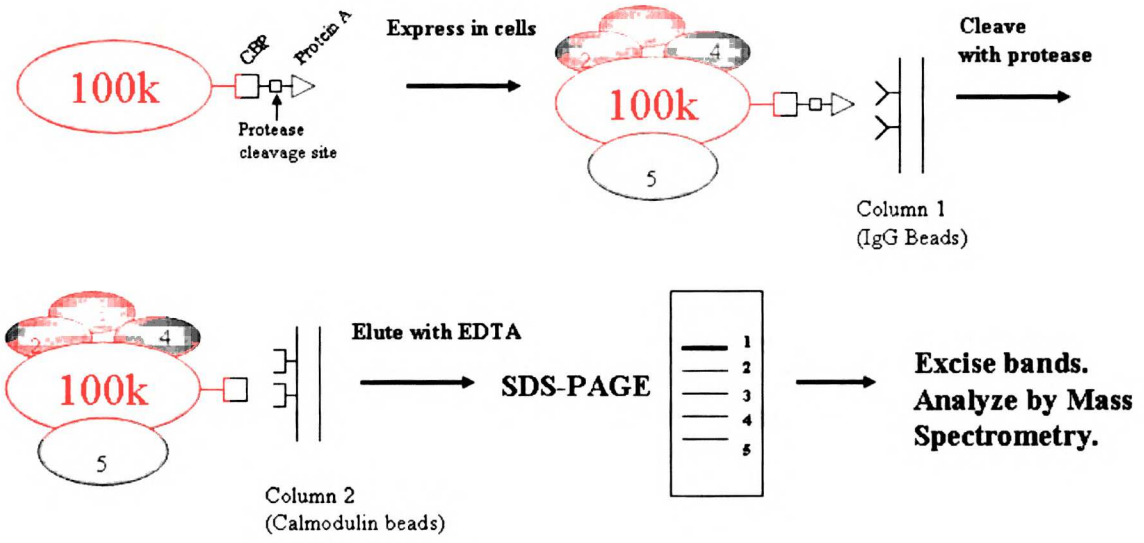


Figure 2.1 L4100k was cloned into an N-TAP construct containing an N-terminal TAP tag, with a protein A domain and a CBP domain. TAP-100k protein complexes were isolated through two consecutive rounds of purification with IgG beads, and beads conjugated to calmodulin molecules. *Adapted from Rodriquez et al, 2004.*

2.15 Reversed-phase liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) analysis

Protein bands were excised from gels and digested in-gel with trypsin as described (J. Rosenfeld, J. Capdeveille, J.C. Guillemot and P. Ferrara. Anal. Biochem. 203 (1992), pp. 173–179) (donatello.ucsf.edu/ingel.html). The extracted digests were vacuum-evaporated and resuspended in 1 µl 0.1% formic acid in water. The digests were separated by nano-flow liquid chromatography using a 75-µm x 150-mm reverse phase C18 PepMap column (Dionex-LC-Packings, San

Francisco, CA) at a flow rate of 350 nL/min in a NanoLC-1D Proteomics high-performance liquid chromatography system (Eksigent Technologies, Livermore, CA, USA) equipped with a FAMOS autosampler (Dionex-LC-Packings, San Francisco, CA). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Following equilibration of the column in 5% solvent B, approximately one-tenth of each digest (1 µl) was injected, then the organic content of the mobile phase was increased linearly to 40% over 30 min, and then to 50% in 1 min. The liquid chromatography eluate was coupled to a microionspray source attached to a QSTAR XL or to a QSTAR Pulsar mass spectrometer (Applied Biosystems/MDS Sciex, South San Francisco, CA, USA). Peptides were analyzed in positive ion mode. MS spectra were acquired for 1s in the m/z range between 310 and 1400. MS acquisitions were followed by 3-s collision-induced dissociation (CID) experiments in information-dependent acquisition mode. For each MS spectrum, the most intense multiple charged peaks over a threshold of 30 counts were selected for generation of CID mass spectra. The most common trypsin autolysis products were excluded (m/z=523.280, 421.750, 737.700). The CID collision energy was automatically set according to mass to charge (m/z) ratio and charge state of the precursor ion. A dynamic exclusion window was applied which prevented the same m/z from being selected for 1 min after its acquisition.

Data were analyzed with Analyst QS software (Applied Biosystems/MDS Sciex, South San Francisco, CA, USA) and peak lists were generated using the mascot.dll script. MS centroiding parameters were 50% peak height and 0.02

amu merge distance. MS/MS centroiding parameters were 50% peak height and 0.05 amu merge distance. The peak list was searched against the NCBI non-redundant database (NCBI nr) as of December 2004 using Mascot (version 2.0, Matrix Science, Boston, MA, USA). Carbamidomethylation and carboxymethylation of cysteine, N-acetylation of the N terminus of the protein, oxidation of methionine, and phosphorylation of serine, threonine or tyrosine were allowed as variable modifications. Peptide tolerance in MS and MS/MS modes was 150 ppm and 0.2 Da, respectively.

Hits were considered significant when three or more peptide sequences matched a protein entry and the MASCOT score was above the significant level [Perkins, D., Pappin, D. J., Creasy, D. M., Cottrell, J. S., Electrophoresis 1999, 20, 3551–3567]. For identifications based on one or two peptide sequence with high MASCOT scores, the MS/MS spectrum was reinterpreted manually by matching all the observed fragment ions to a theoretical fragmentation obtained using MS Product (Protein Prospector) [Clauser, K. R., Baker, P. R., Burlingame, A. L., Anal. Chem. 1999, 71, 2871–2873]

CHAPTER 3

A critical role for arginine methylation in adenovirus-infected cells

3.1 INTRODUCTION

During a productive infection, adenovirus turns the infected cell into an efficient virion-producing factory. To do this, the virus must interfere with and manipulate several cellular processes, to facilitate replication and packaging of its genome into newly synthesized virions. A characteristic of the late phase of infection is the severe inhibition of cellular protein synthesis (Bello and Ginsberg, 1967) and the selective translation of late viral mRNAs (Yueh and Schneider, 1996; Yueh and Schneider, 2000) . At least one viral product has been implicated in this process: the L4100k non-structural protein (Hayes et al., 1990). The ad5 L4100k protein is thought to inhibit host protein synthesis by binding to EIF4G on the EIF4F translation initiation complex in the cytoplasm and preventing its phosphorylation by mnk1 kinase through direct competition for the EIF4G binding site (8, 10). Dephosphorylation of EIF4E is believed to lead to inhibition of cap-dependent cellular protein synthesis (Wang et al., 1998), whereas late viral messages are preferentially translated by a cap-independent process known as ribosome shunting (Dolph et al., 1988).

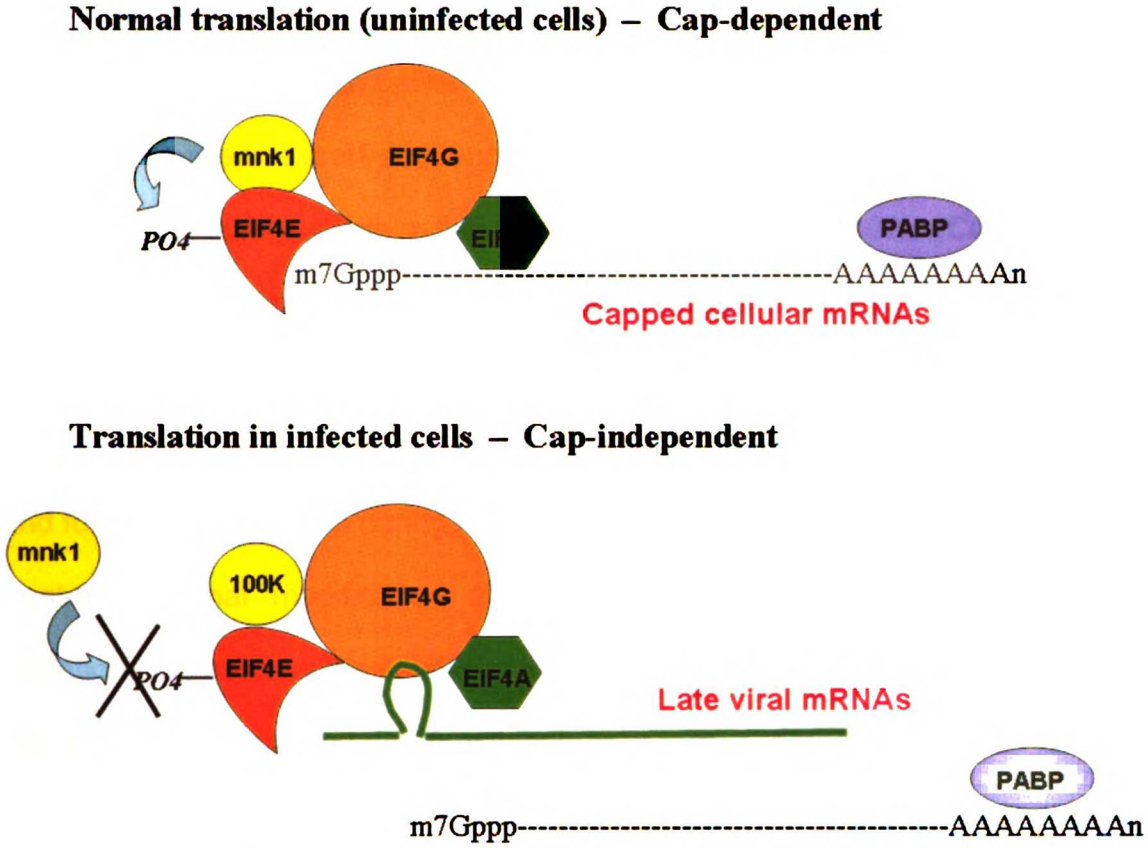


Figure 3.1 In uninfected cells, mnk1 phosphorylates EIF4E and the translation initiation complex binds to the cap structure at the 5' end of cellular mRNAs and initiate translation. During infection, L4100k displaces mnk1 from the complex, which leads to dephosphorylation of EIF4E and inhibition of cap-dependent translation. Viral messages are translated through ribosome shunting.

However, L4100k is also involved in hexon trimerization and assembly, a part of the packaging process of the viral genomes into newly synthesized capsids which occurs in the nucleus (Morin and Boulanger, 1986) and cells infected with conditional ts L4100k mutants show cytoplasmic accumulation of hexon monomers and fail to accumulate hexon trimers in the nucleus at the non-permissive temperature (Cepko and Sharp, 1983) (Oosterom-Dragon and

Ginsberg, 1981). Therefore, L4100k must be able to localize in both sub-cellular compartments and switch between these two functions during infection. Recently a Rev-like nuclear export sequence (NES) and a nuclear localization sequence (NLS) were identified within the carboxy terminus of L4100k (Cuesta et al., 2004). Deletion of the L4100k C-terminus resulted in its cytoplasmic accumulation, suggesting that the NLS sequence is within that part of the molecule. However, the mechanism by which L4100k sub-cellular localization and function is regulated during infection is currently unknown.

Post-translational modification of proteins allows viruses to overcome the limitations imposed by the small size of their genomes, offering each viral product the capacity to expand its functional repertoire. L4100k is known to be a phosphoprotein, although the function and the sites of phosphorylation remain to be elucidated. Moreover, L4100k was recently reported to be methylated during infection of permissive tumor cells and methylation is mediated by PRMT1, the most abundant methylase in the cell (Kzhyshkowska et al., 2004). However, the mechanism by which methylation controls L4100k function and its importance during infection has not been determined. Many proteins are targets of arginine methylation, the majority of which are members of the family of heterogeneous nuclear ribonucleoproteins (hnRNPs; 26, 24) which play roles in pre-mRNA processing and nucleocytoplasmic RNA transport. Most of these proteins are methylated on arginine residues within RGG tripeptides (Kiledjian and Dreyfuss, 1992), although several proteins have also been identified that are asymmetrically dimethylated at RXR, RG and GRG motifs (McBride and Silver,

2001). Although arginine methylation does not alter the overall charge of the protein, addition of methyl groups may alter the overall structure of the molecule due to increased steric hindrance and the reduction of hydrogen bonds as a result of the substitution of hydrogen atoms with methyl groups (McBride and Silver, 2001). Such alterations can affect protein-protein and protein-nucleic acid interactions and several proteins have already been shown to be regulated at that level (Bedford et al., 2000). Arginine methylation has also been shown to regulate nucleocytoplasmic localization. In many cases, methylation acts as a nuclear localization signal and results in the nuclear accumulation of the molecule (Yun and Fu, 2000), whereas in some others the opposite occurs (Gabler et al., 1998).

The C-terminus of L4100k contains an RGG domain and several RXR and RG motifs which are well conserved among different adenovirus serotypes. It is therefore possible that part of the mechanism by which L4100k switches between its two main functions, namely host protein synthesis inhibition and hexon trimerization, is by changing its sub-cellular localization during the course of infection. Since RGG domains have also been implicated in mediating protein-RNA interactions, it is likely that L4100k methylation within the RGG domain may play a role in this process.

The goal of this study was to further define the mechanism by which L4100k exerts its multicomponent function, and examine the role of post-translational methylation in adenovirus-infected cells in the context of L4100k regulation.

3.2 RESULTS

3.2.i. *RGG boxes are well conserved in L4100k and necessary for nuclear accumulation of L4100k during infection.*

L4100k is a multifunctional protein critical for adenovirus functions during the late stages of infection. It is found both in the nucleus and the cytoplasm (Gambke and Deppert, 1981) but it is not clear how L4100k switches between the different functions during infection. Since many multifunctional proteins are controlled by post-translational modifications, we searched the L4100k for sequences that are potential sites for this type of regulation. The main structural characteristics of L4100k include three coil-coil recognition motifs, which mediate protein-protein interactions and an RNA recognition motif (RRM) at the central part of the protein which is involved in RNA binding (figure 1). A Rev-like NES sequence was recently identify within this domain and the EIF4G binding site was mapped to be between amino acids 280-345 (Cuesta et al., 2004). The C-terminus of the protein contains RGG and RGR-like sequences which are well conserved in L4100k proteins from all sequenced serotypes (figure 1) and recent work suggested that this domain contains an NLS sequence (Cuesta et al., 2004). Moreover, most adenovirus serotypes also contain several RG and RXR motifs upstream of the RGG domain within the C-terminus of the protein (data not shown).

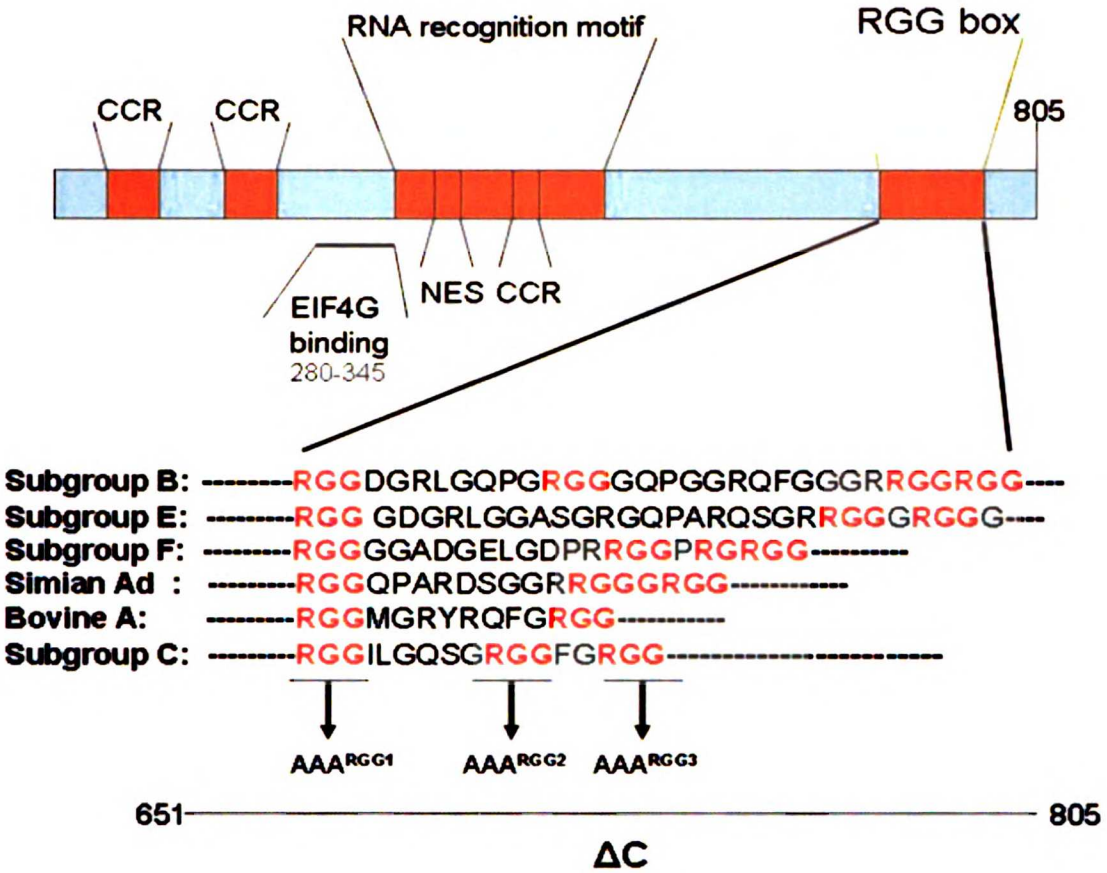


Figure 3.2 The RGG domain is well conserved at the C-terminus of many adenovirus serotypes. The C-terminus of ad5 100k contains several RG and RGR motifs and an RGG domain between amino acids 727 and 743 with three RGG tripeptides. 100kΔC was constructed by deleting amino acids 651 to 805. We also constructed three mutant clones with each of the three RGG motifs with the RGG domain mutated to alanine residues.

