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Transactivation by human Adenovirus Early Region 1A-Conserved Region Three

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology

TRANSACTIVATION BY HUMAN ADENOVIRUS EARLY REGION 1A-CONSERVED REGION THREE

(Transactivation by Human Adenovirus E1A-Conserved Region 3)

(Thesis format: Integrated Article)

by

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Graduate Program in Microbiology & Immunology

A thesis submitted in partial fulfillment
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Abstract

One of the critical functions of human adenovirus (hAd) early region 1A (E1A) protein is to activate transcription of the early viral genes. The largest isoform of E1A contains a unique region termed conserved region 3 (CR3), which includes a Cysteine-4 (C4) zinc finger domain. This region activates viral gene expression by interacting with and recruiting cellular transcription machinery to the regulatory regions of early viral genes. Although this process has been studied at length with hAd type 5 E1A, far less is known about how the E1A proteins from other hAd types activate transcription. There are dramatic differences in the potency of transactivation by E1A CR3s from representative hAd species that cannot be explained by the current model of E1A transactivation. I hypothesized that many of the co-activators targeted by hAd E1A CR3 are conserved between types. However, I also hypothesized that additional cellular factors specific to certain hAd E1A CR3s are also required. The cellular co-activators required by hAd5 E1A CR3 to activate transcription were demonstrated to be conserved among representative members of each hAd species. Furthermore, the cellular lysine acetyl transferase (KAT) GCN5 was identified as a novel negative regulator of E1A CR3 transactivation. The KAT activity of GCN5 was required to exert the effect on E1A CR3. Finally, the C4 zinc finger domain of CR3 is predicted to differ from the rest of E1A by exhibiting a stable structure that is critical for transactivation. A well defined stable solution structure of E1A CR3 was confirmed by NMR spectroscopy. Coordination of a single zinc ion was critical to CR3 structure and folding. Together these observations expand the existing model of E1A CR3 transactivation to not only include representative members of each hAd species, but also implicate a layer of negative regulation and provide structural insight into this paradigm of non-acidic viral transactivators.

Keywords

Human adenovirus, E1A, Conserved Region 3, transactivation, transcriptional control, protein-protein interactions, C4 zinc finger, TBP, MED23, p300/CBP, SUG1, GCN5

Co-Authorship Statement (where applicable)

The following thesis contains material from a published manuscript (Chapter 2). I participated in the design of the study and conducted all of the experiments, with the exception of p300 interaction experiments that were performed by P.Pelka (Figure 2-2C bottom panel and Figure 2-7A).

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List of Abbreviations

Abbreviation	Meaning
19S	19S Ribosomal subunit
20S	20S Ribosomal subunit
AdV	Adenovirus
AMP	Ampicillin
AP2	Activating Protein-2
APIS	ATPase proteins Independent of 20S
AR1	Auxiliary region 1
ATF	Activating Transcription Factor
BAK	Bcl2-homologue Antagonist/Killer
CAM	Chloramphenicol
CAR	Coxsackie and adenovirus receptor
CBP	CREB-binding protein
CDK	Cyclin dependent kinase
ChIP	Chromatin Immunoprecipitation
ChIP-SEQ	Chromatin Immunoprecipitation- Deep Sequencing
CO-IP	Co-immunoprecipitation
COPD	Chronic obstructive pulmonary disease
CPE	Cytopathic effect
CPTH2	cyclopentylidene-[4-(4′-chlorophenyl)thiazol-2-yl)hydrazone
CR	Conserved region
CREB	Cyclic AMP response element-binding protein
CtBP	C-Terminal Binding Protein
DBD	DNA binding domain
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DSS	2,2-dimethyl-2-silapentane-5-sulfonate
DYRK	Dual specificity tyrosine regulated kinase
E1A	Early region 1A
E1B	Early region 1B
FasR	Fas Receptor
GFC	Gel Filtration Chromatography
GST	Glutathione S-Transferase
H3K18	Histone H3 Lysine 18
HA	Hemagglutinin
hAd	Human adenovirus
HDAC	Histone deacetylase
HEK	Human embryonic kidney
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPV	Human papilloma virus
HSQC	Heteronuclear Single Quantum Correlation
hSUG1	Human S8 component of the 19S APIS
HSV	Herpes simplex virus
IEC	Ion Exchange Chromatography
ITR	Inverted Terminal Repeat
KAT	Lysine acetyl transferase
kbp	Kilo-basepair
kDa	Kilo-Dalton
LDS	Lithium Dodecyl sulphate
MED23	Mediator Complex Subunit 23
MEF	Mouse embryonic fibroblast
MET	Mesenchymal to Epithelial Transition
MHC	Major histocompatibility complex
MOI	Multiplicity of Infection
MRN	Mre11/Rad50/NBS1
mRNA	Messenger RNA
NLS	Nuclear localization signal
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
NP-40	Nonidet P-40
ORF	Open reading frame
PBS	Phosphate buffered saline
pCAF	p300/CBP associated factor
PKA	Protein Kinase A
PML	Promyelocytic leukemia
PP2A	protein phosphatase 2A
pRb	Retinoblastoma susceptibility gene product
PVDF	Poly-vinyl Di-fluoride
RGD	Arginine-Glycine-Aspartic Acid aa sequence
RNA	Ribonucleic acid
SAGA	Spt/Ada/Gcn5 containing complex
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEC	Size Exclusion Chormatography
SLIM	Short Linear Interactio Motif
SUMO	Small ubiquitin like protein moiety
SV40	Simian virus 40
SWI/SNF	Mating type switching/sucrose nonfermenting
TAFII	TBP-associated Factor II
TALOS	Torsion Angle Likelihood Obtained from Shift
TBP	TATA-box binding protein

TBS	Tris buffered saline
TEV	Tobacco Etch Virus
TNF-α	Tumor Necrosis Factor Alpha
TP	Terminal protein
TR	Thyroid Hormone Receptor
Tris	Tris(hydroxymethyl)aminomethane
TRRAP	transformation/transcription domain-associated factor
UBC9	Ubiquitin conjugase 9
USF	Upstream Stimulatory Factor
wt	wild-type
X2	Exon 2

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Chapter 1

1 Introduction

1.1 General Introduction

Viruses are obligate intracellular parasites—they must commandeer cellular processes in order to replicate. All viruses must dismantle host cell defenses and hijack the cellular translation machinery during their replication cycle. Despite a sinister reputation as the etiological agents of many—even pandemic—diseases, virus' intimate interaction with their respective host cells make them invaluable and discriminating biochemical tools to dissect and understand cellular processes. Studies of viral genes have led to landmark discoveries in many fields of biology. Some notable examples include, the first T-cell epitope mapped and sequenced, which was the immunodominant T-cell epitope for the vesciular stomatitis virus nucleocapsid protein (144), as well as the process of mRNA splicing, which was first observed and described using viral mRNA from adenovirus infected cells (8). Clearly, hAd as a tool is no exception, but it is the early region 1A (E1A) gene of hAd that has had a tremendous impact on our understanding of cellular processes including, but not limited to, transcription and cell cycle control. E1A is the topic of this thesis and has served as a molecular compass leading the way to a better understanding of key cellular regulatory processes.

1.2 Adenoviruses

1.2.1 Origins of Adenoviruses

Adenovirus was first discovered in the early 1950s by two distinct groups on the hunt for the causative agent of respiratory infections. In 1953, Rowe and colleagues observed unexpected death of primary human adenoid tissue that was the result of pre-existing virus present in the isolated tissues (122). One year later in 1953, while studying respiratory disease in army recruits, Hillman and Werner were able to induce cytopathic effects (CPE) in human cells with respiratory secretions from infected patients (65). In

1956, this new class of infectious agents was collectively called adenoviruses, after the tissue the prototype strain was originally isolated from. Although this virus turned out to be a major causative agent in outbreaks of respiratory disease among military recruits that Hillman and Werner were studying, adenovirus was not the etiological agent of the common cold that both groups set out to find.

Nearly ten years later in 1962, a seminal discovery by Trentin and colleagues re-ignited interest in adenoviruses. Injection of hAd type 12 (hAd12) into newborn baby hamsters, and later baboon retinas, induced malignant tumors. This marked the first human virus to cause cancer (100, 141). However, not all hAds can cause cancer in rodents. This property was originally used to help classify members of the hAd family into subgroups, now referred to as species. As a specific example, hAd5 and other members of species C do not induce tumors when injected into immunocompetent rodents (126). However, all hAds are capable of transforming rodent cells in culture (44, 45, 93, 126). Since the landmark observation that hAds could induce cancer, they have been used as tools to intensively study subversion of normal cell functions in an attempt to decipher the barriers that normally protect cells from aberrant growth. To date, hAds have never been shown to cause cancer in humans.

1.2.2 Taxonomy of Adenoviruses

Adenoviruses belong to the family *Adenoviridae,* which includes well over 100 members, and consist of five genera: *Aviadenovirus, Siadenovirus, Atadenovirus, Ichtadenovirus* and *Mastadenovirus*. The *Mastadenoviruses* include members that infect many species of mammals, but adenoviruses seldom if ever, cross species barriers; for example, human adenovirus is unable to replicate in murine cells and vice versa . Members of this family are icosehedral viral particles ranging in size from 60-90 nm in diameter, and all contain a linear double stranded DNA genome, which in the case of hAd5 is approximately 36 kilobase pairs (kbp) in size (126). There are currently more than 52 serotypes (soon to be called types, because serology is no longer used to distinguish one adenovirus from another) of hAd that are further grouped into six species, A-F, based on a series of biological properties including oncogenic potential (Table 1-1). These species of hAd

Table 1-1: The classification of the different human adenovirus serotypes into six speciess.

Subgroup		Hemagglutination groups	Serotypes	Tumors in animals	Transformation in tissue culture	% of G-C in DNA
A	IV	(little or no agglutination)	12,18,31	High	+	48-49
B	I	(complete agglutination of monkey erythrocytes)	3,7,11,14,16 21,34,35,50	Moderate	+	50-52
C	III	(partial agglutination of rat erythrocytes)	1,2,5,6	Low or none	+	57-59
D	II	(complete agglutination of rat erythrocytes)	8,9,10,13,15 17,19,20,22-30 32,33,36-39, 42-49, 51	Low or none	+	57-61
E	III		4	Low or none	+	57-59
F	III		40,41	Unknown	+	

All 52 serotypes of hAd have been classified into six subgroups based on the criteria shown. The prototypical hAd, hAd5 of subgroup C has been the subject of the vast majority of study to date. Adapted from (126).

are also quite similar at the molecular level, and in the case of E1A and hexon sequence, the original classification into species remains unchanged.

1.2.3 Epidemiology of Adenoviruses

Adenoviruses are ubiquitous in the environment. All humans will have been infected by at least one hAd type in their lifetime. Although initially thought to be linked only to mild disease in humans, more recently emerging hAd strains have been associated with more serious pathogenesis. Recent studies have implicated latent adenovirus infections as co-factors in the development of chronic obstructive pulmonary disease (COPD) (62, 106). According to the National Institute of Health, COPD was the fourth leading cause of death in the United States in 2010 (102). Perhaps more startling was the recent emergence of hAd type 14A, a new variant of hAd14 that caused severe pneumonia and resulted in the hospitalization and death of individuals with otherwise healthy immune systems (90).

1.2.4 Physical Properties of Adenoviruses

Adenoviruses are non-enveloped viral particles consisting of a linear double stranded DNA genome housed in an icosehedral protein shell or capsid. Each viral capsid consists of 252 capsomeric units, of which 240 are hexons and 12 are pentons (52). Hexons are surrounded by six capsomeres, and five capsomeres surround each penton. The protruding fiber protein is attached to a base formed by pentons and varies in length among hAd types (104, 105). The penton base and fiber protein are important for attachment and entry of hAd particles into target cells via phagocytosis. The penton base contains an arginine-glycine-aspartic acid or “RGD” motif that mediates attachment and entry into a broad range of human cells. Attachment is mediated through an interaction with cellular integrins, such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$ and is commonly referred to as fiber-independent entry (154). hAd is also capable of receptor-mediated phagocytic entry through the fiber protein. In the case of hAd5, the receptor is the coxsackie and adenovirus receptor (CAR), which is commonly found on the surface of epithelial cells (94).

All hAds posses a linear double stranded genome ranging in size from 30-38 kbp with a conserved organization. The genome has inverted terminal repeats (ITRs) at both ends and is capped with a 55 kilodalton (kDa) terminal protein covalently attached to the 5' ends of each strand through a phosphodiester bond between the terminal viral nucleotide and a conserved serine residue in the terminal protein (119). The genome is organized into nine transcriptional units: early 1A (E1A), 1B (E1B), E2, E3, E4, pIX, virus-associated RNA (VARNA), U exon and late genes, which are transcribed from both strands of the genome (Figure 1-1) (125, 140). Early genes are defined as those expressed before the onset of viral genome replication and late genes are defined as those whose expression begins with the onset of viral genome replication (126). The early genes are generally responsible for preparing the cell to replicate the viral genome and/or directly participate in the replication of the viral genome. The late transcript codes for primarily structural genes.

1.2.5 The Genes and Life Cycle of Adenoviruses

As mentioned above, the virus life cycle begins with contact between the virus particle and the target cell and internalization by phagocytosis. The adenovirus particle is then uncoated, releasing the viral core, which contains the viral genome, into the cytoplasm (89). The core then traffics to the nucleus, where transcription of the early viral transcripts begins (23).

The first gene expressed from the viral genome is E1A from a constitutively active promoter/enhancer element. E1A is the focus of this thesis, and the details of E1A function will be discussed further in later sections. Briefly, E1A has two critical functions in the viral replication cycle: 1) E1A uncouples the host cell cycle driving the cell into S-phase to create an intracellular milieu conducive to viral replication and 2) activates transcription of the early viral genes, kick-starting the replication cycle (46, 110).

The E1 region of the viral genome is composed of E1A and early region 1B (E1B). In hAd5, the E1B region codes for two proteins of 55kDa and 19kDa, referred to as E1B-55k and E1B 19k, respectively. The major function of these proteins is to counteract the

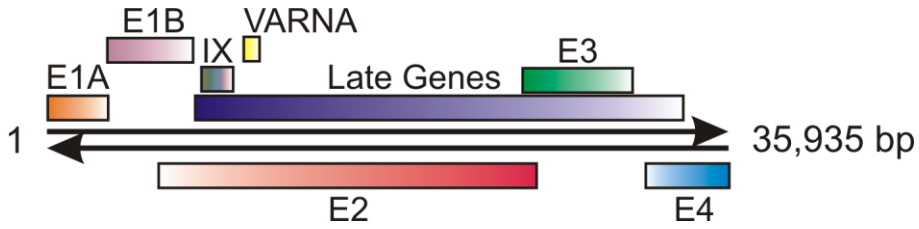

Figure 1-1: Genome Organization of human Adenoviruses

Cartoon representation of the hAd5 dsDNA genome. The hAd5 genome has eight transcriptional units, five or which are expressed early during infection: E1-4, two are expressed delayed early: IX and VARNA, and one late during infection; L. U Exon is not depicted, but located between the E3 region and the fiber gene in the late transcription unit in the left transcription direction.

p53 tumor suppressor pathway and block apoptosis that is stimulated by the effects of E1A (30). Early in infection the E1B-55k protein functions as an E3 ubiquitin ligase in concert with the cellular ubiquitin-dependent degradation pathway to target p53 for degradation, thus inactivating its downstream effects (157). Later in infection, E1B-55k functions in combination with the viral E4 gene product E4ORF6 to export viral mRNA from the nucleus to the cytoplasm to facilitate late mRNA translation (54, 55). The smaller E1B product 19K is a Bcl2-homologue that inhibits apoptosis by interacting with proteins such as BAK (Bcl2-homologues antagonist/killer) and blocking the release of mitochondrial cytochome C (27, 38, 152).

The E2 region codes for three genes critical for replication of the viral genome. The three genes encoded by the E2 region are the terminal protein (discussed above in section 1.2.4), the DNA-dependent DNA polymerase required to replicate the genome, and the single stranded DNA binding protein, which have molecular weights as mature proteins of 55, 140 and 59 kDa, respectively (19-22, 82, 147). Viral DNA replication occurs from the infecting template initially primed by the terminal protein. The 5' phosphate of the first nucleotide is covalently attached to the hydroxyl group of a conserved serine residue of the terminal protein. Viral DNA replication proceeds in the standard 5' to 3' direction by the viral DNA-dependent DNA polymerase. As single-stranded intermediates are produced by displacement of one strand of the duplex, these intermediates are bound by the viral single-stranded DNA binding protein. Progeny genomes are later capped with terminal protein and can then also serve as templates for additional progeny genomes (74).

The E3 region of the adenoviral genome contains genes that code for proteins required for evading and modulating the host immune system, thereby protecting the infected cell from the immune response. The 19k E3 glycoprotein is involved down regulating surface expression of major histocompatibility complex I (MHCI) on infected cells by disrupting endoplasmic reticulum (ER) function (16-18, 80). Moreover, the E3 10.4 and 14.4kDa proteins cooperate to uncouple the Fas signaling pathway from the Fas receptor (FasR) (25). The E3 region is also known as the hypervariable region of the adenovirus genome, as this region exhibits the most genomic sequence variability among hAd types. Based

on these characteristics, it was assumed that the newly emerged hAd14A had evolved a new ORF in the E3 region that was ultimately responsible for the increased pathogenicity. However, this was not the case; sequencing the entire genome revealed only one unique characteristic of hAd14A, a two codon deletion in the fiber gene (67, 73).

The final early region activated by E1A is the E4 region, which contains a collection of genes with quite diverse functions. As previously stated, the E4ORF6 gene product in conjunction with E1B55k plays a role in viral mRNA export. The E4ORF6/E1B55k E3 ligase complex inactivates the Mre11/Rad50/NBS1 (MRN) cellular DNA damage response, preventing the viral genome from being recognized and inactivated by non-homologous end joining (136). The E4ORF3 gene for a long time was an enigma of hAd biology, mainly because the E4ORF3-deleted virus had no detectable phenotype (127). The E4ORF3 protein was originally thought to play a role in suppressing cellular translation late in infection (128). Very recently, it was shown that the E4ORF3 protein is a redundant mechanism for silencing transcription of p53-stimulated genes by heterochromatinization of 265 p53-responsive promoters (132). This tiny protein of only 116 amino acids (aa) is capable of very specific transcriptional silencing of a select subset of genes that appears to rely in part on the SUV39H1/2 methyltransferases (132). E4ORF3 is a shining example of how critical dismantling the p53 pathway is for the viral infection cycle; hAd has at least two distinct mechanisms to inactivate the pathway at two critical points. The E4ORF4 gene codes for a protein that functions to inhibit protein phosphatase 2A (PP2A), thereby inhibiting the family of protein phosphatases that account for the majority of serine/threonine phosphatase activity present in many cells (76, 156). PP2A and E4ORF4 regulate expression of the E4 promoter by dephosphorylating E1A to limit production of the toxic gene products of the E4 region, thereby preventing premature cell death (151). This aspect of E4ORF4 function will be discussed in detail in later sections dealing with E1A-dependent transactivation.

The gene products of the viral early transcription units all cooperate to produce a cellular environment conducive to viral replication that allows expression of the late genes. The adenoviral late gene region generates multiple transcripts from the major late promoter.

The identification of the origin of the multiple late gene transcripts came from an electron microscope-based heteroduplex analysis of viral mRNA from infected cells with DNA from the late regions of the viral genome. This revealed multiple DNA loops of various sizes (8). The explanation for these loops ultimately led to the discovery and characterization of mRNA splicing in mammalian cells (8). The late transcripts code for the both structural and non-structural proteins involved in viral assembly, packaging and egress (126). These final stages of the viral replication cycle take place in the nucleus. Eventually the host cell is lysed, releasing new viral progeny and the virus life cycle repeats (126).

1.3 The Adenovirus E1A Gene and Gene Products

1.3.1 The Adenovirus E1A Gene and Transcripts

The adenovirus E1A gene is approximately 1 kb in length and is located at the extreme left end of the viral genome downstream of the left ITR (Figure 1-1). E1A is absolutely required for productive infection in human cells at low multiplicity of infection (MOI) (72). Immediately upon infection, the E1A gene of hAd is expressed. Indeed, E1A is the first viral gene detectable during infection. E1A expression is controlled by a constitutively active promoter and duplicated enhancer element (63, 64, 103). The E1A gene contains two short introns that give rise to five spliced products. The hAd5 E1A transcript is alternatively spliced to yield five different isoforms, 13S, 12S, 11S, 10S and 9S, that were named based on their sedimentation coefficients (Figure 1.2) (126). The largest E1A single-spliced products (13S and 12S) predominate early in infection, and at late times in infection the smaller double spliced products (11S, 10S and 9S) are observed (114).

1.3.2 The E1A Proteins

In hAd5 the 13S, 12S, 11S, 10S and 9S mRNAs give rise to proteins of 289 residues (R), 243R, 217R, 171R and 55R respectively (Figure 1-2). The two largest E1A isoforms are identical except for a 46 amino acid (aa) region unique to the 289R E1A isoform (114). Both the 289R and 243R isoforms of E1A are phosphorylated and phosphorylated, residues in E1A appear to be involved in protein-protein interactions (59,

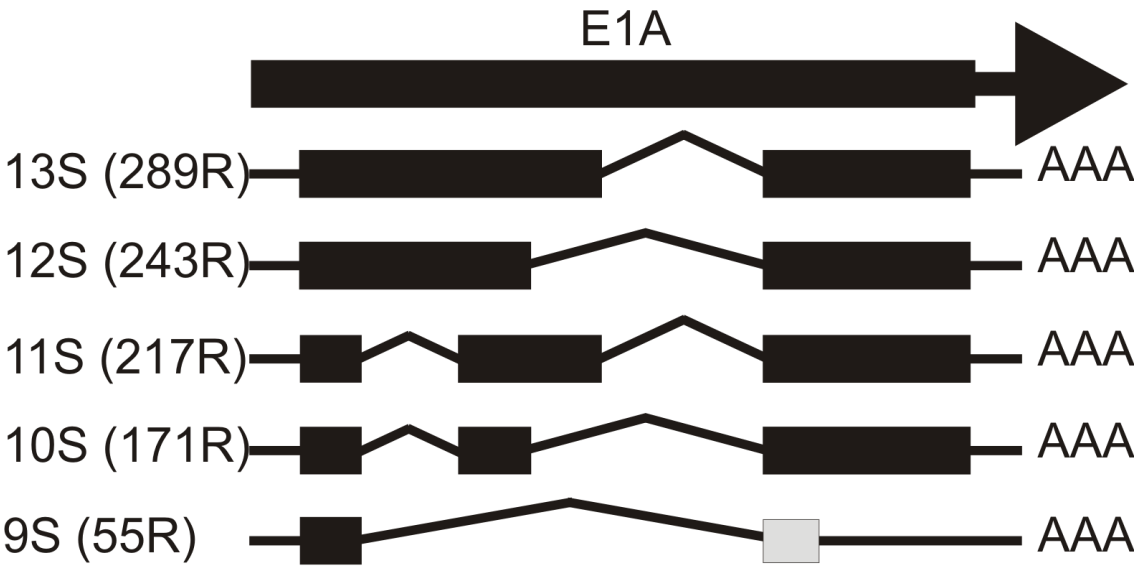

Figure 1-2: Splice products of the hAd5 E1A gene.

The hAd-5 E1A gene is alternatively spliced to yield 5 mRNA products ranging in size from 13S to 9S. These encode proteins ranging in size from 289 residues (R) to 55R. Coding regions are shaded. Note that splicing preserves the translational reading frame in all cases except for the 9S encoded 55R product (grey box).

77, 151). The two largest E1A isoforms localize to both the cytoplasm and the nucleus (121, 142). The remaining three isoforms are produced later in infection, and the precise roles of these proteins in the adenovirus life cycle remain elusive (134, 143).

Early aa sequence alignments of the largest E1A proteins from different hAd types revealed three regions of conservation, aptly named conserved regions (CR) 1, 2 and 3, separated by regions of less conservation (34, 75, 98, 107, 145). Analysis of the aa sequence of 34 human and simian adenovirus E1As refined the boundaries of these conserved regions and also revealed a fourth conserved region, CR4, which is located at the C-terminus of E1A (Figure 1-3) (3, 4). The relatively high degree of conservation in these regions suggests that common functions of different E1As critical to the viral life cycle are mediated by the conserved regions.

1.3.3 The E1A N-terminus/Conserved Region 1

In hAd5 the less conserved N-terminal region spans residues 1-41 and the boundaries of CR1 are aa's 42-72 (4). To date there are 15 cellular proteins known to interact with the 41 amino terminal residues of E1A. The majority of these cellular factors are involved in transcription, including activating protein-2 (AP2) (130), thryroid hormone receptor (TR) (95, 96, 148), p400 (48), transformation/transcription domain-associated factor (TRRAP) (31), p300/CBP associated factor (pCAF) (118), GCN5(81), TATA-binding protein (TBP) (86, 131) and most importantly, p300/CREB binding protein (CBP) (2, 35). The interaction of the N-terminus of E1A and p300/CBP is capable of inducing global changes in p300/CBP occupancy of almost 70% of cellular promoters in infected cells, which ultimately results in a global hypoacetylation of histone H3 lysine 18 (H3K18) (41, 66).

Different classes of cellular proteins also interact with the N-terminus of E1A and associate E1A with protein degradation and signaling. The N-terminus of E1A interacts with the S8 (SUG1) and S4 components of the 19S proteasome subunit. This interaction intimately links E1A function to alterations in the regulated turnover of individual E1A molecules and its cellular targets (57, 117, 142). The N-terminus of E1A also interacts with a series of cellular proteins that feed into protein-protein signaling networks. These

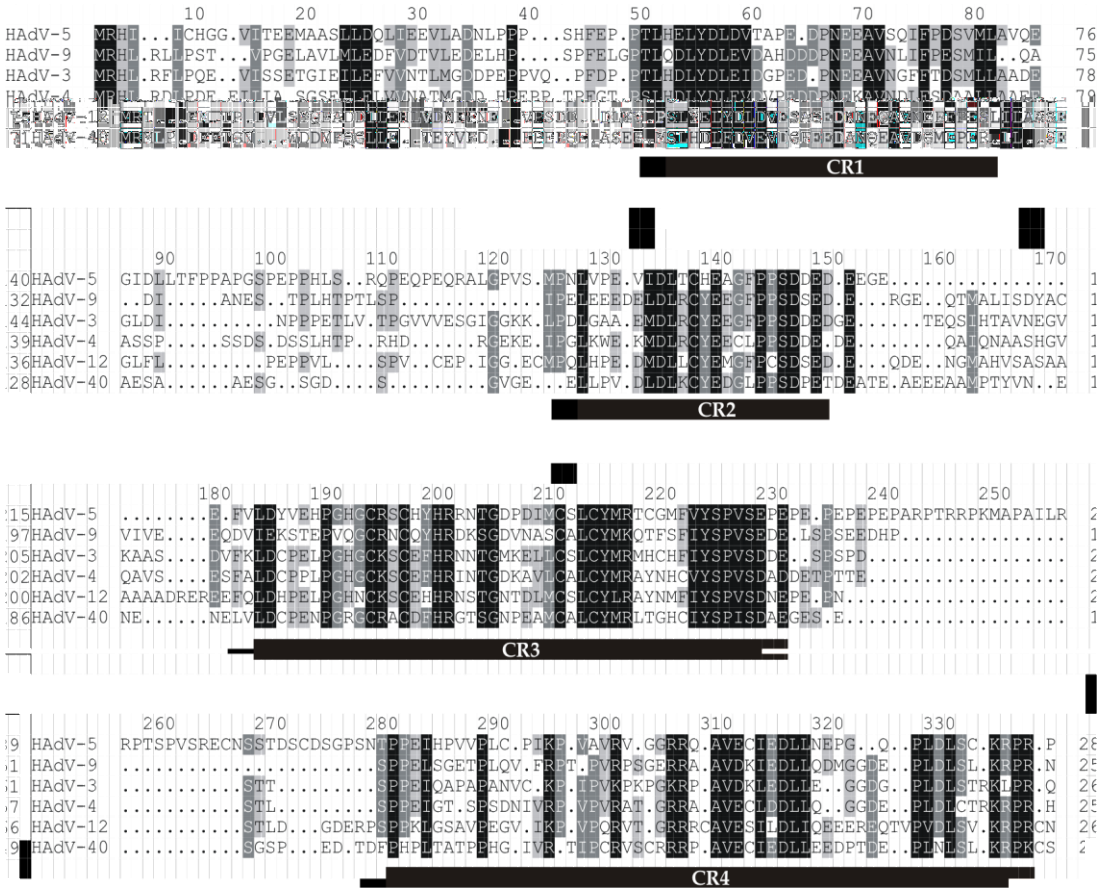

Figure 1-3: Alignment of the E1A proteins of hAd.

The sequences of one representative E1A protein from each of the six hAd subgroups were aligned and shaded for conservation. Darker shading corresponds to higher levels of conservation. Gaps are indicated as dots. The positions of the CRs are indicated as solid bars. Adapted from Avvakumov et al. 2004.

include the Ran GTPase (29), the protein kinases Nek9 (113) and protein kinase A (PKA), via the RIIα subunit (40). These signal transduction proteins interact with conserved elements of the N-terminus of E1A. Upon examination of this impressive list of cellular factors that interact with the first 41 residues of E1A, the model of how E1A facilitates such diverse functions of transformation, S phase induction and transcriptional activation/repression begins to take shape (9, 42, 49). However, the complexity and sheer number of these interactions begs the question, how does E1A bind and organize these factors?

Two independent studies took on the painstaking task of systematically mutating the first 30 residues of hAd5 E1A and subsequently assessing the binding of factors to each mutant (12, 116). For the most part, one or two residues are critical for the interaction of each of the known cellular factors with the N-terminus of E1A. However, three residues, L19, L20, and L23, were shown to be critical to the interaction with a large number of cellular binding partners. The N-terminus of E1A is predicted to form an amphipathic alpha helix from residues 10-27, which likely allows for spatial organization and interaction with the large number of diverse cellular proteins shown to interact within this region (110).

CR1 cooperates with the N-terminus in binding several common cellular targets including p300/CBP and TRRAP (32, 36, 133, 155). CR1 also cooperates with CR2 in binding to the pRb tumor suppressor and family members. The CR1 interaction with pRb is via the E2F binding site of pRb. Although considered a low affinity interaction relative to high-affinity interaction site in CR2, CR1 is critical to dissociating pRb from E2F transcription factors, thereby facilitating expression of not only S-phase genes but also the viral E2 transcription unit (39, 69). It should be noted that the cellular E2F transcription factor family owes its name to the adenoviral E2 transcription unit, where the consequences of pRb loss were first studied (78) Change SHENK for KOVESDI 1986.

As a direct result of the diverse interactions of the N-terminus and CR1 of E1A with cellular proteins, they are both essential to E1A-dependent transformation of rodent cells

in culture (7). These two regions of E1A also constitute a transcriptional activation domain when fused to a heterologous DNA-binding domain (DBD) (11). Based on the transcriptional nature of many of the binding partners of the N-terminus/CR1 and the relative high-affinity of these interactions, this observation is not at all surprising. This same region is also capable of functioning as a generalized transcriptional repressor by sequestering limiting factors, such as p300/CBP (7, 112). In the context of viral infection however, the N-terminus/CR1 of E1A cannot interact with the viral early promoters on its own and is therefore not thought to be sufficient to stimulate transcription of any viral genes.

1.3.4 The E1A Conserved Region 2

CR2 of E1A is perhaps the most well studied region of E1A in terms of transformation and subversion of cellular tumor suppressor gene products. CR2 spans residues 115-137 and includes the DLXCXE motif (corresponding to residues 121-126 in hAd5) that confers high-affinity interaction with pRb (4). This interaction between E1A (CR2) and pRb constituted the first demonstration of a physical interaction between an oncoprotein and a tumor suppressor protein (108). CR2 is required to facilitate efficient and productive infection by providing two functions. Firstly, E1A CR2 forces quiescent cells into S-phase. Secondly, E1A CR2 indirectly activates the viral E2 transcription unit. Both of these functions are mediated through interaction with pRb and family members (6, 115).

Since this landmark role for CR2 was described, additional functions related to cellular transformation have been attributed to this region of E1A. CR2 has additional roles in oncogenic transformation of rodent cells independent of pRb (71, 79, 85, 99, 124, 146, 153), induction of apoptosis and sensitization of cells to tumor necrosis factor (101, 129) as well as stabilization of p53 (162). These pRb-independent functions of CR2 are likely the result of interaction with the three other cellular proteins known to bind this region: BS69, UBC9 and the S2 component of the 19S proteasome (61, 142, 159).

