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The Interaction of the Human Adenovirus E1A Protein with the Human DREF Transcription Factor

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology and Immunology

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**THE INTERACTION OF THE HUMAN ADENOVIRUS E1A PROTEIN WITH THE
HUMAN DREF TRANSCRIPTION FACTOR**

Thesis format: Monograph

by

Kristofer M. James

Graduate Program in Microbiology & Immunology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The human adenovirus (HAdV) E1A protein is the first protein produced post-HAdV infection, and serves two main functions. The first is to modulate host and viral transcription. The second is to induce host cell cycle progression to S phase, to promote an optimal environment for viral replication. E1A performs its functions by binding and manipulating over 50 cellular factors. Interestingly, I found that E1A is capable of interacting with the poorly characterized human DNA replication-related element-binding factor (hDREF). hDREF is a transcription factor involved in the expression of several genes related to the cell cycle. I hypothesized that the interaction between E1A and hDREF would contribute to adenovirus induced transcriptional modulation and viral replication in HAdV-5 infected host cells.

Utilizing co-immunoprecipitation experiments, I discovered that E1A can bind hDREF through residues 15-26. Using quantitative real time polymerase chain reaction (RT-PCR), I found that hDREF also increases expression of HAdV-5 E3 and E4 genes, which are trans-activated by E1A. Finally, hDREF expression increases HAdV-5 replication. Further studies will reveal whether or not the E1A-hDREF interaction is specifically responsible for these observed results.

Keywords

Human adenovirus, HAdV, E1A, hDREF, co-immunoprecipitation, RT-PCR, plaque assay, transcription

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Finally, thanks to all of my friends who have given me some of the most memorable experiences of my life, by diverting me from the academic lifestyle on occasion. Last but not least, the assistance of my cat Dante in writing my thesis was of paramount importance.

Dedicated to my late father, Lyle James.

Your caring heart and sense of humor are dearly missed by all.

You will always exist as the basis of my motivation.

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List of Abbreviations

| | |
|----------------|--|
| Ab | Antibody |
| AdDdDp | Adenovirus DNA-Dependent DNA Polymerase |
| ADP | Adenovirus Death Protein |
| ARD | Acute Respiratory Disease |
| AdV | Adenovirus |
| AVP | Adenoviral Protease |
| BEAF-32 | Boundary Element Associated factor of 32 kDa |
| BED | Boundary Element-Associated Factor and DREF |
| BrdU | Bromodeoxyuridine |
| CAR | Coxsackievirus and AdV Receptor |
| CASTing | Cyclic Amplification and Selection of Targets |
| CBP | CREB-Binding Protein |
| CDK | Cyclin-Dependent Kinase |
| cDNA | Complementary DNA |
| ChIP | Chromatin Immunoprecipitation |
| CKI | CDK Inhibitory Protein |
| CR | Conserved Region |
| CtBP | C-terminal Binding Protein |
| CTL | Cytotoxic T-Lymphocyte |
| dDREF | <i>Drosophila</i> DREF |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DRE | DNA Replication-Related Element |
| DREF | DRE-Binding Factor |
| dsDNA | Double-Stranded DNA |
| DTT | Dithiothreitol |
| eIF-2 α | Eukaryotic Initiation Factor-2- α |
| EMSA | Electrophoretic Mobility Shift/Band Shift Assays |
| ER | Endoplasmic Reticulum |
| E2F | E2 Transcription Factor |
| GAPDH | Glyceraldehyde 3-Phosphate Dehydrogenase |
| Gal4-DBD | Gal4 DNA-Binding Domain |
| GFP | Green Fluorescent Protein |
| GTF | General Transcription Factor |
| HA | Hemagglutinin |
| HAdV | Human Adenovirus |
| hBre1 | Human Brefeldin A Sensitivity 1 |
| hDREF | Human DREF |
| HEK | Human Embryonic Kidney |
| hPaf1 | Human Polymerase II-Associated Factor 1 |
| HPI | Hours Post-Infection |
| HPV | Human Papillomavirus |
| HRP | Horseradish Peroxidase |
| H2B | Histone 2B |
| IFN- α | Interferon α |
| IFN- β | Interferon β |
| IKK | I κ B Kinase |

| | |
|----------------|--|
| IP | Immunoprecipitation |
| IRF1 | IFN-Regulatory Factor 1 |
| ISG | IFN-Stimulated Gene |
| KSR | Kinase Suppressor of Ras |
| LIM | Linear Interaction Motif |
| MED | Mediator |
| MHC | Major Histocompatibility Complex |
| miRNA | Micro RNA |
| MLP | Major Late Promoter |
| MOI | Multiplicity of Infection |
| MRN | Mre11-Rad50-Nbs1 |
| mTOR | Mammalian Target of Rapamycin |
| NF- κ B | Nuclear Factor Kappa B |
| NLS | Nuclear Localization Sequence |
| NPC | Nuclear Pore Complex |
| NURF | Nucleosome Remodelling Factor |
| ORF | Open Reading Frame |
| ORI | Origin of Replication |
| PAR1 | Pseudoautosomal Region 1 |
| PBS | Phosphate-Buffered Saline |
| pCAF | p300/CBP-Associated Factor |
| PCNA | Proliferating Cell Nuclear Antigen |
| PFU | Plaque Forming Units |
| PIC | Pre-Initiation Complex |
| PI3K | Phosphatidylinositol-3-OH Kinase |
| PKR | Protein Kinase R |
| pRb/Rb | Retinoblastoma Tumor Suppressor Protein |
| PVDF | Polyvinylidene Fluoride |
| REP | Rough Eye Phenotype |
| RID | Receptor Internalization and Degradation |
| RISC | RNA Silencing Complex |
| RP | Ribosomal Protein |
| RPS6 | Ribosomal Protein Small 6 |
| RPL10A | Ribosomal Protein Large 10A |
| RPL12 | Ribosomal Protein Large 12 |
| RT-PCR | Real Time Polymerase Chain Reaction |
| SAdV | Simian Adenovirus |
| siRNA | Silencing RNA |
| ssDBP | ssDNA Binding Protein |
| ssDNA | Single-Stranded DNA |
| SUMO | Small Ubiquitin-Related Modifier |
| TBP | TATA-Binding Protein |
| TBS-T | Tris-Buffered Saline Containing 0.1% Tween-20 |
| TFIID | Transcription Factor II D |
| TIR | Terminal Inverted Repeat |
| TNF | Tumor Necrosis Factor |
| TP | Terminal Protein |
| TRAIL | Tumor Necrosis Factor-Related Apoptosis-Inducing |

TRF2
TSS
VA-RNA
UAS
WT
Zen

TBP-Related Factor 2
Transcription Start Site
Virus-Associated RNA
Upstream Activation Sequence
Wild-Type
Zerknüllt

1 Introduction

1.1 Adenoviruses

Adenoviruses (AdV) – named for their discovery in human adenoid tissue – were first isolated and characterized by two independent groups in 1953, who were searching for the causative agent of acute respiratory disease (ARD) plaguing military recruits during World War II (1–3). It is now known, however, that human AdVs (HAdVs) are responsible for only 5-10% of upper respiratory tract disorders in children, and that rhinoviruses are the major causative agents behind the common cold (4, 5). HAdVs have since been confirmed as the etiological agents of many other clinical diseases, most notably conjunctivitis and infantile gastroenteritis (6–8). Although most infections are self-limiting and can be asymptomatic, immunosuppressed patients may experience fatal infections arising through pneumonia, encephalitis, and fulminant hepatitis (9).

The *Adenoviridae* family contains over 100 members spanning five genera, which infect a range of vertebrates from fish to humans (10). All HAdVs are classified within the genus *Mastadenovirus*, which comprises those viruses isolated from mammals (11). There are currently 57 accepted HAdV comprising six species (A-F), with a purported seventh species (G) containing only serotype 52 (12, 13) (Table 1.1). Classification of HAdV into species and serotypes is complicated. Species are classified based on their ability to agglutinate red blood cells, as only antisera against viruses of the same species will prevent the hemagglutination reaction (14). New serotypes are denoted based on the ability of the virus to resist neutralization by antisera against known HAdV types (15). This mainly occurs via exposed virion hexon proteins, as hypervariable regions exist on the hexon surface which are type-specific (16–18). Therefore, individual HAdV types will only neutralize themselves.

The use of HAdVs as experimental models initially became evident in 1962 when Trentin *et al.* discovered that injection of HAdV-12 of species A into infantile hamsters caused

malignant tumors (19). He provided the first recorded evidence of a human virus causing oncogenesis. Only certain species of HAdVs are oncogenic, as tumorigenicity is closely linked to the ability of individual serotypes to evade the host immune system. Indeed, all species can promote tumorigenesis in immune-compromised rodents (20). Furthermore, all species can transform cultured rodent cells, establishing HAdVs as useful model systems for studying the mechanisms of cancer (21). Currently, no HAdVs have been implicated in the oncogenesis of human tumors (22, 23). HAdV infections in rodents are non-productive due to a deficiency in both viral DNA replication and late protein production (24). In contrast, HAdV infections in humans are lytic, and therefore cell death occurs long before tumorigenesis could occur. However, E1A is capable of immortalizing human cells on its own when stably transfected, and in cooperation with a second oncogene such as E1B, can fully transform human cells (25, 26). Human embryonic kidney (HEK) 293 cells were transformed in such a fashion, but transformation of other cell lines with E1A and other oncogenes has proven difficult (25).

Since 1962, the use of HAdVs as molecular tools has gained popularity due to several enticing characteristics. HAdVs are easily grown to high titres, unlike other tumor viruses with more stringent growth conditions, such as human papillomavirus (HPV) (27). They cause synchronous infections in cultured cells, allowing the kinetics of infections to be easily studied and replicated (21). The genomes of many HAdVs are well characterized and easily manipulated, facilitating the study of the functions of both HAdV gene products and the cellular factors that interact with them (4). HAdVs have played a large role in our understanding of DNA replication, cell cycle control, transcription, apoptosis, immunological responses to viral infections, and mRNA processing (21). For example, the discovery that multiple mRNAs are produced from a single transcript – a process we now know as mRNA splicing – as well as the discoveries of introns and pre-mRNA processing to mature mRNA, are all credited to the studies of HAdV infected cells (28–32).

Table 1.1 Classification of Human Adenoviruses

| Species | Serotypes | Tumorigenicity in Rodents | Transformation of Cell Culture |
|----------------|---|----------------------------------|---------------------------------------|
| A | 12, 18, 31 | High | Yes |
| B | 3, 7, 11, 14, 16, 21, 34, 35, 50, 55 | Moderate | Yes |
| C | 1, 2, 5, 6, 57 | Low or None | Yes |
| D | 8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51, 53, 54, 56 | Low or None | Yes |
| E | 4 | Low or None | Yes |
| F | 40, 41 | Unknown | Yes |
| G | 52 | Unknown | Unknown |

Adapted from Berk, AJ., 2007 (4)

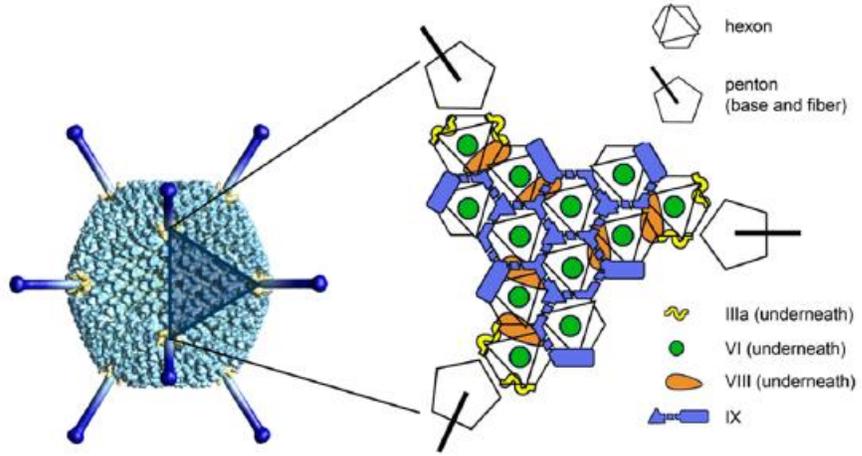
1.1.1 HAdV Virion Structure

All HAdV are 90nm diameter, icosahedral, non-enveloped particles with fibres protruding from each vertex of the virion (33) (Figure 1.1). The virions are made up of a proteinaceous capsid surrounding a core which contains the DNA genome. There are 11 proteins in the virion named/numbered II-IX, IIIa, terminal protein, μ , and p23 viral protease (34). 240 hexon capsomers – which are trimers of polypeptide II – comprise the 20 faces of the virion, with 12 hexons on each face (35). 12 penton capsomers – which are comprised of a penton base made of a pentamer of polypeptide III, and a projecting fibre made of a trimer of polypeptide IV – form the vertices of the icosahedron (36). The fibre contains three major domains: the N-terminal domain contains an invariant FNPVYPY motif found in all HAdV fibres, which connects the fibre to adjacent penton base monomers; a central shaft of 6 to 22 repeating units of a 15-residue motif which forms a “triple β -spiral” fold; and a C-terminal knob domain, which contains a binding site for HAdV receptors on host cells (34, 36–39). Together the flexibility of the shaft and the specificity of the knob domain act as major determinants of viral tropism. The coxsackievirus and AdV receptor (CAR) – a transmembranous immunoglobulin superfamily member found on a vast array of cell types – was discovered as the main, high-affinity receptor for serotypes 2 and 5 of species C (40, 41). It has also been implicated as a main receptor of species A, D, E, and F but not B, as the knob domain in species B is highly divergent in sequence (42, 43). In addition, deviations may occur on an individual basis in each species, as is evidenced by HAdV-37 of species D, which cannot efficiently use CAR as a receptor due to a rigid shaft domain, and instead utilizes sialic acid as a receptor (44, 45). Although most HAdV encode only a single type of fibre, HAdV-40 and -41 of species F encode a second fibre with an unknown receptor which may be responsible for their increased tropism for intestinal cells (46–48).

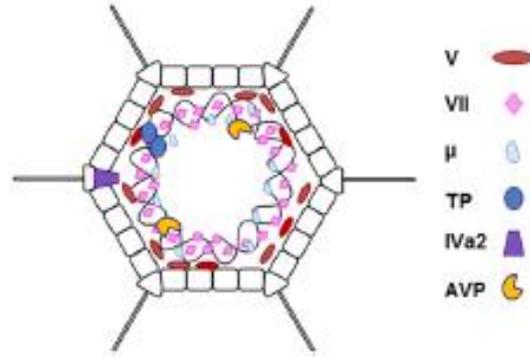
Several minor capsid proteins stabilize protein-protein interactions in the virion. Polypeptides VIII and IX stabilize hexon-hexon interactions (34). Four trimers of IX act as a sort of molecular adhesive to keep the nine central hexons of each icosahedral face in a single plane, promoting virion stability (49–51). Protein IIIa is important for virion assembly, interacting under the capsid with the penton base at each vertex as well as the

Figure 1.1 Cartoon diagram of HAdV virion. A) Enlarged diagram of one icosahedral facet (dark blue triangle), displaying the 12 hexon and 3 penton capsomers as well as the approximate locations of other minor structural proteins that support the major capsid proteins. B) Predicted locations and arrangement of structural proteins and DNA within the core of the capsid (TP = terminal protein; AVP = AdV protease). Adapted from San Martin, C., 2012 (52).

A.



B.



surrounding hexons of each face, essentially holding them together as a scaffold (34, 53, 54). Minor protein VI mediates destruction of the endosomal membrane during internalization, after the capsid has disassembled, and it serves a structural function by tethering the capsid to the protein-DNA core of the virion (39, 55).

Five proteins as well as the DNA genome comprise the virion core. Polypeptides V, VII, and μ are highly basic proteins which contact and condense the viral DNA within the core (56–58). The major core protein is polypeptide VII, which forms dimers that condense the viral DNA into repeating 90-150 base pair nucleoprotein complexes (59, 60). In addition to binding DNA, polypeptide V can bind the penton base and polypeptide VI, participating in the linkage of the core to the capsid (61). The terminal protein is covalently attached to the 5' ends of the viral genome, functioning in DNA replication as a primer for DNA synthesis (62). Finally, the p23 viral protease cleaves several proteins to allow escape from endosomes during infection, as well as several precursors of viral proteins during maturation of the virions (63, 64).

1.1.2 HAdV Genome

Many HAdV genomes have been completely sequenced to date, the first of which were those of HAdV-2, 5, and 12 (65–67). All contain approximately 36000 bp of linear, double-stranded DNA. The genome encodes five early (E1A, E1B, E2 early, E3, E4), three delayed-early (IX, IVa2, E2 late), and one major late transcriptional unit which are transcribed by cellular RNA polymerase II (68). Each of these transcripts is alternatively spliced, giving rise to multiple mRNAs (Figure 1.2). Furthermore, many alternatively spliced products of an individual transcriptional unit encode proteins with similar function. For example, two major E1A proteins – 13S and 12S – activate transcription of HAdV genes and force cells into S phase, and the late transcriptional units encode proteins involved in capsid production and assembly (4). The genome also encodes virus-associated RNAs (VA-RNAs) which are transcribed by RNA polymerase III (10). The ends of the genome contain inverted terminal repeats of 36 to over 200bp, which function as origins of replication (ORI) for DNA synthesis (10). *Cis*-acting packaging sequences

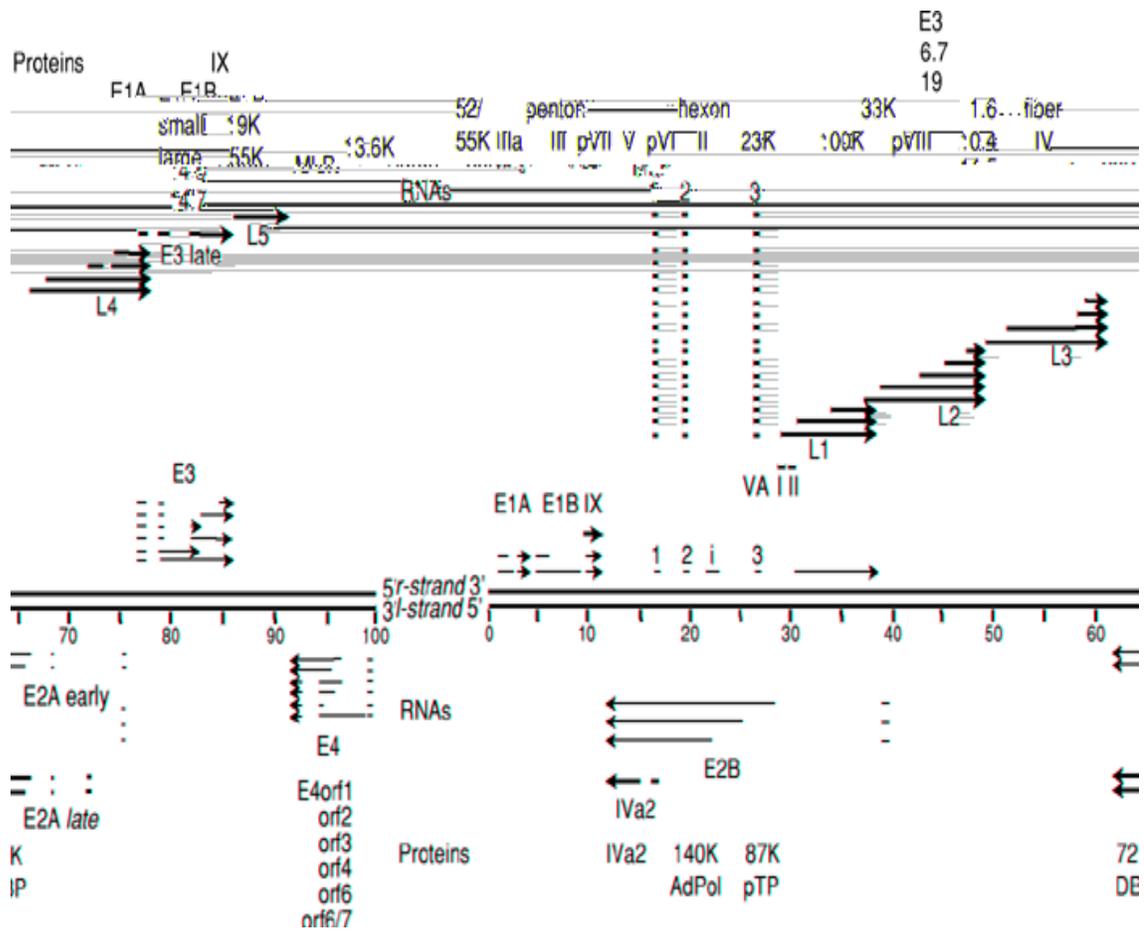
are present between the left terminal repeat and the E1A-coding region, and are essential for proper packaging of replicated DNA into new virions (69, 70).

The genomes of the closely related HAdV-2 and 5 have been extensively manipulated to create viruses which express proteins containing one or more mutations (71). As such, most studies utilize one of these serotypes for studies, including our laboratory which focuses on the study of HAdV-5. By utilizing the natural homologous recombination machinery of *Escherichia coli*, plasmids containing the entire genome of HAdV-5 with mutations of interest can be constructed (72). Plasmids can be amplified to large quantities, isolated, and transfected into HEK 293 cells, which express HAdV-5 E1A and E1B (25). Through transcription of viral genes and replication of viral DNA, viral progeny are created which lyse the cells, and can be plaque purified by further propagation in 293 cells. Many mutant viruses are not as viable as wild type HAdV-5 and thus the complementation of E1A and E1B in 293 cells – particularly for those with mutations in E1A or E1B – serves to increase viral production. In fact, the *dl312* (Δ E1A) strain of HAdV-5 – a common control virus for studying effects of E1A – grows as well as wild-type in these cells, whereas it cannot replicate efficiently in HeLa cervical carcinoma cells (73). Viruses purified from 293 cells can then be used to examine the effects of specific mutations on cellular and viral processes in cells of interest.

1.1.3 HAdV Replication Cycle in Human Cells

Like all viruses, HAdV are obligate intracellular pathogens and therefore must infect a host cell and manipulate the cellular machinery in order to replicate. Most studies of HAdV replication have traditionally been carried out in HeLa cells or their derivatives at high multiplicities of infection (MOI), ranging from 10 to over 200 plaque forming units (PFU) per cell (4, 73–76). HAdV grows very rapidly in these cells, and a high MOI ensures that all cells undergo synchronous infection, enabling the observation of consistently ordered events over time. In contrast, the replication cycle in primary cells, such as IMR-90 lung fibroblasts, is drastically slower (77, 78).

Figure 1.2 Transcription map of HAdV-5 genome. Genes are transcribed from both strands of the genome. Arrows indicate direction of transcription. Breaks in arrows indicate mRNA splice sites. Protein names are indicated above or below their respective coding mRNA. An untranslated tripartite leader sequence indicated by the labels 1, 2, and 3 is spliced onto mRNA transcribed from the major late promoter (MLP). The labelled “i” indicates a leader sometimes transcribed and spliced onto the 52/55K L1-mRNA, which produces a unique but nonessential 13.6kd protein. Adapted from Berk, A.J., 2007 (4).



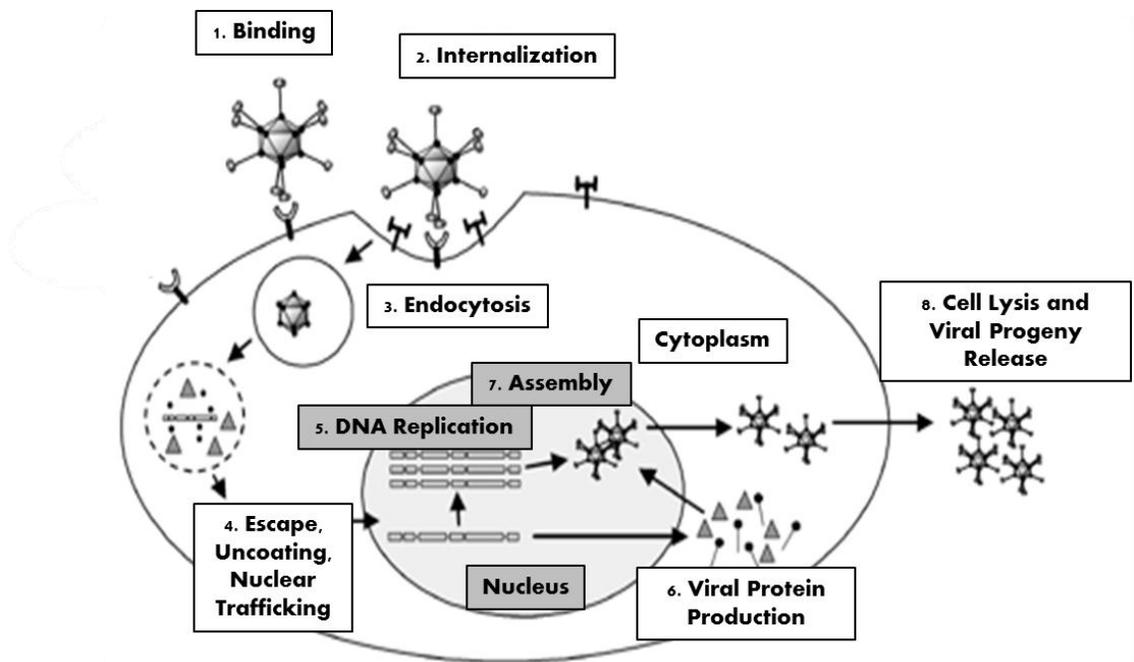
In general, the HAdV replication cycle exhibits an early phase and a late phase, which are separated by the initiation of viral DNA replication (68). Adsorption, entry, uncoating, translocation of viral DNA to the nucleus, and expression of early genes comprise the early phase. At an MOI of 10 in HeLa cells, DNA replication initiates at around six hours post-infection (HPI), at which point the late phase begins and late genes are expressed. This culminates in the production and release of virions, completing the lytic cycle at 24 to 36 HPI (79) (Figure 1.3). At high MOIs, infected cells are completely killed, and thus no further cycles of viral replication can be observed. It is important to note that although early and late phases are convenient categorizations of replicative events, they are not definitive time-points. The major late transcriptional unit is transcribed at low levels in the early phase, the delayed-early genes are expressed intermediately in infection, and the early genes continue to be expressed during the late phase (68).

1.1.3.1 Adsorption, Internalization, Uncoating and Nuclear Import

The information pertaining to viral replication in the proceeding subsections of 1.1.3 will focus on HAdV-2 and 5 – which are 95% homologous at the genomic level – as replication of other serotypes mimic these two prototypes (4, 65). The DNA encoding for the fibre protein accounts for 50% of the differences in HAdV-2 and 5, although both serotypes utilize the same receptor.

Attachment to the CAR is mediated by the knob domain of the fibre protein (80, 81). The receptor is abundantly expressed at epithelial cell tight junctions in most tissues (82, 83). After attachment the penton capsomers at the base of the fibre bind to type $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins through a conserved RGD motif found on the surface of each penton monomer (84). This causes detachment of the fibre, and promotes integrin clustering and activation, leading to downstream signalling of the phosphatidylinositol-3-OH kinase (PI3K) pathway (85, 86). The signalling pathway culminates in actin rearrangement, enabling endocytosis of a fibre-less virion via clathrin-coated vesicles (87–89).