In order to further study the function of this part of the protein in the context of a complete viral infection, we generated a C-terminus truncated form of L4100k (aa651-805) lacking the RGG domain and the upstream RGR and RG containing region of the protein (myc-100kΔC), as well as three additional mutant clones, each of which has one of the three RGG sequences within the RGG box changed to alanine residues (100k^{RGG1}, 100k^{RGG2} and 100k^{RGG3}, figure 1). U2OS

cells were transfected with wild type L4100k epitope tagged at the N-terminus with the myc tag or with myc-tagged mutant clones and infected with wild type ad5. We then performed immunofluorescence studies to determine L4100k localization during the course of infection. As shown in figure 2, non-tagged, viral-coded L4100k is in the cytoplasm during the earlier phase of infection, consistent with its initial involvement in host protein synthesis shutoff. During the later stages of infection L4100k relocalizes to the nucleus (40hrs p.i), consistent with the second L4100k function in hexon trimerization and assembly, a process which is known to occur in the nucleus. In fact, L4100k localization to the nucleus occurs at about the same time as hexon is first expressed (data not shown). Transfected wild-type myc-100k localizes similarly to the L4100k expressed from the virus: At 18hrs and 40hrs p.i. it localizes to the cytoplasm and nucleus respectively. Interestingly, in uninfected cells wild-type myc-100k is both nuclear and cytoplasmic. This suggests that a component of infection acts on wild-type myc-100k and changes its localization to the cytoplasm initially and to the nucleus later. As shown here, transfected myc-100kΔC is exclusively cytoplasmic in uninfected cells, consistent with the absence of the NLS sequence of the protein, recently identified to be within the RGG domain. However, in contrast to wild-type myc-100k, myc-100kΔC remains cytoplasmic throughout infection, suggesting that the RGG domain is necessary for L4100k re-localization to the nucleus. We therefore tested our three RGG mutants for their ability to relocalize to the nucleus after infection. $100k^{RGG1}$ and $100k^{RGG2}$ behaved like wild type L4100k, suggesting that these two boxes are not involved in nuclear

accumulation of the protein (data not shown). However, mutation of the third RGG tripeptide to alanine residues (myc-100k^{RGG3}) resulted in a protein that failed to localize to the nucleus during the late phase of infection. Interestingly, myc-100k^{RGG3}, like wild-type myc-100k, localized both in the cytoplasm and in the nucleus in uninfected cells. These results suggest that even though the whole RGG domain may act as an NLS, the third RGG box may be the important site that regulates L4100k re-localization to the nucleus during the late phase of infection.

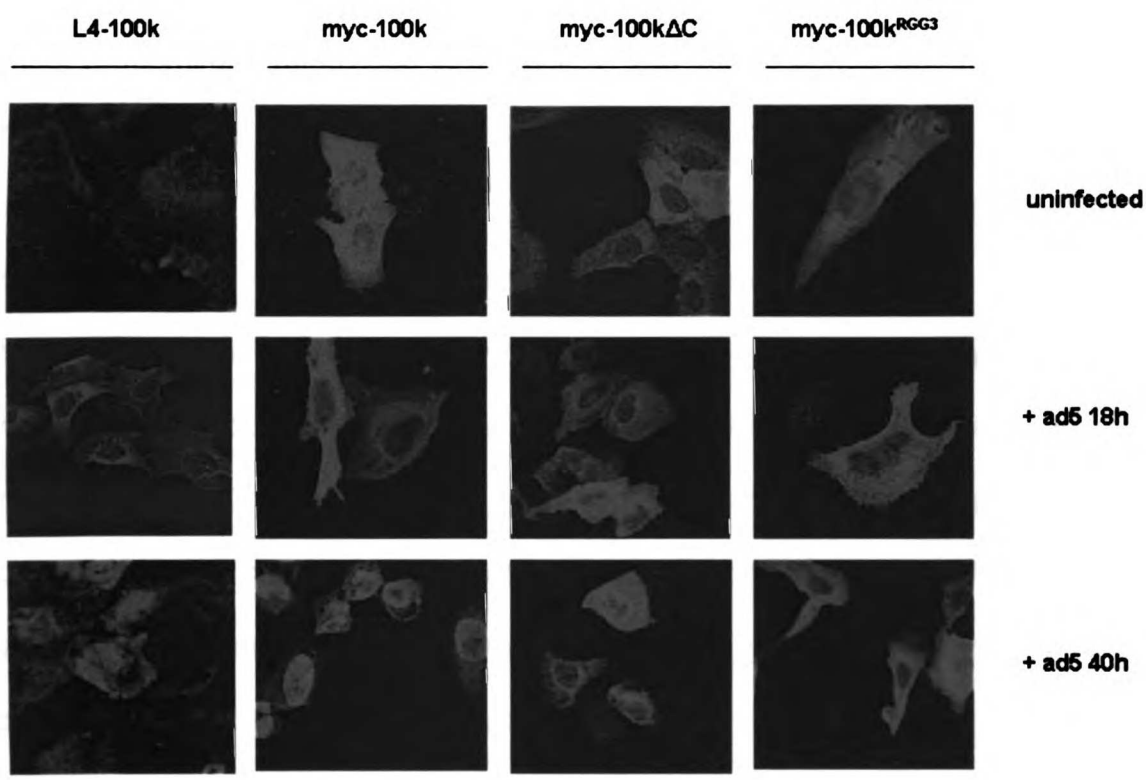


Figure 3.3 RGG mutants fail to localize to the nucleus during infection. U2OS cells were transfected with plasmids expressing myc-tagged wild-type 100k, a C-terminus truncated 100k (100kΔC) or an RGG mutant with positions 741-743 changed to alanine residues. 100kΔC has a deletion of amino acids 680-805 which encompasses the arginine-glycine

rich region with the RGG, RGR and RG motifs. When indicated, untransfected and transfected cells were infected with dl309 (wild-type ad5). Slides were fixed at 18 or 40hrs p.i and stained with a-L4100k or a-myc antibodies as indicated.

3.2.ii L4100k is methylated and binds PRMT1 methylase through its RGG domain

RGG boxes are sequences known in many cases to regulate nucleocytoplasmic localization of the protein, a function modulated by methylation on arginine residues adjacent to glycines. Recently, L4100k was shown to be methylated during infection by PRMT1 methylase (Kzhyshkowska et al., 2004) , which is he most predominant methylase in the cell. We therefore tested whether L4100k is methylated at its RGG domain, and whether this domain is necessary for the interaction with PRMT1. U2OS cells were infected with wild-type ad5 and harvested at 12 and 36hrs p.i. Infected and uninfected cells were lysed in 1% NP40 buffer and total lysates were examined by immunoblotting with an α-methyl-R antibody which recognizes asymmetrically di-methylated arginine residues. L4100k is one of the most heavily methylated proteins in infected U2OS cells and can be detected as early as 12hrs p.i (figure 3A). To determine whether the C-terminus RGG domain is necessary for PRMT1 binding, U2OS cells were transfected with wild-type myc-100k, myc-100kΔC or myc-100k^{RGG3}, infected with wild-type ad5 and subjected to co-immunoprecipitation analysis with α-myc antibodies (see materials and methods). Wild-type myc-100k co-immunoprecipitated with PRMT1 both in infected and uninfected cells. However, myc-100kΔC failed to bind to PRMT1 (figure 3B), suggesting that the C-terminus

of the protein is necessary for PRMT1 binding. Binding of myc-100k^{RGG3} to PRMT1 was greatly reduced compared to the wild-type protein, implicating these residues as one of the points of contact between L4100k and PRMT1.

In order to determine the methylation status of our L4100k mutants, the same samples were also blotted with an α-methyl-R antibody (figure 3C). As shown in figure 3C, transfected myc-100k was not methylated in uninfected cells, which suggests that binding to PRMT1 is not sufficient for L4100k methylation. Presumably an extra component of the infection is required for L4100k methylation. In infected cells wild-type myc-100k, but not myc-100kΔC was methylated. These results show that the C-terminus of L4100k is the site of methylation, and that removing this part of the molecule abolishes this type of post-translational modification. Interestingly, a methylated band of about L4 100 kd co-immunoprecipitated with the truncated myc-100kΔC. Using a monoclonal α-L4100k antibody we show that this band is the viral L4100k. This suggests that L4100k forms oligomers during infection, and that oligomerization does not require the C terminus of the molecule.

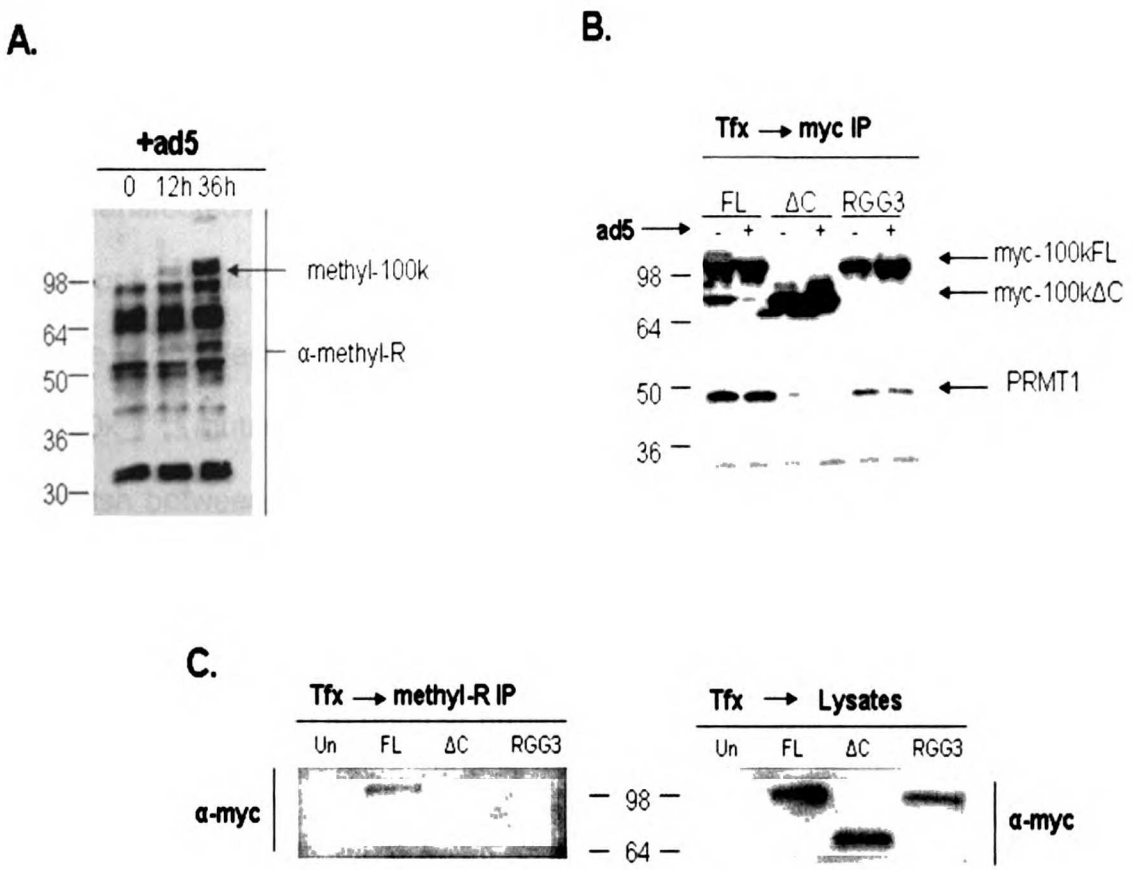


Figure 3.4 100k is methylated and binds PRMT1 methylase through its RGG domain. (**A**) U2OS cells were infected with wild-type ad5, harvested at 12 or 36hrs p.i, lysates prepared and equal amounts resolved by SDS Tris-glycine 4-20% gradient gel and detected by immunoblotting with an a-dimethyl arginine antibody. (**B and C**) Cells were transfected with wild-type myc-100k, myc-100kΔC or myc-100kRGG3, infected with ad5 when indicated and harvested at 24hrs p.i. Equal amounts of lysates were subjected to immunoprecipitation using a-myc antibody or a-dimethyl-R antibody and samples were resolved by SDS Tris-glycine 4-20% gradient gel and detected by immunoblotting with specific antibodies as shown.

To prove that L4100k oligomerizes, we constructed an HA-tagged 100k clone and used it to co-transfect U2OS cells along with the myc-tagged L4100k vector.

We then used an α-myc antibody to immunoprecipitate myc-100k, and blotted the membrane with an α-HA antibody (figure 3, lanes 1-3). In order to test whether L4100k oligomerization only occurs in the context of an infection we also infected the co-transfected cells with ad5 (lanes 4-5). As shown in figure 3D, HA-100k co-immunoprecipitated with myc-100k, with or without infection by ad5 (lanes 3&5). Because of this fact, we were unable to compare the methylation status of the myc-100k^{RGG3} mutant with the wild-type myc-100k, since it was not possible to distinguish between the transfected protein and the co-immunoprecipitated viral L4100k. We are currently investigating the role of L4100k oligomerization and whether L4100k forms complexes directly with itself, or whether monomers interact through binding to another protein.

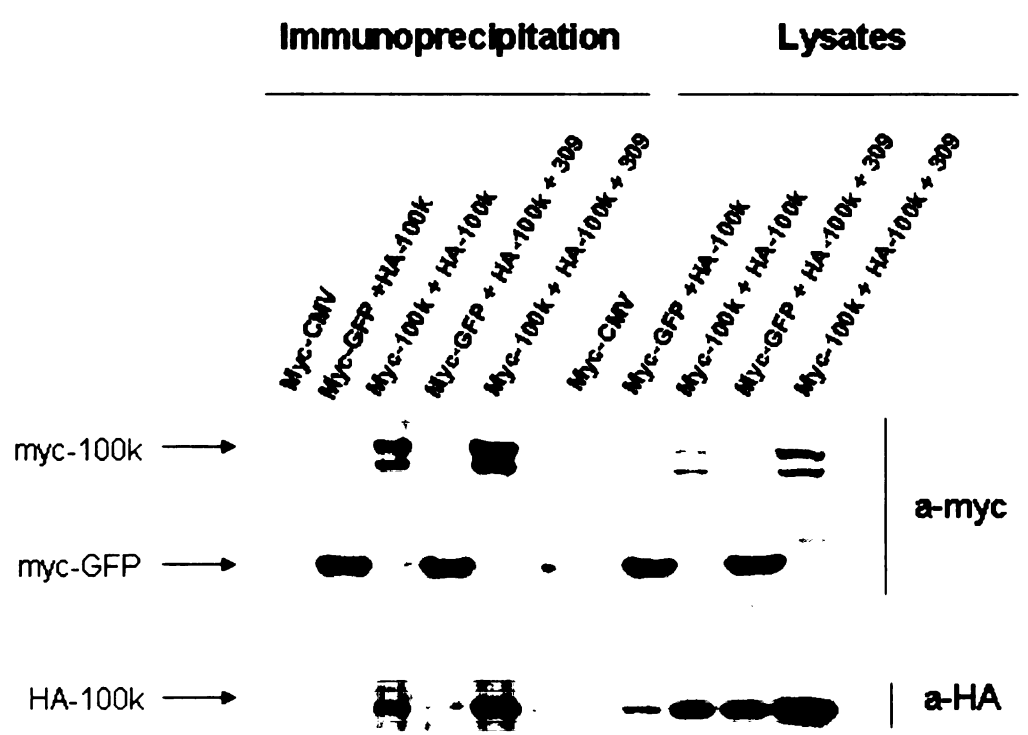
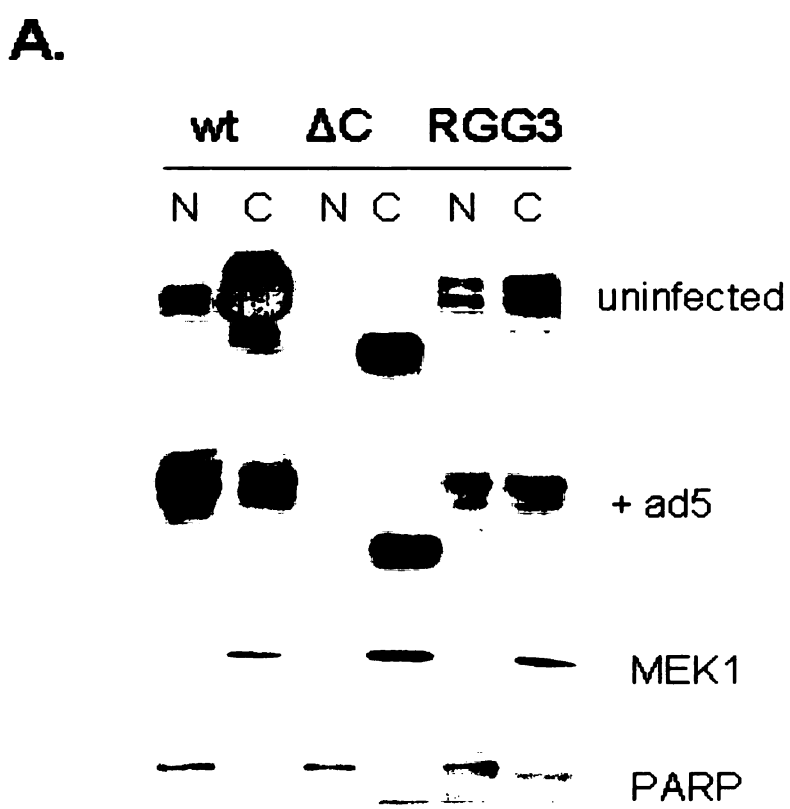


Figure 3.5 U2OS cells were co-transfected with myc-100k and HA-100k, infected with ad5 when indicated and harvested at 24hrs p.i. Equal amounts of lysates were subjected to immunoprecipitation using a-myc antibody and samples were resolved by SDS Tris-glycine 4-20% gradient gel and detected by immunoblotting with specific antibodies as shown.