The cellular protein BS69 was originally identified as a 69 kDa protein that was immunoprecipitated with E1A (61). BS69 was shown to interact with E1A via a PXLXP

motif within CR2 (residues 112-117 in hAd5) that interacted with the MYND domain of BS69, also known as ZYMD11 (1). Interestingly, this PXLXP motif is only found in species A and C hAds (4). BS69 was demonstrated to have a repressive effect on E1A transactivation (61). How BS69 functions as a repressor and what role it plays in E1A-mediated oncogenic transformation is still unclear; however, BS69 is clearly involved in cell cycle control and senescence (68, 109, 161).

CR2 also interacts with the small ubiquitin-like moiety (SUMO) conjugase ubiquitin conjugase 9 (UBC9) via an EVIDLT motif corresponding to residues 118-123 in hAd5. The E residue of this motif actually overlaps with the DLXCXE pRb binding motif of CR2, again highlighting the complexity of E1A interactions with cellular proteins (4, 159). The EVIDLT interaction motif in CR2 contacts the surface of UBC9, which can normally interact non-covalently with SUMO-1. The interaction of E1A with UBC9 displaces SUMO-1 from the N-terminus of UBC9. In this manner E1A effectively blocks SUMO chain formation, which requires the non-covalent interaction between SUMO and UBC9 (159).

Finally, CR2 also interacts with the S2 component of the 19S regulatory subunit of the 26S proteasome. This interaction maps to residues 124-147 and also overlaps the pRb binding motif (4, 162). CR2 effectively stabilizes p53 and sensitizes cells to tumor necrosis factor alpha (TNF-α) by interacting with S2 and blocking degradation of p53 (142).

1.3.5 The E1A Conserved Region 3

Conserved region 3 is the major focus of this thesis. The sequence and function of E1A CR3 is discussed in detail in section 1.4.

1.3.6 The E1A Conserved Region 4

Exon two is the final frontier of E1A biology. Despite 20 years of extensive study, very little is known about the function and cellular targets of the product encoded by the second exon of E1A (4, 46, 110). Conserved region 4 (CR4) lies within exon 2 of E1A, and in hAd5, CR4 spans residues 240-288 (4). The region now identified as CR4 has a

long and bizarre history in saga of E1A-induced oncogenesis. CR4 is required for transformation in combination with E1B (33, 138, 158), but curiously inhibits transformation in cooperation with activated *ras* (13, 139). This region also plays a major role in E1A's ability to promote cells to undergo mesenchymal to epithelial transition (MET). This so called tumor suppressor function of E1A, is mediated by activating transcription of epithelial specific genes (58, 123).

Given the track record of E1A, it is of no surprise that these paradoxical functions are linked to interaction with an eclectic but short list of cellular targets. The first and best characterized target of CR4 is C-terminal binding protein (CtBP). It was first discovered as an E1A-interacting protein and so named because it is the protein that co-precipitated with the C-terminus of E1A (123). The sequence PXDLS is the motif that confers interaction with CtBP. It is present in all known E1A sequences and is also found in cellular proteins that interact with CtBP (4, 110, 123). CtBP is a cellular factor that functions in cells as a transcriptional co-repressor. CtBP interacts with a series of promoter-bound transcription factors and recruits repressive chromatin remodeling complexes to shut off transcription (26). Through its interaction with CtBP, E1A can alter cellular gene expression by two distinct mechanisms that require different isoforms of E1A. First, either 12S or 13S E1A can sequester CtBP through the PXDLS motif, effectively de-repressing promoters that require CtBP to remain repressed (26). Since this model was proposed, a second interaction site for CtBP was identified in CR3 of E1A. The 13S E1A product is capable of interacting with CtBP at repressed promoters. Using the potent transactivation domain of CR3, 13S E1A can directly activate CtBP-silenced promoters (15). Therefore the interaction with CtBP alone is capable of having paradoxical effects on cellular transcription, only adding to the complexity of E1A exon 2 function.

Exon 2 of E1A also interacts with DYRK1A/1B family of dual specificity kinases, and this interaction maps to residues 239-278 of CR4 (4, 158, 163). This interaction is conserved across all six hAd speciess as are the two arginine residues (R262/R263) required for this interaction (4, 158). E1A activates the kinase activity of DYRK1A, resulting in increased phosphorylation of DYRK1A targets including histone H3 and the

transcription factor GLI1 (158, 163). The consequence of increased DYRK kinase activity is increased GLI-dependent transcription and dramatically decreased transformation by E1A in cooperation with E1B. Both of these consequences are not only E1A-dependent, but also require the double arginine binding site found within CR4 (158).

Recently the Forkhead transcription factors FOXK1 and FOXK2 were identified as new targets of E1A exon 2 (77). These transcription factors interact with E1A through S219 (S174 in 12S E1A) in a phosphorylation dependent manner and appear to inhibit E1A transformation in cooperation with activated *ras* (77). Clearly, our understanding of the biology of E1A exon 2/CR4 has identified its involvement in multiple functions of E1A including transcriptional control, the precise role for this region in E1A function is continually evolving.

1.3.7 E1A as a Viral Hub Protein

The model by which E1A performs its functions is a fascinating example of viral subversion of host cell factors. E1A has no DNA binding or enzymatic activities (5, 24, 164). Instead E1A interacts with cellular factors and either modulates or relocalizes their function. This model of cellular subversion related to the high density of sequence conservation in E1A. The residues that are required to interact with key cellular regulators are in general quite conserved across hAd families. After extensive mutational analysis of E1A, it became clear that E1A can tolerate small and large deletions in one region that do not affect the function of adjacent regions (83, 85, 99, 120, 160). Moreover, small fragments of E1A can retain their function when expressed on their own (159). Given these properties of E1A function, much of the E1A protein can be thought of as a series of protein-protein interaction motifs, some of which are capable of interacting with multiple targets (110).

1.4 The Model of E1A-Dependent Transactivation

E1A function can be subdivided into two roles critical to the viral replication cycle. E1A drives the infected host cell into S-phase of the cell division cycle, thus providing an optimal environment for viral DNA replication. This function of E1A does not require

the unique 46 amino acid region within CR3, and thus the 12S E1A product (243R in hAd5) is sufficient to perform this role during infection (46, 110). Perhaps equally important, the E1A protein is required to activate transcription of the early viral genes and thus initiate the viral replication cycle (9, 46). Nearly 25 years ago, this function of E1A was shown to absolutely require the largest E1A isoform, which includes CR3 (10, 60). As a specific example, host range in hAd was originally based on the ratio of virus growth in 293 cells versus HeLa cells. Two classes of mutants were isolated that grew on 293 cells, but failed to grow efficiently (3.5-4 log reduction) on HeLa cells, and these mutants were termed host-range mutants (*hr*) (60). Group II *hr* mutants were able to grow on other tumor cell lines, and the mutations mapped to E1B. Group I *hr* mutants failed to synthesize early viral mRNAs in HeLa cells. All group I *hr* mutants not only mapped to E1A but all of them are single point mutants within CR3. Synthesis of viral early mRNAs could be rescued in 293 cells where E1A is supplied *in trans* (10, 47, 53, 56). Therefore the largest E1A protein which contains CR3 was responsible for early viral mRNA synthesis.

E1A CR3 encodes a four cycteine (C4) zinc finger domain (Culp 1988) and functions as a potent transcriptional activation domain that is critical for activating early viral gene expression (7, 9, 28, 43, 49). CR3 alone is sufficient to potently activate transcription when tethered to a promoter as a fusion to a heterologous DBD (84, 91). Overlapping deletion mutants across the entire E1A coding region were used to deduce sub-regions of CR3 that are required for function. Virtually all deletion mutants within CR3, unlike other regions of E1A, fail to activate transcription, and no short linear interaction motifs (SLIMs) have been identified within this region (37, 70, 110). Furthermore, the CR3s from the six representative hAd E1A proteins are predicted to have a defined structure (110). Taken together, these findings reinforce the concept that CR3 is a more ordered region of E1A, and that this structure is required to activate transcription. However, a three-dimensional structure has yet to be defined for CR3.

Studies of CR3 required a change in tactics from the use of large deletion mutants. Instead, the current model of CR3 function was built from painstaking analysis of point mutants. Indeed, every residue of hAd5 CR3 has been mutated to at least a conservative

aa and assayed for transcriptional activation (51). This effort eventually defined the factors and the key interaction residues of CR3 that are required to activate transcription of the early viral genes and generated a model that serves as the paradigm for non-acidic viral transcriptional activators. In hAd5 E1A, CR3 spans residues 144 to 191 and is comprised of three functional subdomains: an N-terminal zinc binding region mapping between residues 144 and 179, a C-terminal promoter targeting region spanning residues 183 to 188, and an acidic region that extends beyond CR3 and spans residues 189 to 200, termed auxiliary region 1 (AR1) (Figure 1.4) (4, 87, 137, 149). Deletion of the entire C4 zinc binding subdomain (aa 144-179), or any portion of it, results in a complete loss of transcriptional activation function (37, 70). However, mutation of the promoter targeting domain leads to a dominant-negative phenotype. Mutants in the promoter-targeting subdomain continue to bind to limiting cellular factors through the zinc finger subdomain, but are unable to associate with a promoter, resulting in sequestration of limiting cellular factors (squelching) and a loss of activation by wild-type E1A (149). Deletion of AR1, or a decrease in its overall acidic charge, also results in a loss of transcriptional activation (137).

The zinc finger subdomain of CR3 interacts with cellular TBP and MED23 (a component of the mediator adaptor complex) in order to nucleate the transcriptional preinitiation complex. These two targets interact with specific residues found within the zinc finger subdomain (Figure 1-4) (14, 51, 135, 149). Furthermore, these interactions require specific zinc coordination, because a single point mutant in CR3 that converts the zinc finger to a C2H2 type results in a complete loss of transcription activation and binding (150). E1A has no specific DNA binding activity and is recruited to viral and cellular promoters via interaction between the promoter targeting subdomain of E1A CR3 spanning residues 183 to 188 in hAd5 and cellular sequence-specific DNA binding transcription factors (87, 88, 149). The adenoviral early region promoters contain binding sites for many cellular transcription factors that interact with this short region of CR3, including those of the cyclic AMP response element/activating transcription factor (ATF) family, upstream stimulatory transcription factor (USF), and Sp1 (87, 88). This region has also been shown to bind TBP-associated factor II 250 (TAFII250) and TAFII 135 (50, 92). Interestingly, the promoter-targeting region in E1A is predicted to be

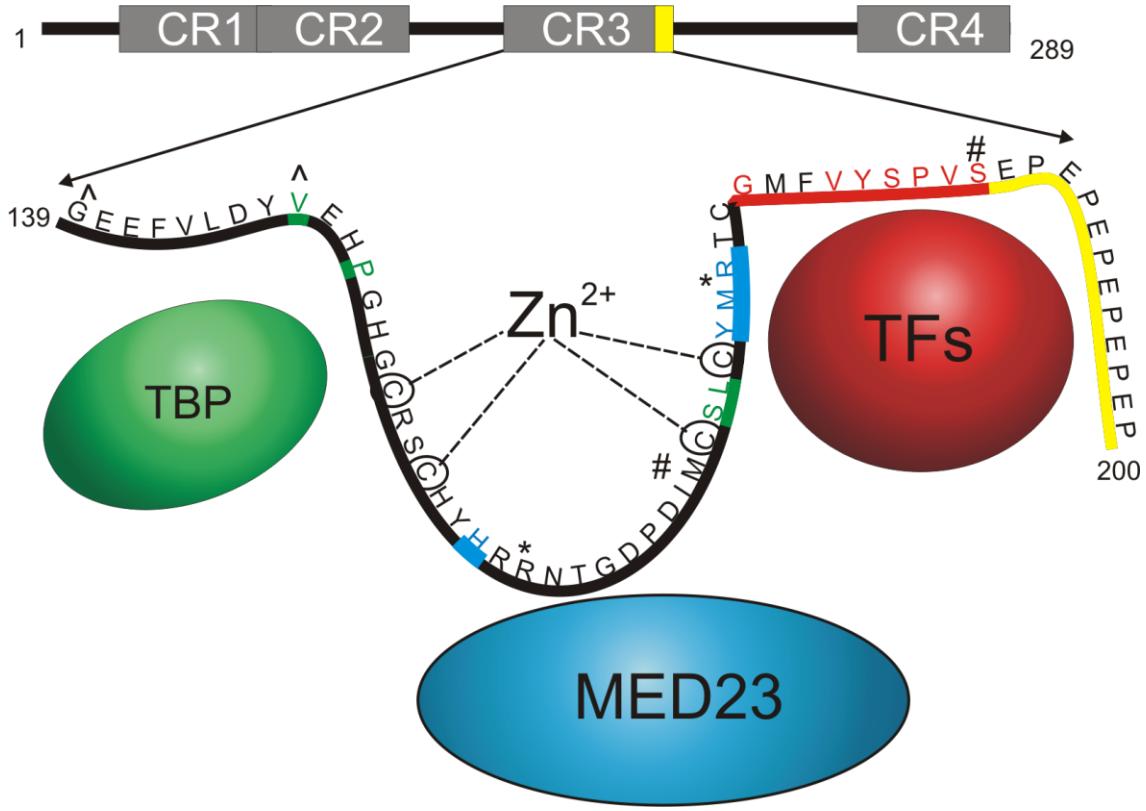

Figure 1-4: The Current Model of E1A Conserved Region 3 Transactivation.

Top: A linear representation of hAd5 289R E1A, conserved regions are labeled and AR1 is denoted in yellow. Bottom: The residues of E1A Cr3 from 139-200 are shown using one letter code. The coordinating Cysteines are circled and the key targets of CR3, TBP, MED23 and ATFs,are shown in green, blue and red respectively. The key residues interacting with each target are indicated by the corresponding color. The boundaries of the residues of CR3 known to be required for interaction with APIS (residues 169-188) and 20S proteosome (residues 161-177) and pCAF (residues 139-147) are marked by #, * and ^ respectively.

unstructured, and the disordered nature of this region of CR3 may contribute to its ability to interact with multiple unrelated transcription factors (110). AR1, which in hAd5 E1A is a series of six repeats of glutamic acid and proline (EP), is consistently predicted to be structurally disordered (110). The target(s) of AR1 remain to be identified, but it is known that the overall negative charge is critical to its function, whereas glycine can substitute for the prolines without a loss of function (137).

At the time I began this project, there existed a model for assembly of an active transcription pre-initiation complex by CR3. E1A CR3 orchestrates the nucleation of multiple key transcriptional regulators via distinct subdomains. E1A is recruited to the viral DNA template via interaction with sequence-specific DNA binding transcription factors through the promoter targeting subdomain, whereas TBP and MED23 are recruited via the zinc finger subdomain. Recruitment of these cellular proteins to a viral promoter is sufficient to stimulate transcription (9). E1A CR3 appears to be an example where an ordered region, required for specific interaction with a few targets (TBP and MED23), is juxtaposed to a disordered region required for promiscuous interaction with multiple promoter targeting transcription factors (110). The result is a compact, yet potent, activation domain capable of activating multiple promoter regions (Figure 1-4).

Since this model was put forth, multiple additional cellular targets have been implicated in CR3-dependent activation of transcription. More recently, components of both major subunits of the proteasome were shown to interact with CR3 of the E1A proteins of each of the six hAd species (117). The S8 component of the 19S ATPase proteins independent of 20S (APIS) complex interacts with hAd5 E1A CR3 via residues 169 to 188 (Figure 1-4). The addition of small amounts of exogenous S8 increases CR3 activity, whereas high levels abrogate transcriptional activation, suggesting that S8 is required in stoichiometric amounts for function. Moreover, small interfering RNA (siRNA) knockdown of S8 results in a loss of CR3-dependent transcriptional activation at levels similar to those seen with siRNA knockdown of other targets of CR3, such as TBP (117). The 20S proteasome subunit has also been shown to interact with CR3 independently of APIS and the 26S proteasome via residues 161 to 177 of hAd5 E1A (Figure 1.4) (117). Chromatin immunoprecipitation experiments show that these proteasome components and E1A are

found at both promoter and transcribed sequences, suggesting a role in both transcription initiation and elongation. Chemical inhibition of the proteasome represses CR3-dependent activation of transcription (117). These findings suggest that the proteasome directly controls E1A-dependent transcriptional activation. Moreover, mutational analysis has also established that the potency of E1A activation is inversely related to stability of the E1A protein, similar to other transcriptional activators, including herpes simplex virus type 1 VP16 (97, 117). These results suggest that degradation of E1A, and potentially other locally associated chromatin bound factors, is required to promote subsequent rounds of transcriptional initiation and contributes to the potency of transcriptional activation by CR3.

Recently three additional cellular transcription factors, previously known to interact with other regions of E1A, have been demonstrated to have an additional interaction site within E1A CR3 and to affect its ability to activate transcription. The cellular co-repressor CtBP interacts with E1A at the extreme C-terminus via the PXDLS motif, and at a second interaction site in CR3 through residues 161-167 (15, 123). This interaction appears to allow the largest E1A product (289R in hAd5) to activate CtBP-repressed promoters, by locally tethering the potent transcriptional activation domain of CR3 to an otherwise inactive promoter (15).

The other two cellular factors recently shown to interact with CR3 are both lysine acetyl-transferases (KATs) and function as co-activators of E1A CR3 transactivation. Both p300/CBP and pCAF have interaction sites in the N-terminus of E1A (as described in section 1.3.3) and were shown by our group to have a second interaction site in CR3 (111, 112, 116, 118). The interaction site for pCAF was mapped to residues 139-147, at the extreme N-terminal portion of CR3, which is predicted to be outside of the zinc finger binding sub-domain (111). Only a subset of E1As from different hAd species (species B, C, D and F) possess this second interaction site for pCAF within CR3, which correlates with the ability of pCAF to function as a co-activator of transactivation by these CR3s (111). Although p300/CBP is a critical co-activator of E1A-CR3, the second interaction site in CR3 has only been shown for hAd5 E1A and, the exact region of CR3 that mediates this interaction remains elusive (112).

1.5 Thesis Overview

The work presented in this thesis is a combination of hypothesis-driven and curiosity-motivated research. E1A provided me the luxury of drawing on the vast collection of literature with respect to E1A transactivation to form my hypotheses and expand our knowledge of this field, while generating exciting and unexpected results that I chose to follow up.

1.5.1 Rationale, Hypothesis and Experimental Approach

Although a model for CR3-dependent activation of early viral gene expression has been proposed, the vast majority of this model was worked out with the one prototypical member of the hAd family, hAd5 of species C. The contributions from Dr. J. Mymryk's laboratory have painstakingly tried to include other E1As wherever possible, but the fact remains that the current model is based largely on hAd5 E1A CR3. Moreover, since the original model was put forth over 10 years ago, multiple additional factors have been identified and implicated in regulating E1A CR3 transactivation. I hypothesized that the model for E1A CR3 is far from complete and that non-prototypical E1A-CR3s may have unique/alternative means to stimulate transcription from their respective promoters. My approach to elucidating a unifying model of E1A-CR3 transactivation involves expanding the existing model of E1A transactivation to representative members of each hAd species. I have chosen a representative member of each species, hAd12 (species A), hAd3 (species B), hAd5 (species C), hAd9 (species D), hAd4 (species E) and hAd40 (species F) as our reference panel of E1As/CR3s based on availability of reagents and presence in the literature. I have endeavored to break new ground with hAd5 E1A CR3 and extend my findings in the context of the whole hAd family. E1A from hAd5 has taught us a great deal about the regulation of cellular processes, and I believe the lessons learned from the hAd family as whole will provide entirely new insight.

1.5.2 Chapter 2: Comparison of E1A CR3-Dependent Transcriptional Activation across Six Different Human Adenovirus Species

This study constitutes the first systematic analysis of the cellular factors required by six different hAd E1A CR3s to activate transcription. First and foremost, there are dramatic differences in how the CR3 regions from six E1As representing each hAd species activate transcription as Gal4 DBD fusions. These differences are conserved across a panel of human cancer cell lines and also in MEFs, and cannot be explained by the existing model of E1A CR3 function. The interactions with all of the known co-activators of E1A CR3 were tested with the panel of representative CR3s, and the interaction with MED23, TBP, SUG1 and p300 were shown to be conserved across all six hAd speciess. Moreover, each of these conserved cellular factors was depleted from cells and the effect on E1A-CR3 transactivation was determined. Importantly, the role played by each cellular factor was conserved across the entire hAd family. In order to determine if the differences among CR3s for activating transcription were due to additional cellular factors, the ability of each CR3 to squelch transcriptional activation by hAd5 CR3 was determined. The competition assay showed conclusively that all six CR3s compete for the same limiting factor(s) and surprisingly, that the potency of transactivation did not correlate with potency of squelching. The squelching assay also demonstrated that there are additional cellular factors required by E1A-CR3 and that at least one is limiting. Therefore, this series of experiments expanded the existing model of E1A-CR3 transactivation and showed us that the model is not yet complete.

1.5.3 Chapter 3: Cellular GCN5 is a Novel Regulator of E1A-CR3 Transactivation

The Mymryk Laboratory has published the identification of a second pCAF binding site within CR3 of E1A, however this interaction was not conserved among all speciess of hAd. I hypothesized that those species of hAd that did not bind pCAF through CR3 could be targeting the closely related KAT GCN5. Furthermore, the Mymryk laboratory previously identified an interaction between CR3 of E1A and yeast GCN5. GCN5 had also been shown previously to interact with the N-terminus E1A. In this study a second interaction site for GCN5 within CR3 of E1A was identified and mapped to residues 178-

184. This interaction through CR3 was conserved across all six hAd species and independent of the pCAF interaction, which binds CR3 through residues 139-147. GCN5 is a negative regulator of E1A CR3 transactivation, as depletion of GCN5 by RNAi results in an increase in E1A CR3 transactivation. Furthermore, overexpression of exogenous GCN5 decreases CR3 transactivation and also transactivation by full length E1A. GCN5 is recruited to the viral E4 promoter in an E1A dependent manner and requires both interaction sites in E1A for proper recruitment. This is the first demonstration of the cooperative recruitment of a cellular factor to a promoter through different regions of E1A and this is likely a recurring theme for many of the cellular factors that interact with multiple regions of E1A. It is the KAT activity of GCN5 that is responsible for the negative effect on E1A-CR3 function, as E1A dependent transactivation increases in *hat/hat* MEFs, where both endogenous copies of GCN5 have point mutations in the active site abrogating KAT activity. In addition, E1A dependent transactivation also increases when cells are treated with a specific inhibitor of GCN5 KAT activity. Virus yield is diminished in the presence of the inhibitor, suggesting that an ideal and balanced level of E1A transactivation is required for optimal virus replication. I have therefore identified GCN5 as a novel negative regulator of E1A dependent transactivation.

1.5.4 Chapter 4: The Structure of E1A CR3

There is currently no structural model of the C4 zinc finger subdomain of E1A-CR3. Unlike the rest of E1A which contains SLIMs, CR3 is completely devoid of these motifs. In addition, CR3 does not tolerate deletions without total loss of function, suggesting this region may in fact be structured. *In silico* predictions of order versus disorder in E1A reveal a potential ordered, or structured domain that corresponds to the zinc finger subdomain of CR3. I undertook the task of generating a 3D structure of E1A CR3 by NMR spectroscopy. E1A CR3 residues 139-204 was overexpressed in *E. coli* and purified. The purified CR3 was of correct mass and could compete for MED23 binding. CR3 was labeled with ^{15}N and a 2D Heteronuclear Single Quantum Correlation (HSQC) spectrum was obtained which demonstrated that CR3 was structured. There was however a very flexible region that obscured several peaks. AR1 was removed from CR3 and a

new 2D HSQC spectrum was collected that again showed CR3 was structured, but the spectra was without sufficient resolution to eventually yield a structure. Further analysis showed that the structure of CR3 was zinc dependent as chelation of zinc with EDTA abolished the structured CR3 signature. Mass spec analysis revealed that CR3 residues 139-190 bound a single zinc ion, however this experiment also revealed a smaller peptide corresponding to residues 139-182, which is most likely the result of proteolytic cleavage which also bound a single zinc ion. To avoid the complications of two species in the structural determination, an even smaller fragment of E1A residues 139-178 was selected for further study. This region would avoid proteolytic cleavage and also removes a non-coordinating cysteine residue that could complicate the analysis. A reliable 3D structure of the zinc finger subdomain of CR3 has not yet been determined. With structural information about the orientation of residues within CR3, a highly detailed model of E1A-CR3 transactivation can be generated.

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Chapter 2

2 Comparison of E1A CR3 Dependent Transcriptional Activation Across Six Different Human Adenovirus Species

2.1 Introduction

The adenovirus (AdV) E1A oncoprotein is the first gene expressed upon infection and performs two essential roles in order to initiate the viral replication cycle. E1A uncouples the cell cycle control program of the host cell, driving it into S-phase to provide an optimal cellular environment for viral replication. This function can be carried out by the smaller major E1A isoform (243 residues in hAd5 E1A) (11, 25). The other function of E1A is to activate transcription of the early viral promoters and is mediated predominantly by the largest E1A isoforms (4, 18, 23, 24). The largest E1A isoform, coding for 289 residues in hAd5 E1A, differs only from the smaller isoform by a unique 46 amino-acid C4 zinc finger domain within conserved region 3 (CR3), which is essential for viral transactivation (4, 18). Single point mutations in CR3 were originally isolated as hAd mutants with a host range limited to HEK 293 cells, which supply *wt* E1A *in trans*. These "host-range" mutations render E1A unable to transactivate viral promoters, thus preventing virus growth in cells at low multiplicity of infection (MOI), unless *wt* E1A is supplied *in trans* (13, 16).

Transactivation by E1A CR3 has been studied predominantly with hAd5, and this has led to the establishment of a model for CR3 function. The region of hAd5 E1A spanning residues 139-204 (which includes CR3) is critical for activating transcription necessary for virus growth and sufficient for potent activation of a minimal Gal4-responsive promoter, as a Gal4-DBD fusion (17, 30). This 65 aa region of E1A CR3 can be further subdivided into the three following subdomains: an N-terminal zinc finger, a promoter targeting region and a region known as Auxiliary Region 1 (AR1) (9, 20, 32). The current model is that the N-terminal zinc finger domain of E1A CR3 activates transcription by interacting with cellular TBP and MED23 proteins (6, 19, 31). The Mediator component MED23 is absolutely critical to E1A CR3 function as CR3 fails to

activate transcription in MED23 null MEFs (31). Furthermore, this requirement is likely shared between the E1A proteins of even very divergent AdVs as mouse adenovirus type 1 is unable to replicate efficiently in MED23 null MEFs (10). In order to nucleate a functional transcription initiation complex at the early viral promoters, the C-terminal promoter targeting subdomain is required. It confers interaction of E1A CR3 with cellular sequence specific DNA binding transcription factors, including members of the ATF family (8, 20, 21). Mutants that delete this promoter targeting domain function as dominant negative mutants unless a second mutation is made in the zinc finger subdomain of CR3, further underscoring the promoter targeting activity of this region (20). The precise role of AR1 in E1A CR3 dependent transactivation remains unclear, however the acidic character of this region is necessary for maximal transactivation by the E1A CR3 region (32).

Since the initial model was put forth, multiple additional cellular factors have been implicated in transcriptional activation by E1A CR3. The S8 component of the 19S ATPase proteins Independent of 20S (APIS), also known as hSUG1, was shown to interact with E1A CR3 and enhance E1A CR3 transactivation (28). A second interaction site for the cellular repressor CtBP was also mapped to the CR3 region of hAd5 E1A, and CtBP appears to repress E1A CR3 transactivation (7). The p300/CBP KAT is critical for hAd5 E1A CR3 transactivation and binds directly to E1A CR3 independently of other interaction motifs in E1A (27). Most recently, the KAT pCAF was identified to interact with the E1A CR3 region and enhance transactivation (26). Many of these cellular factors interact with other regions of E1A besides CR3, further complicating E1A mediated activation of transcription.

The vast majority of the work used to build the initial model was done exclusively with hAd5 E1A CR3. Very little is known regarding how E1A CR3s from other hAd types activate transcription. Alignment of the amino acid sequence of E1A CR3 from representative members of each hAd species, corresponding to the hAd5 E1A residues 139-204, demonstrates their high degree of conservation (Figure 2-1A). The amino acid identities and similarities to hAd5 E1A CR3 range from 34%-41% and 41%-53%, respectively (2). Overall, CR3 is the most conserved domain of E1A, but it is not known

if the model for hAd5 E1A CR3 transactivation will apply universally to the entire hAd family. Our previous work suggests that there may in fact be differences in how specific E1A CR3s activate transcription, as only a subset of the E1A CR3s we tested interact with and are influenced by pCAF (26).

We report here the first comprehensive study of the cellular factors required for E1A CR3 transactivation using representative E1A CR3s from each hAd species. The panel of representative E1A CR3s show dramatic differences in their ability to activate transcription as Gal4-DBD fusions, which cannot be explained by the existing model of E1A CR3 function. Systematic analysis of the roles of MED23, TBP, hSUG1 and p300/CBP, which have been implicated in hAd5 E1A CR3 function, reveals that these interactions are conserved across all hAd speciess, and each representative E1A CR3 can compete for common factor(s) and squelch hAd5 E1A CR3 transactivation. However, the known cellular factors required by E1A CR3 cannot explain the dramatic differences in transactivation observed among these representative E1A CR3s. These results demonstrate that many of the cellular targets utilized by hAd5 E1A CR3 are conserved across the hAd family, expanding the existing model of E1A CR3 transactivation to encompass all six speciess. Importantly, this data also indicates that additional factors influencing CR3 dependent transactivation remain to be discovered.

2.2 Materials and Methods

2.2.1 Cells, Cell Culture and Transfections.

Human A549, HeLa, C33A, U2OS and HT1080 cells, as well as MEFs and MED23-/- MEFs cells were maintained at 37 ^{o}C and 5 % CO_2 in DMEM (Wisent) with 10 % FBS (Gibco) and 100 U/ml of Penicillin/Streptomycin (Wisent). A549 cells and MEFs were transfected with FuGENE HD Reagent (Roche) according to the manufacturer's directions in a ratio of 3 µg to 9 µl per well of a six-well plate. HeLa and HT1080 cells were transfected with Superfect reagent (Qiagen) according to the manufacturer's directions.

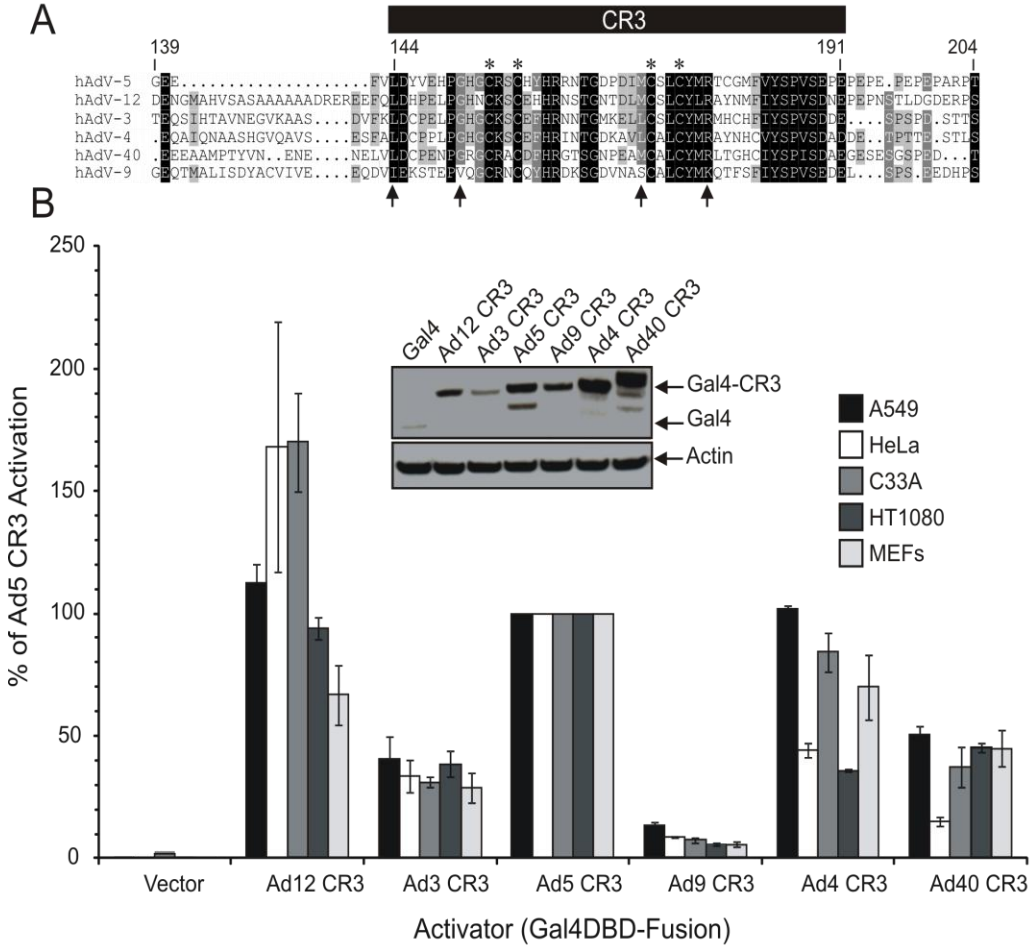


Figure 2-1: Transcriptional Activation By E1A-CR3s From Six Human Adenovirus Species**.**

A) Sequence alignment of human Adenovirus E1A CR3 fragments used from serotypes hAd12 (species A), hAd3 (species B), hAd5 (species C), hAd9 (species D), hAd4 (species E) and hAd40 (species F). Asterisks indicated zinc coordinating cysteines. Arrows indicate residues in hAd9 E1A CR3 that differ from hAd5 E1A CR3 and are critical for transcriptional activation. B) Intrinsic transcriptional activation properties of representative E1A CR3s. A549, HeLa, C33A, HT1080 or MEF cells were co-transfected with a Gal4 responsive luciferase reporter and vectors expressing the indicated Gal4DBD fusions. Luciferase activity expressed as % of hAd5 CR3 fold activation of above empty vector +/-SD. Inset: Western blot for the expression level of Gal4-fused CR3 domains in A549 cells using anti-Gal4 and anti-actin as a loading control.