Figure 1.3 HAdV-5 replication cycle in human cells. HAdV binds to the CAR receptor (horseshoe) via the knob domain of the fibre protein (1). Integrin (boxhead) binding and clustering stimulates receptor-mediated endocytosis (2, 3). Viral uncoating begins to occur as the subviral particle is released from acidified endosomes. The HAdV genome is shuttled to the nuclear pore complex (NPC) on microtubules, where final uncoating occurs as the genome is translocated into the nucleus (4). Early viral gene expression begins, and the host cell is forced into the cell cycle. Next DNA replication and late gene expression begins (5, 6). Viral proteins localize back into the nucleus, where they assemble with the viral genome into virions (7). Cell lysis occurs and new infectious HAdV virions are released (8).



It is currently accepted that clathrin-coated vesicles mature into endosomes, and become acidified. Acidification triggers permeabilization of the membrane and enables translocation of the virus to the cytosol (90–92). However, the mechanisms surrounding this process are not completely understood. In some studies it has been shown that a low pH endosomal environment is not required during an *in vivo* infection (93). The entire process of attachment, internalization, and escape from endosomes is very rapid, and occurs within 15 minutes of initial attachment (94). Partial uncoating of the viral capsid occurs concurrently with endosomal release, and then hexon proteins utilize cellular microtubules to shuttle the subviral core to nuclear pore complex (NPC) (80, 95, 96). At this point – approximately 2 HPI – the remaining proteins of the capsid disassemble at the NPC, and the viral DNA bound to major core protein VII is imported into the nucleus (97).

1.1.3.2 Transcription and Function of Early Genes

Early HAdV gene products are responsible for activating transcription of viral genes, inducing host cell cycle progression, initiating viral DNA replication, and blocking apoptosis and host anti-viral responses (4). The E1A transcriptional unit contains a constitutively active enhancer, and is the first viral gene expressed during infection (75, 98). E1A gene products are the primary trans-activators of the viral genome, and thus the functions of other early genes intimately depend on E1A. Due to the multiple and important functions of E1A, it will be discussed in detail in section 1.1.4.

The E1B transcriptional unit encodes two proteins identified by their molecular weights of 55 and 19kDa. They function to inhibit apoptosis, which would otherwise occur in response to the abnormal cell cycle progression induced by E1A and E4orf4 (99). E1B-55k uses several distinct mechanisms to inhibit E1A-induced p53 tumour suppressor trans-activation of genes involved in apoptosis and cell-cycle arrest. In cooperation with E4orf6 and cellular proteins, E1B-55k forms a large 800kDa ubiquitin ligase complex which binds p53, directing its polyubiquitination and consecutive proteosomal degradation (100–103). E1B-55k on its own can also bind p53 at genes containing p53-

binding sites, functioning to repress these genes (104, 105). E4orf4 induces p53-independent apoptosis through proteosomal degradation of the cellular antiapoptotic MCL-1 protein (106). E1B-19k acts as a viral MCL-1 mimic, which binds BAX and BAK, preventing mitochondrial outer membrane pore formation and release of cytochrome c, thereby inhibiting apoptosis (107).

The E2 transcriptional unit encodes three proteins involved in viral DNA replication: The terminal protein (TP), a 140kDa DNA-dependent DNA polymerase (AdDdDp) of the eukaryotic polymerase- α family, and a 59kDa single stranded DNA (ssDNA) binding protein (ssDBP) (108–110). The TP is post-translationally processed and covalently attached by AdDdDp to the first base – deoxycytidine monophosphate – at either of the 5' ends of the viral genome (111, 112). The TP functions to separate double-stranded DNA (dsDNA) at the ORI, allowing docking of the template strand into AdDdDp, for which TP then acts as a primer (112, 113). Like all DNA polymerases, AdDdDp contains 5' to 3' DNA polymerase activity, and like other polymerase- α family members, it contains 3' to 5' exonuclease activity for proofreading of DNA synthesis (114, 115). The ssDBP functions in chain elongation, driving strand separation through its polymerization on DNA, and replacing the function of DNA helicase in unwinding dsDNA (116). The ssDBP increases the processivity of AdDdDp, allowing complete strand synthesis without re-docking and re-initiation.

The E3 transcriptional unit encodes proteins involved in antagonizing the adaptive cytotoxic T lymphocyte (CTL) response, and the innate apoptosis-inducing cytokine response (117). These include: E3-19K, 14.7K, 14.5K, 10.4K, 6.7K, and adenovirus death protein (ADP/E3-11.6K). E3-19K is a transmembrane protein that localizes to the endoplasmic reticulum (ER) in infection (118). It binds the major histocompatibility complex (MHC) class I peptide-binding groove and through an ER-retention signal, prevents MHC class I from reaching the surface of the host cell (119, 120). In addition it can bind TAP – a protein which loads processed cytosolic peptides onto the MHC class I molecule in the ER – and prevent MHC class I peptide docking (121). Since lysis of an infected cell by CTLs requires antigen presentation, E3-19K can effectively inhibit adaptive immunity. Two E3-10.4K and one E3-14.5K comprise the viral transmembrane

receptor internalization and degradation (RID) complex, which causes endocytosis and subsequent lysosomal degradation of the Fas (CD95) and tumor necrosis factor (TNF)-related apoptosis-inducing (TRAIL) receptors (122–124). These receptors bind Fas ligand on CTLs and TRAIL cytokines secreted by immune cells and subsequently induce apoptosis, and therefore degradation protects from cell death. The transmembrane E3-6.7K protein assists the RID complex in protection against Fas ligand and TRAIL, as well as TNF- α (124, 125). The E3-14.7K protein is found in the cytosol, and inhibits TNF- α , TRAIL, and Fas ligand-induced apoptosis by binding the downstream FLICE caspase and inhibiting its function (126–128). It can also bind members of the I κ B kinase (IKK) complex, blocking nuclear factor kappa B (NF- κ B) activation, and preventing expression of several TNF- α induced chemokines important for immune chemotaxis (129). Contrary to the other E3 proteins, ADP is expressed late during infection (130). It contributes to lysis of cells as it accumulates, through a currently unknown mechanism (131).

The E4 transcriptional unit encodes proteins with a wide array of functions, which are named after their respective genomic open reading frames (ORFs). As previously discussed, E4orf6 cooperates with E1B-55k to inhibit p53-dependent apoptosis, but can also inhibit p53-dependent transcription independently (132). In addition, the E4orf6/E1B-55k 800kDa complex and E4orf3 act to prevent the cellular DNA damage response pathway from acting on the viral genome (133). They function by interfering with the activity and causing the eventual degradation of Mre11-Rad50-Nbs1 (MRN) complexes, which would otherwise recognize the ends of linear HAdV genomes as double-stranded breaks (134). This would result in concatenation of viral genomes by the nonhomologous-end-joining pathway, preventing proper viral DNA replication and subsequent packaging (133, 135, 136). Finally, the E4orf6/E1B-55k 800kDa complex acts to block cellular mRNA export to the cytoplasm, and promote viral late mRNA export to the cytoplasm through the Nxf1/Tap export receptor after the onset of viral DNA replication (137–141). This results in the inhibition of cellular protein synthesis, and promotes viral protein synthesis. E4orf1 and E4orf4 function to activate the protein kinase mammalian target of rapamycin (mTOR) – and thus translation – through distinct pathways (142–145). Activation of mTOR normally occurs in response to nutrient and mitogen signalling, and is required for entry of the host cell into S phase (146). mTOR

phosphorylates p70^{S6K} which in turn phosphorylates ribosomal protein Small 6 (RPS6), leading to a high rate of protein synthesis and allowing mRNA of several cell-cycle regulators such as cyclins to be translated (147–149). Finally, the E4orf6/7 protein binds the E2F transcription factor, and recruits it to the E2 early promoter to provide E1A-independent and E1A-cooperative transactivation of E2 genes (150, 151).

HAdV-5 encodes two VA-RNAs – VA-RNA_I and VA-RNA_{II} – which are transcribed by RNA polymerase III in the nucleus and form stable secondary structures important for their function (152). They act to antagonize host cell viral countermeasures. They are transcribed in the early phase, and reach extremely high levels in the late phase of infection. Both types competitively inhibit cellular micro RNA (miRNA) export and also subsequent processing by Dicer (153, 154). Instead, VA-RNAs are processed by Dicer and then overwhelm the RNA silencing complexes (RISC), which would otherwise seek out and degrade viral mRNA (155). Since VA-RNAs are transcribed from intronic regions in the viral genome, the actions of RISC will only act on subsequently transcribed VA-RNA genes, with no effect on viral fitness (153). In addition, VA-RNA_I binds and inhibits protein kinase R (PKR), which is synthesized in response to interferon α and β (IFN- α , IFN- β) production after viral infection (156, 157). Without this antagonization, PKR would recognize the dsRNA transiently formed by transcription of both strands of the viral genome, and phosphorylate eukaryotic initiation factor-2- α (eIF-2 α), leading to non-specific inhibition of total protein synthesis (158).

1.1.3.3 DNA Replication, Viral Assembly, and Release

Viral genome replication begins when enough E2-encoded proteins have been synthesized to support DNA synthesis. In HeLa cells infected at an MOI of 10, DNA replication occurs between 5 to 8 HPI, and continues until the cell is lysed (4).

There are two stages to HAdV DNA replication. The first stage involves replication of a single parent strand. Replication initiates from either end of the genome at the previously described terminal repeats, which act as ORIs (10, 159). This process displaces the

complementary parent strand, and therefore the first stage ends with a dsDNA genome, and a displaced ssDNA parent strand. The second stage involves replication of the displaced parent strand, however the AdDdDp requires dsDNA and an ORI for DNA replication initiation to occur. The inverted terminal repeats present at each end of the AdV genome alleviate this issue by looping around and basepairing to each other to create a duplex DNA “panhandle”, which is utilized as an ORI by AdDdDp (159).

The late genes are expressed at the onset of viral DNA replication, and many of the proteins they encode were discussed at length in section 1.1.1. They encode proteins involved in virion packaging and assembly (4, 52). Two events are proposed to be responsible for the delayed transcription of late genes. First, a currently unknown change in the viral genome occurring only at the onset of DNA replication allows binding of the USF/MLTF transcription factor to the major late promoter (MLP) (160, 161). Second, the MLP contains a binding site for a transcription factor encoded by the delayed early gene IVa2, which acts in cooperation with USF/MLTF to relieve repression of the MLP (162). Viral structural proteins translocate to the nucleus where final viral assembly occurs (4, 52). Finally, the cell is lysed and at an MOI of 10, around 10,000 viral progeny are released from a single HeLa cell (79).

1.1.4 HAdV E1A Functions and Interactions with Cellular Factors

As discussed, E1A is the first viral gene expressed during infection and is essential for viral replication (75, 163). The various E1A proteins modulate viral and cellular transcription, induce host cell cycle progression to S phase to create an optimal environment for viral replication, and inhibit host anti-viral responses through a variety of intricate mechanisms.

The E1A gene encodes five proteins, termed 9S through 13S based on the sedimentation coefficients of their respective alternatively spliced mRNAs (Figure 1.4). 13S (289 residues) and 12S (243 residues) are the main products expressed early during infection, and are the best characterized (164). They differ only by a 46 amino acid sequence found

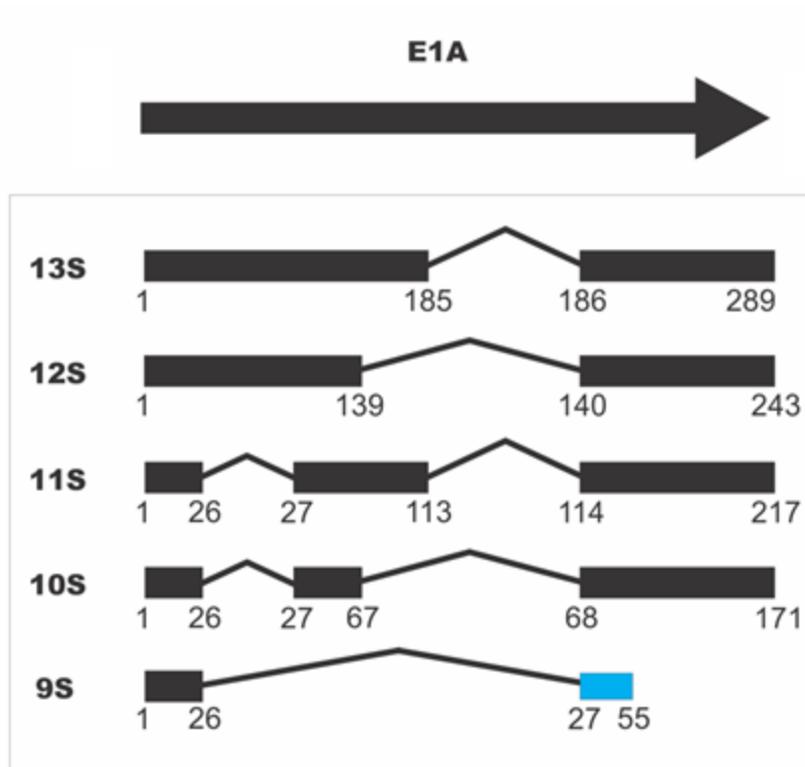
in 13S but not 12S. 11S, 10S, and primarily 9S are expressed later in infection, however their functions are not well understood (165). Recently Miller *et al.* showed that 9S E1A could independently activate expression of viral genes, and promote HAdV replication, and that replication was dependent on an interaction of 9S with the S8 component of the proteasome (77). This is intriguing as only 28 amino acids are common between 9S and the larger forms of E1A.

The structure of E1A has not been solved, proving a difficult process as it is an intrinsically disordered protein (166). However, amino acid sequence alignment of 13S E1A from various serotypes reveals four main conserved regions (CR1-CR4), which are separated by less conserved regions (167, 168). CR3 – the region unique to 13S – contains four cysteines predicted to form a zinc-finger domain, although the structure is still undetermined (164, 169). In addition, residues 16-28 of the HAdV-5 E1A are predicted to form an amphipathic α -helix (170). E1A proteins undergo extensive post-translational phosphorylation at several serine residues, although phosphorylation has little effect on E1A function (171, 172). Finally, a KRPRP nuclear localization sequence (NLS) is found at the C-terminus of E1A proteins, accounting for their nuclear localization (173).

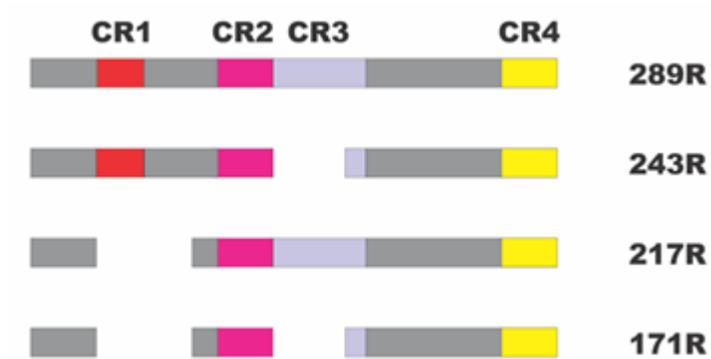
Since E1A proteins have no enzymatic or DNA binding ability, they rely on manipulation of cellular factors to exert their effects (174). The CRs – as well as other areas – participate in important protein-protein interactions with over 50 cellular factors through independently acting short linear interaction motifs (LIMs) (166). The identification of these motifs has been important to HAdV's use as a molecular tool, as many of these linear interaction motifs are also found in cellular factors that bind the same proteins as E1A. Thus, in addition to studying the function of E1A-interacting proteins through mutational studies, their cellular binding partners can also be elucidated through bioinformatic searches for proteins containing the same motifs. For example, both the retinoblastoma tumor suppressor protein (pRb) and the transcriptional co-repressor C-terminal binding protein (CtBP) were discovered via interactions with E1A (175, 176). The linear motifs LXCXE in CR2, and PLDLS in CR4 – used by E1A for interactions

Figure 1.4 Diagram of E1A splice products and proteins. A) The primary E1A transcript is spliced into 5 different isoforms. Boxes indicate coding regions, which are separated by bent lines representing introns. Numbers indicate amino acids present in the translated protein. All proteins besides that encoded by the 9S mRNA are translated within the same reading frames, resulting in identical proteins except for the indicated deleted regions. The splicing of the 9S mRNA results in a coding region that is translated in a different frame than the other four isoforms (indicated by the blue box). B) Alignment of 13S (289R), 12S (243R), 11S (217R), and 10S (171R) proteins and location of conserved regions (CR) 1-4. 9S does not retain any of the conserved regions.

A.



B.



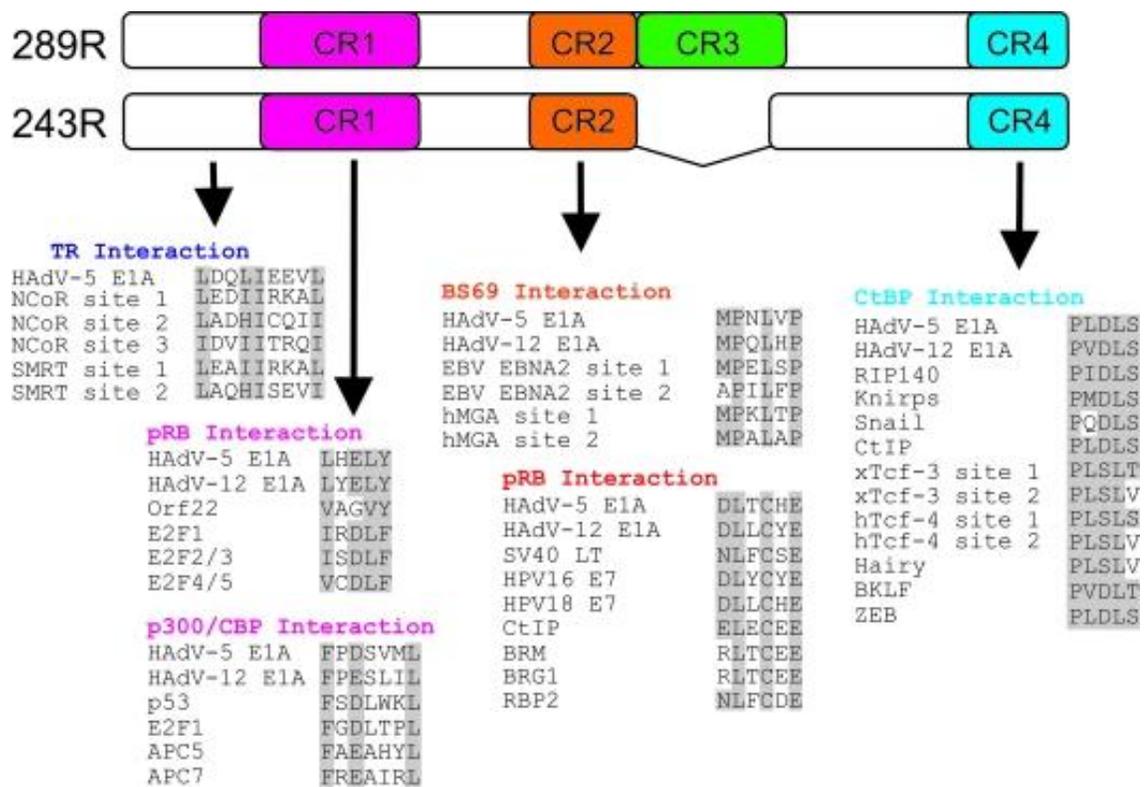
with pRb and CtBP respectively – were subsequently identified in cellular factors later shown to also bind pRb or CtBP (Figure 1.5).

1.1.4.1 Modulation of Viral and Cellular Transcription

E1A modulates transcription of a large portion of the greater than 17000 cellular promoters it is recruited to during infection (177, 178). Through various interactions with cellular sequence-specific DNA-binding transcription factors, or the proteins that regulate them, E1A proteins act as modulators of cellular and viral gene expression (179).

13S E1A is the primary trans-activator at viral early promoters, which all contain TATA-boxes as well as upstream binding sites for cellular trans-activators (180, 181). HAdVs with complete knockout of 13S E1A or mutations in CR3, experience drastically reduced rates of early gene transcription (73, 182). This is because CR3 functions as a potent trans-activator by binding the DNA-binding domains of transcription factors at early HAdV promoters through its C-terminal 10 amino acids, and recruiting transcriptional regulators through its N-terminal zinc-finger domain (183, 184). CR3 binds the MED23 subunit of the Mediator (MED) complex, recruiting this co-activation complex to promoters (185, 186). This interaction is essential for CR3 trans-activation, stimulating the formation of a pre-initiation complex (PIC) – containing RNA polymerase II and the general transcription factors – on promoter DNA (185, 187, 188). Thus, through interactions with MED23, CR3 can aid in recruitment of sequence-specific transcription factors, and complexes essential for initiation of transcription at early viral genes. Interestingly, CR3 interacts with many other transcriptional activators, such as TATA-binding protein (TBP), and the p300/CREB-binding protein (CBP), but also with repressors such as GCN5 and BS69, indicating that CR3 modulation of early gene transcription is complex (189–192). The N-terminus of E1A (residues 1-41) and CR-1 together are also required for optimal early gene transactivation (193, 194). They have been shown to interact with a variety of trans-activators including histone acetylases p300/CBP and p300/CBP-associated factor (pCAF), and TBP, as well as repressors such as the p400 chromatin remodelling complex (166, 195, 196). The exact mechanisms by

Figure 1.5 Cartoon diagram of several linear interaction motifs in HAdV-5 E1A. Locations of several LIMs in E1A are denoted by arrows. The sequence of each LIM is indicated below the respective protein it interacts with. Viral and cellular proteins which contain a similar LIM as E1A, and are known to interact with the same LIM target, are listed below the HAdV-5 LIM. Amino acids highly similar to the consensus LIM binding sequence are shaded in grey. Adapted from Pelka et al., 2008 (166).



which the N-terminus and CR-1 exert their effects on viral early genes are relatively unknown.

12S E1A primarily activates transcription at the E2 early promoter, which contains two E2 transcription factor (E2F)-binding sites (197). It can do so indirectly by freeing bound E2Fs from Rb family members pRb, p107, and p130 (198). Interestingly, the E2Fs are named after their involvement at the E2 promoter, and the pRb proteins were originally identified as E1A-interacting proteins (197, 199). Free E2F subsequently interacts with the E4orf6/7 dimer as discussed previously, increasing E2F affinity for its binding sites in the E2 early promoter (150, 151). In addition, cellular genes – including those required for entry into S phase – containing E2F-binding sites become constitutively activated when E2F is released (198). The liberation of E2Fs from pRb is an important event leading to activation of host cell cycling, and therefore will be discussed more in detail in section 1.1.4.2.

In addition to modulating early gene transcription, and enabling transactivation of E2F-responsive genes, E1A plays a complex role in modulating expression of other cellular genes. Simply stated, E1A recruits and is recruited by activators such as p300/CBP, or repressors such as pRb and CtBP to do so (177, 178). However, its effects are much more complicated. E1A can be recruited by activators such as p300 to active genes, upon which it can then recruit repression complexes to turn expression of those genes off (177, 200). The reverse can also occur, leading to activation of previously inactive genes. One example of this is the interaction of E1A with the CtBP co-repressor. Classically, E1A interacts with CtBP through a conserved PLDLS motif in CR4 (175). This removes CtBP from sequence specific transcription factors, relieving repression of cellular genes. However, CR3 was also recently found to interact with CtBP and through this interaction, direct itself to CtBP occupied promoters (201). CtBP repression is alleviated through E1A binding, and the presence of CR3 then likely causes hyper-activation at these promoters. Although the specific manners in which E1A affects all of its cellular targets are not known, the result of E1As modulation of transcription is a host cell that is optimal for viral replication.

1.1.4.2 Activation of Cell Cycle

The ability of HAdV to stimulate host cell entry into S-phase allows the virus to productively infect G₀-arrested cells found at the sites of infection. It allows for the efficient synthesis of viral DNA and proteins, and therefore replication. Whereas 13S plays a larger role in modulating transcription due to CR3, 12S may primarily promote cell cycling. HAdV mutants expressing only 13S display drastic reductions in viral replication in G₀-arrested cells, owing largely in part to reduced viral DNA synthesis (82, 202).

E2F transcription factors exist as heterodimers of either DP-1 or DP-2 and one of several E2F subunits. They regulate transcription of genes essential for cell cycle progression, such as: DNA polymerase α , cyclin dependent kinase (CDK) 2, cyclin A, and cyclin E (203). pRb proteins directly bind most E2Fs, repressing E2F activation by recruiting histone deacetylase and methyltransferase complexes to E2F-responsive promoters and inhibiting formation of the PIC (204–206). This effectively keeps cells in G₀/G₁. In response to extracellular division signals and mitogens, the G₁ CDKs (cyclin D-CDK4/6 and cyclin E-CDK2) normally phosphorylate and inactivate pRb, relieving E2F-dependent gene repression, and facilitating entry into S phase (205). Both 13S and 12S E1A can bind pRb through CR1, and more importantly, through an LXCXE motif in CR2 (4). This motif binds to the Rb “pocket” domain to displace E2F, bypassing the normal cell cycle regulatory signals, and thereby forcing cell entry into S-phase due to constitutive activation of E2F-responsive promoters (198, 207). The interaction between E1A and pRb was the first example discovered of an oncoprotein interacting with a tumour-suppressor (199). Besides direct binding to pRb, E1A can antagonize CDK inhibitory proteins (CKI) such as p21, which help to keep the cell in G₁ (208). This results in overactive CDKs and thus hyperphosphorylated pRb, which cannot inhibit E2F function. Finally, it was recently shown that E1A can bind to E2F complexes independently of pRb through DP-1, and cooperate with E2F in transactivation of E2F-responsive genes to force cells into S-phase (78).

E1A mutants unable to bind pRb still retain the ability to induce an S-phase transition in host cells, through the N-terminus/CR-1, and therefore other known and unknown targets of E1A must be influencing host cell cycling (195, 209). Recently it was found that the N-terminus/CR1/CR3 interacting protein p300 is removed by E1A from promoters of genes involved in differentiation, and re-directed to promoters involved in the cell cycle (177, 178). Since p300 is an activator that causes local hyperacetylation, the interaction may contribute to expression of genes involved in the G₁-to-S-phase transition (210). Although we have a generally good idea as to how E1A activates the cell cycle, there are still many potentially unknown targets of E1A that may play a role in this process.

1.1.4.3 Subversion of the Host Anti-Viral Response

Although the main functions of the E1A proteins are to modulate viral and cellular transcription, and induce host cells to enter the cell cycle, it is important to note that E1A can also antagonize anti-viral responses. For instance: early after infection, E1A binds the signal transducer and activator of transcription 1 (STAT1) transcription factor through the N-terminus/CR1, blocking its function (211). STAT1 normally binds to p300/CBP and IFN-regulatory factor 1 (IRF1) in response to IFN- α and β signalling due to viral infection (212). This interaction results in expression of anti-viral IFN-stimulated genes (ISGs) such as PKR (213). Therefore E1A attempts to preclude the production of anti-viral factors by blocking IFN signalling. In addition, our lab recently showed that E1A can bind the human Brefeldin A sensitivity 1 (hBre1) ubiquitin ligase and prevent its monoubiquitination of histone 2B (H2B) (214). This modification is necessary for expression of ISGs, and therefore E1A subverts ISG production. Furthermore, Fonseca *et al.* revealed that E1A re-directs hBre1 from cellular promoters to viral promoters, and utilizes it to recruit human polymerase II-associated factor 1 (hPaf1) – an RNA polymerase II elongation factor – which increases transcription from viral early genes (215).

