EXPRESSION, PURIFICATION, AND FUNCTIONAL ANALYSIS OF ADENOVIRUS TYPE 5 E4 ORF3 PROTEIN

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

EMRE KOYUNCU

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE

IN

THE DEPARTMENT OF BIOLOGY

SEPTEMBER 2004

Approval of the Graduate School of Natural and Applied Sciences

_____

Prof. Dr. Canan Özgen
Director

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

_____

Prof. Dr. Semra Kocabıyık
Chair of Biology Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science/Arts/Doctor of Philosophy.

_____	_____
Prof. Dr. Thomas Dobner Co-Supervisor	Prof. Dr. Feride Severcan Supervisor

Examining Committee Members (first name belongs to the chairperson of the jury and the second name belongs to supervisor)

Prof. Dr. Orhan Adalı	_____
Prof. Dr. Feride Severcan	_____
Prof. Dr. Beki Kan	_____
Doç. Dr. Fatih İzgü	_____
Dr. Ayşe Elif Erson	_____

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Emre Koyuncu

Signature :

ABSTRACT

EXPRESSION, PURIFICATION, AND FUNCTIONAL ANALYSIS OF ADENOVIRUS TYPE 5 E4 ORF3 PROTEIN

KOYUNCU, Emre

M.Sc., Department of Biology
Supervisor :Prof. Dr. Feride Severcan
Co-Supervisor :Prof. Dr. Thomas Dobner

September 2004, 119 pages

In this study, structural and functional aspects of adenovirus type 5 (Ad5) E4orf3 protein were analyzed by biophysical and biochemical methods. Ad5 is one of the mostly used gene therapy vectors to date. However, some of its proteins possess oncogenic potential and their presence comprises safety risks. E4orf3 is one of the oncoproteins of Ad5. It also takes important roles in viral infection, and is beneficial for therapy vectors. Therefore, understanding the functions of E4orf3 is very important for developing efficient and safe adenovirus vectors.

Most of the present knowledge about the functions of E4orf3 comes from its mutational analysis. It has never been expressed or purified successfully due to its extreme insolubility. Therefore, this study focused on the optimization of expression of E4orf3 protein. As a result, full-length E4orf3 was obtained in soluble form as a Glutathione-S-transferase (GST) fusion protein and purified

by GST affinity chromatography for the first time. Subsequently, the interaction of E4orf3 with four different proteins, DNA-PK, Aup1, E1B-55 kDa and E4orf6 was analyzed in detail by GST-pulldown technique. In these experiments, E4orf3 was shown to associate with Aup1, E1B-55 kDa and E4orf6 *in vitro*, and the C-terminal of E4orf3 was determined to be responsible for these interactions. Finally, basic structural information about E4orf3 protein was also obtained for the first time by the direct analysis of the fusion protein in glutathione beads with Fourier Transform Infrared (FTIR) spectroscopy. Since the purified E4orf3 protein could not be separated from the glutathione beads due to its hydrophobic regions, the secondary structures in this protein were determined after subtracting glutathione and H2O absorption bands, and the GST moiety.

Key words: adenovirus, gene therapy, oncogenesis, E4orf3, FTIR,

ÖZ

ADENOVİRÜS TİP 5 E4 ORF3 PROTEİNİNİN EKSPRESYONU, PÜRİFİKASYONU VE FONKSİYONEL ANALİZİ

KOYUNCU, Emre

Yüksek Lisans, Biyoloji Bölümü
Tez Yöneticisi : Prof. Dr. Feride Severcan
Ortak Tez Yöneticisi: Prof. Dr. Thomas Dobner

Eylül 2004, 119 sayfa

Bu çalışmada adenovirus tip 5 (Ad5) E4orf3 proteininin yapısal ve fonksiyonel özellikleri biyofiziksel ve biyokimyasal yöntemlerle incelenmiştir. Ad5 günümüzde en çok kullanılan gen terapisi vektörlerinden biridir. Fakat bazı adenovirüs proteinleri onkojenik özellikler taşımaktadır ki bu güvenlik açısından risklidir. E4orf3 Ad5'in onkoproteinlerinden biridir. Bu protein, virüs enfeksiyon döngüsünde de önemli roller üstlenmektedir ve terapi vektörü için yararlıdır. Bu nedenle, bu proteinin fonksiyonlarının aydınlatılması, verimli ve güvenli adenovirüs vektörlerinin geliştirilmesi açısından çok önemlidir.

E4orf3'ün fonksiyonları hakkındaki çoğu bilgi, proteinin mutasyonal analizi sonucu elde edilmiştir. Protein, solüsyonda erimez davranışı nedeniyle daha önce ekspres edilememiş ve saflaştırılamamıştır. Bu nedenle, bu çalışmada E4orf3 proteininin ekspresyonunun optimize edilmesine odaklanılmıştır. Sonuç olarak E4orf3, Glutatyon-S-Transferaz (GST) füzyon proteini halinde

çözünür olarak elde edilmiş ve GST afinite kromatografisi ile ilk kez saflaştırılmıştır. Ardından, E4orf3'ün dört değişik proteinle, DNA-PK, Aup1, E1B-55 kDa ve E4orf6'yla interaksiyonu GST-Pulldown tekniğiyle detaylı bir şekilde incelenmiştir. Bu çalışmalarda E4orf3'ün Aup1, E1B-55 kDa ve E4orf6'yla *in vitro* interaksiyonu gösterilmiş, ve proteinin C-terminalinin bu interaksiyonlardan sorumlu olduğu saptanmıştır. Son olarak, E4orf3 hakkındaki temel yapısal bilgiler, glutatyon sefaroza bağlı füzyon proteininin direk olarak Fourier Transform Infrared (FTIR) spektroskopisiyle analizi sonucu ilk kez elde edilmiştir. Saflaştırılan E4orf3, hidrofobik özellikleri nedeniyle glutatyon sefarozdan ayrılamamış olduğundan, proteinin ikincil yapıları glutatyon, su ve GST bantları ayıklandıktan sonra belirlenmiştir.

Anahtar Kelimeler: adenovirüs, gen terapisi, onkogenez, E4orf3, FTIR

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor Prof. Dr. Feride Severcan and co-supervisor Prof. Dr. Thomas Dobner for their guidance, patience, advice and encouragement throughout this research.

I am thankful to my lab mates, Banu Akkaş, Sevgi Görgülü, Yekbun Adıgüzel, and Güvenç Görgülü for their friendship and supports.

I would like to thank to the Dobner workgroup, “the dicken Dobners” for making me feel at home in Germany.

I wish to express my special thanks to my father Veysel, my mother Nigar and my brother İlker Koyuncu for their patience, support and love.

I would like to send my ultimate appreciation to my love Orkide Dirlik for her endless support, patience and love.

This study was supported by the Institut für Medizinische Mikrobiologie und Hygiene, Regensburg, and the State Planning organization (DPT) Grant No: BAP-2004-07-02-00-09.

To my family,

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER I

INTRODUCTION

Adenovirus (Ad) was first isolated from the human adenoidal tissue of a child having respiratory illness in 1953 (Rowe et al., 1953). After the initial discovery of Ads, it has been realized that they are the only human enteric virus with double stranded DNA (Jiang et al, 2001). They have a genomic size of around 34-44 kb which is relatively large and they do not integrate their DNA into the genome of the host (Danthinne and Imperiale, 2000). Human Ads cause lytic and persistent infections, and show a wide tissue tropism. Interestingly, most of the Ad infections are asymptomatic and cause no illness at all. In rare cases, Ad infections result in mild symptoms that are generally gastrointestinal and respiratory (Horwitz, 1996) which are followed by long-term effective immunity. Fatalities associated with these viruses are extremely rare, except when the patient is immunocompromised. Human Ads are not linked to human cancer, too. However, it is known that a subset of human Ads have the ability to promote tumors in newborn rodents, and all human serotypes can transform primary cells in culture (Nevins and Vogt, 1996). Therefore, human Ads are classified as DNA tumor viruses.

Oncogenic potential of human Ads were first reported in 1962 (Trentin et al., 1962). Following this initial discovery, they provide an excellent tool to analyze fundamental events of normal and malignant cell growth. Much of our current understanding on the molecular mechanisms of virus-mediated

oncogenesis has derived from the study of human Ad oncogenes. Today adenovirus-mediated oncogenesis is a well-understood process. Most of the viral genes and their products involved in Ad transformation are identified (Endter and Dobner, 2003). However, the mechanisms underlying the complex interplay between cellular and viral proteins responsible for oncogenic transformation still remain to be clarified.

Ads comprise over fifty different human serotypes classified into six subgroups (A-F). On the basis of their oncogenicity in animals, human Ad serotypes are subdivided into three classes: highly oncogenic Ads, producing tumors at high frequency within a few months; weakly oncogenic viruses, including tumors inconsistently and after a long incubation time; and nononcogenic Ads causing no tumors (Endter and Dobner, 2003). Among these serotypes, Adenovirus serotype 5 (Ad5) belonging to the nononcogenic subgroup C, is the most widely studied serotype (Endter and Dobner, 2003; Doronin et al. 2001).

Ads, in particular Ad5, exhibit certain properties to be used as a vector in gene therapy. They are able to infect both actively dividing and quiescent cells, show a wide tissue tropism, do not integrate into the genome of the infected cell and have not been associated with major pathologies in human (Doronin et al., 2001). Ads have thus been used to transfer genes of interest into muscle (Ragot et al., 1993), liver (Jaffe et al., 1992) and, nervous system (Akli et al., 1993). Developing Ad vectors for gene therapy requires basically the removal of Early Region 1 (E1) composed of two separate transcription units, E1A and E1B, from the Ad5 genome. This region is essential for virus replication so the resulting vector is replication deficient. The proteins encoded from E1A induce transcription of other viral genes, induce or repress a variety of cellular genes, and in nontransformed cells, they deregulate the cell cycle. E1B proteins inhibit cellular apoptosis (Wickham, 2000; Wienzek et al., 2000). In replication deficient Ad vectors, the E1A and E1B regions of Ad5 are replaced by therapeutic genes. Since the virus can

not replicate, it expresses the therapeutic protein only in the initially infected cells. By having these properties, Ad5 offers a great promise in gene therapy and it is the mostly used *in vivo* gene therapy vector to date (Federal Drug Administration, USA, http://www.fda.gov, 2004).

Moreover, a particular deletion mutant of Ad5, named dl1520 (renamed ONYX-015 for commercial reasons), lacking a functional gene in E1B transcription unit replicates selectively in p53 negative tumor cells and lysis of these cells, whereas, the virus is attenuated in nontransformed cells (Bischoff et al., 1996; Heise, 1997). The lacking gene in E1B region of ONYX-015 encodes E1B-55 kDa which is a multifunctional protein. It binds to the tumor suppressor p53 and neutralizes p53-induced growth arrest and apoptosis during infection (Gabler et al., 1998; Harada and Berk, 1999). Thus, replication of ONYX-015 was originally reported to be restricted to tumor cells lacking wild-type p53. Although today it is known that it can replicate in some cell types with wild-type p53, ONYX-015 has shown clinical benefits in phase I and II clinical trials of head and neck cancer (Ganly et al., 2000; Kirn et al., 1998). Recently, efforts have been made to improve the specificity and efficacy of ONYX-015 and to develop strategies for replication selective oncolytic Ad vectors (Doronin et al., 2001).

As can be deducted from the statements above, Ads bear certain benefits to be used in both gene and tumor therapy. However, oncogenic potential of these viruses bears a safety risk and a major drawback for the therapeutic use of Ad vectors in human. Although Ad5, which belongs to nononcogenic subgroup C, is being used for the recombinant vector development, today it is known that even Ad5 transformed hamster cells can induce tumors in newborn animals from the same species (Täuber and Dobner, 2001). Moreover, human primary cells have been successfully transformed by Ad5 DNA fragments in culture. The oncogenic potential of human Ads, including Ad5, is determined mainly by E1A and E1B transcription units, both of which are necessary and perhaps sufficient for oncogenic transformation of the

cells (Endter and Dobner, 2003). Some proteins of Ad5 encoded by Early Region 4 (E4), in particular E4orf3 and E4orf6 proteins, also have partial transforming activities, but they are not essential for classical Ad transformation. They only contribute to E1 mediated oncogenesis (Öhman et al., 1995). Therefore, it can be assumed that both replication deficient Ad5 vectors for gene therapy and replication selective oncolytic Ad5 vectors for tumor therapy are safe since they lack regions which are strictly necessary for oncogenic transformation. However, Nevels and coworkers recently demonstrated that expression of Ad5 E1A with either E4orf6 or E4orf3 genes can initiate the stable transformation by mutagenesis-based hit-and-run mechanism which is away from classical Ad transformation (Nevels et al., 2001). Hit-and-run mechanism claims that viral molecules are necessary for the initiation but not for the maintenance of cellular transformation (Skinner, 1976). According to their observations, interestingly, majority of the cells transformed by E1A and either E4orf6 or E4orf3 fail to express the viral E1A and E4 gene products and lack E1A- and E4-specific DNA sequences. Moreover, mutagenic effects of E1A plus E4orf6 or E4orf3 have also been proven (Nevels et al., 2001). These results suggest the possibility that even tumors lacking any detectable Ad-specific molecules can be of adenoviral origin. Thus, the Ad gene delivery and oncolytic tumor therapy vectors that are currently being tested in clinical trials or under development comprise safety risks since they express oncogenic E4orf6 or E4orf3 proteins.

Transforming and oncogenic potential of both E4orf6 and E4orf3 proteins are linked to their ability to interact with cellular and viral factors (Nevels et al., 2001). E4orf6 takes part in complete inactivation of p53 function and p53 destabilization (Nevels et al., 2000). E4orf3 co-localizes with PML oncogenic domains (PODs) and reorganizes these structures. E4orf3 induced POD reorganization is thought to trigger a cascade of processes that cause uncontrolled cell proliferation (Endter and Dobner, 2003). Furthermore, each of the E4 proteins is able to interact with the catalytic subunit of DNA-dependent protein kinase (DNA-PK), thereby inhibit double-strand break

repair (Boyer et al., 1999). Considering the mutagenic and oncogenic properties of both E4 proteins listed above, one might argue that deleting the genes expressing E4orf6 and E4orf3 is necessary and sufficient for developing safe Ad vectors. However, these two proteins have functions in Ad infectious cycle. Each E4 protein independently augments viral DNA replication, late viral protein synthesis, shut-off of host protein synthesis, production of progeny virions, and prevents concatemer formation of viral genomes (Täuber and Dobner, 2001). Moreover, Ad mutants that lack the entire E4 region or fail to express both E4orf6 and E4orf3 proteins are severely restricted for growth. Therefore, at least one of these two E4 proteins or both is necessary for efficient adenoviral growth and essential for oncolytic Ad vectors. On the other hand, the replication deficient Ad gene therapy vectors do not require the functions of these proteins in the lytic virus growth. However, the removal of the entire E4 region or genes of E4orf6 and E4orf3 from the Ad vector genome results in highly reduced therapeutic transgene expression. E4 gene products substantially affect transgene persistence (Chirmule et al., 1998), and E4orf3 alone is sufficient to establish and maintain expression of transgenes (Armentano et al., 1999; Lusky et al., 1999). This effect is enhanced in the presence of E4orf6. Thus, these two proteins are also required for Ad gene therapy vectors (Lusky et al., 1999).

As mentioned, E4orf6 and E4orf3 proteins have important functions in the lytic viral infection and at least one of them is essential for replication selective oncolytic Ad vectors. In addition, they are required for efficient transgene expression and especially the presence of E4orf3 is beneficial for replication deficient Ad gene therapy vectors. On the other hand, both proteins bear oncogenic and mutagenic properties. This raises the obvious question of whether the functions of these proteins in Ad infection overlap with their role in transformation. Therefore, in order to unravel how the modifications on E4orf6 and E4orf3 genes can bring clinical benefits, the functions of these proteins in Ad infectious cycle and adenovirus-mediated oncogenesis should be investigated.

Ad5 E4orf3 product was the first protein from early region 4 to be identified in virus-infected cells (Sarnow et al., 1982; Downey et al., 1983). However, less is know about the functions of this protein. The protein contains no similarity to any of the known structural motives or conserved domains. Moreover, E4orf3 could never be expressed or purified in high amounts and soluble form due to the extreme insoluble behavior it shows. Therefore, the functional knowledge about E4orf3 has been restricted to the mutagenesis studies that were introducing random mutations on the E4orf3 gene and characterizing their effects (Dirlik, 2004); a detailed functional analysis could not be performed and no structural data has been available for E4orf3 until this study. For these reasons, the goal of this study was: i) to set a procedure for the successful expression and purification of E4orf3, ii) to analyze the interactions of E4orf3 with other proteins in detail and determine the regions on E4orf3 responsible for those interactions, and iii) to obtain basic structural knowledge about E4orf3 protein by using Fourier Transform Infrared (FTIR) spectroscopy, which is a powerful tool in the study of protein secondary and higher structures.

The results of this research will provide a better understanding of the functions of E4orf3 which is important for developing Ad vectors. Moreover, this study will be a base for all subsequent structural and functional studies concerning this protein, and will also be a model for analyzing the structure and functions of many other proteins that can not be expressed and purified because of their extreme insolubility.

CHAPTER II

GENERAL INFORMATION

2.1. Adenoviruses

2.1.1. Classification

Ads, belonging to Adenoviridae family, are known to infect a wide range of hosts including mammalian and avian species. Adenoviridae family covers over 100 different Ad serotypes which are divided basically into two classes according to the hosts they infect: Avianadenoviruses that are infecting birds and Mastadenoviruses that are infecting mammals. In addition, a third class, Atadenoviruses were suggested recently (Benko and Harrach, 1998).

Human Ads comprise over 50 different serotypes and based on their oncogenicity in immunosuppressed experiment animals, hemagglutination properties and DNA sequence homologies, they are classified into 6 subgroups (A-F) (Horwitz, 1996) (Table 2.1).

Table 2.1. The distribution of Ad serotypes into subgroups according to their oncogenicity and hemagglutination properties (Ustaçelebi, 1992).

	HUMAN ADs	Oncogenicity		Hemagglutination	
Subgroup	**Serotype**	**Oncogenic Potential**	**Tumor Type**	**Rhesus**	**Rat**
A	12, 18, 31	High	Sarcoma	-	+ / -
B	3, 7, 11, 14,16, 21, 34, 35, 50	Moderate	Sarcoma	+	-
C	1, 2, 5, 6	None	-	-	+ / -
D	8, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39 42-49, **51**	None	-	+ / -	+
D	9, 10	Estrogen Dependent	Fibro-adenoma	+ / -	+
E	4	None	-	-	+ / -
F	40, 41	?	?	-	+ / -

2.1.2. Pathogenesis

Human Ads cause lytic and persistant infections. Although most of the Ad infections are asymptomatic (Crabtree, 1997), some serotypes cause mild symptoms that are generally respiratory and gastrointestinal (Table 2.2). The diseases that have been associated with human Ads are acute respiratory disease (usually), pneumonia (occasionally), acute follicular conjunctivitis, epidemic keratoconjunctivitis, cystitis, and gastroenteritis (occasionally). In infants, pharyngitis and pharyngeal-conjunctival fever are common (Baron, 1996). Ads are also the second main causative agent for childhood diarrhea. The symptomatic Ad infections are usually observed in young children and followed by a long term effective immunity. Human Ads are not related to

human cancer. However, some serotypes can induce tumors in newborn rodents and transform primary cells in culture. Thus, human Ads are classified in DNA tumor viruses.

Table 2.2. The diseases that have been associated with different Ad serotypes

Sub-Group	Clinical Syndromes
A, E	Acute respiratory illness, Conjunctivitis, Pharyngitis, Pneumonia, Acute/chronic appendicitis, Respiratory track infections
B (3, 7, 14, 21)	Fever, Pharyngitis, Acute respiratory illness, meningitis
C (1, 2, 5, 6)	Respiratory illness in children, rare in adults
D (8, 19, 37)	Epidemic keratoconjunctivitis
E (4)	Fever, Pharyngitis, Acute respiratory illness
F, G (40, 41)	Gastroenteritis

2.1.3. Structural and Genomic Organization

Ads consist of 80-110 Nm large icosahedral protein capsid which is made up of 252 capsomeres: 240 hexons forming the faces and 12 pentons at the vertices. Each penton bears a slender fiber (Baron, 1996). Ads are non-enveloped viruses containing a linear, double-stranded DNA genome in their capsid (Fig. 2.1). The genome is associated with two major core proteins and carries a terminal 55-kDa protein (TP) covalently attached to the 5′ end of each strand. These TPs are thought to protect the viral DNA.

An important characteristic of Ad capsid is the antenna-like extensions which protrude from each 12 corners of the icosahedron. These extensions take part in the absorption of the virus by the host cell receptor and are also responsible for the hemagglutination (Philipson, 1975). The prime receptor for human adenovirus is identical to that for coxsackie B virus and has been named the coxsackie/adenovirus receptor (CAR). After the attachment step, interaction between the penton base and α_v integrins on the cell surface

leads to internalization of the virus through endocytosis (Fig. 2.2). Once inside the cell, and with help from the penton base, the virus escapes the endosome and translocates to the nuclear pore complex, where the viral DNA is released into the nucleus and transcription begins (Howitt et al., 2003).

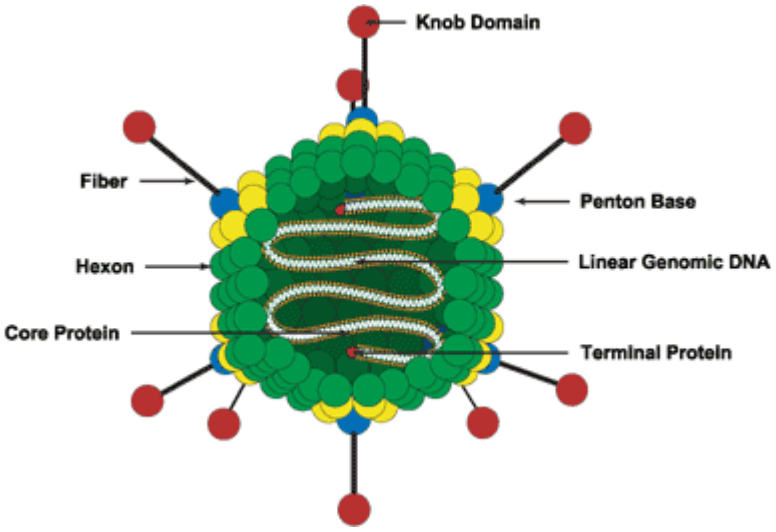


Figure 2.1. Adenovirus morphology (http://www.adenovirus.com, 2002).

Human Ad serotypes have the same genomic organization and express a similar set of RNAs. The viral chromosome of the most widely studied subgroup C Ad serotypes 2 and 5 have characteristic linear, double-stranded DNA genomes of 36kb in length (Shenk, 1996). The genome of Ad5 carries 9 different transcription units which encode 40 different polypeptides and two small RNAs (virus associated RNAs, VA RNAs). The genome of Ad5 is organized into early and late genes. Early genes are designated as E1-E4, and late genes which are under the control of major late promoter (MLP) and designated as L1-L5. In addition, two RNAs encoded from IX and IVa2 genes are observed in intermediate times of infection (Fig. 2.3).

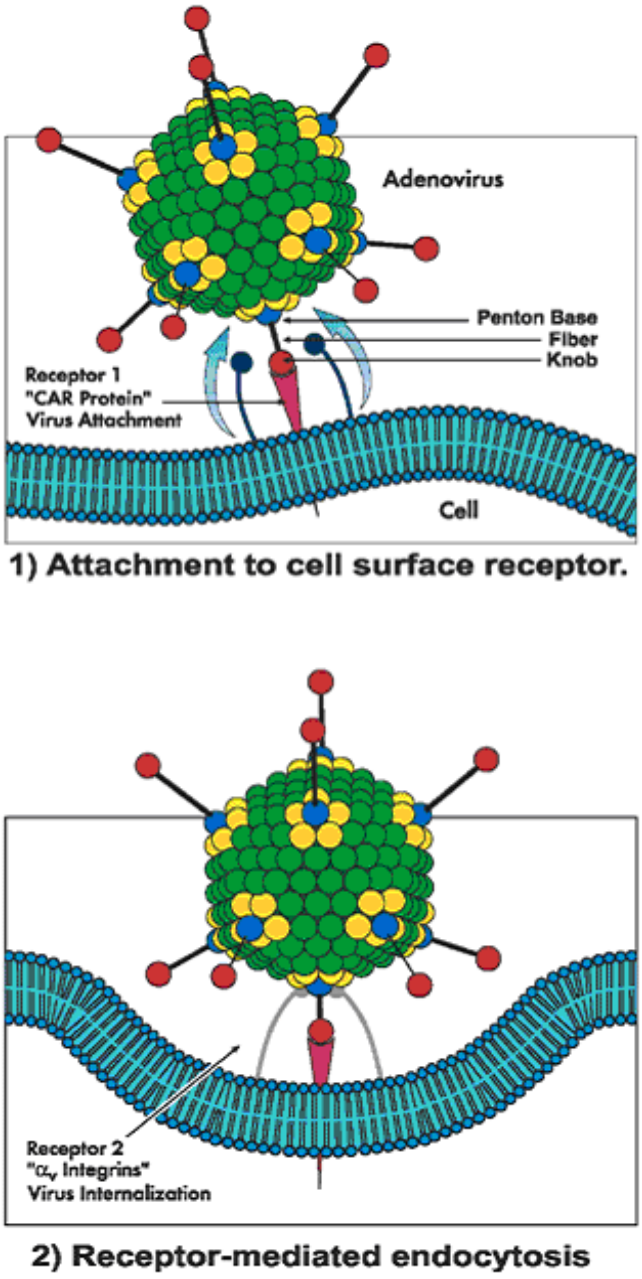


Figure 2.2. Binding and internalization of adenovirus (http://www.adenovirus.com, 2002).

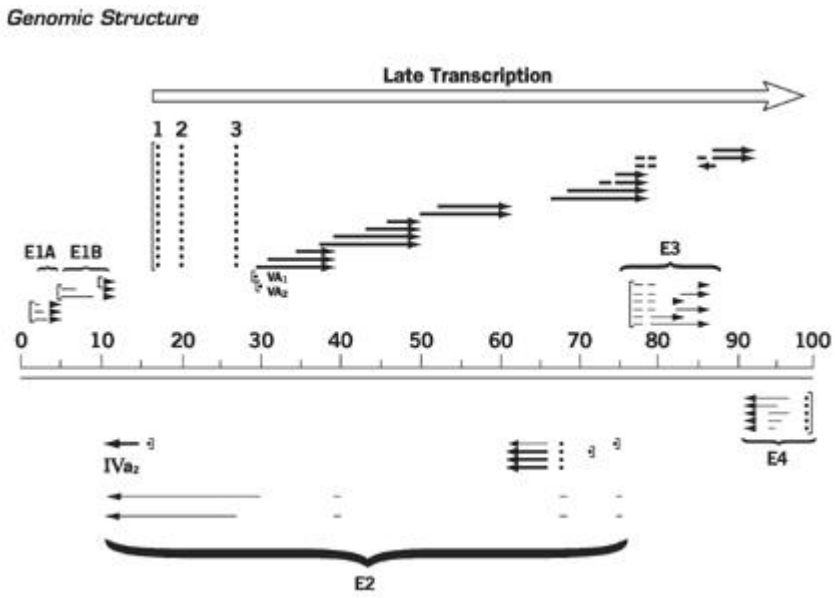


Figure 2.3. Genomic organization of adenovirus (http://adenovirus.com, 01.09.2004)

2.1.4. Productive Infectious Cycle

Ads are able to infect a wide range of cell types but their primary target is post-mitotically resting epithelium cells of neck, nose, throat area, the lung and the digestive tract. Besides, Ads can also infect different types of cultured tumor and primary cells. Human Ads usually go through a productive (lytic) infectious cycle in human cells which results in the death of these cells (Shenk, 1996; Modrow and Falke, 1997).

As for the most DNA viruses, the infectious cycle of Ads is organized into early and late phases (Dobner and Kzhyshkowska, 2001). The early phase of infection is characterized by the production of viral mRNAs from the early transcription units, E1A, E1B, E2, E3 and E4. These transcription units produce multiple differentially spliced and polyadenylated mRNAs encoding a variety of distinct polypeptides. Early viral gene products function in transcriptional and posttranscriptional regulation of viral and host RNA

production, induce cell cycle progression, and antagonize a variety of anti-viral defense mechanisms, such as apoptosis and immune response. Expression of early genes is required to establish an optimal environment for efficient viral DNA replication and the subsequent expression of the late genes.