The U2OS stable cell line containing an integrated Gal4-responsive luciferase reporter (U2OS-UAS) was made by co-transfection of U2OS cells with pGL2-$(Gal4)_6$-Luc and pcDNA3.1-Hygro in a 9:1 ratio and selection on 400 µg/ml of hygromycin. Hygromycin resistant pools were used for all experiments.

2.2.2 Plasmid Construction.

The Gal4-responisve luciferase reporter vector pGL2-$(Gal4)_6$-Luc and Gal4DBD-fusions for each hAd E1A CR3 and *wt* HPV16-E7 have been described previously (1, 30). Expression vectors for EGFP fusions of hAd E1A CR3s and *wt* HPV E7 were cloned from their respective Gal4DBD-fusions into pCANmyc-EGFP with EcoRI and XbaI. The expression vectors for the full length E1A clones of hAd3, 4, 5, 9, 12 and 40 were cloned into pM (Clontech Laboratories Inc.) with EcoRI and SalI. The expression vector for hMED23 (pCS2+-hSur2) was a gift from A. Berk and described previously (6). Expression vectors for TBP (pcDNA4HA-hTBP) and hSUG1 (pcDNA4HA-hSug1) were described previously (28). The p300 expression vector was described previously (27).

2.2.3 Gal4-Fusion Activation Assay.

24 hours prior to transfection $1.5x10^5$ A549 cells/well were seeded on six well plates. Cells were transfected in a 1:1 ratio of reporter vector (pGL2-$(Gal4)_6$-Luc): activator (either pM or pM-CR3). Cells were harvested 48 hours post transfection in 1X Cell Culture Lysis Buffer (Promega) and assayed for Luciferase activity using STEADY-GLO substrate (Promega). Relative Light Units (RLUs) were normalized to protein concentration and plotted as mean fold activation above Gal4-DBD alone (pM) +/- SD.

2.2.4 Co-Immunoprecipitation and Western Blot Analysis.

Typically, $1.5x10^6$ HT1080 cells were seeded into 10 cm plates 24 hours prior to transfection. Cells were transfected in a 1:1 ratio of myc-EGFP fusion and HA-tagged binding partner, or myc-EGFP fusion/E1A alone if using endogenous binding partners. Cells were harvested 24 hours post transfection by scraping and washed once with 1X PBS. Cells were lysed in either NP40 (50 mM Tris-pH7.8, 150 mM NaCl, 0.1 % NP40) or E1A (50 mM Hepes-pH 6.8, 230 mM NaCl and 0.5 % NP40) lysis buffer

supplemented with 1x mammalian protease inhibitor Cocktail (Sigma). Typically, 1 mg of cell lysate was mixed with 100 µl of anti-myc hybridoma (Clone 9E10) or M73 anti-E1A hybridoma supernatant and 125 µl of 10% slurry of ProteinA-Sepharaose resin (Sigma) and incubated at 4 ^{o}C for 1 hour with nutating. Immunoprecipitates were washed five times with lysis buffer, resuspended in 1X LDS sample buffer and boiled for 5 minutes. Samples were then separated by SDS-page, transferred to PVDF membrane (GE) and blocked in 5 % Non-fat Milk in TBS-T. Western blot analysis was carried out with mouse anti-myc hybridoma clone (9E10), rat monoclonal anti-HA (clone 3F10 Roche), monoclonal anti-Rb (clone C36), rabbit anti-actin (Sigma), mouse monoclonal anti-TBP (Millipore), rabbit anti-hSUG1(14) or mouse monoclonal anti-p300 (clone Rw128 Millipore) followed by either rabbit-anti-mouse HRP (Jackson Labs), goat-anti-rat HRP (Pierce) or goat-anti-rabbit HRP (Jackson Labs).

2.2.5 siRNA Knockdown.

Custom siRNA against p300 was used as described previously(27). Silencer select siRNAs against TBP (siRNA ID# s13826) and hSUG1 (siRNA ID# s11381) were purchased from Ambion. siRNA transfections were performed with siLentFECT reagent (BioRad) according to the manufacturer's directions. Typically 1.5x10^{6} HeLa cells were seeded on 10 cm plates for siRNA transfection. At 24 hours post siRNA transfection, cells were re-seeded to 6 well plates at 2x10^{5} cells per well. At 48 hours post siRNA transfection cells were transfected as described above to perform the Gal4-fusion activation assay.

2.2.6 Squelching Assay.

24 hours prior to transfection 1.5x10^{5} A549 cells/well were seeded on six well plates. Cells were transfected in a 1:1:1 ratio of reporter (pGL2-(Gal4)$_{6}$-Luc): activator (either pM or pM-Ad5 CR3): squelcher (myc-EGFP fusion). Cells were harvested 48 hours post transfection for luciferase assay as described above. Relative Light Units (RLUs) were normalized to protein concentration and plotted as a percent of Gal4-Ad5 E1A-CR3 wt with EGFP (empty vector) as competitor +/- SD. The squelching rescue assay was

performed as above at a 1:1:1:1 ratio including pcDNA4HA-hSUG1 or empty pcDNA4HA.

2.2.7 Quantitative RT-PCR.

Human A549 cells were infected with either hAd5 (dl309 or dl312) or *wt* hAd9 virus at MOI of 2.0 (or 200 and 2000 for hAd9 wt). At 16 hrs post infection total RNA was isolated with Trizol (Invitrogen) according the manufacturer's directions. For each sample 1 µg of total RNA was first heated to 70 °C for 5 minutes and subjected to DNaseI treatment (Invitrogen) according to manufacturer's directions. First strand synthesis was performed using OligodT (Inivitrogen) and SuperScriptII (Invitrogen) according to manufacturer's directions. A 15 µl Q-PCR reaction was performed in triplicate using 1X iQ-SYBRGreen SuperMix (BioRad) according to manufacturer's directions in a MyiQ Real Time PCR instrument (BioRad). The following primers for hAd9 targets were used at 200 nM final concentration: RTAd913S E1AF 5'-agctttatttacagtccggtgtcaga-3'; RTAd913SE1AR 5'-acacttgcaggggcgttt-3'; RTAd9E4orf6-7F 5'-cataatactgtgaccttggac-3'; RTAd9E4orf6-7R 5'-tttcctggcgagccaaac-3'. The primers for hAd5 targets and GAPDH were described previously (28). Data was analyzed using BioRad IQ5 sofware (BioRad); briefly, E4ORF6/7 mRNA levels were normalized to GAPDH as an internal control and the respective E1A mRNA level for each sample. E1A and E4ORF6/7 mRNA levels in dl312 (E1A deleted) infected cells were set to 1.

2.3 Results

2.3.1 E1A CR3 mediated transactivation differs greatly between hAd types.

E1A is a potent activator of transcription and hAd5 E1A CR3 (residues 139-204) is sufficient to activate transcription when fused to a heterologous DBD in mammalian and yeast cells (26-30, 34). We tested whether the corresponding E1A CR3 portions of hAd 3, 4, 9, 12 and 40 E1A, which represent the other five hAd speciess were capable of activating transcription as Gal4-DBD fusions in the A549 human alveolar basal epithelial cell line (Figure 2-1B). A549 cells were chosen as they are commonly used as a diagnostic cell line for clinical hAd infections. All six E1A CR3s activated a Gal4

responsive promoter in A549 cells. Interestingly, there were dramatic differences in their relative activities (Figure 2-1B). Given that E1A is the first gene expressed during adenovirus infection and is responsible for activating viral gene expression, the current model would predict that all E1A CR3s should strongly activate transcription. However, there appeared to be three classes of E1A CR3s with respect to activation of transcription: species A (hAd12), C (hAd5) and E (hAd 4) E1A CR3s were the most potent activators of transcription, species B (hAd 3) and species F (hAd 40) E1A CR3s exhibited an intermediate ability, while species D (hAd 9) E1A CR3 was a weak activator. To determine if these differences in activity were specific to A549 cells, we repeated these experiments in HeLa and C33A human cervical carcinoma cells, HT1080 human fibrosarcoma cells and mouse embryonic fibroblasts (MEFs). Although some differences in activation were seen between cell types, hAd 9 CR3 was consistently the weakest activator in all cells tested (Figure 2-1B).

2.3.2 Diverse E1A CR3s share common cellular targets.

Since little is known about the mechanism by which the E1A CR3 regions of any hAd type other than hAd5 activate transcription, we first determined if there are common cellular factors targeted by the different E1A CR3s. We designed a competition, or squelching assay to determine if there is functional overlap of cellular targets required for transactivation. A549 cells were co-transfected with a Gal4-responsive luciferase reporter, an activator and a competitor. In this assay the readout is the activation by Gal4-Ad5 E1A-CR3 wt above Gal4 alone and is expressed as a percentage of Gal4-Ad5 E1A-CR3 *wt* with EGFP (empty vector) as competitor. If a competitor does not target factors required by the DNA bound transactivator, the level of transcriptional activation should remain at 100 % regardless of the level of competitor present. However, if the competitor targets a factor(s) required for function by the activator, a dose dependent reduction of transactivation will be observed as the level of the competitor is increased. As expected, *wt* hAd5 E1A CR3 fused to EGFP potently squelched the activity of Gal4-Ad5 E1A-CR3 *wt* in a dose dependent manner (Figure 2-2A). This clearly demonstrates that soluble EGFP hAd5 E1A CR3, which is unable to bind the promoter,

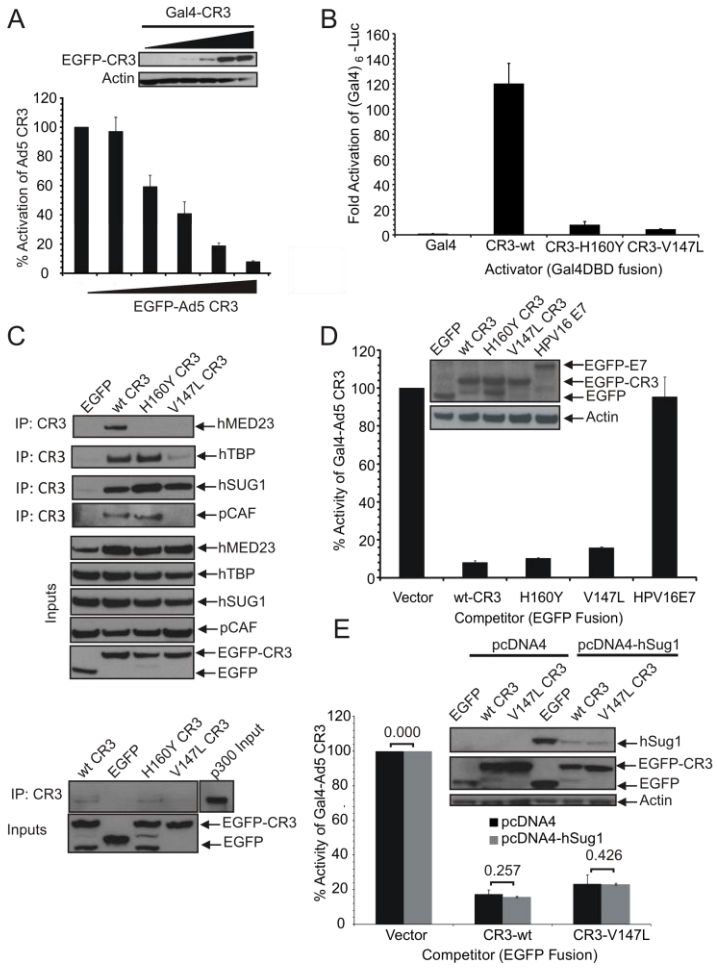


Figure 2-2: Squelching of hAd5 E1A CR3 function.

A) Design and titration of squelching assay. A549 cells were co-transfected with a Gal4 responsive luciferase reporter, vectors expressing Gal4-alone or Gal4-Ad5 CR3 as activator and either EGFP or increasing amounts of vector expressing EGFP-Ad5 CR3 fusion as competitor. Luciferase activity is expressed as % fold activation above vector alone of Gal4-Ad5 CR3 with EGFP as competitor +/-SD. Inset: Western blot of EGFP-CR3 expression levels and actin loading controls. B) Activation of established mutants of E1A-CR3 as Gal4-fusions. A549 cells were co-transfected with a Gal4 responsive luciferase reporter and vectors expressing the indicated Gal4 fusions to E1A-CR3 or empty vector. Luciferase activity expressed as fold activation of over vector +/-SD. C) Interaction of mutants of Ad5 E1A CR3 with known cellular targets of CR3. E1A CR3 fusions were CoIP'd with anti-myc antibody and blotted with anti-HA antibody for the indicated targets. Inputs are probed with anti-myc antibody for CR3s or anti-HA antibody for MED23, TBP, hSUG1 or p300. D) Squelching of E1A CR3 dependent transactivation by hAd5 CR3 mutants, A549 cells were co-transfected as in A) but with equal amounts of vectors expressing Gal4 fused to the indicated Ad5 CR3 mutants or HVPE7. Inset: Western blot of EGFP-fusion expression levels and actin loading controls. E) Sequestration of hSUG1 is not responsible for transcriptional squelching by CR3 V147L. Human A549 cells were co-transfected as in D with either empty vector [pcDNA4 (black bars)] or a hSUG1 expression vector [pcDNA4-hSUG1 (grey bars)] in a 1:1:1:1 ratio. Activation in vector transfected vs. hSUG1 transfected cells were compared by Student's T-Test and P values are indicated. Inset: Western blot of EGFP-fusion expression levels, hSUG1 levels and actin loading controls.

will sequester limiting factors from promoter tethered Gal4-Ad5 E1A CR3, reducing its ability to stimulate transcription of the Gal4 responsive luciferase reporter.

2.3.3 E1A mutants unable to bind multiple factors still squelch *wt* E1A activity.

Two well characterized point mutants of hAd5 E1A CR3 that fail to transactivate, H160Y and V147L, were chosen to validate the squelching assay (12). We confirmed that Gal4 fusions of each of these mutants were unable to activate a Gal4-responsive promoter (Figure 2-2B). hAd5 E1A CR3 interacts with multiple cellular transcriptional regulators in order to orchestrate the activation of gene transcription (25). We determined the interaction profile of these mutants with respect to several of these binding partners. H160Y bound TBP, hSUG1, p300 and pCAF equivalently to *wt* hAd5 E1A CR3, but did not bind hMED23 (Figure 2-2C). V147L bound hSUG1 like *wt* hAd5 E1A CR3, bound TBP only weakly, and did not bind hMed23, p300 or pCAF (Figure 2-2C). Taken together these mutants represent transactivation defective mutants that retained selective interactions with factors that might be rate limiting for E1A CR3 dependent activation of transcription. Despite the inability of these mutants to activate transcription or bind key transcriptional components, both effectively squelched activation by Gal4-hAd5 CR3 *wt* when expressed as soluble EFGP fusions, whereas the human papillomavirus type 16 E7 viral transactivator did not (Figure 2-2D). As both the H160Y and V147L mutants remained capable of interacting with hSUG1 similarly to *wt* hAd5 E1A CR3, it remained possible that hSUG1 is the limiting factor required by CR3 to activate transcription. We tested whether over expression of hSUG1 would reverse the squelching by the V147L mutant. Over expression of hSUG1 resulted in no significant change is the squelching activity of the V147L point mutant (Figure 2-2E). The observation that these mutants lost interaction with multiple cellular factors, yet still managed to squelch *wt* hAd 5 E1A CR3 transactivation demonstrates that E1A CR3 dependent activation of transcription is a complex process requiring the concerted action of multiple factors. Importantly, this data provides evidence that additional factors beyond hMED23, hSUG1, p300, pCAF and TBP are critical for hAd5 CR3 transactivation.

2.3.4 E1A CR3s universally squelch hAd5 E1A CR3 function.

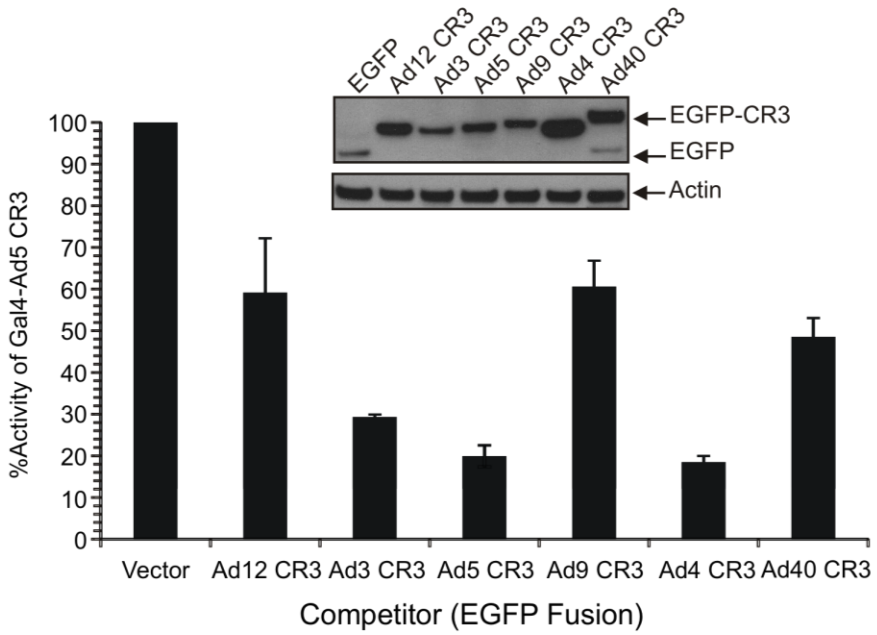
Little is known about the cellular factors required for CR3 dependent transactivation by the E1A proteins of hAd types other than hAd 5. We hypothesized that the squelching assay could be applied to determine if the E1A CR3 regions of other hAd types functioned via interaction with the same set of cellular transcriptional regulators. A549 cells were co-transfected with a Gal4 responsive luciferase reporter and Gal4- hAd5 E1A CR3 *wt* as described before, with EGFP fused to the E1A CR3 regions of chosen representative hAds as competitors. The E1A CR3 regions of all six hAd types were capable of squelching hAd5 CR3 dependent activation, although to varying extents (Figure 2-3A). Three classes of squelching ability were observed: hAd5 and hAd4 E1A CR3s were the most potent, hAd3 E1A CR3 appeared to have an intermediate ability and hAd12, hAd9 and hAd40 E1A CR3 were the least effective squelchers (Figure 2-3A Inset). Weaker squelching was not simply due to low levels of expression as determined by western blot (Figure 2-3A). Interestingly, the effectiveness of an individual CR3 region to squelch was not typically related to its ability to activate transcription as a Gal4-DBD fusion (Figure 2-3B).

Based on the squelching assay, it was clear that the chosen representative E1A CR3s targeted cellular transcriptional regulators also required by hAd5 E1A CR3. We therefore systematically examined their interactions with known coactivators of E1A CR3, including MED23, TBP, hSUG1 and p300, as well as the functional requirements for these interactions.

2.3.5 MED23 is required by all E1A CR3s to activate transcription.

The Mediator component MED23 has been implicated to be the most critical cellular coactivator of hAd5 CR3 function (6, 31). This interaction is also required for murine AdV growth, suggesting that this factor may be universally utilized by different adenovirus types (10). We tested the ability of the full length E1A product from each representative hAd species to interact with hMED23 by Co-IP. Each of the different full length E1A proteins bound hMED23, although none bound it as strongly as hAd5 E1A (Figure 2-4A). Furthermore, the E1A CR3 domains of each representative E1A

A



B

hAd Subgroup	hAd Type	Activation	Squelching	Expression
A	Ad 12	+++	++	+++
B	Ad 3	++	+++	+
C	Ad 5	+++	+++	+++
D	Ad 9	+	++	+++
E	Ad 4	+++	+++	+++
F	Ad 40	++	++	+++

Figure 2-3: Squelching of hAd5 E1A CR3 dependent activation by the CR3 domains from representative hAd types

A) Human A549 cells were co-transfected with a Gal4 responsive luciferase reporter, vectors expressing Gal4-alone or Gal4-hAd5 CR3 as activator and either EGFP or EGFP-fused to each of the indicated E1A-CR3 fusion to mycEGFP as competitor. Inset: Western blot of EGFP-fusion expression levels and actin loading controls. Luciferase activity is expressed as % fold activation of Gal4-hAd5 CR3 above vector alone with EGFP as competitor +/-SD. B) Summary of transcriptional properties of representative E1A CR3s. The transcriptional activation, squelching and expression level of each E1A CR3 are summarized relative to hAd5 E1A CR3.

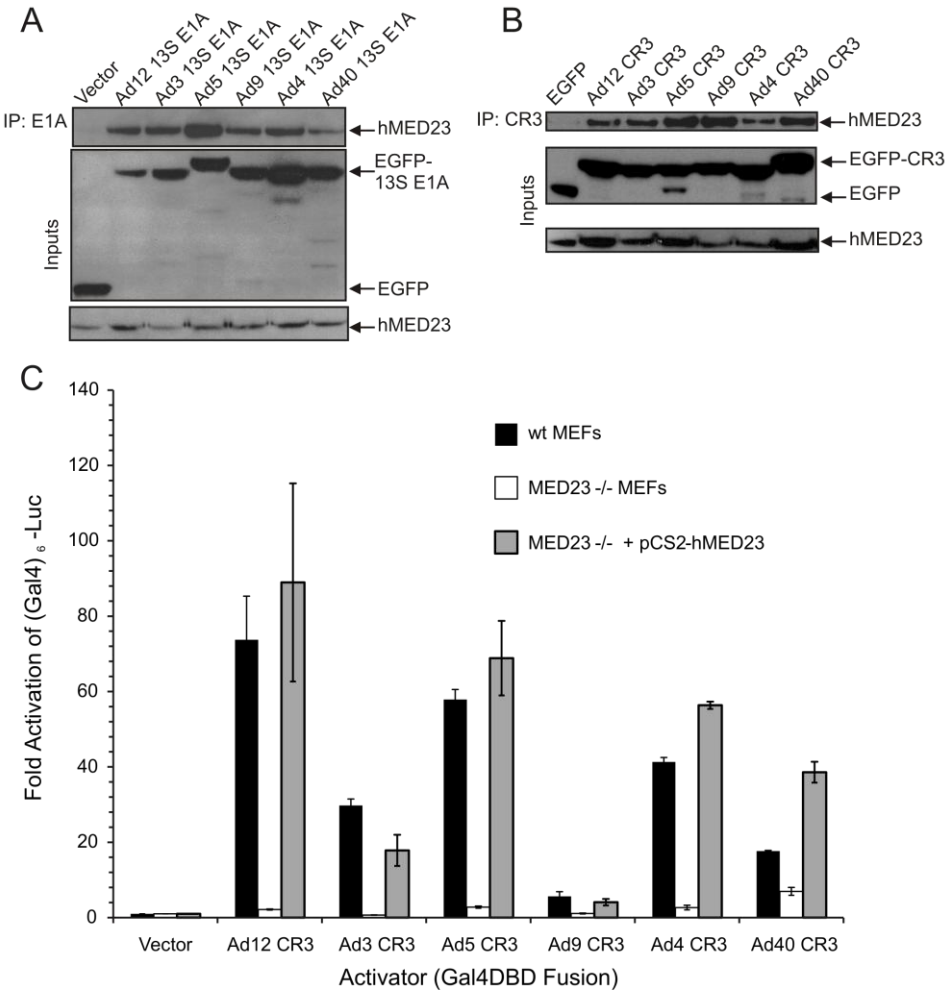


Figure 2-4: MED23 is targeted by E1A CR3s from multiple hAd types.

A) Co-IP of MED23 and the 13S encoded E1A proteins from each hAd subgroup. Human HT1080 cells were co-transfected with a vector expressing HA-tagged human MED23 and vectors expessing either EGFP or an EGFP-fused to the indicated E1A proteins. E1As were immunoprecipitated with anti-EGFP antibody and blotted with anti-HA antibody. Inputs were probed with EGFP antibody for E1As or HA antibody for MED23. B) Co-IP of MED23 with the E1A CR3 domains from each hAd subgroup. Human HT1080 cells were co-transfected with vectors expressing HA-tagged human MED23 and either myc-EGFP or a myc-EGFP-fused to the indicated E1A CR3 domain. E1A CR3 domains were immunoprecipitated with anti-myc antibody for myc-tagged CR3 and blotted with anti-HA antibody for HA tagged MED23. Inputs were probed with anti-myc antibody for CR3s or anti-HA antibody for MED23. C) E1A CR3 activation in MED23 null MEFs. MED23 null MEFs and *wt* littermate derived MEFs were co-transfected with a Gal4 responsive luciferase reporter and vectors expressing Gal4 fused to the indicated E1A CR3 domain (white and black bars respectively). MED23 null MEFs were also transfected with the Gal4 responsive luciferase reporter, vectors expressing the indicated Gal4-E1A CR3 domain fusions and an expression vector for human MED23 (grey bars). Luciferase activity is expressed as fold activation over Gal4DBD alone +/-SD

protein were sufficient for this interaction (Figure 2-4B). In order to determine the functional consequences of this hMED23 interaction, the ability of the representative E1A CR3s to activate transcription in MED23 null (MED23 -/-) MEFs was determined. All six E1A CR3s activated transcription in *wt* MEFs, but failed to activate transcription in MED23 -/- MEFs (Figure 2-4B, black bars and white bars, respectively). The failure to activate transcription was rescued by the expression of exogenous hMED23 in the MED23-/- MEFs (Figure 2-4C, grey bars). We conclude that hMED23 is a common target of each of the six representative CR3s and is absolutely required for transactivation.

2.3.6 TBP binds all E1A CR3s but is not required for transcriptional activation.

The first cellular protein shown to interact with CR3 of hAd5 E1A was TBP (19). We tested if TBP was a conserved interaction among the different E1A CR3s and also determined the requirement for TBP in E1A CR3 dependent activation of transcription. hAd5 E1A CR3 was sufficient to Co-IP TBP, and the five other E1A CR3s interacted with hTBP at least as strongly as hAd 5 E1A CR3 (Figure 2-5A). As a negative control for these interaction studies, we assessed the ability of the E1A CR3 fusions to Co-IP pRb, which binds E1A primarily via CR2. As expected, none of the E1A CR3 fusion proteins bound pRb (Figure 2-5B). To determine the functional role of TBP in E1A CR3 transactivation, we depleted TBP in HeLa cells by RNAi and then examined the ability of each E1A CR3s to activate transcription as a Gal4-fusion in control siRNA vs. TBP specific siRNA treated cells. siRNA knockdown of TBP did not reduce transactivation by any of the six CR3s tested (Figure 2-5C). Based on this observation we conclude that although TBP is a conserved target of the six CR3s, it does not appear to be nearly as critical as hMED23 for transactivation.

2.3.7 hSUG1 binds all E1A CR3s and contributes to transcriptional activation.

The proteasome is a crucial cellular coactivator of hAd5 E1A and CR3 binds the proteasome via the hSUG1 or S8 ATPase proteasome components (28). All six representative E1A CR3s interacted with hSug1 as determined by Co-IP (Figure 2-6A).

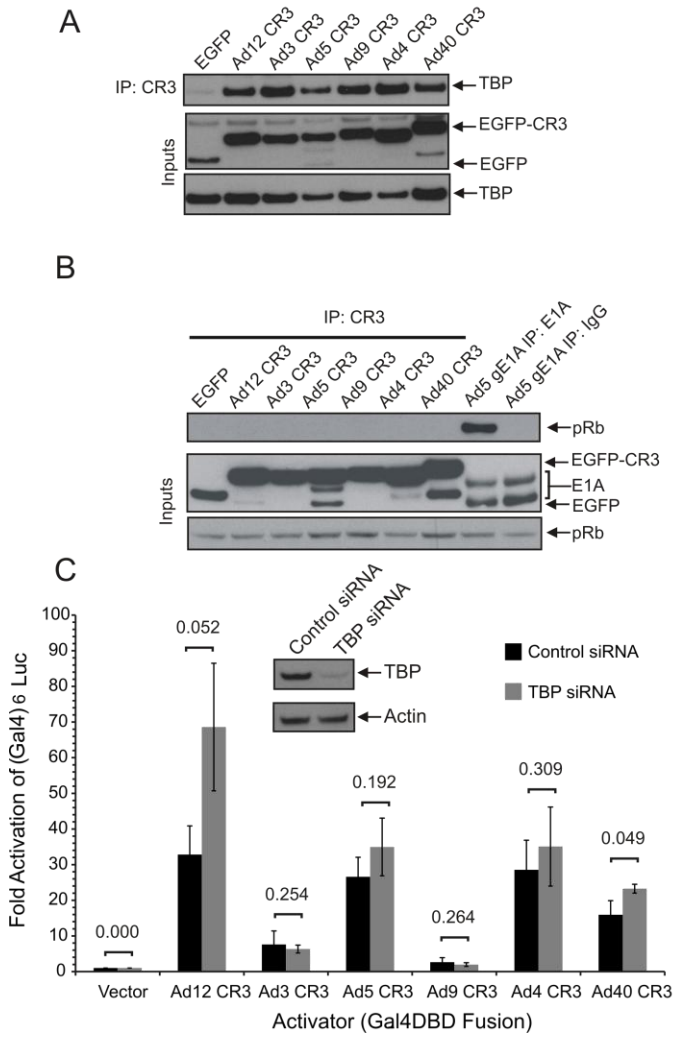


Figure 2-5: TBP is a conserved cellular target of E1A CR3 from multiple hAd types.

A) Co-IP of TBP with representative E1A-CR3s. HT1080 cells were co-transfected with a vector expressing HA-tagged-TBP and vectors expressing the indicated E1A CR3s fused to EGFP. E1A CR3s were immunoprecipitated with 9E10 antibody and blotted for HA (TBP). B) Negative Co-IP of pRb with representative E1A CR3s. HT1080 cells were co-transfected with vectors expressing the indicated E1A CR3s fused to EGFP or genomic E1A as positive control. E1A CR3s were immunoprecipitated with 9E10 antibody and E1As were immunoprecipitated with M73 and blotted for endogenous pRb. C) siRNA knockdown of TBP and the effect on transcriptional activation by E1A CR3. HeLa cells were transfected with 5 nM siRNA (Negative control or TBP specific) and at 2 days post siRNA transfection re-transfected with a Gal4-reponsive luciferase reporter and an expression vector for the indicated Gal4-CR3 fusions. At 48hours post tranfection (120 hours post siRNA transfection) cells were harvested and assayed for luciferase activity. Luciferase activity is expressed as fold above Gal4DBD alone +/- SD. Activation in control vs TBP knockdown were compared by student's T-test and the p-values are indicated. Inset: Western Blot of levels of TBP in knockdown and control cells and actin loading controls.