3.2.iii Methylated L4100k is exclusively nuclear and RGG mutants are defective for nuclear accumulation

Because post-translational methylation modulates sub-cellular localization of several RNA-binding proteins, we next determined where methyl-100k localizes during infection. U2OS cells were transfected with wild-type myc-100k or myc-100kΔC and infected with wild-type ad5. Uninfected and infected (36hrs pi) cells were collected and lysed in a hypotonic lysis buffer to separate nuclear and cytoplasmic fractions. Samples were then examined by immunoblotting with α-myc antibody. Figure 3.5 (A) shows that in uninfected cells, wild-type myc-100k was found both in the cytoplasm and in the nucleus, whereas myc-100kΔC was exclusively cytoplasmic. After infection however there was a dramatic redistribution of wild-type myc-100k to the nucleus whereas myc-100kΔC was still exclusively cytoplasmic, consistent with our previous results. These results lead us to hypothesize that the C-terminal RGG containing region may be involved in L4100k relocalization to the nucleus, possibly as a result of methylation of that domain. To test this hypothesis, we infected U2OS cells with wild-type ad5, collected the cells 18hrs and 36hrs p.i and fractionated the nuclear and the cytoplasmic fractions as described above. Total lysates were subjected

to western blot using an α-methyl-R antibody. Figure 3.5 (B) shows that methylated L4100k is already present at 18hrs p.i and it increases at later stages of infection. More importantly, it remains exclusively nuclear. Thus the function of methylated L4100k must be in the nucleus.



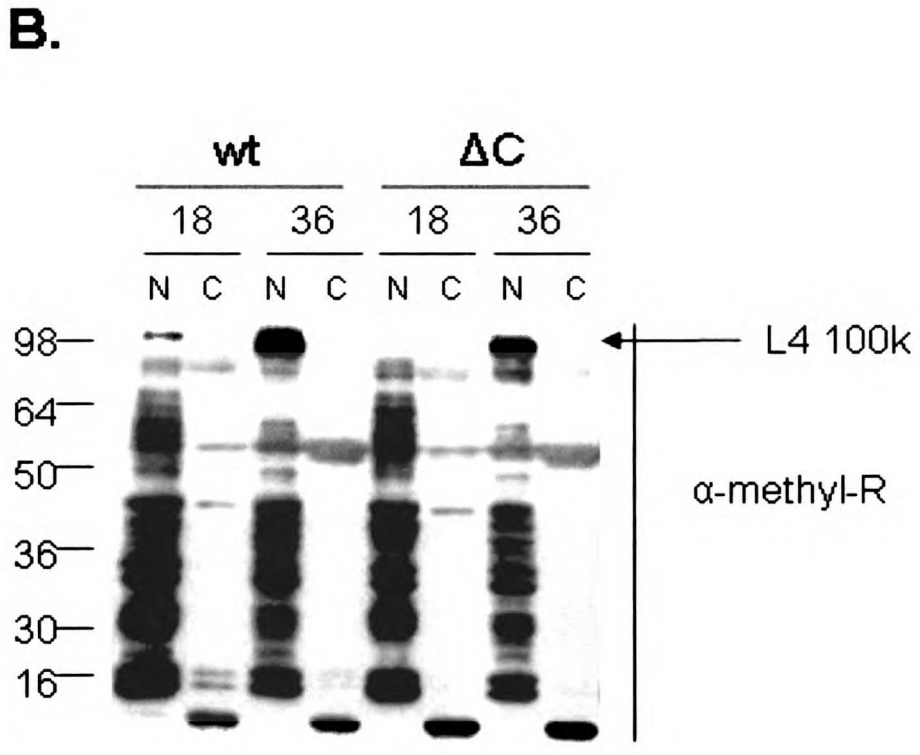


Figure 3.6 Methylated 100k is exclusively nuclear and RGG mutants are defective for nuclear accumulation. (**A**) U2OS cells were transfected with myc-100k, myc-100kΔC or myc-100kRGG3, infected with wild-type ad5 and harvested at 24 hrs p.i., or (**B**) at 18 and 36hrs p.i in a hypotonic lysis buffer, lysates were fractionated into nuclear and cytoplasmic fractions and equal amounts were resolved by SDS Tris-glycine 4-20% gradient gel and detected by immunoblotting using specific antibodies as indicated. N=nuclear fraction, C=cytoplasmic fraction. Immunoblot analysis of MEK1 (always cytoplasmic) and PARP (always nuclear) were included as controls for the nucleocytoplasmic fractionation protocol.

To further test this hypothesis, we treated ad5 infected U2OS cells with methyl-thio-adenosine (MTA) a methylation inhibitor naturally generated in the cells as a metabolic intermediate in the conversion of putrescine to spermidine and of spermidine to spermine (Williams-Ashman et al., 1982). Cells were infected with

ad5, treated with 0.3mM MTA 18hrs p.i, fixed with 4% PFA 36hrs p.i and stained with an α-L4-100k antibody. Using confocal microscopy, we show that in adenovirus-infected cells treated with MTA, L4100k fails to localize to the nucleus and is exclusively cytoplasmic, in contrast to untreated cells where L4100k is already mostly nuclear at 36hrs p.i (figure 3.7).

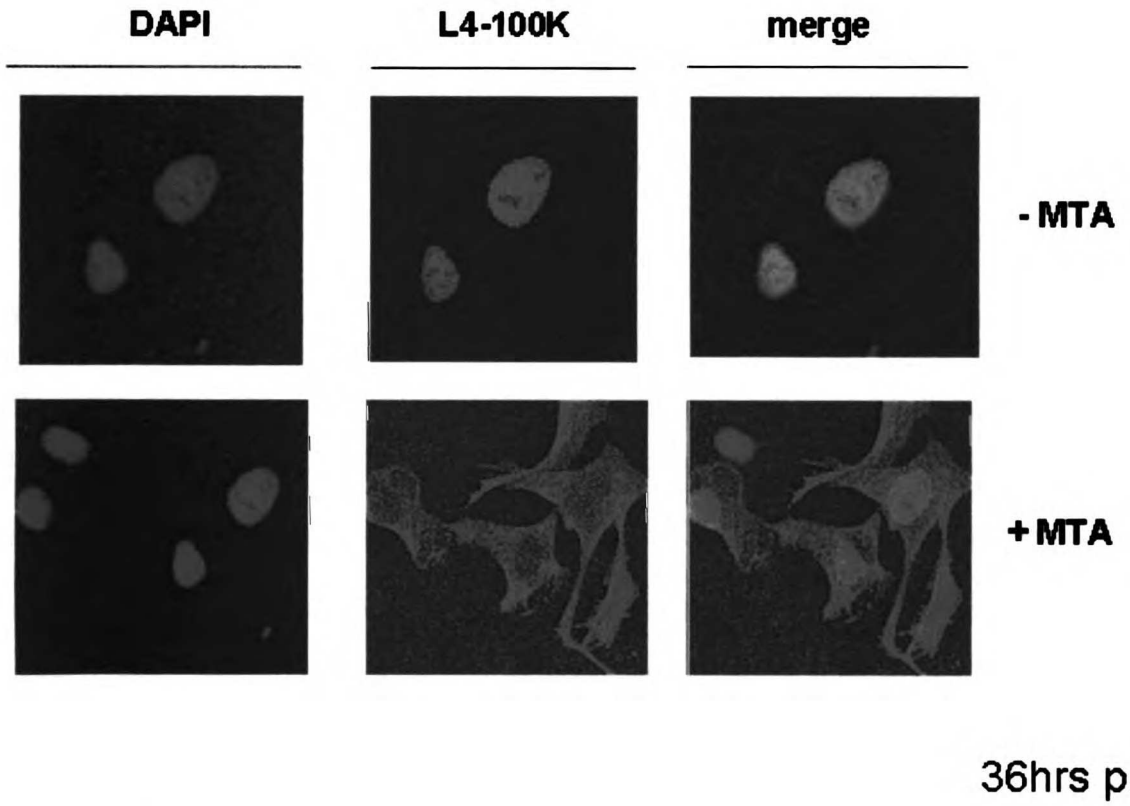


Figure 3.7 Methylation inhibitors prevent L4100k nuclear accumulation. Cells were infected with ad5, treated with 3mM MTA 12hrs p.i, fixed with 4% PFA 36hrs p.i and stained with a monoclonal antibody against L4100K.

3.2.iv L4100k-mediated host protein synthesis shutoff

According to the current model of adenovirus-induced inhibition of cellular protein synthesis, L4100k binds to the translation initiation complex EIF4F, the cap-binding complex required for cap-dependent translation, and displaces mnk1 kinase, which normally phosphorylates EIF4F. Dephosphorylation of EIF4F is thought inhibit the ability of the complex to bind to the cap structure on cellular RNA messages and consequently cap-dependent translation is inhibited. The 100k binding site to EIF4F has been mapped to amino acids 280-365, and this site was shown by others to be sufficient to displace mnk1. Thus, according to this model, a 100k stable cell line should be impossible to make, since inhibition of host protein synthesis would be toxic to the cell. However, we were able to generate such cell lines, by selecting U2OS cells transfected with myc-tagged 100kFL, 100kΔC and $100k^{RGG3}$, which suggests that the above model is not entirely correct. Since we already showed that methylated 100k is nuclear, we next wanted to determine the subcellular localization of L4100k in these cell lines. Cells were plated on chamber glass slides, fixed and stained against the myc tag of the 100k clones. As shown below, 100kFL is exclusively nuclear in the stable cell line, which suggests that the reason 100k is not toxic to these cells is the fact that it does not localize to the cytoplasm were translation occurs. However, 100kΔC was exclusively cytoplasmic, as expected from the results shown in figure 3.3, and the lack of the RGG domain which is necessary for nuclear accumulation. This clone, however, still contains the whole amino

terminus of 100k, which includes the proposed binding site to EIF4F (a.a 280-365), but it nevertheless is not toxic to the cells.

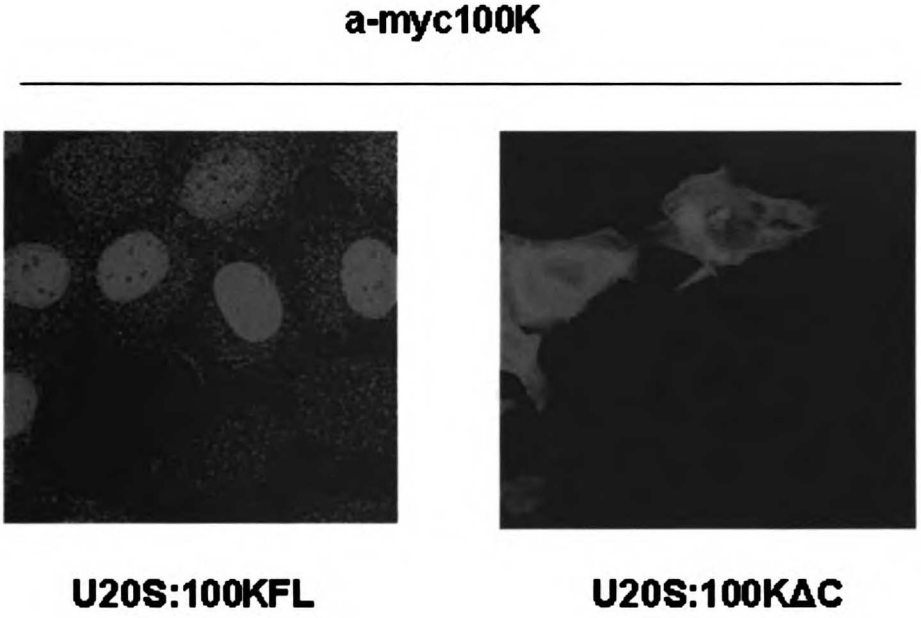
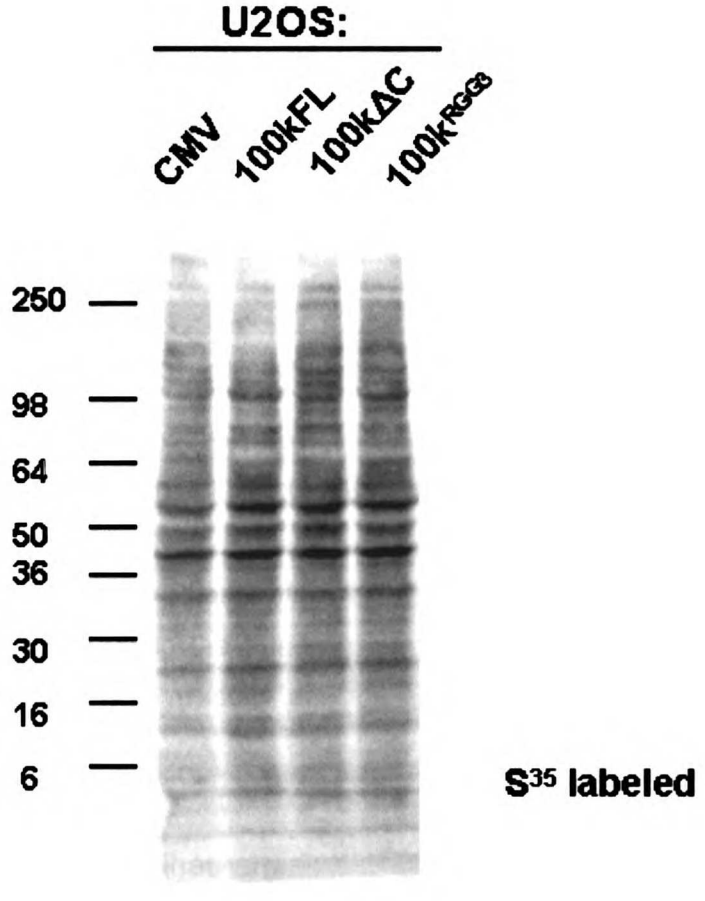


Figure 3.8 U2OS:100kFL and U2OS:100kΔC cells were grown on glass chambers, fixed with 4% PFA and stained with an antibody against the myc tag, and a fluorescently labeled secondary antibody, and visualized by confocal microscopy.

We therefore hypothesized that in these cell lines, 100k is not able to induce host protein synthesis shutoff, which would explain why these cell lines were possible to be generated. To test this hypothesis, we measured total protein synthesis in U2OS:CMV, U2OS:100kFL, U2OS:ΔC and U2OS:100k^{RGG3} cells using S^{35} labeling, as described in the materials and methods section. As shown below, protein synthesis is not inhibited in the U2OS cells expressing the FL, DC, or the RGG3 100k proteins. These results contradict the current model for 100k-

mediated inhibition of host protein synthesis, since L4100k alone is not sufficient to induce shutoff.