Following the onset of viral DNA replication, late phase of infection starts. Ad late transcription is activated at three different transcription units, which encode for structural polypeptides or are involved in packaging of viral genomic DNA. The Ad late coding regions are organized into a single large transcription unit, called major late transcription unit (MLTU). The MLTU is controlled by the MLP and generates a large primary transcript that is processed by differential splicing into different mRNAs grouped into 5 families, L1-L5. Within a few hours of the onset of late phase, several complex metabolic changes occur that ensure preferential synthesis of viral proteins and efficient assembly of progeny virions. The lytic cycle is completed approximately 24h after infection by the production of about 10,000 virus particles per infected cell. A schematic representation of the Ad productive infectious cycle is shown in Fig. 2.4.

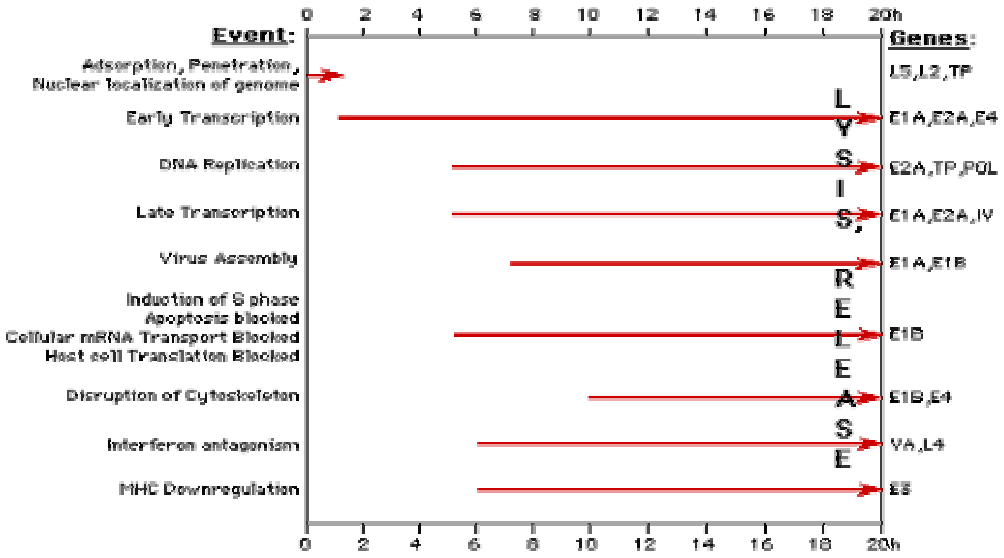


Figure 2.4. Productive infectious cycle of adenovirus (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/00.001.htm, 2003).

2.1.4.1. The Role of E1 Region in Ad Infection

E1 region, which is located at the left end of the Ad genome and transcribed through rightward direction, encodes proteins necessary for the expression of the other early and late genes. The region encodes for two distinct transcription units, E1A and E1B, which are essential for viral growth (Bernards and van der Eb, 1984; Shenk, 1996).

The first virus mRNA which can be observed approximately 1 hour post-infection is transcribed from E1A region and spliced into two transcripts. These transcripts code for two nuclear multi-functional phosphoproteins (E1A 12S and E1A 13S) which are transcriptional regulators inducing the expression of other viral genes. To date, over 40 different E1A binding proteins have been identified including transcriptional coactivators and repressors as well as proteins directly involved in cell cycle regulation such as the retinoblastoma tumor suppressor gene product (pRb). E1A proteins can also affect the proliferative state of the cell. They stimulate entry into S

phase of the cell cycle to create optimal conditions for a productive virus infection in nonproliferating human cells. One of the functions of E1A proteins is down regulating the expression of MHC class I genes. This activity may be used by the virus to escape the host's immune defense mechanism (Pääbo et al., 1989).

The E1B gene lies adjacent to E1A and gives raise to two major mRNA species of 22S and 13S. The 22S mRNA is translated in two unrelated polypeptides called E1B 19-kDa and E1B-55 kDa (Anderson et al., 1984; Takayesu et al., 1994). The 13S E1B mRNA also encodes for the 19-kDa protein and a smaller species containing 75 residues from the amino terminal of the 55-kDa product.

As opposed to the E1A proteins, considerably less is known about the functions of the Ad E1B gene products. However, their primary role in virus infection is antagonizing apoptosis and growth arrest (Wickham, 2000; Wienzek et al., 2000). Among the E1B products, E1B-55 kDa is known to bind to the tumor suppressor p53 and repress p53-responsible promoters. Moreover, by acting together with the Ad E4orf6 protein, it induces degradation of p53 and inhibits p53-induced apoptosis, thereby enabling cells to enter S phase (Gabler et al., 1998; Harada and Berk, 1999). E1B-55 kDa is a multifunctional protein having functions in both nucleus and cytoplasm. It contains a nuclear localization signal (NES), nuclear localization signal (NLS) and a SUMO-1 modification site (Kratzer et al., 2000; Endter et al., 2001). SUMO-1 is a small ubiquitin-like modifier protein that is covalently linked to many cellular and viral protein targets. Modification by SUMO-1 is proposed to play a role in protein targeting and stability (Pilcher and Melchior, 2002). By SUMO-1 modification, E1B-55 kDa protein can accumulate in the nucleus and by its NES, it can leave the nucleus to cytoplasm. In other words, it is an actively shuttling protein. E1B-55 kDa also takes part in efficient viral DNA replication, selective nucleocytoplasmic transport of viral mRNAs, and shut-

off of host cell protein synthesis (Shenk, 1996), and is an important polypeptide for Ad productive infectious cycle.

2.1.4.2. The Role of E4 Region in Ad Infection

In contrast to E1, E4 region is located at the right end of the Ad genome and transcribed through leftward direction. The E4 transcription unit is controlled by E4 promoter (Watanabe et al., 1988). The primary transcript generated by E4 is differentially spliced to produce at least 18 distinct mRNAs that share common 5'- and 3'- terminal sequences (Herisse et al., 1981; Freyer et al., 1984; Virtanen et al., 1984). These mRNAs potentially encode 7 different polypeptides E4orf1, E4orf2, E4orf3, E4orf3/4, E4orf4, E4orf6 and E4orf6/7 (Cutt et al., 1987; Dix and Leppard, 1995; Thomas et al., 2001). Fig. 2.5 shows the reading frames of Ad5 E4 transcription unit. Analysis of other serotypes shows that all human Ads have a homologous E4 region with similar sequence organization.

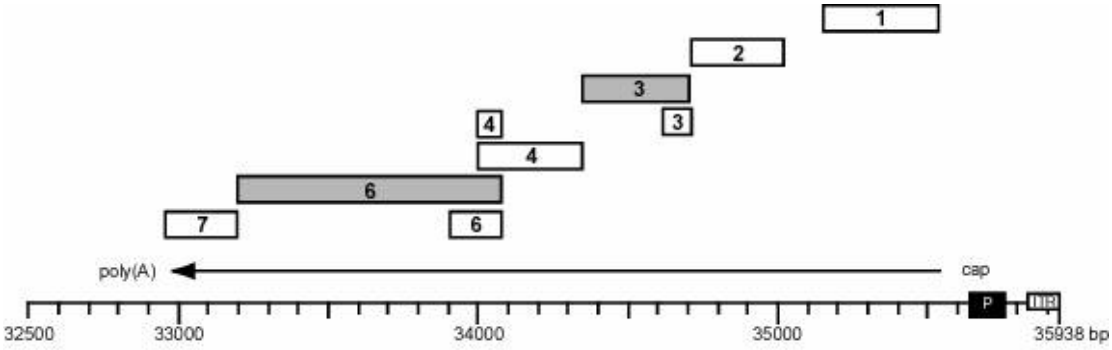


Figure 2.5. Ad5 E4 transcription unit (Based on a previously published figure, Täuber and Dobner, 2001).

In early studies, to elucidate the role of E4 region in virus infection various mutations were introduced into the Ad5 E4 coding region. The

characterization of these mutant viruses showed that none of the individual E4orf proteins contributed significantly to the lytic virus growth (Halbert et al., 1985). However, Ad mutants that lack the entire E4 region or fail to express both E4orf6 and E4orf3 proteins are severely restricted for growth. The mutants expressing only E4orf6 grow to near wild-type levels, while mutants expressing only E4orf3 are moderately defective (Bridge and Ketner, 1989; Huang and Hearing, 1989). Collectively, these genetic studies suggest that E4orf6 and E4orf3 provide redundant functions for efficient viral replication and the other E4 gene products are largely dispensable for lytic growth. E4orf6 and E4orf3 proteins can partially or totally compensate each other's defects. Each E4 protein independently augments viral DNA replication, late viral protein synthesis, shut-off of host protein synthesis, production of progeny virions and prevents concatemer formation of viral genomes (Halbert et al., 1985; Bridge and Ketner, 1989; Huang and Hearing, 1989; Bridge and Ketner, 1990; Weiden and Ginsberg, 1994). These two proteins also enhance late gene expression through nuclear stabilization of late viral mRNAs (Imperiale et al., 1995) and indirectly increase the efficiency of viral DNA synthesis (Medghalchi et al., 1997).

The functions of E4orf6 and E4orf3 proteins which are important for the lytic virus growth are linked to their ability to interact with cellular and viral factors. For instance, each protein has the ability to bind to DNA-PK and inactivate it. DNA-PK is an essential element of double-strand break repair system (DSBR) which repairs the specific double-strand breaks by an end-joining mechanism (Smith and Jackson, 1999). Inhibition of DSBR, is required for the accumulation of monomeric viral DNA genomes since, DSBR recognizes the double-stranded Ad DNA as chromosomal breaks and join them end-to-end to form large concatemers which can not be packaged into the viral capsid (Boyer et al., 1999).

Moreover, these proteins also interact with an important viral factor, E1B-55 kDa protein. The interaction of E4orf6 and E4orf3 with E1B-55 kDa is thought

to be mutually exclusive and regulated in a coordinated manner. Each protein directs the E1B protein to specific locations in the nucleus (König et al., 1999; Leppard and Everett, 1999). The interaction of E4orf6 with E1B-55 kDa promotes cytoplasmic accumulation of viral mRNAs and late viral gene expression, while it inhibits the export of most cellular transcripts (Huang and Hearing, 1989; Bridge and Ketner, 1990). This interaction is also required for the degradation of the tumor suppressor p53 (Wienzek et al., 2000; Querido et al., 2001). E4orf3 interacts with E1B-55 kDa protein in dot-like matrix associated multiprotein complexes known as nuclear bodies or PODs. PODs are linked to the formation of viral replication compartments and efficient viral replication by several viruses (Sternsdorf et al., 1997). E4orf3 induces the reorganization of PODs into track-like formation early after infection (Carvalho et al., 1995; Doucas et al., 1996). Although E4orf3 is alone sufficient for nuclear track formation, other viral proteins, especially E4orf6, also have the limited ability to redistribute the PODs. E1B-55 kDa physically associates with intact PODs and E4orf3 in nuclear tracks. E4orf3 induced reorganization of E1B-55 kDa and PODs is a prerequisite for the efficient viral replication, and late gene expression.

In summary, the redundant functions shared by E4orf6 and E4orf3 proteins nearly accounts for the functions of the whole E4 region in productive infectious cycle. Therefore, at least one or both of E4 proteins is required for efficient virus growth.

2.1.5. Transforming and Oncogenic Potential of Human Ads

To date, Ads could never be convincingly associated with malignant diseases in humans (Mackey et al., 1976; Mackey et al., 1979; Chauvin et al., 1990). However, the oncogenic potential of human Ads was first proposed by Trentin and coworkers. Their studies showed that human Ad serotype 12 (Ad12) can induce malignant transformation in newborn hamsters (Trentin et al., 1962). This initial discovery showing the oncogenic potential of human

Ads inspired an intense research on these viruses which continued to present. Today, most of the genes involved in Ad transformation and their products, and the differences in the oncogenicity among different serotypes have been identified (Endter and Dobner, 2003).

According to the frequency they show and time they consume in producing tumors in rodents, human serotypes can be subdivided as highly oncogenic, weakly oncogenic and nononcogenic Ads. Tumors in rodents induced by highly oncogenic subgroup A and weakly oncogenic subgroup B Ads develop at the site of injection and, depending on the route of administration vary greatly in type, including neurogenic, neuroepithelial, medulloblastic and adenocarcinomatous tumors (Graham, 1984). By contrast, serotypes 9 and 10 belonging to subgroup D develop estrogen-dependent mammary tumors at 100% frequency within 3 months upon subcutaneous or intraperitonal injection in female Wistar-Furth rats (Ankerst and Jonsson, 1989; Javier et al., 1991). The differences in the oncogenicity among different Ad serotypes are related to the genetic background of host animal, as well as the susceptibility of the transformed cell to the host immune system (van der EB and Zantema 1992; Williams et al., 1995).

2.1.5.1. The role of E1 Region in Transformation

Most Ad tumors, tumor cell lines and transformed cell lines are characterized by the persistence of the viral DNA in a chromosomally integrated form and the expression of the viral specific antigens (Graham, 1984). Correspondingly, Ad transformation follows the classical concept of viral oncogenesis where viral genes persist within the transformed cells. From these viral genes, E1 region is almost invariably retained in all virus-transformed cells. Likewise, in most completely transformed cell lines established following transfection with subgenomic viral DNA fragments, the E1 sequences are retained in an integrated form. Therefore, E1 region is assigned to mediate the initiation of transformation and provides functions

required to maintain some of the phenotypic characteristics of the transformed cell (Endter and Dobner, 2003).

As mentioned previously, E1 gene products encoded in two separate transcription units termed E1A and E1B, both of which are necessary and sufficient for oncogenic transformation of primary cells. However, E1A proteins mediate the most critical step in cell transformation. The transforming and oncogenic properties of E1A in nonpermisive cells are derived from their ability to deregulate normal cell growth in order to establish productive infection and their ability to modulate the interaction of the infected cell with the immune system of the host. For example, E1A proteins stimulate entry into S phase of the cell cycle to create optimal conditions for productive virus infection in nonproliferating human cells (Endter and Dobner, 2003).

Accomplishment of cell growth deregulation by E1A proteins depends on their capability of binding to and modulating the functions of key regulators that control cell cycle progression and programmed cell death (Gallimore and Turnell, 2001). Most notable example to those proteins is the retinoblastoma tumor suppressor gene product (pRb). Binding of E1A proteins to Rb family members dissociates them from E2F family of transcription factors, resulting in the activation of the cellular genes containing E2F-binding sites, and induction of cell cycle progression (Cress and Nevins, 1996). In the course of an abortive infection in nonpermisive cells, merely, these same growth deregulatory functions can lead to immortalization or transformation. On the other hand, in the majority of E1A-immortalized cells the transformation is incomplete (Gallimore, 1984), and full manifestation of the transformed phenotype requires the co-expression of E1B subunit (Ruley, 1983).

The studies with virus mutants carrying lesions in the E1B transcription subunit gave rise to the hypothesis that both major E1B gene products, E1B-19 kDa and E1B-55 kDa, are required for complete transformation (Van der Eb and Zantema, 1992). In contrast, some studies suggested that E1B-19

kDa protein is not definitely needed for efficient transformation (Telling and Williams, 1993) but it can be said that both E1B proteins contribute to complete cell transformation at least in part by antagonizing apoptosis and growth arrest (Debbas and White, 1993).

The transforming potential of Ad5 E1B-55 kDa correlates with its ability to act as a direct transcriptional repressor (Yew and Berk, 1992). It binds to the tumor suppressor protein p53, and antagonizes p53-induced apoptosis in the course of the transformation process (Sabbatini et al., 1995; Hutton et al., 2000). In addition, E1B-55 kDa may involve further functions and other protein interactions during transformation (Harada et al., 2002). For example, E1B-55 kDa interacts with the E1B-associated protein 5 (E1B-Ap5) and nuclear body associated promyelocytic leukemia (PML) protein both of which can individually suppress transformation of baby rat kidney (BRK) cells (Nevels et al., 1999b; Kzhyshkowska et al., 2001). Modification of these cellular factors by E1 proteins is required to enhance transformation.

In conclusion, E1 region is the main determinant for the oncogenic potential of Ads, and the E1A and E1B transcription units are essential for Ad-mediated oncogenic transformation.

2.1.5.2. The role of E4 Region in Transformation

Although the expression of E1A and E1B transcription units is sufficient for oncogenic transformation, the gene products of the E4 region also functions in the process of virus-mediated oncogenesis (Graham, 1984; Zalmanzon, 1987). Evidence for the involvement of the E4 region in transformation events stems from the observation that Ad transformed cells often contain viral DNA structures in which E1- and E4- gene segments are joined together (Graham, 1984) and the E4 gene products are present in these cells (Esche and Siegman, 1982). Therefore, it has been suggested that, although not essential for Ad transformation, some E4 proteins may have partial

transforming activities and contribute to E1 mediated oncogenesis (Öhman et al., 1995).

The E4 region of Ad5 expresses at least two proteins, E4orf6 and E4orf3, both of which exhibit transforming and oncogenic potential. Both E4 proteins substantially enhance focus formation in primary BRK cells in cooperation with Ad5 E1 proteins, acting synergistically in the presence of E1B (Nevels et al., 1999b). Ad5 E4orf6 and E4orf3 also initiate focal transformation of primary BRK cells in an E1B-like fashion in cooperation with E1A. Interestingly, however, the majority of BRK cells transformed by E1A plus either E4orf6 or E4orf3 lack both E4- and E1A- specific DNA sequences, whereas, established E1A/E1B-transformed cells consistently maintain and express the viral genes (Nevels et al., 2001). Such observations spawned the hit-and-run model of viral transformation, which claims that viral genes are necessary to initiate but not to maintain cellular transformation. The fact that transient expression of E1A plus E4orf6 or E4orf3 proved to be mutagenic suggests that the viral genes mediate such hit-and-run transformation by inducing oncogenic mutations in cellular genes (Nevels et al., 2001).

The oncogenic and mutagenic properties of both E4 proteins are related in part to their roles in lytic infection (Täuber and Dobner, 2001). For example, E4orf6 expression causes a dramatic reduction in p53 steady-state levels (Nevels et al., 1999a) which is required for the virus to escape apoptosis. Therefore, the ability of E4orf6 to promote oncogenic cell growth is probably linked to modulation of p53 function and stability. The transforming activities of E4orf3, instead, involve binding to Ad5 E1B-55 kDa protein and PODs which in turn trigger a cascade of events leading to oncogenic growth (Nevels et al, 1999b). Furthermore, both E4 proteins associate with the catalytic subunit of the DNA-PK and inhibit the activity of this enzyme having a key function in DSBR system (Boyer et al., 1999).

To sum up, although not essential for Ad transformation, E4 region contribute to Ad-mediated oncogenesis. In particular, the activities of E4orf6 and E4orf3 proteins of Ad5 can be speculated to cause mutations in the cells and by this way, these proteins may contribute to the development of some human tumors through a mutagenesis-based hit-and-run mechanism.

2.2. Infrared Spectroscopy

2.2.1. Basic Theory of the Absorption of Light by Molecules

Light consists of mutually perpendicular electric and magnetic fields oscillating as a sine way as they are propagated through space. The energy (*E*) of the electromagnetic light wave is given by the Bohr equation as:

$$E = h.c/\lambda = h.v = h.\ c.\ \bar{\nu}$$

in which *h* is Planck's constant (h = 6.626 x 10^{-34} J.s), *c* (3.0x108 ms-1) is the velocity of light, λ is the wavelength (cm), and *v* is the frequency (sec^{-1}). The unit wavenumber $\bar{\nu}$ is used for practical reasons. Wavenumber (cm^{-1}) is the number of waves in a length of one centimeter and thus is given by:

$$\bar{\nu} = \frac{1}{\lambda}$$

When such an electromagnetic wave encounters a molecule, it can be either scattered (i.e., its direction of propagation changes), absorbed (i.e., its energy is transferred to the molecule) or emitted (i.e., its energy is released by the molecule). When the energy of the light is absorbed, the molecule is said to be *excited.* An excited molecule can possess any one of a set of discrete amounts (quanta) of energy described by the laws of quantum mechanics. These amounts are called the *energy levels* of the molecule. The major energy levels are determined by the possible spatial distributions of the

electrons and are called *electronic energy levels* ($E_{electronic}$); on these are superimposed *vibrational levels* ($E_{vibration}$) which indicate the various modes of vibration of the molecule. There are even smaller subdivisions called *rotational levels* ($E_{rotation}$). The separations between the neighboring energy levels corresponding to $E_{rotation}$, $E_{vibration}$ and $E_{electronic}$ are associated with the microwave, infrared and ultraviolet-visible region of the electromagnetic spectrum, respectively (Campbell and Dwek, 1984). The means of study for these energy transitions is standard absorption spectroscopy for electronic transition, infrared and Raman spectroscopy for vibrational and rotational transitions, electron spin resonance for electron spin orientation, and nuclear magnetic resonance for nuclear spin orientation. Some of these energy levels are described by an *energy-level diagram* (Fig. 2.6). The lowest electronic level is called the ground state and all others are excited states (Freifelder, 1982).

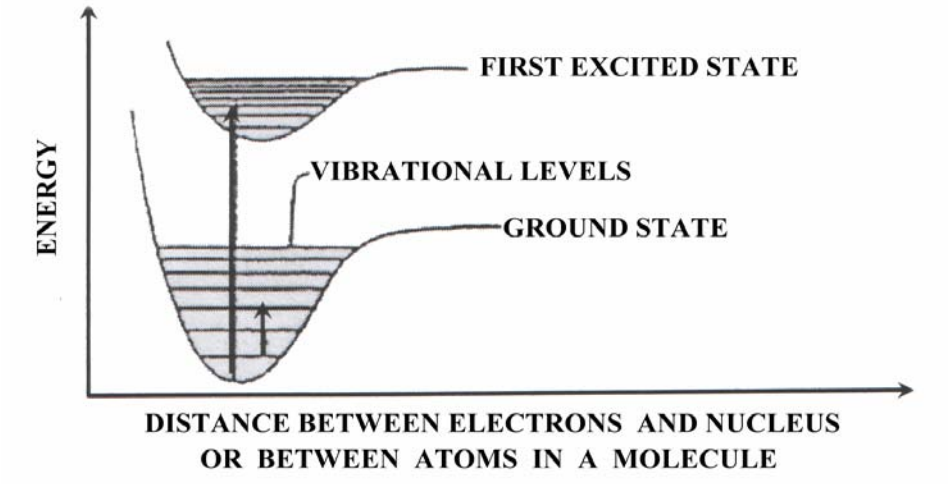


Figure 2.6. Typical energy-level diagram showing the ground state and the first excited state. Vibrational levels are shown as thin horizontal lines. A possible electronic transition between the ground state and the fourth vibrational level of the first excited state is indicated by the long arrow. A vibrational transition within the ground state is indicated by the short arrow (Freifelder, 1982).

2.2.2. Basis of Infrared Spectroscopy

Spectroscopy is defined as the study of interaction of electromagnetic radiation with matter. Spectroscopic techniques involve i) irradiation of a sample with some form of electromagnetic radiation, ii) measurement of the scattering, absorption, or emission in terms of some measured parameters, and iii) the interpretation of these measured parameters to give useful information. Fig. 2.7 represents many of the important regions of the electromagnetic spectrum.

The atoms in a molecule are constantly oscillating around average positions. Bond lengths and bond angles are continuously changing due to this vibration. A molecule absorbs infrared radiation when the vibration of the atoms in the molecule produces an oscillating electric field with the same frequency as the frequency of the incident IR light. The molecule will only absorb radiation if the vibration is accompanied by a change in the dipole moment of the molecule. A dipole occurs when there is charge separation across bond. If the two oppositely charged molecules get closer or move further apart as the bond bends or stretches, the moment will change.

All of the motions can be described in terms of two types of molecular vibrations. One type of vibration, a stretch, produces a change of bond length. A stretch is a rhythmic movement along the line between the atoms so that the interatomic distance is either increasing or decreasing. The second type of vibration, a bend, results in a change in bond angle. Each of these two main types of vibration can have variations. A stretch can be symmetric or asymmetric. Bending can occur in the plane of the molecule or out of plane; it can be scissoring, like blades of a pair of scissors, or rocking, where two atoms move in the same directions (Arrondo et al., 1993). Fig. 2.8 demonstrates the main types of variations schematically.

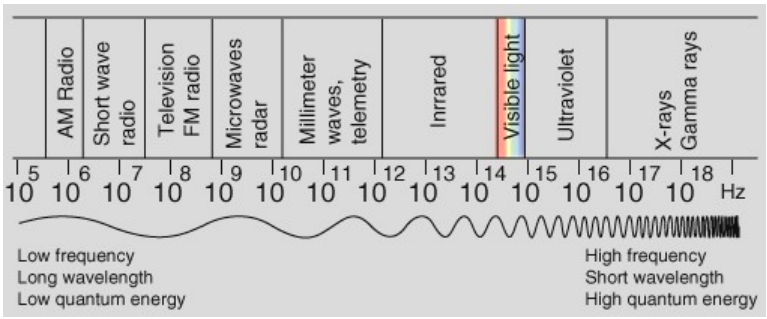


Figure 2.7. The electromagnetic spectrum.

Upon interaction with infrared radiation, portions of the incident radiation are absorbed at particular wavelengths. The multiplicity of vibrations occurring simultaneously produces a highly complex absorption spectrum, which is uniquely characteristic of the functional groups comprising the molecule and of the overall configuration of the atoms as well. The value of infrared spectral analysis comes from the fact that the modes of vibration of each group are very sensitive to the changes in chemical structure, conformation and environment.

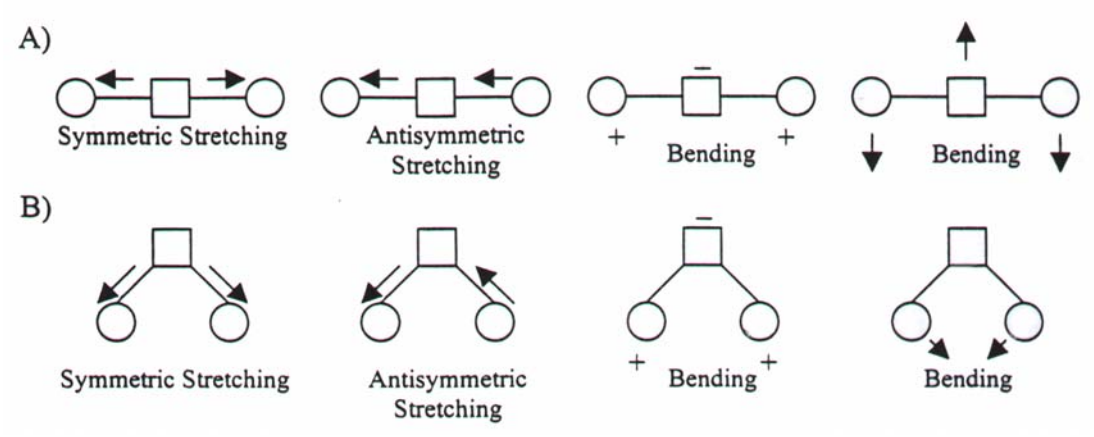


Figure 2.8. Types of normal vibration in a linear and non-linear triatomic molecule (Arrondo et al., 1993).

2.2.3. Fourier Transform Infrared (FTIR) Spectroscopy

The basic components of a Fourier transform infrared spectrometer are visualized in Fig. 2.9. The central apparatus of FTIR instruments is the Michelson interferometer, which causes the incident beam from the source to split into two at a beam splitter. This is followed by the recombination of these two beams at the beam splitter, where they undergo constructive and destructive interference with each other and their transmittance to the sample. This yields an interferogram, which first reaches a detector and then an amplifier. The data from the resulting amplified signal is converted to a digital form via an analog-to-digital converter. The signal is then transferred to a computer, where the Fourier transformation of the signal is carried out in order to convert the intensity falling on the detector to the spectral power density (Stuart, 1997).

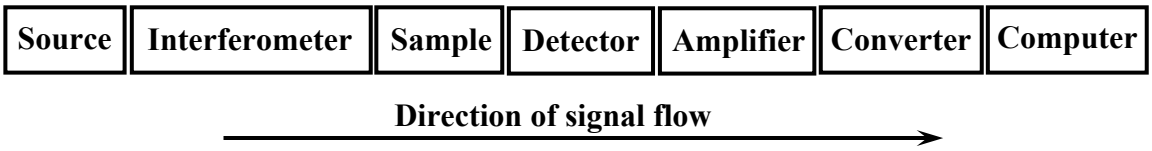


Figure 2.9. The schematic instrumentation of the components of an FTIR spectrometer (Stuart, 1997).