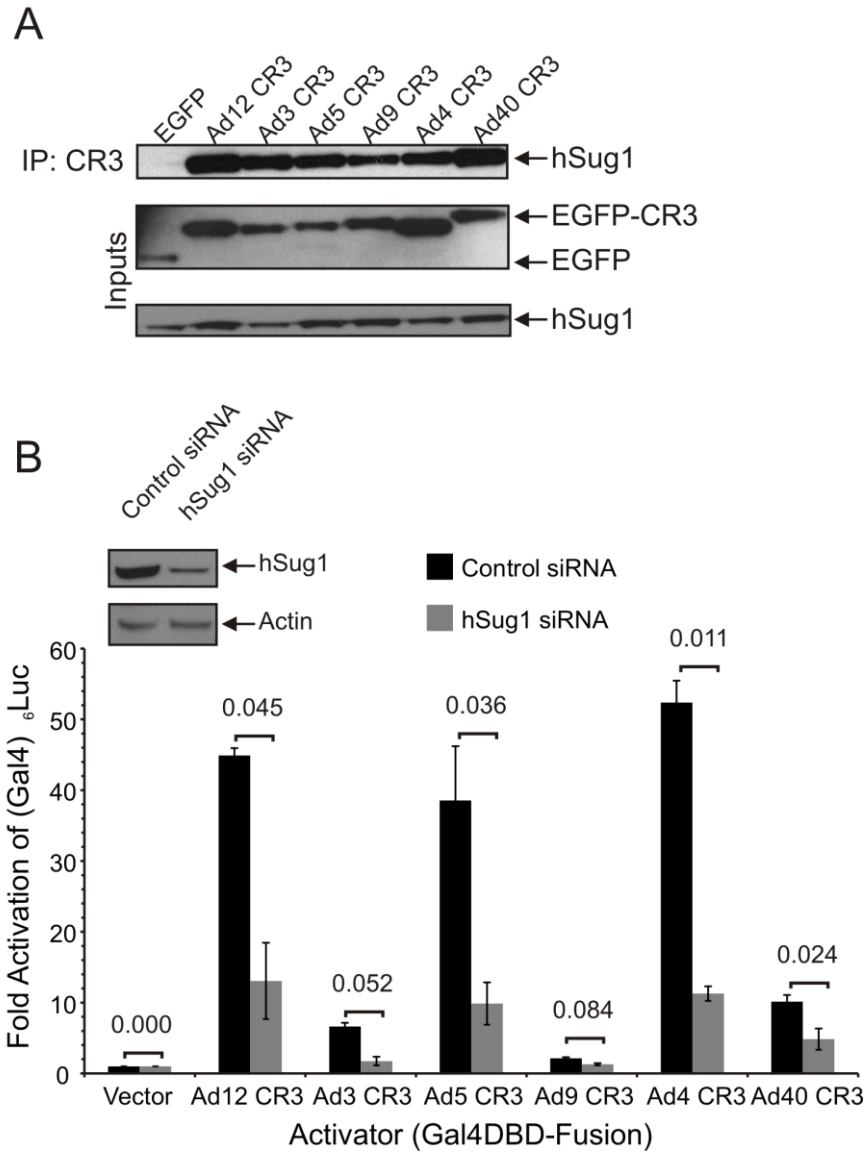


Figure 2-6: Human SUG1 is a conserved target of E1A-CR3 from multiple hAd types.

A) Co-IP of hSUG1 with representative hAd E1A CR3s. HT1080 cells were co-transfected with pcDNA4HA-hSUG1 and vectors expressing the indicated E1A CR3s fused to myc-EGFP. E1A CR3s were immunoprecipitated with anti-myc antibody and blotted for HA. B) siRNA knockdown of hSUG1 and the effect on transcriptional activation by E1A CR3. HeLa cells were transfected with 5 nM siRNA (Negative control or hSUG1 specific) and at 2 days post transfection re-tranfected with a Gal4-reponsive luciferase reporter and an expression vector for the indicated Gal4-CR3 fusions. At 48 hours post transfection (120 hours post siRNA transfection) cells were harvested and assayed for luciferase activity. Luciferase activity is expressed as fold above Gal4DBD alone +/- SD. Fold activation of control vs hSUG1 siRNA treated cells were compared by students t-test and *P* Values are indicated. Inset: Levels of hSUG1 in knockdown and control cells.

RNAi directed knockdown of hSUG1 resulted in a reduction of transactivation by all six different E1A CR3s. In particular, the E1A CR3s capable of potent activation of transcription (hAd12, hAd5, hAd4 and hAd40 CR3s) showed a significant loss of activity in hSUG1 siRNA treated cells relative to control (Figure 2-6B).

2.3.8 p300/CBP is required by all E1A CR3s to activate transcription.

We have previously shown that the p300/CBP KATs also function as a critical co-activator of hAd5 E1A CR3 function (27). All six representative E1A CR3s interacted with p300 as determined by Co-IP, although hAd5 E1A CR3 showed the strongest interaction (Figure 2-7A). Depletion of p300 levels by siRNA resulted in a greater than 50 % reduction in CR3 transactivation for all six E1A CR3s (Figure 2-7B), and this was statistically significant for all but hAd9 E1A CR3, the weakest activator.

Taken together, these data indicate that each of the six different hAd E1A CR3s share these four cellular transcriptional regulators as common targets. Importantly, the relative differences between each of these E1A CR3s to activate transcription or squelch hAd5 E1A CR3 activity cannot be simply explained by differences in their association with these factors.

2.3.9 Transcriptional activation by full-length E1A proteins.

We reasoned that the surprisingly large differences in the intrinsic ability of the different CR3s to activate transcription could be functionally compensated by activities present in other portions of these proteins. Indeed, it is well established that the N-terminal/CR1 region of hAd5 E1A functions as a strong activator of transcription when tethered to a heterologous DNA binding domain (5, 30). We directly compared the ability of the different CR3s with the corresponding full length E1A proteins to activate transcription of a Gal4 responsive luciferase reporter stably integrated into the genome of U2OS human osteosarcoma cells (Figure 2-8A). While there were again marked differences in the activities of the different E1A CR3s, the full-length E1A proteins were all equivalent or superior activators with respect to hAd5 E1A. This was particularly pronounced for hAd9, suggesting that other regions of this E1A protein may compensate for the weak

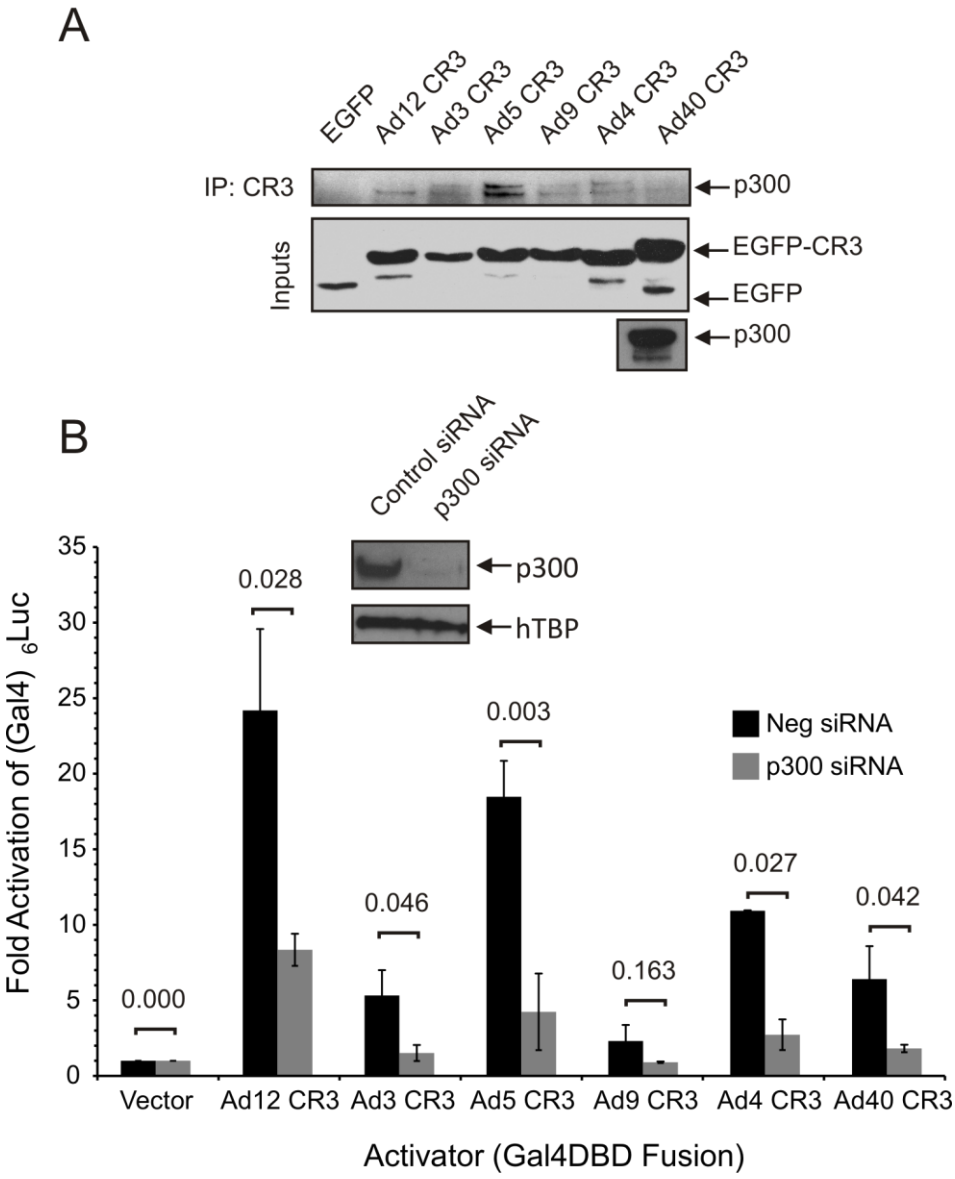


Figure 2-7: p300 is a conserved target of E1A CR3 from multiple hAd types.

A) Interaction of p300 with the E1A CR3 domains of different hAd types. Human HT1080 cells were co-transfected with an expression vector for HA-tagged p300 and expression vectors for the indicated E1A CR3 myc-EGFP fusions. EGFP fusions were immunoprecipitated with a cocktail of 9E10 and GFP antibody and blotted with HA. B) Effect of siRNA depletion of p300 on E1A CR3-dependent activation. HeLa cells were transfected with 20 nM custom siRNA directed against p300 or control siRNA and at 3 days post transfection re-tranfected with a Gal4-reponsive luciferase reporter and an expression vector for the indicated Gal4-CR3 fusions. At 48 hours post transfection (120 hours post siRNA transfection) cells were harvested and assayed for luciferase activity. Luciferase activity is expressed as fold above Gal4DBD alone +/- SD. Fold activation of control vs p300 siRNA treated cells were compared by students t-test *P* Values are indicated. Inset: Levels of p300 in knockdown and control cells.

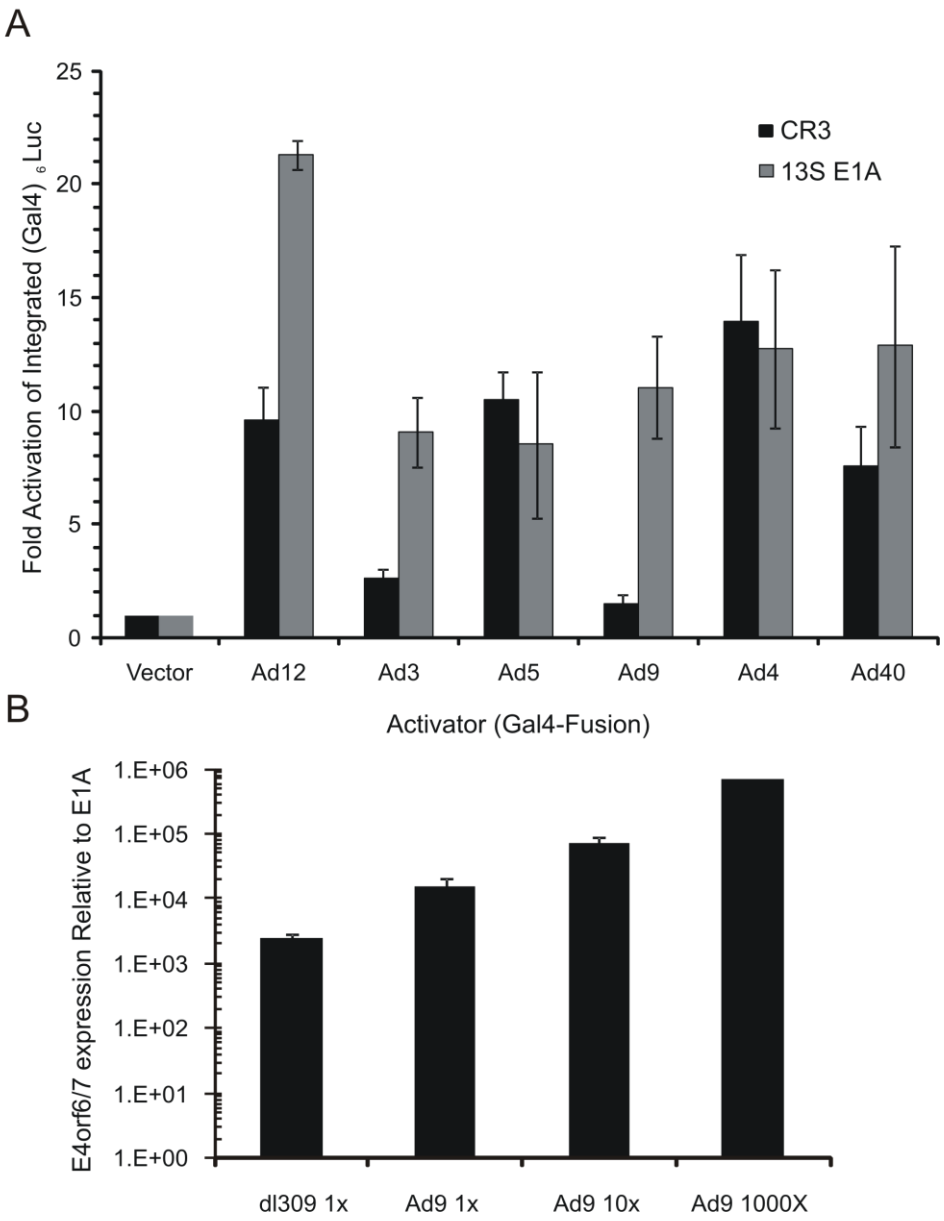


Figure 2-8: Transactivation by Full Length E1A

A) Transactivation by full length E1As in the context of Chromatin. U2OS-UAS cells that contain an integrated Gal4-responsive luciferase reporter were transfected with expression vectors for either the indicated E1A CR3 domains (black bars) or the indicated full length 13S E1As (grey bars). Luciferase activity is expressed as fold activation above Gal4 alone +/-SD. B) Transactivation of the hAd5 and hAd9 E4 promoters. At 16 hrs post infection with the indicated viruses the expression level of E4ORF6/7 mRNA was determined by qRT-PCR. The expression level of E4ORF6/7 relative to GAPDH and E1A is indicated, and the expression level for cells infected with dl312 is set to 1. 1x, 10x and 1000x denote MOIs of 2, 20 and 2000 respectively.

intrinsic transcriptional activation function of hAd9 E1A CR3. We looked further at the activity of the hAd9 E4 promoter in the context of viral infection by qRT-PCR, as E4 is a well documented target of E1A transactivation. During infection, the relative expression level of E4ORF6/7 mRNA was greatly elevated in the presence of *wt* hAd5 E1A as compared to infection with an E1A deleted hAd5 virus (~1500 fold increase). This is expected because E4 expression is highly responsive to full length E1A (22). The relative expression level of the E4ORF6/7 mRNA in hAd9 infected cells exceeds that of *wt* hAd5 infected cells, similar to what was seen with the integrated reporter assay (Figure 2-8B). The expression level of E4ORF6/7 in hAd9 infected cells increased in a dose dependent manner with increased viral inoculum (Figure 2-8B). Assuming that the hAd9 E4ORF6/7 mRNA is similarly regulated by E1A, it is clear that the hAd9 early genes are potently activated upon infection. This supports our observation with an integrated reporter gene (Figure 2-8A) that full length hAd9 E1A retains strong transactivation function, despite the weak activity intrinsic to CR3 alone.

2.4 Discussion

The CR3 portion of hAd5 E1A is a potent transcriptional activation module and serves as a paradigm of viral transactivation (3, 11, 25). CR3 is the most highly conserved of the four conserved regions within E1A (Figure 2-1A) (2). Given this similarity between E1A proteins and their essential role in activating virus early gene expression, one would predict that all E1A CR3s would function as potent activators of transcription. However this is not the case; there are dramatic differences in the potency of representative E1A CR3s to activate transcription (Figure 2-1B). In the experiments described here it was critical to utilize E1A CR3s fused to the Gal4-DBD. Direct tethering of the E1A CR3 activation domain to the transcriptional reporter via fusion to the Gal4DBD allows a direct comparison of the transactivation function by bypassing any differences in affinity between the various E1A CR3s and the sequence specific DNA-binding transcription factors that normally recruit it to the transcriptional template (20, 21). Indeed, our initial experiments revealed that none of the largest E1A products from any of the hAd speciess (except hAd 5) could stimulate transcription of an hAd 5 E4 promoter driven reporter, presumably due to an inability to be targeted to that reporter (unpublished results).

The unexpected and dramatic differences between the different E1A CR3 domains to activate transcription suggest that the hAd lifecycle can initiate and progress efficiently even if the CR3 region of the E1A protein is a relatively weak activator. Indeed, 3 of the 5 other E1A CR3s we tested were less that 50 % as active as the prototype hAd 5 E1A CR3, with hAd9 E1A CR3 being by far the weakest (Figure 2-1B). In agreement with this, previous work using a panel of E1A CR3 mutants found that growth of hAd5 was not significantly reduced unless E1A dependent transactivation was reduced by 5 to 20 fold, which translates to the suggestion that a minimum cut-off of approximately 20 % of hAd 5 E1A CR3 function is critical to virus growth (16).

To understand the molecular basis for the differences in transactivation between hAd types, we initially utilized a transcriptional squelching assay. Despite the inability of the hAd V147L E1A CR3 mutant to interact with multiple cellular proteins targeted by E1A CR3 (MED23, TBP, pCAF and p300), it behaved nearly like *wt* hAd5 E1A CR3 in the squelching assay. This is highly indicative that this mutant retains binding to additional limiting factors necessary for E1A CR3 dependent transactivation that remain to be identified. When tested in the squelching assay, expression of each of the five other representative E1A CR3s as fusions to EGFP reduced activation by Gal4-hAd 5 E1A CR3 (Figure 2-3A). These results confirmed that each of the distinct hAd E1A CR3 domains was capable of competing with Gal4-hAd 5 E1A CR3 for at least one critical factor. Interestingly, there did not appear to be any correlation between the potency of a given E1A CR3 to transactivate (Figure 2-1B) and the ability to squelch hAd5 E1A CR3 (Figure 2-3B). These results suggest that the mechanism of E1A CR3 transactivation is a complex hierarchy of binding kinetics and that multiple cellular factors required by E1A CR3 are limiting *in vivo*. Simply put, the extent to which any given E1A CR3 squelches Gal4-hAd 5 E1A CR3 is based on its ability to sequester one or more of these targets. Furthermore, the ability of hAd 12 E1A CR3 to activate as strongly as hAd 5 E1A CR3 yet squelch Gal4-hAd 5 E1A CR3 poorly, indicates that important mechanistic differences in transactivation exist between at least these two E1A proteins. One explanation for these observations could be that hAd 12 E1A CR3 targets additional factors not utilized by hAd5 E1A CR3 that contribute to its strong transactivation function (15).

We directly tested the ability of each of the six E1A CR3 domains from the different hAd types to bind hMED23 (Figure 2-4B), TBP (Figure 2-5A), hSUG1 (Figure 2-6A) and p300 (Figure 2-7A) and the role that these interacting proteins had on their ability to transactivate (Figures 2-4C, 2-5B, 2-6B and 2-7B, respectively). These data demonstrated, for the first time in most cases, that each of the different E1A CR3s had the ability to interact with these cellular factors, although to varying extents with respect to the hAd 5 E1A CR3 prototype. These data also demonstrate that hMED23, hSUG1 and p300 play vital roles in transactivation by most, if not all, of the different E1A CR3s, as previously described for the hAd 5 E1A CR3 prototype (27, 28, 31). In contrast, the interaction with TBP is not necessary for transcriptional activation by the different CR3s, at least in the context of the Gal4-CR3 fusions.

The binding data demonstrated that hAd 9 E1A CR3 interacted to some degree with all of the known cellular targets of E1A CR3 tested (Figures 2-4B - 2-7B). Indeed, it bound hMED23 (Figure 2-4A) and pCAF far better than all other E1A CR3s except hAd5 (26). Based on the binding data, it is not surprising that hAd9 E1A CR3 squelched hAd5 E1A CR3-dependent activation (Figure 2-3). However, it is surprising that it was the weakest activator of transcription of the six E1A CR3s tested. The existing model of E1A CR3 function cannot explain this phenomenon. According to what is currently known about E1A CR3 function, hAd9 E1A CR3 should be able to potently activate transcription as a Gal4-fusion because it can interact strongly with all of the known cellular coactivators of E1A CR3 so far identified. This line of evidence may indicate that the weak activity of the hAd9 E1A CR3 region results from the involvement of an as yet unidentified cellular co-factor required by E1A CR3 to activate transcription. Alternatively, if E1A CR3 serves as a scaffold to assemble all the factors required for transcriptional activation, hAd 9 E1A CR3 on its own may not properly organize them spatially or temporally.

Interestingly, the hAd9 E1A CR3 sequence has the least identity with hAd5 (47%), as compared to hAds 12, 3, 4 and 40 (60%, 60%, 56% and 54% respectively). The key residues essential for transcriptional activation by hAd5 CR3 have been systematically identified (33). Based on that analysis, inspection of the CR3 sequence of

hAd9 E1A reveals that multiple residues expected to be critical for activation are different. Specifically, individual conservative changes in L144, G151, M170 and R177 significantly impair hAd 5 E1A CR3 activation and these residues all differ in hAd9 E1A CR3 (Figure 2-1A). Despite the pronounced defect in hAd9 E1A CR3 dependent activation, the full length hAd9 E1A protein is a very potent activator (Figure 2-8A), and the hAd9 E4 promoter is highly active upon infection (Figure 2-8B) suggesting that other regions of the protein can effectively complement the deficiency in CR3 to activate viral early gene expression.

From the work presented here, it is clear that there are multiple conserved interactions amongst the representative E1A CR3s with cellular co-factors that are involved in activating transcription. Beyond these conserved interactions, our current and previous work (26) also provides growing evidence that there are selective cellular targets required by the E1A CR3 domains of some hAd types and not others. These may be particularly important for infection of specific tissues types. Perhaps it is the subtle differences in accessory factors that ultimately regulate the potency of a given E1A CR3 to activate transcription, rather than the conserved co-activators. Further studies of these potent transcriptional activation domains may lead to the identification of additional cellular transcriptional regulators and provide novel insight into their mechanism of action.

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Chapter 3

3 Cellular GCN5 is a Novel Regulator of Human Adenovrius E1A-Conserved Region 3 Transactivation

3.1 Introduction

The early region 1A (E1A) gene is the first viral gene expressed in cells upon infection with human adenovirus (hAd). The primary E1A transcript is alternatively spliced to yield two predominant isoforms early in infection that perform the two essential functions required to initiate the viral replication cycle. Firstly, E1A uncouples the cell cycle control program of the host cell, driving it into S phase to provide an optimal cellular environment for viral replication. The smaller major E1A isoform (243 residues in hAd5 E1A) is sufficient to override cell cycle progression and drive cells into S-phase (18, 36). The other critical function of E1A is to activate transcription of the early viral promoters, also known as transactivation. Transactivation of the hAd early genes is predominantly mediated by the product of the largest E1A isoform (7, 24, 33, 34). In hAd5 the 13S E1A mRNA codes for a 289 residues protein that differs from the 243R E1A protein by a unique 46 aa C4 zinc finger domain located within CR3, which is essential for viral transactivation (7, 24). Chemical mutagenesis of hAd originally isolated mutants with a host range limited to HEK293 cells, which supply *wt* E1A in *trans*. These "host range" mutations render E1A unable to transactivate viral promoters, thus preventing virus growth in HeLa cells (which do not supply E1A in *trans*) at a low multiplicity of infection (MOI) (21, 22). Transactivation by E1A CR3 has been studied predominantly with hAd5, and this has established a model for CR3 function. The region of hAd5 E1A spanning residues 139 to 204 (which includes CR3) is not only critical for the 5-20 fold activation of viral transcription necessary for virus growth, but is also sufficient for potent activation of a minimal Gal4-responsive promoter as a Gal4-DNA binding domain (DBD) fusion (23, 44).

This 65 aa region of E1A spanning residues 139-204 which includes CR3 can be further subdivided into the three following subdomains: an N-terminal zinc finger region, a promoter targeting region, and a region known as auxiliary region 1 (AR1) (15, 29, 48).

The existing model of CR3 transactivation states that the N-terminal zinc finger subdomain of E1A CR3 activates transcription by interacting with cellular TBP and MED23 proteins (9, 27, 47). The Mediator component MED23 is absolutely essential to E1A CR3 function, since CR3 fails to activate transcription in MED23-null MEFs (47). The requirement for MED23 by CR3 is shared between the CR3 domains of E1A proteins of closely related hAds. Indeed, the CR3 regions of E1A proteins from hAds representing the six hAd species all bind MED23 and require MED23 to activate transcription as Gal4 DNA Binding Domain (Gal4DBD) fusions (1). Even very divergent Ads require MED23, because mouse adenovirus type 1 is unable to replicate efficiently in MED23-null MEFs (17). Re-targeting of a functional E1A-containing transcription initiation complex to the early viral promoters requires the C-terminal promoter targeting subdomain of CR3. This subdomain confers interaction of E1A CR3 with cellular sequence-specific DNA-binding transcription factors, including members of the ATF family (13, 29-31). Mutants that delete this promoter targeting domain of CR3 function as dominant-negative mutants, unless a second mutation is made in the zinc finger domain of CR3 (29, 50). The precise role of the residues constituting the AR1subdomain of CR3 in transactivation remains unclear; however, the acidic character of this region is necessary for maximal transactivation by E1A CR3 (48).

Since the definition of the initial model of E1A transactivation, several additional cellular factors have been implicated in transactivation by E1A CR3. The S8 component of the 19S APIS, hSUG1, was shown to interact with E1A CR3 and enhance E1A CR3 transactivation (39). A second independent interaction site for the cellular repressor C-terminal Binding Protein (CtBP) was also mapped to the CR3 region of hAd5 E1A, yet CtBP appears to repress E1A CR3 transactivation (11). The p300/ CBP Lysine Acetyl-Transferases (KATs) are critical for hAd5 E1A CR3 transactivation. These KATs were shown to bind directly to E1A CR3 independently of the other two interaction motifs in the N-terminus and CR1 of E1A (38). Most recently, the KAT p300/CBP associated factor (pCAF) was identified to interact with a subset of our panel of representative E1A CR3s in addition to its independent interaction with the N-terminus of E1A and to enhance CR3 transactivation (37). These core co-activators and their functions are conserved across the entire hAd family despite dramatic differences in the magnitude of

E1A transactivation exhibited by representative members of each hAd species (1, 37). Moreover, competition experiments demonstrated that additional as yet unidentified cellular factors are not only involved in E1A transactivation, but and are also limiting in the cell (1).

Many cellular factors that interact with CR3 also independently bind other regions of E1A, further complicating E1A-mediated activation of transcription. Whether these independent interactions function cooperatively or competitively has not been fully elucidated (38, 52). Of the factors that interact with both transactivation regions of E1A, two are KATs (p300/CBP and pCAF). Our lab has identified a second interaction site for both of these KATs within CR3 and characterized their role in E1A transactivation (37, 38). A third KAT and close relative of pCAF, GCN5 also interacts with the N-terminus of E1A but neither a role for GCN5 nor an interaction for GCN5 within CR3 has been demonstrated in mammalian cells (26). In yeast, which lack pCAF, yeast GCN5 interacts with hAd5 CR3 and is a coactivator of CR3 transactivation (44).

The primary function of GCN5 appears to be as a chromatin remodelling factor (5, 10, 35, 42). GCN5 is a catalytic component of the Spt-Ada-GCN5-Acetyltransferase (SAGA) complex in yeast and the Spt-TAF_{II}31-GCN5L (STAGA) complex in mammalian cells. The KAT activity of GCN5 is required to acetylate histone H3 lysine 9 (K9) and K14, and this facilitates transcription elongation by relaxing nucleosomes (5, 10, 35, 42). In mammalian cells, GCN5 plays a major role in acetylation of K14 on H3 and concurrent phosphorylation, so-called tandem phosphoacetylation, of S10 required for transcriptional activation (32). A definitive role for GCN5 in mammalian transcription has remained elusive, mainly due to the developmental phenotype of knockout animals. pCAF null animals (pCAF -/-) are viable due to compensation by increased levels of GCN5, suggesting some functional redundancy between these closely related KATs (55). However, GCN5 null embryos (GCN5-/-) die very early in development, at 10.5 days postcoitum (d.p.c) from massive apoptosis. This results from the loss of the deubiquitination activity of GCN5, and ultimately results in genomic instability and telomere crisis (2, 53). Embryos specifically defective for the GCN5 KAT

activity (GCN5 *hat/hat*) die at 16.5 d.p.c as a result of defects in neural tube closure and encephalopathy (12).

We report here identification of a second independent conserved GCN5 binding site within CR3, mapping to residues 178-184 of hAd5 E1A. GCN5 associated with the viral E4 promoter in an E1A-dependent manner. Moreover, the two interaction sites in E1A cooperate to recruit GCN5 to the transcriptional template. GCN5 functions as a negative regulator of E1A transactivation, because RNAi depletion of GCN5 increased E1A transcriptional activation and ectopic expression of GCN5 repressed it. Pharmacological inhibition or mutation of the KAT activity of GCN5 relieved the repressive effect on E1A transactivation and KAT inhibition also decreased virus yield. We therefore show that GCN5 contributes a new layer of negative regulation to the existing model of E1A transactivation that influences virus production. This activity may balance viral gene expression needed for replication while minimizing production of high levels of toxic gene products that would kill infected cells prematurely.

3.2 Materials and Methods

3.2.1 Cells, cell culture and transfections.

Human A549, HeLa and HT1080 cells, as well as *wt* MEFs and GCN5 *hat/hat* MEFs (12), were maintained at 37 °C and 5 % CO2 in Dulbecco modified Eagle medium (Wisent) with 10 % fetal bovine serum (Gibco) and 100 U/ml of penicillin-streptomycin (Wisent). A549 cells and MEFs were transfected with the FuGENE HD reagent (Roche), according to the manufacturer's directions, in a ratio of 3 µg of total DNA to 9 µl of FuGENE HD per well of a six-well plate. HeLa and HT1080 cells were transfected with the Superfect reagent (Qiagen), according to the manufacturer's directions. Wild type littermate MEFs and GCN5 *hat/hat* MEFs were provided by Sharon Roth-Dent and have been described previously (12).

3.2.2 Plasmid construction.

The Gal4-responsive luciferase reporter vector pGL2-$(Gal4)_6$-Luc and Gal4-DBD fusions for each hAd E1A CR3 and the N-terminus of E1A (residues 1-82) have been described

previously (3, 43). The expression vector for enhanced green fluorescent protein (EGFP) fusions of hAd5 E1A CR3 Δ178-184 was produced by PCR amplification of the CR3 region using the primers CR3N-F 5'- AGACGAATTCGGTGAGGAGTTTGTGTTA-3' and CR3C-R 5'- CGCGGATCCATTAGGTAGGTCTTGCAGGCTC-3' from a 13S E1A *dl*114 clone as template with Phusion polymerase according to the manufacturer's directions. The PCR product was cloned into pCAN-myc-EGFP with EcoRI and XbaI (23). The expression vector for mGCN5 was generated by PCR using the primers hGCN5-F 5'-TCGGAATTCGCGGAACCTTCCCAGGCCCCAAACC-3' and hGCN5-R 5'- GACTCTAGACTACTTGTCGATGAGCCCTCC-3' with Phusion polymerase (NEB) according to the manufacturer's directions using a previously described expression vector for mGCN5 as template (54). The PCR product was digested with EcoRI and XbaI and cloned into the EcoRI and NheI sites of pCMX-FLAG. A correct clone was verified by sequencing.