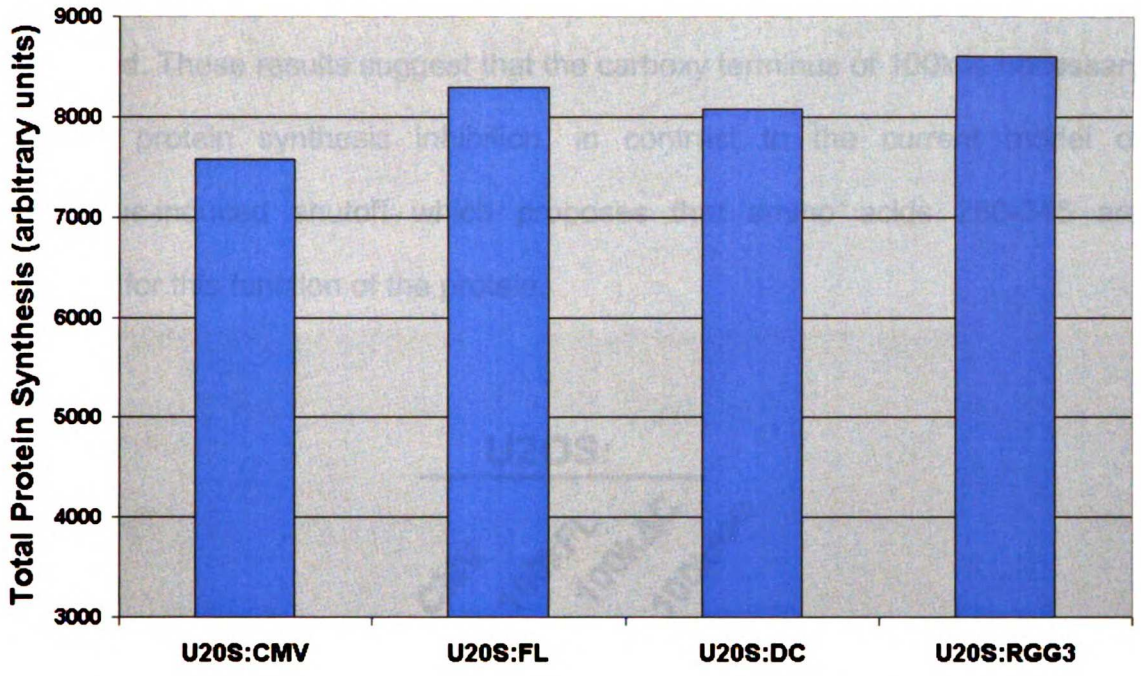
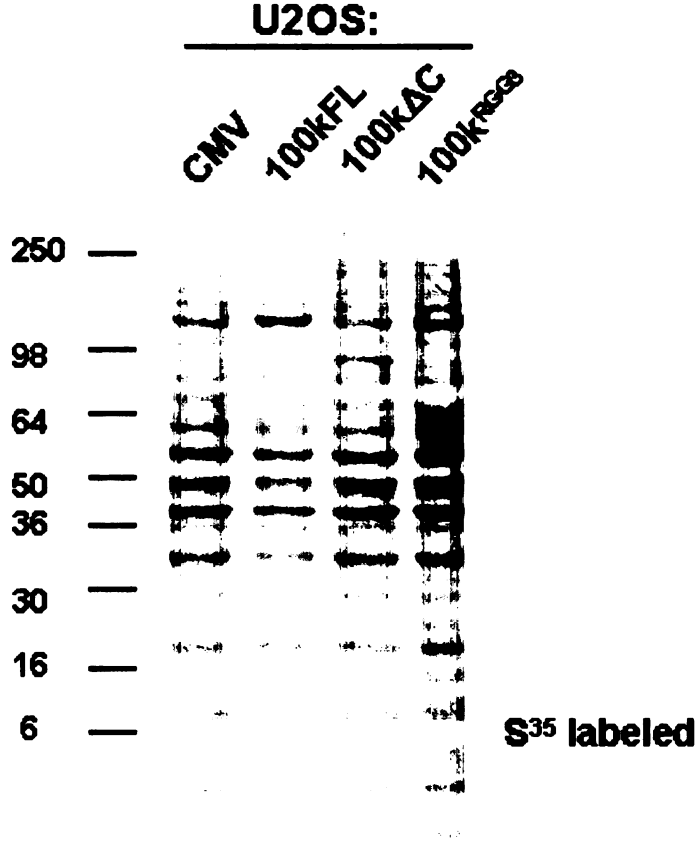


Figure 3.9 (A) U2OS:CMV, U2OS:100kFL, U2OS:ΔC and U2OS:100k^{RGG3} cells were plated in 6-well plates at 5 x 10^4 cells/well and the next day they were labeled with S^{35} for 2hrs, harvested and total lysates were ran on a 4-20% gel, transferred to a PDVF membrane and visualized on PhosphoImager, as described earlier. **(B)** Total protein synthesis was measured using the ImageQuant software, and the absolute value for each cell line is shown here (results are the average value from two independent experiments).

Since we showed earlier that arginine methylation of 100k at the carboxy terminus localizes this protein to the nucleus, we next determined the effects of inhibiting methylation on the ability of 100k to inhibit host protein synthesis, since non-methylated 100k remains in the cytoplasm were translation occurs. U2OS:CMV, U2OS:100kFL, U2OS:ΔC and U2OS:100k^{RGG3} cells were treated with methylation inhibitors for 24hrs and total protein synthesis was measured by S^{35} labeling. Inhibiting methylation in U2OS:100kFL cells resulted in a 2-fold lower amounts of protein synthesized compared to the U2OS:CMV cells.

However, protein synthesis in U2OS:ΔC and U2OS:100k^{RGG3} cells remained unaffected. These results suggest that the carboxy terminus of 100k is necessary for host protein synthesis inhibition, in contrast to the current model of adenovirus-induced shutoff which proposes that amino acids 280-345 are sufficient for this function of the protein.



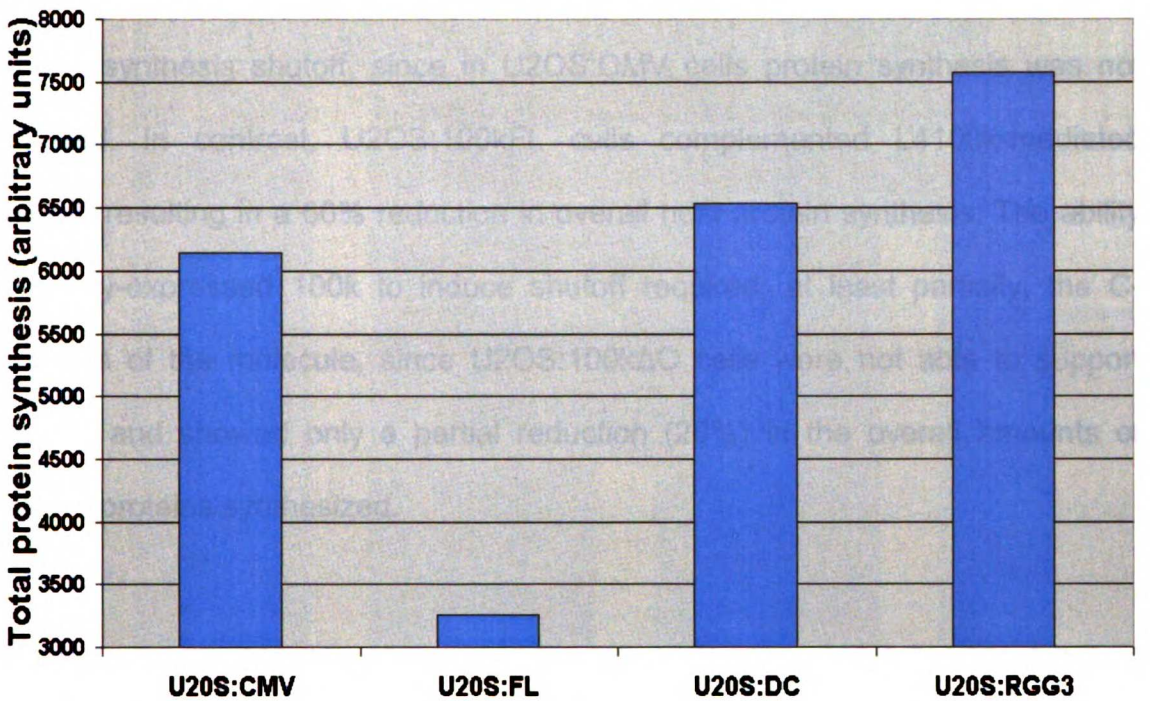
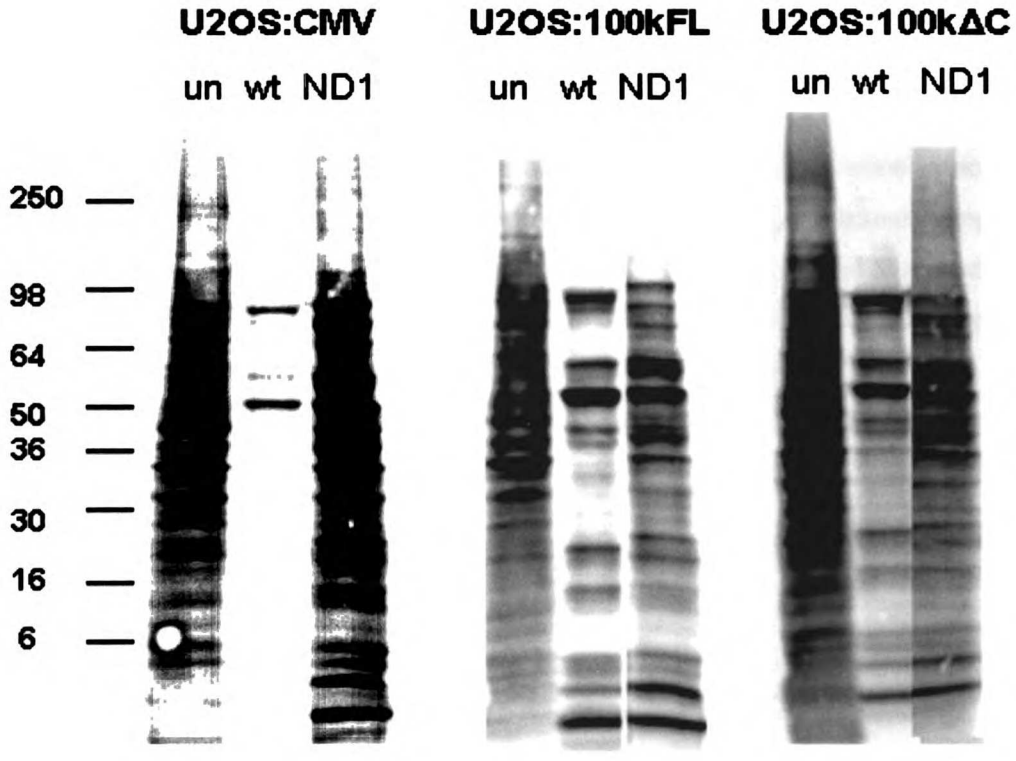


Figure 3.10 (A) U2OS:CMV, U2OS:100kFL, U2OS:ΔC and U2OS:100k^{RGG3} cells were plated in 6-well plates at 5 x 10^4 cells/well and 6hrs later were treated with 30uMof the methylation inhibitor Adenosine Peroxide (AP). Next day cells were labeled with S^{35} for 2hrs, harvested and total lysates were ran on a 4-20% gel, transferred to a PDVF membrane and visualized on PhosphoImager, as described earlier. **(B)** Total protein synthesis was measured using the ImageQuant software, and the absolute value for each cell line is shown here (results are the average value from two independent experiments).

To further determine the importance of the C-terminus of 100k in 100k-mediated shutoff, we used a 100k ts mutant virus, which at the non-permissive temperature (39.4 C) produces a defective 100k protein (reference). Using this virus, and wild-type dl309 as a control, U2OS:CMV, U2OS:100kFL and U2OS:ΔC cell lines were infected at the non-permissive temperature, and 36hrs p.i cells were S^{35} labeled for 1 hr, harvested and total protein synthesis was measured as described in the

materials and methods. The L4100k ts mutant virus was defective for host protein synthesis shutoff, since in U2OS:CMV cells protein synthesis was not inhibited. In contrast, U2OS:100kFL cells complemented L4100k-mediated shutoff, resulting in a 60% reduction in overall host protein synthesis. The ability of stably-expressed 100k to induce shutoff required, at least partially, the C-terminus of the molecule, since U2OS:100kΔC cells were not able to support shutoff, and showed only a partial reduction (20%) in the overall amounts of cellular proteins synthesized.



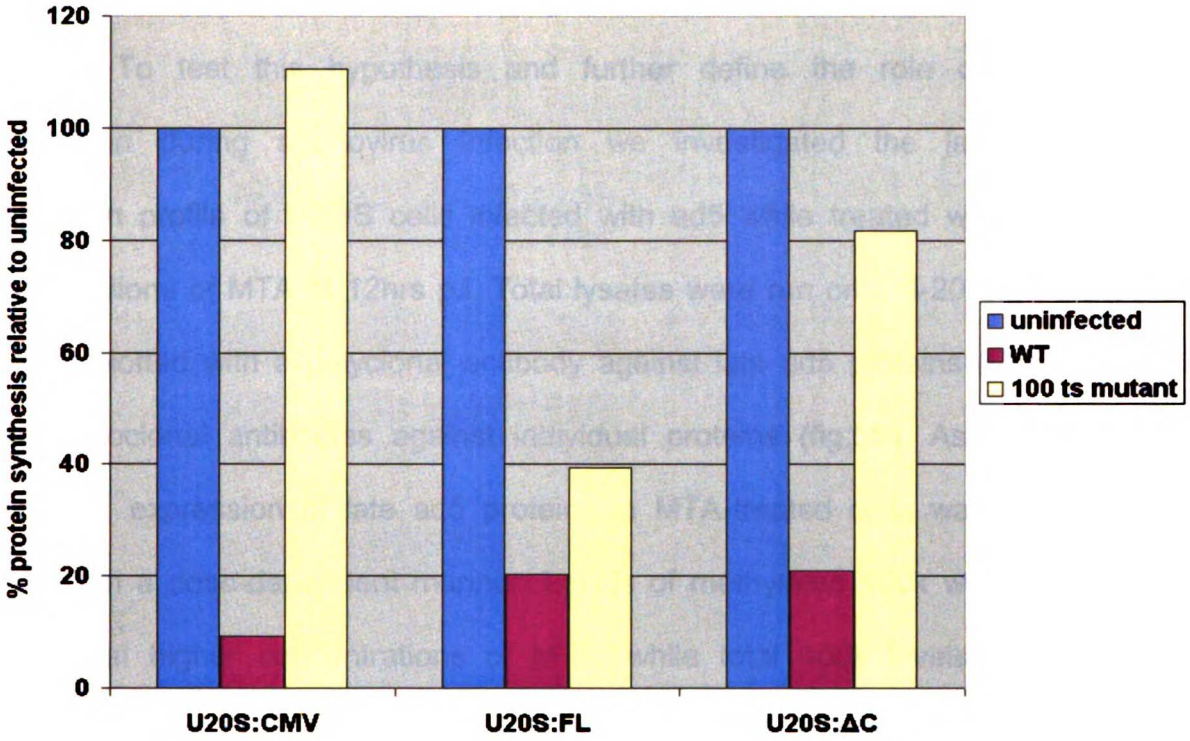
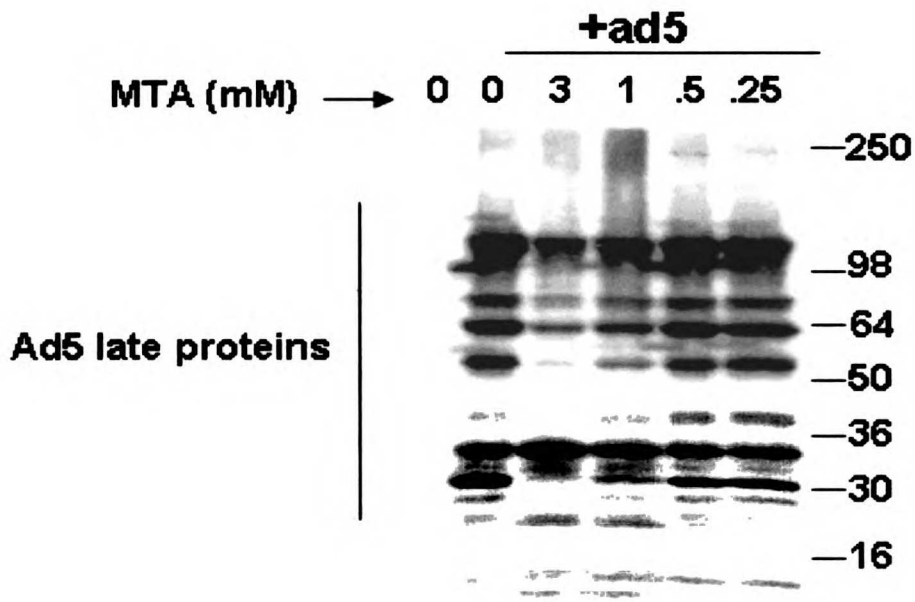


Figure 3.11 (A) U2OS:CMV, U2OS:100kFL and U2OS:100ΔC cells were infected with wild type, or with an L4100k mutant adenovirus at the non-permissive temperature (39.4C). 36hrs post-infection, cells were labeled with S^{35} for 2hrs, harvested, and lysates were ran on a 4-20% Tris-glycine gel. Proteins were transferred onto a PDVF membrane and total labeled protein was visualized using PhosphoImager. **(B)** Total protein synthesis was measured using ImageQuant and depicted on this graph as a percentage relative to protein synthesis in uninfected cells.

3.2.v Expression of late viral protein production is severely defective in MTA-treated cells

Methylation of L4100k may therefore be necessary for localization to the nucleus. L4100k is implicated in RNA transport of late viral messages, and nuclear L4100k is involved in hexon trimerization and assembly, two important functions during infection. Thus, methylation of L4100k may be an important type of post-

translational modification necessary for L4100k function and vital to the virus lifecycle. To test this hypothesis and further define the role of arginine methylation during adenovirus infection we investigated the late protein expression profile of U2OS cells infected with ad5 while treated with several concentrations of MTA at 12hrs p.i. Total lysates were run on a 4-20% gradient gel and blotted with a polyclonal antibody against late ad5 proteins (fig.5A) or with monoclonal antibodies against individual proteins (fig.5B). As shown in figure 5A, expression of late ad5 proteins in MTA-treated cells was severely reduced in a dose-dependent manner. Levels of methylated 100k were greatly reduced at higher concentrations of MTA, while total 100k levels remained constant (figure 5B). Importantly, expression of E1A-an early gene that is constitutively expressed throughout infection- remained stable suggesting that the effect of MTA on these cells is specific to the expression of the late but not the early ad5 proteins.