FTIR spectroscopy has many advantages. First of all, it provides a precise measurement method, which requires no external calibration. It is also a rapid and sensitive technique, which is easy to perform (Manoharan et al., 1993). The system can be applied to the analysis of any kind of material and that is not limited to the physical state of the sample. Samples may be solutions, viscous liquids, suspensions, inhomogeneous solids or powders. Moreover, information about the lipid conformation and the protein secondary

structure can be obtained simultaneously with a single experiment. Furthermore, it is a nondestructive technique (Haris and Severcan, 1999, Çakmak et al., 2003). Samples may be examined in a variety of physical states, such as solids, films, aqueous suspensions. Small sample quantities are sufficient (Mendelsohn and Mantsch, 1986).

2.2.4. Structural Characterization of Proteins by FTIR

During the last years, the use of FTIR to determine the structure of proteins has dramatically expanded. The complete three-dimensional structure of a protein at high resolution can be determined by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, which are the most powerful techniques available by far. However, both techniques has considerable disadvantages: X-ray crystallography requires high-quality single crystals which are not available for many proteins and the structure of a protein in crystal may not always relate to its structure in solution; NMR spectroscopy can determine the structure of proteins in solution, but the interpretation of the NMR spectra of large proteins is complex, and the technique is presently limited to small proteins (~30 kDa) (Haris and Severcan, 1999). These limitations have led to the development of alternative methods that are not able to generate structures at atomic resolution but provide also structural information on proteins (especially on secondary structure) such as FTIR.

The most important advantage of FTIR spectroscopy for structural characterization of proteins is that spectra of almost any protein can be obtained in a wide variety of environments. Spectra of a protein can be obtained in single crystals, aqueous solution, detergent micelles, lipid membranes, etc. Moreover, FTIR requires only small amounts of protein (10 µg), and the size of the protein is not important (Haris and Severcan, 1999). With developments in FTIR instrumentation it is now possible to obtain high quality spectra from dilute protein solutions in H_2O (Haris and Chapman, 1992; Surewicz et al., 1993; Arrondo and Goñi, 1999). The overlapping H_2O

absorption bands can be digitally subtracted from the spectrum of the protein solution. In addition, the broad infrared bands in the spectra of the proteins can be analyzed in detail by using second derivative and deconvolution procedures. These procedures can be utilized to reveal the overlapping components within the broad absorption bands (Haris and Chapman, 1992; Surewicz et al., 1993; Arrondo and Goñi, 1999).

The FTIR spectrum of a protein is characterized by a set of absorption regions known as amide modes. From these modes, Amide I that is located between 1700 and 1600 cm^{-1} and composed mainly (~80 %) by the C=O stretching vibration of the peptidic bond, can be analyzed for structural characterization of proteins. This band can be decomposed into its constituents by curve-fitting procedures and compared with a set of spectra of proteins with known structure (Arrondo and Goñi, 1999). By this way, secondary structure of proteins can be studied by FTIR.

CHAPTER III

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma and Merk, cell culture materials from Falcon, Gibco BRL and Pan, and plastic materials from Falcon, Sarstedt, Greiner, Eppendorf and Nunc.

For the determination of the sizes of the DNA fragments in agarose gel, *1 kb DNA ladder* (Gibco BRL), for the proteins in SDS-PAGE gel either *Broad Range Marker* or *Presicion Protein Standard* (Bio-Rad) were used as markers.

For DNA sequence analysis, *BioEdit version 4.8.5* (Hall, 1999), and for sequence comparisons, *Blast servers* (http://www.ncbi.nlm.nih.gov/BLAST, 2004) were used. Band analysis of SDS-PAGE gels were done by the help of QuantityOne software (Bio-Rad), and the digital immunoflourescence data were obtained by *Meta View 4.6 software* (Universal Imaging Corporation). All the spectral analysis following the FTIR studies was performed with *SpectrumOne* (Perkin-Elmer) and *GRAMS/32* (Galactic Industries, Salem, NH, USA) software.

3.1. Bacterial Culture Techniques

Table 3.1. Different *E. coli* strains used in this study and their genotypic features.

STRAINS	GENOTYPE
DH5α	*sup*E44, Δ*lac*U169, (ϕ80d*lac*ZΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1 (Hanahan, 1983)
DH10B	F'*ara*D139, Δ(*ara*, *leu*)7697, Δ*lac*X174, *gal*U, *gal*K, *mcr*A, Δ(*mrr*$^-$, *hsd*R$^-$, *mcr*BC), *rsp*L, *deo*R, (ϕ80d*lac*ZΔM15), *end*A1, *nup*5, *rec*A1 (Bethesda Research Laboratories)
TOPP™ 3	*rif*^{r} [F'*pro*AB *lac*I^{q}ZΔM15, Tn10 (*tet*^{r})] *kan*^{r} (Hatt et al., 1992)
XL1-Blue	*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, lac, [F'*pro*AB, *lac*I^{q}ZΔM15, Tn10 (*tet*^{r})] (Bullock et al., 1987)

3.1.1. Culture Conditions and Storage

Table 3.2. The chemical composition of LB medium and the antibiotic solutions.

LB-Medium	Trypton	10 g/l
	Yeast extract	5 g/l
	NaCl	5 g/l
	• autoclaved	

Antibiotic Solutions	Ampicillin (500 x)	50 mg/ml in dH_2O
	• filter sterilized	
	• stored at –20 °C	

Liquid cultures of *E. coli* in sterile LB medium with appropriate antibiotics (e.g. Ampicillin) were prepared by single colony inoculation method and incubated overnight at 37 °C in "incubator shaker" (New Brunswick) at 150-220 rpm. If necessary the concentration of the bacteria was determined photometrically at the wavelenght of 600 nm against the medium.

$1\ OD_{600} = 8 \times 108$ cell/ml

After overnight incubation of single colony cultures, plate cultures were prepared on agar plate by streaking a loop-full of liquid culture. Plate cultures can be kept several weeks at 4 °C after covered with parafilm. From liquid cultures, glycerin cultures were also prepared for storage at -80 °C.

Glycerin Culture: Liquid cultures of single colony bacteria which was incubated overnight were centrifuged briefly at 4000 rpm for 10 min (Rotixa 120R, Hettich) at room temperature. The pellets of bacteria were then resuspended in the mixture of 0,5 ml LB medium and 0,5 ml sterile glycerin and were taken in Cryo tubes (Nunc). By this way, bacteria cultures can be stored at -80 °C for a few years.

3.1.2. Transformation of *E. coli* by "Heat Shock" Method

Table 3.3. The chemical composition of NZY+ Broth medium and LB-Amp Agar.

NZY+ Broth (1L)	NZ amine (casein hydrolysate)	10 g
	Yeast extract	5g
	NaCl	5g
	dH_2O	up to 1L
	pH 7,5	by NaOH
	• autoclaved	
	1M $MgCl_2$	12,5 ml
	1M Mg SO_4	12,5 ml
	20% (w/v) glycose	20 ml

LB-Amp Agar (1L)	Tryptone		10 gl
	NaCl		5 g
	Yeast extract		5 g
	Agar		20 g
	dH_2O		up to 1L
	pH 7,5		by NaOH
	Amp		1ml/L
	• autoclaved		
	Pour in petris	~25 ml/100-mm/	petri

For this method, *Epicurian coli XL-1 Blue supercompetent* (Stratagene) strain of *E. coli* were used. 1 µl of DNA to be transformed (~1 µg/µl in concentration) was put in to the Eppendorf tubes and mixed with 1 µl β-Mercapthoethanol (βM) which increases the efficiency of the transformation. Meanwhile *E. coli* cells taken from -80 °C were thawed on ice. Afterwards, 50 µl of the cell suspension was transferred into a *Falcon 2059 polypropylene* tube. The pre-made mixture of DNA and βM was put on these cells, mixed carefully and incubated for 30 min on ice. This mixture was then put in the pre-heated water bath at 42 °C for 45 sec and transferred again on ice for 2 min. By this way the plasmid DNA could enter from the pores of bacteria cell membranes which were occurred by heath shock. Afterwards, the transformed cells were mixed with 500 µl NZY^+ broth which was heated in the water bath at 42 °C. These cells were then incubated at 37 °C for 1h at 150 rpm in order to recover and adapt themselves to the new phenotype.

To measure the efficiency of the transformation, first 100 µl of the 552 µl bacteria culture was spread on LB Amp agar plate by Drigalski method. Then, 10 µl of the culture mixed with 90 µl LB (10^{-1}dilution) and 1 µl of the culture mixed with 99 µl LB (10^{-2} dilution) was spread on agar plates. Rest of the cell culture was then centrifuged at 5000 rpm, supernatant was removed and the cell pellet was resuspended in the supernatant left and spread on the agar plate. The plates were incubated at 37 °C for at least 16h. Since the media of the plates contain ampicillin, only the transformed bacteria could grow because *XL-1 Blue* cells do not possess Amp resistance gene.

3.1.3. Transformation of E. coli by "Electroporation" Method

Table 3.4. The chemical composition of YENB and SOC media.

YENB	Bacto Yeast Extract	7,5 g/l
	Bacto Nutrient Broth	8 g/l
	• autoclaved	

SOC-Medium	Trypton	20 g/l
	Yeast extract	5 g/l
	NaCl	10 mM
	KCl	2,5 mM
	$MgCl_2$	10 mM
	$MgSO_4$	10 mM
	Glucose	20 mM
	• autoclaved	

The production of electrocompetent *E. coli* cells was done according to the protocol of Sharma and Schimke (Sharma and Schimke, 1996). 10 ml of the overnight culture of *E. coli* cells were incubated at 37 °C. After reaching an OD of 0,5-0,9 at 600 nm, they were inoculated into 1 L of YENB medium. Afterwards, the cells were cooled for 5 min on ice and centrifuged for 10 min at 6000 rpm at 4 °C (Centrikon T-124, Kontron of instrument). The bacteria pellet was washed twice by resuspension with 100 ml ice-cold dH_2O and once with 20 ml, 10 % glycerin. It was then resuspended in a final volume of 3 ml, 10 % glycerin, divided into 50 µl aliquots and frozen in liquid nitrogen. The cells were stored at -80 °C.

For the electroporation of the ligation product, first demineralization must be done. For this purpose, DNA to be transformed was mixed with 1/10 volume of 3 M NaOAc and 1 volume of isopropanol. After 10 min centrifugation at 14000 rpm (Eppendorf table centrifuge 5417, Eppendorf) at room temperature, it was washed, dried, pelleted with 75 % EtOH and taken in to 10 µl dH_2O. The electrocompetent cells were thawed on ice; each 50 µl of the cell suspension was mixed with 1-10 µl Plasmid DNA and put into a pre-cooled electroporation cuvette (Bio-Rad) with an electrode gap of 1 mm transfers. The electroporation took place in a *Gene Pulser* machine (Bio-Rad) with a tension of 1,25 kV, a capacity of 25 µF and a bypass resistor of 200 Ω. Thereby, a time constant of approx. 5 ms was obtained. Alternatively cuvettes with an electrode gap of 2 mm were used with a tension of 2,5 kV. After the pulse, the cells were rinsed immediately with 1 ml of SOC medium, transferred into 1,5 ml reaction tubes (Eppendorf) from the cuvettes and incubated 1 h at 37 °C. Then, they were centrifuged, and resuspended in

approximately 50 µl of the supernatant and inoculated on agar plates with LB medium and appropriate antibiotics.

3.2. Mammalian Cell Culture Techniques

Table 3.5. Human cell types used in this study and their properties.

Cell Type	Properties	Reference
991	Human embryonic retinoblasts transformed by Ad5 E1 unit	Graham et al.,1977
A549	Human lung cancer cells, wild type p53	Giard et al., 1973
HeLa	Human cervix cancer cell line	Gey et al., 1952

3.2.1. Culture and Storage of the Cells

Adhesive mammalian cells were cultured as a monolayer in polystyrene cell culture bottles (25-75-175 cm^2 bottles, 6-well-plates or 66 cm^2 petris, Falcon). Dulbecco's Modified Eagle medium (DMEM; Gibco BRL; Dulbecco and Freeman, 1959) with 0,11 g/l sodium pyruvat was used as growth medium. This medium was then enriched with 10 % fetal calf serum (FCS; Pan) and with 1 % of a Penicillin/Streptomycin solution (1000 U/ml penicillin and 10 mg/ml Streptomycin in 0,9 % NaCl; Pan). To inhibit the complement factors in FCS, it was inactivated before use by keeping at 56 °C for 30 min. Cultured cells were incubated at 37 °C in a CO_2-Incubator (Heraeus) with 5% CO_2. Depending on the cell type, in 3-5 days cell monolayers became very dense and they were required to be splitted in a ratio varying 1: 3 to 1:10.

3.2.2. Cell Harvest and Split

Table 3.6. The chemical composition of PBS buffer.

PBS	NaCl	140 mM
	KCl	3 mM
	Na_2HPO_4	4 mM
	KH_2PO_4	1,5 mM
	• pH 7,0 – 7,7	
	• filter sterilized	

To split the confluent cells grew on the cell culture dish, first the medium was vacuumed, then cells were washed with sterile phosphate buffered saline (PBS) and afterwards, Trypsin/EDTA solution (Pan) was added on the cells in order to harvest them. After a few minutes of incubation at room temperature cells detached from the bottom of the dish. This cell suspension was taken up by Pasteur pipette and put into the Falcon tube containing 3-5 ml of FCS. By this way the protease activity of tyripsin was competitively inhibited by the protein content of the FCS serum and cellular proteins were prevented against further destruction. This cell suspension was centrifuged at 1500 rpm for 3-5 min at 20 °C (Hettich Rotixa 50RS, Heraeus Multifuge 3S-R). After the supernatant was vacuumed and removed, cell pellet was resuspended in DMEM in a volume required to achieve desired amount of dilution and put in the cell culture dishes (1ml cell suspention/dish) containing defined volume of DMEM with FCS and Pen/Strep. The cells prepared for transfection were counted before splitting. The dishes were incubated at 37 °C as explained before.

3.2.3. Cell Counting

After the cells were harvested and pelleted as explained in 3.2.2, these cells were resuspended in 1 ml DMEM. 50 µl of this cell suspension was added on 50 µl Trypan-blue in an eppendorf tube and mixed. One drop of this mixture was applied on *Neubauer* cell counting slide. The cells in 4 areas of 16

square quadrates were counted and the total number of the suspension was determined by the formula below.

$$\text{Cell number/ml} = \text{Counted cells} \times 2(\text{dilution factor}) \times 10^4$$

3.2.4. Transfection of Mammalian Cells

Table 3.7. The chemical composition of HeBS and $CaCl_2$ solutions.

2 x HeBS (*HEPES buffered saline*)	HEPES	11,9 g/l
	NaCl	16,4 g/l
	Na_2HPO_4	0,21 g/l
	($Na_2HPO_4 \cdot 2H_2O$)	0,26 g/l
	• pH 7,05	
	• filter sterilized	

2,5 M $CaCl_2$	$CaCl_2$	2,5 M
	• filter sterilized	

For the transfection of mammalian cells with viral DNA fragments, calcium phosphate method was used (Graham and van der Eb, 1973). First, the cells to be transfected were split onto 6-well plates 6-12 h before transfection. The desired viral DNA fragments cloned in pcDNA3 plasmids were prepared by plasmid DNA isolation and purification techniques and their concentrations were determined as described in sections 3.3.1 and 3.3.2. 1-5 µg of these DNAs were solved in dH_2O. 30 µl of CaCl and dH_2O up to a final volume of 300 µl were added on these DNAs. These mixtures were transferred into polystyrene reaction tubes (Falcon). While vortexing the tubes at very low speed, 300 µl of 2 x HeBS solution were added drop by drop to let calcium phosphate crystals form. Tubes were incubated for 20 min at room temperature to let the crystals encapsulate the DNA molecules. Meanwhile 6-well plates which were prepared previously were controlled because cells need to occupy 40-60% of one well. After 20 min, 600 µl from the mixtures were taken up by sterile micro pipettes and given onto the cells drop by drop.

After 16h of incubation, the media of the cells were changed and they were further incubated at least 24h after transfection.

3.3. DNA Techniques

3.3.1. Isolation of Plasmid DNA from *E. coli*

Table 3.8. The chemical composition of buffers used for plasmid DNA isolation.

P1 buffer	Tris/HCl, pH 8,0	50 mM
	EDTA	10 mM
	RNAse A	100 µg/ml
	• stored at 4 °C	

P2 buffer	NaOH	200 mM
	SDS	1 % (w/v)

P3 buffer	Ammonium acetate	3 M
	• stored at 4 °C	PH= 5,5

QC buffer Wash buffer	NaCl	1 M
	MOPS	50 mM
	Isopropanol	15 %
	Distilled water	

QBT buffer Equilibration buffer	NaCl	750 mM
	MOPS	50 mM
	Isopropanol	15 %
	Triton X-100	0,15 %
	Distilled water	

QF buffer Elution buffer	NaCl	1,25 M
	Tris	50 mM
	Isopropanol	15 %
	Distilled water	

In this study, plasmid isolation kits (Qiagen, Stratagene) of different sizes (mini-midi-maxi) were used to prepare plasmid DNA from *E. coli*. In order to purify DNA from bacterial culture, 100 ml of inoculated culture was incubated overnight at 37 °C in shaker incubator (New Brunswick). 50 ml Falcon tubes

were used to centrifuge the cells at 4000 rpm for 10 min at 20 °C (Rotixa 120R, Hettich). After centrifugation, supernatants were removed and pellets were combined in one Falcon after resuspending them in 4 ml of P1 (resuspension buffer) buffer. 4 ml of P2 (lysis buffer) buffer was then added on the resuspended culture, mixed carefully and incubated in water bath for 5 min at 37 °C to lyze the cells completely. Afterwards, 4 ml ice-cold P3 buffer was added on the lysate, mixed carefully and kept on ice for 2-3 min. To clear the lysate, the mixture was centrifuged at 4000 rpm for 30 min at 4 °C. Meanwhile maxi or midi columns were prepared and treated with 2,5 ml QBT (equilibration buffer). After centrifugation, the supernatants between two pellets were applied to the columns and waited till the liquid drain out. Then, the columns were washed immediately without letting the surface get dry, with 5 ml QC (wash buffer) and waited to drain out. This step was repeated two more times. In order to detach the plasmid DNA from filters, 15 ml Falcon tubes were put under the columns and 5 ml QF (elution buffer) was poured on the filters and waited till the DNA in QF gathered in the tube. After drainage, 0,8 volume isopropanol was added on the DNA solution and centrifuged (Hettich Rotixa 50RS, Heraeus Multifuge 3S-R) at 4000 rpm for 30 min at 4 °C to precipitate DNA. After centrifugation, isopropanol was removed, 600 µl, 70 % ethanol was added on the DNA precipitate and the final mixture was centrifuged again at 4000 rpm for 15 min at 4°C. After centrifugation ethanol was removed and the DNA pellet was dried out by vacuumed centrifuge. Pellets were afterwards solved in 50-100 µl 10 mM Tris solutions. The concentrations of these DNA solutions and their purity were then determined. The DNA preparations were kept at 4 °C.

3.3.2. Determination of the DNA Concentration

The concentration of the prepared DNA was determined by the help of UV absorption spectrophotometers (Uvikon Spektralfotometer 930, Kontron and SmartSpec^{TM} 3000 Spectrophotometer, Bio-Rad) at 260 nm wavelength. DNA sample was diluted 20 times in dH_2O and transferred into the quartz

cuvette (1 cm thick). The cuvettes were put into a UV-spectrophotometer scanning wavelengths from 230 nm to 300 nm. Double stranded DNA gives maximum absorption at 260 nm and 1 absorption unit, OD (stands for optical density) shows a DNA concentration of 50 µg/ml (Sambrook et al., 1989).

$$\text{DNA } \mu g/\mu l = \text{OD} \times \frac{50 \times 20}{1000}$$

By this method, the purity of the DNA preparation can also be determined. OD at 260 nm/OD at 280 nm ratio of a DNA solution should be higher than the value of 1,8 for a pure DNA solution (Sambrook et al., 1989).

3.3.3. Agarose Gel Electrophoresis

Table 3.9. The chemical composition of buffers and solutions used for agarose gel electrophoresis.

50 x TAE	Tris/HCl, pH 7,8	2 M
	NaOAc	0,25 M
	EDTA	50 mM

5 x TBE	Tris/HCl, pH 7,8	2 M
	Borate	0,25 M
	EDTA	50 mM

Loading buffer	Bromphenol blue	0,25 % (w/v)
	Xylencyanol	0,25 % (w/v)
	Glycerin	50 % (v/v)
	50 x TAE	2 % (v/v)

Ethidiumbromid-solution	Ethidiumbromid	10 mg/ml
	• stored at 4 °C, in dark	

The investigation of DNA samples by agarose gel electrophoresis is based on the relative speeds of motion of the DNA molecules in an electrical field as a function of their fragment length. To prepare an agarose gel, appropriate quantity (0,6-1 %) of Agarose (Seakem® LE agarose, FMC Bioproducts) was solved in 1 x TAE buffer by boiling the mixture up in a microwave oven for 5 min (Moulinex) and mixed with 50 ng/ml ethidium bromide solution which stains DNA and makes it visible under UV light. The cooled down agarose gel solution was then poured into a gel tray with a comb of appropriate size affixed. When the gel was completely polymerized, 1 x TBE buffer was added on the gel and the comb was removed to form the wells. The DNA samples were loaded into the wells of solidified agarose gel with 1/10 volume of loading buffer. DNA size markers *(Gibco BRL)* were also loaded. Separation of the fragments took place by running the gel with a tension of 5-10 V/cm for several hours depending on the length of the gel tray and the size of the DNA used. Analytical gels were visualized on a UV transmitted light screen (Bachofer) at a wavelength of 312 nm and documented with a gel documentation system (*gel print 2000i*, MWG Biotech). Preparative gels (gels that are prepared if the isolation of DNA from the gel is necessary) were detected with UV light of longer wavelength (365 Nm) for the indulgence of the DNA, and appropriate DNA bands are cut out.

3.3.4. Isolation of DNA from Agarose Gel

The isolation of DNA fragments from Agarose gel was performed with the help of *Qiagen gel extraction kit* (Qiagen). First the DNA bands were cut out by a scalpel under the UV-light (365 Nm) and placed into eppendorf tubes. Then, the procedure of the manufacturer was followed. The concentration of the isolated DNA was measured after preparation.

3.3.5. Polymerase Chain Reaction (PCR)

Table 3.10. DNA primers used in this study and their sequences.

PRIMER	SEQUENCE	PURPOSE
E4orf3 fw	CGC TGC TTG AGG CTG AAG GTG GAG GGC GC	Amplification of E4orf3
E4orf3 rev	CCA AAA GAT TAT CCA AAA CCT CAA AAT GAA G	Amplification of E4orf3
E4orf6 fw	CAC GGA TCC ATG ACT ACG TCC GG	Amplification of E4orf6
E4orf6 rev	CGC GAA TTC GTC GAC GCG CGA ATA AAC TGC TGC	Amplification of E4orf6
E1B-55 kDa fw	GGA GCG AAG AAA CCC ATC TGA GCG GGG GGT ACC	Amplification of E1B-55 kDa
E1B-55 kDa rev	GCC AAG CAC CCC CGG CCA CAT ATT TAT CAT GC	Amplification of E1B-55 kDa
pQE-70 fw	CGG ATA ACA ATT TCA CAC AG	5'-Sequencing of PQE-70-Insertion
pQE-70 rev	GCG TCT CAG GAT AGC AGG CGC CAT TTT AGG ACG G	5'-Sequencing of PQE-70-Insertion
pGEX fw	GGG CTG GCA AGC CAC GTT TGG TG	5'-Sequencing of pGEX-Insertion
pGEX rev	CCG GGA GCT GCA TGT GTC AGA GG	3'- Sequencing of pGEX-Insertion
E4orf3 L103A fw	CCC GAA AAG TGC CAC CTG	Mutagenesis of E4orf3 wt
E4orf3 L103A rev	GGT CAT TAC TGG AGT CTT G	Mutagenesis of E4orf3 wt
E4orf3 E99 Stop fw	CCC GAA AAG TGC CAC CTG	Mutagenesis of E4orf3 wt
E4orf3 E99 Stop rev	GGT CAT TAC TGG AGT CTT G	Mutagenesis of E4orf3 wt

The polymerase nuclear chain reaction (polymerase chain reaction = PCR; Saiki et al., 1988) is a procedure for the amplification of defined nucleic acid sequences *in vitro*, where the specificity of the reaction is guaranteed by the primer oligonucleotides used. For a 50 µl standard reaction, 0,01-1 µg DNA and 0.2 µM desired oligonucleotides (forward and reverse primers), 1 µl dATP, dTTP, dCTP and dGTP, 5 µl 10 x PCR buffer and 0.5 µl *Taq* DNA polymerase (5 U/µl; Roche) enzyme were mixed. This mixture was incubated in thin-walled 0.2 ml reaction containers (Biozym) in a Thermocycler (GeneAmpTM PCR system 9700, Perkin-Elmer) and processed successively as followed: denaturation at 95°C, hybridization (annealing) at 55-70 °C and polymerization at 68-72 °C. Following 20-30 cycles and after the final round the solution was kept at 4 °C. After PCR amplification 5 µl amplified DNA was run electrophoretically in an agarose gel and examined under UV light regarding fragment size and yield. Suitable DNA bands were cut out of the gel and cleaned for sequencing or other processes.

3.3.6. Site-Directed Mutagenesis

In this study, all mutations on Ad5 E4orf3 DNA were done by using *QuickChange™ Site-Directed Mutagenesis Kit* (Stratagene) as reported by the manufacturer. By this method it's possible and easy to mutate one or two defined bases on a desired DNA fragment. First a primer oligonucleotide with a length of 25-45 bases was designed as follows: the Tm value of this oligonucleotide must be higher than 78 °C, the GC content of it must be at least 40 % of its sequence, the altered base/bases must stand approximately in the middle and the oligonucleotide should start and end up with a G or C base.

In this study, designed primers were supplied by Metabion and they were diluted in 10 mM Tris solution to have a final concentration of 125 ng/µl. DNA fragments to be mutated were amplified by PCR which was performed with these primers containing the desired mutations. Therefore, during the

amplification of DNA fragments, they were mutated as well. PCR mixture was prepared in a 0,2 ml PCR tube (Biozym) with 5µl 10x reaction buffer, 1 µl forward and reverse primers, 1 µl dNTPmix (deoxynucleotide triphosphates; dATP, dGTP, dCTP, dTTP; supplied within the kit) and the DNA fragment to be mutated. The reaction tube was filled with dH_2O up to a final volume of 50 µl. Finally 1µl *pFU Turbo DNA Polimerase* was added. Contents were mixed by gentle shaking and spinned down by a desk top centrifuge (Eppendorf desk centrifuge, 5417) and placed in a thermocycler *(GeneAmp™PCR System 9700,* Perkin-Elmer*)*. PCR program for 15 cycles (denaturation at 95 °C, hybridization at 55 °C and polymerization at 72 °C) was performed.

Amplified DNA products were then investigated by agarose gel electrophoresis to confirm if the PCR was successful. Then, in order to digest the template DNA, DPN-I restriction digest was done. DPN-I is a restriction enzyme digesting only the metylated DNA. Therefore, it can only digest the methylated template DNA isolated from an organism but not the DNA prepared by PCR *in vitro*. For the reaction, 50 µl of PCR product and 1 µl of DPN-I is mixed. Reaction took place at 37 °C for at least 2h of incubation. Resulting mutant plasmids were then transferred into bacteria by heath shock transformation or electroporation as explained before (section 3.1.2 and 3.1.3).

3.3.7. Cloning

3.3.7.1. Restriction Digestion of the DNA

Restriction enzymes used in this study and their appropriate 10 x reaction buffers were supplied by New England Biolabs, Boehringer Mannheim and Roche and used in accordance with the data of the manufacturer. For analytic digestion usually 0,5-1 µg DNA and 3-10 U restriction enzyme were used and if not differently indicated, the mixture was incubated at 37 °C for 1h. For preparative restriction digest, 5-20 µg DNA and 50 U enzyme were

used and incubated at 37 °C for at least 2h. PCR products were cleaned and concentrated by isopropanol precipitation to get rid off interfering oligonucleotides and dNTPs before restriction digest for cloning. When necessary, the restricted fragments were seperated by agarose gel electrophoresis and isolated by gel extraction as explained before (section 3.3.4).