1.2 Discoveries in *Drosophila* – an Essential Model System

Since the early 1900's *Drosophila melanogaster* has played a central role in our understanding of the fundamentals of biological processes, particularly at the genetic and molecular level (216). As a small multicellular organism with a short life cycle, rapid reproduction schedule, and development stages which parallel those of humans, *Drosophila* acts as an appropriate model system for scientific discovery (216, 217). For example, T. H. Morgan and his students – the first to consider use of *Drosophila* for genetic studies – used them to discover fundamental concepts such as: recombination of homologous chromosomes, chromosomal sex determination, and the basic realization that genes are arranged linearly on chromosomes (216).

With advances in molecular techniques, *Drosophila* became the first complex multicellular organism to be used for genetic screens of genome-wide mutations affecting biological processes in the 1970s (216). Mutations in the *trp* gene encoding an ion channel gave us insight into sensory and neural processes (218–221). Mutations in the *dunce*, and *rutabaga* genes provided insight into the role of cyclic AMP in memory and behavioural processes (222, 223). Most of our knowledge of how genes regulate the organization of tissues and differentiation of cells within the human embryo is gained from analyses of their mutational effects on *Drosophila* embryo development (224). Indeed, many members of the Hedgehog signalling pathway including Patched, Smoothed, and Hedgehog itself – which plays a key role in embryonic development across all species, and regulates tissue growth in adult vertebrates – were discovered in *Drosophila* mutational screens (225).

It is currently estimated that 60 to 80 percent of genes causally linked to disease in humans have orthologues in *Drosophila* (226, 227). Mutations in *Drosophila patched* were found to alter the differentiation of cells in the epidermis of the embryo (217). Consequently, gene-mapping studies identified that germ-line mutations in the human orthologue of *patched* occur in Gorlin's syndrome – which predisposes an individual to basal-cell carcinoma – and somatic mutations in *patched* are apparent in most cases of

sporadic basal-cell carcinoma (228, 229). Furthermore, the Hedgehog signalling pathway has been implicated in mediating growth of digestive tract tumours (230, 231). In addition to examining functional orthologues of human genes in *Drosophila*, many proteins associated with a disease state in humans can be expressed in the fly to mimic the disease phenotype, thereby providing a model for study of that disease. This approach to modelling disease has been successfully employed to study three major neurodegenerative diseases: Parkinson's, Alzheimer's, and Huntington's (232–234).

One of the most powerful uses of *Drosophila* mutational genetic screens today is through “modifier screens” (216, 235). These screens involve a phenotype caused by a known gene of interest, and search for enhancers which worsen the severity of the phenotype, or suppressors which lessen the phenotype. Most mutations will have no effect on the phenotype, indicating no interplay between the mutated gene and the gene of interest (217). In this respect they can identify gene products which play a role in the gene of interest's molecular pathway, while also further expanding knowledge of the function of the gene of interest. For example, overexpression of kinase suppressor of Ras (KSR) in eye imaginal discs causes a rough eye phenotype (REP) in the adult eye (235). A screen of KSR-induced REP flies with flies carrying random mutations generated around 185000 mutated progeny and led to the identification 15 enhancer genes, and 4 suppressor genes, 10 of which were known components of the Ras1 signalling pathway. This helped to support prior biochemical evidence that KSR was a putative protein kinase functioning downstream of RAS signalling pathways. Similar studies have been used to further our understanding of *Hairless* functions in the Notch pathway, through its overexpression-induced REP (236).

Studies utilizing *Drosophila* as a model organism have been paramount to our understanding of the fundamentals of genetics and molecular biology. They have given us insight into conserved biological processes surrounding diverse systems such as neural, behavioural, and developmental pathways amongst many living species. In addition they can be used as disease models, and through modifier screens, they will continue to provide valuable insight into unresolved molecular pathways well into the

future. The main focus of this thesis, the DNA replication-related element-binding factor (DREF) was in fact discovered and first characterized in *Drosophila* (237).

1.2.1 Discovery of *Drosophila* DRE and DREF

Many proteins involved in DNA replication – such as DNA polymerase α and proliferating cell nuclear antigen (PCNA), which are intimately involved in DNA elongation – are expressed at high levels in proliferating undifferentiated cells, and decrease when differentiation begins (238–240). In 1993, comparison of the PCNA and DNA polymerase α 180kDa subunit promoters in *Drosophila* revealed a common palindromic 8bp sequence (5'-TATCGATA) termed the DNA replication-related element (DRE), which is required for their transcription in both transgenic flies – including embryos and larvae – and cultured *Drosophila* embryonic cells (237, 241–244). Through subsequent electrophoretic mobility shift/band shift assays (EMSA) and then cloning of its complementary DNA (cDNA), *Drosophila* DREF (dDREF) was discovered and initially characterized. dDREF is a 709 amino acid (86kDa) protein, which binds the DRE as a homo-dimer (237, 245). Early studies revealed that mRNA expression of dDREF was temporally similar to the expression patterns of PCNA and DNA polymerase α , and that dDREF protein was found in the nucleus. Although only 71% identical, three conserved regions between *Drosophila virilis* and *D. melanogaster* termed CR1 (14-182; containing many basic residues), CR2 (432-568; containing many prolines) and CR3 (636-730; containing many acidic residues) were discovered. The locations of the conserved regions correspond to their respective positions in the slightly longer *D. virilis* 742 amino acid dDREF protein. CR1 contains a boundary element-associated factor and DREF (BED) zinc finger domain from residues 16-115 essential for homo-dimer formation and DNA binding (237, 246).

1.2.2 Characterization of DRE/DREF Function

Since the initial discoveries of DRE/dDREF involvement in regulating transcription from the PCNA and DNA polymerase α promoters, DREs have been identified in the promoters of over 150 *Drosophila* genes, 61 of which carry the DRE within 600bp upstream of the transcription initiation site (247). DREF transactivation has been linked to expression of additional proteins such as: the DNA polymerase α 73kDa subunit, E2F, DNA primase, raf, ras2/rop, TBP, cyclin A, Orc 2, Orc 5, RFC140, SkpA, *moira*, *osa* and dDREF itself (241–243, 248–259). Common amongst these proteins are their involvement in DNA replication, transcriptional and cell cycle regulation, chromatin remodelling, signal transduction for growth, and protein metabolism. Decreases in levels of functional dDREF through dominant negative mutations or RNA interference in larvae results in: decreased mRNA expression of dDREF-responsive genes, decreased DNA endoreplication in endocycling salivary glands, and decreased DNA replication in mitotic eye imaginal discs leading to an inhibition of G₁-S phase progression, thereby demonstrating its requirement for the transition of these cells through the cell cycle (260, 261). In contrast, overexpression of dDREF in cells that have begun differentiating causes ectopic DNA replication, leading to apoptosis (256). Taken together, most evidence points to the DRE/dDREF system as being a master regulatory system for genes intimately involved in cellular proliferation, which gets shut off at the onset of differentiation (247, 262). However, as is the case with many biological systems – and to complicate matters further – there are exceptions to the rule. Knockdown of dDREF results in irregular vein formation in wing imaginal discs through impaired EGFR signalling, implicating dDREF in the regulation of some differentiation processes. This is thought to be due to the dependence of the raf gene on dDREF for transactivation (263).

1.2.3 Members of the DREF Interactome

The DRE/dDREF system's interaction network stems far beyond the genes described above. The utilization of modifier screens described in section 1.2, as well as yeast two

hybrid screens and co-immunoprecipitation techniques has led to the discovery of many cellular factors which positively or negatively affect dDREF function at the genetic or physical level.

1.2.3.1 Interactions with Positive Effects on DREF Function

In addition to the E2F gene being dDREF responsive, several dDREF responsive genes involved in cell proliferation including: DNA polymerase α 180kDa and 73kDa, raf, and PCNA, contain E2F as well as DRE sites. Therefore, the increase in E2F induced by dDREF may reciprocally act to increase transcription of dDREF-responsive genes (241, 256, 264). TBP-related factor 2 (TRF2) forms complexes with dDREF and the nucleosome remodelling factor (NURF) chromatin remodelling complex, as well as the basal transcription machinery including transcription factors IIA and IIB. This serves to activate transcription of genes distinct from those activated by TBP and TRF1 including PCNA and DNA polymerase 180kDa (265–267).

1.2.3.2 Interactions with Negative Effects on DREF Function

Drosophila Mi-2 normally functions in an ATP-dependent manner to selectively deacetylate histones at homeotic genes (268, 269). Mi-2 can also bind to the DNA-binding domain of dDREF, inhibiting dDREF from functioning (270). Reciprocally, this binding may also inhibit Mi-2's function as a histone deacetylase. Similarly, boundary element associated factor of 32 kDa (BEAF-32) acts to insulate promoters from the effects of distant regulatory elements, and prevent the spread of open and closed chromatin states by binding to the boundary element sequence 5'-CGATA – a sequence found within the DRE (271). dDREF and BEAF-32 antagonize each other and compete for binding at these sites, and thus dDREF may remove BEAF-32 – or vice versa – to shift genes into active or repressed states, modulating transcription (272). *Drosophila* XNP/dATRX is a chromatin remodelling factor which contains a SWI/SNF-like

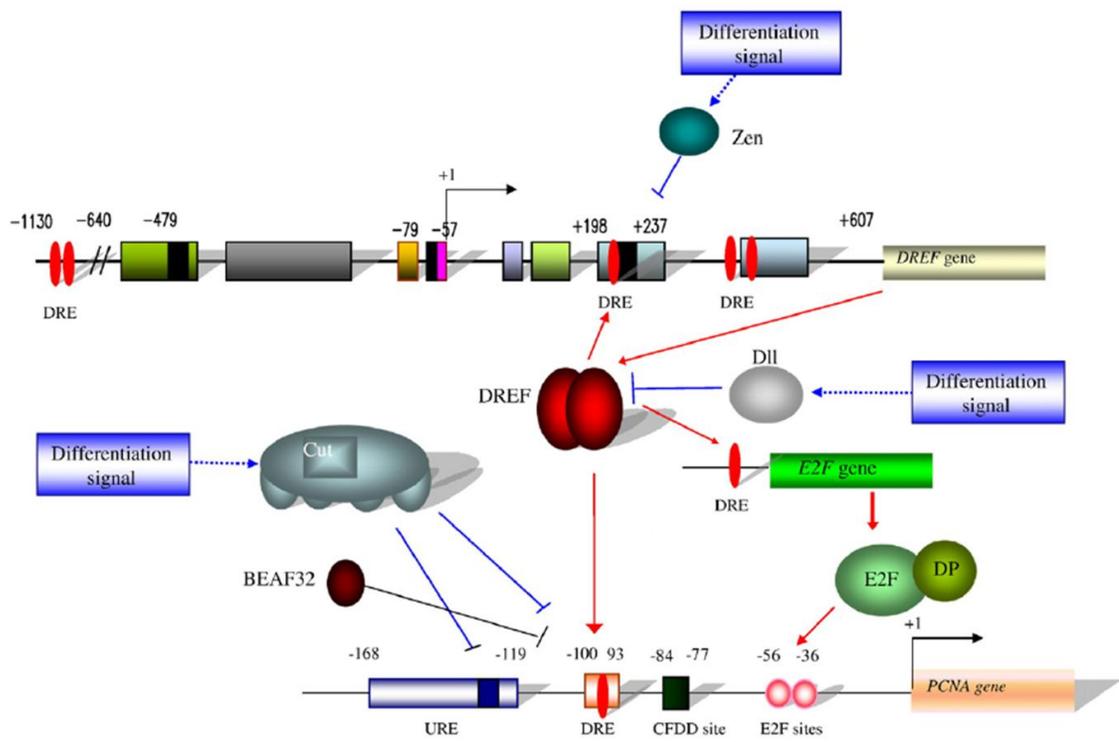
ATPase/helicase domain, but lacks a chromatin binding domain, and therefore relies on recruitment to chromatin through physical interactions with other proteins (273). Recently it was found that XNP/dATR_X binds dDREF at DRE sites and selectively represses transcription of certain dDREF-responsive genes but not others – including E2F, and *osa*, but not PCNA.

1.2.3.2.1 Homeodomain-Containing Proteins

Three *Drosophila* proteins harbouring homeodomains which all convey negative effects on dDREF activity deserve special mention (Figure 1.6). Proteins containing homeodomains act as transcription factors that regulate target genes in a highly regulated temporal and spatial pattern (274). Distal-less (Dll) provides the initial signal for limb formation (275). *Zerknullt* (Zen) is involved in differentiation of certain dorsal tissues in the *Drosophila* embryo, including the optic lobe and amnioserosa (276). Cut expression initiates differentiation of external sensory organs of the peripheral nervous system in embryos, and its expression is maintained in these differentiated cells in adults (277–280). It also determines the specificity of many cells in the central nervous system and ovarian follicles and continues to be expressed in these tissues in adult flies (281). DII binds directly to the DNA binding domain of dDREF, inhibiting dDREF's transactivation ability (275). Zen acts directly at the dDREF promoter, repressing expression of dDREF (282). Cut recognizes and binds with high affinity to all 8bp of the DRE, antagonizing dDREF binding in differentiated cells. It has been shown to repress expression of PCNA and likely does so for other dDREF-responsive genes (277).

As detailed above, extensive studies in *Drosophila* have revealed many genes which are regulated by the DRE/dDREF system, as well as many factors which act to positively or negatively affect dDREF function. The necessity of dDREF for the expression of many genes involved in DNA replication, transcriptional and cell cycle regulation, and protein metabolism indicates that dDREF may act as a master regulator of cellular proliferation. Furthermore, interactions of dDREF with various chromatin remodelling proteins indicates that dDREF may regulate transcription through altering the accessibility and

Figure 1.6 Complex interplay of proliferation and differentiation signals at dDREF responsive genes. dDREF positively regulates several genes related to cell proliferation including E2F, PCNA and dDREF itself. In contrast, homeodomain proteins such as Cut, Zen, and DII which are upregulated in response to differentiation signals can negatively regulate these genes. Zen directly suppresses dDREF expression. DII binds the DNA-binding domain of dDREF, inhibiting its function. Cut competes with dDREF for DRE binding. BEAF32 – although not a homeodomain protein – can also compete for DRE binding, repressing gene expression. Adapted from Matsukage et al., 2008 (262).



conformation of DNA to other transcriptional machinery. Finally, the effects of homeodomain-containing proteins on the DRE/dDREF system provide insight on the repression of dDREF-responsive cell proliferation genes at the onset of differentiation.

1.3 Human DREF

The *Drosophila* DRE/dDREF system was the first and only of its kind to be described, until approximately 10 years ago when a mammalian counterpart was discovered in humans. Since its discovery, only three papers have been published on the human DRE/DREF system, with the most recent being in 2007. Notably, all three are from the same corresponding author – Dr. Fumiko Hirose – in Japan, who discovered *Drosophila* DREF in 1993.

Independently, Esposito *et al.* discovered the gene encoding what we now know as hDREF in 1999, but called it *Tramp* (283). They localized the *Tramp* gene to the pseudoautosomal region 1 (PAR1) area of the X and Y chromosomes, and revealed that the gene encodes a protein with amino acid sequence similarity to the *hAT* family of transposases, for which it was the only member discovered at the time in the human genome. Members of the *hAT* family of transposases share functional and structural characteristics with McClintock's *Activator* in maize, including *Drosophila* hobo, housefly Hermes, and snapdragon Tam3 (284). They discovered that *Tramp* contains all the regions necessary to be an autonomous transposon – two terminal inverted repeats (TIRs) and a transposase – and therefore they proposed that the *Tramp* gene originated as an ancient transposon. However, functional analysis of the Tramp protein revealed an inability to recognize the TIRs of its own gene, indicating that *Tramp* may be a non-autonomous transposon encoding a transposase which recognizes different TIRs. In this respect it may have played a role in acquisition of transposable elements in the human genome, excluding itself. Esposito *et al.* also note that the Tramp protein's amino acid sequence does in fact show similarity with that of *Drosophila* DREF, but they do not

examine any potential biological activity of the protein as a transcription factor similar to dDREF.

1.3.1 Discovery and Characterization of hDREF

Human DNA replication-related element-binding factor (hDREF) was originally identified in 2003 by Ohshima *et al.* by utilizing a BLAST search for proteins sharing sequence similarity with dDREF (285) (Figure 1.7). CR1 of dDREF is 27.7% identical, with 48.1% amino acid similarity in humans. Further analysis revealed that the slightly smaller 694 amino acid (80kDa) human DREF shares an overall 21.3% amino acid sequence identity, and 41.0% similarity with dDREF. CR2 of dDREF shares 29.2% identity with 46.1% similarity, and CR3 shares 21.1% identity with 51.9% similarity to the respective corresponding regions of hDREF (285).

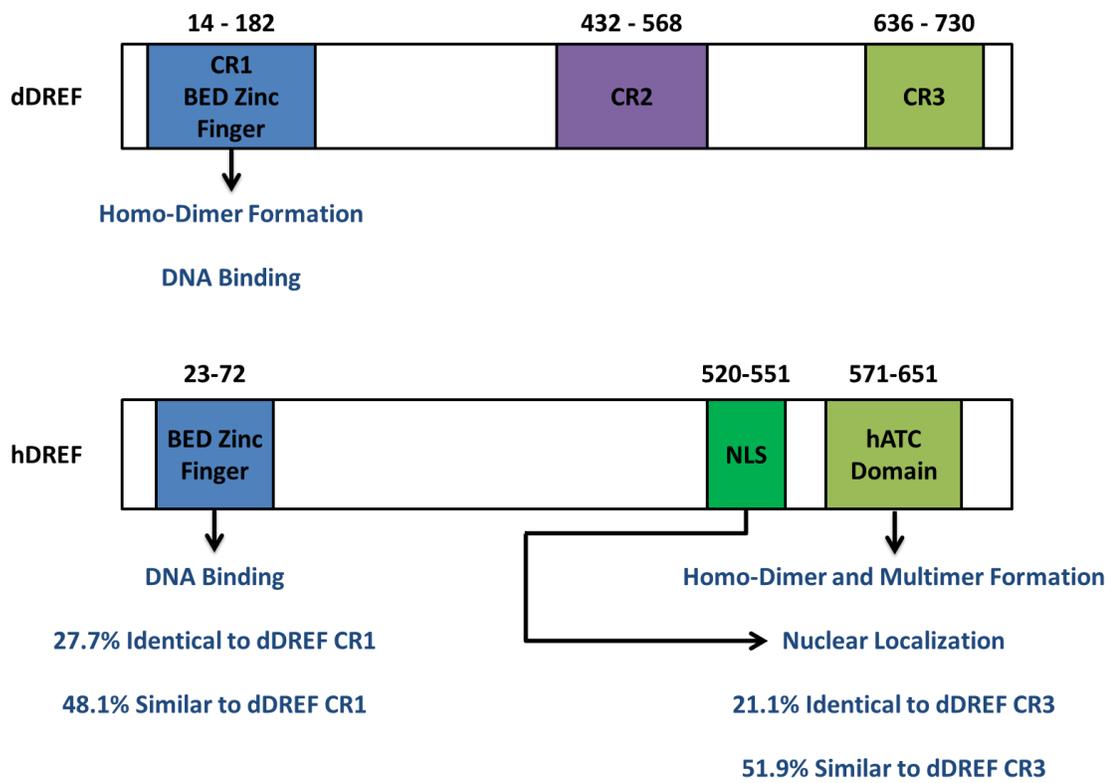
Two major domains exist in the hDREF protein (Figure 1.7). Both domains are conserved amongst members of the *hAT* transposase family, supporting the initial discovery by Esposito *et al.* that the *Tramp* gene encodes such a protein (283, 286). Firstly, a BED zinc finger domain is found at the N-terminus of hDREF in the region corresponding to dDREF CR1, and spans residues 23-72 (286). BED zinc finger domains are known DNA binding domains found in transposases and chromatin boundary element-binding proteins (287). Previous work in *Drosophila*, showed that the BED zinc finger in CR1 of dDREF is responsible for DNA binding (245, 285). Importantly, all cysteine and histidine residues in this domain are conserved between hDREF and dDREF, suggesting a common DNA binding mechanism at the N-terminus through the conserved BED C₂H₂-type zinc finger (285). However, the BED domain of hDREF on its own is not sufficient to bind DNA, nor activate transcription *in vitro* (285, 286).

Second, an hATC domain is found at the C-terminus in the region corresponding to CR3 of dDREF, spanning residues 571-651 (286). hATC domains have previously been shown to be involved in dimerization and multimerization in other hAT transposase family members such as maize Activator and housefly Hermes (288, 289). Yamashita *et*

al. found that the hATC domain in hDREF indeed facilitates and is required for homo-dimerization and multimerization *in vitro* and *in vivo*. A patch of hydrophobic amino acids conserved amongst the hATC domains of several hAT transposase family members proved to be critical for such interactions. Like its counterpart in *Drosophila*, hDREF is primarily found in granular structures in the nucleus (285). hATC domain-mediated self-association is necessary for nuclear localization and granular pattern formation of hDREF. Mutants that fail to self-associate do not interact with importin β 1 and exhibit cytoplasmic localization (286). In addition, residues 520-551 outside the hATC domain contain a classical NLS-like series of basic amino acids, which are also required for nuclear import, but not for self-association (286, 290, 291). Finally, self-association via the hATC domain is necessary to facilitate the interaction between the BED zinc finger in the N-terminus of hDREF and DNA. This indicates that hDREF may bind DNA as a homo-dimer or multimer.

Expression of the hDREF protein was found to be low in serum-starved quiescent WI-38 primary cells, gradually increasing after serum addition, and reaching maximal levels during S phase. siRNA-mediated knockdown of hDREF indicated that it might play an essential role in the transition from G₁ to S phase, as Ohshima *et al.* found that 0% compared to 30% of HeLa cells stained positive for bromodeoxyuridine (BrdU) when treated for 72 hours with hDREF-specific siRNA or scrambled siRNA respectively (285). Further analysis in immortalized human foreskin fibroblasts revealed that cells overexpressing hDREF proliferate faster than cells in which hDREF is knocked down (292). In addition, hDREF was found to exist in high molecular weight complexes, which change in a cell-cycle dependent manner. Residues 652-694 may be critical for these high order interactions. However, the identity of other members of these complexes is currently unknown (286).

Figure 1.7 Cartoon diagram of *D. virilis* and human DREF proteins. Regions of hDREF that correspond to conserved regions of dDREF are shaded with the same colour. Amino acid locations are indicated above each respective domain. Functions of each domain and are listed beneath arrows extending from the respective regions. CR1 of dDREF contributes to homo-dimer formation and DNA binding. The BED zinc finger of hDREF contributes to DNA binding, and shares 27.7% identity and 48.1% similarity to CR1 of dDREF. A functional consequence for the hDREF region corresponding to CR2 of dDREF has not been identified, so it is not depicted. The NLS of hDREF contributes to nuclear localization, but a dDREF counterpart has not been identified. The hATC domain of hDREF contributes to homo-dimer and multimer formation, as well as nuclear localization. It shares 21.1% identity and 51.9% similarity to CR3 of dDREF.



1.3.2 hDREF/hDRE Functional Analysis

Using the cyclic amplification and selection of targets (CASTing) method, a palindromic 10bp consensus recognition sequence (5'-TGTCG(C/T)GA(C/T)A) for hDREF was identified, and termed the hDRE. Five bases of this sequence (5'-CGATA) directly overlap with those in the dDRE and the *Drosophila* BEAF-32 insulator recognition site (271, 285). Mutational analysis of the hDRE revealed that the central six bases (5'-TCG(C/T)GA) are the most important for hDREF binding. Over 500 hDRE-like sequences matching at least 7 of 10 bases of the hDRE exist in the human genome, and many of these are found within promoter regions for genes encoding proteins involved in cell proliferation. This finding indicates that hDREF may be functionally conserved from *Drosophila* to humans. Table 1.2 lists several candidate hDREF target genes, which contain hDRE-like sequences in their promoter regions, and classifies them based on their involvement in: DNA replication and metabolism, cell cycle regulation, transcription, or protein synthesis.

Table 1.2 Select human genes containing hDRE-like sequences

| Category | Gene | hDRE Base Pair Matches (/10) |
|---------------------------------------|-----------------------------------|------------------------------|
| DNA Replication and Metabolism | Histone H1 | 10 |
| | Deoxycytidine Kinase | 9 |
| | Deoxyguanosine Kinase | 8 |
| | Thymidine Kinase | 7 |
| | Topoisomerase IIa | 7 |
| | DNA Polymerase δ | 7 |
| | DNA Polymerase γ | 7 |
| Cell Cycle Regulation | p14ARF | 9 & 8 |
| | p21WAF1 | 8 & 7 |
| | CDC25C | 8 |
| | CDC25A | 7 |
| | CDK6 | 8 |
| | Cyclin D3 | 7 |
| | Cyclin T1 | 7 & 7 |
| Transcription | RNA polymerase II large subunit | 9 & 8 |
| | RNA polymerase III 48-kDa subunit | 7 |
| | NF- κ B | 9 & 7 |
| Protein Synthesis | TAFII55 | 8 & 7 |
| | Surf-5 | 8 |
| | Asparagine Synthetase | 8 & 7 |
| | rRNA | 7 |
| | EF1A-2 | 7 |
| | RPS6 | 8 & 8 |
| | RPL10A | 9 |
| RPL12 | 9 | |

Note: Genes with blue letters were selected in previous studies to examine whether their transcription is affected by hDREF (285, 292). Genes with two numbers listed contain two hDRE-like sequences.

1.3.2.1 hDREF and Histone H1 Gene Expression

Histone H1 is responsible for the higher order structure of DNA, binding the core nucleosomes and linker DNA between them, and compacting the chromatin into a 30nm fibre from a simple “beads-on-a-string” form (293, 294). Its expression is tightly regulated by the cell cycle and intricately tied to DNA replication (295). It is expressed at low levels in serum-starved and differentiated cells, and transiently increases during the G₁ to S phase transition (295–297). This expression pattern mimics that of hDREF. Intriguingly, the histone H1 promoter (FNC16/H1.5) contains an hDRE matching all 10 bases of the consensus sequence. hDREF was found to specifically bind the H1 promoter at this site *in vitro*. Transient luciferase assays in HeLa cells using a luciferase reporter plasmid driven by the histone H1 promoter co-transfected with hemagglutinin (HA)-tagged hDREF revealed that hDREF may possibly activate transcription at the H1 promoter (285). In support of this, Ohshima *et al.* showed that an 88% knockdown of hDREF protein resulted in a 74% reduction in histone H1 mRNA levels in asynchronous HeLa cells using semi-quantitative real time polymerase chain reaction (RT-PCR). However, the reduction in histone H1 mRNA levels resulting from hDREF knockdown could be indirect and due to other factors, such as inhibited progression into S phase.

1.3.2.2 hDREF and Ribosomal Protein Gene Expression

Ribosomes are responsible for protein synthesis in all organisms, and are composed of two subunits containing rRNA and 79 ribosomal proteins (RPs), all of which are essential for ribosomal function (292, 298, 299). Much like hDREF and histone H1, rRNA and RP levels are tightly linked to progression through the cell cycle, with transcription initiating and increasing during G₁ phase, reaching maximal expression in S and G₂ phases (300–302). Ribosome biogenesis has even been implicated as a proliferative checkpoint in the cell (302). The basic theory behind this concept is as follows: In mitogen stimulated cells, ribosome biogenesis is upregulated via the preferential loading of RP mRNAs onto pre-existing ribosomes. This generates more ribosomes, increasing the translational capacity

of the cell, thereby allowing the translation of mRNAs less preferred by the ribosomes, such as those for the cyclin cell cycle regulators (302). The process of ribosome biogenesis in fact consumes 80% of the energy of a proliferating cell. Thus, a cell-cycle checkpoint exists until enough ribosomes have accumulated, which will not occur unless the cell has grown enough and accumulated enough nutrients to support division. In support of this, less than 50% knockdown of a single RP – RPS6 – has been shown to impair cellular proliferation (292).