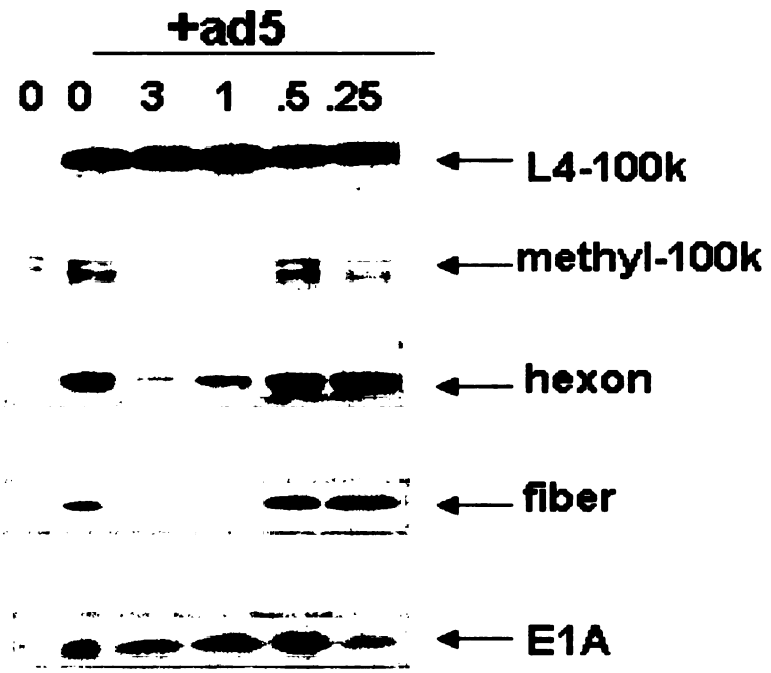


Figure 3.12 Expression of late viral protein production is severely defective in MTA-treated cells. U2OS cells were infected with wild-type ad5, treated with various concentrations of MTA at 12hrsp.i, lysates normalized and resolved by SDS Tris-glycine 4-20% gradient gel at 36hrs p.i, and detected by immunoblot analysis using a polyclonal antibody against late adenovirus proteins **(A)** or monoclonal antibodies against L4100k, hexon and fiber **(B)**. Methyl-100k was detected using an α-methyl arginine antibody. E1A was also included in the experiment as a control for the early expressed viral genes whose expression does not depend on L4100k.

To further determine the effects of inhibiting arginine methylation on adenovirus infection, we measured the viral yield produced from infected U20S cells treated with 3mM MTA at 12hrs p.i, using an ELISA assay as described previously (Johnson et al., 2002). Consistent with our results in figure 5, the viral yield produced from the MTA- treated cells was at least 1000-fold lower when

compared to the yield produced from the untreated cells. Finally, we examined the effects of methylation inhibitors on the ability of wild-type adenovirus to induce CPE. U2OS cells were infected with dl309 and either treated with AP 12hrs post infection, or left untreated. As a control, we also included non-infected cells treated with the inhibitor, to test the effects of inhibiting methylation on infected cells. As shown below, inhibition of arginine methylation protects U2OS cells from adenovirus-induced CPE. These results, confirm our previous findings that arginine methylation is a critical cellular function during adenovirus infection, and necessary for adenovirus-induced CPE in infected cells.

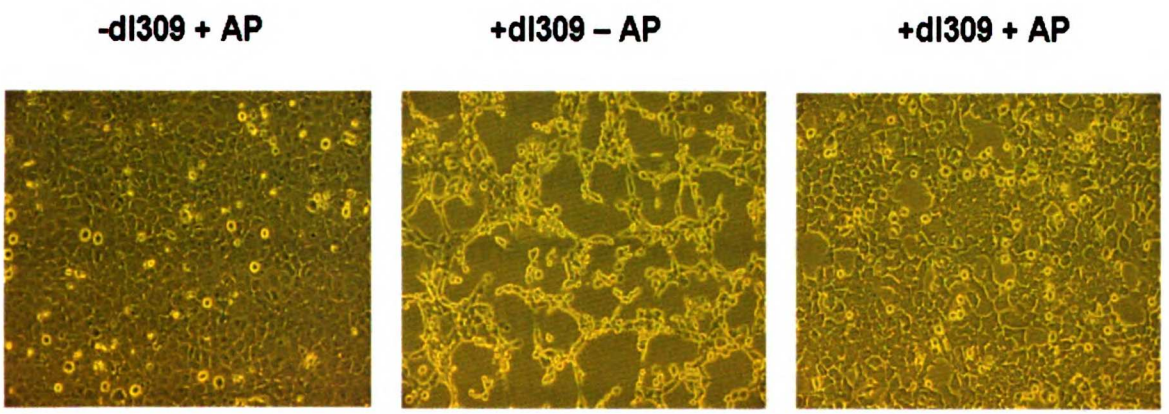


Figure 3.13 U2OS cells were infected with dl309 in the presence or absence of the methylation inhibitor (added 12hrs post infection), and visualized under a light microscope 48hrs post-infection. As a control, non-infected U2OS cells treated with the inhibitor were also included.

3.3 DISCUSSION

L4100k is a multifunctional protein vital to the late phase of adenovirus infection. Its expression is a key event in the early-late switch in MLTU expression (Farley et al., 2004) and in viral morphogenesis. L4100k is involved in host protein synthesis shutoff: it directly competes for the mnk1 binding site on EIF4G, resulting in displacement of the kinase and dephosphorylation of EIF4E (Cuesta et al., 2000). In some systems, dephosphorylation of EIF4E correlates with a decrease in the rate of cap-dependent translation and inhibition of most cellular protein synthesis (Scheper and Proud, 2002) (Wang et al., 1998), although the functional significance of EIF4E phosphorylation has been controversial (Knauf et al., 2001), (Morley and Naegele, 2002) (Scheper and Proud, 2002). Late adenovirus messages possess a unique 5' UTR which allows their translation through a cap-independent mechanism called ribosome shunting (Yueh and Schneider, 1996; Yueh and Schneider, 2000). In addition to its involvement in host protein synthesis shutoff, L4100k is involved in hexon transport to the nucleus, hexon trimerization and assembly (Cepko and Sharp, 1982) and because of its RNA binding activity (Riley and Flint, 1993), though without any apparent sequence specificity (Adam and Dreyfuss, 1987), it may also play a role in RNA transport. The mechanism by which L4100k switches between these functions remains unknown. In this study, we demonstrate that ad5 L4100k localization is controlled by post-translational methylation within its RGG domain, and we show that arginine methylation is a necessary cellular component for a productive adenovirus infection.

Nuclear localization of L4100k depends on arginine methylation

Recently Cuesta *et al* showed that C-terminal truncation of L4100k resulted in cytoplasmic accumulation of the protein, suggesting that this part of the molecule contains an NLS sequence (Cuesta et al., 2004). However cells transfected with wild-type L4100k show both nuclear and cytoplasmic accumulation of the protein suggesting that there is an additional level of complexity in the regulation of L4100k localization. Using the methylation inhibitor MTA we show here that in the absence of arginine methylation, L4100k is exclusively cytoplasmic, suggesting that methylation may be critical for its nuclear accumulation. In support of this hypothesis, we show that methyl 100k is exclusively nuclear, along with most of the methylated proteins in the cell.

A critical RGG sequence necessary for methylation and nuclear import

To date several other cellular and viral proteins have been found to be controlled by post-translational methylation, mostly at the level of sub-cellular localization and RNA-binding activity (McBride and Silver, 2001) for review). Post-translational methylation mainly occurs on arginine residues within RGG and RG motifs collectively called RGG boxes. The C-terminus of ad5 L4100k contains an arginine-glycine rich domain with three RGG tripeptides. As shown in figure 1, the RGG domain, as well as many upstream RG, RGR and RXR motifs, are very well conserved across many adenovirus serotypes, which suggests that these sequences are functionally important. Using an antibody that recognizes asymmetrically di-methylated arginine residues, we show that L4100k is

methylated within its C-terminus and that this part of the molecule is necessary for binding to PRMT1 methylase, since a C-terminal truncated L4100k clone (myc-100kΔC) failed to bind to the methylase and was not methylated. Myc-100kΔC clone localized exclusively in the cytoplasm, further implicating the RGG domain in nuclear accumulation of the protein. Transfected wild-type myc-100k, as well as a mutant clone with the third RGG tripeptide (amino acids 741-743) changed to alanine residues (myc-100k^{RGG3}), localized both in the nucleus and the cytoplasm in uninfected cells. However, even though the wild-type myc-100k shuttled into the nucleus after infection, the myc-100k^{RGG3} mutant did not. Thus, our results suggest that even though the whole RGG domain may act as an NLS sequence, the third RGG box (amino acids 741-743) is important in L4100k transport from the cytoplasm to the nucleus during infection and post-translational methylation of arginine 741 may be a critical modification for this process.

L4100k forms oligomers in infected cells

In order to determine whether methylation of L4100k occurs in several arginine residues, or only on arginine 741, we immunoprecipitated wild-type myc-100k, myc-100kΔC and myc-100k^{RGG3} through the myc-tag and performed western blot analysis using a dimethyl-arginine specific antibody. The 80kD C-terminus truncated protein was not methylated, suggesting that the C-terminal part of the protein is the exclusive site of methylation. During our analysis of truncated

L4100k proteins, we observed that these mutants associated with full length L4100k, and that this association occurred in the absence of other viral proteins. Since L4100k methylation, but not oligomerization, requires another viral component, we conclude that methylation is not necessary for L4100k to form oligomers. In support of this conclusion, the C-terminus deleted L4100k mutant, which lacks the site of methylation by PRMT1, was still able to co-immunoprecipitate with viral-coded L4100k during infection. This is the first demonstration that L4100k forms oligomers *in vivo* and the role of oligomerization remains to be determined. Since L4100k oligomerization can occur in the absence of a viral infection, we conclude that 100k forms homo-dimers, homo-oligomers or complexes with a cellular partner. To date, EIF4G and PRMT1 are the only cellular proteins shown to interact with L4100k. L4100k is thought to bind to EIF4G through a single binding site (the same site that mnk1 utilizes to bind to EIF4G and phosphorylate EIF4E). Thus L4100k may form homo-oligomers, or multimeric complexes through interactions with PRMT1.

Because L4100k forms oligomers *in vivo*, we were unable to compare the methylation status of myc-100k^{RGG3} to that of wild-type myc-100k, since in immunoprecipitation experiments we pulled down viral-coded L4100k along with the myc-tagged molecules. However, our results point to an important role for the third RGG box of L4100k in the accumulation of the protein to the nucleus during infection. This result, along with the fact that arginine methylation seems to be necessary for nuclear accumulation of L4100k suggests that the methylation status of arginine 741 may be the signal for 100k shuttling to the nucleus.

Arginine methylation is a cellular function necessary for productive adenovirus infection

Since nuclear localization of L4100k is important for its function and arginine methylation is necessary for nuclear accumulation of the protein, we hypothesized that arginine methylation must be vital to the late phase of adenovirus infection. Indeed, treatment of ad5-infected U2OS cells with the methylation inhibitor MTA resulted in greatly reduced overall expression of late viral proteins in a dose-dependent manner, whereas the levels of E1A, an early expressed gene that remains constitutively active during infection, remained unaffected. This result, along with the fact that the overall levels of L4100k and total cellular protein synthesis also remained unaffected in MTA-treated cells, suggests that the effect of inhibition of arginine methylation in our cells is a result of inhibition of L4100k methylation and not a non-specific effect on the overall protein synthesis in the cell.

There are several possibilities as to how methylation may alter L4100k function. One would be that merely by changing localization from the cytoplasm to the nucleus, L4100k switches between its cytoplasmic function (host-protein synthesis shutoff) and its nuclear functions (hexon transport to the nucleus and hexon trimerization and assembly). It is also possible that methylation of the protein alters its binding properties thereby affecting protein-protein and protein-RNA interactions, in a way that several other proteins are known to be regulated. For example, the RGG domain of Herpes Simplex Virus (HSV) ICP27 protein mediates binding to viral intronless RNAs (Sandri-Goldin, 1998a; Sandri-Goldin,

1998b) and an ICP27 null virus is defective for nuclear export of these transcripts. ICP27 was also shown to be regulated by post-translational arginine methylation (Hibbard and Sandri-Goldin, 1995), and removing the RGG domain abolishes its RNA-binding activity (Mears and Rice, 1996). It is therefore possible that ad L4100k is regulated in the same way. In support of this hypothesis, methyl-100k can be detected during the early phase of L4100k expression and considerably earlier than when hexon protein can be detected, (unpublished observations) suggesting that L4100k methylation is needed before L4100k is involved in hexon trimerization and assembly, and may therefore also play a role at regulating RNA transport of late viral messages. Even though more experiments are needed in order to fully understand the multicomponent L4100k function and the role that arginine methylation plays in regulating it, our study shows that post-translational methylation of L4100k is necessary for its nuclear accumulation, and that the third RGG box (amino acids 741-743) is necessary for 100k shuttling from the cytoplasm to the nucleus during infection. Moreover, the dramatic effects of inhibition of protein arginine methylation on the late viral protein expression and overall viral yield, suggests that this type of post-translational modification is a critical cellular function necessary for a productive adenovirus infection.

CHAPTER 4

Temporal analysis of L4100K binding partners

4.1 INTRODUCTION

L4100k is a multifunctional protein that is involved in host protein synthesis shutoff, and in hexon trimerization and assembly. These two processes occur in different subcellular compartments, and at different times during infection. Inhibition of translation requires the cytoplasmic accumulation of L4100k, whereas hexon assembly occurs in the nucleus of infected cells. Thus, L4100k is regulated in a spatial and temporal manner. Our previous results identified arginine methylation as a post-translational modification which controls the subcellular localization of L4100k. Asymmetric di-methylation of L4100k on the RGG boxes within its C-terminus by PRMT1 results in nuclear import of this protein. Preventing methylation of L4100k using methylation inhibitors, or by truncating the 100k C-terminus, prevents 100k accumulation to the nucleus. The presence of 100k at different sub-cellular compartments during the early and late phases of infection, the multifunctional character of this protein, and its complex structural organization (figure 4.1) suggest that it may interact with several cellular and viral factors in order to exert its function. However, the only identified L4100k-interacting proteins to date are the EIF4G translation initiation factor, the PRMT1 methylase and the virus-coded hexon polypeptide. The mechanism by which 100k mediates shutoff is thought to involve binding of 100k to EIF4G, a large scaffold protein which bridges together the cap-binding EIF4E, an ATP-dependent RNA helicase (eIF4A) and mnk1 kinase, which phosphorylates EIF4E. Phosphorylation of this complex (collectively termed translation initiation complex) at the EIF4E Ser209 site, is believed to be necessary for efficient

binding of the complex to the cap structure at the 5' end of cellular mRNAs, and dephosphorylation of EIF4E has been correlated with reduced protein synthesis in some systems. However, several studies question the importance of EIF4E phosphorylation in stimulating mRNA translation altogether (Shneider 21, 29), since in certain cases, EIF4E phosphorylation actually decreases the affinity of this protein for the cap structure. Moreover, mnk1/mnk2 double knockout mice are viable, fertile and they develop normally despite the absence of EIF4E phosphorylation at Ser 209 (Ueda et al., 2004), and neither general protein synthesis, nor cap-dependent translation were affected, suggesting that EIF4E phosphorylation is not important for cellular translation in mammals. Thus, L4100k may displace mnk1 from the translation initiation complex for reasons other than inhibiting its phosphorylation, and dephosphorylation of EIF4E may be the indirect consequence and not the purpose of L4100k binding to the complex. For example, L4100k may displace mnk1 and bind to the translation initiation complex in order to recruit additional specific cellular factors that are involved in cap-independent translation, or to exclude proteins that promote cap-dependent translation. The second possibility does not seem to be true, since previous studies have shown that during adenovirus infection, EIF4G, EIF4E and EIF4A are still part of the active translation initiation complex, and their expression levels do not seem to be reduced (Cuesta et al., 2004). Therefore, L4100k may facilitate the recruitment of additional, as of yet unidentified, cellular components that are necessary for L4100k-mediated host protein synthesis shutoff and efficient translation of late viral mRNAs, thereby turning the EIF4F complex into a

virus-specific translation initiation machinery. Identification of these factors is key to our understanding of the molecular mechanism of adenovirus-mediated host protein shutoff, and should provide further insights into the multiple L4100k functions, and the role of this protein in the adenovirus lifecycle.