3.2.3 E1A transactivation assays.

The Gal4 fusion activation assay has been described previously (1). The full length E1A activation assay was performed as follows: At 24 h prior to transfection, 1.5 x 10^5 HT1080 cells/well were seeded on six-well plates. Cells were transfected in a 1:3:4 ratio of E4 reporter pGL2-E4v3: E1A expression vector: GCN5 (either pCMX-FLAG mGCN5 or empty pCMX-FLAG as control) (38). At 6 hours post-transfection cells were washed and fresh media was added. For activation assays involving the GCN5 specific KAT inhibitor cyclopentylidene-[4-(4′-chlorophenyl)thiazol-2-yl)hydrazone (CPTH2) (Sigma), either the indicated final concentration of CPTH2 or equal volume of DMSO (vehicle) was added after washing at 6 hours post-transfection. Cells were harvested at 48 hours post-transfection and assayed for luciferase activity. Luciferase activity is expressed as % of control +/-SD.

3.2.4 Co-immunoprecipitation and Western blot analysis.

Co-immunoprecipitation (Co-IP) of GCN5 with full length E1A from infected HeLa cells was performed as described previously (26). Co-IP of GCN5 with EGFP-fused CR3s was performed as described previously with anti-myc antibody (Clone 9E10) (1). Co-IP

of mycEGFP-fused CR3 and CR3 mutants with FLAG-tagged-GCN5 was performed as described previously but using FLAG-M2 Agarose (Sigma) (1). Western blot analysis was performed as described previously (1). The GCN5 rabbit polyclonal antibody was a gift from Joseph Torchia (UWO).

3.2.5 siRNA knockdown.

Silencer select siRNAs against GCN5 (siRNA ID 5659) was purchased from Ambion. siRNA transfections were performed with siLentFECT reagent (Bio-Rad), according to the manufacturer's directions. Typically, 1.5 x 10^6 HeLa cells were seeded on 10 cm plates for siRNA transfection. At 24 h post-siRNA transfection, cells were reseeded to six-well plates at 2 x 10^5 cells per well. At 48 h post-siRNA transfection, cells were transfected again as described above with the appropriate expression vectors to perform the Gal4 fusion activation assay.

3.2.6 Chromatin Immunoprecipitation (ChIP) assays.

ChIP assays were preformed as described previously (38, 49). M73 hybridoma supernatant was used for ChIP of E1A from infected cells and rabbit polyclonal anti-GCN5 (a gift from Joeseph Torchia) was used for ChIP of GCN5. PCR for a 300 bp region of the Ad5 E4 promoter was performed as described previously (38).

3.2.7 Quantitative Reverse Transcription-PCR (qRT-PCR).

Realtime qRT-PCR of hAd E4ORF6/7 transcripts were performed as described previously (1). Briefly, human A549 cells were infected with either *wt* hAd5 or mutant hAd5 virus at an MOI of 2.0. At 16 h post-infection, total RNA was isolated as described previously and used to generate cDNA. Quantitative PCR was performed in triplicate with a 15 µl reaction mixture and 1x iQ-SYBR green SuperMix (Bio-Rad) according to the manufacturer's directions in a MyiQ real-time PCR instrument (Bio-Rad). The primers for hAd5 targets and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously (39). Data were analyzed using IQ5 software (Bio-Rad). E4ORF6/7 mRNA levels were normalized to GAPDH levels as an internal control and to

the respective E1A mRNA level for each sample. E1A and E4ORF6/7 mRNA levels in cells infected with ΔE1A mutant dl312 were set equal to 1.

3.2.8 Virus Growth Assay

A549 cells were infected at an MOI of 5 with either wt (*dl*309) or E1A deleted (*dl*312) adenovirus for one hour. After one hour adsorption virus innoculum was removed and cells were replenished with fresh media contain either 50μM CPTH2 or an equivalent volume of DMSO (Vehicle). At 96 hours post infection cells and media were harvested and subjected to three freeze-thaw cycles and the yield of virus present was determined by plaque assay on 293 cells. The virus yield from duplicate experiments was averaged and mean virus growth +/-SD was plotted and compared by students T-test.

3.3 Results

3.3.1 There is a second independent interaction site for GCN5 in E1A that maps to the CR3 domain.

Human GCN5 was previously shown to interact with hAd5 E1A through residues 26-35 in its N-terminus (26). We confirmed that 12S (243R) E1A Δ26-35 failed to co-immunoprecipitate (Co-IP) GCN5, yet this mutant can still Co-IP pRb (Figure 3-1A lane 2). Interestingly, E1A Δ26-35 retained the ability to Co-IP GCN5 when both the 12S (243R) and 13S (289R) isoforms of E1A were present (Figure 3-1A lane 3). Moreover, while 12S E1A *wt* and 12S E1A Δ26-35 failed to Co-IP the CR3 specific target MED23, the same mutant Co-IPs MED23 when the 13S (289R) isoform is present (Figure 3-1A lane 3). This suggested that there is an interaction between GCN5 and the unique region of the largest E1A isoform, which corresponds to CR3 (Figure 3-1A). Indeed, the CR3 domain of representative E1As from each hAd species were sufficient to Co-IP GCN5 from cells co-transfected with expression vectors for EGFP-fused E1A CR3 and Flag-tagged mGCN5 (Figure 3-1B). However, the hAd4 E1A CR3 interaction was very weak. Using a collection of E1A CR3 mutants, the interaction of GCN5 was mapped to residues 178-184, since this mutant failed to IP mGCN5 (Figure 3-1C). Mutants that have been characterized previously to lose interaction with pCAF (Δ139-147), MED23 (H160Y), or TBP (V147L) retained interaction with GCN5 (Figure 3-1C) (9, 20, 37, 50).

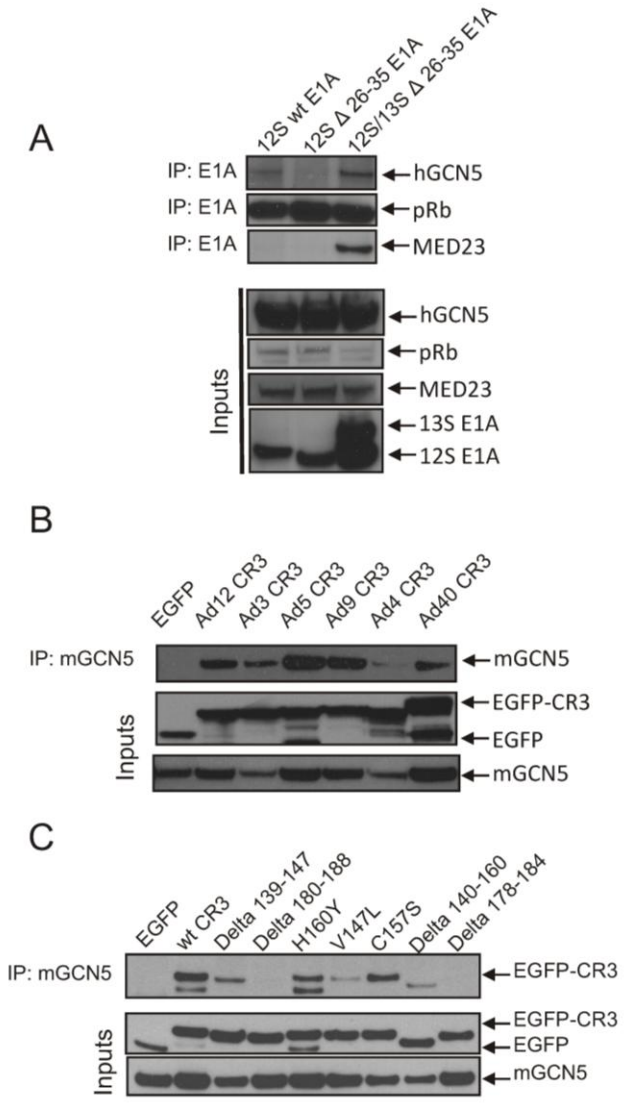

Figure 3-1: E1A-CR3 Interacts with the KAT GCN5.

A) The largest E1A isoform retains a novel interaction site with GCN5. HeLa cells were infected with the indicated viruses at an MOI of 10. At 24 hours post infection, cell lysates were prepared and E1A was immunoprecipitated with M73, separated by SDS-PAGE and transferred to PVDF membrane and subsequently probed with anti-GCN5, anti-pRb, anti-MED23 and anti-E1A antibodies. B) All six representative E1A-CR3s co-immunoprecipitated mGCN5. Human HT1080 cells were co-transfected with expression vectors for myc-EGFP fusions to the indicated E1A-CR3s and an expression vector for FLAG-tagged mGCN5. CR3s were immunoprecipitated with anti-myc and probed for GCN5 with anti-Flag. C) The interaction of E1A-CR3 with mGCN5 mapped to residues 178-184. Human HT1080 cells were transfected as described above, immunoprecipitated with anti-Flag (for GCN5), separated by SDS-PAGE, transferred to PVDF membrane and probed with anti-myc (for CR3).

Furthermore, the interaction of GCN5 with CR3 did not require the zinc finger subdomain, because a point mutant in one of the coordinating cysteine residues (C157S) and a large deletion encompassing the majority of the zinc finger region (Δ140-160) also retained interaction with GCN5 (Figure 1C). However, mutants that contained deletions in the promoter targeting/ATF binding domain lost interaction with GCN5. Therefore, E1A CR3 contains a second independent interaction site for GCN5 that requires at least residues 178-184.

3.3.2 Recruitment of GCN5 to the hAd5 E4 promoter requires both the N-terminal and CR3 interaction domains.

Given the importance of CR3 in activation of viral early gene transcription, we hypothesized that the interaction of the largest E1A isoform with GCN5 would be involved in this process. This would require GCN5 to be associated with viral promoters during infection. The E4 promoter is potently stimulated by E1A during infection and we have shown previously that both E1A and p300 can be found to occupy a 300 bp region of this promoter during infection (Figure 3-2A) (38). In a similar fashion, we determined whether GCN5 was recruited to the adenoviral E4 promoter during infection using chromatin immunoprecipitation (ChIP). Indeed, not only was E1A again shown to occupy the E4 promoter in cells infected with *wt* hAd5 (*dl*309), but GCN5 was also found to occupy the E4 promoter in the context of infection (Figure 3-2B). However, in cells infected with a virus lacking E1A (*dl*312), GCN5 was not found at the E4 promoter. Therefore GCN5 associates with the viral E4 promoter in an E1A-dependent manner. We tested which of the binding sites on E1A were required to recruit GCN5 to the E4 promoter using mutant viruses that have a small deletion in either of the two GCN5 binding sites. Viruses lacking either residues 26-35 or residues 178-184 showed a reduction in recruitment of GCN5 to the E4 promoter, indicating that both sites were required by E1A to effectively recruit cellular GCN5 to the viral promoter. This suggests that the two distinct GCN5 binding sites within E1A function in a cooperative manner to recruit GCN5 to the E4 promoter (Figure 3-2B).

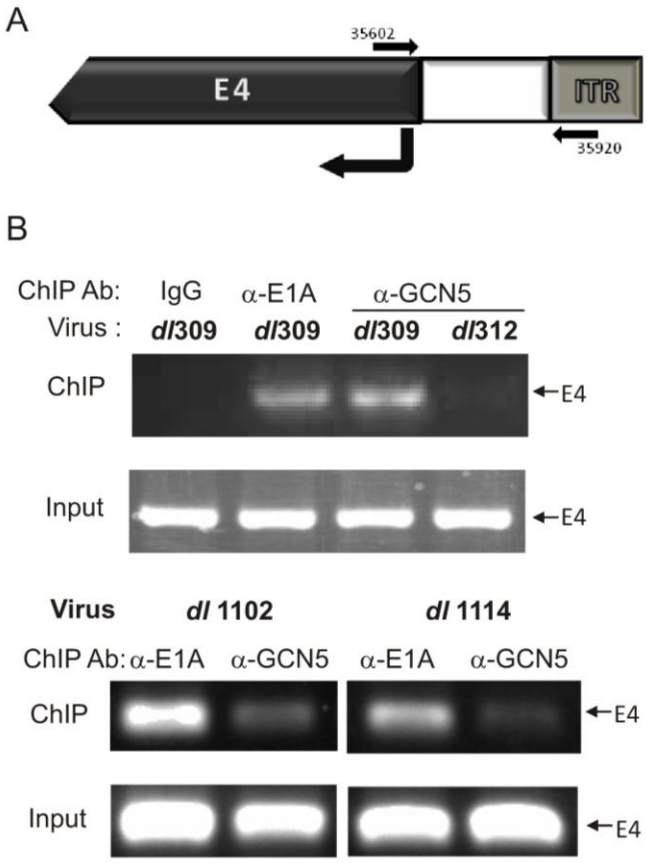

Figure 3-2: GCN5 Occupies the Adenoviral E4 Promoter During Infection

A) Schematic of the right end of the hAd5 genome, showing primer binding sites for ChIP PCR. B) Human A549 cells were infected at an MOI of 5 with the indicated viruses (*dl*309 = wt E1A, *dl*312 = delta E1A, *dl*1102 = E1A delta 26-35, *dl*1114 = E1A delta 178-184). Cells were fixed and chromatin was purified and immunoprecipitated with the indicated antibodies. After washing and de-crosslinking, PCR was performed with a set of primers specific for a 320 bp region of the adenoviral E4 promoter region.

3.3.3 Depletion of GCN5 enhances transactivation by E1A.

To determine the functional role that GCN5 plays in E1A CR3-dependent transactivation, GCN5 was depleted in HeLa cells by specific siRNA (Figure 3-3A), and cells were co-transfected subsequently with a Gal4-responsive luciferase reporter and an expression vector for E1A CR3 fused to the Gal4DBD (Figure 3-3B). Surprisingly, the transactivation function of hAd12, hAd5 and hAd40 E1A CR3 increased in cells treated with GCN5 specific siRNA compared to cells treated with control siRNA (Figure 3-3B). The transactivation function of hAd4 E1A CR3 was unaffected when GCN5 was depleted; this may relate to the fact that the interaction between GCN5 and hAd4 E1A CR3 was the weakest of those tested (Figure 3-3-1B). The ability of hAd3 and hAd9 E1A CR3 to activate transcription was not significantly increased upon depletion of GCN5. These were the two weakest activators of transcription in our panel of E1A CR3s (1). These two CR3s showed an increase in transcriptional activation in GCN5 siRNA-treated cells compared to control siRNA-treated cells; however, the difference was not significant. The N-terminus of E1A is also capable of activating transcription of a Gal4 responsive promoter as a Gal4DBD fusion (8) and it also interacts with GCN5 via residues 26-35 (26). However, the transactivation function of the N-terminus of E1A was unaffected by GCN5 depletion, suggesting that CR3 is using GCN5 in a fashion distinct from the E1A N-terminus (Figure 3-3-3B). Clearly, GCN5 plays a repressive role in E1A CR3-dependent transactivation, because depletion of GCN5 resulted in an overall increase in transcriptional activation.

3.3.4 Overexpression of GCN5 reduces transactivation by E1A.

If GCN5 is a repressor of E1A CR3-stimulated transcription, it follows that overexpression of GCN5 would decrease E1A CR3-dependent transactivation. To test this, we co-transfected human HT1080 cells with a Gal4-responsive luciferase reporter, an expression vector for hAd5 E1A CR3 fused to the Gal4DBD and either increasing amounts of an expression vector for Flag-tagged mGCN5 (pCMX-FLAG mGCN5) or empty vector (pCMX-FLAG) as a control. The fold activation by Gal4-E1A-CR3 over

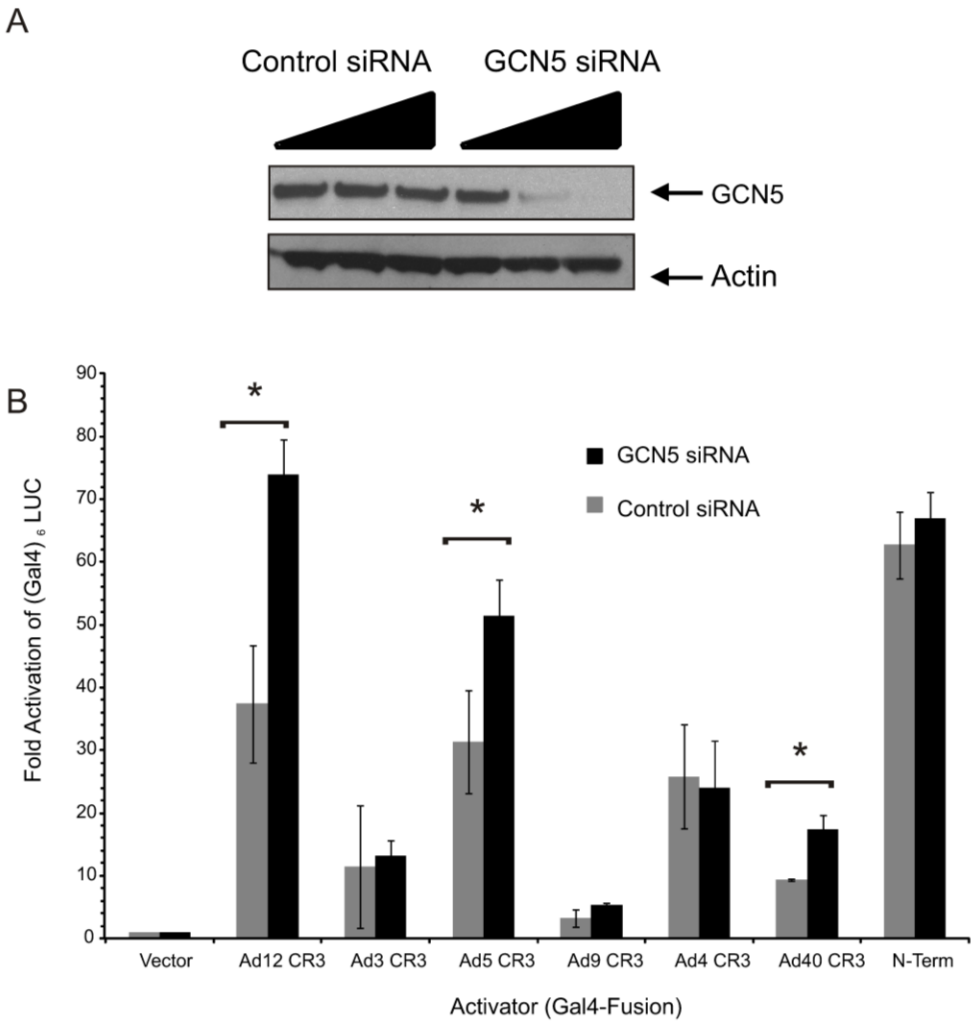

Figure 3-3: Depletion of GCN5 results in an Increase in E1A-CR3 Transactivation

A) Hela cells were transfected with increasing doses of GCN5-specific siRNA, or control siRNA and the levels of GCN5 were determined at 72 hours post-transfection by western blot with the indicated antibodies. B) HeLa cells were transfected with 20 nM siRNA specific for GCN5 or control siRNA. At 48 hrs post-transfection cells were split and co-transfected with equal ratios of a $(Gal4)_6$-Luc reporter and an expression vector for the indicated Gal4-CR3 fusion. 'Vector' denotes cells transfected with Gal4 alone and N-term denotes cells transfected with and expression vector Gal4 fused to residues 1-82 of hAd5 E1A. At 48 hours post-DNA transfection cells were harvested and assayed for luciferase activity. Fold activation in control vs. GCN5 siRNA treated cells were compared by student's *t* test, * indicates $P<0.05$.

Gal4 alone, when co-transfected with empty pCMX-Flag was set to 100 %. Ectopic expression of GCN5 resulted in a dose-dependent repression of E1A CR3 transactivation (Figure 3-4A). At the highest dose used (1:1 ratio of pCMX-Flag mGCN5:pM-hAd5 CR3), a 50 % reduction in E1A CR3 transactivation was observed (Figure 3-4A). In a similar manner, overexpression of GCN5 reduced transactivation of the viral E4 reporter by full length E1A by approximately 40 % (Figure 3-4B). Consistent with the ChIP data, mutations in either binding site for GCN5 (deletion of residues 26-35, or 178-184 respectively) rendered full length E1A significantly less sensitive to overexpression of GCN5 (Figure 3-4B). These data indicate that the repressive effects of GCN5 on transactivation by full length E1A required both binding sites to efficiently interact with and recruit GCN5 to the E4 promoter.

3.3.5 Pharmacological inhibition of GCN5 KAT activity enhances transactivation by E1A.

GCN5 was identified originally as a KAT and has a well established role as a transcriptional co-activator that acetylates H3K14 to relax chromatin and facilitate transcript elongation in the context of the SAGA complex (5, 10, 35, 42). We hypothesized that E1A was retargeting the KAT activity of GCN5 by relocating it to the viral template. Although the viral genome is initially devoid of cellular histones, a recent report demonstrates viral core proteins and histones, including H3, bind to viral DNA during early phases of infection (25). Komatsu and colleagues also demonstrated that H3 is acetylated at K9 and K14 which could be post-translationally modified by GCN5 as a consequence of transcription from the viral template; however, no direct role for E1A was established.

Our data suggest that E1A may be utilizing the GCN5 KAT activity to regulate viral transactivation. To test this pharmacologically, we used a novel small molecule that has been demonstrated to be a specific inhibitor of GCN5 (14). The molecule cyclopentylidene-[4-(4′-chlorophenyl)thiazol-2-yl)hydrazone (CPTH2) specifically inhibits acetylation of histone H3K14 in *vitro* and in *vivo* at μM concentrations (14). We first titrated the effect of CPTH2 on hAd5 E1A CR3 transactivation. Human HT1080 cells were co-transfected with a Gal4-responsive luciferase reporter and an expression

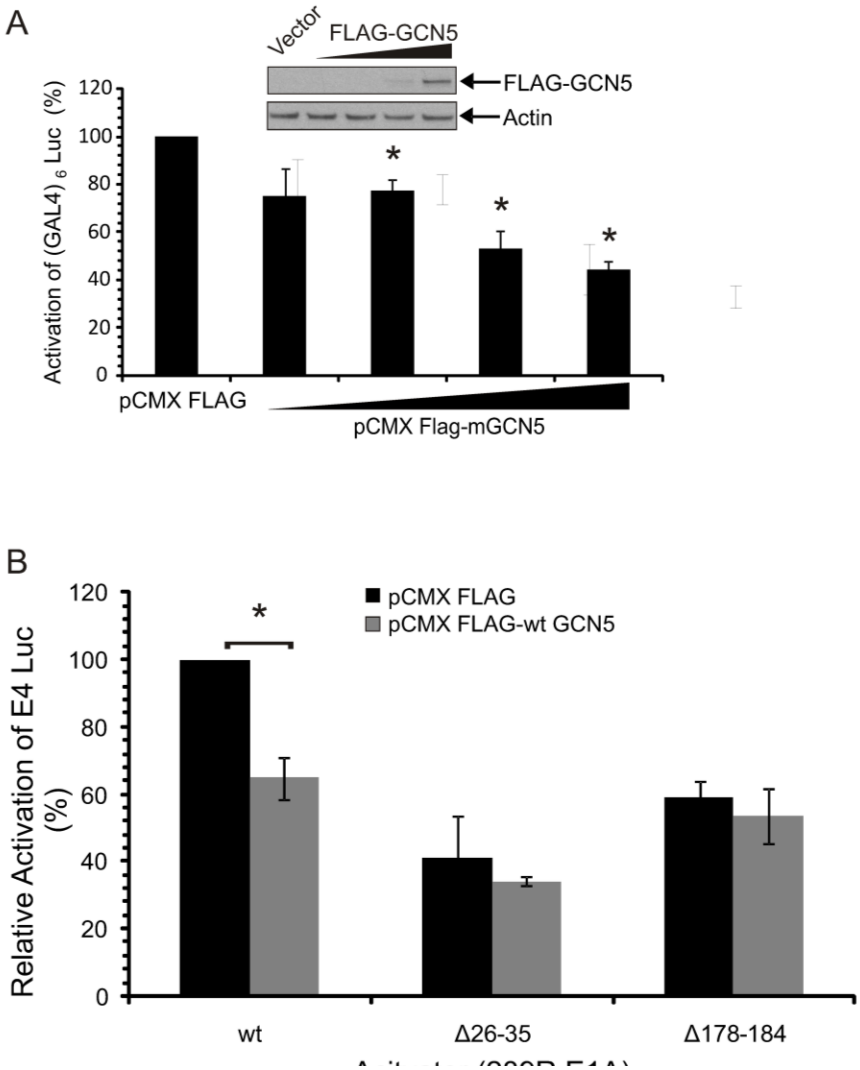

Figure 3-4: Overexpression of GCN5 Results in a Decrease in E1A-CR3 Transactivation

A) Human HT1080 cells were co-transfected with a Gal4-responsive luciferase reporter, and expression vectors for a Gal4-fusion and increasing amounts of Flag-tagged mGCN5. The fold activation by Gal4-E1A-CR3 over Gal4 alone, when co-transfected with empty pCMX-Flag was set to 100 %. INSET: levels of Flag-tagged mGCN5 upon co-transfection of increasing amounts of pMCX-Flag as determined by western blot with the indicated antibodies. B) Human HT1080 cells were co-transfected with equal amounts of an adenoviral E4-responsive luciferase reporter, an expression vector for E1A, and either empty pCMX-FLAG or pCMX Flag-mGCN5. The fold activation of the E4 reporter in cells transfected with *wt* 13S E1A over empty vector, when co-transfected with empty pCMX-Flag was set to 100 %. Fold activation in pCMX-FLAG vs. pCMX FLAG-GCN5 treated cells were compared by students *t* test, * indicates $P<0.05$.

vector for either the Gal4DBD alone or hAd5 E1A CR3 fused to the Gal4DBD. Cells were then treated with either vehicle (DMSO) or a range of concentrations of CPTH2 and assayed for luciferase activity 48 hrs post-treatment. The fold activation by hAd5 E1A CR3 over Gal4DBD alone treated with DMSO was set to 100 %. Treatment of cells with CPTH2 resulted in a dose-dependent increase in E1A CR3-stimulated transcription, which peaked at a dose of 50 μM CPTH2 and increased E1A dependent activation by almost 60 % (Figure 3-5A). Pharmacological inhibition of the KAT activity mimicked RNAi depletion of GCN5, indicating that the KAT activity of GCN5 is required to exert the repressive effect on E1A CR3 transactivation. HT1080 cells co-transfected with an E4-responsive luciferase reporter and an expression vector for *wt* hAd5 E1A (289R) were similarly treated with CPTH2, and this also resulted in an increase in transactivation compared to vehicle control (Figure 3-5B). Moreover, mutants lacking either GCN5 binding site on E1A showed no significant change in transactivation in the presence of CPTH2, further demonstrating the necessity of both interaction surfaces for proper recruitment and utilization of GCN5 by E1A (Figure 3-5B).

3.3.6 E4ORF6/7 gene expression is enhanced in cells lacking GCN5 KAT activity.

To test the repressive role of GCN5's KAT activity in the context of viral infection we used *hat/hat* MEFs in which both copies of GCN5 were replaced with GCN5 that has a point mutant in the catalytic residue of the KAT domain (12). We measured expression levels of hAd5 E4ORF6/7 mRNA during infection by qRT-PCR. The *hat/hat* MEFs or *wt* littermate control MEFs were infected at an MOI of 2 with the following hAds: ΔE1A, *wt* E1A, E1A Δ26-35 and E1A Δ178-184. At 16 hours post-infection there was a significant increase in E4ORF6/7 levels in *wt* hAd5-infected *hat/hat* MEFs relative to the *wt* littermate control MEFs (Figure 3-6A). The adenoviruses harboring E1As with mutations in either GCN5 binding site showed no significant difference in E4ORF6/7 levels between *hat/hat* and control MEFs, indicating that both GCN5 binding sites are important for function. This is consistent with results obtained using the E4 luciferase reporter and the small molecule inhibitor CPTH2 (Figure 3-5B Figure 3-6A). These data

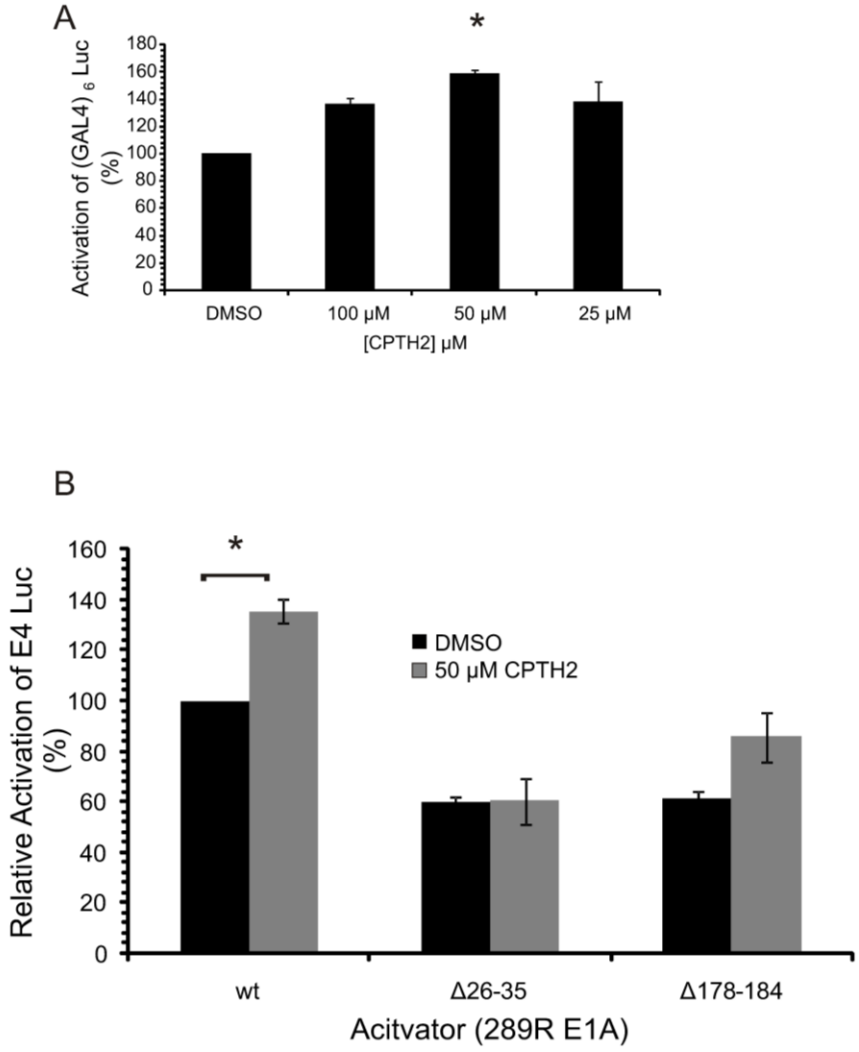

Figure 3-5: Inhibition of GCN5 HAT-Activity Mimics Depletion of GCN5

A) Human HT1080 cells were co-transfected with a Gal4-responsive luciferase reporter and an expression vector for a Gal4-hAd5 CR3 and treated with either DMSO or increasing concentrations of the GCN5-specific KAT inhibitor CPTH2. The fold activation by Gal4-E1A-CR3 over Gal4 alone, when treated with DMSO (vehicle) was set to 100 %. B) Human HT1080 cells were co-transfected with an adenoviral E4 responsive luciferase reporter and with the indicated expression vector for E1A, and treated with either DMSO or 50 µM CPTH2. The fold activation of the E4 reporter in cells transfected with wt 13S E1A over empty vector, when treated with DMSO (vehicle) was set to 100 %. Fold activation in DMSO-vs. CPTH2-treated cells were compared by students *t* test, * indicates $P<0.05$.

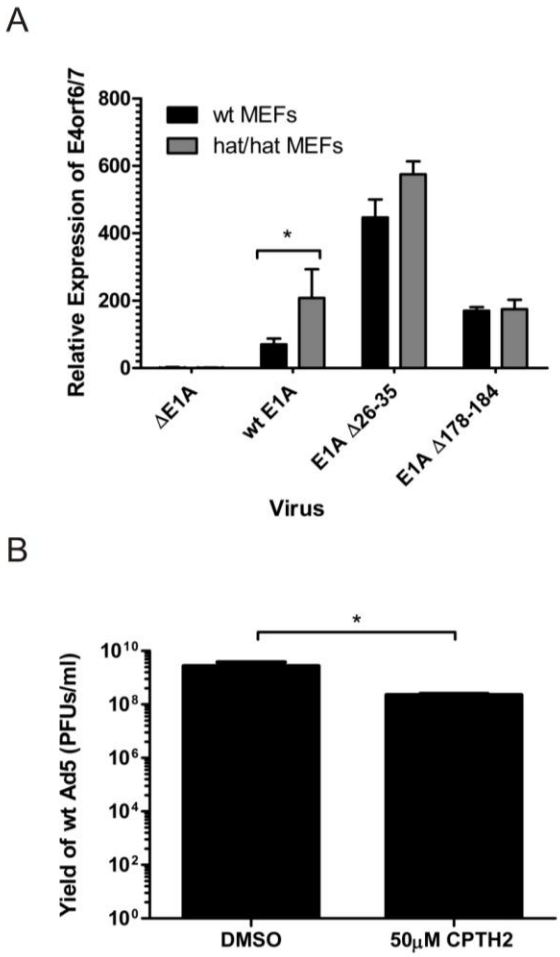

Figure 3-6: The KAT Activity of GCN5 Modulates E1A function During Infection.