Interestingly, 37 of 79 RP genes contain an hDRE-like sequence matching at least eight bases of the hDRE consensus sequence, 22 of which contain it within 200-bp upstream of the transcription start site (TSS) (292). Furthermore, the hDRE in 20 of these genes is centered around 60bp upstream of the TSS. Thus Yamashita *et al.* decided to examine whether hDREF regulates expression of three select ribosomal proteins, as a means of further discovering the role of hDREF in regulating cell proliferation. RPS6 contains two hDRE-like sequences, at 51 and 538 bases upstream of the TSS, each matching eight of ten consensus bases. Ribosomal proteins Large 10A (RPL10A) and Large 12 (RPL12) contain one at 41 and 56 bases upstream of the TSS respectively, matching nine of ten consensus bases (Table 1.2). hDREF was found to bind the hDRE-like sequence of RPS6 *in vitro* via EMSA, and the promoters – albeit weakly – of all three selected RP genes *in vivo* via chromatin immunoprecipitation (ChIP). Transient luciferase assays in HeLa cells using a luciferase reporter plasmid driven by the RPS6, RPL10A, or RPL12 promoter co-transfected with HA-tagged hDREF revealed that hDREF may weakly activate transcription of these genes. Deletion of the hDRE abrogated transcriptional activation, indicating that the increases in luciferase activity observed from co-transfected HA-hDREF were due to the hDREF-hDRE interaction. Additionally, Yamashita *et al.* showed that a 65% knockdown of hDREF reduced mRNA levels of all three genes, and resulted in an overall reduction in protein synthesis by 25%.

1.4 Rationale, Hypothesis, and Objectives

Dr. Peter Pelka – a former post-doctoral fellow in our lab – initially identified an interaction between E1A and hDREF through mass spectrometry analysis of immunoprecipitates of E1A *in vitro*. Although a multitude of potential E1A targets were identified, I decided to pursue hDREF for two main reasons. Firstly, it is a transcription factor involved in the expression of several genes related to cell proliferation, and its expression increases during S-phase. The two main functions of E1A are to modulate cellular and viral transcription, and to induce a G₁-to-S phase transition, and therefore the binding and manipulation of hDREF could be important for both of these functions. Secondly, because hDREF is poorly characterized, studies with the well characterized E1A protein could serve to better elucidate hDREF function. Based on this rationale, we hypothesize that the interaction between E1A and hDREF contributes to adenovirus induced transcriptional modulation and viral replication in HAdV-5 infected host cells.

To test the hypothesis, four main objectives were set:

- 1) Confirm the interaction of E1A with hDREF and map the binding site on E1A
- 2) Examine the effects of hDREF on transcription *in vitro*
- 3) Determine the effects of hDREF on specific viral and cellular gene transcription *in vivo*
- 4) Observe how changes in hDREF expression affect viral replication

2 Materials and Methods

2.1 Cells Cultures

HT1080 human fibrosarcoma cells, HeLa cervical cancer cells, Human Embryonic Kidney (HEK) 293 cells, and IMR-90 primary human lung fibroblast cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Multicell) supplemented with 10% fetal bovine serum (Gibco), 100U penicillin mL⁻¹(Multicell), and 100µg mL⁻¹ streptomycin (Multicell). Cells were grown in a 37°C incubator with 5% CO₂.

2.2 Plasmids

Plasmids 1-24, 26, 27, and 30-32 were constructed and utilized by previous members in our lab (Table 2.1). Gal4 DNA-binding domain (Gal4-DBD) fusion plasmids express proteins with N-terminal Gal4-DBD tags (215, 303). GFP fusion plasmids express proteins with N-terminal GFP tags (78, 304). Plasmid 28 (pGL3-H1p) was constructed as previously described, however the histone H1 promoter sequence was inserted into a promoter-less and enhancer-less pGL3-basic backbone vector (Promega), instead of one containing the SV40 promoter (285). This precluded the need to subsequently remove the SV40 promoter. Plasmid 29 (pcDNA3-HA-hDREF) was obtained by generous donation from Dr. Fumiko Hirose at the University of Hyogo, Japan, and was described previously (285). Plasmid 25 (Gal4-DBD-hDREF) was created by restriction digesting full length hDREF out of plasmid 29 with Sall and HindIII (New England Biolabs) and inserting it into the same restriction sites of the parent vector pM. This generated an N-terminal Gal4-DBD-tagged hDREF. All plasmids were maintained in *Escherichia coli* strain DH5α. Large-scale preparations of plasmid DNA were generated using the HiPure Plasmid Midiprep kit (Invitrogen) according to the manufacturer's guidelines.

Table 2.1. List of Plasmids

| # | Name | Parent Vector | Characteristics |
|----|------------------------------|---------------|--|
| 1 | GFP-13S Ad12 | pGFP | Full length 13S E1A of Ad12 |
| 2 | GFP-13S Ad3 | pGFP | Full length 13S E1A of Ad3 |
| 3 | GFP-13S Ad5 | pGFP | Full length 13S E1A of Ad5 |
| 4 | GFP-13S Ad9 | pGFP | Full length 13S E1A of Ad9 |
| 5 | GFP-13S Ad4 | pGFP | Full length 13S E1A of Ad4 |
| 6 | GFP-13S Ad40 | pGFP | Full length 13S E1A of Ad40 |
| 7 | GFP-12S | pGFP | 12S E1A of Ad5 |
| 8 | GFP-11S | pGFP | 11S E1A of Ad5 |
| 9 | GFP-10S | pGFP | 10S E1A of Ad5 |
| 10 | GFP-9S | pGFP | 9S E1A of Ad5 |
| 11 | GFP-1-82 | pGFP | AA 1-82 of Ad5 E1A |
| 12 | GFP-CR2 | pGFP | AA 93-139 of Ad5 E1A |
| 13 | GFP-CR3 | pGFP | AA 139-204 of Ad5 E1A |
| 14 | GFP-Exon2 | pGFP | AA 187-289 of Ad5 E1A |
| 15 | GFP- Δ 1-82 | pGFP | AA 93-289 of Ad5 E1A |
| 16 | GFP-1-29 | pGFP | AA 1-29 of Ad5 E1A |
| 17 | GFP-30-69 | pGFP | AA 30-69 of Ad5 E1A |
| 18 | GFP-70-82 | pGFP | AA 70-82 of Ad5 E1A |
| 19 | GFP-1-82 (Δ 1-14) | pGFP | Plasmid #11 but Δ 1-14 |
| 20 | GFP-1-82 (Δ 4-25) | pGFP | Plasmid #11 but Δ 4-25 |
| 21 | GFP-1-82 (Δ 26-35) | pGFP | Plasmid #11 but Δ 26-35 |
| 22 | pGFP | N/A | Expresses GFP only; Control |
| 23 | Gal4-DBD-1-82 | pM | AA 1-82 of Ad5 E1A |
| 24 | Gal4-DBD-CR3 | pM | AA 139-204 of Ad5 E1A |
| 25 | Gal4-DBD-hDREF | pM | Full length hDREF |
| 26 | pM | N/A | Expresses Gal4-DBD only; Control |
| 27 | pGL2-(UAS) ₆ -Luc | pGL2-Basic | Luciferase reporter plasmid; Contains 6 UASs and a minimal promoter controlling luciferase gene expression |
| 28 | pGL3-H1p-Luc | pGL3-Basic | Luciferase reporter plasmid; Contains full length histone H1 promoter (-571bp to -1bp) controlling luciferase gene expression |
| 29 | pcDNA3-HA-hDREF | pcDNA3-HA | Full length hDREF |
| 30 | pcDNA3-HA | N/A | Expresses hemagglutinin only; Control and fill-in DNA |
| 31 | pcDNA3-pRb | pcDNA3 | Full length pRb |
| 32 | pcDNA3 | N/A | Non-coding plasmid; Fill-in DNA |

Note: Gal4-DBD = AA1-147 of Gal4; UAS (Upstream Activation Sequence) = The Gal4-DBD binding site

2.3 Plasmid Transfections

10cm² dishes (Sarstedt) were used for all transfections in co-immunoprecipitation experiments, and 1x10⁶ HT1080 or HeLa cells were plated 24 hours before transfection. 6-well plates (Sarstedt) were used for transfections in luciferase assay experiments, and 2x10⁵ HT1080 and HeLa cells were plated 24 hours before transfection. Purified plasmid DNA was transfected into cells using X-tremeGENE HP DNA Transfection Reagent (Roche) as per the manufacturer's protocol.

2.4 Viruses and Infections

All HAdV infections were carried out at a multiplicity of infection (MOI) of 5 PFU per cell. The inoculated cells were incubated at 37°C with 5% CO₂ until their collection at time points indicated by individual experiments. All HAdVs used were derived from the HAdV-5 dl309 (wild-type; WT) background which contains a small deletion in the E3 region, knocking out the E3 14.7K, 14.5K and 10.4K proteins (163, 305). Viruses utilized throughout the experiments, and their respective E1A mutations are as follows: dl312 (Δ E1A), dl343 (Non-functional E1A due to frameshift after the codon for amino acid 20), dl309 (WT), dl1101 (Δ 4-25), dl1102 (Δ 26-35), dl1103 (Δ 30-49). Besides dl312 which expresses no E1A, all viruses express both 13S and 12S E1A.

2.5 Co-immunoprecipitation and Western Blotting

2.5.1 Co-immunoprecipitation of HT-1080 and HeLa Cell Lysates

HT-1080 cells co-transfected with indicated plasmids, and HAdV-infected HeLa cells were collected at 24 and 12 hours post-transfection or post-infection respectively. Cells were washed in 1mL of Phosphate-Buffered Saline (PBS; Multicell), pelleted, and lysed in 1mL of 0.5% NP-40 lysis buffer (0.5% NP-40, 150mM NaCl, 50mM Tris-HCl pH 7.8)

containing 0.5% Complete Protease Inhibitor Cocktail (Sigma-Aldrich). Cells were centrifuged at 13000g to pellet the cell debris, and then 1mL of lysate was transferred to cold 1.5mL Eppendorf tubes. 20 μ L of lysate was transferred to new tubes to be used as a 2% input loading and plasmid expression control. The remaining 980 μ L of lysate was subject to immunoprecipitation. 1 μ L of α -Green Fluorescent Protein (GFP) rabbit polyclonal antibody (Ab) or 80 μ L of the hybridoma-produced M73 α -E1A Ab (Table 2.2), along with 125 μ L of a 10% slurry of protein-A sepharose beads (Sigma-Aldrich) suspended in E1A Buffer (50mM Hepes pH7, 0.1% NP-40, 250mM NaCl) was added to each experimental tube. Samples were incubated with gentle rocking at 4°C for two to four hours. Immunoprecipitated lysates were spun down at 13000g and washed five times in NP-40 lysis buffer to diminish non-specific background binding. Protein-antibody-bead complexes were suspended in 25 μ L of 2X LDS Sample Buffer (Invitrogen) containing 0.1M dithiothreitol (DTT). Input lysates were suspended in 10 μ L of 3X LDS Sample Buffer. All samples were then boiled for five minutes at 100°C to denature proteins and separate them from the antibody-bead complexes. Proteins were separated on NuPAGE 4-12% Bis-Tris polyacrylamide gradient gels (Invitrogen) and then transferred onto a polyvinylidene fluoride membrane (PVDF; Amersham) according to the supplier's protocol. Membranes were blocked in Tris-Buffered Saline containing 0.1% Tween-20 (Sigma-Aldrich) (TBS-T), and 5% weight/volume skim milk powder (BioShop), for one hour at room temperature. The membrane was then incubated in 20mL of the indicated diluted primary antibody (Table 2.2) overnight at 4°C. After washing in TBS-T four times for eight minutes each, the membrane was incubated in 20mL of the indicated diluted horseradish peroxidase (HRP)-conjugated species-specific secondary antibody (Table 2.2) for 45 minutes at room temperature. The membrane was subject to four final TBS-T washes for eight minutes each, before detecting protein bands using the Luminata Forte Western HRP substrate (Millipore) according to the supplier's recommendations. Images were developed on high performance Hyperfilm ECL film (Amersham) using an SRX-101A automated developer (Konica Minolta Medical Imaging).

Table 2.2 List of antibodies

| Specificity | Description | Usage | Dilution Factor from Stock | Company | Catalogue # |
|--------------------|----------------------|--------------|---|-----------------------|--------------------|
| Actin | Rabbit polyclonal | Primary | 1:2000 | Sigma- Aldrich | M-7023 |
| GFP | Rabbit polyclonal | Primary | 1:2000 | Clontech | 632592 |
| GFP | Rabbit polyclonal | IP | 1:1000 (1 μ L) | Clontech | 632592 |
| HA | Rat monoclonal | Primary | 1:2000 | Roche | 11867423001 |
| Gal4-DBD | Mouse monoclonal | Primary | 1:2000 | Santa Cruz | Sc-510 |
| hDREF | Rabbit polyclonal | Primary | 1:1000 | Abcam | Ab48355 |
| E1A (M73) | Mouse monoclonal | Primary | 1:200 (100 μ L) | In house hybridoma | N/A |
| E1A (M73) | Mouse monoclonal | IP | 1:12.5 (80 μ L) | In house hybridoma | N/A |
| Rat IgG | Goat | Secondary | 1:200000 | Thermo Scientific | 31470 |
| Rabbit IgG | Goat | Secondary | 1:100000 | Santa Cruz | Sc-2004 |
| Mouse IgG | Rabbit | Secondary | 1:200000 | Santa Cruz | Sc-358923 |

Note: Primary and secondary antibodies are diluted and applied to membranes in TBS-T containing 5% skim milk powder. Immunoprecipitation (IP) antibodies are added directly to 1mL of lysate

2.5.2 Western Blotting of Other HT-1080, HeLa, and IMR-90 Cell Lysates

All Western blots were performed in identical fashion subsequent to loading the proteins on a gel. There were slight changes in protocol prior to this step, however, for Western blots that were not the product of co-immunoprecipitation experiments. First, the volume of NP-40 lysis buffer varied among experiments. Cells plated on: 10cm² dishes were lysed in 1mL of NP-40 buffer; 6-well dishes were lysed in 200μL; and 12-well dishes were lysed in 100μL. IMR-90 cells plated on 6-well dishes were lysed in 50μL of NP-40 lysis buffer. Next, the protein concentration of each lysate was measured using a Bradford DC Protein assay kit (Bio-Rad), such that 20-30 μg of total protein from each sample could accurately be loaded on a gel.

2.6 Luciferase Assays

2.6.1 Gal4 Luciferase Assays

Cells were transfected with 0.1μg or 1.0μg of the pGL2-(UAS)₆-Luc reporter (Plasmid 27) and indicated Gal4-DBD fusion plasmids (Table 2.1). A total of 2μg of plasmid DNA was transfected into each sample, using empty pcDNA3 plasmid as fill-in to maintain identical total DNA concentrations among all transfections. After 24 hours in HT-1080 cells, or 48 hours in HeLa cells, cells were lysed in 200μL of Reporter Lysis Buffer (Promega E397A) diluted in PBS. To detect luciferase production, 50μL of Luciferase Substrate (Promega E151A) was mixed with 50μL of lysate immediately prior to detection of light by a Lumat LB 9507 luminometer (Berthold). Lysates were also subject to Bradford assays and Western blots as described in section 2.5.2. Results were initially normalized based on protein concentration as determined by Bradford assays, and then related to luciferase activity readout of a Gal4-DBD empty plasmid control.

2.6.2 Histone H1 Promoter Luciferase Assays

Histone H1 promoter luciferase assays were carried out as described in section 2.7.1, however 0.1 μ g of pGL3-H1p-Luc (Plasmid 28) was used as a reporter (Table 2.1). The pcDNA3-HA-hDREF plasmid was co-transfected in increasing concentrations. pcDNA3-HA empty vector was used as fill-in DNA to reach a total of 2 μ g of transfected plasmid DNA. Results were normalized initially based on protein concentration, and then related to luciferase activity readout of samples transfected with only the pGL3-H1p plasmid.

2.7 siRNA Knockdown

Downregulation of hDREF was performed using two combined Silencer Select siRNAs (s17567/s17568; Ambion). siRNA transfections were carried out using Silentfect (Bio-Rad), with slight modifications to the manufacturer's protocol. In HeLa cells used for quantitative RT-PCR experiments, siRNAs were transfected 18 hours after plating 1×10^6 cells on a 10cm² dish, to a final concentration of 15nM (7.5nM of each hDREF siRNA). 15 μ L of Silentfect was used per well. In IMR-90 cells used for viral plaque assay experiments, siRNAs were transfected 18 hours after plating 1×10^5 cells on 6-well dishes, to a final concentration of 10nM (5nM of each hDREF siRNA). 2 μ L of Silentfect was used per well. Scrambled siRNA (Negative Control No. 2; AM4613; Ambion) was used as a control.

2.8 Quantitative RT-PCR

HeLa cells were subject to siRNA knockdown of hDREF as indicated in section 2.7. 24 hours later, the cells were subcultured to 12-well dishes, at 1×10^5 cells per well. After a total of 72 hours post-siRNA knockdown, cells were infected with the indicated viruses at an MOI of 5 PFU per cell. At 6 and 10 HPI, total RNA was prepared from HeLa cells using Trizol reagent (Ambion). 1 μ g of RNA was reverse transcribed to cDNA using the qScript cDNA SuperMix kit (Quanta) as per the manufacturer's instructions. cDNA was

quantified according to the manufacturer's protocol, using PerfeCTa SYBR Green FastMix (Quanta) and oligonucleotide primers that recognize the indicated target gene (Table 2.3). Quantification was performed by the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Results were analyzed using the $2^{-\Delta\Delta CT}$ method, normalizing values first to GAPDH and then to mock infected samples.

2.9 Viral Plaque Assays

IMR-90 cells were transfected with hDREF siRNA as indicated in section 2.7. Cells were then grown to confluence, and then contact inhibited cultures were incubated for an additional 3 days, to allow for complete growth arrest (77, 78). Next, cells were infected with the indicated viruses at an MOI of 5 PFU per cell. IMR-90 cells and supernatant were collected at 4, 48, and 120 HPI. Samples were subjected to three cycles of freeze-thawing between -80°C and 4°C , in order to lyse the cells and release the virus. HEK293 cells were then plated on 48-well dishes and grown to confluence. Next, $100\mu\text{L}$ of the indicated serially diluted virus previously collected from the IMR-90 cells was used to inoculate them. Cells were briefly incubated for 1 hour at 37°C and 5% CO_2 . Subsequently, the inoculum was removed, and $300\mu\text{L}$ of SeaPlaque Low Melting Point Agarose (Lonza) mixed in a ratio of 1.5:1 with 2X DMEM (Gibco) was applied as an overlay. 8 days later plaques were enumerated and used to determine viral titre.

Table 2.3 List of oligonucleotide primers used for Real-Time PCR

| Gene | Forward Primer (5'-3') | Reverse Primer (5'-3') | Amplicon Size (bp) |
|--------------|-------------------------------|-------------------------------|---------------------------|
| GAPDH | ACTGCTTAGCACCCCTGGCAA | ATGGCATGGACTGTGGTCATGAGTC | 79 |
| E2e | GGGGGTGGTTTCGCGCTGCTCC | GCGGATGAGGCGGCGTATCGAG | 138 |
| E3 | GAGGCAGAGCAACTGCGCC | GCTCTCCCTGGGCGGTAAGCCGG | 151 |
| E4 | GCCCCATAGGAGGTATAAC | GGCTGCCGCTGTGGAAGCGC | 129 |
| H1 | TTTCTTGCCACCATGTCGGA | TTGCCTTCTTCTTAGCCGGG | 85 |
| RPS6 | ACGCTCTGGGTGAAGAATGG | AAGACACCCTGCTTCATGGG | 88 |

Note: All primers were supplied by Sigma-Aldrich. Annealing stage of RT-PCR was consistently performed at 55°C.

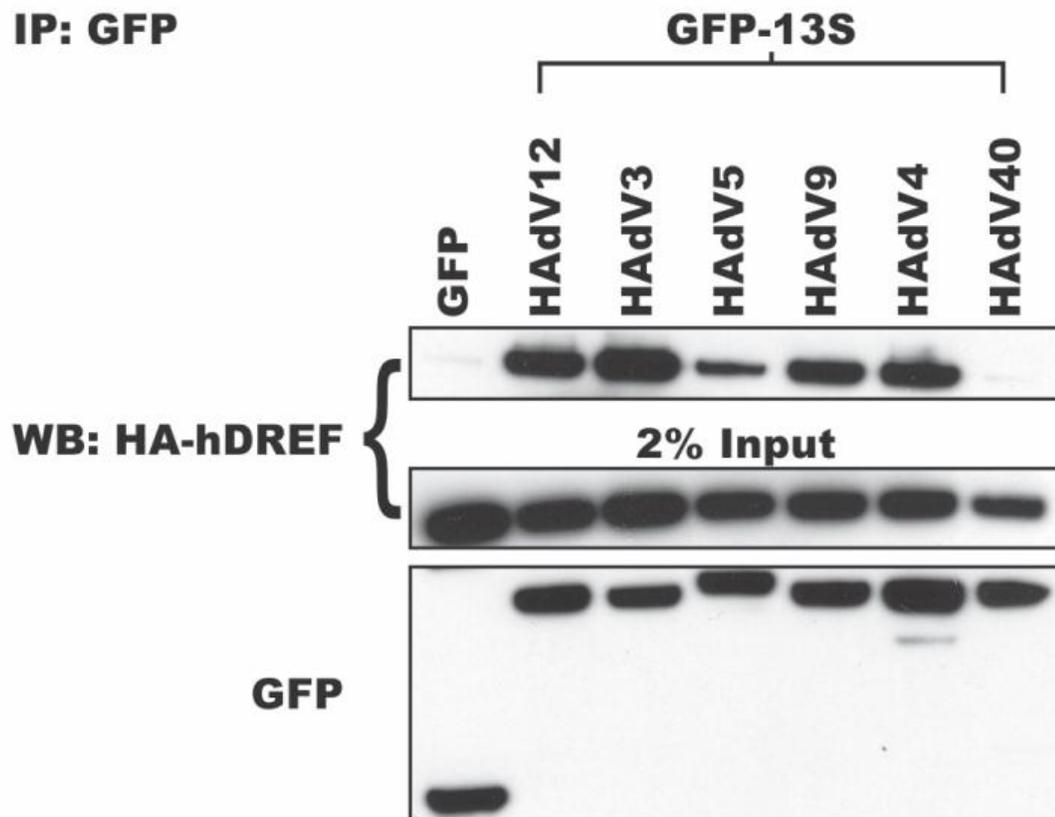
3 Results

3.1 Mapping the Interaction Between E1A and hDREF to Specific Regions of the E1A Protein

3.1.1 Identification of Conserved Interactions between hDREF and 13S E1A of Representative Members of HAdV Species A Through F

Many important interactions with cellular factors such as CtBP, GCN5, pRb, and p300 are conserved amongst various serotypes from the six main species of HAdV (166, 189, 304). Therefore, the identification of new factors displaying conserved interactions with E1A amongst different species possibly indicates an important role for those factors during the HAdV life cycle. The interaction between HAdV-5 13S E1A and hDREF was initially identified in our lab by Dr. Peter Pelka, through mass spectrometry analyses of immunoprecipitates of E1A (Data not shown). To examine whether the interaction with hDREF was conserved across species A through F, we utilized co-immunoprecipitation techniques. HT-1080 cells were co-transfected with plasmids encoding HA-tagged hDREF, and GFP-tagged 13S E1A from representative members of HAdV species A through F: HAdV-12, 3, 5, 9, 4, and 40 respectively. 24 hours later, cells were collected, lysed, and subject to immunoprecipitation using an α -GFP antibody. Western blots were subsequently carried out to determine which serotypes co-immunoprecipitated hDREF through E1A. Results indicated that there is indeed a conserved interaction between hDREF and 13S E1A from serotypes 12, 3, 5, 9, and 4 of species A through E, but not serotype 40 of species F (Figure 3.1). Since HAdV-5 is well characterized, and the majority of molecular tools in our lab were constructed from HAdV-5, it was the serotype of choice for subsequent experiments in this thesis (65).

Figure 3.1 The interaction of hDREF with 13S E1A from various HAdV species. HT-1080 cells were co-transfected with 1 μ g of a plasmid encoding HA-tagged hDREF, and 7 μ g of a plasmid encoding GFP-tagged 13S E1A from representative members of HAdV species A through F: HAdV-12, 3, 5, 9, 4, and 40 respectively. Empty GFP vector was used as a negative control. 24 hours later, cells were collected, lysed, and subject to immunoprecipitation using an α -GFP antibody. Western blots were then carried out using α -HA, and α -GFP antibodies.



3.1.2 Interaction of hDREF with E1A Isoforms

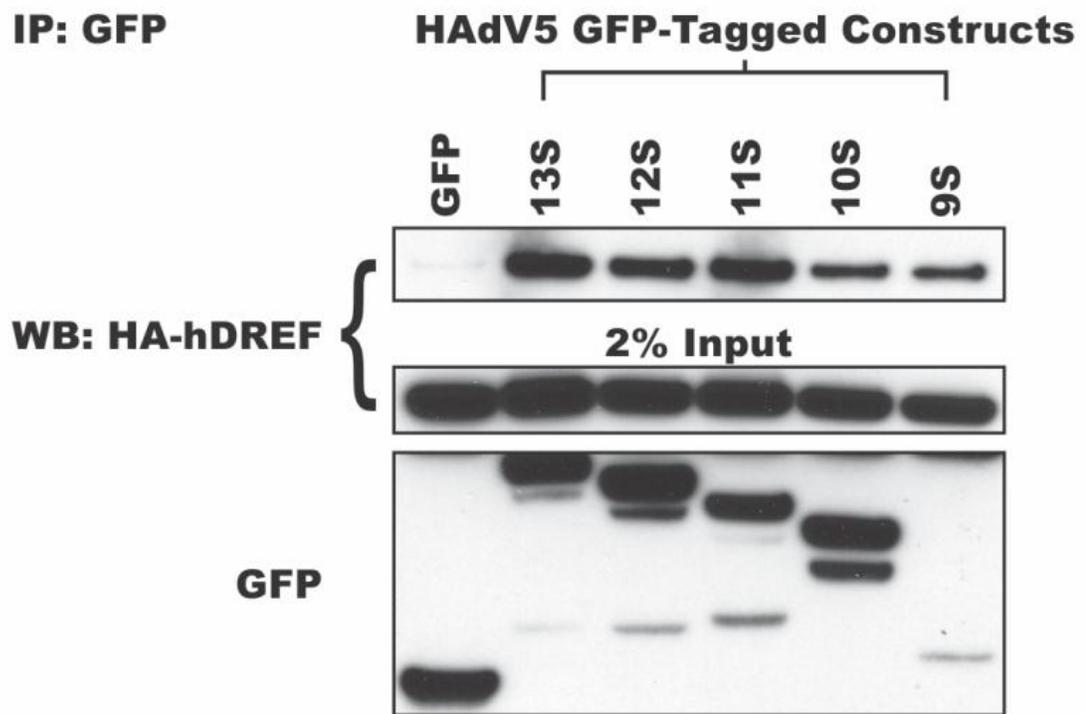
As depicted in Figure 1.4, each E1A protein contains regions shared with 13S, but also contains specifically spliced regions. Binding of proteins to certain E1A isoforms, but not others can be used to expedite the process of mapping an interaction. For instance, binding to 13S but not 12S would indicate potential binding in CR3. Thus, we next examined whether any of the other four isoforms of E1A from HAdV-5 were able to bind hDREF. HT-1080 cells were co-transfected with plasmids encoding HA-tagged hDREF, and GFP-tagged 13S, 12S, 11S, 10S, and 9S E1A from HAdV-5. 24 hours later, cells were collected, lysed, and subject to immunoprecipitation using an α -GFP antibody. Western blots were subsequently carried out to determine which isoforms hDREF co-immunoprecipitated with. Results indicated that all five isoforms of HAdV-5 E1A can bind hDREF *in vitro* (Figure 3.2). Since the only residues common to all five isoforms are the first 26 amino acids, our initial results suggested that hDREF binds to E1A through the N-terminus (77).