3.3.7.2. Ligation and Transformation

After restriction digest linearized vector DNAs were directly reacted with 1 U of Alkaline Phosphatase (SAP, shrimp alkaline phosphatase, Boehringer Mannheim) for 30 min at 37 °C and dephosphorylated in order to prevent religation of the vector onto itself. DNA fragments were cleaned up by isopropanol precipitation as explained before (section 3.3.1) and their concentrations were measured. A standard ligation mixture was prepared with 100 ng vector DNA, 100-500 ng backbone DNA (fragment to be ligated), 2 µl 10 x ligation buffer and 1 U T4-DNA-Ligase (Boehringer Mannheim). For the covalent binding of phosphodiester bridges, mixture was incubated overnight at 13°C. Afterwards, ligated plasmid DNAs were transformed into *E. coli* as explained before.

3.3.7.3. Identification of Recombinant Clones

Transformed *E. coli* cells with ligated recombinant DNAs were inoculated on agar plates containing LB medium with appropriate antibiotics and incubated overnight. Single colonies from these plates were chosen and inoculated in liquid LB medium with suitable antibiotics and incubated overnight again at 37 °C at 220 rpm. Afterwards, plasmid mini-preparations were done as explained before (section 3.3.1) from these cultures and isolated DNAs were dissolved in 50 µl dH_2O. 5 µl of each sample were digested with suitable restriction enzymes and restricted products were then loaded on an agarose gel to analyze the ligation efficiency and accuracy. The plasmid DNA of the

recombinant clones that were containing cloned DNA in right orientation were further examined by DNA sequencing. To check whether the cloned DNA had the desired mutation and no other mutations in the reading frame, the sequence of the original DNA was compared to the mutated DNA sequence by BLAST software of NCBI (www.ncbi.nih.gov/blast, 2004). The right clones were stored as glycerin cultures.

3.3.8. DNA Sequencing

In this study, DNA sequencing was performed by Geneart. Mutated DNAs must be controlled both by agarose gel electrophoresis and also by sequencing. DNA which was mutated, produced in *E. coli,* and purified was put through a PCR mixture with appropriate primers and DNA sequencing was performed by an automated DNA sequencing device. 1 µg recombinant DNA, 10 pmol Ad5 E4 ORF3 forward (fw) primer (primer on the reading frame) and dH_2O up to a final volume of 8 µl were mixed in 0,2 PCR tube (Biozym). Same mixture was prepared again with also 10 pmol Ad5 E4 ORF3 reverse (rev) primer. Other procedures were performed by the company.

3.4. Protein Techniques

3.4.1. Expression of Recombinant Proteins in E. coli

In this study, expression of recombinant proteins in *E. coli* was accomplished by fusing the target proteins with Glutathione-S-Transferase (GST) from *Schistosoma japonicum* (Smith et al., 1986). The GST portion of the fusion protein, so called “GST-tag”, allows the foreign polypeptides to be purified rapidly under nondenaturating conditions from crude cellular lysates. pGEX vectors were designed for this purpose by Smith and Johnson (Smith and Johnson, 1988). pGEX plasmids provide a multiple cloning site for fusing a gene of interest to the C-terminal of GST. GST is a 26 kDa enzyme that binds reversibly and with high affinity to glutathione-containing affinity

matrices such as glutathione coupled sepharose beads. Therefore, the system allows the rapid purification of the target protein by affinity chromatography. Once purified, the protein of interest can also be cleaved away from GST since the pGEX vectors provide a proteolytic cleavage site, such as thrombin, between GST and the target protein (Frangioni et al., 1992). In this study, one of the basic pGEX plasmid vectors, pGEX-2TK (Amersham Biosciences) was used. Fig. 3.1 shows the map of the pGEX-2TK vector. The kinase site that is present in the vector allows the detection of the expressed protein by direct labeling of the fusion products *in vitro* by phosphorilation. Another feature of this pGEX plasmid is, the expression of the fusion protein is under the control of *lac* promoter. This promoter is adjusted by the *lac* operator which binds to the *lacI*^{q} gene product (*lac* repressor) during normal bacterial growth and blocks the transcription of the target gene. Toxic effects of the recombinant gene products which lead to the reduced cell growth and instability of the expression vector can be prevented by this expression control. The pGEX-2TK (Amersham Biosciences) possesses the *trp-lac*-hybrid promoter named as *tac*, which is likewise adjusted by the *lac* repressor. By chemical induction with the lactose analogue Isopropylthio-β-D-galactosid (IPTG) the interaction of the *lac* repressor with its target sequence can be prevented and by this way, expression of the plasmid-coded genes are induced.

Expression of the target protein was started by inoculating a 10 ml of LB medium containing appropriate antibiotics with the recombinant *E. coli* strain. The culture was grown overnight at 37 °C with shaking in a 220 rpm incubator-shaker (New Brunswick). 2 ml of this overnight culture was then transferred into a 100 ml of fresh, pre-warmed medium and grew until the OD_{600} of the culture reached 0,6. 1 ml sample was taken from the culture at this step in order to check whether a “leaky” expression took place before induction. For inducing protein expression, 1 mM IPTG was added to the culture and incubation continued for 4h at 37 °C. Alternatively, 0,1 mM IPTG for induction and 30 °C growth temperature after induction were used for

avoiding the formation of insoluble inclusion bodies (Smith and Corcoran, 1998). Another sample of 1 ml was taken from the 4h induced culture to monitor the level of expression after induction. Afterwards, bacterial culture was harvested by centrifugation (6000 rpm, 10 min, 4 °C; Centrikon T-124, Kontron of instrument) and the pellet was washed with PBS. If necessary, the cells were stored in PBS containing protease inhibitor (*complete™, EDTA-free*, Boehringer Mannheim) at -80 °C. The samples taken before and after induction was centrifuged and the pelleted cells were resuspended in 50 µl SDS-PAGE sample buffer and stored at -20 °C until SDS-PAGE analysis.

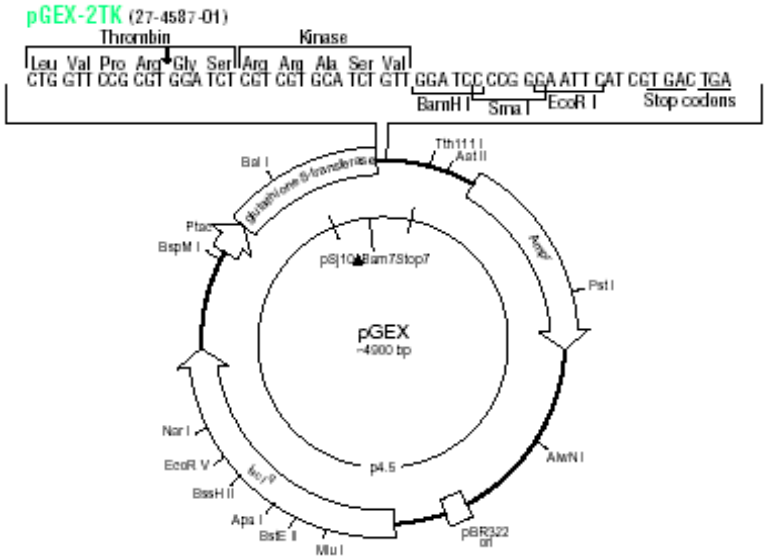


Figure 3.1. Map of GST fusion vectors.

3.4.1.1. Purification of GST Fusion Proteins under Native Conditions

Table 3.11. The chemical composition of elution buffer.

Elution Buffer	Tris/HCl, pH 8,0	50 mM
	NaCl	150 mM
	Reduced glutathione	10 mM
	• stored at -20 °C	

Bacterial expression cultures prepared as described in the previous section (section 3.4.1) were thawed on ice. Then, the cells were lyzed by the addition of 1 mg lysozyme (Sigma), 100 µl DNAse I (1 mg/ml; Sigma), 130 µl $MgCl_2$ and 13 µl $MnCl_2$ (both 1M) per 10 ml of cell suspension. The suspension was incubated for 10 min on ice. Afterwards, in order to obtain a complete cell extract, the suspension was sonicated three times with ultrasound (1 min, output 40, 0,5 Impulse/sec; Branson Sonifier 450) on ice. After sonication, appropriate amounts of 5 M NaCl (to a final concentration of 0,5 M) and 20 % Triton X-100 (to a final concentration of 1 %) were added to the lysate. For the precipitation of the insoluble cell parts, the lysate was centrifuged at 15000 rpm for 30 min at 4 °C (Centrikon T-124, Kontron of instrument) and the supernatant was transferred to a fresh 15 ml falcon tube. The pellet remained was directly resuspended in SDS loading buffer or in 1 ml PBS or in 1 ml of 0,25 % Tween 20, 0,1 mM EGTA buffer. 1 ml sample was also taken from the supernatant. These samples were kept at -20 °C to be analyzed by SDS-PAGE in order to determine whether the protein of interest was in the soluble or insoluble portion of the lysate.

GST fusion proteins were purified from the lysate under nondenaturating conditions by GST affinity chromatography using glutathione immobilized to a matrix such as Sepharose. Batch purification using Glutathione Sepharose 4B (Amersham Biosciences) was performed. First, Glutathione Sepharose 4B beads supplied as 75 % slurry was prepared to give 50 % slurry in PBS according to the instructions of the supplier. 1 ml of the 50 % slurry of Glutathione Sepharose 4B (0,5 bed volume) was added to the supernatant obtained from the lysate of 100 ml culture. The mixture was incubated for 1 h at 4 °C in an overhead incubator (GFL, Society for Laboratory Technology). Afterwards, the mixture was centrifuged at 500 g for 5 min at 4 °C (Rotixa 120R, Hettich) and the pellet was washed three times with 5 ml PBS with protease inhibitors (*complete*™ Edta-free, Boehringer Mannheim). 1 ml sample was taken from both the first flow-through containing the nonbound proteins and the supernatant of each washing steps for SDS-PAGE analysis.

After the last washing step the proteins were either eluted from Glutathione Sepharose 4B or resuspended and kept in 500 µl PBS for GST - pulldown assay. The elution of the fusion proteins were achieved by adding 750 µl elution buffer to the pellet and incubating for 10 min in overhead incubator. The elution step was repeated three times and a sample of 75 µl was taken from each eluate for the detection of the purified proteins by SDS-PAGE analysis. Alternatively, the protein of interest without the GST-tag was eluted from Glutathione Sepharose 4B by thrombin cleavage. For this purpose, 40 µl of thrombin (1 unit/µl, Amersham Biosciences) was mixed with 460 µl of PBS and loaded on the pellet of 0,5 bed volume of fusion protein bound Glutathione Sepharose 4B after the last washing step. The mixture was incubated at room temperature for 16 h. After incubation, the suspension was centrifuged and the supernatant containing the eluted protein and thrombin was transferred into a new tube. The pellet was also resuspended in 500 µl PBS. 50 µl of sample was taken from both the eluate and Glutathione Sepharose for SDS-PAGE analysis.

3.4.1.2. Purification of Proteins under Denaturating Conditions

Table 3.12. The chemical composition of buffers used for protein purification under denaturating conditions.

Homogenization Buffer	Tris/HCl, pH 7,0	100 mM
	EDTA	1 mM

Triton X-100 - EDTA Buffer	Triton X-100	6 %
	EDTA	60 mM
	NaCl	1,5 M
	• pH 7,0	

TE Buffer	Tris/HCl, pH 7,0	100 mM
	EDTA	20 mM

Urea Buffer	Urea	8 M
	Tris/HCl, pH 8,0	100 mM
	EDTA	1 mM
	DTT	100mM

The high-level expression of many proteins can lead to the formation of inclusion bodies, very dense aggregates of insoluble protein and RNA that usually contain most of the expressed protein (Schein, 1989). Precipitation of a protein into inclusion bodies sometimes can be advantageous because inclusion bodies are insoluble and dense, and can be purified relatively easily by centrifugation.

The target proteins were expressed in *E. coli* after induction with 1 mM IPTG and at 37 °C as described in section 3.4.1 were thawed on ice and pelleted by centrifugation (6000 rpm, 10 min, 4 °C; Centrikon T-124, Kontron of instrument). Then, the cells were homogenized in 25 ml of homogenization buffer by vortexing at 4 °C. 1 mg of lysozyme, 250 µl DNAse I (1 mg/ml), 300 µl $MgCl_2$ and 30 µl $MnCl_2$ (both 1M) was added to the homogenate and the mixture was incubated for 30 min at 4 °C. Afterwards, the suspension was sonicated three times with ultrasound (1 min, output 40, 0,5 Impulse/sec; Branson Sonifier 450) on ice and 0,5 volume of Triton X-100 - EDTA buffer was added. After incubation for 30 min at 4 °C, the inclusion bodies (IB) were spinned down by centrifugation at 20000 rpm for 10 min at 4 °C (Centrikon T-124, Kontron of instrument). The IB pellet obtained was washed with 40 ml TE buffer and stored at -20 °C if necessary.

In order to solubilize the inclusion bodies, IB pellet was resuspended in urea buffer and incubated for 2 h at 25 °C. The pH was lowered by the addition of 1 M HCl and the insoluble cell debris was removed by centrifugation (10000 g, 10 min; Centrikon T-124, Kontron of instrument). DTT present in the urea buffer was competely removed by dialyzing the solubilized proteins at 4 °C overnight against 1 L of 6M urea.

Refolding of the denatured proteins in solubilized IBs was performed by dilution. 0,5 ml of solubilized proteins was taken and 9,5 ml of PBS pre-chilled on ice was immediately added. The mixture was first incubated on ice for 15 min and then for 45 min at room temperature. Afterwards, the proteins

that were not folded properly was pelleted by centrifugation at 35000 rpm for 1,5 h at 15 °C (Centrikon T-124, Kontron of instrument; SW 41 rotor).

3.4.1.3. Solubilization and Purification of GST Fusion Proteins

Table 3.13. The chemical composition of STE-buffer

STE-Buffer	Tris/HCl, pH 8,0	10 mM
	NaCl	150 mM
	EDTA	1 mM

In the original description of the pGEX system, it was suggested that most GST fusion proteins would be soluble (Smith and Johnson, 1988). In practice, a major limitation of this system is the fact that many GST fusion proteins are partially or completely insoluble after lysis in nonionic detergents and need to be solubilized by ionic detergents (Frangioni and Neel, 1993).

For the solubilization of GST fusion proteins, bacterial expression cultures were grown as described in section 3.4.1. The cells were pelleted by centrifugation at 7000 g for 7 min at 4 °C (Centrikon T-124, Kontron of instrument). The pellet was then resuspended in 4,5 ml of STE buffer containing 100 µg/ml lysozyme and incubated 15 min on ice. Afterwards, the cells were lyzed by the addition of DTT to a final concentration of 5 mM and *N*-laurylsarcosine (sarkosyl; Roche) to a final concentration of 1,5 %. The cell lysis was completed by sonication (1 min on ice; output 40, 0,5 Impulse/sec; Branson Sonifier 450). The lysate was centrifuged at 10000 g for 5 min at 4 °C (Centrikon T-124, Kontron of instrument; SS-34 rotor) and the supernatant was taken into another tube. In order to sequester sarkosyl, Triton X-100 was added to the suspension to give 2 % final concentration. Then, the mixture was filtered through 0,45 µm pore sized micro-filters (Amicon) and the solution obtained was used for the batch purification of GST fusion proteins as described in section 3.4.1.1.

3.4.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Table 3.14. The chemical composition of solutions and buffers used for SDS-PAGE.

30 % Acrylamide - Stock solution	Acrylamide	29 % (w/v)
	N, N'Methylenbisacrylamide	1 % (w/v)

Stacking Gel (5 %)	Acrylamide - Stock solution	17 % (v/v)
	Tris/HCl, pH 6,8	120 mM
	SDS	0,1 % (w/v)
	APS	0,1 % (w/v)
	TEMED	0,1 % (v/v)

Seperating Gel (8 %)	Acrylamide - Stock solution	27 % (v/v)
	Tris/HCl, pH 8,8	250 mM
	SDS	0,1 % (w/v)
	APS	0,1 % (w/v)
	TEMED	0,04 % (v/v)

Seperating Gel (12 %)	Acrylamide - Stock solution	40 % (v/v)
	Tris/HCl, pH 8,8	250 mM
	SDS	0,1 % (w/v)
	APS	0,1 % (w/v)
	TEMED	0,04 % (v/v)

Seperating Gel (15 %)	Acrylamide - Stock solution	50 % (v/v)
	Tris/HCl, pH 8,8	250 mM
	SDS	0,1 % (w/v)
	APS	0,1 % (w/v)
	TEMED	0,04 % (v/v)

TGS-Buffer	Tris	25 mM
	Glycin	200 mM
	SDS	0,1 % (w/v)

SDS- Loading Buffer	Tris/HCl, pH 6,8	100 mM
	SDS	4 % (w/v)
	DTT	200 mM
	Bromophenol blue	0,2 % (w/v)
	Glycerin	20 %

SDS-PAGE is used for isolating the proteins electophoretically according to their molecular weights in the presence of denaturating SDS detergent. Negatively charged SDS molecules compensate positive charges of denatured proteins and all of the proteins in the gel move through anode

under the voltage applied. The isolation of proteins according to their molecular weights take place in the pores of the polyacrylamide gel since differential velocities of proteins are determined only by their sizes. The quality of protein isolation by this method was increased by Laemmli procedures (Laemmli, 1970) using and intermittent buffer system which concentrates proteins first in a lower percentage collecting or stacking gel and let proteins start migration together before separation. Then, the isolation take place in separating gel.

The compositions of the gel solutions used in this study are given above (Harlow and Lane, 1988). The gel equipments were applied according to the manufacturer. Protein samples were mixed with 2 x SDS Probe buffer (Sambrook et al., 1989) before loading on the gel and denaturated in a thermal block (Eppendorf thermal mixer comfort) by incubating for 5 min at 95 °C. Electrophoresis took place by applying 20 mA/gel volume (ml) in TGS buffer and continued until bromphenol blue band reached to the end of the gel. Following SDS Polyacrylamid Gel Electrophoresis, isolated proteins could be detected by either Coomassie Blue staining or Western Blot immune staining.

3.4.2.1. Staining of Polyacrylamid Gel

For the detection of the proteins on the polyacrylamid gel after electrophoresis colloidal Coomassie System Deep Blue (Ener-Gene) with a sensitivity of around 100 ng was applied as the instructions of the manufacturer. For documentation, gels were dried afterwards on Whatman filters in vacuum dryer (Drystar, H. Hoelzel) for 2h at 70 °C and welded into plastic foil.

3.4.3. Western Blot

Table 3.15. The chemical compositions of Towbin buffer and Ponceau S solution

***Towbin*-Buffer**	Tris/HCl, pH 8,3	25 mM
	Glycin	200 mM
	SDS	0,05 % (w/v)
	Methanol	20 % (v/v)

Ponceau S	Ponceau S	0,2 % (w/v)
	Trichloric acid	3 % (w/v)
	Sulfosalicylic acid	3 % (w/v)

Western blot procedure is carried out to transfer the proteins on nitrocellulose membranes from polyacrylamid gels. By this way, electrophoretically isolated proteins are immobilized on nitrocellulose membranes (Protran, Schleicher & Schuell), which is required for immunological investigations. The transfer took place by the help of a *Trans Blotâ Electrophoretic Transfer Cell* (Bio-Rad) in Towbin buffers with an amper of 400 mA for 80 min. The instructions of the manufacturer about handling the equipment were followed. Subsequently, the proteins transferred on membranes were stained reversibly by *Ponceau S* (Sigma) which was colorizing the protein bands and let the protein and the marker bands marked. Then, the membranes were washed with water before immune staining.

3.4.4. Immune Staining of the Membranes

Before the incubation of the nitrocellulose membranes with specific antibodies, free binding areas of the membranes were saturated by incubating them in 5 % milk powder in PBS for at least 1h at room temperature or overnight at 4 °C. Afterwards, the membranes were washed two times with PBS containing 0,1 % Tween 20 (PBS/Tween) and one time with PBS alone for 5 min each. Subsequently, primary antibodies diluted in

suitable concentrations with PBS plus 1 % milk powder solution were applied on membranes for 1 h at room temperature on a middle speed shaker. After binding of the primary antibodies the membranes were washed again two times with PBS/Tween and one time PBS alone and incubated with horseradish peroxidase (HRP) coupled secondary antibodies (anti-rat, or -rabbit; Amersham Biosciences) which were diluted 1 : 5000 in PBS plus 1 % milk powder for 1h at room temperature on shaker. After three further washing steps with PBS/Tween, nitrocellulose membranes were treated with chemiluminescent substrates for the HRP reaction and color formation. For this specific purpose a commercial kit, *Super Signal West Pico Chemiluminiscent Substrates* (Pierce), was used. This procedure was based on the enhanced chemiluminiscence formation with the participation of HRP and represents a highly sensitive protein detection system. In this system, cyclic Diacylhydrazid Luminol coupled to the anti-immunglobulin antibody is oxidized by peroxidase under alkaline conditions which leas to a maximum light emission at 428 nm. Chemical amplifiers like phenol increase the light intensity approximately 1000 fold and extend the period of the emission. By this way, a maximum intensity is obtained 5-20 min after using the chemicals followed by a reduction of the radiation with a half-life of 1h. For this process, two detection solutions supplied within the kit were mixed as 1:1 ratio just before using and the mixture was applied to the membranes after the last washing step with PBS/Tween in a quantity of approximately 0.125 ml/cm^2. After 5 min of incubation, nitrocellulose filters were dripped off briefly and packed with stretch-film. The autoradiographic detection of the signals was performed by *ChemiDoc System* (Bio-Rad) under exposition lasting 20 sec to 20 min.

3.4.5. Radioactive Labeling after *In vitro* Transcription/Translation

For the coupled transcription and translation of cloned DNA *in vitro*, *TNT T7-coupled Reticulocyte Lysate system* (Promega) was used according to the instructions of the manufacturer. For labeling the proteins, S^{35}-labeled-

Methionine (50 µCi; Roche) was added to the lysate. After the reaction, in order to check whether the protein of interest was expressed and radioactively labeled, 5 µl of sample was examined by SDS-PAGE. Afterwards, the gel was incubated for signal detection for 30 min in *Enlight* (Ener-Gene) and dried on Whatman™ filters in vacuum-dryer (Drystar, H. Hoelzel) for 2h at 70°C. The dried gel was incubated with the phosphor screen in a dark cassette (Bio-Rad). Subsequently the radiographic detection of the radioactively-labeled proteins was done by *Personal Molecular Imaging FX System* (Bio-Rad).

3.4.6. Preparation of Total Cell Extract

Table 3.16. The chemical composition of NP-40 buffer.

NP-40 Buffer	Tris/HCl, pH 8,0	50 mM
	NaCl	150 mM
	EDTA	5 mM
	Nonidet P-40	0,15 % (v/v)

Harvested mammalian cell pellets were resuspended directly in an appropriate quantity of pre-cooled NP-40 buffer and incubated for 30-60 min on ice. In order to obtain a complete cell extraction, the cell lysate were treated with ultrasound as explained before (section 3.4.1.1). Then, these cell lysates were transferred into eppendorf tubes and centrifuged at 12,000 rpm for 10 min at 4 °C (Eppendorf centrifuge 5417R) to separate insoluble cell portions. Afterwards, supernatants were transferred into new eppendorf tubes for further experiments. Before using these protein extracts for experiments their concentrations must be determined.

3.4.7. Quantitative Determination of Protein Concentration

The concentration of soluble proteins in a sample was determined by the help of *Bio-Rad Protein Assay*. The assay is based on protein quantification

procedure of Bradford (Bradford, 1976) and measures the increase in the absorption at 595 nm by using a spectrophotometer (Bio-Rad) after the proteins in the sample bound to the chromogene substrate of the system. Different dilutions of calibration protein, bovine serum albumin (BSA; Sigma), varying from 1 to 20 µg were mixed with 200µl coloring reagent. The mixture was filled with dH_2O up to a final volume of 1 ml in polystyrene cuvettes (Sarstedt). Then, samples were prepared by mixing 1 µl of sample plus 200µl of coloring reagent plus 800µl of dH_2O in cuvettes and incubated for 5-30 min at room temperature. Subsequently the absorptions were determined spectrophotometrically at 595 nm against blank solution (800 µl dH_2O plus 200 µl coloring reagent). According to the calibration curve obtained from different dilutions of BSA, the protein concentrations of the samples were calculated.

3.4.8. GST - Pulldown Assay

Table 3.17. The chemical composition of Dignam buffer.

NETN-40 buffer	Tris/HCl, pH 8,0	20 mM
	NaCl	100 mM
	EDTA	1 mM
	NP-40	0,5 % (v/v)

GST - pulldown is a technique which is used to analyze protein-protein reciprocal effects *in vitro*. First the quantities of the fusion proteins bound to Glutathione Sepharose 4B were obtained by analyzing the stained SDS-PAGE gel with *QuantityOne software* (Bio-Rad). Afterwards, same quantities of proteins were centrifuged (7000 rpm, 5 min, 4 °C; Eppendorf table centrifuge, Eppendorf) and washed with NETN-40 buffer. Then, the pellet was resuspended in 300 µl NETN-40 buffer with a defined quantity of radioactively labeled desired protein which was determined before likewise with SDS-PAGE and following autoradiography. This mixture was then incubated for 2h at 4 °C on a turning rotor (GFL, society for laboratory

technology). The proteins bound to the Glutathione Sepharose subsequently precipitated by centrifugation (6500 rpm, 5 min, 4 °C; Bio joint fresco, Heraeus of instrument), three times washed with NETN-40 buffer, centrifuged and boiled up in 25 µl SDS Probe buffer as explained before. The protein samples were then isolated in SDS-PAGE. Afterwards, the gel was incubated for signal detection for 30 min in *Enlight* (Ener-Gene) and dried for 2h on Whatman™ filters in vacuum-dryer (Drystar, H. Hoelzel) at 70 °C. Subsequently the autoradiographic detection of the radioactively labeled proteins took place during an overnight exposition at -20 °C or -80 °C.

3.4.9. Immunofluorescence Microscopy

3.4.9.1. Fixation with Methanol

Sterile cover glasses had been placed into 6-well dishes before the target mammalian cells were delivered and culture dishes were incubated as explained before (section 3.2.1) for at least 6h or overnight. When the cells reached the desired density they were transfected as explained in section 3.2.4 and after at least 24h their media were vacuumed and they were washed in PBS. Then, pre-cooled methanol was added on the cells and they were incubated for 20 min at -20 °C. Subsequently, methanol was removed and the cover glasses were dried at room temperature. The cells fixed by this way could either be stored at -20 °C for a few weeks or used immediately for immune staining.

3.4.9.2. Immune Staining

Table 3.18. The chemical composition of PBS-Tween solution

PBS-Tween	Tween 20	0,1 % (v/v)
	• in PBS	

After fixation, the cells on cover glasses were washed with PBS for 5 min on a shaker and they were incubated in 5 % milk powder dissolved in PBS/Tween for 1 h at room temperature to eliminate nonspecific reactions with antibodies. Afterwards, they were washed 3 times for 5 min with PBS/Tween and appropriate dilutions of primary antibodies were applied on cover-glasses (30 µl per cover-glass) and they were incubated for 1h at room temperature in a dark and damp chamber in order to prevent drying up of the antibody solution. After incubation, they were washed 3 times with PBS/Tween and then secondary fluorescent-marked antibodies (FITC – anti-rat or -rabbit, Texas Red – anti-rat or –rabbit; Dianova) that were diluted 100 times in PBS plus 5 % milk powder were applied. These secondary antibody solutions were also containing 0.5 µg/ml DAPI (4', 6-Diamidin-2'-phenylindol Dihydrochlorid; stains DNA by forming chromatin complexes). After an hour, unbound secondary antibodies were removed by washing again 3 times with PBS/Tween. Then, cover-glasses were rinsed slightly and with 5 µl *Glow Mounting medium* (Ener-Gene) per cover glass they were sticked on a slide as cells look downward on the slide. The samples prepared in this way could be stored in dark at 4°C for several weeks. Further analysis of immune stained cell preparates were performed by immunefluorescent microscope (Leica) with digital image processing system.

3.4.10. FTIR Spectroscopy

3.4.10.1. Sample Preparation

The spectral analysis was carried out by using a Perkin-Elmer spectrometer equipped with a *MIR TGS* detector (*Spectrum One Instrument*, Perkin-Elmer). The relative concentration of protein samples bound to Glutathione Sepharose 4B beads was determined and the protein concentration of each sample was equalized as described in section 3.4.8. These protein samples of equalized concentration in Glutathione Sepharose 4B beads were placed between water-insoluble CaF_2 temperature dependent window with 12 µm

sample thickness produced by mylar spacers. The volume of 20 µl per sample was used for spectroscopic analysis. Spectra of the samples were recorded from 4000 – 1000 cm^{-1} in a single scan using 2 cm^{-1} resolution. The spectra of free Glutathione Sepharose 4B and buffer suspensions were also recorded under the same conditions with the protein samples. Same amounts (20 µl) of Glutathione Sepharose 4B and buffer were used for scans. Before each set of experiments, the background spectrum of air was also recorded and it was automatically subtracted from the spectra of samples by using *SpectrumOne software* (Perkin-Elmer). This step is required to overcome the atmospheric water vapor absorbance and carbon dioxide interference which overlaps the spectral region of interest. Each scan was performed in a fixed temperature of 25 °C. For this purpose, a digital temperature controller unit (Graseby Specac) was used. Samples were incubated for 5 minutes at 25 °C before data acquisition.