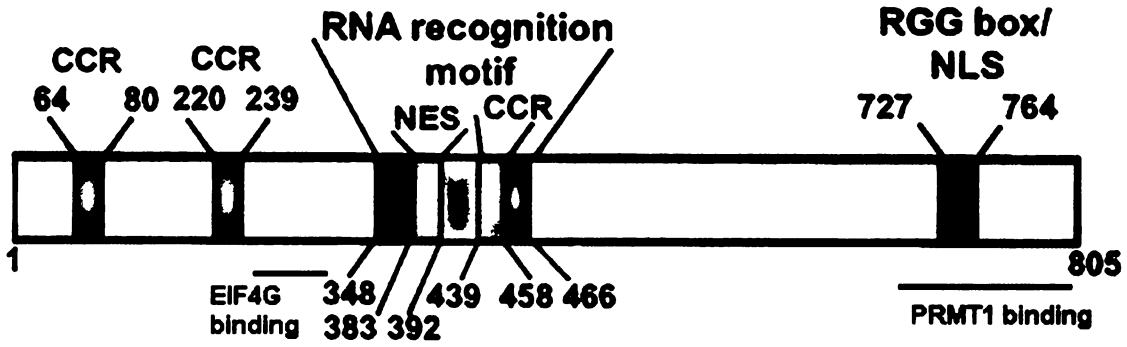


Figure 4.1 L4100k has several structural domains. The N-terminus contains two coil-coil regions (CCR), which are domains that mediate protein-protein interactions, but proteins that bind to these regions are still unidentified. The central part of the molecule contains an RNA recognition motif, within which a nuclear export sequence (NES), and a third CCR domain have been identified. The C-terminus contains a glycine-arginine rich domain, with three RGG tripeptides (RGG box) Recently, this domain was shown to contain a nuclear localization sequence, and this study identified the PRMT1 binding site to also lie within this domain. *Adapted from Questa et al, 2004*

4.2 RESULTS

4.2.i TAP-100k cell line

The TAP-100k cell line was generated by retroviral infection of U2OS cells with a construct that carries 100k fused to the TAP tag, as described in materials and methods. 100k-expressing cells were selected as a pool of cells carrying the selection marker of the construct (neomycin resistance) in DMEM supplemented 10% FBS and 1mg/ml geneticin. To validate TAP-100k expression in this cell line, we grew 2 15-cm dishes of U2OS:TAP100k and U2OS:CMV cells, harvested the cells in 1% NP40 lysis buffer and performed immunoprecipitation with IgG beads, which bind to the protein A part of the TAP tag. IP samples, along with total lysates were ran on a 4-20% tris-glycine gel, transferred on a PDVF membrane and blotted with an HRP-conjugated IgG antibody that binds to the protein A domain, or with an antibody against 100k. As shown below, TAP100k was expressed as a fusion protein of about 120kd, as expected with the addition of the TAP tag, and is recognized by both antibodies.

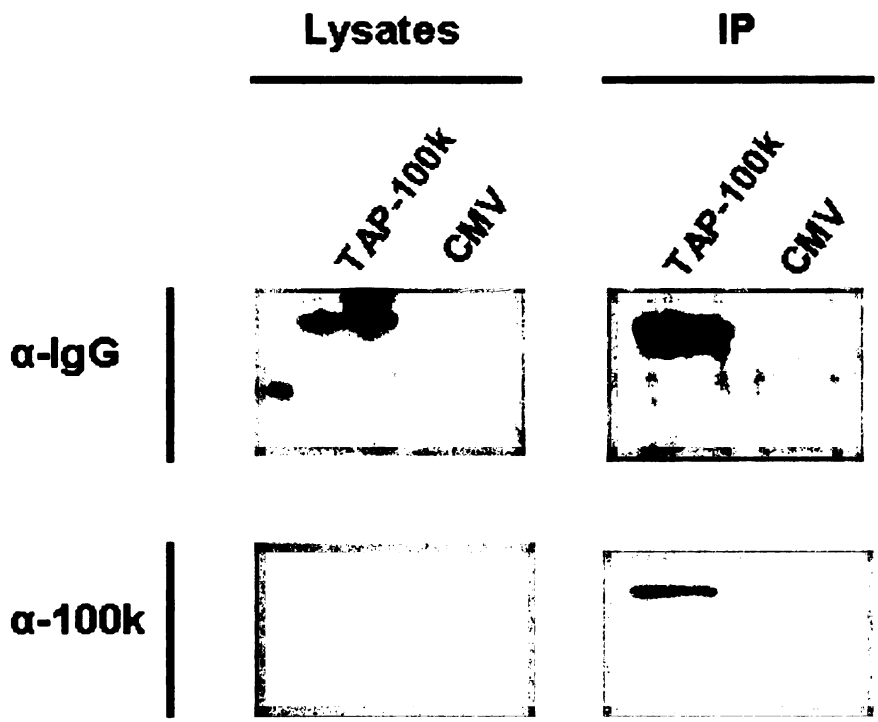


Figure 4.2 Lysates from the U2OS:TAP-100k, or the U2OS:CMV cell lines were subjected to immunoprecipitation with agarose-conjugated IgG beads, and samples were run on a 4-20% gel along with total lysates. Expression of TAP-100k was visualized by blotting with an a-IgG HRP-conjugated antibody (top panels), or an antibody against L4-100k (bottom panels).

4.2.ii Identification of novel 100k-binding proteins using Tandem Affinity Purification

To identify novel cellular and viral L4100k-interacting proteins, we used the U2OS:TAP100k cell line with or without infection with wild-type adenovirus, and the non-expressing U2OS:CMV cell line as a negative control. Infected U2OS:TAP100k cells were harvested at 18hrs and at 36hrs post infection, and

along with uninfected cells and the negative control were subjected to TAP purification (figure 4.3), as described in the materials and methods section. A number of proteins were co-precipitated with TAP100k, in both the uninfected and infected samples, suggesting that these interactions do not depend on infection. These proteins include the ATP-dependent helicase RENT1 (hUpf1), the heterogenous nuclear ribonucleoprotein U (hnrnpU), the Polyadenylate-binding protein 1 (PABP1), and the elongation factor 1-alha-1 (EF1A-1) (figure 4.4 and table 4.2). However, many proteins appear to bind to L4100k only in the context of infection. For example, three RNA helicases, RH II/Gu, DHX15, and DDX5 bound to L4100k in cells infected for 36hrs, but not in uninfected cells, even though in both cases L4100k is localized in the nucleus. Thus, interaction of L4100k with these helicases requires another component of the infection, and must be important for L4100k function during the late phase of infection. PRMT1 methylase was one of the identified proteins that bound to L4100k (figure 4.3), which confirms that the TAP100k fusion protein is functional. PRMT1 bound to L4100k only in the 18hrs p.i sample (figure 4.3), which confirms or previous results that the function of this interaction is to methylate L4100k and facilitate nuclear accumulation of the protein. Hexon polypeptide associated with L4100k (figure 4.3), which confirms L4100k involvement in hexon trimerization and assembly, a process that occurs in the nucleus. However, in this experiment, we were not able to detect interaction of L4100k with EIF4G, despite results from other labs which identified EIF4G as one of the L4100k interacting proteins (Cuesta et al., 2000). Experiments showing association of L4100k with EIF4G in

the past were performed *in vitro*, with purified components, or in transient transfection assays (Cuesta et al., 2000) where the expression levels of EIF4G and L4100k could be higher than the relevant levels *in vivo*. Thus, it is possible that the interaction of L4100k with EIF4G does not occur *in vivo*, or is undetectable in our experimental setting.

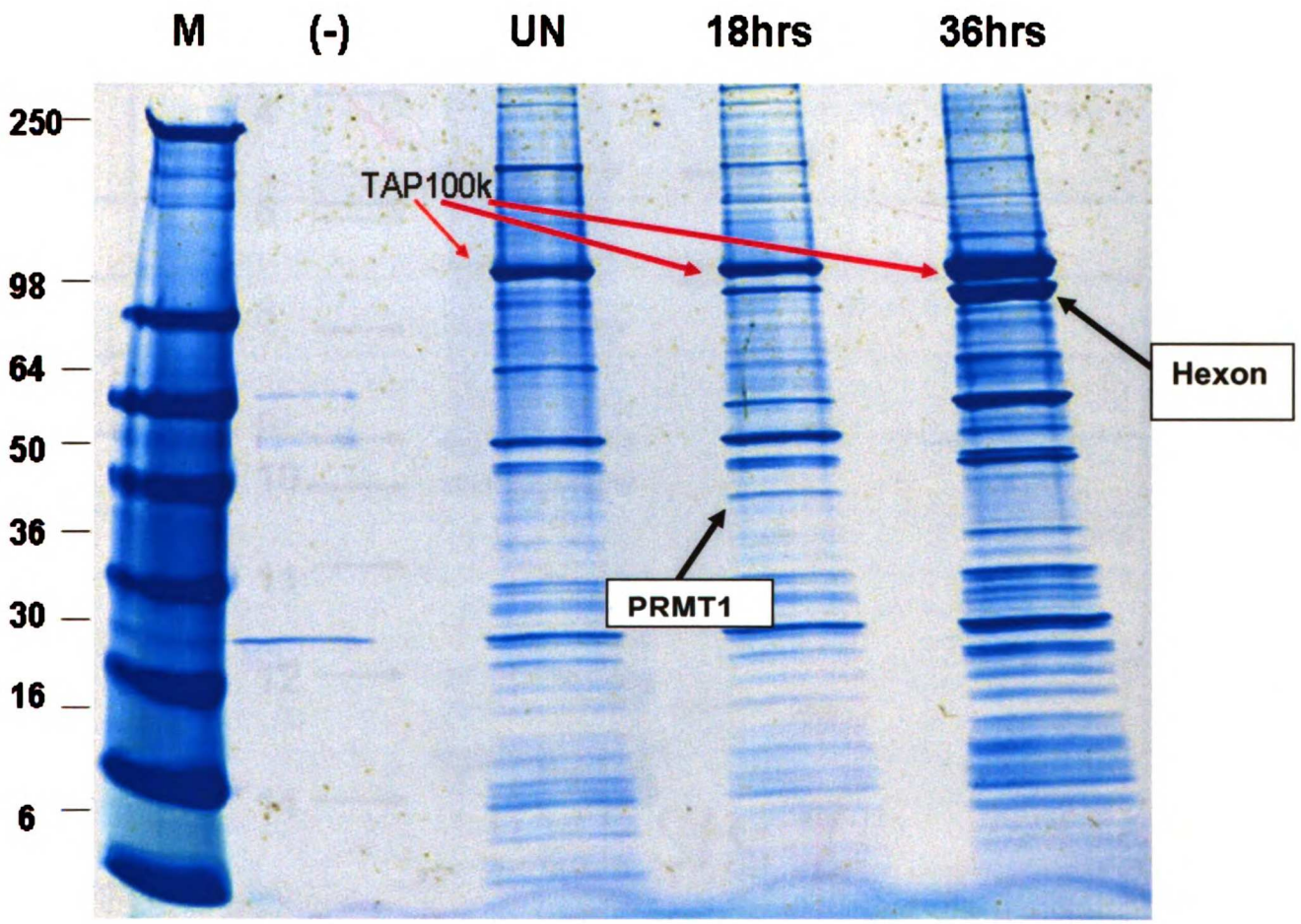


Figure 4.3 TAP-purified L4100k complexes from uninfected and infected cells were ran on a 4-20% gel and stained with SafeBlue Coomasie stain (INVITROGEN). Two of the already identified L4100 binding partners, PRMT1 methylase and hexon, co-purified with TAP-100k and are shown on the figure.

L4100k – interacting proteins that do not depend on infection

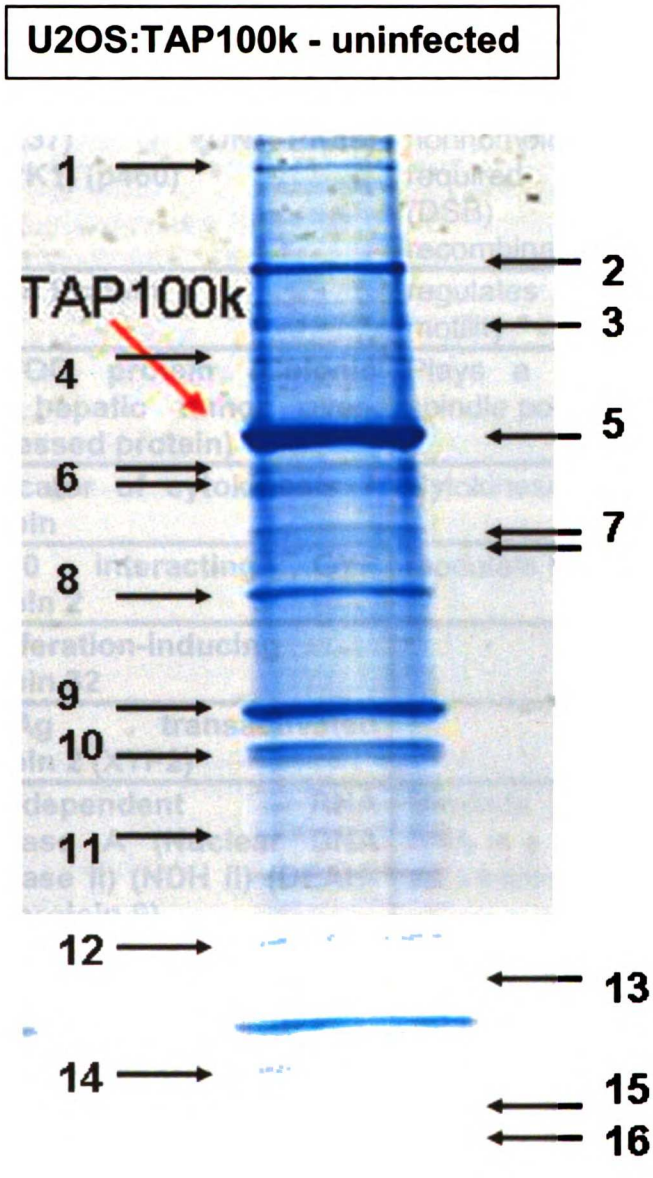
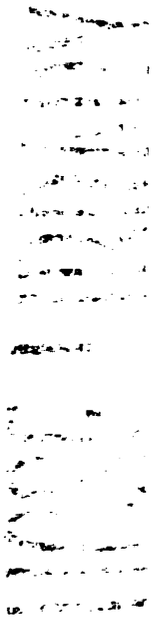


Figure 4.4

Table 4.1

L4100k – interacting proteins that do not depend on infection

BAND	PROTEIN	FUNCTION
1	**DNA-dependent protein kinase catalytic subunit (EC 2.7.1.37) (DNA-PKcs) (DNPK1) (p460)**	Acts as a molecular sensor for DNA damage. Involved in DNA nonhomologous end joining (NHEJ) required for double-strand break (DSB) repair and V(D)J recombination
2	**Girdin Protein**	regulates actin organization and cell motility
2	**CH-TOG protein (Colonic and hepatic tumor over-expressed protein)**	Plays a major role in organizing spindle poles.
2	**Dedicator of cytokinesis 7 Protein**	Cytokinesis
3	**Grb10 interacting GYF protein 2**	modulate IGF-I signaling
3	**Proliferation-inducing protein 32**	
3	**HBxAg transactivated protein 2 (XTP2)**	
4	**ATP-dependent RNA helicase A (Nuclear DNA helicase II) (NDH II) (DEAH-box protein 9)**	Unwinds double-stranded DNA and RNA in a 3' to 5' direction. Functions as a transcriptional activator.
5	**Regulator of nonsense transcripts 1 (Nonsense mRNA reducing factor 1) (NORF1) (Up-frameshift suppressor 1 homolog) (hUpf1)**	Part of a post-splicing multiprotein complex. Involved in nonsense-mediated decay (NMD) of mRNAs containing premature stop codons.
4	**Ubiquitin--protein ligase EDD1**	May be involved in maturation and/or post-transcriptional regulation of mRNA. May play a role in control of cell cycle progression. Regulates DNA topoisomerase II binding protein (TopBP1) for the DNA damage response.
	Poly [ADP-ribose] polymerase 1 (PARP1)	Involved in the base excision repair (BER) pathway, following DNA



6	(ADPRT) (NAD(+) ADP-ribosyltransferase 1)	damages and appears as an obligatory step in a detection/signaling pathway leading to the reparation of DNA strand breaks.
5	Leucine-rich PPR motif-containing protein	
5	DEAH(Asp-Glu-Ala-His) box polypeptide 30, isoform 1	ATP-dependent helicase activity, double-stranded RNA binding
7	Heterogenous nuclear ribonucleoprotein U (hnRNP U) (Scaffold attachment factor A) (SAF-A) (pp120)	Binds to pre-mRNA. Has high affinity for scaffold-attached region (SAR) DNA. Bind to double- and single-stranded DNA and RNA.
7	RelA-associated inhibitor (Inhibitor of ASPP protein) (Protein iASPP) (PPP1R13B-like protein) (NFkB-interacting protein 1)	Plays a central role in regulation of apoptosis and transcription via its interaction with NF-kappa-B and p53/TP53 proteins. Blocks transcription of HIV-1 virus by inhibiting the action of both NF-kappa-B and SP1. Also inhibits p53/TP53 function, possibly by preventing the association between p53/TP53 and ASPP1 or ASPP2, and therefore suppressing the subsequent activation of apoptosis.
7	Ligase III, DNA, ATP-dependent, isoform alpha	DNA ligase involved in DNA recombination and repair
8	ATP-dependent DNA helicase II	NHEJ and DNA repair
8	Sad1/unc-84-like protein 2 (Rab5-interacting protein) (Rab5IP)	Interacts with rab5. Required for homotypic endosome fusion.
9	ATP-dependent DNA helicase II, 70 kDa subunit (Lupus Ku autoantigen protein p70) (Ku70)	Single stranded DNA-dependent ATP-dependent helicase. Single stranded DNA-dependent ATP-dependent helicase and is involved in stabilizing broken DNA ends and bringing them together.
9	Polyadenylate-binding protein 1 (Poly(A)-binding protein 1) (PABP 1)	Binds the poly(A) tail of mRNA. Functions in translational initiation regulation.
	RNA-binding protein 14 (RRM-containing coactivator activator/modulator) (SYT-	Isoform 1 may function as a nuclear receptor coactivator, enhancing transcription through other coactivators such as NCOA6 and