3.1.3 Identification of the hDREF Binding Site on E1A

In order to more precisely define the location of the hDREF binding site on E1A, we performed a series of co-immunoprecipitation experiments, using increasingly specific E1A mutants. HT-1080 cells were co-transfected with plasmids encoding HA-tagged hDREF, and GFP-tagged E1A constructs. 24 hours later, cells were collected, lysed, and subject to immunoprecipitation using an α -GFP antibody. Western blots were subsequently carried out to determine which E1A mutants hDREF could co-immunoprecipitate with.

Initially we examined deletion mutants containing conserved regions of E1A. The following plasmids were transfected, encoding various portions of 13S E1A: AA1-82 which contains the N-terminus and also CR1 spanning residues 42-72; AA93-139 which

Figure 3.2 The interaction of hDREF with different HAdV-5 isoforms. HT-1080 cells were co-transfected with 1 μ g of a plasmid encoding HA-tagged hDREF, and 7 μ g of a plasmid encoding GFP-tagged 13S, 12S, 11S, 10S, or 9S E1A from HAdV-5. Empty GFP vector was used as a negative control. 24 hours later, cells were collected, lysed, and subject to immunoprecipitation using an α -GFP antibody. Western blots were then carried out using α -HA, and α -GFP antibodies.



contains CR2 spanning residues 116-139; AA139-204 which contains CR3 spanning residues 144-191; AA187-289 which contains CR4 spans residues 240-288; or AA93-289 which contains the entire protein besides the N-terminus and CR1 (166). Consistent with Figure 3.2, my results indicated that hDREF binds E1A within the first 82 amino acids (Figure 3.3). In addition, hDREF does not bind regions outside the first 82 amino acids, as indicated by the lack of binding to the construct expressing GFP-tagged residues 93-289. It is important to note that the exact figure displayed was chosen as the clearest representation of multiple replicates of this experiment. The band representing co-immunoprecipitated hDREF through residues 1-82 was consistently as intense as that for 13S E1A, throughout all replicates. Despite the fact that a faint band indicating co-immunoprecipitated hDREF was present for residues 144-191, and even weaker bands were present for binding of residues 93-139 and 93-289, I believe they represent false, non-specific interactions. In other replicates of this experiment these hDREF bands never appeared more intense than those depicted in Figure 3.3, were frequently absent from the blots, and occasionally appeared less intense than the negative control, despite the fact that their respective GFP-tagged E1A constructs were consistently expressed at higher levels than GFP-13S and GFP-1-82.

Next, we transfected plasmids encoding three small, non-overlapping fragments of the first 82 amino acids of E1A. Residues 1-29 displayed hDREF binding, while residues 30-69 and 70-82 were deficient for binding (Figure 3.4). Finally, we transfected plasmids encoding amino acids 1-82 with the following deletions: Δ 1-14; Δ 4-25; Δ 26-35. A deletion of residues 1-14 did not appear to affect binding of the N-terminus to hDREF, as a band nearly as intense as full length 1-82 was detected (Figure 3.5). In contrast, deletions of residues 4-25 or 26-35 severely reduced hDREF co-immunoprecipitation. Low intensity hDREF bands were visible in all replicates of the experiment, indicating weak binding of hDREF to these N-terminal deletion mutants. Collectively the results obtained from figures 3.2 through 3.5 indicate that residues 15-26 of E1A are sufficient for a level of hDREF binding comparable to that of the N-terminal 82 amino acids, and thus full length 13S.

Figure 3.3 The N-terminal 82 amino acids of E1A bind to hDREF. HT-1080 cells were co-transfected with 1 μ g of a plasmid encoding HA-tagged hDREF, and 7 μ g of a plasmid encoding a GFP-tagged fragment of E1A: amino acids 1-82, 93-139, 139-204, 187-289, or 93-289. Empty GFP vector was used as a negative control. GFP-tagged 13S E1A was used as a positive control. 24 hours later, cells were collected, lysed, and subject to immunoprecipitation using an α -GFP antibody. Western blots were then carried out using α -HA, and α -GFP antibodies.

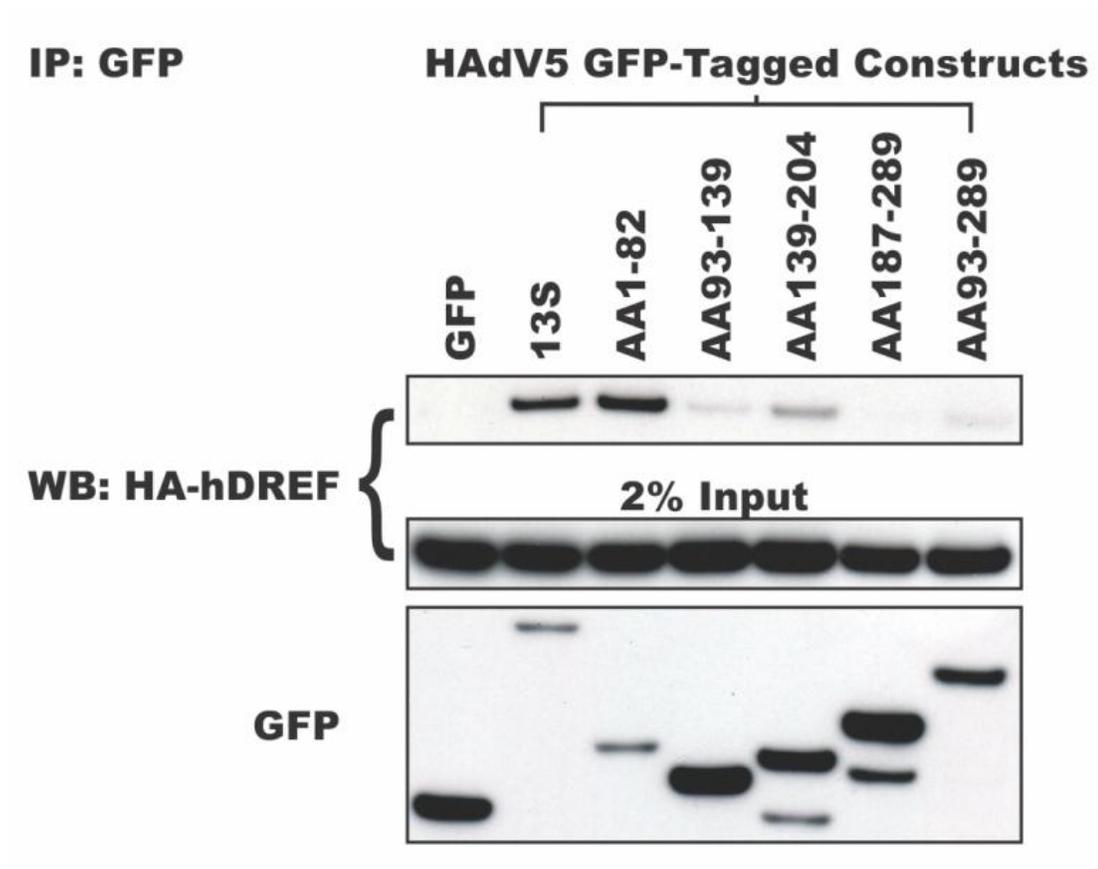


Figure 3.4 The N-terminal 29 amino acids of E1A independently bind to hDREF.

HT-1080 cells were co-transfected with 1 μ g of a plasmid encoding HA-tagged hDREF, and 7 μ g of a plasmid encoding a small GFP-tagged fragment of the first 82 amino acids of E1A: 1-29, 30-69, or 70-82. Empty GFP vector was used as a negative control. GFP-tagged 1-82 of E1A was used as a positive control. 24 hours later, cells were collected, lysed, and subject to immunoprecipitation using an α -GFP antibody. Western blots were then carried out using α -HA, and α -GFP antibodies.

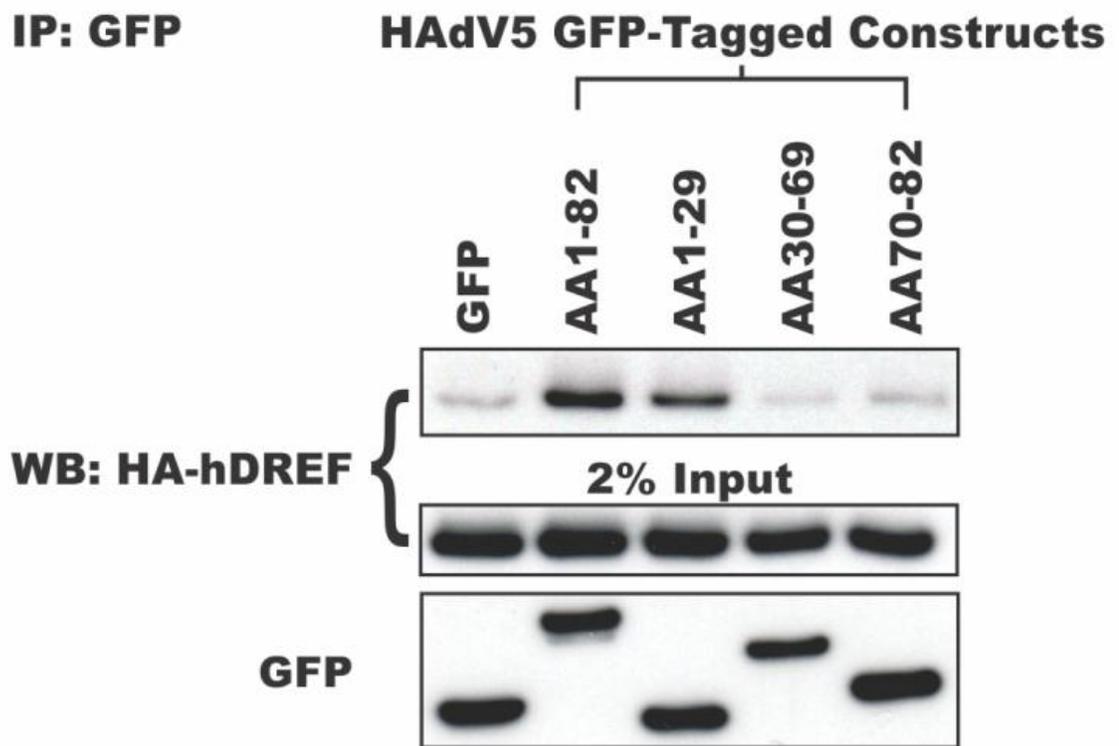
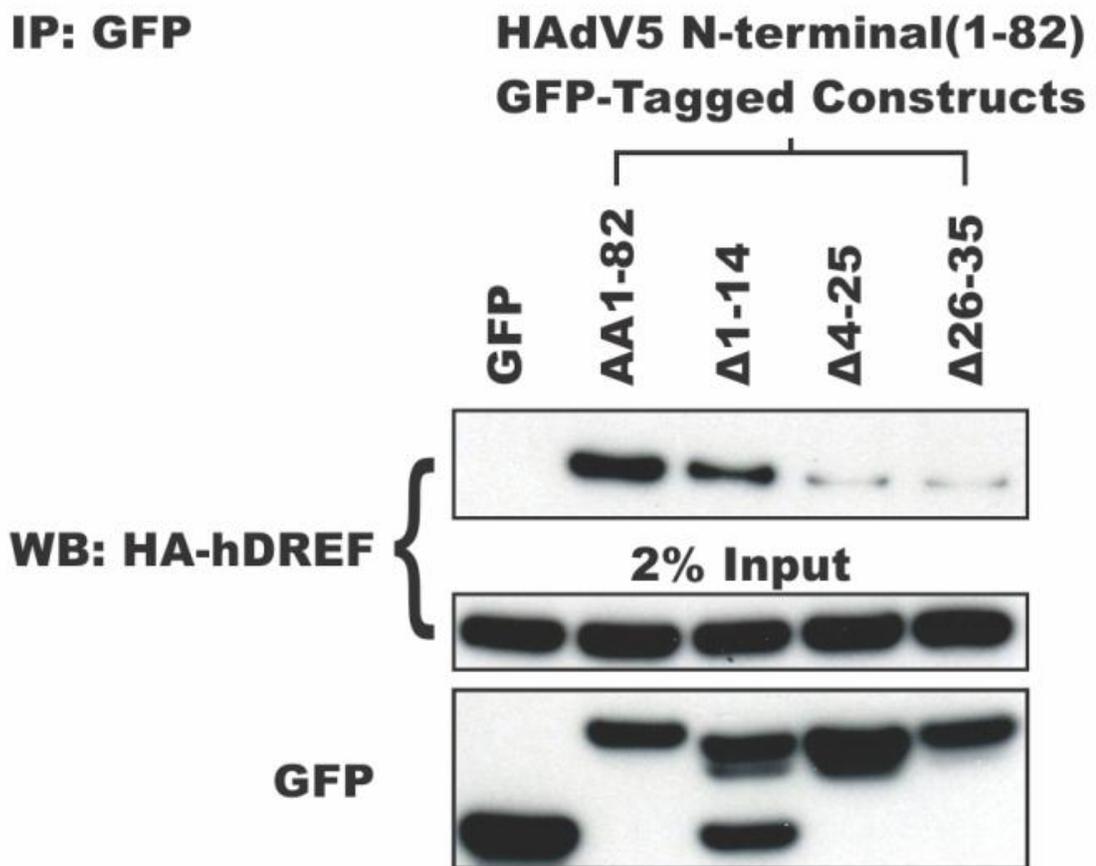


Figure 3.5 Deletion of residues 4-25 or 26-35 reduce the ability of the N-terminal 82 amino acids of E1A to bind hDREF. HT-1080 cells were co-transfected with 1 μ g of a plasmid encoding HA-tagged hDREF, and 7 μ g of a plasmid encoding GFP-tagged amino acids 1-82 of E1A with deletions in residues 1-14, 4-25, or 26-35. Empty GFP vector was used as a negative control. GFP-tagged 1-82 of E1A was used as a positive control. 24 hours later, cells were collected, lysed, and subject to immunoprecipitation using an α -GFP antibody. Western blots were then carried out using α -HA, and α -GFP antibodies.

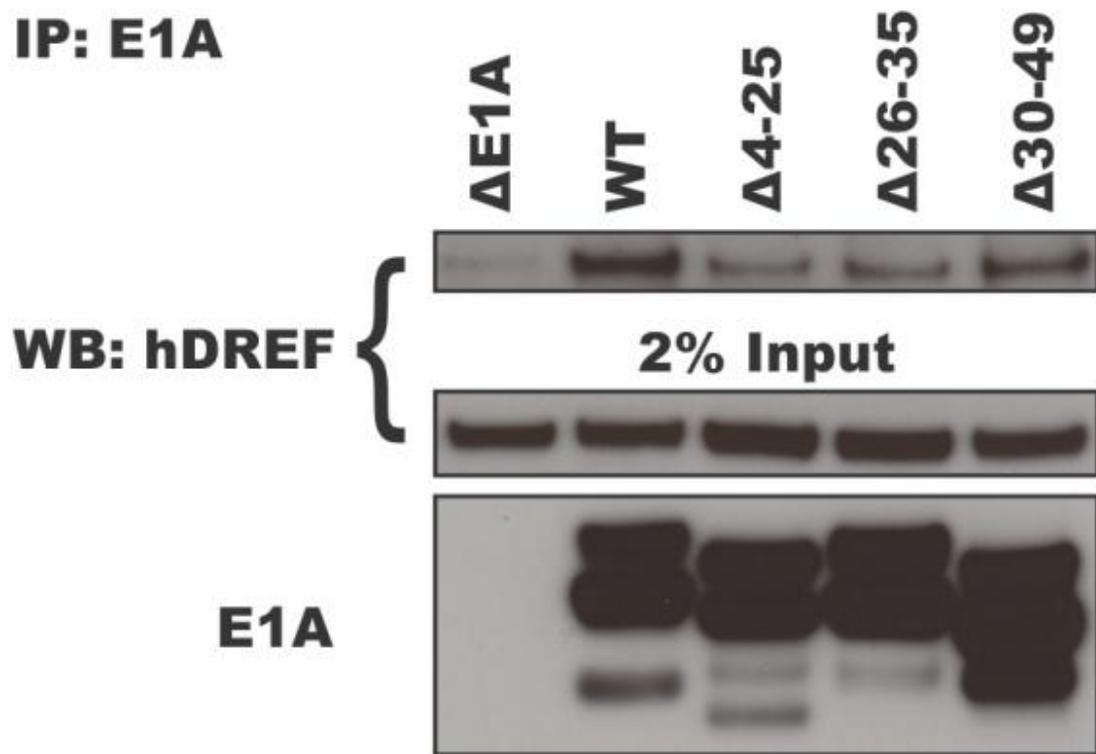


3.1.4 Confirmation of the Interaction between E1A and hDREF during Infection

Although our previous results identified an interaction between E1A and hDREF, they were derived from transfection based assays using overexpressed proteins. To determine whether the interaction between E1A and endogenous hDREF could occur during an actual infection, I decided to use a more relevant model. HeLa cells were infected at an MOI of 5 PFU per cell with HAdV-5 virus expressing wild-type E1A (dl309), E1A mutated in various N-terminal regions (dl1101/ Δ 4-25; dl1102/ Δ 26-35; or dl1103/ Δ 30-49), or not expressing E1A (dl312). 12 hours later, cells were collected, lysed, and subject to immunoprecipitation using the M73 α E1A antibody. Western blots for E1A and endogenous hDREF were subsequently performed. My results indicated that 13S E1A produced during an HAdV-5 infection can interact with endogenous hDREF (Figure 3.6). Results were also consistent with those in Figure 3.5, confirming a greater reduction in the ability of E1A to bind endogenous hDREF when residues 4-25 and 26-35 are deleted, as compared to when residues 30-49 are deleted.

In summary, the results of our co-immunoprecipitation transfection-based and infection-based experiments indicate a definite interaction between E1A and hDREF. The interaction is conserved amongst representative serotypes from HAdV species A through E but not F, and all 5 isoforms of HAdV-5 E1A can bind hDREF. The N-terminal 29 amino acids appear to form a primary binding site for hDREF, of which residues 15-26 are sufficient for binding. The goal of our mapping experiments was to identify a minimal binding region in E1A which was necessary for the hDREF interaction. We then intended to alter that region to create an HAdV-5 derivative encoding an E1A mutant unable to bind hDREF. The virus would have been used to study the effects of the hDREF-E1A interaction on viral and cellular gene transcription, as well as viral replication (objectives three and four). However, due to time restraints for the completion of a Master's degree, this was not feasible. Since both the Δ 4-25 and Δ 26-35 E1A mutants displayed reduced binding – but neither completely abolished binding – a series

Figure 3.6 Deletions in residues 4-25 and 26-35 reduce the ability of 13S E1A produced during infection to bind endogenous hDREF. HeLa cells were infected at an MOI of 5 PFU per cell with HAdV-5 mutants possessing distinct E1A phenotypes: dl312 (Δ E1A), dl309 (WT), dl1101 (Δ 4-25), dl1102 (Δ 26-35), or dl1103 (Δ 30-49). 12 hours later, cells were collected, lysed, and subject to immunoprecipitation using the M73 α -E1A antibody. Western blots for E1A and endogenous hDREF were subsequently performed.



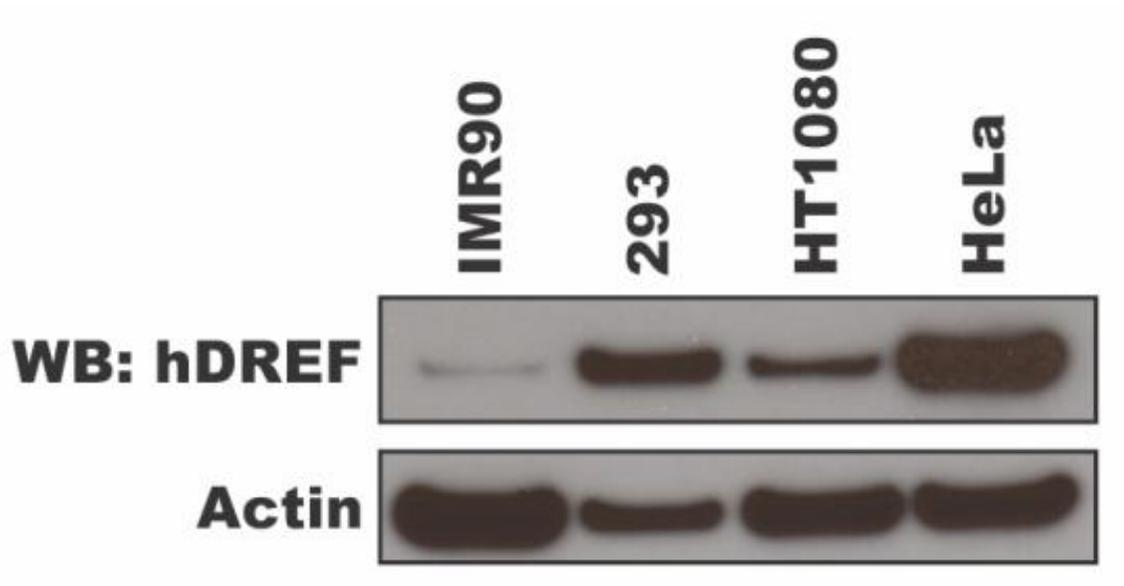
of more refined deletion mutants within these regions would have to be constructed and tested. In lieu of the time restraints, we proceeded to address the second objective of this thesis, and subsequently utilize siRNA against hDREF to specifically address objectives three and four.

3.2 Determining the Effects of hDREF on Transcription *in vitro*

3.2.1 Selecting an Appropriate Cell Line

Previously published luciferase experiments indicated that hDREF was a weak trans-activator in HeLa cells (285, 292). For these experiments, the full length promoters for genes encoding histone H1, RPS6, RPL10A, and RPL12 were cloned into promoter-less and enhancer-less luciferase reporter plasmids. All of the respective promoters contained an hDRE-like sequence, matching 8 to 10 bases of the hDRE consensus. Their results indicated that upon transfection of 100ng of the reporter and 100 to 300ng of a plasmid encoding HA-hDREF, luciferase activity ranged from 1.2 to approximately 2.8 fold higher than when HA-hDREF was not transfected. The authors stated that further investigation of hDREF as a trans-activator was warranted. I decided to repeat one of the previous experiments which examined the histone H1 promoter-containing luciferase reporter. In addition, we performed a series of luciferase assays, in order to examine the trans-activation ability of hDREF through various methods. To select an appropriate cell line, we performed a Western blot on lysates from several asynchronously growing human cell lines (Figure 3.7). Results indicated that out of the four cell lines examined, HeLa cervical carcinoma cells expressed the highest amount of endogenous hDREF, while IMR-90 primary human lung fibroblast cells expressed the least. HT1080 fibrosarcoma cells and transformed HEK293 cells expressed intermediate levels of hDREF. We hypothesized that examining hDREF trans-activation at the histone H1 hDRE-containing promoter using transfection based luciferase assays would yield more significant results in HT1080 cells than HeLa cells. The increased levels of endogenous

Figure 3.7 Comparison of endogenous hDREF levels in asynchronous populations of four cell lines. Asynchronous IMR90, 293, HT1080, and HeLa cells were collected, and lysed. Western blots were performed using α -hDREF and α -actin antibodies.



hDREF in HeLa cells may promote high background activation at hDRE-containing promoters. This could act to obscure any observable differences which would otherwise be obtained from transfecting small amounts of HA-hDREF expression plasmid. Thus we chose to use the easily transfectable HT1080 cell line for all luciferase assays. In addition, all experiments were later repeated separately in HeLa cells, although fewer conditions were tested in each experiment.

3.2.2 Examining hDREF Transactivation of a Simple Gal4 DNA-Binding Domain-Responsive Luciferase Reporter

We initially examined whether hDREF had the ability to act as a trans-activator by recruiting the transcriptional machinery to a minimal promoter. HT1080 cells were co-transfected with increasing amounts of a plasmid encoding Gal4 DNA-binding domain-tagged hDREF (Gal4-DBD-hDREF) and 0.1 μ g of a Gal4-responsive luciferase reporter plasmid (pGL2-(UAS)₆-Luc). The reporter plasmid contains the luciferase gene under the control of a minimal promoter, with six upstream activation sequences (UASs) acting as Gal4 binding sites. A construct encoding Gal4-DBD-tagged amino acids 1-82 of E1A (Gal4-DBD-1-82) was used as a positive control, as the N-terminus of E1A is known to act as a strong trans-activator (166). A construct encoding the Gal4-DBD alone was used as a negative control, since the Gal4-DBD requires an activation domain to trans-activate through the UAS. 24 hours after transfection, cells were collected, lysed, and luciferase activity was measured. Results were initially normalized based on protein concentration as determined by Bradford assays, and then calculated relative to the luciferase activity value of the negative control. When 1.6ng, 8.0ng, 1.0 μ g, 1.5 μ g, or 1.9 μ g of the Gal4-DBD-hDREF plasmid were transfected, significant decreases in luciferase activity relative to the negative control were observed (*=p<0.05; **=p<0.01; ***=p<0.001) (Figure 3.8). A similar lack of activation was observed in HeLa cells; however, no significant reductions in luciferase activity relative to the negative control were observed in these cells (Figure 3.9). In both cell lines, the Gal4-DBD-1-82 fusion activated strongly. Western blots also demonstrated that the Gal4-DBD-hDREF fusion expression

Figure 3.8 Transactivation of a Gal4-DBD-responsive luciferase reporter plasmid containing a minimal promoter by Gal4-DBD-hDREF (HT1080). HT1080 cells were co-transfected with 1.6ng to 1.9 μ g of a plasmid encoding Gal4 DNA-binding domain-tagged hDREF (Gal4-DBD-hDREF) and 0.1 μ g of a Gal4-responsive luciferase reporter plasmid (pGL2-(UAS)₆-Luc). Empty pcDNA3 plasmid was transfected to maintain a total of 2 μ g of transfected plasmid DNA in all samples. 1.9 μ g of a construct encoding Gal4-DBD-tagged amino acids 1-82 of E1A (Gal4-DBD-1-82) was transfected as a positive control. 1.9 μ g of a construct encoding Gal4-DBD alone was used as a negative control. 24 hours after transfection, cells were collected, lysed, and luciferase activity was measured. A) Results were initially normalized based on protein concentration as determined by Bradford assays, and then calculated relative to the luciferase activity present in the negative control. Results are indicative of the means (\pm SEM) of at least three independent experiments (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). B) Western blots were performed using α -Gal4 and α -actin antibodies to examine expression of Gal4-hDREF under each transfection condition.