3.4.10.2. Data Analysis

SpectrumOne (Perkin-Elmer) and *GRAMS/32* (Galactic Industries, Salem, NH, USA) software were used for all data manipulations. The spectroscopic analysis of proteins bound to Glutathione Sepharose 4B was done in aqueous medium. Therefore, the first step of data analysis was consisting of subtracting the strong water absorption bands of buffer which may mask the bands of interest from the sample spectra (Mendelsohn and Mantsch, 1986; Jackson and Mantsch, 1996). The “difference” option of the *SpectrumOne* software was applied for subtracting the spectrum of PBS buffer from the spectra of protein and Glutathione Sepharose 4B samples both of which were present in PBS. In order to obtain the absorption bands of proteins only, resultant spectrum of Glutathione Sepharose 4B was also subtracted from the spectra of protein samples. Fig. 3.2 shows the raw spectrum of a protein sample and its form after subtraction steps. Then, the Amide I region (1600-1700 cm^{-1}) of the final spectrum was resolved into sub-bands by obtaining second derivative (power =2 and breakpoint = 5) spectrum. The baseline was

corrected before starting the fitting procedure (Boyar et al., 2004). The number and positions of the sub-bands of the derivative spectrum were used as initial parameters for curve-fitting analysis. The shapes of the underlying sub-bands were chosen as Gaussian and the frequency of each sub-band was confined to ± 2 cm^{-1} (Paschalis et al, 2001). For the curve-fitting analysis of the Amide I band, a maximum of fifty iterated optimization algorithms were done in each fit routine. The iterations were performed until the correlation was better than 0.995. The output of the analysis was expressed as peak position and relative percentage area. The peak positions corresponding to different secondary structures (α-helix, β-sheet, and so on) in proteins were compared to the previously defined band assignments (Arrondo et al., 1993; Arrondo and Goni, 1999; Severcan et al., 2001) Then, the ratio of the relative percentage areas of the spectral bands showing relative ratio of secondary structures in the protein sample was calculated. By this way, secondary structures present in the protein sample and their relative percentages were determined.

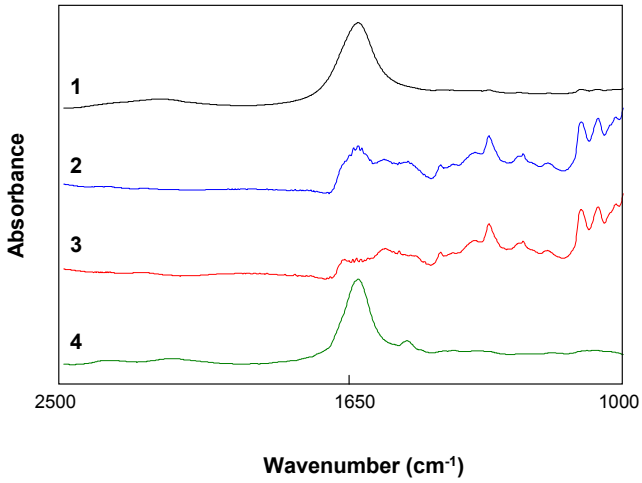


Figure 3.2. FTIR spectra of GST-E4orf3, protein, and glutathione sepharose beads. **1:** Raw FTIR spectrum of GST-E4orf3 in glutathione sepharose beads; **2:** Spectrum of buffer-subtracted GST-E4orf3; **3:** Spectrum of buffer-subtracted glutathione sepharose beads; **4:** Final protein spectrum of GST-E4orf3 obtained after subtracting the buffer and the beads.

CHAPTER IV

RESULTS

4.1. Expression and Purification of Ad5 E4orf3 Protein

In this study, full-length Ad5 E4orf3 protein was expressed as a GST fusion protein in *E. coli* and purified either by GST affinity chromatography or through inclusion body isolation. For affinity purification, the protein was expressed and purified under native conditions. On the other hand, for purification through inclusion body isolation, denaturating conditions were used. Finally, in order to obtain high amounts of GST-E4orf3 protein in soluble and native form for subsequent functional and structural studies the expression conditions were optimized and the fusion protein was solubilized for native purification.

4.1.1. Expression and Purification of E4orf3 Protein under Native Conditions

The standard protocol for isolation of GST fusion proteins works quite well for most of the fusion proteins (Smith and Corcoran, 1998). Therefore, the expression and purification of the GST-E4orf3 protein was first carried out under native conditions with no manipulation on the standard protocol, as described in sections 3.4.1 and 3.4.1.1. The *E. coli* strain, DH5α, carrying the E4orf3 expression plasmid of pGEX-2TK was grown at 37 °C before and

after induction. For inducing protein expression, 1 mM IPTG was applied. However, under these conditions, it was observed that nearly all of the GST-E4orf3 protein remained in the insoluble pellet portion of the lysate (Fig. 4.1). The protein could not be detected at flow-through, washings or eluates, which means that the expressed GST-E4orf3 protein was completely insoluble. If expression of a particular protein produces insoluble aggregates as in this case, purification under denaturating conditions or optimization of the expression conditions for soluble protein production should be performed.

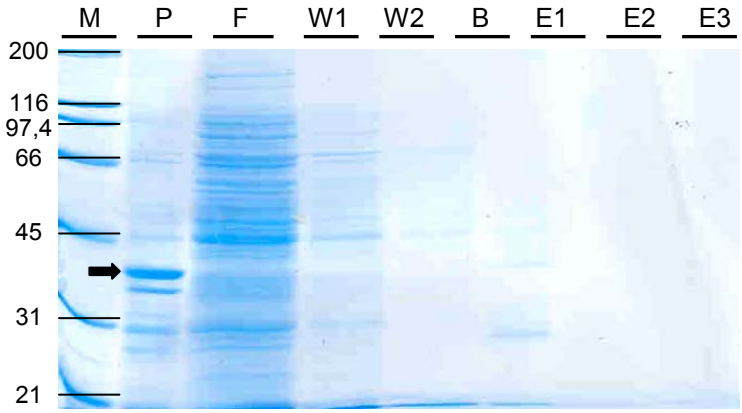


Figure 4.1. Purification of GST-E4orf3 under native conditions. The equal amounts of samples taken at the different stages of purification were analyzed by 12 % SDS-PAGE gel. The proteins were visualized by colloidal Coomassie stain. The arrow indicates the position of 37 kDa GST-E4orf3 protein. **M:** markers; **P:** pellet of lysate; **F:** flow-through; **W1 & W2:** first and second washings; **B:** glutathione sepharose beads; **E1, E2 & E3:** first, second, and third eluates.

4.1.2. Expression and Purification of E4orf3 under Denaturating Conditions

The standard protocol for isolation of GST fusion proteins failed for GST-E4orf3; therefore, the protein was purified after inclusion body isolation under denaturating conditions as described in the section 3.4.1.2. Since this purification method does not require GST affinity tag, E4orf3 protein containing no tag (wild-type E4orf3) was also expressed in *E. coli* by the help of pQE-70 (Qiagen) plasmid in which the affinity tag was removed. As a result, both GST-E4orf3 and wild-type E4orf3 proteins were purified to some extent by the isolation of the inclusion bodies (Fig. 4.2a). GST-E4orf3 protein, the expression level of which was higher when compared to that of wild-type E4orf3 in the gel, was found to be contaminated with the cellular proteins. However, the amount of both proteins in the isolated inclusion bodies was high enough for subsequent refolding experiment. Protein yields per liter culture were determined to be 10 mg for GST-E4orf3 and 4 mg for wild-type E4orf3 by Bio-Rad protein assay.

On the other hand, in addition to the high amount of proteins in the isolated inclusion bodies, it is interesting that a small portion of GST-E4orf3 was also found in the cell debris extracted with Triton X-100 – EDTA buffer (Fig.4.2b). This means that at least some portion of the GST-E4orf3 protein was not present in the insoluble inclusion bodies during the expression and if the conditions are optimized, the amount of this portion can be increased.

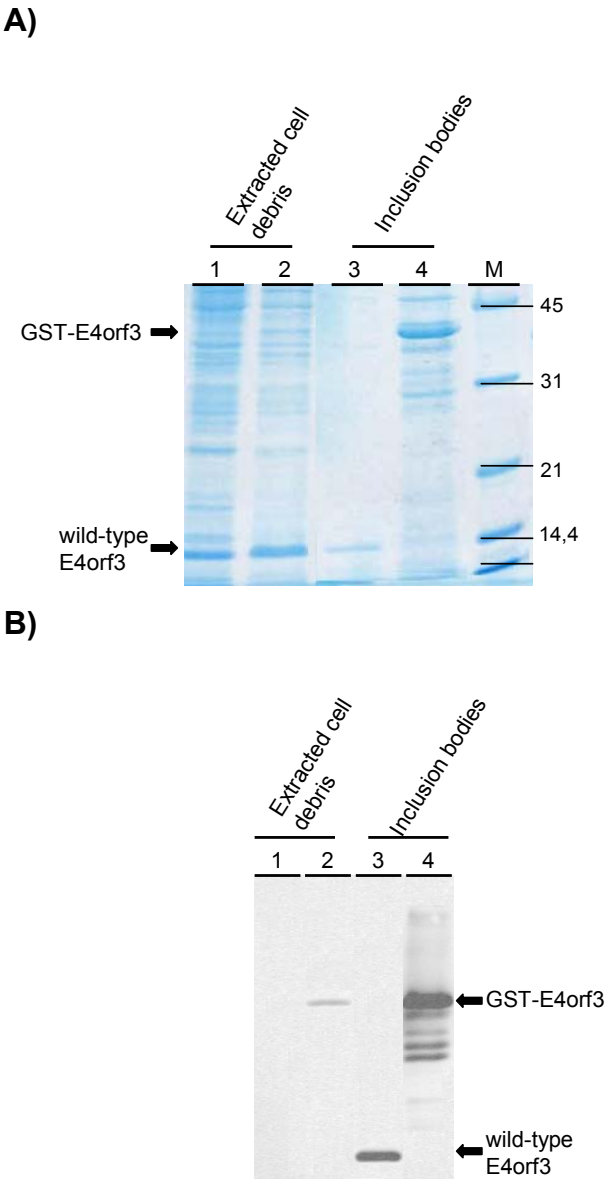


Figure 4.2. Purification of GST-E4orf3 and wild-type E4orf3 proteins under denaturating conditions. Cell debris was extracted with Triton X-100 – EDTA buffer and the remaining inclusion bodies were solubilized in 8 M urea. The proteins were electrophoresed on a 15 % SDS-PAGE gel. The arrows indicate the position of GST-E4orf3 (37 kDa) and wild-type E4orf3 (11 kDa). **(A)** Coomassie blue stain; **(B)** Western blot analysis using anti-E4orf3 antibody (6A11; Nevels et al., 1999b). **1:** GST-E4orf3 in extracted cell debris; **2:** wild-type E4orf3 in extracted cell debris; **3:** wild-type E4orf3 in inclusion bodies; **4:** wild-type E4orf3 in inclusion bodies; **M:** markers.

For refolding of the proteins solubilized by denaturating agents, the protocol described in section 3.4.1.2 was followed. The protocol used involves the rapid dilution of the proteins present in the denaturating solution by a renaturating buffer such as PBS. The following centrifugation step removes the proteins that are not folded properly by aggregating them. The protocol was applied for GST-E4orf3 and wild-type E4orf3, and it was observed that none of these proteins were refolded properly. The proteins aggregated nearly completely after centrifugation in renaturating buffer of PBS, showing that the method was not suitable for these proteins (Fig. 4.3).

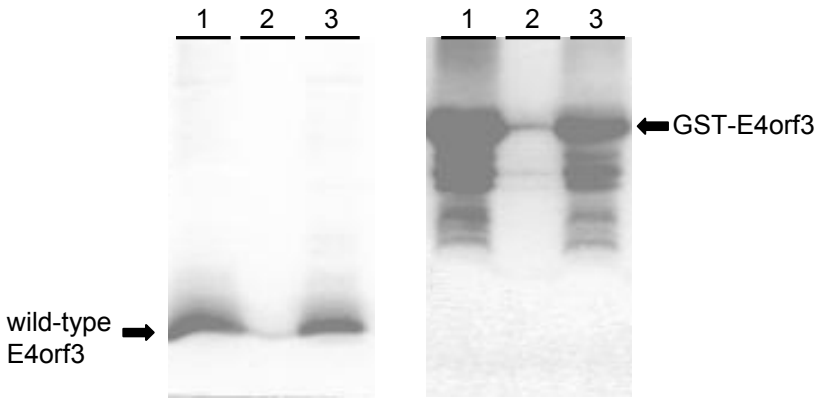


Figure 4.3. Refolding of GST-E4orf3 and wild-type E4orf3 proteins purified under denaturating conditions. Proteins were refolded by rapid dilution of the denaturating solution in PBS. Samples taken before dilution, pellet and supernatant obtained after centrifugation of the diluted solution were electrophoresed on a 15 % SDS-PAGE gel. Protein detection was done after subsequent Western blot by staining the membranes with 6A11 antibody. **1:** proteis in urea; **2:** proteins that remained soluble after renaturation; **3:** proteins aggregated after renaturation.

4.1.3. Optimizing the Conditions for the Expression of E4orf3 Protein

Expression of E4orf3 as a GST fusion protein under standard conditions produced insoluble aggregates and its purification under denaturating conditions failed due to the quite inefficient refolding of the protein. For this reason, in order to obtain soluble E4orf3 protein the conditions for expression were optimized. There are three important variables that may affect the solubility of the protein expressed in *E. coli*: strain selection, temperature, and the level of expression. Therefore, each of these variables were changed to obtain the highest amount of soluble protein.

First of all, four different *E. coli* strains, TOPP™ 2, 3, 4, and 5 were selected for GST-E4orf3 production. Among these strains, TOPP™ 3 was found to be the best for GST-E4orf3 because it produced the highest amount of recombinant protein and at the same time “leaky” expression before IPTG induction was not observed (Fig. 4.4). By the help of this strain, the dependable control of expression was achieved.

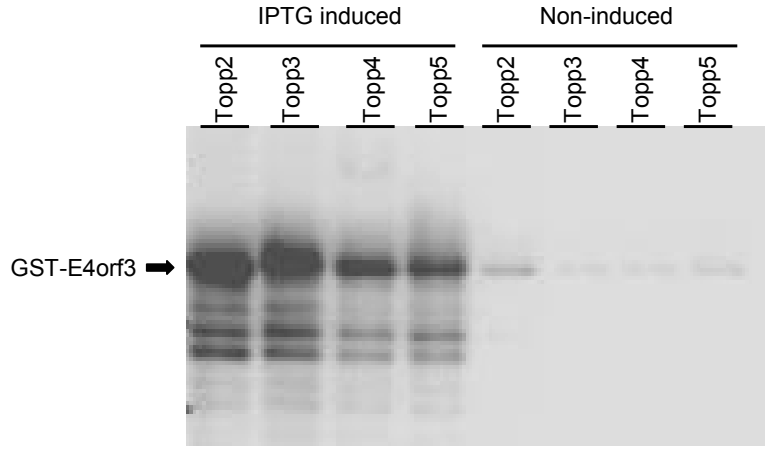
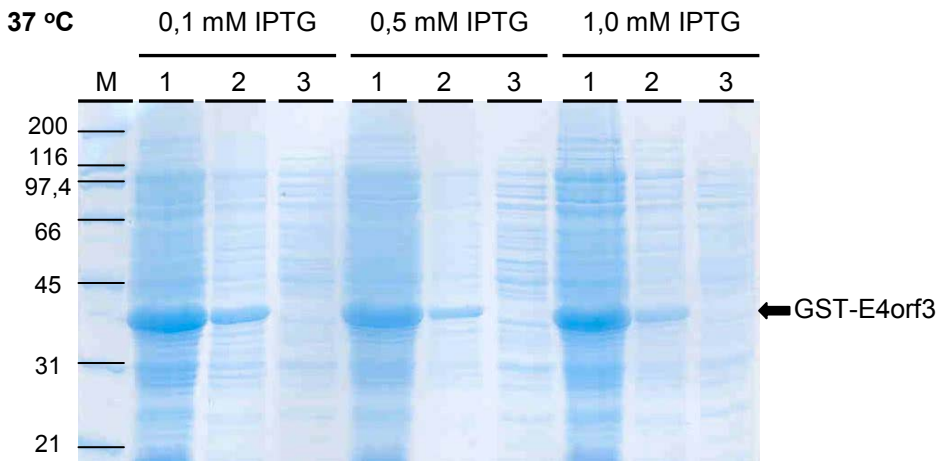


Figure 4.4. Expression of GST-E4orf3 in different *E. coli* strains. The cell pellets obtained before and after 4 h of IPTG induction at 37 °C were loaded into the lanes of 12 % SDS-PAGE gel and run. The proteins were visualized by staining the membranes with 6A11 antibody after Western blot.

Secondly, the effects of reducing the growth temperature and the level of expression on the soluble protein expression were assayed. It has been shown in previous studies that lower temperatures (30 °C) inhibit inclusion body formation (Bishia et al., 1987; Schein, 1989). Sometimes lowering the expression level can also increase the proportion of the protein that is soluble (Schendel, 1998). Thus, GST-E4orf3 protein was expressed in TOPP™ 3 strain by inducing the culture with varying concentrations of IPTG at two different temperatures, 30 ° and 37 °C. As a result, although reducing the growth temperature and expression level did not increase the proportion of the soluble GST-E4orf3 in a huge extent, a detectable amount of soluble recombinant protein was detected in the supernatant of the *E. coli* in which the expression was induced with the lowest IPTG concentration (0,1 mM) at 30 °C (Fig. 4.5).

By optimization of the fermentation conditions, the soluble proportion of GST-E4orf3 protein was increased a little. However, most of the recombinant protein was still in the pellet of the lysate (Fig. 4.5). Therefore, alternative protocols were needed for obtaining soluble GST-E4orf3 protein. It is known that sometimes the proteins expressed in E. coli are not truly insoluble but contain regions that are strongly hydrophobic and associated with the membrane fragments in the pellet (Smith and Corcoran, 1998). If this is the case, extracting the pellet of the lysate with 0,25 % Tween 20, 0,1 mM EGTA buffer may increase the amount of soluble protein obtained (Qiaexpressionist Manual). For this reason, GST-E4orf3 protein was expressed under the optimized conditions and the pelleted bacterial lysate was extracted with the Tween 20 – EGTA buffer as mentioned above. As a result, it was observed that the amount of the soluble recombinant protein was significantly increased by the extraction protocol (Fig. 4.6). However, again considerable amount of the recombinant protein was still in the insoluble pellet and needed to be solubilized.

A)



B)

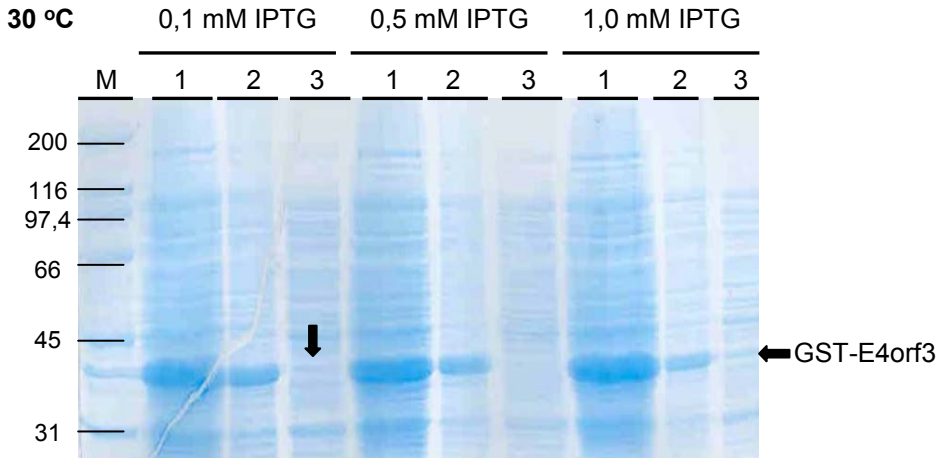


Figure 4.5. Expression of GST-E4orf3 at different temperatures and varying expression levels. The bacteria were incubated at 37 ° **(A)** or 30 °C **(B)** after IPTG induction. The level of expression was changed by applying different concentrations (0,1 mM, 0,5 mM and 1 mM) of IPTG. The cell pellet after 4 h of induction and the samples taken from the pellet and the supernatant of the bacterial lysate were run in a 12 % SDS-PAGE gel. The proteins were visualized by Coomassie blue stain. The arrow indicates the location of soluble GST-E4orf3 protein. **1:** Total cell extract; **2:** pellet; **3:** supernatant; **M:** markers.

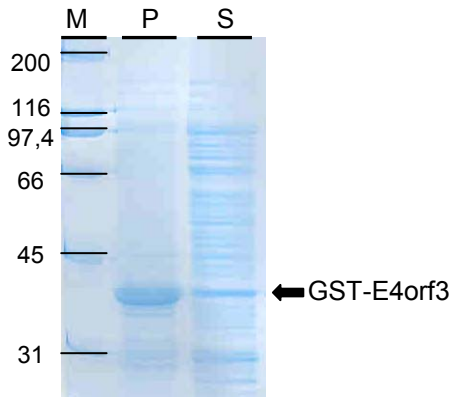


Figure 4.6. The pellet of the bacterial lysate containing GST-E4orf3 protein extracted with Tween 20 – EGTA buffer. GST-E4orf3 protein expressed in TOPP™ 3 strain induced with 0,1 mM IPTG for 4 h at 30 °C and bacteria were lyzed. The samples taken from the pellet and the extracted lysate were run in a 12 % SDS-PAGE gel. The proteins were visualized by Coomassie stain. **P:** pellet of the lysate; **S:** solubilized proteins after extraction; **M:** markers.

4.1.4. Solubilization and Purification of GST Fusion Protein of E4orf3

Optimization of the fermentation conditions and extraction of the pellet of the lysate helped to improve the amount of soluble GST-E4orf3 protein but they were not efficient enough to produce the requested levels of recombinant protein for further biochemical and biophysical studies. On the other hand, increasing the amount of soluble recombinant protein by extracting the cell lysate with a nonionic detergent, Tween 20, which reduces the hydrophobic interactions and a strong chelating agent, EGTA, which reduces the ionic strength of the buffer gave a clue that there might be nonspecific hydrophobic interactions between the GST-E4orf3 protein and the cellular membranes of the bacteria. If this hypothesis is correct, such interactions can be prevented by the use of ionic detergents during cell lysis. The strong ionic detergent

sarkosyl was tested for this purpose because it has been shown to solubilize all components of bacteria except the outer membrane (Filip et al., 1973) and it could solubilize actin which coaggregates with bacterial outer membrane components when overexpressed in *E. coli* (Frankel et al., 1991).

Solubilization and purification of GST-E4orf3 protein was performed as described in section 3.4.1.3. Since sarkosyl is completely insoluble in the presence of divalent cations at room temperature (Helenius et al. 1979), all the procedures were performed in the presence of divalent cation chelator EDTA. The bacteria were lyzed in the presence of sarkosyl and as expected, all proteins in bacteria including the overexpressed GST-E4orf3 were present in the supernatant of the lysate (Fig. 4.7). Pellet was containing only the membrane fragments and no detectable protein content as shown in Fig. 4.7.

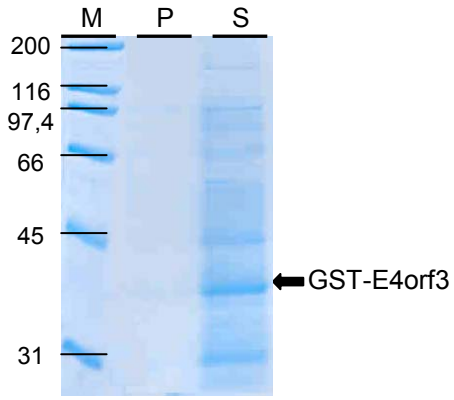


Figure 4.7. Solubilization of GST-E4orf3 protein by an ionic detergent sarkosyl. Protein expression was carried out at the optimum conditions for GST-E4orf3 protein. Cells were lyzed in the buffer containing sarkosyl and the insoluble portion of the lysate was pelleted by centrifugation. Samples from the supernatant and the pellet of the lysate were run in a 12 % SDS-PAGE gel. The proteins were visualized by Coomassie stain. The arrow indicates the location of GST-E4orf3 protein. **P:** pellet of the lysate; **S:** supernatant of the lysate; **M:** markers.

Although the solubilization of the GST-E4orf3 protein was achieved by using sarkosyl, for the subsequent binding of the recombinant protein to glutathione sarkosyl had to be removed from the system. For this purpose, as described in section 3.4.1.3, a nonionic detergent Triton X-100 was added to the lysate since the nonionic detergents were known to sequester sarkosyl in micelles (McNally et al., 1991) and compatible with the binding of GST fusion proteins to glutathione sepharose beads (Smith and Johnson, 1988). Under these conditions, the purification of the full-length E4orf3 as a GST fusion protein was achieved for the first time. As shown in Fig. 4.8 (third lane), GST-E4orf3 protein was found to bind to the glutathione sepharose beads after extensive washing steps. The recombinant protein in the flow-through and washings was significantly less than the protein bound to glutathione sepharose beads showing the efficiency of the solubilization with and the subsequent removal of sarkosyl. Moreover, the presence of GST-E4orf3 in glutathione sepharose beads with nearly no contaminating proteins shows the success of affinity chromatography. However, only a very small amount of protein was able to be eluted from the beads by standard elution buffer (Fig. 4.8; lane 4).

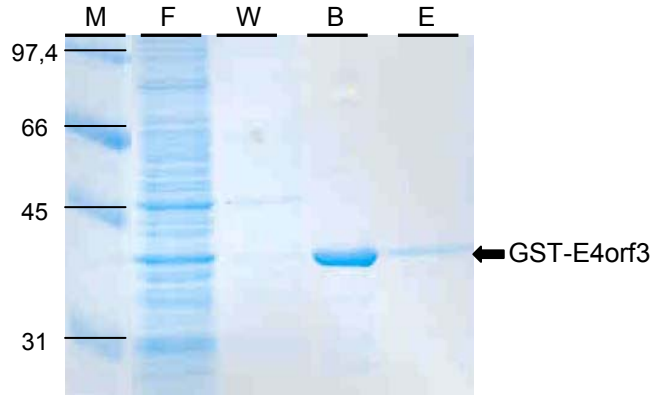


Figure 4.8. Purification of the detergent solubilized GST-E4orf3 protein by GST affinity chromatography. Samples taken from the purification steps were run in a 12 % SDS-PAGE gel. The proteins were visualized by Coomassie stain. The arrow indicate the location of GST-E4orf3 protein. **M:** markers; **F:** flow-through; **W:** washing; **B:** glutathione sepharose beads; **E:** eluate.

In the original description of the pGEX system, the standard elution buffer described (section 3.4.1.1) utilizes reduced glutathione (10 mM) to effectively compete GST from glutathione sepharose. However, this elution buffer was not efficient for eluting the solubilized GST-E4orf3 protein. Increasing the glutathione concentration up to 20 mM and changing the redox potential of the buffer by increasing the concentration of DTT up to 10 mM did not significantly affect the amount of the protein eluted (data not shown). Interestingly, the elution of E4orf3 protein from the glutathione sepharose beads after the cleavage of its GST-tag by thrombin was not successful, neither. As shown in Fig. 4.9, although inefficiently, thrombin was able to cleave some portion of the recombinant protein of GST-E4orf3 to GST and E4orf3. However, cleaved E4orf3 protein containing no GST-tag was not eluted and it remained in the glutathione sepharose beads. Altogether these findings indicated that there was a nonspecific interaction, most probably a hydrophobic interaction, between E4orf3 protein and glutathione sepharose beads which prevented E4orf3 to be eluted from the beads.