A) Either *hat/hat* or *wt* littermate control MEFs were infected with the indicated viruses at an MOI of 2. At 16 hours post-infection total RNA was collected and the level of E4ORF6/7 mRNA relative to E1A mRNA and GAPDH mRNA was determined by qRT-PCR. Mean relative E4ORF6/7 expression between *hat/hat* and *wt* littermate control MEFs were compared by students *t* Test, * indicates $P< 0.01$. B) Human A549 cells were infected with *wt* hAd5 at an MOI of 5 and subsequently treated with either DMSO or 50 μM CPTH2. At 96 hours post-infection cells were harvested and virus yield was determined by plaque assay on HEK293 cells. Mean virus yield in PFUs/ml were compared by students *t* test, * indicates $P<0.05$.

further support a model in which E1A uses the KAT activity of GCN5 to repress transactivation of the viral E4 promoter in the context of infection.

3.3.7 Inhibition of GCN5 KAT activity reduces virus growth.

The results described above suggested that E1A has evolved to utilize GCN5 as a repressor to fine-tune transactivation of the early viral promoters in order to generate and maintain an optimal environment for virus replication. We determined the consequence of blocking GCN5 KAT activity on virus replication in permissive cells. Human A549 cells, the diagnostic cell line for hAd isolation and analysis, were infected at an MOI of 5 with *wt* hAd5 and treated with either vehicle (DMSO) or 50 µM CPTH2. At 96 hours post-infection, cells were harvested, freeze-thawed three times and virus titer was determined on HEK293 cells. Chemical inhibition of the KAT activity of GCN5 resulted in a near 1 log unit reduction in virus growth at 50 µM CPTH2 (Figure 3-6B). These data demonstrate that the KAT activity of GCN5 is required for optimal virus growth, and support a model in which E1A transactivation must be tightly regulated by both co-activators and repressors in order to maximize virus growth.

3.4 Discussion

The CR3 portion of hAd5 E1A is a potent transcriptional activation module and serves as a paradigm of viral transactivation (6, 18, 36). CR3 is the most highly conserved of the four conserved regions within E1A, yet there are dramatic differences in the potency of the CR3 domains from six representative E1As with respect to transactivation (1). The differences in transactivation ability of these representative E1A CR3s was shown to be independent of the conserved co-activators of E1A CR3: MED23, TBP, SUG1 and p300/CBP (1). Moreover, competition experiments suggested that additional cellular factors were involved in E1A CR3 transactivation that may include negative regulators (1). We report here the identification of a second interaction surface in E1A CR3 for the cellular KAT GCN5, and our experiments identify GCN5 as a novel negative regulator of E1A transactivation.

The interaction of GCN5 with the N-terminus of E1A had been reported previously, but all of the experiments used mutant hAds that could only synthesize the 12S (243R)

isoform of E1A and thus did not contain CR3 (26). We repeated the interaction experiment using a virus with the same mutation, i.e, deletion of residues 26-35 of E1A we showed that in the context of the 13S (289R) E1A protein, which contains CR3, a virus with the Δ26-35 mutation in E1A retained interaction with GCN5 (Figure 3-1A). Importantly, it has been established that the E1A of viruses with this Δ26-35 mutation retain interaction with the other cellular proteins shown to interact with the N-terminus of E1A namely, pRb, pCAF and p300 (16, 26, 37, 40). The interaction profile of MED23 with these E1A mutants was also examined as a control for binding of CR3-specific interactors (Figure 3-1A). MED23 is only co-immunoprecipitated with the largest isoform of E1A (289R in hAd5), i.e., when CR3 is present, consistent with previous reports that indicate MED23 only interacts with the largest E1A isoforms (1, 9, 47). Furthermore, the interaction of GCN5 with CR3 was conserved across all six hAd species, suggesting that the functional role of GCN5 is also conserved (Figure 3-1B). The GCN5 interaction site within CR3 was mapped to residues 178-184, which excludes the residues required for interaction with SUG1 (Figure 3-1C) (39). The promoter targeting domain of E1A CR3 was originally shown to be residues 183-188, which does overlap this newly identified GCN5 binding site (20, 28, 50). However, we have shown here that E1A Δ178-184 is still recruited to the E4 promoter (Figure 3-2B).

There is a dichotomy of conservation at the primary amino acid level among hAds in the region required for GCN5 interaction. Residues 174 to 181 of E1A are very poorly conserved among the six representative hAds in our panel (1, 4). However, between residues 182 and 184 there is a much greater degree of conservation. Of the six representative E1As, CR3s from species A through D share a conserved phenylalanine residue at position 182, while species E and F, including hAd4 which binds GCN5 poorly, have a cysteine residue that may contribute to the poor interaction of GCN5 with hAd4 CR3. Residues 183 and 184 are very highly conserved among E1As. Residue 183 is either a valine or isoleucine and residue 184 is an invariant tyrosine residue. These latter conserved residues may make key contacts between GCN5 and E1A CR3.

The primary function of the largest E1A proteins is to activate early viral gene expression by recruiting the cellular transcription machinery to the early viral promoters (6, 18, 36).

Thus, GCN5 would be expected to be present at the viral promoter with E1A, and indeed GCN5 is associated with the viral E4 promoter in an E1A-dependent manner (Figure 3-2B). Interestingly, the recruitment of GCN5 to the E4 promoter requires both GCN5 binding sites in E1A, demonstrating for the first time a physical cooperation between the N-terminus of E1A and CR3 to recruit a cellular protein to the promoter (Figure 3-2B). There is a large body of evidence to suggest that both the N-terminus of E1A and CR3 are transactivation domains. Yet there is very little evidence demonstrating that the N-terminus of E1A and CR3 can cooperate to interact with and recruit cellular proteins (52). Several cellular proteins that bind to CR3 also interact with the N-terminal CR1 domain of E1A including TBP, pCAF, Sug1, and p300/CBP (27, 37-39). There is also evidence that the N-terminus of E1A and CR3 could cooperate functionally. For example, both regions can activate transcription when fused to a heterologous DBD (6, 18, 36), and CR3 can synergize with the N-terminus to activate the E2 promoter, presumably via recruitment of TBP and sequestration of pRb (52). In essence, the interaction of GCN5 with two distinct regions of E1A provides a satisfying mechanism to explain the historical observation that both these regions of E1A are required for efficient activation of early gene expression (46, 52).

RNAi depletion of GCN5 resulted in an increase in E1A CR3 transactivation and provided the first indication that GCN5 is a true negative regulator of E1A transactivation (Figure 3-3B). This role of GCN5 as a negative regulator was conserved among our panel of representative CR3s of each hAd species with the exception of hAd4 E1A CR3 (species D), which also had the weakest physical interaction with GCN5 (Figure 3-3B). Perhaps hAd4 E1A CR3 does not require the same level of negative regulation that the other CR3s display as a consequence of its weaker intrinsic activation function. Depletion of GCN5 resulted in an increase in transcriptional activation (Figure 3-3B), whereas depletion of the other cellular factors required by CR3 (MED23, p300 SUG1 or pCAF) results in a decrease in transactivation (1). This gain of function phenotype suggests that GCN5 is part of another layer of transcriptional control that is recruited to promoters by E1A to optimize early gene expression. This is a rare example where removing a cellular binding partner of CR3 results in enhanced E1A transactivation. The same phenotype has been demonstrated before by depleting CtBP by siRNA or

sequestering CtBP with the C-terminus of E1A (11). In the context of infection, such a paradigm makes complete sense since many of the gene products of the E4 region are toxic and stimulate cellular defenses and/or antagonize survival pathways (45, 51). Therefore it appears that E1A utilizes GCN5 to help balance transactivation of the E4 promoter. Similarly, there may need to be regulated expression of the E1B, E2 and E3 promoters as well.

If GCN5 negatively regulates E1A CR3-dependent transactivation, then ectopic expression of GCN5 should exacerbate the negative effect on CR3 transactivation. This was indeed the case, since overexpression of GCN5 resulted in a decrease in transactivation by CR3 fused to a heterologous DBD, and also by full length E1A acting on an E4 responsive reporter (Figure 3-4). The effect of GCN5 again highlights the cooperative nature of the GCN5 interaction, because mutation of either binding site rendered E1A non-responsive to the repressive effects of GCN5 (Figure 3-4). GCN5 is apparently a limiting factor in this process, since the endogenous levels within cells do not allow for maximal repression (Figure 3-4). Both of these observations suggest that GCN5 is required in stoichiometric, rather than catalytic, amounts to exert its negative effects.

GCN5 is ascribed to have two catalytic functions in the SAGA complexes of both yeast and mammalian cells, a KAT activity and a deubiquitinating activity (5, 10, 35, 42). Our results indicate that the KAT activity of GCN5 is required to negatively affect E1A transactivation on both plasmid and viral genomic templates (Figure 3-5 Figure 3-6A). This is the opposite effect reported for GCN5 on chromatin templates, where acetylation is usually correlated with transcriptional activation (5, 10, 19, 35, 42). The hAd genome has been shown to associate with cellular histones including H3, which is acetylated at K9 and K14, presumably by GCN5 (25), yet no direct role for E1A in this process has been demonstrated. Thus it is possible that E1A is co-opting GCN5 for an alternative purpose. However, there is a single report that links the KAT activity of GCN5 and E1A transactivation that requires the Mediator complex. A sub-Mediator complex, referred to as T/G Mediator, exists that includes MED23, the kinase CDK8 and GCN5 (32). This complex is responsible for histone H3 S10/K14 tandem phosphoacetylation; the KAT

activity of GCN5 and the kinase activity of CDK8 are catalytically responsible for the tandem phosphoacetylation event *in vivo*, which leads to transcriptional activation (32). GCN5 interacts with E1A via its N-terminus, MED23 binds through CR3 and we now show that GCN5 independently binds CR3 (Figure 3-1) (26). Therefore, it is entirely possible that E1A could also recruit the kinase activity of CDK8 to CR3 via MED23 or GCN5. Interestingly, within CR3 two serine residues (S185 and S188) are critical to E1A transactivation and they are hyperphosphorylated *in vivo* (51). S185, but not S188 was reported to be phosphorylated by MAPK (51). S188 is found in a CDK8 consensus phosphorylation site (41). Indeed the sequence found in E1A, YSPVS, is very similar to the consensus sequence of the RNA PolII C-terminal domain (YSPTSPS) which is also phosphorylated by CDK8 in the context of Mediator (41). Therefore the missing link required for phosphorylation of S188, a modification known to affect E4 transactivation, may be the T/G Mediator complex (32, 51).

Importantly, pharmacological inhibition of GCN5 reduced virus yield, suggesting that a balanced level of E1A transactivation is required for optimal virus growth (Figure 3-6B). This same inhibition of GCN5 KAT activity coincides with an increase in E4 promoter activity (Figure 3-6A). Increased transcription of the E4 promoter is known to enhance premature killing of infected cells (51). The adenoviral E4 promoter appears to have redundant levels of regulation. Not only does GCN5 appear to limit E4 promoter activity, but the E4 transcription unit also encodes the E4ORF4 protein, which interacts with cellular PP2A to dephosphorylate S188 of E1A, completing a negative feedback loop for E1A transactivation (51). Therefore the negative effect of GCN5 on E1A-dependent transactivation may provide the optimal intracellular milieu for virus replication by initially maximizing expression of the E4 transcription program needed for the viral replication cycle, then minimizing the transcription of potentially hazardous viral proteins via the E4ORF4 negative feedback loop.

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Chapter 4

4 The Structure of E1A CR3

Despite exhaustive efforts using mutants, a detailed structure-function relationship of E1A CR3 transactivation remains elusive. To date no high resolution structural information is available for E1A CR3. In this chapter I will review the evidence suggesting that CR3 likely has a well defined structure, and I demonstrate experimentally that E1A CR3 is indeed a highly ordered and structured domain.

4.1 Background and Literature Review

The very earliest experiments that implicated CR3 in transactivation also hinted at the structured nature of the domain. Host range (*hr*) in hAd5 was originally defined as the ratio between virus growth on 293 cells, which express E1A and E1B, versus HeLa cells which do not express E1A and E1B (21). All group I *hr* mutants are unable to synthesize early viral mRNAs in HeLa cells, but can be rescued on 293 cells, and the mutations map to residues in E1A CR3 (7, 15). Eventually, the group I *hr* mutants were shown to be single aa substitutions in key residues of E1A CR3 (19). Thus, unlike the remainder of E1A, substitution of a single aa in CR3 is enough to abolish early gene activation and ultimately virus growth, suggesting that the mechanism of CR3-dependent transactivation is distinct from other E1A functions. One interpretation of this critical difference between CR3 function and other functions of E1A is that a complex tertiary structure is required for CR3 function. If CR3 maintained a well defined structure, then mutations in key residues making intra-molecular contacts would result in a phenotype similar to the *hr* mutants.

The classical approach to deciphering E1A function was based on numerous E1A deletion mutants, and these mutants were instrumental in understanding most functions of E1A including oncogenic transformation. The majority of E1A tolerates deletions well, such that small deletions affect interaction with only a subset of binding partners without negating E1A function globally (14, 30). This led to a model of the 12S E1A product being a string of protein-protein interaction motifs. In contrast, CR3, found in the 13S

E1A product, is unique in that all deletion mutants within this region fail to activate transcription, again indicating that CR3 is quite different from the rest of E1A (22). Only when an alternative approach to mutational analysis involving single conservative substitutions was employed was insight into the structure-function relationship of CR3 revealed.

Analysis of the primary amino acid sequence of E1A CR3 revealed two CXXC motifs which are indicative of a C4 zinc finger domain. These four cysteines residues (C154, C157, C171 and C174) were later shown to coordinate a single zinc ion and are absolutely required for E1A CR3 transactivation (10, 39). Interestingly, none of the group I *hr* mutants were point mutations in the coordinating cysteine residues (19). Comprehensive mutational analysis of CR3, where every residue from 140 to 188 was individually substituted to a conservative amino acid, only solidified the view the E1A CR3 is structured (18, 39). Even very subtle conservative changes in select CR3 residues resulted in dramatic loss in transactivation function (18, 39).

The most striking evidence suggesting that CR3 is structured comes from an in-depth analysis of E1A primary sequence. As a whole, the primary amino acid sequence of the 243 E1A is densely packed with short linear interaction motifs or SLIMs (30). In some cases the interaction motifs actually overlap, for example the DLX in the pRb DLXCXE SLIM, corresponds to the DLT sequence in the SUMO EVIDLT SLIM (30, 41). Analysis of the 289R E1A of hAd5, which includes CR3, demonstrates that with the exception of the putative nuclear localization signal (NLS) reported in CR3, this region of E1A is completely devoid of SLIMS; yet CR3 interacts with an equally impressive collection of cellular proteins (30, 35, 36). The non-conventional and developmentally regulated NLS ($FV(X)_{7–20}MXSLXYM(X)_4MF$) reported in CR3 spans residues 140-185. This putative NLS encompasses the entire zinc finger subdomain of E1A CR3. Furthermore, the region spanning residues 140-185 is required for interaction with at least three cellular proteins found in the nucleus (TBP, MED23 and pCAF), which could also mediate the nuclear localization of CR3 (9, 12, 24, 31). I hypothesize this lack of SLIMs and the complexity and features of the NLS in CR3 are indicative of a complex CR3 secondary and/or tertiary structure that would generate non-linear interaction motifs.

Taken together, the literature and sequence of E1A demonstrate that CR3 is not only very different from the rest of E1A in terms of overall function, but also with respect to sequence conservation, distribution of SLIMs and sensitivity to deletion mutations. These observations of E1A CR3's distinct characteristics suggest that unlike the remainder of E1A, this region exhibits a complex secondary and/or tertiary structure to organize cellular transcription factors spatially and temporally to ultimately achieve its unique function of activating early viral genes.

4.2 Materials and Methods

4.2.1 Prokaryotic and Mammalian Cells

Human A549 lung adenocarcinoma cells were a maintained in DMEM containing 10 % FBS 100 U/ml penicillin/streptomycin and grown at 37 °C and 5 % CO_2. Prokaryotic *E. coli* strain DH5-alpha was used to maintain all plasmids while, *E. coli* strain BL-21 RIL (Stratagene) or BL-21 plysys (Novagen) were used for protein production and maintained on 68 µg/ml of chloramphenicol (CAM) (Bioshop).

4.2.2 Plasmid Construction

The prokaryotic expression vector pGEX4T1-TEV was constructed by annealing 10 ng each of two oligos (5`-AATTAGAGAATTTGTATTTTCAGTCTGGAG-3` and 5`-AATTCTCCAGACTGAAAATACAAATTCTCT-3`) at 100 °C in ddH_2O for 10 minutes followed by slowly cooling to room temperature. The parent vector pGEX4T1 (Clontech) was cut with EcoRI (New England Biolabs, NEB), the annealed oligo was ligated into the EcoRI site using T4 DNA ligase (NEB), transformed into competent *E. coli* strain DH5α and selected on 200 µg/ml ampicillin (AMP) (BioShop). Clones were screened by sequencing, and a clone that had only one copy of the oligo inserted was chosen for further manipulation. To generate pGEX4T1-TEV-Ad5 CR3, the DNA sequence encoding hAd5 E1A CR3 residues 139-204 was cut from pM-Ad5 CR3 (34) with EcoRI and NotI and ligated to the same sites of pGEX4T1-TEV. Correct clones were verified by sequencing. The truncations of hAd5 E1A CR3 at residues 190 (pGEX4T1-TEV-Ad5 CR3 190T) and 178 (pGEX4T1-TEV-Ad5 CR3 178T) were generated by PCR using a common forward primer (5`-

AGACGAATTCGGTGAGGAGTTTGTGTTA-3`) and reverse primers CR3190T-R (5`-CTGTCGACTTAAGGTTCAGACACAGGACT-3`) and CR3178T-R (5`-GATGGATCCTTAGGTCCTCATATAGCAAA-3`), respectively with Phusion polymerase (NEB) according to the manufacturer's directions. PCR products were cut with the same enzymes described above and cloned into the same sites used for pGEX4T1-TEV-Ad5 CR3.

4.2.3 Production and Purification of CR3 for NMR Analysis

The expression vectors described above were transformed into *E. coli* strain BL-21 RIL (Stratagene) or BL-21 plysys (Stratagene) and selected on 200 µg/ml AMP and 68 µg/ml of CAM. Individual colonies were selected and grown in LB broth containing AMP and CAM overnight. Overnight cultures were pelleted, washed once in PBS, and then diluted to a starting OD_{600} of 0.2 in M9 media (42 mM Na_2HPO_4, 24 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.3 % glucose, 10 µg/ml thiamine, 10 µg/ml biotin, 50 µg/ml carbenicillin and 34 µg/ml (CAM) and supplemented with 20 µM $ZnCl_2$. Cultures were grown at 37 °C at 250 rpm until a OD_{600} of ~0.9 was reached (mid-log phase), at which point cultures were induced with 1 mM IPTG (Bioshop) and transferred to 16 °C for 18 hours. Cells were harvested by centrifugation (5000 x g for 10 minutes at 4 °C) and lysed in 10 ml of Lysis Buffer (1x PBS pH 7.4, 0.2 % Tween-20, 2 mM DTT and 1.0 mg/ml lysozyme) per gram wet weight of the pellet. The pH of resuspended pellets was raised to 8.15 with 1 M Tris pH 11.0 and incubated at 37 °C for 10 minutes. Lysates were then sonicated for three cycles of 20 seconds at max power with a microtip sonicator (VWR). Lysates were clarified by centrifugation at 30 000xg for 30 minutes at 4 °C. The soluble fraction was collected and the pH adjusted to 7.2 with concentrated HCl. Glutathione S-transferase (GST)-fused proteins were recovered by the addition of 6 ml of 50 % slurry of glutathione sepharose (GE) and incubated at room temperature for two hours with nutating. Glutathione sepharose was washed with 100 column volumes of lysis buffer without lysozyme (~300 ml). CR3 was liberated from GST while bound to glutathione sepharose by the addition of a 4000:1 ratio of GST-protein: TEV protease in lysis buffer. TEV cleavage was performed at 4 °C with nutating overnight (minimum 16 hours). The soluble fraction now containing liberated CR3 was

collected and diluted 10 fold in start buffer (20 mM Tris pH 7.8 + 2 mM DTT) and pumped onto a HiTrapQ FF anion exchange column with a syringe. The column was then connected to an Akta FPLC purifier and CR3 was eluted with a linear salt gradient from 0-100 % of elution buffer (20 mM Tris pH 7.8, 2 mM DTT, 1 M NaCl). CR3 eluted at approximately 40 % NaCl, and fractions were collected and dialyzed into FPLC buffer (20 mM Tris pH 7.8, 2 mM DTT, 150 mM NaCl) and concentrated to a 400 µl volume. The concentrated CR3 was loaded onto a Superdex75 column and separated by gel filtration chromatography (GFC). The peak corresponding to CR3 was collected and dialysed into 50 mM HEPES buffer pH 6.5 plus 2 mM DTT for NMR analysis.

4.2.4 Mass Spectrometry Quality Control of Purified CR3

5 % of the recombinant CR3 purified for NMR analysis (ranging from 500 to 1000 pmoles depending on the protein sample) was sent to the UWO Biological Mass Spectrometry Facility for mass determination.

For mass determination of CR3 residues 139-204 under denaturing conditions, the sample was treated with 50 % acetonitrile. Mass spectrometry was performed on a QTof Micro mass spectrometer (Micromass) equipped with a Z-spray source and run in positive ion mode. Cone voltage was 50 V and a source temperature of 80 °C was used. Calibration was performed with myoglobin with mass error of less than 0.5 Da. Data was acquired using MassLynx 4.1 (Micromass) acquisition software. For mass determination of CR3 190T (residues 139-190 of hAd5 E1A) under non-denaturing conditions, the samples were diluted 10 fold in 10mM ammonium acetate. Mass determination under denaturing conditions was performed in 50 % acetonitrile as above but with variable cone voltage.

4.2.5 Med23 Competition Assay for Quality Control of Purified CR3

Human A549 cell lysate was prepared by lysis in 5 packed cell volumes of E1A buffer (29) containing protease inhibitors (Sigma) on ice for 10 minutes followed by clarification at 20 000 x g for 10 minutes at 4 °C. One mg of A549 total cell lysate was mixed with 1 µg of purified GST or GST-hAd5 CR3 and 20 µl of 50 % glutathione sepharose and incubated at 4 °C for 60 minutes with nutating. Glutathione sepharose was

collected by centrifugation, washed three times with E1A buffer containing protease inhibitors and finally resuspended in 1 ml of E1A buffer with protease inhibitors followed by the addition of increasing amounts of purified CR3. GST alone was treated with the maximum amount of CR3 and incubated for an additional 60 minutes at 4 °C with nutating. Glutathione sepharose was again collected by centrifugation and washed once with E1A buffer containing protease inhibitors. Samples were resuspended in 1x protein loading dye and 1 mM DTT and boiled at 100 °C for 10 minutes. Samples were separated by SDS PAGE, transferred to PVDF membrane and immunobloted as described above in section 2.2.4 with a primary antibody to human MED23 (NOVUS) at a dilution of 1:2000. Input amounts of GST, GST-hAd5 CR3 and purified CR3 were detected by Ponceau stain (Sigma).

4.2.6 NMR Data Collection

Purified CR3 139-204 at a final concentration of 100 µM in 50 mM HEPES pH 6.5 was used to collect 2-Dimensional heteronuclear single quantum correlation (2D HSQC) spectra. Experiments were performed on a Varian Inova 600 MHz spectrometer equipped with a xyz-gradient triple resonance probe (UWO Biomolecular NMR Facility). The spectrum was acquired for 8 hours at 25 °C. All chemical shifts were referenced to the internal DSS (2,2-dimethyl-2-silapentane-5-sulfonate) signal. Data were processed and analyzed using the programs NMRPipe (11) and NMRView (23). Purified CR3 139-190 at a final concentration of 50 µM was used to collect a 2D HSQC spectrum as described above. The 2D HSQC spectrum was acquired for 18 hours at 25 °C. For EDTA chelation of zinc, purified CR3 190T at a final concentration of 100 µM was used to collect a 2D HSQC of CR3. The same sample was subsequently treated with 500 µM EDTA to chelate zinc ions from CR3 and the spectrum was collected under the same conditions. Both spectra were collected for 21 hours at 25 °C

4.3 Results

4.3.1 *In silico* Analysis of E1A Order versus Disorder

I hypothesized that E1A-CR3 was structured or highly ordered, and that this property was unique to CR3 relative to the remainder of E1A. Thus I employed a bioinformatic tool to

analyze the propensity of order versus disorder in E1A. The software tool, Predictor of Naturally Disordered Regions (PONDR), breaks a given amino acid sequence into 9-21 amino acid windows and then takes into account parameters such as net charge and hydropathy index and subsequently assigns each window of the amino acid sequence a score between 0 and 1. A score of 1 indicates a disordered region while a score of 0 indicates order. The threshold value is set to 0.5, so effectively a score above 0.5 is disordered and a score below 0.5 is ordered. The further the score differs from 0.5, the more likely the prediction is correct (1). I put the primary amino acid sequence of hAd5 289R E1A into PONDR and the output is shown in figure 4-1. PONDR clearly predicts an ordered region of E1A that peaks between residues 150 and 175. This region corresponds to the C4 Zinc finger subdomain of CR3, which is bound by coordinating cysteines at residues 154 and 174 (Figure 4-1). PONDR also predicts a disordered region adjacent to CR3 that corresponds to the promoter targeting subdomain of E1A CR3 thought to make promiscuous interactions with cellular DNA-binding transcription factors (Figure 4-1). Therefore PONDR supports the suggestion that there are two functional subdomains of E1A CR3 and that these regions, which appear to have very different propensities to form stable structures, work together to locate a functional transcription initation complex to large variety of promoters.

Apart from the zinc finger domain of CR3, the only other region of E1A predicted to be ordered is the extreme N-terminus and this property is also predicted to be shared by E1A proteins from all six hAd speciess (4, 16, 30). This feature at the extreme N-terminus of E1A is thought to be critical to interacting with and organizing the numerous cellular proteins that interact with this region of E1A (30). In the case of hAd5 E1A this putative alpha helix is predicted to have amphipathic characteristics based on its unique "corner box" motif and the involvement of three leucine residues that when mutated affect binding to multiple cellular partners (8, 30, 32). Curiously, both the N-terminus and CR3 were predicted by PONDR to be ordered, and both share a common theme where single point mutations have been shown to disrupt interaction with multiple cellular binding partners and have dramatic effects on their respective functions.

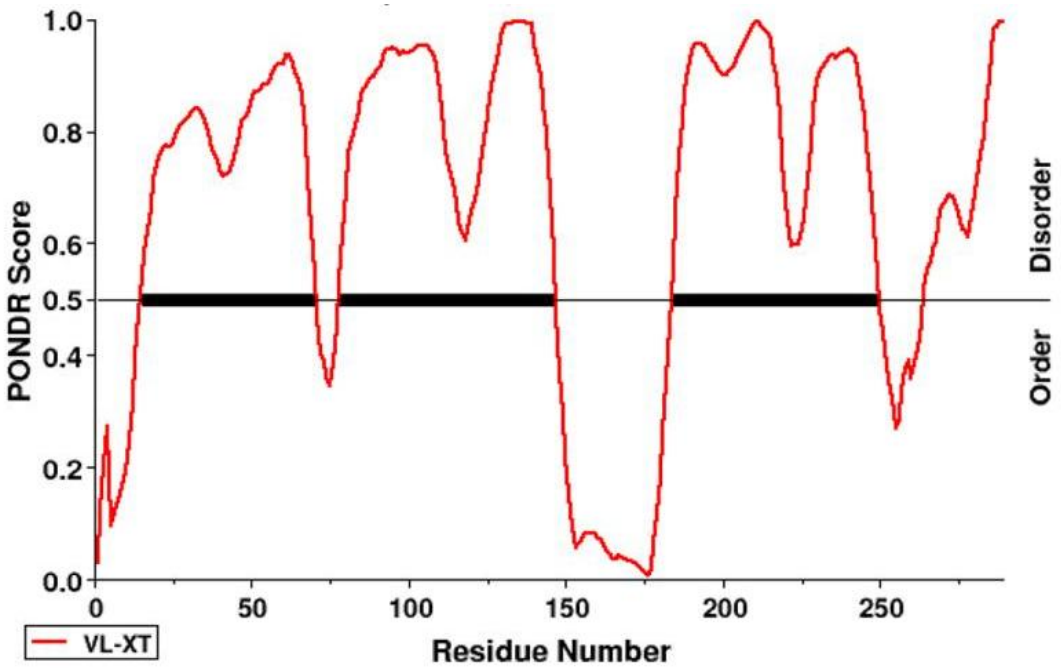

Figure 4-1 *In silico* Prediction of Ordered and Disordered Regions of hAd5 E1A

The amino acid sequence of 289R E1A was submitted to PONDR.com for *in silico* prediction of ordered versus disordered regions within E1A and the output is shown. The X-axis depicts the residues of E1A and the Y-axis plots a measure of ordered/disordred state, VL-XT. The threshold value is set to 0.5; above this value a region is predicted to be disordred, and a value below 0.5 predicts an ordered or structured region. There is a clear region of predicted order between residues 150 to 175 that corresponds to the zinc finger binding subdomain of E1A CR3.

PONDR, predicted the disordered regions of E1A consistent with other findings and predictions. It also supports my hypothesis by predicting that the zinc finger subdomain of E1A CR3 is structured. Based on this *in silico* confirmation, I proceeded to determine the three dimensional structure of the hAd5 E1A transactivation domain within CR3. Due to the relatively small size of the CR3 domain, whose maximum size would be 65 amino acids, and given the expertise and equipment available in the department of Biochemistry at the University of Western Ontario, I chose to pursue the structure of E1A CR3 by nuclear magnetic resonance (NMR) spectroscopy.

4.3.2 Construction of a Prokaryotic Expression Vector for E1A-CR3

Previous studies in Dr. Mymryk's laboratory has successfully expressed E1A and fragments of E1A as GST fusions (5, 31, 33, 34). In order to produce sufficient quantities of the CR3 domain of hAd5 E1A free of any affinity tags, I created a prokaryotic expression vector for GST fusions that integrated a tobacco etch virus (TEV) protease cleavage site between GST and CR3. I designed DNA oligonucleotides that when annealed to each other would base pair to the cleaved EcoRI site of pGEX4T1 (Figure 4-2A) and add the desired sequences. Upon integration of a single copy of the annealed oligonucleotides into EcoRI digested pGEX4T1, the upstream EcoRI site was destroyed and followed by codons for the optimized TEV cleavage site ENLYFQS (Figure 4-2B). These codons were followed by a single glycine codon and finally the EcoRI site was regenerated downstream such that CR3 could be cloned in with EcoRI, in frame with GST and the TEV cleavage site (Figure 4-2B). The newly generated prokaryotic expression vector was called pGEX4T1-TEV and was verified by sequencing. The entire CR3 domain of hAd5 E1A encompassing residues 139-204 was cloned into pGEX4T1-TEV with EcoRI and NotI sites and verified by sequencing. GST-fused E1A CR3 was then produced in BL-21 plysys *E. coli* and purified with glutathione sepharose. Treatment of the purified GST-E1A-CR3 with TEV protease resulted in the liberation of E1A-CR3 from GST, while a control sample without TEV protease showed no cleavage (Figure 4-2C).