9	**interacting protein)**	CITED1. Isoform 2, functions as a transcriptional repressor, modulating transcriptional activities of coactivators including isoform 1, NCOA6 and CITED1.
9	**Calcium-binding mitochondrial carrier protein Aralar2 (Mitochondrial aspartate glutamate carrier 2)**	Calcium-dependent mitochondrial aspartate and glutamate carrier. May have a function in the urea cycle.
10	**Polymerase I and transcript release factor (PTRF protein)**	Termination of transcription by RNA polymerase I involves pausing of transcription by TTF1, and the dissociation of the transcription complex, releasing pre-rRNA and RNA polymerase I from the template. PTRF is required for dissociation of the ternary transcription complex
11	**Elongation factor 1-alpha 1 (EF-1-alpha-1) (eEF1A-1) (Elongation factor Tu) (EF-Tu)**	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis.
11	**Nuclease sensitive element-binding protein 1 (Y-box binding protein 1) (Y-box transcription factor) (YB-1) (CCAAT-binding transcription factor I subunit A) (CBF-A) (Enhancer factor I subunit A) (EFI-A) (DNA-binding protein B) (DBPB)**	Binds to DNA as a homomeric form, (EFI-A)n or as a heteromeric form in association with EFI-B. Binds both RNA and Y box containing DNA. Involved in regulation of transcription.
12	**Import inner membrane translocase subunit TIM50, mitochondrial precursor**	Essential component of the TIM23 complex, a complex that mediates the translocation of transit peptide-containing proteins across the mitochondrial inner membrane. Isoform 2 may participate in the release of snRNPs and SMN from the Cajal body.
12	**Heterogeneous nuclear ribonucleoproteins C1/C2 (hnRNP C1 / hnRNP C2)**	RNA binding proteins. May play a role in ribonucleosome assembly by neutralizing basic proteins such as A and B core hnRNP.
12	**DNA polymerase beta**	Repair polymerase. Conducts "gap-

		filling" DNA synthesis in a stepwise distributive fashion rather than in a processive fashion as for other DNA polymerases. Has a 5'-deoxyribose-5-phosphate lyase (dRP lyase) activity.
13	**Activator 1 38 kDa subunit (Replication factor C 38 kDa subunit) (A1 38 kDa subunit) (RF-C 38 kDa subunit) (RFC38)**	The elongation of primed DNA templates by DNA polymerase delta and epsilon requires the action of the accessory proteins proliferating cell nuclear antigen (PCNA) and activator 1.
14,15	**Melanoma-associated antigen B2 (MAGE-B2 antigen) (DSS-AHC critical interval MAGE superfamily 6) (DAM6) (MAGE XP-2)**	
16	**BAG family molecular chaperone regulator 2 (BCL2-associated athanogene 2) (BAG-2)**	Inhibits the chaperone activity of HSP70/HSC70 by promoting substrate release.
17	**Probable UPF0334 kinase-like protein C1orf57**	
18	**Eukaryotic translation elongation factor 1 epsilon-1 (Multisynthetase complex auxiliary component p18) (Elongation factor p18)**	Positive modulator of ATM response to DNA damage. Positive modulator of ATM response to DNA damage. Translocated into the nucleus when growild-typeh resumes (S phase), and following DNA damage.
18	**ER lumen protein retaining receptor 3 (KDEL receptor 3) (KDEL endoplasmic reticulum protein retention receptor 3)**	Required for the retention of luminal endoplasmic reticulum proteins. Determines the specificity of the luminal ER protein retention system. Also required for normal vesicular traffic through the Golgi. This receptor recognizes K-D-E-L

L4100k-interacting proteins during the early stages of infection

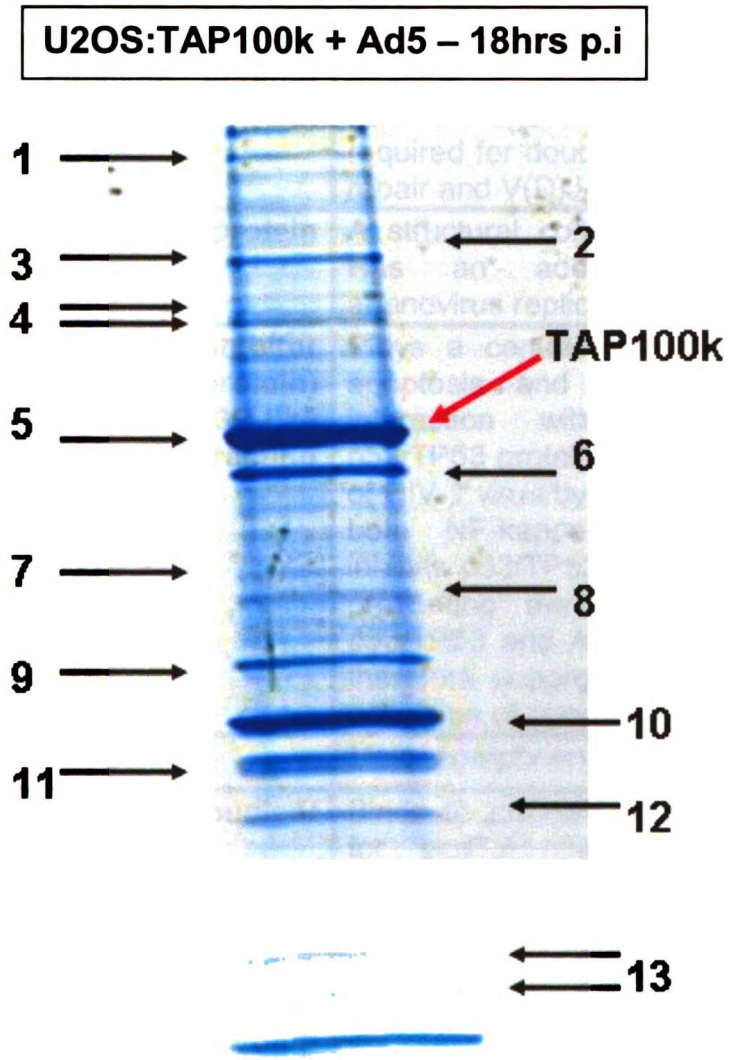
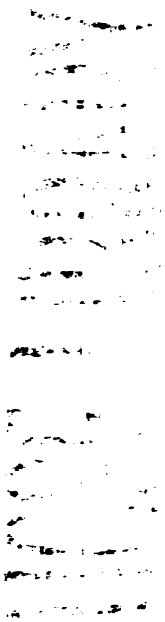


Figure 4.5

Table 4.2

L4100k-interacting proteins during the early stages of infection

BAND	PROTEIN	FUNCTION
1	**DNA-dependent protein kinase catalytic subunit (EC 2.7.1.37) (DNA-PKcs) (DNPK1) (p460)**	Acts as a molecular sensor for DNA damage. Involved in DNA nonhomologous end joining (NHEJ) required for double-strand break (DSB) repair and V(D)J recombination.
4	**Hexon-associated protein (pIX)**	A structural component of the virion. Has an additional role during adenovirus replication.
6	**RelA-associated inhibitor (Inhibitor of ASPP protein) (iASPP) (PPP1R13B-like protein) (NFkB-interacting protein 1)**	Plays a central role in regulation of apoptosis and transcription via its interaction with NF-kappa-B and p53/TP53 proteins. Blocks transcription of HIV-1 virus by inhibiting the action of both NF-kappa-B and SP1. Also inhibits p53/TP53 function, possibly by preventing the association between p53/TP53 and ASPP1 or ASPP2, and therefore suppressing the subsequent activation of apoptosis.
5	**Hexon**	Major component of adenovirus capsid
6	**Heterogenous nuclear ribonucleoproteinU (hnRNPU) (Scaffold attachment factor A) (SAF-A) (pp120)**	Binds to pre-mRNA. Has high affinity for scaffold-attached region (SAR) DNA. Bind to double- and single-stranded DNA and RNA.
7,8	**Polyadenylate-binding protein 1 (Poly(A)-binding protein 1) (PABP 1)**	Binds the poly(A) tail of mRNA. Functions in translational initiation regulation.
9	**Peripentonal hexon-associated protein (pIIIA)**	Structural component of adenovirus.
9	**Lamin-B receptor (Integral nuclear envelope inner membrane protein) (LMN2R)**	Stabilizes the nuclear envelop after cell replication.
10	**ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit,**	ATP synthesis coupled proton transport.



	isoform 1, cardiac muscle	
10	**Polymerase I and transcript release factor (PTRF protein)**	Termination of transcription by RNA polymerase I involves pausing of transcription by TTF1, and the dissociation of the transcription complex, releasing pre-rRNA and RNA polymerase I from the template. PTRF is required for dissociation of the ternary transcription complex
12	**Human arginine methyltransferase (HRMT1L2) PRMT1**	The most predominant methylase in the cell. Methylates 65% of all methylated substrates by asymmetric dimethylation of RGG, RGR and RXR motifs.
11	**(Elongation factor 1 A-1) (eEF1A-1) (Elongation factor Tu) (EF-Tu)**	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis.
11	**60S ribosomal protein L3 (HIV-1 TAR RNA binding protein B) (TARBP-B)**	A component of the large subunit of cytoplasmic ribosomes.
11	**Nuclease sensitive element-binding protein 1 (Y-box binding protein 1) (Y-box transcription factor) (YB-1) (CCAAT-binding transcription factor I subunit A) (CBF-A) (Enhancer factor I subunit A) (EFI-A) (DNA-binding protein B) (DBPB)**	Binds to DNA as a homomeric form, (EFI-A)n or as a heteromeric form in association with EFI-B. Binds both RNA and Y box containing DNA. Involved in regulation of transcription.
11	**Heterogeneous nuclear ribonucleoprotein F (HNRPF protein)**	RNA-binding protein
11	**Minor core protein (pV)**	Structural protein of adenovirus
13	**Splicing factor, arginine/serine-rich 1 (pre-mRNA splicing factor SF2, P33 subunit) (Alternative splicing factor ASF-1)**	Plays a role in preventing exon skipping, ensuring the accuracy of splicing and regulating alternative splicing. Interacts with other spliceosomal components, via the RS domains, to form a bridge between the 5' and 3' splice site binding components, U1 snRNP and U2AF.

L4100k-interacting proteins during the late stages of infection

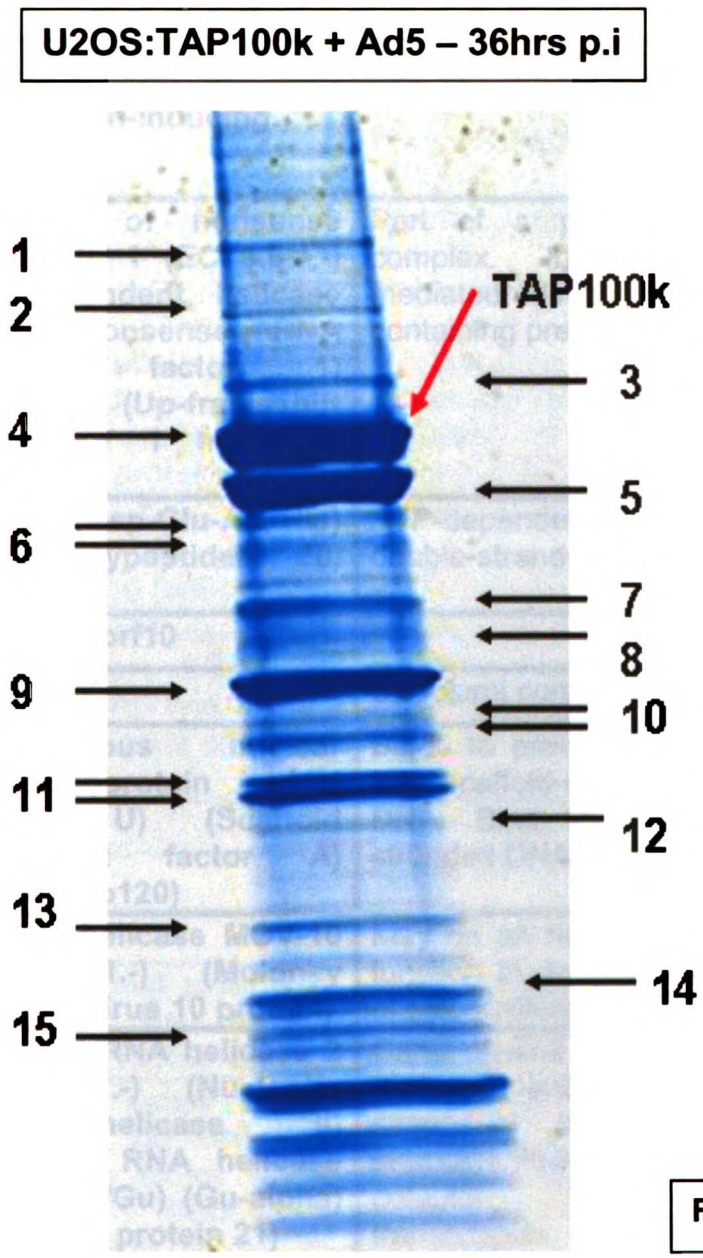


Figure 4.6

Table 4.3

L4100k-interacting proteins during the late stages of infection

BAND	PROTEIN	FUNCTION
2	**Proliferation-inducing protein 32**	
3	**Regulator of nonsense transcripts 1 (EC 3.6.1.-) (ATP-dependent helicase RENT1) (Nonsense mRNA reducing factor 1) (NORF1) (Up-frameshift suppressor 1 homolog) (hUpf1)**	Part of a post-splicing multiprotein complex. Involved in nonsense-mediated decay (NMD) of mRNAs containing premature stop codons.
3	**DEAH (Asp-Glu-Ala-His) box polypeptide 30, isoform 1**	ATP-dependent helicase activity, double-stranded RNA binding
3	**Protein C9orf10**	
4	**L3 pII**	Structural component of the virion
6	**Heterogenous nuclear ribonucleoprotein U (hnRNP U) (Scaffold attachment factor A) (SAF-A) (pp120)**	Binds to pre-mRNA. Has high affinity for scaffold-attached region (SAR) DNA. Bind to double- and single-stranded DNA and RNA.
4	**Putative helicase MOV-10 (EC 3.6.1.-) (Moloney leukemia virus 10 protein)**	May be an helicase with an important function in development and/or control of cell proliferation
5	**Nucleolar RNA helicase 2 (EC 3.6.1.-) (Nucleolar RNA helicase II) (Nucleolar RNA helicase Gu) (RH II/Gu) (Gu-alpha) (DEAD box protein 21)**	Can unwind double-stranded RNA (helicase) and can fold or introduce a secondary structure to a single-stranded RNA (foldase). Functions as cofactor for c-Jun-activated transcription. Involved in rRNA processing.
5	**Hexon protein**	Major component of the adenovirus capsid
	Pre-mRNA splicing factor ATP-dependent RNA helicase DHX15 (EC 3.6.1.-) (DEAH box protein 15) (ATP-dependent RNA	Belongs to the DEAD box helicase family. Pre-mRNA processing factor involved in disassembly of spliceosomes after the release of mature mRNA

6	helicase #46)	
7,8	Polyadenylate-binding protein 1 (Poly(A)-binding protein 1) (PABP 1)	Binds the poly(A) tail of mRNA. Functions in translational initiation regulation
7,8	Heterogeneous nuclear ribonucleoprotein M (hnRNP M)	Pre-mRNA binding protein in vivo, binds avidly to poly(G) and poly(U) RNA homopolymers in vitro. Involved in splicing.
7	RNA-binding protein 14 (RNA-binding motif protein 14) (RRM-containing coactivator activator/modulator) (Synaptotagmin-interacting protein) (SYT-interacting protein)	Isoform 1 may function as a nuclear receptor coactivator, enhancing transcription through other coactivators such as NCOA6 and CITED1. Isoform 2, functions as a transcriptional repressor, modulating transcriptional activities of coactivators including isoform 1, NCOA6 and CITED1.
7,8	Penton protein (Virion component III) (Penton base protein) (pIII)	Structural component of adenovirus capsid
8	Probable ATP-dependent RNA helicase DDX5 (EC 3.6.1.-) (DEAD box protein 5) (RNA helicase p68)	Belongs to the DEAD box helicase family. RNA-dependent ATPase activity. The rate of ATP hydrolysis is highly stimulated by single-stranded RNA.
9	L1 pIIIa	Structural component of the virion
9	L5 pVI	Structural component of the virion
11	L2 pV	Structural component of the virion
11	60S ribosomal protein L3 (HIV-1 TAR RNA binding protein B) (TARBP-B)	A component of the large subunit of cytoplasmic ribosomes.
10	Elongation factor 1-alpha 1 (EF-1-alpha-1) (Elongation factor 1 A-1) (eEF1A-1) (Elongation factor Tu) (EF-Tu)	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis.
11	Nuclease sensitive element-binding protein 1 (Y-box binding protein 1) (Y-box transcription factor) (YB-1) (CCAAT-binding transcription factor I subunit A) (CBF-A) (Enhancer factor I	Binds to DNA as a homomeric form, (EFI-A)n or as a heteromeric form in association with EFI-B. Binds both RNA and Y box containing DNA. Involved in regulation of transcription.

	subunit A) (EFI-A) (DNA-binding protein B) (DBPB)	
12	**Interleukin enhancer-binding factor 2 (Nuclear factor of activated T-cells 45 kDa)**	It can promote the formation of stable DNA-dependent protein kinase holoenzyme complexes on DNA.
15	**L4 pVIII**	Structural component of the virion.
15	**Major core protein precursor (Protein VII) (pVII)**	Structural component of the virion.