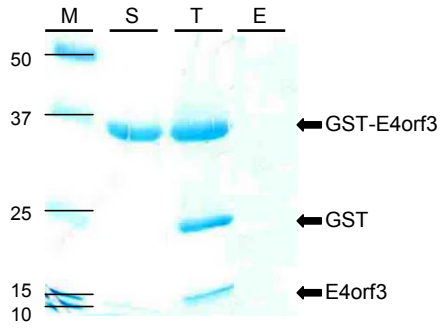


Figure 4.9. Thrombin cleavage of recombinant GST-E4orf3 protein for the removal of GST affinity tag. Thrombin enzyme was used for cleaving GST-E4orf3 protein (37 kDa) containing thrombin site in between GST (26 kDa) and E4orf3 (11 kDa) proteins. Samples were run in a 15 % SDS-PAGE gel. The proteins were visualized by Coomassie stain. The arrows indicate the location of GST-E4orf3, GST, and E4orf3 proteins. **M:** markers; **S:** GST-E4orf3 sample; **T:** thrombin cleaved sample; **E:** eluate after cleavage.

In summary, optimization of the fermentation conditions and solubilization of GST-E4orf3 protein resulted in the successful purification of the full-length recombinant protein. Although the protein could not be eluted from the glutathione sepharose beads, GST-E4orf3 bound to beads was pure enough and suitable for further biochemical and biophysical assays such as GST - pulldown assay and FTIR spectroscopy.

4.2. Functional Analysis of Ad5 E4orf3 Protein

Human Ad5 E4orf3 is a multifunctional protein taking role in efficient viral growth and oncogenic transformation of the cells. These functions of E4orf3 are linked to its ability to interact various cellular and viral proteins. Therefore, in this study, interactions of E4orf3 with other proteins were studied in detail by GST - pulldown assay. Immunoflourescence microscopy was also used as a supportive technique. Moreover, structural analysis of E4orf3 which would further help to understand the functions of this protein was carried out. The secondary structures in E4orf3 protein were assayed by using FTIR spectroscopy.

4.2.1. GST - Pulldown Assay

In this study, the interactions of E4orf3 protein with cellular and viral proteins that were shown or proposed to bind to E4orf3 *in vivo* (Table 4.1) were further studied by GST - pulldown assay.

Table 4.1. Proteins that have been shown to interact with E4orf3.

Proteins	Method	Reference
DNA-PK	Coimmunoprecipitation	Boyer et al., 1999
Aup1	Yeast two hybrid system	Xenia, K., unpublished data
E1B-55 kDa	Coimmunoprecipitation	Leppard and Everett, 1999
E4orf6	Coimmunoprecipitation	Leppard and Everett, 1999

First of all, the proteins given in the table above were radioctively labeled during their expression (section 3.4.5) and used as preys in the assay. As it can be seen in Fig. 4.10, the expression and labeling of these proteins were all successful. However, the expression of E4orf6 in coupled *in vitro* transcription/translation system resulted in three different forms of this protein (lane 4). Two of these were defined as the shorter forms of E4orf6 transcribed from internal start codons and they were assumed not to interfere with the assay.

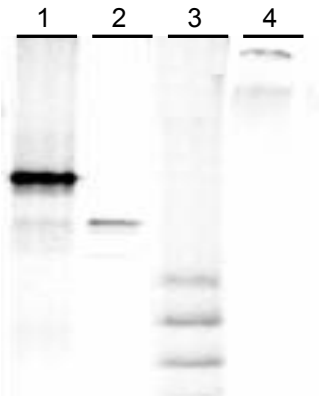
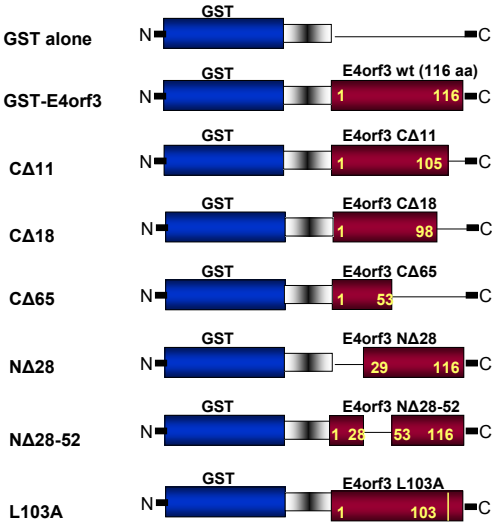


Figure 4.10. Prey protein input. The proteins were expressed by *in vitro*, TNT T7-coupled Reticulocyte Lysate system and radioactively labeled with S^{35}-Methionine added to the lysate. The samples taken from the lysate after the reaction were run in a 12 % SDS-PAGE gel. The proteins were visualized by autoradiography. **1:** E1B-55 kDa; **2:** AUP1; **3:** E4orf6; **4:** DNA-PK.

For showing the interaction of the mentioned proteins with E4orf3, full-length GST-E4orf3 protein present in glutathione sepharose beads were used in the assay. Additionally, for mapping the regions of E4orf3 protein responsible for these interactions, four different mutant GST-E4orf3 proteins that lack varying regions on E4orf3 protein were expressed and purified by the protocol defined for GST-E4orf3. Moreover, the interaction of the prey proteins with two other mutants, one of which was carrying a single amino

acid substitution in the 103rd position (L103A) and the other lacking 18 amino acids from C-terminal (E99s or CΔ18) of E4orf3 protein, were also tested. These two mutant form of E4orf3 were selected because both of them had a different subcellular localization than the wild-type protein in virus infected cells (Dirlik, 2004). Finally, GST protein containing no fusion tag was also purified together with the wild-type and mutant GST-E4orf3 proteins at the same conditions and used as a negative control in the pulldown assay. The schematical representation of these GST fusion proteins and the bait protein input is shown in Fig. 4.11.

A)



B)

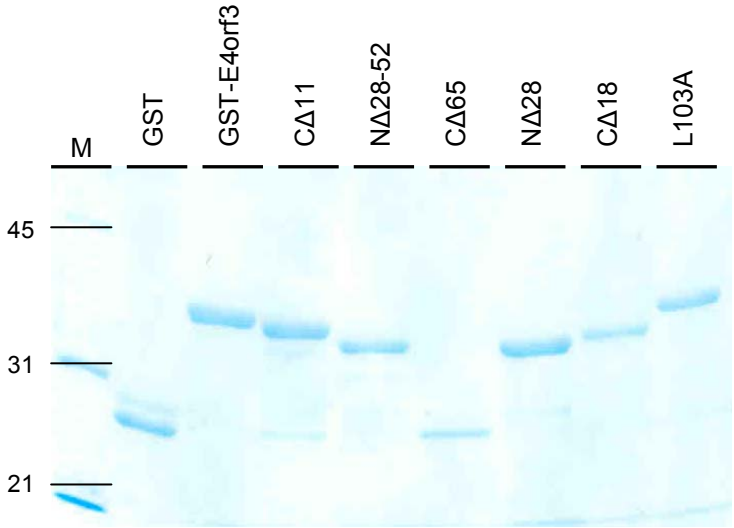


Figure 4.11.(A) The bait proteins. GST containing no tag (GST-alone) was used as a negative, GST-E4orf3 was used as a positive control. CΔ11 lacking 11 amino acids and CΔ65 lacking 65 amino acids from C-terminal, NΔ28 lacking 28 amino acids from N-terminal and NΔ28-52 lacking 24 amino acids between 28th and 52nd residues of E4orf3 were used for mapping the binding regions. CΔ18 and L103A mutants of E4orf3 were used for analyzing the effects of these mutations on the protein interactions of E4orf3; **(B)** Bait protein input. GST-alone, GST-E4orf3 and the mutant proteins of E4orf3 mentioned above were expressed, solubilized and purified by GST affinity

chromatography (section 3.4.1.3). The samples were taken from the glutathione sepharose beads containing these proteins and run in a 12 % SDS-PAGE gel. The proteins were visualized by Coomassie stain.

In Fig. 4.11b, it can be seen that the protocol for solubilization and purification of E4orf3 as a GST fusion protein worked quite well for also its mutant forms. The bait proteins were highly pure and their concentrations in the glutathione sepharose beads were nearly the same. However, for assaying the relative binding efficiencies of prey proteins to GST-E4orf3 and its mutant forms, the concentration of the bait proteins should be exactly the same. For this reason, the relative concentrations of the proteins in the beads were determined and equalized as described in section 3.4.1.3 (data not shown). Afterwards, the lysates containing radioactively labeled DNA-PK, AUP1, E1B-55 kDa and E4orf6 proteins were incubated respectively with these bait proteins and the ones that were pulled down were detected by SDS-PAGE and autoradiography.

First of all, DNA-PK was found to be interacting with none of the GST fusion proteins tested including the full-length GST-E4orf3 (data not shown). The binding of E4orf3 to DNA-PK was shown by coimmunoprecipitation assay (Boyer et al., 1999), however, GST - pulldown assay failed to show this interaction *in vitro*.

On the other hand, the interaction of E4orf3 with AUP1 which was shown previously by yeast two hybrid system (Xenia, K., personal communications) was confirmed by GST - pulldown assay in this study. In Fig. 4.12, it can be observed that AUP1 bound to and pulled down by full-length GST-E4orf3 but not by its negative control, GST alone. Two N-terminal deletion mutants of E4orf3, NΔ28 and NΔ28-52, was also shown to bind to AUP1 as well as wild-type E4orf3 (lanes 4 and 6), whereas, C-terminal deletion mutants CΔ11 and CΔ18 showed significantly reduced binding to AUP1 when compared to the

wild-type protein (lanes 3 and 7); CΔ65 showed no binding at all (lane 5). All together, these results indicate that C-terminal of E4orf3 is responsible for its binding to AUP1. Moreover, L103A mutant of E4orf3 having a point mutation in its C-terminal also reduced the binding of E4orf3 to AUP1 (lane 8) which strengthens the indication above.

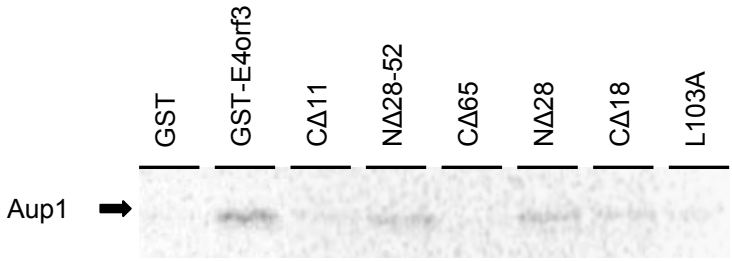


Figure 4.12. Interaction of E4orf3 with AUP1 shown by GST - pulldown assay. Equal concentrations of GST fusion bait proteins in glutathione sepharose beads were incubated with the lysate containing radioactively labeled AUP1 protein. After subsequent washing steps, the beads were loaded into the wells of a 12 % SDS-PAGE gel. The autoradiographic determination of captured proteins took place after western blotting.

The direct physical interaction between E4orf3 and E1B-55 kDa proteins was also shown successfully by the pulldown assay and the regions responsible for this interaction on E4orf3 protein were mapped. Similarly, full-length GST-E4orf3 and its N-terminal deletion mutants NΔ28 and NΔ28-52 were found to bind to E1B-55 kDa protein with similar affinities, however, together with GST-alone all the C-terminal deletion mutants failed in binding (Fig. 4.13). These results indicate the importance of especially the last 11 amino acids of the C-terminal of E4orf3 protein on its interaction with E1B-55 kDa. In addition, the effect of a single amino acid exchange in the C-terminal of E4orf3 protein (L103A) on its association with E1B-55 kDa was also assayed, and interestingly, it was observed that L103A mutant bound to E1B-55 kDa

with an affinity that was significantly higher than the wild-type. In summary, the C-terminal of E4orf3 protein was found to have a key function in its binding to E1B-55 kDa protein.

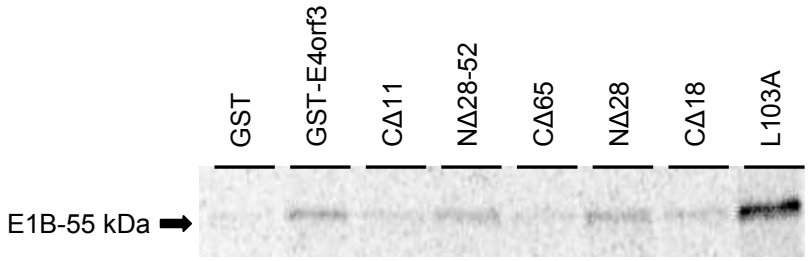


Figure 4.13. Interaction of E4orf3 with E1B-55 kDa shown by GST - pulldown assay. Equal concentrations of GST fusion bait proteins in glutathione sepharose beads were incubated with the lysate containing radioactively labeled E1B-55 kDa protein. After subsequent washing steps, the beads were loaded into the wells of a 12 % SDS-PAGE gel. The autoradiographic determination of captured proteins took place after western blotting.

The interaction between E4orf6 and E4orf3 proteins was first proposed by Leppard and Everett (Leppard and Everett, 1999). In their experiments, they were able to coimmunoprecipitate E4orf3 with E4orf6 in wild-type virus infected cells but not in the cells infected with E1B-55 kDa deleted mutant virus (*dl338*). Therefore, they suggested that this interaction was indirect; via E1B-55 kDa. In contrast, direct interaction of E4orf3 protein with E4orf6 was shown for the first time by GST - pulldown assay in this study. As shown in Fig. 4.14, radioactively labeled full-length E4orf6 and its internally transcribed shorter forms were all captured by GST-E4orf3 protein but not by GST-alone (lanes 1 and 2). This indicated that E4orf3 protein was able to bind to E4orf6 in the absence of E1B-55 kDa *in vitro*. Moreover, the presence of shorter the forms of E4orf6 in the lysate showed that the non-transcribed regions in the N-terminal of E4orf6 protein were not responsible for the binding of this

protein to E4orf3. The N-terminal of E4orf3 was also found to be unimportant for the binding, since N-terminal deletion mutants of E4orf3, NΔ28 and NΔ28-52, showed affinity to E4orf6 as much as the wild-type protein (lanes 4 and 6). However, a retardation of E4orf6 in the gel was observed only for its full-lenght form captured with NΔ28 and NΔ28-52 mutants indicating a modification on E4orf6 protein occurred upon its binding to these N-terminal deletion mutants of GST fused E4orf3. Therefore, it was concluded that N-terminal of E4orf3 was important for the interaction between these two proteins but not for their binding. On the other hand, all C-terminal mutants, CΔ11, CΔ18, CΔ65 and L103A showed significantly reduced affinity against E4orf6 when compared to wild-type E4orf3 and in deletion mutants the effect was observed to be more severe than L103A (lanes 3, 5, 7 and 8). These suggested that the association of E4orf3 with E4orf6 was also due to the C-terminal of E4orf3.

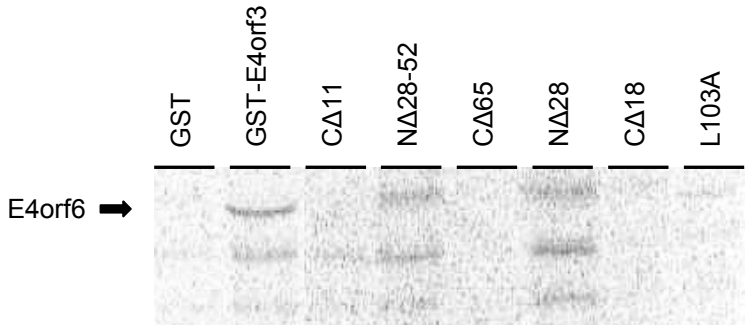


Figure 4.14. Interaction of E4orf3 with E4orf6 shown by GST - pulldown assay. Equal concentrations of GST fusion bait proteins in glutathione sepharose beads were incubated with the lysate containing radioactively labeled E4orf6 protein. After subsequent washing steps, the beads were loaded into the wells of a 12 % SDS-PAGE gel. The autoradiographic determination of captured proteins took place after western blotting.

4.2.3. Immunoflourescence Microscopy Analysis

The interaction between two proteins shown by a single technique is usually untrustworthy and should be confirmed by other methods. Therefore, in this study, immunoflourescence analysis of HeLa cells transfected with E4orf3 was also carried out. However, since the association between E4orf3 and E1B-55 kDa was shown (Leppard and Everett, 1999; Dirlik, 2004) and the binding of E4orf3 to E4orf6 was assayed (Orkide Dirlik, personal communications) previously with this method, only the interaction between E4orf3 and AUP1 was analyzed. As a result, no colocalization of E4orf3 with AUP1 was observed (Fig. 4.15a). E4orf3 was distributed mainly in nucleus in HeLa cells, whereas, endogenous AUP1 was in the cytoplasm. This suggests that the *in vitro* interaction found between these two proteins normally present at different cellular compartments did not really occur in the cell. On the other hand, previous studies confirmed the interaction between E4orf3 and E1B-55 kDa proteins in the nucleus of the cells, as shown in Fig. 4.15b. However, the association of E4orf3 with E4orf6 was not successfully shown by immunoflourescence analysis since E4orf6 showed a diffuse distribution in the nucleus (Fig. 4.15c).

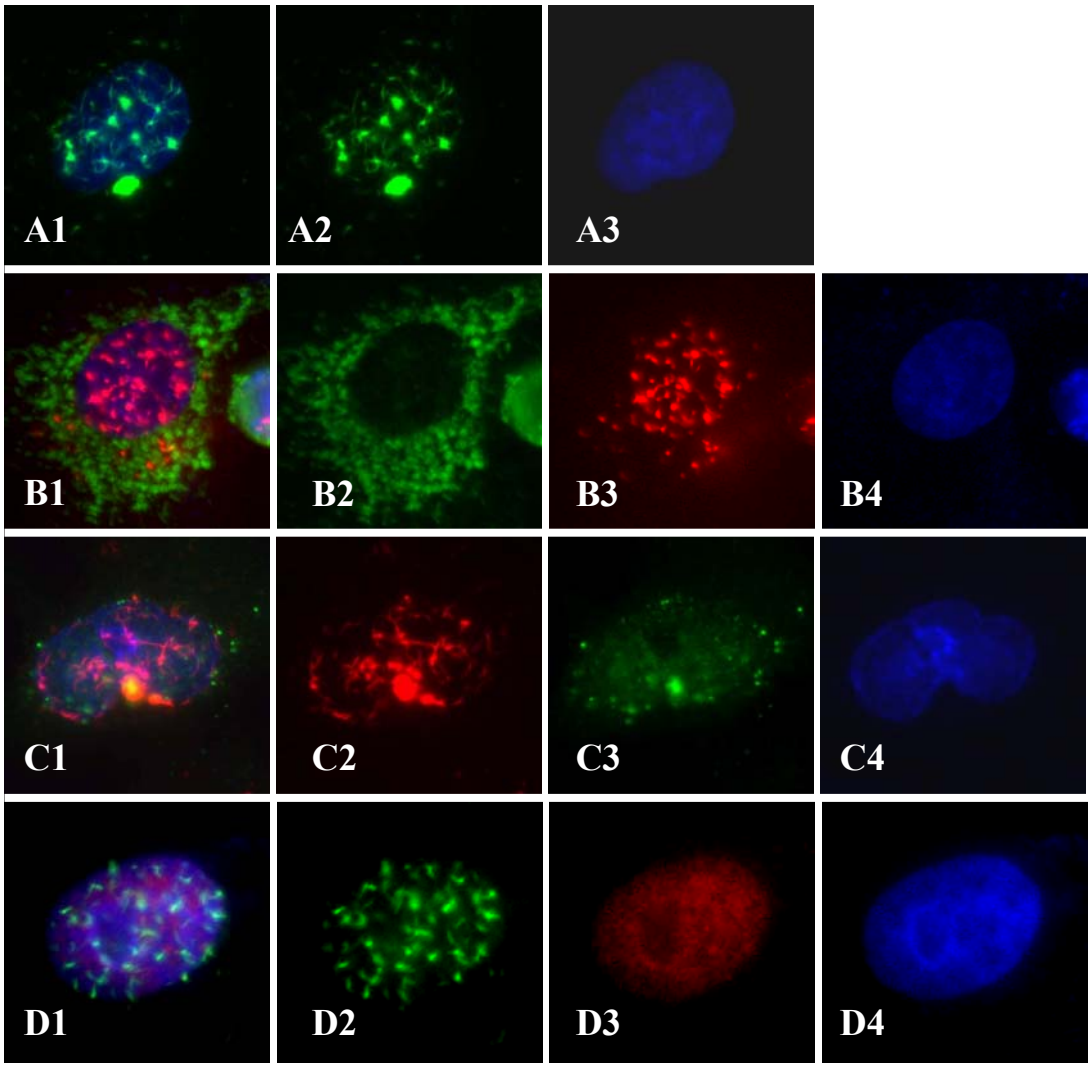


Figure 4.15. Immunoflourescence analysis of HeLa cells transfected with E4orf3. **(A)** Cells were fixed 24 h after transfection and stained with DAPI (A3) and E4orf3 (A2). A1 shows the colocalization of E4orf3; **(B)** Colocalization of E4orf3 with Aup1 (B1). Cells were fixed 24 h after transfection and stained with Aup1 (B2), E4orf3 (B3) and DAPI (B4) antibody and analyzed under immunoflourescence microscope; **(C)** Colocalization of E4orf3 with E1B-55 kDa (C1; Dirlik, 2004). C2 shows E4orf3, C3 shows E1B-55 kDa and C4 shows cell nucleus stained with DAPI; **(D)** Colocalization of E4orf3 with E4orf6 (D1). Cells were fixed 24 h after transfection and stained with E4orf3 (D2), E4orf6 (D3) and DAPI (D4) antibody and analyzed under immunoflourescence microscope.

4.2.4. FTIR Spectroscopy Analysis

For the determination of secondary structures in E4orf3 protein, Amide I band of GST-E4orf3 obtained by FTIR measurements and making appropriate calculations was analyzed. The number and positions of sub-bands under the Amide I band were determined by obtaining the second derivative spectrum. Afterwards, the curve fitting analysis was carried out and the relative percentage areas of the sub-bands were calculated. For determining a secondary structure arising from a specific band, previous band assignments were used (Table 4.2).

Table. 4.2. Assignments of bands in amide I region in H_2O.

Band Position (cm^{-1})	Secondary Structure	Reference
1648-1658	α-Helix	Arrondo et al., 1993
~1645	Open loop	Fabian et al., 1992
1620-1640, 1680	Antiparallel β-sheet	Susi, 1969
<1630	Antiparallel chain pleated sheet	Susi, 1969
1660-1700	Turn, bend, β-turn	Krim and Bandekar, 1986
1615-1616	Tyrosine residue	Severcan et al., 1999

FTIR analysis of GST-E4orf3 fusion protein in glutathione sepharose beads revealed that this protein contains mainly five different types of secondary structures which are: i) α-helix (21 %), ii) β-sheet (27 %), iii) antiparallel chain-pleated sheet (9 %) iv) turn, bend, β-turn (19 %), and v) open loop (22 %, together with overlapping α-helix); (shown in Fig. 4.16). In addition, the absorbance of tyrosine residue was also detected (2 %). These structures could be present in both partners of the fusion protein and in order to determine the secondary structures in E4orf3 protein alone, the contribution of the GST moiety should be eleminated. GST protein has a very well defined

secondary and three dimensional structure (McTigue et al., 1995). It contains 218 amino acid residues that fold to form a small N-terminal α/β domain (residues 1-76), a short linker region (residues 77-84), and a larger C-terminal α domain (residues 85-218). The N-terminal domain contains three α-helices (24 residues) and a four stranded, antiparallel β-sheet (14 residues). The C-terminal domain contains five α-helices (57 residues) and a long coil forming an open loop (residues 195-218). Moreover, the linker amino acids have also shown to form an open loop (residues 218-229; Zhan et al., 2001) before 116 amino acid long E4orf3 protein.

In summary, GST protein mainly consists of α-helix (81 residues), which can be calculated to be the 23,5 % of GST-E4orf3 protein. Therefore, it was concluded that the detected α-helical structure in the GST-E4orf3 was from GST protein. Furthermore, there is an open loop present at the end of the GST protein and in between the two fusion partners. Together with the turn,bend and β-turn structures in GST, the loop can be calculated to cover 39 % of GST-E4orf3. In the curve fitting analysis of GST-E4orf3 the open loop band was found at 1642 $cm^{-}1$. However, the normal assignment of open loop band is 1645 $cm^{-}1$, but this assignment is not ambiguous because other structures, such as α-helices, also give rise to bands in this region (Arrondo and Goni, 1999). The area of this band together with turn, bend, and β-turn bands were found to be 41 % of the total Amide I band. Thus, around 2 % of these bands, corresponding to open loop, turn, bend, β-turn and α-helix can be concluded to arise from E4orf3 alone. Finally, the contribution of GST protein to β-sheet structures of the fusion protein is 4 % which means around 32 % of antiparallel β-sheet and chain-pleated sheet present in the GST-E4orf3 protein arises from E4orf3. In conclusion, the secondary structure of E4orf3 protein was determined to be mainly β-sheet.

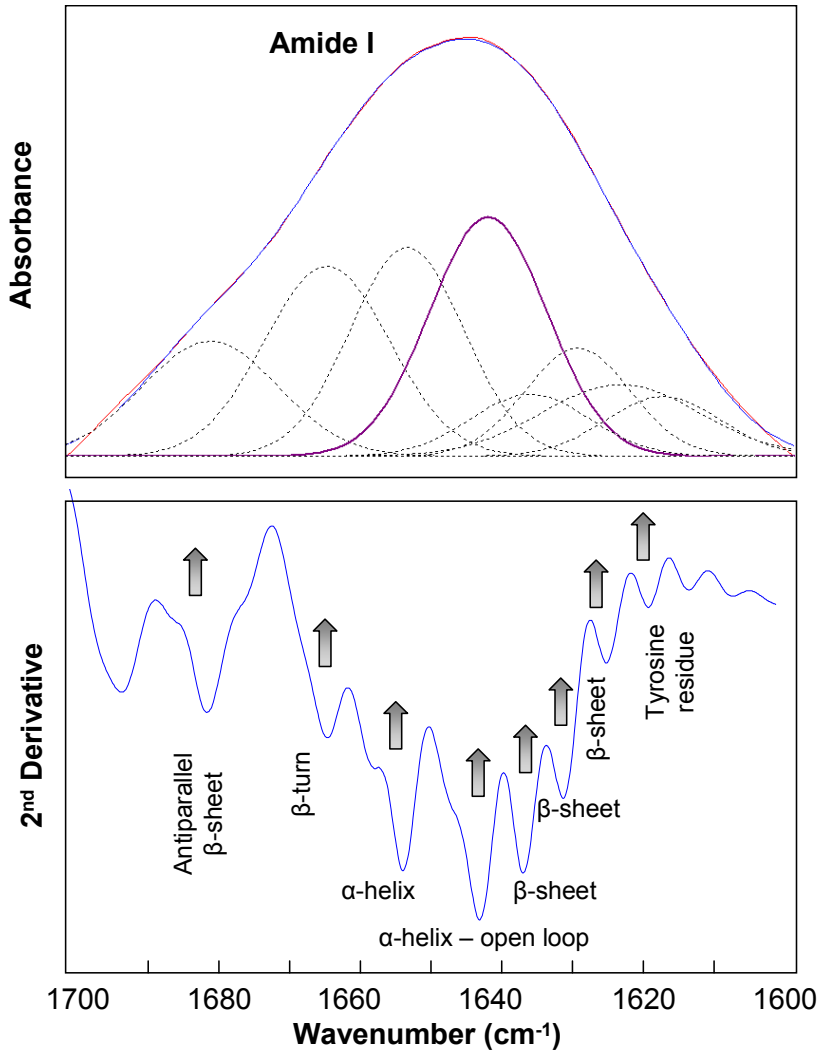


Figure 4.16. Curve fitting analysis (upper part) and second derivative spectrum (lower part) of GST-E4orf3 Amide I band. Arrows indicate the location of the sub-bands and their corresponding secondary structures.

CHAPTER V

DISCUSSION

Much of the recent work on human Ads has been aimed at using these viruses as vectors for gene and cancer therapy due to the certain properties they possess (Benihoud et al., 1999). First of all, they are able to infect a variety of tissue cell types that are dividing or nondividing and they do not cause major pathologies in human (Danthinne and Imperiale, 2000). However, the oncogenic potential of these viruses bears a safety risk and a major drawback for the use of Ads as a vector in gene and cancer therapy. Although a nononcogenic serotype, Ad5, is mostly being used for this purpose, some of the proteins of Ad5 also possess transforming and oncogenic properties (Täuber and Dobner, 2001).