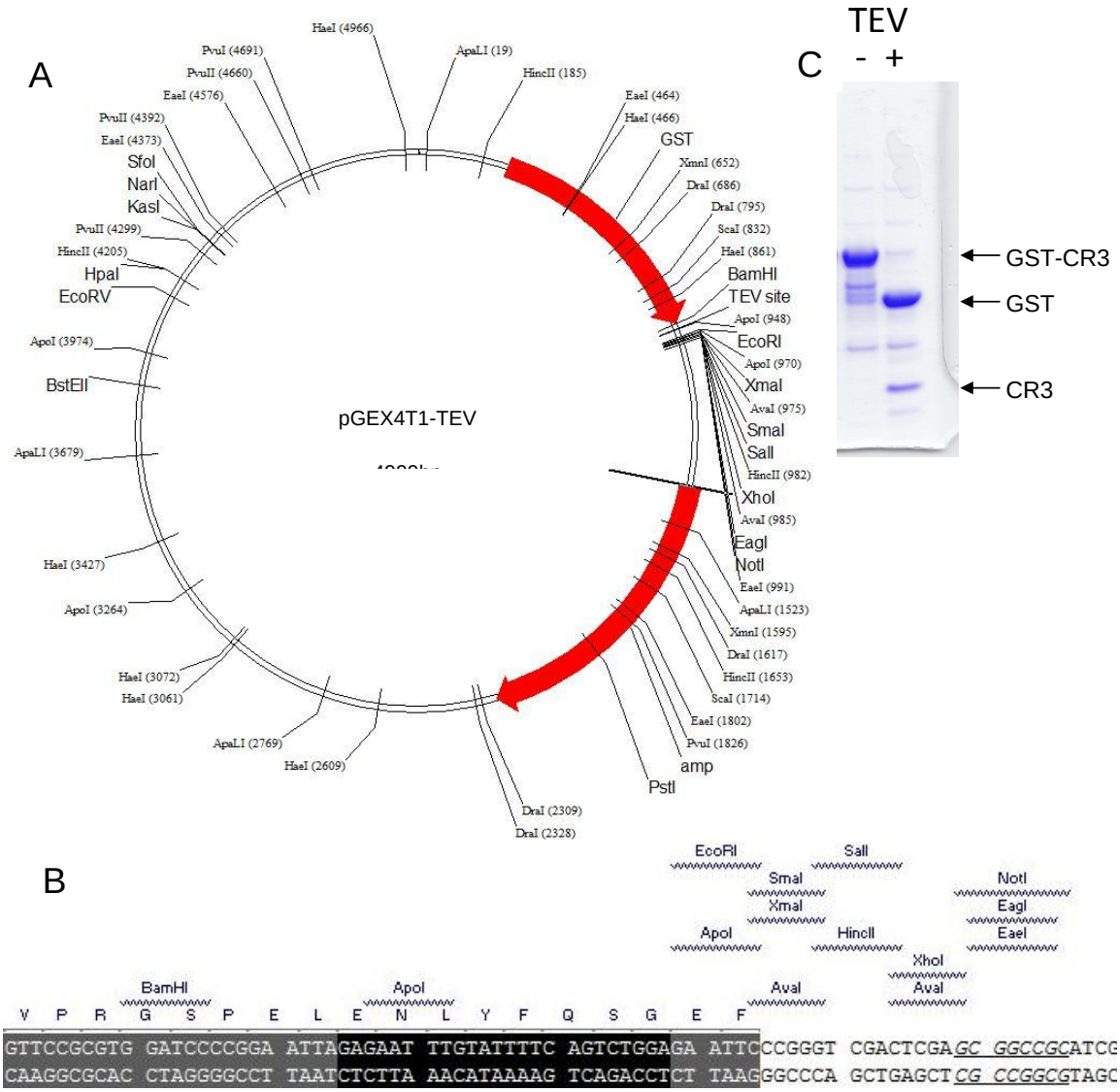

Figure 4-2 Features and Characteristics of pGEX4T1-TEV

A) Vector map of pGEX4T1-TEV. GST and AMP open reading frames are indicated as red arrows. The TEV cleavage site is located between the BamHI and EcoRI restriction sites. B) Sequence of the MCS of pGEX4T1-TEV. The TEV recognition site (shaded black) is shown translated and is in frame with the GST open reading frame upstream (shaded grey) and the EcoRI site downstream (shaded grey). The NotI site is denoted in italics and is underlined. C) The expression vector pGEX4T1-TEV-Ad5 CR3 was transformed into BL-21 *E. coli* and expression induced by the addition of IPTG. The GST fusion was purified with glutathione sepharose affinity resin. The washed resin was incubated with or without TEV protease. Samples were separated by SDS PAGE and stained with coomassie blue. Bands corresponding to GST-CR3, GST alone, or CR3 alone are indicated.

4.3.3 Production and Purification of E1A-CR3 for NMR Analysis

In order to resolve structural information from purified E1A CR3 by NMR the protein must be isotopically labeled to ensure ^{1}H, ^{15}N and ^{13}C atomic nuclei have angular momentum values equal to ½, i.e. an odd number of neutrons. Therefore a strain of BL-21 pLsys *E. coli* harboring pGEX4T1-TEV-CR3 was grown in M9 minimal media with ^{15}N-ammomium chloride as the sole nitrogen source and/or ^{13}C-glucose as the sole carbon source. Labeled CR3 was purified with the optimized purification strategy outlined in Figure 4-3. Briefly, cells were collected by centrifugation at 18 hrs post induction and lysed in lysis buffer containing 1 mg/ml lysozyme, then sonicated and GST-CR3 was purified by the addition of glutathione sepharose to clarified lysates. Glutathione sepharose was washed extensively and CR3 was liberated directly from the resin by addition of TEV protease. Liberated CR3 was then concentrated by Ion Exchange Chromatography (IEC) using HiTRAPPQ anion exchange resin. CR3 was eluted with a linear linear salt gradient, and it eluted at 40 % NaCl. The peak corresponding to CR3 was collected and dialyzed to remove excess salt and then separated by Gel-Filtration Chromatography (GFC) using either Sephadex 75 or Sephadex 200 resin, which separated the small CR3 peptide from larger contaminating proteins (Figure 4-3). The final product was verified by mass spectrometry.

4.3.4 Quality Control of Purified CR3 Residues 139-204

The quality of purified CR3 was verified by mass spectrometry and also assessed functionally using a binding assay with a known cellular binding partner. The theoretical calculated mass for the unlabeled 70 amino acid peptide corresponding to residues of 139-204 of hAd5 E1A is 7960.80 Da (2). The experimental mass of unlabeled CR3 139-204 obtained was 7959.32 Da. Therefore, the CR3 produced was of the appropriate mass and appeared stable because no apparent degradation products were seen (Figure 4-4).

To functionally assess the quality of the purified full length CR3, I developed a competition assay to determine if purified CR3 could compete with GST-CR3 for binding to the well established cellular binding partner MED23 (9, 37). Interaction with MED23

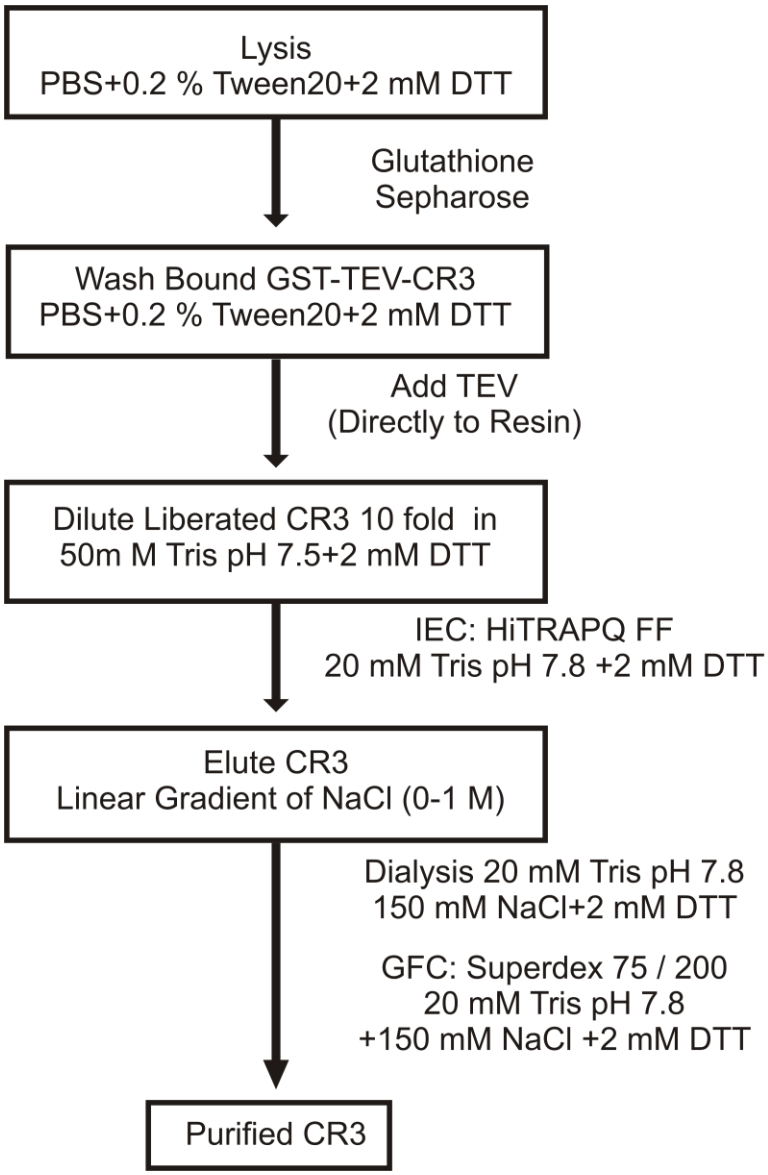

Figure 4-3 Purification Scheme of E1A-CR3

GST-CR3 was grown in *E. coli* Strain BL-21 plysys, bacterial cells were collected subsequently and lysed in lysis buffer. GST-CR3 was then collect with GST affinity resin (glutathione sepharose) and washed extensively. CR3 was then liberated from GST by the addition of TEV protease directly to the GST-affinity resin. Liberated CR3 was concentrated by ion exchange chromatography (IEC) and eluted with high salt. The eluted CR3 was then dialysed to remove excess salt and further purified by gel filtration chromatography (GFC).

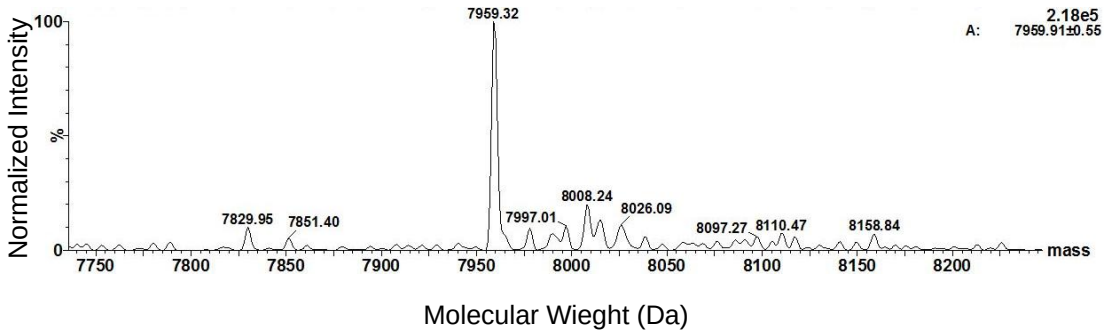

Figure 4-4 Mass Determination of Purified CR3 Residues 139-204

Purified CR3 peptide consisting of residues 139-204 of hAd5 E1A was analyzed by MALDI-TOF mass spectrometry by the University of Western Ontario Biological Mass Spectrometry Laboratory. The observed of CR3 139-204 was 7959.91 Da, consistent with the predicted mass of 7960.8 Da.

requires properly folded E1A CR3 (9). While GST alone failed to interact with MED23, GST-CR3 interacted with and pulled down MED23 from A549 cell lysate (Figure 4-5). Using a fixed amount of GST-CR3 and titrating in the purified CR3, I observed a dose dependent loss of interaction of GST-CR3 with MED23. Therefore, the purified CR3 could interact with MED23 and was likely folded properly, since the interaction of MED23 and E1A requires an intact zinc finger subdomain of CR3 (9, 37). There was an accumulation of GST that appeared with increasing amounts of purified CR3, which was the result of contaminating GST in the purified CR3 preparation that was recovered due to an excess of glutathione sepharose in the reaction.

4.3.5 Two Dimensional HSQC of CR3 Residues 139-204

Satisfied that the purified CR3 was folded properly based on the quality control experiments, I proceeded to acquire a 2D HSQC spectrum and determine experimentally if CR3 is structured. CR3 was overexpressed in *E. coli* using M9 minimal media containing ^{15}N-NH_4Cl and purified as described above. Labeling all of the nitrogen atoms in CR3 with heavy nitrogen amplifies the chemical shift of the amide proton to maximize the NMR signals of the peptide bond. The spectrum collected for CR3 residues 139-204 at 25 °C is shown in Figure 4-6. The plot depicts the chemical shift of amide protons on the X-axis correlated to the chemical shift of the directly bonded amide nitrogen on the Y-axis. The wide dispersion of NMR signals on the spectrum indicates residues that are in confined chemical environments, i.e., residues making intra-molecular contacts and potentially involved in protein folding. Four peaks were shifted to the left of centre in regions of the spectrum usually reserved for tryptophan residues, i.e., between H1 of 10.1 to 9.1 ppm (Figure 4-6), yet there are no tryptophan residues in CR3. Although not conclusive, these dramatic chemical shifts suggest that these peaks correspond to the four coordinating cysteine residues that occupy this region, because the coordination of a single zinc ion presumably locks them in a rigid conformation. Unstructured residues that are flexible in solution fall to the centre of the 2D HSQC spectrum. Clearly with full length CR3, there is substantial signal in this region that cannot be resolved, suggesting that a portion of CR3 is disordered and flexible in solution. Moreover, there are resonant peaks found in this region that are characteristic

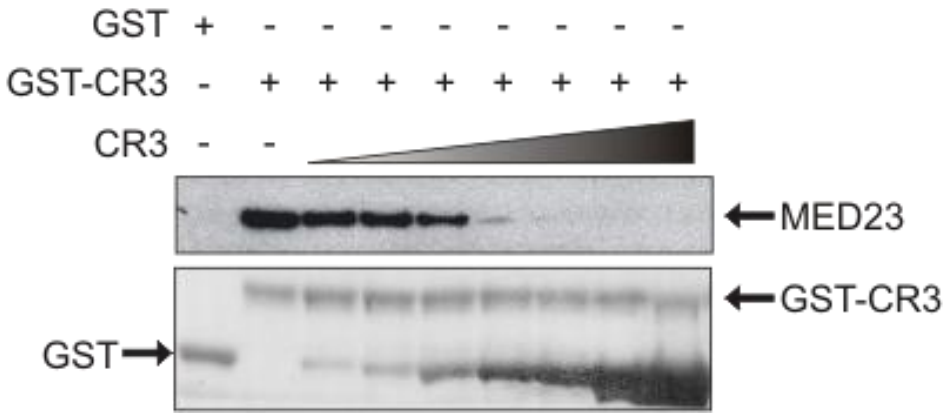

Figure 4-5 Purified CR3 Competes for MED23 Binding

GST or GST-CR3 was mixed with 1 mg of A549 cell lysate and either buffer or increasing amounts of purified CR3. Complexes were allowed to incubate at 4 °C for one hour with nutating and then recovered with glutathione sepharose. Samples were then boiled in sample buffer, separated by SDS PAGE, transferred to membrane and subsequently immunoblotted for hMED23. Immunoblot for hMED23 is shown above and Ponceau stain for GST and GST-CR3 is shown below.

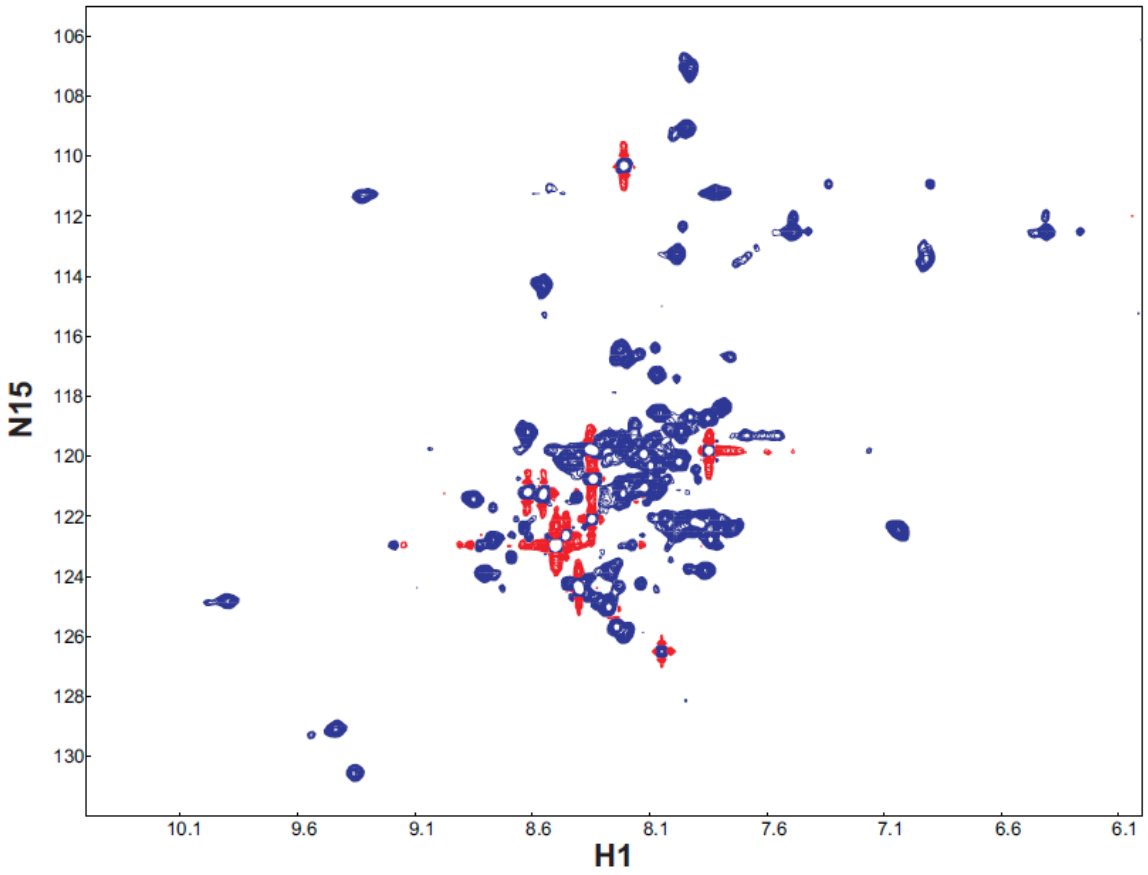

Figure 4-6 2D HSQC of CR3 Residues 139-204

A 2D HSQC of purified ^{15}N-labeled CR3 residues 139-204 was collected. The Y axis depicts the ^{15}N chemical shift and the X axis depicts the proton chemical shift. Each peak represents an amino acid of CR3. Red denotes resonant signals representing extremely flexible residues.

of glutamic acid residues (denoted in red Figure 4-6). The resonant signal is most likely coming from the glutamic acid-proline (EP) repeats of AR-1, because this region was predicted by PONDR to be disordered (Figure 4-1). Overall, initial NMR analysis of CR3 confirmed that at least a subdomain of CR3 has a well defined structure. The 2D HSQC also indicates the presence of a disordered and flexible subdomain that complicates the resolution of the spectrum. Therefore an alternative fragment of CR3 was used for further experiments, with the aim of optimizing the spectrum for subsequent analysis.

4.3.6 Construction and Analysis of CR3 Residues 139-190

The spectrum obtained using residues 139-204 of hAd5 E1A CR3 lacked sufficient resolution to yield reliable structural information. Therefore, I continued my experiments using a truncation of CR3 encompassing residues 139-190 of hAd5 E1A. This particular truncation of CR3 was chosen based on a combination of practical and experimental considerations. First and foremost, residue E189 was included based on previous data obtained from and E189A alanine substitution mutant of hAd5 E1A indicating that this residue is critical to CR3 transactivation as a Gal4DBD fusion (J. Ablack, unpublished data). Evidence suggests that E189 may be involved in the tertiary structure of CR3. Residue P190 was included because together with E189 they reconstitutes the first EP repeat in AR1. Although it is not represented on the 2D HSQC due to a lack of an amide proton, as the terminal residue it can provide chemical shift information on the penultimate residue that could be involved in the structure of CR3. The CR3 peptide fragment truncated at residue 190 was called CR3 190T. CR3190T was purified by the procedure outlined above and a 2D HSQC was collected. As predicted, the 2D HSQC of CR3 190T shows a dramatic increase in the resolution of individual peaks in the centre of spectrum (Figure 4-7). The characteristic NMR signature of E1A CR3 was maintained in CR3190T, including the four characteristic peaks at the extreme left of the spectrum that are believed to be the coordinating cysteines (Figure 4-7).

The literature indicates that the structure and function of CR3 is dependent on the coordination of a single zinc ion (10). In order to test this possibility directly, I compared the 2D HSQC spectrum of CR3 190T in the absence and presence of the zinc chelator

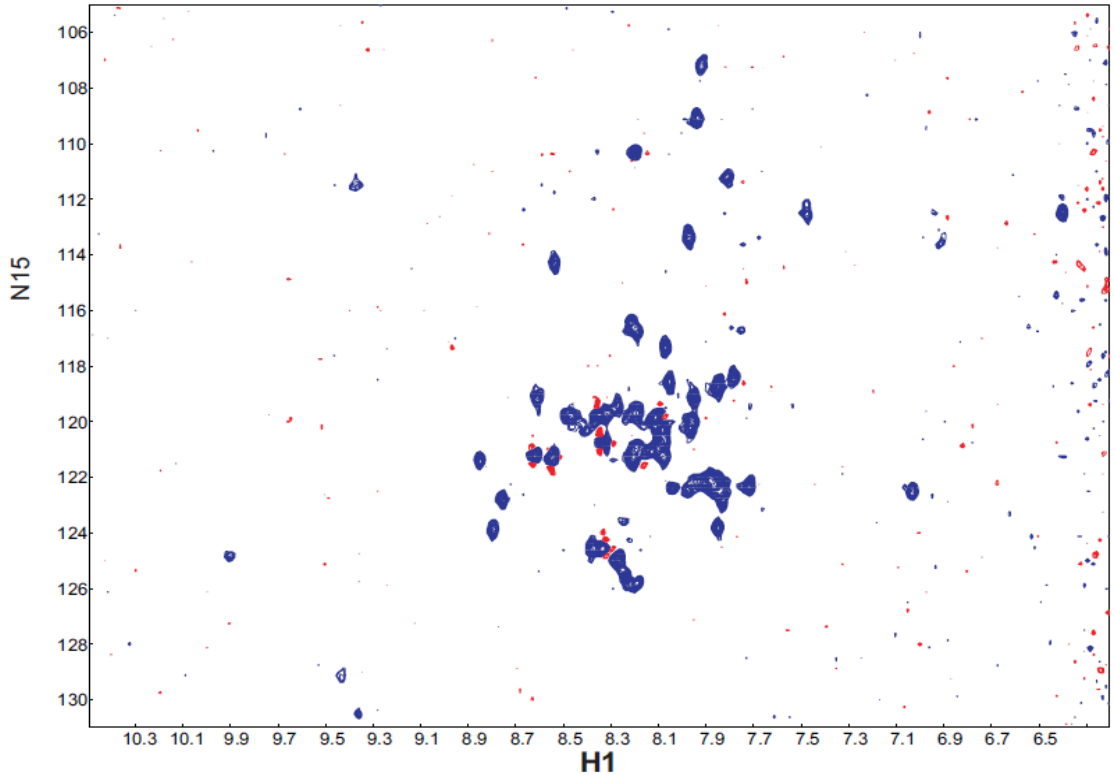

Figure 4-7 2D HSQC of CR3 Residues 139-190

A 2D HSQC of purified ^{15}N-labeled CR3 190T (residues 139-190 of hAd5 E1A) was collected at 25°C. The Y axis depicts the ^{15}N chemical shift and the X axis depicts the proton chemical shift. Each peak represents an amino acid of CR3 190T.

EDTA. I first confirmed the spectrum of properly folded CR3 (Figure 4-8 black peaks). The sample was then treated with a five-fold molar excess of EDTA to chelate the zinc away from CR3 and the spectrum was recollected and overlaid on the spectrum prior to EDTA treatment (Figure 4-8 red peaks). The 2D HSQC of CR3 190T before treatment with EDTA is superimposable on the spectrum collected in Figure 4-7 (Figure 4-8 black peaks). Upon treatment with excess EDTA, the peaks at the exterior of the CR3 190T spectrum collapsed into the centre, indicating a denatured and flexible structure (Figure 4-8 red peaks). This result conclusively demonstrates that the structure of E1A CR3 is dependent on zinc coordination. Based on the EDTA zinc chelation experiment result, I sought to verify the 1:1 molar ratio of zinc ion binding previously reported by mass spectrometry, to ensure that the structure determined by NMR is dependent upon CR3 binding a single zinc ion. The ^{15}N labeled sample of CR3 190T was analysed for mass determination by mass spectrometry under denaturing versus non-denaturing conditions. The predicted denatured mass of ^{15}N labeled CR3 190T is 6480.59 Da and the denatured mass experimentally observed was 6480.24 Da (Figure 4-9). Therefore, the denatured protein is present at the appropriate mass (2). The mass spectrometry data also detected a smaller protein present in relative abundance with a mass of 5613.23 Da. This corresponds to the first 48 residues of CR3190T, and indicates that most likely a proteolytic cleavage occurs after residue 48 of CR3190T in some of the purified material (Figure 4-9). As expected, both of these peptides showed an increase in mass under non-denaturing conditions of approximately 63 Da, which is the mass of a single zinc ion. Therefore both CR3 fragments observed, residues 139-190 and residues 139-182 of hAd5 E1A, are capable of binding a single zinc ion, in agreement with what has been published previously (10). The discovery of the truncated species in the purified CR3 was of concern, since the ratio of the two species was unknown, and the presence of two species would complicate analysis of the NMR data. Since both species could clearly still coordinate a single zinc ion, we decided to truncate CR3 to the smaller fragment in order to obtain a homogeneous population of purified protein. As mentioned above, the mass of the smaller species indicated it terminates at residue F182 of hAd5 E1A (2). Examination of the primary amino acid sequence of CR3 shows that residue 179 is the sole cysteine in CR3 that is not involved in zinc coordination (10). However, this

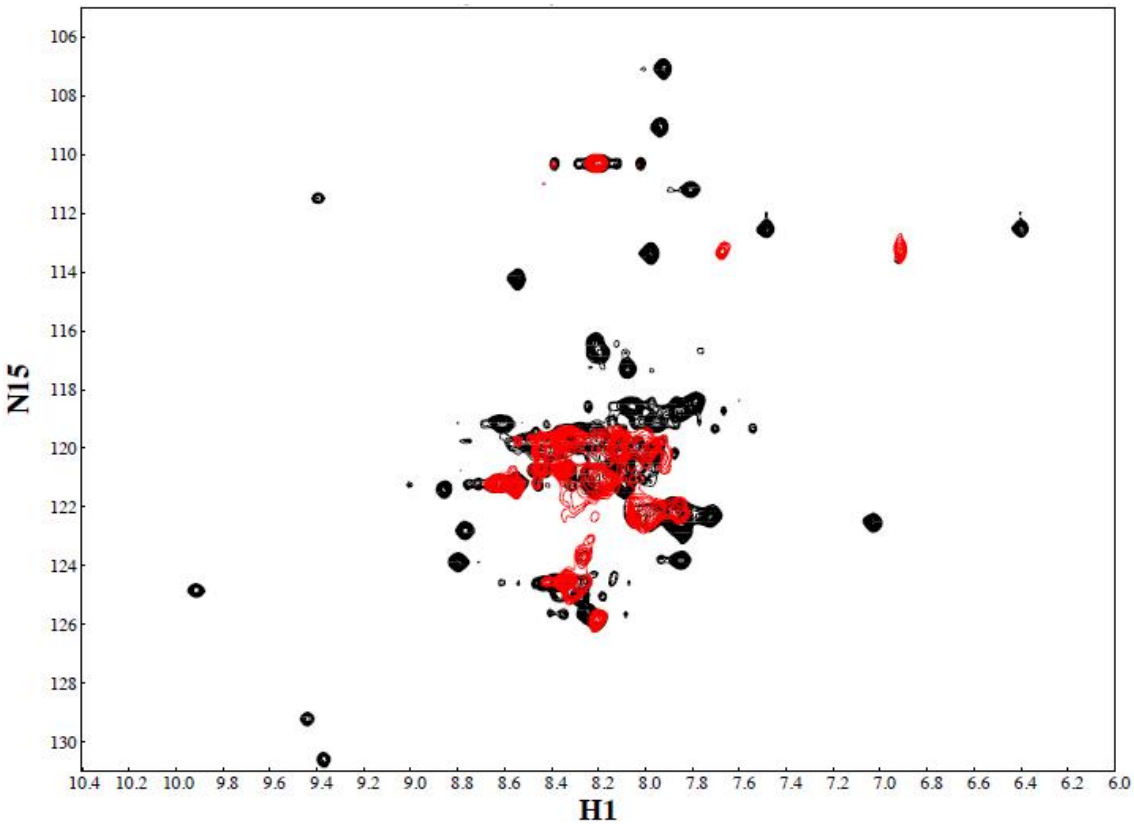

Figure 4-8 2D HSQC of CR3 190T With and Without EDTA

A 2D HSQC of purified ^{15}N-labeled CR3 190T (residues 139-190 of hAd5 E1A) was collected at 25°C. The Y-axis depicts the ^{15}N chemical shift and the X-axis depicts the proton chemical shift. Each peak represents an amino acid of CR3. Black peaks are the spectrum of CR3 190T collected in the absence of EDTA. Red peaks are the spectrum of CR3 190T collected after the addition of 500 mM EDTA.

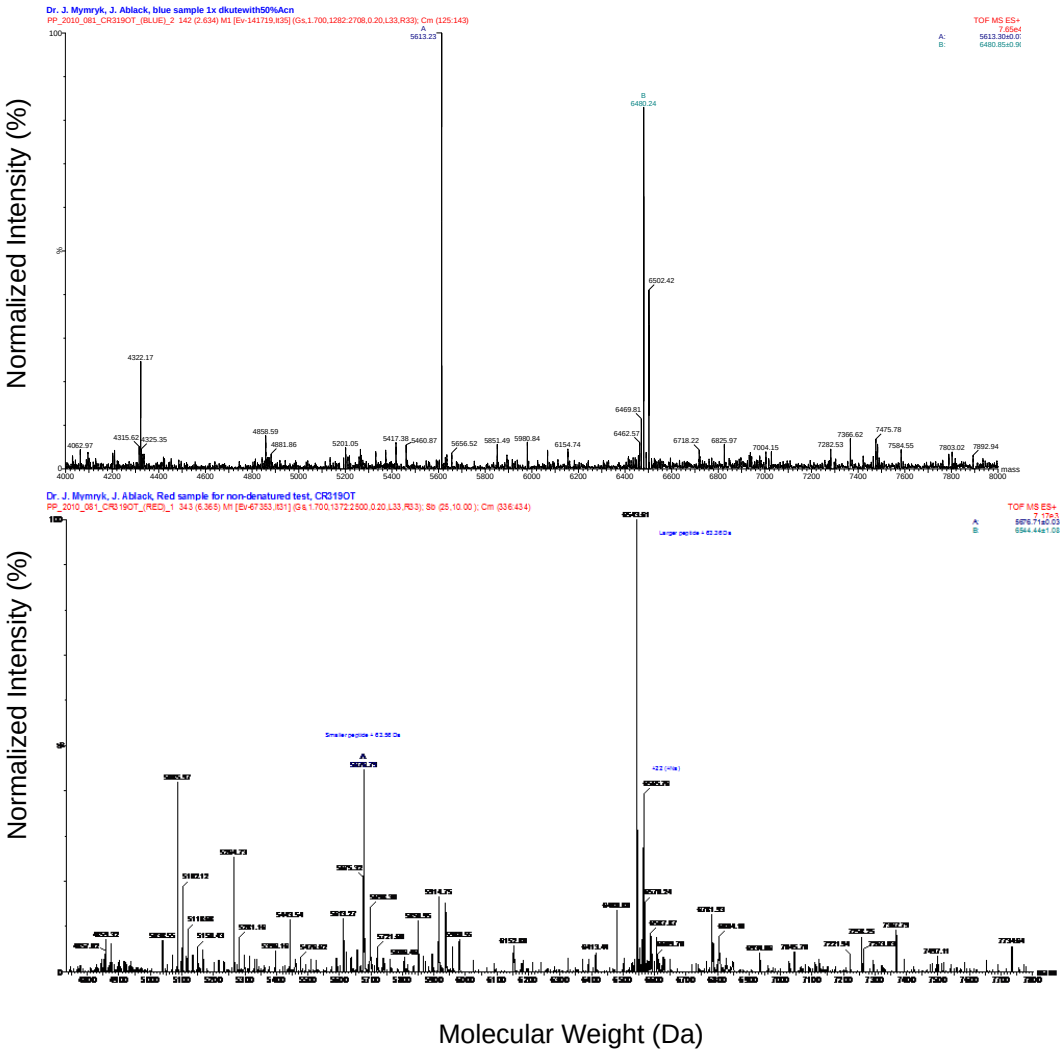

Figure 4-9 Mass Determination of CR3 190T with and without Zinc

Purified CR3 190T (residues 139-190 of hAd5 E1A) was analyzed by ESI QTOF mass spectrometry. The predicted denatured mass of ^{15}N labeled CR3 190T is 6480.59 Da. TOP: Under denaturing conditions (50% acetonitrile) two predominant species of CR3 were observed with masses of 5613.23 Da and 6480.24 Da corresponding to residues 139-182 and 139-190 of hAd5 E1A CR3, respectively. BOTTOM: Under non-denaturing conditions (10 mM ammonium acetate) the mass of both species has increased by 63 Da, which is the mass of one zinc ion.

cysteine could still form disulfide bonds, further complicating purification and interpretation of the NMR spectrum. Therefore I have truncated CR3 at residue 178 to remove this cysteine for all subsequent experiments. This was done by truncating the CR3 domain of hAd5 E1A by PCR and recloning the new fragment into pGEX4T1-TEV; the new construct was designated CR3178T. However, CR3178T has not been used in any of the experiments described in this thesis. Future experiments involving CR3178T are discussed in chapter 5.