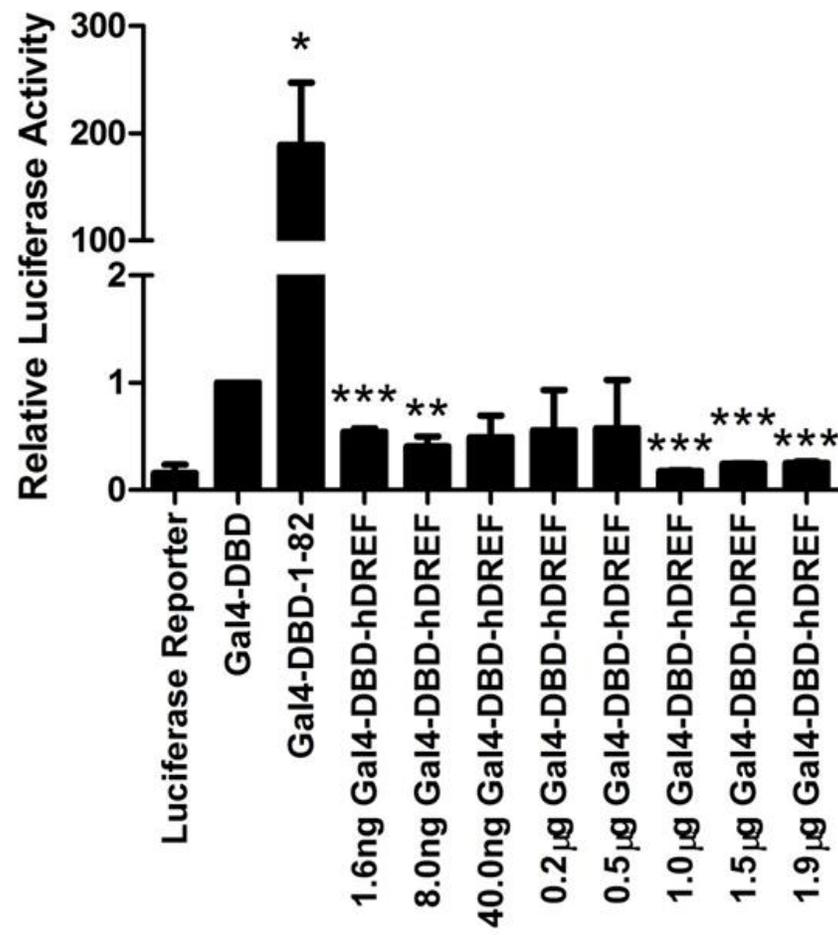
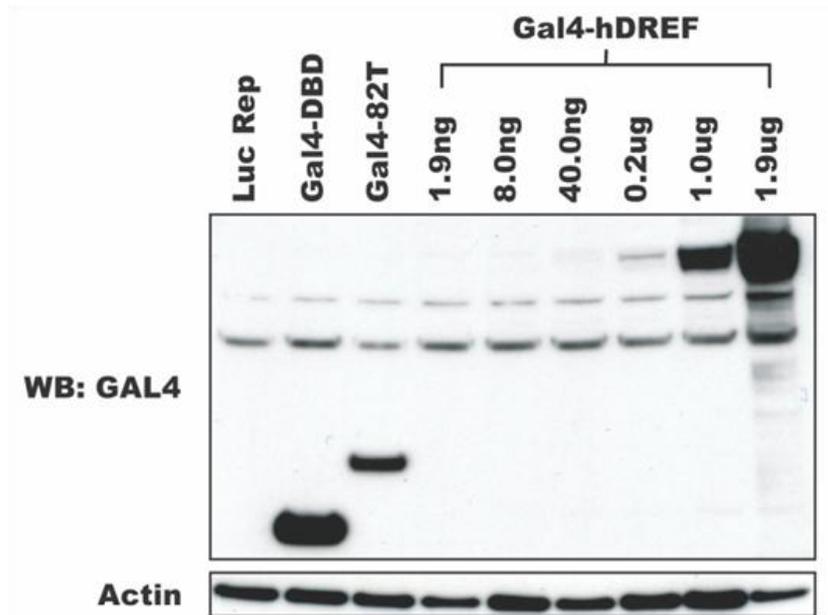
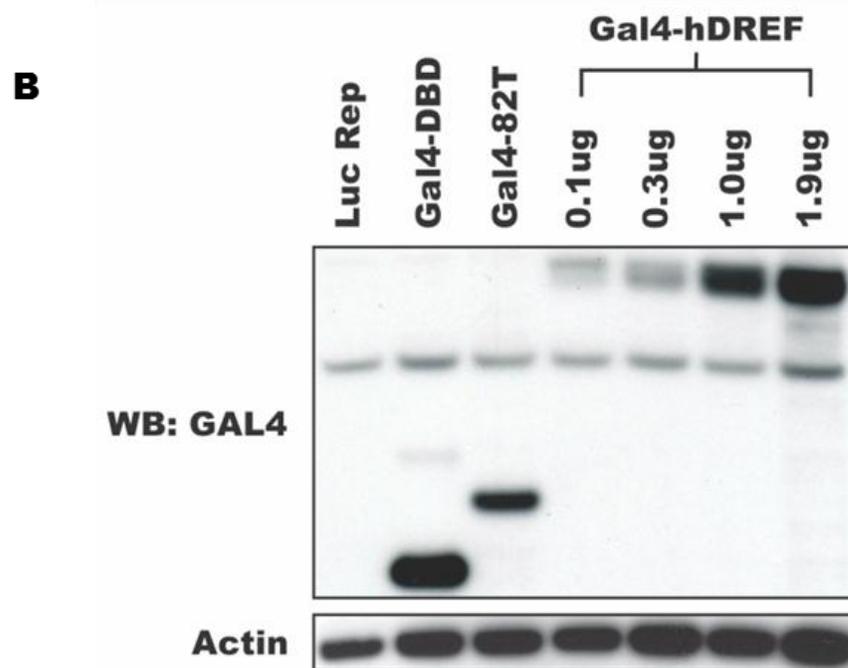
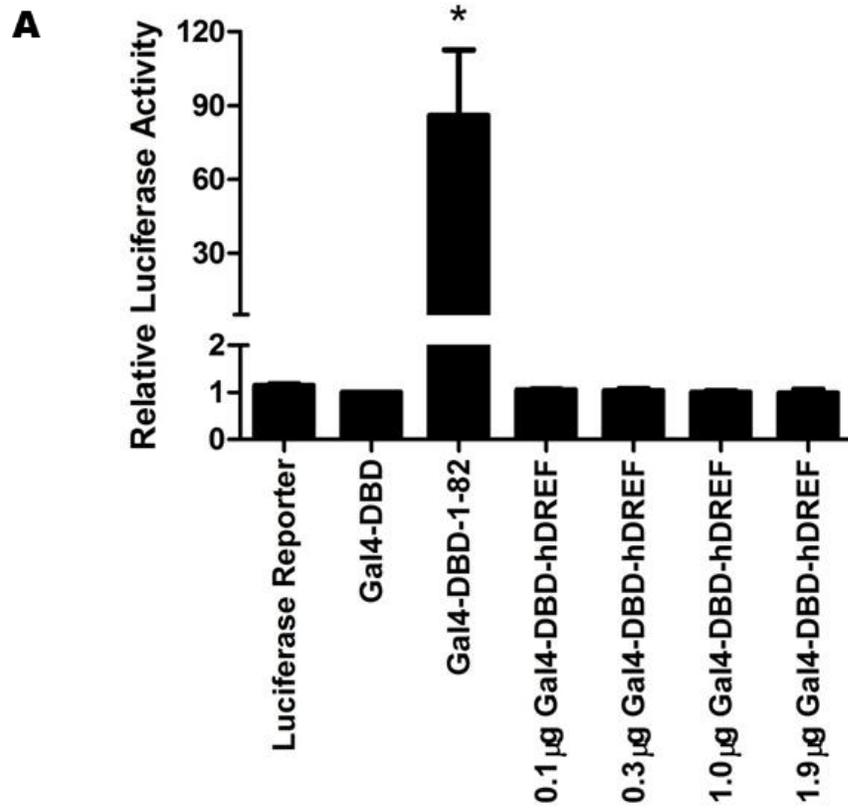
A**B**

Figure 3.9 Transactivation of a Gal4-DBD-responsive luciferase reporter plasmid containing a minimal promoter by Gal4-DBD-hDREF (HeLa). HeLa cells were co-transfected with 0.1 to 1.9 μ g of a plasmid encoding Gal4 DNA-binding domain-tagged hDREF (Gal4-DBD-hDREF) and 0.1 μ g of a Gal4-responsive luciferase reporter plasmid (pGL2-(UAS)₆-Luc). Empty pcDNA3 plasmid was transfected to maintain a total of 2 μ g of transfected plasmid DNA in all samples. 1.9 μ g of a construct encoding Gal4-DBD-tagged amino acids 1-82 of E1A (Gal4-DBD-1-82) was transfected as a positive control. 1.9 μ g of a construct encoding Gal4-DBD alone was used as a negative control. 48 hours after transfection, cells were collected, lysed, and luciferase activity was measured. A) Results were initially normalized based on protein concentration as determined by Bradford assays, and then related to luciferase activity readout of the negative control. Results are indicative of the means (\pm SEM) of at least three independent experiments (* = $p < 0.05$). B) Western blots were performed using α -Gal4 and α -actin antibodies to examine expression of Gal4-hDREF under each transfection condition.



increased in parallel with increased levels of transfected expression vector (Figures 3.8 B and 3.9 B). My results show that hDREF on its own does not have the ability to independently recruit the transcriptional machinery to a minimal promoter and drive luciferase gene expression.

3.2.3 Effect of hDREF on E1A-Induced Transactivation of a Simple Gal4 DNA-Binding Domain-Responsive Luciferase Reporter

Although hDREF did not appear to function as an independent activator of transcription in the experiments described above, it remained possible that its interaction with E1A could modulate E1A's ability to activate transcription. To test this, I next examined whether hDREF specifically enhanced or repressed the ability of Gal4-DBD-tagged E1A trans-activation domains to induce transcription from the pGL2-(UAS)₆-Luc reporter. My previous data indicated that amino acids 1-82 of E1A could bind hDREF strongly, but that CR3 could not bind hDREF strongly (Figure 3.3). Thus we hypothesized that hDREF would enhance Gal4-DBD-tagged transcriptional activation by E1A AA1-82 but not by E1A CR3. HT1080 cells were co-transfected with 0.1µg of the pGL2-(UAS)₆-Luc reporter plasmid, and 0.9µg of plasmids encoding either Gal4-DBD-1-82, Gal4-DBD-CR3, or Gal4-DBD alone, along with increasing amounts of pcDNA3-HA-hDREF plasmid. A plasmid encoding pRb was used as a co-transfected negative control with Gal4-DBD-1-82, as pRb interacts with the N-terminus of E1A and is a transcriptional repressor (306, 307). 24 hours after transfection, cells were collected, lysed, and luciferase activity was measured. Results were initially normalized based on protein concentration as determined by Bradford assays, and then calculated relative to the luciferase activity value of the Gal4-DBD control. Transfection of 1.0µg of pcDNA3-HA-hDREF resulted in significant 3.5 fold (p=0.0421) and 8.2 fold (p=0.0225) reductions of AA1-82 and CR3-induced luciferase activity respectively (Figure 3.10). When lower amounts of hDREF plasmid were transfected, reductions in AA1-82-induced luciferase activity were not observed; however, non-significant concentration-dependent decreases to CR3-induced luciferase activity were consistently observed. In addition,

transfection of 1.0 μ g of pcDNA3-HA-hDREF plasmid significantly reduced the background luciferase activity induced by the Gal4-DBD alone by 1.9 fold ($p=0.0041$). Similar trends were observed in HeLa cells, however no significant changes in transcriptional activation were detected (Figure 3.11). My results indicate that hDREF non-specifically reduces the ability of AA1-82 and CR3 of E1A to activate transcription of a minimal promoter in a concentration-dependent manner.

3.2.4 Evaluating hDREF Transactivation of a Luciferase Gene Under Control of the hDRE-Containing Histone H1 Promoter

My previous results indicated that hDREF does not have the ability to trans-activate a minimal promoter (Figure 3.8/3.9), and that transfection based overexpression of hDREF resulted in a reduction of trans-activation by AA1-82 and CR3 of E1A at a minimal promoter, which occurred irrespective of their individual ability to bind hDREF (Figure 3.10/3.11). It is important to note, however, that the minimal promoter in the pGL2-(UAS)₆-Luc reporter contains only a TATA box. To complete our assessment of the effects of hDREF on transcription *in vitro*, we chose to replicate the original experiment published by Ohshima *et al.*, which examined hDREF transactivation through the full length histone H1 promoter (285). Since the histone H1 promoter contains an hDRE site which matches all ten bases of the consensus sequence, it could potentially provide a more accurate depiction of the role of hDREF in trans-activation than the artificial context of a minimal promoter.

I cloned the histone H1 promoter (-537bp to -1bp) from HeLa cells, into a promoter-less and enhancer-less luciferase reporter plasmid to create pGL3-H1p-Luc as previously described in section 2.2. HT1080 cells were then transfected with 0.1 μ g of the pGL3-H1p-Luc reporter, and increasing amounts of pcDNA3-HA-hDREF. 24 hours after transfection, cells were collected, lysed, and luciferase activity was measured. Results were initially normalized based on protein concentration as determined by Bradford assays, and then calculated relative to the luciferase activity value of the sample containing the reporter plasmid alone. Consistent with previously published data, my

Figure 3.10 Effects of hDREF on the ability of Gal4-DBD-tagged amino acids 1-82 and CR3 of E1A to transactivate a Gal4-DBD-responsive luciferase reporter plasmid (HT1080). HT1080 cells were co-transfected with 0.1 μ g of a Gal4-responsive luciferase reporter plasmid (pGL2-(UAS)₆-Luc) and either: 1.0ug Gal4-DBD plasmid alone, 1.0ug Gal4-DBD-1-82 plasmid, or 1.0ug Gal4-DBD-CR3 plasmid along with 0-1.0ug of pcDNA3-HA-hDREF. Empty pcDNA3-HA plasmid was transfected to maintain a total of 2 μ g of transfected plasmid DNA in all samples. 1.0 μ g of a plasmid encoding pRb was used as a co-transfected negative control with Gal4-DBD-1-82. 24 hours after transfection, cells were collected, lysed, and luciferase activity was measured. Results were initially normalized based on protein concentration as determined by Bradford assays, and then related to luciferase activity readout of the Gal4-DBD negative control. Results are indicative of the means (\pm SEM) of at least three independent experiments (* = p<0.05; ** = p<0.01).

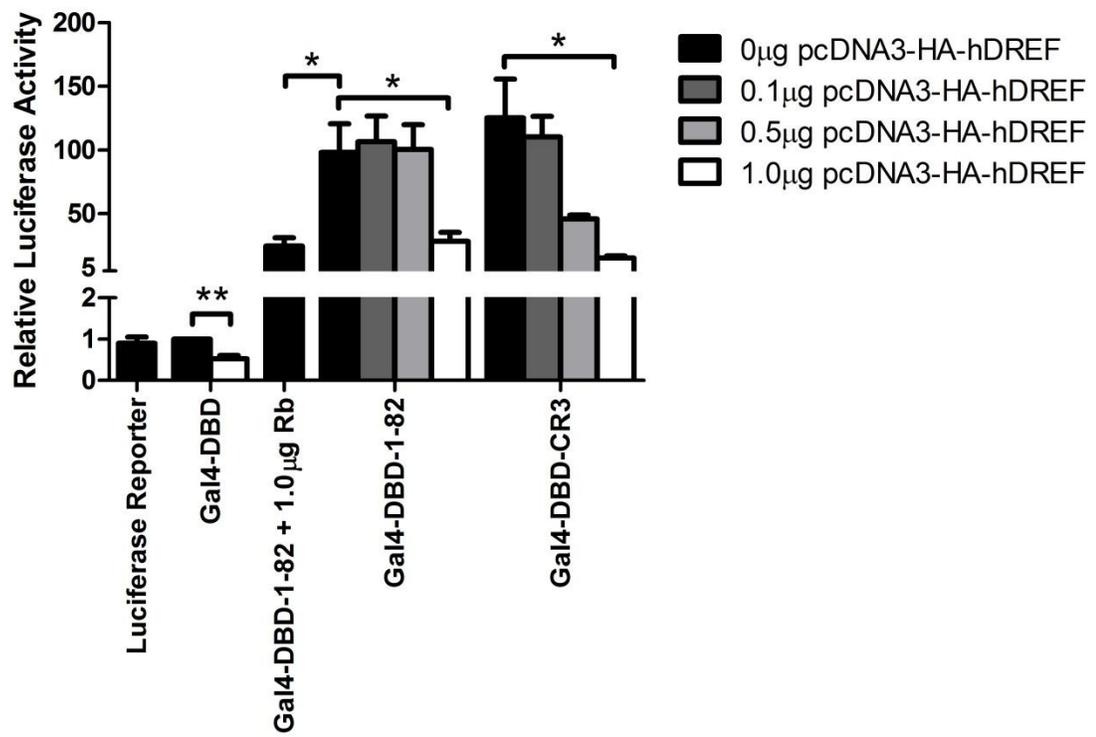
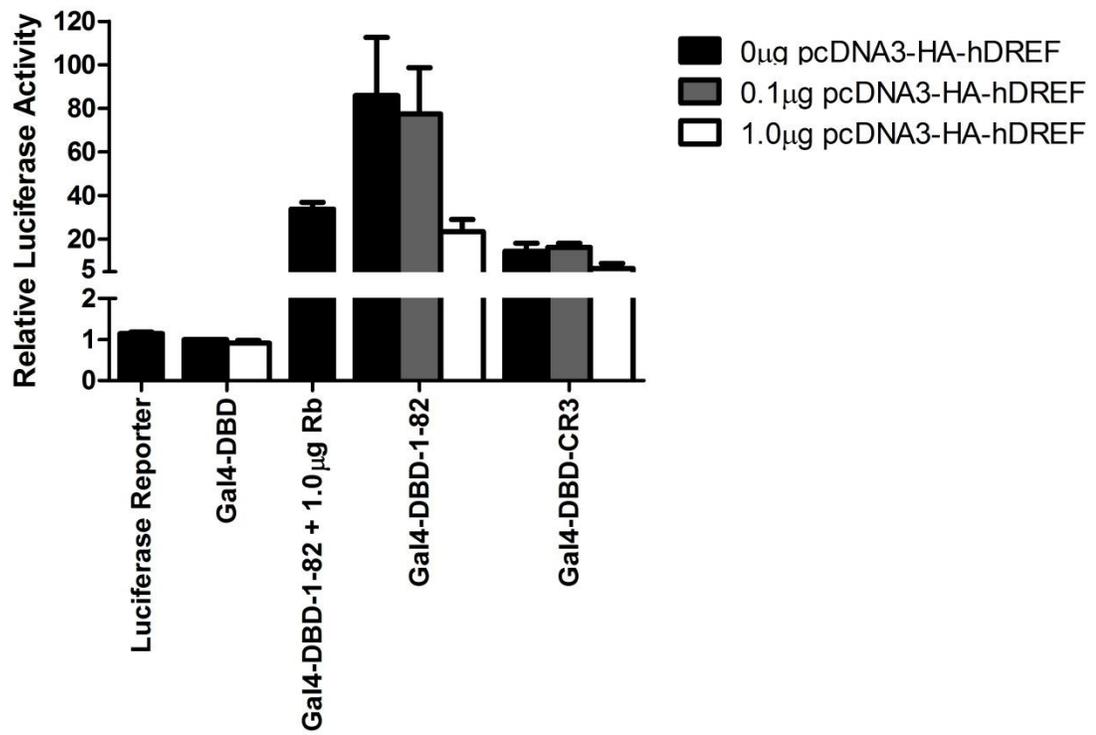


Figure 3.11 Effects of hDREF on the ability of Gal4-DBD-tagged amino acids 1-82 and CR3 of E1A to transactivate a Gal4-DBD-responsive luciferase reporter plasmid (HeLa). HeLa cells were co-transfected with 0.1 μ g of a Gal4-responsive luciferase reporter plasmid (pGL2-(UAS)₆-Luc) and either: 1.0ug Gal4-DBD plasmid alone, 1.0ug Gal4-DBD-1-82 plasmid, or 1.0ug Gal4-DBD-CR3 plasmid along with 0-1.0ug of pcDNA3-HA-hDREF. Empty pcDNA3-HA plasmid was transfected to maintain a total of 2 μ g of transfected plasmid DNA in all samples. 1.0 μ g of a plasmid encoding pRb was used as a co-transfected negative control with Gal4-DBD-1-82. 48 hours after transfection, cells were collected, lysed, and luciferase activity was measured. Results were initially normalized based on protein concentration as determined by Bradford assays, and then related to luciferase activity readout of the Gal4-DBD negative control. Results are indicative of the means (\pm SEM) of at least three independent experiments.



results indicated that hDREF can act as a weak trans-activator at the histone H1 promoter, significantly increasing luciferase activity by 1.21 fold ($p=0.0362$), and 1.11 fold ($p=0.005$) over basal levels when 0.2 and 0.3 μg of pcDNA3-HA-hDREF plasmid were transfected respectively (Figure 3.12A). However, the previous experiments never examined the effects of transfecting more than 0.3 μg of the pcDNA3-HA-hDREF plasmid. My results indicate a significant 1.4 fold ($p=0.0208$) and 4 fold ($p<0.0001$) decrease in luciferase activity compared to basal levels when 1.0 and 1.9 μg of pcDNA3-HA-hDREF plasmid were transfected respectively. A similar trend was observed in HeLa cells (Figure 3.13). A 1.15 fold increase, and a significant 3.3 fold decrease ($p=0.0012$) in luciferase activity compared to basal levels were observed when 0.3 μg and 1.9 μg of pcDNA3-HA-hDREF plasmid were transfected into HeLa cells respectively. Next, I tried increasing the transfected amount of pGL3-H1p-Luc reporter plasmid by 10 fold in HT1080 cells, to assess whether concentration of the reporter was the limiting factor in luciferase production and activity (Figure 3.12C). However, my results indicated a similar trend, whereby increasing the amount of transfected pcDNA3-HA-hDREF significantly reduced luciferase activity.

In summary, my *in vitro* luciferase assay results suggest that hDREF does not possess the ability to independently trans-activate at a basic minimal promoter, but does weakly trans-activate at the hDRE-containing histone H1 promoter when small amounts of pcDNA3-HA-hDREF plasmid are transfected into cells. At high concentrations of transfected pcDNA3-HA-hDREF plasmid, two observable events occur. Firstly, a drastic reduction in trans-activation at the histone H1 promoter is detected. Second, the abilities of E1A AA1-82 and CR3 to trans-activate through a minimal promoter are significantly reduced. Although the results of these luciferase assays served to provide initial insight into the effects of hDREF on transcription, it is important to note that luciferase assays by nature can be highly artificial. This is due to the fact that they are transfection-based experiments, and thus rely heavily on the overexpression of proteins which could prove toxic to cells.

Figure 3.12 Effect of hDREF on transactivation of a luciferase reporter plasmid under the control of the histone H1 promoter (HT1080). HT1080 cells were co-transfected with either 0.1 μ g (A) or 1.0 μ g (C) of the pGL3-H1p-Luc reporter plasmid and 0-1.9 μ g of pcDNA3-HA-hDREF. Empty pcDNA3-HA plasmid was transfected to maintain a total of 2 μ g of transfected plasmid DNA in all samples. 24 hours after transfection, cells were collected, lysed, and luciferase activity was measured. Results were initially normalized based on protein concentration as determined by Bradford assays, and then related to luciferase activity readout of the pGL3-H1p-Luc reporter alone. Results are indicative of the means (\pm SEM) of at least three independent experiments (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). B) Western blots were performed using α -HA, α -hDREF and α -actin antibodies to examine protein expression.