Ad5 E4orf3 protein is one of the oncoproteins of this virus. Although it is not an essential element for the classical Ad transformation, it exhibits partial transforming potential and contributes to E1 mediated oncogenesis (Öhman et al., 1995). However, more importantly, it has been reported that in the absence of E1B proteins E4orf3 acts together with E1A and transforms the cells by mutagenesis-based hit-and-run mechanism (Nevels et al., 2001). The mutagenic properties of this protein have a crucial importance on developing safe Ad vectors because the oncolytic Ad vectors currently being tested in clinical trials for human tumor therapy contain E4orf3 gene together with E1A (Heise et al., 1997). E4orf3 protein has important functions in

productive infectious cycle that are absolutely necessary for the efficiency of these vectors. Moreover, the presence of E4orf3 is also required for the maintenance of transgene expression and beneficial for Ad gene therapy vectors (Armentano et al., 1999). Therefore, the complete removal of the gene encoding for E4orf3 protein is not a solution and in order to develop efficient and safe Ad vectors for gene and cancer therapy, the functions of this protein in Ad infectious cycle and adenovirus-mediated oncogenesis should be well understood.

The studies undertaken to elucidate the functions of E4orf3 protein mostly employed mutant viruses that fail to express E4orf3 for infection or DNA fragments coding wild-type and mutant E4orf3 proteins for transfection and transformation experiments (Dirlik, 2004). The reason for that is the full-length E4orf3 protein could never be expressed and purified in a level required for biochemical and structural studies. Previous attempts for expressing Ad5 E4orf3 with or without a fusion tag in *E. coli* (Barbara Haertl, personal communications) failed due to the aggregation of the overexpressed protein. Same problem was also occurred during the expression of this protein in baculovirus system that was employing eukaryotic insect cells (Birgitte Täuber, personal communications). However, in this study, the expression and purification of E4orf3 as a GST fusion protein in *E. coli* was optimized in a way to obtain large amounts of pure recombinant protein that was suitable for subsequent functional and structural analysis, for the first time.

First of all, GST gene fusion system was chosen for the expression of E4orf3 protein. The system permits rapid and efficient purification of proteins that are fused to the C-terminal of GST protein from the crude cellular lysates since GST is an enzyme that binds to glutathione-containing affinity matrices with high affinity (Smith and Johnson, 1988). Once purified, the fusion protein can be recovered by elution with free reduced glutathione or if necessary, the GST moiety can be removed from the fusion proteins by cleavage with site-

specific proteases. Besides all these properties, the main advantage of this system for expressing and recovering foreign proteins from *E. coli* is that most fusion proteins remain soluble. However, in this study, GST-E4orf3 protein was found to be completely insoluble under the given standard expression conditions (see Fig. 4.1). Inducing the bacterial expression culture of DH5α with 1 mM IPTG at 37 °C when the OD_{600} of the culture reached 0,6 led the high-level expression and the highly insoluble aggregates of GST-E4orf3 protein.

Highly insoluble behavior of a recombinant protein expressed in *E. coli* can be due to the formation of the inclusion bodies that contain most of the expressed protein (Schein, 1989). Eukaryotic proteins – since Ad5 basically infects human cells, E4orf3 protein can be considered as a eukaryotic protein – expressed in *E. coli* are frequently sequestered into insoluble inclusion bodies. Different conditions in the prokaryotic cytosol are believed to play a role in the accumulation and aggregation of the partially folded forms of the recombinant protein by hydrophobic interactions (Mitraki and King, 1989). Improper formation of disulphide bonds in the reducing environment of the *E. coli* cytoplasm may also contribute to incorrect folding and formation of inclusion bodies. However, Ad5 E4orf3 protein contains only three cystein residues (Table 5.1) and the possibility of the latter explanation is low. Moreover, if E4orf3 protein aggregates due to the partial folding of the protein in prokaryotic cytosol, it could be possible to express this protein in a eukaryotic system. Whereas, the expression of E4orf3 in eukaryotic insect cells by using baculovirus vectors also failed because of the insolubility of the expressed protein (Birgitte Täuber, personal communications). Therefore, the reason for the extreme insolubility of E4orf3 is that it may originally contain regions that are strongly hydrophobic.

In this study, the aggregated GST-E4orf3 and wild-type E4orf3 proteins obtained under standard bacterial expression conditions could only be solubilized by the denaturating solution of 8 M urea (see Fig. 4.2). Thus, it

was concluded that E4orf3 was extremely insoluble and its insolubility was regardless of the GST fusion tag. Therefore, in order to obtain functionally active form of this protein in solution the first thing tried was to refold the wild-type and GST-E4orf3 proteins solubilized by denaturation. For the proteins of moderate size refolding was postulated to be relatively easy (Marston and Hartley, 1990). Merely, the refolding of both wild-type and GST-E4orf3 was quite inefficient; refolded proteins aggregated nearly completely soon after the addition of the refolding buffer (see Fig. 4.3). The yield of properly folded proteins after denaturation/renaturation is known to be variable, and sometimes quite low; some proteins, especially large ones (>50 kDa), can not be properly refolded at all (Smith and Corcoran, 1998). Whereas, GST-E4orf3 is a moderately (37 kDa) and wild-type E4orf3 is a small sized protein (11 kDa) but refolding of both of these two proteins failed. This supported the idea that E4orf3 protein might contain strongly hydrophobic regions in its original structure that were responsible for its aggregation.

Table 5.1. Amino acid sequence of Ad5 E4orf3 protein. Cystein residues were written in red.

N-terminal - MIR**C**LRLKVEGALEQIFTMAGLNIRDLLRDILRRWRDENYLGMV
EGAGMFIEEIHPEGFSLYVHLDVRAV**C**LLEAIVQHLTNAII**C**SLAVEFDHATG
GERVHLIDLHFEVLDNLLE - C-terminal

Protein refolding after solubilization by denaturation is a commonly used approach but as indicated above the recoveries after refolding step is usually poor. Moreover, proteins expressed under these conditions are not suitable for structural studies because it is almost impossible to show that a protein of an unknown structure has been precisely refolded after denaturation. Since this study also aimed at doing structural studies on E4orf3 protein, much of the concentration was given on obtaining E4orf3 in a soluble form rather than

refolding it. During the isolation of the inclusion bodies it was observed that a small portion of GST-E4orf3 protein remained in the solution after the extraction of the cell debris with Triton X-100 – EDTA buffer. This finding suggested that the aggregation of GST-E4orf3 was not completely dependent on the inclusion body formation and led the idea that optimization of the expression conditions might help to increase the amount of fusion protein in the soluble portion.

The variables that are optimized to express higher amounts of soluble protein usually concern the level of protein expression. Expression level of a protein in *E. coli* may have direct effects on the solubility of that protein. For instance, some normal prokaryotic proteins were shown to be insoluble when overexpressed in *E. coli*. Therefore, the concentration of IPTG used for inducing protein expression is a variable which controls the level of protein expression. Growth temperature after induction also directly affects both expression levels and protein solubility, and for reasons not well understood, lower temperatures increase the amount of soluble protein (Bishai, et al., 1987; Schein, 1989). However, in order to analyze the effects of these parameters, the dependable control of expression should be assured first by selecting the *E. coli* strain that shows the least “leaky” expression. Therefore, in this study, the optimization of the expression of GST-E4orf3 was carried out in a sequence that, i) the best strain for the expression of GST-E4orf3 was selected, ii) the conditions for obtaining the highest amount of soluble GST-E4orf3 protein were determined by changing the growth temperature and IPTG concentration.

Lowering the growth temperature to 30 °C and IPTG concentration to 0,1 mM reduced the level of expression by 90-95%. This reduction helped to increase the amount of soluble recombinant protein but not to a desired level (see Fig. 4.5). On the other hand, an increase was observed in the soluble proportion of GST-E4orf3 protein due to the extraction of the cell debris with Triton X-100 – EDTA buffer. This suggested a mechanism for the extreme insolubility

of this protein apart from the inclusion body formation. It has been reported previously that extreme insolubility of some proteins are not correlated with the presence of inclusion bodies but due to the coaggregation of the target protein with the outer membrane components (Frankel et al., 1991). The association of the recombinant protein with the vital membrane systems of *E. coli* which probably occurs during lysis is a common problem for membrane proteins or proteins containing hydrophobic regions (Waeber et al., 1993 QE). If this is the case, the effects of lowering the growth temperature and treating the lysate with mild detergents should be investigated. In this study, there were some evidences indicating that E4orf3 protein probably contained strongly hydrophobic regions. Moreover, treating the lysate with Triton X-100 – EDTA buffer helped obtaining a small amount of soluble GST-E4orf3. More importantly, growing the cells at a lowered temperature of 30 °C and the extraction of the cell lysate with Tween 20 – EGTA buffer significantly increased the amount of soluble GST-E4orf3 (see Fig. 4.6). These results have led the conclusion that the insoluble behavior of E4orf3 protein was due to its interaction with the outer membrane components of *E. coli*, and the insoluble aggregates after cell lysis was consisting of E4orf3 together with the membrane fragments.

The coaggregation of E4orf3 with the outer membrane components of bacteria may occur by a mechanism involving the hydrophobic interactions. During the process of cell lysis, the outer membrane components of bacteria are fragmented and these fragments containing extensive hydrophobic regions act as a trap for the hydrophobic surfaces on the expressed protein (Frankel et al., 1991). Once such an interaction occurs, the protein expressed would reflect the highly insoluble characteristic of the outer membrane (Frankel et al., 1991) which appears to be the situation with E4orf3. Therefore in this study, a strong ionic detergent, sarkosyl, was used during the lysis of the bacteria in order to prevent the interaction of E4orf3 with the *E. coli* outer membrane fragments. Sarkosyl has been reported to solubilize all components of bacteria except the outer membrane (Filip et al., 1973).

Moreover, it has been shown to solubilize actin which was also highly insoluble due to the coaggregation with bacterial membrane (Frankel et al., 1991). Many different GST fusion proteins showing highly insoluble behavior when expressed in *E. coli* were able to be purified successfully after sarkosyl solubilization (Frangioni and Neel, 1993). Finally in this study, the complete solubilization of GST-E4orf3 was achieved together with all other proteins of *E. coli* as shown in Fig. 4.7 and only the outer membrane components remained insoluble.

Sarkosyl is an anionic detergent such as SDS and one might argue that solubilization of a protein by this detergent could affect the functions of the protein. However, sarkosyl is not necessary for maintaining the soluble form of the target protein once the outer membrane components are removed (Frankel et al., 1991) and sarkosyl can be sequestered in nonionic detergent micelles (McNally et al., 1991). Moreover, sarkosyl solubilized actin was shown to retain its native functions after nonionic detergent treatment (Frankel et al., 1991). Sarkosyl solubilized GST fusion proteins were also shown to bind with high affinity to glutathione beads after treatment with a nonionic detergent, and these proteins were reported to maintain their enzymatic activities (Frangioni and Neel, 1993). Therefore, it was assumed that GST-E4orf3 protein also retained its native functions after solubilization. High affinity binding of GST moiety to glutathione sepharose beads, the successful purification of the fusion protein (shown in Fig. 4.8), and the binding of E4orf3 to its well-known interaction partner E1B-55 kDa in the subsequent GST - pulldown assays (shown in Fig. 4.10) proved the correctness of this assumption.

In this study, full length E4orf3 was expressed as a GST fusion protein and purified successfully for the first time. Moreover, the method for solubilization and purification of this protein did not employ denaturating agents such as urea and the purified protein was thought to be functionally active. However, the protein could not be eluted from the glutathione sepharose beads most

probably due to the nonspecific hydrophobic interactions occured between sepharose and E4orf3 (see Fig. 4.9). Therefore, the functional analysis of E4orf3 protein was restricted to the assays that could employ the protein in the beads such as GST – pulldown assay and FTIR spectroscopy.

E4orf3 is a multifunctional binding protein acting on efficient viral growth and oncogenic transformation of the cells. The detailed analysis of the interaction between E4orf3 and its binding partners is required to understand the functions of this protein. GST - pulldown assay is a method that employs the GST fusion proteins in the beads and can be used for analyzing protein-protein interactions. The method shows or confirms a possible interaction between two proteins. Moreover, the regions or even single amino acids on the proteins that are responsible for the interaction can be determined if mutant proteins are used. Therefore in this study, four different mutant GST-E4orf3 proteins lacking varying regions (deletion mutants) and two having a single mutation on E4orf3 were purified in addition to the full-length protein. The deletion mutants were selected in such a way to show the region on E4orf3 that was responsible for binding to a specific protein. Each deletion mutant was lacking a different region on the protein and if placed side by side, the lacking regions would cover the whole protein sequence. The other two mutants were selected because they have been shown to differ phenotypically from the wild-type protein in virus infected cells (Dirlik, 2004). The reasons for the differences in phenotype were able to be analyzed further in this study, since these mutants were also expressed and purified successfully for the first time by the method employed for the wild-type GST-E4orf3.

DNA-PK is known to be one of the interaction partners of E4orf3. The physical interaction between these two proteins was first shown by Boyer and coworkers in a coimmunoprecipitation experiment (Boyer et al., 1999). DNA-PK is an essential element of double strand break repair (DSBR) system which is active both on broken chromosomal DNA and on exogenous DNAs

introduced into cells, and linear Ad genome is a potential substrate for concatenation by DSBR system in infected cells. However, concatemeric Ad genome is not a good substrate for packaging (Hearing et al., 1987) and for an efficient DNA replication and packaging of virus concatemer formation should be prevented. E4orf3 protein was reported to be responsible for suppressing the formation of concatemers (Weiden and Ginsberg, 1994) and this function of E4orf3 was linked to its binding to and inhibiting DNA-PK. Thereby the DSBR system is inhibited (Boyer et al., 1999). Inhibition of DNA-PK's activities is one of the functions of E4orf3 protein contributing to its mutagenic and oncogenic potential because this function leads to damages that remain unrepaired on the chromosomal DNA of the host. Thus, determining the region where E4orf3 binds to DNA-PK is important if this protein should be modified in a way to reduce its oncogenic potential. However, in this study, full-length E4orf3 failed to capture *in vitro* transcripted/translated DNA-PK and the binding regions could not be determined. DNA-PK is a large protein having a molecular weight of 400 kDa and failure in capturing this protein with an 11 kDa protein of E4orf3 may be just because of physical reasons. In the report showing the interaction between these two proteins, only the coimmunoprecipitation of E4orf3 with DNA-PK was shown but not vice versa (Boyer et al., 1999). Another possibility for this failure may be that *in vitro* transcripted/translated DNA-PK could not be properly folded because of its size and was functionally inactive. Therefore, if the interaction between these two proteins is aimed to be studied, functionally active DNA-PK should be expressed first and E4orf3 has to be the prey.

The interaction between E4orf3 and Aup1 was previously detected by yeast two hybrid system (Xenia, K., personal communications). Aup1 is a ubiquitously expressed protein that is present in all human cells (Kato et al., 2002). In addition, its amino acid sequence is evolutionary conserved (Jang et al., 1996) and it appears that Aup1 plays an essential role in cell biology. Merely, there is few reports present about this protein and the actual role it

takes in the cell is not clear but it was suggested that Aup1 was involved in integrin signaling since it was found to bind to integrin α subunits (Kato et al., 2002). In the same report, it was also suggested that Aup1 might be responsible for altering the local concentrations of phospatidic acid (PA) and lipophospatidic acid (LPA) which are essential lipid messengers in signal transduction and activators of Ras and the downstream Raf/mitogen-activated protein kinase pathway (Ras/Raf pathway; Moolenaar, 1995). Thus, the interaction between E4orf3 and Aup1 may be necessary for the inhibition of Ras/Raf pathway, and thereby, preventing the apoptosis of the infected cell. If this is the case, this interaction is one of the reasons of E4orf3's being an oncoprotein. In this study, the physical association between Aup1 and E4orf3 was confirmed by GST - pulldown assay and moreover, it was determined that C-terminal of E4orf3 was responsible for this association (see Fig. 4.12). However, Aup1 is originally present in cytoplasm (Kato et al., 2002) and E4orf3 is a nuclear protein (Leppard and Everett, 1999). Therefore, *in vivo* interaction between them was doubtful and in immunoflourescence microscopy analysis, no colocalization between these two proteins was observed (see Fig. 4.15). The positive results obtained from GST - pulldown assay are never definitive; false results (false positives) can be obtained because of the nonspecific interactions. For example, Aup1 contains a highly hydrophobic N-terminal region (Jang et al., 1996) which may interact nonspecifically with E4orf3 protein which is considered to bear hydrophobicity, too. On the other hand, being able to show the association between these two proteins by two different techniques leads to the question of whether the interaction occurs just after the synthesis of E4orf3 in the cytoplasm before it reaches to nucleus. Since the consequences of this possible interaction can be of crucial importance due to the functions of Aup1 in integrin signaling, further studies are necessary to elucidate whether there is a real interaction between these two proteins.

E1B-55 kDa protein is a multifunctional regulator of cell cycle-independent Ad replication that controls several processes, including inhibition of p53-

dependent apoptosis, late viral mRNA transport and efficient viral DNA replication (Dobner and Kzhyshkowska, 2001). Moreover, it is one of the best known interacting partners of E4orf3. The physical interaction between these two proteins was first shown by immunoprecipitation assay and immunoflourescence microscopy (Leppard and Everett, 1999). In the same report, it has been suggested that E4orf3 causes the nuclear matrix localization of E1B-55 kDa. E4orf3 induced nuclear localization of E1B-55 kDa is known to be required for efficient viral DNA replication and expression of Ad late genes (Täuber and Dobner, 2001). If these knowledge is combined with the data showing that the C-terminal region of E4orf3 is responsible for this interaction (section 4.2.1), it can be concluded that N-terminal of E4orf3 plays a role in nuclear matrix association. N-terminal of E4orf3 was found not to be critical for any of the proteins tested (Aup1, E1B-55 kDa, and E4orf6). Therefore, it is tempting to speculate that the hydrophobic domain supposed to be present on E4orf3 is in the N-terminal of the protein and it functions in the attachment of the protein to nuclear matrix. By this way, affinity of E1B-55 kDa to the C-terminal of E4orf3 would result in the nuclear matrix localization of the E1B protein via the N-terminal of E4orf3. Interestingly, in this study, it was observed that L103A mutant of E4orf3 bound to E1B-55 kDa with an affinity that was significantly higher than the wild-type (see Fig. 4.13). The presence of L103A mutant has also been shown to increase the amount of E1B-55 kDa in the nuclear matrix in virus infected cells (Dirlik, 2004), which is consistent with the above speculation. However, the L103A mutant of E4orf3 was reported to show altered subcellular localization in infected cells; it was distributed diffusively both in the nucleus and the cytoplasm and was found to be more in the soluble fraction of the cell lysate (Dirlik, 2004). Thus, it was concluded that the C-terminal of the protein was functioning in the subcellular localization of E4orf3 (Dirlik, 2004). A possible interpretation for the altered subcellular localization of L103A mutant can be that the mutant proteins bind to E1B-55 kDa, a nucleocytoplasmic shuttling protein, with high affinity and some of them lose their nuclear localization by acting together with the E1B protein. If this is the case, then it can be concluded that the effect of the C-

terminal of E4orf3 on its subcellular localization is indirect. At this point, it is convincing to conclude that both N- and C-terminal of E4orf3 may play direct or indirect roles in the subcellular localization of this protein but the main functional domain of E4orf3 protein is its C-terminal which is functioning in protein – protein interactions. Moreover, investigations employing L103A mutant of E4orf3 indicated that C-terminal region of this protein may play a regulatory role in the interaction of E4orf3 with different proteins. For instance, L103A mutation was shown to increase the affinity between E1B-55 kDa and E4orf3 proteins. Conversely, the same mutation was found to reduce the affinity of E4orf3 to Aup1 and E4orf6. It is obvious that substituting the leucine at the 103 position with alanine was enough to alter the conformation of the C-terminal. This altered conformation did not completely inhibit the binding of E4orf3 to Aup1 and E4orf6 but reduced the dissociation constant (K_d), whereas the effect was vice versa for E1B-55 kDa. Taken together, these data suggested that binding and dissociation of the proteins interacting with E4orf3 was regulated by the C-terminal of E4orf3.

Initial attempts to assign specific functions to E4 gene products revealed that E4orf6 and E4orf3 can partially or totally compensate for each other's defects (Täuber and Dobner, 2001). Ad mutants that fail to express E4orf6 and E4orf3 proteins are severely restricted for growth whereas, the mutants lacking only one of these proteins can grow to near wild-type levels at least in standard tissue culture cell lines (Bridge and Ketner, 1989; Huang and Hearing, 1989). The redundant functions shared by both E4 proteins are linked to the ability of E4orf6 and E4orf3 to interact with common cellular and viral factors. For instance, E4orf6 was reported to bind to DNA-PK (Boyer et al., 1999) and to E1B-55 kDa (Ornelles and Shenk, 1991, König et al., 1999). E4orf6 has been indicated to be a nucleocytoplasmic shuttling protein like E1B-55 kDa (Dobbelstein et al., 1997) but it is present mostly in the nucleus (Goodrum et al., 1996). The presence of common partners for both proteins and their sharing the same cellular compartment raises the question of whether there is an interaction between these two proteins. In one study, the

association between E4orf6 and E4orf3 has been shown by immunoprecipitation assay but only in the cells containing E1B-55 kDa protein (Leppard and Everett, 1999). Therefore, the authors have suggested that the interaction between these two proteins was indirect, via E1B-55 kDa. However, they have also indicated that E4orf6 was only extracted from the nucleus into the cytoplasmic or soluble nuclear fractions so that the interaction of E4orf6 with any protein in the nuclear matrix compartment could not be observed in their immunoprecipitation experiments. On the other hand, the direct physical interaction between E4orf6 and E4orf3 has been shown for the first time in this study (see Fig. 4.14). Together with the N-terminal deletion mutants, full-length E4orf3 was able to capture E4orf6 *in vitro*. This finding supports the evidence of association between these two important viral factors and it can be suggested that these two proteins associate most probably in the nuclear matrix. Moreover, if the *in vivo* interaction between these proteins can only be detected in the presence of E1B-55 kDa, it can be concluded that E1B-55 kDa may be responsible for carrying the E4orf6 – E4orf3 complex into the cytoplasm or soluble nuclear fractions, probably by forming a ternary complex. The interaction of E4orf3 with E4orf6 may have significant importance in Ad infectious cycle and Ad mediated oncogenesis. Therefore, the consequences of this interaction should be elucidated in further studies.

It should be noted that obtaining knowledge about the interactions of a protein does not immediately make clear its molecular functions. The structural information has to be obtained also for a better understanding of its functions and is complementary for the functional analysis. However, studying the conformation of proteins is usually difficult, in particular for those containing hydrophobic regions, such as membrane proteins. Since the purification of such proteins often require detergents or organic solvents, the structure of these proteins can only be characterized by the techniques that can be applied in these environments. The most important advantage of FTIR spectroscopy is that spectra of a protein can be obtained in a wide

variety of environments (Haris and Severcan 1999). Moreover, the spectrum of a dilute protein solution can be obtained in high quality, and the absorption of the components in the environment which may interfere with the amide modes can be easily subtracted from this spectrum (Haris and Chapman, 1992; Surewicz et al., 1993; Arrondo and Goñi, 1999). For these reasons, FTIR spectroscopy is a method of choice for studying the secondary structure of E4orf3.

As mentioned above, E4orf3 could be expressed and purified as a GST fusion protein and this fusion protein could only be obtained in an aqueous solution bound to glutathione sepharose beads. However, this environment was not expected to perturb the resolution or sensitivity of the protein's FTIR spectrum because the components in the environment that may interfere with the Amide I region were: i) H_2O, ii) glutathione, and iii) sepharose. From these components, sepharose itself gives no absorption band in none of the Amide modes. Moreover, the overlapping H_2O absorption bands can be subtracted from the spectrum. Thus, only component that can perturb the protein bands was glutathione. FTIR spectroscopic investigation has showed that the absorption bands of glutathione overlaps with the Amide modes. However, if glutathione is in H_2O, the absorption bands can be observed mostly in Amide II and III region; absorption in Amide I can be observed with only a shoulder (Picquart et al., 1999). Nevertheless, the removal of the glutathione bands was required for obtaining the protein band alone and the subtraction procedure to be applied was complex due to the presence of glutathione bound to sepharose in H_2O. In this study, the difference spectrum was obtained by subtracting the buffer-subtracted spectrum of glutathione sepharose beads from buffer-subtracted spectrum of GST-E4orf3 protein in glutathione sepharose beads successfully. The final spectrum obtained was reliably reflecting the absorption spectrum of GST-E4orf3 alone, however, minor variations were possibly produced by the change of the sample in the spectrometer or buffer removal. These variations can be assumed not to affect the results significantly since the same protocol of difference

spectroscopy is applied to study minor conformational changes in a protein upon a reaction (Arrondo and Goñi, 1999).

In this study, the secondary structures in E4orf3 protein could be determined indirectly via GST-E4orf3. The reason for that was the GST moiety could not be completely released from the fusion protein. GST protein has a well-defined three-dimensional structure (McTigue et al., 1995; Zhan et al., 2001), and therefore is suitable for being a carrier protein in structural determination of E4orf3 protein. The secondary structures in E4orf3 protein can easily be derived by eliminating the known structures of GST moiety in GST-E4orf3 fusion protein. However at this point, one might argue that the inter-domain flexibility can be introduced by the GST segment, and for this reason, the determined secondary structures may not reveal the real conformation of E4orf3. Conversly, several reports have recently suggested that GST fusion system can be applied not only as a protein purification method, but also a method to determine structures of small peptides and proteins (Zhan et al., 2001). Several GST fusion proteins have been used successfully to determine the structures of target proteins or peptides, such as GST-gp41 (Lim et al., 1994), GST-MAB (Zhang et al., 1998), and GST-NMTS (Tang et al., 1998). In these studies, the crystal structures of the GST fusion peptides were determined and it has been observed that the crystal structures of the fusion peptide and the GST domain display two well-separated, independently folded motives. Moreover, the structures of the GST domain in the fusion proteins also have been shown not to be affected by the presence of fusion segment. Interestingly, in all of these three studies, target protein fragment was cloned in pGEX-2T vector which contains a thrombin cleavage site between the GST and the target protein. The last eight amino acids in *Schistosoma japonicum* GST forms an open loop and the thrombin site present after this open loop is believed to serve as a flexible linker between the GST domain and the fused protein domains (Zhan et al., 2001). Taken together, those considerations strongly suggest that confirmation of the fused peptide or protein will not be influenced by the presence of GST protein. It

should be noted that, for the expression of GST-E4orf3, a derivative of pGEX-2T plasmid, pGEX-2TK, containing the same thrombin site was used and the open loop structure was detected in the curve fitting analysis (see Fig. 4.16). Therefore, it can be assumed that the conformation of E4orf3 was not affected by its GST partner.

Thus, the secondary structures in Ad5 E4orf3 protein was determined by FTIR spectroscopy and the predicted structures were reliable although the protein was present as a GST fusion in glutathione sepharose beads. In spite of being a low resolution technique that can not resolve whole protein structures at atomic level, FTIR spectroscopy is an extremely powerful tool in study of protein secondary structures. Even if X-ray crystallography is the best method for detailed protein structure analysis, obtaining three-dimensional crystals of a protein is not easy, especially for the proteins containing hydrophobic regions that need special protocols for their expression and purification. For instance, the number of membrane proteins that have been crystallized is very small (Arrondo and Goñi, 1999). NMR spectroscopy offers an alternative to X-ray crystallography for obtaining detailed three-dimensional structures but the interpretation of NMR spectra is very complex (Haris and Severcan, 1999) and the protein in glutathione sepharose beads is probably not a good sample for NMR. Furthermore, both techniques requires proteins in high amounts and high levels of purity. Conversly, the amount of protein required for FTIR spectroscopy is relatively small (10 μg; Haris and Severcan, 1999) and as mentioned above, the spectrum of the proteins can be obtained in glutathione sepharose beads. Under these circumstances FTIR spectroscopy seems to be the best method for studying the conformation of E4orf3 which was able to be expressed and purified for the first time by the protocol given in this study. As a result, this protocol for obtaining pure E4orf3 in soluble form is subject to further improvement and the structural data obtained about this protein will certainly provide a base for analyzing the three-dimensional structure of E4orf3 by high resolution techniques.

In conclusion, the results of this study will contribute to understanding of the structural and functional properties of E4orf3, which is very important for developing adenovirus vectors.

REFERENCES

Akli, S., Caillaud, C., Vigne, E., Stratford-Perricauded, L. D., Poenaru, L., Perricauded, M., Kahn, A., and Peschanski, M. R., 1993, Transfer of a foreign gene in to the brain using adenovirus vectors, Nat Genet., 3, 224-228.

Anderson, C. W., Schmitt, R. C., Smart, J. E., Lewis, J. B., 1984, Early region 1B of adenovirus 2 encodes two coterminal proteins of 495 and 155 amino acid residues, J. Virol., 71, 4599-4613.

Ankerst, J. and Jonsson, N., 1989, Adenovirus type 9-induced tumorigenesis in the rat mammary gland related to sex hormonal state, J Natl Cancer Inst., 81(4), 294-298.