CR3190T was also subjected to circular dichroism (CD) spectropolarimetry to confirm the overall structured nature of E1A CR3 and gain insight into potential secondary structure of E1A CR3. A CD spectrum of the purified CR3 190T sample was collected at 25 °C and is shown in Figure 4-10A (blue curve). CR3 190T showed bands of ellipticity whose minima were between 208 nm and 222 nm, and also some positive character below 200 nm. The spectrum is strongly indicative of a structured protein. However, it is not consistent with an entirely α-helical or β-sheet protein, but rather likely contains a combination of both types of structures. Further insight into the stability of CR3190T was gained by measuring mean residue ellipticity (MRE/[θ]) at 222 nm over a temperature range. The MRE of CR3190T at 222 nm decreased sharply at approximately 70 °C, which indicated that the structure of CR3 unfolded upon heat denaturation (Figure 4-10B). The sample of CR3190T was allowed to cool back to 25 °C and the CD spectrum was recollected. After heat denaturation, the CD spectrum was nearly superimposable on the spectrum collected before heat denaturation (Figure 4-10A red curve). Upon cooling, presumably in the presence of zinc ions, CR3190T was apparently able to refold in a self-directed manner.

4.4 Discussion

Overall, we demonstrated conclusively that CR3 contains a zinc-dependent structured subdomain and have mapped the boundaries of the zinc binding subdomain of CR3 to include up to residue 182 of hAd5 E1A. Moreover, we have confirmed previous reports that E1A CR3 binds a single zinc ion and is capable of self-directed refolding (10, 20, 25). Also my analysis of the purified fragments of CR3 indicates that our material is properly folded and still capable of interacting with key cellular targets of E1A CR3.

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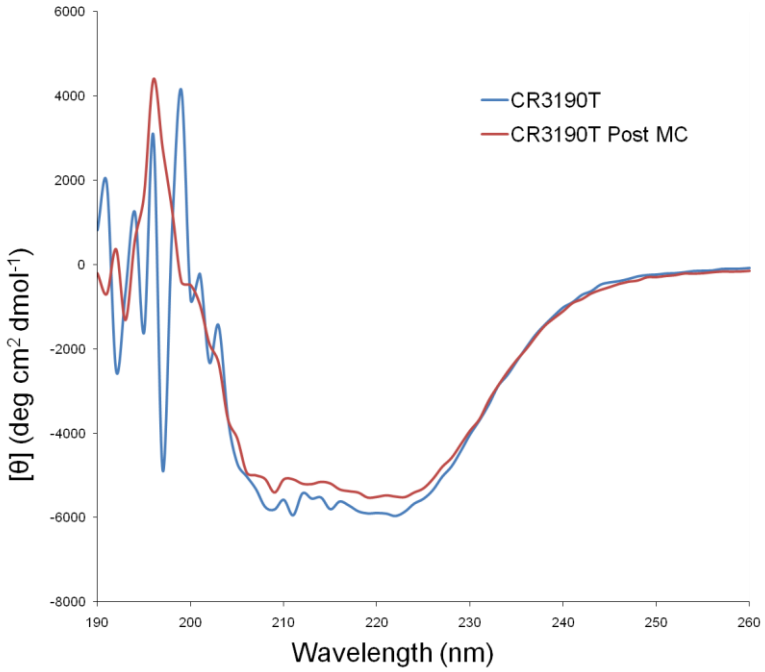

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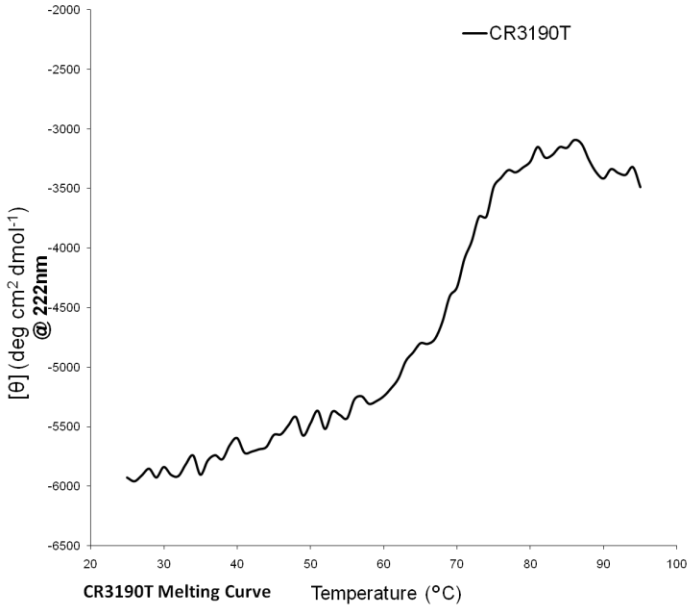

Figure 4-10: Far UV CD Spectra of CR3190T

A) Far UV CD spectra for CR3190T (residues 139-190 of hAd5 E1A) was collected at 25°C in 10 mM PO_4 buffer pH 6.5 at a concentration of 100 µM (blue spectrum). The same sample was denatured by heating (see B), cooled to room temperature and the CD spectrum was recollected at 25 °C (Red spectrum, post MC). B) Melting curve of CR3190T. The MRE ([θ]) was monitored at 222 nm as CR3190T was heated to 100°C.

The bioinformatic tool PONDR predicted two major regions of order in hAd5 E1A. Peaks of order corresponded to the extreme N-terminus and CR3 of hAd5 E1A (Figure 4-1). The region in the N-terminus of hAd5 E1A predicted by PONDR is thought to fold into an amphipathic alpha helix which is critical to the proper organization of the 15 cellular proteins known to interact with this region (30). Furthermore, this amphipathic alpha helix that is predicted to be conserved among representative members of each hAd species (30). As demonstrated above, the prediction of an ordered domain in CR3 of hAd5 E1A has now been verified experimentally by NMR spectroscopy (Figures 4-6 to 4-8).

PONDR also predicted multiple regions of intrinsic disorder within CR1 and CR2 of hAd5 E1A (Figure 4-1). Intrinsic disorder refers to a region of a protein that samples a wide range of conformational space in solution and only takes on a fixed structure when bound to its appropriate partners. The true disordered nature of CR1 and CR2 of hAd5 E1A were recently demonstrated experimentally. The solution structure of hAd5 E1A CR1 and CR2 in complex with the TAZ2 domain of CBP and the pocket domain pRb has been solved (13). The structure shows E1A binding to the TAZ2 domain of CBP and the pocket domain of pRb, through CR1 and CR2, respectively, and independently of each other. There is also an interaction site for pRb in CR1 of E1A that is independent of the TAZ2 interaction site (27). The solution structure also reveals that CR1 and CR2 of E1A take on a helical structure upon interaction with these binding partners (13). My PONDR prediction showed three peaks of intrinsic disorder in regions of E1A that correspond to the interaction sites of TAZ2 and pRb. Each PONDR predicted disordered peak was thus validated experimentally by the work of Ferreon *et al.* (13) as an intrinsically disordered region of E1A critical to the interaction with E1A's cellular partners and essential to E1A performing one of its central functions, uncoupling cell cycle regulation. Therefore overall, PONDR has successfully predicted the landscape of order versus disorder in hAd5 E1A.

The initial NMR experiments using residues 139-204 of hAd5 E1A that includes CR3 and AR1 indicated that this peptide was both structured and unstructured (Figure 4-6). PONDR also predicted that residues 139-204 contained both structured and disordered

regions (Figure 4-1). The dichotomy of the NMR spectrum for CR3/AR1 is not entirely unexpected, since the literature has suggested that CR3 consists of two functional subdomains; a structured zinc finger subdomain and a disordered promoter targeting subdomain. These two subdomains are thought to cooperate in order to activate transcription of the early viral promoters. The zinc finger subdomain makes specific contact with key cellular transcription factors such as TBP and MED23, thus recruiting the cellular transcription machinery (6). Meanwhile, the promoter-targeting subdomain of E1A CR3 can make promiscuous interactions with sequence-specific DNA-binding transcription factors, locating E1A and transcription machinery to the diverse viral promoters (26, 28).

The requirement for zinc in E1A CR3 transactivation has been studied in detail. Single point mutations in any of the coordinating cysteine residues abrogate transactivation by full length E1A on an E4 promoter and also by CR3 when fused to the Gal4DBD (3, 18). Furthermore, conversion of the C4 zinc finger domain of CR3 to a C2H2 zinc finger also abrogates E1A transactivation (40). My mass spectrometry analysis of CR3 190T demonstrated that the zinc finger subdomain of E1A CR3 binds a single zinc ion, consistent with a previous report that showed E1A CR3 bound a single zinc ion by X-ray ion absorption spectroscopy (10). The function of E1A CR3 has already been shown to depend on the coordination of a single zinc ion and my data now demonstrate that there is a correlation between the 3D structure of E1A CR3, coordination of single zinc ion, and transactivation (10, 17, 40).

CD spectropolarimetry demonstrated that residues 139-190 of hAd5 E1A contain both α-helical and β-sheet secondary structures (Figure 4-10). The amino acid sequence of E1A CR3 may also hold some insight into possible secondary structure of this region. Proline residues are unable to form α-helix or β-sheet secondary structures, due to a lack of amide protons to form hydrogen bonds, and pralines would therefore not be found concentrated in regions of secondary structure (38). hAd5 289R E1A contains a total of 46 (15.86%) prolines. Between residues 139-204 within CR3 of hAd5 E1A there are 10 prolines (15.15%), indicating that CR3 has roughly the same proportion of prolines as the rest of E1A (ref protein calculator). However the distribution of proline residues within

CR3 is very uneven. Of the 10 prolines, 8 are found within the promoter-targeting subdomain/AR1, which our NMR analysis suggests is part of unstructured region at the C-terminus of CR3 (Figure 4-6). The remaining two prolines are found at precisely 4 residues upstream of the CXXC zinc binding motifs in the zinc finger subdomain, which may indicate that these two prolines are involved in segregating secondary structures associated with zinc coordination. Mutation of P150 (4 residues upstream of the first CXXC motif) to glycine results in complete loss of CR3 function, while mutation of P167 to glycine appears to have no effect, suggesting that the proline upstream of the first CXXC motif is more important for the folding and structure of CR3 (17, 40). The zinc-binding region of Human papillomavirus E7, another early protein from a small DNA tumor virus that activates transcription, has been crystallized and its structure includes both α-helical and β-sheet secondary structure and bears no homology to other known zinc-binding proteins (27). Perhaps the hAd E1A CR3 zinc binding region, which also bears no homology to known zinc binding proteins, is adopting a similar blend of secondary structures to properly organize its cellular binding partners to initiate transcription.

Curiously, upon refolding of the CR3190T peptide that had been heated to 100 °C, the quality of the signal at the lowest wavelengths improved. I speculate this may be the result of contaminating proteins that were absorbing polarized light before denaturing that were unable to refold after cooling. Thus, the absence of contaminating signal would then allow E1A CR3 to become more prominent in this region of the spectrum. These observations are consistent with self-directed folding of E1A CR3 that has been demonstrated previously. Specifically, microinjection of a peptide corresponding to residues 140-188 of E1A CR3 could activate transcription of an adenoviral E2 promoter even after heating to 100 °C (20, 25).

The structure determination of the zinc finger subdomain of E1A CR3 is ongoing, using E1A-CR3 residues 139-178. The ultimate goal of this project is a high resolution structural model of the ordered zinc finger subdomain of hAd5 E1A CR3. The structural insight into CR3 function is essential to a complete model of E1A CR3 transactivation which serves as a paradigm for non-acidic viral transactivators.

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Chapter 5

5 General Discussion & Future Directions

This body of work describes recent advances in the current understanding of a critical function of hAd E1A—activating transcription of the early viral promoters. This function of E1A 'kickstarts' the viral replication cycle by supplying the early viral mRNAs whose translated products are required for hijacking cellular machinery, preventing antiviral defenses, blocking the host immune response and synthesizing the viral genome (22). The model by which E1A activates transcription of the early viral promoters is that E1A interacts with key cellular proteins and relocates a functional transcription preinitiation complex to the viral template. Within these chapters the model for E1A-CR3 function has been tested, refined and expanded, ultimately furthering our understanding of this paradigm of non-acidic viral transactivators.

5.1 Thesis Summary

The existing model for E1A-CR3 was developed almost exclusively with hAd5 E1A-CR3 of species C, as a prototype for the greater than 51 other types that fall into five additional species (1, 22). Based on the tissue tropism and diverse growth characteristics of the hAd family as a whole, I set out to expand this model to include a representative member of each hAd species and to integrate the function of new cellular factors that have been implicated in E1A transactivation in recent years (3, 18-20). In Chapter 2, I systematically tested the pre-existing model of E1A-CR3 transactivation with a representative E1A-CR3 from each hAd species and demonstrated that the co-activators of E1A-CR3 were conserved across the hAd family, despite dramatic differences in the abilities of the individual CR3s to activate transcription. This work also indicated that additional cellular factors are likely required by E1A-CR3 to activate transcription.

In Chapter 3, I described the role of a novel negative regulator of E1A-CR3 transactivation, the KAT GCN5. I identified a second interaction surface for GCN5 that mapped to residues 178-184 of E1A, which resides within CR3. I also showed that GCN5 was only recruited to the E4 promoter when both binding sites in E1A were

present, conclusively demonstrating that cooperative binding of a factor by the N-terminus and CR3 of E1A was essential for activation. I also established that the KAT activity of GCN5 had a negative regulatory effect on E1A transactivation and that this was required for optimal virus growth. The KAT activity of GCN5 may possibly regulate phosphorylation of S188 in E1A-CR3, which is known to be critical for activation of the viral E4 promoter. The E4 transcription unit contains some of the most toxic viral proteins in hAd genome, and tight control of their expression may be essential in order to maximize virus yield (27). This work provides a glimpse into the levels of control that are coordinated by E1A CR3 to provide the optimal level of early gene expression and suggests that overexpression of at least some early viral genes may be detrimental to virus growth.

Finally, despite anecdotal evidence that a complex secondary and tertiary structure is paramount to the function of E1A-CR3, there was no experimental evidence that E1A CR3 is structured. In chapter 4, my experimental analysis of E1A-CR3 structure by NMR and CD spectropolarimetry revealed that E1A-CR3 is composed of both a structured subdomain and a disordered subdomain. This analysis correlates with the existing functional data regarding E1A CR3-mediated transcriptional activation. Specifically, the structured zinc finger subdomain has been implicated as critical to recruiting the cellular transcription factors necessary to stimulate transcription, while the disordered subdomain has traditionally been associated with dominant negative mutants and known to be essential for targeting E1A to the viral promoters (2, 9, 13, 14, 26). The rigid structure of the zinc finger subdomain of E1A CR3 is dependent upon coordination of a single zinc ion, thereby providing a structural basis for the functional requirement of zinc in E1A transactivation (6, 9, 25). The disordered nature of the C-terminal subdomain of E1A CR3 may be the key to the broad ability of E1A to activate transcription from multiple cellular promoters via promiscuous interactions with numerous cellular transcription factors (15). I have also confirmed the self-directed nature of the zinc finger subdomain folding by CD spectropolarimetry. The results of this analysis were consistent with reports indicating that the presence of zinc is the most critical factor in E1A CR3 folding (Figure 4-8, Figure 4-10) (6, 10). The structure of E1A

CR3 will add a great deal of insight as to how this compact viral domain functions as a potent activator of transcription.

Overall, the work completed here expands our knowledge of E1A function and is the beginning of a highly detailed mechanism for E1A transactivation. Our refined model includes a layer of negative autoregulation and structural insight into the paradigm of E1A transactivation.

5.2 The Co-Activators of E1A CR3 are Conserved Across All Six hAd Speciess

One of the main goals of this research project was to expand our knowledge of E1A-CR3 transactivation with respect to the hAd family as a whole. To this end, I directly compared the ability of E1A CR3s from representative members of each hAd species to activate transcription as Gal4DBD fusions and examined the role of each known cellular co-activator of E1A CR3 in this process. Together, this work not only demonstrated that there are dramatic differences in the magnitude of each E1A CR3's ability to activate transcription, but also showed that the known co-activators of E1A CR3 were not at the heart of these differences in activity. Furthermore, while validating our squelching assay for CR3 transactivation using well defined mutants of CR3, it became apparent that additional potentially rate-limiting cellular factors could be involved in the process of transcriptional activation. Taken together these observations suggest that there could not only be additional cellular factors at work, but these factors could possibly be hAd type-specific, thus tuning the transcriptional output of E1A to suit each hAd's niche. To complete our understanding of this process, all of the players involved need to be identified.

One approach to identifying new cellular factors required by hAd5 E1A CR3 to activate transcription has been undertaken byDr. Mymryk's laboratory in collaboration with Charles Boone at the Banting and Best Institute at the University of Toronto. This project involves using the power of yeast genetics to systematically screen for all of the cellular factors required by hAd5 E1A CR3 to activate transcription. A similar small scale screen of this type has already been completed by my colleagues in Dr. Mymryk's

laboratory (29). E1A's history of targeting highly conserved factors to activate transcription make this sort of screen possible, since it is known that E1A CR3 is an effective transactivator in yeast (18, 23, 29). The purpose of these screens is to identify new genes required by E1A to activate transcription in yeast and then examine the role played by their mammalian orthologues in E1A-transactivation. The simplicity and tools available in the yeast system make this a very attractive approach. However, these screens do not look at E1A transactivation of the viral genome directly, and many cellular genes do not have yeast orthologues.

To address some of the issues raised above, I would approach the problem from an entirely different perspective and directly compare cellular factors required for E1A-dependent transactivation of early viral genes. Initial experiments revealed that incompatibilities between hAd promoter sequences of one type and the E1A proteins of other types precluded direct comparison of representative E1As on the same promoter (Ablack unpublished). Therefore, in order to make direct comparisons, it was imperative to compare CR3 function and the role of cellular factors using GalDBD fusions to CR3 fragments. Later, I was eventually able to develop an assay which directly compares E1A transactivation among hAds by qRT-PCR and measures relative expression of the E4ORF6/7 gene from the E4 promoter during infection (Figure 2-8B). Using this assay, the ability of each E1A to activate its own E4 promoter could be compared in the absence of suspected or implicated cellular transcription factors.

Unfortunately, using RNAi to deplete cellular targets in the context of infection can become problematic, because hAd encodes two virus associated RNAs (VA RNAs) that have been reported to occupy 80% of cellular RISC complexes during infection, effectively blocking the RNAi machinery (28). However, provided the target cellular mRNAs were knocked down prior to infection, the requirement for that particular cellular factor in initiating transcription of the E4 promoter could theoretically be examined. My high throughput qRT-PCR assay for E4 promoter activity could be combined with a large scale siRNA library screen for cellular transcription factors in order to identify genes required by specific hAd species to activate transcription. I propose initially comparing the prototype hAd5 to hAd9, because the CR3 region of hAd9 is the most divergent, and

clearly the weakest activator, yet hAd9 full length E1A is a potent activator in the context of infection (Figure 2-1, Figure 2-8). Genes identified as functionally required by E1A to activate transcription from the screen could easily be validated against the entire panel of representative hAds.

Based on my observations from Chapter 3, there is clearly a role for cellular factors, such as GCN5, to negatively regulate E1A transactivation. The unbiased nature of the E4ORF6/7 qRT-PCR assay allows to us screen both for factors that decrease or increase E1A transactivation. The limitations to these sorts of experiments lie in the lack of reagents for non-hAd5 viruses. With the exception of hAd12, mutants even simply lacking E1A are not generally available. Thus, the screen would compare the relative expression level of E4ORF6/7 in cells infected with *wt* hAd from any species A-F treated with either control siRNA or targeting siRNA. Furthermore, the means to make recombinant non-Ad5 viruses is also unavailable for representative members of each hAd species, hindering screening based on viral reporter systems. At the moment, qRT-PCR of early viral genes such as E4ORF6/7 is the most feasible means to make direct functional comparisons of E1A transactivation among representative hAds. This screen would still be highly relevant to the events occurring early during infection, because E1A is the first viral gene expressed.

Alternatively, a complementary biochemical screen for novel binding partners could be utilized to decipher the differences among representative E1A CR3s. It would require construction of stable cell lines expressing distinctly tagged 12S and 13S E1As from each human hAd species, i.e. Flag-tagged 12S E1A and HA-tagged 13S E1A. Cell lines would be generated using A549 cells, because this cell line supports growth of all hAds, and we would employ cDNA clones for these tagged E1As that are currently available in our lab. The interacting partners of the E1A CR3s of each hAd species could then be determined by comparative proteomic analysis using the *Comp*arative *P*roteomic *A*nalysis *S*oftware *S*uite (CompPASS). The elegance of this approach is that it generates high confidence interacting proteins (HCIPs) based on the frequency of a given peptide identified by mass spectrometry among replicate samples, and it subtracts non-specific peptides found in a control sample where no test protein is present (i.e., from cell lines

harboring empty HA or Flag expression vectors) (24). Therefore, mass spectrometry-based proteomic analysis on HA-IPs would establish the binding partners for 13S E1A after subtracting out binding partners identified in both control IPs and Flag-IPs for the respective 12S E1A. In this way, HCIPs specific to the CR3 region could be determined for each hAd E1A. The role of cellular binding partners identified by CompPASS could then be directly tested using RNAi and the qRT-PCR assay for E4ORF6/7 described above.

The work presented in chapter 3 also demonstrates cooperative binding of cellular targets by the N-terminus and CR3 of E1A. The pliability of the CompPASS approach allows examination and identification of common and/or cooperative interactions between the N-terminus and CR3 of any E1A. Proteomic data could also be mined by subtracting the control IPs and looking for peptides common to both E1A isoforms, but enriched in the HA-IPs for 13S E1A, to identify factors that interact cooperatively with both regions. Since E1A peptides will be present in the IPs, the frequency of peptides enriched by 13S E1A could be normalized to the frequency of E1A peptides found as a relative level of E1A expression. There are already several cellular factors that have binding sites in both the N-terminus of E1A and CR3 that could serve to validate this approach, including p300/CBP, pCAF, TBP and GCN5 (Figure 3-2) (13, 18, 19).

Together these approaches could exhaustively search for and identify all of the cellular factors required by E1A-CR3 to activate transcription of human adenoviral promoters in the context of infection. Although a challenging and a large undertaking, the tools to execute these experiments are currently available in our lab and could be carried with the appropriate collaborations. Using these approaches to identify all of the players involved, a complete and inclusive model for E1A-transactivation across the entire hAd family could be elucidated.

5.3 The KAT-GCN5 is a Novel Negative Regulator of E1A Dependent Transactivation

In Chapter 3, the KAT GCN5 was identified as a novel negative regulator of E1A CR3-dependent transactivation. GCN5 was recruited to the E4 promoter, and RNAi depletion, mutation of the catalytic site of GCN5, or pharmacological inhibition of KAT activity resulted in an increase in transactivation by E1A CR3 and full length E1A. Thus, the KAT activity was necessary to exert the negative effect on E1A CR3. However, a precise mechanism as to how this occurs is not clear. Close examination of the literature revealed a possible link to GCN5 and E1A-transactivation that involved the mediator sub-complex T/G Mediator (composed of Mediator subunits including MED23, GCN5 and CDK8). T/G Mediator is required for tandem phosphoacetylation of S10/K14 on H3 (16). All three of these components can be linked to E1A; MED23 and CDK8 co-purify with E1A-CR3 (2), and I have shown here that E1A-CR3 is sufficient for GCN5 interaction (Figure 3-1B). Furthermore, I identified a potential consensus CDK8 phosphorylation site spanning residues 184-188 (YSPVS) of hAd5 CR3, where the terminal S (S188 in E1A) is phosphorylated. This sequence is highly conserved in our panel of representative E1A CR3s (species F hAds have the sequence YSPIS). Therefore, since all three key players are interacting with E1A via CR3, and the kinase responsible for S188 phosphorylation has yet to be identified, this complex could be responsible for E1A S188 phosphorylation, a modification known to affect E4 transactivation (16, 21, 27). To determine the role of T/G Mediator in E1A transactivation, I would first look at the properties of E1A harboring a single point mutation at S188 to alanine, as well as a mutant that loses interaction with MED23, such as H160Y (Boyer 1999, (2, 9). Then, using the assays presented in Chapter 3, I would determine if these mutants could interact with GCN5 and were no longer responsive to RNAi depletion, overexpression, and pharmacological inhibition of GCN5. These mutants could also be used in kinase assays to determine if S188 was phosphorylated in a manner consistent with GCN5 and MED23 binding. This series of experiments could link phosphorylation of S188 to the Mediator complex and the effect of GCN5. Next, I would test whether CDK8 is the kinase responsible for S188 phosphorylation in two ways: 1) I would look for an interaction between CDK8 and *wt* E1A CR3 by Co-IP. I

could then attempt to correlate CDK8 binding to Mediator and GCN5 binding using CR3 mutants that lose interaction with Mediator and GCN5 (the H160Y mutant and Δ178-184 E1A mutants, respectively) by co-IP. The invariant Y residue of the YSPVS sequence in E1A CR3 corresponds to Y184, which when deleted along with residues 178-183, results in a loss of GCN5 interaction. I will also mutate this residue and examine the effect on GCN5 binding and E1A transactivation. 2) The role of CDK8 in E1A CR3 transactivation will be tested directly by RNAi depletion of CDK8 and pharmacological inhibition of its kinase activity using the same assays described in Chapter 3.

Our working model is that the KAT activity of GCN5 regulates CDK8, which in turn phosphorylates S188 on E1A, which is the most transcriptionally active form of E1A CR3 (27). I suspect that the explanation for repression of E1A-dependent transactivation by GCN5 overexpression is due to the generation of a hypophosphorylated form of E1A at S188. Conversely, knocking-down GCN5 or pharmacologically inhibiting/mutating the KAT of GCN5 indirectly results in hyperphosphorylated E1A CR3 at S188. How the KAT transferase activity is potentially regulating CDK8 would also need to be determined. There is growing evidence that non-histone proteins are acetylated and that this reversible post-translation modification is involved in many cellular processes, including regulating kinases (4). In fact, members of the GCN5-containing STAGA complex (SAGA in yeast) have been shown to be acetylated by GCN5 (4, 8, 17). Once the players involved are determined by the experiments described above, the role for acetylation and which components are acetylated could be determined.

In 2008, the total promoter occupancy of hAd5 243R E1A was shown to result in a global hypoacetylation of histone H3 Lysine 18 (H3K18), an epigenetic mark generally associated with transcriptional repression. Indeed, 12S E1A was shown to occupy greater than 70% of cellular promoters and completely redistribute p300 and pRb promoter occupancy (12). However, very little is known about the 13S E1A product in terms of activating cellular genes during the course of infection. Given that the 13S E1A product has all of the regions in the 12S product and an additional transactivation/promoter targeting domain, it stands to reason that the 13S product could also occupy cellular promoters. Recent evidence indicating that 13S E1A can use repressors such as CtBP to

activate silenced promoters implies that CR3 could be contributing to global changes in cellular gene expression (3). Based on the shocking global changes in cellular promoter occupancy mediated by 12S E1A (7, 12), the global promoter occupancy of 13S E1A is of great interest to the field. Using chromatin immunoprecipitation-deep sequencing (ChIP-Seq) the promoter occupancy of 13S E1A could be determined and compared to that of 12S E1A. There is a monoclonal E1A antibody that specifically immunoprecipitates the 13S E1A of hAd5 (11). Using this monoclonal antibody, the promoter occupancy of 13S E1A in context of infection and in the presence of 12S E1A could be determined. Comprehensive analysis of 12S and 13S E1A function on cellular promoters would provide critical insight to the roles played by each of these two very similar proteins that are both present in abundance at early times during infection.

5.4 The Structure of hAd5 E1A CR3

My progress, in concert with others, in determining the structure of E1A-CR3 is close to yielding a high resolution model. The key to resolving a structure by NMR is the collection of lots of data and satisfying as many experimental and theoretical constraints as possible in order to compute the high resolution final structure. First and foremost, each amino acid of the protein backbone must be assigned chemical shifts by 3D labeling techniques in order to track individual amino acids in subsequent experiments. For this to be completed, E1A CR3 178T will be overexpressed in *E. coli* strain DE3 plysys, with ^{15}N ammonium chloride and ^{13}C glucose as the only nitrogen and carbon sources respectively, to isotopically label the protein. Once double labeled recombinant protein of sufficient quality and quantity is prepared, a series of 3D HSQC experiments can be performed to generate sufficient data such that with the appropriate computation it will be possible to assign each peak to an amino acid in the E1A CR3 sequence. When the protein backbone is assigned, chemical shifts of each aa will provide insight into any secondary structure. In general, α-helices will cause upfield shifts in ^{1}Hα and ^{13}Cβ and downfield shift, in ^{13}Cα ^{13}C', while β-sheets have the opposite effect: downfield shifts in ^{1}Hα and ^{13}Cβ and upfield shifts in ^{13}Cα ^{13}C'. This chemical shift data can be input into programs such as TALOS (Torsion Angle Likelihood Obtained from Shift and sequence similarity) (5) to determined *phi*, and *psi* angles of the protein backbone. In order to

determine the tertiary structure of proteins by NMR, it will be necessary to take advantage of the nuclear overhauser effect (NOE). The NOE states that protons within 5Å of each other will interact and generate cross peaks in a NOESY experiment. Therefore, the amide protons of E1A CR3 that are in close proximity in space but separated in the primary amino acid sequence can be identified. The cross peaks of the NOESY experiment are included in the constraints input into software packages that determine conformers of the aa sequence that fit all of the constraints. The quality of the NMR structure is dependent upon the number of experimental constraints and the flexibility of the protein. In the case of E1A CR3, we are trying to assign only 48 amino acids, which based on our data and predictions thus far, appear to be quite rigid in nature. These properties of E1A CR3 make it a very likely candidate for a solvable solution structure. The ultimate goal of this project is a 3D model of E1A CR3 structure.

Once the structure is available, it will be necessary to test the vast collection of mutants available in CR3 to validate the structure. I will begin by looking at the group I *hr* mutants, which are known to have biological consequences, and determine if any of these mutants are required for structural stability. Furthermore, one can make strategic mutations in CR3 to disrupt key intra-molecular contacts and assay their phenotype by the Gal4DBD transactivation assays described in Chapter 2. Ultimately, fine mapping the contribution of solvent-exposed residues to interaction surfaces on E1A CR3 with the known cellular binding partners, such as MED23, will also be performed using strategic mutations or existing published data. These mutants can also be used in *vitro* to catalogue cellular factors that bind directly to E1A CR3 and those that interact with E1A through large multi-protein complexes. Adding this level of detail to the model of E1A CR3 transactivation will allow for a complete and validated paradigm of E1A CR3 function.

5.5 Concluding Remarks

Viruses are obligate intracellular parasites. The study of viruses including small DNA tumor viruses such as hAd, serves two purposes. Firstly, since many viruses cause disease, it is useful to understand the mechanisms of viral pathogenesis in order to effectively combat these pathogens. Secondly, the complex co-evolution of host and

pathogen can be viewed as a molecular arms race. The tremendous selective pressure exerted on viruses by the host selects for innovation at the molecular level. Thus, viruses can function as discriminating biochemical tools to probe important host pathways. It is in this way that the E1A protein has truly been of value, and studies of this versatile viral protein have propelled our understanding of many cellular processes and will likely continue to do so for many years to come.

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