4.2.iii Comparative analysis of L4100k-interacting proteins during the early and the late phases of infection

As discussed earlier, L4100k has functions both during the early, and the late phase of adenovirus infection, and these distinct functions require differential localization of the protein. During the early phase of its expression, L4100k is localized in the cytoplasm and it later localizes in the nucleus (figure 3.3). Consistent with these results, in the TAP-purified complexes from cells harvested at 18hrs post-infection, L4100k was found associated with cellular proteins with cytoplasmic functions, whereas in cells harvested at 36hrs post-infections, most proteins that bound to L4100k have nuclear functions. As mentioned elsewhere in this chapter, in these cells, most of the bound proteins were also identified as L4100k binding partners in the uninfected cells (figure 4.4), which also exhibit nuclear localization of L4100k.

To further characterize the multiple functions of L4100k, we next identified the L4100k interactions that only occur during the early, or the late phase of infection. Thus, we compared proteins that bound to L4100k in cells harvested at 18hrs with those that bound in cells harvested at 36hrs post infection. As expected from the difference in L4100k sub-cellular localization between these two time points, and the distinct functions of this protein which are temporally regulated, L4100k interacted with a wide spectrum of cellular and viral proteins which varied extensively between 18hrs and 36hrs (table 4.4). Many of the proteins that bound to L4100k during the early phase are molecules with known cytoplasmic localization and functions. For example Rel-A-associated inhibitor (iASPP) functions in the cytoplasm (Slee et al., 2004) and inhibits p53-dependent apoptosis by inhibiting activation of p53 by ASPP (Bergamaschi et al., 2003). Similarly the SF2 pre-mRNA splicing factor, associates with translating ribosomes and enhances translation in the cytoplasm (Sanford et al., 2005). PRMT1 also bound to L4100k at 18hrs p.i, confirming our previous findings that the purpose of this interaction is L4100k methylation and localization to the nucleus. Consistent with this observation, L4100k was not associated with PRMT1 in cells harvested at 36hrs p.i.

In contrast to the proteins that bound to L4100k during the early phase of infection, most of the proteins found in complex with L4100k during the late phase have nuclear functions. These proteins include the nucleolar RNA helicases RH II/Gu alpha (DDX21), DDX5 (p68), and DEAH box protein 15, as well as HNRNPM, a member of the nuclear ribonucleoproteins family. The

function of these factors and the potential significance of their interaction with L4100k is discussed in the next section. Finally, during the late stage of infection L4100k also associated with hexon polypeptide, presumably acting as a scaffold to facilitate hexon trimerization and assembly in the nucleus, as previously reported (Cepko and Sharp, 1982). Association of L4100k with hexon was also seen in the 18hrs time point, which suggests that L4100k binds to hexon immediately after hexon is expressed, and may have a role in hexon transport to the nucleus. In addition to hexon, L4100k was found to interact with many additional structural polypeptides, including L1 pIII, L2 pV, L3 pII and pVII (table 4.3). These results suggest that L4100k may have a more general role in the assembly of the adenovirus capsid, and may act as a scaffold not only for hexon trimerization, but also for the assembly of the rest of the structural components of the virion into mature capsids.

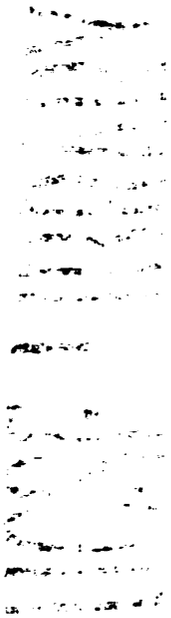


Table 4.4

Comparative analysis of the early (cytoplasmic) and the late (nuclear) L4100k- interacting proteins

18hrs p.i	36hrs p.i
DNA-PKcs	Proliferation-inducing protein 32
Hexon-associated protein pIX	ATP-dependent helicase RENT1
Rel A-associated inhibitor (iASPP)	DEAH box polypeptide 30
Peripentonal protein pIIIA	Adeno L3 pII
Lamin-B receptor (LMN2R)	MOV-10 helicase
ATP synthase, alpha subunit	Nucleolar RNA helicase 2 RH II/Gu alpha (DDX21)
Polymerase I and transcript release factor (PTRF)	DEAH box protein 15, ATP-dependent RNA helicase
HNRNPF	HNRNPM
Minor core protein pV	RNA-binding protein 14
Protein methyltransferase 1 (PRMT1)	Adenovirus hexon
ADP/ATP translocase 2	DDX5 ATP-dependent RNA helicase (p68)
SF2 pre-mRNA splicing factor	Adenovirus L1 pIIIa, L5 pVI, L2 pV, L4 pIIIV, pVII
	Interleukin enhancer-binding factor2

=cytoplasmic =nuclear =shuttles

DISCUSSION

In this chapter, we employed a Tandem Affinity Purification strategy to identify novel L4100k-interacting proteins, in order to further define the multiple roles of L4100k during adenovirus infection. A number of novel cellular and viral proteins were identified as L4100k binding partners during the early and the late phases of infection, including factors that have functions in transcription, translation, DNA damage response, RNA metabolism, and assembly of the virus capsid. The involvement of L4100k in regulation of translation and in hexon assembly has already been reported and studied with some detail. However, in this study we identified many additional L4100k binding proteins, as previously unknown, which add more complexity to the mechanism by which L4100k regulates these processes. Moreover, involvement of L4100k in transcriptional control and regulation of cellular DNA damage response has not been previously reported. Therefore, the results presented here suggest that L4100k has a wider role in adenovirus infection than previously recognized, and additional experiments are needed in order to better understand the multifunctional character of this protein and the mechanisms by which these functions are regulated during infection.

L4100k and regulation of translation

During adenovirus infection, host protein synthesis is inhibited and viral late mRNAs are preferentially translated in the cytoplasm. L4100k mutant viruses fail to induce cellular protein synthesis shutoff and expression of the late proteins is reduced. Thus, involvement of L4100k in regulation of translation during infection

is well documented. The mechanism by which L4100k induces adenovirus-specific translation is thought to involve binding of this protein to the EIF4F translation initiation complex, displacement of mnk1 kinase and dephosphorylation of EIF4E, and consequently inhibition of cap-dependent translation. Late viral mRNAs posses a unique 5' UTR which allows translation via a cap-independent mechanism termed ribosome shunting. However, recent reports from others, and data presented in this work, suggest that the premise of this model has not held true (discussed in chapter 3). The results presented in this chapter suggest that the mechanism of L4100k-mediated regulation of translation is more complex, and likely involves additional cellular proteins that, before this study, had remained unidentified. One of these proteins is the polyadenylate-binding protein 1 (PABP1), the protein that binds to the poly(A) tail at the 3' end of eukaryotic mRNAs. PABP1 interacts with the cap-bound EIF4F translation initiation complex, and this interaction is thought to be necessary to initiate cap-dependent translation (Imataka et al., 1998; Kahvejian et al., 2005; Tarun and Sachs, 1995). L4100k binding to PABP1 may therefore disrupt the interaction between EIF4F and PABP1, thereby inhibiting cap-dependent cellular translation.

L4100k also bound to five cellular RNA helicases: hUpf1, and four members of the DEAD box helicase family, DEAH box polypeptide 30, RH II/Gu alpha, DHX15 and DDX5. hUpf1 (RENT1) is an RNA helicase belonging to the SF1 family of helicases implicated in DNA and RNA metabolism and is conserved from yeast to humans (Tanner and Linder, 2001). It functions as part of a post-

splicing multiprotein complex and is necessary for nonsense-mediated decay (NMD) of mRNAs containing premature stop codons, and for translation termination (Lykke-Andersen et al., 2000). hUpf1 is localized in the cytoplasm, however it was not found associated with L4100k during the early phase of infection (18hrs), when L4100k is in the cytoplasm. Instead, interaction of L4100k with hUpf1 occurred in cells harvested at 36hrs post infection, when L4100k accumulates in the nucleus. Therefore, this result suggests that L4100k may play an inhibitory role in hUpf1 function, by sequestering this protein to the nucleus. Additional experiments are needed to test this hypothesis and determine the role of this interaction in translational regulation during adenovirus infection.

Proteins of the DEAD/H box family of RNA helicases are involved in a variety of biological processes, and have diverse roles in translation, rRNA synthesis, pre-mRNA splicing, transcription, RNA transport, RNA-stability/degradation, and apoptosis (reviewed in (Tanner and Linder, 2001)). These proteins possess RNA-dependent ATPase activity, and they can act as helicases to unwind double stranded RNA, or as foldases to introduce a secondary structure to single stranded RNA, functions that are necessary for translation initiation in all eukaryotic cells. It is therefore not surprising that L4100k interacts with members of the DEAD box family, and these proteins are likely to have an important role in L4100k function in translational control. Even though the specific biological function of these proteins has not been determined *in vivo*, one possibility could be that L4100k recruits these proteins to the polysomes to facilitate cap-

independent translation of late viral mRNAs, at the expense of cellular translation. In support of this hypothesis late viral messages posses a unique 5'-untranslated region (UTR), termed the *tripartite leader*, which allows adenovirus late mRNAs to be translated independent of EIF4F, because the 40s ribosome can scan from cap to AUG by a process called *ribosome shunting* (Yueh and Schneider, 1996), without the need for the EIF4F-associated EIF4A helicase activity. Since EIF4A also belongs to the DEAD box family of RNA helicases, it is possible that recruitment of one or more of the other DEAD box helicases by L4100k may provide a helicase function specific to the unique secondary structure of the viral UTR, and compensate for the loss of EIF4F helicase activity during adenovirus-specific translation.

L4 100k and regulation of transcription

As already mentioned above, in addition to their potential role in translation, proteins of the DEAD/H box family of RNA helicases are also involved in transcriptional control, (reviewed in (Tanner and Linder, 2001)). For example, RHII/GU-alpha, also known as DDX21, is a multifunctional nucleolar DEAD-box helicase that unwinds double-stranded RNA in the 5' to 3' direction *in vitro*. It is also involved in rRNA synthesis and in c-jun-activated transcription (Westermarck et al., 2002), and it has been identified in this study to interact with L4100k. Interestingly, two of the other identified L4100k-interacting proteins, NDHII and hnrnp M, have also been identified as c-jun-interacting proteins (Westermarck et al., 2002). The fact that three previously identified c-jun-

interacting proteins seem to also interact with L4100k strongly suggests a previously unrecognized role for L4100k in c-jun activated transcription. c-Jun plays a pivotal role in the cellular responses to a number of extracellular stimuli, including stress insults, apoptotic and differentiation signals (Leppa and Bohmann, 1999). Adenovirus-mediated induction of c-jun dependent activation of transcription during the late phase of infection was previously documented, and the mechanism involved activation of JNK kinase by E1B19k (See and Shi, 1998). However, c-jun dependent activation of transcription peaks at about 48hrs during infection, which leaves the possibility that another, perhaps late, adenovirus gene product may also contribute to this process. Our results from this study suggest that L4100k may therefore be a second adenovirus protein involved in regulation of c-jun dependent activation of translation during infection. Whether this protein acts in the same signaling pathway with E1B19k or in an independent fashion remains to be determined, but the mechanism could involve one or more of the c-jun-interacting proteins RHII/Gu-alpha, NDHII and hnrnp M, which are shown here to interact with L4100k.

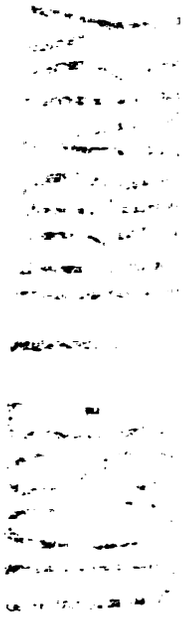
L4100k and regulation of the DNA damage response and apoptosis

During adenovirus infection, the viral DNA genome is freed from the encapsidating structural proteins of the virion and serves as a template for viral genome replication. Thus the viral template and the newly synthesized viral DNA molecules remain unprotected and are viewed by the cellular DNA repair machinery as double stranded DNA breaks that must be ligated and repaired,

which leads to the formation of genome concatemers that are too large to be packaged, resulting in diminished viral replication. Viral concatemerization is mediated by the cellular DNA repair complex Mre11-Rad50-NBS1, which is important for DNA double stranded break repair, and E4orf3/orf6, along with E1B55k were shown to inactivate this complex, and prevent Ad genome concatemerization (Stracker et al., 2002). However, DNA-PK and DNA ligase IV have also been implicated in viral concatemerization (Boyer et al., 1999), and cells with mutations in these genes do not induce formation of viral concatemers. This suggests that additional components of the cellular DNA repair machinery, other than the Mre11-Rad50-NBS1 complex, are important for this process. Several of the proteins that bound to L4100k in our TAP purification experiment have known functions in the cellular response to DNA damage. These include the ATP-dependent DNA helicase II (Ku70) and DNA-PKcs, which are components of the DNA-dependent protein kinase1 (DNA-PK1) complex, PARP-1, the ATP-dependent DNA ligase III, and elongation factor 1-epsilon. It is therefore possible that L4100k may provide a second mechanism by which the DNA damage response to adenovirus infection is inactivated. This mechanism may involve sequestration of some of the proteins that physically interact with double strand DNA breaks, like the ATP-dependent DNA helicase II which binds and stabilizes broken DNA ends and brings them to close proximity for ligation, and DNA ligase III which ligates the broken ends. Another possibility is that L4100k-mediated modulation of the cellular DNA damage response may involve inactivation of factors that are involved in the DNA damage detection/signaling

pathway, like PARP1 and the elongation factor-epsilon, which are positive modulators of the ATM/ATR response to DNA damage, and downstream signaling events that lead to DNA double strand break repair (reviewed in (O'Driscoll and Jeggo, 2006)).

Finally, L4100k was found associated with RelA-associated inhibitor (also known as iASPP). iASPP is an important protein that has a central role in p53-dependent apoptosis. It binds to the p53 associated proteins 1 and 2 (ASPP1/2), which are potent p53 activators, thereby inhibiting their function (Bergamaschi et al., 2003). The reason for the interaction of L4100k with iASPP is unclear, since p53-dependent apoptosis is inhibited early during adenovirus infection by the E1B55k/E4orf6 complex, which targets p53 for degradation (discussed in chapter 1). One possibility is that L4100k interaction with iASPP may simply provide an additional mechanism of p53 inactivation, since inhibition of p53-dependent apoptosis is a very important step in adenovirus infection. However, iASPP has additional functions that are independent of p53, which involve inhibition of the action of NF-kappa-B (Yang et al., 1999). NF-kappa-B is a sequence-specific DNA-binding protein complex which regulates the expression of a variety of cellular genes, particularly those involved in immune and inflammatory responses (reviewed in (Baldwin, 1996)). Therefore, a second possible role for the interaction of L4100k with iASPP may be to regulate NF-kappa-B-dependent activation of transcription, perhaps as a mechanism to inhibit TNF-alpha-mediated antiviral responses.



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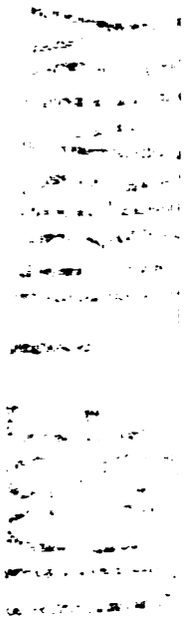

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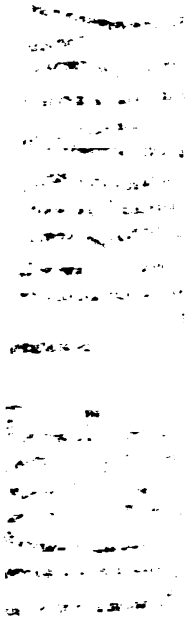

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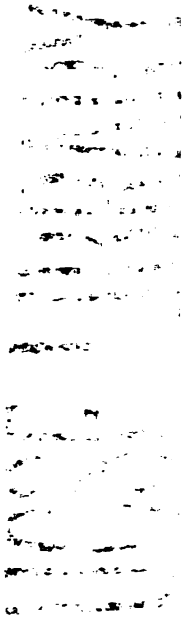

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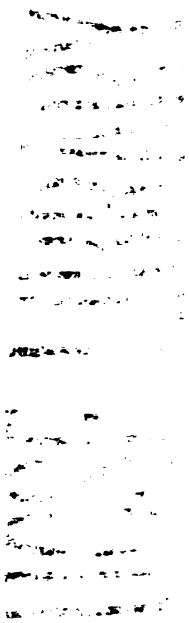

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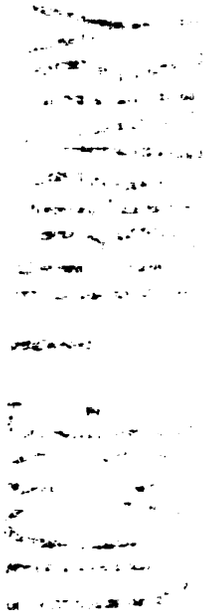

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