Armentano, D., Zabner, J., Sacks, C., Sookdeo, C. C., Smith, M. P., St. George, J. A., Wadsworth, S. C., Smith, A. E. and Gregory, R. J.,1997, Effect of the E4 region on the persistence of transgene expression from adenovirus vectors, J. Virol., 71, 2408-2416.

Arrondo, J. L. R. and Goñi, F. M., 1999, Structure and dynamics of membrane proteins as studied by infrared spectroscopy, Progress in Biophysics and Molecular Biology 72, 365-405.

Arrondo, J. L. R., Goñi, F. M. and Macarulla, J. M., 1984, Infrared spectroscopy of phosphatidylcholines in aqueous suspension a study of the phosphate group vibrations, BBA, 794, 165-168.

Baron, S., 1996, Medical Microbiology, Fourth Edition, University of Texas Medical Branch at Galveston.

Benihoud, K., Yeh, P. and Perricaudet, M., 1999, Adenovirus vectors for gene delivery, Curr. Opin. Biotech., 10, 440-447.

Benko, M. and Harrach, B., 1998, A proposal for a new (third) genus within the family Adenoviridae, Arch Virol., 143(4), 829-837.

Bernards, R. and van der Eb, A. J., 1984, Adenovirus: Transformation and Oncogenicity, Biochim. Biophys. Acta., 783, 187-204.

Bischoff, J. R., Kirn, D. H., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., Nye, J. A., Sampson Johannes, A., Fattaey, A. and McCormick, F., 1996, An adenovirus mutant that replicates selectively in p53-deficient human tumor cells, Science, 274(5286), 373-376.

Bishia, W. R., Rappuoli, R., and Murphy, J. R., 1987, High-level expression of a proteolytically sensitive diptheria toxin fragment in Esherisia coli, J. Bacteriol., 169, 5140-5151.

Boyar, H., Zorlu, F., Mut, M., Severcan, F., 2004, The effects of chronic hypoperfusion on rat cranial bone mineral and organic matrix: A Fourier transform infrared spectroscopy study, Anal Bioanal Chem., 379, 433-438.

Boyer, J., Rohleder, K. and Ketner, G., 1999, Adenovirus E4 34k and E4 11k inhibit double strand break repair and are physically associated with the cellular DNA-dependent protein kinase, Virology, 263, 307-312.

Bradford, M. M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal., Biochem. 72, 248-254.

Bridge, E. and Ketner, G., 1989, Redundant control of adenovirus late gene expression by early region 4, J. Virol., 63(2), 631-638.

Bridge, E. and Ketner, G., 1990, Interaction of adenoviral E4 and E1b products in late gene expression, Virology, 174(2), 345-353.

Bullock, W. O., Fernandez, J. M. and Short, J. M., 1987, XL1-Blue: A high efficiency plasmid transforming recA Escherichia coli strain with b-galactosidase selection, Biotechniques, 5, 376-379.

Cakmak, G., Togan, I., Uguz, C., Severcan F., 2003, FT-IR spectroscopic analysis of rainbow trout liver exposed to nonylphenol, Applied Spectroscopy, 57, 835-841.

Campbell, I. D. and Dwek, R. A., 1984, Biological spectroscopy, Benjamin/Cummings Publishing Company, USA, 1-60.

Carvalho, T., Seeler, J. S., Ohman, K., Jordan, P., Pettersson, U., Akusjärvi, G., Carmo Fonseca, M. and Dejean, A., 1995, Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies, J. Cell. Biol., 131(1), 45-56.

Chauvin, C., Suh, M., Remy, C., and Benabid, A. L., 1990, Failure to detect viral genomic sequences of three viruses (herpes simplex, simian virus 40 and adenovirus) in human and rat brain tumors, Ital. J. Neurol. Sci., 11(4), 347-357.

Chirmule, N., Hughes, J. V., Gao, G. P., Raper S. E., Wilson, J. M., 1998, Role of E4 in eliciting C4 T-cell and B-cell responses to adenovirus vectors delivered to murine and nonhuman primate lungs, J Virol., 72, 6138-6145.

Crabtree, K. D., Gerba, C. P., Rose, J. B., and Haas, C. N., 1997, Waterborne adenovirus: A risk assessment, Water Science and Tech., 35, 1-6.

Cress, W. D. and Nevins, J. R., 1996, Use of the E2F transcription factor by DNA tumor virus regulatory proteins, Curr Top Microbiol Immunol., 208, 63-78.

Cutt, J. R., Shenk, T. and Hearing P., 1987, Analysis of adenovirus early region 4-encoded polypeptides synthesized in productively infected cells, J Virol., 61(2), 543-552.

Danthinne, X. and Imperiale, M. J., 2000, Production of first generation adenovirus vectors: a review, Gene Therapy, 7, 1707-1714.

Debbas, M. and White, E., 1993, Wild-type p53 mediated apoptosis by E1A, which is inhibited by E1B, Genes Dev., 7, 546-554.

Dix, I. and Leppard, K.,1995, Expression of adenovirus type 5 E4 Orf2 protein during lytic infection, J. Gen. Virol., 76, 1051-1055.

Dobbelstein, M., Roth, J., Kimberly, W. T., Levine, A. J. and Shenk, T., 1997, Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a rev-like signal sequence, EMBO J., 16, 4276-4284.

Dobner, T. and Kzhyshkowska, J., 2001, Nuclear export of adenovirus RNA, Curr Top Microbiol Immunol., 259, 25-54.

Doronin, K., Kuppuswamy, M., Toth, K., Toffelson, A., Krajcsi, P., Krougliak, V., Wold, W. S. M., 2001, Tissue-specific, tumor-selective, replication-competent adenovirus vectors for cancer gene therapy, J. Virol., 75, 3314-3324.

Doucas, V., Ishov, A. M., Romo, A., Juguilon, H., Weitzman, M. D., Evans, R. M. and Maul, G. G., 1996, Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure, Genes Dev., 10, 196-207.

Dulbecco, R. and Freeman, G., 1959, Plaque production by the polyoma virus, Virology, 8, 396-397.

Dirlik, O. Ö., 2004, Adenovirüs tip 5 e4 orf3 mutant virüslerinin hazırlanması, üretilmesi ve karakterize edilmesi, Hacettepe Üniversitesi Fen Bilimleri Enstitüsü Yüksek Lisans Tezi.

Endter, C., Kzhyshkowska, J., Stauber, R. and Dobner, T., 2001, SUMO-1 modification required for transformation by adenovirus type 5 early region 1B 55-kDa oncoprotein, Proc Natl Acad Sci U S A, 98(20), 11312-11317.

Endter, C. and Dobner, T., 2003, Cell Transformation by Human Adenoviruses, Curr. Top. Microbiol. Immunol., 273, 163-214.

Esche, H and Siegmann, B., 1982, Expression of early viral gene products in adenovirus type 12-infected and -transformed cells, J Gen Virol., 60, 99-113.

Fabian, H., Naumann, D., Misselwitz, R., Ristau, O., Gerlach, D., Welfle, H., 1992, Secondary structure of streptokinase in aqueous solution: a Fourier transform infrared spectroscopic study, Biochemistry, 31, 6532- 6538.

Federal Drug Administration, USA, 2004, http://www.fda.gov, 2004.

Filip, C., Fletcher, G., Wullf, J. L., and Earhart, C. F., 1973, Solubilization of the cytoplasmic membrane of *Escherischia coli* by the ionic detergent sodium-lauryl sarcosinate, J Bacteriol., 115, 717-722.

Frangioni, J. V., Beahm, P. H., Shifrin, V., Jost, C. A., and Neel, B. G., 1992, The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence, Cell, 68, 545-560.

Frangioni, J. V. And Neel, B. G., 1993, Solubilization and purification of enzymatically active glutathione-s-transferase (pgex) fusion proteins, Analytical Biochem., 210, 179-187.

Frankel, S., Sohn, R., Leinwand, L., 1991, The use of sarkosyl in generating soluble protein after bacterial expression, Proc. Natl. Acad. Sci., USA, 88, 1192-1196.

Freifelder, D., 1982, Physical biochemistry: Applications of biochemistry and molecular biology, Freeman W. H. and Company, USA, 494-536.

Freyer, G. A., Katoh, Y. and Roberts, R. J., 1984, Characterization of the major mRNAs from adenovirus 2 early region 4 by cDNA cloning and sequencing, Nucleic Acids Res., 12(8), 3503-3519.

Gabler, S., Schütt, H., Groitl, P., Wolf, H., Shenk, T. and Dobner, T.,1998, E1B 55-kilodalton-associated protein: a cellular protein with RNA- binding activity implicated in nucleocytoplasmic transport of adenovirus and cellular mRNAs, J Virol., 72(10), 7960-7971.

Gallimore, P. H., 1984, Interactions of adenovirus type 2 with rat embryo cells. Permissiveness, transformation and in vitro characteristics of adenovirus transformed rat embryo cells, J Gen Virol., 25(2), 263-273.

Gallimore, P. H., and Turnell, A. S., 2001, Adenovirus E1A: remodelling the host cell, a life or death experience, Oncogene, 20, 7824-7835.

Ganly, I., Kirn, D., Eckhardt, S. G., Rodriguez, D. S., Soutar, D. S., Otto, R., Robertson, A. G., Park, O., Gulley, M. L., Heise, C., Von Hoff, D. D., Kaye, S. B., 2000, A phase I study pf Onyx-015, an E1B attenuated adenovirus, administrated intratumorally to patients with recurrent head and neck cancer, Clin. Cancer Res., 6, 798-806.

Gey, G. O., Coffman, W. D. and Kubicek, M. T., 1952, Tissue culture studies of a proliferative capacity of cervical carcinoma and normal epithelium, Cancer Res., 12, 264-265.

Giard, D. J., Aaronson, S. A., Todaro, G. J., Arnstein, P., Kersey, J. H., Dosik, H. and Parks, W. P., 1973, In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors, J Natl Cancer Inst., 51(5), 1417-1423.

Goodrum, F., Shenk, T. and Ornelles, D. A., 1996, Adenovirus early region 4 34-kilodalton protein directs the nuclear localization of the early region 1B 55-kilodalton protein in primate cells, J. Virol., 70, 6323-6335.

Graham, F. L. and van der Eb, A. J., 1973, A new technique for the assay of infectivity of human adenovirus 5 DNA, Virology, 52(2), 456-467.

Graham, F. L., 1984, Transformation by and oncogenicity of human adenoviruses, In “The adenoviruses” (H. S. Ginsberg, Ed.), pp. 339-398. Plenum Press, New York.

Graham, F. L., Smiley, J., Russell, W. C. and Nairn, R., 1977, Characteristics of a human cell line transformed by DNA from human adenovirus type 5, J Gen Virol., 36(1), 59-74.

Halbert, D. N., Cutt, J. R. and Shenk, T., 1985, Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff, J. Virol., 56(1), 250-257.

Hanahan, D., 1983, Studies on transformation of Escherichia coli with plasmids, J Mol Biol., 166(4), 557-580.

Harada, J. N. and Berk, A. J., 1999, p53-independent and –dependent requirements for E1B-55K in adenovirus type 5 replication, J Virol., 73(7), 5333-5344.

Harada, J. N., Shevchenko, A., Shevchenko, A., Pallas, D. C. and Berk, A. J., 2002, Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery, J Virol., 76(18), 9194-9206.

Haris, P. I., and Chapman, D., 1992, Does Fourier-transform infrared spectroscopy provide useful information on protein structures?, Trends Biochem Sci., 17, 328.

Haris, P. I., and Severcan, F., 1999, FTIR spectroscopic characterization of protein structure in aqueous and non-aqueous media, J Mol. Catal. B-Enzymatic 7, 207-221.

Harlow, E. and Lane, D. P., 1988, "Antibodies, a laboratory manual." (E. Harlow, and D. P. Lane, Eds.), Cold Spring Harbor.

Hearing, P., Samulski, R. J., Wishart, W. L., and Shenk, T., 1987, Identification of a repeated element required for efficient encapsidation of the adenovirus type 5 chromosome, J. Virol., 61, 2555-2558.

Heise, C., Sampson-Johannes, A., Williams, A., McCormick, F., Von Hoff, D. D. and Kirn, D. H., 1997, ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents, Nat Med., 3(6), 639-645.

Helenius, A., McCaslin, D., Fries, E., and Tanford, C., 1979, Properties of detergents, Methods Enzymol., 56, 734-749.

Herisse, J., Rigolet, M., de Dinechin, S. D. and Galibert, F., 1981, Nucleotide sequence of adenovirus 2 DNA fragment encoding for the carboxylic region of the fiber protein and the entire E4 region, Nucleic Acids Res., 9(16), 4023-4042.

Horwitz, M. S., 1996, Adenoviruses. Third ed. In “Virology” (B. N. Fields, D. M. Knipe und P. M. Howley, Eds.), Vol. 2, pp. 2149-2171. Lippincott-Raven, New York.

Howitt, J., Anderson, C. W., and Freimuth, H., 2003, Adenovirus interaction with its cellular receptor CAR, Curr Top Microbiol Immunol., 272, 331-364.

Huang, M. M. and Hearing, P., 1989, Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection, J. Virol., 63(6), 2605-2615.

Hutton, F. G., Turnell, A. S., Gallimore, P. H. and Grand, R. J., 2000, Consequences of disruption of the interaction between p53 and the larger adenovirus early region 1B protein in adenovirus E1 transformed human cells, Oncogene, 19(3), 452-462.

ICTV, http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/00.001.htm, 2003.

Imperiale, M. J., Akusjiaervi, G., and Leppard, K. N., 1995, Post-transcriptional control of adenovirus gene expression, Curr Top Microbiol Immunol., 199, 139-171.

Jackson, M., and Mantsch, H. H., 1996, Biomedical Infrared Spectroscopy, In: Mantsch, H. H. And Chapman, D. (eds), Infrared Spectroscopy of Biomolecules, Wiley-Liss Inc., USA, 311-340.

Jaffe, H. A., Danel, C., Longenecker, G., Metzger, M., Setoguchi, Y., Rosenfeld, M. A., Gant, T. W., Thorgeirsson, S. S., Stratford-Perricauded, L. D., Perricauded, M., 1992, Adenovirus mediated in vivo gene transfer and expression in normal rat liver, Nat Genet., 1, 372-378.

Jang, W., Weber, J. S., Bashir, R., Bushby, K., and Meisler, M. H., 1996, AUP1, a novel gene on mouse chromosome 6 and human chromosome 2p 13, Genomics, 36, 366-368.

Javier, R., Raska, K., Jr., Macdonald, G. J. and Shenk, T., 1991, Human adenovirus type 9-induced rat mammary tumors. J Virol., 65(6), 3192-3202.

Jiang, S., Noble, R. and Weiping, C., 2001, Human adenovirus and coliphages in urban runoff-impacted coastal waters of southern California, Applied and Environmental Microbiology, 67, 179-184.

Kato, A., Norihiko, K., Kenji, T., Motoki, E., Rika, M., Tsutomu, F., Kimie, M., and Kazuo, O., 2002, Ancient ubiquitous protein 1 binds to the conserved membrane-proximal sequence of the cytoplasmic tail of the Integrin α subunits that plays a crucial role in the inside-out signaling of $\alpha_{IIb}\beta_3$, J Biol Chem., 277, 28934-28941.

Kirn, D., Hermiston, T. and McCormick, F., 1998, ONYX-015: clinical data are encouraging, Nat Med., 4(12), 1341-1342.

König, C., Roth, J. and Dobbelstein, M., 1999, Adenovirus type 5 E4orf3 protein relieves p53 inhibition by E1B-55-kilodalton protein, J. Virol., 73(3), 2253-2262.

Krätzer, F., Rosorius, O., Heger, P., Hirschmann, N., Dobner, T., Hauber, J. and Stauber, R. H., 2000, The adenovirus type 5 E1B-55k oncoprotein is a highly active shuttle protein and shuttling is independent of E4orf6, p53 and Mdm2, Oncogene, 19, 850-857.

Krimm, S., and Bandekar, J., 1986, Vibrational spectroscopy and conformation of peptides, polypeptides and proteins, Adv. Prot. Chem., 38, 181-364.

Kzhyshkowska, J., Schütt, H., Liss, M., Kremmer, E., Stauber, R., Wolf, H. and Dobner, T., 2001, Heterogeneous nuclear ribonucleoprotein E1B-AP5 is methylated in its Arg-Gly-Gly (RGG) box and interacts with human arginine methyltransferase HRMT1L1, Biochem J., 358(Pt 2), 305-314.

Laemmli, U. K., 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, 227(259), 680-685.

Leppard, K. N. and Everett, R. D.,1999, The adenovirus type 5 E1b 55K and E4 Orf3 proteins associate in infected cells and affect ND10 components, J. Gen. Virol., 80(Pt 4), 997-1008.

Lim, K., Ho, J. X., Keeling, K., Gilliland, G. L., Ji, X., Rüker, F., and Carter, D. C., 1994, Three-dimensional structure of *Schistosoma japonicum* glutathione S-transferase fused with a six-amino acid conserved neutralizing epitope of gp41 from HIV, Protein Sci., 3, 2233-2244.

Lusky, M., Christ, M., Rittner, K., Dieterle, A., Dreyer, D., Mourot, B., Schultz, H., Stoeckel, F., Pavirani, A. and Mehtali, M., 1998, In vitro and in vivo biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted, J Virol., 72(3), 2022-2032.

Mackey, J. K., Rigden, P. M. and Green, M.,1976, Do highly oncogenic group A human adenoviruses cause human cancer? Analysis of human tumors for adenovirus 12 transforming DNA sequences, Proc. Natl. Acad. Sci. U S A, 73(12), 4657-4661.

Mackey, J. K., Green, M., Wold, W. S. M. and Ridgen, P., 1979, Analysis of human cancer DNA for DNA sequences of human adenovirus type 4, J. Natl. Cancer Inst., 62, 23-26.

Manoharan, R., Baraga, J. J., Rava, R. P., Dasari, R. R., Fitzmaurice, M., Feld, M. S., 1993, Biochemical analysis and mapping of adherosclerotic human artery using FTIR microspectroscopy, Adherosclerosis, 103, 181-193.

Marston, F. A. O., and Hartley, D. L., 1990, Solubilization of protein aggregates, Methods Enzymol., 182, 264-276.

McNally, E., Sohn, R., Frankel, S., and Leinwand, L., 1991, Expression of myosin and actin in *Escherischia coli*, Methods Enzymol., 196, 368-389.

McTigue, M. A., Williams, D. R., Tainer, J. A., 1995, Crystal structures of a schistosomal drug and vaccine targer: glutathione S-transferase from *Schistosoma japonica* and its complex with the leading antischistosomal drug praziquantel, J. Mol. Biol., 246, 21-27.

Mendelsohn, R., and Mantsch, H. H., 1986, Fourier Transform Infrared Studies of Lipid-Protein Interaction, In: Watts, A. and De Pont, J. J. H. H. M. (eds), Progress in Lipid Interactions, Vol. 2, Elsevier Science Publishers, Netherlands, 103-147.

Mitraki, A., and King, J., 1989, Bio Technology, 7, 690-697.

Modrow, S. and Falke, D., 1997, “Molekulare Virologie.” Spektrum Akademischer Verlag GmbH, Heidelberg.

Moolenaar, W. H., 1995, Lysophosphatidic acid, a multifunctional phospholipid messenger, J Biol Chem., 270, 12949-12952.

National institute of health, http://www.ncbi.nih.gov/blast, 2004

Nevels, M., Spruss, T., Wolf, H., Dobner, T., 1999a, The adenovirus E4orf6 protein contributes to malignant transformation by antagonizing E1A-induced accumulation of the tumor suppressor protein p53, Oncogene, 18, 9-17.

Nevels, M., Täuber, B., Kremmer, E., Spruss, T., Wolf, H. and Dobner, T., 1999b, Transforming potential of the adenovirus type 5 E4orf3 protein, J. Virol., 73(2), 1591-1600.

Nevels, M., Rubenwolf, S., Spruss, T., Wolf, H. and Dobner, T., 2000, Two distinct activities contribute to the oncogenic potential of the adenovirus type 5 E4orf6 protein, J. Virol., 74, 5168-5181.

Nevins, J. R. and Vogt, P. K., 1996, Cell transformation by viruses. Third ed. In “Virology” (B. N. Fields, D. M. Knipe und P. M. Howley, Eds.), Vol. 1, pp. 301-343. Lippincott-Raven, New York.

Nevels, M., Täuber, B., Spruss, T., Wolf, H. and Dobner, T., 2001, "Hit-and-run" transformation by adenovirus oncogenes, J. Virol., 75, 3089-3094.

Öhman, K., Nordquist, K., Linder, S. and Äkusjärvi, G., 1995, Effect of adenovirus-2 early region 4 products on E1 transformation, International Journal of Oncology, 6, 663-668.

Ornelles, D. A. and Shenk, T., 1991, Localization of the adenovirus early region 1B 55-kilodalton protein during lytic infection: association with nuclear viral inclusions requires the early region 4 34-kilodalton protein, J Virol., 65(1):424-429.

Pääbo, S., Severinsson, L., Anderson, M., Martens, I., Nilsson, T., and Peterson, P. A., 1989, Adenovirus proteins and MHC expression, Adv Cancer Res., 52, 151-163.

Philipson, L., Pettersson, U. and Lindberg, U., 1975, Molecular Biology of Adenoviruses, New York, Springer verlag, series title: Virology monographs 14, 115 p.

Paschalis, E. P., Verdelis, K., Doty, S. B., Boskey, A. L., Mendelshon, R., Yamauchi, M., 2001, Spectroscopic characterization of collagen cross-links in bone, J Bone Miner Res., 16, 1821-1828.

Picquart, M., Grajcar, I., Baron, M. H., Abedinzadeh, Z., 1999, Vibrational spectroscopic study of glutathione complexation in aqueous solutions, Biospectroscopy 5, 328-337.

Pilchler, A. and Melchior, F., 2002, Ubiquitin-related modifier SUMO1 and nucleocytoplasmic transport, Traffic, 3(6), 381-387.

Qbiogen, http://www.adenovirus.com, 2002.

Querido, E., Morrison, M. R., Chu-Pham-Dang, H., Thirlwell, S. W., Boivin, D., Branton, P. E. and Morisson, M. R., 2001, Identification of three functions of the adenovirus e4orf6 protein that mediate p53 degradation by the E4orf6-E1B55K complex, J Virol., 75(2), 699-709.

Ragot, T., Vincent, N., Chaffey, P., Vigne, E., Gilgenkrantz, H., Couton, D., Cartaut, J., Briant, P., Kaplan, J. C., Perricauded, M., 1993, Efficient adenovirus mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice, Nature, 361, 647-650.

Rowe, W. P., Huebner, R. J., Gilmore, L. K., Parrot, R. H. and Ward, T. G., 1953, Isolation of a cytopathogenic agent from human adenoids undergoing sponateous degeneration in tissue culture, Proc. Soc. Exp. Biol. Med., 84, 570-573.

Ruley, H. E., 1983, Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture, Nature, 304, 602-606.

Sabbatini, P., Chiou, S. K., Rao, L., White, E., 1995, Modulation of p53-mediated transcriptional repression and apoptosis by the adenovirus E1B 19K protein, Mol. Cell. Biol., 15, 1060-1070.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A., 1988, Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase, Science, 239(4839), 487-491.

Sambrook, J., Fritsch, E. F. and Maniatis, T., 1989, Molecular cloning: A laboratory manual, Ford, N., Nolan, C. ve Ferguson, M. (eds.), 2 Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Schein, C. H., 1989, Production of soluble recombinant proteins in bacteria, Bio/Technology, 7, 1141-1149.

Sharma, R. C. and Schimke, R. T., 1996, Preparation of electrocompetent E. coli using salt-free growth medium, Biotechniques, 20(1), 42-44.

Shenk, T., 1996, Adenoviridae: the viruses and their replication. Third ed. In “Virology”, Fields, B. N., Knipe, D. M. ve Howley, P. M. (eds.), Vol. 2, Lippincott-Raven, New York.

Skinner, G. R., 1976, Transformation of primary hamster embryo fibroblasts by type 2 simplex virus: evidence for a "hit and run" mechanism, Br. J. Exp. Pathol., 57(4), 361-376.

Smith, D. B. and Corcoran, L. M., in Current Protocols in Molecular Biology Vol. 2 (Ausubel, F. M. *et al*., eds.), John Wiley and Sons, Inc., New York, 1998, p. 16.7.1.

Smith, D. B., Davern, K. M., Board, P. G., Tiu, W. U., Garcia, E. G., and Mitchell, G. F., 1986, Mr26000 antigen of Schistosoma japonicum recognized by resistant WEHI 129/J mice is a parasite glutathione S-transferase, Proc Natl Acad Sci., USA, 83, 8703-8707.

Smith, D. B. and Johnson, K. S., 1988, Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase, Gene 67(1), 31-40.

Smith, G. C. M., and Jackson, S. P., 1999, The DNA dependant protein kinase, Genes Dev. 13, 916-934.

Sternsdorf, T., Grothzinger, T., Jensen, K., Wil, H., 1997, Nuclear dots: Actors on many stages, Immunobiology, 198, 307-331.

Stuart, B., 1997, biological applications of infrared spectroscopy, John Wiley and Sons, England.

Surewicz, W. K., Mantsch, H. H., and Chapman, D., 1993, Determination of protein secondary structure by Fourier transform infrared spectroscopy: a critical assesment, Biochemistry, 32, 299.

Tang, L., Guo, B., van Wijnen, A. J., Lian, J. B., Stein, J. L., Stein, G. S., Zhou, G. W., 1998, Preliminary chrystallographic study of glutathione S-transferase fused with the nuclear matrix targeting signal of the transcription factor AML-1/CBF-α2, J Struct Biol., 123, 83-85.

Täuber, B. and Dobner, T., 2001, Molecular regulation and biological function of adenovirus early genes: the E4 ORFs, Gene, 278(1-2), 1-23.

Telling, G. C. and Williams, J., 1993, The E1B 19-kilodalton protein is not essential for transformation of rodent cells in vitro by adenovirus type 5, J Virol., 67(3), 1600-1611.

Thomas, D. L., Schaak, J., Vogel, H. and Javier, R., 2001, Several E4 region functions influence mammary tumorigenesis by human adenovirus type 9, J Virol ., 75, 557-568.

Trentin, J. J., Yabe, Y. and Taylor, G., 1962, The quest for human cancer viruses, Science, 137, 835-849.

Ustaçelebi, Ş., 1992, Genel Viroloji, Hacettepe Taş Kitapçılık Ltd. Şti., 169s.

Virtanen, A., Gilardi, P., Naslund, A., LeMoullec, J. M., Pettersson, U. and Perricaudet, M., 1984, mRNAs from human adenovirus 2 early region 4, J. Virol., 51(3), 822-831.

Waeber, U., Buhr, A., Schunk, T., and Emi, B., 1993, The glucose transporter of *Escherischia coli*: Purification and characterization by Ni^{+2} chelate affinity chromatography of $IIBC^{Glc}$ subunit, FEBS Letters, 324, 109-112.

Weiden, M. D. and Ginsberg, H. S., 1994, Deletion of the E4 region of the genome produces adenovirus DNA concatemers, Proc. Natl. Acad. Sci. USA, 91(1), 153-157.

Wickham, T., J., 2000, Targetting adenovirus, Gene Ther., 7, 110-114.

Wienzek, S., Roth, J. and Dobbelstein, M., 2000, E1B 55-kilodalton oncoproteins of adenovirus types 5 and 12 inactivate and relocalize p53, but not p51 or p73, and cooperate with E4orf6 proteins to destabilize p53, J Virol., 74(1), 193-202.

Williams, J., Williams, M., Liu, C. and Telling, G., 1995, Assessing the role of E1A in the differential oncogenicity of group A and group C human adenoviruses, Curr Top Microbiol Immunol., 199(Pt 3), 149-175.
van der Eb, A. J., and Zantema, A., 1992, Adenovirus Oncogenesis, In: Dörfler, W., Böhm, P. (eds), Malignant transformation by DNA viruses. VCH, Weinheim, 115-140.

Yew, P. R. and Berk, A. J., 1992, Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein, Nature, 357, 82-85.

Zalmanzon, E. S., 1987, Transforming and oncogenic properties of the adenovirus genome, Eksp. Onkol., 9(3), 3-8.

Zhan, Y., Song, X., Zhou, W., 2001, Structural analysis of regulatory protein domains using GST-fusion proteins, Gene, 281, 1-9.

Zhang, Z., Devarajan, P., Dorfman, A. L., and Morrow, J. S., 1998, Structure of ankyrin-binding domain of α-Na, K-ATPase, J Biol Chem., 273, 18681-18684.