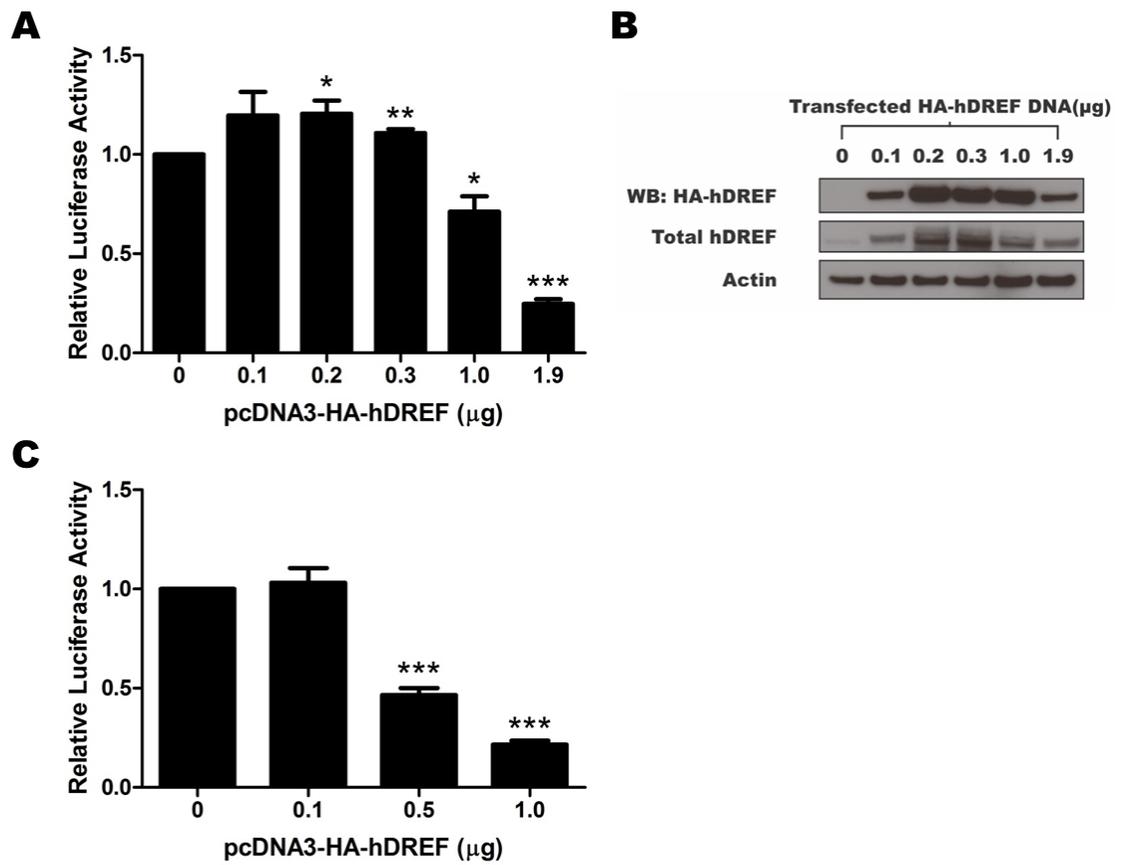
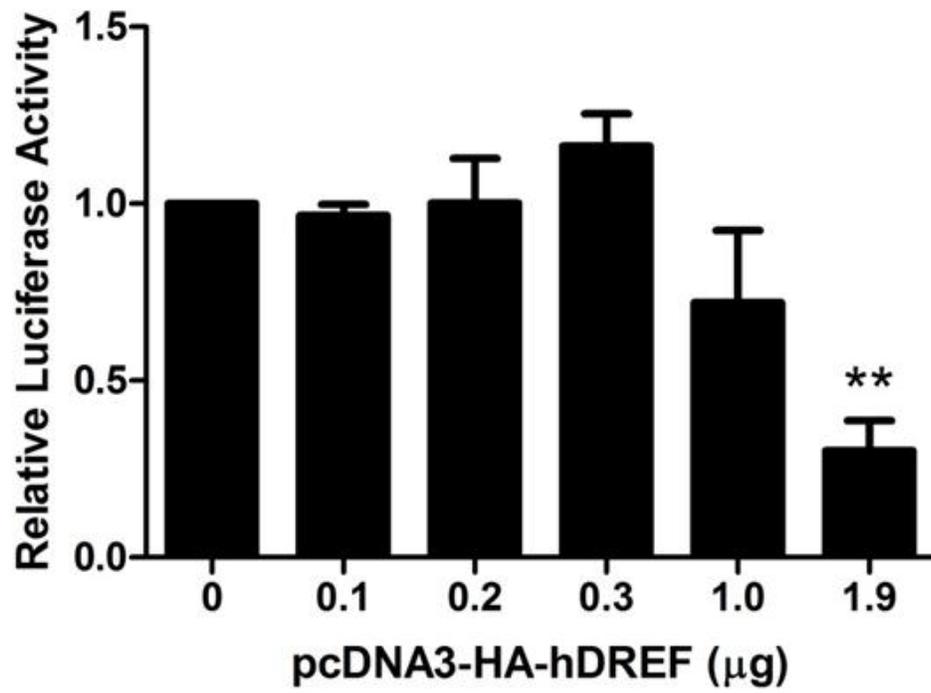
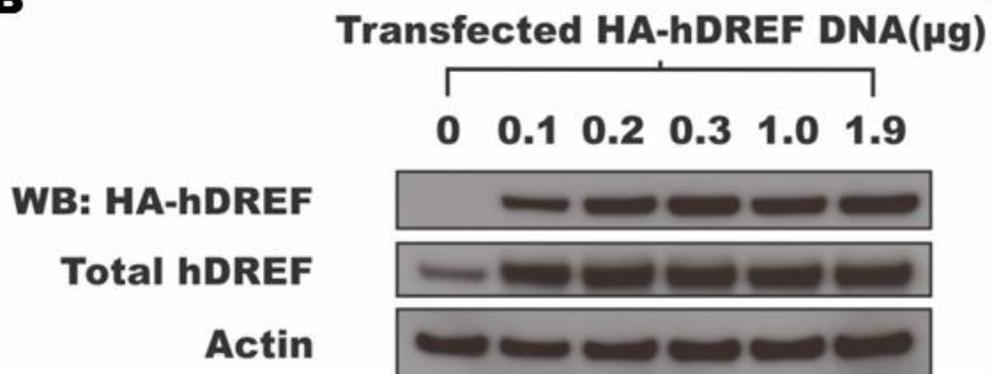


Figure 3.13 Effect of hDREF on transactivation of a luciferase reporter plasmid under the control of the histone H1 promoter (HeLa). HeLa cells were co-transfected with either 0.1 μ g of the pGL3-H1p-Luc reporter plasmid and 0-1.9 μ g of pcDNA3-HA-hDREF. Empty pcDNA3-HA plasmid was transfected to maintain a total of 2 μ g of transfected plasmid DNA in all samples. 48 hours after transfection, cells were collected, lysed, and luciferase activity was measured. A) Results were initially normalized based on protein concentration as determined by Bradford assays, and then related to luciferase activity readout of the pGL3-H1p-Luc reporter alone. Results are indicative of the means (\pm SEM) of at least three independent experiments (** = $p < 0.01$). B) Western blots were performed using α -HA, α -hDREF and α -actin antibodies to examine protein expression.

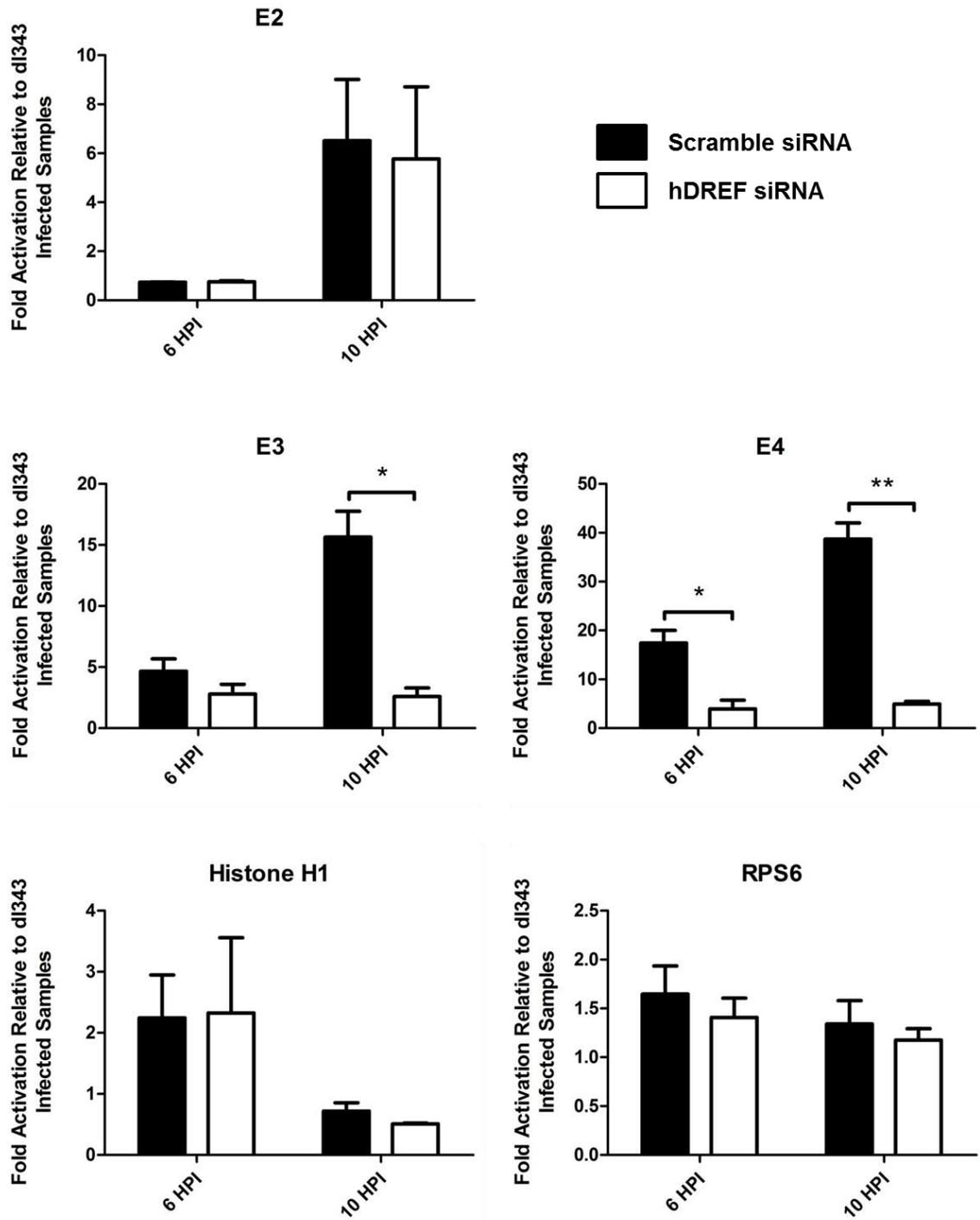
A**B**

3.3 The Effects of hDREF on Viral and Cellular Gene Transcription During an HAdV-5 Infection

To better understand the effects of hDREF on viral and cellular gene transcription in a dynamic system, I decided to utilize an HAdV-5 infection model. HeLa cells were transfected with a combination of hDREF specific siRNAs or scramble siRNA and incubated for 72 hours. On average, a 40% knockdown of hDREF was achieved. We then infected the cells at an MOI of 5 PFU per cell, with HAdV-5 mutants expressing either wild-type E1A (dl309) or E1A that is non-functional due to a frameshift after the codon for amino acid 20 (dl343). At 6 and 10 HPI, total RNA was extracted using Trizol reagent. RNA was reversed transcribed to cDNA, and specific transcripts were quantified using RT-PCR. Results were analyzed using the $2^{-(\Delta\Delta CT)}$ method, normalizing values first to GAPDH and then to dl343-infected samples (Figure 3.14). No significant differences in activation of the E2 early gene were detected between the hDREF knockdown and scramble siRNA-treated samples. However, knockdown of hDREF resulted in decreased activation of the E3 early gene at both time points, with a significant 6 fold decrease ($p=0.0282$) at 10 HPI. Interestingly, hDREF knockdown resulted in significant decreases in activation of the E4 early gene at both time points. A significant 4.4 fold decrease ($p=0.0497$) in E4 activation was observed at 6 HPI, and a significant 7.8 fold decrease ($p=0.0099$) was detected at 10 HPI. No significant differences were observed between the hDREF knockdown and scramble siRNA-treated samples for activation of the cellular histone H1 and RPS6 genes, which contain promoters with hDRE-like sequences. Interestingly however, my results seem to indicate that E1A affects transcription of both of these cellular genes at 6 HPI, as activation of these genes was from 1.4 to 2.3 fold higher than in dl343 infected cells, regardless of the hDREF phenotype.

The results of my quantitative RT-PCR experiments carried out in a HeLa cell infection model indicate that hDREF plays a significant role in activation of the HAdV-5 early genes E3 and E4. However, 40% knockdown of hDREF does not affect activation of viral E2 early, or cellular histone H1 and RPS6 genes during infection.

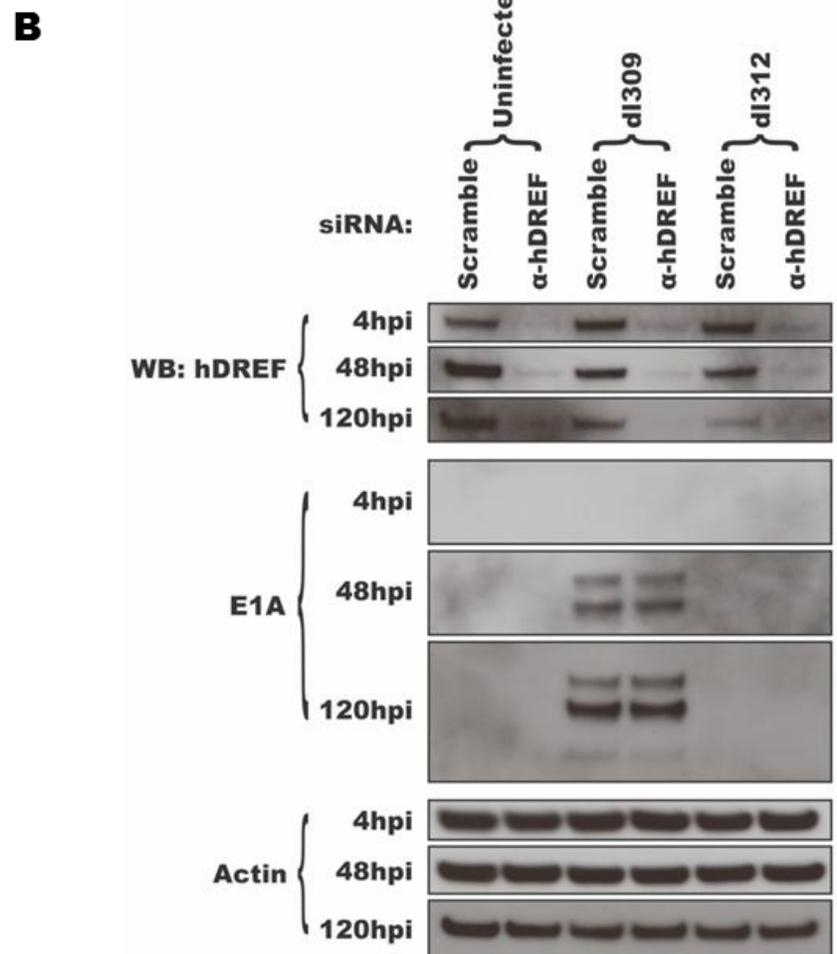
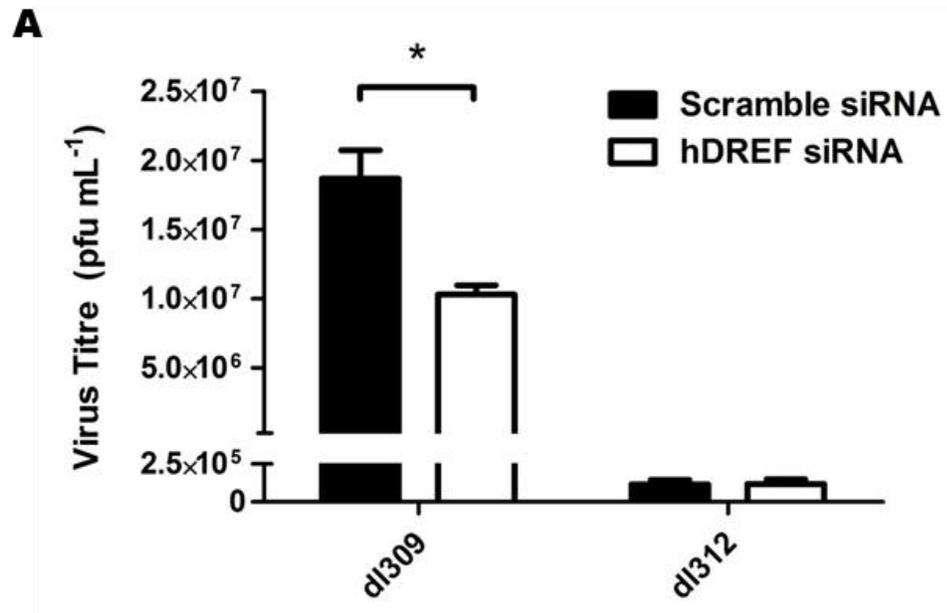
Figure 3.14 Effects of hDREF on early viral gene and cellular gene transcription during an HAdV-5 infection. HeLa cells were transfected with a combination of hDREF specific siRNAs or scramble siRNA and incubated for 72 hours, yielding 40% knockdown on average. Subsequently cells were infected at an MOI of 5 PFU per cell, with HAdV-5 mutants expressing either wild-type E1A (dl309) or E1A that is non-functional due to a frameshift after the codon for amino acid 20 (dl343). Total RNA was extracted at 6 and 10 HPI, and 1 μ g was reverse transcribed to cDNA. HAdV-5 E2, E3, and E4 as well as cellular histone H1 and RPS6 transcripts were quantified by RT-PCR. Results were analyzed using the $2^{-(\Delta\Delta CT)}$ method, normalizing values first to GAPDH and then to dl343-infected samples. Results are indicative of the means (\pm SEM) of at least three independent experiments (* = $p < 0.05$; ** = $p < 0.01$) and are expressed as fold activation of specific genes in dl309-infected samples relative to dl343-infected samples.



3.4 The Effect of hDREF on Viral Replication

Since hDREF significantly affected viral early gene transcription, I decided to examine whether hDREF played a role in overall viral replication. We chose to use IMR-90 primary human lung fibroblasts for studies of viral replication, as they can be easily growth arrested and have been used in previous viral replication studies (77, 78). IMR-90 cells were transfected with a combination of hDREF specific siRNAs or scramble siRNA, and were contact inhibited for 3 days after reaching confluence. The growth arrested cells were then infected at an MOI of 5 PFU per cell with HAdV-5 mutants expressing either wild-type E1A (dl309) or no E1A (dl312). Viruses were collected at 4, 48, and 120 HPI, and serial dilutions of the virus were applied to monolayers of HEK293 cells to assess viral titre via plaque assay. Only data for 120 HPI are shown, as no significant differences in viral titre were observed between dl309 viral samples collected at 4 or 48 HPI (Figure 3.15 A). In addition, the viral titre of dl312 remained at approximately 1.15×10^5 PFU per mL at 4, 48 (Data not shown) and 120 HPI, regardless of hDREF expression (Figure 3.15 B). This indicated that the dl312 virus did not replicate in growth arrested cells as expected, due to a lack of E1A. A significant 1.8 fold decrease ($p=0.0176$) in dl309 viral titre at 120 HPI was detected when samples were treated with hDREF siRNA versus scramble siRNA (Figure 3.15 A), coinciding with an approximate 100% knockdown of hDREF (Figure 3.15 B). Therefore, these results indicate that knockdown of hDREF reduces the overall yield of virus and likely plays a significant role in HAdV-5 replication.

Figure 3.15 Effects of hDREF on HAdV-5 replication. IMR-90 cells were transfected with a combination of hDREF specific siRNAs or scramble siRNA, and then growth to confluence. Cells were incubated for 3 days after reaching confluence, to arrest their growth due to contact inhibition. Subsequently cells were infected at an MOI of 5 PFU per cell, with HAdV-5 mutants expressing either wild-type E1A (dl309) or no E1A (dl312). Viruses were collected at 4, 48 and 120 HPI, and serial dilutions of the virus were applied to monolayers of HEK293 cells to assess viral titre via plaque assay. A) Results for viral samples collected at 120 HPI are shown, and are indicative of the mean viral titres (\pm SEM) of at least three independent experiments (* = $p < 0.05$). B) Western blots were performed using α -hDREF, α -E1A, and α -actin antibodies. Approximately 100% knockdown of hDREF was observed in each sample, at every time points.



4 Discussion

4.1 Mapping the Interaction Between E1A and hDREF

Utilization of co-immunoprecipitation techniques revealed that there is an interaction between 13S E1A and hDREF, and that this interaction is conserved amongst representative serotypes of HAdV species A through E – HAdV 12, 3, 5, 9, and 4 respectively – but not HAdV-40 of species F. My results also indicate that all five isoforms of HAdV-5 E1A – 13S, 12S, 11S, 10S, and 9S – can bind hDREF. Finally, I discovered that the N-terminal 29 amino acids of E1A form a primary binding site for hDREF and that residues 15-26 appear sufficient for binding. Indeed all of my results support this conclusion, as only the first 26 amino acids are common amongst all isoforms of E1A, and a deletion of residues 1-14 does not affect hDREF binding. The final goal of my mapping experiments was to identify specific amino acids in E1A which are necessary for the hDREF interaction, and mutate those residues to create an HAdV-5 derivative encoding an E1A mutant unable to bind hDREF. This virus would allow for careful study of the effects of the E1A-hDREF interaction on viral and cellular events. Unfortunately, due to time constraints, I was unable to identify which specific individual residues of the identified region are necessary for binding hDREF.

A future direction of this project will likely involve the identification of essential residues for hDREF binding. To aid in this process I generated a sequence alignment of residues 15-26 from 13S E1A of HAdV-12, 3, 5, 9, 4, and 40 using Clustal-W (Figure 4.1). Amino acids 19 and 24 are highly conserved in all serotypes, and therefore would be candidate residues to test for deficiencies in binding using point mutations. Interestingly, residues 20 and 21 are highly conserved in species A through E but not F. Since HAdV-40 of species F does not bind hDREF, these residues – which change an aliphatic amino acid to an aromatic one, and an acidic residue to an uncharged residue – may prove to strongly contribute to hDREF binding. The leucine and isoleucine residues at positions 19, 20, 23, and 24 in HAdV-5 E1A have previously been shown to be essential for

interactions with various cellular factors including p300, CBP, TBP, pCAF, and GCN5, as mutation of any one of these residues to alanine severely reduces binding to each protein (308). If any of these four residues is discovered to be essential for hDREF binding, creating a virus encoding E1A that is specifically deficient in hDREF binding for use in functional studies may prove problematic. Co-disruption of the interactions with hDREF as well as other trans-activators like TBP and p300 would preclude the ability to isolate the specific consequences of the E1A-hDREF interaction. It may be possible to instead map the interaction to specific regions of hDREF, as the two major functional and structural domains of the protein have been mapped and therefore mutations in these areas can be avoided (286). The inherent problem with this is that because hDREF is still poorly characterized, deletions in certain areas of the protein could alter its normal function without any possible way of knowing that this had occurred. This would also make it difficult to determine the exact effects of the E1A-hDREF interaction.

To conclusively test whether residues 15-26 are necessary for the hDREF interaction, a full length 13S E1A with residues 15-26 deleted would have to be constructed and tested. However, although residues 15-26 are sufficient for binding, they may not all be required and additional residues outside this region may contribute to binding. Amino acid 26 of E1A is not highly conserved amongst representative members of HAdV species A through F (Figure 4.1). Thus, it is unlikely that deletion of only residue 26 in this binding site would cause such a drastic decrease in hDREF binding, or that deletion of residues 15-25 would still allow for hDREF binding if this were the correct scenario (Figures 3.5 and 3.6). A more probable interpretation of the data is that several residues both within, and external to the 15-26 region act in a cooperative fashion to bind hDREF. This would serve to explain why deletion of residues 15-25 or 26-35 reduces hDREF binding, but does not completely inhibit it. Indeed residues 16-28 are predicted to form an amphipathic α -helix in HAdV-5 (78). Therefore it is possible that deletions or mutations of individual or small groups of residues within this structure act to reduce, but not completely eliminate hDREF binding. Perhaps a deletion of residues 16-28 entirely or

Figure 4.1 Alignment of E1A residues 15-26. Amino acids 15 to 26 of E1A from representative serotypes of HAdV species A through F were aligned using Clustal-W. Darker shaded boxes indicate more conserved residues. Amino acids 20 and 21 of HAdV-40 E1A are highlighted in red to indicate extreme dissimilarity from the highly conserved residues present in the E1A proteins of HAdV-12, 3, 5, 9, and 4.

mutations of key residues to disrupt the amphipathic characteristics of the helix would completely abolish hDREF binding.

Although I have shown an interaction with hDREF and E1A, it is important to note that it has not yet been determined as to whether this interaction is direct or indirect. Since hDREF is a transcriptional regulator and the N-terminus of E1A has been shown to interact with an extensive number of transcriptional regulators, it is entirely possible that E1A indirectly binds hDREF through other factors (214, 308). Indeed, hDREF has been shown to be involved in high molecular weight multiprotein complexes, although the identities of other members of these complexes are unknown (286). To test whether the interaction is indirect or direct, co-immunoprecipitation experiments using purified recombinant E1A and hDREF could be performed. Finally – direct or not – an intriguing future direction of this project would be to identify factors which co-immunoprecipitate in complexes with hDREF and observe whether the identities of factors in these complexes are altered in the presence of E1A. This would not be surprising as E1A alters the abilities of pRb, CtBP, and hBre1 to interact with their normal cellular targets (175, 199, 214). Such studies could provide advanced insight into the normal function of hDREF, as well as the purpose of the hDREF manipulation by E1A.

4.2 Analysis of the Effects of hDREF on Transcription using Luciferase Assays

Based on transient luciferase assays in both HT1080 and HeLa cells, it appears that hDREF does not possess the capability to trans-activate at a minimal promoter containing only a TATA box. In addition, when large amounts of pcDNA3-HA-hDREF plasmid are co-transfected with plasmids encoding Gal4-DBD-tagged AA1-82 or CR3 of E1A, the abilities of these known trans-activators to drive luciferase expression through a minimal promoter are significantly reduced. Consistent with previously published results, hDREF can weakly trans-activate at the hDRE-containing histone H1 promoter when small amounts of pcDNA3-HA-hDREF plasmid are transfected into cells (285). However, at

higher amounts of transfected pcDNA3-HA-hDREF, significant reductions in trans-activation at this promoter were detected.

4.2.1 Gal4-DBD-hDREF Trans-activation of the pGL2-(UAS)₆-Luc Reporter

My observation that Gal4-DBD-hDREF cannot drive expression of the pGL2-(UAS)₆-Luc reporter is very surprising, as it indicates that hDREF lacks the ability to bind and recruit the general transcription factors (GTFs) and RNA polymerase II to the TATA box. Although approximately 76% of human genes lack a TATA box within their core promoter regions, the recruitments of TBP and the rest of the transcription factor II D (TFIID) complex, as well as the other GTFs, are still required to drive expression of these genes (309, 310). Previously published results indicate that hDREF trans-activates – albeit weakly – several genes containing hDRE sequences in their promoter (285, 292). Therefore it seems unlikely that hDREF would be unable to bind at least one general transcription factor to enhance transcription at hDRE-containing genes. Two potential mechanisms may serve to explain the observed results. First, the action of hDREF binding to DNA may serve to induce a conformational change in hDREF, revealing a binding site for TBP or other GTFs. Indeed, conformational changes have been observed in the PhoB transcription factor upon DNA binding, although whether this affects its function or not is unknown (311). In this respect, the Gal4-DBD-hDREF fusion protein would not be able to trans-activate the pGL2-(UAS)₆-Luc reporter, because although it contains Gal4 binding sites, this reporter does not contain an hDRE. To address this hypothesis, a modified luciferase reporter could be constructed, which inserts single, or multiple hDRE sites in tandem upstream of the minimal promoter instead of the existing Gal UASs. One would then expect that trans-activation of the luciferase gene would increase as more hDRE elements were added.

A second explanation is that the fusion of a Gal4-DBD domain to the N-terminus of hDREF inherently prevents activation of the reporter plasmid via steric hindrance. This explanation revolves around the discovery that self-association of hDREF via the C-

terminal hATC domain is necessary for multiple functions of hDREF including DNA binding (286). The fusion of a Gal4-DBD domain to the N-terminus of hDREF could prevent hDREF from properly dimerizing. If dimerization is required for hDREF to interact with the GTFs, this would completely abolish trans-activation. The opposite scenario is also a logical explanation. Since the Gal4-DBD must dimerize to bind DNA, self-association of hDREF could preclude Gal4-DBD dimerization due to steric hindrance, or due to dimerization between Gal4-DBD-hDREF and endogenous hDREF. This would prevent targeting of the Gal4-DBD-hDREF protein to the UAS, resulting in a lack of luciferase expression. In addition, the overexpressed, undirected Gal4-DBD-hDREF protein would be free to bind and sequester putatively targeted GTFs, thereby reducing trans-activation of the pGL2-(UAS)₆-Luc reporter below basal levels, as observed in Figure 3.8.

4.2.2 Alteration of Gal4-DBD-1-82 and Gal4-DBD-CR3 Trans-activation of the pGL2-(UAS)₆-Luc Reporter

Contrary to our hypothesis that the addition of hDREF would enhance Gal4-DBD-tagged transcriptional activation by E1A AA1-82, but not by E1A CR3, it actually appeared to reduce trans-activation by both (Figures 3.11 and 3.12). Importantly, trans-activation by the negative control Gal4 vector was also reduced when the same amount (1 μ g) of pcDNA3-HA-hDREF plasmid was added. This indicates that the apparent repression of transcription occurred in a non-specific fashion. Causation could be similar to what may have been occurring in experiments examining Gal4-DBD-hDREF trans-activation of the same pGL2-(UAS)₆-Luc reporter. It is possible, but unlikely, that the small eight amino acid HA-tag interferes with hDREF dimerization; however, the answer may be that hDREF must bind DNA through the hDRE to induce a self-conformational change, allowing for subsequent GTF binding. This scenario would preclude the ability to identify any interaction specific effects between E1A AA1-82 and hDREF on trans-activation of the luciferase gene, due to a lack of an hDRE in the pGL2-(UAS)₆-Luc reporter. If this explanation is correct, addressing the original hypothesis that hDREF enhances E1A AA1-82 trans-activation but not CR3 trans-activation, would prove

difficult. Since the Gal4-DBD-tagged E1A constructs and HA-hDREF would each target the UAS and hDRE sequences independently in a modified pGL2-(UAS)₆-Luc reporter containing an hDRE, it would be impossible to distinguish whether differences in E1A-induced trans-activation were due to an interaction with hDREF or not. To circumvent this issue, it could be possible to instead examine whether the addition of untagged E1A AA1-82 or CR3 could enhance trans-activation of an hDRE-containing luciferase reporter plasmid. In fact, this is a future direction of the project. Since I already constructed the pGL3-H1p-Luc reporter plasmid, and confirmed previous reports that hDREF can trans-activate at the histone H1 hDRE-containing promoter (285), this experiment could be easily carried out. By co-transfecting reporter, and increasing amounts of untagged AA1-82 or CR3 of E1A, one could observe how the interaction of hDREF with the N-terminus of E1A affects hDREF-induced trans-activation of the H1 promoter.

A second explanation for the non-specific reduction in E1A AA1-82 and CR3-induced trans-activation upon addition of large amounts of pcDNA3-HA-hDREF plasmid is more likely. Perhaps – due to overexpression – the HA-hDREF protein saturates the cellular transcription machinery, binding and sequestering a single, or multiple, rate limiting GTFs, such that they are no longer available for trans-activation of the minimal promoter. This would best explain why addition of 1 μ g of pcDNA3-HA-hDREF plasmid reduces the background activation of luciferase by the Gal4-DBD protein alone, which should not bind hDREF, and on its own does not trans-activate. If this rationale is correct, a combination of several overexpression-related mechanisms may cumulatively contribute to explain why enhanced trans-activation is not apparent when hDREF is co-transfected with Gal4-DBD-AA-1-82 of E1A. As previously mentioned, the first explanation is sequestration of rate limiting GTFs by hDREF. The second explanation is that unbound hDREF protein may compete for E1A binding with hDREF protein that is bound to GTFs. Finally, since hDRE elements are not present in the pGL2-(UAS)₆-Luc reporter plasmid but are present on the chromosomes found in human HT1080 and HeLa cells, it is entirely possible that hDREF is directing transcriptional complexes containing E1A AA1-82 to these promoters, instead of the Gal4-DBD directing the complexes to the

pGL2-(UAS)₆-Luc reporter. The latter two mechanisms would serve to explain the reduction in apparent trans-activation by Gal4-DBD-AA1-82, but not Gal4-DBD-CR3 or Gal4-DBD alone. The first mechanism would explain reductions in all samples.

In retrospect, two extra controls would have helped to elucidate what is happening to the pGL2-(UAS)₆-Luc reporter system upon addition of large amounts of pcDNA3-HA-hDREF plasmid. The pRb tumour suppressor has previously been shown to interact with CR1 and CR2 of E1A, but not CR3 (166). As expected, when I co-transfected 1µg of a plasmid encoding pRb with one encoding Gal4-DBD-1-82, a reduction in trans-activation was observed (Figures 3.10 and 3.11). Co-transfection of the pRb plasmid with the Gal4-DBD-CR3 plasmid would have strengthened the argument that the results observed upon addition of hDREF were occurring in a non-specific fashion. pRb would be expected to have no effect on CR3-induced trans-activation, unless it too was sequestering rate limiting transcriptional machinery in a concentration dependent manner, thereby indicating an inherent problem with utilizing such high amounts of transfected plasmid DNA. Secondly, Western blots of lysates used in these experiments would have helped to understand the effects of hDREF concentration on E1A-induced trans-activation. Finally, it is entirely possible that my choice of using 0.1, 0.5 and 1.0µg of transfected pcDNA3-HA-hDREF completely excluded the appropriate expression level of HA-hDREF which avoids sequestration effects and yields enhanced transactivation by Gal4-DBD-1-82. If this experiment were to be repeated in the future, testing more concentrations of transfected pcDNA3-HA-hDREF DNA, particularly within the range of 0.1 to 0.5µg would be a logical next step.

4.2.3 HA-hDREF Trans-activation of the pGL3-H1p-Luc Reporter

Consistent with previously published results, I've shown that hDREF can weakly, but significantly trans-activate at the hDRE-containing histone H1 promoter when small amounts of pcDNA3-HA-hDREF plasmid are transfected into HT1080 cells (285). Similar trends were observed in HeLa cells as well, however the results across multiple

replicates were less consistent, and significant levels of trans-activation were not observed in these cells. However, my own data indicate that when higher amounts of pcDNA3-HA-hDREF are transfected, significant reductions in trans-activation of the pGL3-H1p-Luc reporter occur in both cell lines. Several explanations may rationalize the observed results, which appear to indicate a biphasic trans-activation response to hDREF. Firstly, hDREF may be negatively auto-regulating itself after it passes a certain threshold of protein expression, thereby reducing hDREF-induced trans-activation at the histone H1 promoter. Auto-regulation of transcription factors is a common mechanism across all eukaryotes and assists in the control of expression of many cell cycle specific transcription factors (312). Negative auto-regulation provides a mechanism to quickly reach steady-state expression levels of individual transcription factors, without having to immediately degrade those proteins when they reach excess cellular amounts (312, 313). Although it is currently unknown whether or not the hDREF gene promoter on human chromosomes contains an hDRE element, five dDRE elements are found within the dDREF promoter in *Drosophila*, and dDREF auto-regulates itself through one of the dDRE elements located 211 to 218 bases upstream of the TSS (259). Indeed the expression pattern of total hDREF within HT1080 cells in my experiments resembles that of negative auto-regulation. Transfection of greater than 0.3 μ g of pcDNA3-HA-hDREF results in levels of hDREF that start to decline (Figure 3.12B). At first glance this seems like a probable answer, but similar effects are not observed in HeLa cells. Results in HeLa cells reveal a similar trend in luciferase activity, but not in hDREF expression (Figure 3.12 and 3.13). Although auto-regulation of transcription factors can be a cell-type specific control mechanism, the discrepancies between protein expression which yield paradoxically similar luciferase activities in HeLa and HT1080 cells indicates that this is likely not the completely correct interpretation. In support of this statement, when either cell line is transfected with 1.9 μ g of pcDNA3-HA-hDREF, there is a significant decrease in luciferase activity compared to the control, whereas hDREF expression is higher. If negative auto-regulation of hDREF was the complete explanation for the observed changes in transcription, trans-activation should not have been reduced below background control levels in HeLa cells.

Perhaps in addition to negative auto-regulation at the genomic level, a molecular switch occurs in the hDREF protein at a certain expression threshold, which changes it from a transcriptional activator to a transcriptional repressor. Recently it has been found that dDREF can recruit the chromatin remodelling complex XNP/dATRX to repress transcription of several transcription factors including dE2F (273). Thus it seems entirely possible as well as intriguing, that hDREF can both activate and repress transcription at a single hDRE in a concentration dependent manner. Indeed many transcription factors display dual activator and repressor functions. Their activities are usually controlled by external factors. For example, the Sp3 transcription factor functions as an activator, unless SUMOylated by SUMO (small ubiquitin-related modifier) (314). In *E. coli*, the ChbR transcription factor binds a regulatory site in the chitobiose operon, which encodes genes for transport and degradation of the chitobiose disaccharide. In the presence of chitobiose it functions as an activator, but in the absence of chitobiose it binds the exact same regulatory site on the operon and represses transcription (315).

The most likely reason that a decrease in trans-activation of the pGL3-H1p-Luc reporter occurs in response to increased amounts of transfected pcDNA3-HA-hDREF is one that is common to all other luciferase assays I performed using hDREF. At high expression levels, hDREF may act to sequester GTFs from the hDRE promoter, or bind the GTFs and compete for hDRE binding with hDREF that is not bound to GTFs. In addition overexpressed hDREF may recruit the GTFs to cellular promoters instead of the reporter plasmid. I attempted – in Figure 3.12C – to provide insight into whether the sequestration of cellular factors was contributing to the reduced luciferase activity when high levels of pcDNA3-HA-hDREF plasmid were transfected. I investigated sequestration effects by increasing the amount of transfected reporter plasmid in HT1080 cells, and therefore increasing the total potential hDREF binding sites. Similar results were observed as with less reporter plasmid, however I utilized a smaller number of transfection conditions and therefore may have overlooked the amount of transfected pcDNA3-HA-hDREF plasmid required to observe significant trans-activation.

Interestingly, the raw output of light units as read by the luminometer in the sample containing only the reporter increased from around 5×10^5 to 5×10^6 when I increased the amount of pGL3-H1p-Luc reporter from 0.1 to 1.0 μg in HT1080 cells. Additionally, in HeLa cells, the raw output of light units in the sample containing 0.1 μg of reporter only was around 6×10^6 . As evidenced by Figure 3.7, HeLa cells express higher levels of endogenous hDREF relative to HT1080 cells. Taken together these results indicate that the endogenous levels of hDREF in both HT1080 and HeLa cells are likely so high that all of the transfected pGL3-H1p-Luc reporter plasmid – which contains only a single hDRE element – became saturated with excess endogenous hDREF in my experiments. If correct, this finding alone would preclude the ability to detect major differences in transactivation when varying amounts of exogenous plasmid encoding HA-hDREF are transfected. The majority of exogenous hDREF would then act in a negative fashion to compete for GTFs and thereby reduce basal levels of transcription below samples expressing only the reporter. In addition, this would explain why other researchers and I have only detected minor levels of transcriptional activation by hDREF using transient luciferase assays in these two cell lines (285, 292). In retrospect, the use of IMR-90 cells – which express the lowest amount of endogenous hDREF out of the four cell lines I tested – would have been a more appropriate cell line choice for all luciferase assays. However, their slow rate of replication and low levels of transcription and protein synthesis might restrict the use of luciferase assays. To enable the use of HT1080 and HeLa cells, in my opinion, a useful future experiment would be to engineer luciferase reporter plasmids which contain only a minimal promoter and a single hDRE site. If hDREF truly activates from the hDRE, then transfecting increasing amounts of this specific plasmid would be expected to increase luciferase activity until the point that endogenous hDREF is no longer present in excess with respect to the reporter plasmid. This would also remove many confounding factors from the system, as the pGL3-H1p-Luc reporter contains the full length histone H1 promoter, of which the single hDRE comprises only 10 bases of the near 550 present (285). It would also remove the need to overexpress hDREF, which may be causing abnormal, unresolvable events to occur with respect to hDREF-induced transactivation. However, these experiments would also

require the construction of negative control luciferase reporter which was otherwise identical except for a mutated hDRE that was unable to bind hDREF.

4.3 The Effects of hDREF on Viral Early Gene and Cellular Gene Transcription during HAdV-5 Infection

Using quantitative RT-PCR experiments in HeLa cells infected with HAdV-5 mutants at an MOI of 5 PFU per cell, I have shown that hDREF plays a significant role in the activation of the HAdV-5 E3 and E4 genes. In addition, a 40% knockdown of hDREF does not affect activation of the early region E2 gene, or the histone H1 and RPS6 cellular genes. The majority of HAdV-5 infection studies in HeLa cells have been carried out at an MOI of 10 or higher, whereby early gene expression begins around 1 hour after infection and DNA replication begins after ~6 hours (75). Each early gene displays different transcriptional kinetics. E3 and E4 transcripts reach maximal transcription around 3 to 4 HPI, and decline over the next 6 hours. Not surprisingly, the E2 transcripts are the last to be expressed, beginning at 2 HPI, reaching maximal transcription at 7 HPI, and then declining. Since I previously detected strong expression of E1A at 12 HPI in HeLa cells infected at an MOI of 5, and showed that an interaction between hDREF and E1A can occur in these cells, I chose to perform quantitative RT-PCR experiments using an MOI of 5 as well. Without any published record of the kinetics of HAdV-5 early gene expression in HeLa cells at this MOI, I had to make an educated guess as to what time points to use. I decided to examine gene expression at 6 and 10 HPI, operating under the assumption that half as much virus per cell would yield 50% slower infection kinetics. My results display trends similar to transcription kinetics at an MOI of 10, as E3 and E4 transcripts are detectable at 6 HPI, and are present at increased levels at 10 HPI. Furthermore, E2 transcripts are delayed, and are detectable only at 10 HPI.

The most significant result of this experiment is the detection of hDREF involvement in early gene E3 and E4 transcription; however, other results deserve individual mention as well. First, hDREF appears to have no effect on E2 transcription. This may be explained

by the fact that the E2 gene is trans-activated in an E1A- independent manner by the E4orf6/7 protein, which recruits E2F to its respective binding sites in the E2 promoter (149, 150). Second, and quite surprisingly, there were also no significant differences in the levels of histone H1 and RPS6 transcripts when hDREF was knocked down. Based on the results of previous researchers and myself, one would expect that since hDREF is a weak trans-activator of both of these genes, at least a small reduction in their transcription would result from hDREF knockdown. This could be the result of an incomplete knockdown. Thus, even with a 40% reduction of hDREF, there still may be sufficient hDREF in the cell to trans-activate genes intimately required for the cell cycle and protein metabolism, such as histone H1 and RPS6 (292, 295). With further optimization of the protocol, it may be possible to achieve close to 100% knockdown of hDREF in HeLa cells, which could resolve this potential issue. In support of this, the histone H1 mRNA levels at 10 HPI, and the RPS6 mRNA levels at 6 and 10 HPI are slightly decreased in the hDREF knockdown samples. Finally, E1A may play a weak role in trans-activation of the histone H1 and RPS6 genes. Figure 3.14 displays results as fold activation of genes in dl309 (WT E1A) infected samples relative to dl343 (non-functional E1A) infected samples. If E1A has no effect on a specific gene, the relative fold activation remains at or around a value of 1. Therefore, relative fold activations above one indicate a role of E1A in transcription of the respective genes during infection, as dl343 encodes a non-functional E1A. The relative fold activations of histone H1 at 6 HPI, and RPS6 at 6 and 10 HPI are all above one, implicating E1A in their trans-activation.

It is important to note that although my results show that hDREF affects viral E3 and E4 transcription, and E1A may affect cellular histone H1 and RPS6 expression, the conclusion that these effects are mediated by the E1A-hDREF interaction cannot yet be made. It is entirely possible that hDREF is affecting viral E3 and E4 transcription independently of its interaction with E1A, and vice versa for cellular genes. However, based on the previous data which shows that E1A interacts with hDREF, a logical hypothesis is that E1A recruits hDREF to early viral promoters and hDREF recruits E1A to the histone H1 and RPS6 promoters. To properly address this question, creation and testing of an HAdV-5 derivative which expresses a mutant form of E1A deficient in

hDREF binding could be performed as discussed in section 4.1. If the results of my experiments can truly be attributed to the E1A-hDREF interaction, then such a mutant virus would yield quantitative RT-PCR results displaying similar trends to when hDREF was knocked down. Furthermore, to determine whether hDREF and E1A are co-localized to the promoters of the E3, E4, and potentially histone H1 and RPS6 genes, a future experiment would be to perform ChIP experiments. ChIP experiments are powerful tools, and recently our laboratory has used ChIP experiments to show that E1A sequesters hBrel from cellular genes, and recruits it to early viral genes to enhance their transcription (214, 215).

To better understand how hDREF may be enhancing transcription of several early viral genes, I searched the HAdV-5 genome for hDRE-like sequences. Interestingly, two hDRE-like sequences which match 8 of 10 consensus bases of the hDRE exist downstream of the E4 gene at around 34,000 base pairs; one exists at around 1450 base pairs which is upstream of the E1B gene. Not surprisingly, these sites are conserved in other members of species C, which share around 99% genomic identity. These sites are also conserved in several simian adenoviruses (SAdVs) closely related to species C, such as SAdV-34, 40.2, 40.1, 42.1, 42.2, and 42.3 which are all about 88-96% similar to HAdV-5 in genomic sequence. These sites however are not found in conserved locations for the less related HAdVs such as 12, 3, 9, 4, and 40, which share about 80% genomic conservation with HAdV-5. Interestingly, HAdV-12, and 3 contain an hDRE-like sequence matching 9 of 10 consensus bases of the hDRE centered around 10,000 base pairs into the genome. This region is upstream of the major late promoter. Perhaps different subgroups have evolved to utilize hDREF to enhance expression of distinct viral genes. Although the two hDRE elements found near the E4 gene are downstream of the TSS, they may still function as hDREF binding and trans-activation sites. This would serve to provide a reasonable explanation as to why E4 transcription appears highly responsive to hDREF. Indeed, trans-activators have been shown to enhance transcriptional activation via enhancers located thousands of bases away, whether upstream or downstream of the promoters they act on (316, 317). Importantly, there may be hDRE-like sequences near the E3 transcriptional unit promoter as well, as my

preliminary scan of the HAdV-5 genome using Clustal-W algorithms may have overlooked more divergent sequences. Finally, my experiments did not look at the effects of hDREF on early gene E1B expression, which was also found to contain an hDRE-like element upstream of the TSS. Thus, E1B expression is an important candidate to monitor in future experiments examining the effects of hDREF on early viral gene transcription.

4.4 The Effects of hDREF on HAdV-5 Replication

The replicative cycle of HAdV-5 experiences vastly delayed kinetics in contact-inhibited IMR-90 cells in comparison to HeLa cells. For example, previously published experiments that examined viral growth in IMR-90 cells were performed at an MOI of 5 PFU per cell, and virus was collected at 4, 48, and 120 HPI (77, 78). I decided to emulate these experimental conditions. The results of plaque assay experiments from HAdV-5 mutants isolated from IMR-90 cells indicate a significant 1.8 fold decrease in dl309 viral titre at 120 HPI when hDREF is approximately 100% knocked down. Similar to the scenario in section 4.3, these differences cannot be attributed directly to the interaction of E1A and hDREF without performing plaque assay experiments using HAdV-5 mutants encoding E1A which does not bind hDREF. Such a mutant would be predicted to display identical growth characteristics regardless of whether hDREF was knocked down or not. No differences were observed in dl309 titre from viruses collected at 4 or 48 HPI. I did not expect any differences at 4 hours, as no E1A was present at this time, and even in HeLa cells, viral DNA replication does not begin until 6 HPI at an MOI of 10 (75). The viral titre of dl309 infected samples only increased minimally by a factor of 2 between 4 and 48 HPI, whereas titres increased by over 10 fold between 48 and 120 HPI (Data not shown). Therefore, minimal viral replication had occurred in dl309 samples after 48 hours, regardless of the status of hDREF. Accordingly, it is not surprising that no differences in viral titre were observed between hDREF siRNA and scramble siRNA-treated samples at 48 HPI.

As expected, dl312 titres remained constant across all time points, indicating the inability of this virus to replicate without E1A, regardless of the presence or absence of hDREF. One final observation can be made; hDREF does not have an effect on E1A expression, as the levels of E1A at individual timepoints between hDREF siRNA and scramble siRNA-treated samples are similar. Therefore, in contrast to what was shown with E3 and E4 genes, hDREF likely does not affect E1A gene transcription. Future experiments should examine viral replication at time points between 48 and 120 HPI, as well as time points after 120 HPI. This would elucidate when the levels of replication start to diverge, and whether or not the growth defect seen in hDREF siRNA-treated samples remedies itself at later time points, or becomes further amplified.

Studies of the effects of hDREF on S phase progression in HAdV-5 infected contact-inhibited IMR-90 cells were also performed, utilizing a dual propidium iodide staining and bromodeoxyuridine incorporation protocol described by Cecchini et al. (318). The expression of hDREF has previously been shown to influence cell cycle progression, and is necessary for entry into S phase (285, 292). Therefore, the goal of these experiments was to examine whether knockdown of hDREF in HAdV-5 infected IMR-90 cells reduced S phase progression, or whether HAdV-5 could compensate for the lack of hDREF during infection. Based on the plaque assay results, I did not expect to see major differences in cell cycle progression at 4 or 48 HPI, but expected to observe differences at 120 HPI. In fact, no noticeable differences at 4, 48 or 120 HPI between hDREF siRNA and scramble siRNA treated samples were observed. However, due to inherent complications in these experiments, the data is not shown, and cannot be properly interpreted. HAdV-5 is a lytic virus, and therefore at 120 HPI, the majority of cells are lysed and cannot be properly assayed for DNA content by a flow cytometer. In the future, transfection based experiments may be required to examine how the E1A-hDREF interaction affects S phase progression. E1A which is sufficient or deficient for hDREF binding could be transfected into contact-inhibited IMR-90 cells treated with either hDREF siRNA or scramble siRNA, and subsequently the dual staining and incorporation protocol could be utilized.

4.5 Summary of Findings and Future Directions

My thesis project has revealed several interesting findings with regards to the functions of the poorly characterized hDREF protein. I have identified that there is an interaction between 13S E1A and hDREF, which is conserved amongst representative serotypes of HAdV species A through E – HAdV 12, 3, 5, 9, and 4 respectively – but not HAdV-40 of species F. In addition, all five isoforms of HAdV-5 E1A – 13S, 12S, 11S, 10S, and 9S – can bind hDREF. Amino acids 15-26 of E1A are sufficient for E1A to interact with hDREF, however other residues may contribute to binding. Therefore, a future direction of the project is to identify all residues essential for hDREF binding, in order to mutate them and generate a HAdV-5 derivative encoding an E1A mutant deficient for hDREF interaction. In addition, it would be interesting to identify factors which co-immunoprecipitate in complexes with hDREF and observe whether the identities of factors in these complexes are altered in the presence of E1A. These studies may help to identify currently unknown cellular binding partners of hDREF.

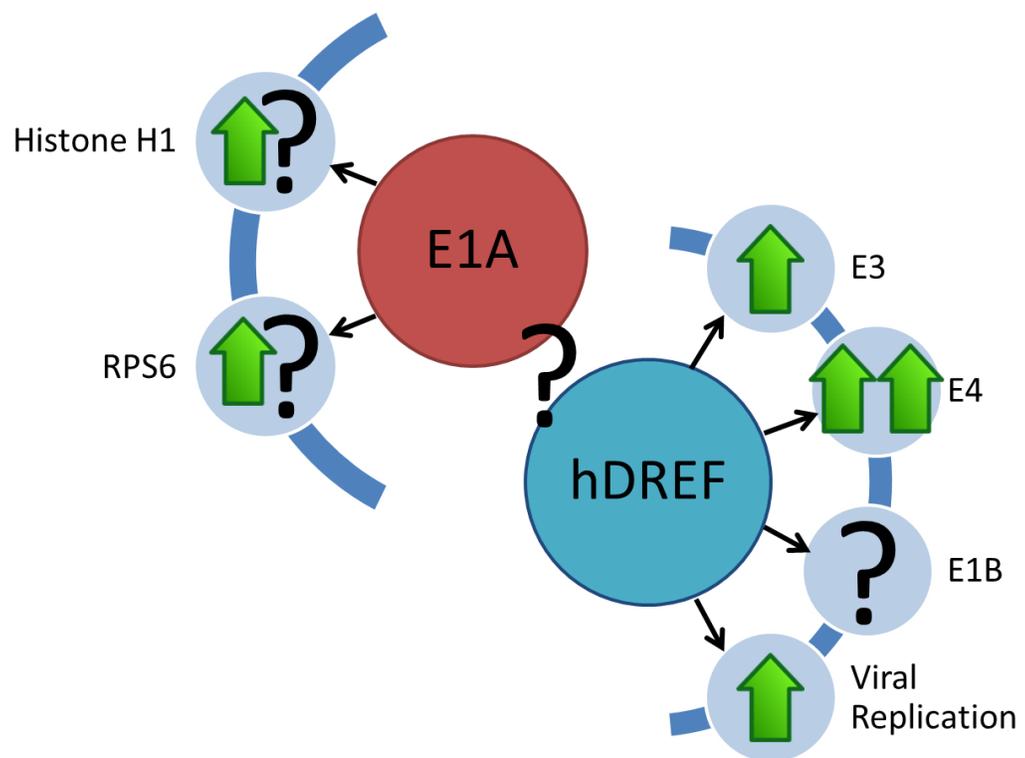
Consistent with previously published results, hDREF appears to be a weak trans-activator at the histone H1 promoter (285). However, it may repress transcription at this promoter as well, through an unknown mechanism. In addition, hDREF does not possess the capability to trans-activate at a minimal promoter containing only a TATA box. Finally, when large amounts of pcDNA3-HA-hDREF plasmid are co-transfected with plasmids encoding Gal4-DBD-tagged AA1-82 or CR3 of E1A, the abilities of these known trans-activators to drive luciferase expression through a minimal promoter are significantly reduced. The probable inherent flaws of my luciferase assay experimental design, as well as how to alleviate their issues for future experiments were addressed in detail in section 4.2. Further examination of hDREF's function as a trans-activator is warranted. Potential solutions involve carrying out the same experiments in different cell lines which express less endogenous hDREF, or utilizing HT1080 and HeLa cells without overexpressing exogenous HA-hDREF. In addition, to truly examine whether hDREF can transactivate through the hDRE, a reporter plasmid should be engineered which contains only a minimal promoter and an upstream hDRE.

I have shown that either directly or indirectly, hDREF plays a significant role in the activation of HAdV-5 early region E3 and E4 genes during infection. As well, E1A may be involved in histone H1 and RPS6 trans-activation during infection. Future directions may include examining the effects of hDREF on all viral early genes during infection when close to 100% knockdown of hDREF is achieved. In addition, once a mutant virus is created which expresses E1A deficient for hDREF binding, it will be possible to determine whether the observed effects are due to the E1A-hDREF interaction, or to some other unknown activity of hDREF in the cell. Finally, ChIP experiments examining whether E1A and hDREF co-localize to early viral promoters or cellular H1 and RPS6 promoters would provide support to my data.

Lastly, I have shown that hDREF plays a significant role in HAdV-5 replication in quiescent, contact-inhibited IMR-90 cells. Future experiments should examine viral titres from 48 to beyond 120 HPI in order to generate a viral growth curve with respect to the presence or absence of hDREF. Finally, once a mutant virus is created which expresses E1A deficient for hDREF binding, it will be possible to determine whether the observed effects on viral replication are due to the E1A-hDREF interaction, or to some other function of hDREF during infection.

My original hypothesis was that the interaction between E1A and hDREF contributes to adenovirus induced transcriptional modulation and viral replication in HAdV-5 infected cells. Although I can't conclusively say whether the E1A-hDREF interaction is contributing to the observed effects, it is clear that my data demonstrates that hDREF contributes to adenovirus early gene transcription and viral replication in HAdV-5 infected cells. Future experiments should determine whether the interaction is responsible for my observed results. Based on all of my observed results, I have synthesized a model which outlines the potential consequences of the E1A-hDREF interaction in an HAdV-5 cell (Figure 4.2).

Figure 4.2 Cartoon diagram of the potential effects of the E1A-hDREF interaction in an HAdV-5 infected cell. Green arrows within circles represent increases in gene expression or viral replication respectively. Question marks within circles represent the unknown or potential effects of hDREF or E1A on genes which warrant further investigation. hDREF increases expression of E3 and to a greater degree, E4. hDREF also increases HAdV-5 replication. hDREF may affect E1B expression through a putative hDRE-like sequence found upstream of the E1B gene. E1A may enhance histone H1 and RPS6 gene expression. The question mark between E1A and hDREF indicates that although E1A and hDREF have been shown to interact, the effects of hDREF on viral gene expression and viral replication have not been directly linked to this interaction.



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